Novel immonium type peptide coupling reagent: 5-(1*H*-benzotriazol-1-yloxy)-3,4-dihydro-1-methyl 2*H*-pyrrolium hexachloroantimonate (BDMP®)

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A novel immonium type coupling reagent, 5-(1*H*-benzotriazol-1-yloxy)-3, 4-dihydro-1-methyl 2*H*-pyrrolium hexachloroantimonate (BDMP) has been designed, synthesized and utilized to synthesize oligopeptides and biologically active peptide both in solution and solid phase with satisfactory yield, low racemization and fast reaction rate. The estimation of racemization and the influence of several reaction parameters were studied by HPLC method using the model reaction: Z-Gly-Phe-OH + Val-OMe·HCl→Z-Gly-*D*/*L*-Phe-Val-OMe. It was shown that the reactivity of BDMP was much higher and the racemization was much lower than those of HOBt-based 'onium' reagents, even though its analogues BOMI. To further verified the effectiveness of BDMP, Leu-enkephalin was synthesized both in solution and solid phase using BDMP as coupling reagent. The proposed mechanism was also speculated.

Keywords BDMP, peptide coupling reagent, immonium salt, racemization, reactivity

Introduction

With the development of peptide chemistry, many new peptide coupling reagents have been designed and synthesized to meet the needs of peptide synthesis. In the past decades, one of the significant development in this field was the exploitation of HOBt-based 'onium'-type reagents, such as BOP,[1] HBTU,[2] PyBOP,[3] HBPyU,[4] HBPipU,[5] HBMDU[6] *et al.*, which were widely used in peptides synthesis both in solution and solid phase. Subsequently those reagents have been modified by replacing HOBt with HOAt to obtain HOAt-based 'onium' agents, such as HATU, HAPyU, AOP and PyAOP.[7] These reagents were more efficient than corresponding HOBt-based reagents due to the anchimeric assistance effect of HOAt.[8] Despite the above mentioned studies, another pathway, enhancing the efficiency and decreasing the racemizaion during coupling, is still assumable by replacing one of the substituted amino groups of central carbon atom of uronium reagents with hydrogen, alkyl or aryl. Thus the electron density of the central carbon of immonium salts was much lower than that of corresponding uronium salts and the immonium molecules were adequately activated due to obvious electronic effect (Scheme 1). Based upon this consideration we designed and synthesized a new series of immonium type coupling reagents. In previous studies,[9] we proved HOBt-based immonium salts BOMI to be very effective, Herein we will report its more efficient analogue BDMP in detail.

Results and discussion

Synthesis and evaluation of BDMP

BDMP can be easily synthesized from inexpensive and nontoxic starting material *N*-methyl pyrrolidone by condensation with bis(trichloromethyl) carbonate yielded the immonium chloride, which was stabilized subsequently with $SbCl_5$ to give the intermediate 5-chloro-3,4-dihydro-1-methyl 2*H*-pyrrolium hexachloroantimonate, followed by reacting with the potassium salt of 1-hydroxyl

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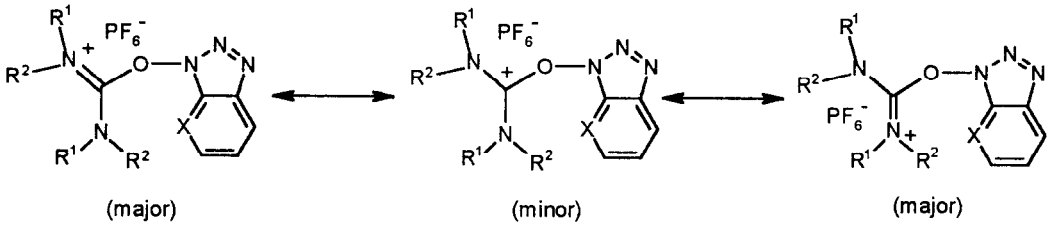
benzotriazole to give the desired compound as a yellowish crystalline and shelf-stable solid (Scheme 2).

Scheme 1 Resonance structures of uronium and immonium type coupling reagents

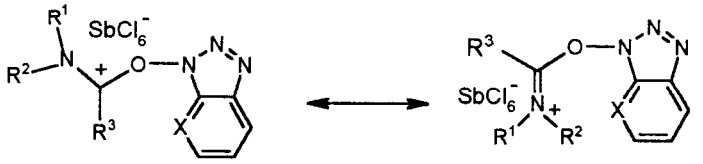


(major) (minor) (major)

Uronium salts: HBTU: $R^1 = R^2 = CH_3$, X = CH; HATU: $R^1 = R^2 = CH_3$, X = N

HBPyU: R^1, $R^2 = (CH_2)_4$, X = CH; HAPyU: R^1, $R^2 = (CH_2)_4$, X = N

HBPipU: R^1, $R^2 = (CH_2)_5$, X = CH

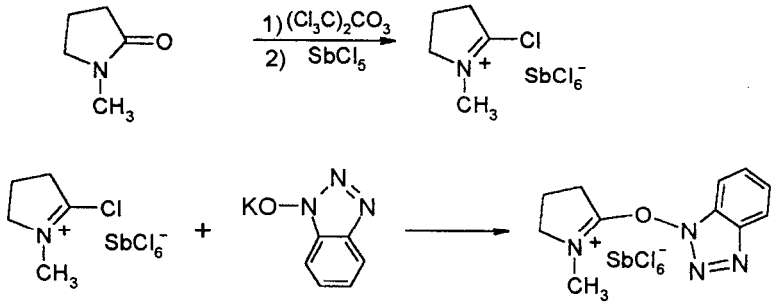


(minor) (major)

