ARTICLE

Peptide nanovaccine conjugated via retro-Diels-Alder reaction linker for overcoming the obstacle in lymph node penetration and eliciting robust cellular immunity

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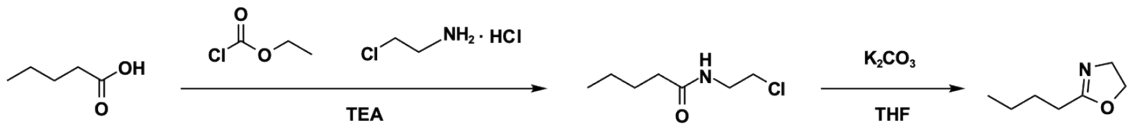
Supplemental methods

Synthesis of 2-butyl-2-oxazoline

Compound **2-butyl-2-oxazoline** was synthesized by a two-step reaction[1] as shown in scheme S1.



Scheme S1. Synthesis of 2-butyl-2-oxazoline.

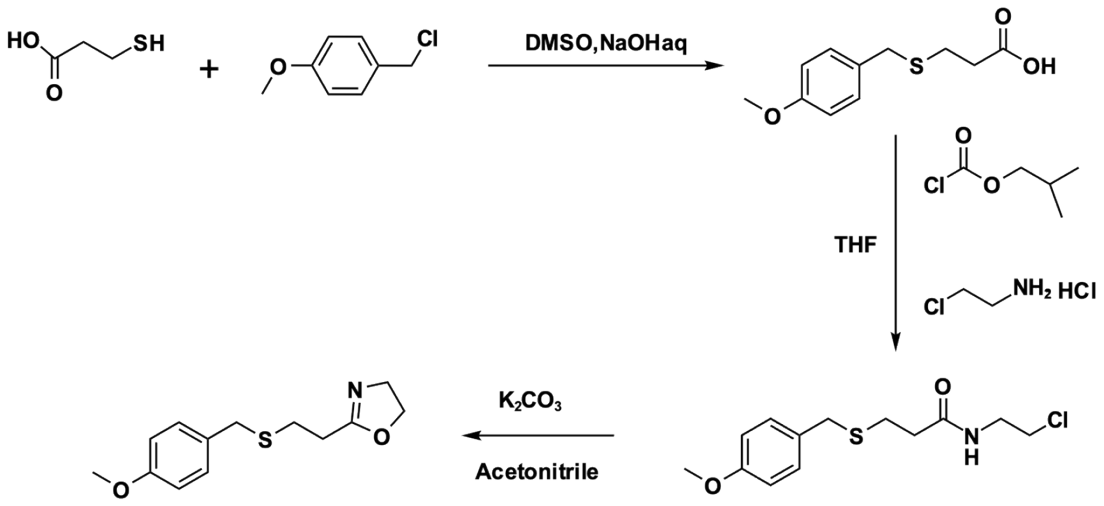
A solution comprising valeric acid (20.0 g, 195.8 mmol) and triethylamine (43.6 g, 430.9 mmol) dissolved in tetrahydrofuran (THF) (300.0 mL) was chilled to 0 °C. Ethyl chloroformate (21.25 g, 195.8 mmol) was then added dropwise to the solution, and the reaction mixture was gradually warmed to room temperature, where it was stirred for 1 hour. Subsequently, the mixture was cooled again to 0 °C, and 2-chloroethylamine hydrochloride (27.25 g, 234.9 mmol) was introduced and stirred for an additional 12 hours. The solvent was removed via rotary evaporation, and the resulting residue was dissolved in dichloromethane and subjected to triple washing with water. The organic layer was dried over sodium sulfate, and the solvent was subsequently evaporated. The product was dissolved in 160 mL of dry THF, followed by the addition of potassium carbonate (13.9 g, 100.9 mmol) under a nitrogen atmosphere. The reaction mixture was stirred at 70 °C for 48 hours. Afterward, the salt was removed by filtration, and the solvent was evaporated. The resulting 2-butyl-2-oxazoline product was purified by vacuum distillation to yield a colorless liquid (15.3 g, yield: 61.3%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 4.16 (t, 2H), 3.87-3.60 (m, 2H), 2.29-2.10 (m, 2H), 1.55 (dq, 2H), 1.43-1.11 (m, 2H), 0.86 (t, 3H).

