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THE REACTION OF CYSTEINE WITH α,β-UNSATURATED ALDEHYDES

H. ESTERBAUER, A. ERTL and N. SCHOLZ
Institut für Biochemie, Universität Graz, A-8010 Graz, Austria

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Abstract—Cysteine adds in a two step reaction to acroleine, crotonaldehyde and 4-hydroxypentenal. The first addition products are the β-cysteinyl-substituted saturated aldehydes **1a**, **1b** and **3**. Only the monoadduct of **3**, which is stabilized through intramolecular hemiacetal formation, could be isolated. The derivatives **1a** and **1b** reacted rapidly with additional cysteine to give the thiazolidine compounds **2a** and **2b**. Whereas **2a** and **2b** were the only products even in reactions carried out with a molar ratio of aldehyde : cysteine ≧ 1, a ratio < 1 was required to obtain a thiazolidine derivative in reactions with hydroxypentenal. The structures of compound **2a**, **2b**, **3** and **4** were ascertained by means of UV, IR and NMR measurements, potentiometric titrations, determination of the rate laws and elemental analysis. All adducts are in solution in equilibrium with cysteine and the parent aldehydes. Rate constants for forward and reverse reactions were estimated. The rate of forward reactions increase approx. 10-fold per pH unit in the pH range 2-10.

INTRODUCTION

It is well established that the reaction of cysteine (CySH) with saturated aldehydes and ketones leads to the formation of 2-substituted thiazolidine-4-carboxylic acids.[1-6] Thiazolidines have also been obtained from various unsaturated carbonyl compounds containing at least two CC double bonds in conjugation to the CO group, e.g. retinal,[8] furfurals,[7] benzaldehydes[1,6,7,11] and other aromatic carbonyl derivatives.[6,10,11] To the best of our knowledge the reaction of CySH with α,β-monounsaturated aldehydes has not been successfully investigated. Schmolka *et al.*[6] failed to obtain chemically defined products upon reaction of CySH with acroleine, crotonaldehyde and other 2-alkenals, in agreement with earlier findings of Liebermann *et al.*[12] that α,β-unsaturated 3-ketosteroids and other unsaturated ketones did not yield thiazolidines. On the other hand, with cinnamaldehyde a crystalline product was obtained[6,12] which was assumed to be a mercaptal and not a thiazolidine derivative.

In our ongoing work on the biochemical effects of conjugated aldehydes we have studied the reactions of cysteine with acroleine, crotonaldehyde and 4-hydroxypentenal and report now certain aspects of the chemistry and the kinetics of these reactions. Besides the general chemical interest the results have biochemical and medical implications. It has been suggested[13-18] that the reactions of the SH groups in enzymes, glutathione and cysteine with α,β-unsaturated carbonyl compounds play an important role in the mechanism by which these compounds exert their antitumor[20-27] and antimicrobial activity[13,14,28-35] and inhibit various metabolic processes.[15,36-40] In addition, a cysteine-hydroxypentenal adduct of undefined structure has recently been found to possess anticancer activity.[41]

RESULTS AND DISCUSSION

When cysteine reacted with acroleine or crotonaldehyde, two moles of the hydrosulfide were consumed for each mole of aldehyde. All of the cysteine required by the reaction was consumed at a rate twice that of the aldehyde consumption (Table 1). The plot of log $[a(b-2x)/b(a-x)]$ vs time, where a and b are the initial concentrations of aldehyde and cysteine, resp., and x the moles of aldehyde consumed, gave a straight line establishing that the reaction is of second order and follows the integrated law for a two step reaction of the general type $A+B \rightarrow AB$, $AB+B \rightarrow AB_2$, in which the formation of AB is the rate limiting step. The reaction with acroleine proceeded approx. 25 times faster than that with crotonaldehyde (Table 2).

