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THERMOSPRAY LIQUID CHROMATOGRAPHIC–MASS SPECTROMETRIC ANALYSIS OF CASTANOSPERMINE-RELATED ALKALOIDS IN *CASTANOSPERMUM AUSTRALE*

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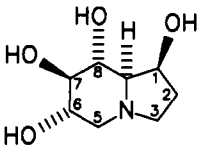
ABSTRACT.—Crude sample solution prepared from the seeds of *Castanosperum australe* was fractionated by preparative liquid chromatography. The fractions thus obtained were subsequently analyzed by means of thermospray liquid chromatography-mass spectrometry (tsp lc-ms). Under the conditions employed, castanospermine [**1**], 6-*epi*-castanospermine [**2**], australine [**3**], 3,8-di-*epi*-alexine [**4**], and fagomine [**5**] could be separated and detected. The possible structures of other castanospermine-related alkaloids found in the crude sample solution are also discussed.

Naturally occurring polyhydroxylated indolizidine (1–3), pyrrolizidine (4–6), piperidine (7), and pyrrolidine (8) alkaloids recently discovered in *Castanospermum australe* A. Cunn. (Leguminosae) and other plants have generated great interest because of their highly potent inhibitory effects on a variety of enzymes such as glucosidases (9), manosidases (10), human immunodefficiency virus (HIV) (11), retroviruses (12), and tumor cells (13). The biologically active polyhydroxylated alkaloids that have been reported in the seeds of *C. australe* are castanospermine [**1**] (the major constituent), 6-*epi*-castanospermine [**2**], australine [**3**], 3,8-di-*epi*-alexine [**4**], and fagomine [**5**]. The isolation and purification of those biologically active alkaloids from the plant material are normally performed by a consecutive procedure of solvent extraction, ion exchange column isolation, and preparative centrifugal tlc purification.

Because of its high speed, efficiency, and resolution, as well as the availability of various detection systems, hplc has become a dominant technique in analytical separations. However, to date this technique has not been applied to analysis of those biologically active alkaloidal constituents in *C. australe* and other plants. The main reason might be lack of a chromophore in the structure of those compounds, which makes them very difficult to detect with common photometric detection.

However, because of the high proton affinity characteristic of nitrogen-containing



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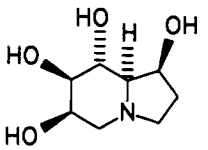

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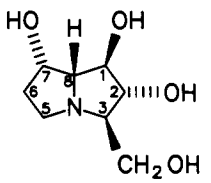

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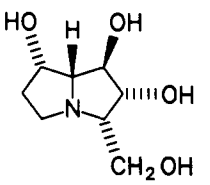

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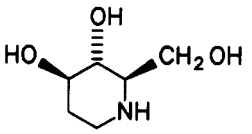

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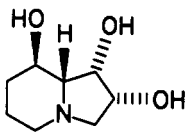

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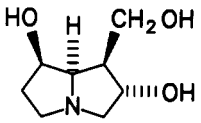

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compounds, the method of thermospray liquid chromatography-mass spectrometry (tsp lc-ms) for the analysis of castanospermine and its structurally related analogues should be very sensitive. In addition, it can provide molecular weight information. Based on these unique capabilities, we sought to use tsp lc-ms to analyze castanospermine and its structurally related alkaloids present in the seeds of *C. australe*.

EXPERIMENTAL

PLANT MATERIAL.—Seeds of *C. australe* (Fruit Spirit Botanical Garden, Dorroughby, Australia 2480) were collected, ground, and extracted with EtOH-H_2O-diatomite (12:16:1) for 2 h at ambient temperature. The seeds were then filtered with a polypropylene (20 μ) filter bag. The extraction filtrates were treated with Dowex HCR-W2-H cation exchange resin to retain the desired products on the resin. After centrifugation, the products were removed from the resin by washing with 2N NH_4OH. The basic eluate was then concentrated by atmospheric distillation and mixed with EtOH to make an EtOH-H_2O (90:10) solution. This solution was further cooled to 5° to crystallize castanospermine and finally filtered with the resulting crude sample solution collected.

