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Bioorganic Chemistry 34 (2006) 49–58
BIOORGANIC CHEMISTRY
www.elsevier.com/locate/bioorg

Synthesis and effects of 3-methylthiopropanoyl thiolesters of lipoic acid, methional metabolite mimics

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Received 5 September 2005
Available online 4 January 2006

Abstract

6*S*,8*S*-Bis(3-methylthiopropanoyl) thiolesters of lipoic acid were synthesized with the carboxyl moiety of lipoate modified as methyl or water soluble choline esters. Evaluation on different cell lines in culture showed that they possessed modest antiproliferative activity. However, the 6-fold decrease in IC_{50} (from 270 to 45 μM) observed with the water soluble 6*S*,8*S*-bis(3-methylthiopropenoyl) thiolester dehydro derivative on a human epithelial prostate cancer cell line (DU145) argues in favor of 3-methylthiopropanoyl metabolites as endogenous growth regulatory (apoptogenic) compounds derived from methionine.
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Keywords: Methionine metabolism; Cancer; Branched chain oxoacid dehydrogenase complex; Lipoic acid; Choline; Synthesis; Antiproliferative activity

1. Introduction

We have previously shown that 3-methylthiopropanaldehyde (methional) is a potent apoptogenic agent for normal and transformed cells in culture [1], except for those

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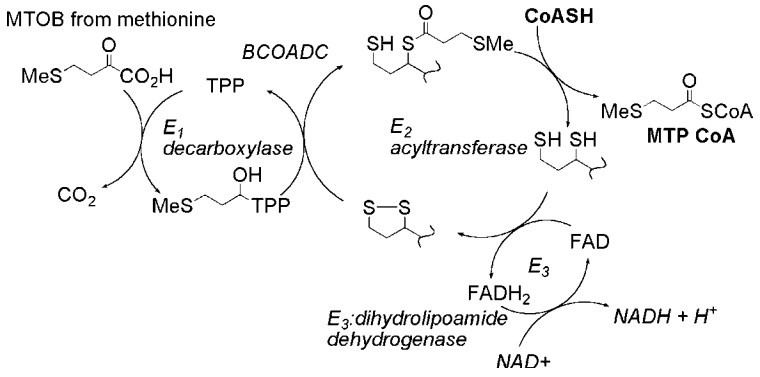
0045-2068/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bioorg.2005.11.001

overexpressing the anti apoptotic gene bcl2 [2]. In vivo, methional is derived from 4-methylthio-2-oxo butanoic acid (MTOB) formed exclusively in the methionine salvage pathway [3,4]. This decarboxylation step is mediated by the E1 component of the intramitochondrial enzyme: branched chain oxoacid dehydrogenase complex (BCOADC) which is composed of three enzymes E1, E2, and E3 working in that sequence. Hence, the decarboxylation of MTOB gives rise to methional as a transient metabolite bound to the thiamine cofactor of E1. Upon the interaction of the E1 component with E2, the thiamine-bound methional is transferred to the lipoic acid cofactor moiety (covalently bound to a lysine residue) situated on the E2 component itself. But E2 is also an acyltransferase, so it transfers the methional (in thiolester linkage to lipoate) to Coenzyme A to form another thiolester compound: 3-methylthiopropanoyl-CoA (MTPCoA), leaving the lipoate moiety in the reduced form. Finally, the disulphydryl lipoate is oxidized by the E3 enzyme, lipoamide dehydrogenase in the presence of FAD and NAD to reform oxidized lipoate.

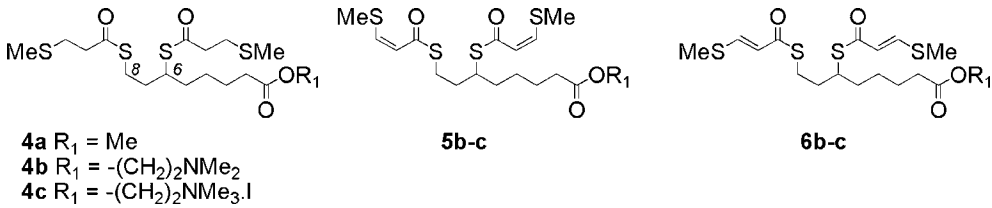
The formation of MTPCoA is therefore strictly dependent on the availability of the precursor: MTOB and on the combined activity of the E1 and E2 components of BCOADC.

