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The Diisopropylcarbodiimide/ 1-Hydroxy-7-azabenzotriazole System: Segment Coupling and Stepwise Peptide Assembly†

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ABSTRACT

For a group of model peptide segments, coupling reactions carried out via solution or solid phase techniques have demonstrated the advantages of the system DIC/HOAt over DIC/HOBt and in addition for systems involving other selected carbodiimides and substituted HOBt derivatives bearing electron-withdrawing substituents. Very little, if any, loss of configuration occurred in DCM regardless of the additive used, although the relative order of efficiency was similar in solvents such as DMF in which more extensive epimerization resulted. In application of DIC/HOAt to stepwise peptide assembly by solid phase techniques, it was found that the hindered pyridine base collidine enhanced the step involving preactivation of the carboxylic acid residue in contrast to the normal situation in which bases such as DIEA, NMM, or non-hindered pyridine bases inhibit this step. These results led to development of a stepwise procedure for peptide assembly in which collidine is added to enhance activation and subsequently DIEA is added to enhance coupling.

Keywords: Carbodiimides; peptide synthesis.

† **Abbreviations used**: ACP = acyl carrier protein decapeptide (65-74); Aib = α-aminoisobutyric acid; *t*-BuTMG = *t*-butyltetramethylguanidine; DB(DMAP) = 2,6-di-*t*-butyl-4-(N,N-dimethylamino)pyridine; DCM = dichloromethane; DIC = diisopropylcarbodiimide; DIEA = diisopropylethylamine; DMAP = 4-(dimethylamino)pyridine; DMF = dimethylformamide; EDC = 1-ethyl-3-[3-(dimethylaminopropyl]carbodiimide; EDC•MH = EDC methohexafluorophosphate; EDC•MI = EDC methiodide; HAPyU = 1-(1-pyrrolidinyl-1*H*-1,2,3-triazolo[4,5-b]pyridin-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate N-oxide; HATU = N-[(dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridin-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate N-oxide; HOAt = 7-aza-1-hydroxybenzotriazole; HOBt = N-hydroxybenzotriazole; HODhbt = 3-hydroxy-3,4-dihydro-4-oxo-1,2,3-benzotriazine; $HONO_2Bt$ = 6-nitro-1-hydroxybenzotriazole; NMI = N-methylimidazole; NMM = N-methylmorpholine; NMP = N-methylpyrrolidone; OHA = 1,2,3,4,5,6,7,8-octahydroacridine; PAL = 5-(4-aminomethyl)-3,5-dimethoxyphenoxy)valeric acid; PEG-PS = polyethylene glycol, polystyrene resin support for solid phase synthesis; PA = preactivation; PEC = phenyl ethyl carbodiimide; PIC = phenyl isopropyl carbodiimide; TCM = trichloromethane = chloroform; TFA = trifluoroacetic acid; TFE = trifluoroethanol; TMP = collidine = 2,4,6-collidine = 2,4,6-trimethylpyridine; Z = benzyloxycarbonyl; $HOCF_3Bt$ = 6-trifluoromethyl-1-hydroxybenzotriazole.

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Following the discovery of the greater effectiveness of HOAt over HOBt as an additive during peptide segment coupling in reducing the extent of configurational loss at the reactive carboxylic acid residue, extensive studies have confirmed the effect for guanidinium salt reagents (e.g., HATU, HAPyU, HBTU, HBPyU) derived from these two species.[1] In the case of carbodiimide-induced coupling reactions the only extensive series of tests involved EDC and EDC•HCl, systems which involve reagents which incorporate a tertiary amino function or the corresponding protonated species.[1e] In the few cases where EDC and EDC•HCl were compared to a neutral carbodiimide such as DCC, the latter proved to be somewhat more effective. To have a more general picture of the expectations for a neutral carbodiimide a more extensive series of tests was undertaken. The present paper details such a comparison with DIC being substituted for DCC in view of the increasing use of the former reagent due to the greater solubility of the by-product urea.[2] The effectiveness of the HOAt additive was confirmed.

While the study was underway, Quibell, Packman and Johnson reported somewhat contrasting results in the case of the [2+1] solid phase coupling leading to tripeptide **1**.[3] Since

H-Asp(O-*t*-Bu)-Phe-Lys(BOC)-OH

1

as noted by Quibell, et al., this sequence appears to be particularly prone to undergo epimerization, it has been adopted as a test model in our studies. In all systems examined, regardless of the conditions, solvents, etc., the use of HOAt or onium reagents derived from HOAt led to less loss of configuration than analogous HOBt systems. In the non-polar solvent DCM, epimerization is minimal in both cases, as has often been observed.[4]

Work in our laboratory has always concentrated on the more polar solvent DMF since in this solvent the challenge to avoid epimerization is greater and differences among various coupling reagents and additives are magnified. The results are collected in Tables 1–6.