FRACTIONATION OF CRUDE SAMPLE SOLUTION BY PREPARATIVE LC.—The crude sample solution was fractionated by preparative lc that included a SepTech ST/800C preparative liquid chromatograph, a Phenomenex IB-SIL 5 NH_2 column (250 × 22.5 mm i.d.), and a step gradient mobile phase consisting of MeCN-H_2O (90:10) as mobile phase A and MeCN-H_2O (50:50) as mobile phase B. Two ml of crude sample solution (containing about 300 mg of solids) diluted to 10 ml with MeCN-H_2O (80:20) was used for each injection. The column was first eluted with mobile phase A at a flow rate of 40 ml/min for 21.5 min, then with mobile phase B using a step gradient for an additional 6.5 min. A total of six fractions was collected according to the elution volumes, i.e., fraction 1, 0–200 ml; fraction 2, 200–360 ml; fraction 3, 360–460 ml; fraction 4, 460–660 ml; fraction 5, 660–860 ml; and fraction 6, 860–1120 ml (mobile phase B elution). The collected fractions were taken to dryness using a rotary evaporator operated at 25° and 3 mm Hg.

TSP LC-MS ANALYSIS.—Tsp lc-ms system used included a Waters 600-MS multisolvent delivery lc system and a Hewlett-Packard 5970 mass-selective detector that was mounted in a Vestec 101 tsp interface. A Hewlett-Packard 59970 ChemStation was used to control the instrument and acquire the ms data.

Fractions 1–6 collected from the crude sample solution by preparative lc were chromatographed further on a Whatman Partisil 10 SCX column (250 × 4.6 mm i.d.) using MeCN-H_2O (5:95) containing 0.015 N of $HCOONH_4$ as mobile phase and operating at 1 ml/min. The temperatures of the tsp vaporizer and ion source block were controlled at 175° and 285°, respectively. The $[NH_4]^+$ ion present in the mobile phase was used to generate tsp ionization.

The compounds isolated from fraction 6 were reanalyzed by different tsp lc-ms conditions: the Whatman Partisil 10 SCX column was replaced by an amino column of IBM Carbo (250 × 4.6 mm i.d.) and a mobile phase of MeCN-H_2O (80:20) operated at 1 ml/min. The temperatures of the tsp vaporizer and ion source block were controlled at 135° and 275°, respectively. The filament-on mode was used to provide tsp ionization.

RESULTS AND DISCUSSION

Since its introduction by Blakley and Vestal (14) and commercial availability in

early 1985, the tsp interface has become a very reliable interface used for on-line lc-ms. The recent refinements in tsp ion source design and the availability of inexpensive dedicated ms systems have made possible the routine application of tsp lc-ms to direct identification of organic compounds in a complex mixture. This technique has also proved to be very sensitive to nitrogen-containing compounds using the $[NH_4]^+$ ion to generate tsp ionization (15). For instance, tsp lc-ms was capable of detecting castanospermine at the high picogram level using single ion monitoring (sim) detection as shown in Figure 1. Under the same chromatographic conditions, the minimum detection of castanospermine using photometric detection at 210 nm was only in the submicrogram region.

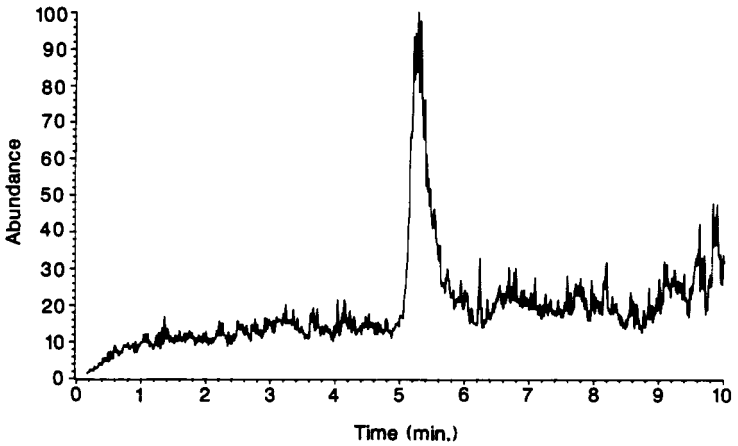

FIGURE 1. Sims (*m/z* 190) detection of castanospermine [**1**] at 500 picograms.

