SOME NEW ALKALOIDS OF LYCOPODIUM ANNOTINUM L.: ISOLATION AND STRUCTURAL STUDIES.

BY

NURUL HOQUE KHAN, M.Sc. (Dacca)

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Department of Chemistry,
Faculty of Pure and Applied Science,
University of Ottawa,
Ottawa, Canada.

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Candidate

Supervisor

N.H. Khan

Prof. F.A.L. Anet D.Phil. (Oxon.)

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

2. PREFACE

The present work is a continuation of that of Mr. C.R. Eves on the alkaloids of Lycopodium annotinum L. During the preliminary investigations, Mr. Eves noted the presence of many bases in small amounts in the crude alkaloidal extract. He isolated several known alkaloids and a new one, lycodine. The present study was designed to isolate some of the other alkaloids detected by Mr. Eves, to characterize these alkaloids and to determine as much as possible about their structures.

My first and foremost sincere thanks are due to Dr. F.A.L. Anet for the stimulating guidance of this research, for discussions of the problems at every stage of this work and for measuring the nuclear magnetic resonance spectra.

I wish to thank Dr. L. Marion of the National Research Council, Ottawa, for the donation of the crude alkaloid mixture used in this study.

Finally, I wish to thank the Colombo Plan authorities for the award of a scholarship and the Pakistan Council of Scientific and Industrial Research for financial support, which enabled me to participate in this work.

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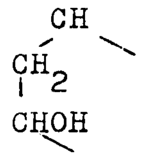
6. ABSTRACT

The present work is a continuation of that of Mr. C.R. Eves on the alkaloids of *Lycopodium annotinum* L., and consists of the isolation, characterization and degradation of some new alkaloids.

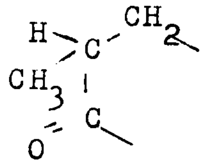
Five new alkaloids were isolated from *Lycopodium annotinum* L. Of these five bases, four were well characterized and were named annofoline, lycofoline, α-lofoline and β-lofoline. The fifth alkaloid, m.p. 117-118°, probably had the formula $C_{17}H_{25}O_3N$ but it was not as well characterized as the other four alkaloids. It contained a hydroxyl group, a C-methyl group and a lactone group.

Annofoline, $C_{16}H_{25}O_2N$, contained a carbonyl group, a hydroxyl group, a C-methyl group and a tertiary nitrogen atom. There was evidence that the hydroxyl and the ketone groups were involved in the formation of a hemiketal in solutions of annofoline. Annofoline formed a perchlorate, $C_{16}H_{25}O_2N.HClO_4$., a methiodide, $C_{16}H_{25}O_2N.CH_3I$; and an O-acetyl derivative which was isolated as the hydrobromide, $C_{18}H_{27}O_3N.HBr$. Annofoline did not form a benzylidene derivative and it was found resistant to chromic acid oxidation. Sodium borohydride reduction of annofoline gave two epimeric alcohols, α-dihydroannofoline, $C_{16}H_{27}O_2N$, and β-dihydroannofoline, $C_{16}H_{27}O_2N$. Annofoline formed a thio-enol ether, $C_{18}H_{29}ONS$, with ethane thiol. Wolf-Kishner reduction of anno-

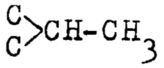
foline gave dihydrodeoxyannofoline, $C_{16}H_{27}ON$. Dihydrodeoxyannofoline formed an O-acetyl derivative which was characterized as the hydrobromide $C_{18}H_{29}O_2N.HBr$. Dihydrodeoxyannofoline on chromic acid oxidation gave a ketone, deoxyannofoline, $C_{16}H_{25}ON$. Preliminary experiments showed that deoxyannofoline can be oxidized with selenium dioxide to a compound which had the properties of an enolic α-diketone. This indicated the probable presence of the group $>CH-CH_2-CO-$ in deoxyannofoline. Selenium dioxide oxidation of annofoline gave an α,β-unsaturated ketone, dehydroannofoline, $C_{16}H_{23}O_2N$. On the basis of the degradative studies and the results of the N.M.R., infrared and ultraviolet spectra, the following partial structure was assigned to annofoline:



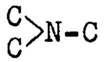
six or larger membered ring



six or larger membered ring

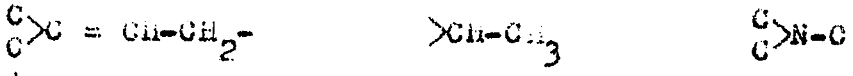


$${}^{C}_{C}{>}CH-CH_3$$

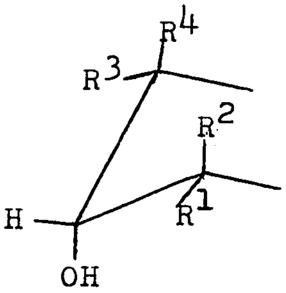


$${}^{C}_{C}{>}N-C$$

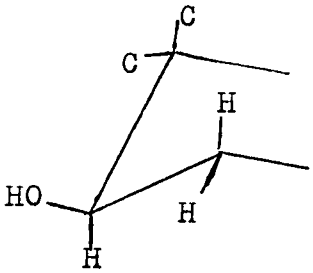
Lycofoline, $C_{16}H_{25}O_2N$, contained two hydroxyl groups, a C-methyl group, a double bond and a tertiary nitrogen. It gave a hydrobromide, $C_{16}H_{25}O_2N.HBr$. and a methiodide, $C_{16}H_{25}O_2N.CH_3I$. Lycofoline formed a mono-O-acetyl derivative, $C_{18}H_{27}O_3N$ and a di-O-acetyl derivative, $C_{20}H_{29}O_4N$. Catalytic hydrogenation of lycofoline gave dihydrolycofoline $C_{16}H_{27}O_2N$. Preliminary experiments indicated that one of the hydroxyl groups of lycofoline can be oxidized to a carbonyl group. The following partial formula was assigned to lycofoline on the basis of the chemical work and the results of the N.M.R. and infrared spectra.



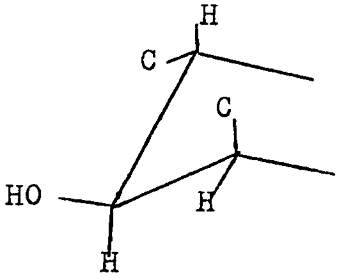
$$\overset{C}{\underset{C}{}}\!\!>\!C = CH{-}CH_2{-} \qquad >CH{-}CH_3 \qquad \overset{C}{\underset{C}{}}\!\!>\!N{-}C$$



where more than two R's are hydrogen atoms



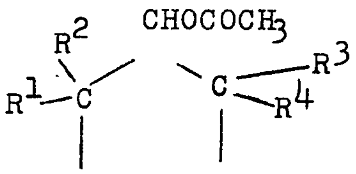
or



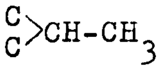
α-Lofoline, $C_{16}H_{29}O_3N$, contained a hydroxyl group, an O-acetyl group, a C-methyl group and a tertiary nitrogen. It formed a methiodide, $C_{16}H_{29}O_3N.CH_3I$.

β-Lofoline, $C_{16}H_{29}O_3N$, contained a hydroxyl group, an O-acetyl group, a C-methyl group and a tertiary nitrogen. It formed a methiodide, $C_{16}H_{29}O_3N.CH_3I$.

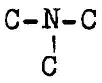
α-Lofoline and β-lofoline on oxidation with chromic acid gave the same product, dehydrolofoline, $C_{16}H_{27}O_3N$, indicating that the two compounds were epimeric alcohols. On the basis of the chemical studies and the results of N.M.R. and infrared spectra the following partial formula was assigned to them



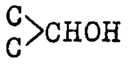
where probably more than two R's are hydrogen atoms.



$\begin{matrix}C\\C\end{matrix}\!>\!CH-CH_3$



$\begin{matrix}C-N-C\\|\\C\end{matrix}$



$\begin{matrix}C\\C\end{matrix}\!>\!CHOH$

Paper chromatography was used extensively during the isolation and degradative work on the alkaloids.

7. INTRODUCTION

(a) Purpose of the work

Alkaloids are nitrogenous bases (usually heterocyclic) widely distributed in plants. Many of these substances have marked physiological effects, a fact discovered by ancient people in many parts of the world, long before organic chemistry developed. To-day, alkaloids with a great variety of structural features are known (1,2).

The presence of alkaloids in Lycopodium plants was detected in the latter part of the nineteenth century (3). Since then a large number of alkaloids has been isolated from various Lycopodium species. These alkaloids were mainly isolated by the tedious and difficultly reproducible methods of fractional crystallization of the bases or of their salts. Eves (4) found countercurrent distribution to be very efficient in isolating lycodine, an alkaloid of Lycopodium annotinum L. which is present in only very small concentration in the crude alkaloid mixture. His paper chromatographic results on the crude alkaloid mixture indicated the presence of many other alkaloids, some in much larger amounts than lycodine. The purpose of the present work was to try to isolate these alkaloids, to characterize them and to compare them with those isolated from this plant by Manske and Marion (5,6), Bertho and Stoll (7) and Achmatowicz and Rodewald (8,9). Although more than thirty alkaloids have been isolated from the different species of *Lycopodium* very little attention was paid to the structural studies of these alkaloids. In fact, annotinine, the major alkaloid of *Lycopodium annotinum* L., is the only *Lycopodium* alkaloid whose structure is known (10,11) and is of a quite novel type. So, it was thought to be of interest, to study the chemical structure of some of the alkaloids of this plant.

(b) Review of pertinent literature

1) General botanical description

Lycopodium annotinum L. belongs to the genus *Lycopodium*. It is one of about one hundred species of this genus (12). They are also known as "Club Mosses", "Ground Pines" or "Christmass Greens". They have creeping under-ground stems.

The genus *Lycopodium* belongs to the group Pteridophyla which also includes the ferns and their allies. The algae and fungi belongs to the group Thallophytes and the higher plants are the members of the group Spermatophytes. Therefore, Pteridophyla occupy a middle position between Thallophytes and Spermatophytes. That they occupy a middle position in the evolution of plant life is shown by their appearance as fossils (13, 14)

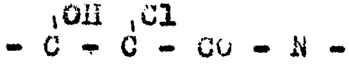
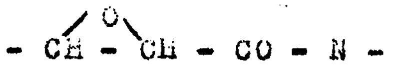
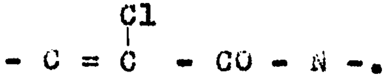
2) Chemistry of *Lycopodium* alkaloids

The presence of alkaloid in *Lycopodium* species was first recorded by Bödekar (3). In 1938, Achmatowicz and

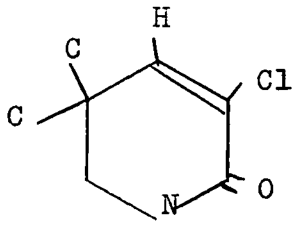
Uzieblo (15) isolated three alkaloids from Lycopodium clavatum C. Following these, Manske and Marion published a series of papers (5,6,16,17,18,19,20,21) reporting the isolation of more than thirty alkaloids from eight different species of Lycopodium. To some of these alkaloids they gave trivial names and the others, less well characterized, were designated by putting a number after the letter L. Recently, Anet and Eves (22) isolated a new alkaloid from Lycopodium annotinum L. Of these alkaloids, the complete structure of only annotinine and the partial structures of lycopodine, acrifoline, α and β obscurine and lycodine are known. Eves (4) made a detail review of the chemistry of Lycopodium alkaloids. His review included the methods of isolation, relative abundance of the alkaloids in the different species and all the reactions carried out so far on the various alkaloids. In the following review, which was written independently from that of Eves, the chemistry of a) annotinine, b) lycopodine, c) acrifoline and d) lycodine are discussed briefly from the structural point of view. The method used by Eves to isolate lycodine will also be presented.

(a) Annotinine, $C_{16}H_{21}O_3N$, the principal alkaloid of Lycopodium annotinum L., was isolated by Manske and Marion (5). It was shown to contain a tertiary nitrogen and a lactone ring. The infrared spectrum of annotinine showed a band at 1762 cm^{-1} indicating the presence of γ-lactone.

Manske and Marion (6) converted annotinine to an amide (I), $C_{16}H_{19}O_{4}N$, [Infrared Spectrum, 1640 cm^{-1}.] by oxidation with potassium permanganate. So annotinine must contain the group $-CH_2 - N -$.

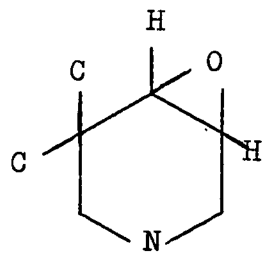
MacLean and Prime (23) converted the amide (I) to a chlorohydrin (II) $C_{16}H_{20}O_{4}NCl$, by refluxing it with concentrated hydrochloric acid. Compound (II) was reconverted to (I) by refluxing with sodium bicarbonate, it was dehydrated with concentrated hydrochloric acid or phosphorous oxychloride to an anhydro compound (III), $C_{16}H_{18}O_{3}NCl$. The compound (II) on hydrogeration in presence of Adams' catalyst produced two compounds, (IV), $C_{16}H_{21}O_{3}N$ and (V), $C_{16}H_{21}O_{4}N$. Compound (IV) was produced by the hydrogenolysis of the chlorine atom and hydroxyl group while compound (V) was formed by the hydrogenolysis of only the chlorine atom. Annotinine chlorohydrin, $C_{16}H_{22}O_{3}NCl$, produced by treatment of annotinine with concentrated hydrochloric acid, did not undergo any reaction upon similar treatment with sodium bicarbonate, concentrated hydrochloric acid, phosphorous oxychloride or hydrogen in presence of Adams' catalyst as did compound (II). The only structural difference between annotinine chlorohydrin and compound (II) is the replacement of $-CH_2-N-$ group by the group $-CO-N-$. The increased reactivity of compound (II) must be due to the group $-CO-N-$, hence the other ring must be attached adjacent to the carbonyl carbon of the amide group of (I). The ready removal of the chloro-and hydroxyl group during the hydrogenolysis and the ring closure with sodium bicarbonate suggests that the chlorine atom must be adjacent to the hydroxyl group. Therefore, compound (II) must contain the group  $-\overset{OH}{C}-\overset{Cl}{C}-CO-N-$, the group  $-CH\overset{O}{-}CH-CO-N-$ must be present in compound (I) and the anhydro compound (III) may be represented by  $-C=\overset{Cl}{C}-CO-N-$.

Henderson, Stonner, Valenta and Wiesner (24) converted the compound (III) into an amino acid (VI), $C_{14}H_{19}O_4N$, by oxidation with potassium permanganate. The pKa (4.03 and 9.30) of (VI) (25) suggested that it must be a β-amino acid. The methyl ester corresponding to the amino acid (VI) was found to be resistant to hydrolysis, which indicated that the carboxylic group was attached to a quaternary carbon atom. The infrared absorption spectrum [1662 cm^{-1}, 1616 cm^{-1}] of the compound (III) suggested that the conjugated amide must be in a six membered ring. Hence, the structure of (III) may further be expanded to (IIIa)



(IIIa)

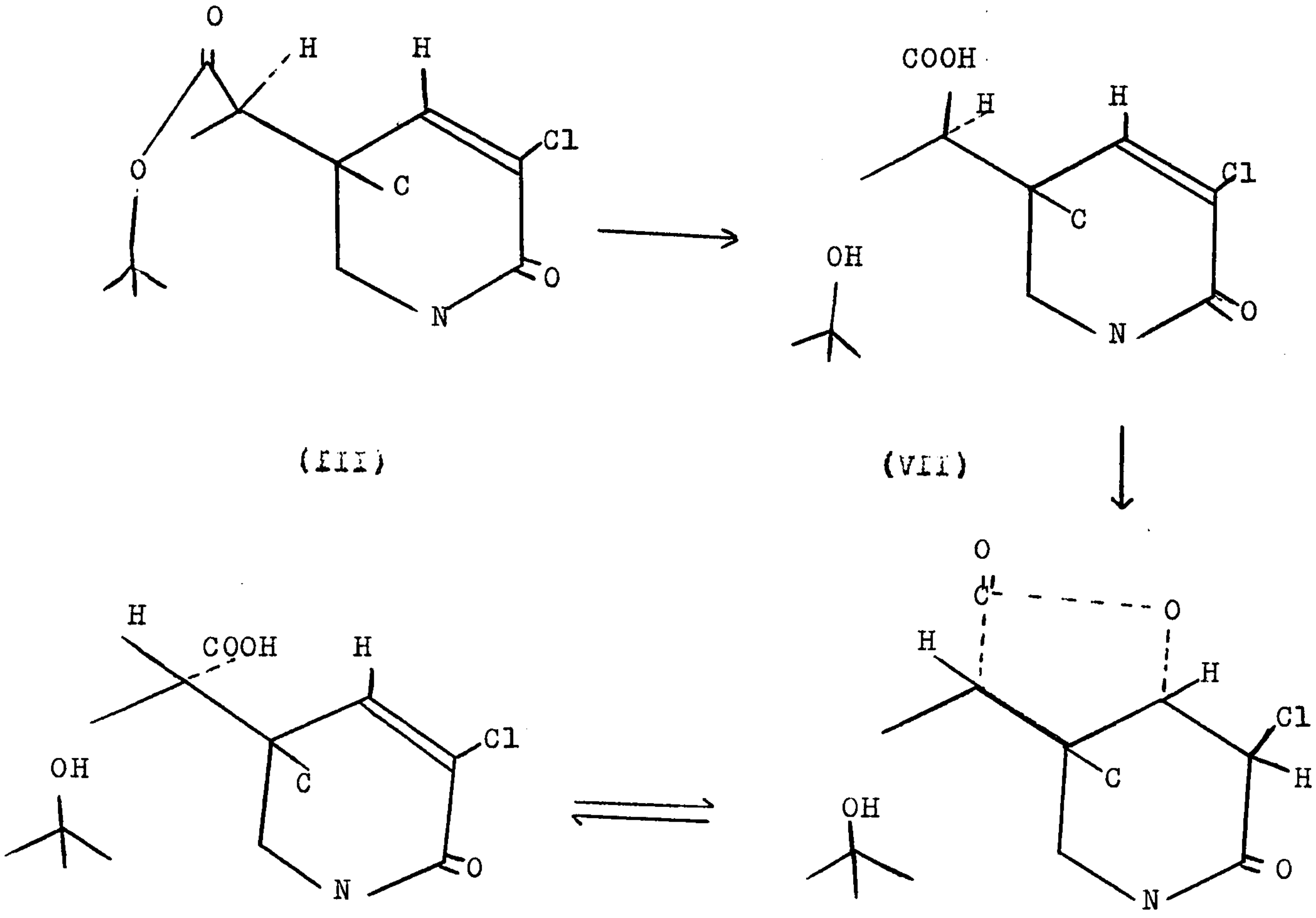
The above discussions indicates that annotinine must have the following partial structure



The same conclusion was also reached by Anet and Marion (26) from the results of the chromous chloride reduction on annotinine chlorohydrin.

The compound (III) on alcoholic KOH hydrolysis (25) gave an acid (VII), $C_{16}H_{20}O_4NCl$. [Infrared spectrum, 1728 cm^{-1}., 1645 cm^{-1}., 1617 cm^{-1}.]. The acid (VII) may be converted to a lactone (VIII), $C_{16}H_{20}O_4NCl$, by treatment with p-toluene sulphonic acid. The ultraviolet spectrum and infrared spectrum [1760 cm^{-1}., 1660 cm^{-1}.] of the compound (VIII) showed the disappearance of the conjugated amide group that was present in the compound (III). Hence, the lactone (VIII) must have been formed by the addition of the carboxylic group across the double bond of the compound (VII). The infrared spectrum of the lactone (VIII) suggested it to be a γ-lactone. The alkaline hydrolysis of the lactone (VIII) gave an acid (IX) $C_{16}H_{20}O_4NCl$, [Infrared spectrum, 1700 cm^{-1}., 1641 cm^{-1}., 1615 cm^{-1}.].

The acid (IX) on treatment with p-toluene sulphonic acid gave the lactone (VIII). The acid (IX) was found to be different from the acid (VII). The reason must lie in the different configurations of the carboxylic groups in the two acids. So isomerisation must have taken place during the process VII → VIII. This indicated that the lactone carboxylic group was attached to a tertiary carbon atom.



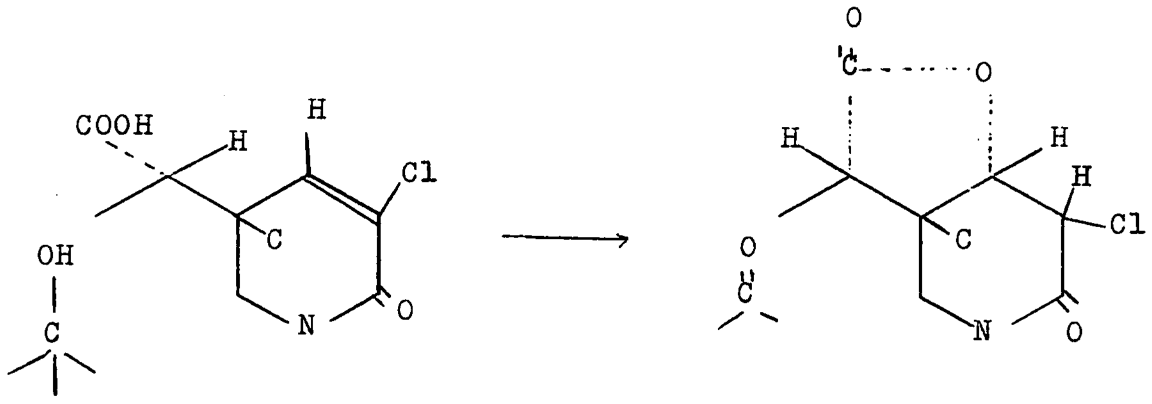
(III)

(VII)

(IX)

(VIII)

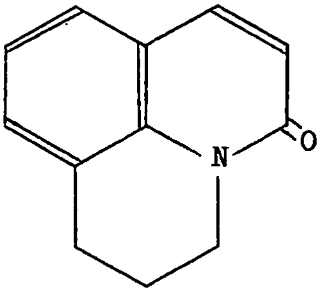
Valenta et al (25) converted the acid (IX) to a keto lactone (X), $C_{16}H_{18}O_4NCl$. [Infrared spectrum, 1790 cm^{-1}, 1725 cm^{-1}, 1665 cm^{-1}] by oxidation with chromic acid in pyridine. In alkaline solution the ultraviolet spectrum of this compound was found to be practically identical with that of (IX) and boiling with alkali did not change the spectrum. This indicated the regeneration of the conjugated amide system in the alkaline solution. The acid corresponding to the keto lactone (X) may be reconverted to the lactone by treatment with p-toluene sulphonic acid. Therefore, the keto group in (X) cannot be in α or β position with respect to the lactam carbonyl, but must be at the site of the original lactone hydroxyl, and the hydroxyl group must be secondary in character.



(IX) (X)

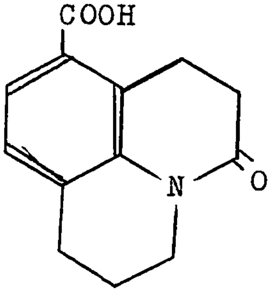
By catalytic hydrogenation, MacLean and Prime (23), converted the compound (III) into the compound (IV), $C_{16}H_{21}O_3N$, [Infrared spectrum, 1775 cm^{-1}, 1625 cm^{-1}]. The alkaline hydrolysis (25) of (IV) with methanolic KOH gave a hydroxy ester (XI) $C_{17}H_{25}O_4N$, [Infrared spectrum, 1730 cm^{-1}, 1660 cm^{-1}] but with ethanolic KOH, an acid (XII) was produced. The acid (XII) may be converted to the corresponding methyl ester (XIII) by treatment with diazomethane. The ester (XIII) isomerised to (XI) on refluxing with methanolic KOH. The molecular rotation difference ($\Delta = 3.0^\circ$) between the ester (XI) and its acetate was shown to be practically identical with that of (XIII) and the corresponding acetate ($\Delta = 3.5^\circ$) both in sign and magnitude. Therefore, the difference between the esters (XI) and (XIII) must be due to the difference in configuration of the carbomethoxy group. It is most probable that during the hydrolysis of (IV) with methanolic KOH, the lactone ring was opened by methoxide ion, forming the methyl ester which then isomerised to the ester (XI). In case of ethanolic KOH, the lactone ring was opened by hydroxide ion and the carboxylate ion was resistant to isomerisation. These findings of Valenta et al (25) indicate that the natural configuration of the carboxylic group is less stable than the epimeric one.

The acid (IX) when treated with diazomethane gave the corresponding hydroxy ester (XIV) (25). Wiesner, Valenta, Ayer, Fowler and Francis (11) dehydrated the hydroxy ester (XIV) to the anhydro ester (XV), $C_{17}H_{20}O_3NCl$. The anhydro ester (XV) on palladium dehydrogenation produced the quinolone acid (XVI), which on decarboxylation gave the trimethylene quinolone (XVII).

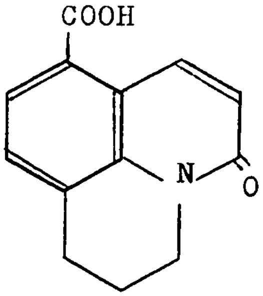


(XVII)

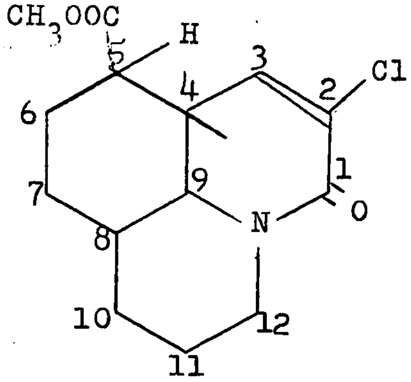
The quinolone acid (XVI) may be reduced with sodium amalgam to dihydroquinolone acid (XVIII). Valenta, Wiesner, Bankiewicz, Henderson and Little (27) showed by synthesis that the dihydroquinolone acid (XVIII) had the following structure



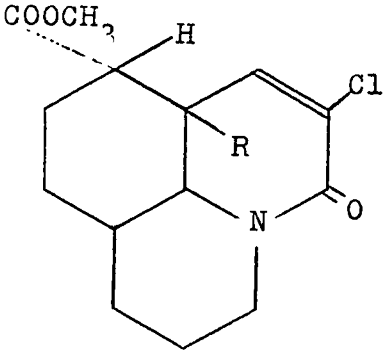
Hence the quinolone acid (XVI) must have the following structure:



On the assumption that no rearrangement has taken place during the palladium dehydrogenation of the anhydro ester (XV), the carbon skeleton in the hydroxy ester (XIV) should be the following;

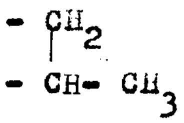


This accounts for three out of four rings present in annotinine. The fourth ring must be added by attaching a bridge at two points in the above skeleton. Valenta et al (25) showed that C_4 is quaternary, so it must be one of the points at which the bridge is attached. Hence, the structure of the hydroxy ester (XIV) may further be evolved to (XIVa)



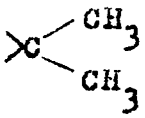
(XIVa)

Since annotinine is known to have only one C-methyl group, by Kuhn-Roth analysis, this bridge must have the following structure:



$\begin{array}{l} -\mathrm{CH_2} \\ -\mathrm{CH}-\mathrm{CH_3} \end{array}$

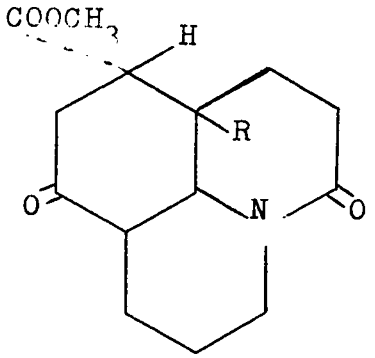
or



$>\mathrm{C}(\mathrm{CH_3})_2$

It is evident from the results of the palladium dehydrogenation of the anhydro ester (XV) that the second point of attachment of the bridge cannot be on C_{10}, C_{11} or C_{12}. Annotinine is γ-lactone and the lactone hydroxyl group is secondary in character (25), the hydroxyl group can hardly be placed in any position other than C_7. This leaves only C_6, C_8 or C_9 as the possible positions for the second point of attachment of the bridge.

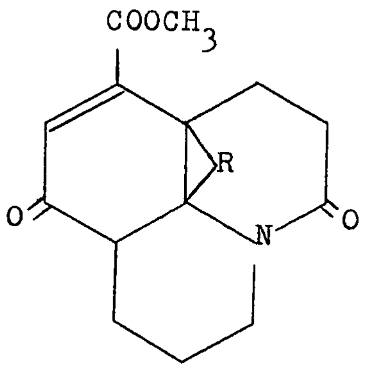
The hydroxy ester (XI) may be oxidized to the corresponding keto ester (XIX) [Infrared spectrum, 1735 cm^{-1}, 1720 cm^{-1}, 1640 cm^{-1}] which on the basis of above discussions must be represented by the structure:



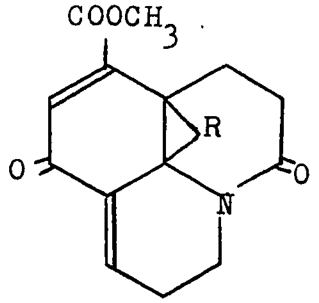
The keto ester (XIX) formed a benzylidene derivative (11) indicating the presence of a reactive methylene group. This excludes the position C_6 as the possible position of the second point of attachment of the bridge.

The following results may be well explained, if it is assumed that the second point of attachment of the bridge is at C_9 rather than C_8.

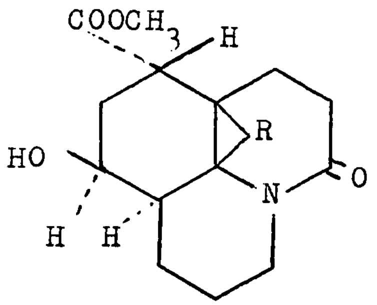
The selenium dioxide oxidation (11) of the keto ester (XIX) gave four products. The infrared spectrum [1725 cm^{-1}., 1698 cm^{-1}., 1640 cm^{-1}.] and ultraviolet spectrum [λ_{max}; 242 mμ, log ε, 3.9] of the first oxidation product (XX), $C_{17}H_{21}O_4N$, were in good agreement with a conjugated keto ester chromophore but did not exclude the possibility of an α, β-unsaturated ketone with an unconjugated ester group. The sodium borohydride reduction of (XX) gave the corresponding alcohol (XXI). The alcohol (XXI) may be reconverted to the keto ester (XIX) by refluxing with sodium methoxide. Such a conversion may only be explained on the assumption that the compound (XXI) is a conjugated allylic alcohol. Hence, the structure of (XX) must be the following:



The ultraviolet spectrum [λ_{max}; 251 mμ, log ϵ, 4.0] and infrared spectrum [1731 cm^{-1}., 1672 cm^{-1}., 1625 cm^{-1}.] of the second product of oxidation (XXII), $C_{17}H_{19}O_4N$, were in good agreement with the structure:

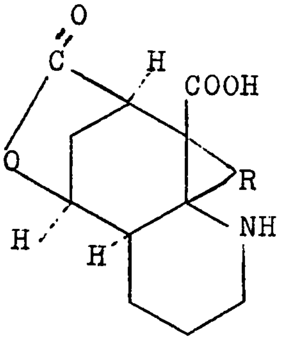


The compound (XXII) on vigorous hydrogenation over platinum oxide in glacial acetic acid gave a hydroxy ester (XXIII). The hydroxy ester (XXIII) was shown to be identical with the hydroxy ester (XIII) of Valenta et al (25). It is quite reasonable to assume that during the hydrogenation of (XXII), all the hydrogen atoms have been added from one side of the ring and opposite the C_4-C_9 bridge. Valenta et al (25) showed that the carbomethoxy group of (XIII) has the natural configuration of annotinine, so the lactone group must be cis to the C_4-C_9 bridge. Hence the hydroxyesters (XXIII) and (XIII) should have the following structure:

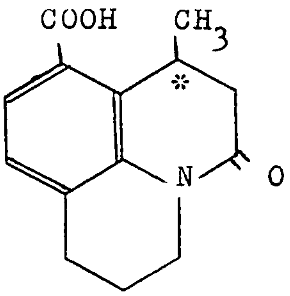


The third and fourth oxidation products were shown to be isomeric hydroxy esters. They differ in the configuration of the hydroxy group at C_8.

