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Original article

Synthesis and biological evaluation of cyclic nitrogen mustards based on carnitine framework

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ABSTRACT

Two series of cyclic nitrogen mustards structurally related to L-carnitine have been prepared. The cytotoxic activity of these compounds was evaluated by using Chlorambucil as a reference. In accordance with earlier report, the cytotoxicity is in direct correlation with the lipophilicity of the introduced alkyl chains. Among the cyclic nitrogen mustards synthesized, the most cytotoxic compounds were the one acylated with a palmitoyl side chain, which showed activities comparable to that of Chlorambucil.

1. Introduction

Nitrogen mustards like Chlorambucil are commonly used as anticancer agents. These cytotoxic compounds are believed to exert their biological activity by inducing interstrand cross-links in the major groove of DNA. This linkage represents the most toxic of all alkylation events [1,2]. During the past years, many nitrogen mustard structural modifications have been envisioned to increase their cytotoxicity and especially their specificity towards tumor cells [3]. A number of reports indicate that mitochondrial DNA are over-expressed in tumor cells making them an attractive loci for selective tumor cell targeting using multiple classes of DNA damaging agents [4–6]. These findings have led to the mitochondrial DNA being targeted for antitumoral treatment [7–10]. We hypothesized that L-carnitine, an essential cofactor that transfers long chain fatty acids to the mitochondria for β-oxidation, could be considered as an appropriate vector to target these organelles. Our work is based on previous research carried out in our laboratory concerning nitrogen mustard derivatives of L-carnitine, for which the trimethylammonium moiety of L-carnitine was replaced by the appropriate bis(2-chloroethyl) amine group of nitrogen mustard [11]. The alcohol function of the mustard-containing carnitine was acylated by alkyl chains of various lengths (acetyl, propionyl, palmitoyl…). The acylated mustards showed high cytotoxic activities on MCF-7, H460 and A375 cell lines comparable to that of chlorambucil. As cyclic analogues of L-carnitine were shown to be very effective mimics of L-carnitine, we designed and prepared new cyclic nitrogen mustards based on L-carnitine specifically aimed at tumor cell mitochondrial targeting. Indeed, the over-expression of the L-carnitine transporter in cancer cells provides an opportunity to selectively alkylate mitochondrial DNA of tumor cells [12,13].

2. Targets design

We herein report the synthesis of two series of racemic cyclic nitrogen mustards **1a**–**d** and **2a**–**d** (Fig. 1) structurally related to L-carnitine, where the original trimethylammonium moiety is replaced by a bis(2-chloroethyl)amine. We believed that the in vivo protonation of the bis(2-chloroethyl)amino group or the nitrogen quaternarisation during the formation of the requisite alkylating aziridinium cation will give a positive ion corresponding to the trimethylammonium moiety of carnitine.

The first series of nitrogen mustards **1a**–**d** was designed on the 1,2- and 2,3-trans relative configurations of the tetramethylammonium and the hydroxyl group along with the hydroxyl and the carboxylic acid moiety of cyclic carnitine. Another series of mustards (**2a**–**d**), which differ from **1a**–**d** by different relative configuration was also designed and prepared. Biological studies would shed more light on the effect of this configuration on the cytotoxic activity of these compounds. As in our previous study, acyl groups with various saturated carbon chains were introduced on these mustards to study the impact of chain length on

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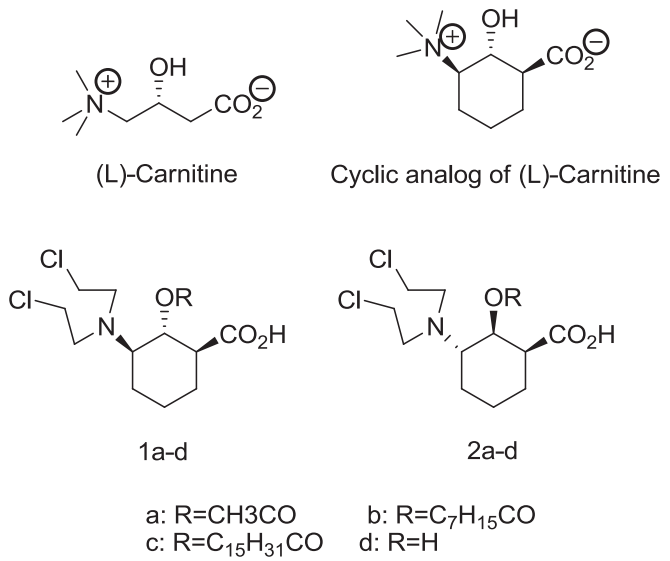


Fig. 1. Synthetic targets **1a**–**d** and **2a**–**d**.

cytotoxicity. We anticipate that the activity should increase with the lipophilicity of these molecules by improving their cell penetration capacity.

3. Synthetic studies

Our retrosynthetic approach (Fig. 2) follows the synthetic pathway reported for the preparation of cyclic L-carnitine analogues [12,13]. The key steps are the regioselective openings of the *trans*-epoxide **3** and *cis*-epoxide **4** which allow the preparation of the two series of targeted molecules. *Trans*- and *cis*-epoxides (**3** and **4**) were prepared by a multi-step sequence from methyl ester **7**, synthesized from 3-bromocyclohexene following the synthetic pathway reported by Hutchison et al. (Scheme 1) [12,13]. The nucleophilic substitution of 3-bromocyclohexene by KCN in presence of 18-crown-6 gave nitrile **6** in good yield (75%) [13,14]. The corresponding methyl ester **7** was obtained in almost quantitative yield by the treatment of **6** with methanolic HCl [14,15]. According to Davies and Whitham's procedure, the *tert*-butyl ester **5** was then prepared by acidic hydrolysis of **7** with APTS, leading quantitatively to carboxylic acid **8**, followed by treatment with isobutylene in catalytic presence of H_2SO_4 [14]. The desired *cis* and *trans*-epoxides **3** and **4** were prepared in 3:2 ratio by the usual epoxidation procedure using *m*-CPBA in CH_2Cl_2. The two stereoisomers were successfully purified and separated by chromatography on silica gel with a global yield of 71%.

The nitrogen mustards **2a**–**d** were obtained by regioselective ring-opening of the *cis*-epoxide **4** with bis(*tert*-butyldiphenylsilyloxyethyl)-amine, prepared previously in our laboratory [11]. The

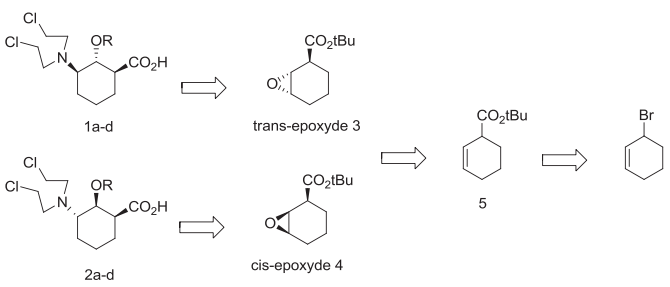


Fig. 2. Retrosynthetic analysis.

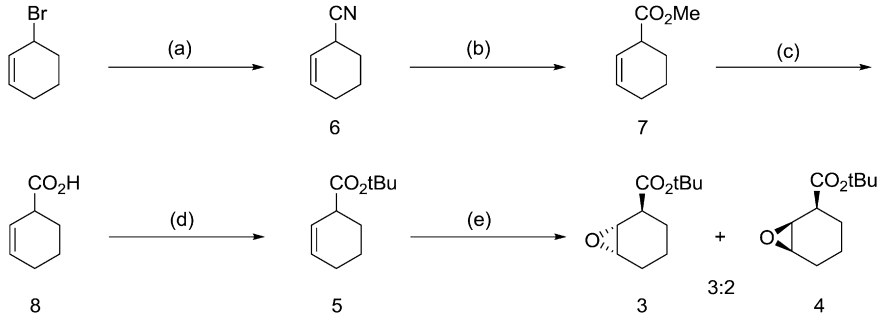
silica gel-mediated epoxide opening of **4** (Scheme 2) did not give any reaction. This problem was overcome by using lithium triflate LiOTf, well known to favour the regioselective opening of epoxides [16]. In this way the γ-amino-β-hydroxyester **9** was obtained in 75% yield. At this stage, different acyl groups (acetyl, capryloyl and palmitoyl) were introduced using appropriate anhydrides or acid chlorides. The expected *O*-acyl aminohydroxyesters **10a**–**c** were obtained in yields ranging from 78 to 97%.

