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Introduction of a Strong Binding Site for Lanthanides at the *N*-Terminus of Peptides and Ribonuclease A

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A new reagent, phthalyl-4-isothiocyanate, reacts specifically at the N-terminus of a peptide or protein with the production of a phthalic acid 4-thiocarbamyl derivative. The adjacent carboxyl groups of the phthalyl group serve as a strong binding site for lanthanides at pH 5 with a stability constant for praseodymium about 220 times that of a single carboxyl group.

Reaction of phthalyl-4-isothiocyanate with α-amino groups of peptides at 25 °C in ^2H_2O at pH meter readings of 8.7 and 7.2 is complete in 15 min and 24 h respectively, without modification of histidine or tyrosine side chains. At the lower pH there is no reaction of the ε-amino group of lysine. The phthalic acid 4-thiocarbamyl derivative of ribonuclease A is cyclised and cleaved by the normal Edman degradation in acid to produce the phthalic acid 4-thiohydantoin of lysine. The α-CH proton of lysine-1 in ribonuclease A produces a triplet nuclear magnetic resonance that has been assigned on the basis of its coupling constant, the pH dependence of its chemical shift (pK' is 7.6) and its disappearance on reaction of ribonuclease A with phthalyl-4-isothiocyanate.

The reaction of phthalyl-4-isothiocyanate with a protein allows the introduction of a strong lanthanide binding site specifically at the N-terminus, as a starting point for structural studies using nuclear magnetic resonance spectroscopy.

It is possible to obtain information about the structure of a molecule in solution by the use of NMR spectroscopy, particularly in the presence of bound paramagnetic species [1–5]. In studies on small molecules the binding site of the paramagnetic species is usually known and the assignment of the NMR resonances to particular nuclei in the molecule can be determined without difficulty. However, in complex macromolecules such as proteins, the correct assignment of resonances can be a very difficult procedure, as shown by the fact that it has taken seven years to obtain the correct assignment for two of the four C-2 histidine resonances of RNase-A [6–11]. Furthermore, locating the position of the binding site(s) for paramagnetic species (usually ions) in proteins is even more difficult (except in those cases where the ion is bound at a known metal ion site in the molecule) and has only been solved satisfactorily by X-ray structure analysis of crystals. In a case such as that of lysozyme [12], where the structure and the binding site for lanthanides in the crystal is known, the problem is greatly simplified, since this information can be used for assignment of resonances [4, 13].

In considering the problem of locating the position of a paramagnetic binding site in a protein of known sequence but of unknown three-dimensional structure, it seemed useful to develop chemical methods for the insertion of strong binding sites at specific locations in the molecule [2]. This would then solve the location problem, providing the introduced binding site was much stronger than any other in the molecule. Because of the importance of lanthanide ions in the shifting and broadening of NMR resonances [1, 14], it was decided to introduce a pair of closely spaced carboxyl groups, which was known from studies with simple compounds to produce a strong binding site [15]. Unsuccessful attempts were made to introduce two carboxyl groups on methionine side chains [16], and also at the N-terminus. Success was achieved with phthalylisothiocyanate, that reacts specifically at the N-terminus and introduces adjacent carboxyl groups on an aromatic ring. While this work was in progress,

Abbreviations. NMR, nuclear magnetic resonance; ^1H NMR, proton magnetic resonance; RNase-A, bovine pancreatic ribonuclease A; PhtNCS, phthalyl-4-isothiocyanate (structure 1, Fig. 1); PhtNCS-Lys, phthalic acid 4-thiocarbamyl derivative of lysine (structure III).

Enzyme. Ribonuclease A (EC 3.1.4.22).

Marinetti *et al.* [17,18] have shown that tyrosine side chains may be nitrated to form a useful binding site for lanthanides.

MATERIALS AND METHODS

Materials

All peptides used in this work were obtained from the Fox Chemical Co., and their purity was checked routinely by examination of ^1H NMR spectra. Praseodymium nitrate (Koch Light) was used without further purification. RNase-A was purchased from Worthington Biochemical Corp., and also from the Sigma Chemical Co. (type II-A). ^2H_2O (>99.7%) was obtained from the Australian Atomic Energy Commission.

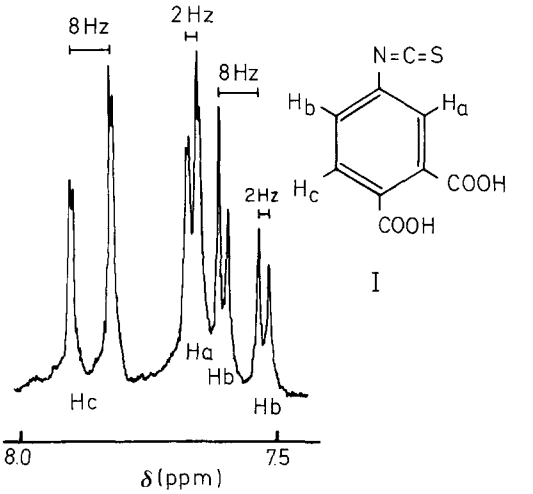


Fig. 1. *^1H NMR spectrum at 100 MHz of phthalylisothiocyanate (I) in [$^2H_6$] acetone*

