ELSEVIER

Journal of Chromatography A, 942 (2002) 107–114

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Analytical and semipreparative enantiomeric separation of azole antifungal agents by high-performance liquid chromatography on polysaccharide-based chiral stationary phases Application to in vitro biological studies

R. Cirilli[a], R. Costi[b], R. Di Santo[b], R. Ferretti[a], F. La Torre[a,*], L. Angiolella[c], M. Micocci[c]

[a]*Istituto Superiore di Sanità, Laboratorio di Chimica del Farmaco, Viale Regina Elena 299, 00161 Rome, Italy*
[b]*Istituto Pasteur–Fondazione Cenci-Bolognetti, Dipartimento di Studi Farmaceutici, Università di Roma "La Sapienza", Piazzale Aldo Moro 5, I-00185 Rome, Italy*
[c]*Istituto di Microbiologia, Università di Roma "La Sapienza", Piazzale Aldo Moro 5, I-00185 Rome, Italy*

Received 25 June 2001; received in revised form 1 October 2001; accepted 12 October 2001

Abstract

High-performance liquid chromatography (HPLC) was used for the enantiomeric separation of chiral imidazole derivatives endowed with antimycotic activity. Enantioselective columns, containing carbamates of cellulose and amylose, were used. The influence of the nature and content of an alcoholic modifier in the mobile phase was studied. The isolated enantiomers, separated on semipreparative columns, were submitted to in vitro biological investigations. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Enantiomeric separation; Chiral stationary phases, LC; Azoles

1. Introduction

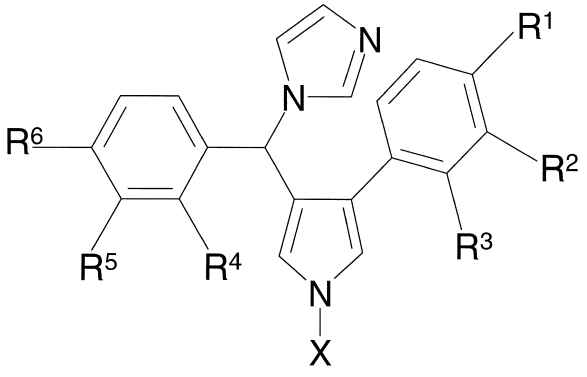
In the last few years there has been an increasing interest in the development of new antimycotic compounds belonging to the azole class. In particular, imidazole derivatives have become an attractive target of synthetic and pharmacological studies because of their effectiveness in the treatment of fungal diseases in immunocompromised patients observed with AIDS or after transplantation.

A variety of new imidazole derivatives, structurally related to bifonazole, were previously synthesised by our group as candidate antifungal agents [1,2] (Fig. 1). In such azole compounds, 1-arylmethylimidazole and 3-arylpyrrole moieties are linked through a stereogenic centre.

In initial in vitro screening, azole racemates exhibited high activity against *Candida albicans* and *Candida* spp., showing potency comparable to or somewhat higher than that of miconazole, ketoconazole or bifonazole used as reference drugs. The aim of this paper is to continue previous studies [1,2] dealing with the stereochemical influence at the biological level. It has been extensively established

*Corresponding author. Fax: +39-06-4938-7100.
E-mail addresses: roberto.disanto@uniroma1.it (R. Di Santo), latorre@iss.it (F. La Torre).

0021-9673/02/$ – see front matter
PII: S0021-9673(01)01401-7



Compound	X	R^1	R^2	R^3	R^4	R^5	R^6
1	CH_3	Cl	H	H	H	H	H
2	H	Cl	H	H	H	H	H
3	H	Cl	H	H	Cl	H	Cl
4	H	Cl	Cl	H	H	H	H
5	H	Cl	H	Cl	H	H	H
6	H	H	Cl	H	H	H	H
7	H	H	H	Cl	Cl	H	Cl
8	H	Cl	H	Cl	Cl	H	Cl
9	H	Cl	Cl	H	H	Cl	Cl
10	H	H	H	Cl	H	H	CH_3
11	H	Cl	H	Cl	H	H	CH_3
12	H	Cl	Cl	H	H	H	CH_3
13	H	Cl	Cl	H	H	H	F
14	H	Cl	H	H	H	H	NO_2
15	H	Cl	H	Cl	H	H	NO_2

Fig. 1. Structures of the investigated azoles.

that a racemic compound can be regarded as a 50:50 mixture of two different molecules which can potentially interact in a different way with a biological system [3]. Notwithstanding growing knowledge in this field, there are only a few reports describing pharmacological profiles of both enantiomers of chiral antifungal drugs [4–6]. Such a lack is probably due to the limited availability of large amounts of enantiomerically pure compounds for use in stereoselective biological and toxicological trials. In this context, HPLC on chiral stationary phases (CSPs) emerges as a competitive method in the separation and analysis of components of a racemic mixture and may constitute a valid alternative to asymmetric synthesis in the production of optically pure compounds [7].

