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MIXED ANHYDRIDES IN PEPTIDE SYNTHESIS. FACTORS AFFECTING URETHANE FORMATION AND RACEMIZATION

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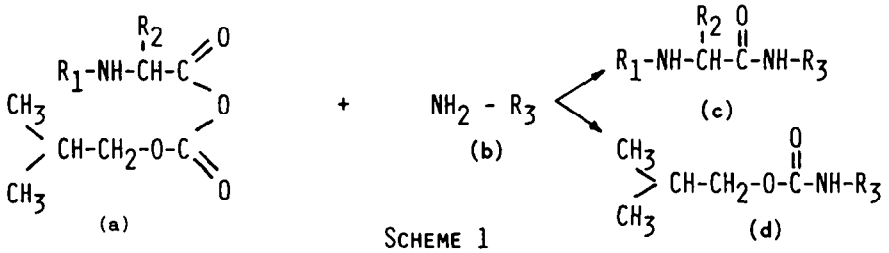
Abstract.- A comparative study on the effectiveness of two mixed anhydrides procedures in the synthesis of a series of hydrophobic tripeptides by using three solvents and four different hydrophobic nucleophiles has been carried out. Dicyclohexylcarbodiimide (DCC) method has also been used as reference for comparative purposes. The results show that despite the system Dpp/DMF provides the best chemical yields with these specific couplings, when retention of configuration is concerned the coupling method of choice should be DCC/HOBt using either THF or DMF as solvent system.

It is well known that mixed carboxylic-carbonic anhydrides (a) are very useful for acylation of amines (1). Aminolysis by the nucleophile (b) yields the peptide (c) but unfortunately, a second acylation product (d), an urethane, produced by the attack of the nucleophile on the carbonic acid carbonyl (ii), can be formed.



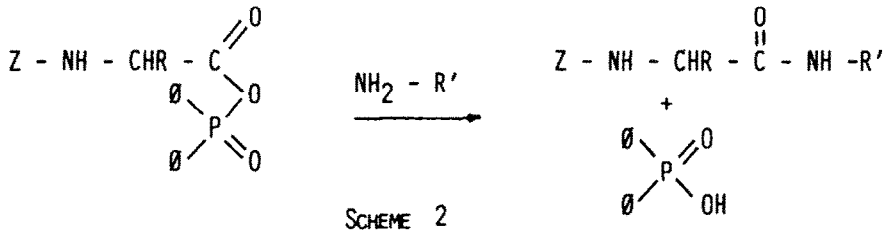
SCHEME 1

The amount of urethane can be controlled, to some extent, by the proper election of the mixed anhydride reagent. Thus, isobutyl chloroformate proved to be more efficient than ethyl chloroformate in its ability to yield the peptide. This is due to the decrease in the electrophilic character of the adjacent carbonyl carbon (ii), induced by the electron release of the isobutyl group, favouring as a consequence the formation of the peptide (c). Secondary amines usually react with mixed carboxylic-carbonic anhydrides to form a mixture of urethane and amide; the former may predominate depending on the compounds involved. In this respect, when the nucleophile is proline, which is a secondary amine with its nitrogen atom situated in a five membered ring, a considerable amount of urethane can be reasonably expected.

Another factor affecting the extent of urethane formation could be the solvent polarity. Tarbell et al. (2) studied the influence of this factor in a series of mixed anhydrides reactions, but they failed to find a striking change that could be related to the dielectric constant of the solvent. However, Benoiton et al. (3), found that the extent of urethane formation is primarily dictated by the amine-solvent combination. By comparing three solvents: tetrahydrofuran (THF), dimethylfor-

mamide (DMF) and dichloromethane (DCM), he concluded that the DCM was the best solvent for minimizing the urethane formation, the N-methylpiperidine (NMP) being slightly better than the more usual N-methylmorpholine (NMN).

To overcome urethane formation, Jackson et al. (4), introduced a new class of mixed anhydrides, the diphenylphosphinic mixed anhydride (Dpp), which could react with nucleophiles specifically, giving only the peptide, being in consequence particularly useful for the activation of hindered amino acids (5).



