Lamellarin D Bioconjugates II: Synthesis and Cellular Internalization of Dendrimer- and Nuclear Location Signal-Derivatives

Daniel Pla, Marc Martí, Josep Farrera-Sinfreu, Daniel Pulido, Andrés Francesch, Pilar Calvo, Carmen Cuevas, Miriam Royo, Rosa Aligué, Fernando Albericio, and Mercedes Álvarez

mercedes.alvarez@irbbarcelona.org

* Corresponding author: MA and FA, Institute for Research in Biomedicine, Barcelona Science Park-University of Barcelona, Baldiri Reixac 10, E-08028 Barcelona, Spain Tel.: +34934037086; Fax: +34934037126.

‡ Department of Organic Chemistry, University of Barcelona, E-08028 Barcelona
ABSTRACT: The design and synthesis of Lamellarin D conjugates with a nuclear localization signal peptide and a poly(ethylene glycol)-based dendrimer are described. Conjugates 1-4 were obtained in 8-84% overall yields from the corresponding protected Lamellarin D. Conjugates 1 and 4 are 1.4 to 3.3-fold more cytotoxic than the parent compound against three human tumor cell lines (MDA-MB-231 breast, A-549 lung, and HT-29 colon). Besides, conjugates 3, 4 showed a decrease in activity potency in BJ skin fibroblasts, a normal cell culture. Cellular internalization was analyzed and nuclear distribution pattern was observed for 4, which contains a nuclear localization signalling sequence.

KEYWORDS: Lamellarin D; PEG; DTPA; Nuclear localization signal; Dendrimer
INTRODUCTION

Actively mediated cellular delivery of biomolecules (1) has garnered great interest as a strategy for delivering cancer chemotherapeutics (2-5). Conjugates of a drug and a macromolecular vehicle such as NLS\(^1\) peptidic sequences (5-8), PEG carriers (9) and dendrimers (10) may have better cellular internalization than the drug alone, and in some cases, may produce passive accumulation of the drug in tumors by the EPR effect (11). In addition, the therapeutic activity of these conjugates is associated to their capacity to release the drug at a specific subcellular target. Thus, the suitability of macromolecules as vehicles also extends to their propensity to deliver the drug to a predetermined intracellular location.

The marine alkaloid Lam-D (12-15) is a promising drug candidate due to its Topo I inhibition activity. Topoisomerases are nuclear enzymes crucial in cellular replication. They change the topology of DNA before and after the replication and transcription processes. Therefore, they are especially attractive targets for cancer therapy (16-19). Lam-D is limited by its insolubility in common solvent media, especially in water. Therefore, it has been used to investigate its conjugation to macromolecules. In the previous paper we have described the first generation of Lam-D-bioconjugates based on PEG esters such as 1 (9). In this paper we describe a second generation of Lam-D conjugates (Figure 1) based on esterification with either a poly(ethylene glycol)-based dendrimer (in 2) or NLS oligopeptide sequences (in 3 and 4). The peptide NLS H-Pro-Pro-Lys-Lys-Lys-Arg-Lys-Val-OH, which has been demonstrated (20) to shuttle compounds to the nucleus, was used in the present work.

The introduction of such oligomeric systems to Lam D demanded an integrated and robust synthetic scheme with a collection of suitable orthogonal protecting groups, in terms of selective removal and compatibility with the presence of other functional groups (21).

EXPERIMENTAL PROCEDURES

General Data. Reagents and solvents were purified according to Purification of Laboratory Chemicals, Armarego, W. and Chai C., Elsevier (2003). Melting points (m.p.) were determined in a
Büchi Melting Point B540 in open capillaries and are uncorrected. Automatic flash chromatography was done in an Isco Combiflash medium pressure liquid chromatograph with Redisep silica gel columns (47-60 µm). Sonication was performed in a Branson ultrasound bath. $^1$H NMR and $^{13}$C NMR spectra were recorded on a Varian Mercury 400 MHz spectrometer and a Gemini 200 MHz spectrometer. Multiplicity of the carbons was assigned with DEPT and gHSQC experiments, using standard abbreviations for off-resonance decoupling: (s) singlet, (d) doublet, (t) triplet, (q) quartet. The same abbreviations were also used for the multiplicity of signals in $^1$H-NMR, plus (m) multiplet, (bs) broad singlet, (bd) broad doublet. Spectra were referenced to appropriate residual solvent peaks (d$_6$-acetone, d$_6$-DMSO, d$_4$-MeOH or CDCl$_3$). IR spectra were obtained on a Thermo Nicolet FT-IR spectrometer. HRMS were performed on a Bruker Autoflex high resolution mass spectrometer by Unidad de Espectrometría de Masas (Universidad de Santiago de Compostela) and by Servei d'Espectrometria de Masses (Universitat de Barcelona). Microwave-assisted reactions were carried out in a CEM Discover microwave. The automatic syringe pump was used as specified for controlled addition of some reactants. Reversed phase analytical HPLC was performed on a Waters Alliance separation module 2695 using a Waters Xterra MS C$_{18}$ column (150 x 4.6 mm, 5 µm) and a Waters 996 PDA detector at 254 nm.

**General Procedures:**

**A) General Method for Simultaneous Removal of TBDPSO and N-Boc.**

HF (5 mL) at -196 ºC was poured over solid 11, 12 or 14 (0.05-0.08 mmol). The solution was stirred for 1 min and the solvent was immediately removed under vacuum at low temperature. MeCN was added to the crude, and the deprotected compound was precipitated by addition of MTBE, cooling to 0 ºC and centrifugation (10 min, 2000 r.p.m.). The residue was dried in vacuo to give the final Lam-D conjugates 1-3.

**B) General Method for MOMO deprotection**
Me$_3$SiI (142 µL, 3.00 mmol) was added at r.t. to a solution of 10a, or 10b (1.00 mmol) in CH$_2$Cl$_2$ (225 mL), and the resulting orange solution was stirred at r.t. for 20 min. The solvent was removed in vacuo, and the residue was dissolved with EtOAc and then washed three times with sat. NH$_4$Cl and brine. The organic phase was dried over anhydrous MgSO$_4$ and the solvent was removed in vacuo. Purification by column chromatography on silica gel by elution with hexane/EtOAc (80:20 to 60:40) gave the title compounds (84%-quant. yield).

C) General Method for Esterification. Synthesis of Conjugates. DMAP (0.6 mmol) and 5 (1 mmol) in dry CH$_2$Cl$_2$ (45 mL) were added to a solution of NHBocPEG$_6$-OH, or Boc-NLS-Gly-OH (4 mmol), and EDC·HCl (4 mmol) in dry CH$_2$Cl$_2$ (5 mL). The resulting solution was stirred at r.t. for 2 h. The reaction mixture was diluted with CH$_2$Cl$_2$ and washed with sat. NaHCO$_3$ solution and brine. The organic phase was dried over anhydrous MgSO$_4$ and the solvent removed under vacuum, to provide the title compounds 11 and 13 (89%-quant. yield).

3-[3-(2-(2-(2-(2-(2-Aminoethoxy)ethoxy)ethoxy)ethoxy)ethoxy)ethoxy]propanoyloxy]Lam-D (22) (1). Following the general procedure A and starting from 11 (24, 23 and 23 mg, 0.05 mmol), a yellow solid (35 mg, 84%) was obtained. The spectroscopic data are in accordance with previous reports (9).

