(2 × 10 mL). Usual workup of the filtrate gave a solid, which, upon recrystallization in ether, gave compound 34 (762 mg): mp 126–127 °C; IR (CCl_4) 1765 (C=O lactone), 1670 (C=C) cm^{-1}; NMR δ ($CDCl_3$) 0.99 (3 H, s, C_{10} CH_3), 2.54 (AB q, 2 H, C_4 OCH_2, J_{AB} = 4.9 Hz), 2.87 (1 H, m, H_7), 4.43 (H, br t, H_8, $J_{7,8}$ = 5.3 Hz), 5.51 (1 H, s, H_{13a}), 6.07 (1 H, s, H_{13b}); MS *m/e* 248 (M^+), 238, 137, 107. Anal. Calcd for $C_{15}H_{20}O_3$: C, 72.49; H, 8.05. Found: C, 72.41; H, 8.03.

Preparation of 4-Ketonorisoalantolactone (35). To a solution of 34 (1.03 g, 4.14 mmol) in anhydrous ether (3 mL) and chloroform (1 mL) at room temperature was added a solution of H_5IO_6 (0.343 g) in anhydrous ether (60 mL). The mixture was stirred for 48 h in the dark, extracted with ether, washed with sodium metabisulfite (10% solution), sodium bicarbonate (5% solution), and water, dried, and evaporated to dryness. The residue was chromatographed on a silica gel column (25 g, 24-cm high with a 1.8-cm diameter). Elution with ether and recrystallization from ethanol gave 35 (0.885 g) as colorless needles: mp 153–155 °C; IR ($CHCl_3$) 1760 (C=O lactone), 1720 (C=O ketone), 1670 (C=C) cm^{-1}; NMR ($CDCl_3$) δ 0.89 (3 H, s, C_{10} CH_3), 2.1–2.4 (3 H, m, CH_2COCH), 2.93 (1 H, m, H_7), 5.60 (1 H, s, H_{13a}), 4.47 (1 H, dt, H_8, $J_{7,8}$ = 4.7 Hz, $J_{7,9}$ = 2 Hz); MS *m/e* 234 ($M^{+\cdot}$), 219, 191, 111. Anal. Calcd for $C_{14}H_{18}O_3$: C, 71.69; H, 7.68. Found: C, 71.71; H, 7.78.

Acknowledgment. Financial support of this work from the Institut National de la Santé et de la Recherche Médicale (Contract libre 77-1-0973) and the Délégation Générale à la Recherche Scientifique et Technique is acknowledged. We also thank Drs. S. G. Klečak and L. Kinnen (Hoffman La Roche, Basel) for providing us with "Füllingsdorf" Himalayan spotted guinea pigs and J. P. Corbet for providing us with α-methylene-γ-butyrolactone. An "allocation d'études" (DGRST) to J. L. Stampf is acknowledged.

Metabolic N-Hydroxylation. Use of Substituent Variation to Modulate the in Vitro Bioactivation of 4-Acetamidostilbenes

Patrick E. Hanna,* Richard E. Gammans,[1] Russell D. Sehon, and Man-Kil Lee[2]

Departments of Medicinal Chemistry and Pharmacology, University of Minnesota, Minneapolis, Minnesota 55455.
Received December 19, 1979

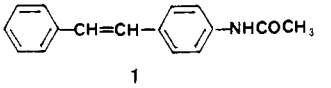
N-Hydroxylation is an obligate step in the bioactivation of carcinogenic aryl amides. Previous reports from this laboratory demonstrated that variation of the 4′ substituent of *trans*-4-acetamidostilbene (1) has a marked effect on the rate of its in vitro microsomal N-hydroxylation. In order to further investigate the effects of electronegative and aliphatic substituents, the 4′-CN, 4′-CH_3, 4′-$C(CH_3)_3$, and 4′-CF_3 analogues of 1 were synthesized and subjected to metabolic transformation by hamster hepatic microsomes. Each compound was synthesized in radiolabeled form, and the metabolites were identified and quantified by TLC, mass spectrometry, and liquid scintillation counting. The V_{max} for N-hydroxylation of the 4′-CN analogue was 24% and the K_m was 11% of that of 1. The glycolamide was a minor metabolite of the 4′-CN compound. The principal metabolite of the 4′-CH_3 compound was the 4′-CH_2OH derivative, the N-hydroxylated product being formed in small quantities. Similarly, the 4′-$C(CH_3)_3$ analogue was metabolized to yield *trans*-4′-[2-(hydroxymethyl)-2-propyl]-4-acetamidostilbene (**26**) along with trace quantities of the hydroxamic acid. The 4′-CF_3 substrate yielded small amounts of the N-hydroxylated material as the only detectable metabolite. Thus, introduction of a 4′ substituent into 1 resulted in a decreased rate of N-hydroxylation for all compounds studied. The reduction in N-hydroxylation depends on both the physicochemical properties of the 4′ substituent and upon the susceptibility of the substituent to metabolic oxidation.

The *N*-aryl amide class of chemical carcinogens is one of the several types of organic chemical carcinogens which require metabolic activation before carcinogenic activity can be manifested. The initial process in the bioactivation of carcinogenic *N*-aryl amides (I) is metabolic hydroxylation of the amide nitrogen atom to yield an *N*-arylhydroxamic acid (II).[3] Although N-hydroxylation is an

I II

obligate step in the induction of carcinogenesis by *N*-aryl amides, the available evidence indicates that, in most cases, further bioactivation of the resultant hydroxamic acid is required. Indeed, *N*-arylhydroxamic acids are potentially capable of undergoing several types of "second step" biotransformations to form electrophilic intermediates which are believed to covalently bind to critical nucleophilic target sites in cellular macromolecules and thereby initiate the carcinogenic process.[4,5]

Because N-hydroxylation, which is mediated by the microsomal mixed function oxidases, is the single biochemical activation process which is common to all members of the *N*-aryl amide class of chemical carcinogens, it is reasonable to expect that an understanding of the molecular characteristics which influence the tendencies of these compounds to serve as substrates for the microsomal *N*-hydroxylase systems would contribute to the development of means to reduce their potential for being metabolically converted to carcinogens. Such information should be useful in the design of new drugs, pesticides, herbicides, and other environmental chemicals to which man and animals are exposed. Previous work conducted in this laboratory demonstrated that variation of the 4′ substituent of *trans*-4-acetamidostilbene (1) has a marked



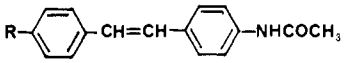
1

effect on its rate of in vitro microsomal N-hydroxylation.[6,7]

(1) Department of Toxicology and Pathology, Mead Johnson Co., Evansville, IN 47721.
(2) College of Pharmacy, Yeungnam University, Gyongsan 632, Korea.
(3) J. A. Miller, *Cancer Res.*, **30**, 559 (1970).
(4) E. C. Miller, *Cancer Res.*, **38**, 1479 (1978).
(5) E. Kriek, *Biochim. Biophys. Acta*, **355**, 177 (1974).
(6) R. E. Gammans, R. D. Sehon, M. W. Anders, and P. E. Hanna, *Drug Metab. Dispos.*, **5**, 310 (1977).

