A New Class of Antifungal Agents. Synthesis and Antimycotic Activity of Disubstituted *N*-Azolylamines

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Summary

In this study we extended our exploration of the *N*-azolylamine moiety for its antifungal activity. We prepared a number of *N*-azolylamino derivatives. The synthetic sequence includes the preparation of aminoazole Schiff bases, and the reduction and the alkylation of the corresponding secondary amines. The title compounds were evaluated *in vitro* against several pathogenic fungi responsible for human disease. The most potent antimicrobial compound was the *N*-(biphenyl-4-yl)methyl-*N*-(2,4-dichlorophenyl)methyl-1*H*-imidazol-1-ylamine (**21**), which was found to be active against yeasts and dermatophytes; its potency and selectivity were comparable to those of miconazole.

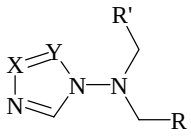
Introduction

During the past 20 years an increase of invasive fungal infections has been observed, particularly in immunosuppressed patients, which are now causes of morbidity and mortality. Autopsy data in fact indicate that more than half of the patients who die with malignancies are infected with *Candida* spp and increasing numbers with other fungi. Since the discovery of amphotericin B a number of different classes of antifungal agents have been discovered. However, there is still a critical need for new antifungal agents to treat life-threatening invasive mycoses[1].

The route by which fungal cells synthesize ergosterol certainly offers a number of possible targets. One of them, lanosterol C-14α-demethylase, has continued to be intensively studied because of the success use of its inhibitors, the azoles. Furthermore, one of the very early enzymes in the pathway of ergosterol synthesis, squalene epoxidase, is the target of other antimycotics, namely the allylamines and the benzylamines[2].

Recently, we reported the synthesis and the antimycotic activity of compounds which simultaneously contain the structural characteristics of both azole and allylamine (or benzylamine) antifungals. These substances were devoid of activity against yeasts; on the other hand, however, some 1-aminoazole derivatives inhibited the growth of dermatophytes, showing an activity comparable with that of naftifine[3].

In order to explore the importance of the *N*-azolylamine moiety for antifungal activity, we here report the synthesis and the antimycotic activity of the disubstituted *N*-azolylamines **1**–**23**, which are structurally related to the previously described compounds[3] and contain the most important and typical portions of the azole and non-azole antifungals (Scheme 1). Moreover, we thought that this preliminary investigation could help us to find a lead compound for the development of a new class of antifungal agents.



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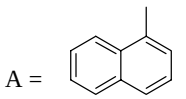

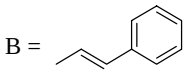

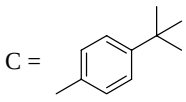

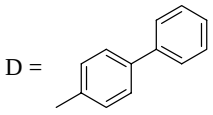

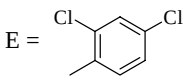

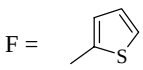

No.	X	Y	R	R'
1	CH	CH	D	C
2	N	CH	D	C
3	CH	N	D	C
4	CH	CH	F	C
5	N	CH	F	C
6	CH	N	F	C
7	CH	CH	E	C
8	N	CH	E	C
9	CH	N	E	C
10	N	CH	B	C
11	CH	N	B	C
12	N	CH	D	B
13	CH	N	D	B
14	N	CH	E	B
15	CH	N	E	B
16	CH	CH	E	A
17	N	CH	A	E
18	CH	N	A	E
19	N	CH	A	A
20	CH	N	A	A
21	CH	CH	D	E
22	N	CH	D	E
23	CH	N	D	E

Scheme 1. Structure of the disubstituted *N*-azolylamines **1–23.**

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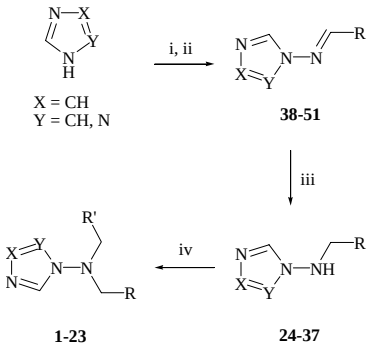
Chemistry

