Reaction of Endocyclic α,β-Unsaturated γ-Lactones with Thiols[1]

S. MORRIS KUPCHAN,[2] THOMAS J. GIACOBBE,[3] IRA S. KRULL,[3] A. M. THOMAS,[3] M. A. EAKIN, AND DYRAL C. FESSLER[3]

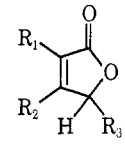
Department of Chemistry, University of Virginia, Charlottesville, Virginia 22901, and Department of Pharmaceutical Chemistry, University of Wisconsin, Madison, Wisconsin 53706

Received April 2, 1970

The reactions of the α,β-unsaturated lactones, $\Delta^{\alpha,\beta}$butenolide (1), α-methyl-$\Delta^{\alpha,\beta}$-butenolide (2), β-methyl-$\Delta^{\alpha,\beta}$-butenolide (3), and γ-methyl-$\Delta^{\alpha,\beta}$-butenolide (4) with the thiols, 1-propanethiol, α-toluenethiol, L-cysteine, and N-acetyl-L-cysteine methyl ester were investigated. The products were identified as the thio ethers resulting from Michael-type addition of the thiols across the conjugated carbonyl systems. The dissociations of the L-cysteine adducts were followed kinetically and their instability contrasted with an exocyclic α-methylene lactone thiol adduct.

The reaction of α,β-unsaturated lactones with thiols has been suggested to play a key role in several biological growth-regulatory phenomena. The selective growth-inhibitory action of δ-hexenolactone on certain animal tissures was shown to be antagonized by cysteine.[4] Spectrophotometric and colorimetric studies showed that direct and reversible reaction took place between the lactone and the thiol grouping, and it was proposed that δ-hexenolactone exerts its effect on cellular proliferation mainly through its reactivity with sulfhydryl groups essential to enzyme function. Similar studies of a variety of unsaturated lactone antibiotics led to similar proposals concerning their mode of action.[5-7] The inhibition of plant growth by protoanemonin,[8] heliangine,[9] and vernolepin[10] is prevented by BAL and other sulfhydryl compounds, and has been attributed to reaction of the inhibitors with sulfhydryl enzymes. Very recently, a study of the reactions of tumor-inhibitory α-methylene lactones with model biological nucleophiles revealed that thiols were the most reactive of the nucleophiles investigated, and that successive thiol addition to bis unsaturated lactones resulted in a marked diminution in the biological activity of the adducts.[11] The tumor-inhibitory α-methylene lactones were shown to inhibit the sulfhydryl enzyme, phosphofructokinase, and evidence was presented to indicate that the inhibition resulted from their reaction with the sulfhydryl groups of the enzyme.[12] A possible relationship between the carcinogenicity of certain unsaturated lactones and their reactivity with sulfhydryl cell components or metabolites has been suggested.[13,14] However, the advisability of using such data as criteria for predicting the carcinogenicity of lactones has been questioned following a more recent study.[15]

In the course of our continuing studies of the reactions of unsaturated lactones with model biological nucleophiles, we have investigated the reactions of substituted endocyclic α,β-unsaturated γ-lactones with thiols. In 1945, Cavallito and Haskell reported the formation of an amphoteric compound by reaction of cysteine with γ-methyl-$\Delta^{\alpha,\beta}$-butenolide (**4**), but the product was not identified.[6] The same authors reported that β-methyl-$\Delta^{\alpha,\beta}$-butenolide (**3**) showed no measurable reaction with cysteine, and that the lactone could be recovered quantitatively unchanged. The product, **4c**, of S-alkylation of cysteine by **4** has recently been isolated and characterized by Black.[16] We report herein the results of a study of the addition of various thiols to α-methyl-, β-methyl-, and γ-methyl-$\Delta^{\alpha,\beta}$-butenolides, and the isolation and characterization of the previously elusive cysteine adducts of α-methyl-, and β-methyl-$\Delta^{\alpha,\beta}$-butenolides.

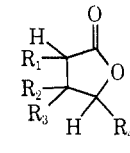


1, $R^1 = R^2 = R^3 = H$

2, $R^1 = CH_3$; $R^2 = R^3 = H$

3, $R^1 = R^3 = H$; $R^2 = CH_3$

4, $R^1 = R^2 = H$; $R^3 = CH_3$



1a, $R^1 = R^2 = R^4 = H$; $R^3 = SCH_2CH_2CH_3$

2c, $R^1 = CH_3$; $R^2 = R^4 = H$; $R^3 = SCH_2CH<^{NH_3^+}_{COO^-}$

3c, $R^1 = R^4 = H$; $R^2 = CH_3$; $R^3 = SCH_2CH<^{NH_3^+}_{COO^-}$

4a, $R^1 = R^2 = H$; $R^3 = SCH_2CH_2CH_3$; $R^4 = CH_3$

4b, $R^1 = R^2 = H$; $R^3 = SCH_2C_6H_6$; $R^4 = CH_3$

4c, $R^1 = R^2 = H$; $R^3 = SCH_2CH<^{NH_3^+}_{COO^-}$; $R^4 = CH_3$

4d, $R^1 = R^2 = H$; $R^3 = SCH_2CH<^{NHCOCH_3}_{COOCH_3}$; $R^4 = CH_3$

Treatment of $\Delta^{\alpha,\beta}$-butenolide (**1**), α-methyl-$\Delta^{\alpha,\beta}$-butenolide (**2**), β-methyl-$\Delta^{\alpha,\beta}$-butenolide (**3**), and γ-methyl-$\Delta^{\alpha,\beta}$-butenolide (**4**) with the thiols, 1-propanethiol, α-toluenethiol, L-cysteine, and N-acetyl-L-cysteine methyl ester, made possible an evaluation of the effect of a methyl substituent on the reactivity of the

(1) Tumor Inhibitors. LVII. Part LVI: S. M. Kupchan, R. M. Smith, Y. Aynehchi, and M. Maruyama, *J. Org. Chem.*, **35**, 2891 (1970). This work was supported by grants from the National Institutes of Health (HE-12957 and CA-11718) and the American Cancer Society (T-275).

