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An Alternative Solid Phase Peptide Fragment Condensation Protocol with Improved Efficiency

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Abstract: The success of solid phase peptide synthesis is often limited by the aggregation of the growing peptide chains on the resin. Working from the results of a study of model coupling reactions in solution between Z-Gly-Phe-OH and H-Phe-OBzl, we have achieved higher efficiency in the repetitive solid phase fragment condensation of VGVAPG, in a 3:1 chloroform-phenol solvent system, using diisopropylcarbodiimide (DIC) as coupling agent, and a combination of 3-hydroxy-3,4-dihydro-4-oxo-1,2,3-benzotriazine (HODhbt) and its tetrabutyl ammonium salt as additive, than in DMF with DIC and HODhbt alone.

Keywords: solid phase peptide synthesis; fragment condensation; solvent system; additives

INTRODUCTION

The major problem in the synthesis of large peptides in solution is the decreasing solubility of the growing sequence and on solid phase the strong association between the peptide chains on the resin. In both cases the difficulties are caused partly by van der Waals interactions and partly by intermolecular hydrogen bonds formed between the peptide chains [1]. In solution peptide synthesis, aggregation of the growing peptide chains also arises when the maximum protection strategy of Sakakibara [2] is used. When applying this strategy, it may happen that fully protected Boc-peptides are insoluble in polar organic solvents such as DMF, 1-methyl-2-pyrrolidinone (NMP) or even DMSO. After systematic investigations, powerful solvent systems with heteroselective solvation capacity (mixtures of TFE-chloroform, TFE-DCM and phenol-chloroform) have been proposed for the synthesis of large peptides in solution [3,4].

Several attempts have been made to disrupt internal aggregation during stepwise solid phase synthesis as well, by using other solvents than DMF or DCM. Using symmetrical anhydrides, the formation of error sequences decreases, when coupling has been started in pure DCM and 10% TFE or phenol has been added to the reaction mixture only in the last third of the whole reaction time [5]. In another solvent study, using pentafluorophenyl ester derivatives, DMSO seems to be outstandingly powerful in disrupting peptide chain association in stepwise SPPS, although its application is limited by its oxidizing capacity [6].

In model experiments, using the powerful chloroform-phenol or chloroform-TFE solvent system the effect of various coupling agents and additives was tested on the yield of peptide bond and ester bond formation and racemization. The best results in the case of fragment condensations in solution were achieved in the 3:1 chloroform-phenol mixture by the use of a water-soluble carbodiimide, 1-ethyl-3-

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(3-dimethylaminopropyl)carbodiimide (EDC) and 3-hydroxy-3,4-dihydro-4-oxo-1,2,3-benzotriazine (HODhbt) [4].

MATERIALS AND METHODS

General

The protected amino acids were purchased from Reanal (Budapest, Hungary) the Boc-Gly-PAM resin (0.2 mmol/g 200–400 mesh, polystyrene cross-linked with 1% divinylbenzene) was obtained from Bachem Feinchemikalien AG (Bubendorf, Switzerland), DIC, HOBt, HODhbt, TFA were from Chem-Impex International (Wood Dale, IL, USA), chloroform and phenol were from Fluka Chem. AG Hungary.

Analytical Methods

Analytical reversed phase-high performance liquid chromatography (RP-HPLC) was run on a Knauer instrument with a Phenomenex Jupiter C18 5 μm, 300 Å, (150 × 4.6 mm) column, using a linear gradient (10→60%, 35 min); (A) 0.1% TFA in water, (B) 0.08% TFA in acetonitrile, flow rate 1 mL/min. Electrospray ionization-mass spectrometry (ESI-MS) spectra were obtained on a PE SCIEX API-2000 spectrometer.

Model Experiments

A typical procedure: to the solution of Z-Gly-Phe-OH and H-Phe-OBzl (0.025 mmol/mL) in 3:1 chloroform-phenol mixture different ratios of HODhbt and TBA*ODhbt (see Table 1) were added. The solution was cooled and EDC or DIC (1.05 eq.) was added and it was stirred at room temperature for 1 h, then diluted with AcOH and injected onto the HPLC column.

Table 1 The Effect of HODhbt and TBA*ODhbt on the Amide and Ester Bond Formation in the Coupling of Z-Gly-Phe-OH with H-Phe-OBzl in 3:1 Chloroform-Phenol Solvent System

Coupling agent (1.05 eq.)	HODhbt: TBA*ODhbt (eq.)	Amide bond (%)[a]	Ester bond (%)
	1:0	98	2
EDC	0:1	94	6
	0.5:0.5	98	2
	1:0	>98	<2
DIC	0:1	98	2
	0.5:0.5	99	1
	2:0	99	1

[a] No racemization has been detected.

Solid Phase Syntheses

(A) Fragment condensation on resin in DMF: Boc-VGVAPG-PAM (which was prepared by coupling Boc-VGVAP-OH to H-Gly-PAM (0.2 mmol/g)) was treated with 35% TFA/DCM for 25 min, washed with DCM (3 ×), neutralized with 5% DIEA/DMF (3 ×), washed with DMF (3 ×). 1.5 fold excess of Boc-VGVAPG-OH was preactivated with equivalent amount of HODhbt and DIC for 7 min and the solution was added to the resin. The coupling was allowed to proceed for 5 h.

