Synthesis and Cytotoxic Activity of Hydroxylated Derivatives of Olivacine in Relation with Their Biotransformation

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The chemical synthesis of 9-hydroxyolivacine and 7-hydroxyolivacine based on a biomimetic approach is described. These two hydroxylated derivatives have been found as main in vitro metabolites of olivacine after incubation with rat hepatic microsomes. The pretreatment of animals with benzo[*a*]pyrene caused a large increase in both microsomal hydroxylations, whereas the pretreatment with phenobarbital caused a weak increase, with a preservation of 9-hydroxylation/7-hydroxylation ratio >1 in both cases. The two hydroxyolivacines have been also found as principal in vivo metabolites of olivacine in rat bile as glucuronide and sulfate conjugates. The pretreatment of animals with benzo[*a*]pyrene reverses the 9-hydroxyolivacine/7-hydroxyolivacine ratio excretion in bile to a value that is <1. In both in vitro and in vivo experiments, the free metabolites were identified by HPLC and UV–visible, MS, and ^{1}H NMR spectra. Hydroxylation at position 9 increases the in vitro cytotoxicity against leukemia L1210 cells (ID_{50} = 0.06 μM compared to 2.03 μM for olivacine) and an opposite effect is observed for hydroxylation at position 7 (ID_{50} = 12.8 μM). On the other hand, hydroxylation at position 9 has no effect on the in vivo antitumor activity against L1210. This might be related to the oxidative and conjugative metabolic pathways that play an important role in antitumor activity and deactivation of olivacine and its hydroxy metabolites.

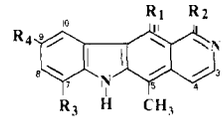
In the last two decades considerable works on chemical syntheses[1] and biological effects[2] of ellipticine (**1**, Scheme I) have been reported. Recently, attention was focused on the hydroxylated derivatives of ellipticine[3] and N^2-methylellipticinium,[4] which are the main rat hepatic metabolites of these compounds. One of these hydroxylated derivatives of ellipticine, the N^2-methyl-9-hydroxyellipticinium acetate, is currently used in the treatment of osteolytic metastases of breast cancer.[5,6] The recent clinical results with this hydroxyellipticine compound attracts attention to olivacine (**2**), a natural isomer of ellipticine and its derivatives.[7] Olivacine shows significant differences[7] in in vitro cytotoxicity (ID_{50} = 1.72 μM, L1210 cells) and in vivo toxicity (LD_0 = 250 mg/kg, mice) compared to ellipticine itself[5] (ID_{50} = 0.99 μM, LD_0 = 50 mg/kg) and this in spite of their great structural similarity (a simple shift of one methyl group from position 11 to 1).

In this paper, we report the chemical syntheses of 9-hydroxyolivacine (**3**) and 7-hydroxyolivacine (**4**), which are the major metabolites of olivacine in rat liver microsomes and bile. The metabolic activation and deactivation pathways of these olivacine compounds in relation with their toxicities and antitumor activities are also discussed.

Results and Discussion

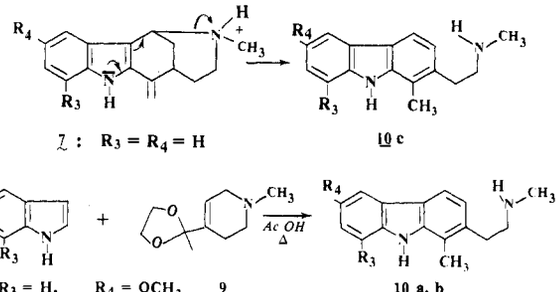
Chemistry. In the early approaches[8] for synthesis of the ellipticine and olivacine derivatives, no attention was paid to the application of the syntheses based on biogenesis and use of other available natural products as starting materials. Previous to this report we proposed a general scheme for the synthesis of ellipticine and olivacine based on the biomimetic acid-catalyzed fragmentation of desethyluleine (**7**) to the carbazole derivative **10c** (Scheme II).[9] The present syntheses of 9-hydroxyolivacine (**3**) and 7-hydroxyolivacine (**4**) were achieved according to this procedure (Schemes II and III). The key intermediates in the synthesis of **3** and **4** that have structures **10a** and **10b** were obtained in a one-pot reaction by condensation of the required methoxyindoles **8a** and **8b** with Δ^3-piperidine **9** (Scheme II). For this purpose equimolecular amounts of **8a** or **8b** and **9** were refluxed for 56 h in 50% AcOH–H_2O under an argon atmosphere. After the usual workup, the carbazoles **10a** and **10b** were obtained in high yields (**10a**,

Scheme I. Formula of the Olivacine and Ellipticine Derivatives



$R_1 = CH_3$, $R_2 = H$, $R_3 = H$, $R_4 = H$: ellipticine **1**
$R_1 = H$, $R_2 = CH_3$, $R_3 = H$, $R_4 = H$: olivacine **2**
$R_1 = H$, $R_2 = CH_3$, $R_3 = H$, $R_4 = OH$: 9-OH-O **3**
$R_1 = H$, $R_2 = CH_3$, $R_3 = OH$, $R_4 = H$: 7-OH-O **4**
$R_1 = CH_3$, $R_2 = H$, $R_3 = H$, $R_4 = OH$: 9-OH-E **5**
$R_1 = CH_3$, $R_2 = H$, $R_3 = OH$, $R_4 = H$: 7-OH-E **6**

Scheme II. Synthesis of Carbazoles **10** from Fragmentation of Desethyluleine (**7**) or Condensation of Methoxyindoles **8** with Δ^3-Piperidine **9**



7 : $R_3 = R_4 = H$ — **10c**

8a : R_3 = H, $R_4 = OCH_3$ — **9** — **10a, b**
8b : $R_3 = OCH_3$, R_4 = H

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(2) Kohn, K. W.; Ross, W. E.; Glaubiger, D. "Antibiotics"; Han, F. E., Ed.; Springer Verlag: Berlin, 1979; Vol. 5(2), p 195.
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(7) Husson, H. P.; Besselievre, R.; Potier, P.; Le Pecq J. B.; Paolleti, C. *Eur. Pat. Appl.* EP 42 438, 1981.
(8) Woodward, R. B.; Jacobucci, G. A.; Hochstein, F. A. *J. Am. Chem. Soc.* **1959**, *81*, 4434.
(9) Besselievre, R.; Husson, H. P. *Tetrahedron, Suppl.* **1981**, *37*, 241.

