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The Antibacterial Principle of *Arctium minus*. I. Isolation, Physical Properties and Antibacterial Action

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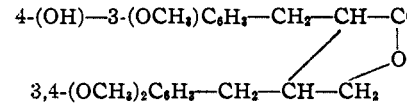
In the course of pursuing a program of investigations among the higher plants for antibacterial substances, it was observed that an aqueous infusion of leaves of the common burdock, *Arctium minus*, showed some antibacterial activity against Gram-positive bacteria when tested by the Oxford cup-plate method. The activity was of a relatively low order and corresponded to that observed by Osborn[1] with extracts of *Arctium lappa*. However it is presumptuous to eliminate a plant from interest merely because of low order antibacterial activity, inasmuch as crude infusions may contain only small quantities of active substances.

From aqueous infusions of ground burdock leaves there has been isolated a colorless crystalline substance, I, possessing some antibacterial activity against Gram-positive bacteria. It is found in relatively large quantities in burdock, almost exclusively in the green leaves. The product is relatively stable in dried leaves, and is present up to approximately 1.8% in small young leaves and as low as 0.3 to 0.5% in large older leaves (content on a dry leaf basis). The fresh leaf contains approximately 75% moisture. Compound I has a mildly bitter flavor resembling that of the fresh plant.

I crystallizes as large prisms from a chloroform–Skellysolve B solution. The compound undergoes decomposition when heated slowly, but within no well-defined temperature range. By placing fresh samples in a slowly rising temperature bath I was found to soften above 110° and melt sharply at 115–117°, then resolidify, the resulting product having no antibacterial activity. Compound I is optically active, $\alpha^{25}D$ in acetone is +120°, in ethanol is +100° and in 10% ethanol–90% water is +92°. It is readily soluble in methanol, ethanol, chloroform, ethyl acetate, dioxane and acetone, slightly to moderately soluble in ether, benzene and water (0.2% at 25°) and insoluble in the Skellysolves. Spectrographic examination in U.S.P. alcohol shows no selective absorption in the ultraviolet between λ 320 mμ and 240 mμ, showing only end absorption with ϵ = 1,680 at λ 270 mμ and 13,400 at λ 240 mμ.

Analyses show the presence in I of only carbon, hydrogen and oxygen in the ratio $(C_3H_4O)_x$. Cryoscopic molecular weight determinations have not been sufficiently reproducible to be satisfactory. I is not readily soluble in aqueous alkalies, but upon standing, slowly goes into solution. Addition of alkali to an alcohol solution of the substance results in a disappearance of titratable alkali. Extraction of a chloroform solution with aqueous sodium bicarbonate solution leaves the active principle in the chloroform. It thus appears that I has a potential, but not a free carboxyl group. On the basis of neutral equivalents obtained by potentiometric titration of the potential carboxyl group (end-point *p*H 8.0), *x* in the empirical formula is 5 and I becomes $C_{15}H_{20}O_5$.

Investigation of the scientific literature shows that there has been isolated from seeds of *Arctium lappa* a glycoside, arctiin,[2] the aglucone of which, arctigenin, has been shown by Omaki[3] to be



A related substance, matairesinol, isolated from the wood of the matai tree, *Podocarpus spicatus*,[4] has been shown[5] to be the 4,4′-dihydroxy-3,3′-dimethoxy compound. On the basis of the melting points, analytical data and optical activity, I cannot be either of these; furthermore, I gives no color change with ferric chloride and contains no methoxyl groups.

The two compounds described above are lactones, and I also behaves as some type of ester. Treatment with an excess of isopropylamine yields a compound $C_{18}H_{29}O_5N$, indicating that no loss of elements occurs in the reaction. The hydrolyzable group thus appears to be an inner ester or lactone type. The reaction product obtained with isopropylamine supports the conclusion that in the formula $(C_3H_4O)_x$ for I, *x* is five.

The results of serial dilution tests in which the minimum bacteriostatic and bactericidal concentrations were determined are shown in Table I.

In a concentration of 1.0 mg. per cc. I was ineffective against the following Gram-negative organisms: *Proteus vulgaris*, *B. morgani* I, *B. typhosus*, *B. paratyphosus* A and B, *B. enteriditis*, *B. typhi murium*, *B. coli*, *B. dysenteriae* Flexner and *B. dysenteriae* Sonne. It thus appears that I is active only against the Gram-positive bacteria. As a comparative substance, citrinin under similar test conditions shows a minimum bacteriostatic concentration of 0.50 mg. per cc. against *S. aureus*.

Biological tests[6] indicate that the intravenous

(1) Osborn, *Brit. J. Exptl. Path.*, **24**, 227 (1943).