(2) Author to whom inquiries should be directed: Department of Chemistry, University of Virginia, Charlottesville, Va. 22901.

(3) National Institutes of Health Postdoctoral Fellow,.

(4) T. S. Hauschka, G. Toennies, and A. P. Swain, *Science*, **101**, 383 (1945).

(5) W. B. Geiger and J. E. Conn, *J. Amer. Chem. Soc.*, **67**, 112 (1945).

(6) C. J. Cavallito and T. H. Haskell, *ibid.*, **67**, 1991 (1945).

(7) *Cf.* L. J. Haynes, *Quart. Rev. (London)*, **2**, 46 (1948).

(8) K. V. Thimann and W. D. Bonner, Jr., *Proc. Nat. Acad. Sci. U. S.*, **35**, 272 (1945).

(9) H. Shibaoka, *Plant Cell Physiol.*, **2**, 175 (1961); *cf.* H. Shibaoka, M. Shimokoriyama, S. Iriuchijima, and S. Tamura, *ibid.*, **8**, 297 (1967).

(10) L. Sequeira, R. J. Hemingway, and S. M. Kupchan, *Science*, **161**, 789 (1968); L. Sequeira and S. M. Kupchan, unpublished observations.

(11) S. M. Kupchan, D. C. Fessler, M. A. Eakin, and T. J. Giacobbe, *Science*, **168**, 376 (1970).

(12) R. L. Hanson, H. A. Lardy, and S. M. Kupchan, *ibid.*, **168**, 378 (1970).

(13) F. Dickens, *Brit. Med. Bull.*, **20**, 96 (1964).

(14) F. Dickens and J. Cooke, *Brit. J. Cancer*, **19**, 404 (1965).

(15) J. B. Jones and J. M. Young, *J. Med. Chem.*, **11**, 1176 (1968).

(16) D. K. Black, *J. Chem. Soc. C*, 1123 (1966).

butenolide toward S-alkylation in a Michael-type reaction.

The results are summarized in Table I. It is apparent that a methyl substituent in the α or β position markedly reduces the reactivity of the butenolide toward Michael-type addition of thiols. The low reactivity of **3** with cysteine is interesting in view of the earlier observation that treatment of strophanthidin (a β-substituted butenolide) with an excess of cysteine did not affect its ultraviolet absorption.[17] The apparent failure of strophanthidin to undergo facile addition of sulfhydryl compounds *in vitro* has been interpreted as rendering improbable the hypothesis that such reactions may play a role in the mode of action of cardiotonic steroids. However, the possibility that polyfunctional enzymatic interactions *in vivo* could favor the addition reaction cannot be excluded from consideration.

TABLE I

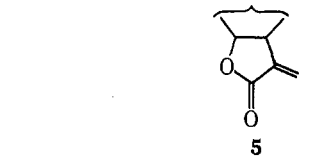
THIOL ADDUCTS OF SUBSTITUTED $\Delta^{\alpha,\beta}$-BUTENOLIDES

Lactone	Thiol[a]	Conditions[b]	Product	Yield, %
1	a	3	1a	65
2	a	3, 4	None	
2	c	1	2c	38
2	d	7	None	
3	a	3, 5, 7	None	
3	b	6	None	
3	c	2	3c	12
3	d	7	None	
4	a	3	4a	20
4	b	3	4b	44
4	c	1	4c[c]	94
4	d	7	4d	60

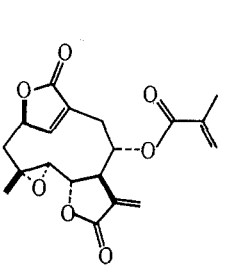
[a] a, $HSCH_2CH_2CH_3$. b, $HSCH_2C_6H_5$. c, $HSCH_2CH<^{NH_3}_{COO^-}$. d, $HSCH_2CH<^{NHCOCH_3}_{COOCH_3}$. [b] 1: pH 7, adjusted to pH with NaOH solution, room temperature, 18 hr. 2: pH 7.4, adjusted to pH with NH_4OH, room temperature, 4 days. 3: pH 7.4, phosphate buffer, 40°, 20 hr. 4: pH 7.4, phosphate buffer, 40°, 7 days. 5: pH 8, phosphate buffer, 60°, 7 days. 6: pH 8, phosphate buffer, 40°, 20 hr. 7: Triethylamine as base, in ether, room temperature, 14 hr. [c] See ref 16.

