Full Paper

Synthesis and Evaluation of Novel *E*-2-(2-Thienyl)- and *Z*-2-(3-Thienyl)-3-Arylacrylonitriles as Antifungal and Anticancer Agents

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A series of 3-aryl-2-(2-thienyl)acrylonitriles **7** and 3-aryl-2-(3-thienyl)acrylonitriles **8** were synthesized by the reaction of aromatic aldehydes **6** with 2- and 3-thienylacetonitriles **4** and **5,** and evaluated for antifungal and cytotoxic activities against a panel of opportunistic and pathogenic fungi and three different cancer cell lines, respectively.

Keywords: Antifungal and cytotoxic activities / Benzaldehydes / Thienylacrylonitriles / 2-Thienylacetonitrile / 3-Thienylacetonitrile

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Introduction

Substituted acrylonitriles have been found to possess interesting biological properties which range from spasmolytic [1], antioxidative [2], insecticidal [3], and antitubercular [4, 5] to cytotoxic [6, 7] activities. Among them, structures with heterocyclic residues such as 1,2,4-triazoles **1**, benzimidazoles **2**, or 1,3,5-triazines **3** at position 2 of the acrylonitrile system (Fig. 1), have showed potent cytotoxic activities against a tumor-cell line screen [7, 8]. Particularly, compounds of the general structures **1** and **2**, possessing a thienyl ring in position 3, showed the best cytotoxic activities [7].

With the aim of analyzing the role played by the position of the thienyl ring in the cytotoxic activity of acrylonitriles, we present here the synthesis of a new series of acrylonitrile derivatives characterized by the presence of a thienyl group in position 2 and a substituted phenyl group as ring B in position 3. They were evaluated for their cytotoxic behavior against three human cancer cell lines and for antifungal properties against a panel of ten human opportunistic pathogenic fungi.

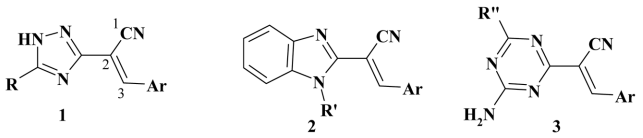

Figure 1. Some 2-heteroarylacrylonitriles with anticancer activity.

Results and discussion

Chemistry

The desired acrylonitriles **7a – k** and **8a – e** were prepared by the Knoevenagel condensation of the corresponding 2-

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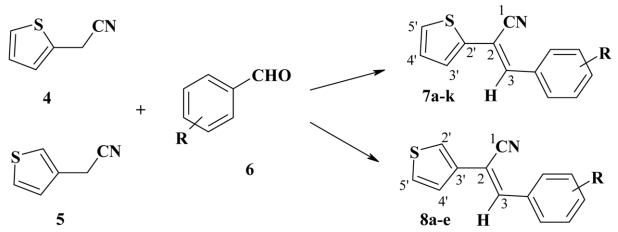
Abbreviations: sulforhodamine B (SRB); minimal inhibitory concentration (MIC)



Comp.	R	Yield (%)	Comp.	R	Yield (%)
7a	4-Cl	76	**7i**	2-F	55
7b	H	65	**7j**	3,4,5-tri-CH_3O	70
7c	4-F	51	**7k**	2-CF_3	73
7d	4-CH_3O	75	**8a**	4-Cl	50
7e	4-Br	68	**8b**	H	60
7f	4-CH_3	55	**8c**	4-F	50
7g	4-CF_3	78	**8d**	4-CH_3O	50
7h	3,4-OCH_2O	63	**8e**	4-Br	70

Scheme 1. Synthesis of 3-aryl-2-thienylacrylonitriles.

and 3-thienylacetonitriles with aldehydes, in the presence of potassium *tert*-butoxide in anhydrous ethanol stirred at room temperature (Scheme 1). The choice of potassium *tert*-butoxide was due to the fact that longer times of the reaction were achieved and the processes of purification were harder when the bases used in the reaction were piperidine [7], sodium methoxide in methanol [9], sodium ethoxide [10], or triethylamine [4] at reflux.

The NMR spectroscopic data of the synthesized compounds were consistent with structures **7** and **8**. For example, compound **7a** exhibited a ^{1}H-NMR spectrum with three double doublets at 7.08, 7.32, and 7.39 ppm, which could be assigned to H-4′, H-5′, and H-3′, respectively. The other two couples of doublets appearing at 7.42 and 7.70 ppm, were assigned to the H_m and H_o, respectively and the singlet at 7.31 ppm was assigned to the vinyl proton. The ^{1}H-NMR spectra of the resting compounds of the series possessed similar signals, differing only in those corresponding to the aryl ring.

