A rapid and efficient synthesis of β-casomorphin employing Boc-amino acids and 9-fluorenylmethyl chloroformate as a coupling agent

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The synthesis of β-casomorphin H-Tyr-Pro-Phe-Pro-Gly-OH employing Boc group for Nα-protection and 9-fluorenylmethyl chloroformate (Fmoc-Cl) for the formation of peptide bond is described. The protocol employing Fmoc-Cl as coupling reagent is found to be simple, efficient and rapid. All the intermediate peptides as well as the final protected peptide Boc-Tyr(Bu)-Pro-Phe-Pro-Gly-OMe have been isolated and fully characterized. They have been obtained in good yield and with high purity.

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The most popular method in contemporary practice, owing to its simplicity and rapidity, is the use of coupling reagents for amide bond formation. The coupling, in the majority of the cases, is complete in less than an hour and is often significantly faster. Dicyclohexylcarbodiimide (DCC, DCCI), introduced by Sheehan, continues to be the most widely used coupling agent. Its utility in the large-scale production of peptides has contributed to its persistent popularity. The suppression of racemization and N-acylation formation during coupling by the addition of an auxiliary nucleophile like 1-hydroxy-benzotriazole (HOBT) or 1-hydroxy-7-azabenzotriazole (HOAt) has further enhanced its utility in both solutions as well as in solid phase peptide synthesis. However, the continuous use of DCC is known to be allergic to some individuals. Further, the separation of the side product dicyclohexylurea (DCU), due to its insolubility, is known to pose practical problems in the work-up as well as in the isolation of the pure peptides. This has been overcome by using more expensive carbodiimide such as diisopropylcarbodiimide (DIPCDI) which is equally effective as coupling reagent and forms a more soluble urea bi-product.

Note

Over the last decade, the use of phoshonium and uronium reagents in peptide chemistry has increased dramatically and several such reagents are commercially available. Some of the important ones include 1H-benzotriazol-1-ylxy-tris(dimethylamino)-phosphonium hexafluorophosphate (BOP), N,N-bis(2-oxo-3-oxazolinylid)phosphinic chloride (BOP-Cl), 1H-benzotriazol-1-ylxy-tris(pyrrolidino)-phosphonium hexafluorophosphate (PyBOP), N-[(1H-benzotriazol-1-yl)(dimethylamino)methylene]-N-methylmethanaminium hexafluorophosphate-N-oxide (HBTU), N-[(dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridin-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate-N-oxide (HATU), and N-[(dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridin-1-ylmethylene]-N-methyl methanaminium hexafluorophosphate-N-oxide (TBTU), etc. Although phosphonium salt and uronium salt based reagents are the powerful addition to the peptide chemists, arsenals, their cost certainly limited their utility in the production of simple peptides. On the other hand, the use of (Boc)2O as a coupling agent in peptide synthesis has been recently demonstrated. The use of 9-fluorenylmethylchloroformate (Fmoc-Cl) for the synthesis of peptides, to the best of our knowledge, is yet to be demonstrated.

It has been now found that Fmoc-Cl can be used as a coupling reagent in peptide synthesis. In a typical reaction, to a well stirred mixture of Boc-amino acid 1, Fmoc-Cl and a base like N-methyl morpholine (NMM) in THF at −15°C was added a solution of amino acid ester 3 in THF and then continued to stir at the same temperature (Scheme 1). The coupling, as monitored by TLC using the solvent systems, i) chloroform-methanol-acetic acid; 40:2:1 and ii) ethyl acetate-n-hexane; 65:35 as well as by RP-HPLC, was found to be complete in about 30 min. The coupling using Fmoc-Cl involves the formation of a mixed anhydride of the type Boc-NH-CO-O-CO-Fm 2. This has been clearly confirmed by the IR analysis of the reaction mixture before the addition of amino acid ester solution. It is found to have a strong vibrational stretching band at around 1820 cm⁻¹ which is characteristic of a mixed anhydride. The versatility of the protocol has been demonstrated by the synthesis of a series of Boc-protected dipeptide esters 4a-s (Table I). All the peptides made have been
obtained in good yield with high purity. They have been fully characterized by $^1$H NMR and mass spectral data. The protocol under the employed conditions, was found to be completely free from racemization. This has been confirmed by the synthesis of the diastereomeric dipeptides Boc-Phg-Ala-OMe 4a and Boc-D-Phg-Ala-OMe 4b by present method and by the $^1$H NMR analysis which revealed that the methyl ester singlets have clear differences in them [4a: 3.57 (OCH$_3$) and 4b: 3.71 (OCH$_3$)]. Further, the HPLC of these peptides 4a and 4b has also confirmed the same (Figures 1 and 2).

