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IDENTIFICATION OF NEW CACTUS ALKALOIDS IN *BACKEBERGIA MILITARIS* BY TANDEM MASS SPECTROMETRY[1]

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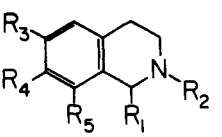
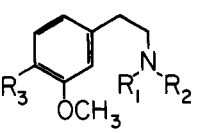
ABSTRACT.—Tandem mass spectrometry, applied to simple extracts of *Backebergia militaris*, indicated the presence of a number of new alkaloids, including fully aromatic oxygenated isoquinolines, their di- and tetra-hydro analogs, and β-phenethylamines. These conclusions were supported by separation using radial tlc and comparison with authentic compounds. Traces of seven alkaloids new to this cactus species were identified; four (**3**,**4**,**8**,**11**) were known previously from other cacti. Three novel cactus alkaloids were identified and confirmed by synthesis as 7,8-dimethoxy-3,4-dihydroisoquinoline (**12**, dehydrolemaireocereine), 6,7-dimethoxyisoquinoline (**13**, backebergine), and 7,8-dimethoxyisoquinoline (**14**, isobackebergine). The last two compounds are the first simple, fully aromatic, isoquinoline alkaloids to be reported from the Cactaceae. The sensitivity of this approach to new alkaloid discovery is emphasized; the entire project consumed only 10 g of dried plant material.

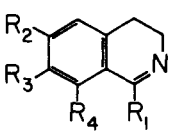
Tandem mass spectroscopy (ms/ms) employs a mass spectrometer as a device for both separation and identification (1-3). Mass spectrometric studies on alkaloids are facilitated by the ease with which they can be protonated under chemical ionization conditions. The combination of chemical ionization and ms/ms has proven valuable in several earlier alkaloid studies (4-6). Several advantages are offered by ms/ms over traditional methods of alkaloid discovery, which typically involve chromatographic detection followed by laborious large-scale isolation and characterization. Of primary importance is the ability to analyze small quantities of crude samples directly with little or no sample preparation. This permits rapid characterization of plant samples of limited availability and also avoids the possible formation of extraction artifacts.



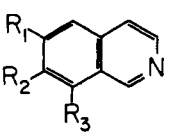
1 R_1,R_2,R_3=H
2 R_1,R_2=H; R_3=CH_3O
3 R_1=CH_3; R_2=H; R_3=CH_3O
4 R_1,R_2=CH_3; R_3=CH_3O



5 R_1=CH_3; R_2=H; R_3,R_4=CH_3O; R_5=H
6 R_1,R_2=H; R_3,R_4=CH_3O; R_5=H
7 R_1,R_2,R_3=H; R_4,R_5=CH_3O
8 R_1=H; R_2=CH_3; R_3,R_4=CH_3; R_5=H
9 R_1=H; R_2=CH_3; R_3=H; R_4,R_5=CH_3O



10 R_1=CH_3; R_2,R_3=CH_3O; R_4=H
11 R_1=H; R_2,R_3=CH_3O; R_4=H
12 R_1,R_2=H; R_3,R_4=CH_3O



13 R_1,R_2=CH_3O; R_3=H
14 R_1=H; R_2,R_3=CH_3O

[1]Part LVIII in the series "Cactus Alkaloids." For part LVII, see N.R. Ferrigni and J.L. McLaughlin, *J. Pharm. Sci.* (submitted for publication).

Other advantages of ms/ms include low detection limits with high sensitivity and selectivity. The technique is particularly appropriate for surveying complex mixtures for targeted compounds or for specific compound classes. The selectivity of the method allows the detection of trace alkaloids not observable when crude extracts are subjected to the usual chromatographic procedures. Some of these advantages were utilized in an earlier chemotaxonomic investigation of several giant Mexican cereoid cacti (7). In this previous work, ms/ms was used to provide a rapid, tentative identification of alkaloids at MH^+ *m/z* 194 and *m/z* 208 in *Backebergia militaris* (Andot) Bravo ex Sanchez Majorada. Subsequently, 3-methoxytyramine (**1**) (MH^+ *m/z* 168), 3,4-dimethoxy-β-phenethylamine (**2**) (MH^+ *m/z* 182), and heliamine (**6**) and lemaireocereine (**7**) (both with MH^+ *m/z* 194) were isolated from the plant material (8,9). Similarly, *m/z* 208 was proposed to be a mixture of the isomers *N*-methylheliamine (8) and *N*-methyllemaireocereine (9) in work that represented a follow-up on the tandem mass spectrometry study (9). The present investigation was initiated to examine further the trace alkaloids of this cactus species using ms/ms in combination with radial tlc.

