

## Lipid tucaresol as an adjuvant for methamphetamine vaccine development

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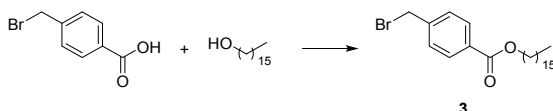
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### Experimental

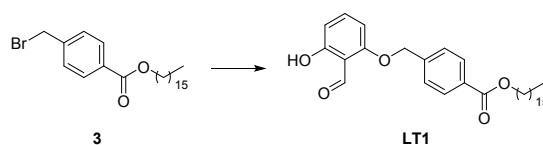
Reactions were performed under an inert atmosphere at rt using flame-dried glassware with dry solvents. Reagents were used as commercially supplied. RP-HPLC was performed using a Grace Vydac C18 column, 10-15  $\mu$ m, 250  $\times$  22 mm on an Agilent Technologies 1260 Infinity system using method [A = 0.1% TFA/H<sub>2</sub>O, B = 0.1% TFA/MeCN;  $\lambda$  = 254 and 210 nm; gradient 1% B (5 min), 1-15% B (15 min), 15-75% B (35 min), 75-95% B (10 min), 95% B (5 min)]. TLC was performed on glass-backed plates pre-coated with silica (EMD 60 F<sub>254</sub>, 0.25-1 mm) and developed using standard visualising agents: UV fluorescence (254 nm) and KMnO<sub>4</sub>, cerium ammonium nitrate or ninhydrin with appropriate heating. <sup>1</sup>H and <sup>13</sup>C-NMR were performed on Bruker spectrometers, with the reference from the residual solvent peak for <sup>1</sup>H-NMR (7.26 ppm for CDCl<sub>3</sub>, 3.31 ppm for CD<sub>3</sub>OD), and the solvent peak for <sup>13</sup>C-NMR (77.1 ppm for CDCl<sub>3</sub>, 49.0 ppm for CD<sub>3</sub>OD), coupling constants (*J* values) are given in Hz. HRMS (ESI) were performed on an Agilent 1100 Series LC/MSD-TOF.

### Hexadecyl 4-(bromomethyl)benzoate, **3**



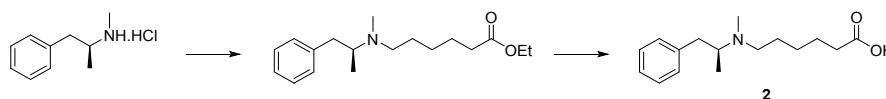
DIC (43  $\mu$ L, 0.275 mmol) and DMAP (3 mg, 0.0250 mmol) were added to a solution of 4-(bromomethyl)benzoic acid (54 mg, 0.250 mmol) and 1-hexadecanol (67 mg, 0.275 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (2.5 mL) at rt and the solution stirred (18 h). The resulting suspension was filtered and the solvent removed *in vacuo*. Purification by preparative TLC using EtOAc/hexane (1:4) gave ester **3** as a white solid (65 mg, 59%), *R*<sub>f</sub> 0.31 (1:9 EtOAc/hexane);  $\delta_{\text{H}}$  (CDCl<sub>3</sub>, 400 MHz) 8.03-7.99 (2H, m), 7.47-7.43 (2H, m), 4.50 (2H, s), 4.31 (2H, t, *J* 6.7), 1.80-1.71 (2H, m), 1.47-1.39 (2H, m), 1.38-1.21 (24H, m), 0.90-0.86 (3H, m);  $\delta_{\text{C}}$  (CDCl<sub>3</sub>, 101 MHz) 166.2, 142.6, 130.6, 130.2, 129.1, 65.4, 32.4, 32.1, 29.8, 29.8, 29.8, 29.8, 29.8, 29.7, 29.7, 29.5, 29.4, 28.8, 26.2, 22.8, 14.3. HRMS (ESI): *m/z* calc'd for C<sub>24</sub>H<sub>40</sub>BrO<sub>2</sub> [MH<sup>+</sup>] 439.2206, found 439.2193.