The kinetic measurements and the results of the structure analyses of the reaction products indicate that the first and rate limiting step is the addition of cysteine to the CC double bond of the α,β-unsaturated aldehyde leading to the monoadducts **1a** and **1b** which, however, react very fast with additional cysteine yielding the products **2a** and **2b**. Whereas the diadducts were easily obtained as solid substances we were unable to isolate the monoadducts **1a** and **1b**. Even when cysteine reacted with an equimolar amount or an excess of aldehyde no indication of the presence of monoadducts was obtained by TLC.

However, the determination of the stoichiometry of the reaction between hydroxypentenal and cysteine revealed that up to a mixing ratio of aldehyde : CySH = 1 only one mole of cysteine was consumed for each mole of aldehyde

Table 1. Reaction of cysteine with crotonaldehyde and 4-hydroxypentenal resp. The reactions were carried out in 0·066 M phosphate buffer pH 7·0, 20°, initial concentrations of the reactants as indicated for zero time

Time (min)	Concentration of the reactants (mM)			
	Crotonal	CySH	Hydroxy-pentenal	CySH
0	1.00	1.00	0.65	0.65
1	0.85	0.64	0.42	0.44
1.5	0.78	0.56	-	-
2.0	0.74	0.48	0.31	0.33
3.0	0.69	0.39	0.26	0.27
5.0	0.60	0.26	0.18	0.20
7.0	0.55	0.14	0.15	0.16
16.0	0.50	0.00	0.07	0.07
32.0	0.50	0.00	0.03	0.03

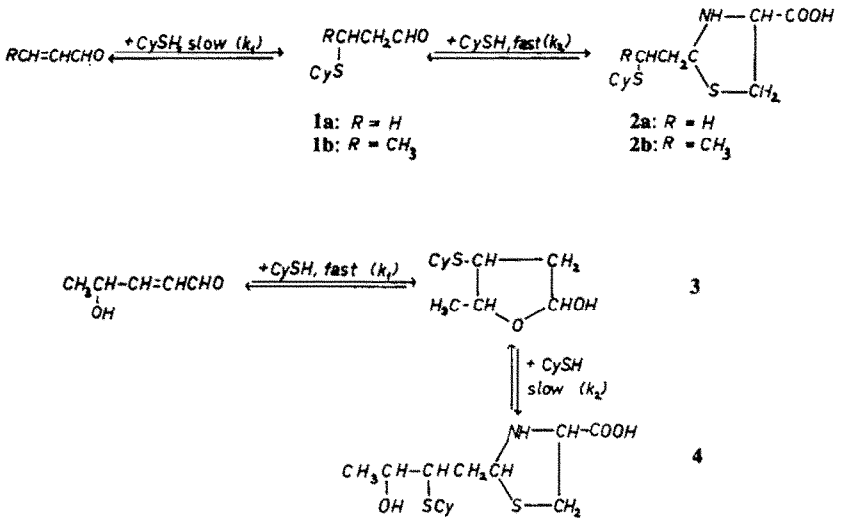

Table 2. Rate and equilibrium constants for the reactions of cysteine with acroleine, crotonaldehyde and hydroxypentenal (20°, 0·066 M phosphate buffer pH 7·5)

Aldehyde $\underset{k_1'}{\overset{k_1}{\rightleftharpoons}}$ Monoadduct (1a, 1b, 3) $\underset{k_2'}{\overset{k_2}{\rightleftharpoons}}$ Diadduct (2a, 2b, 4)

Aldehyde	k_1 ($M^{-1}s^{-1}$)	k_1' (s^{-1})	K $-k_1/k_1'$	k_2 ($M^{-1}s^{-1}$)	k_2' (s^{-1})	K $-k_2/k_2'$
Acrolein	220	5.0×10^{-6}	4.4×10^{7}	$\gg 220$	3.6×10^{-4}	$\gg 6.0 \times 10^{5}$
Crotonaldehyde	8.9	1.7×10^{-4}	5.2×10^{4}	$\gg 8.9$	6.6×10^{-4}	$\gg 1.3 \times 10^{4}$
Hydroxypentenal	25.0	1.0×10^{-5}	2.4×10^{6}	1.1	8.7×10^{-4}	1.2×10^{3}

