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.

Acknowledgment. This work was supported by NIH Grant GM 25136 and Biomedical Research Development Grant 1508 RR09082.

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.

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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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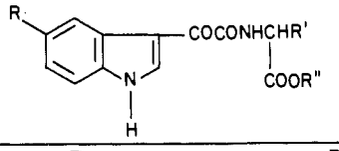
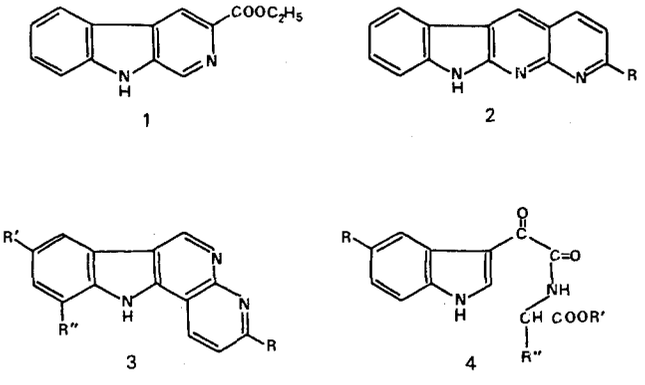
Table I. *N*-[(5-Substituted-indol-3-yl)glyoxylyl]amino Acid Esters



no.	R	R′	R″	inhibn,[a] % (250 μM)	IC_{50},[b] μM
1	H	H	C_2H_5	84 ± 9	
2	H	CH_3	C_2H_5	80 ± 7	
3	H	$CH(CH_3)_2$	C_2H_5	77 ± 8	
4	H	$CH_2C_6H_4OH$-*p*	CH_3	50 ± 6	
5	H	indol-3-ylmethyl	C_2H_5	70 ± 8	
6	H	(6-nitroindol-3-yl)methyl	C_2H_5	48 ± 4	
7	Cl	H	C_2H_5	100	5.4 ± 0.5
8	Cl	CH_3	C_2H_5	100	0.5 ± 0.04
9	Cl	$CH(CH_3)_2$	C_2H_5	62 ± 5	
10	Cl	$CH_2C_6H_4OH$-*p*	CH_3	89 ± 9	80.0 ± 6.0
11	Cl	indol-3-ylmethyl	C_2H_5	55 ± 6	
12	Br	H	C_2H_5	100	2.0 ± 0.2
13	Br	CH_3	C_2H_5	100	4.8 ± 0.5
14	Br	$CH(CH_3)_2$	C_2H_5	88 ± 5	
15	Br	$CH_2C_6H_5$	C_2H_5	65 ± 4	
16	Br	$CH_2C_6H_4OH$-*p*	CH_3	68 ± 5	
17	Br	indol-3-ylmethyl	C_2H_5	100	22.0 ± 2.0
18	NO_2	CH_3	C_2H_5	100	0.2 ± 0.02
19	NO_2	$CH_2C_6H_4OH$-*p*	CH_3	100	20.0 ± 3.0
20	NO_2	indol-3-ylmethyl	C_2H_5	59 ± 7	
21	OCH_3	$CH(CH_3)_2$	C_2H_5	66 ± 3	
22	OCH_3	$CH_2C_6H_5$	C_2H_5	60 ± 2	
diazepam					0.041 ± 0.004
nitrazepam					0.038 ± 0.002
chlordiazepoxide					1.1 ± 0.2

[a] Percents of inhibition of specific [^{3}H]flunitrazepam binding at 250 μM compound concentration are means ± SEM of five determinations. [b] Concentration necessary for 50% inhibition (IC_{50}) are means ± SEM of four determinations.

Scheme I

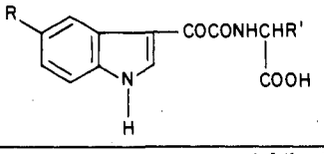


potent. However, this compound has not yet been found "in vivo".[3] While the complete biosynthetic pathway of β-carbolines in animals or in man is not known, it is quite clear that these compounds are derivatives of tryptophan, 5-hydroxytryptophan, tryptamine, 5-hydroxytryptamine, or more generally, of an (2-aminoethyl)indolic structure. We have recently[6] examined the reactivity of some 6*H*-indole[2,3-*b*][1,8]naphthyridines (**2**, Scheme I) and 11*H*-indole[3,2-*c*][1,8]naphthyridines (**3**, Scheme I) in displacing specific [^{3}H]diazepam binding from bovine brain membranes. All the indolenaphthyridines tested (**2**, **3**, Scheme I) are active and showed a higher activity than indole and tryptophan.