### Hexadecyl 4-((2-formyl-3-hydroxyphenoxy)methyl)benzoate, lipid tucaresol, LT1



Cs<sub>2</sub>CO<sub>3</sub> (28 mg, 0.0869 mmol) was added to a solution of 2,6-dihydroxybenzaldehyde<sup>1, 2</sup> (10 mg, 0.0724 mmol) in DMF (750  $\mu$ L), and the solution stirred at rt (15 min), before being added dropwise to a solution of bromide **3** (32 mg, 0.0724 mmol) in DMF (500  $\mu$ L) at 0 °C. The solution was allowed to slowly warm to rt and stirred (2 h) whereupon CH<sub>2</sub>Cl<sub>2</sub> (1.5 mL) was added. After further stirring at rt (16 h), the solution was filtered and the solvent removed *in vacuo*. Purification by preparative TLC using EtOAc/hexane (3:17) gave lipid tucaresol, **LT1** as a white solid (24 mg, 67%), *R*<sub>f</sub> 0.41 (3:17 EtOAc/hexane);  $\delta_{\text{H}}$  (CDCl<sub>3</sub>, 400 MHz) 11.97 (1H, s), 10.43 (1H, s), 8.08 (2H, d, *J* 8.1), 7.48 (2H, d, *J* 8.0), 7.39 (1H, t, *J* 8.4), 6.56 (1H, d, *J* 8.4), 6.41 (1H, d, *J* 8.2), 5.20 (2H, s), 4.32 (2H, t, *J* 6.7), 1.80-1.74 (2H, m), 1.47-1.40 (2H, m), 1.38-1.23 (24H, m), 0.87 (3H, t, *J* 6.9);  $\delta_{\text{C}}$  (CDCl<sub>3</sub>, 151 MHz) 194.2, 166.3, 163.9, 161.3, 140.8, 138.5, 130.7, 130.1, 127.1, 111.1, 110.6, 102.2, 70.1, 65.5, 32.1, 29.8, 29.8, 29.8, 29.8, 29.8, 29.7, 29.7, 29.4, 28.8, 26.2, 22.8, 14.3. HRMS (ESI): *m/z* calc'd for C<sub>31</sub>H<sub>45</sub>O<sub>5</sub> [MH<sup>+</sup>] 497.3261, found 497.3267.

### (S)-6-(Methyl(1-phenylpropan-2-yl)amino)hexanoic acid, **2**



Ethyl 6-((methylsulfonyl)oxy)hexanoate<sup>3</sup> (58 mg, 0.242 mmol) was added to a suspension of (+)-methamphetamine.HCl (30 mg, 0.162 mmol) and K<sub>2</sub>CO<sub>3</sub> (67 mg, 0.485 mmol) in MeCN (2 mL) and the resulting mixture was heated to 80 °C (72 h). Upon cooling, CH<sub>2</sub>Cl<sub>2</sub> (20 mL), H<sub>2</sub>O (10 mL) and brine (10 mL) were added and the aqueous layer extracted with CH<sub>2</sub>Cl<sub>2</sub> (2  $\times$  15 mL). The organic layer was dried (MgSO<sub>4</sub>), filtered and the solvent removed *in vacuo*. Partial purification by column chromatography eluting with MeOH/EtOAc/hexane (0:1:1  $\rightarrow$  3:17:0) gave the intermediate ester as a pale yellow oil.

4M aq. LiOH (200  $\mu$ L, 0.800 mmol) was added to a solution of ester in MeOH (2 mL) and the solution stirred at rt (4 h), whereupon further LiOH (200  $\mu$ L, 0.800 mmol) was added and the solution stirred at rt (4 h). Purification by preparative RP-HPLC (*R*<sub>t</sub> = 35.6 min) followed by lyophilisation gave MH6t-CO<sub>2</sub>H, **2** as a colourless oil (25 mg, 59%), [ $\alpha$ ]<sub>D</sub><sup>25</sup> +8.5 (*c* 1.0, MeOH);  $\delta_{\text{H}}$  (CD<sub>3</sub>OD, 500 MHz) 7.38-7.34 (2H, m), 7.31-7.26 (3H, m), 3.74-3.66 (1H, m), 3.30-3.07 (3H, m), 2.88 (3H, d, *J* 8.3), 2.86-2.76 (1H, m), 2.35 (2H, t, *J* 7.3), 1.88-1.71 (2H, m), 1.69 (2H, p, *J* 7.5), 1.50-1.41 (2H, m), 1.22 (3H, dd, *J* 14.9, 6.7);  $\delta_{\text{C}}$  (CD<sub>3</sub>OD, 151 MHz, mixture of rotamers) 177.2, 137.3, 130.4, 130.0, 128.4, 64.4, 63.8, 55.3, 53.9, 38.6, 37.1, 35.8, 34.4, 27.1, 25.7, 25.6, 25.4, 13.9, 12.1. HRMS (ESI): *m/z* calc'd for C<sub>16</sub>H<sub>26</sub>NO<sub>2</sub> [MH]<sup>+</sup> 264.1958, found 264.1955.

