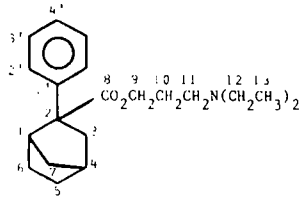
Microbial Metabolism of Bornaprine, 3-(Diethylamino)propyl 2-Phenylbicyclo[2.2.1]heptane-2-carboxylate

SAYED A. ELMARAKBY*, ALICE M. CLARK*, JOHN K. BAKER‡, AND CHARLES D. HUFFORD*x

Received December 20, 1985, from the *Department of Pharmacognosy and the ‡Department of Medicinal Chemistry, School of Pharmacy, University of Mississippi, University, MS 38677. Accepted for publication March 18, 1986.

Abstract □ Metabolism studies of the anticholinergic drug, bornaprine [3-(diethylamino)propyl 2-phenylbicyclo[2.2.1]heptane-2-carboxylate, an epimeric mixture (**1**)], in rats, dogs, and humans have been conducted previously, but the identities of the metabolites were not established. Using an in vitro microbial system to study the metabolism of bornaprine resulted in the isolation of four metabolites whose structures were rigorously established using spectroscopic techniques, especially ^{13}C NMR. The four metabolites found (**2**, **3**, **4**, and **5**) were hydroxylated at C-5 or C-6 in the bicyclic ring.

Bornaprine hydrochloride [3-(diethylamino)propyl 2-phenylbicyclo[2.2.1]heptane-2-carboxylate hydrochloride (**1**)] is a synthetic anticholinergic drug used in the treatment of Parkinsonism.[1] The metabolism of [^{14}C]bornaprine has been investigated in rats, dogs, and humans.[2] Several hydroxylated metabolites were detected, but the products obtained were not identified. Since microbial systems have demonstrated usefulness and versatility as in vitro models for drug metabolism studies,[3] this approach was used with bornaprine in an attempt to produce larger quantities of the unidentified metabolites. The isolation and structure determination of four microbial metabolites of bornaprine are reported in this paper.



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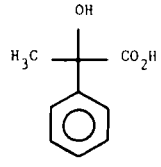

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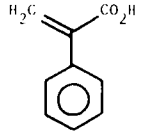

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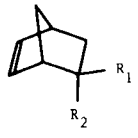
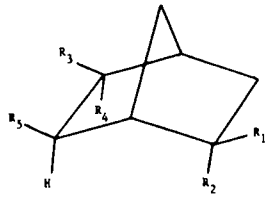


11 $R_1 = CO_2H$, $R_2 = C_6H_5$

12 $R_1 = C_6H_5$, $R_2 = CO_2H$

$HO(CH_2)_3N(CH_2CH_3)_2$

15



	R_1	R_2	R_3	R_4	R_5
2	$CO_2(CH_2)_3N(C_2H_5)_2$	C_6H_5	OH	H	H
3	$CO_2(CH_2)_3N(C_2H_5)_2$	C_6H_5	H	H	OH
4	C_6H_5	$CO_2(CH_2)_3N(C_2H_5)_2$	OH	H	H
5	C_6H_5	$CO_2(CH_2)_3N(C_2H_5)_2$	H	OH	H
6	C_6H_5	$CO_2(CH_2)_3N(C_2H_5)_2$	=O		H
7	$CO_2(CH_2)_3N(C_2H_5)_2$	C_6H_5	=O		H
8	$CO_2(CH_2)_3N(C_2H_5)_2$	C_6H_5	H	H	=O
13	CO_2H	C_6H_5	H	H	H
14	C_6H_5	CO_2H	H	H	H
16	$CO_2(CH_2)_3N(C_2H_5)_2$	C_6H_5	H	H	H
17	C_6H_5	$CO_2(CH_2)_3N(C_2H_5)_2$	H	H	H

Results and Discussion

Screening using 31 microorganisms was conducted to identify organisms capable of metabolizing bornaprine (**1**). Thin-layer chromatographic observation showed that several organisms converted **1** to more polar metabolites (Table I). Of these, *Cunninghamella echinulata* (ATCC 9244) was chosen for the preparative scale fermentation. Incubation of 2.81 g of **1** with stirred cultures of *C. echinulata* for 10 d, followed by extraction of the combined culture broth with ethyl acetate, afforded 2.72 g of crude extract which showed one spot on TLC. HPLC analyses, however, clearly indicated the presence of four metabolites. Also, use of ^{13}C NMR showed that bornaprine (**1**) was a nearly 50:50 mixture of two epimers,[4] which we were not able to separate.[5] The pure metabolites were obtained by column chromatography and preparative HPLC.

To fully characterize the metabolites, it was deemed necessary to synthesize the *exo*- and *endo*-esters (**16**, **17**) using an established procedure.[6,7] This also allowed us to fully characterize by ^{13}C NMR all intermediates which then led to

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unambiguous spectral assignments for **16** and **17**. With these data, the structure of the metabolites was established.

