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AQUATIC PLANT CHEMISTRY. ITS APPLICATION TO WATER POLLUTION CONTROL

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AQUATIC PLANT CHEMISTRY
ITS APPLICATION TO WATER POLLUTION CONTROL

by

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For the

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27 Abstract

Possibly aquatic plants can play a role in aquatic ecology through the production and release of biologically active agents. The research objective was to determine the presence, chemical nature and biological activity of plant-produced agents. Nuphar luteum subsp. variegatum and macrophyllum produce both C-15 alkaloids and C-30, sulfur-containing alkaloids. Two of the latter type were isolated. Both were determined as stereoisomeric biscarbinolamines incorporated into two deoxynupharidine moieties linked together at C-7 through a thiaspirane ring. Two new C-15 alkaloids, 7-epideoxynupharidine and 3-epinuphamine, were discovered in N. luteum subsp. variegatum. Both C-30, sulfur-containing alkaloids were active against Corynebacterium michiganense. The more abundant of the two C-30 alkaloids was tested further against five additional phytopathogenic bacteria and was active against four of these. The same alkaloid was inactive against three bacteria taken from the site where the plant material was harvested. C-15 alkaloids, nupharidine and deoxynupharidine, were inactive against C. michiganense, the bacteria most sensitive to the sulfur-containing alkaloids. There is now sufficient indication that Nuphar produces agents which are active against some phytopathogenic bacteria. The extent of the release of Nuphar-produced agents into the surrounding aquatic environment should be studied.

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Abstractor

Institution

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SECTION 1

INTRODUCTION

Scope, Purpose and Relation to Water Quality Control

The several reports that some aquatic plants produce protistostatic materials suggested an ecological relationship, based on chemical interaction, between these aquatic plants and other water organisms. Such an ecological relationship could effect water quality. Thus an infestation of antibacterial-producing plants could have an adverse effect on water quality through the poisoning of bacteria which ordinarily consume organic material in the water. On the other hand, such aquatic plants could be introduced and managed in some areas to eliminate undesirable organisms which in high populations are making water unsuitable for consumption, recreation or support of wildlife.

While the biological action of these reported protistostatic materials seemed secure, their chemical nature has remained obscure. The primary objectives of the research described herein was to isolate the new and previously described antibacterial materials in their pure form, verify their antibacterial activity, determine their chemical structures and determine their distribution in aquatic plants.

General Background and a General Description of Various Phases of the Project

Preparation of the roots of the aquatic species Nuphar luteum - the yellow water lily - have been used in folklore medicine for the cure of any number of ailments, among them diseases now known to be of bacterial origin. Likely these reports served as the impetus which led a group of Russian workers to investigate the antibiotic properties of various species of the aquatic family Nymphaeaceae. A 1962 British patent (1), in the name of three Russian workers, described the isolation of a $C_{30}H_{42}O_5N_2$ alkaloid from Nuphar which was active against fungi and gram positive bacteria. The reported physical properties helpful in identifying this substance are meager and further studies dealing with the nature of the $C_{30}H_{42}O_5N_2$ alkaloid have not appeared in the chemical literature. However a member of this same group of workers subsequently disclosed (2) the isolation and partial structure determination of a $C_{30}H_{42}O_4N_2S$ alkaloid from Nuphar but antibacterial properties were not revealed. About the same time, a second group of Russians described (3) the isolation of a crystalline substance named nupharine which was active against twenty-three of forty-five phytopathogenic bacteria. Corynebacteria were especially sensitive; xanthomonads were less sensitive. Unfortunately, no physical and chemical properties of this crystalline material were disclosed.

Thus it seemed quite clear that antibacterials were being produced by Nuphar luteum of east European origin but the chemical nature of the specific agent, or agents, responsible for this activity had not been elucidated nor were the physical properties adequately described so that the substances could be subsequently identified with certainty when isolated from other species of Nuphar. Therefore our first concern was

to correlate well characterized substances with specific antibacterial activity. Involvement in this area constituted the initial phase of research which has been conducted during the grant period January 1, 1968 - December 31, 1969.

SECTION 2

APPROACH AND METHODS

Isolation

Since various antibacterials had been isolated by the Russian workers, the most expeditious treatment of the problem seemed to be an attempt to repeat this work using the reported isolation procedures. Many of these procedures were similar and we picked the one which had been used to isolate the $C_{30}H_{42}O_5N_2$ alkaloid. The procedure involved the following steps. Dried, powdered *Nuphar luteum* was treated with 10% aqueous ammonia. The resulting mixture was shaken a number of times for periods of one to two hours with dichloroethane and the separate extracts were treated with 10% sulfuric acid. The combined acid extracts were made alkaline with aqueous ammonia and the resulting precipitate was dissolved in ethanol and treated with 15% alcoholic methylenebissalicyclic acid. The precipitated alkaloid methylenebissalicylate was basified with aqueous ammonia and the solution was extracted with ether. The extract was dried and chromatographed on alumina. Repeated column chromatography, monitored by thin layer chromatography, was used to isolate pure alkaloids.

Later when the presence of the carbinol amine functional group in these alkaloids had been established, the methylenebissalicyclic acid precipitation step and the use of alcohol or ether solvents was abandoned. It seemed likely that the carbinol amine ethyl ethers isolated were really artifacts, being formed by facile etherification of the carbinol amine by the ethanol used in the precipitation step. Accordingly in later extractions using dichloroethane, the extract was concentrated and treated with 5% aqueous sulfuric acid. The aqueous solution was basified with aqueous ammonia (pH 9) and extracted with chloroform. The chloroform extract yielded a mixture of the free carbinol amine alkaloids which were separated by column chromatography. Methylene chloride could be used as the extracting solvent, in place of dichloroethane with no apparent modification in the array of alkaloids. The dichloroethane and methylene chloride extraction procedures were used for extracting solvents for the greens of *Nuphar luteum* subsp. *variegatum* and rhizomes of *N. luteum* subsp. *macrophyllum*.

In addition to using the rather specific extraction procedures described above, a more general isolation procedure was employed. As is revealed in a later section of this report, the two different isolation procedures furnished different alkaloids. Extraction of plant material with methylene chloride or dichloroethane afforded the C_{30}, sulfur-containing alkaloids, deoxynupharidine and 7-epideoxynupharidine. The last two alkaloids belong to the C_{15} class. The second isolation procedure, described immediately below,

produced the C_{15} quinolizidine and piperidine type alkaloids only.

The second procedure involved the following steps. Dried powdered Nuphar luteum was extracted several times with methanol. The combined methanol extracts were concentrated at reduced pressure at 35°. The concentrate was treated with 10% aqueous acetic acid solution and suspended solids were removed by filtration. The clear acetic acid solution was extracted with hexane, benzene and methylene chloride. Each of these extracts was dried, concentrated and set aside for investigation. The acetic acid solution was brought to pH 7, extracted with chloroform, then brought to pH 12 and again extracted with chloroform. pH 7 and pH 12 chloroform extracts were set aside for purification. Isolation of pure alkaloids was achieved through column chromatography on alumina. Chromatographic fractions were monitored by thin layer chromatography (tlc).

Identification and Structure Determination

Known pure alkaloids were identified by tlc R_f values, optical rotation, the melting points and optical rotations of solid derivatives, comparison spectra and chemical correlation with other known alkaloids.

The structures of new alkaloids were elucidated largely through spectral evidence and chemical correlation. The specific methods employed, information obtained and conclusions are covered in Section 3 (Results and Discussion) and Section 4 (Conclusions).

Mass spectra were obtained on a RMU6-Perkin-Elmer-Hitachi mass spectrometer with an all glass heated inlet, and determined at 70eV and a chamber temperature 160-165°. Nuclear magnetic resonance spectra were obtained on Varian A-60 and HR 100 spectrometers and a Joelco HNM-4H 100 spectrometer. Spectra were determined in chloroform and carbon tetrachloride solution, 2% TMS (10τ). Infrared spectra were obtained on a Perkin-Elmer 137. Melting points reported were determined on a Köfler micro-hot stage and are uncorrected. Optical rotations were obtained from a Perkin-Elmer 141 polarimeter. The elemental analyses were performed by Galbraith Laboratories. Ultraviolet spectra were obtained on a Cary 14.

Screening for Antibacterial Properties

Two routine methods for determining antibacterial susceptibility were used. The disc plate method was employed for semi-quantitative determinations and preliminary tests, while the serial dilution-turbidimetric method was used for more quantitative studies.

The phytopathogens chosen for study were Corynebacterium michiganense, C. flaccumfaciens, Xanthomonas phaseoli, X. vesicatoria, and Erwinia caratovora and were obtained from Dr. Robert Dickey, Department of Plant Pathology, Cornell University. Arthobacter (TH-1) was also tested and was obtained from Dr. Robert Baum, Chemistry Department, SUNY College of Forestry. A number of bacteria were isolated from the water and mud of the southeastern corner of Green Lake, Tully, New York, the same area from which Nuphar luteum subsp. variegatum was collected. Three of these bacteria were tested for sensitivity to WPC 45.1.

All bacteria were grown and maintained at 30°C in shake flasks and cultures were transferred directly from these. Disc plate cultures were grown for 48-72 hours and the serial dilution-turbidimetric cultures were grown for 24 hours. All organisms were tested first by the disc plate method. In the case of C. michiganense and Arthrobacter (TH-1) further studies were carried out by the serial dilution-turbidimetric method. The number of organisms tested by the second method was limited primarily by the amount of alkaloid available.

Nupharidine and dexoynupharidine were dissolved in distilled water and WPC 45.1, VPM 74.15 and CFW 225.1 in 0.01M acetic acid.

Plant Material

Nuphar luteum is widely distributed throughout the northern hemisphere. Subspecies obtained for study and their origin are as follows:

1. Nuphar luteum subsp. variegatum (Beal)
   Green Lake, Tully, Onondaga County, New York

2. N. luteum subsp. macrophyllum (Beal)
   Tidal flats along the lower Hudson River near Columbianville, New York

3. N. luteum subsp. ozarkanum (Beal)
   South central Missouri

4. N. luteum
   The Netherlands

Greens and rhizomes of the first listed subspecies and rhizomes of the second listed subspecies have been examined to date. Plant materials were air dried then powdered prior to extraction.

SECTION 3

RESULTS AND DISCUSSION

Research in aquatic plant chemistry carried out at the College of Forestry have been supported by FWPCA (#16020DHV) and the McIntire-Stennis Cooperative Forestry Research Program of the USDA. Research in the different programs occasionally was closely related, particularily in the case of the C_{15} Nuphar alkaloids. Results reported herein are largely those obtained under the FWPCA program but also include results from the USDA program which are closely related and assist in the development of a meaningful discussion.

Alkaloids Isolated by the "Methylenebis(salicyclic) Acid Method"

Alkaloids WPC 45.0 and VPM 74.15 from Rhizomes of Nuphar luteum subsp. macrophyllum.

The dried powdered rhizomes of the sub-title species were

treated by the "methylenebis(salicylic) acid procedure" in the usual manner. Thereby, an ether extract was obtained which when concentrated under vacuum gave a thick paste representing 2.5-5% of the weight of the dry plant material. A preliminary survey of alkaloid content by tlc (thin layer chromatography), using several solvent systems, indicated that the crude mixture contained deoxynupharidine and 7-epideoxynupharidine. The identification is based on the coincidence of R_f values with R_f values of authentic samples. In addition, these preliminary tlc studies showed the presence of no less than three additional alkaloids whose R_f values were always lower than those of deoxynupharidine and 7-epideoxynupharidine.

Cc (column chromatography) on alumina, using ether-hexane, was carried out to first remove deoxynupharidine and 7-epideoxynupharidine. The mixture remaining then was chromatographed repeatedly; the more mobile fractions were recombined after each chromatogram and from them was isolated alkaloid VPM 74.15 giving a single spot on tlc. In the same manner the less mobile fractions were recombined and from them was isolated alkaloid WPC 45.1, a liquid giving a single spot on tlc: $[\alpha]_D^{25}$ + 86.9 (c 19mg/cc, MeOH). No attempt has been made to isolate the alkaloids of intermediate mobility.

The Nature of WPC 45.1 and CFW 225.1

Analysis for nitrogen gave values of 4.97 and 4.73% (N calc'd for $C_{34}H_{50}N_2O_4S$: 4.80). Analysis for sulfur gave a value of 7.27 (% S calc'd for $C_{34}H_{50}N_2O_4S$: 5.50) which was unsatisfactory for the molecular formula consistent with the nitrogen analysis and the ms (mass spectrum) but nevertheless established the presence of sulfur. The ir (infrared spectrum) showed bands at 6.67 and 11.48μ thus indicating the presence of a furan ring. Bohlmann bands, and bands characteristic of carbonyl and hydroxyl groups were absent. WPC 45.1 was recovered unchanged after shaking with palladium on charcoal under an atmosphere of hydrogen. The uv (ultraviolet spectrum) showed only end absorption beyond 215 nm. The 100 MHz nmr (nuclear magnetic resonance spectrum) confirmed the presence of a furan ring (or rings) in showing a doublet at 7.27 and a singlet at 6.35δ whose integrated intensities were in the ratio of 2:1. In addition the nmr showed a) a band of overlapping multiplets in the region of 3.36-3.9δ which indicated protons alpha to nitrogen or oxygen, b) a band of overlapping multiplets in the 2.0-2.6δ region which suggested protons alpha to nitrogen or sulfur and c) a band between 0.9 and 2.0δ corresponding to methyl, methylene and methylnyl protons. The integrated intensities of peaks in the 7.27-6.48δ, 3.36-2.0δ, and 2.0-0.9δ regions was 6:8:36 assuming the presence of two furan rings. Of special interest was the appearance of a methyl doublet at 0.9δ (J=5.5Hz) and a methyl triplet at 1.12δ (J=7Hz) both of which were imposed on the envelope of other protons. The 1.12δ methyl triplet, in conjuction with a methylene quartet at 3.35δ (J=7Hz), indicated the presence of one or more ethoxy groups.

