

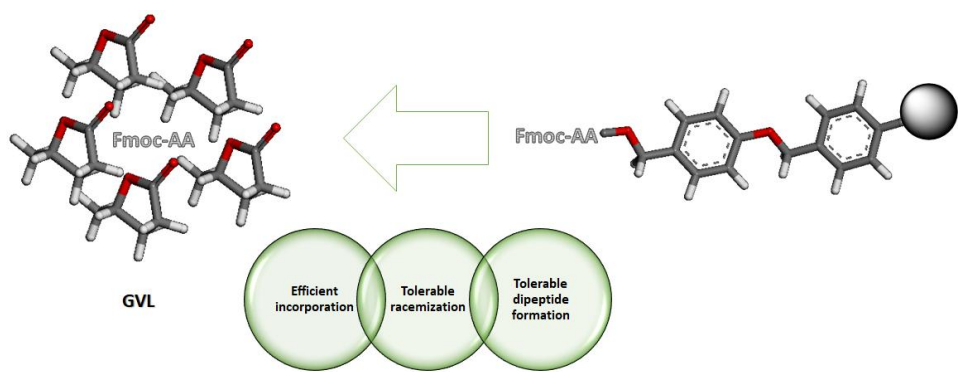
Graphical Abstract

To create your abstract, type over the instructions in the template box below.
Fonts or abstract dimensions should not be changed or altered.

γ -Valerolactone (GVL): An eco-friendly anchoring solvent for solid-phase peptide synthesis

Leave this area blank for abstract info.

Othman Al Musaimi, Ayman El-Faham, Alessandra Basso, Beatriz G. de la Torre, Fernando Albericio





γ -Valerolactone (GVL): An Eco-Friendly Anchoring Solvent for Solid-Phase Peptide Synthesis

Othman Al Musaimi,^{1,2} Ayman El-Faham,^{3,4} Alessandra Basso,⁵ Beatriz G. de la Torre^{2*} Fernando Albericio^{1,3,6,7*}

¹Peptide Science Laboratory, School of Chemistry and Physics, University of KwaZulu-Natal, Durban 4001, South Africa; ²KwaZulu-Natal Research Innovation and Sequencing Platform (KRISP), School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban 4041, South Africa; ³Department of Chemistry, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia; ⁴Department of Chemistry, Faculty of Science, Alexandria University, P.O. Box 426, Alexandria 21321, Egypt; ⁵Purolite, Llantrisant Business Park, Llantrisant, CF72 8LF, United Kingdom; ⁶CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, Barcelona Science Park, University of Barcelona, 08028 Barcelona, Spain; ⁷Department of Organic Chemistry, University of Barcelona, 08028 Barcelona, Spain

ARTICLE INFO

Article history:

Received
Received in revised form
Accepted
Available online

Keywords:

Green chemistry
Wang resin
Racemization
Dipeptide formation

ABSTRACT

Due to the hazardous nature of CH_2Cl_2 , regulatory authorities have imposed restrictions to minimize or even stop its use. It has therefore become imperative to identify environmentally benign solvents to replace it. Here we report on a bio derived solvent, γ -valerolactone, for the incorporation of the first amino acid onto p-alkoxybenzyl alcohol resin in solid-phase peptide synthesis. Satisfactory loading values (by a spectrophotometric method) were achieved. Furthermore, racemization and dipeptide formation were also checked and found to be acceptable.

