Biosynthesis of Deuterated Riboflavin: Structure Determination by NMR and Mass Spectrometry

PAUL L. PLUTA *, HENRY L. CRESPI ˣ, MICHAEL KLEIN ‡, MARTIN I. BLAKE §, MARTIN H. STUDIER, and JOSEPH J. KATZ

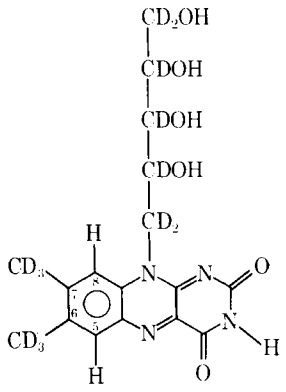
Abstract □ The riboflavin-producing fungus *Eremothecium ashbyii* was cultured in various growth media containing high concentrations of deuterium, and the product was isolated. The structures of highly deuterated riboflavin, in which at least 13 of 15 nonexchangeable hydrogens were replaced by deuterium, and fully deuterated riboflavin, in which all 15 nonexchangeable sites contained deuterium, were established by NMR and mass spectrometry. The aromatic protons (C-5 and C-8) were partially substituted in the highly deuterated molecule. Information regarding three areas of the biosynthetic pathway within the microorganism was obtained as a result of the formation of these compounds. Extensive solvent interaction, possibly due to passage of sugar through the transaldolase–transketolase pathway, occurs during formation of the ribityl chain. Limited solvent participation takes place during formation of 6,7-dimethyl-8-ribityllumazine, the immediate precursor of riboflavin. Deuteration of the riboflavin C-6 and C-7 methyl groups indicates significant solvent exchange during the final step of the biosynthetic process.

Keyphrases □ Riboflavin—deuterated, biosynthesis by fungus *Eremothecium ashbyii*, isolation, structure determination by NMR and mass spectrometry □ Biosynthesis—deuterated riboflavin by fungus *Eremothecium ashbyii* □ NMR—structure elucidation of biosynthesized deuterated riboflavin □ Mass spectrometry—structure elucidation of biosynthesized deuterated riboflavin

Deuterated drugs have proven useful in the study and measurement of isotope effects (1, 2), the elucidation of metabolic and biosynthetic pathways (3, 4), the determination of pharmacological mechanisms of drug action (5, 6), and other experimental investigations (7).

The biochemical reactions within various microorganisms that lead to the production of riboflavin have been the subject of extensive research for many years. The pathway has been almost completely determined and was reviewed (8–12). The final reaction of the biosynthetic process also was explained recently (13–16).

The production and isolation of deuterated riboflavin are reported in this study. Proof of deuterium incorporation at specific molecular sites by means of NMR and mass spectrometry is presented, and the implications of these results on the biosynthetic pathway of riboflavin are considered.



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EXPERIMENTAL

Production and Isolation of Deuterated Riboflavin—The medium used for the *Eremothecium ashbyii*[1] culture in deuterium oxide is listed in Table I. Production cultures contained 1 liter of medium in cotton-plugged 2-liter flasks. All cultures were grown on a rotary shaker[2] at 120 rpm under subdued light at 30°.

Maximum riboflavin production with this organism was obtained in 30 days under these conditions. According to Mohammed *et al.* (17), atmospheric dilution of medium deuterium is less than 10% during this period. Riboflavin was measured[3] by the USP method (18).

The preparative method used here is a combination of methods reported earlier (19–21). Column chromatography was followed by solvent extraction. The purification procedure was monitored by optical spectrometry. Purity of the final product was established by TLC (22), NMR, and mass spectrometry.

NMR Spectroscopy (^{1}HMR)—^{1}HMR spectra were obtained by use of a spectrometer[4] modified for pulse operation. A 5-mg sample was dissolved in 1 ml of solution containing equal parts of deuterated trifluoroacetic acid and trifluoroacetic acid anhydride. Hexamethyldisiloxane was added for internal standardization. Chemical shifts are reported in delta, δ, parts per million downfield from hexamethyldisiloxane.

Mass Spectrometry[5]—Samples were dissolved in ethanol for application to a platinum ribbon, which was placed directly into the ionization source and heated electrically.