Preparation of Phthalyl-4-isothiocyanate

Initially an attempt was made to prepare 3-isothiocyanato-phthalic acid from 3-amino-phthalic acid. However, it was not possible to isolate the isothiocyanate because it cyclised with the adjacent carboxyl group. The 4-isothiocyanato-phthalic acid (phthalylisothiocyanate, PhtNCS) was then prepared in four stages. The first two steps involved the esterification of 4-nitro-phthalic acid to dimethyl 4-nitro-phthalate followed by its reduction to dimethyl 4-aminophthalate [19]. The ester groups were next removed to produce 4-amino-phthalic acid [20]. In the final step, 2 g (11 mmol) of 4-amino-phthalic acid was dissolved in water and 22 mmol of thiophosgene (Fluka) added over a period of 30 min with stirring at room temperature [21]. The mixture was stirred for another 30 min and the precipitate formed was filtered off. Yield of PhtNCS (I) about 1.4 g, m.p. 166–168 °C. The crude light-yellow-coloured material was purified by boiling in ether with activated charcoal. The material (m.p. 167–168 °C) was soluble in ether, tetrahydrofuran, dioxan, acetone and pyridine, but only slightly soluble in water, chloroform and toluene. Elemental analysis gave the following results: C=48.3%, H=2.2%, N=6.4%, S=14.3%; calculated for PhtNCS C=48.4%, H=2.3%, N=6.3%, S=14.4%. The analysis of the crude product was also found to be well within the limits of accuracy of the analytical procedure. The infrared spectrum showed the presence of a characteristic isothiocyano absorption at 2100 cm^{-1} and mass spectrometry gave the correct parent peak at $m/e=223$. The ^1H NMR spectrum of the material in [$^2H_6$]acetone is shown in Fig. 1. The assignment of the resonances is clear from the coupling of the system. The 8-Hz coupling between H_b and H_c is consistent with normal *ortho* coupling and the 2-Hz coupling between H_a and H_b is normal for *meta* coupling. The *para* coupling between H_a and H_c is 0.5 Hz [22].

Nuclear Magnetic Resonance Spectroscopy

For ^1H NMR spectra at 100 MHz a Jeol MH-100 instrument was used with an ambient probe temperature of 26 °C. Chemical shifts were referenced to external tetramethylsilane by using a capillary of tetramethylsilane in carbon tetrachloride. ^1H NMR spectra at 270 MHz were obtained using a 270-MHz Brüker NMR spectrometer fitted with an Oxford Instruments magnet with a probe temperature of 20 °C and located at the National NMR Centre in Canberra. Chemical shifts were measured in ^2H_2O solutions from the H^{2}HO resonance, which occurs 4.83 ppm downfield from internal sodium 2,2-dimethyl-2-silapentane-5-sulphonate. Difference spectroscopy was carried out as described previously [7,23]. The pH was measured using a Beckman research pH meter fitted with pH 0–14 microelectrodes and adjustments of pH made with ^{2}HCl (Stöhler) and KO^{2}H. Values quoted for pH are meter readings uncorrected for deuterium isotope effects.

Determination of Dissociation Constants

Determinations of the apparent dissociation constants K' of the carboxyl groups were made from a plot of log $[(\delta_A-\delta)/(\delta-\delta_B)]$ against pH as described previously [13,24], where δ_A, δ_B and δ are the observed chemical shifts in ppm in the acid and basic forms and at the particular pH of the measurement respectively. The pK' is the value of the pH at which the log term is zero. For the simple Henderson-Hasselbach treatment to be applicable, the equation requires that the graph be linear with a gradient of unity.

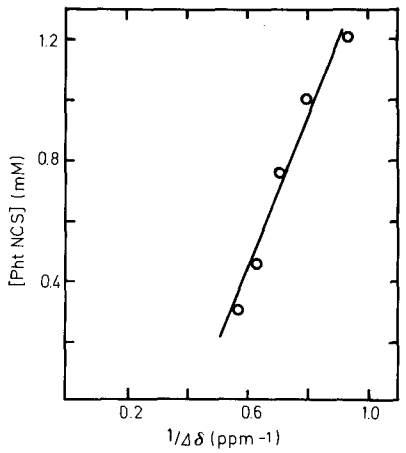


Fig. 2. *Plot of [PhtNCS] vs $1/\Delta\delta$, where $\Delta\delta$ is the shift of the H_a resonance of PhtNCS, at constant lanthanide concentration ($[Pr^{3+}] = 0.095$ mM) in ^2H_2O at pH meter reading 5.0 and 20 °C*

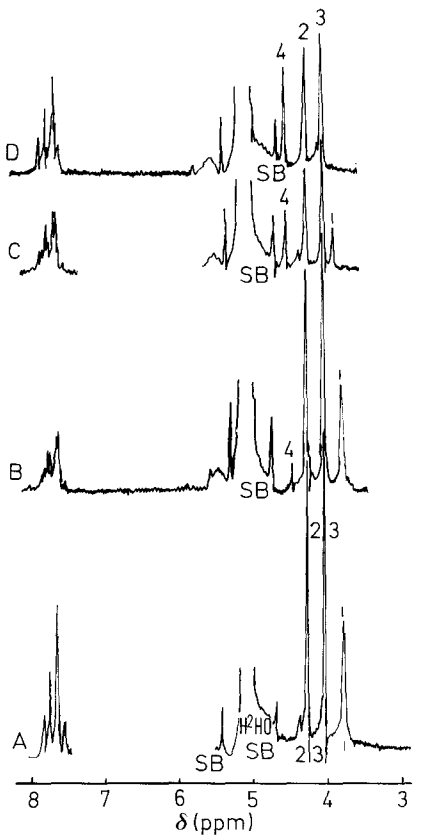


Fig. 3. *^1H NMR spectra at 100 MHz of the reaction in ^2H_2O at pH meter reading 8.7 and 25 °C of stoichiometric amounts (about 0.1 M) of Gly-Gly-Gly and PhtNCS (A) before mixing, separate spectra of each and (B) 1 min, (C) 2 min, (D) 15 min after mixing.* In (A) the singlet CH_2 resonances of triglycine are labelled 1, 2 and 3 from the N-terminus as previously assigned [27,28]. H^2HO resonance not shown in full; sb = spinning side band

RESULTS

Determination of Stability Constant of Phthalylisothiocyanate with Lanthanides

Preliminary experiments showed that addition of small amounts of Pr^{3+} to dilute solutions of PhtNCS in ^2H_2O at pH meter reading 5 caused precipitation. This difficulty was overcome by use of a dilute solution of PhtNCS (1.213 mM) containing Pr^{3+} (0.095 mM) in ^2H_2O at pH 5 and the measurement of the ^1H NMR spectrum at 270 MHz. To the solution was added known volumes of 0.095 mM Pr^{3+} and the spectra measured again at five different PhtNCS concentrations down to 0.303 mM. The PhtNCS spectrum shown in Fig. 1 shifted progressively downfield as [PhtNCS] decreased at constant $[Pr^{3+}]$. The shift of the aromatic protons was $H_c > H_a > H_b$ which is consistent with binding of Pr^{3+} at the carboxyl sites.

