Pergamon

Tetrahedron Letters, Vol. 38, No. 27, pp. 4853-4856, 1997

0040-4039/97 $17.00 + 0.00

PII: S0040-4039(97)01011-3

On the Use of PyAOP, a phosphonium salt derived from HOAt, in Solid-Phase Peptide Synthesis[1]

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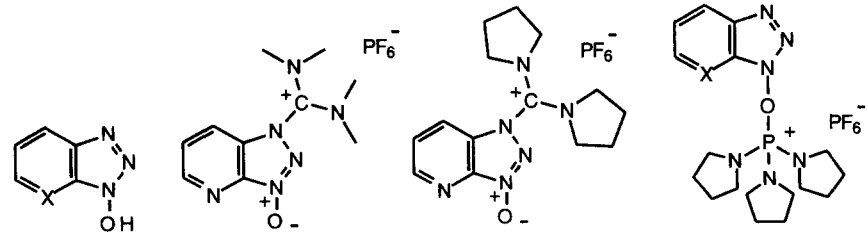
Abstract: Phosphonium derivatives of HOAt such as PyAOP are useful for the solid-phase preparation of a range of peptides that include those incorporating hindered amino acids, difficult short sequences, and cyclic peptides. An advantage relative to uronium salts is that excess PyAOP does not undergo the detrimental side-reaction at the amino terminus which blocks further chain assembly. © 1997 Published by Elsevier Science Ltd.

During the past two decades, improved methods have been reported for peptide bond formation in both solution and solid-phase syntheses. Recently, uronium salts based on 1-hydroxy-7-azabenzotriazole (HOAt)[2] such as {*N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-b]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU), and 1-(1-pyrrolidinyl-1*H*-1,2,3-triazolo[4,5-b]pyridin-1-yl methylene) pyrrolidinium hexafluorophosphate *N*-oxide (HAPyU)} have shown significant advantages for the coupling of hindered amino acids, protected segments and hydrophobic sequences in terms of coupling yields, reduced racemization and faster acylation rates.[2,3] Phosphonium derivatives of 1-hydroxybenzotriazole (HOBt) such as benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP)[4] and benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP)[5] are also commonly used for peptide assembly. This report discusses the extension of these techniques to a phosphonium-based analog of HATU, 7-azabenzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate (PyAOP) and describes practical aspects of the utility of this reagent.[6]



PyAOP as a solid is rather stable; at 55 °C it is completely stable for two days and less than 2 % decomposition occurs over a period of one week, as shown by HPLC analysis. Solutions of PyAOP (0.5 M) in DMF in the reservoir of a PerSeptive 9050-Plus continuous-flow synthesizer are usable for one week (less than

10% decomposition). In these tests PyBOP is more stable than PyAOP, suggesting that the latter is the more reactive of these two species.

According to a method used earlier, the effectiveness of PyXOP where X=A, B as a coupling reagent was examined in the solid-phase assembly of the decapeptide H-Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-NH_2, ACP (65-74).[7] In previous work the deletion by-products, des-Asn^{73}, des-Ile^{72}, des-Ile^{69}, des-Val^{65}, des-Ile^{69}, Ile^{72} had been characterized.[3a] Coupling times were intentionally decreased to 1.5 min and the excesses of reagents were reduced to 1.5 equiv in order to exaggerate the differences among the various phosphonium reagents. Peptide elongation was carried out in the C→N direction on a polyethylene glycol-polystyrene graft (PEG-PS) resin[8] using a PAL linker[9] on a PerSeptive 9050-Plus continuous-flow synthesizer. Release of peptide from the solid support and removal of side-chain protecting groups with TFA-H_2O (9:1) for 2 h at 25 °C was followed by reversed-phase HPLC to determine the purity of the crude products (Table 1).

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

Entry	Coupling method	Equiv	Time/min	ACP	-2Ile	-Ile^{72}	-Ile^{69}	-Val	-Asn
1	PyBOP	1.5	1.5	10	22	13	13	3	<1
2	PyBOP-HOAt	1.5	1.5	19	13	12	12	3	2
3	PyBOP-HOBt	1.5	1.5	11	21	14	14	3	<1
4	PyAOP	1.5	1.5	46	3	5	5	2	18
5	**PyAOP-HOAt**	**1.5**	**1.5**	**60**	**3**	**6**	**7**	**4**	**2**
6	PyAOP-HOBt	1.5	1.5	45	9	12	12	2	2

