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CYANOGENESIS IN DERMATOCARPON MINIATUM (L.) MANN.

(TITLE)

BY

DANIEL LEE BERGMAN

B.S., Eastern Illinois University, 1986

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

Master of Science

IN THE GRADUATE SCHOOL, EASTERN ILLINOIS UNIVERSITY
CHARLESTON, ILLINOIS

1988
YEAR

I HEREBY RECOMMEND THIS THESIS BE ACCEPTED AS FULFILLING
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CYANOGENESIS IN DERMATOCARPON MINIATUM (L.) MANN.

by

Daniel Lee Bergman

B.S., Eastern Illinois University, 1986

ABSTRACT OF A THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science at the Graduate School of
Eastern Illinois University

CHARLESTON, ILLINOIS
1988

ABSTRACT

The lichen species Dermatocarpon miniatum (L.) Mann. was found to be cyanogenic. This umbilicate lichen, which is common on limestone and sandstone outcrops throughout the United States, is the first lichen to be reported as cyanogenic. This species were examined for cyanogenesis using population from within the confines of Kankakee River State Park, Kankakee and Will Counties, Illinois. Of the nine populations examined six of the population (testing 30 to 150 individuals in each population) were 100% strongly cyanogenic. In the three remaining population cyanogenesis ranged from 67% to 97%. In addition D. miniatum, two other lichen species also were found to be occasional cyanogenic. Both Dermatocarpon fluviatile (G. Web.) Th. Fr. and Usnea cavernosa Tuck. gave slightly positive tests for cyanide.

The isolation of the cyanogenic compound in D. miniatum was attempted. A cyanogenic extract from this species was purified using paper chromatography. This extract was analyzed using Nuclear Magnetic Resonance techniques (NMR). The results of this analysis shows a prominent sugar peak between 3.0-4.0 on the NMR spectrum which fits well with the idea that the compound responsible is a cyanogenic glycoside. A mass spectrum analysis was also attempted on the sample, but gave little information concerning the compounds molecular weight. This was probably due to impurities in the sample.

ACKNOWLEDGMENTS

The author would like to thank Dr. John E. Ebinger for his tireless support and motivation. Also, I would like to thank Dr. David S. Seigler, Plant Biology Department, University of Illinois, Urbana, Illinois for the use of his laboratory and his knowledge and support of this work. I would also like to express thanks to my mother and father for their continued support, and to Dr. Wesley Whiteside, Dr. Douglas Zimmerman, and Dr. Andrew Methven for serving on my graduate committee.

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INTRODUCTION

Many plants have the ability to synthesize compounds which liberating hydrogen cyanide gas (HCN) upon hydrolysis through a process known as cyanogenesis. These biochemical reactions have been recognized for more than a century, and are not restricted to a particular plant group, being reported in bacteria, fungi, ferns, fern-allies, gymnosperms and angiosperms. Cyanogenesis results from the enzymatic breakdown of cyanogenic glycosides or cyanolipids. The amount of HCN released depends on several intrinsic (genetics, plant parts, plant age, and sometimes sex) and extrinsic factors (climate, moisture supply, soil fertility, and freeze damage) (Kingsbury 1964). More than 30 different cyanogenic compounds have been isolated, but specific compounds have been reported from less than 200 plant species (Gibbs 1974, Seigler 1976, Tjon Sie Fat 1979).

A recent study (Bergman and Ebinger 1987) detected the release of HCN from specimens of *Dermatocarpon miniatum* (L.) Mann. The present study was undertaken to determine the extent of cyanogenesis in populations of *D. miniatum*, and to isolate the cyanogenic compound involved.

Dermatocarpon miniatum is an umbilicate lichen which is common throughout the United States on limestone and sandstone outcrops. The pale brown to whitish gray thallus is 2-5 cm broad with more or less wavy-convoluted margins; the white medulla often has black, flask-shaped perithecia embedded in it, which are responsible for the black dotted appearance on the upper surface of fertile specimens; the lower cortex is

deep brown to black; rhizines are lacking on the smooth to papillate lower surface except on the central umbilicus which attaches the thallus to the rock substrate.