Several lines of evidence support a role for both MTOB and BCOADC in apoptosis. With regard to MTOB, it has been clearly shown that a deficiency of MTOB occurs in methionine-dependent transformed cells with impaired methylthioadenosine (MTA) phosphorylase activity [5]. Indeed, in these cells, the addition of MTOB to the methionine-deprived culture medium alleviates methionine-dependence and restores cell growth to the rate seen in a medium with the normal concentration (0.1 mM) of methionine [5]. Further, when the transamination of MTOB in these cells is inhibited, they cease to proliferate [5] and die by apoptosis [2]. As cell death cannot be prevented by adding methionine [5], it suggests that apoptosis was due, not to a lack of methionine, but to the MTOB which had accumulated. In such cells with a blocked glutamine/MTOB transaminase, the only metabolic route into which MTOB can be shunted is that mediated by BCOADC (Scheme 1).

With regard to BCOADC, there are two pertinent reports in the literature: it has been reported that the addition to cells of dexamethasone, a potent apoptogenic glucocorticoid, increases the synthesis and activity of the E2 component of BCOADC [6] and that the



Scheme 1. 3-Methylthiopropanoyl-CoA (MTPCoA) formation by oxidative decarboxylation of 4-methylthio-2-oxo-propanoic acid (MTOB) by the branched chain oxoacid dehydrogenase.



Scheme 2. Bisthiolesters derivatives of lipoic acid.

addition of 4-methyl-2-oxopentanoate(oxoleucine) to NIH3T3 cells at 13 mM induces these 3T3 cells into apoptosis, whereas leucine is ineffective at equimolar concentrations [7]. 4-Methyl-2-oxopentanoate, like MTOB and the other branched-chain oxoacids, activates BCOADC [8] by inhibiting (IC_{50}: 20 μM) the specific BCOADC kinase responsible for the inactivation of BCOADC [9].

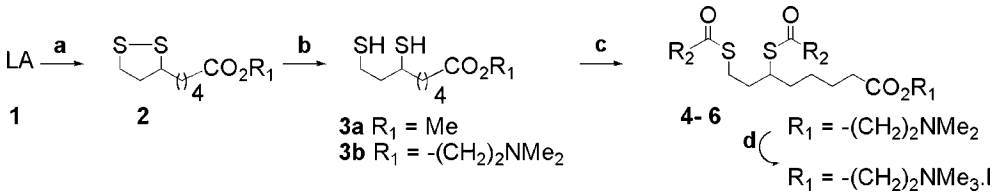
In view of this evidence, it is reasonable to propose that the endogenous apoptogenic MTOB-derived metabolite could be MTPCoA. Thus, it would be ideal to add chemically synthesized MTPCoA to the culture medium of transformed cells in attempt to induce apoptosis. The problems with this approach are 2-fold. CoA is synthesized endogenously in cells from pantothenic acid, 2-mercapto-ethylamine and adenosine 3′-phosphate 5′-pyrophosphate. Further, the membrane is not permeable to phosphorylated compounds.

To try to circumvent these obstacles, we used the fact that lipoic acid is easily taken up by cells [10] and undertook the preparation of a family of compounds i.e., MTP thiolesters of lipoic acid in the hope that they would be able to compensate for the deficiency of MTOB-derived apoptogenic metabolites. It is the details of the synthesis of these MTP-Lipoate mimics and the results of their evaluation as selective anti proliferative compounds which are reported here Scheme 2.

