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THE MECHANISM OF THE BIOSYNTHETIC OXIDATION OF CARANINE
AND DERIVATIVES

by

Norman Eugene Heimer

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INTRODUCTION

Lycorine, the major alkaloid of the Amaryllidaceae, was first isolated in 1897 (1). Since that time its structure as well as those of many related alkaloids has been established and their chemical interconversions explored (2).

Several theories have been proposed to explain the biosynthesis of the carbon skeleton of these alkaloids (3, 4, 5, 6). The hypothesis of Barton and Cohen (5) that the alkaloids were built up by oxidative coupling of phenolic intermediates of the norbelladine type, has been experimentally substantiated by feeding experiments with radioactively labeled materials. Experiments with radioactively labeled hydroxynorbelladine have shown it to be a poor precursor of lycorine (7). With the finding that both norpluviine (7) and caranine (8) were converted to lycorine it became evident that hydroxylation at C-2 could occur at a relatively late stage in the biosynthetic process. Since the timing of the hydroxylation had been discovered, a remaining problem was the determination of the mechanism of the hydroxylation process. It is with this problem that this thesis is involved.

The method used to examine the mechanism of the hydroxylation reaction was stereochemical because it must reflect the process being studied. Examination of the stereochemistry would allow detection of three different mechanisms: a retention mechanism, an inversion mechanism, and an oxidation-reduction mechanism in which there was a ketone intermediate. Since the C-2 hydroxyl group in lycorine has the β configuration, feeding 2- α labeled caranine would result in labeled lycorine only if an overall

inversion process were operating. Similarly, feeding 2- β labeled caranine would result in labeled lycorine if an overall retention process occurred. The third alternative would be evident if neither of the C-2 labeled species were converted to lycorine.

This thesis describes the preparation of specifically labeled caranine, its feeding to Zephranthes candida and Hymenocallis americana, and the degradation of the lycorine isolated from these plants to obtain the data necessary to decide among the three possible stereochemical processes.

HISTORICAL

Structure and Chemistry of Lycorine and Related Alkaloids

Lycorine (I) was first isolated and characterized in 1879 (1). In 1913 its molecular formula was established as $C_{16}H_{17}NO_4$ (9). It was further characterized as the picrate, hydrochloride, and perchlorate salts and color reactions (10). Upon catalytic reduction it took up one equivalent of hydrogen to give a dihydro derivative which demonstrated the presence of one double bond (11, 12).

The ring system of lycorine was established by several degradative studies. Distillation of lycorine from zinc dust gave phenanthridine and distillation of dihydroanhydrolycorine methine (II) from zinc dust gave a mixture of phenanthridine, 1-methylphenanthridine and 1-ethyl-6,7-methylenedioxyphenanthridine (13). Treatment of lycorine with methyl iodide gave a mixture of methiodides which yielded lycorine anhydromethine (III) upon Hofmann degradation (14). The structure of the anhydromethine (III) was assigned by hydrogenation to the dihydro derivative (II) and distillation from zinc dust. The methine could be oxidized by permanganate to give a carboxylic acid. Decarboxylation of this acid gave 6,7-methylenedioxy-N-methylphenanthridone (IV). These degradations located the vinyl group in the anhydromethine and suggested that the ring system was a hexahydro-[d,e]pyrrolophenanthridine.

The results of the Emde degradation of lycorine methiochloride were in agreement with this assignment. This degradation gave a base (V) of formula $C_{17}H_{17}NO_2$ which was isomeric but not identical with dihydroanhydrolycorine methine (II). Ozonolysis of the Emde base gave formaldehyde;

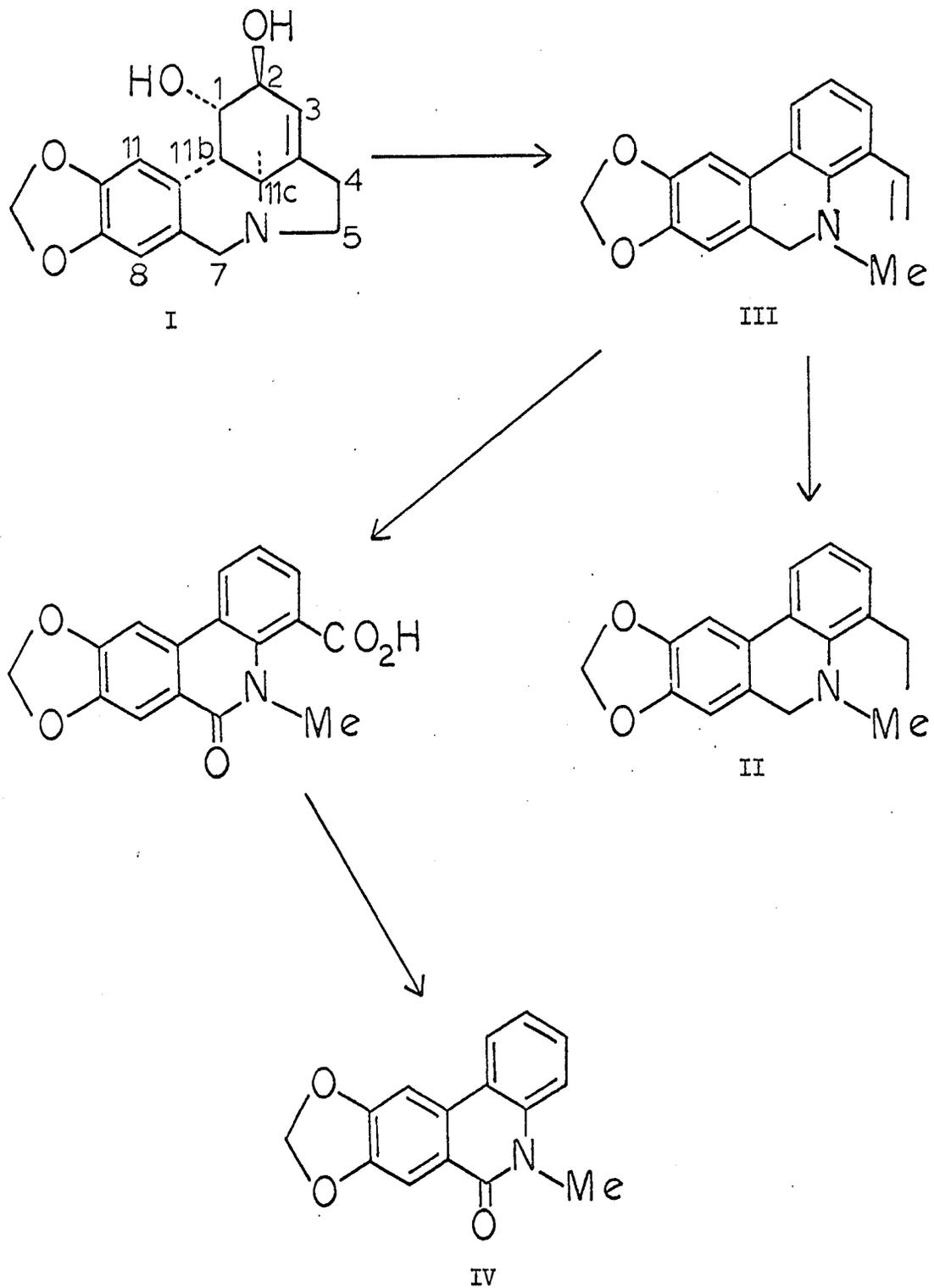


Figure 1. Hofmann degradation of lycorine

however, ozonolysis of II also gave formaldehyde. This suggested that the formaldehyde from the Emde base was probably derived from the methylenedioxy group. Kuhn-Roth oxidation of the Emde base showed the presence of a C-methyl group (15).

Evidence that the aromatic ring had substituents in the 1,2,4, and 5 positions was found in the formation of 1-ethyl-6,7-methylenedioxyphenanthridine (VI) from the zinc dust distillation of II (13). Further proof was found in the oxidation of both lycorine and the Emde base to give hydrastic acid (14).

The remaining two oxygen atoms were present as hydroxyl groups because lycorine formed an O,O-diacetate upon treatment with acetic anhydride in pyridine (11). The hydroxyl groups were determined to be vicinal because treatment of dihydrolycorinone (VII) with lead tetracetate (16), or periodic acid gave a dialdehyde which was isolated and characterized as its dioxime. However, oxidation of VII with lead tetracetate was reported to require two moles of oxidant (16). This was confirmed when the product from the periodate cleavage was identified as VIII (17). This observation required that the hydroxyl groups be at positions 1 and 2.

Further support that the hydroxyl groups were at C-1 and C-2 had been obtained from the oxidation of lycorine with a variety of mild oxidizing agents (18). Oxidation of lycorine with mercuric acetate in aqueous acetic acid gave a mixture of two phenanthridium betaines, X and XI. These degradation products were identified by comparison of their physical properties with those of model synthetic compounds XII and XIII.

Of the four possible configurations for the hydroxyl groups it was possible to eliminate both of the possible cis-configurations because con-

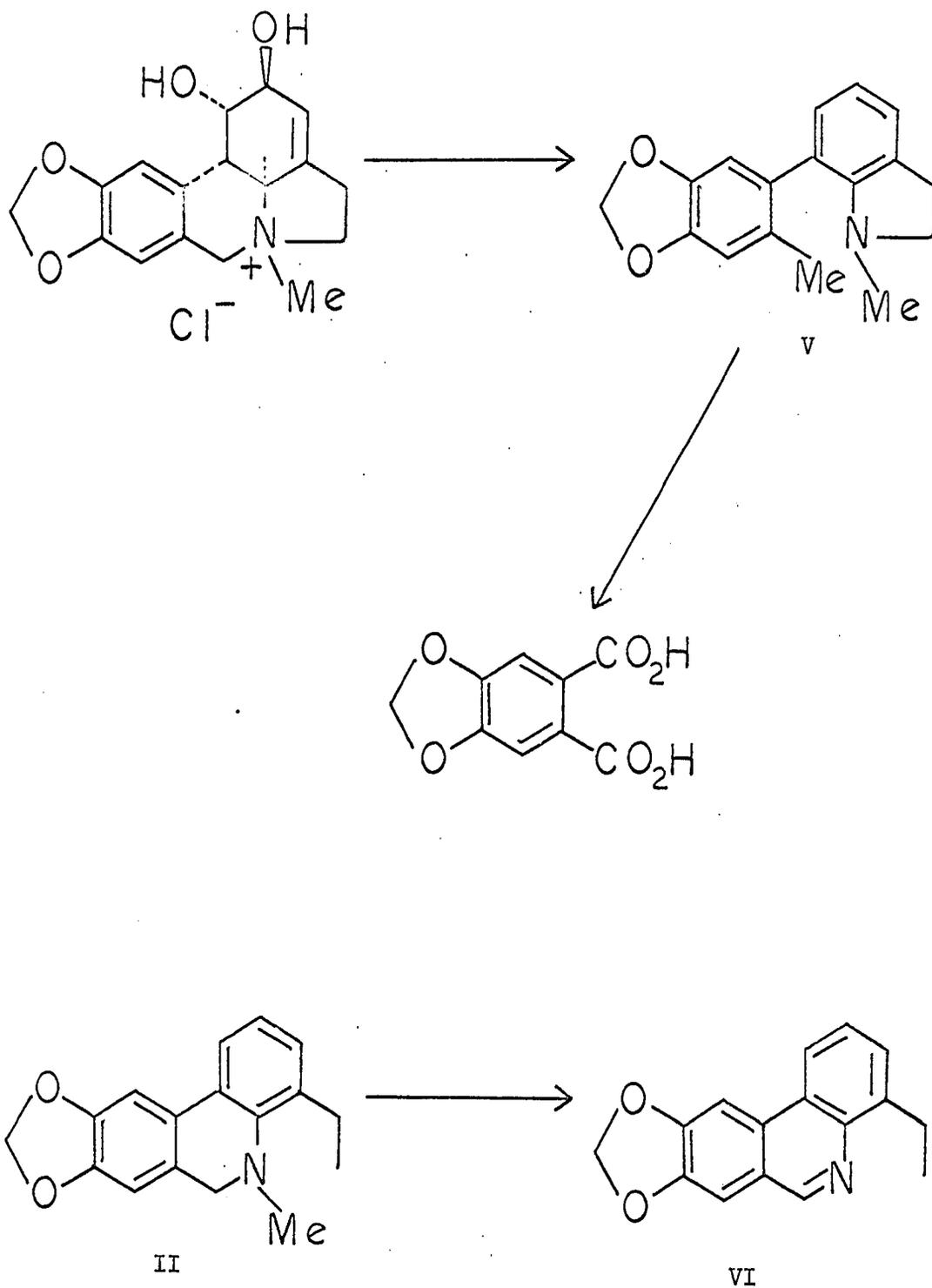


Figure 2. Emde degradation of lycorine and chemistry of the degradation products

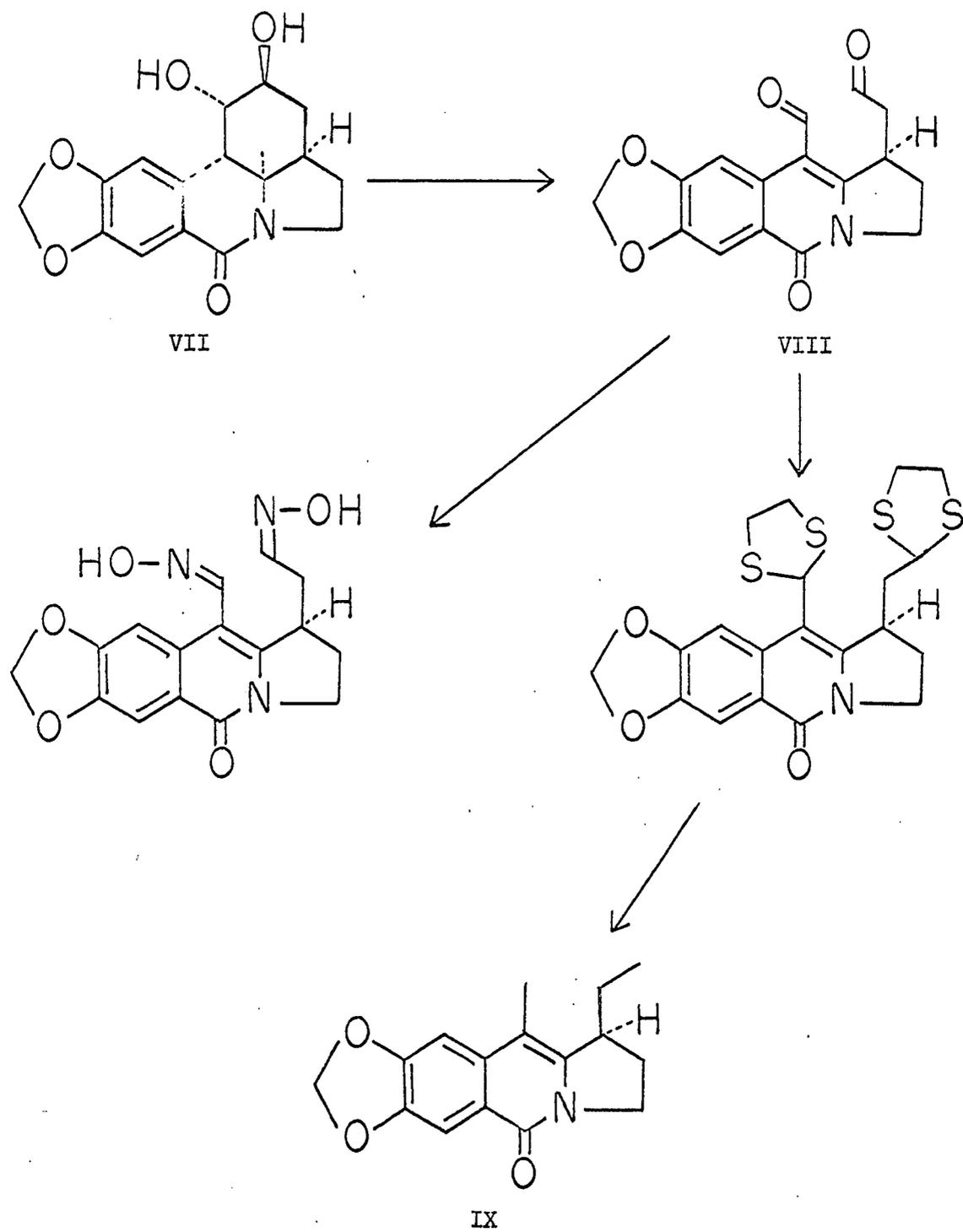


Figure 3. Glycol cleavage of lycorine and chemistry of the product

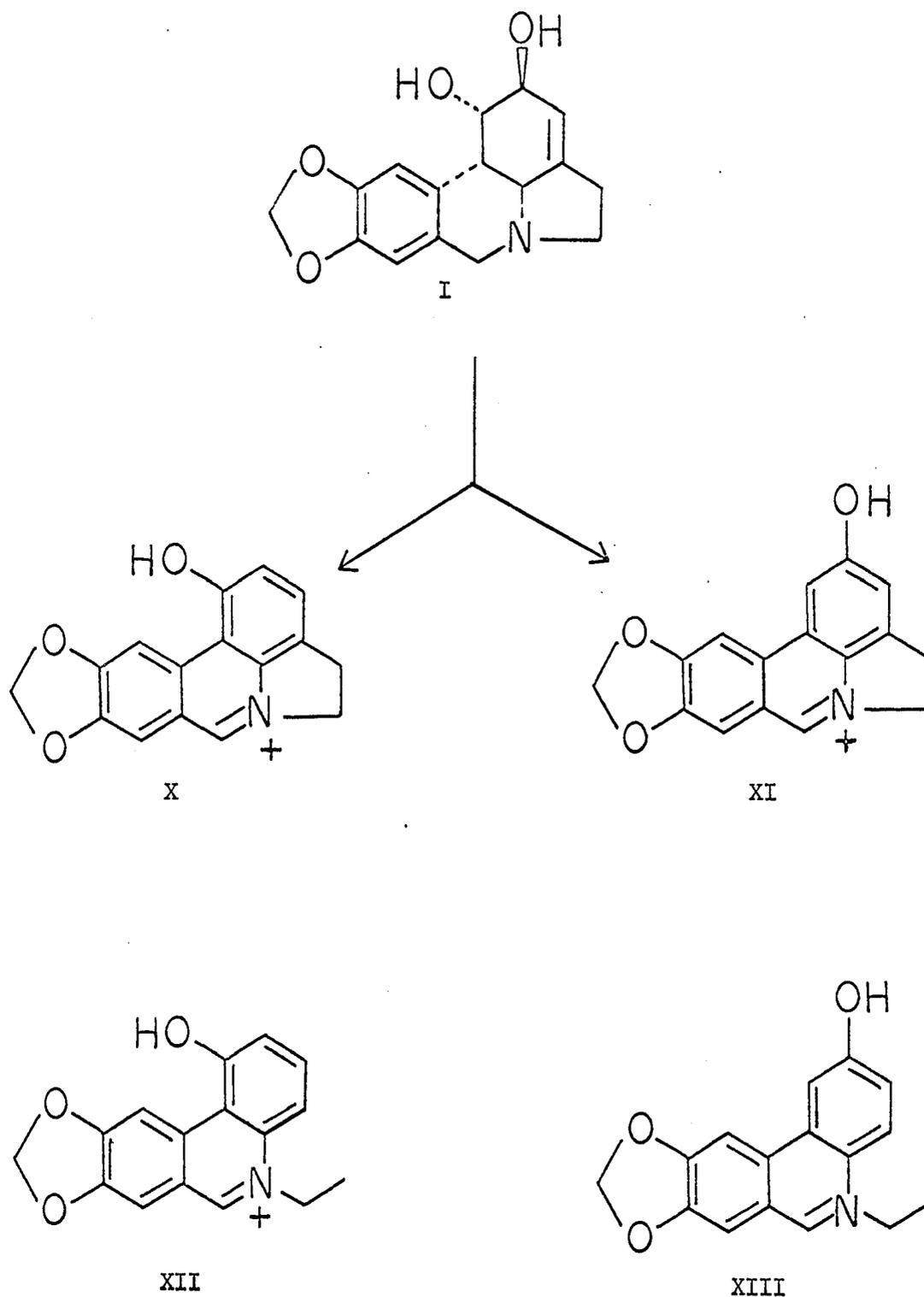


Figure 4. The products from mild oxidation of lycorine

version of dihydrolycorine (XIV) to the 2-tosylate (XV) followed by treatment with mild base resulted in formation of the 1,2-epoxide (XVII) (19a, 19b). The epoxide could also be formed by treatment of dihydrolycorine with phosphoryl chloride to give the trans-chlorohydrin (XVI). The chlorohydrin could be converted to XVII either by treatment with mild base in methanol or by chromatography on alumina (20). Hydrolysis of XVI in aqueous acid gave dihydrolycorine. This indicated that the hydroxyl groups in dihydrolycorine were trans and diaxial (19a, 19b). Reduction of XVII with lithium aluminum hydride gave the C-1 hydroxylated product (XVIII). This was shown by dehydration of XVIII to give anhydrodihydrocaranine (XIX) in which the double bond was conjugated with the aromatic ring (20). Both of these reactions gave the products predicted from the Fürst-Plattner rule (21). In addition to locating the hydroxyl group at C-1, the ease of dehydration suggested that the proton at C-11_b and the C-1 hydroxyl were trans and diaxial to each other (19a, 19b).