The samples used for ms/ms analysis were the crude, ground cactus material, the alkaloid fraction A, subfractions of which had been purified by chromatography, and the synthesized standards. Daughter ion spectra were recorded by collision-induced dissociation of selected ions present in the chemical ionization (ci) mass spectrum of the cactus material. Some of the ions chosen for ms/ms analysis exhibited low ($\leq$ 10) signal-to-noise ratios in the ci mass spectrum, which contained peaks at every mass. Initial identification was via comparison with the ms/ms spectra of alkaloids examined previously and was refined by obtaining spectra on additional authentic compounds. The spectra of the various plant material preparations were similar in appearance; however, those taken using fraction A were of better quality than those obtained using the ground plant material and were used for comparison with the standards. This procedure, using either the crude mixture or fraction A without benefit of data from other spectroscopic or chromatographic characterization or separation, allowed tentative identification of seven new alkaloids in *B. militaris*. Figures 1-3 are examples of the comparison of the mass-analyzed ion kinetic energy (mike) spectra of the ions *m/z* 190, 192, and 196 with those of authentic compounds. The agreement between spectra such as these allowed identification of the alkaloids, although ring substitution patterns could not be assigned. It should be noted that single-stage mass analysis can be used to make these assignments. For example, the primary eims fragmentations of heliamine [*m/z* 164 (100%), 193 (65%), 192 (57%), 121 (24%), 149 (33%), and 77 (18%)] and lemaireocereine [*m/z* 149 (100%), 193 (84%), 164 (79%), 192 (55%), 121 (40%), and 77 (35%)] can be used to differentiate the substitution patterns. However, standards or purified compounds need to be used to obtain these spectra to avoid the presence of interfering ions. As a consequence, these eims spectra are not useful for the study of complex mixtures.

The choice of authentic compounds for comparison with and, hence, characterization of mixture components, was based on two considerations: (a) knowledge of the ms/ms spectra of previously studied alkaloids in cactus species and (b) *a priori* interpretation of the ms/ms spectra. Interpretation of the ms/ms spectra drew on data already available for isoquinoline and β-phenethylamine alkaloids. In each of the ms/ms spectra taken, a sharp charge stripping peak is expected and observed for each alkaloid. These peaks are caused by doubly charged ions, formed from singly charged ions that are further ionized by collision gas (10). These peaks, appearing at one-half the mass of the protonated molecules, are characteristically very sharp, as there is essentially no kinetic energy release associated with their formation (rendering their intensities more variable than those of peaks associated with fragment ions). The β-phenethylamine derivatives are

