Supplementary Materials for

**Substrate-bound and substrate-free outward-facing structures of a multidrug ABC exporter**

Vincent Chaptal, Veronica Zampieri, Benjamin Wiseman, Cédric Orelle, Juliette Martin, Kim-Anh Nguyen, Alexia Gobet, Margot Di Cesare, Sandrine Magnard, Waqas Javed, Jad Eid, Arnaud Kilburg, Marine Peuchmaur, Julien Marcoux, Luca Monticelli, Martin Hogbom, Guy Schoehn, Jean-Michel Jault, Ahcène Boumendjel, Pierre Falson*

*Corresponding author. Email: pierre.falson@univ-lyon1.fr

Published 26 January 2022, *Sci. Adv.* 8, eabg9215 (2022)

DOI: 10.1126/sciadv.abg9215

The PDF file includes:

- Supplementary Text
- Figs. S1 to S19
- Tables S1 and S2
- Legends for data S1 and S2
- Legend for Rhodamine6G.itp
- Legend for ForcefieldR6G.itp
- References

Other Supplementary Material for this manuscript includes the following:

- Data S1 and S2
- Rhodamine6G.itp
- ForcefieldR6G.itp
Chemistry

Solvents and reagents were purchased from commercial sources and used without further purification. Reactions were monitored by thin layer chromatography (TLC) using commercial silica gel 60 F_{254} coated plates from Macherey-Nagel. Visualization was carried out under UV light at 254 and 365 nm and/or heating with a solution of sulfuric acid/acetic acid/water or phosphomolybdic acid/cerium sulfate/sulfuric acid/water or ninhydrin stain or iodine vapor. Purifications were performed by gravity column chromatography using silica gel 60 (230-400 mesh) from Macherey-Nagel or by automatic Reveleris® X2 flash chromatography system. MPs were measured using a Büchi B540 melting point apparatus and are uncorrected. Electrospray ionization (ESI) mass spectra were obtained on an Esquire 3000 Plus Bruker Daltonis instrument with a nanospray inlet. Accurate mass measurements (HRMS) were carried out on an ESI/QTOF with the Waters Xevo G2-S QTof device. Analyses were performed by the analytical service of Institut de Chimie Moléculaire de Grenoble (ICMG). Spectra were recorded in deuterated solvents on Bruker Avance spectrometers at 400 or 500 MHz for $^1$H and 100 or 125 MHz for $^{13}$C NMR, respectively. Chemical shifts (δ) are reported in parts per million (ppm) relative to the solvent [$^1$H: δ(acetone-$d_6$) = 2.05 ppm, δ(DMSO-$d_6$) = 2.50 ppm, δ(CD$_3$OD) = 3.31 ppm, δ(ClC$_6$H$_4$) = 7.26 ppm; $^{13}$C: δ(DMSO-$d_6$) = 39.5 ppm, δ(ClC$_6$H$_4$) = 49.0 ppm, δ(ClC$_6$H$_4$) = 77.2 ppm, δ(acetone-$d_6$) = 206.3 ppm]. Multiplicity of signals is reported as followed: s (singlet), bs (broad singlet), d (doublet), t (triplet), q (quartet), qt (quintet), st (septet), dd (doublet of doublet), ddd (doublet of doublet of doublet), dt (doublet of triplet), ddt (doublet of doublet of triplet) and m (multiplet). Coupling constants (J) are given in Hertz (Hz). When direct signal assignments were difficult, additional spectra were acquired (J-mod, COSY, HMQC or HMBC).

Synthesis of amphiphiles 3a-3e as crystallization additives

Crystallization additives were obtained according to the synthetic scheme shown below.
Reagents and Conditions. i. TBTU, DIEA, DMF; ii. Et₂NH, CH₂Cl₂; iii. R-CO-Cl, DMAP, pyridine, CH₂Cl₂; vi. H₂, Pd/C, MeOH; v. TFA, CH₂Cl₂.

Synthesis of compound 1. Dibenzyl (R)-2-[(S)-2-(((9H-fluoren-9-yl)methoxycarbonyl) amino)-3-tert-butoxypropanamido] glutarate.

To a solution of protected serine (3.5 g, 9.12 mmol) in anhydrous DMF (15 mL/mmol) were successively added the glutamic acid diester (9.0 g, 18.24 mmol, 2 equiv.), TBTU (1.2 equiv.) and DIPEA (5 equiv.). The mixture was stirred at room temperature (rt) under N₂ atmosphere for 3 h. After completion of the reaction, water (15 mL/mmol) was added. The compound precipitated and was crystallized in a mixture of CH₂Cl₂/Et₂O to provide compound 1 (5.14 g, 81% yield).

\[ R_f = 0.50 \text{ (cyclohexane/EtOAc 7:3)}; \text{MP} = 126-128 ^\circ C; \text{^1}H \text{ NMR (400 MHz, CDCl}_3 \text{) \delta ppm 1.19 (s, 9H), 1.99-2.11 (m, 1H), 2.22-2.35 (m, 1H), 2.32-2.55 (m, 2H), 3.41 (dd, } J = 8.3, 8.3 \text{ Hz, 1H), 3.75-3.87 (m, 1H), 4.23 (t, } J = 7.1 \text{ Hz, 1H), 4.24-4.33 (m, 1H), 4.40 (d, } J = 6.8 \text{ Hz, 2H), 4.68-4.76 (m, 1H), 5.09 (s, 2H), 5.17 (s, 2H), 5.78 (bs, 1H), 7.21-7.46 (m, 15H), 7.61 (d, } J = 7.0 \text{ Hz, 2H), 7.76 (d, } J = 7.5 \text{ Hz, 2H). ^13C \text{ NMR (100 MHz, CDCl}_3 \text{) \delta ppm 27.4 (3xCH}_3 \text{), 27.5 (CH}_2 \text{), 30.0 (CH}_2 \text{), 47.1 (CH), 51.8 (CH), 54.6 (CH), 61.7 (CH}_2 \text{), 66.5 (CH}_2 \text{), 67.2 (CH}_2 \text{), 67.4 (CH}_2 \text{), 74.3 (C), 120.0 (2xCH), 125.1 (2xCH), 127.1 (2xCH), 127.7 (2xCH), 128.2-128.7 (10xCH), 135.1 (C), 135.7 (C), 141.3 (2xC), 143.7 (2xC), 156.1 (C), 170.1 (C), 171.2 (C), 172.3 (C); MS (ESI+) \text{ m/z} \text{ (%) 426 (100), 570 (3), 715 (1) [M+Na]^+}; \text{HRMS (ESI+) m/z, calculated for C}_{41}H_{45}N_2O_8 693.3176, found 693.3156.}

Synthesis of compounds 2.

