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Synthesis of 3,6,7-substituted-quinoxalin-2-ones for evaluation of antimicrobial and anticancer activity. Part 2

Paolo Sanna [a,*], Antonio Carta [a], Mario Loriga [a], Stefania Zanetti [b], Leonardo Sechi [b]

[a] *Dipartimento Farmaco Chimico Tossicologico, via Muroni 23/a, I-07100 Sassari, Italy*
[b] *Dipartimento di Scienze Biomediche, viale S. Pietro, I-07100 Sassari, Italy*

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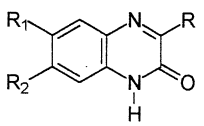
Abstract

A new set of 35 3-alkyl and 3-ethoxycarbonylalkyl 6- and/or 7-substituted-2-quinoxalinones was prepared and submitted to a preliminary in vitro investigation of their antimicrobial, anticancer and anti-HIV activities. Results are referred.

Keywords: 2-Quinoxalinones; Antimicrobial activities; Anticancer activities

1. Introduction

In continuation of our interest in the chemistry and biological properties of the quinoxaline derivatives, we have recently reported the synthesis and antimicrobial and anticancer activities of a series of 3-quinoxalinones [1]. A moderate antibacterial response was evidenced when an ethoxycarbonyl group was present at the 2 position of the heterocyclic along with a trifluoromethyl substituent in the 6 or 7 position. On the basis of these considerations we have planned the preparation of a new series of 2-quinoxalinones of formula **I**, in order to explore further the biological properties of these compounds.



I

R=CH_3, C_2H_5, CO_2Et, CH_2CO_2Et, $CH(CH_3)CO_2Et$
R_1,R_2=F, CF_3, NO_2, morpholine, 4-methylpiperazine.

All these derivatives bear one or two electron-withdrawing groups in the benzene moiety, while the substituents (R) at the 3 position were selected in order to evaluate the effect on the biological activities of the increased lipophilicity and/or steric hindrance. It was also deemed of interest to extend the investigation to some substituents (R_1/R_2) which are favorably present in antibacterial quinolones used in therapy. Preliminary results on their antimicrobial activity have been previously communicated [2,3].

2. Chemistry

Preparation of quinoxalinone esters **2–21**, listed in Table 1, was accomplished by a synthetic approach previously described [1,4] and depicted in Scheme 1.

Condensation of the appropriate 1,2-diaminobenzene **1a–e** with the suitable α-keto diester in ethanol or in a mixture of ethanol–acetic acid (20:1 ratio) under reflux gave in good yields quinoxalinones **2–6** in a mixture with their tautomers **7–11**. Compounds **12–20** have been similarly prepared; however, among these compound **15** was the only one obtained in a mixture with its tautomer **21**. The tautomeric equilibrium observed in the above-mentioned compounds was in accordance with that reported by Chapman for similar cases [5]. Separation of the single compounds from the complex mixtures of isomers **2–6** with their tautomers **7–11** was successful in a few cases (**6**, **8**, **11**) after repeated fractional crystallizations, whereas in the case of mix-

* Corresponding author.

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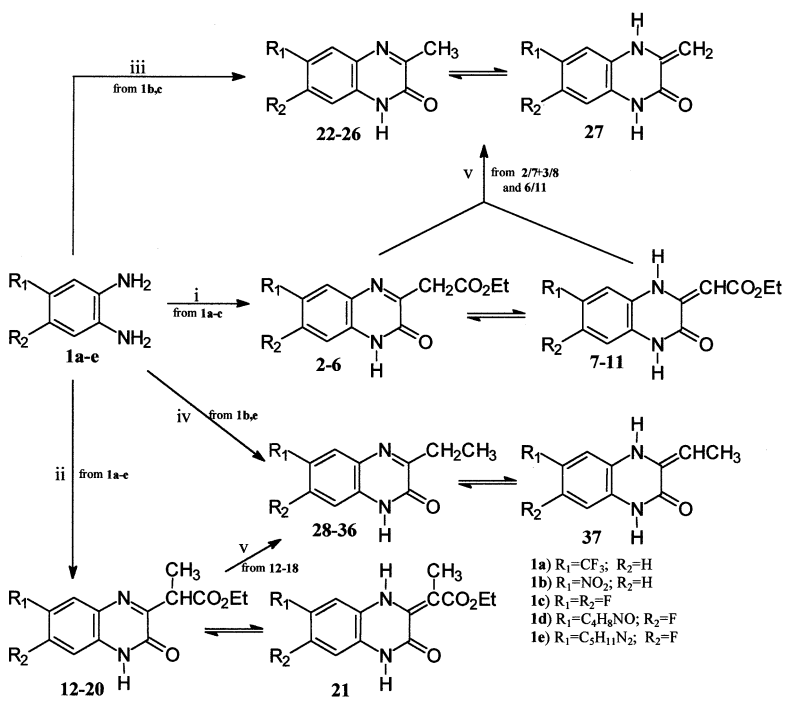
Table 1
Compounds of Scheme 1[a]

Compd	R_1	R_2	Compd	R_1	R_2	Compd	R_1	R_2
2	CF_3	H	**14**	NO_2	H	**26**	F	F
3	H	CF_3	**15**	H	NO_2	**27**	H	NO_2
4	NO_2	H	**16**	F	F	**28**	CF_3	H
5	H	NO_2	**17**	F	C_4H_8NO	**29**	H	CF_3
6	F	F	**18**	C_4H_8NO	F	**30**	NO_2	H
7	CF_3	H	**19**	F	$C_5H_{11}N_2$	**31**	H	NO_2
8	H	CF_3	**20**	$C_5H_{11}N_2$	F	**32**	F	F
9	NO_2	H	**21**	H	NO_2	**33**	F	C_4H_8NO
10	H	NO_2	**22**	CF_3	H	**34**	C_4H_8NO	F
11	F	F	**23**	H	CF_3	**35**	F	$C_5H_{11}N_2$
12	CF_3	H	**24**	NO_2	H	**36**	$C_5H_{11}N_2$	F
13	H	CF_3	**25**	H	NO_2	**37**	NO_2	H

[a] C_4H_8NO = 4-morpholinyl; $C_5H_{11}N_2$ = 4-methylpiperazinyl.

tures of isomers **12–20** and the tautomeric pair **15/21** the purification was accomplished by chromatography.

