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Articles

Products of Metabolic Activation of the Antitumor Drug Ledakrin (Nitracrine) in Vitro

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The aim of this work was to characterize the products of metabolic activation of the antitumor drug ledakrin (Nitracrine) in model metabolic systems, where formation of drug−DNA adducts was previously discovered. The metabolic products obtained in different biological systems were compared with those obtained in experiments where chemical reducing agents were applied. Therefore, activation products were obtained in the presence of the microsomal fraction of rat liver and in the experiments with the reducing agents dithiothreitol, hydrazine hydrate, and $SnCl_2$. Furthermore, transformations of the drug with oxidoreductase enzymes DT-diaphorase and xanthine oxidase were observed. The ledakrin transformation products were separated and analyzed by HPLC with diode array detection. Structural studies of the products were performed by means of ESI-MS and NMR. Proton, carbon, and nitrogen assignments were made based upon DQF-COSY, ROESY, TOCSY, HSQC, and HMBC experiments. It was demonstrated during the reduction of ledakrin that a key metabolite, a compound with an additional five-membered ring attached to positions 1 and 9 of the acridine core and with the retained 9-aminoalkyl side chain, was formed in all the systems that were studied. It was determined that the reactive nitrogen atoms of this additional ring underwent further transformations resulting in the formation of a six-membered ring produced by the addition of a carbon atom to the dihydropyrazoloacridine ring. Furthermore, it was observed that positions 2 and 4 of ledakrin's acridine ring are susceptible to nucleophilic substitution as revealed by the studies with dithiothreitol. Additionally, although most products from the reduction of ledakrin were extremely unstable, 1-aminoacridinone, produced enzymatically and with dithiothreitol, exhibited persistent stability under the studied conditions.

Introduction

Ledakrin, **1** (WHO,[1] recommended Nitracrine), 1-nitro-9-[3′-(dimethylamino)propylamino]acridine (Figure 1), is an antitumor drug that has been used clinically for several years (*1*−*4*). It belongs to a group of 1-nitro-9-

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[1] Abbreviations: DQF-COSY, double-quantum-filtered correlation spectroscopy; DTT, dithiothreitol; ESI-MS, electrospray ionization mass spectrometry; HMBC, heteronuclear multibond correlation; HSQC, heteronuclear single-quantum correlation; ROESY, rotating frame enhanced spectroscopy; TOCSY, total correlation spectroscopy; RP-HPLC, reversed-phase HPLC; WHO, World Health Organization.

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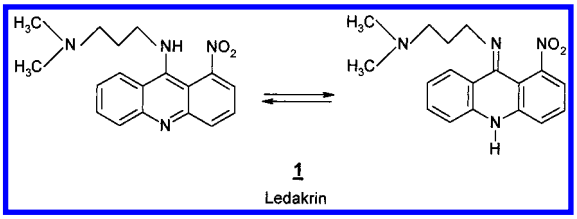

Figure 1. Tautomeric structures of ledakrin (Nitracrine), 1-nitro-9-[3′-(dimethylamino)propylamino]acridine.

aminoacridine derivatives developed in our laboratory, which exhibit exceptionally high cytotoxic and antitumor properties (*5*−*8*). This unusual biological activity relates only to 1-nitroacridine derivatives, whereas the remaining nitro isomers exhibit significantly weaker action (*5*).

The studies on the "mode of action" showed that ledakrin is the latent form of the drug and requires metabolic activation before it exhibits any cytotoxic and antitumor activity. Activation results in covalent binding of the drug to DNA and other cellular macromolecules (*10*−*12*). The formation of the adduct between DNA and the activated form of ledakrin was shown by ^{32}P-postlabeling analyses in cells as well as in other activation systems (*13*). Specifically, ledakrin was able to induce covalent interstrand cross-links in the DNA of mammalian and bacterial cells (*14*) as well as DNA−protein cross-links in leukemia cells (*15*). It was suggested that these cross-links play a crucial role in the mode of antitumor action of this drug (*15*, *16*).

Previously, it was shown that the inhibitory effect of ledakrin on DNA transcription with RNA polymerase significantly increased in the presence of reducing agents such as thiols (*17*). Likewise, the covalent binding of ledakrin to DNA was observed to take place under the reducing environment of microsomes (*10*, *11*). Furthermore, DNA adduct formation was demonstrated not only in experiments performed in the cell but also after reducing activation occurring with microsomal enzymes and after reduction with dithiothreitol (DTT) (*13*). Wilson et al. suggested that nitro reduction is the major route of ledakrin metabolism in hypoxic cells, as the drug is selectively toxic to the AA8 cell line growing under hypoxic conditions (*18*, *19*).

All of the results listed above clearly indicated that the reduction of ledakrin is the necessary activation step that leads to the covalent binding of the drug to DNA and/or proteins in cellular systems.

Several attempts at synthesis, isolation, and identification all the reduced metabolites of ledakrin have been undertaken. It was expected that the *N*-hydroxy-1-amino analogue of ledakrin would be formed, based on previous observations from metabolic reduction of the majority of nitroaromatic compounds (*20*, *21*). Accordingly, Wilson et al. presented the HPLC peak of one main metabolite obtained after anaerobic incubation of AA8 cells with ledakrin, and its structure was suspected to also be the *N*-hydroxyamino analogue of the drug (*19*). Reduction of ledakrin with glutathione resulted in several acridine-containing products; however, none were characterized due to their high level of instability (*22*).

Common reducing agents such ethanethiol in pyridine (*23*), mercaptoethanol (*24*), and hydrogen sulfide (*25*) were used to simulate metabolic reduction of ledakrin. It turned out to be strongly reactive under the applied reducing conditions. The majority of the products obtained in these reactions were 1-amino derivatives missing the aminoalkyl side chain in position 9 of the acridine core. Reduction of the drug with sodium borohydride (*26*) and hydrazine hydrate (*27*) was also investigated; however, no clear evidence of the existence of the *N*-hydroxy-1-amino analogue of ledakrin was provided.

The results described above, although insightful, have yet to substantiate the mode of action of ledakrin due to insufficient structural evidence of the drug's metabolites. This prompted our group to attempt to isolate and structurally identify the products from reductive metabolic activation of ledakrin obtained in such systems where the formation of drug−DNA adducts has been observed (*13*). The following questions were posed: (i) what are the chemical structures of the metabolites; (ii) which elements of those structures could be responsible for the high reactivity of ledakrin under reducing conditions; and (iii) which elements of the metabolites' structure might create the covalent bonds between ledakrin and DNA?

In this work, we present the results from activation of ledakrin in the presence of the microsomal fraction of rat liver and with a chemical reducing agent dithiothreitol. These systems were considered ideal since they have been shown to activate ledakrin, resulting in the formation of DNA−drug adducts (*13*). We also investigated the activation of ledakrin with two oxidoreductase enzymes: DT-diaphorase [NAD(P)H:quinone oxidoreductase] and xanthine oxidase. Furthermore, comparative studies were performed on products obtained in the reaction of ledakrin with chemical reducing agents. The reactions were followed and their products isolated with liquid chromatography. Structural studies of the reaction products were accomplished by employing ESI-MS and the multidimensional NMR techniques of DQF-COSY, ROESY, TOCSY, HSQC, and HMBC.

