Advantageous Applications of Azabenzotriazole (Triazolopyridine)-based Coupling Reagents to Solid-phase Peptide Synthesis

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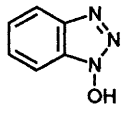
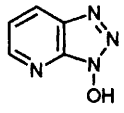
1-Hydroxy-7-azabenzotriazole (HOAt) and its corresponding uronium and phosphonium salts are shown to be superior to their benzotriazole analogs in solid-phase peptide synthesis, thereby making possible the automated synthesis of peptides containing hindered amino acids.

Since the development by König and Geiger[1] in 1970 of 1-hydroxybenzotriazole (HOBt) as a peptide coupling additive, most common methods for peptide bond formation are carried out in its presence.[2] HOBt is used either in conjunction with carbodiimides or active esters, or built into a stand-alone reagent in the form of phosphonium[3] or uronium[4] salts. Recently, 1-hydroxy-7-azabenzotriazole (HOAt)[5] has been described as a superior peptide coupling additive, which enhances coupling yields in solution by about 6–32 times, reduces the loss of chiral integrity by up to 50%, and provides visual indication (yellow-to-colourless) of the reaction endpoint. In this communication, the application of HOAt, as well as its uronium and phosphonium salt derivatives† (Fig. 1) to solid-phase peptide synthesis is described.

In order to demonstrate the suitability of these derivatives and compare their performance relative to that of the corresponding benzotriazole analogs by a solid-phase strategy, several syntheses of fragment 65–74 (H-Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-NH_2)[8] of the acyl carrier protein were carried out by a fluorenylmethyloxycarbonyl (Fmoc)/*tert*-butyl protection scheme [Bu^t for Tyr and Asp, and Trityl (Trt) for Asn and Gln]. Polyethylene glycol-polystyrene (PEG-PS)-resin[9] bearing a peptide amide linker derived from 5-[4-(Fmoc)-aminomethyl-3,5-dimethoxyphenoxy]valeric acid (PAL)[10] was used as a solid support along with a continuous-flow Millipore 9050Plus synthesizer.‡ Coupling times were shortened and excesses of reagents were reduced in order to emphasize the differences between HOAt- and HOBt-mediated couplings. Under these conditions, incomplete incorporations were detected for Asn onto Gly, Ile^{72} onto Asn, Ile^{69} onto Asp, and Val onto Gln. The purity of the peptides was judged, after cleavage of the peptides from the resin with trifluoroacetic acid (TFA)–H_2O (9:1) for 2 h at 25 °C, by reversed-phase high performance liquid chromatography (HPLC). The results, collected in Table 1, indicated clearly that (*i*) HOAt, HATU, and PyAOP are superior to HOBt, HBTU, PyBOP, and PyBroP, respectively (entries 1, 4 *vs.* 2, 5; 9 *vs.* 10; 11 *vs.* 12, 13); (*ii*) uronium and phosphonium salts are preferred to carbodiimide/active ester methods (entries 7, 8 *vs.* 1–6); (*iii*) addition of HOXt to HXTU (X = A, B) couplings did not significantly improve the coupling yields, with the exception of Asn coupling (entries 14, 15 *vs.* 9, 10); (*iv*) uronium salts are slightly better than phosphonium analogues (entries 9, 10 *vs.* 11, 12); and (*v*) uronium salts differ little among themselves (entries 9, 17–20).§



HOBt



HOAt

Equally satisfactory results were obtained when HATU was applied to the solid-phase synthesis of the pentapeptide

Table 1 Distribution of products, including various deletion peptides, according to HPLC analysis[a] of the assembly of ACP(65–74) *via* HOAt- and HOBt-based coupling reagents

Entry	Coupling method	Equiv.[b]	Time/min	ACP	-2Ile	-Ile^{72}	-Ile^{69}	-Val	-Asn
1	DIPCDI[c]–HOAt	4	3	65	2	7	9	1	2
2	DIPCDI–HOBt	4	3	31	13	15	18	3	1
3	DIPCDI	4	3	14	4	7	22	2	32
4	Pfp[d]–HOAt	4	3	82	—	—	15	1	1
5	Pfp–HOBt	4	3	53	1	1	33	5	1
6	Pfp	4	3	0[e]	—	—	—	—	—
7	HATU	4	3	84	—	—	—	3	8
8	HBTU[f]	4	3	73	—	1	2	3	5
9	HATU	1.5	1.5	53	3	6	12	3	16
10	HBTU	1.5	1.5	18	16	11	19	2	7
11	PyAOP	1.5	1.5	45	5	7	16	1	12
12	PyBOP[g]	1.5	1.5	12	19	12	16	1	2
13	PyBroP[h]	1.5	1.5	0[e]	—	—	—	—	—
14	HATU–HOAt	1.5	1.5	50	5	9	12	2	8
15	HBTU–HOBt	1.5	1.5	17	18	12	18	3	3
16	AOP	1.5	1.5	49	3	6	13	1	18
17	HAPyU	1.5	1.5	50	4	7	15	2	13
18	HAPipU	1.5	1.5	50	1	7	14	3	13
19	HAMDU	1.5	1.5	53	1	6	13	3	13
20	HAMTU	1.5	1.5	41	1	7	12	7	16