3-[2-(Bis(2-(2-(2-(2-(2-Aminoethoxy)ethoxy)ethoxy)ethoxy)propylamino)2-oxoethyl)amino)ethyl]amino)acetyl]Lam-D (22) (2). Following the general procedure A and starting from 12 (17 and 15 mg, 0.12 mmol), 2 as a yellow solid (8 mg, 36%) was obtained. $^1$H NMR (D$_2$O, 400 MHz) $\delta$ 1.54 and 1.67 (2h, $J = 6.6$ Hz, 8H); 1.76 and 1.83 (2h, $J = 6.6$ Hz, 8H); 2.93 (2t, $J = 7.2$ Hz, 4H); 2.97 (2t, $J = 7.2$ Hz, 4H); 3.02-3.10 (m, 8H); 3.16-3.29 (m, 11H, OMe); 3.33-3.43 (m, 43H, OMe); 3.52-3.56 (m, 18H); 3.70 (br, 3H, OMe); 6.43 (s, 1H); 6.57 (br, 1H); 6.71 (br, 1H); 6.81-6.88 (m, 4H); 6.99 (br, 1H); 8.35 (br, 1H). $^{13}$C NMR (D$_2$O, 100 MHz) $\delta$ 26.6 (t); 26.7 (t); 28.5 (t); 28.6 (t); 36.4 (t); 36.5 (t); 37.7 (t); 37.8 (t); 55.2 (q); 55.5 (q); 57.6 (t); 57.7 (t); 68.3 (t); 68.4 (t); 68.5 (t); 69.4 (t); 69.5 (t); 69.6 (t); 69.7 (t); 107.7 (d); 111.5 (d); 111.7 (d); 122.2 (d); 122.4 (d); 122.8 (d); 127.6 (d); 145.6 (d); 146.7 (d); 147.4 (s); 149.5 (s); 149.9 (s); 150.3
(s); 151.1 (s); 171.0 (s); 171.1 (s). MS (MALDI-TOF) 1684 (M+1, 100), 1685 (M+2, 93) 1686 (M+3, 49). HRMS m/z calcd. for C_{82}H_{131}N_{12}O_{25} 1683.9342, found 1683.9346. HPLC analysis: 6.9 min retention time (94% purity), with a gradient of 0 to 100% of eluent B over 15 min using the solvent system: H_{2}O/0.045% TFA (A) and MeCN/0.036% TFA (B).

3-[Gly-Gly-NLS]Lam-D (22) (3). Following the general procedure A and starting from 14 (28 and 17 mg, 0.16 mmol), 3 as a white solid (10 mg, 38%) was obtained. \(^1\)H NMR (DMSO-d_6, 400 MHz) \(\delta\) 0.83-0.88 (m, 6H, 2CH_3); 1.22-1.36 (m, 10H, 5CH_2); 1.42-1.54 (m, 8H, 4CH_2); 1.56-1.62 (m, 8H, 4CH_2); 1.88-1.93 (m, 4H, 2CH_2); 2.05 (br, 2H, CH_2); 2.07 (br, 2H, CH_2); 2.33 (t, \(J = 1.8\) Hz, 2H, CH_2); 2.55 (br, 1H, CH); 2.67 (t, \(J = 1.8\) Hz, 2H, CH_2); 2.69-2.75 (m, 16H, 3CH_2); 3.36 (2s, 6H, 2OMe); 3.75 (s, 3H, OMe); 3.99-4.05 (m, 3H, CH); 4.14-4.25 (m, 7H, 3CH, 2CH_2); 4.58 (br, 1H, CH); 5.93 (br, 1H); 5.76 (br, 1H); 5.47 (br, 1H); 6.70 (s, 1H); 6.87 (s, 1H); 7.01 (dd, \(J = 8.0, 1.7\) Hz, 1H); 7.10 (d, \(J = 8.0\) Hz, 1H); 7.12-7.13 (m, 2H); 7.19 (s, 1H); 7.21 (br, 1H); 9.00 (d, \(J = 7.4\) Hz, 1H, H8). MS (MALDI-TOF) 1575 (M+1, 100), 1576 (M+2, 92). HRMS m/z calcd. for C_{77}H_{111}N_{18}O_{18} 1575.8324, found 1575.8319. HPLC analysis: 1.8 min retention time (96.7% purity), with 15 min isocratic MeCN/0.036% TFA.

4’-[Gly-NLS]Lam-D (22) (4). Peptide 8 (392 mg, 0.23 mmol) was pre-activated for 15 min at r.t. with TCFH (63 mg, 23 mmol) and NEt_3 (32 µL, 23 mmol) in CH_2Cl_2 dry (15 mL). Compound 6 (53 mg, 0.08 mmol) and DMAP (9 mg, 0.08 mmol) in CH_2Cl_2 dry (5 mL) were then added. The resulting solution was stirred at r.t. for 120 h. The reaction mixture was diluted with CH_2Cl_2 and washed with sat. NaHCO_3 solution and brine. The organic phase was dried over anhydrous MgSO_4 and the solvent was removed under vacuum. The residue was purified by flash chromatography: elution with CH_2Cl_2/MeOH (99:1 to 95:5) gave 15 (45% yield based on 40% transformation of 6). The Boc protected compound 15 was then treated with 40% TFA in CH_2Cl_2 (10 mL) at r.t. for 1 h. The solvent was removed under reduced pressure, and the residue was purified by reverse phase chromatography. Elution with H_2O/MeCN (60:40 to 40:60) gave 4 as a yellow solid (3 mg, 17%). \(^1\)H NMR (D_2O, 500 MHz) \(\delta\) 1.04 (d, \(J = 6.5\) Hz, 3H, CH_3); 1.16 (d, \(J = 6.5\) Hz, 3H, CH_3); 1.39-1.56
(m, 8H, 4CH$_2$); 1.66-1.77 (m, 10H, 5CH$_2$); 1.78-1.88 (m, 8H, 4CH$_2$); 2.00-2.12 (m, 4H, 2CH$_2$); 2.17 and 2.36 (2br, 2H, CH$_2$); 2.44 and 2.58 (2br, 2H, CH$_2$); 2.84 (br, 1H, CH); 2.96-3.05 (m, 10H, 5CH$_2$); 3.40 (br, 3H, OMe); 3.44 (s, 3H, OMe); 3.45 (s, 3H, OMe); 3.22 (t, $J$ = 7.5 Hz, 1H, CH); 3.59 (t, $J$ = 7.0 Hz, 1H, CH)$_2$; 3.81 (br, 2H, CH$_2$); 4.23-4.42 (m, 5H, 5CH); 4.51 (t, $J$ = 8.3 Hz, 1H, CH); 4.65 (dd, $J$ = 9.0, 8.4 Hz, 1H, CH); 6.38 (s, 1H); 6.64-6.87 (m, 4H); 6.99 (br, 1H); 7.01 (br, 1H); 7.32 (br, 1H); 8.54 (br, 1H). MS (MALDI-TOF) 1518 (M+1, 100), 1519 (M+2, 82). HRMS m/z calcd. for C$_{75}$H$_{108}$N$_1$O$_{17}$ 1518.8109, found 1518.8114. HPLC analysis: 6.9 min retention time (95% purity), with a gradient of 50 to 100% of eluent B over 15 min using the solvent system: H$_2$O/0.045% TFA (A) and MeCN/0.036% TFA (B).

4',11-Bis(tert-butyldiphenylsilyl)Lam-D (22) (5). Following the general procedure B and starting from 10b (1.06 g, 1.04 mmol), 5 as a yellow solid (856 mg, 84%) was obtained. mp (MeCN) 278-280 °C. IR (film) ν 1704, 1487, 1428, 1284, 1111 cm$^{-1}$. $^1$H NMR (CDCl$_3$, 200 MHz) δ 1.15 (s, 9H); 1.17 (s, 9H); 3.18 (s, 3H, OMe); 3.47 (s, 3H, OMe); 3.64 (s, 3H, OMe); 5.89 (s, 1H); 6.68 (d, $J$ = 7.4 Hz, 1H, H9); 6.69 (s, 1H); 6.91-6.93 (m, 3H); 6.95 (s, 1H); 7.05 (br, 1H); 7.06 (br, 1H); 7.37-7.48 (m, 10H); 7.71-7.79 (m, 10H); 9.04 (d, $J$ = 7.4 Hz, 1H, H8). $^{13}$C NMR (CDCl$_3$, 100 MHz) δ 19.9 (s); 26.6 (q); 26.7 (q); 54.5 (q); 55.5 (q); 55.7 (q); 103.4 (d); 104.7 (d); 105.9 (d); 107.6 (s); 109.8 (s); 111.0 (s); 112.1 (d); 115.4 (d); 116.5 (d); 119.7 (s); 120.3 (d); 122.7 (d); 123.7 (d); 124.4 (s); 127.5 (d); 127.6 (d); 128.5 (s); 129.1 (s); 129.7 (d); 129.9 (d); 132.9 (s); 133.0 (s); 133.4 (s); 134.1 (s); 135.0 (d); 135.1 (d); 143.1 (s); 144.9 (s); 146.1 (s); 146.8 (s); 150.7 (s); 151.1 (s); 155.3 (s). MS (MALDI-TOF) 976 (M+1, 100), 977 (M+1, 57), 978 (M+2, 38).