In order to simplify the crude sample solution for tsp lc-ms analysis, the solution was first fractionated by preparative lc on a Phenomenex IB-SIL 5 NH_2 column. Six fractions were collected based on their elution volumes and subsequently analyzed by tsp lc-ms using a cation exchange column of Partisil SCX for separation. Mass spectral detection was operated in a scan mode (*m/z* 120–600). Except for fraction 1, all fractions were found to contain compounds structurally related to castanospermine. Due to the "soft" ionization characteristic of tsp lc-ms, the compounds of interest exhibited only protonated molecular ions in the spectrum, and no fragment ions were observed.

Four types of major components were found to be present in fraction 2; they exhibited $[MH]^+$ ions at *m/z* 132, 148, 174, and 190, as shown in Figure 2. The major peak at *m/z* 190 was suggested to be 6-*epi*-castanospermine [**2**] by comparison of the lc retention time with an authentic sample. Two peaks at *m/z* 148 were the stereoisomers of fagomine [**5**]. Fagomine had been discovered in *C. australe* and *Xanthoceris zambesiaca* (4,7). Based on the $[MH]^+$ obtained, two peaks at *m/z* 174 corresponded to trihydroxyindolizidine and hydroxymethyl-dihydroxypyrrolizidine alkaloids such as swainsonine [**6**] and rosmarinecine [**7**]. However, to date, swainsonine and rosmarinecine alkaloids have not yet been reported to occur in the seeds of *C. australe*. Compounds corresponding to the peaks at *m/z* 132 might possess one hydroxyl group less than fagomine. These compounds are probably not biologically interesting because it has been postulated that polyhydroxylated alkaloids require at least three hydroxyl groups located in β position relative to the nitrogen atom to possess glycosidase inhibitory properties (16).

Fraction 3 mainly contained 6-*epi*-castanospermine [**2**] and a compound that had a mol wt corresponding to that of fagomine [**5**], as shown in Figure 3. However, no au-