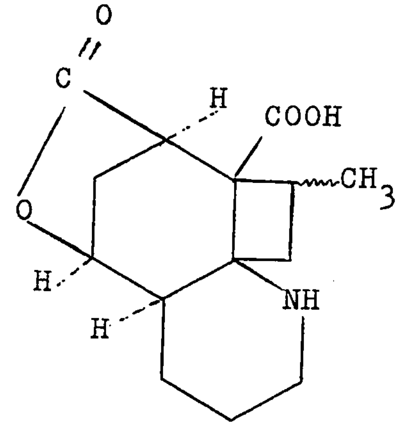
From the above discussion it is evident that the amino acid (VI) should have the following structure:



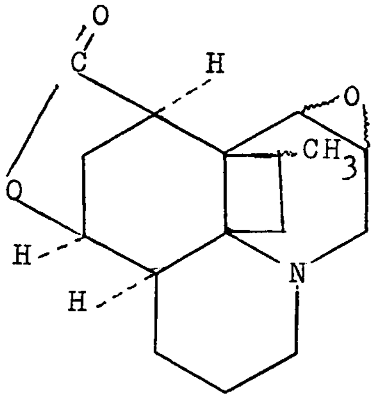
The evidence for the nature of the group R came from the dehydrogenation studies of the amino acid (VI). The amino acid (VI) was dehydrogenated by Anet and Marion (26). They isolated an acid (XXIV), $C_{14}H_{15}O_3N$., as the major product of the dehydrogenation. Valenta et al (27) showed by synthetic means that the compound (XXIV) has the following structure:



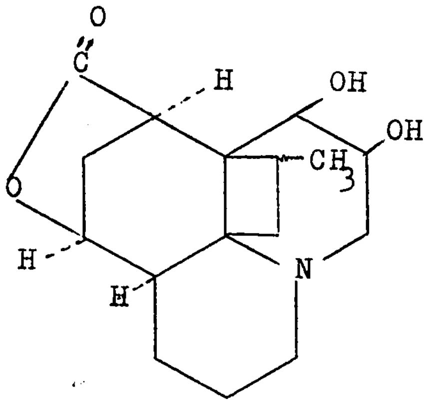
The compound (XXIV) was optically active due to the carbon atom marked by an asterisk. So the acid (XXIV) must have been formed by rearrangement of the amino acid (VI). The formation of the compound (XXIV) may be explained only on the assumption that the amino acid (VI) has the following structure:



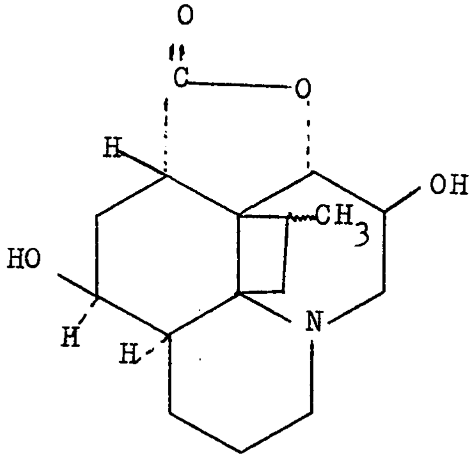
Therefore annotinine may be represented by the following structure:



Meier, Meister and Marion (28) converted annotinine into annotinine hydrate (XXV), $C_{16}H_{23}O_4N$ [infrared spectrum, 3500 cm^{-1}, 1762 cm^{-1}]. On the basis of the above arguments annotinine hydrate (XXV) should have one of the following structures:



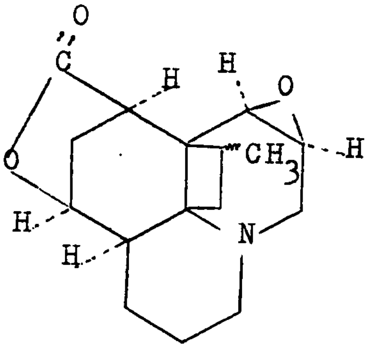
(XXVa)



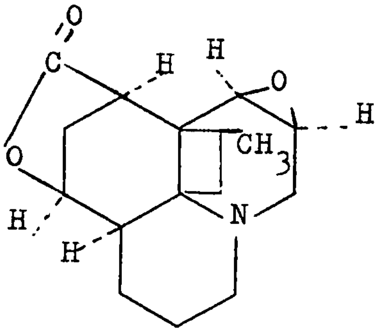
(XXVb)

Annotinine hydrate (XXV) was converted to a hydroxy ketone (XXVI), $C_{16}H_{21}O_4N$, [Infrared spectrum, 3390 cm^{-1}, 1768 cm^{-1}, 1712 cm^{-1}]. The ketone (XXVI) lacks the properties of a-ketol. Hence the structure (XXVb) is the best representation of the annotinine hydrate.

The formation of the annotinine hydrate (XXV) involved the opening of the lactone ring, epimerization of the carboxylic group and the opening of the epoxide ring by means of an intramolecular attack by the carboxylate ion. The recent work of Martin-Smith, Greenhalgh and Marion (29) is in good agreement with this type of transformation during the formation of annotinine hydrate from annotinine. Hence, the epoxide ring must on the same side as the lactone ring so annotinine must be represented by the following structure:



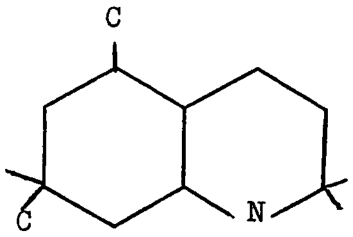
Finally, the X-ray study on the crystal structure of annotinine bromohydrin by Przybylska and Marion (10) supported the above structure and also showed that the methyl group must be on the same side as the lactone ring. Hence annotinine must be represented by the following structure:



(b) Lycopodine is present in almost all the Lycopodium plants. It was first isolated by Bödeker (3) from Lycopodium complanatum L. but he assigned a wrong formula to it. Achmatowicz and Uzieblo (15) isolated lycopodine from Lycopodium clavatum C. and assigned the formula $C_{16}H_{25}ON$, to it.

They showed that lycopodine did not contain any $-OCH_3$, $-N-CH_3$ or active hydrogen.

Manske and Marion (30) showed that lycopodine was resistant to hydrogenation. They dehydrogenated lycopodine with selenium and isolated five bases as picrates. Two of these dehydrogenation products were identified as 7-methylquinoline (I) and 5,7-dimethylquinoline (II) respectively. A treatment of lycopodine with Pd-$BaSO_4$ at 250° yielded 7-methylquinoline (I). 7-Methylquinoline (I) was also produced when lycopodine was treated with phthalic anhydride in vacuum at 350°. So lycopodine probably contained a fully hydrogenated quinoline structure:



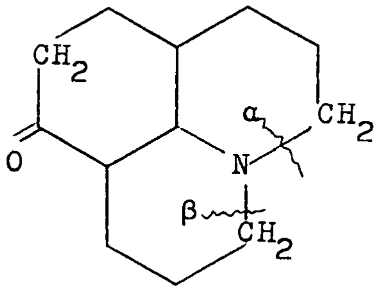
MacLean, Manske and Marion (31) studied the nature of the oxygen atom in lycopodine. The infrared spectrum of lycopodine showed a band at 1695 cm^{-1}. indicating the presence of a carbonyl group. MacLean, Manske and Marion (31) prepared a hydrazone of lycopodine, reduced lycopodine with lithium aluminium hydride to dihydrolycopodine (III), $C_{16}H_{27}ON$, m.p. 168° (Infrared spectrum, 3625 cm^{-1}.) and converted lycopodine to a tertiary alcohol (IV), $C_{22}H_{31}ON$, by treatment with phenyl lithium. All these reactions confirmed the presence of a keto group in lycopodine. Lycopodine reacted with cyanogen bromide to form two isomeric (α and β) cyanobromolycopodines. α-Cyanobromolycopodine (V), $C_{17}H_{25}ON_2Br$, m.p. 140°, was converted to α-cyanolycopodine (VI), $C_{17}H_{26}ON_2$, m.p. 130-131°, by catalytic hydrogenation.

Barclay and MacLean (32) brominated α-cyanolycopodine (VI) to give a dibromoderivative (VII). The compound (VII) on alkaline hydrolysis gave a diketone (VIII), $C_{17}H_{24}O_2N_2$. The compound (VIII) showed the properties of an enol and its ultraviolet spectrum [λ_{max} 280 mμ, log ε 4.0] and infrared spectrum [bands at 3340 cm^{-1}., 1660 cm^{-1}., 1640 cm^{-1}.] were consistent with the presence of the chromophore -C = COH - CO -. This compound must have arisen from an α,α-dibromoketone containing the group $-CH-CBr_2-CO-$. Therefore, α-cyanolycopodine (VI) and hence lycopodine must contain the group $>CH-CH_2-CO-$. The presence of a reactive methylene group was also supported by the fact that α-cyanolycopodine (VI) formed a mono-benzylidene derivative. From the study of pKa of lycopodine and its different derivatives, Barclay and MacLean (32) suggested that the nitrogen atom and the carbonyl group must be relatively close to one another in the molecule.

MacLean, Manske and Marion (31) converted α-cyanobromolycopodine (V) into α-cyanoacetoxylycopodine (IX), $C_{19}H_{29}O_3N_2$, m.p. 112-113°, by treatment with potassium acetate. Compound (IX) on alkaline hydrolysis gave an alcohol (X) $C_{17}H_{26}O_2N_2$, m.p. 97°, which was converted to an acid (XI) by chromic acid oxidation. The acid (XI) on treatment with diazomethane gave an ester (XII), $C_{18}H_{26}O_3N_2$, m.p. 82-83°. Since there was no loss of carbon atom in the above reactions, the bromo group in the compound (V) must be primary.

MacLean and Harrison (33) converted the acid (XI) into a hydroxy acid (XIII), $C_{17}H_{26}O_3N_2$, m.p. 193-194°, by sodium borohydride reduction. The hydroxy acid (XIII) did not yield any lactone on treatment with a catalytic amount of p-toluenesulphonic acid. This indicated that there was no close relationship between the carbonyl group and the nitrogen in the direction of α-cleavage. By hydrolysis with methanolic hydrochloric acid, compound (XI) was converted to an impure aminoacid hydrochloride, which on treatment with diazomethane gave a mixture of compounds from which a lactam (XIV), $C_{16}H_{23}O_3N$, m.p. 159-161°, was isolated by chromatography. The infrared spectrum [bands at 1700 cm^{-1}. (carbonyl), 1635 cm^{-1}. (lactam)] indicated that the compound (XIV) was a six membered or larger ring lactam. MacLean and Harrison (33) converted the compound (XIV) to dihydrolycopodine (III) by lithium aluminium hydride reduction. This led them to conclude that no rearrangement took place in the above sequence of reactions.

MacLean and Harrison (33) treated β-cyanobromolycopodine (31) with silver acetate. From the product of this reaction, they isolated two compounds by subsequent hydrolysis and chromatography on alumina, one of which was an alcohol (XV), $C_{17}H_{26}O_2N_2$. The compound (XV) was oxidized by chromic acid to yield an acid (XVI) which on treatment with diazomethane gave a crystalline methyl ester (XVII), $C_{18}H_{26}O_3N$, m.p. 127-129°. As there was no loss of carbon atom during the above sequence of reactions, so MacLean and Harrison (33) concluded that the alcohol (XV) was primary. The acid (XVI) when reduced with sodium borohydride yielded a lactone (XVIII), $C_{17}H_{24}O_2N_2$, m.p. 201-203°. From the results of the infrared spectra [in Nujol, band at 1743 cm^{-1}; in CCl_4, band at 1761 cm^{-1}] MacLean and Harrison (33) concluded that the lactone must be five or six membered, and that the carbonyl group was separated from the nitrogen atom by three or four carbon atoms in the direction of β-cleavage, and they proposed the following relative position for the carbonyl group and the nitrogen atom.



(c) Acrifoline. Manske and Marion (6) isolated acrifoline, $C_{16}H_{23}O_2N$, from the plant Lycopodium annotinum var. acrifolium Fern. Bertho and Stoll (7) showed that acrifoline did not contain any N-methyl group. Acrifoline reacted with phenyl lithium to form compound (I), $C_{22}H_{29}O_2N$. Acrifoline was resistant to Hofmann degradation but the methiodide of compound (I) underwent Hofmann degradation to produce compound (III), $C_{23}H_{31}O_2N$, m.p. 192-193°. Acrifoline did not form a benzylidene derivative.

Perry and MacLean (34) studied the nature of the functional groups in acrifoline. The infrared spectrum of acrifoline in Nujol mull showed bands at 3310 cm^{-1}. (-OH), and 1670 cm^{-1}. (-C = C-) and a very weak band at 1700 cm^{-1}. ($>C = O$). In chloroform solution there was a strong band at 1700 cm^{-1}. and 1675 cm^{-1}.. Acrifoline was converted to dihydroacrifoline (III), $C_{16}H_{25}O_2N$, by catalytic reduction. By lithium aluminium hydride reduction, compound (III) was converted to α-dihydroacrifolinol (IV), $C_{16}H_{27}O_2N$, m.p. 195.2°. The infrared spectrum of compound (IV) showed the presence of hydroxyl group (band at 3300 cm^{-1}.) but no carbonyl group or unsaturation.

Perry and MacLean (34) converted acrifoline to acrifolinol (V), $C_{16}H_{25}O_2N$, m.p. 192°, by lithium aluminium hydride reduction. Catalytic hydrogenation of compound (V) yielded β-dihydroacrifolinol (VI), $C_{16}H_{27}O_2N$, m.p. 165.5-167.5°.

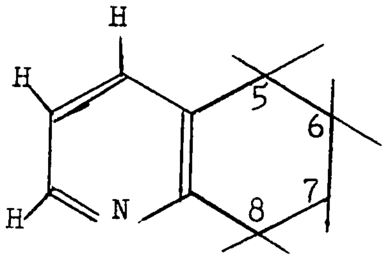
The infrared spectrum of this compound showed absorption in the hydroxyl region (3290 cm^{-1}). Compound (IV) was isomeric with compound (VI). Perry and MacLean (34) were of the opinion that the isomerism could either be about the newly-formed hydroxyl group or about the centers of the original double bond in acrifoline.

Acrifoline was acetylated to mono-O-acetyl acrifoline (VII), $C_{18}H_{25}O_3N$, m.p. 119-120°. The infrared spectrum of compound (VII) showed bands 1740 cm^{-1} (acetoxy group) and 1715 cm^{-1} (carbonyl) but no band in the hydroxyl region. The above studies showed that acrifoline contained a double bond, a carbonyl group, a hydroxyl group and that the nitrogen atom was tertiary in character.

From a study of pKa of acrifoline (pKa 8.34) and of dihydroacrifoline (pKa 9.13), Perry and MacLean (34) concluded that acrifoline had an allylamine structure.

(d) *Lycodine* was isolated by Anet and Eves (22) from *Lycopodium annotinum* L. It was concentrated by two successive countercurrent distributions of the alkaloid mother liquors from the crystallizations of annotinine. The first countercurrent distribution was carried out in six large separatory funnels using the extraction pattern of Bush and Densen and the solvent system chloroform and buffer of pH 6.0. By this fractionation, they obtained six "chloroform soluble" fractions (C_1 to C_6) and six "buffer soluble" fractions

(B_1 to B_6). They partitioned the chloroform soluble fractions (C_1 to C_4) through 60 transfers in a Craig machine between chloroform and buffer of pH 5.2. The alkaloidal fraction containing lycodine was then chromatographed on alumina and finally, they isolated lycodine as a dipicrate. Lycodine, $C_{17}H_{24}N_2$, melted at 117-118°, had $[\alpha]_D - 10^\circ$, pKa 3.97 and 8.08 and contained a C-methyl group. The infrared spectrum (in Nujol) showed a band at 3270 cm^{-1}. indicating the presence of -N-H group. They acetylated lycodine to a N-acetyl derivative which was isolated as a monopicrate. It had a pKa of 4.95. They hydrolysed N-acetyl-lycodine back to lycodine. This proved the presence of a secondary nitrogen atom in lycodine. From the ultraviolet spectra of lycodine and its salts, they concluded that lycodine contained an unconjugated pyridine chromophore. To determine the substitution on the pyridine ring, they examined the N.M.R. spectrum of lycodine. From the analysis of the spectrum, they concluded that lycodine must be a 2,3 disubstituted pyridine. On the basis of chemical and spectral analysis, Anet and Eves (22) assigned the following partial structure to lycodine



$>NH$ not attached to C_5 or C_8

$-CH_3$ attached to a quaternary carbon atom.

During the course of their preliminary investigation on the crude alkaloid of Lycopodium annotinum L, Anet and Eves (35) used paper chromatographic method for the detection of the various alkaloidal constituents present in the mixture. Whatman no. 1 paper impregnated with buffer was used and the chromatograms were developed with 1-butanol saturated with water. Their results indicated that satisfactory separation between the different known alkaloids may be obtained on paper buffered to pH 6.0. The modified Dragendorff reagent (36) and the u.v. absorption or fluorescence were used by them to reveal the presence of the alkaloids on the chromatograms.

8. EXPERIMENTAL

(a) Description of General Methods

1) Paper chromatography - The paper chromatographic method used by Anet and Eves (35) was used to determine the composition of the different fractions obtained by counter-current distribution and by alumina chromatography. The composition of a reaction mixture was also determined with the help of paper chromatography. Buffer of different ph values (as mentioned within the text) were used depending on the nature of alkaloidal mixture. The alkaloidal spots were revealed by spraying with a modified Dragendorff reagent (36). The absolute R_f values of the different alkaloids varied appreciably but their relative values were constant.

2) Spectra - The infrared spectra were measured in carbon tetrachloride solution (1%) in a 1 mm. sodium chloride cell on a Perkin-Elmer single-beam double-pass instrument unless otherwise stated. Infracord refers to a Perkin-Elmer double-beam instrument.

Ultraviolet spectra were measured using a Beckman DK-2 spectrophotometer in ethanol solution in 1 cm. cells.

The nuclear magnetic resonance (N.M.R.) spectra were recorded on a Varian Model V-4302 spectrometer operating at 60 Mc./sec and fitted with a superstabilizer and a Varian recorder. Solutions of the samples were placed in precision thin walled 5 mm. external diameter tubes (Wilmad Corporation). When the volume of the sample was less than 0.4 ml. (minimum 0.2 ml.) Nylon plugs were used as described in the Varian Technical Bulletin. In cases where tetramethylsilane was used the resolution was checked by observing the band width at half intensity of the signal due to this compound. This width was 0.6 c.p.s. or less. Spectra were calibrated by the side-band technique. All the spectra were taken by Dr. F.A.L. Anet. The chemical shifts of particular groups in cycles per second (c.p.s.) with reference to chloroform are shown in Table I and the τ values (37) are shown in Table II. [τ (in p.p.m.) = $10.0 - 10^6(H_{Me_4Si} - H_{obs.})/H_{Me_4Si}$; where H = magnetic field at resonance]

3) Determination of Equivalent weight and pKa - The equivalent weights (E.W.) and pKa of the alkaloids were determined by microtitrations (5-8 mg. of the alkaloid) with 0.1N hydrochloric acid in 50% aqueous methanol solution with the help of Beckman pH meter, model G. A microburette (1 ml., Manostat Corporation) and micro-electrodes were used (The diameter of the electrodes were about 5 mm.). A typical titration curve for lycofoline is shown in Fig. 1.

4) Elementary and group analyses - Elementary and group analyses and molecular weight (M.W.) determinations were carried out at the Geller microanalytical laboratories, Bardonia, New York.

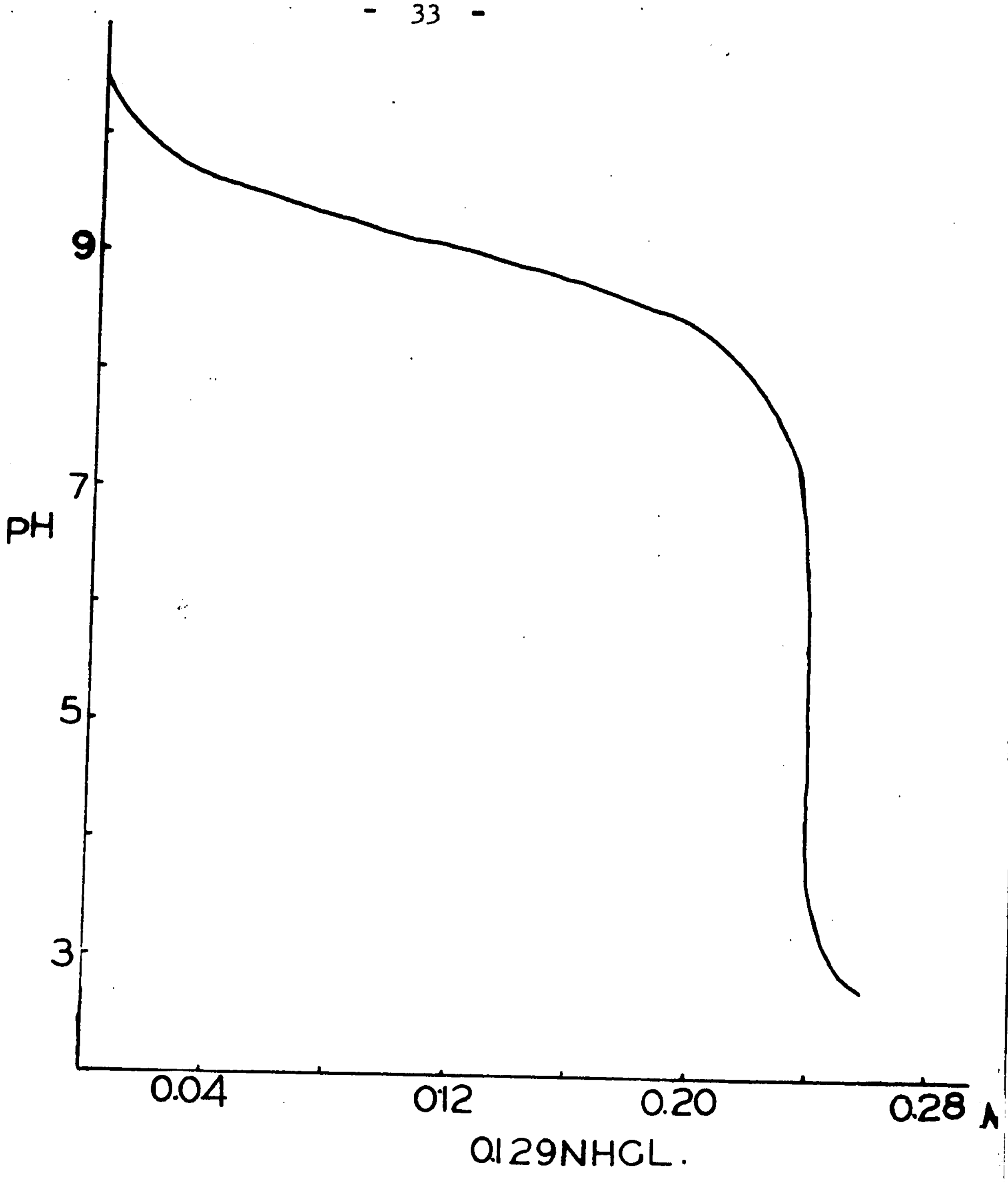


Fig. 1. Microtitration (electrometric) of lycofoline with hydrochloric acid in 50% aqueous methanol.

5) Preliminary Experiments - Preliminary experiments were carried out using 2-10 mg. of the alkaloid to find suitable conditions for carrying out a particular reaction on a larger scale. A typical example has been described on page no. 54 in case of acetylation of annofoline.

6) Sublimation - Sublimations were carried out in glass tubes (8 - 10 mm. diameter) having a small bulb at the end. Heating was by means of an air bath. In some cases the process was actually evaporative distillation.

(b) Isolation of alkaloids

1) Preliminary Separation of Crude Alkaloids - The plant material had been extracted at the National Research Council, Ottawa and most of the annotinine had been removed by crystallization. The mother liquor from the crystallization of annotinine was kindly donated by Dr. L. Marion. It was divided by Anet and Eves (22) into six "chloroform soluble" fractions (C_1 to C_6) and six "buffer (pH 6.0) soluble" fractions (B_1 to B_6). The alkaloid in the first four buffer phases (B_1 to B_4) were combined for the present work and will be referred to as fraction B.

A second lot of alkaloid mother liquors from a different extraction of plant material was also investigated, but was treated differently from the above in view of the results of obtained with fraction B.

2) <u>Countercurrent Distribution with the System chloroform-Buffer of pH 7.0</u> - Fraction B (8.6 g.) was dissolved in chloroform (120 ml.) and the solution was placed in the first three tubes of a 60-tube Craig countercurrent distribution apparatus (38) and partitioned between chloroform and citrate-phosphate buffer of pH 7.0 with the buffer as the moving phase. Fifty seven transfers were made. Every third tube, starting from tube no. 1, was selected for analysis and an aliquots of 2 ml. of each phase from each of these tubes were removed with the help of a hypodermic syringe, mixed, made alkaline and extracted with chloroform. The alkaloid left after the removal of the solvent was weighed. In this way the distribution pattern shown in Fig. 2A was obtained.

The infrared spectra of these fractions were measured in carbon tetrachloride solution. The following results were obtained.

Tube No.	Infrared Spectra, Relative Absorbance		
	$1740\ cm^{-1}$.	$1716\ cm^{-1}$.	$1700\ cm^{-1}$.
10	0.38	-	-
13	0.46	-	-
16	0.86	-	-
19	0.89	-	-
22	0.80	0.30	-
25	0.35	0.45	-
28	-	0.43	-
31	-	0.57	-
34	-	0.51	-
37*	-	0.61	0.50
40*	-	0.55	0.60
43*	0.50	0.33	0.55
46	0.85	-	0.23
49	0.95	-	0.15
52	0.51	-	0.18
55	0.21	-	0.19
58	0.19	-	0.14

The tube no. marked with asterisk (*) had a weak absorption at $1650\ cm^{-1}$.

Alkaloid of melting point 117-118° was isolated from the tube no. 19 (see page 39). α-Lofoline was crystallized from the alkaloidal contents of tube no. 49. Paper chromatography (pH 6.0) on the various fractions gave the following results:

Tube no.	R_f, Relative amount					
	0.12 0.17	0.20 0.25	0.27 0.30	0.36 0.47	0.50 0.52	0.62 0.63
Acrifoline	-	-	(R_f 0.30)	-	-	-
13	-	-	-	1	10	-
16	-	-	-	1	10	-
19*	-	-	-	-	(R_f 0.52)	-
22	-	-	1	2	8	-
25	-	-	8	1	2	-
28	-	-	6	-	1	-
31	-	-	5	-	1	-
34	-	-	5	1	-	-
37	-	-	3	3	-	-
40	-	-	3	1	-	2
43	-	1	-	3	-	2
46	1	2	2	2	-	-
a-Lofoline	-	-	-	(R_f 0.36)	-	-
52	1	3	-	-	-	-
55	1	3	-	-	-	-
58	3	3	-	-	-	-

---

* Crystalline alkaloid which had been isolated from this tube was used.

3) Isolation of Alkaloid of melting point 117-118° - The alkaloid from the tubes 10-22 of the above distribution was isolated in the usual way and when crystallized from ether-pentane gave an alkaloid, m.p. 97-112°. After four recrystallizations it melted at 115-116°. The crystalline alkaloid was slightly yellow in colour and colourless crystals, m.p. 117-118°, were obtained after sublimation under vacuum, (90-110° at 0.05 mm.)

Calc. for $C_{17}H_{25}O_3N$: C, 70.07; H, 8.65; N, 4.8; C-CH_3, 5.14%; m.w. 291.4.

Found: C, 70.14, 70.35; H, 8.47, 8.69; N, 3.90, 4.13; C-CH_3, 4.30%; M.W. (Rast), 314, 304; E.W., 299.7; pKa, 8.20.

The infrared spectrum in Nujol mull showed bands at 3473 cm^{-1}., 3116 cm^{-1}., 1740 cm^{-1}.; in carbon tetrachloride solution bands at 3662 cm^{-1}., 1740 cm^{-1}..

4) Treatment of the Alkaloid of m.p. 117-118° with alkali. - A mixture of 20 mg. of alkaloid of m.p. 117-118° and 1 ml. of aqueous sodium hydroxide (5%) was heated. The alkaloid at first melted and then dissolved. The solution was refluxed for one hour. The light yellow solution was cooled and extracted with chloroform. The chloroform extract did not contain any base. The aqueous solution was made acidic with dilute sulphuric acid and warmed. The acidic aqueous solution was made alkaline in the cold and extracted

with chloroform. The chloroform extract gave a compound which after crystallization from ether-pentane melted at 209-210°. The infrared spectrum of this compound in carbon tetrachloride solution showed band at 1740 cm^{-1}, but no band in the hydroxyl region.

5) <u>Isolation of Acrifoline</u> - The alkaloid in the tubes 26 to 34 was isolated in the usual way. It was dissolved in acetone and concentrated hydrobromic acid was added to this solution until it was slightly acidic. The hydrobromide crystallized immediately, it was filtered off and the hydrobromide was recrystallized from methanol-acetone. The hydrobromide was converted to the free base. The free base had a similar R_f value, infrared spectrum and melting point as acrifoline. Furthermore, it was known from previous work (35) that acrifoline was present in the sample used.

6) <u>Countercurrent Distribution with the System Chloroform-Buffer of pH 8.0</u> - The alkaloid was recovered from the tubes 46 to 57 of the first countercurrent distribution in the usual way and was submitted to fifty eight transfers in the above system. Every third tube, starting from tube no. 1, was analysed as before but this time 4 ml. of each phase was used. The distribution pattern shown in Fig. 28. was obtained. Paper chromatography (pH 7.0) and infrared spectra (1% solution in carbon tetrachloride) gave the following results:

Tube no.	Infrared spectra cm^{-1}	R_f, relative amount		
		0.35-45	0.52-0.57	0.60-0.68
a-Lofoline	1740	-	(R_f 0.52)	-
22	1740, 1700	-	10	-
25	1740	-	8	-
28	1740	-	10	-
31	1740	-	12	-
34	1740, 1700(v.w)	2	8	-
37	1740(w), 1700(w)	8	-	-
40	1700	10	-	-
43	1700(w)	6	-	2
46	1700(v.w)	5	-	8
49	1700(v.w)	2	-	6
52	1740(v.w), 1700(v.w)	3	-	12
55	1740(v.w)	10	-	1

w = weak, v.w = very weak.

Note: 0.22-0.24 mg. of the alkaloidal material was used for paper chromatography.

7) Isolation of a-Lofoline - The alkaloid in tubes 21 to 32 of the above distribution was isolated and was crystallized from methanol to give thick colourless prisms, m.p. 211-212°. To obtain a better yield, it was found desirable to chromatograph the mother liquor on alumina (containing 5% water). The solvents used for elution were benzene-chloroform, chloroform and chloroform-methanol; 40 ml. fractions were collected. Paper chromatography (pH 7.0) gave the following results:

Fraction no.	Eluant	Paper chromatography R_f, relative amount		
		0.20	0.35	0.59-0.67
1-2	Benzene-chloroform (9:1)	-	-	-
3-12	Benzene-chloroform (1:1)	-	-	-
13-31	Benzene-chloroform (1:1)	-	-	3-5
34-43	Chloroform	-	-	2-15
44	Chloroform-methanol (19:1)	-	-	-
45	Chloroform-methanol (19:1)	-	-	20
46	Chloroform-methanol (19:1)	1	3	1
a-Lofoline	-	-	-	(R_f 0.62)

The fractions 13 to 45 gave a-lofoline on crystallization from methanol-ether. After two crystallizations it melted at 195-200° and when recrystallized from benzene it melted at 211-212°.

8) Isolation of Annofoline - The alkaloid in the tube 36 to 41 of the above distribution (with buffer of pH 8.0) was isolated in the usual way. To a viscous solution of the alkaloid in methanol, ether was added and annofoline crystallized on scratching. It melted at 115-130°. After three recrystallizations, it melted at 148-150°. The melting point changed to 156-157° on sublimation under vacuum (120-130° at 0.05 mm.). A better yield of annofoline was obtained by chromatography of the mixture of annofoline and lycofoline as described on page 46 .

9) Isolation of Lycofoline - The alkaloid in the tubes 42 to 51 of the above distribution (with buffer of pH 8.0) was isolated and converted to the hydrobromide by adding concentrated hydrobromic acid to the methanolic solution of the alkaloid. Addition of ether gave a crystalline salt, which after three crystallizations from methanol-ether was converted to the base. The base when sublimed (110-130° at 0.05 mm.) melted at 136-138° and when crystallized from ether it melted at 144-145°. For analysis it was sublimed under vacuum; m.p. unchanged. A better yield of lycofoline was obtained by chromatography of the mixture of annofoline and lycofoline as described on page 46 .

10) Improved Separation of Crude Alkaloid - A second batch of crude alkaloid (after removal of the annotinine) (60 g.) was partitioned between chloroform and citrate-phosphate buffer of pH 7.0 using the modified Bush and Densen extraction pattern of Fig. 3. This was carried out in eight large separatory funnels marked 1 to 8. In each funnel 1 litre of citrate-phosphate buffer solution of pH 7.0 was placed. The chloroform solution (1 litre) of the alkaloidal mixture was placed into the first separatory funnel and equilibrated with the buffer solution. The separatory funnel was then allowed to stand for some time so as to form two separate layers. The chloroform layer was then transferred to the next separatory funnel, the whole procedure was repeated as in the case of the first separatory funnel and the chloroform phase was then transferred to the next separatory funnel. These procedures were repeated until the chloroform phase reached the last separatory funnel (no. 8) and finally emmerged out as a single chloroform phase, C_1'. The pH of the buffer phases was checked with the help of a Beckman pH meter (model H2) and readjusted to 7.0 by adding solid citric acid. One litre of chloroform was then introduced in the first separatory funnel, eight transfers were made as described above and finally the chloroform phase (C_2') was obtained The separatory funnels marked 4,5,6,7 and 8 were then set aside and the buffer phases in them were called B_4', B_5', B_6'

B_7' and B_8' respectively. The pH of the buffer phases in the separatory funnels 1, 2 and 3 was adjusted to 7.0. Fresh chloroform (1 litre) was introduced in the first separatory funnel and three transfers were made when the chloroform phase, C_3', was obtained. Similarly C_4', C_5', C_6', C_7' and C_8' were obtained and the buffer phases in the separatory funnels 1, 2 and 3 were marked B_1', B_2' and B_3' respectively. The buffer phases, B_1', B_2' and B_3' were mixed, made alkaline and the alkaloid was extracted. Yield 18.1 g. This fraction was called the buffer fraction. The fractions C_1' and C_2' were combined and the solvent was removed. Yield 21.0 g. This was called the chloroform fraction. The fractions B_4' to B_8' were combined, made alkaline and the alkaloid extracted. The alkaloid obtained was mixed with the alkaloid obtained from fractions C_3' to C_8'. Yield 19.3 g. This fraction was called the middle fraction

11) *Countercurrent Distribution with the System Chloroform and Buffer of pH 8.2* - A boric acid-sodium hydroxide buffer was used. To prepare the buffer, boric acid was suspended in water so as to give a 1M. solution and to this solid sodium hydroxide was added with stirring just to produce a clear solution. The pH of this solution was then adjusted to 8.2 with the help of a Beckman pH meter.

