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Synthesis of new *N*-isobutyryl-L-cysteine/MEA conjugates: Evaluation of their free radical-scavenging activities and anti-HIV properties in human macrophages

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Abstract

Four novel *N*-isobutyryl-L-cysteine/2-mercaptoethylamine (MEA, cysteamine) conjugates have been designed and synthesized. The antioxidant activities of these new series were evaluated by three different free radical scavenging methods (DPPH test, ABTS test, and deoxyribose assay) and their metal binding capacity was evaluated by the ethidium bromide fluorescence binding assay. These results were compared with those obtained with their pro-GSH acetyl analogues recently developed in our laboratory. We observed that most of these compounds exhibit free radical-scavenging activities similar to those of Trolox, but always superior than NAC. While none of these new derivatives had pro-GSH activities, they displayed anti-HIV properties in human monocyte-derived macrophages infected *in vitro*. The present study demonstrates that these new *N*-isobutyryl derivatives, which are expected to have a greater bioavailability than their acetyl analogues, may have useful applications in HIV infection in respect to their antioxidant and anti-HIV activities.

Keywords: Cysteine derivatives; Lipophilic compounds; Radical scavenging; Antioxidant activity; DNA damage; Anti-HIV activity

1. Introduction

Reactive oxygen species (ROS), resulting from excitation or incomplete reduction of molecular oxygen, are highly reactive, toxic oxygen moieties that affects numerous critical cellular functions. The absence of efficient cellular detoxification mechanisms which remove these radicals can lead to proteins and DNA oxidation, lipid peroxidation, and ultimately cell death [1,2].

To counteract these damaging radicals, antioxidant systems have evolved, including enzymes such as glutathione peroxidase, glutathione reductase, superoxide dismutase, *S*-methyl transferase, and catalase. The cellular arsenal for scavenging ROS and toxic organic radicals also include smaller molecules such as ascorbate, glutathione, tocopherol, carotenoids, polyphenols, alkaloids, and other compounds. Thiol-containing compounds have an essential role in many biochemical reactions due to their ability to be easily oxidized and then quickly regenerated. Main representatives are glutathione, lipoic acid, and thioredoxin, which are synthesized *de novo* in mammalian cells. On the other hand, cysteine-derived compounds such as *N*-acetyl-L-cysteine (NAC) and bucillamine are synthetic thiols which have been administered in experimental and clinical studies for treatment of conditions associated with oxidative stress [3,4]. *N*-Acetyl-L-cysteine acts as an indirect precursor of glutathione (GSH) by raising intracellular level of cysteine, a precursor of glutathione. NAC is being studied and utilized in conditions characterized by decreased GSH or oxidative stress such as in HIV infections, cancer, and heart disease [5]. These properties

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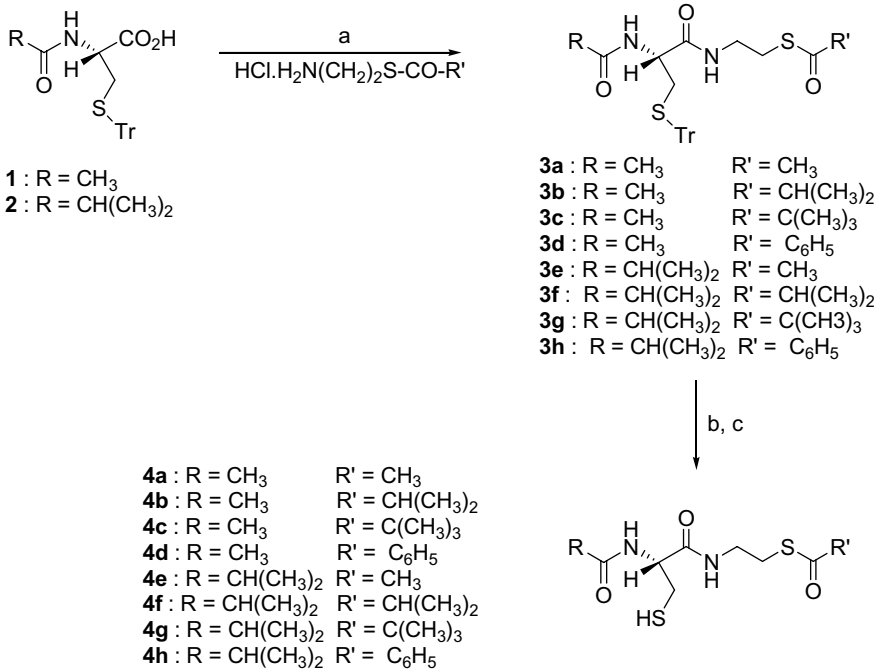
prompted several research groups, including ours to investigate the antioxidant potential of different conjugated cysteine derivatives [6–11]. Recently, we reported that various NAC analogues and notably *N*-(*N*-acetyl-L-cysteinyl)-*S*-acetylcysteamine) **4a**, increased intracellular GSH levels in various cell lines and displayed interesting antiviral activities [6–8]. We postulated that these compounds are *S*-deacetylated upon esterase activation to their corresponding dithiol derivatives which may be responsible for *in situ* release of NAC and cysteamine. GSH-deficiency is associated with impaired survival rates in HIV-infected patients and the administration of pro-GSH drugs such as NAC, has been shown to decrease mortality [12]. However, in the case of compound **4a** and its analogues **4b–d** (Scheme 1), we were unable to correlate their pro-GSH activities with their good anti-HIV effects. We thus suspected, that, due to its sulfhydryl group, **4a** may have antioxidant properties of its own and therefore may function by either directly scavenging free radicals, by inhibiting their generation, or by chelating metals which are known to induce free radicals formation by a variety of processes including the Fenton reaction. In view of the importance of antioxidants in chemoprevention of a number of degenerative diseases, we decided to evaluate the antioxidant activities of *N*-acetyl thiols **4a–d** using various antioxidant assays, including 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\cdot}$), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS$^{\cdot+}$), deoxyribose degradation, and the ethidium bromide fluorescence binding assay. Following this work, compounds **4e–h** (Scheme 1) were designed and synthesized with the aim of producing efficient antioxidant compounds which were expected to be much more biologically stable than their acetyl counterparts, while keeping their antioxidant potency [13,14]. The results obtained are compared with well-known antioxidants such as cysteine, NAC, GSH, and Trolox (a polar analogue of vitamin E). The anti-HIV properties in human monocyte-derived macrophages infected *in vitro* of this new *N*-isobutyryl series were evaluated in parallel.