Fmoc deprotection. To a solution of compound 1 (1 equiv.) in anhydrous dichloromethane (20 mL/mmol) was added diethylamine (20 equiv.). The reaction mixture was stirred at rt under N₂ atmosphere overnight. The volatiles were removed under reduced pressure. To eliminate the residual diethylamine, the crude product was diluted in dichloromethane, washed with a saturated sodium bicarbonate (NaHCO₃) solution, dried over MgSO₄, filtered, and concentrated under reduced pressure and used for the next steps without further purification.

Amide formation. The crude compound obtained in the previous step (1 equiv.) was dissolved in anhydrous dichloromethane (30 mL/mmol). The acyl chloride derivative was added (2 equiv.), together with dimethylaminopyridine (DMAP) (0.5 equiv.) and pyridine (34 equiv.). The reaction mixture was stirred at rt under N₂ atmosphere overnight. The reaction mixture was acidified to pH = 3 with an aqueous solution of HCl 10% and extracted with dichloromethane. The combined organic layers were washed with brine and dried over MgSO₄, filtered, and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography.

Compound 2a. Dibenzyl (R)-2-[[S]-3-tert-butoxy-2-(decanamido)propanamido]glutarate

The crude product was prepared starting from 1 (500 mg, 0.70 mmol) and commercially available decanoyl chloride (267 mg, 1.40 mmol). After purification by column chromatography on silica gel (cyclohexane/EtOAc 8:2 to 7:3), the pure product 2a (195 mg, 0.31 mmol, 45%) was obtained as a white solid.
Rf = 0.18 (cyclohexane/EtOAc 7:3); MP = 82-85 °C; 

1H NMR (400 MHz, CDCl3) δ ppm 0.87 (t, J = 6.9 Hz, 3H), 1.16 (s, 9H), 1.19-1.35 (m, 12H), 1.55-1.66 (m, 2H), 1.96-2.07 (m, 1H), 2.21 (t, J = 7.6 Hz, 2H), 2.21-2.30 (m, 1H), 2.32-2.50 (m, 2H), 3.31 (dd, J = 8.7, 8.7 Hz, 1H), 3.80 (dd, J = 8.7, 4.2 Hz, 1H), 4.44-4.50 (m, 1H), 4.65-4.72 (m, 1H), 5.08 (s, 2H), 5.15 (s, 2H), 6.40 (d, J = 6.4 Hz, 1H, NH), 7.27-7.36 (m, 11H); 

13C NMR (100 MHz, CDCl3) δ ppm 14.1 (CH3), 22.7 (2xCH2), 25.6 (CH2), 27.4 (3xCH3), 27.5 (CH2), 29.3-29.44 (4xCH2), 30.0 (CH2), 31.9 (CH2), 36.6 (CH2), 51.8 (CH), 53.0 (CH), 61.3 (CH2), 66.5 (CH2), 67.3 (CH2), 74.3 (C), 128.3-128.7 (10xCH), 135.2 (C), 135.8 (C), 170.4 (C), 171.2 (C), 172.3 (C), 173.3 (C); MS (ESI+) m/z (%) 626 (30) [M+H]+, 648 (100) [M+Na]+; HRMS (ESI+) m/z, calculated for C36H53N2O7 625.3853, found 625.3846.

**Compound 2b.** Dibenzyl (R)-2-[(S)-3-tert-butoxy-2-(dodecanamido)propanamido]glutarate

The crude product was prepared starting from 1 (400mg, 0.58 mmol) and commercially available dodecanoyl chloride (252 mg, 1.15 mmol). After purification by column chromatography on silica gel (cyclohexane/EtOAc 8:2 to 7:3), the pure product 2b (182 mg, 0.28 mmol, 48%) was obtained as a white solid.

Rf = 0.12 (cyclohexane/EtOAc 8:2); MP = 67-69 °C; 

1H NMR (400 MHz, CDCl3) δ ppm 0.87 (t, J = 6.9 Hz, 3H), 1.17 (s, 9H), 1.19-1.36 (m, 16H), 1.56-1.67 (m, 2H), 1.96-2.08 (m, 1H), 2.21 (t, J = 7.6 Hz, 2H), 2.21-2.32 (m, 1H), 2.32-2.50 (m, 2H), 3.30 (dd, J = 8.7, 8.7 Hz, 1H), 3.80 (dd, J = 8.7, 4.2 Hz, 1H), 4.44-4.49 (m, 1H), 4.64-4.72 (m, 1H), 5.09 (s, 2H), 5.15 (s, 2H), 6.38 (d, J = 6.3 Hz, 1H, NH), 7.23-7.39 (m, 11H); 

13C NMR (100 MHz, CDCl3) δ ppm 14.2 (CH3), 22.8 (CH2), 25.6 (CH2), 27.5 (3xCH3), 27.6 (CH2), 29.4-29.7 (6xCH2), 30.0 (CH2), 32.0 (CH2), 36.7 (CH2), 51.9 (CH), 53.1 (CH), 61.4 (CH2), 66.6 (CH2), 67.5 (CH2), 74.4 (C), 128.4-128.8 (10xCH), 135.2 (C), 135.9 (C), 170.4 (C), 171.3 (C), 172.4 (C), 173.4 (C); MS (ESI+) m/z (%) 131 (30), 199 (40), 654 (50) [M+H]+, 677 (100), 699 (20); HRMS (ESI+) m/z, calculated for C38H57N2O7 653.4166, found 653.4158.

**Compound 2c.** Dibenzyl (R)-2-[(S)-3-tert-butoxy-2-(tridecanamido)propanamido]glutarate

The crude product was prepared starting from 1 (500mg, 0.70 mmol) and commercially available tridecanoyl chloride (326 mg, 1.40 mmol). After purification by column chromatography on silica gel (cyclohexane/EtOAc 8:2 to 7:3), the pure product 2c (233 mg, 0.35 mmol, 50%) was obtained as a white solid.

