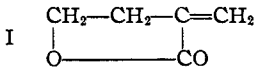
[CONTRIBUTION FROM THE STERLING-WINTHROP RESEARCH INSTITUTE]

Lactone Aliphatic Acids as Antibacterial Agents

BY CHESTER J. CAVALLITO, DOROTHY MCKENICA FRUEHAUF AND JOHN HAYS BAILEY

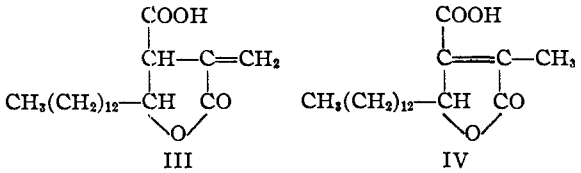
The antibacterial activity of aqueous extracts of *Erythronium americanum*[1] has been shown to be accounted for by the presence in the plant of a precursor, which, upon hydrolysis, yields α-methylene butyrolactone, I. Investigation of the chemical structure of the antibiotic isolated from *Arc-*



I

tium minus[2,3] has indicated that this substance, II, also may contain an α-methylene lactone structure and that this feature endows it with antibacterial properties.

A survey of the scientific literature showed that protolichesterinic acid, a metabolic product of certain lichens, such as *Cetraria islandica* (Iceland Moss), had been shown to possess the α-methylene butyrolactone type of structure, III, which, upon suitable treatment, could be isomerized to lichesterinic acid, IV.



III IV

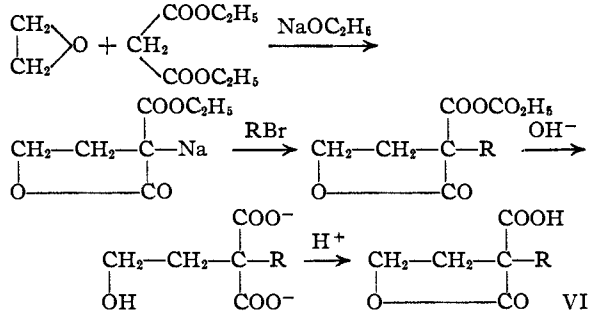
Several optical isomers of protolichesterinic acid have been found in nature and lichesterinic (IV) acid may result from rearrangement of III.[4]

The antiseptic action of certain lichen phenolic acid derivatives has been observed[5]; however, only very recently has there been mention of antibacterial tests[6] with protolichesterinic acid, which, on the basis of analogy to I and II, might be expected to show some antibacterial activity. Screening tests with lichens have indicated the frequent occurrence of antibacterial substances[7] although relatively few of the many lichen metabolic products[8] have been tested as antibiotics.[9]

From a commercial Iceland moss[10] there has been prepared *l*-protolichesterinic acid which was found to be much more effective than I or II in inhibiting the growth of gram positive bacteria and was relatively inert toward gram negative organisms. It was of interest to observe that the isomeric *l*-lichesterinic acid and the hydrogenated derivative, *l*-dihydro-protolichesterinic acid (V), were about equally effective as inhibitors. Surface tension measurements showed that III, IV and V were all anionic detergents. It is known that, in general, anionic detergents inhibit primarily the gram positive group of micro-organisms. It has been shown that the antibacterial activity of these three compounds can be antagonized by the presence of the cationic detergent, cetyldimethylbenzylammonium chloride.

The observations indicate that the double bond in III is of secondary, if of any, importance in contributing to the antibacterial activity. That III is capable of reacting with sulfhydryl groups was shown by preparing the cysteine derivative which formed as a crystalline compound by addition through the —SH group to the α-methylene double bond in III without involvement of the amino group of cysteine in a secondary reaction. In this respect, the α,β-unsaturated lactone differs from the β,γ-type.[11] The ring α,β-unsaturated lactone, IV, did not react with cysteine and it is believed that the reaction is prevented by the presence of the carboxyl group in the β-position. The cysteine derivative of III was much less inhibitory to bacteria than III; however, the derivative also was much less surface-active.

In order to throw more light on the relationship of lactone structure to possible antibiotic activity, it seemed desirable to prepare a series of lactone aliphatic acids of Type VI, where R is a long aliphatic chain.



VI

The reaction of ethylene oxide with malonic ester has been adequately described.[12] Substi-

(1) Cavallito and Haskell, THIS JOURNAL, **68**, 2332 (1946).

(2) Cavallito and Kirchner, *ibid.*, **69**, 3030 (1947).

(3) Sinnhold, *Arch. Pharm.*, **236**, 504 (1898); Böhme, *ibid.*, **241**, 1 (1903); Zopf, *Ann.*, **324**, 39 (1902).

