Carbodiimide-Mediated Amide Formation in a Two-Phase System. A High-Yield and Low-Racemization Procedure for Peptide Synthesis

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The prevention of racemization is one of the major challenges in peptide synthesis.[1] The addition of *N*-hydroxy compounds,[1c] such as 1-hydroxybenzotriazole (HOBt),[2] suppresses side reactions and reduces racemization. Unfortunately, in certain circumstances, racemization still occurs even in the presence of the additive.[3] Most recently, it has been shown that 1-hydroxy-7-azabenzotriazole (HOAt) is superior to HOBt as an additive in coupling efficiency and preservation of chiral integrity during the coupling reactions.[4] However, racemization still occurs to a certain extent even with this new additive.[4] We now report on a simple and efficient two-phase coupling method which affords the di- or tripeptides in high yields with low degrees of racemization. Furthermore, it was found that 2-hydroxypyridine *N*-oxide (HOPO)[5] showed a more significant effect of reducing racemization than the more common *N*-hydroxy derivatives in this two-phase coupling system.

A two-phase approach for oligopeptide synthesis was reported in which the coupling reactions were carried out in dichloromethane using the water soluble *N*-ethyl-*N'*-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) as the coupling reagent.[6] The side products of the reactions were then removed by aqueous extraction. However, the EDC-mediated coupling reaction in dichloromethane sometimes resulted in considerable racemization, even with HOBt as the additive (see below). Nozaki *et al.* reported on a "hold-in-solution" method for oligopeptide synthesis, in which the reaction was carried out in a two-phase mixture of dichloroethane and water using EDC as the coupling reagent and HOBt as the additive. The extent of racemization, however, was not reported, and the yields were not optimized.[7] Furthermore, only *N*-Boc amino acids were used for the peptide elongation, and it is known that the Boc protecting group suppresses the racemization during peptide coupling.[1a] We have examined the degree of racemization of this two-phase coupling reaction in solvent mixtures of dichloromethane or isopropyl acetate (*i*-PrOAc) and water (1:1, v/v). Typically, the reaction was carried out using equimolar amounts of the protected acid and amine components and the additive, with a 10–15% molar excess of EDC at ~0 °C.[8] *N*-Acetyl amino acids were chosen for this study since the acetyl group lacks the ability to suppress racemization.[1a] This coupling method generally gives a high yield and, more importantly, a very low degree of racemization of the di- or tripeptide. The results of the coupling reactions in dichloromethane/water and in *i*-PrOAc/water are shown in Tables 1 and 2, respectively. For comparison, results of analogous reactions in DMF are listed in Table 3. Coupling reactions in the two-phase mixture generally afforded yields

Table 1. Coupling Reaction in Dichloromethane/Water Mixture[a,b]

entry	AA_1	AA_2[c]	additive	yield (%)	*d,l* isomer %
1	Ac-Val	ValOBn	HOBt	93	0.1
2	Ac-Val	ValOBn	HOPO	93	0.2
3	Ac-Val	LeuOBn	HOBt	99	0.3
4[d]	Ac-Val	LeuOBn	HOBt	92	2.8
5	Ac-Val	LeuOBn	HOPO	94	0.1
6[e]	Ac-Val	LeuOBn	HOPO	84	2.2
7	Z-Phg	LeuOBn	HOBt	88	~0.07
8[d]	Z-Phg	LeuOBn	HOBt	93	0.6
9	Z-Phg	LeuOBn	HOPO	94	nd[f]
10	Z-Phg	ValOBn	HOPO	99[g]	nd[f,g]
11[d]	Z-Phg	ValOBn	HOAt	98	0.2
12	Z-Phg	ValOBn	HOAt	97	nd[f]
13	Z-Gly-Val	ValOBn	HOBt	97	nd[f]
14	Z-Gly-Val	ValOBn	HOPO	92[g]	nd[f,g]
15	Z-Gly-Val	ValOBn	HOAt	95	nd[f]

[a] Abbreviations: AA_1, AA_2 = amino acid or dipeptide fragment; Ac = acetyl; Z = (benzyloxy)carbonyl; OBn = benzyloxy. [b] Reactions (0.2 mmol scale) were carried out in 4 mL of 1:1 dichloromethane/water (v/v) with 1 equiv of the additive using 1.1 equiv of EDC at 0–5 °C for 24–40 h and then assayed by HPLC; see the Experimental Section. [c] Solution of the amino acid benzyl ester solution in dichloromethane. [d] Reaction in dichloromethane only, no water added. [e] Reaction in dichloromethane only, HOPO not completely dissolved. [f] Not detected, <0.05 %. [g] Average of three runs.

