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Papers

HOAt.DCHA as co-coupling agent in the synthesis of peptides employing Fmoc-amino acid chlorides as coupling agents: Application to the synthesis of β-casomorphin

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A simple, efficient and racemization free method for the synthesis of peptides employing Fmoc-amino acid chlorides mediated by HOAt.DCHA as a co-coupling agent has been described. This protocol is successfully employed in the synthesis of the pentapeptide H-Pro-Gly-Val-Gly-Val-OH (PGVGV), and β-casomorphin (H-Tyr-Pro-Phe-Pro-Gly-OH) in 85 and 80% yields, respectively.

Keywords: Amino acid chlorides, 1-hydroxy-7-azabenzotriazole, co-coupling agent, non Schotten-Baumann condition, racemization free coupling

In the long journey of about 105 years of peptide chemistry, Emil Fischer in 1901, successfully made the famous octadecapeptide {L-Leu-$(Gly)_3$-L-Leu-$(Gly)_3$-L-Leu-$(Gly)_8$-Gly}using ethoxycarbonyl amino acid chlorides[1]. Leuch's studies on acid chlorides gave birth to *N*-carboxyanhydrides derived from amino acids[2]. This study had made Boc- as well as Z-amino acid chlorides obsolete in aminolysis of amino acid esters. There are sporadic reports on 'acid chlorides-peptide synthesis' combination making use of non-urethane type *N*-protecting groups trifluoroacetyl and phthaloyl, but are far from being used in practice[3,4]. 'Acid chlorides-Fmoc chemistry' as partners, one for activation and the other for amino protection has percolated down as an efficient tool for 'peptide bond formation'. They have occupied a place of their own along with the four pillars namely acid azides, mixed anhydrides, active esters and coupling agents in the construction of peptides, *i.e.*, as the C-component required for the condensation process in the coupling.

Fmoc-amino acid chlorides were discovered by Louis Carpino of University of Massachusetts, U.S.A[5]. Their properties have made them useful intermediates for both solution and solid phase synthesis of peptides[6-9]. The high reactivity, shelf stability, stereomutation free coupling, ease of preparation are some of their characteristic features. In addition, the low cost of thionyl chloride, particularly in comparison with each one of all the other peptide coupling agents, has enhanced the popularity of acid chlorides[10]. They are the first and best choice of reagents for the incorporation of hindered amino acids such as dialkylamino acids, N-methylamino acids as well as N-methyl dialkyl amino acids[11] (eg., N-Me-Aib). Enkephalins[12], dermorphins[12], deltrophins[13], acyl carrier protein fragment (ACP 65-74) (ref. 9), prothrombin (1-9) (ref. 9), eledoisin[9], substance P analogs[9], β-casomorphin[14], laminin pentapeptide fragment[14], cyclosporine fragments[15,16] and oxytocin and its analogs[17] are some of the biologically active peptides that have been made by the acid chloride method. Sureshbabu *et al*., recently accomplished the total synthesis of the immunosuppressant drug cyclosporin O by the stepwise assembly in solution using Fmoc-amino acid chlorides[18]. All the ten protected fragments, starting from di to undecapeptide fragments were isolated and characterized. The protocol for the combinatorial synthesis of indole and indoline alkaloids[19], insertion of sterically hindered piperazic acid into cyclic peptides[20] as well as in the synthesis of pseudopeptides containing N-(hydroxyl)amide bond and hydroxamate peptides {H-Aaa_1-ψ[CON(OH)]-Aaa_2-}[21] also involved Fmoc-amino acid chlorides as key components.

Hydrogen chloride liberated during the coupling process employing Fmoc-amino acid chlorides, needs to be captured which otherwise will react with unreacted amino component resulting in decrease in the rate of acylation as well as incomplete couplings leading to loss of yields of the condensation products. Initially, Carpino *et al*., accomplished coupling reaction of Fmoc-amino acid chlorides in a two phase system using an inorganic base (5% Na_2CO_3 or 10% $NaHCO_3$) in the aqueous layer[6,7]. Under 1these conditions, the reaction was complete within a few minutes and proceeded without loss of configuration at the carboxyl component. As in regular organic synthesis, the use of an equimolar quantity of organic base (hindered tertiary base) for HCl abstraction has been tried during peptide bond formation to accomplish the synthesis in homogeneous one phase system or under solid phase conditions[8]. It resulted in two side reactions namely, racemization *via* oxazole-5(4*H*)-one and premature de-blocking of Fmoc moiety leading to the formation of higher homologous peptide sequences. To improve the product yield as well as purity, a hindered base 2,6-di-*tert*-butyl-pyridine was tried but with limited success[9]. Alternatively, our group introduced the concept of the use of co-coupling agent along with acid chlorides to accomplish the coupling in homogeneous systems (CH_2Cl_2 or $CHCl_3$ or THF) under non Schotten-Baumann conditions.