Immonium salts: BOMI: $R^1 = R^2 = CH_3$, R^3 = H, X=CH

BDMP: R^1, $R^3 = (CH_2)_3$, $R^2 = CH_3$, X = CH

Scheme 2 Synthesis of immonium type peptide coupling reagent BDMP



In previous study,[9] we have optimized the reaction conditions and investigated the influence of solvent, base and temperature during peptide synthesis by HPLC using the model reaction: Z-Gly-Phe-OH + Val-OMe·HCl→Z-Gly-Phe-Val-OMe.[10] It was found that less polar THF and the hindered, less basic tertiary amine, 2,6-lutidine were suitable for these HOBt-based immonium type coupling reagents based on the yield and reaction rate comparing to other solvents and bases which were commonly used in peptide synthesis. Owing to the high reactivity of these reagents, the reaction can be conducted in reduced temperature resulting in higher yield.

In attempt to evaluate the extreme reactivity of BDMP the model reaction: Z-Gly-Phe-OH + Val-OMe·HCl → Z-Gly-Phe-Val-OMe was adopted using HPLC method by comparing to the HOBt-based uronium reagent HBPyU, which was shown to be the most efficient among HOBt-derived 'onium' salts, such as HBTU, HBPipU, BOP and PyBOP.[7b,11] As the results shown in Fig. 1 and Table 1, it is obviously that BDMP is superior to

HBPyU and BOMI by comparison of coupling yield and racemization during different time course of reaction using same reaction condition (THF, 2,6-lutidine, -10℃). For example after 35 min reaction, the yield is up to 94% for BDMP, meantime 80% for BOMI only 8% for HBPyU.

Table 1 Reaction speed and racemization of BDMP, BOMI and HBPyU

Coupling reagent	Reaction conditions			Reaction time (min)	Yield (%)	*D*-isomer (%)
	T (℃)	Base	Solvent			
BDMP	-10	2,6-lutidine	THF	1	46.0	1.11
				10	89.6	
				25	93.1	
				35	93.1	
				120	95.3	
BOMI	-10	2,6-lutidine	THF	1	4.98	1.55
				10	20.1	
				25	61.5	
				35	79.5	
				120	93.4	
HBPyU	-10	2,6-lutidine	THF	1	1.57	14.6
				10	3.29	
				25	5.43	
				35	7.69	
				120	16.1	

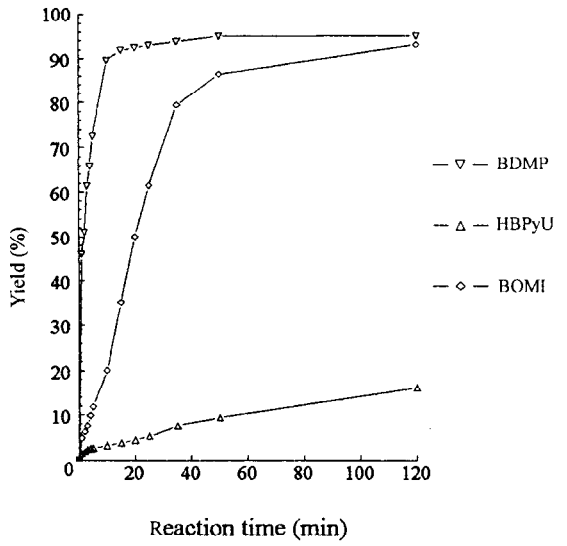


Fig. 1 Comparison of reactivity of BDMP with BOMI and HBPyU. (Reaction conditions: *T*: -10℃; Base: 2, 6-lutidine; Solvent: THF; Substrate ratio: *N*-protected amino acid: amino acid ester hydrochloride: coupling reagent: base = 1:1.1:1.1:3. The reactions were monitored by HPLC, the reaction mixture was sampled at 1′, 2′, 3′, 4′, 5′, 10′, 15′, 20′, 25′, 35′, 50′, 120′ and 180′, respectively. Part of these data were listed in Table 1.)

It is likely that the higher reactivity of BDMP than HBPyU may attribute to the two pyrrolindino groups of the central carbon atom in the HBPyU molecule providing two equal resonances to stabilize uronium salt, but greatly decrease the reactivity of the reagent; Whereas BDMP provides with the structural feature by replacing one of the substituted amino group of the uronium reagent HBMDU with alkyl to dislocate the two equal resonance structures. In addition the higher reactivity of BDMP than its analogues BOMI was probably due to the tension of intra-annular imide bond. By calculation using PCMODEL software[12] it was found that the angle of N—C^{sp2}—C^{sp3} in the molecule of BDMP having a value of 113.7°, indicating the five number ring of 2*H*-pyrrolium was to some extent constraint. The higher reactivity of BDMP were also attribute to the relatively low electron density in central carbon of BDMP.