The cleavage of the silyl ethers **10a**–**c** was performed by using TBAF and imidazole in THF (Scheme 2) [11,17]. However, the obtained bis(2-hydroxyethyl)amine derivatives were prone to very fast acyl migration or degradation on standing and appeared to be really sensitive to silica gel acidity. These difficulties were circumvented by flash chromatography on florisil and immediate conversion of the dihydroxyl compounds to their corresponding dichloro derivatives **11a**–**c**. The chlorination of diols was performed using *p*-TsCl in presence of TEA and DMAP [11,18,19], leading to chlorinated compounds **11a**–**c** in quite good yields (42–65% over two steps) for such derivatives (Scheme 2.). The obtained dichloro-compounds, unstable and sensitive to silica gel, were quickly purified by chromatography on a florisil column. The final acidic hydrolysis step, gave, according to the experimental conditions, either the fully deprotected nitrogen mustard **2d** (20% aq HCl/anisole) [11,20,21] (41% yield) or the *O*-acyl nitrogen mustards **2a**–**c** (TFA/anisole) [11,22–25] (62–79% yields) (Scheme 2.).

The soft reaction conditions leading to regioselective opening of *cis*-epoxide **4** were unsuccessful on the *trans*-epoxide **3**. Stronger conditions were required to obtain γ-amino-β-hydroxyester **12** finally in 25% yield (Scheme 3). Acylation of **12** by the procedure described above led to compounds **13a**–**c.** These were then converted to the corresponding mustards **14a**–**c** by silyl cleavage followed by chlorination in good yields (42–65% over two steps) (Scheme 3). The low yields associated with the synthesis of **12** led us to consider the opening of **3** by using a less sterically hindered nucleophile, such as NaN_3 [26]. This reaction was not regioselective, since a 3:2 mixture of expected γ-azido-β-hydroxyester **15** and its regioisomer β-azido-γ-hydroxyester was obtained. Purified **15** was acylated to afford **16a**–**c** in yields ranging from 75 to 98%. Azides **16a**–**c** were reduced with H_2 and Pd/C to give the corresponding amines which were immediately used for the next step due to their unstable nature. The bis(2-hydroxyethyl)amine derivatives were obtained by reaction with ethylene oxide in a sealed tube [27–32]. The obtained unstable diols were then converted to their corresponding chloro derivatives by the conditions described above. After purification on florisil the chloro derivatives **14 a**–**c** were obtained in 39–58% yields from azides **16a**–**c**. Fully deprotected nitrogen mustard **1d** (20% aq HCl/anisole) (47% yield) or the *O*-acyl nitrogen mustards **1a**–**c** (TFA/anisole) (65–75% yields) were successfully obtained by the same procedure used for the preparation and isolation of compounds **2a**–**d**.

4. Biological studies and discussion

The cytotoxicities of the prepared nitrogen mustards **1a**–**d** and **2a**–**d** were evaluated on two tumor cell lines: A375 (human melanoma) and H460 (adenocarcinoma) (Table 1 and Fig. 3). *O*-acyl derivatives **1b**–**c** and **2b**–**c** exhibited significant cytotoxic activity, especially **1c** and **2c** with CC_{50} respectively of 164 μM (A375)/210 μM (H460) and 310 μM (A375)/298 μM (H460), which are comparable to that of Chlorambucil (CC_{50} = 127 μM for A375 and CC_{50} = 104 μM for H460). A fairly good correlation appears between the antitumor activity of compounds **1a**–**c** and **2a**–**c** and the lipophilicity of their alkyl chains; the presence of either a long alkyl chain (C_7H_{15}, **1b** or **2b**) or a very lipophilic moiety ($C_{15}H_{31}$, **1c** or **2c**) proved to be effective in improving the cytotoxic activity (the



Scheme 1. Synthesis of epoxides **3** and **4.** Reagents and conditions: (a) KCN, 18-crown-6, CH_2Cl_2, r.t., 4 days, 75%; (b) HCl, MeOH, reflux, 2 h, 95%; (c) APTS, 9:1 dioxane/H_2O, reflux, overnight, 100%; (d) Isobutylene, H_2SO_4, CH_2Cl_2, r.t., 12 h, 90%; (e) *m*-CPBA, CH_2Cl_2, 25 °C, 12 h, **4** (28%) and **3** (42%).

cytotoxic activity increased by a factor of four) when compared with acetylated products **1a** and **2a.** These results could suggest that the nitrogen mustards are entering the cells by passive diffusion as it was proposed previously [11]. However, it seems that compounds **1** of the "*trans*" series (with stereochemistry corresponding to the L-carnitine) are more potent than compounds **2** of the "*cis*" series. Moreover, cyclic nitrogen mustard with free hydroxyl **1d**, which displays the same relative configuration as cyclic carnitine, showed relative good cytotoxic activities for H460 cell line (CC_{50} 304 μM) compared to its stereoisomer **2d** which was not significantly cytotoxic.

Therefore, these results could suggest an active transport into the cell possibly by L-carnitine transporters. In order to verify this hypothesis, further experiments with labelled compounds **1** and **2** are planned and will be published in due course.

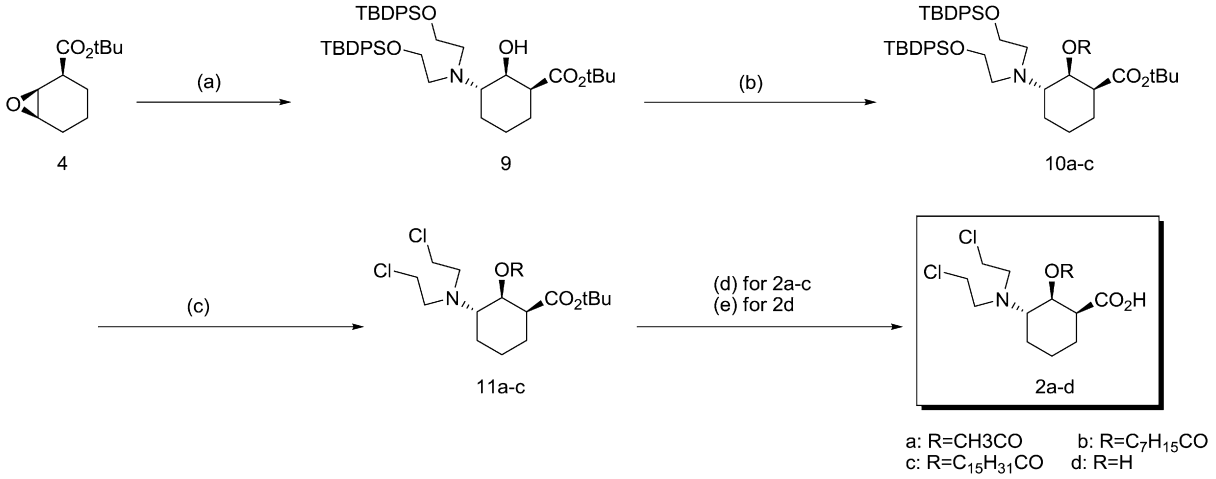
5. Conclusion

In conclusion we synthesized two series of cyclic nitrogen mustards structurally related to L-carnitine. Biological studies identified the best cytotoxic candidate to be **1c** which exhibits better cytotoxic activity than the most potent acyclic nitrogen mustard carnitine analogue we previously reported [11]. Moreover, products **1a**–**d** ("*trans*" series), presenting the same relative configuration than L-carnitine, showed better cytotoxicities than compounds **2** ("*cis*" series). In order to determine which transport pathways are involved, labelled compounds are now under development.