2. Materials and methods

2.1. General

Unless otherwise stated, materials were purchased from commercial suppliers and used without further purification. Melting points were determined on a Büchi capillary melting point apparatus and are uncorrected. Nuclear magnetic resonance spectra (^{1}H NMR) were recorded on a Brüker AC 250 in $CDCl_3$. Chemical shifts are reported in ppm and given in δ units with respect to TMS, used as an internal standard. FAB mass spectra were recorded on a JEOL DX 300 mass spectrophotometer in the positive ion mode, using 1:1 glycerol/thioglycerol. Specific optical rotations were recorded on a Perkin-Elmer 241 polarimeter. Elemental analysis was carried out by the Service Central de Microanalyses du CNRS de Vernaison (France). Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel 60 F_{254} plates. Spots were visualized by exposure to ultraviolet light (254 nm), iodine vapor, or



(a) (i) AcOEt, IBC, NMM, -15 °C, (ii) HOSu, -15 °C, (iii) NMM, -15 °C-rt, 12 h; (b) MeOH, $CHCl_3$, mixture of MeOH/$AgNO_3$/pyridine, rt; (c) $CHCl_3$, HCl 37%, rt..

Scheme 1. Synthesis of compounds **4a–h**.

by spraying with ninhydrin solution. Flash column chromatography was conducted with Merck silica gel 60 (230–400 mesh ASTM). All the commercial reagents and solvents were of analytical grade and were purchased from Aldrich or Fluka. *N*-Isobutyryl-L-cysteine was obtained from Fluka. *S*-Acetylcysteamine hydrochloride and *S*-benzoylcysteamine hydrochloride were prepared as previously described [15]. UV spectra were recorded on a Varian Cary 300 Bio UV/Vis spectrometer. Fluorescence spectra were acquired on a SML AMINCO 8100 spectrofluorometer (slit width 4 nm for excitation and detection). Studies were carried out at 20 °C.

2.2. Synthesis

2.2.1. *N*-Isobutyryl-*S*-trityl-L-cysteine (**2**)

A suspension of *N*-isobutyryl-L-cysteine (4.1 g, 21.5 mmol) and triphenylmethanol (5.6 g, 21.5 mmol) in acetic acid (16 mL) was stirred at room temperature and boron trifluoride diethyl etherate (4.1 mL, 32.2 mmol) was added dropwise while maintaining the temperature at about 20–25 °C. After the addition was complete, stirring was continued at room temperature for 3 h and the solution was cooled to 0 °C and then added of saturated aqueous sodium acetate (70 mL) and water (140 mL). An immediate colourless paste formed. After 12 h at 0 °C, the reaction mixture was vigorously stirred and partitioned between diethyl ether (120 mL) and ice-water (150 mL). The organic layer was separated and the aqueous layer was further extracted with Et_2O (4× 80 mL). The combined organic fractions were washed with ice-water (4× 60 mL), dried (Na_2SO_4), filtered, and evaporated under to dryness under vacuum. The residue was then purified by trituration in hexane to yield **2** (7.54 g, 81%) as gum. TLC and NMR analysis indicated that the product was sufficiently pure and could be used without further purification. TLC (CH_2Cl_2/MeOH/AcOH, 9.4:0.6:0.07) $R_f = 0.53$. α_D^{20} +21.4° (*c* 1.12, $CHCl_3$). ^{1}H NMR: 1.12, 1.14 (2d, $J = 2\times$ 6.9 Hz, 2× 3H, C(C*H*$_3$)$_2$), 2.24–2.42 (m, 1H, C*H*(CH$_3$)$_2$), 2.64–2.28 (m, 2H, C*H*$_2$), 4.31–4.43 (m, 1H, α *H*), 5.67–6.15 (m, 1H, CO_2H), 5.90 (overlapping d, $J =$ 7.1 Hz, 1H, N*H*); 7.18–7.33, 7.37–7.46 (2m, 15H, Ar*H*). MS *m*/*z* 867 $(2M+H)^+$, 434 $(M+H)^+$, 865 $(2M-H)^+$, 432 $(M-H)^+$. Anal. Calcd for $C_{26}H_{27}NO_3S$: C, 72.06; H, 6.24; N, 3.23. Found: C, 72.24; H, 6.22; N, 3.28.

