Synthesis of biotin and fluorescein labeled (–)-lentiginosine

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Dedicated to Prof. Pierre Vogel on the occasion of his 70th birthday

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Abstract

The important proapoptotic activity of (–)-lentiginosine, the enantiomer of a natural glycosidase inhibitor, associated with its low cytotoxicity, suggests the study of the unknown receptor responsible for the triggering of the proapoptotic cascade. To this purpose derivatives of (–)-lentiginosine **7** and **8**, which contain the biotin moiety as an affinity label and fluorescein as fluorophore, have been synthesized. Significantly, the compounds maintain a good activity as the hydroxylentiginosine precursor.

Keywords: Synthesis, conjugated iminosugars, hydroxyindolizidines, acyl azides, apoptosis

Introduction

Iminosugars have attracted a great deal of interest for their numerous potential applications as pharmacological tools in diseases involving the activity of glycosidases, glycosyltransferases and glycoprotein, in general.[1-5]

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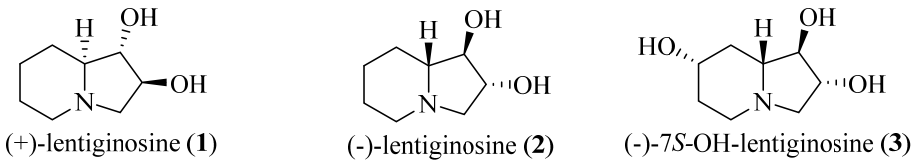

Figure 1. Structures of lentiginosine and 7-hydroxylentiginosine.

Among the different iminosugars (+)-lentiginosine (**1**) (Figure 1), a natural alkaloid,[6] has attracted our interest for the simplified structure, with respect to other iminosugars, compared to the important glycosidase inhibitory activity,[7,8] and the more recently discovered activity as a good inhibitor of Hsp90 (Heat Shock Protein 90).[9] This interest was reinforced by the discovery that the non-natural enantiomer (–)-lentiginosine (**2**, Figure 1) is a potent proapoptotic agent against tumor cells with low toxicity towards normal cells.[10] This activity is also shared by the corresponding synthetic 7*S*-OH derivative **3** (Figure 1).[11]

Apoptosis, a form of programmed cell death that proceeds by a highly regulated mechanism, plays an important role in numerous diseases including cancer. Accordingly, the development of new pro-apoptotic molecules and the knowledge of their mechanism of action are major challenges in organic chemistry and biomedical sciences. Initial studies have disclosed that the activity of **2** is caspase dependent and involves activation of the intrinsic pathway of apoptosis,[12] but still many aspects of the mechanism of action of **2** need to be investigated. In particular, the specific receptor of the molecule that is able to start the cascade of events leading to apoptosis has yet to be identified.

Modification with molecular probes such as biotin and fluorophores provides a useful technique to investigate permeability through cell membranes, intracellular distribution, and cellular targets of biologically active compounds.[13-15] We therefore decided to synthesize labeled lentiginosines and verify the effect of the structural modification on bioactivity.

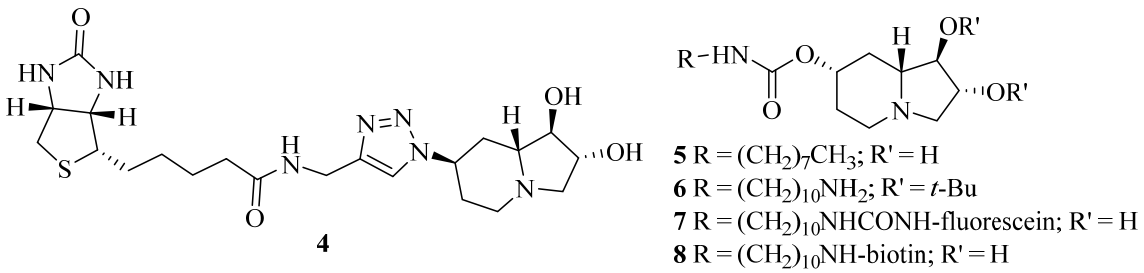

Figure 2. Lentiginosine derivatives

Since the 7-hydroxy substituted lentiginosine **3** retains the pro-apoptotic activity of the parent compound, we envisioned using the hydroxyl group as a handle to introduce biotin and fluorescein tags. The choice of the connecting moiety at indolizidine C-7 required a preliminary

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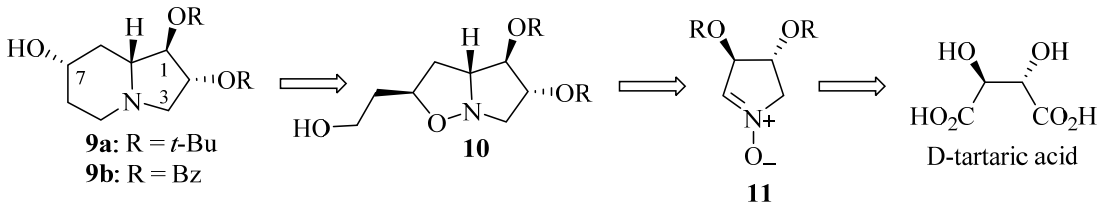
study. Our earlier effort to develop a biotin-labeled lentiginosine resulted in a triazole derivative **4** (Figure 2) that was inactive.[16] The loss of activity in **4** could be ascribed to various factors such as the inverted configuration at C-7, the large biotin tag linked to the indolizidine via a simple triazole unit, and the triazole ring itself that is considered an isostere of the amide bond.[17] The synthesis and biological evaluation of the model 1,2-dihydroxy-3-[(octylcarbamoyl)oxy]-indolizidine **5** (Figure 2) revealed that an alkyl carbamate could be a suitable spacer between the indolizidine ring system and the chosen tags. Accordingly, a flexible C-10 carbon chain α,ω-functionalized with an isocyanate and an amino group was chosen as linker.