[a] Reversed-phase C-18 columns were used with elution by a linear gradient over 20 min of 0.1% TFA in MeCN and 0.1% aqueous TFA from 1:19 to 1:4, flow rate 1.0 ml min^{-1}. [b] Couplings were carried out in DMF in the presence of 2 equiv. of DIEA per equiv. of Fmoc-amino acid/coupling reagent. [c] *N,N'*-diisopropylcarbodiimide. [d] Pentafluorophenyl ester. [e] None of the desired product was obtained. [f] *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate. [g] Benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate. [h] Bromo tris(pyrrolidino)phosphonium hexafluorophosphate.

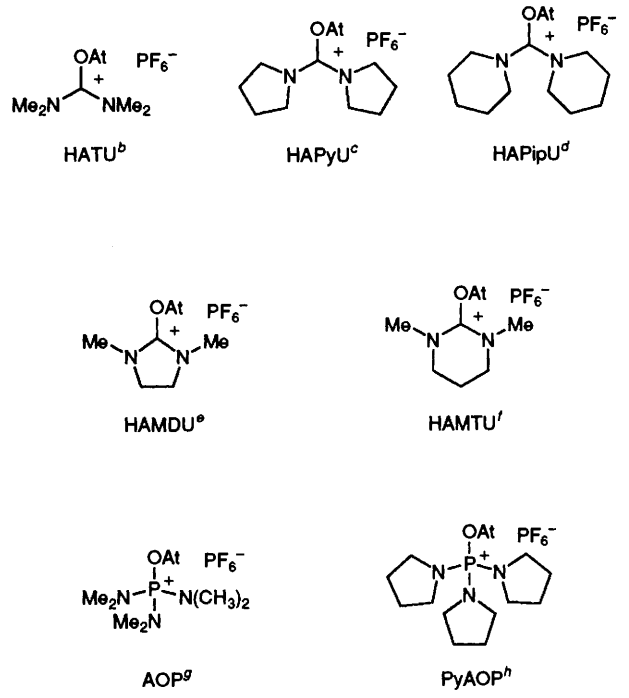


Fig. 1 Structures of HOAt-based coupling reagents. [Abbreviations for these compounds have generally kept to the style previously used for the HOBt analogues; [b]*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; [c]*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-bis(tetramethylene)uronium hexafluorophosphate; [d]*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-bis(pentamethylene)uronium hexafluorophosphate; [e]*O*-(7-azabenzotriazol-1-yl)-1,3-dimethyl-1,3-dimethyleneuronium hexafluorophosphate; [f]*O*-(7-azabenzotriazol-1-yl)-1,3-dimethyl-1,3-trimethyleneuronium hexafluorophosphate; [g]7-azabenzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; [h]7-azabenzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate.]

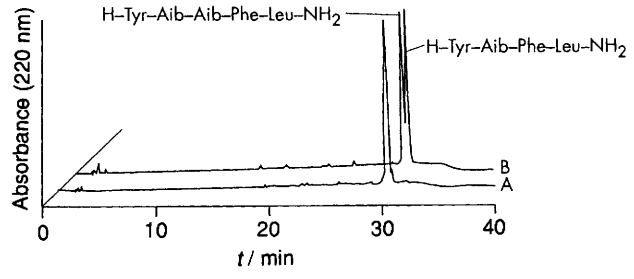


Fig. 2 HPLC chromatograms of crude peptide mixtures containing H-Tyr-Aib-Aib-Phe-Leu-NH_2 directly after extraction with chloroform and H_2O–acetic acid (7 : 3) of the cleavage reagent [TFA–H_2O (9 : 1)]. (A) HATU–HOAt; (B] HBTU–HOBt. Synthetic strategies were as specified in the text and footnote (‡). Reversed-phase C-18 columns were used for the analysis with elution by a linear gradient, over 20 min of 0.1% TFA in MeCN and 0.1% aqueous TFA from 1 : 19 to 3 : 7, flow rate, 1.0 ml min^{-1}.

H-Tyr-Aib-Aib-Phe-Leu-NH_2 under the same conditions. This peptide contains the sequence Aib-Aib, which is described as being particularly difficult for both solution[11] and solid-phase strategies.[11,12] After 2 h of coupling using 4 equiv. of either Fmoc-Aib-OH or Fmoc-Tyr(Bu^t)-OH, 4 equiv. of HXTU, and 8 equiv. of DIEA (all remaining couplings were carried out for 30 min), the pentapeptide was obtained with a purity of 94% for the HATU synthesis, and only 43% for HBTU (Fig. 2).