2009 Elsevier Ltd. All rights reserved.

1. Introduction

Peptides have gained much attention due to their multiple roles and applications, particularly in the pharmaceutical sector, where they are used as either directly as drugs or as carriers of the same [1,2]. During the last two years (2017 and 2018), seven peptides and two oligonucleotides were approved as drugs by the US Food and Drug Administration (FDA) [3,4]. In this new scenario, novel synthetic strategies are needed to handle the large-scale production of these molecules. Solid-phase peptide synthesis (SPPS), which was introduced by Merrifield in 1963 [5], is considered the method of choice for such purposes. This strategy was enhanced by the introduction of a range of polymeric supports, on which the first amino acid residue is anchored. This step is considered important as it drives the reaction either to success or failure, with the final outcome being determined by the loading test [5]. Given the regulations currently imposed by legal authorities regarding the non-green solvents used in various synthetic protocols [6,7], it is timely to develop green alternative chemicals and thus protect the environment and human health.

p-Alkoxybenzyl alcohol (Wang) resin is a commonly used for the preparation of C-terminal acid peptides following a 9-fluorenylmethyloxycarbonyl (Fmoc)-*tert*-butyl (tBu) strategy [8]. CH_2Cl_2 is the main solvent used for anchoring the first amino acid residue onto Wang resin. However, it is considered as a hazardous substance [9] and it has a low boiling point (40°C) [6,7]. Furthermore, it has been classified as a carcinogen by the Occupational Safety and Health Administration (OSHA) [10], as well as by the US Environmental Protection Agency (EPA) [11].

In this regard, EPA now prohibits its use [11]. Hence, the development of alternative solvents is of utmost important. We have successfully used 2-methyltetrahydrofuran (2-MeTHF) as an eco-friendly alternative to CH_2Cl_2 for the incorporation of the first amino acid onto Wang resin and for the peptide precipitation steps [12].

Here we present γ -valerolactone (GVL) as an eco-friendly alternative to CH_2Cl_2 for anchoring the first amino acid residue onto Wang resin. GVL has several favorable characteristics; it is non-toxic, renewable, and biodegradable [13], and it has a pleasant smell and good thermal stability [14,15]. In addition, GVL has a considerably high boiling and flash point, 207°C and 96°C, respectively [14-16].

We first tested GVL for the incorporation of the first amino acid onto 2-chlorotriyl chloride (CTC) resin. However, the study was not successful as only negligible loading was observed (e.g. Fmoc-Gly-OH, 0.10 mmol/g; Fmoc-Arg-OH, 0.08 mmol/g), while the supplier's specification was 1.0 mmol/g. This result could be attributed to the solvolysis phenomenon caused by the high dielectric constant (36.5) and thus the polarity of GVL [17].

In contrast, GVL performed well for amide bond formation in SPPS, and was found to be compatible with microwave-assisted automatic synthesis [18,19].

2. Results and discussion

Eighteen Fmoc-amino acids, namely alanine (Ala), arginine [Arg(Pbf)], cysteine [Cys(Acm)], glutamine [Gln(Trt)], glutamic acid [Glu(OtBu)], glycine (Gly), histidine [His(Trt)], isoleucine (Ile), leucine (Leu), lysine [Lys(Boc)], methionine (Met), phenylalanine (Phe), proline (Pro), serine [Ser(tBu)], threonine [Thr(tBu)], tryptophan [Trp(Boc)], tyrosine [Tyr(tBu)], and valine (Val), were separately incorporated onto Wang resin using GVL. Satisfactory loading values were observed (Table 1).