On the basis of reports in the literature and previous experience, we have now prepared the indole derivatives of general formula **4** (Scheme I) as shown in Tables I and II.

These compounds were prepared from the indoleglyoxylyl chloride and the appropriate amino acid ester hydrochloride in the presence of triethylamine. The saponification of the ester gave the amino acid derivatives. Their preparation and physical properties have been

Table II. *N*-[(5-Substituted-indol-3-yl)glyoxylyl]amino Acid



no.	R	R′	inhibn,[a] % (250 μM)	IC_{50},[b] μM
23	H	H	20 ± 2	
24	H	CH_3	72 ± 4	
25	H	$CH(CH_3)_2$	28 ± 2	
26	H	$CH_2C_6H_4OH$-*p*	27 ± 2	
27	H	indol-3-ylmethyl	11 ± 1	
28	H	(6-nitroindol-3-yl)methyl	31 ± 3	
29	Cl	H	82 ± 8	38 ± 10.1
30	Cl	CH_3	93 ± 8	22 ± 2.0
31	Cl	$CH(CH_3)_2$	68 ± 7	
32	Cl	$CH_2C_6H_4OH$-*p*	67 ± 7	
33	Cl	indol-3-ylmethyl	61 ± 5	
34	Br	H		
35	Br	CH_3		
36	Br	$CH(CH_3)_2$	72 ± 6	
37	Br	$CH_2C_6H_5$	62 ± 4	
38	Br	$CH_2C_6H_4OH$-*p*	37 ± 2	
39	Br	indol-3-ylmethyl	66 ± 5	
40	NO_2	CH_3	94 ± 7	6.2 ± 0.6
41	NO_2	$CH(CH_3)_2$	83 ± 5	60 ± 5.1
42	NO_2	$CH_2C_6H_4OH$-*p*	81 ± 7	75 ± 6.3
43	OCH_3	$CH(CH_3)_2$	12 ± 2	
44	OCH_3	$CH_2C_6H_5$	38 ± 5	

[a,b] See footnotes *a* and *b* in Table I.

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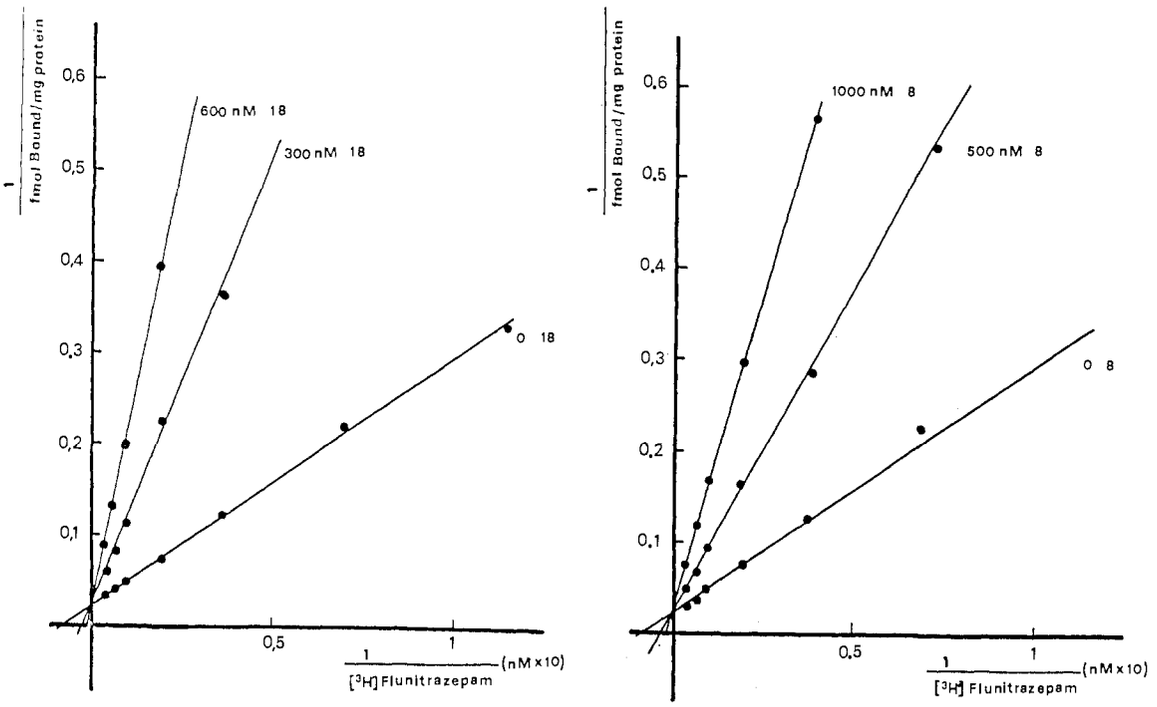

