

Chloroform-Phenol Mixed Solvent Essential for Segment Condensation Reaction Performed in Solution or on a Solid Support

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Introduction

The segment condensation strategy using either the solution or solid-phase method is one of the most promising procedures for synthesizing large peptides or proteins. We previously demonstrated the utility of a combined solid-phase and solution approach for protein synthesis by synthesizing muscarinic toxin 1 (66 AAs) [1]. The procedure is based on performing the segment condensation in solution employing a maximum protection strategy with Boc chemistry, followed by the final deprotection using HF. Each segment used in the subsequent segment condensation is prepared by solid-phase assembly on a base labile *N*-[9-(hydroxymethyl)-2-fluorenyl] succinamic acid (HMFS) linker [2]. To establish this procedure for general protein synthesis, however, we needed to solve several problems encountered during preparation of the protected segments as well as the segment coupling in solution and HF treatment. Among them was the solubility problem of the intermediates, which hampers chemical synthesis of large peptides or proteins.

A β (1-40): DAEFRHDSGY EVHHQKLVFF AEDVGSNKGAIIGLMVGGVVV
A β (1-42): DAEFRHDSGY EVHHQKLVFF AEDVGSNKGAIIGLMVGGVVV IA

Fig. 1. Amino acid sequences of A β s.

Results and Discussion

The insolubility problems can become intractable as the size of the target peptide increases, when using the segment condensation method, either on a solid support or in solution. To overcome this obstacle, we demonstrated the usefulness of a mixture of CHCl₃ and 2,2,2-trifluoroethanol (TFE) as a β -sheet disrupting solvent for segment condensation of sparingly soluble protected peptides in solution [3]. However, in the course of assembling the protected green fluorescent protein (GFP, 238 AAs) and amyloid β -peptides (A β s) (1-40, 1-42, 3-42 or 1-43) (Fig. 1), none of the routinely used solvents, including of CHCl₃-TFE mixed solvent, could dissolve the protected peptide intermediates. Therefore, we had to find a solvent system possessing much higher solubilizing potential than CHCl₃-TFE. After testing various systems, we found that CHCl₃-phenol mixed solvent could dissolve almost all sparingly soluble protected peptides.

In order to examine the utility of the CHCl₃-phenol mixed solvent in the segment condensation reaction mediated by 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), a model coupling reaction of Boc-Phe-Ile-OH with H-Phe-OBzl·TosOH was carried out in the presence of 1-hydroxybenzotriazole (HOBt), 1-hydroxy-7-azabenzotriazole (HOAt) or 3,4-dihydro-3-

hydroxy-4-oxo-1,2,3-benzotriazine (HOOBt) (Table 1). HOOBt was the most effective additive for suppressing not only ester formation from the carboxyl component but also epimerization of the C-terminal amino acid residue during the segment condensation in CHCl₃-phenol. With the use of no, or less than equimolar, HOOBt (0-0.5 eq), coupling reactions were accompanied by considerable amounts of D-isomer and phenyl ester. On the other hand, the extent of epimerization and ester formation decreased as amounts of HOOBt increased. In particular, the amounts of both side products were negligible when employing HOOBt beyond one equimolar. Although the addition of a large excess HOOBt was essential for reducing the formation of side products, it resulted in a low yield of the desired peptide. Therefore, to improve the coupling yield, we tried adding excess EDC corresponding to the increment of HOOBt in the model experiment where the carboxyl component was used in 10% molar excess against the amino component to complete the coupling reaction promptly. From these results, we concluded that the optimal amounts of EDC/HOOBt should be 2 eq/3 eq against the amino component. The present strategy used with CHCl₃-phenol has been successfully applied to the synthesis of protected intermediate of GFP [4] and A β (1-42) [5]. As an example, the final coupling between protected A β (1-33) and (34-42), both of which were insoluble in CHCl₃-TFE, is presented in Fig. 2.

Table 1. Effects of additive (1 eq) and solvents on the yield, epimerization and ester formation in the coupling mediated by EDC (1 eq) between Boc-FI (1 eq) and F-OBzl/TosOH (1 eq).

Solvent	Additive	Yield (%)	Epimerization ^a (%)	Ester formation (%)
DMF	HOBt	95	24	-
	HOAt	96	7.6	-
	HOOBt	98	4.4	-
CHCl ₃ -phenol (v/v, 3/1)	HOBt	78	2.0	15
	HOAt	88	0.5	1.2
	HOOBt	83	0.2	0.8

^aDefined as 100[Boc-F(D-*allo*)IFG] / {Boc-F(IFG) + [Boc-F(D-*allo*)IFG]}.

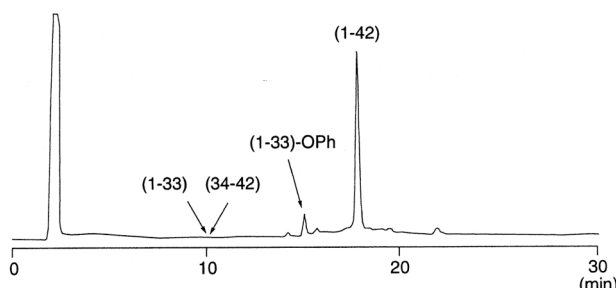


Fig. 2. HPLC profile of the crude HF-product of A β (1-42). Elution conditions: column, PLRP-S (4.6 x 150 mm); gradient, 20-45% MeCN in 0.1% TFA (25 min); flow, 1 ml/min; temp., 60°C; detection, 220nm.

