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Vommina V. Sureshbabu [a], Naremaddepalli S. Sudarshan [a] & G. Chenna Krishna [a]
[a] Department of Studies in Chemistry, Central College Campus, Bangalore University, Bangalore, India
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HOBt·DCHA-Mediated Synthesis of Sterically Hindered Peptides employing Fmoc-Amino Acid Chlorides in Both Solution-Phase and Solid Phase Methods

Vommina V. Sureshbabu, Naremaddepalli S. Sudarshan, and G. Chenna Krishna
Department of Studies in Chemistry, Central College Campus, Bangalore University, Bangalore, India

Abstract: The synthesis of peptides employing Fmoc-amino acid chlorides in presence of HOBt·DCHA salt in solution as well as by the solid-phase methods is described. The coupling was found to be complete in 30 min and free from racemization. The synthesis of β-casomorphin by solid-phase protocol employing Fmoc-amino acid chloride/HOBt·DCHA in DMF–CH_2Cl_2 has also been outlined. The final peptide was obtained in 80% yield and was fully characterized.

Keywords: Fmoc-amino acid chloride, fmoc-amino acid-OBt ester, HOBt·DCHA

INTRODUCTION

One of the many contributions of Louis Carpino, University of Massachusetts, is the discovery of shelf-stable Fmoc-amino acid chlorides,[1,2] which have been demonstrated to be rapid, efficient, acylating agents useful in peptide chemistry. Because of the high degree of activation associated with them, the acylation using acid chlorides[3,4] makes coupling of very hindered or weakly nucleophlic systems possible, whereas the use of other methods gives inferior results. Those Fmoc-amino acid chlorides bearing a tert-butyl type side chain functional group such as Asp(tBu),

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Address correspondence to Vommina V. Sureshbabu, Department of Studies in Chemistry, Central College Campus, Bangalore University, Dr. B. R. Ambedkar Veedhi, Bangalore 560 001, India. E-mail:hariccb@rediffmail.com

Cys(tBu), Lys(Boc), Ser(tBu), Thr(tBu), Trp(Boc), Tyr(tBu) and Asp(Pmc) can also be generated using in situ activation via bis(trichlorophenyl)carbonate. The acid chloride coupling mediated by an organic tertiary amine (pyridine, diisopropylethylamine (DIEA)), an essential component of such a reaction protocol, is known to result in the formation of oxazol-5(4H)-one,[5] leading to stereomutation and/or premature deblocking of Fmoc-group, due to the prolongation of the course of the reaction (usually required for the incorporation of hindered amino acids). This has been circumvented by the use of co-coupling agents such as silver cyanide[6,7] potassium salts of 1-hydroxybenzotriazole (KOBt)[8,9] and 1-hydroxy-7-aza-benzotriazole (KOAt),[10,11] t-butyldimethylsilyloxy benzotriazole (TBDMS) derivatives of HOBt (TBMS-OBt) and HOAt (TBDMS-OAt),[12,13] and zinc dust.[14,15] The use of KOBt/KOAt/TBDMS-OBt or TBDMS-OAt converts acid chloride to its benzotriazole/azabenzotriazole ester, which acts as an acylating agent, whereas in the presence of zinc dust–mediated coupling, the liberated HCl is directly abstracted. There are few limitations in the use of some of these reagents. The formation of KCl as a side product avoids the use of KOBt or KOAt in solid phase. The zinc dust–mediated coupling is limited to acylation reactions in solution phase only. TBDMS derivatives cannot be prepared and stored for long periods and need to be freshly prepared every time. Although the use of these co-coupling agents is satisfactory in solution phase, their utility in solid phase is not a straightforward protocol.

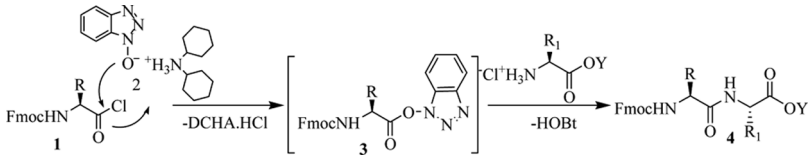
This article demonstrates the efficient synthesis of peptides employing Fmoc-amino acid chlorides mediated by HOBt·DCHA salt. Employing the present protocol, the synthesis of H_2N-Val-Pro-Gly-Val-Gly-OH (VPGVG)[16] in solution phase and β-casomorphin (H-Tyr-Pro-Phe-Pro-Gly-OH) in solid phase have been accomplished. 1-Hydroxybenzotriazole (HOBt), introduced by Konig and Geiger,[17] is well known to suppress racemization in carbodiimide-mediated couplings. Further, the other popular coupling agents 1-[bis(dimethylamino)-methylene]-1H-benzotriazolium hexaflur ophosphate-3-oxide (HBTU)[18] and 2-(1H-benzotriazole-1-yl)1,1,3,3-tetramethylluroniumtetrafluoroborate (TBTU)[19] also possess HOBt as part of their structure. Our interest in developing a simple non–Schotten–Baumann[20] protocol for acid chloride coupling has led to explore the use of HOBt·DCHA salt as a co-coupling agent in base-free conditions. Katritzky et al. have employed the benzotriazole amide derivatives of N-protected amino acids for the acylation of amino acids in an aqueous medium in the presence of triethyl amine.[21] In contrast to this, the present synthesis uses the rapidly and in situ generated –OBt ester as acylating agent in a completely base-free organic medium. The Bt-amide derivatives of amino acids are prepared

