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A New Method for Rapid Solution Synthesis of Shorter Peptides by use of PyBOP®.[1]

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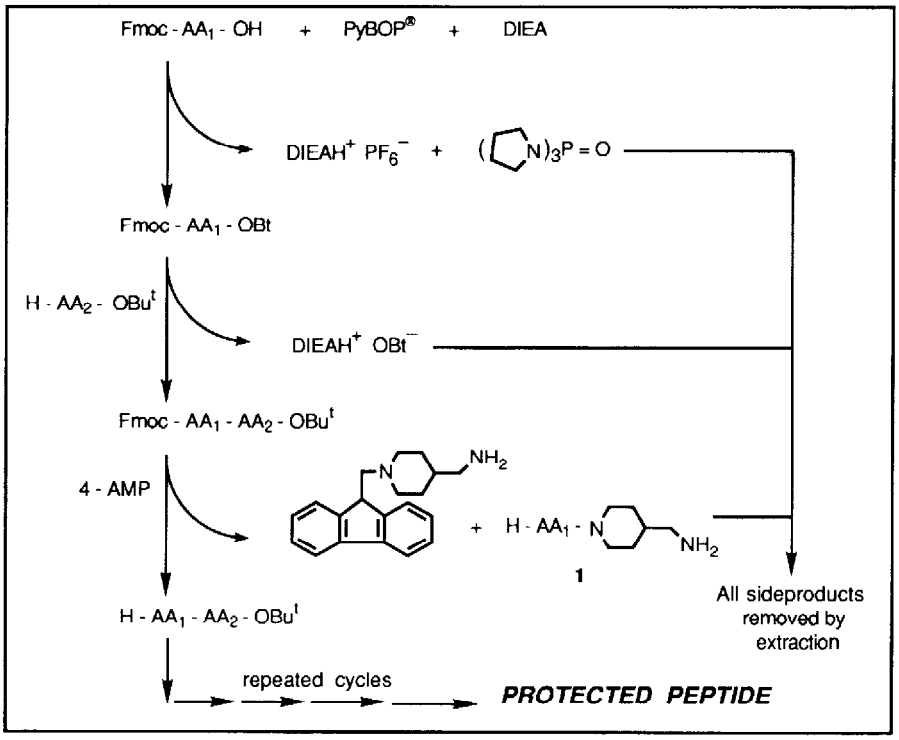
Abstract: *By using PyBOP® promoted coupling of Fmoc protected amino acids, and adopting the deprotection / washing procedure of the* Fmoc Amino Acid Chloride Solution Technique *(FAACST), a sample of [leucine[5]] enkephalin has been synthesized in a rapid, continuous solution phase method in 56 % yield. The method is well applicable for the synthesis of large amounts of shorter peptide fragments.*

In undertaking investigations of the biological activity of both naturally occurring and purely synthetic peptides, only minute amounts are often required. These may be obtained by conventional solid phase peptide synthesis.[2] If gram-scale amounts are needed, on the other hand, classical solution peptide synthesis may be considered, but this involves the well-known problems of many manipulations and loss of material during the isolation and purification of intermediates. Some of these problems have recently been circumvented by using continuous solution techniques, which may be used with both the Boc and the Fmoc strategies.[3,4] In the *Fmoc Amino Acid Chloride Solution Technique* (FAACST)[5] a two phase system is used. In the first step of a reaction sequence, an amino acid ester and a Fmoc amino acid chloride (1.1 - 1.5 equivalent) are coupled in $CHCl_3$ and aqueous $NaHCO_3$. In the second step the aqueous phase is removed and 4-(aminomethyl)piperidine (4-AMP) is added. This di-amine acts both as deprotection reagent for the Fmoc-group and as a quencher of excess acylating agent. In the last step by-products and excess reagents are removed by repeated washings with an acidic aqueous buffer, while the growing peptide remains in the organic phase. This cycle is repeated until the desired chain-length is obtained. About one hour is needed for one coupling, deprotection and washing cycle.