(Fig. 1 and Table 1). The kinetics of the reaction was found to follow a second order rate law. The product formed in this reaction was isolated and identified as the Michael type adduct **3**. When, however, one mole of hydroxypentenal reacted with two or more moles of cysteine the thiazolidine derivative **4** was obtained exclusively. Reaction mixtures consisting of aldehyde and cysteine in ratios between 1 : 1 and 1 : 2 yielded mixtures of the monoadduct **3** and the diadduct **4** (Fig. 1). The formation of the thiazolidine ring requires, that a part of the monoadduct **3** is present as the free aldehyde. The oxo-cyclo equilibrium favors the hemiacetal form and the free aldehyde is available only in very low concentration.[17,19] Consequently, the second cysteine molecule reacts considerably slower than the first one (Table 2). The second order rate constant for the formation of **4** from **3** was estimated by following the disappearance of cysteine added to **3** and was found to be 1·1 $M^{-1}sec^{-1}$ at pH 7·5 compared to 25 $M^{-1}sec^{-1}$ for the formation of **3** (at pH 7·0 the rate constants are 0·204 and 13·6 $M^{-1}sec^{-1}$ resp.). Since the equilibrium constant for the reaction **3** + CySH $\rightleftarrows$ **4** has the small value of $1{\cdot}2 \times 10^{3}$ M^{-1} (Table 2) high concentration of reagents (*ca.* 300 mM) have to be used in order to achieve 95% conversion of **3** to **4**.

The structure of the compounds **2a**, **2b**, **3** and **4** were determined by UV, IR, PNMR and ^{13}C NMR techniques, determination of rate laws and elemental analyses. All compounds prepared were in the zwitter ionic form as it is clearly shown by the results of potentiometric titrations. To assign the NMR signals of our compounds reference

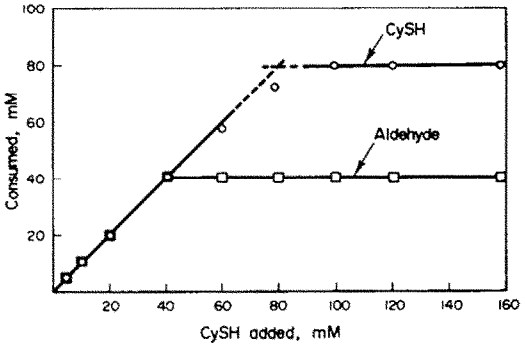

Fig. 1. Stoichiometry of the reaction of cysteine and 4-hydroxypentenal. N_2 degassed mixtures containing aldehyde (40 mM) and cysteine (concentrations as indicated) were allowed to react for 12 hr (20°, phosphate buffer 55 mM, pH 7·0). From the initial and end values the consumed amounts of cysteine (estimated by DTNB) and aldehyde (estimated by UV spectrum) were calculated.

spectra were recorded with cysteine, cystine and 2-alkylthiazolidine-4-carboxylic acids. The ^{13}C NMR spectra of cysteine, cystine and some of their derivatives have recently been reported by Flohe *et al.*[40] The postulated structures of the products **2a**, **2b**, **3** and **4** are consistent with their NMR spectra and the known chemical facts that α,β-unsaturated aldehydes easily add thiols to give saturated aldehydes,[19,43,44] that saturated aldehydes condense with cysteine yielding thiazolidine-4-

carboxylic acids[1-6] and that thiol adducts of 4-hydroxy-alkenals form cyclic hemiacetals.[17,19,45]