As shown in Scheme 2, tertiary amines **1**–**23** were prepared starting from the secondary amines **24**–**37** and the appropriate alkyl halides, using three different methods according to the substrate and the reagent: (a) anhydrous THF in the presence of NaH[4]; (b) anhydrous diethyl ether in the presence of potassium *tert*-butoxide and 18-crown-6[5]; (c) dichloromethane/50% sodium hydroxide aqueous solution in the presence of tetrabutylammonium hydrogen sulfate[6]. Secondary amines **24**–**37** were generated by reducing the corresponding Schiff bases **38**–**51** with $NaBH_4$ in methanol. All the imidazole and triazole Schiff bases but *N*-(biphenyl-4-yl)-methylene-1*H*-imidazol-1-ylamine (**40**) (see Experimental part), were prepared according to a procedure previously reported[3].



No.	X	Y	R
24, 38	CH	CH	F
25, 39	CH	CH	A
26, 40	CH	CH	D
27, 41	CH	CH	E
28, 42	N	CH	A
29, 43	CH	N	A
30, 44	N	CH	D
41, 45	CH	N	D
32, 46	N	CH	E
33, 47	CH	N	E
34, 48	N	CH	B
35, 48	CH	N	B
36, 50	N	CH	F
37, 51	CH	N	F

Scheme 2. Synthesis of tertiary amines **1**–**23**. Reagents and reaction conditions: (i) NH_2OSO_3H, water, $NaHCO_3$; (ii) RCHO, ethanol, HCl; (iii) $NaBH_4$, methanol; (iv) method a (for compounds **2**, **12**, **17**, **22**): $HalCH_2R'$, NaH, THF; method b (for compounds **1**, **4**): $HalCH_2R'$, 18-crown-6, diethyl ether, ^tBuOK; method c (for compounds **3**, **5**–**11**, **13**–**16**, **18**–**21**, **23**): $HalCH_2R'$, dichloromethane/water, NaOH, TBAHS. Hal = Br (for compounds **1**–**15**), Cl (for compounds **16**–**20**, **22**, **23**), I (for compound **21**).

Results and Discussion

The title compounds were evaluated *in vitro* against several pathogenic fungi including representatives of yeasts (*Candida albicans*, *Candida parapsilosis*, *Criptococcus neoformans*), dermatophytes (*Trichophyton verrucosum*, *Trichophyton rubrum*, *Microsporum gypseum*), and molds (*Aspergillus fumigatus*). Miconazole was used as reference drug. The title compounds were also tested for *in vitro* cytotoxity in a lymphoid cell line (MT-4). Cytotoxicity evaluation was performed in order to determine whether test compounds were endowed with selective antimicrobial activity.

None of the compounds was active against the mold strain tested (*A. fumigatus*).

All triazole derivatives were inactive against yeasts and dermatophytes. On the other hand, under the same experimental conditions imidazole derivatives were inhibitory to the growth of yeasts and dermatophytes, although generally with lower potency than that of miconazole. These results are in agreement with literature. In a study on the antifungal activity of aromatic ethers of 1-aryl-2-(1*H*-azolyl)ethanol (which are analogs of miconazole) Y. Wahbi and coworkers also found that derivatives with an imidazole portion were more potent than triazolic counterparts. This may be related to the difference between the lipophilic parameters of the two groups[7]. Among the imidazole derivatives, the best antifungal activity was obtained with compound **21**, which contains the 4-biphenyl and 2,4-dichlorophenyl portions, and whose potency and selectivity were comparable to those of miconazole (Table 1).

Compound **4** was also found to inhibit the growth of yeasts and dermatophytes, although with low potency. Compounds **16**, **7**, and **1** (data not shown) follow in order of decreasing potency.

The substitution of R or R′ in compound **21** turned out to seriously decrease the activity of the parent compound. In particular, compound **1**, containing the 4-biphenyl and 4-*tert*-butylphenyl portions, proved to be inactive against yeasts and poorly active against dermatophytes. It may be assumed that the steric hindrance of the substituent at position 4 might play an important role in the activity.

To conclude, in our opinion 1*H*-imidazol-1-ylamine could represent a pharmacophore group for antimycotic activity.