## Conjugation of haptens

A solution of MH6t-CO<sub>2</sub>H (3.0 mg, 0.0114 mmol), EDC.HCl (6.6 mg, 0.0342 mmol) and sulfo-NHS (3.7 mg, 0.0171 mmol) in 10% H<sub>2</sub>O/DMF (75 µL) was agitated (1 h, rt), whereupon the solvent was removed *in vacuo*. PBS (333 µL) was added to the activated hapten, and the above solution was split into two, adding 167 µL to each of a solution of diphtheria toxoid (DT, Statens Serum Institut, predialysed into in PBS (pH 7.2), 1.5 mg, 4.5 mg/mL, 333 µL) or BSA (Pierce Imject®, Thermo Scientific in PBS (pH 7.2), 1.5 mg, 4.5 mg/mL, 333 µL) to give a final concentration of 3 mg/mL in PBS (pH 7.2) and the solutions mixed (4 °C, 18-40 h). The protein conjugates were subsequently dialysed into PBS (pH 7.4) at 4 °C. DT conjugates were used for immunisation; BSA conjugates were used for ELISA plate coating.

## BCA assay for protein concentration determination

Protein concentrations were determined *via* BCA assay using bicinchoninic acid (Pierce® BCA Protein Assay Kit). Samples were incubated at 37 °C for 30 min before analysis on a plate reader (Molecular Devices SpectraMax 250) at 562 nm.<sup>4</sup>

## MALDI-TOF MS for determination of hapten density

MALDI-TOF MS were performed using sinapinic acid as the matrix on an Applied Biosystems Voyager-DE STR MS. Comparative analysis was performed to determine the hapten density.<sup>5</sup>

## Liposome Preparation

Required volumes of stock solutions of 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), cholesterol, 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (DMPG) and monophosphoryl lipid A (MPLA, PHAD™) (all purchased from Avanti Polar Lipids (Alabaster, AL)) or lipid tucareol **LT1** in CHCl<sub>3</sub>/MeOH were combined and the solvent removed *in vacuo* to give a lipid film. Hydration into PBS (pH 7.2) followed by four freeze-thaw-vortex cycles gave the final liposome suspensions containing DMPC (90 mM), cholesterol (75 mM), DMPG (10 mM) and either MPLA (0.454 mM) or tucareol (0.454 mM), named liposomal MPLA or liposomal tucareol, respectively. The liposomes were either prepared freshly before vaccination or stored at -20 °C, whereupon two freeze-thaw-vortex cycles were performed before vaccination.

## Immunisation

All experiments were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Scripps Institutional Animal Care and Use Committee. MH6t-DT (25 µL, 1 mg/mL) was mixed with either liposomal MPLA (25 µL) or liposomal tucareol (25 µL) and subsequently used to immunise groups of six Swiss Webster mice *via* subcutaneous injection on days 0, 14 and 35. Serum was collected *via* tail bleed on days 21 and 42, and *via* cardiac bleed on day 63.

## Immunologic Assays

### ELISA

Production of anti-methamphetamine IgG was evaluated by ELISA. Microtiter plates (#3690, Costar) were incubated with MH6t(CO<sub>2</sub>H)-BSA in PBS (5 µg/mL, 25 µL) at 37 °C and the solution evaporated overnight. The plates were MeOH-fixed before non-specific binding was blocked with 5% non-fat milk in PBS (30 min, 37 °C). Mouse sera in 1% BSA (25 µL) were serially diluted across the plate before incubation in a moist chamber (1.5 h, 37 °C). After washing with dH<sub>2</sub>O, peroxidase-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) was added before further incubation in a moist chamber (30 min, 37 °C). After subsequent washing with dH<sub>2</sub>O, the plates were developed with the TMB substrate kit (Thermo Pierce). The absorbance at 450 nm was measured on a microplate reader (SpectraMax M2<sup>e</sup> Molecular Devices). Titres were calculated using GraphPad Prism 5 from a plot of the absorbance versus log(dilution) as the dilution corresponding to 50% maximum absorbance.

