Electronic Supplementary Information

Mass spectrometry enables the discovery of inhibitors of an LPS transport assembly via disruption of protein-protein interactions

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Methods

Cloning and protein expression

The plasmids used for over-expression of the protein was obtained by inserting the gene fragment encoding the predicted mature part *Pseudomonas aeruginosa* LptH (amino acids 28-175) into a modified pET-28a expression vector (Novagen) between BamHI and XhoI cloning sites, using an In-Fusion cloning kit (Clonetech). The modified pET-28a contained an N-terminal 10x His affinity tag followed by a TEV protease cleavage site.
Plasmid was transformed in *E. coli* BL21(DE3) (New England Biolabs). Cells were grown in LB media supplemented with 50 µg/ml kanamycin at 37°C. When cultures reached optical density at 600 nm (OD600) of ~0.6, isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added at a final concentration of 0.5 mM and cultures were further grown for 16 h at 20°C.

Protein purification

Cell pellets were collected by centrifugation at 5,000g for 10 min at 4 °C and resuspended in buffer containing 250 mM NaCl, 25 mM Tris-HCl (pH 7.5), EDTA-free protease inhibitor cocktail (Roche) and 10 µg ml^{-1} DNaseI. The cell suspension was passed several times through an M-110 PS microfluidizer (Microfluidics) at 15,000 p.s.i. Insoluble material was pelleted by centrifugation at 20,000g for 20 min at 4 °C.
The supernatant was filtered before loading onto a 5 ml HisTrap-HP column (GE Healthcare, Piscataway, NJ) equilibrated in 250 mM NaCl, 25 mM Tris-HCl (pH 7.5), and 20 mM imidazole. After the clarified supernatant was loaded, the column was initially washed with 50 ml of 250 mM NaCl, 25 mM Tris-HCl (pH 7.5) and 20 mM imidazole, and washed again with 50 ml of 2250 mM NaCl, 25 mM Tris-HCl (pH 7.5) and 80 mM imidazole. The bound protein was eluted with 250 mM NaCl, 25 mM Tris-HCl (pH 7.5) and 500 mM imidazole. Peak fractions

were pooled, incubated with TEV protease and dialysed against 250 mM NaCl, 25 mM Tris-HCl (pH 7.5). The solution was then loaded again onto a 5 ml HisTrap-HP column equilibrated in 250 mM NaCl, 25 mM Tris-HCl (pH 7.5) and the flow through, containing cleaved LptH, was collected, while His-tagged TEV and the His-tagged cleaved peptide remain bound to the resin.
The protein was concentrated in an Amicon Ultra-15 concentrator unit (Millipore) with a molecular cutoff of 3 kDa to 3-4 mg/ml. Protein concentration was measured using a DS-11 FX spectrophotometer (DeNovix). Protein purity was checked by SDS-PAGE analysis using MES buffer and mass spectrometry.

Compounds preparation

Thanatin was purchased from Biomatik and diluted in H_2O to a stock concentration of 2.5 mM. Compounds **1a-1e** were purchased from Life Chemicals.
Compound **2a** was synthesised as previously described by Hopkinson et al[1]. Compounds **2b-2i** were synthesised as previously described by Rotili et al[2]. Compound **2j** was synthesised as previously described by Benedetti et al[3].
Compounds **1a-2j** were prepared as 5 mM stocks in 100% DMSO, diluted with 200 mM ammonium acetate (pH 8.0) and mixed with LptH in appropriate amounts prior to analysis. Final DMSO concentration was 0.1%, therefore we have included 0.1% DMSO in all drug binding experiments.
LogP values were calculated using ChemDraw 19.0.

