Articles

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

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Several optically active *N*-(indol-3-ylglyoxylyl)amino acid derivatives were synthesized and tested for [^{3}H]flunitrazepam displacing activity in bovine brain membranes. IC_{50} values were measured and revealed that the D form of the amino acid moiety of the compounds was more potent than both the L form and racemic form, suggesting a key role of the amino acid stereochemistry on the affinity to the benzodiazepine receptors. GABA ratio and proconvulsant/convulsant data reported for the most active compounds reveal they behave as inverse agonists at the benzodiazepine receptor.

The demonstration of high-affinity, saturable, and stereospecific binding sites for benzodiazepine in the central nervous system[1,2] has radically altered the concepts of the molecular mechanism of benzodiazepine action and stimulated a search for an "endogenous ligand" that physiologically acts on these receptors. The initial observations that β-carboline derivatives like harmane and norharmane and esters of β-carboline-3-carboxylic acid bind to the benzodiazepine receptor[3] have activated a vigorous search for structurally related compounds.[4-12]

In the past few years we examined some racemic *N*-(indol-3-ylglyoxylyl)amino acid derivatives **1** that contain an aminoethylindolic flexible structure analogous to that of β-carboline **2**. The ability of these compounds to inhibit specific [^{3}H]flunitrazepam binding from bovine cortical membranes is dependent on their hydrophobic and electronic properties as well as on amino acid molecular size.[13] Interaction of compounds with protein molecules, receptors, or metabolizing enzymes always implies the possibility of stereoselective action. In fact, stereoselectivity in receptor function is much more the rule than the exception, and stereoselective binding is sometimes used as one of the criteria for identifying an interaction with the receptor.[14,15] On the basis of these concepts we have now prepared some *N*-(indol-3-ylglyoxylyl)amino acid derivatives that have in their structures optically active amino acid moieties to study the influence of the amino acid stereochemistry on the affinity to the benzodiazepine receptors. An inhibition specificity of [^{3}H]diazepam binding was already demonstrated by Cain et al.[4] for geometric isomers and by Lippke et al. for enantiomeric compounds.[6]

In the present paper we report on the synthesis and interaction of optically active forms of those amino acids that had shown the greatest activity in the racemic form.[13] So L- and D-alanine derivatives were examined, while L- and D-phenylalanine compounds were examined to evaluate if the bigger lipophilic area may influence the enantioselective action of chiral compounds.

Chemistry

The general synthetic procedure used in the preparation of compounds **5–24** involved the acylation of the appropriate indole **3** with oxalyl chloride.[16] The resulting indoleglyoxylyl chlorides **4** were allowed to react in mild conditions with L- and D-alanine and with L- and D-phenylalanine ethyl ester hydrochlorides in benzene solution in the presence of triethylamine. The obtained glyoxamides **5–14** (Table I) were then converted to the acid derivatives **15–24** (Table II) by alkaline hydrolysis (see

Scheme I

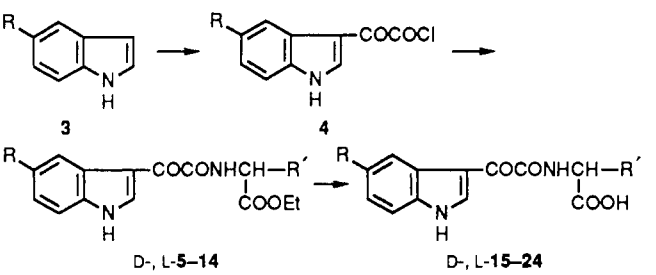

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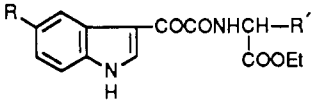
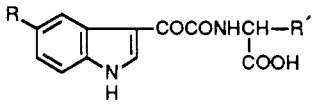
Table I. *N*-[(5-Substituted indol-3-yl)glyoxylyl]amino Acid Esters



