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AMIDE HYDROGEN EXCHANGE RATES OF MODEL PEPTIDES IN DIMETHYL SULPHOXIDE SOLUTIONS

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Abstract - Proton transfers from and to the nitrogen atom (s) α (or α and β) of a doubly protected dipeptide. Gly-Gly or PG_2. (or tripeptide : Gly-Gly-Gly or PG_3) in anhydrous DMSO was followed by DMNR under conditions of both acid (sulphuric) and base (tetramethylguanidine) catalyzed exchange. Two kinetic processes are observed. one catalyzed by the hydrogen ion and the other by the conjugate base (P^-) of the peptide (P). The results at 25°C are summarized by the equation. rate of peptide proton exchange = $k_H[H^+] + k_B[P]/[H^+]$. with k_H = 6.3 ; 17.1 ; 8.85 $M^{-1}s^{-1}$ and k_B = 1.45 ; 5.62 ; 9.55 x $10^{-13}s^{-1}$ for $PG_2(\alpha)$ and PG_3 (α and β). respectively. This allows us to predict maximum half-reaction times for NH-NH (or NH-ND) exchanges of *ca.* 10^4 mn at pH 7 to 8 in DMSO. pK values of N-protonation ($pK_{NH_2^+}$) are estimated from log k_H. and pK values of N-deprotonation (pK_{NH}) are measured by three independent methods. pK_{NH} = 19.4 ; 19.7 ; 19.1 (± 0.2) for PG_2 and PG_3 (α and β). Compared to N-methylacetamide. $pK_{NH_2^+}$ and log k_H of peptides are decreased by *ca.* two units. while pK_{NH} and log k_B are decreased and increased by *ca.* four and two units. respectively.

Résumé - La protonation et la déprotonation de la liaison NHα d'un dipeptide Gly-Gly doublement protégé (PG_2) ou des liaisons NHα et β d'un tripeptide Gly-Gly-Gly (PG_3) sont étudiées par RMN dynamique dans le DMSO acide (H_2SO_4) ou basique (tétraméthylguanidine). Deux processus cinétiques sont mis en évidence : l'un catalysé par l'ion hydrogène et l'autre par la base conjuguée (P^-) du peptide (P). Les résultats à 25°C sont représentés par l'équation : vitesse d'échange protonique = $k_H[H^+] + k_B[P]/[H^+]$ où k_H = 6.3 ; 17.1 ; 8.85 $M^{-1}s^{-1}$ et k_B = 1.45 ; 5.62 ; 9.55 x $10^{-13}s^{-1}$ pour PG_2 (α) et PG_3 (α et β). respectivement. Ces valeurs permettent de prédire des temps de demi-réaction maximaux d'environ 10^4 mn à pH 7 à 8 dans le DMSO. Les pK de protonation sur l'azote ($pK_{NH_2^+}$) sont estimés à partir des constantes k_H et les pK de déprotonation pK_{NH}) sont mesurés selon trois méthodes indépendantes. pK_{NH} = 19,4 ; 19,7 ; 19,1 (± 0.2) pour PG_2 et PG_3 (α et β). Comparativement à la N-méthylacétamide, les valeurs $pK_{NH_2^+}$ et log k_H des peptides sont diminuées d'environ 2 unités, tandis que pK_{NH} et log k_B sont diminuées de quatre unités et augmentées de deux unités environ. respectivement.

INTRODUCTION

Acid-base properties of nitrogen atoms in amides and peptides are still poorly documented[1-3]. pK values for deprotonation (pk_{NH}) or protonation ($pK_{NH_2^+}$) of nitrogen in these compounds cannot be measured by standard methods in aqueous solutions due to the levelling power of water and to the predominant protonation of the adjacent carbonyl in acidic conditions. $pK_{NH_2^+}$ is estimated to be strongly negative while pK_{NH} is assumed to lie above the limiting value of 14. In a previous paper[4]. we reported on the acid and base catalyzed proton exchange rates of N-methylacetamide (NMA) in water and in dimethyl sulphoxide (DMSO). Kinetic data obtained in DMSO allowed us to estimate $pK_{NH_2^+}$, while pK_{NH} was measured directly as the pH at half neutralization using dimsyl anion as a base.

The wealth of information obtained from the study of NMA in DMSO prompted us to extend these investigations to two simple peptides assembling two or three glycyl residues. DMSO is commonly used for NMR

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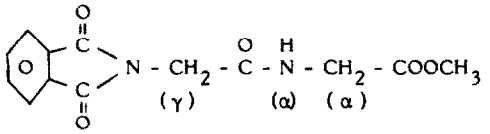
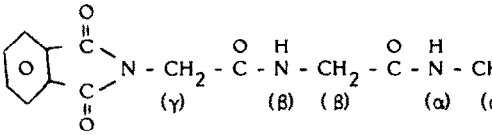
studies of peptides since amide exchange is slow in this medium[5]. DMSO/H_2O mixtures have been used to characterize strong internal or loose intermolecular hydrogen bonds in polypeptides by observing slow or fast NH-ND exchange rates, respectively[5-8]. In our investigations, parasitic proton transfers on both ends of the above peptides were prevented by protecting the terminal amine function as a phthaloyl derivative and the terminal carboxyle as a methylic or ethylic ester. Two doubly protected peptides were studied, the phthaloyl-diglycil methyl ester (PG_2) and the phthaloyl-triglycyl ethyl ester (PG_3) :



(PG_2)



(PG_3)

Protection of the basic and acidic ends of peptides by N-acetylation and N-amidation, respectively, as described by Molday [9] in previous investigations of aqueous solutions of peptides, was not adopted for our studies so as to avoid the simultaneous occurence of proton transfers on the terminal NH bonds of the protecting groups and on the central peptide NH bonds.

This was also the reason why we did not use the more classical benzyloxycarbonyl[10] (Z) and ter- butyloxycarbonyl[11] (BOC) protecting groups. Phthalimide derivatives are however also employed in a great number of syntheses [12-16], being removed by hydrazinolysis without damage to the peptides[17]. The N-phthaloyl group also contributed to make the peptide compounds insoluble in water, this was of a great help to extract the pure compounds from their aqueous solutions. In counterpart, the phthalimide derivative proved to be very fragile in strongly basic solutions, making pK measurements very delicate. We describe in the following the behaviour of the above two peptides in acidic and basic DMSO from spectrophotometric and NMR measurements.

EXPERIMENTAL SECTION

The Peptides

The procedures used for peptides synthesis derive from those worked out by Castro *et al.*[18], using the BOP reagent. This reagent allowed us to prepare relatively large quantities of products due to short reaction times and high yields (more than 80 %). Microanalyses of the products are in accord with molecular formulae. ^1H chemical shifts are in ppm from internal TMS in DMSO-d_6 solutions.

Preparation of Pht-Gly-Gly-OMe (PG_2)

To a solution of phthaloyl-glycine (0.5 mole, obtained according to reference 19) and glycyl methyl ester (0.5 mole) in acetonitrile (200 cm^3) are added triethylamine (1 mole), and then a solution of the BOP reagent [18] (0.5 mole) in acetonitrile (80 cm^3). After stirring the mixture for six hours at room temperature, the solvent is removed by evaporation under reduced pressure. The residue is added with 1N aqueous hydrochloric acid (150 cm^3) and is filtered off, washed twice with 2N hydrochloric acid (100 cm^3), twice with saturated aqueous sodium hydrogen carbonate (120 cm^3), and finally with distilled water (120 cm^3). The solid product is recrystallized from methanol. Yield : 110.4 g (80 %) ; m.p. 202°C (lit.[20] 201-202°C); NMR : δ 3.65 (s ; OCH_3). 3.9 (d ; J = 6 Hz ; α-CH_2). 4.3 (s ; γ-CH_2). 7.9 (m ; Pht.). 8.7 (t ; NH). The same procedure was employed to prepare the peptides mentioned below.

