Azulene Analogs of Pharmacological Agents III: Acute Toxicity and Local Anesthetic Activity of Azulylamides and Azulenecarboxamides

P. H. DOUKAS ˣ, T. J. SPEAKER, and R. S. THOMPSON

Abstract □ This paper describes the acute toxicity of two azulylamides and six azulenecarboxamides and the nerve conduction-inhibiting properties of some of these compounds. The azulene derivatives are compared to their benzenoid prototypes, lidocaine and procainamide, as part of a continuing investigation of the biological properties of nonbenzenoid aromatic compounds.

Keyphrases □ Azulene analogs (azulylamides and azulenecarboxamides)—acute toxicity and local anesthetic activity □ Azulylamides and azulenecarboxamides—acute toxicity and local anesthetic activity □ Structure–activity relationships—azulene analogs–local anesthetic activity

Previous papers (1, 2) reported the synthesis of azulene analogs of procainamide and lidocaine. The acute toxicity and nerve conduction-inhibiting properties are now reported for several of these analogs.

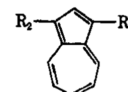

Table I—LD_{50} Values of Some Azulylamides and Azulenecarboxamides[a,b]

Compound	R_2	R_1	LD_{50} (95% Confidence Limits) mg/kg	LD_{50} (95% Confidence Limits) mmoles/kg	LD_{50} Ratios[c] Azulenoid Lidocaine	LD_{50} Ratios[c] Azulenoid Procainamide
I-1	H—	$-NH-C(=O)-CH_2CH_2N(C_2H_5)_2$	229 (212–252)	0.749 (0.69–0.82)	1.1	0.45
I-2	H—	$-NH-C(=O)-CH_2CH_2N(CH_2CH_2)_2O$	210 (171–264)	0.737 (0.60–0.93)	1.1	0.46
II-1	H—	$-C(=O)-NH-CH_2CH_2N(C_2H_5)_2$	178 (150–220)	0.582 (0.49–0.66)	0.85	0.35
II-2	O_2N-	$-C(=O)-NH-CH_2CH_2N(C_2H_5)_2$	221 (148–338)	0.629 (0.41–0.96)	0.92	0.38
II-3	$H_3C-C(=O)-NH-$	$-C(=O)-NH-CH_2CH_2N(C_2H_5)_2$	367 (212–575)	1.00 (0.58–1.57)	1.46	0.61
III-1	H—	$-C(=O)-N(CH_2CH_2)_2N-CH_3$	268 (243–289)	0.926 (0.84–1.02)	1.36	0.57
III-2	O_2N-	$-C(=O)-N(CH_2CH_2)_2N-CH_3$	188 (131–275)	0.561 (0.39–0.82)	0.82	0.34
III-3	$H_3C-C(=O)-NH-$	$-C(=O)-N(CH_2CH_2)_2N-CH_3$	>485[d]	>1.5[d]	>1.4	>0.9
Lidocaine			160 (135–198)	0.682 (0.57–0.77)		
Procainamide			447 (407–486)	1.64 (1.49–1.81)		

[a] All values are for the hydrochloride salts. [b] The chemical names of the compounds are as follows: I-1, 1-(3-diethylaminopropionamido)azulene; I-2, 1-(3-morpholinopropionamido)azulene; II-1, 1-(2-diethylaminoethyl)azulenecarboxamide; II-2, 1-(2-diethylaminoethyl)-3-nitroazulenecarboxamide; II-3, 1-(2-diethylaminoethyl)-3-acetamidoazulenecarboxamide, III-1, 1-(*N*-methylpiperazine)azulenecarboxamide; III-2, 1-(*N*-methylpiperazine)-3-nitroazulenecarboxamide; and III-3, 1-(*N*-methylpiperazine)-3-acetamidoazulenecarboxamide. [c] Calculated on a millimoles per kilogram basis. [d] Insufficient compound was available to allow close estimation of the LD_{50} values or of the 95% confidence limits.

