

Preliminary antifungal and cytotoxic evaluation of synthetic cycloalkyl[*b*]thiophene derivatives with PLS-DA analysis

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A series of 2-[(arylidene)amino]-cycloalkyl[*b*]thiophene-3-carbonitriles (**2a-x**) was synthesized by incorporation of substituted aromatic aldehydes in Gewald adducts (**1a-c**). The title compounds were screened for their antifungal activity against *Candida krusei* and *Cryptococcus neoformans* and for their antiproliferative activity against a panel of 3 human cancer cell lines (HT29, NCI H-292 and HEP). For antiproliferative activity, the partial least squares (PLS) methodology was applied. Some of the prepared compounds exhibited promising antifungal and proliferative properties. The most active compounds for antifungal activity were cyclohexyl[*b*]thiophene derivatives, and for antiproliferative activity cycloheptyl[*b*]thiophene derivatives, especially 2-[(1*H*-indol-2-yl-methylidene)amino]-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carbonitrile (**2r**), which inhibited more than 97 % growth of the three cell lines. The PLS discriminant analysis (PLS-DA) applied generated good exploratory and predictive results and showed that the descriptors having shape characteristics were strongly correlated with the biological data.

Keywords: cycloalkyl[*b*]thiophene derivatives, antifungal activity, antiproliferative activity, PLS-DA, Pentacle program

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2-Aminothiophene derivatives are an important class of heterocycles found in several biologically active and natural compounds. This class of compounds has demonstrated a broad spectrum of activities and applications as pharmaceuticals and agrochemicals, dyes, biodiagnostics, and electronic and optoelectronic devices (1).

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Substituted 2-aminothiophenes are a class of heterocycles that have attracted a great deal of research interest due their great usefulness as precursors of molecules with pharmacological properties. They have been reported to exert antitubercular (2), anti-inflammatory (3), antimicrobial (4) and antianxiety (5) properties. A survey of the literature also reveals that substituted 2-aminothiophenes are potent and selective inhibitors of human leukocyte elastase (6), kinesin spindle protein (KPS) (7) and adenosine A1 receptor allosteric enhancers (8). Antifungal (9) and antitumor (10) properties have also been extensively described, resulting in marketed antifungal agents such as sertaconazole.

The above prompted us to synthesize a new series of 2-[(arylidene)amino]-cycloalkyl[*b*]thiophene-3-carbonitrile derivatives by incorporating different substituted aromatic aldehydes at the 2-amino position of the thiophene ring, thereby affording Schiff bases. These were screened for their *in vitro* antifungal and antiproliferative activities. Partial Least Squares discriminant analysis (PLS-DA) was applied, using Pentacle, to the antiproliferative activity of cycloalkyl[*b*]thiophene derivatives.

EXPERIMENTAL

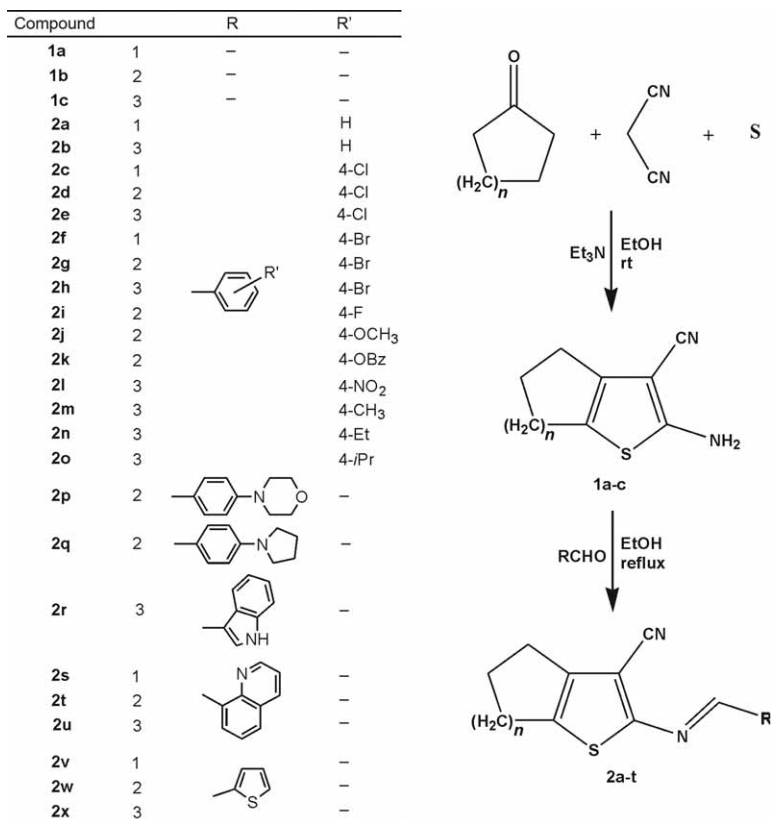
All melting points were measured on a Quimis-340.27 apparatus (Quimis, Brazil) and are uncorrected. IR spectra were recorded using potassium bromide pellets on a Bruker IFS-66 IR spectrophotometer (Bruker, USA). NMR were recorded on a Unity Plus-300 MHz-Varian spectrometer (Varian, USA) using tetramethylsilane as internal standard. Chemical shifts are reported in ppm (δ), and coupling constants (*J*) are reported in Hz. HRMS were recorded on a Delsi-Nermag R1010C mass spectrometer (Delsi-Nermag, France) with 70 eV electron impact. Elemental analyses were performed using an EA 1110 CHNS-O elemental analyzer (CE instruments, UK). The results were found to be in accord ($\pm 0.4\%$) with the calculated values. All reactions were monitored by TLC on 0.25 mm silica gel plates (60F₂₅₄, Merck, Germany) using binary mixtures of hexane/ethyl acetate in different proportions (from 9:1 to 1:1, *V/V*). Spectral, physical and analytical data of all newly synthesized compounds are listed in Tables I–III.