### Table I — Physical constants of Boc-protected dipeptide esters

<table>
<thead>
<tr>
<th>Compd</th>
<th>Peptide</th>
<th>Coupling time (min)</th>
<th>m.p. $^\circ$C</th>
<th>$[\alpha]^{25}_D$</th>
<th>Yield (%)</th>
</tr>
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<tbody>
<tr>
<td>4a</td>
<td>Boc-Ala-Phg-OMe</td>
<td>35</td>
<td>113-15</td>
<td>-125.1 (c, 1.2, MeOH)</td>
<td>80</td>
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<tr>
<td>4b</td>
<td>Boc-Ala-D-Phg$^\delta$-OMe</td>
<td>35</td>
<td>116-18</td>
<td>+125.1 (c, 1.2, MeOH)</td>
<td>90</td>
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<tr>
<td>4c</td>
<td>Boc-Phe-Gly-OEt</td>
<td>30</td>
<td>90-91</td>
<td>-4.3 (c, MeOH)</td>
<td>70</td>
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<tr>
<td>4d</td>
<td>Boc-Pro-Phe-OMe</td>
<td>30</td>
<td>65-66</td>
<td>-50.4 (c, MeOH)</td>
<td>85</td>
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<tr>
<td>4e</td>
<td>Boc-Leu-Leu-OMe</td>
<td>35</td>
<td>140-41</td>
<td>-50.3 (c, 0.7, MeOH)</td>
<td>80</td>
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<tr>
<td>4f</td>
<td>Boc-Phe-Val-OMe</td>
<td>35</td>
<td>116-18</td>
<td>-10.8 (c, 2, DMF)</td>
<td>88</td>
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<tr>
<td>4g</td>
<td>Boc-Gly-Gly-OBzl</td>
<td>30</td>
<td>82-84</td>
<td>-</td>
<td>79</td>
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<tr>
<td>4h</td>
<td>Boc-Ile-Val-OMe</td>
<td>35</td>
<td>165-66</td>
<td>-15.7 (c, MeOH)</td>
<td>80</td>
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<td>4i</td>
<td>Boc-Lys(Z)-Leu-OMe</td>
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<td>85-87</td>
<td>-3.2 (c, DMF)</td>
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<td>4j</td>
<td>Boc-Pro-Tyr-OMe</td>
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<td>63-64</td>
<td>-36.0 (c, MeOH)</td>
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<td>4k</td>
<td>Boc-Ser-Met-OMe</td>
<td>30</td>
<td>66-68</td>
<td>-29.1 (c, MeOH)</td>
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<td>4l</td>
<td>Boc-Lys(Z)-His-OMe</td>
<td>30</td>
<td>95-97</td>
<td>-6.1 (c, EtOH)</td>
<td>82</td>
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<td>4m</td>
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<td>-3.9 (c, MeOH)</td>
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<td>4n</td>
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<td>4o</td>
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<td>137-38</td>
<td>-25.1 (c, MeOH)</td>
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<tr>
<td>4p</td>
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<td>35</td>
<td>139-41</td>
<td>+6.5 (c, AcOH)</td>
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<tr>
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<td>30</td>
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<td>135-36</td>
<td>+54 (c, CHCl$_3$)</td>
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<tr>
<td>4s</td>
<td>Z-Trp-Gly-OMe</td>
<td>30</td>
<td>156-58</td>
<td>-11.2 (c, AcOH)</td>
<td>83</td>
</tr>
</tbody>
</table>

# 2-aminophenylacetic acid

### Scheme I — Synthesis of Boc-/Z-protected peptides using Fmoc-Cl as coupling agent

X = Boc- or Z-group
Analytical RP-HPLC of pure Boc-Phg-Phe-OMe: C-18 deltapak column (3.9x300 mm, 15μ); Flow rate 1.0 mL/min; eluant, acetonitrile-H₂O (65:35; isocratic; monitoring at 220 nm).

Analytical RP-HPLC of pure Boc-D-Phg-Phe-OMe: C-18 deltapak column (3.9x300 mm, 15μ); Flow rate 1.0 mL/min; eluant, methanol-H₂O (65:35; isocratic; monitoring at 220 nm).

