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Identification of two metabolites of the cholinesterase reactivator HI-6 isolated from rat urine

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Two metabolites, isolated from the urine of rats given the cholinesterase reactivator HI-6 intravenously, still contained quaternary nitrogen atoms and therefore could not be extracted from aqueous solutions by organic solvents. Both metabolites were isolated by preparative high performance liquid chromatography and were identified using mass spectrometry, gas chromatography, infrared spectrometry, ultraviolet spectrometry and proton nuclear magnetic resonance spectrometry. The structures were confirmed by in-vitro preparation of the compounds. Both metabolites contained 2-pyridone moieties. One had an intact pyridinium-aldoxime moiety, and therefore could still be therapeutically active. The excretion of unchanged HI-6 together with the two identified metabolites does not provide for a 100% mass balance, indicating that in the rat, other, as yet unidentified, metabolites must be formed.

Organophosphate intoxications are usually treated with a combination of atropine and an acetylcholinesterase (AChE) reactivator, e.g. pralidoxime or obidoxime (Grob & Johns 1958: Namba & Hiraki 1958; Namba 1971; Schenk et al 1976; Erdmann 1976). However, reactivation of soman-inhibited AChE is difficult. Indeed, with conventional oximes, like pralidoxime or obidoxime, no therapeutic effect can be achieved with soman intoxication. A new generation of asymmetric bisquaternary monooximes, amongst which HS-6 and HI-6 are the most well known, has been synthesized by the group of Hagedorn (Schoene 1967). These oximes have proved to be relatively efficient reactivators of soman-inhibited AChE in-vitro (Schoene 1973; De Jong & Wolring 1980). They have also shown therapeutic efficacy in soman-intoxicated animals in-vivo (Erdmann 1969; Boskovic & Stern 1970; Oldiges & Schoene 1970; Schenk et al 1976; Kepner & Wolthuis 1978; Wolthuis & Kepner 1978; Hauser & Weger 1979; Wolthuis et al 1979), HI-6 being one of the most promising compounds against soman intoxication (Wolthuis et al 1981).

Only 57% of HI-6 was recovered from rat urine 24 h after its intravenous administration (Ligtenstein & Kossen 1983). No faecal excretion was observed, indicating HI-6 to be metabolized to approximately 43%. Metabolism of pyridinium-aldoximes has been reported by several investigators (for review see Way & Way 1968).

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In this paper we report the characterization of two metabolites of HI-6, isolated from rat urine by high performance liquid chromatography (HPLC). The chemical structures of the compounds, which both possess a 2-pyridone structure, were characterized by mass spectrometry (MS), ultraviolet spectrometry (UV), gas chromatography (GC), infrared spectrometry (IR) and nuclear magnetic resonance spectrometry (NMR).

MATERIALS AND METHODS

Chemicals

HI-6 dichloride monohydrate (M = 377·22; for structures of the relevant compounds see Fig. 1) was prepared in our laboratory according to Schoene (1967). Isonicotinamide and pyridine-2-aldoxime were from Aldrich (Beerse, Belgium).

Compound A, prepared for the confirmation of the structure of metabolite A, was produced by adding 2 equivalents of NaOH (5·3 mg) to HI-6 dichloride monohydrate (50 mg) in methanol (25 mL). The reaction was immediately stopped by the addition of an excess of concentrated HCl. Thereafter the mixture was dried by evaporation and compound A collected in ca 25% yield via preparative HPLC. The compound proved unstable in methanol as well as in crystalline form, but was reasonably stable in dilute aqueous solutions.

Compound B, prepared for the confirmation of the structure of metabolite B, was obtained by dissolving HI-6 dichloride monohydrate (400 mg) in a 0·1 M phosphate buffer of pH 7·0 at 37 °C (25 mL).

