FULL PAPER

Propylphosphonic Anhydride (T3P®) as Coupling Reagent for Solid-Phase Peptide Synthesis

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Abstract: Amidation is the predominant reaction within the pharmaceutical setting, and it is attracting greater attention due to the increased demand for therapeutic peptides. The high therapeutic efficacy and safety profile of peptides have placed these molecules in prime position within the pharmaceutical arena, which is reflected by these molecules receiving several approvals from various regulatory agencies each year. In this context, the demand for developing efficient strategies for peptide synthesis has also risen.

Although propylphosphonic anhydride (T3P®), which has been recently proposed as a green coupling reagent, has shown good performance in solution, it has never been applied to solid-phase peptide synthesis (SPPS). Here we test the use of T3P® for SPPS. Satisfactory yields were achieved with a mild activation protocol. Various green solvents were tested and proved to be compatible with this coupling reagent.

Several commonly used reagents cause allergic reactions or are susceptible to explosion under certain conditions. To overcome these issues, we propose T3P® as a potential alternative coupling reagent in SPPS.

Introduction

Peptides are gaining ground in the biomedical and pharmaceutical arena. Back in 1954, the first biologically active peptide (Oxytocin) was synthesized by Noble laureate Vincent Du Vigneaud.[1] Continuous efforts are now being channelled into peptide synthesis approaches. Of note is the innovative work of Noble laureate Merrifield in 1963,[2] who developed solid-phase peptide synthesis (SPSS), the current method of choice for preparing peptide entities in both research and production settings. This approach transformed the field, allowing the preparation of virtually any peptide in high purity and satisfactory yield. The increased presence of peptides is reflected by several of them receiving authorization from the US Food and Drug Administration (FDA) each year.[3-5]

Given that the amide bond is the main linkage that forms peptides, chemists are continuously looking for efficient, convenient, and safer amidation protocols/routes. In addition, amide bonds account for 54% of the base bond for medicines in big pharmaceutical companies (Pfizer, GSK, and AstraZenca).[6]

In 1955, the coupling concept was first introduced by Sheehan and Hess.[7] For amide bond formation, the carboxylic acid moiety needs to be activated in an appropriate manner.[8] In this regard, various coupling reagents and strategies are currently available for this purpose.[9, 10] The activation process can affect the enantiomeric purity of the product which can be fuelled depending on how harsh the activation procedure is. Hence, access to coupling reagents with a mild activation mechanism and minimal effects is an important goal, especially for preparing biologically active compounds.

Various coupling reagents are currently used in SPPS, carbodiimide, including dicyclohexylcarbodiimide (DCC) and diisopropylcarbodiimide (DIC), being the most common. The reaction of amino acids with any of these coupling reagents generates a highly reactive O-acylisoaurea. Of note, DIC is more favoured than DCC due to the soluble diisopropylurea rather than the insoluble dicyclohexylurea in the solvents commonly used, namely *N,N*-dimethylformamide (DMF) and dichloromethane (DCM), especially when automatic peptide synthesizers are used. Furthermore, both coupling reagents are allergens, which must also be considered.[11]

To avoid undesired side reactions, the high reactivity of O-acylisoaurea must be reduced. Various nucleophiles can be added for this purpose, namely 1-hydroxybenzotriazole (HOBt)[12] and 1-hydroxy-7-azabenzotriazole (HOAt)[13], where a less reactive benzotriazolyl ester is formed. However, due to the explosive property of these nucleophiles[14], ethyl-2-cyano-2-hydroxyimino acetate (OxymaPure) is considered a safer alternative. In addition, this coupling reagent shows higher efficiency for the previous purpose and excels in suppressing racemization.[15-18]

Uronium/aminium salts were later introduced as stand-alone coupling reagents, the most common being (N-[(1H-benzotriazol-1-yl)(dimethylamino)methylene]-N-methylmethanaminiumhexafluorophosphate N-oxide) (HBTU)[19, 20] and (N-[(dimethylamino)-1H-1,2,3-triazole[4,5-b]pyridine-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate N-oxide) (HATU). However, consideration must be given to the fact that these compounds cause a life-threatening allergic reaction (anaphylaxis) [21]. With the same skeleton as HBTU/HATU, N,N,N′,N′-tetramethylformamidinium hexafluorophosphate (TFFH) is an unique coupling reagent that renders the

corresponding acid fluoride after reaction with the carboxylic acid.[22]

Albericio and co-workers introduced another generation of coupling reagent, (1-(1-(cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylaminomorpholino-methylene) methaminium hexafluorophosphate) (COMU), which shows very high coupling efficiency[23, 24]. The absence of benzotriazolyl moieties in the COMU structure is also an advantage in terms of safety. Figure 1 shows the chemical structures of the aforementioned coupling reagents and additives.

Phosphonium salts such as PyBOP, PyClock, and PyOxim are another important class of coupling reagents[25-27]. Both phosphonium and uronium/aminium derivatives render the corresponding active esters when reacted with the carboxylic group to be activated.[26] However, uroniums/aminiums can react with the amino function, rendering guanidine moieties and therefore capping elongation of the peptide.[26] Phosphonium salts do not give this side reaction and therefore are the coupling reagent of choice in slow kinetic reactions such as cyclization.[28]

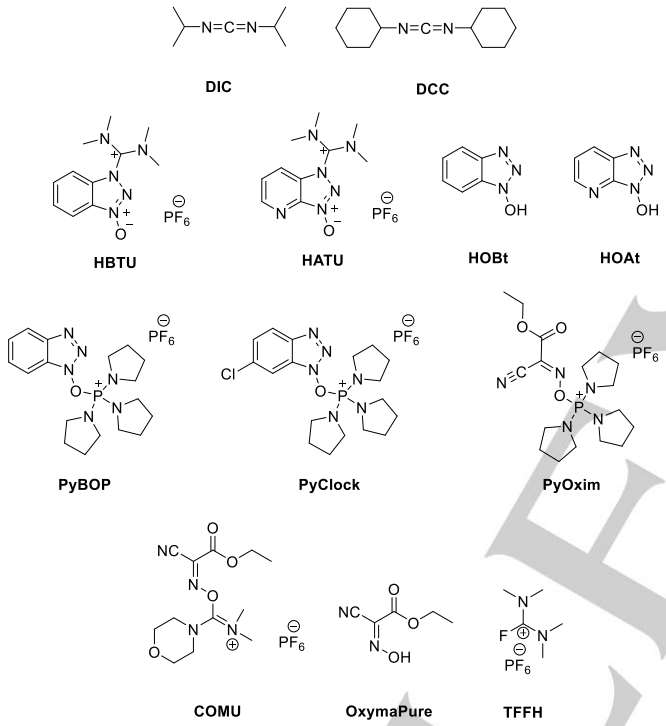

Figure 1. Chemical structures of various common coupling reagents and additives.

Recently, a report from the ACS Green Chemistry Institute® Pharmaceutical Roundtable (GCIPR) declared that OxymaPure, COMU, TFFH, propylphosphonic anhydride (T3P®) are green coupling reagents.[29]

T3P® is a phosphorous-containing cyclic anhydride that was introduced by Wissmann and Kleiner as a condensation reagent for peptide synthesis[30] (Figure 2). Unlike other common coupling reagents, T3P® has a low toxicity and allergy profile, as demonstrated by taxicological studies.[31, 32] A significant advantage in the use of T3P®, is that the by-products of the reaction are highly water soluble and can readily be washed away from products with less good water solubility. This often results in simple work-up procedures.

As an exceptional and safe reagent for use in condensation reactions, T3P® has attracted the attention of almost all innovative pharmaceutical companies worldwide during the past decade. Whilst the major applications for T3P® use are in amidation and peptide coupling reactions, this green reagent may readily be used for various other functional group transformation reactions including nitrile formation from carboxamides, alcohol oxidation (Swern type oxidation), ester formation, Lossen rearrangement, Beckmann rearrangement and heterocycle formation.

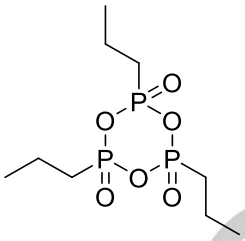

Figure 2. Chemical structure of Propylphosphonic anhydride (T3P®).

T3P® is commercialized as a 50% (w/w) solution in a wide range of different solvents (DMF, DCM, 2-methyltetrahydrofuran (2-MeTHF), tetrahydrofuran (THF), ethyl acetate (EtOAc), butyl acetate) at affordable price and shows appreciable stability.[31, 33] It has been extensively used in solution synthesis[34, 35] and also in amide bond-forming reactions.[33, 36] Moreover, it is preferred for large-scale amidation reactions.[37] Despite the efficient coupling capacity of T3P® and low racemization rates, to the best of our knowledge, no SPPS using this coupling reagent has been reported to date.

Here we developed a straightforward SPPS protocol using T3P® under mild conditions. Various peptides were synthesized with satisfactory purity either in common or green protocols.[38]

Results and Discussion

Several experiments were carried out to establish an optimum coupling procedure with respect to equivalent amounts of each reactant, additives, order of addition, and temperature.

Initial Leu-enkephalin synthesis

As a model, Leu-enkephalin pentapeptide was synthesized on ChemMatrix (CM resin) as follows: fluorenylmethoxycarbonyl-amino acid (Fmoc-amino acid (AA)-OH) - diisopropylethylamine (DIEA) - T3P® (3:6:4) with respect to the amino functional of the resin. A final purity of 69%, as determined by HPLC (Figure S1), was obtained. A rational strategy was then developed to fine tune the use of T3P®.

Screening experiments

[1+4] protocol to optimize the synthesis of H-Tyr-Gly-Gly-Phe-Leu-NH_2 (Leu-enkephalin)

A sufficient amount of H-Gly-Gly-Phe-Leu-NH-Rink-amide-resin was synthesized using the conventional Fmoc/*tert*-butyl (*t*Bu) procedure (Figure S2). Afterwards, [1+4] experiments were carried out, where the Fmoc-Tyr(*t*Bu)-OH was coupled using T3P® in acetonitrile (ACN) under various conditions (Table 1, AA for Fmoc-Tyr(*t*Bu)-OH).

Table 1. Stoichiometric [1+4] screening experiments on H-Tyr-Gly-Gly-Phe-Leu-NH_2

Exp#	Reagents & Order of addition*	Equivalents	Solvent	Temperature	%
1	AA-DIEA-T3P®-OxymaPure	6:6:4:6	DMF	RT	99.1
2	AA-DIEA-T3P®	6:6:4			99.1
3	AA-DIEA-T3P®	3:6:4			98.7
4	AA-DIEA-T3P®-OxymaPure	6:12:4:6			98.0
5	AA-DIEA-T3P®	6:12:4			98.0
6	AA-KOxyma-T3P®	6:6:4			30.7

*AA: Fmoc-Tyr(*t*Bu)-OH; T3P® in ACN

Table 1 shows the results obtained for the incorporation of Fmoc-Tyr(*t*Bu)-OH using slightly different conditions (presence or absence of OxymaPure, stoichiometry, and use of a different base). The different reagents were successively added to the resin with intervals of 15 sec, and the coupling was carried out for 1 h at room temperature (RT). OxymaPure was added to form the oxime active ester from the unsymmetrical anhydride resulting from the reaction of the protected amino acid with T3P®.

The following stoichiometric amounts AA-DIEA-T3P®-OxymaPure (6:6:4:6) achieved an excellent conversion yield (99.1%) (#1, Table 1) (Figure S3). To check whether OxymaPure influences the synthesis, we performed the synthesis without it. The same conversion yield was observed (99.1%) (#2, Table 1). Therefore, the addition of OxymaPure could be beneficial, but it is not crucial (Figure S4). To adhere to green principles, we attempted to decrease the amount of reactants used in the synthesis. In this regard, we tested AA-DIEA-T3P® (3:6:4). The results obtained were also encouraging (98.7%) (#3, Table 1) (Figure S5). An additional excess of DIEA was also used (with and without OxymaPure) and the results remained acceptable (98.0%) (#4, 5, Table 1) (Figures S6 and S7).

The potassium salt of OxymaPure (KOxyma) serves as a coupling additive and a base.[39] We therefore envisaged its use here to replace DIEA. Surprisingly, a dramatic drop in the conversion yield was observed (30.7%) (#6, Table 1) (Figure S8). In conclusion, KOxyma cannot replace DIEA.

Full Leu-enkephalin synthesis

Leu-enkephalin was fully synthesized under the optimized conditions, using T3P® in ACN. The results were promising (a purity of 80.1%) but further optimization was still needed (Figure S9).

[1+4] protocol to optimize the synthesis of H-Tyr-Ile-Gly-Phe-Leu-NH_2

For this second optimization round, a highly demanding coupling was chosen. Thus, Gly^2 residue was substituted with Ile^2, which is a β-branched amino acid, therefore posing a more challenging acylation. A sufficient amount of the H-Ile-Gly-Phe-Leu-NH-Rink-amide-resin was synthesized using the DIC/OxymaPure protocol (Figure S10).

Table 2. Stoichiometric [1+4] screening experiments on H-Tyr-Ile-Gly-Phe-Leu-NH_2

Exp#	Reagents & Order of addition*	Equivalents	Solvent	Temperature	%
1	AA-DIEA-T3P®-OxymaPure	5:5:5:5	DMF	RT	44.4
2	AA-OxymaPure-DIEA-T3P®	5:5:5:5			24.6
3	AA-DIEA-T3P®	5:5:5			41.5
4	AA-KOxyma-T3P®-OxymaPure	5:5:5:5			12.8
5	AA-DIEA-T3P®-KOxyma	5:5:5:5			45.8
6	AA-DIEA-T3P®-OxymaPure	5:5:10:5			57.1
7	AA-DIEA-T3P®-OxymaPure	5:10:5:5			91.7
8	AA-DIEA-T3P®-OxymaPure	5:10:10:5			57.4

*AA: Fmoc-Tyr(*t*Bu)-OH; T3P® in ACN

Again, T3P® in ACN was used and the sequential addition of AA, DIEA, T3P®, OxymaPure, and/or KOxyma was carried out. First, from the overall results of Table 2, this coupling appeared to be more demanding than that carried out in Table 1. Thus, AA, DIEA, T3P®, and OxymaPure (5 equiv. of each) were sequentially added to the peptide resin. HPLC showed a low conversion yield (44.4%) (#1, Table 2) (Figure S11). The experiment was repeated by changing the order of addition. To this end, OxymaPure was added after the AA. However, this change in order led to a reduction in the conversion yield (24.6%) (#2, Table 2) (Figure S12). Another experiment was done without OxymaPure, but the results were almost as same as with it (41.5%) (#3, Table 2) (Figure S13). These three experiments (#1-3, Table 2) reaffirmed that the presence of OxymaPure is not crucial but that its order of addition is. Thus, when it is added after the protected amino acid and before T3P®, the yield is much lower (#2, Table 2) (Figure S12). This order of addition is applied in couplings where DIC-OxymaPure and HBTU/HATU/COMU-HOBt/HOAt/-OxymaPure-DIEA are used and therefore this result is a little surprising.

Next, DIEA was substituted with KOxyma and OxymaPure was maintained, but again the conversion yield dropped even further (12.8%) (#4, Table 2) (Figure S14). We tested whether KOxyma offers any advantage over OxymaPure, achieving a conversion of 45.8% (#5, Table 2) (Figure S15), which is very similar to (#1, Table 2), where OxymaPure was used. From these two experiments, it can be concluded that KOxyma can substitute OxymaPure, but it cannot replace DIEA as a base.

It is known that T3P® could react with oximes, as demonstrated by Augustine et al.[40] through the reaction of T3P® with aldoxime at 100º C. In this regard and to gain a better understanding of the influence of the order of addition, a computational study was carried out and the transition state was determined for the main reaction (Fmoc-AA with the T3P®) and for the one we suspected of hampering/competing with the main reaction, namely the side reaction of OxymaPure with T3P® (Figure 3).

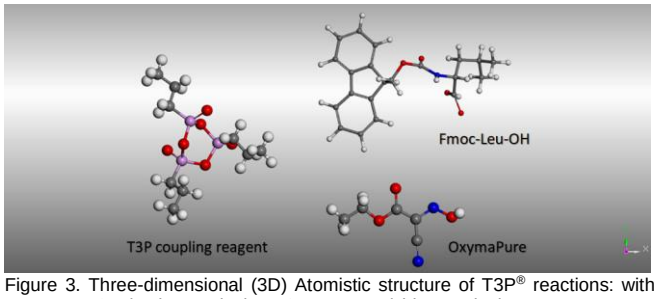

Figure 3. Three-dimensional (3D) Atomistic structure of T3P® reactions: with Fmoc-Leu-OH (main reaction), or OxymaPure (side reaction).

The computational data confirmed that the reaction of OxymaPure is thermodynamically more favoured than the main reaction. In this regard, the Gibbs free energy (ΔG) for the OxymaPure side reaction with T3P® was 26.423 Kcal/mol, whereas for the main reaction it was 31.886 Kcal/mol. Thus, the formation of the OxymaPure-T3P® adduct would explain the low conversion yields when OxymaPure is added directly after the amino acid (2nd position). However, it is worth mentioning that the main reaction is faster than the side reaction, as reflected by the energy of activation (ΔG^{++}) values. Indeed, ΔG^{++} of the main reaction was 7.602 Kcal/mol vs. 35.273 Kcal/mol for the OxymaPure side reaction (Figures S16 and S17). Of note, this type of preference of an oxime derivative for the coupling reagent was previously observed when the oxime derived from Meldrum's acid reacted faster than the Fmoc-amino acid with DIC and therefore that oxime deactivated DIC.[41]
These results confirm the experimental finding that neither OxymaPure nor KOxyma can be used in the second position due to their higher tendency to react with T3P®.

Three syntheses were carried out where excesses of T3P®, DIEA, or both were used. Using double the amount of T3P® (10 equiv.), a conversion of 57.1% was achieved (#6, Table 2) (Figure S18). Next, with a double amount of DIEA, a dramatic increase in the conversion yield was observed (91.7%) (#7, Table 2) (Figure S19). Finally, the increase of both was attempted, but only 57.4% of conversion was detected (#8, Table 2) (Figure S20). These results highlight the need for excess base with respect to the coupling reagent.
The previous screening experiments revealed that that the following stoichiometry and order of addition are optimum: AA-DIEA-T3P®-OxymaPure (5:10:5:5), and these conditions were mostly considered for the rest of this part of the work.

Coupling reagent screening

As mentioned in the introduction, T3P® is commercially available as a 50% (w/w) solution. To examine the optimum reagent for SPPS, we synthesized H-Tyr-Ile-Gly-Phe-Leu-NH_2, following the [1+4] protocol using three different T3P® solutions in 2-MeTHF, ACN, and DMF (Table 3).

Table 3. T3P® solution [1+4] screening experiments on H-Tyr-Ile-Gly-Phe-Leu-NH_2

Exp#	Reagents & Order of addition*	Equivalents	Solvent	Temperature	%
1	AA-DIEA-T3P®(2-MeTHF)-OxymaPure	5:10:5:5	DMF	RT	93.8
2**	AA-DIEA-T3P®(ACN)-OxymaPure				91.7
3	AA-DIEA-T3P®(DMF)-OxymaPure				77.0
4	AA-DIEA-T3P®(DMF)-OxymaPure (regenerated coupling reagent)***				83.4

*AA: Fmoc-Tyr(*t*Bu)-OH; **#7, Table 2; *** See below for the discussion of #4

T3P® in 2-MeTHF showed the best performance, with a conversion yield of 93.8% (#1, Table 3). In ACN, the results were very close, achieving a yield of 91.7% (#2, Table 3), while DMF came in third position at 77.0% (#3, Table 3) (Figures S18, S21 and S22). Given these results, T3P® in 2-MeTHF was used for the rest of the study.
Given that T3P® is a stable compound with a long shelf life, the low conversion yield using T3P® in DMF encouraged us to carry out a computational study to gain a greater understanding of its poor performance.[32] However, T3P® can be influenced by the type of solvent in which it has been prepared. Some literature mentions that the presence of dimethylamine (DMA) impurity in DMF can hamper several reactions.[42]
A study performed by Magtaan J. K. *et al.*[43] showed how aged DMF can influence peptide synthesis as a result of evolving dimethylamine and formic acid impurities. Given these reported observations, we suspected the occurrence of a side reaction between the protected amino acid-T3P® mixed anhydride and the dimethylamine impurity that competes with the reaction with the amino function of the peptide anchored on the resin.
Of note, a computational test was carried out considering solution phase settings for both reactions (Figure 4). Accordingly, in SPPS, the main reaction is expected to have even more difficult kinetics than in solution phase, thereby making it an even greater challenge.

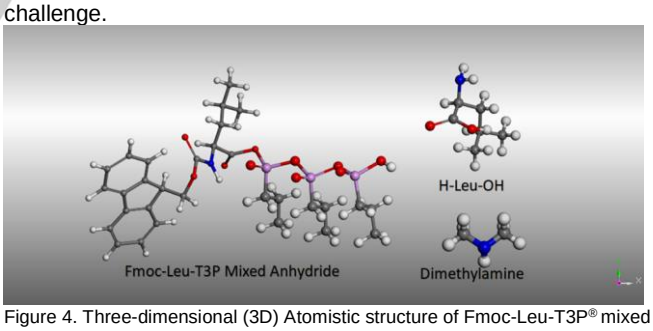

Figure 4. Three-dimensional (3D) Atomistic structure of Fmoc-Leu-T3P® mixed anhydride reactions: with H-Leu-OH (main reaction), or dimethylamine (side reaction).

