The Metabolism of C^{14} 2-PAM in the Isolated Perfused Rat Liver

IV. 1-Methyl-2-Pyridone

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SUMMARY

The metabolic disposition of radioactive 1-methyl-2-aldoximinopyridinium iodide-1-^{14}C (2-PAM) was investigated in the isolated perfused rat liver. In addition to the two metabolites already reported by our laboratory, a third metabolite of 2-PAM, 1-methyl-2-pyridone, has been isolated from the liver perfusate. The isolation procedures employed were ethanol and chloroform extraction, charcoal adsorption chromatography, paper electrophoresis, paper chromatography, and ion exchange chromatography. Characterization of the metabolite was accomplished by comparing its chemical, spectral, chromatographic, and electrophoretic properties with those of authentic 1-methyl-2-pyridone. The chemical, spectral, chromatographic and electrophoretic properties of this metabolite indicate that the compound is 1-methyl-2-pyridone. Metabolic pathways for the formation of 1-methyl-2-pyridone from 2-PAM and the possible molecular mechanisms involved in the various biotransformation reactions were proposed. The role of 1-methyl-2-pyridone and its intermediate in the conversion of 2-PAM to a 2-*O*-conjugate pyridinium ion was discussed.

INTRODUCTION

In earlier studies (1–4), the 1-methyl-2-*O*-conjugate pyridinium ion and the 1-methyl-2-cyanopyridinium ion were shown to be metabolites of the alkylphosphate antagonist, 1-methyl-2-aldoximinopyridinium ion (2-PAM), by the isolated perfused rat liver. Since this antagonist was found to be extensively metabolized by the liver (1–5), other metabolites were sought. Way *et al.* (4) suggested that the 2-*O*-conjugate pyridinium ion may be formed from 1-methyl-2-cyanopyridinium ion via the intermediate, 1-methyl-2-pyridone cyanohydrin, or from 1-methyl-2-pyridone (6). Conjugation then would occur on either the cyanohydrin derivative or 1-methyl-2-pyridone to form the 2-*O*-conjugate product.

This report herein describes the isolation and characterization of 1-methyl-2-pyridone as a third metabolite of 2-PAM in the isolated perfused rat liver. These studies are aiding in the establishment of the sequence of the reaction pathways and the elucidation of the molecular mechanism(s)

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for the formation of a 1-methyl-2-*O*-conjugate pyridinium ion.[4]

MATERIALS AND METHODS

Materials

Pyridine 2-aldoxime, 2-cyanopyridine, and 1-methyl-2-aldoximinopyridinium iodide were obtained from the Aldrich Chemical Company. These compounds were carefully recrystallized at least two times prior to their use. Extreme care was exercised in the handling of 2-cyanopyridine due to the high vapor pressure exerted by this crystalline material. The initial samples of 1-methyl-2-pyridone were kindly furnished byProfessor E. M. Kosower. Subsequent samples of 1-methyl-2-pyridone were prepared by the alkaline hydrolysis of 1-methyl-2-cyanopyridinium iodide (4, 6), and more recently, the 1-methyl-2-pyridone was obtained from the Aldrich Chemical Company. This commercial preparation was purified by vacuum distillation and subsequently precipitated from diethyl ether with hydrogen chloride gas. The resulting 1-methyl-2-pyridone hydrochloride was recrystallized two times with absolute ethanol. Methyl iodide-1-^{14}C (1 mC/mmole) was obtained from the Volk Radiochemical Company. Isotopically labeled 1-methyl-2-aldoximinopyridinium iodide-1-^{14}C was prepared as previously described (2). All other chemicals were of the highest grade and purity which were commercially available.

Perfusion Procedures

Details on the construction of the perfusion apparatus were obtained from Dr. J. A. Miller (personal communication). The isolated perfused rat liver preparation previously described (2, 3) was used to investigate the metabolic disposition of 2-PAM. The hydrostatic pressure of the perfusate entering the portal vein was maintained at approximately 120 mm. The perfusion medium consisted of 90% whole blood, 10% isotonic sodium chloride, and 500 USP units of heparin. Donor blood was obtained by cardiac puncture from adult female Holtzman rats. To each 100 ml of perfusate were added 100,000 units of crystalline penicillin G, 5 mg of streptomycin sulfate, and 100 μmoles of isotopically labeled 2-PAM. The volume of the perfusate was maintained between 150 and 300 ml.