Herein, we report on the synthesis of the common intermediate **6** and its coupling with biotin and fluorescein. The proapoptotic activity of deprotected indolizidines **7** and **8** was also evaluated.

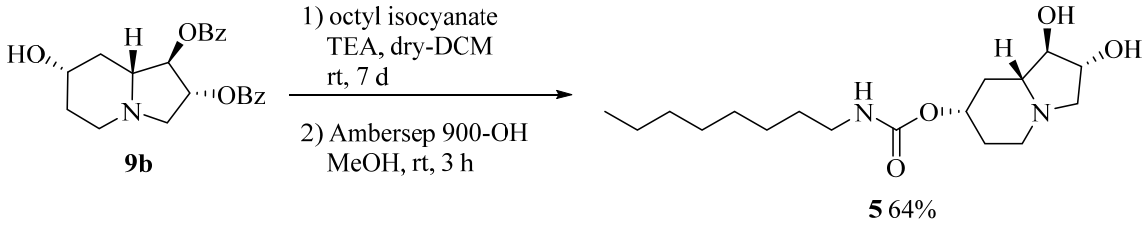
Results and Discussion

1,2-Diprotected 7-hydroxylentiginosine **9** was employed in this study as the key intermediate to produce lentiginosine conjugates. Enantiopure indolizidine **9** was synthesized starting from D-tartaric acid following recently published procedures (Scheme 1).[11,18]



Scheme 1. Synthetic approach to 7-hydroxylentiginosine **9**.

The introduction of a carbamate group in the 7-position was performed by nucleophilic addition of the free hydroxy group of **9** to a suitable isocyanate. Intermediate **9b** was reacted with the commercially available octyl isocyanate in the presence of TEA in anhydrous dichloromethane for 7 hours at room temperature. Simple removal of the solvent and treatment with the strongly basic resin Ambersep 900-OH in methanol to hydrolyze the benzoyl ester groups gave the deprotected carbamate **5** in 64% yield over two steps (Scheme 2).



Scheme 2. Synthesis of the model compound **5.**

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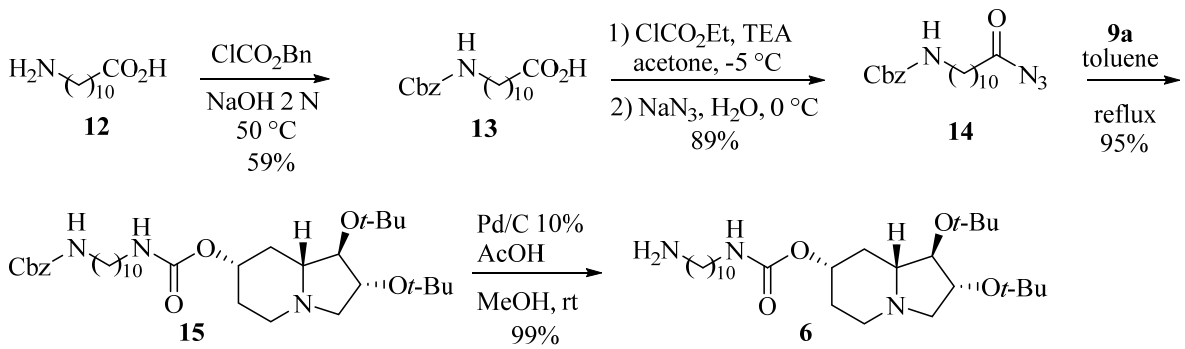
Having established the possibility of the reaction of **9** with a long chain isocyanate, and encouraged by the conservation of the biological activity in the model compound **5** (see below), the next step was to prepare a long chain carbon isocyanate with a terminal amino group to link the molecular probe on lentiginosine. To this end, the inexpensive 11-aminoundecanoic acid (**12**) was protected with the Cbz group,[19] and then the free carboxylic group was converted into isocyanate through Curtius rearrangement of the corresponding acyl azide.

Following analogous approaches reported in the literature,[20-22] the formation of a carbamate in a one-pot reaction was attempted. Heating a solution of **9a** and **13** in the presence of diphenylphosphoryl azide (DPPA) and a base afforded only traces of the desired product along with 7-(diphenoxyphosphoryloxy)-lentiginosine. No improvement was observed by adding alcohol **9a** after acyl azido or isocyanate formation.[23-25]

Fortunately, the stepwise approach was successful. Activation of **13** with ethyl chloroformate followed by treatment with sodium azide gave acyl azide **14** in good yield (Scheme 3).



Scheme 3. Synthesis of the key intermediate **6.**

Compound **14** was heated in toluene to generate the corresponding isocyanate by Curtius rearrangement and then alcohol **9a** was added. Pure carbamate **15** was obtained in 95% yield after chromatography. Deprotection of **15** by hydrogenation (Pd/C 10%, rt) afforded amine **6**, a suitable intermediate for coupling with different probes, in excellent yield (Scheme 3).

The reaction of amine **6** with fluorescein 5-isothiocyanate (FITC) occurred at room temperature in DMF/ Et_3N to give the addition product that could not be purified and was in sequence treated with TFA to remove *tert*-butoxy protection quantitatively (Scheme 4). This product, that was submitted to tests as such, gave a correct ESI-MS (see Experimental Section), but an unresolved NMR spectrum, because of the broadening caused by the acid form. Removal of acid by basic treatment gave the free indolizidine base with correct NMR data.

The reaction of amine **6** with biotin under standard coupling reaction conditions afforded amide **16** in 79% yield (Scheme 4). Deprotection of biotinylated compound **16** by TFA, followed by basic treatment, gave the deprotected labelled (–)-lentiginosine **8**, which was submitted as such for biological tests.