Table 2. Coupling Reactions in Isopropyl Acetate/Water Mixture[a,b]

entry	AA_1	AA_2[c]	additive	yield (%)	*d,l* isomer %
1	Ac-Leu	LeuOBn	HONb	93	0.3
2	Ac-Leu	LeuOBn	HOPO	94	0.1
3[d]	Ac-Leu	LeuOBn	HOBt	96	0.7
4[d]	Ac-Val	ValOBn	HOBt	95	1.7
5	Ac-Val	ValOBn	HONb	68	0.1
6	Ac-Val	ValOBn	HOPO	85	0.1
7	Z-Phg	ValOBn	HOPO	90	0.2
8	Z-Phg	LeuOBn	HOPO	92[e]	0.2[e]
9	Z-Phg	ValOBn	HOAt	70	0.6
10	Z-Gly-Val	ValOBn	HOPO	89[e]	0.2[e]
11	Z-Gly-Val	LeuOBn	HOPO	91	0.2

[a] Abbreviations: AA_1, AA_2 = amino acid or dipeptide fragment; Ac = acetyl; Z = (benzyloxy)carbonyl; OBn = benzyloxy. [b] Reactions (0.2 mmol scale) were carried out in 4 mL of 1:1 *i*-PrOAc/water (v/v) with 1 equiv of the additive using 1.1 equiv of EDC at 0–5 °C for 24–40 h and then assayed by HPLC; see the Experimental Section. [c] Solution of the amino acid benzyl ester solution in *i*-PrOAc. [d] 1 equiv of HOBt was added, resulting in a suspension in the solvent mixture. [e] Average of three runs.

(1) (a) Bodanszky, M. *Principles of Peptide Synthesis*; Springer-Verlag: Berlin, 1984; pp 158–172. (b) Benoiton, N. L. In *The Peptides*; Gross, E., Meienhofer, J., Eds.; Academic Press: New York, 1983; Vol. 5, Chapter 4. (c) Rich, D. H.; Singh, J. In *The Peptides*; Gross, E., Meienhofer, J., Eds.; Academic Press: New York, 1979; Vol. 1, Chapter 5. (d) Kemp. D. S. In *The Peptides*; Gross, E., Meienhofer, J., Eds.; Academic Press: New York, 1979; Vol. 1, Chapter 7.

(2) König, W.; Geiger, R. *Chem. Ber.* **1970**, *103*, 788.

(3) Sieber, P.; Kamber, B.; Hartmann, A.; Jöhl, A.; Riniker, B.; Rittel, W. *Helv. Chim. Acta* **1977**, *60*, 27.

(4) Carpino, L. A. *J. Am. Chem. Soc.* **1993**, *115*, 4397.

(5) The esters of 2-hydroxypyridine *N*-oxide have been used to form dipeptides. The preparation of these esters first required the formation of the acyl chloride or the reaction of the thallium salt with *N*-hydroxypyridine in the presence of thionyl chloride: (a) Paquette, L. A. *J. Am. Chem. Soc.* **1965**, *87*, 5186. (b) Sarantakis, D.; Sutherland, J. K.; Tortorella, C.; Tortorella, V. *J. Chem. Soc. C* **1968**, 72. (c) Taylor, E. C.; Kienzle, F.; McKillop, A. *J. Org. Chem.* **1970**, *35*, 1672.

(6) Sheehan, J. C.; Preston, J.; Cruickshank, P. A. *J. Am. Chem. Soc.* **1965**, *87*, 2492.