Experimental Procedures

Chemicals and Enzymes. Ledakrin (Nitracrine), 1-nitro-9-[3′-(dimethylamino)propylamino]acridine (C-283), was synthesized as a dihydrochloride in the Department of Pharmaceutical Technology and Biochemistry, Technical University of Gdańsk. The following enzymes and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO): DT-diaphorase (EC 1.6.99.2), xanthine oxidase (EC 1.1.3.22), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), dithiothreitol (DTT), tris(hydroxymethyl)aminomethane (Trizma base), 3-methylcholanthrene, nicotinamide, glucose 6-phosphate, NADPH, and xanthine. Polyethylene glycol 6000 was obtained from Serva Feinbiochemica , and DMSO, hydrazine hydrate, $SnCl_2$, THF, $FeSO_4$, Pd/C, and HPLC grade methanol were obtained from Fluka (Buchs, Switzerland). BHT (2,6-di-*tert*-buthyl-*p*-krezol) and ammonium formate (AnalaR) were obtained from BDH Ltd. (Poole, England). All chemicals were used without further purifications.

Instrumentation. Reversed-phase HPLC with UV/vis detection was performed with a Waters Associates HPLC system equipped with a model 600E solvent delivery system and a model 991 UV/vis photodiode array detector. HPLC analyses were carried out with the following systems: (A) an isocratic elution at 40% methanol in ammonium formate (50 mM, pH 3.5) for 15 min followed by a linear gradient from 40 to 100% methanol in ammonium formate for 10 min, followed by a linear gradient from 100 to 40% methanol in ammonium formate, and (B) an isocratic elution at 40% methanol in ammonium formate (50 mM, pH 3.5) for 15 min followed by a linear gradient from 40 to 100% methanol in ammonium formate for 15 min, followed by a linear gradient from 100 to 40% methanol in ammonium

formate at a flow rate of 1 mL/min using a 5 μm Suplex pKb 100 analytical column (0.46 cm × 25 cm) (Supelco, Bellefonte, PA).

The products for NMR analysis were isolated by means of low-pressure liquid chromatography using a reversed stationary-phase C_{18} from Merck (Darmstadt, Switzerland).

LC/MS analysis of the products was accomplished by electrospray ionization with positive ion detection performed on a Finnigan MAT TSQ 700 tandem mass spectrometer equipped with a Finnigan electrospray ionization source interfaced with the HPLC system and a Dionex GP40 gradient pump. N_2 was used as the sheath gas (386 kPa/56 psi). The HPLC apparatus was a Waters Associates System model 600-MS with a 484-MS tuneable absorbance detector operated at 254 nm.

^{1}H NMR measurements with COSY and ROESY techniques, ^{13}C NMR measurements with HSQC and HMBC techniques, and ^{15}N NMR measurements were carried out at 500.13 MHz in deuterated dimethyl sulfoxide (DMSO-d_6) using a Varian 500 spectrometer. Chemical shifts are reported in parts per million for ^{1}H relative to the internal standard $(CH_3)_4Si$.

Activation of Ledakrin with the Microsomal Fraction of Rat Liver. Six-week-old male rats (Wistar) were injected with 3-methylcholanthrene (4 mg/animal) for 3 days before decapitation. Livers were removed, homogenized in a Potter-Elvehjem homogenizer, and centrifuged for 30 min at 10000*g*. The supernatant was mixed 1:1 with 20 mM sodium pyrophosphate. Polyethylene glycol (50%) was added to obtain a concentration of 5% (w/v) and mixed for 10 min. The mixture was then centrifuged for 10 min at 10000*g*. The resulting pellet was then placed in a homogenizer with a solution of 0.1 M Tris acetate, 1 mM EDTA, and 20% glycerine (pH 7.4) and homogenized. All of the operations were carried out between 0 and 4 °C. Protein concentrations were determined by the Lowry method.

The activation of ledakrin with the microsomal fraction of rat liver was carried out in 0.16 M Trizma base buffer (pH 7.4). The incubation mixture consisted of 125 mM nicotinamide, 100 mM glucose 6-phosphate, 10 mM NADPH, glucose-6-phosphate dehydrogenase (40 units), rat liver microsomes (5 mg/mL), and ledakrin (1 mmol). The incubation was carried out at 37 °C in air. RgThe reaction was initiated by addition of the compound from the initial incubation after 5 min. After appropriate periods of time, the incubation mixture was centrifuged for 5 min at 12000*g*. The supernatant was analyzed directly by means of HPLC/UV/Vis, HPLC/MS, and MS/MS. The pellet was extracted with chloroform, dried, then dissolved in water and methanol (1:1), and analyzed by HPLC and MS.

Activation of Ledakrin with DT-Diaphorase. Activation of ledakrin (1 mM) with DT-diaphorase (500 μg/mL) in the presence of 5 mM NADPH was carried out in 0.16 M Trizma base buffer (pH 7.4) at 37 °C in air. The reaction was initiated by the addition of ledakrin. The products were separated by means of HPLC (elution system A) and analyzed by mass spectrometry.

Activation of Ledakrin with Xanthine Oxidase. Activation of ledakrin (1 mM) by xanthine oxidase (5.3 mg/mL) in the presence of xanthine (0.3 mg/mL) was carried out in 0.16 M Trizma base buffer (pH 7.4) at 37 °C in air. The products were separated by HPLC and analyzed by mass spectrometry.

Reduction of Ledakrin by Dithiothreitol. The reaction of ledakrin (1 mM) with DTT (20 mM) was carried out in 0.16 M Trizma base buffer (pH 7.4) at room temperature for 45 min. The products of the reaction were separated by HPLC (elution system B). Mass spectra were recorded directly from the incubation mixture during the reaction progress, after 5, 15, 30, and 60 min. Products **2**−**4** were isolated from the reaction mixture after separation by means of low-pressure liquid chromatography. Products **3** and **4** were eluted from the column with an isocratic elution at 40% methanol in ammonium formate (50 mM, pH 3.5), and product **2** was eluted at 50% methanol in ammonium formate (50 mM, pH 3.5). Ammonium formate was removed from the isolated fractions by separation on the same column with water and methanol as the mobile phase. The samples were lyophilized and redissolved in DMSO for NMR analysis.

Reduction of Ledakrin by Hydrazine Hydrate. Ledakrin (1 mM) was dissolved in 25 mL of THF, and 50 mg of Pd/C (10% v/w) was added. The reaction mixture was placed in the ice bath and bubbled with argon for 5 min. Two hundred microliters of the hydrazine hydrate solution was added in small portions of 50 μL every 2 min. The reaction was monitored spectrophotometrically, and the products were separated and analyzed by HPLC and off-line mass spectrometry. One of the products (**6**) was isolated by means of low-pressure liquid chromatography and analyzed by NMR.

Reduction of Ledakrin by $SnCl_2$. $SnCl_2$ (1.75 g) and $FeSO_4$ (0.35 g) were dissolved in 20 mL of concentrated HCl. The temperature was raised to 70 °C, and 0.5 g of ledakrin (dissolved in concentrated HCl) was added after 15 min. The high reaction temperature was maintained for 30 min and then slowly reduced to −10 °C. The pellet was dissolved in water and alkalized with NaOH. The pellet was extracted with ether, and the extract was dried over P_2O_5. Recrystallization was performed on the solution of methanol and ether, and the product was subjected to NMR analysis. Product **7** was identified with a combination of UV/vis spectra, NMR spectra (including COSY, ROESY, HSQC, HMBC, and ^{15}N), and electrospray ionization MS/MS.