SCHEME 2

In our hands and despite the absence of urthane formation no higher chemical yields when compared with the above mentioned procedures were obtained.

As we were interested in the couplings:

Z-GLY-L-PHE + L-PRO-R ⟶ Z-GLY-L-PHE-L-PRO-R

R: OME, $NH(CH_2)_5\ CH_3$, $NH(CH_2)_9\ CH_3$, $NH(CH_2)_{13}\ CH_3$

SCHEME 3

which are intermediates in the synthesis of a series of hydrophobic enkephalin derivatives, that can suffer from partial racemization in the Phe residue, and since in our preliminary work we found considerable differences in the tripeptide's yields and byproduct formation, depending on the coupling method, solvents and the alkyl chain length, we decided to undertake an exhaustive study of this coupling step.

The design of our experiment was directed to determine the influence of several parameters: solvents, terminal chain length and acylation reagents, on the effectiveness of the afore mentioned coupling, considering both the chemical yields and the stereochemical purity of the tripeptides involved. The effect of temperature was not considered, since in order to prevent disproportionation and racemization, experiments were carried out at low temperatures (-15 or -20ºC). As far as the election of solvents was concerned, we decided to use DCM, THF and DMF whose dielectric constants (ε) and dipolar moments (μ) are: DMF (ε=36.7, μ= 3.8), DCM (ε = 8.9, μ = 1.5), THF (ε= 7.4, μ=1.7), (μ values refered in Debyes). Hexyl, decyl and tetradecyl amines were previously linked to the proline's carboxyl in order to determine how the terminal alkyl chain length could influence the reactivity of a secondary cyclic amine. In this respect, a non sterically hindered derivative, Pro OMe, was used as a reference for comparative purposes. We chose the standard DCC (dicyclohexyl carbodiimide) and two mixed anhydrides: the classical one, isobutylchloroformate and the diphenylphosphinic anhydride, as coupling reagents. Using these methods the expected byproducts were: the acylurea in the case of DCC and the corresponding urethane with the isobutyl chloroformate procedure. No byproducts can be formed with the Dpp anhydride. Nevertheless, with the DCC method and due to the fact that the experiments were carried out in the presence of HOBt and at low temperature, formation of acylurea was not detected.

The results of the coupling step for the three proline alkyl amides as well as for the Pro-OMe according to the different procedures and for the three solvents, are given in the Table 1.

TABLE 1

Formation of tripeptides and second acylation products in the reaction Z Gly Phe + Pro-R

Compounds	Solvents	Methods				
ZGly-Phe-Pro-R		Isobutyl chloroformate			Dpp	DCC/HOBt
		% PEPT	% URETH	RATIO	% PEPT	% PEPT
R= OMe	THF	85.3	13.9	14.0	71.6	80.9
	DCM	76.4	16.6	17.8	69.9	87.9
	DMF	86.0	13.0	13.1	85.0	88.0
R= $NH(CH_2)_5\ CH_3$	THF	78.0	20.1	20.4	74.0	78.0
	DCM	75.0	24.0	24.2	70.0	77.0
	DMF	77.0	19.0	19.8	87.0	89.0
R= $NH(CH_2)_9\ CH_3$	THF	68.1	26.5	28.0	65.8	77.2
	DCM	65.0	29.5	31.2	63.8	72.7
	DMF	67.0	29.8	30.7	80.0	79.1
R= $NH(CH_2)_{13}CH_3$	THF	68.5	28.5	29.4	66.0	77.0
	DCM	66.7	30.5	31.4	62.5	70.5
	DMF	65.7	33.1	33.5	84.0	77.6

The data shown in Table 1 clearly show that taking into account only chemical yields, DMF is the best solvent when using the Dpp anhydrides as activating agents for the four couplings studied. It must also be pointed out that in the case of the more hydrophobic compound the system Dpp/DMF is the one providing better chemical yields. On the other hand, DMF also gave good yields when using the DCC/HOBt method, the results for the less hydrophobic compounds being within the same range of those obtained with the Dpp anhydrides, though a slight decrease in chemical yields for the more hydrophobic derivatives can also be observed. In the case of the isobutyl chloroformate the three solvents proved to be quite similar, increasing the percentage of urethane with the hydrophobicity of the peptide.