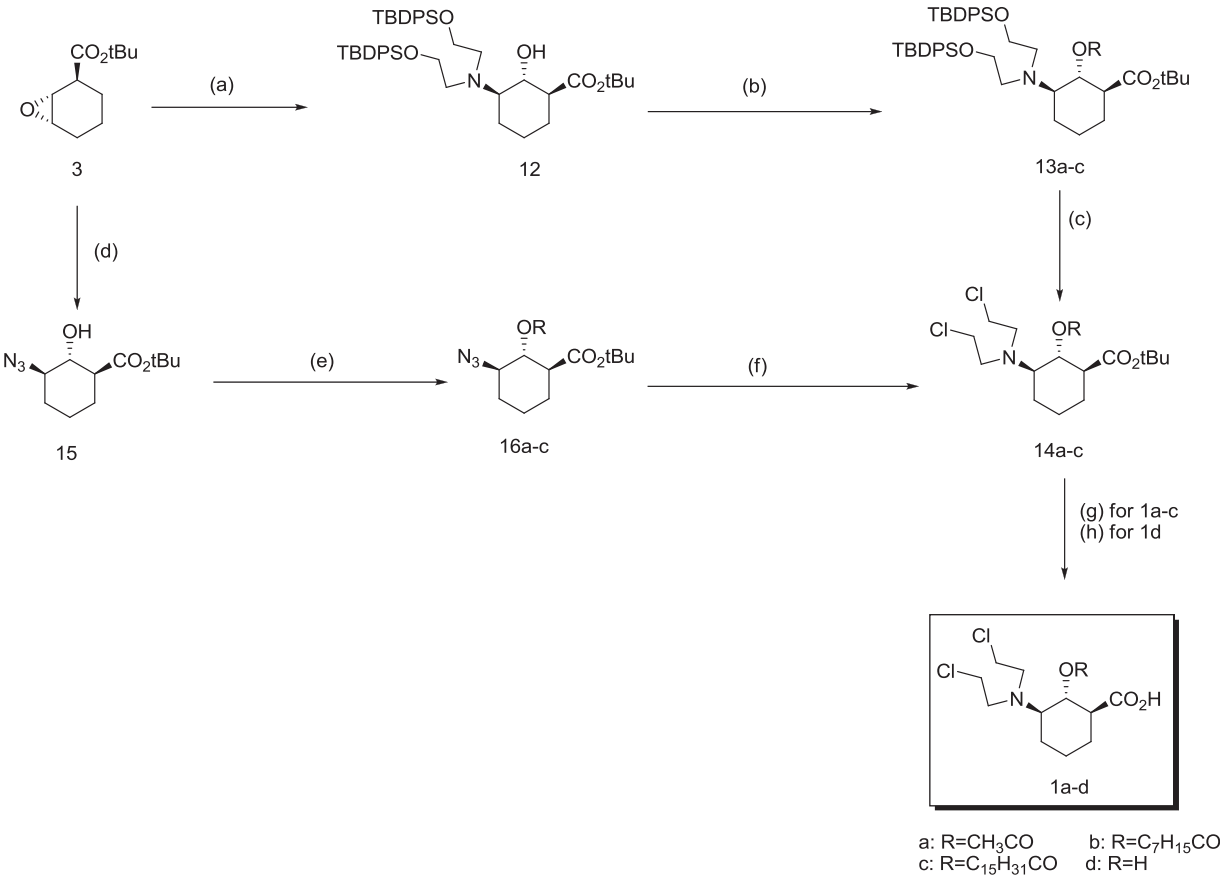
6. Experimental

6.1. Chemistry

Chemical and solvents were of reagent grade or distilled using the procedures described in "D.D. Perrin et W.L.F. Amarego, "Purification of Laboratory Chemicals", *Pergamon Press*, **3**rd **edition**, *London*, **1988**". Anhydrous grade solvents were purchased from Aldrich and VWR. All reactions involving air or moisture sensitive reagents or intermediates were performed under an argon atmosphere. Chromatographic separations were performed on Carlo Erba (Silica Gel 60A, 35–70 μm) or C18 reverse phase silica gel. Elemental analyses were carried out by the service de microanalyses du CNRS, division de Vernaison (France). TLC was performed on Merck 60 F_{354} (art. 5554) brand silica gel plates that were visualized by irradiation (254 nm) or by staining with *p*-anisaldehyde stain. ^1H and ^{13}C NMR spectra were obtained using a Bruker DRX-400 (400 MHz) instrument. Chemical shifts were reported in parts per million (ppm, δ) referenced to the residual ^1H resonance of the solvents ($CDCl_3$, 7.26 ppm). ^{13}C spectra were referenced to the residual ^{13}C resonance of the solvent ($CDCl_3$, 77 ppm). Samples recorded in D_2O were referenced to either H_2O (^1H NMR) or dioxane (^{13}C NMR). Splitting patterns were designated as follow: s, singlet; br, broad; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet. Elemental analyses were carried out by the Service de Microanalyses du CNRS, Division de Vernaison (France). Mass spectra were recorded by electrospray on a Waters micromass ZQ spectrometer.



Scheme 2. Synthesis of mustards **2a**–**d** Reagents and conditions: (a) $NH(CH_2CH_2OTBDPS)_2$, LiOTf, CH_3CN, reflux, 2 days, 75%; (b) Acyl chloride or anhydride, TEA, DMAP, CH_2Cl_2, reflux, 12 h, 78–97%; (c) (i) TBAF, Imidazole, THF, r.t., 30 min; (ii) *p*-TsCl, TEA, CH_2Cl_2, r.t., 12 h, 42–65% over 2 steps; (d) TFA, anisole, CH_2Cl_2, r.t., 8 h, 62–79%; (e) HCl, THF, r.t., 5 days, 41%.



Scheme 3. Synthesis of mustards **1a**–**d**. Reagents and conditions: (a) $NH(CH_2CH_2OTBDPS)_2$, LiOTf, CH_3CN, reflux, 2 days, 25%; (b) Acyl chloride or anhydride, TEA, DMAP, CH_2Cl_2, reflux, 12 h, 74–96%; (c) (i) TBAF, Imidazole, THF, r.t., 30 min; (ii) *p*-TsCl, TEA, CH_2Cl_2, r.t., 12 h, 42–65% over 2 steps; (d) NaN_3, NH_4Cl, MeOH, reflux, 12 h, 51%; (e) Acyl chloride or anhydride, TEA, DMAP, CH_2Cl_2, reflux, 12 h, 75–98%; (f) (i) H_2, Pd/C, THF, r.t., 6 h (ii) Ethylene oxide, EtOH, sealed tube, r.t., 24 h (iii) *p*-TsCl, TEA, DMAP, CH_2Cl_2, r.t., 12 h, 39–58% over 3 steps; (g) TFA, anisole, CH_2Cl_2, r.t., 8 h, 65–75%.; (h) HCl, THF, r.t., 5 days, 47%.

6.1.1. Cyclohex-2-enecarbonitrile (**6**)

Compound **6** was prepared according to the method of Hutchison et al. [13]. To a solution containing 14.0 g (87.0 mmol) of 3-bromocyclohexene in 50 mL of CH_2Cl_2 were added 22.0 g (348 mmol) of KCN and 300 mg (1.10 mmol) of 18-crown-6. The reaction mixture was stirred at 25 °C for 4 days. The unreacted KCN was filtered, washed with CH_2Cl_2 and excess solvent was removed under diminished pressure to afford a crude oil. The residue was purified by chromatography on a silica gel column. Step gradient elution with petroleum ether → 95:5 petroleum ether/ethyl acetate gave **6** as a colourless oil: yield 6.97 g (75%); silica gel TLC R_f 0.84 (9:1 petroleum ether/ethyl acetate); bp 84 °C/20 mm Hg ([33] bp 89 °C/23 mm Hg); ^{1}H NMR ($CDCl_3$) δ 1.40–2.24 (m, 6H), 3.21 (m, 1H) and 5.61–5.80 (m, 2H); MS (ESI): *m*/*e* 108 $(M + H)^+$.

Table 1
Human cell line cytotoxicity activities (CC_{50}) in μM of compounds **1a**–**d** and **2a**–**d** determined by MTT assay.[a]

Compound	A375	H460
1a	>1000	>1000
2a	>1000	>1000
1b	295 ± 19	414 ± 12
2b	322 ± 21	>1000
1c	164 ± 17	215 ± 13
2c	311 ± 25	298 ± 15
1d	857 ± 27	304 ± 17
2d	>1000	>1000
Chlorambucil	127 ± 9	104 ± 11

[a] Cell type: A375 (human melanoma); H460 (human adenocarcinoma).

6.1.2. Methyl cyclohex-2-enecarboxylate (**7**)

Compound **7** was prepared according to the method of Hutchison et al. [13]. Anhydrous hydrogen chloride was bubbled into a refluxing solution containing 5.00 g (46.0 mmol) of **6** in 40 mL of MeOH for 1 h. The reaction mixture was heated at reflux for an additional hour and stirred at room temperature for 12 h. The reaction mixture was poured into ice and extracted with diethyl ether. The combined organic phase was washed with 5% $NaHCO_3$, dried (Na_2SO_4), filtered and concentrated under diminished pressure to afford **7** as a colourless oil which was used without further purification in the next step: yield 6.10 g (95%); silica gel TLC R_f 0.78 (9:1 petroleum ether/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 1.41–2.24 (m, 6H), 2.92 (m, 1H), 3.72 (s, 3H) and 5.60–5.79 (m, 2H); MS (ESI): *m*/*e* 141 $(M + H)^+$ and 163 $(M + Na)^+$.