As expected, the computational data confirmed that this side reaction was thermodynamically more favoured than the main reaction. In this regard, the ΔG of the DMA side reaction with the mixed anhydride was -29.432 Kcal/mol vs. -27.880 for the main reaction. It should be noted that the ΔG^{++} for the side reaction was 124.432 Kcal/mol vs. 33.524 Kcal/mol (Figures S23 and S24). These findings are also consistent with the fact that secondary amines are much more reactive than primary ones.

To further consolidate our findings, we sonicated the T3P® in DMF under a vacuum experiment, which could help remove the DMA, as suggested in the study by Magtaan J. K. *et al.*[43] An increase in the conversion yield was increased from 77.0% using the

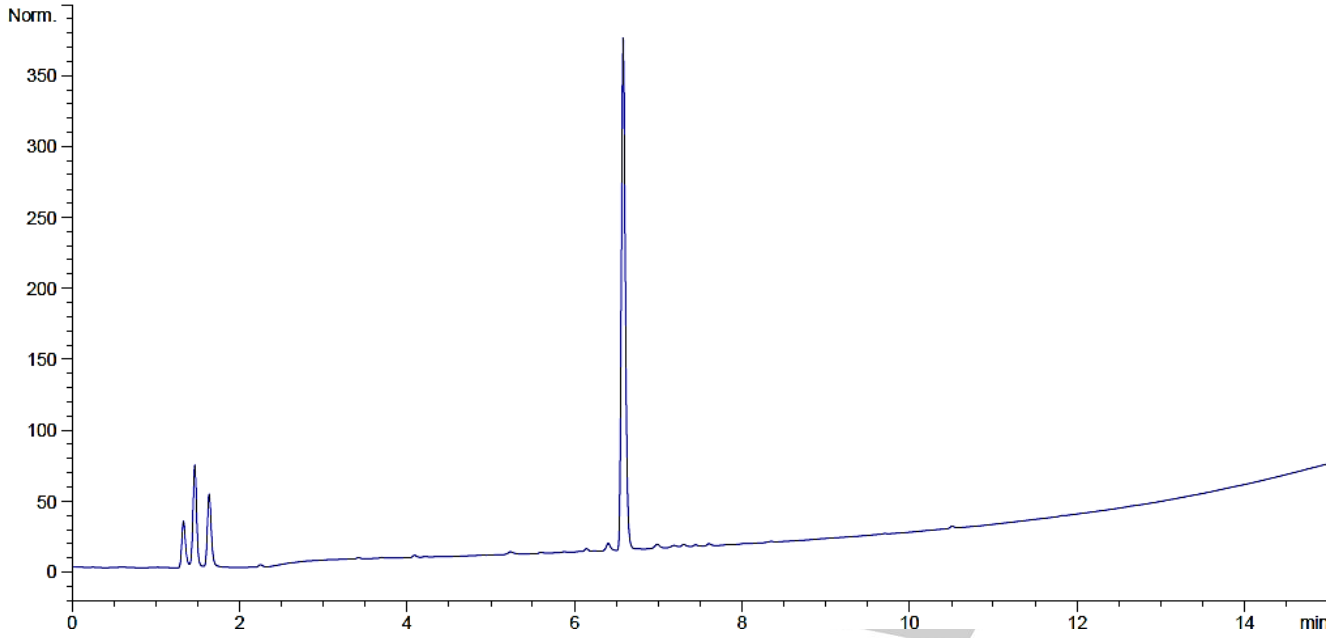

Figure 5. Full synthesis of H-Tyr-Ile-Gly-Phe-Leu-NH_2 pentapeptide on CM resin. AA-DIEA-T3P®(2-MeTHF)-OxymaPure (5:10:5:5) at 60º C. 5−95% gradient elution in 15 min. λ = 220 nm. Mobile phase A: 0.1% TFA in H_2O; mobile phase B: 0.1% TFA in ACN; Phenomenex Luna C_{18} (3.6 µm, 4.6 × 150 mm) column.

reagent directly to 83.4% after sonication (#3, 4, Table 3) (Figure S25). However, while the increase was not remarkable, it did confirm our thoughts that an additional treatment and regeneration of the DMF may overcome such a problem, or that the use of fresh DMF in which to prepare T3P® could be a much safer scenario.

Reaction temperature screening

Unlike classical solution chemistry, SPPS requires yields as high as 99% for each step to be considered acceptable. Thus, several additional experiments involving an increase in temperature and using alternative base such as *N*-methylimidazole (NMI) were carried out to enhance the yield. (Table 4).

Table 4. Temperature [1+4] screening experiments on H-Tyr-Ile-Gly-Phe-Leu-NH_2

Exp#	Reagents & Order of addition*	Equivalents	Solvent	Temperature	%
1	AA-DIEA-T3P®-OxymaPure **	5:10:5:5	DMF	RT	93.8
2	AA-DIEA-T3P®-OxymaPure	5:10:5:5		40º C	99.3
3	AA-DIEA-T3P®-OxymaPure	5:10:5:5		60º C	98.3
4	AA-NMI-T3P®	5:10:5		60º C	8.1
5	AA-NMI-T3P®	5:20:5		60º C	20.3

*AA: Fmoc-Tyr(*t*Bu)-OH; **#1, Table 3; T3P® in 2-MeTHF

We performed [1+4] syntheses at 40º and 60º C (#2, 3, Table 4) (Figures S26 and S27), achieving superior yields (99.3% and 98.3%, respectively) than that obtained at RT (93.8%) (#1, Table 4).

NMI has shown excellent results for amide bond formation between the poor nucleophile 4-chloroaniline and a carboxylic acid.[44] Furthermore, it was previously used by our group along with DIC for ester bond formation.[45] Hence, considering the optimum stoichiometry, coupling reagent, equivalents, and temperature, we tested NMI as an alternative base for DIEA. Unexpectedly, overall discouraging results were obtained, achieving a conversion yield of only 8.1% (#3, Table 4) (Figure S28). The same reaction was repeated with an excess amount of NMI (20 equiv. instead of 10), which allowed an increase in the conversion yield, although still unsatisfactory (20.3%) (#4, Table 4) (Figure S29).

A full H-Tyr-Ile-Gly-Phe-Leu-NH_2 pentapeptide was synthesized using T3P® for the coupling of all amino acid residues (including the first one) onto the resin. At 60º C (Figure 5), this approach yielded a purer peptide (97%) than at 40º C (50.6%), as determined by HPLC (#1, 2, Table 5) (Figure S30).

Table 5. Full synthesis of H-Tyr-Ile-Gly-Phe-Leu-NH_2

Exp #	Reagents & Order of addition*	Equivalents	Solvent	Temperature	%
1	AA-DIEA-T3P®-OxymaPure	5:10:5:5	DMF	40º C	50.6
2	AA-DIEA-T3P®-OxymaPure			60º C	97.0

AA: Fmoc-Leu-OH, Fmoc-Phe-OH, Fmoc-Gly-OH, Fmoc-Ile-OH, Fmoc-Tyr(*t*Bu)-OH; T3P® in 2-MeTHF

The effect of the different temperatures was now more pronounced due to the larger number and diversity of the couplings, as already observed for the anchoring of the first amino acid (Leu) onto the resin. Anchoring reaction is likely to be more demanding than other peptide bonds because of the poor nucleophilicity of the amino function (benzydrylamine) of the Rink-amide-resin. Thus, the loading value of the first amino acid onto the resin was only 50% at 40º C (0.2 mmol/g out of 0.45 mmol/g, supplier's specification), and a double incorporation was needed to achieve the full incorporation capacity. In contrast, at 60º C, full loading was achieved with a single coupling. In this regard, the rest of the experiments were carried out at 60º C.

Green solvent screening

As T3P® is a green coupling reagent, it is highly relevant to check whether a full green SPPS protocol can be followed. To this end, various green solvents were tested. Using the optimized conditions, the following solvents were selected for a [1+4] protocol: γ-valerolactone (GVL); 2-MeTHF; *N*-Butylpyrrolidinone (NBP); EtOAc; EtOAc-NBP (3:1); and EtOAc-

DMF (3:1) and ACN, which is not green, but it is considered less environmental aggressive than DMF (Table 6) (Figures S31-37).

Table 6. Green solvent [1+4] screening experiments for the synthesis of H-Tyr-Ile-Gly-Phe-Leu-NH_2

Exp#	Reagents & Order of addition*	Equivalents	Solvent	Temperature	%
1	AA-DIEA-T3P®-OxymaPure	5:10:5:5	GVL	60º C	84.4
2			2-MeTHF		77.5
3			NBP		63.2
4			EtOAc		96.4
5			ACN		97.9
6			EtOAc-NBP (3:1)		97.4
7			EtOAc-DMF (3:1)		92.6

*AA: Fmoc-Tyr(*t*Bu)-OH; T3P® in 2-MeTHF

On the basis of the results shown in the previous table, EtOAc, ACN, and EtOAc-NBP (3:1) emerge as potential green alternatives for DMF. A full synthesis was also carried out in ACN to confirm these findings (see the next section).

Solid-phase peptide synthesis (SPPS)

Various model peptides were synthesized to demonstrate the coupling capacity of T3P®. ACP decapeptide (Figure 6) (Figure S38), a difficult molecule that has various hydrophobic amino acid residues in its sequence, was synthesized under the optimized conditions. Only two deletion sequences were observed. They were identified by LCMS with the following m/z masses 496.48 and 432.13, which represent the mass of the doubly protonated peptides with the following percentages: 5.8% of des-Gln-Ala and 8.6% of des-Ala (Figures S39 and S40). The overall purity of the final product was considered satisfactory (74.7%) for such a difficult type of peptide. H-Tyr-Ile-Gly-Phe-Leu-Tyr-Ile-Gly-Phe-Leu-NH-Rink-amide-resin decapeptide was also synthesized,

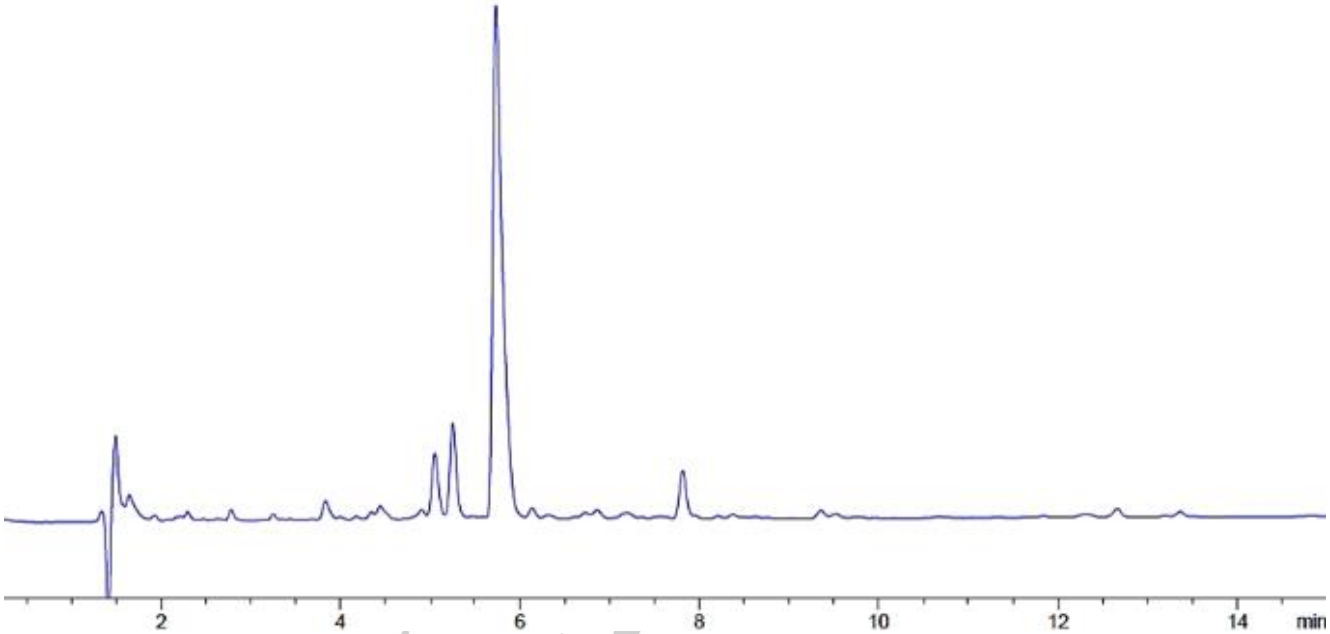

Figure 6. Full synthesis of ACP decapeptide on CM resin. AA-DIEA-T3P®(2-MeTHF)-OxymaPure (5:10:5:5) at 60º C. 15−25% gradient elution in 15 min. See legend of Figure 5 for column and rest of chromatographic conditions.

once in DMF as a solvent and 20% piperidine in DMF for Fmoc removal, and once using ACN as a solvent and 4-methylpiperidine in GVL for Fmoc removal. Satisfactory results were obtained in both cases (Figures 7, A and B).

Microwave study

To demonstrate the coupling capacity of T3P® under the microwave conditions (75º C), two [1+4] coupling reactions at different time points were done (3 min and 60 min). Complete conversion was observed after the first 3 min. This result confirms the compatibility of T3P® with microwave-assisted syntheses (Figures S41 and S42).

Racemization study

When a new coupling reagent is tested, racemization should be analysed. However, the synthesis of the Leu-enkephalin peptide did not show an indication of racemization (Figure 5), racemization of Fmoc-Cys(Acm)-OH and Fmoc-Ser(tBu)-OH, which are prone amino acids to racemize, was studied using the SPPS of dipeptides Xxx-L-Phe-NH_2 with *in situ* activation protocol and adding OxymaPure just before the coupling reagent, AA-DIEA-OxymaPure-T3P (5:10:5:5). While for Ser no racemization was detected, for the case of Cys(Acm) tolerable epimerization in agreement with the reported in the literature for this residue in other coupling reagents (%DL = 0.4) (Figures S43-46).[46]

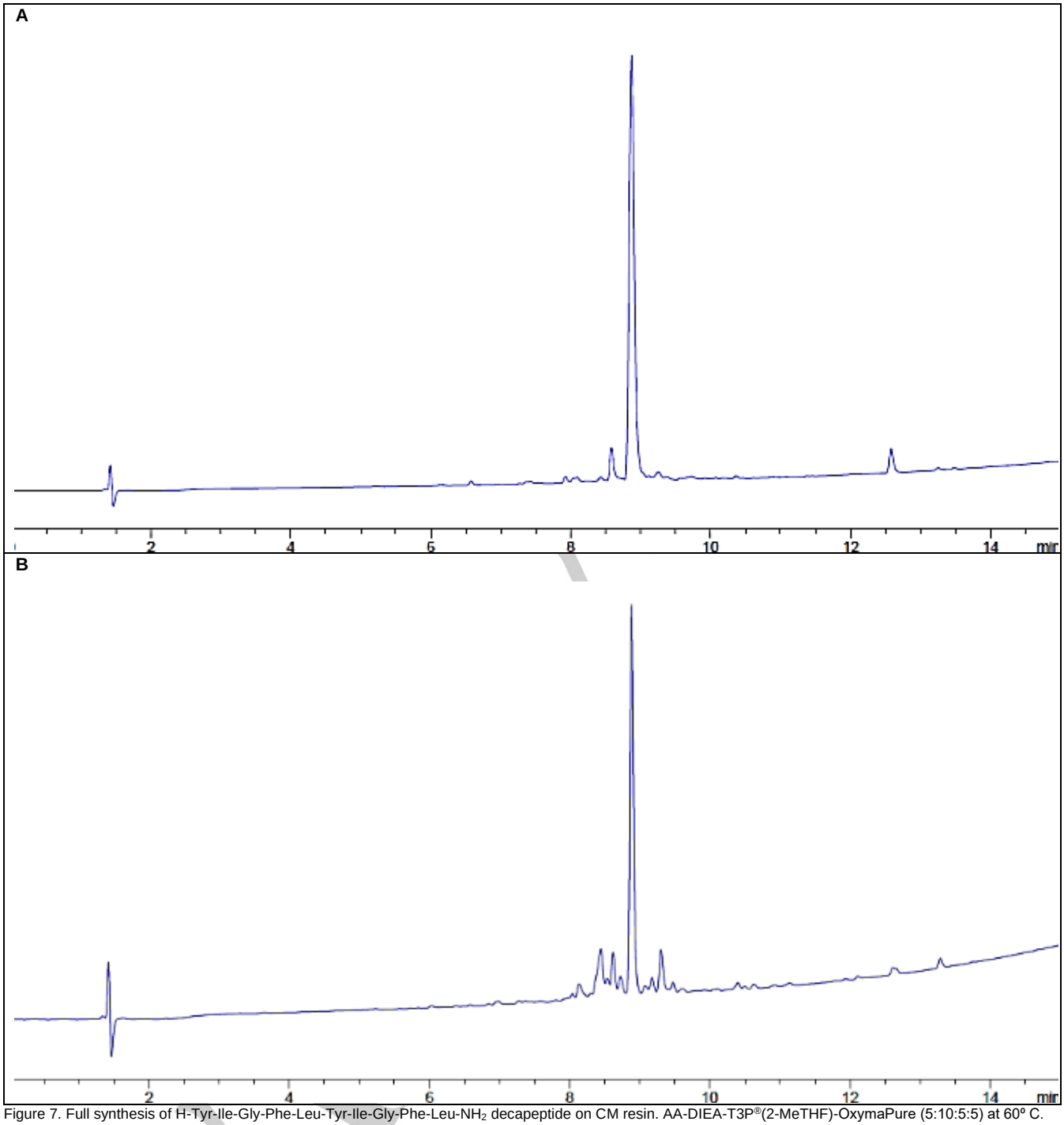

Figure 7. Full synthesis of H-Tyr-Ile-Gly-Phe-Leu-Tyr-Ile-Gly-Phe-Leu-NH_2 decapeptide on CM resin. AA-DIEA-T3P®(2-MeTHF)-OxymaPure (5:10:5:5) at 60º C. A. Solvent: DMF, B. Solvent: ACN. See legend of Figure 5 for column and chromatographic conditions.

Conclusion

After demonstration that T3P® is an efficient coupling reagent in solution phase amidation reactions in general, specifically in peptide synthesis, here we optimized the reagent for SPPS. Several peptides were achieved without the use of common coupling reagents such as carbodiimide and uronium/aminium-based coupling reagents. Here we have shown that T3P® is a green and efficient alternative coupling reagent for SPPS. Satisfactory conversion yields were achieved in a straightforward protocol. Various green solvents were tested and proved also to be compatible with this coupling reagent. It is important to bear in mind that the order of addition of the different reagents is of utmost importance, since in some cases no reaction was achieved when the order was changed. In the case of using OxymaPure as additive, it should be added once the Fmoc-AA-OH has reacted with T3P®. If OxymaPure is added before T3P®, it competes with the Fmoc-AA-OH in reacting with T3P®, resulting in a lower coupling yield, as confirmed by computational calculations.
We envisage that T3P® as an alternative coupling reagent in SPPS. Provided that, some of the common coupling reagents have shown allergic reactions or explosive tendency under certain conditions.

Supporting Information Summary

For the experimental section, chromatograms, mass spectra, computational figures, refer to the the supppрting information.

Acknowledgements

The work was funded in part by the following: the National Research Foundation (NRF) (# 105892 and Blue Sky's Research Programme # 120386) and the University of KwaZulu-Natal (South Africa); and the Spanish Ministry of Science, Innovation, and Universities (RTI2018-093831-B-100) and the Generalitat de Catalunya (2017 SGR 1439) (Spain).

Conflict of interest

RW and PT work for Euticals GmbH, which has provided for free the T3P reagents.

Keywords: Amidation • Green chemistry • Peptides • SPPS • T3P®

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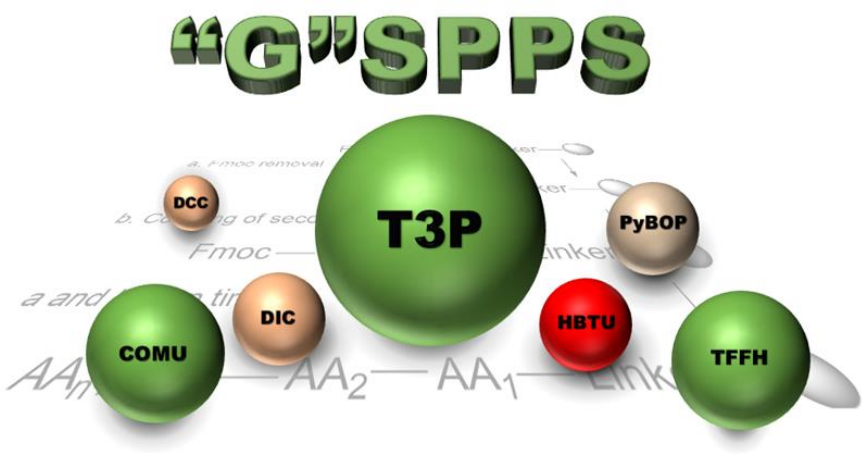

T3P has been introduced as a green alternative coupling reagent for SPPS. Efficient conversion yields were obtained when various peptides were synthesized in this work. The driving force for this study is the health and environmental concerns associated with the common coupling reagents in use.

