Supporting Information

Novel Toll-like Receptor 2 Ligands for Targeted Pancreatic Cancer Imaging and Cancer Immunotherapy

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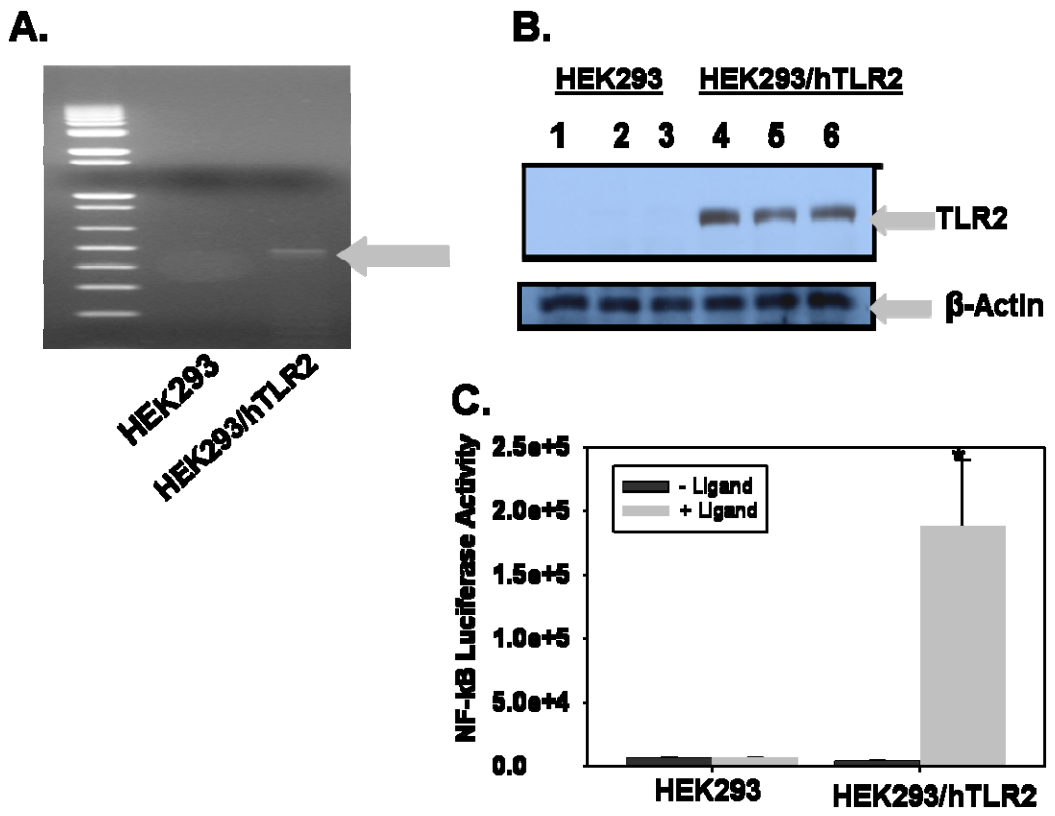
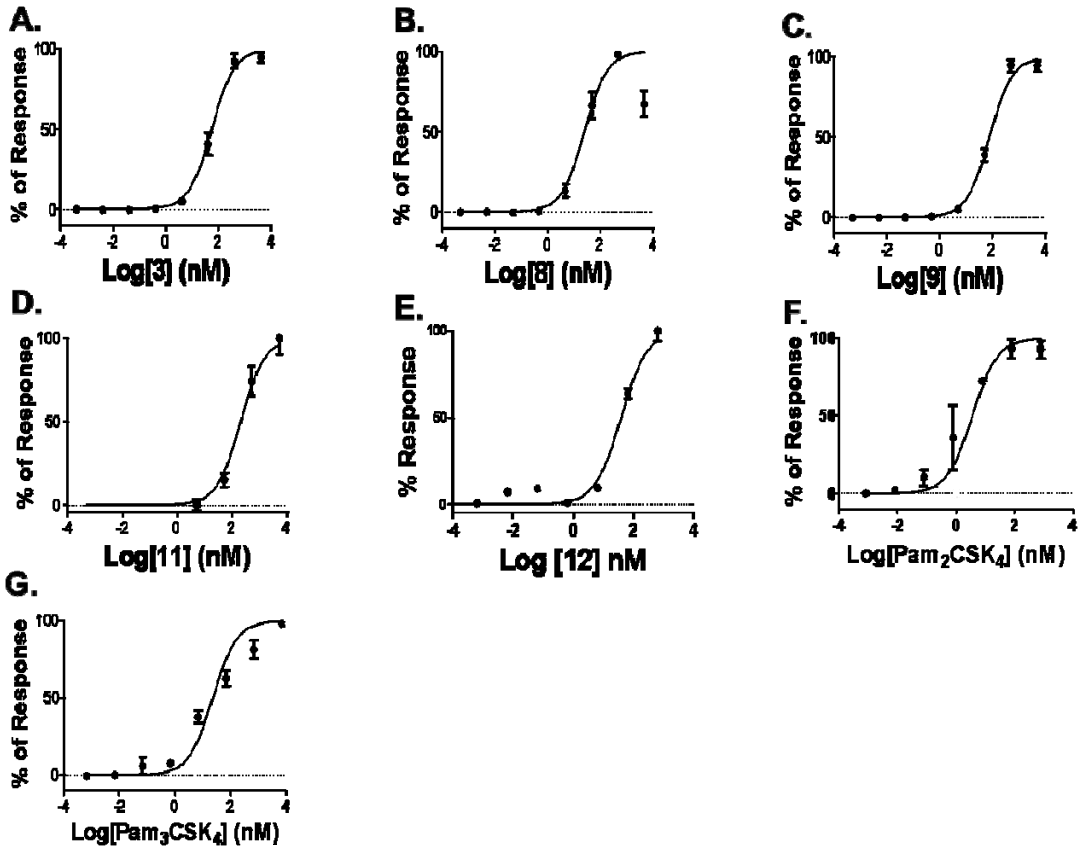
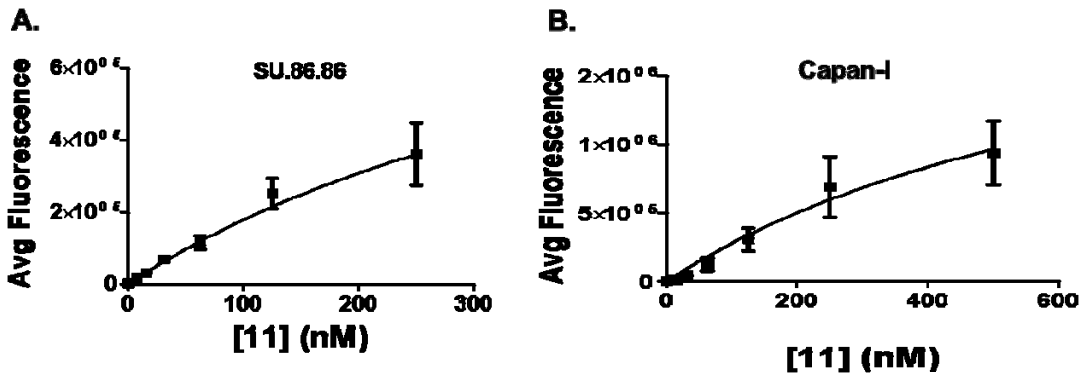


