Stereoselective Metabolism of Conformational Analogues of Warfarin by β-Naphthoflavone-Inducible Cytochrome P-450

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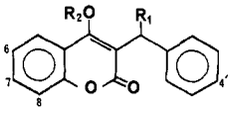
Previous studies have shown that the structurally related oral anticoagulants warfarin and phenprocoumon are regioselectively hydroxylated in the 6- and 8-positions by hepatic microsomes obtained from 3-methylcholanthrene (3-MC) or β-naphthoflavone (BNF) pretreated rats. Stereoselectivity for hydroxylation is also observed and favors (*R*)-warfarin but (*S*)-phenprocoumon. The possibility that the stereoselectivity of warfarin hydroxylation is a function of the solution conformation of the drug was tested with conformationally restricted analogues. In these experiments the analogues were incubated with microsomes obtained from BNF-pretreated rats and any stereoselectivity associatiated with 6- and 8-hydroxylation was determined. The *R* enantiomer of cyclocoumarol, the cyclic ketal analogue of warfarin, was found to be selectively hydroxylated, in contrast to the *S* enantiomer of warfarin 4-methyl ether, the ring-opened analogue. The latter compound is known to have a preferred solution conformation similar to that of phenprocoumon. The results suggest that at the active site of BNF-induced cytochrome P-450 (*R*)-warfarin is metabolized in its cyclic hemiketal tautomer, a form which spatially mimics the preferred solution conformation of (*S*)-phenprocoumon.

Warfarin, (**1a** and **1b**) and phenprocoumon (**2**) are structurally related coumarin anticoagulants. Both are 3-substituted derivatives of 4-hydroxycoumarin which only differ in the side chain attached to the benzylic carbon. Previous metabolic studies have shown both compounds to be hydroxylated in the 4′-, 6-, 7-, and 8-positions by rat liver microsomes.[1,2] The effect of inducing agents on their metabolic profile has also been examined.[3,4] In particular, 3-methylcholanthrene (3-MC) and β-naphthoflavone (BNF) have been found to induce a form or forms of cytochrome P-450 which not only display unusually high activity for warfarin and phenprocoumon but also metabolize the two compounds with a high degree of regio and stereoselectivity.[4,5] These agents selectively induce formation of the 6- and 8-hydroxy metabolites; however, the stereoselectivity associated with these processes is for opposite enantiomers of the two drugs. Although for a given absolute configuration both warfarin and phenprocoumon are spatially related about the asymmetric center, i.e., topologically (*R*)-warfarin corresponds to (*R*)-phenprocoumon, 6- and 8-hydroxylation of warfarin is stereoselective for the *R* enantiomer while the analogous hydroxylations of phenprocoumon are stereoselective for the *S* enantiomer. Considering the close structural similarity of warfarin and phenprocoumon and the fact that the analogous metabolites for each compound are selectively induced suggests that both warfarin and phenprocoumon are biotransformed by the same isozyme(s). The question then arises as to why the enzyme selectively hydroxylates opposite enantiomers of the two drugs.

One possible explanation is provided by studies on the preferred solution conformations of warfarin and phenprocoumon.[6-8] Warfarin is known to exist in solution as an equilibrium mixture of a ring-opened keto tautomer, **1a**, and the diastereomeric ring-closed cyclic hemiketal tautomers, **1b**, with the equilibrium strongly favoring the latter two compounds in all solvents examined. It was further observed that the preferred solution conformation of (*R*)-warfarin hemiketal and (*S*)-phenprocoumon share the same spatial arrangement of the phenyl ring relative to the coumarin nucleus (Figure 1). Therefore, if (*R*)-warfarin were metabolized in its hemiketal conformation, it would spatially mimic (*S*)-phenprocoumon at the active site of the enzyme, resulting in the observed stereoselectivity for opposite enantiomers of the two drugs. In this report conformationally restricted analogues of warfarin were used to probe the effect of conformation on the stereoselectivity of 6- and 8-hydroxylation as catalyzed by hepatic microsomes obtained from BNF-pretreated rats.