The whole carbon skeleton was assigned using ^{13}C-NMR spectra, (including Distortionless Enhancement by Polarization Transfer (DEPT)) and two dimensional HSQC (Heteronuclear Single Quantum Correlation), and HMBC (Heteronuclear Multiple Bond Correlation) experiments. The ^{13}C-NMR spectra of compounds **7a–k** and **8a–e** showed the signal of CN at δ values 116.0–118.3 ppm and the signal of C-2 and =CH at 136.1–146.1 ppm. The ^{13}C-NMR spectra of the rest of the compounds of the series possess similar signals, differing only in those corresponding to the aryl ring.

The configuration of the acrylonitrile double bond could not be established by NMR methods. So, X-ray diffraction analysis [11] was performed with compound **7a**, **7e**, and **8b** from which the isolation of unique crystals was possible. X-ray crystal structures of **7e** and **8b** (Fig. 2)

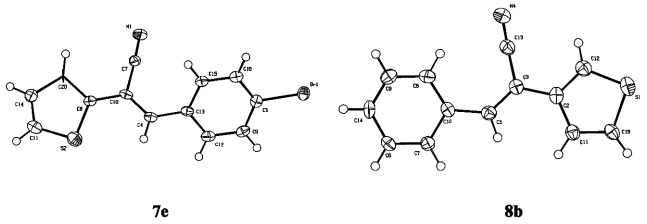


Figure 2. Ortep drawing of structures for compounds **7e** and **8b**, displacement ellipsoids are drawn at the 50% probability level and H are represented as small spheres at arbitrary radii. Note that atom numbering used here is different from that shown at Scheme, 1.

allowed the establishment of the *E*- and *Z*-configuration for the double bond for compounds **7** and **8**, respectively.

Regarding to mass spectra, all products **7** and **8** exhibit a similar pattern of fragmentation, showing the molecular peak along with a typical loss of the substituents of each aryl group.

Biology

The compounds of this series were tested for their cytotoxic activities against breast (MCF-7), lung (H-460), and central nervous system (SF-268) human cancer cell lines, according to the NCI methodology [12a] and for antifungal properties against a panel of ten human opportunistic pathogenic fungi including yeasts, hialohyphomycetes, and dermatophytes following the guidelines of the Clinical and Laboratory Standards Institute (CLSI; formerly National Committee for Clinical and Laboratory Standards (NCCLS)) (Table 1) [13].

Results showed that six out of the fourteen tested compounds were active (IC_{50} < 10 μg/mL) against MCF-7, H-460 and SF-268 cancer cell lines (Table 1). The activity appears not to be completely related to the joint position of the thienyl group to the acrylonitriles C-2 since five out of ten 2-thienyl- and three out of four 3-thienyl- derivatives showed cytotoxic activities. The lowest values of IC_{50} were displayed by **7f** (IC_{50} = 0.10–0.18 μg/mL) which possesses a 2-thienyl ring at the 2-position. This activity is similar to that shown by adriamycin, the standard drug used in the assays (IC_{50} = 0.14–0.18 μg/mL).

Regarding the role played by the substituents of the aromatic ring in the cytotoxic activity, electron-donor substituents appear to exert more influence than electron-withdrawing ones in 2-thienyl derivatives (compare activities of **7f** and **7h** with those of **7c**, **7e**, **7g**, **7i**, and **7k**). Nevertheless, the activity shown by **7a** possessing a 4-chlorophenyl group is not in accordance with this observation.

In turn, the cytotoxic activities of 3-thienyl derivatives **8** seem not to be related to the electronic properties of

Table 1. *In vitro* cytotoxic (IC_{50} in μg/mL) activities of synthetic acrylonitrile derivatives.

	MCF-7	H-460	SF-268
7a	1.30 ± 0.1	2.10 ± 0.2	2.20 ± 0.1
7b	>10	>10	>10
7c	>10	>10	>10
7d	n.t.	n.t.	n.t.
7e	>10	>10	>10
7f	0.11 ± 0.1	0.18 ± 0.1	0.10 ± 0.0
7g	8.40 ± 0.4	4.90 ± 0.3	5.20 ± 0.3
7h	0.39 ± 0.1	0.66 ± 0.2	1.76 ± 0.2
7i	>10	>10	>10
7j	4.4 ± 0.3	>10	>10
7k	>10	>10	>10
8a	5.1 ± 0.4	>10	>10
8c	>10	>10	>10
8d	0.80 ± 0.1	1.40 ± 0.0	3.10 ± 0.1
8e	1.70 ± 0.2	2.30 ± 0.3	1.90 ± 0.2
Adri	0.16 ± 0.1	0.18 ± 0.2	0.14 ± 0.1

Cytotoxic analyses were made in 96-well microtiter plates using the SRB assay. Cell lines used: breast MCF-7, lung H-460, and central nervous system SF-268; n.t.: not tested

the R substituent [compare activities of **8d** (R = 4-OCH_3) and **8e** (R = 4-Br) with those of **8a** (R = 4-Cl)].

