PyClocK, the phosphonium salt derived from 6-Cl-HOBt

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ABSTRACT

Novel phoshphonium salts of benzotriazoles, which are highly reactive coupling agents, and are particularly useful in peptide synthesis, are described. Further described is a process of preparing the novel phosphonium salts and methods for preparing peptides.

INTRODUCTION

The peptide coupling reagent field has clearly evolved in the last decade from carbodiimides to "onium" (phosphonium and uronium) salts (1). The era of industrial coupling reagents began in 1955 with the introduction of dicyclohexylcarbodiimide (DCC) (2), which at that time was already known and well studied, as a reagent for the formation of amide bond. Unfortunately, carbodiimides did not comply with the concept of ultimate coupling reagents because its high reactivity provokes racemization and side reactions during the coupling reaction (1).

In the early 70's, 1-hydroxybenzotriazole (HOBt) (3) was proposed as an additive to DCC to reduce racemization and from then on other benzotriazole derivatives such as 1-hydroxy-7-azabenzotriazole (HOAt) (4) and more recently 1-hydroxy-6-chlorobenzotriazole (6-Cl-HOBt) (5) have also been used (Figure 1). The OBt active esters are less reactive than the O-acylisourea, but are less prone to racemize and more stable.

The predominance of carbodiimides has been overshadowed by the stand-alone reagents, such as the above mentioned immonium and phosphonium salts (1). Among the former, the use of the most reactive of this class, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-yl-methylene)-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU) (1, 6), is problematic because of its price, which makes its use impractical for industry. *N*-[(1*H*-6-chlorobenzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate/tetrafluoroborate *N*-oxide (HCTU/TCTU) (7), based on 6-Cl-HOBt, are good alternatives to *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino) methylene]N-methylmethanaminium hexafluorophosphate/ tetrafluoroborate *N*-oxide (HBTU/TBTU) (8), because of the presence of the chlorine atom that stabilizes the structure, hence, making these reagents less hazardous and more reactive (Figure 1). However, the use of these salts as peptide coupling agents was found to have several limitations. Thus, during the activation of hindered carboxylic components, such as those involved in cyclization reactions of hindered amino acids, the immonium salts can react with a more available amino component, leading to guanidine derivative, a process that terminates the peptide chain (9).

The most prevalent phosphonium salts of benzotriazoles currently used as peptide coupling agents are benzotriazol-1-yl-N-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP) (10) and the more recently discovered azabenzotriazol-1-yl-N-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate (PyAOP) (11), derived from HOAt and considered the most reactive phosphonium salt known (Figure 1). PyAOP and PyBOP are particularly useful as coupling reagents in cyclization steps or for the activation of hindered amino acids, where the use of the immonium salts can lead to the formation of guanidine derivatives. Since it was found that PyAOP and PyBOP do not terminate the peptide chain (9), these coupling agents can be used in excess and be added during the coupling step. However, these compounds too have their limitations, the former due to its relatively high cost and the latter to its somewhat lower reactivity.

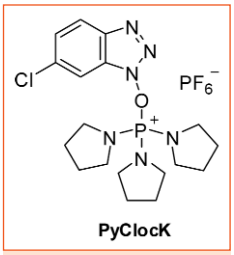

Figure 2. Structure of the new phosphonium salt PyClock.

Due to the ongoing development of peptide-based drugs and in view of the limitations associated with the above mentioned peptide coupling reagents, it would be highly advantageous to have a novel, efficient peptide coupling agent free of the above limitations (12). In the quest for such a coupling agent, 6-chloro-benzotriazole-1-y-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyClocK) was designed (Figure 2), prepared and successfully implemented. , This novel compound was found to be both more effective than other commonly used coupling agents and simple to produce and use in modern peptides synthesis techniques.

RESULTS AND DISCUSSION

Design and Synthesis

PyClocK is derived from Cl-HOBt. The acidity of Cl-HOBt is similar to that of HOAt (pKa: 3.35 for Cl-HOBt: 3.28 for HOAt, and 4.60 for HOBt). Although Cl-HOBt does not present the assisted basic catalysis of HOAt, coupling is faster than with HOBt (7). PyClocK is industrially prepared in multiKg batches by our own technology following previously known methods reported in the literature (1), which assure a high purity and total absence of pyrrolidine. Pyrrolidine can react with the carboxylic acid giving the corresponding pyrrolidide derivatives, jeopardize cyclizations and coupling fragments (13).

