O-Benzotriazolyl-*N*,*N*,*N*′,*N*′-tetramethyluronium Hexafluorophosphate as Coupling Reagent for the Synthesis of Peptides of Biological Interest

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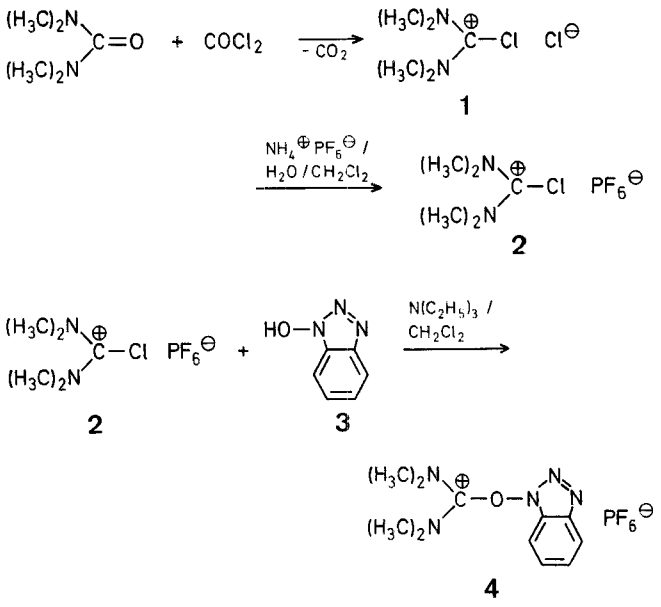
In a previous publication[1], we provided evidence for the use of *O*-benzotriazolyl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU; **4**) in peptide synthesis. This coupling method proved to be very easy and efficient for the synthesis of di- and tripeptides and has the following advantages when compared with other methods:

(a) The amount (%) of racemization of the synthetic peptides as measured by literature methods [2,3,4] is very low;

(b) The coupling conditions are very simple: the coupling is achieved by mixing a solution of the *N*-protected amino acid and of the amino acid ester with the reagent in stoichiometric amounts in the presence of a tertiary base such as *N*-methylmorpholine or triethylamine;

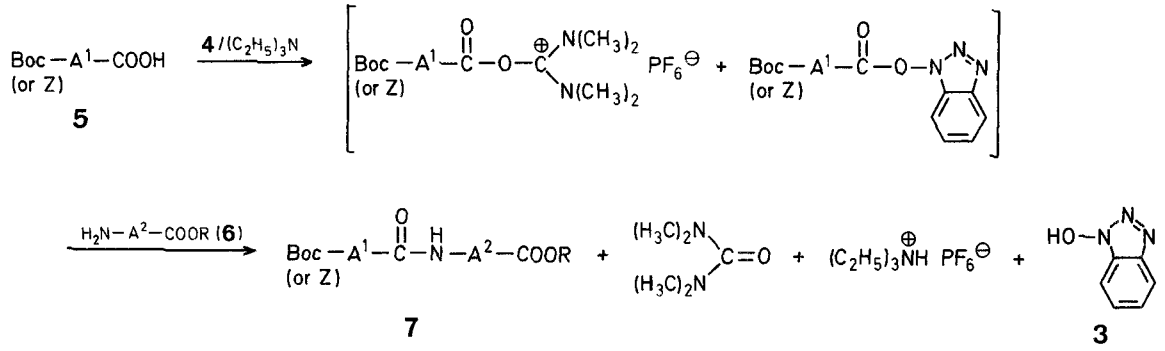
(c) The reaction time is very short (~ 15 min) with yields not less than 87%.

In this work we present a new route for the synthesis of the coupling reagent **4** (Scheme **A**) and its use for the easy synthesis of tetra- or pentapeptides with biological activity.



Scheme **A**

The peptides **7** are prepared by condensing the *N*-protected amino acids **5** with an amino acid ester or peptide **6** in the presence of reagent **4** (Scheme **B** and Table). The mechanism of the peptide bond formation by the action of **4**[5] (Scheme **B**) goes through the intermediate active esters of benzotriazole (isolable) and of the tetramethyluronium ion whose low stationary concentrations are due to the poor solubility of **4** in most solvents.



