PYY(3-36) analogues: Structure-activity relationships in energy homeostasis

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Abstract

The developed world is currently in the grip of an obesity epidemic. As a result, there is much ongoing research into the development of an effective anti-obesity agent. Peptide YY (PYY) is a 36 amino acid gastro-intestinal hormone released post-prandially by L-cells in the gastro-intestinal tract in proportion to the calorie content of a meal. The predominant form of the hormone found in circulation is the truncated PYY(3-36). Administration of PYY(3-36) at physiological doses to humans has been shown to reduce food intake. However, due to enzymatic degradation these effects are short lived, reducing the hormone's utility as an anti-obesity pharmaceutical agent. A series of analogues of PYY(3-36) were designed either with amino acid substitutions in specific parts of the peptide sequence and/or with chemical modifications to the native sequence with the aim of increasing resistance to enzymatic activity whilst retaining or even enhancing the peptide's bioactivity. The analogues were tested for resistance to degradation by different proteolytic enzymes in comparison to natural PYY(3-36). Their affinity to the Y2 receptor, for which PYY(3-36) is a natural agonist was then investigated. Finally, the effects of peripheral administration of selected analogues on food intake in overnight fasted mice were investigated. These studies suggest that PYY(3-36) analogues may be a useful approach for the treatment of obesity, but further development work is required.

Declaration of Contributors

The majority of the work described in this thesis was performed by the author. Any collaboration and assistance is described below.

Chapter 2 – Synthesis and purification of one set of PYY(3-36) analogues were carried out with the assistance of Philip Metcalfe and John Fitzmaurice at Bachem UK Ltd.

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Abbreviations

125I	Iodine-125
ACN	Acetonitrile
AgRP	Agouti-related peptide
Ala	Alanine
Aloc	Allyloxycarbonyl
ANOVA	Analysis of variance
AP	Area postrema
ARC	Arcuate nucleus
Arg	Arginine
Asn	Asparagine
Asp	Aspartic Acid
BBB	Blood brain barrier
Boc	t-Butoxycarbonyl
BSA	Bovine serum albumin
CCK	Cholecystokinin
Cit	Citrulline
CNS	Central nervous system
Cpm	Counts per minute
Cys	Cysteine
Dap	α, β-diaminopropionic acid
DCC	N,N'-Dicyclohexylcarbodiimide
DIC	N,N'-Diisopropylcarbodiimide
DIPEA	N, N'-diisopropylethylamine
DMEM	Dulbecco's modified eagle medium
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
DPP-IV	Dipeptidyl peptidase IV
GI	Gastrointestinal
Gly	Glycine
Gln	Glutamine
GLP-1	Glucagon-like peptide 1
Glu	Glutamic acid
GPCR	G protein coupled receptor
HATU	2-(1H-7-Azabenzotriazol-1-yl)--1,1,3,3-tetramethyluronium hexafluorophosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	Histidine
HOAt	1-Hydroxy-7-azabenzotriazole

HOBt	1-Hydroxybenzotriazole
HPLC	High-performance liquid chromatography
ICV	Intracerebroventricular
Ile	Isoleucine
IP	Intraperitoneal
IV	Intravenous
Leu	Leucine
MALDI MS	Matrix assisted laser desorption ionisation mass spectrometry
Me	Methyl
Mtt	4-methyl trityl
NMP	N-methyl pyrrolidone
NPY	Neuropeptide Y
OAll	Allyl ester
OPip	2-phenylisopropyl ester
Pbf	2,2,4,6,7-pentamethyldihydrobenzofurane
PBS	Phosphate Buffer Solution
PEG	Polyethylene glycol
Phe	Phenylalanine
POMC	Proopiomelanocortin
PP	Pancreatic polypeptide
Pro	Proline
PVN	Paraventricular nucleus
PYY	Peptide YY
SC	Subcutaneous
Ser	Serine
tBu	Tertiary butyl
TBTU	2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
TEAP	Triethylammonium phosphate
TFA	Trifluoroacetic acid
Thr	Threonine
Trp	Tryptophan
Trt	Trityl
Tyr	Tyrosine
UV	Ultraviolet
Val	Valine

1 General Introduction

1.1 Obesity

Obesity is a major world health problem. An individual is classed as overweight if their Body Mass Index (BMI) is greater than 25.0 and obese if their BMI is greater than 30. The World Health Organisation (WHO) projects that by 2015, 2.3 billion adults will be overweight and 700 million will be obese worldwide (World Health Organization, 2009). Historically thought of as a disease affecting only Western countries, in particular the United States of America, there is clear evidence of obesity levels increasing across the globe, including in some of the world's poorest countries.

Weight gain occurs when energy intake exceeds energy expenditure. The thrifty gene hypothesis, first posited in the 1960's by James Neel, proposed that humans are preconditioned to put on weight to protect against starvation (Watve and Yajnik, 2007). Whilst this would have been advantageous in an environment of limited resources when an individual may not have known where their next meal was coming from, in the modern developed world in which food is abundant, the hypothesis suggests it has resulted in excess consumption becoming the norm. The fostering of a "fast food" culture in many modern societies compounds this problem. The convenience and relative cheapness of these high calorific foods promote excessive energy intake. Coupled with the reduced energy expenditure observed in societies with low levels of physical work and mechanised transport, this has easily led to widespread weight gain and obesity.

People who are overweight or obese are at a greater risk of suffering from serious medical conditions such as diabetes, cardiovascular disease, strokes and some cancers. Many patients suffer from a combination of different disorders and are often described as having metabolic syndrome. According to the WHO a person is said to have metabolic syndrome if they have type 2 diabetes, impaired glucose tolerance or

are insulin resistant plus they also meet at least two of the following criteria; they have hypertension, elevated plasma triglyceride levels, reduced high density cholesterol concentration, microalbuminuria or a BMI greater than 30 (Groop, 2000). Obesity can also have serious psychological effects, with subjects often suffering from depression and low self esteem.

The obesity epidemic is thus placing a strain on healthcare systems across the world. In the UK it was estimated that the annual cost to the NHS in England alone of treating obesity and its associated disorders was £4.2 billion in 2007 and could potentially reach £9.7 billion by 2050 (Swanton, 2008). Additionally it is estimated that 18 million sick days are lost annually to UK businesses as a result of obesity related problems (Foresight, 2006).

There would thus be significant health and economic benefits to the successful prevention or treatment of obesity. For many years, health professionals and governments have expounded the benefits of a healthier lifestyle incorporating increased exercise and improved diet. To date, this educational strategy has proven unsuccessful; obesity rates have continued to rise. Identifying novel therapeutics to reduce food intake and limit weight gain is thus a major focus for academic and industrial research.

1.2 Treatments for obesity

1.2.1 Bariatric Surgery

Gastro-intestinal surgery, in the form of gastric banding or gastric and intestinal bypass successfully treats obesity. Gastric banding is the less invasive option and involves the placement of a constrictive band around the top portion of the stomach. This creates a small pouch in the upper region of the stomach which can only hold a

small amount of food. Patients thus feel ‘full’ having eaten only small portions of food. NHS guidelines only recommend gastric banding for those patients with a BMI in excess of 40 or with a BMI between 35 and 40 who also have a co-morbidity that poses a serious health risk, such as diabetes (National Institute for Health and Clinical Excellence, 2006). Studies have shown that patients with a BMI of 30 – 35 fitted with a gastric band experienced more prolonged weight loss than patients with comparable BMI values who underwent non-surgical therapies (O’Brien et al., 2006). Subsequent investigations also showed effective weight loss following gastric band surgery in patients with a BMI greater than 50, the so called super obese. Four years after surgery, an average excess weight loss of 60% was reported (Fielding, 2003). However, to remain effective a gastric band requires post operative readjustment at regular intervals to maintain optimal restriction of food intake (O’Brien et al., 2006).

Gastric bypass surgery is a more invasive and therefore riskier procedure. There are a number of different forms of this surgery with the most commonly performed in the USA being the Roux-en-Y Gastric Bypass (Saliba et al., 2009). It is estimated that 120 thousand people underwent this particular operation in the USA in 2007 (Couzin, 2008). The procedure has two elements to it. Firstly, a small gastric pouch is created at the top of the stomach that restricts the patient’s capacity for food. Secondly, a so called Roux limb is created which connects this pouch directly with the jejunum, thus bypassing a considerable section of the small intestine (Saliba et al., 2009). Average weight loss following Roux-en-Y Gastric Bypass has been reported to be 66% of a patient’s excess weight (Balsiger et al., 2000). Initially it was thought that this profound weight loss was due to the combination of reduced gastric volume coupled with reduction in nutrient and calorie absorption by the intestine. However, the recorded weight loss following gastric bypass surgery exceeded that for gastric

banding, which suggests that restriction of volume was not the primary cause of the weight loss (Morínigo et al., 2006). It was also discovered that the gut adapts following surgery so as to protect against malabsorption of nutrients (le Roux and Bloom, 2005). Studies have since shown that the levels of some gut derived hormones are altered following gastric bypass surgery. Ghrelin is a known orexigenic peptide that is produced in the stomach. Circulating levels are increased by fasting and drop following food intake (Chandarana et al., 2011). Several studies reported that gastric bypass patients have lower circulating levels of ghrelin than patients who had achieved the equivalent weight loss by diet alone. However, other studies have shown either no change in ghrelin levels or, in some cases, an increase (Saliba et al., 2009). The effect of gastric bypass surgery on circulating ghrelin levels remains inconclusive, with some suggesting that the discrepancies between studies may be due to the different surgical techniques employed (Saliba et al., 2009). Ghrelin is a so called “hunger hormone” which increases food intake following peripheral administration in animals and man (Cummings and Shannon, 2003). The theoretical reduced circulating levels of ghrelin may therefore contribute to the reduced appetite of these patients. In contrast, Peptide YY (PYY) is an anorexigenic peptide released postprandially by L-cells in the distal gut (le Roux and Bloom, 2005). Circulating levels of PYY are higher in gastric bypass patients compared with subjects of comparable weight (le Roux and Bloom, 2005). PYY is released from enteroendocrine L cells in the gut and is processed to form PYY(3-36), the major circulating form (Chandarana et al., 2011). Peripheral administration of PYY(3-36) inhibits appetite in animals and man (Morínigo et al., 2006). The elevated PYY levels observed following gastric bypass are thus likely to enhance the feeling of satiety in these patients and so lead to reduced food intake.

Growing evidence demonstrates that gastric bypass surgery can also have profound benefits for patients who suffer from type 2 diabetes, and that this effect may be at least partly independent of its effects on body weight (Hayes et al., 2011). Studies have reported remission levels of 76.8% of type 2 diabetes in post-operative subjects. The number increases to 85.4% when those patients who showed improvement in their diabetes are included (Buchwald et al., 2007). The improvements in glucose homeostasis are observed within a week of the operation in many cases, often before there has been significant weight loss (Hayes et al., 2011). Glucagon-like peptide-1 (GLP-1) is another hormone released post-prandially by the L-cell. The active forms of GLP-1 inhibit appetite and promote insulin release when peripherally administered in animals and man (Saliba et al., 2009). Circulating GLP-1 levels are also increased following gastric bypass (Vidal et al., 2009). As GLP-1 is an incretin, it is hypothesised that this may be the mechanism by which bypass surgery increases insulin secretion and facilitates the remission of diabetes.

Despite the well reported benefits of these surgical procedures, drawbacks remain. Patients must follow a difficult post-operative regime. Typically, patients can only consume fluids for at least one week after the operation. They then move onto purees for another four weeks before slowly progressing onto more solid food types (British Obesity Surgery Patient Organisation, 2011a).

There also exists a small risk of mortality following surgery. A systematic review of bariatric surgery mortality rates published in 2007 reported a total mortality rate of 0.28% in the first 30 days following surgery and 0.35% in the first 2 years (Buchwald et al., 2004). There are a number of other post-operative complications, including vomiting, diarrhoea, anastomotic complications, hernia and infection.

There is also a significant cost implication to these surgeries. In the UK the cost per operation has been estimated at £5000 - £7000 for gastric banding and £8000 - £14000 for gastric bypass (British Obesity Surgery Patient Organisation, 2011b). These costs restrict the number of operations that can be performed under the NHS and prove prohibitive to many people seeking private treatment. Other resources are also limited; there are insufficient operating theatres or qualified surgeons to realistically treat all of the obese patients who would qualify for surgery. However, there is a need to take into account the long term health economics, particularly the costs of not performing these surgeries and having to fund the treatment of obesity related disorders.

1.2.2 Pharmaceutical therapies

Using a drug to treat obesity would be much simpler and would avoid the inconvenience, costs and risks of surgery. Such a product would have huge market potential, and much effort has therefore focused on the development of an anti-obesity pharmacotherapy. Despite this effort, only a relatively small number of therapies have made it onto the market and none have acquired blockbuster status.

There is currently only one prescription anti-obesity medication approved for use in the UK(National Institute for Health and Clinical Excellence, 2006). Orlistat was launched in 1998 by Roche under the trade name Xenical, with worldwide sales in 2005 totalling $450million (Melnikova and Wages, 2006). Orlistat works by inhibiting pancreatic lipase, and thus the breakdown and absorption by the gut of approximately 30% of dietary fats (Lean and Mullan, 2007). It has been demonstrated to produce an additional weight loss of approximately 3% when compared with patients attempting weight loss through changes to their diet (Cooke and Bloom,

2006). Orlistat can improve glycaemic control in type-2 diabetics and also reduce the risk of overweight and obese individuals with impaired glucose tolerance developing type-2 diabetes (Lean and Mullan, 2007). However there are several unwanted side effects associated with the use of Orlistat, including faecal urgency and oily stools (Drew et al., 2007). There is also the potential for the unwanted reduction in absorption of the fat soluble vitamins A, D and E (Drew et al., 2007; Cooke and Bloom, 2006). Approval was granted by the U.S. Food and Drug Administration (FDA) in 2007 and the European Medicines Agency (EMA) in 2009 for the over the counter sale of Alli, a low dosage form of Orlistat produced by GlaxoSmithKline (Steffen et al., 2010).

Until relatively recently other obesity drugs were licensed for use in the UK and the rest of the EU. Rimonabant acts as an antagonist of the cannabinoid-1 receptor (CB1), which was identified and cloned during investigations into the effects of cannabis usage (Foster-Schubert and Cummings, 2006). One of the well reported side effects of cannabis use is an increase in appetite. Identification of ligands for this receptor led to the development of several antagonists, of which Rimonabant was the lead compound (Foster-Schubert and Cummings, 2006). Major trials with Rimonabant were carried out in both Europe and the USA (Pi-Sunyer et al., 2006). In all, modest yet significant weight losses were recorded in comparison with placebo groups. In 2006 the use of Rimonabant for the treatment of obesity was approved in Europe despite concerns that patients might develop psychological problems (Cooke and Bloom, 2006; Lean and Mullan, 2007). However, because of these concerns the drug was never approved for use in the USA, and in 2008 the EMA suspended its use due to the risk of users developing serious psychiatric problems and suicidal impulses (European Medicines Agency, 2008).

Sibutramine was approved by the FDA in 1997 and the EMA in 1999, and recorded sales of $230million in 2005 (Melnikova and Wages, 2006). Sibutramine is a serotonin and noradrenalin reuptake inhibitor that enhances post-prandial satiety and attenuates the fall in metabolic rate that occurs with weight loss (Smith and Goulder, 2001). It is structurally linked to amphetamines which were once widely used as weight loss agents before being banned in the 1970's because of the associated side effects, which include hypertension, cardiac valvulopathy and the potential for dependence (Astrup, 2010; Cooke and Bloom, 2006). Sibutramine elicited weight loss of around 5% more than in a placebo group (Finer, 2002). Sibutramine usage also helped maintain weight loss compared with subjects assigned to a placebo. Over a two year period, placebo patients regained nearly 80% of the initial weight loss despite adhering to an improved diet and exercise regime, whereas those subjects who continued to take Sibutramine retained 85% of their weight loss (Finer, 2002). Sibutramine was initially reported to lack the severe side-effects of amphetamines, but does increase heart rate and hypertension (Cooke and Bloom, 2006). These side effects were hypothesised to lead to adverse cardiovascular effects, and in 2009, data from the Sibutramine Cardiovascular Outcome Trial (SCOUT) showed an increased risk of non-fatal cardiovascular episodes from Sibutramine usage when compared to placebo groups (Haehling et al., 2007). As a result, in 2010 both the FDA and the EMA recommended it be withdrawn from the market (European Medicines Agency, 2010; U.S. Food and Drug Administration, 2010).

Despite the limited success thus far of anti-obesity drugs there continues to be extensive research in this field. Such is the complex nature of the human energy homeostasis system that there exist these many different pathways for scientists to investigate and target. There are many drugs in the pipeline, at various stages of the

drug development process. A major difficulty is that the signalling molecules that regulate appetite often have other physiological roles, making the specific targeting of food intake extremely challenging, and increasing the risk of side effects.

Another difficulty is that body weight regulation is a homeostatic process that has evolved to preserve nutritional status under conditions of limited food availability. Even if a particular anorectic pathway can be successfully and specifically targeted to reduce food intake and body weight, compensatory changes in other appetite-regulating pathways may limit the efficacy of this approach. This may explain the modest weight loss reported with previously developed therapies.

It is thus important to understand the physiological mechanisms regulating food intake and body weight in order to develop anti-obesity agents.

The neuronal and hormonal systems involved in the regulation of energy homeostasis work through both long and short term control mechanisms, regulating food intake and energy expenditure in response to, for example, adipose tissue mass or acute nutritional status, respectively.

1.3 Long term regulation of energy homeostasis

1.3.1 Insulin

Insulin is produced by the ß-cells of the Islets of Langerhans in the pancreas. It is released in response to an increase in blood glucose levels following food intake (Taubes, 2009). Insulin is an anabolic signal. Insulin stimulates the uptake of glucose by muscle and liver cells where it is then stored as glycogen for use by the body as a source of energy for periods of sudden and strenuous activity (Berg, et al., 2002). Insulin also prevents the use of fat tissue as an energy source by inhibiting the release

of the hormone glucagon. A 29 amino acid peptide hormone, glucagon is released into circulation following a decrease in blood glucose levels and stimulates hepatic glucose release via two different mechanisms (Jiang and Zhang, 2003). The first is glycogenolysis which is the process by which glycogen is broken down into glucose. The second mechanism is gluconeogenesis whereby glucose is generated from non-carbohydrate sources. Other roles for glucagon within the body include the regulation of plasma lipid levels and food intake (Habegger et al., 2010).

There are several disorders linked to abnormalities of the body's insulin system. The most widely recognised is diabetes mellitus, the name given to any condition characterized by hyperglycaemia (World Health Organization, 1999). The two most common forms of the disease are type 1 and type 2 diabetes mellitus. Type 1 diabetics are unable to produce sufficient insulin due to autoimmune destruction of their pancreatic ß-cells (World Health Organization, 1999). They are required to regularly check their blood glucose levels and administer exogenous insulin accordingly. Failure to do so adequately can lead to high levels of blood sugar which over the long term can cause organ damage and death (van Belle et al., 2011). Conversely, overdosing with insulin can cause blood glucose levels to fall, putting the patient at risk of hypoglycaemic coma. The cause of type 1 diabetes is still unknown but several risk factors have been identified (van Belle et al., 2011). There is currently no cure for the condition, though many different avenues including ß-cell transplantation, vaccine development and the inhibition of the actions of glucagon are being investigated as potential treatments (van Belle et al., 2011).

Type 2 diabetes is the most prevalent form of diabetes and is characterised by insulin resistance (Taubes, 2009). Sufferers are still able to produce their own insulin but the body becomes relatively unresponsive to its actions. The exact cause of type 2

diabetes is also unknown, but it is strongly associated with obesity (Taubes, 2009). It has been suggested that their insulin resistance is caused by overloading or inflammation of the body's fat cells leading to a breakdown in the mechanisms which mediate the actions of insulin (Taubes, 2009). Type 2 diabetics are encouraged to make lifestyle changes to help manage the condition. It has been shown that regular exercise and dietary improvements increases insulin sensitivity and so help lower blood glucose. If these changes are made early enough they reduce the incidence of at-risk patients going on to develop type 2 diabetes. In 2011, a group from Newcastle University reported the results of a study in which a small group of type 2 diabetics were placed on a restricted (600 kcal/day) diet for 8 weeks. At the end of this period the patients were found to have normalised beta cell function and insulin sensitivity, thus giving rise to the hope that type 2 diabetes could be a potentially reversible condition for many sufferers (Lim et al., 2011).

As type 2 diabetics are still able to produce insulin there is a greater potential to develop effective treatments than with type 1 diabetes.

Those treatments currently on the market can be classified into three classes according to their action. The first are insulin secretagogues which stimulate release of insulin by the pancreas. Sulfonylureas were the first such compounds used to treat diabetes, although they have now been superseded by second generation compounds. Common side effects from these treatments include weight gain and hypoglycaemia (Rendell, 2004). Some of the newest diabetes treatments on the market and in clinical trials are drugs based on natural incretins (hormones within the body that promote insulin release). One such endogenous incretin is Glucagon-like peptide-1 (GLP-1) (discussed in more detail in sections 1.2.1 and 1.5.3), and two drugs based on its

structure, Exenatide and Liraglutide, have been approved for use in the treatment of type 2 diabetes (Nachnani et al., 2010; Parks and Rosebraugh, 2010).

The second class of anti-diabetic drugs are insulin sensitizers which increase the sensitivity of peripheral tissue to the effects of insulin (Lu et al., 2011). One example is Metformin, one of the biggest selling drugs in the field and the first line drug of choice for overweight and obese patients as weight gain is not an associated side effect of its use (Rendell, 2004). Avandia, another insulin sensitizer and one of the Thiazolidinedione group of compounds was also heavily prescribed for diabetes treatment until it was recommended for removal from the European market in 2010 amid concerns over cardiovascular risks.

The final class of anti-diabetic treatment has no direct effect on insulin levels or action but instead slows the rate of glucose absorption into the bloodstream. Examples of this type of treatment are Miglitol and Acarbose (Van de Laar et al., 2005). They have the effect of artificially lowering blood sugar levels, enabling endogenous insulin to have an affect even if the body has begun to develop a resistance to it. However as levels of insulin resistance increase, this type of drug becomes less useful. Reported side effects of their use include flatulence, diarrhoea and stomach pains (Van de Laar et al., 2005).

In addition to these drugs, many type 2 diabetics are prescribed insulin itself as a treatment for their diabetes, often in combination with other treatments (Swinnen et al., 2009). Although these patients have insulin resistance, administering exogenous insulin at supra-physiological levels can activate their insulin-responsive signalling pathways, at least to some limited degree (Swinnen et al., 2009).

1.3.2 Leptin

Leptin was identified following studies on two strains of mutant mice that were extremely obese (Friedman, 2009). The mutant genes were labelled as *ob* and *db* and the similarities in phenotypes between the two strains suggested that the genes were involved in the same physiological process. Further studies led to the proposal that the *ob* gene encoded an as yet unknown substance that reduced food intake whilst the *db* gene encoded its receptor (Friedman, 2009).

Cloning of the mutant gene led to identification of the *ob* gene and the discovery that it was expressed by adipose tissue. The gene was predicted to encode a 167 amino acid protein which later became known as leptin (Friedman and Halaas, 1998). This was subsequently found to circulate in the plasma of a number of mammals, including humans. Circulating leptin levels increase with adipose tissue mass and decrease when adipose tissue is lost (Oswal and Yeo, 2010). The leptin receptor is expressed in regions of the hypothalamus known to be involved in energy homeostasis (Scott et al., 2011). Central or peripheral leptin administration reduces food intake and body weight in both wild type and *ob/ob* mice (Friedman, 2009). Leptin was thus hypothesised to act as an adipose tissue derived hormone that operated in a negative feedback loop to help maintain levels of adipose tissue by influencing the neuronal mechanisms that control food intake and energy consumption (Oswal and Yeo, 2010). As adipose tissue mass increases, leptin levels rise, causing a reduction in food intake to help reduce adiposity.

This led to the hope that leptin had therapeutic utility in the treatment of obesity (Friedman, 2009). However, it was subsequently discovered that most obese humans already had high circulating levels of plasma leptin and were relatively leptin insensitive, meaning that supra-physiological doses of leptin were required to reduce

food intake (Heymsfield, et al., 1999). These findings suggested that obese humans were resistant to the effects of leptin and led some to hypothesise that the development of this resistance was a primary cause of obesity (Friedman and Halaas, 1998). This theory has been challenged, with others proposing that leptin resistance is a consequence, rather than the cause, of obesity (Arch et al., 1998). The mechanism behind leptin resistance is not fully understood. As fat tissue increases rapidly with sustained over-eating so the levels of plasma leptin increase in response. This has been suggested to lead to the saturation of the leptin responsive circuits in the hypothalamus, causing an unknown disturbance in the leptin pathway that leads to a reduced effect. This then results in a compensatory increase in leptin secretion which drives a cycle of chronic weight gain (Oswal and Yeo, 2010). It has been shown in rats that chronic leptin infusion eventually causes leptin resistance. It has also been proposed that leptin resistance is caused by a breakdown in the transport of leptin into the hypothalamus via the blood brain barrier (Oswal and Yeo, 2010). This followed studies that showed that diet induced obese mice developed resistance to peripherally administered leptin but retained sensitivity to centrally administered leptin. Further studies have suggested that these defects in the leptin transport mechanism are a consequence of obesity rather than a cause.

As leptin is secreted by adipose tissue and has been shown to reduce food intake it was widely believed that its physiological role was that of a satiety signal. However, subsequent studies and the discovery of leptin resistance mean that it is now thought of as a starvation signal, with its main purpose to retain sufficient fat stores in readiness for periods of inadequate food supply (Oswal and Yeo, 2010). This would be a vital in an evolutionary context for organisms that live in an insecure nutritional environment but for the many humans living in a world where high calorie food is

readily available, it is less important, and may actually play a role in driving the development of obesity.

Although the majority of obese humans have high circulating levels of leptin, a small number do not. These humans have leptin signalling defects similar to those in the *ob/ob* mouse, and display a similar phenotype. It has been shown that in these individuals, leptin treatment has a beneficial effect on body weight (Friedman, 2009).

There exist in humans other conditions associated with low levels of plasma leptin and which have been shown to be receptive to leptin treatment. Lipodystrophy is characterised by complete or partial absence of normal adipose tissue depots. It can be caused by mutations of the genes that regulate adipose tissue development or by alterations in the immune system, as is the case with some HIV patients. This results in lipids being deposited in other organs, particularly the liver, and the subsequent development of insulin resistance. As leptin is secreted by adipose tissue, lipodystrophic patients have extremely low leptin levels. It has been found that leptin treatment prevents lipid deposition in the liver and corrects insulin resistance in these patients (Friedman, 2009).

Loss of leptin signalling in animals or man results in infertility. It is thought that leptin acts as a signal to the reproductive axis that sufficient energy stores are available to support reproduction; low leptin levels signal that it is likely a period of limited nutritional availability, and hence reproduction should be delayed. Hypothalamic amenorrhea is a condition characterised by suspension of the female menstrual cycle. It can result from weight loss, typically caused by periods of extreme exercise or an eating disorder. The resulting energy deficit leads to disruption in the secretion of gonadotropin-releasing hormone and it is hypothesised that this disruption is caused by a decrease in circulating leptin levels. In accord with this,

recombinant leptin administration to patients with hypothalamic amenorrhea improved reproductive function in just a few months, even in subjects who had not menstruated for several years (Welt, et al., 2004).

Although the phenomenon of leptin resistance prevents the development of solely leptin based therapies for the treatment of obesity, development work has continued on leptin combinatorial treatments. Perhaps the most promising of these was until recently the combination of amylin with leptin. Amylin is a 37 amino acid peptide hormone that is secreted with insulin from the pancreatic ß-cells, and which helps to regulate glucose and energy homeostasis (Roth et al., 2008). Studies into the effects of peripheral administration of the amylin analogue pramlintide (produced by Amylin Pharmaceuticals) in obese subjects found it caused average weight loss of 8% over a 24 week period. In the same study a similar result was achieved with those people receiving metreleptin (an analogue of human leptin) treatment. However, when the two therapies were combined a significantly greater average weight loss of 12.7% was recorded, without weight loss plateauing before the end of the study (Roth et al., 2008). The exclusivity of this enhancing effect on leptin was tested by combining leptin with other known anorexigenic peptides PYY and GLP-1. The weight loss recorded with these combination therapies was not as great as observed with the amylin/leptin treatment. It has therefore been suggested by Amylin Pharmaceuticals that amylin enhances central leptin signalling that is usually diminished in the obese state (Turek, et al., 2010). However, clinical trials on the combination of pramlintide and metreleptin have very recently been abandoned at phase 2.

1.4 The central nervous system centres controlling energy homeostasis

Both long and short term signals of the body's nutritional state are integrated by the Central Nervous System (CNS). Most regions of the brain and the neurons within them in the CNS are protected from exposure to endogenous signals and potentially toxic exogenous substances by the blood brain barrier (BBB) (Norsted et al., 2008). The BBB is a specialised vascular structure that prevents diffusion of all but the smallest lipophilic molecules into the CNS (Price et al., 2008). Larger hydrophilic molecules such as peptides and proteins usually require selective transport mechanisms (Norsted et al., 2008). However, there exist regions of the CNS where the BBB is incomplete. These regions are referred to as circumventricular organs (CVO), and allow the brain to directly sample peripherally circulating signals (Price et al., 2008).

The hypothalamus and the brainstem are the two main regions of the brain responsible for the regulation of energy homeostasis, and both contain circumventricular organs – the median eminence and the area postrema respectively – allowing the sampling of circulating signals of the nutritional state (Price et al., 2008).

1.4.1 The hypothalamus

The hypothalamus is centrally located near the base of the brain below the thalamus. In humans the hypothalamus is about the size of an almond and is extensively connected to the pituitary gland (Cone et al., 2001). It functions as a link between the nervous and endocrine systems and plays a role in a number of metabolic processes. It has long been recognised as being involved in the control of appetite (Arora and Anubhuti, 2006). Initial studies concluded that the lateral hypothalamic area was the "hunger centre" and the ventromedial hypothalamus was a "satiety centre" (Suzuki et

al., 2010). It has since been discovered that a number of different hypothalamic nuclei and neuronal circuits are involved in appetite regulation (Figure 1.1).

1.4.2 The arcuate nucleus

The arcuate nucleus is located at the base of the hypothalamus and is critical to the regulation of energy homeostasis (Cone et al., 2001; Berthoud and Morrison, 2008). It contains two distinct populations of neurons which are responsive to the hormone leptin, which, as described above, acts as a peripheral signal of adipose tissue mass. Within the ventromedial section of the nucleus are located a population of neurons that express the orexigenic peptides neuropeptide Y (NPY) and agouti-related peptide (AgRP). Another neuronal population in the ventrolateral section of the nucleus express the protein pro-opiomelanocortin (POMC), a precursor of the anorectic neuropeptide alpha- melanocyte-stimulating hormone (α-MSH) (Suzuki et al., 2010).

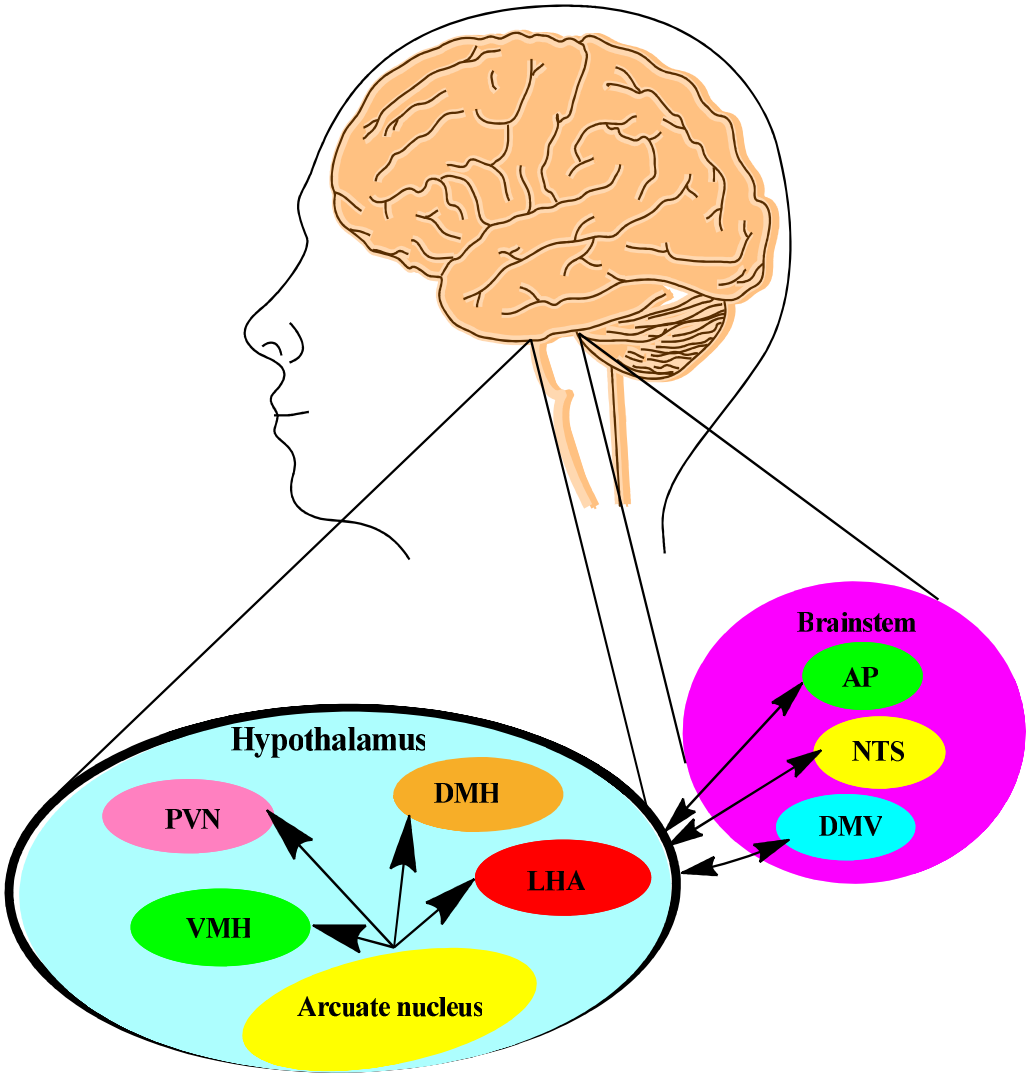


Figure 1.1 A schematic diagram showing the nuclei and regions of the brainstem involved in energy homeostasis.

A simplified representation of how different areas of the brain are linked for the regulation of appetite. Abbreviations: PVN, paraventricular nucleus; VMH, ventromedial nucleus of hypothalamus; LHA, lateral hypothalamic area; DMH, dorsomedial hypothalamic nucleus; AP, area postrema; NTS, nucleus of tractus solitarius; DMV, dorsal motor nucleus of vagus.

1.4.3 NPY

NPY is a 36 residue peptide and a member of the PP fold family of peptides which also includes Pancreatic Polypeptide (PP) and Peptide YY (PYY). All three PP fold peptides have a C-terminal amide moiety and conserved proline residues in positions

2, 5 and 8 which help to maintain their characteristic PP fold, hairpin like structure (Nygaard et al., 2006). They mediate their effects via a family of G-protein coupled receptors called the Y receptors. Thus far, five separate Y receptors have been identified; Y_1, Y_2, Y_4, Y_5 and Y_6 (Wraith et al., 2000). The existence of a Y_3 receptor has been proposed based upon binding studies but it has yet to be successfully cloned (Lee and Miller, 1998). With the exception of Y_6, found only in rat and rabbit tissue, the Y receptors are expressed in various human tissues (Wraith et al., 2000). The PP fold peptides each have different affinities for various Y receptors. NPY has high affinities for all of the Y receptors with the exception of the Y_4 receptor (Wraith et al., 2000).

Central administration of NPY into rats potently and powerfully increases food intake, and chronic administration causes prolonged hyperphagia and the development of obesity (Arora and Anubhuti, 2006). Hypothalamic NPY gene expression is higher in the leptin deficient *ob/ob* mice than in wild type controls, suggesting that leptin inhibits NPY expression (Erickson et al., 1996). In accord with this, NPY knockout *ob/ob* mice are less obese than *ob/ob* mice due to reduced food intake and increased energy expenditure (Erickson et al., 1996).

NPY knockout mice display no profound changes in feeding behaviour or body weight. It was originally thought that AgRP signalling might compensate for the lack of NPY in this model.

NPY is thought to mediate its orexigenic effects via the Y_1 and Y_5 receptors (Turnbull et al., 2002). However, studies with selective Y_1 and Y_5 receptor agonists and antagonists have thus far given contrasting results. The Y_5 receptor was believed to the most important facilitator of NPY induced feeding. Central administration of the NPY analogue [Ala^{31}, Aib^{32}]NPY, a Y_5 agonist, significantly increases food intake in

rats (Cabrele et al., 2000). However a number of studies with non-peptidic Y_5 antagonists have shown no significant reduction in food intake (Turnbull et al., 2002; Della-Zuana et al., 2004). Further to this, Y_5 receptor knock out mice display normal feeding and growth patterns, and counter-intuitively develop mild late-onset obesity. The importance of the Y_1 receptor in the orexigenic action of NPY was confirmed by the observation that Y1 knockout mice consumed less food than wild type controls, following central administration of NPY (Kanatani et al., 2000).

NPY is also implicated in a number of other physiological functions, including anxiety, memory, vasoconstriction and the regulation of alcohol consumption (Arora and Anubhuti, 2006).

1.4.4 AgRP

AgRP is a 132 amino acid peptide that was discovered in 1997 and quickly identified as a mediator of energy homeostasis. Like NPY, AgRP expression is inhibited by leptin. However, unlike NPY, which is widely expressed in the brain and peripheral tissues, AgRP expression is much more limited; within the brain, AgRP is only synthesised within the arcuate nucleus (Arora and Anubhuti, 2006).

Chronic central administration of AgRP in rats increases daily food intake and adipose tissue mass, and causes a decrease in energy expenditure independent of effects on food intake (Small et al., 2001).

Mice bred to over-express AgRP are hyperphagic and exhibit severe obesity (Ilnytska and Argyropoulos, 2008). However, like NPY knockout mice, AgRP knockout mice display no profound changes in feeding behaviour or body weight (Ilnytska and Argyropoulos, 2008). NPY/AgRP double knockouts also lack a profound energy homeostasis phenotype, suggesting that these signals are not required to compensate

for the loss of one another. However, post-embryonic ablation of AgRP neurons in mice has been shown to reduce food intake (Bewick et al., 2005). This suggests that although developmental processes can compensate for the loss of NPY and AgRP early in life, this neuronal population is vital in the physiological regulation of energy homeostasis in adult animals. However, it does not reveal the relative importance of the signalling factors expressed by these neurons.

AgRP works as a potent and selective antagonist of the melanocortin-3-receptor (MC3R) and melanocortin-4-receptor (MC4R), which inhibits the binding of the anorexigenic POMC product α-MSH to these receptors (Rossi et al., 1998). Recent evidence also suggests AgRP can act as an inverse agonist in another melanocortin-independent pathway (Arora and Anubhuti, 2006).

1.4.5 POMC

POMC is a functionally inert precursor protein that undergoes tissue specific translation to yield a number of different biologically active molecules (Pritchard et al., 2002). The POMC derived peptides, which include adrenocorticotropin (ACTH), ß-endorphin and α-, ß- and γ-melanocyte stimulating hormones (MSH), have a wide range of functions within the body that are largely mediated through melanocortin receptors (MCR) (Coll et al., 2005). Of the five identified MCR's, only the MC3R and MC4R are thought to be involved in energy homeostasis (Pritchard et al., 2002). MC4R is densely expressed in those regions of the hypothalamus known to control appetite. Mice deficient in MC4R are obese due to hyperphagia, as are mice bred to over express AgRP, the natural antagonist of MC4R (Pritchard et al., 2002). Mutations of the POMC gene in both humans and mice result in a phenotype of hyperphagia and obesity, and also cause glucocorticoid deficiency and altered

pigmentation due to the role of POMC products in the hypothalamo-pituitary-adrenal axis and in skin and hair colouration (Coll et al., 2005). Fasting down regulates, and over feeding increases hypothalamic POMC expression in rodents. This effect is believed to be partially mediated by leptin, as POMC levels are significantly decreased in *ob/ob* and *db/db* mice, and increased by leptin administration (Pritchard et al., 2002).

These results support involvement of the melanocortin system, and most importantly the MC4R in appetite regulation. In humans it has been found that mutations in MC4R are the most common monogenetic cause of human obesity, being found in up to 4% of adults with severe childhood-onset obesity (Hsiung et al., 2005).

Of the POMC translational products, α-MSH is thought to be the primary ligand for MC4R. Intracerebroventricular administration of α-MSH decreases food intake in rodents (Hsiung et al., 2005). This inhibitory effect is blocked by the administration of AgRP, further emphasising the dynamic relationship between AgRP and POMC neurons in the hypothalamus (Arora and Anubhuti, 2006).

Aside from α-MSH, desacetyl-α-MSH and ß-MSH have been shown to bind to MC4R suggesting a role for these and possibly other POMC derived peptides in energy homeostasis (Hsiung et al., 2005).

1.4.6 Other hypothalamic regions

In addition to the arcuate nucleus, a number of other hypothalamic regions play an important role in energy homeostasis. The paraventricular nucleus (PVN) is a site of convergence of number of different neuronal projections including NPY/AgRP and POMC neurons from the arcuate nucleus. The PVN integrates nutritional signals with

the regulation of the thyroid and hypothalamic-pituitary-adrenal axes (Arora and Anubhuti, 2006).

The dorsomedial hypothalamic nucleus (DMH) is thought to act as an integration centre for information sent from other hypothalamic nuclei due to its extensive network of neuronal connections (Arora and Anubhuti, 2006).

The ventromedial nucleus of hypothalamus (VMH) is also a major site via which leptin mediates its effects. Ablation of the VMH results in hyperphagia, and it is considered to be an important satiety centre (Arora and Anubhuti, 2006).

The lateral hypothalamic area (LHA) contains glucose sensitive neurons that respond to states of hypoglycaemia by inducing hyperphagia. It also expresses the orexigenic neuropeptides orexin and melanin-concentrating hormone (MCH). Ablation of the LHA results in hypophagia, and it is therefore considered a feeding centre (Arora and Anubhuti, 2006).

1.4.7 The brainstem

The brainstem is the region of the brain that connects the cerebrum to the spinal cord and is linked extensively to the hypothalamus (Neary, et al., 2004). The vagus nerve, the longest of the cranial nerves, extends from the brainstem to the neck, chest and abdomen. The brainstem receives gut-derived neural and hormonal signals via this nerve, which plays an important role in controlling individual meal size. Rats that have their connections between the brain stem and the forebrain severed are unable to compensate for decreases in meal frequency (Neary, et al., 2004).

1.4.8 The nucleus tractus solitarius (NTS)

The NTS is the site of extensive neuronal connections between the brainstem and the hypothalamus. The NTS has been found to contain dense populations of the Y receptors Y_1 and Y_5 (Arora and Anubhuti, 2006). NPY is expressed by neurons within the NTS and in rats its levels rise before and fall following a meal (Yoshihara, et al., 1996). There is also evidence of a melanocortin pathway within the NTS that is independent of that found in the arcuate nucleus. These neurons express POMC and extend into the VMH and spinal cord (Ellacott and Cone, 2004). The leptin receptor Ob-R was found to be expressed within the NTS, suggesting it is a target for leptin, and plays a role in its appetite-inhibiting effects.

1.4.9 The area postrema (AP)

The AP is a sensory circumventricular organ which allows for direct sampling of the circulation. Neurons within the AP express a number of different receptors for peptides and hormones involved in energy homeostasis. These include the Y_1, Y_2 and Y_4 receptors, the receptor for the orexigenic gastric hormone ghrelin and for the anorexigenic gut hormones GLP-1 and cholecystokinin (Price et al., 2008).

1.4.10 The dorsal motor nucleus of vagus (DMV)

The DMV is largely made up of vagal preganglionic neurons that project onto postganglionic neurons in the gastrointestinal tract. These consist of both excitatory and inhibitory pathways and exert an influence on gastric motility, gastric secretion, pancreatic secretion and liver gluconeogenesis (Cruz et al., 2007; Niedringhaus et al., 2008).

1.5 Gut hormones

The gastro-intestinal tract is the largest endocrine organ in the body and is responsible for the secretion of over 20 regulatory hormones. The physiological role of the gut is to digest and absorb nutrients. These regulatory hormones act in concert with the CNS to optimize this process. Many of these hormones have well characterised roles in the regulation of gut motility and secretion, and are known to act upon, for example, smooth muscle and the peripheral nervous system. Whereas leptin and insulin serve as long term regulators of the body's nutritional state, many of the gut derived hormones respond acutely to individual meals, and therefore act as short term regulators, conveying feelings of hunger or satiety (Figure 1.2).

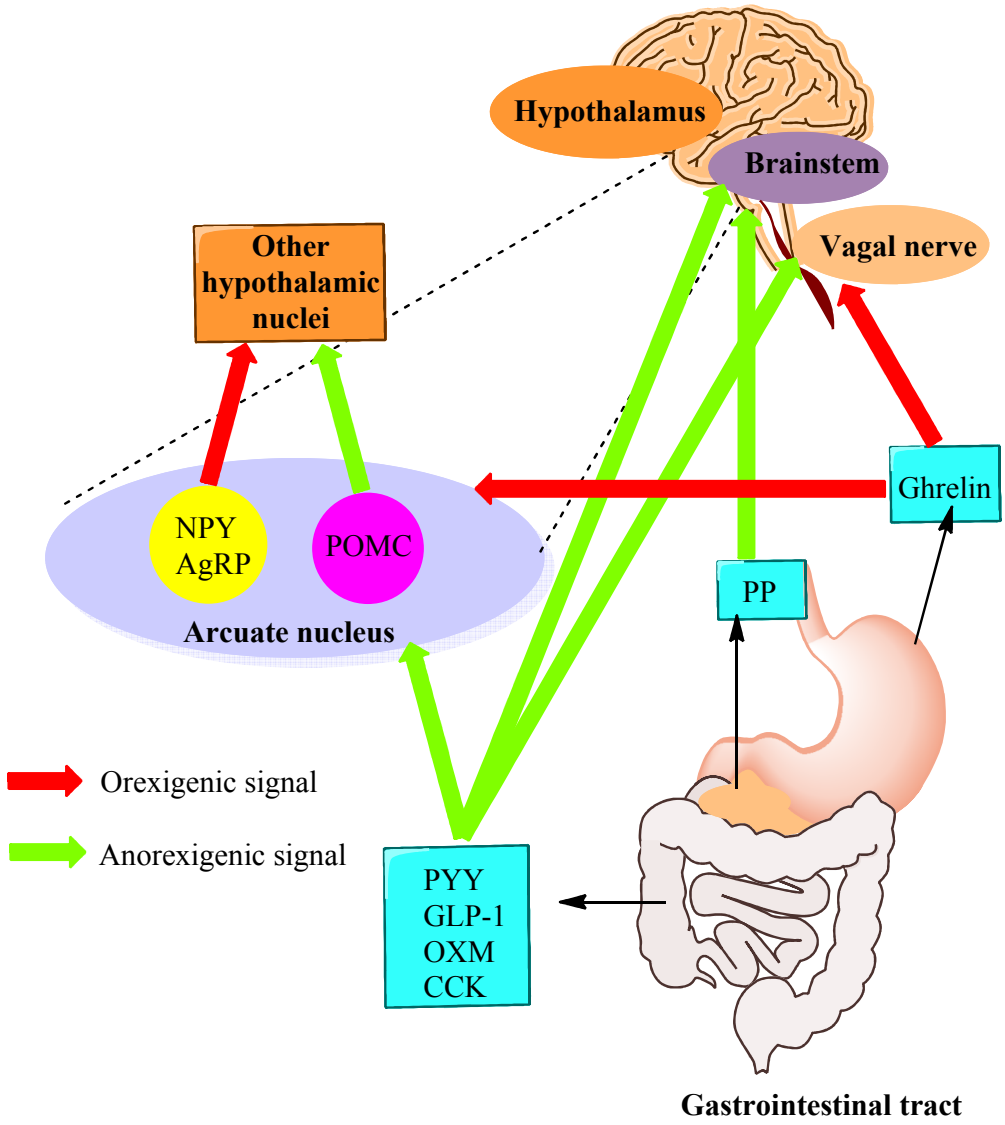


Figure 1.2 A schematic diagram showing how the different gut derived hormones influence energy homeostasis via the CNS.

Abbreviations: NPY, neuropeptide Y; AgRP, agouti-related peptide; POMC, pro-opiomelanocortin; PP, pancreatic polypeptide; PYY, peptide YY; GLP-1, glucagon-like peptide-1; OXM, oxyntomodulin; CCK, cholecystokinin.

1.5.1 Ghrelin

Ghrelin was originally isolated from rat stomach as the endogenous ligand for the growth hormone secretagogue receptor (GHS-R) (Kojima et al., 1999). It is a 28 amino acid peptide with a unique acylation of the Serine^{3} residue with an octanoyl group and which is highly conserved across species; rat and human forms differ by

just two amino acids (Chollet et al., 2009; Wren, et al., 2001). The stomach is the primary site of ghrelin secretion but other areas of the gastro-intestinal tract also contain ghrelin secreting cells. Acylation of the Serine^{3} residue occurs post translationally and is driven by the enzyme ghrelin-O-acyltransferase (GOAT) (Barnett et al., 2010). Acyl and desacyl ghrelin are both found in the circulation but the octanoyl group is required for binding to the GHS-R and may also facilitate ghrelin transfer across the blood brain barrier. Although initially discovered as a stimulator of growth hormone release, the role of ghrelin in the regulation of feeding soon became apparent (Chollet et al., 2009).

Circulating ghrelin levels were found to be raised during periods of fasting and decreased post-prandially, suggesting a role for ghrelin as an initiator of feeding (Chaudhri et al., 2006). Acute central or peripheral administration of ghrelin increased food intake in rodents. Chronic administration of ghrelin to rats was shown to significantly increase food intake and to lead to obesity (Wren, et al., 2001). Intravenous administration of ghrelin in humans was subsequently shown to increase the amount of food consumed in a free choice buffet (Wren, et al., 2001). Studies with ghrelin and GHS-R knockout mice have challenged the importance of ghrelin in the maintenance of energy homeostasis. Both strains of mice were found to display similar phenotypes to wild type mice as regards food intake, body size and growth rate. However, later studies have suggested that the loss of ghrelin signalling is associated with resistance to diet induced obesity; whether these results are due to the strain or age of the mice is unclear (Murphy and Bloom, 2006). It may be that in the absence of ghrelin, compensatory pathways develop to maintain the process of meal initiation and energy homeostasis.

Ghrelin mediates its orexigenic effects by activating NPY/AgRP neurons within the arcuate nucleus, in opposition to leptin which inhibits these neurons. While ghrelin and leptin modulate food intake via shared pathways, there appears to be no feedback by which they regulate the levels of one another (Klok et al., 2007). Ghrelin may activate NPY/AgRP neurons directly, but there is also evidence that it may work via vagal pathways.

Ghrelin levels are low in the obese and rise following weight loss, suggesting they may be regulated to limit weight gain in the obese state (Chaudhri et al., 2006). Ghrelin may rise with weight loss to preserve fat levels and thus to increase survival chances during periods of famine.

Since its discovery, the ghrelin system has been the subject of extensive research as a possible therapy for obesity. Synthetic GHS-R antagonists have been developed which reduce food intake in both lean and obese mice (Chollet et al., 2009). However, so far clinical trials of GHS-R antagonists have proven unsuccessful in the treatment of obesity. As ghrelin is the only hormone known to contain an octanoyl group, inhibition of the GOAT enzyme has emerged as another potential therapeutic avenue. Chronic peripheral administration of a GOAT antagonist to mice reduced circulating ghrelin levels and blunted the high fat diet induced weight gain observed in control animals (Barnett et al., 2010). Conversely, ghrelin and other GHS-R agonists may prove beneficial in the treatment of disorders characterised by anorexia, such as kidney disease, HIV and some cancers. Ghrelin increased food intake in cancer patients with appetite loss and malnourished dialysis patients (Neary, et al., 2004; Wynne, et al., 2005a).

1.5.2 Cholecystokinin (CCK)

Cholescystokinin (CCK) was the first gut hormone implicated in the control of appetite. As early as 1973 it was reported that exogenous administration of CCK to rats inhibited food intake (Moran, 2000). This has since been proven in other species, including humans (Wynne et al., 2004). There are a number of biologically active forms of CCK found in circulation, but all share the common C-terminal heptapeptide which contains a sulphated Tyrosine residue (Crawley and Corwin, 1994). CCK is produced by I-cells in the small intestine and released post-prandially, stimulating the release of digestive enzymes and bile, increasing intestinal motility and inhibiting gastric emptying (Chaudhri et al., 2006).

CCK mediates its effects via two distinct G-protein coupled receptors; CCK-1 and CCK-2 (Wynne et al., 2004). Both receptors are expressed in multiple sites within the brain and gut, but it is the receptors expressed on the afferent vagus nerve that are critical to the effects of CCK on feeding (Chaudhri et al., 2006). Antagonist experiments confirmed that these effects are mediated through the CCK-1 receptor. Administration of a CCK-1 receptor antagonist to rats results in a dose dependent increase in food intake, suggesting a physiological role for CCK-1 receptor signalling in food intake (Lo et al., 2010). In contrast, a CCK-2 receptor antagonist had no effect on food intake (Lo et al., 2010). Otsuka Long Evans Tokushima Fatty (OLETF) rats lack CCK-1 receptors and are hyperphagic and obese (Murphy and Bloom, 2006). However, CCK-1 receptor knockout mice display normal daily food intake, suggesting that CCK may not be crucial for controlling appetite in some species, or that the activation of developmental pathways can compensate for its loss (Moran and Kinzig, 2004). It is a matter of debate whether CCK mediates its effect on food intake through endocrine, paracrine and/or neurocrine actions (Wynne et al., 2004). This

follows reports that the physiological levels of circulating CCK are insufficient to activate the necessary vagal circuits.

The effects of CCK are short lived; when administered just 30 minutes prior to feeding, no anorectic effect was observed (Arora and Anubhuti, 2006). Continuous administration of CCK to rodents inhibited food intake for 24 hours, after which this effect ceased (Murphy and Bloom, 2006). Intermittent pre-prandial injections did reduce subsequent meal size but were compensated for by an increase in meal frequency (Murphy and Bloom, 2006). This suggests that CCK may not be a useful target for drugs to alter long term energy homeostasis.