(B) Fragment condensation on resin in chloroform-phenol: starting with the neutralized resin, the coupling steps were carried out with 1.5 eq. BOC-peptide fragment and 2.5 eq. HODhbt, 0.5 eq. TBA*ODhbt which were dissolved in 3:1 chloroform-phenol, then added to the resin and 3 min later the DIC was added in solution, and then coupling was allowed to proceed for 5 h.

Peptides were cleaved from the resin by a usual liquid HF procedure.

RESULTS AND DISCUSSION

In the present paper we report our attempts focused on the application of the optimized reaction conditions described above in the course of solid phase fragment condensation. We performed further model experiments in solution taking the 3:1 chloroform-phenol solvent system as a basis, and tried to find the best conditions for the solid phase technique. Beside EDC we tested the well-proved DIC as coupling agent as well to avoid the presence of a tertiary base which may enhance phenyl ester formation. The coupling efficiency of DIC in combination with different additives has been studied previously in detail, both in stepwise peptide assembly and in segment coupling, in solution and on solid phase, in DMF and in DCM as well [7]. We found practically no difference in the yield of the tripeptide formed in the coupling reaction between Z-Gly-Phe-OH and H-Phe-OBzl at room temperature, in the presence of one equivalent HODhbt, either with EDC or with DIC in the chloroform-phenol system. In the case of EDC 2% of Z-Gly-Phe-OPh was formed. When DIC was used the ester

production slightly decreased. Since in SPPS reagent excess is applied in the coupling step, we performed an experiment with the application of two equivalents of the additive, too. Ester production did not increase, but in this case HODhbt was not dissolved properly, and due to the increased acidity of the reaction mixture the coupling time increased as well.

To enhance the solubility of HODhbt and decrease the acidity at the same time, we prepared its tetrabutyl ammonium salt, $Bu_4N^+ODhbt^-$ (TBA*ODhbt) and tested it as a peptide coupling additive at different HODhbt-salt ratios (see Table 1) with EDC and DIC as well. The increasing amount of the salt is advantageous for solubility, but at the same time it enhances ester formation, which reached a maximum at about 6%. On the other hand one has to consider that in SPPS a multiple excess of the carboxyl component is used, which would make fragment condensation very costly. Therefore, in our solid phase fragment condensation procedure for 1 eq. amino component we chose only 1.X eq. of the carboxyl component, $2 \times 1.X–0.X$ eq. HODhbt and 0.X eq. TBA*ODhbt. In this ratio the salt not only increases the solubility of the additive, but at the same time it neutralizes the excess of the carboxyl component, too.

For our solid phase fragment condensation experiments we chose one of the repeating hydrophobic sequences in elastin, VGVAPG, which is repeated six times in human elastin. The conformation of the VGVAPG oligomers measured by CD spectroscopy has already been reported [8] and their chemotactic activity will be reported elsewhere.

Convergent solid phase peptide synthetic strategy is especially suited to the synthesis of peptides with repetitive sequences because only one protected segment has to be prepared and purified for the assembly of the target molecule. Accordingly, we synthesized Boc-VGVAPG-OH stepwise, with Fmoc technique on 2-chlorotrityl resin with 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and purified it with HPLC.

We compared the efficiency of repetitive couplings of the monomer on PAM resin, using Boc strategy with the aid of DIC in DMF in the presence of 1 eq. of HODhbt additive and in chloroform-phenol system with different carboxyl fragment excess using the above mentioned ratio of HODhbt:TBA*ODhbt as additive.

The MS analysis of the crude hexamer peptide synthesized in DMF showed the presence of $6 + 1$ oligomer as an impurity. Therefore, we investigated the efficiency of each coupling step more thoroughly: after the incorporation of each monomer we cleaved a sample from the resin for HPLC analysis. Dimer and trimer were formed quantitatively, but in the latter case the crude product contained a significant amount of monomer, too. In the next coupling reaction, beside the monomer unreacted trimer (6%) was also detected and this may lead to the formation of $n + 1$ oligomers in the following steps. The results indicate that the remainder of the carboxyl component, which had not been removed perfectly by the standard washing procedures, might have caused the formation of the $n + 1$ monomers.

In contrast no $n + 1$ oligomers were formed in the 3:1 chloroform-phenol solvent system when 1.5 eq. of the carboxyl fragment, 2.5 eq. of HODhbt and 0.5 eq. of TBA*ODhbt additives were used. These results indicate that under these experimental conditions no improper washing step occurred.

CONCLUSION

In summary, although the nature and the solubility of 'difficult sequences' may be different due to their amino acid composition, here we have shown on the basis of model experiments that the combination of a powerful solvent system capable to remove excess reagents perfectly, and of an appropriate additive especially favourable for amide bond formation, provides an alternative possibility for improving coupling efficiency in solid phase peptide fragment condensation.

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