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DIKETOPIPERAZINE FORMATION IN ACETAMIDO- AND NITROBENZAMIDO-BRIDGED POLYMERIC SUPPORTS.

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Summary: Diketopiperazine formation rates in deprotected Boc-D-Val-L-Pro-OCH_2-resin(**1**), Boc-D-Val-L-Pro-OCH_2-Pab-resin(**2**) and Boc-D-Val-L-Pro-OCH_2-Nbb-resin(**3**) have been studied under a variety of experimental conditions including those used on a solid phase peptide synthesis cycle.

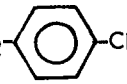
The formation of diketopiperazines (cyclic dipeptides) is a frequent secondary reaction in solid phase peptide synthesis[1-6]. In a classical study, Gisin and Merrifield[7] established that the reaction takes place during the incorporation of the third amino acid into the peptide sequence via a carboxyl-catalyzed intramolecular aminolysis. The rate of diketopiperazine (DKP) formation is strongly sequence-dependent. For most dipeptide sequences no care need be taken during the synthesis since the extension of this side reaction is virtually null. On the contrary, sequences like D-Val-L-Pro[7] , L-Pro-L-Pro[3,5,7] or L-Ala-L-Pro[3] form DKPs much more easily. DKP formation not only causes a decrease in the peptide synthesis yield but also generates hydroxyl sites on the polymer which can give rise to other secondary reactions[8]. Gisin and Merrifield focused their study on dipeptide-oxymethyl-polystyrenes. More recently, new polymer supports which improve the performance or the flexibility of the former oxymethyl-resin have been developped[9]. Thus, phenylacetamido-type bridges have been used to increase the acid stability of the peptide-resin linkage[10-13] avoiding secondary trifluoroacetylation of growing peptide chains[8] and improving synthetic yields for large peptides[14] , and nitrobenzamido-type bridges[15-17] allow the synthesis of fully protected peptides by photolytical cleavage of the peptide-resin bond[18]. The electron-withdrawing character of these spacers would at first sight seem to favor DKP formation. This work intends to evaluate the extent of this reaction in this kind of polymers at several instances of a standard synthetic cycle.

Pab-resin (α-(phenylacetamido)benzylpolystyrene)[12,13] and Nbb-resin (α-(3-nitrobenzamido) benzylpolystyrene)[17] have been chosen respectively as representative examples of phenylacetamido- and nitrobenzamido-type supports mentioned above. Samples of Boc-D-Val-L-Pro-OCH_2-resin(**1**), Boc-D-Val-L-Pro-OCH_2-Pab-resin(**2**) and Boc-D-Val-L-Pro-OCH_2-Nbb-resin(**3**) were deprotected and treated with a variety of reagents (Table 1) reported to form DKP, including those used in a solid phase peptide synthesis cycle, and the rate of formation of cyclo(D-Val-L-Pro) was monitored. Whereas the kinetic study of

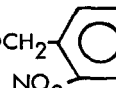
Table 1: Apparent rate constants (min^{-1}) for diketopiperazine formation in polymers[20]:

Boc-D-Val-L-Pro-OCH_2-resin

1

Boc-D-Val-L-Pro-OCH_2-  -CH_2-CO-NH-CH-resin

2

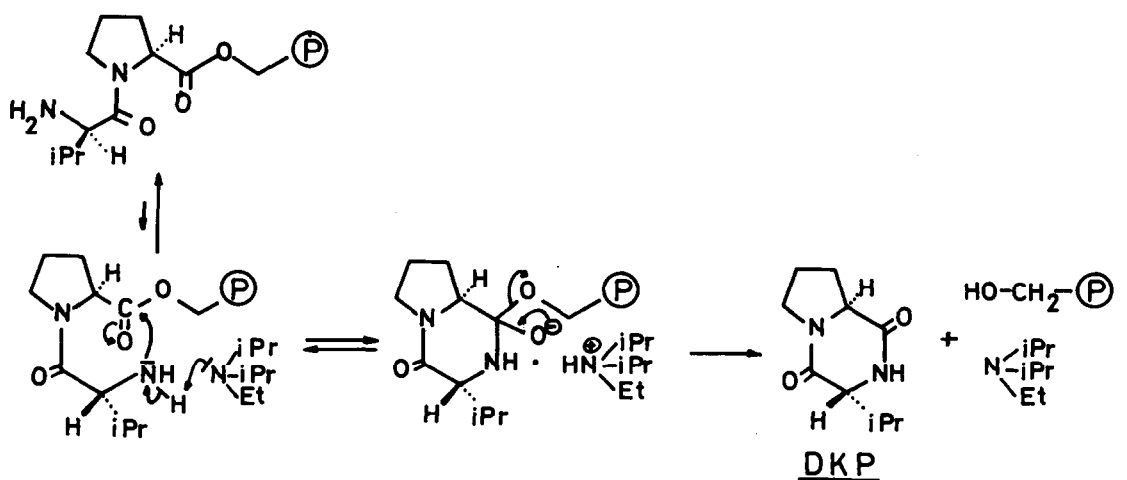
Boc-D-Val-L-Pro-OCH_2-  -CO-NH-CH-resin

(NO_2)

3

Reagent	Compound **1** (k_{rel}^{21})	Compound **2** (k_{rel}^{21})	Compound **3** (k_{rel}^{21})
0.06M trifluoroacetic acid in CH_2Cl_2	1.2×10^{-5} (0.021)	8.4×10^{-5} (0.14)	1.1×10^{-4} (0.19)
0.05M Boc-L-Pro in CH_2Cl_2	2.7×10^{-2} (47)	7.6×10^{-2} (130)	1.4×10^{-1} (240)
0.05M acetic acid in CH_2Cl_2	3.8×10^{-2} (66)	1.1×10^{-1} (190)	1.6×10^{-1} (280)
CH_2Cl_2	5.8×10^{-4} (1.0)	7.4×10^{-4} (1.3)	9.2×10^{-3} (16)
0.3M diisopropylethylamine in CH_2Cl_2	3.5×10^{-4} (0.60)	2.7×10^{-3} (4.7)	1.0×10^{-1} (170)
0.0005M acetic acid in CH_2Cl_2	3.4×10^{-3} (5.9)	2.0×10^{-3} (3.4)	2.3×10^{-2} (40)
0.01M acetic acid in CH_2Cl_2	2.7×10^{-2} (47)	5.0×10^{-2} (86)	1.5×10^{-1} (260)
0.05M acetic acid in CH_2Cl_2	3.8×10^{-2} (66)	1.1×10^{-1} (190)	1.6×10^{-1} (280)
0.1M acetic acid in CH_2Cl_2	4.0×10^{-2} (69)	7.5×10^{-2} (130)	1.6×10^{-1} (280)
1M acetic acid in CH_2Cl_2	7.3×10^{-3} (13)	2.0×10^{-2} (34)	5.8×10^{-2} (100)
5M acetic acid in CH_2Cl_2	6.0×10^{-4} (1.0)	2.4×10^{-3} (4.1)	8.0×10^{-3} (14)

Scheme 1



Gisin and Merrifield on oxymethyl-resins was carried out mainly by picric acid titration[19] of the basic groups remaining on the resin after DKP formation, in our case we have prefered direct gas chromatographic determination of the DKP amount detached from the polymer along time. The advantadges of this direct procedure are: i) Increased selectivity, which allows to discriminate DKP formation from other side reactions also modifying the amine-content of the resin; ii) the experimental data need not be corrected for DKP formation during the picric acid titration procedure (this formation is specially important in the case of Nbb-resin). All kinetic data were treated to give apparent first-order rate constants as shown in table 1. Some discrepancies with the previously reported rate constants[7] were observed when our analytical procedure was applied, for comparison purposes, to the standard oxymethyl-resin, specially in the presence of 0.06M trifluoroacetic acid (reaction 12-times slower) and 5M acetic acid (reaction 28-times slower). This difference probably arises from the acidolytical cleavage of the linear dipeptide from the resin, a reaction which, under these conditions, takes place simultaneously with DKP formation. Using the picric acid method, a combined rate constant for these two reactions higher than that of DKP formation is obtained.