Thus, the potassium salt of 1-hydroxybenzotriazole (KOBt) and 1-hydroxy-7-aza benzotriazole (KOAt) were employed in peptide synthesis for the first time in 1992 (Ref. 22) and in 1998 (Ref. 23) respectively. It was reasoned that the coupling mediated by these salts, on reaction with acid chloride, results in *in situ* generation of benzotriazole ester (Fmoc-AA-OBt) which participates rapidly as an efficient coupling agent during aminolysis. The other product KCl precipitates out from the reaction-mixture and was removed by filtration, hence ensuring easy peptide isolation. On the other hand, the base catalysed side reactions namely stereomutation and premature deblocking of Fmoc group were circumvented. Consequently, by extending the duration of the reaction, the coupling of sterically hindered amino acids was accomplished successfully, namely the assembly of Aib tetramer (Fmoc-Aib-Aib-Aib-Aib-OMe) was carried out[11,24]. Few years later, 1-(t-butyldimethylsilyloxy)benzotriazole (TBDMS-OBt) and 1-(t-butyldiphenylsilyloxy) benzotriazole (TBDPS-OBt)[25,26] were also employed in acid chloride coupling. It was envisaged that the reaction product TBDMS-Cl/TBDPS-Cl dissolves in organic solvent and the product isolation in such coupling reaction becomes much easier. In addition, the protocol can be applied to solid-phase conditions as well. An off shoot of these studies is the synthesis, isolation and final structural determination of HBTU and HATU as two distinguishable unambiguous forms by Carpino group[27]. Thus the use of KOBt/KOAt and HOBt/HOAt-base mixture in separate experiments lead to the isolation of guanidinium and uronium forms of HBTU and HATU.

Parallely, the metal mediated Fmoc-amino acid chloride coupling was also initiated by Sureshbabu and Gopi[28,29]. The commercially available, activated zinc dust mediated acid chloride coupling was carried out. It permits the direct coupling of Fmoc-amino acid chloride to amino acid ester to obtain the desired peptide along with the formation of $ZnCl_2$ and H_2 gas. The use of such non Schotten-Baumann condition in peptide bond formation circumvents the formation of HCl as one of the products. Moreover, highly hindered couplings can also be accomplished in presence of zinc dust. A recent example in this category is the synthesis of Fmoc-Phe-*N*Bn-Aib-OEt in 61% yield under microwave irradiation condition[30]. In an altogether different approach, there are several reports on the use of AgCN in acid chloride coupling but the exact mechanism is not clear[31-33]. HOAt introduced by Carpino, compared to that of HOBt is proved to be more effective additive which accelerates the peptide coupling and reduces the loss of chiral integrity because of anchemeric assistance of 7-nitrogen atom in the active HOAt-ester intermediate. The derivatives of HOAt such as HATU are used to couple sterically hindered *N*-methyl and α,α-disubstituted aminoacids. In this paper, we describe the utility of dicyclohexylamine salt of 1-hydroxy-7-aza benzotriazole (HOAt.DCHA, **Figure 1**) as an efficient co-coupling agent in the peptide synthesis using Fmoc amino acid chlorides under non Schotten-Baumann conditions.

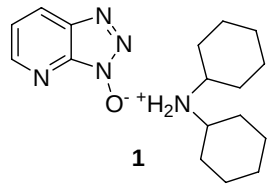
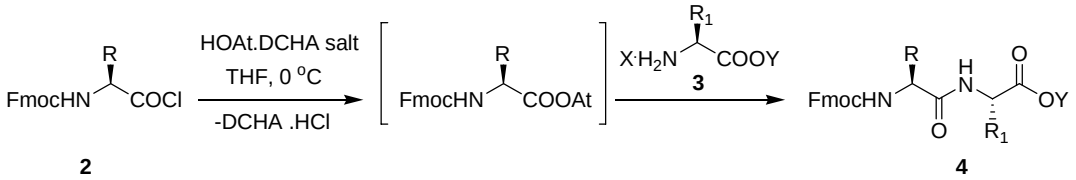


Figure 1— Dicyclohexylamine salt of 1-hydroxy-7-aza benzotriazole

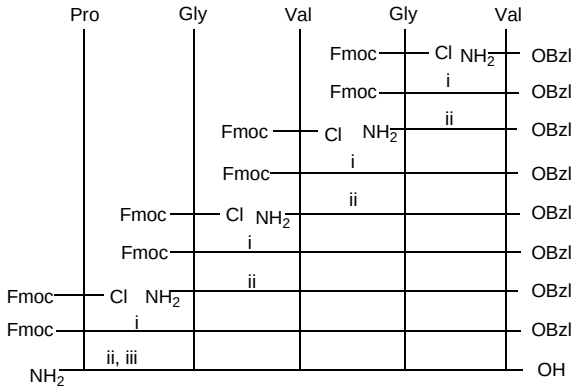


Scheme I — Synthesis of peptides employing HOAt.DCHA

Table I — N^{α}-Fmoc-peptide esters

Peptide	m.p. (ºC)	Yield (%)	Mass[a] $[M+Na]^+$ (calcd/found)
Fmoc-L-Phg-Phe-OMe (**4a**)	193-95	97	557.2 / 557.1
Fmoc-D-Phg-Phe-OMe (**4b**)	194-96	94	557.2 / 557.2
Fmoc-Ac_7c-Dbg-OMe (**4c**)	131-33	79	653.3 / 653.4
Fmoc-Dpg-Dpg-OMe (**4d**)	128-30	77	559.3 / 559.1
Fmoc- Pro-Gly-OBzl (**4e**)	110-12	80	507.2 / 507.5
Fmoc-Tyr(Bzl)-Pro-OMe (**4f**)	198-00	75	537.2 / 537.2
Fmoc-Aib-Aib-OMe (**4g**)	74-76	72	523.2 / 523.3
Fmoc-Leu-Val-OBzl (**4h**)	110-02	95	565.3 / 565.2