Applying the treatment of Armitage *et al.* [25] to this system gives the equation

$$[\text{PhtNCS}]_0 = ([Pr^{3+}]_0 \Delta\delta_{max}/\Delta\delta) - ([Pr^{3+}]_0 + K_B^{-1})$$

where the subscript zero refers to total concentrations, $\Delta\delta$ and $\Delta\delta_{max}$ refer to the shifts of the resonances from their positions in the absence of lanthanide due to complexing with Pr^{3+}. K_B is the stability constant. This equation is derived using the assumptions that $\Delta\delta \ll \Delta\delta_{max}$ and $1/K_B \geqq [\text{PhtNCS}]_0$. Hence a graph of $[\text{PhtNCS}]_0$ against $1/\Delta\delta$, in an experiment in which ligand concentration is varied at constant $[Pr^{3+}]_0$, should give a straight line (see Fig. 2).

The plot was fitted by least squares, with a slope of 2.57×10^{-2}, from which the chemical shift of the H_a proton of PhtNCS when bound to Pr^{3+} was calculated to be 27.1 ppm. The y intercept gives the values of the stability constant, $K_B = 960$ M^{-1} [25]. This is to be compared with a value of 4.3 M^{-1} obtained by the same method in ^2H_2O at pH 5 for the binding of Pr^{3+} to the single carboxyl group of diglycine [26]. Thus the *ortho*-dicarboxyl group of the aromatic nucleus is a strong binding site for lanthanides, relative to the single carboxyl groups available in proteins, hence it is worthwhile to now consider the reaction of PhtNCS with peptides and proteins.

Reaction of PhtNCS with Glycine, Diglycine and Triglycine

The reaction of stoichiometric amounts of PhtNCS with glycine, Gly-Gly and Gly-Gly-Gly, in ^2H_2O was followed by NMR spectroscopy at pH 8.7 (adjusted during the reaction by addition of base as necessary). The results of a typical experiment are shown in Fig. 3. It is noted that the spectrum of PhtNCS in ^2H_2O in Fig. 3A is similar but not identical to that in [$^2H_6$]acetone (Fig. 1), since in the former the downfield doublet of the H_b proton is obscured beneath the large singlet H_a resonance. After mixing the reagents there is a progressive decrease in resonance 1

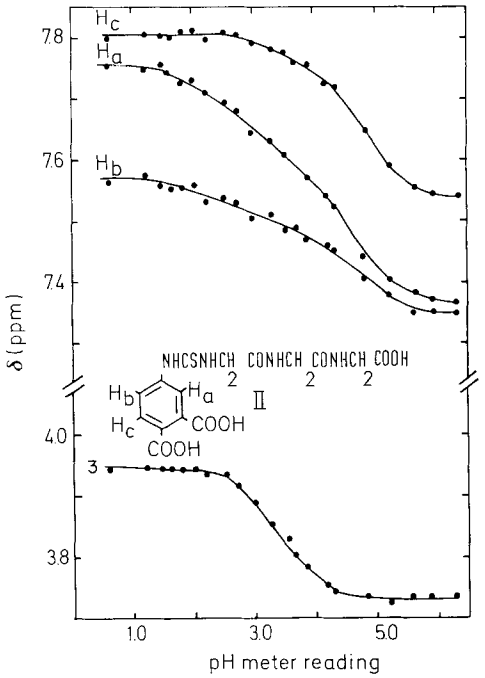


Fig. 4. *Plot of chemical shift against pH meter reading of the proton resonances labelled in structure II and of resonance 3, Fig. 3*

(the resonance from the CH_2 group at the N-terminus) and increase in a new peak labelled 4. There is also a slight downfield shift of peaks 1 and 4 due to a small decrease in the pH during the reaction. In the downfield region of the spectrum there is the progressive appearance of resonances about 2–4 Hz downfield from those of the original spectrum of PhtNCS. These changes reflect the gradual disappearance of the spectrum of the reactants (Fig. 3A) and the appearance of the spectrum of the product (Fig. 3D). The latter spectrum shows the complete absence of resonance 1 from triglycine and indicates that the reaction is complete after 15 min. By comparison the hydrolysis of PhtNCS requires 50 h at pH 8.7 and 40 °C and thus does not interfere with the reaction with amino groups. The thiocarbamyl derivative of triglycine, structure II (Fig. 4) was isolated as a precipitate by cooling to 4 °C overnight, after acidification to pH 1.5. The product II gave a satisfactory analysis for C, H, N and S, on the assumption of an impurity of about 12% NaCl. The NMR spectrum was identical with that of Fig. 3D.