Pht-Gly-Gly-Gly-OEt (PG_3) (From 0.5 mole PG_2). Yield : 141.6 g (82 %) ; m.p. 230°C (lit.[21] 232°C) ; NMR : δ 1.2 (t ; J = 7.5 Hz ; CH_3) 3.85 (d ; J = 5.5 Hz ; α-CH_2). 3.79 (d ; J = 5.5 Hz ; β-CH_2). 4.1 (q ; J = 7.5 MHz OCH_2). 4.3 (s ; γ-CH_2). 7.9 (m ; Pht.). 8.33 (t ; J = 5.5 Hz ; α-NH). 8.57 (t ; J = 5.5 Hz ; β-NH)

Phthaloyl-glycyl-N-methylamide, Pht-Gly-NMA (PGM) (From 0.02 mole PG_2). Yield : 7.1 g (94 %) ; m.p.252°C NMR : δ 2.63 (d ; J = 5 Hz ; CH_3). 4.2 (s ; CH_2). 7.91 (m ; Pht.). 8.15 (m ; NH).

Phthaloyl-glycyl-glycyl-L-alanine methyl ester, Pht-Gly-Gly-Ala-OMe (PG_2A) (From 0.02 mole PG_2). Yield : 6.1 g (88 %) ; NMR : δ 1.32 (d ; J = 7.5 Hz ; CH_3(Ala)). 3.7 (s ; OCH_3). 3.8 (m ; CH(Ala)). 3.85 (d ; J = 7.5 Hz ; α-CH_2). 4.3 (s ; γ-CH_2), 7.95 (m ; Pht.). 8.36 (d ; J = 7.5 Hz ; α-NH). 8.57 (t ; J = 6 Hz ; β-NH).

Materials

DMSO solutions were prepared according to procedures described previously[4,22]. Acidic and basic solutions used to promote proton transfers were obtained by addition of H_2SO_4 or CF_3SO_3H and of tetramethylguanidine, respectively. pH measurements require larger quantities of strong bases, namely dimsyl anion, or tert-butyllithium (Aldrich), or sodium hexamethyldisilazane (Fluka) and lithium diisopropylamide. The latter

base was prepared by slowly adding n-butyllithium in hexane (28 cm^3) to a solution of diisopropylamine (4.6 g) in tetrahydrofuranne (20 cm^3) at - 50°C under argon. The base was titrated using a standardized solution of menthol in tetrahydrofuranne and O-phenanthroline as an indicator.

pH Measurements

pH measurements were carried out using an absolute pK scale in DMSO described previously[4]. The Hammett indicators required for the present investigations were : 3-nitroaniline (pK = 0.9) in the acidic range. and 6-bromo-3.4-dinitro- or 2.4-dinitro-. or 4-nitroanilines (pK = 13.4. 14.8 and 19.26. respectively) in the basic range[23].

NMR Spectroscopy

Proton spectra were recorded on a CAMECA spectrometer operating at 250 MHz and 25°C. Kinetic data were obtained as described in a previous publication . The coalescence of the N-methylene doublet (α) as a function of the pH was used to obtain rate constants for the exchange of the amide proton in PG_2. In the case of PG_3. two groups of closely spaced signals were obtained. corresponding to the existence of two peptide bonds, two sharp doublets and two broad triplets for α and β methylenic and NH protons. respectively (Fig. 1a). The two triplets were identified by comparing the spectrum of PG_3 with that of phthaloyl-glycyl-glycyl-alanine methyl ester (PG_2A). in which the α-NH is represented by a doublet and the β-NH by a triplet (Fig. 1b). This allowed us by comparison to assign the higher and lower field triplets in PG_3 to α and β-NH protons respectively. Each of these triplets could be associated with the lower and higher field doublets by double resonance experiments. The high field doublets thus refers to β-methylene. this is consistent with the components of the corresponding doublet being less sharp than those of the α doublet. as the consequence of unresolved weak long range couplings of β-methylenic protons with α-NH. The coalescence of the two doublets as a function of the pH can then yield proton exchange rates on each peptide bond separately.

RESULTS AND DISCUSSION

Proton Transfers in Acidic DMSO

Exchange rates and pH values were measured at 25°C for two concentrations of peptide. $[PG_2]$ = 0.1 and 0.2 M and $[PG_3]$ = 0.05 and 0.1 M, and. in each case. for three or four concentrations of added sulphuric acid (Table I). Using an indicator of the electrical type I/IH^+ allowed us to deduce hydrogen ion concentration from the experimental ratio $r_I = [I]/[IH^+]$ and the knowledge of the ionization constant K_{IH} :

$$K_{IH} = \frac{[H^+]\,[I]}{[IH^+]} \cdot \frac{\gamma_{H^+}\,\gamma_I}{\gamma_{IH^+}} \simeq [H^+].r_I$$

if we assume $\gamma_{H^+} \simeq \gamma_{IH^+} + \gamma_{\pm}$ and $\gamma_I \simeq 1$ (a piece of evidence suggesting the validity of these assumptions is the independence of r_I upon the (small) concentration of the indicator in the range of *ca.* 10^{-4}-10^{-5}M for a given solution). Large quantities of sulphuric acid were necessary to bring exchange rates to the NMR timescale. i.e. to reduce the mean lifetime τ of peptide molecules between two successive proton exchanges to the order of one tenth of second. $[H^+]$ concentrations required for coalescence in peptides are thus *ca.*50 times larger than those used with NMA. This shows a ***considerable decrease in proton transfer rates from amides to peptides.*** Quantities of added acid are still larger since sulphuric acid is moderately strong only in DMSO[24]. (We gave up the idea of using a strong acid. CF_3SO_3H. since some degradation of DMSO solutions seems to occur for large additions of this acid).

As in the case of NMR. the NMR exchange rate $1/\tau$ is found to be strictly proportional to $[H^+]$ and independent of the peptide concentration :

$$1/\tau = k_H[H^+]$$

We found rate constants

$$k_H^{\alpha}(PG_2) = 6.31 \;;\; k_H^{\alpha}(PG_3) = 17.1 \text{ and } k_H^{\beta}(PG_3) = 8.85\ M^{-1}s^{-1}$$

(the indexes α and β refer to α-NH and β-NH bonds). The generally accepted mechanism, first proposed by Berger *et al.*[25]. involves protonation of the amide nitrogen according to the following scheme (for a discussion. see ref. 4) :

$$\text{R-CO-NH-R' + DMSO ...}H^+ \underset{k_d}{\overset{k_1}{\rightleftharpoons}} \text{R-CO-NH}_2^+\text{-R' + DMSO}$$

followed by a fast diffusion-controlled deprotonation ($k_d \sim 5\times10^9\ M^{-1}s^{-1}$ at 25°C in DMSO[4]). The chemical exchange rate k_1 is consequently twice as fast as the NMR site exchange $1/\tau$. Hence : k_1 = 12.6, 34.1 and 17.7 $M^{-1}s^{-1}$ for $PG_2(\alpha)$ and $PG_3(\alpha$ and $\beta)$, respectively. Experimental errors are difficult to evaluate but should not exceed ± 10 %.