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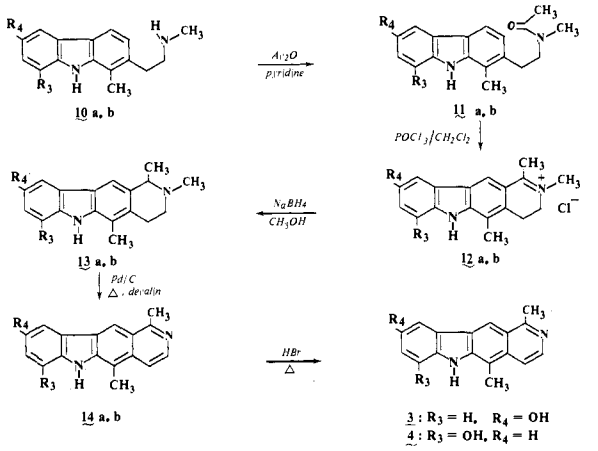
Scheme III. General Scheme for the Syntheses of 9-Hydroxyolivacine (3) and 7-Hydroxyolivacine (4) from Carbazole Derivatives **10**



90%; **10b**, 82%). Compounds **10a** and **10b** were acetylated to amides **11a** and **11b**, which were then cyclized via a Bischler–Napieralski reaction to **12a** and **12b**. Sodium borohydride reduction of these iminium salts yielded methoxyguatambuine **13a** and **13b**. Demethylation and dehydrogenation in boiling Decalin in the presence of 10% palladium–charcoal then led to 9-methoxyolivacine (**14a**) and 7-methoxyolivacine (**14b**). This reaction was performed preferably on the tetrahydro derivatives **13** to the dihydro derivatives **12** for better yields of **14**. Only, in the case of 7-methoxyolivacine (**14b**), the yield of the aromatization reaction was relatively low (22%). The final products, 9-hydroxyolivacine (**3**) and 7-hydroxyolivacine (**4**) were obtained via demethylation of **14a** and **14b**, respectively, in boiling HBr for 1 h.

Metabolism of Olivacine in Vitro. Nature of Metabolites Generated in Vitro. Metabolism of olivacine in vitro has been carried out with three different types of microsomes from (i) nonpretreated, (ii) phenobarbital (PB)-pretreated, and (iii) benzo[*a*]pyrene (BP)-pretreated rats (for a review on the induction of cytochrome P-450 monooxygenases by exogenous compounds, see ref 10). In all cases, besides the unmetabolized olivacine (peak **2**), two main metabolites (peaks I and II) are detected in the HPLC chromatograms of 1-butanol extracts (Figure 1). Metabolites I and II possess the same HPLC behaviors as the authentic 9-hydroxyolivacine (**3**) and 7-hydroxyolivacine (**4**). The ratio of the two metabolic products depends on the nature of microsomes used. For non- and PB-induced microsomes, only a small amount of olivacine is transformed; but with BP-induced microsomes, a near complete conversion of the starting drug is observed (Figure 1).

As observed for ellipticine,[11,12] the main microsomal drug oxidation products result from hydroxylation of the aromatic nucleus of olivacine at either the para or the ortho position to indolic ring nitrogen. It can be noticed that another minor metabolite is also formed with BP-induced microsomes, which is marked by an asterisk in Figure 1.

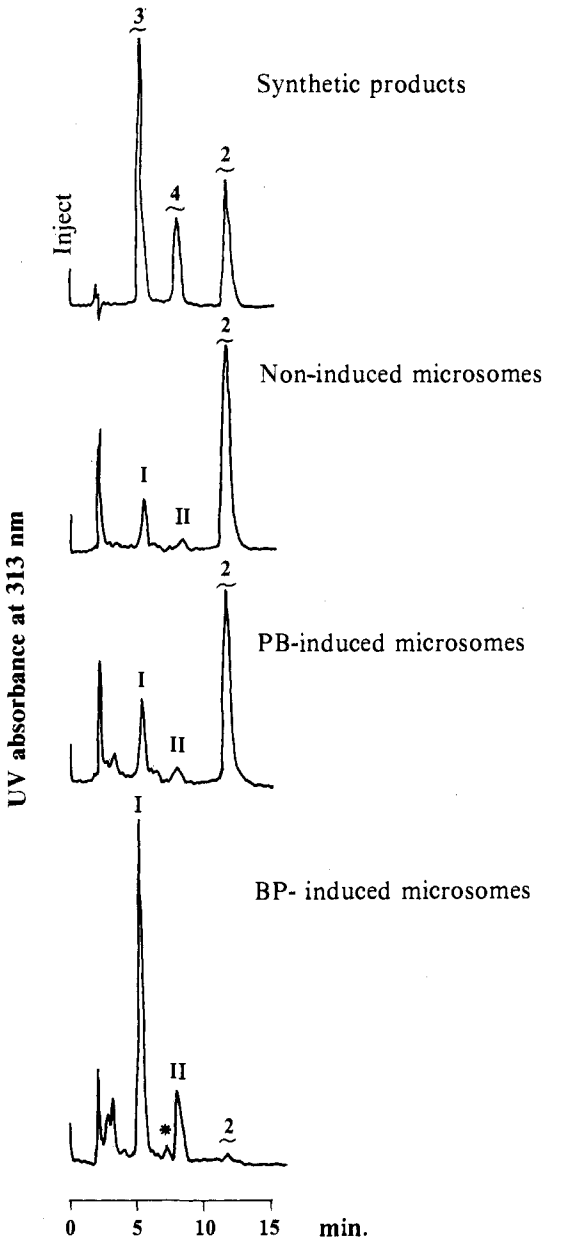


Figure 1. In vitro HPLC metabolic profiles of olivacine in rat hepatic microsomes. **2**–**4** represent the references olivacine, 9-hydroxyolivacine, and 7-hydroxyolivacine. I and II represent the metabolites formed after incubation (5 min) of olivacine (50 μM) with non-, PB-, or BP-induced microsomes (0.5 mg of protein/mL). The asterisk represents a nonidentified minor metabolite.

Table I. Effects of Enzyme Inducers on Kinetic Parameters of Rat Hepatic Microsomal Hydroxylation of Olivacine "in Vitro"[a]

pretreatment	metabolite I (9-hydroxyolivacine)		metabolite II (7-hydroxyolivacine)	
	K_m	V_{max}	K_m	V_{max}
noninduced	25.0 ± 8.8	0.58 ± 0.09	29.4 ± 7.6	0.13 ± 0.02
PB induced	40.0 ± 10.6	1.28 ± 0.29	41.4 ± 9.9	0.35 ± 0.08
BP induced	22.2 ± 5.2	7.14 ± 0.54	29.0 ± 10.3	3.64 ± 0.60

[a] For determination of kinetic parameters, five substrate concentrations in the range of 5–50 μM, 0.5 mg of protein/mL, and 5-min incubation time were used. Results were obtained with use of Lineweaver–Burk plot and are expressed as μM for K_m and nmol min^{-1} (mg of protein)$^{-1}$ for V_{max}. Data are the mean values ± SD with microsomes from two rats.

