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Synthesis and HIV-1 inhibitory properties of new tetrahydrobenzoquinazolinedione and tetrahydrobenzocycloheptenuracil derivatives and of their thioxo analogues

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Abstract

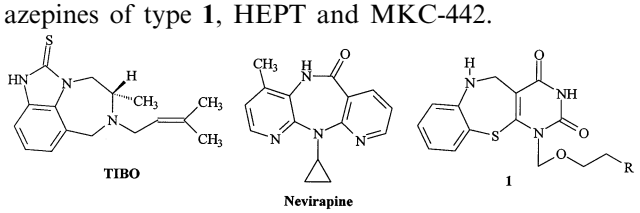
Some new tetrahydrobenzoquinazolinediones **2a**–**4a**, tetrahydrobenzocycloheptenuracils **5a**, **6a** and their thioxo analogues **2b**–**6b** were synthesized within a project aimed at obtaining new HIV-1 tricyclic inhibitors whose scaffold includes a pyrimidine and a phenyl ring, which are present in various HIV-1 non-nucleoside inhibitors. Among the tetrahydrobenzoquinazolinediones **2a**–**4a**, compounds **3a** and **4a**, in which the tricyclic system is respectively in an angular or linear arrangement, proved to possess a HIV-1 inhibitory activity which was in the micromolar range, while compound **2a**, in which the tricyclic system is in the angular arrangement opposite to that of **3a**, was found to be completely inactive. As regards the tetrahydrobenzocycloheptenuracil derivatives (**5a** and **6a**), only **5a** showed an inhibitory activity similar to that of **3a** and **4a**. Furthermore, all thioxo analogues **2b**–**6b** were found to be devoid of any activity.

Keywords: HIV-1; HIV-1 reverse transcriptase; Tetrahydrobenzoquinazolinedione derivatives; Tetrahydrobenzocycloheptenuracil derivatives; Thioxo analogues

1. Introduction

The transcription of viral RNA to proviral DNA by virally encoded reverse transcriptase (RT) is an essential step in the replication of human immunodeficiency virus 1 (HIV-1), the causative agent of AIDS. In these last years, structurally different compounds, including nucleoside and non-nucleoside compounds, have been reported to possess a highly specific activity against HIV-1 RT. In particular, the nucleoside analogues AZT, d4T and 3TC are nowadays used for the treatment of HIV-1-infected patients in combination therapy with protease inhibitors such as Saquinavir, Ritonavir and Indinavir [1,2]. HIV-1 non-nucleoside RT inhibitors are a family of structurally different aromatic compounds, including the tricyclic derivatives TIBO [3], Nevirapine [4] and benzothiazepines **1** [5] and the 6-phenylthio-pyrimidine derivative HEPT [6] and its 6-benzyl analogue MKC-442 [7].

As a part of a project aiming to design new HIV-1 non-nucleoside inhibitors, we focused our attention on the study of new tricyclic compounds whose scaffold was characterized by the presence of a pyrimidine and a phenyl ring, which are common structural features of various non-nucleoside inhibitors such as benzothiazepines of type **1**, HEPT and MKC-442.



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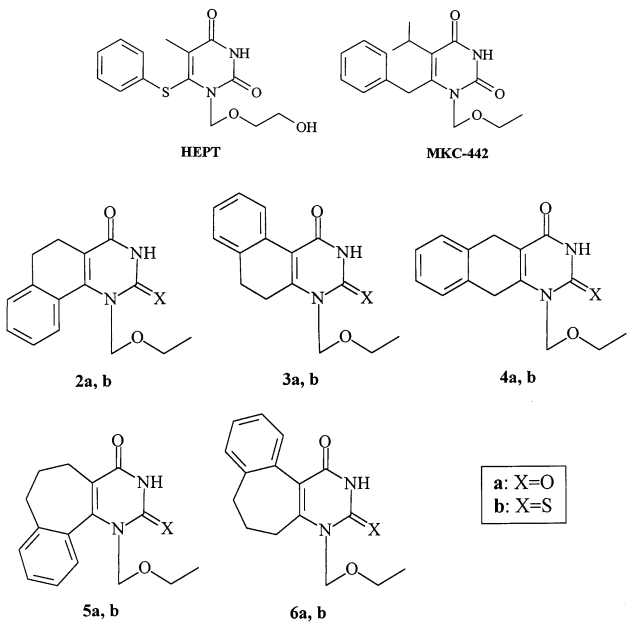
We report here the synthesis and the anti-HIV-1 properties of some tetrahydrobenzoquinazolinedione (**2a**–**4a**) and tetrahydrobenzocycloheptenuracil derivatives (**5a**, **6a**), in which the phenyl and pyrimidine rings are a constituent part of 6,6,6 (**2a**–**4a**) or 6,7,6 (**5a**, **6a**) tricyclic frameworks. In compounds **2a**–**6a**, the ethoxymethyl side-chain of MKC-442 was inserted on the pyrimidine ring.