As suggested by the high $[H^+]$ concentrations required for coalescence. these rate constants are smaller than those for NMA[4] by two orders of magnitude (k_1(NMA) = $8.72\times10^2\ M^{-1}s^{-1}$). The ionization constant of

protonated peptides can be estimated as $K_{NH_2^+} = k_d[DMSO]/k_1$ = 5.62 ; 2.04 and 3.16×10^{9} M or $pK_{NH_2^+}$ = -9.75 -9.31 and -9.50 for PG_2 and PG_3 (α and β), respectively. These results show that peptides are less basic (towards protonation) than amides by about 2.0 pK units ($pK_{NH_2^+}$(NMA) = -7.90 [4]). These observations are in agreement with those obtained by H-D exchange measurements in aqueous solutions of (a) small doubly protected peptides where the rate decrease is about of one decade [9] (b) random-chain unprotected model polypeptides. such as poly-D,L-alanine (PDLA)[1,2,2,0]. where the decrease reaches more than two decades. This change in rate is generally assigned to inductive effects exerted by neighbouring peptide groups[9,27]. It may be expected that. conversely. neutral peptides are more easily converted to their conjugate base than are amides. as shown in the next sections. From this point of view. and with some ambiguity. peptides are said to be more acidic than amides. Using the terminology . peptide bonds of PG_2 are seen to be more acidic than those of PG_3 and the β-NH bond of PG_3 more acidic than the α-NH bond. The presence of an adjacent phthaloyl protecting group thus seems to decrease rate constants from *ca.* 30 to 10 $M^{-1}s^{-1}$. This may be due to a strong delocalization of the nitrogen doublet of the N-phthaloyl structure. which then behaves as a strong electron-withdrawing substituent.

Proton Transfers in Basic DMSO

Three sets of kinetic experiments were performed using three different concentrations of peptide.[PG_2]= 0.15 . 0.24 and 0.33 M. [PG_3] = 0.1 . 0.15 and 0.2 M. and up to five pH values (Table II). Plots of $(1/\tau)$ *versus* $[H^+]^{-1}$ are straight lines of slope p with a zero intercept and correlation coefficients larger than 0.99. These slopes are in turn proportional to the concentration of peptide (Fig.2). with a proportionality coefficient taken as the overall base catalyzed rate constant k_B for each NH bond (α or β) :

$$k^{\alpha}_{B(PG_2)} = 1.45\times10^{-13} \quad ; \quad k^{\alpha}_{B} \text{ and } k^{\beta}_{B(PG_3)} = 5.62 \text{ and } 9.55\times10^{-13}\,s^{-1}$$

at 25°C. As in the case of NMA [4]. these observations are consistent with proton abstraction by the conjugate base of the peptide :

$$RCONHR'(P) + RCONR'(P^-) \underset{k_2}{\overset{k_2}{\rightleftharpoons}} P^- + P$$

and a kinetic law

$$1/\tau = k_2[P^-] = k_B[P]/[H^+]$$

with $k_B = k_2K_{NH}$

K_{NH} is the ionization constant for deprotonation from nitrogen atom (Owing to the low ionic content of solutions activity corrections are unnecessary). The mechanism for deprotonation is then different in water and in DMSO, since in the first case. the exchange involves the hydroxide ion. The present conclusion is supported by another observation. The broad NH triplet in PG_2 remains unaltered all along the coalescence of the N-methylene doublet. this shows that the peptide hydrogen is exchanging between like molecules. thus excluding any other basic partner than its own conjugate base. This result means unfortunately that comparisons of k_B values for various substrates in DMSO do not refer to a constant antagonistic reactant as it is the case in water. As a counterpart, rate constant k_B are in fact the product of two parameters K_{NH} and k_2 which are expected to be inverse functions of structural factors. Thus an increased acidity of the NH bond (K_{NH} large) is probably accompanied byh a decreased nucleophilicity (k_2) of the conjugate base. possibly making predictions over the overall rate constant k_B uncertain. The separation of these two parameters requires measuring K_{NH}. This is the advantage of using DMSO as a solvent which permits pK measurements of very weak acids.

These simple laws actually hold as written above only in the case of PG_2. If two exchangeable hydrogens are simultaneously present as in the case of a tripeptide (PG_3). then four kinds of proton abstraction are possible,namely of α-NH (rate constants $k_{\alpha\alpha}$ and $k_{\alpha\beta}$) and β-NH ($k_{\beta\alpha}$ and $k_{\beta\beta}$) by the conjugate bases at nitrogens α and β (α-N^- and β-N^-). respectively. Two NMR exchange rates are measured in this case for each of the two methylene doublets :

$$(1/\tau)_{\alpha} = (k_{\alpha\alpha}K^{\alpha}_{NH} + k_{\alpha\beta}K^{\beta}_{NH})\,[P]/[H^+]$$

$$(1/\tau)_{\beta} = (k_{\beta\alpha}K^{\alpha}_{NH} + k_{\beta\beta}K^{\beta}_{NH})\,[P]/[H^+]$$

where K^{α}_{NH} and K^{β}_{NH} are the ionization constants of nitrogens α and β respectively. Steady-state concentrations of each peptide bond require that :

$$k_{\alpha\beta}K^{\beta}_{NH} = k_{\beta\alpha}K^{\alpha}_{NH}$$

This allows us to write the base catalyzed rate constants k_B^α, k_B^β for each bond under the simple form :

$$k_B^\alpha = k_2^\alpha K_{NH}^\alpha \quad \text{and} \quad k_B^\beta = k_2^\beta K_{NH}^\beta$$

where :

$$k_2^\alpha = k_{\alpha\alpha} + k_{\beta\alpha} \text{ and } k_2^\beta = k_{\alpha\beta} + k_{\beta\beta}$$

Again, the knowledge of rate constants k_2^α and k_2^β requires the determination of K_{NH}^α and K_{NH}^β separately (see next section). Overall rate constants k_B are smaller than those obtained with NMA[4] ($k_B = 8.50 \times 10^{-15} s^{-1}$) by *ca.* two orders of magnitude. As the acid catalyzed rate constants are themselves decreased by *ca.* two orders of magnitude from NMA to PG_2 and PG_3, the pH at which a minimum of the overall proton transfer rate occurs is itself decreased by *ca.* 2.0 units. Its value can be predicted by considering the complete kinetic law in DMSO solutions[4]

$$1/\tau = k_H[H^+] + k_B[P]/[H^+]$$

whence $(pH)_{min.} = -0.5 \log(k_B[P]/[H^+])$

Numerical values are pH = 6.98 and 7.32 in PG_2 and PG_3, to be compared to 8.85 in NMA[4] (Table III). A shift of about two units towards the acidic end of the pH scale is thus observed from NMA to peptides. Similar shifts have been observed for hydrogen-deuterium exchanges[28] in basic aqueous (D_2O) solutions of NMA[29-31] and of PDLA[1,2,26] or of small peptides[32], from pD $\sim$ 5 to pD $\sim$ 3. As mentioned above, this shift is tentatively assigned to inductive effects in peptides resulting in both a decrease and an increase of the acid- and base-catalyzed reaction rates, respectively.

In this respect, we may also notice the coherence between k_H and k_B values in PG_3, the larger k_H value ($k_H^\alpha > k_H^\beta$) being associated to the smaller k_B value ($k_B^\alpha < k_B^\beta$), and consequently to a shift of $(pH)_{min.}$ towards increasing acidities, from 7.24 for α-NH to 6.98 for β-NH. The latter sequence is in line with that observed in aqueous solution of tripeptides, e.g. Ala-Gly-Gly in DMSO[32], where $(pD)_{min.}$ = 2.2 and 3.0 for the N-terminal (β) and C-terminal (α) peptide groups, respectively.

Finally, another point of interest is the order of magnitude expected for the minimum exchange rate ($1/\tau$) $< \sim 10^6 s^{-1}$ equivalent to half-times for NH-ND exchanges of more than 10^4mn, which are thus longer than those reported for aqueous solutions of peptides[1,2] by about two decades.

pK of Peptides in DMSO

Classical methods using pH values at half neutralization ($pH_{1/2}$) resulted into inconsistencies in pK determinations. This is especially clear when using dimsyl ($DMSO^-$) or hydroxide ions as bases and PG_2 as a model peptide. $pH_{1/2}$ values of *ca.* 15 units were obtained (Table IV), which are surprisingly low compared to pK_{NH} (NMA) = 23.4. Suspecting a degradation of the N-phthaloyl group led us to use less nucleophilic bases, such as lithium ter-butyl (LTB), lithium diisopropylamide(LDA) and sodium hexamethyldisilazane amide (SHA), and peptide derivatives bearing a terminal methyle instead of either a N-phthaloyl(acetyl-glycyl-methyl ester, or AGM) or a C-methoxycarbonyl (N-phthaloyl-glycyl-N-methylamide or PGM) protecting group. The corresponding set $pH_{1/2}$ values (Table IV) undoubtedly shows that the N-phthaloyl group is responsible for abnormal $pH_{1/2}$ values when dimsyl anion is used as a base. The three other bases show internally consistent results suggesting that the pK of PG_2 should be close to 19 in DMSO.

