Tetrahedron Vol. 43, No. 17, pp. 4031 to 4034, 1987
Printed in Great Britain.

0040-4020/87 $3.00+.00

MIXED ANHYDRIDES IN PEPTIDE SYNTHESIS. INFLUENCE OF THE SOLVENT AND THE AMINE ON THE RACEMIZATION OF DMet RESIDUE OF AN ENKEPHALIN SEQUENCE.

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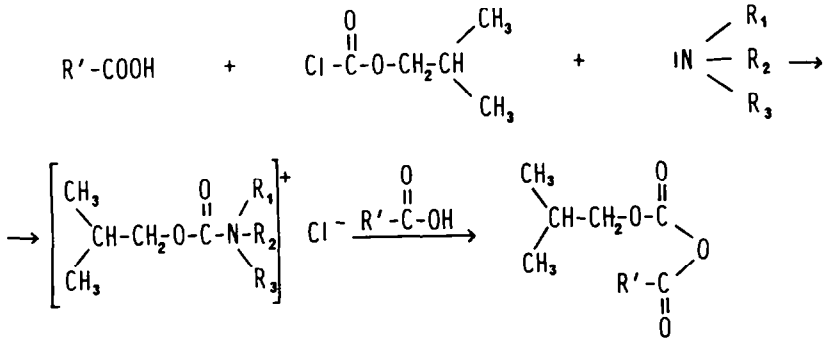
(*Received in UK* 9 *June* 1987)

Abstract - A comparative study on the effectiveness of all possible amine/solvent combination with three different solvents (THF, DCM, DMF) and three different bases (NMM, NMPi, NMPo) in avoiding the racemization of the DMet residue during a mixed anhydride coupling has been carried out. The results show that the best combination is THF/NMM. Though, when more polar solvents are required, the system DMF/NMPo is recommended.

Since its simultaneous introduction in 1951 by Wieland and Bernhard (1) and Boissonnas (2) and Vaughan (3) mixed anhydrides have been widely used in peptide synthesis.

One of the main advantages of this method is the high reaction rates at low temperatures and the comparatively high purity of the products. But despite these useful features racemization and urethane formation can represent a serious problem in some sequences owing to the high reactivity of the mixed anhydrides.

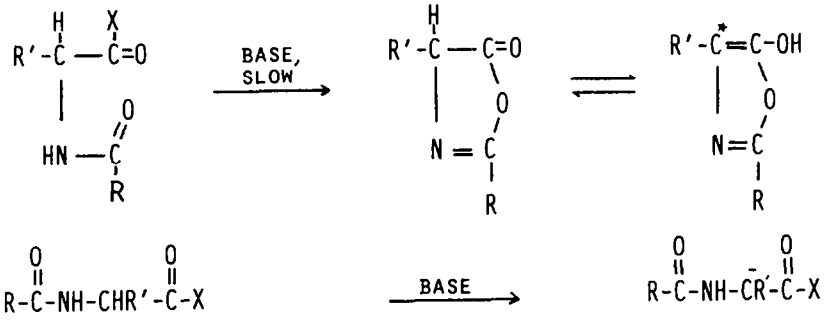


Scheme 1

Among the factors affecting the optical purity of the resulting peptide, amine basicity and solvent polarity are the easiest to manipulate in order to prevent this unwanted side reaction. Amine plays in the reaction a double role,because it affects two separate processes: anhydride generation and

racemization. As Anderson (4) pointed out, the tertiary amine is not merely a hydrogen chloride aceptor, but reacts with the chlorocarbonate to form an alkyloxicarbonylammonium ion complex which subsequently reacts with the carboxylate. Thus, sterically hindered amines give lower yields than less hindered ones.

Regarding steric requirements it has been demonstrated that amines with at least one methyl group turned out to be the best suited for avoiding racemization, but the main factor to affect optical purity of the resulting peptide is the amine basicity. Scheme 2 shows the two main mechanisms proposed for amine induced racemization.



Scheme 2

In a detailed study carried out by Benoiton (5) on this subject, it was shown that NMPi leads to less racemization during coupling of protected peptide acids than NMM. But in general the overall yield of the reaction and the optical purity of the products seem to be strongly dependent on the amine/solvent combination (5,6).