In need of a protected analogue of [leucine[5]] enkephalin, Fmoc-Tyr(OBu^t)-Gly-Gly-Phe-Leu-OBu^t, as a reference in another study,[6] we considered building this fragment by use of a modification of the FAACST : The Fmoc amino acid chlorides are substituted with the Fmoc amino acids, and PyBOP® is used for activation. Fast couplings with low levels of racemisation are generally performed with this coupling agent.[7] Shifting to PyBOP® activation eliminates the need for preparing and purifying the sensitive chlorides, as well as the need for a two phase system during coupling.

A schematic representation of this new synthesis scheme is shown in scheme 1. The C-terminal amino acid is preferably protected as its tert-butyl ester, as tert-butyl protected dipeptides do not undergo intramolecular ring-closure to diketopiperazines.[8] After the final coupling and the Fmoc deprotection step, all other protecting groups are removed with TFA. Unlike in the FAACST it is possible to use a Boc protected amino acid for the last acylation step, leaving a fully protected peptide with only acid labile protecting groups.



Scheme 1. *Rapid route to protected peptide fragments*. Compound **1** results from the quenching of excess HOBt ester (the active ester is probably not the only acylating agent in PyBOP® activation; also acyloxyphosphonium ions and symmetric anhydrides could be involved [9]).

The synthesis of Fmoc-Tyr(OBu^t)-Gly-Gly-Phe-Leu-OBu^t was carried out by dissolving HCl·H-Leu-OBu^t [10] (0.5 mmol), Fmoc-Phe-OH (0.55 mmol), and PyBOP® (0.55 mmol) in $CHCl_3$ (10 ml). DIEA (1.25 mmol) was added and the mixture stirred for 30 min (PyBOP® / BOP couplings are often close to quantitative within minutes [7]). 4-AMP (5 ml) was added and the mixture stirred for 30 min. The mixture was diluted with $CHCl_3$ (40 ml) and extracted with 10 % phosphate buffer, pH = 5.5 (5 x 25 ml), followed by reduction of the volume of the organic phase (to 10 ml). The next coupling cycle was started by adding Fmoc-Gly-OH (0.55 mmol), PyBOP® (0.55 mmol), and DIEA (0.75 mmol). After stirring for 10 min, 4-AMP (5 ml) was added followed by the described extraction procedure with phosphate buffer. Following this protocol, a further Fmoc-Gly-OH and a Fmoc-Tyr(OBu^t)-OH, respectively, were coupled to yield the desired protected pentapeptide, omitting deprotection in the last sequence. The protected peptide was isolated by adding chloroform (40 ml),

extracting with the phosphate buffer as described and washing the organic phase with water (25 ml). After drying with $MgSO_4$, the chloroform was evaporated *in vacuo* to yield the crude peptide, which on TLC (R_f = 0.35, eluent $CHCl_3$ / Bu^tOH, 6:1, Merck 60 F_{254} silica gel sheets) proved to be the major component . It was purified by silica gel flash chromatography ($CHCl_3$ / Bu^tOH, 6:1) to give a white powder (0.302 g, 68 % from H-Leu-OBu^t). LSIMS, $[MH^+]$ = 890.5, ^1H- and ^{13}C-NMR are in agreement with the assumed structure. HPLC (**A**: 0.1 % TFA in H_2O / **B**: 0.1 % TFA in H_2O + 90 % CH_3CN; linear gradients: 0-70 % **B** in 20 min, then 70-100 % **B** in 1 min, then isocratic **B**; C-18 Bondapack™ column) showes one peak (> 98 % , R_f = 24.02 min) (Fig. 1). Mp 166-69 °C, softening at 125 °C (ethanol). $[\alpha]^{22}_D$ - 7.5 ° (c 1, $CHCl_3$).

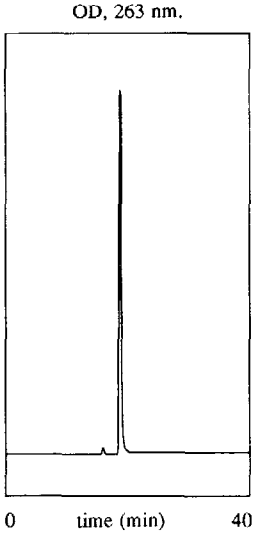


Fig. 1. Analytical HPLC chromatogram of Fmoc-Tyr(OBu^t)-Gly-Gly-Phe-Leu-OBu^t.