Analysis of the chromatograms indicated that the azabenzotriazole based phosphonium salts were as effective as the analogous uronium salts and were superior to the corresponding benzotriazole derivatives (entries 1,2,3 vs. 4,5,6).[3a] Furthermore, the addition of HOXt, where X=A, B was not required except for Fmoc-Asn(Trt)-OH (entries 1 vs. 2,3 and 4 vs. 5,6). However, the use of HOBt in PyAOP-mediated reactions lowers the coupling efficiency of other amino acids (entries 6 vs. 4,5). If no side-chain protection is used for Asn, no incorporation of this residue was detected presumably due to rapid aspartimide formation.[10] To confirm that hydrated amino acids were compatible with azabenzotriazole reagents, 1.5 equiv of Boc-Ile-OH·1/2H_2O and PyXOP and 3 equiv of DIEA were added to H-Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-PAL-PEG-PS for 1.5 min, the peptide was released from the solid support with TFA-H_2O (9:1) for 2 h at 25 °C and the ratio of *N*-terminal Ile to the des-Ile product was examined by HPLC. A 3:1 and 1:1 ratio of the *N*-terminal Ile:des-Ile product was obtained for PyAOP and PyBOP, respectively, confirming that the aza derivative was not affected by hydrated reagents and performed better than the analog which lacked the pyridine *N*-atom.

As a more challenging model the pentapeptide H-Tyr-Aib-Aib-Phe-Leu NH_2[11] was assembled on Fmoc-PAL-PEG-PS using 2-h [Fmoc-Aib-OH and Fmoc-Tyr(tBu)-OH] and 30-min [Fmoc-Phe-OH and Fmoc-Leu-OH] couplings in the presence of 4 equiv of amino acid, 4 equiv of PyXOP and 8 equiv of DIEA. Cleavage of the peptide from the solid support with TFA-H_2O (9:1) followed by HPLC analysis gave the desired pentamer:des-Aib sequence in ratios of 3:1 and 1:3 for PyAOP and PyBOP, respectively. These results were similar to those obtained using HOXt-based uronium salts as coupling reagents.

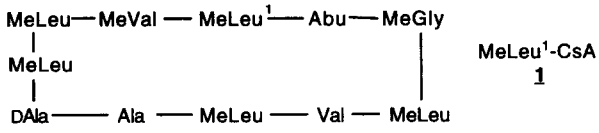
Interest in cyclic peptides has stimulated the development of new methods for the preparation of this family of peptides in which all steps, including the cyclization, have been carried out in the solid-phase mode.[12] Unfortunately, resin-bound cyclizations can be plagued by sluggish kinetics. In slow uronium salt-mediated couplings, a detrimental side-reaction involves formation of a guanidine residue at the amino terminus thereby

blocking further reaction.[13] Phosphonium reagents do not take part in an analogous phosphine imine-forming side reaction and therefore may be advantageous reagents for resin-bound or solution cyclizations.[14] As a model, tachykinin antagonist *cyclo*(Tyr-DTrp-Val-DTrp-DTrp-Arg-Asp) was chosen for assembly. The first amino acid, Fmoc-Asp-OAl, was incorporated onto a PAC-PEG-resin through its free β-carboxyl side-chain. After elongation of the peptide chain using the Fmoc/*t*Bu strategy, allyl removal was accomplished with $Pd(PPh_3)_4$ in $CHCl_3$–HOAc–NMM (20:1:0.5) for 2 h at 25 °C. Following N^{α}-Fmoc removal by treatment with piperidine–DMF (1:4), cyclization was carried out with either PyXOP or HXTU/HOXt/DIEA (5:5:10) in DMF for 2 min at 25 °C. After cleavage of the anchoring linkage and removal of the side-chain protecting groups with reagent R,[9] the crude product was analyzed by HPLC (Table 2).

Table 2. A comparison of different reagents for the cyclization of H-Tyr-DTrp-Val-DTrp-DTrp-Arg-Asp(*O*-PAC-PEG-PS)-OH.