In order to evaluate the extent of the racemization during coupling using BDMP an HPLC method (coupling of Z-Gly-Phe-OH and Val-OCH_3)[10] and Young's test[13] (coupling of Bz-Leu-OH and Gly-OEt·HCl) were adopted. It was observed that the racemization with BDMP was the lowest comparing to other coupling reagents

(Table 2). The reduced racemization with BDMP may be due to the mild reaction conditions, the weak basicity as well as the hindrance of the tertiary amine 2,6-lutidine.

Synthesis of oligopeptides, Leu-enkephalin using BDMP

In order to assess the effectiveness of BDMP the syntheses of a number of oligopeptides were accomplished using the above optimized reaction conditions with satisfactory yield (83—93%) and negligible racemization (Table 3). The reaction can be carried out by one pot procedure without the pre-activation of the carboxylic component.

An biologically active peptide Leu-enkephalin[15] was also synthesized to confirm further the efficiency of BDMP on peptide coupling in solution (Fig. 2). The protected Leu-enkephalin was obtained via seven coupling steps in 51.4% overall yield. The product of each step was confirmed by elemental analysis and other criteria. Using HPLC and ESI-MS the final product was shown to be identical with an anthentic sample from Sigma.

BDMP can also be used in the ester formation with excellent yield, especially the preparation of active esters such as benzotriazolyl ester, pentafluorophenyl ester and succminyl ester which were often used during the synthesis of lactone and lactam. The utilization of BDMP to synthesize cyclopeptide, such as biologically active peptide cyclosporins which bear potent immunosuppressive activity, is underway.

Table 2 Comparison of racemization of BDMP with different coupling reagents[a]

Reagent[14]	HPLC method *DL*(%)[b]	Young's test *DL*(%)[b]
DCCI	19.7	72.1
BOP	9.6	39.6
HBTU	9.8	24.3
HBPyU	7.9	18.0
HBPipU	8.9	20.5
BOMI	6.4(3.1[c])	8.8[c]
BDMP	4.6(2.2[c])	5.3[c]

[a] All reactions were carried out in the same conditions which were commonly used for the HOBt-derived uronium reagents such as BOP, HBTU and HBPyU.[4a]

[b] *DL*(%) equal to *D*-isomer(%) multiplied by two.

[c] Reaction conditions were the same as those used in Fig. 1 which were suitable for the HOBt-derived immonium coupling reagent—BOMI, BDMP.

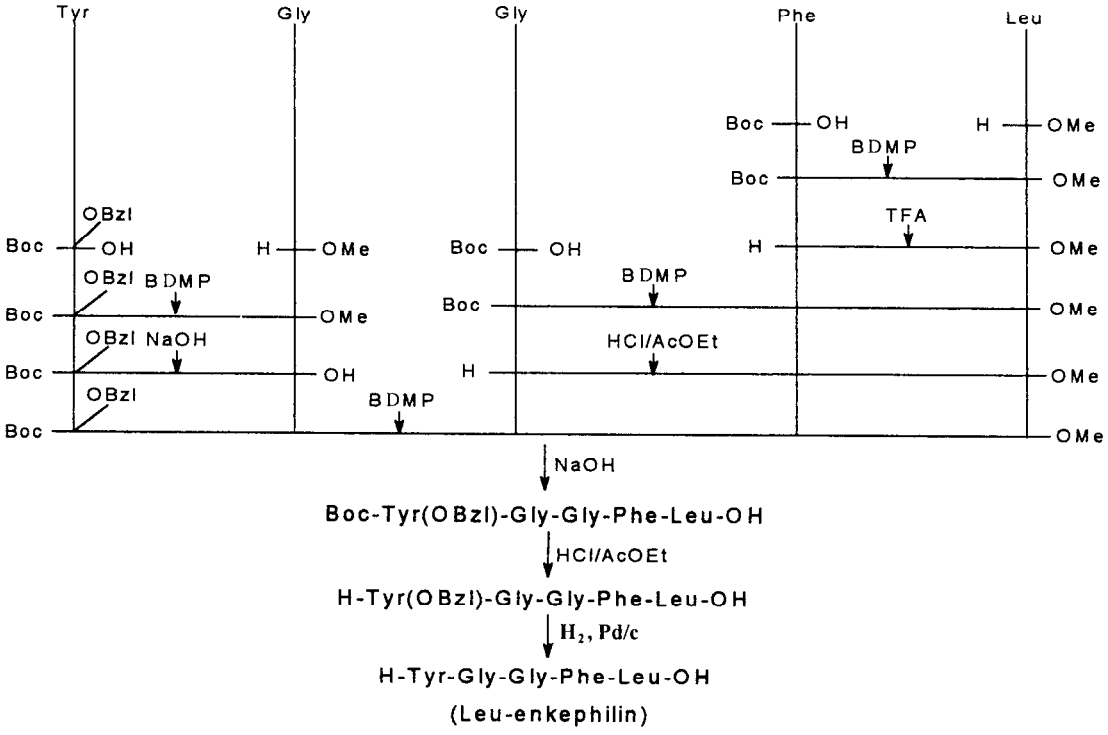


Fig. 2 Synthesis of Leu-enkephalin in solution using BDMP as coupling reagent.

