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Enantiomers of C_5-Chiral 1-Acetyl-3,5-diphenyl-4,5-dihydro-(1*H*)-pyrazole Derivatives: Analytical and Semipreparative HPLC Separation, Chiroptical Properties, Absolute Configuration, and Inhibitory Activity against Monoamine Oxidase

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ABSTRACT The HPLC enantiomer separation of a novel series of C_5-chiral 1-acetyl-3-(4-hydroxy- and 2,4-dihydroxyphenyl)-5-phenyl-4,5-dihydro-(1*H*)-pyrazole derivatives, with inhibitory activity against monoamine oxidases (MAO) type A and B, was accomplished using polysaccharide-based chiral stationary phases (CSPs: Chiralpak AD, Chiralcel OD, and Chiralcel OJ). Pure alcohols, such as ethanol and 2-propanol, and typical normal-phase binary mixtures, such as *n*-hexane and alcohol modifier, were used as mobile phases. Single enantiomers of several analytes examinated were isolated on a semipreparative scale, and their chiroptical properties were measured. The assignment of the absolute configuration was established for one compound by single-crystal X-ray diffraction method and for the other three by CD spectroscopy. The inhibitory activity against MAO of racemic samples and single enantiomers were evaluated in vitro. *Chirality 16:625–636, 2004*.

KEY WORDS: MAO inhibitors; chiral HPLC; polysaccharide chiral stationary phases; semipreparative scale separation; chiroptical properties; X-ray crystallography; absolute configuration

Chiral HPLC is one of the most rapid and efficient methods for obtaining both enantiomers of a chiral compound in high optical purity in a single operation. This approach has now become an established procedure for preliminary comparative biological testing, where a few milligrams of both enantiomers are needed. In addition, the isolation of single enantiomers represents a focus for rational drug design. The structural analysis of enantiomerically pure drug or enantiomerically pure ligand–receptor complexes by X-ray crystallography and computerized molecular modeling techniques allows the clarification of the pivotal role of stereochemistry in molecular interactions involving biological macromolecules.

Monoamine oxidases (MAOs) catalyze oxidative deamination of monoamine neurotransmitters in the central nervous system and peripheral tissues. In mammals, MAO exists in two forms, MAO-A and MAO-B, which are encoded by different genes and differ by preferential substrate oxidation.[1] Altered MAO activity is observed in several psychiatric and neurological diseases. The use of MAO inhibitors in the treatment of these disorders has made this enzyme a very popular target for basic and pharmaceutical studies. Reversible and selective MAO-A inhibitors are employed clinically as antidepressant and antianxiety drugs,[2] whereas selective MAO-B inhibitors are co-adjuvants in the treatment of Parkinson's disease.[3]

Our recent investigations in this area resulted in the synthesis of a series of C_5-chiral 1-acetyl-3-(4-hydroxy- and 2,4-dihydroxyphenyl)-5-phenyl-4,5-dihydro-(1*H*)-pyrazole derivatives **1–10** (Fig. 1), which have been shown to have reversible, potent, and selective in vitro activity against MAO-A.[4]

Thus, in order to evaluate selective effects in molecular activity of the individual enantiomers, we now report on the direct enantioseparation of the chiral compounds **1–10** by chiral HPLC on three polysaccharide-based chiral sta-

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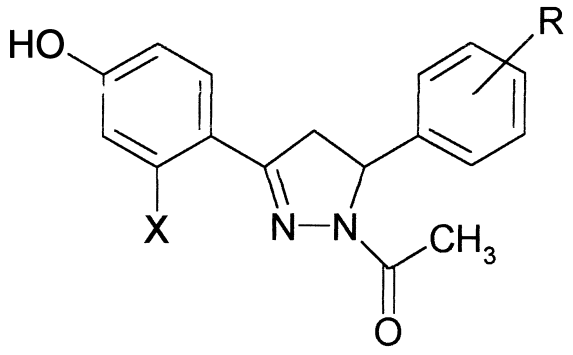

Compound	X	R
1	H	2 OCH_3
2	OH	2 OCH_3
3	H	4 OCH_3
4	OH	4 OCH_3
5	H	2,4 OCH_3
6	OH	2,4 OCH_3
7	H	4 CH_3
8	OH	4 CH_3
9	H	2 Cl
10	H	3 Cl

Fig. 1. Structures of the chiral analytes **1–10**.

tionary phases (CSPs): Chiralpak AD, Chiralcel OD, and Chiralcel OJ.[5] Changes in mobile-phase composition were studied to determine the effect of solvents on the column selectivity. Analyte detection following enantioseparation used both UV and polarimetric detection.

To obtain data for a mechanistic description of the stereoselective ligand–receptor interactions and improve the pharmacological profile of these compounds, the absolute configuration of enantiomers separated on a semipreparative scale was established by X-ray crystallography and CD spectroscopy. The conformational features of the 1-acetyl-3-(2,4-dihydroxyphenyl)-5-(2-methoxyphenyl)-4,5-dihydro-(1*H*)-pyrazole derivative **2** were also evaluated by computational methods.

MATERIALS AND METHODS

Chiral Liquid Chromatography

Racemates **1–10** (Fig. 1) were synthesized by a chemical pathway reported elsewhere.[4] Chiral HPLC of the analytes was performed by using stainless-steel Chiralcel OD (250 × 4.6 mm i.d. and 250 × 10 mm i.d.), Chiralpak AD (250 × 4.6 mm i.d.), and Chiralcel OJ (250 × 4.6 mm i.d.) (Daicel Chemical Industries, Tokyo, Japan) columns. HPLC-grade solvents were supplied by Carlo Erba (Milan, Italy). Chiral HPLC was performed by using a PerkinElmer (Norwalk, CT) 200 lc pump equipped with a Rheodyne (Cotati, CA) injector, a 1-ml sample loop, a PerkinElmer HPLC oven and a Waters (Milford, MA) Model 996 diode array detector (DAD). The sign of the optical rotations of enantiomers of **1–10** was measured online at a wavelength of 365 nm by a PerkinElmer model 241 polarimeter equipped with Hg/Na lamps and a 40-μl flow cell. The system was kept at a constant temperature of 25°C. The signal was acquired and processed by Millenium 2010 software.

The normal-phase eluents for chromatographic separations were prepared by mixing an alcohol modifier (ethanol or 2-propanol) with *n*-hexane at defined ratios. The mobile phases were filtered and degassed by sonication immediately before use. Flow rates of 0.3, 0.5, or 1.0 ml/min for analytical separations and 1.5, 2.0, or 3.0 ml/min for semipreparative separations were used (see Table 4). The UV detector was set at 254 nm and 340 nm for analytical and semipreparative separations, respectively.

Analytical separations were performed at 25°C constant temperature. Standard solutions, prepared by dissolving 1–3 mg of each analyte in 25 ml of chromatographic eluent, were employed. The injection volume was 20 μl. The semipreparative resolution of compound **2** was performed by using the solid injection technique described elsewhere.[6] In the semipreparative run of **1**, **9**, and **10**, 4–10 mg of racemate, dissolved in 500–1000 μl of ethanol or *n*-hexane–ethanol 50:50 (v/v), was injected on to a 10-mm i.d. OD CSP at 25°C (see Table 4). After semipreparative separation, the fractions collected were analyzed on chiral analytical columns to determine their enantiomeric excess (ee).