through a two-step protocol comprising activation of benzotriazoles with thionyl chloride followed by treatment of N-protected amino acids with activated Bt. However, the Fmoc-acid chlorides are prepared easily in a single step by treating the Fmoc-amino acids directly with thionyl chloride. Also, preparation of the HOBT–DCHA reagent is simple, involving the addition of an equimolar quantity of dicyclohexylamine to anhydrous HOBt in tetrahydrofuran (THF) solution. Upon standing, the salt separates out, which can be crystallized and stored for a long time.

RESULTS AND DISCUSSION

In a typical reaction, the mixture of Fmoc-amino acid chloride (1 equivalent) and HOBt·DCHA (2.2 equivalents) in THF was initially stirred for about 5 min to generate–OBt ester (which is evidenced from IR absorption spectrum by the disappearance of the band at 1786 cm^{-1} pertaining to acid chloride and the appearance of the band at 1820 cm^{-1} pertaining to –OBt ester formation), and then amino acid methyl ester salt was added and stirred. As monitored by thin layer chromatography (TLC), high pressure liquid chromatography (HPLC), and infrared spectrum (IR) analysis (Scheme 1), the coupling was found to be complete in about 30 min.

The reaction mixture was filtered to remove the precipitated DCHA·HCl salt, and after simple workup, the peptide was isolated. The efficacy of HOBt·DCHA-mediated coupling is further demonstrated by the synthesis of dipeptides containing $C^{\alpha\alpha}$-dialkylglycines[22,23] (H_2N-CR^1R^2-COOH) with linear substitutents [α-aminoisobutric acid (Aib), diethylglycine (Deg), dipropylglycine (Dpg), and dibutylglycine (Dbg)] and cyclic substitutents [1-aminocyclopentane-1-carboxylic acid (Ac_5 c), and 1-aminocyclo hexane-1-carboxylic acid (Ac_6c), and 1-aminocycloheptane-1-carboxylic acid (Ac_7c)]. The duration of such couplings required about 2 h, and the resulting peptides were obtained in 65–72% yield (Table 1). Further, the coupling of Fmoc-amino acid–chloride mediated using HOBt·DCHA, and using an aqueous $NaHCO_3$ solution of amino acid, resulted in Fmoc-peptide acid with moderate yield. On the other hand, the use of bis-N,O-trimethylsilyl amino acid[24] gave good yields with analytically pure compounds (Scheme 2).



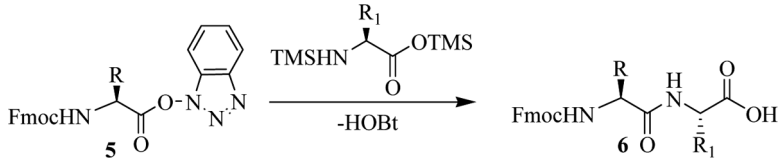
Scheme 1. Synthesis of N^{α}-Fmoc-protected peptide esters.

Table 1. List of peptides made by the HOBt·DCHA method

SI. no.	Peptide	Yield (%)	Mp (°C)	$[\alpha]_D^{25}$	(c1.0, DMF)
1	Fmoc-Phg-Phe-OMe	86	194–196		+24.0
2	Fmoc-D-Phg-Phe-OMe	87	194–194		−23.4
3	Fmoc-Leu-Ala-OMe	88	160–162		−28.0
4	Fmoc-Dpg-Dbg-OMe	72	gum		—
5	Fmoc-Aib-Aib-OMe	72	70–71		—
6	Fmoc-Ac_6c-Ac_6c-OMe	70	164–165		—
7	Fmoc-Deg-Leu-OMe	70	55–57		—
8	Fmoc-Ac_7c-Ac_5c-OMe	71	207–209		—
9	Fmoc-D-Phg-Phe-OH	82	175–177		+43.6
10	Fmoc-Tyr(Bzl)-Pro-OH	76	112–114		+24.6
11	Fmoc-Asp(OBn)-Ala-OH	80	148–149		+38.6
12	Fmoc-Ser(OBn)-Val-OH	78	120–122		−28.4