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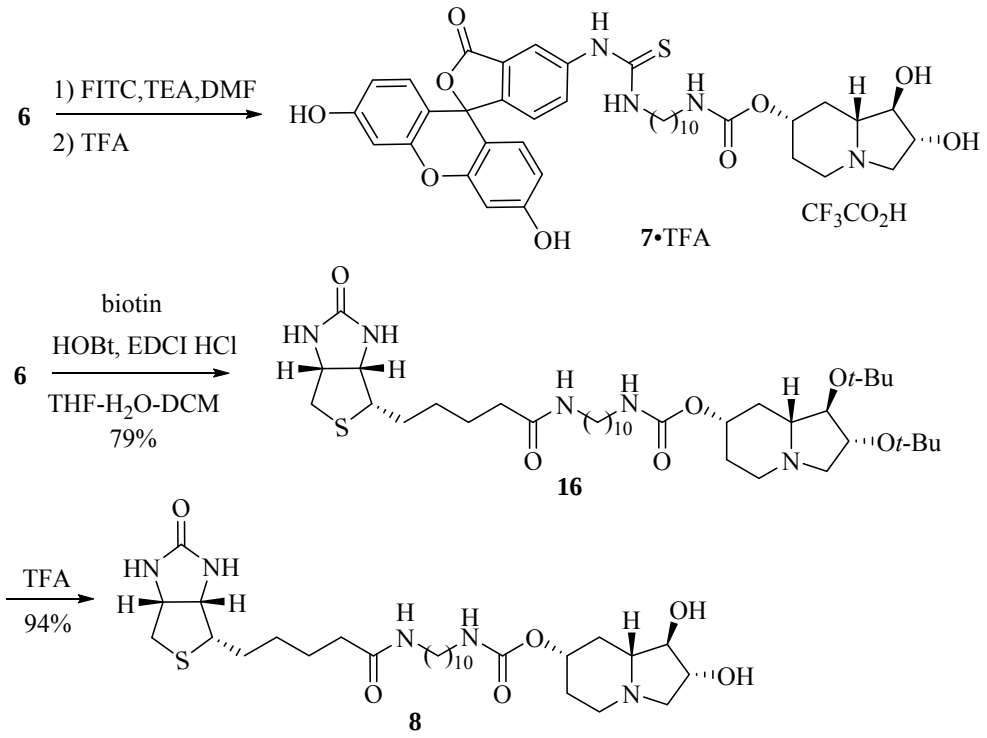

Scheme 4. Synthesis of fluorescein and biotin labelled (-)-lentiginosines **7** and **8.**

Biological testing

Selected data on the effects of **4**, **5**, and **8** on both cell viability and apoptosis of MOLT-3 tumor cells are reported in Table 1 (for dose-effect data see Supporting Information). The three lentiginosine derivatives **4**, **5**, and **8** were not cytotoxic toward MOLT-3, exhibiting CC_{50} (cytotoxic inhibitory concentration 50%) values >1000 μM. Conversely, the well-known cytotoxic chemotherapeutic agent 7-ethyl-10-hydroxycamptothecin (SN38), used as a positive control, was highly toxic. Apoptosis was assayed by flow cytometric quantification of hypodiploid nuclei.[26] Derivative **4** was unable to induce apoptosis, whereas **5** and **8** induced, respectively, 23% and 71% hypodiploid nuclei at a concentration of 500 μM. The colored fluorescein derivative **7**·TFA could be tested only at low concentration and different assays will be necessary to get accurate results, but preliminary tests indicated a good pro-apoptotic activity as well.

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Table 1. Effects of lentiginosine derivatives and **SN38** on viability and apoptosis in MOLT-3

	CC_{50} ±SD (μM)[a]	% hypodiploid nuclei (μM conc.)[b]
SN38	14±2	80 (10)
4	> 1000	0 (1000)
5	> 1000	23 (500)
7·TFA	ND[c]	74 (100)
8	> 1000	71 (500)

[a] CC_{50}: metabolic activity cytotoxic inhibitory concentration 50%. [b] Apoptosis was evaluated as a percentage hypodiploid nuclei by flow cytometry analysis after 18 h of incubation with lentiginosine derivatives and SN38 at the μM concentration reported in brackets. [c] Not determined.

Conclusions

Lentiginosine derivative **6** decorated with a C-10 chain functionalized at the terminal position with an amino group was synthesised in good yield from 7-hydroxylentiginosine **9a** and the amino acid **12**. Intermediate **6** was coupled with FITC and biotin to get labeled (–)-lentiginosines **7** and **8** that showed an adequate pro-apoptotic activity on MOLT-3 tumor cell to be used as fluorescent and affinity probes in the identification of the molecular target of (–)-lentiginosine. The present study also validated the applicability of the carbamate group as a suitable tether at C-7 of lentiginosine with preservation of the pro-apoptotic activity and low cytotoxicity.

Experimental Section

General. R_f values refer to TLC on 0.25 mm silica gel plates. Melting points (m. p.) were determined on a Thiele Electrothermal apparatus. Polarimetric measurements were performed on a JASCO DIP-370. NMR spectra were measured on Varian Gemini (^{1}H, 200 MHz, ^{13}C, 50 MHz), Varian Mercury (^{1}H, 400 MHz, ^{13}C, 100 MHz) and Varian INOVA (^{1}H, 400 MHz, ^{13}C, 100 MHz), nuclear magnetic resonance spectrometers; $CDCl_3$ was used as solvent in NMR analyses. NMR data are reported in δ (ppm) from TMS at 25 °C and peak assignments were made on the basis of ^{1}H–^{1}H COSY, HMQC and HMBC experiments. IR spectra were recorded with a Perkin-Elmer Spectrum BX FT-IR System spectrophotometer on $CDCl_3$ solutions. Elemental analyses were performed with a Perkin-Elmer 2400 analyzer. Accurate mass spectra were recorded on a LTQ-Orbitrap high-resolution mass spectrometer (Thermo, San Jose, CA, USA), equipped with a conventional ESI source. MS (ESI) were recorded on a LCQ Fleet Ion Trap Mass Spectrometer with Surveyor Plus LC System (Thermo Scientific) operating in positive ($^+$ESI) and negative ($^-$ESI) ion mode by direct infusion of a methanolic solution of the sample.