Polarimetry and Circular Dichroism

Optical rotations of enantiomers of chiral analytes **1**, **2**, **9**, and **10**, dissolved in chloroform, were measured at a wavelength of 589 nm by a PerkinElmer Model 241 polarimeter equipped with an Na lamp. The volume of the cell was 1 ml, and the optical path was 10 cm. The system was kept at a constant temperature of 23°C.

The circular dichroism (CD) spectra of enantiomers of chiral analytes **1**, **2**, **9**, and **10**, dissolved in ethanol (concentration about 0.3 mmol/l), in a quartz cell (1-cm path length) at room temperature, were measured using a Jasco (Jasco, Ishikawa-cho, Hachioji City, Tokyo, Japan) Model J-700 spectropolarimeter. The spectra are computer averages over three instrument scans, and the intensities are presented in terms of $\Delta\varepsilon$ values.

Computational Analysis

The calculations were performed on a personal computer. Semiempirical and DFT ab initio methods as implemented with the Titan 1.0.1. package were used throughout this work. A preliminary conformational search on pyrazole derivative **2** was carried out by a Monte Carlo stochastic procedure, with the structure energy calculated at the semiempirical level (Himiltonian AM1). All the rotatable bonds were explored up to obtain 300 least-energy structures. Low-energy structures

found within 1 kcal/mol were optimized at the SCF level by the DFT B3LYP ab initio method and 6-31G** basis set.

X-Ray Crystal Structure Analysis

The (+)-**9** enantiomer crystallized from a solution of acetone and water as colourless elongated thin prisms. Crystal data: $C_{17}H_{15}ClN_2O_2$, M = 314.76, monoclinic, a = 10.702(4) Å, b = 4.8734(8) Å, c = 15.231(3) Å, β = 95.50(2)°, V = 790.7(4), space group $P2_1$, Z = 2, D_c = 1.322 Mg/m^{-3}, $F(000)$ = 328, μ (Cu Kα) = 2.209 mm^{-1}, T = 293 K. A crystal of dimensions 0.8 × 0.2 × 0.1 mm was selected and fixed on a glass fiber. Diffraction data were collected on a Rigaku four-circle diffractometer equipped with a rotating anode (graphite-monochromated Cu Kα radiation) by the θ–2θ scan method in the range $2.9° \le \theta \le 62.1°$. Data were corrected for Lorentz and polarization effects and from absorption by the semiempirical ψ-scan method. The structure was solved by direct methods using the SIR2002 package of crystallographic programs[7] and refined using the program SHELXL-97.[8] Non-hydrogen atoms were refined anisotropically, while hydrogens were included at calculated positions and refined in a riding mode. Correction for extinction was applied (coefficient 0.047(4)). The refinement of 199 parameters, carried out on F^2, with the molecule in the *R*-configuration converged at R_F = 0.0395 for 1,359 reflections with $F_o > 4\sigma(F_o)$, S = 1.037. Refinement of the absolute structure Flack[9] factor x for the *R*-configuration gave the expected value for the right solution, x = 0.00(3), while for the *S*-configuration, x = 0.93(3). Such values, together with a significantly low standard deviation due to the large anomalous scattering of the chlorine atom present in the molecule, allow us to assign unambiguously the *R*-configuration to the (+)-**9** derivative.

In Vitro Biochemistry

All chemicals were commercial reagents of analytical grade and were used without further purification. Bovine brain mitochondria were isolated according to Basford.[10] In all experiments, the MAO activities of the beef brain mitochondria were determined by a fluorometric method, according to Matsumoto et al.[11] using kinuramine as a substrate at final concentration of 0.1 mM. The incubation mixtures contained 0.25 M potassium phosphate buffer (pH 7.4), 0.3 mg mitochondria, and drug solutions with final concentration ranging from 0 to 10^{-3} μM.

The solutions were incubated at 38°C for 30 min. Addition of perchloric acid terminated the reaction. The samples were centrifuged at 10,000*g* for 5 min, and the supernatant was added to 2.7 ml of 0.1 N NaOH. The pyrazole derivatives **9** and **10** were dissolved in dimethyl sulfoxide (DMSO) and added to the reaction mixture from 0 to 10^{-3} μM. In order to study the inhibition of both racemic and enantiomeric forms of chiral compounds **9** and **10** on the activities of both MAO-A and MAO-B separately, the mitochondrial fractions were pre-incubated at 38°C for 30 min before the specific inhibitors were added 0.5 μM L-deprenyl to estimate MAO-A activity, and 0.05 μM clorgyline to assay MAO-B), considering that MAO-A is irreversibly inhibited by a low concentration of clorgyline, but is unaffected by a low concentration of L-deprenyl, which inhibits MAO-B. The solutions were incubated at 38°C for 30 min. Fluorometric measurements were recorded with a PerkinElmer LS 50B spectrofluorimeter. The protein concentration was determined according to Bradford.[12] The data are the means of three or more experiments performed in duplicate. The IC_{50} values were obtained plotting the % of the specific activity versus −Log of the drug concentrations.

RESULTS AND DISCUSSION

Analytical Enantioseparation of 1–10 on Polysaccharide-Based OD, AD, and OJ CSPs

The basic material of polysaccharide-type CSPs consists of modified cellulose or amylose polymers. Cellulose is a linear polysaccharide containing (+)-D-glucose units linked by β-glycosidic bonding, whereas amylose contains α-glycosidic linkages and adopts a helical structure. The most effective polysaccaride-type CSPs are based on phenylcarbamate or benzoate derivatives.[13] Among many semisynthetic polymeric selectors prepared so far, the 3,5-dimethylphenylcarbamates of cellulose (Chiralcel OD) and amylose (Chiralpak AD) and the 4-methylbenzoate of cellulose (Chiralcel OJ) represent efficient CSPs for HPLC enantioseparations of a broad range of chiral compounds, including drugs.[14–17]

The chromatographic enantioseparation of analytes **1–10** was studied using the aforementioned polysaccharide-based OD, AD, and OJ CSPs in combination with normal-phase and polar organic eluents. In particular, four types of mobile phases were adopted: binary mixture *n*-hexane–ethanol 70:30 (v/v), binary mixture *n*-hexane–2-propanol 60:40 (v/v), pure ethanol, and pure 2-propanol. In order to analyze the chromatographic data obtained, we divided the chiral compounds **1–10** into two groups. Group 1 comprises compounds **1**, **3**, **5**, **7**, **9**, and **10**, which contain one hydroxyl group in para position on the phenyl ring in position 3 of pyrazoline moiety, and group 2 comprises the analytes **2**, **4**, **6**, and **8** with two hydroxyl groups in ortho and para positions of same phenyl ring.

