Journal of Natural Products
Vol. 47, No. 3, pp. 453-458, May-Jun 1984

POTENTIAL ANTICANCER AGENTS XXXI. *N*-DEMETHYLATION OF FAGARONINE[1]

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ABSTRACT.—Fusion of fagaronine (**1**) afforded *N*-demethyl fagaronine (**2**) and two minor desmethyl products. Through examination of spectral properties and derivatization, the structures were deduced to be **3**, a tetramethoxy derivative, and **5**, a derivative bearing a hydroxy (rather than a methoxy) group at position -8. Acetylation of **2** afforded a monoacetate derivative (**4**), and similarly, a diacetate (**6**) was produced from **5**. Compounds **2-6** were substantially less cytotoxic than **1**, as judged by KB or P-388 cell culture assays, supporting the functional importance of the quaternary nitrogen atom. The results obtained to date for fagaronine in tumor panel-testing are also presented, and the marginal cytotoxic activity demonstrated by compounds **5** and **6** against cultured P-388 cells is discussed in terms of mechanism of action of the parent compound.

Fagaronine (**1**), a benzophenanthridine alkaloid from *Fagara zanthoxyloides* Lam. (Rutaceae), is a potent antineoplastic agent (2). As part of the evidence accumulated for the structure, spectral studies of *N*-demethyl fagaronine (**2**) were carried out. This compound was formed from **1** through pyrolysis at 270° (3). In the process of repeating this reaction, it was noted that a number of other products were formed. An investigation of these products is the subject of this report.

Chromatography of the crude pyrolysis product on silica gel afforded three products, the major of which was **2** (89.5% yield). The least-polar minor product (2.0% yield) displayed a molecular ion at *m/z* 349, 14 amu more than **2**. In the pmr spectrum (Table 1), the aromatic region was virtually unchanged in terms of chemical shift and

TABLE 1. Proton Nuclear Magnetic Resonance Spectra of Fagaronine and Derivatives[a]

Proton	Compound					
	1[b]	**2**	**3**	**4**	**5**	**6**
1	7.66	7.40	7.29	7.58	7.39	7.61
4	7.94	8.74	8.75	8.80	8.71	8.83
6	9.97	9.25	9.25	9.18	9.20	9.32
7	8.13	7.89	7.90	7.77	8.03	8.27
10	8.36	7.39	7.39	7.31	7.35	7.49
11	8.86	8.29	8.30	8.18	8.23	8.23
	d J=9.0	d J=9.2	d J=8.8	d J=8.9	d J=9.0	d J=8.9
12	8.16	7.81	7.85	7.74	7.78	7.84
	d J=9.0	d J=9.2	d J=8.8	d J=8.9	d J=9.0	d J=9.1
2-OCH_3	—	—	4.08	—	—	—
3-OCH_3	4.04	4.08	4.08	4.04	4.07	4.03
8-OCH_3	4.24	4.19	4.20	4.11	—	—
9-OCH_3	4.11	4.16	4.16	4.11	4.16	4.13
-N-CH_3	5.11	—	—	—	—	—
OAc	—	—	—	2.40	—	2.41, 2.42

[a]Data obtained at 60 MHz in $CDCl_3$, δ ppm, δ_{TMS}=0 ppm.
[b]In DMSO-d_6, data are from Messmer *et al.* (2).

[1]For the previous paper in this series, see Arisawa *et al.* (1).

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multiplicity from that of **1** except for a small upfield shift ($\Delta\ \delta$ - 0.11 ppm) in H-1. However, in the methoxyl region, a new three-proton singlet at 4.08 ppm was observed, suggesting that this product has the structure **3**.

The more polar minor product (4.0% yield) displayed a molecular ion at *m/z* 321, 14 amu less than **2**. A quite strong hydroxyl absorption was observed at 3450 cm^{-1}, together with characteristic imine bands at 1520 and 1540 cm^{-1}. It was thought that this compound could be a demethyl derivative of **2**, the question then being to assign the position of the second hydroxyl group.