Estimation of 2-PAM in the liver perfusate was carried out at periodic time intervals by the method of Way (2). At the termination of the liver perfusion, the perfusate was collected and immediately treated with three volumes of cold (—20°) acidified ethanol (ethanol: 0.5 *N* HCl, 100:1, v/v).

Isolation of Metabolite III

All isolation procedures were performed at 0–2° except when stated otherwise. Initial extraction of the liver perfusate was carried out with acidified ethanol, and the residue was removed by centrifugation, washed, and recentrifuged three times in 200 ml of chilled acidified ethanol. The supernatant fluids were pooled and the ethanol was removed with a rotary evaporator at 10°. The concentrated extract was filtered through Celite and subsequently extracted three times with equal volumes of chloroform. Chloroform extracts were pooled, passed through a sintered glass filter, and the chloroform was removed with a rotary evaporator at 10°. The concentrated extract was adjusted to a pH value of 7.0 or higher, made up to a volume of 30 ml with water and passed through a Darco-S-51 column (15 cm $\times$ 5 cm^2) at a rate of 5 ml/min. After washing the column with three bed volumes of water, the compound was eluted with eight bed volumes of 50% ethanol at a rate of 3 ml/min. The ethanol was removed with a rotary evaporator and the concentrated eluate was further purified by passing it through a mixed bed resin column (3 cm $\times$ 3.2 cm^2) containing equal parts of Dowex 50-W-hydrogen (12% cross-link, 200–400 mesh) and Dowex 1-chloride (12%

[4] A preliminary report of this work was presented at the annual Fall Meetings of the American Society for Pharmacology and Experimental Therapeutics in San Francisco, California, August 1963.

cross-link, 200–400 mesh). The effluent from the column was lyophilized and stored as a dry powder.

In order to ascertain that 1-methyl-2-pyridone was not formed as an artifact from the isolation procedure, control experiments were carried out with 1-methyl-2-aldoximinopyridinium iodide-1-^{14}C and 1-methyl-2-cyanopyridinium iodide-1-^{14}C. These isotopically labeled compounds were added to a solution of the same composition as the liver perfusate. The material was subjected to the same isolation procedures as were employed in the isolation of the radioactive metabolite. The final product isolated was subjected to paper chromatography, paper electrophoresis, ultraviolet absorption spectrophotometry, and radioactive measurements. These procedures indicate that no 1-methyl-2-pyridone was formed as an isolation artifact.

Characterization Procedures

Paper chromatography. Ascending chromatography was carried out on Whatman No. 1 and Whatman No. 3 MM filter paper sheets. The chromatographic sheets were equilibrated with the vapor phase of the solvent system for 1 hr and subsequently immersed in the solvent and developed at 0–2° for 15–24 hr. The solvent systems employed were *a*, *n*-butanol, ethanol and water (4:1:1 v/v), and *b*, *n*-propanol and water (7:3 v/v). The spots containing 1-methyl-2-pyridone, the isolated metabolite and related materials were detected by illumination with ultraviolet light and by radioassay. Liquid nitrogen was poured over the chromatographic sheet in order to enhance the fluorescent properties of 1-methyl-2-pyridone and the metabolite under ultraviolet light.

Paper electrophoresis. Horizontal paper electrophoresis was carried out on Whatman No. 3 MM paper with a Research Specialties Company Model 1910 power supply for 4 hr at 2°, 400 volts and 7 milliamperes. The two buffers employed in these studies were sodium acetate, pH 4.5, 0.05 M; and sodium phosphate, pH 7.2, 0.05 M. Caffeine was employed as the electroneutral prototype. Electrophoretic migrations of the metabolite and related compounds were determined by ultraviolet absorption and radioassay.

Ultraviolet spectrophotometry. Ultraviolet spectrophotometric measurements were performed as previously described (2–4), using a Beckman Model DU spectrophotometer with a photomultiplier and a Pyrocell microattachment and/or a Beckman Model DB spectrophotometer with a Sargent SRL Linear Log Recorder and a microattachment designed in this laboratory.