Solid phase peptide synthesis using BDMP *reagent*

The solid phase synthesis of Leu-enkephalin on Merrifield's resin using BDMP was performed according to the general SPPS principle. It was observed that the trifluoroacetate of amino component on resin can be used directly during coupling without pre-neutralization by adding one more equivalent base, thus the synthetic cycle could be simplified to some extent (the standard synthetic cycle was shown in Table 4). The final product was purified and characterized by HPLC and ESI-MS and shown to be identical with the product obtained from the solution method and an authentic sample.

Table 3 Synthesis of oligopeptides using BDMP as coupling reagent

Entry	Peptide[a]	Yield[b] (%)	Mp (℃)	$[\alpha]_D$[d] (conc., solv., temp.)
1	Boc-Phe-Leu-OMe	91.1	102—103	-26.4°(1, MeOH, 22℃)
	↓			
2	Boc-Gly-Phe-Leu-OMe	92.4	43—44[c]	-22.9°(1, MeOH, 22℃)
3	BOC-Tyr(Bzl)-Gly-OEt	83.3	127—128	1.1°(1, MeOH, 22℃)
	↓			
4	(Boc-Tyr(Bzl)-Gly-Gly-Phe-Leu-OMe	84.1	147—148	-9.1°(1, MeOH, 19℃)
5	Bz-Leu-Gly-OEt	86.9	154—155	-32.2°(3.1, EtOH, 19℃)
6	Fmoc-MeLeu-Ala-OBzl	91.3	—	-43.0°(0.88, MeOH, 20℃)
	↓			
7	Fmoc-Meleu-Val-Meleu-Ala-OBzl	92.9	44—46	-91.8°(0.5, MeOH, 20℃)

[a] The CO-NH bond formed in the peptides is indicated by ↓.

[b] Isolated yields based on *N*-protected amino acid except entry 7 which based on the amino component.

[c] The peptide, which used to be an oil product, was firstly obtained as crystalline solid.

[d] Melting points and $[\alpha]_D$ values are in accord with the reported values.

Table 4 Standard protocol for a synthetic cycle using BDMP

Step	Reagent	Time
1	CH_2Cl_2	1 × 1 min
2	50% TFA/CH_2Cl_2	1 × 1 min
3	CH_2Cl_2	1 × 1 min
4	50% TFA/CH_2Cl_2	1 × 20 min
5	CH_2Cl_2	4 × 1 min
6	3 equiv. Boc-AA--OH/CH_2Cl_2 + 3 equiv. BDMP reagent + 7 equiv. 2,6-lutidine	2 h
7	DMF	2 × 1 min
8	EtOH	2 × 1 min
9	CH_2Cl_2	3 × 1 min

Speculated reaction mechanism for BDMP *coupling reagent*

Based on the previous studies, a mechanism for BDMP during coupling was proposed. We assumed that the first step during carboxylic activation by BDMP involves the formation of an unstable (acyloxy) immonium salt **I** intermediate as shown in Fig. 3. Then, the intermediate was attacked by the highly nucleophilic oxybenzotriazolyl anion to form a benzotriazolyl ester of *N*-protected amino acid accompanied by releasing of the corresponding substrate amide *N*-methyl pyrrolindone. Subsequently the active benzotriazolyl ester reacted with *C*-protected amino acid to afford the final amide product. The proposed mechanism was supported by the isolation of the intermediate HOBt ester from reaction mixture in the absence of amino component or much hindered such as *N*-alkyl or α-*C*-dialkyl amino acid ester and dicyclohexylamine being used. It is also noteworthy that the intermediate **I** seems not to react directly with amino component to afford final product because the reaction rate was very slow accompanied by considerable racemization when 5-chloro-3,4-dihydro-1-methyl 2*H*-pyrrolium hexachloroantimonate was used as coupling reagent for peptide synthesis.

Comparing to the HOBt-based uronium type coupling reagents, the high reactivity of BDMP may attribute to the instability of the immonium salt and the reactivity of (acyloxy) immonium salt **I** was much higher than those

of (acyloxy) uronium salts due to obvious electronic effect.

In conclusion, HOBt-based immonium salt BDMP, which can be easily prepared from easily available starting materials, was proved to be a very efficient novel peptide coupling reagent with respect to the high reactivity, low racemization and excellent yield comparing to other conventional HOBt-derived uronium and phosphonium reagents. A number of oligopeptides and Leu-enkephalin were synthesized both in solution and solid phase in assessing the effectiveness of BDMP. The proposed mechanism was speculated and discussed.

