Bile Acids with a Cyclopropyl-Containing Side Chain. 1. Preparation and Properties of 3α,7β-Dihydroxy-22,23-methylene-5β-cholan-24-oic Acid

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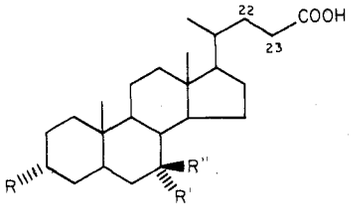
3α,7β-Dihydroxy-22,23-methylene-5β-cholan-24-oic acid (**5**), a side-chain cyclopropyl analogue of ursodeoxycholic acid (**1b**), has been prepared by the cyclopropanation of 3α,7β-diacetoxy-24-nor-5β-chol-22-ene (**2**) with ethyl diazoacetate, followed by saponification of the resulting cyclopropyl ester (**3**). The new bile acid presents similar properties to **1b** in water. The sodium salt self-aggregates to form micelles at a concentration of 15.5 mM. The cyclopropane ring does not modify the pK_a with respect to compound **1b**. Cyclopropyl acid **5** is taken up efficiently by rat liver and promptly secreted into bile. It is partially (70%) conjugated with taurine. The bile flow and bile acids and phospholipids secretion are stimulated by **5**, while the cholesterol secretion is stimulated by **5** to a lesser extent.

In the last 10 years, a renewed interest, for physiological as well as for physicochemical properties, in bile acids (BA) has largely been motivated by the introduction in the therapeutic practice of several countries of natural representatives of this class of compounds, such as chenodeoxycholic acid (**1a**) and ursodeoxycholic acid (**1b**), for



1a, R = OH; R' = OH; R'' = H
b, R = OH; R' = H; R'' = OH
c, R = OAc; R' = H; R'' = OAc

the treatment of cholesterol gallstones.[1,2]

Although it has been reported that a successful therapy is strictly related to the size and chemical composition of the stones,[3,4] the mechanism by which stone dissolution is induced by bile acids **1a** and **1b** is still poorly understood. Most probably a selective inhibition of cholesterol biosynthesis and secretion occasioned by **1a** and **1b** leads to a bile undersaturated in cholesterol.[5-8] As a result, being enriched in these bile acids the bile is able to solubilize more cholesterol via mixed-micelles formation.[9,10] Recently, more controlled and comparative clinical studies have advocated the clinical use of ursodeoxycholic acid (UDCA, **1b**) over that of chenodeoxycholic acid (CDCA, **1a**) in the treatment of cholesterol gallstones, in view of a minor incidence of side effects, such as diarrhea, and more efficiency at lower doses.[11,12] Several factors, however, such as the relatively long time (6-12 months) and the high doses [7-15 mg/(kg day)] required for the treatment are still limiting the therapeutical use of this natural compound.

A review of the publications in the bile acid field reveals that very few attempts to design analogues with the aim to gain insight into the structure–activity relationships of bile acid induced bile flow and lipid secretion and to increase the medical value of this class of compounds have been reported.[13,14] In recent years, a growing interest in the chemistry and unusual electronic characteristics of the cyclopropane structure has led to the development of several cyclopropyl analogues of biologically active compounds.[15-17] As a part of a broad program directed toward the preparation of structurally modified analogues of ursodeoxycholic acid (**1b**), in the present paper we report the synthesis and preliminary physicochemical and biological evaluation of 3α,7β-dihydroxy-22,23-methylene-5β-cholan-24-oic acid (CUDCA, **5**), in which the C_{22}–C_{23} side-chain bond forms one side of the three-membered ring.

Chemistry. 3α,7β-Dihydroxy-22,23-methylene-5β-cholan-24-oic acid (**5**) was prepared as follows. Ursodeoxycholic acid 3α,7β-diacetate (**1c**) was converted into the corresponding Δ^{22}-olefinic derivative (**2**) by oxidative decarboxylation with lead tetraacetate. Carbethoxycarbenoid, generated by the dirhodium(II) tetraacetate catalyzed decomposition of ethyl diazoacetate, smoothly added to olefin **2** to give the corresponding cyclopropyl ester **3** in 92% yield, besides minor amounts of diethyl fumarate and maleate dimers (**4**) arising from self-condensation of excess ethoxycarbonylcarbenoid.

