O-(*N*-Succinimidyl)-1,1,3,3-tetramethyluronium Tetrafluoroborate–*N*-Hydroxysuccinimide–$CuCl_2$: A Facile and Reliable System for Racemization-Free Coupling of Peptides Having a Carboxy-terminal *N*-Methylamino Acid[1)]

Yasuhiro NISHIYAMA,* Sou ISHIZUKA, Tomonori MORI,[2)] and Keisuke KURITA

Department of Industrial Chemistry, Faculty of Engineering, Seikei University, Musashino-shi, Tokyo 180–8633, Japan.
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Simultaneous use of *N*-hydroxysuccinimide (HOSu) and $CuCl_2$ with a HOSu-based uronium coupling reagent, *O*-(*N*-succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate, has been found to eliminate the racemization of the carboxy-terminal *N*-methylamino acid residue during the segment condensation.

Key words *N*-methylamino acid; racemization-free coupling; copper(II) chloride; racemization; segment condensation; peptide synthesis

In peptide synthesis by the convergent strategy the carboxy-terminal amino acid residue of the peptide segment is exposed to the risk of racemization.[3)] Extensive studies on the prevention of racemization have thus been continued, and some compounds, *e.g.*, *N*-hydroxysuccinimide (HOSu),[4)] 1-hydroxybenzotriazole (HOBt),[5)] *N*-hydroxy-5-norbornene-2,3-dicarboximide,[6)] 1-hydroxy-7-azabenzotriazole (HOAt),[7)] and $CuCl_2$,[8,9)] were found to reduce the racemization in the carbodiimide-mediated coupling effectively. However, the situation is quite different when the carboxy-terminal residue is an *N*-methylamino acid, which is considerably more susceptible to racemization than a common amino acid. For instance, McDermott and Benoiton reported that the dicyclohexylcarbodiimide (DCC)–HOSu coupling of (*N*-benzyloxycarbonyl-alanyl)-*N*-methylleucine and H–Gly–OBzl (Bzl, benzyl) in tetrahydrofran at room temperature was accompanied by 11% racemization of the *N*-methylleucine residue.[10)] Though the racemization decreased to an undetectable level on cooling to $-5\,^{\circ}C$,[11)] more convenient and reliable methods are desirable.

Recently, we have reported that $CuCl_2$ could eliminate the racemization of the carboxy-terminal *N*-methylamino acid residue in the 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSCI)-mediated segment coupling at room temperature,[12)] but could not sufficiently suppress the racemization in the coupling with onium-based reagents including benzotriazolyl-*N*-oxytris(dimethylamino)phosphonium hexafluorophosphate (BOP)[13)] and *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU).[7)] This class of coupling reagents is popularly used in peptide synthesis because of its advantages over carbodiimides; compared to carbodiimides, onium reagents usually complete couplings in a short time without any serious side reactions, give no troublesome by-products such as urea derivatives, and are safe to use from the standpoint of allergenicity. If racemization can be suppressed sufficiently, therefore, onium-based reagents would be convenient for use in the segment coupling of peptides having the carboxy-terminal *N*-methylamino acid and could facilitate efficient synthesis of *N*-methylamino acid-containing peptides by the convergent strategy. We have thus directed our study to the development of a racemization-free coupling system using an onium-based coupling reagent.

Leading studies by McDermott and Benoiton[10)] and Davies and Mohammed[11)] suggested that racemization of the carboxy-terminal *N*-methylamino acid in the DCC–HOBt coupling occurs by two major routes as outlined in Fig. 1. Elimination of the racemization by $CuCl_2$ in the carbodiimide-mediated coupling[12)] reveals that $CuCl_2$ inhibits almost completely the oxazolonium pathway (Fig. 1a). On the contrary, insufficient suppression of the racemization by $CuCl_2$ in the BOP-mediated coupling[12)] implies that $CuCl_2$ cannot inhibit the active ester pathway (Fig. 1b). Racemization by pathway b, however, is known not to appear in the HOSu ester coupling.[11)] This suggests that pathway b can possibly be omitted when a HOSu-based onium salt is used as a coupling reagent. Taking this knowledge of racemization-mechanisms into consideration, we have designed a novel coupling system consisting of *O*-(*N*-succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TSTU)[14)] and $CuCl_2$ to achieve racemization-free segment coupling at the *N*-methylamino-acyl bond, and evaluated it in a simple model reaction between Boc–Phe–MeAla–OH (Boc, *tert*-butoxycarbonyl; MeAla, *N*-methylalanine) and H–Phe–OBzl.

Boc–Phe–MeAla–OH[12,15)] was coupled with H–Phe–OBzl in *N*,*N*-dimethylformamide (DMF) by TSTU in the presence or absence of additives, HOSu, HOBt, and $CuCl_2$. After storage of each reaction mixture at room temperature for >24 h, a portion was subjected to HPLC analysis, and % L–D–L isomer was determined as (peak area of the L–D–L isomer)$\times$ 100/[(peak area of the L–L–L isomer)$+$(peak area of the L–D–L isomer)]. Coupling yields were determined from the sum of peak areas of diastereomers in comparison with a standard solution of the L–L–L authentic sample. Partial HPLC profiles of samples from coupling with TSTU, TSTU–$CuCl_2$, and TSTU–HOSu–$CuCl_2$ are shown in Fig. 2.

