The Nature of the Cross-linking of Proteins by Glutaraldehyde. Part 2.[1] The Formation of Quaternary Pyridinium Compounds by the Action of Glutaraldehyde on Proteins and the Identification of a 3-(2-Piperidyl)-pyridinium Derivative, Anabilysine, as a Cross-linking Entity [2]

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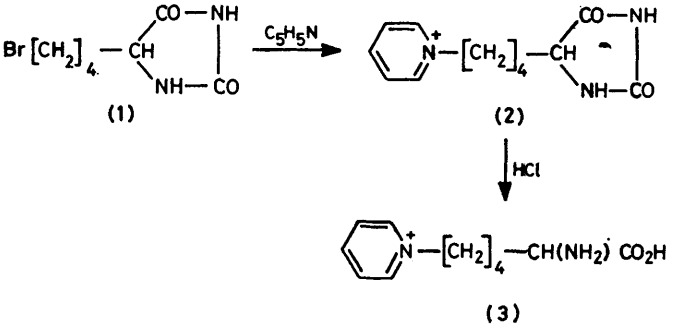
1-(5-Amino-5-carboxypentyl)pyridinium chloride (3) and 1-(5-amino-5-carboxypentyl)-3-[1-(5-amino-5-carboxypentyl)-2-piperidyl]pyridinium chloride, anabilysine (4), have been isolated from acid hydrolysates of glutaraldehyde-treated ovalbumin and their structures confirmed by synthesis. It is concluded that the strong chromophore at *ca.* 265 nm shown by glutaraldehyde-treated proteins is due to the formation of such quaternary pyridinium compounds and that anabilysine residues are important in the cross-linking of proteins by glutaraldehyde.

DESPITE the ever-increasing use of glutaraldehyde for the modification of proteins for both scientific and technological purposes (for some references see Part 1) the chemical nature of the modification remains unknown. It is generally accepted that it involves lysine side-chains and various conjectures as to its chemistry have been made.[1,3-6] In no case, however, have these been supported by the isolation of a modified lysine and the determination of its structure. We now present evidence in support of our earlier proposal [1] that the modification involves the formation of quaternary pyridinium compounds.†

As a result of exploratory experiments with several commercially available proteins, ovalbumin was chosen for study. The reaction was carried out at room temperature using a 4% solution of the protein in sodium acetate buffer, pH 4.7, and an equal volume of an aqueous 25% solution of the aldehyde. The characteristic absorption at *ca.* 265 nm developed rapidly and the reaction appeared to be substantially complete in 90 min. The modified protein was isolated by dialysis, which caused its precipitation, and oxidised with performic acid to destroy any residual aldehyde groups and so simplify the isolation of modified lysines.‡ An amino-acid analysis after complete acidic hydrolysis showed that *ca.* 50% of the lysine residues had been modified. Chromatography of the hydrolysed protein on Dowex 50W-X8 gave two main products with characteristic absorption maxima near 265 nm. One of these (Product *A*) was eluted from the resin by 0.35M-ammonium acetate buffer, pH 5.25, and the other (Product *B*) by 2M-ammonium hydroxide; both were purified by further chromatography.

Product *A* was purified by successive chromatography on Dowex 50W-X8, G15 Sephadex, and carboxymethylcellulose (CM 52) and finally desalted on Dowex 50W-X8 (ammonium form); it was homogeneous by t.l.c. in eight systems. Its absorption spectrum in aqueous solution showed $\lambda_{max.}$ 257 nm (ε 4 200) with shoulders at 254 and 265 nm and was unaffected by pH, as expected for a quaternary salt. The ^{1}H n.m.r. spectrum was typical of a 1-substituted pyridine, showing a doublet at δ 8.85 (2- and 6-H), a triplet at 8.65 (4-H), and a triplet at 8.16 (3- and 5-H) (relative areas 2 : 1 : 2), and consistent with its formulation as 1-(5-amino-5-carboxypentyl)pyridinium chloride (3). The crude yield from ovalbumin, based on the lysine residues modified, was 7% and the final yield of pure material 0.7%.

The structure (3) was confirmed by synthesis from 5-(4-bromobutyl)hydantoin (1),[7] by heating with pyridine followed by hydrolysis of the resulting pyridinium hydantoin (2) with hot 6M-hydrochloric acid. The synthetic



(1) (2) (3)

specimen of (3) showed u.v. absorption and ^{1}H n.m.r. spectra identical with those of the product from ovalbumin and the two were inseparable by t.l.c. in six systems.

Product *B*, hereafter referred to as anabilysine (to signify its derivation from two lysine residues and its relationship to anabasine), was purified in the same way as Product *A*, except that G25 Sephadex was used in the second stage; it, too, was homogeneous on t.l.c. (four systems). The crude yield based on the structure (4) finally established for anabilysine was 13.5%, and the final yield of pure material 5.5%. The absorption spectrum in aqueous solution showed $\lambda_{max.}$ 263 nm (ε 4 000) with a shoulder at 258 nm; as expected for a quaternary salt it was unaffected by change in pH. The ^{1}H n.m.r. spectrum, in CF_3CO_2D, showed three signals attributable to an unsymmetrically substituted pyridine ring, *viz.* a singlet at δ 9.28, a doublet at 9.00, and a triplet

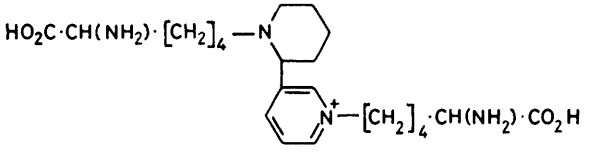
† The formation of such compounds, as one of several possibilities, was also suggested in ref. 4.