Despite the solvent effect does not seem to be very critical with this mixed anhydrides, the high percentage of urethane formation that we have found compared to that obtained by Bodanszky et al. (6) for hindered amino acids, under the same conditions, is not only due to the hydrophobic C-terminal chain, but to the secondary cyclic amine character of Proline itself. On the other hand our results are more close to those obtained by Benoiton et al. (3) with more sterically hindered amino acids.

Nevertheless, as far as optical purity is concerned, both Dpp and isobutyl chloroformate mixed anhydrides induced a partial racemization of the Phe residue in the four couplings studied. In the case of the C_{14} derivative(the more hydrophobic one) the extent of racemization is given in Table 2.

TABLE 2

Racemization of the Phe residue in the synthesis of ZGly-L-Phe-L-Pro-NH$(CH_2)_{13}$-CH_3

Coupling Method	Solvent	% racemization
Isobutyl chloroformate (MA)	THF DCM DMF	2 6.0 5.0
Dpp (MA)	THF DCM DMF	6.0 6.2 6.0
DCC/HOBt	THF DCM DMF	1 2 2

On the contrary, DCC/HOBt provides an almost complete absence of racemization in the three solvents and for the four derivatives. Thus, regardless of the system Dpp/DMF provides the best chemical yields with these specific couplings, when a complete retention of configuration is concerned, the coupling method of choice should be DCC/HOBt; the suitable solvent, according to the chemical yields shown in Table 1, being either THF or DMF, especially for the more hydrophobic compounds.

EXPERIMENTAL

Melting points were determined in a Kofler apparatus and are reported uncorrected. Thin layer chromatography was performed on silica gel plates (0.25 mm) from Merck. The following solvent systems were used: 1) Chloroform/Acetic acid/MeOH (95:3:25) (CAM); 2) Butanol/Acetic acid/H_2O (4:1:1) (BAW) 3) Ethyl acetate.

Spots were detected by reaction with ninhydrin or chlorine followed by tolidine solution. Optical rotations were measured in a P.E. Spectropolarimeter 141. Elemental analysis were done in the Microanalytical Laboratory of the Bio-Organic Chemistry Institute. Amino acid analysis were performed in a Beckman 119 C instrument.

^1H n.m.r. spectra were obtained with a Brucker (80 MHz) spectrometer in $CDCl_3$. Chemical shifts are reported in δ units using tetramethylsilane as the internal standard. THF and DCM were distilled over CaH_2 and $CaCl_2$ respectively. DMF was dried over molecular sieve. In all cases the crude material after filtration of DCU or NMM hydrochloride was subjected to flash chromatography using ethyl acetate as eluent. Recoveries were practically quantitative and each value represents the average of three parallel experiments. The homogeneity of the tripeptides was assessed by reversed phase HPLC (ODS, 5 μm column, solvent system CH_3CN/H_2O-0.05% TFA, gradient elution from 9 to 100% CH_3CN at a linear rate of 3.5% min, λ = 220).

The standard procedure for the preparation of the three Prolin alkylamides have been described in a previous paper (7) and their analytical data are shown in Table 3.

TABLE 3

Physical properties of Proline alkylamides

Compound	m.p. ºC	$[\alpha]_D^{25}$ *	Rf_1	Rf_2
Z-Pro-C_6	67-68	+ 43.92	0.59	0.70
Z-Pro-C_{10}	87-88	+ 40.28	0.65	0.78
Z-Pro-C_{14}	80-81	+ 32.59	0.66	0.78
H-Pro-C_6	oil	+ 42.90	--	0.45
H-Pro-C_{10}	oil	+ 42.4	—	0.47
H-Pro-C_{14}	oil	+ 32.18	--	0.49