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(1*R*,2*R*,7*S*,8a*R*)-1,2-Dihydroxyoctahydroindolizin-7-yl octylcarbamate (**5**). Triethylamine (TEA, 1 μL, 0.008 mmol) and 1-isocyanatooctane (0.084 mL, 0.48 mmol) were added to a solution of **9a** (30 mg, 0.08 mmol) in 0.7 mL of dry CH_2Cl_2. The reaction mixture was stirred at rt for 7 d, then concentrated under reduced pressure. The residue dissolved in MeOH (4 mL) was treated with ion-exchange resin Ambersep 900-OH. The mixture was shaken at rt for 3 h on a flat shaker at 150 rpm. The reaction mixture was filtered through cotton wool and concentrated under reduced pressure. Chromatography on silica gel (eluent: CH_2Cl_2/MeOH 5:1) afforded **5** (16.5 mg) in 64% yield over two steps. ^{1}H NMR (400 MHz, CD_3OD): δ 4.53 (pseudo tt, *J* 11.1, 4.6 Hz, 1H, 7-H), 3.99 (ddd, *J* 7.0, 3.3, 1.3 Hz, 1H, 2-H), 3.63 (dd, *J* 8.3, 3.3 Hz, 1H, 1-H), 3.07 (t, *J* 7.0 Hz, 2H, CH_2NH), 2.97 (ddd, *J* 11.3, 4.1, 2.5 Hz, 1H, 5-Ha), 2.85 (d, *J* 10.6 Hz, 1H, 3-Ha), 2.55 (dd, *J* 10.5, 7.1 Hz, 1H, 3-Hb), 2.28 (dm, *J* 11.7 Hz, 1H, 8-Ha), 2.10 (pseudo dt, *J* 2.5, 11.9 Hz, 1H, 5-Hb), 1.98-1.89 (m, 2H, 8a-H and 6-Ha), 1.61 (pseudo dq, *J* 4.4, 12.1 Hz, 1H, 6-Hb), 1.52-1.42 (m, 2H, CH_2CH_2NH), 1.35 (pseudo q, *J* 11.5 Hz, 1H, 8-Hb), 1.35-1.27 (m, 10H, CH_2 × 5), 0.90 (t, *J* 7.0 Hz, 3H, CH_3) ppm. ^{13}C-NMR (100 MHz, CD_3OD): δ 158.5 (s; C=O), 84.8 (d; C-1), 78.6 (d; C-2), 72.8 (d; C-7), 69.3 (d; C-8a), 61.9 (t; C-3), 51.2 (t; C-5), 41.7 (t; NHCH_2), 35.6 (t; C-8), 33.0 (t; CH_2), 31.9 (t; C-6), 31.0 (t; CH_2), 30.4 (t; 2C, CH_2 × 2), 27.9 (t; CH_2), 23.7 (t; CH_2), 14.5 (q; CH_3) ppm. MS ($^+$ESI): *m/z* 351 $[M + Na]^+$. Anal. Calcd for $C_{17}H_{32}N_2O_4$ (328.45): C, 62.17; H, 9.82; N, 8.53%. Found: C, 61.84; H, 9.84; N, 8.64%.

Benzyl (11-azido-11-oxoundecyl)carbamate (**14**). Freshly distilled triethylamine (0.067 mL, 0.48 mmol) was added to a solution of acid **13** (100 mg, 0.298 mmol) in anhydrous acetone (2.7 mL) cooled at -5 °C. Ethyl chloroformate (0.046 mL, 0.477 mmol) was added dropwise to the cold solution, then the reaction mixture was stirred for 30 min at 0 °C. A solution of sodium azide (39 mg, 0.596 mmol) in water (0.175 mL) was slowly added and the mixture was stirred for 1 h at 0 °C. The mixture was treated with ice and water (5 mL), then extracted with diethyl ether (5 mL × 3). The combined organic layers were sequentially washed with water (5 mL), saturated sodium bicarbonate (5 mL), and water (5 mL), dried with Na_2SO_4, filtered and concentrated under reduced pressure to afford **14** (96 mg, 89%) as a white solid, which was used in the next step without further purification. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.43-7.28 (m, 5H, H_{Ar}), 5.09 (s, 2H, CH_{2Ar}), 4.80-4.64 (br s, 1H, NH), 3.18 (pseudo q, *J* 6.6Hz, 2H, 1-H), 2.33 (t, *J* 7.4 Hz, 2H, 10-H), 1.71-1.41 (m, 4H, 2-H, 9-H), 1.38-1.19 (br s, 12H, 3-H, 4-H, 5-H, 6-H, 7-H, 8-H) ppm. IR ($CDCl_3$):ν_{max} 3452, 2930, 2857, 2137, 1714, 1515, 1227, 1156 cm^{-1}. HRMS ($^+$ESI): calcd. for $C_{19}H_{29}N_4O_3$ $[M+H]^+$: 361.22342, found: 361.22270. Acyl azide **14** slowly rearranges in $CDCl_3$ at rt, but can be stored in at - 30°C (freezer) for several days without alteration.