(2) Shinoda and Kawagoye, *J. Pharm. Soc. Japan*, **49**, 565 (1929), *Chem. Abst.*, **23**, 4707 (1929).

(3) Omaki, *J. Pharm. Soc. Japan*, **55**, 159 (1935); **57**, 22 (1937).

(4) Easterfield and Bee, *J. Chem. Soc.*, **97**, 1028 (1910).

(5) Haworth and Richardson, *J. Chem. Soc.*, 635 (1935).

(6) Animal tests by Drs. L. C. Miller and T. J. Becker, Pharmacodynamics Laboratory, Research Department, Winthrop Chemical Company, Inc.

TABLE I

Organism	Minimum bacteriostatic concn., mg. per cc.	Minimum bactericidal concn., mg. per cc.
Staphylococcus albus	0.25	0.25
Staphylococcus aureus (209)	.25	1.0
Streptococcus hemolyticus (C203)	.075	>1.0
Streptococcus viridans	1.0	>1.0
Pneumococcus type I	0.075	0.075
Pneumococcus type III	.25	>1.0
Pneumococcus type XIX	.05	0.05
Bacillus subtilis	.10	>1.0
Bacillus botulinus A	.70	0.90
Bacillus tetani	.70	..
Bacillus oedematiens	.70	.70
Vibrion septique	.70	.90
Bacillus welchii	.50	..

LD_{50} of I in mice is approximately 90 mg. per kg. During eight days of daily oral administration mice showed no weight loss at 100 mg. per kg., slight loss at 250 mg. per kg. and loss at 500 mg. per kg. with no deaths. Subcutaneous administration of 50 or 250 mg. per kg. in mice infected with *Strep. hemolyticus* C203 did not protect the animals.

The uses of burdock in folk-medicine have been many[7]; however, there is practically no literature of a scientific nature dealing with the rationale for its application.

Experimental

Isolation of I.—The isolation was carried out with both fresh and desiccated burdock leaves. The original procedure used consisted of extraction of the powdered dry leaf with ethyl acetate, evaporation of the solvent and recrystallization of the residue from benzene. This procedure has been discontinued since the product thus obtained was difficult to purify and usually had a greenish color as a result of the presence of chlorophyll. The following procedure was found to be satisfactory and may be carried out using the indicated proportions with various size batches of material.

The center rib of fresh burdock leaves was cut out. The rib contained practically no product and its removal facilitated grinding. To 5.4 kg. of finely ground leaves was added 5 liters of water and the mixture was allowed to stand for an hour. If long standing became necessary, ice was added or the mixture was refrigerated. The mash was pressed or suction filtered and to the filtrate was added 20% aqueous lead acetate solution until no further precipitate formed. After being stirred for thirty minutes, the precipitate was filtered off. To the clear filtrate was added 5% aqueous sodium bicarbonate solution to precipitate any excess lead which was filtered off. At this point approximately 5.4 liters of solution was obtained which was extracted, by shaking or stirring vigorously, once with 600 cc. and three times with 400 cc. of ethyl acetate. The ethyl acetate extracts were combined and evaporated under reduced pressure. A sirupy residue remained which crystallized on standing. The yield on the basis of dry burdock leaves varied from 0.3 to 1.8%, depending upon the age and condition of the leaves.

The crude white crystalline mass (7 g. in this instance) was dissolved in 40 cc. of chloroform; 80 cc. of benzene was added at room temperature and the solution allowed to stand for several hours. Impurities settled out as a sticky precipitate and the clear supernatant solution was decanted. Skellysolve B was added to this solution until no further precipitation occurred and the mixture was allowed to stand overnight. A mass of large colorless prisms was formed, and this was dried *in vacuo* over phosphorus pentoxide and paraffin. If the product were not sufficiently pure, the procedure was repeated with a smaller initial volume of chloroform. The m. p. of 115–117° dec. was determined as previously mentioned. The $\alpha^{25}D$ values for I were found to be +100° in absolute ethanol (5 mg. per cc. concentration) and +120° in acetone (66 mg. per cc. concentration).

Anal. Calcd. for $(C_5H_4O)_x$: C, 64.29; H, 7.14. Found: C, 64.38; H, 7.06; Zeisel methoxyl, negative.

Alkaline Hydrolysis.—Three 50-mg. samples of I were dissolved each in 5 cc. of ethanol. To one was added 20 cc. of 0.02 *N* sodium hydroxide solution, to another, 50 cc. and to the third, 80 cc., then each made to 100 cc. volume with water. After standing overnight, the solutions were back titrated with 0.02 *N* hydrochloric acid solution to *p*H 8.0. The neutral equivalents observed were 277, 271 and 263, respectively. The neutral equivalent for $(C_5H_4O)_5$ would be 280. The alkali uptake from such solutions was rapid, usually going to completion in less than five minutes. When I was added in the solid state to dilute aqueous alkali, solution required an hour or longer.

