Lamellarin D Bioconjugates I: Synthesis and Cellular Internalization of PEG-Derivatives

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ABSTRACT.

Herein is reported the design and synthesis of poly(ethylene glycol) derivatives of Lamellarin D with the aim of modulating their physicochemical properties, and improving the biological activity. Mono-, di- and tri-PEG conjugates with improved solubility were obtained in 18-57% overall yields from the corresponding partially protected phenolic derivatives of Lamellarin D. Conjugates **1-9** were tested in a panel of three human tumor cell lines (MDA-MB-231 breast, A-549 lung and HT-29 colon) to evaluate their cytotoxicity. Several compounds exhibited enhanced cellular internalization, and more than 85% of the derivatives showed a lower GI_{50} than Lam-D. Furthermore, cell cycle arrest at G2 phase, and apoptotic cell-death pathways were determined for Lamellarin D and these derivatives.

KEYWORDS. Lamellarin D, anti-cancer drugs, polyethylene glycol, bioconjugation, polymeric prodrugs.

INTRODUCTION

Compounds with a good therapeutic profile often do not reach the drug market because they show poor pharmacokinetics, mainly due to poor solubility. Attachment of well-defined, water-soluble, biocompatible, non-toxic, and non-immunogenic polymeric systems to drug candidates can overcome this limitation. Furthermore, these chemical modifications can significantly improve tumor targeting, in addition to therapeutic efficacy, by EPR[1] effect of the drug in tumor tissue. Advances in this field require robust and reliable chemical strategies for the preparation of such derivatives of key pharmacological lead compounds, because very often the polymeric backbone should be incorporated to the chemical structure in the middle of the synthetic strategy.

Lam-D (*1-6*) (**1**) is a marine alkaloid with a wide range of biological activities, such as anti-proliferative activity against various tumor cell lines in the low nanomolar concentration. Lam-D inhibits the Topo I (*3*), thereby disrupting the potential of mitochondrial transmembrane (*4*) which promotes apoptosis (*7*). The conjugation of water-soluble biocompatible polymers to low molecular weight anti-cancer drugs has been reported to enhance the solubility of the drugs, enhance their accumulation in tumors, and prolong their retention in the circulatory system, thereby improving the drugs' pharmacodynamics and pharmacokinetics (*8, 9*). Notwithstanding, further controlled drug delivery by means of a readily hydrolyzable conjugation bond is required for the appropriate release of the bioactive drug (*10*). Therefore, modulation of biopolymers that bind drugs is of paramount importance for preclinical research and developments with new lead compounds (*11*). Recently, PEGylated compounds used in cancer therapy have been shown to promote the EPR effect (*12, 13*), raising the local concentration of drug accumulated in targeted solid tumors. It has been proposed that

this effect is caused by vascular leakage in the tumor and a reduction in the tumor's lymphatic drainage (*14*).

Our group sought to modulate the physicochemical properties of Lam-D by creating a series of mono-, di- and tri-PEG ester conjugates of this drug. Herein are described the syntheses using modified synthetic strategies of these conjugates, as well as their biological activity.

EXPERIMENTAL PROCEDURES

General Procedures and Product Characterization

A) General Procedures for Cross-Coupling Reactions.

A solution of bromide derivative **14** (1.0 mmol) in DMF (20 mL) was purged with Ar for 10 min, and boronate **12** (1.0 mmol), $Pd(PPh_3)_4$ (10%) and 2M K_3PO_4 (3.0 mmol) were then added. The reaction mixture was stirred at 115 ºC, and then another portion of boronate **12** (2.0 mmol) was added dropwise using a syringe pump during the first hour of reaction. The solvent was removed and the residue was dissolved in EtOAc. The organic solution was washed with water and brine, dried, and concentrated to give a crude material, which was later purified by column chromatography on silica gel. Elution with hexane/EtOAc (75:25 to 60:40) gave **15a-c** (69-77% yield).

B) General Procedure for Oxidation. Synthesis of compounds 16. A mixture of **15** (1.0 mmol) and DDQ (1.0 mmol) in dry $CHCl_3$ (15 mL) was purged with Ar in a sealed vessel and heated in a microwave at 120 ºC for 10 min. The organic solution was washed with water and brine and then dried ($MgSO_4$), filtered and evaporated in vacuum. The crude material was purified by column chromatography on silica gel. Elution with hexane/EtOAc (85:15 to 60:40) gave **16a-c** (65-93% yield).

C) General Method for Benzyloxy Removal

Pd/C (10%) was added to a solution of **16** (1.0 mmol) in MeOH (50 mL). The suspension was purged with H_2 and stirred for 16 h. The reaction mixture was filtered through a Celite pad, which was washed with CH_2Cl_2. The solvent was removed under vacuum to provide the compounds **17a-c** (yield 70-95%).

D) General Method for Lactonization

NaH (60% dispersion, 1.1 mmol/mmol free OH) was washed twice with dry THF and a solution of **17** (1 mmol) was added in THF (60 mL). The mixture was stirred for 3 h at r.t. The solvent was then removed under reduced pressure, and EtOAc was added to the residue. The organic solution was washed with saturated aq. NH_4Cl, water, and brine, then dried and concentrated to provide the compounds **18a-c** (86%-quant. yield).

E) General Method for Esterification.

EDC·HCl (4 mmol x free OH) was added in one solid portion to a solution of DMAP (0.6 mmol/mmol free OH in SM) PEG-COOH (4 mmol x free OH), and either **Lam-D** (15) or **18** (1 mmol) in dry CH_2Cl_2 (45 mL). The resulting solution was stirred at r.t. for 3 h. The reaction mixture was diluted with CH_2Cl_2 and washed with sat. aq. $NaHCO_3$ and brine. The organic phase was dried over anhydrous $MgSO_4$, and the solvent was removed under vacuum. The residue was directly purified on silica gel to provide compounds **8, 9, 19a-f** (40%-quant. yield).

F) General Method for Isopropoxy Deprotection.

Anhydrous $AlCl_3$ (1.3 equiv.) was added to a solution of compound **19** (1 mmol), in dry CH_2Cl_2 (1 mL). The mixture was sonicated for 10 min, quenched with sat. NH_4Cl, then washed with water and brine. Compounds **19b** and **19d** were treated only with brine, and the aqueous solution was extracted with EtOAc. The organic extracts were dried and evaporated. The crude product was purified by flash chromatography to give compounds **2-7** (yield 19-59%).

Methyl 1-(4-benzyloxy-3-methoxyphenyl)-8-isopropoxy-9-methoxy-5,6-dihydropyrrolo[2,1-*a*]isoquinoline-3-carboxylate (13a)

A solution of methyl 1-bromo-8-isopropoxy-9-methoxy-5,6-dihydropyrrolo[2,1-*a*]isoquinoline-3-carboxylate (543 mg, 1.4 mmol) in DMF (40 mL) was purged with Ar, and 2-(4-benzyloxy-3-methoxyphenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (**11a**) (703 mg, 2.1 mmol), $Pd(PPh_3)_4$ (159 mg, 0.1 mmol) and 2M K_2CO_3 (2.8 mL, 5.5 mmol) were added. The reaction mixture was stirred at 125 ºC for 16 h. The solvent was removed after cooling to r.t. and the residue was dissolved in EtOAc. The organic solution was washed with water and brine, dried ($MgSO_4$) and concentrated to give a crude material, which was later purified by column chromatography on silica gel. Elution with hexane/EtOAc (90:10 to 75:25) gave **13a** (543 mg, 1.38 mmol) as a yellow oil (727 mg, quant.). IR (film) ν 1670, 1464, 1440, 1242. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.37 (d, *J* = 6.0 Hz, 6H); 2.98 (t, *J* = 6.4 Hz, 2H); 3.31 (s, 3H, OMe); 3.84 (s, 3H, OMe); 3.85 (s, 3H, OMe); 4.52 (h, *J* = 6.0 Hz, 1H, OCH); 4.60 (t, *J* = 6.4 Hz, 2H); 5.19 (s, 2H); 6.73 (s, 1H); 6.86 (s, 1H); 6.89 (s, 1H); 6.93 (dd, *J* = 8.0, 1.8 Hz, 1H); 6.96-7.00 (m, 2H); 7.31 (d, *J* = 7.2 Hz, 1H); 7.35 (dd, *J* = 7.2, 7.2 Hz, 2H); 7.44 (d, *J* = 7.2 Hz, 2H). ^{13}C NMR ($CDCl_3$, 50.3 MHz) δ 22.2 (2q); 29.0 (t); 42.6 (t); 51.1 (q); 55.4 (q); 56.1 (q); 71.0 (t); 71.4 (d); 109.1 (d); 113.1 (d); 114.1 (d); 114.7 (d); 119.2 (d); 121.1 (s); 121.47 (s); 121.54 (s); 125.6 (d); 127.1 (2d); 127.8 (d); 128.5 (2d); 129.8 (s); 137.2 (s); 146.5 (s); 146.8 (s); 148.5 (s); 149.5 (s); 161.7 (s). MS (ESI) 528 (M+1, 100); 529 (M+2, 37). HRMS *m/z* calcd .for $C_{32}H_{34}NO_6$ 528.2381, found 528.2373.

Methyl 1-(4-benzyloxy-3-methoxyphenyl)-2-bromo-8-isopropoxy-9-methoxy-5,6-dihydropyrrolo[2,1-*a*]isoquinoline-3-carboxylate (14a)

NBS (82 mg, 0.46 mmol) was added in one portion to a solution of **13a** (245 mg, 0.46 mmol) in THF (10 mL). The mixture was stirred at 70 ºC under Ar for 90 min. The

solvent was removed under reduced pressure. The residue was dissolved in CH_2Cl_2 and filtered through a pad of neutral alumina. **14a** was obtained as a yellow oil (278 mg, 99%). IR (film) ν 1693, 1462, 1112 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz) δ 1.35 (d, *J* = 6.0 Hz, 6H); 2.98 (t, *J* = 6.4 Hz, 2H); 3.20 (s, 3H, OMe); 3.86 (s, 3H, OMe); 3.90 (s, 3H, OMe); 4.51 (h, *J* = 6.0 Hz, 1H, OCH); 4.59 (t, *J* = 6.4 Hz, 2H); 5.20 (br, 2H); 6.54 (s, 1H); 6.70 (s, 1H); 6.85-7.00 (m, 3H); 7.28-7.47 (m, 5H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 22.0 (2q); 28.6 (t); 43.4 (t); 51.1 (q); 55.0 (q); 56.0 (q); 70.7 (t); 71.2 (d); 107.6 (s); 108.9 (d); 113.0 (s); 113.7 (d); 113.9 (s); 114.3 (d); 114.5 (d); 119.1 (s); 122.2 (s); 123.2 (d); 125.3 (s); 126.9 (2d); 127.6 (d); 128.4 (2d); 131.8 (s); 137.0 (s); 146.6 (s); 147.3 (s); 148.3 (s); 149.4 (s); 161.1 (s). MS (ESI) 606 (MBr^{79}+1, 100); 607 (MBr^{79}+2, 23); 608 (MBr^{81}+1, 95). HRMS *m/z* calcd. for $C_{32}H_{33}NO_6Br$ 606.1486, found 606.1485.

Methyl 2-(2,4-dibenzyloxy-5-methoxyphenyl)-8-isopropoxy-1-(4-isopropoxy-3-methoxyphenyl)-9-methoxy-5,6-dihydropyrrolo[2,1-*a*]isoquinoline-3-carboxylate (15a)

Following the general procedure **A** and starting from **14b** (*16*) (674 mg, 1.21 mmol) and 2-(2,4-dibenzyloxy-5-methoxyphenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (**12b**), a yellow oil (719 mg, 75%) was obtained. IR (film) ν 1691, 1254, 1212. ^1H NMR ($CDCl_3$, 200 MHz) δ 1.31 (d, *J* = 6.2 Hz, 6H); 1.35 (d, *J* = 6.0 Hz, 6H); 3.03 (t, *J* = 6.4 Hz, 2H); 3.32 (s, 3H, OMe); 3.47 (s, 3H, OMe); 3.53 (s, 3H, OMe); 3.59 (s, 3H, OMe); 4.35-4.65 (m, 4H, 2OCH); 4.74 (s, 2H); 5.01 (s, 2H); 6.46 (s, 1H); 6.55 (s, 1H); 6.61 (br, 1H); 6.69-6.79 (m, 4H); 7.08-7.13 (m, 2H); 7.17-7.26 (m, 4H); 7.27-7.35 (m, 4H). ^{13}C NMR ($CDCl_3$, 50.3 MHz) δ 21.8 (2q); 22.0 (2q); 28.9 (t); 42.8 (t); 50.7 (q); 54.9 (q); 55.5 (q); 56.2 (q); 71.0 (t); 71.2 (d, t); 71.5 (d); 102.8 (d); 109.1 (d); 114.6 (2d); 115.8 (d); 115.9 (d); 118.2 (s); 118.7 (s); 121.1 (s); 121.5 (s); 122.9 (d); 125.4 (s); 126.5 (2d); 127.1 (2d); 127.5 (2d); 128.0 (2d); 128.2 (2d); 128.6 (s); 130.8 (s); 136.8 (s); 137.6 (s);

143.3 (s); 145.4 (s); 146.0 (s); 146.8 (s); 148.2 (s); 149.8 (s); 150.3 (s); 162.2 (s). MS (MALDI-TOF) 797 (M, 100), 798 (M+1, 54).

