Journal of Peptide Science
J. Peptide Sci. 2006; **12**: 147–153
Published online 13 June 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.689

JOURNAL OF PEPTIDE SCIENCE

Delay of coupling caused by excess additives

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Received 7 March 2005; Accepted 21 April 2005

Abstract: Use of excess quantity of additives such as 1-hydroxybenzotriazole, 1-hydroxy-7-azabenzotriazole, or 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine could retard carbodiimide-mediated peptide coupling or aminolysis of the active esters in organic solvents generally employed in peptide chemistry. Solubility test and infrared spectroscopic data suggested that the amine components interacted with the additives to form some aggregates, which were less reactive toward acylation. The use of 0.1 equimolar additives against carboxyl components or carbodiimides was advantageous for rapid and efficient coupling reaction, especially when the carboxyl components were amino acids carrying urethane type *N*-protecting groups.

Keywords: peptide synthesis; coupling rate; carbodiimides; HOAt; HOBt; HOOBt

INTRODUCTION

In carbodiimide-additive procedures for peptide synthesis, the amount of additives employed has been almost exclusively 1 equimolar against the carboxyl components or carbodiimides. To our knowledge, studies concerning variation of the amount are apparently quite limited [1,2]. Recently, however, the author found that the amount considerably affects the yields of peptides prepared by the procedures in some unusual solvents such as aqueous or alcoholic ones [3,4]; the use of around 0.1 equimolar 1-hydroxybenzotriazole (HOBt) [5], 1-hydroxy-7-azabenzotriazole (HOAt) [6,7], or 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (HOOBt) [8,9] suppressed side reactions more effectively than 1 equimolar ones, affording better yields of the desired peptides. In this paper, the author wishes to report that the use of such abundant additives could slow down the coupling reactions also in commonly used organic solvents, interfering in the aminolysis of the intermediary active esters.

EXPERIMENTAL

Materials and Methods

All reactions were carried out at ice-bath temperature.

Carbodiimide-additive Procedure

Into a mixture of a carboxyl component, an amine component (1 mmol each) and an appropriate amount of an additive in a solvent (5 ml, or 4 ml when dicyclohexylcarbodiimide (DCC) was used), was added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) (1 mmol), or 1 mol/l DCC in CH_2Cl_2 (1 ml). The mixture was stirred for 24 h. When a hydrochloride or a tosylate was used as the amine component, triethylamine (1 mmol) was added prior to the addition of the carbodiimide.

Aminolysis of Active Ester

Into a mixture of a Boc-amino acid and an additive (1 mmol each) in $CHCl_3$ (1 ml), was added EDC·HCl (1 mmol) and the mixture was stirred for 30 min. A chilled solution of an amine component (1 mmol) in a solvent (4 ml) with or without an additive (1 mmol) was added to the mixture, and the mixture was stirred for 24 h.

Monitor of Reaction

From the reaction mixture, samples of a known volume were taken out at appropriate intervals and were diluted immediately with MeOH/acetic acid (1 : 1) to a known volume. The samples were analyzed quantitatively on a high-performance liquid chromatography (HPLC) apparatus (a TSK-gel ODS 80 Ts column, 4.6 mm × 15 cm; MeOH/1%$NaClO_4$ aq, 3.5 : 1, 2 : 1, or 1.7 : 1; flow rate 0.5 ml/min; detected at 220 nm). The yields of compounds formed during the reaction were determined by comparing retention times and area of peaks on HPLC profiles of the samples and of the standard compounds.

Racemization Test

Into a mixture of a carboxyl component, an amine component hydrochloride, *N*-methylmorpholine (1 mmol each) and an appropriate amount of an additive in a solvent (3.5 ml), was added EDC·HCl (1 mmol) and the mixture was stirred for 18 h. After addition of acetic acid (5 ml), the mixture was diluted to a known volume with MeOH and was quantitatively analyzed on the HPLC.