In a recent work carried out in our laboratory (7), we found that DMF and THF in combination with NMM gave better chemical yields than DCM, when the amino component was proline methyl ester, and regarding the optical purity THF/NMM was superior to both DMF and DCM. So it seems that an intermediate value of solvent polarity could be the best choice for obtaining good yields and opticaly pure products.

The negative effect of DMF on the racemization process was also detected by Kemp working with p-nitrophenyl esters of protected aminoacids and could be a serious drawback when strongly polar solvents are needed (8).

Trying to know deeply the effect of the solvent and the amine basicity on the yields and racemization in peptide synthesis we were engaged in the study on the influence of these factors on the synthesis of an enkephalin analogue fragment:

Boc-D-Met + Gly-Phe-OMe ⟶ Boc-D-Met-Gly-Phe-OMe

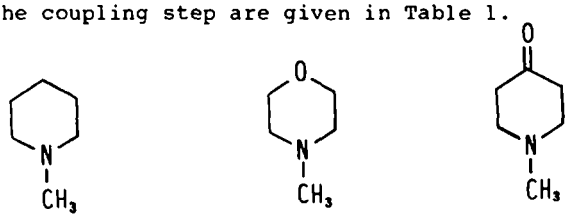
This tripeptide was previously synthesized by the REMA method (repetitive excess mixed anhydride),and racemate analysis showed a 3-4% of L-Methionine isomer.

In the present work in order to separate steric effects from basicity ones we have chosen three amines having nearly the same steric pattern but different pK values: NMM 7.4; NMPo 7.9; NMPi 10.1. (9) (Scheme 3).

In a similar way the solvents of choice have been: DCM (ϵ= 8.9, μ=1.5), THF (ϵ=7.4, μ=1.7) and DMF (ϵ=36.7, μ=3.8); (values refered in Debyes).

The experiments were designed in the following manner. Couplings of Boc-D-Met + Gly-Phe-OMe, were carried out by means of isobutylchloroformate mixed anhydrides, in triplicate runs, for each pair of variables: solvent and amine. After each coupling the amine hydrochloride was filtered, the reaction solvent was evaporated in vacuum and the remaining oily residue was disolved in ethyl acetate and submitted to Flash Chromatography. In this way we were able to quantify by weight the formed tripeptide, and the corresponding urethane formed as byproduct. No other byproducts except in some cases small amounts of the initial products were observed.

The results of the coupling step are given in Table 1.



NMPi NMM NMPo

Scheme 3

Table 1. Yields of both peptide and urethane and racemization of D Met.

Amine	pKa	Solvents	%Pept	%Urethane	%Racemization	
		THF	91.6	2.7	0.3	0.1
NMM	7.4	DCM	95.5	0.9	0,7	0.1
		DMF	77.7	7.7	1.4	0.2
		THF	91.6	2.4	0.93	0.04
NMPo	7.9	DCM	76.6	6.7	4.3	1.7
		DMF	77.5	7.9	0.6	0.1
		THF	84.6	3.6	0.7	0.2
NMPi	10.1	DCM	90.0	1.8	0.56	0.04
		DMF	70.4	10.7	0.9	0.1

The results show that it is possible to find an ideal combination of solvents and bases that afford the desired peptide in a degree of racemization lower than 1% , being the THF/NMM the best of all (0.3% L-DMet).

However when a strongly polar solvent would be necessary the use of NMPo is recomended. In any case the use of DMF as solvent implies chemical yields lower than 80%.

The statistical analysis of the data show that DCM and NMPo give the greatest dispersion, being on the contrary THF, DMF and the base NMPi the reactants that give lower dispersion of results.

As conclusion we can say that as far as this sequence and this method of coupling are concerned the optimal combination is THF / NMM.

Moreover, as in this case the steric characteristics of the amines are very similar we can conclude that amine basicity influences not only chemical yields but also the optical purity of the final peptides.

EXPERIMENTAL

Thin layer chromatrography was performed on silica gel plates (0.25mm) from Merck. The following solvent systems were used 1) CMA, Chloroform/Acetic Acid/MeOH (95:3: 5), 2) Ethyl Acetate. Spots were detected by reaction with ninhydrin or chlorine followed by toluidine solution.

Optical rotations were measured in a P.E.141. Spectropolarimeter. Aminoacid Analysis were performed in a Beckman 119C instrument. ^{1}H-NMR spectra were obtained with a Brucker (80 MHz) spectrometer in $CDCl_3$. Chemical shifts are reported in δ-units, using tetramethylsilane as internal standard. THF was distilled and stored over molecular sieve and inmediately before use a stream of helium was passed through for 10 minutes.