By the same strategy, the synthesis of β-casorphin 6, H-Tyr-Pro-Phe-Pro-Gly-OMe has also accomplished (Scheme II). Thus, its synthesis was carried out by using appropriate Boc-amino acids Boc-Cl as a coupling agent. All the three protected peptides Boc-Pro-Gly-OMe 5a, Boc-Pro-Phg-Phe-OMe 5c, Boc-Tyr(Bzl)-Pro-Phe-Pro-Gly-OMe 5g, Boc-Pro-Phe-Pro-Gly-OMe 5h, and pentapeptide H-Tyr-Pro-Phe-Pro-Gly-OMe 5j were obtained in good yield and purity (for the trace of the final peptide see Figure 3). The synthesized β-casomorphin 6 has been found to have biological activity similar to that of the natural one.25, 26

Figure 3—Analytical RP-HPLC of pure β-casomorphin: Waters C-18 deltapak column (3.9x300 mm, 15μ); Flow rate 1.0 mL/min; eluant, acetonitrile-H₂O with 0.1% TFA (65:35; isocratic; monitoring at 220 nm).

Scheme II—Synthesis of β-casomorphin
Experimental Section

The melting points were determined by using Leitz-Wetzlar melting point apparatus and are uncorrected. Optical rotations were measured with an automatic AA-10 polarimeter (Optical Activity, U.K.). IR spectra were recorded on a Nicolet model impact 400D FT-IR spectrometer (KBr pallets, 3 cm⁻¹ resolution); and ¹H NMR spectra on a Brucker 400 MHz spectrometer with TMS as an internal standard. Unless otherwise mentioned, all amino acids used, have L-configuration. Analytical RP-HPLC was performed on Waters LC 3000 system equipped with Waters 484 tunable absorbance UV detector and millipore 745 data module. A C-18 deltapack column (3.9 mm × 300 mm, 15μ spherical) was used for analysis. TLC analysis was carried out using the precoated silica gel G plates using i) CHCl₃-methanol-acetic acid; 40:2:1; ii) ethyl acetate-n-hexane; 65:35 and the Rf values designated as Rf A and Rf B.

General procedure for the synthesis of Boc protected peptide esters 4a-s. To a well stirred solution of Boc-protected amino acid 1 (1 mmole) and NMM (0.11 mL, 1 mmole) in dry THF (5 mL) maintained at -15°C was added Fmoc-Cl (0.258 g, 1 mmole) and stirred for 5 min and then, the amino free amino acid ester 3 (1.2 mmole) was added in one portion at that temperature. The stirring was continued at the same temperature till the completion of the reaction. The organic layer was evaporated and the residue was diluted with EtOAc (15 mL), washed with 10% citric acid (5 mL×2), 10% aqueous NaHCO₃ (5 mL×2) and water (5 mL×2). The organic layer was dried over anhydrous Na₂SO₄ and evaporated under reduced pressure. The resulting residue was crystallized from n-hexane-EtOAc (3:1) to obtain the protected peptide as a crystalline solid.

General procedure for the deprotection of Boc group. The Boc-peptide ester (2 mmole) was dissolved in 1N HCl-EtOAc (5 mL) and stirred for 30 min. After the completion of the reaction the solution was evaporated under vacuum and triturated with dry ether to get the peptide ester hydrochloride salt.

General procedure for the deporationation of hydrochloride salt using zinc dust.²⁷ To a suspension of amino acid hydrochloride salt (2 mmole) in dry THF (10 mL) was added zinc dust (100 mg) in one portion. The mixture was stirred for 10 min at r.t. After completion of the deprotonation, the reaction mixture was filtered and solvent evaporated under reduced pressure. The resulting residue was precipitated using dry diethyl ether to obtain amino free amino acid ester or peptide ester as a crystalline solid.

Synthesis of β-casomorphin

Boc-Gly-OMe 5a. To a well stirred solution of Boc-Pro (1.05 g, 5 mmole), Fmoc-Cl (1.29 g, 5 mmole) and NMM (0.55 mL, 5 mmole) in dry THF (10 mL) at -15°C, a solution of H-Gly-OMe (1.1 g, 12 mmole) in THF (10 mL) was added and the mixture stirred to yield 1.25 g (90%) of the peptide 5a as a foam; Rf A, 0.75; [α] D⁰²⁵ -17.5 (c=1, CHCl₃).

H-Pro-Gly-OMe 5b. Boc-Pro-Gly-OMe (5a, 1.25 g, 4.5 mmole) was deprotected using 1N HCl-EtOAc (10 mL) and then deprotonated using zinc dust following the general procedures to yield 0.7 g (86%) of the peptide 5b; m.p. 174-77°C; Rf A, 0.55; Rf B, 0.56; [α] D⁰²⁵ -28.4 (c=1, CHCl₃); IR (ν max in cm⁻¹): 3125, 3195.