Furthermore, the thioxo analogues of **2a**–**6a** (**2b**–**6b**) were also synthesized and tested for their HIV-1 inhibitory properties.



2. Chemistry

Compounds **2**–**6** were synthesized as shown in Scheme 1. The α-tetralone **7**, α-benzosuberone **8** and β-tetralone **9** were converted into the appropriate β-ketoesters **11**–**14** [8–11] following synthetic procedures previously described in literature (for **11**–**13**, by reaction of α-tetralone **7**, α-benzosuberone **8** and β-tetralone **9** with dimethylcarbonate in the presence of NaH [8–10]; for **14**, by treatment of β-tetralone **9** with magnesium methyl carbonate followed by esterification of the resulting β-ketoacid with diazomethane [11]). Treatment of β-benzosuberone **10** [12] with dimethyl carbonate, using the same procedure as for the preparation of **11**–**13**, yielded the β-ketoester **15** [13].

The β-ketoesters **11**–**15** were condensed with thiourea at 180°C to give the corresponding 2-thiouracil derivatives **16**–**20**, which were then refluxed with a 10% aqueous solution of chloroacetic acid to yield the appropriate uracil derivatives **21**, **22**, **23** [14], **24** and **25**. The 2-thiouracil (**16**, **17**, **19**, **20**) and uracil (**21**, **22**, **23** and **25**) derivatives were silylated with 1,1,1,3,3,3-hexamethyldisilazane and then condensed with diethoxymethane, using trimethylsilyl trifluoromethanesulfonate as the catalyst, to give the desired compounds **2**, **5**, **4** and **6**. Compounds **3** were obtained in situ by silylation of **18** and **23** with *N*,*O*-bis-(trimethylsilyl)-acetamide, followed by reaction with chloromethyl ethyl ether.

3. Biological results and conclusions

Compounds **2**–**6** were tested for their HIV-1 inhibitory activity at a concentration of 5 μM, using lymphoblastoid $CD4^+$ cells (C8166), infected with the HIV strain HTLV-IIIB derived from chronically infected H9 cells [15].

Among the tetrahydrobenzoquinazolinedione derivatives **2a**–**4a**, compounds **3a** and **4a** showed a certain activity, with inhibition percentages of 41 and 37%, respectively, while compound **2a**, at the concentration used, proved to be inactive. As regards the tetrahydrobenzocycloheptenuracil derivatives (**5a**, **6a**), only **5a** showed an appreciable inhibition percentage of 36%.

Furthermore, all the thioxo analogues **2b**–**6b** were found to be devoid of any activity.

The tetrahydrobenzoquinazolinediones **2a**–**4a**, tetrahydrobenzocycloheptenuracils **5a**, **6a** and their thioxo analogues **2b**–**6b** were synthesized within a project aiming to design new HIV-1 tricyclic derivatives in which a pyrimidine and a phenyl ring were present.

An analysis of the HIV-1 inhibitory properties of compounds **2a**–**6a** indicates that in the case of tetrahydrobenzoquinazolinedione derivatives **2a**–**4a**, while compounds **3a** and **4a**, in which the tricyclic system is in an angular or linear arrangement, respectively, possess a certain HIV inhibitory activity in the micromolar range, compound **2a**, in which the tricyclic system is in the angular position opposite to that of **3a**, proved to be completely inactive. Among the tetrahydrobenzocycloheptenuracil derivatives (**5a** and **6a**), only **5a** showed an inhibitory activity similar to that of **3a** and **4a**.

