6-Alkyl-12-formylindolo[2,1-*a*]isoquinolines. Syntheses, Estrogen Receptor Binding Affinities, and Stereospecific Cytostatic Activity

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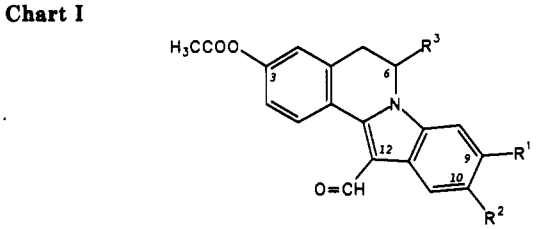
A number of 6-alkyl-12-formyl-5,6-dihydroindolo[2,1-*a*]isoquinolines were synthesized by the Bischler–Napieralski reaction from the respective 1-alkyl-2-(3-methoxyphenyl)ethylamines and bromo-substituted (methoxyphenyl)acetic acid chlorides followed by a second ring closure reaction involving a base-generated benzyne intermediate. The methoxy functions in positions 3 and 9 or 10 were cleaved with BBr_3 and the free hydroxy groups converted into the acetates. The enantiomers of the most potent derivatives were separated by liquid chromatography on triacetylcellulose. All of the compounds tested bind to the calf uterine estrogen receptor. The relative binding affinities (RBA) ranged from 0.5 to 3.9 (17β-estradiol: RBA = 100) and were dependent on the position of the oxygen function in the indole moiety. The 3,10-diacetoxy derivatives showed higher RBA values than the corresponding 3,9-substituted tetracycles. There was no major difference in binding affinity between (+)- and (−)-enantiomers. Computer-assisted molecular modeling studies showed that the chiral carbon atom 6 of the indoloisoquinoline is likely to mimic the C-11 atom of estradiol. In the mouse uterine weight test, only the 3,10-diacetoxy series exhibited weak estrogenic activity at higher doses. The antiestrogenic effects found with all the compounds varied considerably. Maximum inhibition of estrone-stimulated uterine growth was found for the ethyl derivative **7d** (80% with 250 μg/animal per day). All derivatives strongly inhibited the growth of human breast cancer cells in vitro. There was no significant difference between hormone-sensitive MCF-7 cells and hormone-independent MDA-MB 231 cells. Cytostatic activity was higher for the 3,9-substituted indoloisoquinolines than for the 3,10-analogues and dependent on the length of the alkyl group at C-6. The maximum effect was found with the butyl derivative **7g**. When the enantiomers of the ethyl (**7c**), propyl (**7e**), and butyl derivative were studied, a strong difference in activity was observed between the stereoisomers. The IC_{50} values of the (+)-forms were usually only a tenth of those of the levorotatory isomers. Maximum cytostatic activity was found with (+)-**7g**: 85% inhibition at 1×10^{-7} M in MCF-7 cells and 94% inhibition at 2.5×10^{-7} M in MDA-MB 231 cells. This stereospecificity indicates a selective action on a biochemical target. Since no interaction with DNA was observed, the precise mode of action still remains to be elucidated.

The therapeutic efficacy of antiestrogens, particularly that of tamoxifen, in the therapy of breast cancer, both in advanced disease and in the adjuvant treatment of primary breast cancer, is well established.[1] However, approximately 40% of patients with estrogen receptor positive breast tumors do not show any clinical response to tamoxifen treatment.[2] In patients responding to this treatment, the average period of remission is only 14 months.[1] Therefore, it is desirable to search for new drugs with higher efficacy or a more prolonged duration of response. In a number of studies we used different approaches for the improvement of endocrine therapy of hormone-dependent carcinomas of the breast. We have investigated the therapeutic potential of number of different nonsteroidal heterocyclic systems including 2-phenylindoles,[3] -benzofurans,[4] -benzothiophenes,[5] and benzocarbazoles.[6] We were able to increase the antiestrogenic potency of nonsteroidal agents considerably by the introduction of basic side chains into some of these heterocycles.[7] Other groups used triphenylethylene,[8,9] diphenylnaphthalene,[10] and 3-aroyl-2-phenylbenzo[*b*]-thiophene[11] as the nonsteroidal structure or estradiol[12–14] as the receptor binding moiety for the development of potent antiestrogens. In order to improve antitumor activity we linked the 2-phenylindole-based antiestrogen zindoxifene to different diaminodichloroplatinum(II) complexes.[15,16] Following this line, we have also been interested in compounds deriving from tetracyclic alkaloids with inherent cytostatic activity. We modified these structures in a way that they bind to the estrogen receptor and act possibly via this target protein.[17,18]

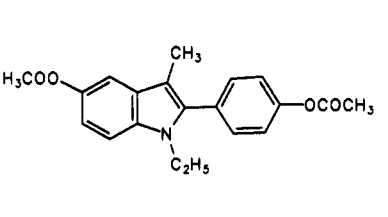
One of the structures under consideration has been the tetracyclic indolo[2,1-*a*]isoquinoline. In a previous paper, Ambros et al. reported on the binding affinities of indoloisoquinolines with a varying number of acetoxy groups for steroid hormone receptors.[19] They also showed that

Chart I

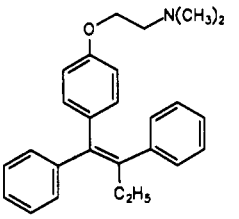


R^1, R^2 = H, $OCOCH_3$

R^3 = CH_3 – C_5H_{11}



ZINDOXIFENE



TAMOXIFEN

† Institut für Pharmazie.
‡ Institut für Organische Chemie.

(1) Furr, B. J. A.; Jordan, V. C. The Pharmacology and Clinical Uses of Tamoxifen. *Pharmacol. Ther.* **1984**, *25*, 127–205.
(2) Maas, H.; Jonat, W.; Stolzenbach, G.; Trams, G. The Problem of Nonresponding Estrogen Receptor-positive Patients with Advanced Breast Cancer. *Cancer* **1980**, *46*, 2783–2787.
(3) von Angerer, E.; Prekajac, J.; Strohmeier, J. 2-Phenylindoles. Relationship between Structure, Estrogen Receptor Affinity, and Mammary Tumor Inhibiting Activity in the Rat. *J. Med. Chem.* **1984**, *27*, 1439–1447.

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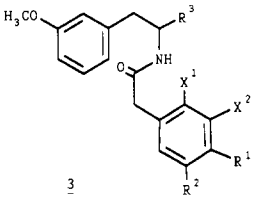
Table I. 2-(Methoxyphenyl)-*N*-[2-(methoxyphenyl)ethyl]acetamides **3** and 1-Benzyl-1,2,3,4-tetrahydroisoquinolines **4**



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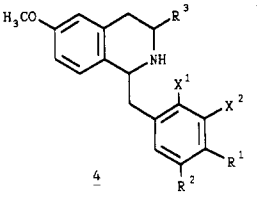
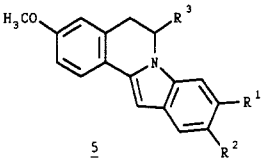


4

compd	R^1	R^2	R^3	X^1	X^2	formula[a]	mp (°C)	compd	formula[b]	bp (°C/Torr)
3a	OCH_3	H	CH_3	H	Br	$C_{19}H_{22}BrNO_3$	89–91	**4a**	$C_{19}H_{22}BrNO_2$	–
3b[c]	H	OCH_3	CH_3	Br	H	$C_{19}H_{22}BrNO_3$	111–113	**4b**[c]	$C_{19}H_{22}BrNO_2$	–
3c[c]	OCH_3	H	C_2H_5	H	Br	$C_{20}H_{24}BrNO_3$	110	**4c**[c]	$C_{20}H_{24}BrNO_2$	158/0.2
3d[c]	H	OCH_3	C_2H_5	Br	H	$C_{20}H_{24}BrNO_3$	108–110	**4d**[c]	$C_{20}H_{24}BrNO_2$	156/0.2
3e	OCH_3	H	C_3H_7	H	Br	$C_{21}H_{26}BrNO_3$	102	**4e**	$C_{21}H_{26}BrNO_2$	160–162/0.15
3f	H	OCH_3	C_3H_7	Br	H	$C_{21}H_{26}BrNO_3$	93–95	**4f**	$C_{21}H_{26}BrNO_2$	144/0.15
3g	OCH_3	H	C_4H_9	H	Br	$C_{22}H_{28}BrNO_3$	82–84	**4g**	$C_{22}H_{28}BrNO_2$	146/0.15
3h	H	OCH_3	C_4H_9	Br	H	$C_{22}H_{28}BrNO_3$	92–93	**4h**	$C_{22}H_{28}BrNO_2$	134/0.2
3i	OCH_3	H	C_5H_{11}	H	Br	$C_{23}H_{30}BrNO_3$	76–78	**4i**	$C_{23}H_{30}BrNO_2$	135/0.1
3j	H	OCH_3	C_5H_{11}	Br	H	$C_{23}H_{30}BrNO_3$	92–94	**4j**	$C_{23}H_{30}BrNO_2$	146/0.15

[a] Analyzed for C and H within ±0.4% of the calculated values. [b] All compounds were obtained as oils, except **4a** (yellow resin) and **4b** (solid, mp 58 °C). [c] Reference 19.

