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Assessment of new 6-Cl-HOBt based coupling reagents for peptide synthesis. Part 1: Coupling efficiency study

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Summary

The efficiency of a series of well-known coupling reagents (TBTU, HATU, and PyBOP) and of new *in situ* activating reagents (TCTU, HCTU, and DMTMM) was compared by synthesizing the 65–74 fragment of the Acyl Carrier Protein (H-Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-NH_2), containing 'a difficult sequence', as a test peptide, in a multiple peptide synthesizer. The longer sequence rMOG(35–55) was also synthesized. It was clear that the aminium salts are more efficient than the phosphonium salt (PyBOP) and that the new 6Cl-HOBt based reagents (HCTU and particularly TCTU) are very efficient, while DMTMM appeared to be not suitable for SPPS.

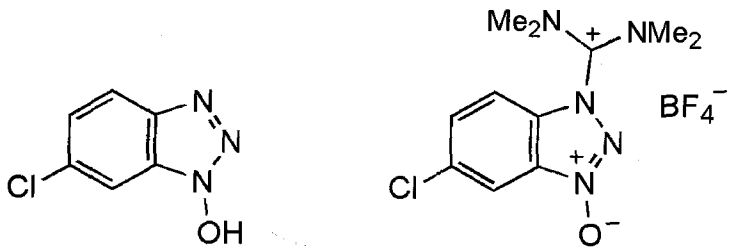
Abbreviations: DMF, *N,N*-dimethylformamide; DMTMM, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride; DMSO, dimethylsulfoxide; 6Cl-HOBt, 6-chloro-1-hydroxybenzotriazole; EDT, 1,2-ethanedithiol; HATU, 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo(4,5-*b*) pyridinium 3-oxide hexafluorophosphate; HCTU, 1-[bis(dimethylamino)methylene]-5-chloro-1*H*-benzotriazolium 3-oxide hexafluorophosphate; HOAt, 1-hydroxy-7-azabenzotriazole; HOBt, 1-hydroxybenzotriazole; LC-ESIMS, liquid chromatography electron spray-ionization mass spectrometry; NMM, *N*-methylmorpholine; NMP, 1-methyl-2-pyrrolidinone; PyBOP, benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate; RP-HPLC, reverse phase high-performance liquid chromatography; SPPS, solid-phase peptide synthesis; TBTU, 1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium 3-oxide tetrafluoroborate; TCTU, 1-[bis(dimethylamino)methylene]-5-chloro-1*H*-benzotriazolium 3-oxide tetrafluoroborate; TFA, trifluoroacetic acid.

Introduction

The growing request of synthetic peptides from biomedical and pharmaceutical researches produced, in the last years, the diffusion of many automatic solid-phase peptide synthesizers. The successful use of these instruments depends mainly on the efficiency of the coupling reaction. A further development of efficient coupling methodologies was prompted by combinatorial chemistry that requires the simultaneous synthesis of a large number of compounds.

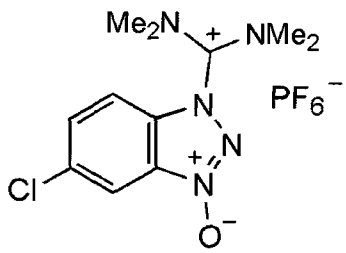
The protocols routinely used on an automatic multiple peptide solid-phase synthesizer take advantage of potent coupling reagents and large excess of the acylating mixture. On the other hand it is well known that over-activation may lead to undesired side reactions [1]. For example, aminium salts can react with N-terminal amino component giving a guanidine derivative [2]. Moreover, the high cost of some protected amino acids and coupling reagents suggests maintaining their consumption to a reasonably low level, compatibly with the success of the synthesis.

In order to prepare peptides of different length and sequences, the coupling reagents to be used in the solid-phase strategy must be efficient, in terms of yield and enantioselectivity; moreover they must be applied

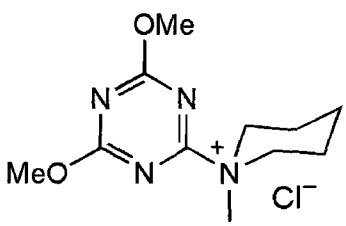


6Cl-HOBt

TCTU



HCTU



DMTMM

Figure 1.

repetitively to a wide range of substrates, as in the case of hindered amino acids [1]. In addition, when using an automatic peptide synthesizer, coupling reagents should be preferably commercially available and easy-to-use with the following characteristics: fast reactions at room temperature, a good solubility in the common solvents and the solution must be stable for several days.

During the last years several studies have been presented about the relative efficiencies of different coupling techniques [2, 3]. The carbodiimide reagents, used in the presence of HOBt [4] or HOAt [5] for the inhibition of side reactions such as racemization, are mostly replaced by phosphonium and aminium salts of HOBt or HOAt [6].

