SUPPORTING INFORMATION

1. FLUORESCENT LABELING OF ESKAPE PATHOGENS......................................................... 2
   FLOW CYTOMETRY................................................................................................. 2
   CONFOCAL MICROSCOPY..................................................................................... 2
   FIGURE S1. LABELLING OF ESKAPE PATHOGENS BY 5 OR BODIPY-FL-N3.................... 3
   DISCUSSION ON FLUORESCENT LABELING OF ESKAPE PATHOGENS....................... 4
2. GROWTH RECOVERY ASSAYS.................................................................................... 4
3. DEACETYLATION AND IRON COMPLEXATION.......................................................... 4
   ANALYSIS BY LC-MS............................................................................................ 4
   TABLE S1: ION TRANSITIONS OF MRM ANALYSIS.................................................. 5
   FIGURE S2. ISOTOPIC MASS PATTERN OF 1 + Fe...................................................... 5
   ANALYSIS OF Fe-COMPLEXATION BY CAS ASSAY.................................................. 6
   TABLE S2: CAS ASSAY CONCENTRATIONS............................................................ 6
   FIGURE S3: ABSORBANCE SPECTRUM FOR Fe(CAS)+2/3/ENTEROBACTIN.................... 7
4. DOTAM AS CARRIER FOR AMPICILLIN.................................................................... 7
5. FAP-SYSTEM FOR VISUALIZING CYTOPLASMIC UPTAKE.......................................... 8
   FAP6.2 EXPRESSION IN E. COLI AND PURIFICATION.............................................. 8
   FLUOROSPECTROMETRY....................................................................................... 8
   FLOW CYTOMETRY AND CONFOCAL MICROSCOPY................................................... 9
   FIGURE S4. EXPRESSION OF FAP AND MG-FLUORESCENCE OF CONJUGATES............. 9
   FIGURE S5. MG-CONJUGATE UPTAKE QUANTIFIED BY FLOW CYTOMETRY.................. 10
   FIGURE S6. CO-INCUBATION OF 11 AND PROPIDIUM IODIDE..................................... 11
6. IN VIVO IMAGING OF P. AERUGINOSA INFECTIONS............................................... 11
   FIGURE S7. ADMINISTRATION REGIMEN FOR IN VIVO IMAGING OF BACTERIAL INFECTIONS 12
SYNTHESIS OF COMPOUNDS..................................................................................... 13
   SCHEME 1: SYNTHESIS OF SIDEROPHORE ARMS AND LINKERS......................... 13
   SCHEME 2: SYNTHESIS OF MALACHITE GREEN DERIVATIVES............................... 13
   SCHEME 3: SYNTHESIS OF 1-4............................................................................... 14
   SCHEME 7: SYNTHESIS OF 11-13........................................................................... 15
7. 1H-NMR, 13C-NMR AND HIGH RESOLUTION MASS SPECTRA.................................... 33
8. ABBREVIATIONS AND GENERAL METHODS............................................................ 82
9. AUTHOR CONTRIBUTIONS...................................................................................... 83
10. REFERENCES............................................................................................................ 83
1. Fluorescent labeling of ESKAPE pathogens

The ability of DOTAM conjugates to label medically important bacterial pathogens of the ESKAPE panel was probed by flow cytometry and confocal microscopy. Bacterial cultures were grown in LB-medium (Gram-negative: *E. coli*, *P. aeruginosa*, *A. baumannii*, *K. pneumoniae*, *E. cloacae*) or TSY-medium (Gram-positive: *S. aureus*, *E. faecium*) under standard conditions.

**Flow cytometry**

Flow cytometric analysis and confocal microscopy of ESKAPE pathogens was performed after 4h incubation with S or Bodipy-FL-N$_3$ (both 10µM). Cells were washed twice with PBS and the OD$_{600}$ was adjusted to 0.025 with sterile-filtered PBS. 100000 cells per sample were analyzed using aLSRFortessa™ with FACSDiva™ software (BD Biosciences, Heidelberg, Germany). Detection of Bodipy was performed with the 488nm laser in combination with 525/50nm band pass filter. Data was evaluated using FlowJo software (FLOWJO LLC, Oregon, US).

**Confocal microscopy**

For confocal microscopy cells were treated with S or Bodipy-FL-N$_3$ (both 10µM) for 4h and were washed twice with PBS before analysis. The cells were then placed into chamber slides (Ibidi GmbH, Martinsried, Germany) and covered with a thin layer pad consisting of 1% agarose. Imaging was performed using the confocal microscope ECLIPSE Ti (Nikon) equipped with UltraVIEWVoX spinning disc (Perkin Elmer, Waltham, US), ORCA-R2 camera (Hamamatsu Photonics, Japan) and Volocity software 6.1.1 (Perkin Elmer, Waltham, US).
Figure S1. Labelling of ESKAPE pathogens by 5 or Bodipy-FL-N₃

Confocal imaging and flow cytometric analysis of ESKAPE pathogens. Scale bars 11µm, bl525-A Bodipy fluorescence intensity.
Discussion on fluorescent labeling of ESKAPE pathogens

As mentioned in the main text we observed various labelling efficiencies within the respective bacterial populations. E.g. some cells display a high fluorescent signal, whereas others show lower signal intensities. This might be attributable to different growth phases of the bacteria and has to be addressed in further studies. For all bacteria besides \textit{E. faecium}, we observed an increased fluorescence signal by confocal microscopy and flow cytometry upon incubation with 5 compared to Bodipy-FL. In repeated experiments we observed that incubation with Bodipy-FL resulted in a strong and homogeneous labelling of \textit{E. faecium}. \textit{E. faecium} and \textit{S. aureus} are the Gram positive members of the ESKAPE panel. As \textit{S. aureus} does not display the intensive fluorescent signal upon incubation with free Bodipy as seen for \textit{E. faecium}, this may not be due to the Gram positive nature in general. Further studies focused on \textit{Enterococci} species have to address this effect.

2. Growth recovery assays

Bacterial strains used for the assay were \textit{E. coli} BW25113 (wildtype), \textit{E. coli} JW0588-1 (Δ\textit{entA}), \textit{P. aeruginosa} PAO1 (wildtype) and \textit{P. aeruginosa} PAS283 (Δ\textit{pydFΔpchA}). The latter was obtained from Isabell Schalk (IREBS, Strasbourg). \textit{E. coliΔentA} mutant was always cultured in presence of 50 µg/ml Kanamycin. Of all strains overnight cultures were grown to full confluency in Mueller-Hinton-Broth (MHB). These were then again diluted in MHB and grown to exponential phase (\textit{OD}_{600nm} of 0.5). \textit{E. coli} were pelleted by centrifugation, washed two times with LMR-medium \cite{1} and adjusted to a density corresponding to an \textit{OD}_{600nm} of 0.01. \textit{P. aeruginosa} were adjusted to a density corresponding to an \textit{OD}_{600nm} of 0.01 by using MHB/H\textsubscript{2}O (50/50) supplemented with 600 µM Bipyridin. Bacterial cultures were then dispensed onto 96-well plates (150 µl/well) and test compounds were added as double replicates. All compounds were dissolved in DMSO and used at final concentrations of 10 µM/well. DMSO served as negative control and Enterobactn (for \textit{E. coli}) or Pyoverdin (for \textit{P. aeruginosa}) served as positive controls. As blank controls corresponding compounds were diluted to 10 µM/well in growth medium. Eventually, plates were sealed with parafilm and incubated for 48 h (\textit{E. coli}) or 24 h (\textit{P. aeruginosa}) at 37 °C. Hereafter, \textit{OD}_{600nm} was determined. All values obtained were blank corrected by subtraction and plotted onto a bar chart.

3. Deacetylation and iron complexation

\textbf{Analysis by LC-MS}

A culture of \textit{E. coliΔentB} grown in LB was washed twice with LMR medium and adjusted to \textit{OD}_{600} = 1. 6\textbf{w} was added to the culture or to blank LMR medium for 0, 4 or 24h at RT at a final concentration of 10µM. The bacteria were removed by centrifugation, and 4 Vol icecold acetonitrile were added to the bacterial supernatant or the blank LMR. After 15 min vortexing and centrifugation (13000rpm, 10min, 4°C), the respective supernatant was used for measuring the ion transitions described in Table S1 in the MRM-mode of AB SciexQTrap 6500 (AB Sciex Germany GmbH, Darmstadt, Germany) coupled to Agilent 1290 UHPLC (Agilent Technologies, Santa Clara, CA, USA). Data were analysed using the MultiQuant software (AB Sciex Germany GmbH, Darmstadt, Germany).
For 1 complexed with iron there was no reference compound available for setting the MRM parameters for AB SciexQTrap analysis. We therefore validated the existence of this molecule upon incubation of equimolar amounts of 1 or 6 with FeCl₃ for 24h in an ACN/water solution at pH 8.2 by measuring the exact mass with a UPLC-ESI-Q-TOF-MS instrument that consisted of an UltiMate 3000 (Thermo Fisher Scientific, Waltham, USA) UPLC coupled to a maxisIITM HD mass spectrometer (Bruker Daltonic, Billerica, USA). The detected isotopic mass pattern was identical to the one predicted by the software (Figure S2). The sample and the molecular weight information was then used to set up an MRM-method on the AB Sciex triple quadrupole mass spectrometer.

*Table S1:* Ion transitions of MRM analysis

Ion transitions used for identification of acetylated, deacetylated and Fe-complexed DOTAM conjugates by tandem mass spectrometry.

<table>
<thead>
<tr>
<th>MOI</th>
<th>Precursorionmass in Da*</th>
<th>Fragment ionmass in Da*</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 (6fold acetylated)</td>
<td>Quantifier</td>
<td>638.9</td>
</tr>
<tr>
<td></td>
<td>Qualifier</td>
<td>638.9</td>
</tr>
<tr>
<td>1 (fully deacetylated)</td>
<td>Quantifier</td>
<td>512.8</td>
</tr>
<tr>
<td></td>
<td>Qualifier</td>
<td>512.8</td>
</tr>
<tr>
<td>1 + Fe (fully deacetylated and Fe-complexed)</td>
<td>Quantifier</td>
<td>539.3</td>
</tr>
<tr>
<td></td>
<td>Qualifier</td>
<td>539.3</td>
</tr>
</tbody>
</table>

* All ions were doubly charged, as verified by full scan experiments

*Figure S2.* Isotopic mass pattern of 1 + Fe.

Isotopic mass pattern of the doubly charged ion derived from 1 complexed with iron. The upper panel depicts the experimental mass spectrum, and the lower panel the one predicted by the software.
**Analysis of Fe-complexation by CAS assay**

The Chrome azurol S (CAS) assay was performed for assessing iron complexation. All used glassware was previously cleaned with 12 M HCl and milliQ water. The stock solutions of hexadecyltrimethylammonium bromide (HDTMA, 10 mM in water) FeCl$_3$ (1 mM in a 10mM HCl solution) and CAS (2 mM in water) were obtained by adding 3.64 mg in 1mL H$_2$O, 0.1622 mg in 1mL HCl (10 mM) and 1.211 mg in 1 mL water, respectively.