‡ The same products were obtained, although less conveniently, when residual aldehyde groups were modified by a Strecker synthesis.

at 8.30 (relative areas 1 : 2 : 1). The presence of a one-proton singlet with a chemical shift (9.28) appropriate to a 2-H is strongly indicative of 1,3-disubstitution; anabasine (5), in the same solvent, shows a singlet at δ 9.44, a doublet at 9.14, and a triplet at 8.42 (relative areas 1 : 2 : 1). Acetylation gave a product whose higher R_F values on t.l.c. afforded better evidence of homogeneity; its ^{1}H n.m.r. spectrum confirmed the 1,3-disubstitution pattern, the allocation of the signals being further supported by double-resonance experiments.

The isolation of anabilysine from glutaraldehyde-treated ovalbumin was tedious and time-consuming and we therefore sought an alternative source to provide sufficient material for ^{13}C n.m.r. studies. Experiments with lysine derivatives attached to various insoluble supports culminated in the use of α-acetyl-lysine coupled with *NN'*-dicyclohexylcarbodi-imide to controlled porosity glass beads treated with 3-aminopropyltriethoxysilane.[8] Using loadings of 0.03—0.04 mmol g^{-1} and reaction conditions similar to those used with ovalbumin in solution, anabilysine was isolated in 8% yield as the major product, accounting for 65% of the initial light absorption in the 265 nm region. The cleaner crude reaction product, allowing the first fractionation on Dowex to be dispensed with, and the relative ease of scaling up the process enabled us to isolate sufficient material spectroscopically and chromatographically identical with anabilysine from ovalbumin to obtain a good ^{13}C n.m.r. spectrum.

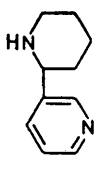
The ^{1}H n.m.r. spectrum of anabilysine and the formation of both a mono- and a bis-*N*-benzyloxycarbonyl derivative were in accord with structure (4), derived from



(4)

two lysine side-chains and two molecules of glutaraldehyde. The ^{13}C noise-decoupled n.m.r. spectrum in D_2O (see Table) completely confirms this structure, showing 17 separate peaks for the 22 carbon atoms; the two carboxy-carbons and the two lysine α-carbons gave only one peak for each pair and six (β-, γ-, and δ-) lysine side-chain carbons two doublets and a singlet.

Structure (4) was confirmed by synthesis from the tobacco alkaloid anabasine (5), which contains the same 3-(2-piperidyl)pyridine skeleton. Reaction of anabasine with 5-(4-bromobutyl)hydantoin (1) in refluxing methanol, followed by acidic hydrolysis, gave a product chromatographically indistinguishable from anabilysine from glutaraldehyde, together with the mono-alkylation product (6). The u.v.–visible and ^{1}H n.m.r. spectra of the synthetic anabilysine were almost identical with those of the product from glutaraldehyde, and the ^{13}C n.m.r. spectrum (see Table) differed only in that the six lysine side-chain carbons referred to in the preceding paragraph gave five singlets instead of two doublets and a singlet. Complete identity of the synthetic anabilysine and that from glutaraldehyde is not to be expected since the two samples will be different mixtures of diastereoisomerides. Anabilysine contains three chiral centres, *viz.* the two α-carbons of the lysine side-chains and the C-2 of the piperidine ring. In the synthetic material only the piperidine C-2 will be stereochemically homogeneous and this material must therefore be a mixture of four diastereoisomers; the material from glutaraldehyde is likely to be a mixture of two diastereoisomers at the piperidine C-2. On the basis of the evidence, therefore, there is no reasonable doubt as to the structural identity of the two products and hence of the correctness of structure (4) for anabilysine.



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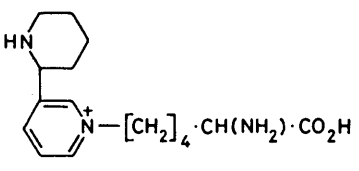

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^{13}C N.m.r. spectrum of anabilysine (13.3 mg ml^{-1} in D_2O at 30 °C)

Chemical shift (p.p.m. from MeOH) Synthetic	*ex* Glutaraldehyde	Assignment *	No. of C atoms
123.6	123.5	Carboxy C	2
96.9	96.7	Pyridine C-4	1
95.9, 95.2	95.8, 95.0	Pyridine C-2 and -6	2
87.7, 80.7	87.5, 80.5	Pyridine C-3 and -5	2
16.6	16.5	ε-C adjacent to pyridine N$^+$	1
13.1	12.9	Piperidine C-2	1
5.3	5.2	Piperidine C-6	1
4.8	4.7	ε-C adjacent to piperidine N	1
4.1	4.1	α-C	2
−16.9, −18.9, −19.6	−17.0, −19.1, −19.7	Other piperidine C	3
−26.1, −26.3, −27.1, −27.3, −27.7	−26.5(d), −27.5(d), −27.8	Other side-chain C	6

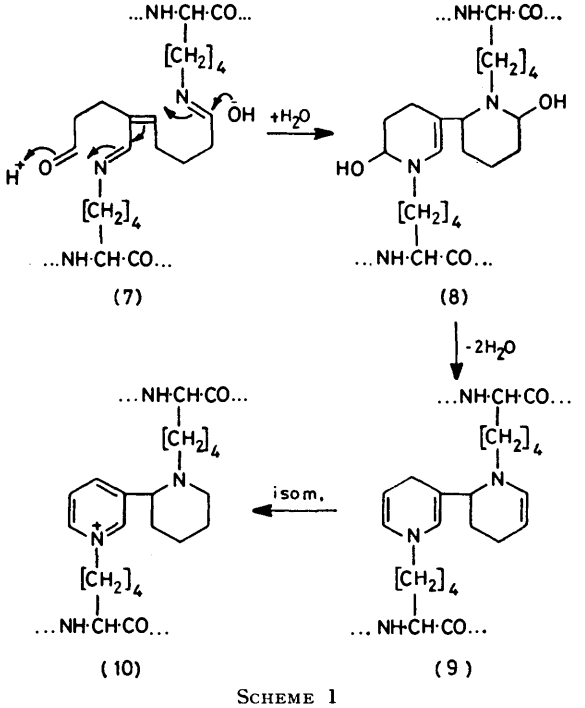
* The ^{13}C n.m.r. spectrum of anabasine shows peaks at 97.0, 93.1, 91.7, 87.5, and 19.8 p.p.m. from MeOH for the pyridine carbons and at 8.2, −3.0, −20.3, −27.1, and −27.7 for the piperidine carbons.

