Oxidation of 4-Aryl- and 4-Alkyl-Substituted 2,6-Dimethyl-3,5-bis(alkoxycarbonyl)-1,4-dihydropyridines by Human Liver Microsomes and Immunochemical Evidence for the Involvement of a Form of Cytochrome P-450

Ronald H. Böcker† and F. Peter Guengerich*‡

Department of Biochemistry and Center in Molecular Toxicology, Vanderbilt University, Nashville, Tennessee 37232.
Received December 3, 1985

4-Substituted 2,6-dimethyl-3,5-bis(alkoxycarbonyl)-1,4-dihydropyridines are important because of their roles as calcium channel blockers. The mixed-function oxidation of 14 4-aryl- and four 4-alkyl-substituted derivatives by human liver microsomes was examined. The major product of enzymatic oxidation of all the 4-aryl compounds was the pyridine derivative containing the 4-aryl group. The 4-alkyl compounds, in contrast, formed a pyridine derivative in which a hydrogen atom was present at the 4-position and the alkyl group was lost; these compounds also inactivated cytochrome P-450 and caused the loss of nifedipine oxidase activity after enzymatic oxidation. All of these reactions were extensively inhibited by an antibody raised to purified human liver nifedipine oxidase cytochrome P-450 ($P\text{-}450_{NF}$), indicating a major role for this enzyme in the oxidation of these compounds. Oxidation of the 4-alkyl compounds led not only to the loss of $P\text{-}450_{NF}$ but also to decreases in catalytic activities of cytochrome P-450 isozymes catalyzing other reactions (phenacetin O-deethylation and hexobarbital 3'-hydroxylation). The results indicate that $P\text{-}450_{NF}$ (or closely related enzyme forms) is responsible for the oxidation of these nifedipine-related compounds in human liver microsomes and that metabolism is highly dependent upon 4-substitution; with alkyl substituents, radicals are postulated to leave $P\text{-}450_{NF}$ to attack other proteins.

Nifedipine (**1a**)[1] and other related dihydropyridine compounds have been widely used in the treatment of hypertension and angina pectoris.[2] These compounds bind to specific receptors and are Ca^{2+} channel blockers.[3] The use of these compounds is generally beneficial, although undesired side effects have been noted in some individuals.[4]

Nifedipine undergoes first pass metabolism in the liver to the 1,4-dehydro derivative, which can be measured in plasma.[5] Further metabolism involves hydrolysis of an ester group (ring 3-position), hydroxylation of the 2-methyl group, and formation of the lactone from these latter two moities.[6] These oxidized compounds are largely devoid of the pharmacological activity of the parent compound. Kleinbloesem et al.[7] reported that one-sixth of the Dutch Caucasian subjects in an in vivo metabolism study segregated as phenotypic slow metabolizers, and a genetic deficiency was suggested.[8] Moreover, the slow metabolizers also showed hypotensive effects of nidefipine for longer periods that did the normal subjects.[7,8]

A number of enzymes with oxidoreductase capability might be suspected of oxidizing nifedipine and other dihydropyridines. We found that rat and human liver microsomes catalyze the NADPH-dependent mixed-function oxidation of nifedipine and isolated the forms of rat and human cytochrome P-450 that catalyze the reaction.[9] Antibodies raised against this cytochrome P-450 inhibited >90% of human liver microsomal NADPH-dependent nifedipine oxidase activity. Cytochrome b_5 also appears to be involved in the reaction, probably by facilitating electron transfer to oxyferrous cytochrome P-450. Immunochemical studies also suggest that this enzyme, termed $P\text{-}450_{NF}$ because of its activity toward nifedipine, is also largely responsible for aldrin epoxidation, *d*-benzphetamine N-demethylation, 17β-estradiol 2- and 4-hydroxylation, and the 6β-hydroxylation of testosterone.[9] In this report we consider the catalytic activity of human liver cytochrome P-450 toward other pharmacologically active dihydropyridines of varying structure.

Synthesis

The 1,4-dihydropyridine compounds used were either supplied by various individuals or synthesized via the Hantzsch method by condensation of the appropriate aldehyde, NH_4OH, and methyl or ethyl acetoacetate.[10] Three of these compounds appear to be new, and physical properties are presented in Table I. With all of the 1,4-dihydro compounds we examined, the UV spectra showed a broad peak near 340 nm (Table I).

Oxidation of 1,4-dihydro-2,6-dimethyl-4-phenyl-3,5-pyridinedicarboxylic acid diethyl ester (**13a**) was compared with use of HNO_3,[11] HNO_2,[12] and *p*-chloranil.[13] HNO_3 oxidation was most satisfactory of the three methods and was used for all of the syntheses. Although the Hantzsch synthesis was first published over a century ago,[10] many

† On leave from the Institute für Pharmakologie and Toxikologie, Lehrstuhl für Toxikologie, Universität Erlangen-Nürnberg 22, D-8520 Erlangen, Federal Republic of Germany.
‡ Burroughs-Wellcome Scholar in Toxicology, 1983-1988.

(1) Throughout the paper **a** refers to the 1,4-dihydropyridine derivative of each pyridine, which is designated **b**.
(2) Flaim, S. F.; Zelis, R. *Fed. Proc.* **1981**, *40*, 2877.
(3) Triggle, D. J.; Janis, R. A. In *Modern Methods in Pharmacology*; Spectro, S., Back, N., Eds.; Alan R. Liss: New York, 1984; Vol. II, p 1.
(4) Jariwalla, A. G.; Anderson, E. G. *Br. Med. J.* **1978**, *1*, 1181. Rodger, C.; Stewart, A. *Br. Med. J.* **1978**, *1*, 1619. Boden, W. E.; Korr, K. S.; Bough, E. W. *JAMA, J. Am. Med. Assoc.* **1985**, *253*, 1131.
(5) Walter, D. G.; Renwick, A. G.; Gruchy, B. S.; George, C. R. *Br. J. Clin. Pharmacol.* **1984**, *18*, 951.
(6) Raemsch, K. D.; Sommer, J. *Hypertension (Suppl. II)* **1983**, *5*, 18.
(7) Kleinbloesem, C. H.; van Brummelen, P.; Faber, H.; Danhof, M.; Vermeulen, N. P. E.; Breimer, D. D. *Biochem. Pharmacol.* **1984**, *33*, 3721.
(8) Kleinbloesem, C. H. Doctoral Thesis, University of Leiden, 1984.
(9) Guengerich, F. P.; Martin, M. V.; Beaune, P. H.; Kremers, P.; Wolff, T.; Waxman, D. J. *J. Biol. Chem.* **1986**, *261*, 5051.
(10) Hantzsch, A. *Ann.* **1882**, *215*, 1. Hantzsch, A. *Ann.* **1882**, *215*, 72. Hantzsch, A. *Ber.* **1885**, *18*, 1744. Hantzsch, A. *Ber.* **1886**, *19*, 289.
(11) Chennot, T.; Eisner, U. *J. Chem. Soc., Perkin Trans. 1* **1975**, 926.
(12) Loev, B.; Snader, K. M. *J. Org. Chem.* **1965**, *30*, 1914.
(13) Braude, E.; Hannah, J.; Linstead, R. *J. Chem. Soc.* **1960**, 3249.

