A Racemization-Free Coupling Method for Peptides Having *N*-Methylamino Acids at the Carboxy-Termini[1,2)]

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To search for racemization-free coupling conditions for peptides having *N*-alkylamino acids at the carboxy-termini, a model coupling using Boc-Phe-MeAla-OH (MeAla, *N*-methylalanine) and H-Phe-OBzl was studied. When benzotriazolyl-*N*-oxytris(dimethylamino)phosphonium hexafluorophosphate or *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate was employed as a coupling reagent, no additives so far examined could sufficiently suppress the racemization of the carboxy-terminal MeAla residue. Though *N*-hydroxy compounds were not satisfactorily effective also in 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSCI)-mediated coupling, $CuCl_2$ could eliminate the racemization of MeAla in the coupling with WSCI even at room temperature.

Key words *N*-methylamino acid; racemization-free coupling; $CuCl_2$; racemization; segment condensation; peptide synthesis

N-Alkylamino acids are ubiquitous in microorganism-produced peptides, which include pharmaceutically important compounds, such as actinomycins and cyclosporins. Furthermore, *N*-alkylamino acids have often been incorporated into various biologically active peptides for studying relationships between their structures and activities. Despite wide attention to *N*-alkylamino-acid-containing peptides, studies on the synthesis of this class of peptides are limited in number in comparison with those of common peptides. *N*-Alkylamino acids are considerably susceptible to racemization, and this is one of the most serious problems in the synthesis of *N*-alkylamino acid-containing peptides. McDermott and Benoiton reported significant extents of racemization in saponification and acidolysis of *N*-methylamino acid derivatives under mild conditions, where common peptides do not undergo racemization.[3)] They pointed out also that peptides with *N*-methylamino acids at their carboxy-termini have a great tendency to be racemized in segment coupling, and proposed a mechanism *via* a pseudoaromatic oxazolonium ion,[4)] whose structure closely resembles the oxazolone anion formed in activation of common peptides (Fig. 1). A model coupling study using benzoyl-*N*-methylalanine (Bz-MeAla-OH) and H-Ala-OMe revealed that *N*,*N*-dicyclohexylcarbodiimide (DCC)–*N*-hydroxysuccinimide (HOSu)[5)] and DCC–1-hydroxybenzotriazole (HOBt)[6)] methods, which have been established as racemization-free coupling methods for common peptides, gave siginificant amounts of the D–L diastereoisomer.[7)] In the same reaction system, however, racemization was eliminated by cooling to −5 °C. Although this is a valuable option for segment condensation or intramolecular head-to-tail cyclization of *N*-methylamino-acid-containing peptides, racemization-reducing methods effective even at ambient temperature are more desirable. We have thus carried out a screening study using a simple coupling of Boc-Phe-MeAla-OH and H-Phe-OBzl, and report here about a substantially racemization-free procedure with $CuCl_2$ in 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSCI)[8)]-mediated coupling even when *N*-methylalanine (MeAla) is the carboxy-terminal residue.

Boc-Phe-MeAla-OH was prepared by the DCC–HOBt coupling of Boc-Phe-OH and H-MeAla-OBzl followed by hydrogenation over Pd–carbon. Authentic samples of the reaction products, Boc-Phe-MeAla-Phe-OBzl[9)] and Boc-Phe-D-MeAla-Phe-OBzl, were prepared by successive DCC–HOBt couplings of Boc-(L or D)-MeAla-OH and Boc-Phe-OH with H-Phe-OBzl. Retention times of diastereomeric products on HPLC differed sufficiently from each other.

Boc-Phe-MeAla-OH was coupled with H-Phe-OBzl in *N*,*N*-dimethylformamide (DMF) by a carbodiimide, a benzotriazole-based onium salt, and an azabenzotriazole-based onium reagent, *i.e.*, WSCI, benzotriazolyl-*N*-oxytris(dimethylamino)phosphonium hexafluorophosphate (Bop),[10)] and *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU).[11)] After storage at room temperature for >24 h, a portion of each reaction mixture was subjected to HPLC analysis, and % L–D–L isomer was determined as (peak area of the L–D–L isomer)×100/[(peak area of the L–L–L isomer)+(peak area of the L–D–L isomer)]. Coupling yields were also determined from the sum of peak areas of diastereomers in comparison with a standard solution prepared from the L–L–L authentic sample. The results are listed in Table 1. Onium-type reagents, Bop and HATU, gave lower levels of racemization than WSCI, but the values were still unsatisfactory.