Table II. 5,6-Dihydro-dimethoxyindolo[2,1-*a*]isoquinolines **5** and 12-Formyl-5,6-dihydro-dimethoxyindolo[2,1-*a*]isoquinolines **6**



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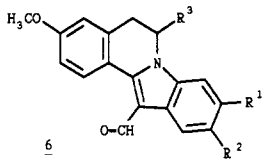

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compd	R^1	R^2	R^3	yield (%)	formula[a]	mp[b] (°C)	compd	yield (%)	formula[a]	mp[c] (°C)
5a	OCH_3	H	CH_3	32	$C_{19}H_{19}NO_2$	167–168	**6a**	91	$C_{20}H_{19}NO_3$	172
5b[d]	H	OCH_3	CH_3	35	$C_{19}H_{19}NO_2$	160–161	**6b**	91	$C_{20}H_{19}NO_3$	152–153
5c[d]	OCH_3	H	C_2H_5	55	$C_{20}H_{21}NO_2$	137–138	**6c**	81	$C_{21}H_{21}NO_3$	196
5d[d]	H	OCH_3	C_2H_5	36	$C_{20}H_{21}NO_2$	103	**6d**[d]	88	$C_{21}H_{21}NO_3$	116
5e	OCH_3	H	C_3H_7	42	$C_{21}H_{23}NO_2$	97–98	**6e**	90	$C_{22}H_{23}NO_3$	177
5f	H	OCH_3	C_3H_7	34	$C_{21}H_{23}NO_2$	142–143	**6f**	87	$C_{22}H_{23}NO_3$	114–115
5g	OCH_3	H	C_4H_9	57	$C_{22}H_{25}NO_2$	89	**6g**	93	$C_{23}H_{25}NO_3$	160
5h	H	OCH_3	C_4H_9	33	$C_{22}H_{25}NO_2$	137–138	**6h**	86	$C_{23}H_{25}NO_3$	101
5i	OCH_3	H	C_5H_{11}	35	$C_{23}H_{27}NO_2$	86–87	**6i**	80	$C_{24}H_{27}NO_3$	97
5j	H	OCH_3	C_5H_{11}	32	$C_{23}H_{27}NO_2$	111–112	**6j**	92	$C_{24}H_{27}NO_3$	103

[a] Analyzed for C and H within ±0.4% of the calculated values. [b] Recrystallization from EtOH. [c] Recrystallization from MeOH. [d] Reference 19.

the introduction of a formyl group into position 12 of the tetracycle improved the cytostatic potency of this system.[17]

These findings prompted us to synthesize a number of 12-formyl-5,6-dihydroindolo[2,1-*a*]isoquinolines (Chart I)

(4) Erber, S.; Ringshandl, R.; von Angerer, E. 2-Phenylbenzo[b]-furans: Relationship between Structure, Estrogen Receptor Affinity and Cytostatic Activity against Mammary Tumor Cells. *Anti-Cancer Drug Des.* **1991**, *6*, 417–426.

(5) von Angerer, E.; Erber, S. 3-Alkyl-2-phenylbenzo[b]-thiophenes: Nonsteroidal Estrogen Antagonists with Mammary Tumor Inhibiting Activity. *J. Steroid Biochem. Mol. Biol.* **1992**, *41*, 557–562.

(6) von Angerer, E.; Prekajac, J. Benzo[*a*]carbazole Derivatives. Synthesis, Estrogen Receptor Binding Affinities, and Mammary Tumor Inhibiting Activity. *J. Med. Chem.* **1986**, *29*, 380–386.

(7) von Angerer, E.; Knebel, N.; Kager, M.; Gan*β*, B. 1-(Aminoalkyl)-2-phenylindoles as Novel Pure Estrogen Antagonists. *J. Med. Chem.* **1990**, *33*, 2635–2640.

(8) Bignon, E.; Pons, M.; de Paulet, A. C.; Doré, J. C.; Gilbert, J.; Abecassis, J.; Miguel, J. F.; Ojasoo, T.; Raynaud, J. P. Effect of Triphenylacrylonitrile Derivatives on Estradiol-receptor Binding and on Human Breast Cancer Cell Growth. *J. Med. Chem.* **1989**, *32*, 2092–2103.

(9) Murphy, C. S.; Parker, C. J.; McCague, R.; Jordan, V. C. Structure-activity Relationship of Nonisomerizable Derivatives of Tamoxifen: Importance of Hydroxyl Group and Side Chain Positioning for Biological Activity. *Mol. Pharmacol.* **1991**, *39*, 421–428.

(10) Jones, C. D.; Blaszczak, L. C.; Goettel, M. E.; Suarez, T.; Crowell, T. A.; Mabry, T. E.; Ruenitz, P. C.; Srivatsan, V. Antiestrogens. 3. Estrogen Receptor Affinities and Antiproliferative Effects in MCF-7 Cells of Phenolic Analogues of Trioxifene, [3,4-Dihydro-2-(4-methoxyphenyl)-1-naphthalenyl][4-[2-(1-pyrrolidinyl)ethoxy]phenyl]methanone. *J. Med. Chem.* **1992**, *35*, 931–938.

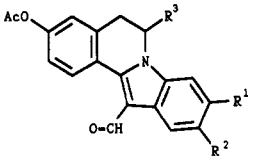
(11) Jones, C. D.; Jevnikar, M. G.; Pike, A. J.; Peters, M. K.; Black, L. J.; Thompson, A. R.; Falcone, J. F.; Clemens, J. A. Antiestrogens. 2. Structure-activity Studies in a Series of 3-Aroyl-2-arylbenzo[*b*]thiophene Derivatives Leading to 6-Hydroxy-2-(4-hydroxyphenyl)benzo[*b*]thien-3-yl-[4-[2-(1-piperidinyl)ethoxy]phenyl]methanone Hydrochloride (LY156758), a Remarkably Effective Estrogen Antagonist with Only Minimal Intrinsic Estrogenicity. *J. Med. Chem.* **1984**, *27*, 1057–1066.

(12) Wakeling, A. E.; Bowler, J. Novel Antiestrogens without Partial Agonist Activity. *J. Steroid Biochem.* **1988**, *31*, 645–653.

(13) Wakeling, A. E.; Dukes, M.; Bowler, J. A Potent Specific Pure Antiestrogen with Clinical Potential. *Cancer Res.* **1991**, *51*, 3867–3873.

(14) Levesque, C.; Merand, Y.; Dufour, J.-M.; Labrie, C.; Labrie, F. Synthesis and Biological Activity of new Halo-steroidal Antiestrogens. *J. Med. Chem.* **1991**, *34*, 1624–1630.

Table III. Diacetoxy-12-formyl-5,6-dihydroindolo[2,1-a]isoquinolines 7



compd	R^1	R^2	R^3	yield (%)	formula[a]	mp[b] (°C)	RBA[c]
7a	OAc	H	CH_3	61	$C_{22}H_{19}NO_5$	166	1.7
7b[d]	H	OAc	CH_3	59	$C_{22}H_{19}NO_5$	227–228	3.9
7c	OAc	H	C_2H_5	65	$C_{23}H_{21}NO_5$	198	1.3
7d[e]	H	OAc	C_2H_5	67	$C_{23}H_{21}NO_5$	177	3.3
7e	OAc	H	C_3H_7	77	$C_{24}H_{23}NO_5$	197	0.5
7f	H	OAc	C_3H_7	59	$C_{24}H_{23}NO_5$	195–196	2.6
7g	OAc	H	C_4H_9	78	$C_{25}H_{25}NO_5$	150–151	0.7
7h	H	OAc	C_4H_9	75	$C_{25}H_{25}NO_5$	167	2.1
7i	OAc	H	C_5H_{11}	74	$C_{26}H_{27}NO_5$	157	0.6
7j	H	OAc	C_5H_{11}	85	$C_{26}H_{27}NO_5$	162	1.9

[a] Analyzed for C and H within ±0.4% of the calculated values, except where noted. [b] Recrystallization from MeOH. [c] Relative binding affinities for the calf urine estrogen receptor = ratio of molar concentration of 17β-estradiol (E2) and inhibitor required to decrease the amount of bound [^{3}H]E2 by 50%, × 100. [d] C: calcd 70.02; found 69.57. [e] Reference 19.

Table IV. Chromatographic Separation of the Enantiomers of **7c**, **7e**, and **7g** on Cellulose Triacetate

compd	k^a	temp (°C)	$[\alpha]^b$ (deg) 436 nm	546 nm	578 nm	P^c (%)
(+)-**7c**	2.43	25	+330° ± 20	+120° ± 10	+100° ± 10	97
(−)-**7c**	3.16	25	−380° ± 30	−130° ± 20	−110° ± 10	70
(+)-**7e**	2.78	22	+460° ± 30	+170° ± 10	+140° ± 10	100
(−)-**7e**	3.30	22	−440° ± 40	−160° ± 20	−130° ± 10	86
(+)-**7g**	2.45	22	+430° ± 20	+170° ± 10	+140° ± 10	97
(−)-**7g**	2.81	22	−340° ± 20	−130° ± 10	−110° ± 10	60

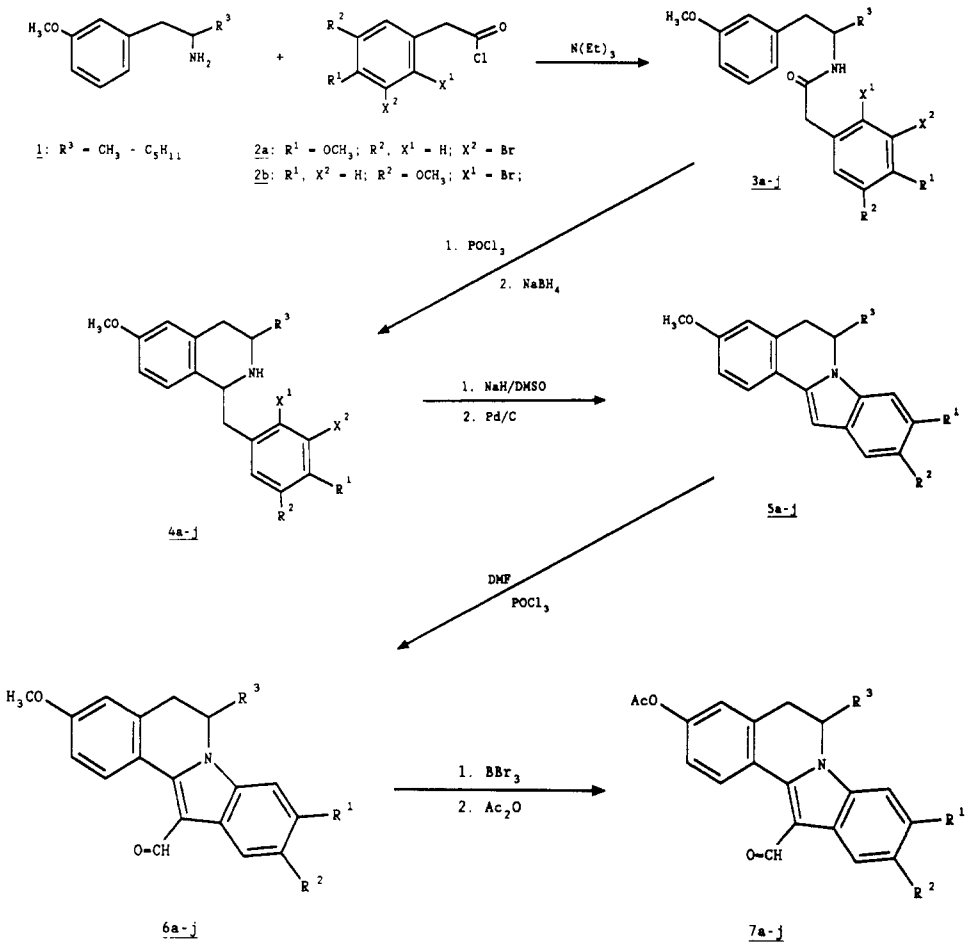
[a] The capacity factor k of the d- and l-enantiomers; $k = (t_i - t_0)/t_0$, where t_i = retention time and t_0 = void volume elution time. [b] Specific rotation at stated wavelengths; differences between (+)- and (−)-enantiomers are due to a partial overlap of peaks. [c] Optical purity, calculated from a plot of rotation angle α versus UV absorbance according to ref 39.

with various alkyl groups in position 6 and two acetoxy groups, one at C-3 and the other at C-9 or C-10. All of these derivatives were studied in respect to binding affinities for steroid hormone receptors, endocrine properties, and cytostatic activity in hormone-sensitive MCF-7 and hormone-independent MDA-MB 231 human mammary tumor cells. Due to the substituent at C-6, these molecules possess a chiral center. Since these agents interact with biochemical targets, it was likely that the enantiomers display a different spectrum of activity. Therefore, the enantiomers of the most active racemates were separated and studied.