0022-2623/80/1823-1038$01.00/0

Table I. Physical Properties of *trans*-4-Acetamidostilbenes



no.	R	mp, °C	% yield	sp act.,[a] mCi/mmol	formula[a]
2	CN	250-253	65	0.09	$C_{17}H_{14}N_2O$
3	$C(CH_3)_3$	201-203	73	0.59	$C_{20}H_{23}NO$
4	CH_3	248-249	80	1.80	$C_{17}H_{17}NO$
5	CF_3	269-271	62	0.10	$C_{17}H_{14}F_3NO$
22	CH_2OH	256	37	1.22	$C_{17}H_{17}NO_2$

[a] Analyses for C, H, and N were within 0.4% of the theoretical values.

The presence of halogen substituents reduced the rate of N-hydroxylation and, in certain instances, resulted in a small amount of carbon hydroxylation. Both a reduction in the rate of N-hydroxylation and a significantly enhanced production of various carbon oxidation products occurred when 4'-alkoxy substituents were present.

This report describes the results of an investigation of the in vitro microsomal metabolism of the 4'-cyano, 4'-methyl, 4'-*tert*-butyl, and 4'-(trifluoromethyl) derivatives (**2–5**, Table I) of **1**. These compounds were selected for study in order to obtain additional information regarding the influence of hydrophilic and hydrophobic electronegative substituents, as well as hydrophobic aliphatic substituents, upon the metabolic N-hydroxylation of acetamidostilbenes.

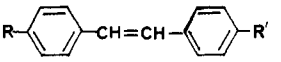
Synthesis. The *trans*-4-acetamidostilbenes **2–5** and **22** (Table I) were prepared by the reduction of the nitrostilbenes **6–9** and **20** (Table II) to the amines **10–13** and **21** (Table II), followed by acetylation with acetic anhydride. The amides **2–5** and **22** were also prepared in radiolabeled forms by treatment of the appropriate amines with [1-^{14}C]acetic anhydride.

The nitrostilbene intermediates **6–9** and **18** (Table II) were synthesized by condensation of the appropriate 4-substituted benzaldehyde with diethyl 4-nitrobenzylphosphonate, a modification of the Wittig reaction which yields exclusively the *trans*-stilbene product.[8] The isomeric purity of the stilbene derivatives was confirmed by thin-layer chromatography and ultraviolet spectroscopy.[8] *trans*-4'-(Hydroxymethyl)-4-nitrostilbene (**20**; Table II) was prepared by conversion of the carboxylic acid **18** to the corresponding ethyl ester **19**, followed by reduction with calcium borohydride.

The hydroxamic acids **14–17** (Table III) were formed from the corresponding nitrostilbenes **6–9** using a modification of the method of Smissman and Corbett, which involves reduction of the nitro compounds to hydroxylamines in the presence of zinc and ammonium chloride followed by acetylation with acetyl chloride.[9] Radiolabeled forms of **14–16** were prepared by acetylation of the unpurified hydroxylamine intermediates with [1-^{14}C]acetyl chloride.

The glycolamides **23–25** (Table III) were prepared by condensation of the appropriate 4-aminostilbenes with ethyl glycolate as described by Shapiro and co-workers.[10]

Table II. Physical Properties of 4-Nitro- and 4-Amino-*trans*-stilbenes



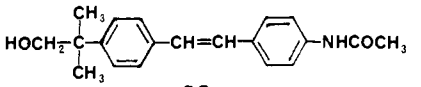
no.	R	R'	mp, °C	% yield	formula[a]
6	CN	NO_2	259-261[b]	83	$C_{15}H_{10}N_2O_2$
7	$C(CH_3)_3$	NO_2	162-164	87	$C_{18}H_{19}NO_2$
8	CH_3	NO_2	149[c]	88	$C_{15}H_{15}NO_2$
9	CF_3	NO_2	171-172	86	$C_{15}H_{10}F_3NO_2$
18	COOH	NO_2	341 (dec)[d]	55	$C_{15}H_{11}NO_4$
19	$CO_2C_2H_5$	NO_2	165.5-166.5	74	$C_{17}H_{15}NO_4$
20	CH_2OH	NO_2	179-181	24	$C_{15}H_{13}NO_3$
10	CN	NH_2	226-228	76	$C_{15}H_{12}N_2$
11	$C(CH_3)_3$	NH_2	141-142	56	$C_{18}H_{21}N$
12	CH_3	NH_2	157-158	57	$C_{15}H_{15}N$
13	CF_3	NH_2	203	59	$C_{15}H_{12}F_3N$
21	CH_2OH	NH_2	210-212	72	$C_{15}H_{15}NO$

[a] Analyses for C, H, and N were within 0.4% of the theoretical values. [b] Lit.[28] mp 255-256 °C. [c] Lit.[28] mp 150-150.5 °C. [d] Lit.[28] mp 333-334 °C.

Metabolite Identification. The ^{14}C-labeled acetamidostilbenes were incubated with hamster hepatic microsomes, and the metabolites were extracted, purified by TLC, and quantified according to the procedures described under Experimental Section. Total recovery of radioactivity from the incubation mixtures usually exceeded 80%, and all of the metabolites which were extracted from the incubation mixtures were identified. The efficiencies of extraction of the metabolites and unmetabolized substrates were determined as previously described, and the values for metabolite formation were appropriately corrected.[6,7] The structures of the metabolites were determined by mass spectral analysis following purification by preparative TLC.