The chromatographic results obtained on Chiralcel OD CSP are shown in Table 1. The racemates were resolved with at least one of the four eluents employed. The nature and position of substituents on the phenyl ring in position 5 of the pyrazoline moiety strongly influenced the enantioseparation factor values. The CSP OD exhibited high chiral recognition ability for compounds **1** and **2** (Table 1, entries 1, 2, 11, 12, 21, 22, 31, and 32) in all the conditions used. By moving the 2-OCH_3 of the phenyl group from the ortho (compounds **1** and **2**) to the para position (compounds **3** and **4**), either low resolution (Table 1, entry 4) or no resolution (Table 1, entries 3, 13, and 14) was observed using binary mixtures of *n*-hexane–alcohol modifier as eluents. Within group 1, when the 2-OCH_3 group (compound **1**) was replaced by a chlorine atom (compound **9**), a consistent reduction in resolution was observed with all mobile phases. By substitution of the 2-OCH_3 (compounds **1** and **2**) group with a 4-CH_3 group, poor enantioseparation for compounds **7** and **8** was achieved (Table 1, entries 7 and 8) using a mixture of *n*-hexane–ethanol 70:30 (v/v) as

TABLE 1. Retention factors (k'_1) for the first eluting enantiomer, enantioseparation (α), and resolution (*Rs*) factors of 1–10 on Chiralcel OD (250 × 4.6 mm I.D.)*

Entry	Compound	Eluent	k'_1[d]	α	*Rs*
1	**1**	*n*-Hexane–ethanol (70:30, v/v)[a]	0.82 (–)	1.83	3.84
2	**2**		1.87 (–)	2.21	6.22
3	**3**		0.99	1.00	—
4	**4**		2.12 (+)	1.06	0.45
5	**5**		0.93 (+)	1.16	0.25
6	**6**		1.85	1.00	—
7	**7**		0.61 (–)	1.11	0.53
8	**8**		1.31 (–)	1.13	0.63
9	**9**		1.22 (–)	1.20	1.09
10	**10**		1.02 (–)	1.35	1.68
11	**1**	*n*-Hexane–2-propanol (60:40, v/v)[a]	1.22 (–)	1.65	2.36
12	**2**		2.35 (–)	2.26	3.85
13	**3**		1.23	1.00	—
14	**4**		3.14	1.00	—
15	**5**		1.36	1.00	—
16	**6**		2.71	1.00	—
17	**7**		0.88	1.00	—
18	**8**		1.90 (–)	1.14	0.50
19	**9**		1.82 (–)	1.09	0.42
20	**10**		1.73 (–)	1.58	0.83
21	**1**	Ethanol[b]	0.33 (–)	2.45	4.40
22	**2**		0.54 (–)	3.44	8.51
23	**3**		0.34 (+)	1.12	0.54
24	**4**		0.61	1.00	—
25	**5**		0.30 (+)	1.40	1.37
26	**6**		0.52 (–)	1.21	0.98
27	**7**		0.27 (–)	~1	—
28	**8**		0.48 (–)	~1	—
29	**9**		0.50 (–)	1.60	2.25
30	**10**		0.37 (–)	1.30	0.95
31	**1**	2-Propanol[c]	0.54 (–)	2.44	4.80
32	**2**		1.05 (–)	3.27	7.86
33	**3**		0.63 (+)	1.16	0.64
34	**4**		1.32	1	—
35	**5**		0.61 (+)	1.38	1.27
36	**6**		1.16 (+)	1.20	0.65
37	**7**		0.48 (–)	~1	—
38	**8**		0.81 (–)	1.17	0.70
39	**9**		0.93 (–)	1.60	2.17
40	**10**		0.69 (–)	1.42	1.33

*Eluent, *n*-hexane–alcohol modifier (ethanol or 2-propanol) or pure polar organic solvent (ethanol or 2-propanol); temperature, 25°C; detector, UV at 254 nm and polarimeter at 365 nm.
[a]Flow rate 1 ml/min.
[b]Flow rate 0.5 ml/min.
[c]Flow rate 0.3 ml/min.
[d]Sign of optical rotation at 365 nm.

mobile phase. In normal-phase conditions, the analytes of the group 2, with the exception of compound **6** (Table 1, entry 6), were resolved better than the corresponding monosubstituted analytes of group 1, while the data obtained using the polar organic mode were more variable (for example, see Table 1, entries 21 vs. 22 and 35 vs. 36). The binary mixture of *n*-hexane–2-propanol 60:40 (v/v) appeared to be the least suitable mobile phase among those examined. Thus, in this case, no enantioseparation of compounds **3**, **4**, **5**, **6**, and **7** was observed. As a general trend, the resolving ability of the OD CSP was increased passing from normal-phase conditions to polar organic mode conditions, and the retention factors of analytes of group 1 were shorter than those of analytes of group 2.

Chiralpak AD, which has same 3,5-dimethylphenylcarbamate structural unit as Chiralcel OD but bonded to amylose instead of cellulose, exhibited a complementary resolving ability for many of the analytes examined with

respect to its cellulosic analogue (Table 2). For example, compound **3** was well resolved on Chiralpak AD with *n*-hexane–ethanol 70:30 (v/v) (Table 2, entry 3) and *n*-hexane–2-propanol 60:40 (v/v) (Table 2, entry 13), but no enantioseparation was observed on Chiralcel OD with the same eluents (Table 1, entries 3 and 13). Of 10 chiral compounds examined on the AD CSP, no enantioselectivity was observed for one (Table 2, compound 7, entry 7) using the *n*-hexane–ethanol mobile phase, for five (Table 2, compounds **1**, **2**, **5**, **6**, and **9**, entries 11, 12, 15, 16, and 19) with the *n*-hexane–2-propanol mobile phase, for four (Table 2, compounds **7**, **8**, **9**, and **10**, entries 27, 28, 29, and 30) in pure 2-propanol, and for five (Table 2, compounds **1**, **2**, **5**, **6**, and **9**, entries 31, 32, 35, 36, and 39) in pure ethanol.

The contribution of functional groups on both phenyl rings of the analytes toward chiral recognition is unclear. For example, the chiral 1-acetyl-3-(2,4-dihydroxy-phenyl)-5-(4-methylphenyl)-4,5-dihydro-(1*H*)-pyrazole derivative **8**

TABLE 2. Retention factors (k'_1) for the first eluting enantiomer, enantioseparation (α), and resolution (*Rs*) factors of 1–10 Chiralpak AD (250 × 4.6 mm I.D.)*

Entry	Compound	Eluent	k'_1[d]	α	*Rs*
1	**1**	*n*-Hexane–ethanol (70:30, v/v)[a]	0.56 (–)	1.62	3.17
2	**2**		1.53 (–)	1.33	2.71
3	**3**		0.80 (+)	1.69	2.54
4	**4**		2.11	1.55	3.85
5	**5**		0.84 (–)	1.67	2.50
6	**6**		2.09 (–)	1.14	0.96
7	**7**		0.81	1	—
8	**8**		1.32 (+)	1.45	3.44
9	**9**		0.51 (–)	1.10	0.54
10	**10**		0.62 (–)	1.26	1.47
11	**1**	*n*-Hexane–2-propanol (60:40, v/v)[a]	0.33 (–)	~1	—
12	**2**		0.58	1	—
13	**3**		0.47 (+)	1.83	3.38
14	**4**		0.73 (+)	2.01	4.26
15	**5**		0.48	1	—
16	**6**		0.85	~1	—
17	**7**		0.30 (+)	1.93	3.15
18	**8**		0.45 (+)	2.07	3.43
19	**9**		0.37 (+)	1	—
20	**10**		0.39 (–)	1.64	1.84
21	**1**	Ethanol[b]	0.12 (–)	1.42	0.62
22	**2**		0.32 (–)	1.34	1.32
23	**3**		0.24 (+)	1.29	0.96
24	**4**		0.75 (+)	1.12	0.60
25	**5**		0.18 (–)	1.40	0.93
26	**6**		0.45 (–)	1.27	1.25
27	**7**		0.18	1	—
28	**8**		0.54 (+)	~1	—
29	**9**		0.17	1	—
30	**10**		0.23 (–)	~1	—
31	**1**	2-Propanol[c]	0.12 (–)	~1	—
32	**2**		0.20	1	—
33	**3**		0.18 (+)	1.67	1.31
34	**4**		0.25	1.96	1.60
35	**5**		0.19	1	—
36	**6**		0.30	1	—
37	**7**		0.13 (+)	1.7	0.99
38	**8**		0.18 (+)	1.8	1.56
39	**9**		0.14	1	—
40	**10**		0.14 (–)	1.71	0.99

*Eluent, *n*-hexane–alcohol modifier (ethanol or 2-propanol) or pure polar organic solvent (ethanol or 2-propanol); temperature, 25°C; detector, UV at 254 nm and polarimeter at 365 nm.
[a]Flow rate 1 ml/min.
[b]Flow rate 0.5 ml/min.
[c]Flow rate 0.3 ml/min.
[d]Sign of optical rotation at 365 nm.