1.5.3 Glucagon-like peptide-1 (GLP-1)

Preproglucagon is a large precursor protein produced in pancreatic α cells, the L-cells of the small intestine and within the CNS (Stanley et al., 2004). It undergoes tissue specific enzymatic cleavage to yield a number of biologically active peptides, some of which are implicated in the regulation of appetite. GLP-1 is generated by preproglucagon processing in the small intestine, and is released into the circulation after a meal (Wynne et al., 2004). A number of different forms are present in the circulation, with the most predominant being GLP-1 (7-36) amide (Murphy and Bloom, 2006). GLP-1 is highly conserved across different species, suggesting an important physiological role. The primary role of GLP-1 within the body is as an incretin; and central and peripheral administration of GLP-1 potently increases insulin secretion (Murphy and Bloom, 2006). In addition to its incretin effects, GLP-1 has also been shown to inhibit glucagon release, gastric emptying, gastrointestinal fat absorption and appetite (Arora and Anubhuti, 2006).

GLP-1 exerts these effects through the GLP-1 receptor (GLP-1R). This is a G-protein coupled receptor found in the gut and CNS (Knudsen et al., 2007).

Administration of GLP-1 (7-36) amide intracerebroventricularly or directly into the PVN inhibits food intake in rats. Chronic intracerebroventricular administration reduces weight gain in rats (Flint et al., 1998). In humans, intravenous administration of GLP-1 (7-36) amide reduces food intake in both lean and obese volunteers (Flint et al., 1998).

It has also been reported that obese humans have lower circulating levels of GLP-1 and that weight loss subsequently increases GLP-1 levels. This suggests that a reduction in GLP-1 secretion could promote obesity. However, other studies have disputed these findings (Chaudhri et al., 2006).

The discovery that obese humans were sensitive to the effects of exogenously administered GLP-1 led to hope that a GLP-1 based therapy could be developed for the treatment of metabolic disorders such as obesity and type-2 diabetes. However, GLP-1 (7-36) amide has a relatively short circulating half life of approximately two minutes (Chaudhri et al., 2006). This is predominantly due to the action of the enzyme dipeptidyl peptidase IV (DPP-IV), which cleaves the first two N-terminal amino acids from GLP-1 (7-36) amide; the resulting peptide, GLP-1 (9-36) amide, has been found to act as an antagonist, rather than an agonist for the GLP-1R (Stanley et al., 2004). The circulating half-life of GLP-1 is extended to 10 minutes in DPP-IV deficient rats (Chaudhri et al., 2006).

The development of GLP-1 based therapies has thus far followed two main avenues; the development of DPP-IV resistant analogues, and the use of DPP-IV inhibitors.

The venom of the Gila monster lizard contains a number of biologically active peptides. One of these peptides, exendin-4, has 53% sequence homology with GLP-1

(7-36) amide and is a potent GLP-1R agonist (Göke et al., 1993). It is also resistant to degradation by DPP-IV due to the nature of its N-terminal dipeptide sequence. Because of its potent incretin effects, exendin-4 or exenatide was developed as a therapy to improve glycaemic control in type-2 diabetics. Marketed as Byetta, exenatide is licensed for use in the USA and Europe as an injectable treatment for type-2 diabetes (Nachnani et al., 2010). Alongside the beneficial effects on glycaemic control, treatment with exenatide also elicits decreased food intake and moderate weight loss. However, recent studies in rats have suggested that there may be an increased risk of developing pancreatitis associated with exenatide treatment (Nachnani et al., 2010).

Liraglutide is a synthetic analogue of GLP-1 incorporating a palmitoyl functional group that aids binding of the peptide to albumin in the circulation. The resulting albumin-peptide conjugate is resistant to degradation by DPP-IV, and has a circulating half life of up to 13 hours (Cummings et al., 2010). Liraglutide, marketed as Victoza, was licensed for use in Europe in 2009 and USA in 2010 as a once daily injectable treatment for type-2 diabetes (Parks and Rosebraugh, 2010). As with exenatide, patients taking Victoza experienced moderate weight loss and a reduction in hyperglycaemia (Cummings et al., 2010). Concerns have been raised over the safety of liraglutide. An FDA review in 2010 highlighted the risk of developing cancer of the thyroid C-cells, after studies in rats and mice showed an increase in occurrence of benign adenomas and malignant carcinomas following prolonged exposure to liraglutide (Parks and Rosebraugh, 2010). Concern was also raised over the risk of developing pancreatitis as it has a similar mechanism of action to exenatide.

Other GLP-1 analogues are in clinical development, including Taspoglutide which has amino-isobutyric acid (Aib) substitutions in two amino acid positions prone to enzymatic cleavage. The bulky side chain of Aib confers steric hindrance to these positions, making the molecule resistant to enzymatic degradation whilst also stabilizing the peptide's conformation to improve receptor binding. Taspoglutide has a plasma half life of 9 hours in rats (Sebokova et al., 2010). When taken in combination with Metformin, Taspoglutide improves both glycaemic control and body weight in type 2 diabetics that were not being adequately controlled by treatment with Metformin alone (Nauck et al., 2009).

A number of different DPP-IV inhibitors are either in clinical development, or have been approved for release and are currently on the market for the treatment of type-2 diabetes. Sitagliptin and vildagliptin are the most established of those currently available (White, 2008). Although they produce the desired effects on glycaemic control, neither significantly reduces body weight (Murphy and Bloom, 2007). This may be because inhibition of DPP-IV causes only a modest increase in circulating GLP-1 levels. DPP-IV is implicated in the enzymatic degradation of a number of other biologically active peptides, which may be related to the reported side effects of DPP-IV inhibitors, which include nasopharyngitis, headaches and dizziness (White, 2008). The gut hormone PYY is cleaved to its anorectic form, PYY(3-36), by DPP-IV, so DPP-IV inhibitors may increase levels of one anorectic hormone only to reduce the levels of another, limiting effects on appetite and body weight (Mentlein, 1999).

In the light of the difficulties facing the development of peptide based therapeutics, non-peptidic GLP-1R agonists have become an attractive concept. Several different lead compounds have been developed, including 'compound 2' and 'Boc5'. These compounds are said to be allosteric ligands which bind to GLP-1R at sites distinct

from the binding site of the natural ligand (Koole et al., 2010). Allosteric ligands may act as an agonist themselves and/or enhance the natural ligand's affinity for the receptor. However, there have been no clinical trials of these molecules reported to date.

1.5.4 Oxyntomodulin (OXM)

Oxyntomodulin (OXM) is another post-translational product of preproglucagon and is released by the L-cells of the distal small intestine following food intake in proportion to the calorie content of the meal (Wynne et al., 2006). It is 37 amino acids long and contains the entire 29 amino acid sequence of glucagon with the remaining 8 amino acids extending the C-terminal end. This change in structure reduces the affinity of the molecule for the glucagon receptor and increases its affinity for the GLP-1R.

Intracerebroventricular, direct PVN or peripheral administration of OXM reduces food intake in rats, and peripheral administration reduces food intake in rats and mice (Chaudhri et al., 2006). Chronic peripheral administration of OXM in rats significantly reduced body weight gain compared to saline controls and to pair fed saline controls (Dakin et al., 2004). As the calorie intake of the pair fed and oxyntomodulin groups were very similar, this experiment suggests in addition to reducing food intake, OXM also increases energy expenditure.

Pre-prandial intravenous administration of OXM into healthy humans significantly decreased food intake by 19% at a buffet meal (Cohen et al., 2003). Repeated pre-prandial sub-cutaneous injection of OXM three times a day over 4 weeks to overweight and obese subjects was shown to produce an average weight loss of 2.4% of body weight, compared to 0.5% in a saline treated control group (Wynne, et al., 2005b). In a separate study, OXM was shown to increase energy expenditure in obese

humans, further enhancing its potential as an anti-obesity therapeutic (Wynne et al., 2006). In particular its more limited incretin effect compared to GLP-1 makes it a potential treatment for those obese subjects without type-2 diabetes.

However, the mechanisms by which OXM facilitates its anorectic effects are not yet fully understood. OXM has been shown to bind to both the GLP-1 and glucagon receptors, but with less affinity than the natural ligands. However, the anorectic effect of OXM is comparable to that of GLP-1, which suggests that it may in part act through a separate pathway (Stanley et al., 2004). Alternatively, the pharmokinetics or tissue availability of OXM may increase its anorectic activity out of proportion to its receptor affinity. Administration of the known GLP-1R antagonist exendin (9-39) directly into the arcuate nucleus reduced the anorectic effect of peripherally administered OXM but not GLP-1 (Murphy and Bloom, 2006). These results suggest that although the GLP-1R is present in the arcuate nucleus, there may also exist an as yet unidentified OXM specific receptor that is also antagonised by exendin (9-39). However, OXM does not reduce food intake in GLP-1R knockout mice (Chaudhri et al., 2006). It may be that the presence of specific receptor-associated proteins are required to mediate the differential effects of GLP-1 and OXM via the GLP-1R, perhaps similarly to the effect of receptor-activity-modifying proteins on the adrenomedullin/calcitonin gene-related peptide receptor. Downstream of the receptor involved, OXM may work in part by suppressing ghrelin secretion. OXM infusion reduced pre-prandial levels of ghrelin in rats following an overnight fast, and OXM reduces circulating ghrelin levels in rats via the hypothalamus (Patterson et al., 2009).

1.5.5 Pancreatic polypeptide (PP)

Pancreatic Polypeptide (PP) is a member of the PP fold family of peptides, which also includes NPY and PYY. PP is 36 amino acids long and is the least conserved of these peptides between species. It is produced predominantly in the endocrine pancreas and is released in response to food intake at levels proportional to the calorie content of the meal (Batterham, et al., 2003a). It functions by delaying gastric emptying, suppressing the release of ghrelin and increasing vagal tone. It is also believed to increase energy expenditure, thus helping to promote a state of negative energy balance (Chaudhri et al., 2006).

The role of PP in appetite regulation has been known for over 30 years. In 1977 it was discovered that the genetically obese *ob/ob* mice lacked the pancreatic cells needed to produce PP, and that twice daily peripheral administration of bovine PP to these mice reduced food intake and body weight gain (Stanley et al., 2004). Later studies with normal mice confirmed this potent inhibitory effect and showed that it lasted for 24 hours after administration (Wynne et al., 2004). Transgenic mice that over express PP are lean and display reduced food intake compared with wild type mice (Asakawa et al., 2003).

Intravenous administration of PP reduced ad libitum food intake two hours later by 22% in healthy human volunteers. This effect was sustained over a 24 hour period resulting in a cumulative reduction in daily food intake of 25% (Batterham, et al., 2003a).

Prader-Willi syndrome is a genetic disorder characterised by hyperphagia and obesity from an early age (Jin, 2011). Sufferers of Prader-Willi syndrome have low basal and postprandial levels of PP and intravenous infusion of PP to Prader-Willi patients significantly reduces their food intake (Balasubramaniam et al., 2006). It has been

reported that non-syndromic obese humans also have reduced circulating levels of PP, whilst anorexic subjects have elevated levels, thus suggesting PP may have a causal influence on the development of obesity (Chaudhri et al., 2006).

As a member of the PP fold family, PP binds to the G-protein-coupled Y receptors. It has been found to have greatest affinity for the Y_4 receptor (Balasubramaniam et al., 2006). Due to the nature of the N-terminal region of the PP sequence, it is unable to cross the blood brain barrier. However, the Y4 receptor is highly expressed in the area postrema of the brainstem, a circumventricular organ, and it is thus believed that PP facilitates its anorectic effect via the Y_4 receptors in this region (Kojima et al., 2007).

PP has been shown to have conflicting effects on food intake depending upon the route of administration. Central administration of PP in rats increased food intake and stimulated gastric emptying. These inimical effects may be due to different sites of receptor activation, or may reflect pharmacological levels of PP binding to the Y_5 receptor. Studies showed the orexigenic effect of central PP was blunted in Y_5 knock out mice, but not by Y_5 antisense oligonucleotides, leaving the issue unresolved (Wynne et al., 2004).

1.5.6 Peptide YY (PYY)

PYY is also a member of the PP fold family of peptides and is named for the presence of tyrosine (Y) residues at both terminals of its amino acid sequence. PYY is synthesised by intestinal L-cells and is widely expressed throughout the gastrointestinal tract, although it is most densely expressed in the distal gut (Batterham and Bloom, 2003). Following food intake, PYY is released into the circulation in proportion to the calorie content of the meal (Batterham and Bloom,

2003). It delays gastric emptying and inhibits gallbladder and pancreatic enzyme secretion.

Once it is released into the circulation, PYY undergoes rapid enzymatic cleavage by DPP-IV to yield the predominant circulating form, PYY(3-36) (Batterham and Bloom, 2003). Unlike GLP-1, PYY(3-36) retains biological activity after this enzymatic degradation. As a member of the PP fold family, PYY binds to the Y receptors. PYY(1-36) has been shown to have greatest affinity for the Y_1 and Y_5 receptors but PYY(3-36) is a selective agonist for the Y_2 receptor (Batterham and Bloom, 2003).

It was reported in 2002 that intraperitoneal administration of PYY(3-36) acutely reduced food intake in rats, and that intermittent seven day administration reduced cumulative food intake and reduced weight gain in rats (Batterham et al., 2002). A number of independent research groups originally failed to reproduce these results, with some actually recording an increase in food intake (Chaudhri et al., 2006). However, subsequently a number of these researchers have found PYY(3-36) to have an anorectic effect. It has been suggested that these discrepancies may be due to the effects of stress masking the anorectic effects of PYY(3-36) (Abbott et al., 2006). PYY knockout mice are hyperphagic and develop obesity, suggesting a physiological role for PYY in appetite (Boey et al., 2006).

In humans it was reported that intravenous administration of PYY(3-36) to healthy volunteers, 2 hours prior to being given access to a buffet lunch, reduced food intake by 36% compared with saline controls. Food intake was recorded for 24 hours after infusion and the PYY(3-36) group showed a 34% decrease in total daily food intake (Batterham et al., 2002). The Y_2 receptor mediates the effects of PYY(3-36) on food intake; Y_2 knockout mice are immune to the anorectic effects of peripheral PYY(3-

36) administration (Batterham and Bloom, 2003). It is proposed that PYY(3-36) facilitates its anorectic effect by freely crossing the blood brain barrier into the arcuate nucleus and binding to the Y_2 receptors which are highly expressed there. Direct injection of PYY(3-36) into the arcuate nucleus reduces food intake in rats (Batterham et al., 2002). The arcuate Y_2 receptors act as presynaptic inhibitors which suppress NPY/AgRP neurons, which in turn disinhibits POMC neurons and increases their activity (le Roux and Bloom, 2005). However, PYY(3-36) reduces food intake in POMC and MC4 receptor deficient mice, suggesting the melanocortin system is not necessary for the actions of PYY(3-36) (Chaudhri et al., 2006). There is also evidence that PYY(3-36) may act through vagal signalling pathways to reduce food intake. Like PP, PYY has also been shown to have opposing effects on food intake depending upon the route of administration. When administered intracerebroventricularly, PYY(3-36) increases food intake in mice. It has been suggested that this orexigenic effect is the result of pharmacological levels of PYY(3-36) binding to Y_1 and Y_5 receptors, for which PYY(3-36) retains a degree of affinity. Both Y_1 and Y_5 receptors are thought to mediate the orexigenic effects of NPY (Wynne et al., 2004).

PYY(3-36) may represent a potential therapeutic agent for the treatment of obesity. Obese humans have been reported to have lower fasting levels of PYY than lean humans, suggesting that reduced levels of PYY may be a causal factor for obesity. However, this finding has been challenged by a number of other researchers. Obese humans retain sensitivity to the effects of exogenously administered PYY(3-36). At a buffet lunch, 2 hours after receiving an intravenous infusion of PYY(3-36), the obese group had a 30% decrease in food intake, compared with 31% decrease in the lean

group. The fact that obesity did not appear to be characterised by PYY resistance suggests that PYY has utility as an anti-obesity medication (Batterham, et al., 2003b). However, there are difficulties in using PYY(3-36) as a drug to reduce appetite and body weight. The circulating half life of PYY(3-36) is very short, thus to be used as an effective drug, PYY(3-36) would need to be administered on a regular basis or in high doses. However, it has been reported that administration of supra-physiological doses of PYY(3-36) causes nausea suggesting it only has a narrow therapeutic window (Murphy and Bloom, 2006). There is also the possibility of patients developing tachyphylaxis to its effects.

A number of hormones have been shown to elicit anorectic effects. Co-administration of these hormones may result in an additive or even synergistic inhibitory effect on food intake. By combining different hormones, it may also be possible to reduce the doses of individual hormones and thus potentially reduce related side effects. Consequently, a number of groups have investigated the effects of co-administering PYY with other known anorexigenic hormones. The effect on food intake of co-administration of PYY(3-36) and GLP-1(7-36) amide was tested in both rodents and humans (Neary et al., 2005). It was reported that dual PYY(3-36) and GLP-1(7-36) amide treatment resulted in an enhanced inhibitory effect on food intake when compared to the effect of single peptide infusion in humans and in lean and obese mice. Similarly co-administration of PYY(3-36) with the long acting GLP-1R agonist exendin-4 had an increased anorectic effect compared with the individual treatments. The reduction in food intake for the combination treatment was greater than the effects of the two single treatments added together, suggesting that PYY(3-36) and GLP-1(7-36) amide may operate in a synergistic manner (Neary et al., 2005). Although PYY and GLP-1 are co-secreted by the L-cells of the small intestine, they

then act through independent mechanisms. The pathways mediating this synergistic effect are as yet unclear.

It was then hypothesised that that the combination of PYY(3-36) and PP may also show enhanced inhibition of food intake, as both act via different Y receptors. However, in human studies, volunteers treated with both PP and PYY(3-36) consumed more food than those receiving PP alone (Neary et al., 2008). In mice the combination treatment led to a statistically insignificant decrease in food intake compared to the single treatments (Neary et al., 2008).

It has been reported by Amylin Pharmaceuticals that the combination therapy of amylin and PYY(3-36) resulted in a synergistic reduction in food intake compared with treatment with either peptide alone (Roth et al., 2007). This has been proposed to be due to amylin induced signalling in the area postrema positively influencing upstream PYY(3-36) signalling in the arcuate nucleus.

The effects of PYY(3-36) on food intake are short lived. Fasting, or a reduction in body weight causes a drop in the circulating levels of leptin which elicits a subsequent increase in appetite, which may oppose any longer acting effects of PYY(3-36). It was thus suggested that co-administration of leptin might extend the anorectic effects of PYY (3-36) (Unniappan and Kieffer, 2008). This combination did prolong the anorectic effects of PYY(3-36) in ad libitum fed rats, but not in 24 hour fasted rats, suggesting that decreased circulating leptin is not the only factor behind the short lived effects of PYY(3-36).

Another possible approach to producing a successful PYY(3-36) based therapeutic would be to increase its selectivity for the anorectic Y_2 receptor over other Y receptors. The C-terminal tyrosine residue of PYY is thought to be essential for binding as an alanine scan of PYY(3-36) found that (Ala^{36})PYY(3-36) had a complete

loss of affinity for the Y_2 receptor (Pedersen et al., 2009). A number of PYY(3-36) analogues have been produced with altered aromatic side chain functionalities of the tyrosine[36] residue. Analogues with the hydroxyl group replaced by an amino or halogen functionality, retained affinity for the Y_2 receptor but had greatly increased selectivity for the Y_2 over the Y_1 receptor (Pedersen et al., 2009).

Pegylation is the process by which a polyethylene glycol (PEG) group is attached to a peptide, usually in an attempt to improve its potency as a drug within the body. The large size of the PEG group hinders enzymatic degradation and slows renal clearance (Elinav et al., 2009). The chemical nature of the PEG group greatly increases solubility and stabilises the drug over a greater pH and temperature range. PEG displays minimal toxicity and is expelled from the body intact either through the kidneys or in faeces, depending upon the size of the PEG unit. PEG is therefore approved by the FDA for use as a vehicle or base for pharmaceuticals, cosmetics and foods (Harris and Chess, 2003).

A number of studies have been conducted on pegylated PYY and Y_2 agonists. PYY(24-36)-L31 has been shown to be a selective Y_2 agonist. Studies were performed on a series of C and N-terminal pegylated cysteine analogues of PYY(24-36)-L31 (DeCarr et al., 2007). It was found that any C-terminal pegylation was detrimental to receptor binding, but that all N-terminal pegylations decreased Y_2 affinity by less than a factor of 10. In the subsequent feeding studies, PEG20-PYY(24-36)-L31 was shown to induce a greater reduction in food intake than native PYY(3-36) in mice (DeCarr et al., 2007). Another group developed a pegylated form of PYY(3-36) in which the PEG moiety was attached to the N-terminal of PYY(3-36) via an FMS linker that would be spontaneously and slowly hydrolysed under physiological conditions to yield the free peptide (Shechter et al., 2005). The group

had previously shown that irreversibly bound PEG40-PYY(3-36) was biologically inactive. Feeding studies in mice with the reversibly bound PEG40-PYY(3-36) found it had a circulating half life of 24 hours and that it resulted in a reduction in food intake comparable to that seen with continuous infusion of PYY(3-36), the effects of which only last for a few days (Shechter et al., 2005). These results support the concept of pegylation as a means of improving PYY based drug delivery.

To improve the utility of peptide based drugs, it may be necessary to change the route of entry into the body. One alternative to intravenous or subcutaneous injection is to administer the peptide orally so that it can be absorbed into the circulation in the small intestine thus mimicking its physiological release (Beglinger et al., 2008). The main drawback to this strategy is that peptides do not readily survive the acidic conditions and proteolytic enzymes found in the stomach. Novel drug formulation technology (details not supplied) were used to develop a form of PYY(3-36) suitable for oral administration. Oral administration of this molecule results in a rapid, dose dependent increase in plasma concentrations of PYY(3-36) and a suppression of ghrelin secretion (Beglinger et al., 2008). No information was supplied regarding subsequent effects on feeding behaviour, but this study demonstrates that a peptide can be successfully delivered into the circulation via oral administration.

Another possible route of entry for peptide drugs is through the nasal mucosa, which acts as an absorptive surface for the transfer of peptides into the circulation. Nasal administration can also potentially facilitate direct transfer into the CNS. An intranasal formulation of PYY(3-36) was used in a 12 week study in obese subjects (Gantz et al., 2007). The study showed no significant weight loss at low doses and a high drop out rate at high doses caused by intolerance of the adverse events including nausea and vomiting. It is possible that the failure to promote weight loss at low doses

was the result of PYY(3-36) binding to central Y_5 receptors following the theoretical transfer of PYY(3-36) directly into the CNS via the nasal mucosa. It may also be that the sharp but short-lived rise in circulating PYY(3-36) was insufficient to chronically reduce appetite and body weight.

1.6 Gut hormones as obesity therapeutics

Current anti-obesity therapies are insufficient to stem the obesity epidemic. There is thus much academic and industrial research into potential anti-obesity agents. Gut hormone systems offer a promising pharmaceutical target. As these hormones are released daily, it seems less likely that a patient would develop tachyphylaxis to their effects. They may also be less likely to cause side effects. Gut hormones are endogenous peripheral factors evolutionarily "designed" to influence central appetite-control circuits. A drug based on gut hormones would therefore target specific neuronal pathways and may therefore be less likely to cause side effects. In contrast, agents such as Sibutramine have sympatho-mimetic side-effects because of their non-specific targeting of neurotransmitter systems.

Using a small molecule agonist for a gut hormone receptor would have advantages over the use of the peptide hormone itself. Such molecules are more likely to be orally active and so could be used in tablet form as opposed to requiring injection. However, designing small molecules so that they will act as agonists for gut hormone receptors is inherently difficult. The receptors often have large interaction sites and the amino acids important for receptor binding in the peptide can be dispersed throughout the sequence (Murphy and Bloom, 2007).

It therefore seems logical to modify the hormone molecule itself, rather than trying to design a small molecule mimetic. Several modified peptide hormones are in widespread clinical use in injectable form e.g. modified forms of vasopressin for

diabetes insipidus, and of somatostatin for neuroendocrine tumours. Such modified hormones are synthetically produced using chemical techniques.

1.7 Peptide chemistry

Natural peptides can be extracted from tissue and in the past, many peptides were characterised and obtained for experimental and even clinical use in this manner. Subsequent developments in peptide chemistry meant that peptides needed for experimental work could be made by chemical means; in particular, this was the only way of obtaining unnatural sequences. In recent years, the development of recombinant DNA technology has provided the option of producing natural peptides and proteins of a hundred amino acids or more using biological methods. However, chemical synthesis is still the most important and widely used method for producing peptides, in particular those without more complex secondary structures.

Peptide chemistry can be said to date back to the beginning of the 20th Century with the synthesis of the first peptide bond by Fisher. For the first half of the century peptides were synthesised using classical organic chemistry methods. This strategy is now referred to as solution phase peptide synthesis as all of the reagents are held within the liquid phase. Coupling reactions are generally performed in an organic solvent. Upon completion of the reaction, the desired compound is separated from unused reagents and by-products via a series of extractions and phase separations with different organic solvents and aqueous solutions. The process is cumbersome, time consuming and requires a high level of skill. The synthesis of simple peptides can still take many days to complete, whilst longer sequences can take months due to the need to perform multiple purification steps on intermediate products. However, solution

phase synthesis methods are still applied commercially in the large scale production of certain peptides.

In 1963 the biochemist Bruce Merrifield reported a new method for the synthesis of peptides that he named Solid Phase Peptide Synthesis (SPPS) (Merrifield, 1963). His approach was to build a peptide chain in a stepwise process whilst it was anchored at the carboxyl terminus via a covalent bond to an insoluble solid support. Merrifield initially reported the synthesis of a model tetrapeptide (H-Leu-Ala-Gly-Val-OH) which was identical to a sample produced using the classical solution method. He later reported the successful solid phase synthesis of bradykinin that had full biological activity (Merrifield, 1964). Crucially Merrifield reported a production time of 8 days and a final yield of 68%.

Merrifield's method was initially met with widespread scepticism within the scientific community but in time became accepted as the most efficient way to synthesise peptides, in particular those with long sequences.

The method involved the use of an insoluble but porous polymer resin onto which the peptide is anchored through the carboxyl group of the C-terminal amino acid. The peptide is then built up via the coupling of the subsequent residue to the free α-amino group of this amino acid. The α-amino group of the new amino acid is masked with a temporary protecting group which is not affected by the conditions of the coupling reaction, but which can subsequently be selectively removed to allow addition of the next residue in the sequence.

Any amino acid containing a reactive side chain needs to have an additional protecting group present. This needs to be stable not only during the coupling reaction but also during the reaction to remove the temporary α-amino protecting group. Once

the peptide has been fully assembled, the side chain protecting groups are removed and the peptide simultaneously cleaved from the resin (Figure 1.3)

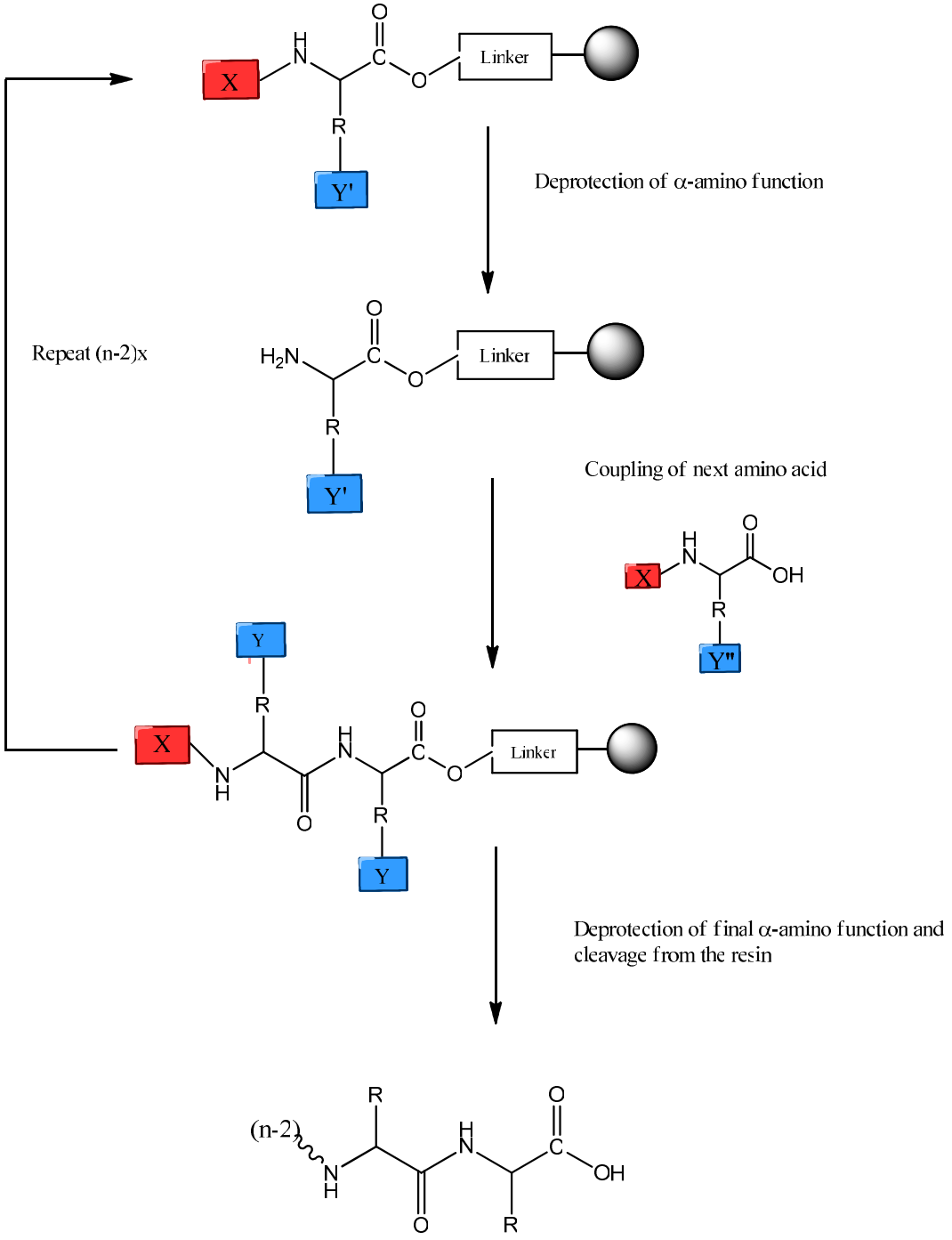


Figure 1.3 The mechanism behind the principle of solid phase peptide synthesis (SPPS)

X = temporary α-amino protecting group; R = amino acid side chain; Y = temporary side chain protecting group; n = number of amino acids in the peptide sequence.

The main advantages of this method are that the peptide can easily be separated from all soluble reagents by simple filtration instead of time consuming extractions and separations. Washes can be performed quickly and easily, and excesses of reagents can be incorporated to speed reactions up, as they can also be easily removed at the end of the synthetic process. As the peptide is anchored to the solid support, loss of peptide is greatly reduced. Finally, Merrifield was quick to realise the potential for his methods to be amendable to automated synthesis (Merrifield, 1963).

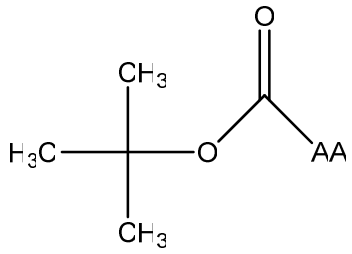
There are some drawbacks to this method. Incomplete couplings and incompletely blocked side reactions cause undesired products to accumulate on the resin. These affect the purity and yield of the resulting crude peptide, and have to be removed using one or more of the available purification methods.

The basic principles of Merrifield's method remain at the core of modern day solid phase peptide synthesis, but it has evolved over 40 years and now exists in two distinct forms.

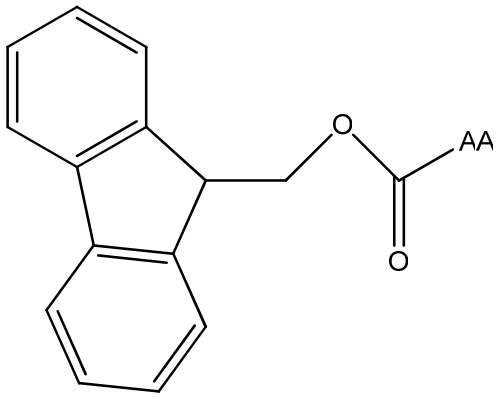
1.7.1 Boc/Bzl (Merrifield) SPPS

This version of SPPS is very similar to the original method pioneered by Merrifield and works on the principle of increasing acidity for the removal of the α-amino protecting group, and for release of the final peptide and removal of the side chain protecting groups. The tertiary butoxycarbonyl (Boc) group (Figure 1.4) is used to protect the α-amino group of the amino acid residues and is removed by treatment with trifluoroacetic acid (TFA). This imparts a positive charge on the exposed α-amino group and a neutralisation step is therefore required following each deprotection. A variety of TFA stable, benzyl based compounds are used to protect the reactive amino acid side chain groups. Final peptide release is brought about with

the very strong acid, anhydrous hydrogen fluoride (HF). However, HF is highly toxic and its use requires skill. Glass is not stable to HF and specialised HF resistant equipment is therefore required. Another drawback with Boc SPPS is the effect that repeated exposure to harsh acidic conditions has on the resin bound peptide. Certain peptide bonds are more sensitive than others to low pH and a number of by products are often produced following Boc SPPS of some peptides. In addition, the principle of Boc SPPS is reliant on the selectivity of the acidolysis reactions. However, these reactions were not always precisely selective, resulting in the accumulation of impurities on the resin, or loss of product due to premature cleavage of the peptide-resin linker. As soon as these limitations were recognised, work began on devising alternative methods of SPPS.



tertiary butoxycarbonyl (Boc)



9-fluorenylmethyloxycarbonyl (Fmoc)

Figure 1.4 The chemical structures of the Boc and Fmoc α-amino protecting groups.

AA = position at which the protecting group attaches to the amino acid.

1.7.2 Fmoc/tBu SPPS

In 1970 Louis Carpino reported the development of a new amino protecting group, 9-fluorenylmethyloxycarbonyl (Fmoc) that was completely stable to acidic conditions but was readily cleaved in mild basic conditions (Carpino and Han, 1972). His study reported the successful synthesis and subsequent deprotection of a number of Fmoc protected amino acids and also the synthesis of some Fmoc dipeptides using solution phase methods.

It was not until 1978 that two independent research groups reported the successful application of Fmoc protected amino acids in solid phase peptide synthesis (Meienhofer et al., 1979; Atherton et al., 1978). Both employed an orthogonal protecting strategy whereby reactive amino acid side chain groups were protected with tertiary butyl based structures that were acid labile. Removal of the Fmoc group was performed using the secondary amine piperidine, a basic substance in the presence of which the other protecting groups and the resin linker were completely stable.

Final removal of the peptide from the resin together with the remaining protecting groups was facilitated by treatment with TFA. The use of a less harsh acid than that used with the Merrifield method meant that the cleavage reaction could be performed using standard laboratory glassware.

In the thirty years that followed, Fmoc SPPS grew in popularity and, aided by a decrease in the cost of commercially available Fmoc protected amino acids, is now the synthesis method of choice in the majority of peptide laboratories. In 1992 the Association of Biomolecular Core Facilities (ABRF) conducted a study in which they asked their associate peptide facilities to synthesise the same peptide using their individual method of choice and submit a crude sample for analysis (Smith et al.,

1992). Of the samples submitted exactly half were prepared by Boc SPPS and the other half by Fmoc SPPS. Although it was feasible to synthesise the sequence in question by either method, on average those facilities conducting Fmoc SPPS achieved greater success than those using Boc SPPS. All of the Fmoc crude samples contained at least 25% of the desired product, but only 56% of the Boc samples did.

All of the peptides in the studies described in this thesis are synthesised using the Fmoc strategy, and all subsequent peptide chemistry information will thus relate to this strategy.

1.7.3 Resins

Polystyrene based resins cross linked with 1 – 2% divinylbenzene are still the most commonly employed solid supports in SPPS. It was on this same structure that Merrifield conducted his first successful synthesis of the tetrapeptide H-Leu-Ala-Gly-Val-OH (Merrifield, 1963). The nature of the polymer matrix is such that 99% of the coupling sites on each resin bead lie within the bead and not on the surface (Amblard et al., 2006). Therefore to achieve optimum permeability for reagents to access the growing peptide chain, as well as accommodating the increasing size of the peptide, the resin has to sufficiently swell in the solvents in which reactions are performed. Polystyrene based resins display a good degree of swelling in both dimethylformamide (DMF) and particularly dichloromethane (DCM), but as the common peptide synthesis reagents are more soluble in polar solvents it is DMF that has become the primary solvent in Fmoc SPPS. DCM is still utilised in specific reactions and also to perform certain wash cycles. Alcohols and aqueous solutions have been found to be inadequate for most SPPS procedures as they lack the desired resin swelling properties (Amblard et al., 2006). In the presence of alcohols,

polystyrene based resins shrink and this property is now utilised as a means of adequately washing away unused reagents by alternating a swelling wash with a shrinking wash. The most commonly used alcohol for this is iso-propanol.

In later years, alternative polymer supports were developed. The most commonly used was a polystyrene–polyethylene glycol (PEG) hybrid whereby PEG molecules were grafted onto the polystyrene matrix. PEG imparts a hydrophilic character to the resin support which increases its swelling properties across a wider range of solvents. This, together with a decrease in the loading capacity, make PEG based hybrid resins useful for the synthesis of long and difficult sequences (Novabiochem, 2010). Disadvantages of using these resins include their high cost and the propensity for PEG leaching to occur during cleavage owing to the lability of the bond between the PEG and polystyrene molecules. Recently, a number of different pure PEG based resins have become commercially available that claim to overcome these disadvantages (Novabiochem, 2010).

The peptide is attached to the polymeric support through a linker molecule. The nature of the linker determines both the required final cleavage conditions and also the functionality of the C-terminal residue. The two most common C-terminal functional groups are the free acid (COOH) and amide ($CONH_2$). In 1973 Wang devised a TFA labile resin for the production of protected peptides in Boc SPPS (Wang, 1973). This polystyrene based resin has a *p*-alkoxybenzyl alcohol linker and is now the standard resin in many peptide laboratories for the production of peptide acids in Fmoc SPPS. Other commonly used resins for the production of peptide acids are 2-chlorotrityl chloride resin and Super Acid Sensitive Resin (SASRIN). The linker group in both only requires a low concentration of TFA to be cleaved, so these resins are used for the production of protected peptide acids whereby the peptide is released

from the resin, but all amino acid and N-terminal protecting groups are retained. This approach is necessary when reactions with the C-terminal carboxylic acid group are required.

The most frequently used resins for producing peptide amides are Rink amide resin, Ramage amide resin and 4-Alkoxy-2,6-dimethoxybenzylamine (PAL) resin. Sieber developed an acid sensitive, peptide amide yielding resin that can be used for the production of protected peptide amides (Figure 1.5)

For peptide acids and amides there exist a number of commercially available PEG based resins that carry these same linkers. These include TentaGel resins supplied by Rapp Polymere (Tübingen, Germany) and NovaSyn resins supplied by Novabiochem (Nottingham, UK).

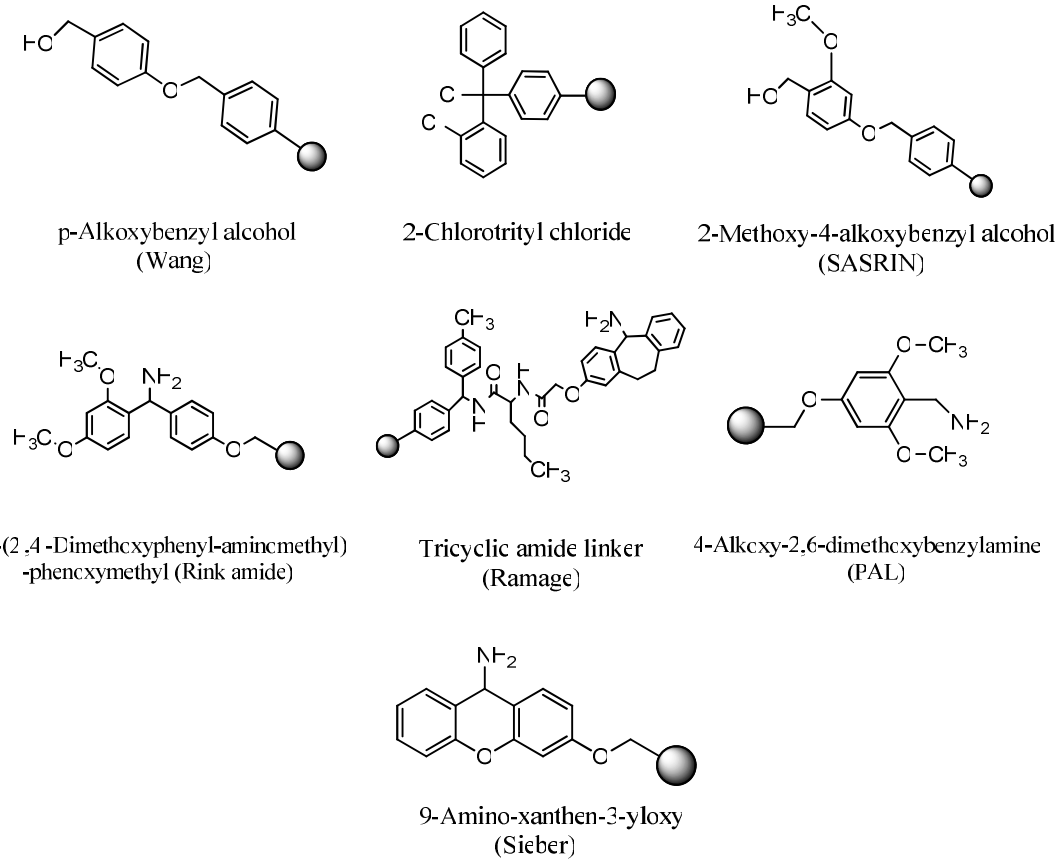


Figure 1.5 The chemical structures of the most commonly used resin linker compounds for the synthesis of peptide acids and amides.

Peptides can be produced with a number of other C-terminal functionalities such as alcohols, aldehydes and alkyl esters. There are specialised resins for the production of these peptides that are often compatible with standard TFA cleavage methods. Alternatively, 2-chlorotrityl chloride and SASRIN resins can be used and specific cleavage conditions applied to produce the desired functionality (Mergler, 1999).

The attachment of the first amino acid to the resin is carried out under a variety of conditions dependent upon the nature of the resin. For the production of a peptide amide, attachment to the resin of the first residue uses the same conditions as for all subsequent amino acid couplings. Thus there is no need to perform a separate resin loading reaction and the base resin can be purchased in bulk and used directly in an

automated process. When a peptide acid is required, loading of the 2-Chlorotrityl resin is a relatively straightforward procedure involving the use of an excess of amino acid in solution in the presence of a base and mixing for up to 4 hours. For the alkoxybenzyl based resins such as Wang resin, attachment of the first amino acid is a more problematic and inconsistent procedure (Chan and White, 2000). It is necessary to form an activated species of the amino acid prior to loading. The activation procedure utilised serves to increase the risk of epimerization. This is a particularly problem with the amino acids histidine (His) and cysteine (Cys). With peptides that contain a C-terminal His or Cys residue, many laboratories will choose to use a 2-Chlorotrityl based resin as the epimerizations levels are much lower than with alkoxybenzyl based resins. Due to the specificity of the loading reaction and the problems associated with it, many choose not to load the resin themselves and instead purchase alkoxybenzyl based resins that are pre-loaded with the relevant Fmoc amino acids.

1.7.4 Fmoc deprotection

The most common method for removing the Fmoc group from a resin bound peptide is to wash the resin with a solution of 20-50% piperidine in DMF. Piperidine deprotonates the fluorene ring of Fmoc, which leads to removal of the molecule from the peptide as dibenzofulvene and carbon dioxide. The dibenzofulvene intermediate is then scavenged by piperidine to form an adduct that strongly absorbs UV radiation (Figure 1.6).

This property enables the monitoring of Fmoc deprotection reactions by certain automated peptide synthesisers which have built in UV monitoring systems. This measures the UV absorbance of a sample of the deprotection solution during the

deprotection reaction. If the UV absorbance reading is above a preset level, the assumption is made that this is due to the presence in the solution of the dibenzofulvene-piperidine adduct, and thus the deprotection reaction is not yet complete. The reaction is then repeated until the UV absorbance reading falls below the preset level and the deprotection is considered complete. In comparison, those conducting manual syntheses, or who do not have access to such synthesisers, at least two consecutive washes, of between 2 and 30 minutes, are performed as standard to ensure effective deprotection.

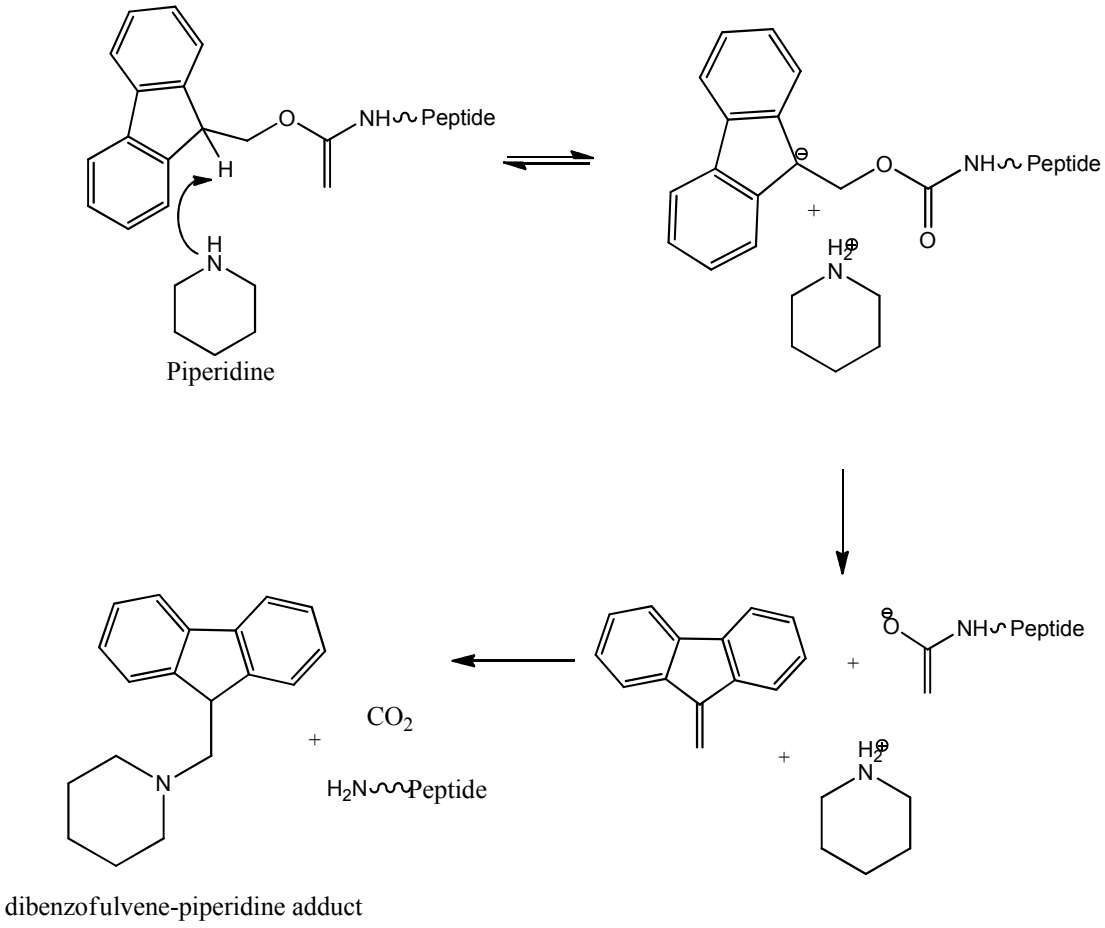


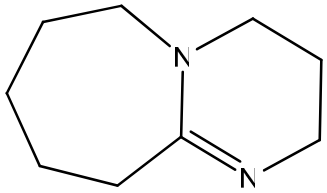
Figure 1.6 The reaction mechanism of Fmoc group removal by piperidine.

There are a variety of chemical tests that can be conducted on a sample of resin to help the chemist determine whether the Fmoc deprotection reaction has been

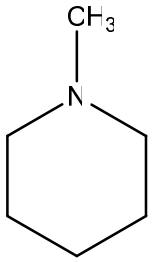
successful. The most commonly used of these is the Kaiser test. This involves washing a sample of resin in iso-propanol and then adding three drops each of ninhydrin in butan-1-ol, phenol in butan-1-ol and pyridine. The sample is then heated to 100°C for 5 minutes; the resin beads turn blue if free α-amino groups are present. The colour results from the formation of the chromophore molecule Ruhemann's Purple (Bottom et al., 1978). As the chromophore is formed by the reaction of ninhydrin with primary amines, a blue colour is not seen when the amino acid being tested is proline. As proline is a secondary amine, an orange/brown colour is usually recorded. The resin beads will not change colour in the event of a negative result. This same test can also be applied to the monitoring of the subsequent coupling reaction.

For some long and difficult peptides, piperidine has been shown to be ineffective in completely removing the Fmoc group, even when longer deprotection times and higher piperidine concentrations are used. In these cases it is often beneficial to use a stronger base such as 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (Figure 1.7), which has been shown to improve the synthesis of, for example, amyloid β protein (1-42) which is known to be a difficult sequence to synthesise (Tickler et al., 2001).

In contrast, certain sequences can contain amino acids that are sensitive to piperidine and as such require the use of a milder base such as n-methylpiperidine (Figure 1.7).



1,8-Diazabicyclo[5.4.0]undec-7-ene
(DBU)



N-Methylpiperidine

Figure 1.7 Chemical structures of alternative basic substances to piperidine for the removal of Fmoc groups.

There are a number of different side reactions that can occur during Fmoc deprotection. Certain C-terminal dipeptide sequences are susceptible to base catalysed diketopiperazine formation (Chan and White, 2000). In this process the N-terminal amino group of the penultimate amino acid cleaves the resin ester linkage, releasing a cyclic dipeptide and leading to a loss of yield. The reaction is more likely to occur when the dipeptide contains proline (Pro) and glycine (Gly) residues and the resin is alkoxybenzyl based. It is therefore recommended that trityl based resins are used for C-terminal proline and glycine peptides, as the bulkier trityl group hinders diketopiperazine formation. Alternatively, the second and third amino acids can be coupled together as a dipeptide.

In basic conditions aspartic acid (Asp) residues are prone to aspartimide formation. This is a sequence dependent reaction, with certain Asp-xxx sequences more prone than others. The problem occurs when the α nitrogen of the xxx residue reacts with the protected Asp side chain group in a base catalysed reaction. This forms the cyclic aspartimide compound and leads to subsequent loss of the aspartic acid protecting group. Aspartimides readily undergo ring opening reactions that subsequently produce

a number of different products, most commonly the α and β aspartyl isomers and, in the presence of piperidine, the α and β piperidide adducts (Mergler, et al., 2003b) (Figure 1.8). The most likely xxx residues to facilitate aspartimide formation are glycine, aspartic acid, asparagine and serine (Mergler, et al., 2003a). The use of strong bases such as DBU exacerbate aspartimide formation, as does prolonged exposure to basic conditions, meaning that longer sequences are more susceptible to higher levels of aspartimide derived impurities.

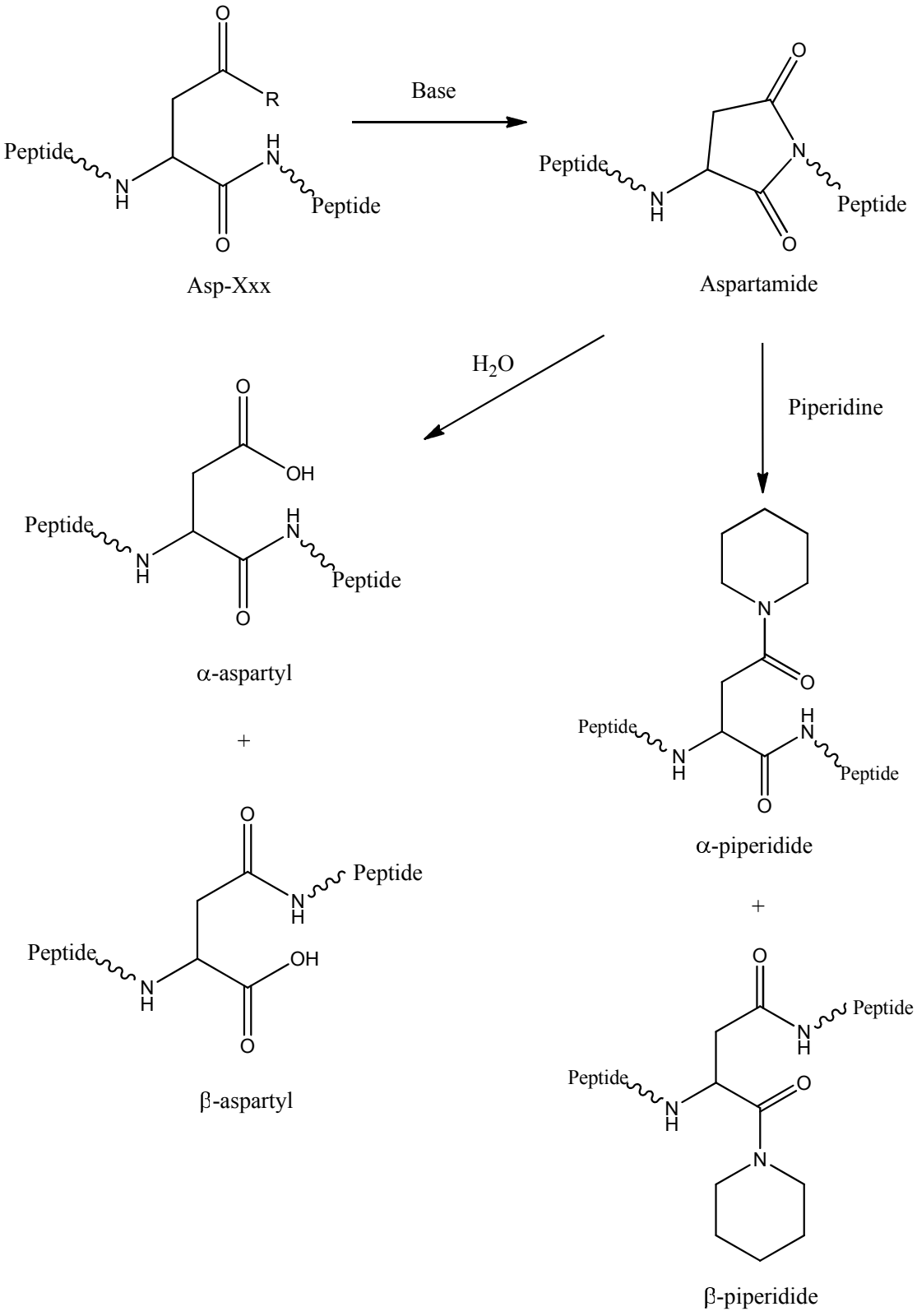
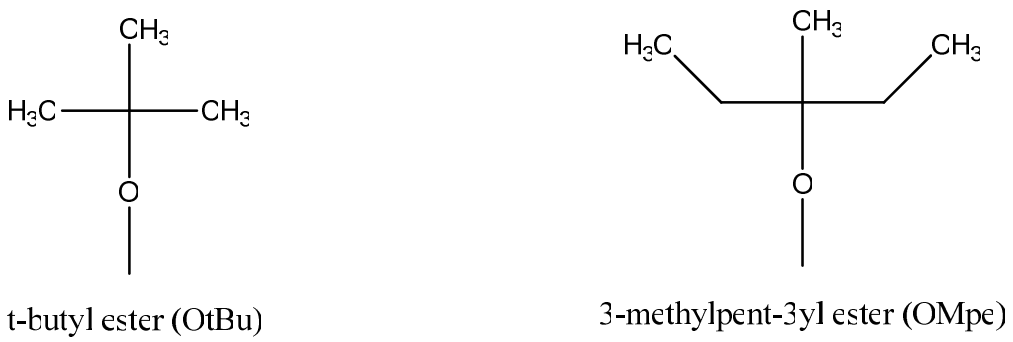


Figure 1.8 The reaction mechanisms behind aspartimide formation and the subsequent conversion to aspartyl and piperidide impurities.