The ^{1}H NMR analysis of Carpino's diastereomeric dipeptides Fmoc-L or D-Phg-Phe-OMe[2] prepared by employing the present protocol indicated that the C-methylene doublets of phenylalanine and methyl ester singlets are distinct. Thus the coupling is free from racemization. Further, their HPLC analysis [R_t-values: L,L-isomer (13.19) and D,L-isomer (14.26)] also confirmed the same. Employing the present protocol for coupling and tris (2-aminoethyl) amine (TAEA) for deprotection of the Fmoc-group, the pentapeptide Fmoc-VPGVG synthesis has been accomplished. The purity of the final peptide Fmoc-VPGVG was satisfactory and was fully characterized by ^{1} H NMR and mass spectra. The yield was 72%.

Although Fmoc-amino acid fluorides[25–27] have been used with or without any organic base successfully as coupling agents in SPPS, the use of Fmoc-amino acid chlorides invariably requires an equimolar quantity of a base. Carpino et al. intially employed a 1:1 mixture of HOBt and pyridine [or diisopropylethylamine (DIEA) or N-methylmorpholine (NMM)] in the coupling. The addition of a base is well known to promote



Scheme 2. Synthesis of N^{α}-Fmoc-protected peptide acids.

one or more side reactions in Fmoc chemistry. The use of already known co-coupling agents (KOBt, KOAt, etc.) is not satisfactory in SPPS.

In the present studies, HOBt·DCHA can be used in SPPS also. This is mainly feasible because of two reasons: 1) HOBt·DCHA dissolves in DMF (or NMP) : CH_2Cl_2 (2 : 8) and converts Fmoc-amino acid chloride to its–OBt ester, and 2) the side product DCHA·HCl salt can be easily and completely removed, after coupling, by washing with a 1:1 mixture of $CHCl_3$ and CH_2Cl_2. The solid-phase synthesis of β-casomorphin using Wang resin as solid support and Fmoc-amino acid chlorides (3 equiv.) and HOBt·DCHA (3 equiv.) in DMF:CH_2Cl_2 (2:8) for coupling starting from Fmoc-Gly-O-CH_2-C_6H_4-O-resin was carried out. The crude peptide, on HPLC analysis, was found to be about 91% pure. It was further purified by RP-HPLC and found to be more than 99% pure. A simple coupling for 30 min was found to result in the completion of the coupling. Fmoc group was deprotected using 20% piperidine. After the removal of Fmoc group from Fmoc-Tyr(Bzl)-Pro-Phe-Pro-Gly-OCH_2-C_6H_4-O-resin, the peptide was cleaved by using TFA/H_2O/thioanisole/phenol mixtures at room temperature for 1.5 h and subjected to catalytic hydrogenation using 10% Pd/C/methanol/hydrogen gas to remove the Bzl group from the peptide, which was purified.

CONCLUSION

HOBt·DCHA is a stable and crystalline solid. Its preparation, isolation, and purification is very simple. It has been now demonstrated that the coupling of Fmoc-amino acid chlorides can be accomplished employing HOBt·DCHA under non–Schotten–Baumann conditions. The acylation was efficient, resulting in good yields. The scaling of coupling up to 10 mmol has not posed any practical difficulties. Thus, it has now been demonstrated that HOBt·DCHA finds utility in a solid-phase method for the synthesis of peptides employing Fmoc-amino acid chlorides under non–Schotten–Baumann conditions.

EXPERIMENTAL

The melting points were determined by the capillary method and are uncorrected. Specific rotations were recorded on a Rudolf Research Autopol IV automatic polarimeter. IR spectra were recorded on a Nicolet model impact 400D FT-IR spectrometer (KBr pellets, 3 cm^{-1} resolution) and ^1H NMR spectra on a Bruker 400-MHz instrument. Unless otherwise mentioned, all amino acids used have L-configuration. Analytical HPLC was performed on a Waters LC

3000 system equipped with Waters 484 tunable absorbance UV detector and millipore 745 data module. Water RP 48-deltapack column (3.9 mm × 300 mm, 15 μ spherical) was used for analysis. Thin-layer chromatography (TLC) analysis was carried out using the precoated silica gel G_{254} plates using 1) R_fA; $CHCl_3$–methanol–acetic acid 40:2:1; 2) R_fB; ethyl acetate–*n*hexane (35:65). Deprotonation of Fmoc group used TAEA, and mass spectra were obtained using a Kratos PCKompact SEQ V1.2.2 spectrometer.