Anabilysine is the first cross-linking entity to be isolated from a glutaraldehyde-treated protein and its isolation and identification provide *prima facie* evidence for the presence in such proteins of cross-linkages of type (10). These could easily arise by internal oxidation–reduction of the isomeric cross-linkages (9), derived from two lysine side-chains and two glutaraldehyde molecules by way, for example, of formation of the Schiff base (7), cyclisation to the dihydroxy-compound (8), and dehydration (Scheme 1). Un-cross-linked pyridinium side-chains giving rise to (3) on hydrolysis

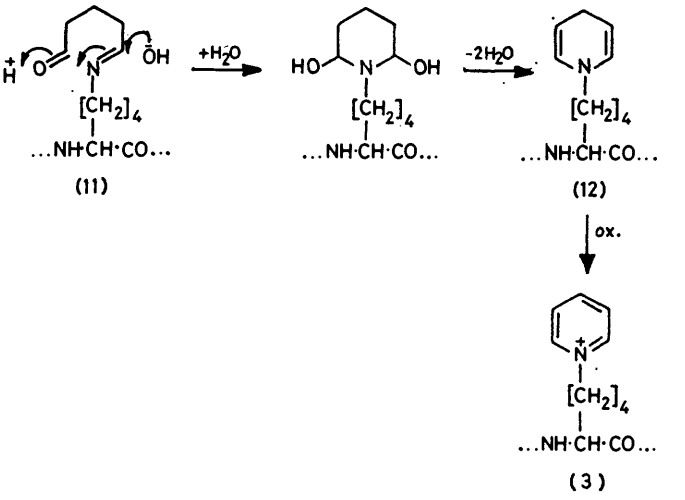
could be formed by a similar process from the simple Schiff base (11) by way of the easily oxidisible dihydropyridine (12) (Scheme 2).

The observation that anabilysine is the major product from the reaction of glutaraldehyde with α-acetyllysine attached to glass beads, but is accompanied by much (3) in the reaction with ovalbumin in solution, is probably due to the proximity of the lysine side-chains (average separation 20 Å) in the former case. This is borne out by the finding that anabilysine is similarly the major product when glutaraldehyde reacts with the tetrapeptide *N*-acetyl-L-lysylglycyl-L-lysylglycine (synthesised for the purpose by conventional methods as described in the Experimental section) attached to glass beads, since here, too, the lysine residues are sufficiently close together to favour greatly the cross-linking reaction.

Although we believe that our work demonstrates the presence of substantial numbers of anabilysine cross-linkages in glutaraldehyde-treated proteins, we do not claim that these are the only cross-linkages present; indeed, we have encountered other, unidentified, pyridinium compounds in hydrolysates of glutaraldehyde-treated ovalbumin in the course of the present work. As in the case of elastin, where desmosine and isodesmosine cross-linkages are known to be accompanied by others involving reduced pyridinium residues,[9] so in the case of glutaraldehyde-treated proteins it is likely that anabilysine cross-linkages will be accompanied by others based on the same 2,3′-bipyridyl skeleton in different oxidation states. Furthermore, the intensity of absorption around 265 nm in acid hydrolysates of glutaraldehyde-treated ovalbumin is considerably greater (1.66 times) than it would be if every modified lysine



SCHEME 1



SCHEME 2

side-chain had been converted into a pyridinium residue as in (3) or anabilysine; the discrepancy would be accounted for by the presence of the more intensely absorbing[10] 2,3′-bipyridinium analogue of (10). The observation that the absorption around 265 nm becomes more intense on acidic hydrolysis[11] could be accounted for by aromatisation of reduced pyridine nuclei under the acidic conditions of the hydrolysis.[4]

EXPERIMENTAL

Solutions were concentrated or evaporated on a rotary evaporator at temperatures as near ambient as practicable. The purity of all compounds was confirmed by t.l.c. in at least two of the following systems: (*A*) C_5H_5N–AcOH–H_2O (50 : 30 : 15) on silica gel; (*B*) aqueous NH_4OH (*d* 0.880)–AcOH–H_2O (10 : 10 : 80) on silica gel; (*C*) MeCOEt–AcOH–H_2O (10 : 30 : 25) on silica gel; (*D*) the same (30 : 10 : 30) on silica gel; (*M*) Bu^nOH–AcOH–H_2O (3 : 1 : 1) on cellulose; (*N*) Bu^nOH–C_5H_5N–AcOH–H_2O (15 : 10 : 3 : 12) on cellulose; (*O*) as (*A*) on cellulose; (*P*) as (*D*) on cellulose. Chromatographic separations were monitored with an L.K.B. Uvicord at 255 nm; the absorbances, *A*, of bulked fractions, corrected for background end-absorption, were measured with a Pye–Unicam SP 800B recording spectrophotometer.

^1H N.m.r. spectra were recorded at 100 MHz on a JEOL MH-100 spectrometer or (spectra marked FT) on a JEOL PFT-100 spectrometer; all synthetic compounds had ^1H n.m.r. spectra in accordance with their structures. ^{13}C N.m.r. spectra were recorded at 25 MHz on a JEOL PFT-100 spectrometer. Amino-acid analyses were carried out with a JEOL JLC-5AH amino-acid analyser.