The pmr spectrum (Table 1) indicated the presence of only two methoxy groups (4.07 and 4.16 ppm), and one of the aromatic singlets (H-7) was shifted downfield by 0.14 ppm; the remaining chemical shifts were very similar to those of **2**. Acetylation of **2** afforded a monoacetate derivative **4** in which the adjacent proton (H-1) was shifted downfield by 0.18 ppm, and the *meta* proton (H-4) was shifted downfield by 0.06 ppm.

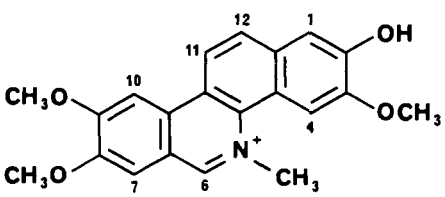
When the polar fusion product was acetylated, a diacetate derivative was produced, showing a molecular ion at *m/z* 405 and two successive losses of 42 amu. In the pmr spectrum, H-1 and H-4 were shifted downfield by 0.22 and 0.12 ppm, but three other protons were also substantially shifted. Thus, a marked downfield shift of 0.24 ppm was observed for H-7, and lesser shifts were observed for H-6 (0.12 ppm) and H-10 (0.14 ppm). These data suggest that the hydroxy group in the most polar fusion product is *ortho* to H-7 and *meta* to H-10, i.e., is at H-8. On this basis, the compound is proposed to have the structure **5**, its diacetate having the corresponding structure **6**.

The cytotoxic potential of the desmethyl derivatives was then evaluated utilizing KB and P-388 cells. As anticipated, in both test systems the cytotoxic activity of compounds **2-6** was substantially reduced in comparison with fagaronine (**1**) (Table 2).

TABLE 2. Evaluation of the Cytotoxic Activity of Fagaronine (**1**) and Derivatives

Compound	Test System	
	KB (ED_{50}, =μg/ml)	P-388 (ED_{50}, =μg/ml)
1	0.57	0.18
2	NA[a]	36.5
3	NA	NA
4	NA	NA
5	22.4	13.0
6	50	12.5

[a]NA, not active. Due to limited quantities of sample, the highest concentration tested was 25 μg/ml. At this (or lower) concentration, the results did not differ from solvent-treated controls (i.e., 100% survival).



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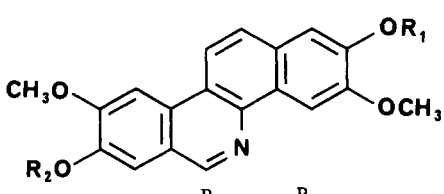

	R_1	R_2
2	H	CH_3
3	CH_3	CH_3
4	Ac	CH_3
5	H	H
6	Ac	Ac

Surprisingly, however, although compounds **5** and **6** would not be classified as "active" by established criteria (4), cytotoxic activity was demonstrated, and this was greater than that of compounds **2-4**.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were determined by means of a Kofler hotplate and are uncorrected. The uv spectra were obtained with a Beckman model DB-G grating spectrometer. The ir spectra were determined on a Beckman IR-18A spectrometer or a Nicolet MX-1 interferometer. Pmr spectra were recorded in $CDCl_3$ on a Varian T-60A instrument with a Nicolet Fourier-Transform attachment. TMS was used as an internal standard and chemical shifts are reported in δ-units (ppm). Mass spectra were obtained with a Varian MAT 112S double focusing spectrometer operating at 70 eV.

ORIGIN OF STARTING MATERIAL.—Fagaronine (**1**) was isolated as the chloride salt from the roots of *F. zanthoxyloides* (Rutaceae) as described previously and showed spectral properties in agreement with those of an authentic sample (2).