Chemistry. The synthesis of the tetracyclic indolo[2,1-a]isoquinolines **5a–j** was performed as outlined in Scheme I. The 1-alkyl-2-(3-methoxyphenyl)ethylamines **1** were reacted with the respective (bromo-methoxyphenyl)acetic acid chloride **2** to yield the amides **3a–j** (Table I). The isoquinoline ring was formed by the Bischler–Napieralski reaction using $POCl_3$ in MeCN. In some cases the reaction products could be isolated as hydrochlorides. The crude benzyldihydroisoquinolines were treated with $NaBH_4$ to give 1-benzyl-1,2,3,4-tetrahydroisoquinolines **4a–j** (Table I). Thin-layer chromatography of the products revealed mixtures of two isomers in a ratio of approximately 20:1. The main product was the cis-configured stereoisomer.[20] The second ring closure reaction was accomplished by treating the bromo-substituted benzyltetrahydroisoquinolines **4a–j** with sodium (methylsulfinyl)methanide[21] as base. The cyclization occurred via a benzyne intermediate as demonstrated by converting both *o*- and *m*-bromo derivatives to the same tetracycle.[22] Under working up conditions, the primarily formed tetrahydroindolo[2,1-a]isoquinolines were partly oxidized by air to the corresponding dihydro derivatives. due to this instability, no effort was made to isolate the tetrahydro intermediates. The mixtures were purified by chromatography and dehydrogenated with palladium to afford the dihydroindolo[2,1-a]isoquinolines **5a–j** (Table II). The formyl group was introduced by the Vilsmeier–Haack reaction with $DMF/POCl_3$ to give the 12-substituted derivatives **6a–j** (Table II). In the last two steps of the synthesis, the ether functions were cleaved with BBr_3 and

(15) von Angerer, E.; Birnböck, H.; Knebel, N. Platinum Complexes with a Selective Action on Estrogen Receptor Positive Mammary Tumors. *Anti-Cancer Drug Des.* **1989**, *4*, 21–35.
(16) Knebel, N. G.; von Angerer, E. 2-Phenylindole Linked [2-(Aminoalkyl)pyridine]dichloroplatinum(II): Complexes with a Selective Action on Estrogen Receptor Positive Mammary Tumors. *J. Med. Chem.* **1991**, *34*, 2145–2152.
(17) Ambros, R.; von Angerer, S.; Wiegrebe, W. C-12-Substituted indolo[2,1-a]isoquinolines at Estrogen Receptor Affinic Cytostatic Agents. *Arch. Pharm. (Weinheim)* **1988**, *321*, 743–747.
(18) Weimar, C. Doctoral Thesis, Univ. of Regensburg, 1990.
(19) Ambros, R.; Schneider, M. R.; von Angerer, S. Indolo[2,1-a]-isoquinolines. Syntheses, Steroid Hormone Receptor Binding Affinities, and Cytostatic Activity. *J. Med. Chem.* **1989**, *33*, 153–160.
(20) Gal, J.; Wienkam, R. J.; Castagnoli, N., Jr. Synthesis of *cis*- and *trans*-1-(2,3-Dimethoxybenzyl)-3,7-dimethyl-5,8-dimethoxy-1,2,3,4-tetrahydroisoquinoline. Observation on the Mechanism of the Bischler-Napieralski Reaction. *J. Org. Chem.* **1974**, *39*, 418–421.
(21) Corey, E. J.; Chaykorsky, M. Methylsulfinyl Carbanion (CH3-SO-CH2-), Formation and Applications to Organic Synthesis. *J. Am. Chem. Soc.* **1965**, *87*, 1345–1353.
(22) Kano, S.; Komiyama, E.; Takahagi, Y.; Shibuya, S. Syntheses of Dibenzo[b,f]azonines and Dibenzo[b,g]azecines through Ring Expansion of 1-Substituted Isoquinolines. *Chem. Pharm. Bull.* **1976**, *24*, 648–654.

Scheme I



	R¹	R²	R³
7a	OAc	H	CH_3
7b	H	OAc	CH_3
7c	OAc	H	C_2H_5
7d	H	OAc	C_2H_5
7e	OAc	H	C_3H_7
7f	H	OAc	C_3H_7
7g	OAc	H	C_4H_9
7h	H	OAc	C_4H_9
7i	OAc	H	C_5H_{11}
7j	H	OAc	C_5H_{11}

the free phenolic groups converted into the acetates **7a–j** to protect them from oxidation and degradation by air (Table III). Due to the chiral center at C-6, all indoloisoquinolines of this study were obtained as racemic mixtures. On the basis of data from the biological evaluation, three derivatives (**7c,7e,7g**) were selected and submitted to a semipreparative chromatographic separation on triacetylcellulose (Table IV) to obtain the enantiomers. Due to a partial overlap of peaks, only the (+)-enantiomers were isolated with an optical purity of 97% and higher.

Binding Affinity for the Estrogen Receptor. As in previous studies,[19] the acetates were used for determination of the relative binding affinities for the estrogen receptors instead of the free hydroxy derivatives, because in solution the latter compounds are sensitive to air oxidation. Calf uterine cytosol was used as receptor source and the dextran-coated charcoal (DCC) method was applied.[3] All of the derivatives tested were able to displace estradiol from its receptor (Table III). The relative binding affinities (RBA) based on estradiol = 100 ranged from 0.5 to 3.9 and are similar to those found with antiestrogens such as tamoxifen (RBA = 1.8[16]) and zindoxifene (RBA = 1.7[23]) (chemical structures are presented in Chart I). The binding affinities for derivatives with the second acetoxy group in position 10 were generally higher than those for the 9-acetoxy compounds. The introduction of the formyl group into position 12 had caused a decrease of binding affinity of the indoloisoquinoline structure. When the formyl group in derivative **7b** was replaced by hydrogen, the RBA value increased from 3.9 to 12.[19] Similar differences were noted for other derivatives with longer alkyl

(23) von Angerer, E.; Engel, J.; Schneider, M. R.; Sheldrick, W. S. D-16726. *Drugs Future* **1985**, *10*, 281–285.

groups in position 6.[24] The interaction of drugs with biochemical targets such as enzymes or receptors is often characterized by a high degree of stereospecificity. Therefore, we had assumed that the enantiomers of 6-alkylindoloisoquinolines possess different binding affinities. When we determined the RBA values, we did not find major differences between the stereoisomers: RBA: (+)-**7c**, 1.5; (−)-**7c**, 0.6; (+)-**7e**, 1.6; (−)-**7e**, 1.2; (+)-**7g**, 0.5; (−)-**7g**, 0.4.

In a previous study, some 5,5-dihydroindolo[2,1-*a*]isoquinolines were shown to possess affinity for androgen receptors.[19] We therefore tested all of the formyl derivatives for their binding affinities for both the androgen and the progesterone receptor. The result was negative: none of the compounds binds to these two steroid hormone receptors at concentrations of 10^{-8} to 10^{-5} M.

Endocrine Activity. The affinity of the 12-formyl-5,6-dihydroindolo[2,1-*a*]isoquinolines for the estrogen receptor made hormonal effects on estrogen-sensitive target tissues likely. In order to evaluate estrogenic and antiestrogenic activity of these agents we studied their effects on the uterine growth in immature mice. In this assay the animals received various doses of the drug, and the increase in uterine dry weight was measured.[25] Antagonistic activity was determined by simultaneous administration of the test compound and a standard dose of estrone (0.4 μg) and calculation of the inhibition of estrone-stimulated uterine growth. The dose of 0.4 μg was chosen on the basis of the dose–response curve of estrone and is high enough to produce full uterotrophic response.[26]

Estrogenic activity of all derivatives was very low in comparison to the standard drug estrone (Table V). Even at 250 μg/animal (14 mg/kg) none of the compounds gave rise to full estrogenic response. The 3,9-diacetoxy-substituted derivatives usually showed no significant estrogenic activity whereas the 3,10-substituted compounds produced weak but significant effects at higher doses. The estrogenicity of the later derivatives decreased with increasing length of the alkyl chain. This is in accord with the decreasing binding affinities for the estrogen receptor.

All compounds of this study showed antagonistic activity. The antiestrogenic effects however varied considerably. An inverse correlation between estrogen antagonistic and agonistic activity, which is usually found in other classes of hormonally active drugs, was not evident. For example, derivative **7d** strongly suppressed uterine growth, but when administered alone it increased uterine weight to about 66% of the weight of estrone-treated animals. Since this was a rare example of uterotrophic and antiuterotrophic dose–response curves that do not merge but cross, we repeated this experiment several times with the same result. It seems to be a rather unique attribute of the structure of **7d** because the indoloisoquinoline with the identical substitution pattern but without the formyl group showed the same course of uterotrophic dose–response curves.[19]

Cytostatic Activity. Cytostatic activities of the new compounds were determined in a microplate assay.[4] Cells were incubated under appropriate conditions with or without the drug for several days, followed by a staining procedure with crystal violet as dye. Optical density of single wells was measured in a microplate reader and used as parameter for cell mass. Initial cell density was obtained by addition of vinblastine (10^{-7} M) as a selective inhibitor of mitosis and was used for correcting the values for other drugs.