Pure metabolites I (4.4. mg) and II (3.5 mg) have been isolated by semipreparative HPLC from BP-microsomal incubations and compared with authentic compounds **3** and **4** by MS, ^{1}H NMR, and UV-visible analysis. The data

(10) (a) Mannering, G. J. "Fundamentals of Drug Metabolism and Drug Disposition"; La Du, B. N.; Mandel, H. G.; Way, E. L. Eds.; Williams and Wilkins: Baltimore, 1972; Chapter 12. (b) Gelboin, H. V. "Fundamentals of Drug Metabolism and Drug Disposition"; La Du, B. N.; Mandel, H. G.; Way, E. L., Eds.; Williams and Wilkins: Baltimore, 1972; Chapter 14.
(11) Lesca, P.; Lecointe, P.; Paoletti, C.; Mansuy, D. *Biochem. Pharmacol.* **1977**, *26*, 2169.
(12) Lesca, P.; Monsarrat, B.; Cros, S.; Paoletti, C. *J. Natl. Cancer Inst. (U.S.)* **1981**, *67*, 871.

reported in the Experimental Section confirm the identity of I and II as 9- and 7-hydroxyolivacine, respectively.

Kinetic Experiments. The protein, time, and substrate linearities of the hydroxylating enzyme with olivacine were examined by HPLC analysis of the 1-butanol extracts. The reactions were linear for at least 10 min, to at least 0.5 mg of protein/mL and to substrate concentration from 5 to 50 μM. The rates of hydroxylation of olivacine (**2**) by microsomal proteins from non-, PB-, and BP-pretreated rats were determined and K_m and V_{max} values are indicated in Table I. The K_m values for both hydroxylations are in the range 22–41 μM, indicating a good affinity of olivacine for all cytochrome P-450 hydroxylating systems. However, BP-induced microsomes are most efficient in enhancing the V_{max} values; they increase these values from 0.58 to 7.14 (12 times) nmol min^{-1} (mg of protein)$^{-1}$ for 9-hydroxylation and from 0.13 to 3.64 (28 times) nmol min^{-1} (mg of protein)$^{-1}$ for 7-hydroxylation reactions. For PB-induced microsomes these values are only slightly increased to 1.28 (2 times) nmol min^{-1} (mg of protein)$^{-1}$ for 9-hydroxylation and to 0.35 (3 times) nmol min^{-1} (mg of protein)$^{-1}$ for 7-hydroxylation (Table I). The low values for V_{max} are obtained when noninduced microsomes are employed.

Furthermore, when these data are compared with the corresponding ones obtained for ellipticine,[11] the K_m values for both molecules are found to be of the same order of value (30–75 μM for 9-hydroxylation of ellipticine), while the V_{max} values are significantly lower for ellipticine: 0.09 (6 times), 0.08 (16 times), and 0.14 (51 times) nmol min^{-1} (mg of protein)$^{-1}$ for 9-hydroxylation of ellipticine, when non-, PB-, or BP-induced microsomes are respectively employed. The K_m and V_{max} values for 7-hydroxylation of ellipticine have not been reported in the literature. This large difference in V_{max} values between olivacine and ellipticine might be related to the reduced interactions between the pyridine nitrogen of olivacine with the iron atom of reduced cytochrome P-450 compared to ellipticine.[13] The absence of a methyl group in position 11 for ellipticine forms the possible coordination of the pyridine moiety onto the metal of the metalloenzyme. Substituted pyridine derivatives with minimal steric hindrance near the nitrogen were recently found to be potent inhibitors of cytochrome P-450 hydroxylases, compared to those with substituents in the carbon adjacent to the nitrogen atom.[14]

As for ellipticine,[11,12] the 9-hydroxyolivacine is the major drug oxidation product of olivacine, formed with non-, PB-, and BP-induced microsomes. A second metabolite, 7-hydroxyolivacine, is also produced with all types of microsomes, in opposition to 7-hydroxyellipticine,[12] which is a minor metabolite only observed with polycyclic aromatic hydrocarbon induced microsomes.

Metabolism of Olivacine in Vivo. After intravenous administration of olivacine acetate, bile was collected over a 24-h period from non-, PB-, and BP-pretreated rats, and the metabolic products in 1-butanol extracts were analyzed by HPLC. The data in Figure 2 clearly demonstrate that the metabolites I and II are detected after treatment with mixed β-glucuronidase and arylsulfatase. The chromatographic behaviors of metabolites I and II are identical with those of authentic samples of 9- and 7-hydroxyolivacine. To establish their identity more conclusively, I (3.7 mg) and II (2.0 mg) were isolated from nonpretreated rat bile by semipreparative HPLC and compared with authentic

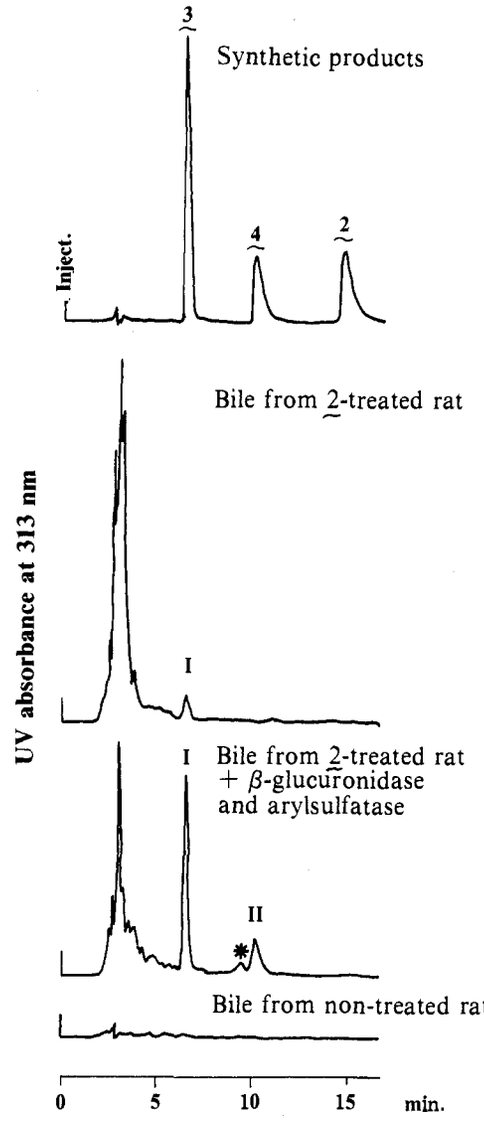


Figure 2. In vivo HPLC metabolic profile of olivacine in nonpretreated rat bile. 2–4 represent the references olivacine, 9-hydroxyolivacine, and 7-hydroxyolivacine. I and II represent the free (before mixed β-glucuronidase/arylsulfatase treatment) or free plus deconjugated metabolites (after enzyme treatment) excreted in 0–1-h bile collection period from rat dosed with olivacine acetate (25 mg/kg). The asterisk represents a nonidentified minor metabolite.