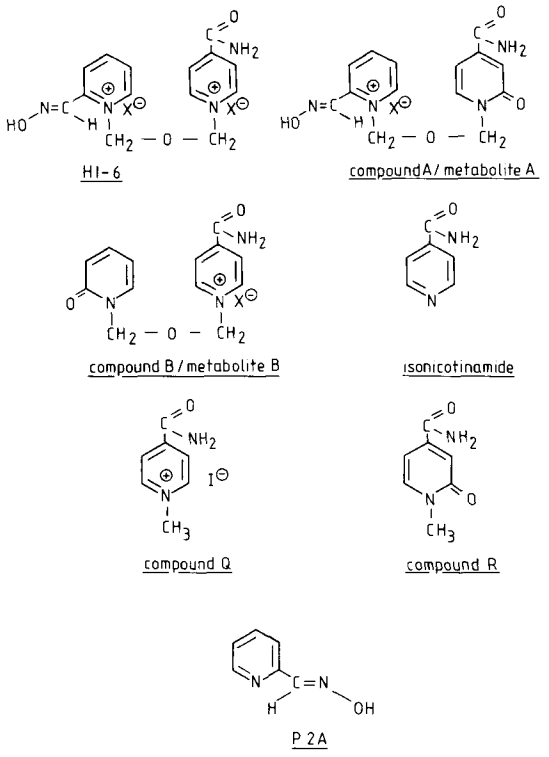

FIG. 1. Chemical structures of HI-6 and relevant derivatives.

After 40 h the solution was freeze-dried and the phosphates precipitated by adding methanol. After centrifugation and subsequent decantation of the supernatant, the methanol was evaporated, yielding a sticky brown residue. By preparative HPLC, followed by addition of 4 M HCl in excess, freeze-drying and redissolution in methanol, 75 mg (19%) of compound B was collected. However, we did not succeed in quantitatively removing all potassium formate originating from the eluent used for HPLC. Purity, as estimated by HPLC: >80%.

Compound Q was prepared by combining equimolar amounts of isonicotinamide (12·2 g) and methyl iodide (12·6 g) in a mixture of 75 mL methanol and 150 mL acetone. After stirring for 16 h the yellow reaction product was precipitated with ether. After filtration and purging of the filter 3 times with 25 mL ether, 11·4 g (46%) compound Q was obtained; m.p. >250 °C; purity by HPLC 99%.

Compound R was prepared by adding two equivalents of KOH (0·3 g) to substance Q (2 g) in 75 mL methanol. After 2·5 h the solvent was evaporated, yielding a red-brown precipitate consisting of a mixture of at least three compounds. Additionally, there were indications of the presence of polymers (Klingsberg 1962). After dissolution in methanol the mixture was injected into the preparative HPLC system, and substance R collected. The eluent was evaporated, the aqueous precipitate freeze-dried and subsequently dissolved in a mixture of 5 mL methanol and 10 mL acetone. The salts of the eluent were precipitated by dropwise addition of ether. Potassium formate could not be quantitatively removed. After filtration, the solvents were evaporated under vacuum. The red-brown oily residue contained, as estimated by HPLC, >90% substance R. Compound Q was found to be absent. Yield 1·3 g/65%.

Compounds A, B, Q and R gave mass spectra that were in accordance with their chemical structure.

Pentobarbitone sodium (Nembutal) was procured from Ceva (Neuilly-sur-Seine, France; 60 mg mL^{-1}). 2-Pyridone was obtained from Merck (Darmstadt, Federal Republic of Germany).

All other reagents were of analytical grade.

High performance liquid chromatography (HPLC)

The HPLC equipment consisted of a Waters 6000A Delivery System, a Waters WISP 710B Automatic Sample Injector, a Tracor 970A variable wavelength UV detector, and an LDC 304 Computing Integrator, coupled to a Kipp BD 41 recorder. Columns were packed according to Linder et al (1976).

Analytical HPLC system. The stationary phase R-SILCAT, 5 μm particle size (Alltech, Eke, Belgium), was packed into a 5 × 250 mm stainless steel column. The eluent was a mixture of water and methanol (1 : 5) containing 0·25 M potassium formate, resulting in a pH of approximately 5 (the high methanol content hampers a reliable pH determination). The column was maintained at 80 °C. The flow rate was 0·7 mL min^{-1}, yielding a pre-column pressure of 1 MPa. Injection volume was 10 μL. This system was described by Kientz et al (1983). The plate number for the compounds investigated was approximately 12 000/25 cm. The UV-detector was set at 304 nm for HI-6, at 263 nm for compound/metabolite B, and at 260 nm for all other compounds.

Preparative HPLC system. A 10 × 250 mm stainless steel column was packed with Lichrosorb SI-6, 10 μm particle size (Merck, Darmstadt, Federal Republic of Germany). The eluent consisted of a mixture of water and methanol (1 : 5) in which 5 mM potassium formate was dissolved. The flow rate was

3 mL min^{-1}, yielding a pre-column pressure of 1 MPa, at room temperature (20 °C). The plate number for the compounds investigated amounted to approximately 10 000/25 cm. Injection volume was 3 mL. Detection was as described for the analytical HPLC system. To remove potassium formate, an excess of concentrated HCl was added and, after freeze-drying, the residue was taken up in methanol. Trace amounts of potassium formate remained. After decantation and evaporation of the solvent under nitrogen at room temperature, the residue was taken up in water.