Benzyl (1*R*,2*R*,7*S*,8a*R*)-1,2-di-*tert*-butoxyoctahydroindolizin-7-yl decane-1,10-diylbis-carbamate (**15**). A solution of acyl azide **14** (59 mg, 0.16 mmol) in anhydrous toluene (6.8 mL) was heated at reflux for 30 min. A solution of **9a** (42 mg, 0.15 mmol) in toluene (1.3 mL) was added, and the resulting reaction mixture heated at reflux for 5 h, then allowed to cool to rt. The solvent was evaporated under reduced pressure and the dry residue was purified by flash chromatography on silica gel (eluent: petroleum ether/AcOEt from 2:1 to 1:1) to obtain **15** (87

mg, 95%) as a white solid. R_f 0.31 (petroleum ether/AcOEt 3:2). $[\alpha]_D^{29}$ –24.9 (c 0.90, $CDCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.36-7.28 (m, 5H, H_{Ar}), 5.09 (s, 2H, CH_{2Ar}), 4.82-4.71 (br s, 1H, NH), 4.68-4.52 (m, 2H, NH e 7-H), 3.83(ddd, *J* 6.9, 3.9, 1.6 Hz, 1H, 2-H), 3.65 (dd, *J* 8.5, 3.9 Hz, 1H, 1-H), 3.21-3.09 (m, 4H, CH_2N × 2), 2.92 (dm, *J* 11.5 Hz, 1H, 5-Ha), 2.88 (br d, *J* 10.0 Hz, 1H, 3-Hb), 2.43 (dd, *J* 10.0, 7.1 Hz, 1H, 3-Ha), 2.19 (dm, *J* 10.7 Hz, 1H, 8-Hb), 2.06-1.97 (m, 1H, 5-Hb), 1.97-1.82 (m, 2H, 6-Hb, 8a-H), 1.64 (pseudo dq, *J* 3.9, 11.6 Hz, 1H, 6-Ha), 1.52-1.41 (m, 4H, CH_2CH$_2$N × 2), 1.35-1.22 [m, 13H, (CH_2)$_6$CH$_2$CH$_2$N e 8-Hb], 1.18 (s, 9H, CH_3 × 3), 1.16 (s, 9H, CH_3 × 3) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.4 (s; C=O), 156.0 (s; C=O), 136.7 (s; C_{Ar}), 128.5 (d; 2C, CH_{Ar}x2), 128.1 (d; 2C, CH_{Ar} x2), 128.0 (d; CH_{Ar}) 83.3 (d; C-1), 77.9 (d; C-2), 73.8 (s; *C*Me$_3$), 73.7 (s; *C*Me$_3$), 72.0 (d; C-7), 66.5 (t; *C*H$_2$Ph), 65.2 (d; C-8a), 61.1 (t; C-3), 50.3 (t; C-5), 41.1 [t; (*C*H$_2$N)$_{chain}$], 40.9 [t; (*C*H$_2$N)$_{chain}$], 34.5 (t; C-8), 30.7 (t; C-6), 29.9 [t; 2C, (*C*H$_2$)$_{chain}$ × 2], 29.4 [t; 2C, (*C*H$_2$)$_{chain}$ × 2], 29.2 [t; 2C, (*C*H$_2$)$_{chain}$ × 2], 29.2 (q; 3C, CH$_3$ × 3), 28.7 (q; 3C, CH$_3$ × 3), 26.7 [t; 2C, (*C*H$_2$)$_{chain}$ × 2] ppm. IR ($CDCl_3$):ν_{max} 3452, 2977, 2932, 2857, 1714, 1515, 1234, 1066 cm^{-1}. MS (ESI) *m/z* 640 $[M+Na]^+$; 618 $[M+H]^+$. Anal. Calcd for $C_{35}H_{59}N_3O_6$ (617.44): C, 68.04; H, 9.62; N, 6.80%. Found: C, 67.74; H, 9.39; N, 7.03%.

(1*R*,2*R*,7*S*,8a*R*)-1,2-Di-*tert*-butoxyoctahydroindolizin-7-yl (10-aminodecyl)carbamate (6). To a mixture of **15** (41 mg, 0.07 mmol) and 10% Pd/C (4.4 mg) in MeOH (0.7 mL) was added AcOH (0.04 mL, 0.7 mmol) at 0 °C. The reaction mixture was stirred under H_2 (1 atm) at rt for 1 h and filtered through a short pad of Celite. The solution was concentrated under reduced pressure, dissolved in MeOH and filtered through a short column of strongly basic ion-exchange resin (Amberlyst A-26) to give crude **6** (32 mg, quantitative yield) as a waxy solid {HRMS ($^+$ESI): calcd. for $C_{27}H_{54}N_3O_4$ $[M+H]^+$: 484.41088, found: 484.41094}, which was used in the next step without further purification.

