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Short communication

2-Triazenopyrroles: synthesis and biological activity

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Abstract – 2-Triazenopyrroles were synthesized by coupling the corresponding 2-diazopyrroles with secondary amines and tested for antiproliferative, antifungal, antiviral and antibacterial activities. Derivative **9m** was the most cytotoxic, showing, against leukaemia, lymphoma and carcinoma cell lines, IC_{50} 3.9–21 μM and inhibited Cox-B2 and VSV with EC_{50} 10 μM. Derivative **9j**, instead, was active against *C. albicans* with a selectivity index higher than that of miconazole. © Elsevier, Paris

2-triazenopyrroles / synthesis / cytotoxic activity / antifungal activity

1. Introduction

Dacarbazine, 5-(3,3-dimethyl-1-triazenyl)imidazole-4-carboxamide (**1**, DTIC), is the only triazene derivative used in anticancer chemotherapy and is the most active drug available for treatment of malignant melanomas and Hodgkin tumours resistant to MOPP therapy, a combination of mechlorethamine, oncovine® (vincristine), prednisone and procarbazine [1, 2].

However, DTIC is not the sole dialkyltriazene having antineoplastic activity. In fact, among compounds of type **2** which have been examined extensively in the search for analogues more potent than DTIC [3], 1-(4-carboxyphenyl)-3,3-dimethyltriazene (**2**, R = COOH, R^1 = Me) emerged [4].

A widely accepted mode of action for dimethyltriazenes encompasses the following steps: a) oxidative metabolism, in the liver, of one of the methyl groups at N-3 leading to α-hydroxymethyltriazenes of type **3;** b) loss of the α-hydroxymethyl group as formic aldehyde generates monomethyltriazenes of type **5** which tautomerise to unstable 3-aryl-1-alkyltriazene forms **6** [5]; c) DNA alkylation by the latter [6], as demonstrated by high levels of N^7- and O^6-methylguanine in the DNA of cells from cancer patients treated with dacarbazine [7].

More recently, imidazotetrazinone **4** (temozolomide), has been developed. At present this compound is in phase II clinical trials due to its efficacy in patients affected by malignant melanomas, mycosis fungoides and brain tumours [8–11]. Although **4** itself is not an acyclic triazene, its mode of action has been shown to involve ring opening of the tetrazinone moiety following nucleophilic attack at C-4 by a molecule of water to afford the same monomethyltriazene of type **5** produced by dacarbazine [12–15].

Although aryl and heteroaryl rings can be considered carrier groups for the triazene moiety; and in spite of the fact that structure-activity relationships are not straightforward (in fact, efficacy parallels toxicity so closely that the latter often masks the antineoplastic activity [16]), in the triazenoazole series the activity increases as the rings become more electron-rich, although at the expense of stability. Therefore, triazenopyrroles, which contain the most electron-rich azole ring could be expected to display good antineoplastic activity.

Here we report on the biological activity of triazenopyrroles which were synthesised to verify the above assumption.