The $\alpha^{25}D$ for I (5 mg. per cc. concentration) in 10% ethanol–90% water was +92°. When to 10 cc. of a 2% solution of this type was added 0.2 cc. of 50% aqueous sodium hydroxide solution, $\alpha^{25}D$ became +66° in two minutes and no further change occurred on standing.

Stability of I.—Aqueous solutions showed almost no loss in antibacterial activity in twenty days at 25°. Alkalies caused rapid loss of activity, dilute acids had no effect. Heating an aqueous solution for fifteen minutes at 100° or for twenty-four hours at 60° caused only slight loss of activity.

Vigorously bubbling air into a solution of I in an organic solvent (chloroform, methanol, dioxane) at room temperature led to a slow loss in activity, formation of less soluble products and a gain in weight of residue was observed after evaporation of the solvent. The change was more rapid at 60° and the products formed were independent of the nature of the solvent used. I appeared to react with oxygen of the air and with itself under these conditions.

Isopropylamine Derivative.—To 300 mg. of I was added 1 cc. of cold isopropylamine and the solution allowed to stand for one hour, then diluted with 20 cc. of water. A precipitate slowly formed as long prisms or needles, yield 301 mg. This was dissolved in a few cc. of ethanol and diluted with water, from which the product re-crystallized. After drying *in vacuo*, the m. p. was 174°. *Anal.* Calcd. for $C_{18}H_{29}O_5N$: C, 63.71; H, 8.62; N, 4.13. Found: C, 63.64; H, 8.60; N, 4.03. In absolute ethanol (7 mg. per cc. concentration) $\alpha^{25}D$ was +48°. This product was relatively insoluble in water or alkalies but was readily soluble in acids.

Iodine Number.—The iodine number of I was found to be 89 (Hanus method). This showed the presence of one unsaturated bond for a compound of molecular weight of 286; $C_{15}H_{20}O_5$ molecular weight is 280.

Antibacterial Tests.—The inhibiting action of I was tested by adding decreasing quantities of it to series of tubes containing such quantities of broth that, when each tube received 1 cc. of a 1:1000 dilution of a twenty-four-hour broth culture of the organism to be tested, the final volume in all tubes was 5 cc.

Streptococcus hemolyticus, Streptococcus viridans, Pneumococcus types I, II, and XIX were grown and tested in dextrose veal infusion broth to which had been added sterile horse serum to give a 5% concentration. All other organisms were grown in the usual nutrient broth (peptone 1%, meat extract 0.3%, NaCl 0.5%).

The test for bactericidal action was performed by inoculating 5 cc. of fresh culture medium with 0.1 cc. of medium from tubes showing no growth after twenty hours of incubation. In the bacteriostatic tests, the concentration showing complete absence of growth after twenty hours was taken as the inhibiting level.

(7) Bascompte, *Rev. Española De Med. y Cir.*, **10**, 210 (1927).

Tests with the anaerobes were conducted in Bacto-Anaerobe Medium with Dextrose. Cotton-stoppered tubes were filled with 9 cc. of the medium and placed in a boiling water-bath for twenty minutes and allowed to cool without agitation. To a series of 8 tubes the antibacterial solution was added in amounts to give 0.9, 0.7, 0.5, 0.25, 0.09, 0.07, 0.05 and 0.025 mg. per cc. of medium when all tubes received 1 cc. of a 1:1000 dilution of the organism to be tested in the anaerobe medium.

Bactericidal action was determined by removing 0.2 cc. of the medium from all tubes showing no growth after twenty hours of incubation and adding this to tubes containing 10 cc. of freshly heated and cooled Anaerobe Medium.

Discussion

The newly isolated compound from *Arctium minus* has a relatively low order of antibacterial activity. It is of interest, however, because of its presence in such relatively large quantities in burdock leaves and also from the standpoint of discovering new antibiotic types, a study of which may give evidence of their mode of action. The antibacterial activity is destroyed by treatment with cysteine or N-acetylcysteine but not with S-methylcysteine,[8] and in this respect resembles the behavior of certain other antibacterial agents.[8,9] It is also of interest to observe the frequency of unsaturated lactone structures among antibiotics, notably in anemonin,[10] patulin,[11] penicillic acid,[12] and now apparently in I.

Summary

A new antibacterial agent has been isolated from the leaves of *Arctium minus*. It has a relatively low order of activity against Gram-positive bacteria and is inactive against the Gram-negative group. The compound appears to be a lactone of empirical formula $C_{15}H_{20}O_5$.