3,11-Di-tert-butoxycarbonyl-Lam-D (22) (6) A solution of TBAF in THF (1 M, 1.64 mmol) was added to a -78 °C solution of 10d (771 mg, 0.82 mmol), in MeOH-THF (100 mL, 80:20). The mixture was stirred at -78 °C for 15 min. Solvents were removed under vacuum, and the residue was dissolved in CH$_2$Cl$_2$. The organic solution was washed with water and brine. The organic phase was dried over anhydrous MgSO$_4$ and the solvent removed under vacuum. The residue was purified by flash chromatography: elution with hexane/EtOAc (60:40 to 40:60) gave 6 as a white solid (491
mg, 85%). mp (MeCN) 149-150 °C. IR (film) ν 1760, 1709, 1275, 1255 cm⁻¹. ¹H NMR (CDCl₃, 400 MHz) δ 1.54 (s, 9H); 1.55 (s, 9H); 3.455 (s, 3H, OMe); 3.463 (s, 3H, OMe); 3.91 (s, 3H, OMe); 5.93 (br, 1H, OH); 6.83 (s, 1H); 6.97 (d, J = 7.4 Hz, 1H, H9); 7.11 (br, 1H); 7.15 (dd, J = 8.0, 1.8 Hz, 1H); 7.18 (br, 1H); 7.24-7.26 (m, 2H); 7.44 (br, 1H); 9.14 (d, J = 7.4 Hz, 1H, H8). ¹³C NMR (CDCl₃, 100 MHz) δ 27.5 (q); 55.3 (q); 55.6 (q); 56.3 (q); 83.89 (s); 83.95 (s); 106.4 (d); 106.6 (d); 108.8 (s); 111.8 (d); 112.6 (d); 113.0 (s); 113.5 (d); 115.4 (d); 115.7 (s); 120.3 (d); 123.0 (d); 123.6 (s); 123.8 (s); 124.0 (s); 124.4 (d); 128.1 (s); 128.3 (s); 133.6 (s); 140.0 (s); 141.0 (s); 145.4 (s); 145.9 (s); 147.7 (s); 147.9 (s); 150.9 (s); 151.0 (s); 151.1 (s); 155.0 (s). MS (MALDI-TOF) 699 (M, 56), 700 (M+1, 100).

**Boc-Pro-Pro-Lys(Boc)-Lys(Boc)-Lys(Boc)-Arg(Boc₂)-Lys(Boc)-Val-Gly-OH (8):** The Boc protected peptide was synthesized manually on solid-phase in a polypropylene syringe fitted with a porous polyethylene disc. Solvents and soluble reagents were removed by suction. Washings between deprotection, coupling and subsequent deprotection steps were carried out with DMF (5 × 1 min) and CH₂Cl₂ (5 × 1 min) using 10 mL solvent/g resin each time. Standard Fmoc/tBu chemistry and chlorotrityl resin (0.5 g, 1.5 mmol/g) were used. The resin was pre-swollen in anhydrous CH₂Cl₂ and then in DMF. The first Fmoc-protected amino acid (Fmoc-L-Gly-OH) (155 mg, 0.7 equiv) was introduced in the presence of DIEA (635 µL, 5 equiv) in DMF. After one hour, MeOH (0.5 mL) was added and the mixture was stirred for 30 min. The resin was then washed with DMF and CH₂Cl₂, and the synthesis continued as described below. The peptide was elongated through successive iterations of Fmoc removal and amino acid coupling. The Fmoc protecting group was removed with several treatments of 20% piperidine in DMF (1× 1 min + 2 × 15 min). The resin was then washed with DMF and CH₂Cl₂. The corresponding Fmoc-protected amino acid (5 equiv) was introduced using DIPCDI (310 µL, 5 equiv) and HOBt (305 mg, 5 equiv) as coupling agents. After 2h, the resin was washed with DMF and CH₂Cl₂, and the coupling was monitored using the Kaiser test. Re-couplings were done when needed. Boc-L-Pro-OH (430 mg, 5 equiv) was used as a last amino acid. The peptide was finally cleaved from the resin using 3% TFA in CH₂Cl₂,
(5 x 1 min). Washes were collected in a flask containing 50 mL of water. The CH₂Cl₂ was then evaporated under reduced pressure, MeCN (30 mL) was added to the aqueous solution, and the resulting mixture was then lyophilized. Peptide 8 (590 mg, 94%) was obtained as a white solid. HPLC analysis: 6.2 min retention time (92% purity), with a gradient of 50 to 100% of eluent B in 7 min using the solvent system: H₂O/0.045% TFA (A) and MeCN/0.036% TFA (B). HPLC ESI-MS calcd. for C₈₂H₁₄₄N₁₆O₂₄ [M + H]⁺ 1738, found: [M+2]²⁺/2, 870.

11-Benzyl-4'-tert-butyldiphenylsilyl-3-methoxymethyl-Lam-D (22) (10). A mixture of 9 (27) (1.54 g, 1.76 mmol) and DDQ (400 mg, 1.76 mmol) in dry CHCl₃ (25 mL) was purged with Ar in a sealed vessel and microwaved at 120 ºC for 10 min. The organic solution was washed with water, and brine, dried over MgSO₄, filtered, and then concentrated in vacuo. Purification by column chromatography on silica gel by elution with hexane/EtOAc (80:20 to 60:40) gave 10 as a white solid (2.27 g, 81%). mp (MeCN) 144-145 ºC. IR (film) ν 1705, 1429, 1267, 1223 cm⁻¹. ¹H NMR (CDCl₃, 200 MHz) δ 1.17 (s, 9H); 3.43 (s, 3H, OMe); 3.46 (s, 3H, OMe); 3.50 (s, 3H, OMe); 3.65 (s, 3H, OMe); 5.23 (s, 2H); 5.24 (s, 2H); 6.77 (s, 1H); 6.92-6.96 (m, 3H); 7.06-7.08 (m, 2H); 7.19 (s, 1H); 7.25 (d, J = 5.4 Hz, 1H); 7.32-7.47 (m, 11H); 7.75-7.79 (m, 4H); 9.16 (d, J = 7.2 Hz, 1H, H8). ¹³C NMR (CDCl₃, 50.3 MHz) δ 19.9 (s); 26.7 (q); 55.2 (q); 55.5 (q); 55.7 (q); 56.3 (q); 70.7 (t); 95.4 (t); 105.1 (d); 105.5 (d); 105.6 (d); 109.4 (d); 111.3 (s); 111.4 (s); 112.3 (d); 115.4 (d); 119.3 (s); 120.3 (d); 123.1 (d); 123.7 (d); 124.5 (s); 127.2 (d); 127.7 (d); 128.0 (d); 128.4 (s); 128.5 (s); 128.6 (d); 128.9 (s); 129.9 (d); 133.3 (s); 134.1 (s); 135.1 (d); 136.2 (s); 145.0 (s); 146.0 (s); 146.2 (s); 146.6 (s); 149.0 (s); 149.5 (s); 151.2 (s); 155.3 (s). MS (MALDI-TOF) 871 (M, 18), 872 (M+1, 100).

4’-tert-Butyldiphenylsilyl-3-methoxymethyl-Lam-D (22) (10a). Pd/C (10%) was added to a solution of 10 (509 mg, 0.58 mmol) in MeOH/EtOAc (2:1, 58 mL), the mixture was purged with H₂, and the resulting suspension was stirred at r.t. for 16 h. The reaction mixture was filtered through a pad of Celite, which was then washed with CH₂Cl₂. The solvent was removed under vacuum to provide the 10a as a brown solid (433 mg, 95%). mp (MeCN) 129-130 ºC. IR (film)
v 3213, 1680, 1425, 1222 cm\(^{-1}\). \(^1\)H NMR (CDCl\(_3\), 400 MHz) \(\delta\) 1.16 (s, 9H); 3.44 (s, 3H, OMe); 3.47 (s, 3H, OMe); 3.51 (s, 3H, OMe); 3.65 (s, 3H, OMe); 5.24 (s, 2H); 5.92 (br, 1H, OH); 6.77 (s, 1H); 6.93-6.94 (m, 2H); 6.98 (d, \(J = 7.4\) Hz, 1H, H9); 7.05 (br, 1H); 7.13 (s, 1H); 7.16 (s, 1H); 7.25 (d, \(J = 5.6\) Hz, 2H); 7.38-7.47 (m, 5H); 7.75-7.78 (m, 4H); 9.18 (d, \(J = 7.4\) Hz, 1H, H8). \(^{13}\)C NMR (CDCl\(_3\), 100 MHz) \(\delta\) 19.8 (s); 26.7 (q); 55.3 (q); 55.5 (q); 55.7 (q); 56.3 (q); 95.5 (t); 105.0 (d); 105.3 (d); 105.6 (d); 110.8 (d); 111.2 (s); 111.5 (s); 112.4 (d); 115.4 (d); 118.8 (s); 120.4 (d); 123.3 (d); 123.7 (d); 125.3 (s); 127.7 (d); 128.6 (s); 129.0 (s); 129.96 (d); 129.98 (d); 133.37 (s); 133.43 (s); 134.4 (s); 135.2 (d); 135.3 (s); 145.1 (s); 146.1 (s); 146.3 (s); 146.66 (s); 146.69 (s); 146.8 (s); 151.3 (s); 155.4 (s). MS (MALDI-TOF) 781 (M, 60), 782 (M+1, 100).

