[CONTRIBUTION FROM THE LABORATORY OF CHEMISTRY OF NATURAL PRODUCTS, NATIONAL HEART INSTITUTE, NATIONAL INSTITUTES OF HEALTH]

Alkaloids of the Amaryllidaceae. IX. On the Structure of Galanthine[1]

BY H. M. FALES AND W. C. WILDMAN

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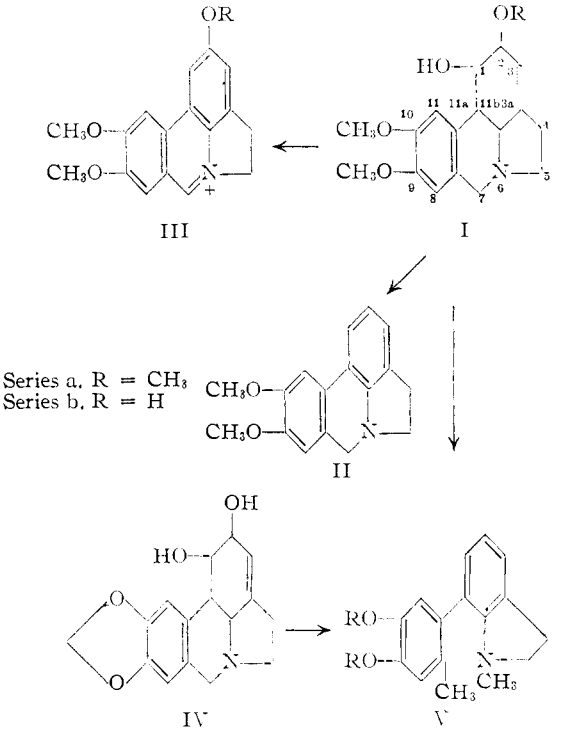
The ring system and functional groups of the alkaloid galanthine have been determined. It has been shown that the aliphatic methoxyl group of galanthine is located in the 2-position. It is suggested that the alkaloid possesses the structure Ia.

Galanthine was isolated by Proskurnina and Areshkina from the Caucasian snowdrop (*Galanthus woronowii* Losinsk.) in 1947.[2] Characterized by its analysis and the analysis of the chloride, bromide and perchlorate salts, the base was assigned the molecular formula $C_{16}H_{23}NO_4$. Galanthine was reported to contain three methoxyl groups and one hydroxyl group, although no proof of the latter was offered. More recently galanthine has been isolated from several of the garden varieties of *Narcissus pseudonarcissus* L.[1,3] These isolation and characterization studies confirmed the revised formula $C_{18}H_{23}NO_4$ for galanthine which had been presented by Proskurnina.[4] While galanthine (like lycorine) appears to undergo the Hofmann and Emde degradations with the formation of a second aromatic ring,[2,4] no chemical proof of the double bond or a hydroxyl group has been given although such groups usually are present in ring C if aromatization occurs. In this paper we wish to prove the presence of these groups. From oxidative degradations we have been able to assign a position to the aliphatic methoxyl group and derive a tentative structural formula (Ia) for the alkaloid.

The presence of both a hydroxyl group and a double bond in galanthine was shown by the formation of a dihydromonoacetylgalanthine when galanthine was hydrogenated in glacial acetic acid and perchloric acid using platinum oxide catalyst. In the absence of perchloric acid, dihydrogalanthine was formed. With this information, the functional groups of galanthine suggested by Proskurnina have been demonstrated experimentally.

Formula Ia with the positions of the double bond, hydroxyl and aliphatic methoxyl unassigned was suggested for galanthine by Proskurnina.[4] The parent ring system, as well as the location of the two aromatic methoxyl groups, was indicated in two ways. Emde degradation of lycorine (IV) and subsequent ether cleavage gave a base (Vb) which was identical with the material derived from galanthine in a similar manner.[4] Furthermore, it seems likely that the Emde base Va from galanthine is identical with the product from methylation of Vb derived from the Emde degradation of lycorine.[5] An alternative proof of the ring system and the position of the aromatic methoxyl groups has been found in the pyrolysis of galanthine. When galanthine was heated above its melting point *in vacuo*, aromatization of ring C occurred to form II. The product was identical with that obtained by a similar treatment of methylpseudolycorine (Ib).[1]



When an ethanolic solution of galanthine was oxidized with selenium dioxide, a quaternary base was obtained. Characterized as its nitrate and perchlorate salts, the alkaloidal moiety possessed the molecular formula $C_{18}H_{18}NO_3^+$ and contained three methoxyl groups. In contrast to the oxidation products of lycorine[6] and methylpseudolycorine,[1] no free betaine was obtained upon gradual basification of a solution of the nitrate salt. Instead, the nitrate of IIIa was precipitated from solution, presumably by a salting-out process. In acid solution the ultraviolet absorption spectrum of IIIa was nearly identical with that of IIIb.[1] In basic solution IIIb showed a drastic change in the positions of its maxima due to betaine formation. As expected, the maxima of the methyl ether IIIa did not change in wave length in basic solution. A slight diminution of extinction coefficients was observed. The ultraviolet spectra of synthetic