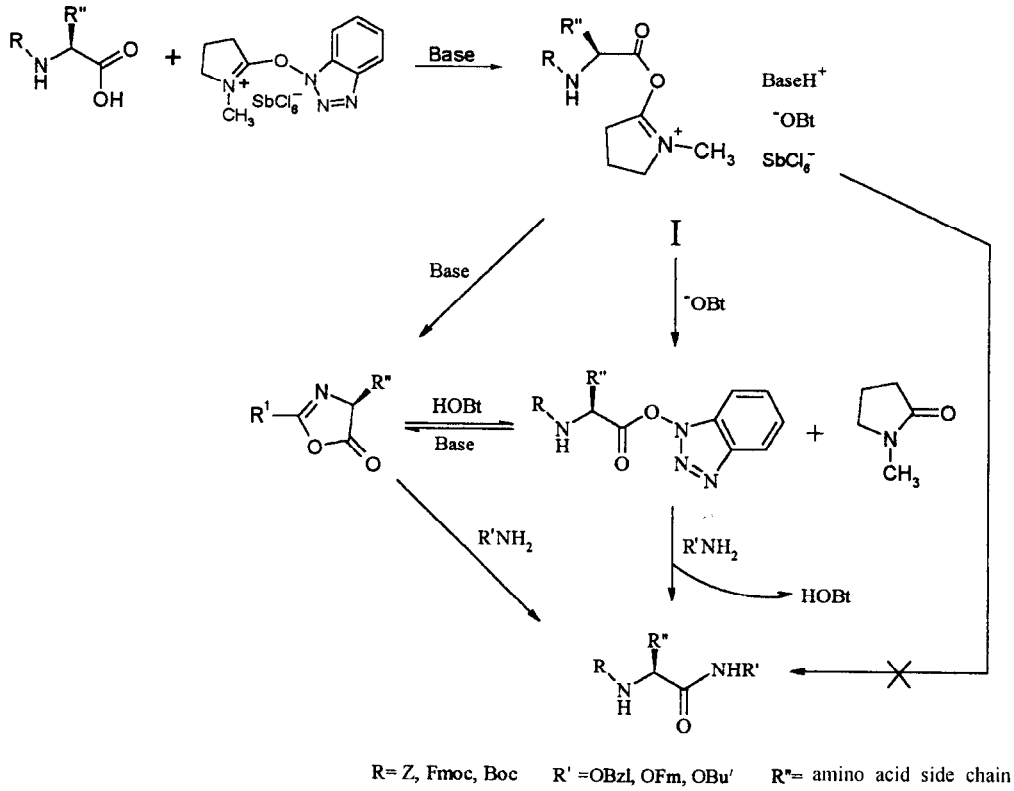


Fig. 3 Proposed reaction mechanism for coupling reagent BDMP.

Experimental

Amino acid derivatives were obtained from commercial sources or synthesized according to the literature. Melting points were determined in capillary tubes and are uncorrected. IR spectra were measured with IR-440 spectrometer. ^{1}H NMR spectra were recorded on Bruker AM 300 MHz and are reported as parts-per-million downfield from a tetramethylsilane internal standard. The following abbreviations are used: singlet(s), doublet(d), triplet(t), quartet(q), multiplet(m), broad(br). Mass spectra were taken with HP5890A, and VG QUATTRO mass spectrometers. Elemental analyses for carbon, hydrogen and nitrogen were determined on an MOD-1106 elemental analyzer. Optical rotations were determined using a Perkin-Elmer 241 MC polarimeter. HPLCs were carried out on Varian-SY-5000 with Kromasil RP-18 (0.5 × 25 cm) column. Flash column chromatography was performed with 300—400 meshes silica gel, and analytical thin layer chromatography was performed on precoated silica gel plates (GF-254) with the systems (v/v) indicated. Solvents and reagents were purified by standard methods as necessary. Amino acids were L-configuration if not otherwise stated.

Synthesis of coupling reagent BDMP

5-Chloro-3, 4-dihydro-1-methyl 2H-pyrrolium hexachloroantimonate (CDMP) A solution of *N*-

methyl pyrrolidone (0.96 mL, 10 mmol) in CH_2Cl_2 was added dropwise to a solution of bis(trichloromethyl)carbonate (0.989 g, 3.33 mmol) in CH_2Cl_2 (25 mL) at 0℃ under nitrogen atmosphere. After approximately 1 h, when the evolution of carbon dioxide has ceased, a 0.887 mol/L solution $SbCl_5$ in $CHCl_3$ (10.7 mL) was added dropwise at −30℃ under vigorous stirring. The reaction mixture was stirred at 0℃ for 2 h, then the resultant suspension was filtered under nitrogen atmosphere, washed with cold CH_2Cl_2 and dried *in vacuo*. Recrystallization from CH_3CN-CH_2Cl_2 gave 4.31 g product as colorless crystals. Yield 90.7%. mp 191—193℃(dec). ν_{max}(KBr): 1661 (C = N) cm^{-1}. δ_H ([d_6]acetone, 25℃, TMS): 3.60(t, J = 7 Hz, 2H, α-CH_2), 2.91(s, 3H, CH_3), 2.59(t, J = 8 Hz, 2H, γ-CH_2), 2.04—2.16(m, 2H, β-CH_2). FABMS: 118 (M − $SbCl_6^-$). Anal. $C_5H_9C_{17}NSb$. Calcd: C, 13.24; H, 1.99; N, 3.09; Cl, 54.83. Found: C, 13.47; H, 2.06; N, 3.03; Cl, 54.99.