Standard Compounds

Compounds [10–20] listed in Table 1 were synthesized by the usual methods and were used as the standards for the HPLC

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Table 1 Properties of synthetic standard compounds

Compound	Melting point (°C)[a]	HPLC retention time (min)	Formula	Mass spectra (M^+)[b] (calculated)
Boc-Ile-Ile-OBzl	88–88.5[c]	29.1[d]	$C_{24}H_{38}O_5N_2$	434.2776 (434.2781)
Boc-Ile-Gly-OBzl	97–98[e]	12.6[d]	$C_{20}H_{30}O_5N_2$	379.2235[f] (379.2233)
Boc-Gly-Ile-OBzl	oil[g]	15.3[d]	$C_{20}H_{30}O_5N_2$	378.2162 (378.2155)
Boc-Gly-Gly-OBzl	84.5–85[h]	16.1[i]	$C_{16}H_{22}O_5N_2$	323.1603[f] (323.1607)
Boc-Ile-Pro-OBzl	oil[j]	19.1[d]	$C_{23}H_{34}O_5N_2$	418.2466 (418.2468)
Boc-Val-Leu-OBzl	89—90[k]	25.8[d]	$C_{23}H_{36}O_5N_2$	420.2617 (420.2624)
Boc-Val-Pro-OBzl	oil[l]	14.8[d]	$C_{22}H_{32}O_5N_2$	404.2306 (404.2311)
Boc-Val-N⟨⬡⟩	53–56	25.6[m]	$C_{15}H_{28}O_3N_2$	284.2091 (284.2100)
Boc-Val-NH-⟨⬡⟩	201–203	29.0[m]	$C_{16}H_{30}O_3N_2$	298.2248 (298.2256)
Boc-Phe-Val-OMe	118–120[n]	28.0[m]	$C_{20}H_{30}O_5N_2$	378.2159 (378.2155)
Boc-D-Phe-Val-OMe	108–110	30.1[m]	$C_{20}H_{30}O_5N_2$	378.2162 (378.2155)
Z-Val-Val-OMe	104–109[o]	19.9[m]	$C_{19}H_{28}O_5N_2$	364.2005 (364.1998)
Z-D-Val-Val-OMe	158—159[p]	21.3[m]	$C_{19}H_{28}O_5N_2$	364.1997 (364.1998)

[a] Melting points are uncorrected. Satisfactory microanalysis data were obtained within an error range of 0.3% for solid compounds.
[b] Data of electron impact mass spectroscopy.
[c] [10] m.p. 89.5–91.5 °C.
[d] MeOH/1% $NaClO_4$, 3.5 : 1.
[e] [11] m.p. 99–100 °C.
[f] Data of fast atom bombardment mass spectroscopy; $(M + H)^+$.
[g] [12] oil.
[h] [13] m.p. 77–79 °C; [14] m.p. 83–84.5 °C.
[i] MeOH/1% $NaClO_4$, 1.7 : 1.
[j] [15] oil.
[k] [16] m.p. 74–78 °C; [17] m.p. 90–90.6 °C.
[l] [18] oil.
[m] MeOH/1% $NaClO_4$, 2 : 1.
[n] [19] m.p. 117.5–118 °C.
[o] [20] m.p. 100–103 °C.
[p] [20] m.p. 163–163.5 °C.

analysis. Microanalyses and measurement of mass spectra were carried out by the staff of the Analytical Laboratory of the University. The properties of Z-Gly-Phe-Val-OMe [21] and Z-Gly-D-Phe-Val-OMe [21] were reported previously [4].

Infrared Spectroscopy

A part (1.50 ml) of a stock solution (0.4 mol/l butylamine or piperidine in $CHCl_3$) was diluted with $CHCl_3$ to 3.00 ml, and an infrared spectrum of the solution (spectrum a) was recorded on a Jasco FT-IR 480 plus spectrometer at resolution of 4 cm^{-1} using a 0.1 mm KBr cell. Into other parts of the stock solution (1.50 ml each), HOBt or Boc-Val-OH (0.3, 0.6, 0.9, 1.2, and 1.5 mmol) was dissolved, and each solution was diluted to 3.00 ml with $CHCl_3$ to record spectra b, c, d, e, and f, respectively. The differential spectra b-a, c-b, d-c, e-d, and f-e are shown in Figures 1 and 2 as spectra A1 or A2, B1 or B2, C1 or C2, D1 or D2, and E1 or E2, respectively (not shown in the cases of Boc-Val-OH). A spectrum of HOBt saturated in $CHCl_3$ was obtained using a 1 mm KBr cell.