Z–Phe–Val–Pro–NH_2
2

Z–Gly–Gly–Val–Ala–Gly–Gly–OMe
3

Z–Gly–Phe–Pro–NH_2
4

Z–Phe–Val–Ala–OMe
5

Z–Phe–Val–Pro–O-*t*-Bu
6

Fmoc–Phe–Ser(*t*-Bu)–Pro–NH_2
7

Table 1

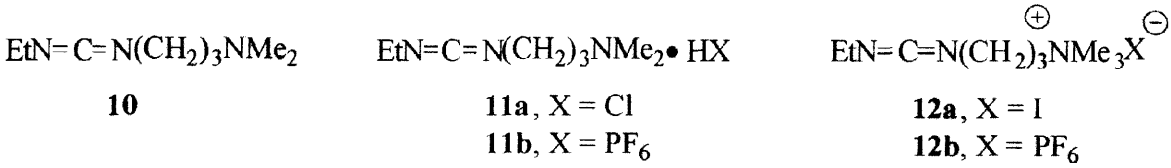
Effect of Identity of Coupling Reagent, Additive, Base, Preactivation Time, and Solvent on the Preservation of Configuration during Formation of Fmoc-Asp-Phe-Lys-NH_2 (QPJ sequence) via [2+1] Coupling under Solid Phase Conditions [a]

Coupling Reagent	Additive	Base	Solvent	Yield (%)	LDL- (%)
DIC	HOAt		DMF	90.0	23.1, 24.3
DIC	HOBt		DMF	89.0	30.2, 28.9
DIC [b]	HOAt		DMF	89.0	26.9 [b]
DIC [b]	HOBt		DMF	86.0	33.9 [b]
DIC	HOAt		DCM	85.0	0.1, 0.1
DIC	HOBt		DCM	86.0	0.9, 0.8
HBTU		TMP (2)	DMF	93.0	26.8
HBTU		DIEA (2)	DMF	95.0	52.9
HBTU [b]		TMP (2)	DMF	92.0	34.9 [b]
HATU		TMP (2)	DMF	95.0	20.3
HATU [b]		TMP (2)	DMF	93.0	25.3 [b]
HATU		DIEA (2)	DMF	93.0	51.5
HATU [b]		DIEA (2)	DMF	93.0	58.3 [b]
HATU	HOAt	DIEA (2)	DMF	94.0	36.7
HATU [b]	HOAt	DIEA (2)	DMF	93.0	42.7 [b]

[a] Coupling reactions were carried out by deblocking 50 mg of Fmoc-Lys(Boc)-PAL-PEG-PS resin by means of 20% piperidine/DMF for 7 min, washing the resin with DMF, DCM and DMF (3 x 5 mL each) and then adding a 5-fold excess (0.0475 mmol) of Fmoc-Asp(O-*t*-Bu)-Phe-OH (26.5 mg), a 5-fold excess of the coupling reagent and 11.5 mg (0.095 mmol) of TMP or 12.3 mg of DIEA (10-fold excess) of the base, if any. In each case the coupling reagent and the base were dissolved in 0.2 mL of the solvent and the resulting solution added to the resin in a small syringe which served as the reactor. Dissolution required about 1 min or less and care was taken to add the solution as soon as possible after everything dissolved. This method is referred to as the "low preactivation" method. Where preactivation was involved, the times are recorded. The mixture was stirred gently every 10 min with a teflon rod for approximately 1 h and then let stand for 12 h after which the resin was washed with DMF and DCM (3 x 5 mL each) and deblocked by treatment with 3 mL of TFA/H_2O (9:1) for 1 1/2 h at room temperature. The solvent was removed *in vacuo* and the residue dissolved in CH_3CN for direct injection onto an HPLC column under the following conditions: 4 μ, 60Å, C_{18} Waters Nova-pak column, 3.9 x 150 mm; flow rate 1 mL/min; Waters 996 PDA detector; linear gradient 10/30 in 20 min and then isocratic 30/70 for 20 min with CH_3CN/H_2O/0.1% TFA; R_t (LLL-) 28.5 min, (LDL-) 30.5 min. Other conditions, reagents, and instrumentation were as described in footnote *a* of Table 2, ref. 2b.

[b] In these cases the dipeptide acid, coupling reagent and base, if any, were allowed to react for 7 min prior to addition of the resulting solution to the resin.

In a few cases for the tripeptide reported by Quibell, et al., tests were run at different preactivation times. Considering that our previous studies have emphasized onium-style reagents, we chose to concentrate in the present study mainly on DIC-based systems although we took the opportunity to compare DIC with a number of other carbodiimides. A special group of carbodiimide reagents substituted with amino or quaternary ammonium functions **10**–**12** offers more ready removal of by-products for solution syntheses than DIC due to exceptional water solubility. The most common such system is EDC **10** or its hydrochloride **11a**. The related methiodide **12a**[5] has also been used.



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11a, X = Cl
11b, X = PF_6

12a, X = I
12b, X = PF_6

Comparisons between EDC and EDC•HCl on the one hand and DCC or DIC on the other in segment condensations often showed cleaner reactions in the latter two cases. This may be related to the presence in the former of an unhindered basic amino function or such a base in equilibrium with the corresponding hydrochloride. Such a negative effect of a base on loss of configuration would be particularly important for slow coupling processes as for example, for solid phase vs. solution systems. Thus for the solid phase coupling to give test peptide **4** the following results were observed: EDC/HOAt (29.8% LDL-); EDC•HCl/HOAt (24.1% LDL-); DIC/HOAt (4.2% LDL-). Capping the amino function as the methiodide (**12a**) was helpful, but less satisfactory than the non-ionic DIC case: 15.3% LDL-. However, it has been observed previously that halide ions may induce epimerization due to their basic character in non-aqueous solvents.[6] Still, the hexafluorophosphate salt **12b**, bearing a non-basic anion, was no better (17.5% LDL-) and the culprit may be the positively charged nature of the carbodiimide moiety of these various salts which could interfere in the preactivation step which requires protonation of the carbodiimide residue.[7] Solution phase coupling reactions to give **4** are significantly faster than the solid phase analogs and differences between the reactions of charged and uncharged species are significantly less: EDC/HOAt (4.7% LDL-); EDC•HCl/HOAt (4.1%);