Spectral Characterization of the Reaction Products. (1) 1-Amino-9-acridinone (2): ^{1}H NMR (in DMSO) δ 6.25 (d, 1H, 2-H), 6.45 (d, 1H, 4-H), 7.18 (m, 1H, 7-H), 7.28 (m, 1H, 3-H), 7.4 (d, 1H, 5-H), 7.6 (m, 1H, 6-H), 8.17 (d, 1H, 8-H), 11.27 (s, 1H, 10-NH); UV (in MeOH) λ_{max} 245, 260, 320, 420 nm; MS $[M + H]^+$ for $C_{13}H_{10}N_2O$ *m*/*z* 211 (found).

(2) N^2-[3′-(Dimethylamino)propyl]-4-(1″,4″-disulfanyl-2″,3″-dihydroxybutane)-1,9-diazane-1,10-dihydroacridine (3): ^{1}H NMR (in DMSO) δ 2.04 (m, 2H, 2′-H), 2.24 (d, 6H, 4′-CH_3), 2.42 (m, 2H, 3′-H), 2.54 (m, 2H, 4″-H), 2.64 (dd, J = 5.3 Hz, 1H, 1″-H), 2.83 (dd, J = 5.3 Hz, 1H, 1″-H), 3.61 (m, 1H, 2″-H), 3.63 (m, 1H, 3″-H), 4.6 (m, J = 6.8 Hz, 2H, 1′-H), 6.5 (d, J = 8.7 Hz, 1H, 2-H), 7.04 (m, 1H, 3-H), 7.06 (m, J = 7.3 Hz, 1H, 7-H), 7.32 (m, J = 5.8 Hz, 1H, 6-H), 7.54 (d, J = 8.3 Hz, 1H, 5-H), 7.8 (d, J = 7.3 Hz, 1H, 8-H), 9.85 (s, 1H, 10-NH); ^{13}C NMR δ 26.0 (4″-C), 26.8 (2′-C), 39.0 (1″-C), 44.8 (4′-C), 48.7 (1′-C), 54.0 (3′-C), 70.0 (3″-C), 72.5 (2″-C), 93.0 (4-C), 103.0 (2-C), 114.0 (12-C), 117.0 (5-C), 118.5 (13-C), 121.0 (7-C), 122.5 (8-C), 129.0 (6-C), 131 (9-C), 137.5 (3-C), 140.0 (1-C), 141.0 (11-C); ^{15}N NMR δ −354.7 (4′-N), −272.5 (10-N), −175 (9-N), −95.5 (1-N); UV [in a 60:40 ammonium formate (pH 3.5)/MeOH mixture] λ_{max} 230, 260, 295, 305, 405 nm; MS $[M + H]^+$ for $C_{22}H_{28}N_4O_2S_2$ *m*/*z* 444 (found), fragment ions at *m*/*z* 400 and 372.

(3) N^2-[3′-(Dimethylamino)propyl]-2-(1″,4″-disulfanyl-2″,3″-dihydroxybutane)-1,9-diazane-1,10-dihydroacridine (4): ^{1}H NMR (in DMSO) δ 2.04 (m, 2H, 2a′-H), 2.24 (d, 6H, 4a′-CH_3), 2.42 (m, 2H, 3a′-H), 2.5 (m, 2H, 4a″-H), 2.89 (dd, 1H, 1a″-H), 3.0 (dd, aH, 1a″-H), 3.60 (m, 1H, 2a″-H), 3.60 (m, 1H, 3a″-H), 4.60 (m, 2H, 1a′-H), 5.79 (d, J = 7.3 Hz, 1H, 4a-H), 7.01 (m, 1H, 7a-H), 7.07 (m, 1H, 3a-H), 7.11 (d, J = 7.8 Hz, 1H, 5a-H), 7.30 (m, J = 5.8 Hz, 1H, 6a-H), 7.78 (d, J = 7.3 Hz, 1H, 8a-H), 10.3 (s, 1H, 10a-NH); ^{13}C NMR δ 36.0 (1a″-C), 69.0 (4a″-C), 70.0 (3a″-C), 72.5 (2a″-C), 93.0 (4a-C), 104.5 (2a-C), 113.5 (12a-C), 116.0 (5a-C), 118.5 (13a-C), 120.5 (7a-C), 122.5 (8a-C), 129.0 (6a-C), 131.0 (9a-C), 135.0 (3a-C), 141.0 (11a-C); ^{15}N NMR δ −354.7 (4a′-N), −272.5 (10a-N), −97.9 (1a-N); UV [in a 60:40 ammonium formate (pH 3.5)/MeOH mixture] λ_{max} 230, 260, 295, 305, 405 nm; MS $[M + H]^+$ for $C_{22}H_{28}N_4O_2S_2$ *m*/*z* 444 (found), fragment ions at *m*/*z* 400 and 372.

(4) N^2-[3′-(Dimethylamino)propyl]-1,9-diazane-1,10-dihydroacridine (5): UV [in a 60:40 ammonium formate (pH 3.5)/MeOH mixture] λ_{max} 225, 365, 430 nm; MS $[M + H]^+$ for $C_{18}H_{20}N_4$ *m*/*z* 293 (found), fragment ions at *m*/*z* 248 and 220.

(5) N^2-[3′-(Dimethylamino)propyl]-N^1,N^2-diaminoethylacridine (6): ^{1}H NMR δ 1.36 (m, 3H, b-CH_3), 2.38 (m, 2H, 2′-H), 3.1 (m, 2H, 3′-H), 4.15 (m, 2H, 1′-H), 5.4 (m, 1H, a−H), 6.6 (d, 1H, 2-H), 7.12 (d, 1H, 4-H), 7.5 (t, 1H, 7-H), 7.68 (t, 1H,

3-H), 7.95 (t, 1H, 6-H), 8.02 (d, 1H, 5-H), 8.2 (d, 1H, 8-H), 8.3 (s, 1H, 1-NH), 10.95 (m, 1H, 4′-NH), 14.38 (s, 1H, 10-NH); ^{13}C NMR δ 19.8 (b-C), 53 (1′-C), 66 (a-C), 103.9 (4-C), 104 (2-C), 106 (13-C), 112.5 (12-C), 119 (5-C), 124 (7-C), 127 (8-C), 136 (6-C), 137.5 (3-C), 139 (14-C), 141 (11-C), 143 (1-C), 155 (9-C); MS $[M + H]^+$ for $C_{20}H_{24}N_4$ *m/z* 320 (found).

(6) N^2-[3′-(Dimethylamino)propyl]-N^1,N^2-diamino-1″-methylethylacridine (7): ^{1}H NMR δ 1.68 (s, 6H, b-H), 2.32 (m, 2H, 2′-H), 2.72 (m, 6H, 5′-H), 3.1 (m, 2H, 3′-H), 4.04 (m, 2H, 1′-H), 6.62 (d, 1H, 2-H), 7.1 (d, 1H, 4-H), 7.56 (t, 1H, 7-H), 7.72 (t, 1H, 3-H), 7.95 (d, 1H, 5-H), 7.95 (t, 1H, 6-H), 8.0 (s, 1H, 1-NH), 8.2 (d, 1H, 8-H), 10.9 (m, 1H, 4′-NH), 14.04 (s, 1H, 10-NH); ^{13}C NMR δ 24 (2′-C), 24 (b-C), 41 (5′-C), 48 (1′-C), 52 (3′-C), 104 (2-C), 104 (4-C), 106 (13-C), 111 (12-C), 119 (5-C), 123 (7-C), 127 (8-C), 135 (6-C), 137 (3-C), 138 (1-C), 140 (11-C), 143 (14-C), 155 (9-C); MS $[M + H]^+$ for $C_{21}H_{26}N_4$ *m/z* 334 (found).