Native mass spectrometry

Protein samples were buffer exchanged into 200 mM ammonium acetate (pH 8.0) using Micro Bio-Spin 6 columns (BioRad) prior to mass spectrometry analyses. These samples were directly introduced into the mass spectrometer using gold-coated capillary needles prepared in-house[4]. Data were collected on a Q Exactive UHMR Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher) in negative polarity. The instrument parameters used for MS1 spectra collection were: capillary voltage 0.9 kV, S-lens RF 100%, quadrupole selection from 1000 to 20,000 m/z range, HCD collision energy 0 V, source fragmentation 0 V, in-source trapping 0 V. The ion transfer optics was set as follows: injection flatapole -5 V, inter-flatapole lens -4 V, bent flatapole -2 V, transfer multipole 0 V. The resolution of the instrument was 8,750 at m/z = 200 (transient time of 64 ms), nitrogen pressure in the HCD cell was maintained at approximately at 3×10^{-10} mbar and source temperature was kept at 50°C.
The noise level was set at 3 rather than the default value of 4.64. Calibration of the instruments was performed using 10 mg/ml solution of caesium iodide in water. Data were analysed using the Xcalibur 3.0 (Thermo Scientific) and UniDec[5] (www.unidec.chem.ox.ac.uk) software packages.
To obtain the monomer-dimer equilibrium K_D, native mass spectra of LptH at increasing concentrations were recorded. To obtain EC_{50} and E_{max} values for compounds binding, each molecule was added in increasing amounts while keeping the protein concentration constant (8 μM total LptH concentration). Thanatin was tested at concentrations ranging between 0 and 32 μM. IMB-881 (**1a**) was tested at concentrations ranging between 0 and 40 μM. All other

compounds were tested at concentrations ranging between 0 and 200 μM. All measurements were performed in triplicate (n = 3).

Data analysis

The relative intensities of monomers and dimers were obtained by deconvoluting the nMS data using UniDec. To calculate the K_D value, the average values of monomer and dimer concentrations from three independent experiments were fitted on a monomer-dimer binding model[6] using a user-defined function in GraphPad Prism 8.0.
Calculation of EC_{50} and E_{max} values was performed through conversion of monomer and dimer concentration in mole fraction (χ):

$$\chi_M = \frac{[M]}{[M] + [D]} \; ; \; \chi_D = \frac{[M]}{[M] + [D]}$$

Where *M* refers to monomer and *D* refers to dimer.

Average values from three independent experiments were plotted as a function of molecule concentration and fitted globally using GraphPad Prism 8.0 with the equation:

$$y = B + \frac{T\text{-}B}{1 + \frac{EC_{50}}{x}}$$

where x refers to compound concentration, y is monomer/dimer mole fraction, B is the basal monomer/dimer mole fraction, T is the maximal response expressed in terms of monomer/dimer mole fraction, EC_{50} is the concentration of molecule that induces a response halfway between T and B. E_{max} is maximum achievable monomer mole fraction in the presence of each compound, therefore it coincides with T when the equation is fitted to monomer mole fractions.
Error bars in the plots indicate SD (n = 3). K_D, EC_{50} and E_{max} values are expressed as mean ± SEM.

References

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Supplementary Table

Compound	LogP	EC_{50} (μM)	E_{max} (monomer χ_{max})
Thanatin	-	1.12 ± 0.15	1.00 ± 0.02
IMB-881 (**1a**)	4.54	3.97 ± 0.76	0.73 ± 0.02
1b	4.28	50.1 ± 10.5	0.69 ± 0.03
1c	4.38	28.6 ± 5.3	0.59 ± 0.01
2b	0.71	42.4 ± 10.3	0.61 ± 0.02
2c	0.97	19.6 ± 1.6	0.75 ± 0.01
2d	1.09	19.6 ± 5.9	0.60 ± 0.02
2e	1.36	17.5 ± 4.3	0.73 ± 0.02
2f	1.13	23.9 ± 5.0	0.62 ± 0.02
2g	1.39	17.3 ± 4.7	0.65 ± 0.02
2h	1.54	31.2 ± 9.0	0.56 ± 0.02
2i	1.81	38.4 ± 8.3	0.74 ± 0.03
2j	4.03	16.2 ± 2.3	0.63 ± 0.01

Table S1. EC_{50} and Emax values for the molecules assayed in this study.