Incubation of the 4'-cyano analogue (**2**) with hepatic microsomes resulted in the production of two ether-extractable metabolites which were identified as the hydroxamic acid **14** and the glycolamide **23** (Table III). The mass spectra and chromatographic properties of the metabolites were identical with those of the synthetic samples. These metabolites, which are positional isomers, are readily distinguishable on the basis of their mass spectra. The mass spectrum of the N-hydroxylated compound **14** exhibits a weak molecular ion at *m/e* 278 with a characteristic M - 16 peak at *m/e* 262 (Table IV). The M - 16 peak is typical of *N*-arylhydroxamic acids.[6,7] The glycolamide **23** also exhibited a weak molecular ion at *m/e* 278; however, this compound produces characteristic weak fragment peaks at M - 2 and M - 16 rather than a strong peak at M - 16.

The 4'-*tert*-butyl compound **3** underwent both N-hydroxylation, producing the hydroxamic acid **15** (Table III), and hydroxylation of the *tert*-butyl substituent, producing the primary alcohol **26**. The chromatographic



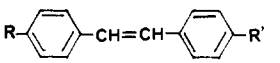
26

and mass spectral properties of the N-hydroxylated metabolite were identical with those of a synthetic sample. The mass spectrum contained a weak molecular ion at *m/e* 309, as well as the expected M - 16 fragment at *m/e* 293 (Table IV).

As no synthetic sample of **26** was available, the structure of this metabolite was assigned primarily on the basis of its mass spectral properties. The mass spectrum exhibited a weak molecular ion at *m/e* 309 and a strong peak at *m/e* 278 (M - 31) (Table IV). The M - 31 peak represents loss

(7) R. E. Gammans, R. D. Sehon, M. W. Anders, and P. E. Hanna, *Drug Metab. Dispos.*, **6**, 226 (1978).
(8) O. W. Wheeler and H. N. Batle de Pabon, *J. Org. Chem.*, **30**, 1473 (1965).
(9) E. E. Smissman and M. D. Corbett, *J. Org. Chem.*, **37**, 1847 (1972).
(10) S. Shapiro, I. Rose, and C. Freedman, *J. Am. Chem. Soc.*, **81**, 6322 (1959).

Table III. Physical Properties of Hydroxamic Acids and Glycolamides



no.	R	R'	mp, °C	yield, %	sp act., mCi/mmol	formula[a]
14	CN	$NOHCOCH_3$	176-177	45	0.91	$C_{17}H_{14}NO_2$
15	$C(CH_3)_3$	$NOHCOCH_3$	186-187	46	1.07	$C_{20}H_{23}NO_2$
16	CH_3	$NOHCOCH_3$	203-204	30	1.90	$C_{17}H_{17}NO_2$
17	CF_3	$NOHCOCH_3$	215-218	44		$C_{17}H_{14}F_3NO_2$
23	CN	$NHCOCH_2OH$	212-213	12		$C_{17}H_{14}N_2O_2$
24	CH_3	$NHCOCH_2OH$	255-257	38		$C_{17}H_{17}NO_2$
25	$C(CH_3)_3$	$NHCOCH_2OH$	210-212	50		$C_{20}H_{23}NO_2$

[a] Analyses for C, H, and N were within 0.4% of the theoretical values.

Table IV. Characteristic Mass Spectral Fragmentations of *trans*-4'-Substituted-4-acetamidostilbene Metabolites[a]

	substrate			
metabolite	2, R = CN	3, R = $C(CH_3)_3$	4, R = CH_3	5, R = CF_3
14 (hydroxamic acid)	278 (1.3), 262 (41.6), 220 (100)			
23 (glycolamide)	278 (2.6), 276 (0.6), 262 (1.3), 220 (47.6), 44 (100)			
15 (hydroxamic acid)		309 (2.1), 293 (100), 278 (71.4), 236 (42.1)		
26 (hydroxymethyl)		309 (3.5), 293 (0.7), 278 (100), 236 (32.7)		
16 (hydroxamic acid)			267 (5.9), 251 (71.6), 225 (5.7), 209 (100)	
22 (hydroxymethyl)			267 (89.9), 265 (28), 251 (18.9), 226 (18.7), 225 (100), 223 (49)	
17 (hydroxamic acid)				321 (1.0), 305 (54.8), 264 (18.2), 263 (100), 262 (24.2)

[a] Relative intensities are shown in parentheses.

of CH_3O from the hydroxylated *tert*-butyl group, a fragmentation pattern which is characteristic of this type of metabolite.[11] In order to eliminate the possibility that the substrate might have been hydroxylated on the acetyl methyl rather than on the *tert*-butyl group, the glycolamide **25** (Table III) was synthesized and was found to differ from **26** in both its chromatographic and mass spectral properties. In particular, the molecular ion (*m/e* 309) is the base peak in the spectrum of **25** and this compound exhibits a very weak ion at *m/e* 278 (M - 31), whereas the M - 31 peak is the base peak in the spectrum of metabolite **26**.

The possibility that metabolite **26** might be the product of epoxidation of the ethylene linkage of the *trans*-stilbene system was investigated by treatment of **26** with the pyridine/trimethylchlorosilane reagent which was used previously to convert stilbene epoxides to chlorohydrin trimethylsilyl ether derivatives.[7] However, **26** could not be converted to such a derivative. Therefore, it was concluded that the carbon oxidation metabolite of the 4'-*tert*-butyl substrate **3** was the primary alcohol **26**. Metabolic hydroxylation of the *tert*-butyl substituent has also been reported for other compounds.[11-13] The hydroxamic acid **15** was identified after isolation from a large-scale preparative incubation mixture, but it frequently could not be detected in the smaller scale incubations used for kinetic experiments.

trans-4'-Methyl-4-acetamidostilbene (**4**) was transformed in the presence of hepatic microsomal enzymes to a product which was identical in all respects with a synthetic sample of *trans*-4'-(hydroxymethyl)-4-acetamidostilbene (**22**; Table I). The mass spectrum of metabolite **22** exhibited a molecular ion at *m/e* 267, a weaker ion at *m/e* 251 (M - 16), and a prominent fragment at *m/e* 225 (M - 42) (Table IV). The glycolamide **24** (Table III) was synthesized for the purpose of comparison and was found to have different chromatographic and mass spectral properties from those of the hydroxymethyl metabolite **22**.