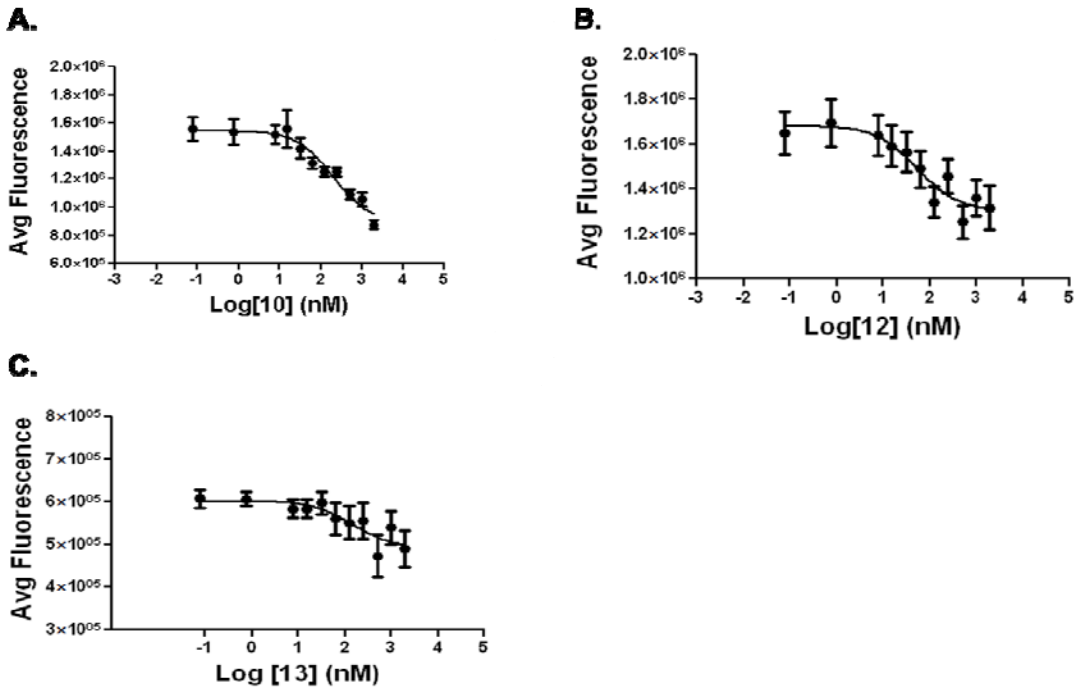
Supporting Figure 1. TLR2 functional bioassay validation and optimization. A) RT-PCR and B) western blot expression of TLR2 on the cell surface of HEK-293/hTLR2 expressing cells with no expression in the parental HEK-293 cells. Band observed at approximately 90 kDa. C) Optimal NF-κB induced expression of luciferase led to observed luminescence 48 h post transient transfection and 24 h post ligand stimulation. Luminescence intensity was significantly greater (50 fold, n = 6, $p<0.001$) in TLR2 ligand (Pam_3CSK_4) stimulated HEK293/hTLR2 cells relative to cells incubated with no ligand. No activity was observed with the HEK293 TLR2 negative expressing cells (n=3 assays with quadruplicate wells).