Results

Activation of Ledakrin with the Microsomal Fraction of Rat Liver. Studies on formation of DNA−ledakrin adducts with ^{32}P-postlabeling methods showed that identical adducts were formed in tumor cells and after activation of ledakrin with the microsomal fraction of rat liver (*13*). Therefore, we began the studies on the metabolism of ledakrin from the experiments with the microsomal fraction of rat liver.

The products from various periods of incubation of ledakrin with this enzymatic system were separated by HPLC and identified by ESI-MS operating in the positive ion mode. The chromatograms displaying the contents of the incubation mixture after activation for 5 and 30 min are shown in panels A and B of Figure 2, respectively. The peaks with retention times of 8.6 (M1) and 28.6 (M2) min primarily exhibited ions in the mass spectrum at *m/z* 293 and 211, respectively (data not shown). However, the M1 HPLC fraction exposed to air revealed many other molecular species with MS ions at *m/z* 307 (M3), 321 (M4), and 335 (M5) (Figure 3).

Further investigations of the structure of M1 (*m/z* 293) were performed by means of ESI-MS/MS. The mass spectrum in Figure 4 shows a fragment ion at *m/z* 248 formed by the loss of 45 mass units. These data will be applied later to determine the structure of M1.

The spectral data (MS and UV/vis) of product M2 turned out to be identical to those of the standard 1-aminoacridinone. Its structure (**2**) was confirmed by ^{1}H NMR as presented in Figure 5.

Product M3 was formed when M1 was exposed to air for 10 min in solution. M3 displays an $[M + H]^+$ value at *m/z* 307, 14 units greater than the M1 ion at *m/z* 293. Products M4 and M5 gave ions at *m/z* 321 and 335, 28 and 42 units greater than M1, respectively. Therefore, M4 and M5 are possibly the result of addition of two and three methylene groups to M1, respectively. The analysis of the structures of M4 and M5 will be discussed later.

Activation of Ledakrin with DT-Diaphorase and Xanthine Oxidase. The second enzymatic system applied for studies on the activation of ledakrin was DT-diaphorase [NAD(P)H:quinone oxidoreductase], an enzyme commonly used in activation of nitroaromatic compounds (*28*, *29*). The incubation of the drug with DT-diaphorase resulted in two main products. Analysis of the HPLC retention times and UV/vis spectra of chromatographic peaks D1 and D2 (Figure 6) revealed that they were identical to products M1 and M2, respectively, from microsomal activation (see Figure 2B). Likewise,

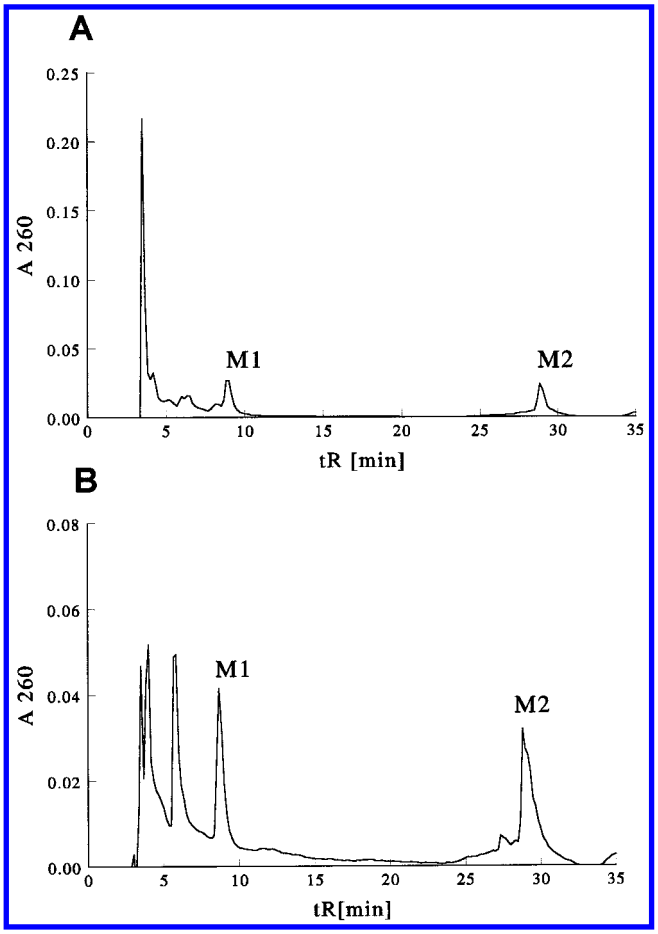

Figure 2. RP-HPLC (see Experimental Procedures for conditions) profile of products obtained by incubation of ledakrin with the microsomal fraction of rat liver after incubation for (A) 5 and (B) 30 min.

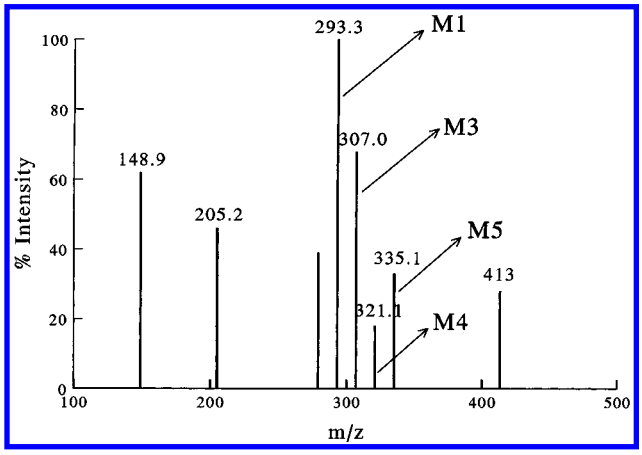

Figure 3. Positive ion ESI-MS spectrum of the products formed during incubation of ledakrin with the microsomal fraction of rat liver. Ions $[M + H]^+$ at *m/z* 293, 307, 321, and 335 correspond to products M1, M3, M4, and M5, respectively.

activation of ledakrin with the third enzymatic system, metalloflavoprotein enzyme, xanthine oxidase (*30*, *31*), gave a product (out of several others), named O1, which exhibits chromatographic and spectral properties identical to those of the microsomally activated product, M1 (data not shown).

The quantities of the products formed during enzymatic activation of ledakrin were not sufficient for structural elucidation by NMR. What is more, standards for the reduced products of ledakrin were not available

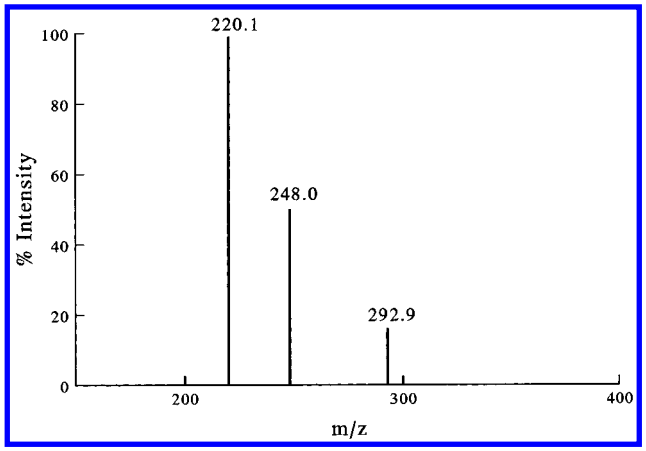

Figure 4. Positive ion ESI-MS/MS spectrum of product M1 (ion at *m*/*z* 293). The mass spectrum shows fragment ions at *m*/*z* 248 and 220 formed by the loss of 45 and 73 mass units, respectively.