Mass spectrometry (MS)

Electron impact (EIMS) and isobutane chemical ionization (CIMS) mass spectra were obtained with a VG 7070F mass spectrometer. The EIMS spectra were recorded at an electron energy of 70 eV. The source temperature was 200 °C. Samples were introduced using a direct insertion probe without sample heating.

Exact mass measurements were carried out by peak matching at a resolution of 10 000 (10% valley).

Field desorption spectrometry (FDMS) was performed at the Central Institute for Nutrition and Food Research TNO (Zeist, The Netherlands) with a Varian MAT 731 mass spectrometer. Emitters were activated using indene as activation substance (Rabrenovic et al 1981). The needle length was typically 20–40 μm, and the samples were loaded by means of the syringe technique.

Gas chromatography (GC)

An Intersmat GC 120 gas chromatograph with a flame ionization detector was coupled to a Kipp BD 8 recorder. The mobile phase was helium (Loosco, Amsterdam, The Netherlands), flowing at 15 cm s^{-1} through a home-made 25 m × 0·7 mm capillary column, roughened by NaCl (De Nijs et al 1979), packed with Hi-eff-8BP (cyclohexane dimethanol succinate; Chrompack, Middelburg, The Netherlands), deactivated in gas phase with Carbowax 1540 (also from Chrompack) (Franken et al 1977). The injection block was heated at 300 °C. The column temperature was programmed starting from 100 °C, increasing by 8 °C min^{-1} for 10 min to 180 °C. Samples were introduced by a glass solid injector device. The temperature of the injection block causes dealkylation of 1-alkylpyridinium compounds.

Infrared spectrometry (IR)

IR spectrometry was performed on a Grubb Parsons Spectromajor IR spectrometer. Spectra were recorded as KBr discs.

Ultraviolet spectrometry (UV)

UV spectra were recorded on the HPLC UV-detector in the eluent of the preparative procedure.

Nuclear magnetic resonance spectrometry (NMR)

Proton NMR spectra were recorded in D_2O at a frequency of 100·1 MHz in the Fourier transform mode on a Varian XL-100-12 NMR spectrometer system. The spectral width was 1024 KHz. A 90° pulse (pulse width 20 μs) and an acquisition time of 4 s were applied. Data accumulation in the time domain and Fourier transformation of the free induction decay were carried out by a Varian 620/L-100 computer, interfaced to the spectrometer. Chemical shifts are expressed as δ-values relative to sodium 3-(trimethylsilyl)propane sulphonate.

Animal procedures

Male 'Small Wistar' rats, strain WAG/MBL, bred at the Medical Biological Laboratory TNO, 180–210 g, were used. Before the experiment the animals were fasted for 24 h, but had free access to water. During anaesthesia with pentobarbitone sodium (75 mg kg^{-1}, intraperitoneally) HI-6 dichloride monohydrate was administered intravenously via the dorsal penile vein in a dose of 50 mg kg^{-1} (0·1325 mmol kg^{-1}). The animals were subsequently placed in metabolism cages, and urine was collected for 24 h. To prevent degradation of HI-6 and the metabolites collected, and to minimize the risk of infection, urine was collected in 4 mL of 50% acetic acid. After determination of the volume of urine produced, this solution was injected into the HPLC system in undiluted form. When intended for application to preparative HPLC, samples were freeze-dried before chromatography and analysed in methanol.

Control experiments

To 6 mL of rat urine, containing 4·4 mM HI-6 dichloride monohydrate, 4 mL of 50% acetic acid was added. This mixture underwent the same procedures as rat urine obtained after intravenous injection of HI-6 to the animals.

RESULTS AND DISCUSSION

Detection and work-up from rat urine of two metabolites of HI-6 by means of HPLC

After intravenous injection of HI-6, two metabolites of HI-6 could be detected in rat urine by HPLC (Fig. 2). That with the lowest retention ($k' = 1·45$) is