RESULTS AND DISCUSSION

Structure Determination—Evidence of deuterium participation during biosynthesis was obtained by a spectral comparison of isolated biosynthesized deuterated riboflavin with known riboflavin. The inability of deuterium to produce a ^{1}HMR signal makes this technique ideal for the determination of deuterium incorporation. The capability of making multiple comparisons of parent and fragment masses makes mass spectrometry equally valuable, especially when high sensitivity is required.

Consideration of deuterium participation will be limited to nonexchangeable (carbon-bound) hydrogen sites only. Of the 20 hydrogen sites in the molecular structure of riboflavin, 15 are of this type. The other five sites are bound to oxygen or nitrogen and are, therefore, exchangeable. Since the last solvent contact was with large volumes of water during purification, these sites contained ^{1}H at the conclusion of the isolation procedure. All further references to the hydrogen sites of riboflavin are to nonexchangeable sites.

Figure 1 shows the ^{1}HMR spectrum of riboflavin. The use of tri-

[1] *Eremothecium ashbyii* (NRRL Y 136) was obtained from the U.S. Department of Agriculture, Northern Regional Research Laboratory, Peoria, Ill.

[2] Eberbach Corp., Ann Arbor, Mich.

[3] Hitachi Perkin-Elmer model MPF 2A fluorescence spectrometer.

[4] Varian HR 220 spectrometer.

[5] Mass spectra were obtained with a modified Bendix Time-of-Flight model 12 mass spectrometer (23).

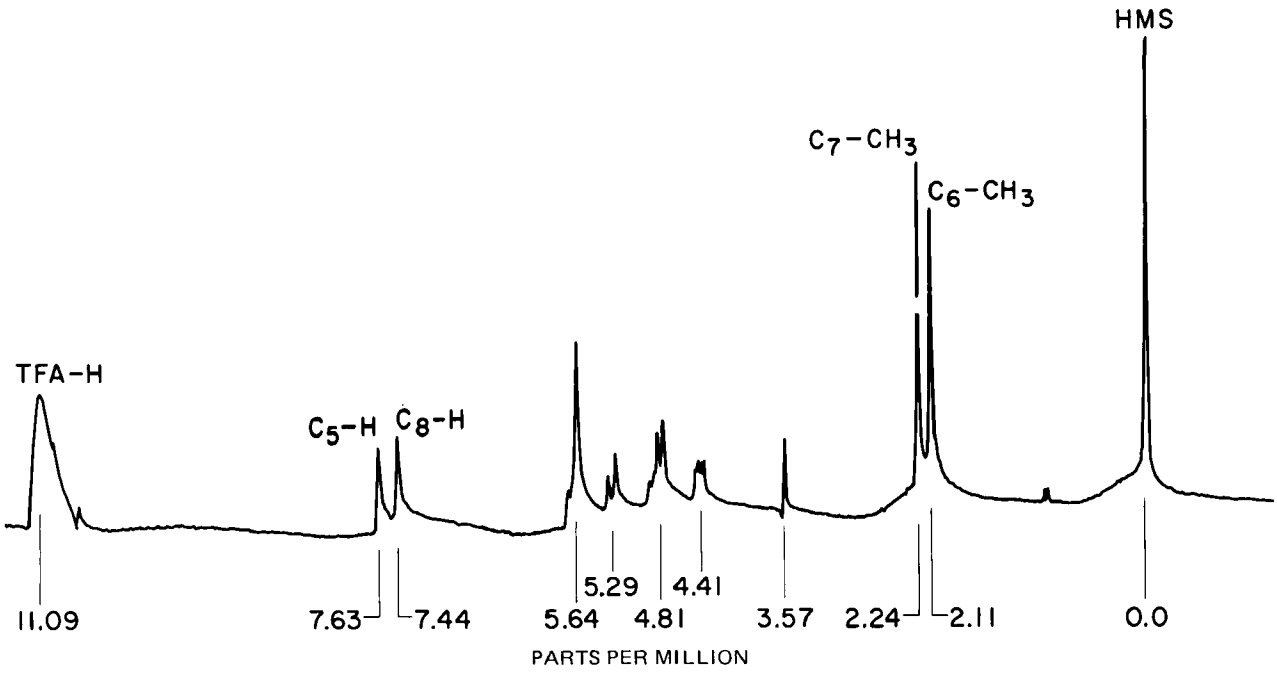

Figure 1—*^{1}HMR spectrum of riboflavin.*

fluoroacetic acid anhydride along with deuterated trifluoroacetic acid as the solvent enabled the aromatic protons of riboflavin to be resolved completely. Proton assignments are analogous to those of other investigators for riboflavin (13) and riboflavin phosphate (24–26). The high sensitivity of the instrument used, attained by operating in pulse mode with computer assistance, enabled procurement of spectra with a minimum of material.