FUSION OF FAGARONINE.—Fagaronine (**1**, 450 mg) was heated at 270° for 5 min, during which time the original orange-yellow color changed to a greyish white. The product after cooling was dissolved in $CHCl_3$ and chromatographed on silica gel PF_{254}[3] (50 g) eluting with $CHCl_3$ and $CHCl_3$-MeOH mixtures. Elution with $CHCl_3$ afforded **3** (8 mg, 2.0% yield), and elution with $CHCl_3$-MeOH (99:1) gave **2** (350 mg, 89.5%) and **5** (15 mg, 4.0%).

SPECTRAL PROPERTIES OF THE FUSION PRODUCTS.—The isolates displayed the following physical and spectral properties: 2-hydroxy-3,8,9-trimethoxy-benzo[*c*]phenanthridine (**2**, *N*-demethyl fagaronine), mp 264°; ir ν max (KBr) 3525, 1640, 1610, 1535, 1495, 1430, 1285, 1235, 1220, 1200, 1180, 1112, 1030, 945, 940, 900, 865, 825, and 810 cm^{-1}; uv λ max (EtOH) 231 (log ε 4.76), 253 (4.74), 276 (4.96), 285 (4.96), and 315 nm (4.63); pmr (see Table 1); ms, *m/z* (%) 335 (M^+, 100%), 334 (42.7), 306 (8), 292 (10.2), 277 (9.3), 276 (3.0), 262 (3.5), 261 (2.5), 249 (5.2), 248 (6.8), 234 (4.2), 233 (2.1), 232 (2.1), 220 (4.6), 219 (4.9), 206 (6.0), 191 (3.0), 190 (3.5), 177 (3.5), 168 (18.3), 151 (2.5), 150 (3.2), 139 (4.4), 131 (3.6), 125 (5.9), 124 (3.1), and 110 (5.7).

2,3,8,9-Tetramethoxy-benzo[*c*]phenanthridine (**3**), mp 308°; ir ν max (KBr) 1620, 1585, 1510, 1498, 1464, 1407, 1260, 1164, 1095, 1008, 880, 850, and 807 cm^{-1}; uv λ max (EtOH) 231, 274, 282, 300 (sh), 335, 350, and 370 nm; pmr (see Table 1); ms, *m/z* (%) 349 (M^+, 100%), 348 (20.8), 334 (18.5), 333 (57.4), 320 (5.1), 319 (3.1), 318 (12.3), 304 (4.2), 303 (9.5), 291 (3.0), 290 (8.8), 289 (3.4), 231 (2.7), 219 (2.7), 181 (4.2), 169 (4.9), 131 (2.9), 119 (5.2), and 69 (28.0).

2,8-Dihydroxy-3,9-dimethoxy-benzo[*c*]phenanthridine (**5**), mp 215-255° (dec.); ir ν max (KBr) 3450, 1640, 1672, 1540, 1522, 1465, 1430, 1290, 1225, 1188, 1115, 1040, 890, 872, 825, and 774 cm^{-1}; uv λ max (EtOH) 233, 253 (sh), 270 (sh), 277, 284, 313 (sh), 335, and 370 nm, λ max (EtOH+KOH) 215, 248, 258, 284 (sh), 298 (sh), 309, and 360 nm; pmr (see Table 1); ms *m/z* (%) 321 (M^+ 100), 320 (45.4), 306 (12.4), 292 (8.9), 291 (4.6), 278 (14.5), 277 (5.5), 263 (9.5), 249 (4.4), 235 (12.0), 220 (2.6), 206 (4.5), 190 (2.0), 178 (3.1), 161 (17.2), 153 (6.8), 139 (9.7), 132 (7.4), 125 (3.2), 124 (2.1), and 118 (19.0).