Figure 2 shows the pH dependence of the rate constants for the reactions of cysteine with crotonaldehyde and 4-hydroxypentenal. In the case of hydroxypentenal the rate increases with increasing pH, approaches a maximum at pH 10 and remains thereafter constant to pH 12. This rate increase parallels the increase of the relative concentration of the sulfhydryl anions [$^-SCH_2CH(NH_3^+)COO^- + {}^-SCH_2CH(NH_2)COO^-$], indicating that at any pH the rate controlling step is the addition of the sulfhydryl anions to the CC double bond as it has also been found for reactions of other thiols with conjugated carbonyls.[17,19] The rate for crotonaldehyde increases up to pH 9·5 and then decreases rapidly at higher pH, suggesting a change in the rate determining step. It is most probable that up to pH 9·5 the rate is also controlled by the addition of CyS^- (formation of **2b**) whereas at higher pH the formation of the thiazolidine derivative becomes rate limiting. A bell-shaped pH rate profil was also found by Kallen[49] for the reaction of cysteine with formaldehyde. The strong pH dependence of the formation of those compounds may also explain why other researchers[6,12] using much more acidic reaction media did not obtain any definite products from reactions between cysteine and 2-alkenals.

It was observed that compounds **2a**, **2b** and **4** are rather unstable in aqueous solution. Their exposure to a moist atmosphere, storage of their aqueous solutions or their recrystallizations from aqueous ethanol yielded products with a high content of cystine. This instability is caused by the dissociation of the Michael type adducts[19] as well as the thiazolidines[1,2,7,10,47,48] to their parent compounds. Consequently the adducts break down, when the thiol or aldehyde present in equilibrium is consumed by side reactions such as oxidation. The stability of the adducts **2a**, **2b**, **3**, **4** and some other thiazolidines was determined by measuring the rate of the reverse reaction using Ellman reagent, 5,5'-dithiobis-(2-nitrobenzoic acid) (= DTNB), to reduce the cysteine concentration and thus shifting the equilibrium to the left until a part or all of the adduct has been dissociated.[19] As shown in Fig. 3 the rate and extent of the reverse reaction depends on the nature of the adduct. Within 3 hr at pH 7·5 the crotonaldehyde diadduct **2b** loses per mole two moles of cysteine indicating a complete dissociation. The acrolein and hydroxypentenal compounds **2a** and **4**, however, release only approx. 1 mole cysteine, whereas the remaining cysteine cleaves with a much slower rate. The hydroxypentenal adduct **4** loses the second cysteine with exactly the same rate as it was found for adduct **3**, this strongly proves the reaction sequence for the reverse reaction. The thiazolidine derivatives **2a**, **2b** and **4** lose first rapidly the cysteine residue, which has been used to form the heterocyclic ring. The resulting monoadducts **1a**, **1b** and **3** dissociate then further in a slower reaction to aldehyde and cysteine. The stability of the adducts as determined by the cysteine cleavage reaction (Table 2) decreases in the order **3** ~ **1a** > **2a** > **1b** > **4** ~ **2b**. The high stability of compounds **1a** and **3** compared to **1b** is in agreement with earlier results[19] according to which Michael type thiol adducts of acrolein and 4-hydroxy-alkenals are 10–100 times more stable than analogous adducts of higher 2-alkenals. The stability of the thiazolidines is mainly governed by the substituent in the 2 position. Thus the unsubstituted thiazolidine-4-carboxylic acid is stable, whereas the 2-propyl and 2-butyl thiazolidinic-4-carboxylic acids are rather unstable as shown in Fig. 3.

As a gradual release of the active carbonyl *in vivo* is expected to provide a prolonged action, the reversible binding to cysteine also offers a promising means of latentiating biologically active α,β-unsaturated aldehydes and ketones.

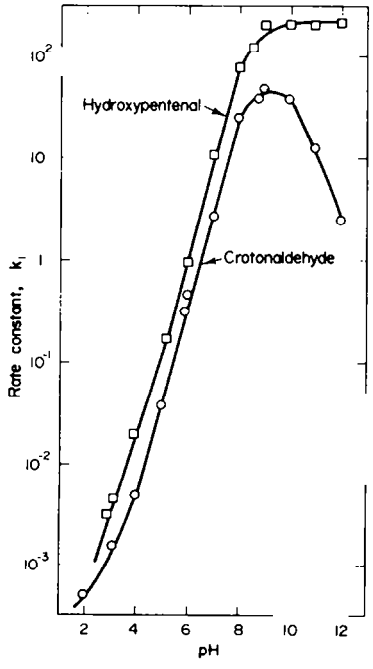

Fig. 2. Effect of pH on the second order rate constants of the reactions of cysteine with crotonaldehyde and 4-hydroxypentenal resp.