EDC•MI/HOAt (4.7%); EDC•MH/HOAt (4.6%); DIC/HOAt (2.1%). For the analogous solid phase coupling leading to hexapeptide **5** the results were in rough agreement with expectations: EDC/HOAt (7.1% DL-); EDC•HCl/HOAt (3.6%); EDC•MI/HOAt (2.0%); EDC•MH/HOAt (1.7%); DIC/HOAt (1.0%). If in these runs HOAt is omitted the results are much poorer: EDC•HCl (26.3%); EDC•MI (33.0%); EDC•MH (28.8%); DIC (22.9%).

Following up on a report[8] that unsymmetrical alkyl aryl carbodiimides offered advantages over the dialkyl analogs in terms of loss of configuration at the carboxylic acid residue, a few such systems were compared to the DIC/HOAt case. Unfortunately, in no case did the ethyl phenyl or isopropyl phenyl carbodiimide systems lead to any improvement over the DIC system (Tables 2-5).

When a carbodiimide such as DCC is used in solid phase peptide assembly, the activation process is believed to involve conversion first to an O-acylurea by reaction with the carboxylic acid followed by reaction of the O-acylurea with a second equivalent of the acid to give the symmetric anhydride which is the active acylating species. Activation is rapid in a non-polar solvent such as DCM but slow in the polar solvent DMF, in which, however, the coupling step is more effective, especially for longer peptides.[9] These considerations led to the development of an early protocol for automated syntheses which involved activation in DCM, filtration of the precipitated DCU and replacement of the volatile solvent DCM by DMF. An alternative protocol involves carrying out the activation step in the presence of HOBt in the mixed solvent DCM-DMF (1:1). Under these conditions the highly reactive OBt[10] esters are generated as the key reaction intermediates.

Such OBt (or OAt in cases where HOAt is substituted for HOBt) esters are also the active intermediates in the case of the newer onium techniques for peptide coupling which involve reagents such as BOP reagent,[11] HBTU[12] and HATU,[1a] **13-15**, respectively. These reagents lead to highly efficient coupling partly because of rapid activation by a base such as DIEA or NMM followed by rapid coupling in the presence of the same base. Beyermann and coworkers

Table 2

Effect of Identity of Carbodiimide Reagent, Additive, Base, and Solvent on the Preservation of Configuration during Formation of Z–Phe–Val–Pro–NH_2 via [2+1] Coupling in Solution [a]

Carbodiimide Reagent	Additive	Base	Solvent	Yield (%)	LDL- (%)
DIC			DMF	73.8	49.4
DIC			NMP	70.1	51.7
DIC	HOAt		DMF	86.3	2.1
DIC	HOBt		DMF	78.8	8.6
DIC	HODhbt		DMF	83.1	5.8
DIC	$HOCF_3Bt$		DMF	85.7	7.8
DIC	$HONO_2Bt$		DMF	86.7	16.3
DIC	HOAt		DCM	91.8	0.5
PEC	HOAt		DMF	91.2	5.6
PIC	HOAt		DMF	88.9	9.6
PEC	HOAt		DCM	91.3	0.7
PIC	HOAt		DCM	90.9	1.1
EDC[b]	HOAt		DMF	84.8	4.7
EDC[b]	HOBt		DMF	86.7	18.9
EDC[b]	HODhbt		DMF	89.1	7.3
EDC•HCl	HOAt		DMF	81.2	4.1
EDC•HCl	HOAt	TMP(1)	DMF	88.9	5.3
EDC•HCl	HOBt		DMF	80.9	16.4
EDC•HCl	HOBt	TMP(1)	DMF	86.7	19.8
EDC•HCl	HODhbt		DMF	82.1	6.3
EDC•HCl	HODhbt	TMP(1)	DMF	89.1	7.6
EDC•HCl	$HOCF_3Bt$		DMF	85.7	13.7
EDC•HCl	$HONO_2Bt$		DMF	85.6	18.9
EDC•MI	HOAt		DMF	83.9	4.7[c]
EDC•MH	HOAt		DMF	84.6, 82.9	4.4, 4.8[c]
EDC•MI			DMF	61.3	51.7[c]
EDC•MH			DMF	53.9	51.9[c]

[a] All coupling reactions and HPLC analyses were carried out as described in footnote *a* and *b* of Table 2, ref. 2e.

[b] EDC free base was a fresh sample kindly provided by JBL Scientific, Inc., San Luis Obispo, CA 93401.

[c] In these cases extra peaks were observed in the HPLC traces, indicative of side product formation.