(group 2) was resolved (α = 1.45) into its enantiomers with *n*-hexane–ethanol 70:30 (v/v) as eluent while no enantioseparation was achieved for the corresponding 1-acetyl-3-(4-hydroxy)-5-phenyl-4,5-dihydro-(1*H*)-pyrazole derivative **7** (group 1). In contrast, using the same mobile phase, the enantiomers of analyte **5** of group 1 and the corresponding analyte **6** of group 2 were separated with enantioseparation factors of 1.67 and 1.14, respectively. Enantioselectivity factor values greater than **2** were observed for compounds **4** and **8** (Table 2, entries 14 and 18).

In contrast to OD CSP, the chiral resolving ability of AD CSP decreased passing from normal-phase conditions to polar organic mode conditions.

Table 3 summarizes the chromatographic data obtained on the non-carbamate type CSP, cellulose tris(4-methylbenzoate) (Chiralcel OJ). The experimental results (Tables 1–3) indicate that the OJ CSP exhibited higher

TABLE 3. Retention factors (k'_1) for the first eluting enantiomer, enantioseparation (α), and resolution (*Rs*) factors of 1–10 on Chiralcel OJ (250 × 4.6 mm I.D.)*

Entry	Compound	Eluent	k'_1[d]	α	*Rs*
1	**1**	*n*-Hexane–ethanol (70:30, v/v)[a]	1.40 (+)	1.89	4.44
2	**2**		3.34 (+)	1.32	1.93
3	**3**		2.29 (+)	5.31	11.95
4	**4**		5.18 (+)	2.71	7.37
5	**5**		2.07 (+)	1.66	0.78
6	**6**		5.30 (+)	1.13	0.86
7	**7**		1.05 (+)	6.15	10.80
8	**8**		2.30 (+)	3.03	7.39
9	**9**		1.77 (+)	4.14	9.63
10	**10**		1.46 (+)	3.11	8.74
11	**1**	*n*-Hexane–2-propanol (60:40, v/v)[a]	0.90 (+)	1.38	1.12
12	**2**		2.46 (+)	~1	—
13	**3**		1.51 (+)	3.28	4.80
14	**4**		3.52 (+)	2.25	3.66
15	**5**		2.44 (+)	1.52	0.84
16	**6**		4.32 (+)	~1	—
17	**7**		0.77 (+)	2.80	4.12
18	**8**		2.07 (+)	1.78	2.24
19	**9**		1.18 (+)	1.73	2.17
20	**10**		1.00 (+)	1.93	2.99
21	**1**	Ethanol[b]	0.34 (+)	1.76	2.28
22	**2**		0.64 (+)	1.25	1.1
23	**3**		0.54 (+)	3.8	7.8
24	**4**		0.93 (+)	2.24	4.83
25	**5**		0.46 (+)	1.59	1.98
26	**6**		0.90 (+)	1.19	0.89
27	**7**		0.32 (+)	5.00	7.78
28	**8**		0.53 (+)	2.62	4.66
29	**9**		1.62 (+)	3.56	6.48
30	**10**		0.36 (+)	2.69	4.66
31	**1**	2-Propanol[c]	0.34 (+)	1.32	0.63
32	**2**		0.69 (+)	1.12	0.41
33	**3**		0.56 (+)	2.66	3.68
34	**4**		1.07 (+)	2.06	2.94
35	**5**		0.50 (+)	1.38	0.69
36	**6**		1.03 (+)	1.21	0.58
37	**7**		0.32 (+)	2.5	2.7
38	**8**		0.67 (+)	1.88	2.11
39	**9**		0.71 (+)	1.51	1.32
40	**10**		0.36 (+)	1.72	1.68

*Eluent, *n*-hexane–alcohol modifier (ethanol or 2-propanol) or pure polar organic solvent (ethanol or 2-propanol); temperature, 25°C; detector, UV at 254 nm and polarimeter at 365 nm.
[a]Flow rate 1 ml/min.
[b]Flow rate 0.5 ml/min.
[c]Flow rate 0.3 ml/min.
[d]Sign of optical rotation at 365 nm.

TABLE 4. Chromatographic, quantitative, and polarimetric analyses of fractions F1 and F2 recovered during the enantioseparation of the pyrazole derivatives 1, 2, 9, and 10

		F1			F2		
Compound	MP/Col/SR/T/FR[a]	ee (%)	Yield (%)	$[\alpha]_D^{23}$	ee (%)	Yield (%)	$[\alpha]_D^{23}$
1	A/OD/4/25/3.0	>99.0	80	−387 (c = 0.11, $CHCl_3$)	98.8	70	+375 (c = 0.15, $CHCl_3$)
2	B/OD/10/40/2.0	>99.0	55	−330 (c = 0.12, $CHCl_3$)	>99.0	50	+325 (c = 0.10, $CHCl_3$)
9	B/OD/10/25/3.0	>99.0	80	−454 (c = 0.45, $CHCl_3$)	>99.0	75	+443 (c = 0.38, $CHCl_3$)
10	A/OD/5/25/1.5	>99.0	75	−238 (c = 0.28, $CHCl_3$)	>99.0	70	+243 (c = 0.16, $CHCl_3$)

[a]MP/Col/SR/T/FR refers to the combination of experimental conditions used for semipreparative enantioseparations: MP, mobile phase (A, *n*-hexane–ethanol 70:30; B, ethanol); Col, column (Chiralcel OD 250 × 10 mm i.d.); SR, semipreparative run (mg); T, temperature (°C); FR, flow-rate (ml/min).

chiral resolving ability in comparison with the OD and AD CSPs. Only for compound **2** did OD CSP evidence higher enantioselectivity compared to the OJ CSP. All analytes were resolved on OJ CSP using *n*-hexane–ethanol, pure ethanol, and pure 2-propanol as eluents. Only for compounds **2** and **6** did the enantioseparation disappear when the organic modifier ethanol was replaced with 2-propanol in a binary mixture with *n*-hexane. The mixture *n*-hexane–ethanol 70:30 (v/v) appeared to be the most suitable mobile phase for all compounds examined. Figure 2 shows typical chromatograms illustrating the resolution of pyrazole derivatives **3** and **9** on the OJ CSP using *n*-hexane–ethanol (70:30, v/v) as an eluent. Thus, from 10 chiral analytes examined, enantioselectivity factor values superior to 3 were observed for five (Table 3, compounds **3**, **7**, **8**, **9**, **10**, and entries 3, 7, 8, 9, and 10). The highest enantioselectivity factor (α = 6.15) was achieved for compound **7** using *n*-hexane–ethanol 70:30 (v/v) as a mobile phase. The chiral resolving ability of OJ CSP decreased passing from normal-phase conditions to polar organic mode conditions. All compounds of group 1 were resolved better than compounds of group 2, using both normal-phase eluents and polar organic mode. The enantiorecognition ability of the OJ CSP was strictly dependent on position and nature