2. Chemistry

The mechanism of E2 suggests a 6S-acylation of lipoic acid derivatives but synthesis of such 3-methylthiopropanoyl thiolesters was not straightforward and we turned first our attention to 8S mono thiolesters. As mentioned [11], acylation occurred in good yield with 3-methylthiopropionic acid but purification of products proved to be difficult. Effectively, 8*S* acyl derivatives were always contaminated by compounds resulting from 6S-acylation. Although acetylation of lipoic acid with acetic anhydride is known to be selective [12], similar acylation with 3-methylthio acetic anhydride always produced 6S-acylation compound as a by-product. For these reasons we decided to prepare bis 3-methylthiolesters of lipoic acid. As shown in Scheme 3, racemic lipoic acid was first esterified and the disulfide bond was reduced by sodium borohydride. The dithiol obtained was acylated by a slight excess of 3-methylthiopropanoic acid using DCC activation. In the case of (dimethylamino) ethyl esters the acylation step may be completed by ammonium formation using methyl iodide.

The two families of dehydrogenated compounds **5** and **6** were synthesized to evaluate the influence of the double bond stereochemistry. For this purpose the required 3-methylthiopropanoyl derivatives Z and E of acrylic acid were prepared from propiolic acid by known methods [13]. Corresponding bisthiolesters were synthesized by the method used for the preparation of compounds **4a**–**c** with poor yields (29 and 35%).



Scheme 3. Reagents and conditions: (a) RCO_2H, DCC, 4-DMAP, Et_2O or MeOH, H_2SO_4 for R = Me, (b) $NaBH_4$, MeOH, THF, (c) 3-methylthiopropanoic acid or 3-methylthiopropenoic acid (E or Z), DCC, 4-DMAP, CH_2Cl_2, (d) MeI, EtOAc.

3. Results and discussion

The first family of compounds tested was that of the bisthiolesters of methional-lipoate methyl ester **4a** because all attempts at preparing the *8S* mono thiolesters and 6*S*,8*S*-bithiolesters with the carboxyl group of lipoate non-esterified, gave rise to a mixture containing both the mono and bisthiolester derivatives which proved very difficult to isolate. The high IC_{50}: 400 μM of **4a** (Table 1) indicated that this compound was not very active as a growth inhibitor of human epithelial prostate cancer cells (DU145) but showed good selectivity, as less than 15% inhibition was observed on normal human embryonic lung fibroblasts (MRC5) at the highest concentration tested (600 μM), however, the tendency to precipitate when more concentrated solutions of **4a** in ethanol were added to the cell culture medium precluded further experiments at higher concentrations. We therefore tried to enhance its water-solubility by increasing the polarity of the ester moiety on the lipoate carboxyl.

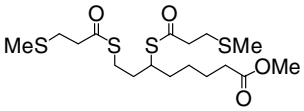
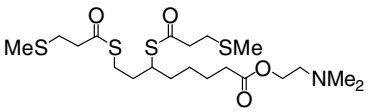
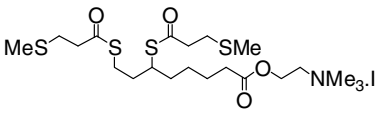
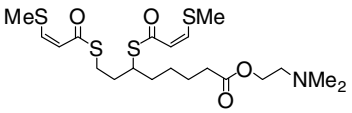
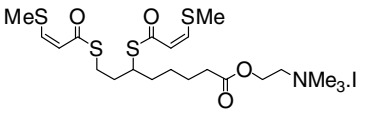
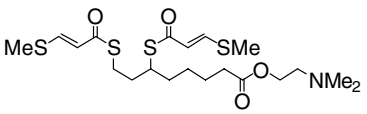
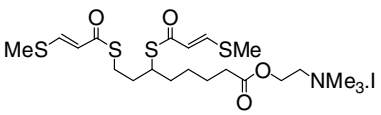
In doing so, we were guided by literature reports on choline which is commonly used to render some lipophilic compounds water soluble [14]. In addition, choline entry into cells is mediated by both an active uptake mechanism [15] and passive diffusion [15]. Hence, it might also serve as a vector to facilitate the passage into cells of compounds bound to it. Accordingly, **4a** was converted to **4b**, the dimethylamino ethyl intermediate and finally to **4c** the trimethylammonium (choline) derivative as described in Section 2. The results in Table 1 show that **4b** has lost its selectivity, because a similar IC_{50} was obtained on MRC5 and DU145 with only a slight gain in activity. The choline derivative **4c** shows no increase in inhibitory activity on both cell types and only a moderate gain in selectivity when compared to the dimethyl intermediate **4b**. This result suggests that selectivity is associated primarily with the hydrophilic/hydrophobic nature of the ester on the carboxyl group of lipoate. In the absence of any improvement in selectivity we concentrated on the amelioration of the anti proliferative activity.