The results are listed in Table 1 with some data selected from our previous report[12)] for comparison. A significant amount of the L–D–L product was detected in the coupling with TSTU alone, and also in the presence of HOSu and HOBt. However, $CuCl_2$ could reduce the amount of the L–D–L product in the TSTU coupling, whereas it was not sufficiently effective in the BOP- and HATU-mediated couplings. These results indicate that the racemization by both pathways

* To whom correspondence should be addressed.

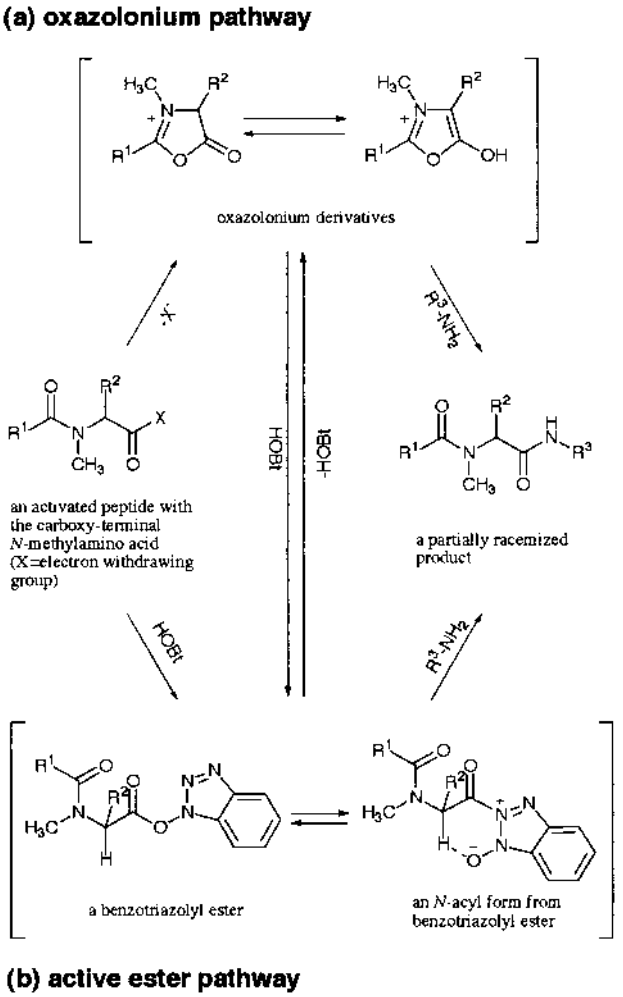

Fig. 1. Two Major Pathways for Racemization of the Carboxy-Terminal *N*-Methylamino Acids in Segment Condensation

Table 1. Extents of Racemization during the Coupling of Boc–Phe–MeAla–OH with H–Phe–OBzl

Additive[a]	% L–D–L[b]			% yield[b]		
	TSTU[c]	Bop[c,d]	HATU[c,d]	TSTU[c]	Bop[c,d]	HATU[c,d]
—	18.1	13.6	9.5	80	95	103
HOSu	14.0	14.5	11.1	73	95	97
HOBt	18.9	15.9	20.9	92	97	102
$CuCl_2$	0.4	2.7	4.7	47	35	52
HOSu–$CuCl_2$	<0.2	3.5	8.1	45[e]	27	46

a) One eq. *b*) Average of two independent experiments. *c*) Coupling reagents, 1.25 eq.; diisopropylethylamine, 2 eq. *d*) Ref. 12. *e*) 60% with 2.5 eq. of TSTU.

a and b can be suppressed effectively by a combination of TSTU and $CuCl_2$. A small amount of the L–D–L product was, however, detected even in the presence of $CuCl_2$. In the segment condensation of common peptides, BOP–HOBt was reported to result in a lower racemization level than BOP alone.[16] We thus examined the simultaneous use of HOSu and $CuCl_2$ in the TSTU coupling. No appreciable racemization was detected by HPLC at an identification limit of 0.2%, and TSTU–HOSu–$CuCl_2$ proved to be an appropriate combination to suppress the racemization in the coupling of peptide segments having a carboxy-terminal *N*-methylamino