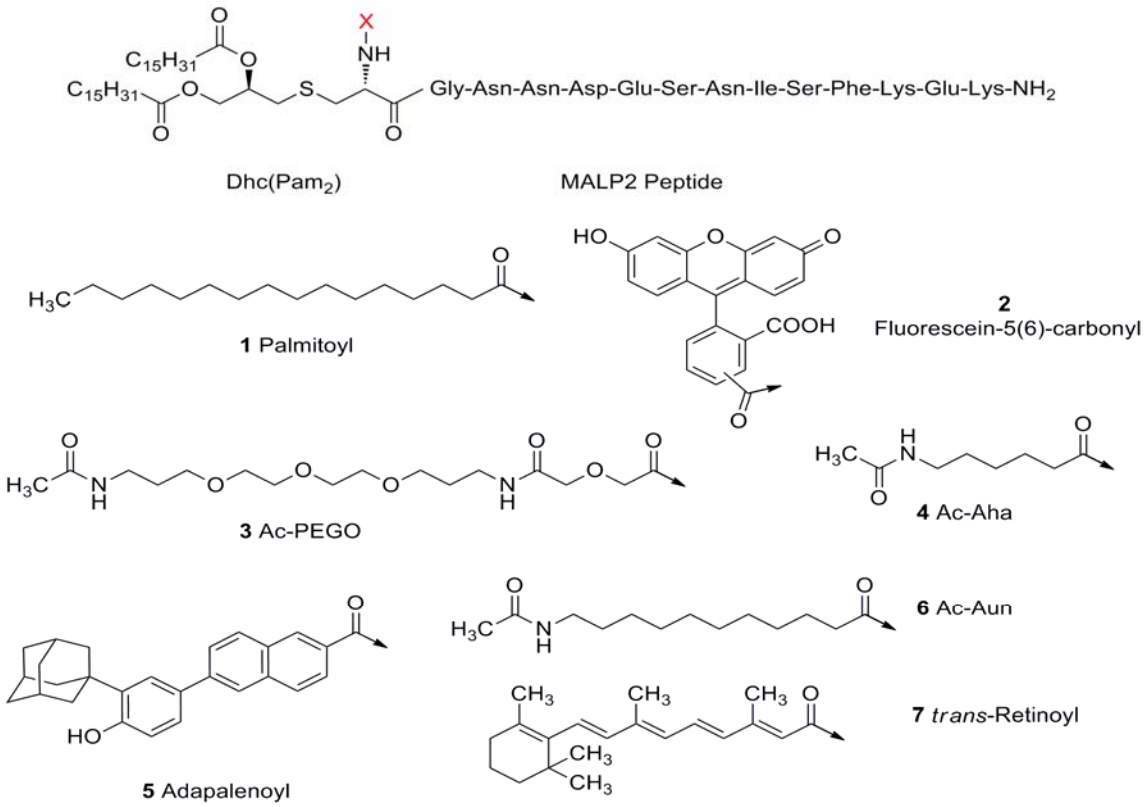
Supporting Figure 2. Dose-response curves generated by measuring the TLR2 agonistic activity of the following compounds: A) **3**, B) **8**, C) **9**, D) **11**, E) **12** and TLR2 reference controls F) Pam_2CSK_4 and G) Pam_3CSK_4. The TLR2 functional bioassay was performed by serially adding (0.001 ng/mL to 10 μg/mL) compound to HEK-293/hTLR2 expressing cells ($n \geq 3$ assays with quadruplicate wells). The EC_{50} values are reported in Table 2**.**



