Journal of Chromatography, 614 (1993) 205–212
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6747

Confirmation of a dopamine metabolite in parkinsonian brain tissue by gas chromatography–mass spectrometry

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(First received October 14th, 1992; revised manuscript received January 11th, 1993)

ABSTRACT

Gas chromatography–mass spectrometry was used to identify a dopamine metabolite isolated from the substantia nigra of parkinsonian brain tissue. Incubation of dopamine with monoamine oxidase B gave the same product which was identified as 3,4-dihydroxyphenylacetaldehyde. The structure of the compound was established by chemical synthesis, metastable ion measurement and high-resolution mass spectrometry.

INTRODUCTION

A variety of theories have been proposed to explain the mechanism underlying the neuronal degeneration in Parkinson's disease (PD). The causes that have been suggested include abiotrophy, metabolic abnormality, autoimmune phenomenon and cytotoxicity due to environmental neurotoxin [1–5]. The cytotoxicity to dopaminergic neurons resulting from generation of reactive oxygen species like hydrogen peroxide, superoxide and hydroxy radicals, resulting from autoxidation and production of dopa quinones which kill cells through inhibition of sulfhydryl enzymes, also have been suggested [6–10]. Since PD is intimately associated with selective neuronal loss, studies have been focused on the mechanism by which nerve cells die in the course of the disease process. To this end, neuroscientists have used toxins, such as 6-hydroxydopamine and more recently 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which can selectively ablate monoaminergic neurons in experimental animals, to study the brain dopaminergic system and its role in PD [11]. The specific nature of the neuronal degeneration precipitated by parenteral or intracerebral injection of these or related toxins has led to the suggestion that a similar mechanism(s) may underlie the loss of nerve cells in PD and its animal models. Consequently, the production of endogenous 6-hydroxydopamine-related metabolites has been linked to the pathogenesis of idiopathic parkinsonism [3].

The primary metabolite resulting from the oxidation of dopamine by monoamine oxidase B (MAO-B) is an aldehyde, namely 3,4-dihydroxyphenylacetaldehyde (Dopal). Recent studies by us and others have shown that the endogenous aldehyde metabolites are neurotoxic and may be involved in the neuronal degeneration [5,11–17].

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0378-4347/93/$06.00

This hypothesis is supported by the fact that some aldehydes are cytotoxic [17], exert an inhibitory effect on cell functions [18], attack both sulfhydryl and amino groups of biomolecules, influence cell division, deactivate enzymes, interfere with microtubule assembly–disassembly, inhibit metabolism, and inhibit biosynthesis of proteins and nucleic acids [13–16,19,20]. These findings suggested that Dopal, an endogenous metabolite of dopamine produced by MAO-B, most probably is a neurotoxin and also is involved in the degeneration of dopaminergic neurons. Hence, separation and identification of Dopal in parkinsonian brain tissue and assessing its reaction with tissue nucleophiles may provide the valuable information leading to clarifying the mechanisms underlying neuronal degeneration in PD. For the first time in this study, we report the isolation and characterization of Dopal in parkinsonian brain tissue by gas chromatography–mass spectrometry (GC–MS). The reaction of this metabolite with cellular nucleophiles is also assessed.

EXPERIMENTAL

Reagents and chemicals

Dopamine (3,4-dihydroxyphenethylamine) hydrochloride, tris(hydroxyphenyl)aminoethane hydrochloride (Tris–HCl) buffer, norepinephrine hydrochloride, double-stranded DNA, bovine serum albumin (BSA) and catalase (bovine liver) were purchased from Sigma (St. Louis, MO, USA). Tubulin protein was a gift from Dr. James C. Lee, St. Louis University (St. Louis, MO, USA). Reactivials, pyridine and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were purchased from Supelco (Bellefonte, PA, USA). HPLC-grade methanol was purchased from J. T. Baker (Phillipsburg, NJ, USA). [2,5,6-^{3}H]Dopamine (57 mCi/mmol) was purchased from Dupont (Boston, MA, USA).

