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Effect of Solvent on Racemization in Carbodiimide Mediated Solid Phase Fragment Condensations

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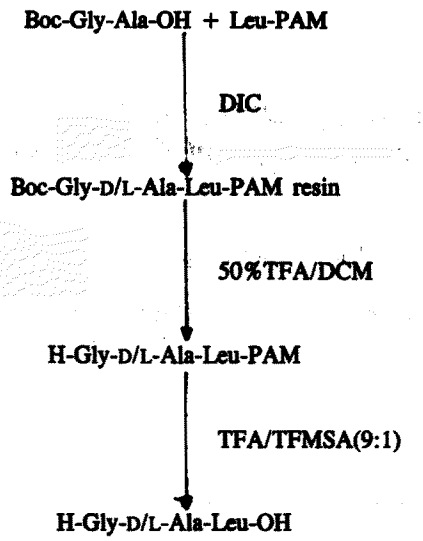
Abstract: *The Izumiya tripeptide was used to assess racemization in solid-phase fragment condensations. Boc-Gly-Ala-OH[1] was coupled to Leu-PAM resin with DIC in a variety of solvents, both with and without HOBt. After cleavage from the resin, the extent of racemization was determined using C18 RP-HPLC to separate the epimers. Solvents used were DMF, NMP, TFE, and each of these as mixtures with DCM. Also tested was a mixture of NMP and DMSO (85:15). Couplings in DMF/DCM (1:1) and NMP/DCM (1:1)in the presence of HOBt were in excess of 98% and racemization was undetectable (<0.1%).*

One of the major obstacles of the fragment condensation approach to peptide synthesis is racemization which occurs in the coupling reaction. Most commonly, the abstraction of a proton by a base present in the coupling solution or the conversion of the activated carboxyl function to an oxazolone is responsible for this racemization.[2]

Previous studies have used the fragment synthesis of model tripeptides to examine the extent of racemization. A protected dipeptide fragment is coupled to a protected amino acid, either in solution[3-6] or bound to an insoluble resin,[7,8] and the two resulting epimers are separated by chromatography. Several tripeptide models have been used in numerous coupling environments.[9-11] RP-HPLC is now frequently used to separate both protected and deprotected peptide epimers.[12,13]

The Izumiya tripeptide[10] was used in conjunction with RP-HPLC to assess the extent of racemization during solid phase fragment condensations. H-Gly-Ala-Leu-OH and H-Gly-D-Ala-Leu-OH were synthesized by stepwise solid phase methods, purified by gel filtration and RP-HPLC and characterized by amino acid analysis and mass spectrometry. Mixtures of these standards were separated on a Vydac C18 column, eluted isocratically with the solvent mixture 0.1% TFA in water/methanol (92:8). Peptides were detected at 208nm and the response was linear over the concentration range 0.012-12mM. These conditions allowed the detection of 0.1% of one epimer in the presence of the other.

Boc-Gly-Ala-OH was prepared by the hydroxysuccinimide method[14] and coupled to Leu-PAM resin in various solvent systems according to the scheme presented below. Solvents used were DMF, NMP, TFE, DMF/DCM (1:1), NMP/DCM (1:1) and TFE/DCM (1:1). In addition, the solvent mixtures NMP/DMSO



Scheme. Coupling, Deprotection, and Cleavage of H-Gly-D/L-Ala-Leu-OH

(85:15) and TFE/DCM (1:4), which have been reported to improve coupling efficiencies in solid phase peptide synthesis, were used.[15,16] Each of these systems was also tested with HOBt present. Unacceptable coupling yields in the solvent mixtures NMP, NMP/DMSO (85:15), and TFE led to the utilization of preformed active esters for coupling to improve yields. All coupling yields were determined by the quantitative ninhydrin method.[17] Tripeptides were cleaved from the resin with TFA/TFMSA (9:1), and the amount of H-Gly-D-Ala-Leu-OH was determined using RP-HPLC. Yield and racemization data are in the table.

Couplings in DMF, NMP and TFE resulted in unacceptably low yields and high levels of racemization. The levels of racemization in DMF were in agreement with those previously reported for DCCI mediated couplings on the solid phase.[7] Apparently, the use of DIC instead of DCCI as the carbodiimide does not significantly affect the extent of racemization. Upon addition of HOBt, yields increased and racemization decreased. The highest yield and lowest racemization was obtained using DMF (97.1% and 0.1% respectively). Interestingly, in NMP, while the addition of HOBt raised the yield to 77.6% and lowered the racemization to 2.7% with *in situ* activation, the preformed active ester predictably raised the yield to an acceptable level (>90%) but left racemization at an unacceptable level (>0.1%). TFE proved to be unsuitable, with or without HOBt, for solid phase fragment condensations.