4',11-Bis(tert-butyldiphenylsilyl)-3-methoxymethyl-Lam-D (10b). TBDPSCI (389 µL, 1.07 g/mL, 1.47 mmol) was added to a solution of 10a (768 mg, 0.98 mmol), imidazole (135 mg, 1.96 mmol) and DMAP (120 mg, 0.98 mmol) in dry DMF (30 mL). The mixture was stirred for 24 h under Ar. DMF was removed under reduced pressure and the residue was dissolved in CH\(_2\)Cl\(_2\). The organic phase was washed with water and brine, and then dried over anhydrous MgSO\(_4\). The solvent was removed under vacuum and the residue was purified by flash chromatography. Elution with hexane/CH\(_2\)Cl\(_2\) (40:60 to 20:80) gave 10b (584 mg, 58%) as a brown oil. IR (film) v 1679, 1426, 1113 cm\(^{-1}\). \(^1\)H NMR (CDCl\(_3\), 200 MHz) \(\delta\) 1.11 (s, 9H); 1.15 (s, 9H); 3.17 (s, 3H, OMe); 3.42 (s, 3H, OMe); 3.49 (s, 3H, OMe); 3.61 (s, 3H, OMe); 5.21 (s, 2H); 6.71 (d, 1H, \(J = 7.4\) Hz, H9); 6.74 (s, 1H); 6.90 (2s, 2H); 6.92 (s, 1H); 7.01-7.05 (m, 2H); 7.22 (br, 1H); 7.10-7.19 (m, 10H); 7.69-7.77 (m, 10H); 9.06 (d, \(J = 7.4\) Hz, 1H, H8). \(^{13}\)C NMR (CDCl\(_3\), 100 MHz) \(\delta\) 19.9 (s); 26.6 (q); 26.7 (q); 54.5 (q); 55.4 (q); 55.7 (q); 56.3 (q); 95.4 (t); 105.1 (d); 105.5 (d); 105.9 (d); 107.9 (s); 111.3 (s); 111.4 (s); 112.3 (d); 115.3 (d); 116.5 (d); 119.7 (s); 120.3 (d); 122.7 (d); 123.6 (d); 124.4 (s); 127.4 (d); 127.6 (d); 127.7 (d); 127.8 (d); 128.4 (s); 128.8 (s); 129.5 (d); 129.7 (d); 130.0 (d); 132.9 (s); 133.0 (s); 133.4 (s); 134.7 (d); 135.0 (d); 135.1 (d); 144.9 (s); 145.9 (s); 146.2 (s); 146.5 (s); 150.7 (s); 151.1 (s); 155.3 (s). MS (ESI) 1020 (M+1, 100).
4'-tert-Butyldiphenylsilyl-Lam-D (22) (10c). Following the general procedure B and starting from 10a (639 mg, 0.81 mmol), 10c as a white solid (603 mg, quant) was obtained. mp (MeCN) 275-276 °C. IR (film) ν 3415, 1679, 1429, 1271 cm⁻¹. ¹H NMR (CDCl₃, 400 MHz) δ 1.17 (s, 9H); 3.46 (s, 3H, OMe); 3.47 (s, 3H, OMe); 3.65 (s, 3H, OMe); 6.71 (s, 1H); 6.93-6.94 (m, 2H); 6.98-7.05 (m, 3H); 7.13 (s, 1H); 7.17 (s, 1H); 7.39-7.47 (m, 6H); 7.76-7.79 (m, 4H); 9.17 (d,  𝐽 = 7.4 Hz, 1H, H8).

¹³C NMR (CDCl₃, 100 MHz) δ 19.9 (s); 26.7 (q); 55.3 (q); 55.5 (q); 55.7 (q); 103.6 (d); 104.7 (s); 104.8 (d); 105.0 (d); 107.8 (s); 109.9 (s); 110.8 (d); 110.9 (s); 112.3 (d); 115.5 (d); 118.8 (s); 120.4 (d); 123.4 (d); 123.8 (d); 125.4 (s); 127.7 (s); 127.9 (d); 129.3 (s); 130.0 (d); 133.4 (s); 133.5 (s); 135.2 (d); 143.2 (s); 145.1 (s); 146.2 (s); 146.7 (s); 146.8 (s); 147.0 (s); 151.3 (s); 155.5 (s). MS (ESI-TOF) 737 (M, 73), 738 (M+1, 100).

4'-tert-Butyldiphenylsilyl-3,11-bis(tert-butoxycarbonyl)Lam-D (22) (10d). (Boc)₂O (536 mg, 2.45 mmol) and DMAP (30 mg, 0.25 mmol) were added to a solution of 10c (605 mg, 0.82 mmol) in CH₂Cl₂ (130 mL). The reaction mixture was stirred at r.t. under Ar for 16 h. The mixture was washed with a sat. NaHCO₃ and brine. The organic layer was dried over anhydrous Na₂SO₄, filtered and concentrated to dryness to give 10d as a yellow solid (767 mg, quant). mp (MeCN) 188-189 °C. IR (film) ν 1760, 1710, 1486, 1430, 1274, 1255 cm⁻¹. ¹H NMR (CDCl₃, 400 MHz) δ 1.15 (s, 9H); 1.56 (s, 9H); 1.57 (s, 9H); 3.40 (s, 3H, OMe); 3.42 (s, 3H, OMe); 3.64 (s, 3H, OMe); 6.83 (s, 1H); 6.90-6.93 (m, 2H); 7.01 (br, 1H); 7.04 (d,  𝐽 = 7.4 Hz, 1H, H9); 7.23 (s, 1H); 7.25 (d,  𝐽 = 5.8 Hz, 2H); 7.38-7.46 (m, 6H); 7.74-7.77 (m, 4H); 9.22 (d,  𝐽 = 7.4 Hz, 1H, H8). ¹³C NMR (CDCl₃, 100 MHz) δ 19.9 (s); 26.7 (q); 55.3 (q); 55.6 (q); 55.7 (q); 83.96 (s); 84.02 (s); 84.02 (s); 106.6 (d); 106.7 (d); 108.9 (s); 111.9 (d); 112.7 (d); 113.1 (s); 115.0 (d); 115.8 (s); 120.3 (d); 120.5 (d); 123.3 (d); 123.4 (d); 123.7 (s); 123.9 (s); 127.7 (s); 127.9 (d); 128.3 (s); 130.0 (d); 133.4 (s); 133.6 (s); 135.1 (d); 140.0 (s); 141.0 (s); 145.3 (s); 145.5 (s); 147.7 (s); 150.8 (s); 151.0 (s); 151.2 (s); 151.5 (s); 155.1 (s). MS (MALDI-TOF) 938 (M+1, 100), 939 (M+2, 63).