In order to prepare the alkyl derivatives **22–26** and **28–36** two different methods were used. Compounds **28–34** were obtained both by concomitant alkaline hydrolysis and decarboxylation of the parent esters **12–18** as reported for similar cases [6]. Compounds **22** and **23**, previously described in [7], and **26** were obtained in the same way starting from the tautomeric pairs **2/7**, **3/8** and **6/11**, respectively, thus showing that the tautomeric equilibrium was reversed during hydrolysis. Attempts at saponification of the mixture of isomers **4** and **5** along with their tautomers **9** and **10**, and of the single isomers **19** and **20** to give **24**, **25**, **35** and **36**, respectively, failed. These results induced us to find an alternative method for the preparation of these compounds. Straightforward condensation of diamine **1b** with pyruvic acid in the presence of 10% sulfuric acid afforded, with poor yields, compounds **24** (6%) and **27** (13%), while the expected known compound **25** [8], a tautomer of **27**, was not isolated. Compound **24** was previously described by an alternative route [8], while the formation of **27** was not observed in any of the previous papers [8,9]. Via the same route from **1e**



Scheme 1. Preparation of substituted quinoxalin-2-ones: (i) $EtO_2CC(ONa)=CHCO_2Et$, $EtOH/H_2O/CH_3CO_2H$; (ii) $EtO_2CCOCH(CH_3)CO_2Et$/EtOH; (iii) $CH_3COCOOH$/10% H_2SO_4; (iv) $CH_3CH_2COCOOH$/10% H_2SO_4; (v) OH^-/H^+.

with 2-oxobutanoic acid only compound **35** (39%) was obtained.

In order to compare the effectiveness of the two methods, we deemed it of interest to react **1c** with pyruvic acid and **1b** with 2-oxobutanoic acid, thus obtaining **26** (21%), **30** in a mixture with its tautomer **37** (68%), and **31** (7%). From these results it appears evident that straightforward condensation is a profitable way to prepare compounds **24**, **27**, **30** and **35**, while the hydrolysis–decarboxylation method is more productive for compounds **26** and **31** (70 and 33% overall yield, respectively).

Interestingly in the cases of the tautomeric couple of the nitro derivatives **15/21** and **30/37** complete interconversion of the β-enaminic form into the thermodynamically more stable forms **15** and **30** took place after heating the mixture in refluxing acetone. This was not observed for the complex mixture of isomers **2** and **3** with their corresponding tautomers **7** and **8** or in the case of **27**.

Structures of all new synthesized compounds were consistent with both spectroscopic and analytical data. In particular, the correct assignment of the resonances of the single tautomers in the mixtures, as previously observed by us [1], was deduced by the ^{1}H NMR spectrum, which showed, for 3-alkylsubstituted quinoxalinones, a signal consistent with CH_3 or CH_2–R resonances located at δ 2.5–2.9, while in the methylidenic derivatives a vinyl proton located at δ 5.5–5.6 is clearly evident, besides a second NH signal which collapses after exchange with D_2O. Moreover UV–Vis spectra of the latter showed a characteristic absorption in the visible region (370–420 nm), with a bathochromic shift (50–80 nm) in comparison with its parent tautomers, thus indicating the presence of a chromophore due to the anilinoacrylate moiety. This absorption around 320–330 nm was in line with the literature reports [10].

3. Experimental

Melting points were determined by a Kofler hot stage or Digital Electrothermal apparatus, and were uncorrected. IR spectra are for Nujol mulls and were recorded using a Perkin–Elmer 781 spectrophotometer. UV–Vis spectra are qualitative and were recorded in nm for solutions in ethanol with a Perkin–Elmer Lambda 5 spectrophotometer. ^{1}H NMR spectra were recorded on a Varian XL-200 (200 MHz) instrument, using TMS as internal standard. Column chromatography was performed using 70–230 and 230–400 mesh silica gel (Merck Silica Gel 60) in the case of flash chromatography. Elemental analyses were performed by the Laboratorio di Microanalisi, Dipartimento di Scienze Farmaceutiche, Università di Padova (Padua). Analytical results for C, H, N, and halogen when present, were within $\pm$0.4% of the theoretical values.

3.1. Intermediates

The diaminobenzene derivatives **1a–e** were prepared according to the procedures previously described by us [1].

3.2. General procedure for preparation of 6- and 7-substituted-3-(1-ethoxycarbonylmethyl)-2(1H)-quinoxalinones (2–6) and their tautomers 6- and 7-substituted-3-ethoxycarbonylmethylene-1,2,3,4-tetrahydroquinoxalin-2-ones (7–11)

A solution of 2.9–3.5 g (13.8–16.7 mmol) of diethyl oxalacetate as sodium salt in water (5 ml) was added to a stirred solution of 2 g (11.4–13.9 mmol) of **1a–c** in a mixture of 20 ml of ethanol and 1 ml of acetic acid. The mixture was then refluxed for 2 h. After cooling to room temperature, the precipitate was filtered, washed, dried and purified by fractional crystallization from a suitable solvent as indicated below.