(1*R*,2*R*,7*S*,8a*R*)-1,2-Dihydroxyoctahydroindolizin-7-yl [10-({[(3',6'-dihydroxy-3-oxo-3*H*-spiro[2-benzofuran-1,9'-xanthen]-4-yl)amino]carbonothioyl}amino)decyl]carbamate-· trifluoroacetic acid (7·TFA). Fluorescein-5-isothiocyanate (FITC, 17 mg, 0.047 mmol) and freshly distilled TEA (0.007 mL) were added to amine **6** (21 mg, 0.043 mmol) in dry DMF (1.4 mL) at 0 °C. The reaction mixture was stirred at rt for 4 h. The solvent was removed under reduced pressure to afford **(1R,2R,7S,8aR)-1,2-Di-tert-butoxyoctahydroindolizin-7-yl [10-[[[(3',6'-dihydroxy-3-oxo-3H-spiro[2-benzofuran-1,9'-xanthen]-4-yl)amino]carbonothioyl]amino]decyl]carbamate** as an orange solid (37 mg). ^{1}H NMR (400 MHz, CD_3OD): δ 8.07 (d, *J* 1.7 Hz, 1H, H_{Ar}), 7.74 (br d, *J* 8.2 Hz, 1H, H_{Ar}), 7.16 (d, *J* 8.2 Hz, 1H, H_{Ar}), 6.90 (d, *J* 8.9 Hz, 2H, H_{Ar}), 6.67 (d, *J* 2.2 Hz, 2H, H_{Ar}), 6.59 (dd, *J* 8.9, 2.2 Hz, 2H, H_{Ar}), 4.57-4.45 (m, 1H, 7-H), 3.88 (ddd, *J* 7.2, 4.0, 1.9 Hz, 1H, 2-H), 3.67 (dd, *J* 8.5, 4.0 Hz, 1H, 1-H), 3.64-3.55 (m, 2H, CH_2NH), 3.07 (t, *J* 7.0 Hz, 2H, CH_2NH), 3.02-2.95 (m, 1H, 5-Ha), 2.93 (dd, *J* 10.5, 1.9 Hz, 1H, 3-Ha), 2.56 (dd, *J* 10.5, 7.2 Hz, 1H, 3-Hb), 2.25-2.18 (m, 1H, 8-Ha), 2.16 (pseudo dt, *J* 12.2, 2.6 Hz, 1H, 5-Hb), 2.02 (ddd, *J* 11.4, 8.5, 2.5 Hz, 1H, 8a-H), 1.95-1.89 (m, 1H, 6-Ha), 1.71-1.54 (m, 3H, 6-Hb, CH_2), 1.54-1.25 (m, 15H, 8-Hb, CH_2 × 7), 1.19 (s, 9H, CH_3 × 3), 1.17 (s, 9H, CH_3 × 3) ppm. ^{13}C NMR (50 MHz, CD_3OD): δ 182.2 (s; C=S), 171.8 (s; C=O), 167.4 (s; C_{Ar}), 158.2 (s; C=O), 156.1 (s; 2C, C_{Ar}), 142.0 (s; C_{Ar}), 133.9 (s; C_{Ar}), 131.3 (d;

2C, CH_{Ar}), 131.2 (s; 2C, C_{Ar}), 129.2 (d; CH_{Ar}), 127.7 (d; CH_{Ar}), 122.0 (d; CH_{Ar}), 116.9 (d; 2C, CH_{Ar}), 113.3 (s; 2C, C_{Ar}), 103.7 (d; 2C, CH_{Ar}), 83.6 (d; C-1), 78.5 (d; C-2), 75.3 (s; *C*Me_3), 75.1 (s; *C*Me_3), 72.4 (d; C-7), 66.2 (d; C-8a), 61.4 (t; C-3), 51.1 (t; C-5), 45.7 (t; CH_2NH), 41.7 (t; CH_2NH), 34.9 (t; C-8), 31.2 (t; C-6), 30.9 (t; CH_2), 30.8 (t; CH_2), 30.6 (t; CH_2), 30.4 (t; CH_2), 30.3 (t; CH_2), 29.9 (t; CH_2), 29.6 (q; 3C, Me × 3), 29.0 (q; 3C, Me × 3), 28.0 (t; CH_2), 27.8 (t; CH_2) ppm. MS ($^+$ESI): *m/z* 895 $[M+Na]^+$, 873 $[M+H]^+$; MS ($^-$ESI): *m/z* 871 $[M-H]^-$.

The protected fluoresceine derivative was dissolved in trifluoroacetic acid (0.239 mL) at 0 °C. The reaction mixture was stirred at rt for 3 h, then TFA was removed under reduced pressure and removal brought to completion by co-evaporation with acetone. The salt **7**·TFA {MS ($^+$ESI): *m/z* 783 $[M+Na]^+$, 761 $[M+H]^+$; MS ($^-$ESI): *m/z* 759 $[M-H]^-$} was used in the biological tests without further purification. The product was neutralized by treatment with a 0.02 M solution of MeONa in MeOH (2 mL) at 0 °C to give **7**: ^{1}H NMR (400 MHz, CD_3OD): δ 8.15 (br s, 1H, H_{Ar}), 7.75 (dd, *J* 8.3, 1.7 Hz, 1H, H_{Ar}), 7.15 (d, *J* 8.3 Hz, 1H, H_{Ar}), 6.74 (d, *J* 8.8 Hz, 2H, H_{Ar}), 6.68 (d, *J* 2.4 Hz, 2H, H_{Ar}), 6.56 (dd, *J* 8.8, 2.4 Hz, 2H, H_{Ar}), 4.65-4.55 (m, 1H, 7-H), 4.06 (ddd, *J* 7.0, 3.2, 1.7 Hz, 1H, 2-H), 3.74 (dd, *J* 7.9, 3.2 Hz, 1H, 1-H), 3.65-3.55 (m, 2H, CH_2NH), 3.17-3.10 (m, 1H, 5-Ha), 3.07 (t, *J* 7.0 Hz, 2H, CH_2NH), 3.02-2.97 (m, 1H, 3-Ha), 2.88-2.80 (m, 1H, 3-Hb), 2.47-2.25 (m, 3H, 5-Hb, 8-Ha, 8a-H), 2.06-1.96 (m, 1H, 6-Ha), 1.74-1.59 (m, 3H, 6-Hb, CH_2), 1.56-1.42 (m, 3H, 8-Hb, CH_2), 1.42-1.23 (m, 12H, CH_2 × 6) ppm. ^{13}C NMR (100 MHz, CD_3OD): δ 182.7 (s; C=S), 171.4 (s; C=O), 163.1 (s; C_{Ar}), 158.2 (s; C=O), 154.7 (s; 2C, C_{Ar}), 147.4 (s; C_{Ar}), 142.5 (s; C_{Ar}), 131.2 (d; CH_{Ar}), 130.6 (d; 2C, CH_{Ar}), 130.3 (s; C_{Ar}), 126.2 (d; CH_{Ar}), 120.5 (d; CH_{Ar}), 114.5 (d; 2C, CH_{Ar}), 112.1 (s; 2C, C_{Ar}), 103.6 (d; 2C, CH_{Ar}), 83.7 (d; C-1), 77.9 (d; C-2), 71.6 (d; C-7), 69.0 (d; C-8a), 61.1 (t; C-3), 50.6 (t; C-5), 45.7 (t; CH_2NH), 41.7 (t; CH_2NH), 34.3 (t; C-8), 30.8 (t; C-6), 30.7 (t; CH_2), 30.5 (t; 2C,CH_2), 30.4 (t; CH_2), 30.3 (t; CH_2), 29.9 (t; CH_2), 28.0 (t; CH_2), 27.8 (t; CH_2) ppm. HRMS ($^+$ESI): calcd. for $C_{40}H_{49}N_4O_9S$ $[M+H]^+$: 761.32148, found: 761.32092.