Figure 1. Lineweaver–Burk analysis of compounds 8 and 18 inhibition of [^{3}H]flunitrazeapm binding. Each of the compounds tested was incorporated at the indicated concentration in the [^{3}H]flunitrazepam receptor binding assay at four to seven different concentrations of [^{3}H]flunitrazepam.

previously described.[7] These molecules were of interest because they contain an (aminoethyl)indolic structure, analogous to that of β-carbolines, and are more flexible than **1–2** and should have a higher affinity for the benzodiazepine binding site. The interaction of the indole derivatives (Table I) with the benzodiazepine receptor was investigated in order to delineate structure–activity relationships.

Binding to the Benzodiazepine Receptor. The ability of indole derivatives to displace specific [^{3}H]flunitrazepam binding was tested. Initially a single concentration (250 μM) of the potential displacing agent was examined, followed by determination of IC_{50} values from log–probit plots for the more active compounds.

The data generated are shown in Table I. In this series the most active compounds inhibiting [^{3}H]flunitrazepam binding had a Cl, Br, or NO_2 group in the 5-position, while hydrogen or methoxy substitution in this position decreased the inhibitory potency of the compounds.

Esterification of the free carboxylic acid is accompanied by an increase of the activity in the binding assay, indicating that the incorporation of suitable substituents with the masking of the ionizable groups might be the crucial step for the dramatic increase in receptor affinity. The nature of the amino acid present in the molecule is also very important. The data presents evidence that the size of this substituent is quite limited since alanine and glycine are present in the most active compounds.

The nature of the [^{3}H]flunitrazepam binding inhibition was determined by Lineweaver–Burk analysis in the presence of a fixed concentration of the compound. The results shown in Figure 1 indicate that in all cases the K_m for [^{3}H]flunitrazepam binding is increased while the maximal binding (B_{max}) remains unchanged for either of the two tested compounds, indicating that these compounds inhibit specific [^{3}H]flunitrazepam binding in a competitive manner.

Two types of benzodiazepine receptors have been described in the brain: the "central type", labeled by the benzodiazepine, clonazepam, and the "peripheral type" labeled by the benzodiazepine Ro 5-486, which is also present in various peripheral tissues.[8]

To investigate the nature of the sites inhibited by these indole derivatives, we studied the binding of [^{3}H]clonazepam in the presence of the compounds. There was an excellent correlation between the inhibitory effectiveness of the compounds on [^{3}H]clonazepam binding and [^{3}H]-flunitrazepam binding, showing that these indole derivatives interact with "central type" benzodiazepine sites. The data reported clearly indicate that these indole derivatives interact with the benzodiazepine receptor with affinities higher than that of L-tryptophan.[9] In all cases lower affinities than that of β-carboline-3-carboxylic acid ethyl ester were found;[9] however, compounds 8 and 18 are more active than chlordiazepoxide, a classical benzodiazepine.

Some important conclusions can be drawn from the data: the ethyl ester formation from indoleglyoxylic amino acid derivatives and the presence of an electron-attractive group led to a pronounced increase of the affinity for the benzodiazepine receptor. The size of the aminoacid must be quite limited (alanine or glycine).

The higher activity of these compounds than the parent structure tryptophan indicates that the present study should be extended to other indole structures and that the search for the still unknown endogenous ligand of the benzodiazepine receptor should also include the indole derivatives and related compounds found in the body.

Experimental Section

Tritiated flunitrazepam was obtained from New England Nuclear (Dreieichenhain, West Germany) and had a specific activity of 76.9 Ci/mmol and a radiochemical purity >99%.

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[^{3}H]Clonazepam (16.2 Ci/mmol) was a gift of Hoffmann-La Roche (Basel, Switzerland). All other chemicals were of reagent grade and obtained from commercial suppliers.

Bovine brains were obtained from a local slaughterhouse and stored at −20 °C after dissection of the cortex. Membranes were prepared by homogenization in 10 vol of ice-cold 0.32 M sucrose containing protease inhibitors[10] in an Ultra-Turrax for 30 s. The homogenate was centrifuged at 1000*g* for 5 min at 4 °C and the supernatant was recentrifuged at 50000*g* for 30 min at 4 °C. The pellet was osmotically shocked by suspension in 20 vol of 50 mM Tris-HCl buffer at pH 7.4 containing protease inhibitors[10] and recentrifuged at 50000*g* for 30 min at 4 °C; the pellet was resuspended in 10 vol of 50 mM Tris-HCl buffer at pH 7.4.