Institute and/or researcher Twitter usernames: @OMusaimi, @FAlbericio, @amriglobal

1. Experimental Section

1.1 Materials and methods

CM-resin was used for all syntheses. All reagents and solvents were obtained from commercial suppliers and were used without further purification unless otherwise stated.

Analytical HPLC was performed on an Agilent 1100 system using Chemstation software for data processing. Column: Phenomenex Luna C_{18} (3.6 µm, 4.6 × 150 mm) column, with flow rate of 1.0 mL/min, 30 ºC column oven, and UV detection at 220 nm. Mobile phase A: 0.1 % TFA in H_2O; mobile phase B: 0.1 % TFA in ACN. 5-95% in 15 min gradient elution was considered in all peptides. Except with ACP decapeptide (15-25% in 15 min). LC-MS was performed on Ultimate™ 3000, AerisTM 3.6 µm-wide pore column, Phenomenex C_{18} (150 × 4.6 mm) column. Buffer A: 0.1% formic acid in H_2O; buffer B: 0.1% formic acid in ACN.

1.2 Peptide synthesis

1.2.1 Tetrapeptide synthesis for screening

Tetrapeptides H-Gly-Gly-Phe-Leu-NH_2 and (H-Ile-Gly-Phe-Leu-NH_2), for the initial screening study, were synthesized following a protocol developed in our laboratory based on Fmoc/*t*Bu-AA-OH, DIC, OxymaPure (5 equiv. each) in GVL, and shaking for 30 min. Fmoc was removed using 20% freshly prepared 4-methylpiperidine in GVL.

1.2.2 First AA anchoring and sequence elongation using T3P®

CM resin was swelled in DMF for about 10 min. The Fmoc-amino acids (5 equiv.) were dissolved in DMF (1.0 mL/100 mg resin) and sonicated for 10 min. *N,N*-diisopropylethylamine (DIEA) (10 equiv.) was then added to the solution followed by T3P® (5 equiv.). The resulting mixture was in turn added to the previously swollen resin. At last, OxymaPure (5 equiv.) was added and the mixture was allowed to react for 1 h at 60º C. After this, acetylation step was carried out to endcap any unreacted NH_2 group with the aid of acetic anhydride-DIEA (10:20 equiv.) in DMF with respect to the amino functional of the resin. Fmoc was then removed using 20% piperidine in DMF for 1 and 7 min. Next, the sequence was elongated by repeating the previous steps until the desired peptide had been assembled.

In the case of green SPPS, ACN was used instead of DMF, and the Fmoc was removed using 20% of a freshly prepared 4-methylpiperidine in GVL.[1]

1.3 Racemization study

A total of four dipeptides were synthesized: (H-L-Ser-L-Phe-NH_2, H-L-Cys-(Acm)-L-Phe -NH_2, in addition to their DL epimers). The first amino acid was attached to the Rink-amide resin (100 mg) using Fmoc/*t*Bu-AA-OH, DIC, OxymaPure (5 equiv. each) in DMF protocol.

The second amino acid of the dipeptides was coupled following *insitu* activation protocol, as follows: Once the Fmoc of the first amino was removed Fmoc-amino acid (5 equiv.) dissolved in DMF (1.0 mL) and added to the preloaded resin, followed by DIEA (10 equiv), and OxymaPure (5 equiv.). At last, T3P (5 equiv.) added. The mixture was allowed to react for 1h at RT. Finally, the peptide resin was washed twice with DMF. Fmoc was removed, the resin washed with DMF then with DCM, and the peptides cleaved from the resin as described in the corresponding section.

1.4 Cleavage of peptides from the resin

The synthesized peptides were cleaved from the resin using a cocktail solution of trifluoroacetic acid (TFA)-triisopropylsilane (TIS)-H_2O (95:2.5:2.5), and then shaken for 1 h at RT. Next, they were precipitated by chilled ether, dissolved in H_2O-ACN (1:1), and then analyzed by HPLC.

1.5 Computational Study

Computational studies were carried out using BIOVIA Materials Studio 2020, Accelrys Inc. (San Diego, CA, USA).

All molecules were generated using simple atomistic representation in the materials studio interface. In this regard, T3P® was reacted once with AA (main reaction) and once with OxymaPure (expected side reaction). Furthermore, the mixed anhydride of Fmoc-Leu-OH-T3P® was reacted once with H-Leu-OH (main reaction) and once with dimethylamine (DMA) impurity (expected side reaction).

All the assemblies were energy-minimized, and the geometry was optimized using the “Forcite module” when considering the universal Forcefield and smart algorithm.

The transition state search was simulated using “$DMol^3$ linear synchronous transit (LST) and quadratic synchronous transit (QST) tools”. A path between the reactants and the products was established via the reaction preview function, where all the atoms in their structures were paired.

Subsequently, under the setup tab, the task was set to “TS search”, when considering Hybrid/B3LYP functional, where the basis set was fixed to “DND”. Complete LST/QST was selected. Under the electronic tab, “use smearing” was activated to optimize convergence and set to 0.05 for the T3P® reactions and 0.01 Ha for the Fmoc-Leu-O-T3P® mixed anhydride reactions. After locating the transition state, it was consequently confirmed using DMol3 module as well, which uses the nudged elastic band (NEB) method for minimum energy path calculations. The process ends up with establishing the intrinsic reaction coordinate (IRC). The optimization is done through two phases, namely (macroiteration and microiteration). Under the setup tab, the task was changed to “TS confirmation”, and keeping the functional to match those that were selected in the TS search settings.

1.6 References

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2. Chromatograms, mass spectra, computational figures

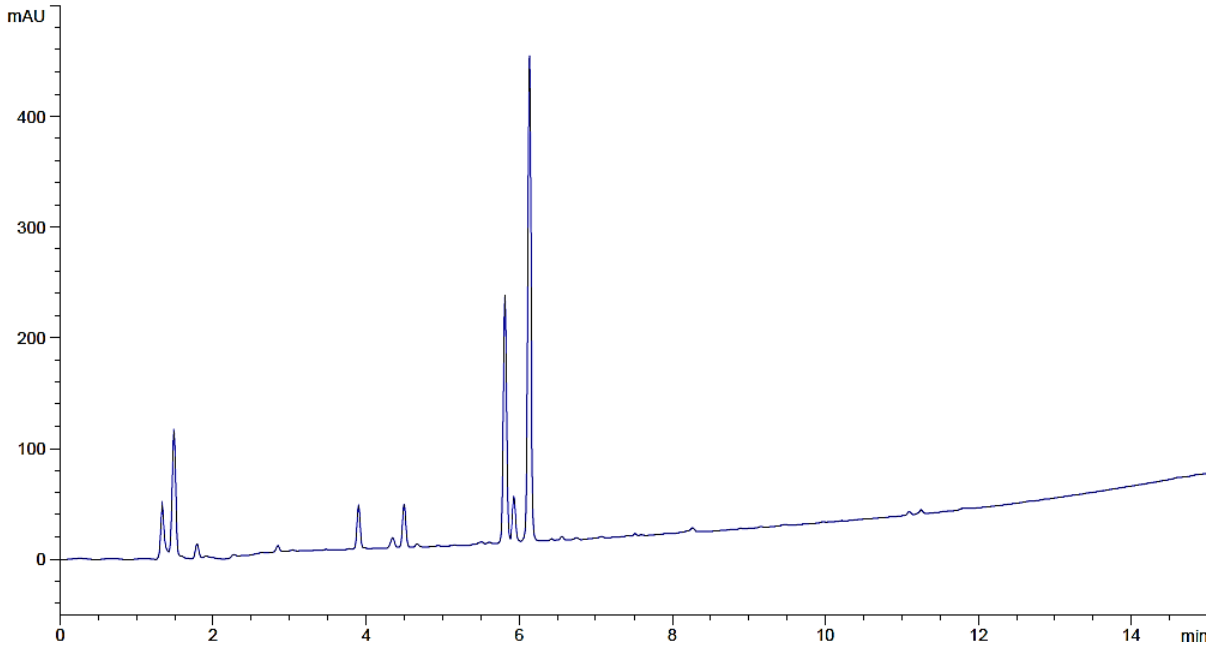

Figure S1. Leu-enkephalin pentapeptide on CM resin. AA-DIEA-T3P in ACN (3:6:4). 5−95% gradient elution in 15 min. λ = 220 nm. Mobile phase A: 0.1% TFA in H_2O; mobile phase B: 0.1% TFA in ACN; Phenomenex Luna C_{18} (3.6 µm, 4.6 × 150 mm) column.

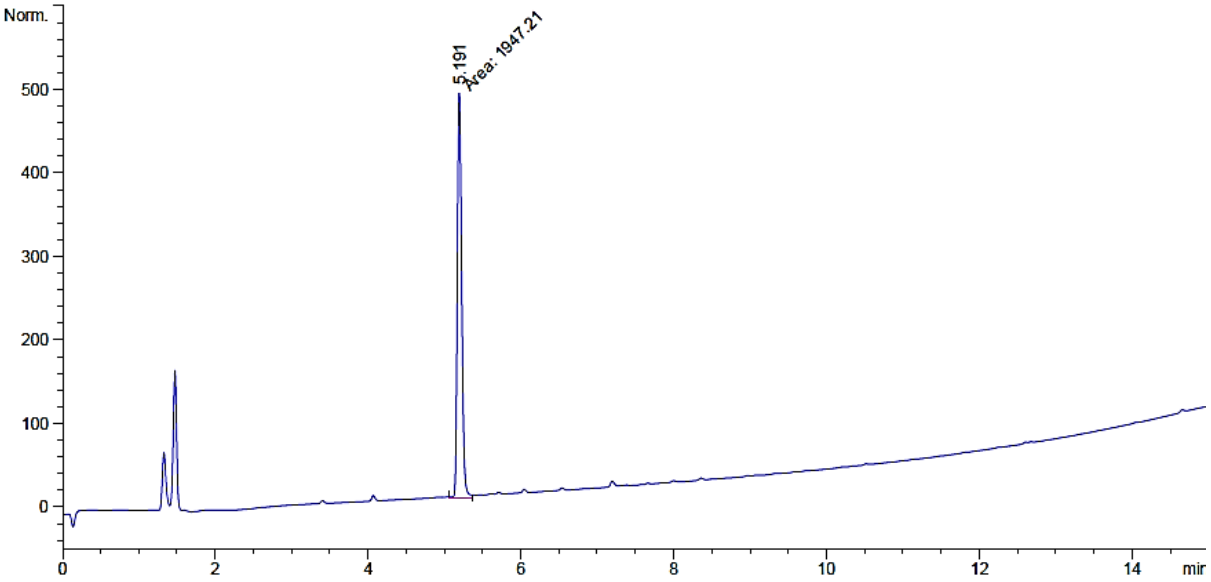

Figure S2. H-Gly-Gly-Phe-Leu-NH_2 tetrapeptide on CM resin. DIC/OxymaPure protocol was used. See legend of Figure S1 for chromatographic conditions.

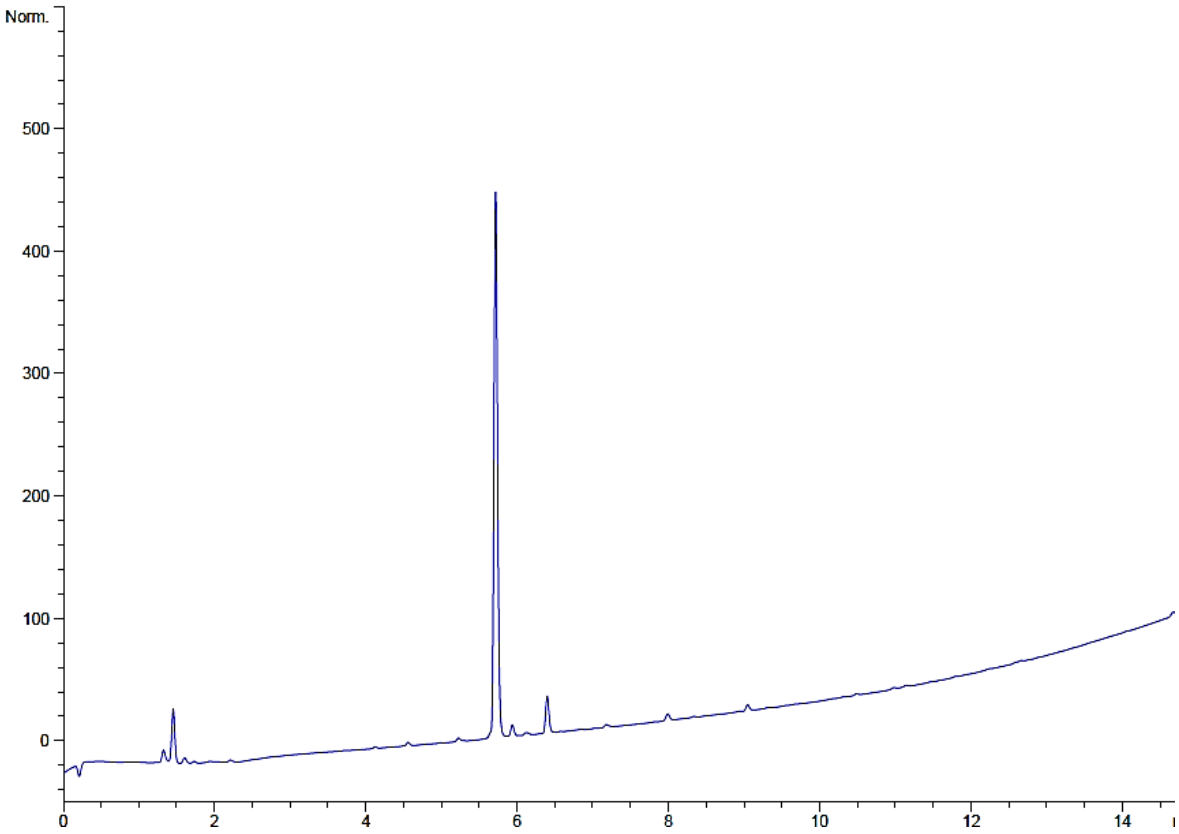

Figure S3. Leu-enkephalin pentapeptide on CM resin. AA-DIEA-T3P(ACN)-OxymaPure (6:6:4:6). See legend of Figure S1 for chromatographic conditions.

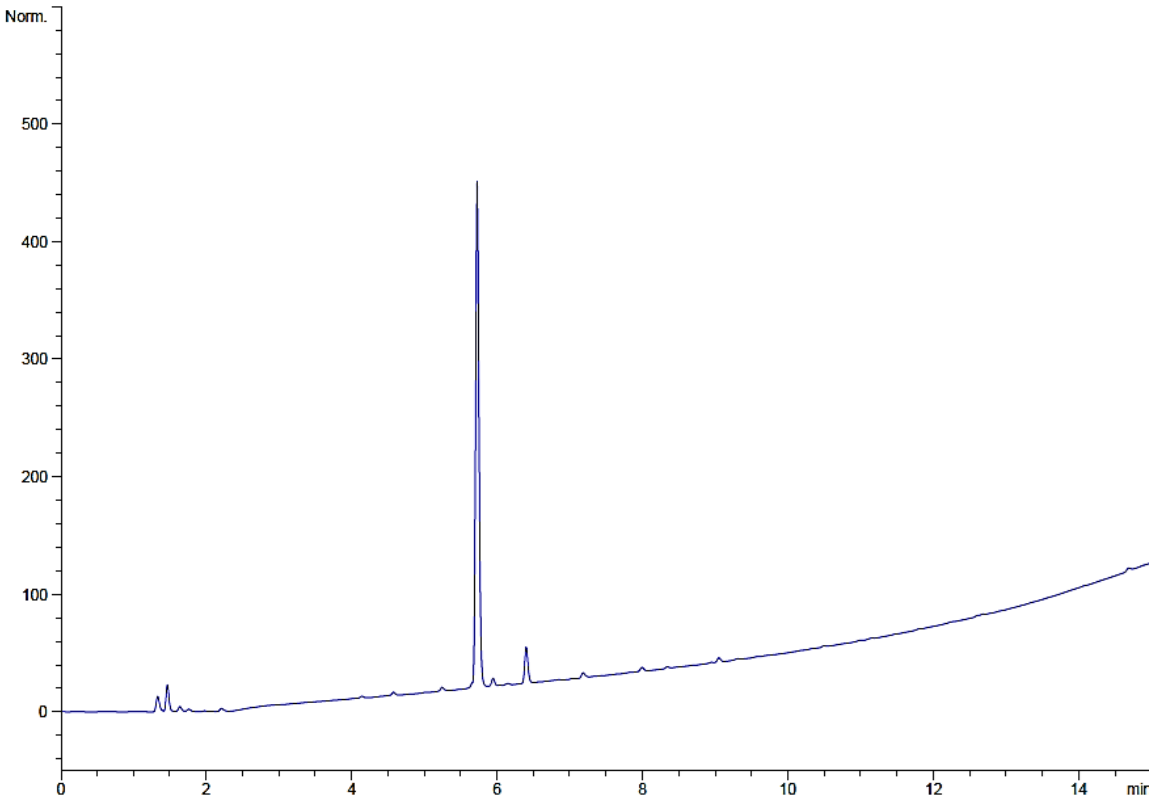

Figure S4. Leu-enkephalin pentapeptide on CM resin. AA-DIEA-T3P(ACN) (6:6:4). See legend of Figure S1 for chromatographic conditions.

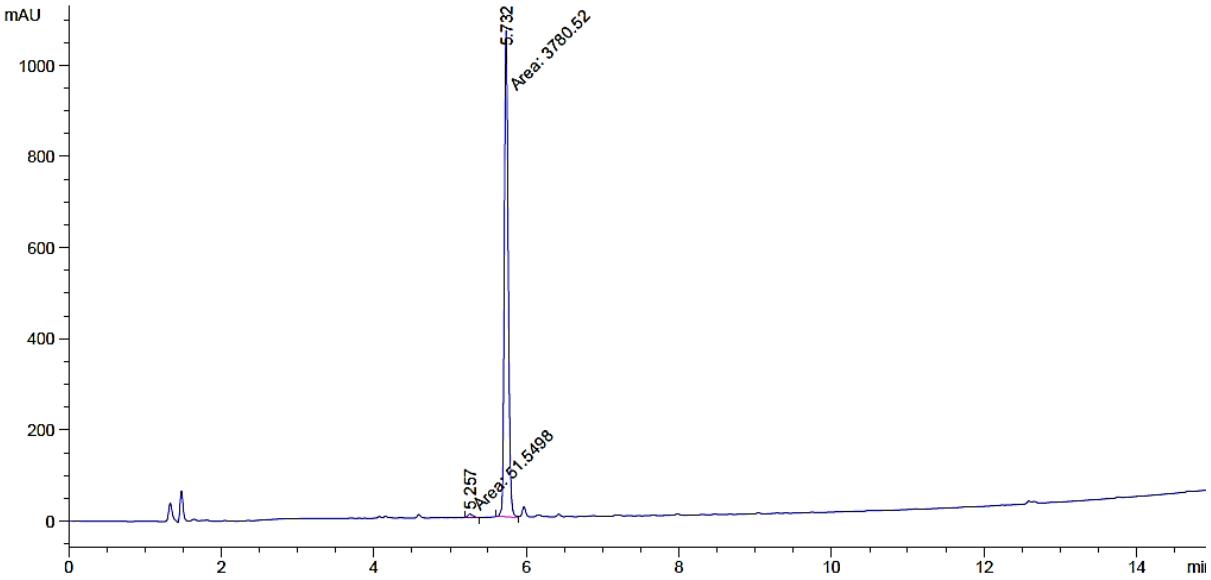

Figure S5. Leu-enkephalin pentapeptide on CM resin. AA-DIEA-T3P(ACN) (3:6:4). See legend of Figure S1 for chromatographic conditions.

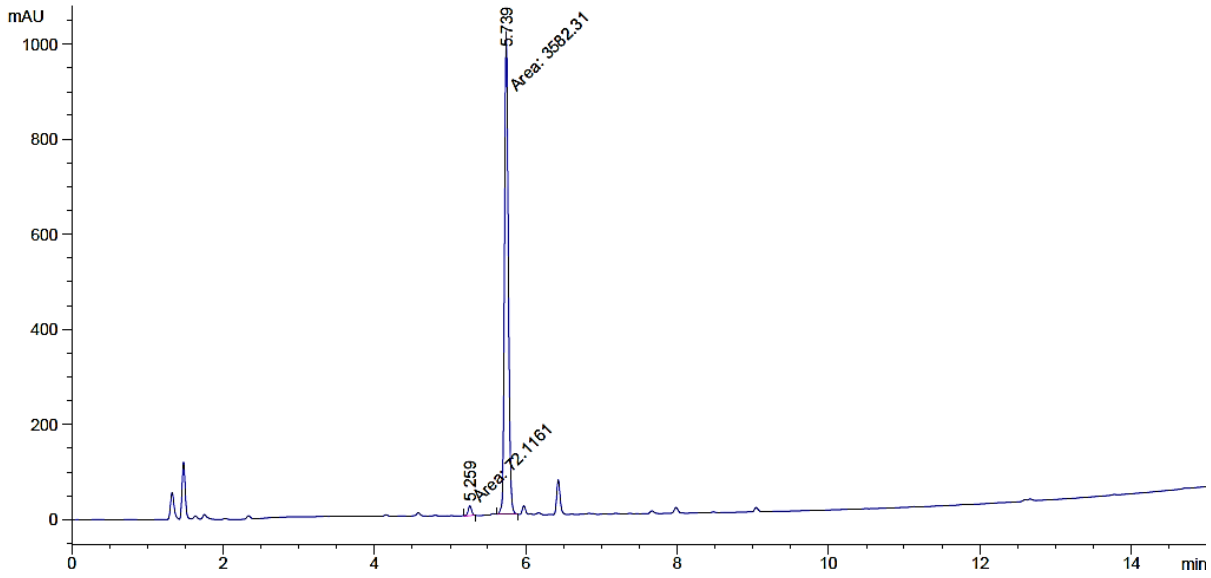

Figure S6. Leu-enkephalin pentapeptide on CM resin. AA-DIEA-T3P(ACN)-OxymaPure (6:12:4:6). See legend of Figure S1 for chromatographic conditions.