The principal peaks of the mass spectrum and their possible assignment are given in Table 1. Possible modes of

fragmentation follow from a gross structure, 1, assigned on the basis of studies on the biscarbinolamine diethyl ether and the parent biscarbinolamine described below. Plausible fragmentation routes to ions a-i are given in Figure 1.

The mass spectrum of WPC 45.1 was of interest in the following respects. The parent ion at 582 indicated a molecular formula consistent with the number of hydrogens suggested by the nmr studies and the percentage nitrogen. The presence of ethoxy groups was indicated by m/e 554, 538, 510 and 509. The loss of ethylene from ethyl ethers is a well known mode of fragmentation (4). The generation of m/e 538 ion through the loss of C_2H_4O was established by the high resolution ms study of this ion: m/e 538 calculated for $C_{32}H_{46}O_3SN_2$; 538.3229; m/e 538 observed, 538.3144 (Δ=0.0085). The presence of m/e 230 strongly suggested that WPC 45.1 was composed of two deoxynupharidine moities. The presence of peaks corresponding to ions d-i strengthened this suggestion. Furthermore, the appearance of this latter group of peaks suggested a dimeric deoxynupharidine connected through ring B, a structural feature which was reinforced by the absence of a peak at m/e 98. Mass spectral studies of deoxynupharidine-6,7-d_2 carried out in our laboratory have shown that m/e 98 originates from ring B. Therefore, if WPC 45.1 contained two deoxynupharidine moities linked through ring A, the presence of a peak at m/e 98 might be expected. Thus the appearance of the e-i group of peaks and the absence of m/e 98 suggested that the gross structural framework of rings A and A' of WPC 45.1 was the same as in deoxynupharidine. This meant the ethoxy substituents must be in ring B and B' and considering the likely identity of m/e 222 with ion d, the ethoxy groups could be located specifically at carbons 6 and 6'.

The only remaining structural feature not yet accounted for was the place of sulfur. This was incorporated into the structure as a thiaspirane involving two methylenes (carbons 17 and 18) and linking the two quinolizidine ring systems through carbons 7 and 7'. At this point in the structure determination, the evidence for the thiaspirane linkage was by no means compelling. However, the appearance of m/e 230 was consistent as was the lack of any singlet methyl groups in the nmr. Since the nmr revealed only split methyl resonance, the C_7 methyls of both deoxynupharidine components must be incorporated into the structure in a higher oxidation state. The involvement of the thiaspirane linkage was substantiated in the work which followed.

Treatment of WPC 45.1 in anhydrous ether with a 50:50 solution of 70% perchloric acid in ethanol gave a diperchlorate salt, mp (melting point) 233-234°, which analyzed for $C_{30}H_{38}N_2O_2S \cdot 2\ HClO_4$. The ms, which did not show a parent peak, was very much the same as the ms of Δ^6-dehydrodeoxynupharidine, thus again suggesting the symmetrical juxtaposition of two deoxynupharidine components possessing unrearranged carbon skeltons. The ir showed a strong band at 6.0μ, due to the presence of $>C = N^+<$, and furan bands at 6.65 and 11.43μ. Strong broad adsorption in the 8.5-9.5μ region was attributed to the perchlorate ion. The uv showed λ_{max} 208 (ϵ 12,000) and λ_{max} 291 nm (ϵ 2,300).

The conversion of WPC 45.1 to the diperchlorate salt with concomitant loss of two ethoxy groups confirmed the presence of the biscarbinolamine ether function. Still further confirmation

Table 1

The Mass Spectrum of WPC 45.1
Principal Peaks, % Relative Intensity and Possible Assignment

m/e	% Rel. Int.	M^+-m/e	Possible Assignment[a]
582	5.5	0	M^+, $C_{34}H_{50}O_4N_2S$
554	12.5	28	$M^+-C_2H_4$, Ion a
538	100	44	$M^+-(C_2H_4+O)$, Ion b
510	50	72	$M^+-(C_2H_4x2+O)$
509	17	73	$M^+-(C_2H_5+C_2H_4+O)$
491	28	91	
490	17	92	
462	7	120	$M^+-(C_2H_5Ox2+CH_3x2)$
230	50		Ion c
222	27		Ion d
178	7		Ion e
176	11		Ion f
164	12		Ion g
107	8		Ion h
94	4		Ion i

[a] Structures of possible ion fragments (a-i) corresponding to m/e are given in Figure 1.

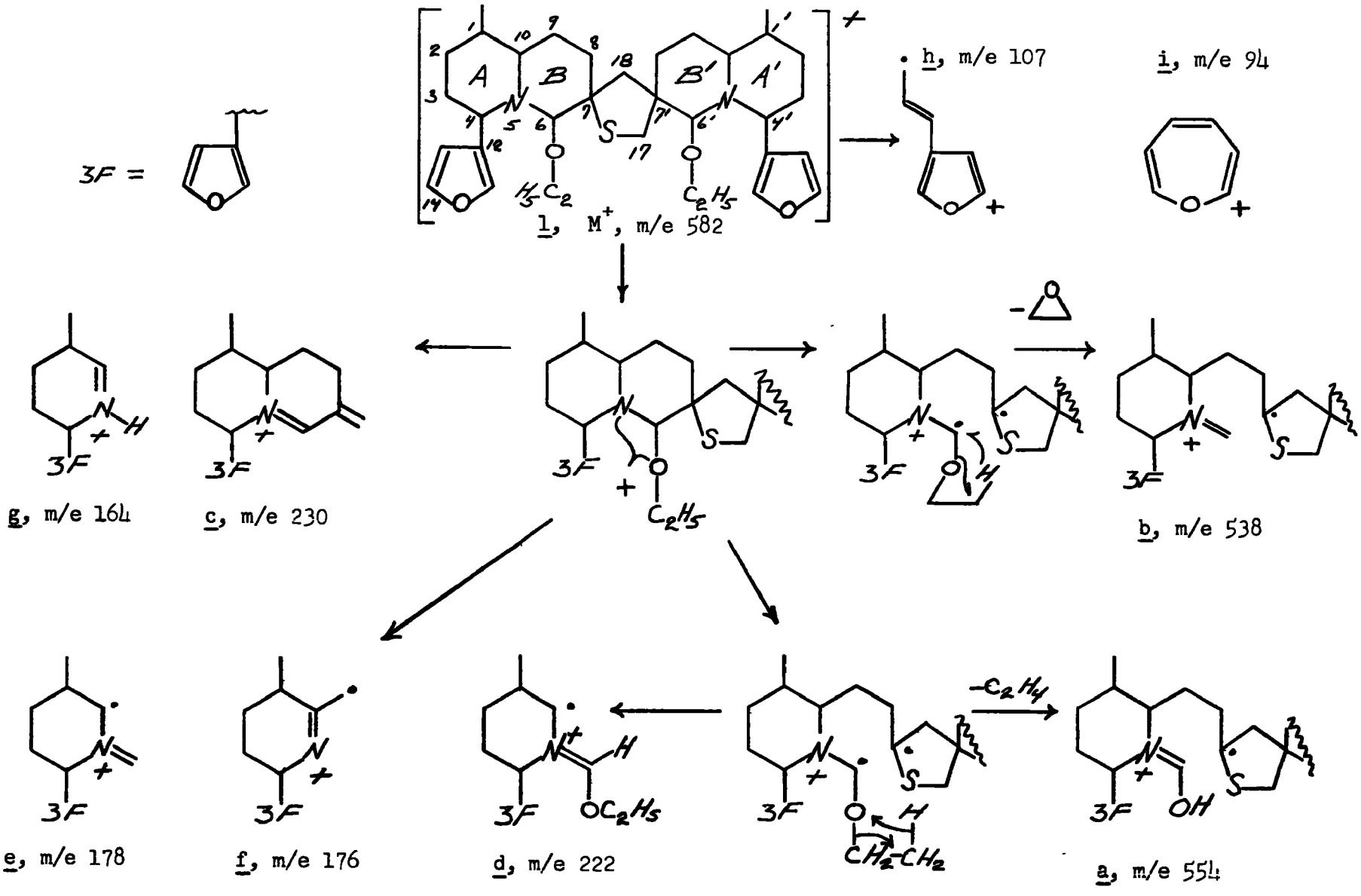
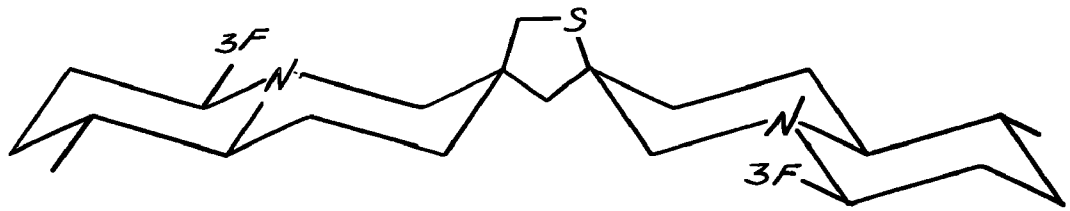


Figure 1. Possible Mass Spectral Fragmentation Routes of WPC 45.1.

was furnished by the conversion of WPC 45.1, with sodium borohydride in methanol, to an amorphous solid whose ms showed M^+ at m/e 494. Reduction with sodium borodeuteride gave a product having M^+ 496. Principal peaks in the ms are correlated, along with the reported ms of neothiobinupharidine, in Table 2. Interestingly, the ms of the product of hydride reduction was virtually identical with the published (5) ms of neothiobinupharidine, an alkaloid having the structure shown in 2 and whose gross structure differs from 1



2

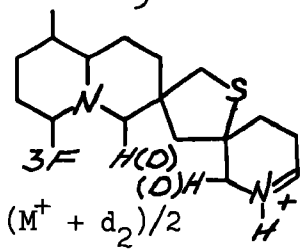
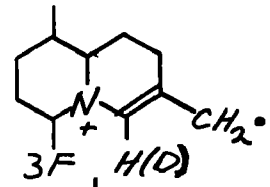
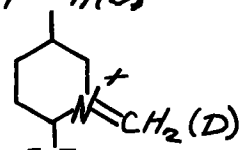
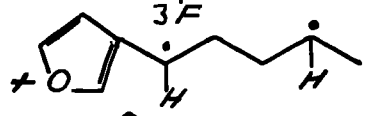
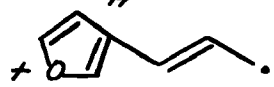
through the lack of ethoxy groups at 6 and 6'. The structure 2 was determined by x-ray analysis (6) and is to date the only structure of the thiobinupharidine class of alkaloids which is secure. Although the ms of the two materials were the same, other physical properties, notably the nmr, were not. Therefore, the reduction product of WPC 45.1 and neothiobinupharidine possessed the same skeletal framework but were stereoisomers. Noteworthy in connection with the mention of the nmr of the reduction product, is the two proton, AB pattern at 2.32δ. Its presence substantiated the presence of the CH_2-S group proposed as part of the thiaspirane linkage.

At this point in the investigation, it seemed likely that the true natural product was really the parent biscarbaniolamine. The biscarbinolamine had undergone ether formation with the ethanol employed in the precipitation step. Therefore, the extraction was repeated but the methylenebissalicylic acid step was omitted as was the use of ethanol as a solvent. Under these conditions was isolated liquid CFW 225.1 ($[\alpha]_D^{25}$ + 44.5, c 1.2, CH_2Cl_2), the free biscarbinolamine corresponding to the ethyl ether form - WPC 45.1, 1.

The ir of CFW 225.1 showed an OH band at 2.9μ, furan bands at 6.64 and 11.43μ. No Bohlmann bands were observed. The ms displayed a parent peak at m/e 526 and peaks at 509, 508, 492, 491 and 490 which corresponded to the loss of OH, H_2O and the combination of any two of these fragments. The uv showed λ_{max} 208 nm (12,200) when netural but an additional λ_{max} at 291 (1850) when acidified; this new band disappeared when the solution was made basic. The nmr (60 and 100 MHz) displayed resonance bands as follows: 0.92δ (d, 5.5Hz, 6H, $C\underline{H}_3CH$), 4.33 (d, 5Hz, 1H, $\underline{H}C_6OH$), 4.1 br m, 1H, $\underline{H}C_{6'}$ OH), 3.74 (q, 1H, $\underline{H}C_4$), 3.61 (q, 1H, $\underline{H}C_{4'}$), 2.49 (AB q, 2H, $C\underline{H}_2S$), 6.50 (m, 2H, furan β$\underline{H}$), 7.48 (m, 4H, furan α$\underline{H}$). The presence of a single methyl resonance indicates both methyl groups are to be found in the same type of environment. Judging from the chemical shift and the magnitude of the coupling constant, both methyl groups are equatorial. Based on earlier studies in the deoxynupharidine series (7) the presence of an axial methyl group would have given a signal at a somewhat lower field (~ 1.1δ) with a larger coupling constant ($>$ 5.5Hz).