GLC analysis of cyclopropyl ester **3** showed that it was a mixture of four stereoisomers, as could have been expected by the formation of two new chiral centers at C-22 and C-23. Hydrolysis of cyclopropyl ester **3** yielded the corresponding 3α,7β-dihydroxy-22,23-methylene-5β-

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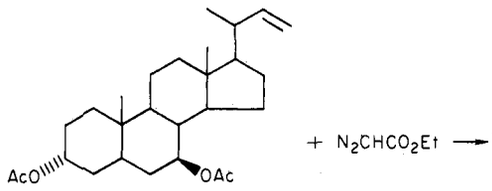

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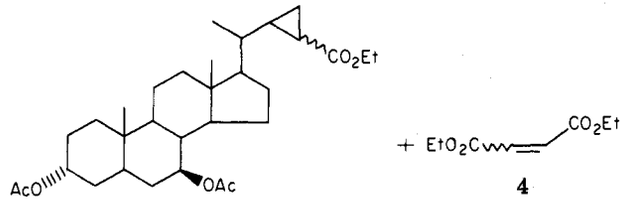

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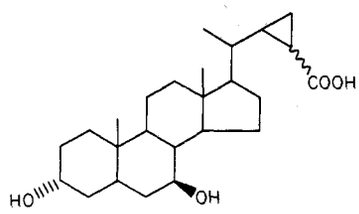
cholan-24-oic acid (**5**) as a stereoisomeric mixture, which



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was subjected as such to physicochemical and preliminary biological evaluation. The separation and determination of the absolute configuration of the four cyclopropyl acids is in progress.

Physicochemical Properties in Water. Critical Micellar Concentration. The physiological bile salts are detergent-like molecules and in water self-aggregate to form micelles.[11,14] The critical micellar concentration (CMC) is an important parameter to be considered, since it is related to the capacity to form micelles in bile, which is composed of cholesterol, lecithins, and BA. The CMC depends on the hydrophilic/hydrophobic balance of the molecule, which in turn is a function both of the ring hydroxyls (number, orientation, and position) and of the nature of the side chain. The CMC of **5** was assessed by a dye solubilization method.[14] The resulting data were compared with physiological BA (UDCA), and the CMC values are given in Table I. The introduction of a cyclopropane ring into the BA side chain reduces the CMC, since the side chain is lengthened, and the side chain is therefore more hydrophobic. A value of 15.5 mmol/L for CUDCA, however, is still a safe one, and possible membrane damage due to its detergency is excluded.

Dissociation Constants. The bile salts' pK_a is an important parameter from which it is possible to calculate the ratio of dissociated (highly water soluble) to dissociated (poorly soluble) forms at different pH values of the medium. The pK_a values for CUDCA and other bile acids have been determined in two different mixed solvents,[18] and it was possible to obtain reliable values when the data were extrapolated to pure water, using linear correlations. The pK_a values are reported in Table I. The presence of a cyclopropane ring close to the carboxylic group does not modify the pK_a value when compared with that of UDCA. The values are 5.01–5.08 for both CUDCA and UDCA.

Solubility. The water solubility of both the acid **5** and the corresponding sodium salt is strictly related to its choleretic effect (water phase secretion).

Table I. Physicochemical Properties of 3α,7β-Dihydroxy-22,23-methylene-5β-cholan-24-oic Acid (CUDCA) and 3α,7β-Dihydroxy-5β-cholan-24-oic Acid (UDCA)

	CUDCA	UDCA
CMC, mM (bile salt)	15.5 ± 0.5	19 ± 0.5
pK_a	5.01 ± 0.03	5.08 ± 0.06
water solubility (bile acid) (25 °C), μM	11.8 ± 1.5	9.0 ± 1.0

[a] Each value represents the mean plus or minus the standard deviation of five determinations. [b] CMC, critical micellar concentration.

The solubility of the sodium salt is extremely high, while that of the undissociated acid is relatively low and in the order of 1×10^{-5} mol/L (Table I). No significant differences have been observed between CUDCA and UDCA.