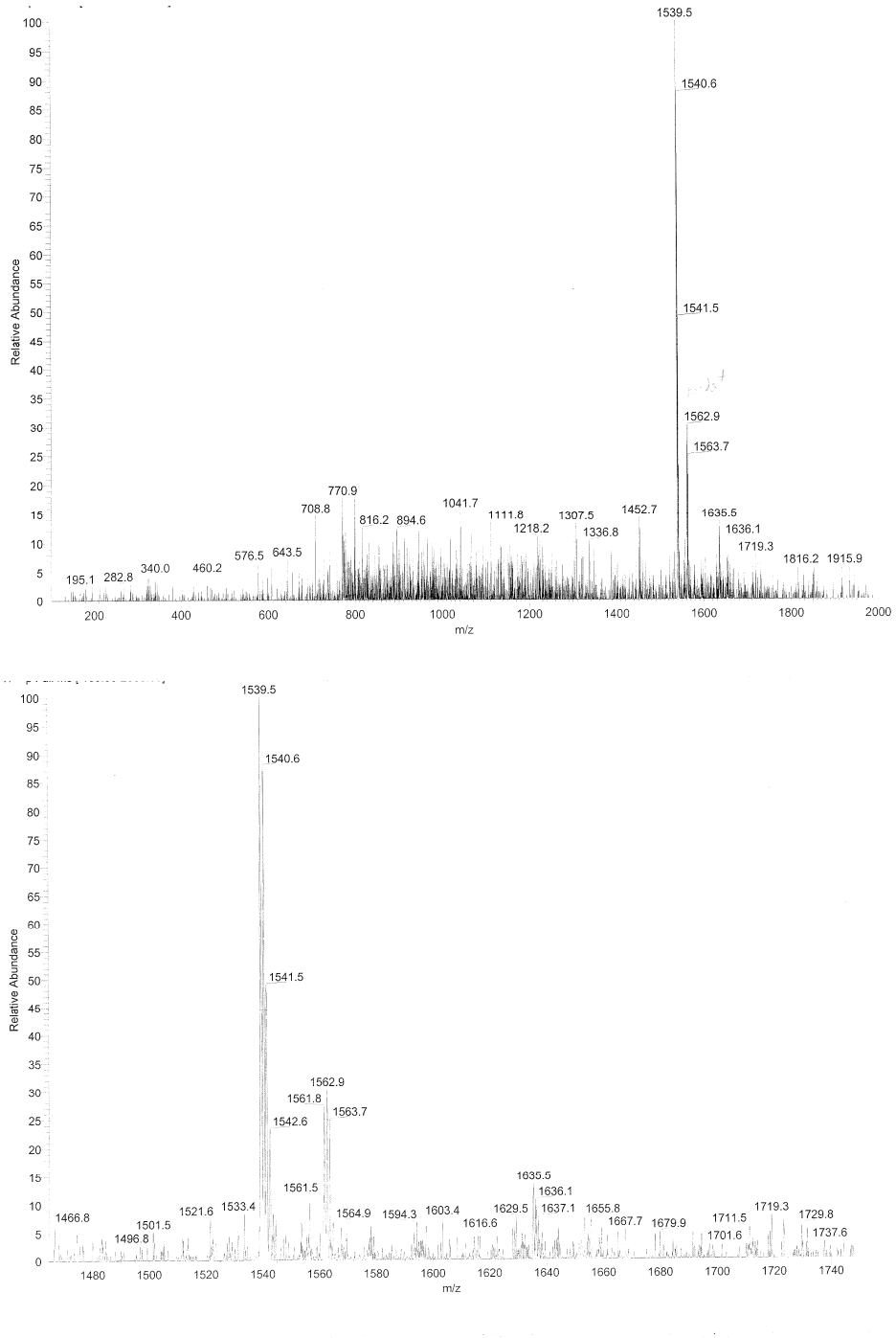
Supporting Figure 3. Saturation binding analysis of Eu-DTPA-labeled compound **11** to TLR2. The saturation binding curves show TLR2 specific binding (total–nonspecific) curves of **11** to TLR2 using the following TLR2-expressing cancer cell lines: A) SU.86.86 with a K_d of 74 nM and B_{max} of 269,878 AFU (n=3 assays, R^2 values >0.99) and B) Capan-I with a K_d of 78 nM and B_{max} of 951,170 AFU (n=3 assays, R^2 values >0.96).



Supporting Figure 4. Competition binding analysis, in which increasing concentrations of test compound were added in the presence of 90 nM compound **11** using SU.86.86 cells to determine TLR2 binding activity. A) Compound **10** had a K_i of 91 nM (n=3 assays, R^2=0.95). B) Compound **12** had a K_i of 25 nM (n=4 assays, R^2=0.90). C) Compound **13** had a K_i of 67 nM (n=4 assays, R^2=0.78).



