99.5 for simazine, atrazine, and propazine, respectively) and are of great analytical importance. The precursors for these ions could not be detected by the MS/MS method. Presumably, these ions are formed from the doubly-charged molecular ions through two α-cleavage reactions very rapidly inside the ion source. The extended conjugation for the above structure seems to counter-balance the strong Coulombic repulsion within the ion and stabilizes the structure.

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Application of BMPI/HOBT Reagent in Solid-Phase Peptide Synthesis

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The suitability of BMPI (2-bromo-N-methyl pyridinium iodide) for solid-phase peptide synthesis was investigated. The coupling rate of BMPI/HOBT procedure. BMPI/HOBT was superior to DCC/HOBT couplings using the solid-phase peptide bond formation proceeded to a greater degree of completion than DCC/HOBT method did. Double couplings with 2 equiv. of Boc-amino acids and 1.5 equiv. of BMPI and NEt_3 and 2 equiv. of HOBT in DMF/MC (1 : 1 v/v) gave the best result for the preparation of a model compound. Stepwise solid phase peptide synthesis using BMPI/HOBT procedure was successfully utilized for the preparation of (D-Ala)^2-dynorphine A. BMPI/HOBT procedure for the synthesis of (D-Ala)^2-dynorphine gave better yield (20%) than DCC/HOBT procedure did.

Introduction

The use of dicyclohexylcarbodiimide (DCC) proposed in 1955 by Sheehan and Hess[1] has since been widely adopted for peptide bond formation. However, this reagent could not be used without shortcomings[2] and side reactions such as racemization or intramolecular rearrangement of the O-acylisourea derivative have been reported during the activation step. Nevertheless, DCC has been widely used for peptide bond formation during Solid Phase Peptide Synthesis (SPPS).[3] Repetitive couplings are often required for the complete introduction of a residue in the peptide chain during SPPS, resulting in the consumption of protected amino acids and time consuming cycles of synthesis. Among the several coupling reagents suggested for the replacement of DCC in SPPS, benzotriazole-1-yl-oxy-tris (dimethyl amino) phosphonium hexafluorophosphate (BOP) was proposed by Fournier *et al.* in recent year.[4] BOP reagent has been used in SPPS for

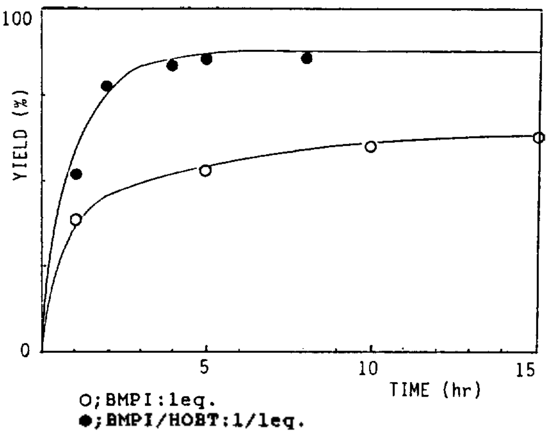


Figure 1. Comparative data of yield vs reaction time using BMPI (1 eq.) and BMPI/HOBT(1/1 eq.) in the synthesis of dipeptide by solution method. Model compound; Boc-Ile-Gly-OMe.

fragment coupling[5] and for stepwise synthesis[6] as well. We have investigated another coupling agent, 2-bromo-N-methyl pyridinium iodide (BMPI). This is nonhygroscopic salt, very stable and soluble in the usual organic solvents used in SPPS as well as easy to synthesize in laboratory. BMPI was first introduced in the synthesis of carboxylic ester,[7] β-lactam and lactone.[8] In this paper, we report details on the use of BMPI/HOBT reagent in solid phase peptide synthesis and on the advantages for the stepwise synthesis of peptides using BMPI/HOBT in the solid phase peptide synthesis. As the first step, we explored the coupling properties of BMPI/HOBT by evaluating its rate for dipeptide formation of Boc-Ile-Leu-Resin in solution and in solid phase method. The efficacy of BMPI/HOBT as a condensing reagent in SPPS was also demonstrated by the successful stepwise preparation of (D-Ala)^2-dynorphin A analogue.