[a] ESI-MS



(i) For coupling of amino acids - HOAt.DCHA;
(ii) For deprotection of Fmoc group - 50% (v/v) DEA: CH_2Cl_2;
(iii) For benzyl deprotection - 10% Pd-C/MeOH

Scheme II — Schematic representation of the synthesis of H-Pro-Gly-Val-Gly-Val-OH

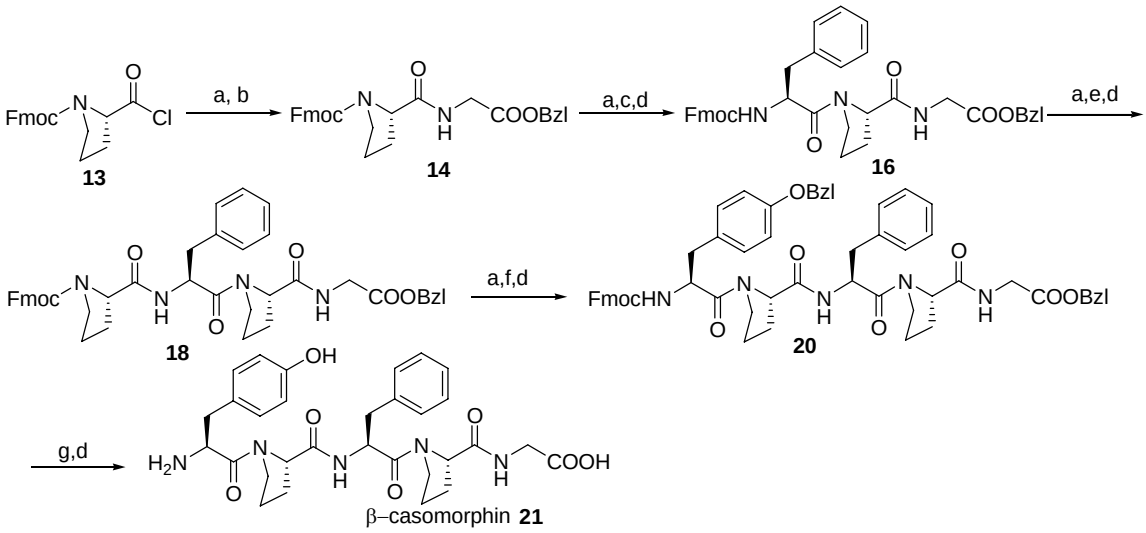
Results and Discussion

Initially, the co-coupling agent **1** was prepared by adding dicyclohexylamine (DCHA) to a solution of 1-hydroxy-7-aza benzotriazole (HOAt) in dry CH_2Cl_2 and stirred at RT for 2 hr. The resulting salt was filtered and recrystallized. In a typical reaction, to a solution containing Fmoc-amino acid chloride **2** (1.0 eq) in dry THF was added HOAt.DCHA (1.2 eq) and stirred for 5 min during which the –OAt ester was formed as confirmed by the appearance of a peak at 1820 cm^{-1} and simultaneous disappearance of acid chloride peak around 1786 cm^{-1} in the IR spectrum. Amino acid ester salt **3** was then added, stirred and the coupling was found to be complete in about an hour (**Scheme I**) (as monitored by TLC).

The precipitated DCHA.HCl salt was filtered and washed with THF. The resulting filtrate was extracted into ethyl acetate. A simple work-up and recrystallization afforded the desired peptide **4** in excellent yield. A series of dipeptides **4a-h**, containing sterically hindered amino acids that are difficult to assemble were synthesized in good yield (**Table I**).

Further, to demonstrate the efficacy and applicability of HOAt.DCHA mediated peptide coupling, a pentapeptide H-Pro-Gly-Val-Gly-Val-OH **12** was synthesized in solution phase. The pentapeptide synthesis began with the coupling of Fmoc-Gly-Cl with valine benzyl ester mediated by HOAt.DCHA. The dipeptide Fmoc-Gly-Val-OBzl **5** was obtained in 95% yield. Next, the Fmoc group was deprotected using 50% diethylamine in CH_2Cl_2 and coupled with Fmoc-Val-Cl to obtain the tripeptide **7** (Fmoc-Val-Gly-Val-OBzl). Again, the protocol was repeated to extend the length of the peptide to tetra **9** and finally to pentapeptide Fmoc-Pro-Gly-Val-Gly-Val-OBzl **11** in good yield. Then both the terminals of the peptide **11** were made free by removing the benzyl ester by catalytic hydrogenation and Fmoc-group using 50% DEA in CH_2Cl_2. The free peptide H-Pro-Gly-Val-Gly-Val-OH **12** was obtained in 85% yield (**Scheme II**). The purity of the obtained peptide **12**, as adjudged by HPLC, was found to be above 98%.