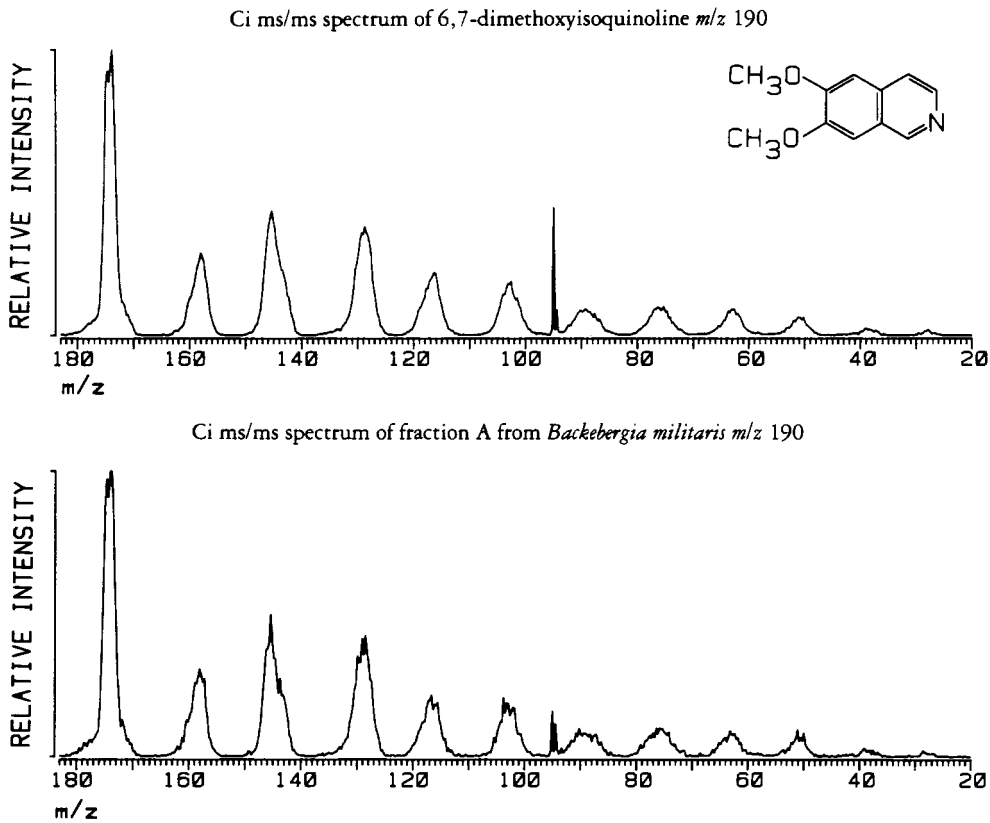


FIGURE 1. Comparison of the ms/ms spectra of (upper) protonated 6,7-dimethyoxyisoquinoline, **13**, and (lower) *m/z* 190 derived from the *Backebergia militaris* alkaloid sample.

characterized by peaks due to β-cleavage with formation of immonium ions. Those at *m/z* 58 and *m/z* 44 correspond to $(CH_3)_2\overset{+}{N}=CH_2$ and $CH_3\overset{+}{N}H=CH_2$, respectively. The presence of this type of side-chain is confirmed by ions due to loss of dimethylamine and methylamine to give the predominant peak at *m/z* 165 in the spectra of compounds **3** and **4**. The cyclic alkaloids fragment extensively, showing groups of ions at masses corresponding to every carbon number. The presence of a peak at *m/z* 30 ($CH_2=\overset{+}{N}H_2$), and the absence of a peak at *m/z* 44 ($CH_2=\overset{+}{N}HCH_3$ or $CHCH_3=\overset{+}{N}H_2$) is indicative of the lack of a methyl group in either the *N*- or 1- position.

Although ms/ms can be used to distinguish between the *N*-methyl and 1-methyl isomers of the tetrahydroisoquinolines (by the respective presence or absence of a peak at *m/z* 44) (7), it is difficult to distinguish between the various positional isomers associated with methoxyl substitution on the aromatic ring. For example, the 6,7- and 7,8-dimethoxy derivatives (**6,7**) cannot be distinguished using the ms/ms spectra of the protonated forms (9). In addition, the loss of hydrogen (H_2) in the ion source from the ionized tetrahydro- and dihydro-derivatives contributed to the spectra of both the dihydro- and anhydro-derivatives; thus, only tentative identification of such isomers at a given mass was possible with ms/ms alone.

Confirmation of the alkaloid identifications made initially by ms/ms was obtained by preparative radial tlc purifications followed by chromatographic and additional spectrometric comparisons with the authentic compounds. (Note that *only* the techniques referred to in the Experimental section were used in making these assignments.) Four of the alkaloids identified—*N*-methyl-3,4-dimethoxy-β-phenethylamine (**3**) (MH^+ *m/z*