Rf = 0.24 (cyclohexane/EtOAc 7:3); MP = 68-71 °C; 

1H NMR (400 MHz, CDCl3) δ ppm 0.87 (t, J = 6.9 Hz, 3H), 1.17 (s, 9H), 1.20-1.36 (m, 18H), 1.55-1.67 (m, 2H), 1.96-2.08 (m, 1H), 2.21 (t, J = 7.6 Hz, 2H), 2.21-2.32 (m, 1H), 2.32-2.50 (m, 2H), 3.30 (dd, J = 8.7, 8.7 Hz, 1H), 3.80 (dd, J = 8.7, 4.2 Hz, 1H), 4.44-4.49 (m, 1H), 4.64-4.72 (m, 1H), 5.09 (s, 2H), 5.15 (s, 2H), 6.41 (d, J = 6.4 Hz, 1H, NH), 7.25-7.37 (m, 11H); 

13C NMR (100 MHz, CDCl3) δ ppm 14.1 (CH3), 22.7 (2xCH2), 25.5 (CH2), 27.4 (3xCH3), 27.5 (CH2), 29.3-29.7 (7xCH2), 29.9 (CH2), 31.9 (CH2), 36.5 (CH2), 51.8 (CH), 53.0 (CH), 61.3 (CH2), 66.5 (CH2), 67.3 (CH2), 74.2 (C), 128.3-128.6 (10xCH), 135.2 (C), 135.8 (C), 170.4 (C), 171.2 (C), 172.3 (C), 173.3 (C); MS (ESI+) m/z (%) 668 (20) [M+H]+, 690 (100) [M+Na]+; HRMS (ESI+) m/z, calculated for C39H59N7O7 667.4322, found 667.4334.
**Compound 2d.** Dibenzyl (R)-2-[(S)-3-t-butoxy-2-(tetradecanamido)propanamido] glutarate.

\[
\begin{align*}
\text{O} & \quad \text{O} \\
\text{t-BuO} & \quad \text{NH} \\
(\text{CH}_2)_2 & \quad (\text{CH}_2)_2 \\
\text{COOBn} & \quad \text{COOBn}
\end{align*}
\]

The crude product was prepared starting from 1 (400 mg, 0.58 mmol) and commercially available tetradecanoyl chloride (285 mg, 1.15 mmol). After purification by column chromatography on silica gel (cyclohexane/EtOAc 8:2 to 7:3), the pure product 2d (187 mg, 0.27 mmol, 48%) was obtained as a white solid. 

\(R_f=0.07\) (cyclohexane/EtOAc 8:2); MP = 71-73 °C; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) ppm 0.88 (t, \(J = 6.9\) Hz, 3H), 1.17 (s, 9H), 1.20-1.36 (m, 20H), 1.56-1.67 (m, 2H), 1.97-2.08 (m, 1H), 2.21 (t, \(J = 7.6\) Hz, 2H), 2.23-2.50 (m, 3H), 3.29 (dd, \(J = 8.7, 8.7\) Hz, 1H), 3.80 (dd, \(J = 8.7, 4.2\) Hz, 1H), 4.41-4.49 (m, 1H), 4.63-4.72 (m, 1H), 5.09 (s, 2H), 5.14 (d, \(J = 12.3\) Hz, 1H), 5.18 (d, \(J = 12.3\) Hz, 1H), 7.25-7.39 (m, 11H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) ppm 14.2 (CH\(_3\)), 22.8 (CH\(_2\)), 25.6 (CH\(_2\)), 27.5 (3xCH\(_3\)), 27.6 (CH\(_2\)), 29.4-29.8 (8xCH\(_2\)), 30.0 (CH\(_2\)), 32.0 (CH\(_2\)), 36.7 (CH\(_2\)), 51.9 (CH), 53.1 (CH), 61.4 (CH\(_2\)), 66.6 (CH\(_2\)), 67.5 (CH\(_2\)), 74.4 (C), 128.4-128.8 (10xCH), 135.2 (C), 135.8 (C), 170.5 (C), 171.3 (C), 172.4 (C), 173.4 (C); MS (ESI+) \(m/z\) (%) 131 (65), 199 (100), 682 (60) \([\text{M}+\text{H}]^+\); HRMS (ESI+) \(m/z\) calculated for C\(_{40}\)H\(_{61}\)N\(_2\)O\(_7\) 681.4479, found 681.4447.

**Compound 2e.** Dibenzyl (R)-2-[(S)-3-tert-butoxy-2-(hexadecanamido)propanamido]glutarate

\[
\begin{align*}
\text{O} & \quad \text{O} \\
\text{t-BuO} & \quad \text{NH} \\
(\text{CH}_2)_{14} & \quad (\text{CH}_2)_2 \\
\text{COOBn} & \quad \text{COOBn}
\end{align*}
\]

The crude product was prepared starting from 1 (300 mg, 0.43 mmol) and commercially available hexadecanoyl chloride (238 mg, 0.87 mmol). After purification by column chromatography on silica gel (cyclohexane/EtOAc 8:2), the pure product 2e (93 mg, 0.13 mmol, 30%) was obtained as a white solid.

\(R_f=0.11\) (8:2 cyclohexane/EtOAc). MP = 69-71 °C. \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) ppm 0.88 (t, \(J = 6.8\) Hz, 3H), 1.17 (s, 9H), 1.20-1.37 (m, 24H), 1.55-1.68 (m, 2H), 1.97-2.09 (m, 1H), 2.21 (t, \(J = 7.6\) Hz, 2H), 2.16-2.51 (m, 3H), 3.29 (dd, \(J = 8.7, 8.7\) Hz, 1H), 3.81 (dd, \(J = 8.7, 4.2\) Hz, 1H), 4.39-4.49 (m, 1H), 4.62-4.72 (m, 1H), 5.09 (s, 2H), 5.14 (d, \(J = 12.3\) Hz, 1H), 5.18 (d, \(J = 12.3\) Hz, 1H), 6.36 (d, \(J = 6.3\) Hz, 1H), 7.20-7.40 (m, 11H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) ppm 14.3 (CH\(_3\)), 22.8 (CH\(_2\)), 25.6 (CH\(_2\)), 27.5 (3xCH\(_3\)), 27.6 (CH\(_2\)), 29.4-29.8 (8xCH\(_2\)), 30.0 (CH\(_2\)), 32.0 (CH\(_2\)), 36.7 (CH\(_2\)), 51.9 (CH), 53.1 (CH), 61.4 (CH\(_2\)), 66.6 (CH\(_2\)), 67.5 (CH\(_2\)), 74.4 (C), 128.4-128.8 (10xCH), 135.2 (C), 135.8 (C), 170.5 (C), 171.3 (C), 172.4 (C), 173.4 (C); MS (ESI+) \(m/z\) (%) 199 (15), 710 (100) \([\text{M}+\text{H}]^+\), 732 (15) \([\text{M}+\text{Na}]^+\); HRMS (ESI+) \(m/z\) calculated for C\(_{42}\)H\(_{65}\)N\(_2\)O\(_7\) 709.4792 \([\text{M}+\text{H}]^+\), found 709.4805.