Although from the results obtained it is difficult to establish a general relationship between chemical structure and antifungal activity, some preliminary conclusions can be the following: a) the presence of the 4-biphenyl portion seems to be a determinant factor for the observed activity; b) in these derivatives, increase in size of the substituent at position 4 of the second aryl portion, appears to have a marked negative effect on biological activity.

Table 1. *In vitro* antifungal activity of compounds **4**, **21** and miconazole.

Compound	MIC [a]					
	C. albicans	*C. parapsilosis*	*C. neoformans*	*T. verrucosum*	*T. rubrum*	*M. gypseum*
4	67	67	67	25 (1.6)	50	6 (4.8)
21	2.5 (19)	1.2 (40)	2.5 (19)	0.3 (160)	0.15 (320)	1.5 (32)
miconazole	5 (3.6)	2.5 (7)	1.2 (15)	0.15 (120)	0.15 (120)	1.2 (15)

[a] Minimum inhibitory concentration (μM). Values in brackets represent the CC_{50}/MIC ratio (selectivity index). Data represent mean values from three independent determinations.

Table 2. Structure, synthetic and analytical data of the disubstituted *N*-azolylamines **1**–**23.**

No.	X	Y	R	R′	Mp (°C) Recrystallization solvent	Formula (MW)*	MS: *m/z* [M^+]	Method	Yield (**)
1	CH	CH	D	C	114–116	$C_{27}H_{29}N_3$	395	b	67
					benzene/petroleum ether	(395.54)			(I)
2	N	CH	D	C	131	$C_{26}H_{28}N_4$	396	a	73
					benzene/cyclohexane	(396.54)			(II)
3	CH	N	D	C	102–103	$C_{26}H_{28}N_4$	396	c	76
					ligroin	(396.54)			(III)
4	CH	CH	F	C	66	$C_{19}H_{23}N_3S$	325	b	49
					benzene/ligroin	(325.47)			(I)
5	N	CH	F	C	glass	$C_{18}H_{22}N_4S$	326	c	85
						(326.45)			(II)
6	CH	N	F	C	oil	$C_{18}H_{22}N_4S$	326	c	90
						(326.45)			(III)
7	CH	CH	E	C	153–154	$C_{21}H_{23}N_3Cl_2 \cdot (CO_2H)_2$	387	c	20
					ethanol/diethyl ether	(478.37)	$[M^+-(CO_2H)_2]$		(I)
8	N	CH	E	C	125	$C_{20}H_{22}N_4Cl_2$	388	c	70
					benzene/ligroin	(389.32)			(II)
9	CH	N	E	C	64	$C_{20}H_{22}N_4Cl_2$	388	c	59
					ligroin	(389.32)			(III)
10	N	CH	B	C	129	$C_{22}H_{26}N_4$	346	c	65
					benzene/ligroin	(346.47)			(II)
11	CH	N	B	C	61	$C_{22}H_{26}N_4$	346	c	60
					petroleum ether	(346.47)			(III)
12	N	CH	D	B	164	$C_{24}H_{22}N_4$	366	a	78
					benzene/ligroin	(366.46)			(II)
13	CH	N	D	B	133	$C_{24}H_{22}N_4$	366	c	74
					ligroin	(366.46)			(III)
14	N	CH	E	B	125	$C_{18}H_{16}N_4Cl_2$	358	c	69
					benzene/petroleum ether	(359.25)			(II)
15	CH	N	E	B	88	$C_{18}H_{16}N_4Cl_2$	358	c	43
					ligroin	(359.25)			(III)
16	CH	CH	E	A	125–127	$C_{21}H_{17}N_3Cl_2 \cdot (CO_2H)_2$	381	c	25
					ethanol/diethyl ether	(472.32)	$[M^+-(CO_2H)_2]$		(I)
17	N	CH	A	E	151	$C_{20}H_{16}N_4Cl_2$	382	a	17
					toluene/petroleum ether	(383.27)			(II)
18	CH	N	A	E	120	$C_{20}H_{16}N_4Cl_2$	382	c	64
					ligroin	(383.27)			(III)
19	N	CH	A	A	215	$C_{24}H_{20}N_4$	364	c	74
					ethanol	(364.44)			(II)
20	CH	N	A	A	135–136	$C_{24}H_{20}N_4$	364	c	47
					benzene/ligroin	(364.44)			(III)
21	CH	CH	D	E	117	$C_{23}H_{19}N_3Cl_2$	407	c	32
					benzene/ligroin	(408.33)			(I)
22	N	CH	D	E	178	$C_{22}H_{18}N_4Cl_2$	408	a	26
					benzene/ligroin	(409.31)			(II)
23	CH	N	D	E	155–156	$C_{22}H_{18}N_4Cl_2$	408	c	88
					ligroin	(409.31)			(III)