MATERIALS AND METHODS

Numerous individuals from nine populations of Dermatocarpon miniatum were tested for the presence of HCN. Several additional species of Dermatocarpon, as well as other lichen taxa, from the E. L. Stover Herbarium (EIU), and from personal collections were also tested for HCN production.

Specimens were tested using the procedure developed by Feigl and Anger (1966) as modified by Tantisewie et al. (1969). Small amounts of lichen material (about 200 mg) is crushed, placed in a straight-sided vial, and moistened with distilled water. A strip of filter paper impregnated with copper ethylacetoacetate and tetra base (4,4'-tetramethyldiaminodiphenylmethane) is added to the vial without touching the sample, and held in place with a cork. The presence of HCN is indicated when the filter paper turns deep blue within 12 hours. A negative test indicates that the specimens does not contain a cyanogenic compound, or that it lacks the enzyme capable of hydrolyzing cyanogenic compounds, or both.

A quantitative determination of cyanide content was not made in this study. A quantitative indication of HCN production, however, can be made by observing the intensity of the color change in the filter paper (Dickenmann 1982). The reaction was considered weak if only part of the filter paper turned light blue, moderate if most of the paper turned a light to medium blue, and strong if the paper turned a deep blue

throughout. According to Dickenmann (1982), the weak reaction contains approximately 2-20 mg HCN per kg fresh weight, the moderate reaction produces 21-50 mg HCN per kg fresh weight, and the strong reaction produces more than 50 mg HCN per kg fresh weight.

Specimens of Dermatocarpon miniatum used for the population studies, and to extract and isolated the cyanogenic compound were collected from December 1986 to May 1988 from limestone outcrops at Kankakee River State Park, Kankakee and Will Counties, Illinois. Identifications were verified by Dr. Wesley C. Whiteside, of Eastern Illinois University, and voucher specimens deposited in the Eastern Illinois University Herbarium, Charleston, Illinois (EIU). Nomenclature follows Hale (1979).

The procedure used for the isolation and identification of the cyanogenic compound are included in the experimental part of this paper.

RESULTS AND DISCUSSION

Prior to the discovery of cyanogenesis in Dermatocarpon miniatum by Bergman and Ebinger (1987), there had been no reports of cyanogenesis in lichens. In contrast, cyanogenesis is known to be relatively common in fungi. Saupe (1981) who reviewed much of the early cyanogenic literature on fungi, reported that some fungi are probably cyanide accumulators, and that stable cyanogenic glycosides probably do not exist among these organisms. Initial studies indicated that a stable cyanogenic compound may exist in D. miniatum. Attempts were made to isolate and identify the compound and to determine the extent of cyanogenesis in several populations of D. miniatum (Table 1).

Cyanogenesis in other lichen taxa: In addition to Dermatocarpon miniatum, two other species of this genus were tested for HCN production. Dermatocarpon fluviatile (G. Web.) Th. Fr. was found to be weakly cyanogenic in two of the four specimens examined, while the one specimen of D. moulinsii (Mont.) Zahler tested negative for cyanogenesis. (Table 2). Of the 32 additional species of lichens tested only Usnea cavernosa Tuck. gave a weak positive test (Table 3). In this species, however, a 24 hour testing period was required before positive results were obtained.

Population Studies of Dermatocarpon miniatum: Nine populations of D. miniatum were tested for HCN production (Table 1) as were numerous specimens from the Eastern Illinois University Herbarium (Table 2). These tests indicate that D. miniatum is polymorphic for cyanide production. Of the herbarium specimens examined seven gave a strong positive test for HCN production, six gave a moderate test, four gave a weak test, and 2 tested negative.

