SYNTHESIS INDUCED BY MICROWAVE IRRADIATIO AND *IN VITRO* ANTIFUNGAL EVALUATION OF NEW DIHYDROPYRAZOLO[3,4-*b*] [1,4]DIAZEPINES

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Abstract: New 2- and 4-methylenedioxyphenyl-8-phenyl-2,3-dihydropyrazolo[3,4-b]diazepines **3** were obtained from the reaction of 4,5-diamino-3-methyl-1-phenylpyrazole **1** with one equivalent of the methylenedioxychalcones **2** in both absolute ethanol and under solvent-free conditions microwave irradiation. The structures of diazepines **3** were determined by detailed nmr measurements. These compounds does not showed any antifungal *in vitro* activity up to 250 μg/ml.

Introduction

In recent years, the synthesis and pharmacological properties of benzodiazepines analogues containing heterocycles fused to a seven-member diazepine ring have appeared in the literature [1]. Particularly, good CNS activity was reported for various pyrazolodiazepines [2], some of them acting as psychotropics agents [3].

Patients suffering anxiety disorders usually suffer an immunodepression that makes them susceptible to fungal infections among other complaints [4]. Although drugs of the azole type (ketoconazole, itraconazole, fluconazole) are usually employed for treating systemic and superficial mycoses, it is well known that concurrent use of azole antifungals and certain benzodiazepine tranquillizers is not recommended, since this combination may result in elevated serum benzodiazepine levels and enhanced or prolonged central nervous system depression. [5].

Considering that recent reports have claimed the use of 1,5-benzodiazepines as fungicides and antimicrobial agents [6], it was though of interest to find new diazepines analogues which, apart from their possible psychotropic properties, possess themselves antifungal properties.

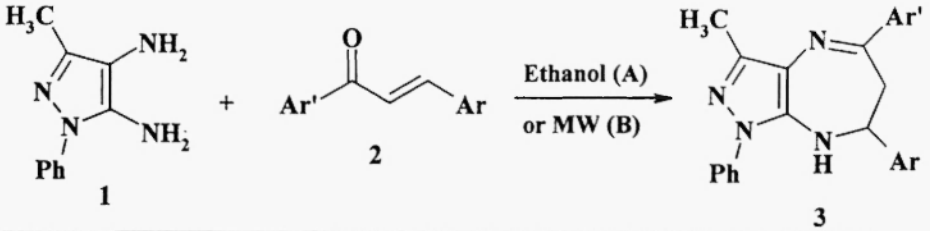
On the other hand, the potential application of microwave technology in organic synthesis is increasing rapidly because of its reaction simplicity, less pollution, and minimum reaction time. A wide range of reactions can be performed in the solid state without the need of solvents, in short times and with high yield and regioselectivity, providing large libraries of diverse molecules. Recent developments in microwave solventless organic syntheses were acquainted previously [7].

We report herein the synthesis of eight new pyrazolo[3,4-b][1,4]diazepines (**3a-3h**), by the reaction of chalcones with 1,2-diamines [8-14] by using two different methods, one of them including microwave irradiation. The reaction of both type of compounds is a convenient and versatile way of synthesizing fused diazepine systems.

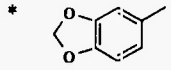
All these compounds were screened for antifungal properties against a panel of 12 human opportunistic pathogenic yeasts, hialohyphomycetes as well as dermatophytes.

Results and Discussion

In this work we have studied the reaction of 4,5-diamino-3-methyl-1-phenylpyrazole **1** with chalcones **2** by refluxing in ethanol with catalytic amounts of acetic acid (Method A) or by microwave irradiation (Method B). Pyrazolo[3,4-b][1,4]diazepines **3** were formed from both approaches in good to excelent yields (Scheme 1).