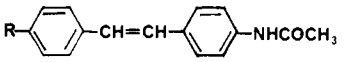
The N-hydroxylated metabolite **16** (Table III) of the 4'-methyl compound was produced in very small amounts and it was not possible to characterize it unequivocally by extraction of large-scale incubation mixtures followed by thin-layer chromatographic purification of the extracts. However, a compound which could be partially purified by preparative TLC and which exhibited a mass spectral fragmentation pattern similar to that of a synthetic sample of **16** was detected. In addition, a metabolite which cochromatographed with synthetic **16** in several solvent systems was detected in the extracts of the small-scale incubation mixtures used in the kinetic experiments.

Hepatic microsomes converted the 4'-(trifluoromethyl) compound **5** to the hydroxamic acid **17** (Table III) which, although formed in small amounts, was identified on the basis of its mass spectral and chromatographic properties, which were identical with those of the synthetic standard.

Kinetics of Metabolism. The apparent K_m and V_{max} values (±SE) for the formation of the hydroxamic acid metabolite **14** from the 4'-CN analogue **2** were 0.31 ± 0.15 mM and 677 ± 221 pmol (mg of microsomal protein)$^{-1}$ min^{-1} (N = 3), respectively (Table V). The glycolamide **23** was formed from the 4'-CN substrate **2** at such a low

(11) J. J. Kamm, A. Szuna, and R. Kuntzman, *J. Pharmacol. Exp. Ther.*, 182, 507 (1972).
(12) J. J. Kamm and A. Szuna, *J. Pharmacol. Exp. Ther.*, 184, 729 (1973).
(13) J. J. Kamm, V. B. Bastone, E. Mohacsi, and F. M. Vane, *Xenobiotica*, 1, 273 (1971).

Table V. Apparent Kinetic Constants for the Microsomal Oxidation of *trans*-4'-Substituted-4-acetamidostilbenes[a]



substrate	R	metabolite	K_m[b]	V_{max}[c]	V/K
2	CN	hydroxamic acid (14)	0.31 ± 0.15	677 ± 221	2.18
3	$C(CH_3)_3$	hydroxymethyl (26)	0.09 ± 0.03	1129 ± 218	12.54
4	CH_3	hydroxamic acid (16)	0.06 ± 0.02	243 ± 47	4.05
	H[d]	hydroxamic acid	2.80 ± 0.20	2767 ± 84	0.99
		4'-hydroxy	0.50 ± 0.03	467 ± 19	0.93
	Br[d]	hydroxamic acid	1.90 ± 0.50	457 ± 67	0.24
	Cl[d]	hydroxamic acid	3.30 ± 0.30	98 ± 13	0.03
		glycolamide	3.50 ± 0.20	17 ± 2	0.005
	F[d]	hydroxamic acid	2.90 ± 0.20	88 ± 0.5	0.03
		glycolamide	3.30 ± 0.90	27 ± 3	0.008
	$O(CH_2)_3CH_3$[e]	hydroxamic acid	1.20 ± 0.40	330 ± 170	0.28
		$\omega - 1$ hydroxy	1.10 ± 0.40	530 ± 220	0.48

[a] Only those metabolites are listed for which kinetic constants could be determined. [b] Values are mM ± SE (N = 3–5). [c] Values are pmol (mg of microsomal protein)$^{-1}$ min^{-1} ± SE (N = 3–5). [d] From ref 6. [e] From ref 7.

rate that it was not possible to reliably determine the apparent kinetic constants for its production. At substrate concentrations of 0.03–1.0 mM, the rate of glycolamide production from **2** ranged from 10 to 50 pmol (mg of microsomal protein)$^{-1}$ min^{-1}.

The apparent kinetic constants for the formation of the hydroxamic acid **15** from the 4'-*tert*-butyl compound **3** could not be determined because the metabolite was formed in only trace quantities. At a substrate concentration of 0.5 mM, 10–20 pmol (mg of microsomal protein)$^{-1}$ min^{-1} was produced. The kinetic constants for the formation of the hydroxymethyl metabolite **26** were as follows: K_m = 0.09 ± 0.03 mM, V_{max} = 1129 ± 218 pmol (mg of microsomal protein)$^{-1}$ min^{-1} (N = 3).

Attempts to examine the kinetics of hydroxylation of the 4'-CH_3 group of **4** were not successful. At substrate concentrations of 0.03–1.0 mM, the rate of product formation ranged from 500 to 1300 pmol (mg of microsomal protein)$^{-1}$ min^{-1}, but the double-reciprocal plots of the data were not interpretable. In order to examine the possibility that metabolite **22** was undergoing further oxidation to the corresponding carboxylic acid, incubation mixtures were acidified and extracted with organic solvents. However, significant amounts of radioactivity were not recovered, indicating that the hydroxymethyl metabolite **22** is not oxidized to the carboxylic acid under the experimental conditions.

Although an unequivocal chemical characterization of the N-hydroxylated metabolite (**16**) of the 4'-CH_3 analogue was not achieved, this metabolite was characterized chromatographically as described above and it was readily quantified in the kinetic experiments. Its formation followed Michaelis–Menten kinetics, and the apparent kinetic constants which were determined for **16** were as follows: K_m = 0.06 ± 0.02 mM, V_{max} = 243 ± 47 pmol (mg of microsomal protein)$^{-1}$ min^{-1} (N = 5).

Microsomal N-hydroxylation of the 4'-CF_3 analogue proceeded at a rate which was so low that the hydroxamic acid **17** was not detected in the kinetic experiments.

Discussion

The data presented herein, as well as in previous reports,[6,7] clearly demonstrate that relatively modest substituent variations have a profound effect on both the quantitative and qualitative aspects of the in vitro metabolism of *trans*-4'-substituted-4-acetamidostilbenes by hamster hepatic microsomes. The two electronegative 4' substituents, CN and CF_3 (compounds **2** and **5**), both caused a reduction in the rate of N-hydroxylation. This result is similar to that observed previously with substrates containing the electronegative halogen atoms in the 4' position.[6] In addition to reducing the rate of N-hydroxylation, the presence of metabolically stable electronegative 4' substituents brings about a general decrease in the overall metabolism of the acetamidostilbenes. Thus, the compounds bearing 4'-CF_3, 4'-CN, 4'-Br, 4'-F or 4'-Cl substituents were not converted in detectable quantities to aromatic ring hydroxylated products or to products resulting from oxidation of the ethylene linkage of the stilbene system. The only measurable carbon-hydroxylated metabolites formed from this group of compounds consisted of minor amounts of the 4'-fluoro-, 4'-chloro-, and 4'-cyanoglycolamides. It is reasonable to conclude that this reduction in metabolic carbon and nitrogen oxidation is at least partially related to the electron-withdrawing influence of the 4' substituents, since the CF_3, CN, F, Cl, and Br groups differ widely in their lipophilic and steric characteristics while each of them is electronegative, as reflected by their positive σ values.[14] Previous reports also indicate that CF_3, CN, and other electronegative substituents reduce metabolic hydroxylation.[15,16a,b] A further indication of the role of electronic effects on N-hydroxylation of acetamidostilbenes is derived from preliminary results obtained with *trans*-4'-hydroxy-4-acetamidostilbene, which is N-hydroxylated at a rate only slightly lower than that of **1** (data not presented).