Cyclization Method	Linear (%)	Cyclic (%)	Purity (%)
PyAOP/HOAt	5	74	61
PyBOP/HOBt	5	69	51
HATU/HOAt	5	70	50
HBTU/HOBt	5	63	44

N-substituted amino acids represent an important class of hindered amino acids. Coupling reactions involving these amino acids represent a continuing challenge with the difficulty increasing enormously when several such *N*-substituted amino acids are linked to each other.[3a,15] The cyclic undecapeptide cyclosporin A contains seven *N*-methylamino acids, four of them in succession. For the present studies MeLeu[1]-cyclosporine (**1**) was chosen for assembly. In this model, (4R)-4((E)-2-butenyl)-4-methyl-L-threonine, present in the natural peptide, was substituted by MeLeu.[16] The synthesis of the linear sequence was carried out using a HMPB-PEG-PS-resin[17] starting with DAla. This highly acid-sensitive resin was chosen because amide bonds between hindered amino acids are prone to undergo hydrolysis under strong acidic conditions.[18] D-Ala was chosen as the *C*-terminal amino acid to avoid the presence of any *N*-methylamino acids in the two first positions of the sequence since it is well known that these residues favor the formation of diketopiperazines during deprotection of the second residue.[19] Fmoc-D-Ala-OH (5 equiv) was incorporated *via* DIPCDI (5 equiv) in the presence of DMAP (0.5 equiv) in DMF, using double coupling for 1 h at 25 °C. The remaining Fmoc-amino acids (5 equiv) were coupled *via* HATU (4.75 equiv) in the presence of DIEA (10 equiv) in DMF for 1 h at 25 °C.[17] For coupling to *N*-methylamino residues arbitrary double couplings were used (2 x 2 h), since application of the ninhydrin test for complete coupling to *N*-methyl amino acids was not possible. Following linear assembly and removal of the terminal Fmoc group, release of the peptide from the solid support was performed with TFA–CH_2Cl_2 (1:19) for 1 h at 25 °C. The solvent was removed by a stream of nitrogen, water was added, and the solution was lyophilized. Analysis by HPLC revealed a single peak (> 97% purity) with the correct FAB-MS (1164.8). The linear MeLeu[1]-cyclosporine was dissolved in DMF and PyAOP (5 equiv) and HOAt (5 equiv) in DMF and DIEA (10 equiv) were added. After 10 min, HPLC analysis showed that all of the linear peptide had undergone cyclization. The solution was evaporated to dryness to give crude MeLeu[1]-CsA (**1**), which was purified by HPLC and showed the expected molecular weight by FAB-MS (1146.9).



In conclusion, phosphonium derivatives of HOAt such as PyAOP are useful for the solid-phase preparation of a range of peptides, including those incorporating hindered amino acids, difficult short sequences, and cyclic systems. An advantage relative to the corresponding uronium salts is that excess PyAOP does not participate in any chain-terminating side reactions at the amino terminus.

Acknowledgments. This work was partially supported by CICYT (PB95-1131 and PB96-1490) and Generalitat de Catalunya [Grup Consolidat (1995SGR 494) i Centre de Referència en Biotecnologia], and the National Institutes of Health (GM-09708) and the National Science Foundation (CHE-9314038). J. A. thanks the Ministerio de Educación y Ciencia, Madrid for financial support.

References and Notes

1. Abbreviations used in this article: ACP, acyl carrier protein; Al, allyl; Aib, aminoisobutyric acid; BOP, benzotriazol-1-yloxy-tris-(dimethylamino)phosphonium hexafluorophosphate; CsA, cyclosporine; DIEA, *N,N*-diisopropylethylamine; DIPCDI, *N,N'*-diisopropylcarbodiimide; DMAP, *N,N*-dimethylaminopyridine; DMF, *N,N*-dimethylformamide; FAB-MS, Fast atom bombardment mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; HAPyU, 1-(1-pyrrolidinyl-1*H*-1,2,3-triazolo[4,5-b]pyridin-1-ylmethylene) pyrrolidinium hexafluorophosphate *N*-oxide; HATU, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-b]pyridin-1-ylmethylene]-*N*-methylmethan-aminium hexafluorophosphate *N*-oxide; HBTU, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide, HMPB, 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid; HPLC, high performance liquid chromatography; HOAc, acetic acid; HOAt, 1-hydroxy-7-azabenzotriazole; HOBt, 1-hydroxybenzotriazole; NMM, *N*-methylmorpholine; PAL, 5-(4-aminomethyl-3,5-dimethoxyphenoxy)valeric acid; PAC, 3-(4-hydroxymethylphenoxy) propionic acid; PEG-PS. polyethylene glycol-polystyrene graft; PyAOP, 7-azabenzotriazol-1-yl-oxytris(pyrrolidino)phosphonium hexafluorophosphate; PyBOP, benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate; Reagent R, TFA–1,2-ethanedithiol–*p*-cresol–anisole (90:5:3:2); TFA, trifluoroacetic acid; Trt, triphenylmethyl (trityl). Amino acid denotes L-configuration unless otherwise noted.
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(*Received in UK* 1 *May* 1997; *accepted* 23 *May* 1997)