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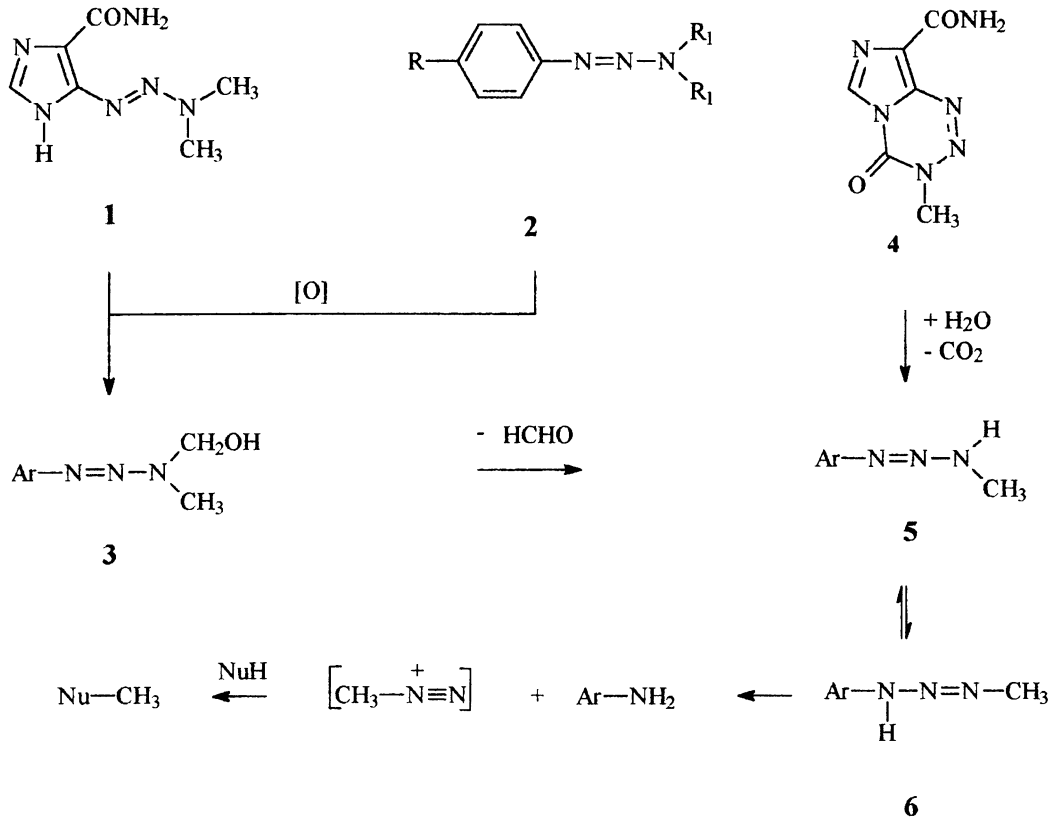


Scheme 1.