Table 2

Principal Peaks, % Relative Intensity and Possible Assignments. The MS of WPC 45.1, Hydride Reduction Products and Neothiobinupharidine (NTBN).

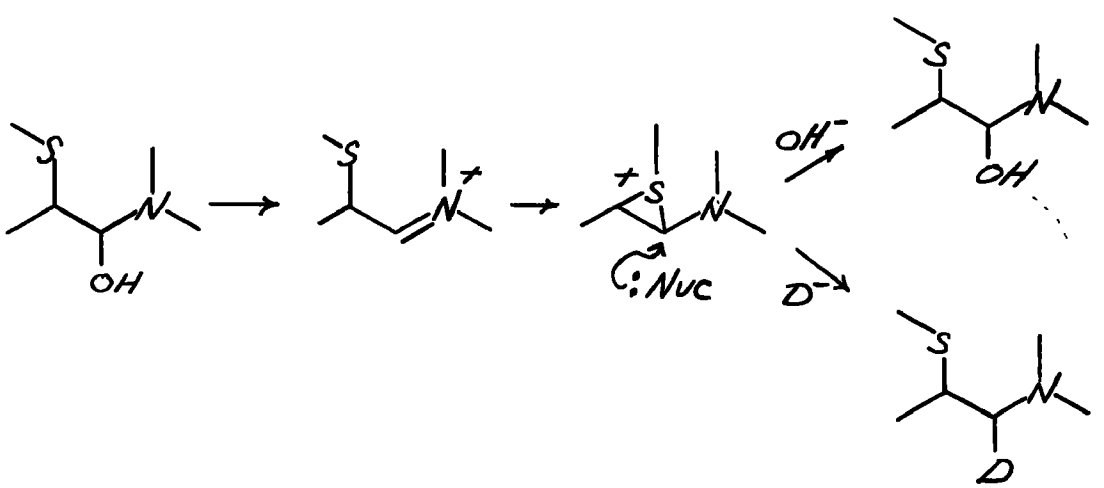
	% Relative Intensity			
m/e	$NaBH_4$ Redn.	NTBN	$NaBD_4$ Redn.	Assignment
496			35	$M^+ + d_2$
494	19.5	39.2		M^+
463			2.4	$(M^+ + d_2)$-SH
461	1.6	2.9		M^+ - SH
449			2.4	$(M^+ + d_2)$-SCH_3
447	1.6	2.6		M^+ - SCH_3
361			12.6	
359	5.9	10.2		
248			2.1	$(M^+ + d_2)/2$
247	1.2	1.6		$M^+/2$
231			34.5	
230	46.6	40.6		
179			100	
178	100	100		
136	4.6	7.2	7.8	
107	11.1	7.3	21	
94	10	24.6	19.2	

The appearance of the 2.49δ AB quartet clearly revealed the involvement of sulfur with one of two methylenes. Since the quartet was not split further nor were there any more than two hydrogens α to sulfur, the involvement of sulfur in a five-membered thiaspirane which linked carbons 7 with 7' was beyond doubt. That this thiaspirane linked the two quinolizidine systems in a symmetrical fashion was indicated by the following nmr features. 1) Only single kinds of α- and β-furan protons were observed. 2) The chemical shifts of protons substituted on carbons 4 and 4' were very nearly the same. 3) A single kind of split methyl resonance was observed. That the resonance of protons at carbons 6 and 6' are so dissimilar is due very likely to one proton being axial and the other being equatorial. Thus the overall shape of the structure was envisioned as possessing a pseudo element of symmetry-i.e., pseudo C_2, pseudo σ, etc.- but lacking local symmetry in the region of the CH_2-S group and, therefore, lacking true symmetry throughout.

Biscarbinolamine CFW 225.1 was converted to a diperchlorate salt identical in all respects with the diperchlorate obtained from the biscarbinolamine ethyl ether, WPC 45.1.

As expected, reduction of CFW 225.1, with sodium borohydride in methanol, gave the dehydroxy counterpart, a liquid $[\alpha]_D^{25}$ + 7.8 (c 18 mg/cc, MeOH). The uv (λ_{max} 215 nm, 13,000) remained unchanged on addition of acid or base.

In addition to single kinds of α and β furan protons, the most significant features of the nmr were a single methyl resonance at 0.93 (br d, 6H, $C\underline{H}_3CH$), the $-CH_2S-$ AB quartet at 2.32 and a 2.7-3.1δ four proton multiplet. The last named signal was assigned to the two protons at carbons 4 and 4' plus two equatorial protons at carbons 6 and 6'. Other work (7) has shown that the C_6 equatorial proton of deoxynupharidine is at 2.7δ while the C_6 axial proton is located considerably upfield, near 1.8δ. The nmr of the C_6 protons is of considerable importance as it relates to the nmr of the product obtained from sodium borodeuteride reduction of CFW 225.1. The nmr of the latter product showed only three protons in the 2.7-3.1δ region and diminished intensity in the 1.75δ region. This result could only mean that deuterium entered equatorially at one center but axially at the other. We have interpreted this result in terms of sulfur participation in reduction and it is depicted in the reaction equation below.



Considering the various shapes of all the possible stereoisomers of 1, there is no reason why the reducing agent should approach C6 and C_6' from opposite directions unless some agent shields the axial approach at C_6 but not C_6'. The proximity of the sulfur atom to C_6 and the energetic and entropy factors more favorable to three-membered ring participation than four-membered ring participation (8) leads us to postulate an episulfonium ion intermediate which acts as the shielding agent and prevents C_6 axial attack. As shown in the reaction equations above, the episulfonium ion possibly may be formed subsequent to the formation of an immonium ion. Deuteride displacement proceeding with inversion leads to C_6 equatorial substitution. In terms of the episulfonium ion intermediate, it follows that the C_7 sulfur atom must be equatorial to account for equatorial deterium substitution.

The Nature of VPM 74.15

The ir spectrum of VPM 74.15 was similar to that of WPC 45.1, there being no significant difference in the position of bands. The uv in neutral solution showed only end absorption beyond 210 nm but in acid a new band appeared at 295 nm (ϵ 1,000). The ms was similar to that of WPC 45.1. Although there were no peaks at m/e 554 and 526, corresponding to partially ethylated biscarbinolamine, the peak at 564 (582-H_2O) suggested that perhaps this material was not completely ethylated, a suggestion which was reinforced by the nmr spectrum which revealed 5 protons in the region 3.22-4.25δ. Judging from the nmr of the biscarbinolamine, which displayed three protons in the same region, a fully ethylated biscarbinolamine should have contained 7 protons in the 3.22-4.25δ region.

Thus the isolation of the partially ethylated biscarbinolamine was ample demonstration of the disadvantages of using the Russian isolation procedure. Treatment of WPM 74.15 with dilute aqueous hydrochloric acid then aqueous base produced the free liquid, biscarbinolamine: $[\alpha]_D^{25}$- 69° (c 10 mg/cc, CH_2Cl_2). Its nmr was significantly different from that of the free biscarbinolamine (CFW 225.1) corresponding to WPC 45.1, in a number of respects. Firstly, the former showed two kinds of furan rings as evidenced by α(4H) signals at 7.37 and 7.27δ and and β(2H) signals at 6.47 and 6.27δ. Secondly, the chemical shifts of protons attached to C_4 and C_4' were widely separated ($\Delta\tau$ = 0.7 ppm) whereas the same protons of the free biscarbinolamine CFW 225.1 were found at nearly the same field strength ($\Delta\tau$ = 0.13). Thirdly, the chemical shifts of protons attached to C_6 and C_6' were found at 4.10 (d, 5Hz) and 3.92δ (d of m, 10Hz) whereas the counterparts in biscarbinolamine CFW 225.1 were observed at 4.10 (m, $W1/2h$ ~ 3Hz) and 4.33δ (d 5Hz). The first two of these features showed that the furan rings, and the carbon atoms to which they are attached, C_4 and C_4', must be in different environments in biscarbinolamine VPM 74.15 but in nearly the same environment in biscarbinolamine CFW 225.1. The third significant difference in nmr properties strongly suggested that the stereochemistry at C_6 and C_6' of biscarbinolamine VPM 74.15 was the same but the stereochemistry at these two centers in CFW 225.1 was different.

VPM 74.15 was treated with sodium borohydride in methanol to obtain the C_4, C_4' dehydroxyamine. The ms, ir and uv were consistent with the same gross structure possessed by neothiobinupharidine, 2. The nmr showed: 1) two kinds of furan signals for α protons (2H, 7.31 and 7.22δ). 2) a total of four protons in the 2.42-3.15δ region corresponding to two protons at C_4 and C_4' and two equatorial protons at C_6 and C_6' and 3) an AB quartet at 2.33δ corresponding

to-CH_2-S. The latter signal and the corresponding signal in the nmr of dehydroxylated CFW 225.1 are found at the same field strength which is higher than that (2.69δ) observed for the CH_2S group of neothiobinupharidine, 2. Applying the same rule for distinguishing axial from equatorial quinolizidine methyls to CH_2S groups, the 2.69δ signal is down field and would correspond to axial substitution of the methylene at C_7' in neothiobinupharidine. The 2.33δ signal of dehydroxy CFW 225.1 and VPM 74.15 are upfield and correspondingly would mean equatorial substitution of the methylene at C_7'.

Sodium borodeuteride reduction of VPM 74.15 produced the deuterated analog of the dehydeoxy VPM 74.15. Interestingly the deuterated analog showed simplified but undiminished intensity of the signals in the equatorial C_6 and C_6' region. However, the signals in the axial C_6 and C_6' region were of diminish intensity. This result must mean that deuterium atoms at both C_6 and C_6' are axial. As described earlier biscarbinolamine CFW 225.1 gave equatorial and axial deuterium at C_6 and C_6' respectively. This was ascribed to participation in reduction by equatorially substituted sulfur at C_7. Using the same rationale, the observation of only axial deuterium at C_6 and C_6' in the product from biscarbinolamine VPM 74.15 must mean the sulfur at C_7 is axially substituted. It should be noted that both VPM 74.15 and WPC 45.1 give at least one axial deuterium. This finding means that without sulfur participation axial attack is the normal mode.

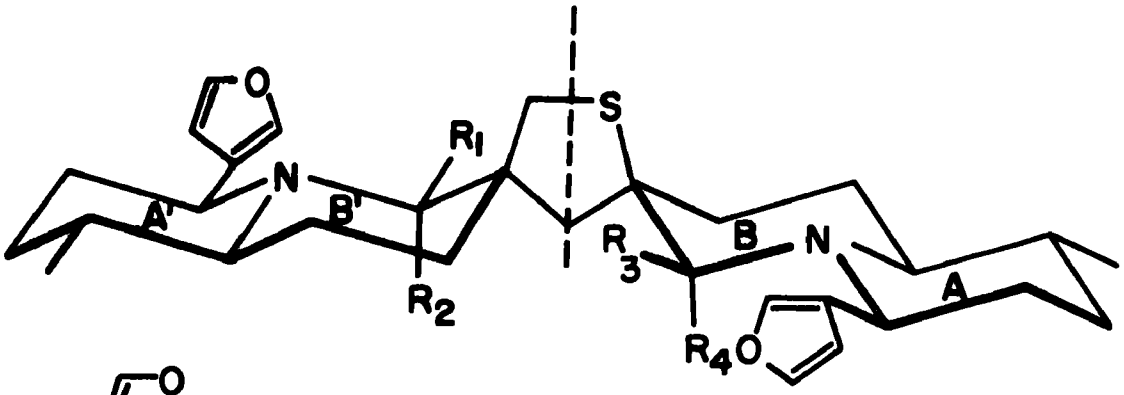
The Structure of WPC 45.1 and VPM 74.15

Results disclosed in previous sub-sections establish that WPC 45.1 and VPM 74.15 are 6,6' biscarbinolamine ethyl ethers. These have been converted by hydrolysis to the free 6,6'-biscarbinolamines. The nmr studies clearly showed that the biscarbinolamines contain secondary hydroxyl groups adjacent to fully substituted carbon. The biscarbinolamines and/or ethyl ethers are reduced by sodium borohydride to two different bisdeoxynupharidine thiaspiranes neither of which is identical to neothiobinupharidine. Sodium borodeuteride reduction of WPC 45.1 produced one equatorial and one axial deuterium while the same treatment of VPM 74.15 produced two axial deuteriums. This result has been interpreted in terms of sulfur atom participation in the course of reduction. Thus the deuteride attacks at C_6 from the side opposite the sulfur atom. This must mean that the C_7 sulfur is axial and equatorial in reduction products of WPC 45.1 and VPM 74.15 respectively.

The nmr of the two reduced products show the CH_2-S-signal at higher field than the signal for the same group in neothiobinupharidine, 2. Therefore, the CH_2-S-group is attached equatorially to the second quinolizidine ring system. Thus on the basis of the evidence obtained to date, the reduction product of WPC 45.1 (CFW 225.1) is assigned structure 4 while the reduction product of VPM 74.15 is assigned structure 5. These are tentative assignments of stereostructure. The gross structural type can be assigned with confidence. Also included for comparison with structure 2, 4 and 5 is the fourth possible stereostructure 3. The structure of WPC 45.1 (CFW 225.1) is related to its reduction product 4 and the structure of VPM 74.15 is related to its reduction product 5.