Table 1. Extent of Racemization during Coupling of Boc-Phe-MeAla-OH with H-Phe-OBzl in the Absence of Additives

Coupling reagent	% L–D–L[a)]	% yield[a)]
WSCI	22.8	80
Bop[b)]	13.6	95
HATU[b)]	9.5	103

a) Average of two independent experiments. *b*) Two equivalents of DIEA were added.

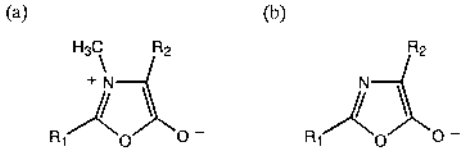

Fig. 1. Structures of (a) an Oxazolonium Ion and (b) an Oxazolone Anion from R_1–CON(CH_3 or H)–CH(R_2)–CO_2H

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Table 2. Extent of Racemization during Coupling of Boc-Phe-MeAla-OH with H-Phe-OBzl in the Presence of *N*-Hydroxy Compounds

Coupling reagent	% L–D–L[a]			% yield[a]		
	HOSu	HOBt	HOAt	HOSu	HOBt	HOAt
WSCI	13.2	13.3	4.4	87	86	66
Bop[b]	14.5	15.9	11.2	95	97	102
HATU[b]	11.1	20.9	13.2	97	102	99

a) Average of two independent experiments. *b*) Two equivalents of DIEA were added.

Table 3. Extent of Racemization during Coupling of Boc-Phe-MeAla-OH with H-Phe-OBzl in the Presence of $CuCl_2$

Coupling reagent	% L–D–L[a]		% yield[a]	
	No additive	$CuCl_2$	No additive	$CuCl_2$
WSCI	22.8	0.2	80	46
Bop[b]	13.6	2.7	95	35
HATU[b]	9.5	4.7	101	52

a) Average of two independent experiments. *b*) Two equivalents of DIEA were added.

The efficiency of *N*-hydroxy compounds, which reduce racemization of common peptides in combination with carbodiimide-type reagents in segment coupling, was then tested, and the results are shown in Table 2. Whereas no *N*-hydroxy compounds suppressed racemization in combination with Bop and HATU, all these additives reduced the amounts of the L–D–L product in combination with WSCI. This is consistent with the observations in segment coupling of common peptides. However, the resulting racemization levels with WSCI–HOSu, WSCI–HOBt, and WSCI–1-hydroxy-7-azabenzotriazole (HOAt)[11] were much higher than those in the segment coupling of common peptides. These results indicate that even the most effective additive to reduce racemization of common peptides, HOAt, cannot sufficiently prevent the racemization of carboxy-terminal *N*-methylamino acids, because of their high susceptibility to racemization.

Previously, Miyazawa and co-workers reported another type of racemization-reducing additive, $CuCl_2$, which could eliminate the racemization in carbodiimide-mediated coupling of common peptides.[12] We then tested this additive in our model reaction system. As concluded from data in Table 3, $CuCl_2$ could suppress the racemization of the MeAla residue in combination with all coupling reagents examined. In particular, racemization was eliminated almost completely in WSCI–$CuCl_2$ coupling. However, coupling yields were lowered to *ca*. 50% or less by adding $CuCl_2$, as previously observed in the DCC–$CuCl_2$ coupling of common peptides.[12]