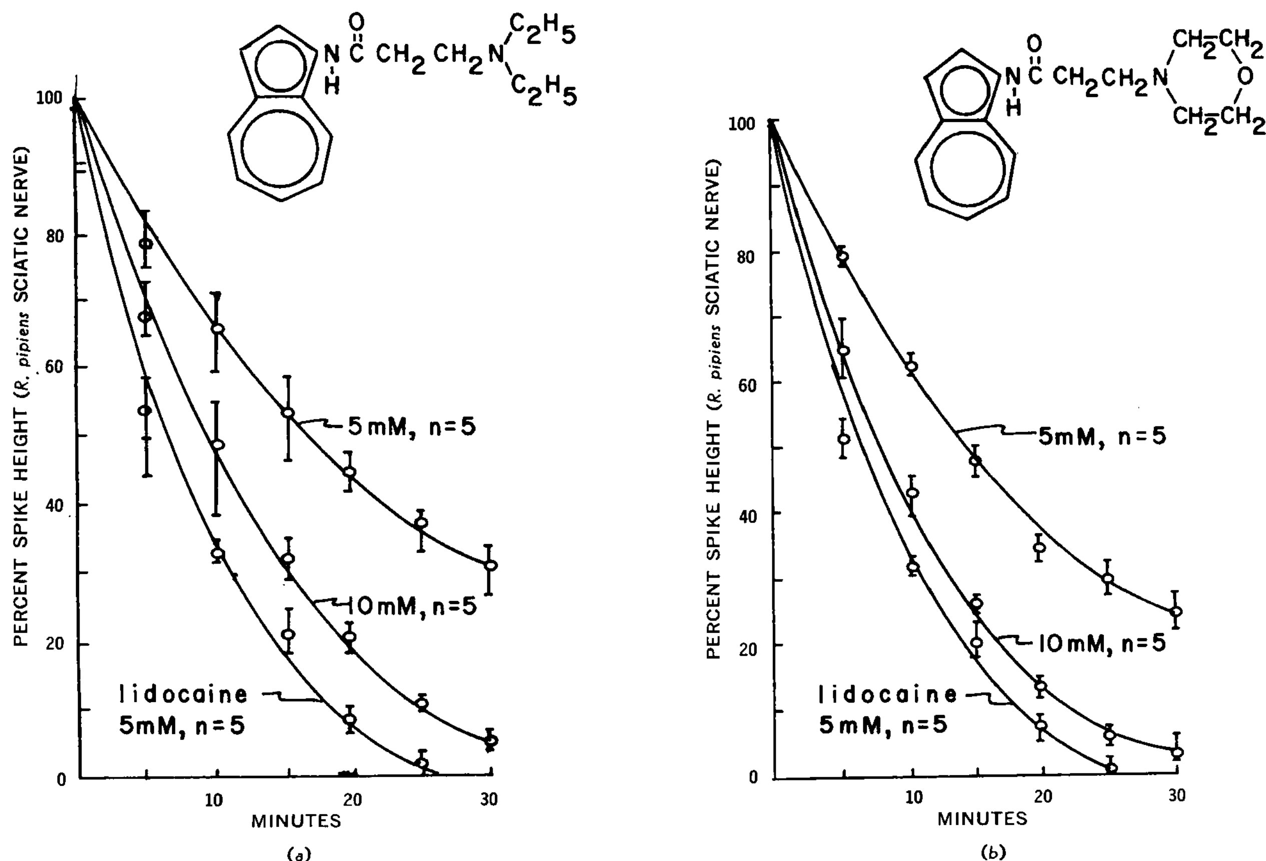

Figure 1—*Inhibition of sciatic nerve action potential* (R. pipiens) *with time at 5- and 10-mmole concentrations of:* (a) *1-(3-diethylaminopropionamido)azulene (I-1), and* (b) *1-(3-morpholinopropionamido)azulene (I-2). Both compounds are compared to 5 mmoles of lidocaine. Each experimental point represents the mean of five experiments; the standard deviation for each point is also shown.*

The compounds included in this study were of three classes: azulylamides (Type I), secondary azulenecarboxamides (Type II), and tertiary azulenecarboxamides (Type III). Intraperitoneal LD_{50} values determined in male Swiss–Webster mice, according to the method of Weil (3), are given in Table I. On the basis of the LD_{50} values, candidate compounds from each series were selected for evaluation as nerve blocking agents. Isolated frog sciatic nerve preparations were employed to determine the degree of inhibition of nerve conduction relative to the pattern compounds. The changes in percentage inhibition as a function of time and of drug concentration are presented in Figs. 1 and 2.