Synthesis of 2-[2-(4-Methoxybenzylsulfanyl)ethyl]-2-oxazoline (MOB-SOx)

Compound **MOB-SOx** was synthesized by a three-step reaction[2] as shown in scheme S2.

3-(4-Methoxybenzylsulfanyl)propionic acid: A solution containing 6.0 g of 3-mercaptopropionic acid (56.5 mmol) and 4.5 g of NaOH (113.0 mmol) was prepared by dissolving them in 80 mL of water and 80 mL of dimethyl sulfoxide (DMSO). To this solution, 4.4 g of 4-methoxybenzyl chloride (28.3 mmol) was added dropwise at room temperature. After 2 hours, the resulting slurry was filtered, and the precipitate was washed with 30 mL of cold chloroform ($CHCl_3$) before being dissolved in 150 mL of 3 N hydrochloric acid (HCl). The mixture was then extracted with dichloromethane (CH_2Cl_2), and the organic extracts were washed with brine before being dried over sodium sulfate (Na_2SO_4). Upon solvent removal under reduced pressure, 2.2 g of colorless solid was obtained, corresponding to a yield of 35%. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.23 (d, 2H), 6.96-6.70 (m, 2H), 3.80 (s, 3H), 3.70 (s, 2H), 2.75-2.52 (m, 4H).

N-(2-Chloroethyl)-3-(4-methoxybenzylsulfanyl)propionamide: In this procedure, 2.0 g (8.8 mmol) of **3-(4-Methoxybenzylsulfanyl)propionic acid** and 1.8 g (18.0 mmol) of triethylamine were dissolved in 50 mL of anhydrous THF.



Scheme S2. Synthesis of MOB-SOx.

Subsequently, 1.2 g (8.8 mmol) of iso-butyl chloroformate was added under a nitrogen atmosphere at 0 °C, and the mixture was stirred for 10 minutes at this temperature, followed by an additional 15 minutes at room temperature. After cooling the mixture to 0 °C once more, 1.2 g (10.6 mmol) of 2-chloroethylamine hydrochloride was added, and the resulting mixture was stirred for 1 hour at room temperature. Upon completion of the reaction, the solvent was evaporated, leaving behind a residue that was dissolved in dichloromethane (CH_2Cl_2) and filtered. The product was then precipitated by the addition of diethyl ether (100 mL). The final product, **N-(2-Chloroethyl)-3-(4-methoxybenzylsulfanyl)propionamide**, was obtained as a colorless solid with a yield of 1.7 g (67%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.26-7.18 (m, 2H), 6.91-6.80 (m, 2H), 5.98 (s, 1H), 3.79 (d, 3H), 3.70 (d, 2H), 3.60 (dt, 4H), 2.72 (td, 1.8 Hz, 2H), 2.38 (td, 2H).

MOB-SOx: In this process, 1.45 g (5.0 mmol) of **N-(2-Chloroethyl)-3-(4-methoxybenzylsulfanyl)propionamide** was dissolved in 20 mL of dry acetonitrile, followed by the addition of 1.40 g (10.1 mmol) of dry potassium carbonate (K_2CO_3). The resulting solution was stirred for 12 hours at 70 °C under a nitrogen atmosphere. After completion of the reaction, the solvent was removed under reduced pressure, leaving behind solid residue, which was dissolved in 20 mL of dichloromethane (CH_2Cl_2). The solution was then filtered successively to remove the excess K_2CO_3. After evaporation of the solvent, the crude product was purified by flash chromatography. This purification process yielded 0.9 g of MOB-SOx as a colorless oil (yield: 71%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.27-7.17 (m, 2H), 6.91-6.74 (m, 2H), 4.21 (t, 2H), 3.89-3.72 (m, 5H), 3.68 (s, 2H), 2.68 (td, 2H), 2.50 (ddd, 2H).

Synthesis of poly{(2-methyl-2-oxazoline)$_{80}$-*co*-[(2-butyl-2-oxazoline)$_{15}$-*r*-(MOB-SOx)$_8$]} (PMBSOx)

The polymerizations were conducted in sealed vessels capable of withstanding elevated pressures, following the methodology outlined in a prior study[1], as depicted in Fig. 1a.