Countercurrent distribution. A twelve-plate countercurrent distribution analysis was performed employing methylene chloride and water as the lower and upper phases, respectively. The molecular extinction coefficient and the ultraviolet absorption curve of 1-methyl-2-pyridone were determined in both methylene chloride and water after the two phases had been saturated with each other. Concentrations of 1-methyl-2-pyridone and the metabolite were determined by measuring the absorbancy of the aqueous phase at 297 mμ and the methylene chloride phase at 305 mμ. The derived partition ratio of 1-methyl-2-pyridone between methylene chloride and water was employed for calculating the theoretical distribution. Theoretical values were obtained by using the formula for the expansion of the binomial expression (7, 8) as simplified by the method of Way and Bennet (9).

Gas chromatography. A Model 600 Aerograph (Wilkens Instruments & Research Inc.) was employed with a coiled aluminum column (5 ft ⅛ in O.D.) which was packed with Anakrom U (100/110 mesh) with 20% Carbowax 20 M as the liquid stationary phase. The operating temperature employed in the column was 165°. Nitrogen was employed as the carrier gas at a flow rate of 26 ml/min. Inlet pressure was 14 psig, and the outlet pressure was atmospheric pressure. A hydrogen flame ionization detector was coupled to a Leeds and Northrup Company recorder. Approximately 1–5 μl of the sample in methanol were employed in these determinations. Concentrations of 1-methyl-2-pyridone and metabo-

lite were determined spectrophotometrically and correlated with the peak heights.

Spectrophotofluorometry. The fluorescent emission spectra of 1-methyl-2-pyridone and the isolated radioactive metabolite in methanol were determined in an Aminco-Keirs spectrophotofluorometer. The activating wavelength at 325 mμ, which is the maximum of the excitation spectrum, gave a fluorescent emission spectrum with a maximum at 370 mμ.

Measurement of radioactivity. Radioactivity measurements were carried out in a Packard windowless gas flow proportional counter and a Nuclear Chicago Automatic Low Background Counter. The samples were prepared on aluminum planchets and counted over a sufficient period of time to give a counting error of 5% or less.

Cocrystallization. The isolated radioactive metabolite, which was suspected of being 1-methyl-2-pyridone, was mixed with nonradioactive carrier 1-methyl-2-pyridone picrate and recrystallized several times from isopropanol. After each crystallization, the crystals were made into a slurry with ether, deposited on an aluminum planchet, and assayed for radioactivity. Radioactivity measurements were extrapolated to infinite thinness by the use of a self-absorption curve prepared for 1-methyl-2-pyridone picrate-1-^{14}C (Fig. 1). After each crystallization the average specific activity was determined for three consecutive samples. The cocrystallization data were subjected to statistical analysis according to the method described by Calvin *et al.* (10).

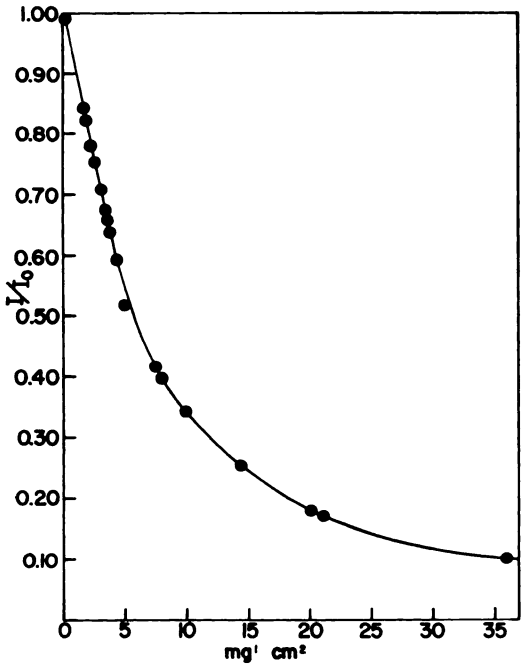

FIG. 1. *Self-absorption curve of 1-methyl-2-pyridone picrate-1-^{14}C*

I_0 = maximum activity at infinite thinness; I = measured activity; I/I_0 = absorption coefficient.

RESULTS

Characterization of Metabolite III

The radioactive metabolite which was isolated from the liver perfusion of radioactive 2-PAM was identified by comparing with authentic 1-methyl-2-pyridone with respect to chemical, chromatographic, electrophoretic, and spectral properties.