The buffer fraction obtained by the above modified Bush and Densen extraction pattern was divided into three equal lots and each was subjected to a 60-tube countercurrent distribution with the solvent system chloroform and boric acid-sodium hydroxide buffer of pH 8.2. Every third tube, starting from tube no. 1 was analysed as described on page no. 35 . The distribution pattern shown in Fig. 20, was obtained.

12) Improved Separation of Annofoline and Lycofoline - The alkaloid mixture (3.2 g.) from the tubes 28 to 43 of the above distribution was chromatographed on alumina (65 g., Fisher Scientific Company; deactivated with water, 5%). Benzene, benzene-chloroform, chloroform and chloroform-methanol were used as eluant; 50 ml. fractions were collected. Paper chromatography (pH 7.0) gave the following results:

Fraction no.	Eluant	Paper chromatography R_f, Relative Amount		
		0.36 0.46	0.57 0.61	0.77 0.82
Annofoline	-	(R_f 0.36)	-	-
2-5	Benzene	40	-	1-2
8-47	Benzene	3-10	-	-
50-63	Benzene-chloroform (4:1)	10-40	-	-
66-72	Benzene-chloroform (3:2)	40	-	-
75-90	Benzene-chloroform (3:2)	2-30	1-15	-
93	Benzene-chloroform (3:7)	1	10	-
96-114	Benzene-chloroform (3:7)	-	20	-
117-119	Benzene-chloroform (3:7)	1	8	-
121	Benzene-chloroform (3:7)	-	3	-
124-130	Chloroform	1-3	1-5	-

Annofoline was crystallized from the fractions 1 to 73, whilst lycofoline was obtained from the fractions 94 to 115. Lycofoline was further purified through the hydrobromide as described on page no. 43.

13) <u>Isolation of β-Lofoline</u> - The alkaloidal material of the tubes 16 to 26 did not give α-lofoline in pure state easily. In fact, paper chromatography (pH 7.0) showed two spots of very similar R_f values (0.58 and 0.51). The mixture was therefore chromatographed on activated alumina. The solvents used for elution were benzene (100 ml.), benzene-chloroform (4:1; 1200 ml.) benzene-chloroform (1:1, 1250 ml.), benzene-chloroform (1:4; 400 ml.) and chloroform (1050 ml.); 50 ml. fractions were collected. The fractions (16 to 78), containing the alkaloidal material, were divided into five different lots. α-Lofoline was crystallized from the fourth (fractions 52 to 66) and fifth (fractions 67 to 78) lots while the second lot (fractions 30 to 36) on crystallization from methanol-ether gave β-lofoline, m.p. 160-166°. After two recrystallizations from ether, the melting point changed to 166-167°.

(c) Chemistry of Annofoline

1) Characterization of Annofoline - Annofoline, m.p. 156-157°, was readily soluble in methanol, chloroform or benzene but sparingly soluble in ether or petroleum ether. A sublimed sample was used for analysis.

Calc. for $C_{16}H_{25}O_2N$: C, 72.96; H, 9.57; N, 5.32; C-CH_3, 5.96%; M.W., 263.4.

Found: C, 72.85, 73.05; H, 9.51, 9.59; N, 4.91; C-CH_3, 6.91%; M.W. (Rast), 270, 283; E.W. 259, 267; pKa, 9.1, 9.2; $[\alpha]_D$ - 131.4° (c, 2.0 in ethanol). The infrared spectrum (Infracord), Fig. 4, in Nujol, showed bands at 3400 cm^{-1}, 1700 cm^{-1} and in carbon tetrachloride solution it showed bands at 3600 cm^{-1}, 3400 cm^{-1} and 1700 cm^{-1}. A comparison of the spectra of lycopodine, annofoline and acrifoline of the carbonyl region in carbon tetrachloride solution is shown in Fig. 5.

N.M.R. spectrum, Fig. 6, was measured in chloroform solution (40 mg in 0.35 ml.) with tetramethylsilane as internal reference. The τ values are shown in Table II. When a solution of annofoline in carbon tetrachloride was allowed to evaporate in the presence of air an intense orange-yellow residue was left.

2) Annofoline Methiodide - The methiodide of annofoline crystallized on addition of methyl iodide (0.8 ml.) to a solution of the alkaloid (60 mg.) in acetone (2 ml.). The mixture was refluxed for five minutes, cooled and the methiodide

collected. After recrystallization from water it melted at 308-309°. Yield, 40 mg.

Calc. for $C_{16}H_{25}O_2N.CH_3I$: C, 50.37; H, 6.94; I, 31.31%.
Found: C, 50.30; H, 6.70; I, 31.50%.

3) Annofoline Perchlorate - A methanolic solution of perchloric acid was added to a solution of annofoline in methanol until a slight excess of the acid was present. Ether was added to the solution and the perchlorate crystallized on scratching. After recrystallization from methanol-ether it melted at 234-236°.

Calc. for $C_{16}H_{25}O_2N.HClO_4.H_2O$: C, 50.30; H, 7.65%.
Found: C, 50.08; H, 7.35%.

4) Treatment of Annofoline with Acid

a) Boiling 10% Hydrochloric Acid - Annofoline (21 mg.) was refluxed with 2 ml. hydrochloric acid (10%) for five hours. The solution was cooled, made alkaline and the alkaloidal material was extracted with chloroform. The infrared spectrum in carbon tetrachloride solution showed bands at 3594 cm^{-1}, 3473 cm^{-1} and 1700 cm^{-1}. Paper chromatography (pH 7.0) gave the following results:

Sample	R_f	Relative amount
Annofoline	0.37	
Reaction product		
	0.43	75
	0.85	1

The compound that had the R_f, 0.43, was probably unchanged annofoline. The difference in R_f values was probably due to the large differences in concentration.

b) Boiling concentrated Hydrochloric Acid

1) For five hours - Annofoline (16 mg.) was refluxed with 1 ml. concentrated hydrochloric acid for five hours. The solution was cooled, made alkaline and extracted with chloroform. Paper chromatography (pH 7.0) on the chloroform extract gave the following results:

Sample	R_f	Relative amount
Annofoline	0.37	
Reaction product		
	0.43	10
	0.85	1

The compound that had R_f value of 0.43 was probably unchanged annofoline and the difference in R_f values was probably due to the larger concentration difference.

2) For twenty hours - A mixture of annofoline (60 mg.) and concentrated hydrochloric acid (6 ml.) was refluxed under nitrogen for twenty hours. The solution was cooled, diluted with water, made alkaline and extracted with chloroform. Paper chromatography (pH 6.0) on the chloroform extract gave the following results:

Sample	R_f	Relative amount
Annofoline	0.31	
Reaction product		
	0.23	40
	0.43	50
	0.63	5
	0.73	2
	0.79	1
	0.82	2

5) Attempted Dehydration of Annofoline

a) Treatment with Phosphorous Oxychloride and Pyridine - To a solution of annofoline (11 mg.) in dry pyridine (0.2 ml.) phosphorous oxychloride (0.1 ml.) was added. The mixture was heated on a water bath for one hour. Most of the pyridine was removed by evaporation under a stream of nitrogen. The phosphorous oxychloride was decomposed with water. The solution was made alkaline and the alkaloid was extracted with chloroform. The chloroform extract was sublimed under vacuum (110-120° at 0.05 mm.). The infrared spectrum of the sublimate in carbon tetrachloride solution showed bands at 3594 cm^{-1}. and 1700 cm^{-1}..

b) Treatment with p-toluene Sulphonic Acid - A mixture of annofoline (22 mg.), xylene (1 ml.) and p-toluenesulphonic acid was refluxed for one hour. The solvent was removed, water was added to the residue. The mixture was made alkaline and extracted with chloroform. Infrared spectrum in carbon tetrachloride solution showed bands at 3594 cm^{-1} and 1700 cm^{-1}. Paper chromatography (pH 6.0) gave the following results:

Sample	R_f	Relative amount
Annofoline	0.18	
Reaction product		
	0.11	1
	0.19	1
	0.24	1
	0.33	1
	0.43	1
	0.48	1
	0.56	1

6) Attempted Hofmann Degradation of Annofoline Methiodide - Annofoline methiodide (3 mg.) was treated with sodium hydroxide solution (2 ml., 10%). The solution was extracted with chloroform. The chloroform extract did not give any test with Dragendorff's reagent. The aqueous solution was boiled for ten minutes, cooled and extracted with chloroform. The chloroform extract showed absorption at 257 mμ in the ultraviolet spectrum (in ethanol). The aqueous extract was refluxed for a further period of two hours, cooled and extracted with chloroform. The chloroform extract did not show any selective absorption in the ultraviolet region.

7) Attempted Preparation of Benzylidene Derivative of Annofoline - Annofoline (5 mg.) was dissolved in methanol (0.5 ml.). To this solution was added benzaldehyde (0.01 ml.) (freshly distilled) and sodium methoxide solution (0.1 ml.) (prepared by dissolving 0.2 g. metallic sodium in 2 ml. methanol). The mixture was refluxed for half an hour and then allowed to stand at room temperature for sixteen hours. The solvent was removed, water was added to the residue and the alkaloid was extracted with chloroform. Paper chromatography (pH 7.0) on the chloroform extract gave the following results:

Sample	R_f
Annofoline	0.31
Reaction product	0.31

8) Acetylation of Annofoline

a) First attempt - A mixture of annofoline (5 mg.), acetic anhydride (0.1 ml.) and anhydrous sodium acetate (2 mg.) was heated on a water bath for seven hours. The dark brown solution was cooled, excess of acetic anhydride was decomposed with methanol. The solvent was removed. Paper chromatography (pH 7.0) gave the following results:

Sample	R_f	Relative amount
Annofoline	0.42	
Reaction product		
	0.43	3
	0.73	2
	0.82	1
	0.88	1
	0.93	1

b) Second attempt - Acetic anhydride (0.1 ml.) was added to a solution of annofoline (26 mg.) in benzene (1 ml.) and the mixture was refluxed for ten hours. The dark solution was cooled, benzene was removed under a stream of nitrogen and excess of acetic anhydride was decomposed with methanol. The residue left after the removal of methyl acetate was sublimed under vacuum (110-120° at 0.05 mm.). The infrared spectrum of the sublimate in carbon tetrachloride solution showed bands at 1742 cm^{-1}. and 1712 cm^{-1}. Paper chromatography (pH 7.0) on the sublimate gave the following results:

Sample	R_f	Relative amount
Annofoline	0.37	
Reaction product		
	0.38	3
	0.71	2

c) Third attempt - A mixture of annofoline (100 mg.), acetic anhydride (0.5 ml.) and toluene (3 ml.) was refluxed for ten hours. The solution was cooled and excess of acetic anhydride was decomposed with methanol. The solvent was removed. Paper chromatography (pH 7.0) on the residue gave the following results:

Sample	R_f	Relative amount
Annofoline	0.36	
Reaction product		
	0.36	1
	0.70	30
	0.78	1
	0.86	1

The above dark brown residue gave an oily material on sublimation under vacuum (110-120° at 0.05 mm.). Yield, 61 mg. The infrared spectrum of the sublimate in carbon tetrachloride solution showed bands at 1745 cm^{-1}. and 1712 cm^{-1}.. The sublimate was chromatographed on alumina (15 g., deactivated with water, 5%). The solvents used for elution were benzene-chloroform, chloroform and chloroform-methanol; 20 ml. fractions were collected. Paper chromatography (pH 7.0) gave the following results:

Fraction no.	Eluant	Paper chromatography R_f, relative amount		
		0.35	0.65-0.69	0.82-0.83
Annofoline	-	(R_f, 0.33)	-	-
1-4	Benzene-chloroform (4:1)	-	-	-
5-9	Benzene-chloroform (4:1)	-	20	1-2
10-13	Benzene-chloroform (4:1)	-	1-10	-
14-16	Benzene-chloroform (4:1)	-	-	-
17-31	Benzene-chloroform (3:2)	-	-	-
32-40	Benzene-chloroform (1:4)	-	-	-
41-48	Chloroform	-	-	-
49	Chloroform-methanol (19:1)	-	1	1
50	Chloroform-methanol (19:1)	1	1	1
51-52	Chloroform-methanol (19:1)	-	-	-

The fractions 5 to 14 were combined. The residue left after the removal of the solvent did not crystallize from methanol, benzene, ether, benzene-pentane or benzene-petroleum ether (b.p. 30-65°). It was dissolved in acetone and a solution of hydrobromic acid in acetone was added to the alkaloidal solution until a slight excess of the acid was present. O-Acetylannofoline hydrobromide crystallized immediately. The crystals of the hydrobromide were collected and washed with cold acetone. Paper chromatography (pH 7.0) gave the following results:

Sample	R_f
Annofoline	0.37
O-Acetylannofoline hydrobromide	0.67

The crystalline hydrobromide was recrystallized from methanol-ether. Yield, 17 mg. For analysis the sample was dried under vacuum (100° at 0.05 mm.)

Calc. for $C_{18}H_{27}O_3N.HBr$: C, 55.65; H, 7.03%.

Found: C, 55.72; H, 7.28%.

The infrared spectrum of the free base in carbon tetrachloride solution showed bands at 1750 cm^{-1} and 1712 cm^{-1}.

9) <u>Hydrolysis of O-Acetyl Annofoline</u> - The solvent was removed from the mother liquor of the second crystallization of O-acetyl annofoline (page 58) and the free base was regenerated in the usual way. O-Acetylannofoline did not crystallize from ether-pentane, benzene-pentane or benzene-petroleum ether

(b.p. 30-65°). It was dissolved in methanol (1 ml.) and refluxed with 5% aqueous sodium hydroxide solution (2 ml.) for two hours. Most of the methanol was removed by evaporation and the alkaloidal material was extracted with chloroform. The chloroform extract when sublimed under vacuum (110-120° at 0.05 mm.) melted at 150-152°. The mixed melting point of the sublimate with annofoline was 150-154°. The sublimate when crystallized from ether, melted at 140-142°. The melting point changed to 154-155° after sublimation in vacuum.

10) Attempted Chromic acid Oxidation of Annofoline

Annofoline (2 mg.) was dissolved in dry pyridine (0.04 ml.). To this solution chromium trioxide (2 mg.) was added with shaking. The mixture was allowed to stand at room temperature. After twenty four hours the chromium trioxide-pyridine complex was decomposed with water. The solution was made alkaline with sodium hydroxide and the alkaloidal material was extracted with chloroform. The chloroform extract was sublimed under vacuum (110-120° at 0.025 mm.). The infrared spectrum (infracord) in carbon tetrachloride solution showed bands at 3600 cm^{-1}., 3400 cm^{-1}. and 1700 cm^{-1}. Paper chromatography (pH 7.0) gave the following results:

Sample	R_f	Relative amount
Annofoline	0.51	
Reaction product		
	0.29	1
	0.54	90
	0.87	1

11) Sodium borohydride Reduction of Annofoline - Annofoline (150 mg.) was dissolved in ethanol (10 ml.) and sodium borohydride (0.3 g.) in ethanol (40 ml.) was added to the above solution. The mixture was refluxed for two hours. The solution was cooled, excess of sodium borohydride was decomposed with dilute acetic acid (1:1) and most of the ethanol was distilled off. The residue was made alkaline and the alkaloid was extracted with chloroform. Paper chromatography (pH 7.0) gave the following resuls:

Sample	R_f
Annofoline	0.37
Reaction product	
	0.27
	0.55

The infrared spectrum in chloroform showed no absorption in the carbonyl region.

The reduction product was chromatographed on alumina (15 g., containing 5% water). The solvents used for elution were benzene-chloroform, chloroform and chloroform-methanol;

20 ml. fractions were collected. Paper chromatography (pH 7.0) gave the following results:

Fraction no.	Eluant	Paper chromatography R_f, relative amount	
		0.24-0.31	0.42-0.57
Annofoline	-	(0.37 R_f)	
1-9	Benzene-chloroform (3:2)	-	-
10-14	Benzene-chloroform (1:1)	-	-
16-31	Benzene-chloroform (1:1)	-	10-20
34	Benzene-chloroform (3:7)	-	4
37	Benzene-chloroform (3:7)	1	2
40	Benzene-chloroform (1:9)	20	1
43	Benzene-chloroform (1:9)	20	-
46-58	Chloroform	5-20	-
60-61	Chloroform-methanol (9:1)	15	-
62-67	Chloroform-methanol (9:1)	-	-

The fractions 15 to 35 were combined. The residue left after the removal of the solvent gave α-dihydroannofoline on crystallization from methanol-actone. After sublimation in vacuum (140-150° at 0.025 mm.), it melted at 264-265° (recrystallized at 190°). Yield, 26 mg.

Calc. for $C_{16}H_{27}O_2N$: C, 72.41; H, 10.26%.

Found: C, 71.63, 72.66; H, 10.14, 10.17%; pKa, 10.05;

$[\alpha]_D$ 0.0 (c, 2.0 in ethanol).

The infrared spectrum (Infracord) in chloroform did not show any band in the carbonyl region. N.M.R. spectrum was measured in chloroform solution (Fig. 7). τ values are shown in Table II.

The fractions 41 to 62 were combined and the residue left after the removal of the solvent was dissolved in methanol-benzene. This solution when subjected to slow spontaneous evaporation gave crystalline β-dihydroannofoline, m.p. 198-200°. β-Dihydroannofoline after sublimation in vacuum (140-150° at 0.025 mm.) melted at 200-203°. Yield, 12 mg.

Calc. for $C_{16}H_{27}O_2N$: C, 72.41; H, 10.26%

Found: C, 71.63, 72.77; H, 10.02, 9.82%; pKa, 10.00;

$[\alpha]_D$ -8.3° (c, 2.0 in ethanol).

The infrared spectrum in chloroform did not show any band in the carbonyl region.

12) Chromic acid Oxidation of α-Dihydroannofoline

α-Dihydroannofoline (2 mg.) was dissolved in dry pyridine (0.1 ml.) and chromium trioxide (2 mg.) was added to the above solution with shaking. The mixture was allowed to stand at room temperature for 19 hours. After this period, chromium trioxide-pyridine complex was decomposed with water.

The solution was made alkaline and the alkaloidal material was extracted with chloroform. The chloroform extract was sublimed under vacuum (110-120° at 0.03 mm.). The infrared spectrum (Infracord) of the sublimate in carbon tetrachloride solution showed bands at 3600 cm^{-1}., 3350 cm^{-1}. (broad), 1700 cm^{-1}.. Paper chromatography (pH 7.0) gave the following results:

Sample	R_f	Relative amount
Annofoline	0.44	
α-Dihydroannofoline	0.53	
Oxidation product		
	0.30	1
	0.53	3
	0.43	30

13) <u>Chromic acid Oxidation of β-Dihydroannofoline</u>

β-Dihydroannofoline (2 mg.) was oxidized in exactly the same way as described above for α-dihydroannofoline. The infrared spectrum (Infracord) of the oxidation product in chloroform solution showed a band at 1700 cm^{-1}.. Paper chromatography (pH 7.0) gave the following results:

Sample	R_f	Relative amount
Annofoline	0.44	
β-Dihydroannofoline	0.37	
Oxidation product		
	0.64	20
	0.82	1

14) Selenium dioxide Oxidation of Annofoline

Annofoline (200 mg.) was dissolved in dioxane (30 ml.) and to this solution was added a solution of selenium dioxide (200 mg.) in aqueous dioxane (water 0.5 ml., dioxane 30 ml.). The mixture was refluxed for twenty hours, cooled and filtered through a layer of Celite. The solvent was removed by evaporation and the residue was dissolved in dilute hydrochloric acid. The aqueous solution was extracted with chloroform and the chloroform extract was in turn extracted with dilute acid. All the aqueous extracts were combined, made alkaline and the alkaloidal material was extracted with chloroform. The chloroform extract was sublimed under vacuum (110-120° at 0.03 mm.). Yield 100 mg. Dehydroannofoline was crystallized from ether. After three crystallizations from ether, it melted at 162-164°. Yield 16 mg. For analysis the sample was sublimed.

Calc. for $C_{16}H_{23}O_2N$: C, 73.53; H, 8.87%.

Found: C, 73.23; H, 8.73%.

The infrared spectrum (Infracord) in carbon tetrachloride solution showed bands at 3550 cm^{-1}, 3400 cm^{-1}, 3000 cm^{-1}, 1670 cm^{-1}. Ultraviolet spectrum, Fig. 8, (λ_{max} 241 mμ; log ϵ 3.8) was measured in ethanol. N.M.R. spectrum, Fig. 9, was measured in $CDCl_3$ solution. Paper chromatography (pH 7.0) gave the following results:

Sample	R_f
Annofoline	0.38
Dehydroannofoline	0.69

The mother liquors from the crystallizations of dehydroannofoline were combined and paper chromatography (pH 7.0) was carried out on the combined mother liquors. The sample was applied on the paper as two spots side by side. After developing the chromatogram, it was divided into two halves each corresponding to one of the samples. The alkaloidal spots on one of these papers when revealed by the modified Dragendorff's reagent showed the presence of eight compounds. The second part of the chromatogram was divided into eight parts each corresponding to one of the compounds on the first half of the chromatogram. Each part of paper was extracted separately with methanol. The residue left after the removal of methanol was extracted with dilute hydrochloric acid. The acid extract was made alkaline and extracted with chloroform. Ultraviolet spectra in ethanol solution on the different fractions were measured. The following results were obtained.

Fraction no.	R_f	Relative amount	λ_{max} (mμ)
1	0.87	8	
2	0.72	1	
3	0.69	16	241
4	0.56	4	
5	0.48	8	
6	0.39	4	252
7	0.29	2	254
8	0.22	2	253
Annofoline	0.38		

15) <u>Treatment of Dehydroannofoline with Acid</u>

Dehydroannofoline (2 mg.) was dissolved in 10% hydrochloric acid (1 ml.) and the solution was refluxed for ten hours. The solution was cooled, made alkaline with sodium hydroxide solution and extracted with chloroform. The chloroform extract showed bands in the infrared region (Infracord) at 3500 cm^{-1}., 3400 cm^{-1}., 1700 cm^{-1}., 1650 cm^{-1}.. Paper chromatography (pH 7.0) gave the following results:

Sample	R_f	Relative amount
Dehydroannofoline	0.76	
Reaction product		
	0.75	10
	0.86	1

16) Treatment of Dehydroannofoline with alkali

Dehydroannofoline (2 mg.) was dissolved in ethanol (0.1 ml.) and it was refluxed with a 10% aqueous sodium hydroxide solution for a period of nine hours. The solution was cooled and the alkaloidal material was extracted with chloroform. The infrared spectrum (Infracord) in carbon tetrachloride solution on the chloroform extract showed bands at 1700 cm^{-1}., 1650 cm^{-1}., 3500 cm^{-1}., 3400 cm^{-1}.. Paper chromatography (pH 7.0) gave the following results:

Sample	R_f	Relative amount
Dehydroannofoline	0.76	
Reaction product		
	0.77	10
	0.87	1

17) **Attempted Hofmann degradation of Dehydroannofoline Methiodide** - To a solution of dehydroannofoline (2 mg.) in acetone methyl iodide was added. The methiodide crystallized immediately. The solvent was removed, and the residue was dissolved in water. The methiodide was converted to the corresponding hydroxide by passing the aqueous solution of

the methiodide through an anion exchange column (Dowex-1 in hydroxide form). The eluate showed a maximum at 245 mμ in the ultraviolet region. The solution was concentrated to 0.5 ml. and refluxed for two hours, cooled and diluted. The ultraviolet spectrum of it showed a maximum at 245 mμ. The volume of the solution was again reduced to 0.5 ml., added 2 drops of sodium hydroxide solution (5%) and the mixture was refluxed for ten hours. The solution was cooled, extracted with chloroform. The ultraviolet spectrum (in ethanol) of the chloroform extract did not show any selective absorption.

18) Annofoline ethylthio-enol ether - To a solution of annofoline (200 mg.) in glacial acetic (2 ml.), ethanethiol (3 ml.) and anhydrous zinc chloride (300 mg.) were added. The mixture was allowed to stand at room temperature with frequent shaking. After a period of thirty four hours, the mixture was made alkaline with a solution of sodium hydroxide and the alkaloidal material was extracted with chloroform. Yield, 220 mg. Paper chromatography (pH 5.0) gave the following results:

Sample	R_f
Annofoline	0.17
Reaction product	0.63

Annofoline ethylthio-enol ether was crystallized from methanol-pentane, m.p. 140-150°. After two recrystallizations from ether it melted at 152-153°. Yield, 50 mg.
Calc. for $C_{18}H_{29}ONS$: C, 70.32; H, 9.51; S, 10.41%.
Found: C, 69.89; H, 9.65; S, 9.28%.
The infrared spectrum in chloroform solution showed a faint band at 1625 cm^{-1}., in carbon tetrachloride solution it showed a band at 3600 cm^{-1}. N.M.R. spectrum, Fig. 10, was measured in carbon tetrachloride solution (25 mg. in 0.35 ml.). The τ values were calculated, Table II.

19) Desulphurization of Annofoline ethylthio-enol ether

All the mother liquors from the crystallization of annofoline ethylthio-enol ether (see above) were combined and the solvent was removed. The residue (160 mg.) was dissolved in ethanol (25 ml.) and refluxed with Raney nickel (2 g.) for sixteen hours. The Raney nickel was filtered and washed with ethanol until the washings gave no alkaloid test. All the ethanol extracts were combined and the solvent was removed. The residue was sublimed under vacuum (110-120° at 0.03 mm.). Yield, 100 mg. The sublimate did not crystallize from methanol, methanol-ether, methanol-acetone or methanol-pentane. It was chromatographed on alumina (15 g., containing 5% water). The solvents used for elution were petroleum ether (b.p. 30-65°)-benzene, benzene, benzene-chloroform and chloroform-methanol; 10 ml. fractions were collected and paper chromatography (pH 7.0) gave the following results:

Fraction no.	Eluant	Paper chromatography R_f, relative amount		
		0.62 0.67	0.71	0.82
Annofoline ethyl thio-enol ether	-		R_f, 0.71	
1-20	Pet. ether-Benzene (1:1)	-	-	-
21-41	Pet. ether-Benzene (3:7)	-	-	-
42-74	Benzene	-	-	-
75-88	Benzene-chloroform (4:1)	-	-	-
92-101	Benzene-chloroform (4:1)	10-20	-	4-2
107	Benzene-chloroform (4:1)	1	-	-
113-116	Benzene-chloroform (1:1)	-	1-4	-
119-129	Benzene-chloroform (1:1)	-	-	-
130-141	Chloroform	-	-	-
142-146	Chloroform-methanol (9:1)	-	-	-

The fractions 96-105 were combined and the solvent was removed. The residue was dissolved in methanol and converted into the hydrobromide. The hydrobromide crystallized when ether was added to the above solution. Yield, 5 mg., m.p. 298-300°.

20) Wolf-Kishner Reduction of Annofoline - A mixture of annofoline (102 mg.), diethylene glycol (1 ml.) and hydrazine (95%, 1 ml.) was placed in a flask fitted with a reflux condenser. The solution was heated at 130° for half an hour. The condenser was then removed, potassium hydroxide (0.2 g.) was added to the solution, the temperature of the solution was raised to 200° and the condenser was replaced. The mixture was heated at this temperature for an hour. It was cooled, diluted with water and the alkaloidal material was extracted. Yield, 93 mg. The infrared spectrum (Infracord) in carbon tetrachloride solution showed a band at 3600 cm^{-1}. and no band in the carbonyl region. Paper chromatography (pH 7.0) gave the following results:

Sample	R_f
Annofoline	0.37
Reaction product	0.66

The chloroform extract was sublimed under vacuum. The sublimate was slightly yellow in colour. It was chromatographed on alumina (15 g. containing 5% water). The solvents used for elution were benzene (100 ml) and benzene-chloroform (1:1; 500 ml.). Dihydrodeoxyannofoline was crystallized from ether. After two recrystallizations from the same solvent it melted at 189-190°. For analysis the sample was sublimed under vacuum (120-130° at 0.02 mm).

Calc. for $C_{16}H_{27}ON$: C, 77.06; H, 10.91%.

Found: C, 76.68, H, 10.87%; pKa, 10.40; $[\alpha]_D$ -42.1° (c, 2.0 in ethanol).

The infrared spectrum (Infracord) in carbon tetrachloride solution showed band at 3600 cm^{-1}. but no band in the carbonyl region. N.M.R. spectrum, Fig. 11, was measured in chloroform solution (20 mg. in 0.2 ml. $CHCl_3$) with tetramethylsilane as internal reference. The τ values were calculated, Table II.

The above Wolf-Kishner reduction was also carried out at a higher temperature and for a longer period. The mixture of annofoline, diethylene glycol and hydrazine was heated at 130° for five hours, potassium hydroxide was added to the above mixture and the temperature of the solution was raised to 230°. The mixture was heated at this temperature for a period of three and a half hours and the alkaloidal material was extracted as in the above case. Paper chromatography (pH 7.0) on the chloroform extract gave the following results:

Sample	R_f	Relative amount
Annofoline	0.39	
Reaction product		
	0.70	4
	0.87	1

The infrared spectrum in chloroform solution showed a weak band at 1700 cm^{-1}.

The chloroform extract was chromatographed on alumina (15 g., containing 5% water). The solvent used for elution were benzene, benzene-chloroform, chloroform and chloroform-methanol; 20 ml. fractions were collected and the following results were obtained by paper chromatography (pH 7).

Fraction no.	Eluant	Paper chromatography R_f, relative amount			
		0.48	0.65 0.71	0.84 0.89	0.97
Annofoline	-	(R_f 0.37)			
1-2	Benzene	-	-	-	-
3-6	Benzene	-	-	4-20	-
9-15	Benzene	-	1-4	10-15	-
18	Benzene-chloroform (9:1)	-	20	3	-
21-24	Benzene-chloroform (9:1)	-	30-50	-	10
27-39	Benzene-chloroform (9:1)	-	50-70	-	-
42	Benzene-chloroform (7:3)	-	10	-	-
45	Benzene-chloroform (7:3)	5	-	-	-
50-56	Benzene-chloroform (2:3)	-	-	-	-
57-60	Chloroform	-	-	-	-
61-65	Chloroform-methanol (19:1)	-	-	-	-

The fractions 3 to 12 were combined. The residue left after the removal of the solvent was sublimed under vacuum (120-130° at 0.05 mm.). The light yellow sublimate did not crystallize from ether, benzene, methanol or pentane. It was converted to hydrobromide by adding concentrated hydrobromic acid to a solution of the base in methanol. The hydrobromide crystallized when ether was added to the above solution. The crystalline hydrobromide was converted to the free base in the usual way but the base could not be induced to crystallize. The infrared spectrum of the free base in carbon tetrachloride solution showed band at 1700 cm^{-1}. but no band in the hydroxyl region. Paper chromatography (pH 7.0) showed that it had R_f value (0.65) similar to deoxyannofoline (R_f, 0.64; page 77). Dihydrodeoxyannofoline was isolated from the fractions 27 to 43.

21) O-Acetyldihydrodeoxyannofoline - Dihydrodeoxyannofoline (50 mg.) was dissolved in benzene (2 ml.) and the solution was refluxed with acetic anhydride (0.1 ml.) for ten hours. The solution was cooled and excess of acetic anhydride was decomposed with methanol. The solvent was removed and the dark brown residue when sublimed under vacuum (110-120° at 0.03 mm.) gave a colourless oily sublimate. Yield, 58 mg. The sublimate did not crystallize from methanol, ether, benzene, ether-pentane, acetone and acetone-pentane. Paper chromatography (pH 4.0) on the sublimate gave the

following results:

Sample	R_f
Dihydrodeoxyannofoline	0.71
Acetylated product	0.78

The infrared spectrum (Infracord) in carbon tetrachloride solution showed band at 1730 cm^{-1}. but no band in the hydroxyl region. N.M.R. spectrum, Fig. 12, was measured in chloroform solution.

The oily O-acetyldihydrodeoxyannofoline was then converted to hydrobromide by adding a solution of hydrobromic acid in acetone to a solution of the base in acetone. The salt, which crystallized immediately, was filtered off and washed with cold acetone. Yield, 30 mg., m.p. 302-304°. For analysis the sample was dried under vacuum (117°, 0.03 mm.).
Calc. for $C_{18}H_{29}O_2N \cdot HBr$: C, 58.04; H, 8.12%.
Found: C, 58.20; H, 8.27%.