A solution of HDTMA (10 mM, 600 µL) was placed in a 10 mL volumetric flask and diluted with water. A mixture of a FeCl$_3$ solution (1mM in 10mM HCl, 150 µL) and an aqueous CAS solution (2 mM, 750 µL) was slowly added under stirring. Anhydrous piperazine (431 mg) was dissolved in water, and HCl (12 M, 625 µL) was carefully added. The buffer solution (pH = 5.56) was rinsed into the volumetric flask which was filled with water to afford a 10 mL of CAS assay solution. 5-sulfosalicylic acid (10.2 mg) was dissolved in the solution to form the Fe(CAS) solution.

15 µM stock solutions of enterobactin and 1-4 were prepared and added at different concentrations into a 96 well plate (Corning CELLBIND Surface, black, transparent flat bottom) according to the following scheme in triplicate:

<table>
<thead>
<tr>
<th>Volume of 1-4 or enterobactin (µL)</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>60</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume of Fe(CAS) (µL)</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Total volume (µL)</td>
<td>20</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Concentration of 1-4 or enterobactin (µM)</td>
<td>0</td>
<td>0.75</td>
<td>1.5</td>
<td>2.25</td>
<td>3.0</td>
<td>4.5</td>
<td>9.0</td>
</tr>
<tr>
<td>Concentration of Fe(CAS) (µM)</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Equivalent of 1-4 or enterobactin (eq)</td>
<td>0</td>
<td>0.25</td>
<td>0.5</td>
<td>0.75</td>
<td>1.0</td>
<td>1.5</td>
<td>3.0</td>
</tr>
</tbody>
</table>

The mixed solutions were analyzed after 17 h to assure that the equilibrium was reached. The absorbance of each solution was measured with anTecan Infinite 200 Pro reader (Tecan, Switzerland). The full spectra of 3 representative experiments were first analyzed between 300 and 800 nm.
Absorbance spectra of three representative samples were recorded to determine the optimal wavelength for the analysis of the CAS assay.

At the wavelength of 620 nm, the absorbance is mainly depending on the concentration of Fe(CAS) in solution. This wavelength was therefore used for the CAS assay experiments.

4. DOTAM as carrier for ampicillin

To evaluate the potential of DOTAM as a carrier for ampicillin, antimicrobial activity assays were performed. Bacterial strains used were *E. coli* BW25113 (as wildtype), JW0587-1 (ΔentB), JW0588-1 (ΔentA), JW5086-3 (ΔfepA), all obtained from Coli Genetic Stock Center (Yale, USA). Bacteria were cultured at 37°C in a shaker at 150rpm. Overnight cultures contained 5ml of LB medium and 30µg/ml Kanamycin for ΔentA/ΔentB/ΔfepA-mutants. Overnight cultures were diluted 1:100 with 5ml LB-medium and grown to an OD$_{600}$ of 0.5-0.8. Cultures were then diluted with MH medium to a final OD$_{600}$ of 0.01 and transferred to 96well half area plates. Test compounds dissolved in DMSO were added to a maximum final concentration of 1% of DMSO and serially diluted. The plate was wrapped with parafilm and incubated in a shaker at 150rpm and 37°C for 24h. Bacterial growth was determined by measuring OD$_{600}$ using the microplate spectrophotometer power wave XS2 (Bio Tek, Winooski, US). Each concentration was measured in triplicate in two biological replicates. IC$_{50}$ values were calculated using Sigma Plot (Systat Software GmbH, Erkrath, Germany) and four parameter logistics curve regression. Mean values of IC$_{50}$ obtained from two biological replicates are reported.
5. FAP-system for visualizing cytoplasmic uptake

FAP6.2 expression in E. coli and purification

The sequence of FAP6.2 \(^{[3]}\) was synthesized by GenScript (NJ, USA) and cloned into a pET23-expression vector (Novagen/Merck, Darmstadt, Germany). Chemically competent E. coli Origami™ B (DE3) pLysS (Novagen/Merck, Darmstadt, Germany) were transformed with pET23_FAP6.2_His and selected for ampicillin resistance yielding E. coli_FAP6.2. The expression of FAP6.2 under different concentrations of IPTG was monitored by Western Blot using His-tag specific antibody (Relia Tech, Wolfenbüttel, Germany). Purification of HIS-tagged FAP6.2 was performed using Ni-NTA-agarose (Qiagen, Hilden, Germany). 500ml LB-Medium containing selection antibiotics tetracycline, kanamycin and ampicillin were inoculated from a 5ml overnight culture of E. coli_FAP6.2. At OD\(_{600}\) of 0.8 the expression of FAP6.2 was induced by the addition of 1mM IPTG and the culture was kept under continuous agitation overnight. For protein purification the cells were centrifuged (5000xg, 10min, 4°C) and resuspended in 20mM Tris, 500mM NaCl pH7.9. After driving of the bacteria through a French press (1500psi, Thermo Scientific, Waltham, US) and centrifugation (20000xg, 30min, 4°C) imidazole was added to the supernatant to a final concentration of 30mM. The supernatant was then incubated with 1ml prewashed Ni-NTA-agarose (washed with 1ml H\(_2\)O followed by 5x1ml 20mM Tris, 500mM NaCl, pH7.9) for 2h at 4°C. Using a poly-prep chromatography column (BioRad, Hercules, US) the Ni-NTA-agarose was washed with 20ml of 20mM Tris, 500mM NaCl, 30mM imidazole, pH7.9. FAP6.2-His was eluted with 2ml 50mM Tris, 150mM NaCl, 5mM CaCl\(_2\), 300mM imidazole, pH 7.5 and dialyzed for 2h against 50mM Tris, 150mM NaCl, 5mM CaCl\(_2\), pH 7.5. Glycerol was added to a final concentration of 10% for storage of FAP6.2 at -20°C. Protein concentration was determined using a NanoDrop™ 2000 (Thermo Scientific, Waltham, US).

Fluorospectrometry

An overnight culture of E. coli_FAP6.2 was diluted 1:100 in MH2 medium and grown until an OD\(_{600}\) of 0.8 was reached. IPTG was added at 1µM final concentration and after 4h the OD\(_{600}\) was adjusted to 2. For assessing uptake of MG-conjugates black 96 well plates with clear bottom with 100µl of bacterial culture each well were used. Compounds were added at 10µM final concentration. Fluorescence was monitored over 16h post compound addition using a Tecan Infinite 200 pro reader (Tecan, Switzerland) in bottom read mode. Each test was measured in triplicate. Szent-Gyorgyi et al. already described that chemical derivatization of MG led to compounds with different binding affinities, fluorescence intensities and excitation/emission spectra \(^{[4]}\). Likewise conjugation of different DOTA-cores to MG yielded conjugates possessing different fluorescence intensities upon binding to FAP6.2. To enable comparison with regard to bacterial uptake, recombinantly expressed FAP6.2 (S3,B) was incubated with the different MG-conjugates in a molar ratio of 4:1 and the fluorescence emission at 665nm with excitation at 610nm were recorded (S3, C). The fluorescence values obtained for FAP + MG-conjugates were corrected by the values obtained from a non-FAP-protein + MG-conjugates (S3,D). The different values for MG-conjugates were used for calculating the ratio between free MG and the respective MG-conjugate. All fluorescence values measured during live kinetics were corrected by this factor afterwards.
Flow cytometry and confocal microscopy

Flow cytometric analysis and confocal microscopy of *E. coli_FAP6.2* was performed upon 16h incubation with MG-conjugates. For flow cytometry cells were washed once with PBS and the OD$_{600}$ was adjusted to 0.025 with sterile-filtered PBS. At least 10000 cells per sample were analyzed using aLSRFortessa™ with FACSDiva™ software (BD Biosciences, Heidelberg, Germany). For detection of MG-derived fluorescence the 640nm laser was used in combination with 670/14nm band pass filter. Data was evaluated using FlowJo software (FLOWJO LLC, Oregon, US). For confocal microscopy cells were placed into chamber slides (Ibidi GmbH, Martinsried, Germany) and covered with a pad consisting of 1% agarose. Imaging was performed using the confocal microscope ECLIPSE Ti (Nikon) equipped with UltraVIEWVoX spinning disc (Perkin Elmer, Waltham, US), ORCA-R2 camera (Hamamatsu Photonics, Japan) and Volocity software 6.1.1 (Perkin Elmer, Waltham, US). Propidium iodide was added at final concentration of 50µg/ml for 20min and cells were washed once with PBS before confocal analysis.

**Figure S4. Expression of FAP and MG-fluorescence of conjugates**

A. Fluorescence emission spectra of MG, FAP6.2 and MG + FAP6.2 with excitation at 610nm. Only upon binding to FAP6.2 a fluorescence signal of MG can be detected. B. *E. coli* FAP6.2were induced by addition of 1mM IPTG for different timespans and lysates were probed by α-His antibody on Western Blot. C. Recombinantly expressed FAP6.2 protein was incubated with MG-conjugates in a molar ratio of 4:1, and fluorescence was recorded with an excitation wavelength of 610nm and an emission
wavelength of 665nm. D. The correction factor corresponds to the ratio of MG-fluorescence intensity divided by fluorescence intensity of the respective MG-conjugate.

*Figure S5.* MG-conjugate uptake quantified by flow cytometry

Flow cytometric analysis of *E. coli_FAP6.2* upon incubation with **11** or **10** at a concentration of 10µM for 16h. FSC forward scatter, SSC side scatter, rd670 MG fluorescence intensity.
Figure S6. Co-incubation of 11 and propidium iodide

Confocal microscopy of E. coli parent strain or E. coli_FAP6.2 upon incubation with 11 at a concentration of 10 µM for 16h after an additional 20min incubation with 50µg/ml propidium iodide (PI). BF brightfield. Scale bars 11 µm.

6. In vivo imaging of P. aeruginosa infections

A culture of P. aeruginosa (PA01) was streaked on LB agarose plates and incubated at 37°C overnight. Single colonies were picked and inoculated in LB media. Inoculated cultures were incubated on shakers at 180 rpm at 37°C until the optical density (OD600) of the culture reached 0.1. In vivo monitoring of the host immune response to bacterial infections was performed by using heterozygous WT/IFN-β-luciferase reporter mice. The dorsal side of the animal was partially shaved, and 5 µl suspension containing 5x10^5 colony forming units (CFU) of P. aeruginosa bacteria was injected subcutaneously. Immediately after infection, 12 or 13 were injected intravenously in mice (3 animals per group) through the tail vein (20µg/kg of mouse body weight). In two control groups that were treated with a solvent control (without bacteria), 12 or 13 were injected intravenously in mice (2 animals per group) through the tail vein (20µg/kg of mouse body weight). Fluorescent imaging was performed at excitation and emission wavelengths of 675 and 694 nm, respectively. To determine luciferase activity in vivo, 150 µl of (30mg/kg) luciferin was injected in the reporter mice after 5 hours of infection, and bioluminescent imaging was subsequently performed. Both bioluminescence and fluorescence were recorded by an in vivo imager instrument (IVIS 200, Xenogen/Caliper Life Sciences, Germany) as previously described. Fluorescent images were
processed by subtracting the autofluorescence background of the tissue using the Living image software VR 4.3.1 (Caliper Life Sciences).

