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Evaluation of Carbodiimides Using a Competition Method

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Abstract: A competitive reaction of activated Boc-Ala-OH and Boc-Phe-OH with H-Leu-resin has been developed for assessing the relative efficiencies of different carbodiimides. This allowed a comparison of the efficiency of the carbodiimides *N,N′*-dicyclohexylcarbodiimide,*N,N′*-diisopropylcarbodiimide, *N-tert*-butyl-*N′*-methylcarbodiimide and *N-tert*-butyl-*N′*-ethylcarbodiimide. Comparable results were obtained when these reagents were used for the preformation of symmetrical anhydrides or of 1-hydroxybenzotriazole esters *in situ*. Differential incorporation was observed when asymmetrical carbodiimides were used for peptide bond formation by the direct carbodiimide procedure. © 1997 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: peptide coupling; mechanism of coupling; symmetrical anhydrides; *N-tert*-butyl-*N′*-ethylcarbodiimide; *N-tert*-butyl-*N′*-methylcarbodiimide

Many procedures have been developed for evaluation of methods for solid-phase peptide synthesis. The most important have involved quantification of unreacted amino component ($HClO_4$ titration, uptake of picrate, ninhydrin analysis), HPLC separation of the products after cleavage of a model test peptide from the support, and quantification of peptide by-products by mass spectrometry [1]. The competition method [2] developed by Hudson is attractively simple: it enables the evaluation of coupling reagents using a simple model reaction. In this method Boc-Tyr(Bzl)-OH and Boc-Phe-OH are activated separately, mixed and immediately added to Leu-resin. The ratio of Tyr to Phe is then determined after hydrolysis of the peptide–resin conjugate so obtained. When the same reagent and procedure are used for the activation of both the Tyr and Phe components, the incorporation ratio is 30:70. When one of the activation reagents or methods of activation is altered, then a variation in the incorporation ratio may be observed, reflecting the difference in the efficiency of activation.

Since the focus of our recent work has been to evaluate the efficiency of novel carbodiimides, we realized that the competition method could be advantageously used for this purpose, and, indeed, we have found it useful for the evaluation of new reagents and known coupling procedures [3]. In the present work we preserve the essential principle of the method, but we employ a new pair of carboxyl components. Based on our previous results concerning propensity for *N*-acylurea formation, we selected Boc-Ala-OH and Boc-Phe-OH, which are similar in this respect [4]. The studies of Ragnarsoon *et al.* [5] on the relative reactivities of amino acids in solid-phase peptide synthesis were also helpful in this selection. In our preliminary experiments with symmetrical anhydrides, the incorporation of these two protected amino acids was close to 1:1.

Equimolar amounts of Boc-Ala-OH and Boc-Phe-OH were activated separately, then mixed and immediately added to H-Leu-resin. For activation in this study two commonly used carbodiimides,

Abbreviations: BEC, *N-tert*-butyl-*N′*-ethylcarbodiimide; BMC, *N-tert*-butyl-*N′*-methylcarbodiimide; C/HOBt, carbodiimide plus HOBt procedure; DCC, *N,N′*-dicyclohexylcarbodiimide; DCM, dichloromethane; DIC, *N,N′*-diisopropylcarbodiimide; NMP, *N*-methylpyrrolidone; pSA, preformed symmetrical anhydride.

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namely *N,N′*-dicyclohexylcarbodiimide (DCC) and *N,N′*-diisoproplycarbodiimide (DIC), and two carbodiimides developed in this laboratory, namely *N*-*tert*-butyl-*N′*-methylcarbodiimide (BMC) [6] and *N*-*tert*-butyl-*N′*-ethylcarbodiimide (BEC) [7] were used for activation. The couplings were performed according to the direct carbodiimide procedure, with preformed symmetrical anhydrides (pSA), or with preformed 1-hydroxybenzotriazole esters (C/HOBt). The results are summarized in Table 1.