0022-2623/86/1829-1596$01.50/0

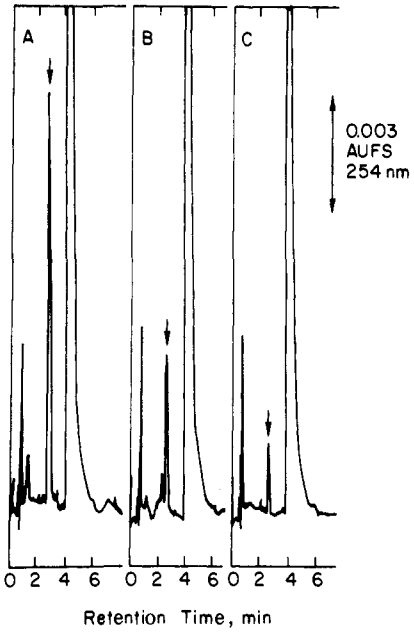


Figure 1. Effect of anti-P-450$_{NF}$ on the oxidation of **9a**. Assays were done as described in the Experimental Section. HPLC chromatograms are shown for incubations (human liver microsomal preparation HL 94) done in the presence of (A) 10 mg of preimmune antibody/nmol of microsomal cytochrome P-450, (B) 5 mg of anti-P-450$_{NF}$/nmol of microsomal cytochrome P-450, and (C) 10 mg of anti-P-450$_{NF}$/nmol of microsomal cytochrome P-450, with preincubation at 23 °C for 20 min in every case. The large peak eluting with a retention time of 4–4.5 min is residual **9a**, and the earlier peak marked with an arrow in each case is the 1,4-dehydro product **9b**.

of the pyridines we synthesized appear to be new compounds, and their physical properties are listed in Table I. All of the pyridines yielded a single HPLC peak in the appropriate solvent system (Table II). Compounds **16b**, **17b**, and **18b** did not crystallize readily, and no melting point data have been reported previously;[14-18] these pyridines were identified by their spectral properties.

The dimethyl esters **2a**, **3a**, and **4a** all lost the 4-alkyl substituent upon oxidation, even when chloranil was used.[13] The product was identified as compound **5b** as judged by the EI MS, HPLC analysis, and UV spectroscopy.

Biological Results

Oxidation of 4-Aryl-Substituted Dihydropyridines (1a, 6a, 7a, 8a, 9a, 10a, 11a, 12a, 13a, 14a, 16a, 17a, 18a, 19a). The oxidation of 14 different 4-aryl-substituted dihydropyridines was examined in liver microsomal preparations prepared from three different individual humans (Table II). In every case the only major product detected was the pyridine compound containing the 4-aryl substituent (compound designated **b**). Some variation in the rates of oxidation among the structures and the microsomal samples was found, although all rates were within 1 order of magnitude.

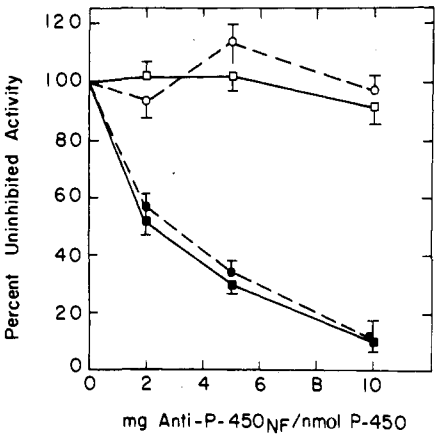


Figure 2. Inhibition of human liver microsomal oxidation of **1a** and **11a** by varying amounts of anti-P-450$_{NF}$. Microsomes (samples HL 95 and HL 96) were incubated with the indicated amounts of preimmune antibody (○) or anti-P-450$_{NF}$ (●) for 20 min at 23 °C, and then the oxidation of **11a** was measured as described under the Experimental Section. Similar assays were done with **1a** in the presenceof preimmune antibody (□) or anti-P-450$_{NF}$ (■). Duplicate assays were done with each microsomal preparation, and the data reflect the mean ± SD (range) of the data for the two samples.

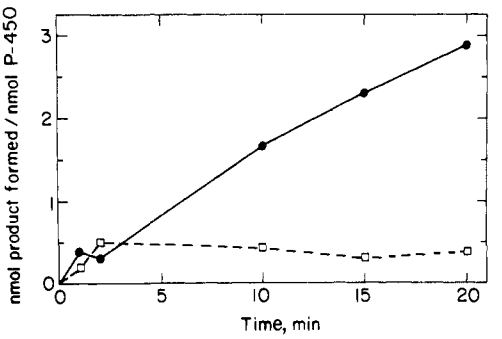


Figure 3. Oxidation of **3a** to **5b** by microsomes. Human liver microsomes (sample HL 94) were incubated with 0.6 mM **3a** and an NADPH-generating system at 37 °C for varying amounts of time in the presence (●) or presence (□) of anti-P-450$_{NF}$ (10 mg/nmol of cytochrome P-450). The data points corrected for the amount of **5b** formed in similar incubations devoid of NADPH, which was about 10% of that formed in the absence of antibody here.

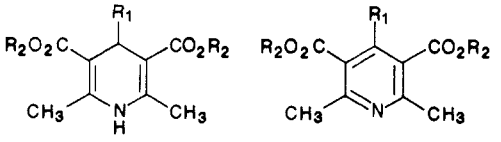
Anti-P-450$_{NF}$ was found to inhibit the oxidation of **9a** and **11a** nearly completely when added at a ratio of 10 mg of immunoglobulin G fraction protein/nmol cytochrome P-450 (Figures 1 and 2). The same amount of anti-P-450$_{NF}$ extensively inhibited the oxidation of all of the 4-alkyl-substituted dihydropyridines examined (Table II).

Oxidation of 4-Alkyl-Substituted Dihydropyridines (2a, 3a, 4a, 15a). The oxidation of these compounds was more complex than in the case of the 4-aryl-substituted compounds. **2a** and **3a** were oxidized to form mainly 2,6-dimethyl-3,5-pyridinedicarboxylic acid dimethyl ester (compound **5b**) with loss of the 4-alkyl substituent. Compound **4a** was not separated from the putative oxidation product **5b** with the HPLC conditions we tried. Some additional products were also formed from compounds **2a** and **3a** but were not identified.

The formation of **5b** from the 4-alkyldihydropyridines **2a** and **3a** was observed, but significant levels of oxidation to form **5b** also occurred in the absence of NADPH. This oxidation was dependent upon the presence of microsomal protein and varied with the particular microsomal sample that was used. The fraction of total oxidation to **5b** due to the NADPH-independent reaction varied from 10% to

(14) Meyer, H. German Patent DE 3 209 276, 1984; *Chem. Abstr.* 1984, *100*, P22580z.
(15) Meyer, H.; Wehinger, E.; Bossert, F.; Scherling, D. *Arzneim-Forsch.* 1983, *33*, 106.
(16) Krol, G. J.; Noe, A. J.; Yeh, S. C.; Raemsch, K. D. *J. Chromatogr.* 1984, *305*, 105.
(17) Janis, R. A.; Krol, G. J.; Noe, A. J..; Pan, M. *J. Clin. Pharmacol.* 1983, *23*, 266.
(18) Eiden, F.; Braatz-Greeske, K. *Dtsch. Apoth.-Ztg.* 1983, *123*, 2003.

Table I. New 2,6-Dimethyl-3,5-bis(alkoxycarbonyl)-1,4-dihydropyridines and Pyridines Used in This Work