Interesting enough, results of the antifungal assays showed that all compounds of the series were devoid of antifungal properties against *Candida, Cryptococcus, Saccharomyces*, and *Aspergillus* spp (MIC = 250 μg/mL). Regarding the activity shown by all compounds against dermatophytes, only compound **8d** showed MICs = 31.2 μg/mL when faced to *M. gypseum*, *T. mentagrophytes*, and *T. rubrum*. This concentration is ten to forty times higher than that at which it showed cytotoxic activity. Particularly, it is noteworthy that the most cytotoxic compound **7d** is completely devoid of antifungal properties up to 250 μg/mL. The comparison of the antitumor and antifungal activities of the 2-thienyl and 3-thienyl acrylonitriles presented here, clearly suggests that they could be interesting compounds for further development as antitumor agents since many of them show interesting cytotoxic activities against three tumor cell lines and do not show any activity against an ample panel of opportunistic and pathogenic fungi.

Conclusions

We report here an efficient synthetic route to prepare thienylacrylonitrile derivatives by using both, potassium *tert*-butoxide and absolute ethanol in the reaction. These conditions allowed us to obtain the compounds in short times and acceptable yields. The cytotoxic evaluation of 2- and 3-thienylcrylonitrile derivatives **7** and **8** revealed that compounds **7** (particularly **7f**) exhibited strong activity against three cell tumor lines and some structure-activity relationships could be extracted from their cytotoxic behavior. Interesting enough, active compounds did not show the capacity of inhibiting a panel of 10 fungal strains up to 250 μg/mL, suggesting that the cytotoxic activity could be selective. These results add valuable information regarding the structural features of cytotoxic acrylonitrile derivatives and could open a new avenue for the development of structurally new antitumor agents.

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Experimental

Chemistry

Melting points (Mp) were taken in open capillaries on Thomas Hoover melting point apparatus (Thomas Hoover Capillary Apparatus , Philadelphia, PA, USA) and are uncorrected. The ^{1}H- and ^{13}C-NMR spectra were run on a Bruker DPX 400 spectrometer operating at 400 MHz and 100 MHz respectively, using dimethyl sulfoxide-d6 as solvent and tetramethylsilane as internal standard (Bruker BioSpin GmbH, Rheinstetten, Germany). The mass-spectra were scanned on a Hewlett Packard HP Engine-5989 spectrometer (equipped with a direct inlet probe) operating at 70 eV (Hewlett Packard, Palo Alto, CA, USA). High Resolution Mass Spectra (HRMS) were recorded in a Waters Micromass AutoSpec NT spectrometer (Waters, Manchester, UK) (STIUJA, Servicios Técnicos de Investigación de la Universidad de Jaén). The elemental analyses have been obtained using a LECO CHNS-900 (Leco, St. Joseph, MI, USA) and a Thermo Finnigan FlashEA1112 CHNS-O (Hemel, Hempstead, UK) (STIUJA) elemental analyzers.

*General procedure for the synthesis of 2-(2-thienyl) and 2-(3-thienyl)-3-arylacrylonitriles **7a–k** and **8a–e***

A solution of 2-thienylacetonitrile **4** (1 mmol) or 3-thienylacetonitrile **5** (1 mmol) and potassium *tert*-butoxide (0.2 mmol) in anhydrous ethanol (3 mL) was stirred at room temperature for 15 min; a solution of benzaldehyde **6** (1 mmol) in anhydrous ethanol was then added. The resulting precipitate was collected by filtration, washed with ethanol, dried; and recrystallized from ethanol.

*E-3-(4-Chlorophenyl)-2-(2-thienyl)acrylonitrile **7a***

This compound was obtained according to general procedure as yellow crystal. Mp. 97 – 98°C; Yield 76%. MS EI (30 eV) *m/z* (%): 248 (6), 247/245 (38/100) [M^+], 218 (7), 212 (8), 211 (17), 210 (74) [M^+-Cl], 209 (61), 183 (11), 177 (22), 166 (12), 139 (13), 91(14), 45 (15). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.08 (dd, 1H, H-4′), 7.31 (s, 1H, =CH), 7.32 (dd, 1H, H-5′), 7.39 (dd, 1H, H-3′) 7.42, (d, 2H, H_m), 7.70 (d, 2H,

H_o); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 106.7 (C-2′), 116.6 (CN), 126.6 (C-5′), 127.6 (C-3′), 128.2 (C-4′), 129.3 (C_o), 130.3 (C_m), 131.8 (C_p), 136.4 (C_i), 137.9 (=CH), 138.9 (C-2). Anal. Calcd. for $C_{13}H_8ClNS$ 6½ H_2O: C, 61.30; H, 3.56; N, 5.50. Found: C, 61.37; H, 3.51; N, 5.41.