Synthesis of HOBt·DCHA Salt (2)

Dicyclohexylamine (DCHA) (0.238 mL, 1.2 mmol) was added to a suspension of anhydrous HOBt (0.135 g, 1 mmol) in CH_2Cl_2 (5 mL) at rt and stirred for 1–2 h. Hexane (2 mL) was added and left for recrystallization. The resulting HOBt·DCHA crystals were filtered, washed with 10% CH_2Cl_2 in hexane (10 mL), and dried thoroughly to obtain 0.284 g (90%) of the title compound,[28] mp 137–139 °C; ^{1}H NMR ($CDCl_3$): δ 1.0–1.7 (12H, m), 1.7–2.1 (8H, m), 3.05 (2H, t), 7.25 (2H, m), 7.6–7.8 (2H, m);^{13}C NMR ($CDCl_3$):δ 24.8, 25.0, 29.3, 52.8, 53.0, 111.3, 118.4, 123.4, 127.7, 143.3; FAB $(M+1)^+$: m/z 317.4.

General Procedure for the Synthesis of N^{α}-Protected Peptide Esters (4)

To a previously stirred solution of Fmoc-amino acid chloride (1 mmol) and HOBt·DCHA (0.695 g, 2.2 mmol) in dry THF (5 mL), a suspension of amino acid ester hydrochloride salt (1 mmol) in THF (5 mL) was added, and the reaction mixture was stirred. The completion of the reaction was monitored by TLC. The reaction mixture was filtered and washed with organic solvent (THF, 10 mL) to remove DCHA·HCl as a white precipitate. The filtrate was concentrated, and CH_2Cl_2 (15 mL) was added. It was washed with 20% sodium bisulphate (10 mL × 3), to remove excess DCHA, water (10 mL × 2), 5% HCl (10 mL × 3), and water, then dried over anhydrous Na_2SO_4. The solvent was removed by evaporation under reduced pressure and product was crystallized using CH_2Cl_2–hexane (2:8) to yield the peptide.

Data

Fmoc-Phg-Phe-OMe: ^{1}H NMR ($CDCl_3$): δ 2.91 (d, 2H), 3.10 (t, 1H), 3.62 (s, 3H), 4.21 (t, 1H), 4.53 (d, 2H), 5.20 (d, 1H), 6.23 (d, 1H), 6.91 (1H, d),

7.30–7.90 (m, 18H);^{13}C NMR ($CDCl_3$): δ 38.2, 47.3, 52.5, 54.4, 66.8, 67.8, 120.1, 124.1, 126.3, 126.5, 127.2, 127.5, 128.2, 128.7, 130.9, 133.4, 135.8, 141.5, 144.0, 155.6, 167.5, 171.9; ES MS: m/z 535.3 $[M+H]^+$, Anal. calcd. for $C_{33}H_{30}N_2O_5$: C, 74.14; H, 5.66;N, 5.24. Found: C, 74.04; H, 5.61; N, 5.28%.

Fmoc-(D)Phg-Phe-OMe: ^{1}H NMR ($CDCl_3$): δ 2.81 (d, 2H), 3.10 (t, 1H), 3.62 (s, 3H), 4.20 (t, 1H), 4.51 (d, 2H), 5.21 (d, 1H), 6.22 (d, 1H), 6.90 (d, 1H), 7.28–7.90 (m, 18H); ^{13}C NMR ($CDCl_3$): δ38.3, 47.3, 52.3, 54.4, 66.8, 67.8, 120.0, 124.0, 126.2, 126.5, 127.2, 127.5, 128.2, 128.7, 130.9, 133.4, 135.8, 141.5, 144.0, 155.6, 167.5, 171.9; ES MS: m/z 535.3 $[M+H]^+$, Anal. calcd. for $C_{33}H_{30}N_2O_5$: C, 74.14;H, 5.66;N, 5.24. Found: C, 74;02; H, 5.60; N, 5.30%.

Fmoc-Leu-Ala-OMe: ^{1}H NMR ($CDCl_3$): δ 0.97 (d, 6H), 1.62 (m, 3H), 3.75 (s, 3H), 4.25 (t, 1H), 4.41 (t, 1H), 4.53 (d, 2H), 5.20 (m, 1H), 6.33 (m, 1H), 7.27–7.80 (m, 8H); ^{13}C NMR ($CDCl_3$): δ 17.4, 22.4, 22.9, 38.3, 46.5, 47.5, 52.3, 52.9, 66.8, 120.0, 125.1, 127.1, 141.5, 144.0, 155.6, 172.0, 173.1; ES MS: m/z 439.4 $[M+H]^+$, Anal. calcd. for $C_{25}H_{30}N_2O_5$: C, 68.47; H, 6.90; N, 6.39. Found; C, 68.41; H, 6.70; N, 6.21%.