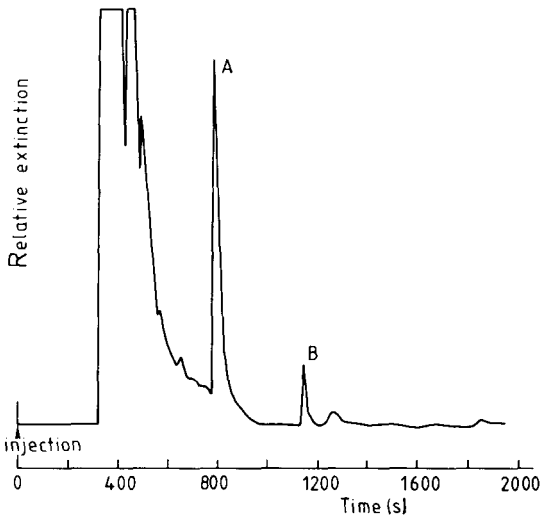

FIG. 2. HPLC chromatogram of rat urine. A = metabolite A; B = metabolite B.

designated metabolite A, that with k′ = 2·48, metabolite B.

No interfering decomposition products were formed, even after prolonged storage at room temperature. HI-6 was always quantitatively recovered from control solutions.

The HPLC behaviour of both compounds was indicative of a monoquaternary chemical structure, rendering them unextractable from aqueous solutions by organic solvents. The metabolites were purified by preparative HPLC and were obtained on a milligram scale.

The isolation of metabolite A in a sufficiently pure form was particularly difficult. It proved to be soluble only in water and methanol, and unstable in the latter solvent. In case of work-up from aqueous solutions, potassium formate remained as an impurity. A small but sufficient sample was isolated and purified for structural analysis. The compound was stored in dilute aqueous solution.

Metabolite A could not be separated by HPLC from *N*-methyl-β-acetylhistidine and its methyl ester, both histidine metabolites being present in rat urine.

To verify the structure of metabolites A and B, these, together with compounds Q and R, were synthesized. Compounds A and R were prepared by addition of potassium hydroxide to the corresponding pyridinium salts. The formation of the carboxamido-pyridone ring system occurs, as is usual for this kind of disproportionation reaction, without a formal oxidation step (Klingsberg 1962). Large amounts of the compounds A and B were difficult to obtain. An estimation of the metabolized fraction of HI-6 present in rat urine was made by comparison of the molar extinction coefficients of metabolites A and B with those of pralidoxime and obidoxime. On these grounds we suggest a total mass balance of 60% unchanged HI-6, 20% metabolite A and B and 20% unrecovered other products.

Structural analysis of metabolite A

Under electron impact the ion *m/z* 122 indicated the presence of a pyridine 2-aldoxime moiety, *m/z* 104 being a dehydrated form (Plucinski et al 1977). The

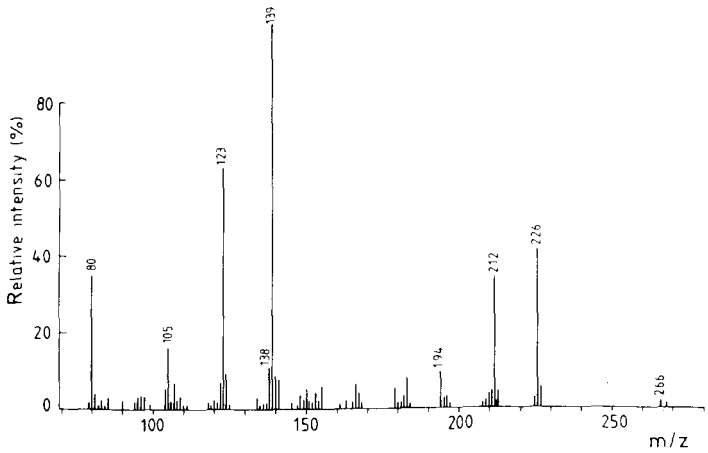

FIG. 3. Isobutane chemical ionization mass spectrum (CIMS) of metabolite A. The ions at *m/z* 212, 226 and 266 originate from *N*-methyl-β-acetylhistidine and its methyl ester.