Table II lists the respective masses and relative abundances of fragments in the first reported mass spectrum of riboflavin. The decomposition properties of this compound, which heretofore prevented obtaining its mass spectrum, were overcome by the inlet system of the instrument. A solution of the sample was applied to a thin metal ribbon, which was inserted directly into the ionizing region of the spectrometer. Passage of a variable electric current through this filament enabled heating to be controlled exactly. As the temperature was increased slowly, vaporized samples were ionized by electron impact prior to major decomposition; mass spectra of components of the sample were obtained sequentially.

Highly Deuterated Riboflavin—The product isolated from cultures grown in the medium listed in Table I was composed of a mixture of compounds containing a minimum of 13 nonexchangeable deuterium atoms and a maximum of two nonexchangeable aromatic hydrogen atoms at C-5 and C-8. The ^{1}HMR spectrum of this compound showed only a singlet peak at 7.50 ppm, indicating the presence of hydrogen in the aromatic region of the molecule and the presence of deuterium in both methyl and ribityl regions.

The chemical shift of this peak was between those of the known aromatic peaks of riboflavin, and the peak height suggests the presence of two protons. However, without a quantitative proton reference within the molecule, a more specific determination of the extent of deuterium incorporation was not possible. Resolution of this peak was not realized, apparently due to the isotope effect related to deuteration in the methyl groups (27). The congruence of spectra of known riboflavin in protio and deuterated solvent systems showed that exchange of protons with deuterium did not occur while the ^{1}HMR spectra were being obtained.

Comparison of the mass spectrum of highly deuterated riboflavin with that of riboflavin USP (Fig. 2) substantiates the ^{1}HMR findings by indicating that at least 13 deuterium atoms participated in biosynthesis, the only remaining hydrogens being located in the isoalloxazine ring. The conclusions are based on the following major peak analyses:

1. 389 (*versus* 376), parent peak: difference of 13 amu indicates 13 of 15 possible deuterium atoms in the molecule.
2. 370 (*versus* 358), loss of HDO (*versus* loss of H_2O): 12 of 14 possible D in fragment.
3. 356 (*versus* 345), loss of CD_2OH (*versus* loss of CH_2OH): 11 of 13 possible D in fragment.
4. 325 (*versus* 315), loss of CDOH—CD_2OH (*versus* loss of CHOH—CH_2OH): 10 of 12 possible D in fragment.
5. 294 (*versus* 285), loss of $(CDOH)_2$—CD_2OH [*versus* loss of $(CHOH)_2$—CH_2OH]: nine of 11 possible D in fragment.
6. 262 (*versus* 256), loss of $(CDOH)_3$—CD_2OH and H/D transfer [*versus* loss of $(CHOH)_3$—CH_2OH and H transfer]: eight of 10 possible D in fragment.
7. 249 (*versus* 243), loss of CD_2—$(CDOH)_3$—CD_2OH and H/D transfer [*versus* loss of CH_2—$(CHOH)_3$—CH_2OH and H transfer]: six of eight possible D in fragment.
8. 219 (*versus* 213), loss of CD_2—$(CDOH)_3$—CD_2OH and CO [*versus* loss of CH_2—$(CHOH)_3$—CH_2OH and CO]: six of eight possible D in fragment.
9. 191 (*versus* 185), loss of CD_2—$(CDOH)_3$—CD_2OH and N—CO—NH and H transfer [*versus* loss of CH_2—$(CHOH)_3$—CH_2OH and N—CO—NH and H transfer]: six of eight possible D in fragment.
10. 177 (*versus* 171), loss of CD_2—$(CDOH)_3$—CD_2OH and CO—NH—CO and H transfer [*versus* loss of CH_2—$(CHOH)_3$—CH_2OH and CO—NH—CO and H transfer]: six of eight possible D in fragment.