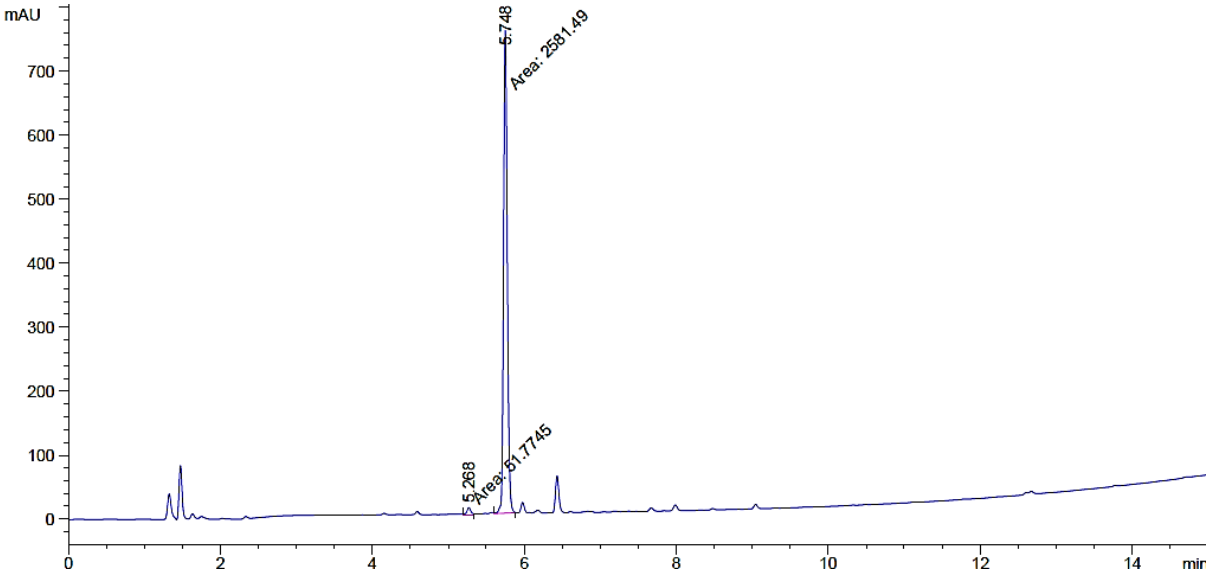

Figure S7. Leu-enkephalin pentapeptide on CM resin. AA-DIEA-T3P(ACN) (6:12:4). See legend of Figure S1 for chromatographic conditions.

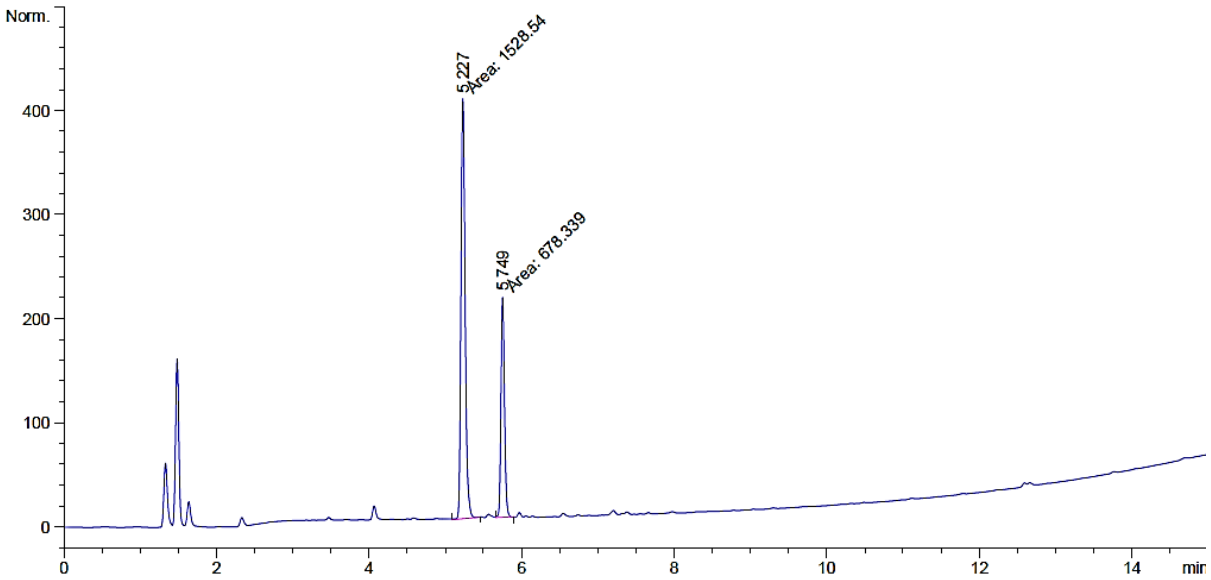

Figure S8. Leu-enkephalin pentapeptide on CM resin. AA-K-Oxyma-T3P(ACN) (6:6:4). See legend of Figure S1 for chromatographic conditions.

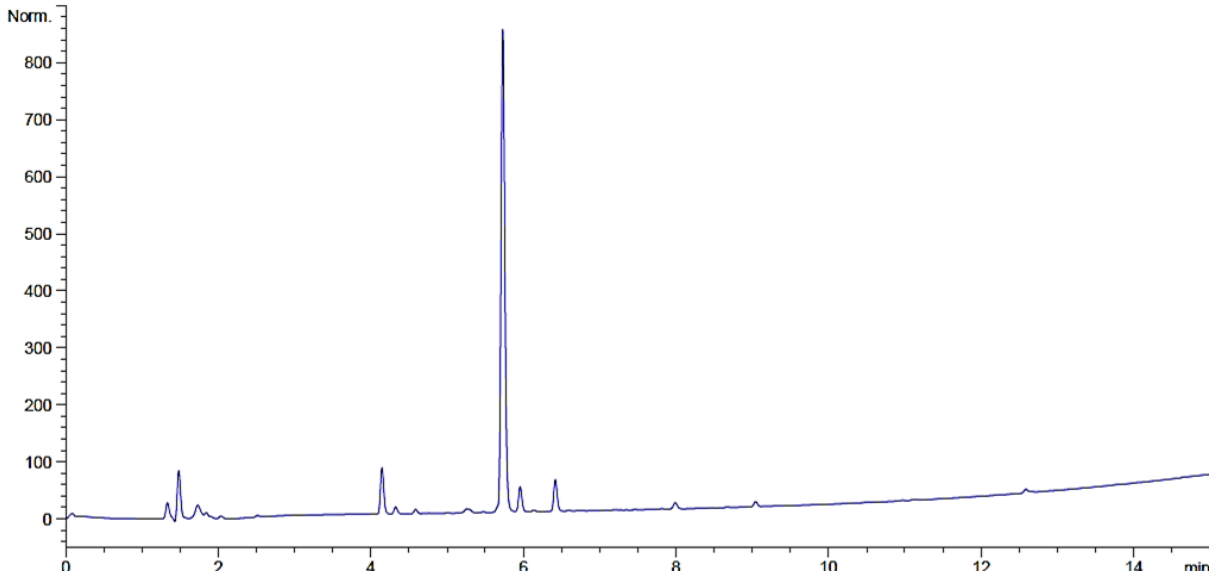

Figure S9. Leu-enkephalin pentapeptide on CM resin. DIC/OxymaPure protocol was used for the first amino acid incorporation, then AA-DIEA-T3P(ACN)-OxymaPure (6:6:6:6) for the rest of AAs. See legend of Figure S1 for chromatographic conditions.

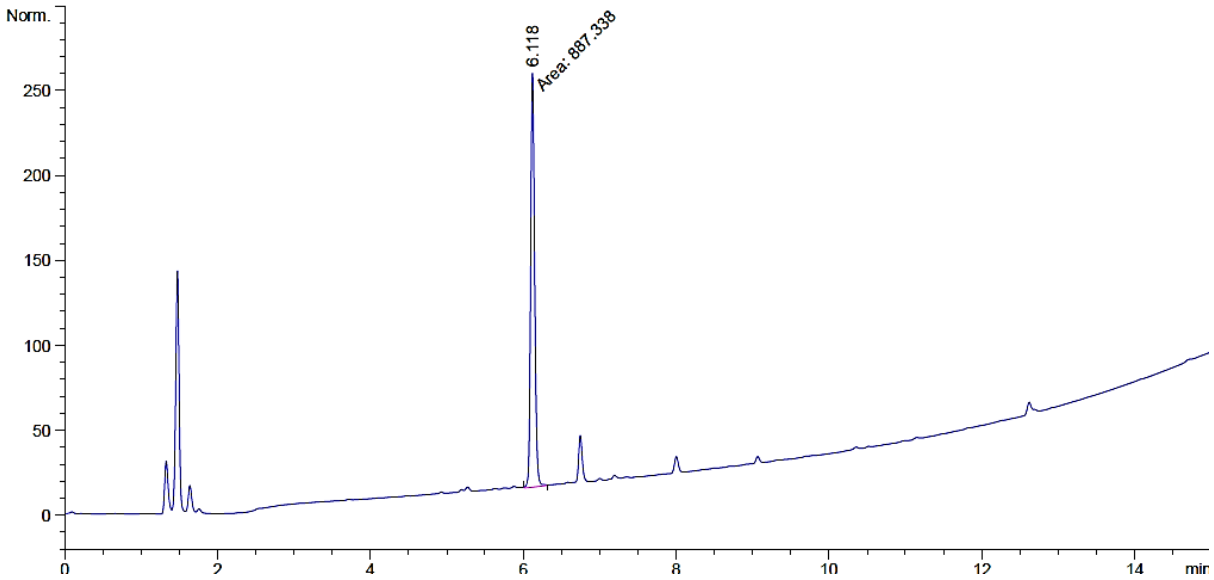

Figure S10. H-Ile-Gly-Phe-Leu-NH_2 tetrapeptide on CM resin. DIC/OxymaPure protocol was used. See legend of Figure S1 for chromatographic conditions.

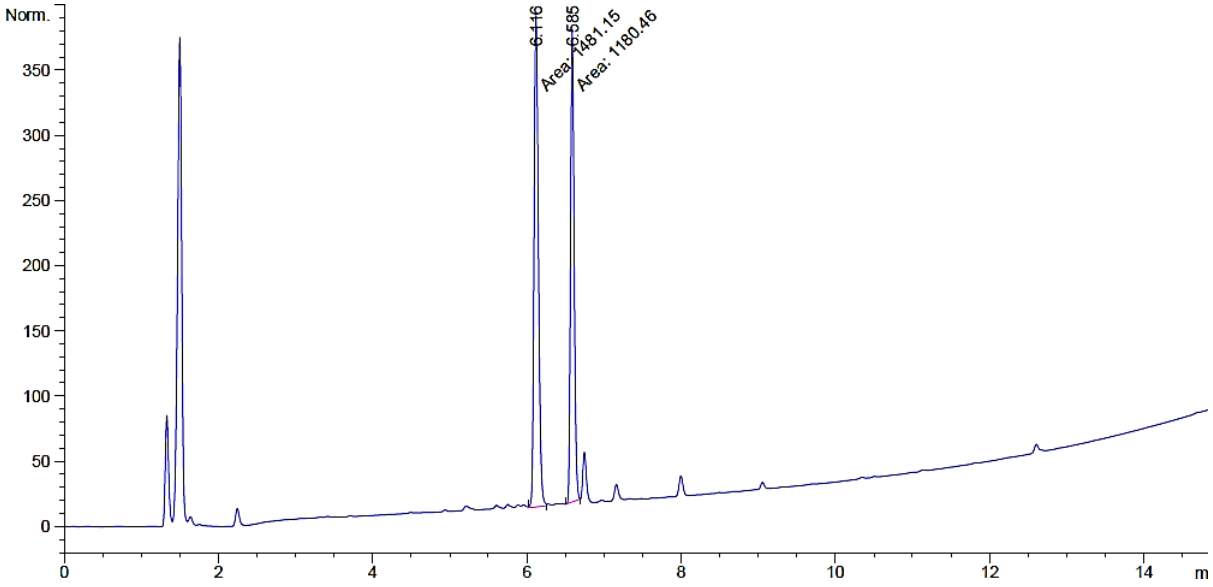

Figure S11. H-Tyr-Ile-Gly-Phe-Leu-NH_2 pentapeptide on CM resin. AA-DIEA-T3P(ACN)-OxymaPure (5:5:5:5). See legend of Figure S1 for chromatographic conditions.

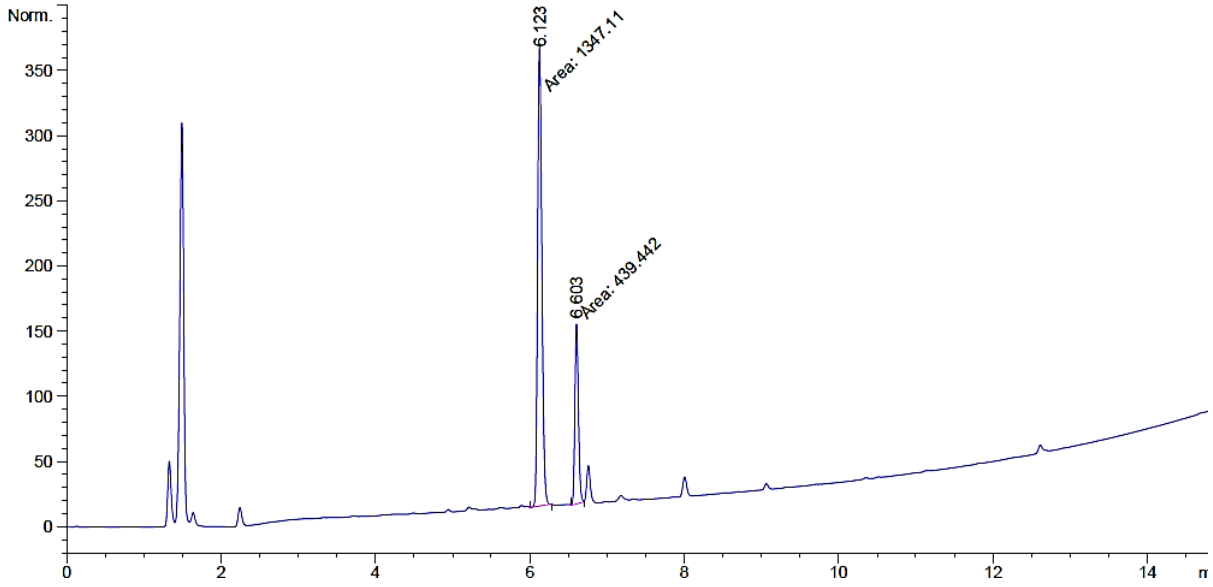

Figure S12. H-Tyr-Ile-Gly-Phe-Leu-NH_2 pentapeptide on CM resin. AA-OxymaPure-DIEA-T3P(ACN) (5:5:5:5). See legend of Figure S1 for chromatographic conditions.

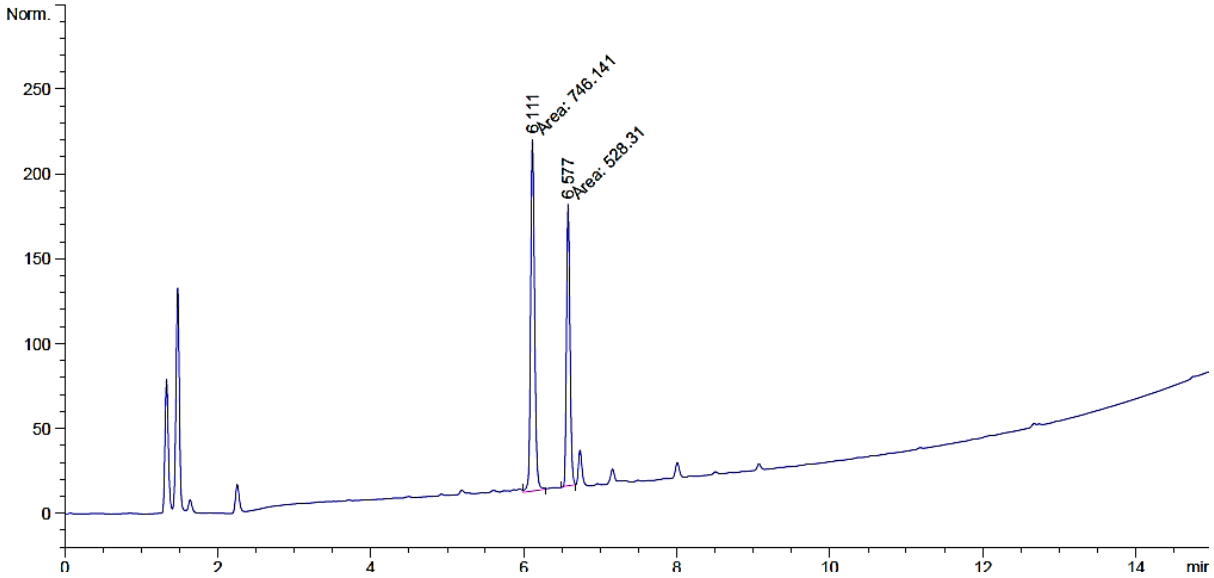

Figure S13. H-Tyr-Ile-Gly-Phe-Leu-NH_2 pentapeptide on CM resin. AA-DIEA-T3P(ACN) (5:5:5). See legend of Figure S1 for chromatographic conditions.

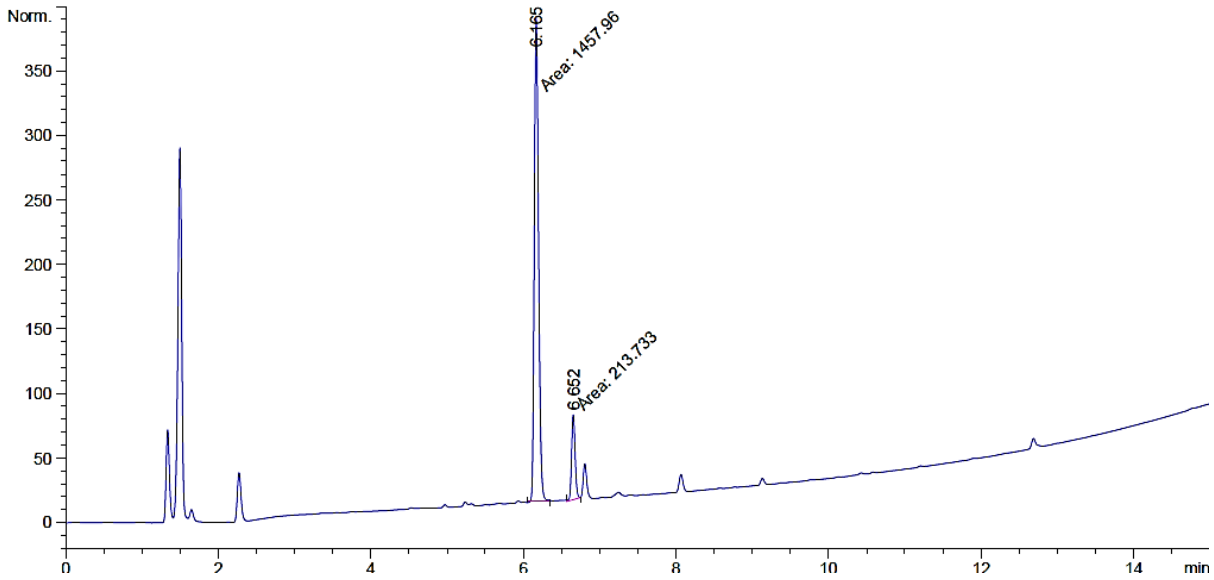

Figure S14. H-Tyr-Ile-Gly-Phe-Leu-NH_2 pentapeptide on CM resin. AA-K-Oxyma-T3P(ACN) (5:5:5:5). See legend of Figure S1 for chromatographic conditions.