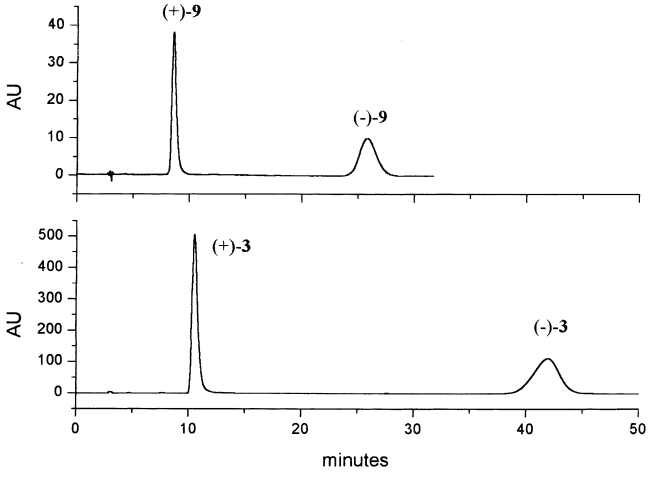

Fig. 2. HPLC resolution of compounds **3** (bottom) and **9** (top): column, Chiralcel OJ (250 × 4.6 mm I.D.); eluent, *n*-hexane–ethanol 70:30 (v/v); flow rate, 1 ml/min; detection wavelength, 254 nm; column temperature, 25°C.

of substituents on phenyl moiety. Thus, when the electron-donating 2-OCH_3 group (compound **1**) was replaced by the electron-withdrawing 2-Cl atom (compound **9**), the enantioselectivity factor increased from 1.89 to 4.14 using an *n*-hexane–ethanol mixture as a mobile phase. This behavior was much less manifest in the presence of pure 2-propanol (α = 1.32 vs. α = 1.51) and an *n*-hexane–2-propanol 60:40 mixture (v/v) (α = 1.38 vs. α = 1.73). Again, moving the OCH_3 group from the 2-position (compound **1**) to the 4-position (compound **4**) increased the enantioselectivity factor from 1.89 to 5.31 with *n*-hexane–ethanol (70:30, v/v).

The mechanism of chiral discrimination on polysaccharide-based CSPs has not yet been established at the molecular level, although some hypotheses based on chromatographic, computational, and spectroscopic approaches have been advanced.[18] It has been generally thought that the chiral selectivity is based on the combination of different factors that control the degree of fit of enantiomers into the chiral cavities of the CSP. This fit is affected by attractive or repulsive interactions of different magnitude and origin. The most important adsorbtion sites for the enantiorecognition process of phenylcarbamate derivatives of cellulose and amylose are probably the polar carbamate residues. These groups interacting with enantiomers through intermolecular dipole–dipole interactions and hydrogen bonds with donor hydrogen bonding NH groups or acceptor hydrogen bonding C=HO groups. Differently, the docking ester sites on benzoate derivative of cellulose provide only acceptor hydrogen bondings with chiral analyte.

Besides these interactions, the π–π interaction between complementary aromatic moieties of polysaccharide-type CSPs and solute may play an important role for chiral discrimination on these phases.

In our case, the solutes have two aromatic moieties and several H-bonding sites, such as the nitrogen atom on the pyrazoline ring, the oxygen atom of the carbonyl group and the hydroxyl on the phenyl ring. Thus, it was difficult to identify any clear structure–enantioseparation factor dependency for structurally related compounds **1–10** resolved on polysaccharide-type CSPs used. On the other hand, the chromatographic data indicate that the chiral selectivity depends not only on the structure of analytes or on the characteristics of polymeric selector (backbone conformation and type and position of substituents on phenyl moiety) but also on the composition of mobile

phase. The different sizes and bulkiness of ethanol and 2-propanol may generate different steric environments of the chiral cavities into the CSP and give rise to different solvation of solutes.[19] The consequent change of inclusion phenomenon of the solvates into the chiral cavity of the OD CSP may significantly affect the accessibility to the selective adsorption sites of the polymeric selector.

Enantiomer Elution Order

Enantiomer elution order is a very important topic in the determination of enantiomeric purity of chiral compounds as well as in the study of enantiorecognition mechanism.[20] The polysaccharide-type CSPs are based on natural materials and are available only in one configuration. Although it is difficult to control the enantiomer elution order with these CSPs, examples of inversion enantiomer retention order have been reported by alteration of the mobile phase composition or enantioseparation temperature.[20,21]

The elution order of enantiomers of the racemic compounds **1–10** on the OD, AD, and OJ CSPs were determined by means of a polarimeter equipped with a 40-μl flow cell. The measurements were performed at 365 nm. The polarimetric detection system was effective to determine enantiomer elution order even under conditions where the enantioresolution was low or apparently nonexistent (Fig. 3).

Analysis of results listed in Table 3 reveals that, under each eluting condition, the enantiomeric elution order on Chiralcel OJ remained constant, with the (+)-enantiomer eluting always first. On cellulose and amylose phenylcarbamates-based OD and AD CSPs a different enantiomer elution order for many chiral analytes was observed. For example, the (−)-form of compounds **1**, **2**, **6**, and **10** was the first eluted enantiomer on OD (Table 1, entries 21, 22, 26, and 30) and AD (Table 2, entries 21, 22, 26, and 30) using ethanol as the mobile phase. These results appear to be consistent with the assumption that the acceptor hydrogen bonding ester groups of OJ CSP play a dominant role in determining both enantiomer elution order and high enantioselectivity. On the other hand, structural differences between the analytes determined the reversal of the enantiomer elution order. Thus, from eight separated compounds on AD in *n*-hexane–ethanol 70/30 (v/v), five (compounds **1**, **2**, **6**, **9**, and **10**, Table 2, entries 1, 2, 6, 9, and 10) exhibited higher affinity of the (−)-form over the (+)-form. Again, the elution order for three of nine resolved chiral compounds on OD CSP with 2-propanol as eluent was (+)- before (−)-enantiomer, and for the remaining six it was the opposite. In Figure 4 is shown the reverse elution order of the enantiomers of **9** on OJ CSP using the same mobile-phase composition. In all cases, the enantiomer elution order of compounds of group 1 was the same of those of the corresponding compounds of group 2. It is interesting to note that enantiomer elution order was not influenced by changing the alcohol (ethanol or 2-propanol) used in the mobile phase in both normal-phase and polar organic modes.

Chromatographic Semipreparative Separation of Enantiomers of 1, 2, 9, and 10

The first step in any preparative separation is optimization, on an analytical scale, of the resolution factor by screening different experimental conditions, such as stationary phase, mobile phase, and temperature. However, in the scaling-up of an analytical enantioseparation to preparative loadings, an additional crucial step is the application of the sample onto the column.[22] To minimize the potential reduction in efficiency and resolution, the sample should be dissolved in a minimal volume of mobile phase. When dealing with compounds exhibiting low solubility (<1 mg/ml) in the mobile phase, this is not possible.