The estimation of proteins was based on the method of Lowry[11] after membrane solubilization with 0.75 N NaOH. Bovine serum albumin was utilized as a standard.

Benzodiazepine receptor binding studies were performed by using a filtration technique and [^{3}H]flunitrazepam and [^{3}H]clonazepam as ligands. The membrane suspension (0.5 mg of protein) was incubated in triplicate together with approximately 0.9 nM [^{3}H]flunitrazepam or 0.9 nM [^{3}H]clonazepam and various concentrations of the displacers for 30 min at 0 °C in 500 μL of 50 mM Tris-HCl buffer at pH 7.4. After incubation the samples were diluted with 5 mL of assay buffer and immediately filtered under reduced pressure through glass fiber filter disks (Whatman GF/B) and then washed with 5 mL of the same buffer. Nonspecific binding was determined by parallel experiments containing diazepam (10 μM) and accounted for less than 10% of total binding.

Water-insoluble ester derivatives were dissolved in 50% ethanol/buffer and the same mixture was present in blank experiments.

The concentrations of the indole derivatives that inhibit specific [^{3}H]flunitrazepam binding by 50% (IC_{50}) were determined by log-probit analysis with four to six concentrations of the displacers, each performed in triplicate.

Registry No. 1, 94732-17-9; 2, 94732-18-0; 3, 94732-19-1; 4, 94732-20-4; 5, 94732-21-5; 6, 94732-22-6; 7, 94732-23-7; 8, 94732-24-8; 9, 94751-05-0; 10, 94732-25-9; 11, 94732-26-0; 12, 94732-27-1; 13, 94732-28-2; 14, 94732-29-3; 15, 94732-30-6; 16, 94732-31-7; 17, 94751-06-1; 18, 94732-32-8; 19, 94732-33-9; 20, 94732-34-0; 21, 94732-35-1; 22, 94732-36-2; 23, 94732-37-3; 24, 94732-38-4; 25, 94732-39-5; 26, 94732-40-8; 27, 94732-41-9; 28, 94732-42-0; 29, 94732-43-1; 30, 94732-44-2; 31, 94732-45-3; 32, 94732-46-4; 33, 94732-47-5; 36, 94732-50-0; 37, 94751-07-2; 38, 94732-51-1; 39, 94732-52-2; 40, 94732-53-3; 41, 94732-54-4; 42, 94732-55-5; 43, 94732-56-6; 44, 94732-57-7.

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Conformational Factors in Cardiac Glycoside Activity

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Gomphoside, a 5α-H cardiac glycoside isolated from *Asclepias fructicosa*, has an unique double glycosidic linkage to the aglycon through oxygen atoms at 2α and 3β of the steroid. The 3′-axial hydroxyl of its conformationally rigid sugar residue appears to be the functional group responsible for its potent inotropic activity. With use of gomphoside as the model compound, the conformation of the flexible glycosidic linkage of the 5β-H cardenolides, digitoxigenin α-L-rhamnoside and digitoxigenin β-D-digitoxoside, and the 5α-H cardenolides, uzarigenin α-L-rhamnoside and uzarigenin β-D-6-deoxyalloside, were investigated with the aid of computer graphics and conformational potential energy calculations. The relative inotropic potencies of these cardenolides can be accounted for by considering their active binding conformations with their potential energy distributions. The conformational distribution of the glycosidic moiety was postulated to be the major determinant of the biological activity of these cardenolides.

Cardiac glycosides are potent cardioactive agents that exert a positive inotropic effect unique to this class of compounds. Studies on the structure–activity relationships (SAR) in these compounds has long been a subject of interest and active research by many groups over many years.

It is generally accepted that an α,β-unsaturated lactone at 17β, a 14β-hydroxyl at the C/D cis ring junction, and a sugar residue at 3β of the C19 steroid nucleus are essential for optimal inotropic activity.[1] Introduction of extra hydroxyls to the aglycon generally reduces activity.

Gomphoside (I), first isolated in the Department of Pharmacy, University of Sydney,[2] was shown to have a unique chemical structure.[3,4] Apart from the A/B trans junction (where A/B cis is the more common configuration), the glycoside moiety is rigidly linked to the steroid through oxygen atoms at 2α and 3β of the steroid (Figure 1a). Several closely related cardenolides isolated later[5] were found to differ only in the configuration of substituent groups in the sugar moiety. The inotropic activity of these compounds was evaluated with use of isolated guinea pig atria, which is a commonly accepted technique that has been used by a number of groups to obtain comparable values for a wide variety of cardiac glycosides.[6-10] The results given (Table I)[11] are the potency of these compounds relative to digitoxigenin as the standard. The actual values, expressed as the molar concentration required to increase the force of contraction of the isolated

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