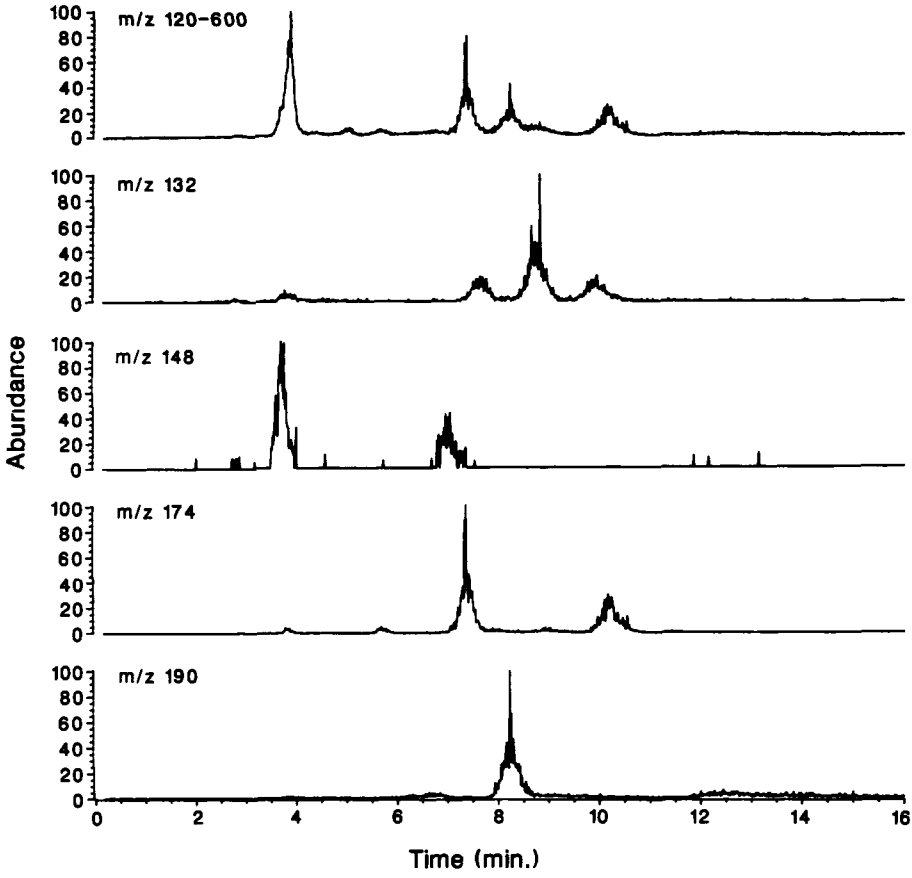

FIGURE 2. Tsp lc-ms analysis of fraction 2: the peak at *m/z* 190 = 6-*epi*-castanospermine [**2**].

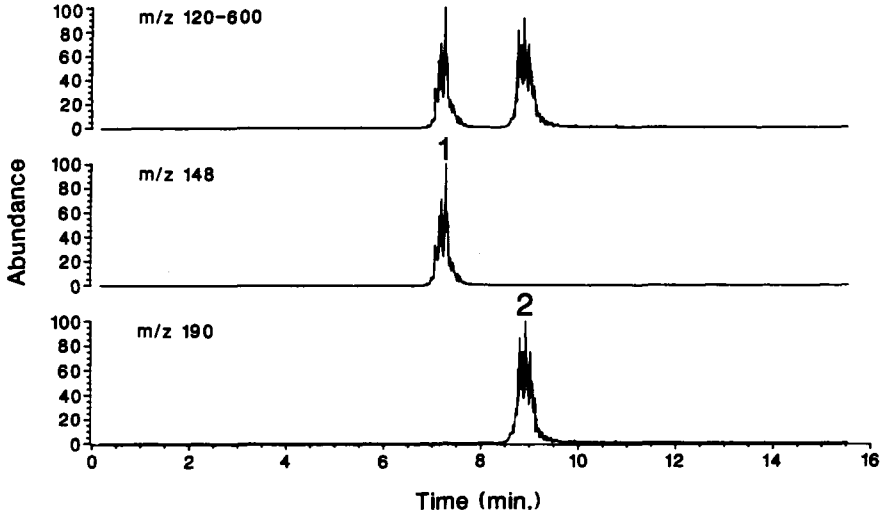

FIGURE 3. Tsp lc-ms analysis of fraction 3: peak 1 (*m/z* 148) = fagomine [**5**] (proposed); peak 2 (*m/z* 190) = 6-*epi*-castanospermine [**2**].

thentic sample of fagomine was available for chromatographic comparison and confirmation.

A large quantity of castanospermine [**1**] was found to dominate in fraction 4, as shown in Figure 4. This indicates that a high concentration of castanospermine still remained in the crude sample solution after solvent crystallization. The solvent crystallization procedure described in this report was capable of generating highly pure castanospermine with an overall yield equal to 0.47% of the wet wt of nuts. Fraction 4 also contained two minor peaks that had been described in fraction 3.

Fraction 5 contained two major components with an $[MH]^+$ at *m/z* 190 as shown in Figure 5. They corresponded to castanospermine [**1**] and australine [**3**] based on comparison of the lc retention times with authentic samples. This fraction also contained a small amount of a compound that exhibited an $[MH]^+$ at *m/z* 206, which is 16 daltons higher than that of castanospermine. This indicates that the compound at *m/z* 206