(1*R*,2*R*,7*S*,8a*R*)-1,2-Di-*tert*-butoxyoctahydroindolizin-7-yl [10-[[5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanoyl]amino]decyl]carbamate (16). To a solution of amine **6** (32 mg, 0.07 mmol) in CH_2Cl_2 (0.50 mL), THF (1 mL) and water (1 mL), biotin (17.7 mg, 0.07 mmol) was added, the solution was cooled at 0 °C, then HOBt (13.4 mg, 0.10 mmol) and EDCI HCl (19 mg, 0.10 mmol) were added. The reaction mixture was stirred at rt for 12 h. The solvent was removed under reduced pressure, and the residue was purified by flash column chromatography on silica gel [eluent: CH_2Cl_2/MeOH (5% NH_4OH) 10:1] to afford **16** (37 mg, 79%) as a waxy solid. R_f 0.3 [DCM/MeOH (5% NH_4OH) 10:1]; ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.34-6.13 (m, 1H, NH_{biotin}), 6.03-5.88 (m, 1H, *NH*CH_2), 5.55-5.33 (m, 1H, NH_{biotin}), 4.79-4.70 (m, 1H, *NH*CH_2), 4.63-4.51 (m, 1H, 7-H), 4.50 (dd, *J* 7.6, 5.0 Hz, 1H, 6a-H_{biotin}), 4.30 (dd, *J* 7.6, 4.5 Hz, 1H, 3a-H_{biotin}), 3.86-3.80 (m, 1H, 2-H), 3.65 (dd, *J* 8.3, 3.8 Hz, 1H, 1-H), 3.20 (pseudo q, *J* 6.6 Hz, 2H, CH_2NH), 3.18-3.08 (m, 3H, CH_2NH, 4-H_{biotin}), 2.96-2.85 (m, 2H, 3-Ha, 5-Ha), 2.90 (dd, *J* 12.8, 5.0 Hz, 1H, 6-Ha_{biotin}), 2.73 (d, *J* 12.8 Hz, 1H, 6-Hb_{biotin}), 2.43 (dd, *J* 9.9, 7.3 Hz, 1H, 3-Hb), 2.19 (t, *J* 7.4 Hz, 2H, CH_2CO), 2.23-2.15 (signal hidden by the triplet at 2.19 ppm, 1H, 8-Ha), 2.07-1.82 (m, 3H, 5-Hb, 6-Ha, 8a-H), 1.80-1.56 (m, 5H, 6-Hb, CH_2 × 2), 1.52-

1.22 (m, 19H, 8-Hb, C*H*$_2$ × 9), 1.18 (s, 9H, C*H*$_3$ × 3), 1.16 (s, 9H, C*H*$_3$ × 3) ppm. ^{13}C NMR (50 MHz, $CDCl_3$): δ 173.1 (s; CH_2*C*=O), 164.0 (s; NCON), 156.0 (s; OCON), 83.1 (d; C-1), 77.8 (d; C-2), 73.8 (s; *C*Me$_3$), 73.7 (s; *C*Me$_3$), 71.8 (d; C-7), 65.2 (d; C-8a), 61.7 (d; C-3a$_{biotin}$), 60.9 (t; C-3), 60.1 (d; C-6a$_{biotin}$), 55.6 (d; C-4$_{biotin}$), 50.2 (t; C-5), 40.9 (t; CH_2NH), 40.5 (t; C-6$_{biotin}$), 39.4 (t; CH_2NH), 36.0 (t; *C*H$_2$CO), 34.3 (t; C-8), 30.6 (t; C-6), 29.9 (t; CH_2), 29.5 (t; CH_2), 29.3 (t; 2C, CH_2 × 2), 29.1 (q; 3C, Me × 3), 29.1 (t; 2C, CH_2 × 2), 28.6 (q; 3C, Me × 3), 28.1 (t; CH_2), 28.0 (t; CH_2), 26.8 (t; CH_2), 26.6 (t; CH_2), 25.7 (t; CH_2) ppm. IR ($CDCl_3$): ν_{max} 3631, 3457, 2977, 2932, 2857, 1708, 1661, 1516, 1265 cm^{-1}. MS ($^+$ESI): *m/z* 732 [M+Na]$^+$; 710 [M+H]$^+$. HRMS ($^+$ESI): calcd. for $C_{37}H_{68}N_5O_6S$ [M+H]$^+$: 710.48848, found: 710.48875.