samples of **3** and **4** by UV–visible, ^{1}H NMR, and MS analysis (see Experimental Section). The data completely confirm the identity of metabolites I and II as 9- and 7-hydroxyolivacine, respectively. As mentioned for BP-induced microsomes, another minor metabolite was detected in rat bile after deconjugation with mixed β-glucuronidase/arylsulfatase (Figure 2). 8-Hydroxyolivacine might be considered as a possible candidate for this nonidentified metabolite. In the case of the ellipticine series, the synthesis of 8-hydroxyellipticine has been recently achieved[15] and this compound was previously identified as a minor metabolite of ellipticine in microorganism *Aspergillus alliaceus*.[16]

Both 9- and 7-hydroxyolivacine metabolites are excreted as conjugates (93 ± 3% for I and 96 ± 2% for II, see Figure 3) as previously observed for hydroxyellipticine derivatives.[3,17,18] The free metabolites are minor excreted forms

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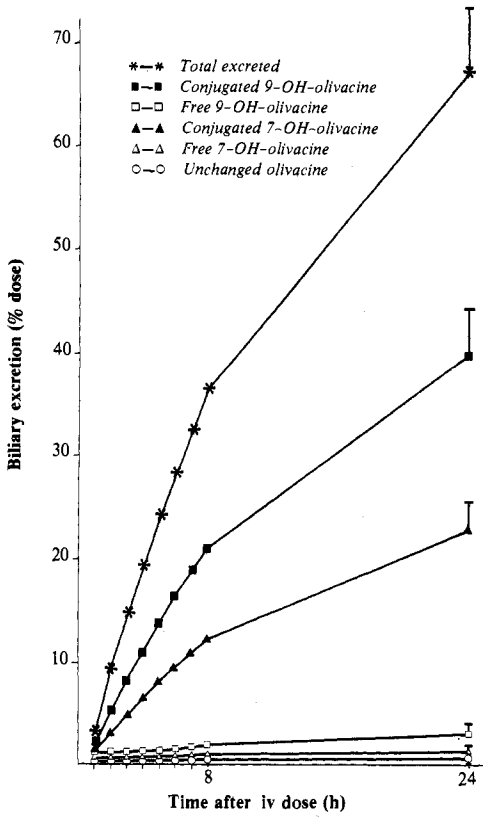


Figure 3. Cumulative biliary excretion of olivacine and its metabolites by nonpretreated rats. In each bile collection period, the unchanged drug olivacine and free 9- and 7-hydroxyolivacine were quantified by HPLC before mixed β-glucuronidase/arylsulfatase treatment and the conjugated 9- and 7-hydroxyolivacine were quantified as equivalent quantities of free 9- and 7-hydroxyolivacine appearing after enzyme hydrolysis. Data are the mean values ± SD (for cumulative 0–24-h bile collection period) from three rats.

Table II. Cytotoxic and Antitumor Activity of Olivacine and Its Hydroxylated Derivatives against L1210 Leukemia Cells

compd	ID_{50},[a] μM	LD_o,[b] mg/kg	ILS,[c] %
olivacine (2)	2.03	250	35
9-hydroxyolivacine (3)	0.06	50	39
7-hydroxyolivacine (4)	12.80	*d*	*d*

[a] Dose that reduces by 50% the L1210 cell growth as compared to controls, 48 h after drug exposure. [b] Highest nonlethal dose (ip treatment, mice). [c] Increase in life span over controls. A single ip dose (= 0.8 LD_o) was injected to DBA/2 mice, 24 h after ip L1210 graffting (10^5 cells). Significant antitumor activity for ILS > 25%. [d] Not tested due to the ID_{50} high value and insufficient supply of 4.

(7 ± 3% for I and 4 ± 2% for II). Only traces of unchanged drug are detected. In all cases, the total yield of excreted compounds reached 62–67% of the administered drug in a 24-h period of bile collection (Figure 4). But in contrast to in vitro metabolism, the sum of the two hydroxylations is not increased in BP-pretreated rats (Figure 4). The hydroxylation ratio is reversed in the case of BP-pretreated animals, since 7-hydroxyolivacine is the major metabolite (Figure 4). This fact might be explained by a further oxidation of 9-hydroxyolivacine and the co-

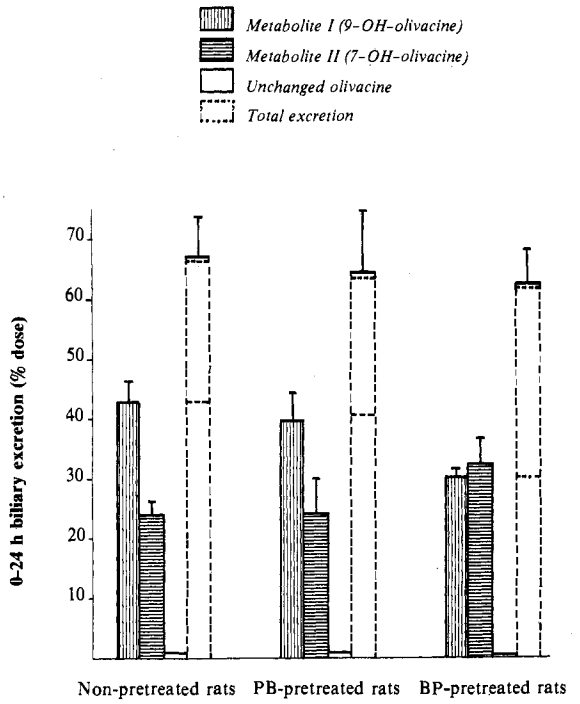


Figure 4. Effects of enzyme inducers on hydroxylation of olivacine in vivo. In each bile collection period, the unchanged drug olivacine and 9- and 7-hydroxyolivacine were quantified by HPLC after mixed β-glucuronidase/arylsulfatase treatment. Data are the mean values λ SD from three rats.