6.1.3. Cyclohex-2-enecarboxylate (**8**)

To a solution containing 2.00 g (14.0 mmol) of **7** in 75 mL of 9:1 dioxane/H_2O were added 500 mg (2.86 mmol) of APTS. The reaction mixture was warmed to reflux and stirred overnight. The reaction was quenched with H_2O and the mixture was extracted with diethyl ether. The combined organic phase was washed with brine and satd aq $NaHCO_3$. The aqueous phase was acidified until pH = 3 with 2 N HCl and the mixture extracted with diethyl ether. This combined organic phase was dried (Na_2SO_4), filtered and concentrated under diminished pressure to give **8** as a colourless oil: yield 1.76 g (100%); silica gel TLC R_f 0.21 (9:1 petroleum ether/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 1.41–2.24 (m, 6H), 3.15 (m, 1H), 5.61–5.79 (m, 2H) and 12.1 (br. s, 1H); Anal. Calcd for $C_7H_{10}O_2$: C,

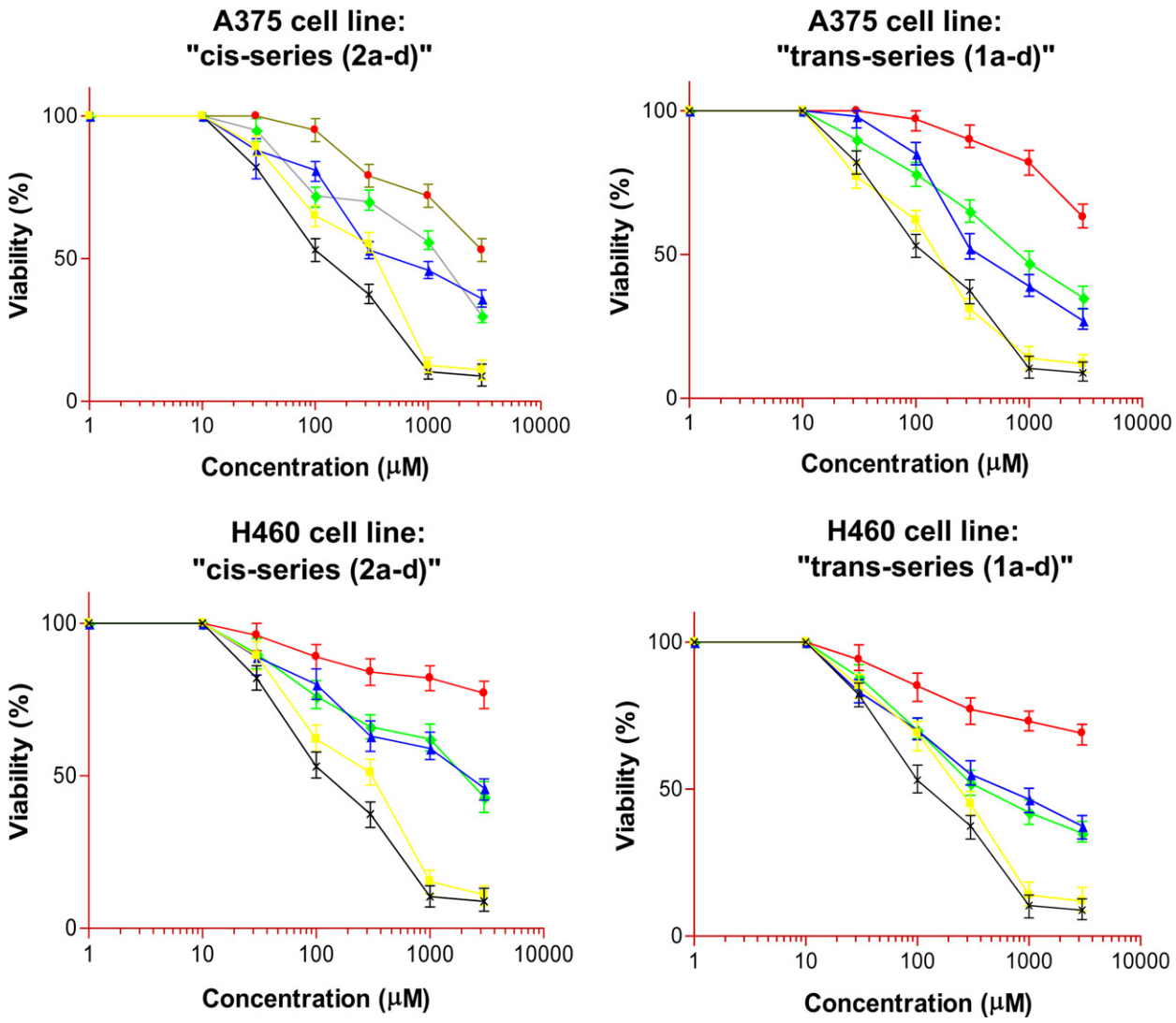


Fig. 3. Viabilities of A375 and H460 cell lines exposed to **1a**–**2a** (●), **1b**–**2b** (▲), **1c**–**2c** (■) and **1d**–**2d** (◆) for 24 h. Chlorambucil (×) was chosen as a reference. All experiments were triplicated.

66.65; H, 7.99; Found C, 66.69; H, 7.82; MS (ESI): *m*/*e* 127 $(M + H)^+$, 149 $(M + Na)^+$ and 125 $(M - H)^-$.

*6.1.4. tert-Butyl cyclohex-2-enecarboxylate (**5**)*

To a cooled (−78 °C) solution containing 2.50 g (20.0 mmol) of **8** in 100 mL of CH_2Cl_2 was bubbled ~10 mL of isobutylene. The mixture was warmed up to room temperature and 100 μL of H_2SO_4 were added. The reaction mixture was stirred at room temperature for 12 h. Excess isobutylene was removed and the organic phase was washed with satd aq $NaHCO_3$ and brine, dried (Na_2SO_4), filtered and concentrated under diminished pressure to give **5** as an oil: yield 3.28 g (90%); silica gel TLC R_f 0.87 (9:1 petroleum ether/ethyl acetate); ^1H NMR ($CDCl_3$) δ 1.41 (s, 9H), 1.41–2.24 (m, 6H), 2.23 (m, 1H) and 5.62–5.81 (m, 2H); Anal. Calcd for $C_{11}H_{18}O_2$: C, 72.49; H, 9.95; Found C, 72.44; H, 9.91; MS (ESI): *m*/*e* 183 $(M + H)^+$.

*6.1.5. tert-Butyl trans-2,3-epoxycyclohexanecarboxylate (**3**) and tert-butyl cis-2,3-epoxycyclohexanecarboxylate (**4**)*

To a cooled (0 °C) solution containing 1.40 g (7.70 mmol) of **5** in 50 mL of CH_2Cl_2 was added dropwise a solution containing 2.80 g (16.0 mmol) of *m*-CPBA in 120 mL of CH_2Cl_2. The reaction mixture was stirred at 25 °C for 12 h. The organic phase was washed with 5% Na_2CO_3 and brine, dried (Na_2SO_4), filtered and concentrated under diminished pressure to give a crude oil. The residue was purified by chromatography on a silica gel column. Step gradient elution with petroleum ether → 97:3 petroleum ether/ethyl acetate gave **3** as a colourless oil: yield 648 mg (42%); silica gel TLC R_f 0.52 (4:1 petroleum ether/diethyl ether). Further elution gave **4** as a colourless oil: yield 432 mg (28%); silica gel TLC R_f 0.49 (4:1 petroleum ether/diethyl ether).

Compound **3**: ^1H NMR ($CDCl_3$) δ 1.41 (s, 9H), 1.42–1.96 (m, 6H), 2.71 (m, 1H), 3.12 (m, 1H) and 3.31 (d, 1H, J = 3.8 Hz); ^{13}C NMR ($CDCl_3$) δ 17.2, 24.2, 24.3, 28.3, 41.9, 52.6, 52.8, 81.4 and 173.2; Anal. Calcd for $C_{11}H_{18}O_3$: C, 66.64; H, 9.15; Found C, 66.67; H, 9.20; MS (ESI): *m*/*e* 199 $(M + H)^+$ and 221 $(M + Na)^+$.

Compound **4**: ^1H NMR ($CDCl_3$) δ 1.41 (s, 9H), 1.4–1.96 (m, 6H), 2.66 (m, 1H), 3.12 (m, 1H) and 3.36 (t, 1H, J = 3.4 Hz); ^{13}C NMR ($CDCl_3$) δ 19.3, 21.7, 23.8, 28.5, 42.1, 52.6, 52.8, 81.4 and 173.2; Anal. Calcd for $C_{11}H_{18}O_3$: C, 66.64; H, 9.15; Found C, 66.68; H, 9.17; MS (ESI): *m*/*e* 199 $(M + H)^+$ and 221 $(M + Na)^+$.