When DMF, NMP, and TFE were used in 1:1 mixtures with DCM, with or without HOBt, all coupling yields increased and racemization decreased. Highest yields and undetectable racemization were obtained with DMF/DCM (1:1) and NMP/DCM (1:1) in the presence of HOBt. The utility of these latter solvent mixtures for solid phase fragment condensations will greatly depend on their ability to dissolve the

fragments.

Contrary to previous studies using the BOP reagent,[15] NMP/DMSO (85:15) was found to be unsuitable for carbodiimide mediated solid phase fragment condensations. Couplings in TFE/DCM (1:4), in the presence of HOBt, led to yields >90% with undetectable racemization. The instantaneous dissolution of the dipeptide fragment in this solvent mixture together with its desirable effects on coupling yields and racemization make it a promising solvent mixture for solid phase fragment condensations.

General Procedure to Measure Racemization: Coupling reactions were carried out in a 15 mL sintered glass funnel (fine porosity) with no shaking or stirring of contents. Boc-Leu-PAM resin (122 mg, 0.41 mmol/g) was placed in the sintered glass funnel and subjected to the following protocol. All washing volumes were 1mL. 1) Washed with DCM (2 x 3 min). 2) Removed Boc group with 50% TFA/DCM (v/v) (1 x 20 min). 3) Washed with DCM (5 x 1 min). 4) Neutralized with 5% DIEA/DCM (v/v) (1 x 2 min) and 5% DIEA/coupling solvent (v/v) (1 x 2 min). 5) Washed with coupling solvent (5 x 1 min). 6) Added a solution of Boc-Gly-Ala-OH (25 mg, 100 μmol) and HOBt (13 mg, 100 μmol), if used, in the coupling solvent (800 μL) and left to stand for 3 min. Added a solution of DIC (17 μL, 100 μmol) in the coupling solvent (200 μL) and left to stand for 1.5 h. 7) Removed solvent and washed with DCM (5 x 1 min). 8) Removed sample of resin (ca. 5-10 mg) for quantitative ninhydrin test.[17] 9) Removed Boc group with 50% TFA/DCM (v/v) (20 min). 10) Washed with DCM (5 x 1 min). The peptide-resin was dried *in vacuo* (18 h, 20 mm Hg) prior to cleavage.

To a sample of the peptide-resin (10 mg) in a test tube (16 x 100 mm) was added TFA/TFMSA (9:1) (100 μL) and the reaction mixture was left to stand for 25 min. The reaction was terminated by addition of water (700 μL) and raising the pH to approximately 3 with ammonium hydroxide solution (200 μL). Resin was removed by filtration and three aliquots of the filtrate were subjected to analytical RP-HPLC on a Vydac C18 (4.6 x 250 mm) column. The epimers were eluted isocratically with 0.1% TFA in water/methanol (92:8) and detected at 208 nm. % D epimer =(D/D+L) x 100. Reported % racemization is the mean of three trials.

Table. Coupling Yields and Extent of Racemization During the Synthesis of Boc-Gly-Ala-Leu-PAM Resin.

Solvent System	% Yield		% D Epimer	
	DIC	DIC+HOBt	DIC	DIC+HOBt
DMF	67.7	97.1	18.0	0.1
DMF/DCM (1:1)	89.9	98.7	11.8	<0.1
NMP Preformed ester	24.3 ---	77.6 93.0	16.8 ---	2.7 2.0
NMP/DCM (1:1)	95.6	98.5	11.7	<0.1
NMP/DMSO (85:15) Preformed ester	--- ---	25.2 74.9	--- ---	1.1 4.6
TFE	18.2	54.1	8.7	2.0
TFE/DCM (1:4)	78.9	92.2	1.6	<0.1
TFE/DCM (1:1)	18.7	97.1	3.5	0.2

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1. Symbols and abbreviations are in accord with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.*, **1972**, *247*, 977). All optically active amino acids are of the L variety unless otherwise stated. Other abbreviations include the following: Boc, tert-butyloxycarbonyl; BOP, benzotriazol-1-yl-tris(dimethylamino)phosphonium hexafluorophosphate; DCCI, dicyclohexylcarbodiimide; DCM, dichloromethane; DIC, diisopropylcarbodiimide; DIEA, N,N-diisopropylethylamine; DMF, N,N-dimethylformamide; DMSO, dimethyl sulfoxide; HOBt, 1-hydroxybenzotriazole; NMP, N-methylpyrrolidinone; PAM, phenylacetamidomethyl; RP-HPLC, reverse phase high performance liquid chromatography; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; TFMSA, trifluoromethanesulfonic acid.
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