* c= 1, MeOH 1: CAM 2:BAW

Standard procedure for the preparation of tripeptides and for the isolation of second acylation products

a) Isobutyl chloroformate Method

Samples of Z-Gly-Phe (0,5 mmol) were disolved in the corresponding solvent (DMF or THF or DCM) 10 ml. After cooling the solution to -15ºC (dry ice/acetone), isobutyl chlorocarbonate (0,5 mmol) was added; the mixture was stirred at -15ºC for 90 seconds. A precooled (-15ºC), solution of the Proline derivative (0,5 mmol) in 2 ml, was then added to the formed mixed anhydride. The reaction mixture was stirred for 1 h at -15ºC and for 3 h at room temperature, filtered, and the evaporated residue submitted to flash chromatography. The recoveries were practically quantitative.

b) Diphenylphosphinic Mixed Anhydride Method

For this reaction we followed essentially the same steps described in a). The activation time was 20 min. and the reaction temperature was -20ºC for mixed anhydride formation. After adding the amine, the reaction mixture was kept for 3 h at room temperature and subjected to flash chromatography.

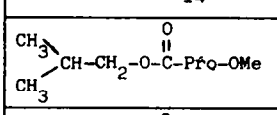
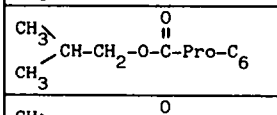
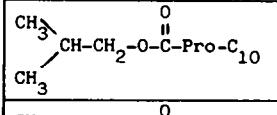
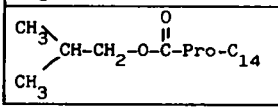
c) Dicyclohexylcarbodiimide Method

To a chilled solution (-20ºC) of Z-Gly-Phe (0,5 mmol) and Pro-R (0,5 mmol) in 10 ml of the corresponding solvent (THF, DCM or DMF), 1-hydroxybenzotriazole (0,5 mmol) and NN-dicyclohexylcarbodiimide (0,5 mmol) were added. The mixture was kept for 1 h at -20ºC and overnight at room temperature. NN -dicyclohexylurea was filtered and the solvent evaporated in vacuo and subjected to flash chromatography.

The four tripeptides and the corresponding proline-urethane were characterized by NMR, TLC; elemental analysis and amino acid analysis. The most important data are given in Table 4.

TABLE 4

Physical properties of peptides (Z-Gly-Phe-Pro-R) and urethanes formed

Compound	$[\alpha]_D^{22}$ (a)	Elem. Anal.			TLC		
		C	H	N	Rf_1	Rf_2	Rf_3
Z-Gly-Phe-Pro-OMe	-39.6	C: 64.24 F: 64.24	6.21 6.20	8.99 9.00	0.51	0.81	0.38
Z-Gly-Phe-Pro-C_6	-43.10	C: 67.16 F: 67.10	7.46 7.51	10.45 10.35	0.60	0.72	0.43
Z-Gly-Phe-Pro-C_{10}	-40.30	C: 68.91 F: 68.80	8.11 8.12	9.46 9.36	0.60	0.74	0.40
Z-Gly-Phe-Pro-C_{14}	-37.19	C: 70.37 F: 69.80	8.64 8.93	8.64 8.96	0.65	0.74	0.40
	-65.92	C: 57.64 F: 57.60	8.29 8.30	6.11 6.07	0.55	--	0.64
	-35.8	C: 64.43 F: 64.40	10.07 9.98	9.39 9.37	0.76	--	0.66
	-35.3	C: 67.79 F: 67.80	10.73 10.70	7.90 7.89	0.76	--	0.66
	-34.7	C: 70.24 F: 70.20	11.22 11.10	6.83 6.80	0.76	--	0.68