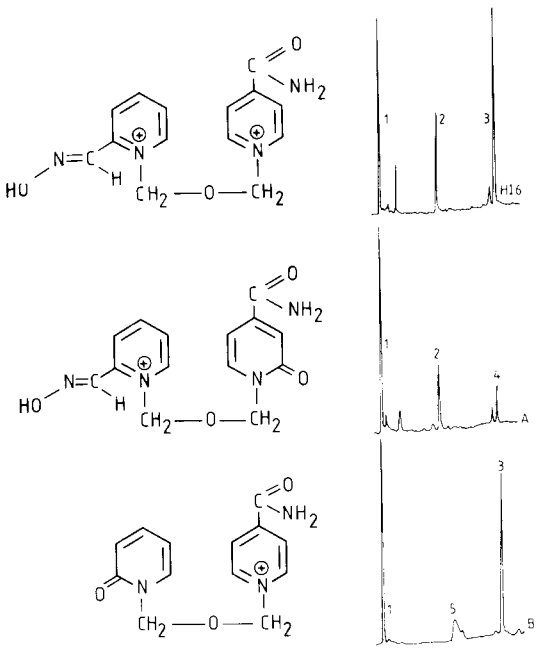

FIG. 4. Gas chromatograms of HI-6 and metabolites A and B, using the dealkylating GC system. The upper chromatogram shows HI-6, yielding P2A(2) and isonicotinamide(3). Metabolite A yields an identical chromatogram, but isonicotinamide(4) in this case has been formed in the chromatograph by reduction of 4-carboxamido-2-pyridone. Metabolite B (lower Fig.) is dealkylated into 2-pyridone(5) and isonicotinamide(3). Peaks labelled (1) originated from volatile products.

second ring was found at *m/z* 138 from 4-carboxamido-3-pyridone.

In the CIMS spectrum (Fig. 3) the same ions were found in protonated form at *m/z* 123, 105 and 139 together with *m/z* 107 (*m/z* 123 − NH_2). The 4-carboxamido-2-pyridone ion was confirmed by mass measurement ($C_6H_7N_2O_2$ requires 139·0507; found 139·0513). *m/z* 80 represents the protonated pyridinium ion. No molecular ion was observed and no additional information was obtained from FDMS.

Under conditions of GC, metabolite A yielded 2 major peaks viz. P2A and isonicotinamide (Fig. 4), their identities being confirmed by GC–MS. Such behaviour mirrors that of HI-6 (Fig. 4 upper chromatogram). It is unexpected that metabolite A yields isonicotinamide but no 4-carboxamido-2-pyridone. We believe that this last compound is reduced to isonicotinamide in the injection block. This was supported by the formation of isonicotinamide likewise, when compound R is injected under the same GC conditions.

Such reduction also occurs during CIMS analysis when source temperatures higher than 200 °C are used. At 200 °C the ratio between *m/z* 139 (protonated 4-carboxamido-2-pyridone) and *m/z* 123 (protonated P2A) is roughly 2 (Fig. 3). This ratio is reversed at a source temperature of 240 °C, whilst at a source temperature of 300 °C *m/z* 139 has almost completely disappeared. Such reduction reactions have been reported earlier (Brophy et al 1979; Soine

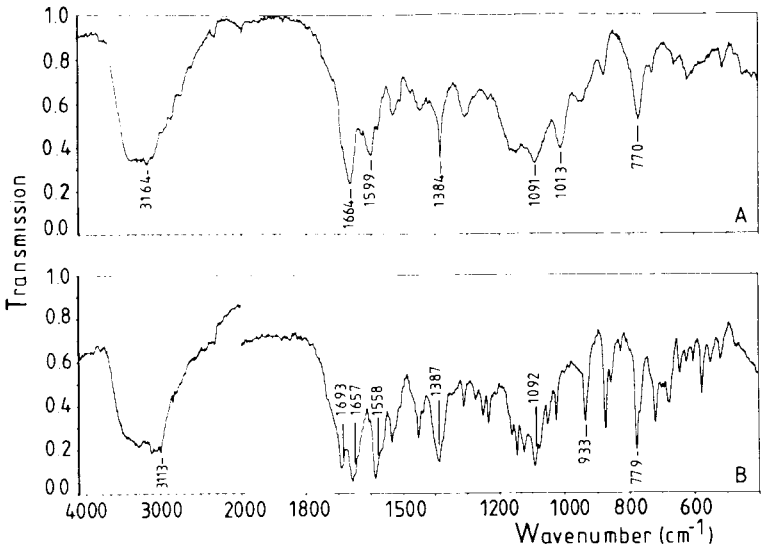

FIG. 5. The IR spectra of metabolite A (A) and metabolite B (B), obtained by KBr disc technique.

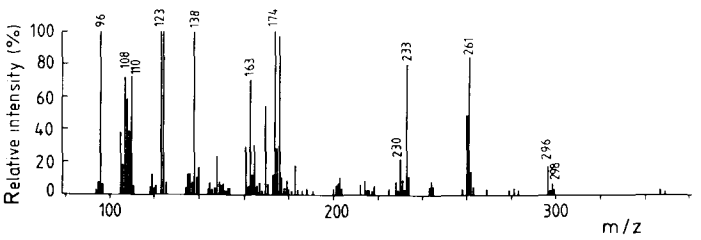

FIG. 6. Isobutane chemical ionization mass spectrum (CIMS) of metabolite B. Masses measured: 174·0322 ($C_7H_9NO_2Cl$ requires: 174·0322), 233·0917 ($C_{12}H_{13}N_2O_3$ requires: 233·0926), 296·0810 ($C_{13}H_{15}N_3O_3Cl$ requires: 296·0802).

et al 1983), being ascribed to catalysis by metal ions present in the system.