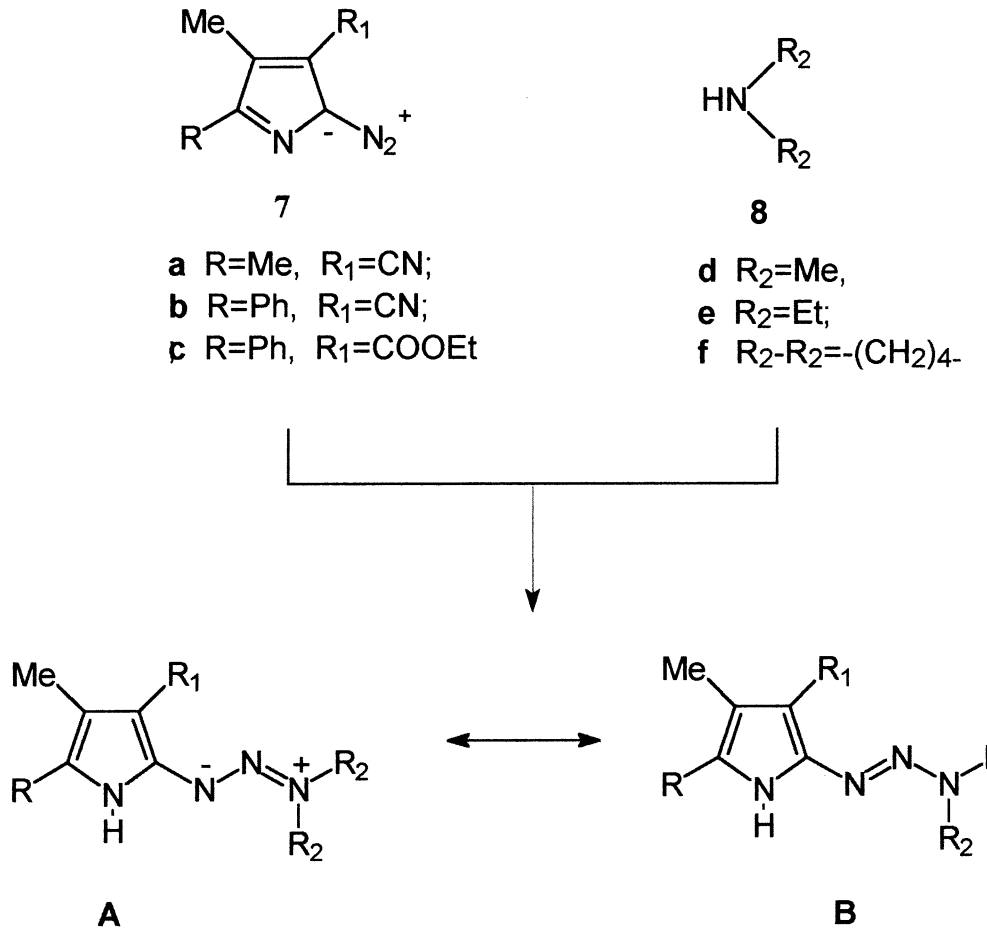
2. Chemistry

Triazenoazoles are prepared by a coupling reaction of diazoazoles and suitable amines. Actually the conjugate acids of the diazoazoles, i.e. the azole diazonium salts which show the typical reactivity of aromatic diazonium salts, are much more reactive than the neutral species. However, in the case of coupling with amines it is impossible to react the diazonium species because, in the presence of the amine, the latter lose the imine proton becoming diazo compounds. It was observed that some diazo forms are not sufficiently electrophilic to undergo coupling reactions even with the most reactive nucleophiles. This reactivity failure was observed in the diazoazoles lacking a ring nitrogen adjacent to the reactive centre, i.e. 3-diazopyrroles, 3-diazoindoles and 4-diazopyrazoles [17].

However it was possible to obtain 3-triazenopyrroles using an inert solvent in which the species were not extensively solvated and the coupling reaction could take place more easily [18].

Approaching the synthesis of 2-triazenopyrroles, difficulties were connected with the availability of 2-diazopyrroles. In fact, in the past, synthesis, of 2-diazopyrroles in preparative yield were unknown and the very few 2-diazopyrroles known were prepared by direct introduction of the diazo group into the pyrrole ring, having the 2-position unsubstituted, in very low yields (2–6%) [19]. Only recently we reported a synthesis of 2-diazopyrroles, in preparative yield, by diazotization of the corresponding 2-aminopyrroles [20].

Such a synthesis was achieved following studies demonstrating that 2- and 3-aminopyrroles behave similarly towards the protonation [21] and that 2-aminopyrroles do react as primary aromatic amines towards nucleophiles [22] and do not behave like enamines as previously reported [23]. Thus, it was assumed that the failure of the 2-aminopyrroles in giving the pyrrole diazonium salts upon diazotization was due to the instability of the final products, rather than to lack of reactivity as primary aromatic amines. In fact under suitable reaction condi-



g R=R_2=Me, R_1=CN; **h** R=Me, R_1=CN, R_2=Et; **i** R=Me, R_1=CN, R_2-R_2=-$(CH_2)_4$-; **j** R=Ph, R_1=CN, R_2=Me; **k** R=Ph, R_1=CN, R_2=Et; **l** R=Ph, R_1=CN, R_2-R_2=-$(CH_2)_4$-; **m** R=Ph, R_1=COOEt, R_2=Et.

Scheme 2.

tions it was possible to obtain the 2-diazopyrroles in 40–76% yield.

Once obtained in preparative yields the 2-diazopyrroles, the coupling reaction with secondary amines was carried out without any difficulty. In fact 2-triazenopyrroles **9g–m** were isolated in quantitative yield by reacting, in the dark, at room temperature, 2-diazopyrroles **7** with a large excess of secondary amines **8e** and **f** (ratio 1:10) in dry dichloromethane, or with bubbling anhydrous dimethylamine **8d**. Disappearance of the diazo band at ca. 2 110 cm^{-1} indicated the completeness of the reaction (2–3 h). The structure of the

2-triazenopyrroles was confirmed by IR and NMR spectral data. Infrared spectra, in fact, showed at 3 436–3 447 cm^{-1} and 3 235–3 287 cm^{-1} the free and associated pyrrole NH stretching bands respectively, while the N=N band was observed at 1 599–1 605 cm^{-1}. The ^1H NMR spectra, beside the signals due to the pyrrole substituents, showed the imine proton at 8.25–11.74 ppm testifying the conversion of the 2H-pyrrole structure of the starting 2-diazopyrroles to the 1H-pyrrole structure of the 2-triazeno derivatives. The 1H-pyrrole structure was confirmed by ^{13}C NMR spectra. In fact the pyrrole ring carbon resonances were found in the usual range for an aromatic structure, in particular, the C-2 carbon resonances were found at 143.9–148.1 ppm, typical of a pyrrole α-carbon affected by the deshielding effect of ca. 22 ppm of the triazene function [24], and the C-3 carbon resonances were found, with the exception of the ethoxycarbonyl derivative **9m**, at 81.9–84.9 due to the shielding effect of ca. 16 ppm of the cyano group [25]. The NMR spectra also indicate a substantial contribution of the form **A** to the resonance hybrid of the triazene in which the N-2-N-3 bond has a strong character of double bond hindering the free rotation and generating two sets of signals for the substituents on the N-3 nitrogen of the triazene chain. For example, in the 3-cyano-5-methyl series, such sets of signals are only present in the ^{13}C NMR spectra, whereas, in the 3-cyano-5-phenyl series, due to a higher delocalization of the negative charge of the N-1 nitrogen because of the phenyl ring, the contribution of the form **A** is higher and the sets of signals are also present in the ^1H NMR spectra.