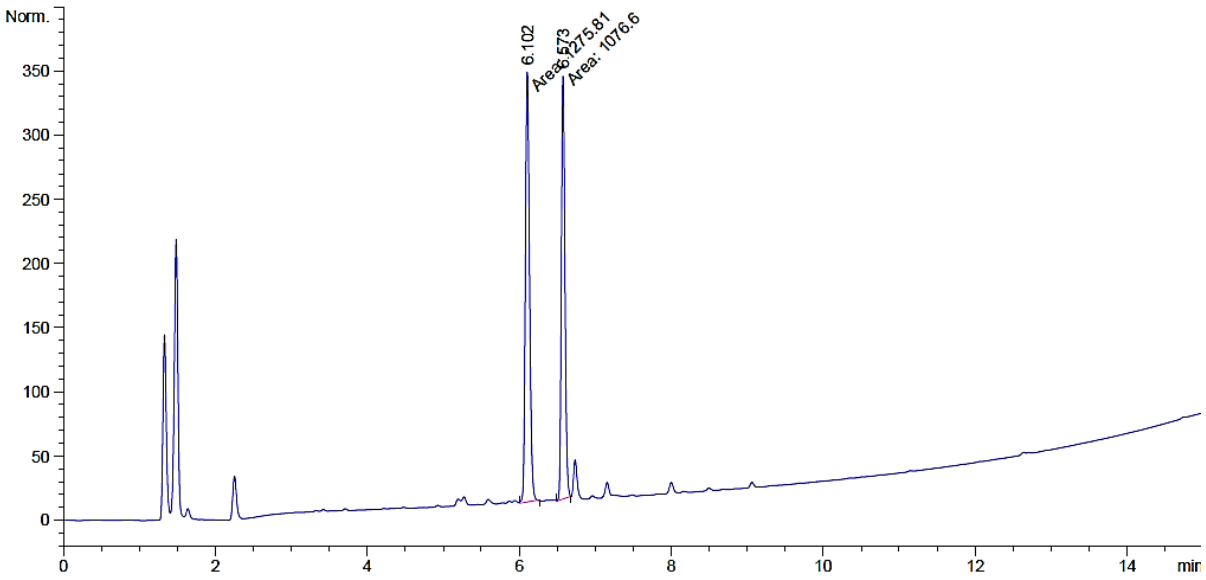

Figure S15. H-Tyr-Ile-Gly-Phe-Leu-NH_2 pentapeptide on CM resin. AA-DIEA-T3P(ACN)-K-Oxyma (5:5:5:5). See legend of Figure S1 for chromatographic conditions.

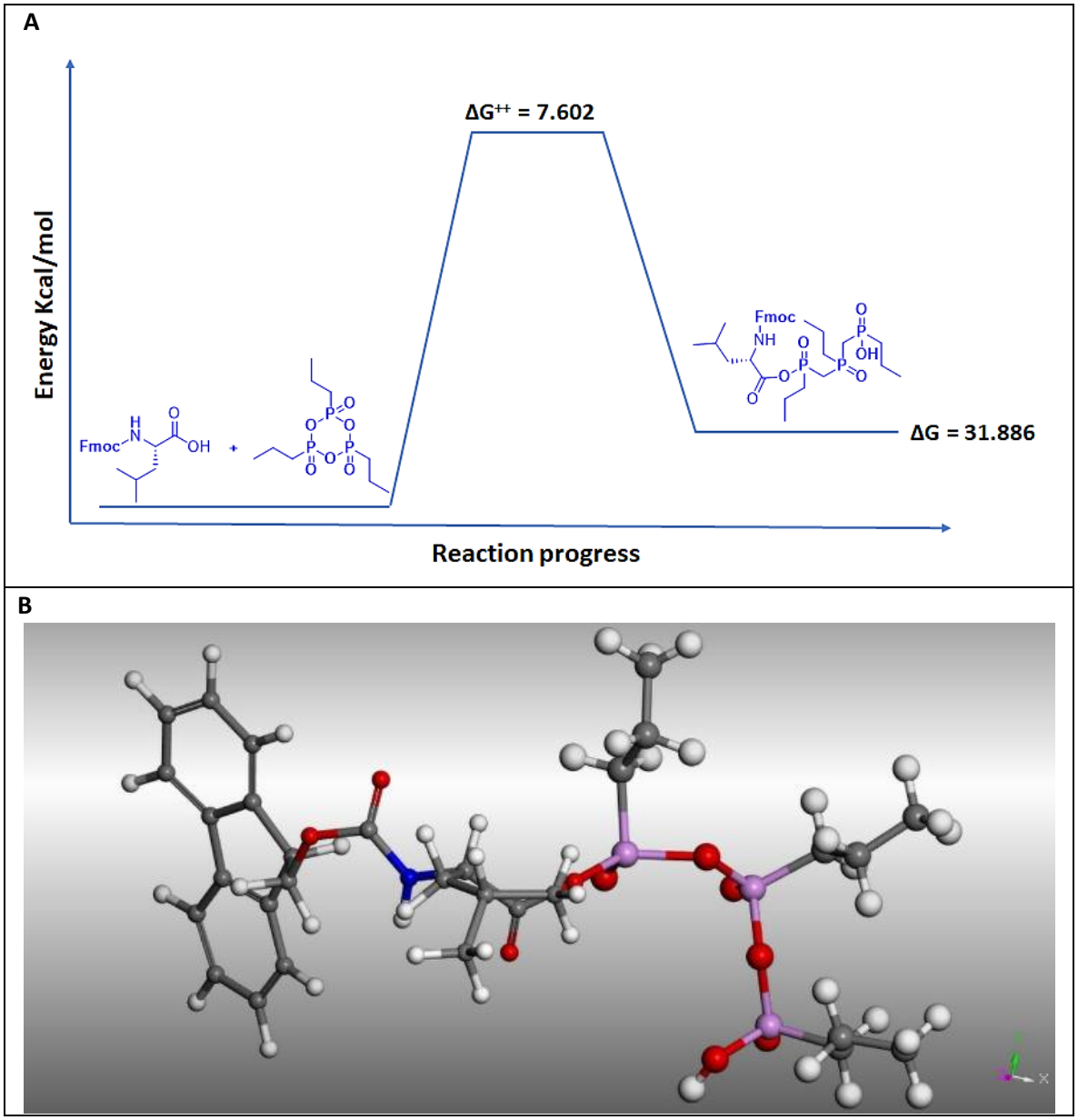

Figure S16. A. Reaction pathway showing energy of reaction (ΔG) and energy of barrier (ΔG^{++}) for the main reaction. B. Transition state structure of the main reaction.

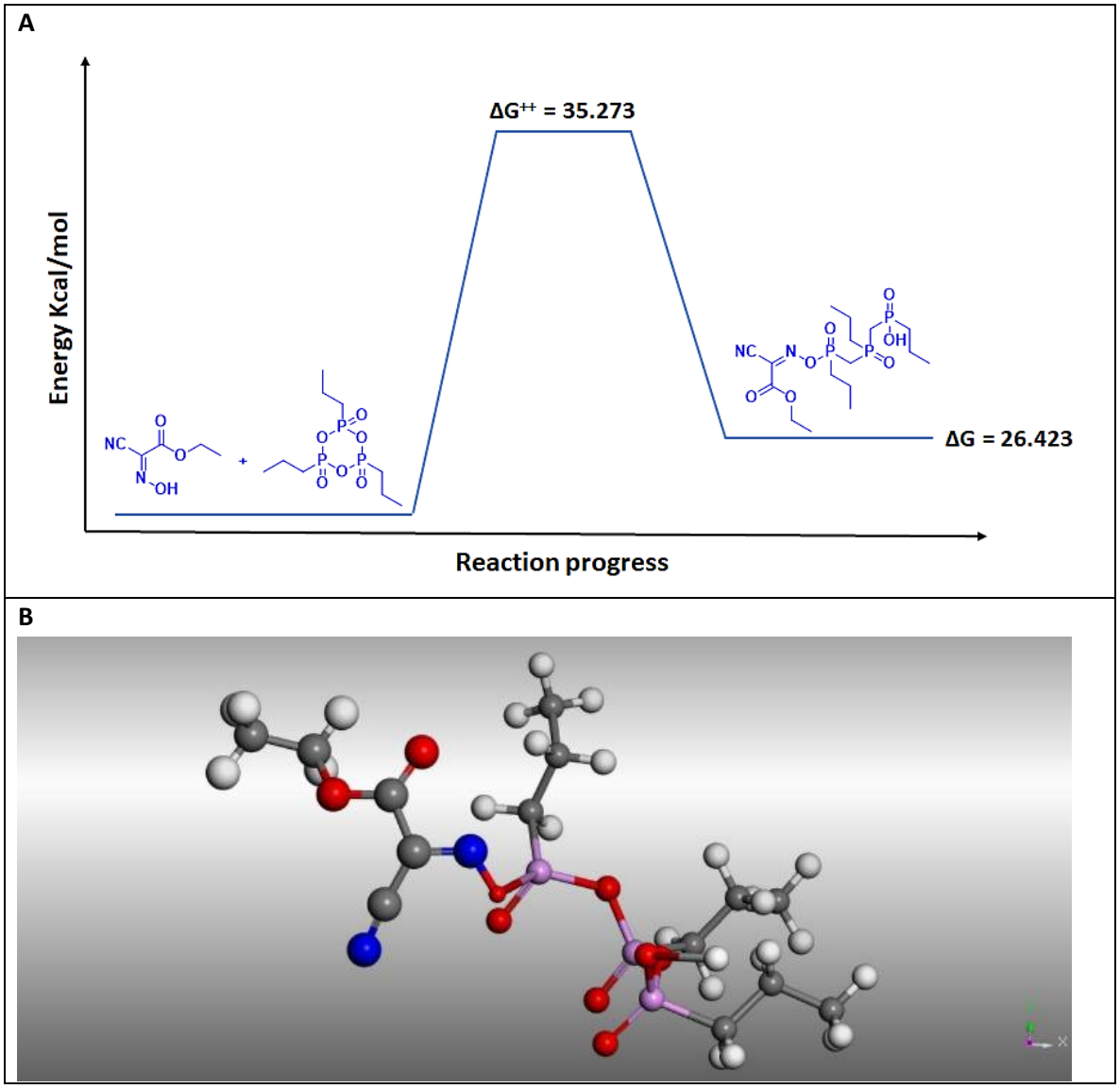

Figure S17. A. Reaction pathway showing energy of reaction (ΔG) and energy of barrier (ΔG^{++}) for the OxymaPure side reaction. B. Transition state structure of the OxymaPure side reaction.

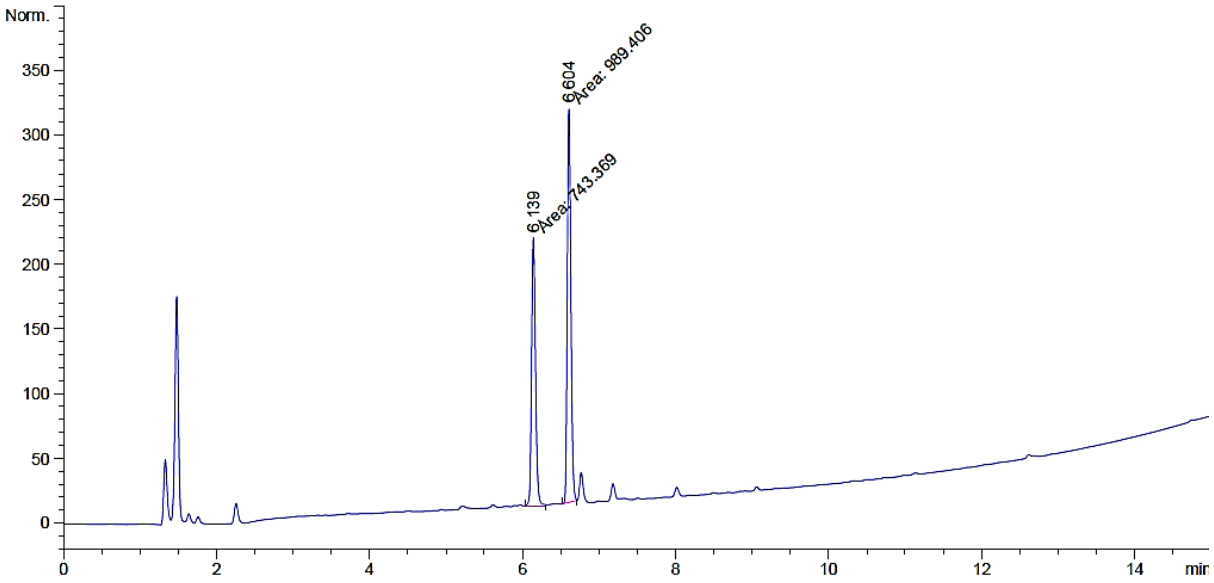

Figure S18. H-Tyr-Ile-Gly-Phe-Leu-NH_2 pentapeptide on CM resin. AA-DIEA-T3P(ACN)-OxymaPure (5:5:10:5). See legend of Figure S1 for chromatographic conditions.

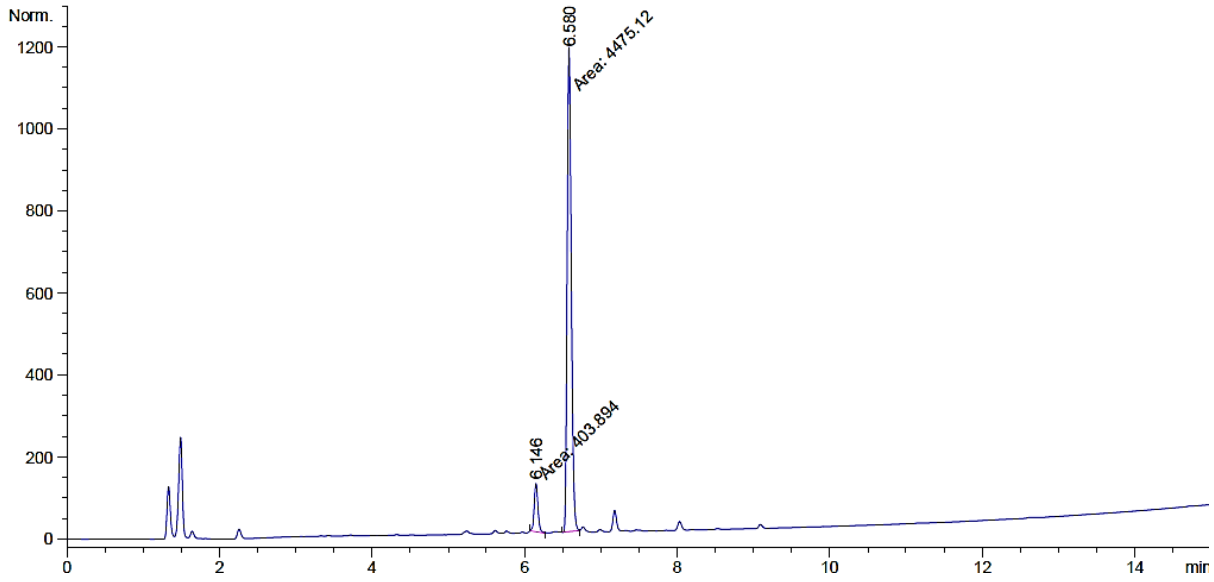

Figure S19. H-Tyr-Ile-Gly-Phe-Leu-NH_2 pentapeptide on CM resin. AA-DIEA-T3P(ACN)-OxymaPure (5:10:5:5). See legend of Figure S1 for chromatographic conditions.

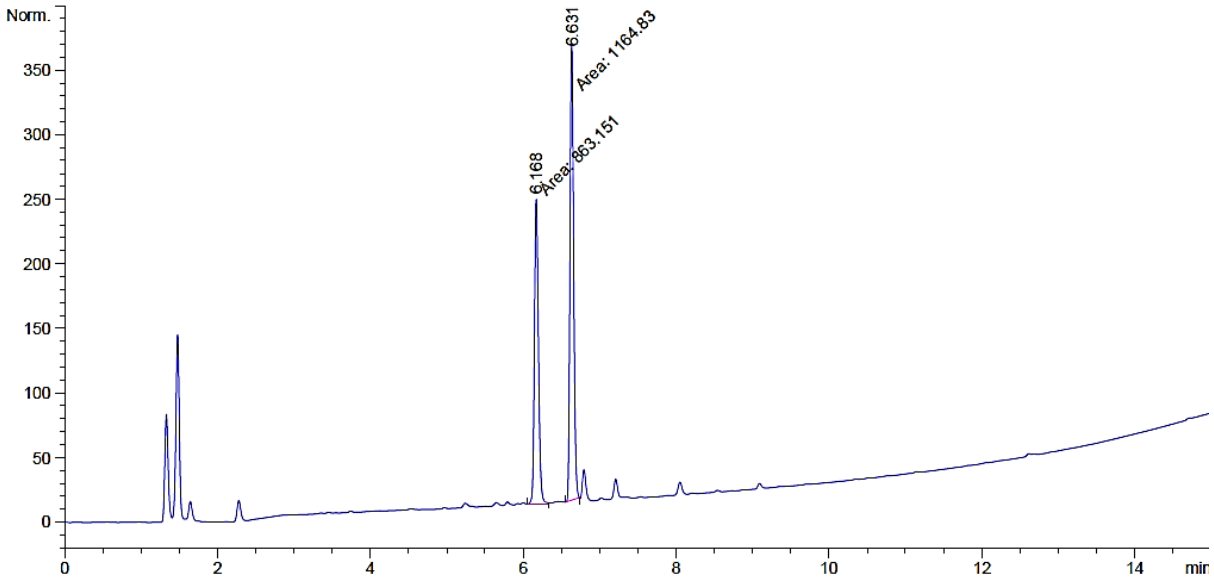

Figure S20. H-Tyr-Ile-Gly-Phe-Leu-NH_2 pentapeptide on CM resin. AA-DIEA-T3P(ACN)-OxymaPure (5:10:10:5). See legend of Figure S1 for chromatographic conditions.

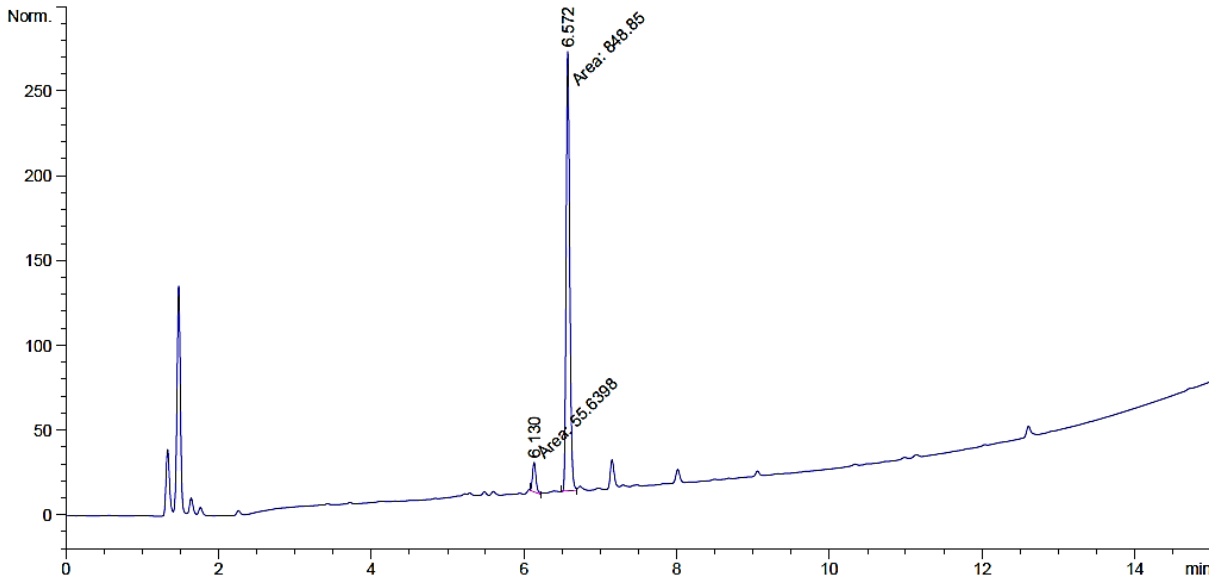

Figure S21. H-Tyr-Ile-Gly-Phe-Leu-NH_2 pentapeptide on CM resin. AA-DIEA-T3P(2-MeTHF)-OxymaPure (5:10:5:5). See legend of Figure S1 for chromatographic conditions.

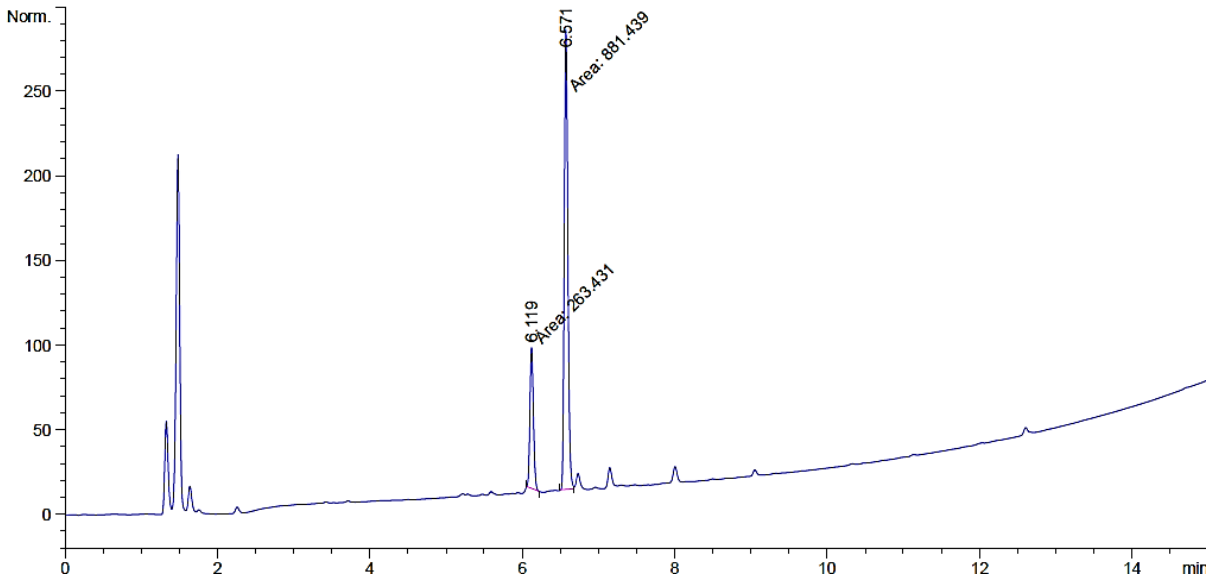

Figure S22. H-Tyr-Ile-Gly-Phe-Leu-NH_2 pentapeptide on CM resin. AA-DIEA-T3P(DMF)-OxymaPure (5:10:5:5). See legend of Figure S1 for chromatographic conditions.