Abbreviation: Asp, aspartic acid.

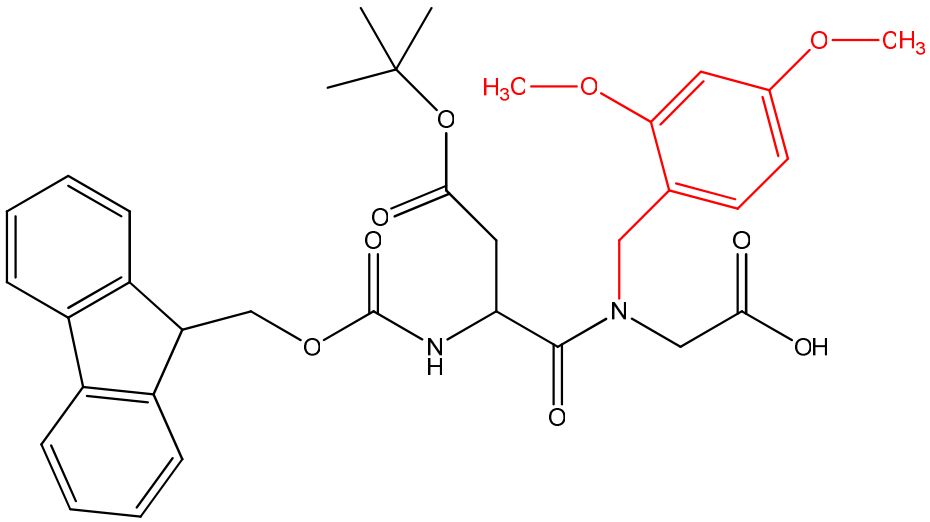
There are a number of different strategies to overcome aspartimide formation. One is to use an alternative aspartic acid side chain protecting group other than the standard t-butyl ester (OtBu) group. Studies have demonstrated that employing the bulkier 3-methylpent-3yl ester (OMpe) protecting group greatly reduced aspartimide formation when compared with t-butyl (Mergler, et al., 2003a) (Figure 1.9). The only drawback to this is the relatively high cost of this Fmoc derivative compared to the standard derivative.



t-butyl ester (OtBu) 3-methylpent-3yl ester (OMpe)

Figure 1.9 The chemical structures of two aspartic acid protecting groups

As stated above, Asp–Gly sequences are particularly prone to aspartimide formation, but using the dipeptide building block Fmoc-Asp(OtBu)-(Dmb)Gly-OH greatly reduces aspartimide formation in Asp-Gly containing sequences. It contains the group, 2,4-dimethoxybenzyl (Dmb) on the α-amino group of the glycine residue (Figure 1.10). Its presence hinders aspartimide formation due to its bulky nature; it is stable to Fmoc deprotection and amino acid coupling conditions and is completely removed during TFA cleavage. Again, the cost of purchasing this derivative is high, thus preventing its routine use by all in the field.



Fmoc-Asp(OtBu)-(Dmb)Gly-OH

Figure 1.10 The chemical structure of a dipeptide for use in the reduction of aspartimide formation in Asp-Gly containing sequences

Abbreviations: Fmoc, 9-fluorenylmethyloxycarbonyl; Asp, aspartic acid; Dmb, 2,4-dimethoxybenzyl; Gly, glycine.

Finally, specific additives in the deprotection solution can also help to suppress aspartimide formation. The addition of 1-hydroxybenzotriazole (HOBt) has been reported to be particularly effective in this role.

1.7.5 Coupling

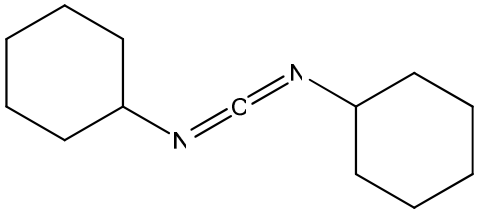
A solid phase peptide coupling reaction involves the reaction of the resin bound α-amino group with the carboxylic acid of the next Fmoc protected amino acid in the sequence. A molar excess of amino acid, relative to the scale of the synthesis, can be used to drive the reaction to completion, as any unused reagent is subsequently washed away. In classic organic chemistry, it has long been established that amide bond formation requires activation of the carboxylic acid group to drive the reaction. However, the commonly used activating methods in organic chemistry are deemed

too harsh for peptide synthesis, due to the generation of over activated intermediate products that can facilitate multiple side reactions (Chan and White, 2000). A number of milder activation strategies have thus been devised specifically for use in peptide synthesis. In the initial SPPS of his tetrapeptide, Merrifield employed N, N'-dicyclohexylcarbodiimide (DCC) as an activating agent, forming the symmetric anhydride of the protected amino acid (Merrifield, 1963). This method of activation is still widely used today, but has its limitations. Two molar equivalents of the protected amino acid and one equivalent of DCC are needed to generate one equivalent of the symmetric anhydride (Pennington and Dunn, 1994). It is best to form the anhydride 20-30 minutes prior to it being added to the peptide bound resin, which increases the synthesis time per amino acid cycle. Activation proceeds more rapidly in the apolar solvent DCM as opposed to the polar solvent DMF. However, some reagents are insoluble in DCM, meaning that a compromise has to be reached whereby a certain concentration of DMF is present in the activation reaction. A by product of the activation process is dicyclohexylurea, and although this is inert during the coupling reaction, it is also insoluble. This usually requires that the reaction mixture be filtered prior to being added to the resin (Pennington and Dunn, 1994). This problem can be overcome by the use of other carbodiimides such as diisopropylcarbodiimide (DIC), which produces a completely soluble urea.

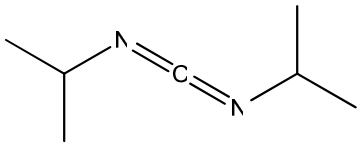
Carbodiimides can also be used in combination with the triazole based compounds HOBt and 1-hydroxy-7-azabenzotriazole (HOAt) to activate amino acids. In this process the OBt or OAt active ester form of the amino acid is produced. This form of activated amino acid has been reported to improve coupling reactions by reducing the amount of racemization that occurs (Pennington and Dunn, 1994). Activation proceeds smoothly in both DCM and DMF, and as with the symmetrical anhydride

method, is best occurring 20-30 minutes prior to addition to the resin. If DIC is the carbodiimide species being used then there is no risk of precipitate forming during activation. Several automated peptide synthesiser models are thus able to perform HOBt/DIC mediated couplings.

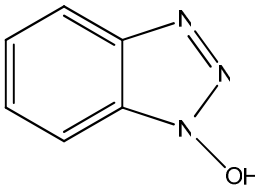
As solid phase peptide synthesis evolved, so a new class of coupling reagents became available. The aminium and phosphonium class of coupling reagents were first reported in the 1970's and have now become popular in many peptide laboratories. The main advantage with these reagents is that the activation reaction can take place *in situ* with no need for separate pre-activation. This speeds up the coupling cycle, with reactions generally complete within 30 minutes of activation. These reagents all share some degree of structural homology with HOBt, but also contain either an aminium or phosphonium salt which in the presence of a tertiary base such as N,N-diisopropylethylamine (DIPEA) will convert a protected amino acid to its OXt ester. The most commonly used reagents within this class are Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP), 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HBTU) and 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate (TBTU), all of which generate OBt active esters. All are widely available, readily soluble in DMF and stable in solution for a number of days, making them compatible with automated synthesisers. Reagents that generate the more reactive OAt ester are also available. The most effective of these is O-(7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU), but its high cost prevents its widespread use as a standard coupling reagent (Albericio, F. and, 1997). Instead, it is utilised for specific coupling reactions that are known to be difficult (Figure 1.11).



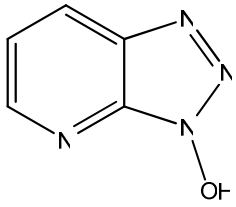
Dicyclohexylcarbodiimide (DCC)



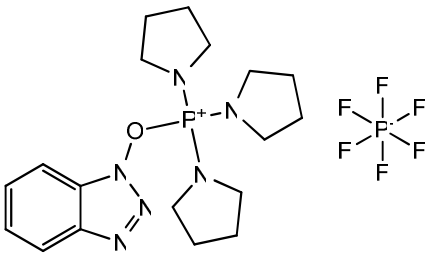
Diisopropylcarbodiimide (DIC)



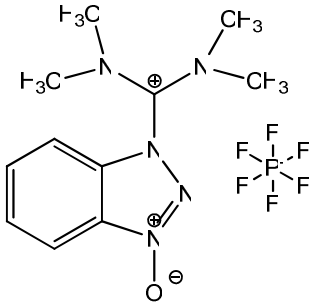
1-Hydroxybenzotriazole (HOBt)



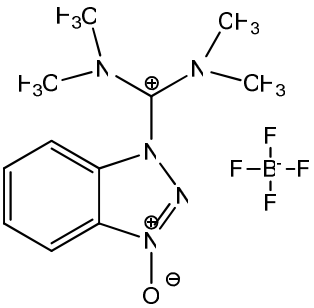
1-Hydroxy-7-azabenzotriazole (HOAt)



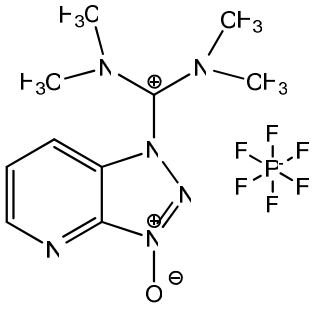
Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP)



2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HBTU)



2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethylaminiumtetrafluoroborate (TBTU)



O-(7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU).

Figure 1.11 The chemical structures of the most commonly used amino acid activating agents

With the exception of PyBOP, all of these reagents are capable of reacting with amino groups, meaning that a potential side reaction during their use is N-terminal guanidation of the peptide chain (Gausepohl et al., 1992). To prevent this, a slightly lower molar excess of coupling reagent compared with protected amino acid is employed. In theory this ensures that all of the coupling reagent will be exhausted during the activation process.

Aminium and phosphonium reagents are reported to increase the propensity for certain amino acids to racemize during coupling. The most susceptible residues are cysteine and histidine (Chan and White, 2000). In the case of cysteine, many recommend the use of carbodiimides for activation as opposed to aminium and phosphonium reagents. An alternative option is to include HOBt in the activation reaction as this can help to suppress the racemization of susceptible amino acids.

1.7.6 Difficulties in peptide synthesis

During elongation the peptide chain can form secondary structures such as β sheets, or can aggregate with other peptide chains or with the polymer support on which they are anchored (Mutter, 1985). These interactions can lead to difficulties in the α-amino deprotection and/or coupling reactions. These problems are entirely sequence dependent, but are most commonly experienced with sequences containing clusters of hydrophobic amino acids. In the most extreme cases, aggregation is such that the resin bed is seen to shrink in size, making further couplings very difficult (Chan and White, 2000).

A number of different techniques have been developed to help overcome these difficulties. The simplest technique is to cap any unreacted amine groups at the end of each coupling step with a small reactive group such as acetyl. This will prevent the

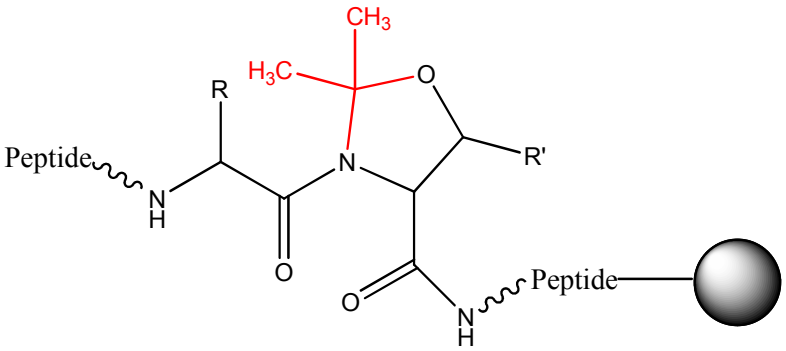
generation of single amino acid deletion impurities. Another method is to use a PEG based resin instead of the standard polystyrene based support. The benefits of this are twofold. Firstly, the presence of the PEG group will increase the swelling properties of the resin, thus making the α-amino group readily accessible. Secondly, PEG based resins generally have lower loading values than the standard resins, and individual peptide chains are therefore spaced further apart and are less prone to intermolecular interactions.

Other techniques that can be employed are to use a more effective activating reagent such as HATU and to increase coupling times. In both cases this can also lead to an increase in undesirable side reactions occurring.

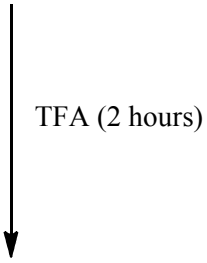
In recent years a number of "secondary amino acid surrogates" have been developed to improve the synthesis of difficult peptides by suppressing aggregation. Insertion of the temporary protecting groups, 2-hydroxy-4-methoxybenzyl (Hmb) and Dmb, onto the backbone of the peptide chain helps to disrupt secondary structure formation, due to their bulky nature (Novabiochem, 2010). The protecting groups are attached to the α-nitrogen of an Fmoc protected amino acid and can be incorporated as a single residue or within a dipeptide. In the case of the single residues, coupling times have been found to be sluggish. The Hmb/Dmb group is released from the peptide chain during the final cleavage reaction, leaving behind the native peptide sequence.

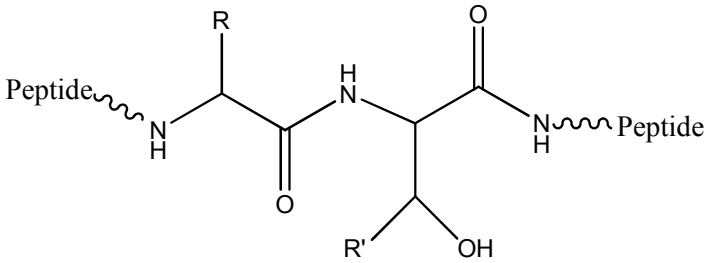
A peptide sequence rich in proline residues is naturally resistant to aggregation due to the rigidity conferred on the peptide chain by the cyclic structure of proline. In 1992, Mutter developed pseudoproline dipeptides which are held in a temporary oxazolidine "proline like" structure and so can be incorporated into peptide sequences containing little or no proline residues to confer the same aggregation disrupting properties

(Mutter et al., 1995). The temporary structure then breaks down upon cleavage with TFA, returning the peptide to its natural configuration (Figure 1.12).



Resin bound peptide with pseudoproline insertion
R' = H (Ser), R' = CH_3 (Thr), R = any amino acid side chain





The native sequence is regenerated upon cleavage with TFA

Figure 1.12 The chemical structure and mechanisms behind the use of pseudoproline dipeptides

Abreviations: Ser, serine; Thr, threonine; TFA, trifluoroacetic acid.

Pseudoprolines have also been shown to aid in the synthesis of resin bound cyclic peptides whereby two residues within the same peptide chain are linked (Novabiochem, 2010). The nature of the oxazolidine structure means that the carboxyl amino acid of the dipeptide must be either serine or threonine. Pseudoprolines can therefore only be used in sequences containing either of these two residues. Although, in theory there is no restriction on the identity of the second amino acid within the dipeptide, in practice many pseusoprolines are not yet commercially available.

In 2006 a new class of aggregate disrupting dipeptides were reported. Isoacyl dipeptides are similar to pseudoprolines in that serine or threonine residues form a dipeptide with another Fmoc protected amino acid held in an alternative, temporary structure (Novabiochem, 2010). In this case the Fmoc amino acid is acylated to the serine/threonine through its β-hydroxyl side chain. The unused α-amino group of the serine/threonine is protected with a Boc group. Replacing the amide bond with an ester bond radically alters the confirmation of the peptide chain, creating a depsipeptide which helps to disrupt aggregation. Unlike with pseudoprolines, the depsipeptide confirmation is stable to TFA cleavage and has been found to be more soluble than the native sequence. By retaining the temporary structure post-cleavage, the improvement in solubility should in theory aid the purification of the peptide. Once purified, the depsipeptide is converted to the native peptide sequence by exposure to pH7.4, at which spontaneous conversion occurs (Figure 1.13).

1.7.7 Peptide modifications

Solid phase peptide synthesis began as a means of efficiently synthesising naturally occurring peptide sequences, and subsequently for synthesising analogues of these peptides with amino acid substitutions. However the methods employed in SPPS

make it amenable to the synthesis of a wide range of chemically modified peptides that are unlikely to be found in nature.

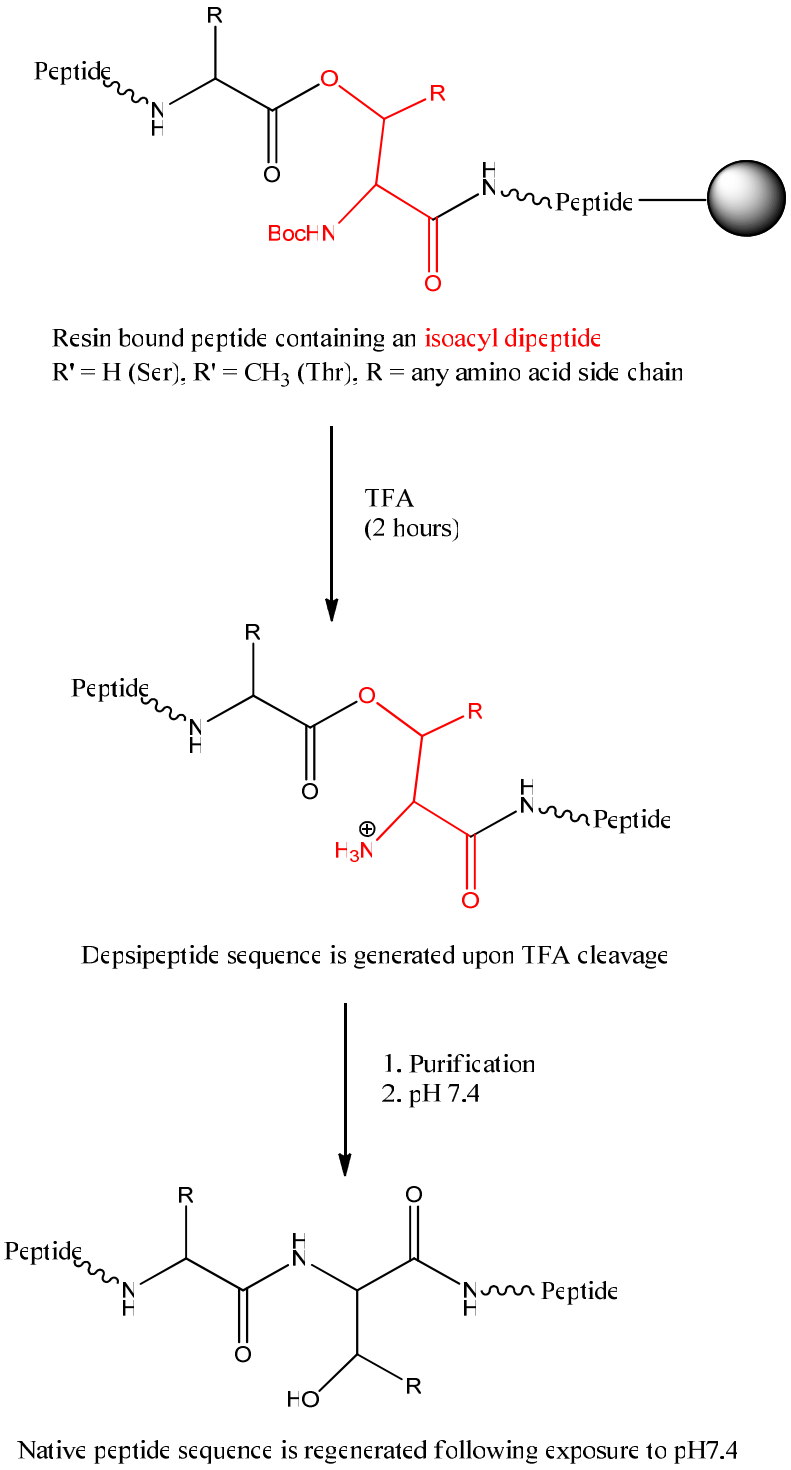


Figure 1.13 The chemical structure, and mechanisms behind the use, of isoacyl dipeptides

Abbreviations: Boc, tertiary butyloxycarbonyl; Ser, serine; Thr, threonine; TFA, trifluoroacetic acid.

The most straightforward modifications are those that extend the N-terminal of the peptide with functional groups not usually associated with peptides. With the peptide bound to the resin and all reactive amino acid side chain groups fully protected, the exposed α-amino group is the only reaction site available. N-terminal modifications can range from simple acetylation, which couples the small acetyl group to the amino group which blocks the amino functionality of the peptide. The reaction usually takes less than 30 minutes to complete and is fully compatible with automated synthesisers. Extension of the N-terminal with larger compounds is also possible using a number of long chain fatty acids, such as palmitic and myristic acid, which are routinely coupled to peptides for use in biological studies (Lumbierres et al., 2005).

A wide range of labelling compounds can also be coupled to peptides via the N-terminal. The most common of these is biotin, which helps the peptide bind to streptavidin and avidin for subsequent use in biological assays. Chromogenic labels which emit at specific wavelengths are useful tools for those working with peptides in enzymology, immunology and histochemistry. In the study of proteolytic enzymes, such labels are utilised to help detect sites of enzymatic cleavage. The enzyme substrate is “double labelled”, with a fluorophore group in one position of the peptide and a quencher molecule in another. Whilst the peptide remains intact the fluorescence of the fluorophore is suppressed by the quencher via a process called Fluorescence Resonance Energy Transfer (FRET). An enzymatic cleavage which separates the two labels will result in a sudden increase in fluorescence.

Spacer molecules are often employed between label and peptide to prevent steric hindrance during coupling of the often bulky label molecule. Common spacers in peptide chemistry are the straight chain hydrocarbon, non-standard amino acids β-

alanine and aminohexanoic acid. Recently, the more hydrophilic compound 8-Amino-3,6-Dioxa-Octanoic Acid (AEEAc) has become a popular alternative spacer molecule as it helps increase the solubility of these labelled peptides.

When modification of a specific amino acid side chain group rather than the N-terminal amino group is required, the strategy of orthogonal protecting groups is applied. This involves protecting the amino acid to be modified with a functional group that can be removed in conditions that also retain the integrity of both the resin linker and all of the other protecting groups. This is most frequently applied to lysine residues as the side chain functionality is as of a primary amine which can be modified in exactly the same way as the N-terminal. There are a number of different lysine protecting groups available, each with their own specific deprotection conditions (Figure 1.14). An example is 4-methyl-trityl (Mtt), a very acid labile protecting group that can be removed by 1-2% TFA, a concentration at which all standard protecting groups are theoretically stable. However, deprotection occurs slowly and requires repeated washes with the TFA solution. The process can take up to an hour to complete and the degree of deprotection cannot be easily quantified.

Alternatively, 1-(4, 4-dimethyl-2, 6-dioxocyclohex-1-ylidene)ethyl (Dde) is an amine protecting group that was introduced in 1993. It is cleaved with 2-5% hydrazine hydrate in DMF and the reaction is usually complete in 10 – 15 minutes. As it is completely orthogonal to the standard protecting groups, the deprotection reaction can be readily followed by High Performance Liquid Chromatography (HPLC) and mass spectrometry. It was later reported that Dde was prone to migration to other amino groups during piperidine mediated Fmoc deprotection (Novabiochem, 2010). To overcome this, the bulkier 1-(4, 4-dimethyl-2, 6-dioxocyclohex-1-ylidene)-3-methylbutyl (ivDde) protecting group was developed. This was proven to be

considerably more stable to piperidine than Dde. The Dde and ivDde derivatives are significantly more expensive than the Mtt derivative, and deprotection can only be carried out once the peptide has been fully assembled, as the Fmoc group is not stable to hydrazine treatment.

Allyloxycarbonyl (Aloc) is a protecting group that is stable to both TFA and piperidine, but can be cleaved by reaction with a palladium(0) catalyst in the presence of acetic acid and N-methylmorpholine in DCM. Drawbacks are that the deprotection reaction can take up to 2 hours, and the palladium catalyst has a short shelf life once exposed to air. However, the derivative is cheaper than both the Mtt and Dde/ivDde versions and the mild deprotection conditions ensure the stability of all other protecting groups.

Sometimes modification of a peptide requires exposure of a carboxyl group as opposed to an amine. In this instance, selective deprotection of either an aspartic acid or glutamic acid residue can be carried out. Both amino acids are commercially available with side chain protecting groups that are complementary to the required deprotection strategy of the previously mentioned amine protecting groups (Figure 1.14). Of these, 2-phenylisopropyl ester (OPip) is removed by treatment with 1-2% TFA, 4{N-[4, 4-dimethyl-2, 6-dioxo-cyclohexylidene)-3-methylbutyl]-amino}benzyloxy (ODmab) is removed with 2-5% hydrazine hydrate, and allyl ester (OAll) is cleaved by exposure to palladium(0) catalyst.

A lactam bridge is an intramolecular bond between the amine group of a lysine and carboxyl group of an aspartic acid or glutamic acid residue that alters or restrains the conformation of the peptide. Such bridges can be formed on the resin by selectively deprotecting the two residues to be linked. If both have complimentary protecting groups then the deprotection can be performed simultaneously. Successful lactam

bridge formation is sequence dependent as it relies upon the two bridge forming functional groups being in close proximity on the resin bound peptide. If the two groups are too far apart on the resin, there is the risk of either intermolecular lactam bridge formation or reaction of the functional groups with the reagents being used to facilitate lactam formation.

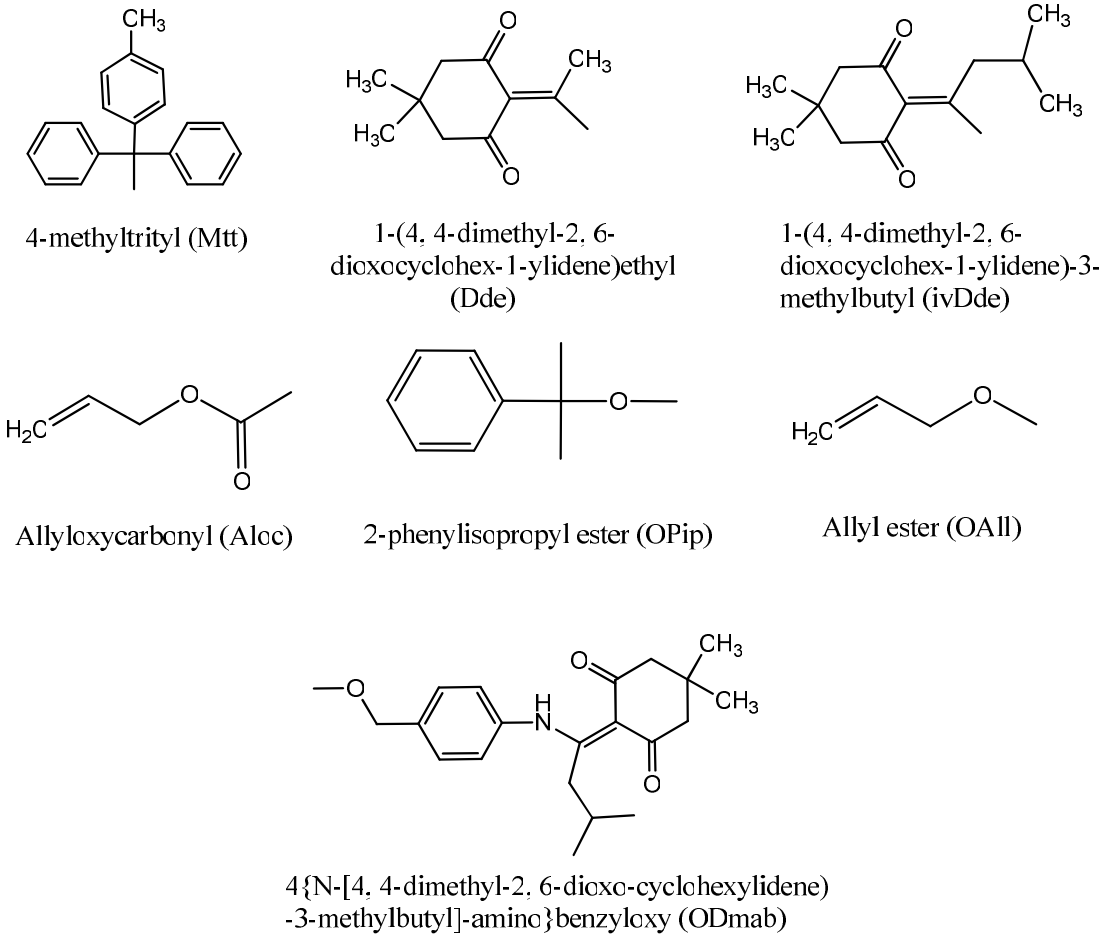


Figure1.14 The chemical structures of alternative protecting groups for lysine, aspartic acid and glutamic acid Fmoc amino acids

A second class of intramolecular bond commonly formed in peptides are disulphide bridges between the thiol side chain groups of cysteine residues. The reaction

conditions for disulphide bridge formation are specific to sulphydryl groups, meaning that they can be formed on fully deprotected peptides, often during the purification stage (Pennington and Dunn, 1994). Disulphide bridge formation is therefore usually more straightforward than lactam formation.

It may be that the carboxyl group required for modification is that at the C-terminal of the peptide. In this case it is necessary to remove the peptide from the resin but to retain all of the amino acid protecting groups. The peptide must therefore be synthesised on an acid sensitive resin such as 2-chlorotrityl chloride or SASRIN. The fully protected peptide can then be obtained either by repeated washes with 1-2% TFA or with 1 – 4 hour treatment with 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) (Mergler, 1999). The TFA method requires that each wash is neutralised with pyridine to prevent prolonged exposure to TFA. This leads to the formation of pyridinium trifluoroacetate which has to be completely removed via extraction or precipitation. Conversely some standard protecting groups are sensitive to HFIP and to ensure that they are retained during cleavage, the HFIP concentration and reaction times must be carefully controlled.

1.7.8 Cleavage

Once the synthesis of the peptide is complete, the next step in the process is to remove the peptide from the resin and remove the protecting groups from the amino acids. Standard resin linkers such as Wang, Rink and Ramage are all cleaved by treatment with TFA at concentrations of 80% or higher. As has been previously mentioned, other resin linkers such as 2-chlorotrityl and SASRIN can be cleaved using much milder conditions. At the higher TFA concentrations, all standard amino acid protecting groups will also be cleaved. Once removed from the amino acid, the

protecting group is released into solution as a highly reactive cation that can modify other amino acids in the sequence (Pennington and Dunn, 1994). To overcome this, scavenger compounds are included in the cleavage solution to remove the reactive cation species. A number of different scavengers are routinely used in peptide chemistry, with each one effective at removing particular cations. It is therefore possible to tailor a cleavage solution for each peptide based upon the amino acids in its sequence. However, more commonly chemists utilise a catch all cleavage cocktail that can be used for the vast majority of peptides synthesised. The most frequently used cocktail is solution K that consists of 82.5% TFA, 5% phenol, 5% H_2O, 5% thioanisole and 2.5% 1,2-ethanedithiol (EDT). Due to their unpleasant odour some users omit both thioanisole and EDT and replace them with the less odorous triisopropylsilane (TIS). It is also necessary to remove these two scavengers from the cleavage solution if the peptide contains residues that are sensitive to thiol compounds.

The dry peptide resin is suspended in the cleavage solution, usually at a concentration of 10 – 25mL per gram of resin, and agitated gently. Some laboratories choose to start the cleavage reaction in an ice bath.

The reaction is left to mix for a minimum of 2 hours, but the actual cleavage time is sequence dependent. Certain amino acid deprotection reactions proceed more slowly than others, with arginine side chain protection groups the slowest. It may thus be necessary to extend the cleavage time for sequences containing multiple arginine residues for up to 24 hours (Chan and White, 2000).

Once the reaction is complete, the resin is filtered away and the peptide that remains in an acidic solution can either be directly precipitated or concentrated under vacuum before precipitation. Concentrating the solution helps to speed up the subsequent work

up. However, cysteine residues are sensitive to the concentration conditions as they are prone to reattachment of their protecting group. Precipitation of the peptide is commonly facilitated with the addition of cold diethyl or tertiary butyl methyl ether. This suspension is usually refrigerated overnight. The peptide is then isolated by filtration before being washed with fresh ether to remove any residual TFA and scavengers, and then being dried to a powder if possible.

Several side reactions, other than reaction with the protecting group cations that reduces the purity of the resulting crude peptide can occur during the cleavage process. If the peptide contains methionine residues, they can undergo acid catalysed oxidation to methionine sulphoxide (Chan and White, 2000), although this reaction can be reversed with the addition of reducing agents prior to the end of the cleavage reaction.

N-terminal glutamine residues also undergo acid catalysed modification, irreversibly forming pyroglutamic acid (Chan and White, 2000). In this case the crude material will contain an impurity that is impossible to separate from the target peptide. Peptides containing N-terminal glutamine are therefore usually acetylated or synthesised with pyroglutamic acid as the final residue.

1.7.9 Purification

For all but the shortest sequences, the quality of the crude peptide material produced from cleavage is seldom good enough for its desired end use. The process of purifying the peptide is therefore as important as the process of synthesising it. A variety of different techniques have traditionally been employed in the purification of peptides including ion-exchange chromatography, countercurrent distribution and gel-filtration chromatography (Miller and Rivier, 1996). However, the development of HPLC has

led to this becoming the standard method of purifying peptides in most laboratories. The specific form of HPLC traditionally used is reverse phase HPLC, in which the stationary phase is either octyl (C8) or octadecyl (C18) modified silica which is tightly packed under pressure into a column of varying diameter and length. The C8 or C18 group confers a hydrophobic nature on the stationery phase to which the peptide binds. To help suppress any hydrophilic characteristics in the peptide and hence increase binding, the mobile phase is generally a low pH solution so as to force the protonation of any carboxyl groups which also contains anions that act as counter ions to any basic amino acids (Miller and Rivier, 1996). The most commonly used buffering compound that meets these requirements is trifluoroacetic acid.

The sample is loaded onto the column in a suitable solution and the HPLC system then begins to pump the mobile phase under high pressure through the column. To begin with, the mobile phase is usually a fully aqueous solution of the buffer which will flush any salts and loading solvent off the column. Unless the peptide is very short and/or hydrophilic it should remain bound to the stationary phase in the column. The system will then run a gradient whereby the organic content of the mobile phase is gradually increased. The most commonly used solvent for peptide purification is acetonitrile, although iso-propanol is an alternative option. As the organic concentration increases, the peptides within the sample will begin to display greater affinity for the mobile phase and so begin to elute from the column. The chemical differences between the components of the sample mean that they will likely each have different affinities for the two phases at any particular solvent concentration. This causes them to elute from the column at different times during the gradient. Within the HPLC system is a UV detector that operates at wavelengths of 200-220nm, the absorption wavelength range of the peptide bond (Mant et al., 2007). Peptides

containing a number of aromatic components, such as protected peptides, can also be detected at longer wavelengths such as 254nm.

Depending upon the size of column and the capacity of the solution pumping system, HPLC systems are used for different tasks. At the smallest scale they are used as analytic tools to produce a representative chromatogram of the peptide sample. As they increase in size, the systems can begin to handle larger amounts of material and so are used to purify the peptide. In this case, as each component elutes from the column and is detected, the eluent is collected in fractions. Each fraction can then be analysed and those containing the desired product can be retained. These fractions can then be combined either for lyophilisation or for further processing.

There are now a wide range of different columns and stationery phase materials available to the peptide chemist to enable them to tailor the purification of any particular peptide.

In some peptide laboratories a second chromatography technique called Flash Chromatography is used alongside reverse phase HPLC. This is complementary to HPLC in that is uses the same buffer solutions and the same stationery phase material. The difference is that Flash Chromatography operates at lower pressures and the particles of the stationery phase are generally larger than those found in HPLC columns. This means that resolution is not as high for Flash Chromatography, but also means material that would damage the HPLC reverse phase column can be loaded without damage to the Flash Chromatography column. This makes it an excellent tool for the initial purification of crude peptide samples, thus preserving the lifetime of the more expensive HPLC column. Flash Chromatography gradients are also generally shorter than HPLC gradients, which helps to reduce overall solvent consumption and

to speed purification. Typical Flash Chromatography gradients are 20 – 30 minutes long compared with typical HPLC gradients of over an hour.

The final analytical tool routinely used in the purification and characterisation of peptides is mass spectrometry. There are a number of different types of mass spectrometers available depending upon the desired method of sample ionization and subsequent mass analysis. The most common types found in peptide laboratories are Matrix Assisted Laser Desorption-Ionization (MALDI) and Electrospray Ionization (ESI) mass spectrometers (Wysocki et al., 2005). For MALDI mass spectrometry, the peptide sample is crystallised onto the sample plate together with a small organic matrix compound such as sinapinic acid or α-cyano-4-hydroxycinnamic acid (CHCA) (Wysocki et al., 2005). The matrix compound contains an aromatic ring structure that absorbs radiation at the wavelength of the laser that is used to ionize the sample (Moore, 1997).

In ESI mass spectrometry, the peptide sample is introduced as a solution which is passed through a high voltage needle. The charged solution is then sprayed from the tip of the needle through an electrostatic field which evaporates the carrier solvent, creating an ionized sample for analysis (Wysocki et al., 2005).

There are a number of different methods for analysing sample masses. The most common method used in tandem with MALDI and ESI, is time of flight (TOF) analysis. In TOF analysis, the ions are accelerated along a flight tube of fixed length using a small voltage gradient. The flight time of an ion is proportional to its mass-to-charge ratio (*m/z*), which allows ions of different masses within the sample to be resolved (Wysocki et al., 2005).

With MALDI mass spectrometry, usually only the singularly charged ion, (M+H) is generated, where M is the molecular weight of the peptide being analysed and H is a

proton. Meanwhile, ESI generates a range of charged species (M+z/z), making the interpretation of the resultant spectra less straightforward.

1.8 Aims

I intend to utilise some of the described SPPS techniques to produce analogues of the gut derived anorectic hormone PYY(3-36) and investigate the effects of these analogues both *in vitro* and *in vivo*. The aim is to produce a PYY(3-36) analogue that has a greater effect in reducing food intake than the endogenous peptide. This can be achieved by increasing the stability of the peptide to enzymatic degradation and/or by increasing the affinity of the peptide to the Y_2 receptor, to which PYY(3-36) is a natural agonist.

Should such an analogue be produced, then it could potentially be developed as an anti-obesity therapeutic.

2 Synthesis and purification of PYY(3-36) analogues

2.1 Introduction

As described in Chapter 1, Peptide YY (PYY) is a hormone released by the L-cells of the small intestine in response to food intake (le Roux and Bloom, 2005). The major circulating form of the peptide is PYY(3-36), formed by the action of the proteolytic enzyme dipeptidyl peptidase IV (DPP IV), which cleaves the N-terminal dipeptide, Tyr-Pro from the parent sequence (Mentlein, 1999). Administration of PYY(3-36) at physiological doses has been shown to reduce food intake in both lean and obese humans (Batterham, et al., 2003b). Thus, extensive research has been conducted with the aim of harnessing the anorectic effects of PYY(3-36) and using it in an anti-obesity therapeutic. To date, although several therapies based on PYY(3-36) have made it to the clinical trial phase, none have yet made it onto the market (Cooke and Bloom, 2006).

The anorectic effects of PYY(3-36) are short lived. The body naturally processes such hormones through enzymatic degradation, allowing the signal that the hormone is conveying to be turned off. Compensating for this by administering supra physiological doses leads to nausea (Murphy and Bloom, 2006). Therefore, chemically modifying the sequence and/or structure of PYY(3-36) to increase its efficacy as an anti-obesity agent is an attractive option. Somatostatin analogues, for example, have a longer half-life than somatostatin and are regularly used in the treatment of acromegaly and neuroendocrine tumours (Pettit and El-Modir, 2011). To succeed in producing PYY(3-36) analogues that have increased efficacy in reducing food intake, there are two options. One is to increase the resistance of the peptide to the actions of proteolytic enzymes, thus extending the circulating half life of the analogue. The second option is to increase the binding affinity to the Y_2 receptor. An

analogue of PYY(3-36) that fulfilled both of these criteria, could prove to be effective in combating the rising obesity epidemic. Currently, peptide drugs are usually administered either intravenously or subcutaneously, but oral administration of a gut derived peptide drug would be preferable; it could be absorbed into the circulation from the small intestine, thus mimicking its physiological release (Beglinger et al., 2008). Also, oral administration would be more convenient and palatable to patients than administration via injection. Unfortunately, the gastro-intestinal tract efficiently breaks down proteins through the actions of a number of different protease enzymes.

2.1.1 Sites of proteolytic enzyme degradation within PYY(3-36)

The sequence of PYY(3-36) contains a pair of basic amino acids in positions 25 (arginine) and 26 (histidine) that are conserved across different species. Basic amino acids are a common site of degradation by proteolytic enzymes (Stoller and Shields, 1989). Many small peptide hormones within the body are synthesised as larger precursor molecules which undergo post-translational cleavage by enzymes to release the active peptide. Within the sequence of the precursor, it is common for the hormone sequence to be flanked by pairs of basic amino acids at which processing by enzymes take place (Stoller and Shields, 1989).

2.1.2 N-terminal modifications of PYY(3-36)

Modifications to the N-terminal of PYY(3-36) have been shown to effect its efficacy both beneficially and detrimentally, depending upon the nature of the modification. Acetylation of the N-terminal of PYY(3-36) has been shown to greatly reduce the potency of the peptide, but studies with PEG modified PYY(3-36) have shown

promising results (Shechter et al., 2005). It appears that the size of the PEG molecule is important to the preservation of activity.

2.1.3 Binding of PYY(3-36) to the Y_2 receptor

As a member of the PP fold family of peptides, PYY(3-36) binds to the NPY Y family of G-protein coupled receptors (Wraith et al., 2000). The C-terminal pentapeptide of the PP fold peptides is highly conserved between peptides and species (Table 2.1) and has been shown to play a crucial role in the binding of these peptides to the Y receptors (Beck-Sickinger et al., 1994; Pedersen et al., 2009).

Table 2.1 The C-terminal pentapeptide sequences of the PP fold family of peptides.

Abbreviations; NPY, neuropeptide Y; PP, pancreatic polypeptide; PYY, peptide YY.

Peptide	**32**	**33**	**34**	**35**	**36**	**C-terminal**
NPY	Threonine	Arginine	Glutamine	Arginine	Tyrosine	NH_2
PP	Threonine	Arginine	*Proline*	Arginine	Tyrosine	NH_2
PYY	Threonine	Arginine	Glutamine	Arginine	Tyrosine	NH_2

The PP fold family peptides have different affinities for the Y receptors. NPY binds with greatest affinity to the Y_1 and Y_5 receptors, PP has greatest affinity with the Y_4 receptor and PYY(3-36) has greatest affinity for the Y_2 receptor, through which it mediates its anorectic effects (Wraith et al., 2000). The specificity displayed by the PP fold peptides for particular Y receptors must be due to the difference in the sequences outside of the C-terminal pentapeptide.

2.1.4 The hairpin like structure of PYY(3-36)

^{1}H NMR studies have determined that PYY(3-36) adopts a hairpin like structure in aqueous solution (Nygaard et al., 2006). This characteristic shape is caused in part by the presence of proline residues in positions 5 and 8, hydrophobic leucine and tyrosine residues in positions 17, 20, 24 and 27, and acidic glutamic acid residues in positions 15 and 16. This all serves to bring the N-terminal and C-terminal regions in close proximity to each other (Figure 2.1). Both of these regions were thought to play an important role in binding to the Y receptors, but subsequent studies suggest that whilst this is true for the Y_1 receptor, it is less so for the Y_2 receptor (Nygaard et al., 2006).

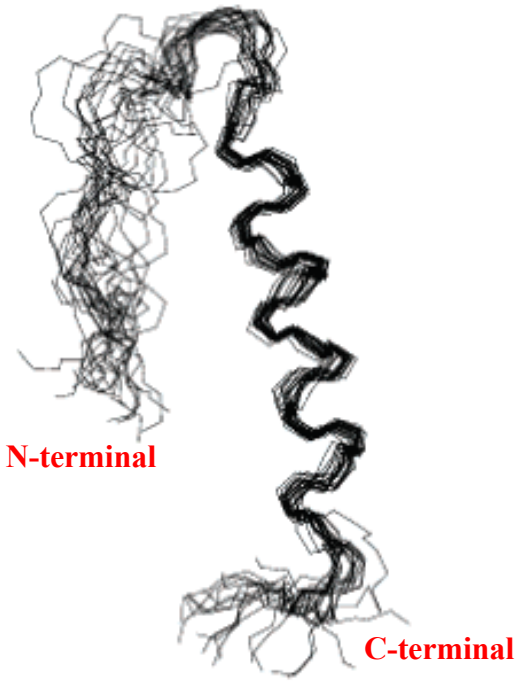


Figure 2.1 The "hairpin like" structure of PYY(3-36) as determined by ^{1}H NMR studies (Nygaard et al.,2006)

2.2 Hypothesis and aims

2.2.1 Hypothesis

Analogues of PYY(3-36) with positional substitutions or structural modifications can be successfully produced using solid phase peptide synthesis (SPPS) techniques.

2.2.2 Aims

1. To produce analogues of PYY(3-36) containing unnatural amino acid substitutions in positions 25 and 26 of the sequence.
2. To produce N-terminal pegylated analogues of PYY(3-36).
3. To produce analogues of PYY(3-36) containing natural amino acid substitutions in the positions 30, 31 and 32 of the sequence.
4. To produce analogues of PYY(3-36) that contain a cross linkage between the amino acids in position 4 and 29 of the sequence.

2.3 Materials and methods

2.3.1 Position 25 and 26 PYY(3-36) analogues

2.3.1.1 Peptide design

Unnatural amino acids, that were structural analogues of the endogenous residues, were chosen so as to retain the structure of the native peptide as much as possible. In doing so, it is hoped that bioactivity is not compromised. For the position 25 substitutes, unnatural amino acids were selected that share structural and/or functional homology with the native arginine residue. The residues selected for position 26 are either isomers, or methylated imidazole ring analogues of the native histidine residue (Table 2.2 and Figure 2.2).

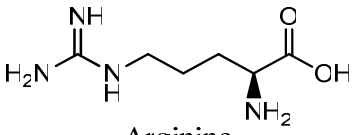
Table 2.2 The peptide sequences of ten PYY(3-36) analogues containing substitutions with unnatural amino acids in positions 25 and 26.

IP codes were assigned to facilitate subsequent biological testing. Residues highlighted in red are those that differ from the endogenous sequence.

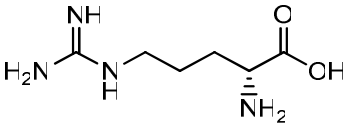
Name	Code	N	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	C
[a] PYY(3-36)	**PYY(3-36)**	H	Ile	Lys	Pro	Glu	Ala	Pro	Gly	Glu	Asp	Ala	Ser	Pro	Glu	Glu	Leu	Asn	Arg	Tyr	Tyr	Ala	Ser	Leu	Arg	His	Tyr	Leu	Asn	Leu	Val	Thr	Arg	Gln	Arg	Tyr	NH_2
(Homoarg)^{25}PYY(3-36)	**IP1**	H	Ile	Lys	Pro	Glu	Ala	Pro	Gly	Glu	Asp	Ala	Ser	Pro	Glu	Glu	Leu	Asn	Arg	Tyr	Tyr	Ala	Ser	Leu	Homoarg	His	Tyr	Leu	Asn	Leu	Val	Thr	Arg	Gln	Arg	Tyr	NH_2
(D-Arg)^{25}PYY(3-36)	**IP2**	H	Ile	Lys	Pro	Glu	Ala	Pro	Gly	Glu	Asp	Ala	Ser	Pro	Glu	Glu	Leu	Asn	Arg	Tyr	Tyr	Ala	Ser	Leu	D-Arg	His	Tyr	Leu	Asn	Leu	Val	Thr	Arg	Gln	Arg	Tyr	NH_2
(Arg(Me)$_2$symm)^{25}PYY(3-36)	**IP3**	H	Ile	Lys	Pro	Glu	Ala	Pro	Gly	Glu	Asp	Ala	Ser	Pro	Glu	Glu	Leu	Asn	Arg	Tyr	Tyr	Ala	Ser	Leu	Arg(Me)$_2$ symm	His	Tyr	Leu	Asn	Leu	Val	Thr	Arg	Gln	Arg	Tyr	NH_2
(Arg(Me)$_2$asymm)^{25}PYY(3-36	**IP4**	H	Ile	Lys	Pro	Glu	Ala	Pro	Gly	Glu	Asp	Ala	Ser	Pro	Glu	Glu	Leu	Asn	Arg	Tyr	Tyr	Ala	Ser	Leu	Arg(Me)$_2$ asymm	His	Tyr	Leu	Asn	Leu	Val	Thr	Arg	Gln	Arg	Tyr	NH_2
(Cit)^{25}PYY(3-36)	**IP5**	H	Ile	Lys	Pro	Glu	Ala	Pro	Gly	Glu	Asp	Ala	Ser	Pro	Glu	Glu	Leu	Asn	Arg	Tyr	Tyr	Ala	Ser	Leu	Cit	His	Tyr	Leu	Asn	Leu	Val	Thr	Arg	Gln	Arg	Tyr	NH_2
(D-Cit)^{25}PYY(3-36)	**IP6**	H	Ile	Lys	Pro	Glu	Ala	Pro	Gly	Glu	Asp	Ala	Ser	Pro	Glu	Glu	Leu	Asn	Arg	Tyr	Tyr	Ala	Ser	Leu	D-Cit	His	Tyr	Leu	Asn	Leu	Val	Thr	Arg	Gln	Arg	Tyr	NH_2
(His(1-Me))^{26}PYY(3-36)	**IP7**	H	Ile	Lys	Pro	Glu	Ala	Pro	Gly	Glu	Asp	Ala	Ser	Pro	Glu	Glu	Leu	Asn	Arg	Tyr	Tyr	Ala	Ser	Leu	Arg	His(1-Me)	Tyr	Leu	Asn	Leu	Val	Thr	Arg	Gln	Arg	Tyr	NH_2
(His(3-Me))^{26}PYY(3-36)	**IP8**	H	Ile	Lys	Pro	Glu	Ala	Pro	Gly	Glu	Asp	Ala	Ser	Pro	Glu	Glu	Leu	Asn	Arg	Tyr	Tyr	Ala	Ser	Leu	Arg	His(3-Me)	Tyr	Leu	Asn	Leu	Val	Thr	Arg	Gln	Arg	Tyr	NH_2
(D-His)^{26}PYY(3-36)	**IP9**	H	Ile	Lys	Pro	Glu	Ala	Pro	Gly	Glu	Asp	Ala	Ser	Pro	Glu	Glu	Leu	Asn	Arg	Tyr	Tyr	Ala	Ser	Leu	Arg	D-His	Tyr	Leu	Asn	Leu	Val	Thr	Arg	Gln	Arg	Tyr	NH_2
(Phe-Gu)^{25}PYY(3-36)	**IP10**	H	Ile	Lys	Pro	Glu	Ala	Pro	Gly	Glu	Asp	Ala	Ser	Pro	Glu	Glu	Leu	Asn	Arg	Tyr	Tyr	Ala	Ser	Leu	Phe-Gu	His	Tyr	Leu	Asn	Leu	Val	Thr	Arg	Gln	Arg	Tyr	NH_2
[b] Biotinyl-PYY(3-36)	**IP11**	Biotin	Ile	Lys	Pro	Glu	Ala	Pro	Gly	Glu	Asp	Ala	Ser	Pro	Glu	Glu	Leu	Asn	Arg	Tyr	Tyr	Ala	Ser	Leu	Arg	His	Tyr	Leu	Asn	Leu	Val	Thr	Arg	Gln	Arg	Tyr	NH_2

[a] PYY(3-36) human was synthesised at the same time for use as a standard in subsequent studies.

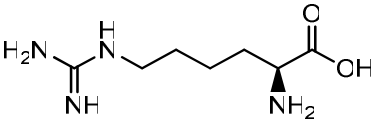
[b] This analogue was synthesised at the same time for potential use in a fluorescence receptor binding assay.