3. Biological results and discussion

When title compounds were screened in MT-4 cells for antiproliferative activity, **9m** was the most potent derivative (data not shown). Therefore, it was further tested against a panel of leukaemia, lymphoma and carcinoma cell lines. As shown in *table I*, **9m** prevented cell growth concentrations ranging from 3.9–21 μM.

Due to the fact that dacarbazine is inactive in vitro, the above results indicate that the antiproliferative activity of 2-triazenopyrroles could be related to the easier breakage of the N-2-N-3 bond. This result is consistent with our findings on the related classes of 3-triazenopyrroles and 3-triazenoindoles which exhibited in vitro anti-leukaemic activity (IC_{50}s were in the range of 1.1–3.1 μM and 0.05–0.34 μM respectively [26, 27]).

In order to investigate whether title compounds were endowed with a broader biological activity, they were tested against eukaryotic and prokaryotic microorganisms. They were found active against fungi at concentrations between 6.2 and 25 μM (MIC/MFC in *table II*). Interestingly, derivative **9j** was active against *C. albicans* at concentrations slightly lower than those of miconazole (3.7 μM) and because of its lower cytotoxicity against Vero cells it also showed a more favourable selectivity index (SI = CC_{50}/MIC). On the contrary, none of the derivatives was significantly active against Gram-negative and Gram-positive bacteria (data not shown).

Furthermore, plaque reduction assays revealed that most compounds were inactive against representative DNA and RNA viruses. The sole exception was **9m**, which inhibited Cox-B2 and VSV at non-cytotoxic concentrations *(table III)*. However, when evaluated for antiretroviral activity, none of the compounds was capable of protecting MT-4 cells from the cytophatic effect induced by HIV-1 (data not shown).

Table I. Antiproliferative activity of **9m** and Cis-platinum.

	[a]IC_{50}[μM]	
Cell lines	**9m**	Cis-platinum
Leukemia/Lymphoma		
Wil2-NS	9.1	32.0
CCRF-SB	6.4	1.3
Raji	10.0	4.8
CCRF-CEM	12.4	1.9
MOLT-4	19.3	32.6
MT-4	11.4	14.8
Carcinoma		
HT-29	19.4	ND
Hela	5.7	1.3
ACHN	3.9	4.7
5637	21.2	8.3

[a]Compound concentration required to reduce cell multiplication by 50% under conditions allowing untreated controls to undergo at least three consecutive rounds of multiplication.
Wil2-NS, human splenic B-lymphoblastoid cells; CCRF-SB, human acute B-lymphoblastic leukaemia; Raji, human Burkitt lymphoma; CCRF-CEM and MOLT-4, human acute T-lymphoblastic leukaemia; MT-4, human $CD4^+$ T-cells containing an integrated HTLV-1 genome; HT-29, human colon adenocarcinoma; HeLa, human cervix carcinoma; ACHN, human renal adenocarcinoma; 5637, human bladder carcinoma.

4. Experimental

4.1. Chemistry

All melting points were taken on a Buchi-Tottoli capillary apparatus and are uncorrected; IR spectra were determined in bromoform with a Jasco FT/IR 5 300 spectrophotometer; ^1H and ^{13}C NMR spectra were measured at 200 and 50.3 MHz respectively in DMSO-d_6

Table II. Antifungal activity of 2-triazenopyrroles.