Accordingly, compounds **5b** and **5c** corresponding to the dimethyl and choline derivatives respectively of the dehydrogenated 3-methylthiopropanoyl moiety were synthesized.

It is apparent (Table 1) that inhibitory activity towards DU145 is sharply increased passing from 240 μM for **4b** to 73 μM for **5b**. A similar trend, but to a less extent, was observed when **4c** was compared to **5c**. Both compounds **5b** and **5c** were also better inhibitors of MRC5 cells than their precursors in the **4** series and were devoid of selectivity.

The clear increase in inhibitory activity prompted us to examine the influence of the stereochemistry of the double bond as the endogenous acyl-lipoate is in the trans configuration. Accordingly, compounds **6b** and **6c** were synthesised and evaluated on the

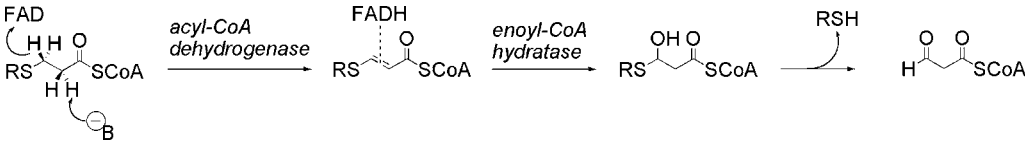
Table 1
Antiproliferative effects of compounds **4–6** on the growth of cells in culture

Compound		IC_{50}			
		Normal cells MRC5	Transformed cells		
			DU 145	BAF3bcl2	HuH7
	**4a**	≫600	400		
	**4b**	290	240	39	
	**4c**	410	270	93	550
	**5b**	110	73	33	
	**5c**	170	100	45	
	**6b**	75	50	20	
	**6c**	113	45	27	100

Values are means of three experiments, IC_{50} values expressed in μM.

same 2 cell types. As can be seen in Table 1 there is a further gain in inhibitory activity towards MRC5 and DU145 for both compounds **6b** and **6c**. However, **6c** with an IC_{50} (113 μM) on MRC5 appears to show slightly better selectivity than **6b** with an IC_{50} of 75 μM.

The work done so far was carried out on only two cell lines. We therefore tried to determine whether similar increases in growth inhibition would be observed on other cell types treated with **4c**, **5c**, and **6c**. In the mouse lymphoid cell line overexpressing the human anti-apoptotic gene bcl_2 ($BAF3bcl_2$) there is a 3-fold increase in inhibitory activity between **4c** and **6c** whereas with the compounds **4b** and **6b** of the dimethyl series the increase was



Scheme 4. Alkanethiol formation from 4-thia-*trans*-2-enoyl-CoA derivative. Based on [17].

almost 2-fold (Table 1). In a human colon carcinoma cell line HuH7 there was also a 5-fold increase in growth inhibition when **6c** was tested in the place of **4c**.