Experiment

Reagents and Solvents. tert-Butoxycarbonyl azide was prepared from phenylcarbazate and sodium nitrate. Boc-protected amino acids were synthesized by the method of schnabel.[9] O-(2,6 Cl_2 Bzl) group was employed in side chain protection for tyrosine. The solid supprot has been used with copoly (styrene-2% divinylbezene) resin, 200-400 mesh, 1.23μ mol of Cl^-/g. Thin layer chromatography was performed on silica gel plate (0.250 mm, 60 F 254, E. Merk) in the following system (v/v): A, 1-butanol/AcOH/water (4:1:1); B, 2-butanol/EtOAc/AcOH (85:10:5); C, Ethanol/AcOH (1:1); D, MC: ether (1:1). Crude and purified peptide products were analyzed by hplc on μ-Bondapark-C_{18} column (4 × 300 mm) using 0.1M NaH_2PO_4 in 25% MeCN/water. Purified products were also characterized by amino acid analyses after hydrolysis in 6 N HCl for 24hr at 110 °C. Optical rotation was measured by Steeg and Reuter GMBH Frankfurt/M-56 polarimeter.

Synthesis of Dipeptides in Solution Phase. Boc, Ac and Phthalyl (Pht) groups were used for N-protection of all amino acids and –OMe, –OEt, and –OBzl groups were also used for C-protection of amino acids. Dipeptides were prepared from the N–, C– protected amino acids by varying the amount of BMPI vs. HOBT (1-hydroxybenzotriazole) and reaction time (Figure 1). Yields of dipeptides were calculated by weighing after drying of the resulted precipitates. The other products were synthesized in methylene chloride (MC) at room temperature for 2 hrs. The reaction mixture was evaporated to dryness to give the dipeptides as solids. The crude products were dissolved in MC/ether (1:1 v/v) and subjected to column chromatography on a column (60 × 1.5 cm) of silica gel 60. The fraction containing the pure product were evaporated and identified with nmr and ir spectra (Table 2).

Synthesis of Dipeptides in Solid Phase. Peptide synthesis was carried out manually by the methods described in Table 3 and Figure 2 using over-stirring vessel.[10] For anchoring of the first amino acid to the resin matrix, Boc-amino acid cesium salt[11] was employed. After proper washings of the resin followed by TFA deprotection and NEt_3 neutralization, coupling of Boc-amino acid using BMPI, BMPI/HOBT were performed in various solvent conditions (Table 3). Coupling yields were measured by weighing the total weight of the final peptide resin after washing with methylene chloride and MeOH and drying over phosphorous pentoxide.

Synthesis of (D-Ala)^2-dynorphine A (1-5). Boc-leucesiun salt, chloromethylated resin and DMF were placed in a reaction vessel. The suspension was stirred overnight while kept at 25 °C. After TFA deprotection of the Boc-group, coupling of next amino acid using BMPI/HOBT (1.5/2 equiv) were performed in DMF/MC (1:1 v/v). Every coupling steps were monitored using the qualitative ninhydrin test by Troll and Cannan.[12] The coupling, deprotection and neutralization steps were performed according to the general method described by Young.[13] All the following residues

Table 1. Comparative Data of Yield vs eq. Ratio of BMPI/HOBT

$$\text{Boc-Ile-OH} + \text{HCl·Gly-OMe} \xrightarrow[\text{2hr}]{\text{BMPI/HOBT}} \text{Boc-Ile-Gly-OMe}$$

Ratio(eq.)	Solvent	Yield(%)
1/1	MC*	77
1/1.2	MC	81
1/1.5	MC	86
1/2	MC	87
1/3	MC	85

*MC: Methylene chloride.

Table 2. Synthetic Dipeptides Data (BMPI/HOBT: 1/1 eq.)

Sample	m.p(°C)	Rf(D)	$[\alpha]^{b)}$	Yield(%) BMPI	Yield(%) DCC$^{a)}$
Boc-Leu-Ala-OMe	113-115	0.73	−31.6	85	81
Boc-Ile-Gly-OMe	79-81	0.63	−13.0	86	32
Boc-Val-Gly-OEt	93-94	0.71	−12.2	85	50
Boc-Val-Val-OMe	171-174	0.68	−15.6	69	
Boc-Ala-Ala-OBzl	67-69	0.61	−27.2	92	86
Pht-Gly-Gly-OMe	202-203	0.35		66	
Pht-Gly-Gly-OEt	132-133	0.38		74	
Pht-Gly-Gly-OBzl	143	0.51		71	
Pht-Val-Phe-OMe	89-90	0.59	+25.4	75	
Pht-Val-Phe-OEt	94-96	0.75	+22.1	93	

$^{a)}$Referance; Synthetic peptides, Vol. 1-4, George R. Pettit, Van Nostrand Reinhold Company, 1976. $^{b)}$c = 0.02 in dichloromethane.