ACETYLATION OF **2**.—A solution of **2** (10 mg) in Ac_2O-pyridine (1:1, 0.5 ml) was heated on a steam bath for 1 h. Work-up in the usual way afforded the monoacetate derivative **4** (8.5 mg) as colorless needles, mp 244-245°, ir ν max (KBr); 3020, 3005, 2975, 2950, 1775, 1640, 1612, 1535, 1525, 1495, 1470, 1460, 1442, 1435, 1395, 1285, 1255, 1245, 1220, 1190, 1185, 1160, 1120, 1040, 1025, 955, 946, 932, 900, 890, 870, 864, 826, and 812 cm^{-1}; pmr (see Table 1); ms, *m/z* (%) 377 (M^+, 27.5), 336 (24.0), 335 (100.0), 334 (42.7), 320 (6.8), 306 (7.3), 305 (3.5), 292 (5.5), 277 (4.5), 263 (3.6), 262 (2.9), 249 (2.6), 248 (3.9), 220 (3.2), 219 (3.2), 190 (3.7), and 177 (3.9).

ACETYLATION OF **5**.—A solution of **5** (8 mg) in Ac_2O-pyridine (1:1, 1 ml) was heated on a steam bath for 1 h. Work-up in the usual way afforded a diacetate derivative **6** (5 mg) as colorless needles, mp 240-242°; pmr (see Table 1); ms, *m/z* (%) 405 (M^+, 65.1%), 364 (28.9), 363 (100.0), 322 (50.0), 321 (100), 320 (68.5), 306 (16.3), 305 (4.2), 292 (18.0), 291 (7.3), 278 (18.6), 277 (13.5), 263 (9.6), 249 (11.5), 248 (6.6), 235 (9.1), 234 (6.0), 220 (3.7), 219 (3.9), 218 (3.0), 206 (9.9), 191 (3.2), 190 (5.3), 178 (3.2), 177 (6.7), 163 (3.8), 151 (3.4), 150 (4.8), and 75 (3.0).

CELL CULTURE ASSAYS.—Cytotoxic activity was assessed using cultured KB cells essentially by the method of Geran *et al.* (4) as described previously (5). Similarly, the protocol utilized for evaluating activ-

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ity with cultured P-388 cells was essentially as established by the NCI. Briefly, cells derived from a 24-h spinner culture were seeded at a concentration of 5×10^4/ml and treated with various concentrations of the test substance, initially dissolved in 0.15 ml of 10% aqueous DMSO (final volume, 3.0 ml). After 48 h at 37° in a humified incubator containing 5% CO_2 in air, the cell number was determined and expressed as a percentage, relative to a control incubation in which the cells were treated with the vehicle only. All assays were performed in duplicate, semilogarithmic plots were constructed from the averaged data, and the effective dose of the substance required to inhibit cell growth by 50% (ED_{50}) was determined.

DISCUSSION

Benzo[*c*]phenanthridine alkaloids display a variety of biological activities (6, 7) of which the antineoplastic activity is currently of greatest interest. Several years ago, we discovered that a member of this group of alkaloids, fagaronine (**1**) (2), was responsible for the antineoplastic activity of extracts derived from the roots of *F. zanthoxyloides*, and this stimulated a number of synthetic endeavors (8-10) to try to enhance the biological activity. Aqueous extracts of the roots of *F. zanthoxyloides* were also reported to possess antisickling activity (11, 12), but we have been unable to reproduce this observation (13).

Clearly, the greatest potential for this compound resides in its activity as an antileukemic agent. Fagaronine (**1**) was shown to possess significant antileukemic activity in the P-388 lymphocytic leukemia (2) and L1210 lymphocytic leukemia (2,14) test systems. This substance has also been evaluated in a number of other systems as a tumor panel-compound (14), and these results are summarized in Table 3. As indicated, fagaronine was evaluated as inactive in all test systems other than P-388 and L-1210. In addition, no reversal in the astrocytoma assay was shown. The biochemical mode of action of fagaronine at the molecular level has been studied by several groups and the results recently summarized (5).