Dehydration of the readily available atrolactic acid (**9**) with polyphosphoric acid led to atropic acid (**10**) which was treated with cyclopentadiene to give the epimeric acids (**11** and **12**) in 88% yield. These were separated by column chromatography, and were identified as the *exo*- and *endo*-isomers, **11** and **12**, respectively, on the basis of a comparison of their melting points with those reported[6] in which the structures of the *exo*- and *endo*-acids were established unambiguously via iodolactone formation. Catalytic hydrogenation over palladium-on-carbon proceeded smoothly to give the corresponding hydrogenated acids (**13** and **14**) in good yield. Refluxing each of the hydrogenated acids with excess thionyl chloride in benzene led to the corresponding acid chlorides which were treated with 3-diethylamino-1-propanol (**15**) to give the corresponding esters (**16** and **17**) in moderate yields. The chemical shifts obtained for the ^1H NMR spectra of the esters and all the intermediates are listed in the *Experimental Section*. Critical proton assignments were made on the basis of homonuclear decoupling experiments. Unambiguous ^{13}C NMR chemical shift assignments for the esters and all the intermediates (Table II) were based on selective heteronuclear decoupling experiments.

The mass spectra of the metabolites, **2**, **3**, **4**, and **5**, showed a molecular ion which was 16 mass units higher than that of **1**. High resolution MS data were consistent with a molecular formula of $C_{21}H_{31}NO_3$ for each of the metabolites suggesting that the metabolites were monohydroxylated derivatives of **1**. The presence of a hydroxyl substituent was also indicated in the IR spectra of the metabolites where distinct absorption signals near 3600 cm^{-1} were observed for **2**, **3**, **4**, and **5**. From ^{13}C NMR, ^1H NMR, and MS data, it was concluded that the hydroxyl group was present in the bicyclic moiety. Comparison of the resonance of C-1′ of **16** and **17** with that of **2**, **3**, **4**, and **5** (Table II) distinguished the metabolites derived from the *exo*-ester from those derived from the *endo*-ester. The resonance of C-1′ of all the *endo*-isomers was consistently 4–5 ppm downfield from that of the corresponding *exo*-isomers (Table II). Thus, **2** and **3** are hydroxylated derivatives of the *exo*-ester, whereas, **4** and **5** are hydroxylated derivatives of the *endo*-ester. The only remaining questions were the position and stereochemistry of the hydroxyl group in the metabolites.

The position of the hydroxyl group in **2** was established by further ^{13}C NMR spectral evaluation. As shown in Table II,

Table I—Cultures Used for Screening Bornaprine

Fungus	Metabolite Production[a]	Fungus	Metabolite Production[a]
1. *Aspergillus alliaceus* (NRRL 315)	(−)	17. *Aspergillus niger* (ATCC 11394)	(−)
2. *Aspergillus flavipes* (ATCC 16795)	(−)	18. *Aspergillus parasiticus* (ATCC 15517)	(−)
3. *Aspergillus flavus* (ATCC 9170)	(−)	19. *Beauveria bassiana* (ATCC 13144)	(−)
4. *Beauveria bassiana* (ATCC 7159)	(−)	20. *Botrytis allii* (ATCC 9435)	(−)
5. *Chaetomium cochloides* (NRRL 2320)	(−)	21. *Cunninghamella elegans* (ATCC 11064)	(+)
6. *Cunninghamella bertholletiae* (NRRL 3644)	(−)	22. *Cunninghamella echinulata* (NRRL 1387)	(−)
7. *Cunninghamella blakesleeana* (ATCC 8688a)	(+)	23. *Cunninghamella echinulata* (NRRL 3655)	(−)
8. *Cunninghamella echinulata* (ATCC 9244)	(+)	24. *Cunninghamella echinulata* (ATCC 11585a)	(−)
9. *Cunninghamella elegans* (ATCC 9245)	(+)	25. *Cunninghamella echinulata* (ATCC 11585b)	(−)
10. *Mucor griseo-cyanus* (ATCC 1207)	(−)	26. *Cunninghamella echinulata* (NRRL 1393)	(−)
11. *Penicillium brevi-compactum* (ATCC 10418)	(−)	27. *Cunninghamella species* (NRRL 5695)	(−)
12. *Penicillium chrysogenum* (ATCC 9480)	(−)	28. *Fusarium oxysporum* (ATCC 26297)	(−)
13. *Rhizopus arrhizus* (ATCC 11145)	(+)	29. *Rhizopus stolonifer* (ATCC 6227b)	(−)
14. *Syncephalastrum racemosum* (ATCC 18192)	(−)	30. *Rhizopus stolonifer* (ATCC 15441)	(−)
15. *Whetzelinia sclerotiorum* (ATCC 18015)	(−)	31. *Whetzelinia sclerotiorum* (ATCC 24156)	(−)
16. *Aspergillus flavus* (ATCC 24741)	(−)		

[a] Metabolite production denoted by (+) indicates one or more metabolites were produced, as shown by TLC.