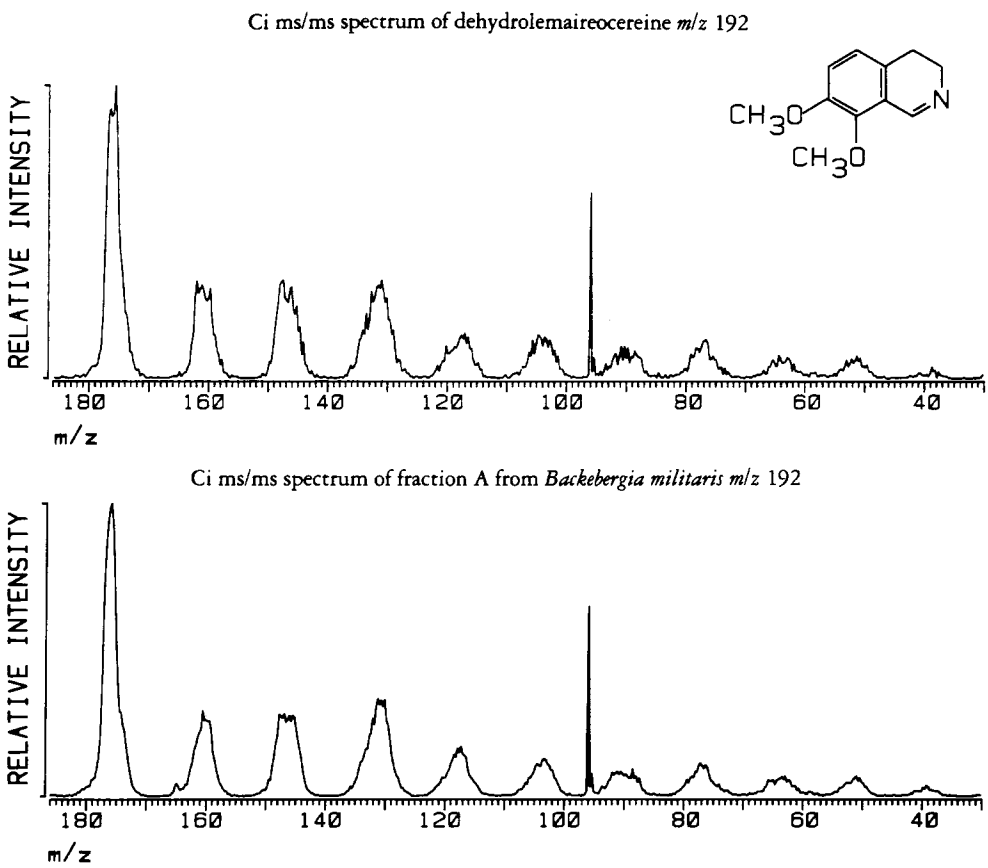


FIGURE 2. The ms/ms spectrum of (upper) protonated dehydrolemaireocereine, **12**, *m/z* 192, compared to that of the ion separated from the ionized alkaloid fraction of *Backebergia militaris*.

196), *N,N*-dimethyl-3,4-dimethoxy-β-phenethylamine (**4**) (MH^+ *m/z* 210), *N*-methylheliamine (**6**) (MH^+ *m/z* 208), and dehydroheliamine (**11**) (MH^+ *m/z* 192)—have been reported previously from other cactus species (11,12). The remaining three alkaloids—dehydrolemaireocereine (**12**) (MH^+ *m/z* 192), 6,7-dimethoxyisoquinoline (backebergine) (**13**) (MH^+ *m/z* 190), and 7,8-dimethoxyisoquinoline (isobackebergine) (**14**) (MH^+ *m/z* 190)—have not been reported heretofore from nature; previously they have been synthesized (13-15). Small amounts of the three new alkaloids were prepared as reference compounds by mild oxidations of the lesser oxidized derivatives.

Five of the compounds (**8,11-14**) are related to the previously observed tetrahydroisoquinoline derivatives, heliamine (**6**) and lemaireocereine (**7**). Both sets of the analogous dihydro- (**11,12**) and anhydro- (fully aromatic) (**13,14**) compounds were found; the fifth (**8**) is the *N*-methyl analog of heliamine (**6**) (9), and the remaining two compounds are the *N*-methyl (**3**) and *N,N*-dimethyl (**4**) analogs of 3,4-dimethoxy-β-phenethylamine (**2**), an alkaloid abundant in the plant (8). The alkaloids identified in this study do not represent the full spectrum of trace alkaloids present in this species, and certain alkaloids, detected by ms/ms, remain unidentified. It is estimated that the identified alkaloids are present at the 0.01% level or higher, while the detection limit for the ms/ms technique is estimated to be an order of magnitude lower, even without more extensive sample preparation.