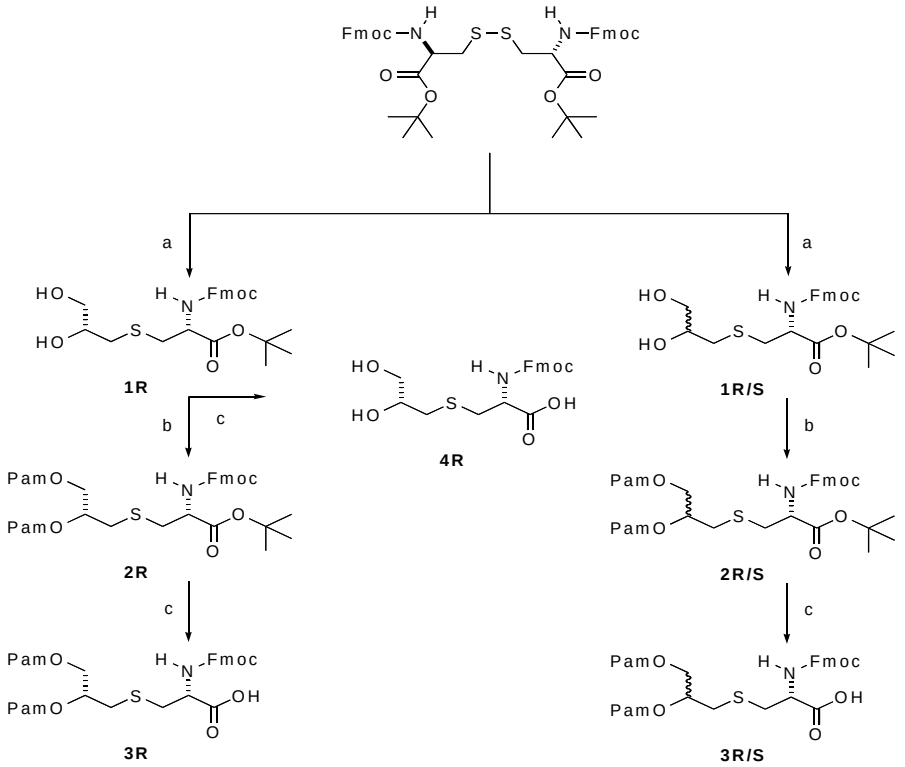
Supporting Figure 5. Structures for the X-MALP-2 peptide derived compounds **1**-**7**.



Supporting Figure 6. Representative mass spectral data for the key compounds **1-13**. The data shown here is for the key intermediate compound **12.**

Supporting Experimental Section

Synthesis of *N*-Fluorenylmethoxycarbonyl-*S*-[2,3-bis(palmitoyloxy)-(2R)-propyl]-(R)-cysteine (3R) (Fmoc-Dhc(Pam_2)-OH)



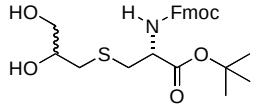
Scheme 1. (a) i) Zn, CH_2Cl_2, MeOH:HCl:H_2SO_4 (100:7:1), r.t. 15 mim. ii) Glycidol, 40 ^oC, 5 hr. (iii) $KHSO_4$, 0 ^oC to r.t. 16 hr. (b) Palmitoyl chloride, DMAP, TEA, CH_2Cl_2:DMF (100:1), 0 ^oC to r.t. 3 hr. (c) TFA, r.t. 1 hr.

General. All reactions were conducted under Ar atmosphere using oven-dried glassware. All chemicals were obtained from commercial sources and used without further purification. ^1H NMR spectra were recorded on Bruker-DRX-300 MHz instrument with chemical shifts reported relative to TMS (0.0 ppm) and residual DMSO (2.50 ppm). Proton-decoupled ^{13}C NMR spectra were referenced to $CDCl_3$ (77.0 ppm) as well as DMSO (39.51 ppm). Low resolution mass spectra were obtained on AGILENT (HP) MDS 1100 using AP-ESI. High resolution mass spectra (HRMS) were recorded on

a Bruker FT-ICR MS 9.4T instrument. Melting points were measured using a Thomas Hoover capillary melting point apparatus and are uncorrected. The starting material *N, N'*-bis-Fmoc-cystine bis-*tert*-butyl ester was prepared as described by Barney *et. al*.[1]

Synthesis of *N*-Fluorenylmethoxycarbonyl-*S*-[2,3-dihydroxy-(2RS)-propyl]-(R)-cysteine *tert*-butyl ester (1R/S)

To a solution of (Fmoc-Cys-O^{t}Bu)$_2$ (1.91 g, 2.4 mmol) in dichloromethane (15 mL) was added Zn (1.10 g, 16.8 mmol). A freshly prepared mixture (8 mL) of MeOH:32 % HCl:concentrated H_2SO_4 (100:7:1=v:v:v) was added under vigorous stirring at room temperature. After 15 m (±)-glycidol (1.78 g, 24 mmol) was added and the resulting mixture was stirred for 5 h at 40 °C. The mixture was concentrated to about half of the original volume and cooled to 0 °C, then 5 % $KHSO_4$ aqueous solution (2 mL) was added and stirred for 16 h with warming-up to room temperature slowly. The mixture was extracted with dichloromethane and the organic layer was washed with water, dried over anhydrous $MgSO_4$, filtered and concentrated. The residue was purified by silica gel column chromatography using a mixture of dichloromethane and methanol (40:1 = v:v) to give *N*-Fluorenylmethoxycarbonyl-*S*-[2,3-dihydroxy-(2RS)-propyl]-(R)-cysteine *tert*-butyl ester (**1R/S**, 2.26 g) as colorless oil in 99 % isolated yield.