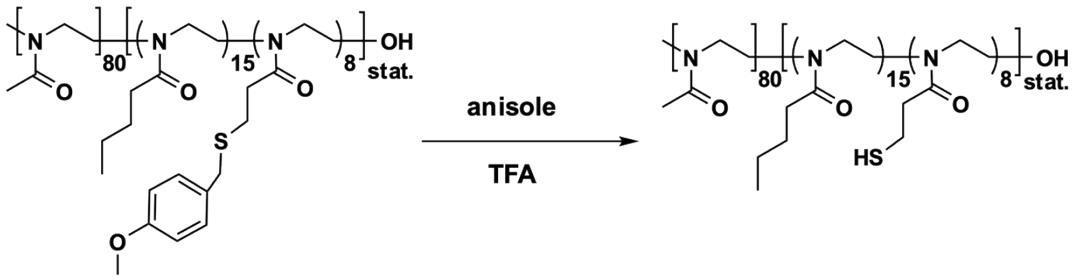
A solution comprising methyl trifluoromethylsulfonate (16.4 mg, 0.1 mmol, MeOTf) dissolved in dry acetonitrile (10.0 mL) was prepared under an inert atmosphere of dry argon. At room temperature, 2-methyl-2-oxazoline (680.4 mg, 8.0 mmol) was introduced into the solution and stirred at 70 °C for 24 hours in the presence of argon. Subsequently, upon cooling the mixture to room temperature, the second block monomers, namely 2-butyl-2-oxazoline (254.2 mg, 2 mmol) and MOB-SOx (376.7 mg, 1.5 mmol), were added. The resulting mixture was stirred at 100 °C for an additional 48 hours. The reaction was quenched by the addition of methanolic NaOH (1 mol/L) upon cooling to room temperature. The product was purified by dialysis against distilled water for 2 days and then recovered through lyophilization. The product yield was 940 mg, corresponding to a yield of 70.5%. ^{1}H-NMR spectrum is shown in Figure 1b.

Deprotection of PMBSOx

We deprotected the polymer according to the methods reported in the literature[2], as shown in scheme S3.
In an argon atmosphere, 500 mg of PMBSOx was dissolved in a mixture of 0.2 mL of anisole and 2.2 mL of trifluoroacetic acid and refluxed for 48 hours. The solvent was removed under vacuum, and the residue was dissolved in $CDCl_3$ and precipitated in diethyl ether, yielding a colorless solid. ^{1}H-NMR spectrum is shown in Figure 1b.

Synthesis of 5-(((2,3-bis(methoxycarbonyl)-7-oxabicyclo[2.2.1]hepta-2,5-dien-1-yl)methyl)amino)-5-oxopentanoic acid (OND Linker)

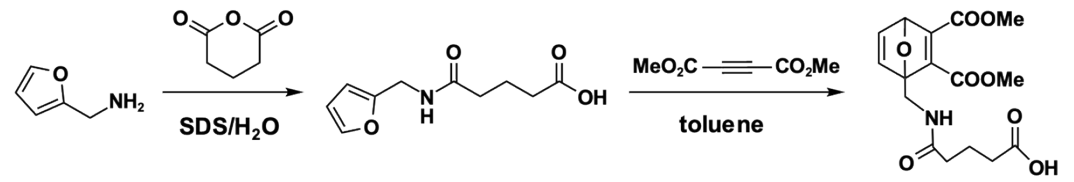
Compound **OND Linker** was synthesized by a two-step reaction[3] as shown in scheme S4.



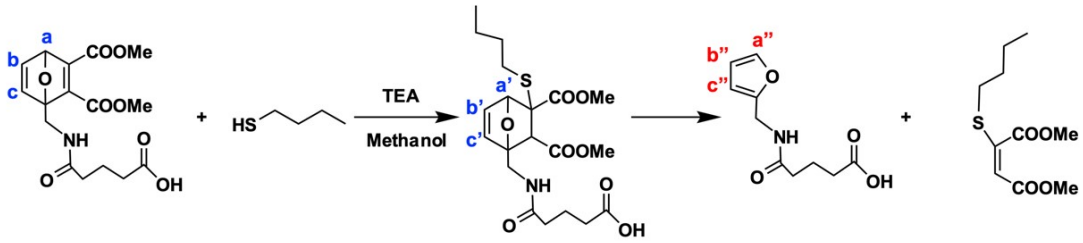
Scheme S3. Deprotection of PMBSOx.