Amino acid incorporation onto Wang resin requires strong carboxylic acid activation by reaction with carbodiimide [*N,N*-diisopropylcarbodiimide (DIC)] in the presence of 10% of either *N,N*-dimethyl-4-aminopyridine (DMAP) or *N*-methylimidazol (NMI) as catalysts [20-22]. The presence of the basic catalyst has two potential drawbacks, namely racemization [23-26] and dipeptide formation [24,27], which were both examined in this study, together with the incorporation yield. First, DMAP was used, but although the incorporation took place with good yields, racemization was very high for several amino acids, which were not considered prone to this process (data not showed). NMI was used to replace DMAP. NMI led to less racemization and maintained good amino acid incorporation capacity. Although it has been described that less racemization is obtained when the first hour of the coupling is carried out at 0°C, in this case the best results were achieved when a double coupling was performed at room temperature (rt). Both couplings were carried out using 2.5 equiv. of Fmoc-aa, 2.5 equiv. of DIC and 0.25 equiv. of NMI [28]. These conditions yielded good results for incorporation and acceptable racemization for all amino acids, except Fmoc-His(Trt)-OH (34.4%, which is close to other values reported in the literature, ref. [22,29]) and Fmoc-Trp(Boc)-OH (15.8%). While it is known that His can easily undergo racemization through the H abstraction of the α -C by the π -nitrogen of its imidazole ring [22], more surprising was the high racemization found for Trp(Boc). It is worth mentioning that high racemization data were reported for Trp(Boc) in solution-phase peptide synthesis under certain conditions [30]. We therefore performed a more in-depth study of these two amino acids either with DMAP or NMI, symmetrical anhydride or equiv. amounts of protected amino acid and DIC, and the first 1 hour at 0°C. Symmetrical anhydride proved to be the best method for both protected amino acids, for His using DMAP and 2 h at 0°C and then 2 h at rt (5.0% of racemization); and for Trp, using NMI and 1 h at 0°C and then 1 h at rt (7.8%) (Supplementary Figures 1-34).

Furthermore, only traces of dipeptide were observed in the cleaved amino acid resin (0.2%) when Fmoc-Gly-OH was incorporated using 2 couplings (1 h at rt each); 2.5 equiv. of Fmoc-Gly-OH, 2.5 equiv. of DIC, and 0.25 equiv. of NMI (Supplementary Figures 35 and 36).

Table 1. Loading, degree of racemization and dipeptide formation when GVL is used in Wang resin

Dipeptide	Loaded 1 st amino acid (mmol/g) ^a	%LD ^a
L-Phe-L-Ala-OH	0.98	0.3
L-Phe-L-Arg-OH	0.51	0.1
L-Phe-L-Cys-(Acm)-OH	0.74	1.1
L-Phe-L-Gln-OH	0.74	1.0
L-Phe-L-Glu-OH	0.75	0.3
L-Phe-L-His-OH	0.63	5.0
L-Phe-L-Ile-OH	0.73	1.2

L-Phe-L-Leu-OH	0.80	0.1
L-Phe-L-Lys-OH	0.69	0.1
L-Phe-L-Met-OH	0.99	1.3
L-Ser-L-Phe-OH	0.78	1.5
L-Phe-L-Pro-OH	0.74	0.01
L-Phe-L-Ser-OH	0.75	0.9
L-Phe-L-Thr-OH	0.80	0.1
L-Phe-L-Trp-OH	0.66	7.8
L-Phe-L-Tyr-OH	0.68	1.0
L-Phe-L-Val-OH	0.96	0.5
Fmoc-Gly-OH	0.82	0.2 % (Dipeptide)

^a See experimental part for method and section 4.3.4 for loading calculation.

3. Conclusion

GVL has been previously used by our group for amide bond formation in SPPS [18,19]. Here we envisaged GVL as an alternative green solvent to CH₂Cl₂ for anchoring the first amino acid onto Wang resin. Satisfactory loading values and acceptable racemization and degree of dipeptide formation were achieved. Our results compete favorably with others described in the literature using Fmoc-amino acid fluoride in CH₂Cl₂ [29]. Given calls for the prohibition of the hazardous solvent CH₂Cl₂ [11], these results support GVL as a green alternative for both incorporation of the first amino acid onto a Wang-type resin and subsequent elongation of the peptide sequence. For the industrial use of GVL for the incorporation of a given Fmoc-amino acid onto the Wang resin, an optimization is desirable.

4. Experimental details

4.1 Materials and methods

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 a Chemstation software for data processing. Column: Phenomenex Luna C₁₈ (3.6 μ m, 4.6 $\times$ 150 mm) column, with flow rate of 1.0 mL/min and UV detection at 220 nm (280 nm was selected in dipeptide ratio determination test). Mobile phase A: 0.1 % trifluoroacetic acid (TFA) in H₂O; mobile phase B: 0.1% TFA in CH₃CN. LC-MS was performed on a Shimadzu 2020 UFLC-MS using an YMC-Triart C₁₈ (5 μ m, 4.6 $\times$ 150 mm) column and data processing was carried out by LabSolution software. Buffer A: 0.1% formic acid in H₂O; buffer B: 0.1% formic acid in CH₃CN. UV tests were performed on Shimadzu UV3600 spectrophotometer.