Scheme **B**

Table. Synthesis of Peptides **7** from *N*-Protected Amino Acids **5** and Amino Acid Esters **6** using Reagent **4**

Peptide[a]		Yield[b] [%]	m.p. [°C]	Molecular formula[c] or Lit. m.p. [°C]	$[\alpha]_D^{20}$ (*c*, solvent) found	reported
7a	Boc-(OBzl)-Tyr*-Gly-Gly*-Phe-Met-OCH_3	90	155°	155–158°[6]	+10° (1, CH_3OH)	+7.8°[6] (0.9, CH_3OH)
7b	Boc-Phe*-Met-OCH_3	98	80°	85°[7]	−22.3° (1, CH_3OH)	−20.7°[7] (1, CH_3OH)
7c	Boc-Gly*-Phe-Met-OCH_3	98	68°	$C_{22}H_{33}N_3O_6S$ (467.6)	−24.35° (1.5, CH_3OH)	–
7d	Boc-Gly*-Gly-Phe-Met-OCH_3	96	106°	$C_{24}H_{36}N_4O_7S$ (524.6)	−16.5° (1.5, CH_3OH)	–
7f	Boc-(OBzl)-Tyr*-Gly-Gly-OCH_3	95	114°	111–114°[8]	+10.5° (5, CH_3OH)	+10°[8] (5, CH_3OH)
7g	Boc-Thr*-Pro-OCH_3	85	–	$C_{15}H_{26}N_2O_6$ (330.4)	−77.88° (1.9, CH_3OH)	–
7h	Boc-Thr*-Leu-OCH_3	95	–	$C_{16}H_{30}N_2O_6$ (346.4)	−39.3° (3, CH_3OH)	–
7i	Boc-Val*-Thr-Leu-OCH_3[d]	96	–	$C_{21}H_{29}N_3O_7$ (445.6)	−55.67° (2.36, CH_3OH)	–
7j	Boc-Leu*-Val-Thr-Leu-OCH_3	90	128°	$C_{27}H_{50}N_4O_8$ (558.7)	−65° (2.5, CH_3OH)	–
7k	Boc-(OBzl)-Ser*-Val-Thr-Leu-OCH_3[e]	92	–	$C_{31}H_{50}N_4O_9$ (622.4)	−45.3° (2, CH_3OH)	–
7l	Boc-Phe*-Leu-Obzl	95	80°	$C_{27}H_{36}N_2O_5$ (468.6)	−25.9° (1, CH_3OH)	–
7m	Boc-Gly*-Phe-Leu-OBzl	94	–	$C_{29}H_{39}N_3O_6$ (525.7)	−25° (1, C_2H_5OH)	–
7n	Boc-D-Ala*-Gly-Phe-Leu-OBzl[f]	98	132°	$C_{32}H_{44}N_4O_7$ (596.7)	−6.5° (1, CH_3OH)	–
7o	Boc-(OBzl)-Tyr*-D-Ala-Gly-Leu-OBzl[g]	90	–	$C_{48}H_{59}N_5O_9$ (850.1)	+11° (1, CH_3OH)	–
7p	Boc-(OCH_3)-Tyr*-D-Ala-Gly-Phe-Leu-OBzl[h]	92	118–122°	$C_{42}H_{55}N_5O_9$ (773.9)	−3° (1, CH_3OH)	–

[a] The CO—NH bond formed in the peptides is indicated by *.
[b] Yield based on the amount of *N*-protected amino acid used.
[c] Satisfactory microanalyses obtained: C ±0.32, H ±0.31, N ±0.35; exceptions: **7c**, C −0.54; **7d**, C +0.58; **7i**, C −0.58; **7b**, C −0.46.
[d] Amino acid ratio = Len : Val : Thr = 1.026 : 1.0 : 0.971.
[e] Amino acid ratio: Leu : Ser : Val : Thr = 1.065 : 0.924 : 1.043 : 0.963.
[f] Amino acid ratio = Ala : Gly : Phe : Leu = 1.036 : 0.964 : 0.982 : 1.020.
[g] Purified by reverse phase H.P.L.C.; column: C18, 250 × 4 mm Perkin Elmer Analytical; eluent: methanol/water (8 : 2); flow: 2 × 5 ml/min; retention time: 5.9 min. Amino acid ratio: Tyr : Ala : Gly : Phe : Leu = 0.992 : 0.972 : 0.982 : 1.061.
[h] Purified be reverse phase H.P.L.C.; column: C8, 250 × 4 mm Perkin Elmer Analytical; eluent: acetonitril/water (8 : 2); flow: 10 ml/min; retention time ≈ 10 min. Amino acid ratio: Tyr : Ala : Gly : Phe : Leu = 0.863 : 1.015 : 1.015 : 1.015 : 1.066.