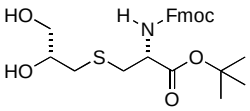


Colorless oil; ^{1}H NMR (300 MHz, $CDCl_3$), two *diastereoisomers* δ 1.50 (s, 9H), 2.04-2.14 (m, 1H), 2.59-3.20 (m, 5H), 3.48-3.59 (m, 1H), 3.65-3.85 (m, 2H), 4.24 (t, J = 6.98 Hz, 1H), 4.35-4.45 (m, 2H), 4.46-4.56 (m, 1H), 5.72-5.84 (m, 1H), 7.32 (t, J = 7.40 Hz, 2H), 7.41 (t, J = 7.41 Hz, 2H), 7.61 (d, J = 7.29 Hz, 2H), 7.77 (d, J = 7.37 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) two *diastereoisomers* δ 28.0, (35.7, 35.8), (36.6, 36.7), 47.0, 54.4, (65.0, 65.2), (67.16, 67.19), (70.3, 70.5), 83.2,

120.0, 125.1, 127.0, 127.7, 141.2, 143.66, 143.72, (156.0, 156.07), (169. 62, 169.65);

LRMS (ESI) *m/z* Calcd for $C_{25}H_{31}NO_6SNa$ $(M+Na)^+$ 496.2, obsd 496.0.

Synthesis of *N*-Fluorenylmethoxycarbonyl-*S*-[2,3-dihydroxy-(2R)-propyl]-(R)-cysteine *tert*-butyl ester (1R)[1-2]

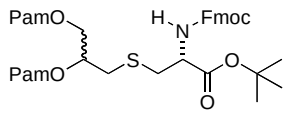


The title compound was synthesized according to the procedure described for compound (**1R/S**) starting from (Fmoc-Cys-O^{t}Bu)$_2$ (5.74 g, 7.2 mmol) and R-(+)-glycidol (5.33 g, 72 mmol). *N*-Fluorenylmethoxycarbonyl-*S*-[2,3-dihydroxy-(2R)-propyl]-(R)-cysteine *tert*-butyl ester (**1R**, 5.70 g) was obtained as a viscous oil in 84 % isolated yield.

Colorless oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.49 (s, 9H), 2.39 (t, *J* = 5.94 Hz, 1H), 2.63 (dd, *J* = 13.7, 8.40 Hz, 1H), 2.75-2.82 (m, 1H), 2.93 (dd, *J* = 14.0, 6.10 Hz, 1H), 3.03 (dd, *J* = 14.0, 4.60 Hz, 1H), 3.33 (d, *J* = 3.63 Hz, 1H), 3.48-3.55 (m, 1H), 3.64-3.72 (m, 1H), 3.75-3.84 (m, 1H), 4.23 (t, *J* = 7.07 Hz, 1H), 4.39 (d, *J* = 7.07 Hz, 2H), 4.49-4.55 (m, 1H), 5.87 (d, *J* = 7.97 Hz, 1H), 7.31 (t, *J* = 7.40 Hz, 2H), 7.41 (t, *J* = 7.36 Hz, 2H), 7.61 (d, *J* = 7.29 Hz, 2H), 7.77 (d, *J* = 7.44 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 28.0, 35.8, 36.8, 47.0, 54.4, 65.2, 67.2, 70.5, 83.2, 120.0, 125.1, 127.1, 127.7, 141.3, 143.66, 143.75, 156.0, 169.7; LRMS (ESI) *m/z* Calcd for $C_{25}H_{31}NO_6SNa$ $(M+Na)^+$ 496.2, obsd 496.0.

Synthesis of *N*-Fluorenylmethoxycarbonyl-*S*-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-(R)-cysteine *tert*-butyl ester (2R/S)[1-2]

To a mixture of compound **1R/S** (1.56 g, 3.28 mmol) and palmitoyl chloride (2.71 g, 9.85 mmol) in a mixture (35 mL) of CH_2Cl_2 and DMF (100:1=v:v) was added triethylamine (997 mg, 9.85 mmol), followed by DMAP (80.3 mg, 0.657 mmol) at 0 °C. The resulting mixture was stirred at 0 °C for 30 m and at room temperature for 3 h. The reaction was quenched by an addition of saturated NaCl aqueous solution and extracted with dichloromethane. The organic layer was washed with water, dried over anhydrous $MgSO_4$, filtered and concentrated. The residue was purified by silica gel column chromatography using a mixture of hexanes and ethyl acetate (9:1 = v:v) to give *N*-Fluorenylmethoxycarbonyl-*S*-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-(R)-cysteine *tert*-butyl ester (**2R/S**, 2.60 g) as white solid in 83 % isolated yield.

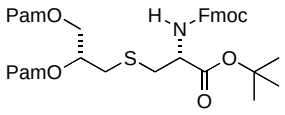


White solid, mp = 52-54 °C (lit.[2] 38-42 °C); ^{1}H NMR (300 MHz, $CDCl_3$), two *diastereoisomers* δ 0.879 (t, *J* = 6.88 Hz, 6H), 1.25 (br, 48H), 1.49 (s, 9H), 1.55-1.64 (br, 4H), 2.26-2.33 (m, 4H), 2.77 (d, *J* = 6.39 Hz, 2H), 3.00-3.13 (m, 2H), 4.12-4.18 (m, 1H), 4.22-4.40 (m, 4H), 4.48-4.55 (m, 1H), 5.10-5.20 (m, 1H), 5.69 (t, *J* = 6.34 Hz, 1H), 7.29-7.34 (m, 2H), 7.40 (t, *J* = 7.39 Hz, 2H), 7.62 (d, *J* = 7.47 Hz, 2H), 7.77 (d, *J* = 7.47 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) two *diastereoisomers* δ 14.1, 22.7, 24.85, 24.87, 28.0, 29.1, 29.3, 29.4, 29.5, 29.66, 29.70, 31.9, (33.17, 33.24), 34.1, 34.2, (35.33, 35.38), 47.1, (54.26, 54.29), (63.40, 63.43), 67.2, (70.19, 70.26), 83.0, 120.0, 125.2, 127.1, 127.7, 141.3, 143.8, (155.66, 155.69), 169.5,