The assignments of protons H_a, H_b and H_c in Fig. 4 are based on the coupling constants observed in a spectrum at pH 2.2 as discussed above for PhtNCS. The titration of the C-terminal carboxyl group of the peptide derivative is monitored by the adjacent α-CH_2 protons labelled 3 and a normal type of titration curve results. The titration curves of the three ring protons are complex because of their proximity to the two carboxyl groups. It is clear that the chemical shift

Table 1. *Titration data for PhtNCS-Gly-Gly-Gly (structure II, Fig. 4)*

Carboxyl group	pK'	Proton used in calculation	Chemical shifts of resonances		
			δ_A	δ_B	$\delta_A - \delta_B$
			ppm		
C-terminal	3.4	α-CH_2 no. 3	3.95	3.74	0.21
para-Carboxyl	4.6	H_c	7.81	7.54	0.27
	4.6	H_a	7.75	7.57	0.18
meta-Carboxyl	2.9	H_a	7.61	7.37	0.24

commences to change as the pH falls below 6, but that whereas H_c is complete at about 2.5, H_a and H_b continue to move down to pH 1.2. Furthermore H_a and H_b show a slight discontinuity at about pH 3.5, and this fact together with the very broad titration curve indicates the sequential titration of two carboxyl groups of appreciably different dissociation constants. Proton H_c is affected greatly by the titration of the carboxyl group of higher pK' and only very slightly by that of lower pK'. We therefore assign the higher pK to the carboxyl group adjacent to H_c and located *para* to the thiocarbamyl group, hereafter called the *para*-carboxyl group. This is consistent with the resonance donation of electrons by the thiocarbamyl group to the *para* position in the ring.

Graphs of $\log[(\delta_A - \delta)/(\delta - \delta_B)]$ against pH for proton 3 (adjacent to the C-terminal carboxyl group) and for proton H_c give straight lines with gradients of 1.26 and 0.84 respectively. This deviation from unity is indicative of an interaction between the carboxyl group and another group, as for example where proton H_c responds not only to the titration of the *para*-carboxyl, but also slightly to the titration of the *meta*-carboxyl group. This broadens out the titration curve somewhat at the low end and has the effect of reducing the gradient below 1.0. The type of interaction that involves the C-terminal carboxyl group is not clear. A reasonable value of the pK' of the two carboxyl groups is obtained from these lines (see Table 1). When the data from protons H_a and H_b is plotted in this way there is clear evidence of interactions since, for example, the graph of H_a is best fitted by two intersecting straight lines [24]. However, it is possible to split up graph H_a in Fig. 4 into two separate titration curves using the information that the titration curve of higher pK is centred around pK 4.6. Reasonable values of δ_a and δ_b can be estimated (see Table 1) and used for the two straight line plots. Satisfactory graphs were obtained of unit gradient which give rise to the pK' values in Table 1. The *meta*-carboxyl group has a low pK' of 2.9 which is identical with the pK_1 for dissociation of phthalic acid in water. However, the pK' of the *para*-carboxyl group of 4.6 is somewhat less than that of pK_2 of phthalic acid (5.5).

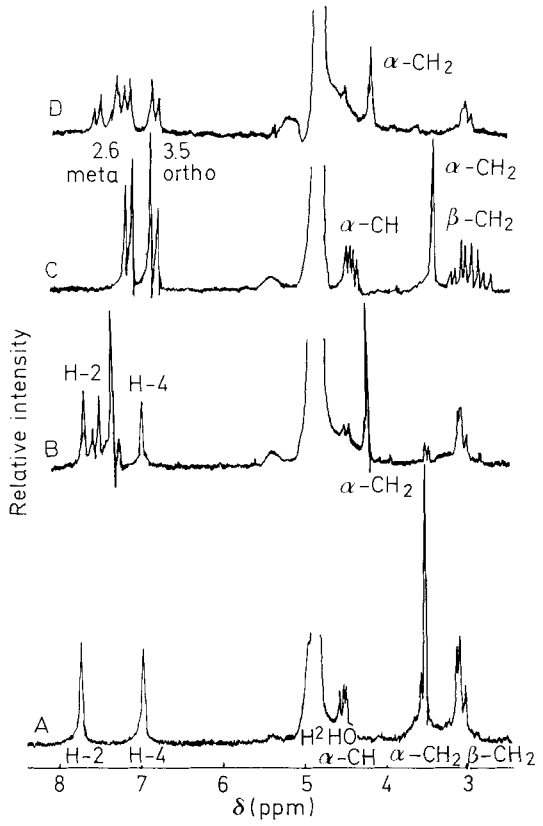


Fig. 5. *^1H NMR spectra at 100 MHz in ^2H_2O at pH meter reading 8.7 of (A) Gly-L-His, (B) the product from the reaction of Gly-L-His with PhtNCS, (C) Gly-L-Tyr and (D) the product from the reaction of Gly-L-Tyr with PhtNCS.* Assignments from [29, 30]

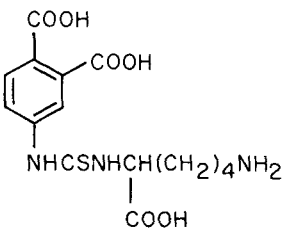
Reaction of PhtNCS with Dipeptides Containing Histidine and Tyrosine

The reaction between equimolar amounts of PhtNCS and glycyl-L-histidine or glycyl-L-tyrosine at 25 °C and pH 8.7 or 7.5 is complete in 0.25 h (see Fig. 5) and 24 h respectively. The reaction with both dipeptides caused the replacement of the α-CH_2 proton resonance of the glycyl residue by another α-CH_2 resonance further downfield, due to the reaction of the α-amino group of the dipeptide. The two singlet H-2 and H-4 resonances of the histidyl side chain [29] remain unchanged after reaction and there is no evidence in Fig. 5B of the occurrence of another pair of shifted resonances, as might be expected if there had been reaction on the imidazole ring. The additional resonances observed in the aromatic region are all due to the three ring protons of the phthalic acid moiety. In the glycyl-L-tyrosine spectra (Fig. 4C and D) the reaction with PhtNCS causes a slight (0.04 ppm) upfield shift of the pair of doublets due to the tyrosyl side chain. There is no evidence of direct reaction of the phenolic group with PhtNCS. The corresponding reactions carried out at pH 7.5 required 24 h to complete and also showed no evidence of reactions with the histidyl or tyrosyl side chains. It is concluded that these side chains do not react with PhtNCS at an appreciable rate compared with an α-amino group.