Action of Glutaraldehyde on Ovalbumin in Solution

(1) *Estimation of Total Yield of Pyridinium Compounds.*—Ovalbumin (400 mg) was treated with aqueous 25% glutaraldehyde and the modified protein (280 mg) isolated as described in the preparative procedure (below) but omitting the treatment with performic acid. A portion of the product (5 mg) was hydrolysed at 110 °C for 24 h with 6M-hydrochloric acid (2 ml) and the resulting solution evaporated to dryness. Amino-acid analysis showed the

modified protein to contain 0.078 3 μmol lysine per mg, corresponding to 0.313 μmol modified lysine per mg (80% reaction); the intensity of absorption at 265 nm corresponded to 0.519 μmol pyridinium compound (ε 4 000) per mg, *i.e.* an apparent yield of 1.66 mol pyridinium compound per mol lysine reacted.

(2) *Preparation of Pyridinium Compounds.*—An aqueous 25% solution of purified glutaraldehyde [12] (100 ml) was added dropwise to a vigorously stirred solution of ovalbumin (Sigma Chemicals) (4 g) in sodium acetate buffer (0.2M; pH 4.7) (100 ml). After stirring for 90 min at room temperature the solution was dialysed (Visking 24/32) for 19 h against running tap water. The precipitated protein was collected by filtration, dried for 3 h over P_2O_5 at 0.5 mmHg, and suspended at 0 °C in aqueous performic acid (90 ml; from 30% w/v hydrogen peroxide and 88% formic acid, 1 : 9 v/v, kept at room temperature for 1 h). The mixture was stirred for 2 h at 0 °C and the excess of peracid then destroyed by addition of aqueous hydrogen bromide (48%; 10 ml). The precipitated protein was collected by filtration, washed with water (200 ml), and dried over P_2O_5 at 50 °C and 0.4 mmHg. The modified protein (3.6 g) was hydrolysed with 6M-hydrochloric acid for 24 h at 110 °C; amino-acid analysis showed that 50% of the lysine residues had been modified.

The hydrolysate was evaporated to dryness and the residue dissolved in water (30 ml) and brought to pH 5.25 by addition of aqueous ammonium hydroxide (30% w/v). The filtered solution was applied to a column (2.5 × 8 cm) of Dowex 50W-X8 equilibrated with 0.35M-ammonium acetate buffer, pH 5.25.

Product A. The same buffer was passed through the column at 80 ml h^{-1}; after 350 ml had been passed through the effluent was almost colourless. The whole eluate ($A_{1\,cm;\ 257\,nm} = 0.70$; 0.058 mmol) was evaporated to dryness and the residue freed from ammonium acetate by sublimation overnight at 50 °C and 0.01 mmHg. The residue was dissolved in water (5 ml) and applied to a column of Dowex 50W-X8 (1 × 20 cm), made up in 0.035M-ammonium acetate buffer, pH 5.25, thermostatted at 40 °C. The column was washed with the same buffer (26 ml) and then eluted at 25 ml h^{-1} with 0.5M-ammonium acetate buffer, pH 9.2. The eluate showed two main peaks absorbing at 257 nm and the fraction (12 ml) containing the larger, faster-running, of these was collected ($A_{1\,cm;\ 257\,nm} = 3.25$; 0.009 3 mmol). After evaporation and removal of ammonium acetate by sublimation, the residue was dissolved in water (2 ml) and applied to a column of G15 Sephadex (1.6 × 100 cm) made up in aqueous 2.5% w/v acetic acid; elution with the same solvent at 13 ml h^{-1} gave a single peak with only traces of other material. The fraction (107—119 ml) containing this peak had $A_{1\,cm;\ 257\,nm} = 2.58$; 0.007 4 mmol). The residue obtained on evaporation was dissolved in 0.035M-ammonium acetate buffer, pH 5.25 (1 ml), and applied to a column (1 × 20 cm) of carboxymethylcellulose (CM 52) made up in the same buffer; elution with this buffer at 25 ml h^{-1} gave a sharp main band with a small faster running band. The eluate (46—58 ml) containing the main band had $A_{1\,cm;\ 257\,nm} = 3.40$ (0.009 7 mmol). It was de-salted by passage through a column (1 × 7 cm) of Dowex 50W-X8 (ammonium form) in 2M-ammonium hydroxide at 25 ml h^{-1} giving a single sharp peak; the eluate (12 ml; $A_{1\,cm;\ 257\,nm} = 2.28$; 0.006 5 mmol) was evaporated and the residue dried overnight at 40 °C and 0.02 mmHg to give Product *A*.

Product *A* so obtained had $\lambda_{max.}$ 257 nm (ε 4 200) with shoulders at 254 and 265 nm in 2M-ammonium hydroxide; the spectrum was unchanged on acidification. The ^1H n.m.r. spectrum, in CF_3CO_2D, was practically identical with that of synthetic (3), showing δ 8.85 (d, pyridine 2- and 6-H), 8.65 (t, pyridine 4-H), 8.16 (t, pyridine 3- and 5-H), 4.75 (t, ε-H_2), 4.45 (t, α-H), 2.24 and 1.84 (br m, β-, γ- and δ-H_2). This product was inseparable from synthetic (3) on t.l.c. in the following systems (R_F values in parentheses): (*A*) (0.42), (*B*) (0.24), (*C*) (0.43), (*D*) (0.44), (*M*) (0.20), (*N*) (0.21), (*O*) (0.28, (*P*) (0.86).