2.2.2. *N*-(*N*-Isobutyryl-*S*-trityl-L-cysteinyl)-*S*-acetylcysteamine (**3e**)

A stirred solution of *N*-isobutyryl-*S*-trityl-L-cysteine (**2**, 1.7 g, 3.93 mmol) in EtOAc (20 mL) was cooled to −15 °C and successively treated with isobutyl chloroformate (IBC, 509 μL, 3.93 mmol), 4-methylmorpholine (NMM, 435 μL, 3.96 mmol), and then (15 min) *N*-hydroxysuccinimide (HOSu, 452 mg, 3.93 mmol). After stirring for 30 min at −15 °C, *S*-acetylcysteamine hydrochloride [15] (672 mg, 4.32 mmol) was added to the mixture, followed by NMM (474 μL, 4.32 mmol), which was added one drop at a time. The reaction mixture was then stirred for 1 h at 0 °C and at room temperature for 12 h. The insoluble salt was collected by filtration and washed with EtOAc (3× 7 mL). The organic phases were pooled, washed [water (2× 25 mL), ice-cold saturated aqueous $NaHCO_3$ (18 mL), water (2× 25 mL), ice-cold 1 N citric acid (20 mL), and water (neutral pH)], dried (Na_2SO_4), filtered, and evaporated to dryness under vacuum. The residue was purified by flash column chromatography (EtOAc/petroleum ether, 6:4) to give **3e** as a white foam (1.41 g, 67%). TLC (EtOAc/petroleum ether, 6:4) $R_f = 0.5$. α_D^{20} +9.3° (*c* 0.97, $CHCl_3$). ^{1}H NMR: 1.10 (d, $J = 6.9$ Hz, 6H, C(C*H*$_3$)$_2$), 2.18–2.36 (m, 1H, C*H*(CH$_3$)$_2$), 2.29 (overlapping s, 3H, SCOC*H*$_3$); 2.50 (dd, $J = 5.6$, 12.8 Hz, 1H, β *H*a cys), 2.72 (dd, $J = 6.7$, 12.8 Hz, 1H, β *H*b cys), 2.88–3.01 (m, 2H, NCH$_2$C*H*$_2$S), 3.29–3.41 (m, 2H, NC*H*$_2$CH$_2$S), 4.06–4.19 (m, 1H, α *H* cys), 5.82 (d, $J = 7.4$ Hz, 1H, N*H* cys), 6.42 (t, $J = 5.5$ Hz, 1H, N*H*CH$_2$), 7.17–7.35, 7.39–7.48 (2m, 15H, Ar*H*). MS *m*/*z* 535 $(M+H)^+$. Anal. Calcd for $C_{30}H_{34}N_2O_3S$: C, 67.42; H, 6.37; N, 5.24. Found: C, 67.05; H, 6.72; N, 5.30.

2.2.3. *N*-(*N*-Isobutyryl-*S*-trityl-L-cysteinyl)-*S*-isobutyrylcysteamine (**3f**)

This compound was prepared according to the general procedure described for **3e**, using **2** (1.7 g, 3.93 mmol) and *S*-isobutyrylcysteamine hydrochloride (obtained by the procedure described for *S*-acetylcysteamine hydrochloride [15], mp 147–148 °C). The crude product was purified by flash column chromatography (EtOAc/petroleum ether, 6.5:2.5) to give **3f** as a colourless foam (1.66 g, 75%). TLC (EtOAc/petroleum ether, 5:5) $R_f = 0.6$. α_D^{20} +7.9° (*c* 1.27, $CHCl_3$). ^{1}H NMR: 1.107, 1.110, 1.159, 1.162 (4d, $J = 4\times$ 6.9 Hz, 4× 3H, 2× C(C*H*$_3$)$_2$), 2.17–2.32 (m, 1H, C*H*(CH$_3$)$_2$: *N*-*i*-but), 2.51 (dd, $J = 5.7$, 12.8 Hz, 1H, β *H*a cys), 2.63–2.79 (m, 1H C*H*(CH$_3$)$_2$: *S*-*i*-but.), 2.72 (overlapping dd, $J = 6.8$, 12.8 Hz, 1H, β *H*b cys), 2.88–2.98 (m, 2H, NCH$_2$C*H*$_2$S), 3.29–3.40 (m, 2H, NC*H*$_2$CH$_2$S), 4.07–4.19 (m, 1H, α *H* cys), 5.81 (d, $J = 7.3$ Hz, 1H, N*H* cys), 6.32–6.43 (m, 1H, N*H*CH$_2$), 7.18–7.35, 7.39–7.47 (2m, 15H, Ar*H*). MS *m*/*z* 563 $(M+H)^+$. Anal. Calcd for $C_{32}H_{38}N_2O_3S_2$: C, 68.33; H, 6.76; N, 4.98. Found: C, 68.27; H, 6.71; N, 4.88.

2.2.4. *N*-(*N*-Isobutyryl-*S*-trityl-L-cysteinyl)-*S*-pivaloylcysteamine (**3g**)

This compound was prepared according to the general procedure described for **3e**, using **2** (1.7 g, 3.93 mmol) and *S*-pivaloylcysteamine hydrochloride (obtained by the procedure described for *S*-acetylcysteamine hydrochloride [15], mp 212–213 °C). The crude product was purified by flash column chromatography (EtOAc/petroleum ether, 7:3) to give a colourless foam. Trituration with hexane gave **3g** as a white powder (1.99 g, 88%): mp 85–88 °C. TLC (CH_2Cl_2/Et_2O, 6:4) $R_f = 0.82$. α_D^{20} +5.9° (*c* 1.02, $CHCl_3$). ^{1}H NMR: 1.11 (d, $J = 6.9$ Hz, 6H, C(C*H*$_3$)$_2$), 1.21 (s, 9H, C(C*H*$_3$)$_3$), 2.20–2.38 (m, 1H, C*H*(CH$_3$)$_2$), 2.51 (dd,

$J = 5.6$, 12.9 Hz, 1H, β *H*a cys), 2.72(dd, $J = 6.7$, 12.9 Hz, 1H, β *H*b cys), 2.84–2.96 (m, 2H, NCH_2CH_2S), 3.27–3.39 (m, 2H, NCH_2CH_2S), 4.03–4.17 (m, 1H, α *H* cys), 5.78 (d, $J = 7.4$ Hz, 1H, N*H* cys), 6.22–6.34 (m, 1H, $NHCH_2$), 7.18–7.35, 7.38–7.48 (2m, 15H, Ar*H*). MS *m/z* 577 $(M+H)^+$. Anal. Calcd for $C_{33}H_{40}N_2O_3S_2$: C, 68.75; H, 6.94; N, 4.86. Found: C, 68.49; H, 6.98; N, 4.93.

