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O-(7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate–1-hydroxy-7-azabenzotriazole–copper(II) chloride: a promising epimerization-free segment coupling system for peptide synthesis

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Abstract—Simultaneous use of 1-hydroxy-7-azabenzotriazole and $CuCl_2$ with *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate has been found to eliminate the enantiomerization of the carboxy-terminal amino acid residue in peptide synthesis by the segment condensation strategy.

For chemical synthesis of large peptides and proteins, the segment condensation method has been employed successfully, where a protected peptide acid (a carboxyl component) and a protected peptide with the unprotected N-terminal amino group (an amine component) are coupled to give a larger protected peptide.[1] The C-terminal amino acid of the carboxyl component, however, is known to enatiomerize at significant levels except the case, in which the C-terminal residue is Gly or Pro. The extent of epimerization of the segment coupling product depends mainly on a ratio of the rate of formation of the 5(4*H*)-oxazolone derivative, which is formed spontaneously from the activated carboxyl component, and the rate of coupling reaction; the 5(4*H*)-oxazolone derivative, the lifetime of which is determined by the coupling rate, readily lose the chiral integrity via resonance-stabilized tautomers of the oxazolone anion, and reacts with the amine component to give the epimerized product.[2] The most common epimerization suppressant for carbodiimide-mediated segment coupling, 1-hydroxybenzotriazole (HOBt), and related compounds permit to trap the vigorously activated intermediate before significant epimerization occurs. Furthermore, these additives are effective to improve the coupling yield, since trapping the acylisourea intermediates inhibits the formation of inert acylurea derivatives. Among this class of suppressants, 1-hydroxy-7-azabenzotriazole[3] (HOAt) appears the most efficient one owing to reaction facilitation by the unique feature to capture the amine component. HOAt-based onium coupling reagents including *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) were reported to give epimerization levels significantly less than do HOBt-based analogous reagents.[4] Even in the presence of these suppressants or HOAt-based coupling reagent, considerable levels of epimerization, however, occur in the segment condensation at consequent bulky amino acids, such as Val-Val, and particularly in the solid-phase segment condensation,[2,3,5] since their epimerization suppression efficiency depends on coupling rate in principle.

$CuCl_2$ was reported to reduce the epimerization effectively not only in the carbodiimide-mediated segment coupling,[6] but also in mixed anhydride-, 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline-, and *N*,*N*′-bis(2-keto-3-oxazolidinyl) phosphinic chloride-mediated coupling.[7] $CuCl_2$ eliminates the epimerization also in the carbodiimide- or *O*-(*N*-succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate-mediated segment condensation of a peptide having a carboxy-terminal *N*-methylamino acid, which is highly prone to epimerization.[8,9] Moreover, no significant loss of chiral

Keywords: copper(II) chloride; epimerization suppressant; 1-hydroxy-7-azabenzotriazole; *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; peptide synthesis.

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integrity of a 5(4*H*)-oxazolone derivative was evident in the presence of equimolar $CuCl_2$,[6] suggesting the thermodynamic stabilization of the oxazolone by $CuCl_2$ to inhibit the enolization, even though the mechanism is not fully understood.

Here we report the efficient epimerization suppression by simultaneous use of an oxazolone-formation inhibitor (kinetic epimerization suppressant), HOAt, and an oxazolone stabilizer (thermodynamic epimerization suppressant), $CuCl_2$, in HATU-mediated segment coupling.

A most epimerization-susceptible coupling system, i.e. the condensation at Val–Val bond was employed as a model to permit generalization of the results. Boc-Phe-Val-OH† was coupled with H-Val-OBn by HATU in the presence of iPr$_2$EtN and additives. An analogous uronium reagent derived from HOBt, *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate[10] (HBTU), was tested for comparison. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC)–HOBt, EDC–HOAt, and EDC–HOBt–$CuCl_2$ were also included as representative epimerization-free coupling systems. In addition to the conventional in situ coupling, the preactivation procedure,[11] where the carboxyl component is allowed to react with an activation reagent and additives in the absence of the amine component, were also tested, because it is favorable for solid-phase coupling and reflects very slow in situ coupling reactions.[12] After storage of each reaction mixture at room temperature for >24 h, a portion was subjected to HPLC analysis.‡ % L–D–L isomer and % yield of the desired product were determined as (peak area of L–D–L isomer)×100/[(peak area of L–D–L isomer)+(peak are of L–L–L isomer)] and (peak area of L–L–L isomer)×100/(expected peak area calculated from peak area on authentic L–L–L isomer analysis), respectively.

Table 1. Extent of epimerization and product yield on the coupling of Boc-Phe-Val-OH and H-Val-OBn

Coupling method[a]		L–D–L (%)[b]	Yield (%)[b]
EDC–HOBt	In situ	9.8	81
EDC–HOAt	In situ	1.6	89
EDC–HOBt–$CuCl_2$	In situ	0.9	62
EDC–HOBt–$CuCl_2$	Preactivation	1.2	64
HBTU–HOBt	In situ	8.2	83
HBTU–HOBt	Preactivation	15.1	76
HATU–HOAt	In situ	0.8	84
HATU–HOAt	Preactivation	6.9	81
HBTU–HOBt–$CuCl_2$	In situ	1.2	62
HBTU–HOBt–$CuCl_2$	Preactivation	1.4	60
HATU–HOAt–$CuCl_2$	In situ	0.4	76
HATU–HOAt–$CuCl_2$	Preactivation	0.2	70

[a] In situ: To an ice-cooling solution of Boc-Phe-Val-OH (2.0 μmol), H-Val-OBn (2.0 μmol), additives (2.0 μmol), coupling reagent (2.5 μmol) in DMF (120 μl) was added 0.1 M iPr$_2$EtN in DMF (40 μl, 4.0 μmol). For EDC-mediated coupling reactions, DMF (40 μl) instead of iPr$_2$EtN solution was added. The mixture was stored at room temperature (ca. 20°C) for >24 h. Preactivation: H-Val-OBn was added after preactivation at an ice-bath temperature for 30 min, then the mixture was stored at room temperature for >24 h.