Table I—Deuterated Riboflavin Production Medium

Ingredient		Amount, g
Sucrose[a]		25.0
Potassium phosphate, dibasic		1.0
Potassium phosphate, monobasic		1.0
Sodium chloride		1.0
Magnesium sulfate heptahydrate		0.7
Ferrous sulfate heptahydrate		0.005
Inositol		0.03
Thiamine hydrochloride		0.0015
Biotin		0.7 μg
Boric acid		0.02
Manganese chloride tetrahydrate		0.02
Calcium chloride		0.5
Yeast extract		0.7
Casein (vitamin free)		0.5
Deuterium oxide	Dilute to	1000.0 ml

[a] Algal deuterated sugars were used for the production of fully deuterated riboflavin (33).

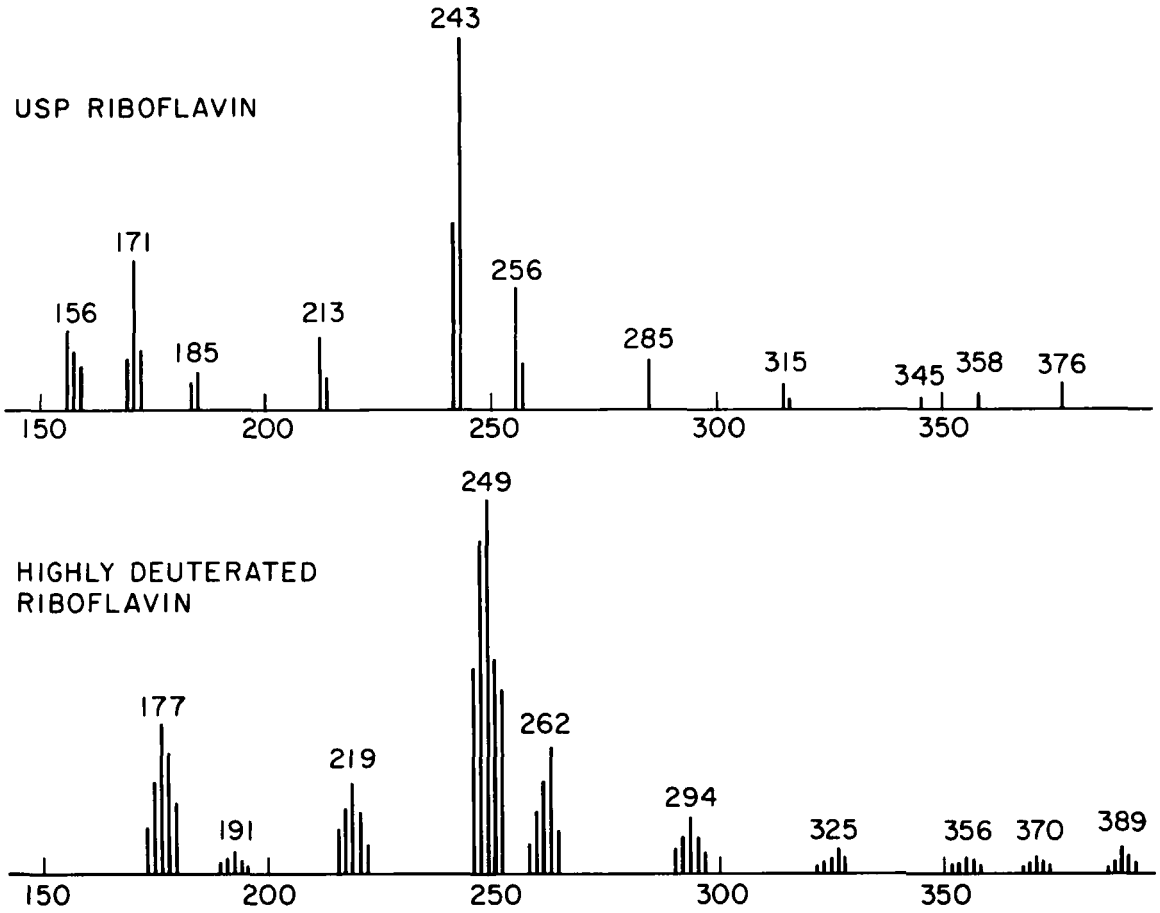

Figure 2—*Comparison of mass spectra of riboflavin USP and highly deuterated riboflavin.*

The range of fragments existing at each highly deuterated riboflavin mass number indicates the presence of minor amounts of compounds containing more than 13 deuterium atoms. This range may also exist due to hydrogen/deuterium transfer to double bonds in the isoalloxazine ring or to hydrogen/deuterium loss from carbon or nitrogen, two processes known to occur under the operating conditions of the spectrometer. The presence of compounds containing less than 13 deuterium atoms was shown not to occur by the previously reported ^{1}HMR data. Therefore, on the basis of data obtained from both spectral techniques, the major compound present appears to be one containing 13 nonexchangeable deuterium atoms in the methyl and ribityl regions and two nonexchangeable aromatic hydrogen atoms at C-5 and C-8 (I).