Cysteine appears to be the most reactive of the thiols investigated. Both the α-methyl- and β-methylbutenolides, **2** and **3**, were found to form adducts only with cysteine and to be unreactive toward N-acetylcysteine methyl ester, 1-propanethiol, and α-toluenethiol within the pH range studied (pH 6–8). The greater reactivity of cysteine may be attributable to the marked acidity of cysteine's sulfhydryl group ($pK_a = 8.5$).[18]

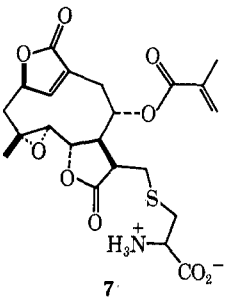
In the preparation of the endocyclic cysteine adducts (**2c**, **3c**, and **4c**), considerable manipulative difficulties were encountered which suggested an inherent instability, and, in fact, successful preparative procedures were developed only after considerable experimentation. This behavior contrasted with the cysteine adduct formed upon reaction of the exocyclic unsaturated γ-lactone function (**5**) present in many sesquiterpene lactones including elephantopin (**6**).[11] The following results indicate that the adducts of the endocyclic compounds (**2**, **3**, and **4**) are indeed much less stable and tend to undergo retro-Michael reactions much more readily than adducts of exocyclic analogs.



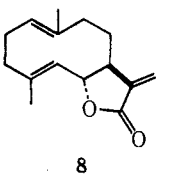
5



6



7



8

The rate of formation of cysteine from the adducts could be monitored under irreversible conditions by means of the thiol reagent 2,2′-dipyridyl disulfide. The endocyclic cysteine adducts **2c**, **3c**, and **4c** underwent retro-Michael reaction at 25° and pH 7.4 with first-order rate constants of 34×10^{-6}, 125×10^{-6}, and 3.0×10^{-6} sec^{-1}, respectively, whereas the rate of dissociation of the exocyclic monocysteine adduct (**7**) of elephantopin was immeasurably slow. Indeed there was no evidence of reversal under such conditions for any of the cysteine adducts of the exocyclic unsaturated γ-lactones.

It is also noteworthy that the exocyclic unsaturated lactone in **6** reacted much faster with cysteine than the endocyclic lactones **2**, **3**, or **4**. The second-order rate constant for the reaction of elephantopin and 1 mol of cysteine at 25° has been reported as 2600 l. mol^{-1} min^{-1}.[11] In contrast the most reactive of the endocyclic lactones, **4**, has been reported to react with cysteine (0.67 *M*) at room temperature and pH 7 in 15 min to afford a 90% yield of adduct **4c**.[15] A second-order rate constant of approximately 1–10 l. mol^{-1} min^{-1} can be associated with this process.[19] This suggests a rate ratio of exocyclic to endocyclic unsaturated lactones of the order of 10^3.

The enhanced reactivity of the exocyclic unsaturated compounds toward thiols compared with that of the endocyclic compounds may find explanation in several factors. The terminal carbon of an exocyclic methylene group should have a lower steric requirement than the corresponding carbon of any of the endocyclic compounds studied. Furthermore, the polarization of the conjugated carbonyl system would be expected to be greater in the exocyclic methylene compounds than in the endocyclic compounds. The inductive effects of

(17) I. M. Glynn, *J. Physiol.*, **136**, 148 (1957).

(18) J. T. Edsall and J. Wyman, "Biophysical Chemistry," Academic Press, New York, N. Y., 1958, p 496.

(19) For a second-order equimolar reaction, $k_2t = {}^1/_A - {}^1/_{A_0}$. Hence k_2 corresponds to approximately 1–10 l. mol^{-1} min^{-1} if this reaction is 90–99% complete after 15 min, since $A_0 = 0.67$ *M*. This is in agreement with a published value of 2.2 l. mol^{-1} min^{-1} [14] for the loss of thiol in the presence of lactone **4**.

the alkyl substituents would be expected to decrease the electrophilic character of the β carbon and thus diminish the reactivity of the endocyclic compounds toward nucleophilic attack. This effect should be most marked in the β-methylbutenolide (**3**) which is the least reactive of all.

The greater stability of the exocyclic adducts, once formed, may be rationalized if the retro-Michael process involves the initial loss of a proton from the α-carbon, for the loss of a tertiary proton is both sterically and inductively less favorable than the loss of a secondary proton. The α-methylbutenolide adduct (**2c**) may be so stabilized, but the subsequent loss of thiol anion from each of the endocyclic derivatives may be associated with a concomitant release of I strain[20] as ionization occurs, thus leading to an enhanced dissociation relative to the exocyclic case.

An entirely analogous situation exists in the interactions of amines with α,β-unsaturated lactones. Of the endocyclic compounds only the γ-methylbutenolide (**4**) has been investigated;[21] addition of dimethylamine has been shown to occur in solution, although the instability of the product precluded isolation and further characterization. In contrast, the exocyclic unsaturated lactone present in costunolide (**8**) has been shown to react with dimethylamine to give a stable Michael adduct.[22]

In a study of the relative nucleophilic reactivities of amino groups and mercaptide ions in addition reactions with α,β-unsaturated compounds, Friedman, *et al.*,[23] found that, at comparable pK_a values and steric environments, sulfur anions are about 280 times more reactive than amino groups. Our results with the amino thiol, cysteine, are in good agreement, for only thiol addition was observed. All the cysteine adducts formed gave negative spot tests on tlc for a thiol group, although some dissociation in aqueous solution was noted.

Further studies of the Michael-type addition of thiols to unsaturated lactones are in progress, and will be reported in due course.