**Synthesis of compounds 3**

*Catalytic hydrogenolysis.* To a degassed solution of a compound 2 (1 equiv.) in MeOH (100 mL/mmol) was added Pd/C 10% (200 mg/mmol). The reaction mixture was stirred at rt under H\(_2\) atmosphere from 4 h to overnight. After filtration over Celite® to remove the catalyst, the solvent was evaporated under reduced pressure. The residue was used directly for the next step or washed with cyclohexane and/or dichloromethane to obtain the product which was used as is for the t-Bu deprotection step.

*t-Butyl deprotection.* To a solution of t-Bu-intermediate, obtained in the previous step (1 equiv.) in anhydrous dichloromethane (12 mL/mmol) at 0 °C was added dropwise TFA (4 mL/mmol). The reaction mixture was stirred at rt under N\(_2\) atmosphere overnight. The
volatiles were removed under reduced pressure and the residue was dissolved in DCM. A solution of NaOH (2 M) was added to pH = 11-12. The aqueous layer was washed with EtOAc before being acidified to pH 1-2 with concentrated HCl and extracted 3 times with EtOAc. The combined organic layers were dried over MgSO$_4$, filtered and concentrated under reduced pressure. The residue was washed with DCM to obtain the pure product.

**Compound 3a. (R)-2-[(S)-2-(Decanamido)-3-hydroxypropanamido]glutaric acid.**

The pure product (white solid, 182 mg, 0.47 mmol, 94%) was prepared starting from 2a (310 mg, 0.50 mmol). MP = 53-57 °C; $^1$H NMR (400 MHz, CD$_3$OD) δ ppm 0.89 (t, J = 6.8 Hz, 3H), 1.22-1.38 (m, 12H), 1.56-1.69 (m, 2H), 1.90-2.03 (m, 1H), 2.14-2.26 (m, 1H), 2.29 (t, J = 7.3 Hz, 2H), 2.36-2.43 (m, 2H), 3.73-3.84 (m, 2H), 4.42-4.52 (m, 2H); $^{13}$C NMR (100 MHz, CD$_3$OD) δ ppm 14.4 (CH$_3$), 23.7 (CH$_2$), 26.8 (CH$_2$), 27.9 (CH$_2$), 30.3-30.5 (4xCH$_2$), 31.1 (CH$_2$), 33.0 (CH$_2$), 36.9 (CH$_2$), 53.3 (CH$_2$), 56.6 (CH), 63.1 (CH$_2$), 172.5 (C), 174.9 (C), 176.5 (C), 176.5 (C); MS (ESI-) m/z (%) 387 (100) [M-H]$, 404 (20); HRMS (ESI-) m/z calculated for C$_{18}$H$_{31}$N$_2$O$_7$ 387.2131 [M-H], found 387.2140.

**Compound 3b. (R)-2-[(S)-2-(Dodecanamido)-3-hydroxypropanamido]glutaric acid.**

The pure product (white solid, 1.68 g, 4.04 mmol, 70%) was prepared starting from 2b (3.76 g, 5.80 mmol). MP = 100-102 °C; $^1$H NMR (400 MHz, CD$_3$OD) δ ppm 0.90 (t, J = 6.9 Hz, 3H), 1.22-1.38 (m, 16H), 1.57-1.67 (m, 2H), 1.91-2.01 (m, 1H), 2.15-2.26 (m, 1H), 2.29 (t, J = 7.2 Hz, 2H), 2.37-2.44 (m, 2H), 3.72-3.81 (m, 2H), 4.43-4.51 (m, 2H); $^{13}$C NMR (100 MHz, CD$_3$OD) δ ppm 14.4 (CH$_3$), 23.7 (CH$_2$), 26.8 (CH$_2$), 27.9 (CH$_2$), 30.4-30.7 (6xCH$_2$), 31.0 (CH$_2$), 33.1 (CH$_2$), 36.9 (CH$_2$), 53.1 (CH), 56.6 (CH), 63.1 (CH$_2$), 172.6 (C), 174.6 (C), 176.4 (C), 176.5 (C); MS (ESI-) m/z (%) 157 (40), 199 (30), 387 (80), 415 (100) [M-H]; HRMS (ESI-) m/z calculated for C$_{20}$H$_{35}$N$_2$O$_7$ 415.2444 [M-H], found 415.2447.

**Compound 3c. (R)-2-[(S)-2-(Tridecanamido)-3-hydroxypropanamido]glutaric acid.**

The pure product (white solid, 115 mg, 0.27 mmol, 89%) was prepared starting from 2c (198 mg, 0.30 mmol). MP = 58-63 °C; $^1$H NMR (400 MHz, CD$_3$OD) δ ppm 0.89 (t, J = 6.9 Hz, 3H), 1.21-1.41 (m, 18H), 1.54-1.68 (m, 2H), 1.90-2.04 (m, 1H), 2.14-2.27 (m, 1H), 2.29 (t, J = 7.4 Hz, 2H), 2.36-2.48 (m, 2H), 3.71-3.83 (m, 2H), 4.43-4.53 (m, 2H); $^{13}$C NMR (100 MHz, CD$_3$OD) δ ppm 14.4 (CH$_3$), 23.7 (CH$_2$), 26.8 (CH$_2$), 27.8 (CH$_2$), 30.3-30.9 (7xCH$_2$), 31.0 (CH$_2$), 33.0 (CH$_2$), 36.9 (CH$_2$), 53.1 (CH), 56.6 (CH), 63.1 (CH$_2$), 172.6 (C), 174.6 (C), 176.4 (C), 176.5 (C); MS (ESI-) m/z (%) 429 (100) [M-H], 446 (30); HRMS (ESI-) m/z calculated for C$_{21}$H$_{37}$N$_2$O$_7$ 429.2599, found 429.2599.