	[a]CC50	[b]MIC/MFC				
Compound	Vero	*C. albicans*	*C. parapsilosis*	*C. paratropicalis*	*C. neoformans*	*A. fumigatus*
9g	100	25	50	50	25	50
9h	100	25	50	25	25	25
9i	50	25	50	25	25	25
9j	> 100	6.2	25	12.5	12.5	25
9k	> 100	25	25	25	25	25
9l	> 100	12.5	25	12.5	25	25
9m	33	100	100	100	66	> 100
Miconazole	18	3.7	0.9	3.7	0.9	3.7

[a]Compound concentration (μM) required to reduce the viability of Vero cells by 50%. [b]Minimum inhibitory concentration (μM). [c]Minimum fungicidal concentration (μM).
MFC = MIC.

solution, unless otherwise specified, using a Bruker AC-E series 200 MHz spectrometer (TMS as internal reference); column chromatography was performed with Merck silica gel 230–400 Mesh ASTM. For all new compounds analyses indicated by the symbols of the elements or functions were within ± 0.4% of theoretical values.

4.1.1. Preparation of 2-Diazopyrroles **7a–c**

2-Diazopyrroles were prepared according to the procedure described previously for **7a** and **b** [19] from the corresponding 2-aminopyrroles [28, 29] (3 mmol) dissolved in glacial acetic acid (6 mL) by addition (dropwise at 0 °C under a nitrogen atmosphere) of a solution of sodium nitrite (3 mmol) in a small amount of water (1 mL). The mixture was neutralised at 0 °C with a saturated aqueous solution of sodium carbonate and extracted with dichloromethane. The organic layer was dried over sodium sulphate and evaporated under reduced pressure to give the diazo compounds **7** which were purified by chromatography on a column of silica gel using dichloromethane:ethyl acetate (9:1) as eluant.

4.1.1.1. 2-Diazo-4-methyl-5-phenylpyrrole-3-carboxylic acid ethyl ester **7c**

Yield 77%, m.p. 77–80 °C; IR: 2 112 (N≡N), 1 713 (CO) cm^{-1}; ^1H NMR ppm: 1.34 (3H, t, J = 7.4 Hz, CH_3), 2.55 (3H, s, CH_3), 4.34 (2H, q, J = 7.4 Hz, CH_2), 7.43 (2H, dt, J = 7.4, 1.8 Hz, H-3′ and H-5′), 7.51 (1H, dt, J = 7.4, 1.8 Hz, H-4′), 7.71 (2H, dd, J = 7.4, 1.8 Hz, H-2′ and H-6′); ^{13}C NMR ppm: 12.9 (q, CH_3), 13. 9 (q, $OCH_2\mathit{CH_3}$), 60.6 (t, CH_2), 75.4 (s, C-2), 127.9 (d, C-3′ and C-5′), 128.3 (d, C-4′), 128.5 (d, C-2′ and C-6′), 130.5 (s, C-1′), 135.9 (s, C-4), 153.9 (s, C-3), 155.3 (s, C-5), 172.0 (s, CO). Anal. ($C_{14}H_{13}N_3O_2$) C, H, N.

Table III. Antiviral activity of 2-triazenopyrroles.

	[a]CC_{50}	[b]EC_{50}			
Compourd	Vero	SB	HSV-1	COxs-B2	VSV
9g	100	>100	100	> 100	25
9h	100	37.5	> 100	50	25
9i	50	37.5	> 50	50	25
9j	> 100	>100	> 100	> 100	> 100
9k	> 100	>100	> 100	50	50
9l	> 100	>100	> 100	22.2	> 100
9m	33	ND	> 33	10	10
ACG	> 100	> 100	0.04	> 100	> 100
Guanidine	> 100	30	> 100	40	> 100

[a]Compound concentration (μM) required to reduce the viability of Vero cells by 50%, as determined by Plaque reduction test. [b]Compound concentration (μM) required to achieve 50% protection of Vero cells from the viral induced cytopathogenicity as determined by PRT method.

4.1.2. Preparation of 2-Triazenopyrroles **9g**–**m**

To a solution of 2-diazopyrroles **7a**–**c** (3 mmol) in dry dichloromethane (30 mL), a solution of secondary amines (30 mmol) **8e** and **f** in dry dichloromethane (50 mL) or bubbling anhydrous dimethylamine **8d** was added. The reaction mixture was kept in the dark at room temperature and under nitrogen atmosphere until the diazo stretching band at 2 110 cm^{-1} disappeared (2–3 h). Removal of the solvent, under reduced pressure gave the 2-triazenopyrroles in quantitative yields.