Fully Deuterated Riboflavin—The compound isolated from cultures grown on medium containing deuterium oxide and deuterated sugars was found to be deuterated in all 15 possible nonexchangeable molecular positions. A mass spectral comparison of major peaks at *m/e* 179, 221, 251, and 264 in this compound with corresponding fragments in ordinary and highly deuterated riboflavin indicates that, in contrast to highly deuterated riboflavin, all hydrogen sites in the isoalloxazine portion of the molecule have been replaced by deuterium and all seven nonexchangeable sites in the ribityl chain have been completely deuterium substituted.

Biosynthetic Implications—As previously mentioned, the biosynthetic pathway of riboflavin has been studied extensively. Most work involved the use of radioactive compounds, which traced the movement of carbon into the vitamin. The method applied in the present study focused on the movement of hydrogen and represents a new approach to the study of riboflavin biosynthesis. Reaction mechanisms in three areas of this pathway were considered in explaining the results.

Synthesis of Ribityl Chain—The exact biosynthetic mode of attachment of the ribityl chain to the riboflavin molecule is unknown, although it is known that the five-carbon moiety is attached by the time 6,7-dimethyl-8-ribityllumazine has been formed (9). Early studies showed that carbon dioxide, formate, glycine, and serine are not incorporated into the ribityl chain (28, 29). Plaut (30) and Plaut and Broberg (29) concluded that at least two carbohydrate pathways, the hexose monophosphate shunt and the transaldolase–transketolase pathway, are involved in the formation of the chain. The results of Ali and Al-Khalidi (31) corroborated this conclusion.

The results of the present study using protiosugars and solvent deuterium oxide, in which substitution occurred in all positions of the chain, suggest that under these experimental conditions the transaldolase–transketolase pathway is of major importance in the synthesis of this moiety. The presence of deuterium at all carbon–hydrogen positions indicates a significant interaction occurring with the solvent. No carbon–hydrogen bond was protected from exchange, as would occur if the hexose monophosphate shunt were involved without further reaction.

For example, if glucose 6-phosphate were metabolized to ribose 5-phosphate *via* this pathway and condensed with the purine, the protons of C-4, C-5, and C-6 of glucose would not be able to exchange with solvent deuterium. Consequently, their presence would be reflected by signals in the corresponding ^{1}HMR spectrum. Extensive interaction with solvent, as is possible in the transaldolase–transketolase pathway, must occur for deuterium to enter all seven nonexchangeable positions.

Synthesis of 6,7-Dimethyl-8-ribityllumazine—This compound, the immediate precursor of riboflavin, is synthesized by condensation of 4-ribitylamino-5-aminouracil with a four-carbon donor molecule. Although various compounds, including acetoin, butanedione, pyruvate, and glucose, have been suggested as the source of the four-carbon fragment, the true identity of this compound remains uncertain (12). The methyl protons of 6,7-dimethyl-8-ribityllumazine are supplied by the four-carbon donor; thereafter, they become the C-5 and C-8 aromatic protons or the C-6 and C-7 methyl protons of riboflavin.

Since the C-5 and C-8 aromatic protons of fully deuterated riboflavin are deuterium substituted when riboflavin is formed in a medium containing deuterium oxide and deuterated sugars and

Table II—Mass Spectrum of Riboflavin

m/e	Percent
55	10.2
56	11.0
57	10.1
60	6.1
61	7.4
69	5.1
70	6.5
71	4.8
74	8.2
77	10.2
78	6.3
91	7.6
116	8.1
117	4.9
130	7.3
131	5.8
143	16.5
144	15.0
145	14.1
156	20.6
157	13.3
158	10.7
170	14.4
171	41.6
172	16.1
184	6.2
185	7.9
213	18.8
214	8.3
242	50.0
243	100.0
256	33.9
257	9.9
285	12.1
315	5.7
316	2.8
345	3.4
358	4.4
376	6.3

are not extensively deuterated in a medium containing only deuterium oxide, it is concluded that these protons must arise directly from the sugar contained in the growth medium. Ali and Al-Khalidi (31) also indicated the likelihood of the sugar being the true four-carbon donor in studies using glucose and ^{14}C-acetate.