a b

no.	R_1	R_2	mp, °C	yield, %	formula (anal.)	MS (rel abund), *m/z*	NMR, δ	UV, λ_{max}, nm (ϵ, mM^{-1} cm^{-1})
2a	cyclohex-3-enyl	CH_3	179–180	84	$C_{17}H_{23}NO_4$ (C, H, N)	CI: 334 (M + 29, 14), 306 (M + H, 12), 304 (4), 275 (17), 274 (91), 225 (11), 224 (100)	1.19 (1 H, m), 1.90 (6 H, m), 2.32 (6 H, s, CCH_3), 3.72 (6 H, s, CO_2CH_3), 4.01 (1 H, d), 5.58 (1 H, s, NH), 5.62 (2 H, m)	340, 274, 235 (ϵ_{340} 9.75, ϵ_{262} 20.3, ϵ_{241} 20.5)
3a	$CH_2C_6H_5$	CH_3	157–158	68	$C_{18}H_{21}NO_4$ (C, H, N)	CI: 344 (M + 29, 13), 317 (2), 316 (M + H, 7), 314 (4), 285 (14), 284 (100), 225 (14), 224 (96)	2.18 (6 H, s, CCH_3), 2.58 (2 H, d, CH_2), 3.61 (6 H, s, CO_2CH_3), 4.19 (1 H, t), 5.20 (1 H, s, NH), 7.02 (2 H, d), 7.17 (3 H, m)	348, 276, 233 (ϵ_{348} 9.75, ϵ_{260} 23.5, ϵ_{240} 24.0)
4a	$CH(CH_3)_2$	CH_3	165–166	77	$C_{14}H_{21}NO_4$ (C, H, N)	CI: 296 (M + 29, 10), 268 (M + H, 4), 266 (4), 237 (16), 236 (100), 224 (54)	0.76 (6 H, d, $C(CH_3)_2$), 1.60 (1 H, m), 2.31 (6 H, s, CCH_3), 3.72 (6 H, s, CO_2CH_3), 3.90 (1 H), d), 5.54 (1 H, s, NH)	338, 272, 258, 232 (ϵ_{338} 8.50, ϵ_{260} 23.3, ϵ_{240} 22.8)
5a	H	CH_3	222–224	45	$C_{11}H_{15}NO_4$ (H, N; C[a])	EI: 225 (M, 9), 224 (M − 1), 14), 210 (100), 194 (41)	2.21 (6 H, s, CCH_3), 3.28 (2 H, s), 3.71 (6 H, s, CO_2CH_3), 5.14 (1 H, s, NH)	368, 271, 258, 226 (ϵ_{368} 8.75, ϵ_{257} 24.7, ϵ_{231} 22.2)
5b	H	CH_3	100–101	14	$C_{11}H_{13}NO_4$ (C, H, N)	CI: 225 (11), 224 (M + H, 100), 192 (8). EI: 224 (19), 223 (M, 85), 193 (11), 192 (100), 191 (38), 165 (10), 164 (28)	2.87 (6 H, s CCH_3), 3.94 (6 H, s, CO_2CH_3), 8.72 (1 H, s)	279, 270, 233 (ϵ_{270} 26.0)
6b	3-NO_2-4-ClC_6H_4	CH_3	121–122	68	$C_{17}H_{15}N_2O_6Cl$ (C, H, N)	EI: 380 (M, 21), 378 (M, 76), 363 (36), 361 (100), 349 (23), 348 (34), 347 (66), 346 (54), 333 (25), 331 (75)	2.62 (6 H, s, CCH_3), 3.67 (6 H, s, CO_2CH_3), 7.40 (1 H, dd), 7.58 (1 H, d), 7.80 (1 H, d)	277, 227 (ϵ_{266} 29.0)
7b	3-CNC_6H_4	CH_3	121–121.5	61	$C_{18}H_{16}N_2O_4$ (H, N; C[b])	EI: 325 (14), 324 (M, 76), 294 (17), 293 (100), 292 (22), 261 (21)	2.62 (6 H, s, CCH_3), 3.57 (6 H, s, CO_2CH_3), 7.51 (3 H, m), 7.68 (1 H, d)	270, 230, 221 (ϵ_{270} 27.2)
8b	4-CH_3-C_6H_4	CH_3	90–91	46	$C_{18}H_{19}NO_4$ (H, N; C[c])	EI: 314 (18), 313 (M, 100), 282 (32), 281 (22), 253 (18), 250 (69), 249 (36)	2.38 (3 H, s, toluene CH_3), 2.58 (6 H, s, CCH_3), 3.57 (6 H, s, CO_2CH_3), 7.16 (4 H, dd)	268, 230 (ϵ_{268} 32.5)
9b	2-Cl-6-FC_6H_3	CH_3	98–99	82	$C_{17}H_{15}NO_4FCl$ (C, H, N)	EI: 353 (M, 1), 351 (M, 3), 320 (11), 317 (17), 316 (100)	2.67 (6 H, s, CCH_3), 3.58 (6 H, s, CO_2CH_3), 7.06 (1 H, m), 7.28 (2 H, m)	278, 268, 233 (ϵ_{271} 30.0)
10b	4-CF_3-C_6H_4	CH_3	77–77.5	60	$C_{18}H_{16}NO_4F_3$ (C, H, N)	EI: 368 (18), 367 (M, 100), 337 (17), 336 (100), 335 (18), 304 (43)	2.62 (6 H, s, CCH_3), 3.54 (6 H, s, CO_2CH_3), 7.38 (2 H, d), 7.67 (2 H, d)	278, 268, 256, 226 (ϵ_{270} 27.5)
11b	2,3,5,6-F_4C_6H	CH_3	87–88	67	$C_{17}H_{13}NO_4F_4$ (C, H, N)	EI: 372 (13), 371 (M, 76), 341 (15), 340 (100), 339 (21)	2.68 (6 H, s, CCH_3), 3.69 (6 H, s, CO_2CH_3), 7.13 (1 H, m)	279, 269, 257 (ϵ_{272} 30.5)
12b	3-ClC_6H_4	CH_3	114–114.5	66	$C_{17}H_{16}NO_4Cl$ (H, N; C[d])	EI: 336 (5), 335 (M, 34), 334 (17), 333 (100), 304 (22), 303 (25), 302 (68), 301 (48), 272 (22), 270 (55)	2.60 (6 H, s, CCH_3), 3.58 (6 H, s, CO_2CH_3), 7.13 (1 H, d), 7.34 (3 H, m)	278, 268, 258 (ϵ_{271} 29.2)
14b	C_6H_5	CH_3	135–136	82	$C_{17}H_{17}NO_4$ (H, N; C[e])	EI: 300 (16), 299 (M, 100), 268 (47), 267 (31), 239 (22), 236 (64), 235 (25)	2.60 (6 H, s, CCH_3), 3.53 (6 H, s, CO_2CH_3), 7.26 (3 H, m), 7.37 (2 H, m)	277, 268 (ϵ_{268} 28.2)

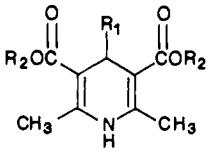
[a] C: calcd, 58.67; found, 59.95. [b] C: calcd, 66.67; found, 66.20. [c] C: calcd, 69.01; found, 66.92. [d] C: calcd, 61.17; found, 59.95. [e] C: calcd, 68.23; found, 66.40.

as much as 50%. More rigorous attempts to exclude light during incubation and extraction did not reduce the extent of the NADPH-independent reaction. (No appreciable reaction was observed with any of the 4-aryl-substituted compounds in the absence of NADPH.) The concentration of spectrally detectable cytochrome P-450 was not reduced when microsomes were incubated with **2a** or **3a** in the absence of NADPH. The NADPH-independent reaction occurred with liver microsomes regardless of whether or not the preparations were made in the presence of butylated hydroxytoluene to inhibit lipid peroxidation.[19]

The portion of the oxidation of compound **3a** to **5b**, which was attributed to the NADPH-dependent pathway,

(19) Welton, A. F.; Aust, S. D. *Biochem. Biophys. Res. Commun.* **1972**, *49*, 661.