5-(1*H*-*benzotriazol*-1-*yloxy*)-3, 4-*dihydro*-1-*methyl* 2*H*-*pyrrolium hexachloroantimonate* (BDMP) KOBt (0.173 g, 1 mmol) was added to a solution of CDMP (0.453 g, 1 mmol) in dry CH_3CN (6 mL) at −30℃ with stirring under nitrogen atmosphere. After the reaction mixture was stirred at room temperature for 2 h, it was filtered and the filtrate was concentrated under reduced pressure, the residue was recrystallized from CH_3CN-Et_2O to give 0.487 g yellowish crystalline compound. Yield 88.2%. mp 165—166℃(dec.). ν_{max} (KBr): 1655, 1496, 1479, 1445, 1165, 1066, 763, 640 cm^{-1}. δ_H([D_6]acetone, 25℃, TMS): 7.34—7.95(m, 4H, aryl), 3.48(t, J = 7 Hz, 2H, α-CH_2), 2.83(s, 3H, CH_3), 2.39(t, J = 8 Hz, 2H, γ-CH_2), 1.98—2.09(m, 2H, β-CH_2). FABMS: 217(M − $SbCl_6^-$). Anal. $C_{11}H_{13}Cl_6N_4OSb$. Calad: C, 23.94; H, 2.36; N, 10.15. Found: C, 23.83; H, 2.13; N, 10.24.

Test of reaction speed and racemization by HPLC *using the model reaction*: Z-Gly-Phe-OH + Val-OMe·HCl→Z-Gly-*D*/*L*-Phe-Val-OMe

Z-Gly-Phe-OH (50 mg, 0.14 mmol) was coupled with Val-OMe·HCl (26 mg, 0.154 mmol) using BDMP (85 mg, 0.154 mmol) or the same equivalent other coupling reagents. Boc-Phe-Val-OMe (66 mg, 0.175 mmol) was added as the internal reference. Test reactions were performed on total 1.5 mL scale. Aliquots (10 μL) from the reaction mixture were quenched and dissolved in 100 μL buffer solution (CH_3CN/H_2O/TFA: 50/50/0.1). The resultant samples were analyzed by HPLC to give the following result: Z-Gly-Phe-OH (t_R = 4.04 min); Z-Gly-*L*-Phe-Val-OMe (t_R = 9.24 min); Z-Gly-*D*-Phe-Val-OMe (t_R = 10.28 min); Boc-Phe-Val-OMe (t_R = 15.82 min). Peak areas were compared in order to obtain the chemical yields (yield (%) = $[(LL/X_1 + DL/X_2)/S] \times 100\%$). Percentage of epimers was calculated according to the equation: $D(\%) = [DL/X_2/(LL/X_1 + DL/X_2)] \times 100\%$; where *LL* refers to the area of Z-Gly-*L*-Phe-Val-OMe, *DL* refers to that of Z-Gly-*D*-Phe-Val-OMe, S refers to that of Boc-Phe-Val-OMe, X_1 = 1.269 and X_2 = 1.254 which are the determined correction factors for absorption difference (220 nm) between the references.

General procedure for peptides synthesis using BDMP *as coupling reagent*

2,6-Lutidine (3.2 equiv.) was added to a cold mixture (−10℃) of *N*-protected amino acid (1 equiv.), amino acid ester hydrochloride (1.1 equiv.), and BDMP (1.1 equiv.) in THF (2—4 mL/mmol), stirred for 1 min cold and for 1 h at room temperature. For large scale and coupling between hindered amino acids, the reaction time should be moderately prolonged. After the completion of reaction, the reaction mixture was diluted with THF, the resultant suspension was filtered and the filtered cake was washed with THF. The filtrate was concentrated under reduced pressure to give the crude product which was purified by flash chromatography on silica gel column to afford the desired product.

Fmoc-Meleu-Ala-OBzl Yield 91.3%. $[\alpha]_D^{20}$ −43.0°(*c* 0.88, MeOH), R_f = 0.59(AcOEt/Pe = 1/2). δ_H(d_6-acetone, 25℃, TMS) 2 conformers: 0.73, 0.85(2d, J = 7 Hz, 6H, $2CH_3^{Leu}$), 1.29—1.42(m, 1H, γ-CH^{Leu}), 1.39(d, J = 7 Hz, 3H, CH_3^{Ala}), 1.63(m, 2H, CH_2^{Leu}), 2.82(s, 3H, N-CH_3), 4.06 (q, J = 7 Hz, 1H, CH^{Ala}), 4.30(t, J = 7 Hz, 1H, 9-CH^{Fmoc}), 4.49(m, 2H, CH_2^{Fmoc}), 4.79(t, J = 7 Hz, 1H, α-CH^{Leu}), 5.12, 5.18(2d, J = 13 Hz, 2H,

CH_2-Ph), 7.32—7.45(m, 9H, 2,3,6,7-CH^{Fmoc} & CH_2**Ph**), 7.68(d, J = 7 Hz, 2H, 1,8-CH^{Fmoc}), 7.75 (d, J = 6 Hz, 1H, NH), 7.89(d, J = 7 Hz, 2H, 4, 5-CH^{Fmoc}). EIMS m/z(%): 529($M+H^+$, 4.79), 421(M^+-CH_2Ph, 0.57), 179[(Fmoc-CO_2)$^+$, 100], 91($PhCH_2$, 28.01).