From the earlier structural work the double bond in lycorine was proposed to occupy one of three positions: 3,3a; 3a,4; 3a,11c (see I). On the basis of pKa measurements with lycorine (pKa=6.4) and dihydrolycorine (pKa=8.4) the enamine possibility was eliminated (22). Since lycorine is stable in hot ethanolic sulfuric acid, it was also assumed that the double bond was not in the 3a,4 position because these conditions should have resulted in isomerization of the double bond into the six-membered ring (23). The ease with which Hofmann and Emde degradations proceed to give the anhydro products III and V is best rationalized if the double bond is present in the six-membered ring since dihydrolycorine does not undergo Hofmann degradation under the same mild conditions (24). The Hofmann degradation

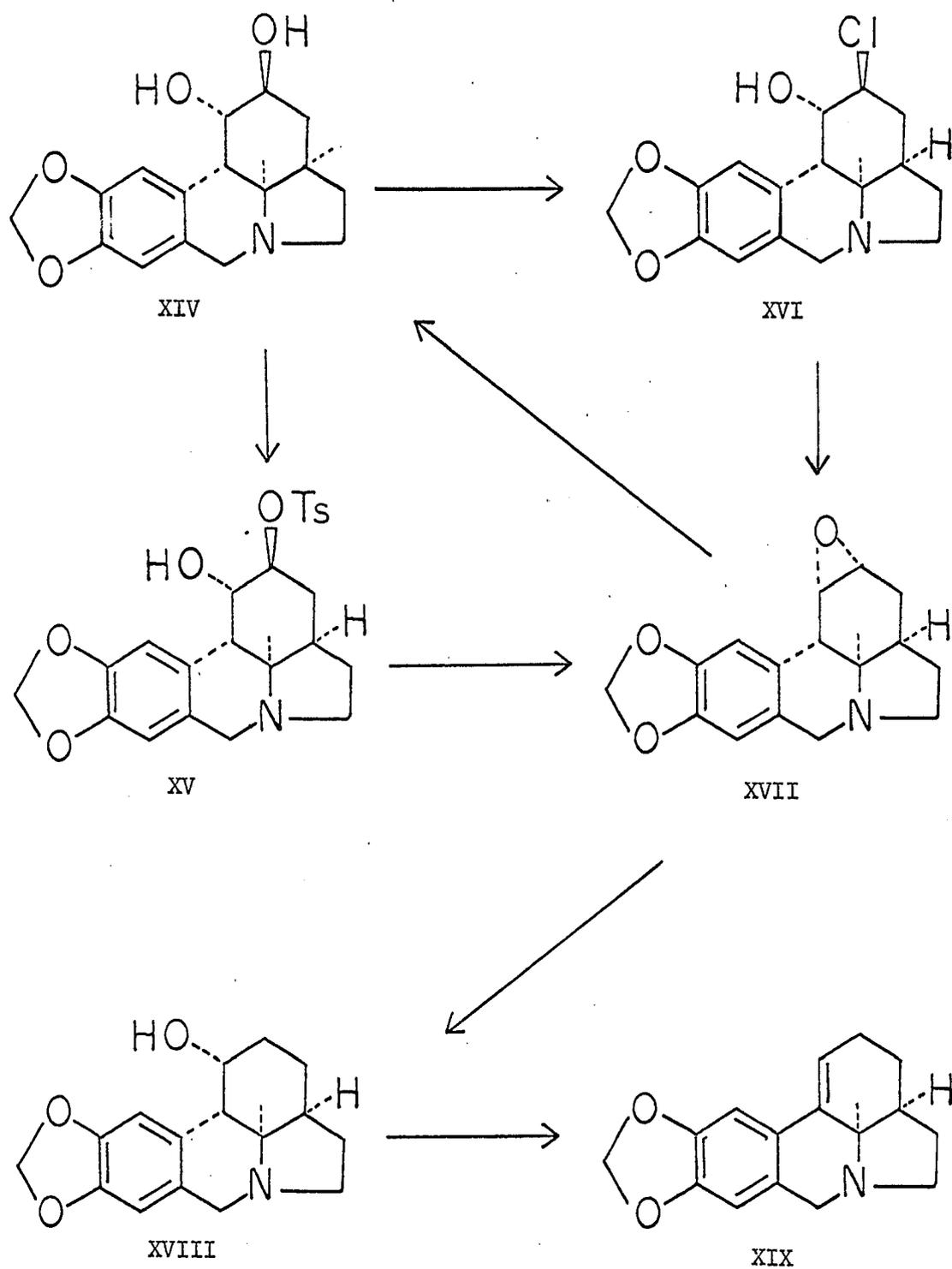


Figure 5. The chemistry of dihydrolycorine and its transformation products

of lycorine can be pictured to proceed by a preliminary aromatization to anhydrolycorine methiodide which then undergoes elimination in the usual manner to give the observed product (25).

The location of the double bond was determined more satisfactorily by the conversion of 1-0-acetyllycorine (XX) to 1-0-acetyllycorin-2-one (XXI) by treatment with manganese dioxide in chloroform at 10°. The ketone could be reduced with lithium aluminum hydride to give largely 2-epilycorine (XXII). Sodium borohydride afforded a mixture of lycorine and 2-epilycorine. Hydrogenation of lycorine provided a single stereoisomer, α -dihydrolycorine, XXIII (11, 12) (Figure 7). Hydrogenation of 2-epilycorine gave a mixture of α - and β -dihydro-2-epilycorine (XXIV and XXV) (25). This undoubtedly resulted from the configuration of the hydroxyl group at C-2. In lycorine the 2-hydroxyl is axial and hinders approach to the β -side of the molecule (steroid convention) causing reduction to occur from the α -side. In 2-epilycorine the 2-hydroxy is quasi-equatorial and reduction could occur from either side of the molecule.

The absolute configuration of lycorine was determined by application of Mill's rule to the pairs, lycorine, 2-epilycorine, 0,0-diacetyllycorine, and 0,0-diacetyl-2-epilycorine. It was shown that the epi series had the more negative molecular rotations; hence, the configuration of C-2 should be as shown in I (25). This assignment was confirmed by examination of ORD and CD spectra of dihydrodesoxylycorin-2-one (XXVI). The spectrum gave a negative Cotton effect at 291 m μ which was attributed to the ketone chromophore. On the basis of the octant rule it was asserted that irrespective of the C-ring conformation, the absolute configuration was the same as that proposed by Mills' rule (26). In a later paper (27) after

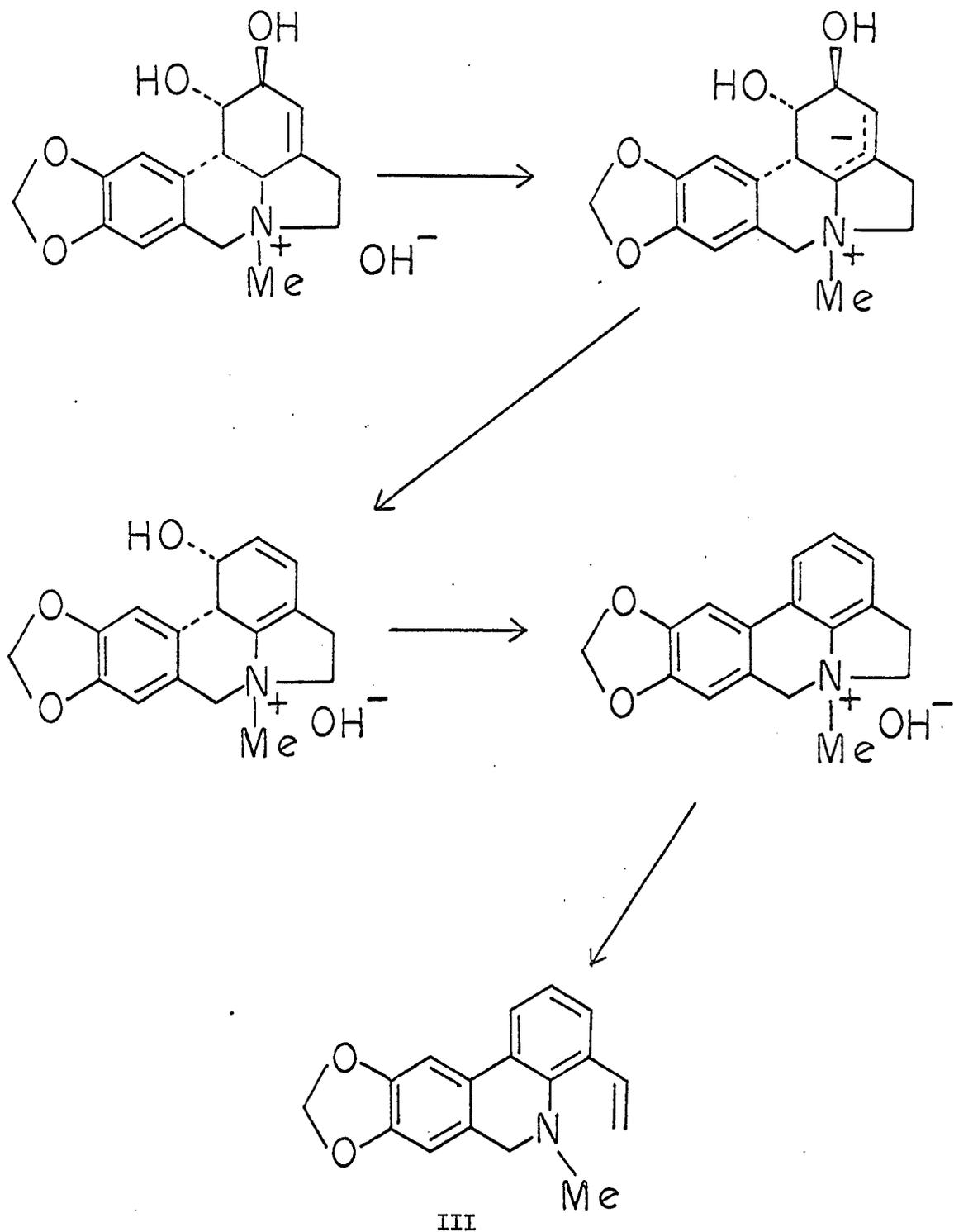


Figure 6. The mechanism of the Hofmann degradation of lycorine

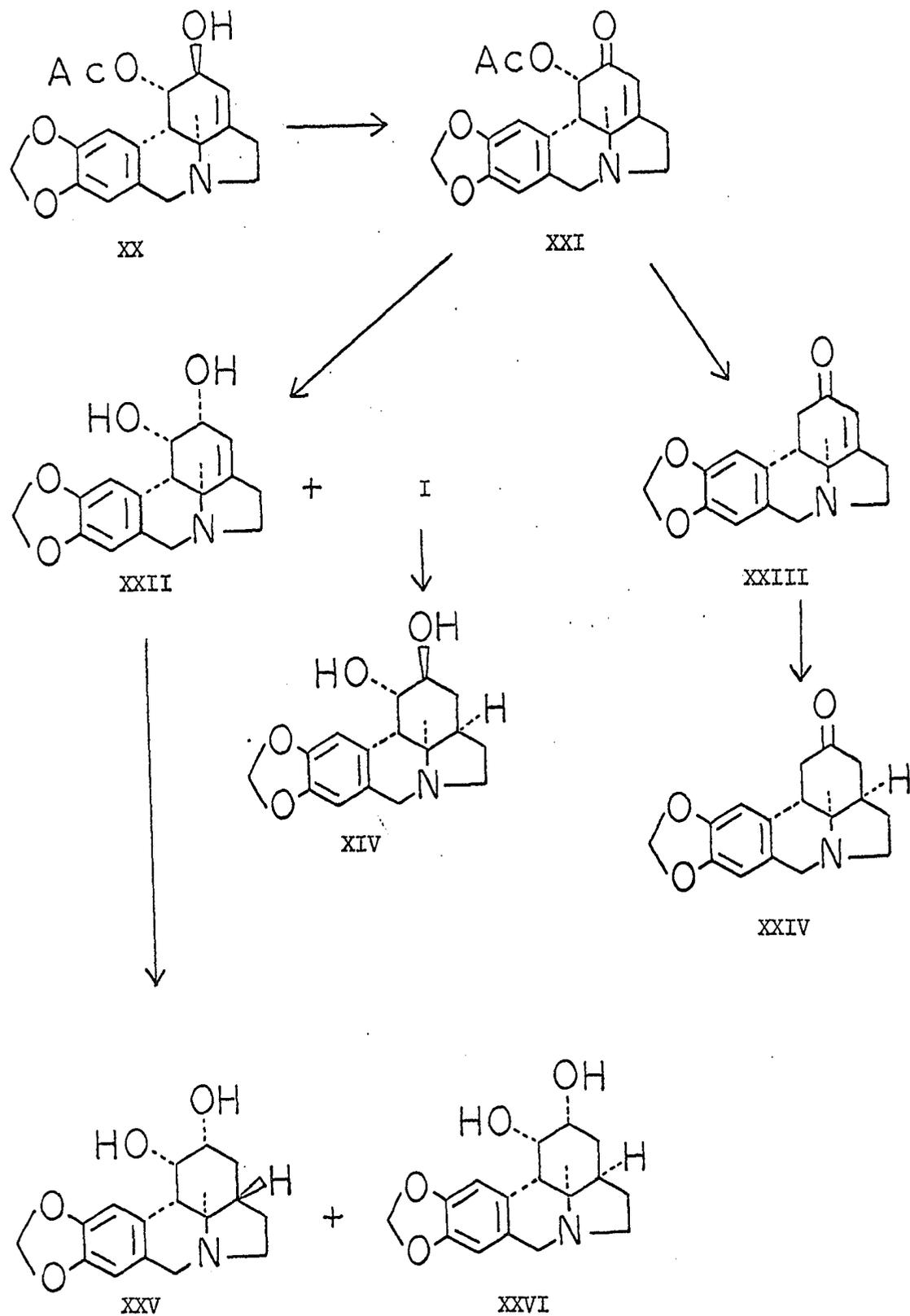


Figure 7. The chemistry of 1-O-acetyllycorine-2-one

considering the symmetry of the transition moments in aromatic rings with C_{2v} local symmetry, a rule was proposed which gave the same result. The absolute and relative stereochemistry in α -dihydrolycorine has been confirmed by X-ray analysis of the hydrobromide salt (28). Further evidence for the relative stereochemistry of ring C in lycorine and its derivatives was obtained by examination of various nmr spectra (26).

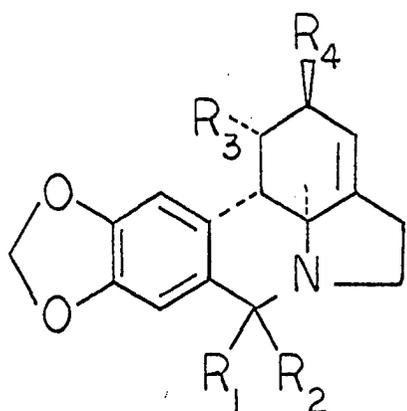
The coupling constant between H_1 and H_2 is less than 4 Hz. The coupling constant between H_1 and H_{11b} is approximately 3 Hz. These values are about the magnitude expected for these coupling constants on the basis of the Karplus relation. The B/C ring junction could be assigned a trans-configuration because the coupling constant found between H_{11b} and H_{11c} was about 12 Hz. The conformation of the C-ring and the orientation of the nitrogen lone pair in lycorine and caranine has been inferred from the examination of their ORD and CD spectra. Because of the similarity of the 290 $m\mu$ Cotton effects found for caranine, lycorine, and β -dihydrocaranine, the conformation of the C-ring and the nitrogen lone pair should be the same. Examination of molecular models of β -dihydrocaranine showed that the C-ring should exist as a twisted chair and the nitrogen lone pair should be anti to the C_{11c} hydrogen (27). This is in agreement with the assignment of the preferred conformation of the nitrogen lone pair in the isomeric lycoranes by the use of Bohlmann bands (29); however, this data must be interpreted with caution. In some cases certainly a mixture of conformers was being observed. Further data on the conformations of the C-ring and the nitrogen lone pair orientation was available from the reaction of lycorine (I), caranine (XXX) and α - and β -dihydrocaranines with methyl iodide. α -Dihydrocaranine yielded only one methiodide,

Table 1. NMR spectral data on lycorine and its related compounds^a

Compounds	Chemical shift (τ) and apparent coupling constants J (Hz, in parentheses)				
	H ₁	H ₂	H ₃	H11b	H11c
XXVIIa	4.29 (t) $W_{\frac{1}{2}}=4$	4.76 (m) $W_{\frac{1}{2}}=7$	4.50(m) $W_{\frac{1}{2}}=7$	6.16 ^b	-b
XXVIIb	4.26 (m) $W_{\frac{1}{2}}=5$	4.71 (m) $W_{\frac{1}{2}}=6$	4.38 (m) $W_{\frac{1}{2}}=6$	3.33 (d) (1.2)	6.97 (d-q) (12.5,2.3,1.2)
XXXVIIIa	4.35 (q) (3.0,2.0)	5.10 (d-q) (10.0,5.5,2.0)		6.94 ^b	-b
XXVIIIb	4.39 (t) (3.2)	4.97 (d-q) (8.7,4.8,3.2)		6.79(d-q)Z (13.0,3.2,1.0)	6.07(d-d) (13.0,9.2)
XXVIIIc	4.14 (t) $W_{\frac{1}{2}}=5$	4.94 (d-q) (11.2,6.5,2.5)		7.09 (d-q) - (12.7,2.2)	6.12 (d-d) (12.7,8.3)
XXIXa	5.39 (q) $W_{\frac{1}{2}}=7$	5.88 (q) $W_{\frac{1}{2}}=8$		6.82 (d-m) (12.2)	-b
XXIXb	4.27 (q) (3.5,2.0) $W_{\frac{1}{2}}=7$	4.80 (q) (3.5) $W_{\frac{1}{2}}=10$		6.56 (d-q) (12.0,2.0,1.0)	6.17 (d-d) (12.0,7.0)
XXXa	4.38 (q) $W_{\frac{1}{2}}=7$			7.08 (d-m) (11.0)	7.56 (d-d) (11.0,4.0)
XXXb	4.27 (q) $W_{\frac{1}{2}}=7$			6.95 (d-q) (13.5,2.7,1.0)	6.15 (d-d) (13.5,4.0)
XXXc	4.35 (t) $W_{\frac{1}{2}}=7$	5.02 (q) (2.7) $W_{\frac{1}{2}}=9$		6.52 (d-q) (13.0,2.7,0.8)	6.11 (d-d) (13.0,3.7)

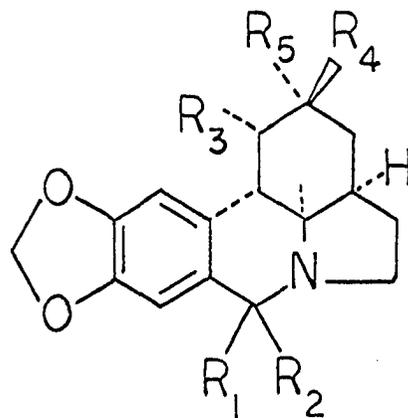
^aPeak multiplicities are represented by s (singlet), d (doublet), q (quartet) and (m) multiplet. $W_{\frac{1}{2}}$ is the half-height band width (Hz).

^bObscured by other signals.



XXVII a $R_1=R_2=H, R_3=R_4=OAc$

b $R_1, R_2=O, R_3=R_4=OAc$



XXVIII a $R_1=R_2=R_3=H,$

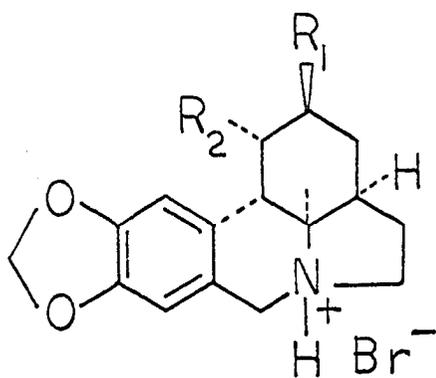
$R_3=R_4=OAc$

b $R_1, R_2=O,$

$R_3=R_4=OAc, R_5=H$

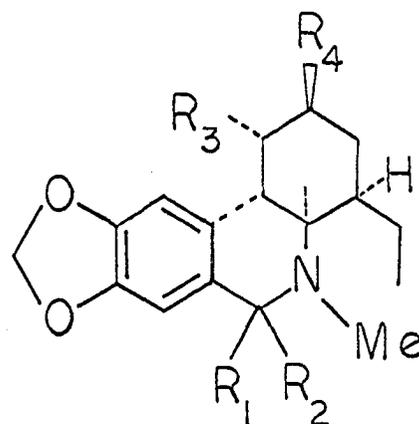
c $R_1, R_2=O,$

$R_3=R_5=OAc, R_4=H$



XXIX a $R_1=R_2=OH$

b $R_1=R_2=OAc$



XXX a $R_1=R_2=R_4=H, R_3=OAc$

b $R_1, R_2=O, R_3=OAc, R_4=H$

c $R_1, R_2=O, R_3=R_4=OAc$

Figure 8. Structures of compounds listed in Table 1

whereas β -dihydrocaranine, lycorine and caranine yielded mixtures of methiodides (24). Caranine (XXXI) was first isolated and characterized in 1956 (30). Elemental analysis showed it to have a formula of $C_{16}H_{17}NO_3$. Two of the oxygen atoms were determined to be present as a methylenedioxy group through infrared analysis and oxidation with neutral permanganate to give hydrastic acid (characterized as its N-ethyl imide derivative). The formation of an O-acetate demonstrated that the remaining oxygen atom was present as a hydroxyl function. The presence of a trisubstituted double bond was demonstrated by the addition of one equivalent of hydrogen upon catalytic reduction to give a mixture of α - and β -dihydrocaranine. α -dihydrocaranine was identical with the product obtained from lithium aluminum hydride reduction of XVI. The size ring B was investigated by oxidation of acetyldihydrocaranine to the lactam (XXII) with permanganate. This lactam showed a carbonyl stretching frequency of 6.13μ in agreement with that found for diacetyldihydrolycorinone (XXX). Ring C was shown to contain six atoms by oxidation of dihydrocaranine to the ketone (XXXIII) via a modified Oppenauer oxidation. The product had a carbonyl stretching frequency of 5.78μ . In contrast to the Hofmann degradation of lycorine methiodide, in which the five-membered ring was opened, Hofmann degradation of caranine gave the o,m-bridged biphenyl (XXXIV) caranine anhydromethine. If the Hofmann degradation was run at a low enough temperature, XXXIV could be obtained optically active. The absolute configuration of the optically active product had been determined to be as shown by ORD studies (31). Further evidence that caranine was 2-desoxylycorine had been obtained from the conversion of lycorine to caranine by two different reaction sequences. Treatment of lycorine with phosphoryl chloride and a trace