Among the many types of CSPs for HPLC developed over the last two decades, polysaccharide-based CSPs showed good chiral recognition ability towards a wide number of different racemic compounds [8].

We selected two polysaccharide-based CSPs, amylose 3,5-dimethylphenyl-carbamate (Chiralpak AD) and cellulose 3,5-dimethylphenyl-carbamate (Chiralcel OD), commercially available in analytical and semipreparative versions, to investigate the enantiomeric separation of the aforementioned antifungal azoles. In the first step of our work, we focused on the optimisation of analytical conditions (concentration, nature of alcoholic modifier in the mobile phase and temperature), for both enantioselective columns, which made it possible to achieve a higher resolution in a shorter analysis time. The best CSP–mobile phase system obtained on an analytical scale was transferred to a larger column (10 mm I.D.) in order to collect single enantiomers in high enantiomeric purity and in semipreparative amounts. The isolated enantiomers were submitted to in vitro biological investigations to delineate the possible difference in antifungal activity between the individual enantiomer and the racemic mixture.

2. Experimental

2.1. Materials

Stainless-steel Chiralcel OD and Chiralpak AD (250×4.6 mm I.D. and 250×10 mm I.D.) (Daicel Chemical Industries, Tokyo, Japan) columns were used. HPLC-grade solvents were supplied by Carlo Erba (Milan, Italy). Diethylamine (DEA) was obtained from Fluka Chemie (Buchs, Switzerland). Analytes **1**–**15** (Fig. 1) were synthesised by chemical pathway as previously reported [2].

2.2. Apparatus

Chromatography was performed using a Waters

(Milford, MA, USA) 510 pump equipped with a Rheodyne (Cotati, CA, USA) injector, a 1-ml sample loop, a Perkin-Elmer (Norwalk, CT, USA) HPLC oven and a Waters model 414 detector. The signal was acquired and processed by Millennium 2010 software.

Optical rotations of enantiomers **1–3**, dissolved in ethanol, were measured on a polarimeter Perkin-Elmer model 241 equipped with a Na lamp, at a wavelength of 589 nm. The volume of the measuring cell was 1 ml and the optical path was 10 cm. The system was thermostated at 23°C.

2.3. Operating conditions

The eluents for the chromatographic separations were mixtures of appropriate percentages of two different alcoholic modifiers (ethanol or 2-propanol) in *n*-hexane, with the addition of 0.1 vol of DEA. The mobile phases were filtered and degassed by sonication immediately before use. The flow-rates were 0.5 and 2.5 ml min^{-1} for analytical and semipreparative separations, respectively. The wavelength of detection was 260 and 310 nm for analytical and semipreparative separations, respectively. All analytical separations were performed at 25°C, except those used for the studies at variable temperature. Semipreparative separations were performed at 40°C.

For the study of the influence of alcoholic modifier type and concentration on enantioselectivity and resolution factors, standard solutions were prepared by dissolving 2–5 mg of each analyte with 25 ml of HPLC-grade ethanol. The injection volume was 20 μl. A 500-μl volume of racemate (10 mg) was applied to the chiral semipreparative column. After semipreparative separation, the collected fractions were analysed by chiral analytical columns to determine their enantiomeric excess (e.e.).

2.4. Microbiological test: anti-Candida in vitro assays

Substances **1**, **2**, **3** and related separated (+) and (−)-enantiomers were tested for antimycotic activities against *C. albicans*. The antifungal potency was evaluated by means of the minimal inhibitory concentration (MIC), using the serial test in the broth microdilution modification method published by the National Committee for Clinical Laboratory Standards (NCCLS) method M27-A [9]. MIC was defined as the lowest concentration of test substances at which there was no visible growth, as compared with a blank experiment, after the present incubation time. For the preparation of the dilution series, the racemic mixture and enantiomers were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mg ml^{-1}, and were stored frozen at −20°C. The solution was added to the medium at the right concentration. Further progressive double dilutions with a test medium furnished the required concentrations in the range from 0.047 to 50 μg ml^{-1}. Blanks were prepared in the test medium, without adding test substance.