RESULTS AND DISCUSSION

Investigations on the rates of various coupling reactions employing carbodiimides and 0.1 or 1 (or 2) equimolar additives (Table 2) showed that the less abundant use of additives is generally advantageous for rapid completion of the reaction, regardless of the amino acids, carbodiimides, or solvents employed. According to the commonly accepted recognition [22,23], the carbodiimide-*N*-hydroxy compound-mediated coupling proceeds predominantly via two steps: formation of an active ester to consume the *N*-hydroxy compound followed by aminolysis of the ester to liberate the additive. The rates of aminolysis of preformed HOBt- or HOAt-active esters with primary or secondary amines decreased with the presence of additional 1 equimolar additives (Table 3), indicating that the use of abundant additives retarded at least the latter step of the carbodiimide-mediated coupling. Addition of a weak acid, Boc-Val-OH (p*K*a in MeOH/water 2 : 1; 5.4), reduced the rate moderately (Table 3; #5, 8, 20, 23,

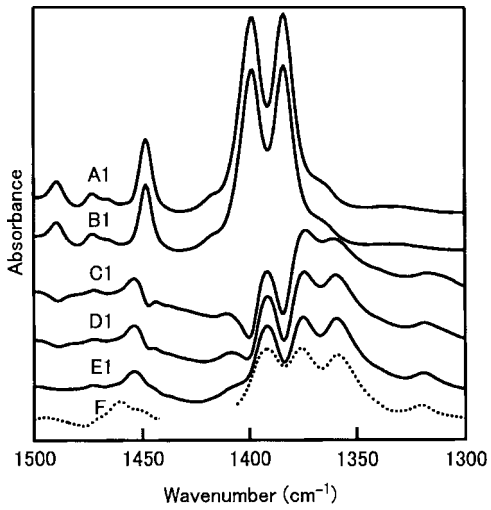

Figure 1 IR spectra of HOBt in $CHCl_3$ containing various amount of butylamine. Spectrum A1 = spectrum b−spectrum a, B1 = c−b, C1 = d−c, D1 = e−d, E1 = f−e, where spectra a, b, c, d, e, and f are those of 0.2 mol/l butylamine solutions in $CHCl_3$ containing 0, 0.5, 1, 1.5, 2, and 2.5 equimolar HOBt, respectively. A spectrum of HOBt saturated in $CHCl_3$ is shown as F (Optical density is uncorrected. The region around $1405 \sim 1445$ cm^{-1} was hindered by the absorbance of the solvent).

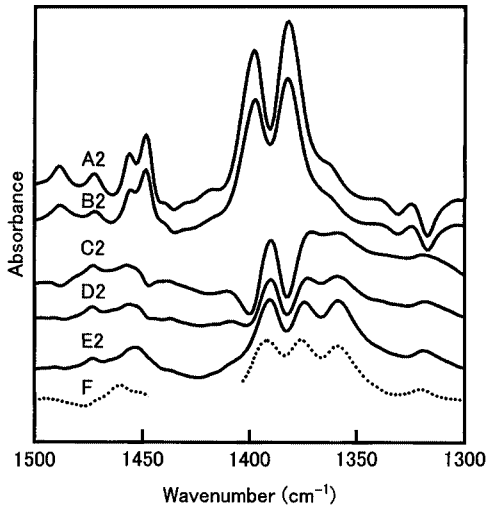

Figure 2 IR spectra of HOBt in $CHCl_3$ containing various amount of piperidine. Spectrum A2 = spectrum b−spectrum a, B2 = c−b, C2 = d−c, D2 = e−d, E2 = f−e, where spectra a, b, c, d, e, and f are those of 0.2 mol/l piperidine solutions in $CHCl_3$ containing 0, 0.5, 1, 1.5, 2, and 2.5 equimolar HOBt, respectively. A spectrum of HOBt saturated in $CHCl_3$ is shown as F (Optical density is uncorrected. The region around $1405 \sim 1445$ cm^{-1} was hindered by the absorbance of the solvent).

26, and 29) and, as described by Beyermann *et al.* [24], addition of triethylamine enhanced the reaction. These facts suggest that salt-formation between the amine components and the acidic additives (p*K*a in MeOH/water 2 : 1; 4.9 for HOBt, 4.4 for HOAt, and 4.8 for HOOBt) caused the retardation, reducing electron density on the nitrogen atoms of the amines. However, in spite of their similar p*K*a values, the extent of hindrance caused by abundant HOBt and HOOBt was rather different (Table 2; #2 *vs* #6, #13 *vs* #16, #32 *vs* #38, and #33 *vs* 39. Table 3; #17 *vs* #19), indicating that the salt-formation is not the sole cause of the retardation.