Boc-Phe-Pro-Gly-OMe 5c. To a well stirred solution of Boc-Phe-OH (1.0 g, 3.85 mmole), Fmoc-Cl (1.0 g, 3.85 mmole) and NMM (0.43 mL, 3.85 mmole) in dry THF (10 mL) at -15°C, a solution of H-Pro-Gly-OMe (5b, 0.7 g, 3.85 mmole) in THF (10 mL) was added and the mixture stirred to yield 1.45 g (87%) of the peptide 5c; m.p. 153-55°C; Rf A, 0.76; [α] D⁰²⁵ -30.5 (c=1, CHCl₃).

H-Pro-Phe-Gly-OMe 5d. Boc-Phe-Pro-Gly-OMe (5c, 1.45 g, 3.37 mmole) was deprotected using HCl-EtOAc (10 mL) and then deprotonated using zinc dust following the general procedure to yield 0.9 g (81%) of the peptide 5d as a foam; Rf A, 0.63; Rf B, 0.61; [α] D⁰²⁵ -36.5 (c=1, CHCl₃); IR (ν max in cm⁻¹): 3219, 3240.

Boc-Pro-Phe-Pro-Gly-OMe 5e. To a well stirred solution of Boc-Pro (1.15 g, 2.75 mmole), Fmoc-Cl (1.41 g, 2.75 mmole) and NMM (0.6 mL, 2.75 mmole) in dry THF (10 mL) at -15°C, a solution of H-Pro-Phe-Pro-Gly-OMe (5d, 0.9 g, 2.75 mmole) in THF (10 mL) was added and the mixture stirred to yield 1.15 g (80%) of the peptide 5e; m.p. 158-59°C; Rf A, 0.86; [α] D⁰²⁵ -10.5 (c=1, CHCl₃).

II-Pro-Phe-Pro-Gly-OMe 5f. Boc-Pro-Phe-Pro-Gly-OMe (5e, 1.15 g, 2.18 mmole) was deprotected using HCl-EtOAc (10 mL) and then deprotonated using zinc dust following the general procedure to yield 0.75 g (80%) of the peptide 5f; m.p. 168-69°C;
RgA, 0.58; Rf B, 0.54; [α]D25 -46.2 (c=1, CHCl3); IR (νmax in cm⁻¹): 3310, 3109.

Boc-Tyr(Bu)₃-Pro-Phe-Pro-Gly-OMe 5g. To a well stirred solution of Boc-Tyr(Bu)₃-OH (500 mg, 1.75 mmoles), Fmoc-Cl (450 mg, 1.75 mmoles) and NMM (0.2 mL, 1.75 mmoles) in dry THF (7 mL) at -15 °C, a solution of H-Pro-Phe-Pro-Gly-OMe (5f, 0.75 g, 1.75 mmoles) in THF (7 mL) was added and the mixture stirred to yield 1.0 g (84%) of the peptide.

H-Tyr-Pro-Phe-Pro-Gly-OMe 5h. Boc-Tyr(Bu)₃-Pro-Phe-Pro-Gly-OMe (5g, 1.0 g, 1.45 mmoles) was deprotected using 1N HCl-EtOAc and then deprotonated using zinc dust following the general procedure to yield 0.65 g (77%) of the peptide 5h; m.p. 150-51°C; Rf A, 0.60; Rf B, 0.42; [α]D25 -66.7 (c=1, CHCl3); IR (νmax in cm⁻¹): 3211, 3198.

Tyr-Pro-Phe-Pro-Gly 6. A solution of peptide ester (5h, 0.59 g, 1 mmole) in methanol (15 mL) and 1N NaOH (3 mL) was stirred for 3 hr by maintaining the pH between 8 to 9. After the completion of the reaction, the solution was neutralized using 1N HCl and the precipitated solid was filtered and dried to get 0.30 g (50%) of the free peptide 6, m.p. 151-53°C; [α]D25 -53.8 (c=0.5, DMF) [Reported25 m.p. 152-54°C; [α]D25 -48.5 (c=0.5, DMF)]; Rf B 0.40; Rf C, 0.42; Anal. Found: C, 62.29; H, 6.02; N, 12.28. Calc. for C30H35N5O7 (578): C, 62.34; H, 6.10; N, 12.17%. Rf 8.36 ([Waters C-18 deltapak column (3.9x300 mm, 15 μ)] using as the eluant acetonitrile -0.1% trifluoroacetic acid (TFA) and water (65:35; isocratic, flow rate 1 mL/min, monitoring at 220 nm).

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References