3.2.1. 6-Trifluoromethyl- (2) and 7-trifluoromethyl-3-ethoxycarbonylmethyl-2(1H)-quinoxalinone (3) in a mixture with 6-trifluoromethyl- (7) and 7-trifluoromethyl-3-ethoxycarbonylmethylene-1,2,3,4-tetrahydroquinoxalin-2-one (8)

The crude precipitate (1.32 g; 38.7%) was constituted by a mixture of **2** and **3** with the respective tautomers **7** and **8** in a 1:1:1:1 ratio, as shown by its ^{1}H NMR spectrum: ($CDCl_3$ + DMSO-d_6): δ 11.84 (4H, br s, NH-1 of **2**, **3**, **7**, and **8**), 11.16 (2H, br s, NH-4 of **7** and **8**), 7.70–7.20 (12H, m, aromatic-H of **2**, **3**, **7**, and **8**), 5.67 (1H, s, CH=C of **7**), 5.62 (1H, s, CH=C of **8**), 4.19 (4H, q, J = 7.0 Hz, CH_2CH_3 of **2** and **3**), 3.87 (4H, s, CH_2CO of **2** and **3**), 1.28 (12H, t, J = 7.0 Hz, CH_3CH_2 of **2**, **3**, **7**, and **8**).

After repeated crystallizations of this mixture from acetone, only compound **8** was isolated in pure form: 0.1 g (3% yield); m.p. 266–268°C; IR: ν 3300–3150, 1700, 1640, 1630 cm^{-1}; UV: λ 384 sh, 366, 350, 295, 225 nm; ^{1}H NMR (acetone-d_6): δ 11.87 (1H, s, NH-1), 11.15 (1H, s, NH-4), 7.56 (1H, d, J = 8.8 Hz, H-6), 7.32 (2H, m, H-5 + H-8), 5.62 (1H, s, *CH*), 4.19 (2H, q, J = 7.0 Hz, CH_2CH_3), 1.28 (3H, t, J = 7.0 Hz, CH_3CH_2).

Finally, evaporation of the hydroalcoholic mother liquors gave 1.15 g (57.5%) of unreacted **1a**. Attempted conversion of the complex mixture of tautomeric pairs **2/7** and **3/8** into **2** and **3** by refluxing in acetone was not successful.

*3.2.2. 6-Nitro- (**4**) and 7-nitro-3-ethoxycarbonylmethyl-2(1H)-quinoxalinone (**5**) in a mixture with 6-nitro- (**9**) and 7-nitro-3-ethoxycarbonylmethylene-1,2,3,4-tetrahydro-quinoxalin-2-one (**10**)*

The crude precipitate resulted in an isomeric mixture (2 g; 56%) of quinoxalinones **4** and **5** with their methylenic tautomers **9** and **10**, respectively. After fractional crystallizations from DMSO this gave 0.2 g (5.6% yield) of a mixture of the two tautomers **5** and **10** in a 1:1 ratio, with no further separation possible: m.p. 280–290°C; IR: ν 3250–3150, 1700, 1670, 1640, 1620 cm^{-1}; UV: λ 397, 383, 316, 263, 217 nm; ^1H NMR (DMSO-d_6): δ 11.93 (2H, br s, NH-1 of **5** and **10**), 11.12 (1H, s, NH-4 of **10**), 7.90–7.40 (6H, m, aromatic H of **5** and **10**), 5.63 (1H, s, *CH*CO of **10**), 4.18 (4H, q, $J = 6.8$ Hz, CH_2CH_3 of **5** and **10**), 2.54 (2H, s, CH_2CO of **5**), 1.25 (6H, t, $J = 6.8$ Hz, CH_3CH_2 of **5** and **10**); and also a mixture of 0.1 g (2.8% yield) of **4** and **9** in a 1:1 ratio: m.p. 270–280°C; IR: ν 3250–3080, 1700, 1640, 1610 cm^{-1}; UV: λ 400, 385, 346, 332, 320 sh, 267, 216 nm; ^1H NMR (DMSO-d_6): δ 12.20 (2H, br s, NH-1 of **4** and **9**), 11.14 (1H, s, NH-4 of **9**), 8.53 (2H, s, H-5 of **4** and **9**), 7.56 (2H, d, $J = 8.4$ Hz, H-7 of **4** and **9**), 7.16 (2H, d, $J = 8.4$ Hz, H-8 of **4** and **9**), 5.57 (1H, s, *CH*CO of **9**), 4.17 (4H, q, $J = 6.8$ Hz, CH_2CH_3 of **4** and **9**), 2.51 (2H, s, CH_2CO of **4**), 1.26 (6H, t, $J = 6.8$ Hz, CH_3CH_2 of **4** and **9**).

The hydroalcoholic mother liquors, after evaporation in vacuo, gave 0.74 g (37%) of unreacted **1b**.

*3.2.3. 6,7-Difluoro-3-ethoxycarbonylmethyl-2(1H)-quinoxalinone (**6**) and 6,7-difluoro-3-ethoxycarbonylmethylene-1,2,3,4-tetrahydroquinoxalin-2-one (**11**)*

The crude precipitate formed of the tautomeric pair **6**/**11**, after fractional crystallization from ethanol, initially afforded 0.71 g (19% yield) of **11**: m.p. 218–220°C; IR: ν 3200, 3180, 1690, 1650, 1630 cm^{-1}; UV: λ 388 sh, 374, 358, 280 sh, 253, 216 nm; ^1H NMR ($CDCl_3$ + DMSO-d_6): δ 11.70 (1H, s, NH-1), 11.04 (1H, s, NH-4), 7.56 (1H, dd, $J = 11.7$ and 7.6 Hz, H-5), 6.97 (1H, dd, $J = 10.6$ and 7.8 Hz, H-8), 5.56 (1H, s, *CH*CO), 4.17 (2H, q, $J = 6.8$ Hz, CH_2CH_3), 1.28 (3H, t, $J = 6.8$ Hz, CH_3CH_2); and then 2.07 g (55.7% yield) of **6**: m.p. 214–216°C; IR: ν 3180, 1730, 1660, 1630, 1610 cm^{-1}; UV: λ 388 sh, 352 sh, 342, 274, 226, 204 nm; ^1H NMR ($CDCl_3$): δ 12.85 (1H, br s, NH-1), 7.66 (1H, dd, $J = 10.0$ and 7.8 Hz, H-5), 7.17 (1H, dd, $J = 10.0$ and 7.2 Hz, H-8), 4.26 (2H, q, $J = 7.0$ Hz, CH_2CH_3), 3.98 (2H, s, CH_2CO), 1.32 (3H, t, $J = 7.0$ Hz, CH_3CH_2).