The present protocol, namely HOAt.DCHA mediated peptide synthesis was then extended for the synthesis of β-casomorphin, an opioid pentapeptide **21**. Initially glycine benzyl ester *p*-toluene sulfonic acid salt was coupled with Fmoc-Pro-Cl **13** in the presence of HOAt.DCHA salt in THF. The dipeptide **14** was obtained in 81% yield with excellent purity. The Fmoc-group was deprotected and coupled with Fmoc-Phe-Cl. Finally the same reaction sequence was repeated to obtain the protected pentapeptide β-casomorphin **20** in 80% yield (**Scheme III**). The



a: HOAt.DCHA; b: H-Gly-OBzl; C: Fmoc-Phe-Cl; d: 50% DEA, DCM; e: Fmoc-Pro-Cl; f: Fmoc-Tyr(Bzl)-Cl; g: 10% pd/C, MeOH

Scheme III — Synthesis of β-casomorphin **21**

benzyl ester of the Fmoc protected pentapeptide **20** was deprotected through catalytic hydrogenation using 10% Pd on carbon for 3 hr. Finally, Fmoc-group was removed by using 50% DEA in CH_2Cl_2 to obtain the β-casomorphin **21** in 80% yield.

Racemization studies were carried out by synthesizing diastereomeric dipeptides, Fmoc-L-Phg-Phe-OMe **4a** and Fmoc-D-Phg-Phe-OMe **4b** wherein the ^{1}H NMR indicated C-methylene doublets and the methyl ester singlet for **4a** at δ 3.26 and 3.28 (d, $CHCH_2C_6H_5$) and 3.67 (s, OCH_3) while for **4b** at δ 3.10 and 3.12 (d, $CHCH_2C_6H_5$) and 3.78 (s, OCH_3) indicating that the coupling was free from racemization.

Conclusion

In summary, the peptide synthesis was carried out efficiently by employing Fmoc-amino acid chlorides in the presence of a new co-coupling agent HOAt. DCHA under non-Schotten-Baumann conditions. The acylation using this method was found to be very efficient leading to the peptide in very good yield which has been well demonstrated through the synthesis of H-Pro-Gly-Val-Gly-Val-OH and β-casomorphin. Moreover, HOAt.DCHA can be easily prepared as a crystalline solid and can be stored for long time. In addition, isolation of the product in this coupling is very easy as the DCHA.HCl salt can be removed by simple filtration and also circumvents the formation of other by-products.

Experimental Section

All chemicals were commercial products of the best grade available. Melting points were determined on a Buchi model 150 melting point apparatus in open capillaries and are uncorrected. IR spectra were recorded on a Nicolet model impact 400 D FT-IR spectrometer (KBr pellets, 3 cm^{-1} resolution). ^{1}H NMR spectra were recorded on a Bruker AMX 300 MHz spectrometer. The microwave reaction was carried out in a LG MS 194A microwave oven producing microwave radiation with a frequency of 2450 MHz.

Procedure for the synthesis of HOAt.DCHA, 1

To a solution of HOAt (1.0 mmole) in dry CH_2Cl_2 (5 mL), was added DCHA (2.0 mL, 1.0 mmole) at RT and stirred for 2 hr. CH_2Cl_2 and *n*-hexane were added and kept for recrystallization. The resulting HOAt.DCHA salt was filtered, washed with hexane (2 × 5 mL) and dried to obtain the title compound in 97% yield. m.p. 145-47°C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.82 - 1.34 (m, 12H), 1.72 - 1.95 (m, 8H), 3.15 (m, 2H), 7.25 - 7.65 (m, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 24.8, 25.0, 29.6, 53.8, 125.2, 127.1, 135.7, 142.3. MS (MALDI-TOF) *m/z* Anal. Calcd for $C_{17}H_{27}N_5O$, $[M + Na]^+$: 340.2. Found: 340.3.

General procedure for the synthesis of N-Fmoc α-amino acid chlorides

To a solution of Fmoc-amino acid (5 mmole) in CH_2Cl_2 (20 mL) was added $SOCl_2$ (5.1 mmole) and

the solution was subjected to ultrasonication for 30 min. Completion of the reaction was monitored by TLC and after completion the solvent was removed under *vacuo* and the product was obtained as a solid.

General procedure for the synthesis of N^{α}–Fmoc-protected peptide esters employing Fmoc-amino acid chlorides mediated by HOAt.DCHA, 4

To a stirred suspension of Fmoc-amino acid chloride (1.0 mmole) and HOAt.DCHA (1.0 mmole) in dry THF (5 mL), was added amino acid ester (1.0 mmole) in THF. The resulting reaction-ixture was stirred at RT for 3-4 hr. The completion of the reaction was identified by TLC. The resulting DCHA.HCl salt was removed by simple filtration, and the filtrate was concentrated *in vacuo.* The crude was taken into 20 mL of CH_2Cl_2, and the organic layer was washed with 5% HCl solution, $NaHCO_3$ (10%) solution, brine and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure to afford the desired peptide, which was recrystallized from CH_2Cl_2 and *n*-hexane.

General procedure for deprotection of Fmoc-group

To a solution of Fmoc protected peptide ester (1.0 mmole) in dry CH_2Cl_2 (4 mL) an equal volume of freshly distilled DEA (4 mL) was added and stirred at RT for about 30 min under nitrogen atmosphere. The completion of the deprotection was monitored through TLC. The reaction mass was then concentrated *in vacuo* at low temperature. The residue was dissolved in 4 mL CH_2Cl_2 and concentrated again to afford peptide ester completely free from DEA, which was further dried under high vacuum for 30 min. It was used directly for the next step without any purification and isolation.