Z-3-(4-Chlorophenyl)-2-(3-thienyl)acrylonitrile ***8a***

This compound was obtained according to general procedure as white solid. Mp. 107–108°C: Yield 50%. MS EI (30 eV) *m/z* (%): 247/245 (24/64) [M^+], 211 (17), 210 (100) [M^+-Cl], 209 (48), 177 (8), 166 (13). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.36 (dd, 1H, H-5′), 7.38–7.46 (m, 4H, =CH, H-4′, H_m), 7.61, (dd, 1H, H-2′), 7.78 (d, 2H, H_o); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 107.4 (C-1′), 117.6 (CN), 121.6 (C-2′), 124.0 (C-3′), 124.1 (C-4′), 129.3 (C_m), 130.3 (C_o), 132.1 (C_p), 136.3 (C_i), 138.8 (=CH). Anal. Calcd. for $C_{13}H_8ClNS$ 6½ H_2O: C, 61.30; H, 3.56; N, 5.50. Found: C, 61.75; H, 3.55; N, 5.37.

Compounds **7b–k** and **8b–e** were prepared by adopting the same procedure.

Cytotoxicity assays

The cytotoxic activity was determined according to the method of Monks *et al*. [12]. Briefly, cells breast (MCF-7), lung (H-460); and central nervous system (SF-268), (obtained from U.S. National Cancer Institute) were counted, diluted with fresh medium, and added to 96-well microtiter plates (100 μL/well) containing test materials (1 mg in 100 μL in DMSO). Test plates were incubated for 2 days at 37°C in a 5% CO_2 incubator. All treatments were performed in duplicate. After the incubation periods, cells were fixed by addition of 50 μL of cold 50% aqueous TCA solution (4°C for 60 min.), washed 4-5 times with tap water and air-dried. The fixed cells were stained with 100 μL sulforhodamine B (SRB) (0.4% wt/vol. in 1% acetic acid) for 15 min. Free SRB solution was then removed by rinsing with 1% acetic acid (×5). The plates were then air-dried, the bound dye was solubilized with 100 μL of 10 mM tris-base, and absorbance was determined at 515 nm using an ELISA plate reader (Model ELX-800, Bio-Tek Instruments, Inc., Winooski, VT, USA). Finally, the absorbance values obtained with each of the treatment procedures were averaged, and the averaged value obtained with the zero-day control was subtracted measuring in this way the relative cell growth or inviability in treated and untreated cells. From the curves, growth inhibition (or growth stimulation) and 50% inhibition of growth (GI_{50}) was calculated. Adriamycin was used as the reference compound.

Antifungal assays

Microorganisms and Media

For the antifungal evaluation, strains from the American Type Culture Collection (ATCC), (Rockville, MD, USA) and CEREMIC (C), (Centro de Referencia en Micología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Suipacha 531-(2000)-Rosario, Argentina) were used: *Candida albicans* ATCC10231, *C. tropicalis* C131, *Saccharomyces cerevisiae* ATCC9763, *Cryptococcus neoformans* ATCC32264, *Aspergillus flavus* ATCC9170, *A. fumigatus* ATTC26934, *A. niger* ATCC9029, *Trichophyton rubrum* C110, *T. mentagrophytes* ATCC9972, and *Microsporum gypseum* C115.

Antifungal susceptibility testing

The minimal inhibitory concentration (MIC) of each extract was determined by using broth microdilution techniques following the guidelines of CLSI (formerly NCCLS) for yeasts and filamentous fungi (M27-A2 and M38-A) [13]. MIC values were determined in RPMI-1640 (Sigma, St Louis, MO, USA) buffered to a pH 7.0 with MOPS (Sigma). The starting inocula, prepared according to reported procedures [13] were 1×10^4 to 5×10^4 CFU/mL. Microtiters trays were incubated at 35°C for yeasts and hialophyphomycetes and at 28–30°C for dermatophyte strains in a moist, dark chamber. MIC values were recorded at 48 h for yeasts, and at a time according to the control fungus growth, for the rest of the fungi. The susceptibilities of the standard drugs amphotericin B (Sigma), ketoconazole (Janssen Pharmaceutica, Beerse, Belgium) and terbinafine (Novartis, Buenos Aires, Argentina) were defined as the lowest concentration of drug which resulted in total inhibition of fungal growth.

For the assay, compound stock solutions were two-fold diluted with RPMI from 250–0.98 μg/mL (final volume = 100 μL) and a final DMSO concentration =1%. A volume of 100 μL of inoculum suspension was added to each well with the exception of the sterility control where sterile water was added to the well instead. The MIC was defined as the minimum inhibitory concentration of the compounds which resulted in total inhibition of the fungal growth.

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