Bifonazole, miconazole and fluconazole were used as standard controls. Bifonazole and miconazole were dissolved in DMSO, while fluconazole was dissolved in distilled water at a concentration of 10 mg ml^{-1}. All the tested microorganisms were preliminarily incubated at 28°C for 24 h on YNB (Difco) or RPMI 1640 (Sigma) medium. Antimicrobial tests were performed on YNB or RPMI 1640 medium using inocula of 10^3 or 10^4 ml^{-1} of cells. Two different concentrations of cells per ml were used, in order to better investigate the antifungal activities in tests with different sensibilities. Readings of MICs were taken at 24 h of growth at 28°C.

MIC values were calculated by the expression $MIC = MIC_i s_t^{-1}$, where MIC_i is the minimal inhibitory concentration values of all strains at the used concentration C_i and s_t is the total number of strains. MIC_{50} and MIC_{90} refer to MIC for 50 and 90% of strains, respectively.

Experiments were carried out in both YNB and RPMI 1640 medium. The first one (YNB) is generally used in experiments to test the activity of antimycotics, while RPMI was a nutrient medium used for tissue culture, to better simulate ''in vivo'' conditions. Eight strains of *C. albicans* (two laboratory strains, four strains isolated from AIDS patients with oropharyngeal candidiasis and two isolated from patients with recurrent vulvovaginitis) were employed in these studies. All strains were identified by conventional diagnostic procedures.

3. Results and discussion

3.1. Optimisation of analytical enantioseparations of compounds *1–3*

The preliminary step of our study was to evaluate the applicability of two CSPs based upon 3,5-dimethylphenylcarbamate of cellulose (Chiralcel OD) and amylose (Chiralpak AD) for the enantiomeric separation of the first three compounds of the series of structurally related azoles reported in Fig. 1. Compounds **1**–**3** exhibited higher antifungal activity compared to that of the other azoles [10] and were therefore chosen as target compounds for analytical and semipreparative separations. The successive objective was to optimise the resolution of selected analytes **1**–**3**, by analytical combinations of two CSPs and different mobile phases, in which the nature and content of the alcohol modifier were changed.

The influence of the nature and content of the alcoholic modifier on the enantioselectivity factor, α, and resolution factor, *Rs*, was studied, using different eluents consisting of mixtures of *n*-hexane–ethanol and *n*-hexane–2-propanol, with the addition of 0.1 vol of DEA. As illustrated in Table 1, the use of ethanol as an organic modifier gave better performances on both enantioselective columns. When *n*-hexane–2-propanol mixtures were employed as mobile phase, compounds **1**–**3** were not resolved on AD CSP and lower enantioselectivity on OD CSP was observed.

The decrease in ethanol content from 25 to 10% led to roughly constant α values, while the resolution increased for both CSPs. Therefore, better resolution of **1**–**3** was achieved using a mobile phase containing 10% of ethanol and 0.1 vol of DEA in *n*-hexane. Of two polysaccharide-based CSPs, Chiralpak AD was more appropriate than Chiralcel OD for the resolution of compounds **1** and **2**, while compound **3** showed greater enantioselectivity on the OD column.

The chromatographic conditions, obtained for compounds **1**–**3**, were applied in the resolution of all other compounds of the azoles series. A review of the experimental data listed in Table 2 reveals the good enantioselectivity power exhibited by both OD and AD towards compounds examined in this study.

Table 1
Effect of nature of the alcohol modifier (ethanol or 2-propanol) on the enantioselectivity factor (α) compounds **1–3**

Compound	Eluent	α	
		OD	AD
1	*n*-Hexane–ethanol (90:10, v/v)	1.29	1.85
1	*n*-Hexane–2-propanol (90:10, v/v)	1.18	1.00
2	*n*-Hexane–ethanol (90:10, v/v)	1.32	2.05
2	*n*-Hexane–2-propanol (90:10, v/v)	1.25	1.00
3	*n*-Hexane–ethanol (90:10, v/v)	2.50	1.20
3	*n*-Hexane–2-propanol (90:10, v/v)	2.43	1.00

Columns: Chiralcel OD and Chiralpak AD (250×4.0 mm I.D.); eluent: *n*-hexane–alcohol modifier (90:10, v/v)+0.1 vol DEA; flow-rate: 0.5 ml min^{-1}; temperature: 25°C; UV detector: 260 nm.