General synthesis procedure

2-[(Arylidene)amino]-cycloalkyl[*b*]thiophene-3-carbonitriles (**2a-x**). – An equimolar mixture of **1a-c** and substituted aromatic aldehyde in ethanol with 0.5 mL of acetic acid was stirred under reflux for 2 h and then cooled to room temperature. Water was added and the solid that precipitated out was filtered under vacuum, washed with water, dried and recrystallized from absolute ethanol (Scheme 1).

Antifungal activity

The *in vitro* antifungal activity of synthesized compounds **2a-o**, **2r-x** was investigated for two species of pathogenic fungi: *Candida krusei* LM08 and *Cryptococcus neoformans* ICB59. These strains were supplied by the URM Culture Collection of the Department of Mycology, Department of Pharmaceutical Sciences of the Federal University of Paraíba, Brazil. Minimum inhibitory concentration (MIC) values were determined by the micro-



Scheme 1.

dilution broth method using microdilution plates according to the guidelines of the National Committee for Clinical and Laboratory Standards (NCCLS). The MIC for all strains was set at the lowest concentration of the antifungal agent that completely inhibited the growth of the organism, as detected by the naked eye when compared to the control group.

All the strains were stored in mineral oil at 18 °C. The viability test and subsequent taxonomic confirmation were carried out according to Barnett *et al.* (12). In order to obtain an inoculum of 2.5×10^3 cells mL⁻¹, each strain was cultured in a tube containing 20 mL of Sabouraud dextrose agar plus yeast extract at 35 °C for two days. After this time, suspensions were prepared in a sterile physiological solution (0.85 %) and mixed in a shaker. The inoculum was adjusted to 90 % transmittance at 530 nm, as measured on a spectrophotometer. Stock solutions of tested compounds were freshly prepared in dimethyl sulfoxide (DMSO), which had no effect on the organism at the concentration studied, aliquoted, and stored at –20 °C in stock solution at a final concentration of 1024 µg mL⁻¹. Decimal dilutions of thiophene stock solutions were prepared in free RPMI 1640 cell cul-

ture medium (Sigma, USA) and buffered to pH 7.0 with 0.165 mol L⁻¹ of morpholinopropanesulphonic acid (MOPS, Sigma, USA). Miconazole and 5-fluorocytosine were used as reference drugs for anti-*Candida* and anti-*Cryptococcus* activity, respectively, and were tested under similar conditions. Microdilution plates containing serial dilutions (from 1024 to 1 µg mL⁻¹) of each compound were inoculated with each organism. Each plate included a positive control (fungi without any compound), negative control (medium only) and reference drugs. The microdilution plates were incubated at 35 °C and were read visually after 24 and 72 h of incubation. All tests were performed in duplicate and the results were expressed as the arithmetic mean of MIC values obtained in the two trials (13). The bands established for antifungal activity were: excellent activity (50 to 500 µg mL⁻¹); moderate activity (600 to 1000 µg mL⁻¹); inactive or poor activity (1000 µg mL⁻¹) (14).

Antiproliferative activity

All synthesized compounds were selected by the National Cancer Institute (NCI) Developmental Therapeutic Program for *in vitro* cell line screening. Antiproliferative assays were performed according to the US NCI protocol as described elsewhere (15). The compounds were evaluated in one primary dose (25 µL mL⁻¹) for three human cancer cell lines: colon carcinoma (HT29), lung cancer (NCI H-292) and laryngeal carcinoma (HEP) cells. The cell lines were supplied by the Rio de Janeiro Cell Bank of the Federal University of Rio de Janeiro (UFRJ), Brazil.

The cytotoxicity/survival of cells in the presence or absence of the experimental agent was determined using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) method, as described previously (16). Cells harvested in the log phase of growth were counted and seeded in triplicate (10⁵ cells per 100 µL per well) in 96-well microculture plates in a complete free Dulbecco's modified eagle's medium (DMEM). After 24 h of incubation at 37 °C and 5 % CO₂, the cultures were treated with the tested compounds at a single concentration of 25 µg mL⁻¹ in DMSO (100 µL per well). Each plate also included a positive control (cells without any compound) and a negative control (medium only). After 72 h of exposure to the compounds, 25 µL of MTT was added to each well. After 4 h at 37 °C, the reaction was stopped and the formazan crystals formed by MTT metabolism were solubilized by adding 50 µL of DMSO to each well. The cellular metabolism of MTT was quantified by reading the absorbance of the solubilized product at 595 nm with a 96-well plate reader attached to a spectrophotometer. Results for each tested compound were reported as the percentage growth of the treated cells compared to the untreated control cells. The mean ± SD of three independent experiments for each compound was calculated. The compounds were classified as possessing no activity (0 % growth inhibition), low activity (up to 30.0 % growth inhibition), moderate activity (between 31.0 % and 70.0 % growth inhibition) and high activity (> 70.0 % growth inhibition) for each cell line tested.

Computational methods

All compounds were drawn (2D structures) using ChemDraw 8.0. They were imported by the Spartan program for Windows 8.0 and were converted into 3D models.

These models were minimized using the MM+ method (17) and atomic partial charges were assigned using the AM1 semiempirical method (18). These methods employed 1000 interactions, 100 cycles of optimization, and 10 conformers of lowest minimum energy. The selected dihedrals were evaluated by rotation in accordance with the standard (default) conditions of the program, in which the number of simultaneous variations was 1 to 8. Acyclic chains were submitted to rotations from 60 to 180° and torsion rings were in the range of 30 to 120°. Conformational search was applied and the E_{MIN} conformer was selected and saved in sdf format (19).