* Analytical results for C, H, N were within ±0.4% of the calculated values; ** Chromatographic conditions: I = silica/ethyl acetate, II = alumina/petroleum ether-chloroform (3:7), III = alumina/petroleum ether-chloroform (7:3).

Table 3. Structure, synthetic and analytical data of the methylazolylamines **24**–**37**.

No.	X	Y	R	Mp (°C) Recryst. solvent	Formula (MW)*	MS: *m/z* [M^+]
24	CH	CH	F	66 toluene/petroleum ether	$C_8H_9N_9S$ (179.23)	179
25	CH	CH	A	102–103[lit.102–103] [3] toluene/petroleum ether	$C_{14}H_{13}N_3$ (223.28)	223
26	CH	CH	D	125 toluene/petroleum ether	$C_{16}H_{15}N_3$ (249.30)	249
27	CH	CH	E	89–90 toluene/petroleum ether	$C_{10}H_9N_3Cl_2$ (242.09)	241
28	N	CH	A	173–175 [lit.171] [8] ethanol	$C_{13}H_{12}N_4$ (224.26)	224
29	CH	N	A	88–89 [88–89] [3] cyclohexane	$C_{13}H_{12}N_4$ (224.26)	224
30	N	CH	D	170–172 toluene	$C_{15}H_{14}N_4$ (250.30)	250
41	CH	N	D	137 ethanol	$C_{15}H_{14}N_4$ (250.30)	250
32	N	CH	E	155 toluene	$C_9H_8N_4Cl_2$ (243.09)	242
33	CH	N	E	102–103 ethanol	$C_9H_8N_4Cl_2$ (243.09)	242
34	N	CH	B	127 ethanol	$C_{11}H_{12}N_4$ (200.24)	200
35	CH	N	B	105–106 ethanol	$C_{11}H_{12}N_4$ (200.24)	200
36	N	CH	F	103–104 toluene	$C_7H_8N_4S$ (180.22)	180
37	CH	N	F	77 cyclohexane	$C_7H_8N_4S$ (180.22)	180

* Analytical results for C, H, N were within ±0.4% of the calculated values.

Further structural modifications of compound **21** are in progress in order to define the structural requirements for optimal activity of this new class of antifungal agents.

Acknowledgements

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Experimental Part

Chemistry

Melting points were determined in open capillary tubes with a Büchi apparatus and are uncorrected. Elemental analyses (C, H, N) were performed by Dr. Emilio Cebulec at the Chemistry Department of the University of Trieste. IR spectra were obtained on a Jasco FT/IR-200 spectrophotometer (KBr). All compounds showed appropriate IR, which are not reported. The ^{1}H-NMR spectra were determined on a Varian 200 instrument. ^{1}H-NMR data were in agreement with the proposed structure and the full set of data is in the PhD thesis of S. Castellano, University of Milan, 1998. Mass spectra data were determined on a V6-Micromass 7070H mass spectrometer. Silica gel chromatography was performed using Merck silica gel 60 (0.015–0.040 mm); alumina chromatography was performed using Merck aluminum oxide 90 (0.063–0.200 mm). Petroleum ether refers to petroleum ether (40–60 °C).