Supplementary Figures

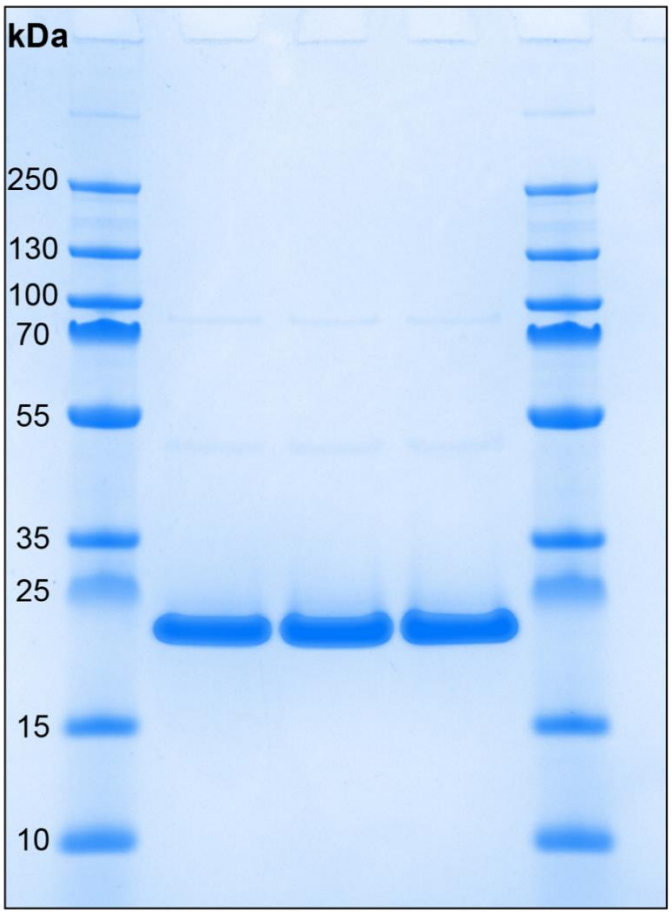

Fig. S1 SDS-PAGE gel of apo LptH following IMAC purification ran in triplicate.

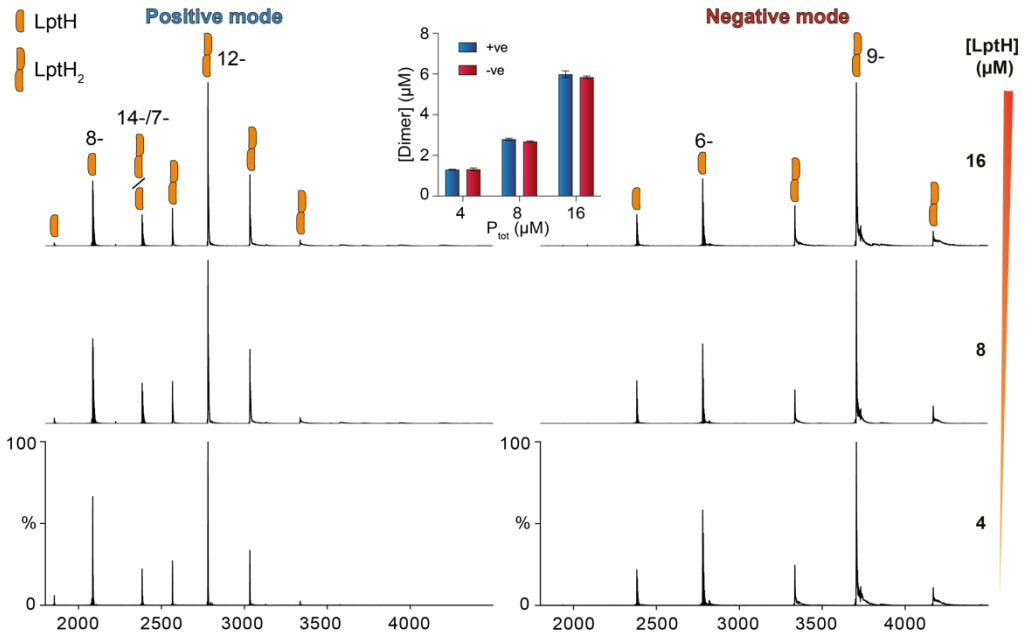

Fig. S2 Native mass spectra of LptH and LptH at different concentrations in positive (left) and negative (right) ion mode. Spectral quantification indicates that there are no major differences in monomer/dimer ratio in different polarities.