The ultraviolet absorption spectra of the isolated radioactive metabolite and of authentic 1-methyl-2-pyridone are shown in Fig. 2. Both compounds exhibited the same absorption maxima and minima under the conditions employed. A strongly acidic solution (1.6 N HCl) produced a hypsochromic shift and an increase in molar absorptivity. The strong acidity necessary to produce these spectral changes would be consistent with the low pK_a (0.32) for 1-methyl-2-pyridone reported by Albert and Phillips (11). The molecular extinction coefficients in 1.6 N HCl at λ_{max} 225 mμ and 280 mμ were 2.30×10^3 and 6.50×10^3, respectively. Spectral curves of these two compounds in water and 0.1 N NaOH were almost identical. The molecular extinction coefficients in water at λ_{max} 225 mμ and 297 mμ were 6.86×10^3 and 5.73×10^3, respectively.

The paper chromatographic properties of the isolated metabolite, authentic 1-methyl-2-pyridone, and related compounds are summarized in Table 1. In both solvent systems *a* and *b*, metabolite III and 1-methyl-2-pyridone show essentially the same R_F values. It should be pointed out

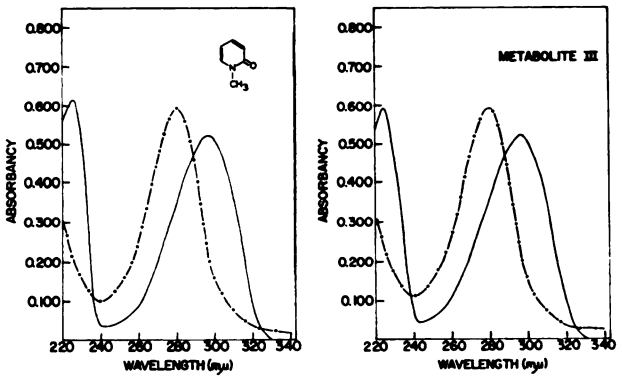

FIG. 2. *Ultraviolet absorption spectra of 1-methyl-2-pyridone and metabolite III* ——— in water and 0.1 N NaOH; .—.—. in 1.6 N HCl.

TABLE 1
Paper chromatography of metabolite III, 1-methyl-2-pyridone, and related compounds in two different solvent systems

Compound	R_F value	
	Solvent system *a*[a]	Solvent system *b*[b]
Metabolite III	0.64	0.76
1-Methyl-2-pyridone	0.64	0.77
1-Methyl-2-aldoximinopyridinium iodide	0.33	0.54
1-Methyl-2-cyanopyridinium iodide	0.27	0.50
1-Methyl-2-carbamidopyridinium iodide	0.20	0.37

[a] Solvent system *a*: *n*-butanol, ethanol, and water (4:1:1, v/v).
[b] Solvent system *b*: *n*-propanol and water (7:3, v/v).

TABLE 2
Paper electrophoresis of metabolite III, 1-methyl-2-pyridone, and related compounds using two different buffers

Compound	Mobility = (cm volt^{-1} hr^{-1} × 10^3)[a]			
	pH = 4.5[b]		pH = 7.2[c]	
	Cathode	Anode	Cathode	Anode
Metabolite III	0	—	0	—
1-Methyl-2-pyridone	0	—	0	—
1-Methyl-2-aldoximinopyridinium iodide	3.87	—	2.50	—
1-Methyl-2-cyanopyridinium iodide	5.51	—	3.79	—
1-Methyl-2-carbamidopyridinium perchlorate	4.75	—	2.27	—

[a] 400 volts for 6 hr.
[b] 0.05 M sodium acetate buffer.
[c] 0.05 M sodium phosphate buffer.

that most of the other derivatives of 2-PAM have R_F values which were much lower than those of 1-methyl-2-pyridone. Furthermore, the areas containing 1-methyl-2-pyridone and metabolite III were eluted from the paper chromatogram with water, and the ultraviolet absorption spectra of the two compounds in neutral, alkaline, and strongly acidic solutions were in satisfactory agreement. The results obtained were comparable to the spectral properties shown in Fig. 2. The relative electrophoretic mobilities of metabolite III, 1-methyl-2-pyridone, and related compounds are presented in Table 2. The lack of electrophoretic mobility of metabolite III and 1-methyl-2-pyridone under these conditions indicates that these compounds behaved as electrically neutral compounds.