1a, $R_1 = -CH_2COCH_3$; $R_2 = -H$

1b, $R_1R_2 = -CH_2C(OH)(CH_3)-$

2, $R_1 = -C_2H_5$; $R_2 = -H$

3, $R_1 = -CH_2COCH_3$; $R_2 = -CH_3$

4, $R_1R_2 = -CH_2C(OCH_3)(CH_3)-$

Experimental Section

Melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. The NMR spectra were recorded on a Varian EM 360A spectrometer using tetramethylsilane as the reference standard. Capillary gas chromatographic mass spectrometric measurements were conducted on a VG micromass 7070H mass spectrometer interfaced to a VG 2000 data system and fitted to a Hewlett-Packard 5700 GC. The capillary gas chromatography column used was a 30-m DB-5 (0.32-mm i.d., 0.25-μm film thickness) from J & W Scientific Co.

Diazald and β-naphthoflavone were purchased from Aldrich Chemical Co. Racemic warfarin was obtained from Sigma Biochemicals and was resolved as previously described.[9] The *R* and *S* enantiomers of cyclocoumarol and warfarin 4-methyl ether were prepared from the separate *R* and *S* enantiomers of warfarin by the method of Link.[10] The major and minor isomers of cyclo-

(1) L. R. Pohl, S. D. Nelson, W. R. Porter, W. F. Trager, M. J. Fasco, F. D. Baker, and J. W. Fenton, *Biochem. Pharmacol.*, **25**, 2153 (1976).
(2) C. R. Wheeler, W. F. Trager, and W. R. Porter, *Biochem. Pharmacol.*, **30**, 1785 (1981).
(3) L. R. Pohl, W. R. Porter, W. F. Trager, M. J. Fasco, and J. W. Fenton, *Biochem. Pharmacol.*, **26**, 109 (1977).
(4) W. R. Porter, C. R. Wheeler, and W. F. Trager, *Biochem. Pharmacol.*, **30**, 3099 (1981).
(5) L. S. Kaminsky, M. J. Fasco, and F. P. Guengerich, *J. Biol. Chem.*, **255**, 85 (1980).
(6) E. J. Valente, E. C. Lingafelter, W. R. Porter, and W. F. Trager, *J. Med. Chem.*, **20**, 1489 (1977).
(7) E. J. Valente and W. F. Trager, *J. Med. Chem.*, **21**, 141 (1978).
(8) E. J. Valente, W. R. Porter, and W. F. Trager, *J. Med. Chem.*, **21**, 231 (1978).
(9) B. D. West, S. Preis, C. H. Schroeder, and K. P. Link, *J. Am. Chem. Soc.*, **83**, 2676 (1961).
(10) M. Ikawa, M. A. Stahmann, and K. P. Link, *J. Am. Chem. Soc.*, **66**, 902 (1944).

0022-2623/85/1828-0503$01.50/0

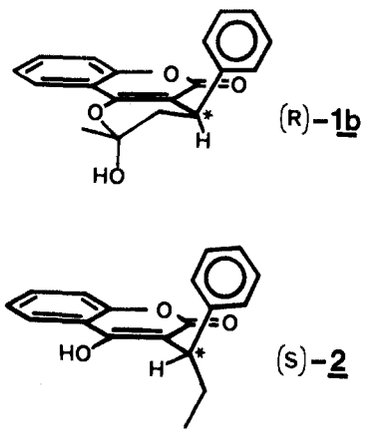


Figure 1. Comparison of the preferred solution conformations of (*R*)-warfarin hemiketal, (*R*)-**1b**, and (*S*)-phenprocoumon, (*S*)-**2**.

coumarol were obtained by the method of Valente.[6,11] Unlabeled phenolic warfarin metabolites (4′-, 6-, 7-, and 8-hydroxywarfarins) were synthesized according to the method of Hermodson et al.[12] The synthesis of 2′,3′,4′,5′,6′-pentadeuterio-6-, -7-, and -8-hydroxywarfarins and 5,6,7,8-tetradeuterio-4′-hydroxywarfarin will be presented elsewhere.

4′-Hydroxycyclocoumarol (5). 4′-Hydroxywarfarin was refluxed in absolute methanol containing 4% HCl for 6 h. After evaporation of the methanol in vacuo, the product was obtained in quantitative yield; white solid, melting point decomposition above 240 °C; ^{1}H NMR (acetone-d_6) δ 1.74 (s, 3 H), 3.31 (s, 3 H), 3.94 (m, 1 H), 7.20 (m, 8 H). Anal. Calcd for $C_{20}H_{18}O_5$: C, 71.0; H, 5.33. Found: C, 71.20; H, 5.49.