2.2.5. N-(N-Isobutyryl-S-trityl-L-cysteinyl)-S-benzoylcysteamine (3h)

This compound was prepared according to the general procedure described for **3e**, using **2** (1.7 g, 3.93 mmol) and *S*-benzoylcysteamine hydrochloride [15]. The crude product was purified by flash column chromatography (EtOAc/petroleum ether, 7:3) to give **3h** as a colourless foam (1.8 g, 77%). TLC (CH_2Cl_2/ Et_2O, 6:4) $R_f = 0.76$. α_D^{20} +7.8° (*c* 1.03, $CHCl_3$). ^1H NMR: 1.09 (d, $J = 6.9$ Hz, 6H, $C(CH_3)_2$), 2.18–2.31 (m, 1H, $CH(CH_3)_2$), 2.51 (dd, $J = 5.6$, 12.9 Hz, 1H, β *H*a cys), 2.74 (dd, $J = 6.7$, 12.9 Hz, 1H, β *H*b cys), 3.07–3.25 (m, 2H, NCH_2CH_2S); 3.40–3.51 (m, 2H, NCH_2CH_2S), 4.07–4.19 (m, 1H, α *H* cys), 5.74 (d, $J = 7.6$ Hz, 1H, N*H* cys), 6.35–6.45 (m, 1H, $NHCH_2$), 7.17–7.33, 7.38–7.47, 7.53–7.62, 7.89–7.96 (4m, 20H, Ar*H*). MS *m/z* 597 $(M+H)^+$. Anal. Calcd for $C_{35}H_{36}N_2O_3S_2$: C, 70.47; H, 6.04; N, 4.70. Found: C, 70.14; H, 6.10; N, 4.79.

2.2.6. N-(N-Isobutyryl-L-cysteinyl)-S-acetylcysteamine (4e)

Compound **3e** (1.367 g, 2.56 mmol) was dissolved in MeOH (20 mL) and $CHCl_3$ (1.4 mL). We added a mixture of $AgNO_3$ (522 mg, 3.07 mmol), pyridine (248 μL, 3.07 mmol) and MeOH (17 mL) at room temperature, in the dark. The reaction mixture was then stirred for 12 h. The resulting silver sulfide precipitate was collected by filtration, washed with MeOH (2× 12 mL), $CHCl_3$ (2× 12 mL) and rapidly dried under vacuum.

This product was then taken up in $CHCl_3$ (17 mL), placed in the dark under argon, and concentrated HCl (450 μL) was added dropwise. The mixture was stirred for 2 h at room temperature and then heated for 3 min at 30–35 °C. The reaction mixture was cooled to room temperature, diluted in $CHCl_3$ (80 mL) and the silver chloride precipitate was removed and washed with $CHCl_3$ (3× 12 mL). The combined filtrates were rapidly washed with iced water (3× 10 mL), dried (Na_2SO_4), filtered, and evaporated to dryness under vacuum. The residual gum was purified by flash column chromatography (CH_2Cl_2/Et_2O, 7:3) to give **4e** as a white solid (523 mg, 70%): mp 117–120 °C. TLC (CH_2Cl_2/Et_2O, 1:1) $R_f = 0.44$. α_D^{20} −36.5° (*c* 1.04, $CHCl_3$). ^1H NMR: 1.19, 1.20 (2d, $J = 2\times$ 6.9 Hz, 2× 3H, $C(CH_3)_2$), 1.62 (dd, $J = 7.5$, 10.3 Hz, 1H, S*H*), 2.37 (s, 3H, $SCOCH_3$), 2.47 (hept, $J = 6.9$ Hz, 1H, $CH(CH_3)_2$), 2.72 (ddd, $J = 6.5$, 10.3, 13.9 Hz, 1H, β *H*a cys), 3.00–3.08 (m, 2H, NCH_2CH_2S), 3.06 (ddd, $J = 4.2$, 7.5, 13.9 Hz, 1H, β *H*b cys), 3.41–3.53 (m, 2H, NCH_2CH_2S), 4.61 (ddd, $J = 4.2$, 6.5, 7.4 Hz, 1H, α *H* cys), 6.46 (d, $J = 7.4$ Hz, 1H, N*H* cys), 6.72–6.85 (m, 1H, $NHCH_2$). MS *m/z* 293 (M+H). Anal. Calcd for $C_{11}H_{20}N_2O_3S_2$: C, 45.20; H, 6.84; N, 9.59. Found: C, 45.22; H, 7.11; N, 9.69.

2.2.7. N-(N-Isobutyryl-L-cysteinyl)-S-isobutyrylcysteamine (4f)

This compound was prepared from **3f** (1.55 g, 2.75 mmol) according to the general procedure described for **4e**. The crude product was purified by flash column chromatography (CH_2Cl_2/Et_2O, 7.5:2.5) to give a colourless gum. Trituration with hexane gave **11** as a white powder (607 mg, 69%): mp 125–127 °C. TLC (CH_2Cl_2/Et_2O, 5:5) $R_f = 0.39$. α_D^{20} −25.7° (*c* 1.05, $CHCl_3$). ^1H NMR: 1.19, 1.20 (2d, $J = 2\times$ 6.9 Hz, 2× 6H, 2× $C(CH_3)_2$), 1.62 (dd, $J = 7.5$, 10.3 Hz, 1H, S*H*), 2.41–2.54 (m, 1H, $CH(CH_3)_2$: *N*-*i*-but.), 2.70–2.84 (m, 1H, $CH(CH_3)_2$: *S*-*i*-but.), 2.72 (overlapping ddd, $J = 6.5$, 10.3, 13.7 Hz, 1H, β *H*a cys), 2.98–3.14 (m, 3H, β *H*b cys, NCH_2CH_2S), 3.42–3.53 (m, 2H, NCH_2CH_2S), 4.54–4.66 (m, 1H, α *H* cys), 5.81 (d, $J = 7.4$ Hz, 1H, N*H* cys), 6.74–6.85 (m, 1H, $NHCH_2$). MS *m/z* 641 $(2M+H)^+$, 321 $(M+H)^+$. Anal. Calcd for $C_{13}H_{24}N_2O_3S_2$: C, 48.75; H, 7.50; N, 8.75. Found: C, 48.45; H, 7.82; N, 8.75.