The desired product was obtained quantitatively when excess amounts of the carboxyl component (1.1 eq) and EDC/HOObt (2 eq/3 eq) were used in CHCl₃-phenol (9:1, v/v).

To grasp the scope and limitations of using the CHCl₃-phenol mixed solvent as a coupling medium in peptide synthesis, we tried using it for convergent solid-phase peptide synthesis (CSPPS) involving the coupling of protected peptide segments on a solid support. The major problem with CSPPS is the solubility of the protected segments. The CHCl₃-phenol mixed solvent proved to be important in CSPPS for achieving high concentrations of the protected segments as well as the increased solvation of the peptide-resin in the coupling medium, both of which are crucial for efficient chain assembly on a solid support. Next, we tried to determine the reaction conditions suitable for CSPPS performed in the CHCl₃-phenol mixed solvent by examining a model coupling of Boc-Phe-Ile onto the Phe-Gly-HMFS resin. After detachment of the resulting tetrapeptide by treatment with 20% morpholine in DMF, the purity and yield of the products were assessed by RP-HPLC. When EDC or EDC·HCl was used for the coupling reaction in CSPPS, the extent of epimerization with Ile was found to be significantly higher than that observed in the solution phase regardless of the additive type, *i.e.* HOBt, HOAt or HOObt. This may be related to the basicity in EDC or EDC·HCl itself, which would be critical for the slow coupling processes in CSPPS (Table 2). Then, we tried to employ diisopropylcarbodiimide (DIC), which has no basicity in its molecule. As expected, the epimerization was suppressed by using DIC instead of EDC (Table 2). In all coupling reagents and solvent systems tested, the ability of additives to suppress epimerization was on the order of HOObt \geq HOAt \gg HOBt. Regarding the amount of additive, the use of more than one equimolar amount was needed to properly suppress the epimerization. On the other hand, the carbodiimide-mediated coupling in the presence of HOObt was accompanied by a considerable amount of azido by-product (0.4-0.8%), although this side reaction was negligible in the solution phase (< 0.1%). From these results, we concluded that the DIC/HOAt method should be used for CSPPS.

In order to demonstrate the utility of CSPPS performed in CHCl₃-phenol with aid of the DIC/HOAt method, this procedure was applied to the synthesis of A β (1-40) [6]. The molecule was divided into five parts, *i.e.* four segments (1-9), (10-19), (20-29) and (30-37), and a PAM resin-bound C-terminal segment (38-40). Each protected segment was synthesized on HMFS resin using Boc chemistry and was detached from the resin by treatment with 20% triethylamine in DMF to obtain it in the form of a fully protected segment with a free α -carboxyl group. This detachment procedure allowed the employment of the regular protecting groups, including the BrZ on Tyr and the 2,4-dimethylpent-3-yloxycarbonyl (Dipmoc or Doc) [7, 8] on His, since these groups remain intact during the detachment reaction. His(τ -Doc), in particular, which is expected to possess less basicity in the imidazole moiety than His(π -Bom), is indispensable for suppressing the risk of ester formation of segments containing it in the coupling reaction using CHCl₃-phenol. The sequential assembly of

four segments onto the resin-bound C-terminal segment proceeded smoothly when a 1.5-equimolar amount of the respective segments was used with the aid of the DIC/HOAt method. In each coupling step, no remaining amino and carboxyl components were observed in the crude products (Fig. 3). This facilitates purification of the final product without being hampered by seeding of the amyloid formation that is caused by contamination with hydrophobic components.

In conclusion, the present synthetic strategy used with the CHCl₃-phenol mixed solvent permits facile synthesis of large peptides, with or without difficult sequences, using both the solution and the solid-phase methods.

Table 2. Effects of additive (1 eq) and solvents on the yield, epimerization and ester formation in the coupling mediated by DIC(1 eq) between Boc-FI (1 eq) and FG-HMFS resin (1 eq).

Solvent	Additive	Yield (%)	Epimerization ^a (%)	Ester formation (%)
DMF	HOBt	95	7.4 (31) ^b	-
	HOAt	97	3.6 (17)	-
	HOObt	92	1.8 (8.8)	-

CHCl ₃ -phenol (v/v, 3/1)	HOBt	85	1.1 (2.0)	17
	HOAt	94	0.3 (1.4)	1.1
	HOObt	95	0.2 (1.0)	0.8

^aDefined as 100[Boc-F(D-*allo*)IFG] / {Boc-FIFG + [Boc-F(D-*allo*)IFG]}.

^b(): These values were obtained by using EDC/HCl instead of DIC.

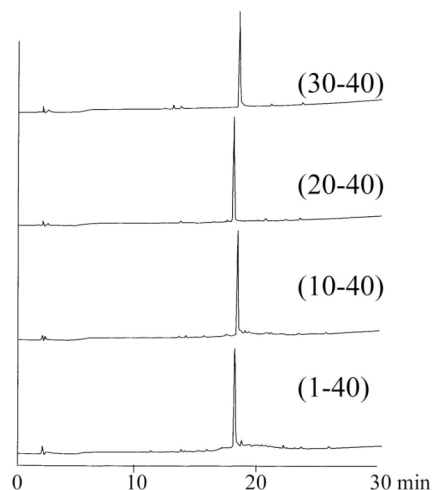


Fig. 3. HPLC profiles of the crude A β (1-40) obtained after sequential assembly of protected segments, followed by HF. Elution conditions: column, YMC Pack A-302 ODS (4.6 x 150 mm); gradient, 10-60% MeCN in 0.1% TFA (25 min); flow, 1 ml/min; temp., 40°C; detection, 220nm.

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