Alkaloids Isolated Through pH Adjustment of a 10% Aqueous Acetic Acid Solution.

Most of the work which would be reported under this sub-heading


O
S
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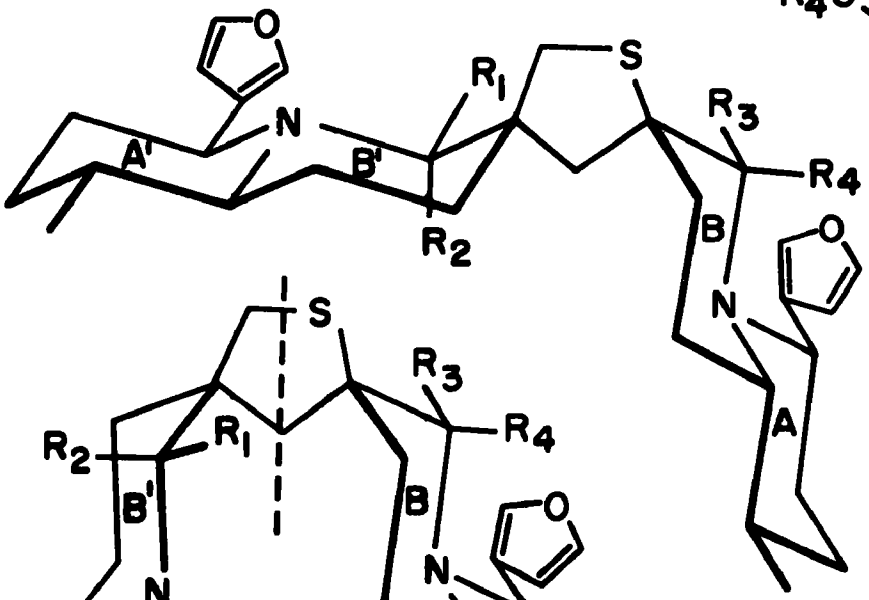
2


O
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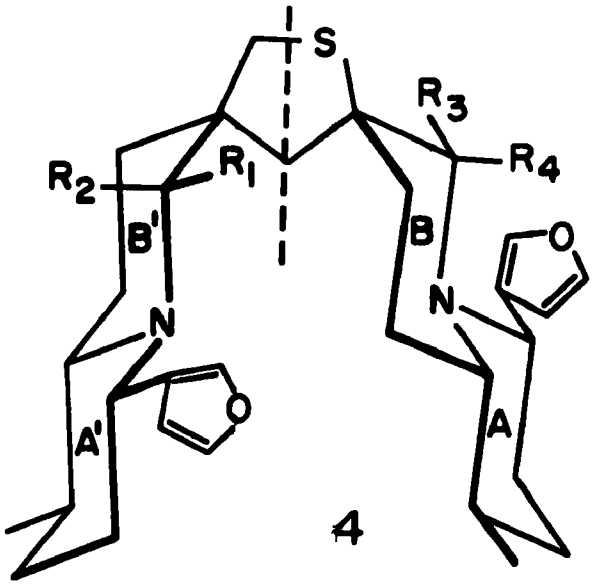
3


S
R3
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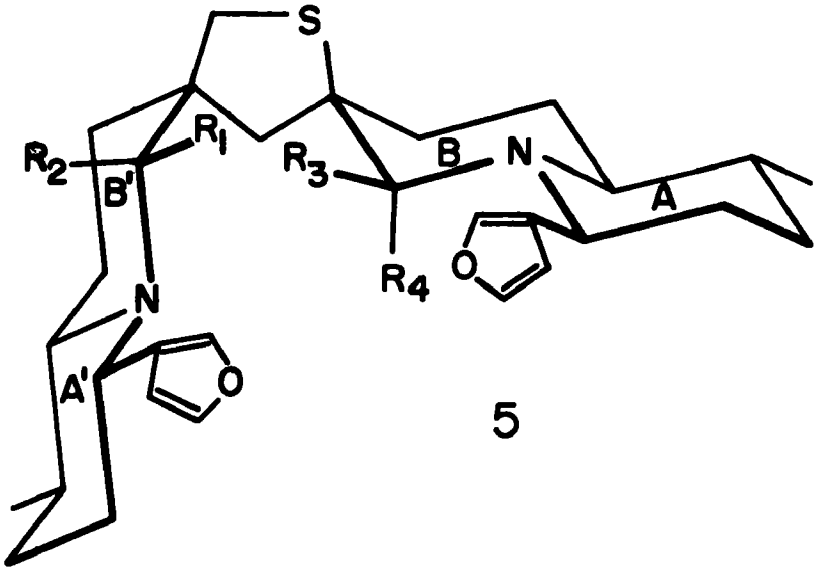
4


S
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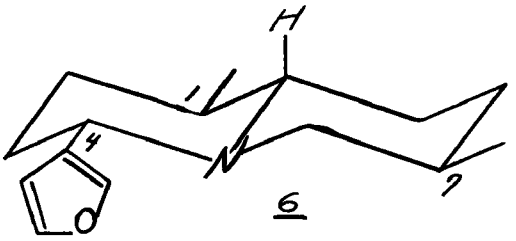
O

5

already has been described in publications which have recently appeared or are now in press and will appear shortly. Since these publications treat the work in detail, only a brief description will be given here. The unpublished portions are treated in more detail and objectives and results are included.

7-Epideoxynupharidine

7-Epideoxynupharidine, 6, was isolated for the first time from natural sources (9). This alkaloid was obtained from a methylene



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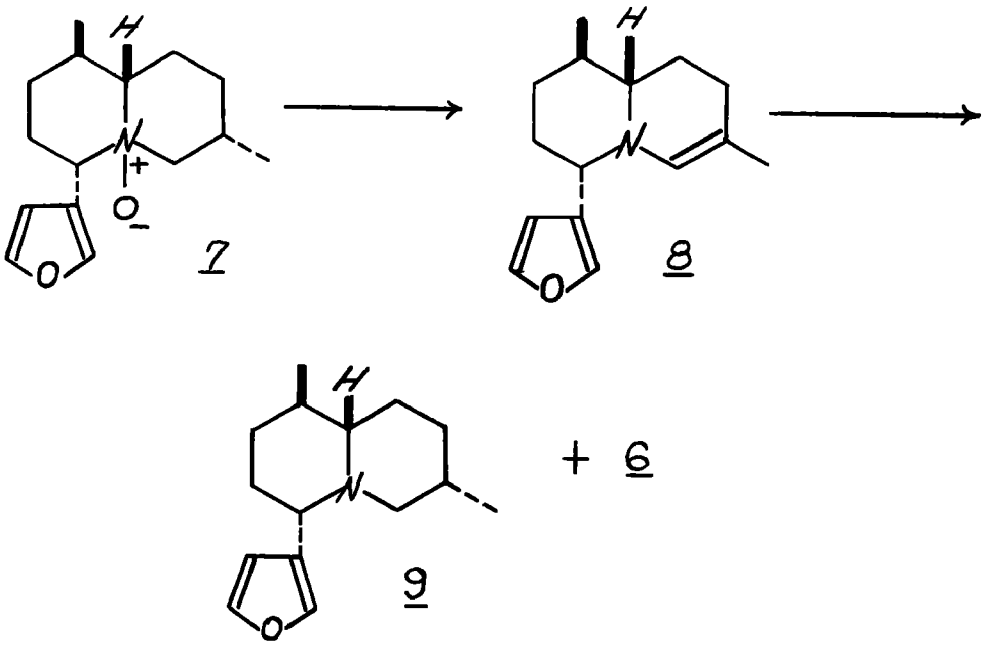
chloride extract of a 10% aqueous acetic acid solution containing the crude mixture from N. luteum subsp. variegatum (rhizomes). Shortly before its isolation from natural sources, the alkaloid had been obtained in our laboratory by synthesis. Thus treatment of nupharidine 7 under Polonovsky reaction conditions produced Δ^6-dehydrodeoxynupharidine, 8, which on catalytic hydrogenation gave a 7:1 mixture of deoxynupharidine, 9, and 7-epideoxynupharidine, 6. This two step reaction sequence is shown in the reaction equation below. The Polonovsky step involved treating 7 in chloroform solution with



acetic anhydride and fused potassium acetate at room temperature (11).

Deoxynupharidine. Relative and Absolute Configuration.

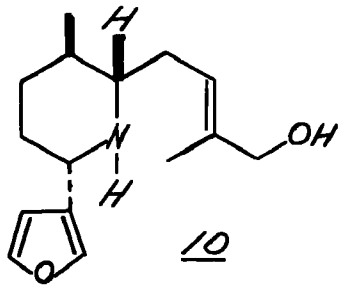
Deoxynupharidine is ubiquitous in Nuphar and its presence in the same methylene chloride extract along with 7-epideoxynupharidine was not unexpected. Although the gross structure and relative configuration of deoxynupharidine had been convincingly established a number of years ago, we wished to obtain corroborative evidence of

the relative stereochemistry of the two methyl groups based on 100 MHz nmr studies. These studies focused on the C_6-methylene group and thereby the axial disposition of the C_7 methyl was confirmed (7). Results from these studies have been helpful in the interpretation of the more complex nmr of the sulfur-bearing C_{30} alkaloids.

In recent times, the previously assigned (11) absolute configuration of deoxynupharidine has been questioned (12). Since the absolute configuration of several other Nuphar alkaloids of both the quinolizidine and piperidine type had been related to deoxynupharidine, the absolute configuration was an important point to establish. The correlation between (-)-deoxynupharidine and (-)-(R)-α-methyladipic acid, carried out in our laboratory (7) revealed that (-)-deoxynupharidine belonged to the enantiomeric series. The correct absolute configuration of deoxynupharidine and correlated Nuphar alkaloids is given in the structures appearing in this report.

Nuphamine

Nuphamine, 10, had been isolated earlier by the Japanese workers from N. japonicum D. C. (13).

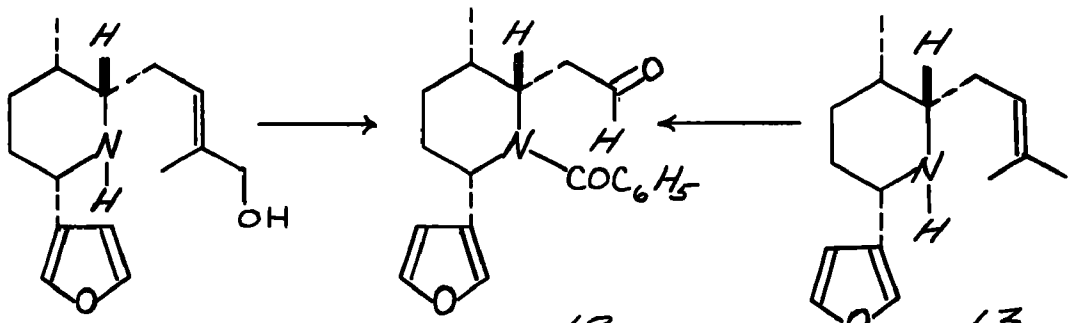


10

N. luteum subsp. variegatum also produces this alkaloid. In our laboratory it was isolated from the pH 12-chloroform extract after elution chromatography on alumina. Fractions removed with ether-hexane 5:95 and benzene contained deoxynupharidine, 7-epideoxynupharidine and other unknown components. Ether elution gave nuphamine: $[\alpha]_D^{25}$ -55.4°(c, 1.22, $CHCl_3$), reported (13) $[\alpha]_D^{19}$ -60.4.° The ir, nmr and ms were consistent with the structure. Treatment of a sample of nuphamine with excess methyl iodide and sodium bicarbonate in methanol afforded the N-methyl methiodide of nuphamine: mp 164-164.5, reported (13) 164°.

3-Epinuphamine

This new alkaloid was isolated from the pH 7-chloroform extract after repeated elution chromatography with cyclohexane-methylene chloride-methanol. The structure of 3-epinuphamine, 11, was assigned on the basis of nmr, ir and mass spectra and correlation with nuphenine, 13 (14), as shown in the reaction equation below. The trans stereochemistry



11 12 13

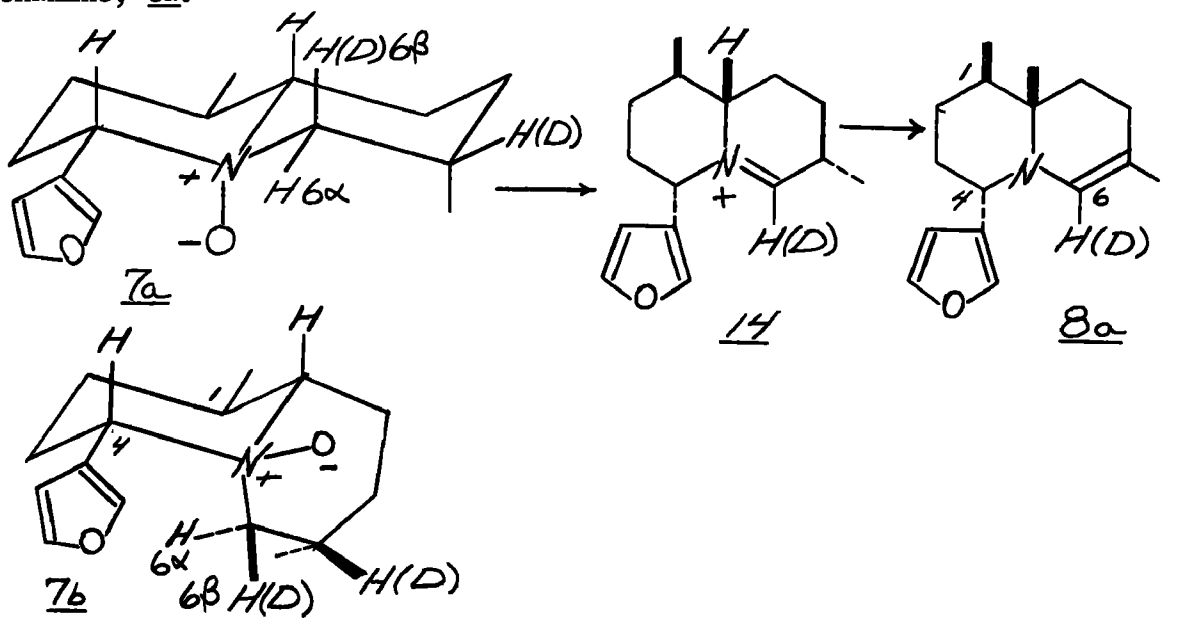
of the allylic side chain was established by nmr studies of the allylic alcohol and uv studies of the corresponding aldehyde obtained on manganese dioxide oxidation of the allylic alcohol, 11.