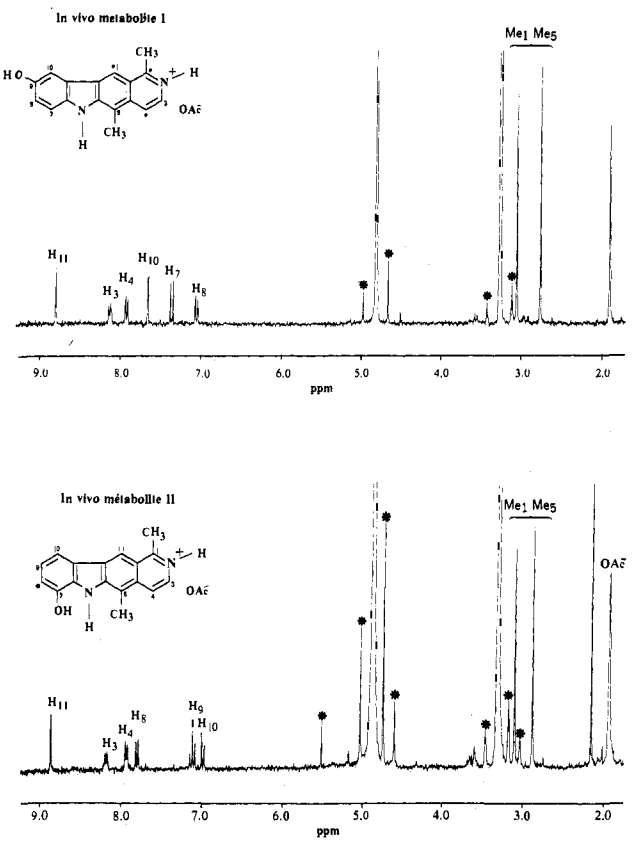


Figure 5. Proton NMR spectra of metabolites I and II (from rat bile), identified as 9-hydroxyolivacine and 7-hydroxyolivacine.

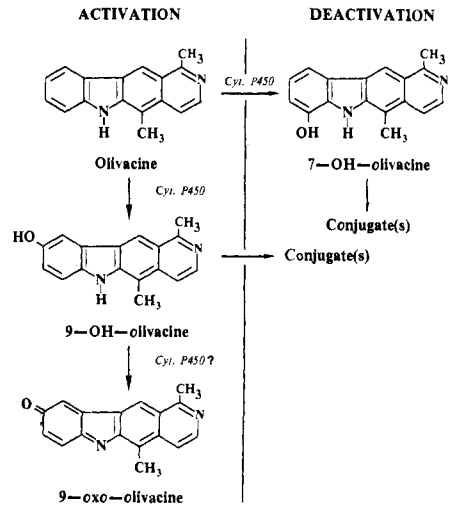
valent binding of the resulting quinone imine intermediate to macromolecules thus reducing its excretion from the animal.

Biological Activities of Olivacine and Its Hydroxylated Derivatives. The cytotoxicity and antitumor activities of olivacine and its hydroxylated derivatives have

(17) (a) Chadwick, M.; Silveira, D. M.; Platz, B. B.; Hayes, D. *Drug Metab. Dispos.* **1978**, *6*, 528. (b) Branfman, A. R.; Bruni, R. J.; Reinhold, V. N.; Silveira, D. M.; Chadwick, M.; Yesair, D. W. *Drug Metab. Dispos.* **1978**, *6*, 542. (c) Lallemand, J. Y.; Lemaitre, P.; Beeley, L.; Lesca, P.; Mansuy, D. *Tetrahedron Lett.* **1978**, 1261.

(18) Paoletti, C.; Cros, S.; Dat-Xuong, N.; Lecointe, P.; Moisand, A. *Chem-Biol. Interact.* **1979**, *25*, 45.

Scheme IV. Proposed Metabolic Pathways of Olivacine



been investigated with L1210 leukemia cells and the data are reported in Table II. In the ellipticine series,[5,18] it is known that hydroxylation at position 9 increases the cytotoxicity, whereas hydroxylation at position 7 largely decreases it (ID_{50} = 0.015 μM for 9-hydroxyellipticine, 5.44 μM for 7-hydroxyellipticine, and 0.99 μM for ellipticine itself). As for ellipticine, hydroxylation of olivacine at position 9 increases the cytotoxicity (ID_{50} = 0.06 μM compared to 2.03 μM for olivacine) and the opposite effect is observed for hydroxylation at position 7 (ID_{50} = 12.8 μM). The increase of life span (% ILS) observed for 9-hydroxyolivacine (39%) is nearly the same as that for olivacine itself (35%), as previously observed for 9-hydroxyellipticine and ellipticine (53% and 68%, respectively).[5,18] The great increase of cytotoxicity for 9-hydroxyolivacine (34 times that of olivacine itself) and for 9-hydroxyellipticine (66 times that of ellipticine itself), as measured in vitro, does not lead to an increase of the in vivo antitumor activity. This might be related to the nearly completed conjugation of the hydroxylated olivacine and ellipticine derivatives in vivo (more than 93% of both hydroxy metabolites are excreted as conjugates), leading to a fast elimination of the drugs.

The difference observed in the LD_0 values for olivacine (250 mg/kg) and ellipticine (50 mg/kg) compared to those of 9-hydroxyolivacine (50 mg/kg) and 9-hydroxyellipticine (50 mg/kg) might be explained by the high rate of hydroxylation of olivacine (compared to that of ellipticine[11]) and the formation of the larger amounts of 7-hydroxyolivacine as metabolite, which is inactive (see Scheme IV for the proposed metabolic pathways of olivacine).

Conclusion

The present data show that olivacine, similar to ellipticine, is metabolized by the same set of hepatic enzymes that produce two main 9- and 7-hydroxy metabolites. However, olivacine appears to be more readily oxidized than ellipticine, as demonstrated by its high V_{max} values compared to those for ellipticine. It is important to note that this enzymatic activation of the administered drug has a considerable consequence on the biological activity of the molecule. As a matter of fact, hydroxylation at position 9 increase the cytotoxicity of the molecule and hydroxylation at position 7 reduces its cytotoxic activity in vitro. This significant difference in biological activity of these two hydroxylation isomers of olivacine might be related to the probable mechanism of its two electron oxidations (see Scheme IV). We know from our studies that peroxidase oxidation of 9-hydroxyellipticines in vitro leads to strong electrophilic quinone imine derivatives[19] which have recently been indirectly shown to be generated in vivo[20], whereas 7-hydroxyellipticines lead to poor electrophilic quinone derivatives.[21] We are currently working in that direction for these hydroxyolivacine compounds.

Experimental Section

Olivacine was prepared according to a previously published method.[7,9] Sodium phenobarbital (PB) and benzo[*a*]pyrene (BP) were obtained from Merck. NADPH and β-glucuronidase and arylsulfatase (both from *Helix pomatia*) were obtained from Sigma Chemical Co. All other chemicals were purchased from Prolabo. Male Sprague–Dawley rats weighing 250–350 g from Evic-Ceba were used for in vitro and in vivo studies.