Fmoc-Dpg-Dbg-Ome: ^{1}H NMR ($CDCl_3$): δ 0.81–2.02 (m, 40H), 3.60 (s, 3H), 4.11 (d, 1H), 4.20 (t, 2H), 6.51 (s, 1H), 6.92 (s, 1H), 7.20–7.81 (m, 8H);[13]C NMR ($CDCl_3$): δ 13.3, 14.0, 18.9, 22.3, 24.7, 37.6, 40.9, 47.4, 52.3, 61.2, 66.7, 66.9, 120.1, 124.0, 127.1, 127.2, 141.4, 144.0, 155.3, 170.2, 175.1; ES MS: m/z 565.6 $[M+H]^+$, Anal. Calcd. for $C_{34}H_{48}N_2O_5$: C, 72.31; H, 8.57; N, 4.96. Found:C, 72.10;H, 8.49;N, 4.90%.

Fmoc-Aib-Aib-Ome: ^{1}H NMR ($CDCl_3$): δ 1.00–1.21 (m, 12H), 3.80 (s, 3H), 4.11 (d, 1H), 4.31 (t, 2H), 6.92 (s, 1H), 7.42 (s, 1H), 7.20–7.80 (m, 8H); [13]C NMR ($CDCl_3$): δ 25.2, 26.9, 47.4, 52.1, 55.9, 60.7, 66.9, 120.0, 124.0, 127.0, 127.6, 141.5, 144.1, 155.3, 171.2, 175.3; ES MS: m/z 425.5 $[M+H]^+$, Anal. calcd. for $C_{24}H_{28}N_2O_5$: C, 67.91; H, 6.65; N, 6.60. Found: C, 67.90; H, 6.54; N, 6.50%.

Fmoc-Ac_6c-Ac_6c-Ome: ^{1}H NMR ($CDCl_3$): δ 1.11–2.30 (m, 20H), 3.71 (s, 3H), 4.20 (d, 1H), 4.52 (t, 2H), 6.50 (s, 1H), 7.01 (s, 1H), 7.20–7.80 (m, 8H);[13] C NMR ($CDCl_3$): δ 20.6, 24.1, 25.5, 33.3, 38.8, 47.5, 52.3, 60.8, 68.5, 66.8, 120.1, 124.1, 126.3, 127.2, 141.5, 144.7, 155.0, 170.1, 174.5; ES MS: m/z 505.5 $[M+H]^+$, Anal. calcd. for $C_{30}H_{36}N_2O_5$: C, 71.41;H, 7.19;N, 5.55. Found: C, 71.01; H, 7.08; N, 5.56%.

Fmoc-Deg-Leu-OMe: ^{1}H NMR ($CDCl_3$): δ 0.81–2.02 (m, 18H), 3.70 (s, 3H), 4.10 (d, 1H), 4.20 (d, 1H), 4.41 (t, 2H), 4.60 (d, 1H), 6.81 (s, 1H), 7.20–7.80 (m, 8H);^{13} C NMR ($CDCl_3$): δ 7.1, 23.0, 24.0, 35.8, 41.2, 47.3, 48.8, 52.3, 66.9, 70.0, 119.9, 124.0, 126.2, 127.3, 141.5,

144.4, 155.0, 171.8, 172.1; ES MS: m/z 481.4 $[M+H]^+$, Anal. calcd. for $C_{28}H_{36}N_2O_5$: C, 69.98; H, 7.55; N, 5.83. Found: C, 69.90;H, 7.51; N, 5.84%.

Fmoc-Ac$_7$c-Ac$_5$c-OMe: ^{1}H NMR ($CDCl_3$): δ 1.51–2.00 (m, 20H), 4.11 (s, 3H), 4.31 (t, 2H), 4.50 (d, 1H), 5.01 (s, 1H), 6.50 (s, 1H), 7.20–8.01 (m, 8H);[13]C NMR ($CDCl_3$): δ 21.6, 23.4, 25.9, 32.7, 39.6, 47.4, 52.3, 63.9, 65.3, 66.9, 120.0, 124.1, 126.2, 127.3, 141.4, 144.7, 155.3, 170.1, 175.1; ES MS: m/z 505.5 $[M+H]^+$, Anal. calcd. for $C_{30}H_{36}N_2O_5$: C, 71.41; H, 7.19; N, 5.55. Found: C, 71.32; H, 7.16; N, 5.60%.

General Procedure for the Synthesis of N^{α}-Protected Peptide Acids (6)

To a stirred solution of Fmoc-amino acid chloride (1 mmol) and HOBt·DCHA (0.695 g, 2.2 mmol) in dry THF (5 mL), a TMS activated amino acid (1.2 mmol) was added. Trimethylsilyl chloride (TMS-Cl, 1.2 mmol) and triethylamine (TEA, 2.5 mmol) were added to a stirred suspension of amino acid (1 mmol) in DCM (10 mL) and refluxed for 2–4 h. The completion of the reaction was monitored by TLC. The reaction mixture was concentrated to remove THF, and the residue was dissolved in sodium carbonate solution (10%, 15 mL). The aqueous phase was washed with ether (2 × 10 mL) and acidified using dil. HCl. The compound was extracted in ethyl acetate (2 × 10 mL). The organic phase was washed with water (2 × 10 mL) and brine (10 mL) and dried over anhydrous sodium sulphate. It was concentrated under vacuum to get the desired compound as a white solid in good yield.