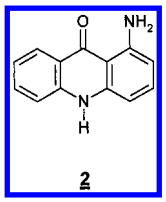

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Figure 5. Structure of 1-amino-9-acridinone, the final product of reduction under the studied conditions.

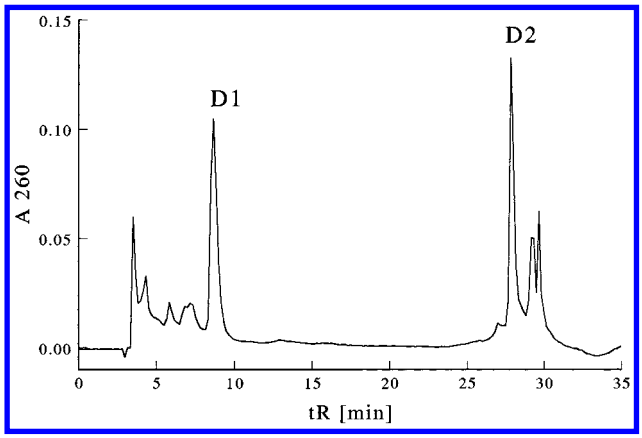


Figure 6. RP-HPLC (see Experimental Procedures for conditions) profile of products obtained by incubation of ledakrin with DT-diaphorase for 30 min. Product D1 (ion at *m*/*z* 293) corresponds to product M1 shown in Figure 2, and product D2 (ion at *m*/*z* 211) corresponds to product M2 shown in Figure 2.

due to their lack of stability under these conditions, and the expected products of ledakrin reduction, *N*-hydroxy-1-amino and 1-amino analogues, have yet to be isolated. Therefore, studies were undertaken on the reductive transformation of ledakrin with the other reducing systems in attempts to produce yields large enough for NMR analysis.

Reaction of Ledakrin with DTT. Like the microsomal fraction of rat liver, it was found by ^{32}P-postlabeling methods that incubation of ledakrin with DTT in the presence of DNA led to the same adduct patterns as that obtained in tumor cells (*13*). Therefore, DTT was chosen as the first nonenzymatic reducing system for the studies on ledakrin metabolic activation.

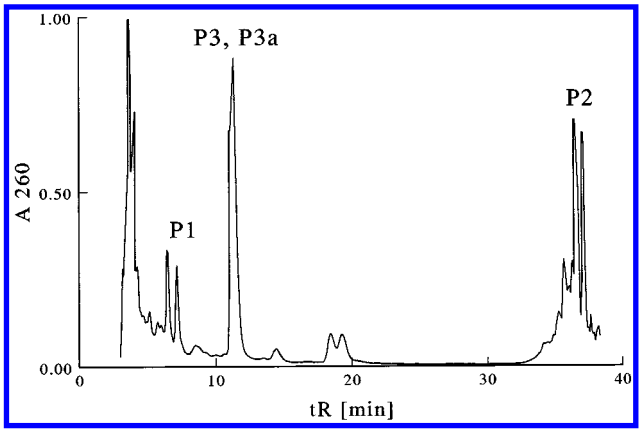


Figure 7. RP-HPLC (see Experimental Procedures for conditions) profile of products obtained by reaction of ledakrin with DTT for 30 min. Products P1 and P2 (ions at *m*/*z* 293 and 211, respectively) correspond to products M1 (D1) and M2 (D2), respectively. Product P3 is an ion at *m*/*z* 445.

Preliminary experiments have successfully established the activation conditions for ledakrin with DTT[2] (*32*). The HPLC analysis (Figure 7) shows that, of the activation products, two compounds, P1 and P2, were identical to products M1 and M2 formed in the microsomal fraction of rat liver and in the presence of reducing−oxidizing enzymes. The MS spectra were obtained from the samples taken after reaction for 5 (Figure 8A), 15 (Figure 8B), 30 (Figure 8C), and 60 min (Figure 8D). The MS spectrum taken after 5 min contains the intensive ion of ledakrin at *m*/*z* 325 and two other ions at *m*/*z* 293 and 445 of low abundance. In the MS spectrum taken after 15 min, besides the two ions at *m*/*z* 293 and 445, an additional ion at *m*/*z* 295 was observed. The third spectrum recorded after reaction for 30 min consists of previously mentioned ions at *m*/*z* 293, 295, and 445 with one added ion at *m*/*z* 447. Last, after reaction for 60 min, an extra ion at *m*/*z* 211 appeared. This product, P2, was isolated, and by NMR, its structure was determined to be identical to M2, which was determined above to be 1-amino-9-acridinone (**2**).

The product termed P1, identical to products M1, D1, and O1 formed in enzymatic systems, could not be isolated due to its instability.

However, product P3 (*m*/*z* 445) could be isolated from the reaction mixture and showed a mass difference of 152 from P1 (*m*/*z* 293). This suggested that P3 was formed as a result of nucleophilic substitution of DTT on P1. Using NMR, the full proton assignment of P3 was made on the basis of DQF-COSY, ROESY, and TOCSY experiments (see Experimental Procedures for detailed results). The ^{13}C assignments were based upon HSQC and HMBC experiments. The results showed that product P3 existed as two isomers, **3** and **4**. Their structures are shown in Figure 9. The DQF-COSY experiment indicated that the nucleophilic substitution at position 4 of the acridine core occurred in compound **3**, whereas substitution in compound **4** was at position 2a. The aliphatic region of the COSY spectrum revealed the presence of both an aminoalkyl side chain and a DTT-derived substituent. Application of ROESY NMR spectra showed that products **3** and **4** possess a hydrogen atom attached to nitrogen at position 10. Furthermore, HSQC and HMBC NMR techniques confirmed the presence of a 9-aminoalkyl side

[2] L. Szmigiero, personal communication.