Binding affinity for the estrogen receptor and antiestrogenic activity are considered prerequisite for selective

Table V. Estrogenic and Antiestrogenic Activity of Diacetoxy-12-formyl-5,6-dihydroindolo[2,1-*a*]isoquinolines **7** in the Mouse Uterine Weight Test

			antiuterotrophic test	
compd	dose[a] (μg)	uterotrophic test: rel uterus weight[b]	rel uterus weight[b,c]	inhibn[d] (%)
control	–	16.4 ± 4.7		
estrone	0.4	53.9 ± 10.9		
7a	2	19.6 ± 4.0	45.3 ± 4.8	20
	10	18.2 ± 1.6	48.5 ± 8.5	14
	50	18.2 ± 3.1	42.6 ± 7.9	30[f]
	250	19.9 ± 5.4	39.7 ± 5.4	38[e]
control	–	12.9 ± 1.2		
estrone	0.4	59.4 ± 8.7		
7b	2	14.8 ± 1.9[f]	60.5 ± 2.5	0
	10	18.2 ± 2.7[e]	42.8 ± 4.1	36[e]
	50	24.6 ± 4.7[e]	36.5 ± 2.6	49[e]
	250	43.2 ± 6.0[e]	44.4 ± 4.9	32[e]
control	–	17.2 ± 3.0		
estrone	0.4	54.2 ± 5.0		
7c	2	15.9 ± 2.4	47.9 ± 9.0	17
	10	17.7 ± 2.6	49.0 ± 5.9	14
	50	18.2 ± 2.9	44.2 ± 4.8	27[e]
	250	22.8 ± 2.3[e]	40.2 ± 3.4	38[e]
7d	2	18.2 ± 4.8	45.3 ± 4.8	25[f]
	10	17.2 ± 2.9	46.1 ± 8.0	22[g]
	50	20.5 ± 3.5	27.0 ± 3.4	74[e]
	250	41.8 ± 6.8[e]	24.6 ± 2.8	80[e]
control	–	16.4 ± 4.7		
estrone	0.4	53.9 ± 5.9		
7e	2	20.4 ± 5.1	39.4 ± 6.5	39[e]
	10	17.1 ± 2.2	38.4 ± 12.6	41[f]
	50	16.9 ± 4.4	40.7 ± 9.6	35[f]
	250	17.4 ± 1.9	29.5 ± 4.7	65[e]
control	–	15.4 ± 3.1		
estrone	0.4	47.6 ± 5.4		
7f	2	16.9 ± 4.1	38.8 ± 6.8	27[f]
	10	21.1 ± 2.9[e]	41.1 ± 4.3	20[f]
	50	24.9 ± 5.0[e]	39.2 ± 3.7	26[e]
	250	32.9 ± 7.7[e]	38.5 ± 2.2	28[e]
control	–	12.9 ± 3.4		
estrone	0.4	55.4 ± 4.7		
7g	2	16.7 ± 2.9[e]	52.8 ± 3.5	6
	10	13.7 ± 3.5	46.7 ± 4.4	20[e]
	50	12.3 ± 3.2	51.8 ± 4.8	8
	250	10.6 ± 3.2	47.5 ± 4.4	19[e]
7h	2	20.2 ± 4.5[e]	46.7 ± 4.8	20[e]
	10	18.6 ± 5.7[g]	48.2 ± 11.1	17
	50	9.1 ± 1.4[g]	47.8 ± 8.8	18
	250	25.5 ± 4.0[e]	34.3 ± 5.9	50[e]
control	–	15.6 ± 3.6		
estrone	0.4	56.9 ± 4.7		
7i	2	16.4 ± 3.6	49.0 ± 6.2	19
	10	16.6 ± 2.9	46.4 ● 7.5	25[e]
	50	15.8 ± 1.7	35.6 ± 6.1	52[e]
	250	19.5 ± 3.0[f]	41.2 ± 7.9	38[e]
7j	2	17.9 ± 2.1	57.3 ± 5.3	0
	10	16.0 ± 4.1	51.0 ± 8.8	14
	50	20.2 ± 3.8[e]	47.1 ± 10.7	24[g]
	250	22.5 ± 4.3[e]	53.8 ± 2.9	8

[a] Dose per animal, administered at three consecutive days sc. [b] Uterus dry weight (mg)/body weight (g) × 100, determined 24 h after the last injection; mean of 6 animals ± SD. [c] Simultaneous administration of 0.4 μg of estrone/animal and day. [d] The U test according to Wilcoxon, modified by Mann and Whitney, was used to determine significance. [e] Significant ($p < 0.01$). [f] Significant ($p < 0.025$). [g] Significant ($p < 0.05$).

(24) Polossek, T. Doctoral Thesis, Univ. of Regensburg, 1991.
(25) Rubin, B. L.; Dorfman, A. S.; Black, L.; Dorfman, R. I. Bioassay of Estrogens using the Mouse Uterine Response. *Endocrinology* **1951**, *49*, 429–439.
(26) Kranzfelder, G.; Schneider, M.; von Angerer, E.; Schönenberger, H. Entwicklung neuer Antiöstrogene vom Typ des 3,3'-Dihydroxy-α,β-diäthylstilbens und ihre Prüfung am DMBA-induzierten, hormonabhängigen Mammacarcinom der SD-Ratte. *J. Cancer Res. Clin. Oncol.* **1980**, *97*, 167–186.

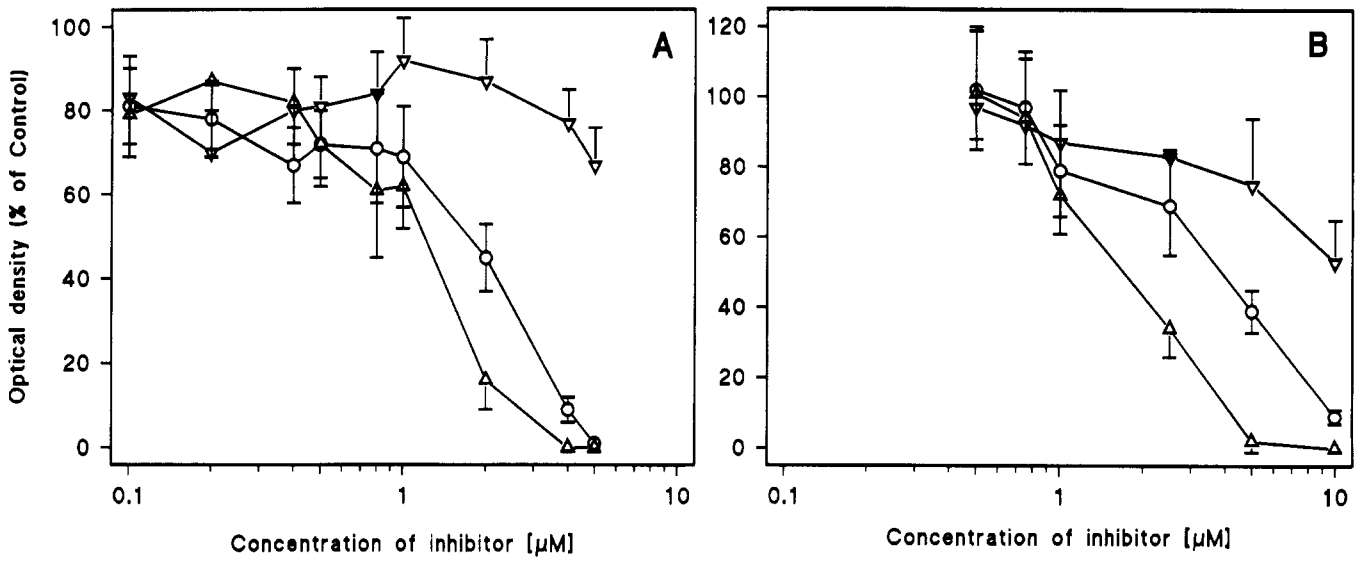


Figure 1. Effect of various concentrations of racemic indoloisoquinoline (±)-**7c** (-○-) and its enantiomers (+)-**7c** (-△-) and (−)-**7c** (-▽-) on the growth of human breast cancer cells. A: hormone-sensitive MCF-7 cells; B: hormone-independent MDA-MB 231 cells. Each point is the mean of 16 replicates; bars represent SD. Values are corrected for initial optical densities.

Table VI. Effect of Compounds **7a–j** and Tamoxifen on the Growth of Hormone-Sensitive MCF-7 Mammary Tumor Cells

	T/C[a] (%)			
compd	1×10^{-7} M	1×10^{-6} M	5×10^{-6} M	1×10^{-5} M
Tam[b]	85 ± 10[c]	57 ± 7[c]	43 ± 8	18 ± 3[c]
7a	97 ± 17	106 ± 15	36 ± 7[c]	1 ± 2[c]
7b	93 ± 13	94 ± 15	54 ± 10[c]	23 ± 6[c]
7c	89 ± 9	83 ± 9[c]	2 ± 3[c]	0 ± 3[c]
7d	85 ± 17	91 ± 18	43 ± 9[c]	12 ± 3[c]
7e	71 ± 12[c]	1 ± 4[c]	0 ± 4[c]	0 ± 3[c]
7f	99 ± 12	86 ± 18	9 ± 3[c]	0 ± 2[c]
7g	66 ± 15[c]	0 ± 2[c]	0 ± 3[c]	0 ± 2[c]
7h	81 ± 9[c]	12 ± 3[c]	0 ± 2[c]	0 ± 2[c]
7i	101 ± 4	90 ± 6[c]	0 ± 2[c]	0 ± 2[c]
7j	102 ± 5[c]	94 ± 10	77 ± 7[c]	37 ± 6[c]

[a] Ratio of optical densities in test wells and control wells after incubation for 3 days; means of 16 replicates ± SD. Values are corrected for initial optical density. [b] Tamoxifen citrate. [c] Significant inhibition of cell growth ($p < 0.01$).