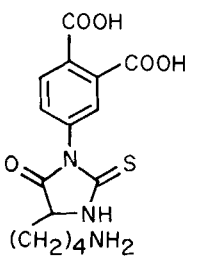
Reaction of PhtNCS with L-Lysine

Lysine monochloride (183 mg, 1 mmol) and PhtNCS (234 mg, 1.05 mmol) were added to 40 ml H_2O at 20 °C. The pH was adjusted to 8.5 with aqueous sodium hydroxide and then kept at this value for 21 h using a pH stat. The pH was adjusted to 6.2 with dilute hydrochloric acid and the solution was lyophilized. A creamy solid was obtained which could not be purified further. The ^1H NMR spectrum in ^2H_2O indicated that the triplet α-CH resonance of lysine ($\delta = 3.77$ ppm) had almost disappeared (less than 20% left); presumably the α-CH resonance of the α-substituted molecule III occurs under the ^{2}HHO signal. A triplet-like resonance at 3.57 ppm is probably due to the ε-CH_2 signal of molecules substituted at the ε-NH_2 group. With this interpretation of the spectrum, the relative amounts of substitution at the α-NH_2 and ε-NH_2 groups is approximately 5:1. This is consistent with the significant difference between the pK of the α-NH_2 and ε-NH_2 groups of lysine, 9.18 and 10.79 respectively [31].

The crude product was heated on a waterbath with 5 ml of 1 M HCl for 15 min and evaporated to dryness to convert compound III to the phthalic acid thiohydantoin derivative of lysine, compound IV. This compound gave a discrete spot on thin-layer chromatography plates. On cellulose with propan-1-ol/acetic acid/water (5/2/1) it had $R_F = 0.42$ and on silica gel (Baker-Flex silica gel IB-F sheets soaked for 30 min in propan-1-ol/acetic acid then washed with acetone and air dried) it had $R_F = 0.74$ with propan-1-ol/acetic acid/water (5/2/1) and $R_F = 0.50$ with butan-1-ol/acetic acid (1/1). Despite these desirable chromatographic properties, it was not possible to purify the product.



(III)



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Reaction of PhtNCS with L-Lysyl-L-tryptophan

Chemical shifts of the α-CH and ε-CH_2 resonances of L-Lys-L-Trp in Fig. 6 show, as well as the major shifts of resonances, second-order perturbations of the

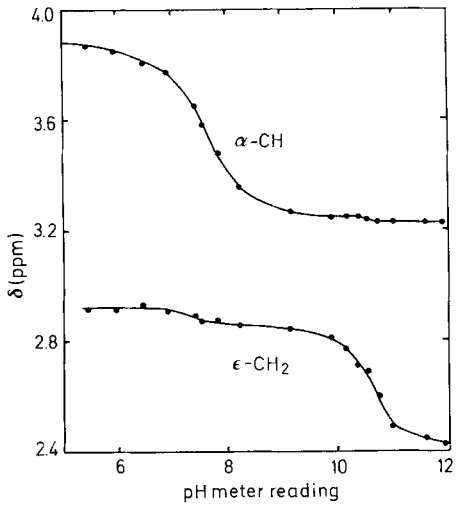

Fig. 6. *Plot of chemical shift against pH meter reading of the α-CH and ε-CH_2 resonances of the lysyl residue of* L-*Lys*-L-*Trp measured at 25 °C in* ^2H_2O

Table 2. *Chemical shift data of Lys-Trp and phthalic acid thiocarbamyl (PhtNCS) derivatives (structure III) measured at pH meter reading 7.2 in* ^2H_2O
Abbreviations used are m multiplet, t triplet, brt broad triplet, dd doublet of doublets.

Assignment of resonances	Chemical shifts, δ, of resonances in		
	Lys-Trp	α-PhtNCS-Lys-Trp	α,ε(PhtNCS)$_2$-Lys-Trp
	ppm		
Lys β,γ,δ-CH_2	1.1 – 1.8 m	1.1 – 1.9 m	1.1 – 1.7 m
Lys ε-CH_2	2.90 t	2.85 t	3.40 brt
Trp CH_2	3.0 – 3.5 m	3.0 – 3.5 m	3.0 – 3.7 m
Lys α-CH	3.71 brt	4.6 – 5.0[a]	4.5 – 5.1[a]
Trp CH	4.61 dd	4.59 dd	4.5 – 5.1[a]
Aromatic H	7.1 – 7.8 m	7.1 – 7.8 m	7.1 – 7.8 m

[a] Obscured by ^{2}HHO signal.

titration curves which result from titration of a charged group that is remote from the proton(s) being observed. pK' values for the α-NH_2 and ε-NH_2 groups are 7.60 and 10.65 respectively.

L-Lysyl-L-tryptophan (22.5 mg, 0.067 mmol) in 0.2 ml ^2H_2O at pH 7.2 was added to PhtNCS (14.9 mg, 0.067 mmol) in 0.2 ml ^2H_2O at pH 7.2 at 25 °C. The reaction was monitored by ^{1}H NMR spectroscopy. After 3 h there had been about 50% substitution of the α-NH_2 groups and after 22 h a plateau of 85% reaction was reached. Another portion of PhtNCS (30 mg, 0.133 mmol) was added to the reaction mixture and the pH adjusted to 9.5 with NaO^{2}H. After 30 min the pH was reduced to 7.2 using ^{2}HCl and the NMR spectrum showed that both the α-NH_2 and ε-NH_2 groups had been substituted by PhtNCS.

The ^{1}H NMR parameters at pH 7.2 for these three compounds are given in Table 2. Satisfactory integrals of the areas under resonances were obtained in all cases. For α-substitution by PhtNCS the Lys α-CH signal shifted downfield by 0.9 to 1.3 ppm and for ε-substitution the Lys ε-CH_2 signal shifted by 0.5 ppm. In the case of the α-CH signal, the resonance shifted under the ^{2}HHO signal and was therefore not observed, the extent of reaction being monitored by the disappearance of the corresponding signal in Lys-Trp.