Table II—^{13}C NMR ($CDCl_3$) Chemical Shift Assignments for Metabolites and Compounds 11–17[a]

Carbon No.	Compounds[b]										
	2	**3**	**4**	**5**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
1	42.5[c] (d)	51.8[c] (d)	42.0[c] (d)	43.6 (d)	49.4[c] (d)	48.3[c] (d)	43.6[c] (d)	42.8 (d)	—	43.6 (d)	42.6 (d)
2	58.7 (s)	57.4 (s)	58.4 (s)	58.9 (s)	59.5 (s)	58.9 (s)	59.9 (s)	59.4 (s)	—	60.1 (s)	59.6 (s)
3	34.0[1] (t)	38.3 (t)	37.8[1] (t)	34.2[1] (t)	37.4 (t)	39.6 (t)	39.4 (t)	42.2[c] (t)	—	39.4 (t)	42.6 (t)
4	44.3 (d)	36.0 (d)	45.9 (d)	44.3 (d)	41.9[c] (d)	43.1[c] (d)	36.5[c] (d)	38.0[c] (d)	—	36.6 (d)	38.1 (d)
5	74.1 (d)	42.6 (t)	73.6 (d)	71.5 (d)	138.7 (d)	140.1[c] (d)	29.6 (t)	28.7 (t)	—	29.8 (t)	28.7 (t)
6	36.1[1] (t)	70.2 (d)	38.3[1] (t)	36.6[1] (t)	134.2 (d)	134.4[c] (d)	23.3 (t)	25.9 (t)	—	23.4 (t)	25.9 (t)
7	35.0[1] (t)	34.8 (t)	34.0[1] (t)	37.5[1] (t)	48.2 (t)	47.8[c] (t)	38.3 (t)	38.0 (t)	—	38.3 (t)	38.0 (t)
8	176.0 (s)	175.8 (s)	175.1 (s)	175.0 (s)	182.9 (s)	181.3 (s)	183.2 (s)	181.4 (s)	—	176.5 (s)	175.4 (s)
9	63.2 (t)	63.7 (t)	63.3 (t)	63.5 (t)	—	—	—	—	63.8 (t)	63.4 (t)	63.0 (t)
10	25.8 (t)	26.2 (t)	26.2 (t)	26.0 (t)	—	—	—	—	28.5 (t)	26.3 (t)	26.2 (t)
11	49.1 (t)	49.3 (t)	49.2 (t)	49.1 (t)	—	—	—	—	53.2 (t)	49.2 (t)	49.3 (t)
12[d]	46.8 (t)	46.8 (t)	46.9 (t)	46.8 (t)	—	—	—	—	47.0 (t)	46.9 (t)	46.9 (t)
13[d]	11.1 (q)	11.6 (q)	11.6 (q)	11.3 (q)	—	—	—	—	11.8 (q)	11.6 (q)	11.5 (q)
1′	141.3 (s)	140.3 (s)	145.0 (s)	145.5 (s)	142.6 (s)	143.5 (s)	140.9 (s)	145.1 (s)	—	141.6 (s)	145.9 (s)
2′[d]	128.5[2] (d)	128.5[2] (d)	128.6[2] (d)	128.7[2] (d)	128.6[2] (d)	128.6[2] (d)	128.8[2] (d)	128.8[2] (d)	—	128.6[2] (d)	128.5[2] (d)
3′[d]	128.3[2] (d)	128.3[2] (d)	126.6[2] (d)	126.5[2] (d)	128.2[2] (d)	127.5[2] (d)	128.2[2] (d)	128.6[2] (d)	—	128.1[2] (d)	126.4[2] (d)
4′	126.8 (d)	126.9 (d)	126.6 (d)	126.3 (d)	126.8 (d)	126.9 (d)	126.8 (d)	126.6 (d)	—	126.5 (d)	126.2 (d)

[a] Assignments bearing the same numerical superscript in any column may be interchanged; s = singlet, d = doublet, t = triplet and q = quartet. [b] Values are in ppm. [c] Assignments are based on heteronuclear selective decoupling experiments involving irradiation at the position of the respective protons. [d] Double intensity signals.

the resonance at 29.8 (t) ppm for C-5 of **16** was not found in the spectrum of metabolite **2**, and a new doublet at 74.1 ppm was present. This suggested that the hydroxyl group was at C-5. The downfield shifts of both carbons 4 and 6 were attributed to the β-effect and confirmed substitution at C-5. The stereochemistry of the hydroxyl group at C-5 was determined to be *exo* since the C-5 proton signal appeared as a doublet (3.67 ppm). Had the stereochemistry been *endo*, this proton signal would have appeared as a double doublet due to coupling with the C-4 and C-6 (*endo*-) protons. The *exo* assignment of the stereochemistry of the OH group at C-5 was further supported by the ^{13}C NMR upfield shifts of resonances for C-7 and C-1 (−4.3 ppm and −1.1 ppm) and a downfield shift for C-4 (+7.7 ppm). Similar shifts have also been reported for other hydroxylated bicyclic systems.[9] Thus the structure of metabolite **2** was established since the OH group was determined to be at C-5 and *exo*.