(4) Asahina and Asano, *C. A.*, **22**, 4470 (1928); Asano and Kanematsu, *Ber.*, **65B**, 1175 (1932); Asahina and Yanagita, *ibid.*, **69B**, 120 (1936); Asahina and Yasue, *ibid.*, **70B**, 1053 (1937).

(5) Huzikawa, *C. A.*, **35**, 445, 8195 (1941).

(6) While this work was in progress, the paper of Stoll, Renz and Brach (*Experientia*, **3**, 111, 115 (1947)) appeared in which they report the antistaphylococcic activity of lichens as resulting from the presence of usninic, vulpinic, physodic and protolichesterinic acids. Usninic acid is indicated to inhibit *S. aureus* in a dilution of 1:100,000; various mycobacteria in 1:300,000–800,000, and as inactive against gram negative bacteria.

(7) Burkholder and Evans, *Bull. Torrey Botan. Club*, **72**, 157 (1945).

(8) Asahina, *Acta Phytochim., Japan*, **8**, 33–45 (1934).

(9) Marshak, *Public Health Reports*, **62**, 3 (1947); Marshak, Barry and Craig, *Science*, **106**, 394 (1947).

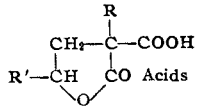
(10) S. B. Penick & Co.

(11) Cavallito and Haskell, THIS JOURNAL, **67**, 1991 (1945).

(12) Traube and Lehman, *Ber.*, **34**, 1977 (1901).

TABLE I

ANALYTICAL, PHYSICAL AND ANTIBACTERIAL PROPERTIES



R'	R	Analyses, % Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found	M. p., °C.[a]	Surface tension,[d] dynes per cm.	Minimum bacteriostatic[e] concn. as mmole. per cc. × 10^4: *Streptococcus hemolyticus* C203	*Staphylococcus aureus* 209	*C. welchii*	*B. typhi*	*B. ranae*	*B. tuberculosis* H37Rv *g*	*B. tuberculosis* H37Rv *h*
H	$C_{10}H_{21}$	66.63	66.66	9.69	9.79	75–77	70.3	7.5	3.7	1.8	>13	1.0	0.5	1.5
H	$C_{12}H_{25}$	68.41	68.51	10.13	10.30	78–79	68.1	1.3	0.7	0.3	>13	0.6	.3	0.3
H	$C_{13}H_{27}$	69.19	69.11	10.32	10.51	69–70	43.3	1.0	0.1	.08	..	.3	.06	.06
H	$C_{14}H_{29}$	69.89	69.88	10.49	10.59	82–83	35.0	1.0	0.1[f]	.03	>13	.3	.05	.06
CH_3	$C_{14}H_{29}$	70.54	70.59	10.66	10.77	64–67	33.2	1.0	0.1	.03	>13	.3	.06	..
H	$C_{16}H_{33}$	71.14	70.95	10.80	10.58	80–82	41.4	10	1	.3	>13	.3	.06	.06
CH_3	$C_{16}H_{33}$	71.69	71.48	10.94	11.13	60–63	37.6	10	1	.03	>13	.6	.05	.06
l-Protolichesterinic		70.33	70.62	9.94	9.97	105	39.2	1.0	0.3	.015	>30	.3	.03	.05
l-cysteine derivative[b]		59.29	59.15	8.82	8.93	185–188 (dec.)	49.5	10	6	>6	..	...	..	..
l-Lichesterinic		70.33	70.44	9.94	10.00	122	41.1	0.6	0.3	0.07	>30	.3	.06	.06
l-Dihydroprotolichesterinic		69.90	70.06	10.50	10.34	99–100	50.2	1.0	.3	0.15	>30	.3	.05	.06
Chaulmoogric[c]		...	...	...	...	.....	..	5	.7	>2	..	...	.06	.15

[a] Corrected. [b] For nitrogen analyses see Experimental. [c] Forms precipitate with media; endpoints difficult to read. [d] Solvent is 0.1 molar potassium phosphate buffer of *p*H 7; solvent surface tension = 71.9 dynes per cm. Measurements made with a Du Nouy interfacial tensiometer. Concentration of the acids = 3×10^{-5} mmole. per cc. [e] Antifungal tests against *Trychophyton mentagrophytes* and *Microsporon audouini* show the synthetic series to be inert whereas inhibitory concentrations for protolichesterinic, lichesterinic and the dihydro acids were 0.4, 0.7 and 0.8×10^{-4} mmole. per cc. respectively. [f] Bacteriostatic concentration in ordinary nutrient broth = 0.5; addition of 10% serum to tryptose phosphate changes endpoint to 1.0. [g] Incubation time eight days. [h] Incubation time fourteen days.

tuted ethylene oxides may also be used. It was found easier to purify the final product when R was substituted after the reaction with ethylene oxide rather than by treating an R-substituted malonic ester with the ethylene oxide. Compounds were prepared in which R ranged from C_{10} through C_{16} carbon atom chains; the C_{14} chain was the optimum in contributing to the antibacterial activity. The derivatives prepared from propylene oxide were essentially of the same activity as those from ethylene oxide.