Experimental Section

Infrared spectra were determined on a Beckman Model IR-5A recording spectrophotometer. Ultraviolet spectra were recorded on a Beckman Model DK-2A spectrophotometer fitted with a thermostated cell compartment and a variable timer interlocked with a repetitive scanning attachment to give fully automatic operation. Nmr spectra were determined on Varian A-60A and HA-100 spectrometers. Evaporations were carried out at temperatures less than 40° under reduced pressure. All melting points were determined on a Thomas–Hoover capillary melting point apparatus and are corrected. Microanalyses were performed by Spang Microanalytical Laboratory, Ann Arbor, Mich. For the mass spectral data, the authors thank the Purdue Mass Spectrometry Center.

Buffers.—The buffers employed were those of Sorensen and Palitzch, described by Kolthoff and Rosenblum.[24]

Deoxygenated Water.—Distilled water was redistilled under deoxygenated nitrogen from potassium permanganate–sodium hydroxide (2 g, 2 g/l.). High purity nitrogen was deoxygenated by bubbling through a solution of pyrogallol [potassium hydroxide (25 g), pyrogallol (100 g), water (250 ml)], and then through a solution of sodium vanadate (*vide infra*).

Sodium Vanadate Solution.[25]—Zinc (125 g) was amalgamated by treatment with 10% mercurous nitrate (125 ml) containing a few drops of concentrated nitric acid. The zinc was washed with water and placed in a gas washing bottle. A solution of 0.1 *M* sodium metavanadate (Alfa Inorganics) in 2 *M* sulfuric acid was added. Nitrogen was bubbled through the yellow-green solution, which became dark blue in a few hours and was then ready for use. A constant flow of nitrogen was maintained through the solution until the color changed to muddy brown, indicating a loss of effectiveness.

Preparative Layer Chromatography on "Avicel."—"Avicel" (American Viscose Corp., Newark, Del.) chromatoplates were prepared (1.25 mm thick) according to Wolfrom's procedure.[26,27] Each chromatoplate was used to separate 50–75 mg of a mixture. The chromatoplates were developed with ethyl acetate:acetic acid:formic acid:water (18:3:1:4, v/v), a system which gave good separation of cystine, cysteine, and the various adducts. Since the butenolide–cysteine adducts usually could not be visualized by uv irradiation, the bands were detected by spraying two narrow strips of the plate with 0.2% ethanolic ninhydrin solution. After the ninhydrin color had developed, the desired band was removed from the plate. The material was eluted with deoxygenated water, and the eluate was lyophilized to give the desired adduct as a powder.

$\Delta^{\alpha,\beta}$-Butenolide (1).—The butenolide **1** was prepared according to the procedure of Price and Judge.[28]

Formation of the $\Delta^{\alpha,\beta}$-Butenolide–1-Propanethiol Adduct: 3-Thiopropyl-4-hydroxybutanoic Acid Lactone (1a).—An aqueous buffered solution (100 ml, pH 7.4) of $\Delta^{\alpha,\beta}$-butenolide (**1**, 250 mg, 0.003 mol) and 1-propanethiol (0.35 ml, 0.0037 mol, Aldrich) was stirred at 40° for 18 hr. The aqueous solution was extracted with chloroform (five 50-ml portions). The combined chloroform extracts were dried over $MgSO_4$ and concentrated under reduced pressure. The residual oil was distilled, bp 95–98° (0.2 mm), to yield a clear, colorless oil (**1a**, 303 mg, 65%): ir λ_{max}^{film} 5.63 μ (lactone carbonyl); nmr ($CDCl_3$) τ 9.02 (3 H, t, J = 7 Hz, $-CH_2CH_3$), 8.8–7.9 (2 H, m, $-CH_2CH_3$), 7.8–6.8 (4 H, m, $-CH_2S-$ and $-CH_2CO_2-$), 6.5–5.3 (3 H, m, *n*-PrSCHCH$_2$O); mass spectrum (75 eV) *m/e* (rel intensity) 160 M^+ (35.5), 102 (62), 85 (22), 84 (20), 74 (11), 73 (34), 61 (15.5), 60 (100), 59 (40), 58 (29).

Anal. Calcd for $C_7H_{12}O_2S$: C, 52.49; H, 7.55; S, 19.98. Found: C, 52.30; H, 7.58; S, 19.89.

α-Methyl-$\Delta^{\alpha,\beta}$-butenolide (2).—The butenolide **2** was prepared according to the reported procedure.[29]

Formation of the α-Methyl-$\Delta^{\alpha,\beta}$-butenolide–L-Cysteine Adduct: 2-Methyl-3-(2-amino-2-carboxyethylthio)-4-hydroxybutanoic Acid Lactone (2c).—A solution of the butenolide **2** (500 mg, 0.005 mol) and cysteine hydrochloride monohydrate (438 mg, 0.0025 mol) in water (15 ml) was adjusted to pH 7.0 with 2 *N* sodium hydroxide solution and kept under a nitrogen atmosphere for 24 hr. The solution was extracted with ether (three 250-ml portions), and the combined ether extracts were dried ($MgSO_4$) and concentrated under reduced pressure to yield recovered butenolide **2** (255 mg, 0.0025 mol). The aqueous solution was concentrated under reduced pressure, and absolute ethanol was added to effect solution of all the precipitated material. The aqueous ethanol solution was cooled, and the white, noncrystalline solid which formed was collected by centrifugation to give the adduct **2c**. Concentration of the mother liquors yielded a second crop, which was shown by its infrared spectrum to be identical with the first material isolated. The total yield of **2c** was 205 mg (38%): mp 188–190° with some softening beforehand and effervescence at the melting point; ir λ_{max}^{KBr} 5.56 μ (lactone carbonyl); nmr (D_2O) τ 8.40 (3 H, d, J = 7 Hz, α-CH_3), 7.4–6.8 (2 H, m, $-C(CH_3)HCHSR$), 6.8–6.6 (2 H, $-SCH_2-$), 6.5–5.5 (3 H, m, SCH_2CH- and $-SCHCH_2-O-$); mass spectrum (75 eV) *m/e* (rel intensity) 219 M^+ (2.5), 174 (5.0), 146 (8.0), 101 (9.3), 100 (7.5),