In the case of metabolite **3** the resonance for C-1 showed a considerable downfield shift, while that for C-4 showed almost no change, relative to **16**. In addition, the C-6 proton of **3** displayed the same pattern observed as that of the C-5 proton in **2** (doublet, 3.71 ppm). Also, the H-1 resonance appeared as a broad singlet (all other metabolites showed H-1 as a doublet). Therefore, it was concluded that the hydroxyl group in **3** was at C-6 and had the same stereochemistry (OH *exo*) as in **2**.

On examination of the ^{13}C NMR spectrum of metabolite **4**, it was evident that a triplet at 28.7 ppm (C-5) in the spectrum of **17** had disappeared and had been replaced by the doublet at 73.6 ppm, which suggested hydroxylation at C-5. The downfield shifts of C-6 and C-4, resonances and the upfield shifts of those for C-1 and C-7 are in agreement with placement of the OH group at C-5. The OH group was determined to be *exo* since the coupling pattern of the C-5 proton signal was identical to that observed for **2**.

As with metabolite **4**, comparison of the ^{13}C NMR spectrum of **5** with that of **17** suggested the presence of the hydroxyl group at C-5, as indicated by the disappearance of 28.7 ppm resonance (C-5) and the appearance of a new doublet at 71.5 ppm. Assignment of the hydroxyl group to the C-5 position was further supported by the downfield shift of the C-4 resonance from 38.1 ppm to 44.3 ppm, while the C-1 resonance was essentially unchanged (Table II). Thus, the hydroxyl group must be *endo* since this metabolite was different from **4**. The ^1H NMR spectrum shows the H-5 resonance as a double doublet, as expected. Also, there was an upfield shift of 2 ppm for the C-5 signal and a downfield shift of 3 ppm for the C-7 resonance, as seen previously[9] (Table II). The ^1H and ^{13}C NMR data of metabolites **4** and **5** indicate, unambiguously, that they are epimers at C-5. Oxidation of **4** and **5** produced the same ketone, confirming their epimeric relationship.

As with other biological hydroxylations of similar drugs,[10] the hydroxylation of bornaprine occurred in the bicyclic moiety in preference to the aromatic ring system resulting in the production of four different positional and configurational isomers. These microbial metabolites produced by *C. echinulata* appear to be similar to the mammalian metabolites of bornaprine. The present study is another example of the potential of microorganisms as an aid in the study of mammalian drug metabolism.

Experimental Section

Melting points (uncorrected) were determined on either a Fisher-Digital melting point analyzer (model 355) or on a Thomas-Hoover Unimelt capillary melting point apparatus. The IR spectra were obtained on a Perkin-Elmer 281B recording spectrophotometer from KBr pellets or $CHCl_3$ solution (0.1-mm NaCl cells). ^1H NMR spectra (90 MHz) were recorded in $CDCl_3$ on a Varian EM390 spectrometer using Me_4Si as an internal standard. Homonuclear selective decoupling experiments were run at ~0.2 mG decoupling power. The ^{13}C NMR spectra (15.03 MHz) were run in $CDCl_3$ on a JEOLFX60 FT spectrometer using Me_4Si as the internal standard. Heteronuclear selective decoupling experiments were performed at 33 db ($\gamma H_2/2\pi$ = 560 Hz). The chemical shift values are reported in ppm and the coupling constants in Hz. Abbreviations for NMR signals are as follows: s is singlet, d is doublet, t is triplet, q is quartet, dd is double doublet, m is multiplet, and br s is broad singlet. Low resolution electron impact mass spectra were obtained using an E. I. Finnigan model 3200 (70 eV ionization potential) with the INCOS data system. High resolution mass spectra were recorded at the University of Utah, Salt Lake City, UT. Combustion analyses for C and H were performed by Scandinavian Microanalytical Laboratories, Herlev, Denmark, and were within ±0.4% of the theoretical values unless otherwise noted.

Thin-layer chromatographic analyses were carried out on precoated silica gel G-25 UV_{254} plates (0.25 mm) and precoated aluminum oxide Alox-25 plates (0.25 mm). The adsorbents used for column chromatography were silica gel 60 (70–270 mesh, Macherey Nagel & Co.) and neutral alumina (alumina for dry column chromatography, activity III/20 mm, ICN Nutritional Biochemicals). Visualization of the TLC plates was done using iodoplatinate spray reagent.

HPLC analyses were conducted as follows: a Waters Associates (Milford, MA) model 6000 A pump, model U6 K injector, and model 440 ultraviolet detector were used. A Whatman (Clifton, NJ) C-18 guard column was used between the injector and the analytical column. A 30 cm × 3.9 mm i.d. octadecyl reversed-phase analytical column (Waters Assoc., μ Bondapak C-18, 10-μm particle size) was used with a flow rate of 1 mL/min. The mobile phase consisted of a solution of 6.6 g K_2HPO_4, 8.4 g KH_2PO_4, 1200 mL CH_3OH, 2800 mL distilled water, and 4 g *N,N*-dimethyloctylamine. Two UV detectors were used in series (254-nm followed by a 280-nm detector).