Table II. Oxidation of Substituted Dihydropyridines to Pyridines by Human Liver Microsomes



no.	R_1	R_2	R_3	HPLC method	HPLC ret time, min: major product	HPLC ret time, min: substr	rate of oxidn, mean (nmol product formed/min per nmol cytochrome P-450) (range)[a]	fraction inhibn, %, by anti-P-450$_{NF}$ (mean, range)[b]
1a	2-NO_2-C_6H_5	CH_3 (nifedipine)	CH_3	1	1.9	3.4	1.4 (1.1–2.18)	88 (85–92)
2a	3-cyclohexenyl	CH_3	CH_3	1	3.0	7.2	0.12 (0.06–0.18)	*c*
3a	$CH_2C_6H_5$	CH_3	CH_3	1	3.0	5.1	0.25 (0.23–0.27)	90[d]
4a	$CH(CH_3)_2$	CH_3	CH_3				*e*	*c*
6a	3-NO_2-4-ClC_6H_3	CH_3	CH_3	2	9.9	12.2	0.61 (0.46–0.69)	91 (89–93)
7a	3-CNC_6H_4	CH_3	CH_3	2	5.3	5.9	0.41 (0.31–0.48)	91 (90–93)
8a	4-CH_3-C_6H_4	CH_3	CH_3	3	6.2	5.2	0.51 (0.47–0.53)	93 (86–94)
9a	2-Cl-6-FC_6H_3	CH_3	CH_3	1	2.7	4.2	0.76 (0.57–0.94)	88 (83–91)
10a	4-CF_3-C_6H_4	CH_3	CH_3	2	8.2	9.3	0.45 (0.38–0.51)	79 (68–90)
11a	2,3,5,6-F_4C_6H	CH_3	CH_3	1	3.8	5.2	0.98 (0.59–1.51)	87 (76–94)
12a	3-ClC_6H_4	CH_3	CH_3	3	8.5	7.3	0.50 (0.44–0.61)	86 (85–87)
13a	C_6H_5	CH_2CH_3	CH_2CH_3	3	10	7.8	0.51 (0.41–0.59)	73 (65–77)
14a	C_6H_5	CH_3	CH_3	3	4.3	3.6	0.53 (0.46–0.58)	90 (85–94)
15a	CH_2CH_3	CH_2CH_3	CH_2CH_3	1	3.0	3.1	0.12 (0.05–0.16)	*c*
16a	2-NO_2-C_6H_4	$CH_2CH(CH_3)_2$ (nisoldipine)	CH_3	1	2.3	3.0	0.90 (0.47–1.38)	88 (84–91)
17a	3-NO_2-C_6H_4	$CH(CH_3)_2$ (nimodipine)	CH_2CH_2-OCH_3	3	5.6	4.8	0.55 (0.44–0.69)	85 (79–91)
18a	3-NO_2-C_6H_4	$CH_2CH_2C_3H_7$ (niludipine)	CH_2CH_2-OC_3H_7	4	5.8	4.6	0.67 (0.61–0.74)	80 (71–86)
19a	3-NO_2-C_6H_4	CH_3 (nitrendipine)	CH_2CH_3	2	8.9	9.8	0.52 (0.35–0.61)	83 (78–86)

[a] Mean and range of assays done with human liver microsomal samples HL 94, HL 95, and HL 96. [b] Mean and range of inhibition of activity of human liver microsomal samples HL 94, HL 95, and HL 96 observed with 10 mg of anti-P-450$_{NF}$/nmol of microsomal cytochrome P-450. [c] Not determined. [d] Inhibition experiments were only done with liver sample HL 94. [e] The product was not separated from the substrate with any of the HPLC systems.

could be nearly completely inhibited by anti-P-450$_{NF}$ (Figure 3).

Inactivation of Cytochromes P-450 Related to Oxidation of Dihydropyridines. Oxidation of any of the four of the 4-alkyl-substituted dihydropyridines led to the loss of nifedipine oxidase activity (Figure 4). Only a slight decrease in activity was observed after similar incubation with the 4-aryl-substituted compound **9a**, which would be expected to have similar properties as a competitive inhibitor, at least as judged by its apparent hydrophobicity. Incubation of the 4-alkyl compounds with microsomes in the absence of NADPH did not necessarily lead to a large extent of inhibition (Figure 4), nor was spectrally determined cytochrome P-450 decreased (vide supra).

Oxidation of any of the four 4-alkyl-substituted dihydropyridines also led to the loss of spectrally detectable cytochrome P-450 (Figure 5). The enzymatic destruction of cytochrome P-450 by compound **15a** could be partially blocked by anti-P-450$_{NF}$ (Figure 5). In other studies, the 4-aryl-substituted compound **9a** did not decrease spectrally detectable cytochrome P-450 when incubated with microsomes and NADPH.

The oxidation of compounds **2a**, **3a**, **4a**, and **15a** also caused the inactivation of hexobarbital 3′-hydroxylase and phenacetin *O*-deethylase activities (Table III) as well as nifedipine oxidase. Other experiments demonstrated that neither of these activities was inhibited by the same amount of anti-P-450$_{NF}$ that nearly completely blocked microsomal nifedipine oxidase activity (results not presented).

Table III. Inhibition of Cytochrome P-450 Mediated Reactions in Microsomes by Incubation with 4-Alkyl-Substituted Dihydropyridines[a]

	% inhibition of catalytic activity			
	nifedipine oxidase		phenacetin *O*-deethylase	hexobarbital 3′-hydroxylase
no.	NADPH absent	NADPH present	(NADPH present)	(NADPH present)
2a	27	86	59	82
3a	25	86	50	80
4a	32	89	55	73
15a	45	89	98[b]	82

[a] Human liver microsomes (sample HL 95) were incubated with each indicated compound (50 μM) for 20 min at 37 °C, and then either nifedipine (**1a**) (200 μM), phenacetin (12 μM), or hexobarbital (500 μM) was added and the respective catalytic activity was measured, as described under the Experimental Section. The results are corrected for the inhibition (<10%) observed in the presence of the solvent (CH_3OH) alone. [b] When the preincubation with **15a** (50 μM) was done in the absence of NADPH, the phenacetin *O*-deethylase activity was inhibited by 40%.

Since data were obtained for both rates of cytochrome P-450 inactivation and formation of the pyridine **5b**, partition ratios can be estimated.[20] If we use the data from

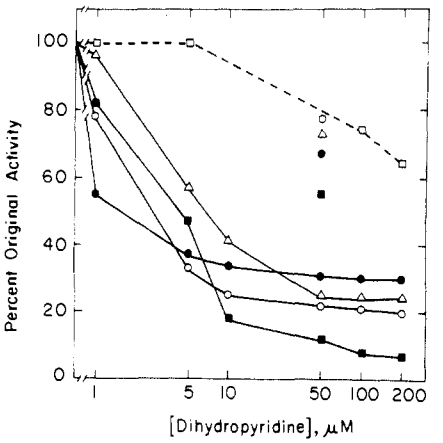


Figure 4. Inhibition of nifedipine oxidase activity by incubation of microsomes with various dihydropyridines. Human liver microsomes (sample HL 95, 500 pmol of cytochrome P-450 in 0.5 mL of 50 mM potassium phosphate buffer, pH 7.85) were incubated for 20 min at 37 °C in the presence of the NADPH-generating system and the indicated concentration of **2a** (△), **3a** (○), **4a** (●), **9a** (□), or **15a** (■). A 50-μL portion of the mixture was diluted 10-fold with 50 mM potassium phosphate buffer (pH 7.85) containing 200 μM **1a** and the NADPH-generating system, and nifedipine oxidase activity was determined. The single points (no connecting lines) at 50 μM show the effects of these compounds in the absence of the NADPH-generating system during the preincubation.

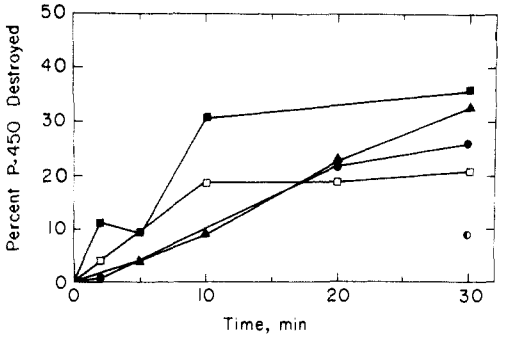


Figure 5. Loss of spectrally detectable cytochrome P-450 heme due to incubation with 4-alkyldihydropyridines. Human liver microsomes (sample HL 95) were incubated in the presence of **2a** (▲), **3a** (□), **4a** (●), or **15a** (■) with the NADPH-generating system for the indicated times. Cytochrome P-450 was determined spectrally using the ferrous carbonyl vs. ferrous difference method.[26] The point designated ◐ shows the percent loss of cytochrome P-450 observed with **15a** in the presence of anti-P-450$_{NF}$ (10 mg/nmol of cytochrome P-450).