5,6,7,8-Tetradeuterio-4′-hydroxycyclocoumarol (6). In the same manner **6** was prepared in quantitative yield from 5,6,7,8-tetradeuterio-4′-hydroxywarfarin; melting point decomposition above 240 °C; MS, m/z 342 [(M + 4)$^+$]; isotopic purity 61.9% (M + 4), 29.2% (M + 3), 6.2% (M + 2), 2.4% (M + 1), 0.3% (M).

6-Hydroxycyclocoumarol (7). In the same manner **7** was prepared in quantitative yield from 6-hydroxywarfarin; white solid, mp 223–226 °C; ^{1}H NMR (acetone-d_6) δ 1.62, 1.71 (2 s, 3 H), 3.20, 3.31 (2 s, 3 H), 4.00 (m, 1 H), 7.15 (m, 8 H). Anal. Calcd for $C_{20}H_{18}O_5$: C, 71.0; H, 5.33. Found: C, 71.11; H, 5.52.

2′,3′,4′,5′,6′-Pentadeuterio-6-hydroxycyclocoumarol (8). In the same manner **8** was prepared in quantitative yield from 2′,3′,4′,5′,6′-pentadeuterio-6-hydroxywarfarin; mp 223–226 °C; MS, m/z 343 [(M + 5)$^+$]; isotopic purity 91.0% (M + 5), 7.6% (M + 4), 1.4% (M + 3).

7-Hydroxycyclocoumarol (9). In the same manner **9** was prepared in quantitative yield from 7-hydroxywarfarin; white solid, mp 229–232 °C; ^{1}H NMR (acetone-d_6) δ 1.65 (s, 3 H), 3.31 (s, 3 H), 4.00 (m, 1 H), 7.20 (m, 8 H). Anal. Calcd for $C_{20}H_{18}O_5$: C, 71.0; H, 5.33. Found: C, 71.05; H, 5.50.

2′,3′,4′,5′,6′-Pentadeuterio-7-hydroxycyclocoumarol (10). In the same manner **10** was prepared in quantitative yield from 2′,3′,4′,5′,6′-pentadeuterio-7-hydroxywarfarin; mp 229–232 °C; MS, m/z 343 [(M + 5)$^+$]; isotopic purity 91.0% (M + 5), 7.6% (M + 4), 1.4% (M + 3).

8-Hydroxycyclocoumarol (11). In the same manner **11** was prepared from 8-hydroxywarfarin in quantitative yield; white solid, mp 214–217 °C; ^{1}H NMR (acetone-d_6) δ 1.61, 1.72 (2 s, 3 H), 3.20, 3.31 (2 s, 3 H), 4.05 (m, 1 H), 7.20 (m, 8 H). Anal. Calcd for $C_{20}H_{18}O_5$: C, 71.0; H, 5.33. Found: C, 70.8, H, 5.41.

2′,3′,4′,5′,6′-Pentadeuterio-8-hydroxycyclocoumarol (12). In the same manner **12** was prepared from 2′,3′,4′,5′,6′-pentadeuterio-8-hydroxywarfarin in quantitative yield; mp 214–217 °C; MS, m/z 343 [(M + 5)$^+$]; isotopic purity 91.0% (M + 5), 7.6% (M + 4), 1.4% (M + 3).

Tissue Preparation. Male Sprague–Dawley rats (140–170 g, Tyler Lab, Bellevue, WA) were used in this study. Induction of microsomal cytochrome P-450 was achieved by administering ip BNF (25 mg/mL in corn oil, 80 mg/kg) once daily for 3 days. On the fourth day three animals were sacrificed and their livers excised and pooled. Microsomes and microsomal stock solutions were prepared as previously described.[4] Cytochrome P-450 determinations were performed with an Aminco DW-2 spectrophotometer according to the method of Omuro and Sato.[13]

Incubations. Incubations were run with a microsomal protein concentration of 2 mg/mL. All incubations were run in triplicate for 20 min at 37 °C in an American Optical Model 2156 shaker bath. After an initial 2-min equilibration period in the shaker bath, the incubation period was initiated by addition of substrate. The separate *R* and *S* enantiomers of warfarin (200 μg) were added to their respective microsomal incubations either in 100 μL of 0.05 M phosphate buffer, pH 7.4, or 20 μL of acetone to give a final incubation volume of 1 mL. The separate enantiomers of warfarin 4-methyl ether and the major and minor isomers of cyclocoumarol were added in 100 μL of acetone to give a final incubation volume of 5 mL and a substrate concentration of 200 μg/mL. The reactions were terminated by addition of 0.6 mL of acetone/mL microsomal incubation.