Product B. The first Dowex column, after removal of crude, *A*, was eluted with 2M-ammonium hydroxide at 80 ml h^{-1}; the effluent containing the major peak (10—70 ml; $A_{1\,cm;\ 263\,nm} = 3.67$; 0.055 mmol) was collected and evaporated to dryness. The residue was dissolved in 0.35M-ammonium acetate buffer, pH 5.25 (5 ml), and applied to a column (1 × 20 cm) of Dowex 50W-X8 made up in the same buffer and thermostatted at 40 °C. After washing the column with the equilibration buffer (26 ml), elution with 0.5M-ammonium acetate buffer, pH 9.2, at 25 ml h^{-1} gave a broad peak. The eluate corresponding to this (70—100 ml; $A_{1\,cm;\ 263\,nm} = 3.53$; 0.026 5 mmol) was evaporated to dryness and freed from ammonium acetate by vacuum sublimation. The residue was dissolved in water (2 ml), applied to a column (1.6 × 100 cm) of G25 Sephadex (superfine), and eluted with 0.2M-ammonium acetate buffer, pH 4.25, at 16 ml h^{-1}; the main absorbing fraction (108—124 ml) had $A_{1\,cm;\ 263\,nm} = 6.25$ (0.025 mmol). After evaporation, the residue was dissolved in 0.035M-ammonium acetate buffer, pH 5.25 (1 ml), and applied to a column of carboxymethylcellulose (CM 52) (1 × 20 cm) made up in the same buffer; after washing with this buffer (80 ml), elution with 0.12M-ammonium acetate buffer, pH 5.25, at 25 ml h^{-1} gave a main peak with two small satellites. The fraction containing the main peak (19—31 ml; $A_{1\,cm;\ 263\,nm} = 8.08$; 0.024 mmol) was collected and applied to a column of Dowex 50W-X8 (ammonium form). Elution with 2M-ammonium hydroxide gave a single sharp peak and evaporation to dryness, followed by drying overnight over P_2O_5 at 40 °C and 0.02 mmHg, afforded Product *B* (0.022 mmol).

Product *B* so obtained had $\lambda_{max.}$ 263 nm (ε 4 000) with a shoulder at 268 nm in 2M-ammonium hydroxide; the spectrum did not change on acidification. The ^1H n.m.r. spectrum, in CF_3CO_2D, was practically identical with that of synthetic (4), showing δ 9.28 (1 H, s, pyridine 2-H), 9.00 (2 H, d, pyridine 4- and 6-H), 8.30 (1 H, t, pyridine 5-H), 4.75 (3 H, m), 4.40 (2 H, m), 4.07 (1 H, m), and 3.1, 2.25, and 1.9 (broad peaks, 27 H in all). This product was inseparable from synthetic anabilysine (4) on t.l.c. in the following systems (R_F values in parentheses): (*A*) (0.16), (*B*) (0.10), (*C*) (0.26), (*D*) (0.19).

Acetic anhydride (250 μl) was added dropwise at 0 °C to a stirred solution of Product *B* (0.018 mmol) in water (25 ml), the pH being kept at 10.5 by addition of 2M-sodium hydroxide (pH-stat). The solution was evaporated to dryness and the product de-salted on a column (1 × 5 cm) of Dowex 50W-X8 (ammonium form) and finally purified by passage through a column (1.6 × 100 cm) of G25-Sephadex (superfine). The resulting acetyl derivative was homogeneous by t.l.c. in four systems: (*A*) (0.39), (*C*) (0.41), (*D*) (0.30), (*N*) (0.10). The ^1H n.m.r. spectrum in CF_3CO_2D showed δ 9.23 (1 H, s, pyridine 2-H), 8.97 (2 H, d sharpened to s on irradiation at 818 Hz, pyridine 4- and

6-H), and 8.26 (1 H, m, sharpened to s on irradiation at 884 Hz, pyridine 5-H).

Action of Glutaraldehyde on Lysine Derivatives attached to an Insoluble Support

(1) *Preparation of Lysine Derivatives.*—Glycine methyl ester hydrochloride (2.6 g, 0.021 mol), triethylamine (2.0 g, 0.02 mol), and *NN'*-dicyclohexylcarbodi-imide (4.3 g, 0.021 mol) in ethyl acetate (10 ml) were added to a stirred solution of N^{α}-benzyloxycarbonyl-N^{ϵ}-t-butoxycarbonyl-L-lysine [13] (8 g, 0.021 mol) in ethyl acetate (100 ml) at −10 °C. After stirring for 1 h at −10 °C and then overnight at room temperature, the mixture was filtered and the filtrate evaporated to 50 ml, washed with 1M-citric acid (20 ml), water (2 × 20 ml), 1M-sodium carbonate (20 ml), and water (2 × 20 ml), dried, and evaporated to dryness. Two crystallisations from butan-1-ol and light petroleum (b.p. 60—80 °C) gave N^{α}-*benzyloxycarbonyl*-N^{ϵ}-t-*butoxycarbonyl*-L-*lysylglycine methyl ester* (4.0 g, 42%), m.p. 88—89.5° (Found: C, 58.5; H, 7.45; N, 9.2. $C_{22}H_{33}N_3O_7$ requires C, 58.5; H, 7.35; N, 9.3%).

Acetic anhydride (6.5 ml, 0.065 mol) was added over 30 min at 0 °C to a stirred solution of N^{ϵ}-benzyloxycarbonyl-L-lysine (6.0 g, 0.021 mol) in 1M-sodium hydroxide (38 ml); the pH was kept at 10.5 by dropwise addition of 4M-sodium hydroxide (pH-stat). At the end of the reaction the pH was brought to 6.0 with 6M-hydrochloric acid and the solution extracted with ether; the pH of the residual aqueous layer was then adjusted to 3.0 and the precipitated oil extracted into ethyl acetate. After washing with water and drying, the extract was evaporated to dryness, giving N^{α}-acetyl-N^{ϵ}-benzyloxycarbonyl-L-lysine, characterised as its dicyclohexylamine salt, m.p. 166—167° (Found: C, 66.7; H, 9.15; N, 8.3. Calc. for $C_{28}H_{45}N_3O_5$: C, 66.75; H, 9.0; N, 8.35%).[14] The free acid was dissolved in ethyl acetate (50 ml) and treated with glycine methyl ester hydrochloride (4.01 g, 0.032 mol), 1-hydroxybenztriazole (7.5 g, 0.049 mol), triethylamine (3.2 g, 0.032 mol), and *NN'*-dicyclohexylcarbodi-imide (6.6 g, 0.032 mol) in ethyl acetate (10 ml). The mixture was stirred at −10 °C for 1 h and then at room temperature overnight. Working up as described in the previous paragraph gave N^{α}-acetyl-N^{ϵ}-benzyloxycarbonyl-L-lysylglycine methyl ester (2.0 g) which was dissolved, without purification, in methanol (10 ml). 1M-Sodium hydroxide (5 ml) was added and the mixture kept for 20 min at room temperature; 1M-hydrochloric acid (5 ml) was then added and the solution evaporated to dryness. The residue was triturated with ethyl acetate (50 ml) and filtered; the filtrate was washed with water, dried, and evaporated to dryness. Precipitation from butan-1-ol with light petroleum (b.p. 60—80 °C), followed by recrystallisation from acetone, gave N^{α}-*acetyl*-N^{ϵ}-*benzyloxycarbonyl*-L-*lysylglycine* (0.9 g, 11% overall), m.p. 141—142° (Found: C, 56.85; H, 6.65; N, 10.9. $C_{18}H_{25}N_3O_6$ requires C, 57.0; H, 6.65; N, 11.1%).