In the coated-type OD, AD, and OJ CSPs, cellulose and amylose derivatives are adsorbed on the silica surface and

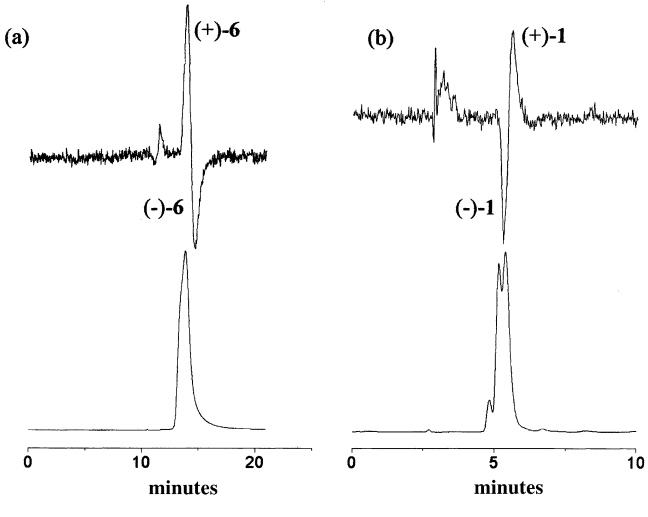

Fig. 3. Chromatograms of compounds **6** (a) and **1** (b) with UV (254 nm) (bottom) and polarimetric (365 nm) (top) detection. (a) Column, Chiralcel OD (250 × 4.6 mm i.d.); eluent, *n*-hexane–ethanol 70:30 (v/v); flow rate, 1 ml/min; column temperature, 25°C. (b) Column, Chiralpak AD (250 × 4.6 mm i.d.); eluent, 2-propanol; flow rate, 0.3 ml/min; column temperature, 25°C.

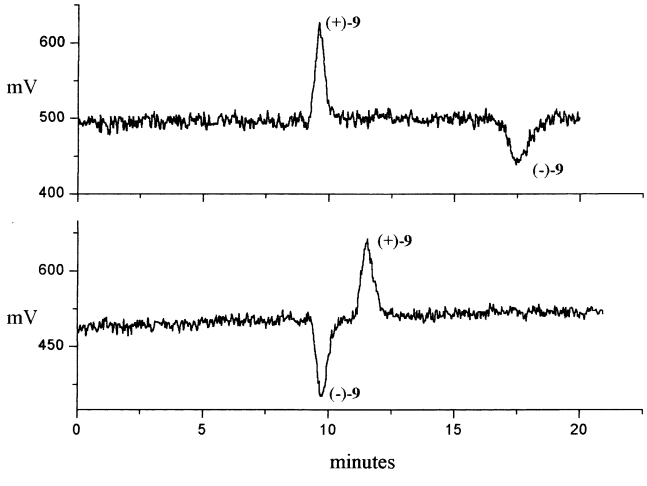

Fig. 4. Polarimetric chromatograms of the enantiomers of **9**. (Top) Column, Chiralcel OJ (250 × 4.6 mm i.d.); eluent, ethanol; flow rate, 0.5 ml/min; detection wavelength, 365 nm; temperature, 25°C. (Bottom) Column, Chiralcel OD (250 × 4.6 mm i.d.); eluent, ethanol; flow rate, 0.5 ml/min; detection wavelength, 365 nm; temperature, 25°C.

not chemically bonded to it. Consequently, several limitations are placed on the choice of mobile phase. The solvents that are recommended as eluents are limited to ethanol, 2-propanol, hexane, or mixtures of them, to prevent irreversible phase damage by dissolving these modified biopolymers. Although a number of publications deal with enantioseparation on polysaccharide-based CSPs using methanol and acetonitrile as mobile phase,[23,24] our choice was to adopt the general operative conditions suggested by the column vendor.

In order to evaluate the quantity of a particular racemate for application to semipreparative polysaccharide-based CSPs, its solubility in ethanol, 2-propanol, and *n*-hexane was examined.

The solubility of compounds of group 2, which contain two hydroxyl groups in the ortho and para positions of the C3 phenyl substituent of the pyrazoline nucleus, proved to be remarkably different from the group 1 analogues. While the 3-(4-hydroxyphenyl)pyrazole derivatives were reasonably soluble in ethanol and 2-propanol at ambient temperature, the corresponding 3-(2,4-dihydroxyphenyl)pyrazole derivatives were almost entirely insoluble. In *n*-hexane, all compounds **1–10** were insoluble. The solubility of **2**, which was selected as a model compound of group 2, was about 0.5 mg per 1 ml of ethanol, while that of the corresponding compound **1** of group 1 was about 5.0 mg per 1 ml; thus **1** is 10 times more soluble than **2**. The striking difference in the behavior was noteworthy in light of their presumed structural similarities. Intuitively, one would expect **2** to be more solvated than **1** as it has an additional phenolic hydrogen donor/acceptor site.

The lower solubility of **2** relative to **1** in ethanol could thus arise from either much less solvated molecules in solution, much stronger intermolecular forces in the solid state, or a combination of these two effects.

The conformational properties of **2** were analyzed by a molecular modeling approach based on DFT ab initio calculations. The results (Fig. 5) clearly point out that, in the more stable conformation of **2** (estimated Boltzmann population greater than 99.99%), the additional 2-OH phenolic group establishes a strong intramolecular hydrogen bond (about 8.5 kcal mol^{-1}) with the aza group of the heterocyclic portion. This conformation was calculated to be of considerably lower energy than to the conformation (b) with the hydrogen bond between the phenolic hydrogen and the methoxy oxygen (about 7.7 kcal/mol). Thus, both the 2-OH hydrogen-donor site and the aza hydrogen-acceptor site are not available to accomplish hydrogen-bond interactions with the alcoholic solvents. It seems reasonable to expect that the unavailability of the basic heterocyclic center in **2** results in a lower solvation capability compared to **1**. Supporting evidence for this conclusion is derived from the behavior of the phenol 2-hydroxybenzaldehyde, which exhibits a significantly lower solubilization rate in alkaline aqueous media with respect to the corresponding 4-hydroxybenzaldehyde, due to the presence of a stable intramolecular hydrogen bond.

To overcome the low solubility of **2** in ethanol, 2-propanol, *n*-hexane, or mixtures of these solvents, a solid injection technique[6] was used to apply the racemate onto the Chiralcel OD column on a semipreparative scale. The powdered sample was mixed with 40-micron silica, and the mixture was dry-packed into a pre-column (30 × 4.6 mm i.d.). Enantioseparation of **2** by the solid injection method is shown in Figure 6. Ethanol as eluent gave a higher resolution factor than *n*-hexane–ethanol eluent (*Rs* = 8.51 vs. *Rs* = 6.22) and had the advantage of increasing the solubility. At a load of 10 mg, no overlap of the peaks was observed. Enantiomeric excess (ee) values >99% and yields of about 50% were obtained for both enantiomers (Table 4).