Animal experiments were done with the ethical approval of the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES), permission number 33.42502/07-10.5.

Figure S7. Administration regimen for in vivo imaging of bacterial infections

Heterozygous interferon-β-luciferase BALB/c reporter mice were infected by injecting a suspension of 5x10^5 colony forming units (CFU) P. aeruginosa bacteria immediately followed by a tail vein injection of 20μg/kg body weight of fluorescent compounds DOTAM-Cy5.5 or DOTA-Cy5.5 solutions, respectively. Fluorescent images were recorded after the four different time points indicated by the marks on the time axis. The luminosity was recorded after 5 hours after intraperitoneal luciferin injection (see text for details).
Synthesis of compounds

Scheme 1: Synthesis of siderophore arms and linkers

Scheme 2: Synthesis of malachite green derivatives
Scheme 3: Synthesis of 1-4

Scheme 4: Synthesis of 5
Scheme 5: Synthesis of 6-9

Scheme 6: Synthesis of 10

Scheme 7: Synthesis of 11-13
**14** was prepared according to the procedure reported by Albrecht et al.\(^7\). 14 (1190 mg, 5 mmol), HATU (2090 mg, 5.5 mmol) and DIPEA (1.7 mL, 10 mmol) were dissolved in DMF (25 mL). After 10 min, N-Boc-ethylenediamine (790 µL, 5 mmol) was added to the reaction mixture and stirred for 30 min under argon at room temperature. Ethyl acetate (50 mL) was added into the reaction mixture followed by an aqueous work up. The organic layer was washed 3 times with 1N HCl and brine then dried over anhydrous sodium sulfate. After filtration and removal of the solvent, the crude compound was purified by flash silica gel column chromatography (eluent: PE : EtOAc = 1:1-1:3) to yield **15** as a white powder (580 mg, 31%). \(^1\)H NMR (500 MHz, CDCl\(_3\)) δ 7.56 – 7.46 (m, 1H, -Ar), 7.25 – 7.24 (m, 1H, -Ar), 7.24 (s, 1H, -Ar), 6.86 (s, 1H, -N\(\text{H}\)), 5.13 (s, 1H, -NH), 3.44 (q, \(J = 5.6\) Hz, 2H, -C\(\text{H}_2\)), 3.35–3.21 (m, 2H, -C\(\text{H}_2\)), 2.29 (s, 3H, -OAc), 2.27 (s, 3H, -OAc), 1.40 (s, 9H). \(^13\)C NMR (126 MHz, CDCl\(_3\)) δ 168.22, 168.20, 165.82, 165.74, 156.69, 142.96, 140.26, 130.22, 129.47, 126.60, 126.40, 126.19, 125.73, 80.29, 79.62, 42.37, 40.75, 40.15, 39.44, 38.58, 29.68, 28.35, 28.31, 20.59, 20.50, 14.13. ESI-HRMS: C\(_{18}\)H\(_{24}\)N\(_2\)O\(_7\)Na\(^+\) m/z = 403.1476 [M+Na]\(^+\), error < 0.1 ppm.

16 was prepared according to the procedure reported by Leydier et al.\(^8\). To a solution of 16 (100 mg, 266 µmol) in DCM (250 µL) cooled to 0°C were added a solution of K\(_2\)CO\(_3\) (44 mg, 319 µmol) in water (250 µL), and a solution of bromoacetyl bromide (28 µL, 319 µmol) in DCM (500 µL). The reaction mixture was stirred at 26 °C for 1 h. The phases were separated, and the aqueous layer was washed with DCM (2×10 mL). The organic layer was dried over magnesium sulfate, filtered and concentrated to yield **17** as a white solid (120 mg, 91 %). \(^1\)H NMR (500 MHz, CDCl\(_3\)) δ 8.19 (bt, 1H, \(J = 5.7\) Hz, NH\(\text{I}\)), 7.72 (dd, 1H, \(J = 2.8\) Hz, J = 6.7 Hz), 7.47 (m, 2H, Ar-H), 7.44–7.31 (m, 9H, Ar-H\(\text{I}\)), 7.19–7.14 (m, 2H, Ar-H\(\text{II}\)), 5.17 (bs, 2H, ArOCH\(_2\)C\(_6\)H\(_5\)), 5.11 (bs, 2H, ArOCH\(_2\)C\(_6\)H\(_5\)), 3.76 (s, 2H, BrCH\(_2\)), 3.38 (m, 2H), 3.29 ppm (m, 2H). \(^13\)C NMR (125 MHz, CDCl\(_3\)) δ 166.6, 166.1, 151.7, 146.9, 136.3, 136.2, 128.9, 128.7, 128.2, 127.6, 126.6, 124.8, 123.2, 117.4, 76.6, 71.3, 41.6, 38.7, 28.8 ppm. ESI-HRMS: C\(_{25}\)H\(_{28}\)N\(_2\)O\(_4\)Br\(^-\) m/z = 497.1076 [M+H]\(^-\), error = 1.2 ppm.
18 was prepared according to the procedure reported by Petrosyan et al.\cite{9}. To a solution of 18 (6.00 g, 16 mmol) in DCM (50 mL) cooled to 0°C were added over 1 h a solution of K$_2$CO$_3$ (4.99 g, 36 mmol) in water (50 mL) and a solution of bromoacetyl bromide (1.72 mL, 20 mmol) in DCM (50 mL). The reaction mixture was stirred at 21 °C for 1.5 h. The phases were separated and the organic layer was washed with water (2×100 mL), brine (2×100 mL), dried over MgSO$_4$, filtered and concentrated to yield 19 as a transparent oil (4.66 g, 90 %). $^1$H NMR (500 MHz, CD$_3$CN). δ 7.42-7.31 (m, 5H, C$_6$H$_5$), 6.95-6.74 (bs, 1H, NH), 5.09 (s, 2H, CO$_2$C$_6$H$_5$), 3.77 (s, 2H, C$_2$H$_2$Br), 3.20 (m, J = 6.9 Hz, 6.0 Hz, C$_2$H$_5$), 2.37 (t, J = 7.4 Hz, CH$_2$), 1.72 ppm (m, C$_2$H$_5$).

$^{13}$C NMR (125 MHz, CD$_3$CN). δ 173.7, 167.1, 137.5, 129.5, 129.0, 128.9, 66.7, 39.7, 31.9, 30.0, 25.3 ppm. ESI-HRMS: C$_{13}$H$_{16}$NO$_3$Na $^+$ m/z = 336.0208 [M+Na]$^+$, error = 0.6 ppm.

20 was prepared according to the procedure reported by Szent-Gyorgyi et al.\cite{10}. To a light blue solution of 20 (200 mg, 462 µmol) in DCM (5 mL) was added p-chloranil (170 mg, 691 µmol). The reaction mixture was stirred at 25 °C for 2 h and diluted in DCM (50 mL) and water (100 mL). The aqueous layer was extracted with DCM (5×100 mL), and the combined organic layers were washed with brine (100 mL), dried over MgSO$_4$, filtered and concentrated to yield a dark blue solid. The crude material was dissolved in ACN:H$_2$O (1:1) and purified by reversed phase column chromatography (column: Phenomenex, C18, 250×21 mm, eluent: ACN/H$_2$O/0.1 % HCOOH). The fractions were lyophilized to yield 21 as a dark blue solid (25 mg, 11 %). $^1$H NMR (500 MHz, CD$_3$OD). δ 7.47 (d, 4H, J = 9.3 Hz, Ar-H), 7.41 (d, 2H, J = 8.9 Hz, Ar-H), 7.22 (d, 2H, J = 8.9 Hz, Ar-H), 7.08 (d, 2H, J = 9.4 Hz, Ar-H), 4.26 (t, 2H, J = 6.3 Hz, OCH$_3$), 3.36 (s, 12H, CH$_2$), 3.13 (s, 1H), 2.58 (t, 2H, J = 7.2 Hz, CH$_2$CO$_2$H), 2.19 ppm (m, 2H, 2H, CH$_2$CH$_2$CO$_2$H). $^{13}$C NMR (125 MHz, CD$_3$OD). δ 179.8, 176.8, 165.7, 158.4, 141.9, 138.9, 133.3, 128.3, 116.0, 114.4, 68.8, 40.8, 31.2, 25.6 ppm. ESI-HRMS: C$_{27}$H$_{31}$N$_2$O$_3$ $^+$ m/z = 431.2335 [M]$^+$, error = 1.4 ppm.
22 was prepared according to the procedure reported by Szent-Gyorgyi et al.\textsuperscript{[10]} To a light blue solution of 22 (10.6 mg, 17 µmol) in EtOAc (1.0 mL) was added \textit{p}-chloranil (9.4 mg, 38 µmol). The reaction mixture was refluxed for 1 h, filtered and concentrated to yield a dark blue oil. The crude material was dissolved in EtOH:HCl (37\%) (1:1 mL) and stirred for 2 h at 25 °C. The reaction was monitored by LC-MS. The dark solution was diluted in water (30 mL) and washed with EtOAc (3×20 mL). The aqueous layer was lyophilized to yield a blue solid (8.0 mg). The crude material was purified by reversed phase column chromatography (column: Phenomenex, C18, 250×10 mm, eluent: ACN/H\textsubscript{2}O/0.1 \% TFA). The fractions were lyophilized to yield 23 as a dark blue solid (6.0 mg, 52 \%). 1\textsuperscript{H} NMR (500 MHz, \textit{D}_6-DMSO). δ 8.12 (br s, 1H, CONH\textsubscript{3}), 7.83 (br s, 3H, NH\textsubscript{3}), 7.33 (dd, 6H, J = 9.2 Hz, J = 8.8 Hz, Ar-H), 7.20 (d, 2H, J = 8.8 Hz, Ar-H), 7.07 (d, 4H, J = 9.4 Hz, Ar-H), 4.17 (t, 2H, J = 6.4 Hz, OCH\textsubscript{3}), 3.33-3.24 (m, 2H, CONH\textsubscript{3}), 3.28 (s, 12H), 2.87 (m, 2H, CH\textsubscript{2}NH\textsubscript{3}), 2.32 (t, 2H, J = 7.4 Hz, CH\textsubscript{2}CONH), 2.02 ppm (m, 2H, CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{2}). 13\textsuperscript{C} NMR (125 MHz, \textit{D}_6-DMSO). δ 176.0, 172.4, 163.5, 156.3, 140.1, 137.4, 131.3, 126.3, 115.0, 113.6, 67.8, 38.7, 36.5, 31.5, 24.5 ppm. ESI-HRMS: C\textsubscript{29}H\textsubscript{37}N\textsubscript{4}O\textsubscript{2}\textsuperscript{+} m/z = 473.2960 [M-H]\textsuperscript{+}. The analyzed data showed accordance to previously published ones.\textsuperscript{[10]}