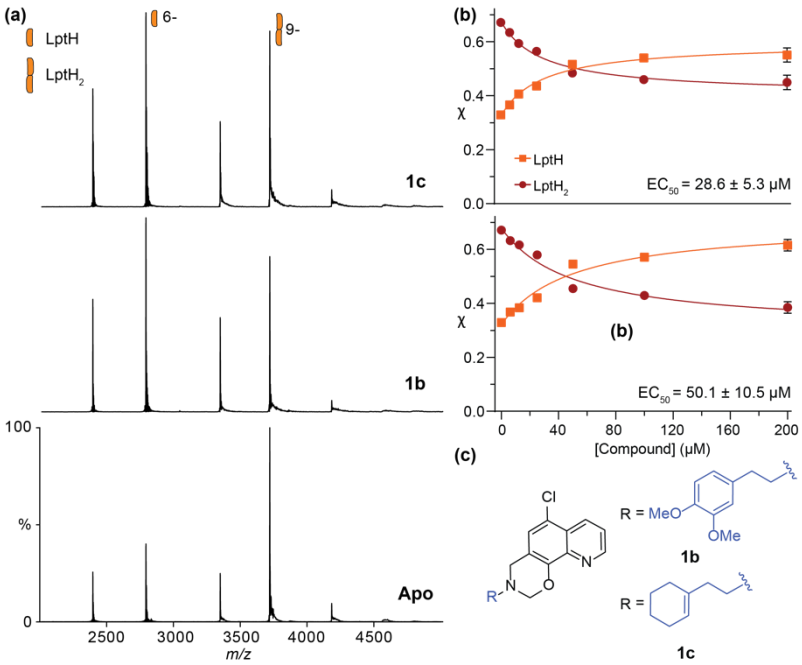

Fig. S3 (a) Native mass spectra of apo LptH and LptH in the presence of compounds **1b** and **1c** at 50 μM concentration. (b) Plot of monomer (orange) or dimer (red) mole fraction (χ) as a function of compound concentration and relative fitting to quantify EC_{50} and E_{max} values. (c) Molecular structures of compounds **1b** and **1c**.

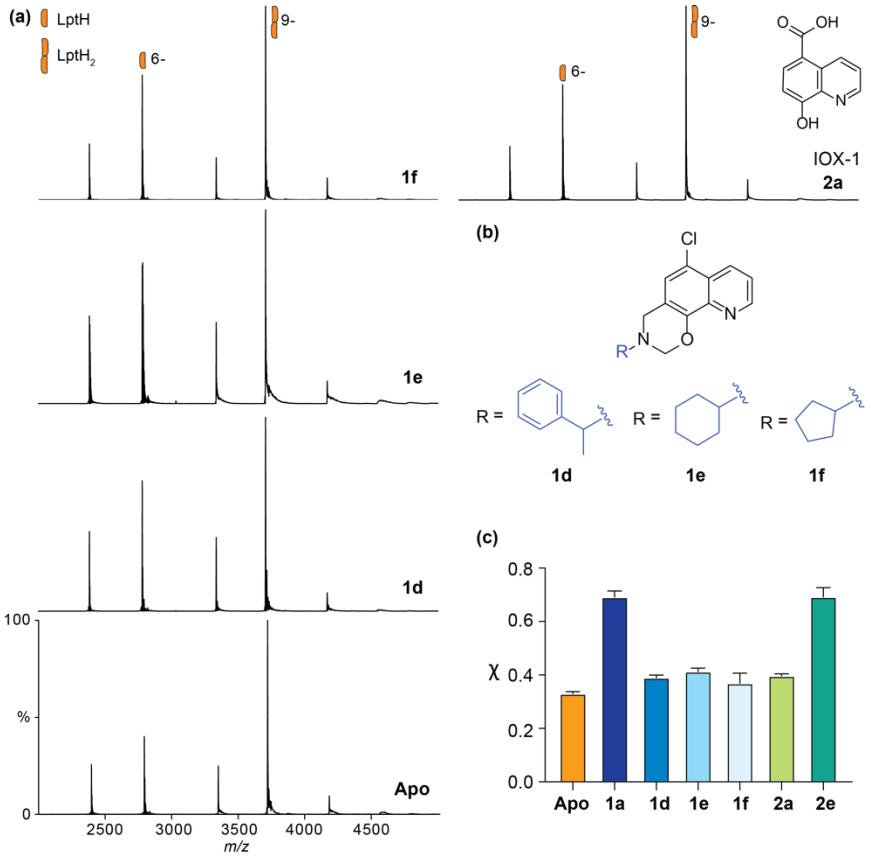

Fig. S4 (a) Native mass spectra of apo LptH and LptH in the presence of compounds **1d**, **1e**, **1f**, and **2a** at 100 μM concentration. (b) Molecular structures of compounds **1d**, **1e**, and **1f**. (c) Bar chart indicating the monomer mole fraction (χ) in apo LptH and in the presence of 100 μM concentration of compounds **1d**, **1e**, **1f**, and **2a**. The effects of IMB-881 (**1a**) at 40 μM and **2e** at 100 μM are also included for comparison.