General Procedure for the Preparation of Compounds **38**–**39**, **41**

A solution of hydroxylamine-*O*-sulfonic acid (11.3 g, 100 mmol) in water (100 ml) was made neutral with $NaHCO_3$ and imidazole (13.6 g, 200 mmol) was added. After stirring for 20 h at room temperature, the mixture was acidified with 2N hydrochloric acid and then was evaporated under reduced pressure. To a stirring suspension of the residue in 150 ml of absolute ethanol, a solution of the appropriate aldehyde (100 mmol) in the same solvent (50 ml) was added and the reaction mixture was stirred at room temperature for 24 h. The solvent was evaporated under reduced pressure and the residue was taken up with water (200 ml). The insoluble portion was filtered off and the filtrate was extracted with diethyl ether (2 × 100 ml). Organic phase was eliminated while aqueous solution was basified with $NaHCO_3$ and extracted with chloroform (3 × 100 ml). The combined extracts were dried over Na_2SO_4, chloroform was evaporated and the residue was crystallized from suitable solvent. Yields, melting points, analytical and spectroscopic data are reported in Table 4.

Table 4. Structure, synthetic and analytical data of the methylenazolylamines **38–51.**

No.	X	Y	R	Yield (%)	Mp (°C) Recryst. solvent	Formula (MW)*	MS: *m/z* [M^+]
38	CH	CH	F	53	84 benzene/petroleum ether	$C_8H_7N_3S$ (177.23)	177
39	CH	CH	A	38	87–88 [lit.87–88] [3] cyclohexane	$C_{14}H_{11}N_3$ (221.26)	221
40	CH	CH	D	50	150–151 ethanol	$C_{16}H_{13}N_3$ (247.29)	247
41	CH	CH	E	33	141 toluene/petroleum ether	$C_{10}H_7N_3Cl_2$ (240.09)	239
42	N	CH	A	13	143 [lit. 142–143] [9] ethanol	$C_{13}H_{10}N_4$ (222.24)	222
43	CH	N	A	13	89 [lit.89] [3] petroleum ether	$C_{13}H_{10}N_4$ (222.24)	222
44	N	CH	D	7	200 [lit. 210–212] [10] methanol	$C_{15}H_{12}N_4$ (248.28)	248
45	CH	N	D	22	133–134 cyclohexane	$C_{15}H_{12}N_4$ (248.28)	248
46	N	CH	E	13	170 [lit. 171–172] [9] toluene/cyclohexane	$C_9H_6N_4Cl_2$ (241.07)	240
47	CH	N	E	15	157–158 ethanol	$C_9H_6N_4Cl_2$ (241.07)	240
48	N	CH	B	10	186–187 [lit. 190] [10] toluene	$C_{11}H_{10}N_4$ (198.22)	198
49	CH	N	B	15	97 ethanol	$C_{11}H_{10}N_4$ (198.22)	198
50	N	CH	F	11	120 toluene	$C_7H_6N_4S$ (178.21)	178
51	CH	N	F	12	94–95 cyclohexane	$C_7H_6N_4S$ (178.21)	178

* Analytical results for C, H, N were within ±0.4% of the calculated values.

*Preparation of N-(Biphenyl-4-yl)methylene-1H-imidazol-1-ylamine (***40***)*

Prepared from imidazole, hydroxylamine-*O*-sulfonic acid, and bipheny-4-carboxaldehyde following the procedure described above. After stirring for 24 h at room temperature, solvent was evaporated under reduced pressure and the residue was taken up with water (200 ml) and filtered. The solid was washed with diethyl ether, treated with boiling ethanol and then filtered. The filtrate was concentrated under reduced pressure, taken up with water, basified with $NaHCO_3$ and extracted with chloroform (3 × 100 ml). The combined extracts were dried over Na_2SO_4, chloroform was evaporated and the residue was crystallized from suitable solvent. Yield, melting point, analytical and spectroscopic data are reported in Table 4.

General Procedure for the Preparation of Compounds **42**–**51**

Prepared from 1*H*-1,2,4-triazole (13.8 g, 200 mmol) following the procedure described above. After stirring for 24 h at room temperature, the solvent was evaporated under reduced pressure and the residue was taken up with water (200 ml). The reaction mixture was basified with $NaHCO_3$ and extracted with chloroform (3 × 100 ml). The combined extracts were dried over Na_2SO_4, chloroform was evaporated and the residue was separated by alumina column chromatography (chloroform-petroleum ether 1:1). First eluates were discarded, then 1*H*-1,2,4-triazole derivatives and after 4*H*-1,2,4-triazole derivatives were recovered. Yields, melting points, analytical and spectroscopic data are reported in Table 4.