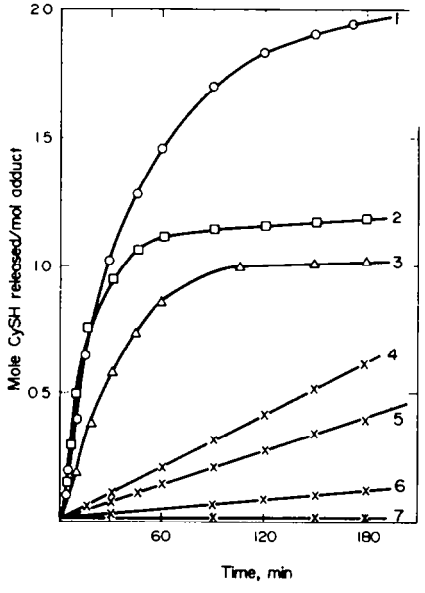

Fig. 3. Rate of cysteine release by various cysteine–aldehyde adducts. 1, crotonaldehyde diadduct **2b**; 2, hydroxypentenal diadduct **4**; 3, acroleine diadduct **2a**; 4, 2-butylthiazolidine-4-carboxylic acid; 5, 2-propylthiazolidine-4-carboxylic acid; 6, hydroxypentenal monoadduct **3**; 7, thiazolidine-4-carboxylic acid. To solutions (phosphate 66 mM, pH 7·4) of the adducts (0·05 mM) excess DTNB (0·5 mM) was added and the increase of absorbance was followed in 1 cm cells at 412 mμ, 20°.

EXPERIMENTAL

Crotonaldehyde and acrolein were obtained from Schuchardt and distilled before use. 4-Hydroxypentenal was prepared according to Esterbauer *et al.*[30] Cysteine (zwitterion) and all other reagents were purchased from Merck. IR spectra were recorded with a Perkin Elmer Model 221 spectrometer. PNMR spectra were taken with a Varian A-60 A or a Varian HA-100 A spectrometer. The ^{13}C NMR spectrum was recorded with the Varian HA-100 A ^{13}C modification Digilab, band width 6250 Hz, number of data points 8192, number of pulses 30,000, delay time 0. All chemical shifts are given in δ values relative to TMS as internal standard. The kinetic measurements were performed as described earlier.[17,19] Silicagel plates, a 2:1:1 butanol–acetic acid–water developer and ninhydrine reagent as detector, were used in the TLC separations.

S - [1 - (4 - *Carboxy* - 2 - *thiazolidinyl*) - 2 - *ethyl*] - *cysteine* (**2a**). To a soln of cysteine zwitterion (0·05 mole) in N_2-degassed water (40 ml) acrolein (0·025 mole) was added. The crystalline adduct **2a** began to precipitate immediately. As the reaction proceeded the pH dropped from initial value of 7·0–3·0 due to the higher acidity of the thiazolidine imino group. After 2 hr at 4° the product was filtered off, washed first with ice water, then with cold EtOH and dried *in vacuo* over P_2O_5. The white, hygroscopic needles (4·9 g, 70% yield) melted at 290° (dec) and were analytically pure without recrystallization (Found: C, 38·51; H, 5·79; N, 9·88; S, 21·83. $C_9H_{16}N_2O_4S_2$ (m.w. 280) requires: C, 38·57; H, 5·71; N, 10·00, S, 22·85%). TLC showed one single spot with R_f 0·20.