All the racemates were resolved with an appreciable enantioselectivity factor on at least one of the two CSPs used. The fact that OD and AD exhibit rather complementary chiral recognition has been reported in several previous papers [11,12]. Initial separations were performed at 25°C and at a flow-rate of 0.5 ml min^{-1}. In order to elucidate the temperature effects on resolutions, compounds **1**–**3** were chromato-

Table 2
HPLC of compounds **1–15**

Compound	OD			AD		
	k_1 [a]	α [b]	*Rs* [c]	k_1	α	*Rs*
1	2.43	1.29	3.09	1.19	1.85	7.48
2	2.41	1.32	3.21	0.69	2.05	7.06
3	0.87	2.50	9.41	1.34	1.20	2.14
4	2.28	1.41	4.83	1.93	1.57	7.00
5	2.18	1.00	–	1.53	1.52	6.36
6	2.00	1.21	2.59	1.90	1.44	6.03
7	1.67	1.80	8.22	1.62	1.45	6.00
8	1.71	1.48	4.97	1.43	1.46	5.42
9	2.11	2.57	12.85	1.72	1.10	1.42
10	3.04	1.63	7.08	2.25	1.22	3.09
11	1.66	1.08	0.86	1.39	1.35	4.01
12	1.99	1.37	4.18	1.84	1.26	3.53
13	2.33	1.49	5.62	1.53	1.52	6.36
14	6.29	1.57	6.05	5.72	1.68	8.27
15	10.13	1.15	2.03	5.35	1.71	8.18

Columns: Chiralcel OD and Chiralpak AD (250×4.6 mm I.D.); eluent: *n*-hexane–ethanol (90:10, v/v)+0.1 vol DEA; flow-rate: 0.5 ml min^{-1}; temperature: 25°C; UV detector: 260 nm.

[a] The retention factor.

[b] The enantioselectivity factor.

[c] The resolution factor.

graphed at different column temperatures, from 25 to 40°C, using a mixture of *n*-hexane–ethanol (90:10, v/v), with the addition of 0.1 vol DEA, as mobile phase. Representative variable-temperature chromatograms for the direct resolution of compound **1** on the AD column are shown in Fig. 2. As expected, the retention factors of both enantiomers decreased as column temperature increased. Furthermore, at 40°C a slight lowering in resolution factor with respect to ambient temperature was noticed. Similar temperature dependence of racemates **2** and **3** on AD and OD CSPs, respectively, were observed. Finally, a higher investigated temperature (40°C) was selected to perform successive semipreparative separations. This choice made it possible to prevent broadening the band, improving sample solubility in the mobile phase and shortening run time, without any notable drop in resolution.

*3.2. Semipreparative enantioseparations of compounds **1–3***

The best CSP–mobile phase system, obtained on an analytical scale for the resolution of compounds **1–3**, was transferred on a semipreparative scale in order to isolate 50–100 mg of individual enantiomer for use in biological investigations. Enantiomeric fractionation was performed by repetitive injections of 10 mg of sample on a 10-mm I.D. AD or OD column. Mobile phases consisting of a mixture of *n*-hexane–ethanol (90:10, v/v) with the addition of 0.1 vol content of DEA were used. The temperature was 40°C and the flow-rate 2.5 ml min^{-1}. Each semipreparative run was cut in three separated fractions. Fig. 3 shows the representative preparative chromatogram obtained from injecting an amount of 10 mg of racemate **1** on AD CSP.

Although the enantioseparations were achieved under overlapping band conditions, the analytical control of the collected fractions (Table 3 and Fig. 4) showed that the more retained enantiomer of **1–3** was isolated with good yield (>65%) and enantiomeric excess (e.e.>99%). The first fraction was practically enantiomerically pure in the case of analyte **3** and showed an e.e. under 90% in the cases of compounds **1** and **2**. These last two fractions were concentrated and rechromatographed to obtain an e.e. greater than 98%.