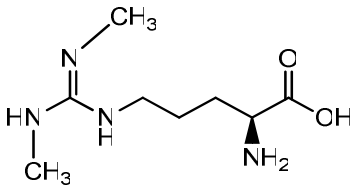
Arginine



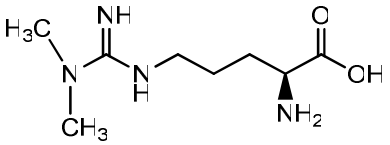
D-Arginine (IP2)



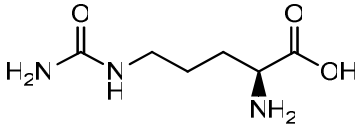
Homoarginine (IP1)



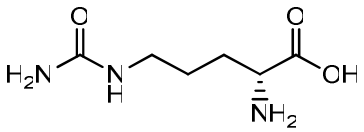
Arginine(Me)$_2$(symmetrical) (IP3)



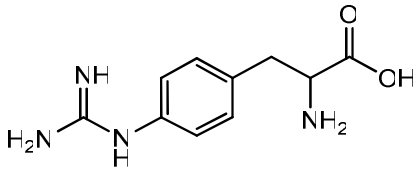
Arginine(Me)$_2$(asymmetrical) (IP4)



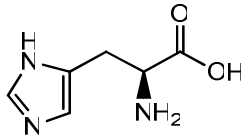
Citrulline (IP5)



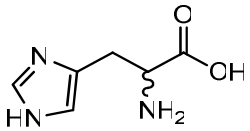
D-Citrulline (IP6)



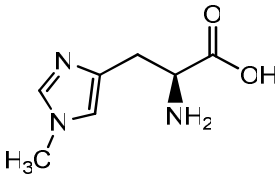
4-Guanidino Phenylalanine (IP10)



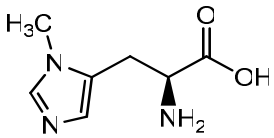
Histidine



D-Histidine (IP9)



Histidine(1-methyl) (IP7)



Histidine(3-methyl) (IP8)

Figure 2.2 The chemical structures of the amino acids used in positions 25 and 26 of PYY(3-36) analogues.

2.3.1.2 Peptide synthesis

All peptides were synthesised on a Symphony multiple peptide synthesiser (Protein Technologies, Tucson, Arizona, USA) using standard Fmoc/tBu chemistry. The amino acids used were Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-D-Arg(Pmc)-OH, Fmoc-Arg$(Me)_2$-OH (symmetrical), Fmoc-Arg$(Me)_2$-OH (asymmetrical), Fmoc-Asn(Trt)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Cit-OH, Fmoc-D-Cit-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Gly-OH, Fmoc-His(1-Trt)-OH, Fmoc-His(1-Me)-OH, Fmoc-His(3-Me)-OH, Fmoc-Homoarg(Pmc)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Pro-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Tyr(tBu)-OH and Fmoc-Val-OH purchased from Bachem AG (Bubendorf, Switzerland) and Fmoc-Phe(4-guanidinoPMC)-OH purchased from Iris Biotech (Marktredwitz, Germany). The resin used was tricyclic amide linker resin (loading of 0.63mmol/g) also purchased from Bachem AG.

All peptides were synthesised at a scale of 0.05mmol (except for PYY(3-36) at 0.1mmol and Biotinyl-PYY(3-36) at 0.6mmol) using double couplings for all residues. Each amino acid was added sequentially to the growing peptide chain from the C to the N terminus applying 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate (TBTU), 1-hydroxybenzotriazole (HOBt) (both Severn Biotech Ltd, Kidderminster, UK) and N,N-diisopropylethylamine (DIPEA) (Apollo Scientific, Stockport, UK) as coupling reagents. Removal of the Fmoc protecting group was carried out with 20% piperidine (Romil, Cambridge, UK) in N-methyl pyrrolidone (NMP) (M56 Chemicals, Runcorn, UK) followed by sequential washes with dimethylformamide (DMF) (Apollo Scientific). All amino acid derivatives (8-fold excess), TBTU and HOBt (7.8-fold excess), and DIPEA (16-fold

excess) were dissolved in DMF. All solvents used were of peptide synthesis grade quality.

For Biotinyl-PYY(3-36), the biotin moiety was coupled manually due to its poor solubility in DMF and the potential for slow coupling. D-(+)-Biotin (Alfa Aesar, Lancaster, UK) was dissolved in 1:1 solution of DMF and dimethylsulphoxide (Sigma Aldrich, Poole, UK) together with O-(7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU) (Novabiochem, Nottingham, UK) and DIPEA. The coupling reaction took 2 hours to complete and was monitored by Kaiser test. This involved taking a sample of resin from the reaction, washing it with iso-propanol, before adding 3 drops each, of the solutions; 42.5M phenol in butan-1-ol, 0.28M ninhydrin in butan-1-ol and pyridine. The resin was then heated to 100°C for 5 minutes, the presence of a blue colour to the solution or any of the resin beads indicated that the coupling was incomplete. No change to the colour of the solution or beads indicated that the coupling was complete.

2.3.1.3 Preparation of solution K

To prepare 100mL of solution K, 7.5g of Phenol (Alfa Aesar) was dissolved in 5mL of water. Thioanisole (5mL) and 1,2-ethanedithiol (2.5mL) (both Alfa Aesar) were added and the solution made up to a final volume of 100mL with the addition of trifluoroacetic acid (TFA) (Romil).

2.3.1.4 Peptide cleavage

The peptides were cleaved from the resin with simultaneous deprotection using solution K (10mL/g of resin) at room temperature for 2½ h.

The cleavage mixture was filtered through a porosity 2 sinter funnel into a round bottom flask. The resin was washed with TFA, then acetonitrile (Avantor, Deventer, Netherlands) and filtered again. The solvent was then removed on a rotary evaporator connected to a high vacuum pump. When all the solvent had been evaporated, the peptide was precipitated with the addition of ice cold diethyl ether (Avantor). The precipitates were stored at 4^{o}C overnight and were collected by filtration through a porosity 3 sinter funnel, washed with ice-cold diethyl ether, and allowed to dry at room temperature. The crude peptides were dissolved in a mixture of 0.1%TFA in 60%acetonitrile/water and lyophilized overnight (-50^{o}C, 6 mbar).

2.3.1.5 Characterisation of crude peptides

Peptides were characterised by reverse phase High Performance Liquid Chromatography (HPLC) (Gilson, Middleton, Wisconsin, USA) using an analytical C-18 column (Vydac 218TP54, 250 x 4.6mm, 5 µm particle size, and 300 Å pore size) and a linear AB gradient of 0–100% for B over 40 min at a flow rate of 1mL/min, where eluent A was 0.1% TFA/water and eluent B was 0.1% TFA in 60% acetonitrile/water. Mass was confirmed using a Kompact Probe Matrix Assisted Laser Desorption Ionisation Mass Spectrometer (MALDI-MS) (Kratos, Manchester, UK).

2.3.1.6 Purification of peptides

Peptides were purified by reverse phase HPLC (Varian, Oxford, UK) using a C-18 column (Vydac 218TP101550, 250 x 50 mm, 10-15 µm particle size and 300Å pore size). A linear AB gradient program of 0-40% B in 4 min followed by 40-90% B in 70 min at a flow rate of 120mL/min was applied. Eluent A was 0.1% TFA/water and

eluent B was 0.1% TFA in 60% acetonitrile/water. The collected fractions were analysed by MALDI MS and analytical reverse phase HPLC. The relevant fractions were then pooled together and lyophilized overnight (-50^{o}C, 6 mbar).

In the case of peptides (Cit)^{25} PYY(3-36) and Biotinyl-PYY(3-36), a second stage of purification was required. This involved the use of a different AB solvent system, where eluent A was 0.2% Triethylamine (TEA) and 0.3% Orthophosphoric acid (both VWR, Leicestershire, UK) in 10% acetonitrile/water and eluent B was 0.2% TEA and 0.3% Orthophosphoric acid in 60% acetonitrile/water. The pH of the system was 2.3.

2.3.1.7 Quality Control (QC)

Full QC was performed on all purified peptides by dedicated QC personnel. Purity was determined by reverse phase HPLC (Series 200, Perkin Elmer, Waltham, Massachusetts, USA) using a C18 column (Vydac 218TP54, 250 x 4.6mm, 5 µm particle size, and 300 Å pore size) and a linear AB gradient of 0–100% for B over 40 min at a flow rate of 1mL/min. The purity was determined in two separate buffer systems. In the first, eluent A was 0.1% TFA/water and eluent B was 0.1% TFA in 60% acetonitrile/water. For the second system, eluent A was 0.2% TEA and 0.3% Orthophosphoric acid in 10% acetonitrile/water and eluent B was 0.2% TEA and 0.3% Orthophosphoric acid in 60% acetonitrile/water. The molecular weights of the peptides were detected by MALDI mass spectrometry (Kratos). Amino acid analysis was performed using a Beckman 6300 amino acid analyzer (High Wycombe, UK), following hydrolysis overnight in 6M hydrochloric acid (P & R Labpack, St Helens, UK).

2.3.2 N-terminal pegylated analogues of PYY(3-36)

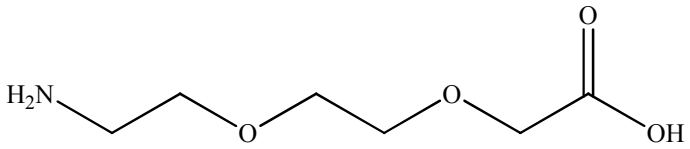
2.3.2.1 Peptide design

The following set of analogues were designed to test the effects of adding relatively small PEG molecules to the N-terminal of PYY(3-36). The peptide sequences and structure of the PEG molecules are shown in Table 2.3 and Figure 2.3.

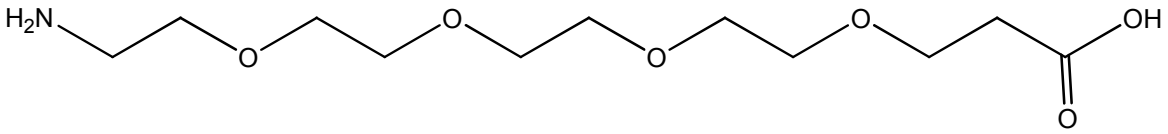
Table 2.3 The peptide sequences of three N-terminally pegylated PYY(3-36) analogues

The numerical codes assigned to the analogues are internal Bachem identification numbers and will be used for identification purposes in subsequent biological studies. Residues highlighted in red are those that differ from the endogenous sequence.

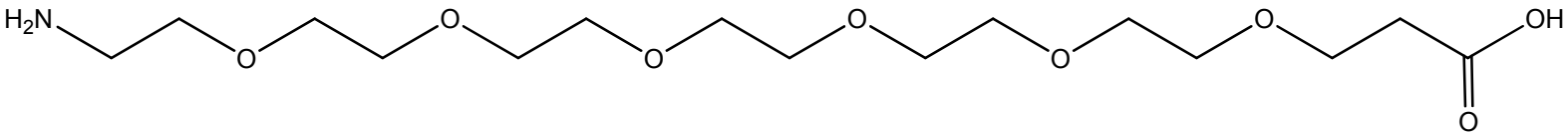
Name	Code	N	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	C
PYY(3-36)	**PYY(3-36)**	H	-	Ile	Lys	Pro	Glu	Ala	Pro	Gly	Glu	Asp	Ala	Ser	Pro	Glu	Glu	Leu	Asn	Arg	Tyr	Tyr	Ala	Ser	Leu	Arg	His	Tyr	Leu	Asn	Leu	Val	Thr	Arg	Gln	Arg	Tyr	NH_2
AEEAc-PYY(3-36)	**4075004**	H	AEEAc	Ile	Lys	Pro	Glu	Ala	Pro	Gly	Glu	Asp	Ala	Ser	Pro	Glu	Glu	Leu	Asn	Arg	Tyr	Tyr	Ala	Ser	Leu	Arg	His	Tyr	Leu	Asn	Leu	Val	Thr	Arg	Gln	Arg	Tyr	NH_2
Aminoethyl-PEG4-carbonyl-PYY(3-36)	**4075005**	H	PEG4	Ile	Lys	Pro	Glu	Ala	Pro	Gly	Glu	Asp	Ala	Ser	Pro	Glu	Glu	Leu	Asn	Arg	Tyr	Tyr	Ala	Ser	Leu	Arg	His	Tyr	Leu	Asn	Leu	Val	Thr	Arg	Gln	Arg	Tyr	NH_2
Aminoethyl-PEG6-carbonyl-PYY(3-36)	**4075006**	H	PEG6	Ile	Lys	Pro	Glu	Ala	Pro	Gly	Glu	Asp	Ala	Ser	Pro	Glu	Glu	Leu	Asn	Arg	Tyr	Tyr	Ala	Ser	Leu	Arg	His	Tyr	Leu	Asn	Leu	Val	Thr	Arg	Gln	Arg	Tyr	NH_2



8-amino-3-6-dioxa-octanoic-acid (AEEAc) (4075004)



aminoethyl-PEG4-carboxylic acid (4075005)



aminoethyl-PEG6-carboxylic acid (4074006)

Figure 2.3 The chemical structures of the PEG molecules to be coupled to the N-terminal of three PYY(3-36) analogues

The internal Bachem identification codes are included to cross reference the PEG molecule with the relevant analogue

2.3.2.2 Peptide synthesis

All peptides were synthesised as per the methods described in section 2.3.1.2. Fmoc-Leu-OH and Fmoc-Gln(Trt)-OH were purchased from Novabiochem. The resin used was tricyclic amide linker resin (loading of 0.66mmol/g) (Bachem AG).

All peptides were synthesised at a scale of 0.1mmol. Amino acids (4-fold excess), TBTU (Bachem AG) and HOBt (3.9-fold excess) and DIPEA (Romil) (8-fold excess) were dissolved in DMF (Romil).

For the N-terminal PEG modifications, AEEAc was introduced as Fmoc-AEEAc-OH (Bachem AG) via an automated process on the Symphony synthesiser, as per the other amino acids. Final Fmoc group removal was also performed on the synthesiser using a solution of 20% piperidine in DMF. The remaining PEG molecules were coupled as Fmoc-NH-PEG3-COOH and Fmoc-NH-PEG5-COOH respectively. Both reagents were purchased from Novabiochem; the discrepancies in the PEG numbering system are due to the different nomenclature protocols at Novabiochem. The PEG molecules were manually coupled with TBTU and DIPEA, and monitored by Kaiser test as described in section 2.3.1.2 in case of slow coupling times. In each case, the N-terminal Fmoc protecting group was then removed with a solution of 20% piperidine in DMF, in a reaction monitored by Kaiser test.

2.3.2.3 Peptide cleavage

Peptides were cleaved as per the methods described in section 2.3.1.4.

2.3.2.4 Characterisation of crude peptides

Crude peptides were characterised as per the methods described in section 2.3.1.5 using a Voyager DE Matrix Assisted Laser Desorption/Ionisation mass spectrometer (MALDI-MS) (Applied Biosystems, Warrington, UK).

2.3.2.5 Purification of peptides

Peptides were purified as per the methods described in section 2.3.1.6.

2.3.3 Position 30, 31 and 32 analogues of PYY(3-36)

2.3.3.1 Peptide design

In human PYY(3-36) the amino acids in positions 30, 31 and 32 are leucine, valine and threonine respectively. Natural amino acids with structural similarities to these amino acids were chosen to replace them in the analogues. The sequences of these analogues are shown in Table 2.4.

Table 2.4 The peptide sequences of eight analogues of PYY(3-36) with amino substitutions in positions 30, 31 and 32

IP codes have been assigned for ease of use in subsequent biological studies. Residues highlighted in red are those that differ from the endogenous sequence.

Name	Code	N	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	C
PYY(3-36)	**PYY(3-36)**	H	Ile	Lys	Pro	Glu	Ala	Pro	Gly	Glu	Asp	Ala	Ser	Pro	Glu	Glu	Leu	Asn	Arg	Tyr	Tyr	Ala	Ser	Leu	Arg	His	Tyr	Leu	Asn	Leu	Val	Thr	Arg	Gln	Arg	Tyr	NH_2
$(Ala)^{30}$PYY(3-36)	**IP12**	H	Ile	Lys	Pro	Glu	Ala	Pro	Gly	Glu	Asp	Ala	Ser	Pro	Glu	Glu	Leu	Asn	Arg	Tyr	Tyr	Ala	Ser	Leu	Arg	His	Tyr	Leu	Asn	**Ala**	Val	Thr	Arg	Gln	Arg	Tyr	NH_2
$(Ile)^{30}$PYY(3-36)	**IP13**	H	Ile	Lys	Pro	Glu	Ala	Pro	Gly	Glu	Asp	Ala	Ser	Pro	Glu	Glu	Leu	Asn	Arg	Tyr	Tyr	Ala	Ser	Leu	Arg	His	Tyr	Leu	Asn	**Ile**	Val	Thr	Arg	Gln	Arg	Tyr	NH_2
$(Val)^{30}$PYY(3-36)	**IP14**	H	Ile	Lys	Pro	Glu	Ala	Pro	Gly	Glu	Asp	Ala	Ser	Pro	Glu	Glu	Leu	Asn	Arg	Tyr	Tyr	Ala	Ser	Leu	Arg	His	Tyr	Leu	Asn	**Val**	Val	Thr	Arg	Gln	Arg	Tyr	NH_2
$(Ala)^{31}$PYY(3-36)	**IP15**	H	Ile	Lys	Pro	Glu	Ala	Pro	Gly	Glu	Asp	Ala	Ser	Pro	Glu	Glu	Leu	Asn	Arg	Tyr	Tyr	Ala	Ser	Leu	Arg	His	Tyr	Leu	Asn	Leu	**Ala**	Thr	Arg	Gln	Arg	Tyr	NH_2
$(Ile)^{31}$PYY(3-36)	**IP16**	H	Ile	Lys	Pro	Glu	Ala	Pro	Gly	Glu	Asp	Ala	Ser	Pro	Glu	Glu	Leu	Asn	Arg	Tyr	Tyr	Ala	Ser	Leu	Arg	His	Tyr	Leu	Asn	Leu	**Ile**	Thr	Arg	Gln	Arg	Tyr	NH_2
$(Leu)^{31}$PYY(3-36)	**IP17**	H	Ile	Lys	Pro	Glu	Ala	Pro	Gly	Glu	Asp	Ala	Ser	Pro	Glu	Glu	Leu	Asn	Arg	Tyr	Tyr	Ala	Ser	Leu	Arg	His	Tyr	Leu	Asn	Leu	**Leu**	Thr	Arg	Gln	Arg	Tyr	NH_2
$(Leu)^{32}$PYY(3-36)	**IP18**	H	Ile	Lys	Pro	Glu	Ala	Pro	Gly	Glu	Asp	Ala	Ser	Pro	Glu	Glu	Leu	Asn	Arg	Tyr	Tyr	Ala	Ser	Leu	Arg	His	Tyr	Leu	Asn	Leu	Val	**Leu**	Arg	Gln	Arg	Tyr	NH_2
$(Ser)^{32}$PYY(3-36)	**IP19**	H	Ile	Lys	Pro	Glu	Ala	Pro	Gly	Glu	Asp	Ala	Ser	Pro	Glu	Glu	Leu	Asn	Arg	Tyr	Tyr	Ala	Ser	Leu	Arg	His	Tyr	Leu	Asn	Leu	Val	**Ser**	Arg	Gln	Arg	Tyr	NH_2

2.3.3.2 Peptide synthesis

All peptides were synthesised as per the methods described in section 2.3.1.2. The resin used was tricyclic amide linker resin (loading of 0.61mmol/g)(Bachem AG).

2.3.3.3 Peptide cleavage

Peptides were cleaved as per the methods described in section 2.3.1.4.

2.3.3.4 Characterisation of crude peptides

Crude peptides were characterised as per the methods described in section 2.3.1.5.

2.3.3.5 Purification of peptides

Peptides were purified as per the methods described in section 2.3.1.6.

2.3.4 Position 4 and 29 cross linked analogues of PYY(3-36)

2.3.4.1 Peptide design

Cross-linkages between amino acid side chains can take different forms, with one of the most common being a lactam bridge, usually formed between the basic side chain of a lysine residue and the acidic side chain of an aspartic acid or glutamic acid residue. The human sequence of PYY contains a lysine residue close to the N-terminal at position 4 and an asparagine residue close to the C-terminal at position 29. As asparagine is the amidated form of aspartic acid, substituting it for an acidic amino acid should not drastically alter the structure of the peptide.

Table 2.5 The peptide sequences of five PYY(3-36) analogues containing cross linking bridges between amino acids in positions 4 and 29

Dap = α,ß-diaminopropionic acid and is an analogue of lysine, containing three less carbons in its side chain functional group. Residues highlighted in red are those that differ from the endogenous sequence. Disulphide and lactam bridges are shown as red horizontal lines between residues in positions 4 and 29.

Name	Code	N	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	C
PYY(3-36)	**PYY(3-36)**	H	Ile	Lys	Pro	Glu	Ala	Pro	Gly	Glu	Asp	Ala	Ser	Pro	Glu	Glu	Leu	Asn	Arg	Tyr	Tyr	Ala	Ser	Leu	Arg	His	Tyr	Leu	Asn	Leu	Val	Thr	Arg	Gln	Arg	Tyr	NH_2
(Cys^4, Cys^{29})-PYY(3-36) (disulphide bridge)	**IP20**	H	Ile	**Cys**	Pro	Glu	Ala	Pro	Gly	Glu	Asp	Ala	Ser	Pro	Glu	Glu	Leu	Asn	Arg	Tyr	Tyr	Ala	Ser	Leu	Arg	His	Tyr	Leu	**Cys**	Leu	Val	Thr	Arg	Gln	Arg	Tyr	NH_2
(Cyclo(Lys^4–Asp^{29}), Asp^{29})-PYY(3-36)		H	Ile	Lys	Pro	Glu	Ala	Pro	Gly	Glu	Asp	Ala	Ser	Pro	Glu	Glu	Leu	Asn	Arg	Tyr	Tyr	Ala	Ser	Leu	Arg	His	Tyr	Leu	**Asp**	Leu	Val	Thr	Arg	Gln	Arg	Tyr	NH_2
Cyclo(Lys^4–Glu^{29}), Glu^{29})-PYY(3-36)	**4060994**	H	Ile	Lys	Pro	Glu	Ala	Pro	Gly	Glu	Asp	Ala	Ser	Pro	Glu	Glu	Leu	Asn	Arg	Tyr	Tyr	Ala	Ser	Leu	Arg	His	Tyr	Leu	**Glu**	Leu	Val	Thr	Arg	Gln	Arg	Tyr	NH_2
(Cyclo(Dap^4–Asp^{29}),Dap^4, Asp^{29})-PYY(3-36)		H	Ile	**Dap**	Pro	Glu	Ala	Pro	Gly	Glu	Asp	Ala	Ser	Pro	Glu	Glu	Leu	Asn	Arg	Tyr	Tyr	Ala	Ser	Leu	Arg	His	Tyr	Leu	**Asp**	Leu	Val	Thr	Arg	Gln	Arg	Tyr	NH_2
(Cyclo(Dap^4–Glu^{29}), Dap^4, Glu^{29})-PYY(3-36)		H	Ile	**Dap**	Pro	Glu	Ala	Pro	Gly	Glu	Asp	Ala	Ser	Pro	Glu	Glu	Leu	Asn	Arg	Tyr	Tyr	Ala	Ser	Leu	Arg	His	Tyr	Leu	**Glu**	Leu	Val	Thr	Arg	Gln	Arg	Tyr	NH_2

The PYY(3-36) analogues described in Table 2.5 were designed to contain cross linkages between different amino acid residues at positions 4 and 29.

To help assess the feasibility of forming a cross linking bridge between positions 4 and 29, an analogue containing a disulphide bridge between cysteine residues in these positions was also synthesised.

2.3.4.2 Peptide synthesis - (Cys^4, Cys^{29})-PYY(3-36) (disulphide bridge)

The peptide was synthesised as per the methods described in section 2.3.2.2. The amino acids in positions 4 and 29 were both coupled as Fmoc-Cys(Trt)-OH (Bachem AG). The resin used was tricyclic amide linker resin (loading of 0.61mmol/g) and was purchased from Bachem AG.

2.3.4.3 Peptide cleavage - (Cys^4, Cys^{29})-PYY(3-36) (disulphide bridge)

The peptide was cleaved as per the methods described in section 2.3.1.4. Due to the presence of cysteine residues in the sequence, the filtered peptide solution was directly precipitated into diethyl ether.

2.3.4.4 Characterisation of crude peptide - (Cys^4, Cys^{29})-PYY(3-36) (disulphide bridge)

The crude peptide was characterised as per the methods described in section 2.3.1.5. In addition, an Ellmans test was performed on a small sample of crude material. This involved dissolving the peptide in 200µL of 5,5′-dithiobis(2-nitrobenzoic acid) in 0.1M disodium hydrogen phosphate (both Sigma Aldrich). A positive result indicates the presence of sulphydryl groups in the peptide.

2.3.4.5 Disulphide bridge formation - (Cys^4, Cys^{29})-PYY(3-36) (disulphide bridge)

The crude peptide was dissolved in 3mL of TFA and diluted with 30mL of 0.1% TFA/acetonitrile/water. This solution was filtered and added to 1.1L of 1.0M Urea solution (VWR). The pH of the solution was measured to confirm it was pH7 and a sample was then analysed by reverse phase HPLC.

A 0.01M solution of potassium ferricyanide ($K_3[Fe(CN)_6]$) (BDH, Poole, UK) was added to the peptide solution in a drop wise manner until the yellow/green colour was retained. The solution was again analysed by HPLC and left to mix at room temperature for 1 hour. After this time had elapsed, a sample of the solution was analysed by HPLC as a co-injection with a sample of the reaction solution at pH7. Further $K_3[Fe(CN)_6]$ solution was added to the reaction and it was left for another hour. At this point the reaction was stopped with the addition of 50% acetic acid (Romil) and the pH reached 4.

To this solution, 80mL of acetonitrile was added and the reaction mixture was then loaded onto a Companion Combi Flash Chromatography system (Teledyne Isco, Lincoln, USA) using a C-18 40g column (Daiso, 50 µm particle size, 200 Å pore size). A linear AB gradient program of 10-35% B in 1 min followed by 35-100% B in 40 min at a flow rate of 40mL/min was applied. Eluent A was 0.1% TFA/water and eluent B was 0.1% TFA in 60% acetonitrile/water.

The collected fractions were identified by MALDI MS and analytical reverse phase HPLC. The relevant fractions were then pooled together and lyophilised overnight (-50^{o}C, 6 mbar).

2.3.4.6 Purification of peptide -(Cys^{4}, Cys^{29})-PYY(3-36) (disulphide bridge)

The peptide was purified as per the methods described in section 2.3.1.6 using a C-18 column (Daiso Bio 250 x 21.2mm, 10µm particle size and 200Å pore size). A second purification step was carried out using a gradient program of 10-50% in 4 min and 50-100% in 70 min.

2.3.4.7 Peptide synthesis - (Cyclo(Lys^{4}–Asp^{29}), Asp^{29})-PYY(3-36)

The peptide was synthesised as per the methods described in section 2.3.2.2. The synthesiser used was a 433A single peptide synthesiser (Applied Biosystems). The residues in positions 4 and 29 were introduced as Fmoc-Lys(Mtt)-OH and Fmoc-Asp(OPip)-OH respectively (both Bachem AG). The final residue was introduced as Boc-Ile-OH.0.5H_2O (Novabiochem). The resin used was tricyclic amide linker resin (loading of 0.61mmol/g) (Bachem AG).

Removal of the Fmoc protecting group was carried out with 20% piperidine in N-methyl pyrrolidone (NMP) (M56 Chemicals) followed by sequential washes with NMP. All amino acid derivatives (10-fold excess), TBTU and HOBt (9-fold excess) and DIPEA (20-fold excess) were dissolved in NMP.

Upon completion of the automated synthesis, a sample of resin was cleaved in TFA for 2 ½ hours. The solution was filtered, diluted with acetonitrile/water and analysed by HPLC and MALDI MS. A second sample of resin was taken and a Kaiser test performed as described in section 2.3.1.2 to confirm the presence of all protecting groups.

2.3.4.8 Cyclisation of resin bound peptide - (Cyclo(Lys^{4}–Asp^{29}), Asp^{29})-PYY(3-36)

The amino acids in positions 4 and 29 were selectively deprotected by performing repeated washes (9 x 5min) with a solution of 1% TFA/ 5% triisopropylsilane (TIS) (Sigma Aldrich) in dichloromethane (DCM) (Avantor). Upon completion of these washes a Kaiser test as described in section 2.3.1.2 was performed to confirm removal of the protecting groups.

Lactam bridge formation was achieved by reacting the resin bound peptide with a solution of 1 molar equivalent of 1-hydroxy-7-azabenzotriazole (HOAt) (Genscript, Piscataway, USA) and 1.5 molar equivalents of diisopropylcarbodiimide (DIC) (Sigma Aldrich) in DMF for 15 hours. A Kaiser test was performed and the reaction was repeated until complete according to a Kaiser test.

2.3.4.9 Peptide cleavage - (Cyclo(Lys^{4}–Asp^{29}), Asp^{29})-PYY(3-36)

The peptide was cleaved as per the methods described in section 2.3.1.4.

2.3.4.10 Characterisation of crude peptide - (Cyclo(Lys^{4}–Asp^{29}), Asp^{29})-PYY(3-36)

The crude peptide was characterised as per the methods described in section 2.3.1.5. A second C-18 column was used for characterisation (Daiso Bio, 250 x 4.6mm, 5 µm particle size, and 200 Å pore size).

2.3.4.11 Purification of peptide - (Cyclo(Lys^{4}–Asp^{29}), Asp^{29})-PYY(3-36)

The peptide was loaded onto a Companion Combi Flash Chromatography system with a C-18 40g column (Daiso, 50 µm particle size, 200 Å pore size). A linear AB

gradient program of 10-85% B in 22 min at a flow rate of 40mL/min. Eluent A was 0.1% TFA/water and eluent B was 0.1% TFA in 60% acetonitrile/water. The collected fractions were identified by MALDI MS and analytical reverse phase HPLC with a Daiso column. The relevant fractions were then pooled together and lyophilised overnight (-50^{o}C, 6 mbar).

2.3.4.12 Peptide synthesis - (Cyclo(Lys^{4}–Glu^{29}), Glu^{29})-PYY(3-36)

The peptide was synthesised as per the methods described in section 2.3.4.7. Residue 29 was introduced as Fmoc-Glu(Opip)-OH (Bachem AG).

2.3.4.13 Peptide synthesis - (Cyclo(Dap^{4}–Asp^{29}), Dap^{4}, Asp^{29})-PYY(3-36)

The peptide was synthesised as per the methods described in section 2.3.2.2. The residues in positions 4 and 29 were introduced as Fmoc-Dap(Mtt)-OH (Novabiochem) and Fmoc-Asp(Opip)-OH (Bachem AG) respectively, whilst the final residue was introduced as Boc-Ile-OH.0.5H_2O (Novabiochem). The resin used was tricyclic amide linker resin (loading of 0.55mmol/g) (Bachem AG). The peptide was synthesised at a scale of 0.05mmol.

2.3.4.14 Cyclisation of resin bound peptide - (Cyclo(Dap^{4}–Asp^{29}), Dap^{4}, Asp^{29})-PYY(3-36)

The resin bound peptide was cyclised as per the methods described in section 2.3.4.8

2.3.4.15 Peptide cleavage - (Cyclo(Dap^{4}–Asp^{29}), Dap^{4}, Asp^{29})-PYY(3-36)

The peptide was cleaved as per the methods described in section 2.3.1.4.

2.3.4.16 Characterisation of crude peptide - (Cyclo(Dap^4–Asp^{29}), Dap^4, Asp^{29})-PYY(3-36)

The crude peptide was characterised as per the methods described in section 2.3.1.5.

2.3.4.17 Peptide synthesis - (Cyclo(Dap^4–Glu^{29}), Dap^4, Glu^{29})-PYY(3-36)

The peptide was synthesised as per the methods described in section 2.3.4.13. The residue in position 29 was introduced as Fmoc-Glu(OPip)-OH (Bachem AG).

2.3.4.18 Peptide synthesis II - (Cyclo(Lys^4–Glu^{29}), Glu^{29})-PYY(3-36)

The peptide was synthesised as per the methods described in section 2.3.4.12. The resin used was TentaGel R RAM (loading of 0.2mmol/g) (Rapp Polymere (Tübingen, Germany). The peptide was synthesised at a scale of 0.05mmol.

2.3.4.19 Cyclisation of resin bound peptide – (Cyclo(Lys^4–Glu^{29}), Glu^{29})-PYY(3-36)

The resin bound peptide was cyclised as per the methods described in section 2.3.4.8.

2.3.4.20 Peptide cleavage - (Cyclo(Lys^4–Glu^{29}), Glu^{29})-PYY(3-36)

The peptide was cleaved as per the methods described in section 2.3.1.4.

2.3.4.21 Characterisation of crude peptide - (Cyclo(Lys^4–Glu^{29}), Glu^{29})-PYY(3-36)

The crude peptide was characterised as per the methods described in section 2.3.1.5.

2.3.4.22 Peptide synthesis III - (Cyclo(Lys^{4}–Glu^{29}), Glu^{29})-PYY(3-36)

The peptide was synthesised as per the methods described in section 2.3.4.13. The residues in positions 4 and 29 were introduced as Fmoc-Lys(Mtt)-OH and Fmoc-Glu(Opip)-OH respectively (Bachem AG). The peptide was synthesised in two different reaction channels on the synthesiser. In one of the reaction channels, the amino acids in positions 22 and 23 were introduced as the pseudoproline dipeptide Fmoc-Ala-Ser(Psi(Me,Me)pro)-OH (Novabiochem). The resin used was TentaGel R RAM (loading of 0.19mmol/g) (Rapp Polymere). The peptide was synthesised at a scale of 2 x 0.05mmol and upon completion of the automated synthesis the two reaction vessels were kept separate

2.3.4.23 Cyclisation of the resin bound peptide - (Cyclo(Lys^{4}–Glu^{29}), Glu^{29})-PYY(3-36)

The resin bound peptide was cyclised as per the methods described in section 2.3.4.8. Lactam bridge formation was achieved by reacting the peptide resins with a solution of 0.98 molar equivalents of TBTU and 2 molar equivalents of DIPEA for 4 hours.

2.3.4.24 Peptide synthesis IV - (Cyclo(Lys^{4}–Glu^{29}), Glu^{29})-PYY(3-36)

The peptide was synthesised as per the methods described in section 2.3.2.2. The residues in positions 4 and 29 were introduced as Fmoc-Lys(Aloc)-OH and Fmoc-Glu(allyl ester)-OH respectively (Bachem AG) whilst the final residue was introduced as Boc-Ile-OH (Bachem AG). The resin used was Xanthenyl linker resin (loading of 0.53mmol/g) (Bachem AG). Also synthesised was the peptide (Glu)^{29}-PYY(3-36) in which both Lys^{4} and Glu^{29} residues were introduced as the standard Fmoc derivatives.

Upon completion, samples of both resins were cleaved in TFA for 2 ½ hours, filtered, diluted and analysed by HPLC and MALDI MS as described in section 2.3.1.5.

2.3.4.25 Partial deprotection of resin bound peptide - (Cyclo(Lys^{4}–Glu^{29}), Glu^{29})-PYY(3-36)

Selective deprotection of the Aloc and allyl ester protecting groups was achieved by dissolving 0.4eq of Tetrakis(triphenylphosphine)palladium(0) and 24eq Phenylsilane (both Sigma Aldrich) in DCM, adding it to the resin and mixing for 2 hours. The reagents were removed by filtration and the resin washed repeatedly with DCM and then 0.03M sodium diethyldithiocarbamate trihydrate (Sigma Aldrich) in DMF to remove any remaining palladium catalyst. Successful removal of both protecting groups was confirmed by Kaiser test as described in section 2.3.1.2, and by cleaving a sample of resin in reagent K for 2 ½ hours, filtering and precipitating into diethyl ether. The precipitate was immediately isolated, washed with diethyl ether and dried under vacuum before being dissolved in 0.1%TFA in acetonitrile/water. This solution was analysed by HPLC and MALDI MS as described in section 2.3.1.5.

2.3.4.26 Cleavage of partially protected peptide - (Cyclo(Lys^{4}–Glu^{29}), Glu^{29})-PYY(3-36)

The partially protected peptide was cleaved by washing the resin with 10mL/g of 2% TFA in DCM for 3 minutes. After this time, the resin was filtered and the filtrate collected in a flask to which 1.01 molar equivalents (relative to TFA) of ice cold DIPEA was added to neutralise. This process was repeated a further six times and the filtrates combined. The resin was then washed repeatedly with DCM until the pH of the filtrate reached 7. Each DCM wash was neutralised and added to the cleavage

filtrate solution. This solution was then extracted three times with water and then three times with saturated sodium chloride solution. The organic layer was dried with sodium sulphate (BDH) and filtered before being concentrated on a rotary evaporator. The resultant residue was precipitated with the addition of ice cold diethyl ether. The precipitate was stored at 4^{o}C overnight and was collected by filtration, washed with ice-cold diethyl ether, and allowed to dry at room temperature.

2.3.4.27 Characterisation of partially protected peptide - (Cyclo(Lys^{4}–Glu^{29}), Glu^{29})-PYY(3-36)

The partially protected peptide was characterised as per the methods described in section 2.3.1.5. Eluent B was 0.1% TFA in 95% acetonitrile/water. Mass was confirmed using a Voyager Matrix Assisted Laser Desorption/Ionisation mass spectrometer MALDI-MS.

2.3.4.28 Cyclisation of partially protected peptide - (Cyclo(Lys^{4}–Glu^{29}), Glu^{29})-PYY(3-36)

The partially protected peptide (304mg) was dissolved in 5mL of DMF and diluted to 25mL with DCM. A solution was made of 10 molar equivalents of HOAt (64mg) and 20 molar equivalents of DIC (143µL) in 300mL of DCM. The peptide solution was added to the HOAt/DIC solution using a Gilson 307 HPLC piston pump at a flow rate of 0.03mL/min. Upon completion of the peptide addition, the reaction solution was analysed by HPLC and MALDI MS. The DCM was then removed via rotary evaporation.

2.3.4.29 Cleavage of peptide - (Cyclo(Lys^{4}–Glu^{29}), Glu^{29})-PYY(3-36)

Following removal of DCM, the remaining residue was dissolved in solution K (100mL) and cleaved as per the methods described in section 2.3.1.4.

2.3.4.30 Characterisation of crude peptide – (Cyclo(Lys^{4}–Glu^{29}), Glu^{29})-PYY(3-36)

The crude peptide was characterised as per the methods described in section 2.3.1.5.

2.4 Results

2.4.1 Position 25 and 26 PYY(3-36) analogues

The ten PYY(3-36) analogues with altered residues at position 25 and 26 were all successfully produced at purities above 95% by reverse phase HPLC on both TFA and triethylammonium phosphate (TEAP) buffer systems. The quantities, purities and peptide content (determined by amino acid analysis) of each analogue produced are summarised in Table 2.6.

Table 2.6 The quantities, purities and peptide content achieved for ten position 25 and 26 PYY(3-36) analogues

% Yield = actual yield (mg)/theoretical yield (mg). Theoretical yield = synthesis scale (mmol) x molecular weight of peptide. Abbreviations: TFA, trifluororacetic acid; TEAP, triethylammonium phosphate.

Analogue	Code	Yield (mg)	Yield (%)	Purity (TFA system) (%)	Purity (TEAP system) (%)	Peptide content (%)
PYY(3-36)	**-**	**83.0**	**20.5**	**98.0**	**96.2**	**76.9**
(Homoarg)^{25}PYY(3-36)	**IP1**	40	19.7	97.7	95.1	67.2
(D-Arg)^{25}PYY(3-36)	**IP2**	40	19.8	96.9	95.2	69.0
(Arg(Me)$_2$symm)^{25}PYY(3-36)	**IP3**	33.5	16.4	97.3	95.6	81.9
(Arg(Me)$_2$asymm)^{25}PYY(3-36)	**IP4**	18.5	9.1	95.0	96.1	72.2
(Cit)^{25}PYY(3-36)	**IP5**	27	13.3	98.4	97.1	75.9
(D-Cit)^{25}PYY(3-36)	**IP6**	40.6	20.0	96.1	95.7	86.2
(His(1-Me))^{26}PYY(3-36)	**IP7**	36.4	17.9	97.0	98.0	76.4
(His(3-Me))^{26}PYY(3-36)	**IP8**	15.2	7.5	97.6	96.4	82.7
(D-His)^{26}PYY(3-36)	**IP9**	33.2	16.4	97.2	97.2	73.4
(Phe-Gu)^{25}PYY(3-36)	**IP10**	31.9	15.6	98.9	95.4	83.3
Biotinyl-PYY(3-36)	**IP11**	189.7	7.4	98.2	95.3	85.0

2.4.2 N-terminal pegylated PYY(3-36) analogues

The three PYY(3-36) analogues were all successfully produced at purities above 95% by reverse phase HPLC in a TFA buffer system. The quantities and purities of each analogue produced are summarised in Table 2.7. Due to financial restraints, it was not possible to submit these and subsequent analogues for full Quality Control (QC)

analysis. Therefore, the same level of analysis as for the previous set of analogues has not been acquired.

Table 2.7 The quantities and purities achieved for three N-terminally pegylated PYY(3-36) analogues.

% Yield = actual yield (mg)/theoretical yield (mg). Theoretical yield = synthesis scale (mmol) x molecular weight of peptide. Abbreviations: TFA, trifluoroacetic acid.

Analogue	Code	Yield (mg)	Yield (%)	Purity (TFA system) (%)
AEEAc-PYY(3-36)	**4075004**	68.0	16.2	98.1
Aminoethyl-PEG4-carbonyl-PYY(3-36)	**4075005**	143.2	33.3	96.7
Aminoethyl-PEG6-carbonyl-PYY(3-36)	**4075006**	111.8	25.4	97.63

2.4.3 Position 30, 31 and 32 PYY(3-36) analogues

The eight PYY(3-36) analogues with altered residues in position 30,31 and 32 were all successfully produced at purities above 95% by reverse phase HPLC in a TFA buffer system. The quantities and purities of each analogue produced are summarised in Table 2.8.

Table 2.8 The quantities and purities achieved for eight position 30, 31 & 32 PYY(3-36) analogues.

% Yield = actual yield (mg)/theoretical yield (mg). Theoretical yield = synthesis scale (mmol) x molecular weight of peptide. Abbreviations: TFA, trifluoroacetic acid.

Analogue	Code	Yield (mg)	Yield (%)	Purity (TFA system) (%)
(Ala)^{30}PYY(3-36)	**IP12**	31.4	15.7	96.7
(Ile)^{30}PYY(3-36)	**IP13**	43.0	21.2	95.0
(Val)^{30}PYY(3-36)	**IP14**	30.2	15.0	98.0
(Ala)^{31}PYY(3-36)	**IP15**	26.9	13.4	98.2
(Ile)^{31}PYY(3-36)	**IP16**	39.3	19.3	98.0
(Leu)^{31}PYY(3-36)	**IP17**	38.0	18.7	97.8
(Leu)^{32}PYY(3-36)	**IP18**	38.0	18.7	95.9
(Ser)^{32}PYY(3-36)	**IP19**	28.3	14.0	98.3

2.4.4 Position 4 and 29 cross linked PYY(3-36) analogues

2.4.4.1 (Cys4, Cys29)PYY(3-36) (disulphide bridge)

This analogue was successfully produced with a final purity of 91% by reverse phase HPLC. The quantities and purities achieved, along with the results of the Ellmans tests, at the different stages of oxidation and purification are summarised in Table 2.9.

Table 2.9 The quantities, purities and Ellmans test results, achieved at the different stages of producing (Cys4, Cys29)PYY(3-36) (disulphide bridge)

% Yield = actual yield (mg)/theoretical yield (mg). Theoretical yield = synthesis scale (mmol) x molecular weight of peptide.

Stage	Yield (mg)	Yield (%)	Purity (TFA system) (%)	Ellmans test
Crude	215	53.6	67.5	+
After oxidation	51.9	12.9	74.4	–
Purification 1	30.4	7.6	91.1	–
Purification 2	24	6.0	90.5	–

2.4.4.2 (Cyclo(Lys4–Asp29), Asp29)-PYY(3-36)

The synthesis of this peptide was unsuccessful. Following purification of the crude peptide via Flash Chromatography, analysis by MALDI MS of the collected fractions failed to detect the presence of the target peptide. All of the fractions containing peptidic material were combined and lyophilised. The HPLC chromatogram of this material is shown in Figure 2.4

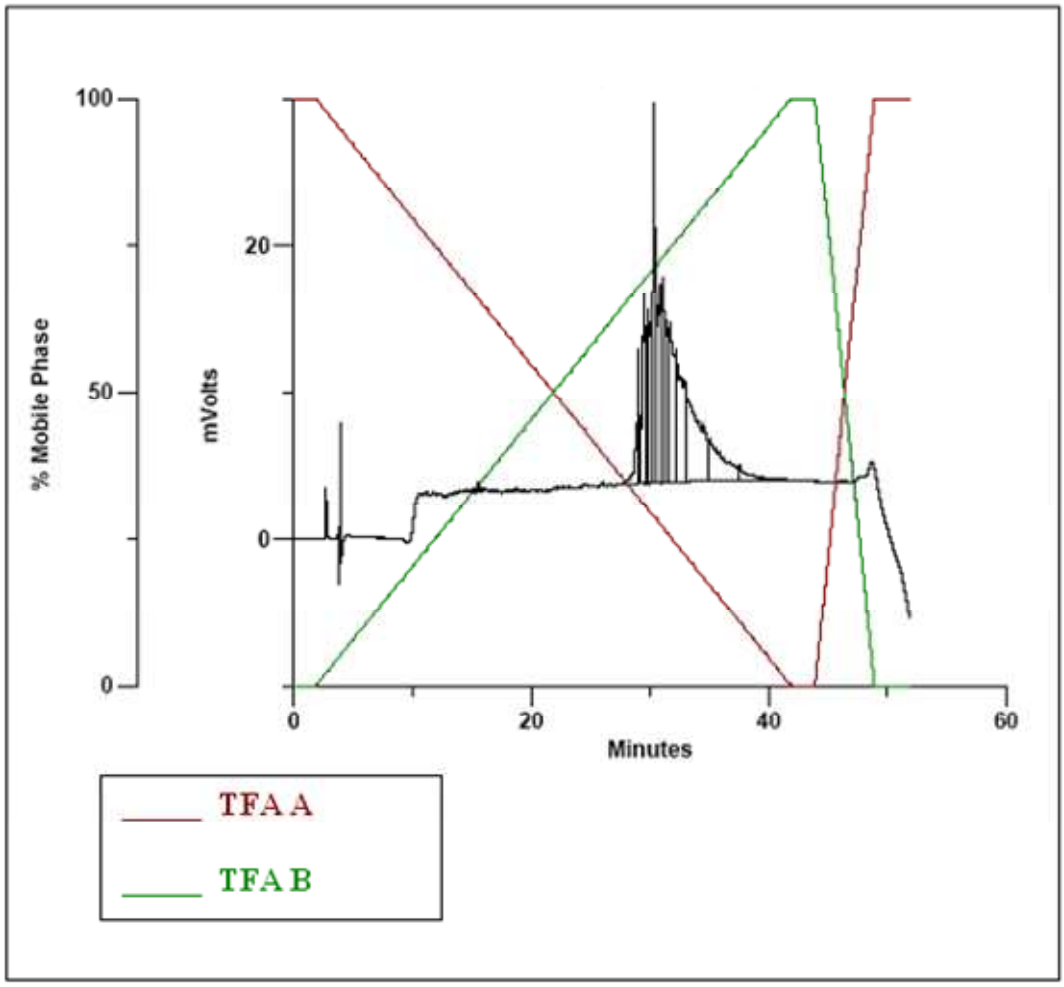


Figure 2.4 Representative reverse phase HPLC absorption profile of the material recovered from the purification of (Cyclo(Lys^{4}–Asp^{29}), Asp^{29})-PYY(3-36)

The peptide was dissolved in 0.1% TFA/water/acetonitrile and analysed by reverse phase HPLC using an analytical C-18 column (Vydac 218TP54, 250 x 4.6mm, 5 µm particle size, and 300 Å pore size) and a linear AB gradient of 0–100% for B over 40 min at a flow rate of 1mL/min, where eluent A was 0.1% TFA/water and eluent B was 0.1% TFA in 60% acetonitrile/water. Peak detection was by spectrophotometry at a wavelength of 210 nm.

2.4.4.3 Cyclo(Lys^{4}–Glu^{29}), Glu^{29})-PYY(3-36)

The synthesis of this peptide was unsuccessful. Upon completion of the automated synthesis, analysis by HPLC and MALDI MS of a cleaved sample of resin showed the quality of the linear peptide to be poor, so synthesis was stopped. The HPLC

chromatogram is shown in Figure 2.5 and the MALDI mass spectrum is shown in Figure 2.6.

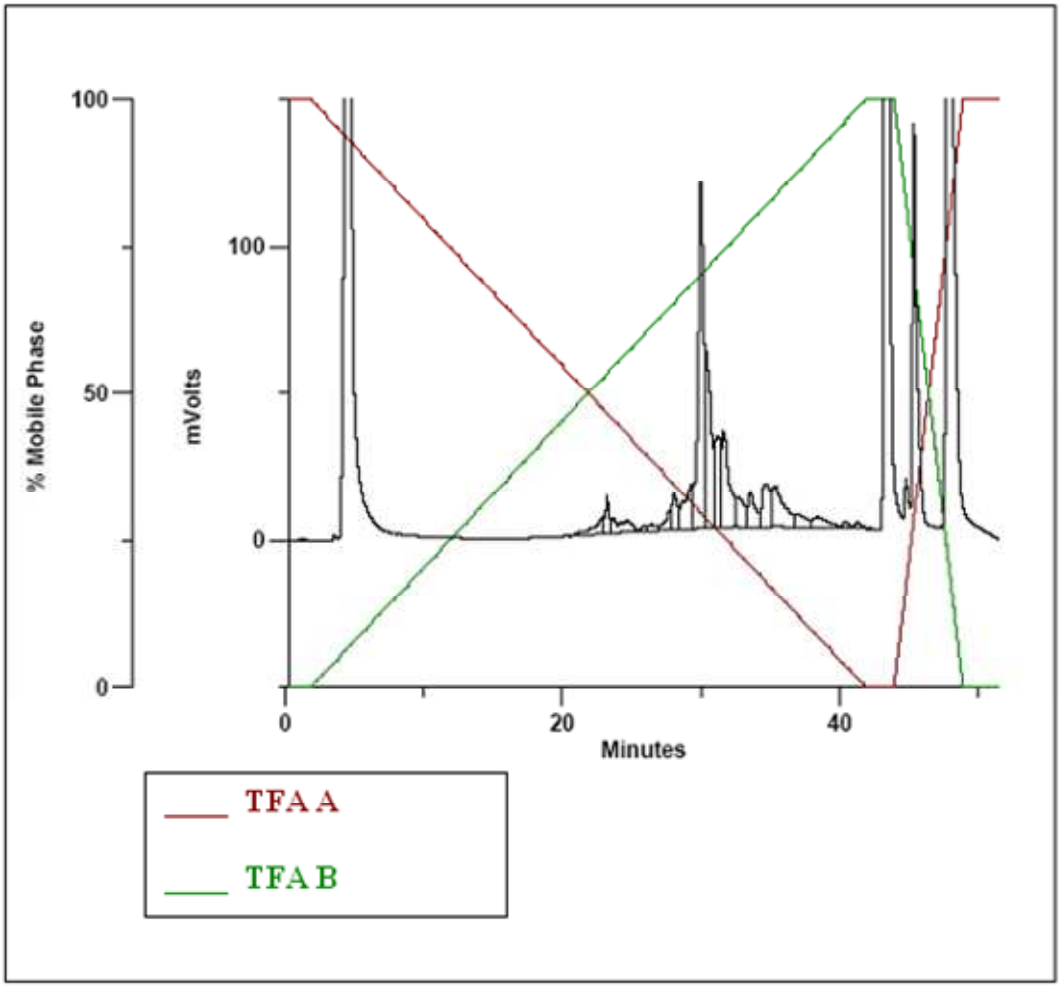


Figure 2.5 Representative reverse phase HPLC absorption profile of the peptide (Glu)^{29}-PYY(3-36) following automated SPPS

A sample of peptide resin was cleaved for 2 hours in conc. TFA. The solution was filtered and diluted with acetonitrile/water. This solution was analysed by reverse phase HPLC using an analytical C-18 column (Vydac 218TP54, 250 x 4.6mm, 5 µm particle size, and 300 Å pore size) and a linear AB gradient of 0–100% for B over 40 min at a flow rate of 1mL/min, where eluent A was 0.1% TFA/water and eluent B was 0.1% TFA in 60% acetonitrile/water. Peak detection was by spectrophotometry at a wavelength of 210nm.

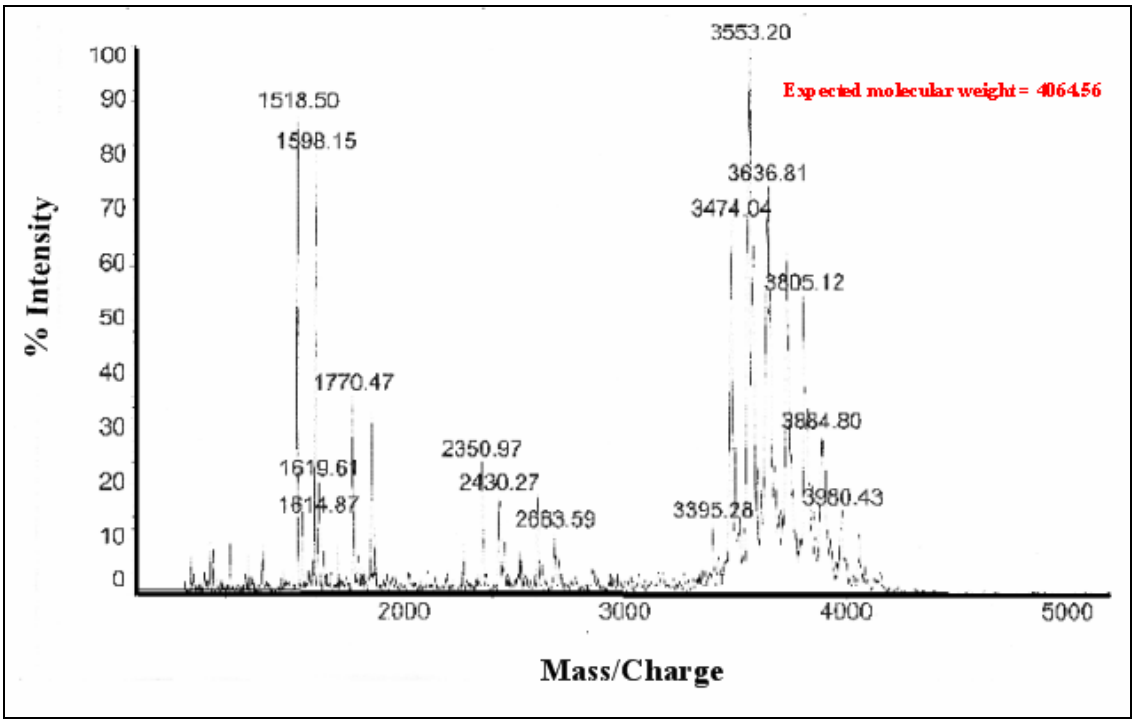


Figure 2.6 Representative MALDI mass spectrum of the peptide (Glu)^{29}-PYY(3-36) following automated SPPS

A sample of peptide resin was cleaved for 2 hours in conc. TFA. The solution was filtered and diluted with acetonitrile/water. The solution was analysed by a Kompact Probe MALDI mass spectrometer in positive voltage mode. The solution was co-crystallised with α-cyano-4-hydroxycinnamic acid (CHCA) matrix compound. The vertical axis represents the relative signal intensity of the molecular ions detected and horizontal axis represents mass to charge ratio (M/Z). The expected molecular weight could not be detected.