Methyl 2-(2-benzyloxy-4-isopropoxy-5-methoxyphenyl)-1-(4-benzyloxy-3-methoxyphenyl)-8-isopropoxy-9-methoxy-5,6-dihydropyrrolo[2,1-*a*]isoquinoline-3-carboxylate (15b)

Following the general procedure **A** and starting from **14a** (277 mg, 0.46 mmol) and 2-(2-benzyloxy-4-isopropoxy-5-methoxyphenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (**12a**), a yellow oil (282 mg, 77%) was obtained. IR (film) ν 1692, 1254, 1212. ^{1}H NMR ($CDCl_3$, 200 MHz) δ 1.26 (d, *J* = 6.0 Hz, 6H); 1.36 (d, *J* = 6.0 Hz, 6H); 3.03 (t, *J* = 6.0 Hz, 2H); 3.21 (s, 3H, OMe); 3.55 (s, 3H, OMe); 3.57 (s, 6H, 2OMe); 4.37 (h, *J* = 6.0 Hz, 1H, OCH); 4.52 (h, *J* = 6.0 Hz, 1H, OCH); 4.63 (br, 2H); 4.82 (s, 2H); 5.12 (s, 2H); 6.46 (s, 1H); 6.55 (s, 1H); 6.66 (s, 1H); 6.72 (br, 4H); 7.16-7.41 (m, 10H). ^{13}C NMR ($CDCl_3$, 50.3 MHz) δ 21.9 (2q); 22.0 (2q); 28.0 and 28.9 (t); 42.2 and 42.8 (t); 50.7 (q); 55.0 (q); 55.5 (q); 56.2 (q); 70.5 (t); 71.1 (d); 71.3 (d); 71.5 (t); 104.5 (d); 109.0 (d); 113.3 (d); 114.3 (d); 114.4 (d); 116.1 (d); 118.2 (s); 118.7 (s); 121.0 (s); 121.4 (s); 122.9 (d); 125.4 (s); 126.5 (2d); 126.8 (2d); 127.1 (d); 127.5 (d); 127.7 (s); 128.0 (2d); 128.2 (2d); 130.8 (s); 137.0 (s); 137.7 (s); 144.0 (s); 145.9 (s); 146.0 (s); 146.1 (s); 148.1 (s); 148.8 (s); 150.2 (s); 162.2 (s). MS (ESI-TOF) 798 (M+1, 100), 799 (M+2, 39), 800 (M+3, 7). HRMS *m/z* calcd. for $C_{49}H_{51}NO_9$ 798.3636, found 798.3621.

Methyl 2-(2,4-dibenzyloxy-5-methoxyphenyl)-1-(4-benzyloxy-3-methoxyphenyl)-8-isopropoxy-9-methoxy-5,6-dihydropyrrolo[2,1-*a*]isoquinoline-3-carboxylate (15c)

Following the general procedure **A** and starting from **14a** (255 mg, 0.42 mmol) and 2-(2,4-dibenzyloxy-5-methoxyphenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (**12b**), a reddish oil (246 mg, 69%) was obtained. IR (film) ν 1692, 1438, 1252, 1212. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.38 (d, *J* = 6.0 Hz, 6H); 3.04 (t, *J* = 6.0 Hz, 2H); 3.24 (s, 3H,

OMe); 3.54 (s, 3H, OMe); 3.56 (s, 3H, OMe); 3.63 (s, 3H, OMe); 4.54 (h, *J* = 6.0 Hz, 1H, OCH); 4.64 (br, 2H); 4.77 (s, 2H); 5.05 (s, 2H); 5.14 (s, 2H); 6.50 (s, 1H); 6.59 (s, 1H); 6.67 (s, 1H); 6.71-6.77 (m, 4H); 7.11-7.14 (m, 2H); 7.21-7.42 (m, 13H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 22.0 (2q); 28.8 (t); 42.8 (t); 50.6 (q); 55.0 (q); 55.6 (q); 56.3 (q); 70.5 (t); 71.0 (t); 71.2 (d); 71.5 (t); 102.9 (d); 109.1 (d); 113.5 (d); 114.46 (d); 114.54 (d); 116.0 (d); 118.3 (s); 118.8 (s); 121.0 (s); 121.5 (s); 122.9 (d); 125.4 (s); 126.6 (2d); 126.8 (2d); 127.19 (d); 127.21 (2d); 127.6 (2d); 127.9 (s); 128.0 (2d); 128.3 (2d); 128.3 (2d); 128.6 (s); 130.8 (s); 136.9 (s); 137.1 (s); 137.7 (s); 143.4 (s); 146.1 (s); 146.3 (s); 146.9 (s); 148.3 (s); 149.0 (s); 150.4 (s); 162.2 (s). MS (ESI-TOF) 846 (M+1, 100), 847 (M+2, 49), 848 (M+3, 12). HRMS *m/z* calcd. for $C_{53}H_{52}NO_9$ 846.3636, found 846. 3621.

Methyl 2-(2,4-dibenzyloxy-5-methoxyphenyl)-8-isopropoxy-1-(4-isopropoxy-3-methoxyphenyl)-9-methoxypyrrolo[2,1-*a*]isoquinoline-3-carboxylate (16a)

Following the general procedure **B** and starting from **15a** (686 mg, 0.86 mmol), a yellow oil (637 mg, 93%) was obtained. IR (film) ν 1682, 1380, 1121 cm^{-1}. ^{1}H NMR ($CDCl_3$, 100 MHz) δ 1.32 (d, *J* = 6.0 Hz, 6H); 1.37 (d, *J* = 6.0 Hz, 6H); 3.42 (s, 6H, 2OMe); 3.59 (s, 3H, OMe); 3.63 (s, 3H, OMe); 4.49 (h, *J* = 6.0 Hz, 1H, OCH); 4.64 (h, *J* = 6.0 Hz, 1H, OCH); 4.78 (br, 2H); 5.03 (s, 2H); 6.48 (s, 1H); 6.54-6.73 (m, 2H); 6.79-6.94 (m, 3H); 7.03-7.11 (m, 3H); 7.15-7.20 (m, 3H); 7.23-7.34 (m, 6H); 9.31 (d, *J* = 7.6 Hz, 1H). ^{13}C NMR ($CDCl_3$, 50.3 MHz) δ 21.7 (2q); 22.0 (2q); 50.6 (q); 54.9 (q); 55.5 (q); 56.3 (q); 70.9 (d); 71.1 (t); 71.2 (d); 71.3 (t); 102.5 (d); 105.4 (d); 110.3 (d); 111.7 (d); 112.7 (s); 115.2 (d); 115.6 (d); 115.7 (s); 115.9 (d); 118.0 (s); 118.4 (s); 119.5 (s); 123.2 (d); 123.6 (d); 126.4 (d); 127.1 (d); 127.2 (2d); 127.5 (2d); 128.0 (2d); 128.2 (2d); 129.0 (s); 130.0 (s); 131.6 (s); 136.8 (s); 137.5 (s); 143.1 (s); 145.7 (s); 147.0 (s);

147.2 (s); 149.6 (s); 149.9 (s); 150.3 (s); 162.3 (s). MS (MALDI-TOF) 795 (M, 100), 796 (M+1, 73).

Methyl 2-(2-benzyloxy-4-isopropoxy-5-methoxyphenyl)-1-(4-benzyloxy-3-methoxyphenyl)-8-isopropoxy-9-methoxypyrrolo[2,1-*a*]isoquinoline-3-carboxylate (16b)

Following the general procedure **B** and starting from **15b** (1.06 g, 1.32 mmol), a yellow oil (685 mg, 65%) was obtained. IR (film) ν 1704, 1377, 1214 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.41 (2d, *J* = 6.0 Hz, 12H); 3.28 (s, 3H, OMe); 3.57 (br, 3H, OMe); 3.62 (s, 3H, OMe); 3.65 (br, 3H, OMe); 4.39 (h, *J* = 6.0 Hz, 1H, OCH); 4.65 (h, *J* = 6.0 Hz, 1H, OCH); 4.86 (br, 2H); 5.14-5.18 (m, 2H); 6.47 (br, 1H); 6.76-6.88 (m, 2H); 6.91 (d, *J* = 7.6 Hz, 1H); 7.03 (s, 1H); 7.14-7.21 (m, 3H); 7.27-7.49 (m, 10H); 9.31 (d, *J* = 7.6 Hz, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.9 (2q); 22.0 (2q); 50.7 (q); 55.2 (q); 55.6 (q); 56.4 (q); 70.6 (t); 70.8 (t); 71.0 (d); 71.5 (d); 104.3 (d); 105.5 (d); 110.3 (d); 111.8 (d); 112.8 (s); 113.3 and 113.6 (d); 115.2 (d); 116.2 (d); 118.5 (s); 119.6 (s); 123.4 (d); 123.7 (d); 127.0 (2d); 127.4 (s); 127.75 (2d); 128.2 (2d); 128.5 (2d); 128.6 (d); 128.7 (s); 128.8 (d); 129.2 (s); 130.2 (s); 131.9 (s); 137.2 (s); 137.7 (s); 144.1 (s); 146.6 (s); 147.4 (s); 149.7 (s); 150.4 (s); 162.6 (s). MS (ESI-TOF) 796 (M+1, 100), 797 (M+2, 54). HRMS *m/z* calcd. for $C_{49}H_{50}NO_9$ 796.3480, found 796.3480.

Methyl 2-(2,4-dibenzyloxy-5-methoxyphenyl)-1-(4-benzyloxy-3-methoxyphenyl)-8-isopropoxy-9-methoxypyrrolo[2,1-*a*]isoquinoline-3-carboxylate (16c)

Following the general procedure **B** and starting from **15c** (244 mg, 0.29 mmol), a yellow oil (181 mg, 74%) was obtained. IR (film) ν 1681, 1374, 1212 cm^{-1}. ^{1}H NMR ($CDCl_3$, 100 MHz) δ 1.43 (d, *J* = 6.0 Hz, 6H); 3.30 (s, 3H, OMe); 3.52-3.65 (m, 9H, 3 OMe); 4.60-4.90 (m, 3H); 5.07 (s, 2H); 5.18-5.21 (m, 2H); 6.50 (s, 1H); 6.59 (br, 1H); 6.74 (br, 1H); 6.82-6.89 (m, 2H); 6.94 (d, *J* = 7.6 Hz, 1H); 7.06 (s, 1H); 7.09-7.15 (m,

2H); 7.20-7.23 (m, 4H); 7.30-7.46 (m, 10H); 9.33 (d, *J* = 7.6 Hz, 1H). ^{13}C NMR ($CDCl_3$, 50.3 MHz) δ 21.9 (2q); 50.7 (q); 55.1 (q); 55.7 (q); 56.4 (q); 70.6 (t); 70.9 (d); 71.2 (t); 71.4 (t); 102.5 (d); 105.4 (d); 110.2 (d); 111.8 (d); 112.8 (s); 113.3 (d); 115.1 (d); 116.0 (d); 118.1 (s); 118.4 (s); 119.5 (s); 123.3 (d); 123.6 (d); 126.5 (d); 126.9 (2d); 127.2 (2d); 127.3 (2d); 127.7 (2d); 128.1 (2d); 128.3 (2d); 128.4 (2d); 129.0 (s); 130.1 (s); 131.7 (s); 136.9 (s); 137.1 (s); 137.5 (s); 143.2 (s); 146.5 (s); 147.1 (s); 147.3 (s); 149.0 (s); 149.6 (s); 150.4 (s); 162.4 (s). MS (MALDI-TOF) 844 (M+1, 100), 845 (M+1, 52), 846 (M+3, 8). HRMS *m/z* calcd. for $C_{53}H_{50}NO_9$ 844.3480, found 844.3467.