HPLC analyses were performed on a Waters M440 liquid chromatograph. Waters μ-Bondapack C_{18} columns, 0.39 × 30 cm and 0.78 × 30 cm, were respectively used for analytical and semipreparative separations with a mixture of methanol–0.01 M ammonium acetate (70:30, v/v) acidified to pH 5.5 with acetic acid as eluent, at a flow rate of 1.2 mL/min. Products were detected by absorption at 313 nm.

Infrared spectra (IR) were recorded in $CHCl_3$ solution on a Perkin-Elmer 257 spectrophotometer. Infrared absorption bands are expressed in reciprocal centimeters with use of polystyrene calibration. Ultraviolet–visible (UV–visible) spectra were run on a Bausch and Lomb spectromic 505 or Beckman Acta III spectrophotometers. Proton nuclear magnetic resonance (^1H NMR) spectra were recorded on a Perkin-Elmer R12 (60 MHz) or a Brucker WH90 (90 MHz) spectrometer. Chemical shift data are reported in parts per million (ppm, tetramethylsilane as an internal standard, δ = 0), where s, d, dd, t, q, and m designate singlet, doublet, doublet of doublet, triplet, quartet, and multiplet, respectively. Electron-impact mass spectroscopy (MS) was performed on a AEI MS50 or a Riber R-1010 instrument.

Chemistry. 1,2,3,6-Tetrahydro-1-methyl-4-(2-methyl-1,3-dioxolan-2-yl)pyridine (9). 4-Acetylpyridine ethylene ketal (6.0 g) was quaternarized with methyl iodide in boiling acetonitrile (150 mL) and the salt (mp 135 °C, from hexane) was reduced with $NaBH_4$ according to usual procedures. The tetrahydropyridine **9** was distilled: bp 114 °C (17 mmHg); overall yield, 81%; $C_{10}H_{17}O_2N$; MS, *m/z* 183 ($M^{+\cdot}$); NMR ($CDCl_3$) δ 1.43 (s, CH_3), 2.34 (s, NCH_3), 3.76 (m, CH_2CH_2), 5.82 (m, 1 H).

2-[2-(Methylamino)ethyl]-6-methoxy-*N*-methyl-9*H*-carbazole (10a). The tetrahydropyridine **9** (1.83 g, 10 mmol) and indole **8a** (1.47 g, 10 mmol) in 50% aqueous acetic acid (50 mL) were refluxed for 56 h under an argon atmosphere. After extraction the amorphous carbazole (2.40 g; yield, 90%) was crystallized as its acetate (mp 250 °C, water): base; $C_{17}H_{20}N_2O$; MS, *m/z* 268 ($M^{+\cdot}$), 225; NMR ($CDCl_3$) δ 2.45 (s, NCH_3), 2.47 (s, CH_3), 3.91 (s, OCH_3).

2-[2-(Methylamino)ethyl]-8-methoxy-*N*-methyl-9*H*-carbazole (10b). The derivative **10b** was prepared according to the procedure used for **10a**: yield, 82% after separation from impurities by chromatography (silica, CH_2Cl_2–CH_3OH); oil; MS, *m/z* 268 ($M^{+\cdot}$), 226, 225, 224, 210, 209; NMR ($CDCl_3$) δ 2.40 (s, NCH_3), 2.47 (s, CH_3), 3.90 (s, OCH_3).

2-[2-(Acetylmethylamino)ethyl]-6-methoxy-*N*-methyl-9*H*-carbazole (11a). The carbazole **10a** (2.14 g, 8 mmol) was stirred for 1 h in acetic anhydride–pyridine (1:1) (20 mL). After extraction, compound **11a** was isolated in 95% yield: mp 171 °C (methanol); MS, *m/z* 310 ($M^{+\cdot}$), 237, 224. Anal. ($C_{19}H_{22}N_2O_2$) C, H, N.

2-[2-(Acetylmethylamino)ethyl]-8-methoxy-*N*-methyl-9*H*-carbazole (11b). The acetyl derivative **11b** was prepared in a similar manner as for **11a**. After purification by chromatography

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(silica, CH_2Cl_2), pure amorphous **11b** was obtained: yield, 65%; MS, m/z 310 ($M^{+\cdot}$), 237, 224.

2,3,4,6-Tetrahydro-9-methoxy-1,2,5-trimethyl-1*H*-pyrido[4,3-*b*]carbazole (13a, 9-Methoxyguatambuine). The amide **11a** (1.86 g, 6 mmol) and $POCl_3$ (4 mL) were refluxed for 10 h in $CHCl_3$. The yellow precipitate was then filtered off and washed with $CHCl_3$. The intermediate iminium salt **12a** was dissolved in a mixture of $CHCl_3$ (30 mL) and CH_3OH (50 mL) and reduced with an excess of$NaBH_4$ at room temperature. After isolation by extraction and crystallization from ethanol, **13a** was isolated in 76% overall yield: mp 95 °C; MS, m/z 294 ($M^{+\cdot}$), 279. Anal. ($C_{19}H_{22}N_2O$) C, H, N.

2,3,4,6-Tetrahydro-7-methoxy-1,2,5-trimethyl-1*H*-pyrido[4,3-*b*]carbazole (13b, 7-Methoxyguatambuine). The compound **13b** was prepared by the same method as **13a**. After purification by chromatography (silica, CH_2Cl_2–CH_3OH, 95:5), **13b** was isolated as an oil: yield, 39%; MS, m/z 294 ($M^{+\cdot}$), 279, 264; NMR ($CDCl_3$) δ 1.65 (d, J = 12 Hz, CH_3), 2.40 (s, NCH_3), 2.60 (s, CH_3), 4.05 (s, OCH_3), 6.8–7.8 (aromatic H), 8.20 (NH). Anal. ($C_{19}H_{22}N_2O$) C, H, N.

9-Methoxy-1,5-dimethyl-6*H*-pyrido[4,3-*b*]carbazole (14a, 9-Methoxyolivacine). A solution of **13a** (0.882 g, 3 mmol) in Decalin (10 mL) was refluxed with 10% palladium–charcoal (0.250 g) for 24 h. The reaction mixture was diluted with hexane (150 mL) and extracted with CH_3OH (5 × 20 mL). The methanol extract was washed with hexane and diluted with aqueous ammonia. Half of the methanol was distilled and the concentrate was then extracted with $CHCl_3$. The $CHCl_3$ extracts were washed, dried, and evaporated, leaving a crude product purified by elution form a silica gel column (yield, 40%). Physical data were identical with those of the natural product.