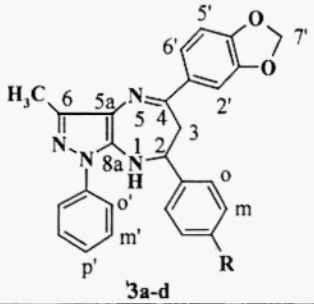


Comp.	Ar	Ar'	Reaction times		Yields, %	
			Method A (hours)	Method B (min)	Method A	Method B
3a	C_6H_5	*	6	10	90	75
3b	4-ClC_6H_4	*	7	8	75	85
3c	4-BrC_6H_4	*	6	8	70	78
3d	4-$O_2NC_6H_4$	*	5.5	6	45	70
3e	*	C_6H_5	8.5	10	70	70
3f	*	4-ClC_6H_4	9	8	90	86
3g	*	4-BrC_6H_4	7	8	85	75
3h	*	4-$O_2NC_6H_4$	8	6	85	80

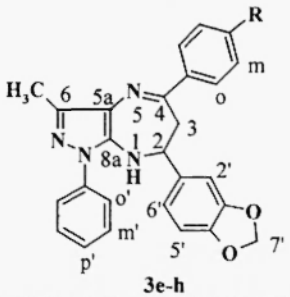


The analytical and spectral data of compounds **3** were consistent with the proposed structures. In the ^{1}H-NMR spectra the proton signals for the CH_2-CH fragment are observed as an AMX system at δ = 2.71-2.83, 3.85-4.08 and δ = 4.88-5.18; the signals for the O-CH_2-O group appear at δ = 5.88-6.07 and finally a multiplet that appears at δ = 6.72-8.15 is assigned to the aromatic protons (Table 1).

Table 1. 1H-NMR Chemical Shift (δ) for compounds **3a-h** (DMSO-d_6, 300 MHz)



3a-d



3e-h

Comp.	3a	3b	3c	3d	3e	3f	3g	3h
CH_3 (s)	2.24	2.24	2.23	2.24	2.27	2.25	2.26	2.28
NH (d)	6.88	6.97	6.93	7.06	6.86	6.93	6.93	7.18
H_a (d)	2.77	2.71	2.72	2.76	2.83	2.77	2.77	2.76
H_m (dd)	3.87	3.95	3.94	4.08	3.83	3.86	3.85	4.00
H_x (t)	4.96	4.99	4.97	5.18	4.88	4.89	4.89	4.97
H2' (d)	7.29	7.31	7.31	7.30	6.79	6.75	6.75	6.75
H5' (d)	6.80	6.82	6.82	6.80	6.75	6.72	6.73	6.73
H6' (d)	7.15	7.18	7.18	7.18	6.68	6.65	6.65	6.63
H7' (d)	5.99	6.00	6.07	5.99	5.89	5.89	5.90	5.88
H_o (d)	7.26	7.20	7.15	7.46	7.42	7.36	7.49	7.95
H_m (d)	7.26	7.26	7.39	7.64	7.74	7.74	7.67	8.15
H_o (d)	7.63	7.62	7.61	7.74	7.66	7.61	7.61	7.60
H_m (t)	7.50	7.50	7.50	7.52	7.54	7.50	7.50	7.53
H_p (t)	7.33	7.34	7.34	7.35	7.33	7.34	7.34	7.37

In the ^{13}C-nmr spectra (DEPT) all signals belonging to tertiary, secondary and primary carbon atoms could be determined for **3a-h** compounds (Table 2).

Table 2. ^{13}C-NMR Chemical Shift (δ) for Compounds **3a-h** (DMSO-d_6, 300 MHz)