Methyl 2-(2,4-dihydroxy-5-methoxyphenyl)-8-isopropoxy-1-(4-isopropoxy-3-methoxyphenyl)-9-methoxypyrrolo[2,1-*a*]isoquinoline-3-carboxylate (17a)

Following the general procedure **C** and starting from **16a** (637 mg, 0.80 mmol), a brown oil (433 mg, 88%) was obtained. IR (film) ν 1685, 1381, 1223 cm^{-1}. ^{1}H NMR ($CDCl_3$, 100 MHz) δ 1.32 and 1.35 (d, *J* = 6.0 Hz, 6H); 1.42 (d, *J* = 6.0 Hz, 6H); 3.43 (s, 3H, OMe); 3.54 (br, 3H, OMe); 3.60 (br, 3H, OMe); 3.75 (s, 3H, OMe); 4.50 (h, *J* = 6.0 Hz, 1H, OCH); 4.67 (h, *J* = 6.0 Hz, 1H, OCH); 6.33 and 6.45 (2br, 1H); 6.57 (s, 1H); 6.82-7.00 (m, 3H); 7.05 (s, 1H); 7.17 and 7.34 (2br, 1H); 7.26 (s, 1H); 9.23 (d, *J* = 7.4 Hz, 1H). ^{13}C NMR ($CDCl_3$, 50.3 MHz) δ 21.6 (2q); 21.8 (2q); 51.0 (q); 54.8 (q); 55.5 (q); 56.2 (q); 70.8 (d); 71.1 (d); 102.5 (d); 105.3 (d); 110.1 (d); 112.1 (d); 112.4 (s); 112.8 (s); 114.1 (s); 115.0 (d); 115.6 (d); 119.0 (s); 119.1 (d); 122.9 (s); 123.2 (d); 123.4 (d); 128.4 (s); 130.2 (s); 130.6 (s); 139.8 (s); 145.5 (s); 145.7 (s); 147.3 (s); 148.2 (s); 149.5 (s); 162.1 (s); 129.0 (s). MS (MALDI-TOF) 615 (M, 100), 616 (M+1, 27).

Methyl 2-(2-hydroxy-4-isopropoxy-5-methoxyphenyl)-1-(4-hydroxy-3-methoxyphenyl)-8-isopropoxy-9-methoxypyrrolo[2,1-*a*]isoquinoline-3-carboxylate (17b)

Following the general procedure **C** and starting from **16b** (680 mg, 0.85 mmol), a brown oil (365 mg, 70%) was obtained. IR (film) ν 3440, 1684, 1381, 1218, 1121 cm^{-1}. ^{1}H NMR ($CDCl_3$, 200 MHz) δ 1.32 (d, *J* = 6.0 Hz, 6H); 1.38 (d, *J* = 6.0 Hz, 6H); 3.41 (s, 3H, OMe); 3.48 and 3.55 (2s, 3H, OMe); 3.57 and 3.68 (2br, 3H, OMe); 3.72 (s, 3H, OMe); 4.50 (h, *J* = 6.0 Hz, 1H, OCH); 4.64 (h, *J* = 6.0 Hz, 1H, OCH); 5.82 (br, 1H, OH); 6.34 and 6.40 (2br, 1H); 6.52 (br, 1H); 6.73 (d, *J* = 7.2 Hz, 1H); 6.84-6.95 (m, 2H); 7.00 (s, 1H); 7.06 and 7.15 (2d, *J* = 2.4 Hz, 1H); 7.26 (br, 1H); 9.13 (d, *J* = 7.2 Hz, 1H). ^{13}C NMR ($CDCl_3$, 50.3 MHz) δ 21.8 (2q); 22.0 (2q); 51.3 (q); 55.2 (q); 55.8 (q); 56.3 (q); 70.9 (d); 71.0 (d); 103.5 (d); 105.5 (d); 110.2 (d); 112.4 (d); 112.5 (s); 113.8 (s); 113.9 (d); 115.2 and 115.4 (d); 119.3 (s); 123.1 (d); 123.4 (d); 124.4 (s); 124.5 (d); 124.6 (s); 127.0 (s); 131.0 (s); 144.7 (s); 146.4 (s); 147.2 (s); 147.7 (s); 147.9 (s); 148.0 (s); 149.8 (s); 162.3 (s). MS (MALDI-TOF) 615 (M, 100), 616 (M+1, 16).

Methyl 2-(2,4-dihydroxy-5-methoxyphenyl)-1-(4-hydroxy-3-methoxyphenyl)-8-isopropoxy-9-methoxypyrrolo[2,1-*a*]isoquinoline-3-carboxylate (17c)

Following the general procedure **C** and starting from **16c** (122 mg, 0.14 mmol), a yellow oil (80 mg, 95%) was obtained. IR (film) ν 3425, 1683, 1219, 1122 cm^{-1}. ^{1}H NMR (acetone-d_6, 400 MHz) δ 1.33 (d, *J* = 6.0 Hz, 6H); 3.38 (s, 3H, OMe); 3.58 (s, 3H, OMe); 3.62 (s, 3H, OMe); 3.73 (br, 3H, OMe); 4.70 (h, *J* = 6.0 Hz, 1H, OCH); 5.60 (s, 1H, OH); 6.37 (s, 1H); 6.57 (br, 1H); 6.82-6.88 (m, 2H); 7.07 (d, *J* = 7.5 Hz, 1H); 7.21 (br, 1H); 7.26 (s, 1H); 7.56 (s, 1H); 9.26 (d, *J* = 7.5 Hz, 1H). ^{13}C NMR (acetone-d_6, 50.3 MHz) δ 21.9 (2q); 51.3 (q); 55.3 (q); 55.9 (q); 56.4 (q); 71.0 (d); 102.5 (d); 105.5 (d); 110.2 (d); 112.5 (d); 112.6 (s); 113.3 (s); 113.9 (d); 114.0 (d); 114.3 (d); 114.4 (s); 119.3 (s); 123.2 (d); 123.5 (s); 124.4 (d); 127.2 (s); 130.1 (s); 131.1 (s); 140.0 (s); 144.6 (s); 145.7 (s); 146.4 (s); 147.7 (s); 148.4 (s); 149.9 (s); 162.2 (s). MS (ESI-TOF) 574

(M+1, 100), 575 (M+2, 23). HRMS *m/z* calcd. for $C_{32}H_{32}NO_9$ 574.2072, found 574.2055.

4',11-Diisopropyl-Lam-D (18a)

Following the general procedure **D** and starting from **17a** (433 mg, 0.70 mmol), a white solid (410 mg, quant.) was obtained. mp (MeCN) 218-220 ºC. IR (film) ν 3359, 1699, 1432, 1258, 1157 cm^{-1}. ^1H NMR ($CDCl_3$, 200 MHz) δ 1.44 (d, *J* = 6.0 Hz, 12H); 3.44 (s, 3H, OMe); 3.51 (s, 3H, OMe); 3.86 (s, 3H, OMe); 4.57-4.77 (m, 2H, 2OCH); 6.72 (s, 1H); 6.98 (br, 1H); 7.02 (s, 1H); 7.09 (s, 1H); 7.15 (s, 1H); 7.16-7.18 (m, 3H); 9.19 (d, *J* = 7.2 Hz, 1H). ^{13}C NMR ($CDCl_3$, 50.3 MHz) δ 21.9 (4q); 55.1 (q); 55.5 (q); 56.2 (q); 71.2 (d); 71.8 (d); 103.5 (d); 104.6 (d); 105.6 (d); 107.7 (s); 109.8 (s); 110.3 (d); 110.8 (s); 112.2 (d); 115.0 (d); 116.9 (d); 118.9 (s); 123.1 (d); 123.9 (d); 124.7 (s); 128.8 (s); 129.3 (s); 134.3 (s); 143.2 (s); 146.2 (s); 146.9 (s); 147.1 (s); 148.4 (s); 150.0 (s); 151.3 (s); 155.4 (s). MS (MALDI-TOF) 583 (M, 100), 584 (M+1, 62).

3,11-Diisopropyl-Lam-D (18b)

Following the general procedure **D** and starting from **17b** (365 mg, 0.59 mmol), a yellow solid (346 mg, quant. yield) was obtained. mp (MeCN) 130-132 ºC. IR (film) 3435, 1704, 1430, 1223 cm^{-1}. ^1H NMR ($CDCl_3$, 200 MHz) δ 1.35 (d, *J* = 6.0 Hz, 6H); 1.39 (d, *J* = 6.0 Hz, 6H); 3.42 (s, 3H, OMe); 3.44 (s, 3H, OMe); 3.85 (s, 3H, OMe); 4.49 (h, *J* = 6.0 Hz, 1H); 4.65 (h, *J* = 6.0 Hz, 1H); 6.73 (s, 1H); 6.87 (s, 1H); 6.94 (d, *J* = 7.2 Hz, 1H); 7.04 (s, 1H); 7.09 (s, 1H); 7.13-7.16 (m, 3H); 9.11 (d, *J* = 7.2 Hz, 1H). ^{13}C NMR ($CDCl_3$, 50.3 MHz) δ 21.7 (2q); 21.8 (2q); 55.1 (q); 55.4 (q); 56.1 (q); 71.0 (d); 71.3 (d); 103.2 (d); 105.4 (d); 105.5 (d); 107.5 (s); 109.7 (s); 110.2 (d); 110.9 (s); 112.1 (d); 113.9 (d); 115.2 (d); 118.8 (s); 122.8 (d); 124.5 (d); 127.1 (s); 129.2 (s); 134.3 (s); 145.7 (s); 146.3 (s); 147.3 (s); 147.6 (s); 148.2 (s); 149.9 (s); 155.3 (s). MS (MALDI-TOF) 583 (M, 100), 584 (M+1, 77).

11-Isopropyl-Lam-D (18c)

Following the general procedure **D** and starting from **17c** (80 mg, 0.14 mmol), a yellow solid (65 mg, 86%) was obtained. IR (film) ν 3312, 1710, 1432, 1264 cm^{-1}. ^{1}H NMR (acetone-d_6, 400 MHz) δ 1.31 (d, *J* = 6.0 Hz, 6H); 3.35 (s, 3H, OMe); 3.39 (s, 3H, OMe); 3.77 (s, 3H, OMe); 4.75 (h, *J* = 6.0 Hz, 1H); 6.73 (s, 1H); 6.87 (s, 1H); 7.01 (dd, *J* = 8.0, 1.7 Hz, 1H); 7.10 (d, *J* = 8.0 Hz, 1H); 7.145 (s, 1H); 7.153 (d, *J* = 1.7 Hz, 1H); 7.29 (d, *J* = 7.4 Hz, 1H); 7.44 (s, 1H); 9.05 (d, *J* = 7.4 Hz, 1H); 9.33 (s, 1H, OH); 9.83 (s, 1H, OH). ^{13}C NMR (acetone-d_6, 100 MHz) δ 21.4 (2q); 54.1 (q); 54.8 (q); 55.7 (q); 69.9 (d); 103.4 (d); 104.9 (s); 105.4 (d); 106.2 (d); 108.0 (s); 110.1 (d); 110.8 (d); 112.4 (s); 114.7 (d); 116.1 (s); 118.0 (d); 121.8 (s); 123.5 (d); 124.1 (d); 125.0 (s); 128.7 (s); 133.5 (s); 144.3 (s); 146.0 (s); 146.5 (s); 147.7 (s); 148.4 (s); 149.4 (s); 154.1 (s). MS (ESI-TOF) 542 (M+1, 100), 543 (M+2, 26). HRMS *m/z* calcd. for $C_{31}H_{28}NO_8$ 542.1809, found 542.1793.

4',11-Diisopropyl-3-(2-(2-(2-methoxyethoxy)ethoxy)acetyl)-Lam-D (19a)

Following the general procedure **E** and starting from **18a** (190 mg, 0.33 mmol), a yellow oil (204 mg, 84%) was obtained. IR (film) ν 1781, 1707, 1485, 1138 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.39 (d, *J* = 6.0 Hz, 12H); 3.35 (s, 3H, OMe); 3.39 (s, 3H, OMe); 3.40 (s, 3H, OMe); 3.52-3.54 (m, 2H); 3.62-3.65 (m, 2H); 3.68-3.71 (m, 2H); 3.78-3.81 (m, 2H); 3.83 (s, OMe); 4.40 (s, 2H); 4.56-4.67 (m, 2H); 6.820 (s, 1H); 6.93 (d, *J* = 7.2 Hz, 1H); 7.03 (s, 1H); 7.05 (s, 1H); 7.0-7.14 (m, 4H); 9.07 (d, *J* = 7.2 Hz, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.69 (q); 21.73 (q); 55.0 (q); 55.3 (q); 56.1 (q); 58.8 (q); 68.1 (t); 70.4 (t); 70.5 (t); 70.8 (t); 71.0 (d); 71.65 (d); 71.72 (t); 105.4 (d); 106.2 (d); 108.0 (s); 110.3 (d); 111.5 (s); 111.7 (d); 112.7 (d); 114.8 (d); 116.1 (s); 116.8 (d); 118.8 (s); 122.7 (d); 123.6 (d); 124.5 (s); 128.0 (s); 128.2 (s); 134.2 (s); 138.8

(s); 145.3 (s); 147.1 (s); 147.1 (s); 148.4 (s); 150.1 (s); 151.3 (s); 154.7 (s); 168.2 (s). MS (MALDI-TOF) 743 (M, 100), 744 (M+1, 90), 745 (M+2, 29).