In conducting the population studies of Dermatocarpon miniatum specimens were randomly collected from limestone cliffs and rocks at the Kankakee River State Park, Kankakee and Will Counties, Illinois (Figure 1). Both fertile and non-fertile forms were collected as well as crowded and isolated individuals, and individuals of different sizes and probably age. In six of the populations all of the individuals tested strongly positive for cyanide production (Table 1). In the other three HCN production varied from 67% to 97%, indicating that this species is polymorphic for cyanogenesis.

Cyanogenic glycoside in Dermatocarpon miniatum: At the present time the cyanogenic compound in Dermatocarpon miniatum has not been identified. The procedure that have been followed, and the results obtained are listed in the experimental section below.

EXPERIMENTAL

Plant Material: Samples of Dermatocarpon miniatum were collected from limestone outcrops in Kankakee River State Park, Kankakee County, Illinois (NE1/4 Sec 23 T31N R11E).

Enzyme Preparation: Samples of Dermatocarpon miniatum (25g) were ground in a blender several times with cold acetone. The suspension was then filtered and allowed to dry in a hood. The remaining solid material was resuspended in pH buffer 6.8 (PO_4) and stirred for 30 minutes and filtered. The filtrate was dialysed against pH 6.8 buffer for 48 hours. The product's hydrolytic activity was confirmed by testing isolated glycosides.

Extraction and Isolation: Fresh lichen material (126 g) was macerated in 800 mls of 50% methanol. The suspension was then filtered and dried to a thick liquid on a rotary evaporator. The extract was washed several times with equal volumes of chloroform to remove the lipid component. The chloroform extract was tested for cyanogenesis (negative) and discarded. The remaining aqueous fraction was concentrated under vacuum and tested for cyanide using Feigl Anger filter paper and prepared enzyme from Passiflora foetida L. and later from Dermatocarpon miniatum. This phase was then streaked on Whatman 3MM paper and run in the

following four solvent systems: acetone:water (1:1), R_f.66; n-butyl: ethanol:water (40:11:19), R_f.1; t-butyl:ethanol:water (1:1:1), R_f.33; acetone:methanol:water (5:3:2), R_f.48. The cyanogenic material was located on the Whatman 3MM paper by cutting 1 cm^2 sections along the paper and testing for HCN production. The band containing the cyanogenic compound was desorbed in water and rechromatographed. The final product as a viscous tan liquid which was freeze dried and dissolved in D_2O for analysis in the NMR.

NMR Determination: A Nuclear Magnetic Resonance (NMR) spectrum was obtained from the extracts of Dermatocarpon miniatum (Figure 2). The compound was examined as a free glycoside in D_2O since they are all readily soluble in water. In the spectrum obtained (Figure 2), peaks between 3.0-4.0 suggest a sugar group, although the determining anomeric proton peaks were obscured by the water peak between 4.4-5.0 . There appears to be no aromatic protons in the extract due to the lack of peaks between 6.0-8.0. One small peak at 8.3 suggests a proton spectra of possibly a phenol or carboxylic acid groups. The phenol group can be eliminated as a possibility due to the lack of aromatic proton signals, while the carboxylic acid groups are also eliminated since a pH test of the extract was not acidic. Peaks between 4.6-6.0 suggest vinyl protons, but this does not seem likely due to the small area under the peak. Numerous peaks between 1.6-2.5 and 0.4-0.9 can not been explained, and probably the result of impurities in the extract. A mass spectrum of the extract was also completed using the Fast Atom Bombardment Method. The results suggest impurities in the extract which probably account for the numerous peaks between 1.6-2.5 and 0.4-0.9 on the NMR spectrum.

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Table 1. Location of Dermatocarpon miniatum populations examined within the confines of Kankakee River State Park (Will and Kankakee Counties, Illinois), date collected, and results of cyanide tests.