Source of postmortem brain tissue

Substantia nigra brain tissue was obtained from St. Louis University Medical Center Brain Bank (St. Louis, MO, USA). In order to obtain brain tissue with a short *post-mortem* interval, we have instituted a brain autopsy acquisition procedure. The brain tissues with *post-mortem* intervals of 3 h or less were routinely obtained. In this study we collected seventeen brain tissue samples pathologically identified as PD. *Post-mortem* interval and age-matched normal brain tissues were used as control. In order to avoid ambiguity in Dopal measurements, only cases ($n = 3$) without L-dopa therapy were included in the study. The ages of the subjects were 75 ± 10 years.

Pathological criteria for PD

Pathological diagnosis is based on neuronal loss and astrocytosis of zona compacta of the substantia innominate and eosinophilic intracytoplasmic (Lewy body) inclusion in some of the remaining neurons of these nuclei. Grossly there may be depigmentation or hypopigmentation of substantia nigra and locus ceruleus.

Instrumentation

High performance liquid chromatography (HPLC). A Beckman (Irvine, CA, USA) Model 332 HPLC system equipped with two Model 100 A pumps, a Model 420 microprocessor and a Model 155 variable-wavelength detector was used for analyses. All purifications were carried out using a Waters (Milford, MA, USA) 30 cm × 10 mm I.D., 10 μm particle size, C_{18} μBondapak semipreparative column which was operated isocratically using methanol–water (10:90, v/v). The flow-rate was 1.5 ml/min.

Gas chromatography–mass spectrometry. All electron impact (EI) mass spectra were obtained at 70 eV. The low-resolution mass spectra were acquired with a Finnigan 3200 GC–MS system equipped with a Teknivent (St. Louis, MO, USA) Vector 1 data system. The separation was performed by a 1.6 m × 2 mm I.D. glass column packed with 3% OV-17 on Chromosorb W-HP (Supelco, Bellefonte, PA, USA). The column was temperature-programmed from 180 to 200°C at 10°C/min. A VG-ZAB-SE (VG Analytical, Cheshire, UK) double-focusing mass spectrometer was used to measure the exact masses by scanning at a resolution of 10 000–12 000 and to observe the metastable peaks by scanning the ac-

celerating voltage or by linked scanning of the electrical sector and magnetic field at constant B/E.

Enzymatic preparation of Dopal from dopamine

A partially purified human adrenal monoamine oxidase was prepared according to the method previously reported [21]. To a 30-ml aliquot of the enzyme preparation (pH 7.4 phosphate buffer, protein 1.0 mg/ml), 6.0 mg of dopamine, 0.8 mg of disodium ascorbate and 5.0 mg of catalase were added. The mixture was incubated at 37°C for 1 h and then quick-frozen and lyophilized. The residue was redissolved in 5.0 ml of water and protein was precipitated by the addition of 2.0 ml of 1.0 *M* hydrochloric acid. After the protein was removed by centrifugation at 10 000 *g* for 10 min, the supernatant was adjusted to pH 6.3 with 0.001 *M* NaOH and lyophilized. The residue was again extracted with 3 × 5.0 ml of acetonitrile–acetone (1:1, v/v). The organic layer was combined, evaporated to 2.0 ml and Dopal was separated by thin-layer chromatography (silica gel, methanol–chloroform, 85:15, v/v; Dopal, R_F 0.50; dopamine, R_F 0.4). The product was extracted with 15 ml of acetone, evaporated to dryness under nitrogen, and an EI mass spectrum was taken. The mass spectrum is shown in Fig. 1. The EI mass spectrum of the trimethylsilyl (TMS) derivative of the compound is shown in Fig. 2. High-resolution mass measurements of the molecular ion and major fragment ions of the derivative are given in Table I. Remainder of the product was used for intracerebral injection studies. In the tissue analyses, the TMS derivative of the compound was prepared.

Extraction of Dopal from PD brain tissue

Substantia nigra brain tissue samples (400–500 mg) were individually homogenized in 10 ml of acetone containing 1.5 ml of 1.0 *M* hydrochloric acid, centrifuged at 20 000 *g* for 15 min, and the clear supernatant was separated. The acetone layer was evaporated to 2.0 ml under nitrogen, the pH was adjusted to 4.2 with 0.001 *M* NaOH, and the layer was mixed with 4 × 1.0 ml of acetone–ethyl acetate (1:2) and centrifuged. The combined organic layer was dried, redissolved in 200 μl of acetone and centrifuged. The acetone layer was removed, evaporated to dryness under nitrogen, and the TMS derivative was prepared for GC–MS analysis.