A crude sample of PhtNCS-Lys-Trp (10 mg) prepared by the above procedure using a slight excess of PhtNCS was warmed in 20 ml of 0.1 M HCl and evaporated to small volume. (This is the Edman degradation procedure which would cleave the peptide and form the thiohydantoin derivative IV.) Thin-layer chromatography of this reaction mixture as described above, gave a spot with the same R_F value as for compound IV prepared from lysine.

^{1}H NMR Study of RNase-A

The ^{1}H NMR spectrum at 270 MHz of RNase-A was examined in ^2H_2O as a function of pH and representative spectra are shown in Fig. 7. In order to follow the movement of the small resonance as a function of pH, difference spectra were collected [7] by subtraction of the spectra as indicated on the figure. These were necessary in order to be able to follow the resonance, particularly at low pH, where it was obscured by other large broad resonances. The small, sharp differences observed at about 3.0 ppm result from the commencement of the titration of the ε-CH_2 lysine residues of RNase-A, which are the major contributors to the large ^{1}H NMR peak at 3.0 ppm. In favourable cases the small resonance was observed as a triplet as shown in spectrum 1 of Fig. 7, in both the presence and absence of phosphate, which causes a slight downfield shift of the resonance. The coupling constant of 6.3 Hz compares favourably with the coupling constants of the α-CH of lysine and of L-Lys-L-Trp at pH 5.5 – 7.5, which were 6.0 Hz and 6.5 Hz respectively.

The chemical shift titration data are plotted in Fig. 8 and the linear plot given in Fig. 9. The measured pK' value of 7.30 is similar to that of 7.60 obtained for the α-NH_2 group of L-Lys-L-Trp (see above). Further confirmation of the assignment of the resonance in RNase-A to the α-CH proton of lysine-1 comes from the similarity of the chemical shift data in Fig. 8 and of the α-CH of L-Lys-L-Trp (Fig. 6). ($\delta_A - \delta_B$) is 0.65 ppm and 0.63 ppm for the RNase-A and the peptide respectively and they both titrate over a similar range of chemical shifts. The gradient of the line in Fig. 9 is 0.87 which is lower than the theoretical value of 1.0. However, since the gradient is very sensitive to the choice of the numerical values of δ_A

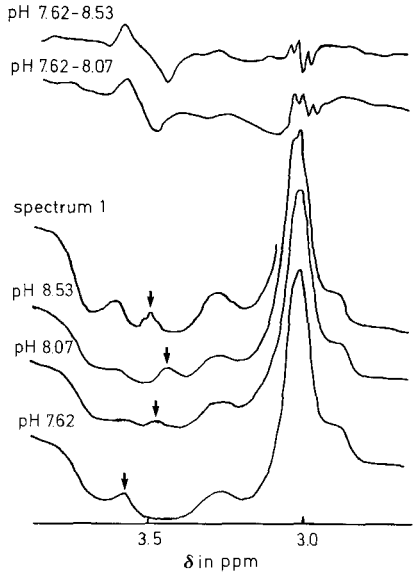

Fig. 7. *^1H NMR spectra at 270 MHz of a 3.5 mM RNase-A solution in ^2H_2O at 25 °C and various pH meter readings.* The resonance marked with an arrow, which appears as a triplet only in the spectrum 1 (obtained at pH meter reading 8.25 in 0.017 M phosphate) is assigned to the α-CH proton of the N-terminal residue, lysine-1. The two spectra at the top are difference spectra at about twice the gain of the normal spectra

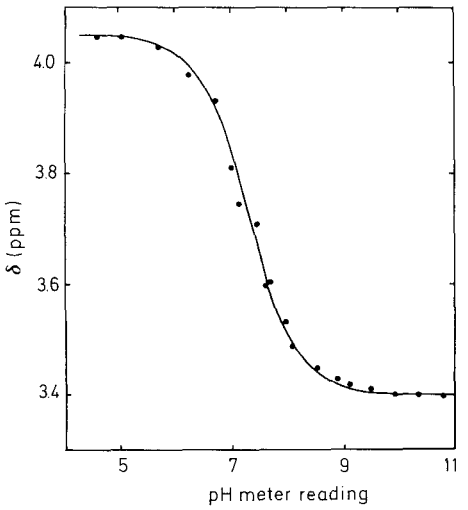

Fig. 8. *Plot of chemical shift against pH meter reading for the titration of the N-terminal α-CH proton of RNase-A in ^2H_2O at 25 °C.* The smooth line is that obtained from the straight line graph of Fig. 9

and δ_B, it is quite possible that it could be within experimental error. The position of the N-terminus is not well defined in RNase-S [32] and in RNase-A [33] residue 1 is projecting away from the molecule into the solution. Thus there is no evidence of interaction between it and the remainder of the molecule and no hydrogen bond is formed that involves lysine-1 [32, 33].

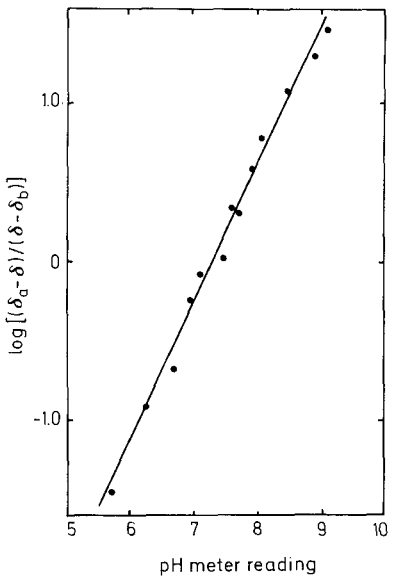

Fig. 9. *Linear plot of log $[(\delta_a-\delta)/(\delta-\delta_b)]$ against pH meter reading for the data points obtained from Fig. 8.* The straight line of best fit to the points gives a pK' of 7.30 and a slope of 0.87