The optical rotations of two enantiomers for compound **1–3** were measured. The isolated enantiomers were characterised as follows: (+)-**1**: e.e.= 99.3%, $[\alpha]_D^{23}$ +72.9 (c=0.46, ethanol); (−)-**1**:

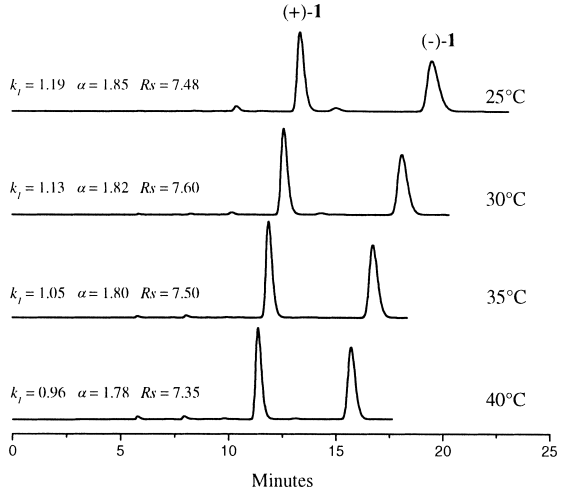


Fig. 2. Variable-temperature HPLC of compound **1**. Column: Chiralpak AD (250×4.6 mm I. D.); eluent: *n*-hexane–ethanol (90:10, v/v)+0.1 vol DEA; flow-rate: 0.5 ml min^{-1}; detection wavelength: 260 nm; column temperature: from 25°C (top) to 40°C (bottom) in 5°C increments.

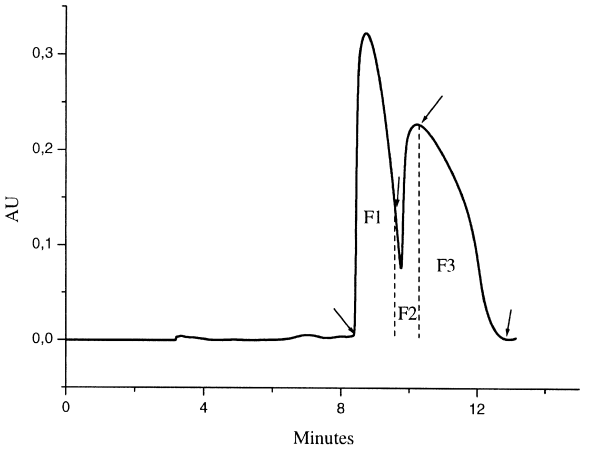


Fig. 3. Fraction collection during the chromatographic semipreparative enantioseparation of 10 mg of racemate **1**. Column: Chiralpak AD (250×10 mm I. D.); eluent: *n*-hexane–alcohol modifier (90:10, v/v)+0.1 vol DEA; flow-rate: 2.5 ml min^{-1}; column temperature: 40°C; detection wavelength: 310 nm.

Table 3
Quantitative analysis of fractions F1 and F3 recovered during the enantioseparation of 10-mg amounts of **1**–**3**

Compound	Column	Yield (%)		e.e. (%)	
		Fraction F1	Fraction F3	Fraction F1	Fraction F3
1	AD	98.0	68.4	86.8[a]	99.7
2	AD	67.0	65.1	82.1[a]	99.4
3	OD	70.4	70.2	99.4	99.5

Columns: Chiralcel OD and Chiralpak AD (250×10 mm I.D); eluent: *n*-hexane–ethanol (90:10, v/v)+0.1 vol DEA; flow-rate: 0.5 ml min^{-1}; temperature: 40°C; UV detector: 260 nm.

[a] Fractions F1 of **1** and **2** were rechromatographed to obtain e. e.=99.3% and e.e.=98.0%, respectively.

e.e.=99.7%, $[\alpha]_D^{23}$ −71.2 (c=0.49, ethanol); (+)-**2**: e.e.=98.0%, $[\alpha]_D^{23}$ +69.7 (c=0.34, ethanol); (−)-**2**: e.e.=99.4%, $[\alpha]_D^{23}$ −68.9 (c=0.30, ethanol); (+)-**3**: e.e.=99.4%, $[\alpha]_D^{23}$ +87.3 (c=0.44, ethanol); (−)-**3**: e.e.=99.5%, $[\alpha]_D^{23}$ −85.6 (c=0.44, ethanol). The (−)-enantiomer of the compounds examined was the more retained isomer on both polysaccharide-based columns and no inversion of elution order resulted in the scale-up procedure.