An identical procedure was used for the preparation of the TMS derivative of normal substantia nigra brain tissue extract. The total ion current (TIC) chromatograms of the TMS derivatives of PD brain tissue extract and normal brain tissue extract are given in Fig. 3. The reconstructed ion chromatograms (RIC) of the metabolite and authentic compound are given in Figs. 3 and 4.

Preparation of TMS derivative

After an aliquot (100 μl) of acetone extract was evaporated under nitrogen in a Reactivial, dry pyridine (25 μl) and BSTFA (25 μl) were added. The reaction mixture was kept at room temperature for 2 h before analysis.

Chemical synthesis of Dopal

Dopal was synthesized from norepinephrine according to the method of Robins [22]. After oxidation, the reaction mixture was extracted with ethyl acetate (3 × 5.0 ml). The combined solution was again extracted with 3 × 3.0 ml of 6 *M* sodium bisulfite. The aqueous layer was pooled, acidified with ice-cold 6 *M* hydrochloric acid and extracted with ethyl acetate (3 × 5.0 ml). The ethyl acetate extract was combined, washed with water, dried with sodium sulfate and evaporated to dryness under nitrogen. The residue was dissolved in 2.0 ml of methanol and purified by HPLC. The peak eluting at 4.5 min was collected. Products from multiple injections were pooled, lyophilized, redissolved in acetone and the TMS derivative was prepared for MS analysis.

Assessment of the reactivity of the dopamine metabolite

To a mixture consisting of MAO-B (human adrenal enzyme preparation, 1.0 mg/ml) and 1.0 mg/ml DNA, BSA or tubulin in a total volume of 0.5 ml of Tris–HCl buffer (pH 7.2) was added 50

μM [^{3}H]dopamine (0.25 μCi) to start the reaction. The reaction was incubated at 37°C for 20 min and terminated by the addition of Tris–HCl buffer (pH 6.0) saturated with ethyl acetate. Measurement of the binding was determined by the previously published method [23].

RESULTS AND DISCUSSION

The EI mass spectrum of the compound isolated from dopamine incubation with adrenal MAO-B shows a molecular ion (Fig. 1) at *m/z* 152 (calculated for $C_8H_8O_3$ 152.0473; found 152.0471) and a base peak at *m/z* 123 representing the $[M - HCO]^+$ ion (calculated for $C_7H_7O_2$ 123.0466; found 123.0466). Major ions are also seen at *m/z* 105, 77 and 51 which corresponds to $[M - \{HCO + H_2O\}]^+$, $[M - \{HCO + H_2O + CO\}]^+$ and $[M - \{HCO + H_2O + CO + C_2H_2\}]^+$ respectively. The identities of the fragment ions were confirmed by the daughter ion scan of the molecular ion. High-resolution mass measurement of the *m/z* 150 ion gives a composition of $C_8H_6O_3$ (calculated 150.0317; found 150.0315), indicating that the ion most probably arises from the rearrangement of molecular ion to tropolone aldehyde, after the loss of two protons. This assumption is based on the daughter ion scan of the *m/z* 150 ion which gives ions *m/z* 122, 94 and 66, possibly from successive losses of CO. The expulsion of CO in tropolone has been previously reported [24]. The above information indicates that the metabolite is Dopal.

The mass spectrum of the TMS derivative of Dopal (Fig. 2) shows the molecular ion and base peak at *m/z* 368. High-resolution mass measurement of the molecular ion gives the composition $C_{17}H_{32}O_3Si_3$, corresponding to three TMS groups. This indicates that enolization has occurred during derivatization. The enolization can be further evidenced by the observation of two peaks in the chromatogram (Figs. 3 and 4). The second peak gives a similar mass spectrum (data not shown), possibly representing a *cis* or *trans* isomer. The formation of silyl enol ethers from active methylene compounds like Dopal has been