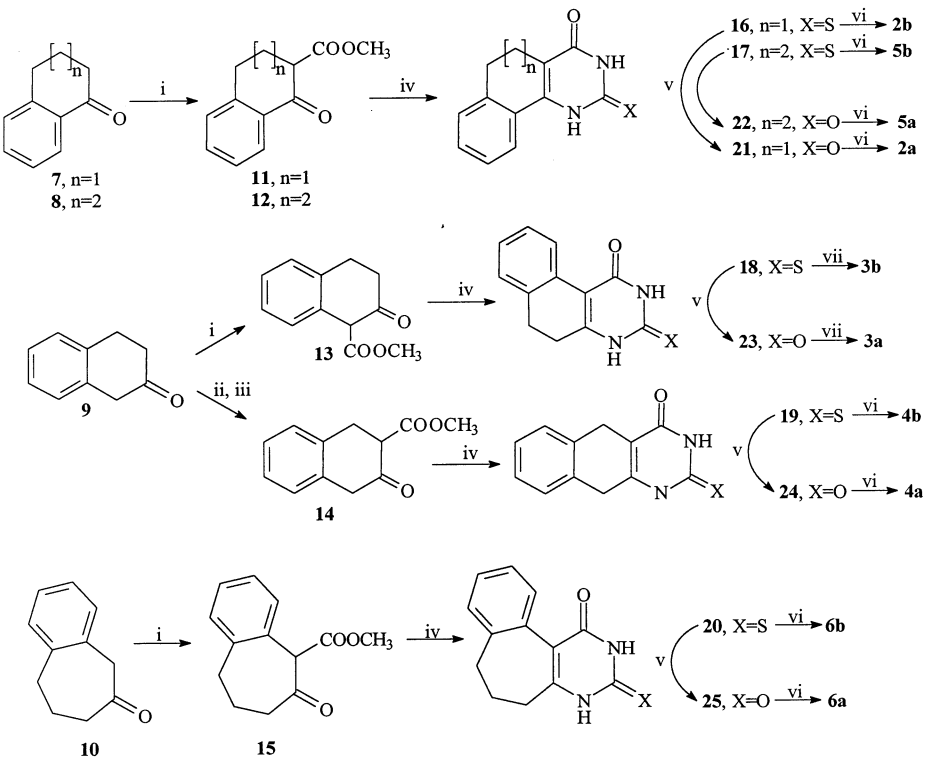
The substitution of the oxygen of the pyrimidine portion of **2a**–**6a** with a sulfur atom (compounds **2b**–**6b**) did not afford any positive result.

On the basis of the modest activity shown, these new tricyclic compounds may be considered to be devoid of any significant interest in the field of anti-HIV compounds.

4. Experimental

4.1. Chemistry

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. IR spectra for



Scheme 1. Reagents and conditions: (i) dimethylcarbonate, NaH (60% dispersion in oil), reflux, 5 h; (ii) magnesium methyl carbonate, anhydrous DMF, 130°C, 12 h; (iii) diazomethane, Et_2O; (iv) thiourea, 180°C, 2 h; (v) 10% chloroacetic acid, reflux, 24 h; (vi) (a) 1,1,1,3,3,3-hexamethyldisilazane (HMDS), Na_2SO_4, 130°C, 6 h and (b) trimethylsilyltriflate, diethoxymethane, CH_3CN, -45°C, 3 h; (vii) (a) bis-(trimethylsilyl)acetamide (BSA), $CHCl_3$, r.t., 24 h and (b) chloromethyl ethyl ether, r.t., 24 h.

comparison of compounds were taken as paraffin oil mulls or as liquid film on a Mattson 1000 FTIR spectrometer. ^{1}H NMR spectra of all compounds were obtained with a Varian CFT-20 instrument operating at 80 MHz or with a Bruker AC-200 instrument operating at 200 MHz, in a ca. 2% solution of $CDCl_3$ or DMSO-d_6, using Me_4Si or $Me_3Si(CH_2)_3SO_3Na$ as the internal standard, respectively. The proton magnetic resonance assignments were established on the basis of the expected chemical shifts and the multiplicity of the signals. For ^{1}H NMR spectra, only the most significant details are reported. Mass spectra were recorded on a HP-5988 A spectrometer. Elemental analyses were carried out by our analytical laboratory and were consistent with theoretical values to within $\pm 0.4\%$. Analytical TLCs were carried out on 0.25 mm layer silica gel plates containing a fluorescent indicator; spots were detected under UV light (254 nm). Column chromatographies were performed using 230–400 mesh silica gel (Macherey–Nagel Silica Gel 60 Art. No. 815381). Magnesium sulfate was always used as the drying agent. Evaporations were performed in vacuo (rotating evaporator). The α-tetralone **7**, the α-benzosuberone **8** and the β-tetralone **9** were commercially available (Aldrich).

