Isolation and Structural Identification of 9-Hydroxy-9-desmethyl-cyclosporine

Larry D. Bowers, Debra D. Norman, Xing-Xian Yan, Douglas Scheeler, and Kathy L. Carlson

A metabolite of cyclosporine has been isolated and its structure identified through use of HPLC and tandem mass spectroscopy. Fast atom bombardment mass spectrometry of an HPLC fraction co-eluting with 9-hydroxy-cyclosporine (M17) indicated that the mass of this metabolite was 2 Da greater than that of cyclosporine. Further isolation by HPLC yielded a pure fraction, which we analyzed with tandem mass spectrometry. Linear acyl fragment ions originating from the metabolite under collision-induced dissociation were consistent with the difference in mass being associated with amino acid 9 in the cyclosporine backbone. We propose a nomenclature system for future discussion of cyclosporine metabolites.

Additional Keyphrases: chromatography, reversed-phase, mass spectrometry, drug metabolites, immunosuppressant drugs

The impact of cyclosporine (CsA) (Figure 1) on transplantation has been well documented. Despite years of experience with the drug, numerous questions remain with regard to its use, including the seemingly poor correlation between its concentration in blood or plasma and its therapeutic or toxic effect, the mechanism of its action, and the mechanism of its toxicity. These questions persist, at least in part, because of the lipophilicity of CsA and the extensive metabolism that it undergoes. The total number of proposed metabolites now exceeds 30, although fewer than six appear to be present in the blood in significant amounts.

Rational study of the activity and toxicity of metabolites requires that their structure and purity be known, and that amounts sufficient for cell culture or animal studies be prepared. The stability and storage characteristics of the materials need to be known so that the material tested can be certified as the material of interest. Controversies surrounding the nature of the metabolites exist, extending even to their molar absorptivity, a characteristic needed in determining the amount of metabolite added to the test system. Some of the controversy apparently originates from the use of materials that are not fully purified and characterized. For example, Zeevi et al. (1) indicate that the material used in their study contained about 10% impurities.

Several techniques have been used to assign structures to CsA metabolites. Maurer et al. (2, 3) made use of fast atom bombardment mass spectrometry (FAB-MS) to determine molecular masses of the metabolites isolated from dog bile and relied heavily on nuclear magnetic resonance (NMR) to make structural assignments. The difficulty with this approach is that about 0.1 mg of sample is required to obtain useful NMR data, which limits the usefulness of the method to those metabolites present in high concentrations in biological fluids. As pointed out by Hartman and Jardine (4), FAB-MS alone can provide some structural information. In addition to the protonated molecular ion, another fragment, \[{}^{[M+H-113]}\], formed from the loss of the C2H2O side chain of N-methyl-4-(2-butenyl)-4-methylthreonine (MeBmt), is observed. Thus, modification of the side chain of MeBmt should be distinguishable from modification of any other amino acid. Unfortunately, modification at other amino acid sites cannot be distinguished from each other by FAB-MS alone. Nevertheless, some investigators have used FAB-MS to determine the structure of their isolated materials. For example, Wallenmaq et al. (5) used FAB-MS to identify metabolites isolated from rabbit and human bile.

FAB-MS can be used in conjunction with other techniques to determine the structural identity of CsA metabolites. Hartman and Jardine (4) used FAB-MS and partial hydrolysis of the metabolite followed by derivatization and analysis by gas chromatography (GC)/MS to identify the carboxylic acid metabolite of CsA. The latter technique requires relatively large amounts of material. In our hands, 100 µg of metabolite and three days of derivatization were required before a GC/MS analysis could be done.

Tandem mass spectrometry (MS/MS) has also been

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1 Nonstandard abbreviations: CsA, cyclosporine; FAB-MS, fast atom bombardment mass spectrometry; GC, gas chromatography; MS/MS, tandem mass spectrometry; MeBmt, N-methyl-4-(2-butenyl)-4-methyl-threonine; Abu, \(\alpha\)-aminobutyric acid; Sar, sarcosine; MeLeu, N-methyl-leucine; MeVal, N-methyl-valine; \(k^*\), capacity factor; and NMR, nuclear magnetic resonance.