For measurement of the biological activity, the peptides **7a** and **7p** were hydrolysed to the corresponding acid derivatives **8** and **10** (see experimental). Subsequently, the protecting group was removed from the *N*-terminal tyrosines. The IC_{50} value of the Tyr (OCH_3)-D-Ala^2-Leu^5-enkephalin for the displacement of ^{3}H-Dalamid from rat brain membranes[9] was found to be 30 μM and that Tyr-D-Ala^2-Leu^5-enkephalin was 1.5 nM. Methylation of the phenolic hy-

droxy of the *N*-terminal tyrosine reduces the binding capacity of the peptide by 200-fold. Beddell at al.[10] report that methylation of the phenolic hydroxy of the *N*-terminal tyrosine of Leu-enkephalin reduces its binding capacity to rat brain membranes by 400-fold. Their measurements refer to the displacement of ^{3}H-naloxone from rat brain membranes. Both unprotected peptides **8** and **10** inhibit the contractions of the electrically stimulated mouse vas deferens[11] in a dose dependent manner and the inhibitory effects are reversed by naloxone.

The *N*-protected amino acids were commercial products except for the *N*-Boc-(OCH_3)-tyrosine which was synthesized as described below.

Boc-(OCH_3)-tyrosine:

Boc-(OCH_3)-tyrosine methyl ester: To a solution of Boc-tyrosine (700 mg, 2.5 mmol) and potassium carbonate (828 mg, 6 mmol) in dimethylformamide/water (4:2, v:v; 10 ml), methyl iodide (0.6 ml, 10 mmol) is added, and the mixture is stirred at room temperature for 2 h. The Boc-(OCH_3)Tyr-OCH_3 is extracted with ethyl acetate (3 × 10 ml), the organic phase is washed with 2 normal hydrochloric acid (10 ml) and 5% aqueous sodium hydrogen carbonate (10 ml), dried with magnesium sulfate, and the solvent is removed under reduced pressure. Pure product is obtained by chromatography on silica gel eluting with ethyl acetate; yield: 703 mg (91%); m.p. 49–50°C; $[\alpha]_D^{20}$: + 5.9° (*c* 2.5, methanol).

$C_{16}H_{23}NO_5$	calc.	C 62.13	H 7.40	N 4.52
(309.4)	found	61.97	7.39	4.53

Hydrolysis of Boc(OCH_3)-tyrosine methyl ester: Boc-(OCH_3)-tyrosine methyl ester (619 mg, 2 mmol) is hydrolysed by stirring at 24°C for 2 h in 1:1 dioxan/1 normal aqueous sodium hydroxide (4 ml). The course of the reaction is followed by T.L.C. on silica gel plates and ethyl acetate as eluent. The mixture is acidified with citric acid (pH = 2) and the Boc-(OCH_3) Tyr-OH is extracted with chloroform (3 × 30 ml). The organic phase is dried with magnesium sulfate and the solvent removed under reduced pressure: yield: 496 mg (84%); m.p. 89–90°C.

$C_{15}H_{21}NO_5$	calc.	C 60.99	H 7.17	N 4.76
(295.4)	found	60.83	7.11	4.65

***O*-Benzotriazolyl-*N,N,N',N'*-tetramethyluronium Hexafluorophosphate (4):**

A 20% solution of phosgene in toluene (100 ml) is added dropwise and under dry and *very efficient ventilating* conditions, to a solution of tetramethylurea (11.6 g) in toluene. After approximately 15 min, when the carbon dioxide evolution has stopped, anhydrous ether (350 ml) is added under vigorous stirring. The precipitated salt **1** is filtered and washed with anhydrous ether (3 × 50 ml). The highly hygroscopic material is immediately dissolved in dichloromethane (500 ml) and to this solution a saturated solution of ammonium hexafluorophosphate (50 ml) is added under continuous stirring. The organic phase is washed with water (40 ml), dried with magnesium sulfate, and the solvent removed under reduced pressure. The solid salt **2** is washed with ether and dried over phosphorus pentoxide in vacuum; yield: 24 g (86%); hygroscopic material.