Data

Fmoc-(D)Phg-Phe-OH: ^{1}H NMR (DMSO): δ 2.90 (d, 2H), 3.70 (m, 2H), 4.25 (m, 3H), 4.90 (d, 1H), 5.31 (d, 1H), 7.10–7.50 (m, 14H), 7.55 (d, 2H), 7.80 (d, 2H), 12.01 (br, 1H); ^{13}C NMR (DMSO): δ 36.6, 47.4, 49.4, 66.4, 74.9, 120.0, 124.0, 125.0, 126.3, 126.4, 126.8, 127.3, 128.4, 129.6, 130.9, 133.6, 140.7, 141.5, 144.5, 155.3, 166.5, 172.7; ES MS: m/z 521.3 $[M+H]^+$, Anal. calcd. for $C_{32}H_{28}N_2O_5$: C, 73.83; H, 5.42; N, 5.38. Found: C, 73.79; H, 5.43; N, 5.40%.

Fmoc-Tyr(Bn)-Pro-OH: ^{1}H NMR (DMSO): δ 2.12 (m, 4H), 3.00 (d, 2H), 3.51 (m, 2H), 3.82 (t, 1H), 4.20 (d, 2H), 4.40 (t, 1H), 5.80 (br, 1H), 7.20–7.90 (m, 17H), 12.5 (br, 1H); ^{13}C NMR (DMSO): δ 20.6, 29.1, 36.7, 47.4, 52.0, 58.4, 60.1, 69.4, 74.4, 118.9, 120.0, 124.2, 127.0, 127.2, 127.6, 128.2, 129.9, 130.6, 136.8, 141.5, 144.7, 153.4, 157.5, 163.6, 175.4; ES MS:

m/z 577.4 $[M+H]^+$, Anal. calcd. for $C_{35}H_{32}N_2O_6$: C, 72.90; H, 5.59; N, 4.86. Found: C, 72.82; H, 5.60; N, 4.88%.

Fmoc-Asp(OBn)-Ala-OH: ^{1}H NMR (DMSO): δ 1.21 (d, 3H), 2.60 (d, 2H), 3.61–3.72 (m, 2H), 4.20 (t, 1H), 4.31 (m, 2H), 4.80 (s, 2H), 5.30 (d, 1H), 5.70 (br, 1H), 7.10–7.52 (m, 9H), 7.61 (d, 2H), 7.80 (d, 2H), 8.00 (d, 1H), 12.4 (br, 1H); ^{13}C NMR (DMSO): δ 17.6, 39.3, 44.9, 47.2, 54.8, 63.8, 66.5, 120.0, 124.0, 126.2, 127.2, 128.1, 128.6, 141.5, 142.8, 144.7, 155.4, 169.9, 171.0, 175.9; ES MS: m/z 517.4 $[M+H]^+$, Anal. calcd. for $C_{29}H_{28}N_2O_7$: C, 67.43; H, 5.46; N, 5.42. Found: C, 67.40; H, 5.47; N, 5.45%.

Fmoc-Ser(OBn)-Val-OH: ^{1}H NMR (DMSO): δ 0.91 (t, 6H), 1.81 (m, 1H), 3.40–3.72 (m, 4H), 4.10 (t, 1H), 4.32 (d, 2H), 4.91 (s, 2H), 5.20 (d, 1H), 5.83 (br, 1H), 7.18–7.54 (m, 9H), 7.62 (d, 2H), 7.85 (d, 2H), 8.00 (d, 1H), 12.6 (br, 1H); ^{13}C NMR (DMSO): δ 18.9, 30.2, 47.4, 52.3, 58.1, 66.5, 69.7, 73.0, 120.0, 124.0, 126.5, 127.2, 127.9, 128.0, 128.3, 137.6, 141.5, 144.7, 155.0, 170.9, 128.3, 137.6, 141.5, 144.7, 155.0, 170.9, 174.8; ES MS: m/z 517.5 $[M+H]^+$, Anal. calcd. for $C_{30}H_{32}N_2O_6$: C, 69.75; H, 6.24; N, 5.42. Found: C, 69.62; H, 6.21; N, 5.39%.