2.2.8. N-(N-Isobutyryl-L-cysteinyl)-S-pivaloylcysteamine (4g)

This compound was prepared from **3g** (1.6 g, 2.78 mmol) according to the general procedure described for **4e**. The crude product was purified by flash column chromatography (CH_2Cl_2/Et_2O, 8.8:2.2) to give a colourless gum. Trituration with hexane gave **4g** as a white powder (650 mg, 70%): mp 120–122 °C. TLC (CH_2Cl_2/Et_2O, 5:5) $R_f = 0.46$. α_D^{20} −25.0° (*c* 1.04, $CHCl_3$). ^1H NMR: 1.194, 1.198 (2d, $J = 2\times$ 6.9 Hz, 2× 3H, $C(CH_3)_2$), 1.23 (s, 9H, $C(CH_3)_3$), 1.61 (dd, $J = 7.6$, 10.2 Hz, 1H, S*H*), 2.47 (hept app., $J = 6.9$ Hz, 1H, $CH(CH_3)_2$), 2.72 (ddd, $J = 6.5$, 10.2, 13.8 Hz, 1H, β *H*a cys), 2.95–3.13 (m, 3H, β *H*b cys, NCH_2CH_2S), 3.40–3.52 (m, 2H, NCH_2CH_2S), 4.54–4.66 (m, 1H, α *H* cys), 6.49 (d, $J = 7.5$ Hz, 1H, N*H* cys), 6.73–6.88 (m, 1H, $NHCH_2$). MS *m/z* 669 $(2M+H)^+$, 335 $(M+H)^+$. Anal. Calcd for $C_{14}H_{26}N_2O_3S_2$: C, 50.30; H, 7.78; N, 8.38. Found: C, 50.19; H, 7.92; N, 8.35.

2.2.9. N-(N-Isobutyryl-L-cysteinyl)-S-benzoylcysteamine (4h)

This compound was prepared from **3h** (1.6 g, 2.68 mmol) according to the general procedure described for **4e**. The crude product was purified by flash column chromatography (CH_2Cl_2/Et_2O, 1:1) to give a colourless gum. Trituration with hexane gave **4h** as a white powder (550 mg, 58%): mp 127–130 °C. TLC (CH_2Cl_2/Et_2O, 6:4) $R_f = 0.35$. α_D^{20} −20.8° (*c* 1.06, $CHCl_3$). ^1H NMR: 1.17, 1.18 (2d, $J = 2\times$ 6.9 Hz, 2× 3H, $C(CH_3)_2$), 1.59 (dd, $J = 7.5$, 10.3 Hz, 1H, SH), 2.44 (hept app., $J = 6.9$ Hz, 1H, $CH(CH_3)_2$), 2.70 (ddd, $J = 6.5$, 10.3, 13.8 Hz, 1H, β *H*a cys), 3.07 (ddd, $J = 4.2$, 7.5, 13.8 Hz, 1H, β *H*b cys), 3.17–3.34 (m, 2H, NCH_2CH_2S), 3.53–3.64 (m, 2H,

NCH_2CH_2S), 4.62 (ddd, $J = 4.2$, 6.5, 8.0 Hz, 1H, α *H* cys), 6.47 (d, $J = 8.0$ Hz, 1H, N*H* cys), 6.80–6.91 (m, 1H, N*H*CH_2), 7.42–7.53, 7.56–7.65, 7.92–8.01 (3m, 5H, Ar*H*). MS *m/z* 709 $(2M+H)^+$, 355 $(M+H)^+$. Anal. Calcd for $C_{16}H_{22}N_2O_3S_2$: C, 54.23; H, 6.21; N, 7.91. Found: C, 54.20; H, 6.18; N, 7.94.

2.3. Scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical

The scavenging effect of the synthesized compounds **4a**–**h** on the DPPH radical was evaluated according to previously published methods [16,17]. Various concentrations of the test compounds were incubated in 1 mL of an 60% ethanolic solution containing the DDPH radical (60 μM). The mixture was shaken vigorously and allowed to stand for 30 min; absorbance at 517 nm was determined, and the percentage of activity was calculated by the following equation: % inhibition $= ((A_{control} - A_{test})/A_{control}) \times 100$.

All tests and analyses were undertaken in triplicate and the results averaged.

2.4. $ABTS^{\bullet+}$ radical cation scavenging assay [18]

The ABTS radical cation ($ABTS^{\bullet+}$) was prepared by mixing ABTS stock solution (7 mM in water) with 2.45 mM potassium persulfate (final concentration). This mixture was allowed to stand in the dark at room temperature for 12–16 h before use. The $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.70 at 734 nm. Diluted $ABTS^{\bullet+}$ solution (0.9 mL) and 100 μL of various concentrations of the test compounds were mixed and measured immediately after 6 min at 734 nm. The antioxidant activity was calculated using the above formula.