Nupharidine. Studies of Stereochemistry and the Course of the Polonovsky Reaction.

Nupharidine, 7, is a well known, naturally occurring, N-oxide related to deoxynupharidine. Possibly this alkaloid is the biogenetic link between quinolizidine, piperidine and the sulfur-bearing C_{30} alkaloids. This proposal, though highly speculative at this point, nevertheless has some basis in the clean and facile Polonovsky type conversion of nupharidine to Δ^6-dehydrodeoxynupharidine, 8 (see Section 3). Moreover with the solitary exception of Δ^3-dehydrodeoxynupharidine (15) all other Nuphar alkaloids, including the C_{30}-sulfur-bearing alkaloids possess a ring B elaborated quinolizidine or a ring B-cleaved quinolizidine, i.e., a piperidine Nuphar alkaloid. Therefore the Polonovsky type transformation may be analogous to what is happening in nature. The resulting Δ^6-dehydrodeoxynupharidine, a ring B enamine, would be susceptible to enamine oxidation or substitution and as such would be a key intermediate in the transformation of quinolizidine to piperidines and the C_{30}-sulfur alkaloids.

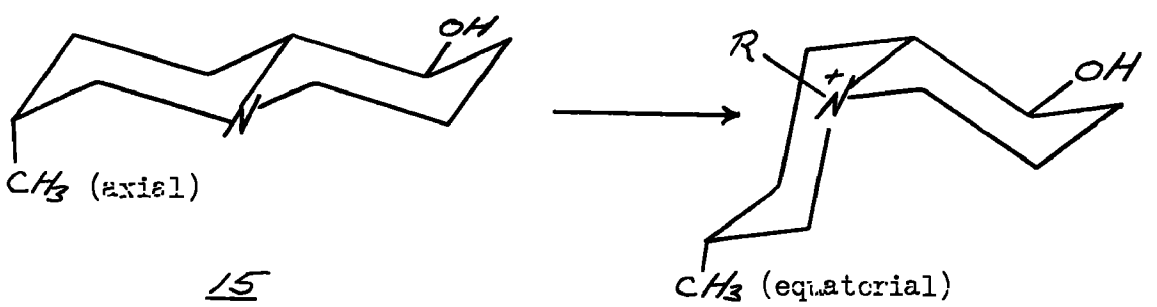
Because of the speculated importance of nupharidine in biogenesis and also because of the realized synthetic entry to the less abundant piperidine alkaloids through nupharidine, a study of the course of the Polonovsky reaction was initiated.

The original rationale for employing nupharidine to obtain a functionalized ring-B quinolizidine was based chiefly on the postulate of Wenkert (16) which treated the Polonovsky reaction as if it were cis-elimination. Thus if nupharidine were to have a trans-quinolizidine N-oxide ring system as is shown in structure 7a, then the only proton cis to the N-oxide is the $C_6\alpha$ proton and Polonovsky elimination would be forced into ring B through the immonium form, 14, of the enamine, 8a.



On the other hand, if nupharidine possessed a cis-fused quinolizidine N-oxide system, cis elimination could possibly remove hydrogens at C_4, $C_{6\beta}$ and C_{10} and thereby produce a mixture of three enamines. However, of the three possible, the Δ^3-enamine seemed unlikely since models showed its formation would involve introduction of an extremely strained double bond between nitrogen and C_4. In any case, a cis Polonovsky elimination from a cis fused quinolizidine N-oxide was not envisioned as giving a single product. However, trans elimination from a cis-fused system could give only the Δ^6 enamine as the $C_6\alpha$ hydrogen now is the only hydrogen trans to the N-oxide.

As results substantiated, nupharidine was converted to Δ^6-dehydrodeoxynupharidine in 84% yield under Polonovsky conditions. Nupharidine used in these and other studies was isolated conveniently as its crystalline acetate salt by concentration of the original methylene chloride extract previously described in the isolation section. The Δ^6-enamine next was converted by catalytic addition of deuterium to deoxynupharidine-6β, 7β-d_2 which in its turn was oxidized with hydrogen peroxide in acetone to the deuterium labelled nupharidine, 7a. The stereochemistry of the deuterium atom was assigned on the basis of preferred cis hydrogenation of the β face of the Δ^6-enamine 8 as evidenced by the formation of deoxynupharidine 7-epideoxynupharidine in a ratio of 7 to 1. Repeating the Polonovsky reaction on the deuterium labelled nupharidine gave a Δ^6-dehydrodeoxynupharidine whose nmr showed no vinyl hydrogen but whose ir and ms were consistent with Δ^6-dehydrodeoxynupharidine-6-d_1 (10). Thus it was clear that the Polonovsky elimination was stereospecific, being either a cis elimination from a trans quinolizidine N-oxide or a trans elimination from a cis quinolizidine N-oxide. Since the stereochemistry of the N-oxide had never been determined it was not possible at this point to make a conclusive distinction between the two stereochemical modes of elimination. However, there was some reason to believe that N-oxidation of deoxynupharidine was equatorial, resulting in the cis fused quinolizidine N-oxide. Thus Fodor has demonstrated the equatorial quarternization of some tropanes (17) and studies by Möhrle (18) and Katritzky (19) have shown that the substituted quinolizidines, such as 15, undergo predominantly equatorial quaternization. Considering an N-oxide to have at least the steric



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requirements of N-alkyl, it would seem reasonable that nupharidine is a cis quinolizidine N-oxide. It an attempt to gain some evidence on this point, the nmr solvent-induced-shifts of equatorial and axial methyls were compared in deoxynupharidine and 7-epideoxynupharidine with shifts in nupharidine and 7-epinupharidine. As the data in Table 3 illustrate, equatorial methyl groups undergo an upfield shift of 3-5 Hz on changing the solvent from chloroform to benzene. The single axial methyl (C_7-CH_3, deoxynupharidine) undergoes a downfield shift of 4.2 Hz. Both methyls of 7-epinupharidine and nupharidine undergo upfield shifts and this result would indicate that these methyl groups are equatorial. To accommodate two equatorial methyl groups in nupharidine, rings A and B must be

Table 3

C_1-C_7 Methyl Resonance, $CDCl_3$ vs C_6H_6 (Rel. To TMS)

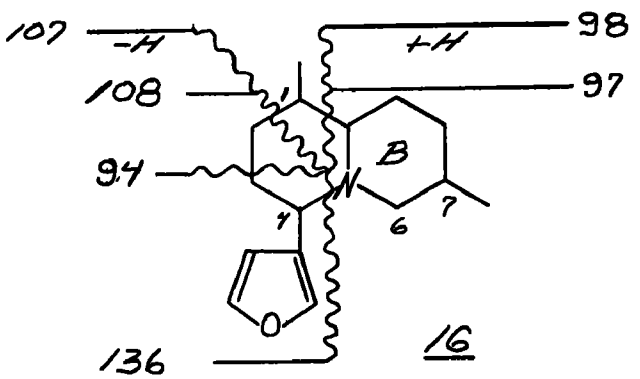
		C_1-CH_3			C_7-CH_3		
Cpd.	Solvent	Conf.	Hz	Δ	Conf.	Hz	Δ
Deoxy-	$CDCl_3$		54.0			59.8	
	C_6H_6	β(e)	49.0	+5.0	α(a)	64.0	-4.2
7-Epi-	$CDCl_3$		55.0			44.0	
	C_6H_6	β(e)	52.0	+3.0	β(e)	41.0	+3.0
7-Epinuph.	$CDCl_3$		55.5			47.8	
	C_6H_6	β(e)	43.5	+12.0	β(e)	37.0	+10.8
Nuph.	$CDCl_3$		59.6			50.6	
	C_6H_6	β(e)	44.0	+15.6		44.0	+6.6

cis-fused. This being the case, the Polonovsky elimination is occurring stereospecifically trans.

The weak point in the above described nmr based studies of nupharidine stereochemistry is the assumption that the magnetic influence of N-oxide oxygen and the nitrogen non-bonding electron pair is the same. This assumption might not be warranted. Therefore, an x-ray analysis of nupharidine hydrobromide has been initiated in order to put the stereochemistry on a firm basis and settle the question of the stereochemical course of the Polonovsky reaction. The x-ray studies are being done in collaboration with Miss Jean Ohrt of the Center for Crystallographic Research, Roswell Park, Buffalo, New York.

The deuterium labelled compounds already mentioned in the paragraphs above, were also useful in confirming nmr assignments of deoxynupharidine and assessing the ms fragmentation routes of deoxynupharidine and Δ^6-dehydrodeoxynupharidine. Thus the 2.7δ quartet (J=12.5, 2.5 Hz) assigned to $C_6\alpha$-H (equatorial) in deoxynupharidine was reduced to a broad singlet in deoxynupharidine -6β, 7β-d_2. The 1.88δ quartet (J=12.5, 2.5 Hz), of which only the lower field half was clearly visible, was assigned to the $C_{6\beta}$-H (axial) in deoxynupharidine. In the labelled deoxynupharidine, the 1.88δ signal was absent.

Correlations of principal ms fragmentation peaks generated by deoxynupharidine, deoxynupharidine-6β, 7β-d_2, Δ^6-dehydrodeoxynupharidine and dehydrodeoxynupharidine-6-d_1 are given in Table 4. The ms of deoxynupharidine always displays intense peaks at m/e 94, 97, 98, 136 and 233. The last peak corresponds to the parent ion which is shifted two m/e units higher in the labelled compound. Neither m/e 94 nor 136 is shifted on labelling. Therefore m/e 94 and 136 does not include C_6 and C_7. Possibly the generation of these two fragments may result as shown in 16 below. It is not surprising that m/e 136 is at best a



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minor peak in the ms of Δ^6-dehydrodeoxynupharidine and nuphenine, 13 a piperidine type nuphar alkaloid. The complement of the m/e 136 mode of the cleavage would generate m/e 97 and 98, but now charge would be located on nitrogen as opposed to oxygen as when m/e 136 is formed. Accordingly, m/e 97 is shifted to m/e 95, a strong peak, in the ms of Δ^6-dehydrodeoxynupharidine.

Peaks which correspond to M^+ - 15, 29, 43, 57, 71 and 85 are of moderate to weak intensity peaks and seem to be generated through the loss of the alkyl groups CH_3-C_6H_{13}. All of the peaks are shifted two mass units higher in the ms of labelled deoxynupharidine. The most intense peak of this type is m/e 190. Possibly generation

of this peak corresponds to the loss of the C_1-methyl, C_1, C_2 and an additional hydrogen from the charged fragment. The genesis of the m/e 107, 108 pair of peaks probably involves fragmentation of ring A, as does the genesis of m/e 136. Significantly, the 107, 108 pair also are found as intense peaks in the ms of nuphenine, 13. The m/e 176-178 group of peaks is shifted to 178-180 in the spectrum of the labelled deoxynupharidine and must therefore, include C_6 and C_7. Additional ms studies with deoxynupharidine labelled at other positions must be carried out before a distinction can be made among the various possible routes consistent with labelling results already obtained.

Significant information relevant to the ms fragmentation could be obtained if ring A of deoxunupharidine could be labelled. Several attempts have been made in our laboratory to achieve this goal. These attempts have focused on the anticipated reactivity of the hydrogen atom at C_4. At first glance, this hydrogen might be activated by the adjacent furan ring and nitrogen atom. This being the case, a Δ^3-enamine, or the corresponding immonium ion, would be formed. To these intermediates could be added deuterium, catalytically to introduce two deuterium atoms, or reductively with sodium borodeuteride to introduce one deterium atom.