4.2 Incorporation procedure

4.2.1 For all amino acids except His and Trp:

The double incorporation scenario was adopted as follows: a known amount of Wang resin (50 mg) was washed and swollen in GVL (2 mL) for 10-20 min, and then 2.5 equiv. of each Fmoc-amino acid was dissolved in a minimum amount of GVL (0.6 mL/50 mg resin) and sonicated for 10 min. Fmoc-amino acid solution was added to the resin, followed by 0.25 equiv. of NMI. Finally, 2.5 equiv. of DIC was added to the resin mixture. The resulting mixture was allowed to react (under mechanical shaking) for 1 h at rt. Immediately after, the resin was washed twice with GVL, and the same reaction was carried out for additional 1 h at rt with a fresh (Fmoc-amino acid, NMI, DIC) solution.

4.2.2 For His:

A known amount of Wang resin (50 mg) was washed and swollen in GVL (2 mL, in an ice bath) for 10-20 min, and then 5 equiv. of Fmoc-His(Trt)-OH amino acid was dissolved in a minimum amount of GVL (0.6 mL/50 mg resin) and sonicated for 10 min. 0.25 equiv. of DMAP was added to the previously swollen resin and the Fmoc-amino acid solution was added to the (resin + DMAP). Finally, 2.5 equiv. of DIC was added to the resin mixture. The resulting mixture was allowed to react (under mechanical shaking) for 2 h at 0°C plus 2 h at rt. Immediately after, the resin was washed twice with GVL and dried under vacuum.

4.2.3 For Trp:

A known amount of Wang resin (50 mg) was washed and swollen in GVL (2 mL, in an ice bath) for 10-20 min, and then 5 equiv. of Fmoc-Trp(Boc)-OH amino acid was dissolved in a minimum amount of GVL (0.6 mL/50 mg resin), sonicated for 10 min and added to the resin. Next, 0.25 equiv. of NMI was added. Finally, 2.5 equiv. of DIC was added to the resin mixture. The resulting mixture was allowed to react (under mechanical shaking) for 1 h at 0°C plus 1 h at rt. Immediately after, the resin was washed twice with GVL and dried under vacuum.

4.3 Loading calculation

4.3.1 Sample preparation

About 10 mg of the loaded resin was weighed in a polypropylene syringe preloaded with a filter. Next, 200 µL of the deprotection solution (20% piperidine/DMF) was added, and the sample was allowed to shake for 10 min. The filtrate was then collected in a 25-mL volumetric flask (another 200 µL was added to repeat this step). Finally, the volume was made up to 25 mL with ethanol.

4.3.2 Standard preparation

About 3 mg of the Fmoc-amino acid was weighed and transferred to a 25-mL volumetric flask. Next, 400 µL of the deprotection solution (20% piperidine/DMF) was added, and the volume was made up to 25 mL with ethanol.

4.3.3 Blank preparation

400 µL of the deprotection solution (20% piperidine/DMF) was transferred to a 25-mL volumetric flask, and the volume was made up to 25 mL with ethanol.

4.3.4 UV spectrophotometer reading

Samples, standards and blank solutions were read at 301 nm. Loading was calculated based on the following formula:

$$L = \frac{\text{sample Abs} \times \text{standard mass} \times 1000}{\text{standard Abs} \times \text{loaded resin mass} \times \text{Fmoc} - \text{amino acid m. wt}}$$

Where; L = Loading, Abs = Absorbance at 301 nm, m.wt = molecular weight.