2.5. Hydroxyl radical scavenging activity [19]

The reactions were performed in 10 mM phosphate buffer (pH 7.4) containing 2.8 mM deoxyribose, 2.8 mM H_2O_2, 12.5 mM $FeCl_3$, 100 μM EDTA, and the test sample (0–15 mM). The reaction was started by adding ascorbic acid to a final concentration of 200 μM. The reaction mixture was incubated for 1 h at 37 °C. After incubation, 1 mL of 1% thiobarbituric acid (w/v) in 0.05 M NaOH was added followed by 1 mL of 2.8% trichloroacetic acid (w/v). This solution was heated 20 min at 100 °C. After cooling, the extent of deoxyribose degradation was measured at 532 nm against a blank. The scavenging activity on hydroxyl radicals was expressed as: % inhibition $= ((A_{control} - A_{test})/A_{control}) \times 100$.

2.6. Ethidium bromide fluorescence binding assay

The method of Stoewe and Pruts was modified to measure DNA damage [20]. A 2 mL mixture containing 20 mM phosphate buffer (pH 7.0), 100 μg/mL calf thymus, 50 μM $FeSO_4$, and 10 mM H_2O_2 was prepared. Various concentrations of the test compounds were added to the reaction mixture before the addition of H_2O_2 and $FeSO_4$. The reaction was carried out at 37 °C for 10 min and terminated by the addition of EDTA to a final concentration of 10 mM. After incubation, 4 μL of a 1 mM EB solution was added and fluorescence intensity was measured at 590 nm ($\lambda_{ex} = 510$ nm). The reduction in fluorescence was used to measure the extent of DNA damage. DNA damage was expressed as the ration $(F_{untreated\ control} - F_{treatment})/(F_{untreated\ control} - F_{FeSO_4-H_2O_2})$. A DNA damage ratio <1 means that the compound inhibits the $FeSO_4$–H_2O_2 induced damage, while a ratio >1 means that the compound enhances the $FeSO_4$–H_2O_2 induced damage.

2.7. Biological methods

Isolation of human monocyte-derived macrophages (MDM), antiviral assay and data analysis were done as previously reported [7].

3. Results and discussion

3.1. Chemistry

N-Acetyl derivatives **4a**–**d** and *N*-isobutyryl analogues **4e**–**h** were synthesized starting, respectively, from commercially available *N*-acetyl-*S*-trityl-L-cysteine **1** or from *S*-tritylated *N*-isobutyryl-L-cysteine derivative **2** [6,7]. A mixed anhydride was first formed *in situ* using isobutyl chloroformate (IBC) and 4-methylmorpholine (NMM) and was allowed to react with *N*-hydroxysuccinimide (HOSu) in order to generate an active *O*-succinimide ester. The latter was then condensed with the appropriate *S*-acylcysteamine hydrochlorides in the presence of NMM to give the expected *S*-trityl derivatives in good to excellent yields. Detritylation was performed in a two steps procedure *via* the corresponding silver sulfides, which were treated with concentrated HCl to give free thiols **4a**–**h** (Scheme 1) [21].

3.2. Antioxidant activity

3.2.1. Free radical scavenging activity on 2,2-diphenyl-1-picrylhydrazyl radical

This assay is based on the measurements of the scavenging ability of compounds towards the stable radical 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) [22]. The disappearance of this commercially available radical is measured spectrophotometrically at 517 nm in a methanolic solution. The antioxidant activity was expressed as the 50% inhibitory concentration (IC_{50}) based on the amount of compound required for a 50% decrease of the initial DPPH radical concentration. The radical-scavenging activities for free thiols, range from 12 μM for **4a** to 33 μM for **4e** (Table 1). Whereas **4a** displays comparable IC_{50} to natural antioxidants such as Trolox and ascorbic acid (11 μM each), compounds **4b**–**4h** activities are closer to the results

Table 1
Antioxidant activities of derivatives **4a**–**h** in DPPH$^{\cdot}$, ABTS$^{\cdot+}$, and OH$^{\cdot}$ radical scavenging assays

Compound	DPPH$^{\cdot}$ IC_{50} (μM)	ABTS$^{\cdot+}$ IC_{50} (μM)	OH$^{\cdot}$ IC_{50} (mM)	Calculated log *P*
Trolox	11 ± 1.9	12 ± 1.4	1.9 ± 0.3	3.09
Ascorbic acid	11 ± 1.7	22 ± 2.1	nd[a]	−1.76
L-Cysteine	25 ± 1.5	6 ± 1.2	nd[a]	−2.35
NAC	38 ± 3.2	27 ± 3.5	7.3 ± 0.4	−0.62
GSH	32 ± 3.7	11 ± 0.4	5.5 ± 0.4	−3.05
Bis-*N*,*S*-acetyl-L-cysteine	<100	<100	nd[a]	−0.29
4a	12 ± 0.3	9 ± 3.2	7.2 ± 0.5	−0.10
4b	22 ± 2.1	10 ± 1.4	8.3 ± 0.8	0.74
4c	23 ± 1.4	11 ± 2.1	8.3 ± 0.7	1.14
4d	22 ± 2.7	8 ± 2.8	7.5 ± 0.6	1.16
4e	33 ± 2.0	6 ± 1.1	9.2 ± 0.5	0.74
4f	27 ± 3.1	6 ± 0.7	9.7 ± 0.6	1.58
4g	25 ± 3.6	5 ± 0.2	9.5 ± 0.6	1.98
4h	26 ± 2.1	7 ± 0.4	11.6 ± 1.1	2.00

[a] Not determined. Values are mean of triplicate determinations ± SD.

obtained with cysteine (25 μM), NAC (38 μM) and GSH (32 μM). Moreover, *N*-acetyl derivatives **4a**–**d** appear to scavenge the DPPH radical more efficiently (although slightly) than the *N*-isobutyryl derivatives **4e**–**h**.