EXPERIMENTAL

LD_{50} Determinations—Naive male Swiss–Webster mice[1], 25 ± 9 g, were randomly divided into five or six groups of four mice each. The groups were housed separately in metabolism cages at 25 ± 2° and provided with food[2] and tap water *ad libitum*, except during the first 4 hr of each experiment when food was withheld. Mice in each group were identified by tail marking. All experimental and control injections were made intraperitoneally.

All drugs for LD_{50} studies were dissolved in enough 0.9% sodium chloride solution that the administered dose was contained in 0.1 ml/10 g of body weight. All solutions were prepared fresh on the day of use, except those of sodium chloride which were obtained from commercial 30-ml multiple-dose vials[3].

All surviving animals were observed over 72 hr; all deaths occurred within 24 hr of dosing.

Nerve Conduction Measurements—Freshly removed sciatic nerves of male frogs (*Rana pipiens*) were used in all measurements of inhibition of nerve conduction. Nerves were kept bathed in oxygenated (95% O_2–5% CO_2) frog Ringer's solution and were maintained at 21 ± 0.5°. All drugs for nerve conduction studies were dissolved in frog Ringer's solution, and all solutions were prepared fresh on the day of use according to the method of Condouris (4).

For measurement of nerve conduction, nerves were suspended across pairs of stimulating and recording platinum electrodes in an oxygenated, humidified nerve chamber. Drug was applied to each nerve by allowing it to dip into a small drug-containing bath positioned between the stimulating and recording electrodes.

Supramaximal stimulation[4] with trains (140 msec; delay = 1 sec) of square wave pulses at a frequency of 89 cycles/sec and a duration of 0.07 msec was utilized. The nerve action potential arising from the recording electrodes was displayed oscillographically[5]. Nerves were permitted to equilibrate in drug-free frog Ringer's solution for 30 min prior to the initiation of an experiment. Measurements of the spike height just prior to drug exposure were taken as individual control values for each nerve.

At the end of the equilibration period, the drug-free Ringer's solution was replaced with drug-containing Ringer's solution and the spike height was measured at 5-min intervals. The nerve remained

[1] West Jersey Biological Supply, Wenonah, N.J.
[2] Purina laboratory chow.
[3] Baxter Laboratories, Division of Travenol Laboratories, Inc., Morton Grove, Ill.
[4] Laboratory stimulator model 104A, American Electronic Laboratories Inc., Colmar, Pa.
[5] Tektronix oscilloscope model 502A, Tektronix, Inc., Beavorton, Ore.