The compounds were imported using the Pentacle program and the PLS methodology was applied. The Pentacle software, produced by Molecular Discovery (Italy), is a computational tool for computing alignment-free molecular descriptors, also called GRid-INDependent descriptors or GRIND (20). The software is based on Molecular Interaction Fields and describes the ability of the molecules to interact with other molecules and does not require superimposition of compounds. The Pentacle program uses the GRID force field to characterize potential polar and hydrophobic interaction sites around target molecules by water (H₂O), hydrophobic (DRY) and carbonyl oxygen (O) and amide nitrogen (N1) probe.

We calculated a binary free PLS-DA for the three cell lines investigated: HT29, NCI H-292 and HEP. The PLS-DA is a partial least squares regression of a set (*Y*) of binary variables describing the categories of a category variable on a set (*X*) of predictor variables. PLS-DA looks for components correlated with the unfolded class while describing a large number of variations in *X*, *i.e.*, directions able to discriminate between the classes. It is a compromise between the usual discriminant analysis and a discriminant analysis of the significant principal components of predictor variables. For the construction of binary PLS matrices the, compounds which showed cytotoxicity greater than 20 % were considered active = 1, and the compounds that did not reach 20 % inactive = 0.

RESULTS AND DISCUSSION

Chemistry

2-Amino-cycloalkyl[*b*]thiophene-3-carbonitriles (**1a-c**) were first synthesized by the reaction of malononitrile with a cyclic ketone and elemental sulphur in the presence of morpholine following the Gewald procedure (9, 10). Treatment of **1a-c** with substituted aromatic aldehydes afforded 2-[(arylidene)amino]-cycloalkyl[*b*]thiophene-3-carbonitrile (**2a-x**). The Schiff bases were prepared in satisfactory yield (except **2q**). Compounds **2a-g** and **2j** were also previously described in the literature (9).

Chemical structures of all newly synthesized compounds (**2h**, **2i** and **2k-x**) were characterized on the basis of their physical, analytical and spectral data, and were in full agreement with the proposed structures (Tables I–III).

The main vibrational bands of the title compounds are given in Table III. IR spectra showed absorption bands at about 2960–2911 and 2223–2212 cm⁻¹, characteristic of C–H and C≡N stretching vibrations, respectively.

¹H NMR and ¹³C NMR data of the compounds obtained are given in Table II and are consistent with the proposed structures. ¹H NMR spectra did not display signs of 2-

Table I. Physical and analytical data of **2h**, *i*, *k-x*

Compd.	Molecular formula (M_r)	M. p. (°C)	Yield (%)	Analysis (calcd./found) (%)		
				C	N	H
2h	C ₁₇ H ₁₅ BrN ₂ S	144	87	56.83/56.83	7.80/7.77	4.21/4.23
2i	C ₁₆ H ₁₃ FN ₂ S	93–95	65	67.58/67.55	9.85/9.85	4.61/4.62
2k	C ₂₃ H ₂₀ N ₂ OS	140–142	92	74.16/74.14	7.52/7.50	5.41/5.43
2l	C ₁₇ H ₁₅ N ₃ O ₂ S	126	90	62.75/62.71	12.91/12.88	4.65/4.66
2m	C ₁₈ H ₁₈ N ₂ S	88–90	92	73.43/73.44	9.51/9.48	6.16/6.19
2n	C ₁₉ H ₂₀ N ₂ S	94–95	84	73.99/73.98	9.08/9.07	6.54/6.54
2o	C ₂₀ H ₂₂ N ₂ S	93–95	70	74.49/74.48	8.69/8.65	6.88/6.91
2p	C ₂₀ H ₂₁ N ₃ OS	159–160	74	68.35/68.34	11.96/11.95	6.02/6.03
2q	C ₂₀ H ₂₁ N ₃ S	211	18	71.61/71.61	12.53/12.51	6.31/6.34
2r	C ₁₉ H ₁₇ N ₃ S	180–182	90	71.44/71.40	13.15/13.12	5.36/5.40
2s	C ₁₈ H ₁₃ N ₃ S	200–202	67	71.26/71.25	13.85/13.85	4.32/4.33
2t	C ₁₉ H ₁₅ N ₃ S	175–177	53	71.90/71.87	13.24/13.21	4.76/4.80
2u	C ₂₀ H ₁₇ N ₃ S	204–206	79	72.48/72.44	12.68/12.67	5.17/5.19
2v	C ₁₃ H ₁₀ N ₂ S ₂	174–175	81	60.43/60.41	10.84/10.80	3.90/3.92
2w	C ₁₄ H ₁₂ N ₂ S ₂	123–125	88	61.73/61.70	10.28/10.25	4.44/4.46
2x	C ₁₅ H ₁₄ N ₂ S ₂	115–117	76	62.90/62.89	9.78/9.77	4.93/4.95

-aminothiophene protons (NH₂), but rather CH=N protons, highlighting the success of the final synthetic step in obtaining the Schiff bases. Signals of the CH=N protons were observed as a singlet in the 8.02–8.95 ppm region, almost always as the stronger deshielding signal.

The strongest shielding signals and peaks in ¹H NMR and ¹³C NMR were attributed to cycloalkyl protons and carbons. These appeared at 1.62–2.99 ppm in ¹H NMR, and at 21.8–32.0 ppm in ¹³C NMR. Further support was obtained from the ¹³C NMR spectra, which exhibited resonance at 114.0–115.9 ppm attributed to the C≡N group.

The HRMS of all synthesized compounds exhibited (M+H)⁺ molecular ion peaks, which is in agreement with the molecular formulas. For compounds **2v** and **2x**, the molecular ion peaks found were [M+Na]⁺.