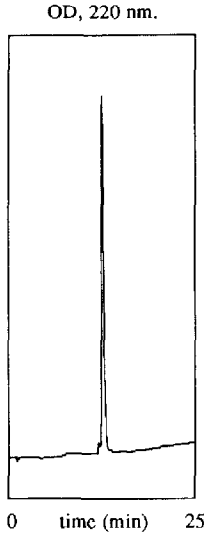


Fig. 2. Analytical HPLC chromatogram of [leucine^5] enkephalin.

The unprotected peptide was obtained by dissolving the protected peptide (0.25 mmol) in chloroform (5 ml) adding 4-AMP (2.5 ml) and leaving the solution for 30 min. The solution was extracted with 10 % phosphate buffer as described above and, after drying with $MgSO_4$, the solvent was removed *in vacuo* to give an oil. TFA (95%, 5 ml) was added and the mixture left for 2 hours. Excess TFA was removed *in vacuo* and, after trituration with ether, the residual peptide TFA-salt was submitted to gel filtration (1 % acetic acid on G-15 Sephadex). The fractions collected were lyophilized to give [leucine^5] enkephalin acetate as a white powder (0.127 g, 82 %; overall yield from H-Leu-OBu^t : 56 %). LSIMS, $[MH^+]$ = 556.6, and ^1H-NMR are in agreement with the assumed structure. TLC showes one spot (n-Butanol / acetic acid / water, 4:1:1, R_f = 0.52. Litt: 0.52 [11]). HPLC (**A**: 0.1 % TFA in H_2O / **B**: 0.1 % TFA in H_2O + 90 % CH_3CN; linear gradients: 0-70 % **B** in 20 min, then 70-100 % **B** in 1 min, then isocratic **B**; C-18 Bondapack™ column) showed one peak (> 97 %, R_f = 11.64 min). (Fig. 2). Mp 208 °C (ethanol). $[\alpha]^{22}_D$ + 24.8 ° (c 1, 95 % AcOH), litt.[11,12] $[\alpha]^{22}_D$ + 25.4 ° (c 1, 95 % AcOH), litt.[13] $[\alpha]^{25}_D$ + 26.4 ° (c 1, 95 % AcOH).

During washing of H-Gly-Gly-Phe-Leu-OBu^t, an emulsion may be formed unless special caution is taken. Tris(pyrrolidino)phosphine oxide is a by-product in PyBOP® promoted couplings. This compound, though partially water-soluble,[14] is not completely removed during the extractions (as detected by TLC), but presents no problem, being inert. Traces of HOBt are also present in the organic phase after completion of the washing procedure, but this substance is an often used additive during peptide synthesis, and even when employing BOP / PyBOP®, HOBt addition is recommended at times.[15,16] Minor impurities such as dibenzofulvene are as well present in the organic phase at the end of each cycle. This is also seen when using FAACST.[17] All of these impurities were easily removed by the chromatography of the protected peptide.

The time required for the above *Fmoc amino acid solution technique* (FAAST) couplings was estimated to 30 min for the relatively bulky phenylalanine and tyrosine and to 10 min for glycine. The time required for one cycle is accordingly between 1 and 1.5 hours permitting synthesis of a pentapeptide in one working day.

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References and notes:

1. Unusual abbreviations not explained in the text: PyBOP®,benzotriazolyloxy-tris(pyrrolidino)-phosphonium hexafluorophosphate. Fmoc, 9-fluorenylmethoxycarbonyl. Boc, tert-butyloxycarbonyl. 4-AMP, 4-(amino methyl)piperidine. BOP, benzotriazolyloxy-tris(dimethylamino)-phosphonium hexafluorophosphate. Bu^t, tert-butyl. HOBt, 1-hydroxybenzotriazole. AA_n, amino acid. DIEA, diisopropylethylamine. TFA, trifluoro acetic acid. LSIMS, Liquid secondary ion mass spectrometry. OD, Optical Density.
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