In the case of common peptides, simultaneous addition of HOBt and $CuCl_2$ was reported to enhance the coupling yield without racemization.[13] Thus we examined the effect of HOBt as well as HOSu and HOAt in the presence of $CuCl_2$, and the results are summarized in Table 4. Addition of HOBt, HOSu, or HOAt did not enhance the yields in WSCI-, Bop-, and HATU-mediated couplings in the presence of $CuCl_2$. Moreover, racemization increased by adding *N*-hydroxy compounds in most cases. Surprisingly, WSCI–HOAt–$CuCl_2$ coupling gave a significant amount of the L–D–L product, although HOAt was the best additive for WSCI in the absence of $CuCl_2$, as shown in Table 2. It is noteworthy that no appreciable amount of the L–D–L product was detected in the WSCI–HOSu–$CuCl_2$ coupling, but the yield was lower than that in the WSCI–$CuCl_2$ coupling. WSCI–$CuCl_2$ would thus be favorable for practical use.

Table 4. Extent of Racemization during Coupling of Boc-Phe-MeAla-OH with H-Phe-OBzl in the Presence of *N*-Hydroxy Compounds and $CuCl_2$ Simultaneously

Coupling reagent[a]	% L–D–L[b]			% yield[b]		
	HOSu	HOBt	HOAt	HOSu	HOBt	HOAt
WSCI	0[c]	1.8	14.6	33	25	39
Bop[d]	3.5	5.0	5.7	27	40	55
HATU[d]	8.1	6.4	15.1	46	35	50

a) One equivalent of $CuCl_2$ was added. *b*) Average of two independent experiments. *c*) Not detected. *d*) Two equivalents of DIEA were added.

Consequently, WSCI–$CuCl_2$ has been found to be a racemization-free coupling agent for segment condensation of peptides with *N*-methylamino acids at their carboxy-termini. The coupling yield by this protocol is not quantitative at present, but would be useful for segment condensation or head-to-tail cyclization, where diastereomeric products are difficult to separate from each other. Improvement of the coupling yield is now under way in our laboratory.

Experimental

On TLC (Kieselgel G, Merck), Rf^1 and Rf^2 values refer to 1) AcOEt–*n*-hexane (1 : 2, v/v) and 2) $CHCl_3$–MeOH–AcOH (90 : 8 : 2, v/v), respectively. Optical rotations were measured with a JASCO DIP-370 polarimeter at room temperature. FAB-MS were recorded on a JEOL JMS-SX102 mass spectrometer using *m*-nitrobenzylalcohol as matrix. In the mobile phase system for HPLC, A and B refer to water and MeCN, respectively, both containing 0.05% (v/v) trifluoroacetic acid (TFA).

Boc-MeAla-OH and its D isomer were purchased from Watanabe Chemical Industries (Hiroshima, Japan) and Sigma (U.S.A.), respectively. DMF was of peptide synthesis grade, perchased from KOKUSAN Chemical Works (Gunma, Japan). Other reagents and solvents were of reagent grade, and used without further purification.

Boc-Phe-MeAla-Phe-OBzl The title compound was prepared by successive DCC–HOBt coupling of Boc-MeAla-OH and Boc-Phe-OH to H-Phe-OBzl·HCl (prepared from Boc-Phe-OBzl[14] and 4 N HCl in AcOEt in the usual manner) according to the published procedure[9] (colorless amorphous powder), $[\alpha]_D$ −31.5° (*c*=1.1, EtOH) [lit.[9] −38.8° (*c*=1, EtOH)], Rf^1 0.27 (lit.[9] 0.23). FAB-MS *m/z*: 588 $(M+H)^+$, 610 $(M+Na)^+$.

Boc-Phe-D-MeAla-Phe-OBzl The title compound was prepared from H-Phe-OBzl·HCl (prepared from Boc-Phe-OBzl[14] and 4 N HCl in AcOEt in the usual manner), Boc-D-MeAla-OH and Boc-Phe-OH in a similar manner to that described for the L–L–L isomer (colorless needles), mp 98—99 °C, $[\alpha]_D$ +50.9° (*c*=1.1, EtOH), Rf^1 0.34. FAB-MS *m/z*: 588 $(M+H)^+$, 610 $(M+Na)^+$. *Anal*. Calcd for $C_{34}H_{41}N_3O_6$: C, 69.5; H, 7.02; N, 7.15. Found: C, 69.6; H, 7.10; N, 7.27.