Antifungal activity

All synthesized compounds (except **2p** and **2q**) were evaluated for their *in vitro* antifungal activity against *Cryptococcus neoformans* and *Candida krusei*. The data presented in Table IV show that the tested compounds more efficiently inhibited the growth of *C. neoformans* than the *C. krusei* strain. The MIC values ranged from excellent (50–500 μg mL⁻¹) to moderate (600–1500 μg mL⁻¹) for half of the tested compounds, although none exhibited greater antifungal activity than the reference drugs 5-fluorocytosine and miconazole.

Table II. NMR data for 2*h*, *i*, *k*-*x*

Compd.	¹ H NMR (δ ppm) ^a	¹³ C NMR (δ ppm) ^a
2 <i>h</i>	1.64–1.74 (m, 4H, CH ₂), 1.84–1.88 (m, 2H, CH ₂), 2.76–2.81 (m, 4H, CH ₂), 7.58 (d, 2H, <i>J</i> = 8.7 Hz, Ar-H), 7.78 (d, 2H, <i>J</i> = 8.7 Hz, Ar-H), 8.34 (s, 1H, CH=N)	157.1, 140.3, 136.7, 133.9, 132.1, 130.5, 126.8, 114.9, 109.7, 31.9, 30.6, 29.1, 27.7, 27.0
2 <i>i</i>	1.84–1.90 (m, 4H, CH ₂), 2.63–2.70 (m, 4H, CH ₂), 7.12 (d, 2H, <i>J</i> = 8.7 Hz, Ar-H), 7.91 (d, 2H, <i>J</i> = 8.7 Hz, Ar-H), 8.36 (s, 1H, CH=N)	166.8, 163.5, 159.4, 157.3, 135.1, 132.5, 131.5, 131.4, 116.2, 115.9, 114.4, 107.0, 25.1, 24.2, 23.0, 21.9
2 <i>k</i>	1.73–1.89 (m, 4H, CH ₂), 2.62–2.69 (m, 4H, CH ₂), 5.12 (s, 2H, OCH ₂), 7.03 (d, 2H, <i>J</i> = 8.7 Hz, Ar-H), 7.34–7.46 (m, 5H, Ar-H), 7.87 (d, 2H, <i>J</i> = 8.7 Hz, Ar-H), 8.32 (s, 1H, CH=N)	162.1, 160.4, 158.2, 136.0, 134.7, 131.5, 131.3, 128.6, 128.1, 128.1, 127.4, 115.1, 114.6, 105.8, 70.0, 25.0, 24.2, 23.0, 21.9
2 <i>l</i>	1.66–1.75 (m, 4H, CH ₂), 1.85–1.89 (m, 2H, CH ₂), 2.79–2.82 (m, 4H, CH ₂), 8.06 (d, 2H, <i>J</i> = 8.7 Hz, Ar-H), 8.28 (d, 2H, <i>J</i> = 8.7 Hz, Ar-H), 8.43 (s, 1H, CH=N)	155.8, 155.0, 149.3, 140.9, 140.4, 138.6, 129.6, 123.9, 114.5, 111.4, 31.8, 30.7, 29.0, 27.6, 26.9
2 <i>m</i>	1.69 (m, 4H, CH ₂), 1.86 (m, 2H, CH ₂), 2.41 (s, 3H, CH ₃), 2.78 (m, 4H, CH ₂), 7.25 (d, 2H, <i>J</i> = 8.1 Hz, Ar-H), 7.81 (d, 2H, <i>J</i> = 8.1 Hz, Ar-H), 8.38 (s, 1H, CH=N)	158.8, 158.1, 143.0, 139.9, 135.7, 132.5, 129.5, 129.3, 115.0, 108.7, 31.9, 30.5, 29.1, 27.7, 27.1, 21.7
2 <i>n</i>	1.26 (t, 3H, <i>J</i> = 7.5 Hz, CH ₃), 1.64–1.74 (m, 4H, CH ₂), 1.84–1.88 (m, 2H, CH ₂), 2.73 (q, 2H, <i>J</i> = 7.5 Hz, CH ₂), 2.75–2.81 (m, 4H, CH ₂), 7.28 (d, 2H, <i>J</i> = 8.1 Hz, Ar-H), 7.84 (d, 2H, <i>J</i> = 8.1 Hz, Ar-H), 8.39 (s, 1H, CH=N)	158.8, 158.1, 149.3, 139.9, 135.7, 132.7, 129.5, 128.4, 115.0, 108.7, 31.9, 30.5, 29.1, 29.0, 27.8, 27.1, 15.2
2 <i>o</i>	1.72 (d, 6H, <i>J</i> = 6.9 Hz, CH ₃), 1.63–1.73 (m, 4H, CH ₂), 1.83–1.91 (m, 2H, CH ₂), 2.75–2.81 (m, 4H, CH ₂), 2.96 (d, 1H, <i>J</i> = 6.9 Hz, CH), 7.31 (d, 2H, <i>J</i> = 8.1 Hz, Ar-H), 7.84 (d, 2H, <i>J</i> = 8.1 Hz, Ar-H), 8.38 (s, 1H, CH=N)	158.7, 158.1, 153.8, 139.9, 135.6, 132.8, 129.4, 126.9, 115.0, 108.7, 34.2, 31.9, 30.5, 29.0, 27.7, 27.0, 23.6
2 <i>p</i>	1.80–1.88 (m, 4H, CH ₂), 2.61–2.67 (m, 4H, CH ₂), 3.30 (t, 4H, <i>J</i> = 4.8 Hz, NCH ₂), 3.84 (t, 4H, <i>J</i> = 4.8 Hz, OCH ₂), 6.88 (d, 2H, <i>J</i> = 9 Hz, Ar-H), 7.80 (d, 2H, <i>J</i> = 9 Hz, Ar-H), 8.26 (s, 1H, CH=N)	161.1, 158.3, 153.7, 134.5, 131.1, 130.8, 125.6, 114.9, 113.9, 105.0, 66.4, 47.4, 25.0, 24.2, 23.0, 21.9
2 <i>q</i>	1.85 (m, 4H, CH ₂), 2.07 (m, 4H, CH ₂), 2.66 (m, 4H, CH ₂), 3.43 (m, 4H, CH ₂), 6.62 (d, 2H, <i>J</i> = 8.4 Hz, Ar-H), 7.94 (d, 2H, <i>J</i> = 8.4 Hz, Ar-H), 8.30 (s, 1H, CH=N)	198.3, 190.2, 158.3, 151.6, 134.5, 133.1, 115.0, 112.3, 111.2, 102.8, 48.0, 47.6, 25.4, 25.3, 24.9, 24.3, 23.1, 22.0
2 <i>r</i>	1.65–1.72 (m, 4H, CH ₂), 1.84–1.88 (m, 2H, CH ₂), 2.75–2.82 (m, 4H, CH ₂), 7.29–7.42 (m, 4H, Ar-H), 7.67 (d, 1H, <i>J</i> = 3 Hz, Ar-H), 8.60 (s, 1H, CH=N)	160.5, 153.4, 139.3, 136.9, 133.5, 132.1, 124.8, 124.2, 123.0, 122.7, 116.0, 115.7, 111.3, 106.7, 32.0, 30.5, 29.1, 27.9, 27.2