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widely used for peptide and protein sequencing, particularly when post-translation modifications have occurred (6). We recently reported our efforts to develop an MS/MS method for sequencing CsA and its metabolites (7). Using structural analogs of CsA, we were able to identify collision-induced fragment (daughter) ions consisting of from one to 10 amino acids. On the basis of mass shifts observed for multiple daughter ions containing the modified amino acid, we could verify the identity of several known metabolites of CsA.

FAB-MS can also be of value in indicating the purity of a preparation (8-10). The finding (8) of two ions in the FAB-mass spectrum of material thought to be pure 1-hydroxy-CsA (M17) initiated the present investigation. The ions were of m/z 1205 and 1219, the latter corresponding to the calculated mass for 1-hydroxy-CsA. The former ion was not likely to originate from the latter, which suggested to us the presence of another metabolite co-eluting in the HPLC fraction with 1-hydroxy-CsA. We report here our efforts to identify this new metabolite.

Materials and Methods

Diethyl ether, methanol, acetonitrile, and KH2PO4 were all HPLC grade (Fisher, Fair Lawn, NJ) and used as received; glacial acetic acid was ACS reagent grade (Fisher). The CsA standard used for the chromatographic standard and the MS/MS studies was obtained from Sandoz (Dr. Winter, East Hanover, NJ), as was a small amount of metabolites 17 and 1 (Dr. Maurer, Basel, Switzerland), used to verify retention times in our chromatographic system.

Human bile from T-tube drainage was extracted as reported earlier (8), with few modifications. We used diethyl ether for the initial extraction instead of ethyl acetate, and obtained a cleaner chromatogram with no apparent lose of metabolite. The final extract was dried and reconstituted in methanol.

Semi-preparative-scale HPLC was carried out with a Varian (Walnut Creek, CA) Model 5560 solvent delivery system, and a DuPont (Wilmington, DE) Model 870 column oven set at 70 ºC and containing a 10 x 250 mm Supelco (Bellefonte, PA) LC-18 preparative HPLC column. Detection was accomplished with a Varian UV-1 detector at 210 nm. Solvent A consisted of a mixture of acetic acid (glacial acetic acid, 10 mL/L, pH 2.7) and methanol (80/20 by vol). Solvent B was a mixture of acetonitrile and methanol (80/20 by vol). The flow rate was 7.0 mL/min. We used the following gradient conditions: initial condition, 42% solvent B, hold for 5 min; ramp to 53% B at 30 min, 80% B at 55 min, 100% B at 60 min; and return to initial conditions at 70 min. One-minute fractions were collected throughout the gradient. Reference points were established with use of purified metabolites supplied previously by Sandoz. The elution times were 1-carboxy-CsA (M203-318), 31 min; 1-hydroxy-CsA (M17), 36 min; 9-hydroxy-CsA (M1), 37 min; and CsA, 43 min.

Fractions collected from the gradient separation during the 36- and 37-min intervals were extracted with diethyl ether, dried under a stream of N2, and reconstituted with 100 µL of methanol followed by 100 µL of water. The fractions were further purified by using the same column and instrumentation, but with reversed-phase isocratic conditions with the same solvents (A/B) at a volume ratio of 53/47. The flow rate for isocratic separation was 3.0 mL/min. Fractions were manually collected according to the peak profile. The metabolites were extracted from the solvent with diethyl ether, dried at room temperature under a stream of N2, and immediately reconstituted in 500 µL of methanol. The quantity of the metabolites was assessed by gradient elution on either a 4 x 150 mm Varian MCH-5 column or 4.6 x 150 mm Supelco LC-18 column, as described earlier (11).

The molecular mass of the isolated material was determined by FAB-MS with either a Model 7070E-HF mass spectrometer (VG Analytical, Manchester, U.K.) or a Model MS60 (Kratos Analytical, Manchester, U.K.). Both were equipped with standard FAB sources (Xe 8-kV saddle field gun). The matrix used for FAB was a mixture of dithiothreitol and dithioerythritol for positive-ion spectra and triethylamine for negative-ion spectra. About 10 µg of metabolite was required for adequate sensitivity in the FAB MS experiments.

For structural identification of the isolated material, we used either a Sciex TAGA 6000E MS/MS or an API III MS/MS (Thornhill, Ontario, Canada). Methanolic solutions of the fractions of interest were introduced continuously by an ion-spray interface (12) in conjunction with the atmospheric pressure ionization source. The rate of introduction was approximately 200 ng/min for a total time of about 12 min. Collision-induced dissociation was achieved with xenon or argon gas and a collision energy of 90 eV. The daughter-ion spectrum was scanned from 40 to 1250 m/z at a rate of 0.050 s/ mass unit. At least three spectra were averaged to obtain a final daughter-ion scan. Daughter-ion fragments were identified on the basis of studies of substituted CsA analogs (7).