Table II and Figure 5 to make calculations, the apparent partition ratios are roughly 10, 13, and 3 for **2a**, **3a**, and **15a**, respectively. These values seem to be relatively low and suggest that a significant fraction of the oxidation of 4-alkyl-substituted dihydropyridines is related to cytochrome P-450 inactivation, although the factors mentioned preclude precise definition of these parameters.

Discussion

We have previously reported that the oxidation of nifedipine is catalyzed by hepatic microsomal cytochromes P-450 and have identified the major forms involved in rat and human liver.[22] The extensive inhibition of microsomal oxidation by a highly specific antibody raised against the major human cytochrome P-450 involved in nifedipine oxidation, P-450$_{NF}$,[9] indicates that the oxidation of a wide variety of dihydropyridine compounds is catalyzed by the same form, or ones that are very closely related. Included in this group of compounds are several with varying carboxylic ester groups and various aryl or alkyl moieties at the 4-position. Nearly all of the 4-aryl substituted compounds considered here have been used as drugs or have been tested for pharmacological activity.[23] Kleinbloesem et al.[7] reported that one-sixth of the Dutch Caucasian population examined in their study was phenotypic poor metabolizers of nifedipine (although it has not been conclusively demonstrated that oxidation and not ester hydrolysis is limiting). These studies would suggest that (if oxidation is indeed the step involved in this apparent polymorphism) this same relatively large segment of the population might also exhibit impaired metabolism of all of these dihydropyridine compounds as well. Individuals deficient in activity show an unusually long duration of the hypotensive effect of nifedipine, and similar effects might also be expected with the other dihydropyridines as well.[7]

The rates of oxidation of the various 4-aryl-substituted dihydropyridines were rather similar when compared to each other; i.e., the 14 measured varied by a factor of 3 in spite of the presence of electron-donating and -withdrawing groups on the aryl moiety (Table II). The rates of oxidation of the 4-alkyl compounds were somewhat less. The alteration of the ester substituents did not seem to alter the oxidation rates, at least when a single high concentration of each substrate was used in the in vitro assays.

A postulated mechanism for the oxidation of the dihydropyridines by cytochrome P-450 is shown in Figure 6. Augusto et al.[24] postulated the mechanism shown for the 4-alkyl-substituted compounds on the basis of experiments in which an alkyl (ethyl) radical reacted with a porphyrin nitrogen or was trapped with nitrones. We have also identified and quantitated the pyridine compound (**5b**) that would be expected in such a mechanism. We also view the oxidation of the 4-aryl-substituted dihydropyridines as resulting from the same initial mechanism, one-electron oxidation of the nitrogen atom. Other evidence for such a generalized mechanism of amine oxidation[25] comes from the rearrangement of cyclopropylamines,[26] kinetic deuterium isotope effects,[27] and consid-

(20) A partition ratio for a mechanism-based enzyme inactivation is the ratio of the rate of formation of a stable product from the reactive intermediate divided by the rate of enzyme inactivation (which involves the same intermediate).[21] Thus, for **2a**, the rate of formation of **5b** (expressed as nmol of product formed/min per nmol of cytochrome P-450, or min^{-1}) divided by the first-order rate of cytochrome P-450 destruction observed in the presence of **2a** and NADPH yields the partition ratio. Such calculations make a number of assumptions, including complete recovery of the pyridine, lack of partitioning of a key intermediate to other major metabolites that might accompany formation of a destructive agent, and eventual inactivation of all of the cytochrome P-450 in microsomes.

(21) Walsh, C. T. *Ann. Rev. Biochem.* **1984**, *53*, 493.

(22) Guengerich, F. P.; Distlerath, L. M.; Reilly, P. E. B.; Wolff, T.; Shimada, T.; Umbenhauer, D. R.; Martin, M. V. *Xenobiotica* **1986**, *16*, 367.

(23) Bolger, G. T.; Gengo, P.; Klockowski, R.; Luchowshi, E.; Siegel, H.; Janis, R. A.; Triggle, A. M.; Triggle, D. J. *J. Pharmacol. Exp. Ther.* **1983**, *225*, 291.

(24) Augusto, O.; Beilan, H. S.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1982**, *257*, 11288.

(25) Guengerich, F. P.; Macdonald, T. L. *Acct. Chem. Res.* **1984**, *17*, 9.

(26) Macdonald, T. L.; Zirvi, K.; Burka, L. T.; Peyman, P.; Guengerich, F. P. *J. Am. Chem. Soc.* **1982**, *104*, 2050. Hanzlik, R. P.; Tullman, R. H. *J. Am. Chem. Soc.* **1982**, *104*, 2048. Guengerich, F. P.; Willard, R. J.; Shea, J. P.; Richards, L. E.; Macdonald, T. L. *J. Am. Chem. Soc.* **1984**, *106*, 6446.

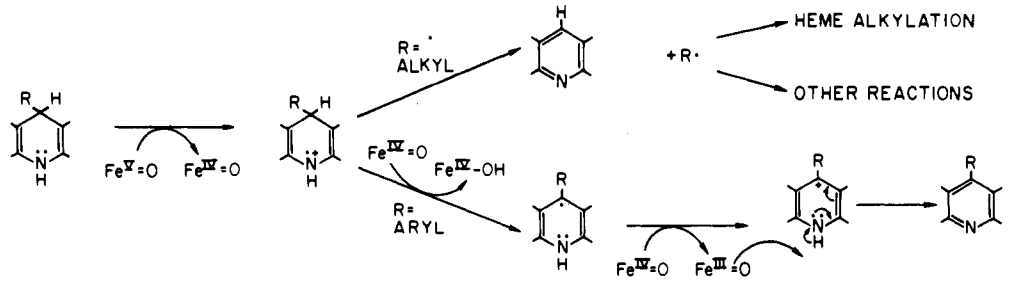


Figure 6. Postulated mechanism for the oxidation of 4-substituted dihydropyridines. See text for further discussion.

eration of Hammett substituent effects on rates of oxidation of aniline derivatives.[28] The pathways of oxidation of the 4-aryl- and 4-alkyl-substituted compounds differ in whether a proton is abstracted or an alkyl radical is split from the ring. With aryl substituents the stability of the benzyl radical is favored and the possibility of phenyl radicals is not very good; thus, abstraction of a proton occurs. With alkyl substituents, the release of radicals is favored. Although we did not have the 4-alkyl-substituted pyridine compounds available as standards, we did not detect major HPLC peaks with the retention times expected for such compounds.

In the pathway shown for the oxidation of the 4-alkyl-substituted dihydropyridines, the iron is left in a formal Fe^{IV}=O form. However, studies with horseradish peroxidase[29] and model metalloporphyrins[30] suggest that the oxidation potentials of the formal Fe^{V}=O and Fe^{IV}=O species may not be very different, and the Fe^{IV}=O might be reduced by the oxidation of a second 4-alkyldihydropyridine molecule. Alternatively the Fe^{IV}=O complex might abstract a proton and then hydroxylate the alkyl radical. However, we have no evidence to support the latter proposal in this case. We also point out that these mechanisms are shown with formal high-valent iron species (Fe^{V}=O, Fe^{IV}=O, etc.); the electronic configurations more likely involve porphyrin radicals[29,30] and perhaps the heme thiolate ligand, but the exact localizations are unknown.[31]

Other oxidation mechanisms can be drawn that involve initial hydrogen abstraction at the 4-position or formation of iron–oxygen–carbon bounds with the olefinic carbons that will yield alkyl radicals and rationalize the observed products.[24] Alternatively, some of the reaction products can be explained by strictly heterolytic mechanisms. We prefer the pathways shown in Figure 6 because of the general case for aminium radical formation.[25-28]

The mechanism-based inhibition of cytochrome P-450 by 4-alkyldihydropyridines has been well documented[32] but to our knowledge has not been directly shown in human systems before. Anti-P-450_{NF} blocked both pyridine formation and cytochrome P-450 heme destruction, indicating that the two processes are linked and that P-450_{NF} (or a closely related variant) is the enzyme in human liver which catalyzes the destructive reaction. We were surprised to find a relatively high (and somewhat variable) rate of nonenzymatic oxidation of the 4-alkyldihydropyridines (but not the 4-aryldihydropyridines). The results are probably not due to oxidation artifacts related to product analysis per se, for the extent of reaction varied with the preparation of microsomes used. We also do not feel that lipid peroxidation is related to this phenomenon. A possible explanation is that 4-alkyldihydropyridines are particularly sensitive to oxidation catalyzed by trace metals. This process might then be distinct from that which is catalyzed by cytochrome P-450, for inactivation did not appear to occur.