Table II. continued

2s	2.48 (q, 2H, $J = 7.6$ Hz, CH ₂), 2.91 (t, 2H, $J = 7.6$ Hz, CH ₂), 2.99 (t, 2H, $J = 7.6$ Hz, CH ₂), 7.79 (t, 1H, $J = 7.6$ Hz, Ar-H), 7.87 (t, 1H, $J = 7.6$ Hz, Ar-H), 8.08 (d, 1H, $J = 4.8$ Hz, Ar-H), 8.40 (d, 1H, $J = 8.4$ Hz, Ar-H), 8.95 (d, 1H, $J = 8.4$ Hz, Ar-H), 8.02 (s, 1H, CH=N), 9.03 (d, 1H, $J = 4.8$ Hz, Ar-H)	163.5, 154.3, 150.0, 149.0, 145.3, 140.1, 137.1, 130.2, 129.7, 128.3, 125.2, 123.9, 122.1, 114.3, 104.7, 30.2, 28.0, 27.3
2t	1.82–1.88 (m, 4H, CH ₂), 2.65–2.74 (m, 4H, CH ₂), 7.91 (d, 2H, $J = 6$ Hz, Ar-H), 8.90 (t, 2H, $J = 11.2$ Hz, Ar-H), 8.95 (s, 1H, CH=N), 9.02 (d, 2H, $J = 6$ Hz, Ar-H)	158.4, 155.5, 150.0, 149.0, 137.1, 135.8, 134.8, 130.2, 129.7, 128.2, 125.2, 123.9, 122.1, 114.0, 109.2, 25.3, 24.2, 22.9, 21.8
2u	1.69–1.72 (m, 4H, CH ₂), 1.88 (m, 2H, CH ₂), 2.79–2.82 (m, 4H, CH ₂), 7.66–7.79 (m, 2H, Ar-H), 7.84 (d, 1H, $J = 8.4$ Hz, Ar-H), 8.17 (d, 1H, $J = 4.5$ Hz, Ar-H), 8.90 (d, 1H, $J = 8.4$ Hz, Ar-H), 8.93 (s, 1H, CH=N), 9.00 (d, 1H, $J = 4.5$ Hz, Ar-H)	156.3, 155.1, 149.8, 148.8, 140.8, 138.7, 137.2, 130.0, 129.7, 128.2, 125.2, 123.9, 122.0, 114.5, 111.4, 31.8, 30.7, 29.0, 27.6, 26.9
2v	2.36–2.45 (m, 2H, CH ₂), 2.80–2.92 (m, 4H, CH ₂), 6.81 (dd, 1H, $J = 1.5, 3.6$ Hz, Ar-H), 7.11 (d, 1H, $J = 3.6$ Hz, Ar-H), 7.64 (d, 1H, $J = 1.5$ Hz, Ar-H), 8.25 (s, 1H, CH=N)	164.9, 151.2, 146.9, 145.2, 144.8, 137.4, 118.0, 114.7, 112.9, 101.6, 30.1, 28.1, 27.2
2w	1.82–1.91 (m, 4H, CH ₂), 2.63–2.69 (m, 4H, CH ₂), 6.58 (dd, 1H, $J = 1.5, 3.3$ Hz, Ar-H), 7.11 (d, 1H, $J = 3.3$ Hz, Ar-H), 7.65 (d, 1H, $J = 1.5$ Hz, Ar-H), 8.23 (s, 1H, CH=N)	159.7, 151.3, 146.9, 146.3, 135.3, 132.3, 117.8, 114.3, 112.9, 106.1, 25.1, 24.2, 22.9, 21.9
2x	1.62–1.72 (m, 4H, CH ₂), 1.82–1.86 (m, 2H, CH ₂), 2.74–2.79 (m, 4H, CH ₂), 6.57 (dd, 1H, $J = 1.5, 3.6$ Hz, Ar-H), 7.10 (d, 1H, $J = 3.6$ Hz, Ar-H), 7.64 (d, 1H, $J = 1.5$ Hz, Ar-H), 8.23 (s, 1H, CH=N)	157.7, 151.2, 146.8, 146.1, 140.3, 136.0, 117.7, 114.9, 112.8, 108.4, 31.9, 30.4, 29.1, 27.7, 27.0