Our results provide no experimental evidence for the particular pathway responsible for the increased activity of **6c** over **4c**. However, it could be postulated that as our compounds are thiolesters, there could easily occur an exchange reaction between the 3-methylthiopropanoyl moiety of the mimics and cellular lipoic acid. Further, if this exchange took place with the lipoic acid cofactor of the E2 component of BCOADC, then the 3-methylthiopropanoyl moiety would then be metabolised to MTPCoA. This compound has been identified as a product of methionine metabolism in rat muscle [16]. MTPCoA has been shown to be a good substrate of medium/short chain acyl-CoA dehydrogenase [17]. Compounds like these, after dehydrogenation to the corresponding 4-thia-*trans*-2-alkenoyl-CoA undergo a further hydroxylation step by enoyl-CoA hydratase to 3-hydroxy-3-methylthiopropanoyl-CoA which then undergoes non enzymic β-elimination, liberating MeSH with the formation of malonyl-semialdehyde-CoA (Scheme 4).

Where **4c** and **6c** to be metabolised via this route, then the dehydrogenated form **6c** would be more readily available for hydroxylation by enoyl-CoA hydratase with enhanced formation of MeSH and malonyl-semialdehyde-CoA. The intracellular fate of malonyl-semialdehyde-CoA is unknown, but it has been clearly shown that MeSH liberated in situ from other sources decreases mitochondrial glutathione (GSH) in BAF_3bcl_2 cells when they are induced into apoptosis [18]. Further, a decrease in mitochondrial GSH is a hall-mark of apoptosis [19]. Experimental verification of the pathways followed by the mimics will be obtained only when the radiolabelled 3-methylthiopropanoyl moiety becomes available.

4. Conclusion

The inhibitory activity of 3-methylthiopropanoyl increase on its dehydrogenation to a 3-methylthiopropenoyl moiety. Nevertheless, the problem of selectivity remains to be solved in order for this family of compounds to be considered for animal experimentation. At this stage of their development they can yet be useful tools for investigating, on cells in culture, the contribution of mitochondrial MTOB metabolism to the intrinsic apoptotic pathway.

5. Experimental

*5.1. 6,8-Bis[[3-(methylthio)-1-oxopropyl]thio] octanoic acid methyl ester (**4a**)*

To a solution of 3-methylthiopropanoic acid (192 mg, 1.6 mmol) in dichloromethane (DCM) (1 mL) was successively added at room temperature: 4-DMAP (8 mg, 0.065 mmol)

and 6,8-dimercapto octanoic acid methyl ester [12] **3a** (355 mg, 1.6 mmol) in DCM (1 mL). The mixture was cooled in an ice bath and dicyclocarbodiimide (DCC) (330 mg, 1.6 mmol) in DCM (1 mL). The mixture was then slowly warmed to room temperature within 3 h and filtered. The filtrate was diluted with ethyl ether, washed with brine and dried over sodium sulfate. Solvents were evaporated under reduced pressure and the crude product was purified by chromatography on silica gel (petroleum ether (PE)/ethyl acetate (EtOAc) = 85/15) to give an oil (yield 60%). IR (KBr) ν 1740, 1590 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.35–1.95 (m, 8H), 2.12 (s, 6H), 2.31 (t, $J = 7.4$, 2H), 2.70–3.10 (m, 10H), 3.55–3.65 (m, 1H), 3.66 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 15.4 ($CH_3 \times 2$), 24.4 (CH_2), 26.0 (CH_2), 26.2 (CH_2), 29.1 (CH_2), 29.2 (CH_2), 33.6 (CH_2), 34.2 (CH_2), 34.6 (CH_2), 43.2 (CH), 43.5 (CH_2),43.6 (CH_2), 51.3 (CH_3), 173.6 (C), 197.07 (C), 197.15 (C). MS (EI) m/z 426 $[M]^+$.

*5.2. 6,8-Bis[[3-(methylthio)-1-oxopropyl]thio] octanoic acid 2-(dimethylamino)ethyl ester (**4b**)*