N^{α}-Benzyloxycarbonyl-N^{ϵ}-t-butoxycarbonyl-lysylglycine methyl ester (440 mg, 1 mmol) was hydrogenated for 2 h over 5% palladised charcoal (100 mg) in methanol (30 ml) containing 6M-hydrochloric acid (0.16 ml). The catalyst was removed by filtration and the filtrate evaporated to dryness. To the residue in dimethylformamide (10 ml) were added N^{α}-acetyl-N^{ϵ}-benzyloxycarbonyl-L-lysylglycine (380 mg, 1 mmol), 1-hydroxybenzotriazole (300 mg, 2 mmol), *NN'*-dicyclohexylcarbodi-imide (206 mg, 1 mmol), and triethylamine (100 mg, 1 mmol). The mixture was stirred overnight at room temperature, filtered, and evaporated to dryness. After washing with ethyl acetate and water, the residue was precipitated from ethanol with ether affording N^{α}-*acetyl*-N^{ϵ}-*benzyloxycarbonyl*-L-*lysyl*-*glycyl*-N^{ϵ}-t-*butoxycarbonyl*-L-*lysylglycine methyl ester* (402 mg, 59%), m.p. 179—180° (Found: C, 56.85; H, 7.65; N, 12.0. $C_{32}H_{50}N_6O_{10}$ requires C, 56.6; H, 7.4; N, 12.4%). 1M-Sodium hydroxide (0.25 ml) was added to this ester (166 mg, 0.24 mmol) in methanol (3 ml). After 1.5 h at room temperature the solution was concentrated to 1.5 ml and kept overnight; 0.1M-hydrochloric acid (2.5 ml) was then added and the mixture evaporated to dryness. The residue was then triturated with water, giving N^{α}-*acetyl*-N^{ϵ}-*benzyloxycarbonyl*-L-*lysylglycyl*-N^{ϵ}-*t-butoxycarbonyl*-L-*lysylglycine* (134 mg 84%), m.p. 144—147°, unchanged by recrystallisation from water (Found: C, 55.55; H, 7.3; N, 12.15. $C_{31}H_{48}N_6O_{10}$ requires C, 56.0; H, 7.3; N, 12.65%).

(2) *Attachment to Support.*—(i) N^{α}-*Acetyl*-L-*lysine*. Corning controlled-porosity glass beads (CPG-250) were heated for several hours at 600 °C, allowed to cool, and then suspended in a solution (2% w/v) of 3-aminopropyltriethoxysilane in anhydrous acetone. The beads were allowed to settle, the supernatant was removed by decantation, and the remaining slurry was kept at 45 °C for 24 h. After thorough washing on a glass sinter with acetone, the pale yellow aminopropylated beads were dried overnight *in vacuo*. The beads (1 g) were suspended in a solution of N^{α}-acetyl-N^{ϵ}-benzyloxycarbonyl-L-lysine (81 mg, 0.25 mmol) and *NN'*-dicyclohexylcarbodi-imide (53 mg, 0.25 mmol) in ethyl acetate (6 ml). After 2 h at room temperature the beads were filtered off, washed with ethyl acetate (3 × 15 ml) and methanol (3 × 15 ml), and dried *in vacuo*. Hydrolysis (6M-hydrochloric acid, 110 °C, overnight) and amino-acid analysis of the hydrolysate for lysine showed the degree of substitution of this product (S1) to be 0.04 mmol g^{-1}.

(ii) N^{α}-*Acetyl*-L-*lysylglycyl*-L-*lysylglycine*. Aminopropylated glass beads (CPG-550) (1 g) were washed thoroughly with dimethylformamide and suspended for 2 h in a solution of N^{α}-acetyl-N^{ϵ}-benzyloxycarbonyl-L-lysylglycyl-N^{ϵ}-t-butoxycarbonyl-L-lysylglycine (69 mg, 0.1 mmol) and *NN'*-dicyclohexylcarbodi-imide (21 mg, 0.1 mmol) in dimethylformamide (5 ml). The beads were filtered off, washed with dimethylformamide (3 × 25 ml) and dried *in vacuo* at 40 °C. Hydrolysis and amino-acid analysis showed the degree of substitution of this product (S2) to be 0.032 mmol g^{-1}.