N-glutaroylfurfurylamine: Glutaric anhydride (2.0 g, 17.7 mmol) was slowly added over 10 minutes to a solution of furfurylamine (1.5 g, 14.9 mmol) in 0.1 wt.% aqueous sodium dodecyl sulfonate (SDS) (50 mL). The resulting solution was then refrigerated at 4 °C overnight. The colorless crystals of the product were isolated by filtration, washed with cold water (50 mL), and subsequently dried under vacuum. This process afforded 1.3 g (42% yield) of **N-glutaroylfurfurylamine** as colorless crystals. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.36 (dd, 1H), 6.32 (dd, 1H), 6.27-6.18 (m, 1H), 5.86 (s, 1H), 4.44 (d, 2H), 2.44 (t, 2H), 2.31 (t, 2H), 1.99 (p, 2H).

OND Linker: **N-glutaroylfurfurylamine** (1.2 g, 5.7 mmol) was mixed with dimethyl acetylenedicarboxylate (1.2 g, 8.6 mmol) and 1 mL of toluene in a sealed vial. The mixture was heated directly on a hot plate at 80 °C with stirring for 12 hours. After completion of the reaction, the mixture was diluted with 15 mL of diethyl ether and triturated to induce precipitation. The resulting off-white precipitate was collected by vacuum filtration and dried under high vacuum until a constant mass was achieved, yielding 1.7 g (83%) of the desired product. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.22 (dd, 1H), 7.00 (d, 1H), 6.18 (t, 1H), 5.64 (d, 1H), 4.17 (dd, 1H), 4.01 (dd, 1H), 3.80 (d, 6H), 2.40 (t, 2H), 2.28 (t, 2H), 1.94 (p, 2H).



Scheme S4. Synthesis of OND Linker.



Scheme S5. Michael addition reaction of OND with n-butyl mercaptan and subsequent r-D-A reaction.

Synthesis of RhoB piperazine amide

We synthesized the compound **RhoB piperazine amide** via a two-step procedure according to the method described by Matthew B. Francis[4], as depicted in Scheme S6.

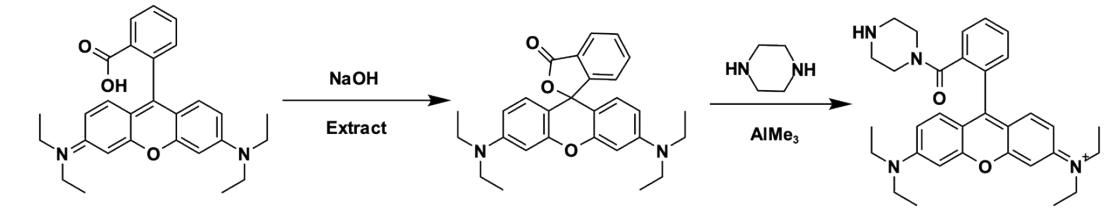
RhoB base: 4.0 g of NaOH (100.0 mmol) was dissolved in 100 mL of water, followed by the addition of 100 mL of ethyl acetate. 3.3 g of RhoB (6.9 mmol) was then added to the solution, and the mixture was stirred at room temperature for 1 hour. The resulting mixture was then transferred to a 500 mL separatory funnel for liquid-liquid extraction, collecting the organic phase. The aqueous phase was subsequently extracted with ethyl acetate. The collected organic phase was washed with 1 mol/L sodium hydroxide solution and saturated saline solution. The organic phase was dried over anhydrous sodium sulfate, filtered, and then subjected to rotary evaporation to yield a pink solid (2.7 g, yield: 90.5%). ^{1}H-NMR (300 MHz, CD_3OD) δ: 8.09 (dd, 1H), 7.64 (pd, 2H), 7.35-7.19 (m, 3H), 7.08-6.85 (m, 4H), 3.65 (q, 8H), 1.29 (t, 12H).