Table 3

Effect of Identity of Carbodiimide Reagent, Additive, Base, and Solvent on the Preservation of Configuration during Formation of Z–Phe–Val–Pro–NH_2 via [2+1] Coupling under Solid Phase Conditions [a]

Carbodiimide Reagent	Additive	Solvent	Yield (%)	LDL- (%)
DIC	HOAt	DMF	93.9	4.2
DIC	HOBt	DMF	94.1	10.6
DIC	HODhbt	DMF	93.2	7.1
DIC	$HOCF_3Bt$	DMF	93.1	12.5
DIC	$HONO_2Bt$	DMF	89.1	28.3
EDC•HCl	HOAt	DMF	89.8	24.1
EDC•HCl	HOBt	DMF	90.2	37.6
EDC•HCl	HODhbt	DMF	89.2	27.9
EDC•HCl	$HOCF_3Bt$	DMF	89.8	30.8
EDC•HCl	$HONO_2Bt$	DMF	88.9	41.5
EDC•MI		DMF	85.1	50.5
EDC•MH		DMF	83.7	50.0
EDC•MI	HOAt	DMF	90.1	15.3
EDC•MH	HOAt	DMF	91.3	17.5
PEC	HOAt	DMF	93.4	7.0
PIC	HOAt	DMF	94.1	9.0
PEC	HOAt	DCM	93.8	1.3
PIC	HOAt	DCM	94.1	2.3
DIC	HOAt	DMF-DCM (1:1)	95.6	1.5
DIC	HOAt	DCM	94.9	1.0

[a] All coupling reactions and HPLC analyses were carried out as described in footnote *a* of Table 1 except that 100 mg of Fmoc–Pro–(PAL–PEG–PS) (0.19 mmol/g) was deblocked via 20% piperidine for 10 min and the resin washed with DMF (3 x 5 mL), DCM (3 x 5 mL) and DMF (2 x 5 mL). For coupling 3 eqs of Z-Phe-Val-OH and coupling reagent and additive, if any, in 0.2 mL of DMF were added to the resin and coupling allowed to proceed for 30 min. After washing with DMF (3 x 5 mL) and DCM (2 x 5 mL) the resin was treated with 2 mL of TFA for 1 h, the mixture filtered and the TFA removed *in vacuo*. The crude sample was dissolved in 2 mL of acetonitrile and 10 μL of this solution injected onto the HPLC column.

Table 4

Effect of Identity of Carbodiimide Reagent, Additive, and Solvent on the Preservation of Configuration during Formation of Z–Gly-Gly-Val-Ala-Gly-Gly–NH_2 by [3+3] Coupling under Solid Phase Conditions [a]

Carbodiimide Reagent	Additive	Solvent	Yield (%)	DL- (%)
DIC	HOAt	DMF	98.9	1.0
DIC	HOBt	DMF	98.7	1.3
DIC	HODhbt	DMF	93.7	0.8
DIC	$HOCF_3Bt$	DMF	97.1	2.9
DIC	$HONO_2Bt$	DMF	55.8	26.7
PEC	HOAt	DMF	92.3	4.7
PIC	HOAt	DMF	93.0	7.1
PEC	HOAt	DCM	91.5	0.6
PIC	HOAt	DCM	93.5	2.3
DIC	HOAt	DCM	94.6	< 0.1
EDC•HCl	HOAt	DMF	96.3	3.6
EDC•HCl	HOBt	DMF	94.6	5.4
EDC•HCl	HODhbt	DMF	97.8	2.1
EDC•HCl	$HOCF_3Bt$	DMF	91.7	8.4
EDC•HCl	$HONO_2Bt$	DMF	63.3	36.7
EDC•MI		DMF	89.1	33.0
EDC•MH		DMF	80.1	28.8
EDC•MI	HOAt	MDF	92.3	2.0
EDC•MH	HOAt	DMF	90.1	1.7

[a] All coupling reactions were carried out under "low preactivation" conditions as noted in footnotes a and b of Table 1. Fmoc–(PAL–PEG–PS) (0.19 mmol/g) was converted in the normal manner to Fmoc–Ala-Gly-Gly–(PAL–PEG–PS) via stepwise coupling with the appropriate isolated Fmoc-substituted acid fluorides (5-fold excess, DIEA, 30-min). It was demonstrated that no loss of configuration had occurred at the Ala residue by extending stepwise assembly to the hexapeptide and demonstrating that, on cleavage from the resin only the LL-hexapeptide was formed. The tripeptide resin was deblocked with piperidine and test couplings made via Z-Gly-Gly-Val-OH in the presence of various coupling reagents. Upon release of Z-Gly-Gly-Val-Ala-Gly-Gly-NH_2 from the resin via TFA treatment for 1 h, the crude product was dissolved in CH_3CN/H_2O (0.05/0.95) for injection onto the HPLC column for which the conditions of separation involved a linear gradient of 5/15 in 10 min followed by isocratic 15/85 for 20 min (CH_3CN/H_2O/0.1% TFA). The remainder of the procedure was the same, t_R (LL-) 18.4 min, (DL-) 21.3; MS(MALDI): 535.8 (calc M+1 536); 558.4 (calc M+Na 559); 574.6 (calc M+K 575). See footnotes *a* and *b* of Table 5, ref. 2b.