*3.3. General procedure for preparation of 6- and 7-substituted 3-(1-ethoxycarbonylethyl)-2(1H)-quinoxalin-2-ones (**12–20**) with 7-nitro-3-(1-ethoxycarbonylethylidene)-1,2,3,4-tetrahydroquinoxalin-2-one (**21**)*

To a solution of the appropriate diamine **1a–e** (1.0 g; 5.7–7.0 mmol) in ethanol (20 ml), diethyl oxalpropionate (1.4–1.7 g; 6.9–8.4 mmol) was added dropwise and the mixture heated under reflux for 2 h. After removal of the solvent in vacuo, the crude residue formed as a mixture of the two isomers was purified by column chromatography on silica gel, eluting with a mixture of diethyl ether/light petroleum in a 70:30 ratio to give compounds **12–16** and **21** or diethyl ether/acetone in a 90:10 ratio to give **17–20**. In general, we observed that the 6-isomers precede the 7-ones in the eluate.

*3.3.1. 6-Trifluoromethyl-3-(1-ethoxycarbonylethyl)-2(1H)-quinoxalinone (**12**)*

0.75 g (42% yield); m.p. 153–155°C; IR: ν 3160, 1740, 1675, 1630 cm^{-1}; UV: λ 317, 258, 218 nm; ^1H NMR ($CDCl_3$): δ 12.66 (1H, s, NH), 8.17 (1H, s, H-5), 7.63 (1H, dd, $J = 8.4$ and 1.8 Hz, H-7), 7.44 (1H, d, $J = 8.4$ Hz, H-8), 4.30 (1H, q, $J = 7.0$ Hz, $CHCH_3$), 4.22 (2H, q, $J = 7.2$ Hz, CH_2CH_3), 1.66 (3H, d, $J = 7.0$ Hz, CH_3CH), 1.25 (3H, t, $J = 7.2$ Hz, CH_3CH_2).

*3.3.2. 7-Trifluoromethyl-3-(1-ethoxycarbonylethyl)-2(1H)-quinoxalinone (**13**)*

0.80 g (45% yield); m.p. 160–162°C; IR: ν 3220, 1760, 1680, 1640 cm^{-1}; UV: λ 323, 316 infl, 258, 240, 212 nm; ^1H NMR ($CDCl_3$): δ 12.36 (1H, s, NH), 7.99 (1H, d, $J = 8.6$ Hz, H-6), 7.58 (1H, d, $J = 8.6$ Hz, H-5), 7.56 (1H, s, H-8), 4.32 (1H, q, $J = 7.2$ Hz, $CHCH_3$), 4.23 (2H, q, $J = 7.0$ Hz, CH_2CH_3), 1.66 (3H, d, $J = 7.2$ Hz, CH_3CH), 1.27 (3H, t, $J = 7.0$ Hz, CH_3CH_2).

*3.3.3. 6-Nitro-3-(1-ethoxycarbonylethyl)-2(1H)-quinoxalinone (**14**)*

0.83 g (44% yield); m.p. 158–160°C; IR: ν 3150, 1700, 1640 cm^{-1}; UV: λ 338, 267, 204 nm; ^1H NMR ($CDCl_3$ + DMSO-d_6): δ 12.92 (1H, br s, NH), 8.60 (1H, d, $J = 2.4$ Hz, H-5), 8.29 (1H, dd, $J = 9.0$ and 2.4 Hz, H-7), 7.48 (1H, d, $J = 9.0$ Hz, H-8), 4.20–4.10 (3H, m, $CHCH_3$ + CH_2CH_3), 1.56 (3H, d, $J = 7.2$ Hz, CH_3CH), 1.22 (3H, t, $J = 7.2$ Hz, CH_3CH_2).

*3.3.4. 7-Nitro-3-(1-ethoxycarbonylethyl)-2(1H)-quinoxalinone (**15**)*

0.80 g (42% yield); m.p. 165–167°C; IR: ν 3200, 1690, 1650 cm^{-1}; UV: λ 361, 281, 222 nm; ^1H NMR ($CDCl_3$ + DMSO-d_6): δ 12.81 (1H, br s, NH), 8.18 (1H, d, $J = 2.4$ Hz, H-8), 8.07 (1H, dd, $J = 8.8$ and 2.4 Hz, H-6), 7.94 (1H, d, $J = 8.8$ Hz, H-5), 4.20–4.05 (3H, m, $CHCH_3$ + CH_2CH_3), 1.53 (3H, d, $J = 7.2$ Hz, CH_3CH), 1.20 (3H, t, $J = 7.2$ Hz, CH_3CH_2).

*3.3.5. 7-Nitro-3-(1-ethoxycarbonylethylidene)-1,2,3,4-tetrahydroquinoxalin-2-one (**21**)*

0.18 g (10% yield); m.p. 220–222°C; IR: ν 3170, 3070, 1670, 1620 cm^{-1}; UV: λ 421, 404, 318, 265, 221, 212 sh nm; ^1H NMR ($CDCl_3$ + DMSO-d_6): δ 12.47 (1H, s, NH-1), 11.53 (1H, s, NH-4), 7.88 (1H, d, $J = 8.8$

Hz, H-7), 7.82 (1H, s, H-5), 7.05 (1H, d, $J = 8.8$ Hz, H-8), 4.25 (2H, q, $J = 7.0$ Hz, CH_2CH_3), 2.39 (3H, s, $CH_3C=C$), 1.35 (3H, t, $J = 7.0$ Hz, CH_3CH_2).