Fmoc-L-Phg-Phe-OMe 4a: m.p. 195-97°C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.27 (d, *J* = 5.8 Hz, 2H), 3.65 (s, 3H), 4.15 (m, 1H), 4.25 (t, *J* = 5.7 Hz, 1H), 4.50 (d, *J* = 5.7 Hz, 2H), 4.85 (m, 1H), 5.95 (m, 1H), 6.26 (br, 1H), 7.20 - 7.95 (m, 18H); ESI-MS *m/z* Anal. Calcd. for $C_{33}H_{30}N_2O_5$, $[M + H]^+$: 535.2, $[M + Na]^+$: 557.2. Found: $[M + H]^+$: 535.4, $[M + Na]^+$: 557.1.

Fmoc-D-Phg-Phe-OMe 4b: m.p. 194-96°C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.11 (d, *J* = 5.8 Hz, 2H), 3.55 (s, 3H), 4.15 (m, 1H), 4.25 (t, *J* = 5.7 Hz, 1H), 4.50 (d, *J* = 5.7 Hz, 2H), 4.85 (m, 1H), 5.95 (m, 1H), 6.26 (br, 1H), 7.20 - 7.95 (m, 18H); ESI-MS *m/z* Anal. Calcd. for $C_{33}H_{30}N_2O_5$, $[M + H]^+$: 535.2, $[M + Na]^+$: 557.2. Found: $[M + H]^+$: 535.4, $[M + Na]^+$: 557.2.

Fmoc-Ac$_7$c-Dbg-OMe 4c: m.p. 131-33°C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.85 - 1.98 (m, 12H), 3.18 (d, *J* = 5.7 Hz, 2H), 3.60 (s, 3H), 3.75 (d, *J* = 5.7 Hz, 2H), 4.29 (t, *J* = 5.6 Hz, 1H), 4.55 (d, *J* = 5.6 Hz, 2H), 5.80 (br, 1H), 7.19-7.90 (m, 18H); ESI-MS *m/z* Anal. Calcd. for $C_{40}H_{42}N_2O_5$, $[M + H]^+$: 631.3, $[M + Na]^+$: 653.3. Found: $[M + H]^+$: 631.5, $[M + Na]^+$: 653.4.

Fmoc-Dpg-Dpg-OMe 4d: m.p. 128-30°C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.85 - 2.05 (m, 18H), 3.65 (s, 3H), 4.21 (d, *J* = 5.8 Hz, 2H), 4.57 (t, *J* = 5.9 Hz, 1H), 6.25 (br, 1H), 7.10-7.95 (m, 8H); ESI-MS *m/z* Anal. Calcd. for $C_{32}H_{44}N_2O_6$, $[M + H]^+$: 537.3, $[M + Na]^+$: 559.3. Found: $[M + H]^+$: 537.4, $[M + Na]^+$: 559.1.

Fmoc- Pro-Gly-OBzl 4e: m.p. 60-62°C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.23 - 2.05 (m, 4H), 3.50 (m, 2H), 4.02 (m, 2H), 4.29 (d, *J* = 5.9 Hz, 2H), 4.55 (t, *J* = 5.8 Hz, 1H), 5.23 (s, 2H), 5.75 (br, 1H), 7.15 - 7.85 (m, 13H); ESI-MS *m/z* Anal. Calcd. for $C_{29}H_{28}N_2O_5$, $[M + H]^+$: 485.2, $[M + Na]^+$: 507.2. Found: $[M + H]^+$: 485.1, $[M + Na]^+$: 507.5.

Fmoc-Tyr(Bzl)-Pro-OMe 4f: m.p. 197-99°C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.25 - 1.75 (m, 4H), 2.75 - 3.10 (m, 4H), 3.65 (s, 3H), 4.25 (d, *J* = 5.8 Hz, 2H), 4.59 (t, *J* = 5.7 Hz, 1H), 5.35 (m, 1H), 5.75 (s, 2H), 6.25 (br, 1H), 7.05 - 7.95 (m, 17H); ESI-MS *m/z* Anal. Calcd. for $C_{30}H_{30}N_2O_6$, $[M + H]^+$: 515.2, $[M + Na]^+$: 537.2. Found: $[M + H]^+$: 515.2, $[M + Na]^+$: 537.2.

Fmoc-Aib-Aib-OBzl 4g: m.p. 129-31°C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.29 (s, 6H), 1.45 (s, 6H), 4.22 (d, *J* = 5.9 Hz, 2H), 4.57 (t, *J* = 5.9 Hz, 1H), 5.15 (s, 2H), 6.45 (br, 1H), 7.10 – 8.05 (m, 13H); ESI-MS *m/z* Anal. Calcd. for $C_{30}H_{32}N_2O_5$, $[M + H]^+$: 501.2, $[M + Na]^+$: 523.2. Found: $[M + H]^+$: 501.1, $[M + Na]^+$: 523.3.