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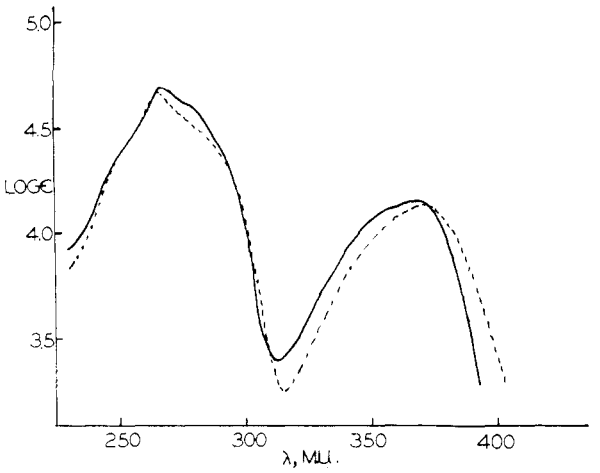

Fig. 1.—Ultraviolet absorption spectra in 0.01 *N* ethanolic hydrochloric acid of: IIIa, —; IIIb, -----.

4,5-dihydro-9,10-dimethoxy-6-(β-hydroxyethyl)-1-hydroxypyrrolo[*de*]phenanthridine[7] in acid and base were quite different from the corresponding spectra of IIIa and IIIb. Thus, the possibility that IIIa might be a 1-oxy derivative was eliminated. From the structure of IIIa, the aliphatic methoxyl group of galanthine must occupy the 2-position in ring C.

Galanthine perchlorate[1] exhibited no strong absorption in the 6 μ region attributable to an enamine salt.[8] The ultraviolet spectra of galanthine and its perchlorate were nearly identical with those of dihydrogalanthine and O-acetyldihydrogalanthine perchlorate, respectively. Since the alkaloid has neither the properties of an enol nor those of an enol ether, the double bond must be situated in a position that is neither α,β to the nitrogen atom, conjugated with the aromatic ring, nor contiguous with the hydroxyl or aliphatic methoxyl group. Since the aliphatic methoxyl group has been assigned the 2-position, the double bond must be located in either the 3–3a or 3a–4 position. The latter is less likely by analogy with other known alkaloids of this family.[9-11] Some spectral evidence for a 3–3a double bond has been found. In carbon tetrachloride solution, galanthine shows a weak absorption band at 3.31 μ which we attribute to =CHR hydrogen stretching when the double bond is part of a six-membered ring.[12-14] This band disappears in dihydrogalanthine.

If the double bond is assigned the 3–3a position, the hydroxyl group must be in position 1 or 11b. The latter is unlikely since no hydrogenolysis occurred in the preparation of dihydrogalanthine. The ease of formation of O-acetyldihydrogalanthine and the stability of galanthine in acid also favor the location of the hydroxyl in position 1.

(7) Prepared from 6-bromoveratric acid by the procedure outlined in reference 6 for 1-oxyphenanthridinium salts.

(8) N. J. Leonard and V. W. Gash, THIS JOURNAL, **76**, 2781 (1954).

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Experimental[15]

Galanthine.—The pure base was obtained by several recrystallizations from water of the crude product isolated from *Narcissus pseudonarcissus* L.[1]; m.p. 134–136°, recrystallized on the hot-stage and remelted at 166–167°; $[\alpha]^{27}D$ −81.6° (*c* 0.21, ethanol).

Dihydrogalanthine.—A solution of 0.738 g. of galanthine in 15 ml. of acetic acid over 0.150 g. of pre-reduced platinum oxide absorbed slightly more than one equivalent of hydrogen at atmospheric pressure. The catalyst was removed by filtration, and the solvents were evaporated in a current of nitrogen. Sodium hydroxide solution was added, and the precipitate which formed was collected and washed with cold water. The product, 0.669 g., m.p. <60°, appeared to be a hydrate. White prisms formed when the compound was recrystallized from benzene–cyclohexane, m.p. 144–147°. A sample was sublimed at 150° (5 μ) and recrystallized from ethyl acetate, m.p. 146–148°, $[\alpha]^{25}D$ −57°, $[\alpha]^{25}_{436}$ −140° (*c* 0.04, ethanol).

Anal. Calcd. for $C_{18}H_{25}NO_4$: C, 67.69; H, 7.89; N, 4.39. Found: C, 67.79; H, 7.81; N, 4.38.