Aim of our study was to compare the performance of different coupling reagents in the case of automatic synthesis, where the reagents are stored in solution for a time depending on the length of the peptide to be synthesized. We compared the most common coupling reagents with the new 6Cl-HOBt-derivatives recently proposed by Luxembourg Ltd. We selected TBTU [7] (HOBt-based aminium salt), HATU [5] (HOAt-based aminium salt), PyBOP [8] (HOBt-based phosphonium salt) and two novel aminium salts based on Cl-HOBt, TCTU and HCTU [9]. These products contain chlorine as an electron withdrawal on the benzotriazole moiety (Figure 1). We included in the study also DMTMM [10], a triazine-based reagent, that was recently proposed as a valuable and cheap alternative to common coupling reagents for SPPS [11].

We chose as a test peptide ACP(65–74) (ACP, Acyl Carrier Protein: H-Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-OH), a sequence derived from a 77-residue single chain protein, containing a prosthetic group and isolated from *Escherichia coli*. This synthesis is a well-known compendium of sequence-dependent problems, due to the development of internal secondary structures that hampers the formation of the desired amide bond. The segment includes sterically hindered couplings and it is prone to interchain aggregation with resulting reduction in amino function accessibility. In the case of Fmoc/tBu strategy, this phenomenon can cause both slow Fmoc-deprotection and poor couplings [12].

We synthesised also rMOG(35–55) (H-Met-Glu-Val-Gly-Trp-Tyr-Arg-Ser-Pro-Phe-Ser-Arg-Val-Val-His-Leu-Tyr-Asn-Gly-Lys-OH), an immunodominant peptide inducing experimental allergic encephalomyelitis in the animal model of multiple sclerosis, in order to compare the coupling reagents in the case of peptide sequences longer than the ACP(65–74).

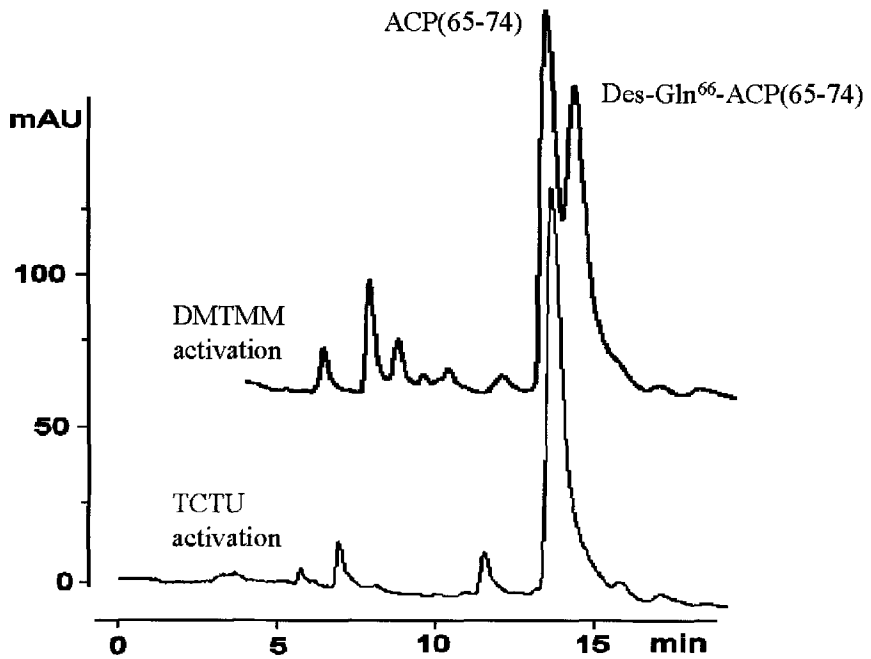


Figure 2. HPLC of the crude peptide ACP(65–74) precipitated with diethyl ether, collected by centrifugation, dissolved in H_2O and lyophilized.

Table 1. Synthesis of ACP(65–74)

Coupling reagent	Yield %
TBTU/HOBt	63
HCTU	73
HCTU/6Cl-HOBt	67
TCTU	87
TCTU/6Cl-HOBt	85
HATU	78
PyBOP	60
DMTMM	38

Table 2. Synthesis of rMOG(35–55)

Coupling reagent	Yield %
TBTU/HOBt	65
HCTU/6Cl-HOBt	67
TCTU/6Cl-HOBt	65
HATU	78

Materials and methods

Materials

Fmoc-Arg(Pbf)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-His(Trt)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Lys(Boc)-Wang resin and Fmoc-Gly-Wang resin were from Novabiochem. HCTU, TCTU and 6Cl-HOBt were from Luxembourg (Israel), HATU was from PerSeptive Biosystem, TBTU was from Chem Impex International, PyBOP from Novabiochem, HOBt from ABI, peptide-synthesis grade DMF was from Scharlau.