Comp.	3a	3b	3c	3d	3e	3f	3g	3h
CH_3	11.5	11.4	11.5	11.5	11.5	11.4	11.5	11.4
C2	55.9	55.2	55.4	55.8	55.6	55.5	55.6	55.5
C3	39.0	39.2	39.2	38.9	40.0	39.6	39.6	38.7
C4	154.7	154.5	154.5	154.4	155.4	153.9	154.0	152.4
C6	147.3	147.3	147.4	147.4	147.7	147.6	147.7	148.0
C5a	115.1	115.0	115.2	115.3	115.2	115.1	115.1	115.4
C8a	139.0	138.7	138.8	138.7	139.0	139.0	139.0	139.4
C1'	135.5	135.2	135.3	135.1	138.0	137.8	137.8	137.6
C2'	106.0	105.9	106.0	105.9	106.4	106.2	106..3	106.1
C3'	147.5	147.5	147.6	147.6	145.7	145.7	145.7	146.5
C4'	147.6	147.6	147.7	147.7	147.0	146.9	147.0	146.9
C5'	107.5	107.4	107.5	107.5	107.8	107.7	107.8	107.8
C6'	120.4	120.3	120.4	120.4	118.9	118.7	118.6	118.6
C7'	101.1	101.1	101.1	101.2	100.7	100.6	100.7	100.7
Ci	143.7	142.5	143.0	146.2	140.8	139.4	139.9	146.5
Co	125.7	125.6	128.0	123.3	128.0	127.9	130.1	126.8
Cm	128.0	127.9	130.9	127.1	126.0	127.7	128.0	123.3
Cp	125.5	131.0	119.6	151.0	128.2	132.9	121.2	138.7
Ci'	139.2	139.0	139.1	139.1	139.1	139.1	139.1	145.7
Co'	123.4	123.3	123.4	123.5	123.4	123.4	123.5	123.6
Cm'	129.2	129.2	129.2	129.3	129.2	129.2	129.3	129.3
Cp'	126.6	126.3	126.4	126.5	126.4	126.4	126.5	126.7

Assignment of the ^{1}H- and ^{13}C- resonances of compounds **3** was deduced from the concerted application of both direct and long range heteronuclear chemical shift correlation experiments. One-bond and two and three bonds proton-carbon chemical shift correlations were established using the HMQC [15] and the HMBC [16] techniques. Molecular structures of compounds **3a** and **3b** were confirmed by X-ray diffraction analysis and have recently been reported [17].

These new compounds were assayed for antifungal properties against a panel of 12 fungal strains comprising human opportunistic pathogenic yeasts, hialohyphomycetes as well as dermatophytes with the agar dilution method [18]. Results showed that none of the compounds tested displayed any activity against the fungi tested up to 250 μg/mL, clearly showing that this series of pyrazolodiazepines are devoid of antifungal properties.

Conclusion

Results demonstrate that both synthetic routes are highly convenient for regioselectively producing fused hidropyrazolo[3,4-*b*]diazepines in good yields, being this work a new example of the utility of microwaves in organic synthesis. Regarding the antifungal properties, this new series of compounds did not show activity when tested against a panel of 12 human opportunistic pathogenic fungi at concentrations up to 250 μg/m in agar dilution assays. Nevertheless, the simple synthesis of hidropyrazolo[3,4-*b*]diazepines using a microwave oven, encourages us to further synthesize new series of diazepine derivatives in order to find the desired analogues that, besides their tranquillizing properties, possess antifungal properties.

Experimental

Melting points were determined in a Buchi Melting Point Apparatus and are uncorrected. The ^{1}H- and ^{13}C nmr spectra were run on a Bruker DPX 300 spectrometer operating at 300 MHz and 75 MHz

respectively, using dimethyl sulfoxide-d_6 as solvent and tetramethylsilane as internal standard. The mass spectra were scanned on a Hewlett Packard HP Engine-5989 spectrometer (equipped with a direct inlet probe) a operating at 70 eV. The elemental analysis have been obtained using a LECO CHNS-900 equipment.

General procedure for the synthesis of 2,3-dihydropyrazolo[3,4-*b*]diazepines **3a-h**.
Method A. A solution of 4,5-diamine-3-methyl-1-phenylpyrazole **1** (2.0 mmol) and chalcone **2a-h** (2.0 mmol) in absolute ethanol (10 mL) with 1 mL of acetic acid was heated to reflux for 5.5-9 h (reaction was monitored by TLC). After cooling and neutralizing with ammonia, the reaction mixture was allowed to stand two hours. The resulting yellow precipitate was filtered off and recrystallized from ethanol.
Method B. Equimolar amounts of amine **1** and chalcone **2** were placed into pyrex-glass open vessels and irradiated in a domestic microwave oven for 6-10 min.(at 600 watts). Products **3** were treated with ethanol, the solid was filtered off and recrystallized from ethanol.