*6.1.6. tert-Butyl 2-hydroxy-3-[N,N-bis(2-tert-butyldiphenylsilyloxyethyl)amino] cyclohexanecarboxylate (**9**)*

To a solution containing 540 mg (2.73 mmol) of **4** in 10 mL of CH_3CN was added 722 mg (4.63 mmol) of lithium triflate LiOTf. The

reaction mixture was stirred at room temperature for 2 h and 2.35 g (4 mmol) of $NH(CH_2CH_2OTBDPS)_2$ were added. The mixture was warmed to reflux and stirred for 2 days. Excess solvent was removed under diminished pressure and the residue was taken up in 20 mL of CH_2Cl_2. The organic phase was washed with water, dried (Na_2SO_4), filtered and concentrated under diminished pressure to afford a crude white solid. The residue was purified by chromatography on a silica gel column. Step gradient elution with petroleum ether→ 9:1 petroleum ether/diethyl ether gave **9** as a colourless oil: yield 1.61 g (75%); silica gel TLC R_f 0.71 (85:15 hexane/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 0.91 (s, 18H), 1.41 (s, 9H), 1.38–1.91 (m, 6H), 2.61 (m, 4H), 2.72 (m, 1H), 2.86 (m, 1H), 3.30 (br. s, 1H), 3.47 (m, 4H), 3.72 (m, 1H) and 7.21–7.55 (m, 20H); ^{13}C NMR ($CDCl_3$) δ 19.1, 22.2, 26.4, 27.3, 28.2, 43.6, 53.5, 58.5, 63.4, 65.0, 81.0, 127.5–137.4 (20C) and 172.9; Anal. Calcd for $C_{47}H_{65}NO_5Si_2$: C, 72.35; H, 8.40; N, 1.80; Found C, 72.32; H, 8.37; N, 1.78; MS (ESI): *m/e* 780 $(M + H)^+$.

6.1.7. *tert*-Butyl 2-hydroxy-3-[*N*,*N*-bis(2-*tert*-butyldiphenylsilyloxyethyl)amino] cyclohexanecarboxylate (12)

To a solution containing 150 mg (0.75 mmol) of **3** in 2 mL of CH_3CN were added 472 mg (3.03 mmol) of lithium triflate LiOTf. The reaction mixture was stirred at room temperature for 2 h and 880 mg (1.51 mmol) of $NH(CH_2CH_2OTBDPS)_2$ were added. The mixture was warmed to reflux and stirred for 2 days. Excess solvent was removed under diminished pressure and the residue was taken up in 20 mL of CH_2Cl_2. The organic phase was washed with water, dried (Na_2SO_4), filtered and concentrated under diminished pressure to afford a crude white solid. The residue was purified by chromatography on silica gel column. Step gradient elution with petroleum ether→ 9:1 petroleum ether/diethyl ether gave **12** as a colourless oil: yield 145 mg (25%); silica gel TLC R_f 0.75 (85:15 hexane/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 0.91 (s, 18H), 1.41 (s, 9H), 1.40–1.92 (m, 6H), 2.59 (m, 4H), 2.70 (m, 1H), 2.89 (m, 1H), 3.31 (br. s, 1H), 3.47 (m, 4H). 3.78 (m, 1H); 7.2–7.55 (m, 20H); ^{13}C NMR ($CDCl_3$) δ 19.1, 22.2, 26.4, 27.2, 28.5, 44.2, 53.0, 58.9, 63.4, 65.0, 81.2, 127.5–137.4 (20C) and 172.9; Anal. Calcd for $C_{47}H_{65}NO_5Si_2$: C, 72.35; H, 8.40; N, 1.80; Found C, 72.37; H, 8.35; N, 1.83; MS (ESI): *m/e* 780 $(M + H)^+$.

6.1.8. *tert*-Butyl 3-azido-2-hydroxycyclohexanecarboxylate (15)

To a solution containing 950 mg (4.80 mmol) of **3** in 20 mL of MeOH were added 530 mg (8.70 mmol) of NaN_3 and 382 mg (7.21 mmol) of NH_4Cl. The reaction mixture was warmed to reflux and stirred for 12 h. The reaction mixture was cooled to room temperature and excess solvent was removed under diminished pressure to give a crude oil. The residue was taken up in 20 mL of H_2O and extracted with ethyl acetate. The combined organic phase was washed with brine, dried (Na_2SO_4), filtered and concentrated under vacuum to give a 3:2 mixture of **17** and its isomer. The residue was purified by chromatography on silica gel column. Step gradient elution with petroleum ether → 4:1 petroleum ether/ethyl acetate gave **17** as an oil: yield 590 mg (51%); silica gel TLC R_f 0.32 (4:1 petroleum ether/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 1.41 (s, 9H), 1.40–1.92 (m, 6H), 2.23 (m, 1H); 3.19 (m, 1H), 3.28 (br. s, 1H) and 3.59 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 23.5, 27.7, 28.2, 30.1, 50.6, 65.2, 74.5, 81.4 and 173.2; Anal. Calcd for $C_{11}H_{19}N_3O_3$: C, 54.76; H, 7.94; N, 17.41; Found C, 54.71; H, 7.89; N, 17.46; MS (ESI): *m/e* 242 $(M + H)^+$ and 264 $(M + Na)^+$.

6.1.9. General procedure for the preparation of *O*-acyl aminohydroxyesters (10a–c, 13a–c, 16a–c)

To a solution containing **9**, **12** or **15** (1 eq.), triethylamine (2 eq.) and DMAP (cat.) in 55 mL of CH_2Cl_2 was added dropwise anhydride (acetic, palmitoic) or acid chloride (capryloyl, lauroyl) (1.2 eq.). The reaction mixture was warmed to reflux and stirred for 12 h. After cooling the organic phase was washed with satd aq $NaHCO_3$ and brine, dried (Na_2SO_4), filtered and concentrated under diminished pressure to afford a crude residue. The residue was purified by chromatography on a silica gel column. Step gradient elution with petroleum ether → 95:5 petroleum ether/ethyl acetate gave *O*-acyl hydroxyesters **10a–c**, **13a–c** and **16a–c**.

6.1.9.1. *tert*-Butyl 2-acetyloxy-3-[*N*,*N*-bis(2-*tert*-butyldiphenylsilyloxyethyl)amino] cyclohexanecarboxylate (10a).

Yield 97%; silica gel TLC R_f 0.65 (9:1 petroleum ether/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 0.91 (s 18H), 1.41 (s, 9H), 1.40–1.92 (m, 6H), 1.81 (s, 3H), 2.61 (m, 4H), 2.72 (m, 1H), 2.86 (m, 1H), 3.47 (m, 4H), 4.84 (dd, 1H, J = 4.8 Hz and J = 8.5 Hz) and 7.21–7.55 (m, 20H); ^{13}C NMR ($CDCl_3$) δ 19.1, 21.4, 26.4, 21.2, 26.8, 28.1, 43.6, 53.5, 58.9, 63.4, 72.1, 81.0, 127.5–137.4 (20C), 170.1 and 172.3; Anal. Calcd for $C_{49}H_{67}NO_6Si_2$: C, 71.58; H, 8.21; N, 1.70; Found C, 71.64; H, 8.26; N, 1.66; MS (ESI): *m/e* 825 $(M + H)^+$.

6.1.9.2. *tert*-Butyl 2-capryloyloxy-3-[*N*,*N*-bis(2-*tert*-butyldiphenylsilyloxyethyl)amino] cyclohexanecarboxylate (10b).

Yield 78%; silica gel TLC R_f 0.78 (9:1 petroleum ether/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 0.81 (t, 3H, J = 6.7 Hz), 0.91 (s, 18H), 1.10–1.71 (m, 10H), 1.41 (s, 9H), 1.40–1.92 (m, 6H), 2.08 (t, 2H, J = 6.6 Hz), 2.64 (m, 4H), 2.81 (m, 1H), 2.96 (m, 1H), 3.46 (m, 4H), 4.87 (dd, 1H, J = 4.6 Hz and J = 7.9 Hz) and 7.21–7.55 (m, 20H); ^{13}C NMR ($CDCl_3$) δ 19.1, 21.2, 21.4, 25.6, 26.4, 27.5–30.7 (5C), 27.1, 33.6, 42.0, 52.3, 57.8, 62.2, 70.9, 79.0, 126.1–137.4 (20C), 171.1 and 171.9; Anal. Calcd for $C_{55}H_{79}NO_6Si_2$: C, 72.88; H, 8.79; N, 1.55; Found C, 72.98; H, 8.85; N, 1.52; MS (ESI): *m/e* 906 $(M + H)^+$.

6.1.9.3. *tert*-Butyl 2-palmitoyloxy-3-[*N*,*N*-bis(2-*tert*-butyldiphenylsilyloxyethyl)amino] cyclohexanecarboxylate (10c).