Biological Properties. In order to assess the biological activity of a BA as a drug useful for cholesterol gallstones' dissolution, several parameters have to be taken into account. Here we want also to point out that the concept of bioavailability completely differs from that of other drugs.[19,20]

The bile acids are mainly localized in the enterohepatic circulation (intestine, gallbladder, and liver) and in the bile exert their physiological functions. The first level screening used to characterize the most relevant biological properties is reported here.

Hepatic Uptake. The hepatic extraction has been evaluated in single pass liver perfusion experiments in rats.[21] The percentage of the bile salt bound to albumin was also evaluated by equilibrium dialysis.[22] Data are reported in Table II. Despite differences in structure, the uptake of CUDCA is similar to that UDCA (50%). Also the interaction with human serum albumin (HSA) is similar to that of physiological bile salts.[22] The affinity constant is 6.8×10^4 L/mol; consequently, under physiological conditions (3% HSA), the percentage of CUDCA bound to HSA is around 70%, and the liver uptake of CUDCA is efficient and comparable with that exhibited by physiological BA.

Bile Lipid Secretion. The evaluation of the effect of this BA on bile lipid secretion gives information on the transport of this BA and indirect evidence of the ability to inhibit the cholesterol secretion and synthesis.[6]

CUDCA was administered in ten rats intravenously at a dose of 50 mg per animal, the bile was collected at 15-min intervals for 5 h, and the bile volume and phospholipid, cholesterol, and BA concentration were measured. The bile acid composition was also analyzed.

CUDCA is promptly secreted into the bile; after 15 min, 80% of the administered dose is recovered. It is secreted into bile 70% conjugated with taurine and 30% as a free acid. In contrast, ursodeoxycholic acid is secreted completely (98%) conjugated with taurine, as commonly observed for all physiological bile acids. CUDCA is the first example of a C-25 BA that can be secreted unmodified, although in moderate amount. These results can be explained by the steric hindrance in the reaction of the carboxyl group exerted by the cyclopropane ring. The bile flow significantly increases with respect to control rats and

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Table II. Biological Properties of 3α,7β-Dihydroxy-22,23-methylene-5β-cholan-24-oic Acid (CUDCA) and 3α,7β-Dihydroxy-5β-cholan-24-oic Acid[a]

	CUDCA	UDCA	controls
hepatic uptake, %	46 ± 1	50 ± 2	
albumin affinity constant, × 10^4 L/mol	6.8 ± 0.4[b]	3.8 ± 0.2	
bile flow, mL/h	1.68 ± 0.20[c]	1.08 ± 0.15[c]	0.55 ± 0.12
BA secretion, μmol kg^{-1} h^{-1}	97 ± 9	98 ± 7	61 ± 8
cholesterol secretion, μmol kg^{-1} h^{-1}	2.70 ± 0.14[c]	2.12 ± 0.09[c]	1.66 ± 0.12
phospholipid secretion, μmol kg^{-1} h^{-1}	9.50 ± 0.43[c]	6.47 ± 0.54	6.92 ± 0.44

[a] The control values were obtained on five rats studied under similar conditions to the BA treated rats. Each value represents the mean plus or minus the SD of five determinations. [b] $p < 0.01$ vs. UDCA. [c] $p < 0.01$ vs. the controls.

also with respect to ursodeoxycholic acid (Table II).

The bile lipid secretion increases, but more selectively than that of phospholipids and BA (Table II). During the maximum effect, we observed an increase both in bile acid and water secretion.

As a result, the cholesterol saturation index fell during the study also at the highest choleretic effect.

These results indicate a potential theoretical value of CUDCA as a cholesterol-dissolving drug, thanks to its capacity to inhibit the cholesterol secretion. In addition, the main characteristics of CUDCA is to stimulate both the bile flow and the bile lipid secretion.

The structure of the rat livers was also evaluated by light microscopy at the end of the study, and the intravenous injection of 50 mg of CUDCA did not alter the basic morphology of the liver.