3-[3-(2-(2-(2-(2-(2-(2-*tert*-Butoxycarbonylaminoethoxy)ethoxy)ethoxy)ethoxy)ethoxy)ethoxy)propanoyl]-4',11-diisopropyl-Lam-D (19b)

Following the general procedure **E** and starting from **18a** (154 mg, 0.26 mmol), a brown oil (205 mg, 76%) was obtained. IR (film) ν 3355, 1709, 1256, 1111 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.42 (d, *J* = 6.0 Hz, 6H); 1.43-1.45 (m, 15H, O*i*Pr, *t*BuOC); 2.88 (t, *J* = 6.5 Hz, 2H); 3.31 (br, 4H, $2CH_2$); 3.40 (s, 3H, OMe); 3.43 (s, 3H, OMe); 3.51-3.54 (m, 4H, $2CH_2$); 3.61-3.66 (m, 16H); 3.75-3.80 (m, 2H, CH_2); 3.88 (s, 3H, OMe); 4.63 (h, *J* = 6.0 Hz, 1H); 4.70 (h, *J* = 6.0 Hz, 1H); 5.16 (br, 1H); 6.86 (s, 1H); 7.06 (d, *J* = 7.6 Hz, 1H); 7.10 (s, 1H); 7.12 (s, 1H); 7.15-7.19 (m, 4H); 9.23 (d, *J* = 7.6 Hz, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.66 (q); 21.69 (q); 28.2 (q); 34.5 (t); 40.1 (t); 54.9 (q); 55.3 (q); 56.0 (q); 66.2 (t); 69.9 (2t); 70.0 (2t); 70.2 (2t); 70.3 (7t); 71.0 (d); 71.6 (d); 105.4 (d); 106.2 (d); 110.3 (d); 111.4 (s); 111.5 (s); 111.8 (d); 111.9 (s); 112.7 (d); 114.7 (d); 115.9 (s); 116.8 (d); 118.8 (s); 122.7 (s); 122.8 (d); 123.6 (d); 124.5 (s); 128.2 (s); 139.3 (s); 147.1 (s); 147.2 (s); 148.4 (s); 150.1 (s); 151.3 (s); 154.8 (s); 156.0 (s); 169.2 (s); 174.7 (s). MS (MALDI-TOF) 919 ([M-Boc], 100), 920 ([M-Boc]+1, 55).

3,11-Diisopropyl-4'-(2-(2-(2-methoxyethoxy)ethoxy)acetyl)-Lam-D (19c)

Following the general procedure **E** and starting from **18b** (104 mg, 0.18 mmol), a yellow oil (127 mg, 96%) was obtained. IR (film) ν 1781, 1704, 1113 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.31 (d, *J* = 6.0 Hz, 6H); 1.35 (d, *J* = 6.0 Hz, 6H); 3.32 (s, 3H, OMe); 3.40 (2s, 6H, 2OMe); 3.45-3.52 (m, 2H); 3.55-3.63 (m, 2H); 3.65-3.71 (m, 2H); 3.74 (s, 3H, OMe); 3.78-3.82 (m, 2H); 4.40-4.49 (m, 3H, OCH, CH_2); 4.61 (h, *J* = 6.0 Hz, 1H); 6.62 (s, 1H); 6.85 (s, 1H); 6.93 (d, *J* = 7.2 Hz, 1H); 7.00-7.03 (m, 2H); 7.16-

7.19 (m, 2H); 7.22 (d, *J* = 8.0 Hz, 1H); 9.11 (d, *J* = 7.2 Hz, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.67 (q); 21.73 (q); 55.4 (q); 55.6 (q); 56.1 (q); 58.9 (q); 68.5 (t); 70.4 (t); 70.5 (t); 70.9 (t); 71.1 (d); 71.3 (d); 71.8 (t); 103.3 (d); 105.1 (d); 105.3 (d); 107.7 (s); 109.5 (s); 110.1 (s); 110.4 (d); 112.3 (d); 115.4 (d); 118.6 (s); 122.8 (d); 123.5 (d); 123.9 (d); 124.5 (s); 129.1 (s); 134.1 (s); 135.0 (s); 139.3 (s); 146.3 (s); 146.6 (s); 147.8 (s); 148.4 (s); 150.3 (s); 151.8 (s); 155.3 (s); 168.2 (s); 170.7 (s). MS (MALDI-TOF) 743 (M, 75), 744 (M+1, 100), 745 (M+2, 45).

4'-[3-(2-(2-(2-(2-(2-(2-*tert*-Butoxycarbonylaminoethoxy)ethoxy)ethoxy)ethoxy)ethoxy)ethoxy)propanoyl]-3,11-diisopropyl-Lam-D (19d)

Following the general procedure **E** and starting from **18b** (82 mg, 0.14 mmol), a brown oil (143 mg, quant. yield) was obtained. IR (film) ν 3362, 1764, 1707, 1268, 1113 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.41 (d, *J* = 6.0 Hz, 6H); 1.42-1.44 (m, 15H, O*i*Pr, *t*BuOC); 2.94 (t, *J* = 6.5 Hz, 2H); 3.29-3.32 (m, 4H, $2CH_2$); 3.48 (s, 6H, 2OMe); 3.61-3.71 (m, 20H, $10CH_2$); 3.81 (s, 3H, OMe); 3.94 (t, *J* = 6.6 Hz, 2H, CH_2); 4.58 (h, *J* = 6.0 Hz, 1H); 4.70 (h, *J* = 6.2 Hz, 1H); 5.09 (br, 1H); 6.72 (s, 1H); 6.98 (s, 1H); 7.05 (d, *J* = 7.4 Hz, 1H); 7.11 (s, 1H); 7.13 (s, 1H); 7.21 (d, *J* = 1.6 Hz, 1H); 7.24 (dd, *J* = 8.0, 1.6 Hz, 1H); 7.30 (d, *J* = 8.0 Hz, 1H); 9.24 (d, *J* = 7.4 Hz, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.6 (2q); 21.7 (2q); 28.3 (q); 34.6 (t); 40.2 (t); 55.4 (q); 55.5 (q); 56.1 (q); 66.3 (t); 70.0 (t); 70.1 (t); 70.2 (t); 70.3 (t); 70.4 (5t); 71.0 (t); 71.3 (t); 103.3 (d); 105.1 (d); 105.3 (d); 107.7 (s); 109.5 (s); 110.2 (s); 110.3 (d); 112.3 (d); 115.3 (d); 118.6 (s); 122.8 (d); 123.7 (d); 123.8 (d); 124.4 (s); 129.1 (s); 134.1 (s); 134.7 (s); 139.8 (s); 146.3 (s); 146.5 (s); 147.7 (s); 148.4 (s); 150.3 (s); 152.0 (s); 155.3 (s); 155.9 (s); 169.2 (s). MS (MALDI-TOF) 919 ([M-Boc], 100), 1018 (M, 35).

11-Isopropyl-3,4'-*bis*(2-(2-methoxyethoxy)acetyl)-Lam-D (19e)

Following the general procedure **E** and starting from **18c** (32 mg, 0.06 mmol), a yellow solid (32 mg, 71%) was obtained. mp (MeCN) 173-175 ºC. IR (film) ν 1781, 1707, 1274, 1116 cm^{-1}. ^{1}H NMR ($CDCl_3$, 200 MHz) δ 1.44 (d, *J* = 6.0 Hz, 6H); 3.41 (s, 3H, OMe); 3.45 (s, 3H, OMe); 3.46 (s, 3H, OMe); 3.49 (s, 3H, OMe); 3.61-3.70 (m, 4H, $2CH_2$); 3.76-3.90 (m, 7H, OMe, $2CH_2$); 4.45 (s, 2H); 4.53 (s, 2H); 4.71 (h, *J* = 6.0 Hz, 1H); 6.81 (s, 1H); 7.04 (d, *J* = 7.4 Hz, 1H); 7.11 (s, 1H); 7.12 (s, 1H); 7.14 (s, 1H); 7.23-7.28 (m, 2H); 7.33 (d, *J* = 8.0 Hz, 1H); 9.19 (d, *J* = 7.4 Hz, 1H). ^{13}C NMR ($CDCl_3$, 50.3 MHz) δ 21.9 (2q); 55.6 (q); 55.8 (q); 56.2 (q); 59.0 (2q); 68.3 (t); 68.4 (t); 70.8 (t); 70.9 (t); 71.2 (d); 71.9 (2t); 105.3 (d); 106.1 (d); 108.3 (s); 110.3 (d); 110.8 (s); 111.9 (d); 112.9 (d); 115.3 (d); 115.9 (s); 118.7 (s); 122.8 (d); 123.7 (d); 123.8 (d); 124.5 (s); 128.1 (s); 134.1 (s); 134.8 (s); 138.9 (s); 139.5 (s); 145.3 (s); 147.4 (s); 148.6 (s); 150.4 (s); 152.0 (s); 154.8 (s); 168.2 (s). MS (ESI-TOF) 774 (M+1, 100), 775 (M+2, 31), 776 (M+3, 7). HRMS *m/z* calcd. for $C_{41}H_{44}NO_{14}$ 774.2756, found 774.2744.

11-Isopropyl-3,4'-*bis*(2-(2-(2-methoxyethoxy)ethoxy)acetyl)-Lam-D (19f)

Following the general procedure **E** and starting from **18c** (32 mg, 0.06 mmol), a yellow solid (50 mg, 97%) was obtained. mp (MeCN) 125-127 ºC. IR (film) ν 1708, 1464, 1275, 1114 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.44 (d, *J* = 6.0 Hz, 6H); 3.39 (s, 3H, OMe); 3.42 (s, 3H, OMe); 3.46 (s, 3H, OMe); 3.49 (s, 3H, OMe); 3.54-3.63 (m, 4H, $2CH_2$); 3.65-3.92 (m, 15H, OMe, $6CH_2$); 4.46 (s, 2H); 4.53 (s, 2H); 4.71 (h, *J* = 6.0 Hz, 1H); 6.82 (s, 1H); 7.06 (d, *J* = 7.4 Hz, 1H); 7.12 (s, 2H); 7.16 (s, 1H); 7.23-7.35 (m, 3H); 9.21 (d, *J* = 7.4 Hz, 1H). ^{13}C NMR ($CDCl_3$, 50.3 MHz) δ 21.9 (2q); 55.6 (q); 55.8 (q); 56.2 (q); 59.0 (2q); 68.3 (t); 68.4 (t); 70.5 (2t); 70.6 (t); 70.7 (t); 71.0 (2t); 71.2 (d); 71.9 (2t); 105.3 (d); 106.1 (d); 108.3 (s); 110.4 (d); 110.8 (s); 112.0 (d); 113.0 (d); 115.3 (d); 115.9 (s); 118.7 (s); 122.8 (d); 123.7 (2d); 124.5 (s); 124.6 (s); 128.1 (s); 134.2 (s); 134.8 (s); 139.0 (s); 139.5 (s); 145.4 (s); 147.4 (s); 148.6 (s); 150.5 (s); 152.0

(s); 154.9 (s); 168.2 (s). MS (ESI-TOF) 862 (M+1, 83), 863 (M+2, 20). HRMS *m/z* calcd. for $C_{45}H_{52}NO_{16}$ 862.3281, found 862.3266.

3,4',11-*tris*(2-(2-methoxyethoxy)acetyl)-Lam-D (8)

Following the general procedure **E** and starting from **Lam-D** (15) (38 mg, 0.08 mmol), elution with hexane/THF (60:40 to 40:60) gave a white solid (26 mg, 40%). mp (MeCN) 148-149 ºC. IR (film) ν 1779, 1707, 1117 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.405 (s, 3H, OMe); 3.410 (s, 3H, OMe); 3.44 (s, 3H, OMe); 3.45 (s, 3H, OMe); 3.46 (s, 3H, OMe); 3.61-3.64 (m, 4H, $2CH_2$); 3.66-3.68 (m, 2H, CH_2); 3.80-3.88 (m, 9H, OMe, $3CH_2$); 4.45 (s, 2H); 4.48 (s, 2H); 4.52 (s, 2H); 6.80 (s, 1H); 7.05 (d, *J* = 7.6 Hz, 1H); 7.16 (s, 1H); 7.21 (s, 1H); 7.24 (d, *J* = 1.6 Hz, 1H); 7.25-7.28 (m, 1H); 7.33 (d, *J* = 8.0 Hz, 1H); 7.42 (s, 1H); 9.22 (d, *J* = 7.6 Hz, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 55.7 (q); 55.8 (q); 56.3 (q); 58.99 (q); 59.02 (q); 59.03 (q) 68.32 (t); 68.34 (t); 68.4 (t); 70.9 (2t); 71.0 (t); 71.9 (2t); 72.0 (t); 106.1 (d); 106.4 (d); 109.1 (s); 112.1 (d); 112.3 (s); 112.8 (d); 115.1 (d); 115.8 (s); 120.7 (d); 123.2 (s); 123.6 (s); 123.8 (2d); 124.0 (d); 128.1 (s); 133.4 (s); 134.5 (s); 139.2 (s); 139.7 (s); 140.4 (s); 145.4 (s); 147.6 (s); 150.8 (s); 152.3 (s); 154.9 (s); 168.3 (s); 168.4 (s). MS (ESI-TOF) 848 (M+1, 100), 849 (M+2, 35). HRMS *m/z* calcd. for $C_{43}H_{46}NO_{17}$ 848.2760, found 848.2751.