The crude 2-triazenopyrroles were crystallized from ethanol (**9h**–**m**) whereas compound **9g** was further purified by column chromatography on silica gel (dichloromethane:ethyl acetate, 9:1).

4.1.2.1. 2-(3,3-Dimethyl-1-triazenyl)-4,5-dimethylpyrrole-3-carbonitrile **9g**

M.p. 189–190 °C, IR: 3 437 and 3 239 (NH), 2 213 (CN), 1 599 (N=N) $cm^{-1;}$ ^{1}H NMR ($CDCl_3$) ppm: 2.06 (3H, s, CH_3), 2.12 (3H, s, CH_3), 3.34 (6H, bs, $N(CH_3)_2$), 8.25 (1H, bs, NH); ^{13}C NMR ($CDCl_3$) ppm: 9.6 (q, CH_3), 10.8 (q, CH_3), 29.7 (q, NCH_3), 30.9 (q, NCH_3), 84.9 (s, C-3), 116.7 (s, CN), 117.1 (s, C-4), 120.9 (s, C-5), 145.6 (s, C-2). Anal. ($C_9H_{13}N_5$) C, H, N.

4.1.2.2. 2-(3,3-Diethyl-1-triazenyl)-4,5-dimethylpyrrole-3-carbonitrile **9h**

M.p. 169–171 °C, IR: 3 436 and 3 266 (NH), 2 211 (CN), 1 603 (N=N) cm^{-1}; ^{1}H NMR ($CDCl_3$) ppm: 1.25 (6H, bs, $N(CH_2\mathit{CH_3})_2$), 2.05 (3H, s, CH_3), 2.12 (3H, s, CH_3), 3.73 (4H, q, J = 7.3 Hz, $N(\mathit{CH_2}CH_3)_2$), 8.30 (1H, bs, NH); ^{13}C NMR ($CDCl_3$) ppm: 9.5 (q, CH_3), 10.7 (q, CH_3), 11.0 (q, $NCH_2\mathit{CH_3}$), 14.2 (q, $NCH_2\mathit{CH_3}$), 41.5 (t, $N\mathit{CH_2}CH_3$), 50.0 (t, $N\mathit{CH_2}CH_3$), 84.0 (s, C-3), 116.3 (s, CN), 117.3 (s, C-4), 120.7 (s, C-5), 146.1 (s, C-2). Anal. ($C_{11}H_{17}N_5$) C, H, N.

4.1.2.3. 2-(3,3-Tetramethylene-1-triazenyl)-4,5-dimethylpyrrole-3-carbonitrile **9i**

M.p. 172–173 °C, IR: 3 439 and 3 235 (NH), 2 211 (CN), 1 599 (N=N) cm^{-1}; ^{1}H NMR ppm: 1.95 (6H, s, 2 × CH_3), 2.04 (4H, bs, 2 × CH_2), 3.52 (2H, bs, NCH_2), 3.84 (2H, bs, NCH_2), 11.15 (1H, s, NH); ^{13}C NMR ppm: 9.5 (q, CH_3), 10.5 (q, CH_3), 23.4 (t, 2 × CH_2), 46.8 (t, NCH_2), 51.1 (t, NCH_2), 81.9 (s, C-3) 115.1 (s, CN), 117.7 (s, C-4), 121.9 (s, C-5), 146.3 (s, C-2). Anal. ($C_{11}H_{15}N_5$) C, H, N.

4.1.2.4. 2-(3,3-Diethyl-1-triazenyl)-4-methyl-5-phenylpyrrole-3-carbonitrile **9j**

M.p. 123–125 °C, IR: 3 437 and 3 270 (NH), 2 213 (CN), 1 605 (N=N) cm^{-1};^{1}H NMR ppm: 2.22 (3H, s, CH_3), 3.19 (3H, bs, NCH_3), 3.53 (3H, bs, NCH_3), 7.27 (1H, dt, J = 7.3, 1.2 Hz, H-4′), 7.42 (2H, dt, J = 7.3, 1.2 Hz, H-3′ and H-5′), 7.53 (2H, dd, J = 7.3, 1.2 Hz, H-2′ and H-6′), 11.72 (1H, s, NH); ^{13}C·NMR ppm: 11.0 (q, CH_3), 35.9 (q, NCH_3), 43.1 (q, NCH_3), 82.2 (s, C-3), 117.1 (s, CN), 117.4 (s, C-4), 125.2 (s, C-5), 126.3 (d, C-4′), 126.7 (d, C-3′ and C-5′), 128.4 (d, C-2′ and C-6′), 131.6 (s, C-1′), 147.3 (s, C-2). Anal. ($C_{14}H_{15}N_5$) C, H, N.