Experimental Section

Melting points were determined with a Kofler apparatus and are uncorrected. ^{1}H NMR spectra were taken on a Varian EM 390 spectrometer. IR spectra were determined with a Perkin-Elmer 1320 spectrometer. Analytical gas–liquid chromatography (GLC) was performed on a Hewlett-Packard 5830 A chromatograph equipped with a flame-ionization detector and a 6 ft × 2 mm i.d. 1% SP2250 on 100/100 chromosorb W column. The oven temperature was 265–275 °C (5 °C/min increase) with nitrogen as the carrier gas at a flow rate of 20 mL/min. Column chromatography was performed with Merck silica gel 60 (0.063–0.200 mm and 0.040–0.063 mm). Elemental analyses were performed with an automatic analyzer Model 1102, Carlo Erba, Italy. All chromatography solvents were distilled prior to use. Ethyl diazoacetate was purchased from Fluka.

3α,7β-Diacetoxy-24-nor-5β-chol-22-ene (2). A mixture of 3α,7β-diacetoxy-5β-cholan-24-oic acid (**1c**; 20 g, 42 mmol) [prepared by acetylation of ursodeoxycholic acid (**1b**) with Ac_2O in the presence of pyridine], lead tetraacetate (40 g, 90 mmol), cupric acetate (1.76 g, 9.67 mmol), benzene (1.7 L), and pyridine (5 mL, 62 mmol) was refluxed under nitrogen and stirring for 8 h. The reaction mixture was then filtered, and the filtrate was evaporated under vacuum. Chromatography of the residue thus obtained on a silica gel column and elution with 6:1 petroleum ether–ether yielded 5.2 g (29%) of olefin **2**: mp 112–114 °C; IR (film) ν 3060, 1730, 1600 cm^{-1}; NMR ($CDCl_3$) δ 0.70 (3 H, s, C-18 Me), 0.98 (3 H, s, C-19 Me), 1.95 (3 H, s, C-7 $OCOCH_3$), 2.00 (3 H, s, C-3 $OCOCH_3$), 4.37–5.00 (4 H, br m, C-3 and C-7 C*H*OAc, C-23 CH_2), 5.35–5.82 (1 H, m, C-22 H). Anal. ($C_{27}H_{42}O_4$) C, H, O.

Ethyl 3α,7β-Diacetoxy-22,23-methylene-5β-cholan-24-oate (3). A solution of ethyl diazoacetate (7.9 g, 69.5 mmol) in anhydrous methylene dichloride (120 mL) was added dropwise evenly during an 18-h period to a stirred suspension of dirhodium(II) tetraacetate (150 mg, 0.34 mmol) and olefin **2** (10 g, 23.3 mmol) in anhydrous methylene dichloride (120 mL) under nitrogen. After the addition was complete, the solvent was removed under vacuum. Flash chromatography of the viscous residue (16 g) on a silica gel column and elution with 1.5:1 petroleum ether–ether yielded 0.52 g of ethyl maleate and ethyl fumarate (**4**) and 0.79 g of unreacted starting olefin **2**. Continuation of the elution with 1.5:1 petroleum ether–ether yielded semisolid cyclopropyl derivative **3** (11 g, 91.6%, calculated from olefin **2**): IR (Nujol) 3070, 1740 cm^{-1}; NMR ($CDCl_3$) δ 0.63 (3 H, s, C-18 Me), 0.97 (3 H, s, C-19 Me), 1.23 (3 H, t, J = 7 Hz, Me of Et), 1.96 (3 H, s, C-7 $OCOCH_3$), 1.98 (3 H, s, C-3 $OCOCH_3$), 4.03 (2 H, q, J = 7 Hz, OCH_2), 4.62 (2 H, br m, C-3 and C-7 C*H*OAc). GC analysis showed the presence of four substances with retention times of 19.38, 21.69, 24.49, and 24.60 min. Anal. ($C_{31}H_{48}O_6$) C, H, O.

3α,7β-Dihydroxy-22,23-methylene-5β-cholan-24-oic Acid (5). A solution of 9.4 N NaOH (60 mL) and the cyclopropyl derivative **3** in ethanol (90 mL) was refluxed for 4 h under stirring. It then was poured into ice–water (300 mL), acidified with 2 N hydrochloric acid, and extracted with ethyl acetate (3 × 100 mL). The organic phase was washed with water, dried ($MgSO_4$), and concentrated under reduced pressure. Chromatography of the residue (4.3 g) on a silica gel column and elution with 4:1 chloroform–methanol yielded the cyclopropyl acid **5** (4 g, 96%): mp 148–158 °C; IR (Nujol) 3380, 1690 cm^{-1}; ^{1}H NMR (CD_3OD) δ 0.63 (3 H, s, C-18 Me), 0.97 (3 H, s, C-19 Me), 3.28–3.67 (2 H, br m, C*H*OH). Anal. ($C_{29}H_{44}O_6$) C, H, O.