4',11-Bis(tert-butylidiphenylsilyl)-3-[3-(2-(2-(2-(2-(2-(2-tert-butoxycarbonylaminoethoxy)ethoxy)ethoxy)ethoxy)ethoxy)propanoyl]Lam-D (22)
(11). Following the general procedure C and starting from 5 (78 mg, 0.80 mmol), 11 as a yellow oil (112 mg, quant.) was obtained. IR (film) ν 1710, 1486, 1429, 1283, 1159, 1114 cm⁻¹. ¹H NMR (CDCl₃, 200 MHz) δ 1.04 (s, 9H); 1.06 (s, 9H); 1.36 (s, 9H); 2.81 (t, J = 6.5 Hz, 2H); 3.07 (s, 3H, OMe); 3.24 (br, 4H, 2CH₂); 3.29 (s, 3H, OMe); 3.43-3.48 (m, 4H, 2CH₂); 3.52-3.60 (m, 19H, 8CH₂, OMe); 3.79 (t, J = 6.5 Hz, 2H); 5.05 (br, 1H); 6.68 (d, J = 7.4 Hz, 1H, H9); 6.75 (s, 1H); 6.81 (br, 2H); 6.85 (s, 1H); 6.91 (br, 1H); 6.95 (br, 1H); 7.03 (s, 1H); 7.24-7.38 (m, 10H); 7.61-7.68 (m, 10H); 9.00 (d, J = 7.4 Hz, 1H, H8). ¹³C NMR (CDCl₃, 100 MHz) δ 19.8 (s); 26.5 (q); 26.7 (q); 28.4 (q); 34.7 (t); 40.3 (t); 54.4 (q); 55.4 (q); 55.6 (q); 66.3 (t); 70.1 (t); 70.5 (t); 105.8 (d); 106.3 (d); 108.1 (s); 111.8 (s); 111.9 (d); 112.6 (d); 115.1 (d); 116.0 (s); 116.5 (d); 119.6 (s); 120.3 (d); 122.5 (d); 123.5 (d); 124.3 (s); 127.4 (s); 127.5 (d); 127.6 (d); 128.0 (s); 128.1 (s); 129.7 (d); 129.8 (d); 132.8 (s); 132.9 (s); 133.3 (s); 134.2 (s); 134.9 (d); 135.1 (d); 139.2 (s); 144.9 (s); 145.3 (s); 146.2 (s); 147.2 (s); 150.8 (s); 151.1 (s); 154.9 (s); 169.2 (s). MS (MALDI-TOF) 1311 (M+1-Boc, 100), 1433 (M+Na, 45).

4',11-Bis(tert-butyldiphenylsilyl)-3-[2-(bis(2-(bis(2-(2-(3-tert-butoxycarbonylamino-propoxy)ethoxy)ethoxy)propylamino)-2-oxoethyl)amino)ethyl]amino)acetyl]Lam-D (22) (12). A mixture of polystyrene solid supported DMAP (6.0 mg, 19 mmol) and 5 (31 mg, 32 mmol) in dry CH₂Cl₂ (2 mL) was added to a solution of 7 (51 mg, 32 mmol) and EDC·HCl (6 mg, 32 mmol) in dry CH₂Cl₂ (2 mL). The resulting solution was stirred at r.t. for 16 h. The reaction mixture was filtered, and the solvent was removed under vacuum. The residue was purified by flash chromatography with neutral alumina. Elution with CH₂Cl₂/MeOH (99:1 to 98:2) gave 12 as a yellow oil (22 mg, 26%). IR (film) ν 3323, 1665, 1548, 1428, 1275, 1112 cm⁻¹. ¹H NMR (CDCl₃, 200 MHz) δ 1.11 (s, 9H); 1.13 (s, 9H); 1.41 (s, 18H); 1.42 (s, 18H); 1.61-1.77 (m, 16H); 2.34 (2t, 4H); 2.71-2.78 (m, 4H); 3.12 (s, 3H, OMe); 3.15-3.31 (m, 26H); 3.35 (s, 3H, OMe); 3.47-3.67 (m, 43H); 5.06 (br, 2H); 5.16 (br, 1H); 5.30 (br, 1H); 6.77 (d, J = 7.4 Hz, 1H, H9); 6.84 (s, 1H); 6.88 (br, 2H); 6.92 (s, 1H); 6.97 (br, 1H); 7.00 (br, 1H); 7.12 (s, 1H); 7.32-7.45 (m, 10H); 7.64-7.73 (m, 10H); 9.08 (d, J = 7.4 Hz, 1H, H8). ¹³C NMR (CDCl₃, 100 MHz) δ 15.4 (q); 19.8 (s); 26.6 (q); 26.7
Following the general procedure C and starting from 5 (99 mg, 1.02 mmol), 13 was obtained as a yellow solid (103 mg, 89%). mp (MeCN) 197-199 ºC. IR (film) ν 1711, 1509, 1486, 1429, 1283, 1158 cm⁻¹. ¹H NMR (CDCl₃, 200 MHz) δ 1.14 (s, 9H); 1.15 (s, 9H); 1.49 (s, 9H); 3.17 (s, 3H, OMe); 3.37 (s, 3H, OMe); 3.62 (s, 3H, OMe); 4.23 (brd, J = 5.0 Hz, 2H); 5.13 (brt, J = 5.0 Hz, 1H); 6.73 (d, J = 7.4 Hz, 1H, H9); 6.85 (s, 1H); 6.91 (br, 2H); 6.93 (s, 1H); 7.02 (br, 1H); 7.05 (br, 1H); 7.12 (br, 1H); 7.36-7.48 (m, 10H); 7.70-7.77 (m, 10H); 9.05 (d, J = 7.4 Hz, 1H, H8). ¹³C NMR (CDCl₃, 100 MHz) δ 19.9 (s); 26.6 (q); 26.7 (q); 28.3 (q); 42.3 (t); 54.5 (q); 55.5 (q); 55.6 (q); 105.8 (d); 106.4 (d); 108.1 (s); 111.7 (d); 111.9 (s); 112.7 (d); 115.2 (d); 116.3 (s); 116.6 (d); 119.7 (s); 120.3 (d); 122.5 (d); 123.5 (d); 124.3 (s); 127.5 (s); 127.6 (d); 127.7 (d); 127.9 (s); 128.1 (s); 129.6 (s); 129.7 (d); 129.9 (d); 132.9 (s); 133.3 (s); 134.2 (s); 135.0 (d); 135.2 (d); 135.4 (s); 138.8 (s); 145.0 (s); 145.3 (s); 146.3 (s); 147.0 (s); 150.8 (s); 151.2 (s); 154.8 (s); 168.3 (s). MS (MALDI-TOF) 1132 (M, 100), 1133 (M+1, 79), 1134 (M+2, 37).

4’,11-Bis(tert-butyldiphenylsilyl)-3-[Gly-Gly-Boc-NLS]Lam-D (22) (14). Lamellarin 13 (32 mg, 29 mmol) was treated with 40% TFA in CH₂Cl₂ (10 mL) at 0 ºC for 10 min. The solvent was removed under reduced pressure, and the crude was used without further purification. A solution of this residue and DIPEA (4.9 µL, 29 mmol) in dry CH₂Cl₂ (3 mL) was added to a solution of 8 (50 mg, 29 mmol), HOBt (5 mg, 34 mmol), DIPEA (4.9 µL, 29 mmol) and EDC·HCl (7 mg, 34 mmol) in dry CH₂Cl₂ (2 mL). The resulting solution was stirred at r.t. for 4 h. The reaction mixture was diluted with CH₂Cl₂ and washed with sat. NaHCO₃ solution and brine. The organic phase was dried
over anhydrous MgSO\textsubscript{4} and the solvent was removed under vacuum. The residue was purified by flash chromatography with neutral alumina: elution with CH\textsubscript{2}Cl\textsubscript{2}/MeOH (98:2 to 95:5) gave 14 as a yellow oil (46 mg, 58%). IR (film) 3328, 1650, 1534, 1429, 1365, 1281, 1166 cm\textsuperscript{-1}. \textsuperscript{1}H NMR (CDCl\textsubscript{3}, 200 MHz) \(\delta\) 0.92-1.00 (m, 6H, 2CH\textsubscript{3}); 1.11 (s, 3H, CH\textsubscript{3}); 1.13 (s, 3H, CH\textsubscript{3}); 1.23-1.33 (m, 8H, 4CH\textsubscript{2}); 1.36-1.37 (m, 4H, 2CH\textsubscript{2}); 1.39 (s, 3H, CH\textsubscript{3}); 1.41 (s, 18H, 6CH\textsubscript{3}); 1.44-1.50 (m, 4H, 2CH\textsubscript{2}); 1.46-1.50 (m, 8H, 4CH\textsubscript{2}); 1.84 (br, 4H, 2CH\textsubscript{2}); 2.07 (br, 4H, 2CH\textsubscript{2}); 2.43 (br, 4H, 2CH\textsubscript{2}); 2.71 (h, \(J = 6.8\) Hz, 1H); 3.00 and 3.07 (2br, 8H, 4CH\textsubscript{2}); 3.14 (s, 3H, OMe); 3.34-3.41 (m, 2H, CH\textsubscript{2}); 3.44 (s, 3H, OMe); 3.59 (s, 3H, OMe); 3.62-3.74 (m, 2H, CH\textsubscript{2}); 3.92-4.23 (m, 5H, 5CH); 4.28 (br, 2H, CH\textsubscript{2}); 4.47-4.53 (m, 2H, CH\textsubscript{2}); 4.67-4.71 (m, 3H, 3CH); 4.77 (br, 1H); 4.86 (br, 1H); 6.67 (s, 1H); 6.71 (d, \(J = 7.4\) Hz, 1H, H9); 6.80-6.89 (m, 2H); 6.91 (d, \(J = 1.8\) Hz, 1H); 6.95-6.99 (m, 2H); 7.02 (d, \(J = 8.2\) Hz, 1H); 7.32-7.47 (m, 10H); 7.56-7.75 (m, 10H); 8.2 (br, 1H); 8.3 (br, 1H); 8.4 (br, 1H); 8.5 (br, 1H); 9.07 (d, \(J = 7.4\) Hz, 1H, H8). \textsuperscript{13}C NMR (CDCl\textsubscript{3}, 100 MHz) \(\delta\) 19.8 (s); 23.6 (t); 23.7 (t); 24.6 (t); 26.2 (t); 26.6 (q); 26.7 (q); 28.0 (q); 28.3 (q); 28.4 (q); 29.5 (t); 29.7 (t); 30.2 (t); 40.4 (t); 47.1 (t); 54.4 (q); 55.48 (q); 55.53 (t); 55.6 (q); 56.4 (d); 56.5 (d); 56.6 (d); 56.7 (t); 63.1 (d); 63.6 (d); 83.2 (s); 103.5 (d); 104.8 (d); 105.9 (d); 109.8 (s); 111.1 (s); 112.2 (d); 115.4 (d); 116.6 (d); 119.7 (s); 120.3 (d); 122.7 (s); 123.7 (d); 124.4 (s); 127.5 (d); 127.6 (d); 127.7 (s); 128.6 (s); 129.2 (s); 129.8 (d); 129.9 (d); 130.0 (s); 132.9 (s); 133.0 (s); 133.4 (s); 135.0 (d); 135.2 (d); 143.3 (s); 145.0 (s); 146.2 (s); 146.3 (s); 146.9 (s); 150.8 (s); 151.2 (s); 153.4 (s); 155.2 (s); 155.9 (s); 156.0 (s); 156.1 (s); 157.4 (s); 170.4 (s); 171.9 (s); 173.1 (s); 174.7 (s); 175.3 (s). MS (ESI) 884 ([M-Boc+3]/3, 24), 1326 ([M-Boc+2]/2, 77).