**Compound 3d. (R)-2-[(S)-2-(Tetradecanamido)-3-hydroxypropanamido]glutaric acid**
The pure product (white solid, 57 mg, 0.13 mmol, quantitative) was prepared starting from 2d (87 mg, 0.13 mmol). MP = 109-112 °C; 1H NMR (400 MHz, CD3OD) δ ppm 0.90 (t, J = 6.8 Hz, 3H), 1.19-1.39 (m, 20H), 1.57- 1.67 (m, 2H), 1.90-2.02 (m, 1H), 2.16-2.26 (m, 1H), 2.29 (t, J = 7.4 Hz, 2H), 2.36-2.45 (m, 2H), 3.71-3.84 (m, 2H), 4.43-4.52 (m, 2H); 13C NMR (100 MHz, CD3OD) δ ppm 14.4 (CH3), 23.7 (CH2), 26.8 (CH2), 27.9 (CH2), 30.4-30.9 (8xCH2), 31.1 (CH2), 33.0 (CH2), 36.9 (CH2), 53.3 (CH), 56.6 (CH), 63.1 (CH2), 172.6 (C), 174.9 (C), 176.5 (C), 176.5 (C); MS (ESI-) m/z (%) 443 (100) [M-H]-; HRMS (ESI-) m/z calculated for C22H39N2O7 443.2757 [M-H]-, found 443.2754.

Compound 3e. (R)-2-[(S)-2-(Hexadecanamido)-3-hydroxypropanamido]glutaric acid

The pure product (white solid, 36 mg, 0.08 mmol, 65%) was prepared starting from 2e (83 mg, 0.18 mmol). MP = 111-113 °C; 1H NMR (400 MHz, CD3OD) δ ppm 0.90 (t, J = 6.8 Hz, 3H), 1.19-1.37 (m, 24H), 1.55-1.68 (m, 2H), 1.90-2.03 (m, 1H), 2.15-2.26 (m, 1H), 2.29 (t, J = 7.4 Hz, 2H), 2.36-2.44 (m, 2H), 3.72-3.82 (m, 2H), 4.43-4.52 (m, 2H); 13C NMR (100 MHz, CD3OD) δ ppm 14.4 (CH3), 23.7 (CH2), 26.8 (CH2), 27.9 (CH2), 30.4-30.8 (10xCH2), 31.1 (CH2), 33.1 (CH2), 36.9 (CH2), 53.2 (CH), 56.6 (CH), 63.1 (CH2), 172.6 (C), 174.7 (C), 176.5 (C), 176.5 (C); MS (ESI-) m/z (%) 471 (100) [M-H]-; HRMS (ESI-) m/z calculated for C24H43N2O7 471.3070, found 471.3057.

Biochemistry

Products. Products were from Sigma except when indicated. SOC medium was from Invitrogen, LB broth medium from Roth, ampicillin and Triton X100 from Euromedex, anti-protease tablets from Roche, Ni2+-NTA resin from Generon, DDM and DM from Anatrace, Amicon Ultra-15 devices from Millipore and Superdex 200 10/300 GL from GE.

BmrA expression. BmrA expression was adapted from methods previously reported (23, 48). The E504A mutant was generated and fused to a 6-histidine N-terminal Nickel-affinity tag in the pET15(+) plasmid and overexpressed in the CD43(DE3)ΔacrB E. coli strain, a gift of Pr. Klaas Martinus Pos. A freshly transformed colony was incubated in 3 mL LB containing 50 µg/mL for 7-8 h at 37 °C. Thirty microliters of this preculture were added to 1 L LB containing 50 µg/mL of ampicillin, which was then incubated at 22 °C until reaching 0.6 OD600. BmrA expression was induced by 0.7 mM IPTG followed by a 5-6 h incubation at 22 °C. Bacteria were collected at 5000 xg for 15 min., 4 °C and then suspended in 10 mL 50 mM Tris-HCl pH 8.0, 5 mM MgCl2 and 1 mM PMSF. Bacteria were lysed by 3 passages at 18,000 psi through a microfluidizer 100 (Microfluidics IDEX Corp). The solution was centrifuged 30 min. at 15,000 xg at 4 °C. The membrane fraction was pelleted by centrifugation for 1 h at 180,000 xg at 4 °C, suspended in 50 mM Tris-HCl pH 8.0, 1 mM PMSF and 1 mM EDTA and centrifuged again. The final pellet was suspended in 20 mM Tris-HCl pH 8.0, 0.3 M sucrose and 1 mM EDTA, frozen in liquid nitrogen and stored at -80 °C.

BmrA purification. Membranes were solubilized at 5 mg/mL in 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 15% glycerol (v/v), anti-protease tablets, 0.1 mM TCEP and 4.5% (w/v)
Triton X100, under gentle agitation for 90 min. and then centrifuged 40 min. at 100,000 xg. The supernatant was loaded onto a Ni\(^{2+}\)-NTA equilibrated in 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 15% (v/v) glycerol, anti-protease tablets, 4.5% Triton X100 and 20 mM imidazole. The resin was washed with 20 mM Hepes-HCl pH 8.0, 100 mM NaCl, 20 mM imidazole, 1.3 mM DDM and 1 mM sodium cholate. The protein was eluted by adding 200 mM imidazole to the same buffer. BmrA fractions were pooled and diluted ten times in the Hepes buffer (same composition as above) without imidazole and loaded again on the same resin for another step of affinity chromatography. The pool of BmrA fractions was concentrated on 50 kDa cutoff Amicon Ultra-15 devices, with the centrifuge speed set at 1000 xg for 10-15 min, and then loaded onto Superdex 200 10/300 using as mobile phase 20 mM Hepes-HCl pH 7.5, 100 mM NaCl, 0.7 mM DDM and 0.7 mM sodium cholate (DDM:cholate molar ratio of 1:1). The same step was also carried out at DDM-cholate ratio of 3:1 or 1:3. Cholate was systematically removed from the Superdex resin by a washing with 1M NaOH. The elution peak was then pooled and stored at 4 °C before use. BmrA was particularly stable when not concentrated as previously reported (34).

**Thermostabilisation assays** were carried out as previously reported (34). Membranes of BmrA diluted at 2 g proteins/L were solubilized with 10 mM DDM, with or without 1 mM of compounds 3a-3e in a final volume of 2 mL, for 2 h at 4 °C. Solutions were clarified by centrifugation at 100,000 xg for 1 h at 4 °C and supernatants were aliquoted (50 μL) and individually submitted 30 min to a temperature of 25 to 90 °C using a PCR thermal cycler (PeqSTAR 2x gradient; Peqlab). Samples were then centrifuged 40 min at 20,000 xg and supernatants were analyzed by SDS-PAGE and Western-blot using anti-His antibody. The relative intensity of BmrA at each temperature was quantified on Western blot using Image Lab software 4.1 (Bio-Rad). Each condition was performed twice or thrice. Intensity was plotted as a function of the temperature and normalized. Data were fitted with equation 5 (see data fit section).