2.4.4.4 (Cyclo(Dap^{4}–Asp^{29}),Dap^{4}, Asp^{29})-PYY(3-36)

The synthesis of this peptide was unsuccessful. Analysis by HPLC and MALDI MS of the crude peptide, failed to detect the presence of the target peptide. The HPLC chromatogram is shown in Figure 2.7.

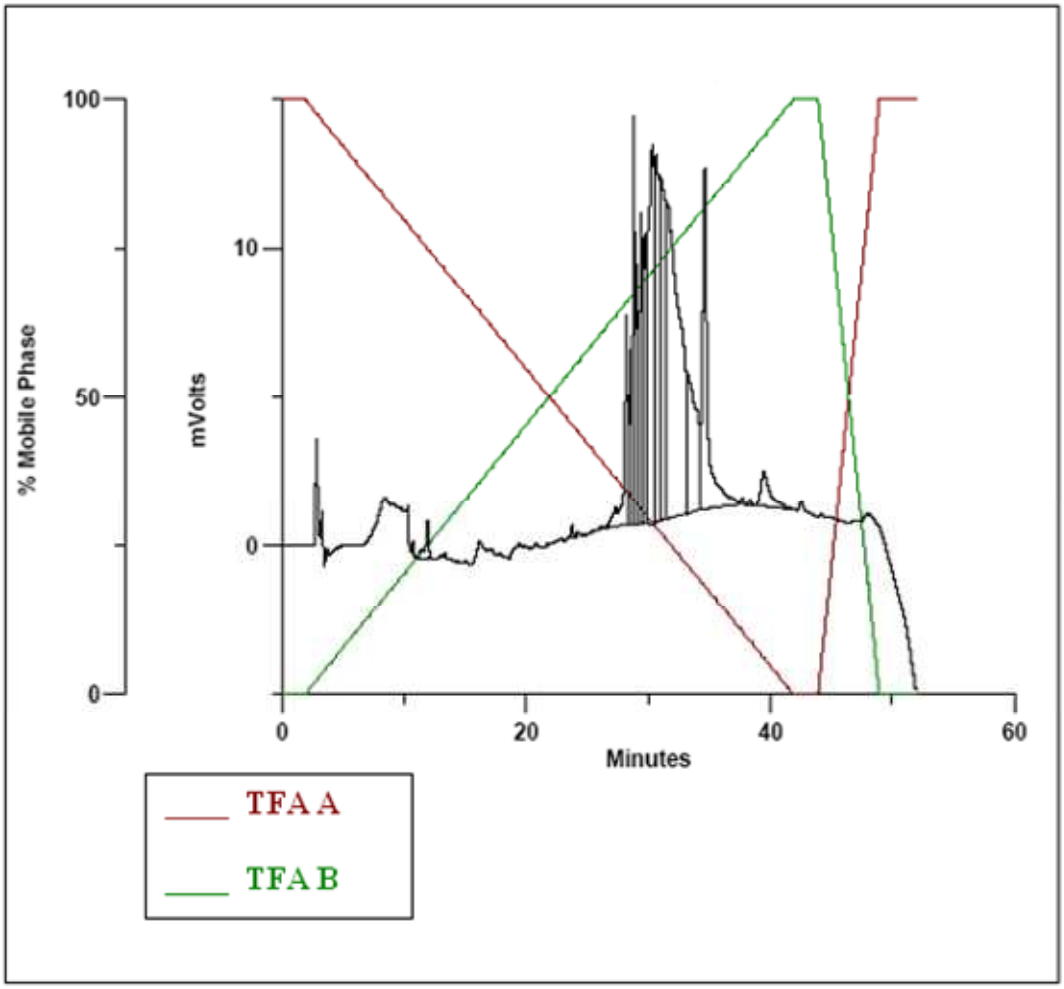


Figure 2.7 Representative reverse phase HPLC absorption profile of the crude peptide (Cyclo(Dap^{4}, Asp^{29}), Dap^{4}, Asp^{29})-PYY(3-36)

A sample of crude peptide was dissolved in 0.1% TFA/water/acetonitrile and analysed by reverse phase HPLC using an analytical C-18 column (Vydac 218TP54, 250 x 4.6mm, 5 µm particle size, and 300 Å pore size) and a linear AB gradient of 0–100% for B over 40 min at a flow rate of 1mL/min, where eluent A was 0.1% TFA/water and eluent B was 0.1% TFA in 60% acetonitrile/water. Peak detection was by spectrophotometry at a wavelength of 210 nm.

2.4.4.5 (Cyclo(Dap^{4}–Glu^{29}), Dap^{4}, Glu^{29})-PYY(3-36)

The synthesis of this peptide was unsuccessful. Upon completion of the automated synthesis, analysis by HPLC of a cleaved sample of resin showed the purity of the

linear peptide to be poor, so synthesis was stopped. The HPLC chromatogram is shown in Figure 2.8.

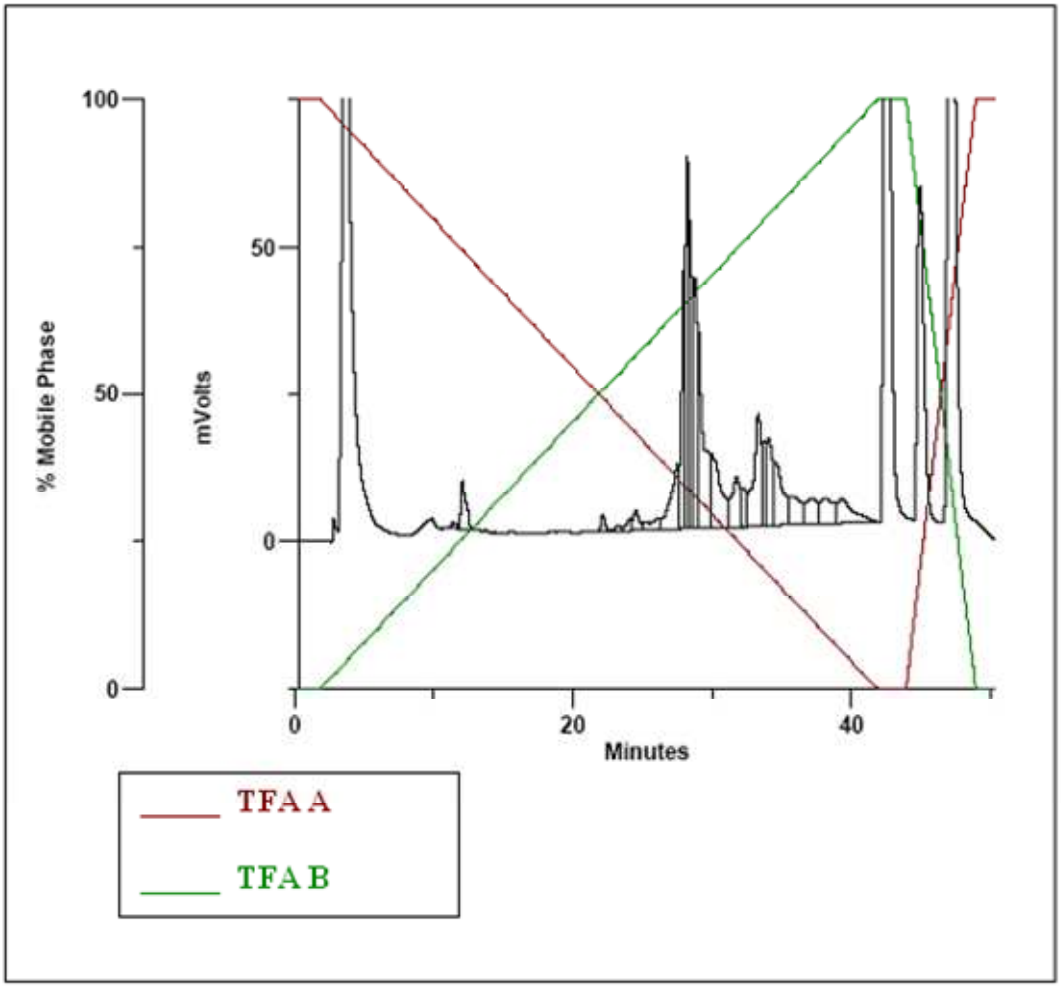


Figure 2.8 Representative reverse phase HPLC absorption profile of the peptide (Dap^{4}, Glu^{29})-PYY(3-36) following automated SPPS

A sample of peptide resin was cleaved for 2 hours in conc. TFA. The solution was filtered and diluted with acetonitrile/water. This solution was analysed by reverse phase HPLC using an analytical C-18 column (Vydac 218TP54, 250 x 4.6mm, 5 µm particle size, and 300 Å pore size) and a linear AB gradient of 0–100% for B over 40 min at a flow rate of 1mL/min, where eluent A was 0.1% TFA/water and eluent B was 0.1% TFA in 60% acetonitrile/water. Peak detection was by spectrophotometry at a wavelength of 210 nm.

2.4.4.6 (Cyclo(Lys^{4}–Glu^{29}), Glu^{29})-PYY(3-36) (Synthesis II)

The synthesis of this peptide was unsuccessful. Analysis by HPLC and MALDI MS of the crude peptide, failed to detect the presence of the target peptide. The HPLC chromatogram of the crude peptide is shown in Figure 2.9 and the MALDI mass spectrum is shown in Figure 2.10.

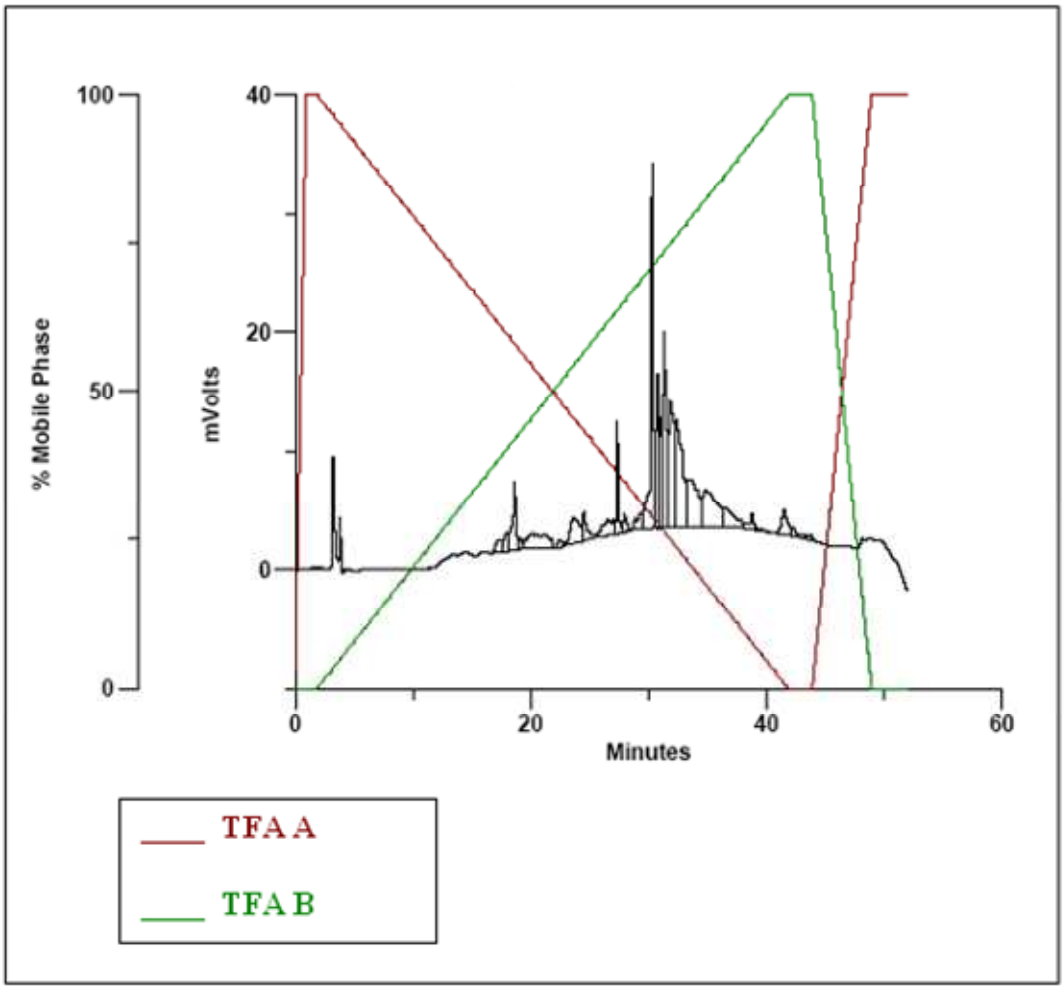


Figure 2.9 Representative reverse phase HPLC absorption profile of the crude peptide (Cyclo(Lys^{4}-Glu^{29}), Glu^{29})-PYY(3-36)

A sample of crude peptide was dissolved in 0.1% TFA/water/acetonitrile and analysed by reverse phase HPLC using an analytical C-18 column (Vydac 218TP54, 250 x 4.6mm, 5 µm particle size, and 300 Å pore size) and a linear AB gradient of 0–100% for B over 40 min at a flow rate of 1mL/min, where eluent A was 0.1% TFA/water and eluent B was 0.1% TFA in 60% acetonitrile/water. Peak detection was by spectrophotometry at a wavelength of 210 nm.

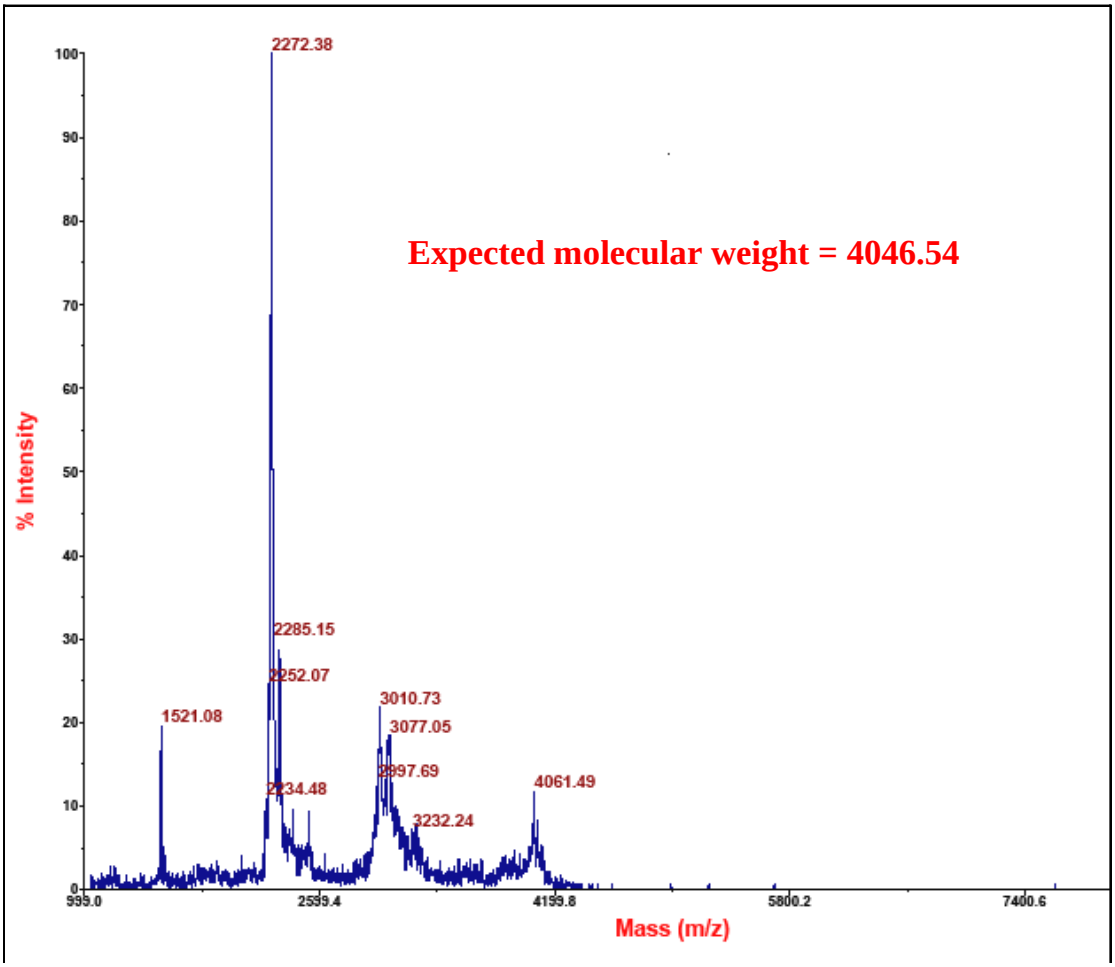


Figure 2.10 Representative MALDI mass spectrum of the crude peptide (Cyclo(Lys^{4}-Glu^{29}), Glu^{29})-PYY(3-36)

A sample of crude peptide was dissolved in 0.1% TFA/acetonitrile/water. The solution was analysed by a Voyager DE MALDI mass spectrometer in positive voltage mode. The solution was co-crystallised with α-cyano-4-hydroxycinnamic acid (CHCA) matrix compound. The vertical axis represents the relative signal intensity of the molecular ions detected and horizontal axis represents mass to charge ratio (M/Z). The expected molecular weight could not be detected.

2.4.4.7 (Cyclo(Lys^{4}–Glu^{29}), Glu^{29})-PYY(3-36) (Synthesis III)

The synthesis of this peptide was unsuccessful. Upon completion of the cyclisation reactions, analysis by HPLC and MALDI MS of cleaved samples of the two resins showed the purity of the crude peptide to be poor. The HPLC chromatograms of both samples are shown in Figure 2.11.

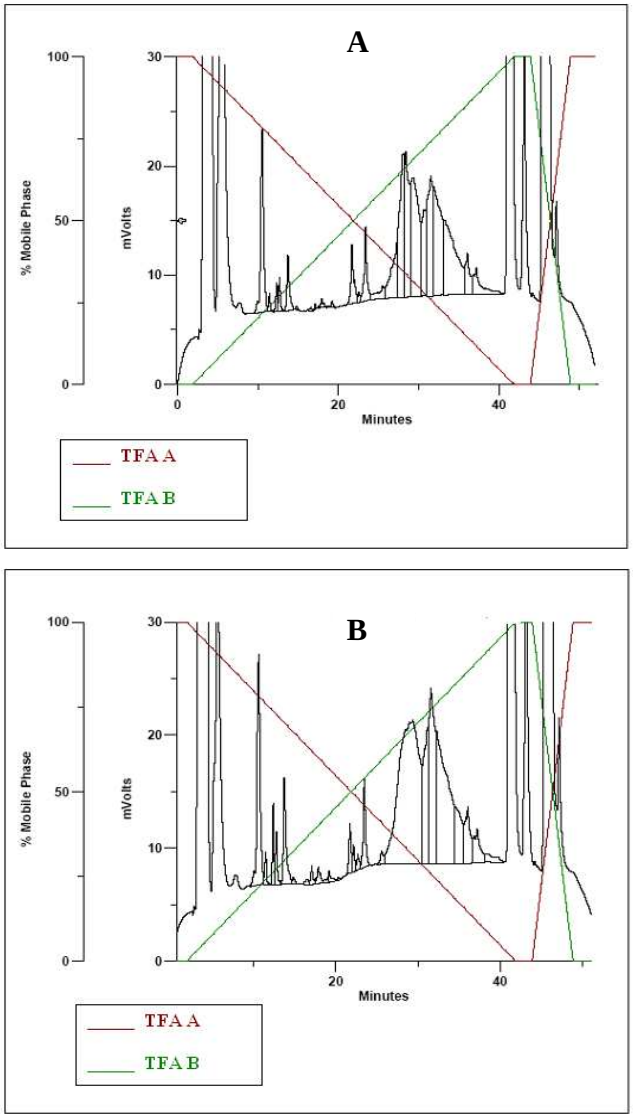


Figure 2.11 Representative reverse phase HPLC absorption profiles of the crude peptide (Cyclo(Lys^{4} – Glu^{29}), Glu^{29})-PYY(3-36) to compare the effect on purity of pseudoproline insertion

A - peptide synthesised with Ala^{22}-Ser^{23} introduced simultaneously as the pseudoproline dipeptide Fmoc-Ala-Ser(Psi(Me,Me)pro)-OH.

B - peptide synthesised with Ala^{22}-Ser^{23} introduced in a stepwise manner as the single, standard Fmoc protected derivatives.

Samples of the crude peptides were dissolved in 0.1% TFA/water/acetonitrile and analysed by reverse phase HPLC using an analytical C-18 column (Vydac 218TP54, 250 x 4.6mm, 5 µm particle size, and 300 Å pore size) and a linear AB gradient of 0–100% for B over 40 min at a flow rate of 1mL/min, where eluent A was 0.1% TFA/water and eluent B was 0.1% TFA in 60% acetonitrile/water. Peak detection was by spectrophotometry at a wavelength of 210 nm.

2.4.4.8 (Cyclo(Lys^{4}–Glu^{29}), Glu^{29})-PYY(3-36) (Synthesis IV)

The Aloc and OAll protecting groups were successfully removed from the Lys^{4} and Glu^{29} residues. This was confirmed by comparing the HPLC chromatograms of samples taken before and after the deprotection (Figure 2.12) and observing a decrease in retention time, following deprotection. Successful deprotection was further characterised by detection of the partially deprotected molecular weight by MALDI MS (Figure 2.13)

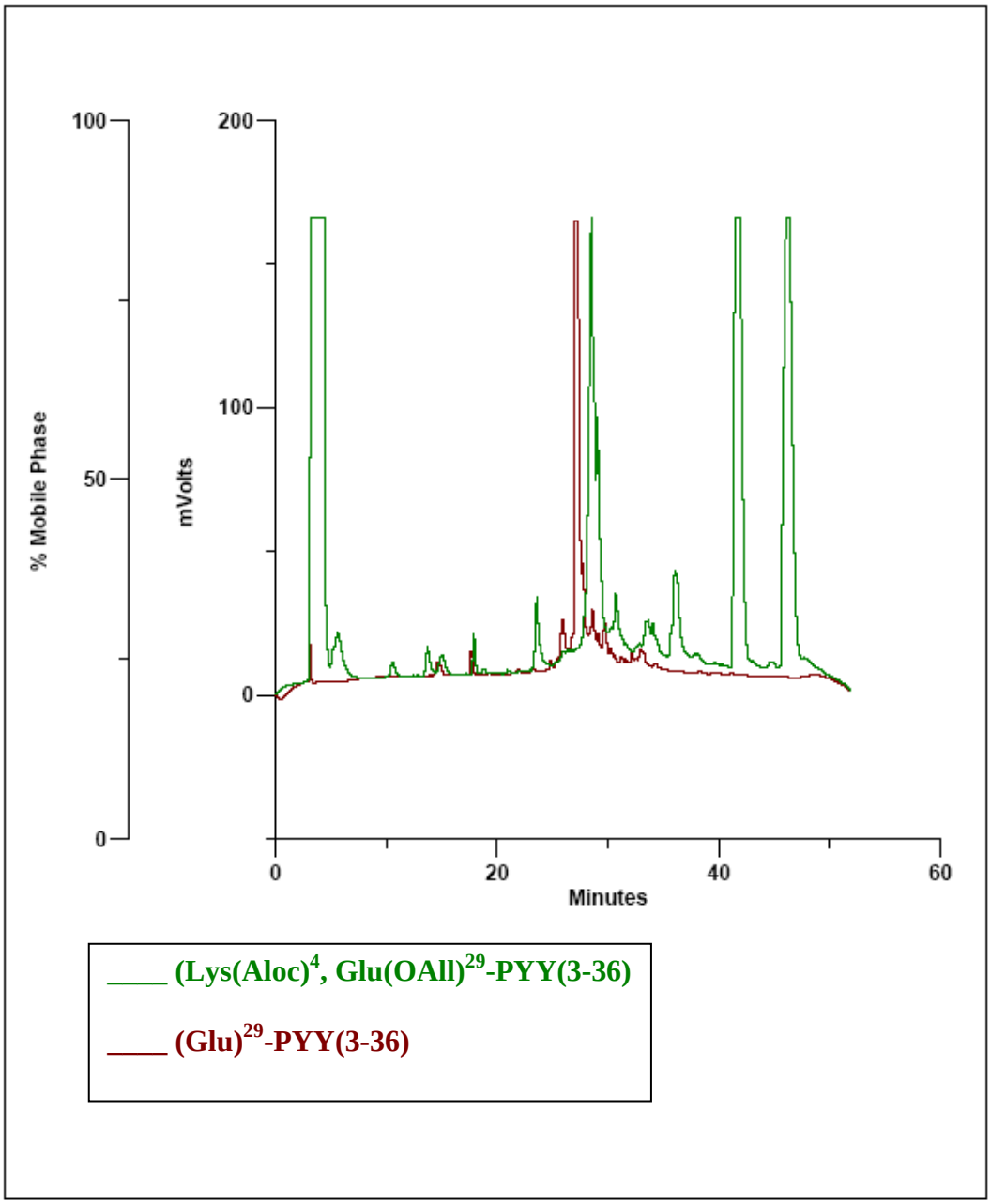


Figure 2.12 Comparison reverse phase HPLC absorption profiles of $(Glu)^{29}$-PYY(3-36) samples taken before and after Aloc/OAll removal.

Samples of the peptide resins were cleaved for 2 hours in conc. TFA. The solutions were filtered and diluted with acetonitrile/water and analysed by reverse phase HPLC using an analytical C-18 column (Vydac 218TP54, 250 x 4.6mm, 5 µm particle size, and 300 Å pore size) and a linear AB gradient of 0–100% for B over 40 min at a flow rate of 1mL/min, where eluent A was 0.1% TFA/water and eluent B was 0.1% TFA in 60% acetonitrile/water. Peak detection was by spectrophotometry at a wavelength of 210 nm.

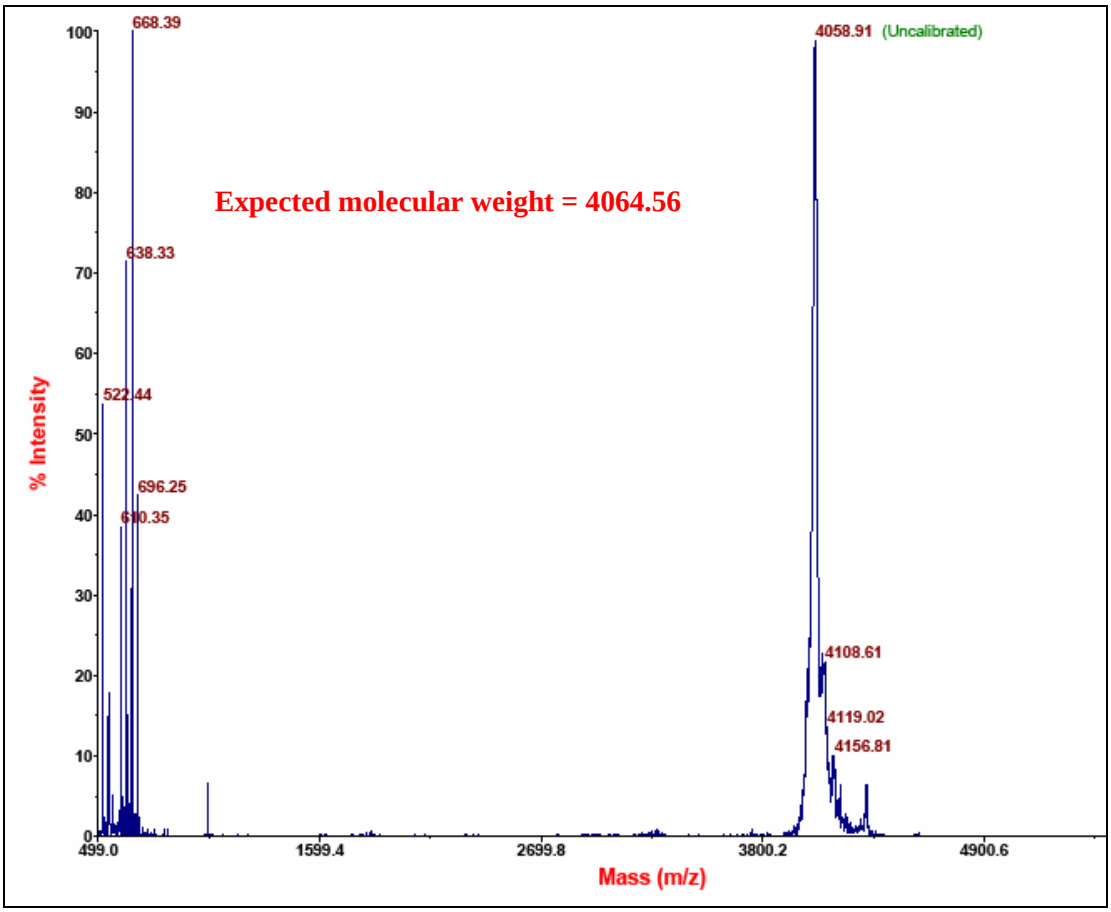


Figure 2.13 Representative MALDI mass spectrum of (Glu)^{29}-PYY(3-36) following Aloc/OAll removal.

A sample of peptide resin was cleaved for 2 hours in conc. TFA. The solution was filtered and diluted with acetonitrile/water. The solution was analysed by a Voyager DE MALDI mass spectrometer in positive voltage mode. The solution was co-crystallised with α-cyano-4-hydroxycinnamic acid (CHCA) matrix compound. The vertical axis represents the relative signal intensity of the molecular ions detected and horizontal axis represents mass to charge ratio (M/Z). The expected molecular weight was detected.

Cleavage of the partially protected peptide yielded 304mg of material. HPLC and MALDI MS analysis of this material was inconclusive. The HPLC chromatogram contained no peaks and in the MALDI mass spectrum, only those masses

corresponding to the matrix compound, α-cyano-4-hydroxycinnamic acid (CHCA) were detected.

Following lactam formation in solution and subsequent cleavage with solution K, 408mg of crude material was obtained. It was not possible to identify the molecular weight of the target peptide in this crude material following analysis by MALDI MS. The HPLC chromatogram of a sample of the crude material is shown in Figure 2.14

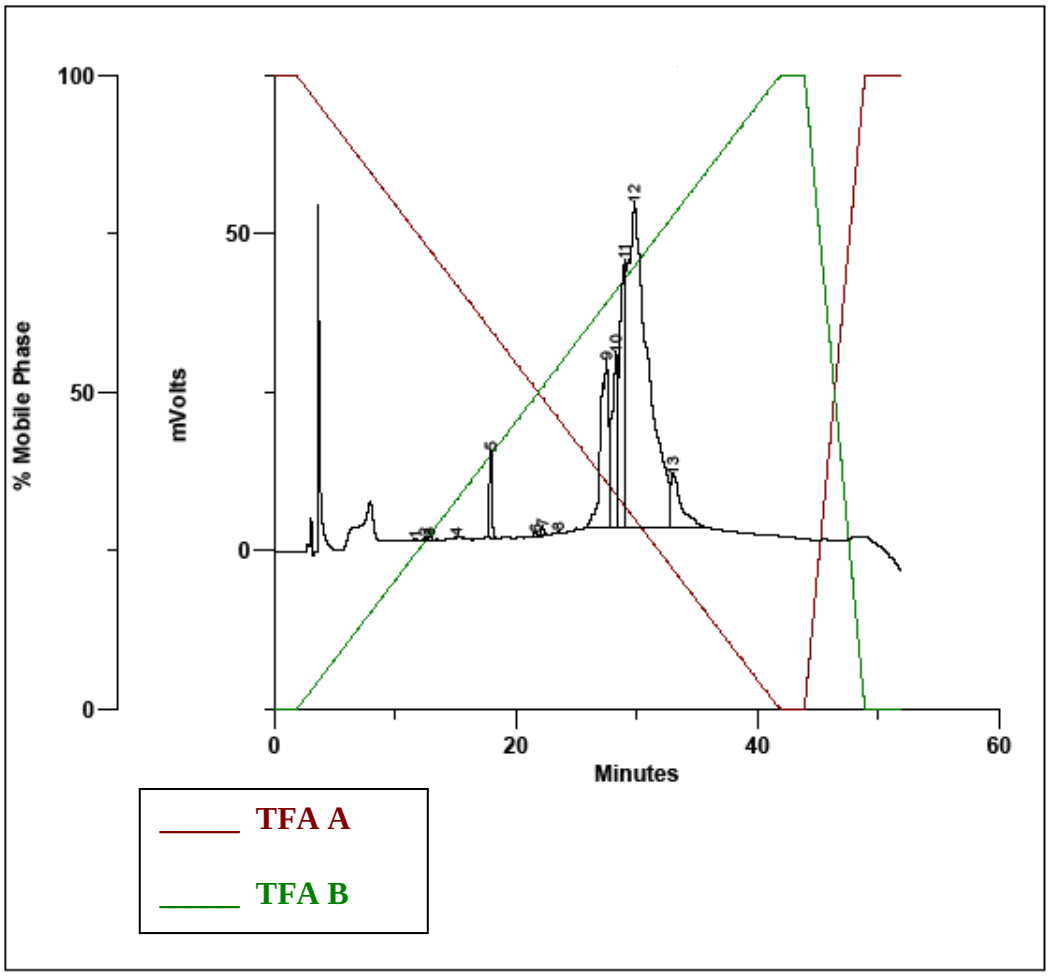


Figure 2.14 Representative reverse phase HPLC absorption profile of the crude peptide (Cyclo(Lys^{4} – Glu^{29}), Glu^{29})-PYY(3-36)

A sample of the crude peptide was dissolved in 0.1% TFA/water/acetonitrile and analysed by reverse phase HPLC using an analytical C-18 column (Vydac 218TP54, 250 x 4.6mm, 5 µm particle size, and 300 Å pore size) and a linear AB gradient of 0–100% for B over 40 min at a flow rate of 1mL/min, where eluent A was 0.1% TFA/water and eluent B was 0.1% TFA in 60% acetonitrile/water. Peak detection was by spectrophotometry at a wavelength of 210 nm.

2.5 Discussion

2.5.1 Position 25 and 26 PYY(3-36) analogues

The ten analogues were produced with final yields that ranged from 7.5% up to 20%. All of the analogues achieved final purities in excess of 95% as assessed by HPLC in two different buffer systems. The disparity in yields cannot, therefore, be attributed to some analogues being produced at a lower purity. The methods used for the synthesis and purification of each of the ten analogues were identical, with the exception of (Cit)^{25}PYY(3-36) which required a second purification step. The material recovered from the first purification step was just less than 95% pure by HPLC on the TEAP buffer system (actual purity of 93.88%). The subsequent re-purification of the material resulted in a loss of a yield (from 30.7mg to 27mg) and contributed to the relatively low final yield of 13.3%. However, there were two analogues that achieved final yields of less than 10%, for which only one purification step was required.

It therefore seems unlikely that the difference in yields seen across the ten analogues was not due to any difference in the synthesis or purification methods used, but rather reflect the effects that the presence of unnatural amino acids in the sequence, had on the synthesis and/or purification. This is supported by comparing the final yield of 20.5% obtained from the production of the native peptide PYY(3-36), with those obtained from the analogues. By comparison, all of the amino acid substitutions had, to some degree, a detrimental effect on the final yield. The analogues with the highest final yields were (D-Arg)^{25}PYY(3-36), (Homoarg)^{25}PYY(3-36) and (D-Cit)^{25}PYY(3-36). In the cases of (D-Arg)^{25}PYY(3-36) and (Homoarg)^{25}PYY(3-36), this would be expected as the unnatural amino acids D-arginine and homoarginine are the closest in structure to the native arginine residue, whilst also retaining the basicity of arginine.

This means that they should display the same coupling tendencies as arginine, and also confer the same character to the peptide once in solution.

Citrulline is also structurally similar to arginine but it does not have a reactive side chain group owing to the presence of an amide group as opposed to a guanidine. This means that the Fmoc derivative contains no side chain protecting group, thus making it a smaller molecule which should allow the coupling reaction to proceed at a faster rate. The potential improvement in the quality of the crude peptide for (D-Cit)^{25}PYY(3-36) may be offset by the loss of the basic functionality in the side chain group. This confers a more hydrophobic nature to the peptide overall, which in turn can affect the purification process. The more hydrophobic a peptide is, the greater its affinity for the stationary phase, resulting in longer retention times. (D-Cit)^{25}PYY(3-36) eluted from the HPLC column 30 seconds later than PYY(3-36) (data not shown). This increase in retention time may result in the analogue co-eluting with an impurity that PYY(3-36) elutes earlier than. Based on the results for (D-Cit)^{25}PYY(3-36), it would be expected that (Cit)^{25}PYY(3-36) would be synthesised in similar quantities and at a similar purity. However, as previously mentioned, a second purification step was required for this analogue. The purity of the material achieved from the first purification step was just below the required specification. Therefore, the loss of material from performing the second purification step was minimal. This suggests that the disparity in yields between the (Cit)^{25} and (D-Cit)^{25} analogues was not simply a result of performing two purification steps on the (Cit)^{25} analogue. Rather, it appears that insertion of D-citrulline in position 25 has a positive stereochemical effect on the peptide, which helps to improve either the remaining synthesis steps and/or the purification.

The unnatural amino acid 4-guaninidino phenylalanine retains the side chain functionality of arginine but is a much bulkier molecule due to the presence of an aromatic ring in the side chain group. This could lead to steric hindrance during coupling and hence explain the lower yield achieved for the synthesis of (Phe-Gu)^{25}PYY(3-36).

The yield achieved for (D-His)^{26}PYY(3-36) was lower than that for the corresponding D-arginine analogue, suggesting that the stereochemical effects of introducing a D configured amino acid at position 26 are more detrimental than introducing one at position 25.

Contrasting results were achieved with the methylated analogues ((Arg(Me)$_2$symm)^{25}PYY(3-36), (Arg(Me)$_2$asymm)^{25}PYY(3-36), (His(1-Me))^{26}PYY(3-36) and (His(3-Me))^{26}PYY(3-36)). For both the methyl arginine and methyl histidine containing analogues, relatively high yields were achieved for one isomer whilst low yields were achieved for the other isomer. In all cases, the presence of the methyl groups removed the need for side chain protecting groups in the Fmoc derivatised amino acid. This makes the molecules smaller and should therefore improve coupling efficiency. The lower yields achieved for these four analogues compared with PYY(3-36) are thus likely due to detrimental effects imparted on the peptide post-synthesis by the presence of the methyl groups. The disparity in yields seen between each set of isomers suggests that the orientation of the methyl groups on the side chains of arginine and histidine is an important factor in dictating the quality of the final peptide.

Alongside production of the ten position 25 and 26 PYY(3-36) analogues, the analogue Biotinyl-PYY(3-36) was produced. The aim was to use this analogue as a label for the potential development of a fluorescence receptor binding assay. The final

yield achieved with this analogue was lower than for any of the other 10 analogues. As with (Cit)^{25}PYY(3-36), a second purification step was required for Biotinyl-PYY(3-36) to meet purity requirements, suggesting that addition of a biotin group to the N-terminal of PYY(3-36) has a negative effect on the quality of the final peptide produced.

2.5.2 N-terminally pegylated PYY(3-36) analogues

Of the three PYY(3-36) analogues produced with an N-terminal PEG functional group, the lowest final yield was obtained for AEEAc-PYY(3-36). This was somewhat surprising, given that the AEEAc molecule is the smallest of the PEG compounds used, and should therefore be the analogue closest in character to the native sequence. However, analysis of the weight of the crude material (data not shown) suggests that material may have been lost during the cleavage process.

The yields obtained for the PEG4 and PEG6 analogues were greater than that obtained for PYY(3-36) during the earlier stage of the investigation. This may be due to the beneficial increase in solubility that PEG molecules can impart onto peptides. However, it may also be a result of the use of more modern HPLC and MALDI MS equipment during the production of this set of analogues. It could also be a reflection of an increase in the skill level and experience of the chemist performing the purification.

2.5.3 Position 30, 31 and 32 PYY(3-36) analogues

The eight PYY(3-36) analogues were all produced with final yields of between 13.4 – 21.2%. This is a more consistent range of yields than for the previous sets of analogues and is almost certainly due to the similarities in the nature of the amino

acids that were used to create the different analogues. In the human sequence of PYY(3-36), the residues in positions 30, 31 and 32 are leucine, valine and threonine respectively. Leucine and valine are hydrophobic amino acids and similarly hydrophobic amino acids were substituted into positions 30 and 31. This should result in there being very little difference in the coupling difficulties for the position 30 and 31 analogue amino acids compared to those used in PYY(3-36). Secondly, the overall hydrophobicity of the peptide is not changed across these analogue sequences, and thus the peptides display the same solubility properties and retention times during the purification process.

The final two analogues in this set contained substitutions of the Thr^{32} residue. Threonine is an uncharged, polar amino acid containing a hydroxyl function in its side chain group. In one analogue, threonine was substituted for leucine which served to increase the overall hydrophobicity of the peptide. However, this analogue was produced with a final yield of 18.7%, one of the highest of all of the analogues. Conversely, the second analogue contained a serine for threonine substitution which resulted in retention of the hydroxyl function but a loss of one carbon atom. The final yield achieved for this peptide was 14%, the second lowest amongst the eight analogues. It would therefore suggest that the number of carbon atoms in the structure is more important than the presence of the hydroxyl function for the successful production of position 32 analogues of PYY(3-36).

The highest yield achieved amongst these analogues was for $(Ile)^{30}$PYY(3-36) which was produced in a 21.2% yield. Leucine and isoleucine contain the same number of atoms and differ only slightly in the structures of their side chain groups. This makes $(Ile)^{30}$PYY(3-36) structurally the closest analogue to PYY(3-36), and may explain the high yield achieved compared to other analogues with more profound changes to

structure and/or chemical property. However, it is also worth noting that the final purity achieved for (Ile)^{30}PYY(3-36) was 95%, the minimum purity requirement set for further studies. This was the lowest purity achieved up until this point in the investigation and may be a contributory factor in the increased yield.

2.5.4 (Cys^{4}, Cys^{29})PYY(3-36) (disulphide bridge)

This analogue was successfully produced and proves that a PYY(3-36) analogue can be made containing a cross linking between amino acids in positions 4 and 29 in the sequence.

This particular analogue was designed to allow the formation of a disulphide bridge when the peptide was in solution following cleavage from the resin. Being in solution enables the peptide to form its natural configuration and eliminates any steric hindrance that may be caused by the presence of side chain protecting groups.

The reaction to form a disulphide bridge can be monitored by reverse phase HPLC, as the change in peptide confirmation upon bridge formation, will usually elicit a change in retention time. However, in the case of (Cys^{4}, Cys^{29})PYY(3-36)(disulphide bridge), no change in retention time was recorded during the oxidation reaction. Taken alone, this could be due to one of two factors; either the formation of the disulphide bridge caused no significant change in peptide confirmation and so did not affect the retention time, or the two cysteine residues are not in close enough proximity to successfully form the disulphide bridge. Once the peptide had been isolated from the reaction solution via Flash chromatography, further tests were performed to determine if the disulphide bridge had been formed. The first of these was an Ellman's test to determine the presence of any sulphydryl (SH) groups within the peptide. If the disulphide bridge had successfully formed then there would be no sulphydryl groups

present and the test result would be negative, as was the case. Secondly, once the peptide had been partially purified it was possible to obtain a more accurate measurement of mass through MALDI MS. In this case, the expected molecular ion was detected.

The findings from the production of this analogue not only prove that amino acids 4 and 29 within PYY(3-36) can be successfully cross linked, but also suggest that these two amino acids are in close proximity to each when the peptide is in solution. This was expected to be the case following the published ^{1}H NMR study data that confirmed the hairpin-like structure of PYY(3-36) (Nygaard et al., 2006). However, the fact that cross linking does not seem to significantly alter the confirmation of the peptide suggests that it should not be detrimental to receptor binding affinity.

The final yield achieved for this analogue was only 6%, but it must to be taken into consideration that the peptide underwent four separate processes following cleavage from the resin, each with associated losses of yield. The final purity achieved was only 90.5% and the purity decreased from 91.1% following the final purification step. It was decided that a purity of 90.5% would be sufficient to conduct further biological testing, and that to perform further purification steps would risk further loss in yield without a significant improvement in purity.

2.5.5 PYY(3-36) analogues containing lactam bridges

As yet, it has not been possible to isolate, at sufficient purity, any of the PYY(3-36) analogues designed to incorporate lactam bridges. Initially, all four analogues were synthesised following the standard methods successfully employed for previous analogues, except for the presence of alternative protecting groups on the two amino acids to be cross linked.

For two of the analogues, (Cyclo(Lys^{4}-Glu^{29}), Glu^{29})-PYY(3-36) and (Cyclo(Dap^{4}-Glu^{29}), Dap^{4}, Glu^{29})-PYY(3-36), the decision was taken to stop the synthesis following automated SPPS of the linear peptides. This was based on the HPLC and MALDI MS analysis of cleaved samples of the resin. In the case of the analogue (Cyclo(Lys^{4}-Glu^{29}), Glu^{29})-PYY(3-36), the HPLC chromatogram suggested that a major product was present in the sample. However, the expected molecular weight was not detected by MALDI MS, but rather that of a number of deletion sequences. This peptide was synthesised on an older synthesiser which may have become unreliable, and hence the decision was taken not to continue with the cyclisation step. For the analogue (Cyclo(Dap^{4}-Glu^{29}), Dap^{4}, Glu^{29})-PYY(3-36), the HPLC chromatogram showed that more than one major product was present in the sample. As negative results had been achieved with other analogues, the decision was made not to proceed with the cyclisation step.

The first of the four analogues to be synthesised was (Cyclo(Lys^{4}-Asp^{29}), Asp^{29})-PYY(3-36), as this was the analogue with the closest sequence homology to PYY(3-36), differing only in an aspartic acid residue instead of asparagine at position 29. The quality of the linear peptide produced was sufficient to continue with the cyclisation. The deprotection of the two amino acids was completed successfully as assessed by Kaiser test. The formation of the lactam bridge was also deemed successful by Kaiser test following three separate overnight couplings. The HPLC chromatogram of the crude peptide displayed a very broad peak which suggested a large number of different products had been formed, and the expected molecular weight could not be detected by MALDI MS. Nevertheless, it was decided to purify the crude material to see if any of the correct peptide could be isolated. No single product could be isolated from this process and the results suggested that instead of forming an intramolecular

lactam bridge, the free amino and carboxyl groups had instead formed a number of different intermolecular products.

While this analogue was being purified, work had begun on the second lactam bridge analogue, (Cyclo(Dap^4-Asp^{29}), Dap^4, Asp^{29})-PYY(3-36). Again, the quality of the linear peptide was deemed good enough to proceed with the cyclisation step. The same methods were employed for this analogue as with the previous one. Results suggested that the lactam bridge had formed following three overnight couplings. However, following analysis of the subsequent crude peptide, it was clear that similar impurities to those formed during the synthesis of (Cyclo(Lys^4-Asp^{29}), Asp^{29})-PYY(3-36) were present.

The four analogues had all been synthesised on tricyclic amide linker resin, which had been successfully used in the production of all of the previous sets of analogues. The loading capacity of this resin was in the range 0.5 – 0.7mmol/g. Other resins are available that have lower loading capacities. One such resin is the TentaGel RAM resin from Rapp Polymere, which has a loading capacity in the range 0.15 – 0.2mmol/g. It was thus decided to use this resin for all subsequent on-resin cyclisation attempts. The lower loading capacity of the TentaGel RAM resin means that the peptide chains are spaced further apart from each other which should hinder intermolecular interactions and promote intramolecular bond formation.

At this point in the investigation it was decided to focus on developing a method for producing one of the analogues and then employ the successful method in the production of the other three analogues. The analogue chosen for this was (Cyclo(Lys^4-Glu^{29}), Glu^{29})-PYY(3-36), as the side chain functional groups of lysine and glutamic acid are longer than those in α,β-diaminopropionic acid and aspartic

acid, and it was hoped that this would further facilitate successful lactam bridge formation.

The second synthesis of (Cyclo(Lys^{4}-Glu^{29}), Glu^{29})-PYY(3-36) was thus conducted on TentaGel RAM resin, with all other parameters kept the same as for the initial synthesis. Lactam bridge formation still required more than one coupling reaction, and the subsequent HPLC chromatogram of the resulting crude material suggested that, once again, a number of intermolecular products had been formed.

The combination of the slow reaction times, and the fact that intermolecular products were still predominantly being formed, even on low loading resins, suggested that the main issue was that the two amino acids were not held in close enough proximity within the resin-bound peptide sequence to react with each other.

One of the proposed advantages in the use of pseudoproline dipeptides in SPPS is that they can assist in the synthesis of cyclic peptides (Novabiochem, 2010). This is due to their ability to alter the confirmation of the resin-bound peptide. It was therefore decided to investigate the synthesis of (Cyclo(Lys^{4}-Glu^{29}), Glu^{29})-PYY(3-36) with a pseudoproline insertion. Within the sequence of PYY(3-36) are three possible sites of pseudoproline insertion. These are Ala^{12}-Ser^{13}, Ala^{22}-Ser^{23} and Val^{31}-Thr^{32}. Pseudoprolines mimic the effect of proline residues and it is therefore recommended to not insert a pseudoproline dipeptide immediately after a proline residue, as the positive effect of pseudoproline insertion will have already been conferred onto the sequence by the proline residue preceding it (Novabiochem, 2010). Amino acid 14 in PYY(3-36) is proline, therefore ruling out Ala^{12}-Ser^{13} as the insertion site. As the aim was to change the confirmation of the peptide so as to bring positions 4 and 29 closer together, it was decided to insert the pseudoproline between these two positions. Therefore position Ala^{22}-Ser^{23} was chosen as the site of insertion.

All previous cyclisations had been attempted with the coupling reagents HOAt and DIC as recommended previously (Novabiochem, 2010). The advantage of using these reagents is that they are unlikely to react with the peptide itself and form impurities, which is a risk when forming on-resin cyclic peptides. However, there are more efficient coupling reagents available which would speed up the cyclisation reaction.

It was thus decided to repeat the synthesis of (Cyclo(Lys^4-Glu^{29}), Glu^{29})-PYY(3-36) employing a pseudoproline insertion for one reaction vessel and standard amino acids in positions 22 and 23 for the other reaction vessel. Both resins would then be cyclised using TBTU and DIPEA activation of the carboxyl group on Glu^{29}. According to the results of the Kaiser tests, the cyclisation reaction was complete in a few hours. However, the HPLC chromatograms of the subsequent crude peptides displayed similar profiles to all previous attempts at producing this set of analogues. There also appeared to be no significant difference in the profiles of the peptide with and without a pseudoproline insertion. In addition, as TBTU was used as the coupling reagent, there is the possibility that the guanidated form of the peptides have been formed alongside other impurities (Gausepohl et al., 1992).

The analogue (Cys^4, Cys^{29})PYY(3-36)(disulphide bridge) was successfully synthesised following formation of the cross linking disulphide bridge in solution post-cleavage. This was possible, as the peptide was able to form its natural hairpin like structure thus bringing the amino acids in positions 4 and 29 close together. It would not be possible to successfully form a single lactam bridge in solution, post-cleavage in PYY(3-36) owing to the presence of multiple amino and carboxyl groups. However, it may be possible to form a lactam bridge in solution if all of the amino acids other than the two lactam forming residues, were still protected. It was therefore decided to synthesise (Cyclo(Lys^4-Glu^{29}), Glu^{29})-PYY(3-36) on an acid sensitive

resin, deprotect the Lys^4 and Glu^{29} residues and then cleave the partially protected peptide from the resin. The protected peptide could then be cyclised in solution as the peptide should be able to form the characteristic hairpin shape and bring amino acids 4 and 29 closer together.

The peptide was synthesised on Xanthenyl resin, which is recommended for the production of protected peptide amides (Mergler, 1999). It was also decided to change the protecting groups on both the Lys^4 and Glu^{29} residues. Previous syntheses had employed the Mtt and OPip protecting groups which are acid sensitive. For this new synthesis, removal of these protecting groups would also cleave the peptide from the resin as the cleavage conditions for the two are identical. The decision was taken to change to the Aloc/OAll protection strategy as the deprotection conditions for these groups would not affect the peptide-resin linker. Another advantage of using these protecting groups is that, as they are TFA stable, samples of the resin can be cleaved with TFA and the amount of Aloc/OAll protected material present determined by HPLC and MALDI MS analysis. Following deprotection of the two amino acids, the partially protected peptide was cleaved from the resin. Unfortunately, the resultant material could not be characterised by either HPLC or MALDI MS. This was to be expected, as protecting groups impart hydrophobicity to a peptide which results in an increase in retention time (Mant et al., 2007). Due to the sheer number of protecting groups still present on the partially protected PYY(3-36) analogue, it is possible that the peptide bound irreversibly to the C18 column. Protected peptides more readily fragment than unprotected peptides during MALDI MS analysis, resulting in a greater range of molecular ions and making it difficult to isolate any one particular mass.

Despite being unable to characterise the protected peptide, it was decided to continue with the solution cyclisation. The theory behind the process employed is that the

peptide is introduced in very small quantities to a concentrated solution of the activation reagents. As the peptide enters this solution, it is far more likely to come into contact with molecules of the activation reagents than it is to encounter another peptide molecule. This should result in a near instantaneous reaction to form the lactam bridge, and thus prevent the formation of intermolecular impurities. As the activation reagent to peptide ratio was so high, use of TBTU and similar activation reagents was not possible.

To date, analysis of the crude material by HPLC and MALDI MS has failed to identify the presence of the target peptide. Further investigations are thus required to determine if the solution cyclisation method has been successful and whether any changes to the method can help to produce the desired lactam bridge-containing analogues.