Finally, the inhibition of catalytic activity observed with the 4-alkyldihydropyridines did not appear to be restricted to P-450_{NF}, the enzyme involved in the oxidation of the compounds. Phenacetin *O*-deethylase and hexobarbital 3′-hydroxylase activities were extensively inhibited when microsomes oxidized compounds **2a**, **3a**, **4a**, or **15a** (Table III). These activities are not catalyzed by P-450_{NF}, as demonstrated by immunochemical studies. The results also indicate that the inhibition is not merely competitive and does require oxidation of the alkyldihydropyridines. Thus, the radicals that are released during oxidation are apparently able to migrate away from the site of formation (i.e., enzyme where they are formed) and destroy other enzymes. This interpretation is consistent with the observation that Augusto et al.[24] were able to trap these radicals with nitrones and isolate the products. Thus, the oxidation of 4-alkyldihydropyridines involves a "mechanism-based" inhibition but is not strictly "suicidal". One might also expect the radicals that are released to attack other (non-cytochrome P-450) proteins and cause other types of damage typical of free radicals in addition to alkylating cytochrome P-450 heme.

Experimental Section

Synthesis. Melting points were determined in open capillary tubes with a Mel-Temp apparatus and are uncorrected. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN; for all compounds that were analyzed, analyses agreed within ±0.4% of calculated values with the exception of the six C values that are indicated (Table I). Mass spectra were obtained with a Ribermag R-10-10C mass spectrometer in the Vanderbilt University facility; samples were introduced by direct inlet. When the electron impact (EI) mode was used, the operating voltage was 70 eV. Chemical ionization (CI) was in the positive-ion mode, and methane was the carrier gas. Proton NMR spectra were obtained (0.2% (w/v) solutions in $CDCl_3$ in 5-mm tubes) on a Bruker AM-400 instrument operating at 400 MHz in the Vanderbilt University facility with the assistance of Dr. M. Stone; shifts are reported as ppm (δ) relative to tetramethylsilane. UV spectra were measured with 100 μM solutions in CH_3OH in a Cary 219 instrument operating in the automatic base-line correction mode; the pathlength was 2 mm. Wavelength maxima were

(27) Miwa, G. T.; Walsh, J. S.; Kedderis, G. L.; Hollenberg, P. F. *J. Biol. Chem.* **1983**, *258*, 14445. Shea, J. P.; Nelson, S. D.; Ford, G. P. *J. Am. Chem. Soc.* **1983**, *105*, 5451.
(28) Burka, L. T.; Guengerich, F. P.; Willard, R. J.; Macdonald, T. L. *J. Am. Chem. Soc.* **1985**, *107*, 2549. Guengerich, F. P.; Shea, J. P.; Willard, R. J.; Schaefer, W. H.; Macdonald, T. L.; Richards, L. E.; Burka, L. T. *Abstracts of Papers*, 190th National Meeting of the American Chemical Society, Chicago, IL; American Chemical Society: Washington, DC, 1985; BIOL 95.
(29) Hayashi, Y.; Yamazaki, I. *J. Biol. Chem.* **1979**, *254*, 9101.
(30) Lee, W. A.; Calderwood, T. S.; Bruice, T. L. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 4301.
(31) Ullrich, V. *Top. Curr. Chem.* **1979**, *83*, 67.
(32) Ortiz de Montellano, P. R.; Correia, M. A. *Ann. Rev. Pharmacol. Toxicol.* **1983**, *23*, 481. Ortiz de Montellano, P. R.; Beilan, H. S.; Kunze, K. L. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 1490. DeMatteis, F.; Gibbs, A. H.; Hollands, C. *Biochem. J.* **1983**, *211*, 455. Tephley, T. R.; Coffman, B. L.; Ingall, G.; Abou Zeit-Hor, M. S.; Goff, H. M.; Tabba, H. D.; Smith, K. M. *Arch. Biochem. Biophys.* **1981**, *212*, 120.

assigned using the first- and second-derivative spectra;[33,34] extinction coefficients are reported, however, only for the *apparent* maxima.

Nifedipine [1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester, **1a**] and its oxidized derivative [2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester, **1b**] were gifts of Dr. J. R. Idle, St. Mary's Hospital Medical School, London, U.K. Compounds **6a**, **7a**, **8a**, **9a**, **10a**, **11a**, and **12a** [1,4-dihydro-2,6-dimethyl-4-(3-nitro-4-chlorophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester; 1,4-dihydro-2,6-dimethyl-4-(3-cyanophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester; 1,4-dihydro-2,6-dimethyl-4-(4-methylphenyl)-3,5-pyridinedicarboxylic acid dimethyl ester; 1,4-dihydro-2,6-dimethyl-4-(2-chloro-6-fluorophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester; 1,4-dihydro-2,6-dimethyl-4-(4-(trifluoromethyl)phenyl)-3,5-pyridinedicarboxylic acid dimethyl ester; 1,4-dihydro-2,6-dimethyl-4-(2,3,5,6-tetrafluorophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester; and 1,4-dihydro-2,6-dimethyl-4-(3-chlorophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester, respectively] were gifts of Dr. D. J. Triggle, State University of New York, Buffalo, NY.[23] Compounds **16a**, **17a**, **18a**, and **19a** [nisoldipine—1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid methyl 2-methylpropyl ester; nimodipine—1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylic acid 2-methoxyethyl 1-methylethyl ester; niludipine—1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylic acid bis(2-propoxyethyl) ester; and nitrendipine—1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylic acid ethyl methyl ester, respectively] were gifts of Dr. A. Scriabine, Miles Laboratories, New Haven, CT.

4-Substituted 1,4-Dihydro-2,6-dimethyl-3,5-pyridinedicarboxylic Acid Esters (2a, 3a, 4a, 5a, 13a, 14a, 15a). These compounds were prepared by the general Hantzsch dihydropyridine synthesis;[10,35] the synthesis of **14a** is representative. Benzaldehyde (21.2 g, 0.2 mol), methyl acetoacetate (46.4 g, 0.4 mol), and concentrated NH_4OH (0.3 mol, 16.7 mL) were dissolved in 40 mL of CH_3OH and heated at reflux for 1 h. The mixture solidified during heating and was cooled to room temperature; 100 mL of H_2O was added, and the precipitate was collected by vacuum filtration. This solid was dissolved in hot ethanol, filtered while hot, and allowed to cool to give crystals that were collected by filtration, washed with water, and dried over silica in vacuo to give 42 g of **14a**: yield 70%; mp 198–198.5 °C (lit.[36] mp 197–198 °C); EI MS, *m/z* (rel abundance) 301 (M, 8), 270 (5), 242 (5), 225 (12), 224 (100). CI MS, *m/z* 330 (M + 29, 11), 302 (M + H, 6), 301 (4), 298 (11), 271 (19), 270 (100), 225 (6), 224 (43). UV λ_{max} 348, 270, 237 (ϵ_{348} 8.25, ϵ_{265} 24.0, ϵ_{241} 27.2 mM^{-1} cm^{-1}). Anal. ($C_{17}H_{19}NO_4$) C, H, N.

1,4-Dihydro-2,6-dimethyl-4-phenyl-3,5-pyridinedicarboxylic Acid Diethyl Ester (13a): yield 67%; mp 156–157 °C (lit.[37] mp 156–157 °C); CI MS, *m/z* 358 (M + 29, 10), 330 (M + H, 5), 329 (M, 5), 328 (2), 312 (5), 300 (21), 285 (19), 284 (100), 252 (34). Anal. ($C_{19}H_{23}NO_4$) C, H, N.