Isolation and Derivatization of Metabolites. The warfarin incubations were worked up and the metabolites extracted and derivatized as previously described.[14] A slightly modified procedure was used for the warfarin analogues. Following termination of the reactions with acetone, 300 ng of each of the deuterium-labeled 4′-, 6-, 7-, and 8-hydroxycyclocoumarols in 50 μL of acetone was added to each of the cyclocoumarol and warfarin 4-methyl ether incubations. Each sample was then vortexed for 30 s and then centrifuged for 10 min at 1000*g*. The supernatant was decanted from the precipitated protein into a fresh vial and adjusted to pH 12 with 1 mL of 1 N KOH. To remove excess substrate, the samples were extracted two times with 5-mL portions of cyclohexane. Next, in order to hydrolyze the cyclocoumarol metabolites and internal standards to the corresponding warfarin compounds, the samples were acidified with 1 mL of 6 N HCl and left at room temperature for 18 h. Finally, the samples were extracted three times with 5-mL portions of ethyl acetate–ethyl ether (1:1). After evaporation of the solvent extract under a stream of dry nitrogen, 2 mL of diazomethane/ethyl ether solution generated from Diazald was added to each sample. The samples were capped and left at room temperature for 36 h, and then the diazomethane/ethyl ether was evaporated under a stream of dry nitrogen, and the samples were stored at −20 °C until analyzed.

Quantitation of Metabolites. The metabolites of warfarin were analyzed by gas chromatography/mass spectrometry (GC/MS) as previously described.[14] After hydrolysis and derivatization, the cyclocoumarol metabolites and the warfarin 4-methyl ether metabolites are identical with the derivatized warfarin metabolites; therefore, the cyclocoumarol and warfarin 4-methyl ether metabolites were also analyzed by the same GC/MS procedure. Standard curves of the weight ratio of unlabeled to labeled metabolite vs. the peak area ratio of the respective ions were constructed as previously described[14] for both the warfarin and the cyclocoumarol metabolites. The deuterated cyclocoumarol metabolites were used as internal standards in the warfarin 4-methyl ether incubations and the amounts of warfarin 4-methyl ether metabolites were calculated with use of the standard curves generated for the cyclocoumarol metabolites. In all cases the amounts of metabolite were calculated by the method of Bush and Trager.[15]

Results and Discussion

While the solution conformation of warfarin at the active site of cytochrome P-450 cannot be determined directly, it is possible to use conformationally restricted analogues of warfarin to mimic the major conformational and tautomeric possibilities. The effect of conformation on the

(11) E. J. Valente, Ph.D. Dissertation, University of Washington, Seattle, WA, 1977.
(12) M. A. Hermodson, W. M. Barker, and K. P. Link, *J. Med. Chem.*, 14, 167 (1971).
(13) T. Omura and R. Sato, *J. Biol. Chem.*, **239**, 2370 (1964).
(14) E. D. Bush, L. K. Low, and W. F. Trager, *Biomed. Mass Spectrom.*, **10**, 395 (1983).
(15) E. D. Bush and W. F. Trager, *Biomed. Mass Spectrom.*, 8, 211 (1981).