The work described in this chapter has demonstrated that it is possible to successfully produce novel analogues of PYY(3-36) utilising different solid phase peptide synthesis techniques. For the development of one or more of these analogues as a potential anti-obesity therapeutic, it would be desirable for the analogue(s) to display increased stability to the actions of proteolytic enzymes.

3 The proteolytic enzyme degradation pattern of PYY(3-36) and its analogues

3.1 Introduction

It is difficult to know which specific enzyme(s) would predominantly drive the degradation of PYY(3-36) were it to be orally administered as an anti-obesity therapeutic. Oral administration would expose the peptide to a number of different proteolytic enzymes.

3.1.1 Gastro-intestinal tract proteolytic enzymes

In the stomach, any ingested proteins and peptides are exposed to gastric juices which largely consist of hydrochloric acid and the protease enzyme pepsin (Schubert, 2010). Pepsin is released into the stomach as its precursor pepsinogen, so as to prevent against auto-digestion of cell proteins (Rajagopalan, 1966). Once in the stomach, pepsinogen is converted to pepsin which then begins to break down proteins by preferentially cleaving peptide bonds at positions containing aromatic and hydrophobic amino acids (Sigma Aldrich, 2011). The high concentration of hydrochloric acid in the gastric juices provides the optimal conditions for this enzymatic cleavage as the optimum pH for pepsin activity is 1.5 to 2.5 (Sigma Aldrich, 2011).

Once a peptide has passed through the stomach it will come into contact with pancreatic-derived enzymes such as trypsin and chymotrypsin. Trypsin is a serine protease enzyme that cleaves the peptide bond at the carboxyl terminus side of arginine and lysine residues, unless the subsequent amino acid is a proline (Beck et al., 2000). It has also been found that the presence of an acidic amino acid either side of a lysine and arginine will slow the rate of hydrolysis (Hedstrom, 1996). Due to this specificity in cleavage site, trypsin is routinely used for protein sequencing (Massolini

and Calleri, 2005). The optimum pH for trypsin activity is pH 8, as found in the small intestine, its endogenous site of action.

Chymotrypsin is co-secreted with, and similar in structure to trypsin. It cleaves the peptide bond at positions containing the amino acids tyrosine, phenylalanine and tryptophan (Hedstrom, 1996).

In the small intestine, remaining proteins and peptides are first broken down into di- and tri- peptides, and then into their constituent amino acids by the actions of different endopeptidase enzymes located on the epithelial cells of the intestinal wall (Taylor, 1993).

3.1.2 Circulatory proteolytic enzymes

Other proteolytic enzymes degrade circulating proteins and peptides for a number of different metabolic processes, including to remove blood clots and to switch off hormonal signalling. Two enzymes known to be involved in the degradation of gut derived hormones are discussed below.

3.1.2.1 Dipeptidyl peptidase IV (DPP-IV)

DPP-IV is an aminopeptidase found on the cell surface of most tissues and also in the circulation (Bermpohl et al., 1998). Many proteolyic enzymes are unable to cleave peptides at proline residues (Mentlein, 1999). However DPP-IV acts by cleaving the N-terminal dipeptide from a peptide where the second amino acid in the sequence is either proline or alanine. Studies have found that it cleaves X-Pro dipeptides more rapidly than X-Ala. Although DPP-IV tolerates a wide range of different amino acids in the X position, the identity of the third amino acid in the sequence can affect

activity. If this residue is proline or hyroxyproline then DPP-IV does not hydrolyse the sequence, as is the case with bradykinin (Mentlein, 1999).

DPP IV has been shown to play a role in the degradation of several regulatory peptides. It is often found in circumventricular organs and close to physiological barriers such as the blood brain barrier. It is the enzyme responsible for the conversion of PYY(1-36) to PYY(3-36) and it also inactivates GLP-1(7-36) amide through removal of the N-terminal His-Ala dipeptide (Mentlein, 1999). The optimum pH for it to operate is between 7.5 and 8.5.

3.1.2.2 Neprilysin

Neprilysin is a member of the M13 zinc metallopeptidase family of enzymes. It is expressed mainly in the kidneys, but has been found in lower concentrations in other tissues, including the brain (Turner et al., 2001).

It has a large number of potential substrates owing to its preference for hydrolysing peptides at the amino terminal of single hydrophobic amino acid residues. In the kidneys, neprilysin constitutes 4% of the brush border membrane protein and facilitates the inactivation of the vasodilator, atrial natriuretic peptide (Turner et al., 2001).

Due to the importance of hydrophobic residues for receptor binding in bioactive peptides, neprilysin has been hypothesised as an important enzyme for turning off neuropeptide signals in the brain. The first such substrates to be identified were the enkephalins and substance P (Shirotani et al., 2001).

Recently the role that neprilysin deficiency plays in the development of Alzheimer's disease has been investigated (Carson and Turner, 2002). Alzheimer's disease is characterised by the accumulation of amyloid β proteins in the brain. These peptides contain regions that are rich in hydrophobic amino acids. Neprilysin was found to be

the most potent amyloid β protein degrading enzyme of those tested *in vivo* (Shirotani et al., 2001). Neprilysin knockout mice displayed levels of endogenous amyloid β proteins comparable to those found in a mouse model of Alzheimer's disease. Mice lacking neprilysin had an impaired ability to break down exogenously administered amyloid β proteins, though as a small proportion of degraded peptide was detected, it seems that neprilysin is not the sole enzyme responsible for amyloid β protein clearance (Carson and Turner, 2002). The pathophysiological role of neprilysin in Alzheimer's, and its utility in the treatment of this disease, are currently being investigated.

3.2 Hypothesis and aims

3.2.1 Hypothesis

Determining the degradation pattern of PYY(3-36) and analogues in the presence of known proteolytic enzymes, will aid the design of further PYY(3-36) analogues that will display greater stability to enzymatic degradation.

3.2.2 Aims

1. To determine the degradation pattern of PYY(3-36) in the presence of trypsin
2. To determine the degradation pattern of PYY(3-36) in the presence of DPP IV and neprilysin.
3. To determine the degradation patterns of ten PYY(3-36) analogues with amino acid substitutions in positions 25 and 26.

3.3 Materials and methods

3.3.1 Trypsin degradation of PYY(3-36)

3.3.1.1 Efficiency trial

Studies were carried out in a 25mM ammonium bicarbonate (Sigma Aldrich) buffer solution at pH 8.0, the optimum pH for trypsin activity. A 0.123mM solution of human PYY(3-36) (produced as described in section 2.3.1) in buffer was prepared. The solution was analysed by reverse-phase High Performance Liquid Chromatography (HPLC) (Gilson) with an analytical C-18 column (Vydac 218TP54, 250 x 4.6mm, 5 µm particle size, and 300 Å pore size) and a linear AB gradient of 0–100% for B over 40 min at a flow rate of 1mL/min, where eluent A was 0.1% TFA/water and eluent B was 0.1% TFA in 60% acetonitrile/water and Kompact Probe Matrix Assisted Laser Desorption/Ionisation mass spectrometer (MALDI-MS) (Kratos). This determined the purity, retention time and molecular weight of the parent peptide. Trypsin (Applied Biosystems), pre-treated with tosyl phenylalanyl chloromethyl ketone (TPCK) to inactivate chymotryptic activity, was prepared at a concentration of 12.5µg/mL in buffer.

Reaction tubes were set up as detailed below, and each combination was run in duplicate (n=2);

1. 300µL Buffer
2. 200µL Buffer + 100µL PYY(3-36)
3. 200µL Buffer + 100µL Trypsin
4. 100µL Buffer + 100µL PYY(3-36) + 100µL Trypsin

The tubes were placed in a water bath set at 37°C and left for 3 hours. The reaction was then stopped with the addition of 100µL of 10% TFA solution. The solutions were then analysed by HPLC and MALDI MS as per the stock peptide solution.

3.3.1.2 Time-course study I

Due to the significant level of PYY(3-36) breakdown recorded after 3 hours incubation in the efficiency trial, a time-course study was set up. Reaction tubes were set up as above and incubated at 37°C for 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours or 6 hours (n=2). At the end of the allotted time period, that particular set of tubes were removed, and the reaction stopped and the solutions analysed as described in section 3.3.1.1.

3.3.1.3 Time-course study II

As significant PYY(3-36) breakdown was observed after only 30 minutes in time-course study I, it was repeated but the experiments terminated at 5 minutes, 10 minutes, 15 minutes and 20 minutes (n=2).

3.3.1.4 Dose response investigation

For this stage of the investigation, digestions were set up as described above, but using different concentrations of trypsin; 25µg/mL, 12.5µg/mL, 6.25µg/mL and 3.125µg/mL (n=2). All of the tubes were incubated at 37°C for 15 minutes, based on the results from time course study II. After this period the reaction was stopped and the solutions analysed as described in section 3.3.1.1.

3.3.2 Dipeptidyl peptidase degradation of PYY(3-36)

DPP IV (Sigma Aldrich) was made up in 100mM TRIS-HCl (Sigma Aldrich) pH8 buffer to a concentration of 37.5µg/mL. A solution of human PYY(3-36) was prepared at a concentration of 0.123mM of buffer. Reaction tubes were set up as detailed below, and each combination was run in triplicate (n=3);

1. 200µL Buffer + 100µL PYY(3-36)
2. 100µL Buffer + 100µL PYY(3-36) + 100µL DPP IV

All of the tubes were incubated at 37°C for time periods of 1 hour, 2 hours, 4 hours, 8 hours, 16 hours and 24 hours. At the end of the allotted time period, that particular set of tubes were removed, and the reaction stopped and the solutions analysed as described in section 3.3.1.1.

3.3.3 Neprilysin degradation of PYY(3-36)

Recombinant human neprilysin (R&D Systems, Minneapolis, Minnesota USA) was made up in 50mM TRIS HCl, 0.25M NaCl (VWR) pH 7.5 buffer solution to a concentration of 5µg/mL. A solution of human PYY(3-36) was made to a concentration of 0.123mM in buffer. Reaction tubes were prepared as detailed below, and each combination was run in triplicate (n=3);

1. 200µL Buffer + 100µL PYY(3-36)
2. 100µL Buffer + 100µL PYY(3-36) + 100µL neprilysin

All of the tubes were incubated at 37°C for time periods of 1 hour, 2 hours, 3 hours, 4 hours, 5 hours and 6 hours.

At the end of the allotted time period, that particular set of tubes were removed, and the reaction stopped and the solutions analysed as described in section 3.3.1.1.

3.3.4 Trypsin degradation of PYY(3-36) analogues

The breakdown by trypsin of the position 25 and 26 PYY(3-36) analogues; IP1 – IP10 was investigated. All peptide solutions were made to a concentration of 0.123mM in ammonium bicarbonate pH8.0 buffer and the trypsin solution was at a concentration

of 12.5µg/mL of buffer. Reaction tubes were prepared as detailed below and each combination was run in duplicate (n=2);

1. 100µL Buffer + 100µL PYY(3-36) + 100µL Trypsin
2. 100µL Buffer + 100µL IP1 + 100µL Trypsin
3. 100µL Buffer + 100µL IP2 + 100µL Trypsin
4. 100µL Buffer + 100µL IP3 + 100µL Trypsin
5. 100µL Buffer + 100µL IP4 + 100µL Trypsin
6. 100µL Buffer + 100µL IP5 + 100µL Trypsin
7. 100µL Buffer + 100µL IP6 + 100µL Trypsin
8. 100µL Buffer + 100µL IP7 + 100µL Trypsin
9. 100µL Buffer + 100µL IP8 + 100µL Trypsin
10. 100µL Buffer + 100µL IP9 + 100µL Trypsin
11. 100µL Buffer + 100µL IP10 + 100µL Trypsin

All tubes were incubated at 37°C for a period of 15 minutes. After this time, 10% TFA was added to stop the reaction and the solutions analysed by HPLC and MALDI MS as described in section 3.3.1.1.

3.4 Results

Unfortunately, due to a hardware failure and the resulting loss of the original data, the HPLC chromatograms for these data are presented in a format of slightly poorer quality than other chromatograms in this thesis.

3.4.1 Trypsin degradation of PYY(3-36)

3.4.1.1 Efficiency trial

The reverse phase HPLC chromatograms for each of the four reaction combinations investigated are shown in Figure 3.1. Although each was run in duplicate, only one

trace for each combination is shown. The peak in chromatogram **C** (ammonium bicarbonate pH8.0 buffer + 0.123mM PYY(3-3-6)) has a retention time of 28 minutes which is consistent with the retention time observed for PYY(3-36). Chromatogram **D** (ammonium bicarbonate pH8.0 buffer + 12.5µg/mL trypsin + 0.123mM PYY(3-36)) contains peaks with retention times of 18.5, 21.5 and 23 minutes.

The MALDI MS facility was unavailable during this stage of the investigation.

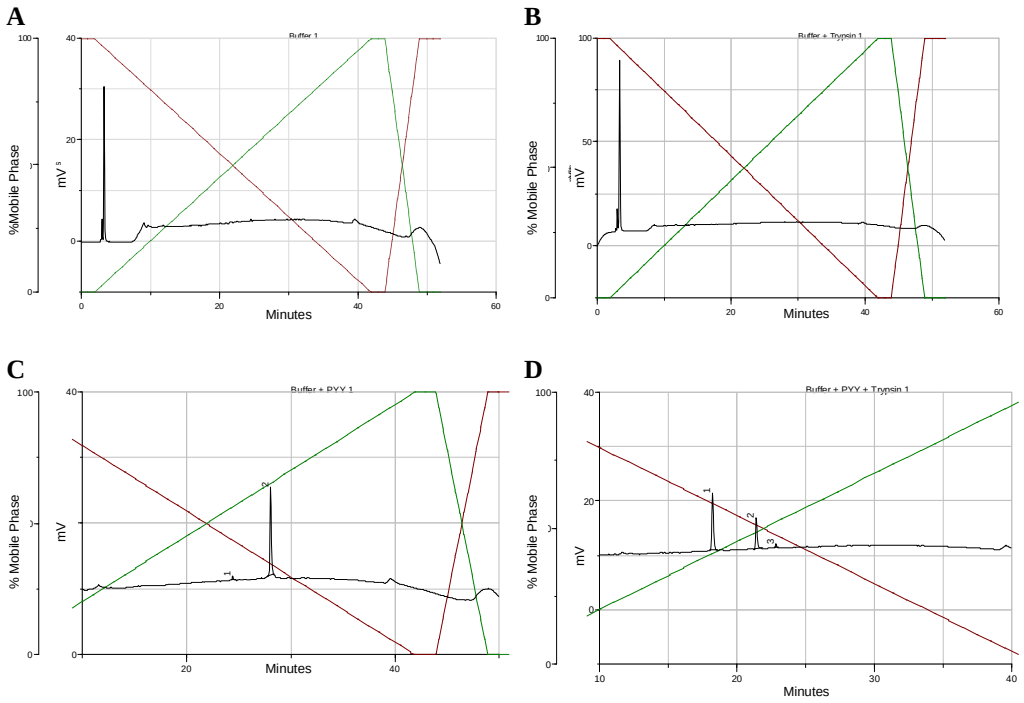


Figure 3.1 Representative reverse phase HPLC absorption profiles following *in vitro* digestion of PYY(3-36) by trypsin over 3 hours at 37°C.

A, ammonium bicarbonate pH8.0 buffer; **B,** ammonium bicarbonate pH8.0 buffer + 12.5µg/mL trypsin; **C,** ammonium bicarbonate pH8.0 buffer + 0.123mM PYY(3-36); **D,** ammonium bicarbonate pH8.0 buffer + 12.5µg/mL trypsin + 0.123mM PYY(3-36).

Digest solutions were analysed by reverse phase HPLC using an analytical C-18 column (Vydac 218TP54, 250 x 4.6mm, 5 µm particle size, and 300 Å pore size) and a linear AB gradient of 0–100% for B over 40 min at a flow rate of 1mL/min, where eluent A was 0.1% TFA/water and eluent B was 0.1% TFA in 60% acetonitrile/water. Peak detection was by spectrophotometry at a wavelength of 210 nm.

3.4.1.2 Time-course study I

The HPLC chromatograms of the solutions collected at the end of this stage of the investigation are shown in Figure 3.2. The chromatograms are just of the solutions containing buffer, PYY(3-36) and trypsin.

The three peaks present in chromatogram **D** (ammonium bicarbonate pH8.0 buffer + 12.5μg/mL trypsin + 0.123mM PYY(3-36)) from Figure 3.1, are visible in all of the chromatograms from this time-course study. Following 30 minute incubation, the peak with retention time 23 minutes is still quite prominent but reduces as time progresses.

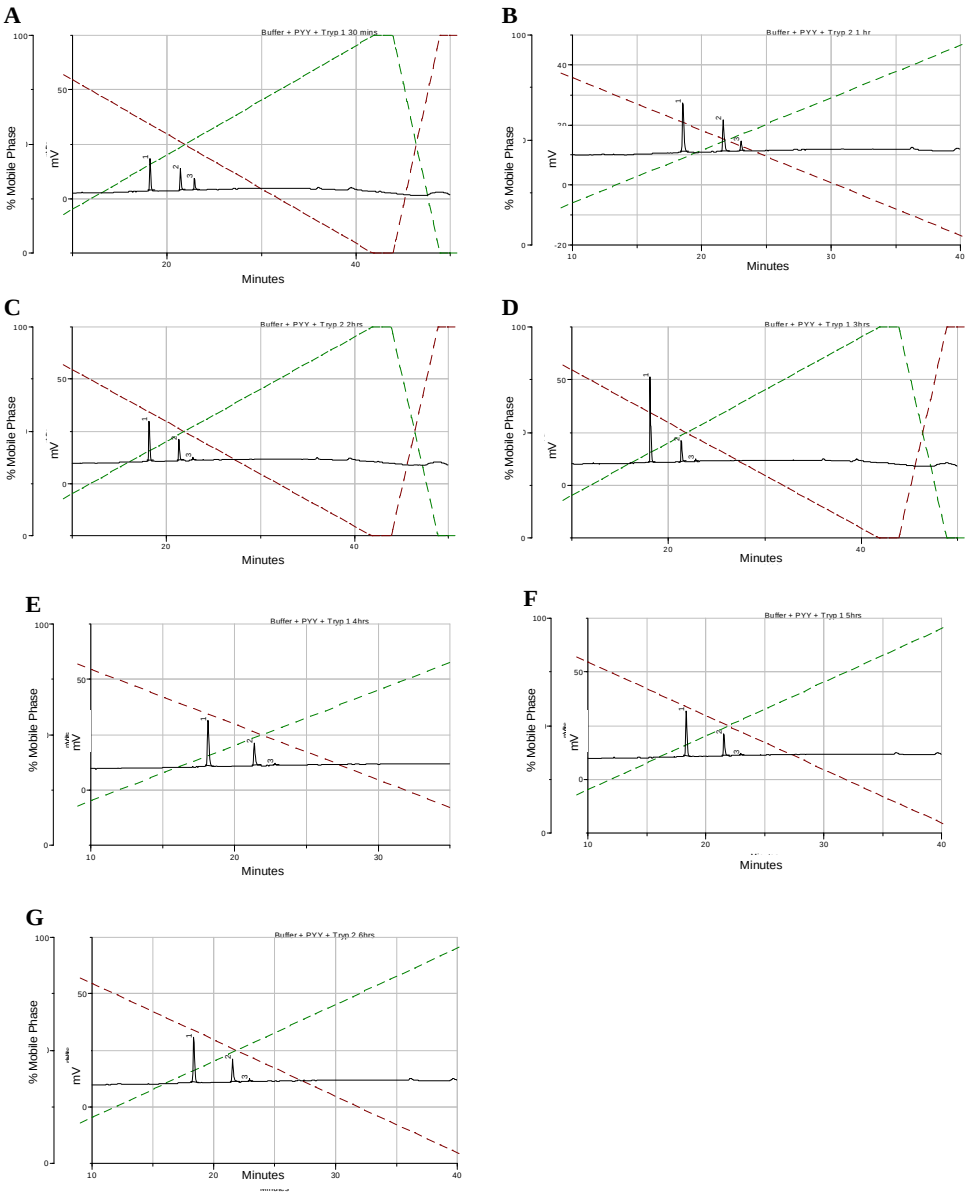


Figure 3.2 Representative reverse phase HPLC absorption profiles following *in vitro* digestion of PYY(3-36) by trypsin at 37°C, over periods of 0.5 – 6 hours incubation

Incubation times; **A** 30 minutes, **B** 1 hour, **C** 2 hours, **D** 3 hours, **E** 4 hours, **F** 5 hours and **G** 6 hours. All reaction tubes contained ammonium bicarbonate ph8.0 buffer, 12.5µg/mL trypsin and 0.123mM PYY(3-36). Digest solutions were analysed by reverse phase HPLC using an analytical C-18 column (Vydac 218TP54, 250 x 4.6mm, 5 µm particle size, and 300 Å pore size) and a linear AB gradient of 0–100% for B over 40 min at a flow rate of 1mL/min, where eluent A was 0.1% TFA/water and eluent B was 0.1% TFA in 60% acetonitrile/water. Peak detection was by spectrophotometry at a wavelength of 210 nm.

3.4.1.3 Time-course study II

Figure 3.3 shows the HPLC chromatograms of solutions obtained after reaction time periods of 5, 10, 15 and 20 minutes. It can be seen from the chromatogram of the 5 minute reaction (**A**), there is a substantial peak at 28 minutes which was not present in the chromatograms for the longer reaction times. Despite the presence of this peak, there are still earlier peaks at retention times of 23, 21 ½ and 18 minutes, though the latter is very small. Also present in this chromatogram is a small peak at retention time 24 minutes.

As the reaction time increases up to 10 then 15 and 20 minutes, the peak with retention time 28 minutes diminishes until it is barely visible in trace **D** (20 minutes incubation). The peak at 24 minutes also diminishes whilst the other three increase in size.

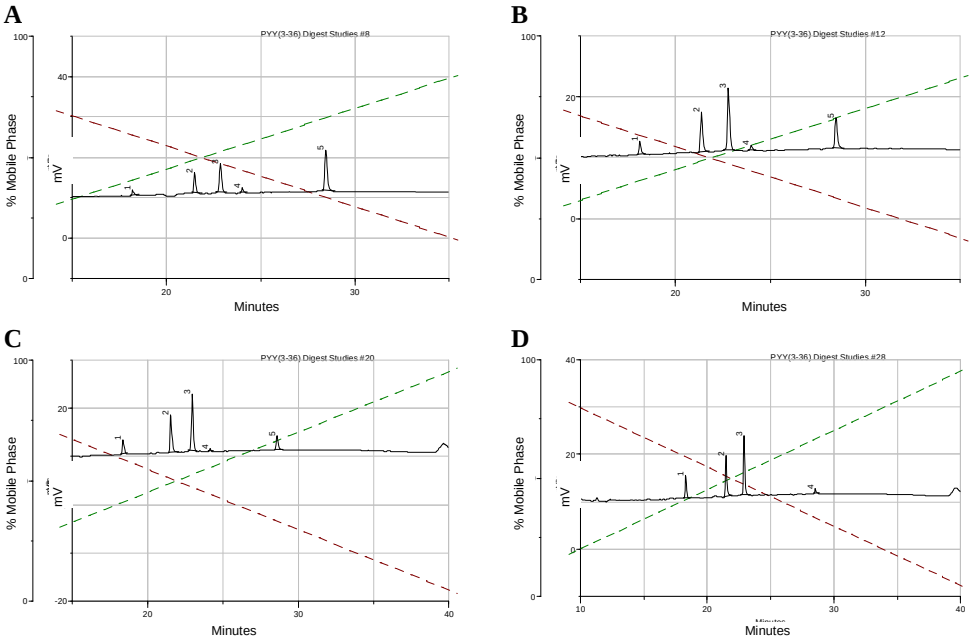


Figure 3.3 Representative reverse phase HPLC absorption profiles following *in vitro* digestion of PYY(3-36) by trypsin at 37°C, over periods of 5 – 20 minutes incubation

Incubation times; **A** 5 minutes, **B** 10 minutes, **C** 15 minutes and **D** 20 minutes. All reaction tubes contained ammonium bicarbonate ph8.0 buffer, 12.5µg/mL trypsin and 0.123mM PYY(3-36). Digest solutions were analysed by reverse phase HPLC using an analytical C-18 column (Vydac 218TP54, 250 x 4.6mm, 5 µm particle size, and 300 Å pore size) and a linear AB gradient of 0–100% for B over 40 min at a flow rate of 1mL/min, where eluent A was 0.1% TFA/water and eluent B was 0.1% TFA in 60% acetonitrile/water. Peak detection was by spectrophotometry at a wavelength of 210 nm.

3.4.1.4 Dose response investigation

The HPLC chromatograms from this stage of the investigation are shown in Figure 3.4. It can be seen that chromatograms **A** (25µg/mL trypsin) and **B** (12.5µg/mL trypsin) display similar peak patterns to each other, with only 3 main peaks present, at retention times 23, 21 ½ and 18 minutes. Chromatogram **C** (6.25µg/mL trypsin) shows a reduction in the size of the peak at 18 minutes, with peaks at 24 and 28 minutes now present. In **D** (3.125µg/mL trypsin), the peak at 28 minutes is now the dominant one, with the other peaks all much smaller in comparison.

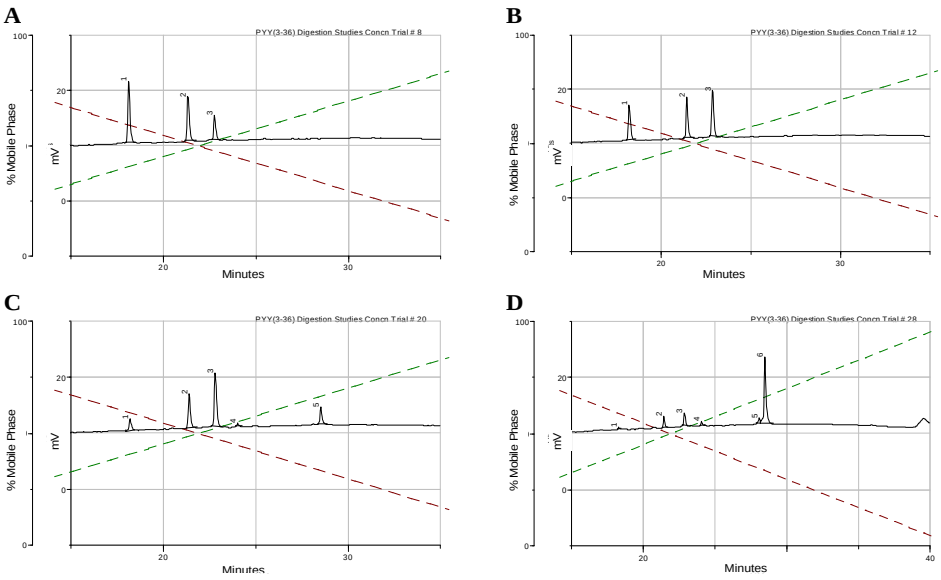


Figure 3.4 Representative reverse phase HPLC absorption profiles following *in vitro* digestion of PYY(3-36) at 37°C by varying concentrations

Trypsin concentrations; **A** 25 µg/mL, **B** 12.5 µg/mL, **C** 6.25 µg/mL and **D** 3.125 µg/mL. All reaction tubes contained ammonium bicarbonate ph8.0 buffer and 0.123mM PYY(3-36). Digest solutions were analysed by reverse phase HPLC using an analytical C-18 column (Vydac 218TP54, 250 x 4.6mm, 5 µm particle size, and 300 Å pore size) and a linear AB gradient of 0–100% for B over 40 min at a flow rate of 1mL/min, where eluent A was 0.1% TFA/water and eluent B was 0.1% TFA in 60% acetonitrile/water. Peak detection was by spectrophotometry at a wavelength of 210 nm.

3.4.1.5 MALDI MS data

All of the solutions from the three different stages of the investigation were analysed by MALDI MS. If there was more than one significant peak on the HPLC chromatogram, the solutions were re-analysed by HPLC and the peaks collected for analysis by MALDI MS. Table 3.1 details the average molecular ion mass detected for each of the peaks seen in the different chromatograms.

Table 3.1 Average molecular ion masses detected by MALDI MS during studies of trypsin digestion of PYY(3-36)

HPLC peak retention time (minutes)	Average mass by MALDI MS [M+H]	Proposed PYY fragment
18	1853.5	PYY(3-19)
21.5	1015.9	PYY(26-33)
23	2606.9	PYY(3-25)
28	3605.1	PYY(3-33)

3.4.2 Dipeptidyl peptidase degradation of PYY(3-36)

The HPLC and MALDI MS data showed no degradation in reaction tubes for time periods up to 8 hours. After this time period, minimal degradation of PYY(3-36) occurred. This was mirrored in the control tubes containing no enzyme.

3.4.3 Neprilysin degradation of PYY(3-36)

The HPLC and MALDI MS data from the solutions from this investigation showed no degradation of PYY(3-36) after each time interval.

3.4.4 Trypsin degradation of PYY(3-36) analogues

Figure 3.5 shows HPLC chromatograms for reaction solutions from each of the PYY(3-36) analogues tested. The chromatogram of the solution containing native PYY(3-36) is not shown as it was consistent with those produced in section 3.4.1.3.

Only two analogues, IP7 and IP8, display similar degradation patterns, as detected by HPLC, to that of native PYY(3-36). The remaining analogues display a different

degradation pattern to native PYY(3-36) and this pattern appears to be consistent throughout the analogues. The average mass of the molecular ions detected in these solutions are shown in Table 3.2.

Table 3.2 Average molecular ion masses detected by MALDI MS during studies of trypsin digestion of PYY(3-36) analogues

Analogue	Average mass by MALDI MS [M+H]	Proposed PYY fragments
IP1	3617.2, 1783.9	(Homoarg)^{25}PYY(3-33), (Homoarg)^{25}PYY(19-33)
IP2	3603.3, 1768.8	(D-Arg)^{25}PYY(3-33), (D-Arg)^{25}PYY(19-33)
IP3	3631.9, 1797.6	(Arg(Me)$_2$symm)^{25}PYY(3-33), (Arg(Me)$_2$symm)^{25}PYY(19-33)
IP4	3632.4	(Arg(Me)$_2$asymm)^{25}PYY(3-33),
IP5	3603.1, 1770.2	(Cit)^{25}PYY(3-33), (Cit)^{25}PYY(19-33)
IP6	3603.6, 1770.1, 1851.9	(D-Cit)^{25}PYY(3-33), (D-Cit)^{25}PYY(19-33), PYY(3-19)
IP7	2606.8, 1853.7, 1029.6	PYY(3-25), PYY(3-19), (His(1-Me))^{26}PYY(3-36)
IP8	2606.0, 1852.1, 1029.6	PYY(3-25), PYY(3-19), (His(3-Me))^{26}PYY(3-36)
IP9	3603.1, 1769.0	(D-His)^{26}PYY(3-33), (D-His)^{26}PYY(19-33)
IP10	3650.6. 1817.9	(Phe-Gu)^{25}PYY(3-33), (Phe-Gu)^{25}PYY(19-33)

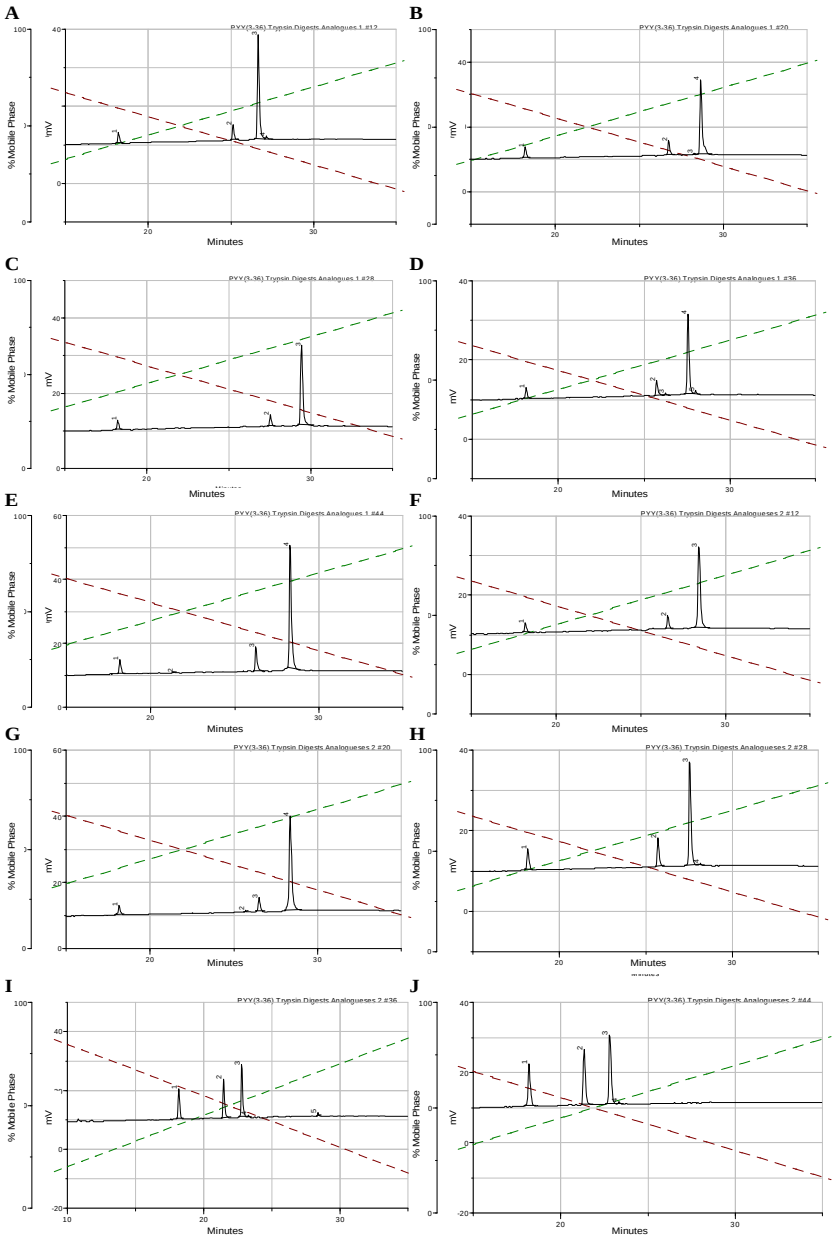


Figure 3.5 Representative reverse phase HPLC absorption profiles following *in vitro* digestion of PYY(3-36) analogues by trypsin at 37°C

PYY(3-36) analogues; **A** IP2, **B** IP1, **C** IP5, **D** IP6, **E** IP10, **F** IP3, **G** IP4, **H** IP9, **I** IP7 and **J** IP8. All reaction tubes contained ammonium bicarbonate ph8.0 buffer, 12.5µg/mL trypsin and 0.123mM PYY(3-36) analogue. Digest solutions were analysed by reverse phase HPLC using an analytical C-18 column (Vydac 218TP54, 250 x 4.6mm, 5 µm particle size, and 300 Å pore size) and a linear AB gradient of 0–100% for B over 40 min at a flow rate of 1mL/min, where eluent A was 0.1% TFA/water and eluent B was 0.1% TFA in 60% acetonitrile/water. Peak detection was by spectrophotometry at a wavelength of 210 nm.

Following the MALDI MS results, solutions were made containing 100µL of the digest solutions for analogues (IP1-6, 9 & 10), and 100µL of 0.123mM PYY(3-36) analogue in buffer. These solutions were analysed by HPLC. The chromatograms produced for analogues (IP1, 3, 4, 5 & 10) are shown in Figure 3.6. The remaining three chromatograms for analogues (IP2, 6 and 9) contained only one main peak and are not shown.

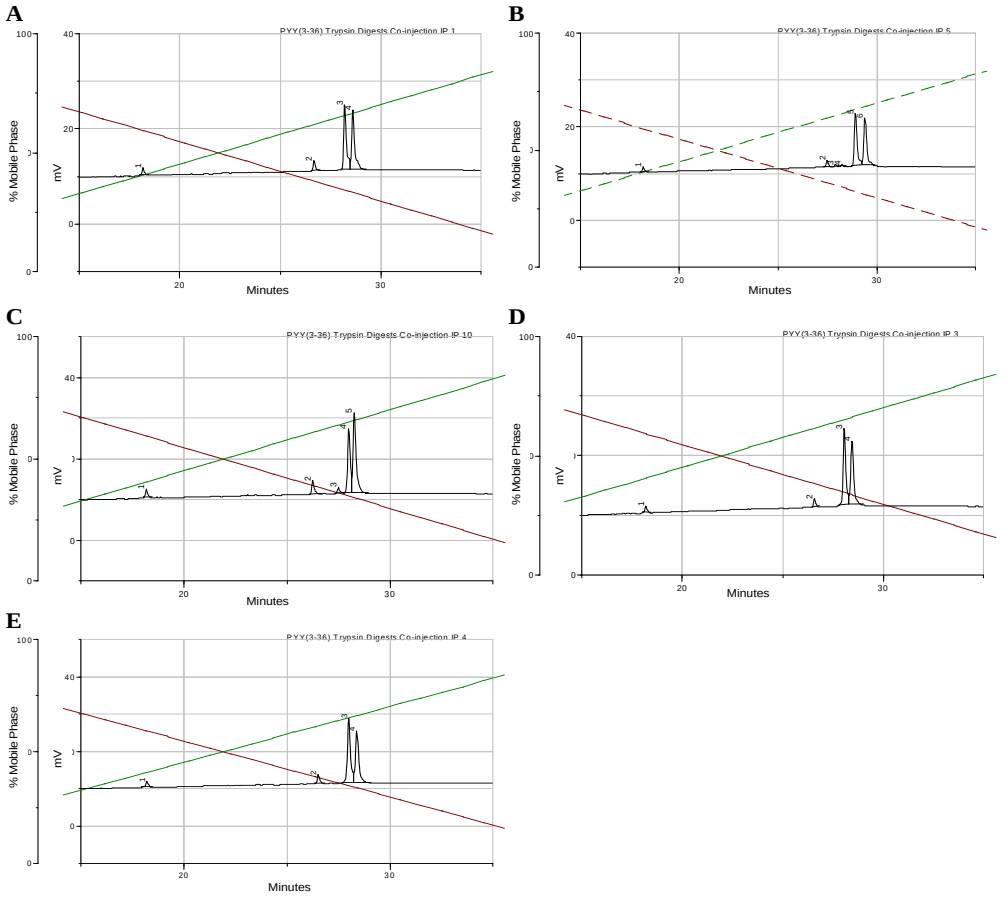


Figure 3.6 Representative reverse phase HPLC absorption profiles of co-injected solutions of PYY(3-36) analogues and the corresponding trypsin digest solution

PYY(3-36) analogues; **A** IP1, **B** IP5, **C** IP10, **D** IP3 and **E** IP4. Each solution consisted of 100µL of digest solution and 100µL of 0.123mM PYY(3-36) analogue. Solutions were analysed by reverse phase HPLC using an analytical C-18 column (Vydac 218TP54, 250 x 4.6mm, 5 µm particle size, and 300 Å pore size) and a linear AB gradient of 0–100% for B over 40 min at a flow rate of 1mL/min, where eluent A was 0.1% TFA/water and eluent B was 0.1% TFA in 60% acetonitrile/water. Peak detection was by spectrophotometry at a wavelength of 210 nm.

3.5 Discussion

A number of interesting and surprising results have been obtained from this part of the investigation. The primary site for trypsin degradation of PYY(3-36) has been shown to be different to that which was hypothesised. Secondly, the extent to which PYY(3-36) is degraded was higher than expected. However, it has been possible to show that some of the unnatural amino acid insertions in positions 25 and 26 of the molecule, conferred stability against trypsin activity in those positions.

3.5.1 Trypsin degradation of PYY(3-36)

From this part of the investigation it is clear that in the presence of trypsin, PYY(3-36) is not simply cleaved at the 25-26 position into two pieces. The results of the initial efficiency trial show three separate peaks on the HPLC chromatogram for the solution containing both PYY(3-36) and trypsin. These peaks all had retention times different to that of the parent peptide. Hence, there must be at least three different peptide fragments present in this solution. If this is so, then there must be more than one trypsin cleavage site within PYY(3-36), or a spontaneous cleavage site that breaks following the initial trypsin cleavage.

The time-course studies suggest that the different cleavages are time dependent. Over the time period 30 minutes to 6 hours, the relative sizes of the three peaks change. The earliest peak increases, whilst the latest peak decreases in size. This suggests that the peptide fragment with retention time 23 minutes is subsequently further fragmented producing the peptide fragment with retention time 18 minutes.

The HPLC analysis of the reaction mixtures following shorter reaction times initially suggested that there is a critical time period for the onset of cleavage, as what appears

to be a peak representing PYY(3-36) occurs in the solutions for the 5,10 and 15 minute reactions. However, this peak, at retention time 28 minutes, does not show the expected mass of the PYY(3-36) (4049.52), but rather that of a fragment (3605.1). This would suggest the presence of another trypsin cleavage site within the sequence of PYY(3-36), possibly that at position 33-34 as the molecular weight of PYY(3-33) is 3602.46.

The results of the dose response investigation suggest that the cleavages occur in a stepwise manner. At high concentrations, the degradation pattern appears to be the same as those from the long reaction times. However, at lower concentrations the pattern mimics that of the shorter reaction times. This suggests that the cleavage site that creates the fragment with mass 3605 is the initial site of cleavage.

Collection of the three peaks that were present in the majority of the HPLC chromatograms enabled definitive mass identification. The masses had to then be matched to fragments of PYY(3-36). The initial belief was that trypsin would preferentially cleave PYY(3-36) at the 25-26 (Arg-His) position. The mass of PYY(3-25) is 2605.87, which suggests this is the fragment with retention time 23 minutes. This fragment appears to further cleaved to produce the peptide with retention time 18 minutes. Within PYY(3-25) there is another arginine residue at position 19. The mass of PYY(3-19) is 1852.0 which correlates with the mass detected when the peak with retention time 18 minutes was collected. Initially it was thought that the fragment PYY(26-36) would be produced. The mass of this peptide is 1461.70, which was not detected by MALDI MS, suggesting that the fragment PYY(26-36) is not formed during trypsin degradation of PYY(3-36)), or that if it is produced, it is only transiently present.

The sequence of PYY(26-36) contains two arginine residues at positions 33 and 35. The mass of PYY(26-35) is 1299.51, whilst that of PYY(26-33) is 1015.19. This second mass is equal to that found when the peak with retention time 21.5 minutes was collected and analysed by MALDI MS, further supporting the concept of position 33-34 being the initial site of trypsin degradation.

From these deductions, it appears that the initial site for trypsin degradation of PYY(3-36), is not between the pair of basic amino acids in positions 25 and 26, but rather between the amino acids in positions 33 and 34. The second cleavage site appears to be between positions 25 and 26. Finally, cleavage occurs between the amino acids in positions 19 and 20.

Due to the difficulty in identifying di- and tri- peptide fragments via both reverse phase HPLC and MALDI MS it is not possible to determine if the PYY(34-36) fragment is itself cleaved between the amino acids in positions 34 and 35. The fragment PYY(20-25) was not detected in any of the reaction solutions.

In conclusion, these data suggest that the degradation of PYY(3-36) by trypsin targets every arginine residue in the sequence. Also, it seems that this digestion proceeds in a particular order, with certain positions in the amino acid sequence cleaved before others. Importantly, the basic amino acid pair at positions 25-26 is not the initial site of cleavage by trypsin.

3.5.2 DPP IV degradation of PYY(3-36)

The lack of degradation found in this investigation was expected. DPP IV is known to cleave N-terminal dipeptides X-Ala and X-Pro and as the N-terminal dipeptide of PYY(3-36) is Ile-Lys, DPP IV action on this peptide would be unlikely.

The signs of degradation observed during the longer reaction times can not be attributed to the action of the enzyme as identical results were seen in the control reactions containing no enzyme. This degradation is therefore likely to be due to the prolonged exposure of the peptide to the reaction temperature and/or pH.

3.5.3 Neprilysin degradation of PYY(3-36)

The lack of degradation caused by neprilysin was unexpected, as this enzyme is known to cleave peptides containing hydrophobic residues (Turner et al., 2001; Shirotani et al., 2001). PYY(3-36) contains a number of hydrophobic amino acids, including a Leu-Val dipeptide at position 30 and 31. The lack of degradation observed may be due to the low concentration of the enzyme solution used, or perhaps due to the enzyme denaturing during storage at -20°C. It would be interesting to check the ability of the batch of enzyme used to degrade a peptide neprilysin is known to target, for example substance P or amyloid ß proteins, to confirm its bioactivity.

3.5.4 Trypsin degradation of PYY(3-36) analogues

It was initially thought that modifying the sequence of PYY(3-36) at positions 25 and 26, would generate PYY(3-36) analogues that are resistant to trypsin-induced degradation. However, the studies with PYY(3-36) and trypsin described above (carried out concurrently with these analogue studies) demonstrate that this position is not the first site targeted by trypsin, though it is subsequently targeted.

Each of the ten analogues produced had a different modification to the sequence in either the 25 or 26 positions. These data suggest that only two of these modifications failed to prevent trypsin cleavage at this bond. These were the substitution of His^{26}

with His(1-Me) (IP7) and His(3-Me) (IP8). In both cases, fragments terminating at positions 25 and 26 were found, consistent with degradation of PYY(3-36).

Of the remaining eight analogues, all of the modifications resulted in stability to trypsin activity at the 25-26 position. This was evident from the results which showed the presence of fragments containing both amino acids at positions 25 and 26. There was also a complete absence of fragments terminating at these positions. There appeared to be little difference in the stabilising qualities of the different modifications. This was surprising, as the modifications ranged from a subtle change in residue (D-Arg for Arg^{25}) (IP2) to more extensive alterations (Phe-Gu for Arg^{25}) (IP10), and suggests that the action of trypsin is highly sensitive to amino acid structure; even small changes in stereochemistry detrimentally affect its effects.

Most interesting was the apparent success of the analogue IP9, in which D-His replaced His at the 26 position. From the investigation into the digestion of PYY(3-36), it appeared that the identity of the amino acid residue directly following an arginine residue was not important for trypsin activity. This hypothesis was supported, first by the facts that Arg-Tyr and Arg-Gln bonds were broken in PYY(3-36), and that substituting His(1-Me) and His(3-Me) for His^{26} failed to prevent cleavage of the 25-26 bond. However, the stability shown by analogue IP9, would suggest that changing the stereochemistry of the residue following an arginine, from the L to the D isomer, stabilises the bond against trypsin activity.

Despite the success of these substitutions at stabilising the 25-26 bond, the masses detected in each of the degradation solutions demonstrated that the 33-34 bond was still being cleaved by trypsin at the same rate as in PYY(3-36). In all cases, the mass corresponding to that of the parent analogue was not detected, whilst the masses of PYY(3-33) analogues were evident. As a final confirmation of this, HPLC co-

injections of the analogue solution together with the digestion solution resulted in two peaks in five of the eight solutions. If the PYY(3-36) analogue had been present in the digestion solution, these additional peaks would not have been seen on the HPLC chromatogram.

The PYY(3-36) analogues were only tested over a period of 15 minutes each and it is possible that over longer periods of time the analogues may begin to display differing fragmentation patterns.

In conclusion, it had been hoped that amino acid substitutions at the 25 and 26 positions would lead to a PYY(3-36) analogue that was more stable than the native sequence to trypsin activity. This has proven not to be the case as this bond is not the primary site for trypsin activity. However, the majority of the substitutions were successful at stabilising the 25-26 bond to trypsin cleavage. There is every possibility that by implementing these same substitutions at other points in the molecule, in particular at the 33 position, the analogue would show increased stability to the action of trypsin.

4 *In vitro* and *in vivo* investigations with PYY(3-36) analogues

4.1 Introduction

PYY(3-36) mediates its inhibitory effects on food intake via the Y_2 receptor, a member of the NPY Y receptor family of G-protein coupled receptors. The Y receptors are distributed throughout both the central and peripheral nervous systems, and are also found in non-neuronal tissues (Parker and Balasubramaniam, 2008).

4.1.1 The Y_2 receptor

The human Y_2 receptor was first reported to have been cloned in 1986 and was found to have only 30% sequence homology with the Y_1 and Y_4 receptors (Parker and Balasubramaniam, 2008). The Y_2 receptor is highly conserved across species, with greater than 90% homology between mammalian species (Berglund et al., 2003). The human and chicken Y_2 receptors share 80% sequence homology (Parker and Balasubramaniam, 2008). This high degree of conservation suggests an important role for the Y_2 receptor in, for example, developmental and metabolic mechanisms.

The Y_2 is largely located pre-synaptically where it acts to inhibit release of neurotransmitters (Berglund et al., 2003).

Aside from its well reported role in the regulation of appetite, the Y_2 receptor has been implicated in a number of other physiological and metabolic functions; Y_2 agonists increase the rate of intestinal absorption of water and sodium. Y_2 knock out mice display slower wound healing than wild type mice, and it has also been proposed that the Y_2 receptor has a role in angiogenesis and bone formation (Parker and Balasubramaniam, 2008).

A number of human neuroblastoma cell lines express NPY Y receptors. In particular, the SMS KAN cell line has been found to abundantly express Y_2 receptors (Shigeri and Fujimoto, 1994).

4.2 Hypothesis and aims

4.2.1 Hypothesis

In vitro and *in vivo* testing of PYY(3-36) analogues will determine their efficacy as potential anti-obesity therapeutics.

4.2.2 Aims

1. To determine the binding affinity of a series of PYY(3-36) analogues for the Y_2 receptor,.
2. To determine the efficacy of a series of PYY(3-36) analogues at reducing food intake *in vivo.*

4.3 Materials and methods

4.3.1 Membrane preparation

For a number of the following receptor binding assays, the SMS KAN human neuroblastoma cell line will be used.

Cells were grown in DMEM containing 0.11g/L Na Pyr with pyroxidone, 10% FCS and 100 units/L penicillin sodium pyruvate and 100µg/L streptomycin sulphate in large flasks (20 X T175cm^2) until confluent. They were then washed with Phosphate Buffered Saline (PBS) and harvested from the flasks using versene alone. Cells were centrifuged at 200xg for 5 minutes, the supernatants discarded and the pellets put onto ice. To the pellets 20mL of ice-cold homogenisation buffer (50mM HEPES buffer pH 7.4 containing 0.25M sucrose, and a range of protease inhibitors (10µg/mL soybean trypsin inhibitor, 0.5µg/mL pepstatin, 0.25µg/mL leupeptin, 0.25µg/mL antipain, 0.1mg/mL benzamidine and 30µg/mL aprotinin)) was added and cells homogenised for

1 minute with an Ultra Turrax homogeniser. Homogenates were poured into ultracentrifuge tubes and centrifuged for 30 minutes at 4^{o}C, 50,000xg in a Sorvall OTD 55B ultra-centrifuge. Supernatants were discarded and pellets resuspended in homogenisation buffer without sucrose using a hand-homogeniser.

The protein content of the membrane produced was assessed by the Biuret method as follows;

A standard curve was constructed using 100, 200, 300, 400 and 500µL of 10mg/mL bovine serum albumin (BSA). Typically 50-200µL of membrane suspension was assayed. All volumes were adjusted to 500µL and 3mL Biuret reagent (1litre: 1.5g $CuSO_4.5H_2O$, 30g NaOH, 6g Na K tartrate, 1g KI, 15g Na deoxycholate) was added to all tubes. The tubes were then incubated at 100^{o}C for 2 minutes. Samples were left at room temperature for 45 minutes before the absorbance at A_{540} nm was measured in a spectrophotometer (Shimadzu UV-160) and a standard curve plotted. The absorbance of the membrane samples was then converted to protein concentration (mg/mL) by comparison with the standard curve.

4.3.2 Iodination of PYY(3-36)

PYY(3-36) was iodinated by the direct iodogen method. PYY(3-36) (5nmol) in 10µl 0.2M phosphate buffer (pH 7.2) was added to a polypropylene vial containing 10µg iodogen reagent together with 37MBq $Na^{125}I$, and incubated for 5 minutes on ice. Iodinated peptide was separated by reversed-phase HPLC on a 20-50%, 90 minute ACN/H_2O/0.05% TFA gradient. Peak fractions containing radioactivity were tested for binding to membranes, freeze-dried overnight (crystalline BSA added to a final concentration of 0.6%) and stored in vacuo at -20^{o}C.

4.3.3 Receptor binding assays

4.3.3.1 PYY(3-36) analogues (IP1 – IP11)

Membranes (human SMS KAN)(100μg) were incubated for 90 minutes in silanised polypropylene tubes together with ^{125}I-PYY(3-36) (500 Bq) at 30^{o}C in binding buffer (HEPES pH 7.4 20mM, $MgCl_2$ 1mM, $CaCl_2$ 5mM, plus 1% BSA) in a final assay volume of 0.5mL. Bound and free label were separated by centrifugation at 15,600xg for 2 minutes followed by washing with 1mL of binding buffer and re-centrifugation. Radioactivity in the pellet was measured using a γ-counter. Specific binding (SB) was calculated as the difference between the amount of ^{125}I-PYY(3-36) bound in the absence (total) and presence (non-specific) of either unlabelled PYY(3-36) (20pM – 2nM) or PYY(3-36) analogues (IP1 – IP11) (2pM – 200nM).

4.3.3.2 PYY(3-36) analogues (IP12 – IP19)

The assays were performed according to the methods described in section 4.3.3.1.

4.3.3.3 PYY(3-36) analogue (IP20)

The assay was performed according to the methods described in section 4.3.3.1. Following the results of the first assay, a second assay was performed using iodinated PYY(1-36) (Iodinated according to methods described in section 4.3.2).

4.3.3.4 PYY(3-36) analogues (4075004, 4075005 & 4075006)

The assays were performed according to the methods described in section 4.3.3.1. The membranes used were HEK 293 cells manipulated to over express mouse Y_2 receptors (a kind gift from Dr. James Gardiner, Section of Investigative Medicine, Imperial College). The radioactive label used was iodinated PYY(1-36) (Iodinated according to

methods described in section 4.3.2). The range of concentrations investigated for both PYY(3-36) and the analogues was 0.01pM to 200nM.