(20) H. C. Brown and G. Ham, *J. Amer. Chem. Soc.*, **78**, 2735 (1956).
(21) J. B. Jones and J. M. Young, *Can. J. Chem.*, **44**, 1059 (1966).
(22) S. V. Hiremath, G. H. Kulkarni, G. R. Kelkar, and S. C. Bhattacharyya, *Indian J. Chem.*, **6**, 339 (1968).
(23) M. Friedman, J. F. Cavins, and J. S. Wall, *J. Amer. Chem. Soc.*, **87**, 3672 (1965).
(24) I. M. Kolthoff and C. Rosenblum, "Acid Base Indicators," Macmillan, New York, N. Y., 1937, p 249.
(25) Private communication from Professor A. J. Krubsack, The Ohio State University.
(26) D. Horton, A. Tanimuri, and M. L. Wolfrom, *J. Chromatogr.*, **23**, 309 (1966).
(27) M. L. Wolfrom, D. L. Patin, and R. M. De Lederkremer, *ibid.*, **17**, 488 (1965).
(28) C. C. Price and J. M. Judge, *Org. Syn.*, **45**, 22 (1965).
(29) C. J. Cavallito and T. H. Haskell, *J. Amer. Chem. Soc.*, **68**, 2332 (1946).

99 (16), 98 (48), 89 (11), 76 (32), 75 (32), 74 (100), 70 (6.2), 69 (47).

Anal. Calcd for $C_8H_{13}NO_4S$: C, 43.83; H, 5.98; N, 6.39; S, 14.45. Found: C, 43.62; H, 5.91; N, 6.49; S, 14.25.

β-Methyl-Δ$^{\alpha,\beta}$-butenolide (3).—The butenolide **3** was prepared according to reported procedures.[30-32]

Formation of the β-Methyl-Δ$^{\alpha,\beta}$-butenolide–L-Cysteine Adduct: 3-(2-Amino-2-carboxyethylthio)-3-methyl-4-hydroxybutanoic Acid Lactone (3c).—A solution of cysteine hydrochloride (158 mg, 0.001 mol) and the butenolide **3** (98 mg, 0.001 mol) in deoxygenated water (1 ml) was adjusted to pH 7.4 with concentrated ammonium hydroxide, while kept under a stream of nitrogen. The flask was sealed and kept at room temperature for 4 days, and then the solution was lyophilized to give a white powder. The powder was dissolved in a minimum of water and chromatographed on "Avicel." A band, R_f 0.45–0.65, was removed and eluted with ice water. The water eluates were immediately frozen and lyophilized to give a white powder. The powder was dissolved in MeOH (4 ml) and precipitated by addition of ethyl acetate (20 ml). The precipitate was collected by filtration and washed with ethyl acetate to give, after drying, a white powder (**3c**, 26 mg, 12.0%): mp 203–205° dec; ir λ_{max}^{KBr} 5.62 μ (lactone carbonyl); mass spectrum (75 eV) *m/e* (rel intensity) 219 M$^+$ (0.23), 98 (43.7), 76 (11.9), 74 (17.6), 69 (100), 68 (17.6), 56 (17.9).

Anal.[33] Calcd for $C_8H_{13}NO_4S$: C, 43.81; H, 5.98; N, 6.38; S, 14.62. Found: C, 39.54 (43.4 corrected); H, 5.31 (5.8 corrected); N, 6.03 (6.6 corrected); S, 13.03 (14.3 corrected).

Adduct **3c** was found to decompose very rapidly in water at room temperature to give cysteine and the starting butenolide.

γ-Methyl-Δ$^{\alpha,\beta}$-butenolide (4).—The butenolide **4** was prepared from α-angelica lactone (Aldrich) according to the reported procedure.[34]

Formation of the γ-Methyl-Δ$^{\alpha,\beta}$-butenolide–1-Propanethiol Adduct: 3-Propylthio-4-hydroxypentanoic Acid Lactone (4a).—An aqueous buffer solution (125 ml, pH 7.4) of the γ-methylbutenolide (0.5 g, 0.005 mol) and 1-propanethiol (0.38 g, Aldrich) were mixed and stirred at 40° for 20 hr. The solution was extracted with ether, and the combined ethereal extracts were dried over anhydrous $MgSO_4$. Concentration of the ethereal solution under reduced pressure yielded an oil which was purified on freshly activated silica gel GF_{254} plates. The plates were eluted with ether:hexane (1:1), and a band R_f 0.4 (visualized with uv light), was removed and eluted with ether. Concentration of the ether eluate yielded a clear, colorless oil (**4a**, 166 mg, 20%): ir $\lambda_{max}^{CHCl_3}$ 5.58 μ (saturated lactone carbonyl); nmr ($CDCl_3$) τ 9.02 (3 H, t, CH_3CH_2–), 8.55 (3 H, d, J = 6 Hz, CH_3CHO), 8.7–8.1 (2 H, m, $CH_3CH_2CH_2S$–), 7.6–7.0 (4 H, m, $-CH_2S$ and $-CH_2CO_2$–), 7.0–6.7 (1 H, m, –SCH–), 5.8–5.4 (1 H, quintet, J = 6 Hz, CH_3CHO); mass spectrum (75 eV) *m/e* (rel intensity) 174 M$^+$ (27.6), 102 (87.5), 99 (17.3), 98 (17.3), 73 (20.7), 61 (17.3), 60 (100), 59 (18.4), 55 (20.7).