Table I. Rates of Metabolism of the *R* and *S* Enantiomers of Warfarin and the Warfarin Conformational Analogues by Liver Microsomes from BNF-Pretreated Rats

substrate	rate of product formation,[a] pmol/nmol of cytochrome P-450 per min			
	8-hydroxy	6-hydroxy	7-hydroxy	4′-hydroxy
cyclocoumarol, major isomer[b]				
R	42.8 ± 7.2	24.1 ± 4.0	14.9 ± 3.4	73.5 ± 10.1
S	14.9 ± 4.3	12.5 ± 3.7	12.8 ± 2.9	52.0 ± 7.5
cyclocoumarol, minor isomer				
R	34.6 ± 6.8	20.3 ± 3.2	19.3 ± 3.0	76.2 ± 8.8
S	15.1 ± 3.9	14.8 ± 2.5	13.9 ± 2.2	64.8 ± 6.3
warfarin 4-methyl ether				
R	16.9 ± 3.8	8.3 ± 2.3	11.5 ± 3.6	85.3 ± 11.8
S	40.5 ± 5.7	31.2 ± 4.0	19.1 ± 3.1	47.8 ± 7.0
warfarin (buffer solution)[c]				
R	871.1 ± 13.1	532.1 ± 10.3	113.7 ± 5.8	22.4 ± 1.6
S	73.3 ± 4.9	216.8 ± 7.5	41.2 ± 1.9	13.7 ± 0.9
warfarin (acetone solution)[d]				
R	809.5 ± 11.0	484.1 ± 8.9	102.3 ± 4.5	20.8 ± 1.2
S	68.9 ± 3.7	195.1 ± 7.1	37.1 ± 1.8	12.6 ± 1.0

[a] Data expressed as the mean ±SD, $n = 3$. [b] In the case of the cyclocoumarols, the absolute configuration designation refers to the absolute configuration about the benzylic carbon. [c] Incubation initiated by addition of warfarin dissolved in phosphate buffer, pH 7.4. [d] Incubation initiated by addition of warfarin dissolved in acetone.

stereoselectivity of 6- and 8-hydroxylation may then be studied by incubating the separate *R* and *S* enantiomers of each conformational analogue with hepatic microsomes obtained from BNF-pretreated rats and determining the stereoselectivity of hydroxylation.

The two analogues used in this study are both methyl derivatives of warfarin. Methylation of the 4-hydroxy group to give warfarin 4-methyl ether (**3**) prevents formation of the cyclic hemiketal and fixes warfarin in the ring-opened keto form. Cyclocoumarol (**4**), the cyclic methyl ketal of warfarin, was chosen to represent the ring-closed hemiketal tautomer. Cyclization of warfarin generates a second asymmetric center in the molecule. The thermodynamically more stable diastereomer and consequently the predominant form of warfarin hemiketal observed in solution has the *R,R* or *S,S* absolute configuration.[6] The first absolute configuration designation refers to the absolute configuration about the benzylic carbon; i.e., both (*R,R*)- and (*R,S*)-cyclocoumarol are derived from (*R*)-warfarin. In this study both the major and minor diastereomers and the individual enantiomers of each diastereomer were incubated separately.

If the stereoselectivity of warfarin hydroxylation is a function of its solution conformation at the active site of the BNF-inducible enzyme(s), then the metabolism of the warfarin conformational analogues should exhibit the following pattern of stereoselectivity. The 6- and 8-hydroxylation of cyclocoumarol which corresponds to the cyclic hemiketal form of warfarin should be stereoselective for the *R* enantiomer. Warfarin 4-methyl ether, which cannot cyclize, would be expected to have a preferred solution conformation similar to phenprocoumon and therefore stereoselectivity for its *S* enantiomer is predicted. These predictions assume that the covalent changes used to restrict conformation do not alter stereoselectivity to any major extent. The results of the microsomal incubations of the warfarin conformational analogues are presented in Table I. Included in this experiment was a set of warfarin control incubations. Warfarin and phenprocoumon are water soluble due to the acidic 4-hydroxy group; however, the warfarin analogues lack this ionizable function. The analogues being largely water insoluble therefore require addition to the microsomal incubation as an acetone solution. To control for any effect the solvent might have on the regio- and stereoselectivity, warfarin was added both as an aqueous and an acetone solution. A comparison of the data for the two sets of warfarin incubations indicate that the solvent depresses metabolism a slight amount but has no significant effect on the regio- and stereoselectivity.

The data for the warfarin incubations is typical of the metabolite levels observed for warfarin metabolism by microsomes from BNF-pretreated rats. Phenprocoumon also typically exhibits metabolite levels which are comparable to that observed for warfarin but with opposite stereoselectivity. In contrast, the levels of metabolites found for the analogues are quite low by comparison. This is probably, at least in part, due to their poor solubility in aqueous media.