Results and Discussion

Identification of the Metabolite Structure

A typical preparative HPLC chromatogram (Figure 2) contained many fractions that cross-reacted with a nonselective monoclonal RIA. On the basis of previous studies...
(8), the fraction from 35–37 min was collected and rechromatographed under isocratic conditions. We observed three peaks: the first corresponded to 1-hydroxy-CsA (k’ = 12.7) and the last to 9-hydroxy-CsA (k’ = 14.0). We achieved baseline resolution of the unknown compound from 1-hydroxy-CsA and 9-hydroxy-CsA (Figure 3). Thus we were able to collect pure material. The fact that we were able to enrich the contents of the fractions also reinforced the fact that a separate material did exist. FAB-MS analysis of the purified material yielded a molecular mass of 1204.6 Da, suggesting either demethylation and hydroxylation of CsA or reduction of the double bond on MeBmt. The presence of a fragment at m/z 1091 suggests that all modifications are on the ring, and not on the side chain of MeBmt. Negative-ion FAB-MS gave a similar 2-mass-unit difference.

We analyzed a sample of the purified metabolite by continuous-flow ion-spray atmospheric pressure ionization MS/MS. The pattern of daughter ions was compared with ions produced by CsA and a number of analogs and metabolites of known structure (7). Changes in the mass of a daughter ion indicate a modification to an amino acid in that peptide fragment. By studying all fragments containing a particular amino acid, we could confirm or rule out the modification of that amino acid. For example, hydroxylation of MeBmt would require a 16-Da increase (or, because of the loss of water, a 2-Da decrease) in mass for all daughter ions containing MeBmt. The daughter-ion spectra (Figure 4) and listing of the peptide fragments responsible for them (Table 1) are given for both CsA and the unknown metabolite. Close inspection of Figure 4 and Table 1 clearly shows that the site of metabolism on the unknown metabolite is not MeBmt (m/z 156, 184, 269, 340, etc.), which supports the FAB-MS findings. This lack of modification would rule out reduction of the double bond, or hydroxylation or demethylation on this amino acid.

Examination of the spectra for other sites of substitution also rules out modification of 4-MeLeu, the only previously reported site of demethylation. It should be pointed out that 4-N-desmethyl-CsA shows a 14-Da decrease in all daughter ions that contain an amino acid (7). Similarly, daughter ions containing 2-Abu, 3-Sar, 4-Val, 5-MeLeu, 7-Ala, and 8-Ala are similar in mass for both CsA and the unknown metabolite. Daughter ions containing 9-MeLeu, however, are in all cases modified. The most obvious change in the spectrum is at m/z 224, which is essentially missing from the metabolite spectrum. We had previously shown that m/z 224 arises from 9-MeLeu-10-MeLeu (7). The difference in mass would be expected to give rise to an ion at m/z 226, and although there is a change in the spectrum in this area relative to the CsA spectrum, the intensity does not reflect the missing

Fig. 3. Chromatogram of preparative HPLC fraction 36 obtained under isocratic conditions

The peak eluted at 32.3 min corresponds to 1-OH-CsA (M17) and that at 35.2 min to 9-OH-CsA (M1), as determined by injection of standard material. The contents of the center peak were isolated and characterized as described in the text.
m/z 224 ion. There is a significant increase in the m/z 210 fragment, but the data are insufficient to conclude that the increase is from the 9MeLeu-10MeLeu dipeptide. In the remainder of the spectrum, daughter peptide fragments arising from 6MeLeu-7Ala-9MeLeu (m/z 397), 6MeLeu-7Ala-9MeLeu-10MeLeu (m/z 524), 9MeLeu-7Ala-9MeLeu-10MeLeu (m/z 637), and 3Ser-9MeLeu-7Ala-9MeLeu-10MeLeu (m/z 822) are all increased by 2 Da, further implicating the modification of 9MeLeu. We emphasize that other daughter ions containing 9MeLeu (m/z 322, 425) do not show mass shifts; therefore, the modification cannot be on 9MeLeu.