Yield 85%; silica gel TLC R_f 0.95 (9:1 petroleum ether/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 0.81 (t, 3H, J = 6.9 Hz), 0.91 (s, 18H), 1.10–1.71 (m, 26H), 1.41 (s, 9H), 1.40–1.92 (m, 6H), 2.08 (t, 2H, J = 6.5 Hz), 2.64 (m, 4H), 2.81 (m, 1H), 2.96 (m, 1H), 3.46 (m, 4H), 4.85 (dd, 1H, J = 4.6 Hz and J = 7.8 Hz), and 7.21–7.55 (m, 20H); ^{13}C NMR ($CDCl_3$) δ 19.1, 21.2, 21.4, 25.6, 26.4, 27.5–30.7 (13C), 27.1, 33.6, 42.0, 52.3, 57.8, 62.2, 70.9, 79.0, 126.1–137.4 (20C), 171.1 and 172.0; Anal. Calcd for $C_{63}H_{95}NO_6Si_2$: C, 74.29; H, 9.40; N, 1.38 Found C, 74.36; H, 9.45; N, 1.34; MS (ESI): *m/e* 1018 $(M + H)^+$.

6.1.9.4. *tert*-Butyl 2-acetyloxy-3-[*N*,*N*-bis(2-*tert*-butyldiphenylsilyloxyethyl)amino] cyclohexanecarboxylate (13a).

Yield 96%; silica gel TLC R_f 0.61 (9:1 petroleum ether/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 0.91 (s, 18H), 1.41 (s, 9H), 1.40–1.92 (m, 6H), 1.83 (s, 3H), 2.19 (m, 1H), 2.43 (m, 1H), 2.50–2.62 (m, 4H), 3.47 (m, 4H), 4.77 (t, 1H, J = 9.8 Hz) and 7.21–7.55 (m, 20H); ^{13}C NMR ($CDCl_3$) δ 19.1, 21.2, 21.4, 25.6, 26.9, 28.1, 43.6, 53.5, 58.9, 63.4, 72.1, 81.0, 127.5–137.4, 170.1 and 172.3; Anal. Calcd for $C_{49}H_{67}NO_6Si_2$: C, 71.58; H, 8.21; N, 1.70; Found C, 71.63; H, 8.23; N, 1.72; MS (ESI): *m/e* 826 $(M + H)^+$.

6.1.9.5. *tert*-Butyl 2-capryloyloxy-3-[*N*,*N*-bis(2-*tert*-butyldiphenylsilyloxyethyl)amino] cyclohexanecarboxylate (13b).

Yield 74%; silica gel TLC R_f 0.75 (9:1 petroleum ether/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 0.78 (t, 3H, J = 6.6 Hz), 0.91 (s, 18H), 1.10–1.71 (m, 10H), 1.41 (s, 9H), 1.40–1.92 (m, 6H), 2.08 (t, 2H, J = 6.6 Hz), 2.20 (m, 1H), 2.42 (m, 1H), 2.49–2.61 (m, 4H), 3.46 (m, 4H), 4.91 (t, 1H, J = 10.1 Hz) and 7.21–7.55 (m, 20H); ^{13}C NMR ($CDCl_3$) δ 19.1, 21.2, 21.4, 25.6, 26.4, 27.5–30.7 (5C), 27.0, 33.6, 42.1, 52.3, 57.8, 62.2, 70.9, 79.0, 126.0–137.4 (20C), 171.1 and 171.9; Anal. Calcd for $C_{55}H_{79}NO_6Si_2$: C, 72.88; H, 8.79; N, 1.55; Found C, 72.97; H, 8.84; N, 1.52; MS (ESI): *m/e* 906 $(M + H)^+$.

6.1.9.6. *tert*-Butyl 2-palmitoyloxy-3-[*N*,*N*-bis(2-*tert*-butyldiphenylsilyloxyethyl)amino] cyclohexanecarboxylate (13c).

Yield 80%; silica

gel TLC R_f 0.92 (9:1 petroleum ether/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 0.81 (t, 3H, J = 6.9 Hz), 0.91 (s, 18H), 1.10–1.71 (m, 26H), 1.41 (s, 9H), 1.43–1.90 (m, 6H), 2.08 (t, 2H, J = 6.5 Hz), 2.15 (m, 1H), 2.39 (m, 1H), 2.42–2.61 (m, 4H), 3.46 (m, 4H), 4.92 (t, 1H, J = 10.3 Hz) and 7.21–7.55 (m, 20H); ^{13}C NMR ($CDCl_3$) δ 19.0, 21.1, 21.3, 25.6, 26.4, 27.5–30.7 (13C), 27.2, 33.6, 42.1, 52.3, 57.8, 62.2, 70.9, 79.1, 126.2–137.4 (20C), 171.1 and 172.5; Anal. Calcd for $C_{63}H_{95}NO_6Si_2$: C, 74.29; H, 9.40; N, 1.38; Found C, 74.36; H, 9.45; N, 1.36; MS (ESI): m/e 1018 $(M + H)^+$.

*6.1.9.7. tert-Butyl 2-acetyloxy-3-azidocyclohexanecarboxylate (**16a**).* Yield 98%; silica gel TLC R_f 0.49 (9:1 petroleum ether/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 1.41 (s, 9H), 1.40–1.91 (m, 6H), 2.23 (m, 1H), 3.19 (m, 1H) and 5.03 (t, 1H, J = 10.1 Hz); ^{13}C NMR ($CDCl_3$) δ 19.4, 21.3, 26.4, 28.2, 50.6, 65.1, 72.2, 81.4, 171.2 and 173.2; Anal. Calcd for $C_{13}H_{21}N_3O_4$: C, 55.11; H, 7.47; N, 14.83; Found C, 55.23; H, 7.52; N, 14.81; MS (ESI): m/e 284 $(M + H)^+$.

*6.1.9.8. tert-Butyl 2-capryloyloxy-3-azidocyclohexanecarboxylate (**16b**).* Yield 75%; silica gel TLC R_f 0.61 (9:1 petroleum ether/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 0.83 (t, 3H, J = 7.0 Hz), 1.10–1.71 (m, 10H), 1.41 (s, 9H), 1.40–1.92 (m, 6H), 2.05 (t, 2H, J = 6.5 Hz), 2.26 (m, 1H), 3.15 (m, 1H) and 5.09 (t, 1H, J = 9.9 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 19.2, 21.2, 22.1, 26.4, 27.5–30.7 (5C), 28.1, 50.6, 65.1, 72.3, 81.1, 170.9 and 173.1; Anal. Calcd for $C_{19}H_{33}N_3O_4$: C, 62.10; H, 9.05; N, 11.43; Found C, 62.19; H, 9.09; N, 11.40; MS (ESI): m/e 368 $(M + H)^+$.

*6.1.9.9. tert-Butyl 2-palmitoyloxy-3-azidocyclohexanecarboxylate (**16c**).* Yield 86%; silica gel TLC R_f 0.89 (9:1 petroleum ether/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 0.81 (t, 3H, J = 7.4 Hz), 1.09–1.71 (m, 26H), 1.41 (s, 9H), 1.38–1.92 (m, 6H), 2.06 (t, 2H, J = 6.9 Hz), 2.27 (m, 1H), 3.10 (m, 1H) and 5.15 (t, 1H, J = 10.1 Hz); ^{13}C NMR ($CDCl_3$) δ 19.1, 21.2, 22.3, 26.4, 27.5–30.7 (13C), 28.2, 50.6, 65.1, 72.3, 81.1, 171.1 and 173.2; Anal. Calcd for $C_{27}H_{49}N_3O_4$: C, 67.60; H, 10.30; N, 8.76; Found C, 67.72; H, 10.35; N, 8.73; MS (ESI): m/e 480 $(M + H)^+$.

*6.1.10. General procedure for the reduction of the azides **16a**–**c** and for the preparation of the corresponding bis(2-hydroxyethyl)amines using ethylene oxide*

To a solution containing 0.71 mmol of **16a**–**c** in 8 mL of THF was added Pd/C (0.150 mmol) and the reaction mixture was purged with hydrogen and stirred for 6 h under hydrogen atmosphere. The reaction mixture was filtered through a celite pad which was washed with ethyl acetate. Excess solvent was removed under diminished pressure to give a crude oil. The residue was taken up in EtOH (2 mL) under an ethylene oxide atmosphere in a sealed tube. The reaction mixture was stirred at room temperature for 24 h and excess solvent was removed under diminished pressure to afford a crude oil. The residue was purified by flash chromatography on a florisil column. Step gradient elution with petroleum ether → 2:3 petroleum ether/ethyl acetate gave expected diols which were immediately chlorinated due to their instabilities by using the procedure reported thereafter in Section 6.1.12.