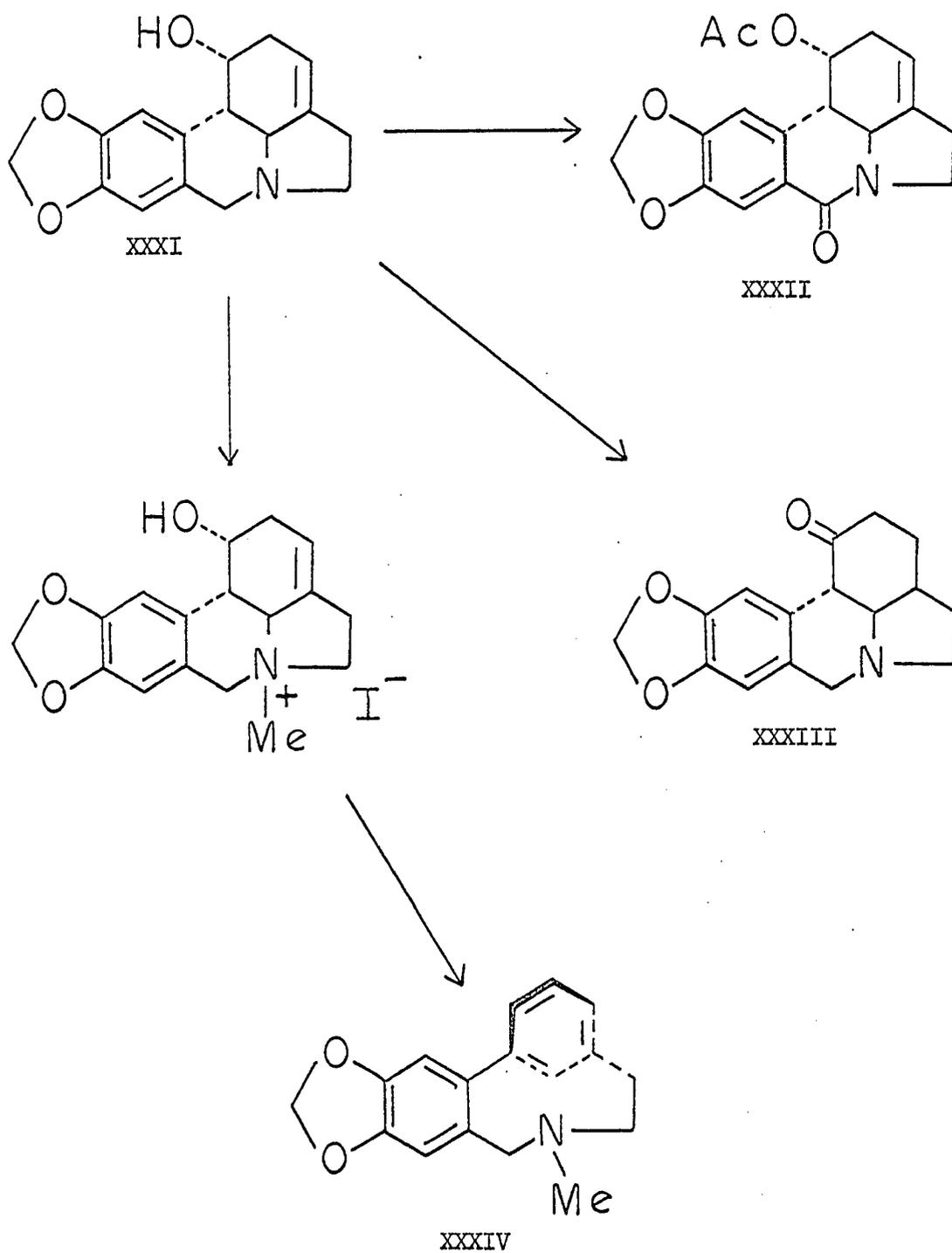


Figure 9. The chemistry of caranine

of hydrochloric acid gave a cis chlorohydrin (XXXV). The chlorohydrin was shown to be cis by its stability to mild base and by its conversion to lycorine-2-methyl ether upon treatment with potassium carbonate in methanol. Reaction of XXXV with sodium acetate in acetone resulted in conversion to 2-O-acetyllycorine. Reduction of the chlorohydrin with lithium aluminum hydride gave caranine (32). Treatment of 0,0-diacetyllycorine with sodium in amyl alcohol gave caranine in addition to other products (33) (see Figure 10).

Recently two new alkaloids have been reported: jonquilline (XXI) and zephyranthine (XXXIV). Jonquilline was isolated and characterized in 1965 (34). The infrared and ultraviolet spectra suggested the presence of an O-acetyl group and an α, β -unsaturated ketone. Since reduction of XXI with lithium aluminum hydride gave 2-epilycorine, jonquilline must be 1-O-acetyllycorin-2-one. Zephyranthine was isolated and characterized in 1964 (35). It formed an 0,0-diacetate and an acetonide derivative. Cleavage of the alkaloid with periodic acid gave a dialdehyde that was identical with the one obtained from the cleavage of α -dihydrolycorine. Zephyranthine has been shown to be identical with α -dihydro-2-epilycorine by a mixture melting point determination.

Biosynthesis of Pyrrolo [d,e] Phenanthridine Alkaloids

Several theories had been proposed to explain the biosynthesis the lycorine ring system (3, 4, 5, 6). The theory that has now gained the most acceptance is that proposed by Barton and Cohen in 1957 (5). According to this proposal, the lycorine type alkaloids should be derived from the ortho-para coupling of a diradical produced by the oxidation of a

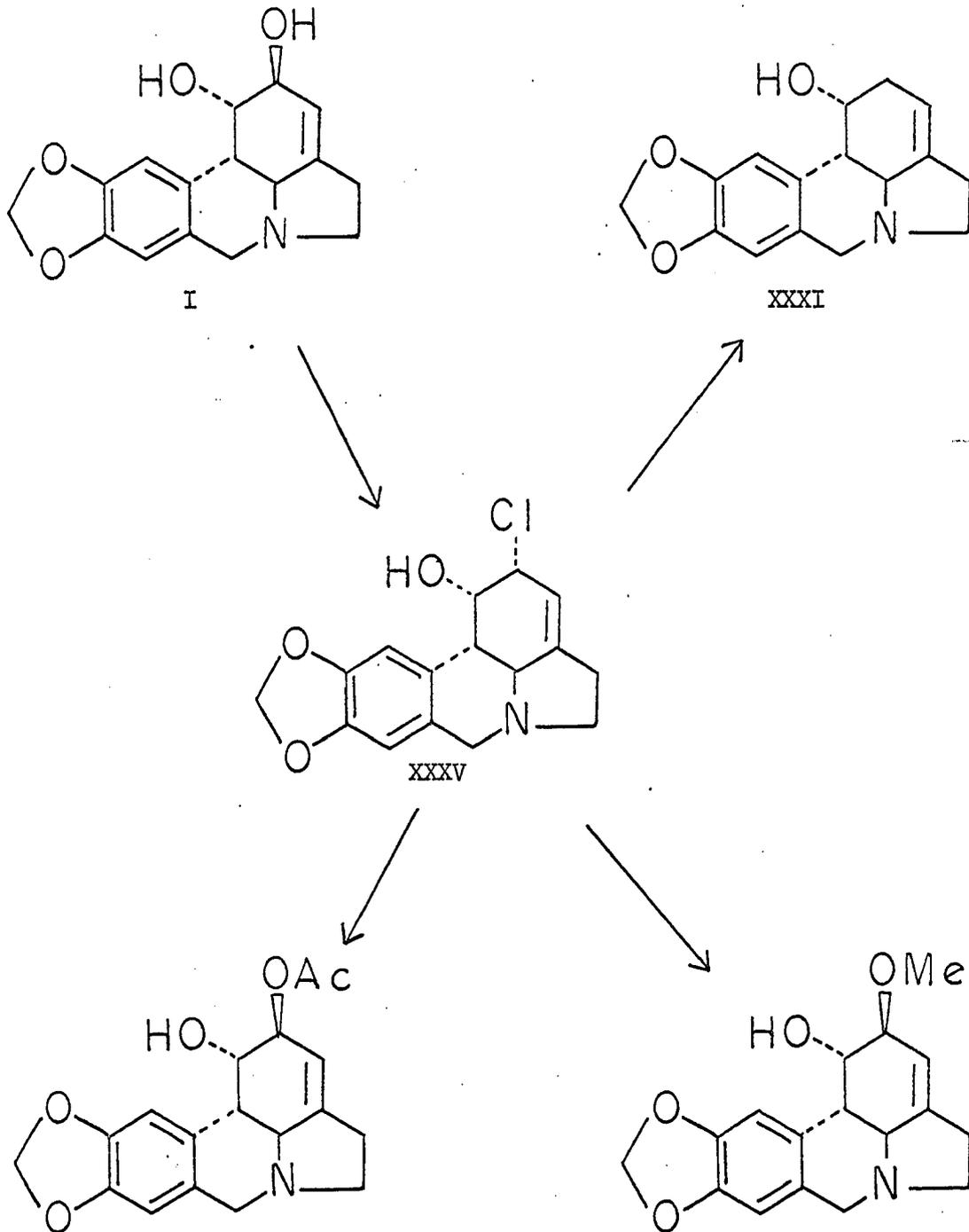


Figure 10. The chemistry of lycorine chlorohydrin

norbelladine intermediate (XXXVI). Further oxidation and cyclization yield norpluviine (XXXVII) as shown in Figure 11. This theory has been substantiated by a number of experiments (36). Administration of doubly-labeled norbelladine to Nerine bowdenii yielded radioactive norpluviine and lycorine (Figure 12). The ratio of activity in the two labeled positions was the same as that in the norbelladine fed. This suggested that the norbelladine was incorporated intact (7).

Other experiments with 0-methylnorbelladine, XXXVIII, have shown that it was also an efficient precursor of haemanthamine, XXXIX, (37, 38) and norpluviine, XXXVII, (39). The origin of the norbelladine intermediate had been demonstrated by experiments in which phenylalanine and tyrosine were shown to be incorporated into norpluviine, lycorine and belladine. Phenylalanine was found to be the precursor of the C₆-C₁ fragment (7) and tyrosine was the precursor of the C₆-C₂ fragment (7, 40). Norbelladine was probably built up by conversion of tyrosine to tyramine (36) which was then condensed with protocatechuic aldehyde, the latter being produced from phenylalanine via cinnamic acid and caffeic acid (41). These results determined the origin of the carbon skeleton and the C-1 hydroxyl group; however, they did not allow any conclusion to be drawn about the origin of the C-2 hydroxyl group. Experiments with labeled hydroxynorbelladine, XL, (7) 3,4-dihydroxyphenylalanine and methoxynorbelladine, XLI, (39) had shown that these compounds were inefficient precursors of lycorine and galanthine respectively.

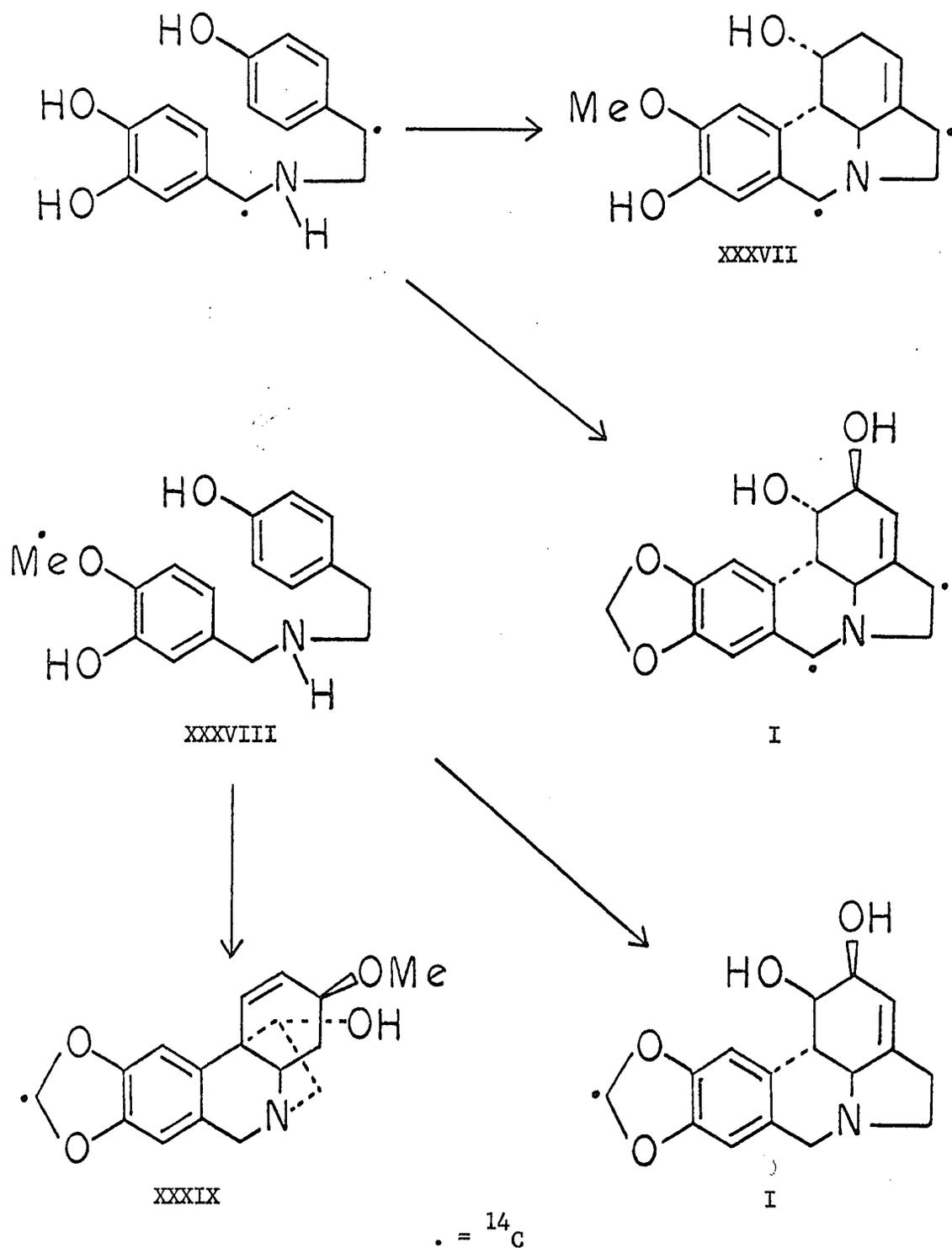
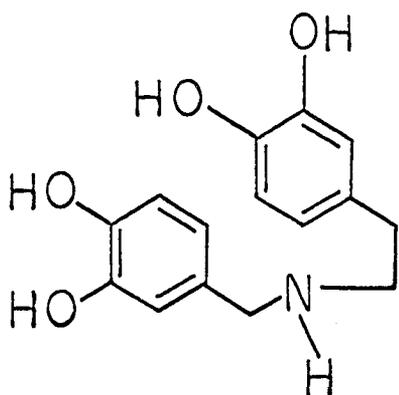
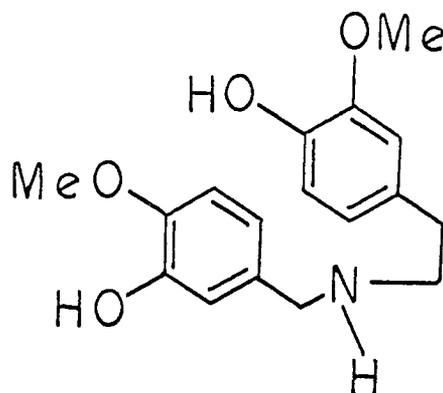


Figure 12. Conversion of norbelladine to norpluviine and lycorine



XL



XLI

Evidence that the C-2 hydroxyl was introduced as a late stage in the biosynthesis was obtained from feeding $[^3\text{H}]$ norpluviine (7) and caranine (8) which were converted to lycorine in good yield in the plant. Similar results had been obtained in the conversion of $[^3\text{H}]$ norpluviine to galanthine (XLII) and narcissidine (39) (Figure 13).

The conversion of C-H to C-OH by microbiological techniques is a well-known process (42). Hydroxylations of a number of alkaloids have been performed by these methods (43, 44, 45). Research on the mechanism of the process has been performed exclusively with steroids (Figure 14). In these cases the oxygen atom is derived from molecular oxygen and not from water (46). The hydroxyl group introduced in the hydroxylation process replaces the hydrogen atom of the same steric configuration (47, 48, 49, 50).

Incubation of *Rhizopus nigricans* with $[11\alpha, 12\beta\text{-}^3\text{H}]$ pregnan-3,20-dione (XLIIIa) resulted in loss of the C_{11} -tritium atom upon conversion to XLIVa (47). In a similar experiment it was shown that incubation of *R. nigricans* with $[11\beta\text{-}^2\text{H}]$ pregnan-3,20-dione (XLIIIb) gave the 11α -hydroxy derivative XLIIIb in which the deuterium atom was retained (48). The action of surviving bovine adrenals on $[11\alpha, 12\beta\text{-}^3\text{H}]$ pregnan-3,20-

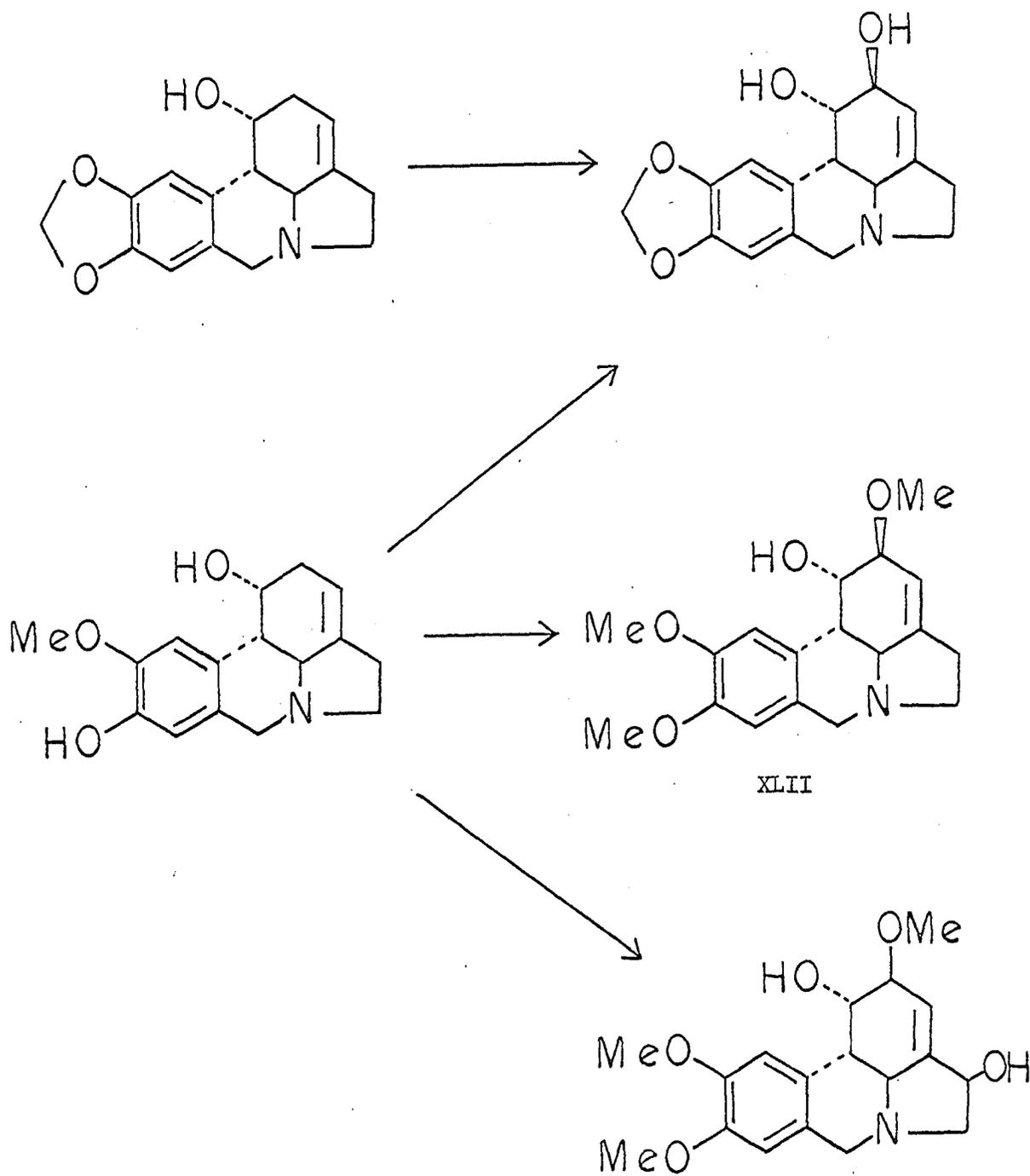
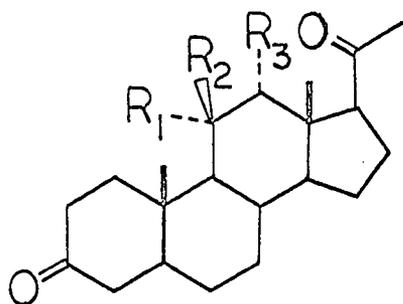
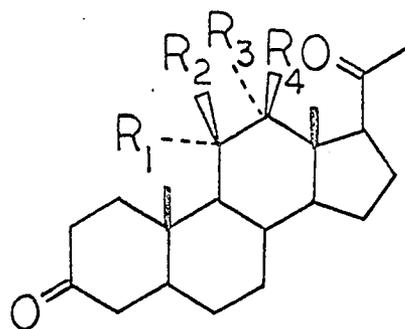


Figure 13. Conversion of norpluviine and caranine to galanthine, lycorine and narcissidine



XLIII a $R_1=R_3=^3\text{H}, R_2=\text{H}$

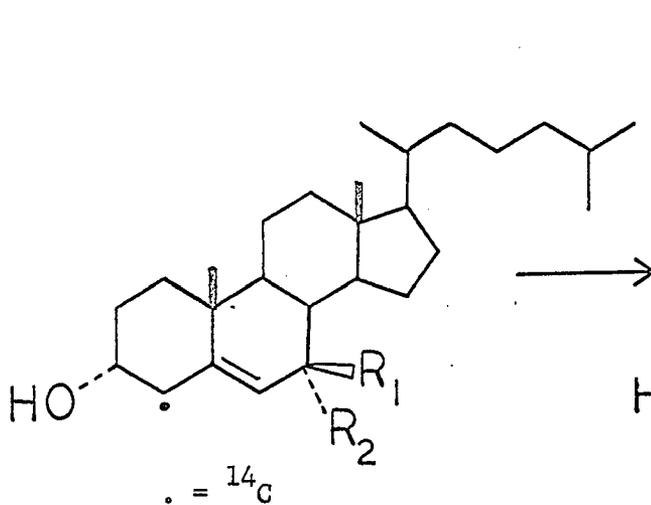
XLIII b $R_1=R_3=\text{H}, R_2=^2\text{H}$



XLIV a $R_1=\text{OH}, R_2=R_4=\text{H}, R_3=^3\text{H}$

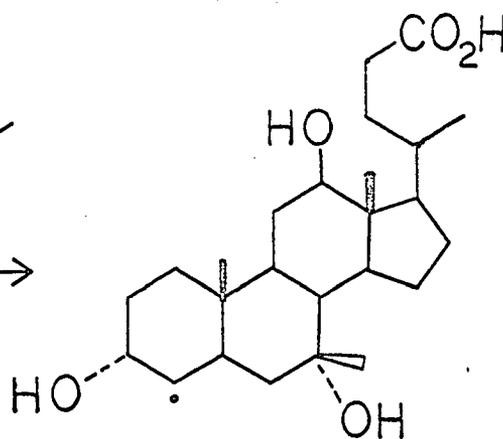
XLIV b $R_1=\text{OH}, R_2=R_3=R_4=\text{H}$

XLIV c $R_1=R_3=^3\text{H}, R_2=\text{H}, R_4=\text{OH}$



XLV a $R_1=\text{H}, R_2=^3\text{H}$

b $R_1=^3\text{H}, R_2=\text{H}$



XLVI a $R_1 = \text{H}$

XLVI b $R_2 = ^3\text{H}$

Figure 14. Hydroxylation of steroids

dione yielded the 11 β -hydroxy derivative (XLIVc) in which all of the tritium at C₁₁ and C₁₂ was retained (49). In another experiment doubly labeled material [4-¹⁴C, 7 α -³H] cholesterol, (XLVa) and the [7 β -³H] epimer (XLVb) were administered to living rats, and cholic acid was isolated. Hydroxylation at C-7 had proceeded with retention as shown by the conversion of XLIVa to XLVa and XLIVb to XLVb (50). It is significant that in these hydroxylation reactions there appears to be no isotope effect. This indicates that either rupture of the C-H bond occurs after the rate determining step or that chemical reaction is preceded by at least one slow physical step, such as adsorption, which is insensitive to the hydrogen isotope (50).