The ultraviolet absorption spectrum (ethanol) showed a maximum at 283 mμ (log ε 3.53).

Dihydrogalanthine Picrate.—The free base was treated with picric acid in ethanol. The resulting precipitate was recrystallized from ethanol, m.p. 182–190° dec.

Anal. Calcd. for $C_{18}H_{25}NO_4 \cdot C_6H_3N_3O_7$: C, 52.55; H, 5.15; N, 10.22. Found: C, 52.74; H, 5.23; N, 10.29.

O-Acetyldihydrogalanthine Perchlorate.—This product resulted when a reduction mixture prepared as above from 0.229 g. of galanthine was treated with 5 drops of 70% perchloric acid during the reduction. The mixture was filtered, concentrated and triturated with water. The perchlorate crystallized and was recrystallized from water, 0.192 g., m.p. 250–269° dec.

Anal. Calcd. for $C_{20}H_{27}NO_5 \cdot HClO_4 \cdot H_2O$: C, 50.06; H, 6.30; 1 CH_3CO, 8.97. Found: C, 49.98; H, 6.31; CH_3CO, 9.31.

The ultraviolet absorption spectrum showed maxima at 236 mμ (log ε 3.90) and 284 mμ (log ε 3.57).

Action of Selenium Dioxide on Galanthine.—A solution of 0.080 g. of galanthine in ethanol was treated with 0.075 g. of selenium dioxide and heated under reflux for two hours. The precipitated selenium metal was removed by centrifugation, and 2 ml. of 10% hydrochloric acid was added. The solution was evaporated in a current of air, and water was added to the residue. A precipitate of pale yellow prisms formed when the solution was carefully made alkaline. The product, m.p. 234–240° dec., undoubtedly was the chloride of IIIa, but attempted recrystallizations were attended by much loss. Therefore, aliquots of the solutions were treated with dilute nitric acid and dilute perchloric acid to precipitate the more insoluble nitrate and perchlorate, respectively. **The nitrate** was recrystallized from water as fine needles, m.p. 240–255° dec., no gas evolution.

Anal. Calcd. for $C_{18}H_{18}NO_3 \cdot NO_3$: C, 60.33; H, 5.06; N, 7.82; 3 OCH_3, 25.98. Found: C, 60.13; H, 5.21; N, 7.83; OCH_3, 25.60.

The perchlorate of IIIa was recrystallized from dimethylformamide and digested with ethanol: short, light tan, strongly birefringent prisms, m.p. 300–302° dec., no gas evolution.

(15) All melting points are corrected and were observed on a Kofler microscope hot-stage equipped with polarizer. Analyses were performed by Dr. W. C. Alford and staff, National Institute of Arthritis and Metabolic Diseases, Bethesda, Md.; the Clark Microanalytical Laboratory, Urbana, Ill.; Dr. W. Manser, Zurich, Switzerland; and Mr. J. F. Alicino, Metuchen, N. J. Ultraviolet absorption spectra were recorded with a Cary model 11 MS spectrophotometer, and infrared absorption spectra were recorded with a Perkin-Elmer model 21 spectrophotometer equipped with sodium chloride optics. High resolution spectra in the 3 μ region were recorded in carbon tetrachloride solution with a Beckman model IR-3 spectrophotometer equipped with sodium chloride optics. The spectral work was performed by Mr. H. F. Byers and Miss Catherine Monaghan.

Anal. Calcd. for $C_{18}H_{18}NO_3 \cdot ClO_4$: C, 54.61; H, 4.58; Cl, 8.96; 3 OCH_3, 23.52. Found: C, 54.59; H, 4.60; Cl, 8.77; OCH_3, 23.49.

Action of Heat on Galanthine.—A sublimation tube was packed with 12 mg. of galanthine and heated to 280° (3 mm.). A fluorescent oil sublimed along with much unchanged galanthine. The total solid was dissolved in benzene, and the solution was chromatographed on a short column of aluminum oxide. A fluorescent band was eluted easily with benzene and 50% benzene-ethyl acetate. The infrared spectrum of the crude product (2–3 mg.) was nearly identical with that of synthetic II. It exhibited the same action during melting point determination as authentic II[1] and, therefore, was converted to the corresponding 7-phenanthridone by air at 150° and sublimed at 200° (0.25 mm.). The m.p. of the crude product, 265–270° (reported[1] 272–274°), was not depressed by admixture with authentic phenanthridone.[1] The infrared spectrum (KBr) also was identical with that of authentic phenanthridone.[1]

BETHESDA 14, MARYLAND

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Formation of Copper Complexes During Tyrosinase-catalyzed Oxidations

BY JETT C. ARTHUR, JR., AND T. A. MCLEMORE

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The formation of Cu complexes with products of catechol, hydroquinone, chlorogenic and caffeic acids, during tyrosinase-catalyzed oxidation, was determined, using radioactive Cu^{64} as a tracer and an ion-exchange method. The most stable Cu complexes formed were with *o*-quinone, with their stability decreasing with the introduction of groups on the initial aromatic ring of the compounds investigated. The maximum amount of Cu was complexed with *o*-quinone at the time of its formation. If the polymerization of the *o*-quinone was allowed to proceed prior to the addition of Cu^{++}, a smaller amount of metal was bound.