Anal. Calcd for $C_8H_{14}O_2S$: C, 55.16; H, 8.10; S, 18.37. Found: C, 55.29; H, 8.08; S, 18.49.

Formation of the γ-Methyl-Δ$^{\alpha,\beta}$-butenolide–α-Toluenethiol Adduct: 3-Benzylthio-4-hydroxypentanoic Acid Lactone (4b).—This adduct was prepared as described for the 1-propanethiol adduct (**4a**) to yield after chromatography a clear, colorless oil (**4b**, 44%): ir $\lambda_{max}^{CHCl_3}$ 5.62 (saturated lactone carbonyl), 6.65–7.70 μ (aromatic); nmr ($CDCl_3$) τ 8.73 (3 H, d, J = 6 Hz, $-CHCH_3{}^a$), 8.0–6.9 (3 H, m, $-SCHCH_2CO_2$–), 6.25 (2 H, s, $-SCH_2C_6H_5$), 5.9–5.4 (1 H, m, CH_3CH^bO–), 2.75 (5 H, s, aromatic protons); H^a and H^b shown to be coupled by double irradiation experiments (J = 6 Hz); mass spectrum (75 eV) *m/e* (rel intensity) 222 M$^+$ (43.7), 150 (15.6), 121 (42), 92 (26), 91 (100), 77 (7.3), 65 (30.2).

Anal. Calcd for: $C_{12}H_{14}O_2S$: C, 64.85; H, 6.35; S, 14.40. Found: C, 64.88; H, 6.36; S, 14.46.

While this work was in progress, adduct **4b**, 3-benzylthio-4-hydroxypentanoic acid lactone, was independently prepared in a nonaqueous medium by Jones and Young.[15]

Formation of the γ-Methyl-Δ$^{\alpha,\beta}$-butenolide–L-Cysteine Adduct: 3-(2-Amino-2-carboxyethylthio)-4-hydroxypentanoic Acid Lactone (4c).—This adduct was prepared according to the procedure published by Black.[16] The product was obtained in 94% yield as a crystalline solid, mp 179–180° dec (lit 193–197° dec).[9]

Anal. Calcd for $C_8H_{13}NO_4S$: C, 43.81; H, 5.98; N, 6.38; S, 14.62; M, 219. Found: C, 43.62; H, 5.89; N, 6.53; S, 14.55; M (mass spectrum), 219.

N-Acetyl-L-cysteine Methyl Ester (d).—A solution of N-acetyl-L-cysteine (1.0 g, 0.006 mol, K & K Laboratories) in methanol (20 ml) was mixed with an ethereal solution of diazomethane. The reaction was monitored by tlc (silica gel, ether), and addition of diazomethane was stopped when only a trace of N-acetyl-L-cysteine remained. (Addition of an excess of diazomethane made the product difficult to purify.) The product was recrystallized from ether–petroleum ether to yield the methyl ester (**d**, 0.79 g, 73%): mp 78–80.5°, and after sublimation [45–50° (bath temperature)(0.01 mm)] mp 81–82° (lit.[35] 79–80°); ir λ_{max}^{KBr} 3.08 (–NHCO–), 3.92 (sh, –SH), 5.78 μ (ester).

Formation of the γ-Methyl-Δ$^{\alpha,\beta}$-butenolide–N-Acetyl-L-cysteine Methyl Ester Adduct: 3-(2-Acetylamino-2-carbomethoxyethylthio)-4-hydroxypentanoic Acid Lactone (4d).—A solution of N-acetyl-L-cysteine methyl ester (583 mg, 0.0033 mol), γ-methyl-Δ$^{\alpha,\beta}$-butenolide (339 mg, 0.0033 mol, 96% pure), and triethylamine (6 drops) in ether (8 ml) was allowed to stand at room temperature for 14 hr. Two layers resulted, and the ethereal layer (upper) was pipetted from the tube. The lower layer was distilled at 205° (bath temperature) and 0.075 mm to yield the adduct **4d** (556 mg, 60%): $[\alpha]^{28}D$ +46° (*c* 1.01, $CHCl_3$); ir λ_{max}^{film} 6.63 (saturated lactone), 5.74, 5.97 μ (amide); nmr ($CDCl_3$) τ 8.55 [3 H, d, J = 6 Hz, $-CH(CH_3{}^a)O$–], 7.93 (3 H, s, –NHCO–CH_3), 6.21 (3 H, s, $-CO_2CH_3$), 5.65 [1 H, quintet, J = 6.5 Hz, $-CH^b(CH_3)O$–], 5.20 [1 H, 6 line multiplet, $-CH(NHCOCH_3)$], 2.85 (1 H, m, –NHCO–); H^a and H^b were shown to be coupled by double irradiation experiments.

Anal. Calcd for $C_{11}H_{17}NO_5S$: C, 47.99; H, 6.23; N, 5.09; S, 11.64. Found: C, 47.84; H, 6.38; N, 5.20; S, 11.52.