3.2.2. ABTS$^{\cdot+}$ radical cation scavenging assay

In this assay the ABTS radical cation is produced chemically by the oxidation of the corresponding colourless sulfonic acid with potassium persulfate [18]. The green-blue ABTS radical cation has excellent spectral characteristics is stable over a wide range of pH and is applicable to the study of both water- and lipid-soluble antioxidants that converts ABTS$^{\cdot+}$ back to the initial sulfonic acid. The disappearance the ABTS$^{\cdot+}$ radical is measured spectrophotometrically at 734 nm in a methanolic solution. The antioxidant activity was expressed as the 50% inhibitory concentration (IC_{50}) based on the amount of compound required for a 50% decrease of the initial ABTS$^{\cdot+}$ radical concentration.

It is important to note that the IC_{50} values for both (DPPH and ABTS) of these assays are dependent on the details on the experiments (rate at which the free radicals are generated; use of aqueous vs. organic solvent; reaction with the species detected by the assay) and need to be in agreement with one another. The DPPH and ABTS assays are generally considered to be useful for ranking the potency of various antioxidants, and we found that the relative IC_{50} values determined using these assays agreed well with one another (Table 1). It was found that all free thiols tested were able to scavenge the ABTS$^{\cdot+}$ radical cation with values in the micromolar range (5–11 μM) while protected thiols such as bis-*N*-*S*-acetylcysteine [23] (Table 1) were not. In this assay, almost no difference was found depending on the nature of the *N*-protecting group. IC_{50} values are comparable with those obtained with cysteine (6 μM), GSH (11 μM) or Trolox (12 μM) but at least two times better than NAC (27 μM) or ascorbic acid (22 μM). Whereas compound **4a** seemed to show a higher scavenging ability in the DPPH assay (12 μM), this dominance was however, not confirmed in the ABTS assay (9 μM). The results indicate that the sulfhydryl group confers to these molecules interesting free radical scavenging properties always higher than NAC. However, these two assays do not support a free radical scavenging ability dominance depending on the nature of the *N*-protecting group of these series.

3.2.3. Deoxyribose assay

The ability of these compounds to scavenge hydroxyl radicals (OH$^{\cdot}$) was assessed using the classic deoxyribose degradation assay described by Halliwell et al. [19]. The principle of this assay is the quantification of the 2-deoxyribose degradation into malonaldehyde which is condensed with thiobarbituric acid. The degree of hydroxyl radicals (OH$^{\cdot}$) scavenging was expressed as the 50% inhibitory concentration (IC_{50}) based on the amount of compound required for a 50% decrease of the initial chromophore absorbance at 532 nm.

Table 1 shows the effect of compounds **4a**–**h** on deoxyribose degradation induced by Fe^{3+}/H_2O_2. The inhibitory effect on sugar degradation was shown to be concentration-dependant, in the millimolar range, Trolox having the highest apparent OH$^{\cdot}$ radical scavenging activity (1.9 mM). All other *N*-acetyl or *N*-isobutyryl derivatives possessed lower scavenging activity (7.2–11.6 mM), very similar to the ones observed with GSH (5.5 mM) and NAC (7.3 mM).

In biochemical systems, superoxide radicals are converted to hydrogen peroxides, which subsequently generate reactive hydroxyl radicals through the Fenton reaction with metal ions [24]. Therefore, the mode of action of these compounds (direct OH$^{\cdot}$ scavenging or metal chelation) was further examined by evaluating their capacity to inhibit the iron-driven Fenton reaction-induced damage of calf thymus DNA.

3.2.4. Ethidium bromide binding assay

The ethidium bromide (EB) binding assay, based on the formation of a fluorescent complex between double-stranded DNA and EB, was used to measure DNA damage [20]. Damaged DNA inhibits the binding of EB to DNA which results in a decrease in intensity of fluorescence. Several forms of DNA damage, including strand scission, base oxidation and base liberation, contribute to the loss of fluorescence. Hence, the assay detects a broad range of different DNA lesions. DNA damage was expressed as the ratio $(F_{\text{untreated control}} - F_{\text{treatment}})/(F_{\text{untreated control}} - F_{\text{FeSO}_4\text{–}\text{H}_2\text{O}_2})$. A DNA damage ratio <1 means that the compound inhibits the $FeSO_4$–H_2O_2 induced damage, while a ratio >1 means that the compound enhances the $FeSO_4$–H_2O_2 induced damage.

The effects of compounds **4a**–**h** to the iron-driven Fenton reaction are presented in Fig. 1. In the absence of any compound, the presence of $FeSO_4$ and H_2O_2 signifi-

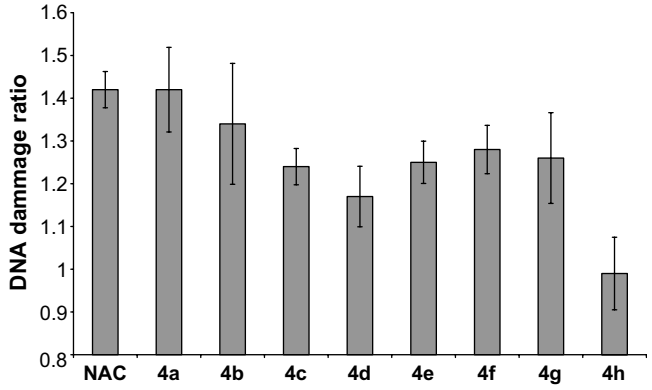


Fig. 1. Effect of compounds **4a**–**h** (7 mM) on $FeSO_4$–H_2O_2 induced damage in calf thymus DNA. The level of DNA damage after treatment was expressed as ratio of DNA damage relative to the $FeSO_4$–H_2O_2 induced damage control, as described in Section 2. Values are mean of triplicate determinations ± SD.