The countercurrent distribution analysis

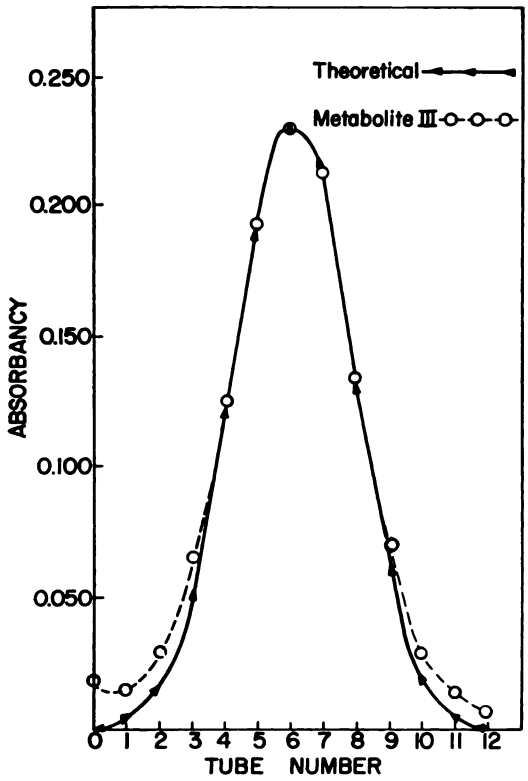

FIG. 3. *Countercurrent distribution analysis of metabolite III*

Pattern of a 12-transfer distribution.

of metabolite III is shown in Fig. 3. Distribution of the radioactive metabolite in each fraction was determined both spectrally and radioactively and found to be similar. Since 1-methyl-2-pyridone distributes equally to methylene chloride and water, that is, the partition ratio is 1.0; the maximal distribution occurred in the middle of the 12-plate countercurrent. It is fairly apparent that both the experimental and theoretical curves coincide. However, it should be pointed out that there are some deviations at both extremities of the curve, with the experimental values exceeding those of the theoretical. These deviations can be ascribed to small amounts of impurities (7) which were not separated by paper chromatography or paper electrophoresis.

Since 1-methyl-2-pyridone could be subjected to gas chromatography, the conditions for the isolation of 1-methyl-2-pyridone and metabolite III from partially purified liver perfusates were investigated. The retention times and peak heights of equimolar amounts of metabolite III and 1-methyl-2-pyridone are shown in Table 3.

TABLE 3

Retention times and peak heights of 1-methyl-2-pyridone and metabolite III by gas chromatography

Compound	Sample size (μg)	Retention time (min)	Peak height (mm)
1-Methyl-2-pyridone	3.3	43	170
Metabolite III	3.3	41	180

Sample size of metabolite III and 1-methyl-2-pyridone was estimated spectrally, assuming that the molar absorptivity of 1-methyl-2-pyridone and metabolite III were identical. It is quite apparent that under these conditions the peak height and retention time of metabolite III were similar to those of authentic 1-methyl-2-pyridone.

TABLE 4

Specific activity of ^{14}C-metabolite III and 1-methyl-2-pyridone picrate after cocrystallization

Number of recrystallizations	Specific activity (cpm/mg) $P = 0.05$
3	78.7 ± 3.70
4	76.8 ± 3.37
5	79.3 ± 3.80

The cocrystallization studies of the radioactive metabolite with carrier 1-methyl-2-pyridone picrate are shown in Table 4. On the third, fourth, and fifth recrystallizations the radioactive metabolite was cocrystallized to a constant specific activity with isopropanol. The ability to cocrystallize a micro amount of radioactive metabolite with a large amount of carrier to a constant specific activity would indicate that the properties of metabolite III

are consistent with those of 1-methyl-2-pyridone.