Table VII. Effect of Compounds **7a–j** on the Growth of Hormone-Independent MDA-MB 231 Mammary Tumor Cells

	T/C[a] (%)			
compd	1×10^{-7} M	1×10^{-6} M	5×10^{-6} M	1×10^{-5} M
7a	109 ± 15	112 ± 13	79 ± 10[b]	45 ± 6[b]
7b	109 ± 11	104 ± 10	94 ± 9	61 ± 7[b]
7c	101 ± 10	92 ± 6	34 ± 3[b]	10 ± 3[b]
7d	106 ± 9	111 ± 8	86 ± 6[b]	52 ± 3[b]
7e	100 ± 12	31 ± 4[b]	15 ± 3[b]	14 ± 4[b]
7f	109 ± 11	106 ± 10	43 ± 5[b]	9 ± 4[b]
7g	104 ± 13	30 ± 4[b]	24 ± 3[b]	16 ± 3[b]
7h	116 ± 11	73 ± 8[b]	12 ± 3[b]	12 ± 3[b]
7i	118 ± 14	105 ± 19	70 ± 17[c]	27 ± 8[b]
7j	105 ± 10	100 ± 5	80 ± 4[b]	47 ± 4[b]

[a] Ratio of optical densities in test wells and control wells after incubation for 2 days; means of 16 replicates ± SD. Values are corrected for initial optical density. [b] Significant inhibition of cellular growth ($p < 0.01$). [c] Significant inhibition ($p < 0.025$).

cytostatic activity in estrogen receptor positive cells. Therefore, it was not unexpected that all of the indoloisoquinoline derivatives tested were able to inhibit the growth of estrogen-sensitive human MCF-7 breast cancer cells (Table VI). There was a significant difference in cytostatic activity between the 9-acetoxy (**7a,7c,7e,7g,7i**) and the 10-acetoxy series (**7b,7d,7f,7h,7j**), the latter being less effective. Besides the position of the oxygen function in the indole part of the molecule, the length of the alkyl chain influenced the cytostatic potency. Activity increased with the length of the side chain and reached a maximum for *n*-butyl derivatives (**7g,7h**). Compounds **7e** and **7g** completely suppressed cellular growth at a concentration of 1×10^{-6} M and showed a significant inhibition at 1×10^{-7} M.

The results with estrogen-sensitive MCF-7 cells showed no correlation with endocrine parameters such as receptor affinity or antiestrogenic activity. This observation prompted us to study the effect of the 12-formyl-substituted 5,6-dihydroindolo[2,1-*a*]isoquinolines in hormone-independent human MDA-MB 231 mammary tumor cells (Table VII). The results obtained in this cell line were rather similar to those in MCF-7 cells except that the cytostatic activities of these compounds were somewhat lower in hormone-resistant cells than in hormone-sensitive cells. The differences of the T/C values between the isomeric 9-acetoxy and 10-acetoxy derivatives and the influence of the side chain at C-6 were about the same in both cell lines. These findings indicated a rather specific mode of cytostatic action of this class of compounds. Therefore, it was of interest to study the stereoisomers of three of the most active racemic derivatives (**7c,7e,7g**).

Cytostatic activity of both the enantiomers and the racemates were determined first in estrogen-receptor positive MCF-7 cells. The dose–response curves are shown on the left-hand side of Figures 1–3. It is obvious that the cytostatic activity exerted by these drugs is mainly due to the (+)-stereoisomers which had been obtained in optically pure form (Table IV). The IC_{50} values differed by 1 order of magnitude between the (+)- and (−)-form. This difference was also observed in hormone-independent MDA-MB 231 breast cancer cells. In this cell line dose–response curves were slightly shifted to higher concentrations indicating a somewhat lower sensitivity in comparison to MCF-7 cells. For comparison the concentration range (10^{-7}–10^{-5} M) was kept constant for all derivatives.

Discussion

This study was aimed to improve the cytostatic activity of 5,6-dihydroindolo[2,1-*a*]isoquinolines which are of interest as drugs for the endocrine treatment of hormone-dependent mammary carcinomas. These agents are characterized by acetylated hydroxy groups in both aro-

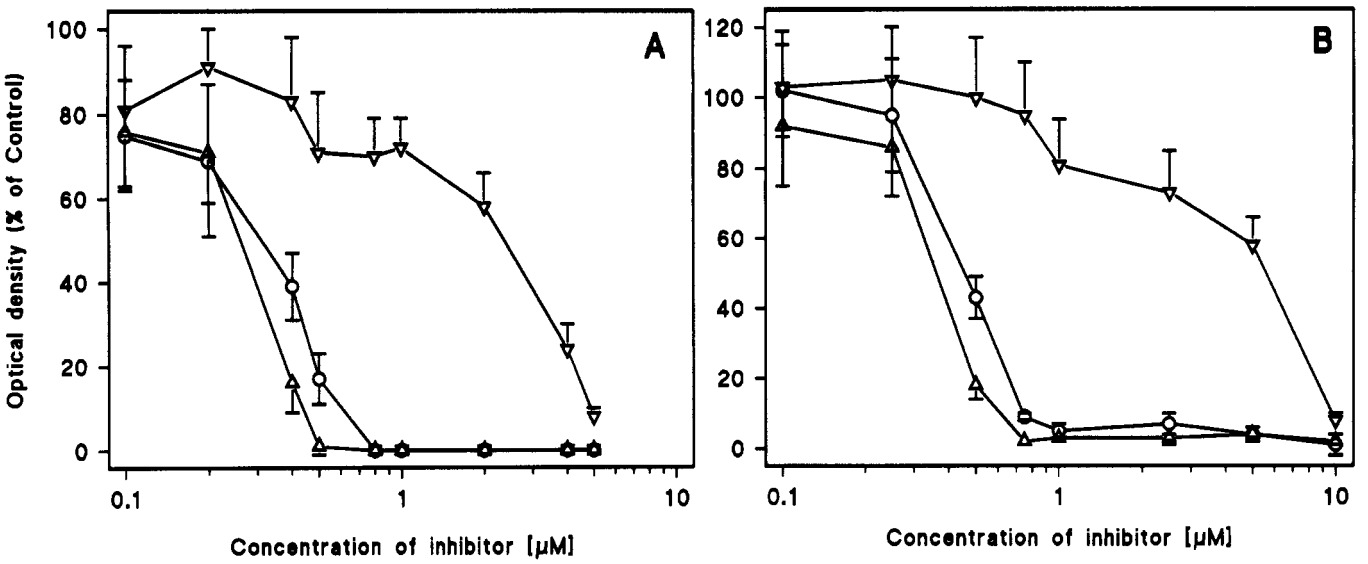


Figure 2. Effect of various concentrations of racemic indoloisoquinoline (±)-**7e** (-○-) and its enantiomers (+)-**7e** (-△-) and (−)-**7e** (-▽-) on the growth of human breast cancer cells. A: hormone-sensitive MCF-7 cells; B: hormone-independent MDA-MB 231 cells. Each point is the mean of 16 replicates; bars represent SD. Values are corrected for initial optical densities.

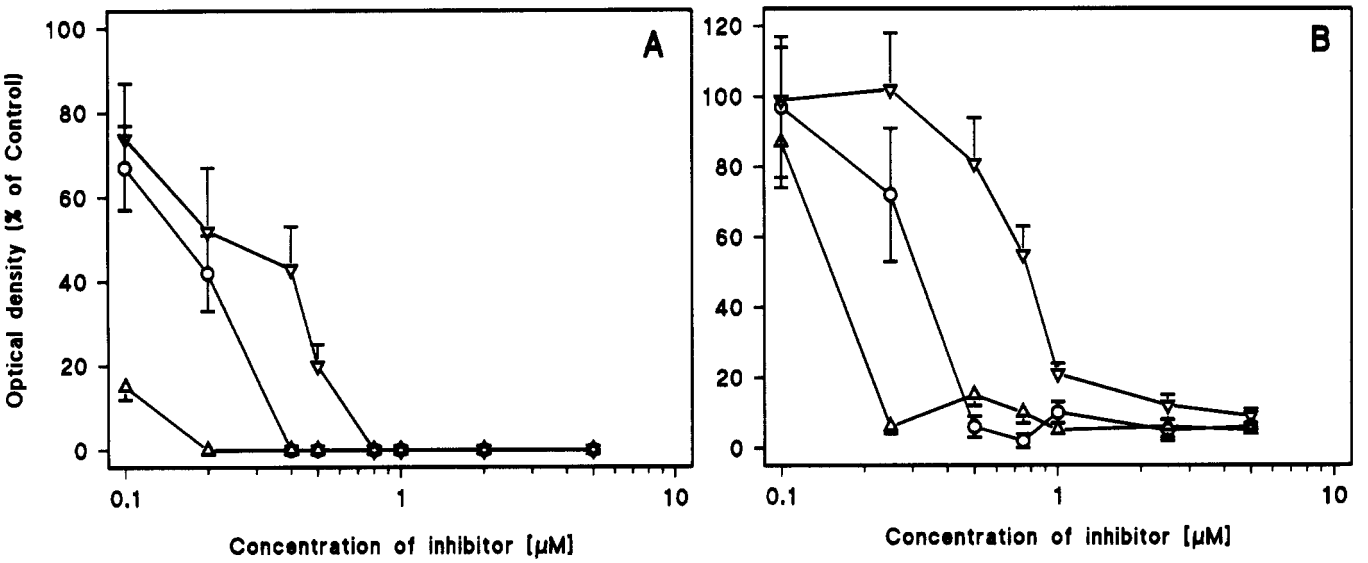


Figure 3. Effect of various concentrations of racemic indoloisoquinoline (±)-**7g** (-○-) and its enantiomers (+)-**7g** (-△-) and (−)-**7g** (-▽-) on the growth of human breast cancer cells. A: hormone-sensitive MCF-7 cells; B: hormone-independent MDA-MB 231 cells. Each point is the mean of 16 replicates; bars represent SD. Values are corrected for initial optical densities.

matic rings and a lipophilic area in center of the molecule. Due to these structural features, they bind very well to the estrogen receptor. From previous investigations,[17,19] we knew that introduction of a formyl group into position 12 of the tetracyclic structure can enhance cytostatic activity of the parent drug. It was of great importance for further development that the binding affinity for the estrogen receptor is not lost by these structural modifications.