Furthermore, since the mass spectral data of the present study indicated the presence of only minor amounts of deuterium at C-5 and C-8 in highly deuterated riboflavin, it is concluded that the pathway from sugar to the lumazine is one in which these positions are protected from extensive solvent interaction. This lack of solvent interaction is a contrast to that observed in the synthesis of the ribityl chain. The conclusion (32) that the four-carbon unit arising from sugar does not pass through an acetate pool is thus confirmed, since the occurrence of pooling would have caused greater deuterium substitution than was actually observed.

Synthesis of Riboflavin—The mechanism in which two molecules of 6,7-dimethyl-8-ribityllumazine are condensed to form riboflavin was explained recently (13–16). In this reaction, the C-6 and C-7 methyl groups of one lumazine molecule (acceptor lumazine) condense with C-7 and C-6, respectively, of a second lumazine molecule (donor lumazine). Lumazine methyl protons thus become the C-5 and C-8 aromatic protons (from acceptor lumazine) and the C-6 and C-7 methyl protons (from donor lumazine) of riboflavin.

In highly deuterated riboflavin (I), the C-5 and C-8 aromatic protons are present as hydrogen and the C-6 and C-7 methyl hydrogens are present as deuterium. Thus, despite a common molecular origin, the hydrogens in these areas of highly deuterated riboflavin may be distinguished. In applying the known reaction mechanism to the formation of highly deuterated riboflavin, it is apparent that this molecule could only have been formed if the following reaction sequence had occurred. Initially, two protiolumazines interacted and the riboflavin C-7 to C-8 (donor lumazine C-6 to acceptor lumazine C-7 methyl) bond formed without extensive solvent interaction. After this bond formed, complete exchange of the riboflavin C-6 and C-7 methyl groups (donor lumazine C-6 and C-7 methyl groups) took place. The biosynthesis of riboflavin was then completed by the formation of the riboflavin C-5 to C-6 (donor lumazine C-7 to acceptor lumazine C-6 methyl) bond, the solvent again being excluded from any significant interaction.

The exclusion of solvent interaction during formation of the riboflavin C-5 to C-6 bond and the C-7 to C-8 bond is necessary to retain the presence of hydrogen at C-5 and C-8. The possibility that exchange of the riboflavin C-6 and C-7 methyl groups occurred after riboflavin synthesis was complete was also considered. An attempted exchange of known riboflavin at pH 5, the pH maintained in the production culture, was performed by treating riboflavin with deuterium oxide at sterilization conditions of 15 psig for 15 min, followed by five repeated additions and evaporations of deuterium oxide. ^{1}HMR data showed that no exchange occurred as a result of this treatment, thereby establishing that once riboflavin was fully synthesized in the culture medium, exchange of both methyl or ribityl groups with solvent deuterium oxide did not occur.

The following summarizes the proposed synthetic sequence. The presence of hydrogen at C-5 and C-8 of highly deuterated riboflavin indicated that this reaction sequence arose from two ^{1}H-lumazine molecules. The congruence of ^{1}HMR spectra of known and deuterium oxide-treated riboflavin indicated that deuterium exchange did not occur after the synthesis was completed. Therefore, it is concluded that incorporation of deuterium into the riboflavin C-6 and C-7 methyl groups occurred at an intermediate stage before total riboflavin synthesis was complete.

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* Argonne Universities Association–Argonne National Laboratory Fellowship appointee. Present address: Abbott Laboratories, North Chicago, Ill.

‡ Present address: U.S. Department of Justice, Drug Enforcement Administration Special Training and Research Laboratory, McLean, VA 22101

§ Present address: Department of Pharmacy, College of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60612

x To whom inquiries should be directed.