no.	amino acid moiety config	R	R′	yield, %	cryst solvent	mp, °C	formula[a]	$[\alpha]_D$, deg
5	L	H	CH_3	56.5	methanol–water	118–120	$C_{15}H_{16}N_2O_4$	−9.63
	D	H	CH_3	65.5	ethanol–water	118–120	$C_{15}H_{16}N_2O_4$	+9.77
6	L	Br	CH_3	71.4	ethanol	182–184	$C_{15}H_{15}BrN_2O_4$	+9.54
	D	Br	CH_3	68.3	ethanol–water	182–184	$C_{15}H_{15}BrN_2O_4$	−9.69
7	L	Cl	CH_3	71.1	methanol–water	197–199	$C_{15}H_{15}ClN_2O_4$	+5.37
	D	Cl	CH_3	64.0	ethanol–water	195–197	$C_{15}H_{15}ClN_2O_4$	−5.49
8	L	NO_2	CH_3	34.1	methanol	219–221	$C_{15}H_{15}N_3O_6$	+17.10
	D	NO_2	CH_3	38.2	dioxane–water	218–220	$C_{15}H_{15}N_3O_6$	−17.30
9	L	OCH_3	CH_3	68.4	methanol	160–162	$C_{16}H_{18}N_2O_5$	+4.83
	D	OCH_3	CH_3	58.6	methanol–water	161–163	$C_{16}H_{18}N_2O_5$	−4.90
10	L	H	$CH_2C_6H_5$	66.6	benzene–pet. ether	148–150	$C_{21}H_{20}N_2O_4$	−8.61
	D	H	$CH_2C_6H_5$	27.9	benzene	150–152	$C_{21}H_{20}N_2O_4$	+8.41
11	L	Br	$CH_2C_6H_5$	52.1	benzene–pet. ether	124–126	$C_{21}H_{19}BrN_2O_4$	−19.51
	D	Br	$CH_2C_6H_5$	29.5	methanol–water	125–127	$C_{21}H_{19}BrN_2O_4$	+19.87
12	L	Cl	$CH_2C_6H_5$	57.1	benzene–pet. ether	120–123	$C_{21}H_{19}ClN_2O_4$	−18.12
	D	Cl	$CH_2C_6H_5$	38.6	methanol–water	119–121	$C_{21}H_{19}ClN_2O_4$	+18.39
13	L	NO_2	$CH_2C_6H_5$	34.7	methanol–water	173–175	$C_{21}H_{19}N_3O_6$	−27.81
	D	NO_2	$CH_2C_6H_5$	22.7	ethanol	174–176	$C_{21}H_{19}N_3O_6$	+27.85
14	L	OCH_3	$CH_2C_6H_5$	74.8	benzene–pet. ether	140–142	$C_{22}H_{22}N_2O_5$	−15.06
	D	OCH_3	$CH_2C_6H_5$	33.3	ethanol–water	139–141	$C_{22}H_{22}N_2O_5$	+14.89

[a] Elemental analyses for C, H, N were within ±0.4% of the calculated values.

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



no.	amino acid moiety config	R	R′	yield, %	cryst solvent	mp, °C	formula[a]	$[\alpha]_D$, deg
15	L	H	CH_3	66.5	methanol–water	203–205	$C_{13}H_{12}N_2O_4$	+5.92
	D	H	CH_3	81.6	methanol–water	204–206	$C_{13}H_{12}N_2O_4$	−5.85
16	L	Br	CH_3	75.6	methanol–water	228–230 dec	$C_{13}H_{11}BrN_2O_4$	+21.63
	D	Br	CH_3	84.0	methanol–water	228–230 dec	$C_{13}H_{11}BrN_2O_4$	−21.46
17	L	Cl	CH_3	80.5	methanol–water	212–215	$C_{13}H_{11}ClN_2O_4$	+19.28
	D	Cl	CH_3	79.4	ethanol–water	213–215	$C_{13}H_{11}ClN_2O_4$	−18.94
18	L	NO_2	CH_3	75.1	methanol–water	218–220	$C_{13}H_{11}N_3O_6$	+29.97
	D	NO_2	CH_3	67.3	ethanol–water	219–221	$C_{13}H_{11}N_3O_6$	−30.11
19	L	OCH_3	CH_3	81.9	methanol–water	208–210	$C_{14}H_{14}N_2O_5$	+18.57[b]
	D	OCH_3	CH_3	79.2	methanol	208–210	$C_{14}H_{14}N_2O_5$	−18.75[b]
20	L	H	$CH_2C_6H_5$	32.5	ethanol–water	98–100	$C_{19}H_{16}N_2O_4$	+9.92
	D	H	$CH_2C_6H_5$	62.5	ethyl acetate-pet. ether	98–100	$C_{19}H_{16}N_2O_4$	−9.87
21	L	Br	$CH_2C_6H_5$	88.1	methanol–water	187–189	$C_{19}H_{15}BrN_2O_4$	−11.76
	D	Br	$CH_2C_6H_5$	78.6	methanol–water	188–190	$C_{19}H_{15}BrN_2O_4$	+11.56
22	L	Cl	$CH_2C_6H_5$	53.3	methanol–water	168–170	$C_{19}H_{15}ClN_2O_4$	−6.85
	D	Cl	$CH_2C_6H_5$	72.2	ethanol–water	170–171	$C_{19}H_{15}ClN_2O_4$	+7.26
23	L	NO_2	$CH_2C_6H_5$	81.4	ethanol–water	118–120	$C_{19}H_{15}N_3O_6H_2O$	−21.78
	D	NO_2	$CH_2C_6H_5$	43.8	methanol–water	117–119	$C_{19}H_{15}N_3O_6$	+21.76
24	L	OCH_3	$CH_2C_6H_5$	78.1	ethanol–water	105–108	$C_{20}H_{18}N_2O_5H_2O$	−1.71
	D	OCH_3	$CH_2C_6H_5$	77.9	methanol-water	104–106	$C_{20}H_{18}N_2O_5$	+1.96