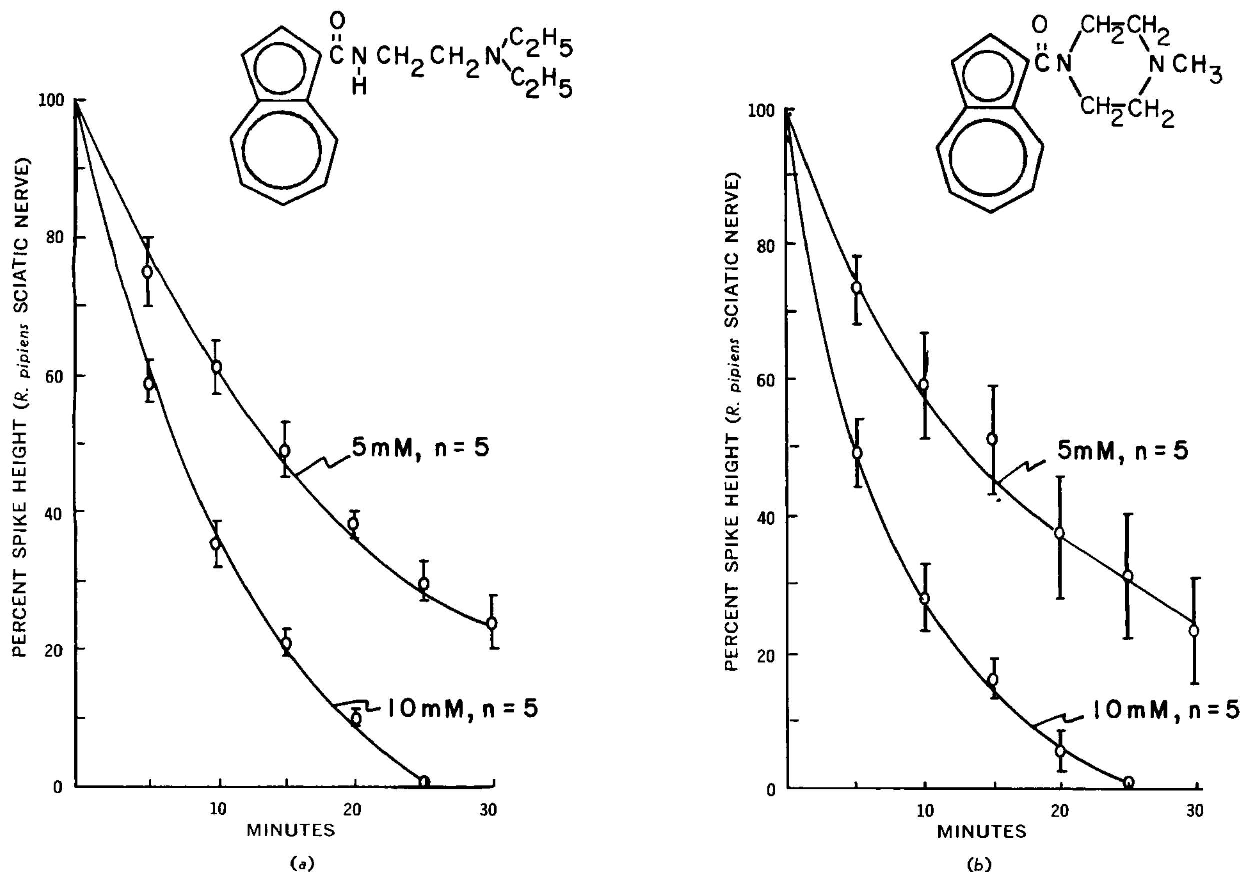

Figure 2—*Inhibition of sciatic nerve action potential* (R. pipiens) *with time at 5- and 10-mmole concentrations of:* (a) *1-(2-diethylaminoethyl)azulenecarboxamide (II-1), and* (b) *1-(N-methylpiperazine)azulenecarboxamide (III-1). Each experimental point represents the mean of five experiments; the standard deviation for each point is also shown.*

in contact with the drug solution for the duration of the experiment. A fresh nerve was used for each experiment, and no nerve was exposed to a second drug-containing solution.

The reversibility of drug effects was assessed by repeated washing of the nerve preparation with drug-free Ringer's solution until the action potential was restored.

Preliminary range-finding experiments established the concentrations at which 50% inhibition of conduction could be established within 30 min. These experimentally determined concentrations for each azulenoid were then used in subsequent replicate experiments. Experiments were replicated five or more times.

All azulenoid compounds tested were synthesized in these laboratories (1, 2). Lidocaine hydrochloride[6] and procainamide hydrochloride[7] were used as comparative controls and 0.9% sodium chloride solution and frog Ringer's solution were used as vehicle controls in LD_{50} and nerve conduction studies, respectively.