RESULTS AND DISCUSSION

Synthesis of Labeled Precursors

Since it had been shown that both caranine and norpluviine were converted to lycorine, one of the remaining problems was to try to determine the mechanism by which this transformation occurred. There are three stereochemically distinguishable pathways that could lead to the product hydroxylated at C-2: retention, inversion and an intermediate ketone. Ideally, to distinguish the possible paths $[2\alpha\text{-}^3\text{H}]$ caranine and $[2\beta\text{-}^3\text{H}]$ caranine would be required. If the $[2\alpha\text{-}^3\text{H}]$ caranine were converted to labeled lycorine, the process would have to proceed by an overall retention process. Similarly, if $[2\beta\text{-}^3\text{H}]$ caranine were converted to labeled lycorine, the process would occur by overall inversion. If neither tracer were converted to labeled lycorine, a ketone intermediate would be probable.

The most attractive route for synthesis of labeled $[2\beta\text{-}^3\text{H}]$ caranine appeared to be via Takeda's chlorohydrin (XXXV) because the preparation and reduction to give caranine had been reported (32). A cis configuration of XXXV was demonstrated by treatment with potassium carbonate in methanol. The only product obtained was lycorine-2-methyl ether. Treatment of XXXV with potassium acetate in acetone yielded 2-O-acetyllycorine. No epoxide was found in either of these reactions (32).

Although Takeda (32) reported the chlorohydrin as crystalline, the chlorohydrin was never obtained in a crystalline state in this laboratory. The infrared spectrum of the crude chlorohydrin did not indicate any major contaminant. Treatment of the amorphous material with methanol and

potassium carbonate gave lycorine-2-methyl ether (LXVII). Reduction of the crude chlorohydrin with lithium aluminum hydride gave caranine. Both reactions proceeded in good yield. To further establish the relative configuration of the hydroxyl group and the chlorine atom, the high dilution infrared spectrum in carbon tetrachloride of the 3600 cm^{-1} region was obtained. The results of this determination and the hydroxyl stretching frequencies found for several related compounds are shown in Table 2.

Table 2. Hydroxyl stretching frequencies for lycorine derivatives

Compound	OH(cm^{-1})	Assignment
Galanthine	3602	OH- π
Lycorine-2-methyl ether	3602	OH- π
Caranine	3595	OH- π
α -Dihydrocaranine	3598	OH- π
β -Dihydrocaranine	3597	OH- π
2- <u>Epilycorine</u>	3578	OH-0
	3619	OH- π
Lycorine chlorohydrin	3585	OH-Cl
Dihydrolycorine chlorohydrin	3605	OH- π

By comparing the hydroxyl stretching frequencies of lycorine chlorohydrin and 2-epilycorine, it can be seen that the cis arrangement is more probable. The corresponding frequency found for dihydrolycorine

chlorohydrin, which had been shown to be trans (20) appears at 3605 cm^{-1} in agreement with that found for both galanthine and lycorine-2-methyl ether, 3602 cm^{-1} . All of the compounds examined showed a low intensity, free hydroxyl stretching frequency near 3625 cm^{-1} . In lycorine chlorohydrin, the shift upon hydrogen bond formation was 40 cm^{-1} . The values found for a series of halohydrins in the ring A of the steroid nucleus ranged from 25 to 48 cm^{-1} (51).

A most curious aspect of this problem was the conversion of lycorine to the cis-chlorohydrin while dihydrolycorine affords a trans-chlorohydrin under essentially the same conditions. The yield of cis-chlorohydrin from lycorine was reported to be about 10% (32) Dihydrolycorine was converted to the trans-chlorohydrin in about 50% yield (20).

The process leading to the trans-chlorohydrin probably does not occur by an S_N1 mechanism. If an S_N1 mechanism were operating, hydrochloric acids should not be needed for the reaction to proceed. It has been reported that the reaction does not proceed if hydrochloric acid is not added (20). Furthermore, if an S_N1 mechanism were operating in this case it should be more favored in lycorine where the hydroxyl group is allylic.

The formation of the trans-chlorohydrin from dihydrolycorine probably proceeds by first formation of the dichlorophosphate ester. This ester is then decomposed by the hydrochloric acid added to the reaction mixture by protonation of the ester and displacement of dichlorophosphoric acid by the C-1 hydroxyl group to give the epoxide. The epoxide would then be opened by the acid present to give the trans-chlorohydrin as shown in Figure 15.

Lycorine is thought to give the cis-chlorohydrin by a simple S_N2 displacement of the protonated dichlorophosphate ester by chloride ion (32).

All attempts to crystallize lycorine chlorohydrin (XXXV) failed to give any product that had properties similar to those reported (32). Finally an attempt was made to purify the chlorohydrin by chromatography over Florisil in ether. The ether eluate from the column, after evaporation to near dryness, deposited crystalline material when allowed to stand overnight at about 5°. This crystalline product proved to be a halogen free compound (XLVIII) with a melting point close to that reported for the chlorohydrin.

The ultraviolet spectrum of XLVIII showed a normal methylenedioxyaryl system. The infrared spectrum (Figure 16) showed no absorption which could be attributed to hydroxyl or carbonyl groups. The mass spectrum showed a parent molecular ion at m/e 269 which confirmed the molecular formula obtained from analytical data, $C_{16}H_{15}NO_3$. The nmr spectrum of XLVIII (Figure 16) showed the presence of two aromatic protons, 7.03δ and 6.57δ ; a methylenedioxy group, 5.90δ ; and a broad olefinic proton, 5.75δ . The remainder of the spectrum was complicated by superposition of coupling patterns. Spin decoupling allowed the assignment of most of the remaining peaks. The 7α and 7β benzylic protons were found as the usual quartet. The lower field doublet was centered at 4.06δ , $J=14.5$ Hz, and the upfield doublet was centered at 3.55δ . The C-1 proton appeared as a doublet centered at 3.95δ , $J=4$ Hz; and the C-2 proton appeared as a triplet centered at 3.47δ , $J=4$ Hz. The multiplet at 3.71 was assigned as the C-11c proton by analogy with previously reported work (26). The remaining five protons were not assigned.

The mass spectrum of XLVIII shows three ions of moderate abundance in addition to the parent molecular ion at m/e 269. The base peak in the

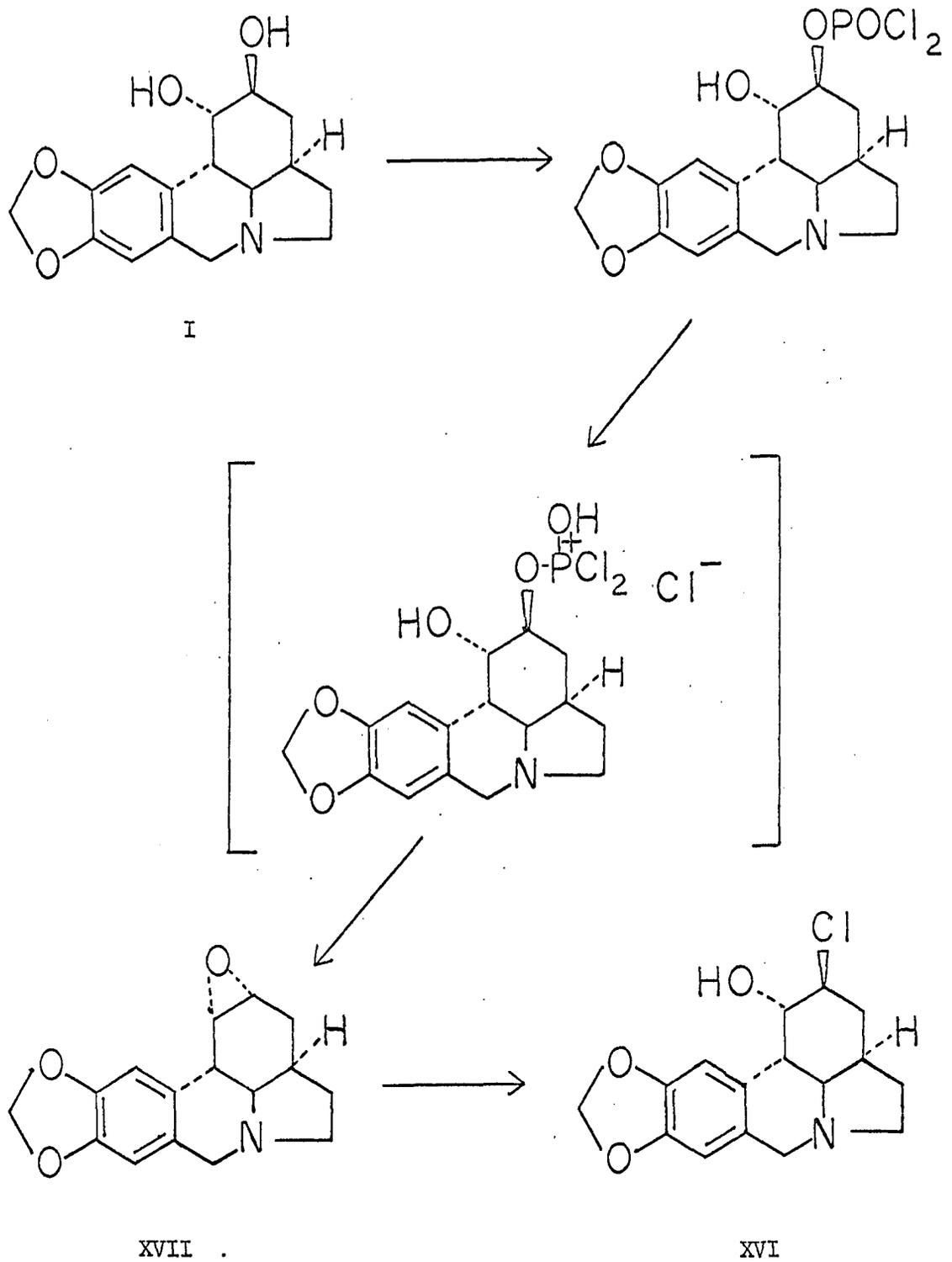
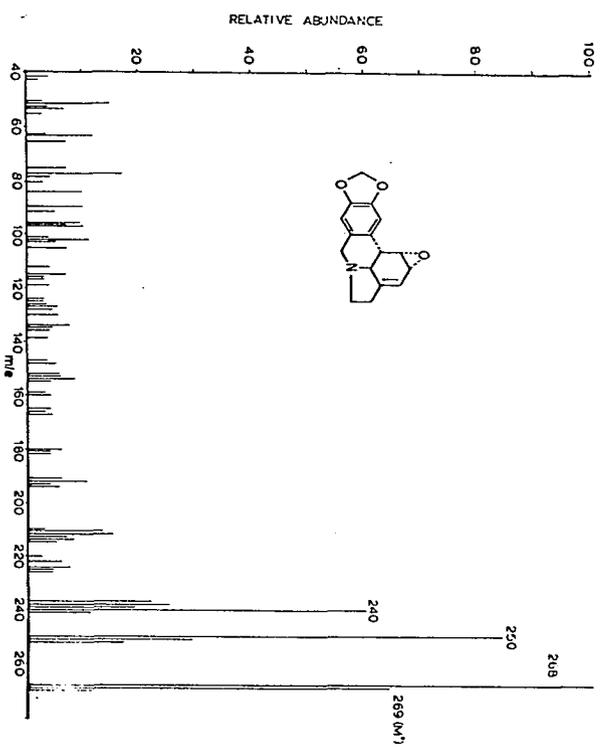
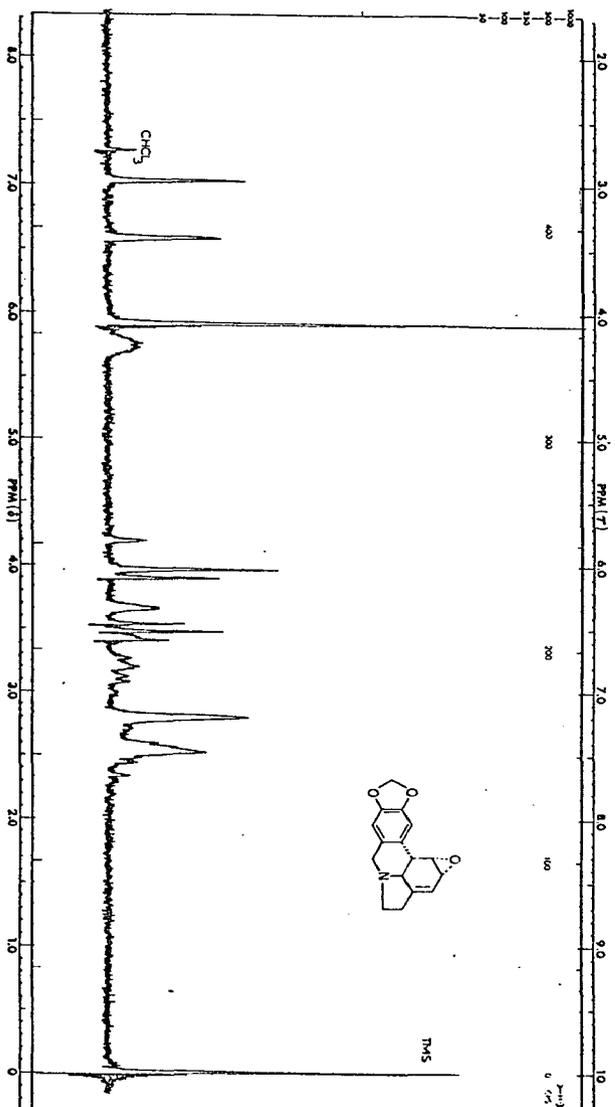


Figure 15. Mechanism for formation of dihydrolycorine chlorohydrin

Figure 16. Spectra of lycorine α -epoxide

top: nmr spectrum of lycorine α -epoxide

bottom: mass spectrum of lycorine α -epoxide



spectrum occurred at m/e 268, resulting from a loss of one proton. The two remaining peaks were at m/e 250 and m/e 240. Possible fragmentation pathways leading to these ions are shown in Figure 17. From this evidence XLVIII was identified as lycorine δ -epoxide.

To further establish the structure of XLVIII, two chemical transformations were undertaken. Reduction of XLVIII with lithium aluminum hydride in ether gave caranine, which was identified by its melting point, infrared spectrum, conversion to the O-acetate, and a mixture melting point determination. This reduction to give caranine established that the epoxide ring was cis to the benzene ring as shown in XLVIII. The epoxide was hydrolyzed in dilute aqueous sulfuric acid to give lycorine. This established that the oxirane ring was located between C-1 and C-2.

The epoxide could be isolated only after column chromatography. There could be two reasons for this observation. It is possible that the epoxide is not present in the crude reaction product and is formed on the column from the trans-chlorohydrin. The other possibility is that the epoxide is present in the reaction mixture, but could not be isolated by crystallization. An nmr spectrum of the crude reaction product (Figure 18) suggests the latter to be correct. A peak occurred at 5.74δ , the position of the olefinic proton resonance in the epoxide. The pattern for the superposition of the low-field benzylic proton and the C-1 proton near 3.9δ is present as is the triplet centered at 3.43δ which corresponds to the C-2 proton in the epoxide. Further evidence that the epoxide is present in the crude product mixture can be seen in its infrared spectrum (Figure 18). The infrared spectrum of the epoxide shows a moderately intense band at 1000 cm^{-1} . Examination of the infrared spectra of a number