6-Methyl-4-(3,4-dioxymethylenphenyl)-2,8-diphenyl-2,3-dihydropyrazolo[3,4-*b*]diazepine **3a**.
This compound was obtained according to general procedure as yellow crystals, mp 177 °C; MS: *m/z* (%) = 423 (42), 422 (M^+, 100), 421 (30), 408 (23), 407 (80), 318 (12), 301 (13), 207 (15), 148 (12), 147 (10), 104 (14), 103 (10), 89 (14), 77 (36).
Anal. Calcd. for $C_{26}H_{22}N_4O_2$: C, 73.92; H, 5.25; N, 13.26. Found: C, 73.96; H, 5.19; N, 13.15.

2-(4-Chlorophenyl)-6-methyl-4-(3,4-dioxymethylenphenyl)-8-phenyl-2,3-dihydropyrazolo[3,4-*b*]diazepine **3b**.
This compound was obtained according to general procedure as yellow crystals, mp 186 °C; MS: *m/z* (%) = 458/456 (M^+, 37/100), 443 (20), 441 (54), 335 (10), 318 (14), 241 (14), 148 (17), 147 (13), 103 (10), 89 (17), 77 (43), 51 (13).
Anal. Calcd. for $C_{26}H_{21}ClN_4O_2$: C, 68.34; H, 4.63; N, 12.26. Found: C, 68.29; H, 4.55; N, 12.31.

2-(4-Bromophenyl)-6-methyl-4-(3,4-dioxymethylenphenyl)-8-phenyl-2,3-dihydropyrazolo[3,4-*b*]diazepine **3c**.
This compound was obtained according to general procedure as yellow crystals, mp 210 °C; MS: *m/z* (%) = 503 (37), 502/500 (M^+, 100/97), 501 (66), 499 (25), 488 (16), 487 (56), 485 (46), 379 (13), 345 (13), 318 (21), 148 (26), 147 (25), 119 (10), 118 (13), 117 (10), 104 (11), 103 (19), 91 (12), 89 (29), 77 (73).
Anal. Calcd. for $C_{26}H_{21}BrN_4O_2$: C, 62.29; H, 4.22; N, 11.17. Found: C, 62.38; H, 4.17; N, 11.22.

6-Methyl-2-(4-nitrophenyl)-4-(3,4-dioxymethylenphenyl)-8-phenyl-2,3-dihydropyrazolo[3,4-*b*]diazepine **3d**.
This compound was obtained according to general procedure as red crystals, mp 229 °C; MS: *m/z* (%) = 467 (M^+, 85), 452 (35), 148 (27), 147 (27), 119 (11), 118 (20), 117 (13), 104 (12), 103 (22), 102 (10), 91 (18), 90 (13), 89 (38), 77 (100), 65 (25), 64 (10), 63 (31), 51 (36).
Anal. Calcd. for $C_{26}H_{21}N_5O_4$: C, 66.80; H, 4.53; N, 14.98. Found: C, 66.74; H, 4.48; N, 14.89.

6-Methyl-2-(3,4-dioxymethylenphenyl)-4,8-diphenyl-2,3-dihydropyrazolo[3,4-*b*]diazepine **3e**.
This compound was obtained according to general procedure as yellow crystals, mp 136 °C; MS: *m/z* (%) = 422 (M^+, 100), 407 (82), 345 (18), 274 (15), 148 (15), 147 (10), 104 (27), 103 (29), 91 (10), 77 (45), 51 (12).
Anal. Calcd. for $C_{26}H_{22}N_4O_2$: C, 73.92; H, 5.25; N, 13.26. Found: C, 73.96; H, 5.29; N, 13.17.

4-(4-Chlorophenyl)-2-(3,4-dioxymethylenphenyl)-6-methyl-8-phenyl-2,3-dihydropyrazolo[3,4-*b*]diazepine **3f**.