(173.0, 173.04), 173.3; LRMS (ESI) *m/z* Calcd for $C_{57}H_{91}NO_8SNa$ $(M+Na)^+$ 972.6, obsd 972.5.

Synthesis of *N*-Fluorenylmethoxycarbonyl-*S*-[2,3-bis(palmitoyloxy)-(2R)-propyl]-(R)-cysteine *tert*-butyl ester (2R)[1-2]

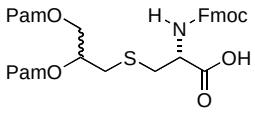
The title compound was synthesized according to the same procedure described for compound (**2R/S**) starting from compound **1R** (3.62 g, 7.64 mmol). *N*-Fluorenylmethoxycarbonyl-*S*-[2,3-bis(palmitoyloxy)-(2R)-propyl]-(R)-cysteine *tert*-butyl ester (**2R**, 5.12 g) was obtained as white solid in 71 % isolated yield.



White solid, mp = 56-57 °C (lit.[2] 45 °C); ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.878 (t, *J* = 6.93 Hz, 6H), 1.25 (br, 48H), 1.49 (s, 9H), 1.55-1.63 (br, 4H), 2.26-2.33 (m, 4H), 2.77 (d, *J* = 6.40 Hz, 2H), 3.02 (dd, *J* = 13.8, 5.24 Hz, 1H), 3.09 (dd, *J* = 13.8, 4.64 Hz, 1H), 4.15 (dd, *J* = 11.9, 5.90 Hz, 1H), 4.21-4.40 (m, 4H), 4.49-4.54 (m, 1H), 5.14-5.18 (m, 1H), 5.71 (d *J* = 7.60 Hz, 1H), 7.32 (t, *J* = 7.39 Hz, 2H), 7.40 (t, *J* = 7.31 Hz, 2H), 7.62 (d, *J* = 7.44 Hz, 2H), 7.77 (d, *J* = 7.44 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.1, 22.7, 24.81, 24.83, 27.9, 29.1, 29.2, 29.3, 29.4, 29.62, 29.66, 31.9, 33.2, 34.0, 34.2, 35.3, 47.0, 54.3, 63.4, 67.1, 70.2, 82.9, 119.9, 125.08, 125.13, 127.0, 127.6, 141.2, 143.7, 155.7, 169.4, 173.0, 173.3; LRMS (ESI) *m/z* Calcd for $C_{57}H_{91}NO_8SNa$ $(M+Na)^+$ 972.6, obsd 972.8.

Synthesis of *N*-Fluorenylmethoxycarbonyl-*S*-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-(R)-cysteine (3R/S)[1-2]

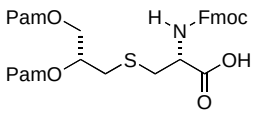
Compound **2R/S** (1.0 g, 1.05 mmol) was dissolved in trifluoroacetic acid (20 mL) and the resulting mixture was stirred at room temperature for 1 h. The reaction mixture was concentrated under reduced pressure and the resulting oily residue was coevaporated from toluene (30 mL) and dichloromethane (30 mL). The residue was lyophilized from *tert*-butyl alcohol to give *N*-Fluorenylmethoxycarbonyl-*S*-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-(R)-cysteine (**3R/S**, 750 mg) as white solid in 84 % isolated yield.



White solid, mp = 82-84 °C (lit.[2] 64-65 °C); ^{1}H NMR (300 MHz, $CDCl_3$), two *diastereoisomers* δ 0.878 (t, *J* = 6.88 Hz, 6H), 1.25 (br, 48H), 1.55-1.65 (br, 4H), 2.27-2.38 (m, 4H), 2.70-2.80 (m, 2H), 3.06-3.21 (m, 2H), 4.10-4.41 (m, 5H), 4.61-4.72 (m, 1H), 5.11-5.22 (m, 1H), 5.78 (d, *J* = 7.81 H, 1H), 7.31 (td, *J* = 7.44, 1.10 Hz, 2H), 7.40 (t, *J* = 7.34 Hz, 2H), 7.61 (d, *J* = 7.29 Hz, 2H), 7.76 (d, *J* = 7.44 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) two *diastereoisomers* δ 14.1, 22.7, 24.84, 24.87, 29.0, 29.09, 29.11, 29.24, 29.29, 29.36, 29.44, 29.50, 29.66, 29.70, 31.9 (32.79, 32.92), 34.1, 34.3, (34.59, 34.74), 47.0, (53.48, 53.61), (63.51 63.56), 67.4, 70.2, 120.0, 125.1, 127.1, 127.7, 141.3, 143.64, 143.68, (155.89, 155.95), (173.41, 173.46), (173.55, 173.62), (174.20, 174.30) LRMS (ESI) *m/z* Calcd for $C_{53}H_{84}NO_8S$ $(M+H)^+$ 894.6, obsd 894.5.