Retro-Michael Reaction Kinetic Measurements. Procedure.—The L-cysteine adduct (~1 μmol) was dissolved in phosphate buffer solution (pH 7.4, 1 ml). A calculated volume (~ 360 μl) was added to a 1-cm cuvette, and diluted with more buffer (thermostated at 25°) to give a final concentration of adduct of 10^{-4} *M*. The cuvette was placed in the thermostated cell holder of the uv spectrophotometer and the reaction was started by the addition of a sufficient quantity (~36 μl) of a freshly prepared solution of 2,2′-dipyridyl disulfide[36] in tetrahydrofuran (22.0 mg in 10 ml) to give an equimolar reaction mixture.

The uv absorption of the solution was measured automatically over the range 300–400 mμ at appropriate time intervals. The rate of liberation of cysteine was measured by monitoring the very fast and irreversible reaction with 2,2′-dipyridyl disulfide to give 2-thiopyridone (λ_{max} 343 mμ, ε 7780). The infinity values were calculated from known starting concentrations since the observed infinity readings were found to be low.

α-Methyl-Δ$^{\alpha,\beta}$-butenolide–L-Cysteine Adduct.—Adduct **2c** (0.235 mg/ml, 335 μl), in the presence of 2,2′-dipyridyl disulfide (1.0×10^{-4} *M*) underwent irreversible retro-Michael reaction in buffered 1% tetrahydrofuran solution. The first-order plot of the kinetic data tapered off with time at about 30% reaction; however, the rate constant, $k_1 = 3.4 \times 10^{-5}$ sec^{-1}, calculated from the straight initial portion of the plots was reproducible to about ±10%.

β-Methyl-Δ$^{\alpha,\beta}$-butenolide–L-Cysteine Adduct.—Adduct **3c** (0.239 mg/ml, 412 μl), at an initial concentration of 1.25×10^{-4} *M*, underwent dissociation in the presence of 2,2′-dipyridyl disulfide (1.25×10^{-4} *M*) with $k_1 = 1.25 (\pm 0.17) \times 10^{-4}$ sec^{-1}.

γ-Methyl-Δ$^{\alpha,\beta}$-butenolide–L-Cysteine Adduct.—Adduct **4c** (0.250 mg/ml, 400 μl), at an initial concentration of 1.27×10^{-4} *M*, underwent dissociation in the presence of 2,2′-dipyridyl disulfide (1.27×10^{-4} *M*) with $k_1 = 3.0 (\pm 0.25) \times 10^{-6}$ sec^{-1}.

Elephantopin–Monocysteine Adduct.—The preparation of this compound (**7**) has been described elsewhere.[11] The adduct **7** was prepared in a 4% tetrahydrofuran–buffer solution (pH 7.4) by allowing an equimolar mixture of L-cysteine (1.0×10^{-4} *M*) and elephantopin (**6**) to react to completion. After 60 min the con-

(30) W. J. Conradie, C. F. Garbers, and P. S. Steyn, *J. Chem. Soc.*, 594 (1964).

(31) J. M. Stewart and D. W. Woolley, *J. Amer. Chem. Soc.*, **81**, 4951 (1959).

(32) C. H. Hoffman, *ibid.*, **79**, 2316 (1957).

(33) The analysis was corrected for 9.8% ash. Attempts to free **3c** from ash using a Bio-Gel column (a successful procedure in these laboratories for purifying stable, high molecular weight, cysteine adducts) were unsuccessful.

(34) J. Thiele, R. Tischbein, and E. Lossow, *Justus Liebigs Ann. Chem.*, **319**, 144 (1902).

(35) J. B. Jones and D. C. Wigfield, *Can. J. Chem.*, **44**, 2517 (1966).

(36) D. R. Grassetti and J. F. Murray, Jr., *Arch. Biochem. Biophys.*, **119**, 41 (1967).

centration of free cysteine was negligible. The solution of adduct **7** (10^{-4} *M*) was placed in a cuvette and 2,2′-dipyridyl disulfide (36 μl., 10^{-2} *M* solution in tetrahydrofuran) was added to give an equimolar mixture. The ultraviolet absorption of the solution was then measured at various times. However, there was no change in the absorption; *i.e.*, there was no thiopyridone produced.

Registry No.—**1**, 497-23-4; **1a**, 25516-01-2; **2**, 22122-36-7; **2c**, 25516-03-4; **3**, 6124-79-4; **3c**, 25516-05-6; **4**, 591-11-7; **4a**, 25516-07-8; **4b**, 25516-08-9; **4c**, 6417-06-7; **4d**, 25516-10-3; 1-propanethiol, 107-03-9; α-toluenethiol, 100-53-8; L-cysteine, 52-90-4; N-acetyl-L-cysteine methyl ester, 7652-46-2.

Synthesis of D-1-Hydroxy-2-amino-3-ketooctadecane-4,5-^{3}H Hydrochloride[1a]

BENJAMIN WEISS AND RICHARD L. STILLER

Division of Neuroscience, New York State Psychiatric Institute, and Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, New York 10032

Received March 13, 1970

N-Trifluoroacetyl- and N-carbobenzoxydihydrosphingosines were oxidized to their corresponding keto analogs with chromic anhydride in pyridine and converted to D-1-hydroxy-2-amino-3-ketooctadecane hydrochloride. It was found from the ease of removal of the N-protective groups that the N-carbobenzoxy compound provided the best yield of the ketoamine salt. N-Carbobenzoxydihydrosphingosine-4,5-^{3}H carried through the same reaction sequence yielded D-1-hydroxy-2-amino-3-ketooctadecane-4,5-^{3}H hydrochloride. The N-acetyl, O,N-diacetyl, and 2,4-dinitrophenylhydrazone derivatives of the ketoamine base were prepared for purposes of characterization. The presence of two forms of D-1-hydroxy-2-acetamido-3-ketooctadecane was suggested by the finding of one band on thin layer chromatography and two bands on gas–liquid chromatography.