Since it has been possible to determine the kinetic constants for N-hydroxylation of only 7 of the 12 4-acetamidostilbenes which have been studied, insufficient data are available for a meaningful quantitative structure–activity correlation. However, the apparent kinetic constants determined for the N-hydroxylation of the 4'-CN compound **2** indicate that factors other than substituent electronegativity also contribute to the abilities of acetamidostilbenes to serve as substrates for the microsomal *N*-hydroxylase system. The K_m for N-hydroxylation of **2** is 6–10 times lower than that of either the unsubstituted compound **1** or the 4'-halogenated compounds (Table V).[6] On the other hand, the apparent V_{max} for N-hydroxylation of **2** is one-fourth of that of **1**, while it is approximately seven times greater than that of the 4'-F and 4'-Cl com-

(14) Y. C. Martin, "Quantitative Drug Design", Marcel Dekker, New York, 1978, pp 378–379.
(15) B. Testa and P. Jenner, "Drug Metabolism. Chemical and Biochemical Aspects", Marcel Dekker, New York, 1976, pp 40–41.
(16) (a) L. M. Hjelmeland, L. Aronow, and J. R. Trudell, *Mol. Pharmacol.*, 13, 634 (1977); (b) J. Daly, D. Jerina, and B. Witkop, *Arch. Biochem. Biophys.*, 128, 517 (1968).

pounds and only about 1.5 times greater than that of the 4′-Br analogue. If electronegativity were the sole contributing factor in determining the N-hydroxylation rate, the 4′-CN group, which has a much larger σ value than the halogens, would be expected to have reduced the V_{max} to a greater extent than the 4′-halogen substituents. Therefore, although the electronegativity of the 4′ substituent apparently is an important factor in reducing the rate of N-hydroxylation, other physicochemical properties must also be considered in the structure–activity analysis. This point is further emphasized when the ratio of V_{max} to K_m (V/K) values for the various substrates are examined. The V/K value is an apparent first-order rate constant, having the units of min^{-1}, which provides a measure of the propensity of a substrate to undergo an enzyme-catalyzed reaction at low substrate concentrations.[17a-c] The V/K for N-hydroxylation of the unsubstituted compound **1** is 0.99, while that of the 4′-CN analogue **2** is 2.18 (Table V). Thus, the hydrophilic electronegative cyano group causes only a fourfold reduction in the V_{max} of N-hydroxylation, and the efficiency of N-hydroxylation is similar for **1** and **2**. This is in contrast to the results obtained with the lipophilic 4′-halogen substituents which reduced the V/K values for N-hydroxylation by a factor of 4–33 and reduced the V_{max} values by a factor of 6–31 (Table V). It is important to note that a discussion of relative V/K values is applicable only to results obtained at low substrate concentrations (initial velocity conditions) and that the conclusions cannot be extrapolated to the in vivo situation where, in some instances, the substrates may accumulate in the cells and the relative efficiences of metabolism may change.

Perhaps the most striking result of these studies is the finding that the presence of the lipophilic aliphatic *tert*-butyl and methyl substituents in compounds **3** and **4** resulted in a switching of the primary site of metabolic oxidation from the amide nitrogen atom to the aliphatic substituents. The K_m for hydroxylation of the *tert*-butyl group of **3** was 0.09 mM and the V_{max} was 1129 pmol (mg of protein)$^{-1}$ min^{-1} (Table V). The V/K value for formation of the hydroxylated *tert*-butyl group (metabolite **26**) was 12.54, which is the largest value determined for this series of compounds. The rate of N-hydroxylation of the *tert*-butyl analogue **3** was so low that, in some incubation mixtures, the hydroxamic acid **15** could not be detected. Therefore, the presence of the highly lipophilic *tert*-butyl group brought about an almost complete diversion of metabolism from the toxification pathway of N-hydroxylation to the detoxification pathway of aliphatic carbon hydroxylation. The 4′-CH_3 compound also was hydroxylated to a considerably greater extent on the CH_3 substituent than on the amide nitrogen, although kinetic constants for the methyl group hydroxylation could not be determined.

Recently, Kapetanovic and co-workers reported that the presence of the 4-*n*-butoxy substituent in acetanilide caused an enhanced rate of production of the N-hydroxylated metabolite by mouse hepatic microsomes.[18] The 4-methoxy-, 4-ethoxy-, and 4-*n*-butoxyacetanilides underwent O-dealkylation at comparable rates, but 4-*n*-butoxyacetanilide was N-hydroxylated more rapidly than either of the other two analogues. When the metabolism of 4′-*n*-butoxy-*trans*-4-acetamidostilbene was studied, the O-dealkylated metabolite was not detected.[7] Therefore, there are qualitative differences in the patterns of metabolism of the 4′-alkoxy-*trans*-4-acetamidostilbenes and the 4-alkoxyacetanilides. It cannot be stated with certainty whether these are due to species differences, differences in substrate structure, or a combination of factors. It is, however, noteworthy that Kapetanovic and co-workers found little difference in the ratio of N-hydroxylated to O-dealkylated metabolites produced in the presence of mouse and hamster hepatic microsomes.[18] This result was interpreted to indicate that the nature of the substrate rather than the type of microsomal preparation governs the metabolic profile of 4-alkoxyacetanilides. Since no mention was made of any attempt to measure other possible metabolites of 4-*n*-butoxyacetanilide, further comparison of the data of Kapetanovic and co-workers with the present results is not warranted.