### Radioimmunoassay (RIA)

Dissociation constants and antibody concentrations were determined using competitive RIA. Mouse sera were pooled and diluted into 2% BSA, giving a concentration that was determined to bind *ca.* 30% (+)-[2',6'-<sup>3</sup>H(n)] methamphetamine tracer (20,000 dpm, 39 Ci/mmol (National Institute on Drug Abuse, Bethesda, MD)). Diluted serum (60 µL) and methamphetamine tracer (60 µL) were added to the sample chamber of a 5 kDa MWCO 96-well Equilibrium Dialyzer (Harvard Apparatus), and unlabelled (+)-methamphetamine (120 µL) at varying concentrations in 1% BSA was added to the buffer chamber. After equilibration on a plate rotator (Harvard Apparatus) at rt (22 h), a sample from each chamber (60 µL) was diluted into Ecolite(+)<sup>™</sup> liquid scintillation cocktail (5 mL, MP Biomedicals) and the radioactivity measured (Beckman LS 6500 Scintillation Counter). Dissociation constants (*K<sub>d</sub>* values) and antibody concentrations were calculated according to the method of Müller.<sup>6</sup>

### References

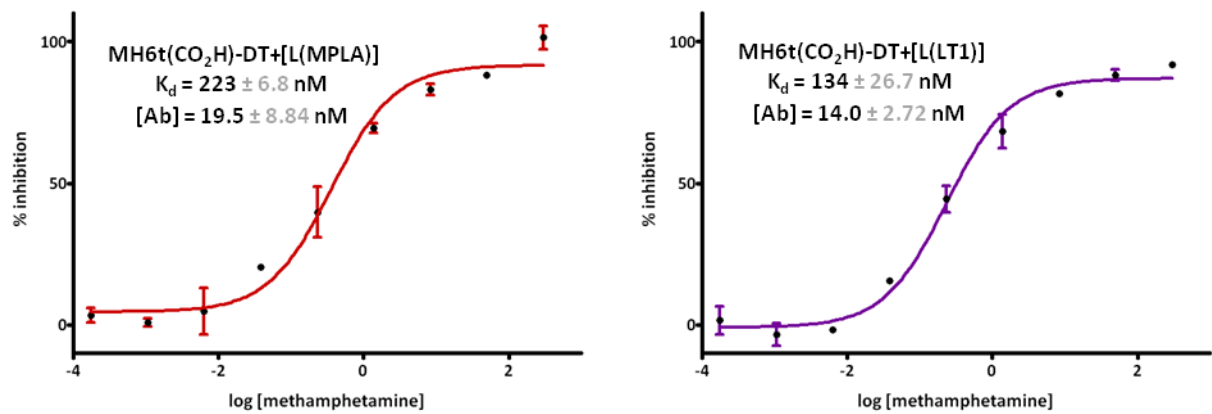
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ELISA data

Vaccine	ELISA titre (MH6t-BSA coated plates)		
	21 d	42 d	63 d
MH6t(CO <sub>2</sub> H)-DT+[L(MPLA)]	14643 ± 1944	11230 ± 2255	7325 ± 2401
MH6t(CO <sub>2</sub> H)-DT+[L(LT1)]	21666 ± 3967	15298 ± 1944	12107 ± 2271

**Table S1** Anti-methamphetamine antibody titres from MH6t(CO<sub>2</sub>H)-DT+[L(MPLA)] and MH6t(CO<sub>2</sub>H)-DT+[L(LT1)] vaccinated mice (n=6) as determined by ELISA. Data were obtained in duplicate; errors represent SEM.

RIA data



**Fig. S1** Anti-methamphetamine antibody affinities and concentrations from MH6t(CO<sub>2</sub>H)-DT+[L(MPLA)] and MH6t(CO<sub>2</sub>H)-DT+[L(LT1)] vaccinated mice (n=6) as determined by competitive RIA using pooled sera. Data were obtained in duplicate; errors represent SEM.

# NMR Spectra