Bornaprine hydrochloride [3-(diethylamino)propyl 2-phenylbicyclo[2.2.1]heptane-2-carboxylate hydrochloride (**1**)] was provided by Dr. M. Hollmann, Knoll AG, Ludwigschafen, West Germany. All the chemicals used were purchased from Aldrich Chemical Company except for the 10% Pd-C which was purchased from Alfa Division (Danvers, MA).

Maintenance of Cultures—Stock cultures of fungi were stored on slants of Mycophil agar at 4 °C. Bacterial cultures were stored on Eugon agar slants at 4 °C. The cultures were obtained from the University of Mississippi, Department of Pharmacognosy Culture Collection and were originally from the American Type Culture Collection (ATCC), Rockville, MD or from Northern Regional Research Laboratories (NRRL), Peoria, IL.

Preliminary Screening—Preliminary screening experiments were performed in 25 mL of medium contained in 125-mL stainless steel capped Delong culture flasks. A two-stage fermentation protocol was routinely employed in which sterile liquid culture medium was inoculated (by means of a sterile wire loop) with surface growth from agar slants. These stage I cultures were incubated (250 rpm, 24–26 °C) for 72 h, at which time 2.5-mL portions were used to inoculate stage II cultures. Drug substrate was added to the stage II cultures following 24-h incubation. The drug was added as a 5% solution in dimethylformamide to give a final concentration of 0.2 mg/mL of stage II medium.

Substrate controls were composed of sterile media to which the same amount of the drug was added. Culture controls consisted of fermentation blanks, in which the organisms were grown under identical conditions, including the addition of dimethylformamide but without the addition of the drug.

Culture Medium—All preliminary screening for microorganisms capable of metabolizing bornaprine hydrochloride was carried out in a medium consisting of: dextrose, 20 g; yeast extract, 5 g; peptone, 5 g; NaCl, 5 g; K_2HPO_4, 5 g; distilled H_2O, 1000 mL.

Fermentation Sampling and Detection of Metabolites—Under aseptic conditions, 5 mL of each culture were withdrawn and filtered. The pH of the broth was adjusted to 9 with 3 M NaOH. The aqueous samples were extracted three times with equal volumes of ethyl acetate (EtOAc). The combined extracts were dried by filtration through absorbent cotton, evaporated (under reduced pressure) at 40 °C, and analyzed by TLC. Silica gel plates were developed in

EtOAc:MeOH:NH_4OH (85:10:5), whereas aluminum oxide plates were developed in MeOH:$CHCl_3$ (1:99).

Microbial Transformation of Bornaprine (1) by *Cunninghamella echinulata* (ATCC 9244)—A total of 2.81 g of bornaprine hydrochloride was distributed evenly among 24-h old stage II cultures of *C. echinulata* (ATCC 9244). The cultures were incubated for 10 d and were harvested by filtration. The pH of the combined aqueous culture broth (14.3 L) was adjusted to 9 with 3 M NaOH, divided into portions, and extracted four times with equal volumes of ethyl acetate. The combined ethyl acetate extracts were dried over anhydrous Na_2SO_4 and were filtered through absorbent cotton. The solvent was evaporated under reduced pressure (40 °C) to leave 2.72 g of a dark brown residue. This residue was chromatographed on neutral alumina (5 × 115 cm column, containing 1.5 kg neutral alumina for dry column chromatography, activity III/20 mm). The column was initially eluted with $CHCl_3$ followed by $CHCl_3$:MeOH mixtures of increasing polarity. After elution of the column with 3.6 L of $CHCl_3$, the eluate gave a positive spot test with iodoplatinate spray reagent and 10 mL fractions were then collected. The composition of the fractions obtained was monitored by HPLC. Fractions of similar composition were pooled giving a total of six fractions: 1 ($CHCl_3$, 0.205 g), 2 ($CHCl_3$, 0.169 g), 3[MeOH:$CHCl_3$ (1:99), 0.157 g], 4 [MeOH:$CHCl_3$ (1.5:98.5) 0.396 g], 5 [MeOH:$CHCl_3$ (2:98), 0.290 g], and 6 [MeOH:$CHCl_3$ (5:95) 0.110 g]. The column was then washed with MeOH.

Fraction 1 was shown to be the starting material 1 by TLC analysis: one spot, R_f 0.3 on silica gel G plates developed in MeOH:$CHCl_3$ (10:90); and one spot, R_f 0.57 on aluminum oxide Alox plates developed in MeOH:$CHCl_3$ (2:98). TLC analysis of fraction 2 showed it to be a mixture of the starting material and a material of a lower R_f value (metabolite 2): R_f 0.23 on silica gel plates and 0.26 on aluminum oxide plates using the same systems as above.