This compound was completely converted into **15** when heated at reflux in acetone for 1 h.

*3.3.6. 6,7-Difluoro-3-(1-ethoxycarbonylethyl)-2(1H)-quinoxalinone (**16**)*

1.33 g (68% yield); m.p. 182–184°C; IR: ν 3440, 1730, 1660, 1630, 1610 cm^{-1}; UV: λ 338, 271, 225, 203 nm; ^1H NMR ($CDCl_3$): δ 12.64 (1H, s, NH), 7.69 (1H, dd, $J = 10.4$ and 8.0 Hz, H-5), 7.14 (1H, dd, $J = 10.0$ and 7.2 Hz, H-8), 4.20–4.15 (3H, m, $CHCH_3 + CH_2CH_3$), 1.63 (3H, d, $J = 7.2$ Hz, CH_3CH), 1.26 (3H, t, $J = 7.2$ Hz, CH_3CH_2).

*3.3.7. 6-Fluoro-7-morpholinyl-3-(1-ethoxycarbonylethyl)-2(1H)-quinoxalinone (**17**)*

0.70 g (42% yield); m.p. 198–200°C; IR: ν 3150, 1710, 1660, 1640, 1630 cm^{-1}; UV: λ 360, 224 nm; ^1H NMR ($CDCl_3$): δ 12.62 (1H, s, NH), 7.50 (1H, d, $J = 13.0$ Hz, H-5), 6.72 (1H, d, $J = 8.0$ Hz, H-8), 4.30–4.10 (3H, m, $CHCH_3 + CH_2CH_3$), 3.92 (4H, t, $J = 4.8$ Hz, CH_2-2′ + CH_2-6′), 3.23 (4H, t, $J = 4.8$ Hz, CH_2-3′ + CH_2-5′), 1.61 (3H, d, $J = 7.2$ Hz, CH_3CH), 1.21 (3H, t, $J = 7.0$ Hz, CH_3CH_2).

*3.3.8. 7-Fluoro-6-morpholinyl-3-(1-ethoxycarbonylethyl)-2(1H)-quinoxalinone (**18**)*

0.67 g (40% yield); m.p. 167–168°C; IR: ν 3150, 1730, 1660, 1650, 1630 cm^{-1}; UV: λ 366, 250, 202 nm; ^1H NMR ($CDCl_3$): δ 12.93 (1H, s, NH), 7.41 (1H, d, $J = 8.6$ Hz, H-5), 7.05 (1H, d, $J = 12.0$ Hz, H-8), 4.20–4.15 (3H, m, $CHCH_3 + CH_2CH_3$), 3.92 (4H, t, $J = 4.8$ Hz, CH_2-2′ + CH_2-6′), 3.12 (4H, t, $J = 4.8$ Hz, CH_2-3′ + CH_2-5′), 1.63 (3H, d, $J = 7.2$ Hz, CH_3CH), 1.26 (3H, t, $J = 7.2$ Hz, CH_3CH_2).

*3.3.9. 6-Fluoro-7-(4-methylpiperazinyl)-3-(1-ethoxycarbonylethyl)-2(1H)-quinoxalinone (**19**)*

0.24 g (25% yield) as oil; IR (film): ν 3320, 1720, 1640 cm^{-1}; UV: λ 332, 288 infl, 244 infl, 228, 204 nm; ^1H NMR ($CDCl_3$): δ 6.77 (1H, d, $J = 13.4$ Hz, H-5), 6.21 (1H, d, $J = 8.4$ Hz, H-8), 4.24–4.11 (3H, m, $CHCH_3 + CH_2CH_3$), 3.13 (4H, t, $J = 4.4$ Hz, CH_2-2′ + CH_2-6′), 2.72 (4H, t, $J = 4.4$ Hz, CH_2-3′ + CH_2-5′), 2.42 (3H, s, CH_3-N), 1.30–1.20 (6H, m, $CH_3CH + CH_3CH_2$).

*3.3.10. 7-Fluoro-6-(4-methylpiperazinyl)-3-(1-ethoxycarbonylethyl)-2(1H)-quinoxalinone (**20**)*

0.43 g (27% yield); m.p. 133–134°C; IR: ν 3250, 1720, 1650 cm^{-1}; UV: λ 366, 251, 204 nm; ^1H NMR ($CDCl_3$): δ 7.41 (1H, d, $J = 8.8$ Hz, H-5), 7.04 (1H, d, $J = 12.2$ Hz, H-8), 4.35–4.15 (3H, m, $CHCH_3 + CH_2CH_3$), 3.16 (4H, t, $J = 4.4$ Hz, CH_2-2′ + CH_2-6′), 2.67 (4H, t, $J = 4.4$ Hz, CH_2-3′ + CH_2-5′), 2.40 (3H, s, CH_3-N), 1.62 (3H, d, $J = 7.0$ Hz, CH_3CH), 1.25 (3H, t, $J = 7.0$ Hz, CH_3CH_2).

*3.4. General procedure for preparation of 6- and 7-substituted 3-alkyl-2(1H)-quinoxalinones (**22–24**, **26** and **28–35**) and 7-nitro-3-alkylidene-1,2,3,4-tetrahydroquinoxalin-2-ones (**27** and **37**)*

*3.4.1. Method A (compounds **22**, **23**, **26**, **28–34**)*

A suspension of 1 g (2.8–3.8 mmol) of the appropriate ester **12–18** or of the tautomeric mixture of **2/7** with **3/8** and **6/11** in 100 ml of 2 M sodium hydroxide aqueous solution was stirred at reflux for 2 h. After cooling to room temperature, the solution was made acidic (pH $\approx$ 2) with conc. hydrochloric acid to give a precipitate which was collected by filtration. Generally, the solid products obtained were pure enough (**26** and **30–34**) or purified by crystallization from diethyl ether (**28** and **29**), or by column chromatography on silica gel eluting with a 70:30 mixture of diethyl ether/light petroleum (**22** and **23**). In this latter case the 6-isomer **22** was collected as first eluate.