Every 4′-substituted analogue of **1** which has been examined to date undergoes microsomal N-hydroxylation at a rate which is lower than that of **1**. However, the reasons for the reduction in the rate of hydroxamic acid production appear to include both the electronegativity of the substituents and the susceptibility of the substituents to metabolic oxidation. No definite conclusions regarding steric or lipophilic effects of the substituents could be drawn from the present study, but the results of other investigations of microsomal oxidation indicate that such factors are important.[19-24]

Ariens has suggested that the presence of readily metabolized alkyl substituents on aromatic ring systems might prevent the formation of toxic metabolites by diverting the oxidative attack away from the aromatic ring.[25,26] The results of the present study indicate that this suggestion also applies to the diversion of metabolism away from the nitrogen atom of N-aryl amides. Although Ariens proposed that branched alkyl groups are not suitable for this purpose because they do not undergo rapid oxidative conversion, the *tert*-butyl substituent of compound **3** was much more readily oxidized than the nitrogen atom. Harfenist eliminated the mutagenic activity of an *N*-aryl amide monoamine oxidase inhibitor by the incorporation of metabolically stable electronegative substituents into the molecule.[27] This molecular modification was based on the assumption that the mutagenicity of the *N*-aryl amide was due to the metabolic formation of an *N*-arylhydroxamic acid. The lack of mutagenicity of the compounds bearing electronegative substituents is consistent with the effects of the CF_3 and CN groups on N-hydroxylation which were observed in the present study. It is reasonable to conclude, therefore, that information such as that derived from the present investigation can be applied to the design of drugs and other environmental chemicals in a qualitative fashion to permit the medicinal chemist to enhance or reduce metabolism rates as well to minimize the possibility that

(17) (a) W. W. Cleland, *Acc. Chem. Res.*, **8**, 145 (1975); (b) I. H. Segel, "Biochemical Calculations", Wiley, New York, 1976, pp 225–229; (c) J. W. Williams and D. B. Northrup, *J. Biol. Chem.*, **253**, 5908 (1978).
(18) I. M. Kapetanovic, J. M. Strong, and J. J. Mieyal, *J. Pharmacol. Exp. Ther.*, **209**, 20 (1979).
(19) B. Testa, *Pharm. Acta Helv.*, **53**, 143 (1978).
(20) C. Hansch, *Drug Metab. Rev.*, 1, 1 (1973).
(21) L. E. Gaudette and B. B. Brodie, *Biochem. Pharmacol.*, **2**, 89 (1959).
(22) Y. C. Martin and C. Hansch, *J. Med. Chem.*, **14**, 777 (1971).
(23) C. F. Wilkinson and K. Hetnarski, *Biochem. Pharmacol.*, **23**, 2377 (1974).
(24) T. D. Rodgerson, C. F. Wilkinson, and K. Hetnarski, *Biochem. Pharmacol.*, **26**, 1039 (1977).
(25) E. J. Ariens and A. M. Simonis, "Drug Design and Adverse Reactions", H. Bundgaard, P. Juul, and H. Kofod, Eds., Academic Press, New York, 1977, p 329.
(26) E. J. Ariens, *Drug Des.*, **9**, 30–32 (1980).
(27) M. Harfenist, *J. Med. Chem.*, in press.

a molecule will be metabolically converted to a toxic or carcinogenic product.

Experimental Section

Metabolism Studies. Enzymatic Assays. Male Golden Syrian hamsters (90–125 g) were sacrificed by decapitation. Livers were excised, perfused with ice-cold 1.15% KCl, and homogenized in 3 mL of 1.15% KCl/g of liver in a Potter-type homogenizer with a motor-driven pestle. The whole homogenate was centrifuged for 20 min at 9000*g* at 5 °C. The supernatant was decanted and centrifuged at 105000*g* for 60 min using a refrigerated Beckman preparative ultracentrifuge. The resultant pellet was resuspended in 1.15% KCl and the protein concentration was determined by the method of Lowry et al.[29] using bovine serum albumin as a standard. Incubation flasks contained 0.5 mL of microsomal preparation (2.3 to 2.8 mg of microsomal protein), 1 mL of 0.1 M phosphate buffer (pH 7.4), 0.96 mL of 1.15% KCl, 15 μmol of $MgCl_2$, 0.313 to 7.5 μmol of ^{14}C-labeled substrate in 40 μL of Me_2SO and an NADPH generating system consisting of 1.6 μmol of NADP, 1 unit of isocitrate dehydrogenase (Sigma Type IV), and 16 μmol of isocitric acid in a total volume of 2.5 mL. Paraoxon (0.25 μmol) was included in all incubations to inhibit enzymatic deacetylation of the acetamidostilbenes and their metabolites; inhibition of deacetylation was indicated by the absence of amines or hydroxylamines in organic extracts of the incubation mixtures. Incubations were carried out for 10 min at 37 °C in a Dubnoff metabolic shaker and were terminated by pouring into centrifuge tubes containing 2.5 mL of ether and 0.5 g of solid sodium chloride. The tubes were shaken for 10 min and centrifuged, and an aliquot of the etherial layer was used for metabolite analysis. Preparative incubations were run on a scale 4–8 times larger than that described above. Neither paraoxon nor Me_2SO affected the rates of metabolite formation in the concentrations used. The rates of formation of those metabolites for which kinetic constants were determined were linear with respect to protein concentration up to at least 1.0 mg/mL and with respect to time up to 10 min. No metabolite formation was observed when heat-inactivated microsomal preparations were used or when the NADPH generating system was omitted.

Metabolite Purification and Quantitation. Extracts of the incubation mixtures were routinely examined by TLC in several solvent systems and, except for metabolite **26**, their chromatographic behavior was compared with that of synthetic material. In the following paragraphs are described the chromatographic systems used for the preparative and analytical (kinetic) experiments. Unless otherwise indicated, preparative TLC was carried out with Quantum PQF 500 silica gel plates, and Eastman 0.1-mm silica gel plates, No. 13181 (with fluorescent indicator), were used in the quantitative (kinetic) experiments. The metabolites and unmetabolized substrates were visualized under 254-nm light.

A. Metabolism of *trans*-4′-Cyano-4-acetamidostilbene (2). The preparative TLC plate was developed twice with ethyl acetate and the metabolite bands were eluted with $CHCl_3$. The analytical TLC plates were developed with ethyl acetate. R_f values: **2** = 0.45; **14** = 0.33; **23** = 0.25.