[a] Elemental analyses for C, H, N were within ±0.4% of the calculated values. [b] $[\alpha]_D$ values recorded in methanol solution.

Scheme I).

As concerns the optical purity of these compounds, the $[\alpha]_D$ reported values clearly indicate that none of the compounds was converted to its racemic form, under the experimental conditions we employed. In fact, although the chiral center is never involved in our reactions, it was thought that enantiomeric compounds **15–24** could be susceptible to racemization under the basic pH conditions used in the hydrolysis of ethyl esters **5–14**. So the optical purity of the enantiomers **15–24** was established by reconversion to the ethyl esters,[17] obtaining $[\alpha]_D$ values practically the same as those registered before hydrolysis.

Binding to the Benzodiazepine Receptors

Results. The ability of chiral indole derivatives to displace specific [^{3}H]flunitrazepam binding was studied in membrane prepared from bovine cerebral cortex with a radioligand concentration of 0.9 nM. The inhibition of [^{3}H]flunitrazepam binding was initially examined at a single concentration (250 μM) of the displacing agent, and then for the most active compounds IC_{50} values were determined from log–probit plots using six to eight concentrations. The resulting data (Tables III and IV) show that 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 de-

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

no.	R	R′	inhibn,[a] % (250 μM)	IC_{50},[b] μM	GABA ratio[d]
5-D,L[c]	H	CH_3	80 ± 7[c]	13.00 ± 1.50[c]	
5-D	H	CH_3		7.50 ± 0.50	
5-L	H	CH_3		17.00 ± 2.00	
6-D,L[c]	Br	CH_3	100[c]	4.8 ± 0.50[c]	
6-D	Br	CH_3		0.18 ± 0.02	0.78
6-L	Br	CH_3		52.00 ± 3.00	0.85
7-D,L[c]	Cl	CH_3	100[c]	0.50 ± 0.04[c]	
7-D	Cl	CH_3		0.15 ± 0.01	0.90
7-L	Cl	CH_3		25.00 ± 2.00	0.92
8-D,L[c]	NO_2	CH_3	100[c]	0.20 ± 0.02[c]	
8-D	NO_2	CH_3		0.07 ± 0.005	0.67
8-L	NO_2	CH_3		3.00 ± 0.20	0.79
9-D	OCH_3	CH_3		0.90 ± 0.1	
9-L	OCH_3	CH_3		110.00 ± 15.0	
10-D	H	$CH_2C_6H_5$	69 ± 5		
10-L	H	$CH_2C_6H_5$	55 ± 4		
11-D,L[c]	Br	$CH_2C_6H_5$	65 ± 6[c]	180.0 ± 15.0[c]	
11-D	Br	$CH_2C_6H_5$		55.0 ± 4.0	
11-L	Br	$CH_2C_6H_5$		220.0 ± 12.0	
12-D	Cl	$CH_2C_6H_5$		30.0 ± 2.0	
12-L	Cl	$CH_2C_6H_5$		45.0 ± 3.0	
13-D	NO_2	$CH_2C_6H_5$		0.10 ± 0.01	
13-L	NO_2	$CH_2C_6H_5$		2.80 ± 0.09	
14-D,L[c]	OCH_3	$CH_2C_6H_5$	60 ± 2[c]		
14-D	OCH_3	$CH_2C_6H_5$	77 ± 5		
14-L	OCH_3	$CH_2C_6H_5$	30 ± 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 determinants. [c] See ref 4. [d] GABA ratio = IC_{50} without GABA/IC_{50} with GABA.