**Detergents quantification.** DDM bound to BmrA was quantified by mass spectrometry as described (33). Cholate was quantified as previously reported (49). Modelling of the detergent belt radius was done following the same protocol and using the DeltBelt server (www.deltbelt.ibcp.fr).

**ATPase activity.** The ATPase activity of BmrA was measured as previously described (34). The protein in solution in 20 mM Hepes-HCl pH 7.5, 100 mM NaCl, 0.7 mM DDM and 0.7 mM cholate was diluted in the ATPase activity assay buffer containing either 1 mM DDM or a mixture of 0.7 mM DDM and 0.7 mM cholate, and the ATPase activity recorded.

**Membrane-scaffold protein (MSP) production and purification.** The MSP1E3D1 protein was expressed in BL21 *E. coli* (p1E3D1 plasmid, Addgene) as previously described (36). Bacteria were suspended in 50 mL of 40 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 % (w/v) Triton X100, 0.5 mM EDTA, 1 mM PMSF. Two microliters of Benzonase (24 U/mL, Merck) were added and the bacteria were lysed by 2 passages at 18,000 psi through a microfluidizer 100 (Microfluidics IDEX Corp) and then centrifuged during 30 min. at 30,000 xg, 4°C. The supernatant was loaded onto a 0.5-mL Ni\(^{2+}\)-NTA column (GE Healthcare) resin pre-equilibrated with 5 resin-volumes of 40 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 % (w/v) Triton X100, 0.5 mM EDTA and 1 mM PMSF. The resin was then washed with 10 resin-volume with 3 different buffers: wash buffer 1 composed of 40 mM Tris-HCl pH 8.0, 300 mM NaCl and 1% (w/v) Triton X100; wash buffer 2 composed of 40 mM Tris-HCl pH 8.0, 300 mM NaCl, 50 mM sodium cholate and 20 mM Imidazole; wash buffer 3 composed of 40 mM Tris-HCl pH 8.0, 300 mM NaCl, 50 mM Imidazole. MSP1E3D1 was eluted with 15 mL of 40 mM Tris-HCl pH 8.0, 300 mM NaCl and 500 mM Imidazole. The factions of the elution pic were pooled and the TEV (2 mg/mL) was added to remove the His tag, at a ratio of 1 mg TEV for 40 mg MSP1E3D1. The mixture was then dialyzed (cutoff 12-14 kDa), a first time
against 300 mL 40 mM Tris-HCl, pH 7.4, 100 mM NaCl and 0.5 mM EDTA for 3 hours and then against 700 mL of the same buffer, overnight at 4 °C. After dialysis 20 mM imidazole was added and the solution loaded on a 0.5 mL Ni²⁺-NTA column equilibrated with 20 mM Tris-HCl pH 7.4 and 100 mM NaCl. The flow-through containing MSP1E3D1 was collected. The uncleaved fraction was eluted with 40 mM Tris-HCl pH 8.0, 300 mM NaCl and 500 mM Imidazole, dialyzed two times as above and finally concentrated spinning at 5,000 xg with a 100 kDa cutoff Amicon Ultra-15. The concentrated samples were frozen in liquid nitrogen and stored at -80 °C.

**BmrA reconstitution into nanodiscs.**

BmrA was reconstituted into nanodiscs as previously described (36) with the following modifications. Six hundred micrograms of purified BmrA E504A in 200 µL of Hepes-HCl pH 7.5, 100 mM NaCl, 0.035% DDM and 0.03% sodium cholate were mixed with 1.4 mg of *E. coli* lipids (Avanti Polar) in 56 µL of 99 mM cholate, 20 mM Hepes-HCl pH 7.5, 100 mM NaCl for 10 min at room temperature. The mix was then added of 665 µg MSP1E3D1 in 35 µL of 40 mM Tris-HCl, pH 7.4, 100 mM NaCl and 0.5 mM EDTA. The volume was completed to 1 mL with Hepes-HCl pH 7.5, 100 mM NaCl and incubated 1 h at room temperature. The final molar ratio of BmrA/MSP/lipids was 1/5/400 in 20 mM Hepes-HCl pH 7.5, 100 mM NaCl. SM-2 biobeads (170 mg/100 µg BmrA, Biorad) were then added to the mixture, placed 3 h under gentle agitation at room temperature. Empty nanodiscs were removed from the BmrA-nanodiscs by Ni²⁺-NTA chromatography. The resin was equilibrated with 20 mM Hepes-HCl pH 7.5, 100 mM NaCl, then loaded with the sample, washed with 20 mM Hepes-HCl pH 7.5, 100 mM NaCl, 20 mM imidazole. The BmrA-nanodiscs complex was then eluted with 20 mM Hepes-HCl pH 7.5, 100 mM NaCl, 200 mM imidazole. Imidazole was then removed from the solution by passing through a HiTrap desalting column equilibrated with 20 mM Hepes-HCl pH 7.5, 100 mM NaCl.

**Ligand binding on BmrA in detergents.**

R6G, ATP-Mg²⁺ and cholate binding was carried out by incubating 15 min on ice 0.5 µM BmrA, WT or E504A mutant prepared in DDM or DDM-cholate with or without 5 mM ATP-Mg²⁺ in 20 mM Hepes-HCl pH 7.5, 100 mM NaCl, including 0.7 mM DDM and/or 0.7 mM cholate depending on the experiments. The binding of R6G was probed by intrinsic fluorescence recorded on a SAFAS Xenius spectrophotofluorimeter set up at a constant photo multiplier voltage of 570 V. Tryptophan residues or N-acetyl tryptophan amide (NATA) used as negative control were excited at 280 nm, and their fluorescence emission spectra were recorded between 310 and 380 nm, with a 5-nm bandwidth for excitation and emission. Experiments were done in a quartz cuvette in a final volume of 200 µL, in which increasing amounts of R6G were added. Resulting emission curves were integrated and deduced from the same experiments carried out with NATA, used at the same concentration than that of BmrA tryptophan residues. Data were plotted as a function of R6G concentration. Binding of ATP-Mg²⁺ was carried out in the same way, pre-incubating BmrA E504A with or without 100 µM R6G for 15 min on ice.