1,4-Dihydro-2,6-dimethyl-4-ethyl-3,5-pyridinedicarboxylic Acid Diethyl Ester (15a): yield 86%; mp 108–109 °C (lit.[38] mp 108–110 °C); CI MS, *m/z* 310 (M + 29, 13), 282 (M + H, 6), 280 (6), 252 (53), 237 (16), 236 (100).

The other 1,4-dihydropyridines synthesized in this work appear to be new compounds, and their yields and physical properties are listed in Table I.

2,6-Dimethyl-3,5-pyridinedicarboxylic Acid Esters (5b, 6b, 7b, 8b, 9b, 10b, 11b, 12b, 13b, 14b, 16b, 17b, 18b, 19b). The general HNO_3 oxidation method described by Chennot and Eisner[11] was used with the oxidation of **13a** to **13b** presented here as an example. **13a** (0.66 g, 2 mmol) was heated in 12 mL of 20% aqueous HNO_3 at 100 °C for 60 min and then cooled. Solid Na_2CO_3 was added to the solution (chilled on ice) until the pH was basic. The product was extracted three times with an equal volume of diethyl ether; the combined ether phases were dried over $MgSO_4$ and reduced in vacuo. The residue was dissolved in 3 mL of ethanol and triturated with water to give 0.52 g of **13b** as white crystals: yield 80%; mp 61–62 °C (lit.[39] mp 66 °C; lit.[12] mp 63–64.5 °C); EI MS, *m/z* 328 (22), 327 (M, 100), 272 (51), 271 (23), 255 (10), 254 (28), 253 (27), 252 (12), 236 (32); UV, λ_{max} 277, 268 (ϵ_{268} 28.2 mM^{-1} cm^{-1}).

2,6-Dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic Acid Methyl 2-Methylpropyl Ester (16b): yield 63% (reported in ref 14 but no mp); UV, λ_{max} 277, 270, 257; NMR δ 0.77 (6 H, t, $C(CH_3)_2$), 1.63 (1 H, m, –CH<), 2.65 (3 H, s, CCH_3), 2.67 (3 H, s, CCH_3), 3.51 (3 H, s, CO_2CH_3), 3.70 (2 H, m, CO_2CH_2), 7.02 (1 H, m), 7.51 (1 H, m), 7.64 (1 H, m), 8.21 (1 H, d); EI MS, *m/z* 371 (M – 1, 1), 355 (5), 341 (15), 340 (100), 341 (18), 284 (100), 267 (12).

2,6-Dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylic Acid 2-Methoxyethyl 1-Methylethyl Ester (17b): yield 71% (reported in ref 15 and 16 but no mp); EI MS, *m/z* 417 (3), 416 (M, 14), 359 (10), 358 (14), 357 (32), 342 (48), 341 (49), 304 (51), 325 (10), 316 (22), 300 (15), 299 (56), 298 (70), 281 (30), 59 (32), 58 (100); UV, λ_{max} 268; NMR, δ 1.02 (6 H, d, $C(CH_3)_2$), 2.62 (3 H, s, CCH_3), 2.64 (3 H, s, CCH_3), 3.24 (3 H, s, OCH_3), 3.34 (2 H, t, OCH_2), 4.13 (2 H, m, CO_2CH_2), 4.95 (1 H, m, OCH<), 7.61 (2 H, m), 8.20 (1 H, s), 8.26 (1 H, d).

2,6-Dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylic Acid Ethyl Methyl Ester (18b): yield 81% (reported in ref 14 but no mp); EI MS, *m/z* 359 (15), 358 (M, 83), 342 (19), 341 (100), 327 (26), 326 (14), 314 (16), 313 (46), 312 (30), 297 (13), 283 (15), 281 (17), 252 (25); UV, λ_{max} 277, 268 (ϵ_{268} 26 mM^{-1} cm^{-1}); NMR, δ 0.89 (6 H, t), 1.52 (4 H, m), 2.66 (6 H, s, CCH_3), 3.29 (4 H, t, OCH_2), 3.40 (4 H, t, OCH_2), 4.14 (4 H, m, CO_2CH_2), 7.60 (2 H, m), 8.22 (2 H, m).

2,6-Dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylic Acid Bis(2-propoxyethyl) Ester (19b): yield 33%; mp 43–45 °C (reported in ref 18 but no mp); EI MS, *m/z* 488 (M, 7), 458 (9), 429 (23), 413 (20), 386 (29), 385 (100), 384 (39), 373 (12), 319 (21), 343 (36), 319 (21), 343 (36), 328 (43), 299 (35), 284 (27), 281 (35), 209 (22); UV, λ_{max} 268; NMR, δ 1.02 (3 H, t), 2.63 (3 H, s, CCH_3), 2.66 (3 H, s, CCH_3), 3.58 (3 H, s, CO_2CH_3), 4.06 (2 H, q), 7.59 (3 H, m), 8.22 (1 H, m). Anal. ($C_{25}H_{32}N_2O_8$) H, N, C: calcd, 61.48; found, 59.17.

2,6-Dimethyl-3,5-pyridinedicarboxylic acid diethyl ester (20b) was prepared by HNO_3 oxidation of 1,4-dihydro-2,6-dimethyl-3,5-pyridinedicarboxylic acid diethyl ester [synthesized via the Hantzsch reaction described above in 43% yield, mp 172–174.5 °C (lit.[13] mp 184–185 °C)]: yield 10%; mp 69–69.5 °C (lit.[13] mp 70.5–72 °C); EI MS, *m/z* 251 (M, 16), 207 (11), 206 (100), 205 (38), 195 (13), 178 (32).

Microsomal Preparations. Human liver samples were obtained from organ donors who met accidental deaths. The processing of these samples and preparation of microsomes are detailed elsewhere.[40] Samples used here (coded by donor number) included HL 94 (0.26 nmol of cytochrome P-450/mg of protein), HL 95 (0.43 nmol of cytochrome P-450/mg of protein), and HL 96 (0.62 nmol of cytochrome P-450/mg of protein).[9] We thank the Nashville Regional Organ Procurement Agency for their assistance in obtaining these samples.

Antibodies. Human liver $P\text{-}450_{NF}$ was purified to apparent homogeneity on the basis of nifedipine oxidase activity as described elsewhere, and antibodies were raised in rabbits.[9] Immunoglobulin G fractions were prepared.[41] Electrophoretic, catlaytic, and other evidence for specificity of the antibody are presented elsewhere.[9]

Dihydropyridine Oxidation Assays. Because of the light sensitivity of nifedipine and some of the other compounds,[42] all assays were done in amber vials.[9] Detailed conditions for the assay of microsomal nifedipine oxidase are described elsewhere,[9] and these general conditions were used with the other dihydro-

(33) O'Haver, T. C.; Green, G. L. *Anal. Chem.* **1976**, *48*, 312.
(34) Guengerich, F. P. *Biochemistry* **1983**, *22*, 2811.
(35) Loev, B.; Goodman, M. M.; Snader, K. M.; Tedeschi, R.; Macko, E. *J. Med. Chem.* **1974**, *17*, 956.
(36) Phillips, A. P. *J. Am. Chem. Soc.* **1949**, *71*, 4003.
(37) Schiff, R.; Puliti, J. *Ber.* **1883**, *16*, 1607.
(38) Roomi, M. W. *J. Med. Chem.* **1975**, *18*, 457.
(39) Skraup, S. *Ann.* **1919**, *419*, 58.
(40) Wang, P. P.; Beaune, P.; Kaminsky, L. S.; Dannan, G. A.; Kadlubar, F. F.; Larrey, D.; Guengerich, F. P. *Biochemistry* **1983**, *22*, 5375.
(41) Kaminsky, L. S.; Fasco, M. J.; Guengerich, F. P. *Methods Enzymol.* **1981**, *74*, 262.
(42) *Merck Index*; Merck and Co.: Rahway, NJ, 1983; p 6368.