**Product distributions following multiple alkylations of cyclen**

Alkylations under the reported conditions were generally slow and tended to become slower with increasing alkylation level; therefore, monitoring and stopping reactions was well possible. By dropping a diluted solution of the alkylating agent (3.3 eq) into a solution of cyclen, an excess of alkylating agent was avoided. In this manner, it was possible to optimize the yields of trisalkylated compounds to levels that are consistent with literature data.\textsuperscript{[11]} However, both bis- as well as tetraalkylated species were observed by LC/MS. The bis-alkylated products were separated during the aqueous workup. The tetra-alkylated side products were separated by RP column chromatography after the subsequent attachment of the fourth arm.
To a stirred suspension of cyclen (1.0 mg, 6 µmol) and sodium acetate (1.6 mg, 19 µmol) in DMA (100 µL) at 26°C under Ar conditions was added dropwise a yellow solution of 17 in DMA (150 µL). The reaction was stopped after 19 h stirring at 26 °C. The yellow solution was diluted in DCM (10 mL) and washed with KHCO₃ (10 mL) and water (10 mL), dried over Na₂SO₄, filtered and concentrated to yield a yellow oil (10 mg). The crude material and K₂CO₃ (3.2 mg, 23 µmol) were suspended in ACN (250 µL), and a solution of 19 (2.4 mg, 8 µmol) in ACN (250 µL) was added dropwise for 5 min under Ar conditions at 0 °C. The suspension was stirred for 4 h at 31 °C. The yellow suspension was filtered and concentrated to yield a yellow oil (15 mg). The crude material was set to a hydrogen atmosphere over 10% Pd/C (2 mg, 2 µmol) in MeOH (500 µL) at 26-32 °C for 15 h. The black suspension was filtered through celite, washed with MeOH and concentrated to yield a pale oil, which was partially dissolved in ACN/H₂O (1000 µL), filtered and purified by reversed phase column chromatography (250×10 mm, 40-min-long gradient from 10 to 25 % ACN in H₂O) to yield pure 1 (3.0 mg, 51 %). ¹H NMR (500 MHz, CD₃CN). δ 12.48 (bs, 3H), 9.19 (bs, 2H), 8.77 (m, 3H), 8.42 (bs, 2H), 8.27 (bs, 1H), 7.90 (bs, 1H), 7.23 (m, 3H, Ar-H), 6.91 (m, 3H, Ar-H), 6.67 (m, 3H, Ar-H), 3.63 (bs, 7H), 3.40 (bs, 7H), 3.28 (m, 13H), 3.13 (m, 9H), 2.23 (t, 2H, J = 7.5 Hz, CH₂CO₂H), 1.65 ppm (m, 2H, CH₂CH₂CO₂H). ¹³C NMR (125 MHz, CD₃CN). δ 174.2, 169.9, 149.4, 146.2, 118.8, 118.0, 117.3, 115.1, 54.7, 54.5, 49.4, 38.4, 38.4, 38.2, 31.0, 24.2 ppm. ESI-HRMS: C₄₃H₆₇N₁₁O₁₅²⁺ m/z = 512.7403 [M+2H]²⁺, error = 0.2 ppm.

To a stirred suspension of cyclen (1.4 mg, 8.1 µmol) and sodium acetate (2.5 mg, 30.5 µmol) in DMA (100 µL) at 22 °C under Ar conditions was added dropwise a transparent solution of
17 (13.8 mg, 27.7 µmol) in DMA (150 µL). The reaction was stopped after stirring for 26 h at 23 °C. The yellow solution was diluted in DCM (10 mL), washed with KHCO₃ (0.5 M in water, 10 mL) and water (10 mL), dried over Na₂SO₄, filtered and concentrated to yield a yellow oil. The yellow oil was diluted in DCM (5 mL), washed with KHCO₃ (0.5 M in water, 6 mL) and water (10 mL), dried over Na₂SO₄, filtered and concentrated to yield a yellow oil (12.4 mg).

The crude material, 19 (3.4 mg, 10.8 µmol) and K₂CO₃ (5.8 mg, 42.0 µmol) were suspended in ACN (0.5 mL) for 5 min under Ar conditions at 0 °C, and the transparent suspension was stirred for 4.5 h at 22 °C. The precipitate was filtered and the filtrate was concentrated. More 4KF09CR (3.3 mg, 10.5 µmol) and K₂CO₃ (5.9 mg, 42.7 µmol) were added, suspended in ACN (250 µL) for 3 min under Ar conditions at 0 °C and the pale yellow suspension was stirred for 18.5 h at 22 °C. The precipitate was filtered, and the filtrate was concentrated. The resulting yellow oil was dissolved in DCM (10 mL), washed with K₂CO₃ (3×10mL), dried over Na₂SO₄, filtered and concentrated to yield a transparent oil (12 mg). The crude material was dissolved in THF (375 µL), NaOH solution (1 M in water, 125 µL) was added and RM was stirred for 4 h at 22 °C. The RM was concentrated to yield a yellow solid, then diluted in ACN (1 mL) and filtered to yield a transparent solution. A few drops of DMSO were added. The solution was purified by reversed phase column chromatography (column: Gemini, C18, 250×10 mm, eluent: ACN/H₂O/0.1 % TFA, 40-min-long gradient from 20 % to 80 % ACN). The fraction of the main peak was concentrated to yield an uncolored oil. The resulting uncolored oil was dissolved in ACN/H₂O (1/1, 0.1 % TFA, 700 µL) and filtered to yield a transparent solution. The solution was purified by reversed phase column chromatography (column: Gemini, C18, 250×10 mm, eluent: ACN/H₂O/0.1 % TFA, 40-min-long gradient from 40 % to 70 % ACN). Fractions containing the expected product were lyophilized overnight to yield 2 as a transparent oil (1.17 mg, 10%).

**¹H NMR** (500 MHz, CD₃CN). δ 8.01-7.90 (m, 4H), 7.54-7.46 (m, 6H), 7.46-7.34 (m, 10H), 7.34-7.26 (m, 15H), 7.25-7.16 (m, 5H), 7.16-7.02 (m, 4H), 5.19-5.01 (m, 11H), 3.94-3.38 (m, 10H), 3.35 (q, 4H), 3.30 (q, 2H), 3.27-3.22 (m, 1H), 3.22-3.16 (m, 4H), 3.16-3.10 (m, 6H), 3.10-2.82 (bs, 11H), 2.30-2.22 (m, 2H), 1.73-1.63 ppm (m, 2H).

**¹³C NMR** (125 MHz, CD₃CN). δ 175.0, 167.0, 166.9, 152.9, 146.9, 137.8, 137.7, 129.9, 129.9, 129.8, 129.6, 129.4, 129.2, 129.0, 125.3, 125.3, 122.8, 76.6, 76.6, 71.7, 56.1, 56.0, 50.9, 40.5, 39.5, 39.4, 31.8, 25.2 ppm. ESI-HRMS: C₈₉H₁₀₃N₁₁O₁₅⁺ m/z = 782.8838 [M+2H]²⁺, error = 3.3 ppm.

24 was prepared according to the procedure reported by N. Raghunand et al. To a stirred solution of 24 (2.00 g, 3.9 mmol, dissolved for 10 min) and K₂CO₃ (2.15 g, 15.6 mmol) in ACN (40 mL), benzyl-2-bromoacetate (0.80 mL, 5.1 mmol) was added dropwise in the suspension under Ar conditions at 0 °C. The reaction mixture was stirred for 3 h at 25 °C. The reaction was monitored by TLC (CH₂Cl₂:MeOH/4:1) and LC-MS. The suspension was filtered, and the filtrate was concentrated to yield a white foaming solid (2.58 g). ESI-MS: C₃₅H₅₉N₄O₈⁺ m/z = 663.5 [M+H]⁺. The crude material (2.58 g, 3.9 mmol) was dissolved in TFA (10 mL) at 0 °C under Ar atmosphere and stirred at 25 °C for 26 h. The reaction mixture was coevaporated with toluene (3×10 mL) to yield a green sticky solid (4.19 g). The crude material was
dissolved in an HCl solution (0.1 M in H₂O, 75 mL) and lyophilized to yield a yellow solid (3.11 g). A part of the crude material (1.00 g) was diluted in ACN/H₂O (1/1), filtered and purified by reversed phase column chromatography (column: Macherey-Nagel, C18, 250×40 mm, eluent: ACN/H₂O/0.1 % HCOOH) to yield 25 as a white solid (250 mg, 40%). ¹H NMR (500 MHz, D₆-DMSO). δ 7.38-7.30 (m, 5H, Ar-H), 5.11 (s, 2H, OCH₂), 3.65 (bs, 2H, CH₂COOBn), 3.49 (2×bs, 6H, CH₂COOH), 2.96 (bs, 8H), 2.82 ppm (bs, 4H). ¹³C NMR (125 MHz, D₆-DMSO). δ 170.9, 170.3, 163.3, 136.0, 128.5, 128.1, 128.0, 65.4, 55.3, 55.0, 54.0, 51.4, 50.4, 49.8, 49.3 ppm. ESI-HRMS: C₂₃H₃₅N₄O₈⁺ m/z = 495.2449 [M+H]⁺, error = 0.2 ppm.

To a solution of 25 (5 mg, 10 µmol) in DMF (200 µL) were added DIPEA (6.2 µL, 36 µmol) and HATU (13 mg, 34 µmol). The reaction mixture was stirred for 15 min. 16 (16 mg, 43 µmol) was added, and the pale solution was stirred at 25 °C for 1 h. The reaction mixture was directly filtered and purified by reversed phase column chromatography (column: Phenomenex, C18, 250×21 mm, eluent: ACN/H₂O/0.1 % TFA) to yield 26 as a crystallized solid (14.5 mg, 91 %). A bigger scale of this reaction (0.25 g of 25 led to a diminished yield (0.47 g, 59 %). ¹H NMR (500 MHz, D₆-DMSO). δ 8.65 (bs, 1H, CONH), 8.26 (t, 2H, J = 5.7 Hz), 8.22 (t, 1H, J = 5.8 Hz), 8.10 (bs, 1H, CONH), 7.74 (bs, 2H, CONH), 7.50-7.45 (m, 6H, Ar-H), 7.43-7.22 (m, 32H, Ar-H), 7.15-7.05 (m, 6H, Ar-H), 5.20-5.15 (2×bs, 6H, ArOCH₂C₆H₅), 5.13-5.05 (2×bs, 2H, CH₂COOCH₂C₆H₅), 5.02-4.96 (2×bs, 6H, ArOCH₂C₆H₅), 3.93 (m, 4H), 3.73 (s, 2H), 3.68-2.91 ppm (m, 32H). ¹³C NMR (125 MHz, D₆-DMSO). δ 171.4, 170.4, 166.1, 166.0, 165.1, 151.6, 145.2, 145.1, 137.0, 137.0, 136.8, 135.6, 130.7, 128.5, 128.4, 128.3, 128.3, 128.2, 128.1, 128.0, 128.0, 127.7, 124.1, 120.9, 120.8, 116.0, 115.9, 75.2, 70.2, 65.9, 54.6, 50.9, 50.7, 48.1, 47.7, 38.4, 38.1 ppm. ESI-HRMS: C₉₂H₁₀₁N₁₅O₁₄⁺ m/z = 1569.7502 [M+H]⁺, error = 0.5 ppm.
26 (100 mg, 64 µmol) was hydrogenolyzed over 10% Pd/C (1 mg, 0.9 µmol) in MeOH (5 mL) for 14 h. The black suspension was filtered through celite, washed and concentrated to yield purple solid which was purified by reversed phase column chromatography (ACN/H₂O/0.1% TFA, linear gradient 10%-30% ACN) to yield 3 as a pale solid (54 mg, 90 %). ¹H NMR (500 MHz, D₆-DMSO). δ 12.51 (bd, 2H), 9.21 (bs, 1H), 8.80 (m, 3H) 8.16 (bs, 2H), 8.26 (bs, 1H), 7.24 (t, 3H, J = 7.9 Hz, Ar-H), 6.91 (t, 3H, J = 6.7 Hz, Ar-H), 6.67 (m, 3H, Ar-H), 4.00-3.76 (bs, 4H), 3.65 (bs, 3H), 3.57-2.91 ppm (m, 29H).