Like warfarin and phenprocoumon, the analogues were also found to be hydroxylated in the 4′-, 6-, 7-, and 8-positions. Of principle interest are the 6- and 8-hydroxy metabolites. The stereoselectivity of these two metabolites was found to be for the *S* enantiomer of warfarin 4-methyl ether and the *R* enantiomers of the cyclocoumarols as predicted. Both the major and minor isomers of cyclocoumarol having the *R* configuration at the benzylic carbon were stereoselectively hydroxylated at the 6- and 8-positions. The absolute configuration of the second asymmetric center, the ketal carbon, had only a minor effect on the metabolic profile. Since the *R* enantiomers of both warfarin and cyclocoumarol are selected for, it would suggest that the compounds share a common solution conformation at the active site(s) of the enzyme(s) catalyzing 6- and 8-hydroxylation; i.e., warfarin is metabolized in its cyclic hemiketal form. This conclusion is supported by studies on the solution chemistry of warfarin which have demonstrated that the hemiketal is the predominant tautomer of warfarin in a hydrophobic environment such as the drug world encounter in the microsomal membrane and the active site of cytochrome P-450.[16]

Stereoselectivity for opposite enantiomers of warfarin and warfarin 4-methyl ether also argues against the possibility that warfarin is metabolized in the ring-opened form. Like phenprocoumon, which cannot cyclize, 6- and 8-hydroxylation of warfarin 4-methyl ether is stereoselective for the *S* enantiomer. Since opposite enantiomers of ring-opened and ring-closed compounds are selectively hydroxylated, it appears that there is a positive correlation between the spatial orientation of the phenyl group relative to the coumarin ring system and the stereoselectivity of

(16) L. D. Heimark and W. F. Trager, *J. Med. Chem.*, **27**, 1092 (1984).

6- and 8-hydroxylation catalyzed by BNF-induced cytochrome P-450.

The one major difference between the metabolism of warfarin and phenprocoumon and the conformational analogues is the regioselectivity. The major metabolite for the analogues is the 4'-hydroxy product, which in the case of warfarin and phenprocoumon is the metabolite produced in the lowest level. This difference in regioselectivity may simply represent preferential binding of the analogues to other isozymes due to the difference in physical chemical properties between warfarin and its analogues. Alternatively, the analogues bind to the same isozyme(s) catalyzing 6- and 8-hydroxylation of warfarin and phenprocoumon, but due to their physical or steric properties the phenyl group is oxidized at a faster rate than the coumarin ring.

Despite the discrepancy in regioselectivity, in general the data presented in this report support the hypothesis that (*R*)-warfarin in the cyclic hemiketal form spatially mimics the preferred solution conformation of (*S*)-phenprocoumon at the active site(s) of the BNF-induced enzyme(s) catalyzing 6- and 8-hydroxylation, therefore resulting in stereoselectivity for opposite enantiomers of the two drugs. However, firm conclusions cannot be made on the basis of this experiment alone. Since liver microsomes from BNF-pretreated rats contain several forms of cytochrome P-450,[5,17] it is not certain that 6- and 8-hydroxylation of the warfarin analogues are due to the same isozyme(s) involved in warfarin and phenprocoumon metabolism. Neither has it been established that warfarin and phenprocoumon are interacting at the same active site. In order to eliminate potential interference from other isozymes, the metabolism of warfarin, phenprocoumon, and the conformational analogues needs to be studied with a purified preparation of the major isozyme induced by BNF or 3-MC. If such a study confirms the results observed with the microsomal preparation, then the correlation of the three-dimensional structure of phenprocoumon, warfarin, and the analogues with the stereoselectivity of 6- and 8-hydroxylation may provide important information as to the stereotopical nature of the active site of this specific isozyme.