Synthesis of Fmoc-Val-Pro-Gly-Val-Gly-OBzl

Fmoc-Val-Gly-Obn (I)

To H-Gly-OBn (1.65 g, 10 mmol) in THF (10 mL), a mixture of Fmoc-Val-Cl (3.6 g, 10 mmol) and HOBt·DCHA (3.5 g, 11 mmol) in THF (20 mL) was added and stirred untill the completion of reaction. The workup of the reaction mixture followed by column purification gave the peptide (I) as a white crystalline solid (4.3 g, 90%); mp 182–184 °C; $[\alpha]^{25}_D + 32.2^\circ$ (c 1.0, $CHCl_3$); R_fA, 0.51; R_fB, 0.65; ^{1}H NMR ($CDCl_3$): δ 0.90–1.01 (d, $J = 6.4$ Hz, 6H), 1.29 (m, 1H), 3.81 (m, 1H), 4.20–4.40 (m, 4H), 5.00 (s, 2H), 6.70 (m, 1H), 7.20–7.43 (m, 9H), 7.58 (d, $J = 6.9$ Hz, 2H), 7.76 (d, $J = 6.8$ Hz, 2H); ^{13}C NMR ($CDCl_3$): δ 18.6, 29.1, 40.4, 47.3, 55.1, 67.4, 69.5, 119.9, 124.1, 126.4, 126.8, 127.2, 127.4, 128.7, 133.5, 141.5, 143.4, 155.2, 169.4, 172.4; ES MS: m/z 509.3 $[M+Na]^+$, Anal. calcd. for $C_{29}H_{30}N_2O_5$: C, 71.59; H, 6.21; N, 5.76, Found: C, 71.55; H, 6.19; N, 5.73%.

Fmoc-Gly-Val-Gly-OBn (II)

Fmoc-Gly-Cl (2.7 g, 8.8 mmol) and HOBt·DCHA (3.1 g, 9.7 mmol) in THF (20 mL), were added to a solution of free peptide H-Val-Gly-OBn

(2.3 g, 8.8 mmol), obtained by the deprotection of I (4.3 g, 9 mmol) using TAEA[4] (45 mL) in THF (10 mL). Workup of the reaction mixture followed by column purification gave the peptide (II) as a white crystalline solid (4.4 g, 88%); mp 176–179 °C; $[\alpha]^{[25]}_D + 30.4°$(*c* 1.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 0.90–1.01 (d, $J = 6.3$ Hz, 6H), 1.30 (m, 1H), 3.67 (m, 4H), 3.93 (m, 1H), 4.20–4.45 (m, 5H), 5.01 (s, 2H), 6.20 (d, 1H), 7.20–7.81 (m, 13H), ^{13}C NMR ($CDCl_3$): δ 18.6, 29.1, 40.4, 46.2, 47.1, 57.5, 67.4, 69.5, 119.9, 124.1, 126.2, 126.9, 127.2, 127.4, 128.7, 133.2, 141.5, 143.4, 157.1, 165.4, 169.4, 173.4; ES MS: m/z 566.2 $[M + Na]^+$, Anal. calcd. for $C_{31}H_{33}N_3O_6$: C, 68.49; H, 6.12; N, 7.73, Found: C, 68.55; H, 6.19; N, 5.79%.

Fmoc-Pro-Gly-Val-Gly-OBn (III)

Fmoc-Pro-Cl (2.6 g, 8.0 mmol) and HOBt·DCHA (2.78 g, 8.8 mmol) in THF (20 mL) were added to a solution of the free peptide H-Gly-Val-Gly-OBn (2.2 g, 8.0 mmol), obtained by the deprotection of the peptide (II) (4.4 g, 8.1 mmol) using tris(2-aminoethyl)amine (TAEA) (40 mL) in THF (10 mL). Workup of the reaction mixture followed by column purification gave the title product (III) as a crystalline solid (4.6 g, 78%) mp 158–60 °C; $[\alpha]^{25}_D + 24.2°$(*c* 1.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 0.90–1.01 (d, $J = 6.7$ Hz, 6H), 1.29 (m, 1H), 1.73–2.24 (m, 6H), 3.67 (m, 4H), 3.93 (m, 1H), 4.11 (t, 1H), 4.20–4.45 (m, 5H), 5.00 (s, 2H), 5.10 (d, 1H), 6.20 (d, 1H), 7.20–7.81 (m, 13H); ^{13}C NMR ($CDCl_3$): δ 18.6, 26.8, 28.4, 29.4, 40.2, 43.6, 46.9, 47.3, 57.5, 59.4, 67.4, 69.6, 119.9, 124.1, 126.4, 127.0, 127.2, 127.4, 128.9, 133.3, 141.5, 143.7, 165.5, 167.8, 167.9, 169.4, 170.2; ES MS: m/z 663.7 $[M + Na]^+$, Anal. calcd. for $C_{36}H_{40}N_4O_7$: C, 67.48; H, 6.29; N, 8.74, Found: C, 67.20; H, 6.29; N, 8.73%.