Metabolite 2: 3-(Diethylamino)propyl (2-*exo*,5-*exo*)-5-Hydroxy-2-phenylbicyclo[2.2.1]heptane-2-carboxylate—Fraction 3 was homogeneous on TLC (one spot, R_f 0.23 and 0.26 on silica gel and aluminum oxide plates, respectively) which corresponds to 2. Metabolite 2 (72 mg) was further purified by column chromatography over silica gel (8 g) using $CHCl_3$ and $CHCl_3$:EtOH mixtures of increasing polarity. The EtOAc:$CHCl_3$ (10:90) eluate (75 mL) gave 50 mg of 2; MS (high resolution): M^+ 345.2296 (calcd. for $C_{21}H_{31}NO_3$: 345.2303); IR ($CHCl_3$): 3600, 2970, 1716, 1600, 1495, 1450, and 1390 cm^{-1}; ^1H NMR (90 MHz, $CDCl_3$): δ 1.00 (t, 6, J = 6.8 Hz, H-13), 2.43 (q, 4, J = 6.8 Hz, H-12), 2.78 (dd, 1, J = 12 and 5.5 Hz, H-3 *exo*), 3.22 (d, 1, J = 3.8 Hz, H-1), 3.65 (d, 1, J = 6.4 Hz, H-5), 4.05 (t, 2, J = 6.0 Hz, H-9), and 7.30 ppm (br s, 5, ArH); ^{13}C NMR (see Table II).

Metabolite 3: 3-(Diethylamino)propyl (2-*exo*, 6-*exo*)-6-Hydroxy-2-phenylbicyclo[2.2.1]heptane-2-carboxylate—Fraction 4 was shown by HPLC to consist mainly of a mixture of metabolites 2 and 3 (major component). In addition, the HPLC chromatogram showed the presence of a small quantity of another minor metabolite. Both 2 and 3 have the same R_f value using silica gel plates [MeOH:$CHCl_3$ (10:90)] or aluminum oxide plates [MeOH:$CHCl_3$ (2:98)] but have different retention times on analysis by HPLC. Separation of these metabolites was achieved by HPLC using a semipreparative column (250 mm × 9.4 mm i.d., Whatman, Clifton, NJ) with a flow rate of 6 mL/min. A total of 340 mg was injected (in five portions). The fractions containing 3 were pooled, concentrated, and extracted (five times) with an equal volume of $CHCl_3$. The combined extracts were washed, dried, and evaporated under reduced pressure to leave 44 mg of 3; MS (high resolution): M^+ 345.2296 (calcd. for $C_{21}H_{31}NO_3$: 345.2303); IR ($CHCl_3$) 3600, 2990, 1715, 1595, 1500, 1450, and 1390 cm^{-1}; ^1H NMR (90 MHz, $CDCl_3$): δ 1.07 (t, 6, J = 6.8 Hz, H-13), 2.43 (q, 4, J = 6.8 Hz, H-12), 3.26 (s, 1, H-1), 3.80 (d, 1, J = 7.0 Hz, H-5), 4.09 (t, 2, J = 6 Hz, H-9), and 7.41 ppm (m, 5, ArH); ^{13}C NMR (see Table II).

Metabolite 4: 3-(Diethylamino)propyl (2-*endo*,5-*exo*)-5-Hydroxy-2-phenylbicyclo[2.2.1]heptane-2-carboxylate—High-performance liquid chromatographic analysis of fraction 5 indicated the presence of a major component with a retention time close to that of 3. Metabolite 3 was also detected as a minor component in this fraction. In an attempt to separate the major component 4 from 3, 100 mg of fraction 5 was chromatographed on silica gel (10 g) eluting successively with $CHCl_3$ and $CHCl_3$ containing increasing volumes of EtOH. The fraction eluted with 60-mL EtOH:$CHCl_3$ (10:90) yielded, after evaporation, 59 mg of 4, (a trace of 3 could still be detected on HPLC). A total of 16 mg of 4 was obtained by semipreparative HPLC of fraction 6 as described above; MS (high resolution): M^+ 345.2304 (calcd. for $C_{21}H_{31}NO_3$: 345.2303); IR ($CHCl_3$) 3605, 3390(br), 2970, 1716, 1660, 1596, 1495, 1458, 1446, and 1380 cm^{-1}; ^1H NMR (90 MHz, $CDCl_3$): δ 0.99 (t, 6, J = 6.6 Hz, H-13), 2.46 (q, 4, J = 6.6 Hz, H-12), 3.16 (d, 1, J = 4.0 Hz, H-1), 3.96 (d, 1, J = 7 Hz, H-5), 4.20 (t, 2, J = 6 Hz, H-9), 7.32 (m, 5, ArH); ^{13}C NMR (see Table II).

Metabolite 5: 3-(Diethylamino)propyl (2-*endo*,5-*endo*)-5-Hydroxy-2-phenylbicyclo[2.2.1]heptane-2-carboxylate—During the course of the preparative HPLC procedures utilized to obtain 3 and 4, another minor component was observed. The fractions containing the minor component were pooled and extracted as described previously. After evaporation, a total of 12-mg residue of metabolite 5 was obtained; MS (high resolution): M^+ 345.2304 (calcd. for $C_{21}H_{31}NO_3$: 345.2303); IR ($CHCl_3$) 3609, 2970, 1715, 1650, 1500, and 1455 cm^{-1}; ^1H NMR (90 MHz, $CDCl_3$): δ 0.94 (t, 6, J = 6.2 Hz, H-13), 2.50 (q, 4, J = 6.2 Hz, H-12), 3.30 (d, 1, J = 3 Hz, H-1), 4.16 (m, 3, H-9, 5), and 7.40 ppm (m, 5, ArH); ^{13}C NMR (see Table II).