H-MeAla-OBzl·HCl To an ice-cooled solution of Boc-MeAla-OH (2.0 g, 9.84 mmol) in MeOH (50 ml) and water (5 ml) was added 20% (w/v) aq. Cs_2CO_3 to adjust the pH to 7. The resultant mixture was stirred for 30 min, and then evaporated to dryness. The residue was dissolved in DMF (30 ml), and the solution was evaporated. To remove water completely, this process was repeated once more. To an ice-cooled solution of the residue in DMF (30 ml) was added benzyl bromide (2.0 g, 11.8 mmol). The reaction mixture was stirred at room temperature for 2 h, and then evaporated. The residual oil was extracted with AcOEt. The extract was washed with water, dried over Na_2SO_4, and then evaporated. An ice-cooled solution of the residue in 4 N HCl in AcOEt (49.2 ml, 0.2 mol) was stirred at ice-bath temperature for 30 min, and then at room temperature for 1 h (after about 10 min, a white precipitate was formed). Diethyl ether (100 ml) was added to the above suspension under cooling with ice to afford a precipitate, which was collected by filtration and washed with diethyl ether, yield 2.0 g

(88.5%), mp 183—186 °C, $[\alpha]_D$ −11.7° (c=1.1, MeOH). *Anal*. Calcd for $C_{11}H_{16}ClNO_2$: C, 57.5; H, 7.02; N, 6.10. Found: C, 57.7; H, 7.08; N, 6.08.

Boc-Phe-MeAla-OH To an ice-salt-cooled solution of H-MeAla-OBzl·HCl (2.00 g, 8.72 mmol) and Boc-Phe-OH (2.30 g, 8.72 mmol) in CH_2Cl_2 (50 ml) was added HOBt (1.18 g, 8.72 mmol) in a small amount of DMF and Et_3N (1.22 ml, 8.72 mmol). DCC (1.80 g, 8.72 mmol) was added to the above solution, and the reaction mixture was stirred at room temperature overnight. After removal of the precipitate by filtration, the filtrate was diluted with $CHCl_3$ (60 ml), washed with 5% (w/v) aq. $NaHCO_3$, 10% (w/v) aq. citric acid and water, successively, dried over Na_2SO_4, and evaporated. Chromatography on a silica gel column with AcOEt–*n*-hexane (1 : 5, v/v) as an eluent yielded a colorless oil, yield 0.89 g (23.2%), Rf^2 0.86. Boc-Phe-MeAla-OBzl thus obtained was dissolved in MeOH–water (20 : 10 ml), and hydrogenated over 5% Pd–carbon catalyst for 3 h. After removal of the catalyst by filtration, the reaction mixture was evaporated to dryness. *n*-Hexane was added to the residue to afford crystals, which were collected by filtration, yield 0.51 g (72.0%), mp 149—151 °C, $[\alpha]_D$ −28.4° (c=1.0, DMF). FAB-MS m/z: 351 $(M+H)^+$, 373 $(M+Na)^+$. *Anal*. Calcd for $C_{18}H_{26}N_2O_5$: C, 61.7; H, 7.48; N, 7.99. Found: C, 61.6; H, 7.51; N, 7.90.

Racemization Test WSCI: To a mixture of 0.04 M Boc-Phe-MeAla-OH in DMF (50 μl, 2 μmol), 0.04 M H-Phe-OBzl (prepared from H-Phe-OBzl·HCl in the usual manner) in DMF (50 μl, 2 μmol), and DMF (50 μl) was added 0.25 M WSCI in CH_2Cl_2 (10 μl, 2.5 μmol).

Bop: To a mixture of 0.04 M Boc-Phe-MeAla-OH in DMF (50 μl, 2 μmol), 0.04 M H-Phe-OBzl (prepared from H-Phe-OBzl·HCl in the usual manner) in DMF (50 μl, 2 μmol), 0.25 M Bop in DMF (10 μl, 2.5 μmol), and DMF (10 μl) was added 0.1 M *N*,*N*-diisopropylethylamine (DIEA) in DMF (40 μl, 4 μmol).