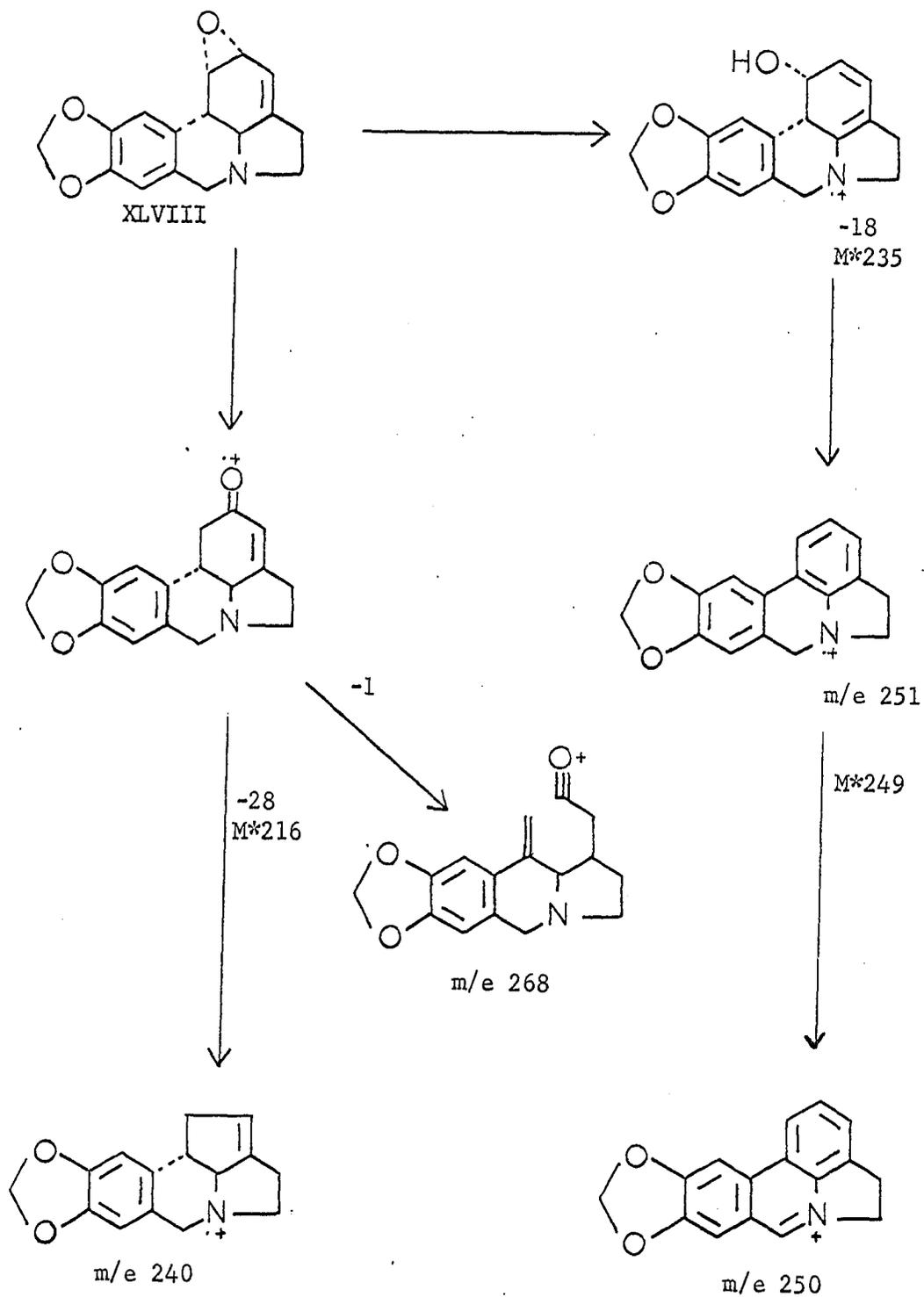


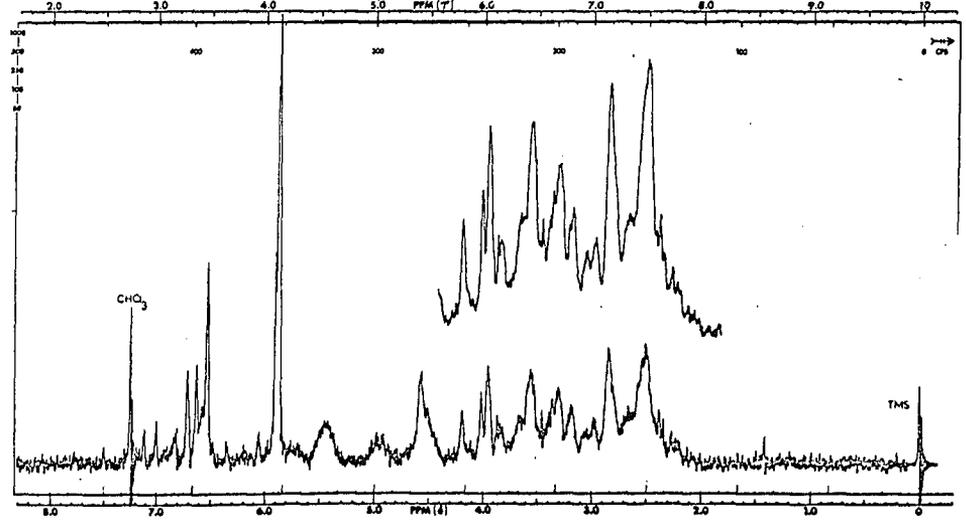
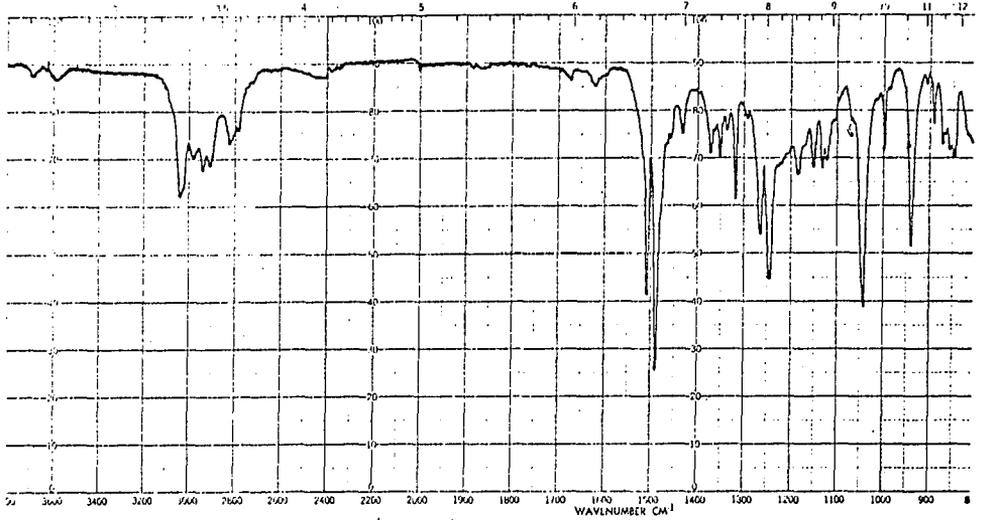
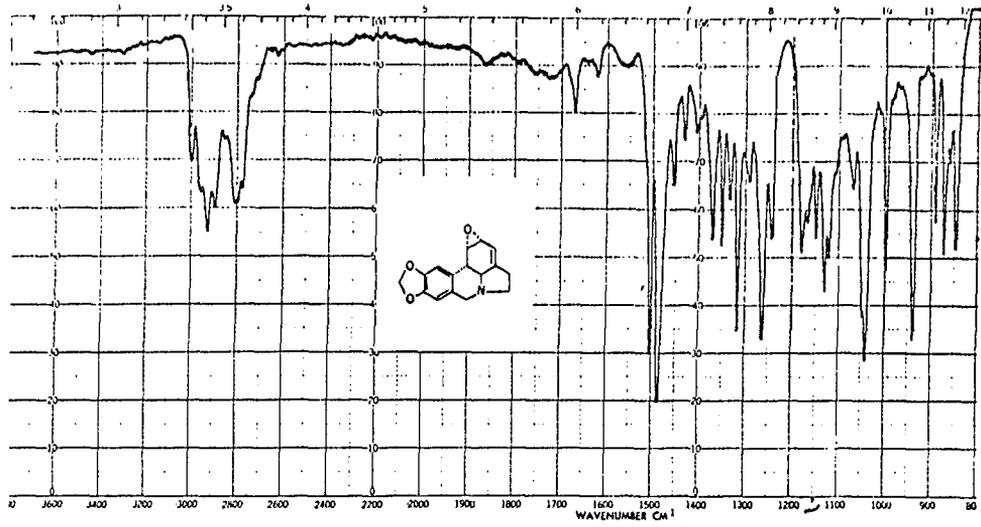
Figure 17. Fragmentation of lycorine α -epoxide

Figure 18. Spectra of crude chlorohydrin

top: ir spectrum of lycorine α -epoxide

middle: ir spectrum of crude reaction product

bottom: nmr spectrum of crude reaction product



of crude chlorohydrin samples showed a band at 1000 cm^{-1} which varied widely in intensity. This suggested that the 1000 cm^{-1} band is not in the spectrum of the pure chlorohydrin and is probably derived from the epoxide.

It was also discovered that the yield of the epoxide could be increased by adding powdered sodium chloride to the reaction mixture. This could result either in formation of the trans-chlorohydrin by halide exchange or by acceleration of the intramolecular process leading to the trans-chlorohydrin. The trans-chlorohydrin would then be converted to the epoxide in the isolation procedure.

With the discovery that the epoxide could be obtained from the chlorohydrin reaction, the problem of preparing the $[2\beta\text{-}^3\text{H}]$ -labeled precursor was solved. Reduction of the epoxide must give the product in which the label is introduced in the position trans to the hydroxyl group.

To substantiate this hypothesis, reduction of XLVIII with lithium aluminum deuteride was carried out to give caranine. The caranine was converted to acetylcaranine because acetylcaranine was more convenient to handle and more stable. The infrared spectrum of the deuterated acetylcaranine was not identical with that of the natural product. Identification was made by comparison of the vpc retention times and a mixture melting point determination with the natural product. Characterization of the deuterated product by mass spectrum was also useful (Figure 20). The parent molecular ion was shifted from m/e 313 to 314. A characteristic fragmentation of the lycorine-type alkaloids has been shown to involve loss of C_1 and C_2 and substituents attached to these atoms as a neutral fragment (52). In acetylcaranine this fragmentation corresponded to a loss of vinylacetate and gave rise to an ion at m/e 227. This loss was then

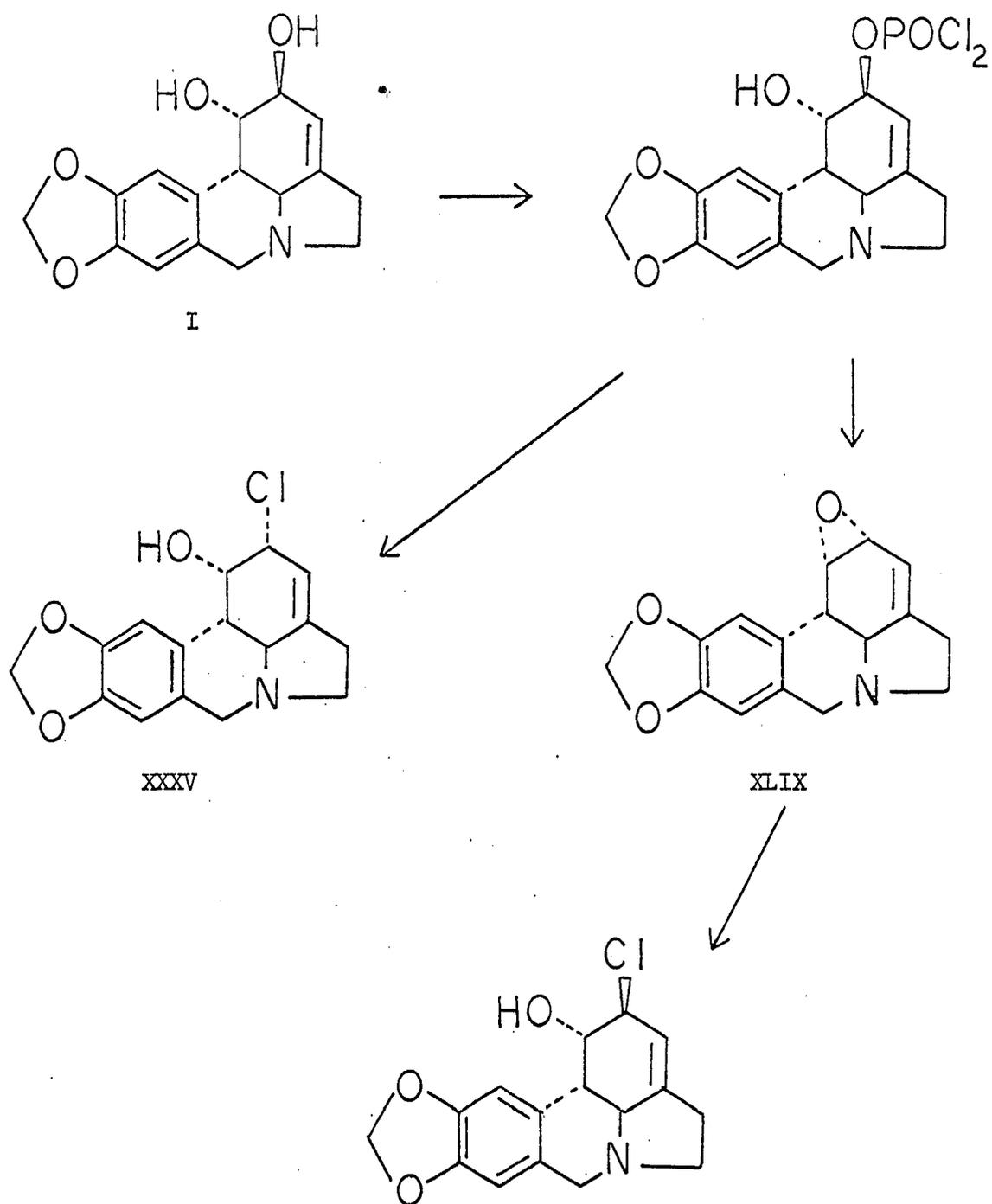
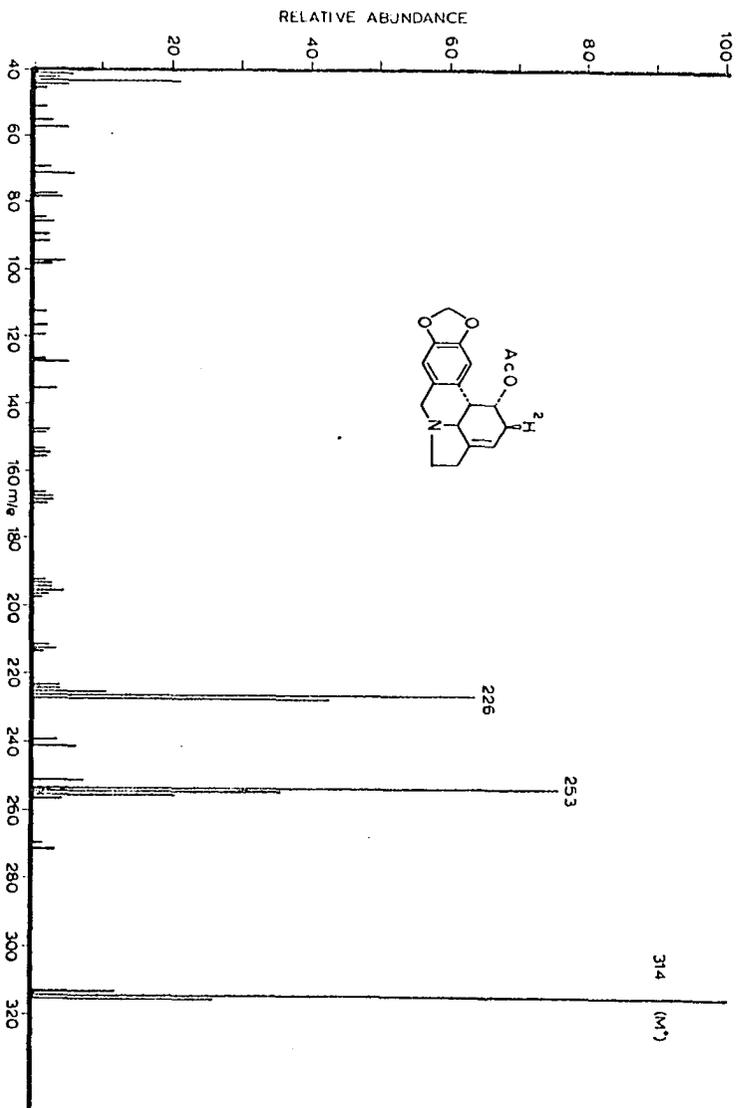
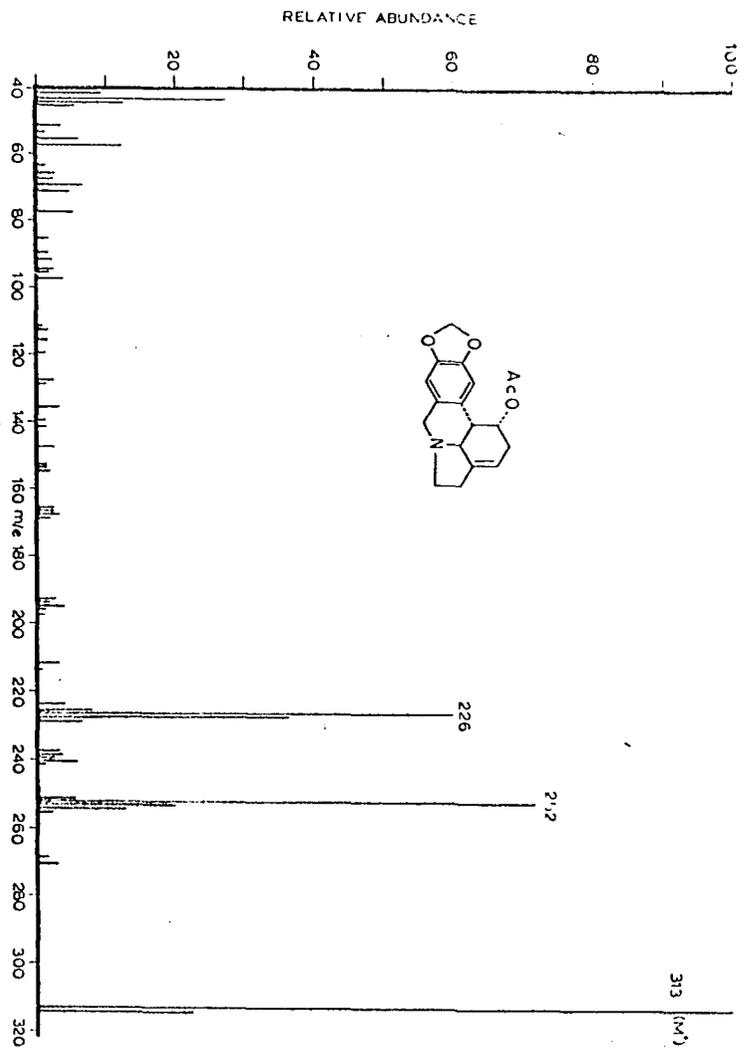


Figure 19. Mechanism for formation of *cis*- and *trans*-chlorohydrins from lycorine

Figure 20. Mass spectra of acetylcaranine

top: mass spectrum of acetylcaranine

bottom: mass spectrum of $[2\beta\text{-}^2\text{H}]$ acetylcaranine



followed by loss of a proton to give an intense ion at m/e 226 (52). The mass spectrum of L showed the same loss with formation of ions at m/e 227 and 226. This indicated that the deuterium atom must have been lost with the two carbon fragment.

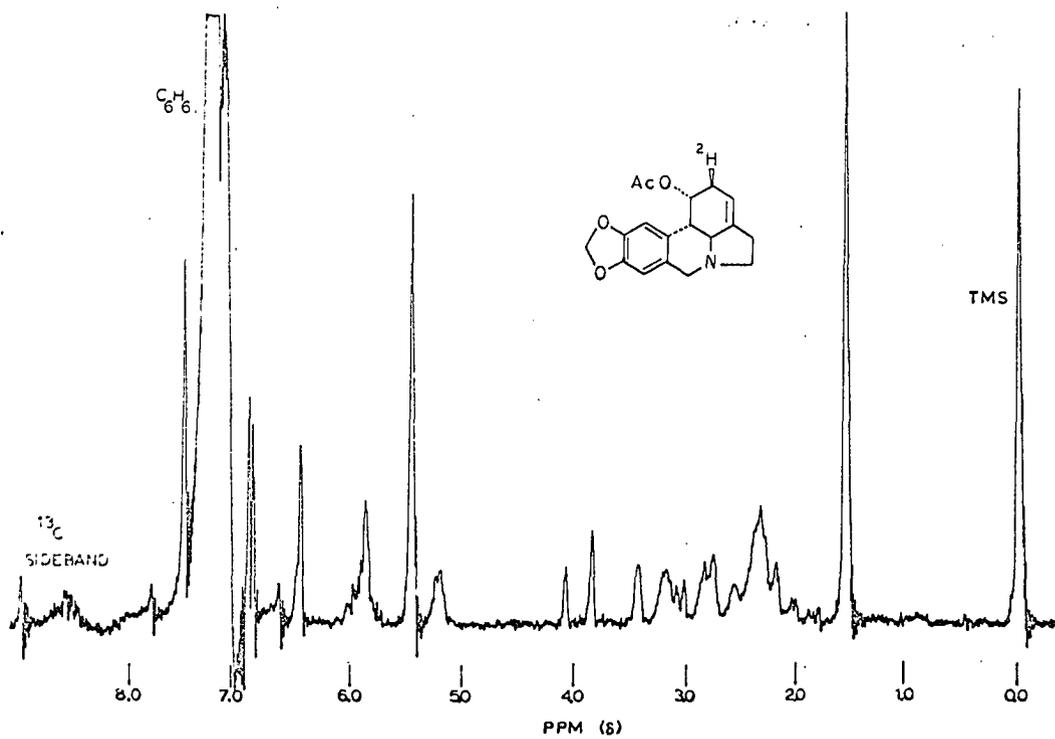
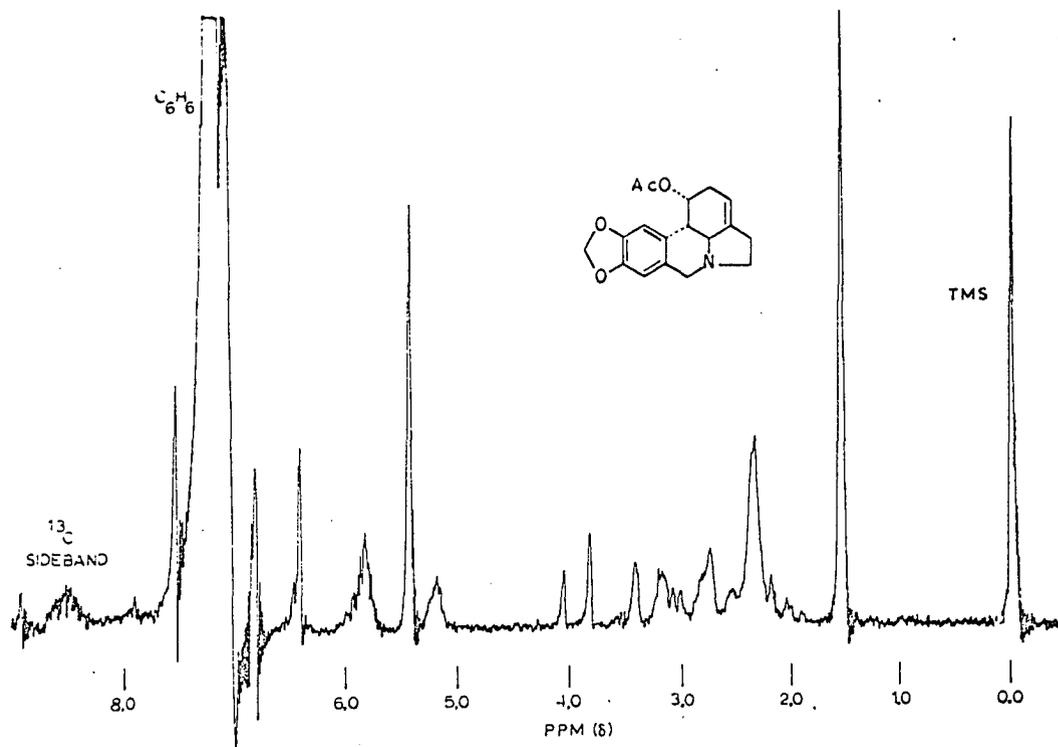
Comparison of the nmr spectra of L and XLIX in 4:1 benzene-deuteriochloroform (Figure 21) showed the absence of one proton in the 0 to 4δ region. A major difference was observed in the shape of the bands which corresponded to the C-1 and C-3 protons. The half-height band width of both was decreased in the deuterated derivative. This showed that the deuterium atom must have been at C-2.