Population Location	Total Tested	% Cyanogenic
Population A. 20 Dec 1986	60	100
NE1/4 S28 T32N R10E, Will Co., IL.		
Population B. 22 Dec 1986	150	100
NW1/4 S32 T32N R11E, Kankakee Co., IL.		
Population C. 24 Dec 1986	60	75
NW1/4 S5 T31N R11E, Kankakee Co., IL.		
Population D. 7 Jan 1987	30	67
SW1/4 S32 T32N R11E, Kankakee Co., IL.		
Population E. 7 Jan 1987	30	100
NW1/4 S36 T32N R10E, Will Co., IL.		
Population F. 7 Jan 1987	30	97
SE1/4 S 36 T32N R10E, Will Co., IL.		
Population G. 7 Jan 1987	30	100
SE1/4 S 36 T32N R10E, Will Co., IL.		
Population H. 7 Jan 1987	30	100
SE1/4 S36 T32N R10E, Will Co., IL.		
Population I. 7 Jan 1987	30	100
NE1/4 S5 T31N R11E, Kankakee Co., IL.		
Totals	450	94

Table 2. -- continued

L. Phillippe (5 Jul 1984)	Jackson Co., TN	positive	M
L. Phillippe (23 Jul 1984)	Pendleton Co., WV	positive	M
L. Phillippe # 2905	Jackson Co., TN	positive	W
L. Phillippe # 3123	Tyler Co., WV	positive	S
L. Phillippe # 3143	Tyler Co., WV	negative	---
L. Phillippe # 3177	Harrison Co., WV	positive	S
R. Vogel (10 Apr 1976)	Pope Co., IL	positive	W
W. Whiteside (Aug 1969)	Clark Co., IL	positive	S
W. Whiteside (9 Jun 1970)	Effingham Co., IL	positive	W
W. Whiteside (28 Sep 1974)	Jersey Co., IL	positive	W
J. Wiedman (7 Sep 1970)	Clark Co., IL	positive	S

Dermatocarpon moulinsii (Mont.) Zahlbr.

W. McClain (Sep 1975)	Alaska	negative	---

Table 3. Additional lichen taxa tested for cyanide production.

Species Tested	Results
Cetrelia chicitae (Culb.) Culb. & Culb.	negative
Cladina subtenuis (Abb.) Hale & Culb.	negative
Cladonia coniocraea (Flk.) Spreng.	negative
Cladonia cristatella Tuck.	negative
Cladonia furcata (Huds.) Schrad.	negative
Cladonia macilenta Hoffm.	negative
Cladonia piedmontensis Merr.	negative
Cladonia polycarpoides Nyl.	negative
Cladonia rei Schaer.	negative
Cladonia verticillata (Hoffm.) Schaer.	negative
Hypogymnia krogii Ohlss.	negative
Lasallia papulosa (Ach.) Llano.	negative
Parmelia rudecta Ach.	negative
Parmelia sulcata Tayl.	negative
Parmelina aurulenta (Tuck.) Hale.	negative
Parmotrema eurysacum (Hue) Hale.	negative
Peltigera canina (L.) Willd.	negative
Peltigera polydactyla (Neck.) Hoffm.	negative
Phaeophyscia cernohorskyi (Nadv.) Essl.	negative
Physcia adscendens (Fr.) Oliv.	negative
Pseudevernia cladonia (Tuck.) Hale & Culb.	negative

Table 3. -- continued

Pseudovernia consocians (Vain.) Hale & Culb.	negative
Pseudevernia intensa (Nyl.) Hale & Culp.	negative
Pseudoparmelia caperata (L.) Hale.	negative
Ramalina americana Hale.	negative
Sticta weigelii (Ach.) Vain.	negative
Teloschistes chrysophthalmus (L.) Th. Fr.	negative
Umbilicaria muhlenbergii (Ach.) Tuck.	negative
Usnea angulata Ach.	negative
Usnea arizonica Mot.	negative
Usnea cavernosa Tuck.	positive (weak)
Usnea strigosa (Ach.) Eaton	negative