^{1}H-N.M.R. (CD_3CN/TMS): δ = 3.25 ppm (s, 6H).

The solid salt **2** (20 g, 0.07 mol) is dissolved in dichloromethane (300 ml) and hydroxybenzotriazole (**3**; 9.45 g, 0.07 mol) is added. After the addition of one equivalent of triethylamine, a white precipitate forms which is filtered and washed with dichloromethane (100 ml). This material **(4)** is recrystalized from acetonitrile, dried, and kept in the cold in the absence of light; yield: 26 g (~ 100%); m.p. 250°C (Lit.[1], m.p. 254°C).

^{1}H-N.M.R. (CD_3CN/TMS): δ = 3.05 (s, 6H); 3.4 (s, 6H); 7.6–8.2 ppm (m, 4H).

Boc-(OBzl)-L-Tyr-Gly-Gly-OCH_3 (7f); Typical Coupling Procedure:

To a solution of Boc-(OBzl)-L-Tyr-OH (925 mg, 2.5 mmol), Gly-Gly-OCH_3·HCl (473 mg, 2.6 mmol) and triethylamine (550 mg, 5 mmol) in acetonitrile (20 ml) is added reagent **4** (983 mg, 2.6 mmol). The mixture is stirred at room temperature for 15 min. After addition of saturated sodium chloride (70 ml), the peptide is extracted with ethyl acetate (3 × 50 ml). The organic phase is washed successively with 2 normal hydrochloric acid (5 ml), water (5 ml), 5% sodium hydrogen carbonate (5 ml), and water (5 ml), and dried with magnesium sulfate. The solvent is removed under reduced pressure to give the crude crystalline peptide **7f**; yield: 1.2 g (95%).

Hydrolysis of Peptides 7a, 7f, and 7p to the Acids 8, 9, and 10 Respectively:

The procedure described above for the hydrolysis of Boc-(OCH_3)-tyrosine methyl ester is used.

Boc-(OBzl)-Tyr-Gly-Phe-Met-OH **(8)**; yield: 50%; m.p. 175°C; $[\alpha]_D^{20}$: − 12.4° (*c* 0.9 DMF).

Amino acid ratio: Tyr: Gly: Phe: Met = 0.52:1.43:0.71:0.73.

$C_{39}H_{49}N_5O_9S$	calc.	C 61.32	H 6.47	N 9.17
(763.9)	found	61.00	5.94	9.21

The acid **8** after hydrogenolysis (palladium on carbon) and removal of the Boc protecting group gives TFA-NH_3-Tyr-Gly-Gly-Phe-Met-OH **(11)** which is purified by reverse phase H.P.L.C.; column: Micro Bondapak-NH_2, 300 × 4 mm Waters; eluent: methanol/water (8:2); flow 1 ml/min; retention tine: 8 min. (TFA = F_3C-$COO^{\ominus}$).

Amino acid ratio: Tyr : Gly : Phe : Met = 0.52 : 1.43 : 0.69 : 0.77.

Boc-(OBzl)-Tyr-Gly-Gly-OH **(9)**; yield: 75%; m.p. 118°C; $[\alpha]_D^{20}$: + 9.65° (*c* 3, ethanol).

$C_{24}H_{31}N_3O_7$	calc.	C 61.84	H 6.44	N 8.65
(485.5)	found	60.74	6.38	8.30

Boc-(OCH_3)-Tyr-D-Ala-Gly-Phe-Leu-OH **(10)**; yield; 79%; oil; $[\alpha]_D^{20}$: − 2.9° (*c* 1.5, methanol).

Amino acid ratio: Tyr: Ala: Gly: Phe: Leu = 0.914:0.997:0.997:0.997:1.080.

$C_{35}H_{49}N_5O_9$	calc.	C 61.48	H 7.22	N 10.24
(683.8)	found	61.30	7.20	10.21

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