Table 2 Time course of formation of dipeptide in coupling using different amount of additive[a,b]

Entry (#)	Coupling	Solvent	Additive[c]	Yield of dipeptide (%)						
				10 min	20 min	40 min	1 h 20 min	2 h 40 min	6 h	24 h
1	Boc-Ile-OH + H-Ile-OBzl	$CHCl_3$	0.1 eq HOBt	58	74	87	95	96	100	—
2			1 eq HOBt	47	62	72	78	85	89	95
3			0.1 eq HOAt	72	87	97	98	99	101	—
4			1 eq HOAt	71	78	84	90	93	97	98
5			0.1 eq HOOBt	65	83	93	99	102	—	—
6			1 eq HOOBt	45	58	66	72	76	81	87
7		DMF	0.1 eq HOBt	29	45	64	79	92	96	99
8			1 eq HOBt	35	53	69	81	87	91	96
9			0.1 eq HOAt	30	46	64	80	91	98	98
10			1 eq HOAt	36	53	69	80	88	93	94
11[d]		CH_2Cl_2	0.1 eq HOBt	—	—	—	93	—	—	100
12[d]			1 eq HOBt	—	—	—	85	—	—	97
13[d]			2 eq HOBt	—	—	—	76	—	—	95
14[d]			0.1 eq HOOBt	—	—	—	96	—	—	100
15[d]			1 eq HOOBt	—	—	—	81	—	—	94

(*continued overleaf*)

Table 2 (*Continued*)

Entry (#)	Coupling	Solvent	Additive[c]	Yield of dipeptide (%)						
				10 min	20 min	40 min	1 h 20 min	2 h 40 min	6 h	24 h
16[d]			2 eq HOOBt	—	—	—	57	—	—	95
17	Boc-Ile-OH + H-Gly-OBzl	$CHCl_3$	0.1 eq HOBt	54	73	87	94	98	100	—
18			1 eq HOBt	49	62	71	78	82	88	92
19	Boc-Gly-OH + H-Ile-OBzl	$CHCl_3$	0.1 eq HOBt	71	84	93	96	98	99	100
20			1 eq HOBt	63	70	75	79	84	87	91
21	Boc-Gly-OH + H-Gly-OBzl	$CHCl_3$	0.1 eq HOBt	66	83	92	97	99	100	—
22			1 eq HOBt	64	71	76	80	85	89	91
23	Boc-Ile-OH + H-Pro-OBzl	$CHCl_3$	0.1 eq HOBt	25	40	58	75	87	95	97
24			1 eq HOBt	14	29	46	60	68	76	83
25			0.1 eq HOAt	43	62	82	92	97	99	99
26			1 eq HOAt	43	57	66	72	77	81	87
27		DMF	0.1 eq HOBt	12	24	42	67	85	95	100
28			1 eq HOBt	20	37	56	71	80	86	97
29			0.1 eq HOAt	14	24	43	68	87	96	100
30			1 eq HOAt	26	42	60	74	82	87	90
31[d]		CH_2Cl_2	0.1 eq HOBt	—	—	73	—	—	—	100
32[d]			1 eq HOBt	—	—	66	—	—	—	91
33[d]			2 eq HOBt	—	—	44	—	—	—	79
34[d]			0.1 eq HOAt	—	—	90	—	—	—	98
35[d]			1 eq HOAt	—	—	75	—	—	—	95
36[d]			2 eq HOAt	—	—	53	—	—	—	95
37[d]			0.1 eq HOOBt	—	—	68	—	—	—	99
38[d]			1 eq HOOBt	—	—	50	—	—	—	74
39[d]			2 eq HOOBt	—	—	16	—	—	—	46
40	Boc-Val-OH + H-Leu-OBzl	CH_3CN	0.1 eq HOBt	—	—	88	—	—	—	100
41			1 eq HOBt	—	—	74	—	—	—	92
42			2 eq HOBt	—	—	65	—	—	—	86
43		*N*-Methyl-	0.1 eq HOBt	—	—	66	—	—	—	98
44		pyrrolidone	1 eq HOBt	—	—	67	—	—	—	92
45			2 eq HOBt	—	—	57	—	—	—	89
46		AcOEt	0.1 eq HOBt	—	—	53	—	—	—	99
47			1 eq HOBt	—	—	70	—	—	—	88
48			2 eq HOBt	—	—	57	—	—	—	83

[a] Each reaction was duplicated. Data are of the mean values.
[b] EDC.HCl was used as the carbodiimide.
[c] Equimolar against other reagents.
[d] DCC was used as the carbodiimide.