When we introduced a formyl group into the parent structure, we observed some decrease of binding affinity, but the RBA values remained in the range of other drugs used in endocrine treatment of breast cancer. The lower RBA values can be rationalized by the presence of the carbonyl group that gives rise to an area with negative potential close to the center of the molecule and is also capable to form hydrogen bridges as an acceptor. When we studied the receptor binding of the enantiomers of the racemic derivatives, we found no major difference between the stereoisomers. This result was unexpected because biomolecules are usually able to discriminate between two optical isomers. We carried out computer-assisted molecular modeling studies to find an explanation for this phenomenon. Molecular comparison showed that the alkyl substituent in position 6 of the indolo[2,1-*a*]isoquinolines adopts an axial position which is favored by approximately 10 kcal/mol in comparison to the equatorial position. An axial orientation for R^3 is also supported by NMR data.[19] In relation to 17β-estradiol, the natural ligand of the estrogen receptor, there are four different options for the indoloisoquinoline to bind to the receptor site: two if the indole part mimics the steroidal A- and B-ring and two if isoquinoline corresponds to this moiety of the steroid. In both cases the formyl group can be either in the vicinity of the B- or the C-ring of estradiol. The molecular modeling studies were based on the assumption that the 3-hydroxyphenyl ring of the isoquinoline represents the steroidal A-ring and serves as a kind of anchor. Only this orientation allows the D-ring to be superimposed properly by the second benzene ring. This assumption is also supported by results obtained with monohydroxylated 2-phenylindoles which suggest that the phenyl ring corresponds to the A-ring of estradiol.[27] Several other studies

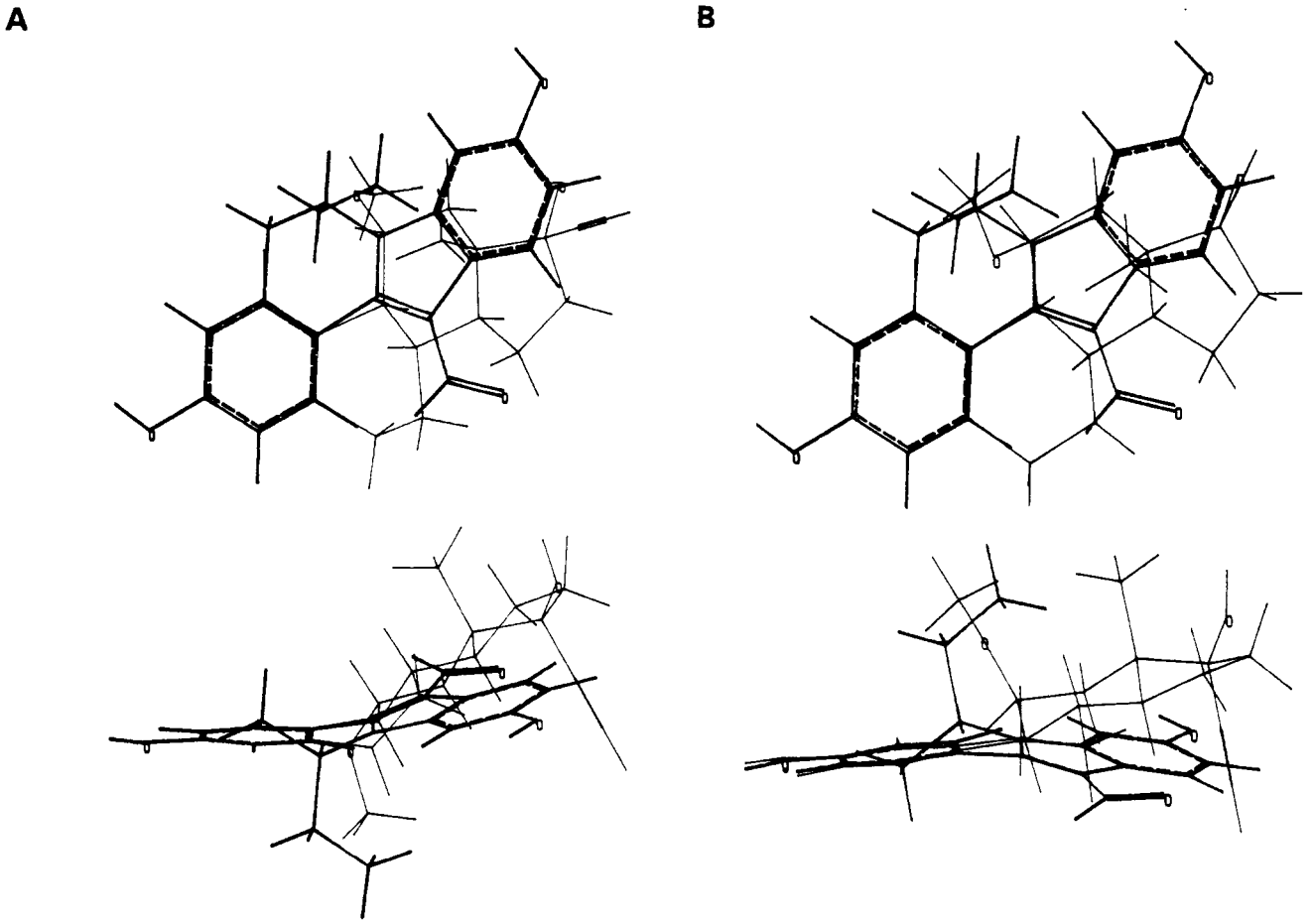
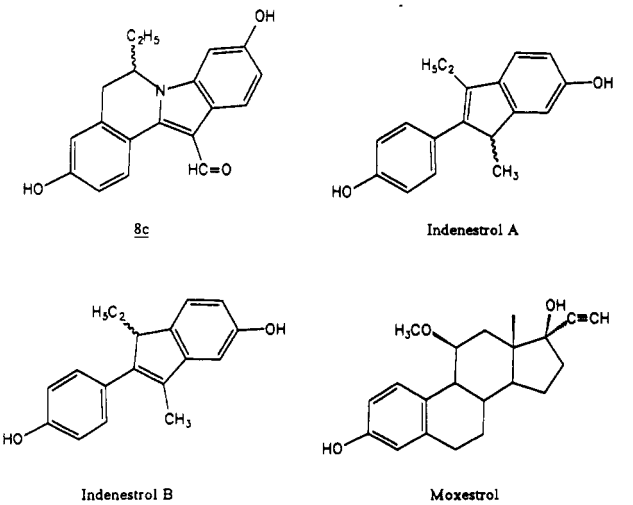


Figure 4. Orthogonal views of a computer-generated alignment of the enantiomers of **8c** (bold lines) and 17α-ethinyl-11-methoxy-17β-estradiol (thin lines). A: (*R*)-6-ethyl-12-formyl-5,6-dihydro-3,9-dihydroxyindolo[2,1-*a*]isoquinoline and moxestrol (11β-methoxy-ethinylestradiol). B: (*S*)-6-ethyl-12-formyl-5,6-dihydro-3,9-dihydroxyindolo[2,1-*a*]isoquinoline and 11α-methoxy-ethinylestradiol. Low-energy conformations were obtained by force-field calculations (MAXIMIM function within SYBYL 5.41, Tripos Assoc.). Superposition was made by the MULTIFIT function (SYBYL 5.41) using the atoms of the steroidal A-ring and its oxygen atom and the corresponding atoms of the indoloisoquinoline.

showed that nonsteroidal ligands for the estrogen receptor with a phenol ring bind in a fashion where the phenol corresponds to the steroidal A-ring.[28-30] When 6-ethyl-3,10-dihydroxyindoloisoquinoline (**8d**) (diacetylated **7d**) was superimposed upon estradiol in this way, the oxygen atom at C-10 is closer (ca. 2.5 Å, mean distance) to the steroidal hydroxy group in position 17 than the indole oxygen in the 3,9-dihydroxy analogue **8c** (ca. 3.4 Å). This probably accounts for the lower binding affinities of the later structure.

Multifit calculations using the A-ring and its oxygen function revealed that the chiral carbon atom bearing the alkyl substituent is either close to C-7 or C-11 of the steroid. Depending on the chirality, the substituent adopts an α- or β-orientation. Since a marked difference in binding affinity for 7α- and 7β-substituted estradiol has been reported,[31] we assume that C-6 of the indoloisoquinoline corresponds not to C-7 but to C-11 of estradiol. For steric considerations we superimposed both stereoisomers of 6-ethyl-12-formyl-5,6-dihydro-3,9-dihydroxyindolo[2,1-*a*]isoquinoline (**8c**) upon an 11-substituted

Chart II



(27) von Angerer, E. Unpublished results.
(28) Anstead, G. M.; Wilson, S. R.; Katzenellenbogen, J. A. 2-Arylindenes and 2-Arylindenones: Molecular Structures and Considerations in the Binding Orientation of Unsymmetrical Nonsteroidal Ligands to the Estrogen Receptor. *J. Med. Chem.* **1989**, *32*, 2163-2171.
(29) Duax, W. L.; Griffin, J. F. Structural Features which Distinguish Estrogen Agonist and Antagonist. *J. Steroid Biochem. Mol. Biol.* **1987**, *27*, 271-280.
(30) Duax, W. L.; Swenson, D. C.; Strong, P. D.; Korach, K. S.; McLachlan, J.; Metzler, M. Molecular Structures of Metabolites and Analogues of Diethylstilbestrol and their Relationship to Receptor Binding and Biological Activity. *Mol. Pharmacol.* **1984**, *26*, 520-525.
(31) Wakeling, A. E.; Bowler, J. Biology and Mode of Action of Pure Antiestrogens. *J. Steroid Biochem. Mol. Biol.* **1988**, *30*, 141-147.

17β-estradiol derivative. As a reference molecule we used moxestrol (17α-ethinyl-11β-methoxyestradiol; Chart II) and its 11α-epimer. In the latter epimer, the methoxy substituent can adopt two orientations, axial or equatorial, which exhibited only minor differences in energy as shown by force-field calculations. The comparison of the molecular structures suggests that the *R*-enantiomer matches moxestrol and the *S*-form the 11α-isomer (Figure 4). Raynaud and colleagues have shown that the RBA values of moxestrol and its 11α-epimer do not differ very much (12% vs 13% of estradiol[32]), despite the fact that the 11α-isomer dissociates much faster from the receptor.[33] The lack of preference in receptor binding of enantiomers was also observed by Korach et al. when they studied the isomers of indenestrol B (Chart II).[34] They suggest an alignment of the nonsteroidal compound where the chiral carbon atom is in the vicinity of C-8 of estradiol when both structures are superimposed. They argue that the stereogenic center C-3 of the isomeric indenestrol A mimics the C-11 of the steroid and therefore gives rise to different binding affinities and, consequently, the chiral atom in indenestrol B must adopt a different position. However, the same argument holds true when the chiral carbon of indenstrol B mimics C-11 of estradiol. Considering both the results from Raynaud's group and those reported by Korach and colleagues for indenestrol B, it is likely that the chiral center C-6 of the indoloisoquinoline corresponds to C-11 of the steroid as shown in Figure 4. The indole double bond would then be close to the steroidal C-8/C-9 bond and possibly match the double bond in the five-membered ring of indenestrol B.