RESULTS AND DISCUSSION

The LD_{50} doses and 95% confidence limits of the several compounds are presented in Table I, both as milligrams per kilogram and as millimoles per kilogram, the latter to provide a rapid method of comparison between the compounds. The ratios of the LD_{50} values, in millimoles per kilogram, of the azulene derivatives and the benzenoid prototypes are also listed in the table.

The two azulylamides have approximately the same LD_{50} values as lidocaine and have about twice the toxicity of procainamide. The azulenecarboxamides, by contrast, have LD_{50} values extending over a threefold range, depending on substitution. The parent and 3-nitro-substituted carboxamides are substantially more toxic than are the corresponding 3-acetamido derivatives; similarly, substitution of an acetamido group on the annular system reduces the toxicity of the parent compound by at least half. In the case of Compound III-3, the LD_{50} value is of the same order as that of procainamide on a millimoles per kilogram basis.

All carboxamides except the 3-acetamidopiperazineamido analog are more toxic than procainamide, but three of these (Compounds II-3, III-1, and III-3) are of lesser toxicity than lidocaine.

Lethal doses of both experimental and reference compounds appeared to produce death by respiratory collapse. The sequence of toxic effects was as follows. In all instances, the mice were initially depressed following administration of the compounds. Subsequently, they suffered prolonged mixed clonic and tonic convulsions. Death, when it occurred, was a result of respiratory failure. The intensity and duration of convulsions were roughly proportional to the administered dose.

The intensity of the convulsions was greatest in the 3-nitro-substituted compounds, intermediate in the 3-unsubstituted compounds, and least in the 3-acetamido compounds. These highly colored compounds and their metabolites were detected in urine soon after administration. The metabolic transformation of selected azulylcarboxamides is currently under investigation. Gross observation upon necropsy showed the large and small intestines, mesentery, and bladder to be deeply stained by the azulenoid compounds.

All 3-unsubstituted azulene derivatives produced reversible graded dose–effect relationships in isolated nerve preparations. Two- to threefold changes in concentration of azulenoid produced about twofold changes in the time required for a 50% inhibition of conduction, with higher concentrations producing more rapid effects.

Figures 1*a* and 1*b* show time–effect relationships on frog nerve conduction produced by the azulylamides I-1 and I-2. Neither of these compounds is as potent on a molar basis as is the prototype compound lidocaine.

Figures 2*a* and 2*b* show the time–effect relationship on frog

[6] Supplied by Dr. Bertil Takman, Astra Laboratories, Worcester, Mass.
[7] K and K Laboratories, Jamaica, N.Y.

nerve conduction produced by the azulenecarboxamides II-1 and III-1. Although the prototype compound for these carboxamides, procainamide, is without effect on frog sciatic nerve, both azulenoid compounds inhibited conduction. Compared with lidocaine, these compounds are about half as active.

The 3-nitro compounds (II-2 and III-2) consistently produced an irreversible inhibition of conduction graded with concentration. However, in contrast with the other compounds, the dose–effect relationships produced by the nitro derivatives were extremely variable from nerve to nerve.

The 3-acetamidocarboxamides are devoid of nerve conduction-inhibiting activity at concentrations up to 50 mmoles.

REFERENCES

(1) P. H. Doukas and T. J. Speaker, *J. Pharm. Sci.*, **60,** 184(1971).

(2) *Ibid.*, **60,** 479(1971).

(3) C. S. Weil, *Biometrics*, **8,** 249(1952).

(4) G. A. Condouris, *J. Pharmacol. Exp. Ther.*, **131,** 243(1961).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 22, 1972, from the *School of Pharmacy, Temple University, Philadelphia, PA 19140*

Accepted for publication July 8, 1974.

Supported in part by Temple University Grant-in-Aid of Research 400-111-24.

The authors thank the following undergraduate students who participated in various phases of this work: S. Havsy, M. Jagani, J. Sipala, B. Stoler, D. Triglia, E. Watkins, and N. Yapsuga.

x To whom inquiries should be directed.