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

no.	R	R′	inhibn[a], % (250 μM)	IC_{50},[b] μM
15-D,L[c]	H	CH_3	72 ± 4[c]	105.00 ± 9.80[c]
15-D	H	CH_3		48.00 ± 3.00
15-L	H	CH_3	32 ± 2	304.00 ± 20.00
16-D,L[c]	Br	CH_3		40.01 ± 3.50[c]
16-D	Br	CH_3		7.10 ± 0.05
16-L	Br	CH_3		80.00 ± 5.00
17-D,L[c]	Cl	CH_3	93 ± 8[c]	22.00 ± 2.00[c]
17-D	Cl	CH_3		5.01 ± 0.30
17-L	Cl	CH_3		46.01 ± 3.90
18-D,L[c]	NO_2	CH_3	94 ± 7[c]	6.20 ± 0.60[c]
18-D	NO_2	CH_3		3.00 ± 0.20
18-L	NO_2	CH_3		19.00 ± 2.00
19-D	OCH_3	CH_3		22.00 ± 2.00
19-L	OCH_3	CH_3		170.00 ± 15.00
20-D	H	$CH_2C_6H_5$	47 ± 5	
20-L	H	$CH_2C_6H_5$	32 ± 2	
21-D,L[c]	Br	$CH_2C_6H_5$	62 ± 4[c]	158.00 ± 12.80[c]
21-D	Br	$CH_2C_6H_5$		60.00 ± 6.00
21-L	Br	$CH_2C_6H_5$	51 ± 4	245.00 ± 21.00
22-D	Cl	$CH_2C_6H_5$		12.00 ± 1.00
22-L	Cl	$CH_2C_6H_5$		48.00 ± 5.00
23-D	NO_2	$CH_2C_6H_5$		4.00 ± 0.30
23-L	NO_2	$CH_2C_6H_5$		21.00 ± 1.80
24-D,L[c]	OCH_3	$CH_2C_6H_5$	38 ± 5[c]	
24-D	OCH_3	$CH_2C_6H_5$	50 ± 3	
24-L	OCH_3	$CH_2C_6H_5$	18 ± 2	

[a-c] See footnotes *a–c* of Table III.

creased 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 masking of the ionizable functions is crucial for the dramatic increase in receptor affinity, as already shown for β-carboline by other authors.[4] The size of the amino acid present in the compound is important as shown by the higher activity of the compounds where alanine is present. These findings were already evidenced in the previous work[13] for the racemic form of the compounds and remain unchanged for the chiral form. The stereoisomer forms showed different quantitative activities. For all the compounds examined, the D form was more active than the L form in displacing [^{3}H]flunitrazepam binding from bovine brain membranes.

Table V. Biological Activity of Some *N*-[(5-Substituted indol-3-yl)glyoxylyl]amino Acid Esters

no.	R	R′	proconvulsant actions:[a] ED_{50}, mg/kg	diazepam antagonism:[b] ED_{50}, mg/kg
6-D	Br	CH_3	40	50
8-D	NO_2	CH_3	150	100

[a] Dose necessary to induce convulsions in 50% of the mice that hade been previously given a subconvulsant dose of PTZ (40 mg/kg). [b] Dose necessary to antagonize the anticonvulsant effects of diazepam (2.5 mg/kg) in mice that had been given a convulsant dose of PTZ (80 mg/kg); see ref 8 for details.