However the full neutralization curve of PG_2 is abnormally flat at the equivalence point (Fig.3) as compared to that of AGM, revealing an additional consumption of the added base. This may be due to either ionization of the N-methylene groups or to the opening of the phthalimide substituent into acidic products. The α-CH_2 methylene is born by a negatively charged nitrogen in the ionized peptide and is consequently weakly acidic ; the absence of ionization is shown by the sharpness of the α-CH_2 lines in the entire pH range explored (Fig.4). On the contrary, the γ-methylene is adjacent to a nitrogen atom bearing a fractional positive charge and is therefore much more acidic. It is observed indeed that the γ-CH_2 singlet progressively broadens and even disappears when equimolecular addition of SHA is achieved (Fig.4). In the same time, additional lines of growing intensity appear in the spectrum, which seems to be duplicate in its α-CH_2, γ-CH_2 and aromatic lines. This suggests a progressive opening of the N-phthaloyl group superimposed to a small degree of ionization of the γ-methylene. The NMR spectra show that this parasitic degradation is of little importance up to half neutralization (pH < $\sim$ 19). However the overall hydrogen ion concentration includes contributions from the degradation products which can still be large compared to that of the weak peptide acid. $pH_{1/2}$ values for PG_2 in Table IV can still be decreased by the presence of acidic impurities which are not detected by NMR.

More reliable results are expected from the intensity of the NH triplet, which can yield unambiguously the ratio r of the ionized to the unionized peptide. Due to the poor accuracy of the method, two convenient pH values (18.80 and 19.03) were selected from which we can deduce the ionization constant $K_{NH} = r/10^{-pH} \times$ pK_{NH} of 19.5 and 19.1 were thus obtained, leading to an average value pK_{NH} (PG_2) = 19.3 ± 0.2.

This conclusion was put on a firm basis by observing the chemical shifts. δ^{α} and δ^{γ}, of the α and γ-CH_2 protons and the pH level as the amount of added base is varied (Table V). Assuming the ionization of the NH bond alone, the observed chemical shifts δ (in fact δ^{α} or δ^{γ}) are expressed in the fast exchange limit as a weighted mean over their values in the unionized (δ_p) and ionized (δ_{p^-}) peptide :

$$\delta([P] + [P^-]) = \delta_p \times [P] + \delta_{p^-}[P^-]$$

Introducing the ionization constant K_{NH} of PG_2 allows us to recast this equation under a more useful form :

$$[H^+] = \delta_o K_{NH}/(\delta_p - \delta) - K_{NH}$$

where K_{NH} and $\delta_o = \delta_p - \delta_{p^-}$ are two unknown parameters to be adjusted (In fact, two δ_o parameters are to be introduced for each of the α and γ lines, $\delta_o^{\alpha} = \delta_p^{\alpha} - \delta_{p^-}^{\alpha}$ and $\delta_o^{\gamma} = \delta_p^{\gamma} - \delta_{p^-}^{\gamma}$). A linear plot of $y = [H^+]$ as a function of $x = (\delta_p - \delta)^{-1}$ is indeed obtained for the α-CH_2 line in the acidic end of the graph (Fig.5), i.e. as long as $y > 1.2 \times 10^{-19}$M. Strong deviations from linearity are observed in the more basic solutions, again revealing an abnormal consumption of the added base. The linear portion of the plot allows us to compute K_{NH} from the intercept of the least squares line :

$$K_{NH} = 0.245 \times 10^{-19} \quad \text{and} \quad pK_{NH} = 19.6$$

The chemical shift in the ionized peptide is also obtained from the slope and the above K_{NH} value. δ_{p^-}=886 Hz or 2.215 ppm. This is an upfield shift from the neutral peptide (δ_o = 84.4 Hz or 0.211 ppm) as expected from the presence of a strong electron-donating adjacent nitrogen atom ($N^{\ominus}$-CH_2). The γ-CH_2 shift seems to follow approximately the same linear plot for small additions of base, but deviations from linearity occur as soon as $y < \sim 1.5 \times 10^{-19}$M. The fact that the linear parts of the α-CH_2 and γ-CH_2 plots seem to be identical is probably fortuitous, since the two lines are expected to have the same intercept ($-K_{NH}$), but different slopes ($\delta_o^{\alpha}K_{NH}$ and $\delta_o^{\gamma}K_{NH}$).

All these observations strongly support a pK value in PG_2 close to 19.5. pK_{NH} = 19.4 ± 0.2. The value of 18.9 in Table IV is presumably too small due to a slight degradation of the phthalimide substituent, as explained above.

If we try to extend these NMR measurements to PG_3, we must restrict ourselves to chemical shift determinations of the α-, β- and γ-CH_2 groups (Table VI).

The chemical shift method is complicated by the existence of two monoanions P_{α}^- and P_{β}^- bearing a negative charge on nitrogens α and β, respectively, and of a dianion P^{--}. This obliges us to consider three ionization constants, K_{NH}^{α}, K_{NH}^{β} and $K_{NH}^{\alpha\beta}$, respectively. The second ionization of diacids is however much weaker than the first one, so that monoanions alone can be considered in the evaluation of the average methylene chemical shift δ (in fact, either δ^{α}, δ^{β} or δ^{γ} for α β or γ-CH) as long as the added base is less than equimolecular. This means that for the less basic solutions, a linear x,y plot should be observed according to a rearranged equation :

$$[H^+] = \frac{\delta_{o\alpha}K_{NH}^{\alpha} + \delta_{o\beta}K_{NH}^{\beta}}{\delta_p - \delta} - (K_{NH}^{\alpha} + K_{NH}^{\beta})$$

where $\delta_{o\alpha} = \delta_p - \delta_{p_{\alpha}^-}$ and $\delta_{o\beta} = \delta_p - \delta_{p_{\beta}^-}$

The denomination $\delta_{o\alpha}$, $\delta_{o\beta}$ recovers in fact six parameters at the rate of two parameters per CH_2 group studied : $\delta_{o\alpha}^{\alpha}$, $\delta_{o\alpha}^{\beta}$, $\delta_{o\alpha}^{\gamma}$; $\delta_{o\beta}^{\alpha}$, $\delta_{o\beta}^{\beta}$, $\delta_{o\beta}^{\gamma}$. All this informations cannot be extracted from three linear plots only. An important point however is that the three straight lines should have a common intercept, $-(K_{NH}^{\alpha}+K_{NH}^{\beta})$. Further additions of bases would result into deviations from linearity as the combined consequence of the quadratic dependence of the dianion on the hydrogen ion concentration and of the degradation of the N-phthaloyl group. In the relatively acidic pH region explored (18<pH<19), we obtained effectively three straight lines whose convergence to a common point on the vertical axis is reasonably good (Fig.6). β- and γ-CH_2 lines again appear to be very close to each other. The intercepts of the least squares lines, -1.641, -0.783 and -0.500 × 10^{-19}M yield the sum $K = K_{NH}^{\alpha} + K_{NH}^{\beta}$ with a high degree of certainty concerning its order of magnitude and a poor accuracy concerning its absolute value taken as the average, K = (0.97±0.44) × 10^{-19}M. This shows again that the pK of the α and β peptide bonds are close to 19.