**Ligand binding to BmrA-nanodiscs complexes and empty nanodiscs.** R6G, DDM and DM binding assays were carried out as above. Assays with empty nanodiscs (without BmrA) were carried out at the same nanodiscs concentration as that of BmrA-nanodiscs, complexes. This allowed to correct the fluorescence quenching due to the interaction between empty nanodiscs alone and ligands. Two cuvettes of NATA were also used: one for BmrA-nanodiscs complex and the other one for the empty nanodiscs. Data were analyzed in the same way as above.

**Doxorubicin transport by BmrA** was recorded as previously described (23). Ten micromolar of doxorubicin and 2 mM ATP were added to 100 µg *E. coli* inverted membrane vesicles containing overexpressed BmrA. Transport was initiated upon addition of 2 mM MgCl₂ and monitored at 25 °C in 1-mL quartz cuvettes recording the fluorescence on a
Photon Technology International fluorimeter at 590 nm with a bandwidth of 4 nm upon excitation at 480 nm with a bandwidth of 2 nm. Transport was initiated by adding 2 mM ATP-Mg^{2+}.

**R6G accumulation in Bacillus subtilis strains.** R6G accumulation assay was performed in *B. subtilis* strain 168 (WT) and 8R (overexpressing *BmrA* (24) kindly provided by Pr. Hans Krügel). Strains were grown overnight in LB medium at 37 °C with agitation, and then diluted to 0.05 OD_{600nm} with fresh medium. Once the culture reached 0.5 OD_{600nm}, they were incubated with 5 μM R6G for 30 min more. Then 2 mL of each culture (~ 1 OD_{600nm}) was centrifuged at 15,000 xg for 10 min at 4 °C. The pellets were washed with 1 mL LB medium and centrifuged. The pellets were suspended in 500 μL of 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mg/mL lysozyme and incubated for 1 h at 37 °C with agitation. The cells were then incubated with 0.5% SDS for 15 min more. R6G fluorescence was recorded with a SAFAS Xenius spectrophotofluorimeter in a black 96 well-plate using 200 μL of cell lysates setting excitation to 526 nm and recording fluorescence between 541 and 650 nm.

**Data fit.** Data were fitted using Microsoft Excel (365), SigmaPlot (v12.5) and GraphPad (v8) using/setting up the following equations:

**Equation 1 (Intrinsic fluorescence quenching, ligand binding, one site saturation):**

\[ f = \frac{F_{max} \cdot abs([L])}{K_D + abs([L])}, \]

where \( F_{max} \) = maximal intrinsic fluorescence without ligand, [L] = ligand concentration, \( K_D \), ligand dissociation constant.

**Equation 2 (allosteric intrinsic fluorescence increase):**

\[ f = \frac{F_{min} + (F_{max} - F_{min})/(1+([L]/K_D)^h)}{F_{min}}, \]

where \( F_{min} \) = minimal intrinsic fluorescence without ligand, \( F_{max} \) = maximal intrinsic fluorescence with ligand, [L] = ligand concentration, \( K_D \), ligand dissociation constant, \( h \) = Hill number.

**Equation 3 (Sigmoidal, 3 parameters):**

\[ f = \frac{F_{max}}{\left(1 + \exp\left(-\frac{([L]-[L]_{50}}{b}\right)\right)} \]

where \( F_{max} \) = maximal intrinsic fluorescence, [L] = ligand concentration, μM, \( [L]_{50} \) = ligand concentration at half-maximal intrinsic fluorescence, μM.

**Equation 4 (Intrinsic fluorescence quenching, ligand binding, two sites saturation):**

\[ f = \frac{F_{max1} \cdot abs([L])}{K_{D1} + abs([L])} + \frac{F_{max2} \cdot abs([L])}{(K_{D2} + abs([L]))}, \]

where \( F_{max1}, F_{max2} \) = maximal intrinsic fluorescence without ligand, [L] = ligand concentration, \( K_{D1}, K_{D2} \), ligand dissociation constants.

**HDX experiments**

HDX-MS experiments were performed using a Synapt G2-Si mass spectrometer coupled to a NanoAcquity UPLC M-Class System with HDX Technology (Waters™).

*BmrA reconstituted in nanodiscs labelling.* All the reactions were carried out manually. Labeling was initiated by diluting 5 μL of 15 μM BmrA WT or 37 μM BmrA E504A in nanodiscs, in 95 μL D_{2}O labeling buffer (5 mM Hepes pD 8.0, 50 mM NaCl). For the ATP/Vi- and ATP-incubated conditions, the labeling buffer additionally contained 10 mM ATP, 10 mM MgCl_{2}, 1 mM Vi and 10 mM ATP, 10 mM MgCl_{2}, respectively. For the drug-bound condition, the labeling buffer additionally contained 10 mM ATP, 10 mM MgCl_{2}, and 100 μM R6G. Prior to labeling, the samples were incubated with the respective ligands for 15 min at 20 °C. Samples were labeled for 2, 5, 15 and 30 min at 20 °C. Subsequently, the reactions were quenched by adding 22 μL of ice-cold quenching buffer (0.5 M glycine, 8 M guanidine-HCl pH 2.2, 0.035% DDM and 0.03% sodium cholate) to 100 μL of labelled sample, in ice bath. After 1 min, the 122-μL quenched sample was added into a microtube containing 200 μg of activated zirconium magnetic beads (MagReSyn Zr-IMAC from Resyn Biosciences, USA), to remove the phospholipids (50). After 1 min magnetic beads were removed, and the supernatant was injected immediately into a 100-μL loop.

*BmrA in detergent mixture labelling.* The reactions were carried out by a Twin HTS PAL dispensing and labelling robot (LEAP Technologies, Carborro, NC, USA) coupled to a Synapt
G2Si mass spectrometer (Waters, Manchester, UK). Labeling was initiated by diluting 5.2 µL, of typically 37 µM protein, in 98.8 µL D₂O labeling buffer (5 mM Hepes pH 8, 50 mM NaCl, 0.035% DDM, 0.03% sodium cholate). For the drug-bound condition, the labeling buffer additionally contained 10 mM ATP, 10 mM MgCl₂, and 100 µM R6G. Prior labeling the samples were incubated with the respective ligands for 15 min at 20 °C. Samples were labeled for 2, 5, 15 and 30 min at 20 °C. Subsequently the samples were quenched by adding 22 µL of ice-cold quenching buffer (0.5 M glycine, 8 M guanidine-HCl pH 2.2) to 100 µL of labelled sample, in ice bath. After 1 min, 105-µL quenched sample were injected immediately into a 100-µL loop.