The behaviour of polymers **1**, **2** and **3** in front of carboxylic acids is qualitatively the same. 0.05M acetic acid or 0.05M Boc-Pro-OH strongly favour DKP formation. On the contrary, 0.06M trifluoroacetic acid inhibits the reaction with respect to data from CH_2Cl_2, probably due to a great extent of protonation of the amino group. Plotting DKP formation rate constant vs acetic acid concentration a Gaussian-like curve with its maximum around 0.05-0.1M acid concentration is obtained for each polymer. All these results are consistent with a bifunctional catalysis by carboxylic acids as proposed by Gisin and Merrifield[17,22].

DKP formation rates for polymers **1**, **2** and **3** in diisopropylethylamine solution differ very acutely. For oxymethyl-resin **1** DKP formation is very small, of the same order that with CH_2Cl_2. On the contrary, rather high rates are found for Pab-resin **2** and even higher for Nbb-resin **3**. In this case the reaction probably proceeds via a base-assisted nucleophilic attack by the amino group, as shown in Scheme I. At first sight it may seem surprising that introduction of electron-withdrawing groups on the resin enhances only moderately the DKP formation rate in acidic medium (4 times from **1** to **3** in 0.5M AcOH) but very strongly in basic medium (300 times from **1** to **3** in 0.3M diisopropylethylamine). Nevertheless, it must be noticed that in basic medium phenylacetamido and nitrobenzamido bridges probably favour strongly both the addition and elimination steps. Nevertheless, even in the most favourable case (polymer **3**) DKP formation rate in diisopropylethylamine is still slightly lower than that found in acetic acid.

As far as application of Pab- and Nbb-resins to solid phase peptide synthesis is concerned two important remarks should be made: i) Since acid-catalysed DKP formation for these supports is a more serious side reaction than in standard oxymethyl-resin care should be taken, regardless of the sequence, at the incorporation of the third amino acid; ii) for Nbb-resin DKP formation may take place whenever the resin is in basic medium. Therefore, neutralization steps using diisopropylethylamine must

be shortened to the least, supressed or replaced by weaker treatments (e.g. reaction with an equimolar amount of N-methylmorpholine[23]. Use of Suzuki's method[24] normally suffices to circumvent both problems[18]. The preceeding remarks need not be strictely circumscribed to Pab- and Nbb-resins but can be more or less extended to other polymer supports having phenylacetamido or nitrobenzamido bridges such as those described by Sparrow[25], Li[26], Mitchell[10,11], Rich[15], Tjoeng[27] and Tam[16]. Finally, the ease of DKP formation in the case of Nbb-resins opens a possibility of using this resin for the synthesis of cyclic peptides which is currently being explored in our laboratory.

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20.- **1**, **2** and **3** were prepared following standard solid phase peptide synthesis procedures. Samples of ca. 100 mg of each polymer were deprotected, neutralized and treated at 20ºC with 3 ml of each reagent shown in the table. After filtering and washing (except for the very fast reactions) the resin was treated again with a fresh bath of the same reagent. 1 ml of a 0.05% n-butyl stearate acetone solution was added as internal standard to the combined filtrate and washings. The resulting solution was evaporated to dryness and analyzed by gas chromatography using a 2mx4mm 1%OV-210,2%OV-17 in Gas-Chrom Q(100-120) glass column at 210ºC flushed with a high He flow rate (100 ml/min) in order to prevent DKP decomposition. Kinetic data were treated as a pseudo-first order process.
21.-Relative to polymer **1** in CH_2Cl_2
22.-In oxymethyl-resin the acceleration found with weak acids without the carboxyl groups was invariably smaller than with carboxylic acids[7]. In our study the rate constant found for polymer **2** with 0.06M p-nitrophenol in CH_2Cl_2 was 1.5×10^{-2}.
23.-For the polymer **3** the rate of DKP formation in 5% N-methylmorpholine/CH_2Cl_2 is 6 times lower than in 5% diisopropylethylamine/CH_2Cl_2 (0.3M)
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