HATU: The reaction was carried out in a similar manner to the Bop method by using 0.25 M HATU in DMF (10 μl, 2.5 μmol).

WSCI–HOSu: To a mixture of 0.04 M Boc-Phe-MeAla-OH in DMF (50 μl, 2 μmol), 0.04 M H-Phe-OBzl (prepared from H-Phe-OBzl·HCl in the usual manner) in DMF (50 μl, 2 μmol), 0.4 M HOSu in DMF (5 μl, 2 μmol), and DMF (45 μl) was added 0.25 M WSCI in CH_2Cl_2 (10 μl, 2.5 μmol).

WSCI–HOBt: The reaction was carried out in a similar manner to the WSCI–HOSu method by using 0.4 M HOBt in DMF (5 μl, 2 μmol).

WSCI–HOAt: The reaction was carried out in a similar manner to the WSCI–HOSu method by using 0.4 M HOAt in DMF (5 μl, 2 μmol).

Bop–HOSu: To a mixture of 0.04 M Boc-Phe-MeAla-OH in DMF (50 μl, 2 μmol), 0.04 M H-Phe-OBzl (prepared from H-Phe-OBzl·HCl in the usual manner) in DMF (50 μl, 2 μmol), 0.25 M Bop in DMF (10 μl, 2.5 μmol), 0.4 M HOSu in DMF (5 μl, 2 μmol), and DMF (5 μl) was added 0.1 M DIEA in DMF (40 μl, 4 μmol).

Bop–HOBt: The reaction was carried out in a similar manner to the Bop–HOSu method by using 0.4 M HOBt in DMF (5 μl, 2 μmol).

Bop–HOAt: The reaction was carried out in a similar manner to the Bop–HOSu method by using 0.4 M HOAt in DMF (5 μl, 2 μmol).

HATU–HOSu: The reaction was carried out in a similar manner to the Bop–HOSu method by using 0.25 M HATU in DMF (10 μl, 2.5 μmol).

HATU–HOBt: The reaction was carried out in a similar manner to the Bop–HOBt method by using 0.25 M HATU in DMF (10 μl, 2.5 μmol).

HATU–HOAt: The reaction was carried out in a similar manner to the Bop–HOAt method by using 0.25 M HATU in DMF (10 μl, 2.5 μmol).

WSCI–$CuCl_2$: To a mixture of 0.04 M Boc-Phe-MeAla-OH in DMF (50 μl, 2 μmol), 0.04 M H-Phe-OBzl (prepared from H-Phe-OBzl·HCl in the usual manner) in DMF (50 μl, 2 μmol), 0.4 M $CuCl_2$ in DMF (5 μl, 2 μmol), and DMF (45 μl) was added 0.25 M WSCI in CH_2Cl_2 (10 μl, 2.5 μmol).

Bop–$CuCl_2$: To a mixture of 0.04 M Boc-Phe-MeAla-OH in DMF (50 μl, 2 μmol), 0.04 M H-Phe-OBzl (prepared from H-Phe-OBzl·HCl in the usual manner) in DMF (50 μl, 2 μmol), 0.25 M Bop in DMF (10 μl, 2.5 μmol), 0.4 M $CuCl_2$ in DMF (5 μl, 2 μmol), and DMF (5 μl) was added 0.1 M DIEA in DMF (40 μl, 4 μmol).

HATU–$CuCl_2$: The reaction was carried out in a similar manner to the Bop–$CuCl_2$ method by using 0.25 M HATU in DMF (10 μl, 2.5 μmol).

WSCI–HOSu–$CuCl_2$: To a mixture of 0.04 M Boc-Phe-MeAla-OH in DMF (50 μl, 2 μmol), 0.04 M H-Phe-OBzl (prepared from H-Phe-OBzl·HCl in the usual manner) in DMF (50 μl, 2 μmol), 0.4 M HOSu in DMF (5 μl, 2 μmol), 0.4 M $CuCl_2$ in DMF (5 μl, 2 μmol), and DMF (40 μl) was added 0.25 M WSCI in CH_2Cl_2 (10 μl, 2.5 μmol).