pyridines. Briefly, NADPH-fortified microsomes (usually 50 pmol of cytochrome P-450 equivalent) were incubated with 0.2 mM dihydropyridine in 100 mM potassium phosphate buffer (pH 7.85) at 37 °C for 10 min (total volume 0.5 mL). A 100-μL portion of 1 M Na_2CO_3 buffer (pH 10.5) containing 2 M NaCl was added, and the solution was extracted twice with 1.0 mL of CH_2Cl_2. Measured portions of the combined CH_2Cl_2 extracts were withdrawn each time and dried under N_2. Residues were dissolved in CH_3OH, and aliquots were assayed by HPLC. Either a 6.2 mm × 8 cm 3 μm DuPont (DuPont Instruments, Wilmington, DE) Zorbax Gold octadecylsilyl (C_{18}) column (1-5, 8, 9, 13-19) or a 4.6 mm × 25 cm 5 μm DuPont Zorbax octylsilyl (C_8) column (6, 7, 10, 12) was used with a Spectra-Physics 8700 system (Spectra-Physics, Piscataway, NJ) and Laboratory Data Control UV II (Rivera Beach, FL) monitor. The solvent systems used for particular compounds included CH_3OH and H_2O, and all separations were performed isocratically. HPLC method 1: C_{18}, CH_3OH/H_2O 60:40 (v/v), flow 2.5 mL min^{-1}. HPLC method 2: C_8, CH_3OH/H_2O 60:40 (v/v), flow 1.3 mL min^{-1}. HPLC method 3: C_{18}, CH_3OH/H_2O 60:40 (v/v), flow 2.0 mL min^{-1}. HPLC method 4: C_{18}, CH_3OH/H_2O 75:25 (v/v), flow 2.0 mL min^{-1}. The detection wavelength was 254 nm for the assays with compounds 1, 6-14, and 16-19 and both 254 and 280 nm (two detectors in line) for the assays with compounds 2-5. Quantitative measurements were generally made with external standards and measurements based upon peak heights.

Other Assays. Cytochrome P-450 measurements were made as described.[43] Phenacetin *O*-deethylase activity was measured by TLC as described elsewhere.[44] Hexobarbital 3′-hydroxylase activity was estimated by HPLC as described by Rummelt et al.[45]

Acknowledgment. This work was supported in part by USPHS Grants CA 30907 and ES 00267 and the Deutsche Forschungsgemeinschaft (Boe 784/1-1).

Registry No. **1a**, 21829-25-4; **2a**, 103026-73-9; **3a**, 103026-74-0; **4a**, 103026-75-1; **5a**, 17438-14-1; **5b**, 27525-74-2; **6a**, 51384-20-4; **6b**, 103026-76-2; **7a**, 32947-20-9; **7b**, 103026-77-3; **8a**, 73257-48-4; **8b**, 103026-78-4; **9a**, 86408-11-9; **9b**, 103026-79-5; **10a**, 103026-84-2; **10b**, 103026-80-8; **11a**, 103026-85-3; **11b**, 103026-81-9; **12a**, 73257-45-1; **12b**, 103026-82-0; **13a**, 1165-06-6; **13b**, 1539-44-2; **14a**, 70677-78-0; **14b**, 77234-00-5; **15a**, 1153-66-8; **16a**, 63675-72-9; **16b**, 103026-83-1; **17a**, 66085-59-4; **17b**, 85677-93-6; **18a**, 22609-73-0; **18b**, 88443-04-3; **19a**, 39562-70-4; **19b**, 89267-41-4; **20a**, 1149-23-1; **20b**, 1149-24-2; $PhCH_2CHO$, 122-78-1; $(CH_3)_2CHCHO$, 78-84-2; CH_2O, 50-00-0; C_6H_5CHO, 100-52-7; C_2H_5CHO, 123-38-6; $CH_3COCH_2CO_2CH_3$, 105-45-3; $CH_3COCH_2CO_2CH_2CH_3$, 141-97-9; 3-cyclohexene-1-carboxaldehyde, 100-50-5.

(43) Omura, T.; Sato, R. *J. Biol. Chem.* **1964**, *239*, 2370.
(44) Larrey, D.; Distlerath, L. M.; Dannan, G. A.; Wilkinson, G. R.; Guengerich, F. P. *Biochemistry* **1984**, *23*, 2787.
(45) Rummelt, V.; Ott, R.; Böcker, R. *Drugs Exp. Clin. Res.*, in press.

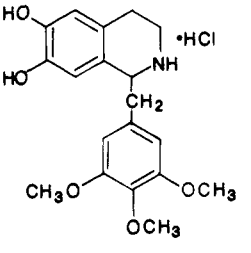
Syntheses and β-Adrenergic Agonist and Antiaggregatory Properties of N-Substituted Trimetoquinol Analogues[1]

Adeboye Adejare,[†] Duane D. Miller,*[†] Joanne S. Fedyna,[‡] Chang-Ho Ahn,[‡] and Dennis R. Feller[‡]

Divisions of Medicinal Chemistry and Pharmacology, College of Pharmacy, The Ohio State University, Columbus, Ohio 43210.
Received September 16, 1985

Trimetoquinol [1-(3,4,5-trimethoxybenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, TMQ] is a potent β-adrenergic receptor agonist and inhibitor of human platelet aggregation. Selective cleavage of *O*-benzyl groups in the presence of an *N*-benzyl group using HCl and formation of a cyclic sulfite ester from the reaction of a catechol with thionyl chloride were achieved. The N-substituents included methyl, benzyl, and β-hydroxy- and β-chloroethyl groups. Each N-substituted TMQ caused a concentration-dependent stimulation of β_2 (trachea) and β_1 (atria) adrenoceptor tissues and inhibition of 15(*S*)-hydroxy-11α,9α-(epoxymethano)prosta-5,13(*E*)-dienoic acid (U46619, a thromboxane A_2 mimetic) mediated human platelet activation. TMQ remained the most potent in the series. Structure-activity results indicated that the larger the N-substituent, the lower the β-adrenergic activity but the higher the inhibition of platelet aggregatory activity. Thus, the structural requirements of these TMQ analogues for the two types of biological activity are different.

Tetrahydroisoquinolines (THIs) represent a class of cyclized phenethylamines whose pharmacological properties include lipolytic,[2,3] bronchial relaxant,[4-7] hypotensive,[8,9] platelet antiaggregatory,[10] cardiostimulant,[4-7,11] and uterine-stimulant[5] properties. Structural requirements for potent bronchodilating activity in the THI series have been proposed to include the 1,2,3,4-THI nucleus, the catechol hydroxy groups at positions 6 and 7, and an arylmethyl group at position 1.[6,12] 1-(3,4,5-Trimethoxybenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (trimetoquinol, TMQ; **1**) is one of the most potent β-adrenoceptor stimulants examined thus far and is approximately 10 times more active than the classical β-adrenoceptor agonist isoproterenol in guinea pig tracheal relaxation.[11] Potent β-adrenoceptor activation residues primarily with the *S*-(-) isomer of TMQ (Inolin) and is currently used in Japan for relief of asthmatic bronchospasms.[11]



1

Trimetoquinol and related analogues[13] also have been found to be potent inhibitors of platelet aggregation. The

[†] Division of Medicinal Chemistry.
[‡] Division of Pharmacology.

(1) (a) Taken in part from the Ph.D Dissertation of A. Adejare, The Ohio State University, 1985. (b) Adejare, A.; Ahn, C.; Fedyna, J. S.; Feller, D. R.; Miller, D. D. Presented at the 189th National Meeting of the American Chemical Society, Miami, FL, April 1985.
(2) Shonk, R. F.; Miller, D. D.; Feller, D. R. *Biochem. Pharmacol.* **1971**, *20*, 3403.

0022-2623/86/1829-1603$01.50/0