Table 5

Effect of Identity of Carbodiimide Reagent, Additive, Base, and Solvent on the Preservation of Configuration during Formation of Z–Gly–Phe–Pro–NH_2 via [2+1] Coupling in Solution [a]

Carbodiimide Reagent	Additive	Base	Solvent	Yield (%)	DL- (%)
DIC			DMF	58.9	5.5
DIC	HOAt		DMF	89.4	0.3
DIC	HOBt		DMF	88.6	1.7
PEC	HOAt		DMF	89.9	0.4
PIC	HOAt		DMF	90.3	1.9
EDC•MI	HOAt		DMF	85.6	1.2
EDC•MH	HOAt		DMF	84.3	0.6
EDC•MI			DMF	80.1	12.3
EDC•MH			DMF	59.8	10.9
DIC	HOAt		DCM	90.3	< 0.1
DIC	HOAt	TMP	DCM	94.8	< 0.1
DIC	HOAt	TMP	DMF	93.1	0.5

[a] All coupling reactions were carried out as described in footnote 14 of ref. 2e.

showed that carbodiimide/HOBt coupling could be made equally efficient by addition of a strong base such as DIEA to the coupling step.[13,14] In this case the activation and coupling steps must be separated since DIEA greatly inhibits the carbodiimide activation process. This

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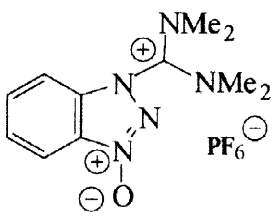

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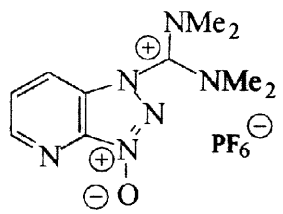

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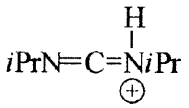
follows from the nature of the activation process which involves protonation by the carboxylic acid to give **16** which is the ultimate source of the symmetric anhydride or active ester depending on which particular protocol is chosen for coupling via the carbodiimide.



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We speculated that since the presence of both the free acid and its anion are important in determining the overall rate of conversion of the acid to its symmetric anhydride or active ester, the use of a weaker base than DIEA (pK_a 10.1) might promote attack by the acid anion in subsequent steps without unduly interfering with the first step of carbodiimide protonation. It was indeed gratifying to find that when DIEA was replaced by collidine (pK_a 7.43) in the preactivation step, conversion of Z-Aib-OH to Z-Aib-OAt was accelerated, not only over the rate in DIEA/DMF but also *over the rate in DMF alone* (Fig. 1). On the other hand the view that this effect is caused only by the influence of the weaker base on the various equilibria involved is not confirmed by substitution of two other bases of pK_a similar to that of collidine, namely NMM (pK_a 7.38), and NMI (pK_a 7.13).[15] Table 6 collects the relevant data for these and other bases. Non-pyridine bases showed effects consistent with their pK_a values. Thus NMM was less effective than DIEA in reducing reactivity in DMF and *t*-butyl tetramethylguanidine was about twice as effective.

Other hindered pyridine bases which were tested accelerated the activation process, but none more effectively than collidine. Pertinent halftimes for conversion to active ester were as follows: no base ($t_{1/2}$ 27 min), collidine (15 min), 2,6-lutidine (16 min), 2,3,5,6-tetramethylpyridine (15 min), DB(DMAP) (15 min), OHA (15 min). Non-hindered pyridines such as 2,4-lutidine showed little effect ($t_{1/2}$ 25 min); 2-picoline was more inhibitory than DIEA and the highly basic DMAP ($t_{1/2}$ 180 min) was highly inhibitory. Because of the effect of protonation on the speed of activation (cf. **16**), the acid-carrying reagents such as EDC•HCl or EDC•HCl/HOAt enhanced preactivation in DMF to a greater extent than DIC/HOAt, an effect which was further boosted by collidine (Table 6).

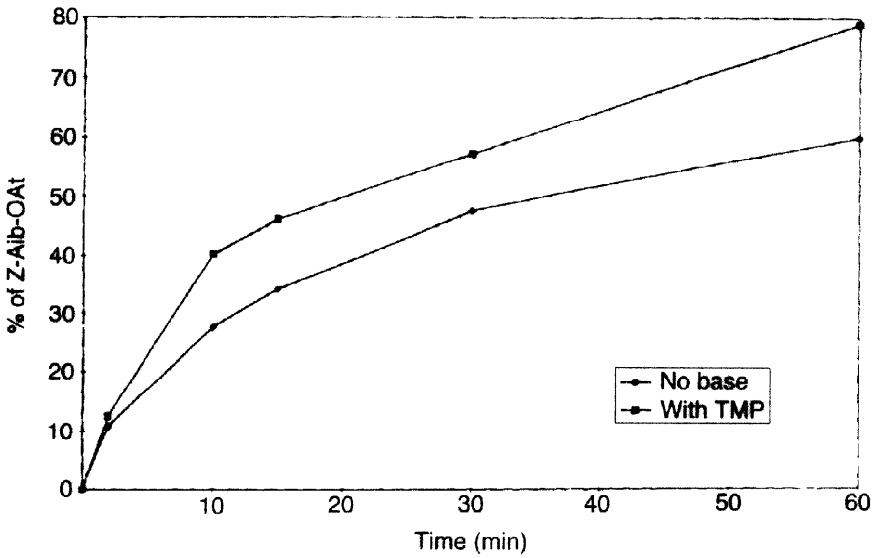
Table 6

Approximate Halftimes for the Conversion of Z-Aib-OH to Z-Aib-OAt via DIC/HOAt or EDC•HCl/HOAt in the Presence of Various Bases (^{1}H NMR Analysis)[a]