To verify the identity of the masses of fragments and to support the universality of the tandem MS approach, we obtained daughter-ion spectra with a Kratos MS 50 three-sector MS/MS. All of the ions present in the daughter spectra of the tandem quadrupole were also present in the spectra obtained on the magnetic sector instrument. The intensities of the ions were different, as expected, with the magnetic instrument showing higher intensities at high mass. Also, because of the energy distribution of the daughter ions produced in the high-energy collision chamber, the resolution on the three-sector instrument was inferior to the quadrupole, making exact mass assignment of some fragments impossible on the former instrument.

The data are consistent with hydroxylation and demethylation on 9MeLeu. Determination of the carbon atom on which the modification occurs is possible only by using NMR spectroscopy. We are currently isolating sufficient amounts of the metabolite to obtain confirmation of the structure by 1H and 13C NMR. Maurer et al. (2) described the loss of the N-methyl group only from 9MeLeu; no other demethylation sites were reported. A metabolite was described with a mass of 1204 Da with the designation M13.

Preliminary proton NMR spectroscopy evidence indicated that the structure was hydroxylated on 9MeLeu and demethylated on 9MeLeu. The assignment was made in the absence of 13C NMR data, which would have confirmed the structure. In any case, M13 does not appear to be the same compound that we report here because HPLC elution is quite different. In Maurer's system, M13 elutes with the dihydroxylated metabolites, considerably before 14OH-CsA. In our HPLC system, which is similar to Maurer's, the demethylated, hydroxylated metabolite reported here elutes with 14OH-CsA (M17). Maurer et al. identified a second hydroxylated, demethylated metabolite designated M25. This molecule was demethylated on 9MeLeu and hydroxylated on 9MeBmt. Although there are no chromatographic data from their study, we present strong evidence above that the material we isolated is not modified on either of those amino acids.

The clinical importance of 9OH,9-desmethyl-CsA is unclear. Our findings indicate that this metabolite will coelute with 14OH-CsA in many analytical HPLC systems, thus making the measurement of the latter compound inaccurate. In fact, we have assayed bile from a number of patients and find different relative amounts of 14OH-CsA and 9OH,9-desmethyl-CsA. We have not extended the analysis to blood or plasma. Preliminary results indicate that 9OH,9-desmethyl-CsA is not toxic in a renal cell culture model system (13). The activity of the compound awaits further testing.

Nomenclature for CsA Metabolites

The role of CsA metabolites in the activity and toxicity associated with CsA therapy is the subject of intensive research. To develop structure/function relationships, one must document the structural identity of the various metabolites. The results of activity and toxicity investigations must then be compiled, and correlations established. The present system of nomenclature is an impediment to such work. The original work of Maurer et al. (2) used a numerical system, the foundation of which remains obscure because in reversed-phase HPLC M17 and M1 elute close together. Subsequent investigators have proliferated their own nomenclature such as R20 (5), MUNDF1 (14), and H2830 (9). Although this serves the function of reporting the preliminary identification of a new metabolite, it poses a problem for comparing research results and determining whether the above three compounds isolated in three different chromatographic systems are the same compound. This problem is exaggerated by the lack of pure metabolite from any source. More importantly, this approach frustrates the development of the aforementioned structure/function relationships. A proposed system to use chromatographic retention times (9) to identify metabolites is fraught with difficulties. As has been shown by Snyder et al. (15, 16), elution time under gradient conditions is a function of several variables, including the rate of solvent strength change, the column size, and the flow velocity. In addition, the differences in columns present a major impediment to this approach. For example, different semi-preparative LC-18 columns obtained from Supelco over the last year showed significant differences in retention time, despite the fact that for a hydrophobic, non-amide compound like cyclosporine one would expect minimal differences in columns. This is also borne out by the work of Rocher et al. (17), who showed significant selectivity differences for CsA and an interference among columns.

We propose here a structural nomenclature system, extending an approach originated by Maurer and Lemaire (3). In this system, the location of the modification of the CsA structure is denoted in two ways. The amino acid modified is denoted by its positional number in the CsA ring. By convention, MeBmt is given the designation of amino acid one. The location of the modification on the amino acid is indicated with standard nomenclature describing the carbon atoms by Greek letters. The carbon atom attached to the carboxyl function is designated the α carbon. This nomenclature is summarized for the relevant amino acids in Figure 1 (right). Both the number and position of the modification would appear as a superscript before the nature of the modification—either hydroxylation or demethylation. If the double bond of MeBmt is reduced, the structure is designated "dihydro" CsA. The names of the known metabolites, along with references to data supporting the structural identification, are summarized in Table 2.