*6.1.11. General procedure for the cleavage of silylated ethers (**10a**–**c** and **13a**–**c**)*

To a solution containing 0.286 mmol of **10a**–**c** or **13a**–**c** in 4 mL of THF were added 136 mg (2.00 mmol) of imidazole and 1.72 mL (1.72 mmol) of TBAF (1 M in THF). The reaction mixture was stirred at room temperature for 30 min and excess solvent was removed under diminished pressure to afford a crude oil. The residue was purified by flash chromatography on a florisil column. Step gradient elution with petroleum ether → 2:3 petroleum ether/ethyl acetate gave expected diols which were immediately chlorinated due to their instabilities by using the procedure reported herein thereafter in Section 6.1.12.

*6.1.12. General procedure for the synthesis of chlorinated products (**11a**–**c** and **14a**–**c**)*

To a solution containing 0.25 mmol of diol in 5 mL of CH_2Cl_2 were added 79.4 μL (0.57 mmol) of triethylamine, 41.9 mg (0.686 mmol) of DMAP and 130 mg (0.686 mmol) of *p*-TsCl. The reaction mixture was stirred for 12 h at room temperature and excess solvent was removed under diminished pressure to afford a crude oil. The residue was purified by flash chromatography on a florisil column. Step gradient elution with petroleum ether → 2:3 petroleum ether/ethyl acetate gave **11a**–**c** and **14a**–**c**.

*6.1.12.1. tert-Butyl 2-acetyloxy-3-[N,N-bis(2-chloroethyl)amino] cyclohexanecarboxylate (**11a**).* Yield 42%; silica gel TLC R_f 0.23 (9:1 petroleum ether/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 1.41 (s, 9H), 1.40–1.92 (m, 6H), 1.81 (s, 3H), 2.97 (m, 1H), 3.15–3.37 (m, 4H), 3.51 (m, 1H), 3.71 (m, 4H) and 4.90 (dd, 1H, J = 5.1 Hz and J = 8.2 Hz); Anal. Calcd for $C_{17}H_{29}Cl_2NO_4$: C, 53.41; H, 7.65; N, 3.66; Found C, 53.50; H, 7.68; N, 3.62; MS (ESI): m/e 382 $(M + H)^+$.

*6.1.12.2. tert-Butyl 2-capryloyloxy-3-[N,N-bis(2-chloroethyl)amino] cyclohexane carboxylate (**11b**).* Yield 51%; silica gel TLC R_f 0.38 (9:1 petroleum ether/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 0.81 (t, 3H, J = 6.9 Hz), 1.10–1.71 (m, 10H), 1.41 (s, 9H), 1.40–1.92 (m, 6H), 2.15 (t, 2H, J = 7.2 Hz), 2.99 (m, 1H), 3.11–3.32 (m, 4H), 3.51 (m, 1H), 3.72 (m, 4H) and 4.91 (dd, 1H, J = 5.7 Hz and J = 10.6 Hz); Anal. Calcd for $C_{23}H_{41}Cl_2NO_4$: C, 59.22; H, 8.86; N, 3.00; Found C, 59.28; H, 8.93; N, 2.98; MS (ESI): m/e 466 $(M + H)^+$.

*6.1.12.3. tert-Butyl 2-palmitoyloxy-3-[N,N-bis(2-chloroxyethyl) amino]cyclohexane carboxylate (**11c**).* Yield 65%; silica gel TLC R_f 0.78 (9:1 petroleum ether/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 0.81 (t, 3H, J = 6.8 Hz), 1.10–1.71 (m, 26H), 1.41 (s, 9H), 1.40–1.92 (m, 6H), 2.22 (t, 2H, J = 7.0 Hz), 2.97 (m, 1H), 3.13–3.35 (m, 4H), 3.56 (m, 1H), 3.72 (m, 4H) and 4.87 (dd, 1H, J = 5.3 Hz and J = 10.2 Hz); Anal. Calcd for $C_{31}H_{57}Cl_2NO_4$: C, 64.34; H, 9.93; N, 2.42; Found C, 64.41; H, 9.97; N, 2.41; MS (ESI): m/e 578 $(M + H)^+$.

*6.1.12.4. tert-Butyl 2-acetyloxy-3-[N,N-bis(2-chloroethyl)amino] cyclohexanecarboxylate (**14a**).* Yield 39% from **16a** and 42% from **13a**; silica gel TLC R_f 0.25 (9:1 petroleum ether/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 1.41 (s, 9H), 1.40–1.92 (m, 6H), 1.81 (s, 3H), 2.28 (m, 1H), 2.39 (m, 1H), 2.62–2.74 (m, 4H), 3.37 (m, 4H) and 5.02 (t, 1H, J = 9.5 Hz); Anal. Calcd for $C_{17}H_{29}Cl_2NO_4$: C, 53.41; H, 7.65; N, 3.66; Found C, 53.52; H, 7.70; N, 3.63; MS (ESI): m/e 382 $(M + H)^+$.

*6.1.12.5. tert-Butyl 2-capryloyloxy-3-[N,N-bis(2-chloroethyl)amino] cyclohexane carboxylate (**14b**).* Yield 49% from **16b** and 61% from **13b**; silica gel TLC R_f 0.64 (9:1 petroleum ether/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 0.82 (t, 3H, J = 6.9 Hz), 1.10–1.69 (m, 10H), 1.41 (s, 9H), 1.40–1.95 (m, 6H), 2.11 (t, 2H, J = 6.4 Hz), 2.25 (m, 1H), 2.39 (m, 1H), 2.59–2.71 (m, 4H), 3.41 (m, 4H), 5.05 (t, 1H, J = 10.2 Hz); Anal. Calcd for $C_{23}H_{41}Cl_2NO_4$: C, 59.22; H, 8.86; N, 3.00; Found C, 59.35; H, 8.91; N, 2.96; MS (ESI): m/e 466 $(M + H)^+$.

*6.1.12.6. tert-Butyl 2-palmitoyloxy-3-[N,N-bis(2-chloroethyl)amino] cyclohexane carboxylate (**14c**).* Yield 58% from **16c** and 65% from **13c**; silica gel TLC R_f 0.78 (9:1 petroleum ether/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 0.81 (t, 3H, J = 7.1 Hz), 1.10–1.72 (m, 26H), 1.41 (s, 9H), 1.40–1.89 (m, 6H), 2.15 (t, 2H, J = 6.0 Hz), 2.28 (m, 1H), 2.49 (m, 1H), 2.68–2.75 (m, 4H), 3.39 (m, 4H) and 5.10 (t, 1H, J = 10.2 Hz); Anal. Calcd for $C_{31}H_{57}Cl_2NO_4$: C, 64.34; H, 9.93; N, 2.42; Found C, 64.35; H, 9.98; N, 2.40; MS (ESI): m/e 578 $(M + H)^+$.

*6.1.13. General procedure for the synthesis of carboxylic acids **(2a–c** and **1a–c)***

To a solution containing 0.12 mmol of **11a–c** or **14a–c** in 1 mL of CH_2Cl_2 were added 13.1 μL (0.12 mmol) of anisole and 92.5 μL (1.20 mmol) of TFA. The reaction mixture was stirred at room temperature for 8 h and excess solvent was removed under diminished pressure. The residue was coevaporated with 20 mL of cyclohexane and purified by flash chromatography on a silica gel column. Elution with ethyl acetate gave **2a–c** and **1a–c** as colourless oils.

*6.1.13.1. 2-Acetyloxy-3-[N,N-bis(2-chloroethyl)amino]cyclohexanecarboxylic acid (**2a**).* Yield 62%; silica gel TLC R_f 0.35 (2:3 petroleum ether/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 1.38–1.89 (m, 6H), 1.83 (s, 3H), 3.21 (m, 1H), 3.15–3.37 (m, 4H), 3.45 (m, 1H), 3.71 (m, 4H) and 4.90 (dd, 1H, J = 5.1 Hz and J = 8.6 Hz); ^{13}C NMR ($CDCl_3$) δ 19.2, 21.2, 22.5, 26.5, 37.1, 41.2, 53.5, 56.1, 72.1, 170.1 and 173.7; Anal. Calcd for $C_{13}H_{21}Cl_2NO_4$: C, 47.86; H, 6.49; N, 4.29; Found C, 47.82; H, 6.54; N, 4.25; MS (ESI): m/e 325 $(M - H)^-$.