The incorporation figures show that the Ala content is always 52–57% when preformed symmetrical anhydrides are used for acylation. These experiments show that all the carbodiimides studied are equally effective in the formation of symmetrical anhydrides. The slightly better acylation with the use of BMC rather than DCC which is observed in practical peptide synthesis [6] was probably due to the favourable effect of soluble dialkylurea. In this model reaction the soluble urea formed during the activation of one reagent is present in the reaction mixture of the two competing reactants. A similar situation is found in experiments with preformed active HOBt esters, where the incorporation of Ala is between 55 and 58%. But when the direct carbodiimide procedure is used, the values for acylation with Boc-Ala-OH vary within a broader range, from 34 to 50%, and depend on the nature of activating carbodiimide. This difference is consistent with the reaction mechanism (Scheme) in which the acylating species in the anhydride procedure are the same symmetrical anhydrides, and in the carbodiimide plus additive method are the same active esters, irrespective of which carbodiimide is used for activation. Interpretation of the differential incorporation in the direct carbodiimide procedure is not obvious because of the complexity of the reaction between equimolar amounts of the reagents [8,9]. There is, however, the possibility that the acylating species in this procedure are the *O*-acylisoureas which are different depending on the carbodiimide used for the activation. It should be mentioned in this context that Rebek and Feitler [10] have presented evidence that in solid-phase peptide synthesis the DCC reaction mechanism follows the alternative path and the actual acylating agent is the symmetrical anhydride. However, the experimental conditions in their experiments did not duplicate standard solid-phase procedures; a large excess of the carboxyl component was favourable to anhydride formation. On the other hand there are reports from the same laboratory [11,12] that the acylating agent in DCC-mediated peptide synthesis in solution is the *O*-acylisourea.

In conclusion a modified competition method has proved to be useful for the evaluation of different carbodiimides for peptide synthesis. Our experiments confirm our expectation that asymmetrical carbodiimides containing a *tert*-butyl group at the N^1 position and methyl or ethyl at the N^2 position (BMC and BEC respectively) are equally effective in the formation of symmetrical anhydrides as commonly used DCC and DIC. Since during activation with BEC and BMC soluble dialkylureas are formed, which simplifies the procedure and has a beneficial effect on the conformation of the acylated peptide

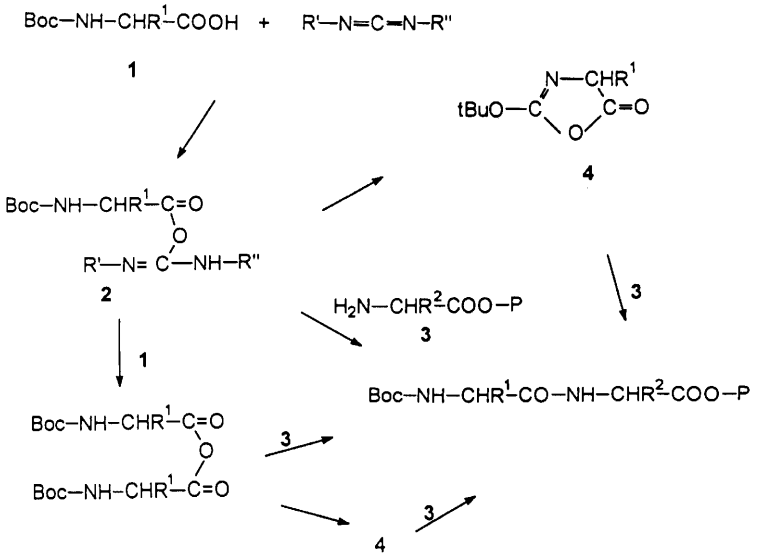

Table 1 Comparison Between Various Carbodiimides and Coupling Procedures Using Competition Experiments[a]

No.	Ala			Phe			% Ala inc.
	Reagent	Activation		Reagent	Activation		
		Method	Time (min)		Method	Time (min)	
1	DCC	Direct	2	DCC	Direct	2	42
2	BEC	Direct	2	BEC	Direct	2	36
3	BMC	Direct	2	BMC	Direct	2	39
4	DIC	Direct	2	DIC	Direct	2	38
5	DCC	Direct	2	BEC	Direct	2	46
6	BEC	Direct	2	DCC	Direct	2	34
7	DCC	Direct	2	BMC	Direct	2	50
8	BMC	Direct	2	DCC	Direct	2	37
9	BEC	Direct	2	DIC	Direct	2	36
10	DIC	Direct	2	BEC	Direct	2	47
11	DCC	pSa	15	DCC	pSA	15	55
12	BEC	pSA	15	BEC	pSA	15	54
13	BMC	pSA	15	BMC	pSA	15	54
14	DIC	pSA	15	DIC	pSA	15	50
15	DCC	pSA	15	BEC	pSA	15	55
16	BEC	pSA	15	DCC	pSA	15	57
17	DCC	pSA	15	BMC	pSA	15	52
18	BMC	pSA	15	DCC	pSA	15	57
19	DIC	pSA	15	BEC	pSA	15	54
20	BEC	C/HOBt	10	BEC	C/HOBt	10	58
21	DIC	C/HOBt	10	DIC	C/HOBt	10	55
22	BEC	C/HOBt	10	DIC	C/HOBt	10	56
23	DIC	C/HOBt	10	BEC	C/HOBt	10	58