The *N*-hydroxy compounds were sparingly soluble in less polar solvents (in $CHCl_3$, less than 5×10^{-3} mol/l for HOBt or HOAt, and less than 2.5×10^{-3} mol/l for HOOBt), while the presence of amines greatly improved the solubility (in 0.2 mol/l butylamine in $CHCl_3$, about 0.5 mol/l for HOBt, about 0.4 mol/l for HOAt, and about 0.3 mol/l for HOOBt; in 0.2 mol/l piperidine, about 0.6 mol/l for HOBt, about 0.4 mol/l for HOAt, and about 0.3 mol/l HOOBt; in 0.2 mol/l triethylamine, about 0.7 mol/l for HOBt, about 0.4 mol/l for HOAt, and about 0.3 mol/l HOOBt), suggesting occurrence of some nonstoichiometric interactions between the additives and the amines. Alteration of the infrared spectra found in a fingerprint region ($1500 \sim 1300\ cm^{-1}$) of HOBt, which was added in 0.5 equimolar portions accumulatively onto amines, also suggested the occurrence of such interactions. Spectrum A1 (Figure 1), which was obtained using a differential spectroscopic methodology [25], shows the profile of the first 0.5 equimolar portion of HOBt added onto a $CHCl_3$ solution of butylamine,

Table 3 Time course of dipeptide formation in aminolysis of active ester[a]

Entry (#)	Aminolysis	Solvent	Additive[b]	Yield of dipeptide (%)						
				10 min	20 min	40 min	1 h 20 min	2 h 40 min	6 h	24 h
1	Boc-Val-OBt + H-Leu-OBzl	$CHCl_3$	None	74	78	82	85	88	91	96
2			1 eq HOBt	51	58	64	69	76	81	88
3			1 eq HOAt	58	64	70	76	81	86	92
4			1 eq HOOBt	49	57	64	70	75	81	89
5			1 eq Boc-Val-OH	55	62	68	73	79	83	90
6	Boc-Val-OAt + H-Leu-OBzl	DMF	None	82	84	86	88	89	91	92
7			1 eq HOAt	67	72	74	76	80	83	85
8			1 eq Boc-Val-OH	78	81	84	86	88	89	90
9	Boc-Gly-OBt + H-Ile-OBzl	$CHCl_3$	None	89	90	92	94	94	96	97
10			1 eq HOBt	77	80	84	87	90	91	93
11	Boc-Val-OBt + H-Pro-OBzl	$CHCl_3$	None	72	76	80	83	86	92	96
12			1 eq HOBt	34	43	50	57	62	71	81
13			1 eq HOAt	41	46	53	60	66	75	84
14	Boc-Val-OAt + H-Pro-OBzl	DMF	None	89	91	93	95	97	100	—
15			1 eq HOAt	69	75	78	81	87	90	90
16	Boc-Val-OBt + cyclohexylamine	$CHCl_3$	None	67	69	72	75	78	85	89
17			1 eq HOBt	33	39	43	49	55	63	72
18			1 eq HOAt	36	40	46	51	58	65	78
19			1 eq HOOBt	31	34	41	44	50	62	64
20			1 eq Boc-Val-OH	41	46	52	59	65	71	81
21		DMF	None	71	74	76	79	83	87	94
22			1 eq HOBt	49	52	57	62	68	78	84
23			1 eq Boc-Val-OH	54	61	67	70	73	76	82
24	Boc-Val-OAt + piperidine	$CHCl_3$	None	69	71	75	77	84	87	92
25			1 eq HOAt	35	40	46	53	64	79	93
26			1 eq Boc-Val-OH	52	59	63	70	72	76	91
27		DMF	None	83	84	87	90	94	96	99
28			1 eq HOAt	64	69	74	79	86	92	98
29			1 eq Boc-Val-OH	81	86	89	93	97	99	102

[a] Each reaction was duplicated. Data are of the mean values.
[b] Equimolar against other reagents.