B. Metabolism of *trans*-4′-*tert*-Butyl-4-acetamidostilbene (3). The preparative TLC plate was developed once with benzene and once with $CHCl_3$–CH_3OH–NH_4OH (97.5:2.0:0.5). The metabolite bands were eluted with ethyl acetate. The analytical TLC plates were developed with $CHCl_3$–CH_3OH–NH_4OH (97.5:2.0:0.5). R_f values: **3** = 0.65; **26** = 0.46; **15** = 0.30.

C. Metabolism of *trans*-4′-Methyl-4-acetamidostilbene (4). The preparative TLC plate was developed once with benzene and once with $CHCl_3$–CH_3CN (75:25). The metabolite bands were eluted with ethyl acetate. The hydroxymethyl metabolite **22** was also purified using a 1.5-mm aluminum oxide TLC plate (EM 5756). The plate was developed once with benzene and once with 2-butanone; the metabolite band was extracted with ethyl acetate. The hydroxamic acid metabolite **16** was quantified using the silica gel analytical TLC plates which were developed with CH_3CN–$CHCl_3$–$N(C_2H_5)_3$ (49:49:2). R_f values: **16** = 0.30; **22** = 0.47; **4** = 0.58. Because metabolite **22** had an R_f value similar to that of the internal standard in the above system, it was quantified using alumina analytical TLC plates (Eastman 6063) which were developed with 2-butanone–ethanol (97.5:2.5). R_f values: **16** = 0; **22** = 0.43; **4** = 0.60.

D. Metabolism of *trans*-4′-(Trifluoromethyl)-4-acetamidostilbene (5). The preparative TLC plate was developed with ethyl acetate and the metabolite band was eluted with $CHCl_3$. The analytical plates were developed with ethyl acetate. R_f values: **17** (synthetic standard) = 0.28; **5** = 0.45.

Mass Spectral Analysis. The mass spectra of the metabolites isolated from the preparative TLC plates were obtained through the Mass Spectrometry Laboratory, Department of Chemistry, University of Minnesota, using an Associated Electronic Industries (AEI) MS 30/PDP 8E mass spectrometer data system. Spectra were run in the electron-impact mode at 70 eV and a probe temperature of 150 °C; samples were introduced by direct inlet.

Quantitative Methods. Unaltered substrate and N-hydroxylated metabolite were quantified by a dual-label radiochemical assay. A constant amount (2.5 μmol) of an internal standard, 4′-chloro-4-[^3H]acetamidostilbene, was added to each sample immediately after the metabolic reaction was stopped. An aliquot (0.1 mL) of the ether layer was applied to a 0.1-mm silica gel TLC plate, which was then developed in the appropriate solvent system. The areas corresponding to the internal standard, unmetabolized substrate, and metabolites were cut out and placed in scintillation vials containing 8 mL of Econofluor (NEN). The disintegrations per minute of ^{14}C or ^3H present in each sample were determined using a Beckman LS-100 liquid scintillation counter. For each compound a standard curve was constructed by adding known quantities of the ^{14}C-labeled amide (0.1 to 7.5 μmol) or the ^{14}C-labeled metabolite (1 to 31 nmol) and internal standard to incubation mixtures containing heat-inactivated microsomes followed by extraction and chromatography as described above. Curves were constructed by plotting the ratio of amide or metabolite to internal standard recovered vs. amount added. Average recoveries of the acetamidostilbenes by this procedure were as follows: **2**, 99%; **3**, 99%; **4**, 99%; **5**, 71%. Average recoveries of the metabolites were as follows: **14**, 83%; **15**, 76%; **16**, 59%; **22**, 23%. It was established that under the conditions of the assay no exchange of ^3H from the internal standard occurred.

Kinetics of Metabolism. The apparent kinetic constants were determined by the method of Wilkinson,[30] with use of a BASIC program.

Chemical Syntheses. Melting points were determined with a Mel-Temp capillary melting point apparatus and are uncorrected. Infrared spectra were obtained with a Perkin-Elmer 237-B spectrophotometer, and ultraviolet spectra were measured with Beckman DB-GT and Beckman Model 24 spectrophotometers. Mass spectra were determined in the manner described above for the metabolites. For each compound the mass spectrum, infrared spectrum, and ultraviolet spectrum were consistent with the assigned structure. Elemental analyses were performed by Midwest Microlab, Inc., Indianapolis, IN. Radiolabeled acetic anhydride (^3H and ^{14}C) and [^{14}C]acetyl chloride were purchased from New England Nuclear (Boston, MA) and ICN (Irvine, CA).

***trans*-4′-Substituted-4-nitrostilbenes (6–9 and 18; Table II).** Diethyl 4-nitrobenzylphosphonate was prepared as previously described.[28] To a solution of 0.134 mol of sodium ethoxide in 125 mL of absolute ethanol was added 0.056 mol of diethyl 4-nitrobenzylphosphonate. The appropriate 4-substituted benzaldehyde (0.056 mol) in 125 mL of absolute ethanol was added dropwise over 30 min with constant stirring. The mixture was stirred at room temperature for 30 min and then was cooled to 0 °C. The crude *trans*-4′-substituted-4-nitrostilbene was collected by filtration, washed with water, and recrystallized from ethanol. Compound **18** was recrystallized from glacial acetic acid.

***trans*-4′-Carbethoxy-4-nitrostilbene (19).** A solution of 0.27 g (1.0 mmol) of **18** was dissolved in 650 mL of absolute ethanol along with 3 mL of concentrated H_2SO_4. The solution was heated under reflux for 24 h and the solvent was evaporated. Addition

(28) A. Yamaguchi and O. Mitsuo, *Nippon Kagaku Zasshi*, **91**, 390 (1970); *Chem. Abstr.*, **73**, 45566*n* (1970).

(29) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

(30) G. N. Wilkinson, *Biochem. J.*, **80**, 324 (1961).

of water to the residue afforded a yellow precipitate, which was collected by filtration and was recrystallized from ethyl acetate–ethanol (1:3) to yield 19 (Table II) which was used without further purification.

***trans*-4′-(Hydroxymethyl)-4-nitrostilbene (20).** A mixture of $CaCl_2$ (0.159 g, 1.41 mmol) and $NaBH_4$ (0.109 g, 2.86 mmol) in dry THF (60 mL) was stirred at room temperature for 1 h. A solution of 19 (0.28 g, 0.955 mmol) in 30 mL of dry THF was added in one portion, and the mixture was stirred at room temperature for 18 h, cooled in an ice bath, and 3.5 mL of water was added dropwise. The solution was acidified to pH 1.5 by the dropwise addition of cold 6 N HCl, and the resulting mixture was stirred at room temperature for 1 h. The THF was evaporated to afford 0.2 g of a solid material, which was purified by column chromatography on silica gel. Elution with $CHCl_3$ yielded 20 (Table II).