N-methylmorpholine, N-methylpiperidine and N-methylpiperidone were purchased to Fluka, and were used directely, without previous purification.

Isolation and purification of the peptides and the corresponding urethane were carried out by "Flash Chromatography" (10), using Silica Gel (40-63 μm), a (15x2 cm) column, and eluting with ethyl acetate, at a flow rate of 5 cm/min.

The fractions collected were of 10 ml; the urethane eluted in the 4-5th fraction and the peptide from the 7th.

The purity and homogeneity of peptide and urethane were checked by HPLC-reversed phase (ODS, 5 μm column, H_2O 0,05% TFA/ ACN, gradient elution from 9 to 100% CH_3CN at a linear rate of 3,5% ACN/min, λ= 220 nm).

Synthesis of the tripeptide BocDMet-Gly-PheOMe.

Samples of Boc-DMet (216 mg, 0.87 mmol) and the same amount of the base were dissolved in 12 ml of the corresponding solvent (DMF, DCM or THF). The solution was cooled to -15° C (dry ice-acethone) and isobutylchlorocarbonate (0.113 ml, 0.87 mmol) was added; the mixture was stirred at -15°C for 90 seconds. A precooled (-15°C) solution of Gly-Phe-OMe derivative (238 mg, 0.87 mmol) and the equimolecular amount of base (0.87 mmol), in 4 ml of solvent was then added to the recently formed mixed anhydride. The reaction mixture was stirred for 1h at -15° C and 3h at room temperature, filtered and after solvent evaporation the remaining oily residue was submitted to flash chromatrography, in the conditions described before.

The two main products obtained were the tripeptide and the urethane.

BocDMet-Gly-PheOMe was an amorphous solid melting between 20-30° C. Aminoacid analysis: Met: 0.89; Gly: 1.00; Phe: 1.05; $[\alpha]$ = +10.1 (c=1, MeOH); TLC: Rf_1=0.5; Rf_2=0.4; HPLC: Retention time 15.2 min, k=6.6. (ODS, 10μ column, H_2O, 0,05% TFA, ACN 70:30, 1ml/min λ=220nm).

$(CH_3)_2$-CH-CH_2-OCO-Gly-PheOMe (urethane). The product was an oil. Aminoacid Analysis Gly: 1.00; Phe: 1.07; $[\alpha]$ = +14.4 (c=1, MeOH); TLC: Rf_1=0.55; Rf_2=0.45; HPLC: Retention time 17.0 min, k=7.5. (the above described conditions).

The ^{1}H-NMR chemical shifts for the peptide and the urethane are as follow: (CDCl , TMS)

Boc-DMet-Gly-PheOMe δ: 7.25 (arom., Phe); 3.85 d (-CH_2, Gly); 3.65 s (-O-CH_3, ester); 3.10 d (-CH_2, Phe); 2.00 s (-S-CH_3, Met); 1.40 s (tertbut, Boc).

$(CH_3)_2$-CH-CH_2-OCO-Gly-PheOMe δ: 7.25 (arom, Phe); 3.85 d (-CH_2, Gly); 3.65 s (-O-CH_3, ester); 3.10 d (-CH_2 , Phe); 2.50 m (-CH_2-O-CO-); 1.20 m (-CH-); 0,9 d ($(CH_3)_2$CH-).

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.

As some amount of racemization can be produced during acid hydrolysis , a paralel set of experiments has been carried out by submitting pure aminoacid to the same hydrolisis process than peptides. In this way we could know more accurately the real extent of the racemization due to the activation and condensation processes.

a) L-AMINO ACID OXIDASE

10 μmol of the corresponding peptide were hycrolysed 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, 100μl 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 oxygen atmosphere at 37°C for 24 hours (11). 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. (12) procedure and subjected to GC in a Hewlett-Packard instrument (HP-5840 A) equipped with a capilary glass column coated with a chiral stationary phase: Cyano-ethyl-siloxane-L-valine-S-α phenyl ethylamide. The analysis was performed in the isothermal moce at 90°C for 5 min., gradient mode up to 170°C at 4°C/min rate, and kept at 170°C for 10 min.

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