*5.2.1. Lipoate **2b***

To lipoic acid (1.00 g, 4.85 mmol), 4-dimethylaminopyridine (DMAP) (0.178 g, 1.46 mmol) and 2-dimethylaminoethanol (0.475 g, 5.33 mmol) in 8.5 mL of Et_2O was added dropwise (DCC) (1.100 g, 5.33 mmol) in Et_2O (3.5 mL) at O °C. The mixture was warmed to room temperature and stirred overnight. The dicyclohexylurea was removed by filtration and the filtrate was diluted with dichloromethane (DCM) (40 mL), washed with water (3× 30 mL). After drying (Na_2SO_4) and concentration on a rotary evaporator the crude product was purified by chromatography on silicagel (DCM/MeOH: 92/8) to give ester **2b** (yield: 83%) as an oil. IR (neat) ν 1740, 1450 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.40–1.75 (m, 6H), 1.91 (qd, $J = 6.6$; 12.9, 1H), 2.28 (s, 6H), 2.35 (t, $J = 7.3$, 2H), 2.46 (dddd, $J = 12.9$; 6.6; 6.6; 5.5, 1H), 2.56 (t, $J = 5.9$, 2H), 3.11 (dt, $J = 11.0$; 6.6, 1H), 3.15 (ddd, $J = 11.0$; 6.6; 5.5, 1H), 3.55 (m, 1H), 4.17 (t, $J = 5.9$, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 24.6 (CH_2), 28.7 (CH_2), 34.0 (CH_2), 34.6 (CH_2), 38.5 (CH_2), 40.2 (CH_2), 45.7 ($CH_3 \times 2$), 56.3 (CH), 57.8 (CH_2), 62.1 (CH_2), 173.5 (C).

*5.2.2. Dithiol **3b***

To lipoate **2b** (560 mg, 2.02 mmol) in MeOH/THF (5/1) (30 mL) was added $NaBH_4$ (86 mg, 2.3 mmol) at 0 °C in two portions. After 40 min, 5N HCl was added until a white precipitate remained. The solvents were evaporated and the residue was taken up in Et_2O. Organic phase was then washed by saturated aqueous bicarbonate solution and brine. Further drying over Na_2SO_4 and concentration under reduced pressure gave **3b** as an oil (yield: 99%). IR (neat) ν 2250, 1730 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.23–1.80 (m, 8H), 1.83–1.97 (m, 2H), 2.28 (s, 6H), 2.35 (t, $J = 7.3$, 2H), 2.56 (t, $J = 5.9$, 2H), 2.63–2.99 (m, 3H), 4.17 (t, $J = 5.9$, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 22.3 (CH_2), 24.5 (CH_2), 26.5 (CH_2), 34.0 (CH_2), 38.7 (CH_2), 39.3 (CH), 42.7 (CH_2), 45.7 ($CH_2 \times 2$), 57.8 (CH_2), 62.01 (CH_2), 173.6 (CH).

*5.2.3. Bisthiolester **4b***

Prepared as described for **4a** starting from dithiol **3b**. Purification on silicagel (DCM/MeOH: 96/4). Viscous oil. Yield: 60%; IR (neat) ν 1735, 1690 cm^{-1}. ^1H NMR

(300 MHz, $CDCl_3$) δ 1.29–1.70 (m, 6H), 1.71–1.95 (m, 2H), 2.13 (s, 3H), 2.14 (s, 3H), 2.29 (s, 6H), 2.33 (t, $J = 7.3$, 2H), 2.56 (t, $J = 5.9$, 2H), 2.75–2.90 (m, 9H), 2.96–3.07 (m, 1H), 3.60 (m, 1H), 4.17 (t, $J = 5.9$, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 15.6 ($CH_3 \times 2$), 24.6 (CH_2), 26.2 (CH_2), 26.4 (CH_2), 29.36 (CH_2), 29.43 (CH_2), 34.0 (CH_2), 34.5 (CH_2), 34.8 (CH_2), 43.5 (CH), 43.7 (CH_2), 43.8 (CH_2), 45.7 ($CH_3 \times 2$), 57.8 (CH_2), 62.1 (CH_2), 173.5 (C), 197.3 (C), 197.4 (C). Anal calcd. for $C_{20}H_{37}NO_4S_4$: C, 49.65; H, 7.71; O, 13.26. Found: C, 49.72; H, 7.57; O, 13.56.