7-Methoxy-1,5-dimethyl-6*H*-pyrido[4,3-*b*]carbazole (14b, 7-Methoxyolivacine). The compound **13b** (0.690 g, 2.35 mmol) was dehydrogenated and demethylated according to the procedure used for **13a**. The desired product (0.143 g) was obtained after separation from a less polar impurity by thin-layer chromatography on silica gel (CH_2Cl_2–CH_3OH, 95:5): mp 257 °C (methanol); MS, m/z 276 ($M^{+\cdot}$), 261; NMR ($CDCl_3$–CD_3OD, 50:50) 2.70 (s, CH_3), 2.95 (s, CH_3), 4.01 (s, OCH_3), 6.85–8.55 (aromatic H); UV–visible (ethanol) λ_{max} (ϵ) 246 (35 530), 285 (59 220), 330 (8980), 400 (8580).

9-Hydroxy-1,5-dimethyl-6*H*-pyrido[4,3-*b*]carbazole (3, 9-Hydroxyolivacine). A solution of **14a** (0.276 g, 1 mmol) in HBr (48%) was refluxed for 1 h under argon atmosphere. The reaction mixture was concentrated under vacuum and poured into a solution of dilute ammonia. The solution was then evaporated to dryness and the residue was extracted with a mixture of CH_2Cl_2–CH_3OH (50:50). The desired product **3** was obtained after filtration of the solution through a short column of silica gel: yield, 75%; mp >260 °C (methanol); MS, m/z 262 ($M^{+\cdot}$), 132; NMR (CD_3OD) δ 2.78 (s, 3p, Me_1 or Me_5), 3.08 (s, 3p, Me_5 or Me_1), 7.06 (dd, J = 8.8 and 2.2 Hz, 1p, H_8), 7.36 (d, J = 8.8 Hz, 1p, H_7), 7.65 (d, J = 2.2.Hz, 1p, H_{10}), 7.92 (d, J = 6.6 Hz, 1p, H_4), 8.11 (d, J = 6.6 Hz, 1p, H_3), 8.75 (s, 1p, H_{11}); UV–visible (ethanol) λ_{max} (ϵ) 226 (13 790), 246 (19 300), 275 (27 580), 298 (35 163), 333 (6670), 376 (2100); UV–visible (methanol) λ_{max} (ϵ) 229 (17 280), 238, (21 360), 271 (36 960), 293 (49 200), 326 (6640), 398 (2960). Anal. ($C_{17}H_{14}N_2O$·CH_3OH) C, H, N.

7-Hydroxy-1,5-dimethyl-6*H*-pyrido[4,3-*b*]carbazole (4, 7-Hydroxyolivacine). The compound **4** was obtained with the same procedure used for **3**: yield, 70%; mp >260 °C (methanol); MS, m/z 262 ($M^{+\cdot}$), 132; NMR (CD_3OD) δ 2.87 (s, 3p, Me_1 or Me_5), 3.09 (5, 3p, Me_5 or Me_1), 6.96 (d, J = 7.3 Hz, 1p, H_{10}), 7.10 (m, 1p, H_9), 7.79 (d, J = 8.1 Hz, 1p, H_8), 7.93 (d, J = 5.1 Hz, 1p, H_4), 8.16 (d, J = 5.1 Hz, 1p, H_3), 8.85 (s, 1p, H_{11}); UV–visible (ethanol) λ_{max} (ϵ) 248 (24 500), 287 (33 220), 331 (4360), 400 (3320); UV–visible (methanol) λ_{max} (ϵ) 228 (8800), 240 (17 440), 282 (34 560), 328 (2440), 384 (1920). Anal. ($C_{17}H_{14}N_2O$·CH_3OH) C, H, N.

Metabolism. Pretreatment of Animals. Nonpretreated rats received sunflower oil or 0.9% NaCl intraperitoneally (ip), PB-pretreated rats received 80 mg/kg per day by ip route in 0.9% NaCl for 3 consecutive days, and BP-pretreated rats received 20 mg/kg per day by ip route in sunflower oil for 2 consecutive days. The animals were either killed (for microsomal preparation) or treated by olivacine (for biliary excretion) 2 days after the last dose.

Hepatic Microsomal Preparation. Rats were anesthetized with ethyl ether inhalation, and livers were perfused with isotonic KCl (1.15%), removed quickly, rinsed three times in ice-cold Tris-HCl buffer 0.02 M (pH 7.4) containing isotonic KCl (1.15%), weighed, and homogenized in Tris-HCl–KCl buffer (3 mL/g of liver) by using a motorized glass–Teflon Eveljehm homogenizer. The homogenates were centrifuged at 10000*g* for 20 min (Beckman J-21C centrifuge). The resulting supernatants were centrifuged at 110000*g* (Beckman L5-50 ultracentrifuge) for 60 min and the microsomal pellets were washed in Tris-HCl–KCl buffer and centrifuged again at 110000*g* for 60 min. The final microsomal pellets were then suspended in Tris-HCl–KCl buffer (1 mL/g of liver) and homogenized by using a hand glass–teflon homogenizer. The protein concentrations were determinated by the method of Lowry et al.[22]

Microsomal Incubation. For enzymatic kinetic constant (K_m and V_{max}) determinations, a standard incubation mixture was used, containing in Tris-HCl–KCl buffer (1-mL final volume) non-, PB-, or BP-induced microsomes (0.5 mg protein/mL) in 100 μL of buffer and olivacine acetate (5–50 nmol/mL) in 10 μL of dimethylformamide; the reaction was started by the addition of cofactor NADPH (1 mg/mL) in 100 μL of buffer. The mixture was shaken in a water bath at 37 °C under air for 5 min. Control incubations in which omission of any one of the components or incubation with boiled microsomes resulted in an absence of reaction. The reaction was stopped by rapid cooling in an ice bath, addition of 2 mL of ice-cold saturated 1-butanol (containing 10% of water), and vortexing for 1 min. After centrifugation, 0.5 mL of the organic phase was lyophilized and the resulting dry residue was dissolved in eluent for analytical HPLC analysis. The unmetabolized drug **2** and metabolic products I and II were quantified from linear calibration curves obtained with microsomal suspensions in buffer after addition of known amounts of synthetic **2**–**4**. Time and protein and substrate concentration linearities were investigated for 20 min and up to 2 mg/mL and 200 μM. For isolation of in vitro olivacine metabolites, duplicate incubation mixtures were used, containing (i) BP-induced microsomes (200 mg protein), (ii) olivacine acetate (6.72 mg, 0.022 mmol), and (iii) NADPH (150 mg) in Tris-HCl–KCl buffer (100-mL final volume). The mixture was shaken at 37 °C under air for 30 min. The extraction of metabolic products was performed by vigorous mixing with 200 mL of 1-butanol. Then, the 1-butanol extracts were subjected to semipreparative HPLC.