This evidence restricts the deuterium atom to C-2; however, it would be desirable to have data (other than chemical precedent) to establish the β -configuration for the deuterium atom. The mass spectrum of L shows an ion derived from loss of acetic acid (m/e 254) which is accompanied by a metastable ion at 205.5 (Calcd. 205.5). The same fragmentation in natural acetylcaranine leads to an ion at m/e 253 which is accompanied by a metastable ion at 204.5 (Calcd. 204.5). If the deuterium atom were in the α -configuration, loss of deuterated acetic acid should occur more readily and give a larger M-61 peak than is observed in the 2β -labeled compound. This seems reasonable in the light of the results obtained for the loss of acetamide from the epimeric 3-acetamidosteroids (53). The elimination of acetamide involved more loss of deuterium from C-2 and C-4 when the acetamido group was equatorial than when it was axial. This should be true when the group is equatorial because it can reach the adjacent protons more easily. To check this postulate, $[2\alpha\text{-}^2\text{H}]$ acetylcaranine was prepared and the mass spectrum was obtained. $[2\alpha\text{-}^2\text{H}]$ Acetylcaranine was prepared

Figure 21. NMR spectra of acetylcaranine

top: nmr spectrum of acetylcaranine

bottom: nmr spectrum of $[2\beta\text{-}^2\text{H}]$ acetylcaranine



by treatment of the cis-chlorohydrin with zinc in deuterated acetic acid. This reaction sequence has been reported to give replacement of halogen by hydrogen with greater than 95% retention of configuration (50). The mass spectrum of the acetate from the reaction product showed an M-61 ion at m/e 253 which had an intensity 80% of the base peak. In $[2\beta\text{-}^2\text{H}]$ acetylcaranine the intensity of this ion was 76% and in natural acetylcaranine 72%. This indicated that the deuterium labels were probably introduced as proposed.

The preparation of tritium labeled caranine was accomplished by reduction of XLVIII with lithium aluminum tritide. The first time the reduction was accomplished by addition of the tritium labeled lithium aluminum hydride to a solution of the epoxide in dry ether. The caranine was isolated as acetylcaranine because the ester was more amenable to crystallization and more stable. A small amount of the $[^3\text{H}]$ acetylcaranine was diluted with non-radioactive acetylcaranine and the diluted mixture was crystallized to constant activity. No decrease in specific activity was observed after two recrystallizations and the tracer must have been chemically pure. The second preparation of labeled caranine was accomplished by addition of the epoxide to an ethereal solution of lithium aluminum tritide. The product from this reaction was isolated as caranine. Analysis for chemical and radiochemical purity by analytical gas phase chromatography and dilution analysis showed the product to be pure by both criteria.

The pattern of labeling in the caranine tracers varies depending on the order of addition of reagents. The different labeling patterns probably result from partial isomerism of the epoxide to the C-1 ketone in

the inverse addition procedure. Reduction of this ketone would then give caranine in which the label was located at C-1. The reduction where the normal addition procedure was used introduced the label into the 2β -position in accord with the usual course of hydride reduction of epoxides.

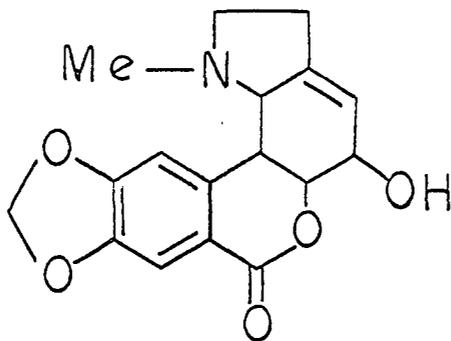
Feeding Experiments and Degradations

Zephyranthes candida was chosen for the first two feeding experiments because it was reported to contain zephyranthine (XXVI) and lycorine (I) (35). There would be four possible results from the isolation of lycorine and zephyranthine. Both alkaloids could be non-radioactive, both could be radioactive, lycorine could be radioactive and zephyranthine non-radioactive, and zephyranthine could be radioactive and lycorine non-radioactive. If neither of the alkaloids were radioactive, no comment could be made on the experiment without a separate, simultaneous experiment showing that caranine was being converted to lycorine in these plants. If both were radioactive, it would suggest that zephyranthine were either being formed from lycorine or from dihydrocaranine by a retention mechanism. If lycorine were radioactive and zephyranthine were non-radioactive, it could be inferred that both hydroxylations proceeded by an inversion mechanism. If zephyranthine were radioactive and lycorine were non-radioactive, it could be inferred that both hydroxylation processes occurred by a retention mechanism.

The tracers, $[2\beta\text{-}^3\text{H}]$ caranine and $[2\beta\text{-}^3\text{H}]$ acetylcaranine were fed to growing Z. candida by injection into the bulb. The plants were allowed to grow for three weeks and then processed in a standard manner. The lycorine isolated from both feeding experiments was radioactive. The

incorporation of acetylcaranine into lycorine (Feeding B) was lower, 0.51%, than the incorporation of caranine into lycorine (Feeding A), 7.05%. All attempts to isolate zephyranthine failed. Finally, isolation by dilution with non-radioactive zephyranthine was attempted. Repeated recrystallization of the zephyranthine gave a continual drop in specific activity. After five recrystallizations only 2.4% of the activity remained and the specific activity was still dropping. Thus, either the Z. candida contained no zephyranthine or the zephyranthine present was not radioactive.

In a later experiment (Feeding C) caranine, obtained from hydrolysis of the acetylcaranine previously used, was injected into the flower stems of two Hymenocallis americana bulbs. After one week of growth one of the bulbs was processed. The other bulb was allowed to grow an additional two weeks before isolation of the alkaloids. The lycorine obtained from both plants was radioactive and the incorporation from the three-week growth period was higher (6.11%) than that obtained from the one-week growth period (1.81%). Hippeastrine (LI) was also isolated from the three-week experiment, but the incorporation from caranine was very low ($5.38 \times 10^{-3}\%$).



LI

This low incorporation of caranine into hippastrine shows that the pathway leading from caranine into the lactone alkaloids are not very efficient, but that they do exist.

The pH 2 extracts from the one-week feeding, both the leaves and the bulbs, showed more radioactivity than expected. Thin-layer analyses of these fractions indicated the presence of a small amount of acetylcaranine. A sample from each of these extracts was withdrawn and counted. Another identical sample was diluted with acetylcaranine, and these acetylcaranine samples were crystallized to constant specific activity. By this method it was established that, within experimental error, all of the activity in these fractions was due to acetylcaranine (a 25% conversion from caranine). This is the highest incorporation yet reported. It suggests that the feeding techniques are reasonably efficient.

The lycorine isolated from Feeding A was degraded to jonquilline (XXI), caranine (XXXI), anhydrolycorine, and anhydrocaranine (LII) (Figures 22 and 23). The specific activities of caranine, anhydrocaranine and anhydrolycorine were within experimental error of the specific activity of the original lycorine degraded (Table 3). The conversion of caranine to anhydrocaranine of the same specific activity showed that the label was not located at C-11b. The conversion of lycorine to anhydrolycorine demonstrated that the label was not located at C-11b or C-11c. The possibility that the label was located at the C-3, C-4, C-5, C-7 or the aromatic positions was not considered since the nmr spectrum of the $[2\beta\text{-}^2\text{H}]$ acetylcaranine did not indicate any label at these positions. The label was located at the C-2 position by oxidation of $[^3\text{H}]$ 1-O-acetyllycorine to jonquilline which was nearly inactive. This oxidation proved that the label was

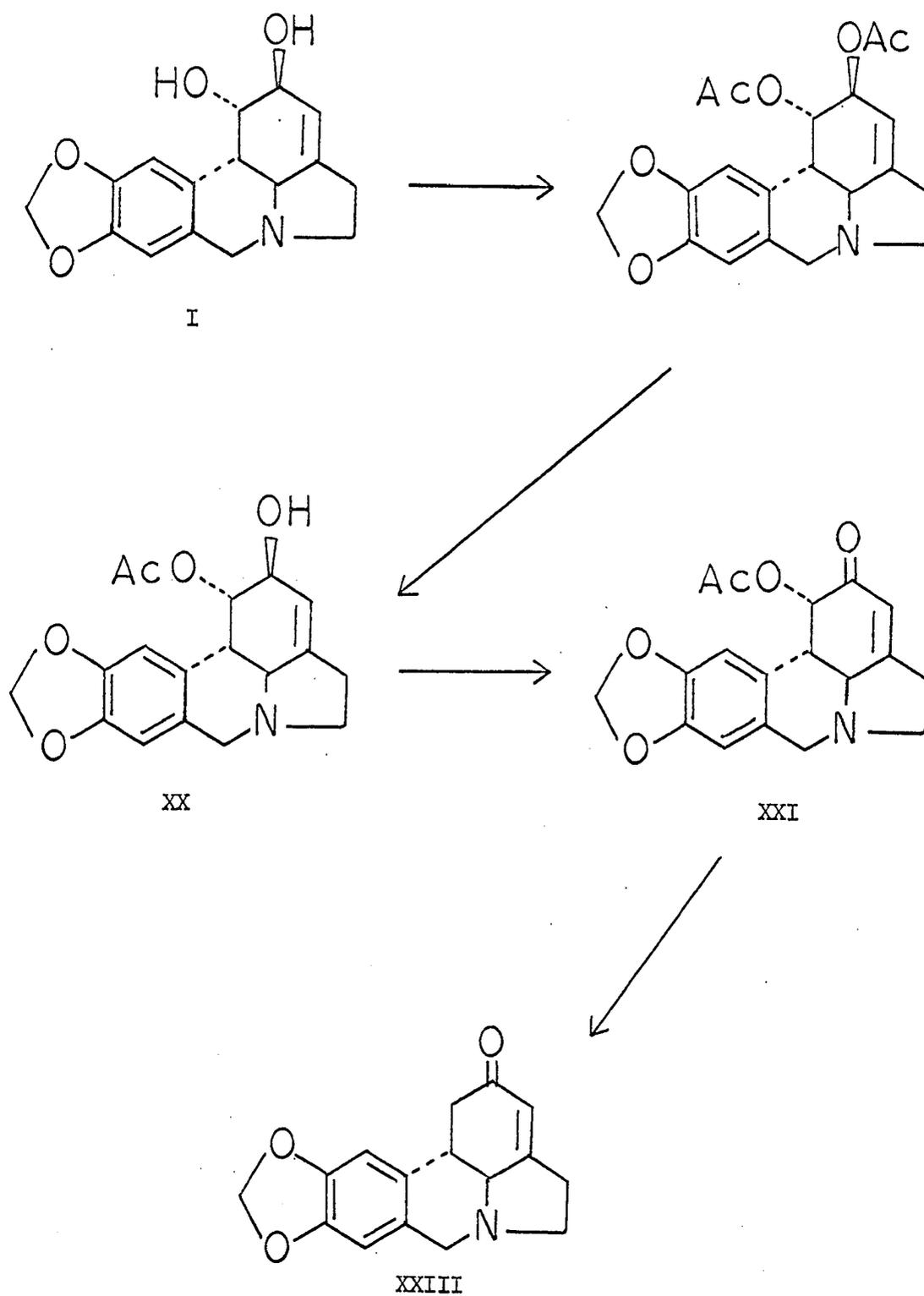


Figure 22. Degradation of caranine to jonquilline

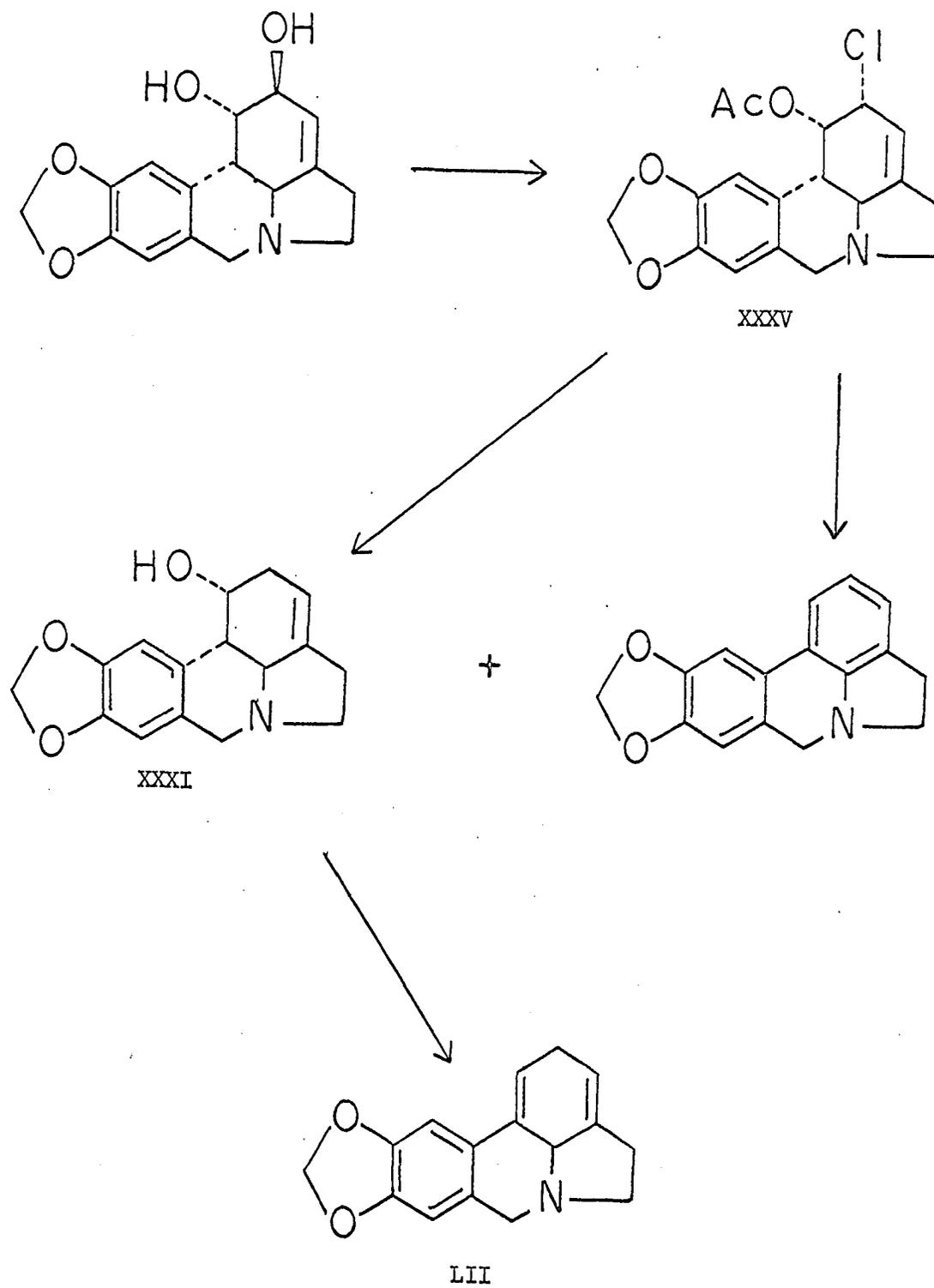


Figure 23. Degradation of lycorine to anhydrocaranine

Table 3. Activities of degradation products

Compound	Feeding A		Feeding B		Feeding C	
	Rel. Act.	Act. ^a	Rel. Act.	Act. ^a	Rel. Act.	Act. ^a
Lycorine	1.00	5.37	1.00	2.79		
O,O-Diacetyllycorine	1.00	5.37	1.01	2.82	1.00	7.54
1-O-Acetyllycorine			0.98	2.74		
Jonquilline	0.04	0.21	0.89 ^b	2.48 ^b	1.04 ^b	7.88 ^b
Anhydrolycorine	0.93 ^b	5.00 ^b				
Anhydrocaranine	1.00	5.37				
Caranine	1.00	5.36				
1-Desoxylycorin-2-one			0.75 ^b	2.01 ^b		

^adpm/mmmole X 10⁻⁴.

^bConstant activity had not been attained, but the lack of material made further work impossible.

located at C-2. The possibility that some label was originally at C-1 and was lost by enolization in jonquilline was rejected because the nmr spectrum of [2β -²H] acetylcaranine clearly showed that the label was not located there.

The experiments in which the precursors prepared by addition of lithium aluminum tritide to the epoxide were used (Feedings B and C) gave lycorine which was not labeled exclusively at C-2. The lycorine obtained in these cases was degraded to jonquilline which retained most of the activity. The label must be in a position that would allow it to be lost upon recrystallization. This could occur readily from position C-1 and

perhaps C-11c. The easiest position to determine would be C-1 since oxidation to the C-1 ketone, caranone (LIII), should result in loss of all tritium from that position.

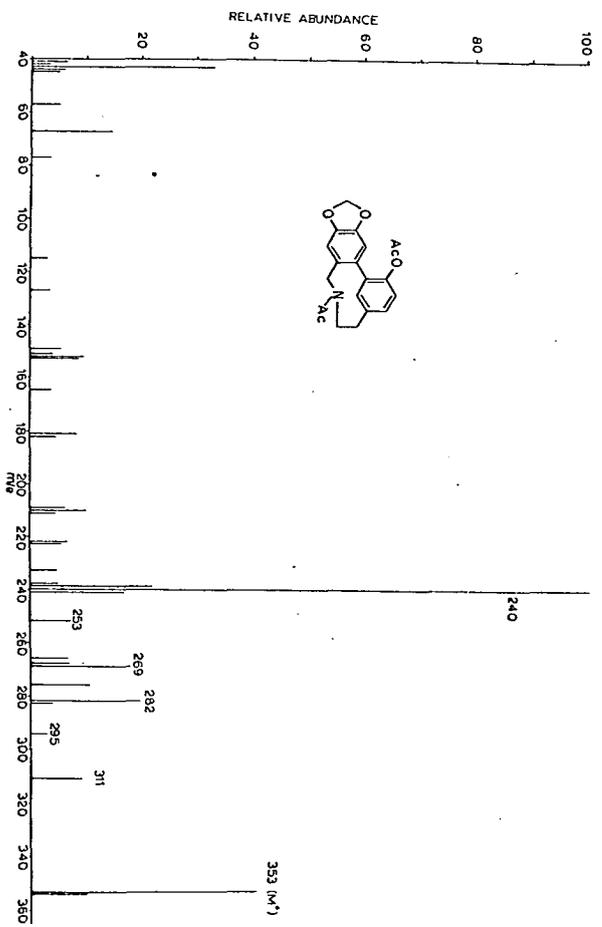
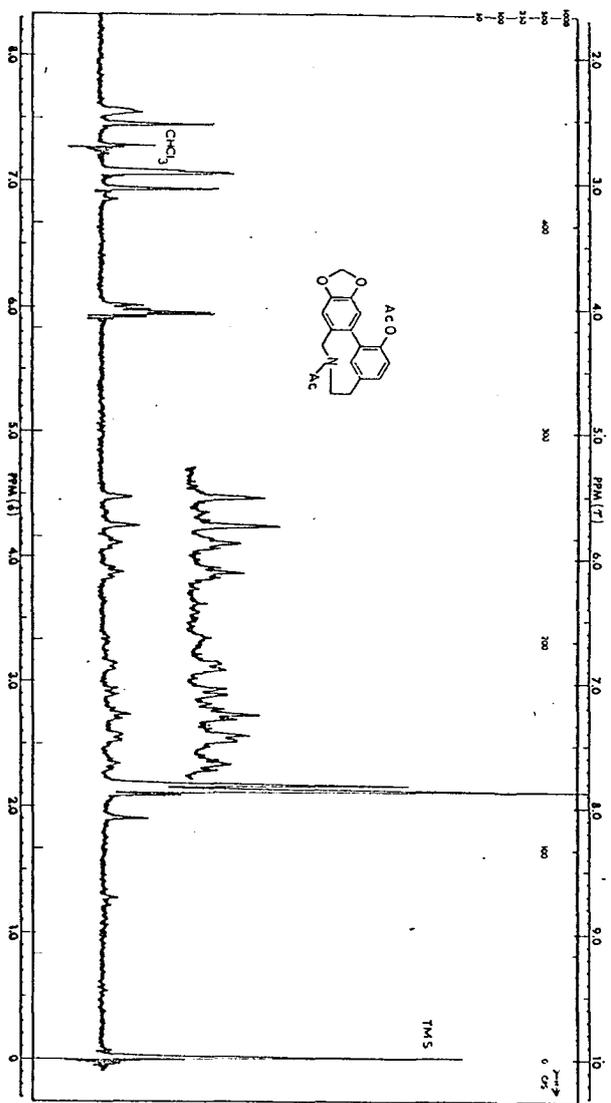
Since the action of most oxidizing agents on caranine and lycorine give rise to oxybetaines (30), an attempt was made to oxidize caranine to the ketone with dimethyl sulfoxide and acetic anhydride. This reaction gave two products which could be separated by dilution of the reaction mixture with water, acidification, and extraction. One of these was shown to be O,N-diacetyloxocaranine (LIV). A second product was presumed to be the thiomethyl methyl ether of caranine.

Combustion analysis of O,N-diacetyloxocaranine indicated a molecular formula of $C_{22}H_{23}NO_{5.5}$. This analysis was in poor agreement with the calculated values for $C_{20}H_{19}NO_5$. The analytical findings corresponded well with the partial solvate with diethyl ether $C_{20}H_{19}NO_5 \cdot \frac{1}{2}C_4H_{10}O$. However this assignment is suspect since no evidence for the presence of diethyl ether was found in the nmr spectrum. The strongest evidence for the assignment of LIV as O,N-diacetyloxocaranine is based on the spectroscopic properties. The infrared spectrum showed bands indicative of a phenol acetate (1745 cm^{-1}) and a tertiary amide (1640 cm^{-1}). The mass spectrum (Figure 24) showed the molecular weight to be 353. The presence of two acetyl functions was confirmed by consecutive loss of two ketene molecules from the parent molecular ion. The only important fragmentation remaining was the loss of 71 mass units from the m/e 311 ion to give the base peak at m/e 240. Loss of the fragment of mass 71 from the parent molecular ion to give an ion at m/e 282 was also observed. This ion then lost ketene to give rise to the base peak at m/e 240. Proposed fragmentation pathways

Figure 24. Spectra of O,N-diacetyloxocaranine

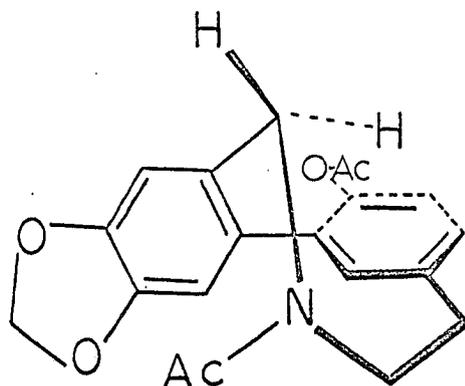
top: nmr spectrum of O,N-diacetyloxocaranine

bottom: mass spectrum of O,N-diacetyloxocaranine



are shown in Figure 25.