**Cell Lines and Culture.** Human-derived established cell lines used in this study were purchased from ATCC (American Type Culture Collection): A-549, human lung carcinoma (ATCC no. CCL-185), BJ, Skin Fibroblast (ATCC no. CRL-2522), HT-29, human colorectal adenocarcinoma (ATCC no. HTB-38), and MDA-MB 231, human breast adenocarcinoma (ATCC no. HTB-26). All cell lines were maintained in DMEM supplemented with 10% FBS and 100 units/mL penicillin and streptomycin at 37 °C and 5% CO\textsubscript{2}.
**GI_{50} Analysis.** Triplicate cultures were incubated for 72 h in the presence or absence of test compounds Lam-D, 1, 2, 4, 15 (at ten concentrations, typically ranging from 0.0026 to 10 µg/mL).

A colorimetric assay using SRB was adapted for quantitative measurement of cell growth and viability, following a previously described method (23). Cells were plated in 96-well microtiter plates at a density of 5 \times 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 compounds at the concentrations indicated. Treated cells were incubated for an additional 72 h, and then evaluated 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 measured at 490 nm. Cell survival is expressed as percentage of control cell growth.

Dose−response curves were obtained by using the NCI algorithm (24): 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} (the concentration that causes 50% cell growth inhibition, compared to control cultures).

**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 replaced by fresh DMEM medium containing either Lam-D, 1 or 4 at 1 µM. Absence of compound was used as a negative control. Cells were then incubated for 12 h at 37 °C.

**Topo I-GFP Visualization.** Procedure for a single microwell dish transfection: A-549, MDA-MB-231 and HT-29 cells were seeded onto a MatTek (Ashland, USA) glass bottom microwell dish
at 30 \(10^3\) cells/cm\(^2\). Culture medium was discarded after 24 h, cells were washed 3 \(\times\) PBS, and 1400 
\(\mu\)L of reduced serum media Opti-MEM I (Invitrogen, U.S.A) were added. The preparation of DNA 
for transfection required previous dilution of 3 \(\mu\)L FuGENE (Roche Molecular Biochemicals, 
Indianapolis, IN) in 100 \(\mu\)L of reduced serum media Opti-MEM I (Invitrogen, U.S.A), and further 
addition of 6.7 \(\mu\)L of the plasmid solution encoding green fluorescent protein GFP with full length 
Topo I (25). The mixture was mixed thoroughly and incubated for 30 min at r.t., before addition to 
the dish. Samples were incubated at 37 °C and 5% CO\(_2\) for 10 h. Afterwards medium was 
discarded, cells were washed three times with PBS, and new DMEM solution containing 1 \(\mu\)M 
Lam-D, 1 or 4 was added. Absence of compound was used as a negative control. The cells were 
incubated for an additional 12 h at 37 °C, and then analyzed by confocal microscopy.

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

**Uptake Measurements by Fluorescence-Activated Cell Sorting 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\(\mu\)M Lam-D, 1, 2 or 4 at a 
Absence of compound was used as a negative control. The cells were then incubated for 12 h at 37 
°C. After incubation, the cells were washed three times with PBS, detached with trypsin-EDTA 
0.25% and centrifuged at 1000 \(\times\) rpm. Finally, the medium was decanted, and the cellular pellet was 
resuspended in PBS and kept at 0 °C until measurements were performed. Fluorescence analysis 
was performed with a MoFlo cytometer (DakoCytomation, Colorado, USA), using the 351 nm 
excitation line of an 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 by dividing the fluorescence value of the test compound by the fluorescence value obtained with the Lam-D control under the same experimental conditions.

RESULTS
Orthogonal protecting groups had to be used for the three phenol groups of Lam-D and for the functional groups presented into the conjugation building blocks. N-Boc was used to protect the amino and guanidino groups of the oligomeric building blocks 7 and 8. Synthesis of compounds 2-4 required the preparation of different building blocks: the protected Lam-D derivatives 5 and 6, the DTPA-PEG dendrimer 7 and the peptide 8 (Figure 2). Compounds 5 and 6 are the precursors for the conjugates at positions 3 and 4’, respectively, and contain two phenolic groups protected as either TBDPS ethers or Boc esters, respectively. In the protected peptide 8 a glycine was introduced as spacer at the C-terminal position to avoid the steric hindrance caused by the C-terminal Val residue during coupling.

Synthesis of Protected Lamellarins 5 and 6:
The protecting groups OiPr/OBn used in earlier strategies (9) required harsh deprotection conditions incompatible with the synthesis of more complex Lam-D conjugates such as 4.² The previously described lamellarin 9 (26, 27), for which three different and orthogonal protecting groups were employed (MOM, Bn and TBDPS), was used as the precursor to Lam derivatives 5 and 6 (Scheme 1). Lam 9 was prepared following Banwell’s strategy for the total synthesis of Lam-K (28). Oxidation of 9 under MW irradiation using DDQ in CHCl₃ gave 10 with good yield. Compound 10 was subjected to changes of protecting groups (see Scheme 1). Catalytic hydrogenation of 10 over Pd-C in methanolic EtOAc gave 10a, which was successfully converted into 10b by TBDPS protection of the phenol. Removal of the MOM protecting group at position 3 of 10b with trimethylsilyl iodide in CHCl₂ gave 5 in excellent yield. Moreover, compound 10a was subjected to
the latter conditions, which afforded the 4’-OTBDPS mono-protected Lam-D \textit{10c} in quantitative yield. The free phenol groups of \textit{10c} were protected as tert-butyl carbonates using Boc\textsubscript{2}O, DMAP and CH\textsubscript{2}Cl\textsubscript{2} to give \textit{10d} in quantitative yield. Finally, OTBDPS deprotection of \textit{10d} with TBAF in MeOH gave the 4’-OH Lam-D intermediate \textit{6} (85%).

As a side note, compounds \textit{10a} and \textit{10c} are privileged synthetic intermediates for the construction of additional mono-, and di-conjugates at positions 11-OH, and 3,11-OHs of Lam-D, respectively.