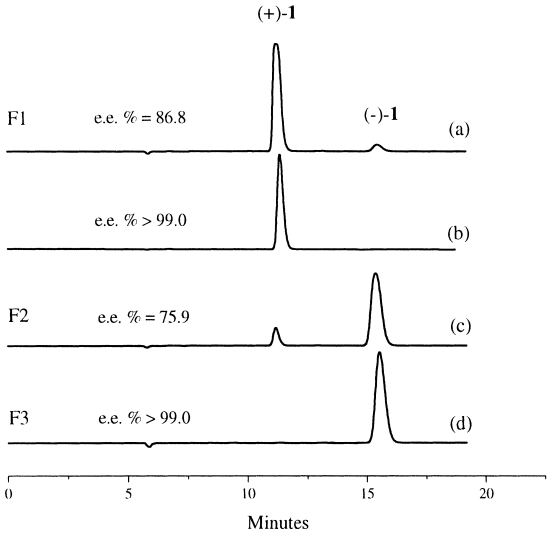


Fig. 4. Analytical investigation of collected fractions F1, F2 and F3 recovered during semipreparative resolution of racemate **1**. Column: Chiralpak AD (250×4.6 mm I. D.); eluent: *n*-hexane–alcohol modifier (80:20, v/v)+0.1 vol DEA; flow-rate: 0.5 ml min^{-1}; column temperature: 40°C; detection wavelength: 260 nm. Trace a, c, d: fractions F1, F2 and F3, respectively. Trace b: rechromatographed F1.

3.3. In vitro anti-Candida albicans activities

The results of the in vitro antifungal activities of racemates **1–3** and separated enantiomers against eight strains of *Candida albicans* in YNB and RPMI 1640 medium are reported in Tables 4 and 5, respectively. Data refer to MIC mean values (MIC), MIC_{50} and MIC_{90}.

Racemic mixtures of compounds **1**, **2** and **3** showed a very good MIC, as we expected from data obtained previously [2]. Unfortunately, the values of MIC, MIC_{50} and MIC_{90} of separated enantiomers did not show a significant increase in antifungal activity compared to racemic mixtures.

In order to study the configurational stability of samples in the mediums used, the enantiomers were dissolved in a mixture of DMSO–water (10:90, v/v) at pH 7 (phosphate buffer). The resulting solutions were maintained at constant temperature (28°C) for 3 h and analysed by enantioselective HPLC. In each case, no decrease in e.e. was observed. This result suggests that no racemization occurred during in vitro tests.

4. Conclusions

The HPLC method described is designed for the enantiomeric separation, at the analytical and semi-preparative level, of chiral azole antifungal agents.

The in vitro antifungal activities of enantiomers of analytes **1–3** were investigated against *Candida albicans* strains, in comparison with the corresponding racemates as well as with reference standards (miconazole, fluconazole, bifonazole). The

Table 4
In vitro antifungal activities of racemic mixtures and enantiomers of analytes **1–3** against *C. albicans* in YNB

Compound	Inoculum 10^3cells ml^{-1}			Inoculum 10^4cells ml^{-1}		
	MIC ($\mu g\ ml^{-1}$)	MIC_{50} ($\mu g\ ml^{-1}$)	MIC_{90} ($\mu g\ ml^{-1}$)	MIC ($\mu g\ ml^{-1}$)	MIC_{50} ($\mu g\ ml^{-1}$)	MIC_{90} ($\mu g\ ml^{-1}$)
1	0.39	0.39	0.39	0.78	0.78	0.78
(+)-**1**	0.41	0.39	0.39	0.58	0.39	0.78
(−)-**1**	0.68	0.78	0.78	1.36	0.78	0.78
2	0.87	0.78	1.56	0.52	0.39	0.78
(+)-**2**	2.18	1.56	3.12	2.34	1.56	3.12
(−)-**2**	0.93	0.78	1.56	1.30	0.78	1.56
3	1.17	0.78	1.56	1.95	1.56	3.12
(+)-**3**	3.31	3.12	3.12	5.46	6.25	6.25
(−)-**3**	1.95	1.56	1.56	3.51	3.12	3.12
Miconazole	0.39	0.39	0.39	1.41	0.78	1.56
Fluconazole	2.08	1.56	3.12	1.49	1.56	3.12
Bifonazole	2.73	3.12	3.12	6.24	6.25	6.25