The binding affinity for the estrogen receptor gave rise to endocrine activity of the formyl-substituted indoloisoquinolines. Significant estrogenic effects were only found with 3,10-diacetoxy derivatives and depend on the length of the alkyl chain as the RBA values do. All of the derivatives inhibited the growth of estrone-stimulated uterine growth. The maximum effect was an inhibition of 80% (**7d**). The antagonistic activity varied among the derivatives and appeared to be independent from the position of the acetoxy group in the indole part and the length of the alkyl chain. This is in accord with findings from our previous studies[3,7] and from other laboratories[31] showing that small alterations of the chemical structure influence estrogen antagonism more strongly than binding affinity for the estrogen receptor. One of the indoloisoquinoline derivatives (**7e**) appears to be a good candidate for the development as pure antiestrogen because it inhibited estrone-stimulated uterine growth by 65% without any estrogenic side effects at a dose of 250 μg/animal.

The most interesting aspect of these investigations was the evaluation of cytostatic activity in hormone-sensitive MCF-7 and hormone-independent MDA-MB 231 human breast cancer cells. In this study it became evident that the cytostatic effect in estrogen receptor positive cells was not mediated by the receptor because there was no significant difference in activity between these two cell lines. The inhibitory effect on cellular growth was strongly dependent on the position of the acetoxy group in the indole part and the length of the alkyl side chain in position 6. The marked influence of structural parameters on cytostatic activity was reflected by the different potencies of the optical isomers. The IC_{50} values of the (−)-enantiomers were about 10-fold higher than those of the (+)-isomers. This discrepancy between the enantiomers could even be more pronounced if one takes in account that the (−)-isomers are still contaminated by the more active (+)-isomers, which were obtained in optically pure form. Such a high degree of stereospecificity is rather rare among cytostatic drugs and is usually restricted to antibiotics such as anthracyclines.[35] The stereospecificity observed for these derivatives makes an interaction with a well-defined biochemical target likely.

Since many cytostatic or cytotoxic polycycles have the ability to intercalate into the DNA and inhibit DNA replication, thereby we studied the interaction of some of the active derivatives with the DNA. The UV difference spectra of DNA in the presence of the indoloisoquinolines gave no hint to an intercalation into the DNA or other major changes of DNA structure (data not shown). Ethidium bromide served as a reference drug in these experiments. Apart from this particular interaction with DNA there are many other possibilities by which cell proliferation can be inhibited. Most of them involve enzymes such as DNA polymerases or topoisomerase II. The protein structure of the enzymes makes a preference for binding one stereoisomer likely. Up to now, we do not know which biochemical target might be involved in this specific antitumor activity of 3,9-diacetoxy-12-formyl-5,6-dihydroindolo[2,1-*a*]isoquinolines. Further studies on the mode of action are in progress. The strong cytostatic effect of (+)-**7g** (85% inhibition at 1×10^{-7} M in MCF-7 cells; 94% inhibition at 2.5×10^{-7} M in MDA-MB 231 cells) warrants investigations on the in vivo antitumor activity of the formyl-substituted indoloisoquinolines.

Experimental Section

Melting points were determined on a Büchi 510 apparatus and are uncorrected. Elemental analyses were performed by Mikroanalytisches Laboratorium, University of Regensburg, and were within ±0.40% of the calculated values except where noted. NMR spectra were obtained on a Varian EM 360 and a Bruker WM 250 spectrometer and are consistent with the assigned structures. Mass spectra of all final products were recorded on a Varian MAT 112S spectrometer and showed correct molecular weights.

General Procedure for the Synthesis of the Acetamides 3a–j. The methoxyphenylethylamine 1 (150 mmol) was dissolved in dry CH_2Cl_2 (350 mL) containing 150 mmol of NEt_3. 2-(Bromophenyl)acetic acid chloride (150 mmol) in 150 mL of dry CH_2Cl_2 was added. After stirring for 1 h at room temperature, the mixture was poured into 2 N HCl and extracted with CH_2Cl_2. The organic layer was washed with water and dried (Na_2SO_4). After evaporation of the solvent, the products crystallized at 4 °C from ether or suitable Et_2O/EtOAc or Et_2O/EtOH mixtures. Recrystallization from EtOH yielded colorless crystals. The yields were 35–55%. Melting points are reported in Table I. Compounds **3b–d** have been described previously.[19]

General Procedure for the Synthesis of the 1-Benzyl-1,2,3,4-tetrahydroisoquinolines 4a–j. A mixture of the acetamide 3 (54 mmol), $POCl_3$ (15 mL), and dry MeCN (85 mL) was refluxed for 3 h. Then the mixture was poured into ice–water (600 mL). The free base was liberated with 40% NaOH and extracted with CH_2Cl_2. The organic layer was washed with water and saline and dried (Na_2SO_4). The 3,4-dihydroisoquinolines obtained after evaporation of the solvent were used without further

(32) Raynaud, J. P., T. Ojasso, M. M. Bouton, Philibert, D. Receptor Binding as a Tool in the Development of New Bioactive Steroids. In *Drug Design*; Ariens, E. J., Ed.; Academic Press: New York, 1979; Vol. 8, pp 169–214.

(33) Bouton, M. M.; Raynaud, J. P. The Relevance of Interaction Kinetics in Determining Biological Response to Estrogens. *Endocrinology* **1979**, *105*, 509–515.

(34) Korach, K. S.; Chae, K.; Levy, L. A.; Duax, W. L.; Sarver, P. J. Diethylstilbestrol Metabolites and Analogs. Stereochemical Probes for the Estrogen Receptor Binding Site. *J. Biol. Chem.* **1989**, *264*, 5642–5647.

(35) von Angerer, E., E. Holler, H. Schönenberger, Schönenberger, R. Antineoplastic Drugs. In *Handbook of Stereoisomers: Therapeutic Drugs*; Smith, D. F., Ed.; CRC Press: Boca Raton, 1989; pp 247–284.

purification. The yields were 85–95%.

The 3,4-dihydroisoquinoline (26 mmol) was dissolved in 110 mL of MeOH and treated with $NaBH_4$ (3.2 g, 84 mmol) at 0 °C. The mixture was stirred for 45 min at 0 °C and room temperature and then refluxed for further 30 min. After the solvent had been removed, the residue was treated with 150 mL of water and extracted with CH_2Cl_2. The organic layer was washed with water and dried (Na_2SO_4). After evaporation of the solvent, the residue was purified by chromatography (SiO_2, CH_2Cl_2/Et_2O 1:1) to give a colorless oil. The yields were 60–85%. Boiling points are reported in Table I. Compounds **4b–d** have been described previously.[19]

General Procedure for the Ring Closure Reaction of (Bromobenzyl)tetrahydroisoquinolines to 5,6-Dihydroindolo[2,1-*a*]isoquinolines 5a–j. A solution of (bromobenzyl)tetrahydroisoquinoline **4** (19.5 mmol) in 75 mL of DMSO was added to a solution of sodium (methylsulfinyl)methanide prepared from 4.1 g (137 mmol) of NaH (80% in oil dispersion) and 75 mL of DMSO. After stirring for 15 h, the mixture was poured into 300 mL of water containing an excess of NH_4Cl and extracted with CH_2Cl_2. The organic layer was washed with water and saline. After drying (Na_2SO_4) and evaporation of the solvent, an oil was obtained. Chromatography (SiO_2, CH_2Cl_2) yielded a mixture of dihydro- and tetrahydroindolo[2,1-*a*]isoquinolines in a ratio of approximately 1:1. The mixture was used without further purification.

Under an atmosphere of N_2, a mixture of dihydro- and tetrahydroindolo[2,1-*a*]isoquinolines (2.6 g) and Pd/C 10% (940 mg) was mixed thoroughly in a flask, which was then placed in a hot oil bath. The temperature was kept at 120–130 °C for 12 h. After cooling, the mixture was dissolved in CH_2Cl_2 and filtered. The solvent was evaporated and the residue chromatographed (SiO_2; CH_2Cl_2 or suitable CH_2Cl_2/ligroin mixtures). Recrystallization from EtOH afforded colorless crystals. Melting points and yields are reported in Table II. Compounds **5b–d** have been described previously.[19]

General Procedure for the Synthesis of 12-Formyl-5,6-dihydroindolo[2,1-*a*]isoquinolines 6a–j. Dry DMF (3 mL) was added slowly to $POCl_3$ (4.3 mL) at 10–20 °C under N_2. The 5,6-dihydro-dimethoxyindolo[2,1-*a*]isoquinoline **5** (4.7 mmol) dissolved in 30 mL of dry DMF was added slowly to keep the temperature below 35 °C. After stirring for 35 min at 35 °C, the mixture was poured into 150 mL of ice–water. The aqueous layer was made alkaline (NaOH, 40%), and extracted with $CHCl_3$. After washing with water and drying ($NaSO_4$), the solvent was removed in vacuo. The residue was purified by chromatography (SiO_2; suitable $CHCl_3/Et_2O$ mixtures). The products were recrystallized from MeOH to give slightly yellow crystals. Melting points and yields are reported in Table II.

General Procedure for Ether Cleavage and Acetylation. A solution of the methoxy-substituted indoloisoquinoline **6** (0.8 mmol) in dry CH_2Cl_2 (15 mL) was cooled to −50 °C, and BBr_3 (0.3 mL) was added under N_2. After 30 min, the cooling bath was removed and the mixture was stirred for 15 h. The mixture was poured into 35 mL of an ice-cold aqueous solution of $NaHCO_3$. Fifty milliliters of EtOAc was added, and the mixture was stirred for 15 min. The organic layer was separated, and the aqueous phase was extracted with EtOAc. The combined organic layers were washed with saline and dried (Na_2SO_4). After the solvent was removed, the residue was dried in vacuo and treated with a mixture of Ac_2O (14 mL) and pyridine (0.3 mL). After stirring for 30 min at room temperature, the mixture was poured into ice–water, stirred for 10 min, and extracted with CH_2Cl_2. The organic layer was washed with 2 N HCl and an aqueous solution of $NaHCO_3$ and dried (Na_2SO_4). After evaporation of the solvent, the remaining residue was chromatographed (SiO_2; CH_2Cl_2). Recrystallization from MeOH yielded **7** as colorless crystals. Melting points and yields are reported in Table II.

Separation of the Enantiomers of 7c, e, and g. The enantiomers of **7c**, **e**, and **g** were separated semipreparatively by liquid chromatography on triacetylcellulose (20–30 μm)[36] with EtOH as eluent at 22 °C or 25 °C (2–3 bar, flow rate 2–5 mL/min). The chromatogram showed an incomplete separation of the rather broad peaks for the two isomers. However, fractions containing optically pure (+)-enantiomers could be obtained. Since (−)-isomers turned out to be much less active, no further effort was made to increase their enantiomeric purity. Capacity factors, data on specific rotations, and optical purities are reported in Table IV.