¹³C NMR (125 MHz, D₆-DMSO). δ 170.0, 169.9, 149.4, 146.2, 118.8, 1, 117.9, 117.3, 115.1, 54.8, 50.4, 38.6, 38.5, 38.2 ppm. ESI-HRMS: C₄₃H₅₉N₁₀O₁₄⁺ m/z = 939.4207 [M+H]⁺, error < 0.1 ppm.

A solution of Eu(SO₃CF₃)₃ (19 mg, 32 µmol) in MeOH (250 µL) was added to 26 (25 mg, 16 µmol), and the resulting mixture was stirred for 48 h at 29 °C. The reaction mixture was concentrated and hydrogenolyzed over 10% Pd/C (1.0 mg, 0.9 µmol) in MeOH (200 µL) for 41 h at 22 °C. The black suspension was filtered through celite, washed and concentrated to yield yellow solid, which was purified in by reversed phase column chromatography (ACN/H₂O/ 0.1%TFA) to yield 4 as a white solid (12.0 mg, 69 %). ESI-HRMS: C₄₃H₅ₙN₁₀O₁₄Eu²⁺ m/z = 544.1618 [M-H]⁺, error = 1.3 ppm.
27 was prepared according to the procedure reported by Stefankiewicz et al.\cite{13}. To a stirred white suspension of cyclen (61 mg, 0.4 mmol) and sodium acetate (96 mg, 1.2 mmol) in DMA (0.5 mL) at 23 °C under Ar conditions was added a solution of 27 (330 mg, 1.2 mmol) in DMA (1.0 mL, 250 µL/h). The reaction mixture was stirred for 20 h at 23 °C. A KHCO$_3$ solution (100 mL, 0.5 M in H$_2$O) was then added to the yellow solution, which was extracted with DCM (3×50 mL). The combined organic layers were washed with brine (100 mL), dried over Na$_2$SO$_4$, filtered and concentrated to yield a yellow oil. The crude material was suspended with K$_2$CO$_3$ (196 mg, 1.4 mmol) in ACN (0.5 mL). 28 was prepared according to the procedure reported by Abedin et al.\cite{14}. A solution of 28 (81 mg, 0.5 mmol) in ACN (1.0 mL) was added for 1 min under Ar conditions at 0 °C, and the suspension was stirred for 2 h at 23 °C. The precipitate was filtered and the filtrate was concentrated. The resulting yellow oil was dissolved in DCM (50 mL) and washed with H$_2$O (3×25 mL), dried over MgSO$_4$, filtered and concentrated to yield a pale solid (524 mg). The crude material was dissolved in DCM, TFA (1.5 mL, 19.6 mmol) was added at 0 °C under Ar conditions, and the yellow solution was stirred at 22 °C for 4 h. The yellow solution was concentrated to yield a brown oil, which was dissolved in ACN/H$_2$O (1/1, 0.1 % TFA, 1300 µL), purified by reversed phase column chromatography (column: C18, 250×30 mm, eluent: ACN/H$_2$O/0.1 % TFA, 40-min-long gradient from 0 % to 25 % ACN) to yield 29 as a yellow oil (193 mg, 60 %). $^1$H NMR (500 MHz, CD$_3$CN). δ 10.05 (bs, 7H, NH), 8.28-7.14 (m, 9H), 4.05-3.81 (m, 3H), 3.58-3.38 (m, 6H), 3.38 (bs, 4H), 3.21-3.09 (m, 5H), 3.01 (bs, 12H), 2.91 ppm (bs, 9H). $^{13}$C NMR (125 MHz, CD$_3$CN). δ 173.3, 72.8, 55.9, 55.1, 51.9, 50.1, 41.5, 40.7, 38.6, 38.2, 38.0, 35.8, 29.7, 20.7 ppm. ESI-HRMS: C$_{25}$H$_{50}$N$_1$O$_4$+ m/z = 568.4042 [M+H]$^+$, error < 0.1 ppm.

To a transparent solution of 14 (46 mg, 192 µmol) in DCM (200 µL) and DMF (25 µL) was added oxalyl chloride (33 µL, 390 µmol), and the pale solution was stirred at 21 °C for 0.5 h. The reaction mixture was concentrated and dried under vacuum for 0.5 h to yield a yellow
solid. To a solution of 29 (50 mg, 55 µmol) in KHCO$_3$ (1.5 mL, of a 0.5 M solution in H$_2$O) was added dropwise a pale suspension of the acyl chloride in dioxane (1.5 mL) for 1 min at 0 °C. The pale solution was stirred at 22 °C for 30 min. The pale solution was concentrated, diluted in ACN (2 mL), filtered and concentrated to yield a pale oil (110 mg). The yellow oil was diluted in ACN/H$_2$O (600 µL) and purified by reversed phase column chromatography (Phenomenex C18, 250×21.2 mm, 40-min-long gradient from 10 to 40 % ACN in H$_2$O) to yield 30 as a white solid (19 mg, 29 %). $^1$H NMR (500 MHz, CD$_3$CN). $\delta$ 8.02 (bs, 1H), 7.68 (bs, 1H), 7.54 (m, 4H), 7.45 (bs, 1H), 7.33 (m, 9H), 3.92 (m, 2H), 3.80-2.97 (m, 37H), 2.49 (t, J = 1.8 Hz, 1H), 2.25 ppm (m, 18H).

$^{13}$C NMR (125 MHz, CD$_3$CN). $\delta$ 169.6, 169.4, 166.5, 166.5, 144.3, 141.4, 131.4, 127.5, 127.3, 126.8, 126.8, 80.8, 72.5, 56.0, 51.2, 50.8, 47.2, 40.3, 40.2, 29.4, 20.9, 20.8 ppm. ESI-HRMS: C$_{58}$H$_{75}$N$_{11}$O$_{19}$BF$_2$Zn$_2^+$ m/z = 614.7617 [M+2H]$^{2+}$, error = 0.3 ppm.

To a solution of 30 (2.5 mg, 2.0 µmol) and BODIPY FL-azide (0.8 mg, 2.2 µmol) in DMSO (40 µL) and tBuOH (25 µL) was added a solution of TBTA (0.4 mg, 8.1 µmol) in DMSO (10 µL). Zn(OAc)$_2$ (1.1 mg, 6.1 µmol) was added to the solution, which was stirred for 30 min. A premixed yellow solution of CuSO$_4$ (0.36 mg, 2.2 µmol) and Na ascorbate (0.44 mg, 2.2 µmol) in water (25 µL) was added to the orange solution, which was stirred for 15 min. The resulting orange solution was diluted in ACN/H$_2$O (700 µL) and purified by reversed phase column chromatography (Phenomenex C18, 250×10 mm, 2 runs of 40-min-long gradient from 10 to 40 % ACN in H$_2$O) to yield 5 as an orange solid (0.7 mg, 21 %). $^1$H NMR (500 MHz, CD$_3$CN). $\delta$ 7.66 (m, 3H), 7.55 (m, 4H), 7.32 (m, 9H), 6.99 (s, 1H), 6.75 (m, 1H), 6.47-6.21 (m, 3H), 4.40 (s, 1H), 4.27 (s, 2H), 3.54-3.20 (m, 20H), 3.14 (bt, 2H), 3.08 (bs, 3H), 3.00-2.80 (bs, 7H), 2.80-2.60 (bs, 7H), 2.53 (m, 4H), 2.49 (s, 3H), 2.26 (s, 12H, CH$_3$CO$_2$), 2.23 (s, 6H, CH$_3$CO$_2$), 1.00 (s, 3H), 0.21 ppm (s, 3H). $^{13}$C NMR (125 MHz, CD$_3$CN). $\delta$ 173.1, 172.8, 172.4, 172.0, 169.8, 169.7, 169.7, 169.5, 169.3, 166.6, 161.3, 158.4, 146.0, 144.7, 144.4, 144.2, 141.4, 141.3, 136.0, 134.2, 131.4, 131.3, 131.2, 129.5, 127.6, 127.5, 127.4, 127.0, 126.9, 125.8, 124.3, 57.2, 56.8, 51.4, 48.4, 40.8, 40.7, 39.5, 39.5, 36.9, 35.7, 35.0, 30.7, 25.1, 21.1, 20.9, 20.8, 15.1, 11.4, 11.4 ppm. $^{19}$F NMR (CD$_3$CN, 471 MHz, q, $^1$J = 33 Hz). $\delta$ -146.25 ppm. ESI-HRMS: C$_{75}$H$_{96}$N$_{17}$O$_{39}$BF$_2$Zn$_2^+$ m/z = 832.8127 [M]$^{2+}$, error = 2.4 ppm.
To a stirred white suspension of cyclen (371 mg, 2.2 mmol) and sodium acetate (584 mg, 7.1 mmol) in DMA (3 mL) at 21 °C under Ar conditions was added a solution of 27 (2.00 g, 7.1 mmol) in DMA (7 mL, 1.2 mL/h). The reaction was stopped after stirring for 20 h at 21 °C. Potassium bicarbonate solution (350 mL, 0.2 M in H₂O) was then added to the yellow solution, which was extracted with DCM (3×100 mL). The combined organic layers were washed with brine (150 mL), dried over MgSO₄, filtered, concentrated and coevaporated with ACN (20 mL) to yield a pale solid.