This compound was obtained according to general procedure as yellow crystals, mp 194 °C; MS: *m/z* (%) = 458/456 (M^+, 33/95), 443 (25), 441 (65), 345 (20), 308 (15), 251 (13), 148 (20), 147 (12), 119 (16), 118 (13), 104 (14), 103 (30), 102 (19), 101 (15), 91 (18), 89 (14), 77 (100).
Anal. Calcd. for $C_{26}H_{21}ClN_4O_2$: C, 68.34; H, 4.63; N, 12.26. Found: C, 68.38; H, 4.53; N, 12.34.

4-(4-Bromophenyl)-2-(3,4-dioxymethylenphenyl)-6-methyl-8-phenyl-2,3-dihydropyrazolo[3,4-*b*]diazepine **3g**.
This compound was obtained according to general procedure as yellow crystals, mp 198 °C; MS: *m/z* (%) = 502/500 (M^+, 100/87), 487 (78), 485 (74), 345 (34), 251 (28), 148 (29), 147 (17), 119 (15), 118 (14), 117 (10), 103 (28), 102 (31), 91 (13), 89 (11), 77 (59), 51 (14).
Anal. Calcd. for $C_{26}H_{21}BrN_4O_2$: C, 62.29; H, 4.22; N, 11.17. Found: C, 62.22; H, 4.30; N, 11.09.

6-Methyl-4-(4-nitrophenyl)-2-(3,4-dioxymethylenphenyl)-8-phenyl-2,3-dihydropyrazolo[3,4-*b*]diazepine **3h**.
This compound was obtained according to general procedure as red crystals, mp 265 °C; MS: *m/z* (%) = 467 (M^+, 100), 452 (98), 345 (27), 251 (13), 148 (14), 147 (10), 119 (15), 118 (12), 103 (23), 102 (10), 91 (10), 89 (10), 77 (42), 51 (10).
Anal. Calcd. for $C_{26}H_{21}N_5O_4$: C, 66.80; H, 4.53; N, 14.98. Found: C, 66.73; H, 4.57; N, 14.87.

Microorganisms and media

The microorganisms used for the fungistatic evaluation were purchased from the American Type Culture Collection (Rockville, MD, USA): *Candida albicans* ATCC 10231, *Saccharomyces cerevisiae* ATCC 9763, *Cryptococcus neoformans* ATCC 32264, *Aspergillus flavus* ATCC 9170, *Aspergillus fumigatus* ATCC 26934, *Aspergillus niger* ATCC 9029 and *Trichophyton mentagrophytes* ATCC 9972. Strains were grown on Sabouraud-chloramphenicol agar slants for 48 h at 30°C. Cell suspensions in sterile distilled water were adjusted to give a final concentration of 10^6 viable yeast cells/mL [19]. Dermatophytes: *Microsporum canis* C 112, *Trichophyton rubrum* C 113, *Epidermophyton floccosum* C 114 and *Microsporum gypseum* C 115 as well as *Candida tropicalis* C131 are clinical isolates and were kindly provided by CEREMIC, Centro de Referencia Micológica, Facultad de Ciencias Bioquímicas y Farmaceuticas, Suipacha 531-(2000)-Rosario, Argentina. The strains were maintained on slopes of Sabouraud-dextrose agar (SDA, Oxoid) and subcultured every 15 days to prevent pleomorphic transformations. Spore suspensions were obtained according to reported procedures [19] and adjusted to 10^6 spores with colony forming ability/mL.

Antifungal assays.

The antifungal activity was evaluated with the agar dilution method by using Sabouraud-chloramphenicol agar for yeasts, hialohyphomycetes and dermatophyte species as previously described [20]. Stock solutions of compounds (10 mg/mL in DMSO) were diluted to give serial two-fold dilutions that were added to each medium resulting in concentrations ranging from 0.10 to 250 μg/mL. MIC for each compound was defined as the lowest concentration that produces no visible fungal growth after the incubation time. Ketoconazole (Sigma, St Louis, Mo), Terbinafine (Novartis, Bs. As) and Amphotericin B (Sigma , St Louis; Mo), were used as positive controls. Tests were performed by duplicate.

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