**Synthesis of poly(ethylene glycol)-based dendrimer 7**

PEG-based dendrimer \textit{7} was synthesized by coupling 1-(tertbutyloxy carbonylamino)-4,7,10-trioxa-13-tridecanamine to monobenzyl-protected diethylenetriamine pentaacetic acid with PyBOP and DIPEA. The tetracarboxylic moiety derivative was prepared from an orthogonally protected DTPA derivative (one benzyl and four tert-butyl protecting groups). The \textit{iBu} groups were eliminated using 4.0 M HCl in dioxane, and then the benzyl group was eliminated by hydrogenolysis with Pd/C to render dendrimer \textit{7} (29).

**Synthesis of the peptide NLS 8**

The protected peptide \textit{8} was synthesized on chlorotrityl resin following standard Fmoc/iBu solid-phase chemistry, with 20% piperidine-DMF for the deprotection steps, and DIPCDI and HOBT as coupling reagents.

\textit{N-\alpha-Fmoc-N-\omega,N-\omega'-bis-tert-butoxycarbonyl-L-arginine} was used for the synthesis of the NLS peptide sequence. Attempts to use Fmoc-\textit{N-\omega-Pbf-L-arginine}, resulted in harsher deprotection conditions and complex reaction crudes. The last amino acid used was Boc-L-Pro-OH (as the desired building block had to be completely protected). The peptide was cleaved from the resin using 3% TFA in CH\textsubscript{2}Cl\textsubscript{2}, and after solvent evaporation, it was lyophilized.

**Esterification and Synthesis of Conjugates 1-4**

To test the efficacy of the protecting scheme, the synthesis of bioconjugate \textit{1} was repeated using the following strategy (21). An ester bond between \textit{5} and BocNH-\text{CH\textsubscript{2}(CH\textsubscript{2}OCH\textsubscript{2})\textsubscript{6}CH\textsubscript{2}COOH} was formed using EDC·HCl with a catalytic amount of DMAP to afford compound \textit{11} (Figure 1) in
quantitative yield. Compound 11 was considered a good model for the deprotection assays, because it contains the critical protecting groups and the ester functional group. Hence, it was used for the optimization of procedures and to test the success of the deprotection steps. Initial assays of sequential TBAF-TFA, two-step deprotection led to complex crude products. Furthermore, purification on SiO$_2$ gave low yields of 1. The best results were obtained via simultaneous deprotection of both groups, using liquid HF at low temperature. Highly pure final product was obtained from the reaction crude by precipitation with MTBE. Notwithstanding, the scope of the reaction was limited to small amounts of starting material.$^4$ Compound 1 was synthesized in 84% overall yield from its precursor 5. This is a major improvement over previous OiPr/OBn strategies (45% yield, from 4’,11-diisopropyl-Lam-D) ($^9$).

Formation of an ester bond between 5 and 7 ($^29$) afforded compound 12 (Fig. 1) in 26% yield, using EDC·HCl with a catalytic amount of PS solid supported DMAP in CH$_2$Cl$_2$. Deprotection of compound 12 using HF provided 2 in 36% yield.

Ester bond formation between 8 and protected lamellarin 5 or 6 using EDC·HCl was unsuccessful. The inaccessibility of the carboxylic acid in the N-Boc protected oligopeptide sequence 8, or steric hindrance of the free phenolic group in Lam-D building blocks 5 and 6, may have been decisive to the lack of reaction. Various attempts at ester bond formation between 8 and the scaffold 5 were also unsuccessful.$^5$ Therefore, taking advantage of the relatively easy amide bond formation (i.e. compared to ester bonds), an N-Boc-Gly-OH spacer was introduced at position 3 of 5 (affording 13 in 89% yield, Fig. 2) for subsequent amide bond formation with 8. N-Boc deprotection of 13 with 40% TFA followed by reaction with 8 in EDC·HCl, HOBt, and DIPEA as base, gave 14 (Fig. 1, 58% yield). Deprotection of 14 with HF under standard conditions afforded the NLS peptidic conjugate 3 in 38% yield.

The NLS conjugate at position 4’ of Lam-D could not be formed using the same conditions employed for ester bond formation between 6 and 8.$^{6,7}$ Instead, pre-activation of 8 with TCFH ($^30$) and NEt$_3$, followed by the addition of a solution of 6 and DMAP were required, affording 15 (Fig. 1, 55% yield).
1) in 45% yield (relative to 40% transformation of 6). Elimination of the nine Boc protecting groups with 40% TFA in CH₂Cl₂ gave compound 4 in 17% yield.

The ester bond of compound 3, which has a double Gly spacer, is more susceptible to nucleophilic attack by nucleophiles from the medium than that of compound 4, which has a single Gly spacer. Thus, the final conjugate 3 (derived from 14) was water labile. The rapid degradation of 3 made biological tests with this compound impossible.⁸

**Cytotoxicity and Cellular Uptake.**

The cytotoxicity of Lam-D and its analogs (1, 2, 4 and 15) was evaluated against BJ human skin fibroblasts, and a panel of three human tumor cell lines: A-549, HT-29, and MDA-MB-231 (Table 1). A conventional colorimetric assay was used to estimate values of GI₅₀ (defined here as the drug concentration that causes 50% of cell growth inhibition after 72 h of continuous exposure to the test molecule). Lam-D was included for comparison. The results are shown in Table 1. The tested compounds in the tumor cell lines exhibited cytotoxicities from 4 µM to 40.7 nM, except for the Boc protected derivative 15, which only had micromolar activity for the HT-29 and MDA-MB-231 cell cultures. 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, conjugate 1 citotoxicity was similar to that of Lam-D, or even 2.4-4.9-fold less for 2 and 4.

FACS flow cytometry was used to measure cellular uptake quantification (9). The results are shown in Table 2. Interestingly, the cellular internalization quotient for the PEGylated derivatives 1 and 2 were higher than that of Lam-D in all cancer cell lines. Indeed, compound 4, with an NLS sequence, was 10 times more active than Lam-D in A-549 and MDA-MB-231 cell lines and retained CIQ, despite having the highly charged NLS peptide. CIQ of conjugates in BJ cellular culture were from 76.8 to 128.6%.

**Cellular Distribution of Lam-D, 1 and 4. Tracking in GFP-Topo I Transfected Cell Cultures.**
Lam-D is a Topo I inhibitor. To determine whether Lam-D, 1 and 4 localize to the same subcellular compartment as Topo I, a cellular localization assay was performed. A functional chimera of the green fluorescent protein EGFP with full length Topo I (GFP-Topo I) was expressed in HT-29, A-549 and MDA-MD-231 cells, which were then treated with either Lam-D, 1 or 4. As described, GFP-Topo I was located in the nucleus (25) in all cell types (Fig.3, positive control). Conjugate 4, carrying the NLS signal, was localized to the nucleus in HT-29 and A-549 cells (Fig. 3, j1, j2, k1 and k2), suggesting that its higher activity could correlate to subcellular co-localization with its target, Topo I.

Interestingly, Lam-D and 1 showed both higher activity and greater nuclear localization in MDA-MB-231 cells than 4 did (Fig. 3, f1, f2, i1 and i2)

DISCUSSION
We have described the synthesis of Lam-D conjugates with well-defined, water-soluble peptidic and dendritic systems as potential nontoxic drug delivery vehicles. Interestingly, the Lam-D conjugate containing a single backbone attached to a phenolic residue of Lam-D has very different solubility compared to Lam-D alone. Scaffold 10, with three orthogonal protecting groups, has proven to be a good starting material, enabling synthesis of 5 and 6, via protection group interchange, on a multigram scale and in good overall yield. New protected derivatives of Lam-D, which can be conjugated to one (C\textsuperscript{11}-OH, 10a) or two (C\textsuperscript{3}-OH and C\textsuperscript{11}-OH, 10b) phenol groups, have been isolated in good yields with this strategy. Compound 1 was synthesized in 84% overall yield from its precursor 5; this constitutes an important improvement over previous strategies (45% yield, from 4’,11-diisopropyl-Lam-D) (9). Simultaneous removal of TBDPSO and NBoc with HF is a new and highly efficient deprotection scheme for compounds with labile ester bonds. The OBoc and TBDPSO protecting groups used in lamellarins 5 and 6 permitted optimization of the final deprotection conditions and represented adequate choices for total deprotection in the last step.
Thus, single step HF deprotection was the cleanest method for preparing Lam-D 1-3 (9-84% overall yields from 5).