19 (264 mg, 0.8 mmol) and K₂CO₃ (358 mg, 2.6 mmol) were added and suspended in ACN (5.0 mL) for 5 min under Ar conditions at 0 °C, and the yellow suspension was stirred for 3 h at 21 °C. The precipitate was filtered and the filtrate was concentrated. The resulting yellow oil was dissolved in DCM (50 mL) and washed with H₂O (3×50 mL), dried over MgSO₄, filtered and concentrated to yield a pale oil. The crude material was dissolved in DCM (4.0 mL), TFA (1.3 mL, 11.6 mmol) was added at 0 °C under Ar conditions and the yellow solution was stirred at 21 °C for 2 h and concentrated to yield a yellow oil. The crude material was dissolved in ACN/H₂O (1/1, 0.1 % TFA, 1250 µL) purified by reversed phase column chromatography (column: Phenomenex, C18, 250×40 mm, eluent: ACN/H₂O/0.1 % TFA, 40-min-long gradient from 10 % to 20 % ACN). Fractions containing the expected product were lyophilized overnight to yield 31 as a pale oil (403 mg, 75 %).

**¹H NMR** (500 MHz, CD₃CN). δ 7.87 (bs, 2H), 7.52 (m, 5H), 7.44-7.23 (m, 11H), 5.11 (s, 2H, CH₂C₆H₅), 3.94 (bs, 2H), 3.83 (bs, 2H), 3.54 (q, 2H, J = 5.5 Hz), 3.52-3.38 (m, 8H), 3.39-3.19 (m, 10H), 3.06-2.93 (m, 8H), 2.41 (t, 2H, J = 7.3 Hz, CH₂CH₂CO₂Bn), 1.80 ppm (m, 2H, CH₂CH₂CO₂Bn). **¹³C NMR** (125 MHz, CD₃CN). δ 174.2, 137.5, 129.6, 129.3, 129.1, 67.1, 56.2, 56.1, 55.1, 52.4, 49.9, 41.8, 40.8, 39.9, 38.3, 38.1, 32.0, 25.1 ppm. ESI-HRMS: C₃₃H₆₀N₁₁O₆⁺ m/z = 706.4723 [M+H]⁺, error < 0.1 ppm.
To a yellow solution of 14 (249 mg, 1.0 mmol) in DCM (2.5 mL) and DMF (0.25 mL) was added oxalyl chloride (177 µL, 2.1 mmol), and the orange solution was stirred at 20 °C for 1 h. The reaction mixture was concentrated and dried under the vacuum line for 1 h to yield a brown oil. To a solution of 31 (300 mg, 0.3 mmol) in KHCO₃ (7.8 mL, 0.5 M in H₂O) was added dropwise an orange suspension of the acyl chloride in dioxane (7.8 mL) for 1 min in a 0 °C bath. After addition, the mixture was stirred at 20 °C for 0.5 h. After concentration the orange solid was suspended in ACN, filtered and concentrated in the vacuum line for 30 min to yield an orange solid. The crude material was set to a hydrogen atmosphere over 10% Pd/C (32 mg, 0.03 mmol) in MeOH (5.0 mL) at 21 °C for 2 h. The black suspension was filtered through celite, washed and concentrated to yield a transparent oil, which was purified by reversed phase column chromatography (column: Phenomenex, C18, 250×40 mm, eluent: ACN/H₂O/0.1 % HCOOH, 40-min-long gradient from 20 to 50 % ACN) to yield 6 as a white solid (135 mg, 35 %). ¹H NMR (500 MHz, D₆-DMSO). δ 8.39 (m, 3H), 8.19 (m, 2H), 8.13 (m, 1H), 7.47 (dd, 3H, J = 5.3 Hz, J = 1.3 Hz, Ar-H), 7.39-7.33 (m, 6H, Ar-H), 3.43-2.77 (m, 38H), 2.28 (s, 9H, CH₃CO₂), 2.22 ppm (s, 9H, CH₃CO₂). ¹³C NMR (125 MHz, D₆-DMSO). δ 174.2, 172.0, 169.5, 169.1, 168.4, 168.3, 167.8, 164.8, 142.8, 140.1, 139.1, 130.7, 126.1, 126.1, 125.5, 117.7, 56.2, 51.0, 38.3, 38.0, 31.0, 24.5, 21.0, 20.4, 20.3, 20.2 ppm. ESI-HRMS: C₅₉H₇₈N₁₁O₂₁⁺ m/z = 1276.5382 [M+H]⁺, error = 1.1 ppm.

To a solution of 6 (10.0 mg, 7.8 µmol) and N-methylmorpholine (4.3 µL, 39 µmol) in anhydrous DMF (100 µL) was added ethyl chloroformate (3.7 µL, 39 µmol) at 21 ºC, and the reaction mixture was stirred for 10 min. A solution of ampicillin (13.7 mg, 39 µmol) and Et₃N (16.4 µL, 118 µmol) in DMF (50 µL) was added, and the mixture was stirred for 15 min at 21 ºC. The transparent solution was purified by preparative HPLC (column: Phenomenex, C18, 250×10 mm, eluent: ACN/H₂O/0.1 % HCOOH, 40-min-long gradient from 20 to 30 % ACN). The fractions were lyophilized to yield 8 as a white solid (7.1 mg, 56 %). ¹H NMR (500 MHz, D₆-DMSO). δ 9.08 (d, 1H), 8.51 (d, 1H), 8.41 (m, 3H), 8.21 (bs, 2H), 8.11 (m, 1H), 7.47 (dd, 3H, J = 5.4 Hz, J = 1.2 Hz, Ar-H), 7.41 (d, 3H, J = 5.2 Hz, Ar-H), 7.38-7.30 (m, 8H, Ar-H), 5.71 (d, 1H, J = 5.9 Hz), 5.49 (m, 1H), 5.37 (d, 1H, J = 2.9 Hz), 4.16 (d, 1H, J = 9.6 Hz), 3.42-3.13 (m, 24H), 3.13-3.00 (m, 3H), 2.95-2.79 (m, 11H), 2.25 (s, 18H, CH₂CO₂), 1.53 (s, 3H, CCH₃), 1.40 ppm (s, 3H, CCH₃). ¹³C NMR (125 MHz, D₆-DMSO). δ 173.3, 173.2,
171.6, 170.2, 169.7, 168.9, 164.7, 160.5, 142.8, 140.1, 138.3, 138.1, 138.0, 130.7, 128.2, 127.6, 127.5, 127.2, 127.1, 126.9, 126.1, 125.5, 70.8, 70.6, 67.2, 63.8, 58.0, 56.2, 55.4, 53.8, 51.1, 38.8, 38.2, 30.4, 26.7, 26.7, 25.4, 20.3, 20.2 ppm. ESI-HRMS: C\textsubscript{75}H\textsubscript{96}N\textsubscript{14}O\textsubscript{24}S\textsuperscript{2+} m/z = 804.3216 [M+2H]\textsuperscript{+}, error < 0.1 ppm.

To a solution of 8 (4.0 mg, 2.5 µmol) in dried MeOH (200 µL) was added anhydrous CuSO\textsubscript{4} (1.1 mg, 6.9 µmol) under Ar, and the blue solution was stirred for 15 min at 26 °C. The blue solution was concentrated, rediluted in ACN/H\textsubscript{2}O (600 µL), filtered and purified by reversed phase column chromatography (column: Phenomenex, C18, 250×10 mm, eluent: ACN/H\textsubscript{2}O, 40-min-long gradient from 10 to 40 % ACN in water) to yield 9 as a blue solid (3.5 mg, 85 %). ESI-HRMS: C\textsubscript{75}H\textsubscript{96}N\textsubscript{14}O\textsubscript{24}SCu\textsuperscript{2+} m/z = 834.7787 [M]\textsuperscript{2+}, error = 0.2 ppm.

To a solution of 26 (50 mg, 32 µmol) in THF (300 µL) was added a LiOH solution (1 M in H\textsubscript{2}O, 100 µL). The reaction was monitored by TLC (CH\textsubscript{2}Cl\textsubscript{2}:MeOH/8:2, UV), and the solution was stirred for 4 h at 25 °C. The reaction mixture was concentrated, rediluted in ACN (1.3 mL), filtered and purified by reversed phase column chromatography (column: Phenomenex, C18, 250×21 mm, eluent: ACN/H\textsubscript{2}O/0.1 % TFA) to yield 32 as a colorless oil (40.7 mg, 87 %). \textsuperscript{1}H NMR (500 MHz, D\textsubscript{6}-DMSO). δ 8.52 (bs, 2H, CONH), 8.27 (t, 2H, J = 27
5.9 Hz, CONH), 8.23 (t, 1H, J = 5.9 Hz, CONH), 8.18 (bs, 1H, CONH), 7.80 (bs, 1H), 7.51-7.45 (m, 6H, Ar-H), 7.43-7.37 (m, 6H, Ar-H), 7.37-7.31 (m, 9H, Ar-H), 7.31-7.24 (m, 12H, Ar-H), 7.15-7.06 (m, 6H, Ar-H), 5.21-5.16 (2×bs, 6H, ArOC6H5), 5.02-4.97 (2×bs, 6H, ArOC6H5), 4.07-2.94 ppm (m, 36H, DOTAM + NHCH2CH2NH).

13C NMR (125 MHz, D<sub>6</sub>-DMSO). δ 166.1, 166.0, 151.6, 145.2, 145.2, 137.0, 136.8, 130.8, 128.5, 128.4, 128.2, 128.0, 127.7, 124.2, 120.9, 116.0, 75.2, 75.1, 70.2, 38.8, 38.1 ppm. ESI-HRMS: C<sub>85</sub>H<sub>95</sub>N<sub>10</sub>O<sub>14</sub> + m/z = 1479.7032 [M+H]<sup>+</sup>, error = 0.5 ppm.