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Registry No. (*R*)-**1a**, 5543-58-8; (*S*)-**1a**, 5543-57-7; (*R*)-**1a** (8-OH), 63740-77-2; (*S*)-**1a** (8-OH), 63740-82-9; (*R*)-**1a** (6-OH), 63740-75-0; (*S*)-**1a** (6-OH), 63740-80-7; (*R*)-**1a** (7-OH), 63740-75-0; (*S*)-**1a** (7-OH), 63740-81-8; (*R*)-**1a** (4'-OH), 63740-78-3; (*S*)-**1a** (4'-OH), 68407-05-6; **1a**-*5,6,7,8-d*$_4$ (4'-OH), 94820-63-0; **1a**-*2',3',-j',5',6'-d*$_5$ (6-OH), 94820-64-1; **1a**-*2',3',4',5',6'-d*$_5$ (7-OH), 94820-65-2; **1a**-*2',3',4',5',6'-d*$_5$ (8-OH), 94820-66-3; (*R*)-**3**, 60431-18-7; (*S*)-**3**, 60431-20-1; (*R*)-**3** (8-OH), 94820-69-6; (*S*)-**3** (8-OH), 94820-70-9; (*R*)-**3** (6-OH), 94820-71-0; (*S*)-**3** (6-OH), 94820-72-1; (*R*)-**3** (7-OH), 94820-73-2; (*S*)-**3** (7-OH), 94820-74-3; (*R*)-**3** (4'-OH), 94820-75-4; (*S*)-**3** (4'-OH), 94820-76-5; (*RR*)-**4**, 64754-01-4; (*SS*)-**4**, 64753-99-7; (*RS*)-**4**, 64754-00-3; (*SR*)-**4**, 94902-10-0; (*RR*)-**5**, 94820-68-5; (*SS*)-**5**, 94902-22-4; (*RS*)-**5**, 94902-23-5; (*SR*)-**5**, 94902-24-6; **6**, 94820-59-4; (*RR*)-**7**, 94902-14-4; (*SS*)-**7**, 94902-15-5; (*RS*)-**7**, 94902-16-6; (*SR*)-**7**, 94902-17-7; **8**, 94820-60-7; (*RR*)-**9**, 94902-18-8; (*SS*)-**9**, 94902-19-9; (*RS*)-**9**, 94902-20-2; (*SR*)-**9**, 94902-21-3; **10**, 94820-61-8; (*RR*)-**11**, 94820-67-4; (*SS*)-**11**, 94902-11-1; (*RS*)-**11**, 94902-12-2; (*SR*)-**11**, 94902-13-3; **12**, 94820-62-9.

(17) P. P. Lau and H. W. Strobel, *J. Biol. Chem.*, **257**, 5257 (1982).

Notes

Specific Inhibition of Benzodiazepine Receptor Binding by Some *N*-(Indol-3-ylglyoxylyl)amino Acid Derivatives

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Several *N*-(indol-3-ylglyoxylyl)amino acid derivatives were synthesized and tested for their affinity for the benzodiazepine receptor in bovine cortical membranes. From these compounds, the *N*-[(5-chloro-, 5-bromo-, or 5-nitroindol-3-yl)glyoxylyl]glycine or -alanine esters were clearly the most potent, while the 5-methoxy analogues were considerably less active. Moreover, esters were more active than the corresponding acids. It is concluded that the affinity of these derivatives for the benzodiazepine receptor is profoundly dependent on amino acid molecular size, as well as the hydrophobic and electronic properties of the compounds.

The existence of specific receptors in the brain for benzodiazepines[1] has raised the intriguing question of whether endogenous compounds exist that interact with these sites in a physiologically relevant manner. Inosine, hypoxanthine, and nicotinamide have had the most attention as possible candidates for the unknown endogenous ligand,[2] though their affinities for the benzodiazepine receptor are very low. Much higher affinities have been found for some β-carboline derivatives,[3-5] norharmane-3-carboxylic acid ethyl ester (**1**, Scheme I) being the most

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(1) Braestrup, C.; Squires, R. F. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 3805.
(2) Marangos, P. J.; Paul, S. M.; Goodwin, F. K.; Solnick, P. *Life Sci.* **1979**, *25*, 1093.
(3) Braestrup, C.; Nielsen, M.; Olsen, C. E. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 2288.
(4) Guzman, F.; Cain, M.; Lerschlid, P.; Hagen, T.; Cook, J. M.; Schweri, M.; Skolnick, P.; Paul, S. M. *J. Med. Chem.* **1984**, *27*, 564.

0022-2623/85/1828-0506$01.50/0