Biliary Excretion. Rats were anesthetized with ip administered 1 g/kg of ethyl carbamate in normal saline. Bile ducts were cannulated with use of Becton-Dickinson polyethylene tubing, and control bile was collected before drug administration. Then, olivacine acetate was administered in DMF/sodium acetate buffer, 0.1 M, pH 5.0 (10:90, v/v) at a dose of 25 mg/kg intravenously, and bile was collected at various times over a period of 24 h. Aliquots (0.2 mL) of bile samples were extracted with saturated 1-butanol and analyzed by analytical HPLC for quantification of free metabolites. The remaining bile samples were treated with β-glucuronidase and arylsulfatase (1000 and 100 units/mL of bile, respectively), in 1:1 vol of sodium acetate buffer, (0.1 M, pH 5.0) and incubated in a shaking water bath at 37 °C for 12 h. After enzymatic hydrolysis, the mixtures were extracted with saturated 1-butanol and analyzed by analytical HPLC for quantification of conjugated metabolites. The unchanged olivacine (**2**) and metabolites I and II were quantified from linear calibration curves obtained with control bile samples after addition of synthetic **2**–**4**.

For isolation of in vivo olivacine metabolites, two nonpretreated rats were used. The bile collected over a 8-period was treated with mixed β-glucuronidase/arylsulfatase, and the 1-butanol extracts were subjected to semipreparative HPLC.

Isolation and Characterization of Metabolites I and II. Microsomal (from BP-pretreated rats) or biliary (from nonpretreated rats) extracts were injected on semipreparative HPLC column, and the collected fractions corresponding to each metabolite I or II were combined and evaporated to dryness. Further purification was accomplished on a Pharmacia Sephadex LH-20

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gel chromatography column (2 × 25 cm) using ammonium acetate buffer (0.01 M, pH 5.5)/methanol (30:70, v/v, for I and 40:60 for II) as eluents. The purity of metabolites was checked by analytical HPLC.

Characterization of I and II (from in vitro and in vivo materials) was made by comparison with UV-visible, EI mass, proton NMR spectra of authentics **3** and **4**. Physicochemical data of I: MS, m/z 262 ($M^{+\cdot}$); NMR (CD_3OD, I was recorded as acetate salt; see Figure 5) δ 2.80 (s, 3p, Me_1 or Me_5), 3.09 (s, 3p, Me_5 or Me_1), 7.05 (dd, J = 8.8 and 2.2. Hz, 1p, H_9), 7.37 (d, J = 8.8 Hz, 1p, H_7), 7.66 (d, J = 2.2 Hz, 1p, H_{10}), 7.92 (d, J = 6.6 Hz, 1p, H_4), 8.12 (d, J = 6.6. Hz, 1p, H_3), 8.75 (s, 1p, H_{11}); UV-visible (methanol) λ_{max} 230, 239, 272, 293, 329, 399. Physicochemical data of II: MS, m/z 262 ($M^{+\cdot}$); NMR (CD_3OD, II was recorded as acetate salt; see Figure 5) δ 2.87 (s, 3p, Me_1 or Me_5), 3.08 (s, 3p, Me_5 or Me_1), 6.96 (d, J = 7.3 Hz, 1p, H_{10}), 7.09 (m, 1p, H_9), 7.79 (d, J = 8.1 Hz, 1p, H_8), 7.91 (d, J = 5.1 Hz, 1p, H_4), 8.16 (d, J = 5.1 Hz, 1p, H_3), 8.84 (s, 1p, H_{11}); UV-visible (methanol) λ_{max} 229, 241, 283, 328, 384.

Cytotoxic and Antitumor Activities. The inhibitory efficiency of cell growth was determined with L1210 leukemia cells in vitro as previously described[18] and expressed in terms of ID_{50} (inhibition dose 50), the drug concentration that reduces the number of cells by 50% as compared to the control, after 48 h to drug exposure. The antitumoral efficiency is expressed in terms of ILS (increase of life span), defined as [(median survival time in treated animals) − (median survival time in controls)/median survival time in controls] × 100. DBA/2 mice were treated with drug 24 h after intraperitoneal inoculation of 10^5 L1210 cells.

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Registry No. **3**, 70173-18-1; **4**, 83201-13-2; **8a**, 1006-94-6; **8b**, 3189-22-8; **9**, 60553-32-4; **10a**, 79853-92-2; **10b**, 95589-85-8; **11a**, 93841-60-2; **11b**, 95589-86-9; **12a**, 94200-72-3; **13a**, 95589-87-0; **13b**, 95589-88-1; **14a**, 16101-08-9; **14b**, 95589-89-2; 4-acetylpyridine ethylene, 60553-33-5; 4-acetylpyridine ethylene ketal methyl iodide, 60553-34-6; olivacine, 484-49-1.

New Antiarrhythmic Agents. *N*-Aryl-8-pyrrolizidinealkanamides[1]

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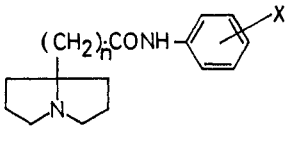
The synthesis and antiarrhythmic activity of *N*-aryl-8-pyrrolizidinealkanamides are described. The target compounds were evaluated for their ability to protect against chloroform-induced fibrillation in mice. Many of them were found to have antifibrillatory activity comparable to that of lidocaine; several are more potent than lidocaine. *N*-(2,6-Dimethylphenyl)-8-pyrrolizidineacetamide (**6n**), which was found to be more potent and less toxic (LD_{50}) than lidocaine, also showed a long duration of action in dogs with ventricular arrhythmias after oral administration.

Much research[2] has been done on the relationships between local anesthetics and their chemically related antiarrhythmic agents, and structural modifications of local anesthetics including amide groups, i.e., lidocaine and procainamide, as well as a large number of compounds[3] are attracting considerable attention.[4] The study of quantitative structure–activity relationships (QSAR)[4b] of the antiarrhythmic activity of aminoxylides has suggested that the pK_a value has an important effect on the biological properties of the molecule.[5] Consequently we have chosen pyrrolizidine as the prospective moiety[6] to be included in the molecule. Our recent synthetic accomplishments[7] that made available a number of bicyclic amines with a bridgehead nitrogen[8] led us to attempt an introduction of these amines as a moiety of lidocaine-type antiarrhythmic agents.

We report here the results of pharmacological screening tests in mice on the antiarrhythmic activity of a new "amide-type" pyrrolizidine series, which is represented by the general formula **1**.



1

n = 1 or 2

Chemistry. The synthetic routes to the target compounds are outlined in Scheme I. Compounds **4** and **5**,

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[‡] Suntory Institute for Biomedical Research.

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