4.1.2.5. 2-(3,3-Diethyl-1-triazenyl)-4-methyl-5-phenylpyrrole-3-carbonitrile **9k**

M.p. 104–106 °C, IR: 3 437 and 3 273 (NH), 2 212 (CN), 1 605 (N=N) cm^{-1}; ^{1}H NMR ppm: 1.19 (3H, bs, $NCH_2\mathit{CH_3}$), 1.28 (3H, bs, $NCH_2\mathit{CH_3}$), 2.22 (3H, s, CH_3), 3.76 (2H, bs, $N\mathit{CH_2}CH_3$), 3.78 (2H, bs, $N\mathit{CH_2}CH_3$), 7.27 (1H, dt, J = 7.3, 1.0 Hz, H-4′), 7.42 (2H, dt, J = 7.3, 1.0 Hz, H-3′ and H-5′), 7.50 (2H, dd, J = 7.3, 1.0 Hz, H-2′ and H-6′), 11.68 (1H, s, NH); ^{13}C NMR ppm: 10.9 (q, $NCH_2\mathit{CH_3}$), 11.2 (q, CH_3), 14.0 (q, $NCH_2\mathit{CH_3}$), 41.6 (t, $N\mathit{CH_2}CH_3$), 49.1 (t, $N\mathit{CH_2}CH_3$), 82.1 (s, C-3), 117.5 (s, CN), 117.6 (s, C-4), 125.4 (s, C-5), 126.5 (d, C-4′), 127.0 (d, C-3′ and C-5′), 128.6 (d, C-2′ and C-6′), 131.7 (s, C-1′), 148.1 (s, C-2). Anal. ($C_{16}H_{19}N_5$) C, H, N.

4.1.2.6. 2-(3,3-Tetramethylene-1-triazenyl)-4-methyl-5-phenylpyrrole-3-carbonitrile **9l**

M.p. 120–123 °C, IR: 3 437 and 3 246 (NH), 2 213 (CN), 1 602 (N=N) cm^{-1}; ^{1}H NMR ppm: 2.01 (4H, bs, 2 × CH_2), 2.23 (3H, s, CH_3), 3.59 (2H, bs, NCH_2), 3.91 (2H, bs, NCH_2), 7.27 (1H, dt, J = 7.3, 1.0 Hz, H-4′), 7.42 (2H, dt, J = 7.3, 1.0 Hz, H-3′ and H-5′), 7.53 (2H, dd, J = 7.3, 1.0 Hz, H-2′ and H-6′) 11.74 (1H, s, NH); ^{13}C NMR ppm: 11.3 (q, CH_3), 23.2 (t, CH_2), 23.6 (t, CH_2) 47.1 (t, NCH_2) 51.4 (t, NCH_2), 82.4 (s, C-3), 117.4 (s, CN), 117.6 (s, C-4), 125.4 (s, C-5), 126.5 (d, C-4′), 126.9 (d, C-3′ and C-5′), 128.6 (d, C-2′ and C-6′), 131.9 (s, C-1′), 148.1 (s, C-2). Anal. ($C_{16}H_{17}N_5$) C, H, N.

4.1.2.7. 2-(3,3-Diethyl-1-triazenyl)-4-methyl-5-phenylpyrrole-3-carboxylic acid ethyl ester **9m**