4.4 Racemization determination

A total of 34 dipeptides were synthesized: (H-L-Phe-L-Ala-OH, H-L-Phe-L-Arg-OH, H-L-Phe-L-Cys-(Acm)-OH, H-L-Phe-L-Gln-OH, H-L-Phe-L-Glu-OH, H-L-Phe-L-His-OH, H-L-Phe-L-Ile-OH, H-L-Phe-L-Leu-OH, H-L-Ser-L-Lys-OH, H-L-Phe-L-Met-OH, H-L-Ser-L-Phe-OH, H-L-Phe-L-Pro-OH, H-L-Phe-L-Ser-OH, H-L-Phe-L-Thr-OH, H-L-Phe-L-Trp-OH, H-L-Phe-L-Tyr-OH and H-L-Phe-L-Val-OH, in addition to their LD epimers). The second amino acid of the dipeptides was coupled as follows: the Fmoc protecting group was removed from the first amino

acid (incorporated onto the resin) by 20% piperidine/DMF for 1 and 7 min. Next, 3 equiv. of the Fmoc-amino acid was dissolved in a minimum amount of DMF (0.5 mL/50mg resin), then 3 equiv. of OxymaPure was added to the mixture, in addition to 3 equiv. of DIC. The resultant solution was then added to the preloaded resins and the mixture was allowed to react for 1 h at rt (under mechanical shaking). Finally, the peptidyl resin was washed twice with DMF. Fmoc was removed by 20% piperidine/DMF for 1 and 7 min.

The synthesized dipeptides were cleaved from the resin using a cocktail solution TFA-Triisopropylsilane (TIS)-H₂O (95:2.5:2.5). They were then precipitated by chilled ether and dissolved in H₂O-CH₃CN (1:1) and then analyzed by HPLC using a 0–20% gradient elution in 15 min for (H-L-Phe-L-Ser-OH, H-L-Phe-L-Lys-OH, H-L-Phe-L-Cys-(Acm)-OH and H-L-Phe-L-His-OH) dipeptides and a 5–40% gradient elution in 15 min for the rest of the dipeptides; λ = 220 nm. They were then compared with samples of the LD epimer previously synthesized on Wang resin.

4.5 Dipeptide ratio determination

Fmoc-Gly-OH amino acid incorporated on the resin (using GVL) was cleaved from the support following the above protocol (racemization determination protocol), precipitated by chilled ether and dissolved in H₂O-CH₃CN (1:1) and then analyzed by HPLC to check for the formation of dipeptide (Fmoc-Gly-Gly-OH). The same synthetic procedure was followed for the preparation of a real sample of the dipeptide. HPLC analysis was carried out using a 5–95% gradient elution in 15 min; λ = 280 nm. Results were compared with a Fmoc-Gly-Gly-OH sample previously synthesized on Wang resin.

Acknowledgment

The work was funded in part by the following: the International Scientific Partnership Program ISPP at King Saud University (ISPP# 0061; Saudi Arabia); the National Research Foundation (NRF) (Blue Sky's Research Programme # 110960) and the University of KwaZulu-Natal (South Africa); and the Spanish Ministry of Science, Innovation, and Universities (CTQ2015-67870-P) and the Generalitat de Catalunya (2017 SGR 1439; Spain).

References

- Uhlig, T.; Kyprianou, T.; Martinelli, F.G.; Oppici, C.A.; Heiligers, D.; Hills, D.; Calvo, X.R.; Verhaert, P. The emergence of peptides in the pharmaceutical business: From exploration to exploitation. *EuPA Open Proteomics* **2014**, *4*, 58-69. 10.1016/j.euprot.2014.05.003
- Patel, A.; Patel, M.; Yang, X.; Mitra, A.K. Recent advances in protein and peptide drug delivery: A special emphasis on polymeric nanoparticles. *Protein Pept. Lett.* **2014**, *21*, 1102-1120. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4407643/pdf/nihms680956.pdf>
- Al Musaimi, O.; Al Shaer, D.; de la Torre, B.G.; Albericio, F. 2017 fda peptide harvest. *Pharmaceuticals (Basel)* **2018**, *11*, 10.3390/ph11020042
- Al Shaer, D.; Al Musaimi, O.; Albericio, F.; Torre, B.G.d.l. 2018 fda tides harvest. *Pharmaceuticals (Basel)* **2019**, *12*, 1-6. 10.3390/ph12020052