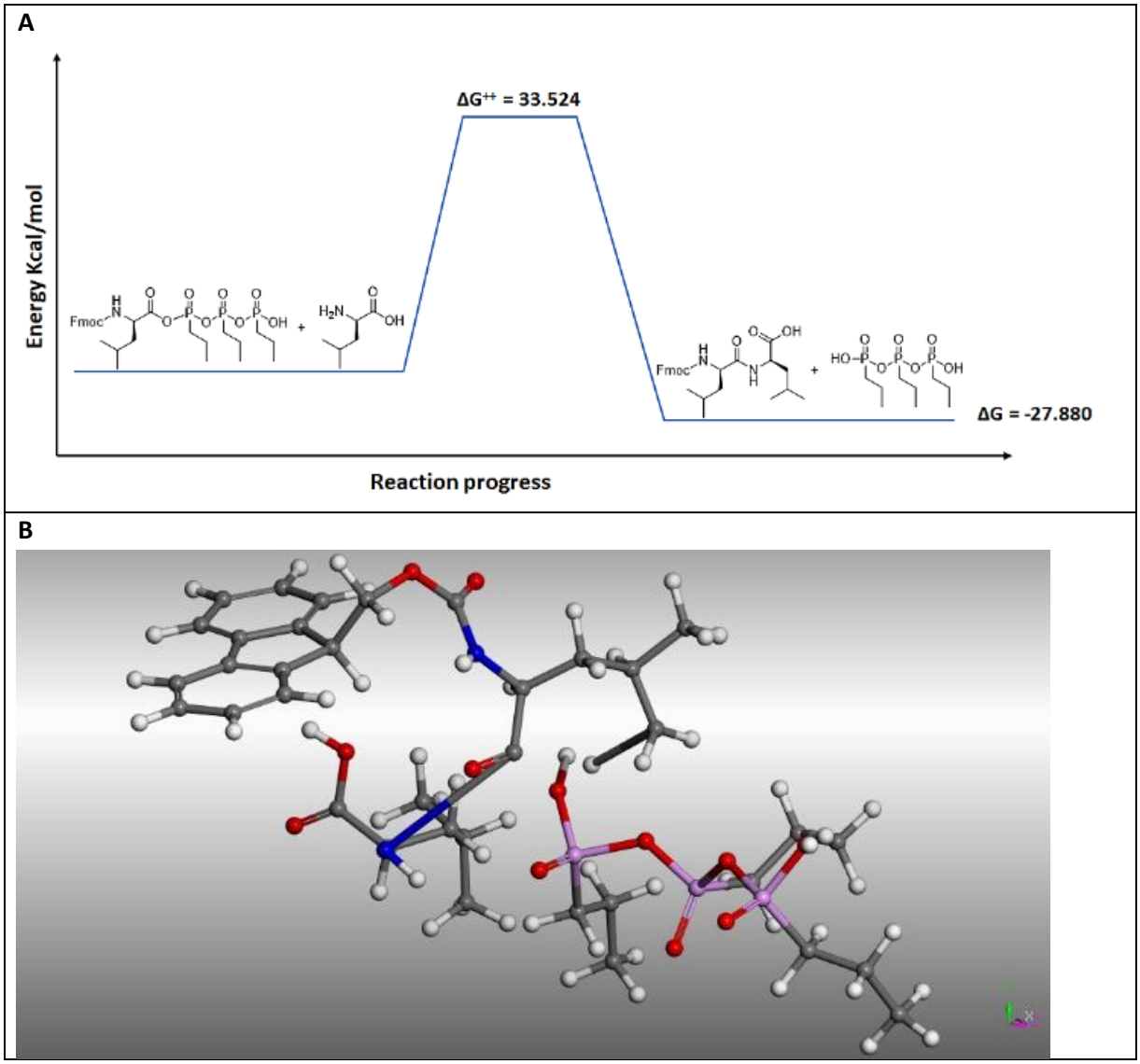

Figure S23. A. Reaction pathway showing energy of reaction (ΔG) and energy of barrier (ΔG^{++}) for the main reaction. B. Transition state structure of the main reaction.

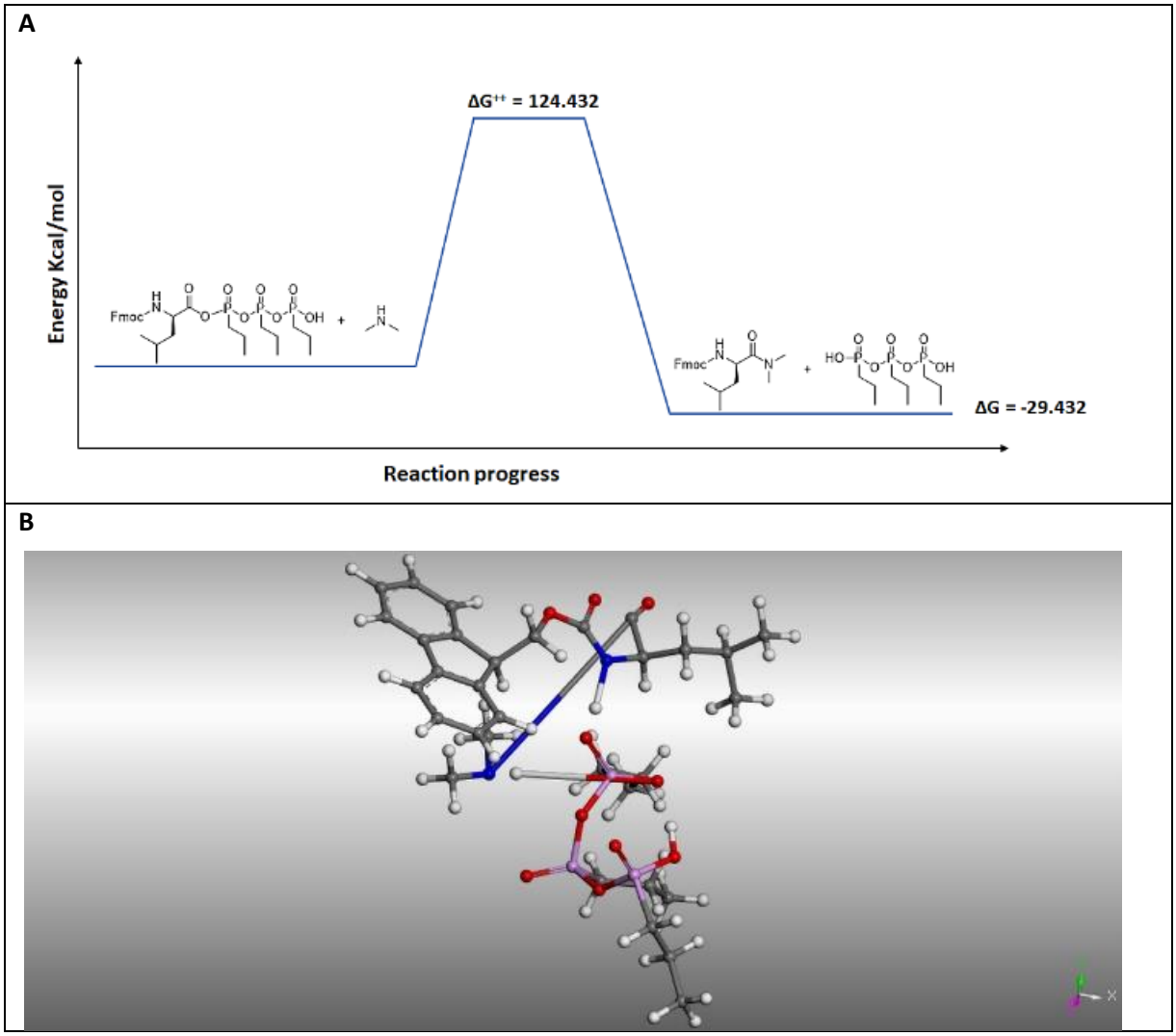

Figure S24. A. Reaction pathway showing energy of reaction (ΔG) and energy of barrier (ΔG^{++}) for the DMA side reaction. B. Transition state structure of the DMA side reaction.

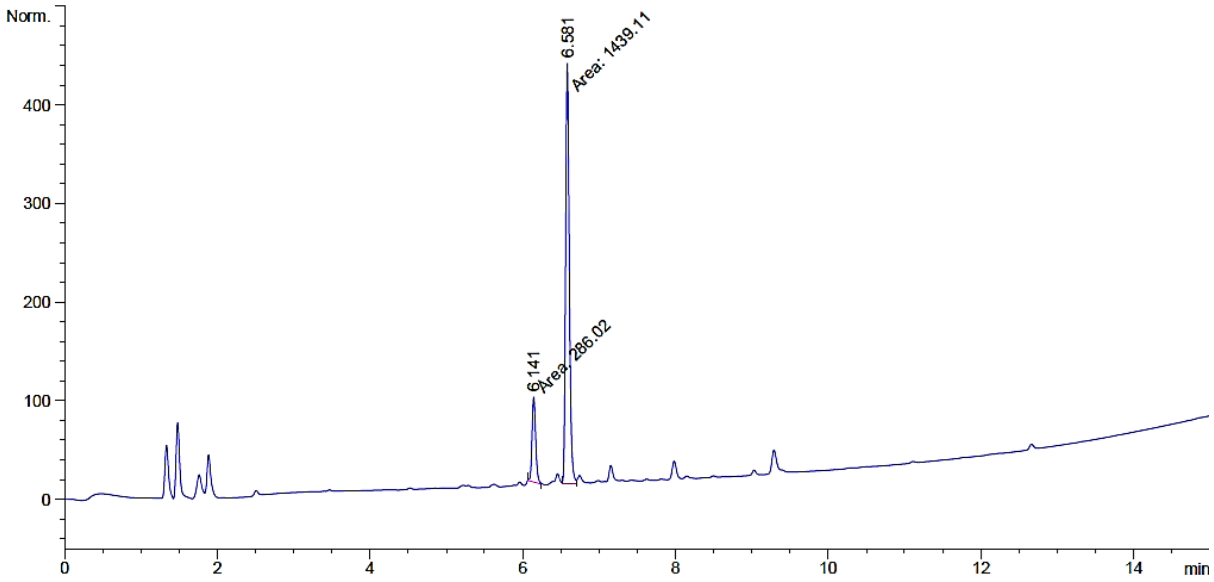

Figure S25. H-Tyr-Ile-Gly-Phe-Leu-NH_2 pentapeptide on CM resin. AA-DIEA-T3P(DMF)-OxymaPure (5:10:5:5). Regenerated T3P(DMF) reagent. See legend of Figure S1 for chromatographic conditions.

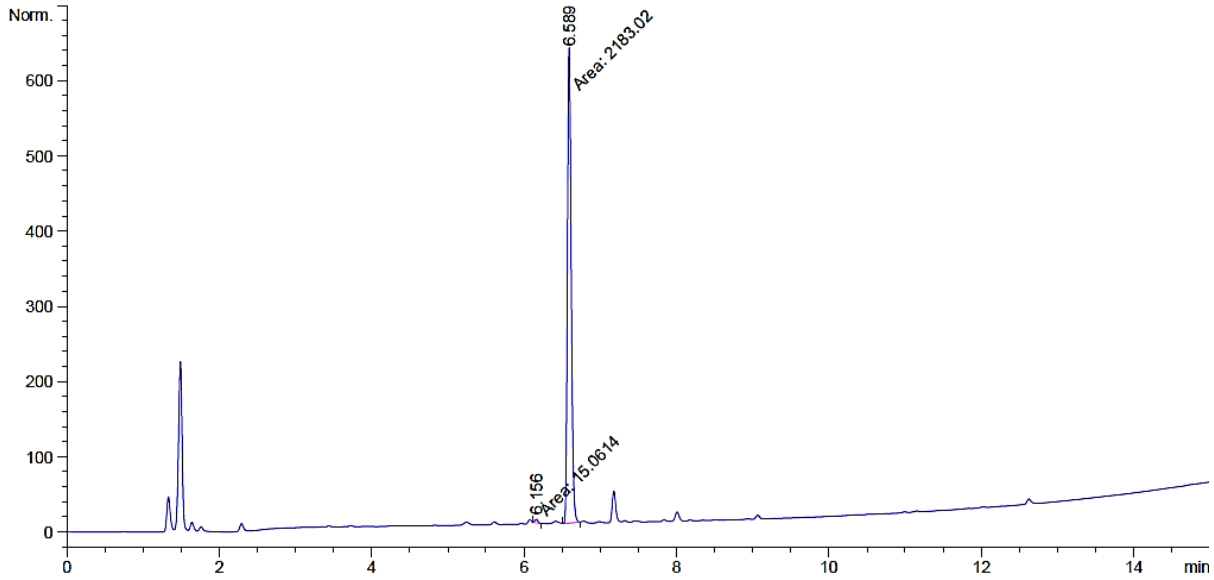

Figure S26. H-Tyr-Ile-Gly-Phe-Leu-NH_2 pentapeptide on CM resin. AA-DIEA-T3P(2-MeTHF)-OxymaPure (5:10:5:5) at 40^{o} C. See legend of Figure S1 for chromatographic conditions.

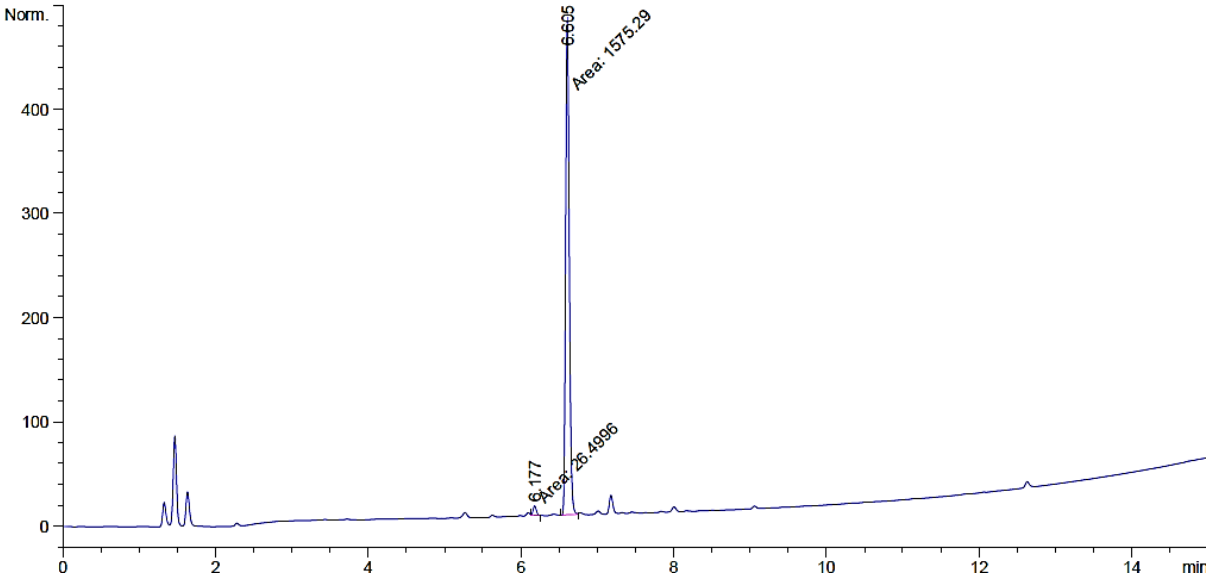

Figure S27. H-Tyr-Ile-Gly-Phe-Leu-NH_2 pentapeptide on CM resin. AA-DIEA-T3P(2-MeTHF)-OxymaPure (5:10:5:5) at 60^{o} C. See legend of Figure S1 for chromatographic conditions.

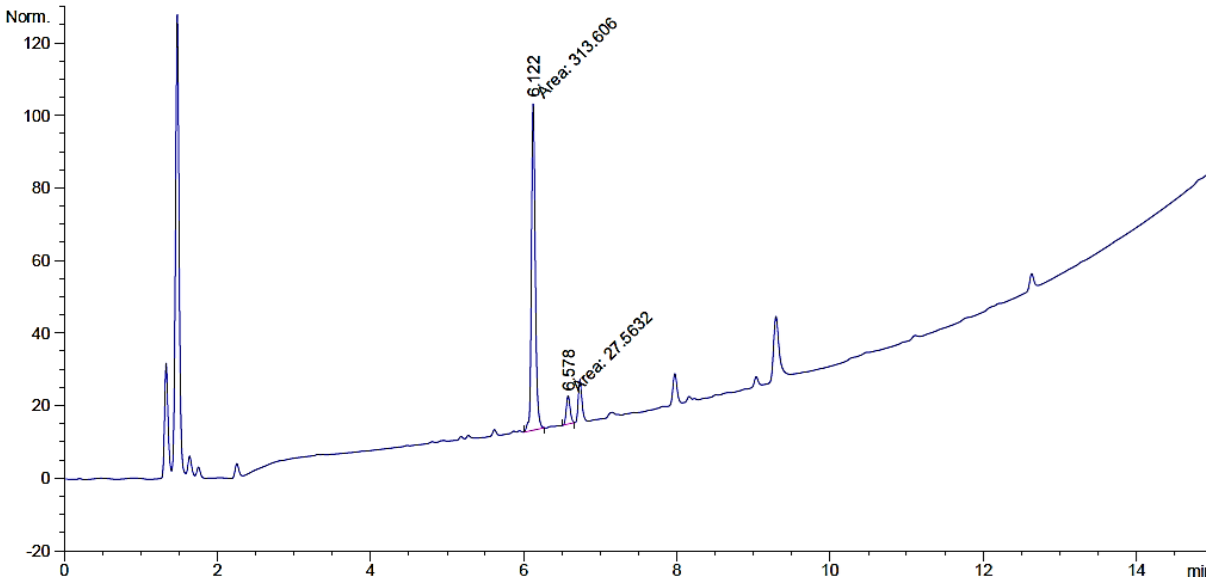

Figure S28. H-Tyr-Ile-Gly-Phe-Leu-NH_2 pentapeptide on CM resin. AA-NMI-T3P(2-MeTHF) (5:10:5). See legend of Figure S1 for chromatographic conditions.

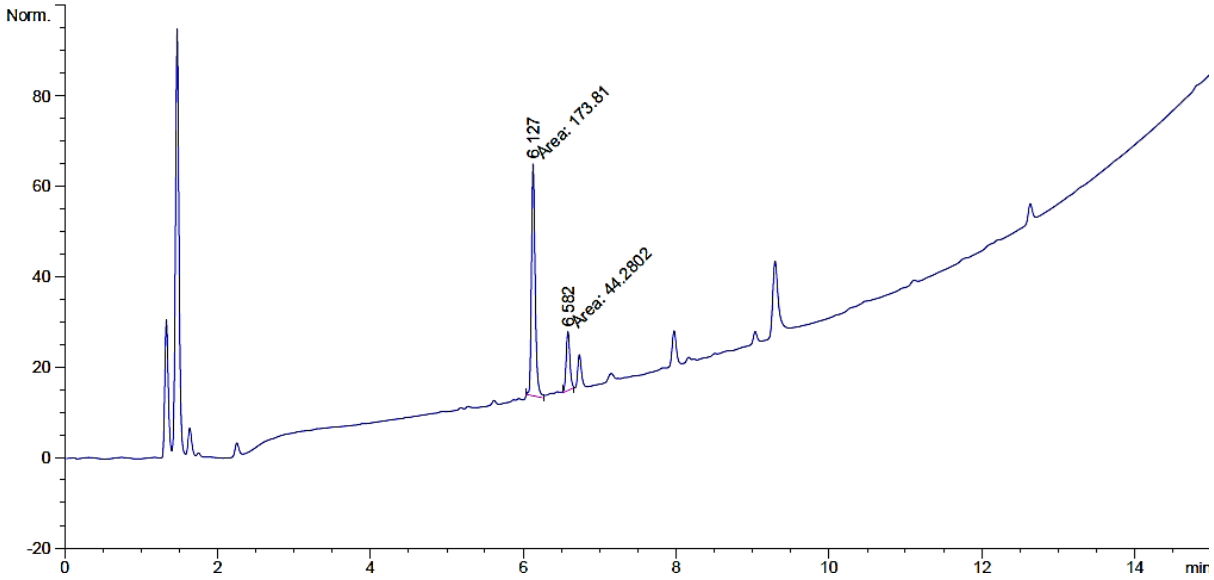

Figure S29. H-Tyr-Ile-Gly-Phe-Leu-NH_2 pentapeptide on CM resin. AA-NMI-T3P(2-MeTHF) (5:20:5). See legend of Figure S1 for chromatographic conditions.