RhoB piperazine amide: In a round-bottom flask, 7.8 g of piperazine (91.0 mmol) and 35 mL of dichloromethane were added. Then, at room temperature, 22.6 mL of a 2 mol/L solution of trimethylaluminum in toluene (45.2 mmol) was added dropwise to the above solution. After stirring at room temperature for 1 hour, 10.0 g of **RhoB base** (22.6 mmol) was dissolved in 20 mL of dichloromethane and then added dropwise to the above solution. After stirring at 40 °C for 24 hours, 0.1 mol/L hydrochloric acid was added dropwise to the reaction mixture until no more gas was evolved. The mixture was filtered, and the residue was washed

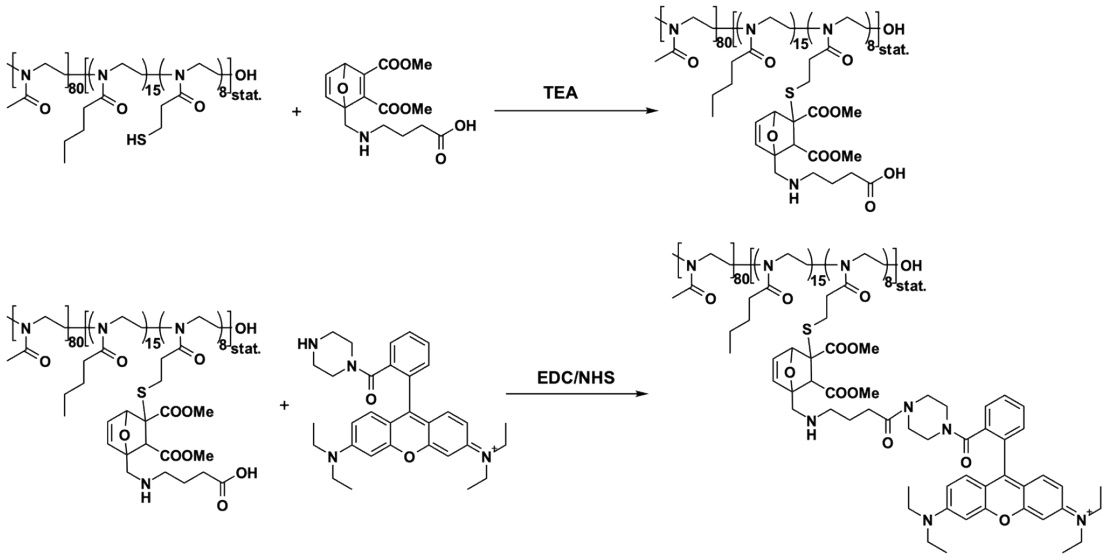
with dichloromethane. The filtrate was collected and subjected to rotary evaporation. The residue was dissolved in

dichloromethane, filtered, and the filtrate was rotary evaporated. To the residual solid, 100 mL of ethyl acetate and 100 mL of

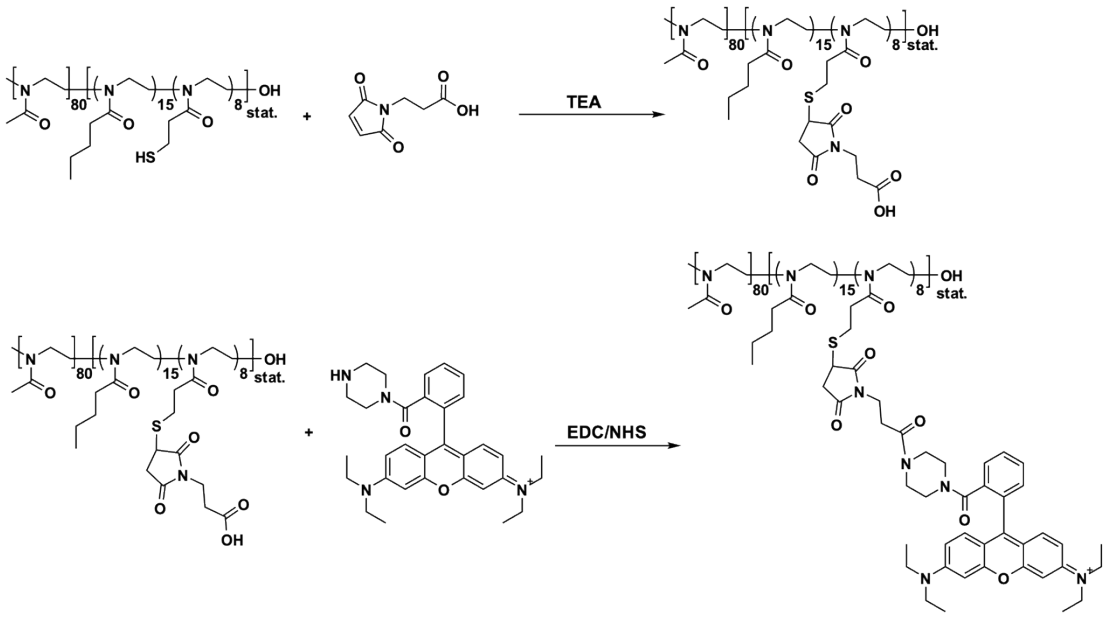
water were added, followed by the addition of 1.5 g of sodium bicarbonate (17.9 mmol), and stirred at room temperature for 1 hour. The solution was then transferred to a 500 mL separatory funnel to remove the organic phase. The aqueous phase was washed with ethyl acetate, and sodium chloride was added to the water phase to saturation. 1 mol/L hydrochloric acid was added dropwise to the water phase until no more bubbles were produced. The mixture was extracted several times with isopropanol/dichloromethane (2:1), and the organic phase was collected and dried over anhydrous sodium sulfate. After filtration, rotary evaporation was carried out, and the solid was dissolved in methanol. Addition of a large amount of ice-cold diethyl ether led to the precipitation of a deep purple solid upon filtration (8.1 g, yield: 65.0%). ^{1}H-NMR (300 MHz, CD_3OD) δ: 7.79 (d, 3H), 7.61-