Pyrrolidine can not be detected by standard analytical methods and Its presence cannot be checked by NMR, since the ^1H chemical shifts of free pyrrolidine are not distinguishable from the ones in the phosphonium salt. Also a lack of a cromophore group also does not enable the HPLC analysis. This can be solved by coupling pyrrolidine with a cromophore-containing molecule, such as Z-Gly-OH (Figure 3). Consequently, a 0.05M solution of

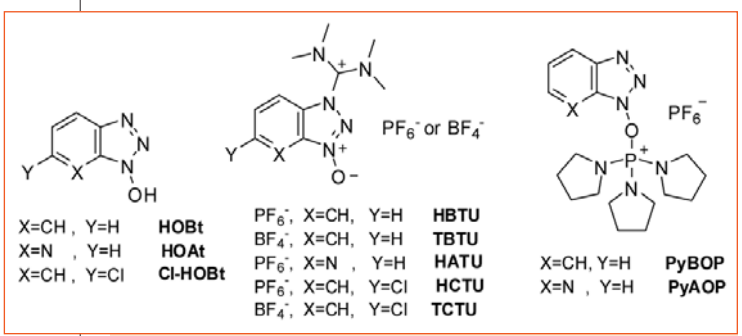

Figure 1. Structures of benzotriazole additives and immonium and phosphonium salts derived there from

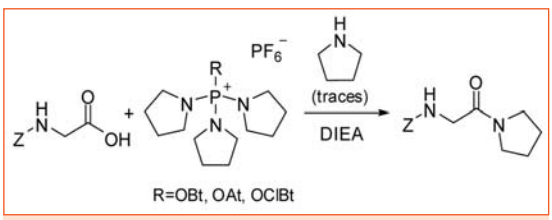

Figure 3. Side-reaction due to the presence of pyrrolidine traces into phosphonium salts.

Time	PyClocK	6-Cl-HOBt	PyBOP	HOBt	PyAOP	HOAt
4 hr	78.74	21.26	83.39	16.61	76.55	23.45
10 hr	44.82	55.18	64.01	35.99	39.65	60.35
24 hr	0.04	99.96	1.09	98.91	0.16	99.84

Table 1. Stability of the phosphonium salts in DMF. Yields were calculated according to the integration of the peak area at 220 nm of the signal associated with phosphonium salt with respect to the corresponding HXBt.

the protected amino acid plus *N,N*-diisopropylethylamine (DIEA) and 10 eq PyClock were checked at 10 min, 1 hr and 2 hr. Then 10 eq PyClock were added and the mixture was again checked at 10 min and 1hr. The analysis by HPLC showed in all cases the total absence of Z-Gly-Py (compared to the retention time of the pure product made beforehand).

Stability

The stability of a phosphonium salt can be correlated with its reactivity. For that purpose, the relative stability of PyAOP, PyBOP and PyClock was measured in DMF. A 0.05 M solution of the corresponding coupling reagent in DMF was prepared and analyzed by HPLC (see Table 1).
PyBOP and PyClock were clearly more stable however less reactive than PyAOP.

Activation of bulky residues

The ability of the phosphonium salt to activate an β-carbon sterically hindered amino acid is taken into account. The activation is slower than with less hindered amino acids and therefore differences among the coupling reagents can be appreciated easily. The chosen amino acid was Fmoc-aminoisobutyric (Fmoc-Aib-OH).
A 0.33 M Fmoc-Aib-OH in DMF solution was prepared. Then, 3 equivalents of DIEA and 1 equivalents of the phosphonium salt were added. The activation process was followed by HPLC analysis (see Table 2). PyAOP was not tested, due to the superiority shown in the previous experiments. The activation of Fmoc-Aib-OH was faster with PyClock than with PyBOP. After 2 hr of setting the mixture, the ratio activated/non-activated aminoacid began to decrease in the PyClock experiment while in the PyBOP one it continued growing. This can be rationalized, bearing in mind that the 6-Cl-HOBt active ester is more reactive (and therefrore unstable) than the HOBt ester. In the absence of a nucleophile, after 2 hr, the rate of active ester formation became lower than its decomposition rate in the PyClock case, and so, the ratio activated/non-activated amino acid diminished.

Coupling efficiency

In order to further test the performance of PyClock relative to its parent phosphonium salts PyAOP and PyBOP, it was interesting to see their efficiency in difficult couplings, in which sterically hindered amino acids were involved.

	PyClock		PyBOP	
Time	Fmoc-Aib-OH	Fmoc-Aib-OClBt	Fmoc-Aib-OH	Fmoc-Aib-OBt
t = 2 min	18.1	81.9	32.5	67.5
t = 1hr	14.1	85.9	31.5	68.6
t = 2hr	21.4	78.6	27.3	72.7

Table 2. Activation of Fmoc-Aib-OH with PyCloCK and PyBOP. Yields were calculated according to the integration of the peak area at 220 nm of the signal corresponding Tp Fmoc-Aib-OXt with respect Fmoc-Aib-OH.