Fmoc-Leu-Val-OBzl 4h: m.p. 125-27°C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.95 - 1.10 (m, 12H), 1.53 - 1.67 (m, 3H), 1.95 (m, 1H), 4.05 (m, 1H), 4.19 (d, *J* = 5.6 Hz, 2H), 4.54 (t, *J* = 5.6 Hz, 1H), 4.95 (m, 1H), 5.34 (s, 2H), 5.90 (br, 1H), 6.35 (m, 1H), 7.20 – 7.85 (m, 13H); ESI-MS *m/z* Anal. Calcd. for $C_{33}H_{38}N_2O_5$, $[M + H]^+$: 543.3, $[M + Na]^+$: 565.3. Found: $[M + H]^+$: 543.1, $[M + Na]^+$: 565.2.

Synthesis of H-Pro-Gly-Val-Gly-Val-OH 12

Fmoc-Gly-Val-OBzl 5: To a suspention of Fmoc-Gly-Cl (3.5 g, 11.1 mmole) and HOAt.DCHA (3.8 g,

12.21 mmole) in THF (10 mL) was added valine benzyl ester (2.3g, 11.1 mmole). After the completion of the reaction followed work-up as mentioned in the general procedure gave the title product **5** as a pale white solid, yield 4.86 g, (90%); m.p. 108-10°C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.06 (d, J = 6.7 Hz, 6H), 1.95 (m, 1H), 3.87 (d, J = 5.6 Hz, 2H), 4.13 (m, 1H), 4.20 (t, J = 5.4 Hz, 1H), 4.45 (d, J = 6.1 Hz, 2H), 5.21 (s, 2H), 6.27 (br, 1H), 7.00 (br, 1H), 7.18 - 7.85 (m, 13H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 16.5, 28.3, 39.8, 46.9, 48.2, 57.4, 60.2, 119.5, 120.1, 120.7, 121.0, 121.4, 123.3, 126.7, 127.4, 137.9. 142.5, 157.4, 169.6, 171.1; ESI-MS *m/z* Anal. Calcd. for $C_{29}H_{30}N_2O_5$, $[M + H]^+$: 486.2, $[M + Na]^+$: 509.2. Found: $[M + H]^+$: 486.1, $[M + Na]^+$: 509.2.

H-Gly-Val-OBzl 6: The N^{α}-Fmoc group of the peptide **5** (4.6 g, 9.4 mmole) was deprotected following the general procedure using DEA (37 mL) and CH_2Cl_2 (37 mL) to yield 2.37 g (95%) of the free peptide **6**.

Fmoc-Val-Gly-Val-OBzl 7: To Fmoc-Val-Cl (2.9 g, 8.3 mmole) and HOAt.DCHA (2.9 g, 9.1 mmole) in THF (25 mL) was added **6** (2.2 g, 8.3 mmole) to obtain title compound **7** as a pale white solid, yield 3.98 g, (82%); m.p. 134-36°C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.98 - 109 (m, 12H), 1.94 - 2.10 (m, 2H), 3.92 (d, J = 6.7, 2H), 4.05 - 4.13 (m, 3H), 4.52 (d, J = 5.8, 2H), 5.25 (s, 2H), 5.75 (br, 1H), 6.29 (m, 1H), 7.18-7.90 (m, 13H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 16.6, 17.9, 28.2, 40.2, 45.4, 49.4, 52.3, 56.7, 59.9, 119.5, 120.1, 120.5, 121.2, 121.9, 124.0, 125.9, 136.2, 139.1, 157.0, 169.8, 170.1, 171.3; MS (MALDI-TOF) *m/z* Anal. Calcd. for $C_{34}H_{39}N_3O_6$, $[M + Na]^+$: 608.3. Found: $[M + Na]^+$: 608.2.

H-Val-Gly-Val-OBzl 8: The Fmoc group of the tripeptide **7** (3.5 g, 5.9 mmole) was deprotected using DEA (23 mL) and CH_2Cl_2 (23 mL) to yield 1.9 g (92%) of the free peptide **8**.

Fmoc-Gly-Val-Gly-Val-OBzl 9: To a solution of Fmoc-Gly-Cl (1.47 g, 4.7 mmole) and HOAt.DCHA (1.64 g, 5.2 mmole) in THF (10 mL) was added amino free tripeptide **8** (1.70 g, 4.7 mmole) and stirred at RT for 30 min. Work-up of the reaction-mixture as mentioned in the general procedure gave the title product **9** as a pale white solid, yield 2.4 g, (80%); m.p. 174-76°C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.95 - 1.10 (m, 12H), 1.97 - 2.13 (m, 2H), 3.80 - 3.95 (m, 4H), 4.10 - 4.21 (m, 2H), 4.45 - 4.60 (m, 3H), 5.20 (s, 2H), 5.56 (m, 1H), 6.21 (br, 1H), 7.15 - 7.95 (m, 13H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 16.9, 17.3, 28.3, 29.1, 40.5, 41.0, 48.4, 51.2, 53.3, 59.7, 62.0, 119.4, 120.6, 120.9, 121.5, 122.4, 123.6, 126.2, 131.0, 133.7, 137.2, 139.1, 156.4, 167.6, 169.1, 170.3, 171.4; MS (MALDI-TOF) *m/z* Anal. Calcd. for $C_{36}H_{42}N_4O_7$, $[M + Na]^+$: 665.3. Found: $[M + Na]^+$: 665.4.