Fmoc-Meleu-Val-Meleu-Ala-OBzl Yield 92.9%. R_f = 0.61(AcOEt/Pe = 1/1), $[\alpha]_D^{20}$ - 91.8°(c 0.5, MeOH). δ_H($CDCl_3$, 25℃, TMS) 2 conformers: 0.82—0.95(m, 18H, 4 CH_3^{Leu} & 2CH_3^{Val}), 1.26, 1.36(2d, J = 7 Hz, 3H, CH_3Ala), 1.33—2.06(3m, 7H, 2 CH_2^{Leu} & 2γ-CH^{Leu} & β-CH^{Val}), 2.78, 2.82, 2.84, 3.01(4s, 6H, 2 N-CH_3), 4.10—4.92(m, 3H, 3 α-$CH^{Val, Leu, Ala}$), 5.06—5.28(m, 3H, α-CH^{Leu} & CH_2Ph), 6.48, 6.63(2d, J = 9 Hz, 2H, 2NH), 7.25—7.43(m, 9H, 2,3,6,7-CH^{Fmoc} & CH_2**Ph**), 7.59(d, J = 7 Hz, 2H, 1,8-CH^{Fmoc}), 7.76 (d, J = 7 Hz, 2H, 4,5-CH^{Fmoc}). EIMS m/z(%): 754(M^+, 0.11), 576(M^+-la-OBzl, 41.74), 179 [(Fmoc-CO_2)$^+$, 100], 91($PhCH_2$, 28.01).

Z-Aib-OBt 2,6-Lutidine (1.0 mmol, 116 μL) was added to a cold mixture (-10℃) of Z-Aib-OH (0.5 mmol, 119 mg) and BDMP (0.55 mmol, 304 mg) in THF (2 mL), stirred for 1 min cold and 1 h at room temperature. The reaction mixture was treated according to the general procedure and purified by flash chromatography on silica gel column eluted with AcOEt/Petroleum ether(1/2) to give Z-Aib-OBt. Yield: 157 mg (88.7%). R_f = 0.53(AcOEt/Pe: 1/2). δ_H($CDCl_3$, 25℃, TMS): 8.02(d, J = 8 Hz, 1H, aryl), 7.20—7.75(m, 8H, aryl), 5.59(s, 1H, NH), 5.21 (s, 2H, $PhCH_2$), 1.78(s, 6H, 2CH_3). ν_{max}(Nujol): 3322, 1817, 1713, 1521, 1270, 1091, 1047, 744 cm^{-1}.

Synthesis of Leu-enkephalin in solution

Boc-Tyr(OBzl)-Gly-OEt Yield 83.3%. mp 127—128℃, $[\alpha]_D^{22}$ + 1.1°(c 1, MeOH). Anal. $C_{25}H_{32}N_2O_6$. Calcd: C, 65.77; H, 7.06; N, 6.14. Found: C, 65.91; H, 7.11; N, 6.22.

Boc-Tyr(OBzl)-Gly-OH Yield 95.0%, mp 149—150℃, $[\alpha]_D^{21}$ + 1.6°(c 1, MeOH). Anal. $C_{23}H_{28}N_2O_6$. Calcd: C, 64.47; H, 6.59; N, 6.54. Found: C, 64.42; H, 6.52; N, 6.47.

Boc-Phe-Leu-OCH_3 Yield 91.1%. mp 102—103℃, $[\alpha]_D^{22}$ - 26.4°(c 1, MeOH). Anal. $C_{21}H_{32}N_2O_5$. Calcd: C, 64.26; H, 8.22; N, 7.14. Found: C, 64.05; H, 8.48; N, 7.19.

TFA·Phe-Leu-OCH_3 Yield 95.5%. mp 163—165℃, R_f = 0.85($CHCl_3$/MeOH: 10/1). $[\alpha]_D^{22}$ - 11.4°(c 1, MeOH).

Boc-Gly-Phe-Leu-OCH_3 Yield 92.4%, mp 43—44℃, $[\alpha]_D^{22}$ - 22.9°(c 1, MeOH). Anal. $C_{23}H_{35}N_3O_6 \cdot H_2O$. Calcd: C, 59.15; H, 7.92; N, 8.99. Found: C, 59.06; H, 7.64; N, 9.02.

HCl·Gly-Phe-Leu-OCH_3 Yield 96.0%, mp 89—91℃, R_f = 0.78 (n-BuOH/HOAc/H_2O: 4/1/2), $[\alpha]_D^{21}$ - 5.5°(c 1, MeOH).

Boc-Tyr(OBzl)-Gly-Gly-Phe-Leu-OCH_3 Yield 84.1%, mp 147—148℃, $[\alpha]_D^{19}$ - 9.1°(c 1, MeOH). Anal. $C_{41}H_{53}N_5O_9$. Calcd: C, 64.78; H, 7.03; N, 9.22. Found: C, 64.48; H, 6.92; N, 9.22.