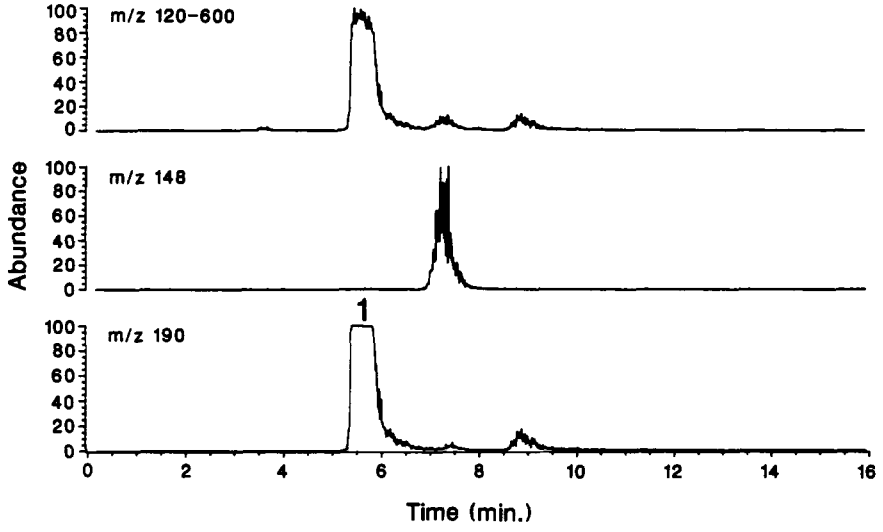

FIGURE 4. Tsp lc-ms analysis of fraction 4: peak 1 (*m/z* 190) = castanospermine [**1**].

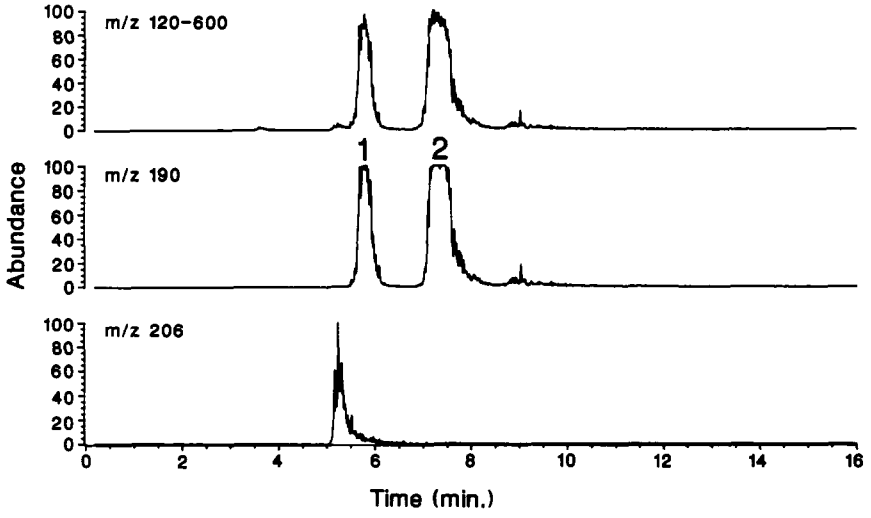

FIGURE 5. Tsp lc-ms analysis of fraction 5: peak 1 (*m/z* 190) = castanospermine [**1**]; peak 2 (*m/z* 190) = australine [**3**].

could be a pentahydroxyindolizidine or a monohydroxymethyl-tetrahydroxypyrrolizidine alkaloid.

Fraction 6 contained compounds that exhibited $[MH]^+$ ions at *m/z* 176 and 190 as shown in Figure 6. The $[MH]^+$ at *m/z* 176 suggests a structure of tetrahydroxypyrrolizidine alkaloid. The major peak at *m/z* 190 (peak 1) was eluted slightly after australine. In order to separate this compound from australine, peak 1 was isolated and reanalyzed using different tsp lc-ms conditions. An IBM Carbo column was used instead of Partisil SCX column for lc separation. The result is shown in Figure 7. Surprisingly, three stereoisomers, peaks a, b, and c, were observed and none of them corresponded to australine according to the retention time. Peak b, the major peak observed, was further isolated and analyzed by direct probe ms using CH_4 ci. The ci spectrum of peak b revealed an $[MH]^+$ (base peak) at *m/z* 190 and fragment ions at *m/z* 172 and 158. Apparently, the ion at *m/z* 158 was derived from the fragmentation of