M.p. 134 °C, IR: 3 447 and 3 287 (NH), 1 680 (CO), 1 603 (N=N) cm^{-1}; ^{1}H NMR ($CDCl_3$) ppm: 1.28 (6H, t, J = 6.7 Hz, $N(CH_2\mathit{CH_3})_2$), 1.37 (3H, t, J = 7.2 Hz, $OCH_2\mathit{CH_3}$), 2.42 (3H, s, CH_3), 3.78 (4H, q, J = 6.7 Hz, $N(\mathit{CH_2}CH_3)_2$), 4.33 (2H, q, J = 7.2 Hz, $O\mathit{CH_2}CH_3$), 7.26–7.43 (5H, m, C_6H_5), 8.62 (1H, s, NH); ^{13}C NMR ($CDCl_3$) ppm: 11.1 (q, $NCH_2\mathit{CH_3}$), 11.7 (q, CH_3), 14.1 (q, $NCH_2\mathit{CH_3}$), 14.4 (q, $OCH_2\mathit{CH_3}$), 41.8 (t, $N\mathit{CH_2}CH_3$), 48.9 (t, $N\mathit{CH_2}CH_3$), 59.2 (t, $O\mathit{CH_2}CH_3$), 106.8 (s, C-3), 118.1 (s, C-4), 125.4 (s, C-5), 126.5 (d, C-4′), 127.2 (d, C-3′ and C-5′), 128.6 (d, C-2′ and C-6′), 132.8 (s, C-1′), 143.9 (s, C-2). 165.9 (s, CO). Anal. ($C_{18}H_{24}N_4O_2$) C, H, N.

4.2. Biology

4.2.1. Compounds

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

4.2.2. Cells

Cell lines were from American Type Culture Collection (ATCC); bacterial and fungal strains were either clinical isolates (obtained from Clinica Dermosifilopatica, University of Cagliari) or collection strains from ATCC. H9/III_B, MT-4 and C8166 cells (grown in RPMI 1 640 containing 10% foetal calf serum (FCS), 100 UI/mL penicillin G and 100 μg/mL streptomycin) were used for anti-HIV-1 assays. Cell cultures were checked periodically for the absence of mycoplasma contamination with a MycoTect Kit (Gibco).

4.2.3. Viruses

Polio-virus (SB), Herpes-virus (HSV-1), Coxakie-B2-virus (Coxs-B2), vesicular stomatitis-virus (VSV) and HIV-1 (strain IIIB) were ATCC strains.

4.2.4. Antiviral assays

The activity of test compounds against Polio-virus, Herpes-virus and Coxakie-B2-virus and vesicular stomatitis-virus were tested in classical plaque reduction assays [30], whereas the anti-HIV-1 activity was evaluated in MTT assays [31].

4.2.5. Antibacterial assays

Staphylococcus aureus, group D *Streptococcus*, *Shigella spp.* and *Salmonella spp.* were recent clinical isolates. Assays were carried out in nutrient broth, pH 7.2, with an inoculum of 10^3 bacterial cells/tube. Minimum inhibitory concentrations (MIC) were determined after incubation at 37 °C for 18 h in the presence of serial dilutions of the test compounds.

4.2.6. Antimycotic assays

Yeast inocula were obtained by properly diluting cultures incubated at 37 °C for 30 h in Sabouraud dextrose broth to obtain 5×10^3 cells/mL. On the contrary, dermatophyte inocula were obtained from cultures grown at 37 °C for 5 d in Sabouraud dextrose broth by finely dispersing clumps with a glass homogenizer before diluting to 0.05 OD_{590}/mL. Then, 20 μl of the above suspensions were added to each well of flat-bottomed microtitre trays containing 80 μl of medium with serial dilutions of test compounds, and were incubated at 37 °C. Growth controls were visually determined after 2 d (yeast) or 3 d (dermatophytes).

MIC was defined as the compound concentration at which no macroscopic signs of fungal growth were detected. The minimal germicidal concentration (MBC or MFC) was determined by subcultivating in Sabouraud dextrose agar samples from cultures with no apparent growth.

4.2.7. Antiproliferative assays

Exponentially growing leukaemia and lymphoma cells were resuspended at a density of 1×10^5 cells/mL in RPMI containing serial dilutions of the drugs. Cell viability was determined after 4 d at 37 °C by the MTT method [31]. Activity against cell lines derived from solid tumours was evaluated in exponentially growing cultures seeded at 5×10^4 cells/mL which were allowed to adhere for 16 h to culture plates before addition of the drugs. Cell viability was determined by the MTT method 4 d later.

4.2.8. Linear regression analysis

Viral, microbial and tumour cell growth at each drug concentration was expressed as the percentage of untreated controls, and the concentration resulting in 50% (EC_{50}, IC_{50}) growth inhibition was determined by linear regression analysis.

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