The data reported indicate that of the stereoisomeric indole derivatives tested the structure–activity relationships (SAR) were similar to those obtained for the racemic form. The higher activity of the D enantiomers shows that chirality has a strong influence on the benzodiazepine receptor affinity. In fact, when moving from racemic compounds to individual enantiomers, the stereochemical requirements of biological activity become apparent over all for the most active *N*-(indol-3-ylglyoxylyl)amino acid derivatives examined, where a higher activity increase in inhibiting [^{3}H]flunitrazepam binding is evidenced. These stereochemical requirements may help to elucidate the structure of specific binding sites that are asymmetric and thus prefer to bind one enantiomer over the other.

Moreover, using an exhaustively washed membrane preparation we evaluated the GABA ratio values as an in vitro indicator of the agonist, inverse agonist, or antagonist properties, according to the suggestions of different authors,[18,19] for our most active compounds. The results, shown in Table III, indicate that the examined compounds behave as inverse agonists.

Compounds 6-D and 8-D were also evaluated in vivo since they showed the most potent binding affinity to the receptor in vitro. These compounds were tested in mice for anticonvulsant properties by using the convulsant pentylenetetrazole (PTZ, 80 mg/kg) and for proconvulsant actions by employing a subthreshold dose of PTZ (40 mg/kg). They were also tested as benzodiazepine antagonists by assessing their ability to disrupt the anticonvulsant actions of diazepam (2.5 mg/kg) against PTZ (80 mg/kg). None of the compounds tested displayed anticonvulsant activity since PTZ (80 mg/kg) produced seizures in all animals even at the highest doses (200 mg/kg) tested. Compound 6-D showed proconvulsant activity with an ED_{50} of 40 mg/kg and antagonized the anticonvulsant effects of diazepam with an ED_{50} of 50 mg/kg. Compound 8-D behaved as a proconvulsant (ED_{50} = 150 mg/kg) and also antagonized the anticonvulsant actions of diazepam with an ED_{50} of 100 mg/kg. These results (Table V) confirm that these compounds behave as inverse agonists as also shown by the in vitro GABA ratio experiments.

Discussion. It has been hypothesized[20] that the recognition site for the β-carboline inverse agonist ligands on

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the GABA/Bz/chloride channel receptor complex is most probably a planar cleft where the main drug–receptor interaction is mediated by the indole N(9)-H, the β-carboline N(2), and the carbonyl group of the ester or amide function at the 3-position. According to this model, the structural requirements for inverse agonists are located on the C=O, N(2), N(9) line.

More recently it has been reported that β-carbolines with a group different from a carbonyl function[11] or devoid of a substituent at the 3-position[9] possess moderate to high affinities to the benzodiazepine receptor. Moreover, completely planar and rigid structures as pyridodiindoles[8] and azapyridodiindoles[12] have been shown to bind to the benzodiazepine receptor with potent affinity. These findings have led to a model with two points of interaction for the benzodiazepine receptor inverse agonist site: a hydrogen bond donor site and a hydrogen bond acceptor site.[11] This supports the involvement of the indole N–H in a hydrogen bond interaction of the ligand with a hydrogen bond donor site and of the β-carboline N(2) with a hydrogen bond acceptor site on the benzodiazepine receptor in a planar or pseudoplanar domain.[4,7,11]

The *N*-(indol-3-ylglyoxylyl)amino acid derivatives described in this paper bind to the benzodiazepine receptor with moderately to high affinities, and on the basis of GABA ratio values and proconvulsant/convulsant paradigm for the most active compounds, they behave as inverse agonists or partial inverse agonists. It is therefore plausible to suppose that they interact with the same receptor site, a planar cleft as hypothesized by Cook and Trudell.[11] This could mean that our compounds, with a more flexible structure than a completely planar β-carboline, assume a planar or pseudoplanar conformation in the interaction with the receptor site. From theoretical calculations[21] the conformation shown in Figure 1 should be one of the favored (the aromatic ring and the glyoxylylamide moiety lie approximately in the same plane). The interaction of our ligands with the receptor area should still occur via the indole NH hydrogen bond acceptor and the C=O(2) of the glyoxylyl bridge as hydrogen bond donor. The glyoxylylamino acid chain in the 3-position of indole nucleus adds a further point of attachment to the receptor and has a tremendous effect on the affinity, considering the very low IC_{50} value of indole itself (IC_{50} = 1.8 mM). In this case the oxygen atom of the second carbonyl function should play the same role as the β-carboline N(2) for a hydrogen bond with the receptor acceptor site. Although this last point need further studies to be confirmed, our hypothesis is consistent with the fact that an electron-withdrawing group (NO_2, Cl, Br) at the 5-position of the indole nucleus increase the affinity of our compounds, enhancing the ability of indole NH to interact with a hydrogen bond donor site on the receptor via polarization of the indole NH bond, as already reported by other authors.[11]