Boc-Tyr(OBzl)-Gly-Gly-Phe-Leu-OH Yield 92.7%, mp 148—150℃, R_f = 0.84 (n-BuOH/HOAc/H_2O: 4/1/2), $[\alpha]_D^{19}$ -1.2°(c 1, MeOH). Anal. $C_{40}H_{51}$-N_5O_9. Calcd: C, 64.41; H, 6.89; N, 9.39. Found: C, 64.21; H, 6.72; N, 9.30.

HCl(Tyr(OBzl)-Gly-Gly-Phe-Leu-OH Yield: 0.126 g(90.0%), mp = 125—127℃, R_f = 0.74 (BuOH/ AcOH/H_2O: 4/1/2). $[\alpha]_D^{19}$ + 9.5°(c 1, MeOH).

Leu-enkephalin HCl·Tyr(OBzl)-Gly-Gly-Phe-Leu-OH (0.110 g, 0.175 mmol) was hydrogenated over 10% Pd/C (0.2 g) in CH_3OH (15 mL) for 6h. The catalyst was filtered off, the filtrate was concentrated under reduced pressure. The residue was precipitated with ether to give the crude product which was purified by preparative HPLC[16] to afford the pure product as white solid. Yield: 52 mg (53%), mp 157—160℃, R_f = 0.56(BuOH/AcOH/H_2O: 4/1/2), $[\alpha]_D^{19}$ + 10.4° (c 1, MeOH). ESI-MS: 278[($M+2H^+$)/2], 557($M+H^+$), 1112($M+M+H^+$).

t_R = 19.53 min. HPLC conditions: Column: Lichrosorb RP-18 (0.5 × 30 cm). Solvent A: 0.1% TFA in water; Solvent B: 75% CH_3CN (0.1% TFA), Gradient: 20% solvent B to 60% solvent B in 20 min. Flow rate: 1.0 mL/min. Detection: 230 nm (0.5 AUFS).

Solid phase synthesis of Leu-enkephalin

Anchoring of Boc-Leu-OH *on merrifield resin*

To a solution of Boc-Leu-Cs (2.022 g, 5.304 mmol) in DMF (35 mL), Merrifield resin (3.040 g, 3.536 mmol) was added. The mixture was gently stirred 4 days at 45—50℃. The resin was filtered, washed successively with DMF (3 ×), MeOH (3 ×), DMF/H_2O 9/1 (v/v, 3 ×), DMF (3 ×) and MeOH (3 ×), and dried under vacuum. Element analysis indicated the quantity of unreacted chloride was less than 0.1%.

General procedure for solid phase peptide coupling using BDMP Starting from Boc-Leu-Merrifield resin (0.3 g, 0.273mmol), the following synthetic cycles were performed according to the standard protocol as shown in Table 4.

The peptide was cleavaged from the resin using HF-anisole to yield 140 mg of crude product. 40 mg of crude product was purified by preparative HPLC[16] to afford 18 mg pure product. Overall yield: ~40%. mp 155—159℃, R_f = 0.54 (BuOH/AcOH/H_2O: 4/1/2). ESI-MS: 278[(M + 2H$^+$)/2], 557(M + H$^+$), 1112(M + M + H$^+$).

t_R = 19.19 min. HPLC conditions: Column: Lichrosorb RP-18 (0.5 × 30 cm). Solvent A: 0.1% TFA in water; Solvent B: 75% CH_3CN (0.1% TFA), Gradient: 20% solvent B to 60% solvent B in 20 min. Flow rate: 1.0 mL/min. Detection: 230 nm (0.5 AUFS).

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14. Abbreviations: AA, AA′: amino acid; BOMI: (1*H*-benzotriazol-1-yloxy)-*N*,*N*-dimethylmethaniminium hexa-chloroantimonate; BDMP: 5-(1*H*-benzotriazol-1-yloxy)-3,4-dihydro-1-methyl 2*H*-pyrrolium hexachloro-antimonate; BOP: (1*H*-benzotriazol-1-yloxy) tris(dimethylamino)-phosphonium hexafluoro-phosphate; DCC: Dicyclohexylcarbodiimide; DIEA: diisopropylethyl amine; HBPipU: *O*-(1*H*-benzotriazol-1-yl)-*N*, *N*, *N*′, *N*′-bis (pentamethylene) uronium hexafluorophosphate; HBTU: *O*-(1*H*-benzotriazol-1-yl-tetramethyluronium hexafluoro-phosphate; HBPyU: *O*-(1*H*-benzotriazol-1-yl)-*N*, *N*, *N*′, *N*′-bis-tetramethylene) uronium hexafluoro-phosphate; HOBt: 1-hydroxy benzotriazole; NMM: *N*-methylmorpholine; SPPS: Solid Phase Peptide Synthesis; Z: benzyloxycarbonyl.
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16. HPLC conditions: Column: REGIS RP-18 (25 cm × 10 mm ID, Spherisorb S50DS, 5 microns). Eluent: 75% CH_3CN (0.1% TFA), Flow rate: 1.0 mL/min. Detection: 230 nm (0.5 AUFS).

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