Fmoc-Val-Pro-Gly-Val-Gly-OBn (IV)

Fmoc-Val-Cl (2.3 g, 7.0 mmol) and HOBt·DCHA (2.43 g, 7.7 mmol) in THF (20 mL)were added to a solution of the free peptide H-Pro-Gly-Val-Gly-OBn (2.91 g, 7.0 mmol), obtained by the deprotection of the peptide (III) (4.6 g, 7.1 mmol) using TAEA (35 mL) in THF (10 mL). Workup of the reaction mixture followed by column purification gave the title product (IV) as a crystalline solid (4.6 g, 67%); mp 98–100 °C; $[\alpha]^{25}_D$–18.7° (*c* 1.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 0.89–1.10 (m, 12H), 1.28 (m, 2H), 1.73–2.24 (m, 6H), 3.65 (d, 1H), 3.95 (m, 1H), 4.02 (d, 2H), 4.13 (d, 2H), 4.18 (m, 3H), 4.20–4.40 (m, 2H), 4.45 (t, 1H), 5.01 (s, 2H), 5.10 (m, 2H), 6.01 (m, 1H), 7.20–7.84 (m, 13H); ^{13}C NMR ($CDCl_3$): δ 18.6, 18.8, 27.0, 27.3, 30.1, 30.2, 40.1, 42.4, 47.1, 48.4, 54.6,

55.9, 58.6, 67.4, 69.4, 119.9, 124.1, 126.2, 127.0, 127.3, 127.5, 128.7, 133.5, 141.5, 143.4, 155.2, 165.4, 167.8, 167.9, 168.4, 170.4; ES MS: m/z 763.7 $[M + Na]^+$, Anal. calcd. for $C_{41}H_{49}N_5O_8$: C, 66.56; H, 6.68; N, 9.47, Found: C, 66.47; H, 6.57; N, 9.40%.

Synthesis of β-Casomorphin (H-Tyr-Pro-Phe-Pro-Gly-OH)

The peptide was synthesized using manual Fmoc-chemistry with a solid-phase synthesis strategy on Wang resin (substitution 0.6 mmol/g) employing Fmoc-Tyr(OBzl)-COCl (0.92 mg, 1.8 mmol, 3 eq), Fmoc-Pro-COCl (0.64 mg, 1.8 mmol, 3 eq), Fmoc-Phe-COCl (0.72 mg, 1.8 mmol, 3 eq), Fmoc-Pro-COCl (0.64 mg, 1.8 mmol, 3 eq), and HOBt·DCHA (0.63 mg, 1.98 mmol, 3.1 eq) in DMF–CH_2Cl_2 (10 mL, 1:1). The synthesis was carried out using 1 g of Fmoc-Gly-Wang resin. A single coupling for 30 min was sufficient for the completion of each coupling. The DCHA·HCl formed as a white precipitate was removed by washings using $CHCl_3$–CH_2Cl_2 (1:1). After deprotection of Fmoc group using 20% pipyridine in DMF, the peptide was cleaved using trifluroacetic acid (TFA) (10 mL)/ H_2O (0.5 mL)/thioanisole (0.5 mL)/ phenol (0.75 g) mixture at room temperature for 1 h. The crude peptide was subjected to catalytic hydrogenation using 10% Pd/C (50 mg)/ methanol (10 mL)/hydrogen gas to remove the benzyl group and was purified by RP-HPLC to obtain the final peptide (275 mg, 79%). Mp 151–153 °C; $[\alpha]^{[25]}_D$–48.5 ° (c 0.5, DMF); ^{1}H NMR ($CDCl_3$): δ 1.10–2.10 (m, 14H), 2.31 (t, 2H), 2.90 (t, 2H), 3.11 (t, 1H), 5.20 (br, 1H), 5.40 (br, 1H), 5.71 (br, 1H), 7.20–8.10 (m, 9H), 12.0–13.0 (1H, COOH); ^{13}C NMR ($CDCl_3$): δ 27.3, 29.0, 33.9, 35.4, 36.1, 50.6, 51.3, 51.8, 54.3, 58.1, 58.8, 127.1, 128.9, 129.4, 129.5, 130.1, 130.2, 140.1, 155.4, 166.0, 168.6, 169.4, 170.3, 177.2; ES MS: m/z 602.65 $[M + Na]^+$, , Anal. calcd. for $C_{30}H_{37}N_5O_7$: C, 62.16; H, 6.43; N, 12.08, Found: C, 62.12;H, 6.40; N, 12.00%; R, 8.45 [(Waters C-18 deltapak column (3.9 × 300 mm, 15μ); using eluent acetonitrile 0.1% trifluoroacetic acid (TFA) and water (65:35; isocratic, flow rater 1 mL/min, monitoring at 220 nm).

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