We already have evidenced the importance of hydrophobic, electronic, and steric factors of the side chain for the analogous racemic compounds.[13] We wish now to stress the importance of the stereochemistry of the chiral center of the amino acid residue on the affinity of our ligands on the benzodiazepine receptor. The D-form compounds show a higher affinity than the L-form compounds, with IC_{50} value differences of 1 or 2 orders of magnitude. It appears therefore that the glyoxylylamino acid chain in the 3-position of the indole interacts with an asymmetric region of the benzodiazepine receptor. The very high affinity of only one of the two enantiomers of chiral 3-substituted benzodiazepines[22] supports the existence of such an asymmetric region. Moreover a stereoselective binding, although not as dramatically, was also reported by Lippke et al.[6] for chiral β-carboline-3-carboxylic acid amides and by Cain et al.[4] for geometric isomer tetrahydro-β-carbolines.

At this time it is not possible to elucidate the nature of this asymmetrical region and its influence on the efficacy of different ligands. Further studies in our Laboratories will be directed to better define the stereochemical requirements of ligands for a high affinity to benzodiazepine receptor.

Experimental Section

Chemistry. All the amino acids were optically active of L and D configurations. Melting points were determined on a Köfler hot-stage apparatus and are uncorrected. IR spectra were recorded with a Pye Unicam Infracord Model PU 9516 in Nujol mulls. ^1H NMR spectra were determined in DMSO-d_6 with TMS as an internal standard on a Varian EM 360 A spectrometer and were also consistent with assigned structures. The $[\alpha]_D$ values were measured with a Perkin-Elmer Model 241 polarimeter in acetone solution, if not otherwise indicated. Magnesium sulfate was always used as the drying agent. Evaporations were made in vacuo (rotating evaporator). Elemental analyses were performed by our Analytical Laboratory and agreed with theoretical values to within ±0.4%.

General Procedure for the Synthesis of the L- and D-*N*-[(5-Substituted indol-3-yl)glyoxylyl]amino Acid Ethyl Esters 5–14. A. Triethylamine (0.11 mol) and 0.55 mol of the appropriate L- or D-amino acid ethyl ester hydrochloride were added to a stirred suspension of 0.05 mol of indol-3-ylglyoxylyl chloride **4**. The reaction mixture was stirred for 24 h at room temperature, refluxed for 2 h, and then filtered. The collected precipitate was washed with water to eliminate the triethylamine hydrochloride, triturated with saturated aqueous $NaHCO_3$ solution, and collected again to give crude **5–14**. The benzene solution was washed with diluted HCl, with 10% aqueous $NaHCO_3$ and water, dried, and evaporated to dryness to yield additional amounts of compounds **5–14**. The quantities obtained from the initial insoluble precipitate or from the benzene solution are variable, depending upon the solubility of the various glyoxamides (Table I).

B. Compounds **15–24** (0.200 g) were added to a cooled (−10 °C) mixture of 20 mL of absolute ethanol and 0.1 mL of thionyl chloride. The reaction mixture was heated at reflux for 2 h, cooled at room temperature, and evaporated to dryness to yield crude **5–14**.

General Procedure for the Preparation of the L- and D-*N*-[(5-Substituted indol-3-yl)glyoxylyl]amino Acids 15–24. A suspension of 1 g of compounds **5–14** in 20 mL of 10% aqueous NaOH was stirred to solution at room temperature (in some cases a gentle warming to 40–50 °C was necessary). The solution was then acidified with hydrochloric acid and the resulting precipitate was collected and treated with 10% aqueous $NaHCO_3$. After filtration, the solution was made acid with HCl and the precipitate solid was collected, washed with water, and purified by crystallization (Table II).

Binding Studies. Tritiated flunitrazepam was obtained from New England Nuclear (Dreieichenhaim, West Germany) and had a specific activity of 76.9 Ci/mmol and a radiochemical purity >99%. All the other chemicals were of reagent grade and obtained from commercial suppliers.