The antibacterial activity of the lactone acids was compared with that of chaulmoogric and dihydrochaulmoogric acids and with alkyl substituted malonic acids. The lactone acids were readily prepared in from 0.2% to 1% solutions in buffers of *p*H 7, whereas chaulmoogric acid, alkyl monocarboxylic acids and alkylmalonic acids were difficult to dissolve at this *p*H and were much less compatible with growth media than were the lactone acids. The solubility difficulty with aliphatic acids in general and its implications in testing for antibacterial activity have been pointed out in previous investigations[13] of agents active against acid-fast microörganisms. Serum and complex media were found to decrease the antibacterial activity of the lactone acids from the values obtained with simple media.

The properties and antibacterial activity of the compounds are outlined in the table. Comparison of antibacterial action with the ability of the compounds to lower surface tension shows a close parallelism in the synthetic series; however, no obvious relationship exists with the protolichesterinic acid and its related modifications. Dihydrochaulmoogric acid and tetradecyl and dodecyl malonic acids could not be dissolved in a sufficient concentration at *p*H 7 to obtain inhibitory levels.

(13) Stanley, Colemen, Greer, Sacks and Adams, *J. Pharmacol. and Exper. Therap.*, **48**, 121 (1932).

Experimental

Synthesis of γ-Lactone Aliphatic Acids.—The sodium salt of α-carbethoxy butyrolactone was prepared as described by Traube and Lehman.[12] To 18 g. (0.1 mole) of the sodium salt dissolved in 250 cc. of absolute ethanol was added 0.1 mole of the appropriate normal alkyl bromide and the mixture refluxed for four hours. The reaction mixture was poured into 500 cc. of water and extracted three times, each with 150 cc. of chloroform. The chloroform was distilled off and the residue saponified with a solution of 8.4 g. of potassium hydroxide in 150 cc. of ethanol. The reaction mixture was diluted with water and acidified with sulfuric acid. The precipitate was filtered off and crystallized several times from Skellysolve B. Yields varied from 20 to 45% depending upon the number of recrystallizations required in the purification.

The γ-methyl derivatives were prepared by using α-carbethoxy-γ-methylbutyrolactone made from propylene oxide and malonic ester.

Protolichesterinic Acid and Lichesterinic Acid.—The protolichesterinic acid was obtained from *Cetraria islandica* as the *l*-isomer by extraction of the dry material with methylene chloride, evaporation of the solvent and crystallization of the residue from benzene; $[\alpha]^{22}D$ (10 mg. per cc. in chloroform), −15°.

When protolichesterinic acid was heated at 100° for twenty hours in a toluene solution containing a few drops of triethylamine, the isomeric *l*-lichesterinic acid was obtained.

Dihydroprotolichesterinic Acid.—The *l*-protolichesterinic acid was hydrogenated with an Adams platinum catalyst by the usual procedures to yield the dihydro derivative.

Cysteine Derivative of Protolichesterinic Acid.—To 1.5 g. of *l*-protolichesterinic acid in 150 cc. of dilute sodium bicarbonate solution was added 1.5 g. of *l*-cysteine hydrochloride dissolved in 10 cc. of water. The *p*H of the reaction mixture was 7.0. After twenty hours at 25°, the solution was acidified strongly with hydrochloric acid and the precipitate which formed was filtered off and washed with water. The compound was crystallized as spherules

from 250 cc. of hot ethanol. The yield of pure product was 1 g., m. p. 185–188° dec.

Anal. Calcd. for $C_{22}H_{39}O_6NS$: N, 3.14. Found: N (Dumas), 2.84, basic N (perchloric acid titration in acetic acid), 2.76.

The product was soluble in sodium bicarbonate solutions and in hot dilute hydrochloric acid solution. Alkaline titration indicated the presence of one carboxyl and one lactone group. The reaction product obtained from cysteine and $\Delta^{\beta,\gamma}$-angelicalactone[11] when titrated with perchloric acid showed the absence of a basic amino group; protolichesterinic acid appears to have added cysteine through the —SH group without secondary involvement of the amino group.