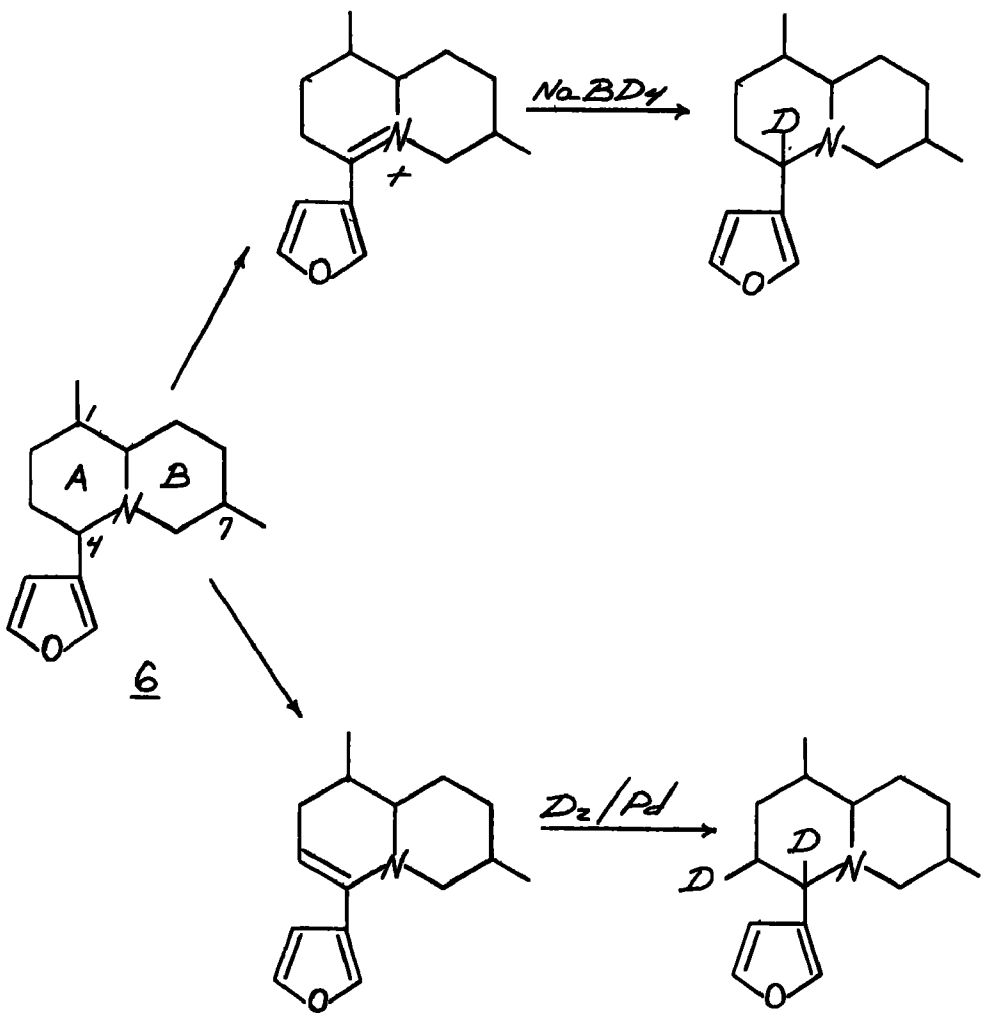


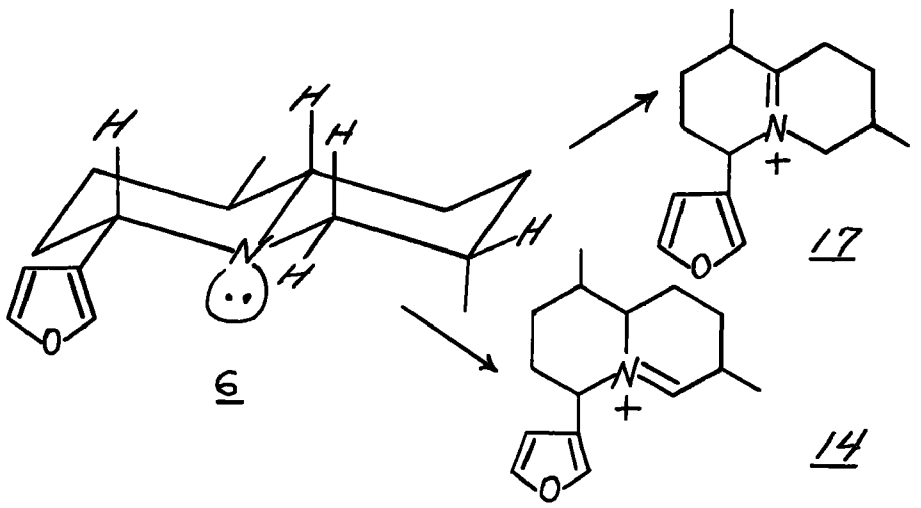
Table 4

Shifts of the Principal Peaks (m/e) in the Mass Spectra of Deuterated Deoxynupharidine and Δ^6-Dehydrodeoxynupharidine.

Peak	Deoxy-nupharidine-d_o	-6β,7β-d_2	Δ^6-Dehydrodeoxy-nupharidine-d_o	-6-d_1
M^+	233	235	231	232
M^+-1	232	234	230	231
M^+-15	218	220	216	217
M^+-29	204	206	204(202)	203
M^+-43	190	192	188	189
M^+-55	178	180	176	177
M^+-56	177	179		
M^+-57	176	178	174	175
M^+-71	162	164	160	161
M^+-85	148	150(50%)	146(148)	149
M^+-97	136	136	136	136
M^+-125	108	108	108	108
M^+-126	107	107	107	107
M^+-135	98	100		
M^+-136	97	99	95	96
M^+-139	94	94	94	94

However, participation of the nitrogen non-bonding electron pair and furan π electrons in stablizing the intermediate, whether a carbonium ion or free radical, would necessarily involve a planar framework. That is, the furan ring, C_3, C_4, N, C_6 and C_{10} would be coplanar. Models show that such a structure is sterically hindered since α and/or β furanyl protons would be forced into the region occupied by the equatorial proton at C_6. This analysis tends to be substantiated by experiments which show that the C_4 proton is inert. Dehydrogenation of deoxynupharidine was attempted using dichlorodicyanoquinone, chloranil and selenium dioxide under standard conditions used for the dehydrogenation of steriodal ketones. In all three cases, unconverted deoxynupharidine was recovered. Treatment of deoxynupharidine with bromine in aqueous solution at pH 5 resulted in electrophilic substitution of the furan ring.

Mercuric acetate oxidation has been used in quinolizidine systems for selective introduction of an enamine double bond (20). The selectivity is believed (21) to stem from a preferential loss of a hydrogen anticoplanar to the nitrogen non-bonding electron pair. In deoxynupharidine there are three hydrogens with the preferred



steric requirement for mercuric acetate oxidation. Oxidation at C_4 would be prohibited for the steric reasons already discussed. However, judging from models, the formation of either immonium ions 17 or 14 could be achieved without introduction of excessive strain. Presumably, it is the latter immonium ion which is formed as an intermediate in the Polonovsky transformation of nupharidine to Δ^6-dehydrodeoxynupharidine. From the actual treatment of deoxynupharidine with mercuric acetate in acetic acid solution, only unconverted deoxynupharidine was detected.

Carbanion oxidation was also tried as a method to introduce functionality into deoxynupharidine. Deoxynupharidine was treated with potassium t-butoxide in dimethyl sulfoxide and t-butyl alcohol solution in the presence of oxygen. Uncoverted deoxynupharidine was recovered in 80%.

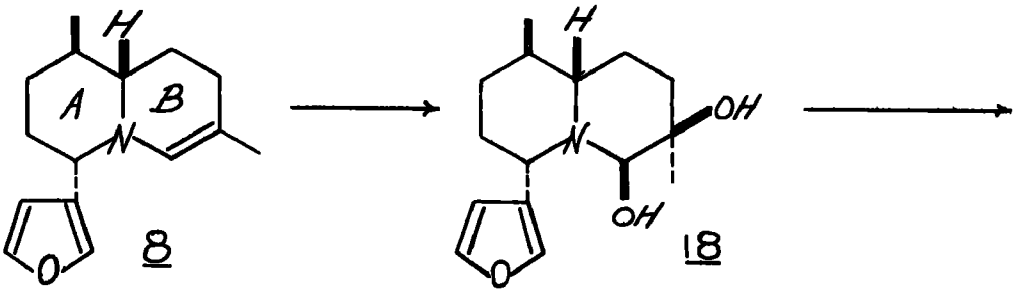
Correlation Studies

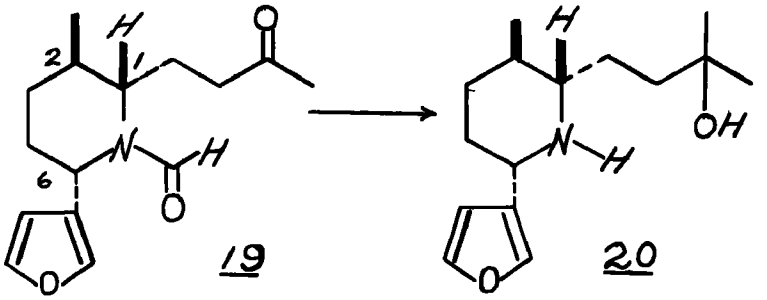
Various transformations and interconversions of piperidine and quinolizidine type alkaloids were attempted in order to: 1) correlate structure, 2) obtain minor alkaloids in sufficient quantity for biological screening and 3) obtain intermediates which could be labelled and used subsequently in ms fragmentation studies.

Conversion of Nupharidine to Nupharamine.

Nupharamine, a piperidine nuphar alkaloid, has been isolated by the Japenese workers from N. Japonicum (22) but has not been isolated to date from any plant material examined in our laboratory. Furthermore, all of the other piperidine alkaloids were isolated only in small amounts. Consequently, a synthesis approach to these less available alkaloids was attempted.

The basis for choosing nupharidine as the starting material was its ample supply and the stereochemical considerations which suggested a singular potential in nupharidine for functionalizing ring B of the quinolizidine ring system by way of the Polonovsky reaction. The latter aspect has been discussed already in this Section, as has the actual conversion of nupharidine to Δ^6-dehydrodeoxynupharidine, 8. Generation of the 6-(3-furanyl)-piperidine skeleton was achieved through oxidation. Sodium dichromate in acetic acid-acetone at 0° for two hours produced the formamido-ketone, 19, $[\alpha]_D^{25}$ -154.6(c 20 mg/cc ethanol), directly in 15% yield. A satisfactory analysis was obtained for $C_{15}H_{21}NO_3$. The ms gave a parent peak at m/e 263 and prominent peaks at 234 and 192 which





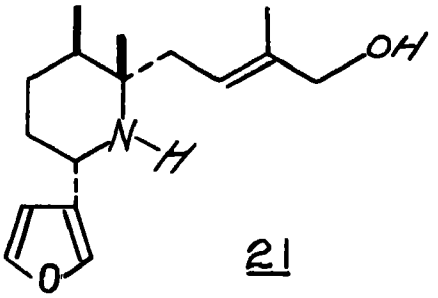
corresponded to the loss of CHO and the four carbon keto side chain. The ir showed carbonyl bands at 5.83 (keto) and 6.03μ (amido), methyl group bands at 7.29 and 7.38μ and furan peaks at 6.66 and 11.45μ. The nmr revealed a doublet methyl at 1.06δ, a keto singlet methyl at 2.01δ, broad triplet pairs centered at 3.18 and 4.15δ and a pair of broad multiplets at 4.7 and 5.55δ. These pairs of triplets and broad multiplets were assigned to C_1 and C_6 protons of 19 respectively. Also observed were a pair of β-furanyl protons at 6.3 and 6.42δ, two α-furanyl protons at 7.37δ and a pair of formyl protons at 8.02 and 8.6δ. The four signals appearing as pairs were attributed to restricted rotation of the formyl groups and the consequent differences in magnetic environments of the four different protons. The restricted rotation of amides is well known (23) and has been encountered previously for acylated piperidines (24).

Other methods of oxidative cleavage of the enamine double bond were explored. Air oxidation in the presence of cuprous chloride at room temperature produced a complex mixture containing only a small amount of the formamido-ketone as evidenced by the relative intensity of 5.8 and 6.0μ bands in the ir. A two step oxidation sequence was explored. Osmium tetroxide oxidation of the Δ^6-enamine was carried out in ether solution at 25°. The osmate ester was decomposed with mannitol. Elution chromatography produced the diol 18. The ir of this material showed strong OH absorption at 2.87μ, furan bands at 6.7 and 11.48μ but no Bohlmann bands in the region of 3.6μ. The nmr revealed furan signals at 7.35 and 6.36δ, a methyl doublet at 0.98δ, a methyl singlet at 1.06δ and a C_4-H quartet at 3.67δ. In addition the nmr showed an OH doublet (J=5.3Hz) at 2.37δ and an OH singlet at 3.08δ, both of which disappeared on addition of D_2O. Corresponding to the OH doublet was an α-proton doublet (J=5.3Hz) at 4.07δ which was reduced to a singlet on addition of D_2O. Cleavage of the diol to the formamido-ketone, 19, was achieved with sodium metaperiodate in aqueous tert-butyl alcohol at 25°. The overall yield from Δ^6-enamine was 38%. An attempt was made to obtain the diol without using expensive osmium tetroxide. Treatment of the Δ^6-enamine with m-chloroperbenzoic acid at room temperature in chloroform solution produced a crude product mixture whose ir and nmr showed no furan signals. In another attempt, rhuthenium tetroxide in carbon tetrachloride solution at 0° produced material whose nmr showed deminished furan signals. Since it appeared that the furan ring had been oxidized in both rhuthenium tetroxide and peroxide procedures, further attempts along these lines were abandoned. Finally, a quantitative conversion of enamine to formamido-ketone was achieved in one step with osmium tetroxide, in small amounts, and sodium metaperiodate in dioxane containing a few drops of pyridine.