*4.1.1. Synthesis of β-ketoesters **11–14** [8–11]*

β-Ketoesters **11**–**13** were prepared as previously reported in literature [8–10] by reaction of α-tetralone **7**, α-benzosuberone **8** and β-tetralone **9** with dimethylcarbonate in the presence of NaH.

The β-ketoester **14** was synthesized as previously reported in Ref. [11] by treatment of β-tetralone **9** with magnesium methyl carbonate followed by esterification of the resulting β-ketoacid with diazomethane.

11 [8]: (86%). ^{1}H NMR ($CDCl_3$, 80 MHz): δ 2.41–2.63 (m, 2H), 2.91–3.01 (m, 2H), 3.74 (s, 1H), 3.80 (s, 3H), 7.15–8.00 (m, 4H).

12 [9]: (84%). ^{1}H NMR ($CDCl_3$, 80 MHz): δ 2.20–3.30 (m, 6H), 3.75 (s, 1H), 3.90 (s, 3H), 7.22–7.64 (m, 4H).

13 [10]: (68%). ^{1}H NMR ($CDCl_3$, 80 MHz): δ 2.40–2.90 (m, 4H), 3.76 (s, 1H), 3.89 (s, 3H), 7.00–7.80 (m, 4H).

14 [11]: (45%). ^{1}H NMR ($CDCl_3$, 200 MHz): δ 3.64 (s, 4H), 3.83 (s, 3H), 7.10–7.36 (m, 4H), 12.24 (s, 1H).

*4.1.2. Synthesis of β-ketoester **15** [13]*

The β-ketoester **15** was prepared using the same synthetic procedure previously reported in literature for the preparation of β-ketoesters **11**–**13**.

Sodium hydride (60%; 8.24 g, 0.21 mol), prewashed with hexane, was treated dropwise, under magnetic stirring and cooling in ice, with β-benzosuberone **10** [12] (0.14 mol) under an argon atmosphere. After the addition had been completed, dimethyl carbonate (342 ml, 4.06 mol) was added dropwise. The reaction mixture was heated to reflux for 5 h under an argon atmosphere, then concentrated to eliminate most of the dimethyl carbonate. The residual mass obtained was cooled and acidified with 10% HCl (500 ml). This solution was extracted with Et_2O (3 × 150 ml). The organic phase was washed with brine (3 × 100 ml), dried and evaporated to dryness under reduced pressure to give a crude oil which was further purified by column chromatography, eluting with a 9:1 hexane/EtOAc mixture, to yield pure β-ketoester **15** as a low-melting solid.

15: (79%) m.p. = 53–55 °C (lit. [13] 55.5–56.5 °C). ^{1}H NMR ($CDCl_3$, 80 MHz): δ 2.08–2.27 (m, 4H), 2.51–2.75 (m, 2H), 3.76 (s, 3H), 7.09–7.28 (m, 4H), 10.05 (s, 1H). *Anal.* $C_{13}H_{14}O_3$ (C, H). MS m/z 218 (M^+).

*4.1.3. Synthesis of 2-thiouracil derivatives **16–20***

A mixture composed of the appropriate β-ketoesters **11–15** (4.60 mmol) and thiourea (0.350 g, 4.60 mmol) was heated to 180°C for 2 h. After cooling at room temperature, the reaction mixture was diluted with acetone (7 ml) and filtered through a frit to give a crude white solid composed mainly of the desired 2-thiouracil derivatives (**16–20**) which were purified by crystallization using absolute EtOH.

16: (33%) m.p. = 245–247°C. ^{1}H NMR (DMSO-d_6, 80 MHz): δ 2.38–2.92 (m, 4H), 7.20–7.45 (m, 3H), 7.90–8.05 (m, 1H), 11.31 (s, 1H), 11.60 (s, 1H). *Anal.* $C_{12}H_{10}N_2OS$ (C, H, N). MS m/z 230 (M^+).