One must conclude from this study that the detection by tandem mass spectrometry of new trace alkaloids, especially if representative of a previously known class of compounds, is a relatively straightforward undertaking. The low levels of alkaloids

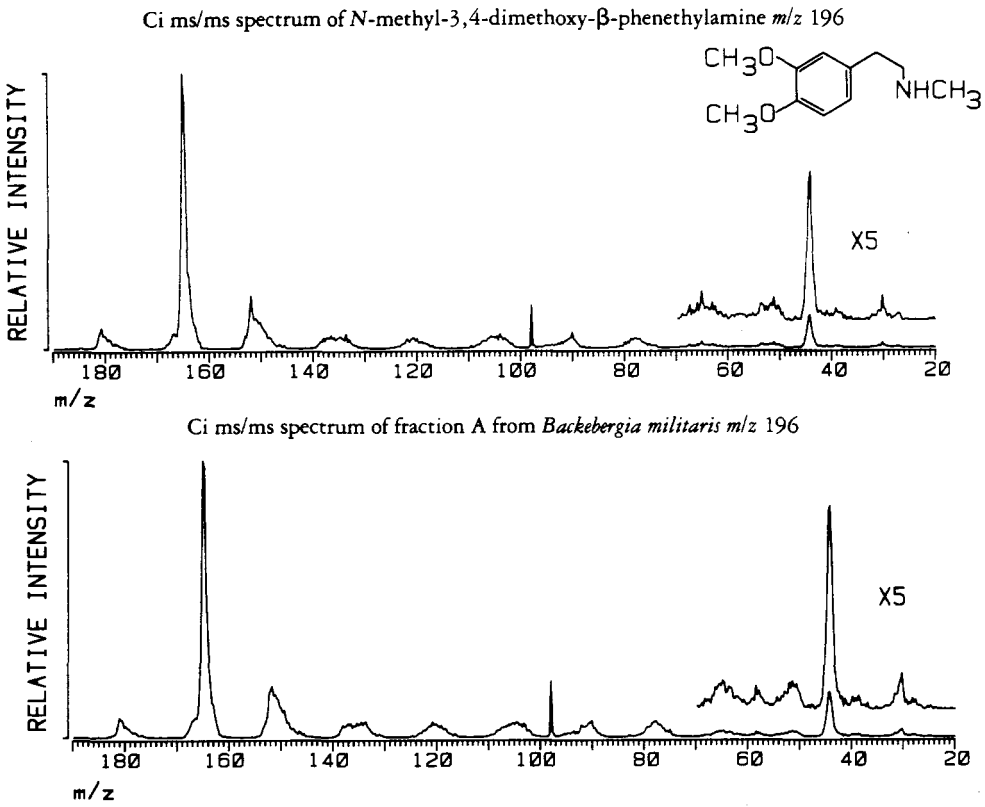


FIGURE 3. The ms/ms spectra of ions *m/z* 196, obtained by protonation of *N*-methyl-3,4-dimethoxy-β-phenethylamine, **3**, (upper), and from the *Backebergia militaris* alkaloid fraction (lower).

that can be identified and the speed of the method mean that a considerable expansion in the number of known alkaloids in individual plant species may be readily achieved, even if only small amounts of plant material are available. This raises important questions for the phytochemist as how best to utilize this new capability. Traces of suspected biogenetic intermediates can be detected (6), chemotaxonomic screenings can be made (7), extraction artifacts can be eliminated (16), plant parts can be mapped for highest alkaloid concentration (17), and quaternary amines can be desorbed and detected (18). Additional applications undoubtedly lie in the near future.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The ms/ms spectra were taken on a mike spectrometer (19). The samples were introduced into the source via a direct insertion probe and ionized with isobutane (~0.15-0.20 torr) to form the protonated molecules. Samples of ~10-15 μg of fraction A and the chromatographically purified extracts and ~ 1 μg of the standards were used. The sample probe was heated to ~150°. The magnetic sector was set to pass the ion of interest, with 7000 eV collisions with air ($\sim 1.5 \times 10^{-5}$ torr nominal pressure) occurring between the two sectors. Peak intensities reported for ms/ms spectra are accurate to about 10%. Melting points were determined on a Mel-Temp apparatus and are uncorrected. Low resolution eims and cims were determined on a Finnigan 4023. Preparative radial tlc was performed on a Chromatotron model 7924 (Harrison Research) on layers of silica gel G. Reference alkaloids were available from previous work and other laboratories: **3** (20), **4** (21), **8** (22), **11** (12), and **12** (23). The tlc solvent systems and spray reagents were employed as previously described (24). Compounds **11-14** were easily distinguished on tlc plates by their fluorescence under long-wave uv, showing, respectively, bright blue, dark yellow, very dark blue, and yellow.