4.3.4 Acute feeding studies

4.3.4.1 PYY(3-36) analogues (IP1 – IP11)

C57BL/6 mice (20-25g), purchased from Harlan UK, were singly-housed and maintained under controlled conditions of temperature (21-23^{o}C) and light (lights on at 0700, lights off at 1900) with *ad libitum* access to RM1 diet (Special Diet Services, Witham, UK). All animal procedures undertaken were approved by the British Home Office Animals (Scientific Procedures) Act 1986. Animals were acclimatised to laboratory conditions for at least one week and handled daily prior to the first study, during which time they received two injections of saline in order to minimize stress on the study days. Stressed animals display anorectic tendencies. Exposure to minor stressful events, including absence of pre-study handling and exposure to a novel environment, inhibits the anorectic effects of PYY(3-36) in rats (Abbott et al., 2006). Intraperitoneal (IP) injections were administered via a 1ml syringe with a 28-gauge needle (maximum injection volume 0.2 ml). For each study animals were fasted from 4pm the preceding day and randomized into groups for injection in the early light phase (0900-1000). Each study consisted of 48 mice randomized into five groups with all the members of each group receiving the same injection of either peptide or control. The peptides used for the studies were PYY(3-36), exendin-4 and the PYY(3-36) analogues (IP1 – IP11). They were dissolved in 0.9% saline with 0.9% saline administered as the control injection. The dosage used for PYY(3-36) and the analogues was 30nmol/kg and for exendin-4 was 3nmol/kg. Following injection, animals were returned to their home cages containing a pre-weighed amount of food.

Food remaining in the cage dispenser was re-weighed at 1, 2, 3, 4, 6, 8 and 24 hours post-injection. Body weight was recorded at t=0 and t=24 hours.

4.3.4.2 PYY(3-36) analogues (4075004, 4075005 & 4075006)

Studies were conducted as per the methods described in section 4.3.4.1. The effect of intraperitoneal (IP) administration of saline control, PYY(3-36), PYY(3-36) analogues (4075004, 4075005 & 4075006) (30nmol/kg) on food intake was investigated. Overnight fasted animals were injected in the early light phase 09.00-10.00hr with the designated treatment (n=9-10). Body weight was measured at 0 and 24 hours and food intake 1, 2, 4, 8 and 24 hours post injection.

4.3.5 Statistical analysis

All receptor binding assay and food intake data are expressed as mean ± standard deviation. Food intake data was also analysed by Analysis of Variance (ANOVA) with a post-hoc Dunnett's test (Systat, Evanston, IL, USA). In all cases, $P < 0.05$ was considered to be statistically significant.

4.4 Results

4.4.1 Receptor binding assays

4.4.1.1 PYY(3-36) analogues (IP1 – IP11)

The specific binding curves for each of the PYY(3-36) analogues (IP1 – IP11) are shown in Figures 4.1 and 4.2. Two analogues were tested in each assay alongside human PYY(3-36), and the IC_{50} value for each peptide was calculated. The IC_{50} value is the concentration of unlabelled peptide required for 50% specific binding of the receptor. These values are summarised in Table 4.1. The IC_{50} value recorded for

human PYY(3-36) is the average value from all assays (n=6). All PYY(3-36) analogues (IP1 – IP11) displayed less affinity for the Y_2 receptor than PYY(3-36).

Table 4.1 The IC_{50} values for the PYY(3-36) analogues (IP1 – IP11)

Analogue	Code	IC_{50} (M)	IC_{50} (nM)
PYY(3-36)	**-**	**5.33E-10**	**0.533 ± 0.054**
(Homoarg)[25] PYY(3-36)	IP1	1.65E-09	1.65
(D-Arg)[25] PYY(3-36)	IP2	3.78E-09	3.78
(Arg(Me)$_2$symm)[25] PYY(3-36)	IP3	2.10E-09	2.10
(Arg(Me)$_2$asymm)[25] PYY(3-36)	IP4	1.45E-09	1.45
(Cit)[25] PYY(3-36)	IP5	3.08E-09	3.08
(D-Cit)[25] PYY(3-36)	IP6	1.30E-08	13.0
(D-His)[26] PYY(3-36)	IP7	9.65E-09	9.65
(His(1-Me))[26] PYY(3-36)	IP8	1.49E-09	1.49
(His(3-Me))[26] PYY(3-36)	IP9	1.77E-09	1.77
(Phe-Gu)[25] PYY(3-36)	IP10	4.38E-09	4.38
Btn-PYY(3-36)	IP11	1.04E-09	1.04

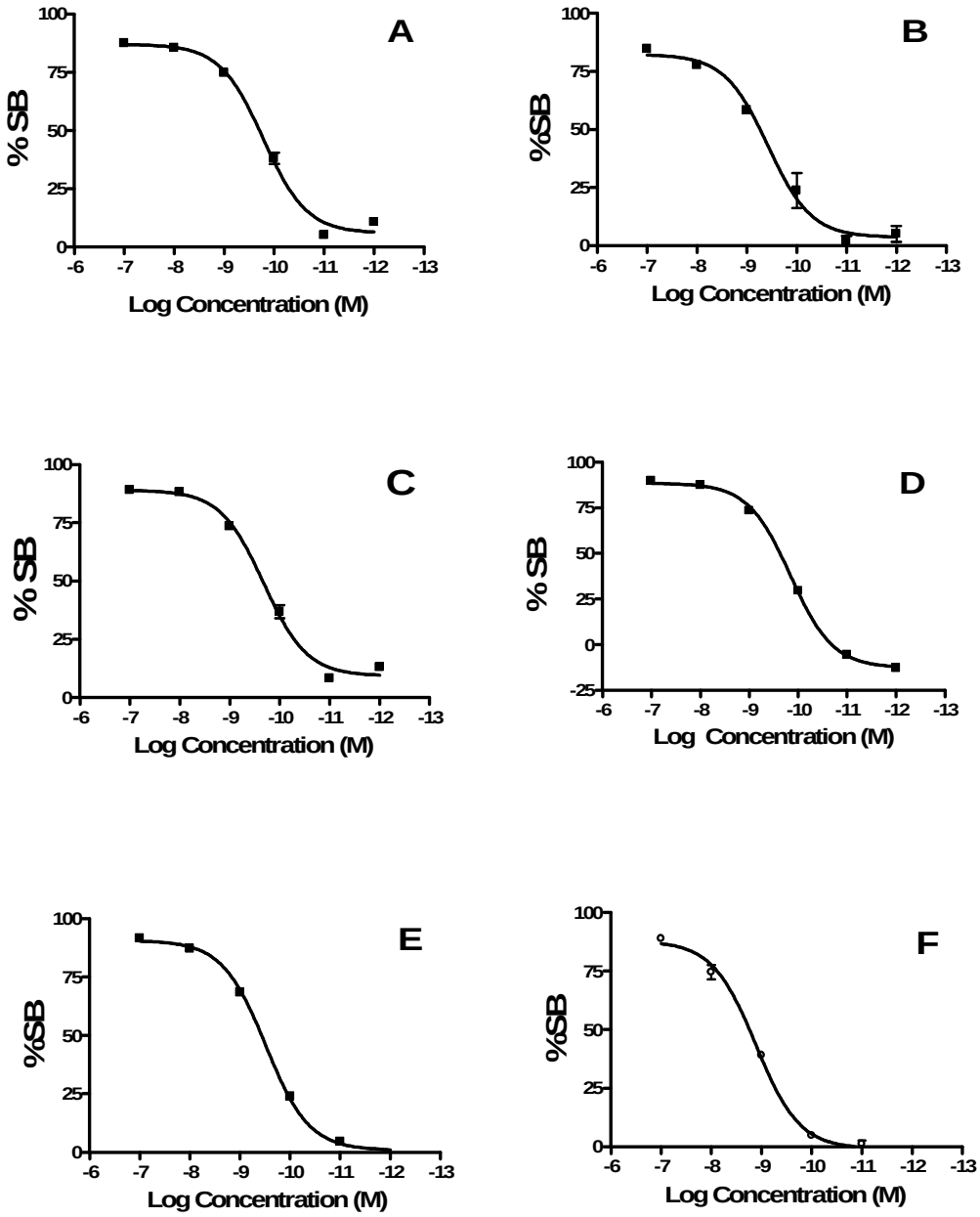


Figure 4.1 Representative competition binding affinity curves of PYY(3-36) analogues IP1 – IP6 to the human Y_2 receptor

A, IP1; **B**, IP2; **C**, IP3; **D**, IP4; **E**, IP5; **F**, IP6. Abbreviations: % SB, specific binding of, unlabelled peptide relative to labelled peptide, to the receptor. Radiolabelled I^{125}-PYY(3-36) was used to compete with unlabelled peptide for human Y_2 receptor binding sites. Cell membranes of SMS KAN cells were the source of receptors.

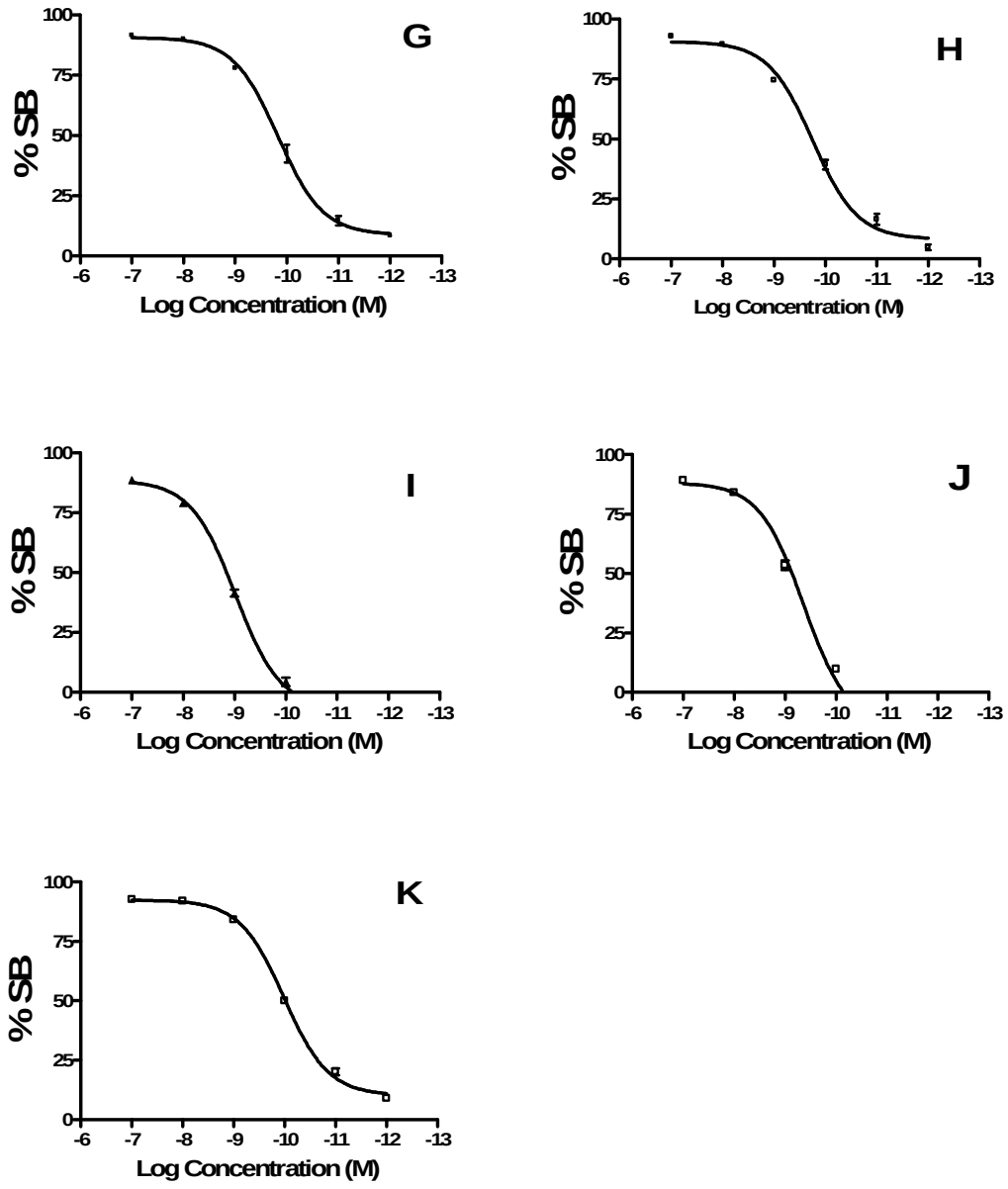


Figure 4.2 Representative competition binding affinity curves of PYY(3-36) analogues IP7 – IP11 to the human Y_2 receptor

G, IP7; **H**, IP8; **I**, IP9; **J**, IP10; **K**, IP11. Abbreviations: % SB, specific binding of, unlabelled peptide relative to labelled peptide, to the receptor. Radiolabelled I^{125}-PYY(3-36) was used to compete with unlabelled peptide for human Y_2 receptor binding sites. Cell membranes of SMS KAN cells were the source of receptors.

4.4.1.2 PYY(3-36) analogues (IP12 – IP19)

The specific binding curves for each of the PYY(3-36) analogues (IP12 – IP19) are shown in Figures 4.3 and 4.4. Two analogues were tested in each assay alongside human PYY(3-36) and the IC_{50} value for each peptide was calculated. These values are summarised in Table 4.2. For all analogues except IP13 and IP18, repeat assays were performed (n=2). The IC_{50} value recorded for human PYY(3-36) is the average value from all assays (n=7). $(Leu)^{31}$PYY(3-36) (IP17) was the only analogue to display greater affinity for the Y_2 receptor than PYY(3-36).

Table 4.2 The IC_{50} values for the PYY(3-36) analogues (IP12 – IP19)

Analogue	Code	IC_{50} (M)	IC_{50} (nM)
PYY(3-36)	**-**	**9.67E-10**	**0.967 ± 0.47**
$(Ala)^{30}$PYY(3-36)	IP12	3.90E-09	3.90
$(Ile)^{30}$PYY(3-36)	IP13	2.45E-09	2.45
$(Val)^{30}$PYY(3-36)	IP14	9.97E-09	9.97
$(Ala)^{31}$PYY(3-36)	IP15	1.36E-08	13.6
$(Ile)^{31}$PYY(3-36)	IP16	1.06E-09	1.06
$(Leu)^{31}$PYY(3-36)	IP17	4.01E-10	0.401
$(Leu)^{32}$PYY(3-36)	IP18	3.15E-09	3.15
$(Ser)^{32}$PYY(3-36)	IP19	1.23E-09	1.23

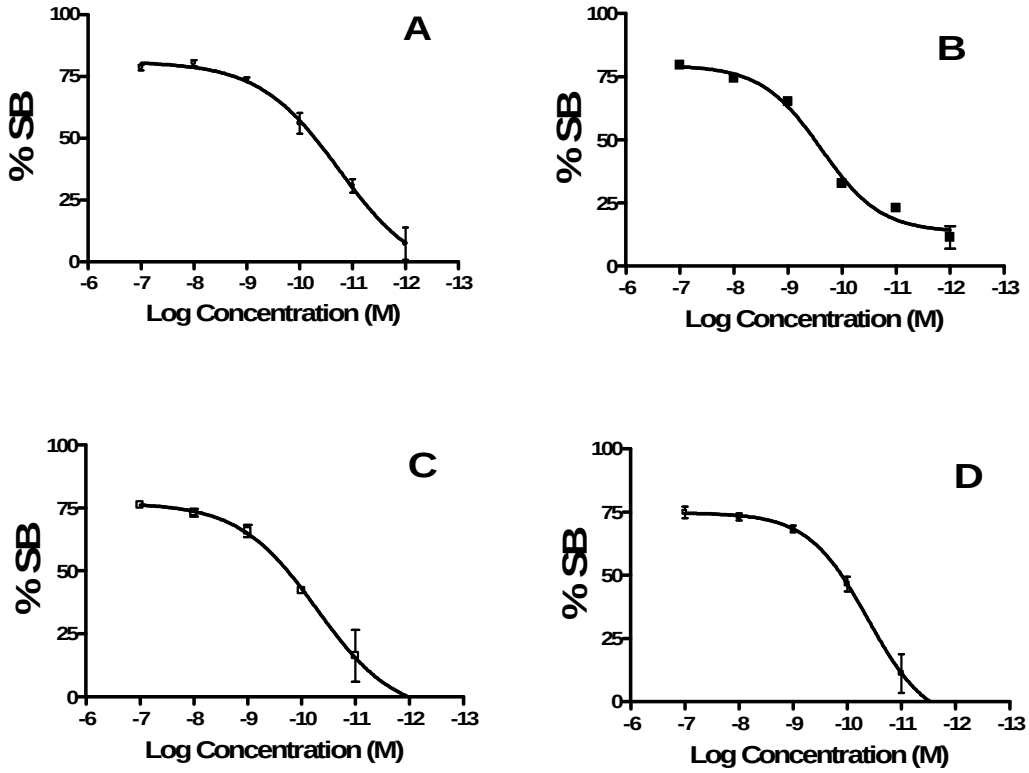


Figure 4.3 Representative competition binding affinity curves of PYY(3-36) analogues IP12 – IP15 to the human Y_2 receptor

A, IP12; **B**, IP13; **C**, IP14; **D**, IP15. Abbreviations: % SB, specific binding of, unlabelled peptide relative to labelled peptide, to the receptor. Radiolabelled I^{125}-PYY(3-36) was used to compete with unlabelled peptide for human Y_2 receptor binding sites. Cell membranes of SMS KAN cells were the source of receptors.

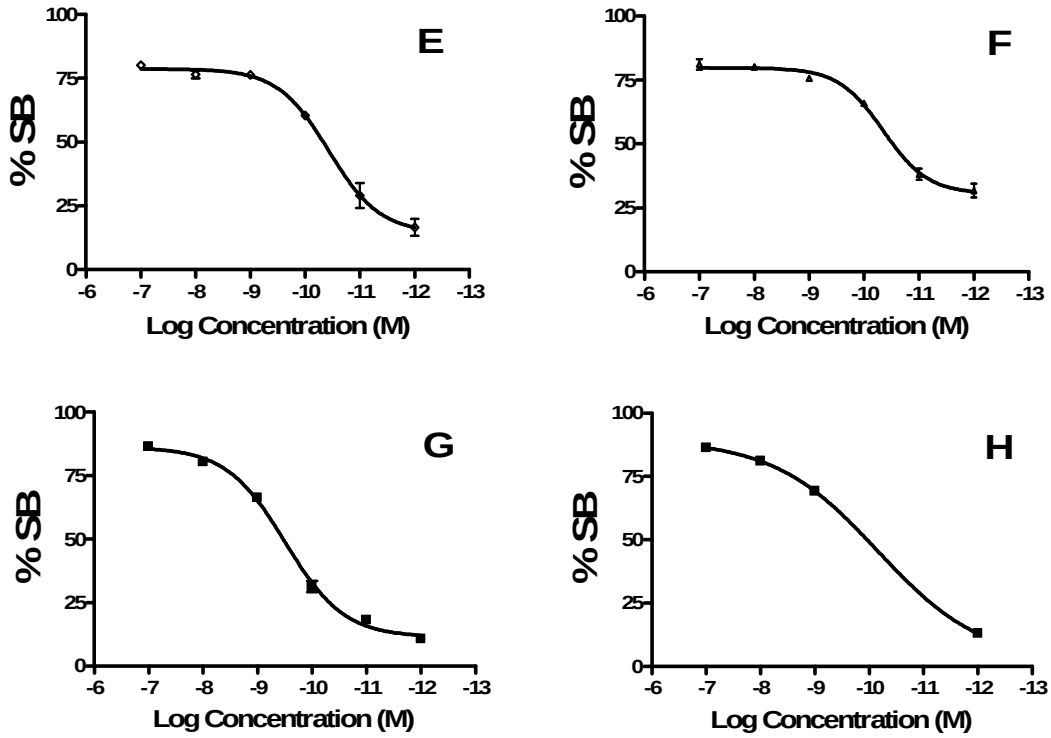


Figure 4.4 Representative competition binding affinity curves of PYY(3-36) analogues IP16 – IP19 to the human Y_2 receptor

E, IP16; **F**, IP17; **G**, IP18; **H**, IP19. Abbreviations: % SB, specific binding of, unlabelled peptide relative to labelled peptide, to the receptor. Radiolabelled I^{125}-PYY(3-36) was used to compete with unlabelled peptide for human Y_2 receptor binding sites. Cell membranes of SMS KAN cells were the source of receptors.

4.4.1.3 PYY(3-36) analogues (4075004, 4075005 & 4075006)

The specific binding curves for the three PYY(3-36) analogues (4075004, 4075005 & 4075006) are shown in Figure 4.5. The analogue 4075006 was tested twice (n=2), as was human PYY(3-36) and the IC_{50} values for each peptide calculated. These values are summarised in Table 4.3, (where n=2 the average IC_{50} value has been calculated).

Table 4.3 The IC_{50} values for the PYY(3-36) analogues (4075004, 4075005 & 4075006)

Analogue	Code	IC_{50} (M)	IC_{50} (nM)
PYY(3-36)		**9.64E-11**	**0.0964**
AEEAc-PYY(3-36)	4075004	4.95E-11	0.0495
Aminoethyl-PEG4-carbonyl-PYY(3-36)	4075005	8.45E-11	0.0845
Aminoethyl-PEG6-carbonyl-PYY(3-36)	4075006	1.92E-10	0.1920

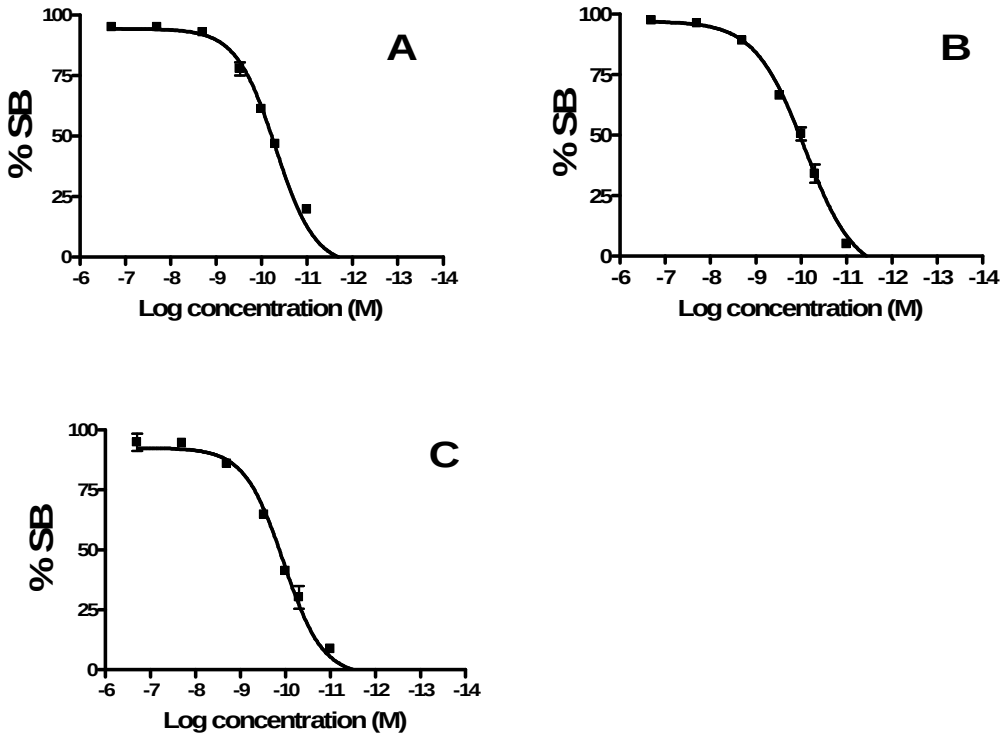


Figure 4.5 Representative competition binding affinity curves of PYY(3-36) analogues 4075004, 4075005 and 4075006 to the mouse Y_2 receptor

A, 4075004; **B**, 4075005; **C**, 4075006. Abbreviations: % SB, specific binding of, unlabelled peptide relative to labelled peptide, to the receptor. Radiolabelled I^{125}-PYY(1-36) was used to compete with unlabelled peptide for mouse Y_2 receptor binding sites. Cell membranes of HEK 293 cells over expressing mouse Y_2 receptors were the source of receptors.

4.4.1.4 PYY(3-36) analogue (IP20)

The results from two separate Y_2 receptor binding assays with analogue (Cys^4, Cys^{29})PYY(3-36)(disulphide bridge) (IP20), were such that a specific binding curve could not be plotted due to an indiscriminate spread of low and negative specific binding values. In both assays, human PYY(3-36) was run alongside IP20. In the first assay, the results for PYY(3-36) were such that a specific binding curve could also not be plotted, due to minimal variation in the specific binding values over the concentrations investigated. However, in the second assay, it was possible to plot a curve for PYY(3-36) and the IC_{50} value was 0.238nM.

4.4.2 Acute feeding studies

4.4.2.1 PYY(3-36) analogues (IP1 – IP11)

4.4.2.1.1 Study 1 – IP1 and IP2 (Figure 4.6)

PYY(3-36) analogue IP2 significantly reduced food intake compared with saline control at 0-1 hour (Saline, 0.96g ± 0.13; IP2, 0.806 ± 0.13; ($p \leq 0.05$)) and reduced food intake over remaining time periods compared with saline, but these effects did not reach statistical significance. Analogue IP1 failed to reduce food intake compared with saline over time periods 0-1 and 0-2 hours and produced a small reduction compared with saline over the time periods 0-8 and 0-24 hours.

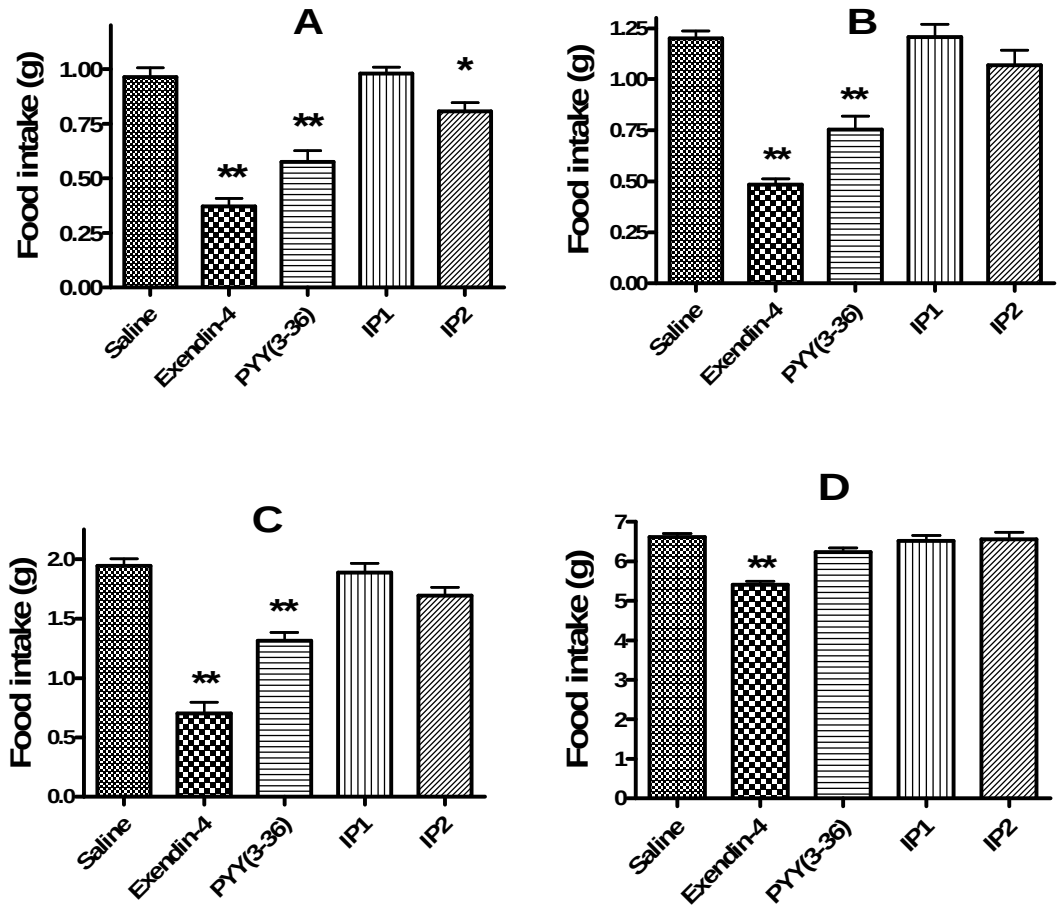


Figure 4.6 The effect of intraperitoneal administration of Exendin-4, PYY(3-36) and PYY(3-36) analogues IP1 and IP2 on cumulative food intake in overnight fasted C57BL/6 mice

A, 0-1 hour; **B,** 0-2 hours; **C,** 0-4 hours; **D**, 0-24 hours post-injection. Doses: Exendin-4, 3nnmol/kg, PYY(3-36) and analogues, 30nmol/kg. (* p≤0.05, **p≤0.01compared to saline using one way ANOVA with post *hoc* Dunnett's test (n=10))

4.4.2.1.2 Study 2 – IP3 and IP4 (Figure 4.7)

PYY(3-36) analogue IP4 significantly reduced food intake compared with saline control at 0-1 hour (Saline, 1.06g ± 0.14; IP4, 0.807 ± 0.15; (p≤0.05)) and reduced food intake over remaining time periods compared with saline, but these effects did

not reach statistical significance. Analogue IP3 reduced food intake compared with saline over time periods 0-1, 0-2 and 0-4 hours but theses effects did not reach statistical significance.

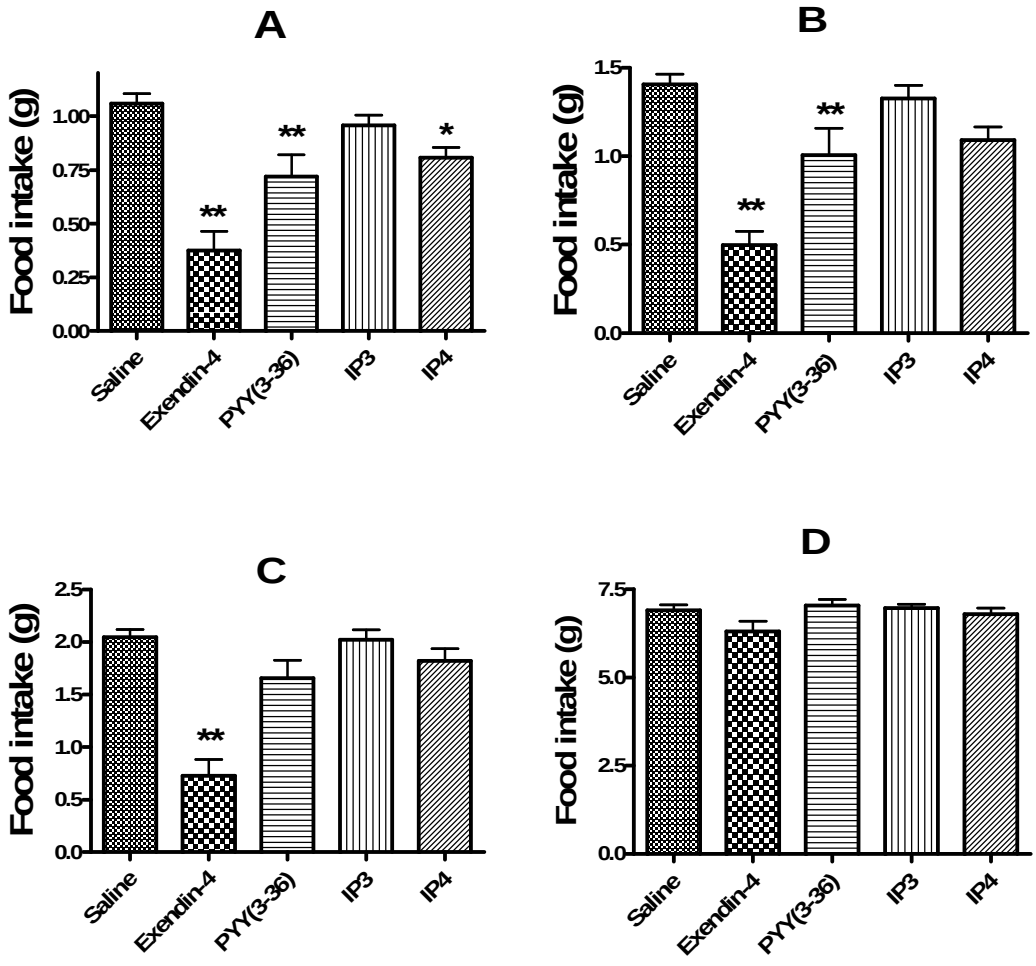


Figure 4.7. The effect of intraperitoneal administration of Exendin-4, PYY(3-36) and PYY(3-36) analogues IP3 and IP4 on cumulative food intake in overnight fasted C57BL/6 mice

A, 0-1 hour; **B,** 0-2 hours; **C,** 0-4 hours; **D**, 0-24 hours post-injection. Doses: Exendin-4, 3nnmol/kg, PYY(3-36) and analogues, 30nmol/kg. (* p≤0.05, **p≤0.01compared to saline using one way ANOVA with post *hoc* Dunnett's test (n=9/10))

4.4.2.1.3 Study 3 – IP5, IP6 and IP7 (Figure 4.8)

All three PYY(3-36) analogues reduced food intake compared with saline control at 0-1 hour, 0-2 hours and 0-4 hours but these effects did not reach statistical significance. Analogue IP5 further reduced food intake compared with saline over time periods 0-8 and 0-24 hours but theses effects also did not reach statistical significance.

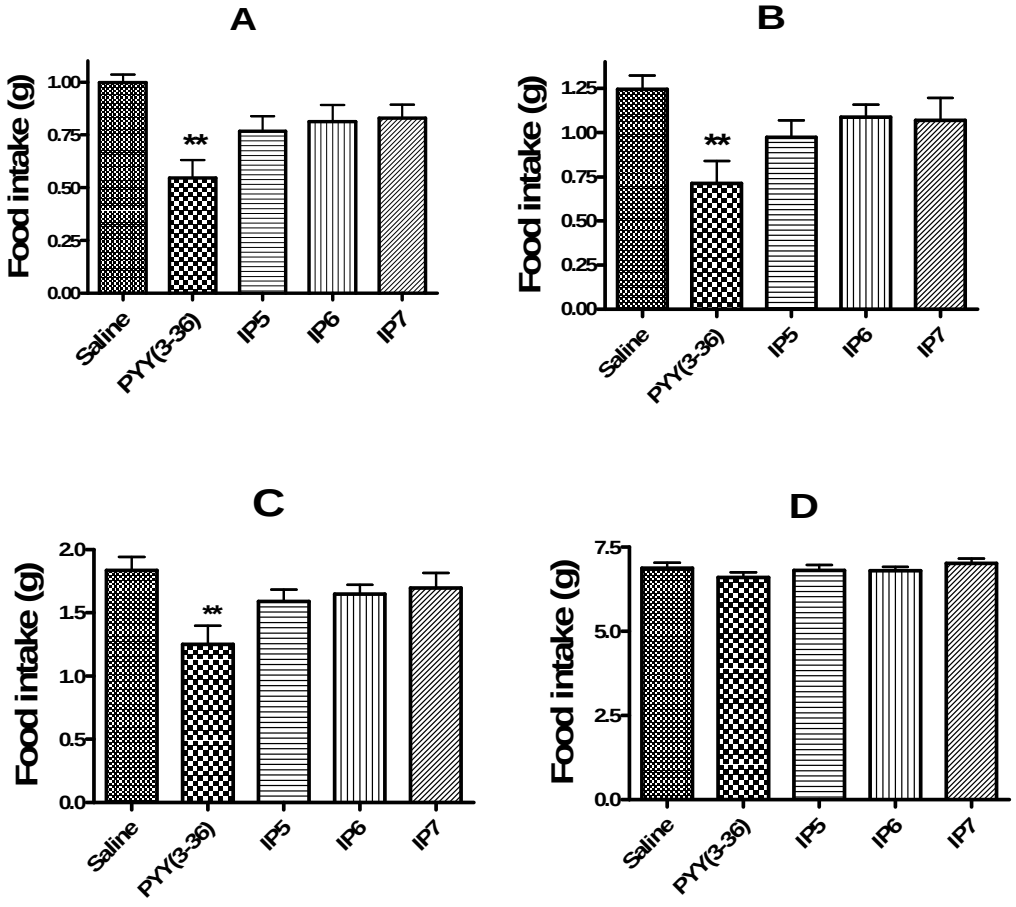


Figure 4.8 The effect of intraperitoneal administration of PYY(3-36) and PYY(3-36) analogues IP5, IP6 and IP7 on cumulative food intake in overnight fasted C57BL/6 mice

A, 0-1 hour; **B,** 0-2 hours; **C,** 0-4 hours; **D**, 0-24 hours post-injection. Dose: PYY(3-36) and analogues, 30nmol/kg. (* p≤0.05, **p≤0.01compared to saline using one way ANOVA with post *hoc* Dunnett's test (n=9/10))

4.4.2.1.4 Study 4 – IP8, IP9 and IP10 (Figure 4.9)

All three PYY(3-36) analogues reduced food intake compared with saline control at 0-1 hour, 0-2 hours and 0-4 hours but these effects did not reach statistical significance. Analogue IP10 further reduced food intake compared with saline over time periods 0-8 and 0-24 hours but theses effects also did not reach statistical significance.

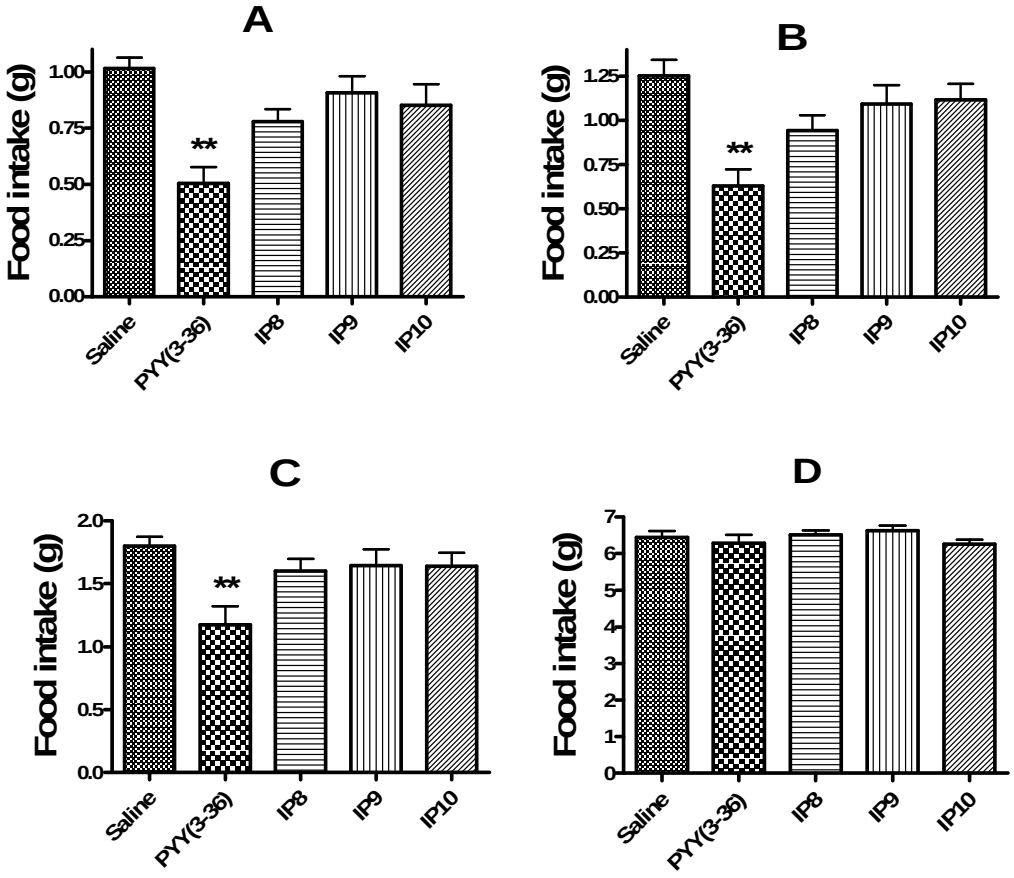


Figure 4.9 The effect of intraperitoneal administration of PYY(3-36) and PYY(3-36) analogues IP8, IP9 and IP10 on cumulative food intake in overnight fasted C57BL/6 mice

A, 0-1 hour; **B,** 0-2 hours; **C,** 0-4 hours; **D**, 0-24 hours post-injection. Dose: PYY(3-36) and analogues, 30nmol/kg. (* p≤0.05, **p≤0.01compared to saline using one way ANOVA with post *hoc* Dunnett's test (n=9/10))

4.4.2.1.5 Study 5 – IP11 (Figure 4.10)

PYY(3-36) analogue IP11 significantly reduced food intake compared with saline control at 0-1 hour (Saline, 0.83g ± 0.2; IP11, 0.46g ± 0.07; p≤0.01), 0-2 hours (Saline, 1.13g ± 0.27; IP11, 0.52g ± 0.07; p≤0.01), 0-4 hours (Saline, 1.67 ± 0.34; IP11, 0.5g ± 0.01; p≤0.01) and 0-24 hours (Saline, 6.41 ± 0.65; IP11, 5.62g ± 0.26; p≤0.01).

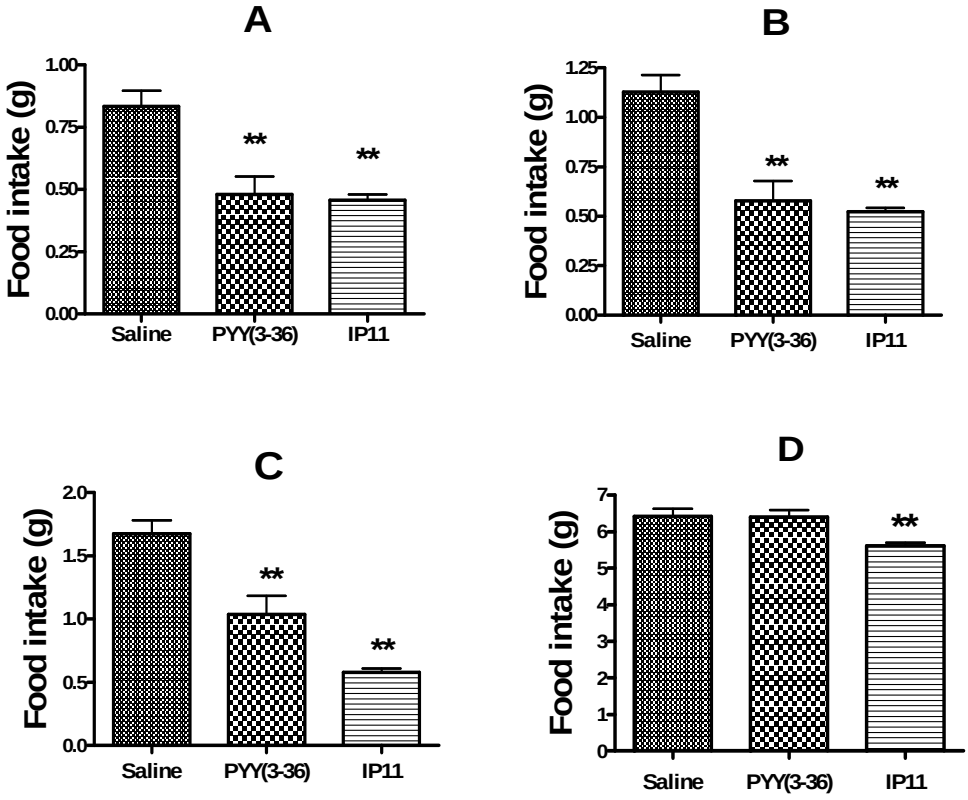


Figure 4.10 The effect of intraperitoneal administration of PYY(3-36) and PYY(3-36) analogue IP11 on cumulative food intake in overnight fasted C57BL/6 mice

A, 0-1 hour; **B,** 0-2 hours; **C,** 0-4 hours; **D**, 0-24 hours post-injection. Dose: PYY(3-36) and analogues, 30nmol/kg. (* p≤0.05, **p≤0.01 compared to saline using one way ANOVA with post *hoc* Dunnett's test (n=10))

4.4.2.2 PYY(3-36) analogues (4075004, 4075005 & 4075006) (Figure 4.11)

PYY(3-36) analogue 4075004 reduced food intake compared with saline control in the time period 0-1 hour but this effect did not reach statistical significance. It significantly reduced food intake compared to saline over the period 0-2 hours (Saline, 0.65g ± 0.25; 4075004, 0.37g ± 0.1; $p \leq 0.01$). Over the time period 0-4 hours, the analogue reduced food intake compared with saline but this effect did not reach statistical significance. Finally, over the time period 0-24 hours, 4075004 slightly increased food intake compared to saline.

PYY(3-36) analogue 4075005 significantly reduced food intake compared with saline over the time period 0-1 hour (Saline, 0.42g ± 0.16; 4075005, 0.28g ± 0.11; $p \leq 0.05$). Over the time period 0-2 hours, the analogue significantly reduced food intake compared to saline control (Saline, 0.65g ± 0.25; 4075005, 0.37g ± 0.12; $p \leq 0.01$). The analogue significantly reduced food intake over the time period 0-4 hours compared with saline (Saline, 1.04g ± 0.33; 4075005, 0.71g ± 0.16; $p \leq 0.05$). Over the total study time period 0-24 hours, the analogue slightly reduced food intake compared with saline but this effect was not statistically significant.

PYY(3-36) analogue 4075006 significantly reduced food intake compared with saline over the time period 0-1 hour (Saline, 0.42g ± 0.16; 4075006, 0.23g ± 0.09; $p \leq 0.01$). Over the time period 0-2 hours, the analogue significantly reduced food intake compared to saline control (Saline, 0.65g ± 0.25; 4075006, 0.32g ± 0.16; $p \leq 0.01$). The analogue significantly reduced food intake over the time period 0-4 hours compared with saline (Saline, 1.04g ± 0.33; 4075006, 0.69g ± 0.27; $p \leq 0.05$). Over the total study time period 0-24 hours, the analogue slightly increased food intake compared with saline.

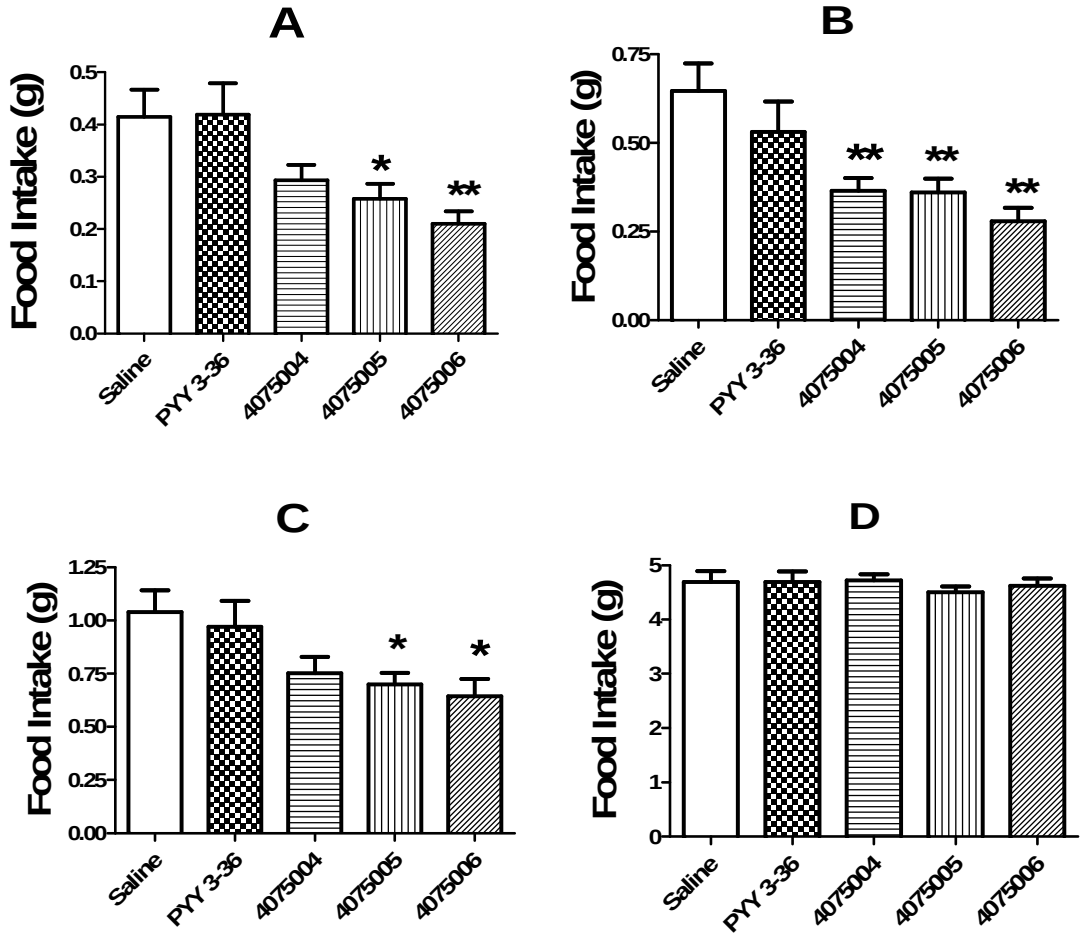


Figure 4.11 The effect of intraperitoneal administration of PYY(3-36) and PYY(3-36) analogues 4075004, 4075005 and 4075006 on cumulative food intake in overnight fasted C57BL/6 mice

A, 0-1 hour; **B,** 0-2 hours; **C,** 0-4 hours; **D**, 0-24 hours post-injection. Dose: PYY(3-36) and analogues, 30nmol/kg. (* p≤0.05, **p≤0.01 compared to saline using one way ANOVA with post *hoc* Dunnett's test (n=9/10))

4.5 Discussion

The series of *in vitro* and *in vivo* tests conducted on a number of PYY(3-36) analogues, have provided some interesting results. The majority of the analogues displayed less affinity for the Y_2 receptor compared with PYY(3-36) but three analogues did record IC_{50} values lower than those recorded for PYY(3-36) in the same series of assays.

Likewise, the majority of the analogues subjected to *in vivo* studies failed to elicit significant inhibition of food intake. However, a number of the N-terminal modified PYY(3-36) analogues did significantly reduce food intake in fasted mice.

4.5.1 Receptor binding studies

Amino acid substitutions at the 25 or 26 position do not eliminate the ability of these peptides to bind to the Y_2 receptor. However, they do reduce the affinity of the peptides for the receptor compared to PYY(3-36). This is clearly seen by comparing the IC_{50} values of the analogues with that of the parent peptide. The IC_{50} for PYY(3-36) was 0.533nM, whilst the analogue with the IC_{50} closest to this was IP11 (1.04nM). This analogue only had an N-terminal modification and so had the closest sequence homology with the native peptide. All the other analogues had IC_{50} values in excess of this, ranging from 1.45 to 13.0nM. This suggests that substituting amino acids in the positions 25 and 26 has a detrimental effect on the ability of the molecule to bind to the Y_2 receptor. It is known that the C-terminal segment amino acids Thr^{32}, Arg^{33}, Arg^{35} and Tyr^{36} are the most important for receptor binding (Beck-Sickinger et al., 1994; Pedersen et al., 2009). It is possible that changes to the amino acids in the 25 and 26 positions elicited a change in the orientation of these important amino acids further along the sequence, thus reducing the binding affinity.

The lowest IC_{50} values were seen for the analogues containing methyl group additions to the standard amino acids arginine and histidine, (IP1, IP3, IP4, IP7 and IP8). Where a more drastic amino acid substitution occurred, this was reflected in the loss of binding affinity. For example, the substitution of guanidino-phenylalanine for arginine (IP10), for this analogue the IC_{50} value increased tenfold compared to PYY(3-36). The IC_{50} values for the analogues with a D for L amino acid substitution (IP2 and IP9), were also high compared to other analogues. This suggests that a change in stereochemistry in these positions has a profound effect on receptor affinity. Finally, the substitution of citrulline for arginine also had a detrimental effect. There is little difference in structure between these amino acids, though crucially the replacement of the amino function NH with oxygen results in a loss of charge. The high IC_{50} value for the $(Cit)^{25}$ substituted analogue suggests that the presence of the base pair in the 25-26 position is also important for receptor binding. This loss of basicity together with the change in stereochemistry is a probable reason why analogue IP6, with a D-citrulline for arginine substitution at position 25, gave the highest IC_{50} value.

In conclusion, amino acid substitutions made at positions 25 and 26 in the PYY(3-36) molecule resulted in decreased affinity for the Y_2 receptor. However, it should be noted that whilst the IC_{50} value calculated for PYY(3-36) was the average from six different assays, the values for the analogues came from one single assay each. It would therefore be desirable to repeat these assays to verify binding affinity. It is also worth considering that peptides with lower receptor affinities can have increased bioactivity compared to the native peptide if they have, for example, longer half-lives or increased tissue permeability. It is therefore important to test the ability of these analogues to reduce food intake in vivo.

In the case of the analogues IP12 – IP19, the substitution of amino acids in the 30, 31 and 32 positions did not eliminate the ability of the peptides to bind to the Y_2 receptor. However, for all but one of the analogues, the binding affinity was lower than that displayed by PYY(3-36) across the same assays. The analogue $(Leu)^{31}$PYY(3-36) (IP17) was the only PYY(3-36) analogue produced in this investigation displaying greater affinity for the Y_2 receptor than native PYY(3-36). Position 31 is adjacent to the C-terminal pentapeptide region that is known to be key for receptor binding (Beck-Sickinger et al., 1994; Pedersen et al., 2009). It is therefore likely that position 31 is also important in the binding affinity of the peptide to the receptor, and that changing the structure of the amino acid in this position will change this affinity. The amino acid in position 31 of PYY(3-36) is valine. Leucine and valine differ in chemical composition by just one methyl group and both side chains terminate in a branched structure. $(Ile)^{31}$PYY(3-36) (IP16) had an IC_{50} value only slightly higher than that of PYY(3-36), but over twice as high as that of $(Leu)^{31}$PYY(3-36). Isoleucine has the same chemical composition as leucine but has a different configuration of its side chain group. These results therefore suggest that it is the combination of an extra carbon atom in the side chain together with retention of the terminal branched structure in the side chain, that facilitates the improved binding of $(Leu)^{31}$PYY(3-36) to the Y_2 receptor. To further support this hypothesis, the IC_{50} value for $(Ala)^{31}$PYY(3-36) (IP15) was 13.6nM, over ten times higher than for analogues IP16 and IP17, and PYY(3-36). Alanine contains two fewer carbon atoms than valine in its side chain group, and has no branching in its structure.

Surprisingly, given that it lies within the C-terminal pentapeptide region of PYY(3-36), substitutions at the 32 position were relatively well tolerated. The IC_{50} values for both analogues with changes as this position were greater than that recorded for

PYY(3-36), but not as high as for some other analogues. Substitution of serine in this position resulted in an IC_{50} value only marginally higher than that of PYY(3-36), whilst substitution with leucine resulted in an IC_{50} value approximately three times that of PYY(3-36). This would suggest that the hydroxyl function in threonine is important for receptor binding at position 32.