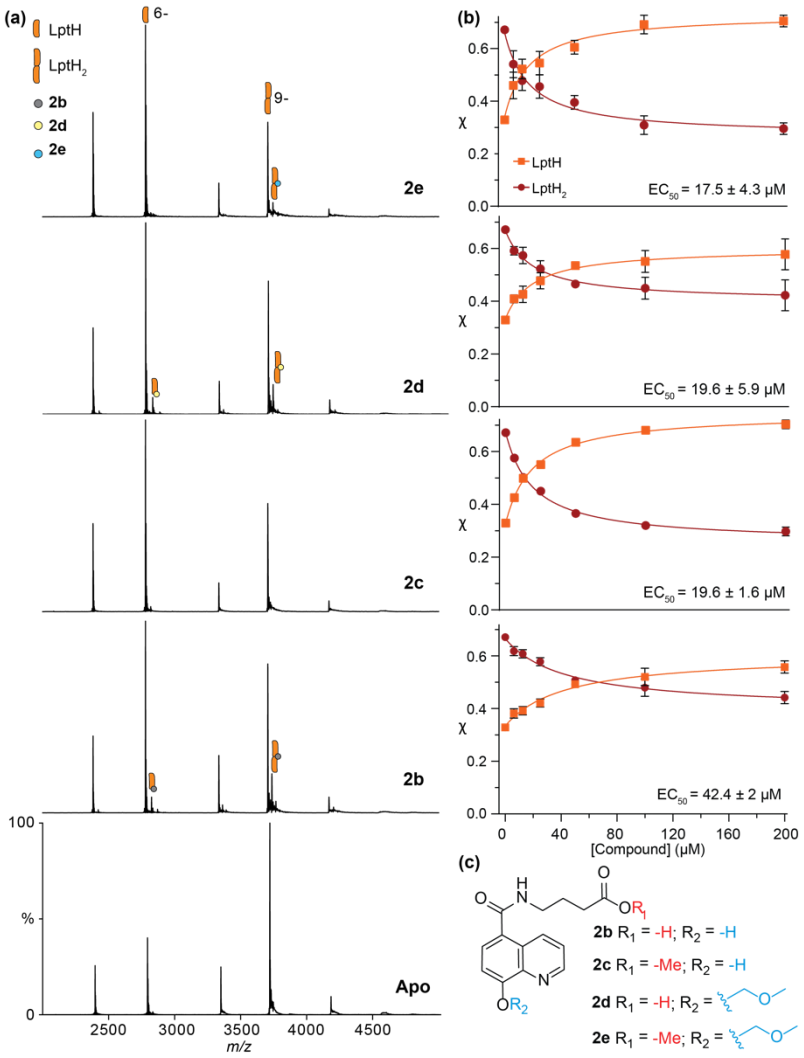

Fig. S5 (a) Native mass spectra of apo LptH and LptH in the presence of compounds **2b**-**2e** at 50 μM concentration, showing their effect on monomer-dimer equilibrium. (b) Plot of monomer (orange) or dimer (red) mole fraction (χ) as a function of compound concentration and relative fitting to quantify EC_{50} and E_{max} values. (c) Molecular structures of compounds **2b**-**2e**.