WSCI–HOBt–$CuCl_2$: The reaction was carried out in a similar manner to the WSCI–HOSu–$CuCl_2$ method by using 0.4 M HOBt in DMF (5 μl, 2 μmol).

WSCI–HOAt–$CuCl_2$: The reaction was carried out in a similar manner to the WSCI–HOSu–$CuCl_2$ method by using 0.4 M HOAt in DMF (5 μl, 2 μmol).

Bop–HOSu–$CuCl_2$: To a mixture of 0.04 M Boc-Phe-MeAla-OH in DMF (50 μl, 2 μmol), 0.04 M H-Phe-OBzl (prepared from H-Phe-OBzl·HCl in the usual manner) in DMF (50 μl, 2 μmol), 0.25 M Bop in DMF (10 μl, 2.5 μmol), 0.4 M HOSu in DMF (5 μl, 2 μmol), and 0.4 M $CuCl_2$ in DMF (5 μl, 2 μmol) was added 0.1 M DIEA in DMF (40 μl, 4 μmol).

Bop–HOBt–$CuCl_2$: The reaction was carried out in a similar manner to the Bop–HOSu–$CuCl_2$ method by using 0.4 M HOBt in DMF (5 μl, 2 μmol).

Bop–HOAt–$CuCl_2$: The reaction was carried out in a similar manner to the Bop–HOSu–$CuCl_2$ method by using 0.4 M HOAt in DMF (5 μl, 2 μmol).

HATU–HOSu–$CuCl_2$: The reaction was carried out in a similar manner to the Bop–HOSu–$CuCl_2$ method by using 0.25 M HATU in DMF (10 μl, 2.5 μmol).

HATU–HOBt–$CuCl_2$: The reaction was carried out in a similar manner to the Bop–HOBt–$CuCl_2$ method by using 0.25 M HATU in DMF (10 μl, 2.5 μmol).

HATU–HOAt–$CuCl_2$: The reaction was carried out similarly to the Bop–HOAt–$CuCl_2$ method by using 0.25 M HATU in DMF (10 μl, 2.5 μmol).

After >24 h reaction at room temperature, each reaction mixture was diluted with DMF (640 μl), and a portion (10 μl) of the resultant solution was analyzed by HPLC [Boc-Phe-MeAla-Phe-OBzl: t_R 24.26 min. Boc-Phe-D-MeAla-Phe-OBzl: t_R 25.44 min. (Waters μBondasphere 5C_{18} 100 Å (3.9×150 mm), A : B=60 : 40 to 20 : 80 in 40 min, flow rate=1.0 ml/min)]. % L–D–L and % yield were determined as follows: % L–D–L=(peak area of the Boc-Phe-D-MeAla-Phe-OBzl)×100/[(peak area of the Boc-Phe-MeAla-Phe-OBzl)+(peak area of the Boc-Phe-D-MeAla-Phe-OBzl)]; % yield=[(peak area of the Boc-Phe-MeAla-Phe-OBzl)+(peak area of the Boc-Phe-D-MeAla-Phe-OBzl)]×100/[peak area of standard solution of Boc-Phe-MeAla-Phe-OBzl (2 μmol/800 μl DMF, 10 μl injection)].

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References and Notes

1) Amino acids used in this study are of L-configuration, unless otherwise noted. Abbreviations used in this report for amino acids, peptides and their derivatives are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochem. J*., **219**, 345—373 (1984). The following additional abbreviations are used: AcOEt, ethyl acetate; Boc, *tert*-butoxycarbonyl; Bop, benzotriazolyl-*N*-oxytris(dimethylamino)phosphonium hexafluorophosphate; Bz, benzoyl; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOAt, 1-hydroxy-7-azabenzotriazole; HOBt, 1-hydroxybenzotriazole; HOSu, *N*-hydroxysuccinimide; MeAla, *N*-methylalanine; OBzl, benzyl ester; WSCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride.
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