The two individual ionization constants, K^{α}_{NH} and K^{β}_{NH}, are close to each other, and the sequence of acid-catalyzed rate constants k^{α}_{H} and k^{β}_{H} led us to predict that $K^{\beta}_{NH} > K^{\alpha}_{NH}$. An estimation of the small pK difference, $\Delta pK = pK^{\alpha}_{NH} - pK^{\beta}_{NH}$, can be made with a reasonable degree of approximation by assuming a Broensted relationship between the sets of K_{NH} and k_H values. The Broensted coefficient α can be computed from the values relative to NMA and PG_2, $\alpha = \log[(k_H(NMA)/k_H(PG_2)] / (pK(NMA) - pK(PG_2)) \; 0.46$, whence :

$$\Delta pK = \frac{1}{\alpha} \log(k^{\alpha}_{H}/k^{\beta}_{H}) = 0.62 \text{ or } K^{\beta}_{N}/K^{\alpha}_{N} = 4.18$$

and, in turn,

$$K^{\alpha}_{NH} \text{ and } K^{\beta}_{NH} = (0.186 \pm 0.09) \text{ and } (0.78 \pm 0.37) \times 10^{-19} M \text{ or}$$

$pK^{\alpha}_{NH} = 19.7 \pm 0.2$ and $pK^{\beta}_{NH} = 19.1 \pm 02$.

Rate Constants in Basic DMSO

True rate constants for depronation of the peptide bond were deduced from the overall base-catalyzed rate constant k_B and the above pK_{NH} values according to $k_2 = k_B \times 10^{pK_{NH}}$.

Triads of k_B, k_2, pK_{NH} values for NMA, PG_2, PG_3 (α and β) are shown in Table VII for the sake of comparison, together with the corresponding sets of k_H, k_1 and $pK_{NH_2^+}$ values obtained in acidic DMSO. The expected errors over k_B and pK_{NH} being of ± 10 % and ± 0.2 unit, respectively. k_2 values are comprised within a large uncertainty range of *ca.* ± 70 %.

While k_2 was found close to the diffusion limit in the case of NMA, the values found for PG_2 and PG_3 are smaller by two to three orders of magnitude. This shows that the more acidic peptide bonds in PG_2 and PG_3 exchange more slowly with their respective conjugate bases than does NMA. This means that the favourable increase of acidity of the peptide (P) is more than counterbalanced by an unfavourable smaller basicity of the conjugate base partner (P^-) in spite of the apparent symmetry of the transition state generally assumed (P...H...P).

CONCLUSION

Besides kinetic data showing quantitatively the interest of using DMSO for slow NH-NH and NH-ND exchange, the pK of amides and peptides are obtained for the first time with a reasonable degree of confidence. Protected di- and tripeptide show relatively similar kinetic and thermodynamic acidities of the peptide bond. The most striking feature seems to be the large differences between data relative to NMA and to PG_2 and PG_3. Parallel decreases are observed for both $pK_{NH_2^+}$ and pK_{NH} values from NMA to peptides, but the magnitude of the variation is nearly double for pK_{NH} than for $pK_{NH_2^+}$. 4.0 against 2.1 units. The reasons for such a decrease, which is in accord with the shift of the log k_1 *vs.* pH curves for NH-ND exchanges mentioned in a previous section, are not so clear as implied by the recourse to simple inductive effects of the substituents. The data of Table IV, in spite of errors inherent to the method of pK measurement , are sufficient to reveal that the central structure of NMA supporting the molecular formulae of AGM, PG_2 and PGM is strongly affected by the presence of one protecting group (pK_{NH} decreases from 23.4 to 19-20), but that the effect over pK_{NH} is not additive as expected from purely inductive effects. Further experiments are therefore necessary to elucidate this point.

pK values for protonation ($pK_{NH_2^+}$) result from an estimation, assuming that the reverse deprotonation is diffusion-limited in all cases. $pK_{NH_2^+}$ values in aqueous solutions are expected to be very close to those presently obtained in DMSO, as it was shown previously in the case of NMA[4].

Experiments using NMA have also shown that pK_{NH} values are decreased by about 5 units from DMSO to H_2O, due to the electrical charge types of the involved species[4]. pKs of 14-5 would then be expected for PG_2 and PG_3 in aqueous solution on this basis, these values are surprinsingly low, just above the upper limit of measurable pKs in water. These pK_{NH} values would in turn result into rate constants for proton abstraction by hydroxide ion, $k_{OH} = K_{NH} k_d/K_W$ (where $K_W = 10^{-14}$ and $k_d = 10^{10} s^{-1}$) , which are close to the diffusion limit $k_d \sim 10^{10} M^{-1} s^{-1}$. Such high values for proton abstraction rates would in fact be in accord with the pK decrease of 4 units from NMA to PG_2 and PG_3, which is expected to produce a parallel increase of log k_{OH} from *ca.* 6 in NMA to 10 in PG_2 and PG_3. Experiments are currently devised in this laboratory to test this prediction by using protected peptides which are soluble in both DMSO and water. While a pK of *ca.* 19 for the NH bond of peptides in DMSO seems to be firmly established on the basis of our experiments, the predictions of pK values of 14 in water will be tested in further work.

Figure 1 : ^1H NMR spectra (N-CH_2 and N-H protons only) at 250 MHz and 25°C of 0.1 M DMSO-d_6 solutions of (a) Phthaloyl-triglycyl ethyl ester (PG_3) (b) Phthaloyl-glycyl-glycyl-alanine methyl ester (PG_2A).