5.3. 2-[[6,8-Bis[[3-(methylthio)-1-oxopropyl]thio]-1-oxooctyl]oxy]-N,N,N-trimethyl ethanaminium iodide **2c**

MeI (0.127 mL, 2.05 mmol) was added to ester **4b** (0.660 g, 1.36 mmol) in ethyl acetate (AcOEt) (14 mL) at room temperature, and stirring was continued for 24 h in the dark. Concentration in vacuo followed by chromatography on silica gel (DCM/MeOH: 9/1)gave an amorphous hygroscopic solid (0.763 g, 89%); IR (neat) ν 1730, 1690 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.32–1.76 (m, 6H) −1.76–1.97 (m, 2H), 2.15 (s, 3H), 2.16 (s, 3H), 2.43 (t, $J = 7.3$, 2H), 2.78–2.93 (m, 9H), 2.99–3.09 (m, 1H), 3.60 (s, 9H), 3.52–3.64 (m, 1H), 4.20 (m, 2H), 4.60 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 15.69 (CH_3), 15.71 (CH_3), 24.3 (CH_2), 26.2 (CH_2), 26.4 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 33.9 (CH_2), 34.4 (CH_2), 34.8 (CH_2), 43.4 (CH), 43.7 (CH_2), 43.8 (CH_2), 54.9 ($CH_3 \times 3$), 57.8 (CH_2), 65.3 (CH_2), 172.5 (CH_2), 197.6 ($CH_2 \times 2$); Anal calcd. for $C_{21}H_{40}INO_4S_4$: C, 40.31; H, 6.44; S, 20.50. Found: C, 39.91; H, 6.41; S, 20.43.

5.4. 6,8-Bis[[3-(methylthio)-1-oxo-2-propenyl]thio] octanoic acid 2-(dimethylamino)ethyl ester (Z,Z) **5b**

Prepared as described for **4a** starting from dithiol **3b** and (Z)-3-(methylthio)acrylic acid [13]. Purification on silicagel (DCM/MeOH: 94/6). Viscous oil. Yield: 29%; IR (neat) ν 1730, 1640, 1540, 870 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.31–1.77 (m, 6H), 1.80–2.06 (m, 2H), 2.28 (s, 6H), 2.34 (t, $J = 7.3$, 2H), 2.40 (s, 6H), 2.55 (t, $J = 5.90$, 2H), 2.92 (m, 1H), 3.09 (m, 1H), 3.66 (m, 1H), 4.16 (t, $J = 5.90$, 2H), 6.19 (d, $J = 9.9$, 1H), 6.2 (d, $J = 9.9$, 1H), 6.86 (d, $J = 9.9$, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 19.7 ($CH_3 \times 2$), 24.7 (CH_2), 26.28 (CH_2), 26.34 (CH_2), 34.0 (CH_2), 34.6 (CH_2), 35.1 (CH_2), 43.5 (CH), 45.7 ($CH_3 \times 2$), 57.8 (CH_2), 62.0 (CH_2), 119.56 (CH), 119.64 (CH), 148.5 ($CH \times 2$), 173.5 (C), 187.2 (C), 187.5 (C); HRMS-FAB$^+$ calcd. for $C_{20}H_{34}NO_4S_4[MH]^+$: 480.1371 found: 480.1367.

5.5. 2-[[6,8-Bis[[3-(methylthio)-1-oxo-2-propenyl]thio]-1-oxooctyl]oxy]-N,N, N-trimethyl ethanaminium iodide (Z,Z) **5c**

Prepared as described for **2c** starting from **5b**. Purification by chromatography on silica gel (DCM/MeOH: 90/10). Hygroscopic amorphous solid. Yield 60%. IR (DCM) ν 1740, 1640, 1540, 870 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.27–1.76 (m, 6H), 1.77–2.03 (m, 2H), 2.41 (t, $J = 7.3$, 2H), 2.42 (s, 6H), 2.90 (m, 1H), 3.05 (m, 1H), 3.54 (s, 9H), 3.60 (m, 1H), 4.13 (m, 2H), 4.58 (m, 2H), 6.18 (d, $J = 9.9$, 2H), 6.89 (d, $J = 9.9$, 1H), 6.91 (d, $J = 9.9$, 1H); HRMS-FAB$^+$ calcd. for $C_{21}H_{36}NO_4S_4[M-I]^+$: 494.1527 found: 494.1527.