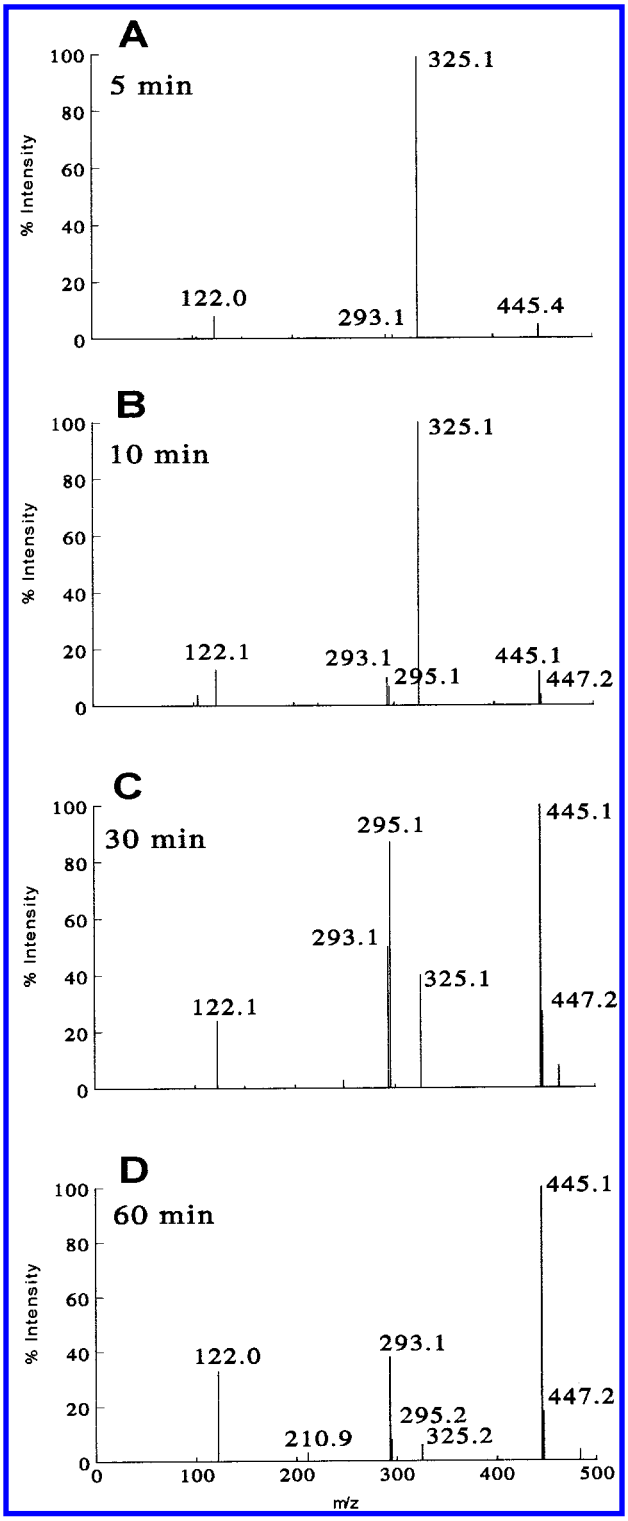


Figure 8. Positive ion ESI-MS spectra of the products of ledakrin reaction with DTT after reaction for (A) 5, (B) 10, (C) 30, and (D) 60 min.

chain and a DTT-derived substituent in positions 4 and 2a for compounds **3** and **4**, respectively. The spectral data extracted from ^{15}N NMR (HSQC and HMBC experiments) were diagnostic for the assignment of the structure of the additional ring. The chemical shifts of appropriate nitrogen atoms [N1, −95.5; N1a, −97.9; and N9, −175] associated with the long-range heteronuclear connectivity of N1 (N1a) to H1′ (H1a′) and N9 (N9a) to H2′ (H2a′) revealed the presence of the five-membered ring. Furthermore, the chemical shift values of N1 in the

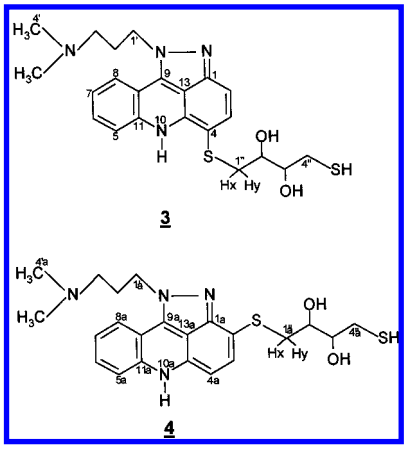


Figure 9. Structures of the two main products formed in the reaction of ledakrin with DTT, N^2-[3′-(dimethylamino)propyl]-4-(1″,4″-disulfanyl-2″,3″-dihydroxybutane)-1,9-diazane-1,10-dihydroacridine (P3) (**3**) and N^2-[3′-(dimethylamino)propyl]-2-(1″,4″-disulfanyl-2″,3″-dihydroxybutane)-1,9-diazane-1,10-dihydroacridine (P3a) (**4**).

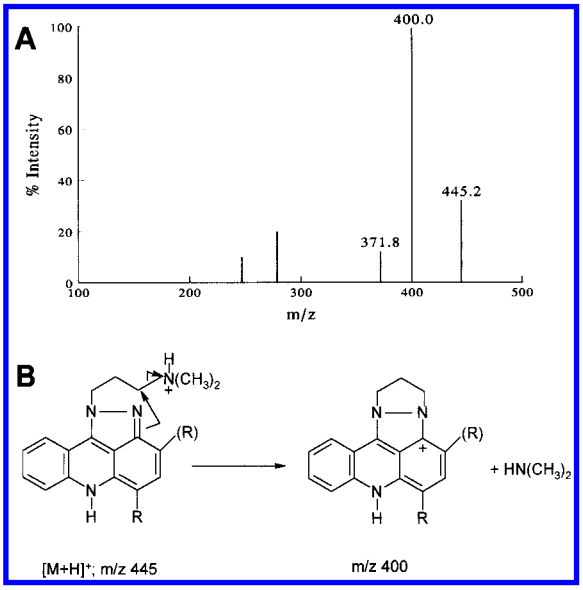
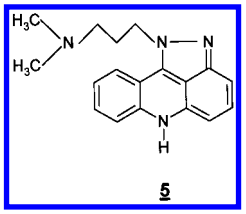


Figure 10. (A) Positive-ion ESI-MS/MS spectrum of product P3 (ion at *m*/*z* 445). The mass spectrum shows fragment ions at *m*/*z* 400 and 372 formed by the loss of 45 and 73 mass units, respectively. (B) Ion formation process observed in the ESI-MS/MS spectrum.

^{15}N spectrum clearly indicate the presence of a double bond between N1 and C1.

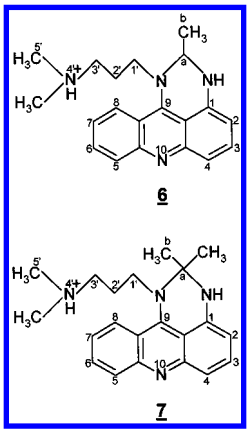
This evidence for a five-membered ring in compounds **3** and **4** clarified the spectral data obtained from the MS analysis of P3. Namely, the MS/MS spectrum obtained for P3 (Figure 10A) shows that the fragment ion at *m*/*z* 400 was formed by loss of dimethylamine from the species at *m*/*z* 445. The driving force for such fragmentation is the formation of a highly stable fragment ion at *m*/*z* 400, the structure of which is shown in Figure 10B. The formation of this fragment ion was only possible when the additional five-membered ring was present in positions 1 and 9 of the acridine ring.

In light of the results described above, it is apparent that the ledakrin activation product M1, formed with the microsomal fraction of rat liver (Figures 2 and 3), undergoes loss of the dimethylamine moiety (Figure 4). The proposed structure of M1 (**5**) is shown in Figure 11. On the basis of the HPLC retention time and UV/vis and



5

Figure 11. Structure of the key product P1 (M1 and D1), N^2-[3′-(dimethylamino)propyl]-1,9-diazane-1,10-dihydroacridine (**5**), obtained with microsomes, DT-diaphorase, and DTT.



6 **7**

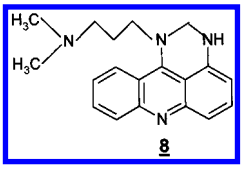
Figure 12. Structures of the products formed by reduction of ledakrin with hydrazine hydrate, N^2-[3′-(dimethylamino)propyl]-N^1,N^2-diaminoethylacridine (**6**), and $SnCl_2$, N^2-[3′-(dimethylamino)propyl]-N^1,N^2-diamino-1″-methylethylacridine (**7**), related to M4 and M5, respectively, obtained with microsomes (see Figure 3).

MS data, product M1 was identical to product P1 formed during reduction of ledakrin with DTT. Similarly, the D1 (Figure 6) and O1 (data not shown) products described above were also identical to the M1 metabolite. Thus, activation product **5** appeared to be a key product, which was observed in all the activation systems that were studied.