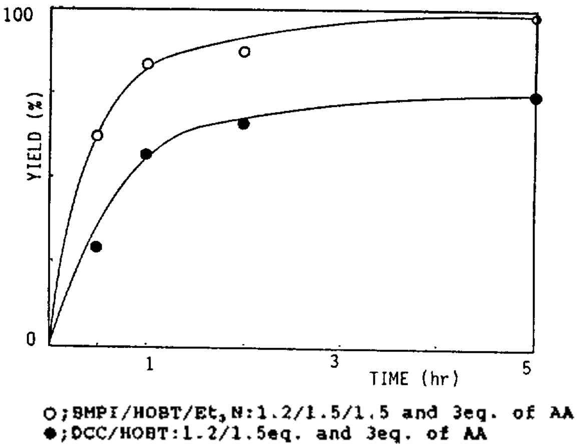
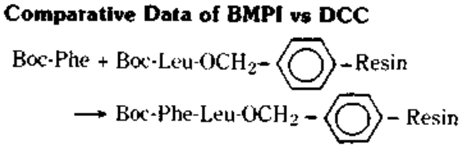


Figure 2. Comparative data of yield vs reaction time using BMPI/HOBT and DCC/HOBT as coupling agent. (Boc-Ile-Leu-OCH_2-C_6H_4-Resin).

Table 3. Comparative Data of BMPI vs DCC



Coupling	Solvent	Time (hr)	Yield(%) Single coupling	Yield(%) Doubble coupling
DCC(1.5 eq.)	MC	2	70.8	75.5
DCC/HOBt (1.5/2.0 eq.)	MC	2	44	50
DCC/HOBt (1.5/2.0 eq.)	DMF	2	64.6	66.6
BMPI/Et_3N (1.5/1.5 eq.)	DMF	2	68.9	86.8
BMPI/HOBt/Et_3N (1.5/2.0/1.5 eq.)	DMF	2	74.4	92.5
BMPI/HOBt/Et_3N (1.5/2.0/1.5 eq.)	MC-DMF (1:1, v/v)	2	84.2	94.2

were coupled to the growing peptide chain by the same protocol using double coupling procedure.

Cleavage of the Peptide from the Support and Identification. After the last amino acid was introduced, peptide-resin (0.5g) was treated with 10 m*l* of TFA/32% HBr-AcOH (1:1 v/v). After stirring for 1 hr at room temperature, the suspension was filtered and washed with 10 m*l* portions of TFA, TFA/MC (1:1 v/v) and MC. The mixture containing crude products was evaporated in vacuo, and the remaining oily material was precipitated with ether (Figure 3,a). A portion of the crude material was dissolved in 0.1N AcOH and subjected to gel filtration on a column (1.5 × 40 cm) of Sephadex LH-20 with 0.1 N AcOH. The fractions containing the pure product were pooled and lyophilized. TLC, Rf 0.91 (C). HPLC elution time: 3.17 min (Figure 3,b). Amino acid analysis: Gly 1.00, D-Ala 0.92, Leu 1.05, Tyr 0.97, Phe 0.96. m.p. 175-177 °C.

Results and Discussion

Synthesis of Dipeptide by Solution Method. Yields *vs.*

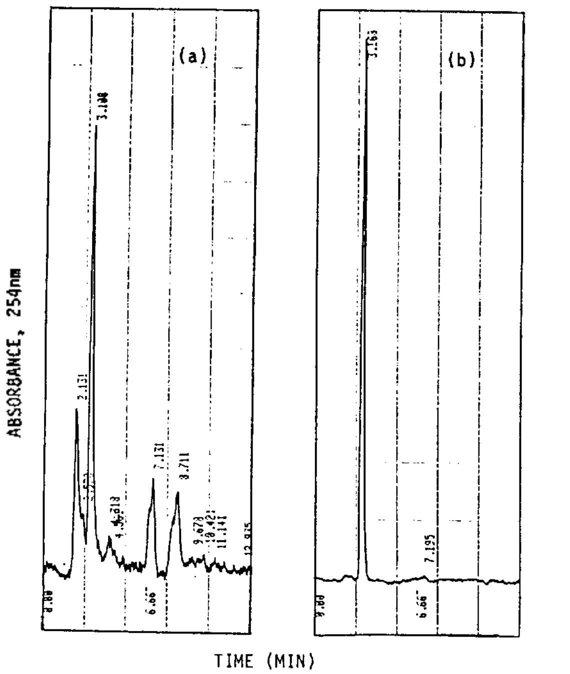


Figure 3. Analytical HPLC of (D-Ala)[2]-dynorphine A (1-5). Waters Bondapak C_{18} column (4 × 300 mm). Eluent; 0.1M NaH_2PO_4 in 25% CH_3CN/H_2O. Flow rate; 0.5 m*l*/min, detection; 254 nm. (a) Crude product (b) Purified product.