*6.1.13.2. 2-Capryloyloxy-3-[N,N-bis(2-chloroethyl)amino]cyclohexanecarboxylic acid (**2b**).* Yield 71%; silica gel TLC R_f 0.59 (2:3 petroleum ether/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 0.81 (t, 3H, J = 6.9 Hz), 1.10–1.71 (m, 10H), 1.41–1.93 (m, 6H), 2.15 (t, 2H, J = 7.2 Hz), 3.22 (m, 1H), 3.21–3.39 (m, 4H), 3.42 (m, 1H), 3.72 (m, 4H) and 4.96 (dd, 1H, J = 5.7 Hz and J = 10.1 Hz); ^{13}C NMR ($CDCl_3$) δ 14.2, 19.2, 20.3, 22.7, 22.9, 24.5, 26.7, 27.2, 29.8, 33.2, 37.8, 41.8, 55.3, 56.1, 71.4, 169.2 and 173.9; Anal. Calcd for $C_{19}H_{33}Cl_2NO_4$: C, 55.61; H, 8.11; N, 3.41; Found C, 55.53; H, 8.15; N, 3.38; MS (ESI): m/e 409 $(M - H)^-$.

*6.1.13.3. 2-Palmitoyloxy-3-[N,N-bis(2-chloroxyethyl)amino]cyclohexanecarboxylic acid **(2c)**.* Yield 79%; silica gel TLC R_f 0.82 (2:3 petroleum ether/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 0.81 (t, 3H, J = 6.8 Hz), 1.10–1.72 (m, 26H), 1.42–1.89 (m, 6H), 2.19 (t, 2H, J = 6.9 Hz), 3.18 (m, 1H), 3.13–3.35 (m, 4H), 3.51 (m, 1H), 3.72 (m, 4H) and 4.87 (dd, 1H, J = 5.3 Hz and J = 10.1 Hz); ^{13}C NMR ($CDCl_3$) δ 14.7, 17.0–29.5 (16C), 34.0, 37.8, 42.5, 53.2, 56.7, 70.3, 169.3 and 173.4; Anal. Calcd for $C_{27}H_{49}Cl_2NO_4$: C, 62.05; H, 9.45; N, 2.68; Found C, 61.97; H, 9.49; N, 2.64; MS (ESI): m/e 520 $(M - H)^-$.

*6.1.13.4. 2-Acetyloxy-3-[N,N-bis(2-chloroethyl)amino]cyclohexanecarboxylic acid **(1a)**.* Yield 65%; silica gel TLC R_f 0.29 (2:3 petroleum ether/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 1.41–1.90 (m, 6H), 1.81 (s, 3H), 2.46 (m, 1H), 2.51 (m, 1H), 2.62–2.74 (m, 4H), 3.37 (m, 4H) and 5.02 (t, 1H, J = 9.5 Hz); ^{13}C NMR ($CDCl_3$) δ 19.0 21.5, 22.5, 27.3, 37.8, 42.4, 54.5, 57.2, 71.8, 169.0 and 172.5; Anal. Calcd for $C_{13}H_{21}Cl_2NO_4$: C, 47.86; H, 6.49; N, 4.29; Found C, 47.79; H, 6.53; N, 4.31; MS (ESI): m/e 325 $(M - H)^-$.

*6.1.13.5. 2-Capryloyloxy-3-[N,N-bis(2-chloroethyl)amino]cyclohexanecarboxylic acid **(1b)**.* Yield 67%; silica gel TLC R_f 0.59 (2:3 petroleum ether/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 0.82 (t, 3H, J = 6.9 Hz), 1.10–1.71 (m, 10H), 1.41–1.93 (m, 6H), 2.11 (t, 2H, J = 6.4 Hz), 2.48 (m, 1H), 2.54 (m, 1H), 2.59–2.71 (m, 4H), 3.42 (m, 4H) and 5.05 (t, 1H, J = 10.0 Hz); ^{13}C NMR ($CDCl_3$) δ 15.5, 17.8–31.2 (8C), 33.4, 38.2, 41.7, 55.4, 56.3, 71.8, 169.4 and 173.1; Anal. Calcd for $C_{19}H_{33}Cl_2NO_4$: C, 55.61; H, 8.11; N, 3.41; Found C, 55.56; H, 8.15; N, 3.39; MS (ESI): m/e 409 $(M - H)^-$.

*6.1.13.6. 2-Palmitoyloxy-3-[N,N-bis(2-chloroethyl)amino]cyclohexanecarboxylic acid **(1c)**.* Yield 75%; silica gel TLC R_f 0.76 (2:3 petroleum ether/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 0.81 (t, 3H, J = 7.1 Hz), 1.10–1.72 (m, 26H), 1.40–1.93 (m, 6H), 2.15 (t, 2H, J = 6.0 Hz), 2.45 (m, 1H), 2.52 (m, 1H), 2.68–2.75 (m, 4H), 3.41 (m, 4H) and 5.1 (t, 1H, J = 10.2 Hz); ^{13}C NMR ($CDCl_3$) δ 15.2, 17.5–29.2 (16C), 34.4, 38.0, 43.4, 53.2, 56.4, 69.7, 169.4 and 173.2; Anal. Calcd for $C_{27}H_{49}Cl_2NO_4$: C, 62.05; H, 9.45; N, 2.68; Found C, 61.89; H, 9.50; N, 2.64; MS (ESI): m/e 520 $(M - H)^-$.

*6.1.14. General procedure for the preparation of **1d** and **2d***

To a solution containing 0.15 mmol of **11a** or **14a** in 150 μL of THF were added 16.0 μL (0.15 mmol) of anisole and 0.3 mL (3.7 mmol) of aqueous HCl (2.4 mol L^{-1}). The reaction mixture was stirred at room temperature for 5 days. The solution was diluted with 5 mL of diethyl ether and the organic phase was washed with water. The aqueous phase was lyophilised to afford a crude oil. The residue was purified by chromatography on a reverse phase silica gel column (C18). Elution with water gave **2d** or **1d** respectively.

*6.1.14.1. 2-Hydroxy-3-[N,N-bis(2-chloroethyl)amino]cyclohexanecarboxylic acid **(1d)**.* Yield 47%; silica gel TLC R_f 0.82 (2:3:1 acetone/ethyl acetate/water); ^{1}H NMR (D_2O) δ 1.41–1.88 (m, 6H), 2.45 (m, 1H), 2.52 (m, 1H), 2.69–2.74 (m, 4H), 3.44 (m, 4H) and 4.15 (t, 1H, J = 9.5 Hz); ^{13}C NMR (D_2O) δ 17.5, 21.2, 29.0, 36.9, 38.7, 51.8, 55.2, 63.1 and 171.9; Anal. Calcd for $C_{11}H_{19}Cl_2NO_3$: C, 46.49; H, 6.74; N, 4.93; Found C, 46.42; H, 6.81; N, 4.90; MS (ESI): m/e 283 $(M - H)^-$.

*6.1.14.2. 2-Hydroxy-3-[N,N-bis(2-chloroethyl)amino]cyclohexanecarboxylic acid **(2d)**.* Yield 41%; silica gel TLC R_f 0.78 (2:3:1 acetone–ethyl acetate/water); ^{1}H NMR (D_2O) δ 1.40–1.89 (m, 6H), 2.45 (m, 1H), 2.52 (m, 1H), 3.21–3.40 (m, 4H), 3.72 (m, 4H), 4.21 (dd, 1H, J = 5.2 Hz and J = 9.8 Hz); ^{13}C NMR (D_2O) δ 17.5, 21.9, 29.4, 37.1, 38.2, 53.6, 55.9, 62.2 and 172.6; Anal. Calcd for $C_{11}H_{19}Cl_2NO_3$: C, 46.49; H, 6.74; N, 4.93; Found C, 46.38; H, 6.77; N, 4.92; MS (ESI): m/e 283 $(M - H)^-$.

6.2. Biological procedure

Cytotoxicity assays for the nitrogen mustards **1a–d** and **2a–d** were carried out in triplicate on two different cell lines (all wild type) obtained from the American Type Culture Collection (Bethesda, MD, USA). Human melanoma cells (A375) grown in DMEM (Dulbecco's Modified Eagle's Medium) and human large cell carcinoma of the lung (NCI-H460) grown in RPMI 1640 (Roswell Park Memorial Institute), both supplemented with 10% fetal calf serum, were selected. Cells were seeded at a density of 3×10^4 cells/well in a 96-well plate and left to adhere for 24 h at 37 °C in the presence of 5% CO_2 for attachment. Culture media containing increasing concentrations (0.1–1000 mM) of test compound was prepared and the cells exposed to it for 24 h. For the viability staining, a neutral red solution (33 mg/L) was added for 4 h and the cells were stained (15 min) with a mixture glacial acetic acid–ethanol [1:50 (v/v)]. Absorbances were read at 540 nm. The cytotoxic activity of the drugs was expressed as CC_{50}, the concentration at which the proportion of death cells was 50% when compared to untreated cells. Chlorambucil was chosen as reference.

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