Membrane preparation was obtained essentially as previously described.[13] Bovine cerebral cortex was homogenized in 10 vol (w/v) of 0.32 M ice-cold sucrose containing protease inhibitors.[23] The homogenate was centrifuged at 1000*g* for 10 min at 4 °C. The resultant pellet was discarded and the supernatant was recen-

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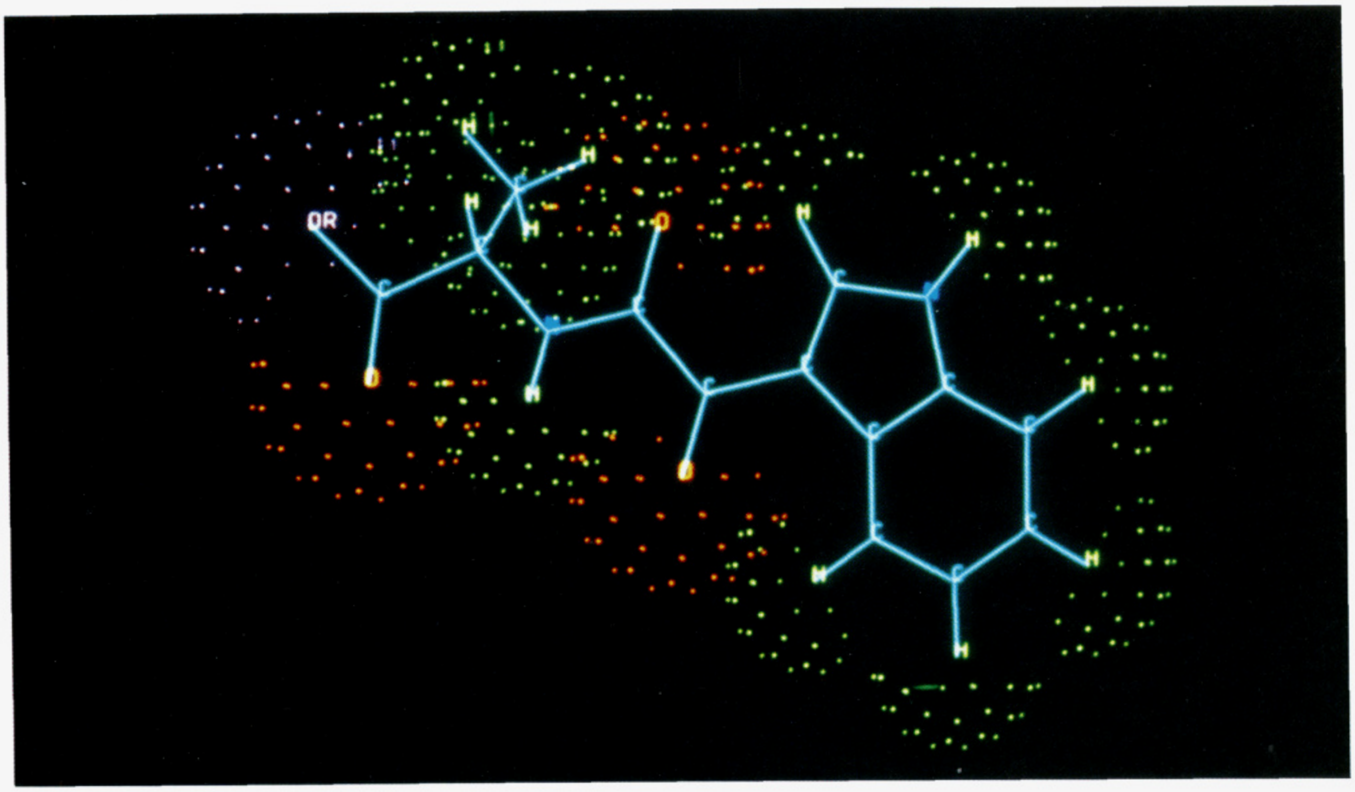

Figure 1. Stereoview of the pseudoplanar conformation of *N*-(indol-3-ylglyoxylyl)alanine derivatives.