22) Attempted Tosylation of Dihydrodeoxyannofoline

To a solution of dihydrodeoxyannofoline (2 mg.) in dry pyridine (0.1 ml.), p-toluene sulphonyl chloride (10 mg.) was added. The mixture was allowed to stand at room temperature. After a period of seventeen hours, the solvent was removed by evaporation under a stream of nitrogen. The residue was dissolved in methanol and the solvent was removed. The procedure was repeated three times more. Sodium hydroxide solution was added to the residue and the alkaloidal material was extracted with chloroform. The infrared spectrum (Infracord) in carbon tetrachloride solution showed band at 3500 cm^{-1}. Paper chromatography (pH 5.0) gave the following results:

Sample	R_f	Relative amount
Dihydrodeoxyannofoline	0.73	
Reaction product		
	0.69	60
	0.79	1

23) <u>Chromic Acid Oxidation of Dihydrodeoxyannofoline</u>

Chromium trioxide (95 mg.) was added portionwise to pyridine (1 ml.) with shaking after each addition. To the slurry thus produced, was added a solution of dihydrodeoxyannofoline (95 mg.) in pyridine (1 ml.). The mixture was allowed to stand at room temperature for thirty five hours. The chromium trioxide-pyridine complex was decomposed by adding water to the above mixture. The mixture was made alkaline and the alkaloidal material was extracted with chloroform. The chloroform extract was sublimed under vacuum (80-90° at 0.025 mm.). Yield, 73 mg. Paper chromatography (pH 4.0) gave the following results:

Sample	R_f
Dihydrodeoxyannofoline	0.75
Oxidation product	
	0.60

The sublimate was chromatographed on alumina (10 g.) using benzene as the eluant. Deoxyannofoline travelled almost along with the solvent front. Deoxyannofoline, obtained after the removal of the solvent, was sublimed under vacuum (80-90° at 0.025 mm.). Yield, 61 mg., m.p. 75-85°. It was converted to the hydrobromide by adding concentrated hydrobromic acid to a solution of deoxyannofoline in acetone. The hydrobromide crystallized immediately, and was recrystallized from methanol-acetone. Yield, 48 mg., m.p. 305° (decomp.). The hydrobromide was converted to the free base which was sublimed under vacuum (80-90° at 0.025 mm.). Yield, 30 mg., m.p. 88-92°. Crystallizations of deoxyannofoline from n-pentane did not change the melting point.

Calc. for $C_{16}H_{25}ON$: C, 77.68; H, 10.19%.

Found: C, 78.00; H, 10.40%. pKa, 8.40.

The infrared spectrum (Infracord), Fig. 13, in Nujol mull showed band at 1690 cm^{-1}. Ultraviolet spectrum, Fig. 14, of the free base [λ_{max} 288 mμ, ϵ, 109] and also of the salt [λ_{max} 284 mμ, ϵ, 33] were measured in ethanol. N.M.R. spectrum, Fig. 15, was measured in chloroform solution (20 mg. in 0.25 ml.) with tetramethyl silane as internal reference. The τ values were calculated, Table II.

24) <u>Treatment of Deoxyannofoline with Acid</u> - Deoxyannofoline was refluxed with 10% HCl (0.5 ml.) for five hours. The solution was cooled, made alkaline with sodium hydroxide

solution and the alkaloidal material was extracted with chloroform. The infrared spectrum (Infracord) in carbon tetrachloride solution showed band at 1690 cm^{-1}. Paper chromatography (5.0) gave the following results:

Sample	R_f
Deoxyannofoline	0.59
Reaction product	
	0.59

25) <u>Treatment of Deoxyannofoline with Alkali</u>

Deoxyannofoline (2 mg.) was dissolved in ethanol (0.1 ml.) and the solution was refluxed with 5% sodium hydroxide solution (0.5 ml.) for five hours. The solution was cooled and the alkaloidal material was extracted with chloroform. The infrared spectrum (Infracord) in carbon tetrachloride solution showed band at 1690 cm^{-1}. Paper chromatography (pH 5.0) gave the following results:

Sample	R_f	Relative amount
Deoxyannofoline	0.61	
Reaction product		
	0.61	99
	0.94	1

26) <u>Sodium borohydride Reduction of Deoxyannofoline</u>

Deoxyannofoline (5 mg.) was dissolved in ethanol (1 ml.). The solution was refluxed for two hours with a solution of sodium borohydride (5 mg.) in ethanol (1 ml.). The solution was cooled and worked up in the same way as described on page no. 60. The alkaloidal extract showed a band at 3500 cm^{-1} in the infrared spectrum (infracord) in carbon tetrachloride solution but no band in the carbonyl region. Paper chromatography (pH 4.0) gave the following results:

Sample	R_f
Dihydrodeoxyannofoline	0.74
Deoxyannofoline	0.63
Reduction product	0.77

27) Attempted Hofmann Degradation of Deoxyannofoline Methiodide - To a solution of deoxyannofoline (2 mg.) in acetone, methyl iodide was added and deoxyannofoline methiodide crystallized immediately. The solvent was removed by evaporation and the residue was dissolved in water. The methiodide was converted to the corresponding hydroxide by passing the aqueous solution of the methiodide through an anion exchange column (Dowex - 1 in hydroxide form). The eluate was concentrated to approximately 3 ml. The ultraviolet spectrum of this solution did not show any selective absorption. The solution was further concentrated to 0.5 ml. and was refluxed with sodium hydroxide solution (5%) for ten hours. The solution was cooled and extracted with chloroform. The chloroform extract still did not show any absorption in the ultraviolet region.

28) Attempted Wolf-Kishner Reduction of Deoxyannofoline

Diethylene glycol (0.1 ml.) was heated to 130° and deoxyannofoline (2 mg.) and hydrazine (0.1 ml.) were added. The flask was fitted with a reflux condenser and the mixture was heated at 130° for half an hour. The condenser was removed, potassium hydroxide (approximately 2 mg.) was added to the above mixture and the temperature of the solution was raised to 200°. The condenser was replaced and the mixture was heated at this temperature for one and half an hour. It was cooled, diluted with water and the alkaloidal material was extracted with chloroform. The infrared spectrum (Infracord) of the chloroform extract in carbon tetrachloride solution showed bands at 3525 cm^{-1}., 3300 cm^{-1}., 1700 cm^{-1}. (weak). Paper chromatography (pH 4.0) showed a long tail.

29) Attempted Preparation of Deoxyannofoline ethylene dithioketal

a) First attempt - A mixture of deoxyannofoline (2 mg.), glacial acetic acid (0.1 ml.), 1,2-ethanedithiol (two drops) and anhydrous zinc chloride (2 mg.) was allowed to stand at room temperature with frequent shaking. After a period eighteen hours, the mixture was made alkaline and the alkaloid was extracted with chloroform. Paper chromatography (pH 4.0) gave the following results:

Sample	R_f	Relative amount
Deoxyannofoline	0.27	
Reaction product		
	0.31	2
	0.48	1
	0.83	6

The infrared spectrum (Infracord) in carbon tetrachloride solution showed bands at 1725 cm^{-1}. and 1690 cm^{-1}. The above experiment was repeated but this time 1,2-ethanedithiol was omitted. The infrared spectrum (Infracord) of the alkaloidal extract in carbon tetrachloride solution showed band at 1690 cm^{-1}. but no band at 1725 cm^{-1}.

b) Second attempt - A mixture of deoxyannofoline (2 mg.), anhydrous zinc chloride (2 mg.), anhydrous sodium sulphate (2 mg.), 1,2-ethanedithiol (0.1 ml.) and dioxane (0.2 ml.) was allowed to stand at room temperature with frequent shaking for 22 hours. The mixture was then made acidic with dilute hydrochloric acid and extracted with chloroform. The chloroform extract was in turn extracted with dilute acid. All the acidic extracts were combined, made alkaline with sodium hydroxide solution and the alkaloidal material was extracted with chloroform. Paper chromatography (pH 5.0) gave the following results:

Sample	R_f	Relative amount
Deoxyannofoline	0.60	
Reaction product		
	0.63	2
	0.82	1
	0.89	1

The infrared spectrum (Infracord) in chloroform solution showed a weak band at 1720 cm^{-1}. and a shoulder at 1700 cm^{-1}.

c) Third attempt - To a solution of deoxyannofoline (2 mg.) in benzene (0.5 ml.) were added 1,2-ethanedithiol (0.2 ml.) and p-toluene sulphonic acid (2 mg.). The mixture was refluxed for five hours and the solvent was removed by evaporation under nitrogen. The residue was dissolved in water. The solution was made alkaline with sodium hydroxide and the alkaloidal material was extracted with chloroform. Paper chromatography (pH 5.0) gave the following results:

Sample	R_f	Relative amount
Deoxyannofoline	0.57	
Reaction product		
	0.91	3
	0.81	1

The infrared spectrum (infracord) in chloroform solution showed a weak band at 1700 cm^{-1}.

30) Selenium dioxide Oxidation of Deoxyannofoline

To a solution of deoxyannofoline (10 mg.) in dioxane (0.5 ml.) was added a solution of selenium dioxide (40 mg.) in aqueous dioxane (water .2 ml, dioxane 0.5 ml.). The mixture was refluxed on a hot plate for 40 hours. The solution was cooled and the black selenium was filtered off. The residue was washed with methanol. The washings were mixed with the main filtrate and the solvent was removed. The dark brown residue was sublimed under vacuum (100-120°, 0.025 mm.). Yield, 4.5 mg. Paper chromatography (pH. 4.0) on the sublimate gave the following results:

Sample	R_f	Relative amount
Deoxyannofoline	0.60	
Reaction product		
	0.50	30
	0.63	1

The infrared spectrum in carbon tetrachloride showed bands at 3400 cm^{-1}, 1710 cm^{-1}, 1660 cm^{-1}, 1640 cm^{-1}. The ultraviolet spectrum in ethanol showed absorption at 2[illegible] mμ. In alkaline solution the position of the maximum shifted to 335 mμ and also the intensity of absorption was greatly decreased. The crude oxidation product gave a greenish-brown colouration with ferric chloride solution.

(d) Chemistry of Lycofoline

1) Characterization of Lycofoline - Lycofoline, m.p. 144-145°, was soluble in methanol, chloroform, benzene, ether and acetone. A sublimed sample was used for analysis.

Calc. for $C_{16}H_{25}O_2N$: C, 72.96; H, 9.57; N, 5.32; C-CH_3, 5.69%; M.W., 263.4.

Found: C, 72.77, 72.66; H, 9.51, 9.59; N, 5.11; C-CH_3, 4.96%; M.W. (Rast), 239; E.W. 264, 267; pKa, 9.05, 9.10; $[\alpha]_D$ -74.0 (c, 2.0 in ethanol).

The infrared spectrum (Infracord), Fig. 16, in Nujol mull showed bands at 3300 cm^{-1}, and a very faint band at 1670 cm^{-1}, and in CCl_4, bands at 3600 cm^{-1}, 3395 cm^{-1}. The N.M.R. spectrum, Fig. 17, was measured in chloroform solution (40 mg. in 0.45 ml.) with tetramethylsilane as internal reference. The τ values were calculated and is shown in Table II. When a solution of lycofoline in carbon tetrachloride was allowed to evaporate in presence of air, an intense crimson-red coloured residue was left.

2) Lycofoline Hydrobromide - For analysis, the hydrobromide was recrystallized from methanol-ether and dried at 117° in vacuum, m.p. 274-275°.

Calc. for $C_{16}H_{25}O_2N.HBr$: C, 56.05; H, 7.61%.

Found: C, 55.64, 55.68; H, 7.55, 7.42%.

3) Lycofoline Methiodide - Lycofoline (34 mg.) was dissolved in methanol (1.5 ml.) and the solution was refluxed for ten minutes with methyl iodide (0.5 ml.). The solvent was removed and the methiodide was crystallized from methanol. Yield, 15 mg., m.p. 263-264°.
Calc. for $C_{16}H_{25}O_2N.CH_3I$: C, 50.37; H, 6.94; I, 31.31%.
Found: C, 50.65; H, 6.99; I, 31.58%.

4) Treatment of lycofoline with Acid - Lycofoline (5 mg.) was refluxed with hydrochlorid acid (10%, 2 ml.) for six hours. The solution was cooled, made alkaline with sodium hydroxide and extracted with chloroform. The infrared spectrum (Infracord) in carbon tetrachloride solution showed a band at 3500 cm^{-1}. and a very faint band at 1700 cm^{-1}.. Paper chromatography (pH 7.0) gave the following results:

Sample	R_f	Relative amount
Lycofoline	0.54	
Reaction product		
	0.87	5
	0.95	1

5) Treatment of lycofoline with Sodium borohydride

A solution of lycofoline (20 mg.) in ethanol (0.2 ml.) was added to a solution of sodium borohydride (50 mg.) in ethanol (2 ml.). The mixture was refluxed for two hours and cooled. The excess sodium borohydride was decomposed with dilute acetic acid (1:1) and most of the ethanol was removed by evaporation. The solution was diluted with water, made alkaline and the alkaloidal material was extracted with chloroform. Paper chromatography (pH 7.0) gave the following results:

Sample	R_f
Lycofoline	0.48
Reaction product	0.48

6) <u>Mono-O-Acetyl Lycofoline</u> - Lycofoline (50 mg.) was dissolved in dry pyridine (0.3 ml), and to this solution acetic anhydride (0.3 ml.) was added. The mixture was allowed to stand at room temperature for five hours. The excess of acetic anhydride was decomposed with methanol and the solvent was removed by evaporation under a stream of nitrogen. The residue was sublimed under vacuum (110-120° at 0.03 mm.). Yield, 45 mg. Paper chromatography (pH 6.0) gave the following results:

Sample	R_f
Lycofoline	0.25
Reaction product	0.66

The sublimate was crystallized from acetone-pentane. Yield, 27 mg., m.p. 159-160°. For analysis the sample was sublimed.

Calc. for $C_{18}H_{27}O_3N$: C, 70.79; H, 8.91%.

Found: C, 71.03; H, 8.86; pKa, 6.70.

The infrared spectrum (Infracord) in carbon tetrachloride solution showed bands at 1730 cm^{-1}., 3600 cm^{-1}.. N.M.R. spectrum

Fig. 18, was measured in chloroform solution (15 mg. in 0.25 ml.) with tetramethylsilane as internal reference. The τ values were calculated, see Table II.

7) Di-O-Acetyl Lycofoline - To a solution of lycofoline (103 mg.) in dry pyridine (1 ml.) acetic anhydride (1 ml.) was added and the mixture was heated under nitrogen at 80-90° for seven hours. The solution was cooled, excess acetic anhydride was decomposed with methanol and the solvent was removed. The residue, when sublimed under vacuum (110-120° at 0.03 mm.), gave an oily sublimate. Yield, 117 mg. The following results were obtained by paper chromatography (pH 6.0):

Sample	R_f	Relative amount
Mono-O-acetyl lycofoline	0.61	
Reaction product		
	0.61	3
	0.83	7

The sublimate was chromatographed on alumina (25 g., containing 5% water). The solvents used for elution were benzene-petroleum ether (b.p. 30-65°), benzene, benzene-chloroform, chloroform and chloroform-methanol; 30 ml. fractions were collected. Paper chromatography (pH 6.0) gave the following results:

Fraction no.	Eluant	Paper chromatography R_f, relative amount	
		0.68	0.85-0.87
Mono-O-acetyl lycofoline		(R_f, 0.64)	
1-10	Benzene-Pet. ether (4:1)	-	-
11-14	Benzene	-	-
15-23	Benzene	-	1-10
25-29	Benzene-chloroform (4:1)	-	3
31-35	Benzene-chloroform (4:1)	1-10	1-10
37	Benzene-chloroform (1:1)	1	-
38-40	Benzene-chloroform (1:1)	-	-
41-45	Chloroform	-	-
46-47	Chloroform-methanol (19:1)	-	-

The fractions, 15 to 29, were combined and the solvent was removed. The residue on sublimation under vacuum (110-120° at 0.025 mm.) gave crystalline di-O-acetyl lycofoline. Yield, 34 mg., m.p. 94-98°. It was crystallized from ether. Yield, 17 mg., m.p. 110-115°. The melting point changed to 113-118° on sublimation. Yield, 12 mg.
Calc. for $C_{20}H_{29}O_4N$: C, 69.13; H, 8.41%.
Found: C, 68.85; H, 8.13%; pKa, 8.05.
The infrared spectrum (Infracord) in carbon tetrachloride solution showed a band at 1725 cm^{-1}., but no band in the hydroxyl region. N.M.R. spectrum (Fig. 19) was measured in carbon tetrachloride solution (5 mg. in 0.2 ml. CCl_4) with tetramethyl silane as internal reference. The τ values, Table II, were calculated.

8) <u>Dihydrolycofoline</u> - A mixture of lycofoline (35 mg.), ethanol (6 ml.) and Adams' catalyst (18 mg.) was shaken in an atmosphere of hydrogen for a period of eight hours. The catalyst was filtered off. The solvent was removed from the filtrate and the residue was sublimed under vacuum (130-140° at 0.025 mm.). Yield, 30 mg. Paper chromatography (pH 7.0) gave the following results:

Sample	R_f	Relative amount
Lycofoline	0.60	
Hydrogenation product		
	0.21	1
	0.37	90
	0.77	2

The sublimate was chromatographed on alumina (8 g., containing 5% water). The solvents used for elution were benzene, benzene-chloroform, chloroform and chloroform-methanol; 20 ml. fractions were collected. Paper chromatography (pH 7.0) gave the following results:

Fraction no.	Eluant	Paper chromatography R_f, Relative amount	
		0.42-0.44	0.36
Lycofoline	-	(R_f, 0.60)	
1-10	Benzene	-	-
11-17	Benzene-chloroform (7:3)	-	-
18-20	Benzene-chloroform (7:3)	3	-
21-28	Benzene-chloroform (2:3)	1-10	-
29-34	Benzene-chloroform (2:3)	-	-
35-41	Benzene-chloroform (1:4)	-	-
42-43	Chloroform	-	-
44	Chloroform	1	1
45-51	Chloroform	-	-
52-53	Chloroform-methanol (9:1)	-	-

Fractions 10-29 were combined and the solvent was removed. The residue after three sublimations under vacuum (130-140° at 0.025 mm.) melted at 146-147°. Yield, 12 mg.

Calc. for $C_{16}H_{27}O_2N$: C, 72.41; H, 10.26%.

Found: C, 72.12; H, 9.99%.

9) Oxidation of Lycofoline

a) Oxidation with Chromic acid - Lycofoline (2 mg.) was oxidized with chromic acid in pyridine in the same way as described in the case of α-dihydroannofoline (page 62). Paper chromatography (pH 7.0) on the oxidation product gave the following results:

Sample	R_f	Relative amount
Lycofoline	0.58	
Oxidation product		
	0.58	1
	0.87	9

The infrared spectrum in carbon tetrachloride solution showed bands at 3500 cm^{-1}, 3300 cm^{-1} and 1700 cm^{-1}.

b) Oxidation with Active Manganese dioxide

To a solution of lycofoline (2 mg.) in benzene (0.5 ml.), active manganese dioxide (40 mg.) (39) was added. The mixture was stirred for a period of sixteen hours. Manganese dioxide was filtered off and washed with methanol. The washings were combined with the main filtrate and the solvent was removed. The residue was sublimed under vacuum (110-120° at 0.025 mm.).

(e) Chemistry of Lofoline

1) Characterization of α-Lofoline - α-Lofoline, m.p. 211-212°, was soluble in methanol, chloroform, acetone, benzene. The sample for analysis was sublimed under vacuum (120-130° at 0.05 mm.).

Calc. for $C_{18}H_{29}O_3N$: C, 70.32; H, 9.51; N, 4.56; 2 C-CH_3, 9.8; O-$COCH_3$, 14.0%; M.W. 307.4.

Found: C, 70.56, 70.56; H, 9.54, 9.54; N, 4.63; C-CH_3, 10.6; $OCOCH_3$, 14.21%; M.W. (Rast), 291, 312; E.W. 310, 310; pKa, 9.70, 9.70; $[\alpha]_D$ -52.2° (c, 2.0 in ethanol).

The infrared spectrum (Infracord), Fig. 20, in Nujol mull showed bands at 3200 cm^{-1}., 1720 cm^{-1}., 1240 cm^{-1}., 1225 cm^{-1}. and in carbon tetrachloride α-lofoline showed bands at 3550 cm^{-1}. and 1725 cm^{-1}..

2) α-Lofoline Methiodide - α-Lofoline (20 mg.) was dissolved in a mixture of methanol (1 ml.) and methyl iodide (0.5 ml.) and the mixture was refluxed for ten minutes. The solvent was removed by evaporation and the methiodide was crystallized from methanol-acetone. Yield, 16 mg., m.p. 266-267°.

Calc. for $C_{18}H_{29}O_3N.CH_3I$: C, 50.78; H, 7.18; I, 28.24%.

Found, C, 51.08; H, 7.04; I, 28.04%.

3) Treatment of α-Lofoline with acid. - α-Lofoline (5 mg.) was dissolved in hydrochloric acid (10%; 2 ml.) and the solution was refluxed under nitrogen for six hours. The

solution was cooled, made alkaline with sodium hydroxide solution, and extracted with chloroform. The infrared spectrum (Infracord) in carbon tetrachloride solution showed weak bands at 1725 cm^{-1} and 1700 cm^{-1}. Paper chromatography (pH 7.0) gave the following results:

Sample	R_f	Relative amount
α-Lofoline	0.50	
Reaction product		
	0.61	10
	0.93	1

4) Treatment of α-Lofoline with alkali. - α-Lofoline was dissolved in ethanol (0.2 ml.), and to this solution, 10% sodium hydroxide solution (1 ml.) was added. The solution was refluxed for two hours, and most of the ethanol was removed by evaporation. The residue was diluted with water (2 ml.) and extracted with chloroform. Paper chromatography (pH 7.0) gave the following results.

Sample	R_f
α-Lofoline	0.50
Hydrolytic product	0.25

The hydrolytic product was dissolved in 10% hydrochloric acid (2 ml) and the solution was refluxed for six hours. The solution was then cooled, made alkaline and extracted with chloroform.

The infrared spectrum (Infracord) in carbon tetrachloride showed bands at 3600 cm^{-1}, 3400 cm^{-1}, 3150 cm^{-1}, and very weak bands at 1730 cm^{-1} and 1700 cm^{-1}. Paper chromatography (pH 7.0) gave the following results:

Sample	R_f	Relative amount
α-Lofoline	0.46	
Reaction product		
	0.46	1
	0.59	50
	0.96	5

5) <u>Characterization of β-Lofoline</u> - β-Lofoline, m.p. 166-167°, was soluble in methanol, chloroform, benzene, acetone. For analysis the sample was sublimed under vacuum (120-130° at 0.05 mm.).

Calc. for $C_{18}H_{29}O_3N$: C, 70.32; H, 9.51; N, 4.56; 2C-CH_3, 9.8; O-$COCH_3$, 14.0%. M.W. 307.4.

Found: C, 70.18, 70.14; H. 9.45, 9.69; N, 4.64; C-CH_3, 8.83; O-$COCH_3$, 15.6%; M.W. (Rast), 322; E.W., 311; pKa, 9.55; $[\alpha]_D$ -5.7 (c, 2.0 in ethanol).

The infrared spectrum (Infracord), Fig. 21, in Nujol mull showed bands at 3400 cm^{-1}, 3100 cm^{-1}, 1700 cm^{-1}, 1730 cm^{-1}, 1225 cm^{-1} and 1270 cm^{-1}, and in carbon tetrachloride solution, β-lofoline showed bands at 3525 cm^{-1} and 1720 cm^{-1}.

6) __β-Lofoline Methiodide__ - β-Lofoline (20 mg.) was dissolved in methanol (2 ml.) and methyl iodide was added to this solution. The mixture was refluxed for ten minutes, the solvent was removed and the methiodide was crystallized from methanol. Yield, 10 mg., m.p. 296-297° (decomp.). Calc. for $C_{18}H_{29}O_{3}N.\ CH_{3}I$: C, 50.78; H, 7.18%, Found: C, 50.90; H, 6.80%.

7) __Treatment of β-Lofoline with Acid__ - β-Lofoline (5 mg.) was treated with acid in the same way as α-lofoline, described on page 94 . The infrared spectrum (Infracord) in carbon tetrachloride solution showed band at 3700 cm^{-1} and a very weak band at 1700 cm^{-1}. Paper chromatography (pH 7.0) gave the following results:

Sample	R_f	Relative amount
β-Lofoline	0.43	
Reaction product		
	0.60	50
	0.51	1

8) __Treatment of β-Lofoline with Alkali__ - β-Lofoline (4 mg.) was hydrolysed in the same way as was α-lofoline (page 95). Paper chromatography (pH 7.0) gave the following results:

Sample	R_f
β-Lofoline	0.43
Hydrolytic product	0.22

The hydrolytic product of β-lofoline was treated with acid in the same way as was the hydrolytic product of α-lofoline (page 95). The infrared spectrum (Infracord) in carbon tetrachloride solution showed bands at 3600 cm^{-1}., 3400-3300 cm^{-1}. (broad) and a very weak band at 1700 cm^{-1}. Paper chromatography (pH 7.0) gave the following results:

Sample	R_f	Relative amount
α-Lofoline	0.46	
Relative product		
	0.46	2
	0.60	20
	0.96	1

9) Oxidation of Lofolines

a) Oxidation of α-Lofoline - α-Lofoline (2 mg.) was dissolved in dry pyridine (0.04 ml.) and chromium trioxide (2 mg.) was added to the solution. The mixture was allowed to stand at room temperature for 16 hours and then the chromium trioxide-pyridine complex was decomposed by adding water to the reaction mixture. The solution was made alkaline and extracted with chloroform. The chloroform extract was sublimed under vacuum (110-120° at 0.03 mm.). The infrared spectrum (Infracord) in carbon tetrachloride solution showed bands at 1740 cm^{-1}., 1700 cm^{-1}. but no absorption band in the hydroxyl region. Paper chromatography (pH 7.0) gave the following results:

Sample	R_f
α-Lofoline	0.55
Oxidation product	0.71

b) <u>Oxidation of β-Lofoline</u> - β-Lofoline (2 mg.) was oxidized exactly in the same way as α-lofoline. The infrared spectrum (Infracord) of the oxidation product in carbon tetrachloride solution showed bands at 1740 cm^{-1}., 1700 cm^{-1}., but no band in the hydroxyl region. Paper chromatography (pH 7.0) gave the following results:

Sample	R_f	Relative amount
β-Lofoline	0.51	
Reaction product		
	0.71	9
	0.51	1

c) <u>Oxidation of a mixture of α- and β-Lofoline</u>

Chromium trioxide (100 mg.) was added portionwise to dry pyridine (1 ml.), with shaking after each addition, whereby a slurry was obtained. A solution of a mixture of α-and β-lofoline (100 mg.) in dry pyridine (1 ml.) was added to the above slurry, and the mixture was allowed to stand at room temperature for a period of 24 hours. The reaction mixture was worked up in the same way as in case of α-lofoline. Yield, 73 mg. Paper chromatography (pH 7.0) gave the following results:

Sample	R_f	Relative amount
(α and β) Lofoline	0.52 (elongated spot)	
Reaction product		
	0.32	1
	0.49	25
	0.70	30
	0.87	1

The oxidation product was chromatographed on alumina (10 gm., containing 5% water). The solvents used for elution were benzene, benzene-chloroform, chloroform and chloroform-methanol; 30 ml. fractions were collected. Paper chromatography (pH 7.0) gave the following results:

Fraction no.	Eluant	R_f
(α and β) Lofoline	-	0.65 (elongated spot)
1-7	Benzene	-
8	Benzene-chloroform (9:1)	-
9-15	Benzene-chloroform (9:1)	0.81
16-18	Benzene-chloroform (9:1)	-
19-26	Benzene-chloroform (7:3)	-
27	Benzene-chloroform (2:3)	-
28-30	Benzene-chloroform (2:3)	0.65
33	Benzene-chloroform	-
34-41	Chloroform	-
42	Chloroform-methanol (9:1)	0.89
43-45	Chloroform-methanol (9:1)	-

The fractions 9 to 16 were combined and the solvent was removed. The residue after two sublimations had m.p. 129-133°. Yield, 29 mg. Paper chromatography (pH 7.0) gave the following results:

Sample	R_f
(α and β) Lofoline	0.57
Dehydrolofoline	0.74

Calc. for $C_{18}H_{27}O_3N$: C, 70.79; H, 8.91%.

Found: C, 70.77; H, 9.06%.

The infrared spectrum (Infracord) in carbon tetrachloride solution showed bands at 1745 cm^{-1}., 1720 cm^{-1}., but no band in the hydroxyl region. N.M.R. spectrum, Fig. 22, was measured in chloroform solution (20 mg., in 0.25 ml.) with tetramethyl silane as internal reference. The τ values are shown in Table II.

α-Lofoline (3.4 mg.) was oxidized in the same way as described under the oxidation of α-lofoline (page 98), except that the reaction time was twenty-four hours. The sublimate melted at 126-134°. The mixed melting point of the oxidation product with dehydrolofoline, obtained from a mixture of α-and β-lofoline, was found to be 129-136°.

β-Lofoline (5 mg.) was oxidized in the same way as described in case of α-lofoline (page 98), but the reaction time was 30 hours. The sublimate melted at 134-137°. The mixed melting point of the oxidation product with dehydrolofoline, obtained from a mixture of α-and β-lofoline was found to be 132-135°. The infrared spectra of the oxidation products of α-lofoline and β-lofoline were superimposable.

9. DISCUSSION OF RESULTS

(a) Isolation of alkaloids

This part of the thesis deals with the isolation of alkaloids from Lycopodium annotinum L. Two different batches of crude alkaloids were investigated, which resulted in the isolation of five new alkaloids. Both the batches of crude alkaloids, from which most of the annotinine had been removed by crystallization, were kindly donated by Dr. L. Marion. The first batch of crude alkaloid had been partioned by Anet and Eves (22) between chloroform and buffer of pH 6.0. The present work was concerned with the alkaloids of partition coefficient greater than one (buffer pH 6.0/chloroform) obtained in the above distribution. This fraction will be referred to as fraction B. It was known from the previous work (35) that acrifoline was present in fraction B but the nature of the other alkaloids was not known. The second batch of crude alkaloid, which had been extracted from a different lot of plant material, was treated in a slightly different way from the first batch in view of the results obtained with fraction B.

It was decided to carry out a countercurrent distribution on fraction B. Acrifoline, which was known to be present in this fraction, had a partition coefficient of about one in the system buffer pH 7.0 - chloroform (4). Therefore, the alkaloid fraction B was partitioned in the above solvent system through 57-transfers in a 60-tube Craig's machine with the buffer as the moving phase. Weight analysis gave the distribution shown in Fig. 2A. Although in this distribution the pH of the buffer changed appreciably due to the comparatively high concentration of the alkaloid, the separation obtained was not far from that expected on the basis of constent partition coefficient.

The results of paper chromatography indicated the presence of at least two alkaloids in the tubes 10-22. Of the two compounds, one was obtained crystalline. It melted at 117-118°. Although the carbon and hydrogen analysis of this compound was in good agreement with the formula $C_{17}H_{25}O_3N$., the nitrogen content was low. It was not possible to settle this point because of the lack of material. It contained at least one C-methyl group and had a pKa of 8.20. The infrared spectrum in Nujol mull showed the presence of at least one hydroxyl group [bands at 3473 cm^{-1}., 3116 cm^{-1}.] and carbonyl group [band at 1740 cm^{-1}.]. The alkaloid was slowly converted to a sodium salt by refluxing with an aqueous solution of sodium hydroxide. The relactonization, however, produced a second lactone, which on crystallization from ether-pentane melted at 209-210°. The second lactone showed a band at 1740 cm^{-1}. [carbonyl] and no band in the hydroxyl region of the infrared spectrum. The absence of the hydroxyl band indicated that during relactonization the alkaloid probably underwent dehydration. On the basis of the present study, it is not possible to say whether the original lactone hydroxyl group or the second hydroxyl group is involved in the relactonization. However, it is evident that this alkaloid contained a hydroxyl group and a lactone group. This accounts for all the three oxygen atoms present in the molecule. The infrared result [band at 1740 cm^{-1}.] suggested that the lactone group was probably present in a six membered ring. Although from the general properties of this alkaloid it appeared to be new, no trivial name was given to it because of the poor nitrogen analysis.

The infrared spectrum and paper chromatography results of the alkaloidal contents of the tubes 25 to 40 suggested the presence of at least three compounds in these tubes. Of the three alkaloids, only acrifoline (6, 34) was isolated as the hydrobromide from the tubes 26-34 and no attempt was made to isolate the other components.

The peak represented by the tubes 46 to 57 was too wide to represent a single alkaloid. The infrared spectrum of these fractions showed bands at 1740 cm^{-1}. and 1700 cm^{-1}.. The results of paper chromatography indicated the presence of at least three components. The various components making up the peak were not well resolved due to their high partition coefficients. However, an alkaloid (a-lofoline, see page 108) was readily crystallized in small yield from the alkaloidal contents of tube 49. The alkaloidal contents of the tubes 46 to 57 were mixed and subjected to another countercurrent distribution in order to improve the separation between the different components. The solvent system used for this distribution was chloroform and buffer of pH 8.0. Weight analysis gave the distribution pattern shown in Fig. 28., which showed three peaks at tube no. 28, 40 and 46. The last two peaks were not well resolved. The partition coefficients of the alkaloids corresponding to the first two peaks were calculated by using the following expression of Williamson and Craig (40)

$$N = \frac{nKr}{Kr + 1} \qquad (1)$$

where N = position of maximum

K = partition coefficient of the solute

n = total number of transfer

r = ratio of the volume of the two phases.