In conclusion, studies on the reaction of ledakrin with DTT allowed us to obtain the structures of the rat liver microsomal metabolites, M1 and M2. The structures of compounds **5** and **2** were determined for M1 and M2, respectively. It was also found that two compounds (**3** and **4**) are isomeric products from the nucleophilic substitution occurring with the reductive transformation of ledakrin by DTT.

Reduction of Ledakrin by Hydrazine Hydrate. The conditions for the formation of activation product M4 were achieved by reducing ledakrin with hydrazine hydrate in the presence of Pd/C. Although preliminary identification of M4 was made with MS (*m*/*z* 321), reduction of ledakrin with hydrazine hydrate provided a large enough yield of M4 to be analyzed by NMR. DQF-COSY, ROESY, HSQC, and HMBC experiments were used to determine the structure of M4 (**6**) which is shown in Figure 12. The presence of the additional carbon atom between N1 and N9 was demonstrated by DQF-COSY and HMBC techniques. The DQF-COSY spectrum revealed the correlation of the C_aH with N1H and C_bH, and the HMBC technique was able to observe the coupling signals between C_a and H1′. The aminoalkyl side chain in position 9 was proven by heterocorrelation HMBC experiments to be a signal between C9 and the aminoalkyl aliphatic protons.

Reduction of Ledakrin by $SnCl_2$. Product M5 (*m*/*z* 335) was formed in experiments where $SnCl_2$ was used as a reducing agent of ledakrin. The reduction product of the drug was isolated and analyzed by ^1H NMR and ^{13}C NMR. The assignment of the structure of M5 was based on the same NMR experiments as presented above for compound M4. The structure of M5 was proven to be **7** and is presented in Figure 12. The ROESY experiment showed the presence of a proton on N1 and the methyl protons on C_b. The C_b proton, in turn, also coupled with H1′, confirming that the aminoalkyl side chain remainded on this structure. The coupling of C_a to the protons on the side chain (C1′) was observed by HMBC.



8

Figure 13. Proposed structure of M3 microsome metabolite **8**, N^2-[3′-(dimethylamino)propyl]-N^1,N^2-diaminomethylacridine.

Table 1. Chemical Structure Identification of the Products Formed during Activation of Ledakrin by the Microsomal Fraction of Rat Liver (M), DT-Diaphorase (D), Xanthine Oxidase (O), and DTT (P)

number	symbol name	number	symbol name
1	ledakrin	5	M1, D1, O1, P1
2	M2, D2, P2	6	M4
3	P3	7	M5
4	P3a	8	M3

Considering that the formation of six-membered ring structures above was evidence for two products of ledakrin reduction, **6** and **7**, we postulate that product M3 found in the incubation mixture with microsomes (*m*/*z* 307, 14 units lower than that of **6**) might also contain the six-membered ring in its structure. The proposed structure of M3 (**8**) is presented in Figure 13.

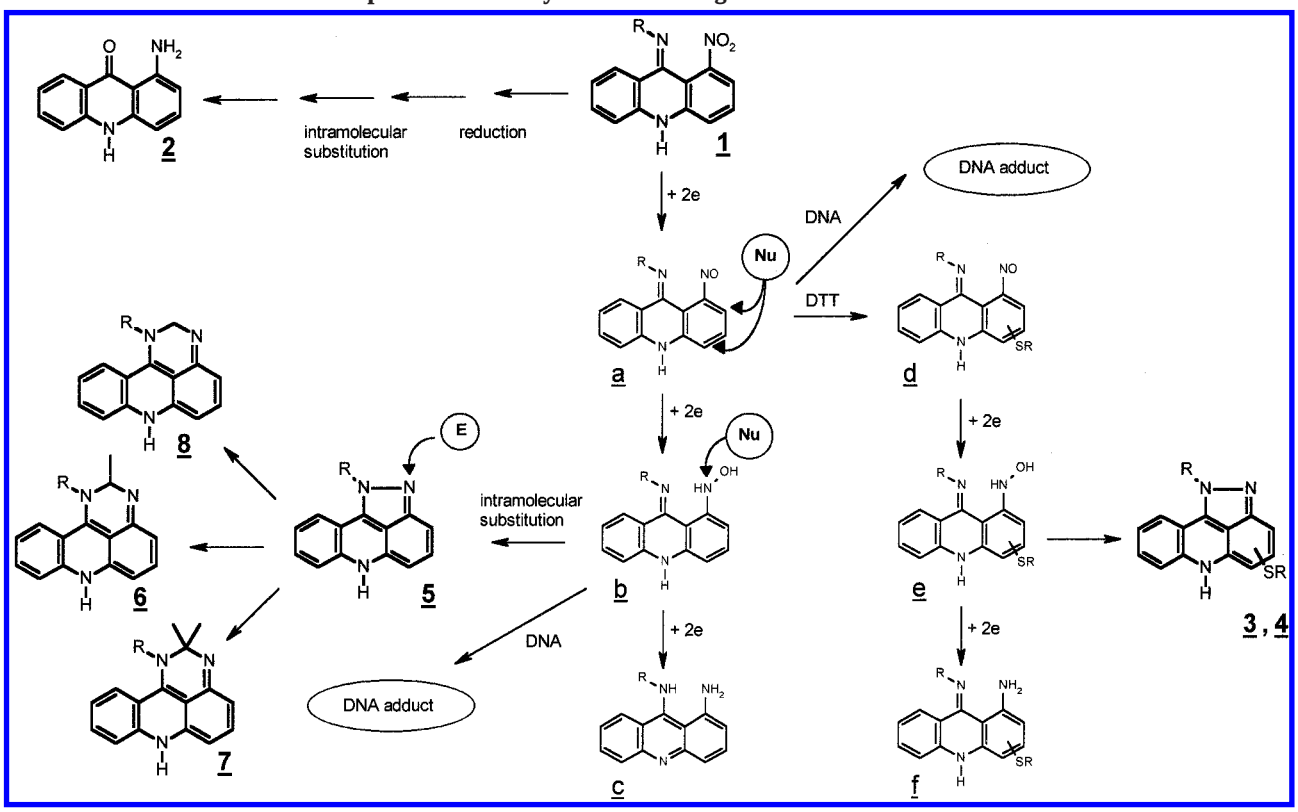
Discussion

The aim of this work was to characterize the products from metabolic activation of ledakrin in systems, where the formation of drug−DNA adducts was previously demonstrated (*13*).

We showed that ledakrin easily underwent metabolic transformations in all studied activation systems. It was reactive in the presence of the microsomal fraction of rat liver and with DTT, a chemical reducing agent. Both activation systems were previously shown to result in similar patterns of drug−DNA adducts. Ledakrin was also easily metabolized with oxidoreductase enzymes: DT-diaphorase and xanthine oxidase. The numbers and the related symbols of the obtained products are presented in Table 1. The structures of the identified metabolites are shown in Scheme 1. The probable pathway of enzymatic and chemical reduction of ledakrin was also proposed in Scheme 1.

One product of metabolic transformations (**5**) was observed in all studied systems. It is an unstable compound obtained as a result of incomplete reduction of ledakrin. A new heterocyclic ring, formed between nitrogen atoms in positions 1 and 9 of the acridine core, distinguishes the structure of this metabolite from the structures of metabolites reported for other biologically active aromatic nitro compounds (*20*, *21*). Furthermore, in contrast to previously reported products of ledakrin reduction (*23*−*25*), this new product (**5**) retained the

Scheme 1. Proposed Pathway for Reducing Transformations of Ledakrin[a]



[a] It was based on the structures determined for the products observed with rat liver microsomes and with chemical reducing agents. The structures, which are drawn in bold lines, were proven in this paper, whereas the structures of the remaining ones related to the hypothesized intermediates were not determined. The most possible directions for the attack of cellular nucleo- and electrophiles to ledakrin metabolites are marked with circle arrows.

aminoalkyl side chain of the parent drug. Product **5** seems to be a key compound of the ledakrin reduction pathway.