Biochemical and Biological Methods. Reagents. [2,4,6,7-^{3}H]-17β-Estradiol (90–115 Ci/mmol) was obtained from New England Nuclear, Dreieich, FRG. Hormones and biochemicals were purchased from Sigma, München, FRG. TEA [Tris buffer (10 mM, pH 7.5) supplemented with EDTA (1.0 mM) and NaN_3 (3 mM)] was used as buffer.

Estradiol Receptor Binding Assay. Fresh calf uteri, stored in ice-cold saline, were freed of adherent fat and connective tissue at 4 °C. After addition of TEA buffer (1 mL/g), the uteri were homogenized by treatment with a ultraturrax mixer (IKA, FRG) and a glass-in-glass homogenizer (Potter S; Braun, FRG) at 4 °C. Lipids were separated by centrifugation at 10500*g* for 10 min and discarded. The homogenate was centrifuged at 105000*g* for 60 min (4 °C). The supernatant (cytosol) was then used for determining the affinity of compounds for the estrogen receptor. The protein concentration of the cytosol was ca. 15 mg/mL leading to a final concentration of 3 mg/mL in the assay.

For the determination of the relative binding affinity (RBA), the described procedure was applied with modifications.[3] The 500-μL incubation mixture was comprised of 5 nM [^{3}H]-17β-estradiol (added in 100 μL TEA), 10^{-9} to 10^{-5} M competing ligand (in 100 μL TEA), 100 μL of uterine cytosol, and TEA. The mixture was incubated for 18 h at 4 °C, 0.5 mL of dextran-coated charcoal (DCC) slurry (0.8% charcoal Norit A and 0.008% dextran in TEA) was added to the tubes, and the contents were mixed. The tubes were incubated for 90 min at 4 °C and then centrifuged at 700*g* for 10 min to pellet the charcoal. An aliquot (100 μL) of the supernatant was removed, and the radioactivity was determined by liquid scintillation measurement after addition of 3 mL of Quickszint 212 (Zinsser). Nonspecific binding was calculated using 4 μM 17β-estradiol as competing ligand. Six concentrations of competitor should cover the range where 10–90% of [^{3}H]estradiol has been displaced from the receptor. Radioactivity was plotted as a function of log concentration of competing ligand in the assay. The RBA was calculated as the ratio of the molar concentrations of estradiol and test compound required to decrease the amount of bound radioactivity by 50%, multiplied by 100.

Determination of Cytostatic Activity. Hormone-sensitive human MCF-7 breast cancer cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were grown in improved minimal essential medium (MEM), as modified by Richter et al.[37] (Biochrom, Berlin, FRG), supplemented with glutamine (0.3 g/L), gentamycin (60 mg/L), and 10% newborn calf serum (NCS) (Gibco). The serum was sterilized through a 0.20-μm filter (Sartorius, Göttingen, FRG) and stored at −20 °C. Cells were grown in a humidified incubator in 5% CO_2 at 37 °C and harvested with 0.05% trypsin–0.02% EDTA in 0.15 M NaCl.

At the start of the experiment, the cell suspension was transferred to 96-well microplates (150 μL/well). After growing them for 3 d in a humidified incubator with 5% CO_2 at 37 °C, medium was replaced by one containing the drug. Control wells (16/plate) contained 0.1% of DMF that was used for the preparation of the stock solution. The initial cell density was determined by addition of vinblastine (10^{-7} M). After incubation for 4–7 d, medium was removed and 100 μL of glutaric aldehyde in PBS (1%) was added for fixation. After 15 min, the solution of aldehyde was decanted. Cells were stained by treating them for 25 min with 100 μL of an aqueous solution of crystal violet (0.02%). After decanting, cells were washed several times with water to remove adherent dye. After addition of 100 μL of ethanol (70%), plates were gently shaken for 2 h. Optical density of each well was measured in a microplate autoreader EL 309 (Bio-tek)

(36) Koller, H.; Rimböck, K. H.; Mannschreck, A. High-pressure Liquid Chromatography on Triacetylcellulose. Characterization of a Sorbent for Separation of Enantiomers. *J. Chromatogr.* **1983**, *282*, 89–94.

(37) Richter, A.; Sandford, K. K.; Evans, V. J. Influence of Oxygen and Culture Medium on Plating Efficiency in Some Mammalian Tissue Culture Cells. *J. Natl. Cancer Inst.* **1972**, *49*, 1705–1712.

at 578 nm. Data calculation and analysis were performed on a Olivetti M24 PC.[38]

Hormone-independent MDA-MB 231 human mammary tumor cells were also obtained from ATCC. Cells were grown in McCOY 5a medium supplemented with 10% NCS and gentamycin (40 μg/mL). Cytostatic activity was determined in a microplate assay as described for MCF-7 cells with one exception: the incubation period was reduced to 2 d.

Mice Uterine Weight Tests. Immature female mice (20 days old, of the NMRI strain) from Charles River, Wiga, Sulzfeld, FRG, were randomly divided into groups of six to ten animals. To determine estrogenic activity, compounds were dissolved in olive oil (100 μL/animal) and injected subcutaneously on three consecutive days. Control animals received the vehicle alone. Twenty-four hours after the last injection, the animals were killed by cervical dislocation and weighed. Uteri were dissected free of fat and fixed in Bouin solution (saturated aqueous picric acid/40% formaldehyde/glacial acetic acid 15:5:1 by vol) for 2 h. Uteri were freed from connective tissue, washed with ethanol, dried at 100 °C for 18 h, and weighed. The relative uterus weight was calculated by the formula: uterine dry weight (mg)/body weight (g), multiplied by 100. To determine the antiestrogenic activity, injections contained a standard dose (0.4 μg) of estrone and increasing doses of the compounds. The inhibition (%) of the estrone-stimulated uterine growth was estimated by the formula: $100 - [(W_{S,T} - W_V)/(W_S - W_V) \times 100]$ ($W_{S,T}$ = rel uterus weight of animals treated with estrone standard (0.4 μg) + test compound; W_V = rel uterus weight of control animals; W_S = rel uterus weight of animals treated with estrone standard).

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Supplementary Material Available: ^{1}H NMR data of 2-(methoxyphenyl)-*N*-[2-(3-methoxyphenyl)ethyl]acetamides (**3a**, **3e–j**), 1-benzyl-1,2,3,4-tetrahydroisoquinolines (**4a**, **4e–j**), 5,6-dihydro-dimethoxyindolo[2,1-*a*]isoquinolines (**5a**, **5e–j**), 12-formyl-5,6-dihydro-dimethoxyindolo[2,1-*a*]isoquinolines (**6a–j**), and diacetoxy-12-formyl-5,6-dihydroindolo[2,1-*a*]isoquinolines (**7a–j**) (6 pages). Ordering information is given on any current masthead page.

(38) Reile, H.; Birnböck, H.; Bernhardt, G.; Spruß, T.; Schönenberger, H. Computerized Determination of Growth Kinetic Curves and Doubling Times from Cells in Microculture. *Anal. Biochem.* **1990**, *187*, 262–267.

(39) Mannschreck, A.; Eiglsperger, A.; Stühler, G. Determination of Enantiomeric Purity in Spite of Incomplete Chromatographic Separation. *Chem. Ber.* **1982**, *115*, 1568–1575.

6-Substituted Decahydroisoquinoline-3-carboxylic Acids as Potent and Selective Conformationally Constrained NMDA Receptor Antagonists

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We have prepared a series of 6-substituted decahydroisoquinoline-3-carboxylic acids, and structurally similar analogs, as potential *N*-methyl-D-aspartate receptor antagonists. There is a large body of evidence to support the use of such compounds as cerebroprotective agents in a variety of acute and chronic neurodegenerative disorders, where some component of glutamate-mediated excitotoxicity may exist. The compounds prepared were evaluated in vitro in both receptor binding assays ([^{3}H]CGS19755, [^{3}H]AMPA, and [^{3}H]kainic acid) and in a cortical wedge preparation (versus NMDA, AMPA, and kainic acid) to determine affinity, potency, and selectivity. The new amino acids were also evaluated in vivo for their ability to block NMDA-induced lethality in mice. We synthesized many of the possible diastereomers of the decahydroisoquinoline nucleus in order to examine the spatial and steric requirements for affinity at the NMDA receptor and activity as NMDA antagonists. From our structure–activity relationship we identified two potent and selective NMDA receptor antagonists, the phosphonate- and tetrazole-substituted amino acids **31a** and **32a**, respectively, that show good activity in animals following systemic administration. For example, **31a** and **32a** selectively displaced [^{3}H]CGS19755 binding with IC_{50}s of 55 ● 14 and 856 ± 136 nM, respectively, and selectively antagonized responses due to NMDA in a cortical wedge preparation with IC_{50}s of 0.15 ± 0.01 and 1.39 ± 0.29 μM, respectively. And compounds **31a** and **32a** blocked NMDA-induced lethality in mice with minimum effective doses of 1.25 and 2.5 mg/kg (intraperitoneal), respectively. These novel amino acids are among some of the most potent NMDA antagonists described thus far, and are excellent candidates for development as neuroprotective agents for a number of CNS disorders.

Glutamic acid is an important excitatory amino acid (EAA) neurotransmitter in the central nervous system and is involved in a myriad of neuronal functions.[1] There are at least four well-characterized EAA receptor subclasses.[1] The ionotropic NMDA (for *N*-methyl-D-aspartic acid), AMPA (for 2-amino-3-(5-methyl-3-hydroxyisoxazol-4-yl)-propanoic acid) and kainic acid (KA) receptors are all linked to ion channels permeable to sodium and, in the case of NMDA and AMPA receptors, calcium. The metabotropic or 1*S*,3*R*-ACPD-sensitive glutamate receptor (for (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid) receptor is coupled via a G-protein to phosphoinositide hydrolysis.

Under certain circumstances, glutamic acid can be lethal to cells[2] (excitotoxicity). For example, in cerebral ischemia,[3,4] when cellular energy stores are diminished, gluta-

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(1) Collingridge, G. L.; Lester, R. A. Excitatory amino acid receptors in the vertebrate central nervous system. *Pharmacol. Rev.* **1989**, *40*, 143–210.

(2) Meldrum, B.; Garthwaite, J. Excitatory amino acid neurotoxicity and neurodegenerative disease. *Trends Pharmacol. Sci.* **1990**, *11*, 379–387.

(3) Meldrum, B. Protection against ischemic neuronal damage by drugs acting on excitatory neurotransmission. *Cerebrovasc. Brain Metab. Rev.* **1990**, *2*, 27–57.

0022-2623/92/1835-3547$03.00/0