5.6. 6,8-Bis[[3-(methylthio)-1-oxo-2-propenyl]thio] octanoic acid 2-(dimethylamino)ethyl ester (E,E) **6b**

Prepared as described for **4a** starting from dithiol **3b** and (E)-3-(Methylthio)acrylic acid [13]. Purification on silicagel (DCM/MeOH: 95/5). Viscous oil. Yield: 35%. IR (neat) ν 1730, 1655, 1555, 935, 700 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.31–1.77 (m, 6H), 1.79–2.01 (m, 2H), 2.28 (s, 6H), 2.29 (t, $J = 7.3$, 2H), 2.34 (s, 6H), 2.55 (t, $J = 5.9$, 2H), 2.92 (m, 1H), 3.10 (m, 1H), 3.68 (m, 1H), 4.16 (t, $J = 5.9$ 2H), 5.97 (d, $J = 5.9$, 2H), 7.73 (d, $J = 14.7$, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.52 (CH_3), 14.53 (CH_3), 24.7 (CH_2), 26.3 ($CH_2 \times 2$), 34.1 (CH_2), 34.8 (CH_2), 35.3 (CH_2), 43.4 (CH), 45.8 ($CH_3 \times 2$), 57.9 (CH_2), 62.1 (CH_2), 120.50 (CH), 120.52 (CH), 144.2 (CH), 144.3 (CH), 173.6 (C), 186.2 (C), 186.4 (C); HRMS-FAB$^+$ calcd. for $C_{20}H_{34}NO_4S_4[MH]^+$: 480.1371 found: 480.1369.

5.7. 2-[[6,8-Bis[[3-(methylthio)-1-oxo-2-propenyl]thio]-1-oxooctyl]oxy]-N,N, N-trimethyl ethanaminium iodide (E,E) **6c**

Prepared as described for **2c** starting from **6b**. Purification by chromatography on silica gel (DCM/MeOH: 90/10). Hygroscopic amorphous solid. Yield 85%. IR (DCM) ν 1740, 1650, 1550, 935 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.26–1.71 (m, 6H), 1.72–1.96 (m, 2H), 2.30 (s, 3H), 2.31 (s 3H), 2.33 (t, $J = 7.35$, 2H), 2.68 (m, 1H), 3.02 (m, 1H), 3.51 (s, 9H), 3.60 (m, 1H), 4.08 (m, 2H), 4.55 (m, 2H), 5.93 (d, $J = 14.7$, 2H), 7.69 (d, $J = 14.7$, 1H), 7.69 (d, $J = 9.93$, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.59 (CH_3), 14.64 (CH_3), 24.3 (CH_2), 26.2 (CH_2), 26.3 (CH_2), 33.9 (CH_2), 34.6 (CH_2), 35.2 (CH_2), 43.4 (CH), 54.8 ($CH_3 \times 3$), 57.8 (CH_2), 65.2 (CH_2), 120.50 ($CH \times 2$), 144.5 (CH), 144.7 (CH), 172.5 (C), 186.4 (C), 186.5 (C); HRMS-FAB$^+$ calcd. for $C_{21}H_{36}NO_4S_4[M-I]^+$: 494.1527 found: 494.1527.

6. Effects of compounds 4–6 on cells in culture

The in vitro toxicity assays were carried out as described before [18]. The panel of tumor cell lines panel was composed of human embryonic lung fibroblasts MRC5; human prostate cancer cells DU145; mouse lymphoid cells overexpressing the bcl_2 gene BAF3 Bcl_2 and the human hepatoma HuH7.

Acknowledgment

We gratefully acknowledge the helpfull discussions and financial support of Dr. Uwe Reichert, Galderma R&D, Sophia Antipolis, France.

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