Coupling Reagent	Solvent	Base	$t_{1/2}$ (min)	PercentCompletion, 1 h
DIC/HOAt	DMF		27	60.1
DIC/HOAt	DMF	TMP (1)	15	72.9
DIC/HOAt	DMF	TMP (2)	15	88.2
DIC/HOAt	DMF	DIEA (1)	130	
DIC/HOAt	DMF	NMM (1)	90	
DIC/HOAt	DMF	NMI (1)	30	63.1
DIC/HOAt	DMF	DMAP (1)	180	
DIC/HOAt	DMF	*t*-BuTMG (1)	240	
DIC/HOAt	DMF	2,6-Lutidine (1)	16	66.7
DIC/HOAt	DMF	2,4-Lutidine (1)	25	57.4
DIC/HOAt	DMF	TEMP (1)	15	73.2
DIC/HOAt	DMF	DB(DMAP) (1)	15	75.6
DIC/HOAt	DMF	OHA (1)	15	72.6
DIC/HOAt	DMF	2-picoline (1)	150	43.7
DIC/HOAt	DMF-DCM(1:1)		8	100
DIC/HOAt	DMF-DCM(1:1)	TMP (1)	5	100
DIC/HOAt	DCM		2	
DIC/HOAt	DCM	TMP (1)	< 1	
DIC/HOAt	DCM	DIEA (1)	1	
DIC/HOAt	DCM	NMM (1)	1	
EDC•HCl/HOAt	DMF		5	100
EDC•HCl/HOAt	DMF	TMP (1)	3	100
EDC•HCl/HOAt	DMF	DIEA (1)	10	73.6
EDC•MI/HOAt	DMF		26	64.3
EDC•HCl/HOAt	DCM		< 1	
EDC•MI/HOAt			4	

[a] A solution prepared from 23.7 mg (0.1 mmol) of Z-Aib-OH, 13.6 mg (0.1 mmol) of HOAt, 0.1 mmol of a base and finally 12.6 mg (0.1 mmol) of DIC in 0.5 mL of either DMF or $CDCl_3$ in an NMR tube was placed in the probe of a 60 MHz NMR spectrometer held at 37°C. The reaction was followed by the decrease in the integral of the benzylic proton of Z-Aib-OH (δ 5.10) and the increase of that of Z-Aib-OAt (δ 5.26).

Fig. 1. **Time Course for the Conversion** of Z-Aib-OH to Z-Aib-OAt via DIC/HOAt in DMF in the Presence or Absence of Collidine[a]



[a] In a series of vials a solution of 23.7 mg (0.1 mmol) of Z-Aib-OH and 13.6 mg (0.1 mmol) of HOAt in 0.3 mL of DMF was treated with 15.5 μL (0.1 mmol) of DIC and the solution let stand at room temperature. At the times given individual reaction mixtures (60 μL) were quenched by addition to 3 mL of a mixture of H_2O-CH_3CN (1:2). Injection of 10 μL of the resulting solution onto a Waters C_{18} Nova-Pak column gave the ratio of active ester (t_R 19.6 min) to acid (t_R 14.2 min) shown using a linear gradient from 5 to 60% acetonitrile in 20 min (CH_3CN/H_2O/0.1% TFA, λ_{max} 214 nm). A second run was made in the presence of 12.9 mg (0.1 mmol) of collidine.

Based on these data, we cannot at this time offer a satisfactory rationale for the influence of collidine, although it was possible to make practical use of the effect to improve peptide assembly carried out by the DIC/HOAt technique. For example, in the assembly of pentapeptide

H–Tyr–Aib–Aib–Phe–Leu–NH_2

17

17[16] by manual solid phase techniques using a two-step protocol with 7-min preactivation in DCM followed by addition of 4 eqs of DIEA in DMF such that in the final solution the ratio of DCM and DMF was 1:1, there was obtained the desired pentapeptide along with the des-Aib tetrapeptide in the ratio 78.9/21.1. If the initial preactivation was carried out in the presence of 1 eq. of collidine and then 3 eqs. of DIEA in DMF added, the ratio of products was raised to 84.4/15.6. With no base added to either step, poor results were obtained with only 5.6% of the desired pentapeptide being formed along with 73.1% of the des-Aib tetrapeptide and 21.2% of a

third unidentified material according to HPLC analysis. For syntheses carried out in the single solvent mixture DCM-DMF (1:1) without any base added, only 4.4% of the pentapeptide resulted whereas if 4 eqs of collidine were present from the beginning 57.6% of the pentapeptide resulted. Results for this and a number of related runs are collected in Table 7. For comparison with these DIC/HOAt runs, pentapeptide **17** is readily obtained via HATU/DIEA with 7-min preactivation and 30-min coupling in DMF alone in a yield of 79.2%.

A second synthetic example of the collidine effect was observed in the assembly of the ACP-decapeptide.[17] The results are presented in Fig. 2.