For the three position 30 analogues, the IC_{50} values were all higher than that of PYY(3-36). This suggests that position 30 is also important for receptor binding. As previously mentioned, there is only a slight change in structure between leucine and isoleucine, yet inserting an isoleucine residue in position 30 decreased receptor binding affinity by a factor of 2.5. The high IC_{50} value of 9.97 for analogue IP14, $(Val)^{30}$PYY(3-36) was surprising given that a valine/leucine substitution at position 31 had been shown to beneficial to binding affinity, but supports the concept that position 30 is sensitive to changes in the amino acid composition.

Once again, the IC_{50} value for PYY(3-36) is more reliable than that for the analogues, as it represents the mean of seven separate assays. It would therefore be desirable to run repeat assays with analogues IP12 – IP19, to verify that the same patterns of binding affinity are observed.

Two of the N-terminally pegylated PYY(3-36) analogues showed greater affinity for the Y_2 receptor than PYY(3-36). These were analogues 4075004 and 4075005, containing N-terminal AEEAc and PEG4 respectively. The analogue containing the larger PEG6 group had twofold lower affinity for the receptor than PYY(3-36). These findings suggest that addition of PEG molecules to the N-terminal of PYY(3-36) can have a beneficial effect on the binding affinity of the peptide to the Y_2 receptor, but that the size of the PEG molecule is important. These results are somewhat surprising, given that that the N-terminal region of PYY(3-36) is believed to be located outside of

the binding site and thus is not thought to play a role in binding to the Y_2 receptor. It is possible that extending the peptide with the addition of a PEG molecule brings the N-terminal region into closer proximity to the binding site and facilitates additional interactions between peptide and receptor that enhance binding. The effects on receptor binding of the PEG6 addition, suggest that there a size constraint to this enhancing effect, and that addition of larger PEG molecules compromises binding affinity.

All of the PEG molecules used in this investigation contained α-amino functional groups (Figure 2.3), but it is possible to obtain PEG molecules with different terminal functional groups, for example, a methyl group. It would therefore be interesting to investigate the effects on receptor binding of attaching methyl-PEG molecules to the N-terminal of PYY(3-36). This would help to determine whether the α-amino group is important in enhancing receptor binding of N-terminally pegylated PYY(3-36) analogues.

It is worth noting that the IC_{50} values for both the analogues and PYY(3-36) are significantly lower in this set of RBA's than those obtained in the previous RBA studies. This is because a different cell line and a different competing radioactive label were used. Previous studies used the SMS KAN cell line, which express the human Y_2 receptor, whilst the latest study used HEK 293 cells that were manipulated to over express mouse Y_2 receptors. Although there is a high degree of homology between the mouse and human Y_2 receptors (Berglund et al., 2003), it is possible that N-terminal pegylated PYY(3-36) analogues have greater affinity for the mouse Y_2 receptor over the human Y_2 receptor. It might therefore be useful to repeat these assays with a human Y_2 receptor expressing cell line to see if the same patterns of binding affinity are observed. However, to date, in all PYY analogue studies carried

out in the Section of Investigative Medicine, the relative affinity of the analogue for the mouse Y2 receptor compared to PYY(3-36) has reflected the relative affinity at the human Y2 receptor. This latest set of assays also used iodinated PYY(1-36) as the competing ligand with the analogues, rather than PYY(3-36). PYY(1-36) has a lower affinity for the Y_2 receptor than PYY(3-36), and would therefore be expected to result in lower IC_{50} values than assays using radiolabelled PYY(3-36). To enable direct comparison between all analogues, it would be beneficial to repeat this set of assays using radioactive PYY(3-36) as the competing ligand.

The results obtained from the two receptor binding assays conducted with the analogue (Cys^4, Cys^{29})-PYY(3-36)(disulphide bridge) suggest that cross linking amino acids 4 and 29 in PYY(3-36) severely impairs binding affinity to the Y_2 receptor. This was a somewhat surprising result, as the synthesis and purification of this analogue suggested that forming the cross linkage did not drastically change the confirmation of the peptide. However, the results from these receptor binding assays would suggest that the confirmation has been sufficiently altered so as to completely prevent the peptide binding to the receptor. It is possible that this inhibitory effect is not due to the cross linkage itself, but rather due to the presence of two cysteine residues in the sequence. The endogenous residues in positions 4 and 29 are lysine and asparagine, and there are significant structural and functional differences between these two amino acids and cysteine. It would therefore be interesting to investigate the receptor binding properties of both (Cys^4, Cys^{29})-PYY(3-36) without a disulphide bridge and a PYY(3-36) analogue containing a cross linkage between amino acids more homologous to lysine and asparagine.

4.5.2 Acute feeding studies

Of all of the first eleven PYY(3-36) analogues synthesised (IP1 – IP11), only one, Biotinyl-PYY(3-36) (IP11), was shown to have a statistically significant effect in reducing food intake compared with saline over the entire 24 hour time period. The receptor binding studies suggest that this increased effect is not due to improved binding affinity to the Y_2 receptor; the IC_{50} value for this analogue was higher than that of natural PYY(3-36). The improved bioactivity may be due to the biotin molecule increasing the half life of the peptide in the circulation. Biotin is a large bulky molecule and it may be that its presence at the N-terminal of the peptide hinders the action of enzymes which naturally degrade PYY(3-36). To test this hypothesis it would be interesting to determine the effects on food intake of administration of both Biotin and Biotin in combination with PYY(3-36), and to assess the ability of this peptide to resist different types of enzymatic degradation.

Of the remaining analogues synthesised and tested *in vivo*, two had statistically significant effects in reducing food intake compared with saline over the first hour of the study. These were the analogues IP2 and IP4 which contain D-Arg and $Arg(Me)_2$asymmetrical in position 25 respectively. These analogues both had relatively low IC_{50} values compared to others and are structurally similar to the endogenous PYY(3-36). Therefore, the positive effect they had in relation to reducing food intake is more likely due to effective binding of the Y_2 receptor, than an increase in stability to enzymatic degradation. This hypothesis is further supported by the observation that the significant effect in reducing food intake was not long lasting.

In conclusion, the analogues (IP1 – IP11), produced with the aim of stabilising the 25-26 position to enzymatic degradation in PYY(3-36), did not demonstrate significantly increased bioactivity *in vivo*. However, following on from the investigation into

trypsin digestion of PYY(3-36), it may worth investigating what effect stabilising position 33-34 in the molecule has both *in vitro* and *in vivo*. The strong inhibitory effect on food intake of Biotinyl-PYY(3-36) (IP11) was an interesting and unexpected result, and suggests further investigation into the effects of N-terminal modifications of PYY(3-36) may yield useful results.

Due to concerns over solubility and regulatory issues, of developing therapeutics containing a biotin group, it was decided to instead develop analogues with N-terminal PEG modifications. Such modifications are already FDA approved for use in pharmaceuticals (Harris and Chess, 2003). The three N-terminal pegylated PYY(3-36) analogues developed significantly reduced food intake in mice compared to saline at specific time points, though there was no significant difference in cumulative food intake between the PEG-PYY(3-36) administered mice and the saline control mice over the total 24 hour time period. During the first hour, the PEG-PYY(3-36) groups all consumed less food than both the saline and PYY(3-36) groups. The analogues containing the largest PEG molecules had the greatest inhibitory effect. The three PEG-PYY(3-36) treated groups continued to consume less food during the second hour of the study. It appears that the inhibitory effects of the PEG-PYY(3-36) analogues wear off after this point.; between 4 and 8 hours post-injection, the three PEG-PYY(3-36) groups consumed more food than either the PYY(3-36) and the saline groups. The mice are likely compensating for the earlier reduction in food intake by consuming more food following cessation of the inhibitory effect. Thus using these molecules for weight control would likely require a frequent dosing regimen or the administration of high doses.

The results from the receptor binding studies with the pegylated analogues suggest that both 4075004 and 4075005 bind to the Y_2 receptor with greater affinity than

PYY(3-36). This would help to explain the greater inhibitory effect on food intake these two analogues show when compared with PYY(3-36). However, the analogue 4075006 had lower affinity for the Y_2 receptor than PYY(3-36) yet had the greatest effect on inhibiting food intake. Thus whilst the presence of the PEG molecules may enhance receptor binding to some degree, it is more likely that an increase in the circulating half life is more of a contributory factor. The PEG molecules are large and therefore could inhibit the action of proteolytic enzymes by blocking some or all of the active site. The analogue 4075006 contained the largest PEG molecule and so by this rationale, would resist enzymatic degradation relatively well. This might explain why of the three pegylated analogues produced, this analogue had the greatest effect on reducing short term cumulative food intake. However, the results in this particular study for the PYY(3-36) group were unexpected. At no stage in the study did the PYY(3-36) mice display a significant reduction in food intake when compared with the saline group as has been widely reported in previous studies. Therefore, the effects of the PEG-PYY(3-36) analogues on cumulative food intake cannot be accurately compared with PYY(3-36) without further investigations.

In conclusion, these studies have provided useful insights into the structure function relationships of the PYY(3-36) molecule, and suggest that PYY(3-36) analogues may be useful tools to reduce food intake. Further work is required to assess their utility in the regulation of body weight.

5 General discussion

5.1 Introduction

The aim of this programme of work was to successfully develop novel analogues of the anorectic gut derived hormone PYY(3-36) that displayed greater inhibitory effects on food intake than the endogenous molecule. Should such an analogue have been developed, there would then be scope to investigate its potential for use in humans an anti-obesity therapeutic. At this stage, no single analogue has been produced that displays a consistent increase in reducing food intake in mice compared to PYY(3-36). However, a number of interesting results have been obtained throughout the various stages of this investigation which will hopefully be useful to those looking to conduct further investigations in this field.

5.2 The use of SPPS techniques to produce PYY(3-36) analogues

My investigations have shown that a number of PYY(3-36) analogues, embodying varied design strategies, can be successfully produced utilising specific solid phase peptide synthesis techniques. A number of unnatural amino acids were successfully incorporated into the peptide sequence at positions 25 and 26. Following this, further analogues were synthesised with natural amino acid substitutions in positions 30, 31 and 32. Whilst the incorporation of some of these amino acids was less successful than others, all nineteen analogues designed were produced in sufficient yields and of suitable purity to enable them to be used for biological testing. The addition of PEG molecules to peptides, particularly those being developed as therapeutics, is an attractive option. This is because the hydrophilic nature of the PEG molecules can increase the solubility of the peptide to which they are attached. However, the hydrophilic nature of PEG can also hinder the work of a peptide chemist. Depending

upon the size of the PEG unit, the starting material can often be in the form of an oil, which makes the handling and accurate weighing of the material difficult. The resulting PEG-peptide can be so hydrophilic that it will resist lyophilisation to a dry powder. This makes the final purification and characterisation processes challenging. Despite these concerns, I was able to successfully produce three novel N-terminal pegylated PYY(3-36) analogues in sufficient yields and of suitable purity to enable biological testing of the compounds.

The concept behind the final set of analogues utilised the natural hairpin shape of PYY(3-36) (Nygaard et al., 2006). The aim was to successfully cross link amino acids in the N and C-terminal regions of the peptide, at positions 4 and 29. Were this approach to prove successful, and the subsequent analogues found to retain biological activity, further manipulations of the peptide sequence were planned. These manipulations would have included the removal of amino acids from non-critical positions within the 5 – 28 loop, with the ultimate goal of producing a small molecule mimetic of PYY(3-36).

The analogue (Cys^{4}, Cys^{29})-PYY(3-36)(disulphide bridge) was successfully produced in sufficient yield and of suitable purity to enable biological testing to be performed. The synthesis of this analogue proved that the amino acid positions 4 and 29 could be successfully cross linked. The HPLC data obtained during the reaction to form the disulphide bridge suggested that the formation of the cross linkage did not significantly change the confirmation of the peptide. This result was promising, as it suggested that the presence of the cross linkage would not be detrimental to receptor binding.

Unfortunately, attempts to synthesise further cross linked analogues of PYY(3-36) were unsuccessful. The aim was to synthesise analogues containing lactam bridges

between the amino acids in positions 4 and 29, as the amino acids used to form the bridges would be more homologous to the endogenous residues in these positions. Lactam bridges are formed between the amino side chain group of one amino acid and the carboxyl side chain group of a second amino acid. PYY(3-36) contains a number of basic and acidic residues, and thus these other amino acids would need to be protected during the formation of the lactam bridge between the two designated amino acids to avoid the formation of multiple bridges.. The most straightforward way of doing this is to perform the reaction on the resin bound peptide and selectively deprotect the two designated amino acid side chains. In total, six attempts were made at synthesising PYY(3-36) analogues containing a lactam bridge between amino acids 4 and 29. A number of different synthesis techniques were utilised to try to improve the process but ultimately without success. The crux of the problem behind the unsuccessful formation of the proposed lactam bridge may be that amino acid positions 4 and 29 are insufficiently close to one another when the peptide is bound to the resin to enable the reaction to proceed.

I thus attempted to form the lactam bridge in solution, on a partially protected peptide. This was based on the successful formation in solution of the disulphide bridge in the analogue (Cys^{4}, Cys^{29})-PYY(3-36)(disulphide bridge), and was based on the idea that the protected peptide would still be able to form the hairpin like shape in solution. Difficulties in producing the analogue in this manner were predicted, mainly linked to the solubility and characterisation of a protected peptide containing 34 amino acids. These expected problems did occur, but it was still surprising that the initial analysis of the resultant crude peptide suggested that the synthesis had failed. It may be that the amino and carboxyl groups of the lysine and glutamic acid residues were sufficiently hindered by the presence of adjacent amino acid protecting groups, to

successfully form the lactam bridge. However, were this to be the case, I would expect to have observed the linear material in the subsequent analysis of the crude material, which was not the case. Therefore, it would be desirable to attempt a repeat synthesis of this analogue, following the same methods, to see if identical results were obtained.

5.3 The effects of PYY(3-36) analogues *in vitro* and *in vivo*

Due to the considerable effort and resources spent working on developing novel synthesis protocols, it was not possible to conduct the extensive *in vitro* and *in vivo* testing of various analogues originally planned. Following the work into trypsin degradation of PYY(3-36) and the analogues IP1 – IP10, it was discovered that position 33-34 within PYY(3-36) is likely the primary site of cleavage by trypsin. I subsequently demonstrated that insertion of specific unnatural amino acids in the 25 and 26 positions succeeded in stabilising the bond between them to trypsin-induced degradation.

The series of receptor binding assays that were conducted showed that, in general, making amino acid substitutions in either the 25 and 26 positions or in the 30, 31 and 32 positions decreased the resulting analogues binding affinity for the Y_2 receptor compared to PYY(3-36). The exception to this was the analogue $(Leu)^{31}$PYY(3-36), which displayed greater affinity for the Y_2 receptor than PYY(3-36). Promising results were also obtained from the receptor binding studies with the PEG-PYY(3-36) analogues. Both AEEAc-PYY(3-36) and PEG4-PYY(3-36) had greater affinity for the Y_2 receptor than PYY(3-36), whilst PEG6-PYY(3-36) displayed a small decrease in binding affinity. It is interesting to speculate whether this increased affinity represents improved binding of the molecule or, for example, increased stability of the receptor-

ligand complex. It would also be helpful to repeat these RBAs in order to confirm some of the conclusions reached in this thesis.

Perhaps the most surprising result was obtained from the receptor binding assays conducted with the analogue (Cys^4, Cys^{29})-PYY(3-36)(disulphide bridge). This suggested that formation of the cross linkage completely eliminated the affinity of the analogue for the Y_2 receptor. This deleterious effect may be due to the nature of the cysteine residues as opposed to the presence of a cross linkage per se. However, due to the failure to successfully synthesise analogues incorporating a lactam bridge, it is not currently possible to test this hypothesis.

In vivo studies with mice, were conducted using the IP1 – IP11 and PEG-PYY(3-36) analogues. From these analogues, only the N-terminally modified peptides; Biotinyl-PYY(3-36), AEEAc-PYY(3-36), PEG4-PYY(3-36) and PEG6-PYY(3-36) were found to significantly reduce cumulative food intake in fasted mice. In the case of the three PEG-PYY(3-36) analogues, the promising results obtained need to be tempered by the fact that PYY(3-36) did not behave as expected in this particular study. Further studies are now required to assess the utility of pegylated PYY(3-36) analogues in the treatment of obesity.

5.4 Further work

The results of the investigations described in this thesis leave scope for further development work with PYY(3-36) analogues. In particular, following the positive biological results that were obtained for the analogues $(Leu)^{31}$PYY(3-36) and the various PEG-PYY(3-36) peptides, it would interesting to synthesise an analogue

incorporating $(Leu)^{31}$ and pegylation to test whether the observed effects on receptor binding and inhibition of food intake are additive. It would also be interesting to study the effects of stabilising the amino acid position 33 in PYY(3-36) following the results obtained from the trypsin degradation studies.

In conclusion, further work is required to determine the utility of PYY(3-36) analogues in the treatment of obesity.

Appendices

Appendix I – List of suppliers

Alfa Aesar, Lancaster, UK

Applied Biosystems, Warrington, UK

Apollo Scientific, Stockport, UK

Avantor, Deventer, Netherlands

Bachem AG, Bubendorf, Switzerland UK

BDH, Poole, UK

Gilson, Middleton, Wisconsin, USA

GraphPad, La Jolla, CA, USA

Iris Biotech, Marktredwitz, Germany

Kratos, Manchester, UK

M56 Chemicals, Runcorn, UK

Novabiochem, Nottingham, UK

Perkin Elmer, Massachusetts, USA

P&R Labpack, St. Helens, UK

Protein Technologies, Tucson, Arizona, USA

Rapp Polymere, Tübingen, Germany

R&D Systems, Minneapolis, Minnesota USA

Romil, Cambridge, UK

SDS UK, Witham, UK

Severn Biotech, Kidderminster, UK

Sigma-Aldrich Company Ltd., Poole, UK.

VWR, Poole, UK

Publications

James R. C. Parkinson, Waljit S. Dhillo, Caroline J. Small, Owais B. Chaudhri, Gavin A. Bewick, Iain Pritchard, Stanley Moore, Mohammed A. Ghatei, and Stephen R. Bloom. **PYY3-36 injection in mice produces an acute anorexigenic effect followed by a delayed orexigenic effect not observed with other anorexigenic gut hormones.** *Am J Physiol Endocrinol Metab* 294: E698–E708, (2008).

References

Abbott,,C. et al. (2006) The importance of acclimatisation and habituation to experimental conditions when investigating the anorectic effects of gastrointestinal hormones in the rat. *International Journal of Obesity*, 288-292.

Albericio, F. and Carpino, L.A. (1997) Coupling reagents and activation. *Methods in Enzymology*, **289**, 104-127.

Alves,L.C. et al. (2001) S1 subsite specificity of a recombinant cysteine proteinase, CPB, of Leishmania mexicana compared with cruzain, human cathepsin L and papain using substrates containing non-natural basic amino acids. *Eur. J. Biochem*, **268**, 1206-1212.

Amblard,M. et al. (2005) Fundamentals of modern peptide synthesis. *Methods Mol. Biol*, **298**, 3-24.

Amblard,M. et al. (2006) Methods and protocols of modern solid phase Peptide synthesis. *Mol. Biotechnol*, **33**, 239-254.

Arch,J.R.S. et al. (1998) Leptin resistance in obese humans: does it exist and what does it mean? *International Journal of Obesity*, **22**, 1159-1163.

Arora,S. and Anubhuti (2006) Role of neuropeptides in appetite regulation and obesity--a review. *Neuropeptides*, **40**, 375-401.

Asakawa,Akihiro et al. (2003) Characterization of the effects of pancreatic polypeptide in the regulation of energy balance. *Gastroenterology*, **124**, 1325-1336.

Astrup,Arne (2010) Drug management of obesity--efficacy versus safety. *N. Engl. J. Med*, **363**, 288-290.

Atherton,E. et al. (1978) Application of polyamide resins to polypeptide synthesis: an improved synthesis of β-endorphin using fluorenylmethoxycarbonylamino-acids. *J. Chem. Soc., Chem. Commun.*, 539.

Balasubramaniam,Ambikaipakan et al. (2006) Neuropeptide Y (NPY) Y4 receptor selective agonists based on NPY(32-36): development of an anorectic Y4 receptor selective agonist with picomolar affinity. *J. Med. Chem*, **49**, 2661-2665.

Balsiger,B.M. et al. (2000) Prospective evaluation of Roux-en-Y gastric bypass as primary operation for medically complicated obesity. *Mayo Clin. Proc*, **75**, 673-680.

Barnett,B.P. et al. (2010) Glucose and Weight Control in Mice with a Designed Ghrelin O-Acyltransferase Inhibitor. *Science*, **330**, 1689 -1692.

Batterham,R L and Bloom,S R (2003) The gut hormone peptide YY regulates appetite. *Ann. N. Y. Acad. Sci*, **994**, 162-168.

Batterham,R. L., Le Roux,C. W., et al. (2003a) Pancreatic Polypeptide Reduces Appetite and Food Intake in Humans. *Journal of Clinical Endocrinology & Metabolism*, **88**, 3989 -3992.

Batterham,Rachel L, Cohen,Mark A, et al. (2003b) Inhibition of food intake in obese subjects by peptide YY3-36. *N. Engl. J. Med*, **349**, 941-948.

Batterham,Rachel L. et al. (2002) Gut hormone PYY3-36 physiologically inhibits food intake. *Nature*, **418**, 650-654.

Beck,A. et al. (2000) Synthesis and characterization of Respiratory Syncytial Virus protein G related peptides containing two disulfide bridges. *J. Pept. Res*, **55**, 24-35.

Beck-Sickinger,A G et al. (1994) Complete L-alanine scan of neuropeptide Y reveals ligands binding to Y1 and Y2 receptors with distinguished conformations. *Eur. J. Biochem*, **225**, 947-958.

Beglinger,C. et al. (2008) Pharmacokinetics and Pharmacodynamic Effects of Oral GLP-1 and PYY3-36: A Proof-of-concept Study in Healthy Subjects. *Clin Pharmacol Ther*, **84**, 468-474.

van Belle,T.L. et al. (2011) Type 1 diabetes: etiology, immunology, and therapeutic strategies. *Physiol Rev*, **91**, 79-118.

Benoit,S.C. and Tschöp,M.H. (2005) PYY3-36 “monkeys around” with energy balance. *Am. J. Physiol. Regul. Integr. Comp. Physiol*, **288**, R358-359.

Berg,J.M. et al. (2002) Glycogen Metabolism. In, *Biochemistry. 5th Edition*.

Berglund,M.M. et al. (2003) Recent developments in our understanding of the physiological role of PP-fold peptide receptor subtypes. *Exp. Biol. Med. (Maywood)*, **228**, 217-244.

Bermpohl,F. et al. (1998) Rat dipeptidyl peptidase IV (DPP IV) exhibits endopeptidase activity with specificity for denatured fibrillar collagens. *FEBS Lett*, **428**, 152-156.

Berntson,G.G. et al. (1993) Pancreatic polypeptide infusions reduce food intake in Prader-Willi syndrome. *Peptides*, **14**, 497-503.

Berthoud,H.-R. and Morrison,C. (2008) The brain, appetite, and obesity. *Annu Rev Psychol*, **59**, 55-92.

Bewick,G.A. et al. (2005) Post-embryonic ablation of AgRP neurons in mice leads to a lean, hypophagic phenotype. *FASEB J*, **19**, 1680-1682.

Biemann,K. (1995) The coming of age of mass spectrometry in peptide and protein chemistry. *Protein Sci*, **4**, 1920-1927.

Bjerre Knudsen,L. et al. (2010) Glucagon-Like Peptide-1 Receptor Agonists Activate Rodent Thyroid C-Cells Causing Calcitonin Release and C-Cell Proliferation. *Endocrinology*, **151**, 1473 -1486.

Bloom,Stephen R et al. (2008) The obesity epidemic: pharmacological challenges. *Mol. Interv*, **8**, 82-98.

Boey,D. et al. (2006) Peptide YY ablation in mice leads to the development of hyperinsulinaemia and obesity. *Diabetologia*, **49**, 1360-1370.

Bottom,C.B. et al. (1978) Mechanism of the ninhydrin reaction. *Biochemical Education*, **6**, 4-5.

British Obesity Surgery Patient Organisation (2011a) Gastric bypass post-op eating plan.

British Obesity Surgery Patient Organisation (2011b) NHS funded surgery.

Brubaker,P.L. (2010) Minireview: update on incretin biology: focus on glucagon-like peptide-1. *Endocrinology*, **151**, 1984-1989.

Buchwald,H. et al. (2004) Bariatric surgery: a systematic review and meta-analysis. *JAMA*, **292**, 1724-1737.

Cabrele,C. et al. (2000) The first selective agonist for the neuropeptide YY5 receptor increases food intake in rats. *J. Biol. Chem*, **275**, 36043-36048.

Carpino,L.A. and Han,G.Y. (1972) 9-Fluorenylmethoxycarbonyl amino-protecting group. *The Journal of Organic Chemistry*, **37**, 3404-3409.

Carson,J.A. and Turner,Anthony J (2002) Beta-amyloid catabolism: roles for neprilysin (NEP) and other metallopeptidases? *J. Neurochem*, **81**, 1-8.

Chan,W.C. and White,P.D. (2000) Fmoc Solid Phase Peptide Synthesis: A practical approach Oxford University Press.

Chandarana,K. et al. (2011) Diet and Gastrointestinal Bypass–Induced Weight Loss. *Diabetes*, **60**, 810 -818.

Chaudhri,O. et al. (2006) Gastrointestinal hormones regulating appetite. *Philos Trans R Soc Lond B Biol Sci*, **361**, 1187-1209.

Chiesi,M. et al. (2001) Pharmacotherapy of obesity: targets and perspectives. *Trends Pharmacol. Sci*, **22**, 247-254.

Chollet,C. et al. (2009) Ghrelin--a novel generation of anti-obesity drug: design, pharmacomodulation and biological activity of ghrelin analogues. *J. Pept. Sci*, **15**, 711-730.

Cohen,Mark A et al. (2003) Oxyntomodulin suppresses appetite and reduces food intake in humans. *J. Clin. Endocrinol. Metab*, **88**, 4696-4701.

Coll,A.P. et al. (2005) Proopiomelanocortin-deficient mice are hypersensitive to the adverse metabolic effects of glucocorticoids. *Diabetes*, **54**, 2269-2276.

Cone,R D et al. (2001) The arcuate nucleus as a conduit for diverse signals relevant to energy homeostasis. *Int. J. Obes. Relat. Metab. Disord*, **25 Suppl 5**, S63-67.

Cooke,D. and Bloom,Steve (2006) The obesity pipeline: current strategies in the development of anti-obesity drugs. *Nat Rev Drug Discov*, **5**, 919-931.

Couzin,J. (2008) Bypassing Medicine to Treat Diabetes. *Science*, **320**, 438-440.

Crawley,J.N. and Corwin,R.L. (1994) Biological actions of cholecystokinin. *Peptides*, **15**, 731-755.

Cruz,M.T. et al. (2007) A reevaluation of the effects of stimulation of the dorsal motor nucleus of the vagus on gastric motility in the rat. *Am. J. Physiol. Regul. Integr. Comp. Physiol*, **292**, R291-307.

Cummings,B.P. et al. (2010) Chronic administration of the glucagon-like peptide-1 analog, liraglutide, delays the onset of diabetes and lowers triglycerides in UCD-T2DM rats. *Diabetes*, **59**, 2653-2661.

Cummings,David E. and Shannon,M.H. (2003) Ghrelin and Gastric Bypass: Is There a Hormonal Contribution to Surgical Weight Loss? *Journal of Clinical Endocrinology & Metabolism*, **88**, 2999 -3002.

Dakin,C.L. et al. (2004) Peripheral Oxyntomodulin Reduces Food Intake and Body Weight Gain in Rats. *Endocrinology*, **145**, 2687 -2695.

Day,J.W. et al. (2010) Charge inversion at position 68 of the glucagon and glucagon-like peptide-1 receptors supports selectivity in hormone action. *J. Pept. Sci*.

Day,J.W. et al. (2009) A new glucagon and GLP-1 co-agonist eliminates obesity in rodents. *Nat Chem Biol*, **5**, 749-757.

DeCarr,L.B. et al. (2007) A long-acting selective neuropeptide Y2 receptor PEGylated peptide agonist reduces food intake in mice. *Bioorg. Med. Chem. Lett*, **17**, 1916-1919.

Della-Zuana,O. et al. (2004) A potent and selective NPY Y5 antagonist reduces food intake but not through blockade of the NPY Y5 receptor. *Int. J. Obes. Relat. Metab. Disord*, **28**, 628-639.

Drew,B.S. et al. (2007) Obesity management: update on orlistat. *Vasc Health Risk Manag*, **3**, 817-821.

Dumont,Y. et al. (2005) Evidence for the existence of an additional class of neuropeptide Y receptor sites in rat brain. *J. Pharmacol. Exp. Ther*, **315**, 99-108.

Elinav,E. et al. (2009) Pegylated leptin antagonist is a potent orexigenic agent: preparation and mechanism of activity. *Endocrinology*, **150**, 3083-3091.

Ellacott KL and Cone RD (2004) The Central Melanocortin System and the Integration of Short- and Long-term Regulators of Energy Homeostasis. *Recent Prog Horm Res*, **59**, 395-408.

Erickson,J.C. et al. (1996) Attenuation of the Obesity Syndrome of ob/ob Mice by the Loss of Neuropeptide Y. *Science*, **274**, 1704 -1707.

European Medicines Agency (2010) European Medicines Agency recommends suspension of marketing authorisations for sibutramine.

European Medicines Agency (2008) The European Medicines Agency recommends suspension of the marketing authorisation of Acomplia.

Fielding,G.A. (2003) Laparoscopic adjustable gastric banding for massive superobesity (> 60 body mass index kg/m2). *Surg Endosc*, **17**, 1541-1545.

Fields,G.B. and Noble,R.L. (1990) Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. *Int. J. Pept. Protein Res*, **35**, 161-214.

Finer,N. (2002) Sibutramine: its mode of action and efficacy. *Int J Obes Relat Metab Disord*, **26**, S29-S33.

Flint,A et al. (1998) Glucagon-like peptide 1 promotes satiety and suppresses energy intake in humans. *J Clin Invest*, **101**, 515-520.

Foresight (2006) Trends and drivers of obesity: A literature review for the Foresight project on obesity.

Foster-Schubert,K.E. and Cummings,David E. (2006) Emerging Therapeutic Strategies for Obesity. *Endocrine Reviews*, **27**, 779 -793.

Friedman,J.M. (2009) Leptin at 14 y of age: an ongoing story. *Am J Clin Nutr*, **89**, 973S-979S.

Friedman,J.M. and Halaas,J.L. (1998) Leptin and the regulation of body weight in mammals. *Nature*, **395**, 763-770.

Gantz,I. et al. (2007) Efficacy and Safety of Intranasal Peptide YY3–36 for Weight Reduction in Obese Adults. *Journal of Clinical Endocrinology & Metabolism*, **92**, 1754 -1757.

Gausepohl,H. et al. (1992) Schiff base analog formation during in situ activation by HBTU and TBTU.

Göke,R. et al. (1993) Exendin-4 is a high potency agonist and truncated exendin-(9-39)-amide an antagonist at the glucagon-like peptide 1-(7-36)-amide receptor of insulin-secreting beta-cells. *J. Biol. Chem*, **268**, 19650-19655.

Grill,H.J. et al. (2002) Evidence That the Caudal Brainstem Is a Target for the Inhibitory Effect of Leptin on Food Intake. *Endocrinology*, **143**, 239 -246.

Groop,L. (2000) Genetics of the metabolic syndrome. *Br. J. Nutr*, **83 Suppl 1**, S39-48.

Habegger,K.M. et al. (2010) The metabolic actions of glucagon revisited. *Nat Rev Endocrinol*, **6**, 689-697.

Haehling,S. von et al. (2007) Sibutramine in cardiovascular disease: is SCOUT the new STORM on the horizon? *European Heart Journal*.

Harris,J.M. and Chess,R.B. (2003) Effect of pegylation on pharmaceuticals. *Nat Rev Drug Discov*, **2**, 215-221.

Hatahet,M.A. and Dhurandhar,N.V. (2002) Antiobesity drugs: current and future issues. *Curr. Diab. Rep*, **2**, 409-415.

Hayes,M.T. et al. (2011) A model for predicting the resolution of type 2 diabetes in severely obese subjects following Roux-en Y gastric bypass surgery. *Obes Surg*, **21**, 910-916.

Hedstrom,L. (1996) Trypsin: a case study in the structural determinants of enzyme specificity. *Biol. Chem*, **377**, 465-470.

Heymsfield,,S.B. et al. (1999) Recombinant Leptin for Weight Loss in Obese and Lean Adults. *JAMA: The Journal of the American Medical Association*, **282**, 1568-1575.

Hsiung,H.M. et al. (2005) A novel and selective beta-melanocyte-stimulating hormone-derived peptide agonist for melanocortin 4 receptor potently decreased food intake and body weight gain in diet-induced obese rats. *Endocrinology*, **146**, 5257-5266.

Ilnytska,O. and Argyropoulos,G. (2008) The role of the Agouti-Related Protein in energy balance regulation. *Cell. Mol. Life Sci*, **65**, 2721-2731.

Iwata,N. et al. (2001) Metabolic regulation of brain Abeta by neprilysin. *Science*, **292**, 1550-1552.

Jeremy M Berg,J.L.T. (2002) Biochemistry.

Jiang,G. and Zhang,B.B. (2003) Glucagon and regulation of glucose metabolism. *Am. J. Physiol. Endocrinol. Metab*, **284**, E671-678.

Jin,D.K. (2011) Systematic review of the clinical and genetic aspects of Prader-Willi syndrome. *Korean J Pediatr*, **54**, 55-63.

Kanatani,A. et al. (2000) Role of the Y1 receptor in the regulation of neuropeptide Y-mediated feeding: comparison of wild-type, Y1 receptor-deficient, and Y5 receptor-deficient mice. *Endocrinology*, **141**, 1011-1016.

Klok,M.D. et al. (2007) The role of leptin and ghrelin in the regulation of food intake and body weight in humans: a review. *Obesity Reviews: An Official Journal of the International Association for the Study of Obesity*, **8**, 21-34.

Knudsen,Lotte Bjerre et al. (2007) Small-molecule agonists for the glucagon-like peptide 1 receptor. *Proceedings of the National Academy of Sciences*, **104**, 937 -942.

Kojima,M. et al. (1999) Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature*, **402**, 656-660.

Kojima,S. et al. (2007) A role for pancreatic polypeptide in feeding and body weight regulation. *Peptides*, **28**, 459-463.

Koole,C. et al. (2010) Allosteric ligands of the glucagon-like peptide 1 receptor (GLP-1R) differentially modulate endogenous and exogenous peptide responses in a pathway-selective manner: implications for drug screening. *Mol. Pharmacol*, **78**, 456-465.

Van de Laar,F.A. et al. (2005) Alpha-glucosidase inhibitors for type 2 diabetes mellitus. In, The Cochrane Collaboration and Van de Laar,F.A. (eds), *Cochrane Database of Systematic Reviews*. John Wiley & Sons, Ltd, Chichester, UK.

Lean,M. and Mullan,A. (2007) Obesity: which drug and when? *Int. J. Clin. Pract*, **61**, 1555-1560.

Lee,C.C. and Miller,R.J. (1998) Is there really an NPY Y3 receptor? *Regul. Pept*, **75-76**, 71-78.

Lim,E.L. et al. (2011) Reversal of type 2 diabetes: normalisation of beta cell function in association with decreased pancreas and liver triacylglycerol. *Diabetologia*.

Lo,C.-M. et al. (2010) Cholecystokinin knockout mice are resistant to high-fat diet-induced obesity. *Gastroenterology*, **138**, 1997-2005.

Lu,M. et al. (2011) Brain PPAR-promotes obesity and is required for the insulin–sensitizing effect of thiazolidinediones. *Nature Medicine*, **17**, 618-623.

Lumbierres,M. et al. (2005) Solid-phase synthesis of lipidated peptides. *Chemistry*, **11**, 7405-7415.

Mant,C.T. et al. (2007) HPLC analysis and purification of peptides. *Methods Mol. Biol*, **386**, 3-55.

Massolini,G. and Calleri,E. (2005) Immobilized trypsin systems coupled on-line to separation methods: recent developments and analytical applications. *J Sep Sci*, **28**, 7-21.

Meienhofer,J. et al. (1979) Solid phase synthesis without repetitive acidolysis. Preparation of leucyl-alanyl-glycyl-valine using 9-fluorenylmethyloxycarbonylamino acids. *Int. J. Pept. Protein Res*, **13**, 35-42.

Melnikova,I. and Wages,D. (2006) Anti-obesity therapies. *Nat Rev Drug Discov*, **5**, 369-370.

Mentlein,R. (1999) Dipeptidyl-peptidase IV (CD26)--role in the inactivation of regulatory peptides. *Regul. Pept*, **85**, 9-24.

Mergler,M. and Dick,F. (2005) The Aspartimide Problem in Fmoc-based SPPS. Part III. *J Peptide Sci*, **11**, 605-657.

Mergler,M., Dick,F., Sax,B., Stahelin,C., et al. (2003a) The Aspartimide Problem in Fmoc-based SPPS. Part II. *J Peptide Sci*, **9**, 518-526.

Mergler,M., Dick,F., Sax,B., Weiler,P., et al. (2003b) The Aspartimide Problem in Fmoc-based SPPS. Part I. *J Peptide Sci*, **9**, 36-46.

Mergler,Monika (1999) SASRIN: A review of its manifold applications including many useful procedures 2nd ed. Bachem.

Merrifield,R.B. (1964) Solid phas synthesis II. The synthesis of bradykinin. *J Amer Chem Soc*, **86**, 304.

Merrifield,R.B. (1963) Solid phase synthesis I. The synthesis of a tetrapeptide. *J Amer Chem Soc*, **85**, 2149.

Miller,C. and Rivier,J. (1996) Peptide chemistry: development of high performance liquid chromatography and capillary zone electrophoresis. *Biopolymers*, **40**, 265-317.

Moore,W.T. (1997) Laser desorption mass spectrometry. *Meth. Enzymol*, **289**, 520-542.

Moran,T H (2000) Cholecystokinin and satiety: current perspectives. *Nutrition*, **16**, 858-865.

Moran,Timothy H (2004) Gut peptides in the control of food intake: 30 years of ideas. *Physiol. Behav*, **82**, 175-180.

Moran,Timothy H and Dailey,M.J. (2009) Minireview: Gut peptides: targets for antiobesity drug development? *Endocrinology*, **150**, 2526-2530.

Moran,Timothy H. and Kinzig,K.P. (2004) Gastrointestinal satiety signals II. Cholecystokinin. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, **286**, G183 -G188.

Morínigo,R et al. (2006) GLP-1, PYY, Hunger and Satiety Following Gastric Bypass Surgery In Morbidly Obese Subjects. *Journal of Clinical Endocrinology & Metabolism*, **91**, 1735-1740.

Morton,G.J. et al. (2006) Central nervous system control of food intake and body weight. *Nature*, **443**, 289-295.

Murphy,K. G. and Bloom,S. R. (2007) Nonpeptidic glucagon-like peptide 1 receptor agonists: A magic bullet for diabetes? *Proceedings of the National Academy of Sciences*, **104**, 689 -690.

Murphy,Kevin G and Bloom,Stephen R (2006) Gut hormones and the regulation of energy homeostasis. *Nature*, **444**, 854-859.

Mutter,M. (1985) The impact of secondary structure formation in peptide synthesis. *Problems in peptide coupling*, 397-405.

Mutter,M. et al. (1995) Pseudo-prolines (psi Pro) for accessing "inaccessible" peptides. *Pept. Res*, **8**, 145-153.

Nachnani,J.S. et al. (2010) Biochemical and histological effects of exendin-4 (exenatide) on the rat pancreas. *Diabetologia*, **53**, 153-159.

National Institute for Health and Clinical Excellence (2006) Obesity: Guidance on the prevention, identification, assessment and management of overweight and obesity in adults and children.

Nauck,M.A. et al. (2009) Treatment with the human once-weekly glucagon-like peptide-1 analog taspoglutide in combination with metformin improves glycemic control and lowers body weight in patients with type 2 diabetes inadequately controlled with metformin alone: a double-blind placebo-controlled study. *Diabetes Care*, **32**, 1237-1243.

Neary,N M et al. (2008) No evidence of an additive inhibitory feeding effect following PP and PYY 3-36 administration. *Int J Obes (Lond)*, **32**, 1438-1440.

Neary,Nicola M. et al. (2005) Peptide YY3–36 and Glucagon-Like Peptide-17–36 Inhibit Food Intake Additively. *Endocrinology*, **146**, 5120 -5127.

Neary,Nicola M., Small,Caroline J., et al. (2004) Ghrelin Increases Energy Intake in Cancer Patients with Impaired Appetite: Acute, Randomized, Placebo-Controlled Trial. *Journal of Clinical Endocrinology & Metabolism*, **89**, 2832 -2836.

Neary,Nicola Marguerite, Goldstone,Anthony Peter, et al. (2004) Appetite regulation: from the gut to the hypothalamus. *Clin. Endocrinol. (Oxf)*, **60**, 153-160.

Niedringhaus,M. et al. (2008) Dorsal motor nucleus of the vagus: a site for evoking simultaneous changes in crural diaphragm activity, lower esophageal sphincter pressure, and fundus tone. *Am. J. Physiol. Regul. Integr. Comp. Physiol*, **294**, R121-131.

Norsted,E. et al. (2008) Protein components of the blood-brain barrier (BBB) in the mediobasal hypothalamus. *J. Chem. Neuroanat*, **36**, 107-121.

Novabiochem (2010) Peptide Synthesis.

Nygaard,R. et al. (2006) The PP-fold solution structure of human polypeptide YY and human PYY3-36 as determined by NMR. *Biochemistry*, **45**, 8350-8357.

O'Brien,P.E. et al. (2006) Treatment of Mild to Moderate Obesity with Laparoscopic Adjustable Gastric Banding or an Intensive Medical Program. *Annals of Internal Medicine*, **144**, 625 -633.

Oswal,A. and Yeo,G. (2010) Leptin and the Control of Body Weight: A Review of Its Diverse Central Targets, Signaling Mechanisms, and Role in the Pathogenesis of Obesity. *Obesity*, **18**, 221-229.

Parker,S.L. and Balasubramaniam,A (2008) Neuropeptide Y Y2 receptor in health and disease. *Br. J. Pharmacol*, **153**, 420-431.

Parks,M. and Rosebraugh,C. (2010) Weighing Risks and Benefits of Liraglutide — The FDA's Review of a New Antidiabetic Therapy. *N Engl J Med*, **362**, 774-777.

Patterson,Michael et al. (2009) Hypothalamic Injection of Oxyntomodulin Suppresses Circulating Ghrelin-Like Immunoreactivity. *Endocrinology*, **150**, 3513 -3520.

Pedersen,S.L. et al. (2009) Modifying the conserved C-terminal tyrosine of the peptide hormone PYY3-36 to improve Y2 receptor selectivity. *J. Pept. Sci*, **15**, 753-759.

Pennington,M.W. and Dunn,B.M. (1994) Peptide synthesis protcols Humana Press.

Pettit,L. and El-Modir,A. (2011) The role of somatostatin analogues in the treatment of advanced malignant thymomas: case report and review of the literature. *Brain J Radiol*, **84**, 7-10.

Pi-Sunyer,F.X. et al. (2006) Effect of rimonabant, a cannabinoid-1 receptor blocker, on weight and cardiometabolic risk factors in overweight or obese patients: RIO-North America: a randomized controlled trial. *JAMA*, **295**, 761-775.

Price,C.J. et al. (2008) The Area Postrema: A Brain Monitor and Integrator of Systemic Autonomic State. *The Neuroscientist*, **14**, 182 -194.

Pritchard,L. et al. (2002) Pro-opiomelanocortin processing in the hypothalamus: impact on melanocortin signalling and obesity. *Journal of Endocrinology*, **172**, 411 -421.

Rajagopalan,T.G. (1966) Pepsin from Pepsinogen. Preparation and Properties. *J Biol Chem*, **241**, 4940.

Rendell,M. (2004) Advances in Diabetes for the Millennium: Drug Therapy of Type 2 Diabetes. *MedGenMed*, **6**.

Rossi,M. et al. (1998) A C-terminal fragment of Agouti-related protein increases feeding and antagonizes the effect of alpha-melanocyte stimulating hormone in vivo. *Endocrinology*, **139**, 4428-4431.

Roth,J.D. et al. (2007) Combination therapy with amylin and peptide YY[3-36] in obese rodents: anorexigenic synergy and weight loss additivity. *Endocrinology*, **148**, 6054-6061.

Roth,J.D. et al. (2008) Leptin responsiveness restored by amylin agonism in diet-induced obesity: Evidence from nonclinical and clinical studies. *PNAS*, **105**, 7257–7262.

le Roux,C W and Bloom,S R (2005) Peptide YY, appetite and food intake. *Proc Nutr Soc*, **64**, 213-216.

le Roux,C. W. and Bloom,S. R. (2005) Why Do Patients Lose Weight after Roux-en-Y Gastric Bypass? *Journal of Clinical Endocrinology & Metabolism*, **90**, 591 -592.

Saliba,J. et al. (2009) Endocrine and metabolic response to gastric bypass. *Current Opinion in Clinical Nutrition and Metabolic Care*, **12**, 515-521.

Schmidt,P.T. et al. (2005) A role for pancreatic polypeptide in the regulation of gastric emptying and short-term metabolic control. *J. Clin. Endocrinol. Metab*, **90**, 5241-5246.

Schubert,M.L. (2010) Gastric secretion. *Curr. Opin. Gastroenterol*, **26**, 598-603.

Scott,M.M. et al. (2011) Leptin receptor expression in hindbrain Glp-1 neurons regulates food intake and energy balance in mice. *J Clin Invest*, **121**, 2152-2155.

Sebokova,E. et al. (2010) Taspoglutide, an analog of human glucagon-like Peptide-1 with enhanced stability and in vivo potency. *Endocrinology*, **151**, 2474-2482.

Shechter,Y. et al. (2005) Reversible PEGylation of peptide YY3-36 prolongs its inhibition of food intake in mice. *FEBS Letters*, **579**, 2439-2444.

Sheikh,S.P. et al. (1989) Binding of monoiodinated neuropeptide Y to hippocampal membranes and human neuroblastoma cell lines. *J. Biol. Chem*, **264**, 6648-6654.

Shigeri,Y. and Fujimoto,M. (1994) Y2 receptors for neuropeptide Y are coupled to three intracellular signal transduction pathways in a human neuroblastoma cell line. *J. Biol. Chem*, **269**, 8842-8848.

Shirotani,K. et al. (2001) Neprilysin degrades both amyloid beta peptides 1-40 and 1-42 most rapidly and efficiently among thiorphan- and phosphoramidon-sensitive endopeptidases. *J. Biol. Chem*, **276**, 21895-21901.

Sigma Aldrich (2011) Enzyme Explorer - Pepsin Sigma Aldrich.

Sloth,B. et al. (2007) Effect of subcutaneous injections of PYY1-36 and PYY3-36 on appetite, ad libitum energy intake, and plasma free fatty acid concentration in obese

males. *American Journal of Physiology - Endocrinology And Metabolism*, **293**, E604 - E609.

Small,Caroline J. et al. (2001) Effects of Chronic Central Nervous System Administration of Agouti-Related Protein in Pair-Fed Animals. *Diabetes*, **50**, 248 -254.

Smith,A.J. et al. (1992) State-of-the-art peptide synthesis: Comparative characterization of a 16-mer synthesized in 31 different laboratories. *Techniques in Protein Chemistry*, **3**, 219-229.

Smith,I.G. and Goulder,M.A. (2001) Randomized placebo-controlled trial of long-term treatment with sibutramine in mild to moderate obesity. *J Fam Pract*, **50**, 505-512.

Smith,S.R. et al. (2010) Multicenter, placebo-controlled trial of lorcaserin for weight management. *N. Engl. J. Med*, **363**, 245-256.

Sowden,G.L. et al. (2007) Oxyntomodulin increases intrinsic heart rate in mice independent of the glucagon-like peptide-1 receptor. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, **292**, R962 -R970.

Stanley,S. et al. (2004) Gastrointestinal satiety signals III. Glucagon-like peptide 1, oxyntomodulin, peptide YY, and pancreatic polypeptide. *Am. J. Physiol. Gastrointest. Liver Physiol*, **286**, G693-697.

Steffen,K.J. et al. (2010) A prevalence study and description of alli use by patients with eating disorders. *Int J Eat Disord*, **43**, 472-479.

Stoller,T.J. and Shields,D. (1989) The role of paired basic amino acids in mediating proteolytic cleavage of prosomatostatin. Analysis using site-directed mutagenesis. *J. Biol. Chem*, **264**, 6922-6928.

Suzuki,K. et al. (2010) The role of gut hormones and the hypothalamus in appetite regulation. *Endocr. J*, **57**, 359-372.

Swanton,K. (2008) Healthy weight, healthy lives.

Swinnen,S.G. et al. (2009) Insulin Therapy for Type 2 Diabetes. *Diabetes Care*, **32**, S253-S259.

Talsania,T. et al. (2005) Peripheral exendin-4 and peptide YY(3-36) synergistically reduce food intake through different mechanisms in mice. *Endocrinology*, **146**, 3748-3756.

Taubes,G. (2009) Prosperity's Plague. *Science*, **325**, 256 -260.

Taylor,A. (1993) Aminopeptidases: structure and function. *FASEB J*, **7**, 290-298.

Tickler,A.K. et al. (2001) Improved preparation of amyloid B peptides using DBU as Fmoc deprotection reagent. *J Peptide Sci*, **7**, 488-494.

Turek,,V.F. et al. (2010) Mechanisms of Amylin/Leptin Synergy in Rodent Models. *Endocrinology*, **151**, 1-10.

Turnbull,A.V. et al. (2002) Selective Antagonism of the NPY Y5 Receptor Does Not Have a Major Effect on Feeding in Rats. *Diabetes*, **51**, 2441 -2449.

Turner,A J et al. (2001) The neprilysin (NEP) family of zinc metalloendopeptidases: genomics and function. *Bioessays*, **23**, 261-269.

U.S. Food and Drug Administration (2010) Meridia (sibutramine): Market Withdrawal Due to Risk of Serious Cardiovascular Events.

Ueno,N et al. (1999) Decreased food intake and body weight in pancreatic polypeptide-overexpressing mice. *Gastroenterology*, **117**, 1427-1432.

Unniappan,S. and Kieffer,T.J. (2008) Leptin extends the anorectic effects of chronic PYY(3-36) administration in ad libitum-fed rats. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, **295**, R51 -R58.

Verdich,C., Flint,A, et al. (2001) A meta-analysis of the effect of glucagon-like peptide-1 (7-36) amide on ad libitum energy intake in humans. *J. Clin. Endocrinol. Metab*, **86**, 4382-4389.

Verdich,C., Toubro,S., et al. (2001) The role of postprandial releases of insulin and incretin hormones in meal-induced satiety--effect of obesity and weight reduction. *Int. J. Obes. Relat. Metab. Disord*, **25**, 1206-1214.

Vidal,J. et al. (2009) Long-term effects of Roux-en-Y gastric bypass surgery on plasma glucagon-like peptide-1 and islet function in morbidly obese subjects. *J. Clin. Endocrinol. Metab*, **94**, 884-891.

Wang,S.S. (1973) p-alkoxybenzyl alcohol resin and p-alkoxybenzyloxycarbonylhydrazide resin for solid phase synthesis of protected peptide fragments. *J. Am. Chem. Soc*, **95**, 1328-1333.

Watve,M. and Yajnik,C. (2007) Evolutionary origins of insulin resistance: a behavioral switch hypothesis. *BMC Evolutionary Biology*, **7**, 61.

Welt,,C.K. et al. (2004) Recombinant Human Leptin in Women with Hypothalamic Amenorrhea. *The New England Journal of Medicine*, **351**, 987-997.

White,J.R. (2008) Dipeptidyl Peptidase-IV Inhibitors: Pharmacological Profile and Clinical Use. *Clinical Diabetes*, **26**, 53-57.

Wieland,H.A. et al. (1998) Subtype selectivity of the novel nonpeptide neuropeptide Y Y1 receptor antagonist BIBO 3304 and its effect on feeding in rodents. *Br. J. Pharmacol*, **125**, 549-555.

Williams,D.L., Baskin,Denis G., et al. (2009) Evidence that Intestinal Glucagon-Like Peptide-1 Plays a Physiological Role in Satiety. *Endocrinology*, **150**, 1680 -1687.

Williams,K.W., Scott,M.M., et al. (2009) From observation to experimentation: leptin action in the mediobasal hypothalamus. *The American Journal of Clinical Nutrition*, **89**, 985S -990S.

World Health Organization (1999) Definition, Diagnosis and Classification of Diabetes Mellitus and its complications.

World Health Organization (2009) Unhealthy diets and physical inactivity.

Wraith,A. et al. (2000) Evolution of the neuropeptide Y receptor family: gene and chromosome duplications deduced from the cloning and mapping of the five receptor subtype genes in pig. *Genome Res*, **10**, 302-310.

Wren,A M, Seal,L.J., et al. (2001) Ghrelin enhances appetite and increases food intake in humans. *J. Clin. Endocrinol. Metab*, **86**, 5992.

Wren,A M, Small,C J, et al. (2001) Ghrelin causes hyperphagia and obesity in rats. *Diabetes*, **50**, 2540-2547.

Wynne,K et al. (2006) Oxyntomodulin increases energy expenditure in addition to decreasing energy intake in overweight and obese humans: a randomised controlled trial. *Int J Obes (Lond)*, **30**, 1729-1736.

Wynne,Katie, Giannitsopoulou,K., et al. (2005a) Subcutaneous Ghrelin Enhances Acute Food Intake in Malnourished Patients Who Receive Maintenance Peritoneal Dialysis: A Randomized, Placebo-Controlled Trial. *Journal of the American Society of Nephrology*, **16**, 2111 -2118.

Wynne,Katie, Park,Adrian J, et al. (2005b) Subcutaneous oxyntomodulin reduces body weight in overweight and obese subjects: a double-blind, randomized, controlled trial. *Diabetes*, **54**, 2390-2395.

Wynne,Katie et al. (2004) The Gut and Regulation of Body Weight. *Journal of Clinical Endocrinology & Metabolism*, **89**, 2576 -2582.

Wysocki,V.H. et al. (2005) Mass spectrometry of peptides and proteins. *Methods*, **35**, 211-222.

Yoshihara T, et al. (1996) Effects of restricted daily feeding on neuropeptide Y release in the rat paraventricular nucleus. *Am J Physiol*, **270**, 589-595.