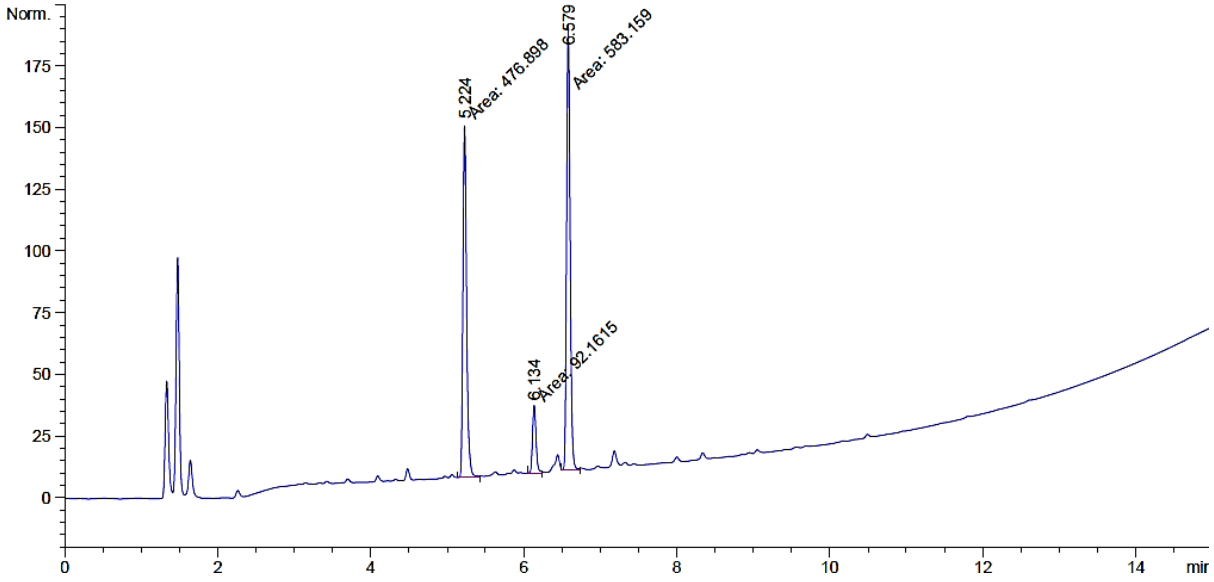

Figure S30. Full synthesis of H-Tyr-Ile-Gly-Phe-Leu-NH_2 pentapeptide on CM resin. AA-DIEA-T3P(2-MeTHF)-OxymaPure (5:10:5:5) at 40^{o} C. See legend of Figure S1 for chromatographic conditions.

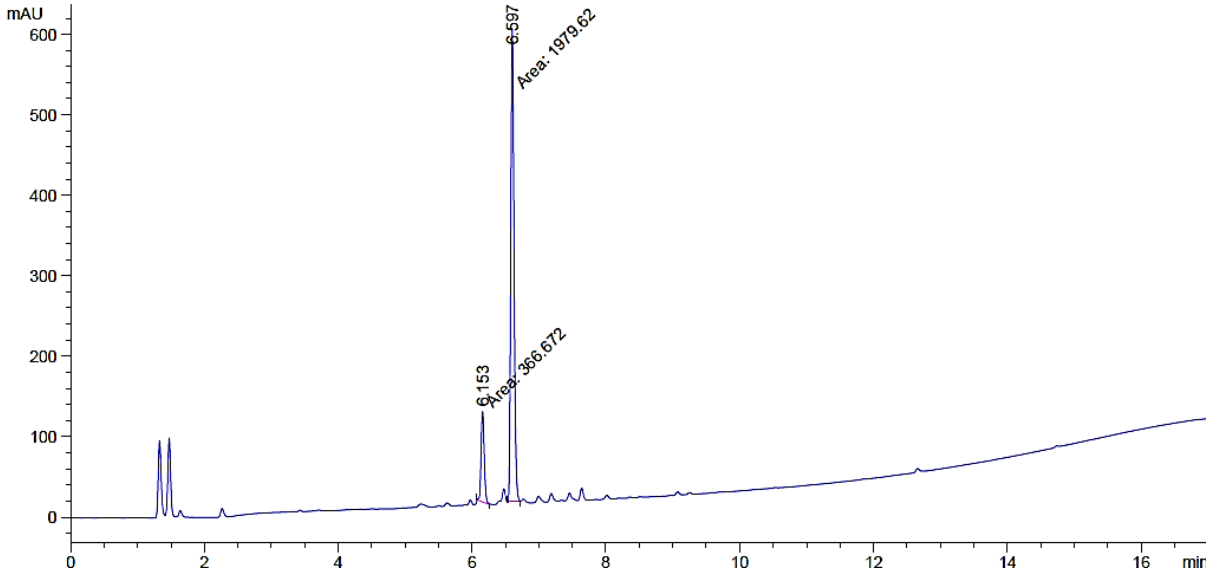

Figure S31. H-Tyr-Ile-Gly-Phe-Leu-NH_2 pentapeptide on CM resin. AA-DIEA-T3P(2-MeTHF)-OxymaPure (5:10:5:5). Solvent: GVL. See legend of Figure S1 for chromatographic conditions.

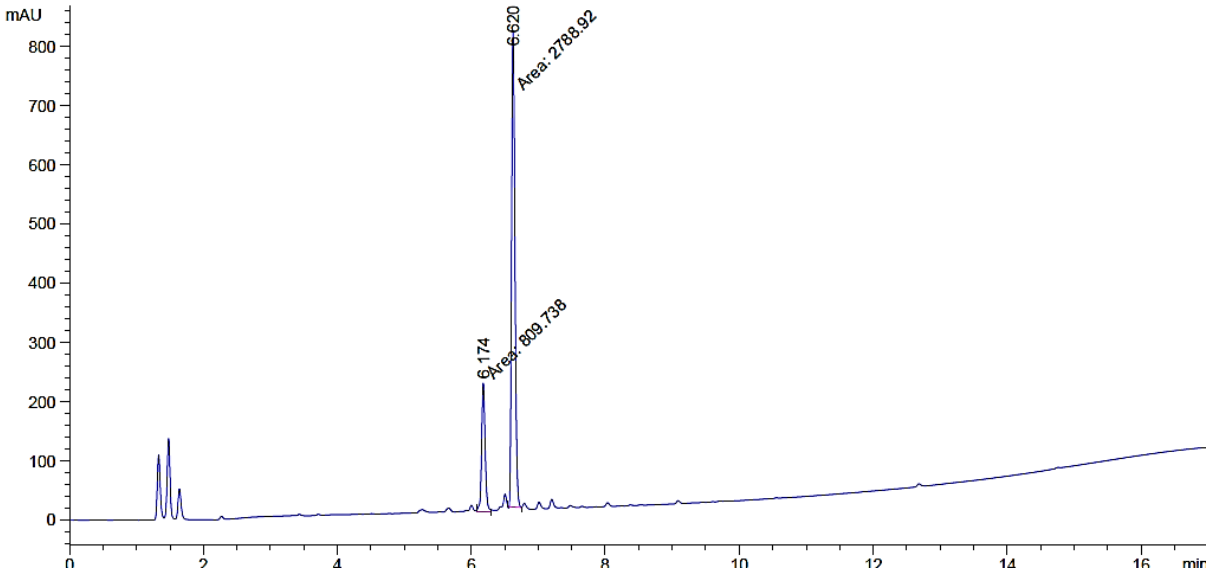

Figure S32. H-Tyr-Ile-Gly-Phe-Leu-NH_2 pentapeptide on CM resin. AA-DIEA-T3P(2-MeTHF)-OxymaPure (5:10:5:5). Solvent: 2-MeTHF. See legend of Figure S1 for chromatographic conditions.

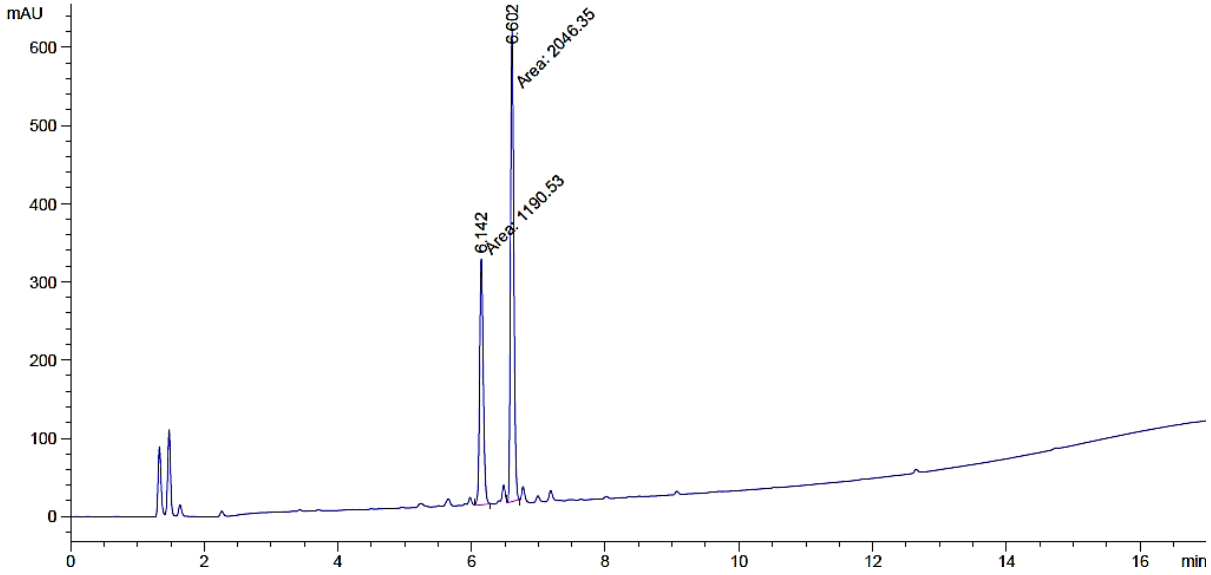

Figure S33. H-Tyr-Ile-Gly-Phe-Leu-NH_2 pentapeptide on CM resin. AA-DIEA-T3P(2-MeTHF)-OxymaPure (5:10:5:5). Solvent: NBP. See legend of Figure S1 for chromatographic conditions.

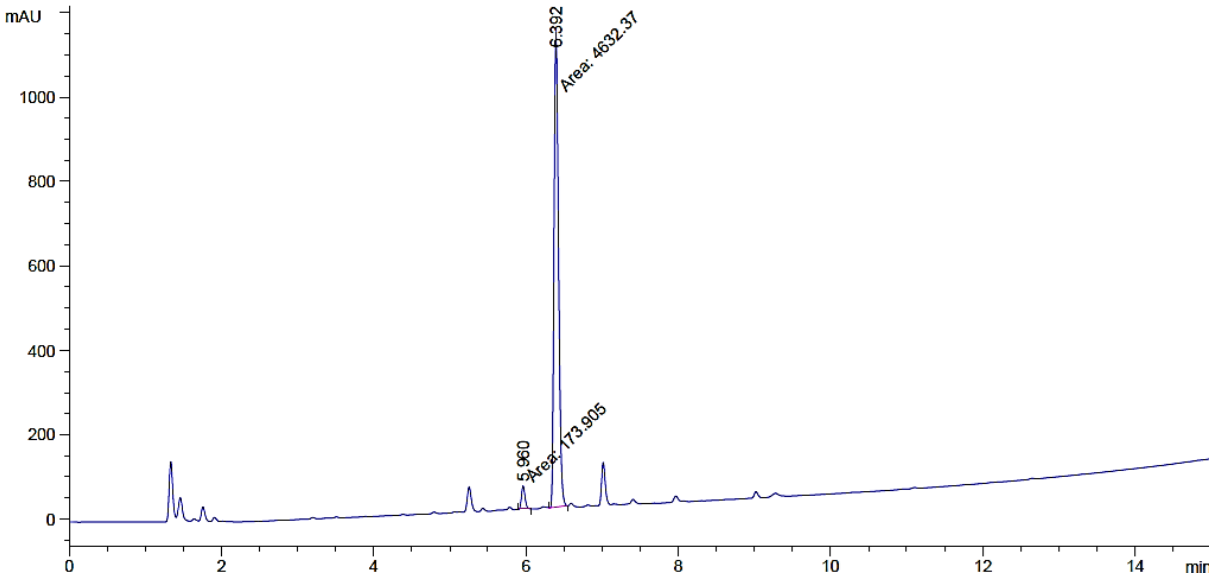

Figure S34. H-Tyr-Ile-Gly-Phe-Leu-NH_2 pentapeptide on CM resin. AA-DIEA-T3P(2-MeTHF)-OxymaPure (5:10:5:5). Solvent: EtOAc. See legend of Figure S1 for chromatographic conditions.

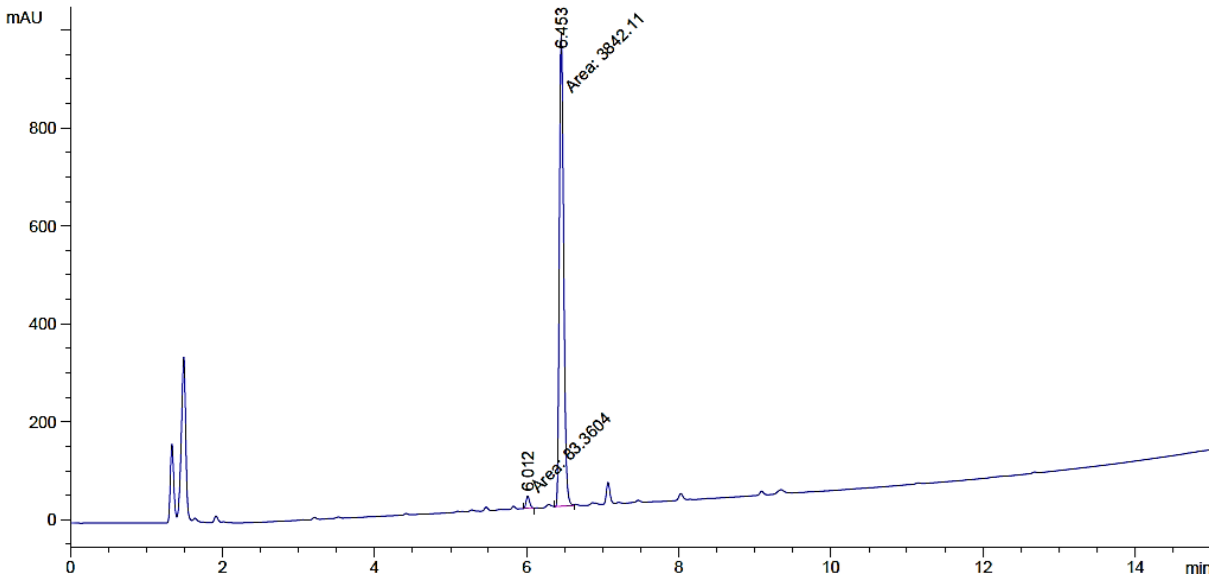

Figure S35. H-Tyr-Ile-Gly-Phe-Leu-NH_2 pentapeptide on CM resin. AA-DIEA-T3P(2-MeTHF)-OxymaPure (5:10:5:5). Solvent: ACN. See legend of Figure S1 for chromatographic conditions.

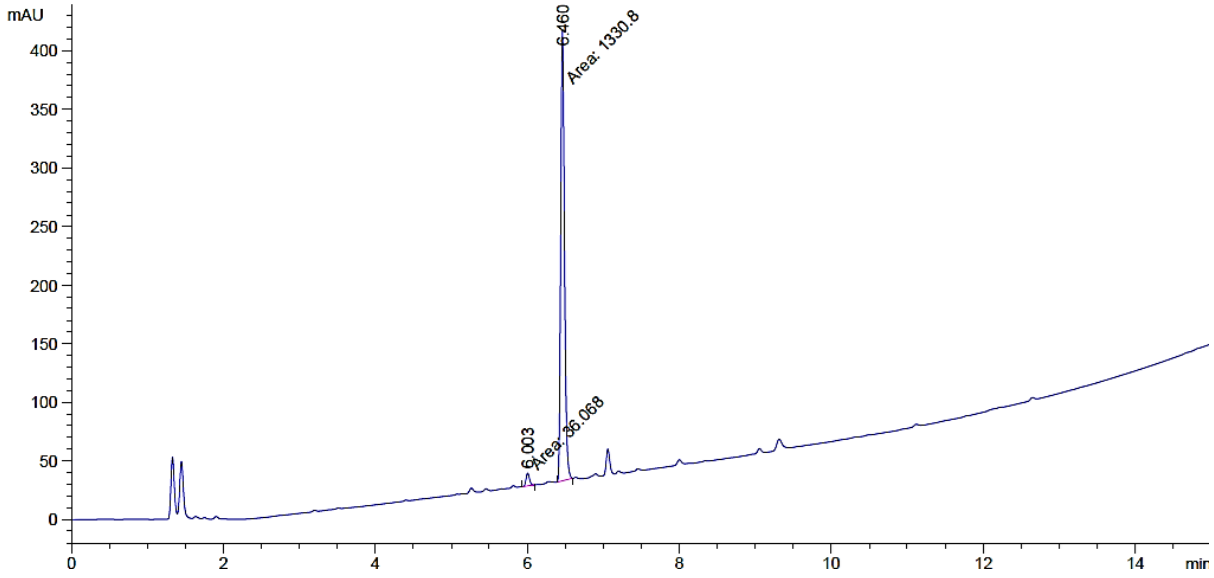

Figure S36. H-Tyr-Ile-Gly-Phe-Leu-NH_2 pentapeptide on CM resin. AA-DIEA-T3P(2-MeTHF)-OxymaPure (5:10:5:5). Solvent: EtOAc-NBP (3:1). See legend of Figure S1 for chromatographic conditions.

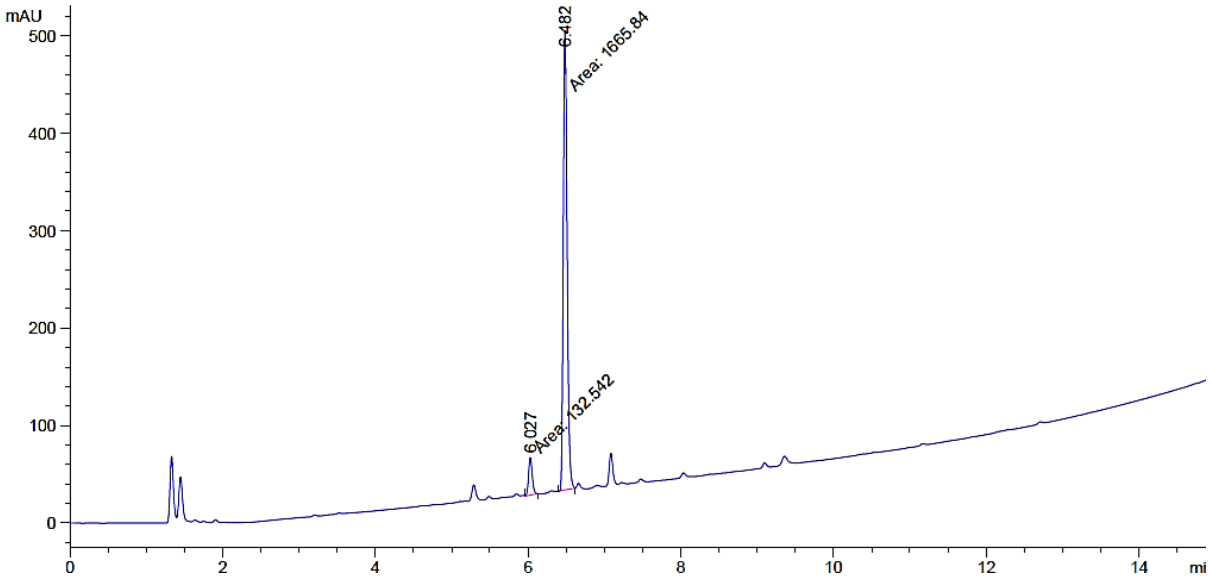

Figure S37. H-Tyr-Ile-Gly-Phe-Leu-NH_2 pentapeptide on CM resin. AA-DIEA-T3P(2-MeTHF)-OxymaPure (5:10:5:5). Solvent: EtOAc-DMF (3:1). See legend of Figure S1 for chromatographic conditions.