^a CDCl₃

Compounds **2t** and **2u**, with MIC values of 64 µg mL⁻¹, and compounds **2d**, **2g**, **2j**, **2k**, **2m**, **2o** and **2v** with MIC values of 128 µg mL⁻¹, exhibited excellent antifungal activity against *Cryptococcus neoformans*. Other tested compounds were inactive, or showed moderate activity (**2a**). The most active compounds for anti-*Cryptococcus* activity were **2t** and **2u**, which have in common the presence of a quinoline moiety linked to the 2-amino position of the thiophene ring. This result corroborates the data of Boateng and collaborators (21) for a series of benzothieno[3,2-*b*]quinoline derivatives.

The compounds showed moderate activity against *C. krusei*. Compounds **2i**, **2k** and **2t** were the most active (MIC = 512 µg mL⁻¹), followed by compounds **2a**, **2g**, **2j**, **2r** and **2s** (MIC values at 1024 µg mL⁻¹).

The present study also confirmed that the presence of the cyclohexyl ring linked to the C-4 and C-5 position of the thiophene ring, as found in compounds **2d**, **2g**, **2i-k** and **2t**, increases antifungal activity. The cyclopentyl (**2a**, **2s** and **2v**) and cycloheptyl (**2l-o** and **2r**) derivatives displayed similar antifungal activity profiles and were, generally, two to four times less active than the cyclohexyl derivatives (9).

Table III. HRMS and IR data for **2h**, **i**, **k-x**

Compd.	IR (KBr) (ν_{\max} , cm^{-1})	HRMS m/z (ES+)	
		Calculated	Found
2h	CH (2916)	358.0139	359.0940
	CN (2223)		
2i	CH (2940)	284.0783	285.0903
	CN (2222)		
2k	CH (2938)	372.1296	373.1119
	CN (2218)		
2l	CH (2929)	325.0884	326.0718
	CN (2220)		
2m	CH (2912)	294.1190	295.1067
	CN (2222)		
2n	CH (2928)	308.1347	309.1455
	CN (2216)		
2o	CH (2927)	322.1503	323.1641
	CN (2216)		
2p	CH (2945)	351.1405	352.1217
	CN (2213)		
2q	CH (2928)	335.1456	336.1291
	CN (2212)		
2r	CH (2911)	319.1143	320.1003
	CN (2220)		
2s	CH (2924)	303.0830	304.0767
	CN (2220)		
2t	CH (2937)	317.0986	318.1099
	CN (2217)		
2u	CH (2930)	331.1143	332.1270
	CN (2219)		
2v	CH (2960)	258.0285	281.0148 ^a
	CN (2220)		
2w	CH (2936)	272.0441	273.0399
	CN (2213)		
2x	CH (2929)	286.0598	309.0227 ^a
	CN (2215)		

^a [M+Na]⁺

Antiproliferative activity

Table V summarizes the *in vitro* antiproliferative effects of cycloalkyl[*b*]thiophene derivatives **2a-x** against the three human cancer cell lines. Compounds **2a**, **2c**, **2f-g**, **2k-l**, **2p-q** and **2s-v** exhibited antiproliferative activity below the concentration of 25 $\mu\text{g mL}^{-1}$.

The results indicate that the size of the cycloalkyl ring coupled at the C-4 and C-5 position of the thiophene ring is essential for antiproliferative activity. Increasing the si-

Table IV. Antifungal activity of the synthesized compounds, miconazole and 5-fluorocytosine

Compd. ^a	MIC ($\mu\text{g mL}^{-1}$)	
	<i>C. neoformans</i>	<i>C. krusei</i>
2a	512	1024
2b	–	–
2c	–	–
2d	128	–
2e	–	–
2f	–	–
2g	128	1024
2h	–	–
2i	–	512
2j	128	1024
2k	128	512
2l	–	512
2m	128	–
2n	–	–
2o	128	–
2r	256	1024
2s	–	1024
2t	64	512
2u	64	–
2v	128	–
2w	–	–
2x	–	–
5-Fluorocytosine	10	NT
Miconazole	NT	50

– No inhibition

NT – Not tested

^a Solvent: DMSO

ze of the cycloalkyl ring helps increase the capacity to inhibit cell growth. Cycloheptyl derivatives **2r**, **2n**, **2o** and **2x** were thus the most active compounds, while all cyclopentyl derivatives were inactive compounds.

The antiproliferative activity of the compounds was greater against the HEP cells compared to the other two cell lines, as can be seen in compounds **2i**, **2n**, **2o** and **2x** with inhibition of 95.4, 57.3, 71.0 and 52.6 %, respectively. The most active cycloalkyl[*b*]thiophene compound identified in this study was **2r**, which inhibited the growth of HT29, NCI H-292 and HEP cancer cell lines by 100.0, 100.0 and 97.5 %, respectively.

Cycloheptyl[*b*]thiophene derivatives also demonstrated that the size of the alkyl group in the *para* position of the benzylidene moiety seemed to be important for anti-proliferative activity. In this context, a comparison of substituent effects revealed that the replacement of *i*-propyl (**2o**) by an ethyl (**2n**) group resulted in a slight reduction in antitumor activity (reduction of 71.0 to 57.3 % for the HEP cell, and of 45.7 to 42.5 % for the NCI H-292 cell), and the replacement of ethyl (**2n**) by a methyl (**2m**) group resulted in a substantial loss of activity (reduction of 57.3 to 22.1 % for the HEP cell, 42.5 to 25.8 % for the NCI H-292 cell and 61.6 to 11.0 % for the HT29 cell).