(8) Cavallito and Bailey, *Science*, **100**, 390 (1944).

(9) Cavallito and Bailey, THIS JOURNAL, **66**, 1950 (1944); Cavallito, Buck and Suter, *ibid.*, **66**, 1952 (1944).

(10) Asahina and Fujita, *Acta Phytochim.* (Japan), **1**, 1 (1922).

(11) Raistrick, *et al.*, *Lancet*, **245** (2), 625 (1943).

(12) Birkinshaw, Oxford and Raistrick, *Biochem. J.*, **30**, 394 (1936).

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The Reaction of Formaldehyde with Proteins

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The reaction of formaldehyde with proteins is of importance in a number of fields, some of which are the tanning of leather, the hardening of tissues, fibers and plastics, and the preparation of toxoids. Much of the extensive earlier literature on the mode of interaction is cited in recent publications.[2-11]

The confusion existing in this field is emphasized by the fact that, in the four most recent papers, the binding of aldehyde by proteins in acid or neutral solution is attributed to the following groups: (1) all basic and phenolic and aliphatic hydroxyl[6b]; (2) imidazole, possibly amide and peptide, but not primary amino[9]; (3) primary amino and peptide[10]; and (4) primary amide and basic groups.[11] In addition, formaldehyde has been shown to react under certain conditions with the thiol,[12] indole,[13] guanidyl,[3] and disulfide groups.[5]

The present investigation was undertaken when it was observed that gliadin and wheat gluten bound more aldehyde than did other proteins, after treatment with 4% formaldehyde solution at 70° and at *p*H 3 to 7.[14] It was possible to demonstrate that the primary amide as well as the amino groups of proteins bind aldehyde under these conditions. On the other hand, the secondary amides of the peptide chain were found not to react appreciably with formaldehyde. These conclusions were derived from experiments with a series of proteins and protein derivatives, and with synthetic polypeptides and simple model substances.

Experimental

Analytical Methods.—Combined formaldehyde was determined by a method involving hydrolysis and distillation,[15] followed by precipitation of the aldehyde in the distillate with dimethyldihydroresorcinol (dimedon).[16] 100 or 200 mg. of the washed and dried protein derivative and 50 ml. of *N* sulfuric acid were placed in a 1000-ml. round-bottom flask, which was connected to a condenser fitted with a trap and an outlet tube dipping into a mixture of 50 ml. of 0.214% dimedon solution and 75 ml. of *p*H 4.6 acetate buffer (1 part *N* hydrochloric acid and 2 parts *N* sodium acetate). Distillation was continued until

(1) Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture. Article not copyrighted.

(2) Theis and Ottens, *J. Am. Leather Chem. Assoc.*, **35**, 330 (1940).

(3) Highberger and Salcedo, *ibid.*, **35**, 11 (1940); **36**, 271 (1941).

(4) Brother and McKinney, *Ind. Eng. Chem.*, **30**, 1236 (1938).

(5) Middlebrook and Phillips, *Biochem. J.*, **36**, 294 (1942).

(6) (a) Carpenter and Lovelace, *Ind. Eng. Chem.* **34**, 759 (1942); (b) **36**, 680 (1944).

(7) Hegman, *J. Am. Leather Chem. Assoc.*, **37**, 276 (1942).

(8) Gustavson, *Kolloid Z.*, **103**, 43 (1943).

(9) (a) Theis, *J. Biol. Chem.*, **154**, 87 (1944); (b) Theis and Lams, *ibid.*, **154**, 99 (1944).

(10) Nitschmann and Hadorn, *Helv. Chim. Acta*, **27**, 299 (1944).

(11) Wormell and Kaye, *Nature*, **153**, 525 (1944).

(12) Ratner and Clarke, THIS JOURNAL, **59**, 200 (1937).

(13) Ross and Stanley, *J. Gen. Physiol.*, **22**, 165 (1938).

(14) The use of elevated temperature for the protein–formaldehyde reaction has been suggested by Ferretti (French Patent 853.123; March 11, 1940), Middlebrook and Phillips,[5] and others.

(15) Highberger and Retzsch, *J. Am. Leather Chem. Assoc.*, **33**, 341 (1939). The modification proposed by Nitschmann (*Helv. Chim. Acta*, **24**, 237 (1941)) was not found suitable for maximal recovery of the aldehyde bound by the casein at 70°, in agreement with a later paper from the same laboratory (Nitschmann, Hadorn and Lauener, *Helv. Chim. Acta*, **26**, 1069 (1943)).

(16) Yoe and Reid, *Ind. Eng. Chem., Anal. Ed.*, **13**, 238 (1941).