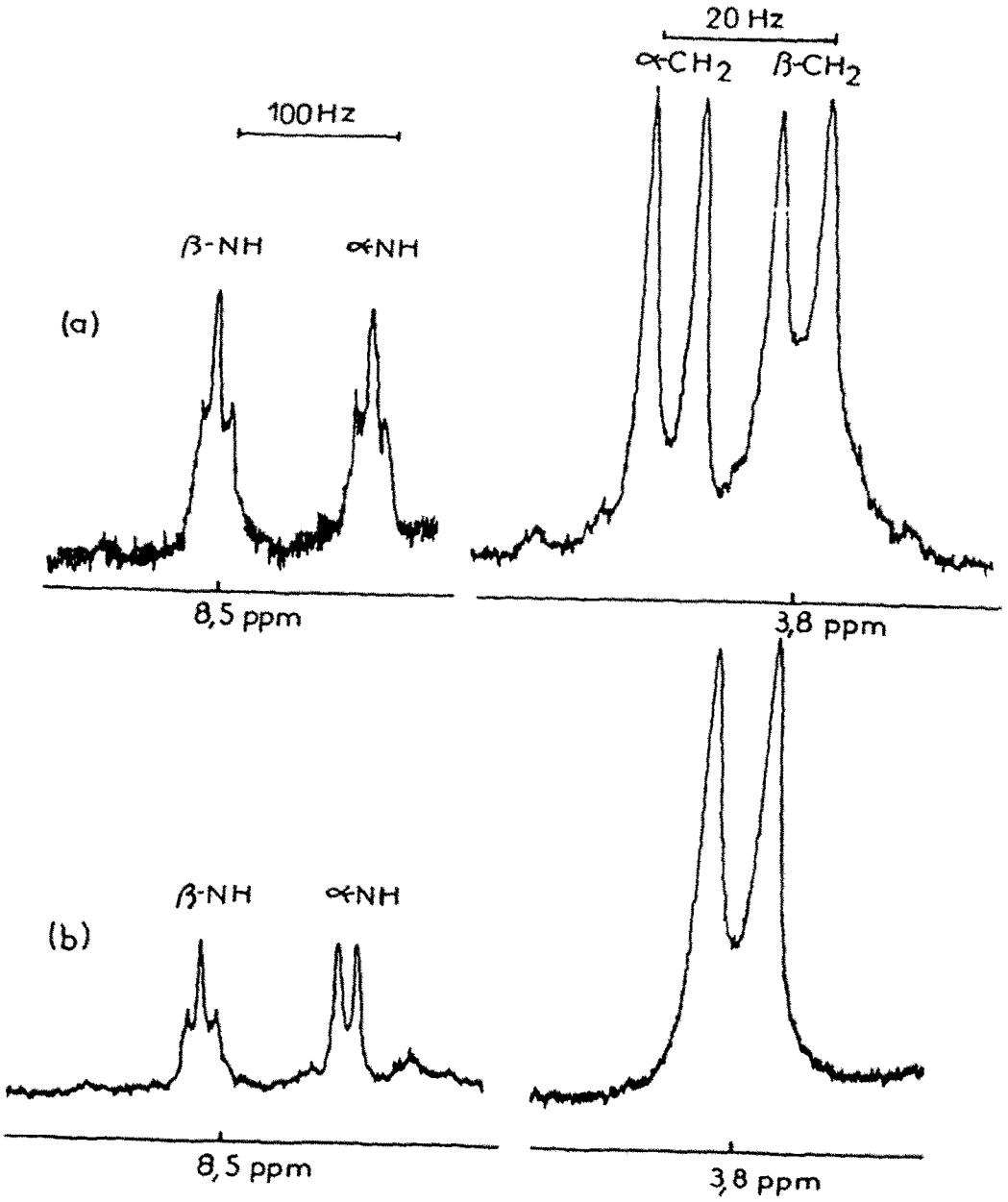


Figure 2 : A plot of slopes p obtained in basic DMSO (see the text) *versus* the concentration of the corresponding peptide PG_2 (O), PG_3 (Δ) and (+).

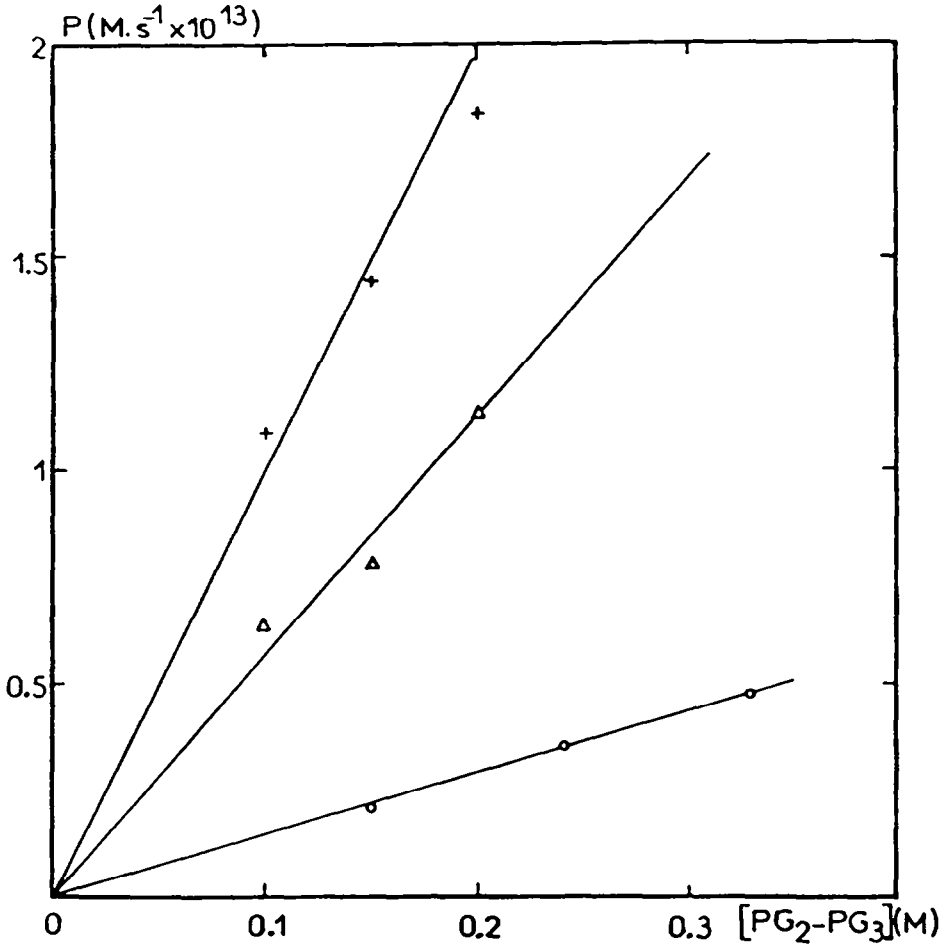


Figure 3 : Neutralization curves of 0.02 M DMSO solutions of (a) acetyl-glycyl-methyl ester (O) and (b) phthaloyl-glycyl-glycyl methyl ester (PG_2) by sodium hexamethyldisilazane (SHA)

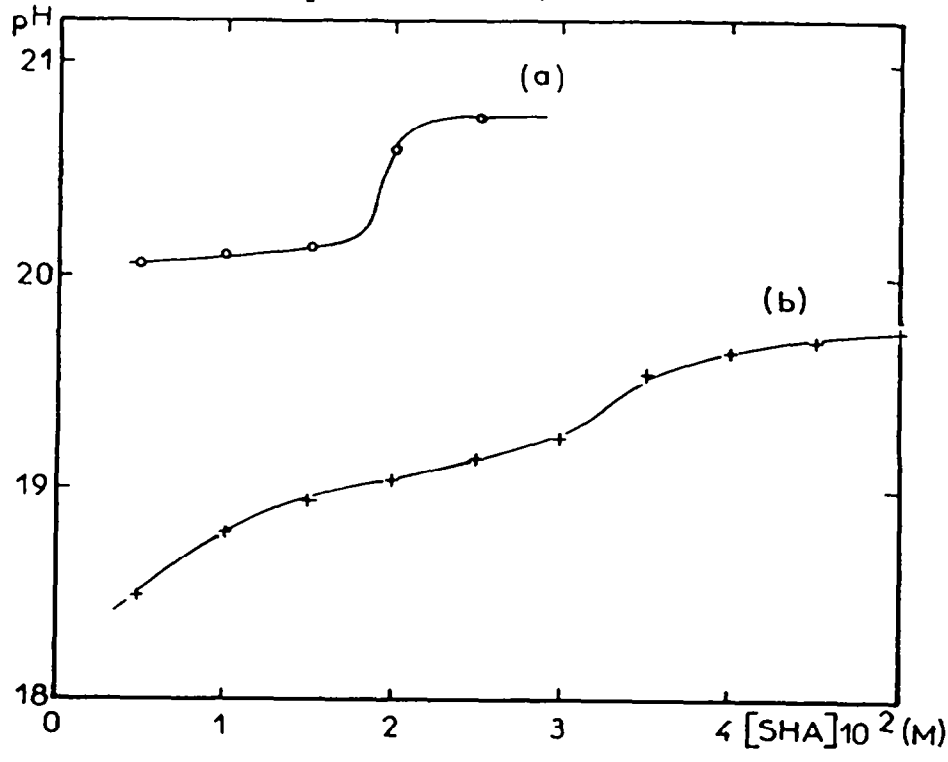


Figure 4 : ^1H NMR spectra (N-CH_2 and NH regions only) at 400 MHz and 25°C of 0.05 M DMSO solutions of phthaloyl-glycyl-glycyl methyl ester (PG_2) (lines shown by arrows) accompanied by degradation products (starred lines) for increasing additions of base : [SHA] = 0 (a), 0.025 (b), 0.045 (c).