The nmr spectrum (Figure 24) in deuteriochloroform showed four peaks plus an additional small peak in the 6.7δ to 7.7δ region. The integral of this region corresponded to slightly less than five protons. The methylenedioxy group appeared as a quartet centered at 5.95δ and was nearly superimposed on a fifth peak at 6.02δ . The integral of this region corresponded to slightly more than two protons. Two singlets, each corresponding to three protons, assigned as the methyl groups on the acetyl functions were found between 2.1δ and 2.2δ . The remainder of the spectrum was difficult to assign. The usual pattern for the C-7 benzylic protons was not evident. Half of the pattern appeared to be located as a one proton doublet centered at 4.4δ , $J=14$ Hz. The other half of the pattern may be located as a doublet centered at 2.0δ . Only half of the pattern could be seen because one of the peaks was obscured by the methyl groups. The large upfield shift results from the 7β -proton being positioned above ring C. The remainder of the protons, those on C-4 and C-5, were assigned to the series of multiplets between 2.2 and 4.3 .



LIV

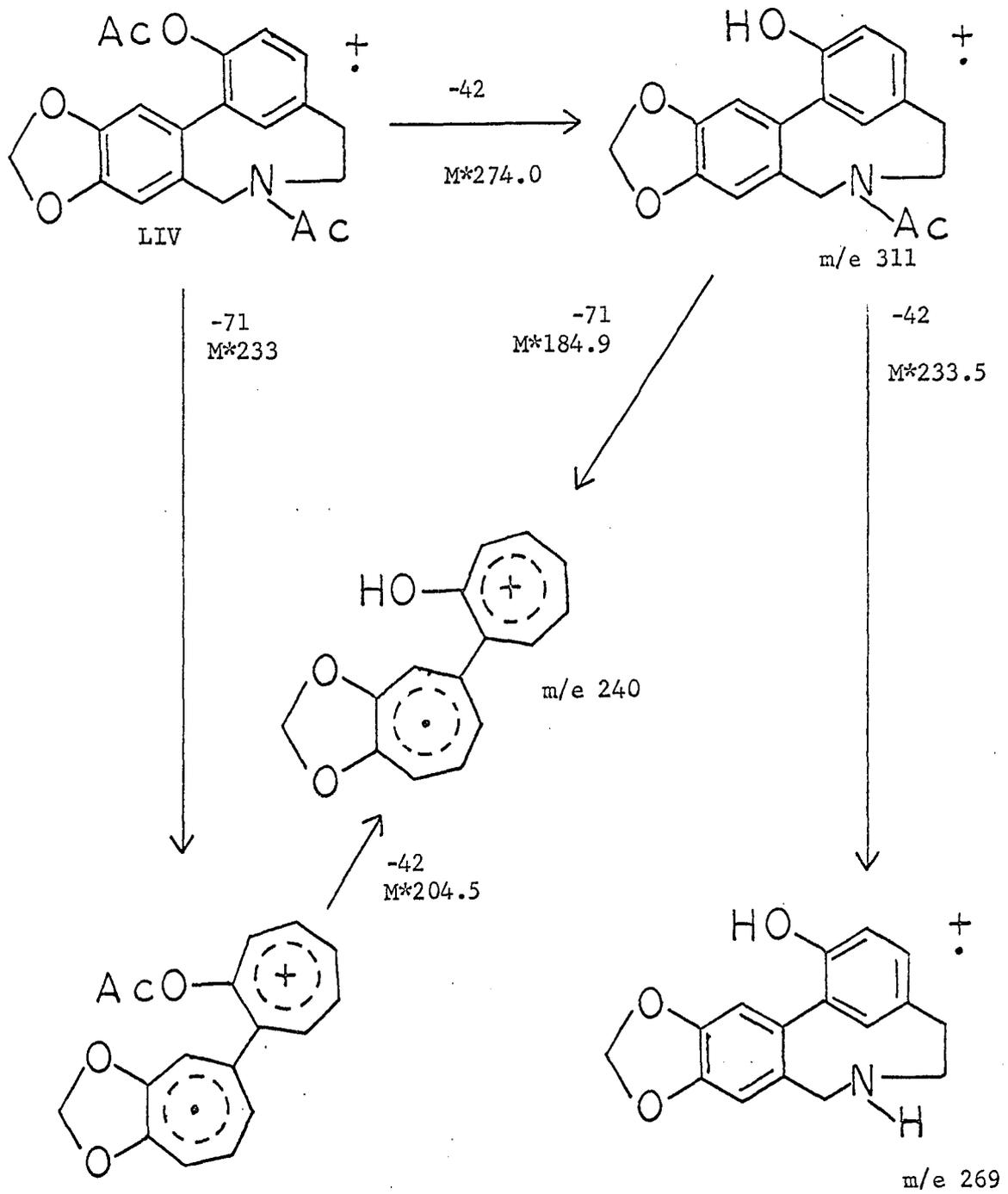


Figure 25. Fragmentation of O,N-diacetyloxocaraine

The mechanism proposed for this conversion is shown in Figure 26. If this mechanism is correct, a conclusion concerning the labeling pattern in the caranine precursors can be made. For caranine labeled only at C-2, the loss of label should reflect the isotope effect for enolization from C-2 to give the phenol. If the isotope effect between hydrogen and deuterium can be obtained, the isotope effect between hydrogen and tritium can be calculated (54). Oxidation of $[2\beta\text{-}^2\text{H}]$ caranine with dimethyl sulfoxide and acetic anhydride gave O,N-diacetyloxocaranine (LIV). Analysis of the product for deuterium content showed that 20% of the deuterium had been lost. From this data a value of 4.0 for the isotope effect ($k_{\text{H}}/k_{\text{D}}$) can be calculated and an isotope effect ($k_{\text{H}}/k_{\text{T}}$) of 7.4 can be calculated from Equation 1.

$$\frac{\log (k_{\text{T}} / k_{\text{H}})}{\log (k_{\text{D}} / k_{\text{H}})} = 1.44 \quad \text{Equation 1 (54)}$$

Oxidation of the caranine obtained from the degradation of Feeding A which was labeled only at C-2 showed a 3% loss of activity. However, because of the low counting rate (39 cpm) the probable error at the 90% confidence level corresponded to a range of isotope effects larger than 8.0. This value is nearly within experimental error of the predicted value.

Oxidation of the caranine used in Feeding C gave O,N-diacetyloxocaranine which showed a 26% loss of tritium label. This corresponded to an isotope effect of 2.78. This value is lower than that found for $k_{\text{H}}/k_{\text{D}}$ in the same reaction so there must be non-specific labeling in the caranine which was fed.

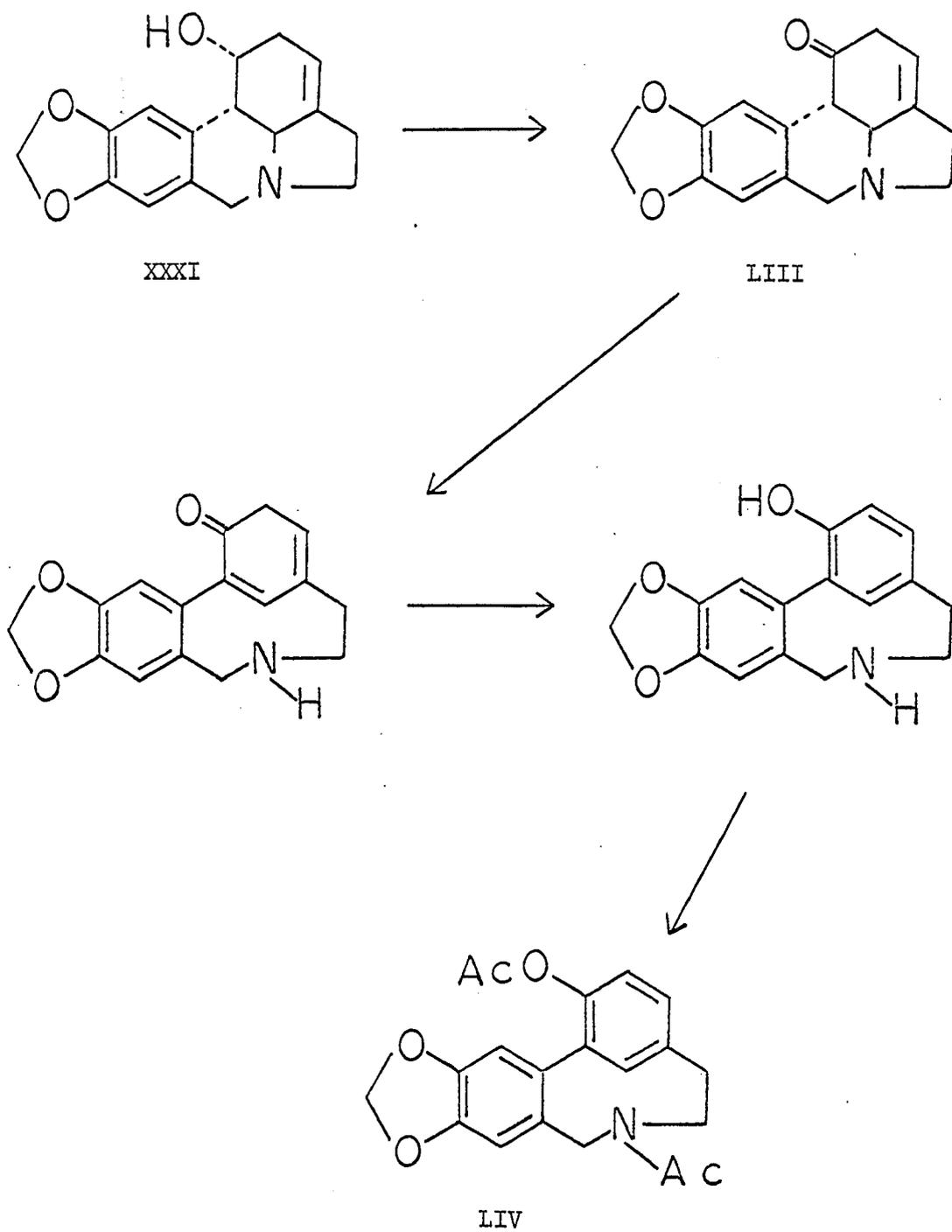


Figure 26. Mechanism for the formation of O,N-diacetyloxocaranine

The isotope effect expected is 7.4 and the fraction of label which should have been lost for the species labeled specifically at C-2 is 11.9%. The remaining 14% of the label which is lost can be located by the following argument. It is known that a portion of the label is lost upon recrystallization the jonquilline obtained in the degradation. This suggests that the label was also located at C-1. Three assumptions were made: the caranine oxidized was a mixture of two tracers, no molecule contains more than one tritium atom, and one of the tracers has the label at C-1 while the other tracer has the label at C-2. In the first step the C-1 hydroxyl is oxidized to the C-1 ketone. The caranine which had tracer only at C-2 should show only a small secondary isotope effect in this step. The caranine which had the tritium atom at C-1 would be oxidized more slowly than either the C-2 labeled species or unlabeled caranine. This slower rate of oxidation at C-1 labeled caranine would result in a ketone intermediate with a lower specific activity than the starting material. Enolization to the phenol results in loss of a portion of the label at C-2. Since 11.9% of the label should have been lost in the final step, the remaining 14% must have been lost in oxidation to the ketone. This corresponds to an isotope effect of 7.5 for the oxidation to the ketone. Unfortunately, there is no data available on isotope effects in this oxidation with which to compare this value.

The conversion of $[2 \beta\text{-}^3\text{H}]$ caranine to lycorine must involve inversion of configuration at C-2. This is not in accord with the results found earlier in which hydroxylation of the steroid molecules occurred with retention of configuration. The present case may be a result of hydroxylation in an allylic position; however, XLVa and XLVb were both

converted to cholic acid with retention of configuration. It might also result from the neighboring hydroxyl group or a combination of these effects.

The mechanism operating in the present case would be more clear if it were known whether the oxygen atom introduced at C-2 were derived from molecular oxygen or water. In the previous work all oxygen atoms introduced came from molecular oxygen. It would be strange if the oxygen atom originated from molecular oxygen in the present case since inversion is observed. It seems more reasonable to assume that the oxygen atom in this case is derived from water. The process operating in the conversion of caranine to lycorine might proceed through the following sequence: attack of an electrophilic oxygen species, derived from molecular oxygen, on the C-2 α carbon hydrogen bond, abstraction of the C-2 hydrogen to give an allylic carbonium ion, capture of the carbonium ion either by the C-1 oxygen atom to give the epoxide or capture by water to give lycorine, hydrolysis of the epoxide in the aqueous plant medium to give lycorine (see Figure 27).

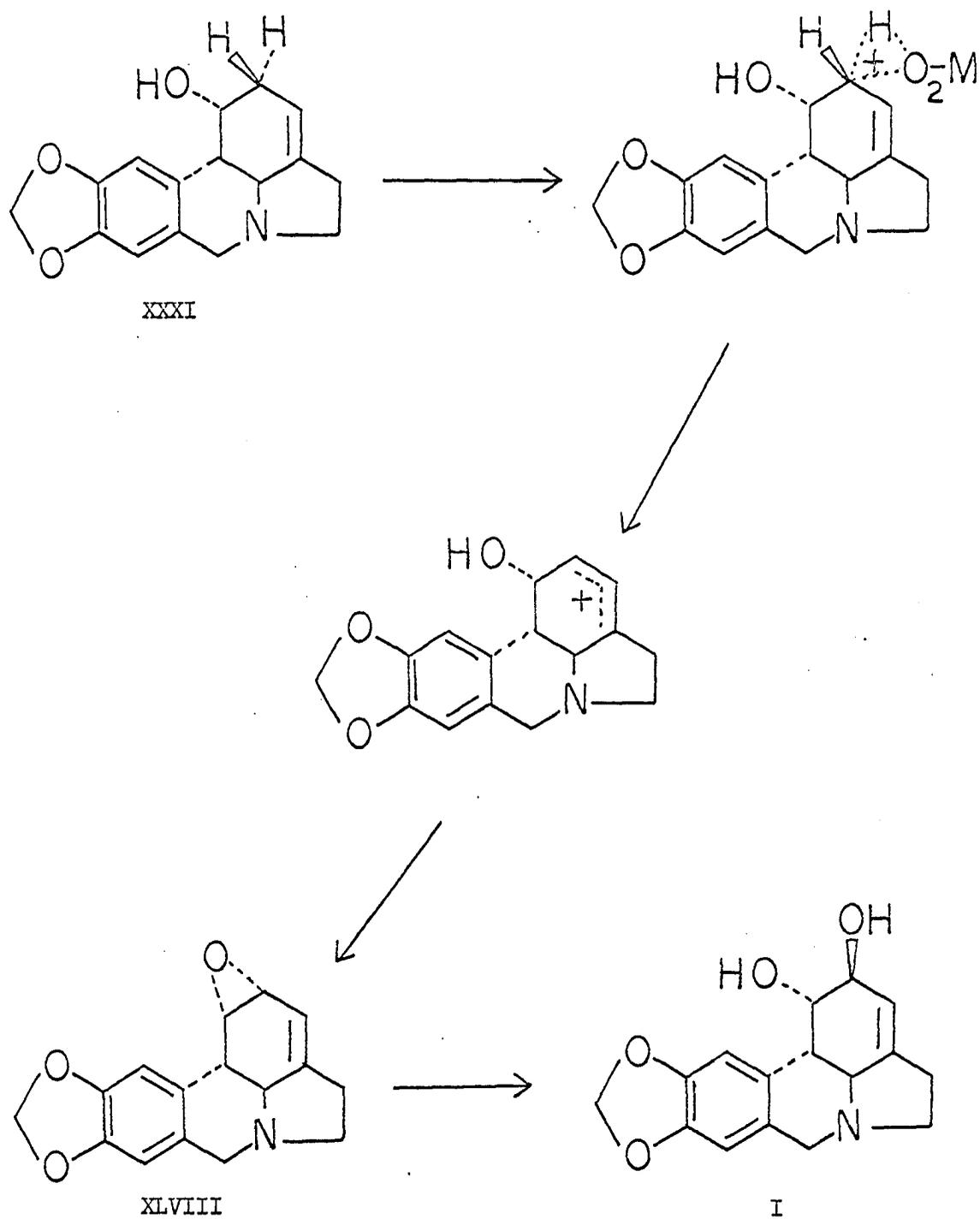


Figure 27. Mechanism for biosynthetic conversion of caranine to lycorine

SUMMARY

The alkaloid caranine was selected as a precursor to examine the stereochemistry of the in vivo oxidation of caranine to lycorine. A synthetic route for the preparation of caranine specifically labeled in the 2β -position was developed from lycorine and used to prepare $[2\beta\text{-}^3\text{H}]$ and $[2\beta\text{-}^2\text{H}]$ caranine. The tritium-labeled caranine was converted to lycorine in good yield by Z. candida. This process was shown to occur with inversion of configuration. A mechanism for the oxidation was proposed which included an epoxide intermediate.

In the course of the degradation of the radioactive products, the oxidation of caranine with dimethyl sulfoxide and acetic anhydride was investigated. The reaction product was found to give a biphenyl resulting from elimination of the nitrogen function from ring C.

The formation of lycorine α -epoxide was reported and its chemistry and spectroscopic properties examined.

INSTRUMENTATION AND METHODS

Melting points were taken on a Kofler hot-stage apparatus and were not corrected. Infrared spectra were obtained with either a Perkin-Elmer Model 21 spectrometer or a Beckman Model IR-12 spectrometer. Hydrogen bonding studies were carried out with a Beckman Model IR-12 spectrometer using 2.00 cm. silica cells in carbon tetrachloride which had been stored over Molecular sieves (Fischer Chemical Company type 4A). The usual scan rate used was less than $8 \text{ cm}^{-1}/\text{min}$. Proton magnetic resonance spectra were obtained either in deuteriochloroform or a 4:1 mixture of benzene and chloroform at 60.0 MHz with either a Varian HR-60 or A-60 spectrometer. Mass spectra were determined with an Atlas CH-4 spectrometer operating at 70-ev electron energy. Vapor phase chromatographic analyses were obtained with a Chromolab A-210 chromatograph containing a 12-ft. glass column packed with 1% SE-30 on Gaschrom Q.

The activities of radioactive materials were measured in Packard Tri-Carb Liquid Scintillation Spectrometer System, Model 314X or Model 3002. The radioactive samples, 0.5 - 5 mg, were counted in either Bray's solution [60g of naphthalene, 4 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene (Packard) and 100 mg of 2,5-diphenyloxazole (Packard) in 20 ml of 1,2-ethandiol, 100 ml of methanol and sufficient dry, peroxide-free dioxane to bring the total volume to 1 l] or toluene POPOP solution [4 g of 1,4-bis-2,5-(5-phenyloxazolyl)-benzene (Packard) and 100 mg of 2,5-diphenyloxazole (Packard) in 1 l of toluene]. The samples were counted for a sufficient period to give a minimum of 1000 total counts (3% error at the 90% confidence level) unless errors were specified. Most samples were counted long

enough to give more total counts; therefore, the error would be less depending on the activity found.

Ultraviolet spectra were obtained using a Beckman DK-2A spectrometer and 1.00 cm cells in 95% ethanol. Analyses were obtained from either Clark Microanalytical Laboratories (Urbana, Illinois) or Ilse Beetz Microanalytical Laboratory (West Germany).

Plant materials were processed by the usual procedures. The complete plant was ground in a Waring Blendor with ethanol. The thick slurry was allowed to stand for a day and filtered. The filter cakes were again ground in ethanol and allowed to stand one day before filtration. This process was repeated again so that the plant material had been extracted a total of three times. The ethanol filtrates were concentrated almost to dryness under reduced pressure at less than 45°. The concentrated extract was acidified to pH 2, diluted with an equal volume of tap water and filtered to remove insoluble materials.

The aqueous solution was extracted with chloroform to remove neutral substances and the alkaloids which form chloroform soluble hydrochlorides. The alkaloids in this class are the non-polar ones, esters and ethers. The chloroform extract was evaporated to dryness, taken up in water and washed with benzene. The solution was made basic with ammonium hydroxide and extracted with chloroform. Evaporation of the chloroform extract to dryness under reduced pressure gave the crude alkaloid mixture as bases.

The pH 2 solution was adjusted to pH 8 with ammonium hydroxide and extracted several times with chloroform to remove the majority of the alkaloids present. This extract usually contains a portion of the non-polar alkaloids as well as the more polar ones. The chloroform extract

was evaporated to dryness under reduced pressure to give the crude alkaloid residue.

The pH 8 solution was made strongly basic (pH 12) with sodium hydroxide and extracted with chloroform several times. The aqueous layer was extracted with 10% ethanol in chloroform until a negative silicotungstic acid test was obtained in the aqueous layer. Evaporation of the combined extracts under reduced pressure gave the crude alkaloid residue.

EXPERIMENTAL

Preparation of Tracer Materials

Lycorine α -epoxide

To a mixture of 1 ml of phosphoryl chloride and 200 mg of powdered sodium chloride was added 330 mg of lycorine. The mixture was heated to 35° in a water bath. After 5 min two drops of 6 N hydrochloric acid was added. The temperature was raised to 40°, and the reaction mixture was maintained at this temperature for 35 min. The reaction mixture was hydrolyzed with ice-water, made basic (pH 8) with sodium hydroxide and sodium carbonate, and extracted with ether. The ether extract was dried with a saturated sodium chloride solution and evaporated under reduced pressure to give 300 mg of non-crystalline residue which was pure by thin-layer chromatographic criteria. The residue was dissolved in a minimum amount of anhydrous ether and passed rapidly through a Florisil column (15 g) which had been packed in ether. Elution with 200 ml of ether provided 80 mg (26%) of crystalline epoxide, mp 148-53°. Recrystallization from methanol gave colorless prisms, mp 148-150°; $[\alpha]_D^{20} - 192^\circ$ (c 0.76, CHCl₃); ultraviolet maxima (95% ethanol) at 290 m μ ($\epsilon = 5500$ and 235 m μ (sh)).