Antibacterial Tests.—Tests against the *Streptococcus*, *Staphylococcus* and *Bacillus typhi* were conducted in tryptose phosphate medium. The inoculum was a 1:1000 dilution of a twenty-four-hour culture of the organism and incubation was for eighteen hours at 37°. The anaerobe was tested in Bacto-Anaerobe Medium with Dextrose. The acid-fast organisms, *B. tuberculosis ranae* and the human tuberculosis strain H37Rv were grown in submerged culture in Youmans' modification[14] of Proskauer–Beck synthetic medium. The inoculum with *ranae* was a 1:100 dilution of a forty-eight hour culture in Long's synthetic medium; incubation was for forty-eight hours at 37°. An inoculum of 0.02 mg. of fresh bacteria per cc. of test medium was used with H37Rv; incubation at 37° was for fourteen days, excellent growth being evidenced after eight days.

(14) Composition: Asparagin, 0.5%; primary potassium acid phosphate, 0.5%, potassium sulfate, 0.05%; magnesium citrate, 0.15%; and glycerol, 2% in distilled water.

Acknowledgment.—We are indebted to M. E. Auerbach and Staff for analytical data, to Dr. F. C. Nachod for surface-tension measurements, and John W. Klimek for antifungal tests.

Summary

The antibacterial activity of *l*-protolichesterinic acid is related to its effect on surface tension and not to any significant extent to the unsaturated system. A series of synthetic lactone aliphatic acids of the approximate molecular size of the lichesterinic acids also demonstrate antibacterial activity, particularly against acid-fast bacteria. The lactone aliphatic acids are more compatible with complex media than are the aliphatic monocarboxylic and malonic acids and are more soluble at neutrality.

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[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF PARKE, DAVIS AND CO.]

Substituted Chlorodiamino-*s*-triazines[1]

BY WILLIAM M. PEARLMAN AND C. K. BANKS

Previous studies have described a number of compounds of pharmacological interest containing the *s*-triazine nucleus.[2-5] Most of these compounds were 2-substituted 4,6-diamino-*s*-triazines. Since unsubstituted amino- and hydroxy-*s*-triazines are theoretically capable of existence in more than one tautomeric form (I–IV), substitution of hydrogen atoms by alkyl groups should alter or completely inhibit the equilibrium of certain of the tautomers and thereby alter the pharmacological properties of the resulting compounds.

As most of the previous compounds had been derived from 2-chloro-4,6-diamino-*s*-triazine, it was considered of interest to prepare representative chlorodiaminotriazines in which the hydrogen atoms of the amine groups had been substituted by alkyl or alkylene groups. Very few compounds of this type had been described, and several of these, on reexamination were found to be mixtures of related products. Cyanuric chloride (2,4,6-trichloro-*s*-triazine) was used as the starting material for the preparation of the desired substituted chlorodiaminotriazines. Fierz-David and Matter[6] had shown that the three halogens of cyanuric chloride were hydrolyzed stepwise depending upon the temperature of the hydrolytic mixture and Diels[7] had demonstrated the stepwise replacement by reaction with amines and ammonia.

In attempting the preparation of unsymmetrically substituted triazines it was found that while stepwise substitution could be achieved, the nature of the reacting group and the order of entry of the groups were highly important in the preparation of unique products. While reaction of ammonia with cyanuric chloride would replace only one chlorine below 0° and two below 100°, interaction with dimethylamine replaced all three halogens at 25°. The behavior of morpholine and piperidine also indicated that the basicity of the amine was not the only factor, since both were practically as reactive as dimethylamine and more reactive than the monoalkyl amines. It was found that only one halogen would be replaced by an amino group if a sufficiently low temperature was maintained during reaction. The second amino group was then introduced at a higher temperature. When mixed diamines were desired, it was found to be more practical to introduce the basic radical of the less reactive amine first, and then that of the more reactive amine. Organic solvents were used in the synthesis of monoaminodichlorotriazines but most of the diamines were prepared in aqueous suspensions. Alcohols, acids and other

(1) Presented before the Division of Medicinal Chemistry, Chicago, Ill., April 20, 1948.

(2) (a) Banks, THIS JOURNAL, **66**, 1127 (1944); (b) Banks, *et al.*, *ibid.*, **66**, 1771 (1944).

(3) Controulis and Banks, *ibid.*, **67**, 1946 (1945).

(4) Friedheim, *ibid.*, **66**, 1776 (1944).

(5) Witt and Hamilton, *ibid.*, **67**, 1078 (1945).

(6) Fierz-David and Matter, *J. Soc. Dyers Colourists*, **426** (1937).

(7) Diels, *Ber.*, **32**, 697 (1899).