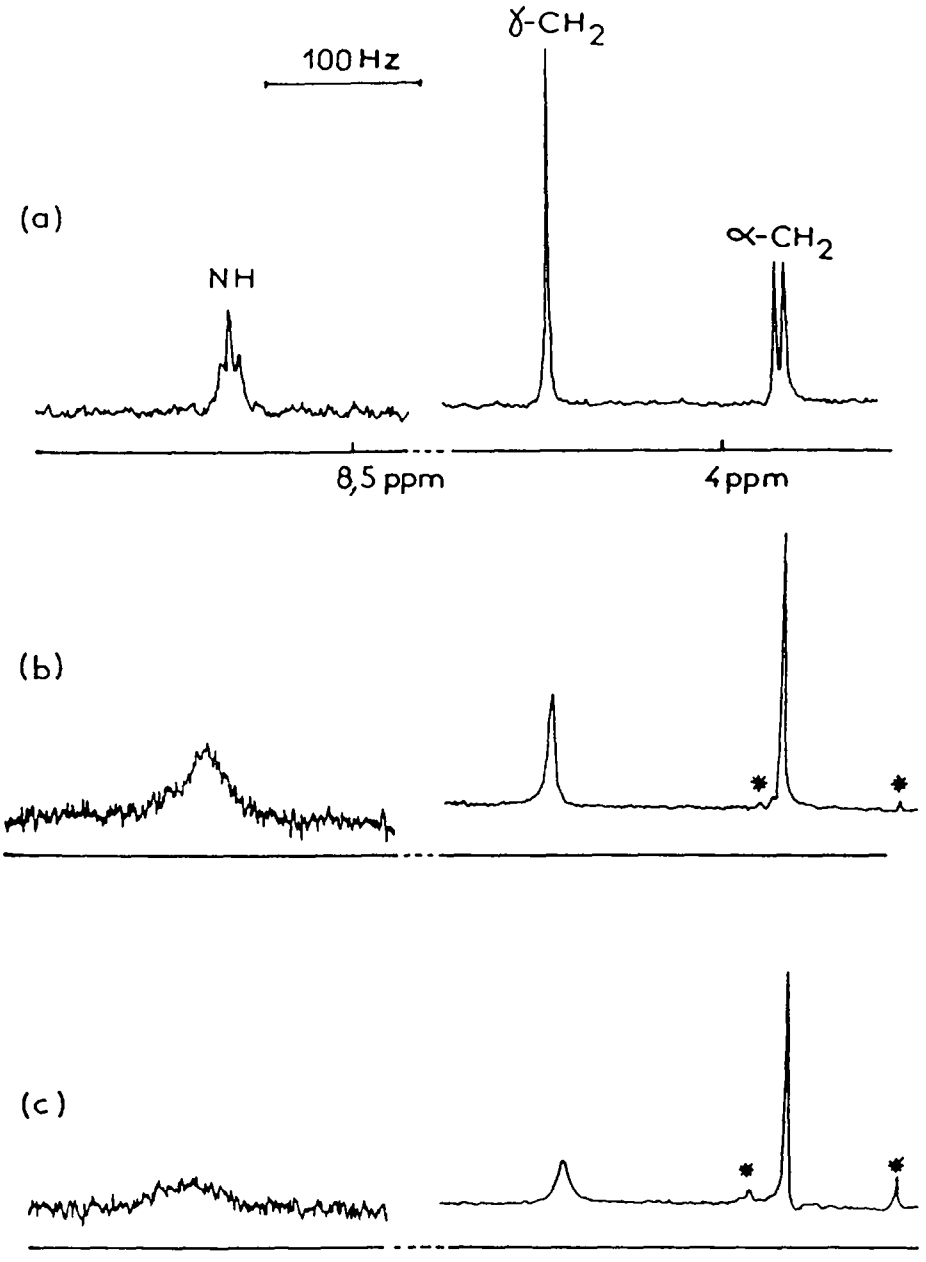


Figure 5 : Plots of $y = [H^+]$ *versus* $x = (\delta_p - \delta)^{-1}$ using the α (·) or γ-CH_2 (Δ) chemical shifts of PG_2. The α-CH_2 line is drawn (least squares eqn : y = 20.68 x - 0.245 with r = 0.9997).

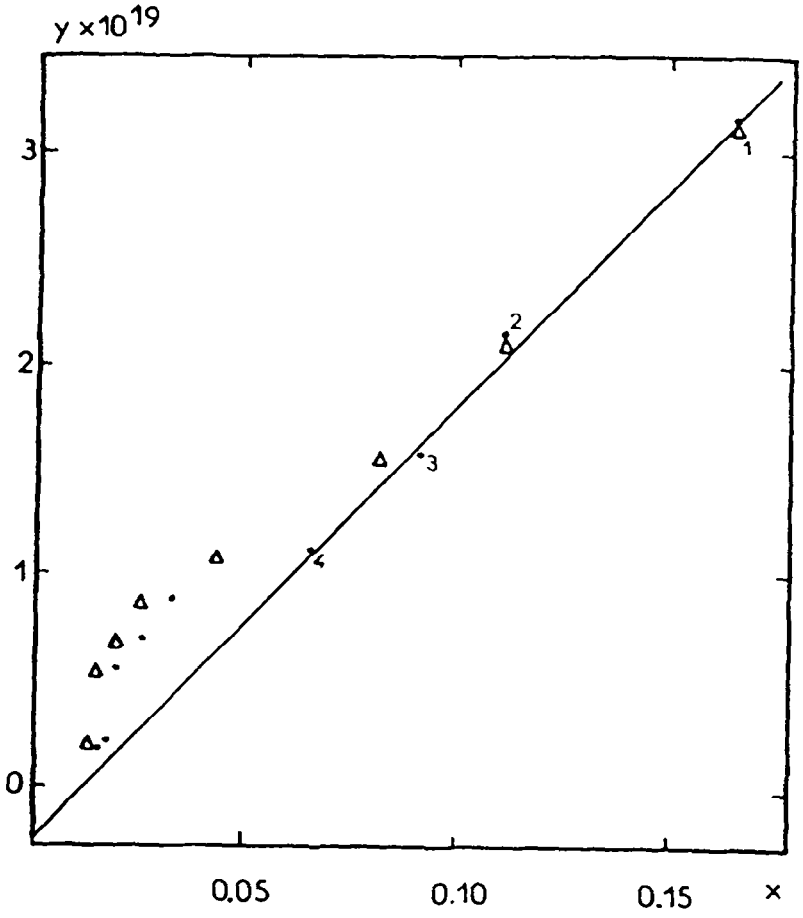


Figure 6 : Plots of $y = [H^+]$ *versus* $x = (\delta_p - \delta)^{-1}$ using the α (·), β (Δ) or γ-CH_2 (O) chemical shifts of PG_3 (least squares eqns : y = ax + b ; a = 48.14, 87.73, 76.70 ; 10^{19} x b = - 1.64 ; - 0.783 ; - 0.500, respectively).