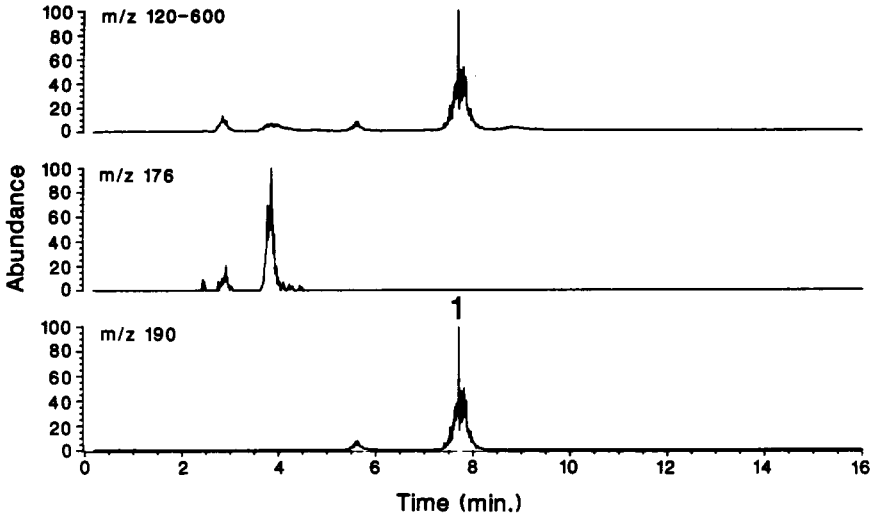

FIGURE 6. Tsp lc-ms analysis of fraction 6: peak 1 (*m/z* 190) was isolated.

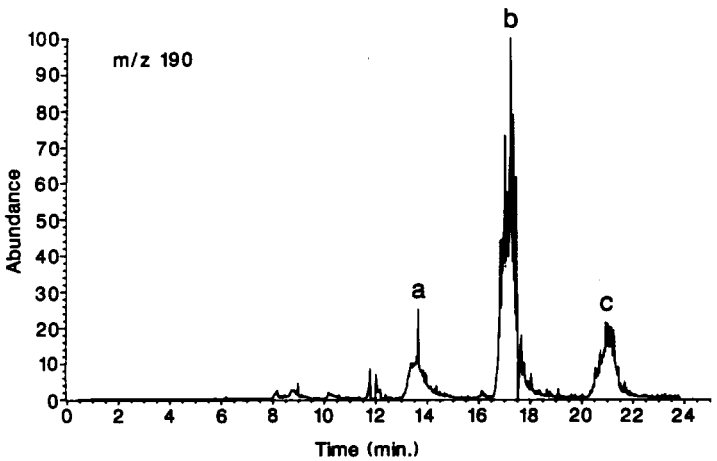

FIGURE 7. Tsp lc-ms analysis of peak 1 (isolated from fraction 6) with sims detection at *m/z* 190.

$[MH - MeOH]^+$, indicative of a monohydroxymethyl-trihydroxypyrrolizidine alkaloid for peak b. As expected, this fragment ion was also observed in the ci spectrum of australine but not in the ci spectra of castanospermine and 6-*epi*-castanospermine. Therefore, based on the information described above, peak b could be 3,8-di-*epi*-alexine [**4**], a new monohydroxymethyl-trihydroxypyrrolizidine alkaloid that has been discovered in *C. australe* recently (6). Because of the low quantity of peaks a and c present in fraction 6, the possible identities of these two compounds have not yet been investigated further.

In conclusion, tsp lc-ms is a unique technique in terms of sensitivity and qualitative capability for analysis of castanospermine and its structurally related alkaloids in *C. australe* and, in principle, other species of plants. The preparative and analytical lc procedures described in this study may open a simpler approach for isolation of new alkaloids structurally related to castanospermine.

ACKNOWLEDGMENTS

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