IR absorption (Fig. 5) is in agreement with the proposed structure: 3164 cm^{-1} (amide NH_2, oxime OH), 1664 cm^{-1} (carboxamide and pyridone CO), 1599 cm^{-1} (pyridinium ring), 1091 cm^{-1} (oxydimethylene bridge), 1013 cm^{-1} (oxime = NO) and 770 cm^{-1} (aromatic C–H).

The UV spectrum of metabolite A shows an absorption maximum at 304 nm indicating the presence of an oxime group (Barkman 1962).

At concentrations in D_2O normally necessary for NMR spectra metabolite A rapidly underwent chemical changes, probably leading to the formation of dimers and polymers (Klingsberg 1962). No differences were found between the analytical results of metabolite A and of the synthesized compound A.

Structural analysis of metabolite B

The mass spectrum (electron impact) shows ions at *m/z* 122, 106 and 78 originating from isonicotinamide (Chen 1976). The absence of *m/z* 104 (122 − H_2O) indicated the absence of the oxime group. Fragments at *m/z* 108 and 109 stemmed from 1-methyl-2-pyridone.

The CIMS spectrum (Fig. 6) shows protonated masses of 2-pyridone (*m/z* 96), 1-methyl-2-pyridone (*m/z* 110), isonicotinamide (*m/z* 123) and that of an ion containing an oxydimethylene bridge (*m/z* 260). Mass measurement gave the value of 269·1029; $C_{13}H_{14}N_3O_3$ requires 260·1035. Other measured masses are represented in the legend of Fig. 6.

Gas chromatography of metabolite B yielded two peaks, 2-pyridone and isonicotinamide (Fig. 4).

The IR spectrum of metabolite B (Fig. 5) shows the following peaks: 3113 cm^{-1} (amide NH_2), 1693 cm^{-1} (pyridone CO), 1657 cm^{-1} (carboxamide CO), 1588 cm^{-1} (pyridinium ring), 1092 cm^{-1} (oxydimethylene bridge) and 779 cm^{-1} (aromatic C-H).

The UV spectrum of metabolite B lacks an absorption band at 304 nm, illustrating the disappearance of the oxime group.

Part of the NMR spectrum of metabolite B (D_2O) is represented in Fig. 7. Based on comparison with 1-methyl-2-pyridone and compound Q the spectrum is in agreement with the proposed structure: two $N–CH_2–O$ substructures with signals at δ 5·72 (2H, s, $CH_2\bar{N}$) and 6·25 (2H, s,CH_2N^+); the 2-pyridone ring indicated by four multiplets (1H each) at δ 6·44 (d 9 Hz, broadened; H-3), δ 6·54 (t 7 Hz and d 1·2 Hz; H-5), δ 7·58 (ddd 9 Hz, 7 Hz and 2 Hz; H-4) and δ 7·76 (ddd 7 Hz, 2 Hz and 0·8 Hz; H-6); the para-substituted pyridinium ring shown by an AA'BB'-pattern around δ 8·43 and 9·19 (4H, J_{AB} = 7 Hz). In the low field region some impurities were observed.

No differences were found between the analytical results of metabolite B and those of the synthesized compound B.

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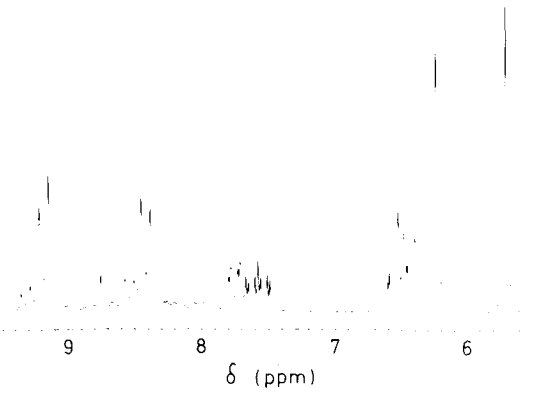

FIG. 7. Proton NMR spectrum of metabolite B, recorded in D_2O.

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