[a] Each value in the table is an average of three determinations (± 2%).

chain [13], these reagents can be used instead of DCC and DIC with advantage. These carbodiimides can also be used in combination with HOBt. The application of BMC and BEC for peptide bond formation according to the direct carbodiimide procedure is not recommended.

EXPERIMENTAL PART

Preformed Symmetrical Anhydrides

Solutions of Boc-AlaOH (1.0 g, 5.28 mmol) and Boc-PheOH (1.4 g, 5.28 mmol) in DCM (10 ml) were prepared. Carbodiimide solutions were prepared as follows: DCC (544 mg, 2.64 mmol), DIC (370 mg, 2.64 mmol), BEC (333 mg, 2.64 mmol) and BMC (296 mg, 2.64 mmol) in DCM (10 ml in each case). For each experiment, an aliquot of the Boc-amino acid solution (0.5 ml. 0.264 mmol) was mixed with an aliquot of appropriate carbodiimide solution (0.5 ml, 0.132 mmol) and allowed to stand for 15 min at room temperature. From each of two solutions so prepared, samples (0.5 ml, 0.066 mmol) were taken, combined and immediately added to the reaction vessel containing a sample of swollen (DCM) standard Merrifield Leu-resin (50 mg, 0.002 meq). The reaction mixture was left for 2 h. The resin was washed with DMF (2 × 2 ml) 10% Et_3N in DMF (1 × 2 ml), DCM/DMF (4 × 2 ml), DCM (6 × 2 ml) and methanol (2 × 2 ml). Dried samples (1.5 mg) were hydrolysed at 130°C for 2 h in 1:1 propionic acid/12 N HCl containing 1% of phenol. Amino acid analyses were performed in triplicate on a Microtechna Praha model T339 Amino Acid Analyzer. Percentages of Ala incorporation are given in Table 1.

Direct Carbodiimide Method

Solutions in DCM:DMF (1:1) (10 ml) were prepared from the following samples: Boc-Ala-OH (500 mg, 2.64 mmol), Boc-Phe-OH (700 mg, 2.64 mmol),

BMC (296 mg, 2.64 mmol), BEC (333 mg, 2.64 mmol), DCC (544 mg, 2.64 mmol) and DIC (370 mg, 2.64 mmol). An aliquot of the Boc-amino acid solution (0.5 ml, 0.066 mmol) was mixed with an aliquot of the solution of the appropriate carbodiimide (0.5 ml, 0.066 mmol). After 2 min of mixing, a portion of this solution (0.5 ml) was combined with a simultaneously prepared solution of the other activated amino acid derivative and immediately added to the reaction vessel containing the Leu-resin (50 mg, 0.02 meq). The reaction and workup procedure were as described above.

Carbodiimide Plus Additive Method

Solutions of Boc-Ala-OH (500 mg, 2.64 mmol) in DCM (10 ml), Boc-Phe-PH (700 mg, 2.64 mmol) in DCM (10 ml), BEC (300 mg, 2.64 mmol) in DCM (10 ml) and HOBt (400 mg, 2.64 mmol) in NMP (10 ml) were prepared. An aliquot of the Boc-amino acid solutions (0.5 ml) was mixed with an aliquot of the solution of appropriate carbodiimide (0.57 ml) and an aliquot of the solution of HOBt (0.5 ml). After 10 ml of mixing an aliquot (0.75 ml) was combined with an aliquot of the solution of other activated amino acid derivative (0.75 ml) and immediately added to the reaction vessel containing the resin. The reaction and workup were as described above. The results are presented in Table 1.

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