trifuged at 48000*g* for 30 min at 4 °C. The resultant pellet was osmotically shocked by suspension in 10 vol of 50 mM Tris-HCl buffer at pH 7.4 containing protease inhibitors and recentrifuged at 48000*g* for 30 min at 4 °C. The pellet was suspended in 50 mM Tris-HCl buffer at pH 7.4 and used for benzodiazepine binding assays as follows: membrane suspension (0.5 mg of proteins) was incubated in triplicate with 0.9 nM [^{3}H]flunitrazepam at 0 °C (90 min) in the buffer in a final volume of 0.5 mL. After incubation the samples were diluted at 0 °C with 5 mL of the assay buffer and were immediately filtered under reduced pressure through glass fiber filter disks (Whatman GF/B). The filters were washed with 5 mL of the buffer, dried, and added to 8 mL of HP Beckman scintillation liquid, containing 0.4 mL of 0.01 N KOH solution in plastic vials. Water-insoluble ester derivatives were dissolved in 50% ethanol buffer, and the same mixture was present in blank experiments. The nonspecific binding was determined by incubating membrane and [^{3}H]flunitrazepam without (control) or with the compound in the presence of 10 μM diazepam. The estimation of proteins was based on the method of Lowry et al.,[24] after solubilization with 0.75 N NaOH. Bovine serum albumin was utilized as standard. The concentrations of D- or L-*N*-(indol-3-ylglyoxylyl)amino acid derivatives that inhibit specific [^{3}H]flunitrazepam binding by 50% (IC_{50}) were determined by log–probit analysis with six to eight concentrations of the displacers, each performed in triplicate. Membranes used for the determination of the GABA ratio were exhaustively washed as described by Martini et al.[25] IC_{50} determinations for GABA ratio values were carried out in the absence and in the presence of 10 μM GABA.

In Vivo Studies. Groups of 10 mice were injected intraperitoneally (0.1 mL) with graded doses of the compounds suspended in 1% (carboxymethyl)cellulose (vehicle) or an equal volume of vehicle, followed 30 min later by PTZ at 80 or 40 mg/kg to assess the anticonvulsant and proconvulsant actions, respectively, as described by Trudell et al.[8]

Antagonism of the anticonvulsant effects of diazepam was carried out as described by Cain et al.[4] Groups of 10 mice were injected with diazepam (2.5 mg/kg ip) followed 15 min later by administration of graded doses of agents or vehicle. Fifteen minutes after injection of the compound, animals were injected with PTZ (80 mg/kg).

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Registry No. **4** (R = H), 22980-09-2; **4** (R = Br), 63843-81-2; **4** (R = Cl), 883-55-6; **4** (R = NO_2), 6953-35-1; **4** (R = OMe), 2426-19-9; L-**5**, 94732-18-0; D-**5**, 122334-57-0; L-**6**, 94732-28-2; D-**6**, 122334-58-1; L-**7**, 94732-24-8; D-**7**, 122334-59-2; L-**8**, 94732-32-8; D-**8**, 122334-60-5; L-**9**, 122334-61-6; D-**9**, 122334-62-7; L-**10**, 122334-63-8; D-**10**, 122334-64-9; L-**11**, 94732-30-6; D-**11**, 122334-65-0; L-**12**, 122334-66-1; D-**12**, 122334-67-2; L-**13**, 122334-68-3; D-**13**, 122334-69-4; L-**14**, 94732-36-2; D-**14**, 122334-70-7; L-**15**, 94732-38-4; D-**15**, 122334-71-8; L-**16**, 122334-72-9; D-**16**, 122334-73-0; L-**17**, 94732-44-2; D-**17**, 122334-74-1; L-**18**, 94732-53-3; D-**18**, 122334-75-2; L-**19**, 122334-76-3; D-**19**, 122334-77-4; L-**20**, 122334-78-5; D-**20**, 122334-79-6; L-**21**, 94751-07-2; D-**21**, 122334-80-9; L-**22**, 122334-81-0; D-**22**, 122334-82-1; L-**23**, 122334-83-2; D-**23**, 122334-84-3; L-**24**, 94732-57-7; D-**24**, 122334-85-4; H-Ala-OEt·HCl, 1115-59-9; H-D-Ala-OEt·HCl, 6331-09-5; H-Phe-OEt·HCl, 3182-93-2; H-D-Phe-OEt·HCl, 63060-94-6; benzodiazepine, 12794-10-4.

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