Critical Micellar Concentration. Sodium salts of CUDCA and UDCA were prepared by neutralization of an alcoholic solution with an equivalent amount of sodium hydroxide. The sodium salts were obtained by crystallization after the addition of diethyl ether.

The CMC's were measured in water by the dye solubilization method[14] and, in particular, with Azulene (Aldrich Chemical Co., Milwaukee WI) and Orange OT (gift from Dr. K. Mysels, Chemistry Department, University of California at San Diego, La Jolla, CA) as water-insoluble dyes. Different bile salt solutions were prepared by weighing in a concentration ranging from 0.1 to 100 mmol, and the pH was adjusted to 8.00 with sodium hydroxide. A sample of the final solution was analyzed for bile acid content by an enzymatic method with 3α-hydroxysteroid dehydrogenase.[23] The sodium content was measured by atomic absorption spectroscopy. Bile salt solutions, 2 mL, were rotated for 1 day at room temperature with an excess of crystalline dyes (kept in the dark). Then the solutions were filtered through a 0.22-μm millipore filter. The absorbance of solutions was recorded, respectively, at 580 and 490 nm for Azulene and Orange OT. The absorbance was plotted against bile salt concentration.

Water Solubility. CUDCA and UDCA (0.2 mmol) were suspended in 100 mL of water. The pH after the addition of water was adjusted to 3.00 with $HClO_4$ (1 M). The solutions were refluxed for 24 h in order to achieve the equilibrium. Two-milliliter aliquots of the saturated solutions were transferred to a thermostat maintained at 25 °C. After 1 month the solutions were filtered on a Millipore (0.22 μm), and 2 mL of methanol was added to prevent crystallization. The concentration of BA was measured enzymatically by using a 3α-hydroxy steroid dehydrogenase enzyme.[23]

pK_a Value Determination. The pK_a* of **5** and UDCA were determined by potentiometric titration (Radiometer PH M26) with a glass electrode and a saturated calomel electrode as reference. The measurements were carried out in dimethyl sulfoxide–water (80:20, w/w) and in methanol–water (50:50, w/w). The pH meter was calibrated with a diluted solution of $HClO_4$ in the solvent mixture Me_2SO–H_2O according to Cox et al.[24] A procedure previously described[18] was followed for the determinations in methanol–water with an oxalate buffer for standardization. The pK_a values estimated in water by means of previously

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assessed correlations from the pK_a* values in mixed solvents are in close agreement with each other and with the value of UDCA. All the measurements have been carried out at 25 ± 0.01 °C.

Hepatic Uptake. Isolated liver perfusions were performed as described by Mortimore.[25] The perfusate consisted of Krebs–Ringer bicarbonate, pH 7.4, containing glucose (100 mg/mL) and bovine serum albumin (3% w/v) (fraction V essentially fatty acid free, Sigma Chemical Co., St. Louis MO). Evans blue (1 mg/g liver) was added as marker of the flow rate and recovery. The outflow was collected at 10-s intervals immediately after injection of a 10-μmol/L solution of CUDCA (200 μL). In all experiments, CUDCA (**5**) and UDCA were randomly injected. The concentrations of BA in the outflow sample were measured enzymatically.[23] The Evans blue was directly evaluated in each sample in a Perkin-Elmer spectrophotometer at 610 nm. Albumin concentration in the perfusate was measured by the method of Lowry et al.[26] The percentage of outflow was corrected for the Evans blue, and the liver uptake was calculated as 100% − outflow.