PLANT MATERIAL.—The freeze-dried and powdered plant material of *B. militaris* was taken from the same collection as previously reported (8,9). A voucher sample [(Gibson 3433 (ARIZ)] is filed at the University of Arizona Herbarium.

ALKALOID EXTRACTIONS.—A 10-g portion of the powdered plant material was defatted by shaking with five 100-ml portions of hexane. The defatted plant material was moistened with $CHCl_3$-MeOH-NH_4OH (2:2:1), and the extraction procedure was repeated with $CHCl_3$. The $CHCl_3$ residue was processed, through acid-base partitioning as previously described (24), to obtain fractions A (alkaloids) (0.16 g), B (nonalkaloids), and C (H_2O soluble alkaloids) (0.15 g). A portion of fraction A (0.04 g) was reserved for ms/ms analysis, and the remainder was resolved into phenolic (0.01 g) and nonphenolic (0.11 g) portions by passing through Amberite IRA 400 anion exchange resin (hydroxide form), as previously described (24).

ISOLATION AND IDENTIFICATION OF 7,8-DIMETHOXYISOQUINOLINE (ISOBACKEBERGINE, **14**).—The nonphenolic alkaloid fraction (72 mg) was separated by preparative radial tlc (Chromatotron) using a stepwise gradient of $CHCl_3$-MeOH, 25 ml of each mixture: 99:1, 95:5, 90:10, 80:20, 50:50, 30:70, 0:100; 35 fractions of 5 ml each were collected. Analysis of the fractions in tlc ($CHCl_3$-MeOH, 9:1) detected a single, yellow fluorescent (long-wave uv) spot in fractions 5-11. The residue (1.2 mg) from these combined fractions was chromatographed ($CHCl_3$-MeOH gradient) over a micro-column (7×0.4 cm) of silica gel G (0.7 g) to obtain a residue of 0.7 mg of the pure compound. This isolated material was identical (ms/ms, eims, cims, and five tlc systems) with synthesized reference **14**. The ms/ms spectrum of the protonated **14** showed fragment ions centered at *m/z* 174 (100%), 158 (29%), 145 (44%), 129 (38%), 116 (22%), 103 (19%), 95 (45%), 89 (10%), 75 (10%), 63 (9%), and 51 (6%).

SYNTHESIS OF 7,8-DIMETHOXYISOQUINOLINE (ISOBACKEGERGINE, **14**) AND 7,8-DIMETHOXY-3,4-DIHYDROISOQUINOLINE (DEHYDROLEMAIREOCEREINE, **12**).—Following the procedure of El-Fishawy *et al.* (23), 20 mg of **7** (22) was dissolved in 5 ml of Me_2CO, and 0.8 ml of 5% aqueous $KMnO_4$ was added slowly over a period of 15 min and stirred for 1.5 h. The solution was filtered and evaporated to yield a residue (11 mg) of two fluorescent compounds (tlc). By using a microcolumn (7×0.4 cm) of silica gel G (0.7 g) developed with $CHCl_3$-MeOH (100:0, 99.9:1, 99.5:0.5, 99:1), 0.7 mg of **14** (MH^+, *m/z* 190) and 8 mg of 12 (MH^+, *m/z* 192) were isolated. Mass spectra (ms/ms and cims for **14** and ms/ms, cims, and eims for **12**) were all consistent with the proposed structures. The eims for **12** gave *m/z* 191 (100%) M^+, 176 (40%) (minus CH_3), and 161 (40%) (minus CH_2O). The ms/ms spectrum of the protonated **12** showed fragment ions centered at *m/z* 176 (100%), 160 (29%), 146 (28%), 131 (33%), 117 (17%), 103 (12%), 96 (65%), 91 (8%), 77 (12%), and 63 (7%) (Figure 2).

ISOLATION AND IDENTIFICATION OF 6,7-DIMETHOXYISOQUINOLINE (BACKEBERGINE, **13**).—The residue from Chromatotron fractions 12 and 13 (see above) (5.5 mg) was rechromatographed over a micro-column (7×0.4 cm) of silica gel G (0.7 g) using a $CHCl_3$-MeOH gradient (99.9:0.1, 99.7:.03, and 99:1). Nine fractions were collected; fraction 4 yielded 0.8 mg of **13**. The isolated material was identical (ms/ms, eims, cims, and five tlc systems) with the synthesized reference **13**. The ms/ms spectrum of the protonated **13** (Figure 1) was indistinguishable from that of **14**.