During our investigation of the *in vitro* biosynthesis of long-chain bases by *Hansenula ciferri*, it was necessary to have D-1-hydroxy-2-amino-3-ketooctadecane[1b] which has been shown to be an intermediate in the biosynthesis of dihydrosphingosine.[2-4] The preparation of D-1-hydroxy-2-acetamido-3-ketooctadecane was reported[5] in which the secondary hydroxyl group of N-acetyldihydrosphingosine, D-*erythro*-1,3-dihydroxy-2-acetamidooctadecane,[6] was oxidized with chromic anhydride in pyridine.[7] It was thought that the free ketoamine or its salt could be obtained by this procedure if the oxidation were performed on the appropriate N-substituted base. N-Trifluoroacetyl- and N-carbobenzoxydihydrosphingosines were selected for this purpose because the trifluoroacetyl group could be cleaved easily under mild alkaline conditions at room temperature, whereas the carbobenzoxy function could be removed by hydrogenolysis.

Oxidation of N-trifluoroacetyldihydrosphingosine gave the expected D-1-hydroxy-2-trifluoroacetamido-3-ketooctadecane in about 35% yield. However, upon treatment with K_2CO_3, little or no free ketoamine was obtained, unlike reaction with the unoxidized parent compound which yielded the free base. The ketoamine hydrochloride was prepared in 24% yield from the N-trifluoroacetyl keto derivative by refluxing with 1.5 *N* HCl in aqueous ethanol. By comparison, oxidation of N-carbobenzoxydihydrosphingosine gave yields of about 45% of the respective keto analog (Scheme I, B, C); reduction over palladium in ethanol containing sufficient hydrochloric acid to neutralize the generated free base resulted in 95% yields of ketoamine hydrochloride. When the N-carbobenzoxy keto compound was reduced in glacial acetic acid, dihydrosphingosine was obtained whose identity was proved by infrared spectroscopy and by the melting point of its N-acetyl derivative. Thin layer chromatography of the ketoamine hydrochloride in chloroform:methanol (95:5) showed a single component, R_F 0.40; gas–liquid chromatography of the trimethylsilyl derivative was unsuccessful. The free ketoamine was obtained by treatment of the hydrochloride with $KHCO_3$ in aqueous methanol followed by extraction into ether and removal of solvent; it changed color rapidly from white to yellow after crystallization from petroleum ether. The yellow ketoamine melted at 49–54°. Thin layer chromatography disclosed one major component, R_F 0.42, along with five minor ones, R_F 0.69, 0.76, 0.83, 0.89, and 0.93. It was concluded that N-carbobenzoxydihydrosphingosine was the substrate of choice for preparation of the ketoamine hydrochloride because of the ease of removal of the protective group and the better overall yield.

The overall yield of D-1-hydroxy-2-amino-3-ketooctadecane-4,5-^{3}H hydrochloride was 30%; the radioactive yield based on N-carbobenzoxydihydrosphingosine-4,5-^{3}H was 15% (Scheme I). Since little or no tritium activity was observed on carbon atoms 1 to 3 in previous preparations[8] of dihydrosphingosine-4,5-^{3}H, the loss of 10.4 μCi after oxidation of N-carbobenzoxydihydrosphingosine (35.9 μCi/mg) to N-carbobenzoxy keto compound (25.5 μCi/mg) was attributed to an impurity of high specific activity which was removed from the keto compound but cochromatographed with N-carbobenzoxydihydrosphingosine-4,5-^{3}H during purification on the silicic acid column, and to removal of tritium on carbon atom 4 by exchange.

Proof that oxidation had occurred at the secondary hydroxyl group was obtained by treating an acetic acid

(1) (a) This investigation was supported in part by Public Health Service Research Grant No. NB 06300-04 from the National Institutes of Neurological Diseases and Stroke. (b) As this work was being prepared for publication, P. B. Mendershausen and C. C. Sweeley, *Biochemistry*, **8**, 2633 (1969), reported the micropreparation of the ketoamine free base from N-carbobenzoxydihydrosphingosine. However, the complete chemical characterization of this compound was not made. Since our efforts were directed at preparing sufficient material to be stored for varying periods of time during radioactive studies, it was decided to isolate the ketoamine as a salt.

(2) R. N. Brady, S. J. DiMari, and E. E. Snell, *J. Biol. Chem.*, **244**, 491 (1969).

(3) P. E. Braun and E. E. Snell, *ibid.*, **243**, 3775 (1968).

(4) (a) W. Stoffel, D. Le Kim, and G. Sticht, *Z. Physiol. Chem.*, **349**, 664 (1968); (b) *ibid.*, **349**, 1637 (1968).

(5) R. C. Gaver and C. C. Sweeley, *J. Amer. Chem. Soc.*, **88**, 3643 (1966).

(6) H. E. Carter and Y. Fujino, *J. Biol. Chem.*, **221**, 879 (1956).

(7) G. I. Poos, G. E. Arth, R. E. Beyler, and L. H. Sarett, *J. Amer. Chem. Soc.*, **75**, 422 (1953).

(8) B. Weiss and R. L. Stiller, *J. Biol. Chem.*, **242**, 2903 (1966).