The overall yields of metabolites 2, 3, 4, and 5 were 10, 5, 8, and 3%, respectively.

Oxidation of Metabolites 2–5—A solution of metabolite 4 (10 mg) in 1 mL of CH_2Cl_2 was added to a stirred solution of pyridinium chlorochromate (PCC, 20 mg) in 1 mL of CH_2Cl_2. The mixture was stirred at room temperature for 1.5 h. After TLC analysis showed that oxidation was complete, the mixture was diluted with three volumes of CH_2Cl_2, filtered through diatomaceous earth, and then passed through a short plug of silica gel. Evaporation of the solvent gave a residue which was redissolved in MeOH, filtered, and evaporated to give 6 [MS m/z = 343; IR ($CHCl_3$) 1725 and 1745 cm^{-1}]. Oxidation of 5, using the same procedure, produced a ketone which was identical to 6 (MS, TLC, HPLC). Oxidations of 2 and 3 were carried out as above and gave ketones 7 and 8, respectively (7: MS m/z = 343; IR ($CHCl_3$): 1723 and 1745 cm^{-1}; 8: MS m/z = 343; IR ($CHCl_3$): 1725 and 1741 cm^{-1}). The absorbance ratio at 254 and 280 nm was also measured for each of the ketones and metabolites. This greatly facilitates identification of unknown compounds.[11] The retention times of the metabolites and ketones relative to 2-pentanone are listed first and absorbance ratios are listed second: 2 (1.71, 17.00); 3 (1.95, 16.00); 4 (1.97, 16.94); 5 (2.35, 15.33); 6 (2.62, 6.25); 7 (2.36, 5.00); and 8 (2.61, 6.50).

Preparation of Atropic Acid[12]—Atrolactic acid 9 (6 g) was added to polyphosphoric acid (60 g) contained in a 250-mL three-necked flask fitted with a mechanical stirrer. The mixture was stirred at room temperature. After 30 min, the mixture developed an intense yellow color. Stirring was continued for another 4.5 h (3 h sufficed in other cases). The resulting mixture was diluted with five volumes of ice water (500 mL), and was extracted five times with $CHCl_3$ (500, 4 × 200 mL). The extracts were washed with water, dried over anhydrous Na_2SO_4, and evaporated to afford 4.749 g of atropic acid 10. This solid could be used without further purification. Crystallization from EtOH gave a white solid 10, mp 105–106 °C, (lit.[13] 106–107 °C); ^1H NMR (90 MHz, $CDCl_3$): δ 6.00 and 6.51 (s, 1, C=CH_2), 7.48 (m, 5, ArH), and 10.53 ppm (s, 1, CO_2H).

Preparation of *exo*- and *endo*-2-Phenylbicyclo[2.2.1]hept-5-ene-2-carboxylic Acids (11, 12)—A procedure was followed as before[6] using freshly distilled cyclopentadiene (3.829 g) and 10 (4.294 g) to give an oily residue (5.430 g). The residue (an epimeric mixture of 11 and 12 by ^1H and ^{13}C NMR) was chromatographed on silica gel 60 (70–270 mesh). Elution was carried out with CH_2Cl_2 containing 0.25% AcOH. Fractions containing 11 were eluted first and were pooled together based on the ^1H NMR spectral data. Subsequent fractions contained various proportions of 11 and 12. The tailing fractions contained mostly 12. Successive recrystallization from EtOAc:hexane afforded 11 and 12. The *exo*-acid (11) was characterized as follows: mp 135–136 °C (lit.[6] 137 °C); ^1H NMR (90 MHz, $CDCl_3$): δ 1.50 (br s, 2, H-7), 1.70 (dd, 1, J = 12.0 and 1.2 Hz, H-3 *endo*), 2.77 (dd, 1, J = 12.0 and 4.0 Hz, H-3 *exo*), 2.83 (br s, 1, H-4), 3.80 (br s, 1, H-1), 5.85 (dd, 1, J = 6.0 and 2.9 Hz, H-6), 6.06 (dd, 1, J = 6.0 and 2.9 Hz, H-5), 7.23 (m, 5, ArH), and 11.70 ppm (s, 1, COOH). These assignments were based on homonuclear decoupling experiments performed by irradiating at the exact frequencies of each H-1 and H-4. ^{13}C NMR (see Table II). Anal. ($C_{14}H_{14}O_2$), C, H. The *endo*-acid was characterized as follows: (12) mp 131–132 °C (lit.[6] 132 °C); ^1H NMR (90 MHz, $CDCl_3$): δ 1.50 (br s, 2, H-7), 2.06 (dd, 1, J = 12.0 and 3.0 Hz, H-3 *exo*), 2.36 (dd, 1, J = 12.0 and 1.2 Hz, H-3 *endo*), 2.90 (br s, 1, H-4), 3.68 (br s, 1, H-1), 6.16 (dd, 1, J = 5.8 and 3.0 Hz, H-6), 6.31 (dd, 1, J = 5.8 and 3.0 Hz, H-5), 7.43 (m, 5, ArH), and 10.75 ppm (s, 1, COOH). As in the case of 11, these assignments were made unambiguously based on irradiation at H-1 and H-4 frequencies. ^{13}C NMR (see Table II). Anal. ($C_{14}H_{14}O_2$) C, H.