[b] Means of four to eight replicates.

The results are compiled in Table 1. A considerable level of epimerization was detected in the coupling with EDC–HOBt, a most common epimerization-free segment condensation method. The EDC–HOAt and EDC–HOBt–$CuCl_2$ methods also gave significant amounts of the L–D–L product, though much lower than that of the ECC–HOBt coupling in consistent with previous reports.[3–6,13] These results validates that the model reaction employed in this study is exceedingly susceptible to epimerization. The HATU–HOAt (in situ) coupling gave a very low level of epimerization, whereas HBTU–HOBt (in situ) gave a considerable amount of L–D–L isomer. The preactivation coupling with HATU–HOAt, however, resulted in a much higher extent of epimerization than the in situ coupling. This result indicates that the HATU–HOAt segment coupling is not suitable for the preactivation procedure, implying that a considerable level of epimerization would occur even in the in situ coupling when the coupling reaction is very slow, as is the case under highly diluted conditions (like the coupling of large segments) and the solid-phase segment condensation. The significantly higher levels of epimerization in preactivation coupling than in situ has realized the limitation of kinetic suppressants. The HATU–HOAt–$CuCl_2$ (in situ) method gave a negligible level of epimerization, whereas HBTU–HOBt–$CuCl_2$ gave a significant level. Furthermore, the HATU–HOAt–$CuCl_2$ system, unlike HATU–HOAt, afforded a negligible level of epimerization also in preactivation coupling. These results indicate that the HATU–HOAt–$CuCl_2$ method is suitable for both in situ and preactivation procedures and is highly reliable even for very slow segment coupling reactions, proving the effectiveness of simultaneous use of kinetic and thermodynamic epimerization suppressants.

Previously, coupling in the presence of $CuCl_2$ was reported to result in considerably low coupling yield.[6–9] Similar phenomena were observed in the present study. New copper(II)-containing epimerization suppressants, such as Cu_2(OBt) and Cu_2(OAt), also appear to diminish coupling yield.[14,15] It is, therefore, noteworthy that HATU–HOAt–$CuCl_2$ gave yields close to those in the

† Amorphous powder; R_f 0.40 ($CHCl_3$–MeOH–AcOH 90:80:2); $[\alpha]_D^{20}$ −2.3 (*c* 1, DMF). C, 62.7; H, 7.85; N, 7.79. $C_{19}H_{28}N_2O_5$ requires C, 62.6; H, 7.74; N, 7.69.

‡ L–L–L isomer: mp 97–101°C; R_f 0.78 ($CHCl_3$–MeOH–AcOH 90:80:2); $[\alpha]_D^{20}$ −14.7 (*c* 1, DMF); t_R 40.42 min [μBondasphere 5μ C_{18} 100 Å (3.9×150 mm), 0.05 % aq. TFA:0.05 % TFA in MeCN 80:20 to 20:80 in 60 min (1.0 cm^3 min^{-1})]. C, 67.4; H, 7.81; N, 7.43. $C_{31}H_{43}N_3O_6$ requires C, 67.2; H, 7.83; N, 7.59. L–D–L isomer: mp 160–163°C; R_f 0.78 ($CHCl_3$–MeOH–AcOH 90:80:2); $[\alpha]_D^{20}$ −34.4 (*c* 1, DMF); t_R 41.01 min (the same conditions to the above). C, 67.1; H, 7.82; N, 7.37.

absence of $CuCl_2$; relative yields of HATU–HOAt–$CuCl_2$ to HATU–HOAt was 90 (in situ) and 86 (preactivation) %, whereas those of EDC–HOBt–$CuCl_2$ (in situ) to EDC–HOBt (in situ) and of HBTU–HOBt–$CuCl_2$ (in situ) to HBTU–HOBt (in situ) were 77 and 75%, respectively.

The above results obtained using an extremely epimerization-susceptible reaction and the preactivation procedure could permit us to conclude that HATU–HOAt–$CuCl_2$ is a reaction rate-independent, thus sequence-independent, epimerization-free segment condensation method. To obtain further evidence, another model reaction, coupling of Boc-Phe-Ala-OH and H-Phe-OBn by HATU–HOAt–$CuCl_2$ (in situ) was studied. Coupling reaction and determination of % L–D–L and % yield were carried out in the same manner.[§] No epimerized product was detected by HPLC (<0.1%) and yield was satisfactorily high (86%; relative yield to HATU–HOAt, 91%).

Consequently, the HATU–HOAt–$CuCl_2$ system developed here can be concluded as a superior epimerization-free segment coupling method. The segment condensation between large protected peptides as in the practical peptide synthesis is occasionally much slower process and more susceptible to epimerization than model reactions using small peptides. The data from preactivation experiments, which simulate such slow reactions, provide us high reliability on practical use of this new method.

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[§] Boc-Phe-Ala-Phe-OBn: L–L–L isomer, t_R 39.34 min [μBondasphere 5μ C_{18} 100 Å (3.9×150 mm), 0.05 % aq. TFA:0.05 % TFA in MeCN 80:20 to 20:80 in 60 min (1.0 cm^3 min^{-1})]; L–D–L isomer, t_R 39.86 min (the same conditions to the above).