General Procedure for the Preparation of Compounds **24**–**37**

A cooled solution of Schiff base (0.050 mol) in methanol (200 ml) was treated portionwise with an excess of sodium borohydride powder until the starting material disappeared (TLC), then the solvent was removed under reduced pressure. The crude residue was taken up with water (200 ml) and extracted with chloroform. The organic phase was dried over Na_2SO_4 and the solvent was evaporated to give a solid which was crystallized from suitable solvent. The yield of the reduction reaction was quantitative. Melting points, analytical and spectroscopic data are reported in Table 3.

General Procedure for the Preparation of Compounds **1**–**23**
General Method a

Sodium hydride (0.96 g, 40.0 mmol) was added to a solution of the appropriate secondary amine (20.0 mmol) in anhydrous tetrahydrofuran (200 ml) under dry nitrogen at room temperature. The mixture was stirred for 24 h then a solution of the appropriate halide (22.0 mmol) in anhydrous tetrahydrofuran (70 ml) was added dropwise over a period of 20 min. After stirring under nitrogen at room temperature for 24 h, methanol (15 ml) was added. The solvent was evaporated and the residue was taken up with water

(100 ml) and extracted with chloroform (3 × 50 ml). The combined organic solution was washed with brine and dried over Na_2SO_4. The solvent was removed by evaporation under reduced pressure and the residue was purified by chromatography.

General Method b

Potassium *tert*-butoxide (2.24 g, 20.0 mmol) was added to a solution of 18-crown-6 (0.53 g, 2.00 mmol) in diethylether (50 ml), then the appropriate secondary amine (20.0 mmol) was added in a single portion. After stirring for 15 min at room temperature under nitrogen, a solution of the appropriate halide (20.0 mmol) in diethylether (30 ml) was added dropwise to the cooled reaction mixture over a period of 20 min. Stirring was continued for 4 h then water (100 ml) was added and the layers were separated. Aqueous phase was extracted with diethyl ether (3 × 50 ml) and the combined extracts were washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by chromatography.

General Method c

A solution of the secondary amine (10.0 mmol) and tetrabutylammonium hydrogen sulfate (3.39 g, 10.0 mmol) in dichloromethane (50 ml) was treated with the appropriate halide (15.0 mmol) and 50% sodium hydroxide aqueous solution (30 ml). The reaction mixture was stirred overnight at room temperature, then treated with water (60 ml) and dichloromethane (60 ml) and shaken. The aqueous layer was extracted with dichloromethane (3 × 50 ml). Organic extracts were collected and the solvent was removed by evaporation under reduced pressure. The residue was taken up with ethyl acetate (150 ml), washed with brine and then dried over Na_2SO_4. The solvent was removed by evaporation under reduced pressure and the residue was purified by chromatography.

Microbiology

Compounds. Test compounds were dissolved in DMSO at an initial concentration of 200 mM and then were serially diluted in culture medium.

Cells. Cell lines were from American Type Culture Collection (ATCC); fungal strains were clinical isolates (obtained from Clinica Dermosifilopatica, University of Cagliari) or collection strains from ATCC.

Antimycotic Assays. Yeast blastospores were obtained from a 30 h old shaken culture incubated at 30 °C in Sabouraud dextrose broth. The dermatophyte inoculum was scraped aseptically with a spatula from a 7 day-old culture on agar and the macerate was finely suspended in Sabouraud dextrose broth using a glass homogenizer. Glycerol, final concentration 10%, was added as a cryoprotective agent to both yeast and dermatophyte suspension, aliquots of which were then stored in liquid nitrogen. Test tubes were inoculated with 10^3 blastospores or colony forming units (CFU)/tube. The minimal inhibitory concentration (MIC) was determined by serial dilutions of compound using Sabouraud dextrose broth (pH 5.7) and incubating at 37 °C. The growth control for yeast was read after 1 day and for dermatophytes after 3 days (5 days for Cryptococcus neoformans). The MIC was defined as the compound concentration at which no macroscopic signs of fungal growth were detected. The minimal germicidal concentration (MGC) was determined by subcultivating negative test tubes in Sabouraud dextrose agar.

The cytotoxity evaluation of compounds was based on the viability of mock-infected cells, as monitored by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method [11].

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