(3) *Reactions with Glutaraldehyde.*—*S1*. Acetic anhydride (1 ml) was added in portions over 30 min to a stirred suspension of S1 (1 g) in 0.2M-sodium hydroxide (10 ml) at 0 °C; the pH was kept at 9.5 with 4M-sodium hydroxide (pH-stat). The beads were then filtered off, washed with water (2 × 15 ml) and acetone (2 × 15 ml), and dried *in vacuo*. The beads were suspended in 10% w/v hydrogen bromide in acetic acid (5 ml) for 1.5 h and then filtered off, washed with acetic acid (3 × 15 ml), water (15 ml), 1M-sodium acetate (15 ml), and water (3 × 15 ml) and suspended in a 12.5% solution of glutaraldehyde in 0.1M-sodium acetate buffer, pH 4.7 (10 ml). After stirring for 36 h at room temperature, the beads were filtered off, washed with water (6 × 15 ml), and suspended at 0 °C in performic acid (10 ml; from 30% hydrogen peroxide and 98% formic acid, 1 : 9 by volume). After stirring for 2.5 h the beads were collected by filtration, washed with water (6 × 15 ml), and

hydrolysed overnight in 6M-hydrochloric acid (10 ml) at 110 °C.

This procedure was carried out with three batches of beads (30 g in all; 1.2 mmol lysine); amino-acid analysis of the hydrolysate showed that 80% of the lysine remained attached to the beads after treatment with hydrogen bromide, and that 90% of this had reacted with the aldehyde. The extinction coefficient at 265 nm showed the final hydrolysate to contain 0.18 mmol of quaternary pyridinium compound (ε 4 000).

The hydrolysate was evaporated to dryness and the residue dissolved in water (4 ml) and applied to a column (1.6 × 100 cm) of Sephadex G25 (superfine) made up in 0.2M-ammonium acetate buffer, pH 4.25. Elution with the same buffer at 11 ml h^{-1} gave a main peak, accounting for *ca.* 65% of the absorption at 265 nm, due to anabilysine; there was a small early peak, due to Product *A*, and small slower-running peaks due to other pyridinium compounds, which were not fully investigated. The fraction (112—129 ml) corresponding to the main peak had $A_{1\,cm;\ 263\,nm} = 27.4$ (0.116 mmol of anabilysine; 19.3%). It was applied to a column (1 × 8 cm) of Dowex 50W-X8 made up in 0.2M-ammonium acetate buffer, pH 4.25; the column was washed with this buffer and then developed with 0.5M-ammonium acetate buffer, pH 9.2, at 45 ml h^{-1}. The anabilysine-containing fraction (15—110 ml; $A_{1\,cm;\ 263\,nm} = 3.05$; 0.072 5 mmol) was evaporated to dryness and the residue applied in water (2 ml) to a column (1.6 × 100 cm) of buffer, pH 4.25. Elution with the same buffer at 10 ml h^{-1} gave a fraction (115—125 ml) consisting almost entirely of anabilysine ($A_{1\,cm;\ 263\,nm} = 19.6$; 0.049 mmol; 8.2%). This was evaporated to dryness and freed from ammonium acetate by sublimation for 48 h at 40 °C and 0.05 mmHg; the product was dissolved in 0.6M-hydrochloric acid, the solution was evaporated, and the residue dried *in vacuo*.

Anabilysine so obtained had $\lambda_{max.}$ 263 nm (ε 4 000) with a shoulder at 268 nm in 0.2M-ammonium acetate buffer, pH 4.25. It was inseparable from anabilysine from ovalbumin in the following systems (R_F values in parentheses): (*A*) (0.13), (*B*) (0.08), (*C*) (0.27). Its ^1H n.m.r. spectrum in CF_3CO_2D was identical with that of synthetic anabilysine and its ^{13}C n.m.r. spectrum (see Table) almost identical with that of the synthetic product. Acetylation by the procedure described above (p. 2285) for the product from ovalbumin gave an acetyl derivative; the two derivatives were inseparable by t.l.c. in systems (*A*) (0.41) and (*C*) (0.44) and their ^1H n.m.r. spectra were identical.

A solution of this specimen of anabilysine (0.05 mmol) in 0.1M-sodium borate (10 ml) was cooled to 0 °C; benzyl chloroformate (350 μl, 2.5 mmol) was added and the pH kept at 10 (pH-stat) by titration with 2M-sodium hydroxide. After 30 min the mixture was washed with ether (3 × 3 ml) and ethyl acetate (3 × 3 ml) and the residual solution evaporated to dryness. The residue was dissolved in water and chromatographed on a column (1.6 × 100 cm) of G25 Sephadex (superfine) in 0.2M-ammonium acetate buffer, pH 4.25. The following three fractions were obtained:

Fraction	Elution vol.	T.l.c. R_F	Ninhydrin
1	114—123 ml	(*A*) (0.12), (*C*) (0.12), (*M*) (0.00)	+ve
2	125—135 ml	(*A*) (0.42), (*C*) (0.45), (*M*) (0.04)	+ve
3	150—161 ml	(*B*) (0.73), (*C*) (0.62), (*M*) (0.36)	−ve

Fractions 2 and 3 were de-salted on Dowex 50W-X8; their ^1H n.m.r. spectra in CF_3CO_2D showed peaks at δ 9.0 (Ph) and 5.9 (benzyl CH_2).

S2. Treatment of S2 (1.0 g) with glutaraldehyde, as described above for S1, followed by eventual hydrolysis, gave a crude product containing 0.002 5 mmol of quaternary pyridinium compounds. Chromatography as before gave as the major product anabilysine, $\lambda_{max.}$ 263 nm (ε 4 000) and 268sh nm (0.001 mmol; 3.1%) identical in all respects with anabilysine from S1.