Anal. Calcd for C₁₆H₁₅NO₃: C, 71.35; H, 5.44; N, 5.20; mol wt 269.

Found: C, 71.09; H, 5.46; N, 5.15 [mass spectrum m/e 269 (M⁺)].

Conversion of lycorine α -epoxide to caranine

To a solution of 100 mg of lithium aluminum hydride in dry ether was added 50 mg of lycorine α -epoxide. The mixture was refluxed for 3 hr,

and the excess lithium aluminum hydride was destroyed with wet ether. The ether layer was separated, washed with a dilute solution of sodium hydroxide, and evaporated to dryness to yield 33 mg of crude caranine. Recrystallization from ethyl acetate gave colorless prisms, mp 174-177° [lit. (32) mp 176-177°]. The infrared spectra (KBr and CHCl₃) were identical with those of authentic caranine in these phases.

Hydrolysis of lycorine α -epoxide

A solution of 20 mg of lycorine α -epoxide in 3.0 ml of water and six drops of 6N sulfuric acid was heated at 95° for 0.5 hr. The reaction mixture was cooled and made basic. The precipitated lycorine (14 mg) was removed by filtration, mp 225-235°(dec). The infrared spectrum (KBr) was identical with that of authentic lycorine.

[2 β -³H] caranine

To a solution of 5 mc of lithium aluminum [³H] hydride (5 mg) and 6 mg of lithium aluminum hydride in dry ether was added 150 mg of lycorine α -Epoxide. After the mixture had refluxed for 2 hr, an additional 25 mg of lithium aluminum hydride was added. The mixture was heated under reflux for an additional 3 hr. Excess lithium aluminum hydride was destroyed with wet ether, water was added, and the mixture was extracted with ether and then chloroform. The combined residues from the extractions were chromatographed on 10 g of alumina (Merck 71707). Elution with benzene and chloroform gave 67 mg of caranine (1.345 X 10⁶dpm/mg). Analysis of the product by gas-phase chromatography showed no chemical impurities. Dilution of a small sample of the [2 β -³H] caranine with nonradioactive caranine followed by repeated recrystallization gave no

change from the initial diluted activity.

[2 β -²H] acetylcaranine

To a solution of 85 mg of lithium aluminum deuteride in 25 ml of dry ether was added 164 mg of lycorine α -epoxide. The reaction was refluxed for 3 hr, and the excess lithium aluminum deuteride was destroyed with wet ether. The ether layer was separated, washed with a dilute solution of sodium hydroxide, and evaporated to dryness to yield 170 mg of crude [2 β -²H] caranine. This caranine was then taken up in acetic anhydride and pyridine and allowed to stand overnight. The solution was then poured into water, neutralized with ammonium hydroxide, and extracted with ether. After chromatography on alumina (Merck 71707), 110 mg of [2 β -²H] acetylcaranine was obtained, mp 187-188°, mixed melting point with authentic acetylcaranine 186-188°, molecular weight calcd. 314; found 314 (mass spectrum).

[2 β -³H] acetylcaranine

To a solution of 49 mg of lycorine α -epoxide in 25 ml of ether (freshly distilled from lithium aluminum hydride) was added a mixture of 5 mg of lithium aluminum tritide (5.0 mc.) and 20 mg. of lithium aluminum hydride. The mixture was refluxed for 2 hr. The excess hydride was destroyed with wet ether, filtered, and evaporated to dryness under reduced pressure. The [³H] caranine was then treated with acetic anhydride at room temperature in pyridine overnight. Standard isolation procedure gave [³H] acetylcaranine (activity 6.74 X 10⁶ dpm/mg). A small amount of this material (1.3 mg) was diluted with 100 mg of non-radioactive acetylcaranine and recrystallized from acetone:hexane to give material with an activity

of 7.85×10^4 dpm/mg. One more recrystallization gave no decrease in specific activity.

Feeding Experiments and Degradations

Feeding of [2β - ^3H] caranine to Zephyranthes candida and isolation of lycorine: Feeding A

An aqueous solution (pH 6, 2 ml) of 19.5 mg of caranine (1.345×10^6 dpm/mg) was introduced into 35 Zephyranthes candida injection into the bulb, $30 \mu\text{l/bulb}$. After 2 weeks of growth, the bulbs (347 g) were processed according to the usual procedure to give 113 mg of lycorine ($1,470 \times 10^4$ dpm/mg, 7.05 % incorporation). To the basic chloroform filtrate, after removal of lycorine, was added 30 mg of zephyranthine [prepared by the procedure of Nakagawa and Uyeo (25)]. This material was recrystallized five times from acetone. The radioactivity fell drastically with each recrystallization. Only 2.6% remained after five recrystallizations, and the activity was still decreasing rapidly.

Table 4. Activities of zephyranthine diluted extract

Recrystallization	Activity ($\times 10^2$ dpm/mg)
1	4.96
2	1.40
3	0.76
4	0.40
5	0.24

Feeding of [^3H] acetylcaranine to *Zephyranthes candida* and isolation of lycorine: Feeding B

A solution of 5.8 mg of [^3H] acetylcaranine [6.74×10^6 dpm/mg] in 3 drops of glycerine and enough water to bring the volume to 1 ml was injected into 40 *Zephyranthes candida* bulbs (avg. $25 \mu\text{l}$ /bulb). The plants were allowed to grow for 2 weeks, and then they (220 g) were processed in the usual manner yielding 109 mg of crude bases (activity 3.15×10^3 dpm/mg). After removal of the Lycorine (60 mg 1.013×10^6 dpm/mole, 0.51% incorporation) the residue activity had increased to 4.59×10^4 dpm/mg. Analysis of this residue by two-dimensional thin layer chromatography (Silica Gel G; solvent 1, chloroform 92:methanol 5:diethylamine 3; solvent 2, diethyl ether 85:methanol 10:diethylamine 5) showed that most of the activity was localized in the area of the plate where acetylcaranine and caranine appear.

Hydrolysis of [^3H] acetylcaranine to [^3H] caranine

To 29 mg of [^3H] acetylcaranine [6.74×10^6 dpm/mg] in 5 ml of 95% ethanol was added 168 mg of potassium hydroxide. The resulting solution was refluxed for 1 day. The cooled reaction mixture was acidified with 4 N hydrochloric acid and washed with 20 ml of benzene, then made basic with 6 ml of 6 N ammonium hydroxide and extracted 5 times with 30-ml portions of chloroform. Concentration of the chloroform extract gave 33 mg of residue. This was recrystallized 4 times from ethyl acetate to give 20 mg of [^3H] caranine (activity 7.42×10^6 dpm/mg).

Feeding of [³H] caranine to *Hymenocallis americana*: Feeding C

A solution of 10 mg of [³H] caranine (activity 7.42×10^6 dpm/mg) in 1.0 ml of water (pH 6) was fed to two blooming *Hymenocallis americana* plants by injection into the stem (0.5 ml, 3.71×10^6 dpm to each bulb). One of the bulbs was allowed to grow for one week, and the other was allowed to grow for three weeks before processing.

Isolation of the alkaloids from the 1 week feeding of [³H] caranine to *H. americana*

The plant was processed in the usual manner to give the results shown below:

Table 5. Yield of alkaloids from the one week feeding

	Wet Weight (kg)	Dry Weight (kg)	Weight of Crude Alkaloid (g)	Total Alkaloid Yield % Wet Weight	% Dry Weight
Bulbs	1.81	0.328	1.474	0.076	0.42
Leaves	2.9	0.170	0.229	0.0079	0.13

Lycorine was isolated from the crude extracts by trituration of the crude alkaloid residue with chloroform. This afforded 580 mg of lycorine (activity 3.32×10^5 dpm/nmole, 1.81% incorporation).

To 1.58×10^5 dpm of the residue from the pH 2 extraction of the leaves was added 50 mg of non-radioactive acetylcaranine. This was re-crystallized from acetone:hexane to give a recovery of 105% of the activity

--- Table 6. Activities and weights of fractions from the one week feeding

Fraction	Weight (g)	% Total Weight	Activity ($\times 10^{-6}$ dpm/mg)	% Total Activity	<u>% Total Activity</u> <u>% Total Weight</u>
Leaves pH 2	0.098	5.8	8.4	68	11.7
Leaves pH 8	0.705	41.6	2.4	19	0.46
Leaves pH 12	0.671	39.6	0.51	4	0.10
Bulbs pH 2	0.091	5.7	0.75	6	1.05
Bulbs pH 8	0.064	3.8	0.56	2	0.54
Bulbs pH 12	0.165	9.8	0.06	0.4	0.04

as acetylcaranine.

The specific activity of the recrystallized acetylcaranine from the pH 2 leaf extract is shown in Table 7.

Table 7. Activities of acetylcaranine from dilution of leaf pH2 extract

Recrystallization	Activity ($\times 10^{-3}$ dpm/mg)
1	3.32
2	2.88
3	3.30
4	3.02
5	3.33

To 7.80×10^3 dpm of the residue from the pH 2 extraction of the bulb was added 50 mg of non-radioactive acetylcaranine. This was recrystallized from acetone:hexane to give a recovery of 110% of the activity as acetylcaranine. The specific activities of the recrystallized acetylcaranine are shown in Table 8.

Table 8. Activities of acetylcaranine from dilution of bulb-pH2 extract

Recrystallization	Activity ($\times 10^{-2}$ dpm/mg)
1	2.14
2	1.62
3	2.74
4	1.75
5	1.73

The total incorporation of caranine into acetylcaranine was 25%.

Isolation of alkaloids from the 3 week feeding of [^3H] caranine to H. americana

The plant was processed in the usual manner to give the results shown below in Tables 9 and 10. Lycorine (620 mg, activity 1.05×10^6 dpm/mole, 6.11% incorporation) was isolated from the crude extract by trituration of the residue with chloroform. Separation of the chloroform soluble filtrate by preparative thick-layer chromatography (Silica Gel PF-254, 0.5 mm thick plates, solvent:8% ethanol in chloroform) yielded

Table 9. Yield of alkaloids from the three week feeding

	Wet Weight (kg)	Dry Weight (kg)	Weight of Crude Alkaloids (g)	Total Alkaloid Yield % Wet Weight	% Dry Weight
Bulbs	2.15	0.534	1.426	0.066	0.27
Leaves	1.67	0.081	1.082	0.065	1.34

Table 10. Activities and weights of fractions from the three week feeding

Fraction	Weight (g)	% Total Weight	Activity ($\times 10^{-5}$ dpm/mg)	% Total Activity	$\frac{\% \text{ Total Activity}}{\% \text{ Total Weight}}$
Leaves pH2	0.175	7.0	3.58	4.8	0.69
Leaves pH 8	0.678	27	5.96	80	2.96
Leaves pH 12	0.229	9.1	9.59	13	1.4
Bulbs pH 2	0.072	2.9	0.085	0.1	0.35
Bulbs pH 8	0.892	36	0.12	1.6	0.04
Bulbs pH 12	0.462	18	0.94	1.3	0.72

330 mg of hippeastrine mp 211-214⁰, infrared spectrum identical with authentic hippeastrine (CHCl₃) (activity 2.10×10^3 dpm/mole, $5.38 \times 10^{-3}\%$ incorporation).

[³H] lycorine dilution: Feeding A

To 3.950 mg of non-radioactive lycorine was added 53 mg of lycorine (1.470×10^4 dpm/mg) which had been obtained from Feeding A. The lycorine was then recrystallized from methanol to give 3.50 g of lycorine

(5.37×10^4 dpm/mmmole).

0,0-Diacetyllycorine: Feeding A

To a mixture of 5 ml of dry pyridine and 3.5 ml of acetic anhydride was added 1.213 g of lycorine (5.37×10^4 dpm/mmmole). The mixture was allowed to stand in the dark for 1 week. The solution was poured into water, neutralized with ammonium hydroxide, and extracted with ether. The ether was evaporated to dryness, and the residue was chromatographed on 20 g of alumina (Merck 71707) in chloroform to provide 1.480 g of 0,0-diacetyllycorine mp 210-212°, [lit. (25) mp 215-216°]; activity 5.37×10^4 dpm/mmmole.

1-0-Acetyllycorine: Feeding A

To a mixture of 1.45 g of 0,0-diacetyllycorine (5.37×10^4 dpm/mmmole) in 63 ml of methanol was added 13 ml of 35% hydrochloric acid. The mixture was heated on a steam bath for 4 min. The solution was cooled as rapidly as possible in an ice bath, and 13 ml of concentrated ammonium hydroxide was added. The resulting mixture was poured into 300 ml of cold water and extracted 6 times with 50-ml portions of chloroform. Evaporation of the chloroform extracts to dryness under reduced pressure gave 1.2 g of material, which was purified by chromatography on alumina (Merck 71707). Elution with chloroform and 10% methanol in chloroform gave 0.254 g of 0,0-diacetyllycorine and 0.850g of 1-0-acetyllycorine respectively, mp 212-213° [lit. (25) mp 215-216°].

Oxidation of 1-0-acetyllycorine: Feeding A

To a solution of 0.805 g of 1-0-acetyllycorine in 85 ml of chloroform was added 8.0 g of manganese dioxide. The reaction mixture was stirred for 19 hr at 13°. The manganese dioxide was removed by filtration, and the chloroform solution was evaporated to dryness under reduced pressure to give 520 mg of crude product. Chromatography of the crude product on 52 g of alumina (Merck 71707) with chloroform provided 300 mg of 1-0-acetyllycorin-2-one, mp 188-192° dec. [lit. mp 191° dec. (25)], activity 0.21×10^4 dpm/mmole.

Caranine and anhydrocaranine from lycorine: Feeding A

To a mixture of 3.0 ml of phosphoryl chloride and 1.0 g of powdered sodium chloride was added 0.977 g of lycorine (5.37×10^4 dpm/mmole). After 10 min at 37°, 1 drop of 35% hydrochloric acid was added. After an additional 10 minutes another drop of 35% hydrochloric acid was added, and after an additional 45 min the mixture was cooled to room temperature and added dropwise to 250 ml of a mixture of water and ice. The solution was made slightly basic with sodium carbonate, allowed to stand 1 hr, and then extracted 4 times with ether. The ether extract was washed with a saturated solution of sodium chloride and evaporated to dryness under reduced pressure. The residue was dissolved in 20 ml of dry ether and added slowly to 125 ml of a refluxing mixture of 600 mg of lithium aluminum hydride in ether. After 3 hr the excess lithium aluminum hydride was destroyed with ethyl acetate, and the reaction mixture was washed with 10% sodium hydroxide. The aqueous solution was extracted several times with benzene:ethyl acetate (1:1). The combined organic extracts were evaporated

to dryness under reduced pressure, and the residue (500 mg) was chromatographed on alumina. Elution with benzene and ethyl acetate afforded 30 mg of anhydrolycorine (4.99×10^4 dpm/mmmole), mp 110-112° [lit (32) mp 110-111°], and 160 mg of caranine mp 176-177° (5.37×10^4 dpm/mmmole).

Anhydrocaranine from caranine

Caranine (5.37×10^4 dpm/mmmole) was converted to anhydrocaranine by the published procedure (32), mp 150-151°, [lit (32) mp 153-154], activity 5.36×10^4 dpm/mmmole.

0,0-Diacetyllycorine: Feeding B

To 55 mg of lycorine (1.013×10^6 dpm/mmmole) was added 1.945 g of lycorine. The resulting lycorine (calcd. activity 2.78×10^4 cpm/mmmole) was treated with 10 ml of acetic anhydride in 100 ml of pyridine for 24 hr. The reaction mixture was worked up in the same manner described for Feeding A, yield 1.835 g 0,0-diacetyllycorine mp 212-215°, 2.82×10^4 dpm/mmmole.

1-0-Acetyllycorine: Feeding B

The partial hydrolysis was carried out on 1.525 g of 0,0-diacetyllycorine in the same manner described for feeding A yielding 400 mg of 0,0-diacetyllycorine and 1.1 g of 1-0-acetyllycorine mp 212-213°, 2.74×10^4 dpm/mmmole.

Oxidation of 1-0-acetyllycorine: Feeding B

The oxidation was carried out using 0.383 mg of 1-0-acetyllycorine, and the same ratio of the other reactants as described for Feeding A yielding 150 mg of 1-0-acetyllycorin-2-one mp 185-190 dec., 2.48×10^4 dpm/mmmole.

1-Desoxylycorin-2-one

To a solution of 125 mg of 1-0-acetyllycorine in 5 ml of xylene and 5 ml of acetic anhydride contained in 50-ml, 3-neck flask equipped with a magnetic stirrer was added 3 g of zinc dust over a 20-minute period. The mixture was refluxed for 10 minutes and maintained at 80° for 40 min. The reaction mixture was filtered, the residue was washed with benzene and ethanol, and then discarded. The filtrate was extracted with 2-25 ml portions of dilute aqueous acid, after which the organic layer was discarded. The aqueous layer was made basic with ammonium hydroxide and extracted with chloroform yielding 50 mg of crude product. Recrystallization of this residue from acetone gave 30 mg of 1-desoxylycorine-2-one, mp 162-163° [lit. (25) 157-158°], 2.10×10^4 dpm/mmole.

0,0-Diacetyllycorine: Feeding C

A portion of the lycorine obtained from Feeding C was treated with 10 ml of acetic anhydride and 10 ml of pyridine by the same procedure described for Feeding A to provide 360 mg of 0,0-diacetyllycorine (activity 1.04×10^4 dpm/mmole).

1-0-Acetyllycorine

0,0-Diacetyllycorine (50 mg, activity 1.04×10^4 dpm/mmole) was hydrolyzed by the same procedure described for Feeding A. The products were separated by preparative thick-layer chromatography on Silica Gel PF 254+366 (solvent:8% ethanol, 92% chloroform) to give 22 mg of 1-0-acetyllycorine, which was identified by its infrared spectrum.

Oxidation of 1-0-acetyllycorine: Feeding C

The oxidation was carried out on 22 mg of 1-0-Acetyllycorine (22 mg 1.04×10^6 dpm/mmmole) as described for Feeding A. The products were separated by preparative thick-layer chromatography to yield 10 mg of 1-0-acetyllycorin-2-one. The product was identified by comparison of its infrared spectrum with that of authentic 1-0-acetyllycorin-2-one. This material was recrystallized once from methanol to give an activity of 1.04×10^6 dpm/mmmole.

Dilution of 0,0-diacetyllycorine and partial hydrolysis

To approximately 20 mg of 0,0-diacetyllycorine (1.04×10^6 dpm/mmmole) was added 135 mg of 0,0-diacetyllycorine. The mixture was subjected to partial hydrolysis as described for Feeding A. The product mixture was separated by preparative thick-layer chromatography (1.0 mm thickness of Silica Gel PF 254+366; solvent 80% chloroform, 12% ethyl acetate, 8% methanol) to give 59 mg of 0,0-diacetyllycorine (7.20×10^4 dpm/mmmole); and 78 mg of 1-0-acetyllycorine.

Oxidation of 1-0-acetyllycorine

The 1-0-acetyllycorine (78 mg), pure by tlc criteria, was dissolved in chloroform and oxidized by the procedure described for Feeding A. The product mixture was separated by preparative thick-layer chromatography (Silica Gel PF 254+366, 80% chloroform, 12% ethyl acetate and 8% methanol) to give 25 mg of 1-0-acetyllycorin-2-one, identified by its infrared spectrum and comparison of R_f value with that of authentic material (activity after 3 recrystallizations 7.88×10^4 dpm/mmmole).

O,N-Diacetyloxocaranine (LIV) from caranine

To a solution composed of 3 ml of dry dimethyl sulfoxide and 2 ml of acetic anhydride was added 1 mmole of caranine, and the mixture was stored under nitrogen. After 24 hours the mixture was poured into water and extracted several times with ether. The ether extract was washed twice with water, dried, and evaporated to dryness under reduced pressure to yield 130 mg of material which crystallized upon standing. Several recrystallizations from ethanol gave 60 mg of white crystals mp 243.5-244°; IR 1760, 1630 cm^{-1} ; UV 235 $\text{m}\mu$ ($\log\epsilon$ 4.105), 262 $\text{m}\mu$ ($\log\epsilon$ 3.588), 300 $\text{m}\mu$ ($\log\epsilon$ 3.725); molecular weight 353, mass spectrum m/e 353; Anal. Calcd. for $\text{C}_{20}\text{H}_{19}\text{NO}_5$ C, 67.98; H, 5.42; N, 3.99; Calcd. for $\text{C}_{20}\text{H}_{19}\text{NO}_{5.5}$ $\frac{1}{2}(\text{C}_2\text{H}_5)_2\text{O}$ C, 67.78; H, 6.15; N, 3.59; Found C, 67.81; H, 6.07 N, 3.61.

O,N-Diacetyloxocaranine from [^3H] caranine used for Feeding C

A portion of the caranine used for Feeding C was diluted to give an activity of 1.195×10^6 dpm/mmole. This material, 600 mg, was then oxidized according to the procedure described above to give 100 mg of O,N-diacetyloxocaranine identified by infrared spectrum, mp 240-243°, activity 8.78×10^5 dpm/mmole.

O,N-Diacetyloxocaranine from [^2H] caranine

The [2β - ^2H] acetylcaranine (80 mg) was hydrolyzed to give 60 mg of [2β - ^2H] caranine which was oxidized by the procedure described above to give O,N-diacetyloxocaranine which contained 0.80 deuterium atoms per molecule as shown by the mass spectrum run at 20 ev electron energy, mp 240-242°.

0,N-Diacetyloxocarane from [2 β -³H] caranine: Feeding A

The caranine, 100 mg, (activity 4.526×10^4 dpm/mole) was oxidized according to the procedure described above to give 0,N-diacetyloxocarane $4.48 \times 10^4 \pm 0.45 \times 10^4$ dpm/mole.

[2 α -²H] Acetylcaranine

The chlorohydrin (300 mg) was reduced with zinc dust in boiling d₁-acetic acid according to the established procedure (32). The product was acetylated by the usual technique and purified by preparative thick-layer chromatography to give [2 α -²H] acetylcaranine identified by mp 184-186, infrared spectrum, and mass spectrum (molecular weight 314).

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