Table 5
In vitro antifungal activities of racemic mixtures and enantiomers of analytes **1–3** against *C. albicans* in RPMI 1640 medium

Compound	Inoculum 10^3 cells ml^{-1}			Inoculum 10^4cells ml^{-1}		
	MIC ($\mu g\ ml^{-1}$)	MIC_{50} ($\mu g\ ml^{-1}$)	MIC_{90} ($\mu g\ ml^{-1}$)	MIC ($\mu g\ ml^{-1}$)	MIC_{50} ($\mu g\ ml^{-1}$)	MIC_{90} ($\mu g\ ml^{-1}$)
1	0.26	0.195	0.195	0.58	0.195	0.39
(+)-**1**	0.65	0.39	0.39	1.58	0.78	1.56
(−)-**1**	0.36	0.195	0.195	1.41	0.78	1.56
2	0.36	0.39	0.39	1.31	0.78	1.56
(+)-**2**	0.68	0.78	0.78	1.95	1.56	1.56
(−)-**2**	0.58	0.39	0.78	1.48	0.78	1.56
3	0.53	0.39	0.78	1.02	0.78	1.56
(+)-**3**	2.04	1.56	3.12	2.24	1.56	3.12
(−)-**3**	0.70	0.78	0.78	2.73	1.56	3.12
Miconazole	0.67	0.39	0.39	2.73	0.78	3.12
Fluconazole	1.07	0.39	0.39	2.92	0.78	0.50
Bifonazole	3.91	3.12	6.25	4.68	6.25	6.25

antifungal profile of the tested compounds was comparable to that of the reference drugs and in no case did the single enantiomer show a significant increase in antifungal activity compared to racemic mixtures.

Acknowledgements

We are grateful to A. Mosca, L. Zanitti and Professor M. Artico for their helpful collaboration. Thanks are due to the Italian MURST for partial support.

References

[1] S. Massa, R. Di Santo, M. Artico, R. Costi, C. Di Filippo, G. Simonetti, A. Retico, M. Artico, Eur. Bull. Drug Res. 1 (1992) 12.
[2] M. Artico, R. Di Santo, R. Costi, S. Massa, A. Retico, M. Artico, G. Apuzzo, G. Simonetti, V. Strippoli, J. Med. Chem. 38 (1995) 4223.

[3] E.J.D. Lee, K.M. Williams, Clin. Pharmacokinet. 18 (1990) 339.
[4] D. Loebenberg, A. Cacciapuoti, R. Parmegiani, E.L. Moss, F. Menzel, B. Antonacci, C. Norris, T. Yarosh-Tomaine, R.S. Hare, G.H. Miller, Antimicrob. Agents Chemother. 36 (1992) 498.
[5] M. Botta, F. Corelli, F. Gasparrini, F. Messina, C. Mugnaini, J. Org. Chem. 65 (2000) 4736.
[6] D. Bianchi, P. Cesti, S. Spezia, C. Garavaglia, L. Mirenna, J. Agric. Food Chem. 39 (1991) 197.
[7] G. Gargaro, F. Gasparrini, D. Misiti, G. Palmieri, M. Pierini, C. Villani, Chromatographia 24 (1987) 505.
[8] Y. Okamoto, Y. Kaida, J. Chromatogr. A 666 (1994) 403.
[9] J. Galgiani, M. Bartlett, M. Espinel-Ingroff, R. Fromtling, M. Pfaller, M. Rinaldi, Reference Method for Both Dilution Antifungal Susceptibility Testing of Yeast. Proposed Standard, Vol. 12, National Committee for Clinical Laboratory Standards, Villanova, PA, 1992.
[10] A. Tafi, J. Anastassopoulou, T. Theophanides, M. Botta, F. Corelli, S. Massa, M. Artico, R. Costi, R. Di Santo, R. Ragno, J. Med. Chem. 39 (1996) 1127.
[11] Y. Okamoto, E. Yashima, Angew. Chem. Int. Ed. 37 (1998) 1020.
[12] E. Yashima, C. Yamamoto, Y. Okamoto, Synlett 1 (1998) 344.