Another structural feature that can be associated with complete loss of activity in these derivatives is the presence of bulky or large arylidenes linked to the 2-amino position, as can be seen in compounds which have a quinoline moiety (**2s**, **2t** and **2u**), and in compounds **2k**, **2p** and **2q**, which represent 4-benzyloxy-benzylidene, 4-morpholinyl-benzylidene and 4-pyrrolidinyl-benzylidene, respectively.

Computational methods

The goal of PLS regression is to provide a dimension reduction strategy in a situation where we want to relate a set of response variables *Y* to a set of predictor variables *X*. The PLS-DA is a PLS regression where *Y* is a set of binary variables describing the effects of a category variable on *X*; *i.e.*, the number of dependent, or response, variables is equal to the number of categories. The best way to extract information from the PLS-DA is graphically, by plotting the obtained matrices.

Table V. Antiproliferative activity of the synthesized compounds

Compd. ^a	Inhibition (%) ^{b,c}		
	HT29	NCI H-292	HEP
2b	11.1 ± 2.7	30.4 ± 1.1	20.8 ± 6.4
2d	24.9 ± 3.8	24.2 ± 5.5	1.6 ± 1.0
2e	0.0 ± 0.0	29.5 ± 3.4	0.0 ± 3.1
2h	11.3 ± 4.8	23.5 ± 1.2	11.3 ± 0.4
2i	53.7 ± 1.9	50.9 ± 4.0	95.4 ± 0.0
2j	4.9 ± 0.6	18.4 ± 0.2	3.7 ± 4.8
2m	11.0 ± 0.6	25.8 ± 1.1	22.1 ± 0.5
2n	61.6 ± 2.0	42.5 ± 2.7	57.3 ± 5.0
2o	28.9 ± 1.5	45.7 ± 3.3	71.0 ± 3.9
2r	100.0 ± 0.5	100.0 ± 0.0	97.5 ± 2.5
2w	34.7 ± 0.7	33.9 ± 3.8	24.1 ± 2.7
2x	25.0 ± 2.0	27.9 ± 2.7	52.6 ± 2.9

^a Solvent: DMSO

^b 25 µg mL⁻¹

^c Mean ± SD, *n* = 3.

PLS-DA was applied in this study to improve the results obtained by the classic image analysis and to identify the significant spots responsible for the differences in anti-proliferative activity against the three cell lines, which were similar to each other. The models presented the following statistical indices:

- $R^2 \sim 0.80$ – coefficient of determination, the squared multiple correlation coefficient, which is the total variance of the response explained by a regression model;
- SDEP ~ 0.70 – standard deviation error of prediction, also known as standard error in prediction SEP or PSE, which is the function of the predictive residual sum of squares;
- $Q^2 \sim 0.60$ – predictive ability of the model.

A large difference was observed between active (triangle up) and inactive (triangle down) compounds for the three cell lines (Fig. 1). This graph plots X-scores (T) against Y-scores (U). It provides a clear idea of the correlation between the X's and the Y's obtained in the model for each of the LVs. LVs are linear combinations of the original X-variables.

The variables highlighted in the studies with the three cell lines differ only by Angstroms but showed that hydrogen bond acceptor regions were characteristic of the active compounds. The plot weight, obtained in the study with the NCI H-292 cell line, reflects the behavior observed in all investigations (Fig. 2). Active compounds showed greater interactions between the probes DRY-DRY, N1-N1 and DRY-N1.

The most active compound (**2r**) was the only one that exhibited an O-N1 interaction (represented by the variable VAR246) (triangle up). In Fig. 3 this variable corresponds to an interaction between 4.40 and 4.80 Å, and characterizes a hydrogen bond acceptor region.

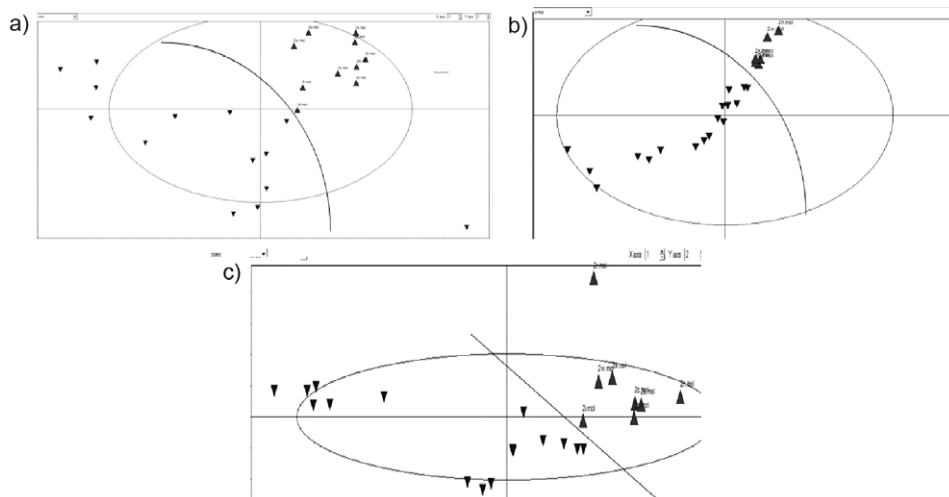


Fig. 1. Plot scores obtained from PLS-DA, distribution and separation of the set by the best model. Active (triangle up) and inactive (triangle down) compounds against the investigated cell lines: a) HT 29 cell line, b) NCI H-292 cell line, and c) HEP cell line.