17: (16%) m.p. = 275–277°C. ^{1}H NMR (DMSO-d_6, 200 MHz): δ 1.97–2.17 (m, 4H), 2.48–2.64 (m, 2H), 7.33–7.50 (m, 4H), 12.44 (s, 1H), 12.49 (s, 1H). *Anal.* $C_{13}H_{12}N_2OS$ (C, H, N). MS m/z 244 (M^+).

18: (27%) m.p. = 326–328°C. ^{1}H NMR (DMSO-d_6, 200 MHz): δ 2.61–2.81 (m, 4H), 7.10–7.25 (m, 3H), 8.33–8.38 (m, 1H), 12.54 (s, 1H), 12.59 (s, 1H). *Anal.* $C_{12}H_{10}N_2OS$ (C, H, N). MS m/z 230 (M^+).

19: (21%) m.p. = > 300°C. ^{1}H NMR (DMSO-d_6, 200 MHz): δ 3.35–3.55 (m, 2H), 3.75–3.79 (m, 2H), 7.18–7.30 (m, 4H), 12.44 (s, 2H). *Anal.* $C_{12}H_{10}N_2OS$ (C, H, N). MS m/z 230 (M^+).

20: (18%) m.p. = 295–299°C. ^{1}H NMR (DMSO-d_6, 80 MHz): δ 2.16–2.68 (m, 6H), 7.15–7.68 (m, 4H), 11.13 (s, 1H), 11.17 (s, 1H). *Anal.* $C_{13}H_{12}N_2OS$ (C, H, N). MS m/z 244 (M^+).

*4.1.4. Synthesis of uracil derivatives **21–23** [14], **24** and **25***

The appropriate 2-thiouracil derivatives (**16–20**) (0.43 mmol) were suspended in 10% aqueous chloroacetic acid (11 ml, 11.54 mmol). The suspension obtained was heated to reflux for 12 h under magnetic stirring. After cooling, the suspension was filtered through a frit and washed with 95% EtOH (10 ml) and with Et_2O (30 ml) to give a crude solid which was purified by crystallization using absolute EtOH.

21: (81%) m.p. = 272–275°C. ^{1}H NMR (DMSO-d_6, 80 MHz): δ 2.37–2.83 (m, 4H), 7.24–7.50 (m, 3H), 7.82–7.98 (m, 1H), 10.91 (s, 1H), 11.08 (s, 1H). *Anal.* $C_{12}H_{10}N_2O_2$ (C, H, N). MS m/z 214 (M^+).

22: (52%) m.p. = 267–269°C. ^{1}H NMR (DMSO-d_6, 80 MHz): δ 2.03–2.11 (m, 4H), 2.42–2.63 (m, 2H), 7.31–7.52 (m, 4H), 10.77–11.12 (m, 2H). *Anal.* $C_{13}H_{12}N_2O_2$ (C, H, N). MS m/z 228 (M^+).

23: (37%) m.p. = 245–247°C. ^{1}H NMR (DMSO-d_6, 200 MHz): δ 2.53–2.85 (m, 4H), 7.09–7.28 (m, 3H), 8.32–8.41(m, 1H), 11.15 (s, 1H), 11.23 (s, 1H). *Anal.* $C_{12}H_{10}N_2O_2$ (C, H, N). MS m/z 214 (M^+).

24: (83%) m.p. = 273–275°C. ^{1}H NMR (DMSO-d_6, 80 MHz): δ 3.45–3.75 (m, 4H), 7.10–7.25 (m, 4H), 10.83 (s, 1H), 10.93 (s, 1H). *Anal.* $C_{12}H_{10}N_2O_2$ (C, H, N). MS m/z 214 (M^+).

25: (79%) m.p. = 270–275°C (dec.). ^{1}H NMR (DMSO-d_6, 200 MHz): δ 2.15–2.51 (m, 6H), 7.13–7.46 (m, 4H), 11.10 (s, 1H), 11.14 (s, 1H). *Anal.* $C_{13}H_{12}N_2O_2$ (C, H, N). MS m/z 228 (M^+).