SYNTHESIS OF 6,7-DIMETHOXYISOQUINOLINE (BACKEBERGINE, **13**).—Following the method of Popp *et al.* (25) to 0.103 g (0.54 mmoles) of **11** (12) in 3 ml of decalin was added 0.10 g of 5% palladium on charcoal. The mixture was refluxed for 2 h and then extracted with 15% aqueous HCl. The filtered acidic extract was basified and extracted with Et_2O. The Et_2O residue yielded 0.07 g (75%) of **13** (ms/ms and cims *m/z* 190), a portion of which was crystallized as the HCl (mp 219°). The eims for **13** gave *m/z* 189 (100%) M^+, 174 (15%) (minus CH_3), and 146 (35%) (minus CH_3 and CO).

ISOLATION AND IDENTIFICATION OF 7,8-DIMETHOXY-3,4-DIHYDROISOQUINOLINE (DEHYDROLEMAIREOCEREINE, **12**).—Fraction 6, from the microcolumn that was used for the isolation of **13**, yielded a residue (0.4 mg) which was identified as **12** (ms/ms of *m/z* 192, cims, eims and co-chromatography with synthesized **12** in five tlc systems).

ISOLATION AND IDENTIFICATION OF 6,7-DIMETHOXY-3,4-DIHYDROISOQUINOLINE (DEHYDROHELIAMINE, **11**).—The residue of fractions 18-22 (6 mg), from the Chromatotron separation of the nonphenolic alkaloids, was rechromatographed on a micro-column (7×0.4 cm) of silica gel G (0.7 g) using a gradient of $CHCl_3$-MeOH (100:0, 99.9:01, 99:1, 98:2, 97:3) to yield bright blue fluorescent fractions containing **11** (4.5 mg). This material was identical to reference **11** (5), (ms/ms of *m/z* 192, cims, eims, and co-chromatography in five tlc systems). The ms/ms spectrum of the protonated **11** was indistinguishable from that of **12**.

IDENTIFICATION OF *N*-METHYL-3,4-DIMETHOXY-β-PHENETHYLAMINE (**3**) AND *N,N*-DIMETHYL-3,4-DIMETHOXY-β-PHENETHYLAMINE (**4**).—The residue (6 mg) of fractions 30-31, from the Chromatotron separation of the nonphenolic alkaloids, was a mixture of several alkaloids. Analytical tlc, visualizing with iodoplatinic acid, detected **4**, which co-chromatographed with reference **4** in five tlc systems. Similarly, the residue (4 mg) of Chromatotron fractions 32-33 contained a trace of **3**, which co-

chromatographed with reference **3** in five tlc solvents. The ms/ms spectrum of the protonated **4** showed fragment ions centered at *m/z* 194 (17%), 178 (14%), 165 (100%), 150 (18%), 105 (18%), 58 (34%), and 44 (9%). The ms/ms spectrum of the protonated **3** (Figure 3) shows fragment ions centered at *m/z* 181 (8%), 165 (100%), 152 (20%), 98 (16%), and 44 (12%).

IDENTIFICATION OF *N*-METHYL-6,7-DIMETHOXY-1,2,3,4-TETRAHYDROISOQUINOLINE (*N*-METHYLHELIAMINE, **8**).—The residue (3.3 mg) of fractions 14-17 from the Chromatotron separation of the nonphenolic alkaloids, contained an alkaloid which co-chromatographed (in five tlc systems) with reference **8**. The ms/ms of the anticipated ionized molecule (*m/z* 208) in the residue gave the expected collision induced dissociation fragments. A reference of the *N*-methylated 7,8-isomer (**9**) of **8** is not available; however, co-chromatography of **8** and **9** in all five tlc systems would be unlikely; thus, the material at *m/z* 208 is concluded to represent **8**, with the presence of **9** remaining a possibility. The ms/ms spectrum of the protonated **8** showed fragment ions centered at *m/z* 193 (100%), 176 (42%), 165 (92%), 161 (20%), 150 (26%), 131 (10%), 104 (49%), 96 (9%), 91 (11%), 77 (11%), and 44 (47%).

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