3,4',11-*tris*(2-(2-(2-methoxyethoxy)ethoxy)acetyl)-Lam-D (9)

Following the general procedure **E** and starting from **Lam-D** (15) (37 mg, 0.08 mmol), elution with hexane/THF (60:40 to 40:60) gave a pink solid (34 mg, 46%). mp (MeCN) 137-139 ºC. IR (film) ν 1779, 1707, 1117 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.39 (s, 6H, 2OMe); 3.41 (s, 3H, OMe); 3.447 (s, 3H, OMe); 3.451 (s, 3H, OMe); 3.54-3.61 (m, 6H, $3CH_2$); 3.66-3.79 (m, 12H, $6CH_2$); 3.82-3.89 (m, 9H, OMe, $3CH_2$); 4.45 (s, 2H); 4.48 (s, 2H); 4.52 (s, 2H); 6.80 (s, 1H); 7.04 (d, *J* = 7.6 Hz, 1H); 7.15 (s, 1H); 7.21 (s, 1H); 7.24-7.27 (m, 2H); 7.32 (d, *J* = 8.0 Hz, 1H); 7.42 (s, 1H); 9.21 (d, *J* = 7.6 Hz, 1H).

^{13}C NMR ($CDCl_3$, 100 MHz) δ 55.7 (q); 55.8 (q); 56.3 (q); 59.0 (3q); 68.29 (t); 68.33 (t); 68.4 (t); 70.53 (2t); 70.60 (t); 70.62 (2t); 70.67 (t); 71.00 (t); 71.01 (t); 71.04 (t); 71.88 (2t); 71.90 (t); 106.1 (d); 106.4 (d); 109.1 (s); 112.1 (d); 112.3 (s); 112.8 (d); 115.1 (d); 115.8 (s); 120.6 (d); 123.1 (s); 123.6 (d); 123.7 (s); 123.8 (d); 124.0 (d); 128.1 (s); 133.4 (s); 134.5 (s); 139.2 (s); 139.7 (s); 140.4 (s); 145.4 (s); 147.6 (s); 150.8 (s); 152.3 (s); 154.9 (s); 168.3 (s); 168.5 (s). MS (ESI-TOF) 980 (M+1, 100), 981 (M+2, 62). HRMS *m/z* calcd. for $C_{49}H_{58}NO_{20}$ 980.3547, found 980.3529.

3-(2-(2-(2-Methoxyethoxy)ethoxy)acetyl)-Lam-D (2)

Following the general procedure **F** and starting from **19a** (187 mg, 0.26 mmol), elution with hexane/THF (60:40 to 40:60) gave a yellow solid (73 mg, 44%). mp (MeCN) 163-164 ºC. IR (film) ν 3284, 1786, 1684, 1424 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.38 (s, 3H, OMe); 3.42 (s, 3H, OMe); 3.50 (s, 3H, OMe); 3.54-3.58 (m, 2H); 3.64-3.69 (m, 2H); 3.70-3.75 (m, 2H); 3.80-3.84 (m, 2H); 3.91 (s, OMe); 4.43 (s, 2H); 6.04 (br, 1H, OH); 6.28 (br, 1H, OH); 6.82 (s, 1H); 6.88 (d, *J* = 7.2 Hz, 1H); 7.04 (s, 1H); 7.09 (s, 1H); 7.10-7.11 (m, 1H); 7.13 (s, 2H); 7.18 (d, *J* = 7.6 Hz, 1H); 9.02 (d, *J* = 7.2 Hz, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 55.3 (q); 55.5 (q); 56.3 (q); 59.0 (q); 68.2 (t); 70.4 (t); 70.6 (t); 70.9 (t); 71.8 (t); 104.9 (d); 106.3 (d); 108.0 (s); 110.9 (d); 111.5 (s); 111.7 (d); 112.7 (d); 113.8 (d); 115.3 (d); 116.1 (s); 118.6 (s); 122.8 (d); 124.5 (d); 125.1 (s); 127.0 (s); 128.1 (s); 134.5 (s); 138.8 (s); 145.3 (s); 145.8 (s); 146.9 (s); 147.1 (s); 147.2 (s); 147.5 (s); 154.8 (s); 168.4 (s). MS (ESI-TOF) 660 (M+1, 100), 682 (M+Na, 80). HRMS *m/z* calcd. for $C_{35}H_{34}NO_{12}$ 660.2075, found 660.2062.

3-[3-(2-(2-(2-(2-(2-(2-Aminoethoxy)ethoxy)ethoxy)ethoxy)ethoxy)ethoxy)propanoyl]-Lam-D (3)

Following the general procedure **F** and starting from **19b** (140 mg, 0.14 mmol), elution with CH_2Cl_2/MeOH (95:5 to 90:10) gave a yellow oil (68 mg, 59%). IR (film)

ν 3127, 1702, 1420, 1278 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.60 and 2.86 (2t, *J* = 6.4 Hz, 2H); 3.20 (br, 2H); 3.43 (s, 3H, OMe); 3.50 (s, 3H, OMe); 3.63-3.70 (m, 20H); 3.83-3.89 (m, 4H); 3.90 (s, 3H, OMe) 6.82 (s, 1H); 6.93 (d, *J* = 7.5 Hz, 1H); 7.01 (s, 1H); 7.10 (dd, *J* = 8.0, 1.6 Hz, 1H); 7.12 (2s, 2H); 7.22 (s, 1H); 7.24 (d, *J* = 8.0 Hz, 1H); 7.92 (br, 4H, NH_2, 2 OH); 9.10 (d, *J* = 7.5 Hz, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 34.6 (t); 40.2 (t); 55.2 (q); 55.5 (q); 56.2 (q); 66.2 (t); 66.6 (t); 69.7 (t); 69.8 (t); 69.9 (t); 70.0 (2t); 70.1 (t); 70.2 (2t); 70.3 (t); 70.4 (t); 104.9 (d); 106.3 (d); 107.8 (s); 111.0 (d); 111.5 (s); 111.7 (d); 112.7 (d); 113.9 (d); 115.6 (d); 115.9 (s); 118.5 (s); 122.7 (d); 124.3 (d); 125.1 (s); 126.7 (s); 128.1 (s); 134.5 (s); 139.2 (s); 145.2 (s); 146.0 (s); 147.2 (s); 147.3 (s); 147.4 (s); 147.7 (s); 154.8 (s); 169.5 (s). MS (ESI-TOF) 835 (M+1, 100); 836 (M+2, 40), 837 (M+3, 8). HRMS *m/z* calcd. for $C_{43}H_{51}N_2O_{15}$ 835.3283, found 835.3277.

4'-(2-(2-(2-Methoxyethoxy)ethoxy)acetyl)-Lam-D (4)

Following the general procedure **F** and starting from **19c** (111 mg, 0.15 mmol), elution with hexane/THF (60:40 to 40:60) gave a yellow solid (55 mg, 55%). mp (MeCN) 201-203 ºC. IR (film) ν 3455, 1679, 1426 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.41 (s, 3H, OMe); 3.54 (s, 3H, OMe); 3.55 (s, 3H, OMe); 3.58-3.62 (m, 2H); 3.68-3.75 (m, 2H); 3.76-3.81 (m, 2H); 3.83 (s, OMe); 3.86-3.91 (m, 2H); 4.53 (s, 2H); 5.88 (br, 1H, OH); 5.98 (br, 1H, OH); 6.66 (s, 1H); 6.93-7.10 (m, 2H); 7.07 (s, 1H); 7.18 (s, 1H); 7.23-7.28 (m, 2H); 7.32 (d, *J* = 8.0 Hz, 1H); 9.17 (d, *J* = 7.2 Hz, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 55.8 (q); 55.9 (q); 56.3 (q); 59.1 (q); 68.5 (t); 70.6 (t); 70.7 (t); 71.1 (t); 71.9 (t); 103.6 (d); 104.4 (d); 104.7 (d); 109.6 (s); 110.0 (s); 110.8 (d); 115.5 (d); 118.6 (s); 123.3 (s); 123.7 (d); 124.0 (d); 125.5 (d); 125.6 (d); 128.2 (s); 129.3 (s); 134.3 (s); 135.4 (s); 135.8 (s); 139.5 (s); 143.5 (s); 146.4 (s); 146.9 (s); 147.0 (s); 147.2 (s); 152.1 (s); 168.5 (s). MS (ESI-TOF) 660 (M+1, 100), 682 (M+Na, 33). HRMS *m/z* calcd. for $C_{35}H_{34}NO_{12}$ 660.2075, found 660.2065.

4'-[3-(2-(2-(2-(2-(2-(2-

Aminoethoxy)ethoxy)ethoxy)ethoxy)ethoxy)ethoxy)propanoyl]-Lam-D (5)

Following the general procedure **F** and starting from **19d** (130 mg, 0.13 mmol), elution with CH_2Cl_2/MeOH (95:15 to 85:15) gave a brown oil (61 mg, 57%). IR (film) ν 3133, 1700, 1425, 1277 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz) δ 2.60 and 2.95 (2t, *J* = 6.4 Hz, 2H); 3.16-3.24 (m, 2H); 3.43 and 3.47 (s, 3H, OMe); 3.52 (s, 3H, OMe); 3.62-3.77 (m, 20H); 3.83 (s, 3H, OMe); 3.89-3.96 (m, 4H); 6.65 (s, 1H); 6.95 (d, *J* = 7.4 Hz, 1H); 6.99 (s, 1H); 7.06 (s, 1H); 7.19-7.24 (m, 2H); 7.25 (d, *J* = 1.6 Hz, 1H); 7.26-7.31 (m, 1H); 9.10 (d, *J* = 7.4 Hz, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 34.7 (t); 40.2 (t); 55.6 (q); 55.8 (q); 56.3 (q); 66.4 (t); 66.7 (t); 69.7 (t); 69.8 (t); 69.9 (2t); 70.0 (2t); 70.1 (t); 70.2 (t); 70.3 (t); 70.4 (t); 103.7 (d); 104.6 (d); 104.8 (d); 107.7 (s); 109.4 and 109.9 (s); 111.0 (d); 112.3 (d); 115.5 (d); 118.4 (s); 123.0 (d); 123.9 (2d); 125.2 (s); 129.2 (s); 134.3 (s); 135.0 (s); 139.9 (s); 143.8 (s); 146.6 (s); 146.8 (s); 147.2 (s); 147.5 (s); 152.1 (s); 155.4 (s); 169.5 (s); 172.1 (s). MS (ESI-TOF) 835 (M+1, 100); 836 (M+2, 44), 837 (M+3, 23). HRMS *m/z* calcd. for $C_{43}H_{51}N_2O_{15}$ 835.3283, found 835.3277.

3,4'-*bis*(2-(2-Methoxyethoxy)acetyl)-Lam-D (6)

Following the general procedure **F** and starting from **19e** (41 mg, 0.05 mmol), elution with hexane/THF (60:40 to 40:60) gave a yellow solid (19 mg, 48%). mp (MeCN) 162-164 ºC. IR (film) ν 3170, 1780, 1681, 1425 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz) δ 3.40 (s, 3H, OMe); 3.44 (s, 3H, OMe); 3.45 (s, 3H, OMe); 3.56 (s, 3H, OMe); 3.61-3.64 (m, 2H); 3.66-3.68 (m, 2H); 3.80-3.82 (m, 2H); 3.83 (s, 3H, OMe); 3.85-3.88 (m, 2H); 4.45 (s, 2H); 4.52 (s, 2H); 6.00 (br, 1H, OH); 6.82 (s, 1H); 6.95-6.99 (m, 1H); 7.02 (d, *J* = 7.2 Hz, 1H); 7.06 (s, 1H); 7.14 (s, 1H); 7.18 (s, 1H); 7.24 (d, *J* = 2.0 Hz, 1H); 7.32 (d, *J* = 8.0 Hz, 1H); 9.17 (d, *J* = 7.2 Hz, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 55.76 (q); 55.80 (q); 56.3 (q); 58.99 (q); 59.04 (q); 68.3 (t); 68.4 (t); 70.9 (t); 71.0 (t); 71.9 (t); 72.0 (t);

104.7 (d); 106.2 (d); 108.4 (s); 110.7 (s); 110.9 (d); 112.0 (d); 113.0 (d); 115.3 (d); 116.0 (s); 118.6 (s); 123.1 (d); 123.8 (d); 125.2 (s); 125.5 (d); 128.1 (s); 134.4 (s); 134.9 (s); 139.1 (s); 139.6 (s); 145.5 (s); 147.0 (s); 147.3 (s); 147.5 (s); 152.2 (s); 154.9 (s); 168.3 (s); 168.4 (s). MS (ESI-TOF) 732 (M+1, 100), 733 (M+2, 32); 734 (M+3, 9). HRMS *m/z* calcd. for $C_{38}H_{38}NO_{14}$ 732.2287, found 732.2307.