The partition coefficient thus obtained was used to calculate the amount of the alkaloid in a given tube relative to the amount present in the tube containing the maximum amount of the alkaloid. The following expression of Williamson and Craig (40) gives the absolute amount of each component in a tube.

$$Y = \frac{1}{2 \quad nK/(K + 1)^2} e^{-x^2/[2nK/(K + 1)^2]} \qquad (2)$$

where Y = amount of solute in a given tube.

n = total number of transfers made.

x = distance of the tube in question from the position of maximum.

K = partition coefficient of the solute.

The following expression was used to calculate the amount of the alkaloid in a given tube relative to the amount present in the tube containing the maximum amount

$$Y^1 = e^{-x^2}/[2nK/(K + 1)^2] \qquad (3)$$

where Y^1 = amount of solute present in a given tube relative to the amount present in the tube containing the maximum amount. The other notations are the same as in expression (2). The theoretical curves a and b (represented by dotted in lines), Fig. 2B, were thus obtained. The theoretical curve c was then fitted by method of trial and error keeping in mind the results of paper chromatography.

The results of infrared spectrum and paper chromatography together with the curve analysis indicated that the tubes 21 to 32 contained one main component. This component was crystallized easily. The alkaloid melted at 211-212° and its properties were unchanged after repeated recrystallizations and chromatography on alumina. The general properties (see page 154) of this alkaloid indicated it to be new, so it was called α-lofoline.

The results of paper chromatography suggested the presence of two alkaloids in the tubes 36 to 51 which were not too well resolved in the distribution. These alkaloids were best separated by chromatography on alumina. The alkaloid that was eluted first melted at 156-157° after recrystallization and sublimation. The properties of this alkaloid (see page 112) indicated it to be new and the name annofoline was given to it. The second alkaloid eluted from the alumina chromatogram when further purified through the hydrobromide, melted at 144-145°. This alkaloid also appeared to be new from its properties (see page 142) so it was named lycofoline. In the last few tubes of the distribution (Fig. 2B.) a small amount of a compound was present that showed absorption at 1740 cm^{-1}. (in CCl_4) in the infrared, but no attempt was made to isolate the compound responsible for this absorption.

It was shown by Anet and Eves (22) that the crude alkaloid mixture did not contain to any great extent alkaloids having partition coefficients near unity (buffer of pH 6.0/chloroform). The distribution pattern of Fig. 2A also showed a pronounced minimum at tube no. 42. With these facts in hand it was possible to modify the original method of preliminary separation of the alkaloid. By the modified method the second batch of crude alkaloid, mentioned earlier, was divided into three groups instead of two which would have been obtained by the original method. This was done by devising the extraction pattern shown in Fig. 3. The solvent system used was chloroform-buffer of pH 7.0. Although the number of transfers were not greatly increased, the separation was such that no major alkaloid was present in more than one group. The buffer soluble fractions B_1' to B_3' were submitted to a 60-tube countercurrent distribution using the solvent system chloroform-buffer of pH 8.2. The distribution pattern obtained is shown in Fig. 2C. The middle fractions (B_4' to B_8' and C_3' to C_8') have been found by Mr. G.W. Dong in these laboratories to contain the same alkaloids as those present in the tubes 10 to 42 of the countercurrent distribution given in Fig. 1A. Also, the chloroform soluble fractions (C_1' to C_2') have been found by Mr. M.V. Rao to contain all the annotinine, lycodine, and lycopodine present in the original mixture. Thus approximately the same separation was obtained as before but with one 60-transfer countercurrent distribution less.

A comparison of the distribution patterns of Fig. 2B and Fig. 2C shows that the peak corresponding to α-lofoline in the former is much more intense than in the latter. Moreover, the peak occuring at tubes 16 to 26 did not give pure α-lofoline easily. In fact, paper chromatography showed the presence of two alkaloids in these tubes. They were separated by careful chromatography on activated alumina. The second alkaloid after repeated recrystallizations melted at 166-167°. The general properties of this alkaloid showed it to be new. It had a close resemblance to α-lofoline so the name β-lofoline was given to it. Furthermore, it will be shown (see on page 159) that α-lofoline and β-lofoline are epimeric alcohols.

(b) Chemistry of Annofoline

Annofoline, m.p. 156-157°, $[\alpha]_D$ -131.4°, had the formula $C_{16}H_{25}O_2N$ and Kuhn-Roth analysis showed the presence of at least one C-methyl group. Titration of the alkaloid in 50% aqueous methanol gave a pKa of 9.15 and an equivalent weight which was in agreement with the above formula. Annofoline gave a perchlorate, $C_{16}H_{25}O_2N.HClO_4$, m.p. 234-236° and a methiodide, $C_{16}H_{25}O_2N.CH_3I$, m.p. 308-309°. The infrared spectrum of annofoline in Nujol mull (Fig. 4) showed bands at 3400 cm^{-1}. (hydroxyl) and 1700 cm^{-1}. (carbonyl, probably a ketone in a six-membered ring) and in carbon tetrachloride solution bands at 3600 cm^{-1}. (free hydroxyl), 3400 cm^{-1}. (bonded hydroxyl) and 1700 cm^{-1}. (carbonyl). In carbon tetrachloride solution lycopodine, acrifoline and annofoline showed bands in the carbonyl region of the infrared spectrum at 1697 cm^{-1}., 1706 cm^{-1}. and 1700 cm^{-1}. respectively. The positions of these bands indicate that the carbonyl groups in these alkaloids may be in six-membered rings. The molecular

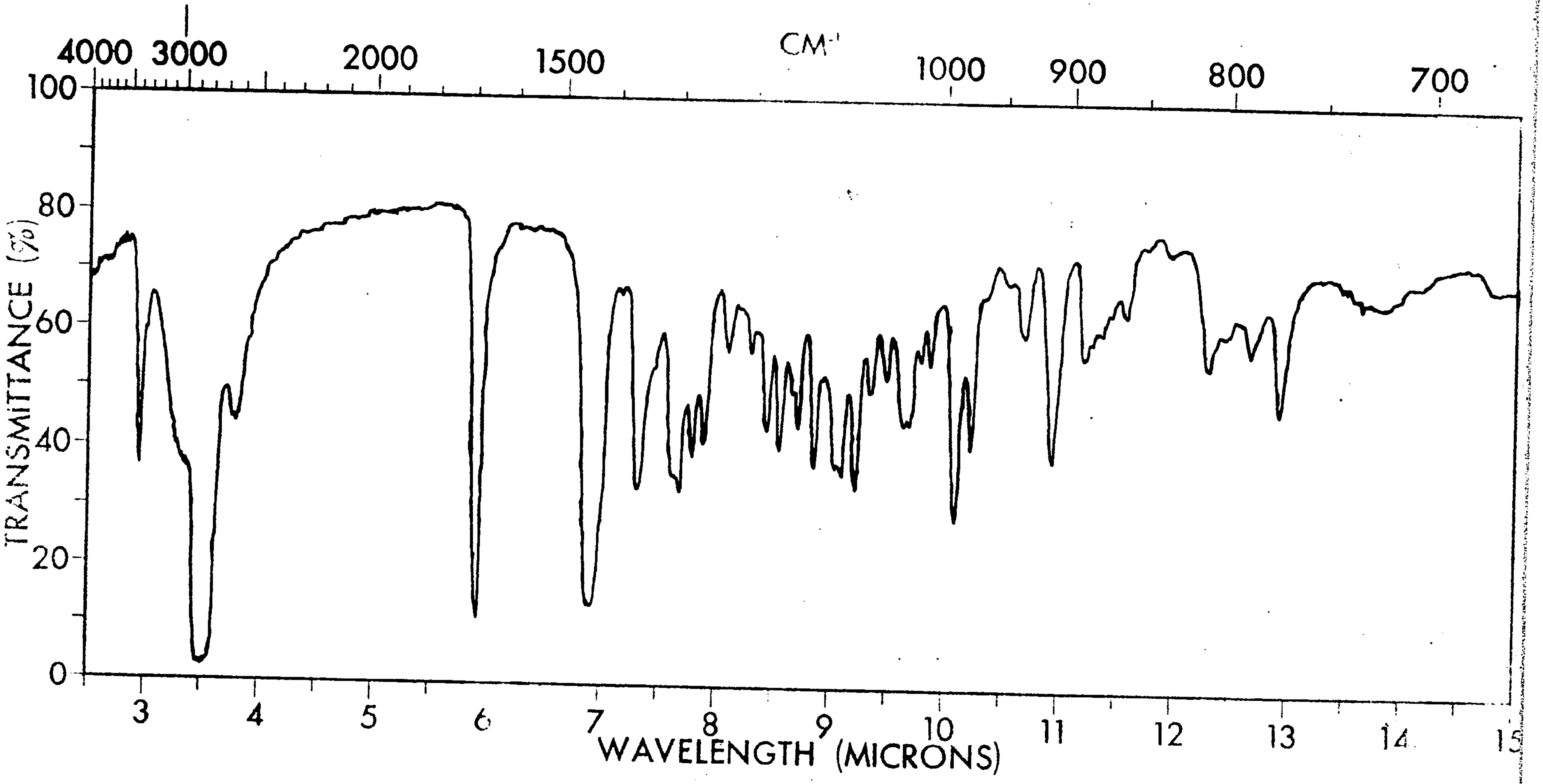


Fig. 4. Infrared absorption spectrum of annofoline in Nujol mull measured on a Perkin-Elmer "Infracord".

extinction coefficients of the carbonyl bands of the alkaloids, lycopodine, acrifoline and annofoline, were found to be 361, 184 and 156 respectively (Fig. 5). A value of 290 to 1335 for the molecular extinction coefficient, had been observed in the case of alkaloids and steroids (41, 42). In some of these compounds the carbonyl groups were present as six membered ketone. Thus, the value of the molecular extinction coefficient of lycopodine is normal. It had been shown by Perry and MacLean (34) that the infrared spectrum of crystalline acrifoline in Nujol mull showed very weak carbonyl absorption while in chloroform solution a strong band appeared in the carbonyl region. These results indicate that acrifoline crystallizes as a hemiketal and in solution, the hemiketal is converted partially into the ketonic form. By measuring the infrared spectrum of acrifoline in carbon tetrachloride solution at different time intervals it was shown by Anet and Aves (43) that the hemiketal form of acrifoline slowly changes to the ketonic form. The low molecular extinction coefficient of the carbonyl band of acrifoline may therefore, be due to the presence of a mixture of a hemiketal and a ketonic form of acrifoline. On the other hand, the infrared spectrum of annofoline in Nujol mull showed carbonyl absorption of apparently normal intensity indicating that annofoline crystallizes as a ketone. Hence, the low molecular extinction coefficient of the carbonyl band

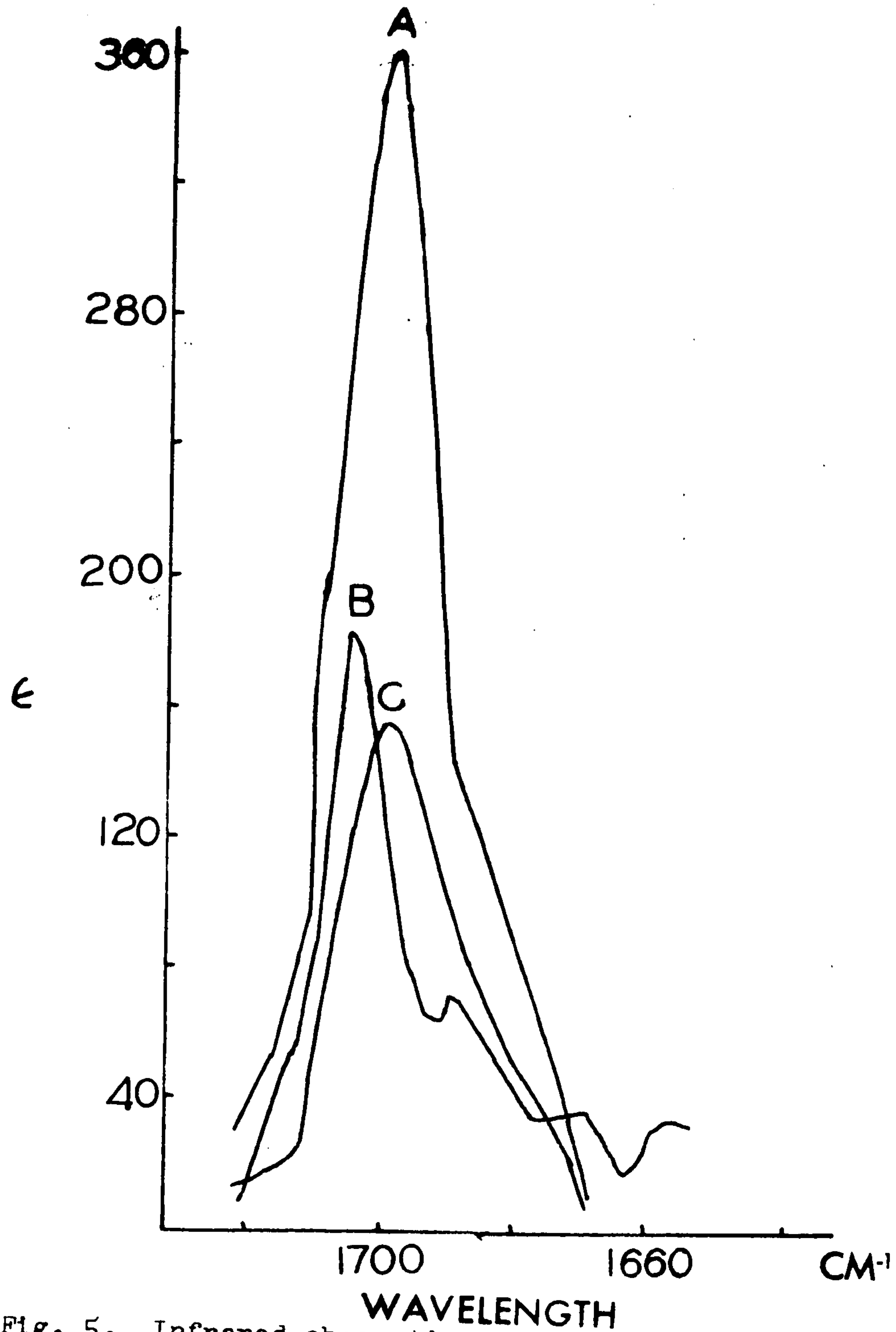


Fig. 5. Infrared absorption spectra of the carbonyl region in carbon tetrachloride solution measured on a Perkin-Elmer single beam double-pass instrument: A, lycopodine; B, acrifoline; C, annofoline. The spectra were measured in a 1 mm. cell.

of annofoline in solution may best be explained as being due to the presence of both the keto and hemiketal forms. This indicates that the carbonyl group and the hydroxyl group in annofoline are close enough to permit the formation of the hemiketal. The infrared spectra in very dilute carbon tetrachloride solution of acrifoline and annofoline in the hydroxyl region were identical. Both the alkaloids showed bands due to a free hydroxyl group and due to a bonded hydroxyl group. It is most likely that the free hydroxyl band is due to the hydroxyl group of the hemiketal form of acrifoline and annofoline. The bonded hydroxyl probably results from the keto form, where intra molecular bonding of the hydroxy and keto group is very likely.

The N.M.R. spectrum of annofoline (Fig. 6) showed a doublet at $8.97\ \tau$. This is a characteristic position for a methyl group on a saturated carbon atom (44, 45). The area under the peaks indicated the prosence of only one such group, in agreement with the Kuhn-Roth determination. The splitting of the peak into a doublet indicated the presence of a hydrogen atom on the carbon bearing the methyl group. The spacing between the peaks gave J_{CH, CH_3} = 6 c.p.s. This is in agreement with the value for the coupling constant (3 - 9 c.p.s.) observed by Slomp and McGarvey (45) in case of 6-methyl storoids. The peak at $6.93\ \tau$ is probably due to the hydrogen attached to the carbon bearing the hydroxyl

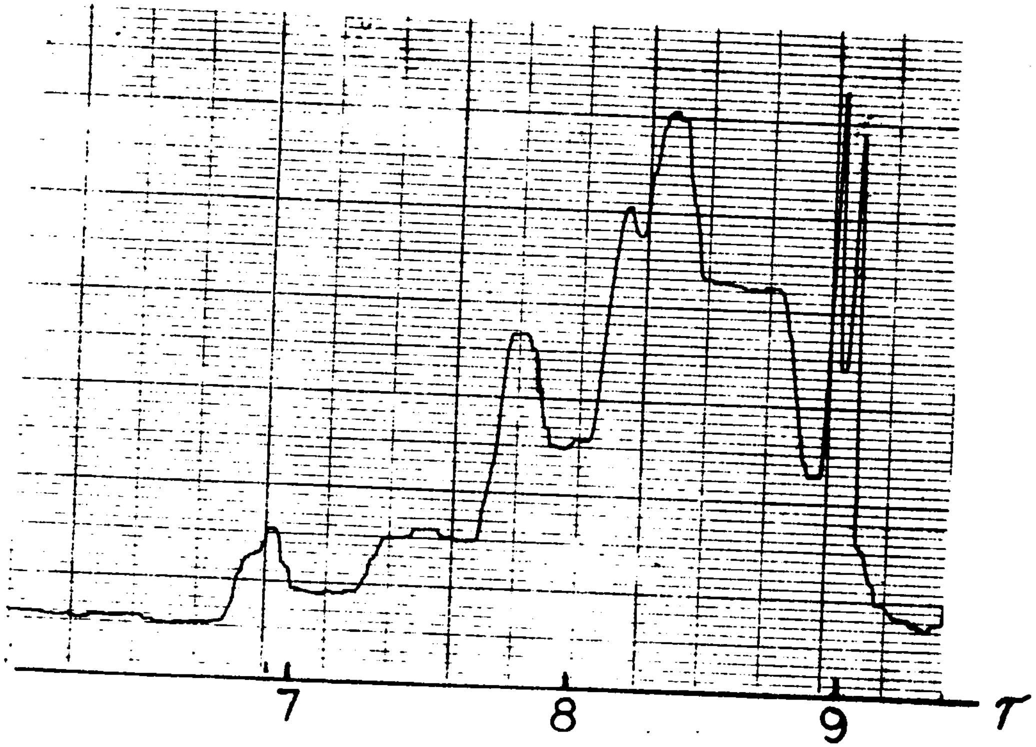


Fig. 6. Nuclear magnetic resonance spectrum of annofoline.

group. This assignment is in agreement with the results of Lemieux, Kullnig, Bernstein and Schneider (46) obtained in case of t-butyl-cyclohexyl alcohol (6.85 τ).

The behavior of annofoline towards acidic reagents was studied with the help of paper chromatography. After treatment with dilute or concentrated hydrochloric acid for five hours, annofoline was found to be mainly unchanged. On prolonged (24 hours) boiling with concentrated hydrochloric acid, annofoline gave a mixture of six compounds as shown by paper chromatography on the reaction product. One intense spot had an R_f value similar to that of annofoline. Paper chromatography on the product, obtained after treatment of annofoline with p-toluene sulphonic acid, indicated the presence of seven compounds in almost equal amounts. The infrared spectrum of the reaction product in carbon tetrachloride solution showed bands at 3594 cm^{-1}. and 1700 cm^{-1}. indicating the presence of hydroxyl and carbonyl groups. The infrared spectrum on the product obtained after treatment of annofoline with phosphorous oxychloride and pyridine showed bands at 3594 cm^{-1}. (hydroxyl), and 1700 cm^{-1}. (carbonyl). The above results indicated that annofoline yielded a complicated mixture with acidic reagents, and therefore these reactions were not further investigated.

Attempted Hofmann degradation on annofoline methiodide gave a very small amount of base which showed absorption at

257 mμ in the ultraviolet region, but most of the alkaloidal material could not be extracted from the aqueous solution. This indicated that Hofmann degradation did not produce an α,β-unsaturated ketone to any appreciable extent.

To find out a suitable method for acetylation of annofoline, various conditions were studied with the help of paper chromatography. Annofoline was refluxed for seven hours with anhydrous sodium acetate and acetic anhydride. Paper chromatography on the reaction product showed the presence of five compounds of which annofoline was present in largest amount. Under milder conditions (benzene, acetic anhydride, reflux for ten hours) about 40% of a new compound was formed and the rest was unchanged annofoline. The infrared spectrum of the reaction product in carbon tetrachloride solution showed a new band at 1742 cm^{-1} in the carbonyl region indicating the formation of an O-acetyl derivative. Therefore, toluene, having a higher boiling point than benzene, was used as a solvent and a solution of annofoline in toluene was refluxed with acetic anhydride for ten hours. Paper chromatography revealed the presence of one major compound and three other minor components in the reaction product. One of these minor compounds was probably unchanged annofoline. Chromatography of the crude acetylation product on alumina gave O-acetyl annofoline which was further purified through hydrobromide. Analysis of the hydrobromide gave the formula

$C_{18}H_{27}O_3N.HBr$. The infrared spectrum of the free base in carbon tetrachloride solution showed bands at 1750 cm^{-1}. (acetyl carbonyl) and 1712 cm^{-1}. (original carbonyl group) but no band in the hydroxyl and amide region. O-Acetyl annofoline was hydrolysed to annofoline with aqueous methanolic sodium hydroxide. This confirmed the presence of a hydroxyl group in annofoline. Since no amide was formed during acetylation and annofoline gave a true methiodide, the nitrogen atom in annofoline must be tertiary in character.

Confirmatory evidence for the presence of a keto group in annofoline came from a study of sodium borohydride reduction of annofoline. Paper chromatography of the reduction product indicated the presence of two compounds and the absence of annofoline. The two compounds were separated by chromatography on alumina, and since they were found to be isomeric alcohols, they will be referred to as α-and β-dihydroannofoline. In the reduction α-dihydroannofoline, m.p. 264-265°, was produced in larger amount (ca. 70%) than β-dihydroannofoline, m.p. 200-203°, (ca. 30%). Analysis of α-dihydroannofoline gave the formula $C_{16}H_{27}O_2N$. It had a pKa of 10.05 and $[\alpha]_D$ 0.0°. The infrared spectrum in chloroform solution did not show any band in the carbonyl region indicating the reduction of the carbonyl group. The N.M.R. spectrum of α-dihydroannofoline (Fig. 7) showed a doublet at 9.09 τ as in annofoline. The peaks at 6.36 τ, 6.27 τ and 5.93 τ

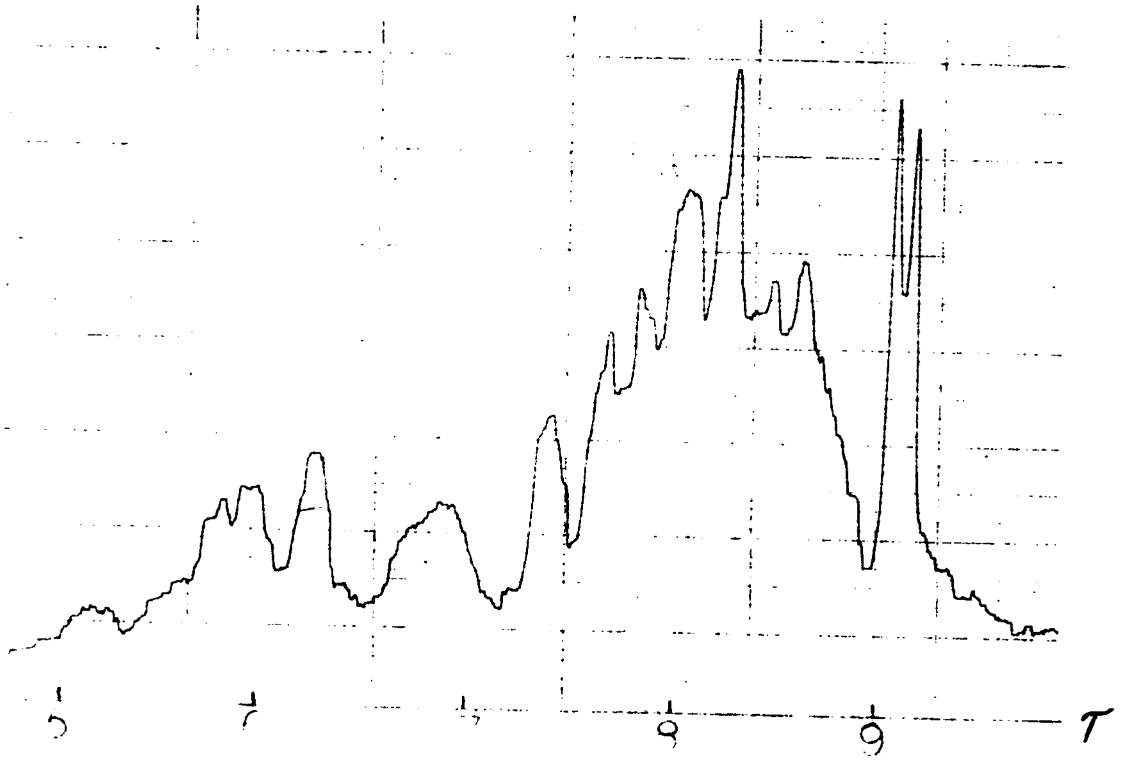


Fig. 7. Nuclear magnetic resonance spectrum of α-dihydroannofoline.

had the half width as 23 c.p.s., 10 c.p.s. and 21 c.p.s., respectively. Due to the low solubility of the compound, it was not possible to obtain a better resolution in this region of spectrum. In fact, the acetates of dihydroannofoline would be better compounds for N.M.R. study. β-Dihydroannofoline, $C_{16}H_{27}O_2N$, had pKa, 10.00 and $[\alpha]_D$ -8.3°. The infrared spectrum in chloroform solution did not show any band in the carbonyl region. The properties of α and β-dihydroannofoline were different from the two isomeric alcohols obtained from acrifoline (34).

Preliminary experiments using paper chromatography showed that chromic acid oxidation product of α-dihydroannofoline contained one major compound and two other minor components. One of the minor compounds (R_f 0.53) was probably unchanged α-dihydroannofoline. The major product of the oxidation had an R_f value similar to that of annofoline. The infrared spectrum (in carbon tetrachloride) of the oxidation product showed bands at 3600 cm^{-1} (free OH), 3350 cm^{-1} (bonded -OH) and 1700 cm^{-1} (carbonyl) indicating that one of the hydroxyl groups was oxidized to a carbonyl group. On the other hand, β-dihydroannofoline under similar conditions gave two products as shown by aper-chromatography. The infrared spectrum in chloroform solution showed band at 1700 cm^{-1} indicating the formation of a carbonyl group. Paper chromatography showed that the oxidation products obtained from β-dihydroannofoline were entirely different from those produced by oxidation of α-dihydroannofoline. Whilst these experiments were not pursued, they are worthy of further study.

Selenium dioxide oxidation of annofoline gave a complicated mixture. However, the major product of this reaction was easily crystallized. It was found to contain two hydrogen atoms less than annofoline and was therefore called dehydroannofoline. Dehydroannofoline melted at 162-164° and had the formula $C_{16}H_{23}O_2N$. The infrared spectrum in carbon tetrachloride solution showed bands at 3550 cm^{-1}. (free hydroxyl group), 3400 cm^{-1}. (bonded hydroxyl group), 3000 cm^{-1}. (olefinic C - H) 1670 cm^{-1}. (conjugated carbonyl group). The ultraviolet spectrum of dehydroannofoline (Fig. 8) showed absorption at 241 mμ (log ε 3.8). Thus, the spectroscopical data indicates that dehydroannofoline is an α, β-unsaturated ketone.

The N.M.R. spectrum of dehydroannofoline (Fig. 9) was most instructive. In the first place, the doublet at about 9 τ, present in the spectrum of annofoline (Fig. 6), was absent but there was a new intense peak at 8.04 τ. This is a typical position for a methyl group attached to a carbon bearing a double bond (44). The shift of the methyl peak towards lower field and the fact that the peak is unsplit, suggests that the carbon atom bearing the methyl group must be

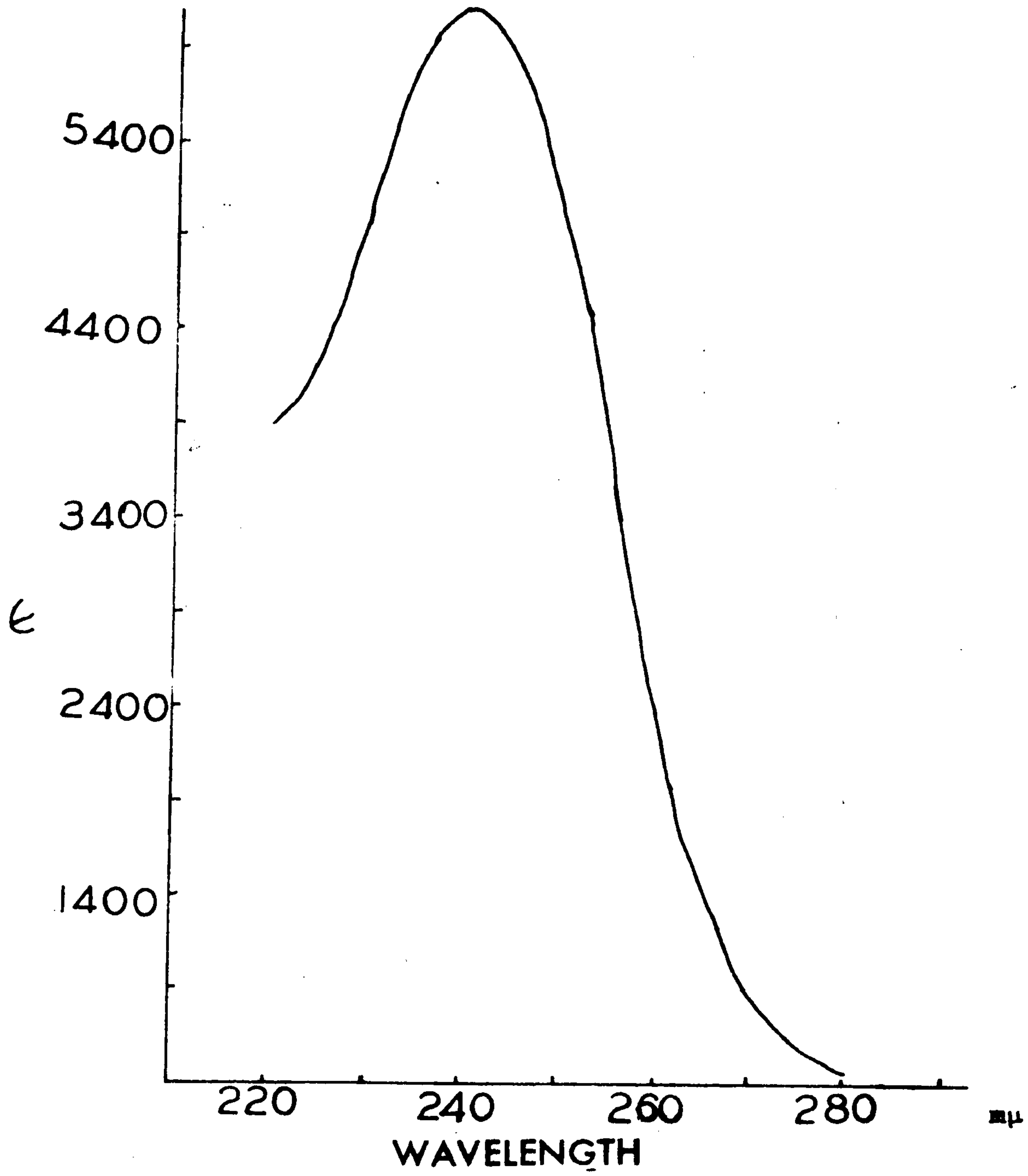


Fig. 8. Ultraviolet absorption spectrum of dehydroannofoline in ethanol solution measured on a Beckman DK-2 spectrophotometer.