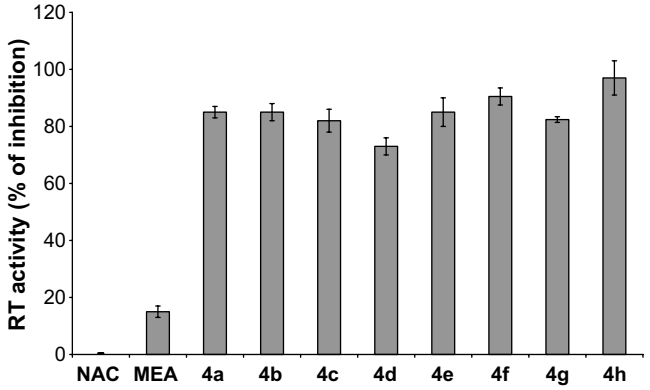


Fig. 2. Anti-HIV effects of **4a**–**h** at a concentration of 150 μM in HIV-1/Ba-L-infected MDM. Cumulative RT activities were used to calculate percentages with respect to untreated control, and the results are expressed as means ± SD.

cantly damaged calf thymus DNA. Addition of 7 mM of compounds **4a**–**h** did not show any protection toward DNA damage and even enhanced the $FeSO_4$–H_2O_2 induced damage of calf thymus DNA, thus suggesting a pro-oxidant effect of these derivatives. Like the Fenton reaction, the deoxyribose assay has been used to assess the pro-oxidant action of many compounds [25,26]. However, no pro-oxidant activity was detected with any of the compounds tested in the deoxyribose assay. The fact that the Fenton reaction is a potent source of hydroxyl radicals, suggest that compounds **4a**–**h** are bad metal chelators and act essentially as hydroxyl radicals scavengers. The results obtained here with *N*-acetyl and *N*-isobutyryl derivatives are in accordance with recently published results demonstrating that NAC could increase the level of DNA double strand breaks instead of inhibiting the damage [27]. Looking at Fig. 1, it seems however that these pro-oxidant activities might be counterbalanced by the steric hindrance brought by large protecting groups, *N*-isobutyryl-*S*-benzoyl derivative **4h** showing no activity whatsoever.

It has long been proposed that the beneficial protective effect of NAC mainly comes from its capacity to directly scavenge radicals by means of its thiol function. Nevertheless, the interaction of thiols with reactive radicals can generate thiol radicals which in turn may impart a pro-oxidant function [28]. Our results support the ability of these *N*-acetyl and *N*-isobutyryl derivatives to scavenge free radicals like $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ radicals and to a lesser extent the hydroxyl radical $OH^{\cdot}$ but enhance the Fe^{2+}–H_2O_2-dependent oxidative actions suggesting a balance between pro- and antioxidant mechanism.

3.2.5. Anti-HIV activities in human MDM

Lipophilicity plays an important role in cell-uptake and may be essential for dispersion of food antioxidants in bulk oils or fats. We therefore confirmed the higher lipophilicity of the *N*-isobutyryl series by calculating their respective log *P* values (Table 1) [29]. On the basis of these result we decided to explore the effects of these compounds in MDM infected *in vitro* with the reference macrophage-tropic HIV-1/Ba-L strain. As a consequence of an expected ineffective hydrolysis of the *N*-isobutyryl group, none of these derivatives were found to increase GSH level (data not shown). However, we were pleased to find that the introduction of the *N*-isobutyryl group did not seem to affect the antiviral effects of derivatives **4e**–**h**. In fact, the anti-HIV-1/Ba-L activity of these thiols in human MDM is comparable to that of the *N*-acetyl series, while NAC and MEA were efficient at millimolar concentration (Fig. 2) [7]. We therefore carried out an experiment to determine and compare more precisely the dose-dependant effect of compounds of the two series (Table 2). Interestingly, compound **4f** was found to be more efficient that **4a** (formerly known as I-152) [6–8] suggesting again the need of a not too bulky R′ group for high anti-HIV activities. These results strongly suggest that no direct correlation can be drawn between pro-GSH and anti-HIV activities of potent molecules.

Table 2
ED_{50}, ED_{70}, and ED_{90} for the anti-HIV-1/Ba-L activity of **4a**, **4b**, **4e**, and **4f** in human MDM

	4a (μM)	**4b** (μM)	**4e** (μM)	**4f** (μM)
ED_{50}	51	110	95	36
ED_{70}	82	175	121	54
ED_{90}	178	380	146	103

4. Conclusions

In conclusion, a series of new *N*-isobutyryl-L-cysteine derivatives was synthesized and their potential as antioxidants was evaluated, and compared with known pro-GSH compounds. These *N*-isobutyryl as well as their *N*-acetyl analogues showed remarkable free radical scavenging abilities always better than NAC and GSH. Moreover, both series displayed similar hydroxyl radical-

scavenging activities. As the *N*-isobutyryl compounds are more lipophilic, they might produce higher levels of free thiols in the body, reducing therefore the damage provoked by oxidative stress. The present study support the stringent sulfhydryl functional group requirement for maximal free radical scavenging efficacy, and the presence of the *N*-isobutyryl group suggests that a better bioactivation of these derivatives might play a major role in the ultimate release of ROS scavenging active compounds. As these compounds display equal or better anti-HIV activities than already reported cysteine derivatives, they could be good candidates to interfere with inflammatory and oxidative disorders associated with HIV infection. Moreover, these results suggest that anti-HIV effects of our compounds are related to their antioxidant effects and not their pro-GSH properties. These lipophilic analogues **4e–h** may therefore, be profitably used, in principle, as food antioxidants.

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