Chromatographic semipreparative enantioseparation of **1**, **9**, and **10** was performed by applying the solution containing the dissolved racemate onto the chiral column

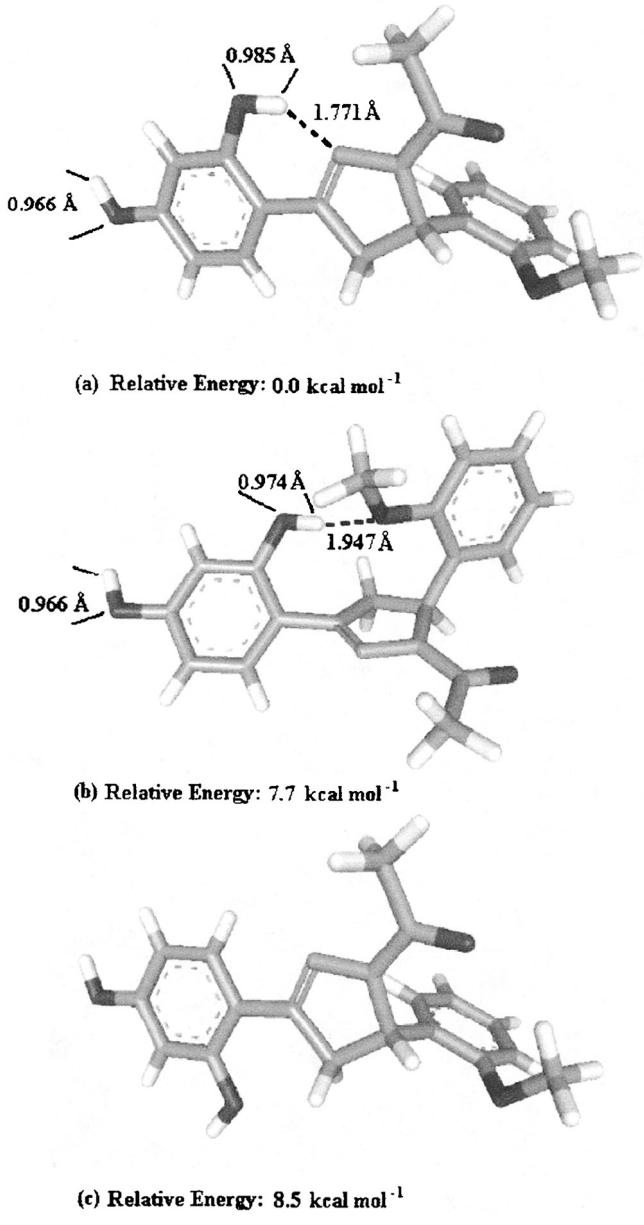

Fig. 5. Evidence of the strength of the intramolecular hydrogen bond in the calculated global minimum conformation of **2**: (a) global minimum; (b) second most stable conformation; (c) conformation of global minimum-like devoid of hydrogen bond.

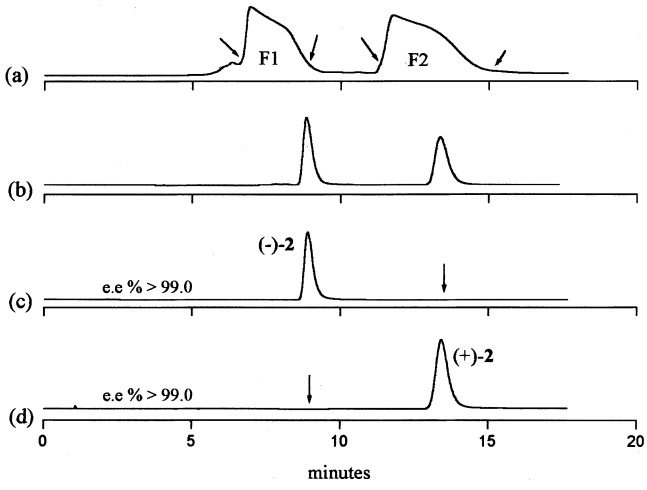

Fig. 6. (a) Semipreparative enantioseparation of 10 mg of **2**: column, Chiralcel OD (250 × 10 mm i.d.); eluent, ethanol; flow rate, 2.0 ml/min; detection wavelength, 340 nm; column temperature, 40°C. (b) Analytical resolution of **2**. (c, d) Purity control of collected fractions F1 and F2, respectively; column, Chiralcel OD (250 × 4.6 mm i.d.); eluent, ethanol; flow rate, 0.5 ml/min; detection wavelength, 254 nm; column temperature, 25°C.

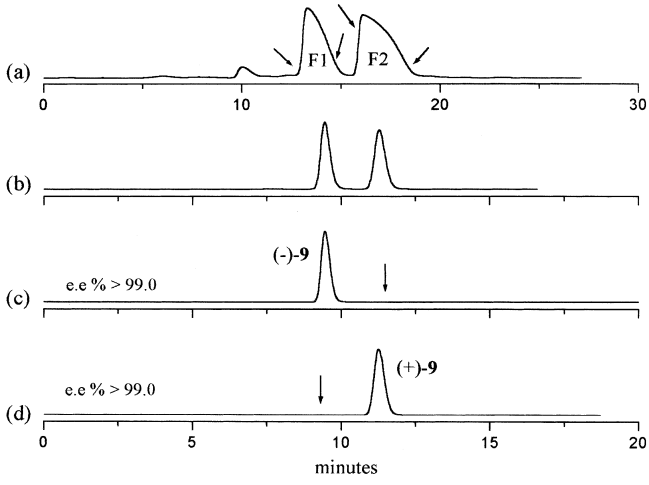

Fig. 7. (a) Semipreparative enantioseparation of 10 mg of **9**: column, Chiralcel OD (250 × 10 mm i.d.); eluent, ethanol; flow rate, 3.0 ml/min; detection wavelength, 340 nm; column temperature, 25°C. (b) Analytical resolution of **9**. (c, d) Purity control of collected fractions F1 and F2, respectively; column, Chiralcel OD (250 × 4.6 mm i.d.); eluent, ethanol; flow rate, 0.5 ml/min; detection wavelength, 254 nm; column temperature, 25°C.

via an injector. Unfortunately, the most effective CSP, Chiralcel OJ, was unavailable for the racemic compounds of interest. Thus, to achieve direct separation of enantiomers of **1**, **9**, and **10** on a semipreparative scale, the available OD CSP was selected. Table 4 shows chromatographic and polarimetric data pertinent to each enantiomer that was collected in a single fraction.

Enantiomeric excess values >98% and yields ranging from 70% to 80% were obtained. Figure 7 shows a typical chromatogram illustrating the semipreparative enantioseparation of **9** on OD CSP and the analytical investigations of the two collected fractions.

Polarimetric analysis indicated that, in all cases tested, the first eluted enantiomer on OD CSP rotated polarized light in the negative direction in chloroform solution. The specific rotations reported in Table 4 were measured at 589 nm. The corresponding optical rotations determined at 365 nm was monitored online during HPLC on the OD CSP using ethanol and 2-propanol as eluents.

Determination of Absolute Configuration

Single-crystal X-ray diffraction analysis showed the (+)-enantiomer of compound **9** to have (*R*) absolute configuration at C_5 (labeled C(1) in Fig. 8) of the pyrazoline moiety.

The enantiomers of chiral analytes **1**, **2**, **9**, and **10**, collected from semipreparative HPLC separation, were dissolved in ethanol, and their CD spectra were measured. The CD spectrum of the (+)-(*R*)-**9** enantiomer (Fig. 9) displays a broad positive Cotton effect around 300 nm, a sharper band of opposite sign at 237.2 nm, and another positive Cotton effect at 219.4 nm. The (−)-(*S*)-**9** enantiomer exhibited the corresponding mirror-image CD.

The assignment of the absolute configuration of enantiomers of compounds **1**, **2**, and **10** could be empirically established according to their CD properties. The shape and the absorption maxima of the CD curves of these chiral compounds (Fig. 9) are strictly correlated with those of compound **9**, indicating that the chromophores contributing to the CD spectra are similar.