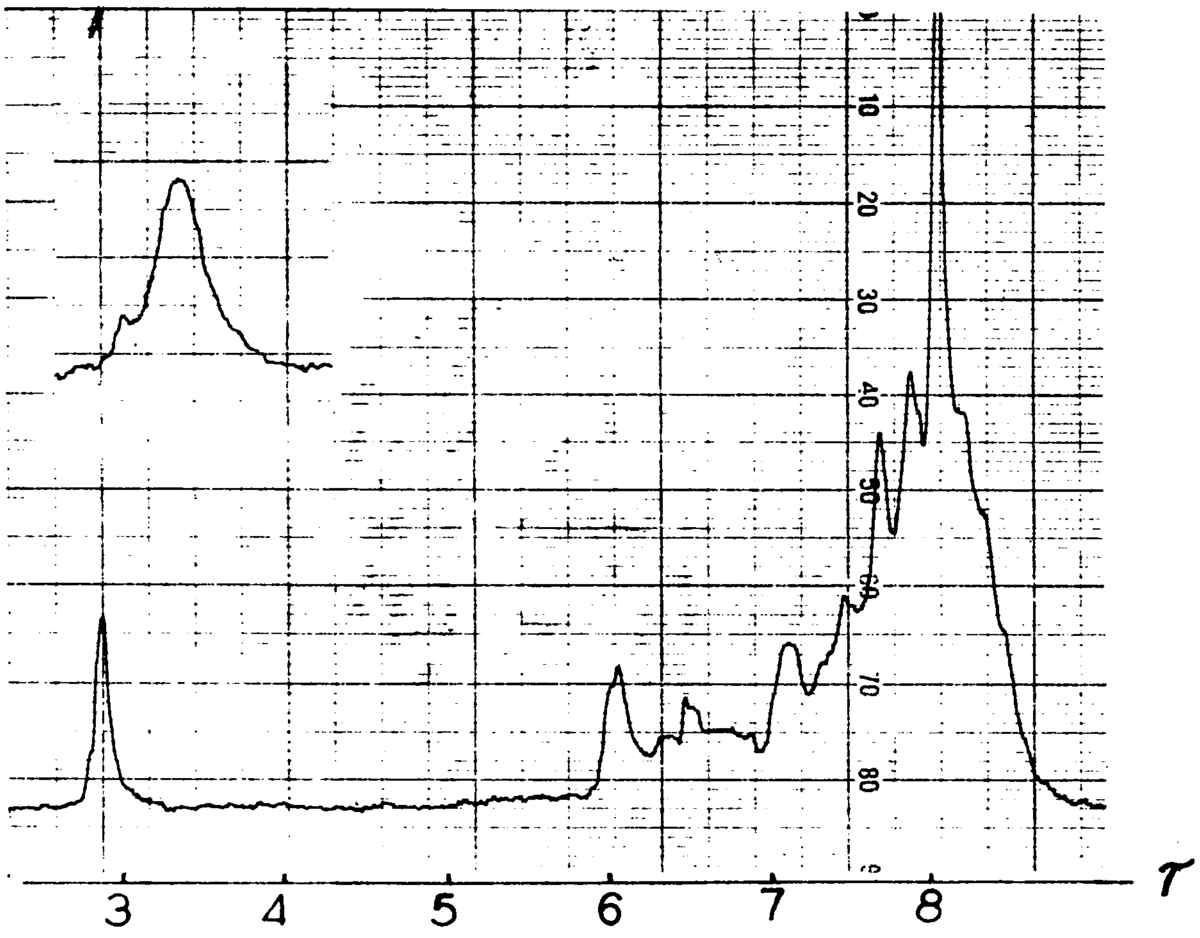
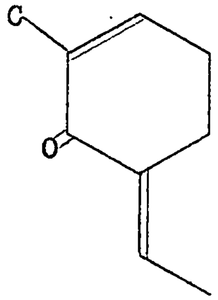


Fig. 9. Nuclear magnetic resonance spectrum of dehydroannofoline. The upper curve represent the peak at 2.85τ of the lower curve at higher resolution.

involved in the formation of the double bond in dehydroannofoline. Since dehydroannofoline is an α,β-unsaturated ketone, the methyl group must be either at an α or β-position to the carbonyl group. Again, in the N.M.R. spectrum there was a new peak at 2.85 τ, a position abnormally low for an ordinary olefinic hydrogen. The half-width of this peak was 2.6 c.p.s. which indicated that there is perhaps no hydrogen adjacent to the olefinic hydrogen. It had recently been shown by Shoolery and Rogers (47) that in case of an α,β-unsaturated keto-steroid, the proton at the β-position to the carbonyl group gave a signal at 2.30 τ whilst that of the α-proton appeared at 4.13 τ. In case of photo-gamma tropolone, it had been noted that the proton at the β-position to the carbonyl group gave signal at 2.62 τ whilst that due to the α-hydrogen occurred at 4.19 τ (48). Recently, Fraser (49) found that in the case of croton aldehyde, the signal due to the β-hydrogen also occurred at lower field than the signal of the α-hydrogen. Hence, in all probability the methyl group and olefinic hydrogen in dehydroannofoline are in α and β-positions, respectively. Confirmatory evidence in support of this conclusion came from the study of the N.M.R. spectrum of annofoline ethyl thio-enol ether (see page 128).

Paper chromatography showed that the mother liquors from the crystallization of dehydroannofoline contained at least eight compounds and three of them showed ultraviolet absorption at 252-254 mμ (in ethanol) but these were not further investigated. They may perhaps contain the following chromophore:



The behavior of dehydroannofoline towards dilute acid and alkali was studied on small scale with the help of paper chromatography. Dehydroannofoline was found to be fairly stable towards dilute acid and alkali.

After establishing the nature of the functional groups in annofoline, attempts were made to prepare an oxygen-free compound with retention of the carbon skeleton of annofoline. As a first step in this direction the conversion of the carbonyl group into a methylene group was considered. The following three methods, namely, (a) hydrogenolysis of a dialkyl thioketal derivative with Raney nickel, (b) Wolf-Kishner reduction and (c) Clemmensen reduction, are generally used for this purpose. It is well known that α-amino ketones undergo Clemmensen reduction with rearrangement. So reduction by Clemmensen method was not considered. Of the remaining two methods, the first method is milder than the second. Therefore, it was tried first. The first step in this method consists in the conversion of a ketone into a dialkyl thioketal derivative. Paper chromatography on the product obtained after treatment of annofoline with ethanethiol, zinc chloride and glacial acetic acid at room temperature, showed the presence of one compound and the absence of annofoline. This compound when crystallized melted at 152-153°. Analysis showed that it was not the diethyl thioketal derivative, the normal product of such reaction. The reaction product had the formula $C_{18}H_{29}ONS$ and it will be referred to as annofoline ethyl thio-enol ether. The infrared spectrum of annofoline ethyl thio-enol ether in carbon tetrachloride solution showed a band in the hydroxyl region ($3600\ cm^{-1}$.) and in chloroform solution there was a very faint band at $1625\ cm^{-1}$. which might indicate unsaturation. There was no band in the carbonyl region of the infrared spectrum, indicating the removal of the keto group of annofoline.

The N.M.R. spectrum of annofoline ethyl thio-enol ether (Fig. 10) did not show any doublet at about 9 τ as in the spectrum of annofoline (Fig. 6). The triplet at 8.79 τ is most probably due to the methyl protons of ethyl thio group. The new unsplit peak at 8.16 τ is probably due to the original methyl group present in annofoline. This position as mentioned before is characteristic of a methyl group attached

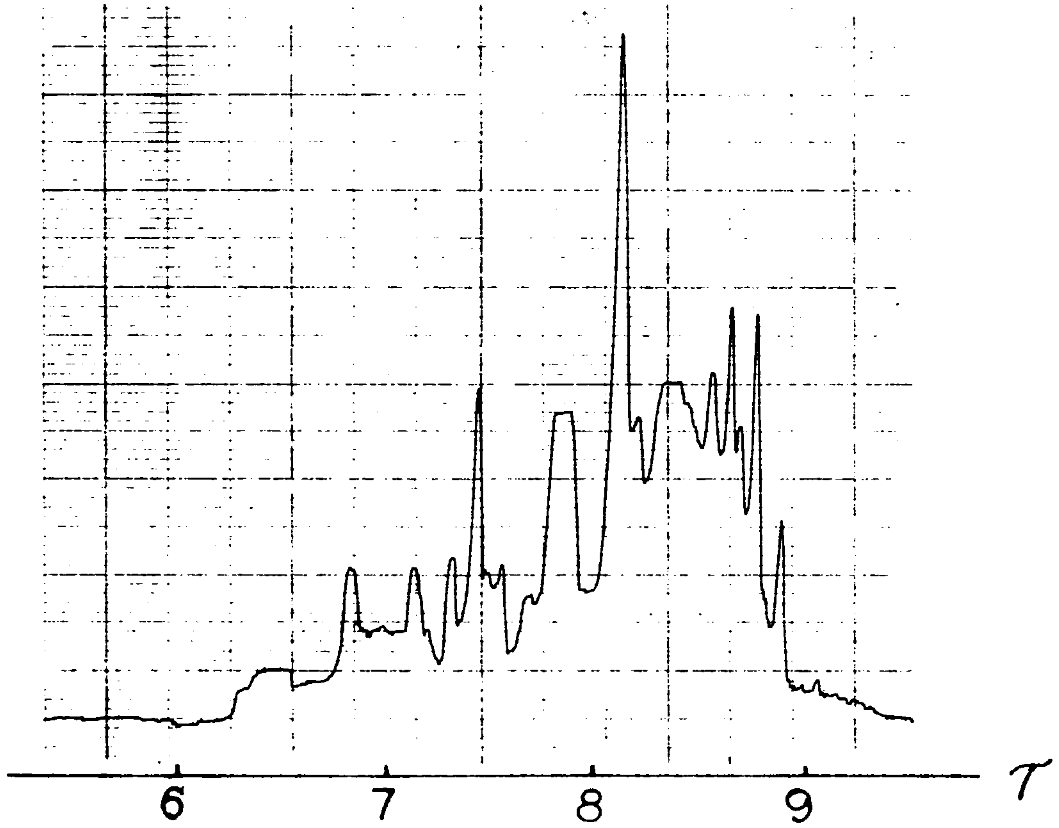


Fig. 10. Nuclear magnetic resonance spectrum of annofoline ethyl thio-enol ether.

to a carbon bearing a double bond. The fact that the peak due to the methyl group is shifted towards the lower field of the spectrum, that the peak is unsplit and that there is no peak corresponding to an olefinic hydrogen, suggests that the newly formed double bond in annofoline ethyl thio-enol ether must be between the carbonyl carbon and the carbon bearing the methyl group. The methyl group in annofoline must therefore be in the α-position to the carbonyl group. This conclusion is in agreement with the fact that annofoline does not form any benzylidene derivative and also with the results of the N.M.R. spectrum of dehydroannofoline.

Desulfurization of annofoline ethyl thio-enol ether with Raney nickel did not proceed very well. A compound was isolated in low yield from the desulfurization reaction but was not characterized. Since the yield of the desulfurization product was very low, it was decided to investigate the Wolf-Kishner reduction.

Wolf-Kishner reduction of annofoline gave a single compound as indicated by paper chromatography. It was purified by chromatography on alumina. The crystalline reduction product melted at 189-190° and analysis gave the formula $C_{16}H_{27}ON$. This compound will be referred to as dihydrodeoxyannofoline. Dihydrodeoxyannofoline had a pKa of 10.4 and $[\alpha]_D$ -42.1°. The infrared spectrum of this compound in carbon tetrachloride solution showed a band in the hydroxyl region (band at 3600 cm^{-1}.) but no band in the carbonyl region, indicating the reduction of the carbonyl group into a methylene group. The N.M.R. spectrum (Fig. 11) showed a doublet at 9.13 τ (methyl group attached to a saturated carbon atom.). The spacing between the peaks gave the value of the coupling constant as 4 c.p.s. The peak at 6.24τ is probably due to the hydrogen attached to carbon bearing the hydroxyl group.

The above Wolf-Kishner reduction was also carried out at a higher temperature and for a longer period. Paper chromatography revealed the presence of two compounds in the reaction product both of which were different from annofoline. The infrared spectrum in chloroform solution showed a weak band in the carbonyl region (band at 1700 cm^{-1}.). The two compounds were separated by chromatography on alumina. One of these compounds was identified as dihydrodeoxyannofoline. The second compound was not fully characterized. It had an R_f value similar to that of deoxyannofoline (see page 135) and its infrared spectrum was similar to that of deoxyannofoline. It is probable that under the strongly alkaline condition and at the high temperature of the reaction, dihydrodeoxyannofoline has undergone dehydrogenation.

Dihydrodeoxyannofoline, when refluxed with acetic anhydride in benzene solution, gave an O-acetyl derivative. O-Acetyl dihydrodeoxyannofoline was isolated and characterized

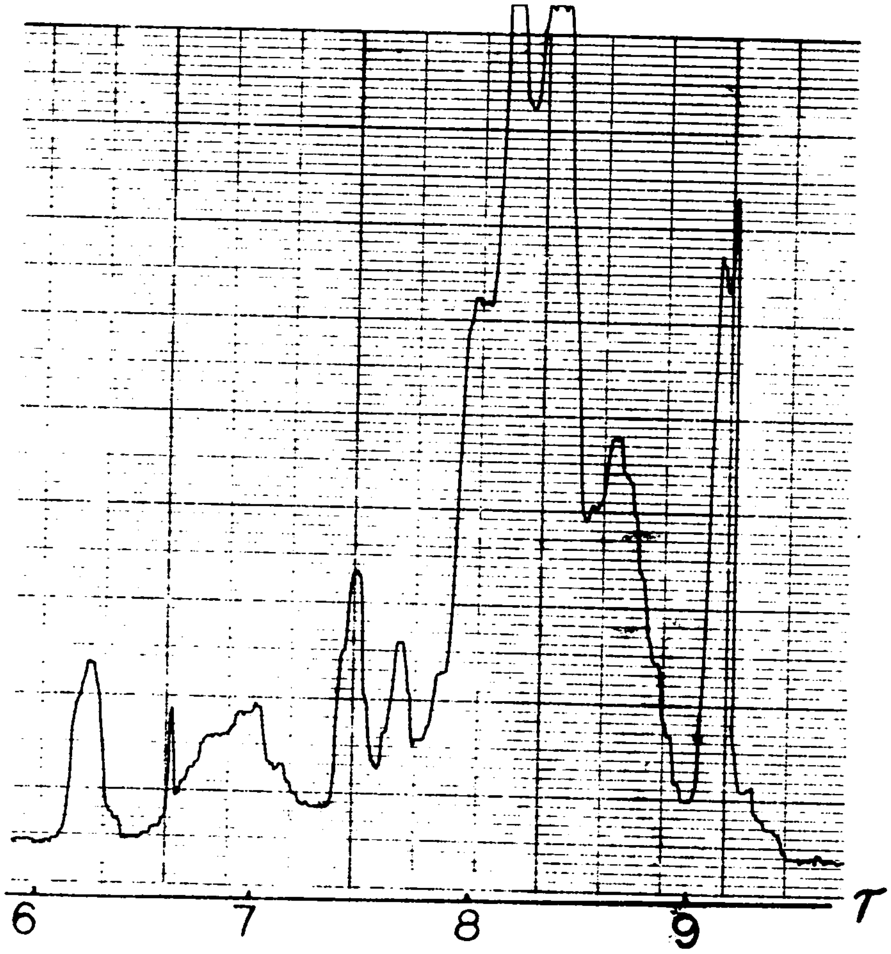


Fig. 11. Nuclear magnetic resonance spectrum of dihydrodeoxyannofoline.

as the hydrobromide, $C_{18}H_{29}O_2N.HBr$. The infrared spectrum of the free base in carbon tetrachloride solution showed a band at 1730 cm^{-1}. (acetoxy carbonyl) but no band in the hydroxyl region. The N.M.R. spectrum of O-acetyldihydrodeoxyannofoline (Fig. 12) showed a doublet at 9.43 τ which must be due to the methyl group. The spacing between the peaks gave the value of coupling constant as 4 c.p.s. The peak at 8.31 τ is due to the methyl group of the acetoxy group. The peak that was present at 6.24 τ in the spectrum of dihydrodeoxyannofoline (Fig. 5) is not present in this spectrum, instead a new peak appears at 5.23 τ which must be due to a hydrogen attached to the carbon bearing the acetoxy group. The half-width of this peak was found to be 8.8 c.p.s. This might have arisen from the coupling of the hydrogen attached to the carbon bearing the acetoxy group with more than two neighbouring hydrogens.

A preliminary experiment was carried out to tosylate dihydrodeoxyannofoline. Paper chromatography and infrared spectroscopy on the reaction product indicated that dihydrodeoxyannofoline was mostly unchanged.

Dihydrodeoxyannofoline was oxidized with chromic acid in pyridine. The oxidation product was purified by alumina chromatography and subsequently through the hydrobromide. The free base melted over a range, m.p. 88-92°, and the melting point could not be improved by repeated recrystal-

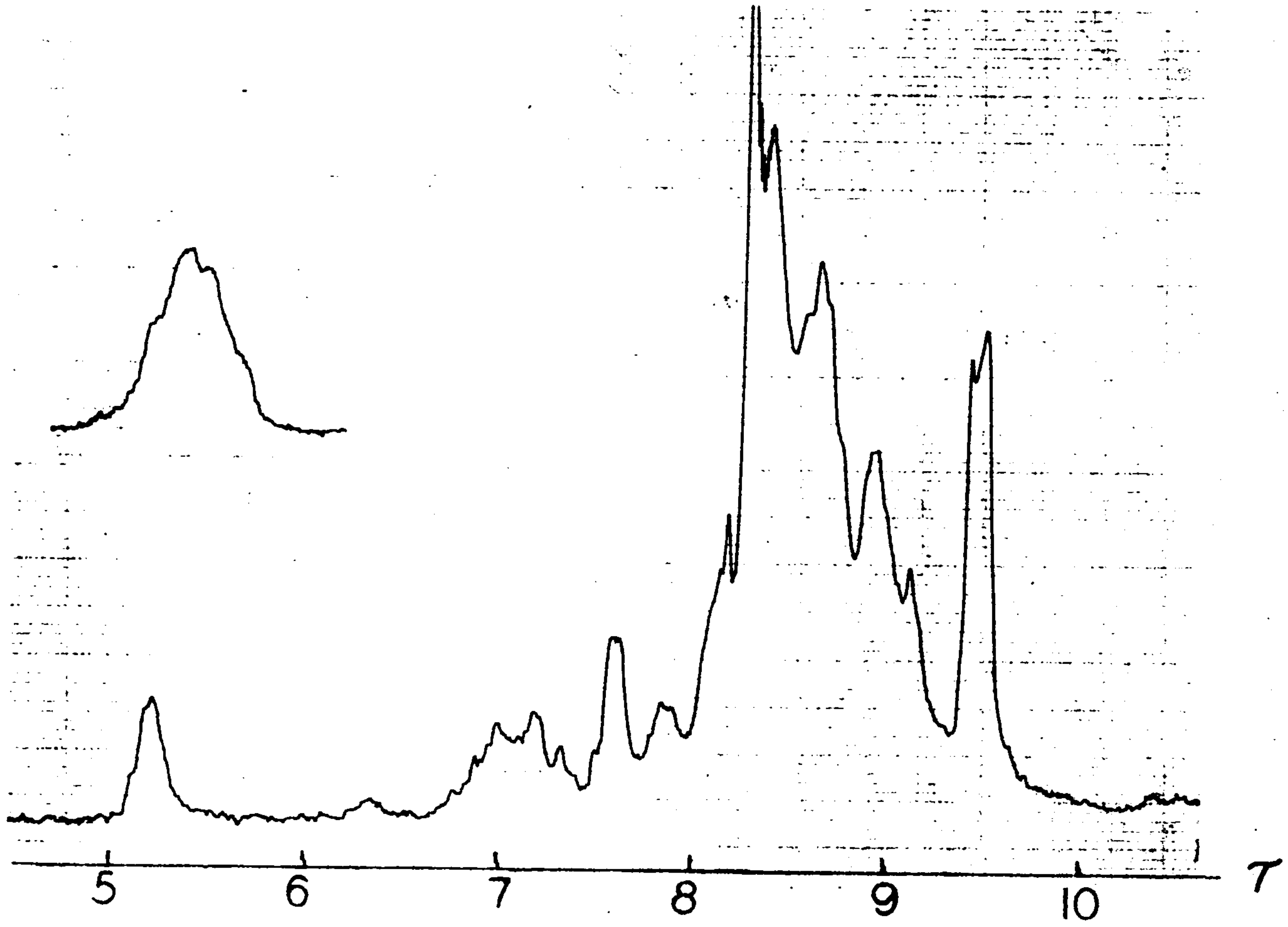


Fig. 12. Nuclear magnetic resonance spectrum of O-acetyl dihydrodeoxyannofoline. The upper curve represent the peak at 6.24 τ of the lower curve at higher resolution.

lizations. Paper chromatography of the compound gave only one spot. The compound had the fromula $C_{16}H_{25}ON$. and it will be referred to as deoxyannofoline. Titration of deoxyannofoline in 50% aqueous methanol gave a pKa of 8.4. The infrared spectrum (Fig. 13) showed no band in the hydroxyl region but a new band appeared at 1690 cm^{-1}. (probably a six membered ketone). Therefore, the hydroxyl group in dihydrodeoxyannofoline and hence in annofoline must be secondary in character.

Annofoline was found to be resistant to chromic acid oxidation under the conditions that dihydrodeoxyannofoline was oxidized. It is most probable that in annofoline the carbonyl and hydroxyl groups are sterically very close to each other and due to the electrostatic effect of the carbonyl group, the oxidation of annofoline is hindered. In dihydrodeoxyannofoline, where the carbonyl group has been reduced to a methylene group, no such effect exists and therefore oxidation take place smoothly.

The ultraviolet spectrum (Fig. 14A) of deoxyannofoline showed an absorption at 288 mμ [ϵ , 109.]. The ultraviolet spectrum of deoxyannofoline hydrochloride (Fig. 14B) was much less intense than that of deoxyannofoline and the position of the maximum [λ_{max} 284 mμ; ϵ, 33] shifted towards lower wavelength, so the basic nitrogen atom in deoxyannofoline may be involved in some way in this absorption. The ultraviolet spectrum of deoxyannofoline

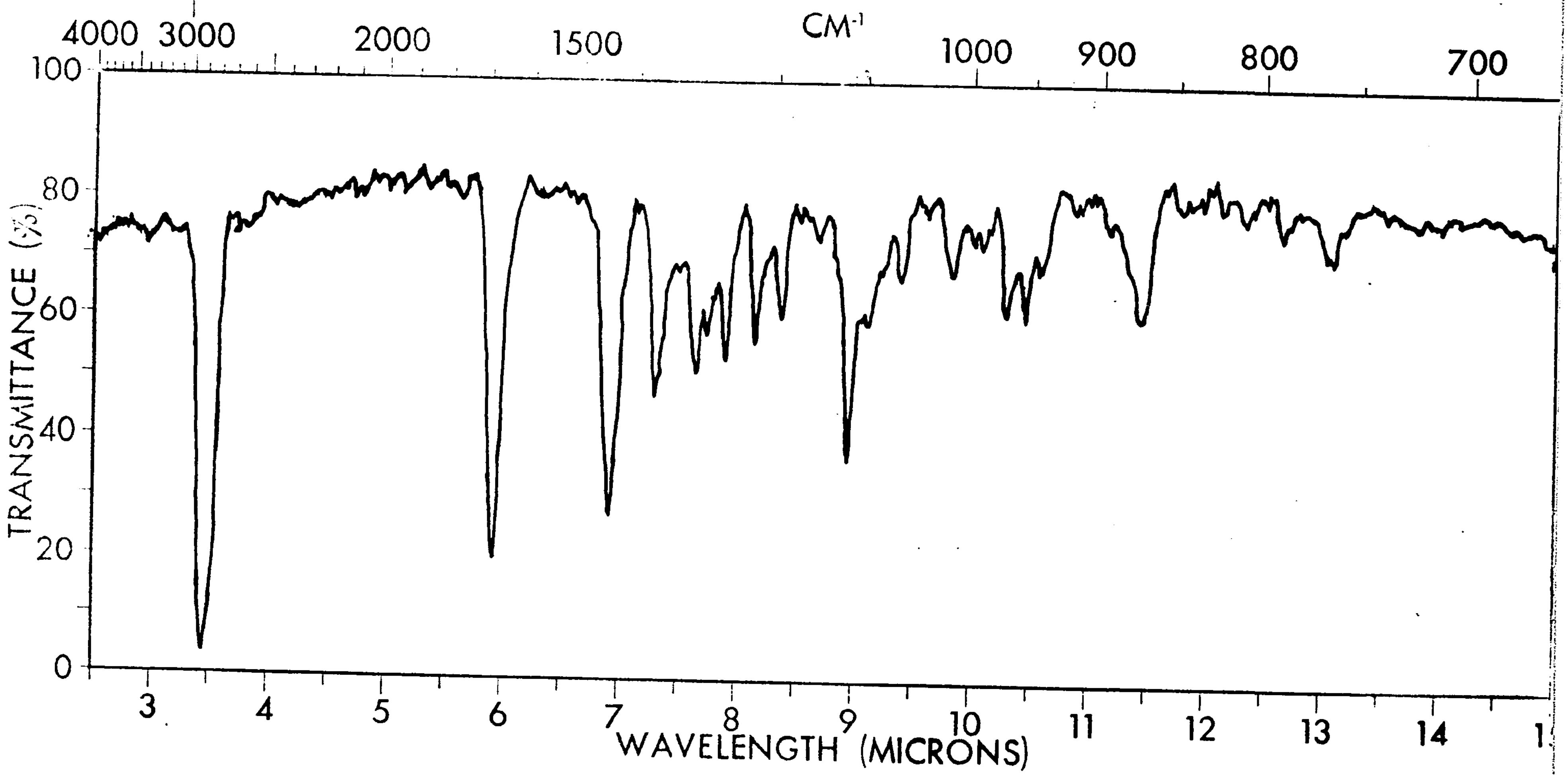


Fig. 13. Infrared absorption spectrum of deoxyannofoline in Nujol mull measured on a Perkin-Elmer "Infracord".

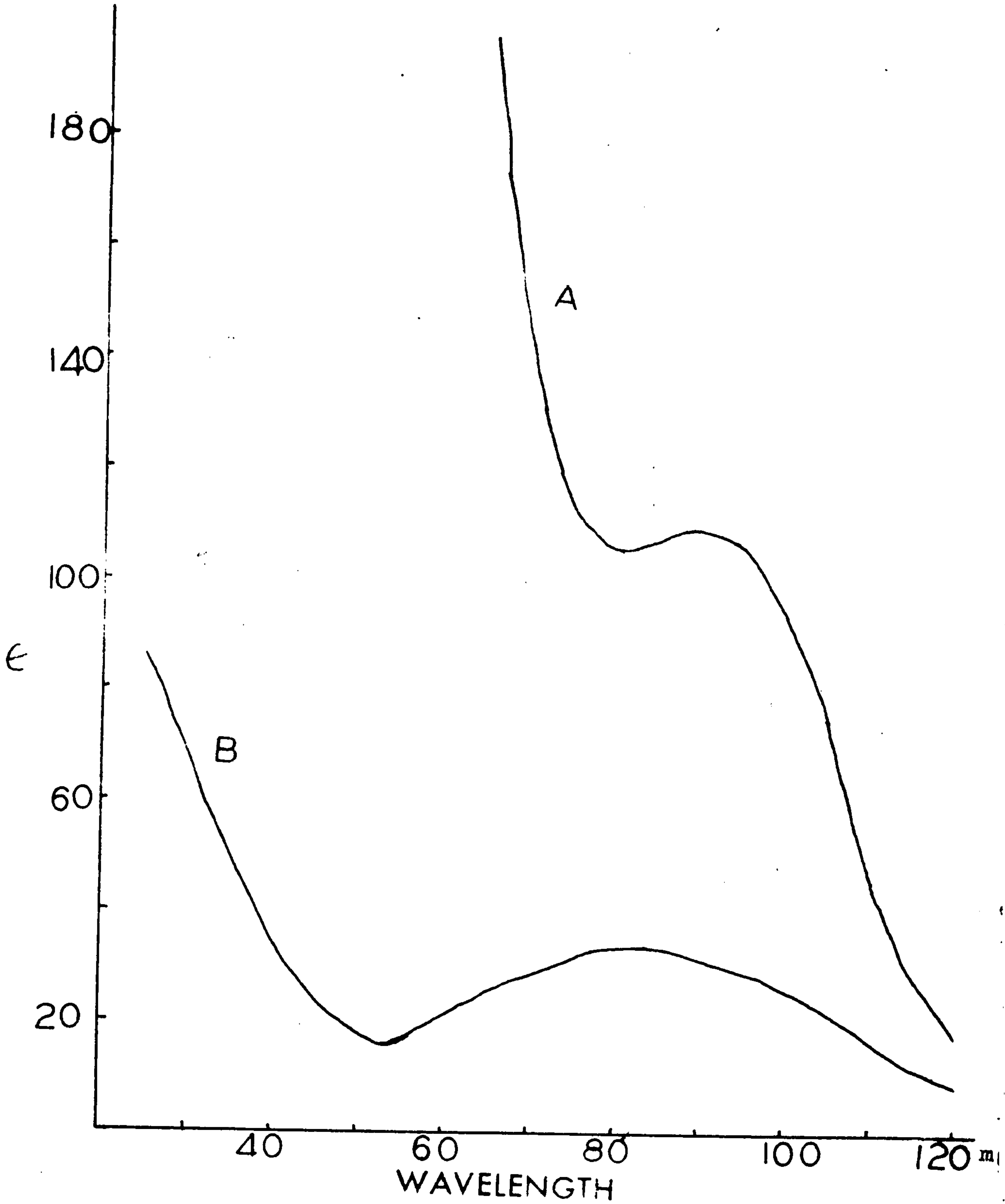


Fig. 14. Ultraviolet absorption spectrum in ethanol measured on a Beckman DK-2 spectrophotomer. A, deoxyannofoline; B, deoxyannofoline hydrochloride.

hydrochloride showed very weak end absorption (ϵ , 86; at 225 mμ) indicating the absence of a tetrasubstituted double bond (50) in deoxyannofoline and therefore also in annofoline. The N.M.R. spectrum of annofoline also showed that the alkaloid does not contain any olefinic hydrogen. Hence, annofoline must be a tetracyclic compound like lycopodine (33), acrifoline (34) and annotinine (apart from oxygen containing rings) (11). It seems that C_{16} alkaloids of Lycopodium annotinum L. may have some structural relationship.

The pKa of dihydrodeoxyannofoline and deoxyannofoline were 10.40 and 8.40 respectively. This decrease in pKa may be interpreted as an increase in the withdrawal of electrons from the nitrogen atom. Thus there is a possibility that the deoxyannofoline is an α-amino ketone. A drop of 2.1 unit in pKa of delpheline due to oxidation to dehydrodelpheline had been observed by Cookson and Trevett (51), although dehydrodelpheline was not an α-amino ketone. Therefore, the second possibility is that the nitrogen atom and the carbonyl group in deoxyannofoline are very close to each other in space even though the compound is not an α-amino ketone. The N.M.R. spectrum of deoxyannofoline (Fig. 15) showed a doublet at 9.20 due to the methyl group attached to a saturated carbon. The methyl peak was split into a doublet (J, 6 c.p.s.) by one adjacent hydrogen.

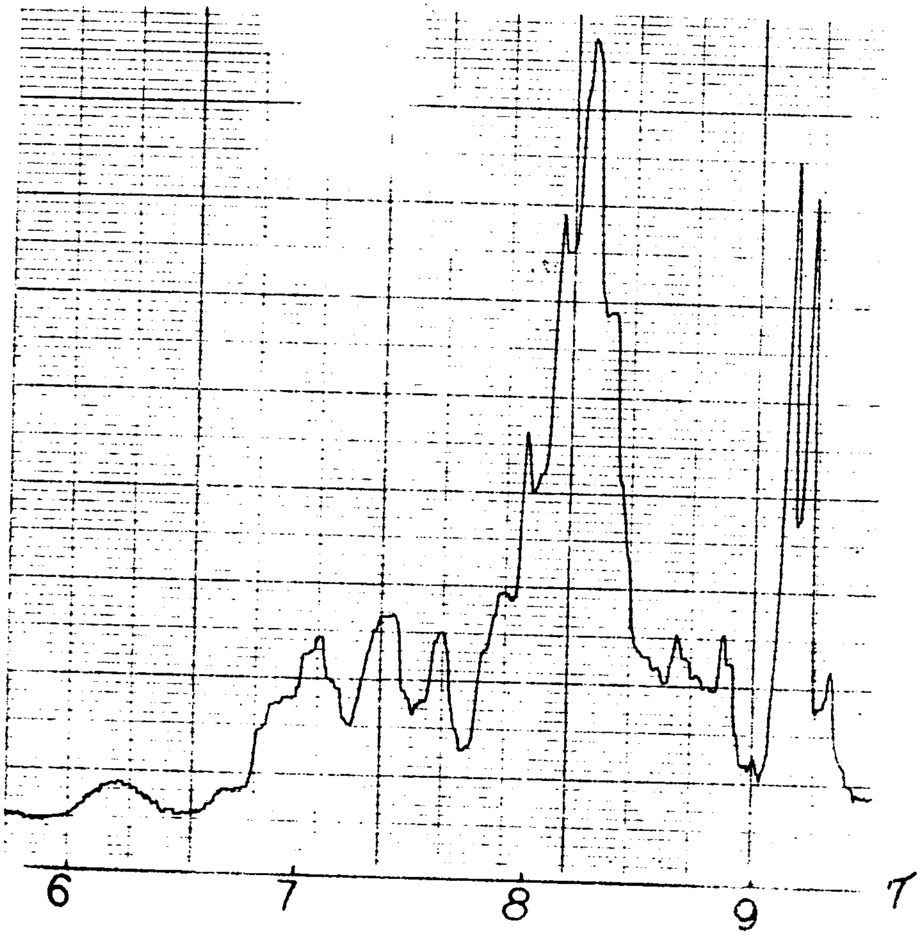


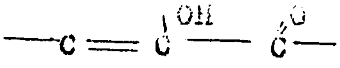
Fig. 15. Nuclear magnetic resonance spectrum of deoxyannofoline.

Deoxyannofoline was found to be fairly stable towards dilute acid and alkali. Its methiodide did not undergo Hofmann degradation to yield α,β-unsaturated ketone. Sodium borohydride reduction of deoxyannofoline was studied on a small scale using paper chromatography. The crude reduction product had an R_f value similar to that of dihydrodeoxyannofoline. The infrared spectrum of the reduction product in carbon tetrachloride solution showed absorption in the hydroxyl region (band at 3500 cm^{-1}.) but no band in the carbonyl region, indicating the conversion of the carbonyl group into a hydroxyl group. It might be of interest to carry out the reduction on a larger scale and to characterize the reduction product.