Introduction

An investigation of the formation of Cu complexes with products of tyrosinase-catalyzed reactions could indicate some differences in the mechanism of oxidation of different substrates, particularly *o*-dihydric and *p*-dihydric phenolic donors. The determination of the formation of Cu complexes using ion-exchange methods,[1,2] radioactive Cu^{64} and catalytic quantities of tyrosinase oxidizing the substrates would offer a new approach to this problem. It is the purpose of this report to present experimental data which indicate formation of Cu complexes with *o*-quinones.

Experimental

The activity of the tyrosinase was measured manometrically (Warburg apparatus) at 25° by determining the rate of oxygen uptake. The flasks, about 17–18 ml., contained two side arms and a center well. The enzyme and $Cu^{64}SO_4$ solutions were usually put in separate side arms. The center well contained a filter paper wick wetted with 0.2 ml. of 10% KOH to absorb CO_2 which might be released. Phosphate buffer (0.3 ml. of 0.5 *M*) and substrate solution (optimum amount) previously determined were placed in the main reservoir of the flask, sufficient Cu-free distilled water being added to bring the total liquid volume to 3 ml.

During the measurements of the rate of oxidation of different substrates by tyrosinase, $Cu^{64}SO_4$ solution was added. After a predetermined time, cation exchange material, 0.2 g. of phosphorylated cotton fabric,[3] was added to remove the copper ions from the solution. The Cu^{64} complexed was determined by drying an aliquot of the solution (which had been extracted with cation-exchange material by shaking the material with the solution for 1 hr.) and measuring its radioactivity in a gas flow counter. The use of this method to determine formation of metal complexes has been outlined by Schubert and Richter.[1,2]

The mushroom tyrosinase used was a commercial preparation. About 0.1 ml. of the preparation as received, about 0.5 mg. of enzyme preparation, was diluted with Cu-free water to 25 ml. One-ml. portions of this diluted preparation, about 20 μg. of enzyme, were used per test.

The Cu^{64} (half-life 12.88 hr.; β-0.57, 0.65; γ-1.34 mev.) was obtained as Cu wire, initial specific activity about 300 mc./g. About 0.64 g. of Cu was dissolved in 5 ml. of concentrated HNO_3, then 3 ml. of concentrated H_2SO_4 were added and the mixture was heated until SO_3 was evolved. The acid $Cu^{64}SO_4$ solution was diluted with Cu-free distilled water to yield a stock solution containing 3–4 μg. of Cu per ml. The other chemicals used were C.P. or reagent grade.

Results

The effect of substrate on tyrosinase oxidation at *p*H 6.7 and 25° with $Cu^{64}SO_4$ equivalent to 3.84 μg. of Cu^{64} added at zero time is shown in Fig. 1. The tyrosinase preparation has *o*-dihydric phenolase activity; however, hydroquinone can be oxidized by the enzyme on addition of a trace of catechol as a mediator. Using about 20 μg. of enzyme per test, the data indicate that initially the mechanism of oxidation of catechol and hydroquinone containing a trace of catechol approximates zero order.

Oxidation was also determined at *p*H 5.6 and 7.8 at 25°. Very little deviation from the data shown in Fig. 1 for *p*H 6.7 was noted.

The effect of *p*H and substrate on the amount of Cu ion complexed is shown in Table I. $Cu^{64}SO_4$, equivalent to 3.84 μg. of Cu, was added to the enzyme–substrate solution at zero time. After 60 minutes, cation-exchange cotton fabric was added. An aliquot of the extracted solution was analyzed for radioactive Cu^{64}, and, after appropriate corrections for decay time, the μg. of Cu associated with

TABLE I

EFFECT OF *p*H AND SUBSTRATE ON Cu^{++} COMPLEXED[a]

Substrate	Cu^{++} complexed, μg. *p*H 5.6	*p*H 6.7	*p*H 7.8
Hydroquinone + trace catechol	0.04	0.19	0.50
Catechol	.77	1.01	.90
Chlorogenic acid	.22	0.08	.38
Caffeic acid	.40	.23	.23
Protocatechuic acid	.00	..	.00
Hydroquinone	.06	.00	.00

[a] Age of diluted enzyme, 24 hr., estimated Cu content of tyrosinase used per test, 0.04 μg.; values reported are in excess of controls.

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