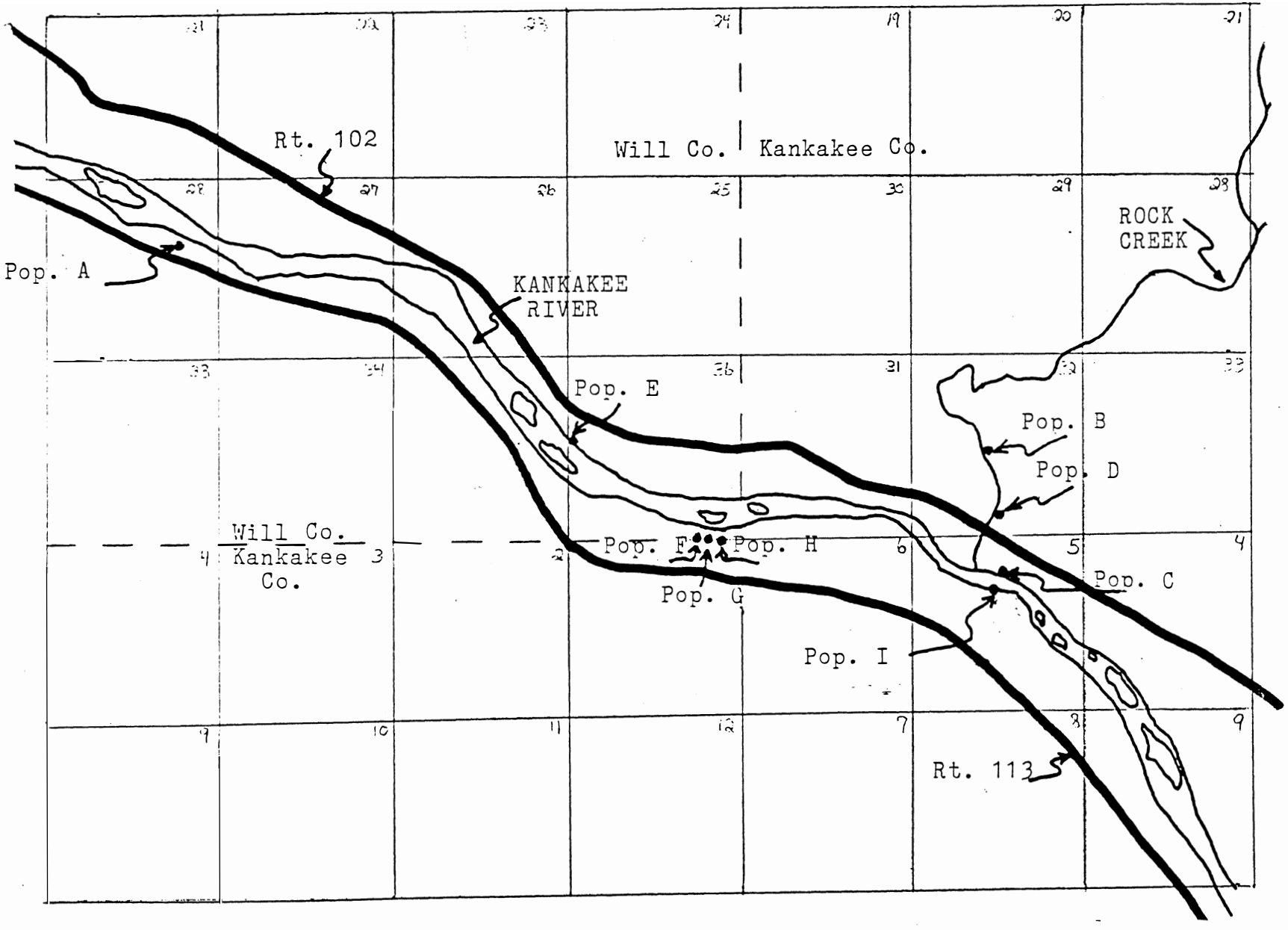

Figure 1. Dermatocarpon miniatum populations, Kankakee River State Park, Kankakee and Wll Counties, IL.