H-Gly-Val-Gly-Val-OBzl 10: Fmoc of the tetrapeptide **9** (2.0 g, 3.1 mmole) was deprotected using DEA (12 mL) and CH_2Cl_2 (12 mL) to yield 1.1 g (85%) of free tetrapeptide **10**.

Fmoc-Pro-Gly-Val-Gly-Val-OBzl 11: To a suspension of Fmoc-Pro-Cl (0.85 g, 2.3 mmole) and HOAt.DCHA (0.80 g, 2.5 mmole) in THF (10 mL) was added amino free tetrapeptide **10** (1.0 g, 2.3 mmole) and stirred at RT for 20 min. Work-up of the reaction-mixture as furnished in the general procedure gave the Fmoc protected pentapeptide ester **11** as a pale white solid, yield 1.3 g, (75%); m.p. 194-96°C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.97 - 1.15 (m, 12H), 1.35 - 2.05 (m, 4H), 2.52 - 2.69 (m, 4H), 3.95 (m, 1H), 4.15 (d, J = 5.8 Hz, 2H), 4.35 (m, 1H), 4.55 (t, J = 5.9 Hz, 1H), 4.95 (m, 1H), 5.20 (s, 2H) 5.59 (m, 1H), 5.60 (m, 2H), 7.18 - 7.79 (m, 13H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 16.7, 17.4, 19.3, 19.7, 27.9, 28.3, 39.3, 40.2, 40.9, 44.2, 49.7, 50.6, 55.4, 59.1, 62.3, 119.7, 120.0, 120.5, 121.1, 121.7, 127.2, 129.2, 132.3, 137.4, 140.1, 156.5, 167.8, 168. 6, 169.2, 170.0, 173. 1. MS (MALDI-TOF) *m/z* Anal. Calcd. for $C_{41}H_{49}N_5O_8$, $[M + Na]^+$: 762.3. Found: $[M + Na]^+$: 762.2.

H-Pro-Gly-Val-Gly-Val-OH 12: The protected pentapeptide **11** (1.2 g, 1.6 mmole) was dissolved in methanol (10 mL) and 10% palladium black (0.12 g) was added. The reaction-mixture was stirred under H_2 pressure (using bladder) for about 2 hr. After the completion of the benzyl ester deprotection (monitered by TLC) the reaction-mixture was filtered over celite bed. The residue was purified with column chromatography using silica gel to afford the penta peptide acid with yield 0.93 g (89%). Then the pentapeptide acid (0.7 g, 1.1 mmole) was stirred with a mixture of CH_2Cl_2 : DEA (8 mL, 50% v/v) for 45 min under nitrogen. After completion of the reaction the solution was concentrated *in vacuo* at RT. The crude was purified by RP-HPLC to obtain the final peptide **12** 0.36 g (80%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.92 - 1.20 (m, 12H), 1.32 - 2.10 (m, 4H), 2.57 –2.69 (m, 4H), 3.85 (m, 1H), 4.09 - 4.21 (m, 4H), 4.75 (m, 1H), 4.95 (m, 1H), 6.54 (br 2H), 10.95 (br, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 16.7, 17.4, 22.4, 23.1, 27.9, 28.3, 39.3, 40.2, 40.9, 50.6, 55.4, 59.1, 62.3, 167.8, 168. 6, 169.2, 170.0. 175.5; MS

(MALDI-TOF) *m/z* Anal. Calcd. for $C_{19}H_{33}N_5O_6$, [M + Na]$^+$: 450.5. Found: [M + Na]$^+$: 450.2.

Synthesis of β-casomorphin

Fmoc-Pro-Gly-OBzl 14: To a stirred solution of Fmoc-Pro-Cl **13** (3.2 g, 9.0 mmoles) and HOAt.DCHA (3.1 g, 9.9 mmoles) in THF (25 mL), was added amino free glycine benzyl ester (1.5 g, 9.0 mmoles). Work-up of the reaction gave the peptide **14** as a pale white solid. Yield 3.3 g (75%); m.p. 61-63°C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.20 – 1.85 (m, 4H), 3.15 (m, 2H), 4.05 (d, *J* = 7.5 Hz, 2H), 4.20 (d, *J* = 5.6 Hz, 2H), 4.55 (m, 2H), 5.14 (m, 1H), 5.25 (s, 2H), 6.16 (br, 1H), 7.20 - 7.95 (m, 13H); ESI-MS *m/z* Anal. Calcd. for $C_{29}H_{28}N_2O_5$, [M + H]$^+$: 485.2, [M + Na]$^+$: 507.2. Found [M + H]$^+$: 485.1, [M + Na]$^+$: 507.5.

H-Pro-Gly-OBzl 15: The Fmoc group of the dipeptide **14** (3.0g, 6.2 mmole) was deprotected using DEA (25 mL) and CH_2Cl_2 (25 mL) to yield 1.5 g (92%) of the amino free peptide ester **15**.