The conversion of the formamido-ketone to nupharamine, 20, was accomplished in one step with a large excess of methyl magnesium iodide. Purification of the product mixture by elution chromatography gave two fractions of pure nupharamine, $[\alpha]_D^{25}$ - 39.5°(c 18 mg/cc, ethanol), which represented a 50% conversion. Other fractions also contained nupharamine. The ms of the synthetic material gave a parent peak at m/e 251. The ir and nmr were identical with the spectra of an authentic sample of nupharamine generated from the perchlorate salt of nupharamine. The latter was kindly provided by Dr. I. Kawasaki, of Osaka University, who had isolated nupharamine from N. japonicum. The synthetic nupharamine was converted to its perchlorate salt: mp 163-166°, mixture mp 163-166°.

Attempted Conversion of Nupharidine to Nuphamine.

Presently, conversion of quinolizidine to piperidine type nuphar alkaloids through the employment of the Δ^6-enamine, and its oxidized transformation products, cannot be claimed as a general procedure. While conversion of nupharidine to nupharamine was achieved in over-all good yield, extending the use of the enamine, 8, diol 18, the formamido-ketone 19 or the further elaboration of the isopentyl side chain skeleton of nupharamine has met only with failure in preparing nuphamine, 21.



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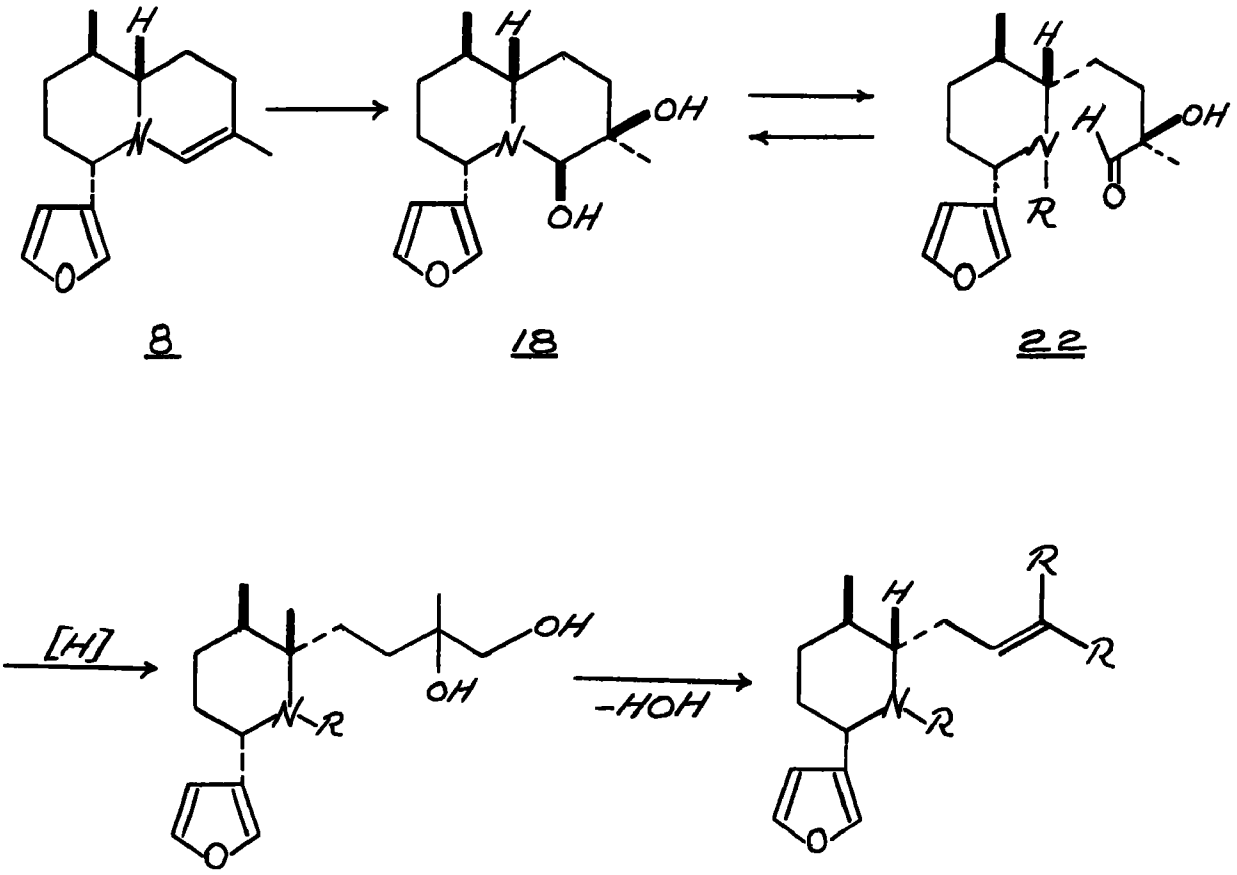
Initially, three schemes involving the use of diol 18, formamido-ketone 19 and nupharamine, 20, were contemplated. In time, there appeared a report (25) which described the conversion of enamines to α-acetoxy ketones with thallium acetate. This report lead us to consider the direct use of the Δ^6-dehydrodeoxy-nupharidine, 8.

A synthesis starting with the formamido-ketone has the advantage that the secondary nitrogen is already protected by the formamido group. However, a carbon atom must be added in order to complete the isopentyl side chain and coincidentally allow the future development of primary allyl alcohol. The use of the diol as a starting point has the advantages of possessing all the requisite number of carbon atoms in elevated oxidation states in the right location for subsequent conversion to the primary allyl alcohol side chain. Another appealing feature was a synthesis starting from enamine and proceeding through oxidation to diol, 18, hydrolysis of the resulting carbinol amine function, reduction of the aldehyde form - 22, and finally dehydration seemed closer to a possible biogenetic transformation of quinolizidine to piperidine type nuphar alkaloids.



8 18 22

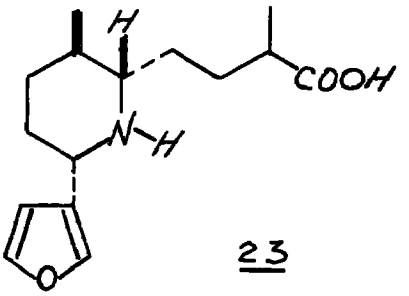
The thallium acetate oxidation of the Δ^6-enamine seemed like a very direct route to an appropriately functionalized isopentyl side chain. However, expections for success were not high since the oxidation procedure does not seem to be generally applicable to all enamines but rather only those which are substituted at the α-position.

The use of nupharamine in theory seemed straightforward. Protection of piperidine nitrogen, dehydration and allylic oxidation at the vinyl methyl group were envisioned as the three basic steps.

Work in our laboratory has proceeded along all four of the principal routes discussed above. The results are treated separately in each of the sub-sections below.

Starting from Diol 18

Although the route from the diol 18 seemed for a number of reasons the most appealing, work proceeding along these lines was met with the earliest failures. Attempts to detect any of the aldehyde form in equilibrium with the carbinol amine form were carried out in a number of solvents of varying polarity. In no case could an aldehydic proton be found in the nmr nor an aldehyde band observed in the ir. Also, oxidation of the carbinol amine 18 with chromic anhydride in the presence of pyridine gave not the desired carboxylic acid, 23.but the formamido-ketone 19. Similar results have already been described



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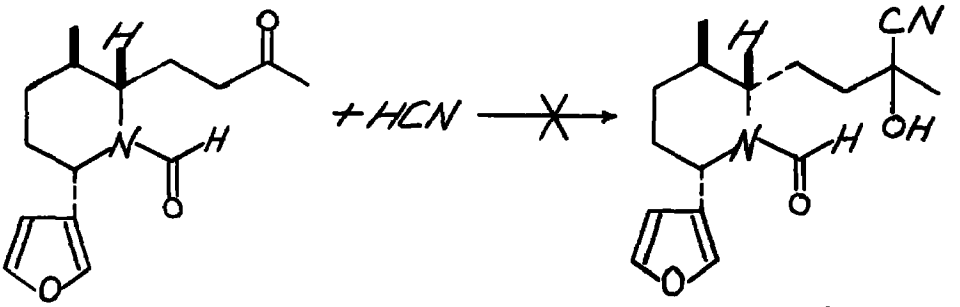
elsewhere in this report for the chromic anhydride oxidation in acid solution.

Starting with Formamido-Ketone 19

Cyanohydrin Formation and Dehydration.

Generation of the isopentyl side chain containing the requisite carbons in an elevated oxidative state was attempted through HCN addition to the formamido-ketone, 19.



19 24

Conditions listed below were employed:

1) Acetone cyanohydrin, neat or in ethanol solution for 15 hr at 25° with and without added ammonia.

2) Acetone cyanohydrin in refluxing methanol for 4 hr.

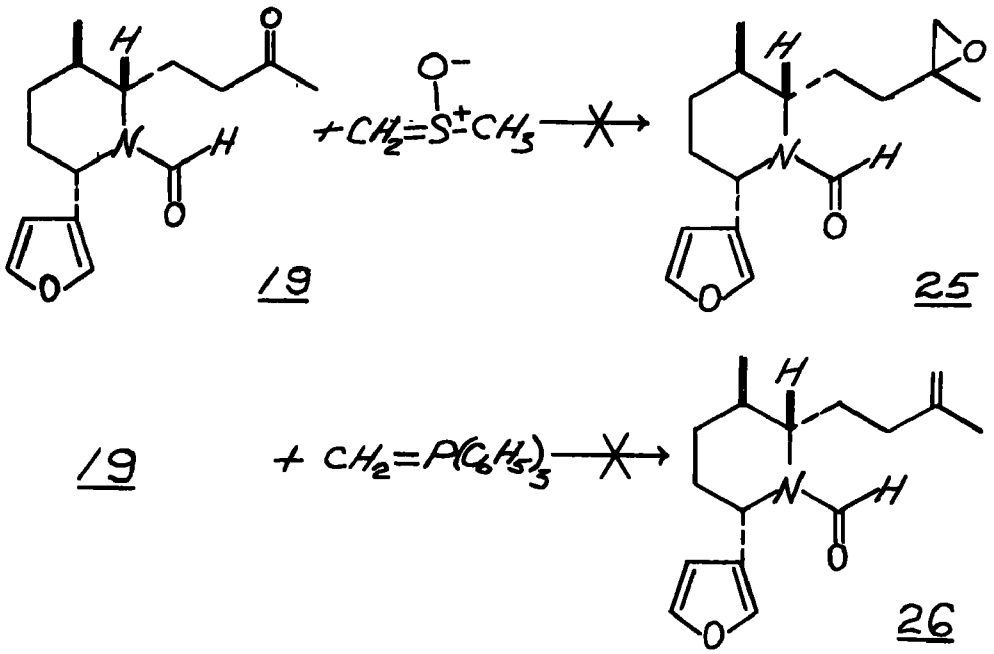
3) Acetone cyanohydrin in diethylamine solution at 25° for 15 hr.

4) Sodium cyanide in sulfuric acid ethanol at 25° for 24 hrs.

In no case could the transformation of formamido-ketone to cyanohydrin, be detected. Starting formamido-ketone could be recovered in nearly quantitative amounts.

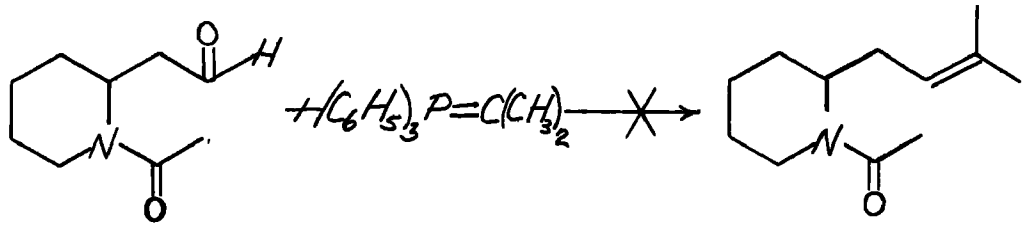
Carbon framework extension by ylide addition.

The formation of an epoxide at the side chain, as depicted below, was attempted by treating the formamido-ketone with the ylide generated from trimethylsulfoxonium iodide. Two products were obtained, each in



15% yield. One of them contained neither ketone nor furan ring as evidenced by the ir. The other contained a furan ring but no ketone group and was suspected as the desired epoxide 25. However, insufficient quantity prevented further studies necessary to confirm the structure. Since the yield of the desired epoxide was poor at best, and there was indication that the furan ring as well as the ketone group reacted with the ylide, the search for better conditions was not attempted. Instead, another Wittig extention reaction was tried. The formamido-ketone was treated with triphenylphosphonium methylide in refluxing ether solution for 10 hr in an attempt to prepare 26. Only unconverted formamido-ketone was obtained.

It is clear from the observations described above that the carbonyl of the 3-ketobutyl side chain is extremely inert to nucleophiles, even the highly nucleophilic dimethylsulfoxonium methylide. In line with this lack of reactivity is the finding that the treatment of N-acetyl-2-[2-ethanal]-piperidine, 27, with triphenylphosphonium isopropylide produced no Wittig product.