*3.4.1.1. 6-Trifluoromethyl-3-methyl-2(1H)-quinoxalinone (**22**).* 0.25 g (33% yield); m.p. 188–190°C; IR: ν 3160, 1670, 1625 cm^{-1}; UV: λ 344 sh, 330, 320, 264, 235, 205 nm; ^1H NMR ($CDCl_3$): δ 12.72 (1H, s, NH), 8.08 (1H, s, H-5), 7.71 (1H, d, $J = 8.4$ Hz, H-7), 7.46 (1H, d, $J = 8.4$ Hz, H-8), 2.66 (3H, s, CH_3).

*3.4.1.2. 7-Trifluoromethyl-3-methyl-2(1H)-quinoxalinone (**23**).* 0.33 g (43% yield); m.p. 271–275°C (Ref. [7], 272–275°C).

*3.4.1.3. 6,7-Difluoro-3-methyl-2(1H)-quinoxalinone (**26**).* 0.70 g (93% yield); m.p. > 300°C (dec.); IR: ν 3170, 1680, 1640, 1620 cm^{-1}; UV: λ 338, 272, 226, 206 nm; ^1H NMR (DMSO-d_6): δ 12.41 (1H, s, NH), 7.77 (1H, dd, $J = 11.0$ and 8.3 Hz, H-5), 7.17 (1H, dd, $J = 11.0$ and 7.7 Hz, H-8), 2.38 (3H, s, CH_3).

*3.4.1.4. 6-Trifluoromethyl-3-ethyl-2(1H)-quinoxalinone (**28**).* 0.67 g (73% yield); m.p. 161–163°C; IR: ν 3160, 1680, 1620, 1580 cm^{-1}; UV: λ 328, 268 sh, 236, 207 nm; ^1H NMR ($CDCl_3$ + DMSO-d_6): δ 12.58 (1H, s, NH), 7.98 (1H, s, H-5), 7.67 (1H, d, $J = 8.2$ Hz, H-7), 7.44 (1H, d, $J = 8.2$ Hz, H-8), 2.92 (2H, q, $J = 7.4$ Hz, CH_2CH_3), 1.32 (3H, t, $J = 7.4$ Hz, CH_3CH_2).

*3.4.1.5. 7-Trifluoromethyl-3-ethyl-2(1H)-quinoxalinone (**29**).* 0.65 g (71% yield); m.p. 206–208°C; IR: ν 3450–3300, 1670, 1630, 1570 cm^{-1}; UV: λ 332, 274, 229, 206 nm; ^1H NMR ($CDCl_3$ + DMSO-d_6): δ 12.49 (1H, s, NH), 7.87 (1H, d, $J = 8.4$ Hz, H-5), 7.58 (1H, s, H-8), 7.47 (1H, d, $J = 8.4$ Hz, H-6), 2.87 (2H, q, $J = 7.4$ Hz, CH_2CH_3), 1.28 (3H, t, $J = 7.4$ Hz, CH_3CH_2).

*3.4.1.6. 6-Nitro-3-ethyl-2(1H)-quinoxalinone (**30**).* 0.60 g (79% yield); m.p. 228–230°C; IR: ν 3170, 1690, 1625, 1600, 1595 cm^{-1}; UV: λ 337, 325 sh, 264, 203 nm; ^1H NMR ($CDCl_3$ + DMSO-d_6): δ 12.79 (1H, s, NH), 8.49 (1H, d, $J = 2.4$ Hz, H-5), 8.26 (1H, dd, $J = 9.0$ and 2.4 Hz, H-7), 7.42 (1H, d, $J = 9.0$ Hz, H-8), 2.87 (2H, q, $J = 7.4$ Hz, CH_2CH_3), 1.28 (3H, t, $J = 7.4$ Hz, CH_3CH_2).

*3.4.1.7. 7-Nitro-3-ethyl-2(1H)-quinoxalinone (**31**).* 0.60 g (79% yield); m.p. 235–237°C; IR: ν 3190, 1690, 1620, 1600 cm^{-1}; UV: λ 358, 279, 222, 202 nm; ^1H NMR (acetone-d_6): δ 11.60 (1H, s, NH), 8.27 (1H, d, $J = 2.4$ Hz, H-8), 8.14 (1H, dd, $J = 8.8$ and 2.4 Hz, H-6), 7.98 (1H, d, $J = 8.8$ Hz, H-5), 2.95 (2H, q, $J = 7.2$ Hz, CH_2CH_3), 1.33 (3H, t, $J = 7.2$ Hz, CH_3CH_2).

*3.4.1.8. 6,7-Difluoro-3-ethyl-2(1H)-quinoxalinone (**32**).* 0.68 g (96% yield); m.p. 216–218°C; IR: ν 3150, 1660, 1610, 1560 cm^{-1}; UV: λ 332, 272, 226, 204 nm; ^1H NMR ($CDCl_3$ + DMSO-d_6): δ 12.40 (1H, s, NH), 7.67 (1H, dd, $J = 11.0$ and 8.2 Hz, H-5), 7.18 (1H, dd, $J = 10.6$ and 7.4 Hz, H-8), 2.81 (2H, q, $J = 7.2$ Hz, CH_2CH_3), 1.24 (3H, t, $J = 7.2$ Hz, CH_3CH_2).