To a solution of 32 (10.0 mg, 6.8 µmol) in DMF (200 µL) were successively added HATU (4.4 mg, 11.6 µmol) and DIPEA (4.7 µL, 27.0 µmol). The reaction mixture was stirred at 25 °C for 10 min. A solution of 23 (11.1 mg, 20.3 µmol) in DMF (50 µL) was added to the reaction mixture and stirred for 1 h at 25 °C. More DIPEA (4.7 µL, 27.0 µmol) and HATU (4.4 mg, 11.6 µmol) were added to the reaction mixture, which was stirred for 3 further hours. The reaction mixture was filtered and purified by reversed phase column chromatography (column: Phenomenex, C18, 250×10 mm, eluent: ACN/H2O/0.1 % TFA, linear gradient from 30% to 80% ACN). The fractions were gathered and lyophilized to yield 10 as a dark blue solid (8.5 mg, 13.8±0.6% of water content assessed by 1H NMR, 56%). 1H NMR (700 MHz, D<sub>6</sub>-DMSO). δ 8.38 (bs, 3H, CONH), 8.26 (m, 3H, CONH), 8.00-7.81 (m, 2H, CONH), 7.46 (d, 6H, J = 7.3 Hz, Ar-H), 7.38 (t, 6H, J = 7.5 Hz, Ar-H), 7.36-7.31 (m, 9H, Ar-H), 7.31-7.24 (m, 18H, Ar-H), 7.15-7.07 (m, 8H, Ar-H), 7.03 (d, 4H, J = 9.3 Hz, Ar<sub>MG</sub>-H), 5.21-5.14 (2×bs, 6H, ArOCH<sub>2</sub>C<sub>6</sub>H<sub>5</sub>), 5.01-4.96 (2×bs, 6H, ArOCH<sub>2</sub>C<sub>6</sub>H<sub>5</sub>), 4.07 (t, 2H, J = 6.3 Hz, MG CH<sub>2</sub>NHCO), 3.77-3.53 (bs, 9H), 3.39-3.28 (m, 10H), 3.26 (s, 13H), 3.22-3.10 (m, 15H), 2.23 (t, 2H, J = 7.5 Hz, CH<sub>2</sub>CONH), 2.02 ppm (m, 2H, J = 7.0 Hz, J = 6.0 Hz, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 13C NMR (175 MHz, D<sub>6</sub>-DMSO). δ 176.0, 171.9, 166.1, 163.4, 156.3, 151.6, 145.1, 140.1, 137.3, 137.0, 136.7, 131.3, 130.8, 128.5, 128.3, 128.2, 128.0, 127.7, 126.2, 124.2, 120.9, 115.9, 114.9, 113.6, 75.1, 70.2, 67.8, 54.5, 49.6, 40.4, 40.0, 38.6, 38.4, 31.6, 24.6 ppm. ESI-HRMS: C<sub>114</sub>H<sub>131</sub>N<sub>15</sub>O<sub>15</sub> 3+ m/z = 645.3300 [M+2H]<sup>3+</sup>, error = 0.2 ppm.
Into a flask were added THF (200 mL, 0.1 M), 4,7,10-trioxa-1,13-tridecanediamine (22.0 mL, 100 mmol), triethylamine (2.77 mL, 20.0 mmol), and MeOH (70 mL, 0.3 M). The flask was fitted with a dropping funnel, maintained under a nitrogen atmosphere, and cooled in an ice bath (0 ºC). Benzyl chloroformate (2.84 mL, 20.0 mmol) was dissolved in THF (100 mL, 0.2 M) and added dropwise (over 45 min) to the reaction mixture, which was allowed to warm to room temperature and stirred (30 min). Volatiles were removed under reduced pressure.

The crude mixture was diluted with brine (200 mL) and sodium carbonate (10% aqueous, 40 mL), extracted with ether (150 + 2 × 100 mL), washed with brine (100 mL), and dried with sodium sulfate. Volatiles were removed under reduced pressure, to yield a mixture (approximately 4:1) of monocarbamate and dicarbonate, which was used directly for the next step. Product analysis is consistent with reported data. Crude monocarbamate: \(^1\)H NMR (CDCl\(_3\), 500 MHz) \(\delta\) 7.36–7.28 (m, 5H, Bn), 5.58–5.52 (brs, 1H, NHZ), 5.09 (s, 2H, Bn), 3.65–3.45 (m, 12H, 6×CH\(_2\)–O), 3.33–3.27 (m, 2H, CH\(_2\)–NHZ), 2.77 (t, J = 6.8 Hz, 2H, CH\(_2\)–NHZ), 1.80–1.66 (m, 4H, 2×C–CH\(_2\)–C). \(^{15}\)Bromoacetyl bromide (287 µl, 3.3 mmol) was added dropwise to the crude material (1062 mg, 3 mmol) and TEA (837 µl, 6 mmol) in 12 ml dichloromethane at 0°C. The reaction mixture was allowed to stir for 2 h, and the temperature gradually rose to room temperature. The solvents were evaporated followed by aqueous work up and extraction with ethyl acetate. The organic layer was washed with 10% citric acid, water and saturated sodium bicarbonate and brine and dried over anhydrous sodium sulfate, filtered and concentrated.

ESI-MS: C\(_{20}\)H\(_{31}\)BrN\(_2\)O\(_6\) m/z = 475.2 [M+H\(^+\)].

To a stirred solution of 24 (925 mg, 1.8 mmol) and K\(_2\)CO\(_3\) (994 mg, 7.2 mmol) in CNCH\(_3\) (50 mL) was added the crude material (1067 mg, 2.25 mmol) in CNCH\(_3\) (20 mL) within 10 min. Stirring was continued overnight under argon at room temperature. The precipitate was filtered and the filtrate concentrated. The resulting oil was purified by reversed phase-HPLC (10% to 90% MeCN) or Flash silica gel column chromatography (eluent: DCM:MeOH = 9:1-4:1) yielding 33 (0.644 g, 39%) as a white solid. \(^1\)H NMR (500 MHz, Chloroform-\(d\)) \(\delta\) 8.30 (s, 1H, -NH), 7.34 (bs, 2H, -Ar), 7.33 (bs, 2H, -Ar), 7.32–7.26 (m, 1H), 5.42 (s, 1H, -NH) 5.07 (s, 2H, -CH\(_2\)–Ar), 4.10 (bs, 2H, -CH\(_2\)–Ar), 3.88 (bs, 2H, -CH\(_2\)–Ar), 3.65–3.44 (m, 2H, -CH\(_2\)–Ar), 3.03 (bs, 8H, -CH\(_2\)–), 1.77-1.75 (m, 4H, -CH\(_2\)CH\(_2\)CH\(_2\)–), 1.48 (s, 9H, -tBu), 1.41 (s, 18H, -tBu). \(^{13}\)C NMR (125 MHz, CDCl\(_3\)) \(\delta\) 169.8, 161.4, 161.1, 160.8, 160.5, 156.6, 136.9, 128.5, 128.1, 119.6, 117.3, 115.0, 112.7, 82.5, 70.5, 70.4, 70.1, 69.5, 68.9, 66.5, 55.3, 55.2, 54.8, 51.9, 48.9, 39.2, 37.3, 29.5, 28.9, 28.1, 28.0. ESI-HRMS: C\(_{46}\)H\(_{81}\)N\(_6\)O\(_{12}\)\(^+\) m/z = 909.5905 [M+H\(^+\)], error = 0.2 ppm.
2eq. of compound 33 (18 mg, 20 µmol) was hydrogenolyzed over 10 % Pd on carbon (4 mg) in MeOH (5 mL) for 12 h. The Pd/C was removed by filtration and the MeOH removed by evaporation. The resulting product was dissolved in 1.5 ml DMF, followed by the addition of 1 equiv. Cy5.5 NHS ester (succinimidyl Ester) (7 mg, 10 µmol) and 5 equiv. DIPEA (10 µl, 50 µmol). The reaction mixture was stirred under argon at room temperature for 12 hours. After the reaction was complete, the DMF was removed by evaporation. 3 mL 95% TFA/H$_2$O was added, and the reaction mixture was stirred for 2 hours under argon at room temperature. After the reaction was complete, the solvent was 3 times co-evaporated with toluene. The reaction solution was purified by HPLC to give 13 (7.8 mg, 58 %) as a blue powder. $^1$H NMR (700 MHz, $D_6$-DMSO). $\delta$ 8.45 (t, 2H), 8.26 (dd, 1H), 8.07 (m, 3H), 7.74 (m, 3H), 7.68 (t, 2H), 7.52 (m, 2H), 6.62 (t, 1H), 6.34 (dd, 2H), 4.22 (t, 2H), 3.73 (s, 3H), 3.66-3.54 (bs, 5H), 3.54-3.47 (m, 6H), 3.47-3.44 (m, 3H), 3.44-3.41 (m, 4H), 3.41-3.33 (m, 6H), 3.33-3.29 (t, 3H), 3.29-3.08 (m, 9H), 3.08-2.97 (m, 7H), 2.04 (t, 2H), 1.96 (d, 8H), 1.74 (m, 2H), 1.64 (m, 2H), 1.54 (m, 4H), 1.37 (m, 2H). $^{13}$C NMR (175 MHz, $D_6$-DMSO). $\delta$ 174.3, 173.5, 171.7, 152.9, 140.4, 139.7, 133.1, 133.0, 131.3, 131.3, 130.3, 130.2, 129.9, 127.7, 127.6, 125.5, 124.8, 122.2, 111.6, 103.1, 102.8, 69.5, 69.5, 68.0, 67.9, 50.7, 43.4, 36.1, 35.7, 35.1, 31.5, 29.4, 29.1, 27.0, 26.8, 26.6, 25.7, 25.0 ppm. ESI-HRMS: C$_{66}$H$_{92}$N$_8$O$_{11}^2+$ m/z = 586.3439 [M+H]$^{2+}$, error = 0.3 ppm.

33 (91 mg, 0.1 mmol) was dissolved in 3 ml 95% TFA/H$_2$O, and the reaction mixture was stirred for 2 hours under argon at room temperature. The reaction was monitored by LC-MS. After the reaction was complete, the solvent was 3 times co-evaporated with toluene. The crude product was used without further purification in the next step. ESI-MS found: [M+H]$^+$ = 741.8. $^1$H NMR (500 MHz, Methanol-d$_4$) $\delta$ 7.42-7.30 (m, 5H, Ar), 5.11 (s, 2H, -CH$_2$-Ar), 3.87-
3.24 (m, 40H, -CH_{2}), 1.80 (m, 4H, -CH_{2}CH_{2}CH_{2}).^{13}C NMR (125 MHz, MeOD) δ 162.95, 162.68, 158.85, 138.50, 129.50, 128.99, 128.75, 119.35, 117.03, 71.50, 71.44, 71.15, 71.08, 69.98, 69.77, 67.32, 55.81, 54.68, 51.13, 39.24, 38.07, 30.85, 30.21. For removal of the Boc protection group, 15 (80 mg, 0.21 mmol) was dissolved in 3 ml 50% TFA/CH_{2}Cl_{2}, and the reaction mixture was stirred for 10 min under argon at room temperature. The reaction was monitored by LC-MS. After the reaction was complete, the solvent was 3 times co-evaporated with toluene. The crude product was used without further purification in the next step. ESI-MS found: [M+H]^{+} = 281.2. 1 equiv. of the unprotected form of 33 (52 mg, 0.07 mmol), 3 equiv. HATU (80 mg, 0.21 mmol) and 3.3 equiv. DIPEA (40 μL, 0.23 mmol) were dissolved in 3 mL DMF/CH_{2}Cl_{2} (1:1). After 10 min, the reaction mixture was added into 3 equiv. of the unprotected form of 15 (66 mg, 0.21 mmol) and stirred 30 min under argon at room temperature. The solution was concentrated in vacuo to remove the CH_{2}Cl_{2}, thendirectly and immediately purified by HPLC to give 34 (40 mg, 37 %) as a white powder. 34 is easily hydrolysed in weakly basic solution. ^{1}H NMR (500 MHz, Methanol-d_{4}) δ 7.58 – 7.50 (m, 3H), 7.41 – 7.31 (m, 10H), 5.09 (s, 2H, CH_{2}C_{6}H_{5}), 3.71 – 3.45 (m, 27H), 3.38 (t, J = 10.0, 8.0 Hz, 6H), 3.26 – 3.19 (m, 6H), 2.35 – 2.28 (m, 18H, CO_{2}C_{6}H_{5}), 1.78 (dp, J = 12.9, 6.4 Hz, 5H).^{13}C NMR (125 MHz, Methanol-d_{4}). δ 170.0, 169.9, 168.3, 158.8, 144.7, 141.8, 138.5, 131.8, 129.5, 129.0, 128.7, 127.7, 127.2, 125.6, 116.5, 71.5, 71.4, 71.2, 70.0, 69.7, 67.3, 56.2, 40.6, 40.0, 39.3, 38.3, 30.9, 30.3, 20.7, 20.4 ppm. ESI-HRMS C_{73}H_{100}N_{12}O_{24}^{2+} m/z = 764.3469 [M+2H]^{2+}, error = 1.7 ppm.