The fluorescent emission properties of 1-methyl-2-pyridone were investigated, and a strong fluorescent spectrum was exhibited when activated at 325 mμ (Fig. 4). In order to compare the fluorescent properties of this compound with those of metabolite III, authentic 1-methyl-2-pyridone was

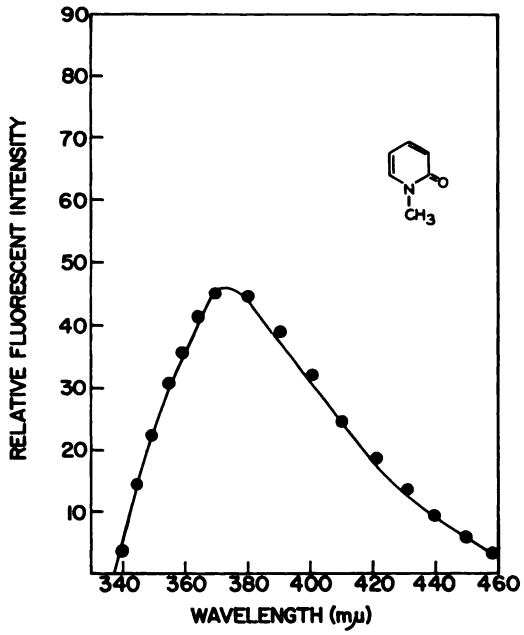

FIG. 4. *Fluorescent emission spectra of metabolite III and 1-methyl-2-pyridone*

——— 1-methyl-2-pyridone; ●●● Metabolite III.

added to a solution with the same composition as the liver perfusate and recovered by the same procedures used in the isolation of the metabolite from the ^{14}C 2-PAM liver perfusate. The fluorescent emission spectra of the isolated authentic 1-methyl-2-pyridone and metabolite III were compared and found to be almost identical. It is to be noted that the spectrophotofluorometric method is sufficiently sensitive to measure 1.0×10^{-6} M 1-methyl-2-pyridone.

DISCUSSION

The characterization of 1-methyl-2-pyridone as a metabolite of 2-PAM raises the question with respect to the molecular mechanism(s) for the formation of 1-methyl-2-pyridone and the biochemical pathways involved in the formation of the 2-*O*-conjugate pyridinium ion. A probable reaction mechanism for the formation of 1-methyl-2-pyridone from 2-PAM involves 1-methyl-2-cyanopyridinium ion as the intermediate (Fig. 5). Alkaline hydrolysis of the 2-cyanopyridinium ion could occur by a bimolecular nucleophilic aromatic substitution mechanism with hydroxyl ion adding to the ring to form 1-methyl-2-pyridone cyanohydrin as the intermediate and the subsequent elimination of cyanide ion to form 1-methyl-2-pyridone (12). Since the pK_a of 1-methyl-2-pyridone cyanohydrin is approximately 10.3 (6), the reactive species under biological conditions is probably in the form of the cyanohydrin rather than the anion of the cyanohydrin. Compounds such as 1-methyl-2-pyridone exist in aqueous solution in equilibrium as an amide or an α hydroxy form, normally with the amide form predominating (13, 14). The equilibrium of 1-methyl-2-pyridone, with a pK_a value of 0.32 (11), is presumably changed to the α hydroxy form only in very strongly acidic solution. The hypsochromic shift in the ultraviolet absorption curve of 1-methyl-2-pyridone in going from an alkaline or neutral solution to a strongly acidic solution would be consistent with the chemistry of 1-methyl-2-pyridone. Similar displacement of the spectral curve of 1-methyl-4-pyridone to a shorter wavelength in acidic media have been reported by Wu Chang and Johnson

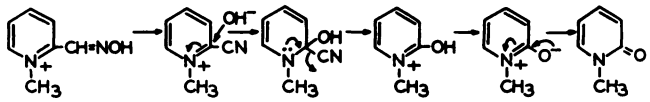

FIG. 5. *Proposed reaction mechanism for the formation of 1-methyl-2-pyridone from 2-PAM*

(15). It should be emphasized that, at the pH values of biological fluids, the hydrolysis of the cyano group by water also may play an important role.

The 1-methyl-2-*O*-conjugate pyridinium ion can now be formed from either 1-methyl-2-pyridone or 1-methyl-2-pyridone cyanohydrin. However, since 1-methyl-2-pyridone exists in biological fluids predominantly as the amide form, it seems more reasonable to assume that the 2-*O*-conjugate pyridinium ion is formed by the conjugation of 1-methyl-2-pyridone cyanohydrin with the subsequent elimination of cyanide ion rather than by the direct conjugation of 1-methyl-2-pyridone. Preliminary data on this point have been reported by Way (16).

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