5. Merrifield, R.B. Solid phase peptide synthesis. I. The synthesis of a tetrapeptide'. *J. Am. Chem. Soc.* **1963**, *85*, 2149–2154. 10.1021/ja00897a025
6. Henderson, R.K.; Jiménez-González, C.; Constable, D.J.C.; Alston, S.R.; Inglis, G.G.A.; Fisher, G.; Sherwood, J.; Binks, S.P.; Curzons, A.D. Expanding gsk's solvent selection guide – embedding sustainability into solvent selection starting at medicinal chemistry. *Green Chem.* **2011**, *13*, 854–862. 10.1039/c0gc00918k
7. Alder, C.M.; Hayler, J.D.; Henderson, R.K.; Redman, A.M.; Shukla, L.; Shuster, L.E.; Sneddon, H.F. Updating and further expanding gsk's solvent sustainability guide. *Green Chem.* **2016**, *18*, 3879–3890. 10.1039/c6gc00611f
8. Wang, S.-S. P-alkoxybenzyl alcohol resin and p-alkoxybenzyloxycarbonylhydrazide resin for solid phase synthesis of protected peptide fragments *J. Am. Chem. Soc.* **1973**, *95*, 1328 - 1333. 10.1021/ja00785a602
9. Prat, D.; Hayler, J.; Wells, A. A survey of solvent selection guides. *Green Chem.* **2012**, *16*, 4546–4551. 10.1039/c4gc01149j
10. OSHA. Methylene chloride. <https://www.osha.gov/SLTC/methylenechloride/>
11. (EPA), E.P.A. Regulation of certain uses under toxic substances control act: Methylene chloride and n-methylpyrrolidone. **2017**. <https://www.regulations.gov/document?D=EPA-HQ-OPPT-2016-0231-0001>
12. Al Musaimi, O.; Jad, Y.E.; Kumar, A.; El-Faham, A.; Collins, J.M.; Basso, A.; Torre, B.G.d.l.; Albericio, F. Greening the solid-phase peptide synthesis process. 2- methf for the incorporation of the first amino acid and precipitation of peptides after global deprotection. *Org. Process Res. Dev.* **2018**, *22*, 1809–1816. 10.1021/acs.oprd.8b00335
13. Fegyverneki, D.; Orha, L.; Láng, G.; Horváth, I.T. Gamma-valerolactone-based solvents. *Tetrahedron* **2010**, *66*, 1078–1081. 10.1016/j.tet.2009.11.013
14. Wong, C.Y.Y.; Choi, A.W.-T.; Lui, M.Y.; Fridrich, B.; Horváth, A.K.; Mika, L.T.; Horváth, I.T. Stability of gamma-valerolactone under neutral, acidic, and basic conditions. *Structural Chemistry* **2016**, *28*, 423–429. 10.1007/s11224-016-0887-6
15. Horváth, I.T.; Mehdi, H.; Fábos, V.; Boda, L.; Mika, L.T. Γ -valerolactone—a sustainable liquid for energy and carbon-based chemicals. *Green Chem.* **2008**, *10*, 238–242. 10.1039/b712863k
16. Alonso, D.M.; Wettstein, S.G.; Dumesic, J.A. Gamma-valerolactone, a sustainable platform molecule derived from lignocellulosic biomass. *Green Chemistry* **2013**, *15*, 584. 10.1039/c3gc37065h
17. Ismalaj, E.; Strappaveccia, G.; Ballerini, E.; Elisei, F.; Piermatti, O.; Gelman, D.; Vaccaro, L. Γ -valerolactone as a renewable dipolar aprotic solvent deriving from biomass degradation for the hiyama reaction. *ACS Sustain. Chem. Eng.* **2014**, *2*, 2461–2464. 10.1021/sc5004727
18. Kumar, A.; Jad, Y.E.; Collins, J.M.; Albericio, F.; de la Torre, B. Microwave assisted green solid-phase peptide synthesis using γ -valerolactone (gvl) as solvent. *ACS Sustain. Chem. Eng.* **2018**, *6*, 8034–8039. 10.1021/acssuschemeng.8b01531