Therefore, based on a comparison with reference CD spectra of enantiomers of **9** of known absolute configuration, the (+)- and (−)-enantiomers of **1**, **2**, and **10** were assigned the *R*- and *S*-configurations, respectively. Again, all analogues reported (**1**–**10**) exhibited same-sense

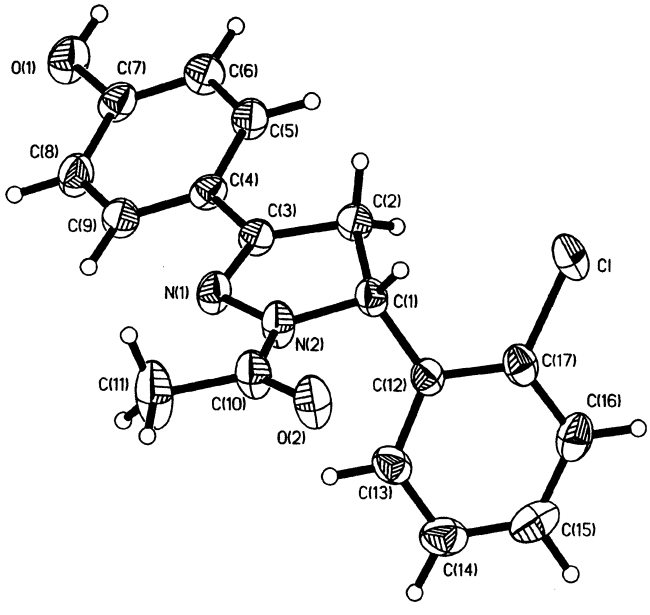

Fig. 8. Perspective view of the molecular structure of the (+)-(*R*)-enantiomer of **9**, showing the labeling of the atoms. Displacement ellipsoids are at 30% probability level. Crystallographic data have been deposited with the Cambridge Crystallographic Data Center (CCDC# 234059).

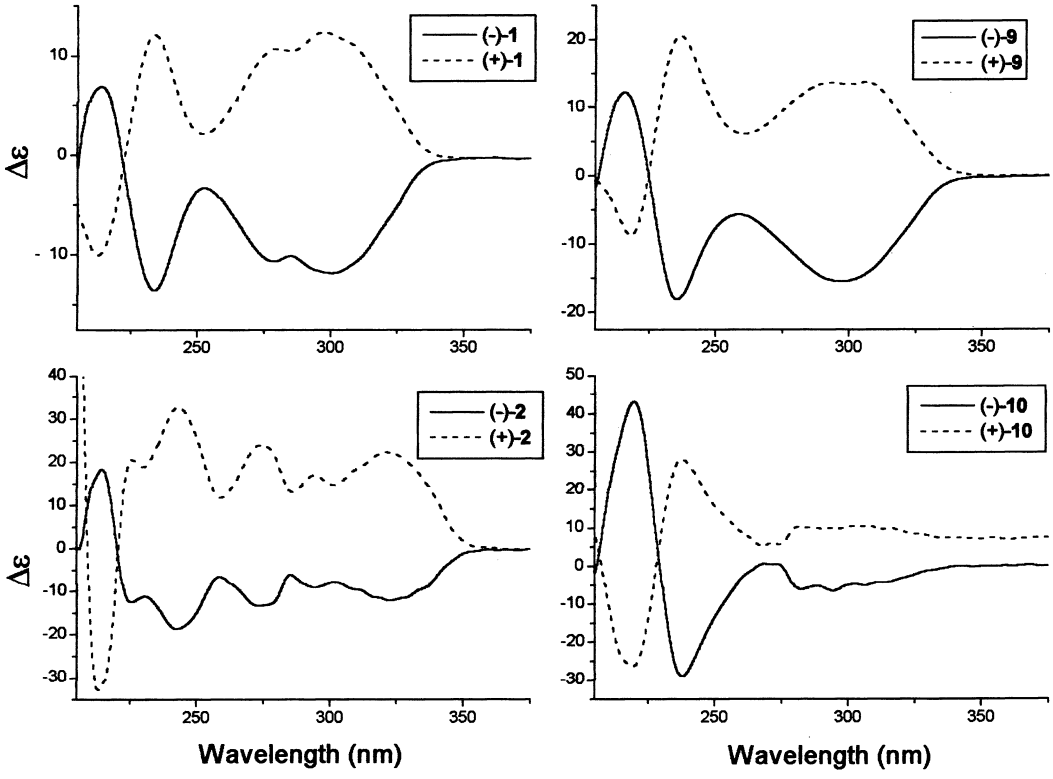

Fig. 9. Circular dichroism (CD) spectra of the enantiomers of the pyrazole derivatives **1**, **2**, **9**, and **10** in ethanol at 25°C. Solid and dashed traces correspond respectively to the first and second eluted enantiomers on OD CSP.

chiral recognition mechanism and, consequently, the same enantiomer elution order on OJ CSP with preferential retention of the (−)-enantiomer. These data support the presumption that all the (+)-enantiomers of chiral analytes **1–10** possess the same absolute *R*-configuration.

In Vitro Anti-MAO Activity of Enantiomers and Racemates of 9 and 10

The results of the in vitro anti-MAO activities of racemates **9** and **10** and their individual enantiomers are reported in Table 5. Data refer to IC_{50} values, and selectivity (SI = selectivity index) was estimated by the ratio of IC_{50} B/A values.

TABLE 5. Monoamine oxidase inhibitory activity of pyrazole derivatives 9 and 10*

Compound	IC_{50} MAO-A (mean ± SD)	IC_{50} MAO-B (mean ± SD)	SI[a]
rac-**9**	8.6×10^{-9} (±0.4)	8.7×10^{-5} (±0.4)	10,116
(−)-(*S*)-**9**	4.8×10^{-9} (±0.4)	6.5×10^{-5} (±0.3)	13,542
(+)-(*R*)-**9**	3.0×10^{-9} (±0.5)	2.0×10^{-5} (±0.6)	6,666
rac-**10**	1.0×10^{-8} (±0.3)	2.3×10^{-5} (±0.2)	2,300
(−)-(*S*)-**10**	6.0×10^{-9} (±0.8)	2.2×10^{-5} (±0.8)	3,666
(+)-(*R*)-**10**	3.0×10^{-9} (±0.2)	1.0×10^{-5} (±0.4)	3,333

*Data represent mean values of at least three separate experiments.
[a]SI: selectivity index = IC_{50} (MAO-B)/IC_{50} (MAO-A).

Enzymatic assays revealed that both racemic mixtures of **9** and **10** were weak MAO-B inhibitors, while, as we expected from data obtained previously, potent MAO-A inhibitory activity at nanomolar concentrations was found. The IC_{50} values of racemic samples were significantly lower than the corresponding individual enantiomers in inhibitory activity (for example, *rac*-**9** $IC_{50} = 8.6 \times 10^{-9}$ M, (−)-(*S*)-**9** $IC_{50} = 4.8 \times 10^{-9}$ M, and (+)-(*R*)-**9** $IC_{50} = 3.0 \times 10^{-9}$ M). This behavior could be due to the presence of some impurities in racemic samples observed during HPLC semipreparative enantioseparation. The inhibitory activity of the (+)-(*R*)-enantiomer of pyrazole derivatives tested proved to be 1.5–3.0 times more potent than parent (+)-(*S*)-form toward both A and B isoforms of MAO. The biological results obtained for enantiomers of compounds **9** and **10** are in agreement with previously reported anti-MAO activity of single stereoisomers of compounds **1** and **2**.[4]

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