Deletion peptides of variable length are usually formed in these experiments. The efficiency is measured in terms of the percentage of target peptide obtained, relative to the different deletion peptides. Syntheses were carried out in solid phase either manually or in an automatic synthesizer.

	Peptides	PyClocK	PyBOP	PyAOP
1	H-Tyr-MeVal-Phe-Leu-NH_2	65.6	48.6	67.4
2	H-Tyr-Aib-Aib-Phe-Leu-NH_2	97.2	85.1	99.5
3	H-Tyr-Arg-Arg-Phe-Leu-NH_2	84.7	74.7	-
4	Human Leptin (150-167)	63.1	48.3	-

Table 3. Coupling efficiency in terms of percentage of target peptide obtained. Experiments 1-3 were carried out manually and experiment 4 in an automatic synthesizer.

Pentapeptides with general sequence H-Tyr-aa_1-aa_2-Phe-Leu-NH_2 (where both aa1 and aa2 are the same residue in a single pentapeptide: Aib, Arg or MeVal) were manually elongated on a Fmoc-RinkAmide-MBHA-PS resin. Coupling time was 30 min and excesses of reagents were 5 eq Fmoc-amino acid-OH (in the case of Arg, the guanidine group was protected with Pbf), 5 eq PyXOP and 15 eq DIEA. Cleavages of the resin-bound peptides were carried out with TFA (1hr). Crudes were precipitated with ether, lyophilized and analyzed by HPLC. Syntheses of Human Leptin (H-LQGSLQDMLWQLSPGC-OH), larger peptides were run in an automatic synthesizer (Results are displayed in Table 3).

The phosphonium salts showed high efficiency in the synthesis of the pentapeptides, generally with high yields, except in H-Tyr-MeVal-MeVal-Phe-Leu-NH_2, where no pentapeptide was obtained at all, and even the deletion tetrapeptide H-Tyr-MeVal-Phe- Leu-NH_2 was found in low yield. PyAOP was the most efficient in all cases and for that reason required no further testing in the rest of the experiments.

PyClock performed better than PyBOP. Reactions in the synthesizer were designed to compare only PyBOP and PyClock. The latter gave higher yield. Thec conclusion can be therefore that PyClock was more efficient than PyBOP.

Conditions		Z-L-Phe-D-Val-L-Pro-NH_2/ Z-L-Phe-L-Val-L-Pro-NH_2
DIEA	PyAOP	27
	PyBOP	43
	PyClock	34
DIEA/collidine (1:1)	PyAOP	8
	PyBOP	18
	PyClock	13

Table 4. Degree of racemization expressed as percentages of Z-D-Phg-Pro-NH_2 vs Z-Phe-D-Val-Pro-NH_2 obtained in the HPLC.

Protein coupling

The use of the PyClock coupling reagent was investigated in the construction of a model biosensor through the attachment of a protein, HRP to an optical fiber transducer. A comparison of protein coupling protocols was undertaken, in which of activation was performed by either PyClockK or EDAC/NHSS.

The use of the PyClockK yielded a 2.6 fold increase in the attachment level of the bioreceptor relative to the EDAC/NHSS activation. The increase in attachment efficiency in turn led to an increase in luminescence level when checked by means of PMT photodetection.

Racemization

The racemization suppression capacity of the phosphonium salts was studied by employing the system **Z-Phe**-Val-Pro-NH_2, which is prone to racemize. Experiments were carried out in solid phase on an Fmoc-Rink amide-MBHA-PS-resin. After introduction of Pro, coupling with either Z-Phe-Val-OH was performed in DMF with 3 equivalents of PyXOP, 3 equivalents HOXt , 3 equivalents Z-Phg/Phe-Val-OH and 6 equivalents of DIEA or DIEA/collidine (1:1) for 1 hr at 0°C and then an additional hour at 25°C. After cleavage of resin-bound peptides with TFA-H_2O (19:1) for 1 hr, crudes were lyophilized and analyzed by HPLC (Table 4). Racemization rates were higher than expected. Couplings effected with PyAOP gave in all experiments much less racemization than with PyClockK and PyBOP, PyClockK being slightly superior in terms of racemization compared to than PyBop.

CONCLUSIONS

PyClockK was found to give better performance than the hydroxybenzotriazole derivative PyBOP in terms of coupling efficiency and of racemization control.

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