Fmoc-Phe-Pro-Gly-OBzl 16: 1.3g of H-Pro-Gly-OBzl **15** was (5.0 mmole) added to a stirred solution of Fmoc-Phe-Cl (2.0 g, 5.0 mmole) and HOAt.DCHA (1.7 g, 5.5 mmole) in THF(15 mL) to obtain the protected tripeptide **16** as a pale white solid. Yield 2.3 g (73%). m.p. 146-48°C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.14 - 2.10 (m, 4H), 2.65 – 2.98 (m, 4H), 3.87 (m, 2H), 4.16 (d, *J* = 5.8 Hz, 3m), 4.35 (m, 1H), 4.56 (t, *J* = 5.8 Hz, 1H), 4.92 (m, 2H), 5.35 (s, 2H), 6.23 (br, 1H), 7.24 - 7.89 (m, 18H); MS (MALDI-TOF) *m/z* Anal. Calcd for $C_{38}H_{37}N_3O_6$, [M + Na]$^+$: 654.3. Found: [M + Na]$^+$: 654.2.

H-Phe-Pro-Gly-OBzl 17: Upon deprotection of Fmoc group from the peptide **16** (2.1 g, 3.3 mmole) using DEA (13 mL) and CH_2Cl_2 (13 mL) gave the free amino free tripeptide benzyl ester **17** was obtained Yield 1.1 g (85%).

Fmoc-Pro-Phe-Pro-Gly-OBzl 18: To a suspension of Fmoc-Pro-Cl (0.8 g, 2.3 mmole) and HOAt.DCHA (0.81 g, 2.5 mmole) in THF (10 mL) was added H-Phe-Pro-Gly-OBzl **17** (0.95 g, 2.3 mmole). Work-up of the reaction-mixture using general procedure gave the title compound **18** as a white soild. Yield 1.2 g (70%). m.p. 162-64°C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.15 - 2.13 (m, 8H), 2.59 - 3.12 (m, 6H), 3.82 (m, 2H), 4.19 – 4.31 (m, 3H), 4.33 (m, 1H), 4.67 (t, *J* = 5.8 Hz, 1H), 5.30 (s, 2H), 6.15 (br, 1H), 7.10 (m, 18H); MS (MALDI-TOF) *m/z* Anal. Calcd for $C_{43}H_{44}N_5O_7$, [M + Na]$^+$: 751.3. Found: [M + Na]$^+$: 751.7.

H-Pro-Phe-Pro-Gly-OBzl 19: Cleavage of Fmoc group from the peptide **18** (1.0 g, 1.4 mmole) was done employing DEA:CH_2Cl_2 (12 ml) mixture to obtain the free peptide ester **19** (0.6 g, 87%).

Fmoc-Tyr(Bzl)-Pro-Phe-Pro-Gly-OBzl 20: To the suspension of Fmoc-Tyr-(Bzl)-Cl (0.50 g, 0.98 mmole) and HOAt.DCHA (0.34 g, 1.0 mmole) in THF (5 mL), which was added the free tetra peptide ester **19**. Work-up was done using general procedure which resulted in protected β-casomorphin **20** as a pale yellow solid 0.67 g (70%). m.p. 160-62°C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.05 - 2.10 (m, 8H), 2.63 - 3.10 (m, 8H), 3.91 (m, 2H), 4.10 (d, *J* = 5.7 Hz, 2H), 4.32 (m, 1H), 4.59 (t, *J* = 5.8 Hz, 1H), 4.92 (m, 1H), 5.18 (s, 2H), 5.34 (s, 2H), 5.99 (br, 1H), 7.14 - 7.86 (m, 27H); MS (MALDI-TOF): *m/z* Anal. Calcd for $C_{59}H_{59}N_5O_9$, [M + Na]$^+$: 1004.4. Found: [M + Na]$^+$: 1004.3.

H-Tyr-Pro-Phe-Pro-Gly-OH 21: First, the benzyl ester of the peptide **20** (0.50 g, 0.51 mmole) was deprotected using 10% palladium black (0.11 g) under H_2 pressure is about 3 hr. After the completion of the reaction (TLC), palladium black was filtered over celite bed and the filtrate was evaporated *in vacuo.* The residue was column purified using silica gel to afford pure Fmoc peptide acid 0.35 g (85%). Then the Fmoc group was deprotected from the pentapeptide acid (0.30 g, 0.37 mmole) using CH_2Cl_2: DEA (3 mL, 50% v/v) within 1 hr under nitrogen. After the completion of the reaction, the solution was concentrated *in vacuo* and the residue was triturated with ether to dissolve 9-methylfluorene, and was precipitated with methanol-ether to obtain the peptide salt. The peptide salt was then stirred with water (2 mL) and 2% aq. Na_2CO_3 (5 mL) for 30 min and extracted with EtOAc. The organic layer was evaporated *in vacuo* to get free β-casomorphin **21** as solid. Yield 0.17 (78%). m.p.152-54°C ; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.28 - 2.19 (m, 9H), 2.67 - 2.92 (m, 4H), 3.20 - 3.48 (m, 4H), 3.92 (m, 1H), 4.35 (d, *J* = 5.9 Hz, 2H), 4.67 (m, 1H), 4.95 (m, 1H), 5.75 (br, 1H), 7.18 7.52 (m, 9H); MS (MALDI-TOF) *m/z* Anal. Calcd for $C_{30}H_{37}N_5O_7$, [M + Na]$^+$: 602.3. Found: [M + Na]$^+$: 602.3

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