The new Lam-D conjugates reported here are excellent candidates for further biological evaluation. The evaluation of conjugates 3, 4 in BJ skin fibroblasts as normal cells was used in the present study. In this cellular culture, no significative variation, or even less citotoxicity was obtained for conjugates 3, 4 than the parent compound Lam-D. PEGylated conjugates 1 and 2 have much higher cytotoxicity to MDA-MB-231 cells than does Lam-D alone. Surprisingly, compound 4 had nanomolar GI₅₀ for MDA-MB-231 and A-549, representing 10-fold-lower GI₅₀ respectively, compared to Lam-D. These results in A-549 cancer cell line could correlate to co-localization of 4 in nuclear regions where GFP–Topo I accumulated (Fig. 3k). The NLS peptidic sequence is at least partly responsible for nuclear import of 4 in A-549 and HT-29 cell lines. Contrariwise, Lam-D and 1 were able to weakly reach the nucleus in only MDA-MB-231 cells. Altogether, we conclude that better cytotoxicity correlates with greater nuclear localization.

In summary, the use of a robust chemistry strategies, with a combination of solid-phase and solution strategies and a myriad of orthogonal and/or compatible protecting groups, has allowed the preparation of several Lam-D bioconjugates. All of them show some improved characteristics when compared with the parent lamellarin D. Particularly 4, which contains the NLS-peptide, shows a clearly improved cytotoxicity and a co-localization in the nucleus. To the best of our knowledge, 4 is one of the first examples of NLS peptide conjugation with small molecules.

Our results (9) indicate that Lam-D derivatives obtained through various chemical modifications may have markedly higher activity than the parent compound in certain tumor cell lines increasing the selectivity between the tumor cell lines.

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Footnotes:

1 Abbreviations: AU, absorbance units; A-549, lung carcinoma cell line; Bn, benzyl; Boc, tert-butoxycarbonyl; Cbz, benzoxycarbonyl; CIQ, cellular internalization quotient; DDQ, 2,3-dichloro-5,6-dicyano-p-benzoquinone; DIPCDI, N,N'-disopropylcarbodiimide; DIPEA, diisopropylethylamine; DMAP, 4-dimethylaminopyridine; DMEM, Dulbecco’s modified Eagle’s medium; DMF, dimethylformamide; DMSO, dimethylsulfoxide; DTPA, diethylenetriamine-N,N,N',N'-pentaacetic acid; EDC·HCl, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride; EPR, enhance permeability and retention; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; GFP, green fluorescent protein; HOAt, 1-hydroxy-7-azabenzotriazole; HOBt, 1-hydroxybenzotriazole; HT-29, colon carcinoma cell line; GI\textsubscript{50}, 50 percent growth inhibition; iPr, isopropyl; Lam, Lamellarin; MDA-MB-231, breast adenocarcinoma cell line, MOM, methoxymethyl; MSNT, 1-(mesitylene-2-sulfonyl)-3-nitro-1H-1,2,4-triazole; MW, microwave; NLS, nuclear location signal; NMI, N-methylimidazole; Pbf, (2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl), PBS, phosphate buffered saline; PEG, poly(ethylene glycol); PS, polystyrene; PyBOP, (benzotriazol-1-yl oxy)tripyrrolidinophosphonium hexafluorophosphate; SRB, sulforhodamine B; TBAF, tetrabutylammonium fluoride; TBDPS, tert-butyldiphenylsilyl; TBME, tert-butyl methyl ether; tBu, tert-butyl; TCFH, N,N,N',N'-tetramethylchloroformamidinium hexafluorophosphate; TFFH, N,N,N',N'-tetramethylfluoroformamidinium hexafluorophosphate; TFA, trifluoroacetic acid; THF, tetrahydrofuran; Topo, Topoisomerase.

2 Conjugate 4 protected with OiPr on positions 3 and 11, and Boc-Lys, Boc-Pro and Pbf-Arg gave only mixtures on deprotection assays.

3 The ester bonds of the protected Lam-D conjugates were labile; hence, to avoid problems with hydrolysis, we minimized the deprotection steps after condensation.

4 The yield for the deprotection was 84% working on a 20-30 mg scale. However, the procedure could not be scaled up. The stability 1 was studied in DMEM supplemented with 10% FBS and 100 units/mL penicillin and streptomycin at 37 °C. HPLC analysis indicates 97% of Lam-D liberation after 360 min of incubation.

5 Esterification of 5 was tested with EDC·HCl, TCFH or N,N,N',N'-tetramethylchloroformamidyl chloride as activating agents.

6 N-Cbz-Gly-OH was anchored to 6 in quantitative yield. However, further Cbz deprotection could not be performed without concomitant hydrolysis of the conjugate ester bond.

7 Other coupling reagents as EDC·HCl, DIPCDI, TFFH, PyBOP with HOAt, and MSNT with NMI failed in ester bond formation.

8 Compound 3 quickly hydrolyzes and liberates Lam-D into the medium, even on the time scale of an HPLC analysis.
Table 1. Cytotoxicity of compounds 1, 2, 4, 15 in three human cancer cell lines.

| Compound | Cytotoxicity (M) |
|----------|------------------|
|          | HT-29 Colon | A-549 Lung | MDA-MB-231 Breast | BJ Skin Fibroblast |
| Lam-D    | $3.00 \times 10^{-6}$ | $1.22 \times 10^{-7}$ | $1.34 \times 10^{-7}$ | $6.37 \times 10^{-9}$ |
| 1        | $1.68 \times 10^{-6}$ | $8.86 \times 10^{-8}$ | $4.07 \times 10^{-8}$ | $6.51 \times 10^{-9}$ |
| 2        | $3.92 \times 10^{-6}$ | $2.20 \times 10^{-7}$ | $8.31 \times 10^{-8}$ | $1.54 \times 10^{-9}$ |
| 4        | $1.01 \times 10^{-5}$ | $4.79 \times 10^{-8}$ | $4.79 \times 10^{-8}$ | $3.14 \times 10^{-8}$ |
| 15       | $1.94 \times 10^{-6}$ | n.d. | $1.24 \times 10^{-6}$ | --- |

Table 2. Cellular internalization as measured by FACS.

| Compound | Cellular Internalization (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    | 82.7 | 328.5 | 443.8 | 259.4 | 100%$^a$ | 100%$^a$ | 100%$^a$ | 100%$^a$ |
| 1        | 88.0 | 393.0 | 527.5 | 333.4 | 106% | 120% | 119% | 128.6% |
| 2        | 83.7 | 374.9 | 455.3 | 199.2 | 101% | 114% | 103% | 76.8% |
| 4        | 88.5 | 377.7 | 434.9 | 240.6 | 107% | 115% | 98% | 92.8% |

$^a$ CIQ was calculated in reference to the cellular uptake of Lam-D.
Reagents and conditions: i: DDQ, CHCl₃, MW, 120 ºC, 10 min (81%); ii: H₂, Pd-C, EtOAc, MeOH, r.t., 16 h (95%); iii: TBDPSCl, Im, DMAP, DMF, r.t., 24 h (58%); iv: Me₃SiI, CH₂Cl₂ r.t., 20 min (84% for 5; quant. yield for 10c); v: Boc₂O, DMAP, CH₂Cl₂ r.t., 16 h (quant.); vi: 1M TBAF in THF, MeOH, -78 ºC, 15 min (85%).
Figure 1.

Structures of Lam-D conjugates
Building blocks for the synthesis
**Figure 3.** Internalization of Lam-D and conjugates 1 and 4 by Topo I-GFP transfected cells.

|          | HT-29 Colon | A-549 Lung | MDA-MB-231 Breast |
|----------|-------------|------------|-------------------|
| GFP channel | Transmission // GFP-UV Overlay | GFP channel | Transmission // GFP-UV Overlay | GFP channel | Transmission // GFP-UV Overlay |
| Positive Control | a1 | a2 | b1 | b2 | c1 | c2 |
| Lam-D | d1 | d2 | e1 | e2 | f1 | f2 |
| 1 | g1 | g2 | h1 | h2 | i1 | i2 |
| 4 | j1 | j2 | k1 | k2 | l1 | l2 |

The UV emissions corresponding to Lam-D, 1 and 4 are arbitrarily represented in red tones. The test compounds were seeded at a concentration of 1 µM and then incubated for 12 h.