The *N*-hydroxyamino derivative, which is usually the common metabolic product of the majority of nitroaromatic agents (*20*, *21*), was not isolated in our studies of ledakrin activation. Nevertheless, the mass ion, *m*/*z* 311 (data not shown), which may relate to 1-hydroxyamino analogue of this drug, *b*, was observed to persist for a short time during chemical and microsomal reductions. On the other hand, incubation of ledakrin with DTT gave rise to the 1-amino analogue of the drug, *c* (*m*/*z* 295, Figure 8), which disappeared after incubation for 60 min. The structure of **5** allowed us to explain why neither the *N*-hydroxy-1-amino, *b*, nor the 1-amino, *c*, derivative of ledakrin was isolated from the mixture of the reduction products. The proximity of nitrogen atoms at positions 1 and 9 of the acridine core seems to make the *N*-hydroxy intermediate, *b*, susceptible to the following cyclization to the dihydropyrazoloacridine ring, **5**, which, in turn, inhibits the formation of the 1-amino derivative.

Although ledakrin was strongly susceptible to cyclization under reducing conditions, the resulting product **5**, bearing a five-membered ring added to the acridine core, also turned out to be the reactive compound, and it was easily transformed to product **8**. The structure of **8** has not been proved by NMR. However, the fact that product **8** exhibited a molecular ion 14 mass units greater than **5** suggests that metabolite **8** possesses an additional one-carbon group. It indicates, in turn, that a six-membered ring was formed between positions 1 and 9. Reactions of this type were reported earlier as a result of radical oxidation of Tris to formaldehyde (*33*). Furthermore, oxidation of Tris to one-carbon compounds was also shown in microsomes (*34*), and with dithiothreitol and xanthine oxidase (*35*). When it is considered that Tris buffer was applied in our experiments, the source of the one-carbon element in **8** might have the same origin.

The reactive dihydropyrazoloacridine (**5**) could also be the precursor for metabolites **6** and **7**. These metabolites were found during microsomal activation as well as from chemical reduction of ledakrin. The source of the two-carbon unit in **6** in the mixture of microsomal enzymes might be an acetyl group transported by acetyl-CoA. The three-carbon unit of **7** might originate from the natural transformation of glucose 6-phosphate to pyruvate, because the former compound was added to the incubation mixture as a component of the reducing−oxidizing system. In conclusion, the transformation of **5** to **6**−**8** likely results from electrophilic attack of the formaldehyde, acetyl, and puryvate groups, respectively, on the nitrogen atom of the dihydropyrazoloacridine ring.

It was established that 1-aminoacridinone **2** was the second-most common product of all the studied reactions. This product seemed to be the result of exhaustive reduction of the nitro group and detachment of the aminoalkyl side chain. However, the appearance of the acridinone derivative under the studied conditions (pH 7.4 and 37 °C) did not result from the hydrolysis of the aminoalkyl side chain of ledakrin, which usually occurs slowly even in boiling acidic medium (*36*). It was shown earlier that the origin of the acridinone oxygen atom in such a reaction was the nitro group of ledakrin (*37*). Therefore, the obtained product had to be a result of intramolecular substitution by the oxygen atom at position 9 of the acridine core. Thus, we observed another

case where the "proximity effect" of positions 1 and 9 dictated the exceptional properties of ledakrin.

The reaction of ledakrin with DTT as a reducing agent yielded not only the two metabolites, **5** and **2**, presented above but also compounds **3** and **4**, which were the products of substitution by the DTT residue at positions ortho and para to the nitro group. Therefore, the nucleophilic attack of DTT on ledakrin most likely occurred before the final reduction of the nitro group, or the reduction and nucleophilic substitution happened simultaneously. Had nucleophilic substitution resulted after the reduction of ledakrin to **5**, it would have substituted into the position meta to the 1-imine group and the nucleophilic attack would have been much more difficult, if at all possible.

In summary, the structural studies presented in this paper have revealed three characteristic chemical properties of ledakrin, which seem to be crucial for its exceptional biological activity. First, ledakrin turned out to be extremely reactive under enzymatic and chemical reducing conditions. We suggest that this trait results more from the specific reactivity of the reduced intermediate species than from the susceptibility of the 1-nitro group to reduction. This was based on the fact that the reduction potential of ledakrin is not significantly lower than that of other nitroacridines (*38*, *39*). The high reactivity of the reduction intermediates seems to result from the proximity of nitrogen atoms in positions 1 and 9 of the acridine core. This proximity, in turn, makes the intermediate molecule strongly susceptible to intramolecular cyclization, giving rise to metabolite **5**. Second, the crucial product (**5**) was also found to be a reactive compound as it easily undergoes transformation in the presence of electrophilic carbon atoms to six-membered ring products (**6**−**8**). The third characteristic chemical feature of ledakrin relates to its high susceptibility to nucleophilic substitution para and ortho to the nitro group.

The specific reduction pathway of ledakrin (Scheme 1) described above should have a significant impact on the reaction of this drug with DNA. The formation of interstrand cross-links between DNA and the activated form of ledakrin was shown in vitro as well as in tumor cells (*14*, *16*). Taking this into account, we have proposed the possible reactive sites of ledakrin that might participate in bond formation with DNA. The first one appeared on the level of the two-electron reduction intermediate, nitroso derivative, *a*. Carbon atoms at position 2 or 4 of this intermediate are capable of reaction with DNA. The second reactive site was formed on the reduction level of four electrons. It is the nitrogen atom of the *N*-hydroxy-1-amino group in species *b*. Both reactive sites of activated ledakrin molecule are the electrophilic centers. Therefore, in the absence of DNA, product **3**, **4**, or **5** was obtained, whereas in the presence of DNA, the nucleophilic attack of the 2-amino group or the N^7 atom of guanine seems to be preferable. The reactions such as these were demonstrated earlier for carcinogenic compounds, where the formation of N^2-guanyl adducts with an aromatic carbon atom (*40*) as well as with a *N*-hydroxyamino group (*41*, *42*) was shown. The N^7-guanyl adduct was postulated to be the intermediate of the C^8-guanyl adduct, which was observed for activated arylamines (*43*, *44*). In this case, the nitrenium ion formed from *N*-hydroxyamine was the reactive species able to bind with the C^8 atom of guanine. We postulate that in the case of the *N*-hydroxy-1-amino derivative of ledakrin the reaction pathway might be similar. On the other hand, carbon atoms 2 and 4, activated during metabolic reduction, might create the C−C bond with the C^8 carbon atom of guanine as previously reported (*44*, *45*).

The above discussion indicates that two reactive regions of the ledakrin molecule are able to bind with guanine. Two bonds might be formed between one activated molecule of ledakrin and two guanine moieties, possibly resulting in cross-links between the drug and DNA. DNA cross-links with ledakrin were discovered earlier in our laboratory (*14*) and were postulated to have a significant impact on the cytotoxic and antitumor action of this drug (*15*, *16*). Because similar patterns of DNA adducts were previously observed under reducing conditions in vitro and in the cell (*13*), we postulate that two reactive centers of activated ledakrin presented above might also react with cellular DNA.

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