Hydrogenated *exo*-2-Phenylbicyclo[2.2.1]heptane-2-carboxylic

Acid (13)—A solution of 110 mg of 11 in 2 mL of C_2H_5OH was stirred with 17 mg of 10% Pd-C in an atmosphere of hydrogen for 1 h at 30 psi. The solution was filtered and evaporated to give 84 mg of 13: mp 160–161 °C (EtOAc:hexane) (lit.[6] 158–159 °C); ^{1}H NMR (90 MHz, $CDCl_3$): δ 1.40 (br m, 6, H-5, 6, 7), 1.73 (dd, 1, J = 13.2 and 1.0 Hz, H-3 *endo*), 2.35 (br s 1, H-4), 2.70 (dd, 1, J = 13.2 and 4.0 Hz, H-3 *exo*), 3.20 (br s, 1, H-1), 7.40 (m, 5, ArH), and 11.36 ppm (s, 1, COOH); ^{13}C NMR (see Table II). Anal. ($C_{14}H_{16}O_2$) C, H.

Hydrogenated *endo*-2-Phenylbicyclo[2.2.1]heptane-2-carboxylic Acid (14)—The acid was prepared as above as a white solid in 93% yield: (EtOAc:hexane), mp 117–118 °C, (lit.[6] 120 °C); ^{1}H NMR (90 MHz, $CDCl_3$): δ 1.48 (br m, 6, H-5, 6, 7), 1.90 (dd, 1, J = 14.0 and 3.5 Hz, H-3 *exo*), 2.30 (br s, 1, H-4), 2.55 (dd, 1, J = 14.0 and 1.2 Hz, H-3 *endo*), 3.15 (br s, 1, H-1), 7.46 (m, 5, ArH), and 10.53 ppm (s, 1, COOH); ^{13}C NMR (see Table II). Anal. ($C_{14}H_{16}O_2$) C, H.

3-(Diethylamino)propyl (2-*exo*)-2-Phenylbicyclo[2.2.1]heptane-2-carboxylate (16)—A solution of 13 (63 mg) in dry benzene (5 mL) was refluxed with thionyl chloride (1–2 mL) for 12 h. After complete removal of thionyl chloride and benzene, the residue (67 mg) was dissolved in dry benzene (3 mL). A solution of 3-diethylamino-1-propanol 15 (38.5 mg) in dry benzene (1 mL) was added in portions, and the mixture was refluxed for 19 h. Removal of the solvent, under reduced pressure, gave an oily residue (108 mg). This material was dissolved in distilled water (7 mL) and was rendered alkaline using conc. NH_4OH. The alkaline solution (pH 11) was extracted five times with $CHCl_3$ (5 × 8 mL). The combined extracts were washed with water, dried, and evaporated to give 16, as an oil (60 mg); MS (high resolution): M^+ at m/z 329.2355 consistent with $C_{21}H_{31}NO_2$ (Calcd. 329.2355); IR ($CHCl_3$): 1722 cm^{-1}; ^{1}H NMR (90 MHz, $CDCl_3$): δ 1.03 (t, 6, J = 6.8 Hz, H-13), 1.20–1.95 (9), 2.36 (m, 3, H-11, 4), 2.45 (q, 4, J = 6.8 Hz, H-12), 2.80 (dd, 1, J = 13.2 and 5.5 Hz, H-3 *exo*), 3.25 (br s, 1, H-1), 4.06 (t, 2, J = 6 Hz, H-9), and 7.40 ppm (m, 5, ArH); ^{13}C NMR (see Table II).

3-(Diethylamino)propyl (2-*endo*)-2-Phenylbicyclo[2.2.1]heptane-2-carboxylate (17)—The acid was prepared as above in 61% yield. MS (high resolution): M^+ at m/z 329.2355 consistent with $C_{21}H_{31}NO_2$ (calcd. 329.2355); IR ($CHCl_3$): 1720 cm^{-1}; ^{1}H NMR (90 MHz, $CDCl_3$): δ 1.02 (t, 6, J = 6.0 Hz, H-13), 1.19–1.99 (9), 2.29 (m, 3, H-4, 11), 2.42 (q, 4, J = 6.0 Hz, H-12), 2.66 (dd, 1, J = 12.0 and 3.0 Hz, H-3 *exo*), 3.16 (br s, 1, H-1), 4.06 (m, 3, H-5, 9), and 7.39 ppm (m, 5, ArH); ^{13}C NMR (see Table II).

References and Notes

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Acknowledgments

The authors gratefully acknowledge partial support of this work by the Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi and Dr. M. Hollman of Knoll for a generous supply of bornaprine hydrochloride.