3,4'-*bis*(2-(2-(2-Methoxyethoxy)ethoxy)acetyl)-Lam-D (7)

Following the general procedure **F** and starting from **19f** (48 mg, 0.06 mmol), elution with hexane/THF (60:40 to 40:60) gave a white solid (8 mg, 19%). mp (MeCN) 151-152 ºC. IR (film) ν 3204, 1780, 1681, 1425 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz) δ 3.39 (s, 3H, OMe); 3.41 (s, 3H, OMe); 3.44 (s, 3H, OMe); 3.55-3.58 (m, 5H, OMe, CH_2); 3.59-3.62 (m, 4H, $2CH_2$); 3.66-3.75 (m, 6H, $3CH_2$); 3.77-3.79 (m, 2H, CH_2); 3.83 (br, 3H, OMe); 3.87-3.90 (m, 2H, CH_2); 4.45 (s, 2H); 4.53 (s, 2H); 5.97 (br, 1H); 6.82 (s, 1H); 6.97-7.08 (m, 2H); 7.15 (s, 1H); 7.20 (s, 1H); 7.23-7.25 (m, 2H); 7.32 (d, *J* = 8.0 Hz, 1H); 9.20 (d, *J* = 7.2 Hz, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 55.76 (q); 55.82 (q); 56.3 (q); 59.1 (2q); 68.3 (t); 68.5 (t); 70.55 (t); 70.62 (t); 70.65 (t); 70.70 (t); 71.0 (t); 71.1 (t); 71.90 (t); 71.93 (t); 103.6 (s); 104.4 (s); 104.7 (d); 106.2 (d); 110.8 (s); 110.9 (d); 112.1 (d); 112.4 (s); 113.0 (d); 115.3 (d); 115.5 (s); 116.0 (s); 118.6 (s); 123.1 (s); 123.8 (d); 123.9 (d); 125.3 (s); 125.5 (d); 135.0 (s); 139.6 (s); 145.5 (s); 147.0 (s); 147.5 (s); 152.2 (s); 155.0 (s); 168.4 (s); 168.5 (s). MS (ESI-TOF) 820 (M+1, 100), 821 (M+2, 39); 822 (M+2, 14). HRMS *m/z* calcd. for $C_{42}H_{46}NO_{16}$ 820.2811, found 820.2797.

Cell Lines and Culture. Established human-derived cell lines used in this study were purchased from ATCC (American Type Culture Collection): A-549, human lung carcinoma, BJ, Skin Fibroblast, HT-29, human colorectal adenocarcinoma, and MDA-MB 231, human breast adenocarcinoma. All cell lines were maintained in DMEM

supplemented with 10% FBS and 100 units/mL penicillin and streptomycin at 37 °C and 5% CO_2.

GI_{50} Analysis. Triplicate cultures were incubated for 72 h in the presence or absence of test compounds **1-9** (at ten concentrations, typically ranging from 0.0026 to 10 μg/mL).

A colorimetric assay using SRB was adapted for a quant. measurement of cell growth and viability, following a previously described method (*16*). Cells were plated in 96-well microtiter plates at a density of 5×10^3/well and incubated for 24 h. One plate from each different cell line was fixed, stained and used for Tz reference (see next paragraph). The cells were then treated with vehicle alone (control) or the test compounds at the concentrations indicated. The treated cells were incubated for additional 72 h, and then assayed for cytotoxicity via colorimetric analysis.

The cells were washed twice with PBS, fixed for 15 min in 1% glutaraldehyde solution, rinsed twice in PBS, and stained in 0.4% SRB solution for 30 min at room temperature. The cells were then rinsed several times in 1% acetic acid solution and air-dried. SRB was then extracted in 10 mM trizma base solution, and the absorbance at 490 nm was then measured. Cell survival is expressed as percentage of control cell growth.

Dose−response curves were obtained by using the NCI algorithm (*17*): Tz = number of control cells at time t_0, C = number of control cells at time t, and T = number of treated cells at time t.

If $Tz < T < C$ (growth inhibition), then the result is $100 \times ([T - Tz]/[C - Tz])$.

If $T < Tz$ (net cell death), then the result is $100 \times ([T - Tz]/Tz)$.

After dose-curve generation, the results were expressed as GI_{50}.

General Treatments for Imaging. A-549, MDA-MB-231 and HT-29 cells were seeded onto MatTek (Ashland, USA) glass bottom microwell dishes at 30 10^3 cells/cm^2. After 24 h, the culture medium was discarded, and then replaced by fresh DMEM medium containing compound **9** at 25 nM. A negative control (no compound) was also used. The cells were then incubated for 48 h at 37 ºC.

The following dyes were used for co-localization experiments: WGA-FITC (Sigma, USA), and Mitotracker Red (Molecular Probes). Standard staining protocols were employed for live-cell imaging. Images were then subsequently acquired within the following 30 min.

Confocal Laser Scanning Microscopy. Confocal laser scanning microscopy was performed with a Leica TCS SPII spectral confocal laser scanning microscope (Leica Microsystems Heidelberg GmbH, Mannheim, Germany), using a 63× objective. FITC fluorescence was excited with an Ar laser at 488 nm. Lam-D and its derivatives were excited at 351 nm. Mitotracker was excited at 543 nm. The same microscope settings were maintained for each conjugate and concentration. To avoid crosstalk, two-fluorescence scanning was performed in a sequential mode.

Apoptosis Assay. The methodology used is based on fluorochrome inhibitors of caspases (FLICA; CaspaTag *in situ* caspase detection kits, Chemicon International, U.S. and Canada). The inhibitors are cell permeable and non-cytotoxic. Once inside the cell, the inhibitor covalently binds to the active caspase. This kit uses a carboxyfluorescein-labeled fluoromethyl ketone peptide inhibitor of caspase-3 (FAM-DEVD-FMK), which fluoresces green. When added to a group of cells, the FAM-DEVD-FMK probe enters each cell and covalently binds to a reactive cysteine residue in the large subunit of the active caspase heterodimer, thereby inhibiting further enzymatic activity (*18*).

Cell cultures previously incubated with Lam-D for 48 h at a concentration of 25 nM were tested for apoptosis determination. After the latter drug treatments, 30 × FLICA reagent was added at 1:15 dilution in culture medium, and then incubated for 1 h at 37 °C. The cells were then washed with PBS, and subsequently analyzed by confocal scanning microscopy. The green fluorescence signal was a direct measure of the amount of active caspase-3/7 present in the cell at the time the reagent was added.

Uptake Measurements by FACS Flow Cytometry. 1 10^6 A-549, BJ, MDA-MB-231 and HT-29 cells were seeded onto 25 cm^2 cell culture flasks (Nalgene Nunc International, Naperville, USA) with 10 mL of DMEM. After 12 h, the culture medium was discarded and replaced by fresh DMEM medium containing compounds **1-9** at a concentration of 1µM. A negative control (no compound) was also used. Cells were then incubated for 12 h at 37 ºC. After incubation, cells were washed 3 × PBS, detached with trypsin-EDTA 0.25% and centrifuged at 1000 × rpm. Finally, the medium was decanted, and the cellular pellet was resuspended in PBS and kept at 0 ºC until measurements were performed. Fluorescence analysis was performed with a MoFlo cytometer (DakoCytomation, Colorado, USA), using the 351 nm excitation line of Ar laser (25 mW) and emission detection at 450 nm (tolerance range ± 65 nm). CIQ is expressed as a percentage value in reference to Lam-D. It was calculated as a division of the fluorescence value obtained by the Lam-D fluorescence control under the same experimental conditions.

Cell Cycle Analysis. Cell cycle analysis were performed by DRAQ5 (Biostatus) staining to determine DNA content. DRAQ5 was added to the previous PBS cell culture suspensions (1 µL / 4 10^5 cells), incubated for 15 min at 37 ºC, and the cells were directly analyzed without any further treatment. Fluorescence analysis was performed with a MoFlo cytometer (DakoCytomation, Colorado, USA), using the 488 nm

excitation line of an Ar laser (150 mW) and emission detection at 670 nm (tolerance range ± 30 nm). Cell cycle profile was analyzed using Cell Quest software.

RESULTS

Lam-D contains three phenolic sites susceptible to modification. Despite previous SAR studies that highlighted the relevance of the phenolic residues at the C-3 and C-11 positions of Lam-D (*3*, *19*), and their essential role for cytotoxicity and topoisomerase I inhibition (*3*), in this study, the free OH groups were chosen for attachment the PEG moiety. As esters readily hydrolyze under physiological conditions, our group envisioned that these derivatives would enable gradual release of the drug. Use of Lam-D derivatives having differently protected phenols allowed selective deprotection and further conjugation with PEG-carboxylic acids to enable synthesis of mono-PEG (**2-5**), di-PEG (**6, 7**) and tri-PEG (**8, 9**) conjugates (**Fig. 1**).

The derivatives prepared contain monodisperse PEG backbones with OMe (**2**, **4**, **6**, **7**, **8**, and **9**) and NH_2 (**3**, **5**) terminal moieties and vary in their chain length. The PEGs used for derivatization contain one, two, or six sequentially linked units of ethylene glycol. Another source of diversity introduced in the PEG carriers was a spacer, acetyl (**2**, **4**, **6**, **7**, **8**, and **9**) or propanoyl (**3**, **5**) moieties were used.

The methodology developed in our group for the total synthesis of Lam-D (*15*) was used for the preparation of conjugates **2-9**. The key steps of the synthesis comprise two successive processes of regioselective bromination and Pd(0)-catalyzed cross-coupling reactions starting from the scaffold **10**.

As shown in **Scheme 1**, several dialkoxy and trialkoxy-phenylboronates **11a-b** and **12a-b** were required. These compounds were obtained with good yields by borylation of the corresponding aryl bromides with pinacolborane using $Pd(OAc)_2$ and DPE-Phos as catalysts (for details, see Supporting Information). The protected alkoxy-aryl bromides

required were obtained by protection of commercially available phenols followed by mild bromination via NBS in THF at -78 ºC (*20*).

Regioselective bromination of **10** (*21*) at position 1 using one equivalent of NBS in THF followed by Pd(0)-catalyzed cross-coupling reaction with the boronic ester **11a** or **11b** afforded the monoaryl scaffolds **13a** and **13b** (*15*), respectively, which were converted into the bromides **14a, 14b** (*15*) by regioselective bromination at position 2. A second cross-coupling reaction, using the trialkoxyphenylboronates **12a** or **12b**, gave the diaryl derivatives **15a-c** (with the desired orthogonal protected phenol groups as *i*Pr/Bn ethers) in 45-64% yields from the scaffold **10**.

Tight control of the oxidation of **15** using 1 equivalent of DDQ and MW irradiation at 120 ºC for 10 min gave **16a-c** (65-93% yield), avoiding the complex mixtures obtained by simultaneous *O*-Bn cleavage using other conditions (*22*).

The *O*Bn protecting group was successfully removed to give **17a-c** in 70-95% yields by hydrogenolysis over Pd-C in methanolic solution. Lactonization in excellent yields (86% to quant.) was achieved by direct treatment of the methyl esters **17a-c** with sodium hydride in THF. Lactonization was needed before PEGylation to protect the phenol required in the lactone formation and to prevent undesired PEG removal caused by the instability of the ester bond under the basic reaction conditions.[2] Compounds **18a** and **18b,** having only one free phenol group (each of which at a distinct position), and **18c,** with two free phenol groups, were then further conjugated. The PEG-groups were successfully introduced by esterification of the free phenols of **18a-c** to give compounds **19a-f** using EDC·HCl with a catalytic amount of DMAP in CH_2Cl_2 (*23*). The same reaction conditions were used for the conjugation of Lam-D, obtained by deprotection of two isopropyl groups of **18c** with $AlCl_3$, to give the triester derivatives **8** and **9**. The isopropyl-protecting groups of **19a-f** were eliminated using $AlCl_3$ in CH_2Cl_2 with

moderate to good yields, with concomitant removal of the *N*-Boc-protecting groups of **19b** and **19d**.

High polarity solvents such as DMSO and PEG-400 were required to dissolve Lam-D (**1**), which is insoluble in most common solvents. The solubility of monoPEG compounds **2-5**, with a single conjugation of one phenolic residue, is much better to that of Lam-D.[3] Conjugate **3**, bearing six units of ethylene glycol and a NH_2 termination, showed 80-fold more solubility in water than Lam-D. The solubility of conjugates **3**, **7** and **9** -mono-, di- and tri-ester, respectively- in PEG-400 solvent was in accordance with the increase of PEG conjugations. Final compounds **2-9** were prepared in 18 to 57% yields from the corresponding phenolic Lam-D precursors.

Lastly, the conjugates were further evaluated biologically and *in vitro* drug delivery assays were carried out.

Cytotoxicity and Cellular Uptake.

The cytotoxicity of Lam-D (**1**) and its analogs (**2-9**) was evaluated against BJ human skin fibroblasts, and a panel of three human tumor cell lines: HT-29, A-549 and MDA-MB-231. A conventional colorimetric assay was used to estimate values of GI_{50} (i.e. the drug concentration that causes 50% of cell growth inhibition after 72 h of continuous exposure to the test molecule (*17*)). The results are shown in **Table 1**.