Synthesis of *N*-Fluorenylmethoxycarbonyl-*S*-[2,3-bis(palmitoyloxy)-(2R)-propyl]-(R)-cysteine (3R)

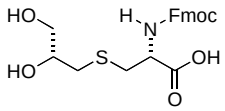
The title compound was synthesized according to the same procedure described for compound (**3R/S**) starting from compound **2R** (1.50 g, 1.58 mmol) and trifluoroacetic acid (30 mL). *N*-Fluorenylmethoxycarbonyl-*S*-[2,3-bis(palmitoyloxy)-(2R)-propyl]-(R)-cysteine (**3R**, 1.34 g) was obtained as white solid in 95 % isolated yield.



White solid, mp = 84-85 °C (lit.[2] 61 °C); ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.877 (t, *J* = 6.91 Hz, 6H), 1.25 (br, 48H), 1.55-1.65 (br, 4H), 2.27-2.34 (m, 4H), 2.77 (d, *J* = 6.33 Hz, 2H), 3.06 (dd, *J* = 13.7, 6.13 Hz, 1H), 3.17 (dd, *J* = 14.0, 4.61 Hz, 1H), 4.13-4.19 (m, 1H), 4.21-4.41 (m, 4H), 4.64-4.70 (m, 1H), 5.16-5.19 (m, 1H), 5.79 (d, *J* = 7.86 H, 1H), 7.33 (td, *J* = 7.45, 0.95 Hz, 2H), 7.40 (t, *J* = 7.30 Hz, 2H), 7.61 (d, *J* = 7.34 Hz, 2H), 7.76 (d, *J* = 7.45 Hz, 2H), 10.0 (brs, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.1, 22.7, 24.81, 24.85, 29.07, 29.09, 29.26, 29.34, 29.5, 29.64, 29.68, 31.9, 32.9, 34.0, 34.2, 34.6, 47.0, 53.6, 63.5, 67.4, 70.2, 120.0, 125.1, 127.0, 127.7, 141.2, 143.61, 143.65, 156.0, 173.45, 173.55, 174.6; HRMS (ESI) *m/z* Calcd for $C_{53}H_{84}NO_8S$ $(M+H)^+$ 894.5912, obsd 894.5900.

Synthesis of *N*-Fluorenylmethoxycarbonyl-*S*-[2,3-dihydroxy-(2R)-propyl]-(R)-cysteine (4R)

Compound **1R** (3.15 g, 6.65 mmol) was dissolved in trifluoroacetic acid (70 mL) and the resulting mixture was stirred at room temperature for 1 h. The reaction mixture was concentrated under reduced pressure and the resulting oily residue was coevaporated from toluene (50 mL) and dichloromethane (50 mL). The residue was purified by silica gel column chromatography using a mixture of dichloromethane and methanol (9:1 = v:v) to give *N*-Fluorenylmethoxycarbonyl-*S*-[2,3-dihydroxy-(2R)-propyl]-(R)-cysteine (**4R**, 1.80 g) as white solid in 65 % isolated yield.



White solid, mp = 54-56 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.49 (dd, *J* = 13.2, 7.05 Hz, 1H), 2.69 (dd, *J* = 13.5, 4.63 Hz, 1H), 2.78 (dd, *J* = 13.5, 9.69 Hz, 1H), 2.98 (dd, *J* = 13.6, 4.54 Hz, 1H), 3.29-3.39 (m, 2H), 3.54-3.61 (m, 1H), 4.10-4.17 (m, 1H), 4.21-4.30 (m. 3H), 4.50-5.20 (br, 2H), 7.33 (t, *J* = 7.32 Hz, 2H), 7.42 (t, *J* = 7.31 Hz, 2H), 7.73-7.78 (m, 3H), 7.89 (d, *J* = 7.44 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 34.9, 35.5, 46.9, 53.8, 64.9, 67.3, 71.1, 120.0, 125.1, 127.1, 127.7, 141.2, 143.47, 143.63, 156.5, 173.7; HRMS (ESI) *m/z* Calcd for $C_{21}H_{23}NO_6SNa$ $(M+Na)^+$ 440.1138, obsd 440.1133.

REFERENCES

1. Barany, G.; Han, Y.; Hargittai, B.; Liu, R. Q.; Varkey, J. T., Side-chain anchoring strategy for solid-phase synthesis of peptide acids with C-terminal cysteine. *Biopolymers* **2003,** *71* (6), 652-666.