Table 7

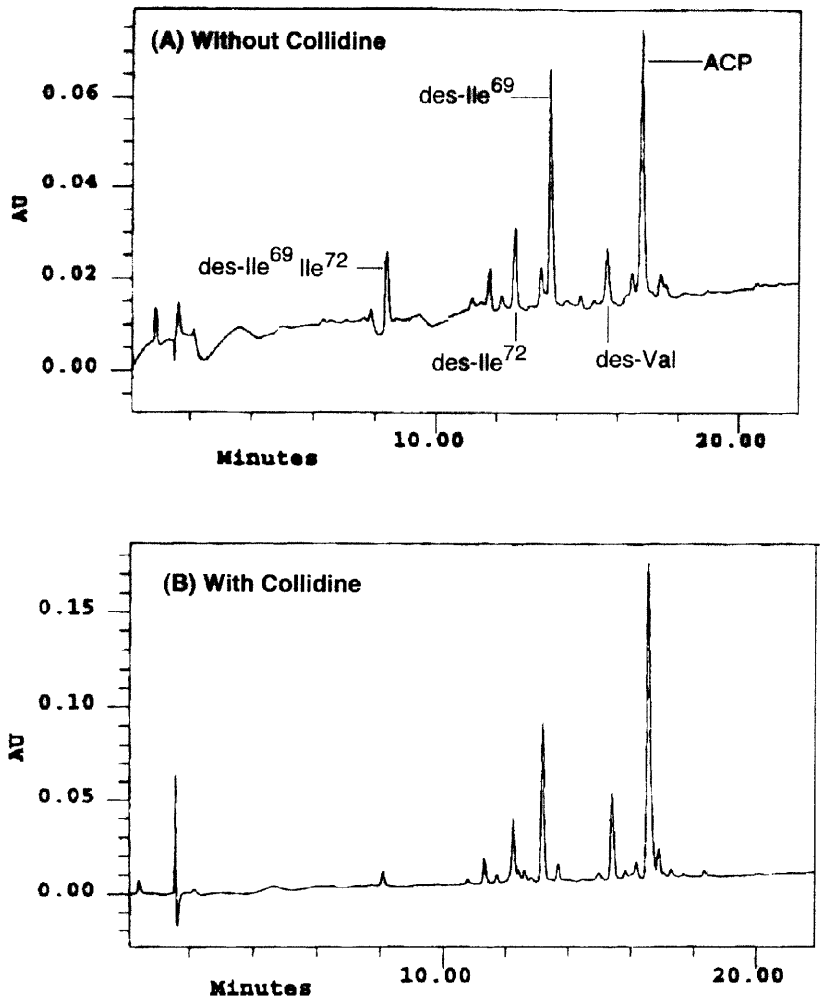
Solid Phase Assembly of H-Tyr-Aib-Aib-Phe-Leu-NH_2 in the Presence or Absence of Various Bases via DIC/HOAt [a,b]

	Conditions	Yield (%)	5-mer (%)	des-Aib-4-mer (%)
1.	PA in DCM-DMF(1:1), coupling in the same solvent	83.2	4.4	95.6
2.	PA in DCM, coupling in DCM-DMF (1:1)	79.9	5.6	73.1
3.	PA in DCM, coupling with 4 eqs TMP in DCM-DMF (1:1)	81.3	26.2	73.8
4.	PA in DCM-DMF(1:1) with 4 eqs TMP, coupling in the same solvent	81.5	57.6	42.4
5.	PA in DCM with 2 eqs TMP, coupling in DCM-DMF (1:1) with 2 eqs DIEA	92.3	75.0	25.0
6.	PA in DCM, coupling in DCM-DMF (1:1) with 4 eqs DIEA	83.9	78.9	21.2
7.	PA in DCM with 1 eq TMP, coupling in DCM-DMF (1:1) with 3 eqs DIEA	89.3	84.4	15.6

[a] The syntheses were carried out manually in a plastic syringe which was attached to a vacuum manifold so as to effect rapid removal of reagents and solvents. The resin [Fmoc-PAL-PEG-PS] with a loading of 0.19 mmol/g (100 mg) was washed with DCM and DMF (2 x 5 mL each), treated with 5 mL of 20% piperidine in DMF for 7 min, washed with DMF, DCM, and DMF again (2 x 5 mL each) and then acylated with 4 eqs of each Fmoc amino acid, 4 eqs of HOAt and 4 eqs of DIC under the conditions given. All preactivation times (PA) were 7 min and all coupling times 30 min. The coupling step was always carried out in the same volume of solvent to maintain the same concentrations. Any second solvent was added at the end of the 7-min preactivation period. Following the coupling step, the peptide was cleaved from the resin by TFA-H_2O (9:1) at room temperature for 2 h. TFA was removed *in vacuo* and the crude peptide was precipitated with ether. The crude weight was recorded and the ratio of the penta- and tetrapeptides determined by HPLC analysis using a Waters Nova-Pak C_{18} column (4 μ, 60Å, 3.9 x 150 mm) with the detector set at 220 nm, flow rate 1 mL/min using a linear gradient of 5/95 in 25 min, CH_3CN/H_2O/0.1% TFA, t_R 11.4 min (pentapeptide), 11.7 min (tetrapeptide). The results are listed in the order of increasing efficiency (% of 5-mer).

[b] In most cases only the penta- and tetrapeptides were observed. Where the percentages do not come to 100%, other by-product peaks were noted in the HPLC traces.

Fig. 2. Solid Phase Assembly of ACP(65-74) in the Absence and Presence of Collidine via DIC/HOAt



Each assembly was carried out as described in ref. 16 and Table 7, ref a with 7-min preactivation using 1.5 eqs of the Fmoc amino acid, 1.5 eqs of DIC and either none or 1.5 eqs of TMP with a coupling time of 1.5 min. These "forcing" conditions are not practical for synthetic purposes but serve to bring out clearly the differences among different protocols. It may be noted that if assembly is carried out in DMF alone with 7-min preactivation, 4 eqs excess of both acid and DIC for a 30-min coupling time an excellent synthesis results: 89.5% yield, purity of ACP, 95.5% with the only observed by-product being 4.4% of the des-Val derivative. With a similar run made with 7-min preactivation in DCM-TMP followed by addition of 3 eqs of DIEA in DMF for the coupling step (30 min), final solution 1:1 in DCM and DMF

the yield was 86.9%, purity of ACP 97.2%, with the only by-products being des-Asn, des-Val and des-^{69}Ile, each in less than 0.8-0.9%.