Conjugates **2-9** are all active against the cell lines: HT-29, the cytotoxicity of **2-9** is similar to that of Lam-D; in A-549 and MDA-MB-231 cell lines, the activity of **2-4** and **6-9** is 1.4-4.3-fold better than Lam-D. Furthermore, BJ skin fibroblasts were used in the present study to evaluate the effects of the drug and its conjugates in normal cells. In this non tumoral cellular culture, conjugates **3**, **6** and **7** maintained the same order of magnitude of cytotoxicities than Lam-D. Besides, conjugates **2**, **4**, **5**, **8**, and **9** showed a decrease of 4 to 500-fold in their cytotoxic activity for BJ skin fibroblasts.

The results of initial cellular internalization experiments in HeLa cells at different compound concentrations and exposure times were encouraging for Lam-D and **7** (see **Fig. SI1** and **Fig. SI2** in the Supporting Information). Compound **9** was selected as one of the best compounds of the series for co-localization experiments. Cell cultures were incubated with compound **9** for 48 h. WGA-FITC and Mitotracker were then employed for the selective staining of cell membrane-Golgi apparatus (green fluorescence) and mitochondria (red fluorescence), respectively. Likewise, UV fluorescence corresponding to **9** was observed in the cytoplasm;[4] however, no fluorescence was observed in the nuclear region of any of the cells for compound **9** (**Fig. 2**).

The internalization of compounds **1-9** was measured by FACS flow cytometry (see **Table 2**). Conjugates **3**, **6** and **9** underwent the greatest cellular uptake in HT-29 and MDA-MB-231. Furthermore, conjugates **2-9** all had higher internalization in A-549 than did Lam-D. Results were normalized in function of Lam-D uptake, thus the CIQ was calculated relative to 100% internalization for Lam-D (**1**). The comparative study showed that all the PEG conjugation motifs introduced enabled greater internalization in A-549, and BJ cell lines. For compounds **4**, **8** and **9**, the ratio was greater than 150%. Although compounds **3**, **6** and **9** exhibited greater internalization than did Lam-D (**1**) in HT-29 and MDA-MB-231, the differences observed for these cell lines were not as dramatic. Likewise, as observed in the results for **3**, monoconjugation enables greater uptake. The introduction of the PEG-NH_2 backbone at phenolic position 3 of Lam-D (compound **3**) led to better GI_{50} cytotoxicity and internalization than at position 4' (compound **5**).

Effects of 1 on Cell Cycle Progression of HT-29 Cells.

Lam-D was tested for effects on cell cycle progression in HT-29 tumor cells (**Fig. 3**). Cells were treated with the product (see Experimental Section) and then analyzed by flow cytometry to determine if the cell cycle had been arrested at a specific phase. A control set of untreated cells was also used.

Compared to the control cells (**Fig. 3,** panels **1a, c**), the cells treated with **1** (**Fig. 3**, panels **1b, c**) showed an arrest in G2/M progression. Hence, it was concluded that Lam-D inhibits cell cycle progression. Furthermore, conjugates **3** and **9** provoked cell cycle arrest in a greater extent. The results showed one single peak corresponding to the G2/M phase (see **Fig. SI3** in the Supporting Information).

Induction of Apoptosis by 1, 3 and 9 in HT-29, A-549, and MDA-MB-231 Cell Lines. Many anticancer drugs induce cell cycle arrest and apoptosis. Apoptotic cells undergo characteristic morphological changes. Among these, the cell surface often bends and breaks up into membrane-enclosed fragments called apoptotic bodies. This process depends on a cascade of proteolytic enzymes called caspases (*24*).

To analyze whether Lam-D induces apoptosis, HT-29, A-549, and MDA-MB-231 cell lines were incubated with a single compound and then qualitatively measured for apoptosis (**Fig. 4**, see Experimental Section). The assay consists of detecting active caspases (caspase-3/7) using a commercially available colorimetric test.

All cell lines showed a high level of apoptotic cell death after treatment with compounds **1**, **3** and **9** (**Fig. 4**, panels **d−l**). A negative control of the assay was performed by analyzing untreated cells under the same experimental conditions (**Figure 4**, panels **a−c**). Altogether, these results indicate that Lam-D and derivatives **3** and **9**, induce cell cycle arrest and apoptosis.

DISCUSSION

A procedure for preparing PEGylated Lam-D pro-drugs has been described for the first time. One, two or three phenol groups were selectively conjugated to the Lam-D in mild reaction conditions employing selective orthogonal protection (benzyloxy and isopropoxy groups).

The feasibility of the selective modification of the phenolic moieties of Lam-D has been demonstrated with readily available and inexpensive PEG-carboxylic acid as starting material. Conjugates functionalized with only a single phenolic residue of **2-5** (C-3 or C-4' OH groups) are much more soluble than Lam-D in various solvents. Notwithstanding, di- and tri-PEG conjugates were also synthesized. Di- and tri-PEG conjugates -which imply greater steric hindrance- were also synthesized, thereby demonstrating the robustness of our method. The pH-labile ester conjugates were prepared in 18 to 57% yields from the corresponding Lam-D phenolic precursors.

Conjugates (**2-9**), screened against normal BJ skin fibroblasts and three human cancer cell lines, exhibited better internalization than Lam-D (**1**) did. Conjugation provided cytotoxicity amelioration for the cancerous cell lines. Indeed, the GI_{50} of conjugates **2-4**, **6**, **7** and **9** (bearing mono-, di- and tri-PEG backbones) in MDA-MB-231 and A549 cancer cell lines were one order of magnitude lower than that of Lam-D (**1**). Comparison of cytotoxic activity in BJ skin fibroblast between Lam-D and compounds **2-9** shown that bioconjugates are equal or less toxic than Lam-D. Compounds **2**, **4**, and **9** with higher cytotoxycity than Lam-D in A-549 and MDA-MB-231 tumor cell lines displayed less cytotoxyc-activity in the BJ normal cell line. Although these compounds **2**, **4**, and **9** possess better internalization in normal cells. These results indicate that these compounds have better affinity to components of tumor cell lines. Confocal microscopy revealed that Lam-D (**1**) and the conjugates (**2-9**) were internalized in the cytoplasmatic

region. Furthermore, Lam-D induces cell cycle arrest at the G2 phase. Lam-D, and derivatives **3** and **9**, produce caspase-3,7-dependent apoptosis in all cell lines tested.

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Supporting Information Available: Materials and methods, experimental procedures, characterization data of the precursor compounds. This information is available free of charge via the internet at http://pubs.acs.org.

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(6) IUPAC nomenclature of Lam-D: 3,11-dihydroxy-2,12-dimethoxy-14-(4-hydroxy-3-methoxyphenyl)-6*H*[1]benzopyrano[4,3;4,5]pyrrolo[2,1-*a*]isoquinoline-6-one.

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Footnotes:

[1] Abbreviations: AU, absorbance units; A-549, lung carcinoma cell line; BJ, skin fibroblast cell line; Bn, benzyl; Boc, *tert*-butoxycarbonyl; CIQ, cellular internalization quotient; DDQ, 2,3-dichloro-5,6-dicyano-*p*-benzoquinone; DMAP, 4-dimethylaminopyridine; DMEM, Dulbecco's modified Eagle's medium; DMF, dimethylformamide; DMSO, dimethylsulfoxide; DPE-Phos, (oxydi-2,1-phenylene)bis(diphenylphosphine); EDC·HCl, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride; EPR, enhance permeability and retention; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HeLa, human cervix carcinoma cell line; HT-29, colon carcinoma cell line; GI_{50}, 50 percent growth inhibition; *i*Pr, isopropyl; Lam, Lamellarin; MDA-MB-231, breast adenocarcinoma cell line; MW, microwave; NBS, *N*-bromosuccinimide; PBS, phosphate buffered saline; PEG, poly(ethyleneglycol); PEGylated, polyethyleneglycolated; SAR, structure activity relationship; SRB, sulforhodamine B; WGA, wheat germ agglutinin; THF, tetrahydrofurane; Topo, topoisomerase.

[2] Lactonization of methyl 2-(2,4-dihydroxy-5-methoxyphenyl)-8-hydroxy-1-(4-(2-(2-(2-methoxyethoxy)ethoxy)acetoxy)-3-methoxyphenyl)-9-methoxypyrrolo[2,1-*a*]isoquinoline-3-carboxylate did not provide compound **4**; rather, it led exclusively to formation of Lam-D.

[3] The conjugates are more soluble in water, EtOH and PEG-400 than is Lam-D. See **Table SI1** in the Supporting Information.

[4] The Lam-D UV spectrum presents four characteristic absorption maxima: 237, 267, 356 and 382 nm. Excitation at 267 nm of **1** led to three emission maximum wavelengths (319, 430 and 490 nm). The authors assumed that any minor deviation in the molar extinction coefficient of **2**-**9** due to ester conjugation of **1** with aliphatic PEG backbones was not important.

Table 1. Cytotoxicity of compounds **1-9** in three human cancer cell lines.

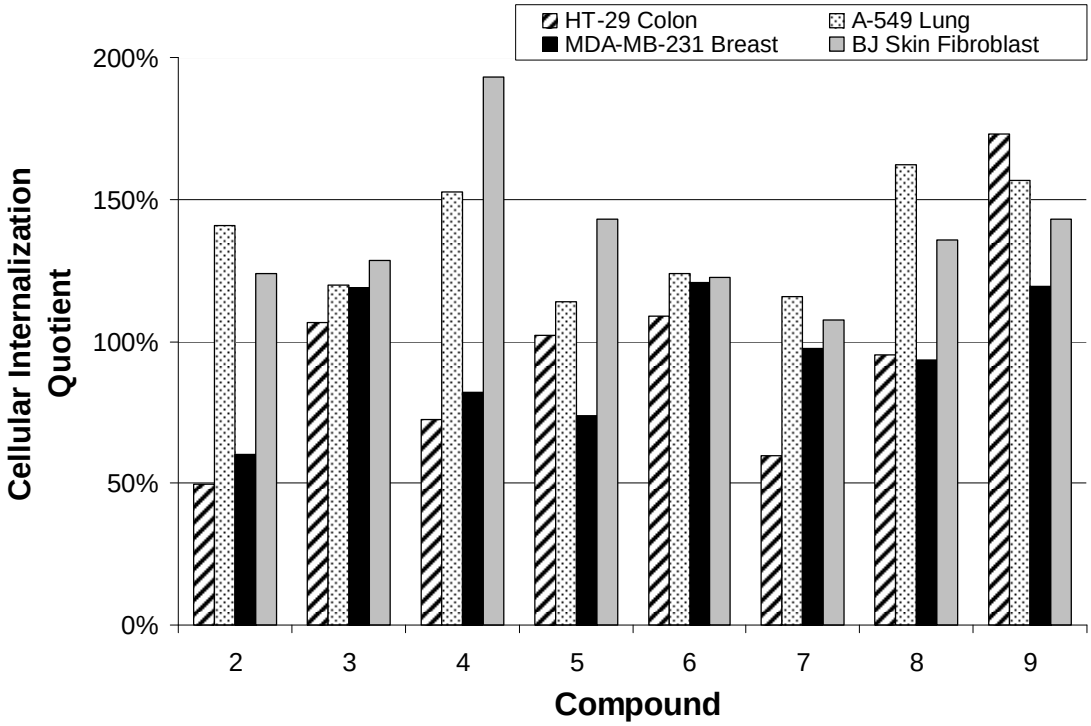
Compound	Cytotoxicity (M)			
	HT-29 Colon	A-549 Lung	MDA-MB-231 Breast	BJ Skin Fibroblast
Lam-D (**1**)	$3.00\ 10^{-6}$	$1.22\ 10^{-7}$	$1.34\ 10^{-7}$	$6.37\ 10^{-9}$
2	$1.97\ 10^{-6}$	$7.13\ 10^{-8}$	$4.55\ 10^{-8}$	$6.28\ 10^{-7}$
3	$1.68\ 10^{-6}$	$8.86\ 10^{-8}$	$4.07\ 10^{-8}$	$6.51\ 10^{-9}$
4	$1.97\ 10^{-6}$	$5.46\ 10^{-8}$	$5.31\ 10^{-8}$	$2.90\ 10^{-6}$
5	$4.79\ 10^{-6}$	$3.47\ 10^{-7}$	$2.52\ 10^{-7}$	$3.51\ 10^{-6}$
6	$1.28\ 10^{-6}$	$6.29\ 10^{-8}$	$3.14\ 10^{-8}$	$1.81\ 10^{-9}$
7	$5.49\ 10^{-6}$	$5.37\ 10^{-8}$	$7.68\ 10^{-8}$	$3.17\ 10^{-9}$
8	$4.60\ 10^{-6}$	$7.31\ 10^{-8}$	$1.04\ 10^{-7}$	$4.90\ 10^{-8}$
9	$4.08\ 10^{-6}$	$4.69\ 10^{-8}$	$8.57\ 10^{-8}$	$2.67\ 10^{-8}$

Table 2. Cellular internalization uptake measured by FACS.