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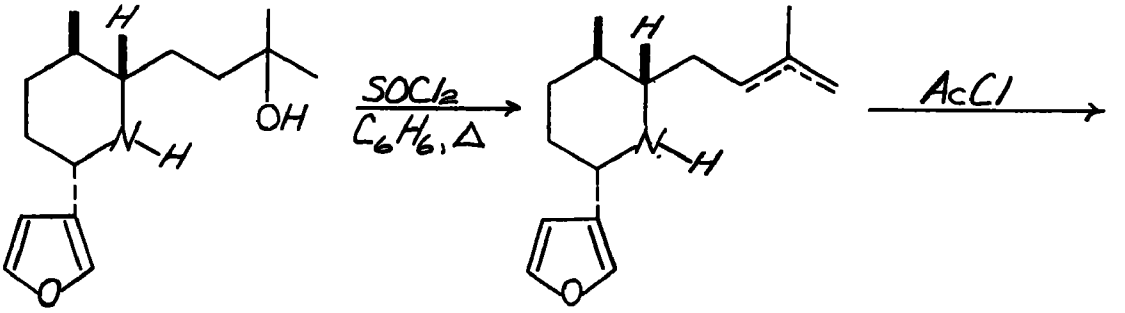
Thallium acetate oxidation of Δ^6-dehydrodeoxynupharidine.

Treatment of Δ^6-dehydrodeoxynupharidine, 8, with thallium acetate in acetic acid solution at room temperature afforded two products. The first gave an ir which showed no carbonyl, olefinic double bonds-other than furan, or hydroxyl group. The ms showed principal peaks at m/e 478 (M^+), 463, 450, 435, 313, 272, 231, 232 which suggested the material possessed a deoxynupharidine dimer structure.

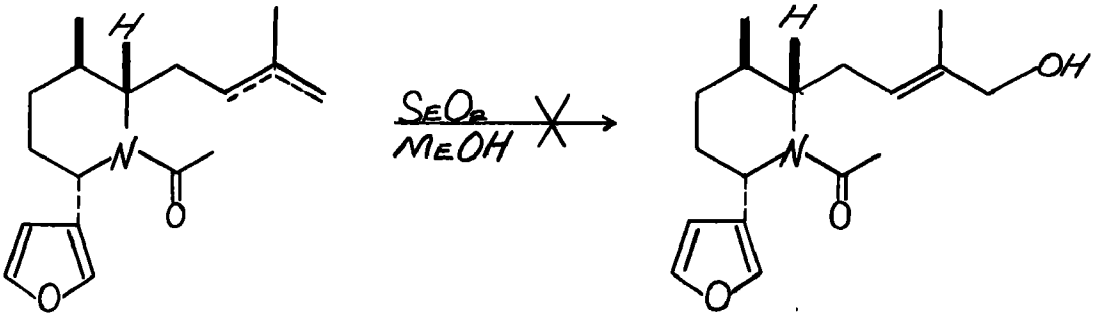
The second product gave an ir which showed a carbonyl band and an ms having M^+ at m/e 230. These products are under continued investigation at this time.

Elaboration of the isopentyl side chain of nupharamine.

Nupharamine, 20, was treated with thionyl chloride in refluxing benzene according to the procedure of Arata (26). Although Arata reports only the formation of a γ, γ -dimethylallyl side chain, the product obtained in our laboratory was a mixture of olefins, 28, as evidenced by the nmr.

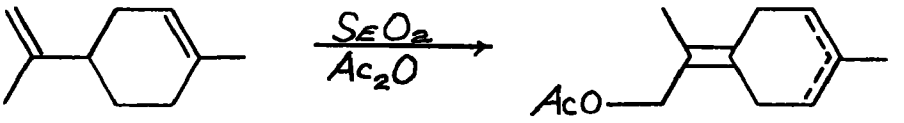


20 28



29 10,N-Ac

The olefin mixture, 28, was acetylated with acetyl chloride in triethylamine to give the acetamide 29 which in turn was treated with selenium dioxide in refluxing methanol for 3 hr. Regardless of the location of the double bond in the side chain of 29, a single allyl alcohol was expected on the basis that selenium dioxide of dipentene, 30, in acetic anhydride at 50° gave the single allyl acetate 31. The ir of the product mixture obtained from the amide derivative of the olefin mixture 29 displayed a hydroxyl band at 2.8μ, a single amide carbonyl band at 6.1μ, and bands at 6.67 and 11.45μ. The tlc



30 31

showed the product mixture to contain four components, no one of which corresponded to the R_f value of N-acetyl nuphamine. The authentic sample of N-acetyl nuphamine was prepared from nuphamine isolated from N. luteum subsp. variegatum.

Antibacterial Properties of WPC 45.1, VPM 74.15 and CFW 225.1

The cumulative results of the inhibition studies are given in Table 5. Specific information about the degree of sensitivity is presented in Tables 6 and 7. The sensitivities of the selected phytopathogens to alkaloids WPC 45.1, VPM 74.15 and CFW 225.1 as determined by the disc plate test are given in Table 6. The sensitivities of C. michiganense and Arthrobacter (TH-1) to WPC 45.1 as determined by the serial dilution-turbidimetric method are given in Table 7. Since the available quantity of pure VPM 74.15 was small, inhibition studies with this alkaloid were carried out on only a limited scale.

Although C. michiganense was particularily sensitive to the sulfur-bearing alkaloids VFW 225.1, WPC 45.1 and VPM 74.15, this bacteria was insensitive to the non-sulfur, C_{15}-alkaloids deoxynupharidine and nupharidine. The lack of activity by the N-oxide alkaloid nupharidine was somewhat surprising since a number of synthetic N-oxides have been employed as bactericides. Possibly screening nupharidine against a broad spectrum of bacteria would show some selective sensitivity.

Section 4

CONCLUSIONS

Two sulfur-containing C_{30}-alkaloids have been isolated in pure form from the rhizomes of Nuphar luteum subsp. macrophyllum by using a modified procedure of the Russian workers (1). The same two alkaloids are present in Nuphar luteum subsp. variegatum. One of these alkaloids is CFW 225.1, the free biscarbinolamine form of the diethyl ether WPC 45.1 which was first isolated by using the Russian procedure precisely as reported.

Alkaloid WPC 45.1 was active against four of six phytopathogenic bacteria. The second alkaloid was VPM 75.15 which was about one-half as active against the same sensitive bacteria. Three other unidentified bacteria were obtained from the region where N. luteum subsp. variegatum was harvested; these bacteria were insensitive to WPC 45.1. While the sulfur-containing C_{30}-alkaloids were active against some phytopathogens, two C_{15} alkaloids, nupharidine and deoxynupharidine, were not. Thus not all alkaloids of Nuphar are active and not all all bacteria tested are sensitive.

Studies directed toward the structure of the two alkaloids showed that both contain the thiaspirane, deoxynupharidine structural framework of structure 1. Reduction of both alkaloids produced the dehydroxylated counterparts, that from WPC 45.1 has tentatively been assigned stereostructure 4. The dehydroxylated counterpart of VPM 75.15 has been assigned structure 5. These assignments are based on the stereochemistry of sodium borodeuteride reduction of VPM 75.15 and WPC 45.1 and n.m.r. results. The stereostructures also assume that both deoxynupharidine moieties are the same and belong to the

Table 5

Cumulative Results of Inhibition. Bacteria *vs* Nuphar Alkaloids

Bacteria	WPC 45.1	VPM 74.15	CFW 225.1	Deoxynupharidine	Nupharidine
Corynebacterium michiganense	pos.	pos.	pos.	neg.	neg.
C. flaccumfaciens	pos.		pos.		
C. insidiosum	pos.		pos.		
Xanthomonas phaseoli	pos.		pos.		
X. vesicatoria	neg.				
Erwinia caratovora	neg.				
Arthrobacter (TH-1)	pos.				
Green Lake Tully #1	neg.			neg.	neg.
Green Lake Tully #4	neg.			neg.	neg.
Green Lake Tully #5	neg.			neg.	neg.

Table 6

Results of Disc Plate Tests. Total Diameter[a] of Zone Inhibition in cm for the Weight in μg Given for WPC 45.1, VPM 74.15 and CFW 225.1

	WPC 45.1		VPM 74.15		CFW 225.1		
Bacteria	1 μg	10 μg	1 μg	10 μg	3 μg	4 μg	10 μg
Corynebacterium michiganese	1.6	3	1.4	2.2		0.0	2.2
C. flaccumfaciens		3			1.6		2.8
C. insidiosum		3			0.0		1.8
X. phaseoli		2.5			0.0		2.1-2.3

[a] The diameter of the disc employed was 1.25 cm. The zone inhibition diameter is the total diameter.

Table 7

Results of Serial Dilution-Turbidimetric Tests Given in "Klett" Units of Transmission. Inhibition by WPC 45.1.

	Concentration of WPC 45.1 in μg/ml							
Bacteria	200	20	10	8	5	2	1	Blank*
C. michiganense	11	11	11	205	315	325	410	420
	11	13	32	22	310	56	435	450
	12	12	42	40	76	315		360
	11	11	37	40	240	280		450
Arthrobacter (TH-1)	34	84	450			520		510
	22	86	72			495		500

same enantiomeric series as deoxynupharidine, the absolute configuration of which was determined as a part of this study.

Work with the C_{15} class of Nuphar alkaloids resulted in the discovery of 7-epideoxynupharidine and 3-epinuphamine, both new alkaloids. The absolute configuration of deoxynupharidine was established and a method of converting quinolizidine to piperidine type C_{15} alkaloids was found.

Section 5

RECOMMENDATIONS

The antibacterial activity of Nuphar now can be linked to the presence of sulfur-containing alkaloids. Therefore, it would be desirable to extend the antibacterial studies to a greater range of bacteria, especially a far greater number of those present in the soil and water in which Nuphar grow. It would also be desirable to determine the activity of the alkaloids against other aquatic protista, the most important of which would seem to be fungi.

The release of the antibacterial alkaloids by the plants into water in control tanks should be studied. In connection with this area of investigation, methods for determining the alkaloid concentrations in water must be found. Once these analytical problems have been solved and the water content of plant-produced alkaloid has been determined, then the biological significance of growing Nuphar should

be established.

Within the area of chemistry, the most important problem remaining is the determination of the stereochemistry of alkaloids WPC 45.1 and VPM 74.15.

Section 6

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Section 7

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Section 8

LIST OF PUBLICATIONS

1. "The Application of Polonovsky Reaction Conditions to Nupharidine." E. Auer and R. T. LaLonde, Abstr. of Papers, 157th Meeting of the American Chemical Society, Minneapolis, Minn., April 1969, ORGN 150.

2. "The Stereochemistry of (-)-Deoxynupharidine. The Synthesis of (-)-(R)-α-Methyladipic Acid," C. F. Wong, E. Auer and R. T. LaLonde, J. Org. Chem., 35, 517 (1970).

3. "7-Epideoxynupharidine from Nuphar luteum subsp. Variegatum," C. F. Wong and R. T. LaLonde, Phytochem., 9, 659 (1970).

4. "The Structure of 3-Epinuphamine, A New Alkaloid from Nuphar luteum subsp. Variegatum," C. F. Wong and R. T. LaLonde, Phytochem., in press.

5. "Sesquiterpenic Alkaloids of Nuphar luteum subsp. Variegatum," C. F. Wong and R. T. LaLonde, Phytochem., in press.

Section 9

GLOSSARY OF ABBREVIATIONS AND SYMBOLS

br m	broad multiplet nuclear magnetic resonance signal
br d	broad doublet nuclear magnetic resonance signal
c	concentration
Cc	column chromatography
cc	cubic centimers
C_2	symmetry designation, a two fold axis of symmetry
CFW 225.1	free biscarbinolamine form of the diether biscarbinolamine WPC 45.1; see WPC 45.1
$CHCl_3$	chloroform
CH_2Cl_2	methylene chloride
d	doublet
eV	electron Volt
Hz	magnetic field strength in Hertz
ir	infrared spectrum (spectra)
J	coupling constant
m	a multiplet nuclear magnetic resonance signal
M^+	molecular ion or parent peak in the mass spectrum
m/e	mass divided by charge
mg	milligrams
mHz	magnetic field strength in mega Hertz
mp	melting point
ms	mass spectrum (spectra)

MeOH	methanol
nm	wavelength in nanometers
nmr	nuclear magnetic resonance spectrum (spectra)
NTBN	neothiobinupharidine
q	a quartet nuclear magnetic resonance signal
(R)	absolute configuration designation
R_f	chromatographic retention
s	singlet
subsp.	subspecies
t	a triplet nuclear magnetic resonance signal
tlc	thin layer chromatography
TMS	tetramethylsilane
uv	ultraviolet spectrum (spectra)
VPM 74.15	one of two pure alkaloids isolated from N. luteum subsp. variegatum
WPC 45.1	one of two pure alkaloids isolated from N. luteum subsp. variegatum
$[\alpha]_D^t$	specific optical rotation measured at temperature t and at the sodium D line
δ	see τ
$\Delta\nu$	frequency difference, but magnetic field strength difference when applied to nmr.
ϵ	molecular extinction coefficient
λmax	wavelength corresponding to the ultraviolet absorption maximum
μ	wavelength in microns
μg	micrograms
σ	symmetry designation, a plane of symmetry

τ	$10-\delta$, where δ = [(H-sample—H-reference)/ H-reference] x 10^6, H-sample is the magnetic field strength for resonance of any given proton in the sample and H-reference is the magnetic field strength for resonance of a reference proton
(-)	levorotatory optical rotation