Potentiometric titration. 84·6 mg of the product dissolved in 40 ml H_2O consumed 0·570 ml 1 N NaOH (calc.: 0·603 ml); pK 5·37 (ring NH_2^+) and pK 8·67 (NH_3^+). $[\alpha]_D^{20}$ −93° (H_2O, c = 1). UV spectrum (H_2O): shoulder at 277 mμ (ϵ = 52), then continuous increase of absorbance, 222 mμ (ϵ = 974). IR spectrum (KBr) showed bands at (s, strong; m, medium; w, weak) 3390 m, 2985 m, 2739 m, 2100 w, 1600 s, 1490 w, 1408 m, 1360 m, 1290 m, 1212 w, 1190 w, 1150 w, 1124 w, 1052 w, 862 m, 847 w, 819 w, 784 w cm^{-1}. NMR data (60 MHz), D_2O + DCl): δ 2·40 (m, $SCH_2\underline{CH_2}$), 2·80 (*ca.* t, 6 Hz, $S\underline{CH_2}CH_2$), 3·23 (d, 5 Hz, $S\underline{CH_2}CH$), 3·50 (d, 7 Hz, ring CH_2), 4·34 (t, 5 Hz, CHCOO), 4·80 (HOD, ring CH and ring CHCOO). In D_2O + DCl + CF_3COOD the NMR spectrum exhibits signals at δ 2·45, 2·80, 3·28, 3·60, 4·40, 4·98 (m, 2 protons, ring CH and ring CHCOO) and 6·20 (HOD).

S - [1 - (4 - *Carboxy* - 2 - *thiyzolidinyl*) - 2 - *propyl*] - *cysteine* (**2b**). Compound **2b** was prepared in a similar manner as described for **2a**. After 12 hr at 4° the ppt was collected, washed with ice water and EtOH and dried *in vacuo* over P_2O_5; yield 5 g (68%) of a white, hygroscopic powder, m.p. 168–171°, dec. (Found: C, 40·52; H, 5·75; N, 9·85; S, 21·52. $C_{10}H_{18}N_2O_4S_2$ (m.w. 294) requires: C, 40·82; H, 6·11; N, 9·51; S, 21·78%). TLC showed one spot with R_f 0·29.

Potentiometric titration. 230 mg in 40 ml water consumed 1·58 ml 1·0 N NaOH (calc. 1·56 ml); pK 5·5 (ring NH_2^+) and pK 9·0 (NH_3^+). $[\alpha]_D^{20}$ −84·6° (H_2O, c = 1·5). UV spectrum (H_2O); sh at 285 mμ (ϵ = 16), then continuous increase of absorbance, 222 mμ (ϵ = 126). IR spectrum (KBr) showed bands at 3390 s, 2940 s, 2700 m, 2100 w, 1600 s, 1480 m, 1380 s, 1340 m, 1300 m, 1120 w, 1050 w, 845 w, 815 w. NMR data (60 MHz, D_2O): δ 1·55 (d, 7 Hz, CH_3), 2·35 (m, $SCH\underline{CH_2}$), 3·10 (m, $S\underline{CH}CH_2$), 3·3 (d, 5 Hz, ring CH_2), 4·1 (t, 1 proton, 5 Hz, CHCOO), 4·75 (HOD, ring CH and ring CHCOO). In D_2O + DCl the following signals were found: δ 1·2, 2·0, 2·8, 2·95, 3·3, 4·1, 4·90 (m, 2 protons, ring CH and ring CHCOO), 6·0 (HOD).

S - [2 - *Methyl* - 5 - *oxy* - 3 - *tetrahydrofuranyl*] - *cysteine* (**3**). To a soln of 4-hydroxypentenal (0·01 mole) in water (40 ml) cysteine zwitterion (0·01 mole) was added. The mixture was degassed with N_2 and kept at 20° in a tightly closed vial for 3 hr. After extracting 3 times with $CHCl_3$ (20 ml) the aqueous phase was freeze dried to give 2·1 g (95%) of a white, highly hygroscopic powder, m.p. 145° (dec). All attempts to crystallize the compound failed (Found: C, 44·00; H, 6·62; N, 6·47; S, 14·10. $C_8H_{15}NO_4S$, m.w. 221, requires: C, 43·43; H, 6·78; N, 6·33; S, 14·47%). TLC showed one major spot with R_f 0·44 and one spot of very low intensity with R_f 0·20 (adduct **4**).