***trans*-4′-Substituted-4-aminostilbenes (10–13 and 21; Table II).** A solution of 0.17 mol of $SnCl_2$ in 40 mL of concentrated HCl was added dropwise to a solution of 0.02 mol of the *trans*-4′-substituted-4-nitrostilbene in 125 mL of glacial acetic acid with constant stirring (20 was dissolved in warm THF) and the reaction mixture was stirred overnight at room temperature. A precipitate formed which was collected by filtration, washed with 50 mL of glacial acetic acid, and suspended in 1.4 L of H_2O. The aqueous suspension was adjusted to pH 9–10 with NaOH pellets and was extracted with $CHCl_3$. The combined $CHCl_3$ extracts were washed with 150 mL of water and dried over $MgSO_4$, and the $CHCl_3$ was evaporated under reduced pressure. Compounds 10, 11, and 13 were recrystallized from ethanol, while compounds 12 and 21 were recrystallized from hexane and benzene, respectively.

***trans*-4′-Substituted-4-acetamidostilbenes (2–5 and 22; Table I).** The appropriate *trans*-4′-substituted-4-aminostilbene (0.5 mmol) was dissolved in 50 mL of benzene. Acetic anhydride (0.5 mmol) was added and the reaction mixture was stirred overnight at room temperature. The precipitate was collected by filtration and recrystallized to a constant melting point, constant specific activity, and homogeneity by TLC.

***trans*-4′-Substituted-4-(*N*-hydroxyacetamido)stilbenes (14–17; Table III).** The appropriate *trans*-4′-substituted-4-nitrostilbene (0.01 mol) was dissolved in 80 mL of DMF, 40 mL of ethanol, and 20 mL of water. Ammonium chloride (0.04 mol) and zinc dust (0.04 mol) were added and the mixture was stirred for 1 h at room temperature. The reaction mixture was filtered and the filter cake was washed with two 50-mL portions of DMF. The washes and the filtrate were combined and poured into 30 mL of water. The hydroxylamine was extracted from the aqueous phase with three 100-mL portions of ether, which were combined and washed with three 50-mL portions of saturated NaCl. The ether solution was cooled to 0 °C and 5 mL of saturated sodium bicarbonate was added. Acetyl chloride (0.01 mol) in 25 mL of ether was added dropwise over 20 min to the reaction mixture. The mixture was stirred for 30 min and the ether was removed under reduced pressure. The residue was stirred with 150 mL of saturated sodium bicarbonate solution for 1 h at 0 °C. The hydroxamic acid was collected by filtration, washed with 100 mL of water, and recrystallized from benzene. The ^{14}C-labeled hydroxamic acids were synthesized using 0.05 mmol of the nitrostilbene and 0.001 mCi of [1-^{14}C]acetyl chloride. The products were recrystallized to constant melting points, constant specific activity, and homogeneity by TLC.

***trans*-4′-Substituted-4-(2-hydroxyacetamido)stilbenes (23–25; Table III).** The appropriate *trans*-4′-substituted-4-aminostilbene (1.0 mmol) was dissolved in 15 mL of dry benzene along with 10 mmol of ethyl glycolate. The solution was heated under reflux for 42 h, cooled to room temperature, and 25 mL of petroleum ether was added. The glycolamide was collected by filtration and was recrystallized from benzene.

Acknowledgment. The authors express their gratitude to Professor M. W. Anders for his advice and encouragement. This research was supported in part by American Cancer Society Grant BC-190. Richard E. Gammans was the recipient of NIH postdoctoral fellowship CA 05746.

Resolution, Absolute Configuration, and Antiarrhythmic Properties of the Enantiomers of Disopyramide, 4-(Diisopropylamino)-2-(2-pyridyl)-2-phenylbutyramide

Terrence R. Burke, Jr., Wendel L. Nelson,*

Department of Medicinal Chemistry, School of Pharmacy, University of Washington, Seattle, Washington 98195

Margaret Mangion, Gilbert J. Hite,*

Section of Medicinal Chemistry and Pharmacognosy, School of Pharmacy and Institute of Materials Science, University of Connecticut, Storrs, Connecticut 06268

Corwin M. Mokler, and Peter C. Ruenitz

Departments of Pharmacology and Medicinal Chemistry, School of Pharmacy, University of Georgia, Athens, Georgia 30602.
Received April 7, 1980

Disopyramide was resolved by fractional crystallization of its diastereomeric bitartrate salts from methanol–acetone. Diastereomeric sulfonamides prepared from (+)-camphor-10-sulfonyl chloride and the primary amines obtained by $LiAlH_4$ reduction of the resolved bases were separable by high-performance LC and were used to show that within experimental error resolution of disopyramide was complete. The absolute configuration was determined by X-ray crystallography. (+)-[(2*R*)-(−)-4-(Diisopropylamino)-2-(2-pyridyl)-2-phenylbutyramide (+)-(2*R*,3*R*)-bitartrate] crystallizes in space group $P2_12_12_1$: a = 14.789 (4), b = 18.151 (4), c = 9.878 (2) Å; Z = 4; D_x = 1.225, D_m (flotation C_6H_6/CCl_4) = 1.226 g cm^{-3}. The structure was solved by direct methods. The enantiomeric bitartrates were tested for antiarrhythmic properties. The enantiomeric bitartrate salts were equally effective in prolonging the effective refractory period (ERP) of rabbit ventricle. At 3×10^{-6} M, the (−)-bitartrate [from (2*S*)-(+)-disopyramide] increased the ERP 21.8 ± 6.3 ms and the (+)-bitartrate [from (2*R*)-(−)-disopyramide] increased the ERP 25.8 ± 3.6 ms. At 1.5×10^{-5} M no significant difference was noted, as the increases in the ERP were 41.2 ± 8.9 and 50.5 ± 6.3 ms for the (−)- and (+)-bitartrate, respectively.

Disopyramide [1; 4-(diisopropylamino)-2-(2-pyridyl)-2-phenylbutyramide][1] is a recently available, orally active, quinidine-like agent used in the treatment of cardiac dysrhythmias.[2,3] Interest in its pharmacokinetic properties

0022-2623/80/1823-1044$01.00/0