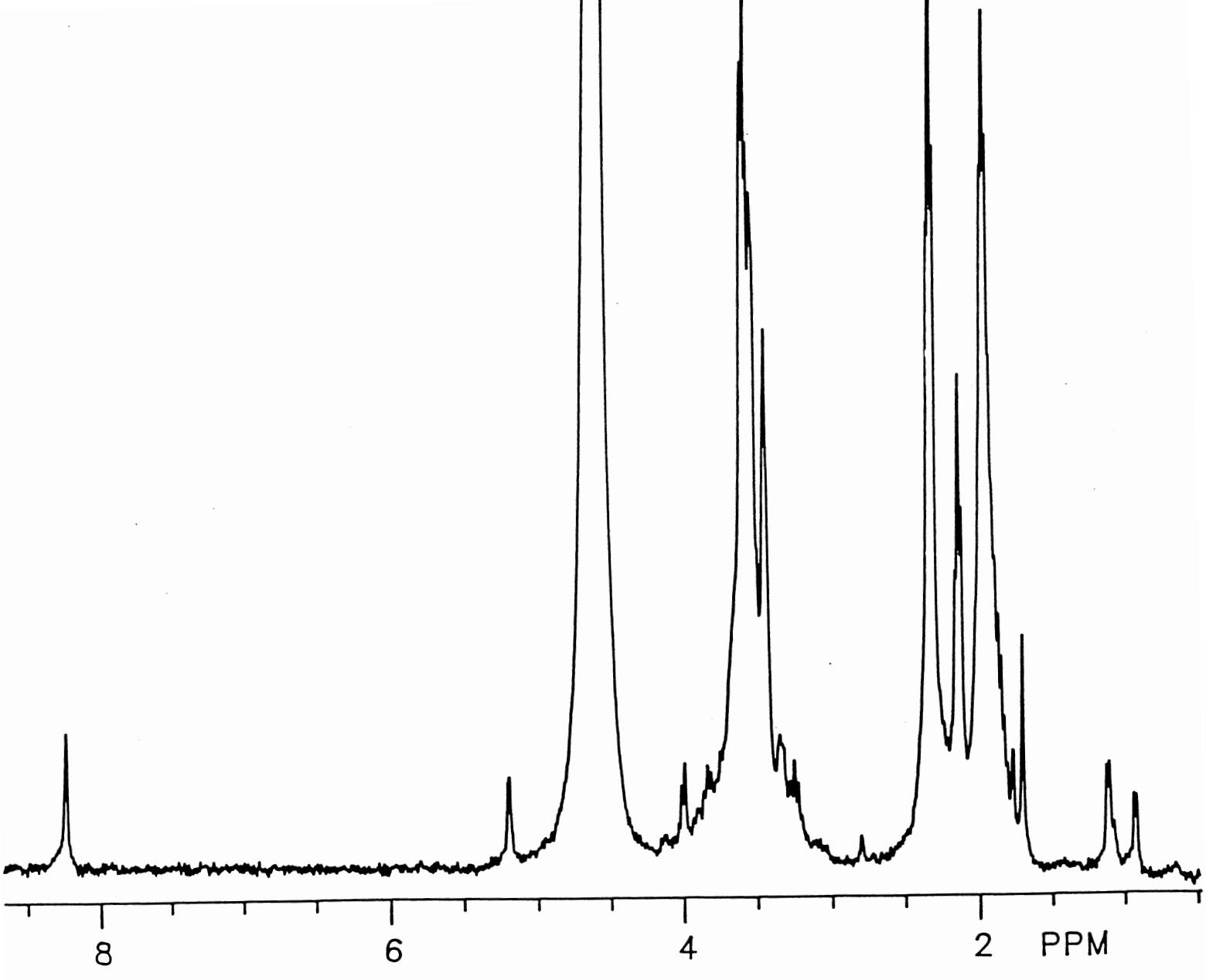

Figure 2. NMR spectrum of Dermatocarpon miniatum extract.