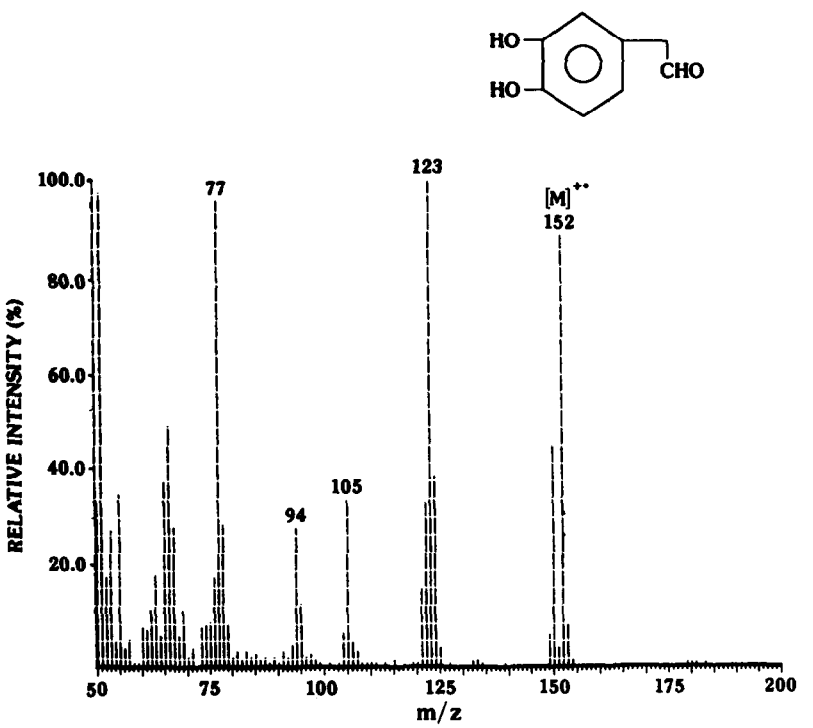

Fig. 1. Mass spectrum of the metabolite isolated from incubation of dopamine with monoamine oxidase B.

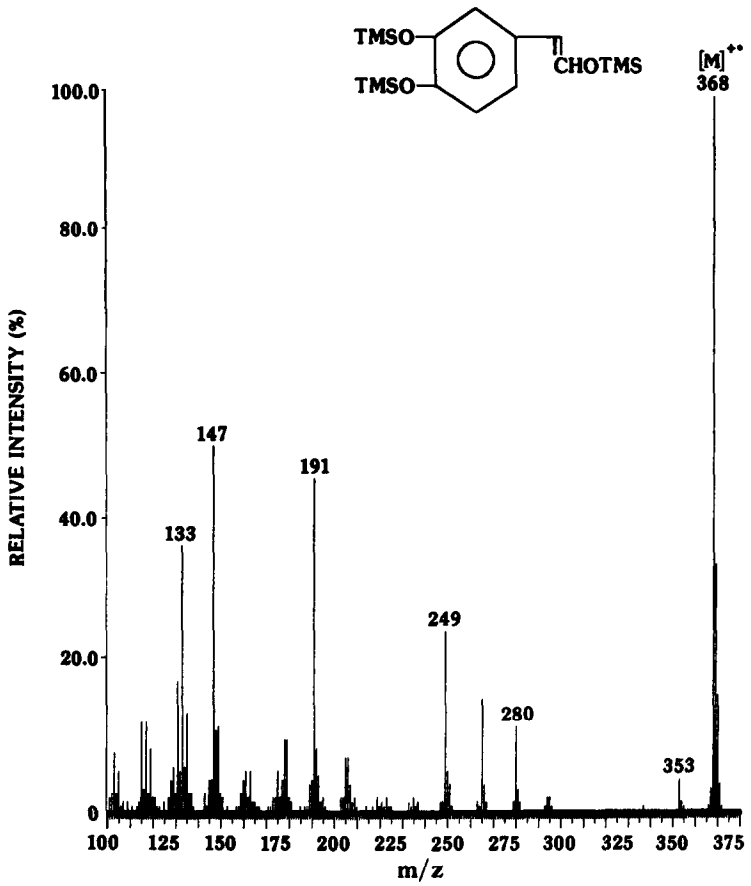

Fig. 2. Mass spectrum of the TMS derivative of the metabolite obtained from incubation of dopamine with monoamine oxidase B.

well documented [25,26]. Table I depicts the most probable structure of the fragment ions which are confirmed by linked scan and high-resolution mass measurement. The above mass spectrum (Fig. 2) is identical to that of the synthetic Dopal (data not shown). There are many possible structures for the ions seen in the mass spectrum and no study has been carried out to confirm the isomeric structures.