*4.1.5. Synthesis of tetrahydrobenzoquinazolinediones **2a**, **4a**, tetrahydrobenzocycloheptenuracils **5a**, **6a** and their thioxo analogues **2b**, **4b–6b***

A mixture of the appropriate 2-thiouracil (**16**, **17**, **19**, **20**) or uracil (**21**, **22**, **23** and **25**) derivatives (0.9 mmol), 1,1,1,3,3,3-hexamethyldisilazane (4.5 ml, 21 mmol) and anhydrous $(NH_4)_2SO_4$ (0.009 g, 0.068 mmol) was heated to reflux for 2 h under magnetic stirring and an argon atmosphere. The mixture was concentrated, under an argon atmosphere, at room temperature under reduced pressure to obtain the silylated base as a pale yellow solid. Anhydrous CH_3CN (7 ml) was added, and the solution was stirred at −45°C. Trimethylsilyl trifluoromethanesulfonate (0.16 ml, 0.9 mmol) was added followed by dropwise addition of diethoxymethane (0.22 ml, 1.8 mmol). The reaction was quenched after 3 h and neutralized by the addition of saturated aqueous $NaHCO_3$ at −45°C and evaporated in vacuo at room temperature to dryness. The residue was diluted with water (40 ml) and extracted with Et_2O (3 × 15 ml). The ether extracts were dried and evaporated under reduced pressure to give a crude solid composed mainly of the desired final compound. This was purified by column chromatography (chromatographic eluants: **2a**, 8:3:0.1 hexane/EtOAc/*i*-PrOH; **2b**, 4:1 hexane/EtOAc; **4a**, 20:4:1 hexane/$CHCl_3$/*i*-PrOH; **4b**, 20:4:1 hexane/$CHCl_3$/*i*-PrOH; **5a**, 9:4:1 hexane/$CHCl_3$/*i*-PrOH; **5b**, 9:4:1 hexane/$CHCl_3$/*i*-PrOH; **6a**, 8:1:0.5 hexane/$CHCl_3$/*i*-PrOH; **6b**, 8:1:0.5 hexane/$CHCl_3$/*i*-PrOH) to yield pure **2a**, **4a–6a** and **2b**, **4b–6b** as a white solid.

2a: (47%) m.p. = 205–207°C. ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.11 (t, 3H, $J = 7.2$ Hz), 2.65–2.83 (m, 4H), 3.66 (q, 2H, $J = 7.2$ Hz), 5.45 (s, 2H), 7.24–7.36 (m, 3H), 7.68–7.72 (m, 1H), 10.14 (m, 1H). *Anal.* $C_{15}H_{16}N_2O_3$ (C, H, N). MS m/z 272 (M^+).

2b: (44%) m.p. = 175–177°C. ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.25 (t, 3H, $J = 6.95$ Hz), 2.79–2.95 (m, 4H), 3.73 (q, 2H, $J = 6.95$ Hz), 5.54 (s, 2H), 7.21–7.40 (m, 4H), 8.16–8.20 (m, 1H). *Anal.* $C_{15}H_{16}N_2O_2S$ (C, H, N). MS m/z 288 (M^+).

4a: (12%) m.p. = 238–240°C. ^{1}H NMR ($CDCl_3$, 80 MHz): δ 1.23 (t, 3H, $J = 7.2$ Hz), 3.51–4.15 (m, 6H), 5.43 (s, 1H), 5.47 (s, 1H), 7.16–7.30 (m, 4H), 10.11 (s, 1H). *Anal.* $C_{15}H_{16}N_2O_3$ (C, H, N). MS m/z 272 (M^+).

4b: (17%) m.p. = 220–222°C. ^{1}H NMR ($CDCl_3$, 80 MHz): δ 1.25 (t, 3H, $J = 7.2$ Hz), 3.45–3.98 (m, 6H), 5.49 (s, 1H), 5.53 (s, 1H), 7.21–7.36 (m, 4H), 11.81 (s, 1H). *Anal.* $C_{15}H_{16}N_2O_2S$ (C, H, N). MS m/z 288 (M^+).

5a: (44%) m.p. = 150–152°C. ^{1}H NMR ($CDCl_3$, 80 MHz): δ 1.15 (t, 3H, $J = 7.2$ Hz), 1.92–3.11 (m, 6H), 3.43–3.72 (m, 2H), 4.90–5.32 (m, 2H), 7.22–7.62 (m, 4H), 8.71–8.94 (m, 1H). *Anal.* $C_{16}H_{18}N_2O_3$ (C, H, N). MS m/z 286 (M^+).