With this approach, a structure/function relationship can begin to be formulated based on the ease with which the site of modification can be located. The work of Quineaux et al. (18) and of Tropschung et al. (19) suggests that binding of compounds to cyclophilin correlates with their immunosuppressive activity. Quineaux et al. found that 14OH-CsA bound to cyclophilin with affinity equivalent to that of CsA, whereas 14COOH-CsA and 9-nor-CsA bind much less strongly. Their results indicate the importance to binding of the amino acids in the 11–2 positions. With the proposed nomenclature system, the location of the modified amino acids will be readily apparent.

After submission of this manuscript, an abbreviated
nomenclature was presented at the Hawk's Cay Meeting on Therapeutic Drug Monitoring of Cyclosporine. This system is based on the approach outlined above, but uses only the number of the amino acid oxidized. The Hawk's Cay nomenclature is also given in Table 2.

We particularly thank Dr. Jack Henion of Cornell University and Dr. Tom Covey of Sciex Instruments, who provided access to the tandem mass spectrometer. Dr. Phil Lyon of 3M Corporation graciously provided FAB-MS data on the Kratos MS-50 and obtained the three-sector MS/MS data both at 3M and at the University of Nebraska NSF mass spectrometry center. Dr. Nancy Ascher, Dr. Dan Canafax, the surgery residents and fellows, and the staff of the University of Minnesota Transplant Program provided bile samples from live transplant patients.

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References


Table 2: Systematic Nomenclature for Cyclosporine Metabolites

<table>
<thead>
<tr>
<th>Proposed name</th>
<th>Abbreviation</th>
<th>Previous name</th>
<th>H.C.* name</th>
<th>Mass, De</th>
<th>Techniques used to characterize</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>$^{1}{H}$Hydroxy-cyclosporine</td>
<td>$^{1}{OH}$-CsA</td>
<td>M17</td>
<td>AM1</td>
<td>1218.64</td>
<td>NMR, MS, GC/MS, MS/MS</td>
<td>2, 4, 5, 7</td>
</tr>
<tr>
<td>$^{1}{H}$Hydroxy-$^{1}$tetrohydrofuryl-cyclosporine</td>
<td>$^{1}{OH}$-$^{1}$THF-CSA</td>
<td>M18</td>
<td>AM1c</td>
<td>1218.64</td>
<td>NMR, MS, MS/MS</td>
<td>2, 7</td>
</tr>
<tr>
<td>$^{1}$Carboxy-cyclosporine</td>
<td>$^{1}$COOH-CSA</td>
<td>203-218</td>
<td>AM1a</td>
<td>1232.62</td>
<td>GC/MS</td>
<td>4</td>
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<tr>
<td>$^{4}$N-Desmethyl-cyclosporine</td>
<td>nor-CSA</td>
<td>M21</td>
<td>AM4N</td>
<td>1188.62</td>
<td>NMR, MS, MS/MS</td>
<td>2, 4, 7</td>
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<tr>
<td>$^{6}$Hydroxy-cyclosporine</td>
<td>$^{6}{OH}$-CsA</td>
<td>—</td>
<td>AM6</td>
<td>1218.64</td>
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<td>3</td>
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<td>M1</td>
<td>AM9</td>
<td>1218.64</td>
<td>NMR, MS, GC/MS, MS/MS</td>
<td>2, 4, 7</td>
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<tr>
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<td>1234.62</td>
<td>NMR, MS, GC/MS</td>
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<td>$^{10}{OH}$-$^{9}$THF-CSA</td>
<td>M26</td>
<td>AM1c,9</td>
<td>1234.64</td>
<td>—</td>
<td>3</td>
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<td>$^{4}{OH}$-CsA</td>
<td>M10</td>
<td>AM4,9</td>
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<td>2</td>
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<td>$^{6}{OH}$-$^{5}$OH2-CSA</td>
<td>M16</td>
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<td>NMR, MS, GC/MS</td>
<td>2, 3</td>
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* Nomenclature presented at Hawk's Cay Meeting. *p No data included on characterization methods.