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DIKETOPIPERAZINE FORMATION IN SOLID PHASE PEPTIDE SYNTHESIS USING p-ALKOXYBENZYL ESTER RESINS AND FMOC-AMINO ACIDS.

Enrique Pedroso*, Anna Grandas, Xavier de las Heras[1], Ramon Eritja[2] and Ernest Giralt.

Departament de Química Orgànica, Facultat de Química, Universitat de Barcelona, Diagonal 645, Barcelona 08028, Spain.

Summary: Diketopiperazine formation rates under the usual conditions of a solid phase peptide synthesis cycle with Fmoc-amino acids have been studied on a p-alkoxybenzyl ester resin. Piperidine has been found to be an extremely efficient catalyst for the intramolecular aminolysis reaction.

Diketopiperazine formation is a well known side reaction in solid phase peptide synthesis using benzyl ester type peptide-resin linkages (3-6). This intramolecular aminolysis has been demonstrated to be either acid- (4) or base-catalyzed (5-8) and it is accelerated by the presence of certain residues such as glycine, proline, N-methylamino acids or a D-amino acid in the dipeptide sequence (4-6).

In the last years, the combined use of Fmoc-amino acids and a p-alkoxybenzyl ester peptide-resin linkage has found wide application to synthesize both free (9-14) and protected peptides (15-17). Although the use of this approach has extended rapidly, very little attention has been paid to the possibility of undesired diketopiperazine formation (16, 18). The main purpose of this work is to evaluate the extension of this side reaction in each step of a synthetic cycle in order to find alternatives to minimize it.

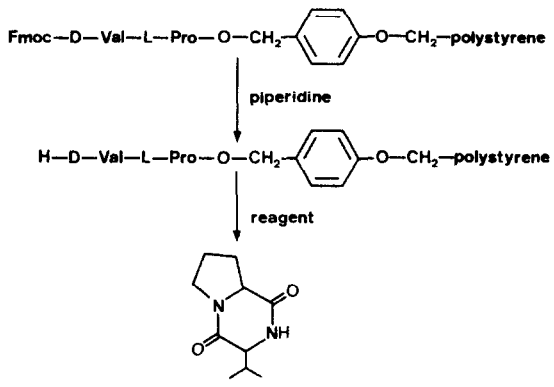
We have synthesized Fmoc-D-Val-L-Pro-O-CH_2-C_6H_4-O-CH_2-polystyrene following standard procedures (16), and the rate of cyclo(-D-Val-L-Pro-) formation has been determined after gas chromatographic quantitation of the diketopiperazine (DKP) formed (19). We have first studied the influence of the Fmoc cleavage reagent, 50% piperidine in DMF, on DKP formation, and we have found a very high rate constant as compared with other reported values under several conditions (tables 1 and 4) (8). For other reagents kinetic data were obtained after partial deprotection with a 20s 20% piperidine/DMF treatment stopped by washing with DMF and the amount of deprotection and DKP formation were determined by the absorbance of N-(9-fluorenylmethyl)piperidine at 301nm (ε=7800 (20)) and gas chromatography respectively. Results are presented in table 1.

Of the reagents tested, 50% piperidine/DMF is by far the best catalyst for DKP formation. The use of 0.1M EDIA/DCM does not represent any problem in synthesis on this resin, probably because it is a weaker base and is employed in a concentration much lower than that

Table 1

Reagent*	k(min^{-1})	k/kDCM
50% piperidine/DMF	$8.7x10^{-1}$	9300
0.05M AcOH/DCM	$2.2x10^{-2}$	230
0.1M Fmoc-Pro-OH/DCM	$2.1x10^{-2}$	220
0.1M EDIA/DCM	$2.3x10^{-4}$	2.4
DMF	$2.1x10^{-3}$	22
DCM	$9.4x10^{-5}$	1

*DMF=N,N-dimethylformamide; DCM=dichloromethane; AcOH=acetic acid; EDIA=ethyldiisopropylamine.



of piperidine (5.1M). The kinetics of DKP formation with acid catalysis is quite similar in the two cases studied (AcOH, Fmoc-Pro-OH), and the cyclisation side reaction will only be an important problem during the coupling step of the third amino acid when the dipeptide sequence strongly favours it. Finally, we can see that intramolecular aminolysis is more rapid in DMF than in dichloromethane.

As it is not possible to avoid the use of piperidine or of similar bases in a synthesis with Fmoc-amino acids, we have found necessary to get a better knowledge of the Fmoc cleavage reaction in order to define experimental conditions which ensure a quantitative deprotection with a minimal loss of peptide chains. For this study, a small amount of Fmoc--dipeptide-resin was suspended in a piperidine solution in an U.V. spectrophotometer cell, periodically stirred, and the deprotection was followed by the 301nm absorption data (table 2). The values of $t_{\frac{1}{2}}$ found for the DMF solutions are of the same order as those described in the literature (21, 22).