5b: (52%) m.p. = 162–164°C. ^{1}H NMR ($CDCl_3$, 80 MHz): δ 1.09–1.40 (m, 3H), 2.12–2.59 (m, 6H), 3.37–3.84 (m, 2H), 4.73 (s, 1H), 4.86 (s, 1H), 5.39 (s, 1H), 7.22–7.72 (m, 4H). *Anal.* $C_{16}H_{18}N_2O_2S$ (C, H, N). MS m/z 302 (M^+).

6a: (46%) m.p. = 195–197°C. ^{1}H NMR ($CDCl_3$, 80 MHz): δ 1.22 (t, 3H, $J = 7.2$ Hz), 2.18–2.71 (m, 6H), 3.71 (q, 2H, $J = 7.2$ Hz), 5.47 (s, 2H), 7.10–7.65 (m, 4H), 10.11 (s, 1H). *Anal.* $C_{16}H_{18}N_2O_3$ (C, H, N). MS m/z 286 (M^+).

6b: (48%) m.p. = 167–169°C. ^{1}H NMR ($CDCl_3$, 80 MHz): δ 1.24 (t, 3H, $J = 7.2$ Hz), 2.20–2.75 (m, 6H), 3.78 (q, 2H, $J = 7.2$ Hz), 5.89 (s, 2H), 7.12–7.62 (m, 4H), 10.13 (s, 1H). *Anal.* $C_{16}H_{18}N_2O_2S$ (C, H, N). MS m/z 302 (M^+).

*4.1.6. Synthesis of tetrahydrobenzoquinazolinedione **3a** and its thioxo analogue **3b***

The appropriate 2-thiouracil derivative **19** or uracil derivative **24** (0.87 mmol) was suspended in anhydrous $CHCl_3$ (10 ml) under magnetic stirring and argon atmosphere. *N,O*-bis-(Trimethylsilyl)acetamide (0.46 ml, 1.85 mmol) was added to this suspension. The reaction mixture was kept under stirring and argon until a limpid solution was obtained. Chloromethyl ethyl ether (0.1 ml, 1.25 mmol) was added to this solution. The reaction mixture was stirred for 24 h at room temperature. After evaporation of the solvent under reduced pressure, the resulting syrup was purified by column chromatography eluting with a hexane/EtOAc/*i*-PrOH mixture (4:1:0.05 in the case of **3a** and 10:3:0.1 in the case of **3b**) to yield pure **3a** and **3b** as a white solid.

3a: (12%) m.p. = 180–182°C. ^{1}H NMR ($CDCl_3$, 80 MHz): δ 1.21 (t, 3H, $J = 7.2$ Hz), 2.88 (s, 4H), 3.65 (q, 2H, $J = 7.2$ Hz), 5.42 (s, 2H), 7.08–7.32 (m, 3H), 8.28–8.48 (m, 1H), 9.10 (s, 1H). *Anal.* $C_{15}H_{16}N_2O_3$ (C, H, N). MS m/z 272 (M^+).

3b: (15%) m.p. = 165–167°C. ^{1}H NMR ($CDCl_3$, 80 MHz): δ 1.23 (t, 3H, $J = 7.2$ Hz), 2.94 (s, 4H), 3.79 (q, 2H, $J = 7.2$ Hz), 6.01 (s, 2H), 7.10–7.25 (m, 3H), 8.25–8.52 (m, 1H), 9.70 (s, 1H). *Anal.* $C_{15}H_{16}N_2O_2S$ (C, H, N). MS m/z 288 (M^+).

4.2. Virus and cells

The HTLV-IIIB strain of HIV-1 derived from chronically infected H9 cells. Acute infection with HIV-1 were carried out in the $CD4^+$ lymphoblastoid cell line C8166 containing the HTLV-I genome and expressing only the *tax* gene [16]. These cells were maintained in RPMI supplemented with 10% fetal calf serum and gentamicin.

4.2.1. Assay of antiviral activity

The antiviral activity of substances was evaluated in terms of inhibition of virus yield in the presence of the drugs. Briefly C8166 cells were incubated at 0°C with HTLV-IIIB at a multiplicity of infection (MOI) of 0.001. 72 h later the cells were subjected to three cycles of freeze–thawing, cells and cells debris were removed by low speed centrifugation and the supernatants were titrated as previously described [15] in C8166 cells by the standard limiting dilution method (0.5 log ratio, three replicates for dilution) in 96-well microtitre plates. Infectious titre, expressed as tissue culture infectious doses ($TCID_{50}$)/ml, was calculated by the method of Reed and Muench.

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