Scheme S6. Synthesis routes of RhoB piperazine amide.

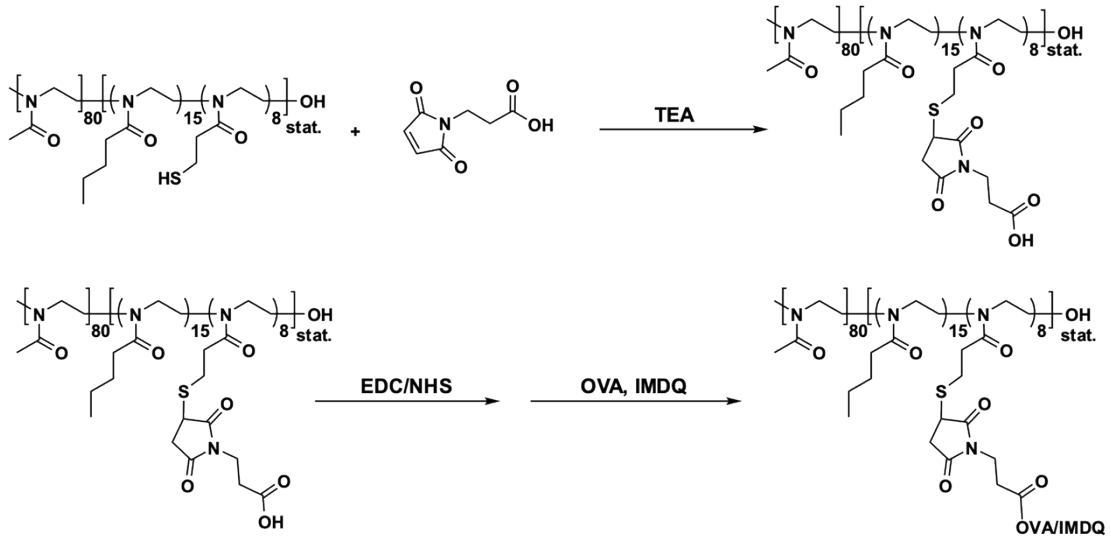


Scheme S7. Synthesis routes of PMBOxS-OND-Rhodamine.



Scheme S8. Synthesis routes of PMBOxS-Mal-Rhodamine.

7.44 (m, 1H), 7.27 (d, 2H), 7.11 (d, 2H), 6.99 (d, 2H), 3.87-3.58 (m, 12H), 3.14 (s, 4H), 1.42-1.26 (m, 12H).



Scheme S9. Synthesis routes of PMBOxS-Mal-OVA/IMDQ.

Supplemental figures

Notes and references

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4. T. Nguyen and M. B. Francis, *Organic Letters*, 2003, **5**, 3245-3248.