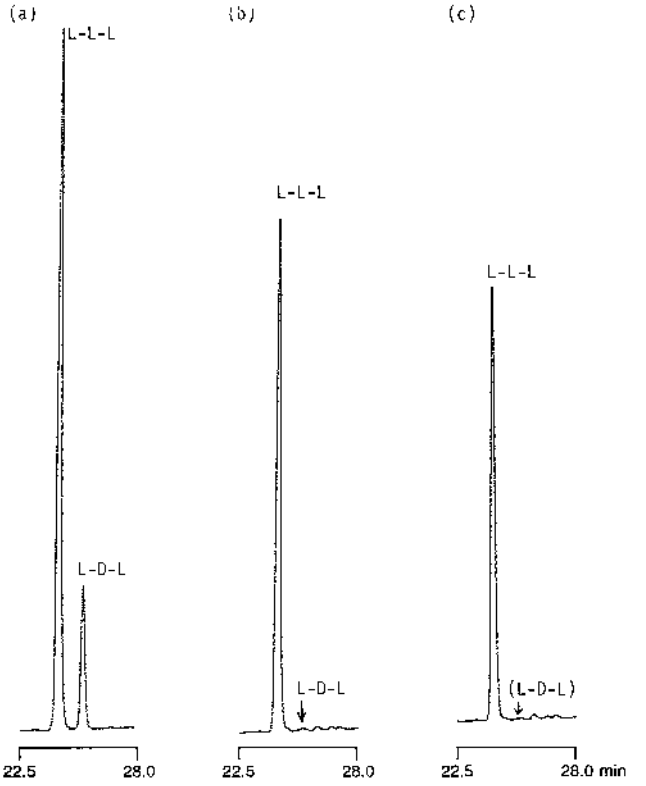

Fig. 2. Partial HPLC Profiles of Samples from Coupling with (a) TSTU, (b) TSTU–$CuCl_2$, and (c) TSTU–HOSu–$CuCl_2$

acid. In the WSCI–$CuCl_2$ coupling of Boc–Phe–MeAla–OH and H–Phe–OBzl, a small amount of the L–D–L product (0.2%) was detected, and the yield was similar (46%) to that of TSTU–HOSu–$CuCl_2$.[12] When HOSu and $CuCl_2$ were simultaneously used in the WSCI-coupling, racemization was not detected, but the yield decreased to 33%.[12] Thus the TSTU–HOSu–$CuCl_2$ system would be more favorable than WSCI–$CuCl_2$ or WSCI–HOSu–$CuCl_2$ systems.

In conclusion, a novel coupling system consisting of TSTU, HOSu, and $CuCl_2$ has proved to completely eliminate the racemization of the carboxy-terminal *N*-methylamino acid residue during segment condensation. This is the first example of an onium-based coupling system which enables the racemization-free segment condensation at the *N*-methylaminoacyl bond. Optimization of the reaction conditions is now under way,[17] facilitating the efficient convergent synthesis or head-to-tail cyclization of *N*-methylamino acid-containing peptides.

Experimental

In the HPLC analysis, elution profiles were monitored at 220 nm by a Waters 480 UV/VIS tunable absorbance detecter and recorded with a SIC Chromatocoder-21.

Boc–Phe–MeAla–OH, Boc–Phe–MeAla–Phe–OBzl, and Boc–Phe–D-MeAla–Phe–OBzl were prepared as previously reported.[12] TSTU was purchased from Novabiochem (U.S.A.). DMF was of peptide synthesis grade, and used without purification. $CuCl_2$ used in this study was its dihydrate.

Racemization Test The coupling reactions were conducted using the following stock solutions: A, 0.04 M Boc–Phe–MeAla–OH in DMF; B, 0.04 M H–Phe–OBzl (prepared from H–Phe–OBzl·HCl in the usual manner) in DMF; C, 0.25 M TSTU in DMF; D, 0.1 M diisopropylethylamine in DMF; E, 0.4 M HOSu in DMF; F, 0.4 M $CuCl_2$ in DMF.

TSTU Method: To a mixture of A (50 μl, 2 μmol), B (50 μl, 2 μmol), C (10 μl, 2.5 μmol), and DMF (10 μl) was added D (40 μl, 4 μmol).

TSTU–HOSu Method: To a mixture of A (50 μl, 2 μmol), B (50 μl,

2 μmol), C (10 μl, 2.5 μmol), E (5 μl, 2 μmol), and DMF (5 μl) was added D (40 μl, 4 μmol).

TSTU–$CuCl_2$ Method: To a mixture of A (50 μl, 2 μmol), B (50 μl, 2 μmol), C (10 μl, 2.5 μmol), F (5 μl, 2 μmol), and DMF (5 μl) was added D (40 μl, 4 μmol).

TSTU–HOSu–$CuCl_2$ Method: To a mixture of A (50 μl, 2 μmol), B (50 μl 2 μmol), C (10 μl, 2.5 μmol), E (5 μl, 2 μmol), and F (5 μl, 2 μmol) was added D (40 μl, 4 μmol).

Determination of % L–D–L: 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 [column, Waters μBondasphere $5C_{18}$ 100 Å (3.9×150 mm); solvent system, 0.05% trifluoroacetic acid (TFA) in water : 0.05% TFA in MeCN 60 : 40 to 20 : 80 in 40 min at a flow rate of 1.0 ml/min. Boc–Phe–MeAla–Phe–OBzl, t_R 24.26 min; Boc–Phe–D-MeAla–Phe–OBzl, t_R 25.44 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)]. The results are summarized in Table 1.

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).
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17) Preliminarily, the coupling yield could be improved to 60% by using 2.5 eq. TSTU without detectable racemization. At present, the reason for the incomplete coupling is unclear; no side products but only Boc–Phe–MeAla–Phe–OBzl and Boc–Phe–MeAla–OH were detected on the HPLC analaysis of the crude product after extraction with ethyl acetate and washing with 1 N HCl.