The possibilities for the removal of the oxygen atom of deoxyannofoline were studied on small scales using paper chromatography. The Wolf-Kishner reduction was tried first. The product of this reaction showed the presence of a hydroxyl group (bands at 3525 cm^{-1}., 3300 cm^{-1}.) and a weak band in the carbonyl region (band at 1700 cm^{-1}.) in the infrared spectrum. The reaction product gave a long tail on the paper. It is most probable that the carbonyl group of deoxyannofoline is reduced to a hydroxyl group and the weak absorption in the carbonyl region might be due to the small amount of deoxyannofoline present.

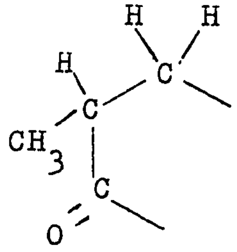
Attempts were then made to convert deoxyannofoline into the corresponding thioketal. The reaction of deoxyannofoline with 1,2-ethanedithiol under various conditions was studied. Paper chromatography showed that in all the cases a mixture of compounds were formed. Owing to the small amount of deoxyannofoline available, this approach was discontinued.

Preliminary experiments were performed on the selenium dioxide oxidation of deoxyannofoline. Paper chromatography indicated the presence of two compounds in the crude oxidation product. The minor compound was probably the starting material. The crude oxidation product showed the properties of an enol. The infrared spectrum in carbon tetrachloride solution showed bands at 3400 cm^{-1}. (hydroxyl group), 1710 cm^{-1}. (carbonyl), 1660 cm^{-1}. (conjugated carbonyl) and 1640 cm^{-1}. (double bond). The ultraviolet spectrum in ethanol showed absorption at 282 mμ. In alkaline solution the position of maximum was shifted to 335 mμ and also the intensity of absorption was greatly decreased. The spectral results indicates that the selenium dioxide oxidation product of deoxyannofoline should have the chromophore

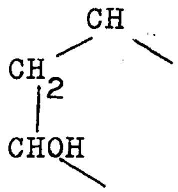


$—C = \overset{OH}{C} — \overset{O}{\overset{\|}{C}}—$. This must have arisen from the group $—CH—CH_2—CO—$ present in deoxyannofoline.

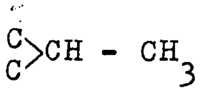
Hence, the present study shows that annofoline is tetracyclic and has the following partial structure:



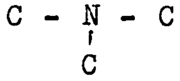
six or larger membered ring



six or larger membered ring



$$\begin{matrix}C\\C\end{matrix}\!>CH - CH_3$$



$$\begin{matrix}C - & N & - C\\ & | & \\ & C & \end{matrix}$$

(c) Chemistry of Lycofoline

Lycofoline, m.p. 144-145°, had the formula $C_{16}H_{25}O_2N$, $[\alpha]_D$ -74.8°, and Kuhn-Roth analysis showed the presence of at least one C-methyl group. Titration of the alkaloid in 50% aqueous methanol gave a pKa of 9.1 and an equivalent weight of 265.5. Lycofoline gave a hydrobromide, $C_{16}H_{25}O_2N.HBr$, m.p. 274-275° and a methiodide, $C_{16}H_{25}O_2N.\ CH_3I$, m.p. 263-264°. The infrared spectrum in Nujol mull (Fig. 16) showed a band at 3300 cm^{-1}. indicating the presence of at least one hydroxyl group. A very faint band at 1670 cm^{-1}. indicated unsaturation. There was no absorption in the carbonyl region. In carbon tetrachloride solution lycofoline showed bands at 3600 cm^{-1}. (free hydroxyl) and 3395 cm^{-1}.

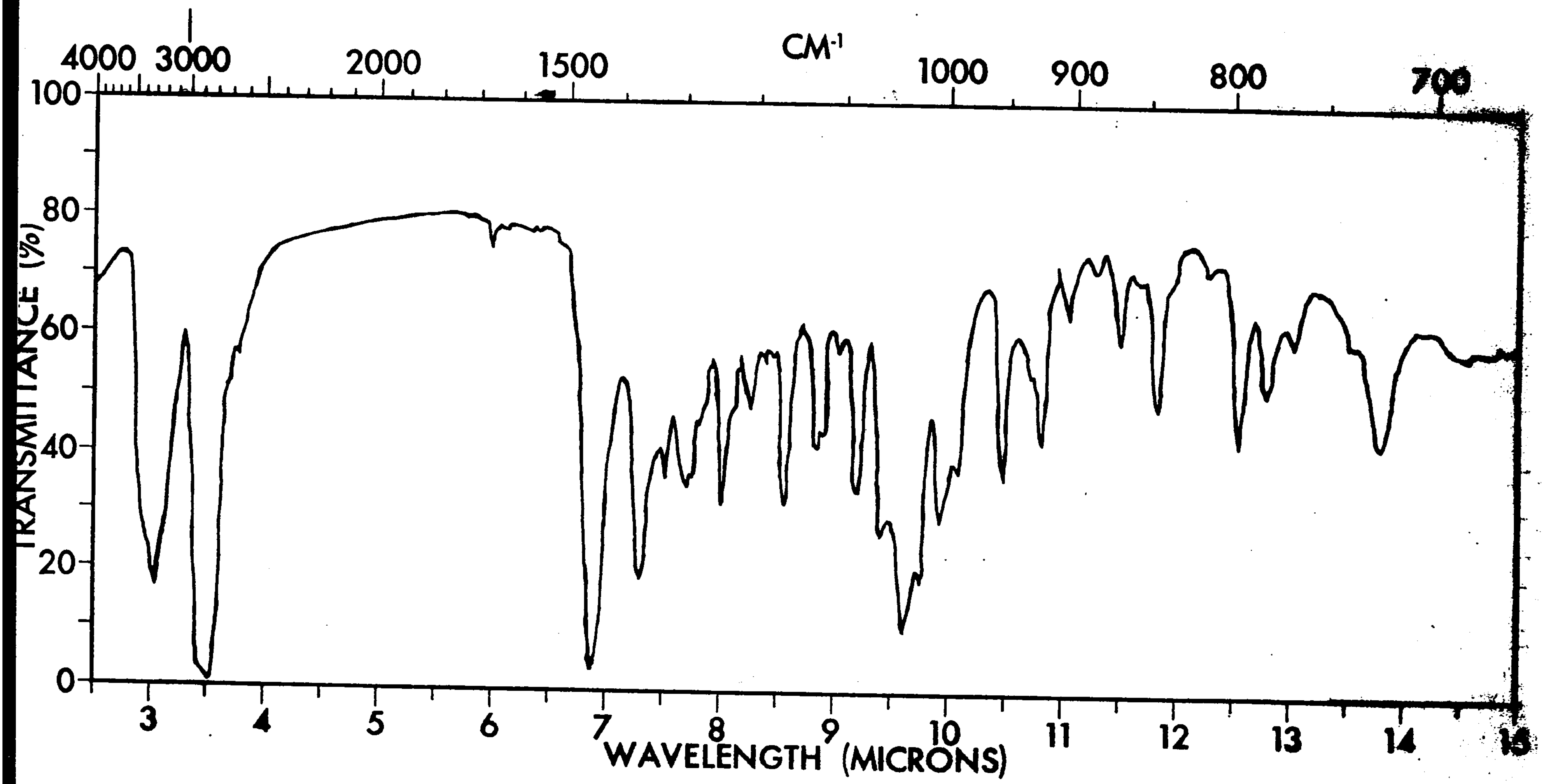


Fig. 16. Infrared absorption spectrum of lycofoline in Nujol mull measured on a Perkin-Elmer "Infracord".

(bonded hydroxyl). The results of the N.M.R. spectra of lycofoline and its derivatives will be discussed together.

Treatment of lycofoline with boiling dilute hydrochloric acid gave a mixture of two products, revealed by paper chromatography (pH 7.0), which possibly are the dehydration products. The infrared spectrum in carbon tetrachloride solution showed a band at 3580 cm^{-1}. and a very faint band at 1700 cm^{-1}.. Reaction of lycofoline with sodium borohydride gave back the starting material. This indicated the absence of a masked carbonyl group such as a carbinolamine group in lycofoline.

Catalytic hydrogenation of lycofoline over Adams' catalyst in ethanol solution gave three compounds as indicated by paper chromatography. The major product was dihydrolycofoline. It was purified by chromatography on alumina. Dihydrolycofoline had the formula $C_{16}H_{27}O_2$, and it melted at 146-147°. This confirmed the presence of a double bond in lycofoline.

The nature of the oxygen atoms in lycofoline was revealed by acetylation studies. Paper chromatography on the product obtained after treatment of lycofoline with acetic anhydride and pyridine at room temperature indicated the formation of a new compound. This compound was crystallized and analysis showed that it was mono-O-acetyl lycofoline. Mono-O-acetyl lycofoline had the formula $C_{18}H_{27}O_3N$, m.p.

159-160° and pKa 8.70. The infrared spectrum of mono-O-acetyl lycofoline in carbon tetrachloride solution showed bands at 3600 cm^{-1}. (hydroxyl group) and 1730 cm^{-1}. (acetoxy carbonyl) but no band in the amide region. To acetylate the second hydroxyl group of lycofoline, a mixture of the alkaloid, acetic anhydride and pyridine was heated at 80-90° for seven hours. Paper chromatography indicated the presence of two products in the reaction mixture. One of these compounds had an R_f value similar to that of mono-O-acetyl lycofoline and the second compound was di-O-acetyl lycofoline. The two compounds were separated by chromatography on alumina. The compound that was eluted first from the column melted at 113-118° after sublimation and crystallization. Analysis showed it to be di-O-acetyl lycofoline. Di-O-acetyl lycofoline had the formula $C_{20}H_{29}O_4N$ and a pKa of 8.05. The infrared spectrum of this compound showed a band at 1725 cm^{-1}. (acetoxy carbonyl) but no band in the hydroxyl or amide region. Hence both the oxygen atoms in lycofoline must be present as hydroxyl groups. The facts that no amide was formed during acetylation of lycofoline and that it gave a true methiodide, establishes the tertiary nature of the nitrogen atom present in lycofoline. A drop of only one unit in pKa due to acetylation of lycofoline (lycofoline pKa, 9.1 and di-O-acetyl lycofoline, pKa, 8.05) indicates that the hydroxyl groups are not in α or β positions to the nitrogen atom.

The N.M.R. spectrum of lycofoline (Fig. 17) showed a doublet at 9.0 τ, a position typical for the methyl group attached to a saturated carbon atom (44, 45). The area under the peaks indicated the presence of one such group already found by the Kuhn-Roth analysis. The splitting of the peak into a doublet indicated that a hydrogen atom is attached to the carbon bearing the methyl group. The spacing in the doublet gave $J_{CH, CH_3} = 6$ c.p.s. This value of the coupling constant is comparable with that of 6-methyl steroids (3 - 9 c.p.s.) (45). The triplet at 4.71 τ may be assigned to olefinic hydrogen by analogy with the substituted ethylenes (44). It is evident from the area under the peaks that one such hydrogen is present in lycofoline. The intensities of the peaks are in the ratio 1:2:1 indicating the coupling of the ethylenic hydrogen with two equivalent hydrogens. The only arrangement that is possible to give rise to two equivalent protons adjacent to the olefinic hydrogen is the presence of a methylene group attached to the carbon bearing the olefinic hydrogen. Hence, lycofoline must contain the group $\begin{smallmatrix}C\\C\end{smallmatrix}\!\!>C = CH - CH_2 -$. The spacing in the triplet gave the value of the coupling constant as 4. c.p.s. The quartet at 6.82 τ and the peak at 6.14 τ are probably due to the hydrogens on the carbon bearing the hydroxyl groups. From the intensities of these bands both hydroxyl groups appear to be secondary. In the following discussion certain stereochemical deductions

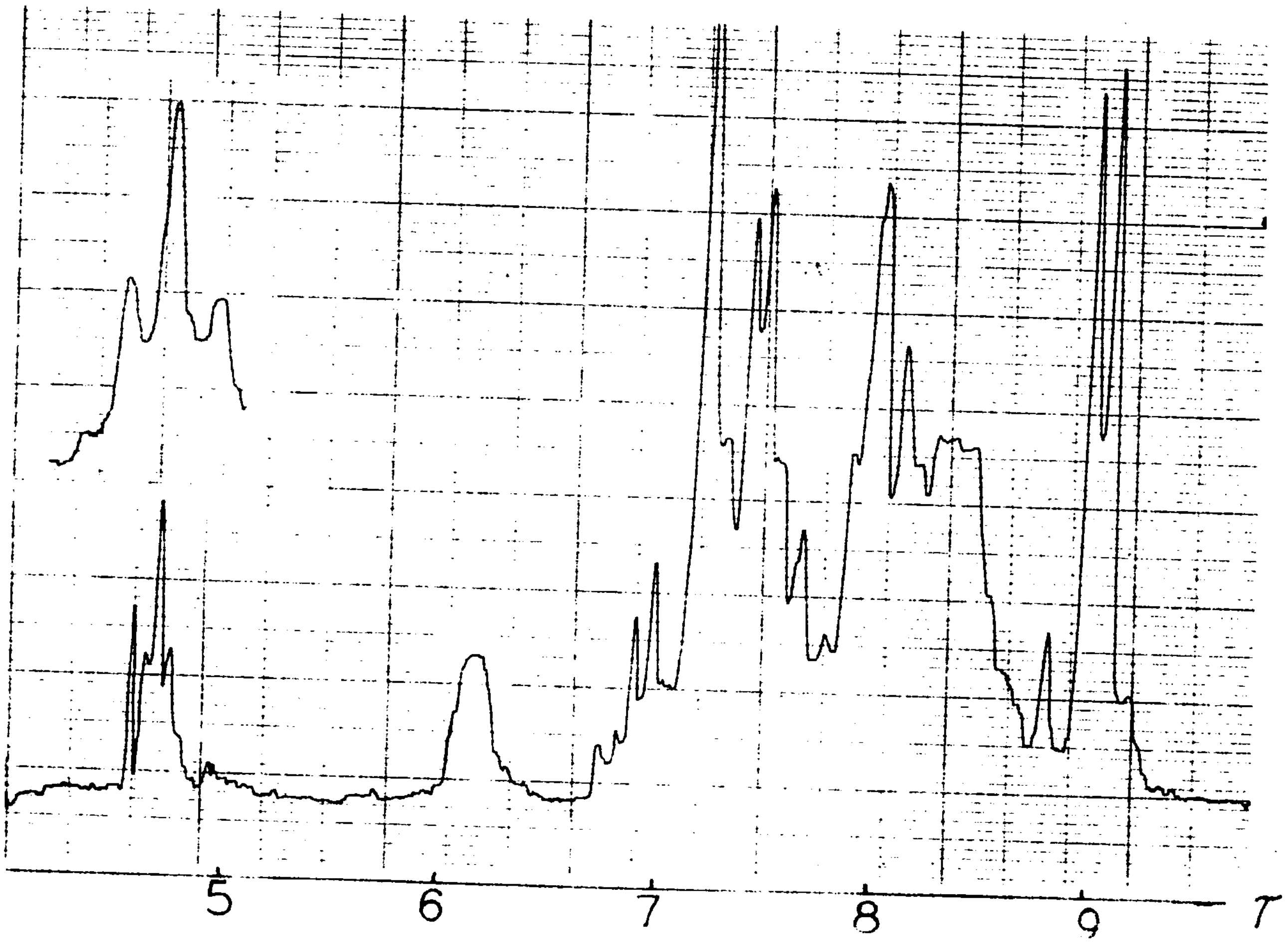


Fig. 17. Nuclear magnetic resonance spectrum of lycofoline. The upper curve represent the peak at 4.71 τ of the lower curve at higher resolution. The sharp peak at 4.59 τ is due to $C^{13}HCl_3$ present in the solvent.

may be made if it is assumed that the carbon atoms bearing the hydroxyl groups are included in six membered rings. This assumption does not exclude the possibility of the presence of a smaller or larger than six membered ring. However, there is some evidence that at least one of the hydroxyl groups is attached to a carbon which is present in a six membered ring (see page 153). Unfortunately, the value of the coupling constants in the case of five or seven membered ring compounds are not known. In any case, it will be of great interest to prove that the carbon atoms bearing the hydroxy groups are included in six membered rings. Lemieux, Kullnig, Bernstein and Schneider (46) found that axial hydrogen attached to a carbon bearing an equatorial hydroxyl group gave a signal at a higher field of the spectrum than the equatorial hydrogen attached to a carbon bearing an axial hydroxyl group. Thus it is possible that the quartet at $6.82\ \tau$ is due to an axial hydrogen and hence the hydroxyl group is equatorial. This conclusion is consistent with the fact that lycofoline forms a mono-O-acetyl derivative under very mild conditions. The N.M.R. spectrum of mono-O-acetyl lycofoline (Fig. 18) did not show the quartet at $6.82\ \tau$ that was present in the spectrum of lycofoline (Fig. 17) but instead a new quartet appeared at lower field, namely at $5.66\ \tau$. This quartet must be due to the hydrogen attached to the carbon bearing the acetoxy group. The area under the peaks indicated

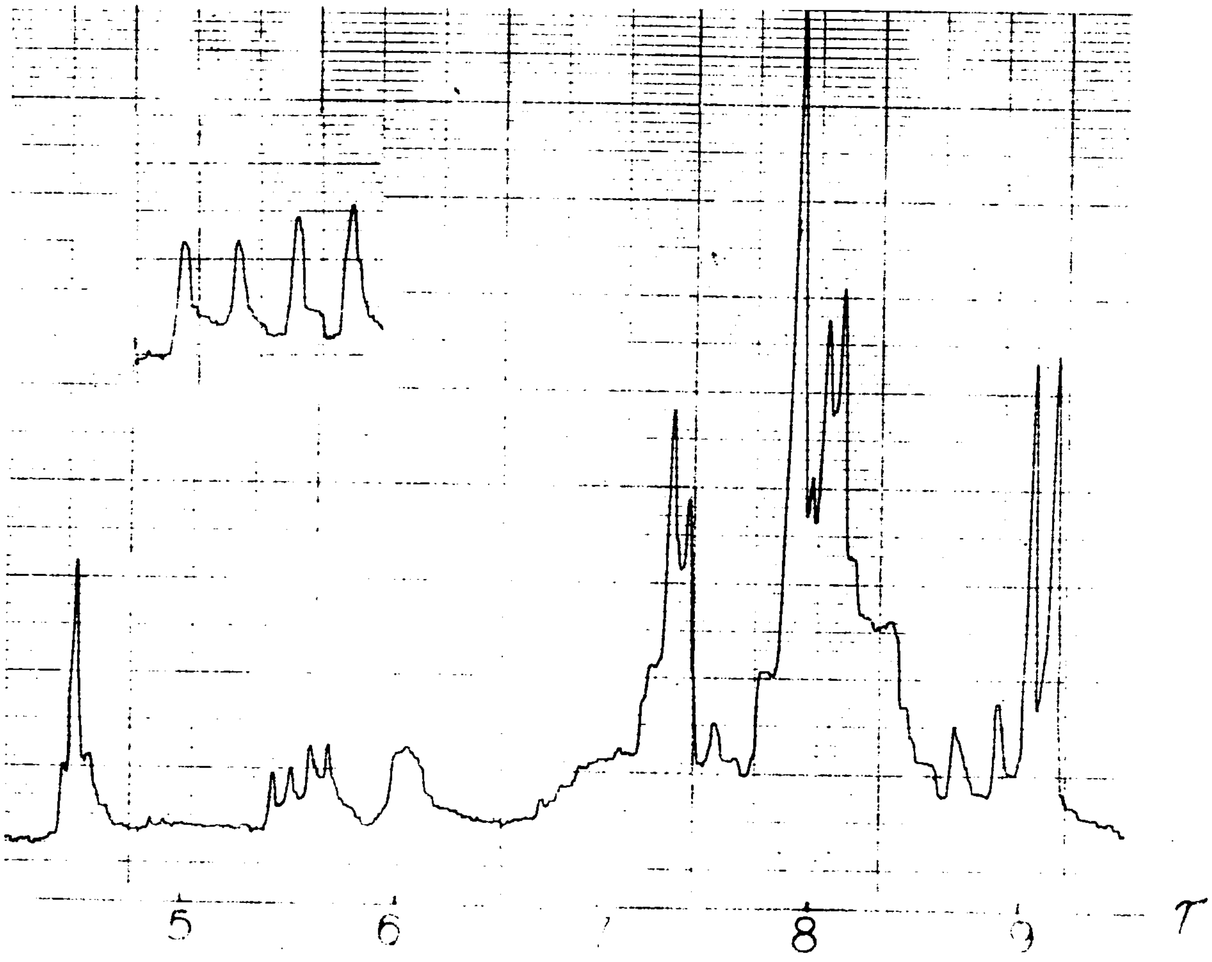
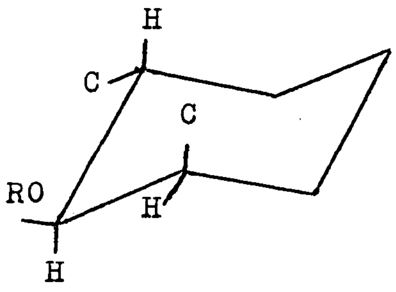
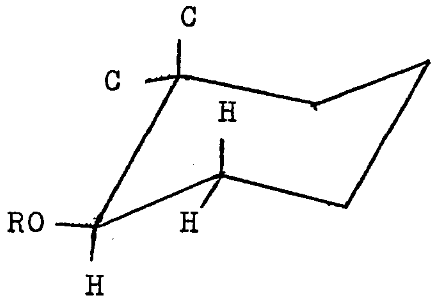


Fig. 18. Nuclear magnetic resonance spectrum of mono-O-acetyl lycofoline. The upper curve represent the peak at 5.66 τ of the lower curve at higher resolution.

the presence of only one such hydrogen, confirming that the hydroxyl group in lycofoline is secondary in character. The pattern of the quartet is the same as the X-portion of the spectrum of an ABX type of Bernstein, Pople and Schneider (52) where X represent the hydrogen attached to the carbon bearing the acetoxy group. The spacing between the first and second peaks (or third and fourth) and that between the first and third peaks (or second and fourth) of this quartet gave the coupling constants 5.4 and 10.8 c.p.s. respectively. Lemieux, Kullnig, Bernstein and Schneider (46) showed that in case of sugar acetates a smaller coupling constant (approximately 3 c.p.s.) was due to the coupling between an axial and an equatorial hydrogen and a larger coupling constant (8. c.p.s.) was observed due to the coupling between two axial hydrogens. In fact, it is a general phenomenon that in case of six membered ring compounds trans conformation of protons have larger coupling constants than the gauche arrangement. Therefore, in the present case, it seems quite reasonable to assume that the larger coupling constant (10.8 c.p.s.) is probably due to the coupling between two axial hydrogens and the smaller coupling constant (5.4 c.p.s.) is due to the coupling between an axial hydrogen and an equatorial hydrogen. This will give the following two possible arrangements (I and II) for the two hydrogens adjacent to the hydrogen attached to the carbon bearing the acetoxy group.



I, R = $COCH_3$
Ia, R = H



II, R = $COCH_3$
IIa, R = H

Hence mono-O-acetyl lycofoline will have the partial structure (I) or (II) and lycofoline will have the partial structure (Ia) or (IIa). The peak at 7.97 τ (Fig. 17) is due to the methyl protons of the acetoxy group. The N.M.R. spectrum of di-O-acetyl lycofoline (Fig. 19) did not contain any peak at 6.14 τ , instead a new peak appeared at 4.94 τ which must be due to the hydrogen attached to the carbon bearing the second acetoxy group. The area under the peak indicated the presence of only one such hydrogen, confirming the secondary character of the hydroxyl group. The peak may perhaps arise from the coupling of the hydrogen attached to the carbon bearing the acetoxy group with more than two hydrogens attached to the neighbouring carbons. It has been shown by Lemieux, Kullnig, Bernstein and Schneider (46) that in the spectrum of t-butyl cyclohexyl acetates, the signal due to the equatorial hydrogen attached to a carbon bearing an acetoxy group was at the lower field than the axial hydrogen. Hence, by analogy, the peak

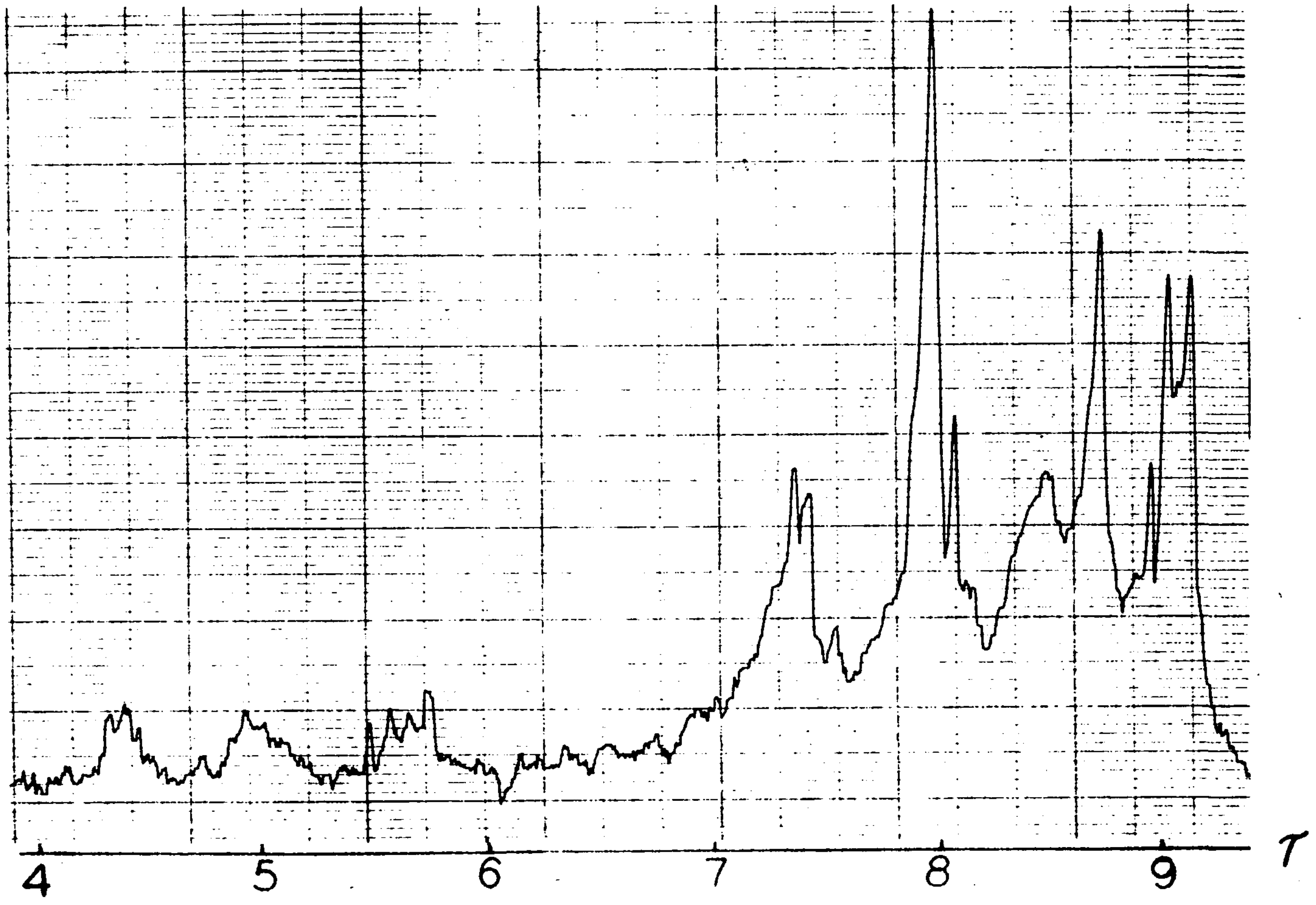


Fig. 19. Nuclear magnetic resonance spectrum of di-O-acetyl lycofoline.

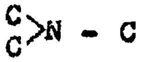
at $4.94\,\tau$ may be assigned to an equatorial hydrogen attached to the carbon bearing the acetoxy group. Thus, the second acetoxy group in di-O-acetyl lycofoline and hence the original hydroxyl group in lycofoline most probably has an axial configuration. It is most probable that due to its axial nature, the hydroxyl group is difficult to acetylate.

Preliminary experiments were carried out on the oxidation of lycofoline with chromic acid and with active manganese dioxide (39). The oxidation with chromic acid in pyridine was carried out at room temperature. Paper chromatography showed that the reaction product contained two compounds. The minor component had the same R_f value as lycofoline and probably it is unchanged lycofoline. The major component is probably the oxidation product. The crude reaction mixture showed bands in the hydroxyl region (bands at 3500 cm^{-1}., 3300 cm^{-1}.) and carbonyl region (1700 cm^{-1}., probably a six membered ketone). This indicates that one of the hydroxyl groups is easily oxidized. The oxidation product obtained with active manganese dioxide contained two compounds, shown by paper chromatography. One of them is perhaps the unchanged lycofoline and the second compound the oxidation product. The crude reaction product showed ultraviolet absorption at 256 mμ (in ethanol). The infrared spectrum(carbon tetrachloride solution)showed bands at 3500 cm^{-1}. and 1700 cm^{-1}., in the hydroxyl and carbonyl regions respectively. In neutral media, active manganese dioxide generally oxidizes an allylic alcohol to the corresponding α, β-unsaturated ketone (39, 53). The above spectroscopical data are a little high for an ordinary α, β-unsaturated ketone. Further investigation on this oxidation product was not possible because of lack of material.

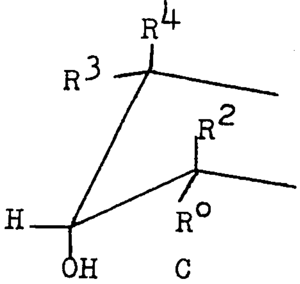
On the basis of the present study, the following partial structure may be assigned to lycofoline

$>CH - CH_3,$

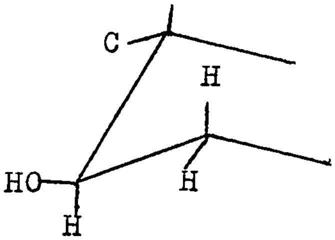
$>C = CH - CH_2 -$



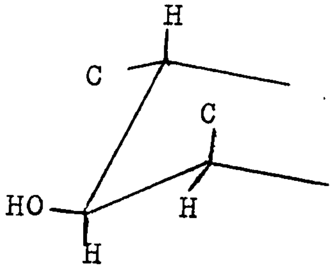
$\overset{C}{\underset{C}{}}{>}N - C$



where more than two R's are hydrogen atoms



or



(d) <u>Chemistry of Lofoline</u>

α-Lofoline had m.p. 211-212°, $[\alpha]_D$ -52.2°. Analysis gave the formula $C_{18}H_{29}O_3N$ and indicated the presence of at least two C-methyl groups and an O-acetyl group. Titration of the base in 50% aqueous methanol gave a pKa of 9.7 and an equivalent weight of 310. α-Lofoline gave a methiodide,

$C_{18}H_{29}O_3N.CH_3I$; m.p. 266-267°. The infrared spectrum of α-lofoline (Fig. 20) in Nujol mull confirmed the presence of an O-acetyl group (bands at 1720 cm^{-1}, 1225 cm^{-1} and 1240 cm^{-1}) and also indicated the presence of a hydroxyl group (band at 3200 cm^{-1}). This accounted for the three oxygen atoms present in α-lofoline.

β-Lofoline, $C_{18}H_{29}O_3N$, m.p. 166-167°, had $[\alpha]_D$ -5.7° and analysis indicated the presence of at least two C-methyl groups and an O-acetyl group. Titration of the alkaloid in 50% aqueous methanol gave a pKa of 9.55 and an equivalent weight of 311. β-Lofoline gave a methiodide, $C_{18}H_{29}O_3N.CH_3I$, m.p. 296-297. The infrared spectrum of β-lofoline (Fig. 21) in Nujol mull indicated the presence of a hydroxyl group [bands at 3400 cm^{-1} (free hydroxyl) and 3100 cm^{-1} (bonded hydroxyl)] and an O-acetyl group (bands at 1730 cm^{-1}, 1700 cm^{-1}, 1225 cm^{-1} and 1270 cm^{-1}). The splitting of the carbonyl band of the O-acetyl group and the presence of two hydroxyl frequencies indicated that the carbonyl groups of some molecules were hydrogen bonded while the carbonyl groups of other molecules were not involved in this type of bonding. The infrared spectra of α-lofoline and β-lofoline in dilute carbon tetrachloride solution showed only one hydroxyl (free) and one carbonyl band. Hence, the hydrogen bonding observed in case of β-lofoline was intermolecular and not intramolecular.