(1*R*,2*R*,7*S*,8a*R*)-1,2-Dihydroxyoctahydroindolizin-7-yl [10-[[5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanoyl]amino]decyl]carbamate (**8**). Compound **16** (36 mg, 0.05 mmol) was dissolved in trifluoroacetic acid (0.28 mL) at 0 °C. The reaction mixture was stirred at rt for 1 h, then TFA was removed under reduced pressure and removal brought to completion by co-evaporation with acetone. The crude was purified by flash column chromatography on silica gel [eluent: CH_2Cl_2/MeOH (5% NH_4OH) 5:1] to afford **8** (28 mg, 93%) as a waxy solid. R$_f$ 0.29 [CH_2Cl_2/MeOH (5% NH_4OH) 5:1]; ^{1}H NMR (400 MHz, $CD_3OD/CDCl_3$ 7:3): δ 4.60-4.48 (m, 1H, 7-H), 4.49 (dd, *J* 7.8, 4.9 Hz, 1H, 6a-H$_{biotin}$), 4.29 (dd, *J* 7.8, 4.4 Hz, 1H, 3a-H$_{biotin}$), 4.01-3.96 (m, 1H, 2-H), 3.67-3.61 (m, 1H, 1-H), 3.22-3.11 (m, 3H, C*H*$_2$NH, 4-H$_{biotin}$), 3.06 (t, *J* 7.1 Hz, 2H, C*H*$_2$NH), 3.00-2.94 (m, 1H, 5-Ha), 2.91 (dd, *J* 12.8, 5.0 Hz, 1H, 6-Ha$_{biotin}$), 2.85 (br d, *J* 10.6 Hz, 1H, 3-Ha), 2.71 (d, *J* 12.8 Hz, 1H, 6-Hb$_{biotin}$), 2.54 (dd, *J* 10.6, 7.0 Hz, 1H, 3-Hb), 2.32-2.24 (m, 1H, 8-Ha), 2.18 (t, *J* 7.2 Hz, 2H, C*H*$_2$CO), 2.13-2.05 (m, 1H, 5-Hb), 2.00-1.88 (m, 2H, 6-Ha, 8a-H), 1.77-1.53 (m, 5H, 6-Hb, C*H*$_2$ × 2), 1.53-1.22 (m, 19H, 8-Hb, C*H*$_2$ × 9) ppm. ^{13}C NMR (100 MHz, $CD_3OD/CDCl_3$ 7:3): δ 175.7 (s; CH_2*C*=O), 165.8 (s; NCON), 158.2 (s; OCON), 84.6 (d; C-1), 78.5 (d; C-2), 72.6 (d; C-7), 69.1 (d; C-8a), 63.2 (d; C-3a$_{biotin}$), 61.7 (t; C-3), 61.4 (d; C-6a$_{biotin}$), 56.8 (d; C-4$_{biotin}$), 51.1 (t; C-5), 41.6 (t; CH_2NH), 41.0 (t; C-6$_{biotin}$), 40.3 (t; CH_2NH), 36.7 (t; *C*H$_2$CO), 35.4 (t; C-8), 31.7 (t; C-6), 30.8 (t; CH_2), 30.6 (t; CH_2), 30.5 (t; 2C, CH_2 × 2), 30.31 (t; 2C, CH_2 × 2), 29.6 (t; CH_2), 29.3 (t; CH_2), 27.9 (t; CH_2), 27.7 (t; CH_2), 26.8 (t; CH_2) ppm. MS ($^+$ESI): *m/z* 598 [M+H]$^+$. HRMS ($^+$ESI): calcd. for $C_{29}H_{52}N_5O_6S$ [M+H]$^+$: 598.36328, found: 598.36316.

Biological assays

Cell culture. The human acute lymphoblastic T cell line MOLT-3 (Zooprofilactic Institute, Brescia, Italy) was cultured in Roswell Park Memorial Institute (RPMI) 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 2 mM glutamine (Hyclone, Cramlington, England, UK), 50 U/mL penicillin and 50 U/mL streptomycin (Hyclone). MOLT-3 was cultured at 37 °C under humidified 5% CO_2 atmosphere, in the presence or absence of **4**, **5**, and **8** at the concentrations of 10, 100, 250, 500 μM. The **SN38** (a metabolite of irinotecan, a topoisomerase I inhibitor) at the concentration of 10 μM was used as positive control in MTS and apoptosis assays.

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Evaluation of apoptosis. Apoptosis was evaluated, after 18 h of incubation, by flow cytometry analysis of hypodiploid events following treatment of the cells with detergent and PI staining, using a method that distinguishes nuclei from apoptotic, necrotic or viable cells, as previously described.[26] Isolated nuclei were analyzed using a FACScan flow cytometry (BD Biosciences, San Josè, CA). Detectors and amplifier gains for forward and orthogonal scatter were adequately selected in order to simultaneously detect nuclei from viable, apoptotic and necrotic cells. Events were gated on forward versus orthogonal scatter in such a way that degraded DNA from cell debris or from doublets was excluded, and nuclei from viable, apoptotic and necrotic cells were assayed. Data acquisition and analysis were performed using CellQuestTM software on a minimum of 5000 events for each sample (BD Biosciences, San Josè, CA).

MTS assay. Cell metabolic activity, measured by reduction of MTS to formazan, was evaluated using a colorimetric commercial kit, MTS (Cell Titer 96 Aqueous One Solution, Promega). The assay was performed by seeding 1×10^4 MOLT-3, in 100 μL in the presence or absence of **4**, **5**, and **8** at seven different concentrations, from 1 to 1000 μM in complete medium RPMI supplemented with 5% FBS. After 24 hours, twenty microliters of "Cell Titer 96 Aqueous One Solution Reagent" was added directly to culture wells at the end of the culture, incubated for 1–4 h and then absorbance was read at 490 nm.

Calculation of inhibitory concentration. Cumulative results from at least three different determinations were used to calculate the drug concentration required to inhibit mitochondrial enzyme activity by 50% (CC_{50}), as evaluated by MTS assay in all cell lines. The CC_{50} were calculated according to the best-fit curve, y value versus log x, where y is the value of the examined function and x is the drug concentration.

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