Reaction of RNase-A with PhtNCS

To a 4 mM solution of RNase-A in ^2H_2O at pH 7.2 was added a solution of PhtNCS in ^2H_2O at pH 7.2 to give a 30% molar excess of PhtNCS. The extent of reaction was estimated from the disappearance of the aromatic ^1H NMR signals of PhtNCS and the appearance at 270 MHz of the slightly downfield signals of the reacted PhtNCS (see also Fig. 3). There was 50% reaction in 2 h and the reaction was complete in 22 h. Two further reactions were carried out as above except that the amount of PhtNCS was varied using (a) 1.1 and (b) 1.75 mol of PhtNCS per mol of RNase-A. In these cases after 24 h the pH was adjusted to 8.3 and NMR spectra run immediately and compared with the spectrum of RNase-A at pH 8.3 (Fig. 7). The size of the small peak due to the α-CH of lysine-1 was reduced to 20 – 30% of its original size in case (a) and to zero in (b). Difference spectroscopy at 270 MHz was used without success to try to find the resonance due to the α-CH of lysine-1 of PhtNCS-RNase-A. The resonance probably disappears under the large ^{2}HHO resonance, since the α-CH of lysine was also shifted under the ^{2}HHO signal by reaction with PhtNCS. No evidence could be found by difference spectroscopy for reaction of PhtNCS at any of the ε-NH_2 lysine sites in RNase-A.

RNase-A (137 mg, 10 μmol) and PhtNCS (3.0 mg, 13 μmol) were mixed in water (3 ml) and sodium hydroxide solution added to keep the solution at pH 7.2 – 7.4 for 24 h. A solution of 1 M HCl (5 ml) was added, the mixture flushed with nitrogen and heated at 80 °C for 10 min. The product was then concentrated to 3 ml and extracted with butan-1-ol (5 × 3 ml). This

was evaporated to dryness and the residue exhaustively extracted with acetone. Comparison with an authentic sample of the thiohydantoin derivative IV by thin-layer chromatography, as described earlier, showed that the extract contained only compound IV and from the intensity of the spots was obtained in $60 \pm 10\%$ yield, based on RNase-A. This provides further proof of the correctness of the assignment of the triplet resonance in Fig. 7 to the α-CH proton of lysine-1 of RNase-A.

DISCUSSION

It is useful to summarise the ^1H NMR evidence for the assignment of the resonance in Fig. 7 to the α-CH proton of lysine-1 of RNase-A, and then to discuss the more general question of the use of the new reagent PhtNCS, for reaction with α-amino groups at the N-terminus of proteins. The resonance in spectrum 1 of Fig. 7 is a triplet with a coupling constant of 6.3 Hz, compared with 6.0 Hz in lysine and 6.5 Hz in L-Lys-L-Trp. The chemical shift of the resonance and its titration curve with change of pH, is very similar to that obtained for the simple model peptide L-Lys-L-Trp; the pK' values are 7.3 and 7.6 respectively. Convincing proof comes from the disappearance of the resonance on reaction of RNase-A with PhtNCS to form Pht-NCS-RNase-A, which is then converted to the corresponding thiohydantoin and cleaved by dilute acid to give compound IV. The pK' of 7.6 is higher than the value of 6.6 obtained from methylation of the amino groups of RNase-A [24], but the need for a correction factor in the earlier work makes it more open to error. In insulin pK' values of 8.0 and about 6.7 have recently been obtained [23] for the two N-terminal amino groups. The value of 7.6 for RNase-A seems to be quite reasonable for an α-amino group that is exposed to solvent [33].

Since an α-NH_2 group has a lower pK than an ε-NH_2 group, it should be possible to obtain preferential reaction of the former with an isothiocyanate by reaction at a pH low enough to ensure that the concentration of the active species, i.e. the uncharged form of the ε-NH_2 group, is very small compared with that of the uncharged form of the α-NH_2 group. For L-lysine, where the pK_a of the α-NH_2 and ε-NH_2 groups are 9.18 and 10.79 respectively, reaction at pH 8.5 gave about 5 times as much of the α-NH_2 as of the ε-NH_2 product. However, in a peptide the pK_a of the α-NH_2 group is reduced to about 8 because of the replacement of the carboxylate anion by a peptide group, without any appreciable change of the pK_a of the ε-NH_2 group. It is therefore possible to obtain essentially complete reaction at the α-NH_2, by reaction at pH meter reading 7.2 in ^2H_2O for about 22 h, without any appreciable amount of reaction at the ε-NH_2 group. This was found for both L-lysyl-L-tryptophan and RNase-A; in the former case it was possible to react the ε-NH_2 group by a subsequent treatment with PhtNCS at pH meter reading 9.5. In the general case of the reaction of the α-NH_2 group of any protein it may be necessary to work at a slightly higher pH (say 7.5), because the pK_a of most α-NH_2 groups is greater than the values of 7.6 and 7.3 which obtain for the α-NH_2 lysyl residues used here.

The lack of reaction of PhtNCS with either histidyl or tyrosyl side chains also shows the specificity of the reagent for the N-terminal α-amino group. The general specificity of the reagent is further confirmed by the fact that complete reaction of the α-NH_2 group of RNase-A can be effected by the addition of about 1.3 mol of PhtNCS per mol of RNase-A. Clarly the amount of PhtNCS consumed in side reactions, including slow hydrolysis of the reagent (see above), is small. We have also confirmed that reaction occurs cleanly on lysine-1 by NMR spectroscopy of the product and also by formation and identification of compound IV produced by cyclisation and cleavage of the N-terminal peptide bond.