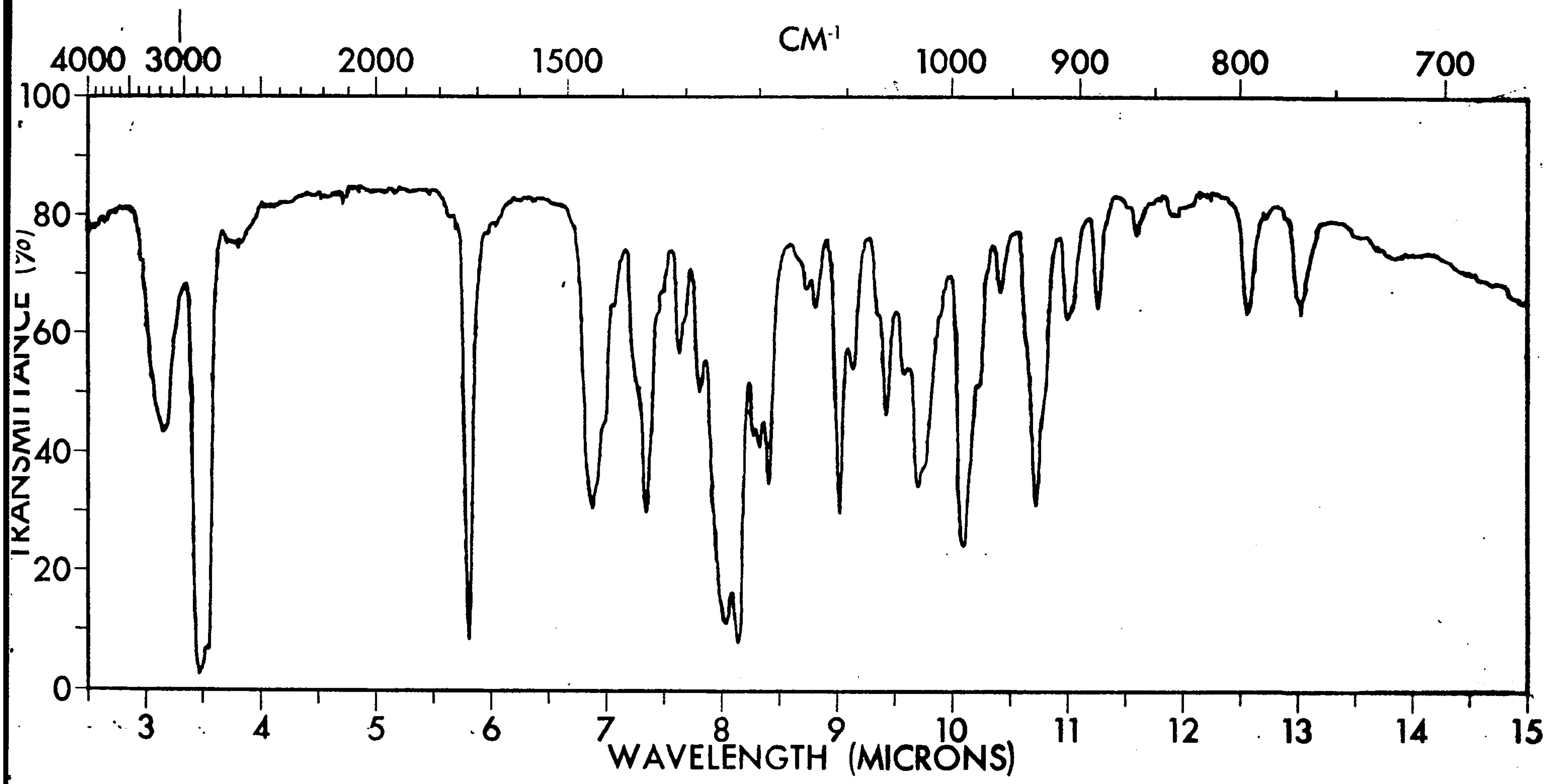


Fig. 20. Infrared absorption spectrum of α-lofoline in Nujol mull measured on a Perkin-Elmer "Infracord".

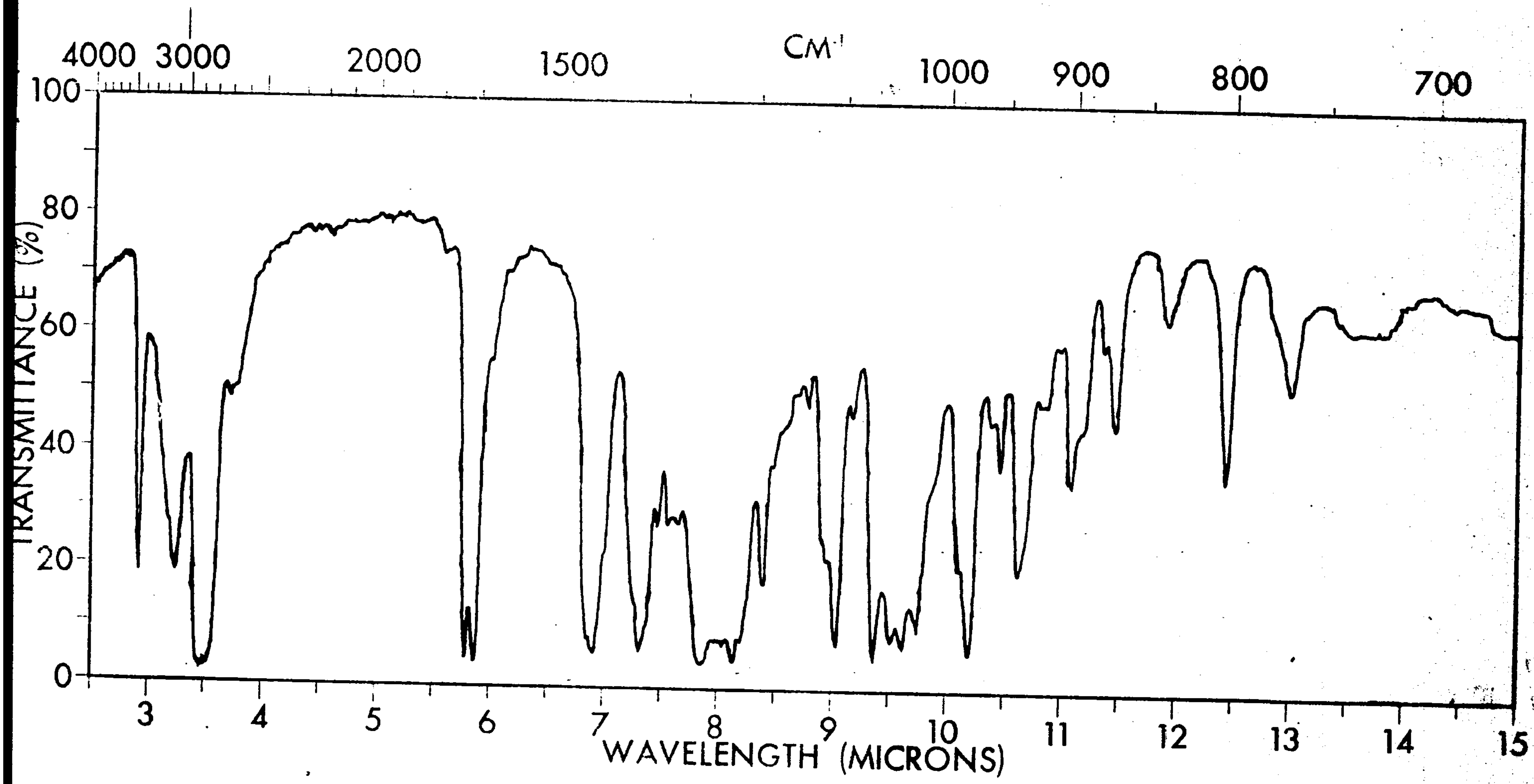


Fig. 21. Infrared absorption spectrum of β-lofoline in Nujol mull measured on a Perkin-Elmer "Infracord".

The amount of pure α-lofoline and β-lofoline available were very small and so preliminary experiments were performed using 2-10 mg. of the alkaloid. The behaviour of α-lofoline and β-lofoline towards the acidic and alkaline reagents was studied on a small scale. The acidic hydrolysis of α-lofoline gave one major product and a minor product. The infrared spectrum of the hydrolytic product showed very faint bands at 1725 cm^{-1}. and 1700 cm^{-1}. but no bands in the hydroxyl region. β-Lofoline, under the same conditions, gave one major product and one minor compound. The infrared spectrum showed a band at 3600 cm^{-1}.. It is most probable that under the condition of the reaction the acetyl group of lofolines were removed by hydrolysis and the corresponding hydroxy compounds underwent dehydration or formation of cyclic ether or chloro-derivatives.

The alkaline hydrolysis of α-lofoline gave one compound as indicated by paper chromatography. The hydrolytic product when refluxed with dilute acid gave a mixture of three compounds. The infrared spectrum of this mixture showed bands at 3600 cm^{-1}., 3400 cm^{-1}. (hydroxyl), 3150 cm^{-1}. (probably unsaturation) and very weak absorption at 1730 cm^{-1}. and 1700 cm^{-1}.. Paper chromatography on the product of alkaline hydrolysis of β-lofoline showed the presence of one product which when treated with acid gave a mixture of three compounds as indicated by paper chromatography. The R_f value of these components were similar to those obtained in case of α-lofoline after similar treatment. The infrared spectrum of the mixture showed bands at 3600 cm^{-1}, 3400 - 3300 cm^{-1} (hydroxyl group) and a very weak band at 1700 cm^{-1}. It is most probable that during alkaline hydrolysis the O-acetyl group of α-lofoline and β-lofoline are removed and the hydrolytic products on treatment with acid undergo dehydration or other reactions.

Chromic acid oxidation of pure α-lofoline and β-lofoline, on a small scale (2 mg.), showed that the oxidation products had the same R_f value and the infrared spectra were superimposable and had bands at 1740 cm^{-1} (acetates), 1700 cm^{-1} (carbonyl) but had no hydroxyl band. Therefore, a mixture of α-lofoline and β-lofoline, which was more readily available than the pure alkaloids, was oxidized with chromic acid in pyridine. Paper chromatography on the oxidation product showed the presence of four components, one of which was probably due to unchanged lofoline. The four components were separated on alumina. The major product of oxidation was crystallized. Analysis showed that it had two hydrogen atoms less than α-lofoline or β-lofoline, therefore, it was called dehydrolofoline. Dehydrolofoline, $C_{18}H_{27}O_3N$., had m.p. 129-133°. Paper chromatography showed only one spot. The infrared spectrum in carbon tetrachloride solution showed bands at 1745 cm^{-1} (carbonyl of O-acetyl group) and 1700 cm^{-1}

(keto group) but no band in the hydroxyl region. The newly formed carbonyl group is probably in a six-membered ring. The chromic acid oxidation product obtained from pure α-lofoline melted at 126-134°. The mixed melting point of the oxidation product, obtained from α-lofoline, with dehydrolofoline was found to be 129-136°. Again, the oxidation product obtained by chromic acid oxidation of pure β-lofoline melted at 134-137°. The mixed melting point of the oxidation product, obtained from β-lofoline, with dehydrolofoline was found to be 132-135°. The infrared spectra of α-dehydrolofoline and β-dehydrolofoline were identical. Hence, α-lofoline and β-lofoline are epimeric secondary alcohols. The N.M.R. spectrum (Fig. 22) of dehydrolofoline showed a doublet at 8.62 τ . This is a typical position for a C-methyl group (44). The area under the peaks indicated the presence of one such group. The splitting of the peak into a doublet indicates the presence of a hydrogen on the carbon atom bearing the methyl group. The spacing between the peaks gave $J_{CH,\ CH_3}$ = 7.8 c.p.s. The single peak at 8.36 τ is due to the methyl group of the acetoxy group. The badly resolved quartet at 4.94 τ is probably due to the hydrogen attached to a carbon bearing the acetoxy group. The area under the peaks indicated that only one such hydrogen is present. The intensities of the peaks are roughly in the proportion 1:3:3:1. The quartet might be due to the coupling of the hydrogen attached to the

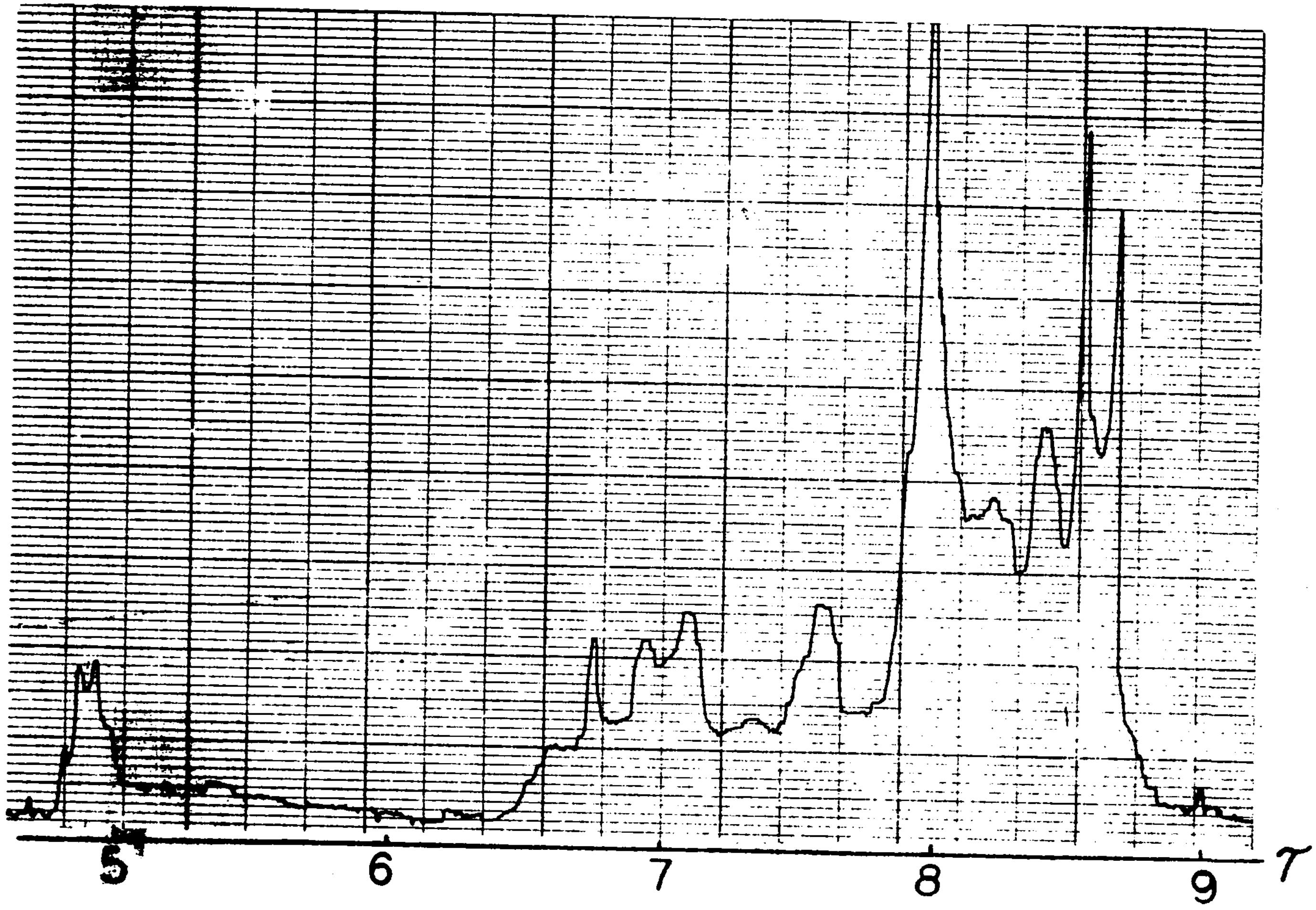
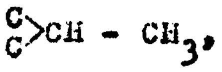


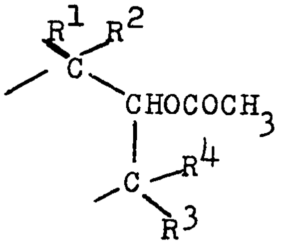
Fig. 22. Nuclear magnetic resonance spectrum of dehydrolofoline.

carbon bearing the acetoxy group probably with more than two neighbouring hydrogen atoms. The spacing between the first and second (or third and fourth) peaks gave the coupling constant as 3 c.p.s. and that between the first and third (or second and fourth) gave the value of the second coupling constant as 6. c.p.s. On the basis of the present studies, the following partial structure may be assigned to lofoline:


$\genfrac{}{}{0pt}{}{C}{C}{>}CH - CH_3,$


$>CHOH$


$N{<}^{C}_{C}C$



where probably, more than two R's are hydrogen atoms.

(e) N.M.R. Spectra of Acrifoline and Annotinine

The N.M.R. spectrum of acrifoline (Fig. 23) showed a doublet at 8.95 τ, characteristic of a methyl group attached to a saturated carbon atom. The area under the peaks indicates the presence of only one such group. The splitting of the band into a doublet indicates the presence of a hydrogen on the carbon bearing the methyl group. The spacing

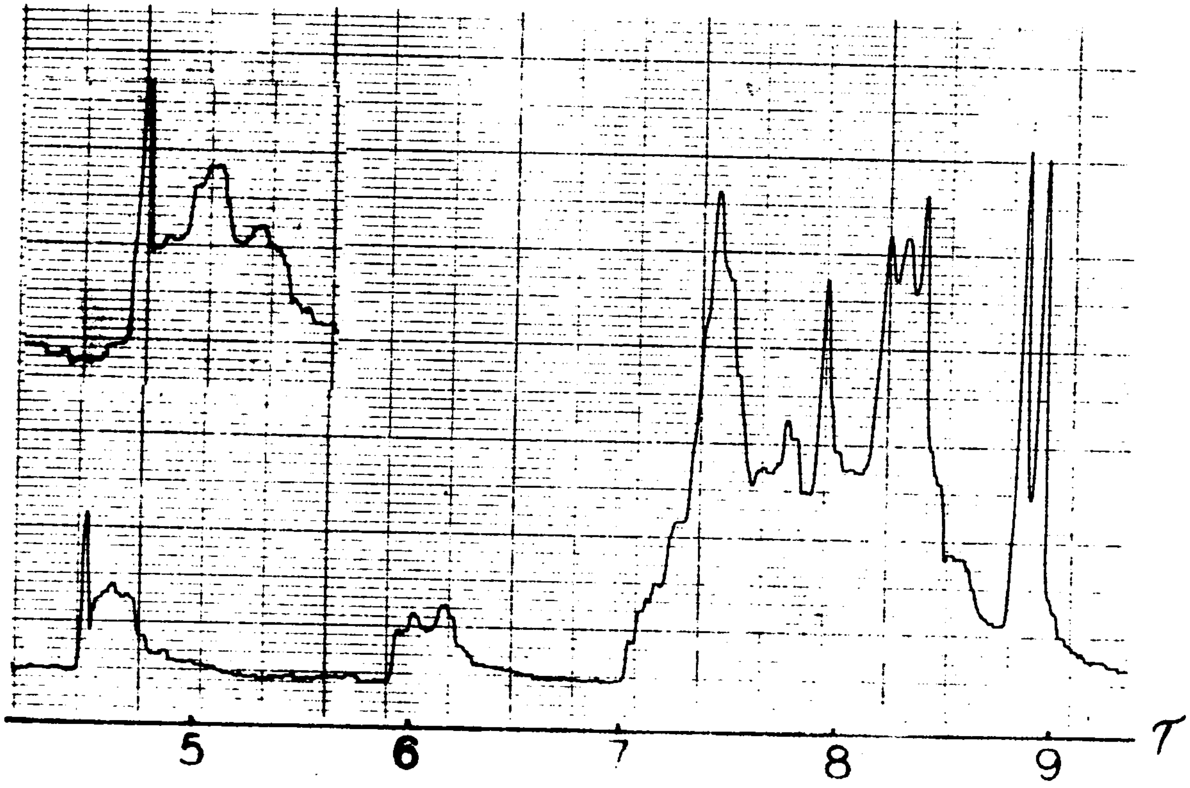


Fig. 23. Nuclear magnetic resonance spectrum of acrifoline. The upper curve represent the peak at 4.60 τ of the lower curve at higher resolution. The sharp peak at 4.50 τ is due to $C^{13}HCl_3$ present in the solvent.

between the peaks gave the value of the coupling constant as 6 c.p.s. By analogy with substituted ethylenes, the peak at 4.6 τ may be assigned to an olefinic hydrogen. The area under the peaks indicates the presence of only one such hydrogen atom in acrifoline. The peak might have arisen due to the coupling of the olefinic hydrogen with at least two neighbouring hydrogens. The triplet at 6.1 τ is perhaps due to hydrogen attached to the carbon bearing the hydroxyl group. The area under the peaks indicates the presence of only one such hydrogen. Hence, the hydroxyl group of acrifoline may be secondary in character.

The N.M.R. spectrum of annotinine (Fig. 24) showed a doublet at 8.71 τ . This must be due to a methyl group attached to the saturated carbon atom and the doublet is due to the coupling of the methyl protons with one hydrogen attached to the carbon bearing the methyl group. The spacing between the peaks gave a coupling constant of 8 c.p.s. The doublet at 5.48 τ is probably due to the hydrogen attached to the carbon which carries the lactone bridge. The spacing between the peaks gave a coupling constant of 6 c.p.s. This hydrogen is expected to couple with three adjacent hydrogens and as such a quartet is expected but instead, a doublet appeared. The peaks are slightly wider than a doublet formed by coupling of a hydrogen atom with an adjacent hydrogen. It is possible that two of the hydrogens in the present case has

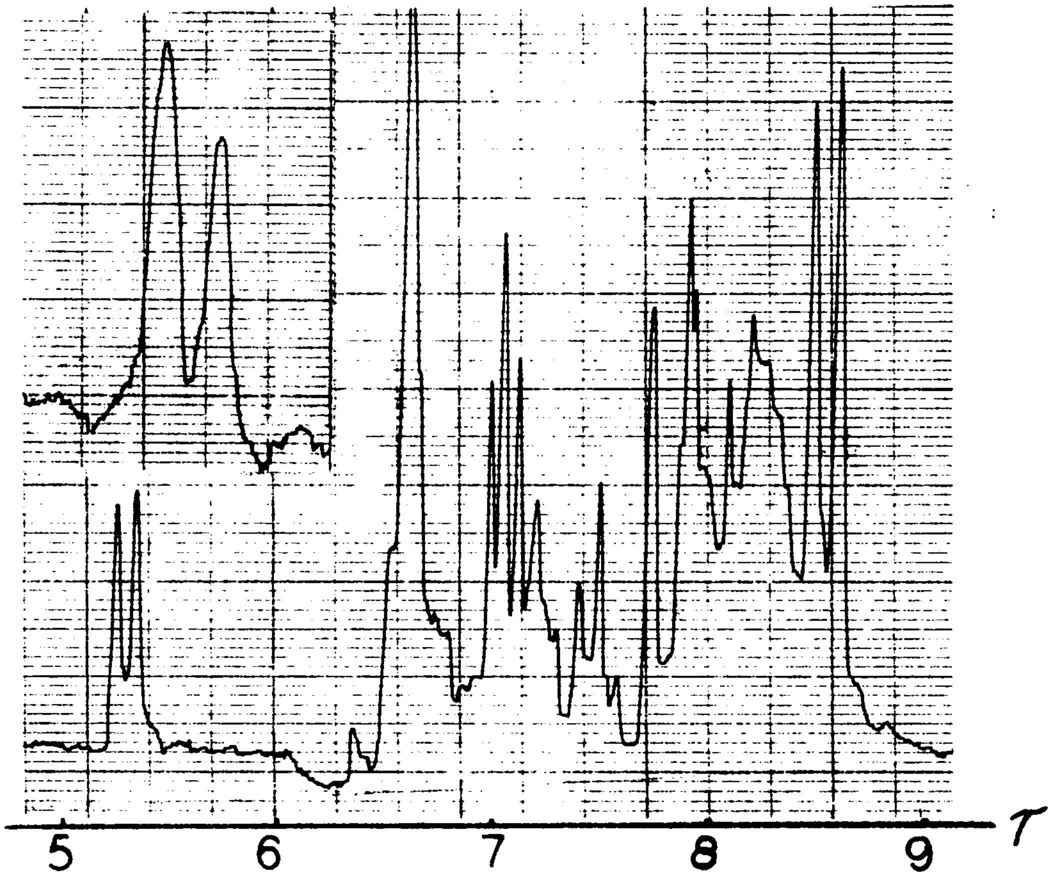


Fig. 24. Nuclear magnetic resonance spectrum of annotinine. The upper curve represent the peaks at 5.8 τ of the lower curve at higher resolution.

very small coupling constants and the third hydrogen has a coupling constant much larger than the other two.

The chemical shifts and the coupling constants of all the compounds whose N.M.R. spectra have been measured are tabulated in Table I (page 167) and Table II (page 168 and 168a).

Table I

Chemical shifts in cycles per second with reference to chloroform

Alkaloid	$C-CH_3$	$CH_{3,acetoxy}$	$>CH_{alcohol}$	$>CH_{acetoxy}$	$>C = CH-$
Annofoline	367	-	206	-	-
α-Dihydroannofoline	384	-	255 288 199	-	-
Dihydrodeoxy-annofoline	376	-	197	-	-
O-Acetyl dihydrodeoxy annofoline	395	328	-	143	-
Deoxyannofoline	383	-	-	-	-
Dehydroannofoline	311	-	190	-	0
Lycofoline	370	-	234 197	-	111
Mono-O-acetyl lycofoline	377	305	194	167	97
Dehydrolofoline	376	329	-	147	-
Acrifoline	371	-	198	-	110
Annotinine	360	-	166	-	-

Table II

Chemical shifts[a] and coupling constants.

	Solvent	$C-CH_3$		$>CH_3$ acetoxy	$>CH$ alcohol	$>CH$ acetoxy		$>C = CH-$	
		τ	J(c.p.s.)	τ	τ	τ	J(c.p.s.)	τ	J(c.p.s.)
Annofoline	$CHCl_3$	8.97	6	-	6.93	-	-	-	-
α-Dihydroannofoline	$CHCl_3$	9.09	6	-	5.93) 6.29)b 6.86)	-	-	-	-
Dihydrodeoxyannofoline	$CDCl_3$	9.13	4	-	6.24	-	-	-	-
O-Acetyl dihydroannofoline[c]	$CDCl_3$	9.43	4	8.31	-	5.23	8.8[e]	-	-
Deoxyannofoline	$CDCl_3$	9.20	6	-	-	-	-	-	-
Dehydroannofoline	$CDCl_3$	8.04	-	-	6.02	-	-	2.85	2.6[e]
Annofoline ethyl thio-enol ether	CCl_4	8.79 8.16	-	-	6.43	-	-	-	-

(continued on next page)

Table II (Cont'd.)

	Solvent	τ_{C-CH_3}		$>CH_3$ acetoxy	$>CH_{alcohol}$	$>CH_{acetoxy}$		$>C = CH-$	
			J(c.p.s.)	τ	τ	τ	J(c.p.s.)	τ	J(c.p.s.)
Lycofoline	$CHCl_3$	9.02	6	-	6.82 6.14	-	-	4.71	4
Mono-O-acetyl lycofoline	$CHCl_3$	9.13	6	7.97	6.12	5.66	5.4 10.8	4.50	4
Di-O-acetyl lycofoline	CCl_4	9.08	6	8.0	-	5.42 4.94	5.4 10.8	4.38	4
Dehydrolofoline	$CHCl_3$	8.62	7.8	8.03	-	4.89	3, 6	-	-
Acrifoline	$CHCl_3$	8.95	6	-	6.1	-	-	4.6	7.8[e]
Annotinine	$CHCl_3$	8.71	8	-	5.48[d] (J = 6)	-	-	-	-

(a) τ (in p.p.m.) $= 10 - 10^6 (H_{Me_4}Si - H_{obs.})/H_{Me_4}Si$. Where H = magnetic field at resonance.

(b) Any two of these peaks were probably due to the hydrogens attached to the carbon bearing the hydroxyl groups. (c) Spectrum was run, so the values are not reliable. (d) hydrogen attached to the carbon bearing the lactone bridge. (e) Half width of the band.

10. CLAIMS TO ORIGINAL RESEARCH

(a) Four new alkaloids have been isolated from Lycopodium annotinum L. by countercurrent distribution followed by alumina chromatography. They have been fully characterized and have been named annofoline, lycofoline, α-lofoline and β-lofoline.

(b) Annofoline has the formula $C_{16}H_{25}O_2N$.

(c) The following derivatives of annofoline have been prepared: (1) annofoline perchlorate, $C_{16}H_{25}O_2N.HClO_4$, (2) annofoline methiodide, $C_{16}H_{25}O_2N.CH_3I$., (3) O-acetyl annofoline, isolated as the hydrobromide $C_{18}H_{27}O_3N.HBr$.

(d) Annofoline has been reduced with sodium borohydride to give two epimeric alcohols, α-dihydroannofoline, $C_{16}H_{27}O_2N$, and β-dihydroannofoline, $C_{16}H_{27}O_2N$.

(e) Annofoline has been converted into annofoline ethyl thio-enol ether, $C_{18}H_{29}ONS$, by treatment with ethane thiol.

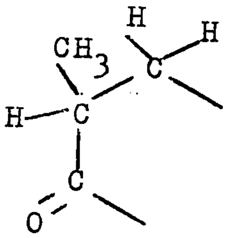
(f) By Wolf-Kishner reduction annofoline has been converted to dihydrodeoxyannofoline, $C_{16}H_{27}ON$.

(g) Dihydrodeoxyannofoline has been converted into an O-acetyl derivative. The O-acetyl dihydrodeoxyannofoline has been isolated as the hydrobromide, $C_{18}H_{29}O_2N.HBr$.

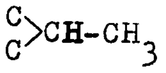
(h) Deoxyannofoline, $C_{16}H_{25}ON$, has been prepared by the chromic acid oxidation of dihydrodeoxyannofoline.

(i) Annofoline has been converted to dehydroannofoline, $C_{16}H_{23}O_2N$, by oxidation with selenium dioxide.

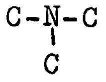
(j) On the basis of the chemical studies and the results of infrared, ultraviolet and N.M.R. spectra the following partial structure has been given to annofoline:



six or larger membered ring



$\genfrac{}{}{0pt}{}{C}{C}{>}CH\text{-}CH_3$



$C\text{-}\underset{\underset{C}{|}}{N}\text{-}C$

$(\text{-}CH\text{-}CH_2)^{*}\text{-}CHOH\text{-}$

*most probable

(k) Lycofoline has the formula, $C_{16}H_{25}O_2N$.

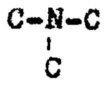
(l) The following derivatives of lycofoline have been prepared: (1) lycofoline hydrobromide $C_{16}H_{25}O_2N.HBr.$, (2) lycofoline methiodide, $C_{16}H_{25}O_2N.CH_3I.$, (3) mono-O-acetyl lycofoline, $C_{18}H_{27}O_3N.$, (4) di-O-acetyl lycofoline, $C_{20}H_{29}O_4N$.

(m) By catalytic hydrogenation, lycofoline has been converted to dihydrolycofoline, $C_{16}H_{27}O_2N$.

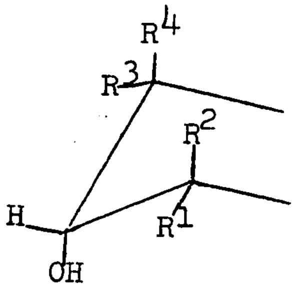
(n) On the basis of chemical studies and the results of the infrared and N.M.R. spectra the following partial structure has been given to lycofoline.

$\begin{matrix}C\\C\end{matrix}\!>C{=}CH{-}CH_2{-}$

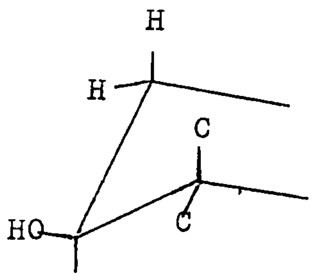
$\begin{matrix}C\\C\end{matrix}\!>CH{-}CH_3$



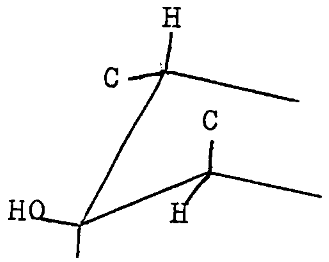
$C{-}\underset{\begin{matrix}|\\C\end{matrix}}{N}{-}C$



where more than two R's are hydrogen atoms.



or



(o) α-Lofoline has the formula $C_{18}H_{29}O_3N$.

(p) α-Lofoline methiodide, $C_{18}H_{29}O_3N.CH_3I$, has been prepared.

(q) β-Lofoline has the formula $C_{18}H_{29}O_3N$.

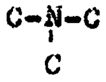
(r) β-Lofoline methiodide, $C_{18}H_{29}O_3N.CH_3I$, has been prepared.

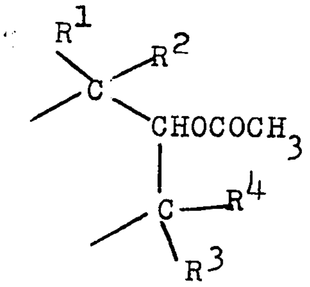
(s) α-Lofoline and β-lofoline have been oxidized to give the same product, dehydrolofoline, $C_{18}H_{27}O_3N$, proving that α-lofoline and β-lofoline are epimeric alcohols.

(t) The following partial structure has been assigned to α-lofoline and β-lofoline on the basis of chemical studies and the results of the N.M.R. and infrared spectra:

$>CHOH$,


$${}^{C}_{C}\!>CH-CH_3$$


$$C-\underset{C}{\overset{}{N}}-C$$



where probably more than two R's are hydrogen.

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