*3.4.1.9. 6-Fluoro-7-morpholinyl-3-ethyl-2(1H)-quinoxalinone (**33**).* 0.76 g (96% yield); m.p. 237–238°C; IR: ν 3150, 1670, 1560 cm^{-1}; UV: λ 357, 244 sh, 223 nm; ^1H NMR ($CDCl_3$ + DMSO-d_6): δ 12.14 (1H, s, NH), 7.37 (1H, d, $J = 13.0$ Hz, H-5), 6.78 (1H, d, $J = 8.4$ Hz, H-8), 3.84 (4H, t, $J = 4.8$ Hz, CH_2-2′ + CH_2-6′), 3.13 (4H, t, $J = 4.8$ Hz, CH_2-3′ + CH_2-5′), 2.83 (2H, q, $J =$ 7.2 Hz, CH_2CH_3), 1.26 (3H, t, $J = 7.2$ Hz, CH_3CH_2).

*3.4.1.10. 7-Fluoro-6-morpholinyl-3-ethyl-2(1H)-quinoxalinone (**34**).* 0.72 g (92% yield); m.p. 222–224°C; IR: ν 3150, 1650, 1570 cm^{-1}; UV: λ 360, 250, 203 nm; ^1H NMR ($CDCl_3$ + DMSO-d_6): δ 12.08 (1H, s, NH), 7.33 (1H, d, $J = 8.2$ Hz, H-5), 7.03 (1H, d, $J = 12.1$ Hz, H-8), 3.88 (4H, t, $J = 4.8$ Hz, CH_2-2′ + CH_2-6′), 3.09 (4H, t, $J = 4.8$ Hz, CH_2-3′ + CH_2-5′), 2.90 (2H, q, $J =$ 7.2 Hz, CH_2CH_3), 1.32 (3H, t, $J = 7.2$ Hz, CH_3CH_2).

*3.4.2. Method B (compounds **24**, **26**, **27**, **30**, **31**, **35**, **37**)*

To a stirred solution of the appropriate diamine **1b**, **1c** and **1e** (3.9–7.0 mmol) in a 10% aqueous solution of sulfuric acid (20 ml), pyruvic acid (8.0–9.0 mmol) or 2-oxobutanoic acid (4.9–8.0 mmol) was added. The mixture was then heated to 70°C for 1 h. On cooling to room temperature a crude precipitate was collected and purified by column chromatography on silica gel, eluting with diethyl ether/light petroleum mixtures with increasing percentage of diethyl ether (**24**, **26**, **27**, **30**, **31** and **37**). The 6-isomers were collected in the eluate before the 7-ones. Compound **35** was obtained by chloroform extraction of the mother liquors made alkaline by addition of conc. ammonia solution.

*3.4.2.1. 6-Nitro-3-methyl-2(1H)-quinoxalinone (**24**).* 0.08 g (6% yield); m.p. 278–280°C (dec.) (Ref. [8], 280°C); IR: ν 3350, 1660, 1600 cm^{-1}; UV: λ 389, 285, 205 nm; ^1H NMR ($CDCl_3$): δ 12.50 (1H, s, NH), 8.10 (1H, d, $J = 2.4$ Hz, H-5), 7.84 (1H, dd, $J = 8.8$ and 2.4 Hz, H-7), 6.59 (1H, d, $J = 8.8$ Hz, H-8), 2.35 (3H, s, CH_3).

*3.4.2.2. 7-Nitro-3-methylene-1,2,3,4-tetrahydroquinoxalin-2-one (**27**).* 0.17 g (13% yield); m.p. 178–180°C; IR: ν 3350, 3200, 1650, 1600 cm^{-1}; UV: λ 401, 273, 224 infl, 203 nm; ^1H NMR ($CDCl_3$ + DMSO-d_6): δ 12.30 (1H, br s, NH-1), 11.40 (1H, br s, NH-4), 7.49 (1H, s, H-8), 7.47 (1H, d, $J = 7.6$ Hz, H-6), 6.57 (1H, d, $J = 7.6$ Hz, H-5), 5.56 (2H, s, CH_2=C).

The attempted conversion of this compound into **25** in refluxing acetone was not successful.

*3.4.2.3. 6,7-Difluoro-3-methyl-2(1H)-quinoxalinone (**26**).* This compound was obtained in 21% yield and its analytical and spectroscopical data are identical to those above described for the compound obtained by hydrolysis of **6**.

*3.4.2.4. 6-Nitro-3-ethyl-2(1H)-quinoxalinone (**30**) and 6-nitro-3-ethylidene-1,2,3,4-tetrahydroquinoxalin-2-one (**37**).* The title compounds were obtained (0.96 g; 68% yield) as a mixture of tautomers identified by their ^1H NMR spectrum: ($CDCl_3$ + DMSO-d_6): 12.79 (2H, s, NH-1 of **30** and **37**), 10.99 (1H, s, NH-4 of **37**), 8.75 (1H, d, $J = 2.4$ Hz, H-5 of **37**), 8.49 (1H, d, $J = 2.4$ Hz, H-5 of **30**), 8.26 (1H, dd, $J = 9.0$ and 2.4 Hz, H-7 of **30**), 7.53–7.42 (2H, m, H-8 of **30** and H-7 of **37**), 6.90 (1H, d, $J = 8.4$ Hz, H-8 of **37**), 5.48 (1H, q, $J = 7.6$ Hz, $CHCH_3$ of **37**), 2.87 (2H, q, $J = 7.4$ Hz, CH_2CH_3 of **30**), 1.75 (3H, d, $J = 7.4$ Hz, CH_3CH of **37**), 1.28 (3H, t, $J = 7.4$ Hz, CH_3CH_2 of **30**).

The conversion of **37** into **30** was complete once this mixture was heated at reflux in acetone for 1 h.

*3.4.2.5. 7-Nitro-3-ethyl-2(1H)-quinoxalinone (**31**).* 0.10 g (7% yield); m.p. 235–237°C, identical to the compound obtained under Method A.