Table 2

	% piperidine/DCM						% piperidine/DMF	
	70	60	50	40	30	20	50	20
$t_{\frac{1}{2}}$(s)	13	15	20	36	50	90	6	10
$t_{0.95}$(s)	65	105	225	360	540	840	33	68

In view of these results, we repeated the determination of the DKP formation rate using 50% piperidine/DCM and 20% piperidine/DMF. DKP formation was also found to be very rapid ($7.5x10^{-1}min^{-1}$ and $9.8x10^{-1}min^{-1}$ respectively). Combining the deprotection and cyclisation kinetic data the amount of DKP formed after $t_{\frac{1}{2}}$ and $t_{0.95}$ can be calculated for each of these reagents (table 3). Considering these data we recommend the use of 50% piperidine/DMF to deprotect Fmoc-dipeptide-resins. A 5min treatment, which corresponds approximately to $10t_{0.95}$ and ensures a quantitative deprotection, is the best choice for most sequences (not particularly prone to DKP formation). This time can even be reduced with sequences which have a higher tendency to cyclisation, e.g. L-Val-L-Pro or L-Pro-Gly, though there is an increasing risk of incomplete deprotection (23).

Table 3

Reagent	$10t_{0.95}$(s)	%DKP	$t_{0.95}$(s)	%DKP	$t_{\frac{1}{2}}$(s)	%DKP
20% piperidine/DMF	680	100	68	67	10	15
50% piperidine/DMF	330	99	33	38	6	8
50% piperidine/DCM	2250	100	225	94	20	25

Finally, it is interesting to compare these results with other kinetic data already reported in the literature (8) (table 4). With the only exception of 50% piperidine/DMF treatment, the lowest DKP formation rates are found for the p-alkoxybenzyl ester resin 4 (the high rate observed in the piperidine treatment of 4 might be related with the possible intervention of an anionic intermediate during the deprotection (24)). This general behaviour points to a direct relationship between the ability to give intramolecular aminolysis and the electron-withdrawing character of the phenyl ring substituents.

Table 4

	H-D-Val-L-Pro-resin(a)			
Reagent	1	2	3	4
50% piperidine/DMF	5.9×10^{-1}	7.1×10^{-1}	-	8.7×10^{-1}(b)
EDIA/DCM (c)	3.5×10^{-4}	2.7×10^{-3}	1.0×10^{-1}	2.3×10^{-4}
Amino acid/DCM	2.7×10^{-2}	7.6×10^{-2}	1.4×10^{-1}	2.1×10^{-2}
0.05M AcOH/DCM	3.8×10^{-2}	1.1×10^{-1}	1.6×10^{-1}	2.2×10^{-2}
DCM	5.8×10^{-4}	7.4×10^{-4}	9.2×10^{-3}	9.4×10^{-5}

(a) 1: resin=O-CH_2-polystyrene; 2: resin=O-CH_2-  -CH_2-CO-NH-CHØ-polystyrene;
3: resin=O-CH_2-  -CO-NH-CHØ-polystyrene; 4: resin=O-CH_2-  -O-CH_2-polystyrene.

(b) From Fmoc-D-Val-L-Pro-O-CH_2-C_6H_4-O-CH_2-polystyrene.

(c) 0.3M EDIA/DCM for 1, 2 and 3; 0.1M EDIA/DCM for 4.

(d) 0.05M Boc-L-Pro-OH/DCM for 1, 2 and 3; 0.1M Fmoc-L-Pro-OH for 4.

Thus, it can be concluded that DKP formation is not a problem under most conditions with p-alkoxybenzyl ester resins. When such resins are used together with Fmoc-amino acids, DKP formation can be avoided or minimized in most cases by using the mild deprotection conditions recommended above. Our experience with the synthesis of some protected peptide segments is that there is either no DKP formation or a very small percentage (less than 4%) (25). In the case of difficult sequences, the problem can be overcome incorporating the second and third amino acids as a dipeptide, or, alternatively, avoiding the use of piperidine by protecting the α-amino function of the second amino acid with the 2-(4-biphenylyl)isopropyloxycarbonyl (Bpoc) group (26, 27).

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2.- Present adress: Department of Molecular Genetics, City of Hope Research Institute, 1450 East Duarte Road, Duarte, CA 91010, USA.
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25.-Deprotection of the dipeptide Fmoc-Tyr(2,6-dichlorobenzyl)-Gly-resin with 50% piperidine/DCM during 30min was accompanied by a loss of less than 2% of dipeptide (16). Using the mild deprotection conditions recommended in this paper DKP formation could not be detected with Fmoc-Ser(benzyl)-Gly-resin and a loss of dipeptide lower than 4% was measured in the case of Fmoc-Tyr(cyclohexyl)-Gly-resin (unpublished results)
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