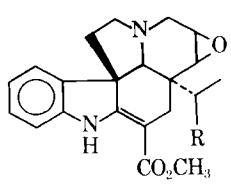
Catharanthus Alkaloids XXXII: Isolation of Alkaloids from *Catharanthus trichophyllus* Roots and Structure Elucidation of Cathaphylline

G. A. CORDELL and N. R. FARNSWORTH x

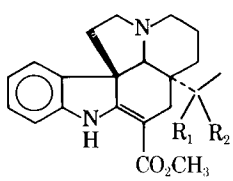
Abstract □ Further examination of the cytotoxic alkaloid fractions of *Catharanthus trichophyllus* roots afforded nine alkaloids. Two of these alkaloids, lochnericine and horhammericine, are responsible for part of the cytotoxic activity. The structure elucidation of cathaphylline, a new β-anilino acrylate derivative, is described.

Keyphrases □ *Catharanthus* alkaloids—isolation of nine alkaloids from *Catharanthus trichophyllus* roots, cytotoxic activity screened, structure elucidation of cathaphylline □ Alkaloids—isolated from *Catharanthus trichophyllus*, screened for cytotoxic activity □ Cathaphylline—isolated from *Catharanthus trichophyllus*, structure elucidated, cytotoxic activity screened □ Cytotoxicity—nine alkaloids isolated from *Catharanthus trichophyllus* screened

The cytotoxic and antitumor activities of the alkaloid fraction of *Catharanthus trichophyllus* were described previously (1, 2). However, the alkaloids isolated from this plant in those studies were devoid of antitumor and/or cytotoxic activity. The present phytochemical study dealt with the isolation of the two known alkaloids, lochnericine (I) and horhammericine (II), which were found to be cytotoxic. In addition, the isolation of several other bases from *C. trichophyllus* is reported, including a new indole alkaloid, cathaphylline, for which Structure X is proposed.



I: R = H
II: R = OH



III: R_1R_2 = O
IV: R_1 = H, R_2 = $OCOCH_3$
V: R_1 = H, R_2 = OH
IX: R_1 = H, R_2 = H

EXPERIMENTAL[1]

Plant Material—The coarsely milled, air-dried roots[2] of *C. trichophyllus* (Bak.) Pich. (Apocynaceae) used were collected in Madagascar during 1969.

Preparation of pH Gradient Fractions—Fractionation of the crude alkaloids from this plant and the pH gradient separation of the Extract N alkaloids were described previously (2).

Chromatographic Separation of N-3.0 Alkaloids—Fraction

[1] Melting points were determined using an American Optical Instrument Co. model 569 instrument and are uncorrected. Specific rotations were measured using a Carl Zeiss optical polarimeter (Merz Optical Instruments, Chicago, Ill.). UV spectra were recorded in ethanol using a Beckman model DB-G grating spectrophotometer. IR spectra were measured as potassium bromide pellets *versus* air with a Beckman model IR-18A spectrophotometer. Low-resolution mass spectra were recorded at 70 ev using a Hitachi Perkin-Elmer model RMU-6D mass spectrometer. High-resolution mass spectra were recorded using a double-focusing mass spectrometer, model 110 (Consolidated Electro Dynamics Co.), operating at 70 ev. NMR spectra were determined in deuterochloroform, with tetramethylsilane as the internal standard, using a Varian model T-60A spectrometer. Column chromatography was carried out using silica gel PF_{254} (E. Merck, Darmstadt, West Germany). Routine analytical TLC was carried out using silica gel PF_{254} on 0.25-mm layers (E. Merck), and preparative TLC was performed using silica gel PF_{254} on 2-mm layers. Resolved components were detected by quenching under 254-nm UV light and visualized with ceric ammonium sulfate reagent. Appropriate zones were removed from the plates and the components were purified by filtered elution with methanol and evaporation of the solvent *in vacuo*. The residue was taken up in chloroform or ethyl acetate and filtered, and the filtrate was evaporated *in vacuo*. Solvent systems used in this study were: A, 1-butanol–acetic acid–water (4:4:1); B, 1-butanol–acetic acid–water (10:1:1); C, benzene–triethylamine (9:1); D, ethyl acetate–absolute ethanol (3:1); and E, 1-butanol–acetic acid–water (10:10:1).

[2] Obtained from the Curran Corp., South Hackensack, N.J. Voucher specimens representing the collection are deposited in the Herbarium of the Department of Pharmacognosy and Pharmacology, College of Pharmacy, University of Illinois at the Medical Center, Chicago, Ill. The identification was confirmed by N. R. Farnsworth.