Peptide synthesis

The peptides were synthesized on an automatic batch synthesizer (APEX 396, Advanced ChemTech) equipped with either a 40 or a 8-wells reaction block, starting from Fmoc-Lys(Boc)-Wang resin and Fmoc-Gly-Wang resin. Fmoc deprotections were performed with 25% piperidine in DMF. Fmoc amino acids were stored as 0.5 M DMF solutions. Coupling reagents were pre-dissolved in DMF (0.5 M solutions), while the activators HOBt, HOAt and 6Cl-HOBt as 2 M solutions.

In all cases we have utilized four equivalents of amino acids and coupling reagents, eight equivalents of NMM and a single 45 min coupling time.

Peptide cleavage from the resin and deprotection of the amino-acids side chains were carried out for 3 h with TFA/anisole/EDT/phenol (94:2:2:2 v/v/v/v) for

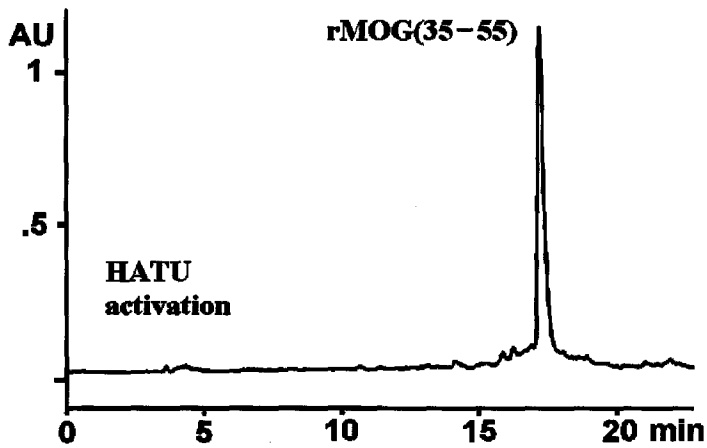


Figure 3. HPLC of the crude peptide rMOG(35–55) precipitated with diethyl ether, collected by centrifugation, dissolved in H_2O and lyophilized.

rMOG(35–55) and TFA/H_2O (9:1 v/v) for ACP(65–74). The resins were washed with TFA and the filtrates partially evaporated. The crude products were precipitated with diethyl ether, collected by centrifugation, dissolved in H_2O and lyophilized. The yields of the peptides were determined by analytical RP-HPLC using a Phenomenex Jupiter C-18 column (250 × 4.6 mm) on a Beckman System Gold apparatus equipped with a diode array detector. The solvent systems used were: A (0.1% TFA in H_2O) and B (0.1% TFA in CH_3CN). Gradients, at 1 ml min^{-1}, were: isocratic 20% B for ACP(65–74) and 10–60% B in 20 min for rMOG(35–55). Characterization of the products was performed by using LC-ESIMS (ESI Ion Trap LCQ Advantage from ThermoFinnigan).

Results and discussion

The aim of the present study was to compare the efficiency of different coupling reagents in order to optimize the yield of automatic peptide synthesis. The results shown in Table 1 indicate that the yield of ACP(65–74) increases in the case of the aminium salts, particularly using TCTU (87%). Therefore, the new Cl-containing coupling reagents appear to be effective in the synthesis of difficult peptide sequences by using an automatic multiple synthesizer. The phosphonium salt PyBOP showed a minor efficiency (60% yield).

The very poor result (38% yield) obtained by using DMTMM as a coupling reagent is probably due to its low solubility in the commonly used solvents for SPPS (DMF or NMP). In fact, DMTMM is soluble only in DMSO, MeOH, H_2O and CH_3CN [13]. On the basis of these results, this reagent, originally proposed for solution synthesis [10], does not appear to be a valuable alternative to common coupling reagents for SPPS, as recently claimed [11].

During the synthesis of ACP(65–74), we have identified, as the main by-product, the deletion peptide des-Gln^{66}-ACP(65–74). This by-product increases in the case of DMTMM, while it decreases with TCTU (Figure 2). Moreover, we observed, to a lesser extent, an incomplete incorporation of Val65, Ile69/72, Ala67/68 and Asn73.

In order to compare the performances of these coupling reagents in the synthesis of long sequences too, we synthesized a second test peptide: rMOG(35–55). In this case we obtained the best yield with HATU (78%) (Table 2). Unlike ACP(65–74), no main deletion by-products were observed in the synthesis of rMOG(35–55) (Figure 3).

In conclusion, all the tested coupling reagents, except DMTMM, can be used in the automatic SPPS. The aminium salts are more efficient than the phosphonium salts, the new 6Cl-HOBt-based reagents are efficient, while the addition of 6Cl-HOBt to HCTU and TCTU decreases the yield.

Acknowledgements

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