Finally, synthesis of hexapeptide H-D-Ala-MeLeu-MeLeu-MeVal-Phe-Val-OH on a hypersensitive acid-labile (HAL)[13-

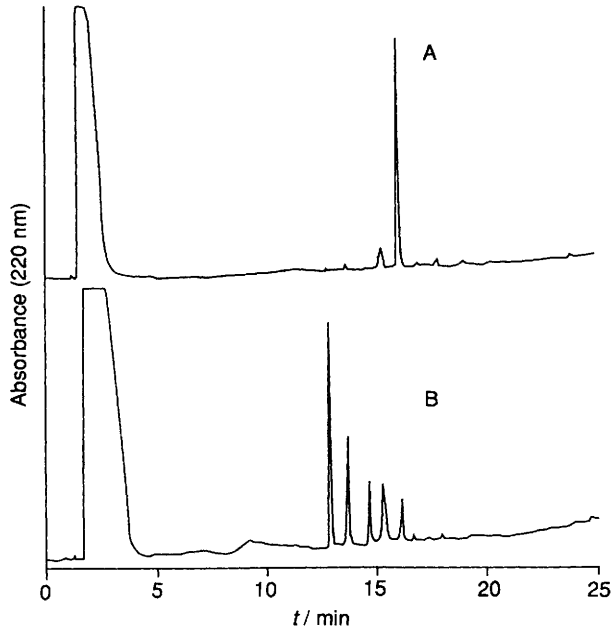


Fig. 3 HPLC chromatograms of crude peptide mixtures containing H-D-Ala-MeLeu-MeLeu-MeVal-Phe-Val-OH directly after evaporation of cleavage reagent. (A) HATU–HOAt; (B) HBTU–HOBt. Synthetic strategies were as specified in the text and footnote (¶). Reversed-phase C-18 columns were used for the analysis with elution by a linear gradient over 20 min of 0.1% TFA in MeCN and 0.1% aqueous TFA from 0 : 1 to 1 : 0, flow rate 1.0 ml min^{-1}. The peak at 16.1 min corresponds to the title peptide. Peaks at 12.9, 13.7, 14.6 and 15.3 min correspond to H-MeVal-Phe-Val-OH, H-D-Ala-MeVal-Phe-Val-OH, H-MeLeu-MeVal-Phe-Val-OH, and H-D-Ala-MeLeu-MeVal-Phe-Val-OH, respectively.

PEG-PS-resin was attempted.¶ The *N*-terminal tetrapeptide section of this peptide, containing three consecutive *N*-methyl amino acids, represents a fragment of cyclosporin.[14] Synthesis of a peptide of this type constitutes an exceptionally difficult challenge even for conventional solution strategies.[15] Incorporation of the last three amino acids required a double coupling protocol for 2 h. Using these conditions and after cleavage of the peptide with TFA–CH_2Cl_2 (5 : 95) for 1 h, the hexapeptide was obtained in a purity of 85% in the case of HATU, and only 8% for HBTU. In the HBTU synthesis, the major peak (48%) corresponded to the tripeptide H-MeVal-Phe-Val-OH, and the rest of the peaks to various deletion peptides (Fig. 3).

In conclusion, the marked efficiency of 7-azabenzotriazole-based coupling reagents in solid-phase peptide synthesis has been demonstrated. These derivatives enhance reactivity and are especially suited to the preparation of peptides containing hindered amino acids. Previously only Fmoc-amino acid fluorides have been used successfully for the solid phase assembly of peptides bearing contiguous Aib units.[16]

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Footnotes

† These derivatives were prepared from 1-hydroxy-7-azabenzotriazole according to published methods.[6,7]

‡ All syntheses performed in this work were carried out automatically. The flow rate of the unit pump was set at 5.0 ml min^{-1} and the following synthetic protocol was used: Fmoc group deprotection with 1,8-diazabicyclo[5,4,0]-undec-7-ene (DBU)-piperidine-*N*,*N*-dimethylformamide (DMF) (2:2:96) (6 min), DMF washing (7 min), Fmoc-amino acid coupling (see text and Table 1), and DMF washings (8 min).

§ A standard coupling protocol for routine peptide synthesis comprises 2 equiv. each of Fmoc-amino acid and HATU, and 4 equiv. of DIEA, for 10 min. For Asn and Gln couplings, 2 equiv. of HOAt are added. Using this protocol ACP (65–74) has been obtained with a purity of 96%. For more complex peptides, extended coupling times and extra equivalents of reagents may be advisable depending on the case.

¶ Incorporation of the first Fmoc-amino acid (4 equiv.) onto the hydroxymethyl resin was performed with DIPCDI (4 equiv.), in the presence of 4-(dimethylamino)pyridine (0.4 equiv.) in DMF for 1 h. Application of HOAt derivatives for this purpose is under study.

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