Compound	Cellular Internalization Uptake (AU)				Cellular Internalization Quotient (CIQ)[a]			
	HT-29 Colon	A-549 Lung	MDA-MB-231 Breast	BJ Skin Fibroblast	HT-29 Colon	A-549 Lung	MDA-MB-231 Breast	BJ Skin Fibroblast
Lam-D (**1**)	82.7	328.5	443.8	259.4	100%[a]	100%[a]	100%[a]	100%[a]
2	41.1	463.1	266.6	321.0	50%	141%	60%	123.8%
3	88.0	393.0	527.5	333.4	106%	120%	119%	128.6%
4	59.8	500.6	364.8	501.5	72%	152%	82%	193.4%
5	84.4	373.7	328.1	371.4	102%	114%	74%	143.2%
6	90.3	407.2	535.0	317.7	109%	124%	121%	122.5%
7	49.3	379.3	432.1	279.3	60%	116%	97%	107.7%
8	78.7	532.6	414.8	352.0	95%	162%	94%	135.7%
9	143.3	514.8	529.7	371.0	173%	157%	119%	143.0%

[a] The CIQ has been calculated in reference to the cellular uptake of Lam-D.

Chart 1. Cellular internalization quotient.



Scheme 1

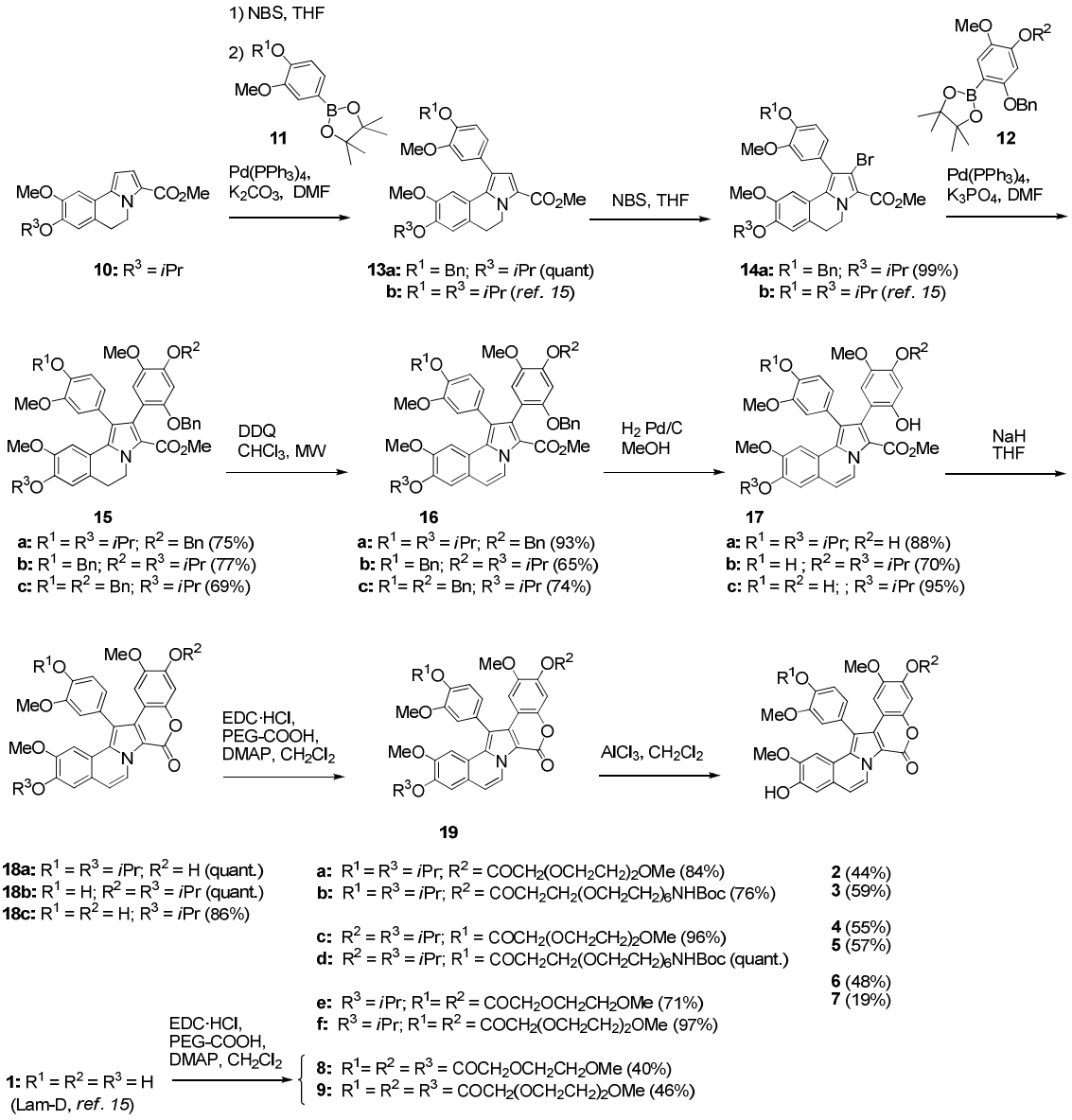

1) NBS, THF
2) R1O
MeO
B-O
O
11
Pd(PPh3)4,
K2CO3, DMF
MeO
R3O
N
CO2Me
10: R3 = iPr
13a: R1 = Bn; R3 = iPr (quant)
b: R1 = R3 = iPr (ref. 15)
NBS, THF
Br
14a: R1 = Bn; R3 = iPr (99%)
b: R1 = R3 = iPr (ref. 15)
MeO
OR2
OBn
12
Pd(PPh3)4,
K3PO4, DMF
DDQ
CHCl3, MW
15
a: R1 = R3 = iPr; R2 = Bn (75%)
b: R1 = Bn; R2 = R3 = iPr (77%)
c: R1= R2 = Bn; R3 = iPr (69%)
16
a: R1 = R3 = iPr; R2 = Bn (93%)
b: R1 = Bn; R2 = R3 = iPr (65%)
c: R1= R2 = Bn; R3 = iPr (74%)
H2 Pd/C
MeOH
OH
17
a: R1 = R3 = iPr; R2= H (88%)
b: R1 = H ; R2 = R3 = iPr (70%)
c: R1 = R2 = H; ; R3 = iPr (95%)
NaH
THF
EDC·HCl,
PEG-COOH,
DMAP, CH2Cl2
19
AlCl3, CH2Cl2
HO
18a: R1 = R3 = iPr; R2 = H (quant.)
18b: R1 = H; R2 = R3 = iPr (quant.)
18c: R1 = R2 = H; R3 = iPr (86%)
a: R1 = R3 = iPr; R2 = COCH2(OCH2CH2)2OMe (84%)
b: R1 = R3 = iPr; R2 = COCH2CH2(OCH2CH2)6NHBoc (76%)
c: R2 = R3 = iPr; R1 = COCH2(OCH2CH2)2OMe (96%)
d: R2 = R3 = iPr; R1 = COCH2CH2(OCH2CH2)6NHBoc (quant.)
e: R3 = iPr; R1= R2 = COCH2OCH2CH2OMe (71%)
f: R3 = iPr; R1= R2 = COCH2(OCH2CH2)2OMe (97%)
2 (44%)
3 (59%)
4 (55%)
5 (57%)
6 (48%)
7 (19%)
1: R1 = R2 = R3 = H
(Lam-D, ref. 15)
8: R1= R2 = R3 = COCH2OCH2CH2OMe (40%)
9: R1 = R2 = R3 = COCH2(OCH2CH2)2OMe (46%)

Figure 1. Structures of Lam-D 1 and PEG derivatives 2-9

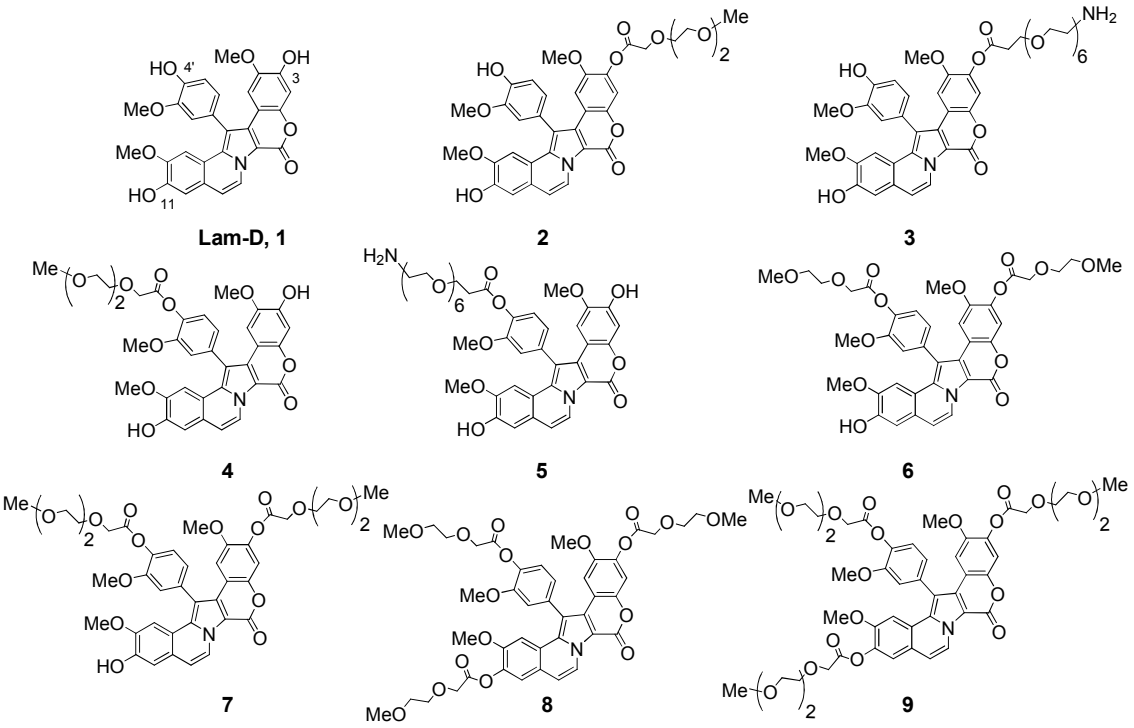
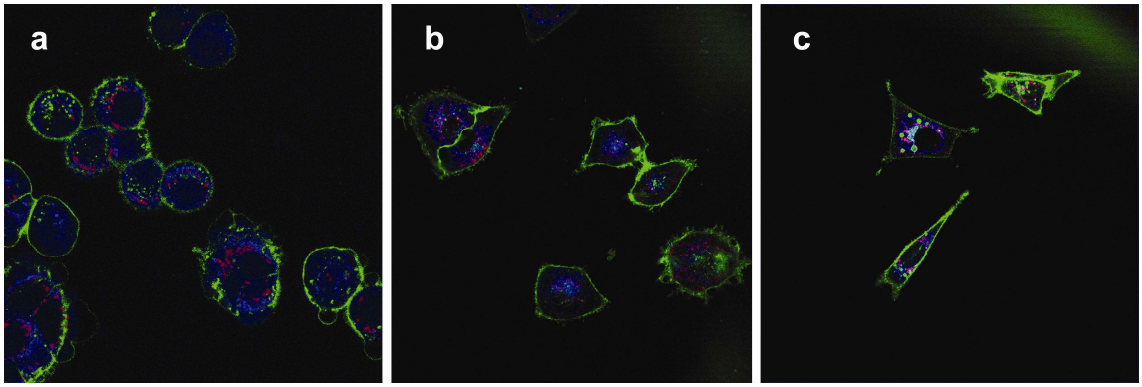
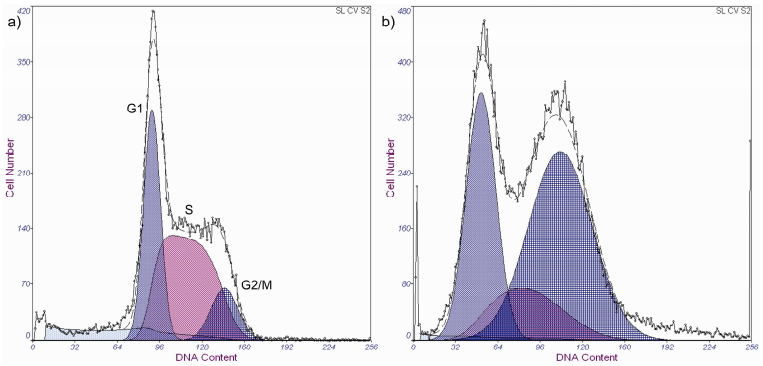


Figure 2. Cellular uptake of **9** and selective staining of mitochondria, Golgi apparatus and cell membrane.



The following cancer cell lines were used for the co-localization experiments. a) HT-29, b) A-549 and c) MDA-MB-231

Figure 3. Cell cycle progression of Lam-D in HT-29 cancer cell line.



c) Phase of Cell Cycle	control	Lam-D
G1 (%)	34.8	31.9
G2/M (%)	12.5	52.2
S (%)	52.6	15.9

a) HT-29 asynchronous colon cell line; b) HT-29 with 1 μM Lam-D for 12 h.

Figure 4. Detection of apoptosis by CaspaTag green fluorescence.