Chromatography on Lipophilic Dextran Gels for Fractionation of Low Molecular Weight Compounds I: Steroid Digitonides

M. M. EL-OLEMY * and S. J. STOHS x

Abstract □ A method for the separation of 3β-hydroxysterols from other sterols is presented. The method involves precipitating 3β-hydroxysterols as the digitonides. The digitonide is then decomposed and separated into components using chromatography on highly cross-linked lipophilic polysaccharide gels. The digitonin in the mother liquor is separated from other sterols using the same chromatographic procedure.

Keyphrases □ Dextran gel chromatography—decomposition of steroid digitonides □ 3β-Hydroxysterols—separation from other sterols by dextran gel chromatography □ Chromatography—separation of 3β-hydroxysterols from other sterols, dextran gel columns

The ability of digitonin to form a sparingly soluble complex with an equivalent amount of 3β-hydroxysteroids was first noted in 1910 (1). The manipulation of concentration (2) as well as the use of gathering agents such as aluminum chloride and aluminum hydroxide was later introduced to permit the rapid and quantitative precipitation of steroid digitonides (3–5). Specificity of digitonide formation to 3β-hydroxysterols (6) and 3β-hydroxysteroidal sapogenins (7) has been shown. Haslam and Klyne (6) showed that 3β-hydroxysterols of the 5α series (A/B *trans*) and Δ^5-3β-hydroxysterols are precipitated at a higher dilution than 3β-hydroxysterols of the 5β series (A/B *cis*). This property can be used to fractionate the 3β-hydroxysterols further (6).

DISCUSSION

Digitonide formation has been used in the quantitative estimation of 3β-hydroxysteroids. A gravimetric assay was proposed (1) while more recently a photometric assay was used for this purpose (2, 4, 8, 9). A radioisotopic assay was also proposed (10).

Decomposition of steroid digitonides has afforded a means for the isolation of 3β-hydroxysteroids. Windaus (1) proposed prolonged boiling with xylene. Schöenheimer and Dam (11) decomposed the digitonides in pyridine. Ether was then added to precipitate digitonin and the steroids were obtained by evaporation of the filtrate.

Mikhelovich (12) showed that the Schöenheimer and Dam (11) method results in less decomposition of either the steroid or digitonin than the Windaus (1) method. Bergmann (13) modified the Schöenheimer and Dam procedure by heating the digitonide with anhydrous pyridine at 70–100°, evaporating pyridine, and extracting the steroid with ether in a soxhlet apparatus. The digitonin was then recovered by extraction with hot 90% alcohol.

Sobel *et al.* (9) extracted the steroids with petroleum ether after digitonide decomposition and demonstrated that pyridine was superior to acetic acid for digitonide decomposition. Issidorides *et al.* (14) used dimethyl sulfoxide to decompose the digitonide. Digitonin remained in solution, while the precipitated steroids were extracted with hexane. The digitonin was subsequently obtained by evaporation of dimethyl sulfoxide.

Digitonide formation has been used to isolate digitonin-like compounds by precipitation with cholesterol. Chanley *et al.* (15) used this procedure in the isolation of holothurin A from the sea cucumber, *Actinopygo agassiza*, while Issidorides *et al.* (14) used this procedure for the recovery of tomatine and honothurin.

^{3}H-Digitonin has been employed to determine the subcellular location of sterols by autoradiography (16). Digitonin has also been used to fragment chloroplasts. The enzymes associated with fragments of various particle sizes can then be examined (17).

Several sources of errors may be encountered when using digitonin to precipitate sterols quantitatively (18). Terpenoids and other products are also precipitated, while excess digitonin cannot be readily removed from the precipitate (18).

Occasionally, the separation of 3β-hydroxysterols from 3α-, 3-dehydro-, or other sterols is required. This report describes a method for effecting such fractionation using digitonide preparations and lipophilic, highly cross-linked dextran gel[1] chromatography. The procedure involves precipitation of 3β-hydroxysterols as the digitonides. The digitonides, in solution, are applied to this column, using benzene–methanol (1:1) as the eluting solvent,