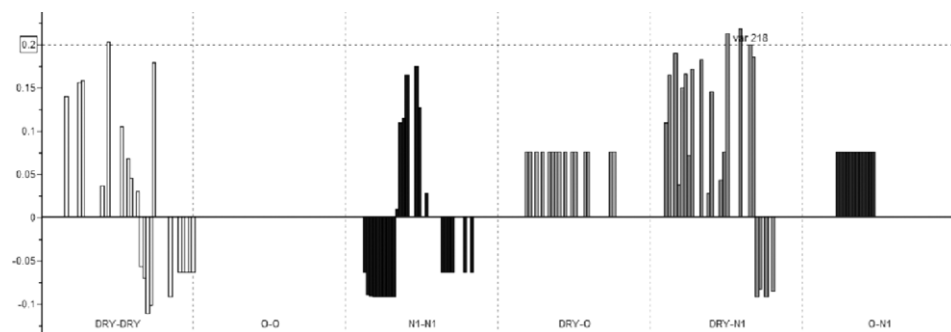


Fig. 2. Plot weight obtained with the NCI H-292 cell line, selecting the active compounds. Greater intensity and number of positive influence of the variables are generated on the interaction of probes DRY-DRY, N1-N1 and DRY-N1 in active compounds.

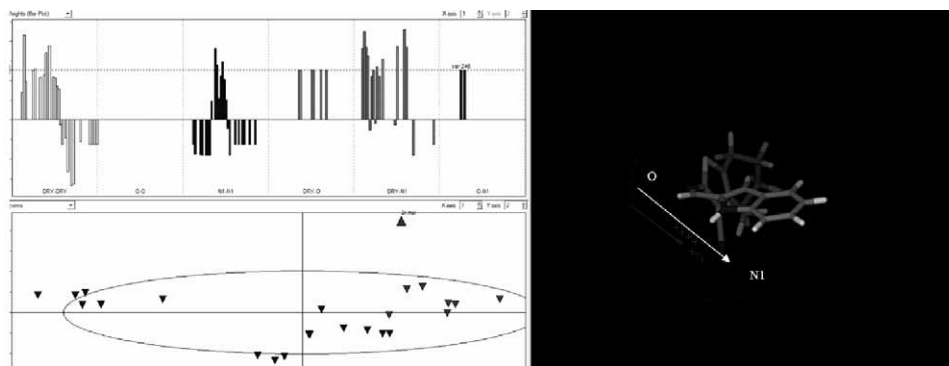


Fig. 3. Disclosure of O-N1 interaction, VAR 246, in the most active compound (**2r**) – (triangle up).

CONCLUSIONS

In short, novel thiophene derivatives substituted with an arylidene moiety were synthesized, characterized on the basis of their physical, analytical, and spectral data, and preliminarily evaluated for their *in vitro* antifungal and antiproliferative properties.

Antifungal studies revealed that the results for the antifungal activity of cycloalkyl[*b*]thiophene derivatives ranged from excellent to moderate and that the most promising compounds were those that had a cyclohexyl ring linked to the thiophene ring (**2d**, **2g**, **2i-k** and **2t**).

The results for antiproliferative activity indicate that the most active compounds are cycloheptyl[*b*]thiophene derivatives, especially 2-[(1*H*-indol-2-yl-methylidene)amino]-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carbonitrile (**2r**).

The chemometric tool (PLS-DA) applied in this study generated good exploratory and predictive results. The behavior of the binary free PLS-DA was similar for the three human cancer cell lines studied. The descriptors having shape characteristics were strongly correlated with the biological data. Higher weight variables highlight the interactions between the DRY-DRY N1-N1, DRY-N1 probes. Hydrogen bond acceptor regions are characteristic of active compounds and the presence of O-N1 interaction, as found in **2r**, increased the antitumor activity.

Finally, it can be concluded that cycloalkyl[*b*]thiophene derivatives could be considered promising compounds for the discovery of new antitumor agents. Further investigation of the mode of action at molecular level needs to be carried out, and more extensive studies are needed to determine additional physicochemical and biological parameters in order to provide a deeper insight into the SAR and to optimize the efficacy and safety of this series of compounds.

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S A Ž E T A K

**Preliminarno ispitivanje antimikotskog i citotoksičnog djelovanja
derivata cikloalkil[*b*]tiofena PLS-DA analizom**

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Koristeći supstituirane aromatske aldehide u Gewaldovim aduktima **1a-c** sintetizirani su derivati 2-[(ariliden)amino]-cikloalkil[*b*]tiofen-3-karbonitrila (**2a-x**). Ispitano je antimikotsko djelovanje tih spojeva na gljivice *Candida krusei* i *Cryptococcus neoformans* te antiproliferativno djelovanje na tri humane tumorske stanične linije (HT29, NCI H-292 i HEP). Za antiproliferativno djelovanje primijenjena je metoda parcijalnih najmanjih kvadrata (PLS) koristeći softverski program Pentacle. Neki od ispitanih spojeva pokazuju obećavajuće antimikotsko i antiproliferativno djelovanje. Najjače antimikotsko djelovanje imaju cikloheksil[*b*]tiofen derivati, a najjače antiproliferativno djelovanje cikloheptil[*b*]tiofen derivati, posebice 2-[(1*H*-indol-2-il-metiliden)amino]-5,6,7,8-tetrahidro-4*H*-ciklohepta[*b*]tiofen-3-karbonitril (**2r**), koji inhibira više od 97 % rast svih triju ispitivanih staničnih linija. Primijenjena PLS diskriminirajuća analiza dala je dobre istraživačke i prognostičke rezultate i pokazala da deskriptori dobro koreliraju s biološkim rezultatima.

Ključne riječi: cikloalkil[*b*]tiofen derivati, antimikotsko djelovanje, antiproliferativno djelovanje, PLS-DA metoda, program Pentacle

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