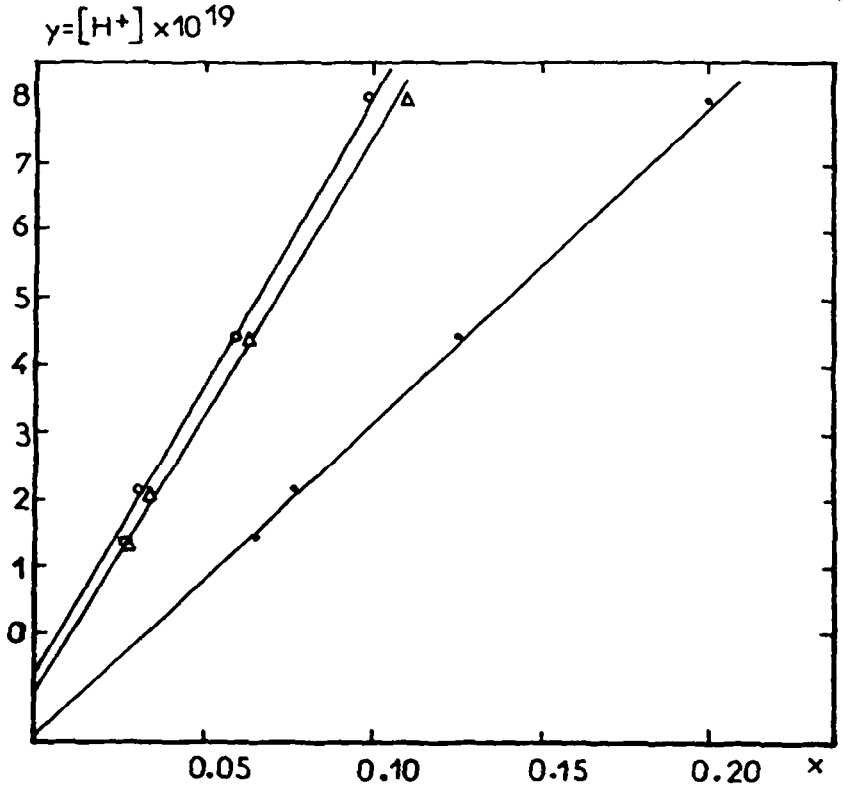


Table 1. pH values, hydrogen ion concentrations $[H^+]$ and NMR exchange rates $(1/\tau)_\alpha$, $(1/\tau)_\beta$ of PG_2 and PG_3 in DMSO at 25°C using two peptide concentrations, $[PG_2] = 0.1$ and 0.2 M, $[PG_3] = 0.05$ and 0.1 M, and three of four additions of sulphuric acid $[H_2SO_4]$.

$[H_2SO_4]$ (M)	$[H^+]$ (M)	pH	$(1/\tau)_\alpha$ (s^{-1})	$(1/\tau)_\beta$ (s^{-1})
PG_2				
0.94	0.40	0.40	2.1	–
1.41	0.56	0.25	3.1	–
1.88	0.78	0.11	4.5	–
PG_3				
0.47	0.20	0.70	4.0	1.3
0.70	0.30	0.52	5.5	2.0
0.98	0.43	0.37	8.0	3.5
1.17	0.50	0.30	9.0	3.8

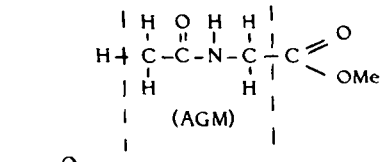
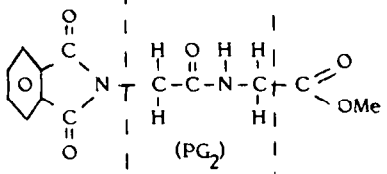
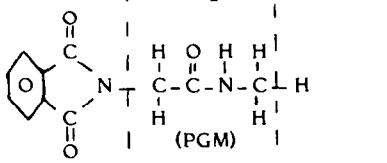
Table II. NMR exchange rates $(1/\tau)_\alpha$, $(1/\tau)_\beta$ (s^{-1}) of PG_2 and PG_3 in basic DMSO at 25°C as a function of the pH and peptide concentration, and the proportionality coefficients p of $(1/\tau)$ to $1/[H^+]$.

$[PG_2] = 0.33$ M			0.24 M			0.15 M		
pH	$(1/\tau)_\alpha$		pH	$(1/\tau)_\alpha$		pH	$(1/\tau)_\alpha$	
14.08	5.6		13.90	2.4		14.28	4.2	
14.33	10.2		14.26	5.4		14.46	5.7	
14.47	14.0		14.41	8.7		14.55	7.7	
$p(M.s^{-1} \times 10^{+14}) = 4.80$			3.50			2.07		
$[PG_3] = 0.2$ M			0.15 M			0.1 M		
pH	$(1/\tau)_\alpha$	$(1/\tau)_\beta$	pH	$(1/\tau)_\alpha$	$(1/\tau)_\beta$	pH	$(1/\tau)_\alpha$	$(1/\tau)_\beta$
13.36	4.4	4.0	13.69	5.0	6.5	13.72	3.0	5.0
13.50	5.5	5.5	13.90	6.5	12.0	13.93	4.0	10.0
13.75	6.7	10.5	14.08	10.7		14.02	5.0	
13.78	7.0	11.5	14.27	15.4		14.22	9.3	
14.13	17.0					14.37	14.3	
$p_\alpha(M.s^{-1} \times 10^{13}) = 1.14$			0.78			0.64		
$p_\beta(M.s^{-1} \times 10^{13}) = 1.84$			1.44			1.09		

Table III. Acid ($M^{-1}s^{-1}$) and base-catalyzed (s^{-1}) reaction rates, minimum exchange rates $(1/\tau)_{min.}$ (s^{-1}) and the corresponding half-times $t_{1/2}$ (mn) expected for NH-ND exchange (in 0.1 M solutions)

System		$k_B(\times 10^{15})$	$(pH)_{min.}$	$(1/\tau)_{min}(\times 10^7 s^{-1})$	$t_{1/2}$ (mn)
NMA/H_2O	470		5.24	2.7×10^4	2.6
NMA/DMSO	436	8.50	8.85	6.1	1.9×10^4
PG_2/DMSO	6.31	145	7.32	3.0	3.8×10^4
$PG_3(\alpha)$/DMSO	17.1	562	7.24	9.8	$1.2\ 10^4$
$PG_3(\beta)$/DMSO	8.85	955	6.98	9.2	1.2×10^4

Table IV. pH half neutralization of three model amides (chosen with a common structural unit substituted by various end groups as shown by the dashed lines) by four bases (see the text). and the corresponding average pK values.

Amide [a]	Base DMSO$^{\ominus}$	LTB	LDA	SHA	$\overline{pK}_{NA}$
 (AGM)	20.4	20.2	20.1	20.1	20.2
 (PG_2)	15.7	18.8	19.2	18.8	18.9[b]
 (PGM)	15.1	18.3	18.8	18.7	18.6[b]

[a] 0.02 M solutions in DMSO

[b] computed over the last three pH values

Table V. pH values and chemical shifts δ^{α} and δ^{γ} of α- and γ-CH_2 protons (in Hz from internal TMS at 400 MHz and 25° C) of PG_2 in 0.02 M DMSO solutions containing various amounts of sodium hexamethyldisilazane (SHA)

[SHA] (M)	pH	δ^{α}	δ^{γ}
0		1065	971
5.0×10^{-3}	18.50	1059	965
7.5×10^{-3}	18.67	1056	962
1.0×10^{-2}	18.80	1051	960
1.5×10^{-2}	18.95	1042	956
2.0×10^{-2}	19.05	1027	941
2.5×10^{-2}	19.15	1016	933
3.0×10^{-2}	19.25	1000	922
4.0×10^{-2}	19.65	992	915
5.0×10^{-2}	19.75	–	912

Table VI. Chemical shifts δ^{α}. δ^{β} . δ^{γ} (in Hz from internal TMS at 250 MHz and 25°C) of the α-. β- and γ-CH_2 protons of PG_3 in 0.05 M DMSO solutions as a function of the pH.

pH	δ^{α}	δ^{β}	δ^{γ}
–	963	947	1073
18.10	958	937	1064
18.35	955	930	1057
18.67	950	916	1043
18.84	948	910	1037

Table VII. Kinetic (k_H . k_1 and k_B . k_2) and thermodynamic ($pK_{NH_2^+}$ and pK_{NH}) data relative to simple amide and peptides in acidic and basic DMSO a 25°C. respectively.

	ACIDIC DMSO			*BASIC DMSO*		
	$k_H(M^{-1}s^{-1})$	$k_1(M^{-1}s^{-1})$	$pK_{NH_2^+}$	$k_B(s^{-1}\times10^{15})$	$k_2(M^{-1}s^{-1}\times10^{6})$	pK_{NH}
NMA	436	872	-7.90	8.50	2130	23.4
PG_2	6.3	12.6	-9.75	145	3.6	19.4
PG_3 (α)	17.1	34.2	-9.31	562	30.2	19.7
PG_3 (β)	8.85	17.7	-9.50	955	12.3	19.1

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