reaction time for the synthesis of model compound, Boc-Ile-Gly-OMe were measured with BMPI and BMPI/HOBT, in MC respectively. As shown Figure 1, BMPI/HOBT procedure gave the better results than that of BMPI method. Optimum equiv. ratio of BMPI *vs.* HOBT (1:1.5) was also obtained from this experiment. On the basis of these results, the following dipeptides (Table 2) were prepared with BMPI/HOBT (1:1.5 equiv.) at room temperature for 2hrs of reaction time. It was certained from Table 2 that BMPI/HOBT procedure gave more favorable yields compared to DCC/HOBT method.

Kinetics of BMPI/HOBT Coupling Reaction. In order to demonstrate the effectiveness of the BMPI/HOBT reagent for other type of difficult peptide bond formation, we evaluate coupling yield after incorporating of the hindered residue, Boc-Ile, which often gives poor coupling yields.[14] A kinetic study was carried out for the coupling of Boc-Ile-Leu-Resin (Figure 2). The use of 1.5 equiv. BMPI reagent made the rate of completion much faster than DCC reagent. After double coupling for 2 hrs, the percent yield of coupling with 1.5 equiv. BMPI was 20% higher than that of DCC reagent. This result suggested that the BMPI/HOBT reagent can be used for more efficient coupling even when the amount of acylating reagent is kept minimal. We compared the coupling yields obtained with DCC with that obtained with BMPI. Double coupling method was also compared with single coupling methods (Table 3). As shown in Table 3, the coupling of Boc-Phe proceeded to a greater extent of completion in single coupling using the BMPI/HOBT procedure (44% vs. 74% and 65% vs. 84%). In case of using MC/DMF (1:1 v/v) as solvent, the better result was obtained. Therefore, these results suggested that BMPI/HOBT procedure for SPPS proceeded more rapidly and to a greater degree of completion than DCC/HOBT method did (Figure 2). Furthermore,

Table 4. Synthetic Protocol for TFA Deprotection-BMPI/HOBT Coupling in Solid Phase Synthesis

Step	Reagent	Vol(ml)	Time(min)
1	MC wash (3 times)	15	5
2	30% TFA-MC	15	1.5
3	30% TFA-MC	15	30
4	MC wash (6 times)	15	5
5	5% NEt_3-MC	15	1.5
6	5% NEt_3-MC	15	1.5
7	MC wash (6 times)	15	5
8	2 eq. Boc-A.A. in MC/DMF (1:1 v/v)	10	2
9	1.5 eq. BMPI-2 eq. HOBT in DMF/MC (1:1 v/v)	10	120
10	Recouple if necessary by repeating steps 4-8		
11	DMF/MC wash (3 times)	15	3
12	MeOH wash (3 times)	15	3
13	MC wash (5 times)	15	3

MC: Methylene chloride, NEt_3: Triethylamine.

BMPI coupling reagent can be synthesized easily in laboratory and the price is inexpensive compared to BOP reagent.[4] Synthesis of (D-Ala)^2-dynorphine A (BMPI/HOBT vs. DCC/HOBT); Starting with Leu-O-CH_2-resin, parallel syntheses of (D-Ala)^2-dynorphine A were carried out under various coupling conditions. Double coupling method with various amounts of Boc-amino acids (1 equiv., 2 equiv., and 3 equiv.) and 1.5 equiv. of NET_3 in DMF/MC (1:1 v/v) were used for the synthesis of (D-Ala)^2-dynorphine A. DCC/HOBT coupling was also carried out with 3 equiv. of Boc-amino acid/cycle (double coupling). It was observed that BMPI/HOBT method gave the overall yields of 24%, 36% and 38%, respectively while DCC/HOBT method resulted in 12% overall yield of the purified products after gel filtration BMPI/HOBT method gave better yield (>20%) than DCC/HOBT method did. Since similar results were obtained from 2 equiv. of Boc-amino acid or 3 equiv. of Boc-amino acid, 2 equiv. of Boc-amino acids are sufficient for every coupling steps, which is different in DCC/HOBT method. The resulting 2-pyridone by-product was removed easily with common solvents used in SPPS. It was concluded that the BMPI/HOBT coupling procedure offers important advantages over DCC/HOBT procedure. Synthetic protocol using BMPI/HOBT reagent for SPPS was described in Table 4.

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