Synthesis of 1-(5-*Amino*-5-*carboxypentyl*)*pyridinium Chloride* (3)

5-(4-Bromobutyl)hydantoin [7] (1.7 g, 7 mmol) was heated overnight at 85 °C, with stirring, in pyridine (6.0 g, 70 mmol). The precipitate was collected by filtration from the cooled mixture and washed with ether (50 ml) and acetone (10 ml). Two recrystallisations from methanol–acetone gave the hygroscopic 5-(4-*pyridiniobutyl*)*hydantoin bromide* (2) (1.54 g, 70%), m.p. 179° (sealed tube) (Found: C, 43.95; H, 4.9; N, 12.75. $C_{12}H_{16}BrN_3O_2 \cdot 0.5H_2O$ requires C, 44.6; H, 5.3; N, 13.0%). This salt (1 g, 3 mmol) was refluxed for 72 h with 6M-hydrochloric acid (50 ml). The solution was evaporated to dryness and the residue de-salted on a column (1 × 5 cm) of Dowex 50W-X8 (ammonium form). The product obtained by elution with 2M-ammonium hydroxide was dried *in vacuo* and dissolved in trifluoroacetic acid (10 ml), and the solution was evaporated to dryness. Drying *in vacuo* gave 1-(5-*amino*-5-*carboxypentyl*)*pyridinium trifluoroacetate* as a hygroscopic red gum which resisted all attempts at crystallisation, $\lambda_{max.}$ 257 nm (ε 4 200) with shoulders at 254 and 265 nm in water (Found: C, 35.95; H, 3.6; N, 4.85. $C_{15}H_{18}F_6N_2O_6 \cdot CF_3CO_2H \cdot H_2O$ requires C, 35.9; H, 3.7; N, 4.95%). The ^1H n.m.r. spectrum in CF_3CO_2D showed δ 8.84 (2 H, d, pyridine 2- and 6-H), 8.64 (1 H, t, pyridine 4-H), 8.15 (2 H, t, pyridine 3- and 5-H), 7.60 (3 H, br, NH_3^+), 4.74 (2 H, t, ε-H_2), 4.44 (1 H, t, α-H), 2.24 and 1.84 (6 H, br, β-, γ-, and δ-H_2). This salt was dissolved in 0.6M-hydrochloric acid (20 ml) and the solution evaporated to dryness; two further evaporations with ethanolic 6M-hydrogen chloride (5 ml) and drying *in vacuo* gave the *chloride hydrochloride* of (3) as a hygroscopic orange solid which resisted all attempts at crystallisation (Found: C, 46.7; H, 6.2; N, 9.4. $C_{11}H_{18}Cl_2N_2O_2$ requires C, 47.0; H, 6.45; N, 9.96%), homogeneous on t.l.c. in the following systems: (*A*) (0.42), (*B*) (0.24), (*C*) (0.43), (*D*) (0.44), (*N*) (0.21), (*O*) (0.28). The noise-decoupled ^{13}C n.m.r. spectrum in D_2O at 30 °C showed the following peaks (p.p.m. from MeOH): 123.2 (carboxy C), 96.8 (pyridine C-4), 95.3 (pyridine C-2 and -6), 79.4 (pyridine C-3 and -5), 12.3 (side-chain ε-C), 3.9 (side-chain α-C), and −18.9, −19.7, and −27.8 (other side-chain C). Numerous attempts to obtain crystalline salts of (3) with other anions failed.

Synthesis of Anabilysine (4)

Anabasine (350 mg, 2.15 mmol) and 5-(4-bromobutyl hydantoin (1.01 g, 4.3 mmol) were refluxed for 48 h methanol (5 ml). The solution was evaporated to dryn and the residue refluxed in 6M-hydrochloric acid (50 ml) 72 h. The hydrolysate was evaporated to dryness and residue chromatographed on a column (2.5 × 15 cm) Dowex 50W-X8 made up with 0.75M-ammonium acet

buffer, pH 9.25. Elution was carried out at 120 ml h^{-1} with a linear gradient between 0.75M-ammonium acetate buffer solutions of pH 9.25 and 10.4. The elution was monitored by both light absorption and t.l.c.

The first fraction containing material absorbing at 265 nm was collected and de-salted on a column (1 × 8 cm) of Dowex 50W-X8 (ammonium form), eluted with 2M-ammonium hydroxide, and evaporated to dryness. The residue was taken up in 6M-hydrochloric acid (0.7 ml), the solution evaporated to dryness, and the residue dried for 24 h at 45 °C and 0.05 mmHg. 1-(5-*Amino*-5-*carboxypentyl*)-3-[1-(5-*amino*-5-*carboxypentyl*)-2-*piperidyl*]*pyridinium chloride trihydrochloride* (*anabilysine*) (4), so obtained (445 mg, 37%) (Found: C, 43.55; H, 6.85; N, 8.65. $C_{22}H_4Cl_4N_4O_4 \cdot 2H_2O$ requires C, 43.85; H, 7.35; N, 9.3%) had $\lambda_{max.}$ 263 nm (ε 4 050) and 268sh nm, in water. The ^1H n.m.r. spectrum (FT) in CF_3CO_2D showed δ 9.62 (1 H, s, pyridine 2-H), 9.03 (2 H, pair of overlapping d, pyridine 4- and 6-H), 8.29 (1 H, t, pyridine 5-H), 4.84 (3 H, m), 4.46 (2 H, m), 4.04 (1 H, m), and 3.2, 2.3 and 2.0 (27 H in all, br); see Table for ^{13}C n.m.r. spectrum.

A second fraction containing material absorbing at 265 nm, which followed that containing anabilysine, was worked up in the same way affording 1-(5-*amino*-5-*carboxypentyl*)-3-(2-*piperidyl*)*pyridinium chloride dihydrochloride* (6) (110 mg, 12%) (Found: C, 44.5; H, 6.75: N, 9.15. $C_{16}H_{28}Cl_3N_3O_2 \cdot 2H_2O$ requires C, 44.0; H, 7.4; N, 9.6%). The ^1H n.m.r. spectrum (FT) in CF_3CO_2D showed δ 9.46 (1 H, s, pyridine 2-H), 8.93 (2 H, d, pyridine 4- and 6-H), and 8.25 (1 H, t, pyridine 5-H).

We thank the S.R.C. for a Research Grant, Dr. R. Casey for exploratory experiments, and the Imperial Tobacco Research Group for a gift of anabasine.

[8/2120 *Received*, 14*th December*, 1978]

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