Potentiometric titration. 38 mg in 25 ml H_2O consumed 0·333 ml 0·5 N NaOH (calc.: 0·343 ml). pK 7·90 (NH_3^+). $[\alpha]_D^{20}$ −21·2° (H_2O, c = 0·91). The IR spectrum showed bands at 3390 m, 2666 m, 1960 w, 1639 s, 1503 m, 1388 m, 1342 m, 1307 m, 1098 w, 1047 w, 1000 w, 896 w cm^{-1}. NMR data (100 MHz, D_2O): δ 1·35 (q, 7 Hz, 3 Hz, CH_3), 2·35 (*ca.* t, 7 Hz, 5 Hz, $SCH\underline{CH_2}$), 3·22 (d, 5 Hz, $S\underline{CH_2}CH$), 3·70 (m, 7 Hz, $S\underline{CH}CH_2$), 4·00 (t, 5 Hz, CHCOO), 4·75 (HOD and CH_3CH), 5·60 (t, 5 Hz, CHOH). ^{13}C NMR data (100 MHz, D_2O): δ −179 (COO^-), −104 (CHOH), −83 (m, $CH_3\underline{CH}$), −61 ($\underline{CH}COO$), −55, −48 ($S\underline{CH}CH_2$), −39 ($S\underline{CH_2}CH$), −23 (m, CH_3).

S - [1 - (4 - *Carboxy* - 2 - *thiazolidinyl*) - 2 - (3 - *oxybutyl*)] - *cysteine* (**4**). 4-Hydroxypentenal (0·01 mole) and cysteine zwitterion (0·02 mole) in water (20 ml) were allowed to react 12 hr under N_2 at 20°. The mixture was extracted 3 times with $CHCl_3$ (20 ml) and freeze dried to give 3·2 g (93%) of a white, very hygroscopic powder m.p. 177°. All attempts to crystallize the product failed. (Found: C, 38·04; H, 6·21; N, 7·90; S, 16·67. $C_{11}H_{20}O_5N_2S_2 \cdot H_2O$, m.w. 342, requires: C, 38·58; H, 6·47; N, 8·18; S, 18·72%). TLC showed one major spot with R_f 0·23 and a second spot of very low intensity with R_f 0·44 (adduct **3**). No excess cysteine (R_f 0·38) or cystine (R_f 0·17) was present.

Potentiometric titration. 81 mg dissolved in 40 ml water consumed 0·475 ml 1 N NaOH (calc.: 0·473); pK 5·25 (ring NH_2^+) and pK 9·10 (NH_3^+). $[\alpha]_D^{20}$ −73·2° (H_2O, c = 0·79). UV spectrum (H_2O): λ_{max} 322 mμ (ϵ = 8·64) then continuous increase of absorbance, 222 mμ (ϵ = 267). IR spectrum (KBr) showed bands at 3350 s, 2900 m, 2100 w, 1630 s, 1490 m, 1390 s, 1340 m, 1290 w, 1260 w, 1120 w, 1060 w, 960 w, 840 w, 800 w. NMR data (100 MHz, D_2O): δ 1·39 (q, 7 Hz, 3 Hz, CH_3), 2·45 (m, $SCH\underline{CH_2}$), 3·05 (m, $SCH\underline{CH_2}$), 3·30 (*ca.* q, $S\underline{CH_2}CH$), 3·65 (m, 6 Hz, ring $\underline{CH_2}$), 4·10 (t, 6 Hz, $\underline{CH}COO$), 4·75 (HOD, ring CH and ring CHCOO), 5·25 (m, 1 proton, CHOH).

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