34 (31 mg, 20 μmol) was hydrogenolyzed over 10 % Pd on carbon (4 mg) in MeOH (5 mL) for 12 h. The Pd/C was removed by filtration, and the MeOH was removed by evaporation. Compounds were analyzed by LC/MS. ESI-MS found: [M+2H]^{2+} = 698.0. This procedure was repeated. To a solution of 21 (25 mg, 54 μmol) in DMF (2 mL) were successively added HATU (21 mg, 55 μmol) and DIPEA (16 μL, 92 μmol). A solution of the crude material (44 mg, 32 μmol) in DMF (3 mL) was added to the reaction mixture and stirred for 1 h at 25 °C. The reaction mixture was filtered and purified by reversed phase column
chromatography (column: Phenomenex, C18, 250×21 mm, eluent: ACN/H$_2$O/0.1 % HCOOH, linear gradient from 10 % to 70 % ACN). The fractions were gathered and lyophilized to yield 11 as a dark blue solid (0.4 mg, 1 %). $^1$H NMR (700 MHz, D$_6$-DMSO). δ 8.42 (m, 4H, CONH), 7.88 (m, 1H, CONH), 7.49 (d, 2H, J = 7.4 Hz), 7.41-7.28 (m, 11H, Ar-H), 7.19 (d, 2H, J = 8.9 Hz, Ar-H), 7.07 (d, 4H, J = 9.4 Hz, Ar-H), 4.14 (t, 2H), 3.76 (m, 2H), 3.52-3.46 (m, 8H), 3.46-3.41 (m, 7H), 3.40-3.35 (m, 7H), 3.34-3.26 (m, 23H), 3.25-3.20 (m, 7H), 3.20-2.20 (2×s, 18H, CH$_3$COO), 1.99 (m, 2H), 1.68-1.59 (m, 4H), 1.31-1.20 ppm.

$^{13}$C NMR (175 MHz, D$_6$-DMSO). δ 176.1, 171.3, 168.3, 167.8, 164.9, 163.5, 156.3, 142.9, 140.1, 137.4, 131.3, 130.6, 126.3, 126.2, 126.1, 125.6, 115.0, 113.6, 69.7, 69.5, 68.1, 68.0, 67.8, 63.3, 54.6, 53.6, 49.6, 42.9, 40.4, 40.0, 38.6, 38.4, 35.8, 31.5, 29.4, 29.0, 24.7, 20.3, 20.3 ppm. ESI-HRMS: C$_{92}$H$_{123}$N$_{14}$O$_{24}$3+ m/z = 602.6272 [M+2H]$_{3+}$, error = 0.1 ppm.

34 (31 mg, 20 µmol) was hydrogenolyzed over 10 % Pd on carbon (4 mg) in MeOH (5 mL) for 12 h. The Pd/C was removed by filtration, and the MeOH was removed by evaporation. Compounds were analyzed by LC/MS and used without further purification. ESI-MS found: [M+2H]$_{2+}$ = 698.0. Half of the crude material (14 mg, 10 µmol) was dissolved in 1.5 ml DMF, followed by the addition of 1 equiv. Cy5.5 NHS ester (succinimidyl ester) (7 mg, 10 µmol) and 5 equiv. DIPEA (10 µl, 50 µmol). The reaction mixture was stirred under argon at room temperature for 12 hours. After the reaction was complete, the reaction solution was directly purified by HPLC to give 12 (11 mg, Yield 56 %) as a blue powder. $^1$H NMR (700 MHz, D$_6$-DMSO). δ 9.05 (bs, 1H), 8.44 (m, 5H), 8.26 (m, 3H), 8.08 (m, 5H), 7.93 (m, 1H), 7.71 (m, 6H), 7.50 (m, 4H), 7.36 (m, 3H), 7.26 (m, 1H), 7.19 (m, 1H), 6.88 (m, 1H), 6.66 (m, 2H), 6.34 (dd, 2H), 5.33 (m, 1H), 4.22 (t, 2H), 3.89-2.76 (m, 54H), 2.26 (m, 9H), 2.21 (d, 5H), 2.02 (m, 4H), 1.96 (d, 12H), 1.73 (m, 2H), 1.62 (m, 2H), 1.54 (m, 2H), 1.54 (m, 5H), 1.46 (m, 2H), 1.37 (m, 3H), 0.85 ppm (t, 2H), 4.00-1.00 (m, 97H). $^{13}$C NMR (175 MHz, D$_6$-DMSO). δ 174.5, 174.3, 173.5, 172.0, 171.7, 169.6, 168.5, 168.3, 167.8, 164.9, 152.9, 142.8, 140.4, 140.1, 139.7, 139.1, 133.1, 130.0, 131.3, 130.3, 130.0, 129.2, 129.9, 129.6, 127.7, 127.6, 127.5, 127.0, 126.2, 126.1, 125.5, 124.8, 124.6, 122.2, 122.1, 118.1, 117.8, 115.9, 111.6, 103.1, 102.8, 72.5, 69.8, 69.7, 69.5, 68.0, 68.0, 63.1, 54.6, 50.7, 49.5, 43.4, 38.6, 38.4, 35.7, 35.1, 33.7, 31.5, 31.3, 29.4, 29.129.1, 29.0, 28.8, 28.7, 28.7, 28.6, 28.6,
27.0, 26.8, 26.6, 26.6, 25.7, 25.1, 25.0, 22.1, 20.4, 20.3, 20.2, 14.0 ppm. ESI-HRMS: C$_{105}$H$_{134}$N$_{14}$O$_{23}^2^+$ m/z = 979.4855 [M+H]$^2^+$, error = 1.3 ppm. A partial deacetylation could be observed in the LCMS and $^1$H NMR data.

7. $^1$H-NMR, $^{13}$C-NMR and high resolution mass spectra
1. \[+\text{MS}, 0.64-1.37\text{min} \] #145-316, -Peak Bkgrnd

2. \[+\text{MS}, 0.62-1.38\text{min} \] #140-319, -Peak Bkgrnd

\[C_{18}H_{24}N_2O_7Na, \quad m/z = 403.1476\]

- Intens.
- m/z

[Graphs and charts showing data and peaks]
2. +MS, 11.83-12.28 min #1402-1456, -Peak Bkgrnd

3. +MS, 11.80-12.48 min #1399-1480, -Peak Bkgrnd

C₁₃H₁₆NO₃BrNa, m/z 336.0206
1. +MS, 0.63-2.95 min #110-652, -Peak Bkgrnd

2. +MS, 0.59-3.00 min #102-665, -Peak Bkgrnd

C₄₇H₆₇N₁₁O₁₅, M, 512.7404
1. +MS, 0.71-2.93 min #164-684, -Peak Bkgrnd

2. +MS, 0.67-3.10 min #155-723, -Peak Bkgrnd

C₂₅H₅₀N₁₁O₄, M, 568.4042
1. +MS, 0.54-1.51 min #89-315, -Spectral Bkgrnd, -Peak Bkgrnd

C₅₈H₇₅N₁₁O₁₉, M, 614.7615

2. +MS, 0.51-1.66 min #84-351, -Spectral Bkgrnd, -Peak Bkgrnd

C₅₈H₇₅N₁₁O₁₉, M, 614.7615

Intens.
1. +MS, 0.55-1.60 min #92-338, -Peak Bkgrnd

2. +MS, 0.52-1.64 min #86-346, -Peak Bkgrnd

C₇₅H₉₄BF₂N₁₇O₂₀Zn, M, 832.8101

m/z 832 833 834 835 836

Intens. 5 x 10^2

m/z 832 833 834 835 836

Intens. 5 x 10^2

m/z 832 833 834 835 836

Intens. 5 x 10^2
1. +MS, 0.60-2.87min #138-668, -Peak Bkgrnd

2. +MS, 0.57-3.03min #132-705, -Peak Bkgrnd

C₃₃H₆₀N₁₁O₆, 706.4723
C₇₅H₉₆N₁₄O₂₄S, M, 804.3216

2.

+MS, 10.7-10.9min #2456-2503, -Peak Bkgrnd

Intens. 0 500 1000 1500 2000

m/z 804.25 804.50 804.75 805.00 805.25 805.50 805.75 806.00 806.25

ppm 200 150 100 50 0
8. Abbreviations and general methods

Boc = tert-butyloxycarbonyl

DIPEA = diisopropyl-ethyl amine

DMA = N,N-dimethylacetamide

DMF = dimethylformamide

DCM = dichloromethane

DOTA = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid

DOTAM = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid amide

DPBS = Dulbecco’s Phosphate-Buffered Saline

ESI-MS = electrospray ionization mass spectrometry

equiv. = equivalents

HATU = 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate.

HPLC = high performance liquid chromatography

HRMS = high resolution mass spectrometry

LC-MS = liquid chromatography mass spectrometry

LRMS = low resolution mass spectrometry

NMR = nuclear magnetic resonance

TEA = triethylamine

TFA = trifluoroacetic acid

Unless otherwise noted, all reagents were purchased from commercial suppliers and used without further purification. All solvents used were of HPLC grade. Reactions were analyzed by LC-MS. Reverse-phase HPLC was performed on a Gemini, 10u, C18, 110A, 250×10.00 mm, 10 µm or a Gemini, 5u, C18, 110A, 250×21.20 mm, 5 µm or a Nucleosil 100-7 VP, C18, 250×40 mm column. Low resolution LC-MS data were acquired using a Agilent 1100/AB Sciex API 150 EX or a Dionex Ultimate
3000/Bruker amazon ion trap system. High resolution LC-MS data were acquired using a Dionex Ultimate 3000/Bruker maXis HD system. NMR-data were recorded on a Bruker Avance III (500 MHz for $^1$H NMR and 125 MHz for $^{13}$C NMR) or a Bruker Avance III HD with cryoprobe (700 MHz for $^1$H NMR and 175 MHz for $^{13}$C NMR) system in $D_6$-DMSO, CDCl$_3$, CD$_3$OD or CD$_3$CN.

9. Author contributions

HH and KF designed and synthesized conjugates.

VF designed and constructed the FAP system and characterized the compounds in cellular experiments.

HP characterized the compounds in cellular experiments.

PPM and BR designed and conducted the in vivo experiments.

MB conceived the study and wrote the manuscript.

10. References