a) (c= 1, MeOH) 1: CAM 2: BAM 3: Ethyl acetate

The ^1H n.m.r. chemical shifts for the peptides and the corresponding urethans are as follow: ($CDCl_3$ TMS) Z Gly Phe Pro OMe δ : 1.9 (m, 4H); 2.8-3.2 (m, 4H); 3.7 (s, 3H); 4.1-4.7 (m, 4H); 5.1 (2, 2H); 5.5-5.8 (m, 3H); 7.2 (s, 5H); 7.3 (s, 5H); Z Gly Phe Pro NH $(CH_2)_5-CH_3$ δ : 0.9 (t, 3H); 1.2 (s, 8H); 1.9-2.1 (m, 4H); 3.0-3.4 (m, 4H); 3.6 (m, 4H); 3.9 (m, 2H); 5.0 (s, 2H); 6.8 (m, 4H); 7.1 (s, 5H); 7.3 (s, 5H); Z Gly Phe Pro NH $(CH_2)_9-CH_3$ δ : 0.8 (t, 3H); 1.3 (s, 16H); 2.0 (m, 4H); 3.0-3.2 (m, 4H); 3.6-4.3 (m, 6H); 5.1 (s, 2H); 6.7 (m, 1H); 6.8 (m, 2H); 7.2 (s, 5H); 7.3 (s, 5H); 7.6 (m, 1H); Z Gly Phe Pro NH $(CH_2)_{13}-CH_3$ δ: 0.8 (t, 3H); 1.2 (s, 24 H); 2.0 (m, 4H); 2.9-3.2 (m, 4H); 3.3-4.0 (m, 6H); 5.0 (s, 2H); 5.5 (m, 2H); 6.7 (m, 2H); 7.2 (s, 5H); 7.3 (s, 5H); $(CH_3)_2$ $CH-CH_2OCO$ NH Pro OMe δ: 0.9 (d, 6H); 1.2 (m, 1H); 2.0 (m, 4H); 3.5 (m, 2H); 3.7 (s, 3H); 3.9 (d, 2H); 4.3 (m, 1H); 4.6 (m, 1H); $(CH_3)_2CH-CH_2-OCONH$ Pro NH $(CH_2)_5CH_3$ δ: 0.9 (d, 6H); 1.2 (s, 9H); 1.9 (m, 4H); 2.5 (m, 2H); 3.0-3.6 (m, 4H); 3.8 (d, 2H); 4.2 (m, 1H); $(CH_3)_2-CH-CH_2-OCONH$ Pro NH $(CH_2)_9$ CH_3 δ: 0.9 (d, 6H); 1.3 (s, 17H); 1.9 (m, 4H), 3.1-3.6 (m, 4H); 3.9 (d, 2H); 4.3 (m, 1H); 7.4 (m, 2H); $(CH_3)_2-CH-CH_2-OCONH$ Pro NH $(CH_2)_{13}-CH_3$ δ: 0.9 (d, 6H); 1.2 (s, 25H); 1.9 (m, 4H); 3.2 (m, 2H); 3.5 (m, 2H); 3.8 (d, 2H); 4.2 (m, 1H); 7.6 (m, 2H).

Racemization tests

Two different racemization tests, the L-amino acid oxidase and gas chromatography on a chiral stationary phase were used. Since the limit of error with the L-amino acid oxidase test is 2%, the precise extent of racemization was ascertained by GC.

a) L-amino acid oxidase

10 µmol of the corresponding peptide were hydrolysed with 6 N HCl and a small crystal of phenol for 24 hours in a sealed glass tube. The dried hydrolysate was dissolved in 2.5 ml of 0.2 M "Tris buffer", pH 7.52. To 100 µl of that solution, 100 µl of the L-amino acid oxidase (Crotalus Adamanteus) solution, 20 mg/ml, 6.8 units/mg. Prot, from Sigma Chem. Company and 10 µl of toluene were added, and the mixture shaken in an oxigen atmosphere at 37ºC for 24 hours (8). 50 µl of this solution were injected into the amino acid analyzer.

b) Gas chromatography

The hydrolysed peptides were derivatized according to the Kaiser et al. (9) procedure and subjected to GC in a Hewlett-Packard instrument (HP-5840 A) equipped with a capilar glass column coated with a chiral stationary phase: Cyano-ethyl-siloxane-L-valine-S-α phenyl ethylamide. The analysis was performed in the isothermal mode at 90ºC for 5 min., gradient mode up to 170ºC at 4ºC/min rate, and kept at 170ºC for 10 min.

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