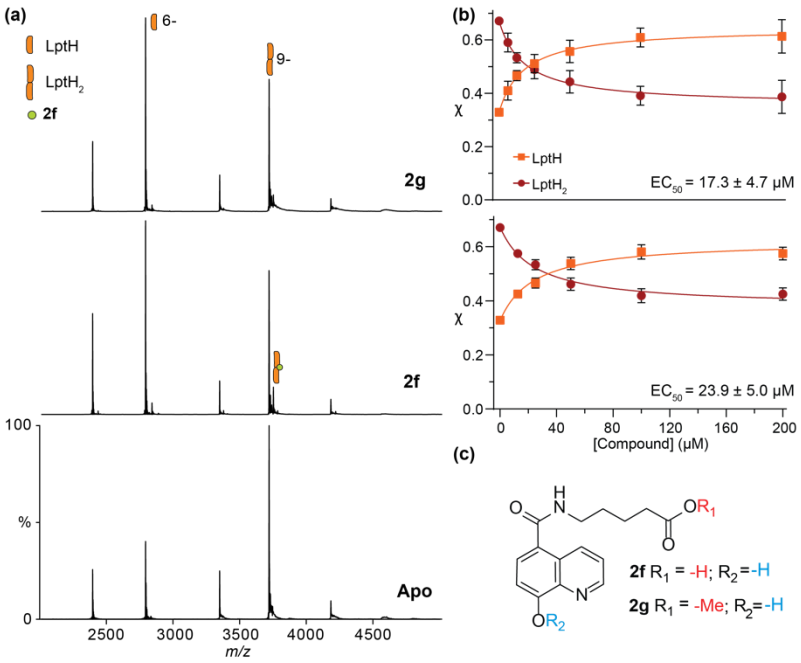

Fig. S6 (a) Native mass spectra of apo LptH and LptH in the presence of compounds **2f**-**2g** at 50 μM concentration, showing their effect on monomer-dimer equilibrium. (b) Plot of monomer (orange) or dimer (red) mole fraction (χ) as a function of compound concentration and relative fitting to quantify EC_{50} and E_{max} values. (c) Molecular structures of compounds **2f** and **2g**.

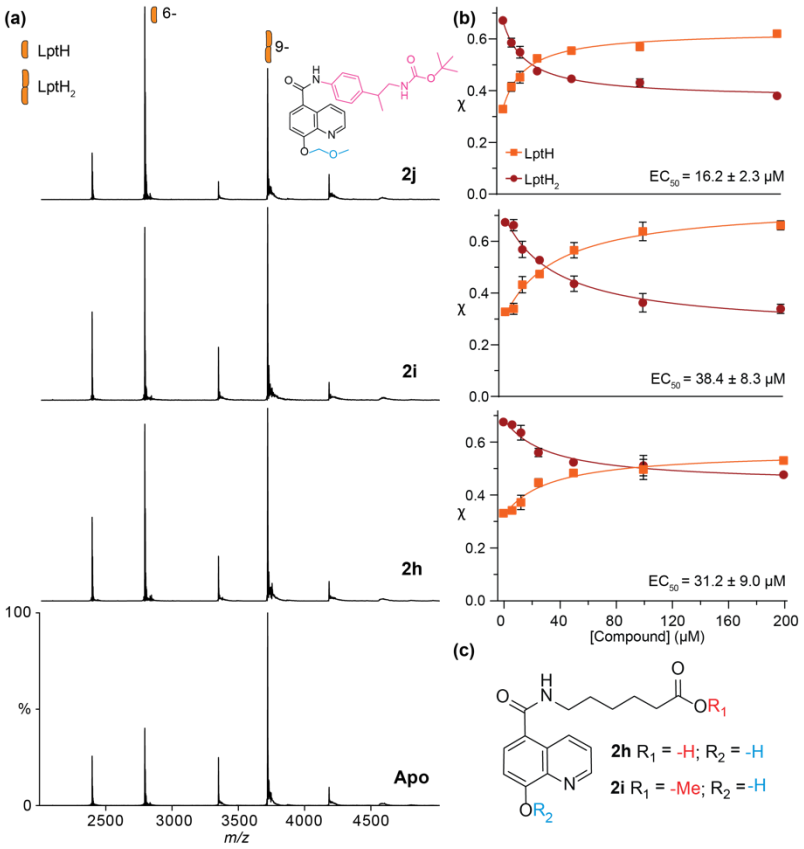

Fig. S7 (a) Native mass spectra of apo LptH and LptH in the presence of compounds **2h**-**2j** at 50 μM concentration, showing their effect on monomer-dimer equilibrium. (b) Plot of monomer (orange) or dimer (red) mole fraction (χ) as a function of compound concentration and relative fitting to quantify EC_{50} and E_{max} values. (c) Molecular structures of compounds **2h** and **2i**.