The identification of Dopal in parkinsonian brain tissue extract is confirmed by the abundance ratio $M^+/[M-CH_3]^+$ and retention time (t_R) of standard and metabolite under the same GC–MS conditions. Figs. 3 and 4 indicate the RIC of ions m/z 368 and 353 for metabolite (ratio = 16.5; t_R 3.7 min) and authentic compound (ratio = 16.3, t_R 3.7 min), respectively. However, the RIC (data not shown) of the same ions plotted from normal brain tissue extract failed to show the presence of Dopal. It should be mentioned that only cases without L-Dopa therapy were included in the study.

Dopal reacts with dopamine non-enzymatically to form 6,7-dihydroxy-1-(3,4-dihydroxybenzyl)isoquinoline (THP). THP has been found in the *in vitro* incubation of dopamine with MAO-B and in the urine of parkinsonian patients receiving L-Dopa therapy [27–29]. Hence, Dopal can be formed intraneuronally and can be built up in neurons in the absence of an efficient removal mechanism. The decline in the level of detoxifying enzymes [30,31] and the increased turnover of dopamine due to hyperactiveness in the neurons of PD patients [8] suggested that a large amount

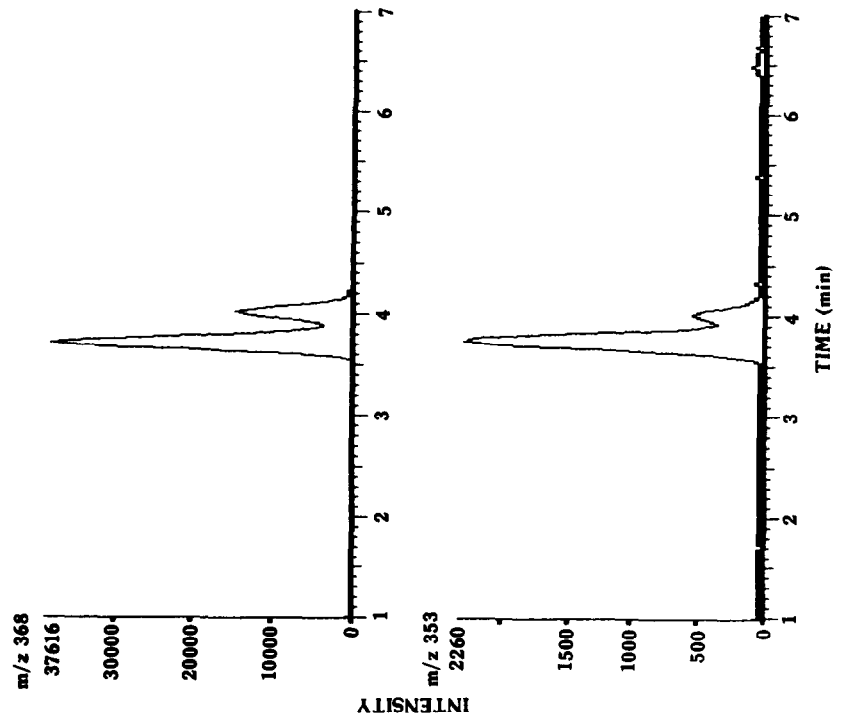

Fig. 3. Reconstructed ion chromatogram (*m/z* 368 and 353) for 3,4-dihydroxyphenylacetaldehyde obtained from parkinsonian brain tissue extract.