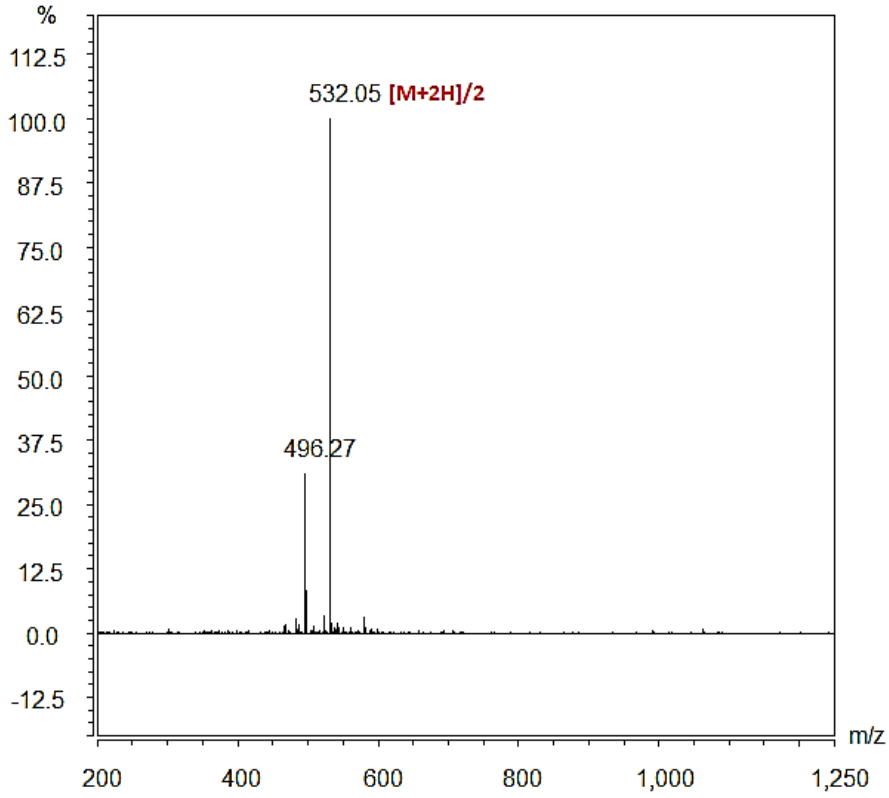

Figure S38. Mass for ACP decapeptide on CM resin.

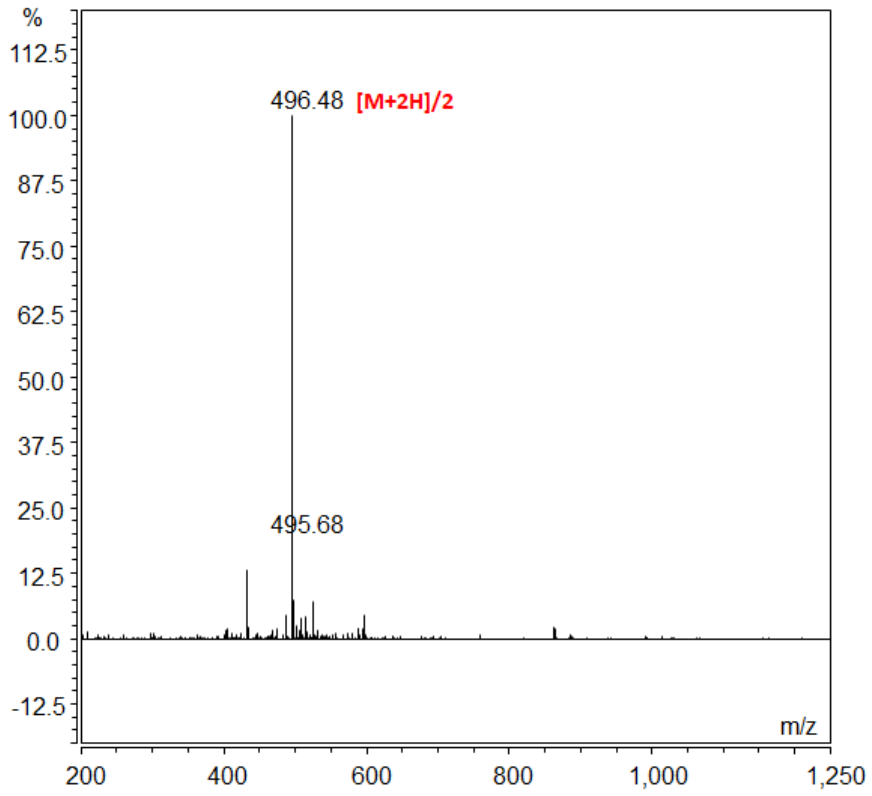

Figure S39. Mass for Des-Ala of ACP decapeptide on CM resin.

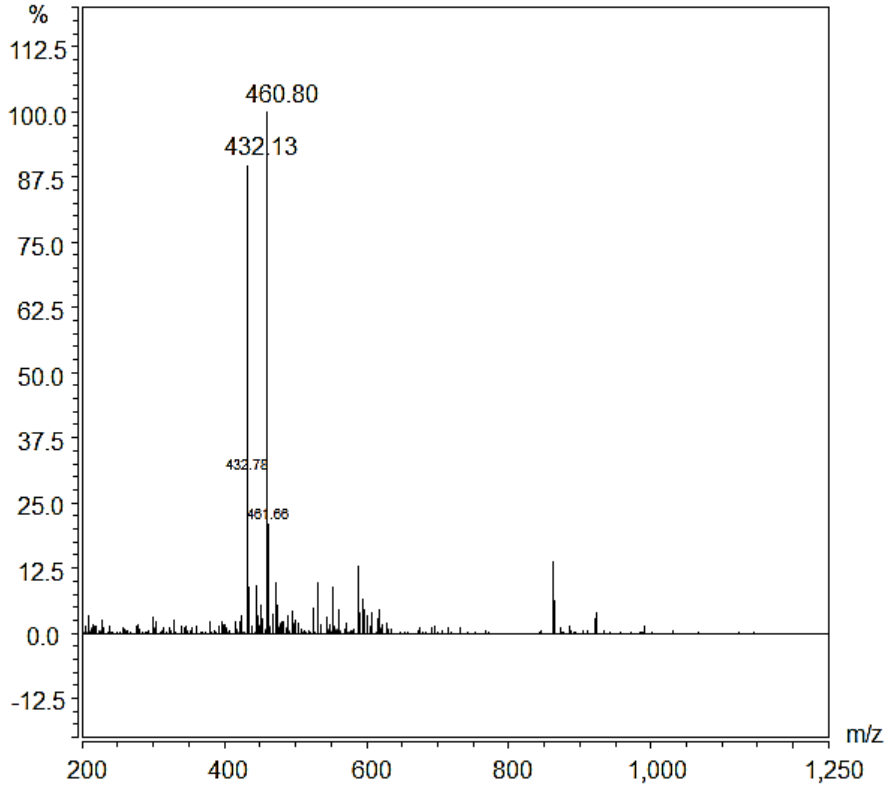

Figure S40. Mass for Des-Gln-Ala of ACP decapeptide on CM resin.

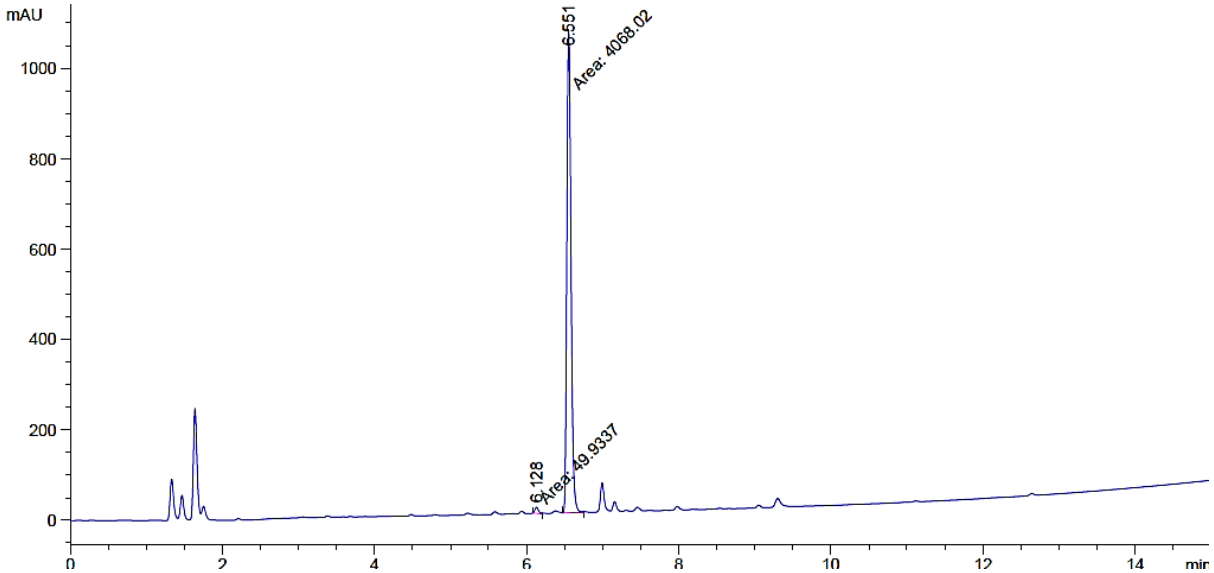

Figure S41. H-Tyr-Ile-Gly-Phe-Leu-NH_2 pentapeptide on CM resin. AA-DIEA-T3P(2-MeTHF)-OxymaPure (5:10:5:5). Coupling for 3 min under microwave irradiation (75^o C). See legend of Figure S1 for chromatographic conditions.

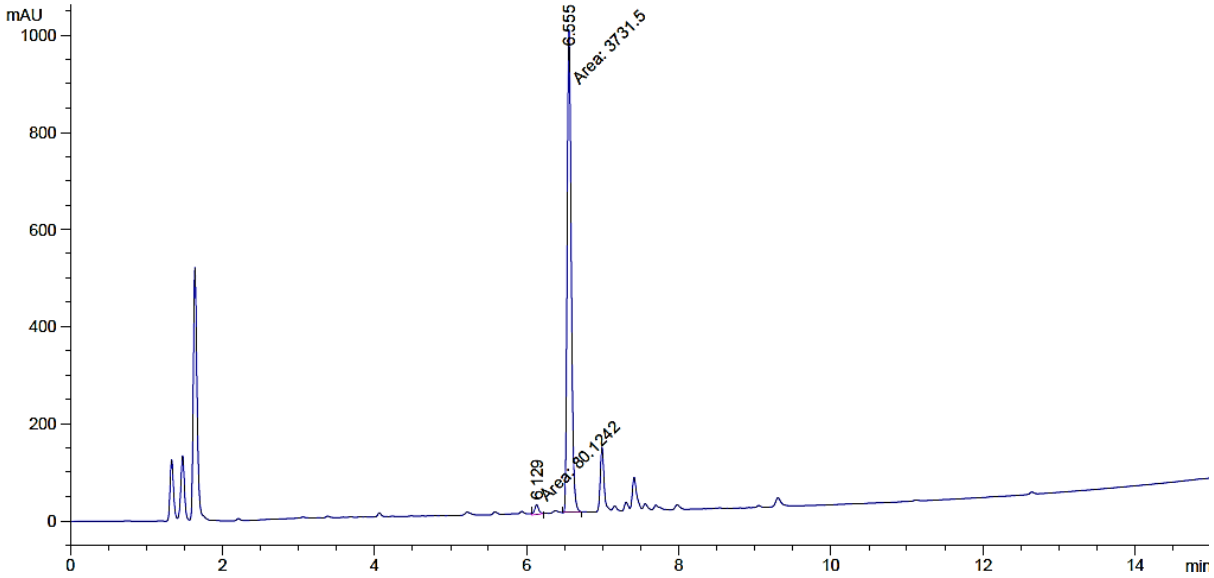

Figure S42. H-Tyr-Ile-Gly-Phe-Leu-NH_2 pentapeptide on CM resin. AA-DIEA-T3P(2-MeTHF)-OxymaPure (5:10:5:5). Coupling for 60 min under microwave irradiation (75^o C). See legend of Figure S1 for chromatographic conditions.

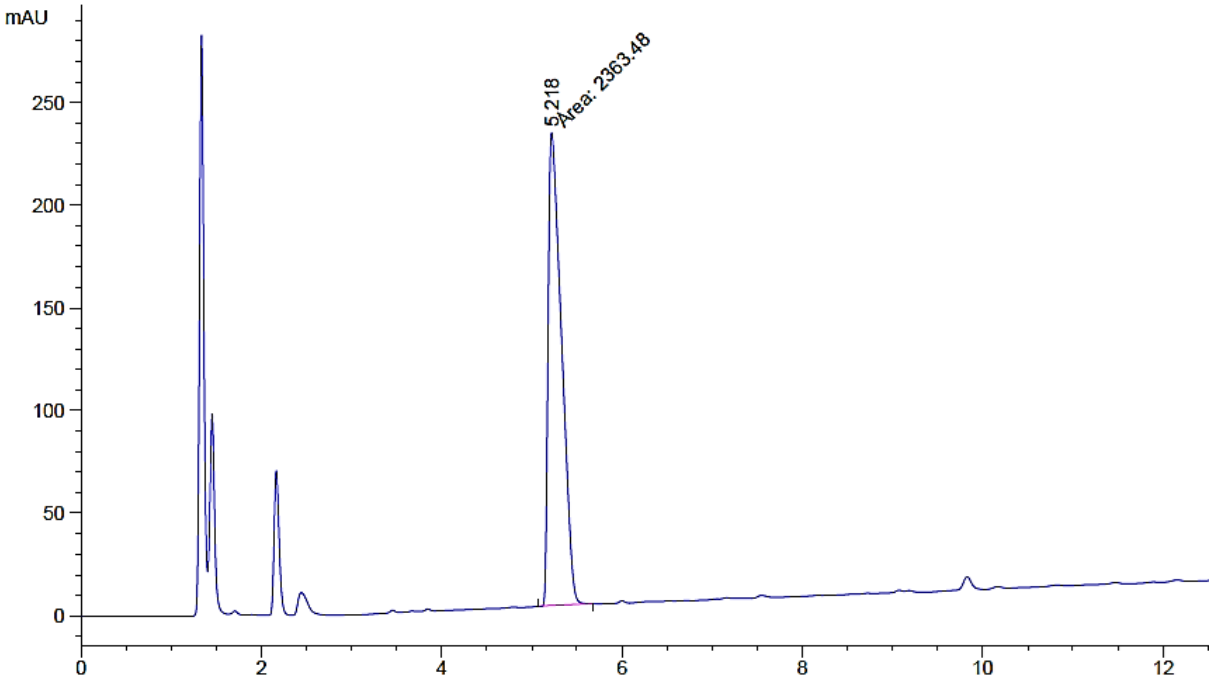

Figure S43. H-LSer-LPhe-NH_2. 0-20% gradient elution in 15 min. t_R (LL) = 5.2 min; (DL) = 6.8. See legend of Figure S1 for the rest of the chromatographic conditions.

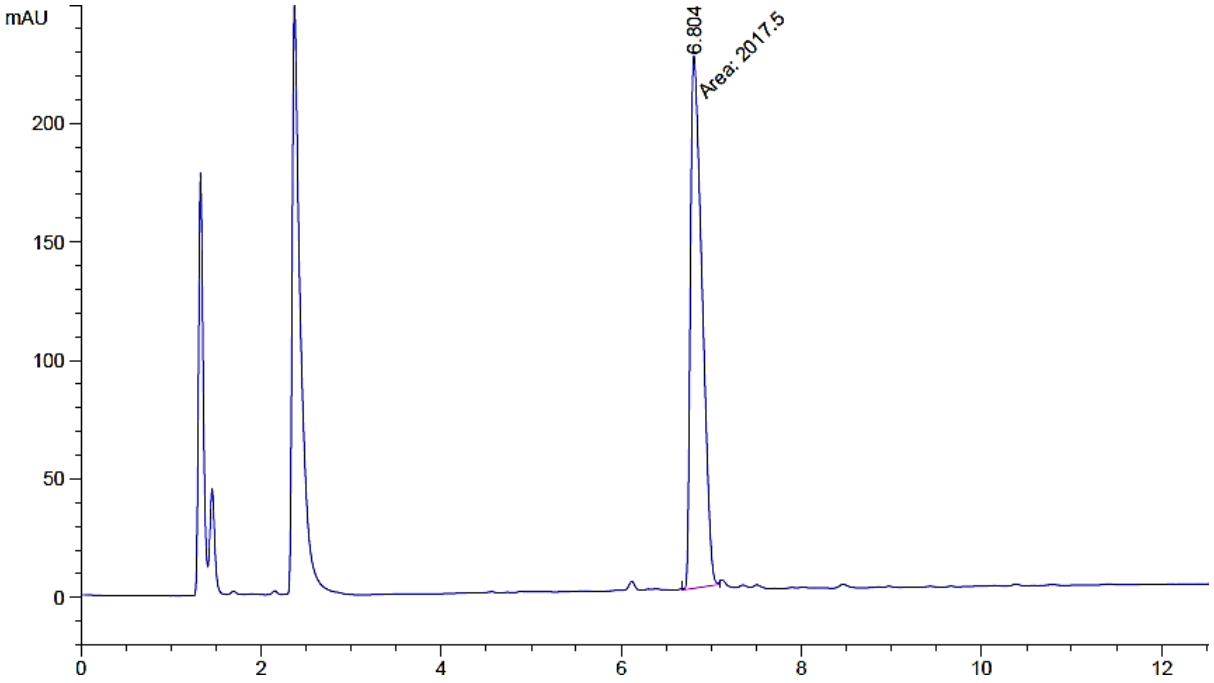

Figure S44. H-DSer-LPhe-NH_2. t_R (DL) = 6.8. See legend of Figure S43 for chromatographic conditions.

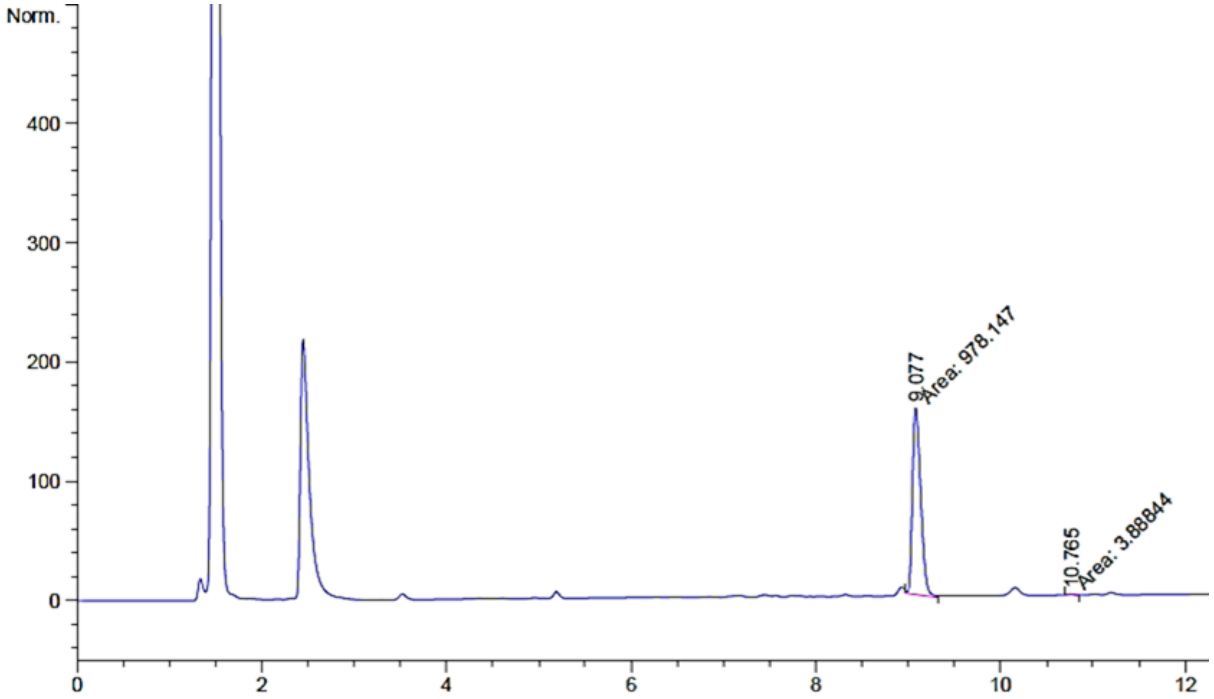

Figure S45. H-LCys(ACM)-LPhe-NH_2. t_R (LL) = 9.1 min; (DL) = 10.8. See legend of Figure S43 for chromatographic conditions.

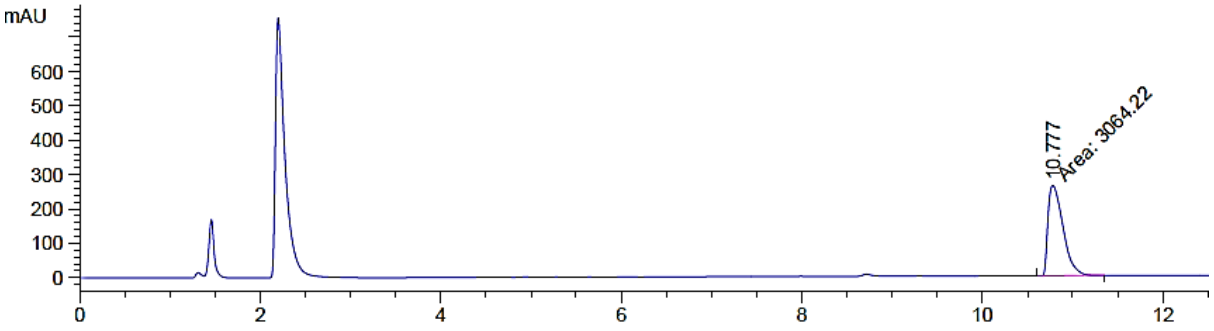

Figure S46. H-DCys(ACM)-LPhe-NH_2. t_R (DL) = 10.8. See legend of Figure S43 for chromatographic conditions.