*3.4.2.6. 7-Fluoro-6-(4-methylpiperazinyl)-3-ethyl-2(1H)-quinoxalinone (**35**).* 0.52 g (39% yield), recrystallized from acetone; m.p. 258–260°C; IR: ν 1670, 1640, 1550 cm^{-1}; UV: λ 359, 244 infl, 224 nm; ^1H NMR (DMSO-d_6): δ 12.20 (1H, s, NH), 7.46 (1H, d, $J = 13.6$ Hz, H-5), 6.78 (1H, d, $J = 8.2$ Hz, H-8), 3.08 (4H, t, $J = 4.4$ Hz, CH_2-2′ + CH_2-6′), 2.74 (2H, q, $J = 7.2$ Hz, CH_2CH_3), 2.47 (4H, t, $J = 4.4$ Hz, CH_2-3′ + CH_2-5′), 2.23 (3H, s, CH_3-N), 1.18 (2H, t, $J = 7.2$ Hz, CH_3CH_2).

4. Microbiology

All the isolated compounds were tested for their in vitro growth inhibitory activity against different bacteria and the yeast *Candida* spp. at the Department of Biomedical Science of Sassari University. The bacterial strains used were *Staphylococcus aureus* ATCC 25923 as Gram positive and *Escherichia coli* ATCC 25922, *E. coli* (hospital isolated), and *Pseudomonas aeruginosa* ATCC 27922 as Gram negative. For antimycotic assay hospital-isolated strains of *Candida* spp. were used. Some compounds were also tested against *Trichomonas vaginalis*, *Leishmania major* and *Acanthamoeba castellanii* as protozoa.

4.1. Antibacterial assay

All bacteria strains were cultured in Lb broth (Luria broth, Difco) and, after overnight incubation at 37°C, were diluted to the optical density of 0.5 McFarland turbidity standard (measured spectrophotometrically at 450 nm). The final inoculum concentration was 10^6 CFU/ml. Each compound was dissolved (1 mg/ml) in dimethyl sulfoxide (DMSO) and then diluted in the test medium. The range of concentrations used for each compound was 500–0.5 μg/ml. MICs were determined by the standard microbroth dilution method [11] as the lowest concentration of the compound which completely inhibited bacteria growth.

4.2. Antimycotic assay

The yeast of *Candida* spp. (hospital isolated) was cultured at 37 ± 1°C in Brain Heart infusion broth (Difco) for 24 h before use. Compounds were dissolved in DMSO. Susceptibility testing was determined using the National Committee for Clinical Laboratory Standards microbroth dilution method [12]. MIC values were determined in the range 500–0.5 μg/ml as the lowest concentration of compound which inhibited *Candida* growth.

4.3. Antiprotozoa assay

T. vaginalis were axenically cultured in Diamond medium [13] containing 10% of heat-inactivated calf serum (Flow, UK) and 5% of carbon dioxide (CO_2) by incubation for 24 h at 37 ± 1°C. Cultures of *L. major* were obtained in 199 medium (Gibco-BRL) Life Technologies containing 10% of heat-inactivated fetal calf serum after overnight incubation at 26 ± 1°C. Stocks of *A. castellanii* cystis, kept at -70°C until used, were slowly thawed and plated on non-nutrient agar seeded with a lawn of *E. coli* ATCC 35218 (NNA-*E. coli*) for 1 day to obtain motile trophozoites. Concentrations of protozoa tested were 2×10^6/ml, as determined using the turbidimetric method.

The compounds tested were dissolved in DMSO (1 mg/ml), then diluted in the test medium by the serial dilution method in order to obtain the required range (100–0.1 μg/ml). MICs were determined as the lowest concentration of the compound which completely inhibited protozoa growth, using both the turbidimetric method and the microscopic determination in the presence of a contrast dye, as described by Zanetti et al. [14].

5. In vitro antitumoral and anti-HIV activity

Some of new compounds synthesized (**6**, **11**, **12**, **13**, **16**, **18**, **19**, **20**, **23**, **26**, **28**, **29**, **33** and **34**) were evaluated for anticancer and anti-HIV activity at the National Cancer Institute (NCI) of Bethesda, MD, following the known in vitro disease-oriented antitumor screening program against a panel of about 60 human tumor cell lines and anti-HIV drug testing system [15,16]. The activity of each compound tested was deduced from the dose–response curve on the basis of the data provided by NCI.

6. Results and discussion

All synthesized quinoxalinones were tested in vitro for antibacterial activity against Gram positive (*S. aureus*) and Gram negative (*E. coli* and *P. aeruginosa*) strains and for antifungal activity against *Candida* spp. (hospital isolated). The results obtained generally indicate that most of these compounds were only poorly active or completely inactive (MIC $\geq$ 500 μg/ml) against bacteria. However, the ethyl derivatives **28** and **29** exhibited a moderate activity against *P. aeruginosa* (MIC = 62.5 and 125 μg/ml, respectively). None showed appreciable activity against *Candida* spp. The derivatives **6**, **11**, **12**, **13**, **14** and **16** have been tested against some protozoa, but only **13** showed a weak appreciable activity against *T. vaginalis* (MIC = 100 μg/ml), while all compounds showed an insignificant activity against both *L. major* and *A. castellanii*. Results of the in vitro anticancer screening showed insignificant activity for most of the compounds tested. Only compounds **12** and **13** exhibited percent growth inhibition on most tumor cell lines at 10^{-4} molar concentration, while compounds **33** and **34** showed, at the same concentration, a moderate activity against leukemia subpanels. Finally, none of the compounds exhibited anti-HIV activity.

Comparison of these results with those previously described by us suggests that, wherever an electron-withdrawing group (CF_3, Cl, F) is present a minimum of biological activity is associated with it. Replacement of the ester group at C-3 with an ethyl seems to retain the antibacterial activity.

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