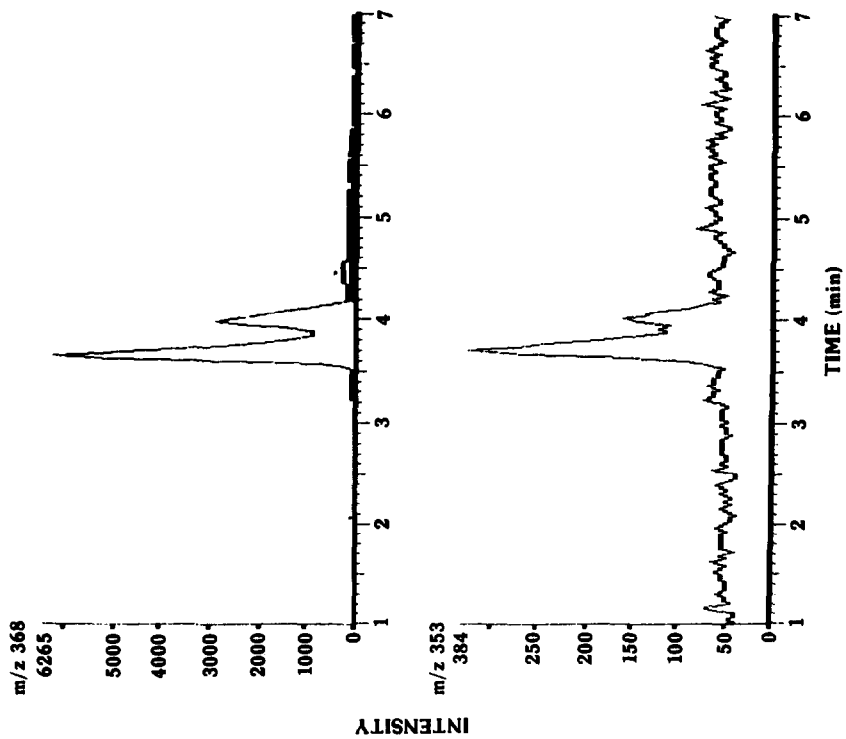

Fig. 4. Reconstructed ion chromatogram (*m/z* 368 and 353) for authentic 3,4-dihydroxyphenylacetaldehyde.

TABLE I

HIGH-RESOLUTION MASS MEASUREMENTS OF THE TMS DERIVATIVE OF 3,4-DIHYDROXYPHENYLACETALDEHYDE

Proposed structure	Formula	Calculated	Found
$M^{+\cdot}$	$C_{17}H_{32}O_3Si_3$	368.1659	368.1660
$[M - CH_3]^+$	$C_{16}H_{29}O_3Si_3$	353.1424	353.1430
$[M - TMS]^+$	$C_{14}H_{23}O_3Si_3$	295.1186	295.1187
$[M - (CH_3 + TMS)]^+$	$C_{13}H_{20}O_3Si_2$	280.0951	280.0954
$[M - (CH_3 + TMS + CH_3)]^+$	$C_{12}H_{17}O_3Si_2$	265.0716	265.0710
$[M - (CH_3 + TMS + CH_3 + CH_4)]^+$	$C_{11}H_{13}O_3Si_2$	249.0403	249.0400
$[M - (CH_3 + TMS + CH_3 + TMSH)]^+$	$C_9H_7O_3Si$	191.0164	191.0170

of Dopal may be produced and accumulated, and may be present in PD brain tissue in a measurable quantity. Indeed, our results demonstrate that Dopal is present. In the normal brain tissues, however, it appears to be beyond the detection limit of our study.

The amounts of covalent binding of the [2,5,6-^{3}H]dopamine metabolite to BSA, DNA and tubulin were 0.62 ± 0.11, 1.21 ± 0.15 and 1.31 ± 0.12 (blank value was 0.10 ± 0.05 for BSA and 0.20 ± 0.10 for DNA and tubulin, n = 5), respectively. These results indicate that incubation of dopamine with MAO-B resulted in a four- or five-fold increase in covalent binding to DNA, BSA and microtubule protein. Although this observation does not prove that the binding itself results in cell death, the reaction of the metabolite with tissue nucleophiles would interfere with normal cellular function, and therefore can be detrimental to neurons. Our preliminary studies also demonstrate that intracerebral injection of Dopal (5 μg) into substantia nigra of rats is toxic to dopaminergic neurons and produces tissue damage (unpublished data).

Dopal has never been identified in parkinsonian brain tissue by MS. In this report, we are able to utilize chemical synthesis and MS techniques to demonstrate the endogenous neurotoxic dopamine metabolite in the substantia nigra of parkinsonian brain tissue.

ACKNOWLEDGEMENTS

This work was supported by the Department of Veterans Affairs. Mass spectra were obtained at the Center of Mass Spectrometry Resource, Washington University School of Medicine which is supported by NIH Grants RR-00954 and AM-20579.

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