The pK' values of the *para*-carboxyl and *meta*-carboxyl groups are 4.6 and 2.9 respectively in PhtNCS-Gly-Gly-Gly (structure II, Fig. 4) and the stability constant of Pr^{3+} to PhtNCS at pH 5 in ^2H_2O is 960 M^{-1}. Since lanthanides precipitate as the hydroxide above about pH 7 and at pH values below about 4 would have to compete with hydrogen ions for the carboxylate anion binding sites, it is seen that the range of pH values over which the binding site is effective is about 4 – 7. The stability constant of 960 M^{-1} is about 220 times the value of 4.3 M^{-1} obtained by similar methods with Pr^{3+} on diglycine [26]. Thus, in a situation in which the protein concentration is in excess over lanthanide, it can be readily shown that the amount of lanthanide bound on the adjacent carboxyl groups of the PhtNCS derivative of the protein is about 100 times that bound on a single carboxyl side chain.

In conclusion, it has been shown that PhtNCS is a simple reagent that is able to react specifically at the N-terminus of a protein, with the introduction of adjacent carboxyl groups that bind lanthanide ions very much more strongly than single carboxyl side chains. It is therefore possible to introduce the binding site at a known location in the molecule. The possibility of mapping the structure of the protein in the vicinity of the N-terminus, by NMR spectroscopy, is being investigated.

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REFERENCES

1. Dwek, R. A. (1973) *Nuclear Magnetic Resonance in Biochemistry*, Clarendon Press, Oxford.
2. Bradbury, J. H., Brown, L. R., Crompton, M. W. & Warren, B. (1974) *Pure Appl. Chem. 40*, 83 – 92.
3. Levine, B. A. & Williams, R. J. P. (1975) *Proc. R. Soc. Lond. A, Math. Phys. Sci. 345*, 5 – 22.
4. Campbell, I. D., Dobson, C. M. & Williams, R. J. P. (1975) *Proc. R. Soc. Lond. A, Math. Phys. Sci. 345*, 23 – 40, 41 – 59.
5. Sullivan, G. R. (1976) *J. Am. Chem. Soc. 98*, 7162 – 7169.
6. Meadows, D. H., Jardetzky, O., Epand, R. M., Rüterjans, H. H. & Scheraga, H. A. (1968) *Proc. Natl Acad. Sci. U.S.A. 60*, 766 – 772.
7. King, N. L. R. & Bradbury, J. H. (1971) *Nature (Lond.) 229*, 404 – 406.
8. Bradbury, J. H. & Chapman, B. E. (1972) *Biochem. Biophys. Res. Commun. 49*, 891 – 897.
9. Patel, D. J., Canuel, L. L. & Bovey, F. A. (1975) *Biopolymers, 14*, 987 – 997.
10. Markley, J. L. (1975) *Acc. Chem. Res. 8*, 70 – 80.
11. Bradbury, J. H. & Teh, J. S. (1975) *Chem. Commun.* 936 – 937.
12. Imoto, T., Johnson, L. N., North, A. C. T., Phillips, D. C. & Rupley, J. A. (1972) in *The Enzymes*, 3rd edn (Boyer, P. D., ed.) vol. 7, pp. 665 – 868, Academic Press, New York.
13. Bradbury, J. H. & Brown, L. R. (1973) *Eur. J. Biochem. 40*, 565 – 576.
14. Reuben, J. (1975) *Naturwissenschaft, 62*, 172 – 178.
15. Moeller, T., Martin, D. F., Thompson, L. C., Ferrus, R., Feistel, G. R. & Randall, W. J. (1965) *Chem. Rev. 65*, 1 – 50.
16. Brown, L. R. (1974) Ph. D. Thesis, Australian National University.
17. Marinetti, T. D., Snyder, G. H. & Sykes, B. D. (1975) *J. Am. Chem. Soc. 97*, 6562 – 6570.
18. Marinetti, T. D., Snyder, G. H. & Sykes, B. D. (1976) *Biochemistry, 15*, 4600 – 4608.
19. Bogert, M. T. & Renshaw, R. R. (1906) *J. Am. Chem. Soc. 28*, 617 – 627.
20. Bogert, M. T. & Renshaw, R. R. (1908) *J. Am. Chem. Soc. 30*, 1135 – 1144.
21. Dyson, G. M. (1961) *Org. Syn. Coll. 1*, 165 – 166.
22. Emsley, J. W., Feeney, J. & Sutcliffe, L. H. (1966) *High Resolution Nuclear Magnetic Resonance Spectroscopy*, vol. 2, Pergamon Press, Oxford.
23. Bradbury, J. H. & Brown, L. R. (1977) *Eur. J. Biochem. 76*, 573 – 582.
24. Brown, L. R. & Bradbury, J. H. (1975) *Eur. J. Biochem. 54*, 219 – 227.
25. Armitage, I., Dunsmore, G., Hall, L. D. & Marshall, A. G. (1972) *Can. J. Chem. 50*, 2119 – 2129.
26. Warren, B. (1976) Ph. D. Thesis, Australian National University.
27. Sheinblatt, M. (1966) *J. Am. Chem. Soc. 88*, 2845 – 2848.
28. Bradbury, J. H., Crompton, M. W. & Warren, B. (1974) *Anal. Biochem. 62*, 310 – 316.
29. Bradbury, J. H. & Scheraga, H. A. (1966) *J. Am. Chem. Soc. 88*, 4240 – 4246.
30. Campbell, I. D., Dobson, C. M. & Williams, R. J. P. (1975) *Proc. R. Soc. Lond. B, Biol. Sci. 189*, 503 – 509.
31. Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. (1969) *Data for Biochemical Research*, 2nd edn, Clarendon Press, Oxford.
32. Richards, F. M. & Wyckoff, H. W. (1971) in *The Enzymes*, 3rd edn (Boyer, P. D., ed.) vol. 4, pp. 647 – 806, Academic Press, New York.
33. Carlisle, C. H., Palmer, R. A., Mazumdar, S. K., Gorinsky, B. A. & Yeates, D. G. R. (1974) *J. Mol. Biol. 85*, 1 – 18.

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