In conclusion, this work shows that the DIC/HOAt system is of greater efficiency in preserving configuration during peptide segment coupling than the analogous DIC/HOBt system in both DMF and DCM. The introduction of electron-withdrawing substituents, e.g., CF_3 or NO_2, into the HOBt nucleus is counterproductive. The use of unsymmetrical alkyl aryl carbodiimides or those bearing a tertiary amino substituent, whether protonated or quaternized, was also less effective than the neutral DIC type. In the case of stepwise peptide assembly the efficiency of the DIC/HOAt system in DMF solvent can be increased by carrying out the preactivation step in the presence of collidine.

EXPERIMENTAL SECTION

HOAt, HATU, and PAL-PEG-PS were obtained from Perseptive Biosystems. Z-Phe-Val-OH and H-Pro-NH_2 were obtained from Bachem. Protected amino acids and amino acid esters were from Novabiochim and Chem-Impex. Other reagents were from Fisher Scientific (Acros) and Aldrich Chemical Co. $HOCF_3Bt$[10] and $HONO_2Bt$[18] were synthesized as described. DB(DMAP) was available from an earlier study.[1f]

Preparation of Fmoc-Asp(O-*t*-Bu)-Phe-OH. A solution of 3.41 g (8 mmol) of H-Phe-OBn•TsOH and 2.06 g (16 mmol) of DIEA in 80 mL of DCM was treated with 3.4 g of Fmoc-Asp(O-*t*-Bu)-F and the mixture stirred at room temperature for 1 h (TLC indicates complete reaction). The solution was diluted with 50 mL of DCM and washed with saturated $NaHCO_3$ solution, 10% citric acid and saturated NaCl solution (2 x 30 mL each) and dried over $MgSO_4$. Removal of solvent and recrystallization of the residue from DCM/hexane gave 4.56 g (89.5%) of the dipeptide benzyl ester, mp 116°C; IR (KBr) 1736, 1696, 1645 cm^{-1}. The ester (3 g) was dissolved in 50 mL of MeOH-THF (1:1) and the solution cooled to 0°C and treated with 0.4 g of ammonium formate and 0.3 g of 5% Pd-C (Acros). The mixture was stirred at 0°C for 15 min and at room temperature for 30 min. TLC indicated that at this time removal of the benzyl

ester function was nearly complete and a small spot for 9-methylfluorene began to appear. The mixture was filtered, washed with MeOH, the solvent removed *in vacuo* and the residue recrystallized from EtOAc/hexane to give 1.93 g (73.7%) of the dipeptide acid, mp 169-171°C (LL-), mp 159-162°C (LD-); ^{1}H-NMR (LL-) ($CDCl_3$-DMSO-d_6) δ 1.5 (s, 9), 2.5 (m, 2), 3.3 (m, 2), 3.8-4.1 (m, 5), 6.9-7.9 (m, 13); ^{1}H NMR (LD-) (DMSO-d_6) δ 1.49 (s, 9), 2.5 (m, 2), 3.27 (m, 2), 3.79-4.1 (m, 5), 6.9-7.9 (m, 13). *Anal.* (LL-) Calcd for $C_{32}H_{34}N_2O_7 \bullet H_2O$: C, 66.66; H, 6.25; N, 4.86. Found: C, 66.90; H, 5.79; N, 4.87.

Test Coupling Reactions. All coupling tests were carried out as described in previous publications. The results are collected in Tables 1-5 in which the methods or appropriate literature references are given in the footnotes. Rough determination of the time course for the activation of model acid Z-Aib-OH was carried out as described in footnote (a) of Table 6. Model peptide assemblies under various conditions are collected in Table 7 and Fig. 2. When different solvents were used for the preactivation and coupling steps care was taken so that the final concentration of acid was always 0.3 M. For example if DCM was used for preactivation the volume of DCM was 0.15 mL and after 7 min 0.15 mL was added for a final total volume of 0.3 mL which was the same as the volume of DMF used if reaction is carried out in this solvent alone.

Preparation of EDC•MH. Potassium hexafluorophosphate (1.01 g, 5.5 mmol) was added to a solution of EDC•MI[5] (5 mmol, 1.49 g) in 30 mL of dry acetonitrile. The reaction mixture was stirred at room temperature for 5 h, filtered, and washed with 10 mL of CH_3CN. The solvent was removed *in vacuo* and the crude product was recrystallized from CH_2Cl_2 and ether to give in 92.3% yield a yellowish white solid, mp 38-40°C. ^{1}H NMR ($CDCl_3$) δ 1.35 (t, 3 H, CH_3), 2.0 (m, 4 H, $2CH_2$-), 3.3-3.95 (m, 15 H, 3 CH_3, 3 CH_2).

Anal. Calcd for $C_9H_{20}N_3PF_6$: C, 34.28; H, 6.35; N, 13.33. Found: C, 34.63; H, 6.39; N, 13.36.

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