19. Kumar, A.; Niyi, A.; Nandhini, K.P.; Collins, J.M.; Albericio, F.; Torre, B.G.d.l. Troubleshooting when using α -valerolactone (gvl) in green solid-phase peptide synthesis. *Org. Process Res. Dev.* **2019**. 10.1021/acs.oprd.9b00073
20. Dryland, A.; Sheppard, R.C. Peptide synthesis. Part 8. A system for solid-phase synthesis under low pressure continuous flow conditions. *J. Chem. Soc. Perkin Trans* **1986**. 10.1039/p19860000125
21. Neises, B.; Steglich, W. Simple method for the esterification of carboxylic acids. *Angew. Chem. Int. Ed. Engl.* **1978**, *17*. 10.1002/anie.197805221
22. Takayama, R.; Hayakawa, S.; Hinou, H.; Albericio, F.; Garcia-Martin, F. Further applications of classical amide coupling reagents: Microwave-assisted esterification on solid phase. *J. Pept. Sci.* **2018**, *24*, e3111. 10.1002/psc.3111
23. Barlos, K.; Chatzi, O.; Gatos, D.; Stavropoulos, G. 2-chlorotrityl chloride resin. Studies on anchoring of fmoc-amino acids and peptide cleavage. *Int. J. Pept. Protein Res.* **1991**, *37*, 513–520. 10.1111/j.1399-3011.1991.tb00769.x
24. Albericio, F.; Barany, G. Application of n,ndimethylformamide dineopentyl acetal for efficient anchoring of n" -9-fluorenylme thyloxycarbonylamino acids as p-alkoxybenzyl esters in solid-phase peptide synthesis. *Int. J. Peptide Protein Res.* **1984**, *23*, 342–349. 10.1111/j.1399-3011.1984.tb02729.x
25. Atherton, E.; Benoiton, N.L.; Brown, E.; Sheppard, R.C.; William, B.J. Racemisation of activated, urethane-protected amino-acids by p-dimethylaminopyridine. Significance in solid -phase peptide synthesis. *J.C.S. Chem. Comm.* **1981**. 10.1039/C39810000336
26. Wang, S.S.; Tam, J.P.; Wang, B.S.H.; Merrifield, R.B. Enhancement of peptide coupling reactions by 4-dimethylaminopyridine. *Int. J. Peptide protein Res.* **1981**, *18*, 459–467. 10.1111/j.1399-3011.1981.tb03007.x
27. Atherton, E.; Logan, C.J.; Sheppard, R.C. Peptide synthesis. Part 2. Procedures for solid-phase synthesis using α -fluorenylmethoxycarbonylamino-acids on polyamide supports. Synthesis of substance p and of acyl carrier protein 65–74 decapeptide. *J. Chem. Soc., Perkin Trans. 1* **1981**, 538–546. 10.1039/p19810000538
28. The use of the symmetrical anhydride method with the concourse of double amount of Fmoc-aa (5 equiv) gave worse results in terms of racemization and therefore was discarded.
29. Green, J.; Bradley, K. Studies on the acylation of hydroxy-functionalized resins using fmoc amino acids activated using diisopropylcarbodiimideihobt or as acid fluorides. *Tetrahedron* **1993**, *49*, 4141–4146. 10.1016/S0040-4020(01)85732-X
30. Griehl, C.; Weigt, J.; Jeschkeit, H. Use of tryptophan peptides for an hplc racemization test with fluorescence detection. *J. High Resolut. Chrom.* **1994**, *17*, 700–704. 10.1002/jhrc.1240171004