(7) (a) Nozaki, S.; Kimura, A.; Muramatsu, I. *Chem. Lett.* **1977**, 1057. (b) Nozaki, S.; Muramatsu, I. *Bull. Chem. Soc. Jpn.* **1982**, *55*, 2165.

(8) In ref 7, the reactions were carried out at room temperature. We found that this often resulted in a 3–4-fold increase in racemization.

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Table 3. Coupling Reactions in DMF[a,b]

entry	AA_1	AA_2[c]	yield (%)	d,l isomer %
1	Ac-Leu	LeuOBn	99	0.4
2[d]	Ac-Val	LeuOBn	70	21
3	Ac-Val	LeuOBn	94	4.6
4	Ac-Val	ValOBn	85	3.5
5[e]	Ac-Val	ValOBn	93	1.0
6[d]	Ac-Val	ValOBn	73	13
7	Z-Phg	ValOBn	95	4.7
8	Z-Phg	LeuOBn	99	4.1
9	Z-Gly-Val	ValOBn	94	1.8
10	Z-Gly-Val	LeuOBn	96	1.6

[a] Abbreviations: AA_1, AA_2 = amino acid or dipeptide fragment; Ac = acetyl; Z = (benzyloxy)carbonyl; OBn = benzyloxy. [b] 1 equiv of HOBt was used as the additive unless specified. Reactions (0.2 mmol scale) were carried out in 2 mL of DMF using 1 equiv of EDC at 0–5 °C for 24–40 h and then assayed by HPLC. [c] The tosylate salts were used with addition of 1 equiv of *N*-methylmorpholine. [d] 1 equiv of HONb was used as the additive. [e] 1 equiv of HOPO was used as the additive.

comparable to those in DMF. In addition, the two-phase procedure provides a more convenient workup, and consequently, higher recovery yields were generally obtained.[9]

Various *N*-hydroxy derivatives were tested as the additive in the two-phase coupling reaction, including HOPO, HOBt, HOAt, *endo*-*N*-hydroxy-5-norbonene-2,3-dicarboximide (HONb),[10] and *N*-hydroxysuccinimide (HOSu).[11] In the absence of an additive, the coupling reactions in the two-phase mixture generally resulted in low yields (<50%) and extensive racemization (up to 15–30% of the *d,l* isomer formed). In the dichloromethane/water mixture, HOBt, HOAt, and HOPO showed significant effects on coupling efficiency and racemization suppression (Table 1). On the other hand, HOBt and HOAt were less effective in reducing racemization in *i*-PrOAc/water because of their poor solubility in this mixture (Table 2).[12] HONb, readily soluble in *i*-PrOAc/water, appears to be a good additive (but not as effective as HOPO) for reactions in this solvent mixture. Finally, HOSu generally afforded the peptides in low yields with significant amounts of side products (detected by HPLC).

As shown in Tables 1 and 2, the racemization-suppressing effect of the two-phase coupling system becomes very significant in cases involving valine[13] or phenylglycine[4,14] as the acid component, since the coupling reactions of these amino acids are very sensitive toward racemization (Table 3). The low racemization observed in the two-phase reaction is not only due to the low polarity of the organic solvent, since coupling reactions in dichloromethane gave higher racemization (Table 1, entries 4, 6, 8, and 11).[15] Therefore, suppression of racemization in the two-phase mixture may be due to the extensive hydrogen-bonding interactions in an aqueous medium which inhibit the intramolecular proton transfer in an activated carboxy component such as an *O*-acylisourea intermediate.[1c] Alternatively, it may be attributed to a reduction in the formation (and deprotonation) of oxazol-5(4*H*)-ones under these reaction conditions.[1a,1d]

Although the mechanistic details leading to the peptide bond formation with little racemization in this heterogeneous system remain unknown, it seems likely that the reaction occurs at the interface of the phases.[16] Carbodiimide-mediated peptide formation in a reversed micelle system has been reported.[17] Similar to the micellar system, it seems reasonable that, in the two-phase mixture, the polar residues of the reacting species, COOH, NH_2, =NOH, N=C=N, would mainly be distributed in the aqueous side at the interface, while the hydrophobic species remain in the organic phase. This model thus affords an explanation for the high efficiency of amide formation. In conclusion, the carbodiimide-mediated amide-coupling reaction in a two-phase solvent mixture provides a convenient method for peptide coupling in high yields with little racemization.