In the present work, a series of semisynthetic *N*-demethyl fagaronine derivatives was prepared and structurally characterized. Consistent with previous studies (5), **2** was not significantly cytotoxic toward KB cells. This observation has now been extended in that **2** demonstrated only weak cytotoxicity when incubated with cultured P-388 cells. Similarly, at the doses tested, compounds **3** and **4** demonstrated no substantial cytotoxic activity in either of these test systems. Taken together, these results substantiate the functional importance of the quaternary nitrogen atom in **1**. As suggested previously (5), this cationic functionality appears to serve as an anchor through interaction with the negatively charged phosphate backbone of nucleic acids. Subsequently, the benzo[*c*]phenanthridine nucleus can intercalate between base pairs.

Interestingly, although compounds **5** and **6** would not be classified as potential antitumor agents on the basis of the current results, cytotoxic activity was significantly greater than that observed with the other derivatives. Parallel, albeit weaker, activity was demonstrated with the cultured epithelial cell line (KB). Assuming the cytotoxicity observed with **5** and **6** was reduced, relative to **1**, solely due to the valence state of the nitrogen atom (i.e., that the mechanism of action involves the same target site affected by **1**), it could be suggested that a substituent pattern other than that of the parent compound could lead to greater cytotoxic activity. This is subject to experimental verification in future studies. Alternatively, the derivatives may have a different mechanism of action which has heretofore been uninvestigated. An additional consideration that could not be overlooked is the role of cellular "metabolic activation" factors. As shown previously, rat liver preparations substantially enhance the bactericidal activity of fagaronine (**1**) (5). Final elucidation of the cytotoxic mechanism facilitated by fagaronine or its derivatives may indicate a rather complex sequence of events involving multiple steps or sites of action.

TABLE 3. Summary of the Antineoplastic Evaluation of Fagaronine (**1**)[a]

Test System	Treatment Schedule	Dose mg/kg	T/C %	Activity Criterion
B16 Melanoma	Days 1-9	400	toxic	≥125
		200	100	
		100	97	
		50	108	
$CD8F_1$ Mammary Tumor	Day 24	2000	121	≤10
		1000	89	
		500	134	
		250	190	
		125	137	
CX-1 Colon Xenograft	Days 1, 5, 9, 13	600	75	≤20
		300	51	
		150	42	
		75	49	
Colon 26	Days 1, 5, 9	320	138	≥130
		160	120	
		80	121	
		40	117	
Colon 38	Days 2, 9	800	toxic	≤42
		400	toxic	
		200	81	
		100	89	
		50	139	
L1210 Lymphoid Leukemia	Days 1-9	320	161	≥125
		160	145	
		80	159	
		40	137	
	Days 1, 5, 9	320	137	
		160	136	
		80	145	
		40	143	
Lewis Lung Carcinoma	Days 1-9	400	toxic	≥140
		200	134	
		100	125	
		50	113	
		25	111	
LX-1 Lung Xenograft	Days 1, 5, 9	2400	65	≤20
		1200	46	
		600	79	
		300	107	
MX-1 Breast Xenograft	Days 1, 5, 9	1200	68	≤20
		600	79	
		300	74	
		150	83	
P-388 Lymphocytic Leukemia	Days 1-9	320	205	≥120
		160	270	
		80	210	
		40	190	
	Days 1-10	100	265	
		50	210	
		25	90	
	Days 1, 5, 9	320	270	
		160	215	
		80	180	
		40	190	

[a]Evaluated according to established protocols (4).

ACKNOWLEDGMENTS

This work was supported, in part, by Contract CM-67295 from the Natural Products Branch, Division of Cancer Treatment, Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD. The authors would like to thank Dr. Suffness of the National Products Branch, NCI, for the information on the *in vivo* biological testing of fagaronine (**1**) and E.G. and G. Mason Research Institute, Worcester, MA, for the supply of KB and P-388 cell lines.

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Received 19 May 1983