Bile Lipid Secretion. Sprague–Dawley male rats (300–330 g) were used. The rats were anesthetized with ethyl carbamate, and the bile was collected. The bile acids, CUDCA and UDCA, were administered intravenously as sodium salts through the femoral vein at a dose of 50 mg per animal (five rats each group). Bile samples were collected at 15-min intervals for a 1-h period. The concentrations of bile acids, cholesterol, and phospholipids in bile samples were determined by enzymatic procedures.[23,27,28] The bile acid compositions were also determined by an HPLC method[29] and by TLC on silica gel G plates, 250-μm thickness. The conjugated bile acids (glycine/taurine) were separated from the corresponding unconjugated BA by the solvent system propionic acid/isoamyl acetate/H_2O/1-propanol (75:100:25:5, v/v). Biliary lipid secretion, calculated from the volume of the excreted bile and from the biliary lipid concentration, was expressed as micromoles per kilogram per hour.

The results of the physicochemical and biological evaluations of **5a** clearly indicate that the cyclopropane ring at C-22 and C-23 exerts an effect that could be explained by the unusual electronic and steric characteristics of the three-membered ring. The new bile acid presents promising properties as a candidate drug both for cholesterol gallstones dissolution and for improving the bile flow and the biliary lipid secretion. The major new features are as follows: (1) Optimum critical micellar concentration: this means that the "coupling" with cholesterol and phospholipid is favorable to the formation of a micellar solution. As a consequence, more cholesterol will be solubilized by this BA. (2) CUDCA is able to inhibit selectively the cholesterol secretion; consequently, the bile secreted during feeding of this BA is less saturated in cholesterol. In addition, CUDCA largely increases the bile flow, and during this effect the bile is still enriched in BA and phospholipids and less in cholesterol.

More detailed biochemical studies devoted to explaining the metabolism in terms of deconjugation and dehydroxylation are in progress.

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Registry No. **1b**, 128-13-2; **1c**, 6533-77-3; **2**, 89414-89-1; **3** (22*R*,23*R*), 89414-90-4; **3** (22*R*,23*S*), 89495-32-9; **3** (22*S*,23*R*), 89495-33-0; **3** (22*S*,23*S*), 89495-34-1; **5** (22*R*,23*R*), 89414-91-5; **5** (22*R*,23*S*), 89495-35-2; **5** (22*S*,23*S*), 89495-36-3; **5** (22*S*,23*R*), 89496-31-1; N_2CHCO_2Et, 623-73-4.

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Formation of a Reactive Iminium Derivative by Enzymatic and Chemical Oxidations of 16-*O*-Acetylvindoline

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16-*O*-Acetylvindoline (**1a**) was oxidatively transformed into an iminium derivative (**2a**) by copper oxidases (laccase and human ceruloplasmin), an unknown enzyme system(s) of *Streptomyces griseus*, and the chemical oxidizing agent 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ). The iminium derivative (**2a**) was isolated from enzymatic and chemical oxidation mixtures and was identified by spectral and chemical techniques. Reduction of the iminium compound with sodium borodeuteride provided monodeuterated 16-*O*-acetylvindoline (**1b**) as the sole product. Mass spectral analysis indicated that the deuterium atom was introduced into position C-3 of the piperidine portion of the alkaloid structure. The location and stereochemistry of the deuterium atom were confirmed by high-field ^1H and ^2H NMR analyses of the deuterated product to be in the $^2H_\alpha$ orientation. Hydrolysis of the 16-*O*-acetyl functional group from the iminium derivative (**2a**) resulted in the production of a previously identified dimer (**5**), which forms by intramolecular etherification through the reactive enamine (**3**). The iminium derivative (**2a**) reacts with cyanide to provide complex mixtures of products, one of which was identified by mass spectrometry as a cyanide addition product. The results confirm the existence of a reactive iminium intermediate formed by all of the biochemical and chemical systems examined.

Vindoline (**1**) is one of the major alkaloids of the plant *Catharanthus roseus*. The structure of this toxic compound is found intact in so-called "dimeric" alkaloids, such as vincristine and vinblastine, which have long been used in the treatment of human neoplastic diseases. These complex alkaloids function through their specific ability to achieve metaphase arrest, but little is known of the molecular events associated with specific chemical interactions of these compounds with macromolecules. Indirect evidence has been used to demonstrate that other nitrogen heterocyclic compounds, such as nicotine, benzylpyrrolidine, and phencyclidine,[1-3] yield iminium derivatives

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