Experimental Section

All amino acid derivatives were purchased from Sigma, except for (*Z*)-phenylglycine (Bachem). All additives (HOBt, HONb, HOPO, and HOSu) were obtained from Aldrich, except for HOAt (test sample from Millipore). EDC was purchased from JBL Scientific. HPLC analyses were carried out using a Zorbax C18 column (4.6 × 250 mm) or an Inertsil ODS-2 column (4.6 × 250 mm) on a Hewlett-Packard 1050 system under the conditions (methanol/water as the eluting solvent, λ = 220 nm) similar to those described by Miyazawa *et al.*[18] The retention times of the diastereomeric peptides were determined by comparing them with those of the racemic amino acid-coupled products.

General Procedure for Two-Phase Peptide-Coupling Reactions. Preparative Experiment. To a solution of the amino acid benzyl ester (AA_2, 4 mmol) in 40 mL of dichloromethane (or *i*-PrOAc)[19] were added sequentially water (40 mL), the acetyl amino acid (AA_1, 4 mmol), and the additive. The mixture was then cooled in an ice bath to 0–5 °C, and EDC (4.4 mmol) was added. The resulting mixture was then stirred for 24–40 h at 0–5 °C. Aqueous hydrochloric acid (2 M, 10 mL) was added, and the layers were partitioned. The organic phase was further washed sequentially with aqueous hydrochloric acid (0.5 M, 20 mL), brine (20 mL), aqueous sodium bicarbonate (1 M, 2 × 20 mL), and brine (2 × 20 mL). It was then dried over sodium sulfate, filtered, and concentrated to dryness.

Analytical Experiment. The reaction was carried out in 0.2 mmol scale in 4 mL of 1:1 dichloromethane/water (or *i*-PrOAc/water). At the end of the reaction, the mixture was diluted with 60:40 MeOH/H_2O (v/v) to 50 mL and assayed by HPLC.

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(9) The loss in the workup of DMF solution ranged from 5–10% of the total amount of the dipeptide if DMF was not removed completely prior to extraction.

(10) Fujino, M.; Kobayashi, S.; Obayashi, M.; Tsunehiko, F.; Shinagawa, S.; Nishimuar, O. *Chem. Pharm. Bull.* **1974**, *22*, 1857.

(11) (a) Weygand, F.; Hoffmann, D.; Wünsch, E. *Z. Naturforsch., B* **1966**, *21*, 426. (b) Wünsch, E.; Drees, F. *Chem. Ber.* **1966**, *99*, 110.

(12) HOBt or HOAt (1 equiv) was not completely soluble in the *i*-PrOAc/water mixture, and a suspension was observed throughout the course of the reaction.

(13) For racemization of activated valine, see refs 1a, p 172, and 1d, p 361.

(14) Carpino, L. A. *J. Org. Chem.* **1988**, *53*, 875.

(15) HOPO is not very soluble in dichloromethane or *i*-PrOAc, and HOBt is not completely soluble in *i*-PrOAc; therefore, a solid remained during the course of the coupling reaction.

(16) The reacting mixture containing the acid, amine, additive HOPO, and EDC is not homogeneous in dichloromethane (ref 15) or *i*-PrOAc; reactions in water resulted in slow rates and low yields of the dipeptides (oily suspension in water).

(17) Ranganathan, D.; Singh, G. P.; Ranganathan, S. *J. Am. Chem. Soc.* **1989**, *111*, 1144.

(18) Miyazawa, T.; Otomatsu, T.; Fukui, Y.; Yamada, T.; Kuwata, S. *Int. J. Pept. Protein Res.* **1992**, *39*, 237.

(19) Prepared by neutralization of the tosylate with 1 M sodium carbonate (40 mL) and washed with saturated brine (2 × 30 mL).