spectrum B1 corresponds to that of the secondary added 0.5 equimolar portion (total amount of HOBt in the solution was 1 equimolar), C1 corresponds to the third portion (total 1.5 equimolar), and so on (see also *Experimental*). Similarly, spectra A2, B2, C2, D2, and E2 in Figure 2 correspond to each profile of 0.5 equimolar portions of HOBt added onto the solution of piperidine. In the two series, the spectra under corresponding conditions fairly resembled each other, in spite of the usage of different amines; the bands of the amines were well canceled in the region. However, spectra A1 and B1 or A2 and B2 were quite different from other spectra, indicating that the mechanism of the interaction concerning HOBt of up to 1 equimolar against the amines differed from that concerning HOBt excessively added. Spectra C1, D1, and E1, or C2, D2, and E2 were virtually similar to spectrum F, a profile of a highly diluted HOBt in $CHCl_3$, except the shapes around 1460 $\sim$ 1450 cm^{-1} and near 1350 cm^{-1}. It is noteworthy that the widths of peaks at around 1360 cm^{-1} and 1320 cm^{-1} slightly decreased as C1, C2 > D1, D2 > E1, E2 $\cong$ F; the intermolecular interactions involving HOBt successively added onto the amines decreased gradually [26]. Such change of peak widths was not observed

in the corresponding experiments employing Boc-Val-OH and butylamine or piperidine. These data suggest the occurrence of two kinds of interactions: stoichiometric interactions perhaps due to salt-formation between the amines and HOBt of up to 1 equimolar (reflected in spectra A and B), and the following nonstoichiometric interactions caused by the excess HOBt (reflected in spectra C, D, and E) perhaps surrounding the salt.

In conclusion, the observations shown in Table 2 can be explained as follows: low concentration of an additive caused slow formation of an active ester, particularly when the reaction was carried out in a polar solvent such as *N,N*-dimethylformamide (DMF), in which the formation is very slow [24]. In the earlier stage of peptide coupling, concentration of an amine component was high and hence aminolysis of the ester should be fast; the formation of the ester was the rate-determining step of the coupling. This could be the reason that the use of 0.1 equimolar additives in polar solvents leads to the comparatively slow coupling rate at beginning of the reaction (Table 2; #7 *vs* #8, #9 *vs* #10, #27 *vs* #28, #29 *vs* #30). As the coupling proceeded, concentration of the amine decreased, while that of the free additive remained almost unchanged because of its liberation from the ester. In the latter stage of the coupling, concentration of the free additive became much higher than those of the remaining reactants, especially when an abundant additive was employed in the coupling. Under such conditions, the remaining amines would interact quite easily with the additive to form a salt and some 'aggregates', both of which would reduce the susceptibility toward attack of the active esters to cause the delayed completion of the coupling.

Table 4 shows the extent of racemization during a model fragment condensation between Z-Gly-Phe-OH and H-Val-OMe [21] with the aid of EDC·HCl and 0.1 or 1 equimolar additives in $CHCl_3$ or DMF. In both solvents, reduction of the amount of additives to one-tenth caused slight increase of racemization, as was observed in aqueous or alcoholic solvents [3,4]. In similar tests using Boc-Phe-OH and H-Val-OMe, or Z-Val-OH and H-Val-OMe in the solvents, the use of 0.1 equimolar HOBt, HOAt, or HOOBt caused no detectable racemization (less than 0.05%), indicating introduction of a single amino acid carrying such protective groups was safe from racemization under the conditions.

Table 4 Effect of amount of additive on racemization in coupling between Z-Gly-Phe-OH and H-Val-OMe

Solvent	Additive[a]	Chemical yield (%)[b]	Extent of racemization (%)[c]
$CHCl_3$	0.1 eq HOBt	98	1.64
	1 eq HOBt	94	0.31
	0.1 eq HOAt	98	0.49
	1 eq HOAt	97	0.22
	0.1 eq HOOBt	95	1.80
	1 eq HOOBt	88	0.24
DMF	0.1 eq HOBt	100	1.61
	1 eq HOBt	98	0.87
	0.1 eq HOAt	98	0.67
	1 eq HOAt	98	0.44
	0.1 eq HOOBt	99	0.56
	1 eq HOOBt	93	<0.1

[a] Equimolar against other reagents.
[b] Z-Gly-D-Phe-Val-OMe + Z-Gly-Phe-Val-OMe.
[c] Z-Gly-D-Phe-Val-OMe/(Z-Gly-D-Phe-Val-OMe + Z-Gly-Phe-Val-OMe).

CONCLUSION

Use of excess additives rather slowed down the reaction in carbodiimide-mediated peptide coupling in organic solvents. The amine components seemed to form some aggregates with the excess additives via two types of interactions, stoichiometric salt-formation and the following nonstoichiometric association, reducing the reactivity toward acylation. Use of 0.1 equimolar additives against the carbodiimides or the carboxyl components was generally advantageous for rapid and efficient reaction in comparison with the traditional use of 1 equimolar additives, although slight increase of racemization was observed during optically sensitive fragment condensation.

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