UPLC-MS. Labelled proteins were then subjected to on-line digestion at 15 °C using a pepsin column (Waters Enzymate™ BEH Pepsin Column 300 Å, 5 µm, 2.1 x 30 mm). The resulting peptides were trapped and desalted for 3 min on a C4 pre-column (Waters ACQUITY UPLC Protein BEH C4 VanGuard pre-column 300 Å, 1.7 µm, 2.1 x 5 mm, 10K - 500K) before separating them with a C4 column (Waters ACQUITY UPLC Protein BEH C4 Column 300 Å, 1.7 µm, 1 x 100 mm) using 0.2% formic acid and a 5-40 % linear acetonitrile gradient in 15 min and then 4 alternative cycles of 5% and 95% until 25 min. The valve position was adjusted to divert the sample after 14 min of each run from C4 column to waste, to avoid a contamination of the mass spectrometer with detergent. Two or three full kinetics were run for each condition, one after the other, to get either duplicate or triplicates of each deuteration timepoint. Blanks (equilibration buffer: 5 mM Hepes pH 8.0, 50 mM NaCl) were injected after each sample injection and pepsin column washed during each run with pepsin wash (1.5 M guanidine-HCl, 4% acetonitrile, 0.8% formic acid pH 2.5) to minimize the carryover. Electrospray ionization mass spectra were acquired in positive mode in the m/z range of 50–2000 and with a scan time of 0.3 s. For the identification of non-deuterated peptides, data was collected in MSE mode and the resulting peptides were identified using PLGS™ software (ProteinLynx Global SERVER 3.0.2 from Waters™). Peptides were then filtered in DynamX 3.0 software (Waters™), with the following parameters: minimum intensity of 1000, minimum products per amino acid of 0.3 and file threshold of 2. After manual curation, Deuteros 2.0 software (51) was used for data analysis, visualization and statistical treatments. The mass spectrometry data have been deposited to the ProteomeXchange Consortium via the PRIDE (52) partner repository with the dataset identifier PXD027447.

Biophysics

Products. Crystallization solutions were from Grenier bio-one. The Mosquito crystallization robot is from TTP Labtech. Crystallization plates and cover were from Grace Bio-Labs. The cryoprotection kit was from Molecular Dimensions. Cryscheme plates were from Hampton Research. Vitrobot grid freezing device is from FEI. The Talos Arctica and Titan Krios G3 are from Thermo Scientific.

X-ray

Protein crystallization. The crystallography step was performed at 19 °C. Crystals were obtained by vapor diffusion on hanging drops. E504A BmrA mutant was concentrated by centrifugation-filtration to 7-10 mg/mL spinning at 500 xg on a 50 kDa cutoff Amicon Ultra-15 at 22 °C. BmrA E504A mutant was then incubated with 5 mM ATP-Mg for 30 min. Crystallogenesis was done by mixing with a Mosquito 450 nL of reservoir solution containing 100 µL 0.1 M Tris-HCl pH 8.5, 23-27% PEG 1000 with 50 nL of compounds 3a-3e and 500 nL of BmrA E504A sample. the mix was deposited on a plastic cover, sealed onto the plate and imaged periodically with a Formulatrix. Crystals appear after 3 days, grown up to 5-8 days and progressively disappeared if the incubation lasted longer.

Crystal cryocooling. As BmrA E504A mutant crystals were sensitive to cryoprotection, it was therefore performed using the CryoProtX MD1-61 kit. Best results were obtained with a final
solution containing 12.5% (v/v) diethylene glycol, 18% (v/v) 2-methyl-2,4-pentanediol, 7% (v/v) ethylene glycol, 12.5% (v/v) 1,2-propanediol, 12.5% (v/v) dimethyl sulfoxide, supplemented with 5 mM ATP-Mg and 1 mM compounds 3a-3e. One microliter of cryosolution was divided in 3 drops under the binocular, close to the drop containing the crystal and then gently brought in contact using the freezing loop, at the opposite side where the crystal was sitting, in the course of 1 min. This operation was performed in Cryochem sitting drop plates, with the drop sitting in the middle of a water-filled reservoir to saturate the solution with humidity. Crystals were then harvested and placed on a fresh drop of cryosolution for 1 min. before harvesting and cryocooling in liquid N\textsubscript{2}. Crystals were stored in liquid N\textsubscript{2} before being analyzed at the synchrotron.

**Diffraction data acquisition.** Diffraction screening has been performed at ESRF and SOLEIL synchrotrons on multiple beamlines over the years. Best data set was collected on PX2 at SOLEIL, consisting of a low-resolution pass at low transmission, and a high-resolution dataset at full transmission collected helicoidally. Crystal polymorphism was strongly present, precluding data merging among several crystals. Crystals diffracted very anisotropically, going to 3.9 Å resolution in the strongest diffracting direction. Data were processed in XDS as spherical to the highest resolution possible (3.9 Å) even though spherical statistics were not usable. Staraniso analysis for diffraction anisotropy revealed that completeness was 78% in the highest resolution shell, therefore revealing that all the data collectable for this crystal had been collected. Data was cut at the diffraction limits suggested by the Staraniso server. Anisotropic diffraction table is available in supplementary Table 1.

**X-ray structure and model building.** Phases were solved by molecular replacement using Phaser on amplitudes, with data corrected for anisotropy using Staraniso, and using the outward facing conformation dimer of Sav1866 (PDB code ID 2hyd) or MsbA (PDB code ID 3b60) as search models. Although Sav1866 and MsbA structures are very similar, the MsbA model yielded higher molecular replacement solution scores. Crystals belonged to the P21 space group with 2 dimers in the asymmetric unit. The molecular replacement solution was clear, but the electron density was very noisy due to the large conformational changes observed on BmrA, and that resulted in poor overall phases. The core of the protein was nevertheless clearly visible with helices as tubes. The nucleotide-binding domain was very blurry as well as external loops. The model was turned into poly-Ala to place helices of the transmembrane region, and initial movement of the TM1-TM2 hinge. Refinement was carried out in autoBUSTER using corrected amplitudes, applying strict NCS. Iterative manual building in Coot followed by refinement resulted in visible continuous electron density with decreasing R-factors. Density for large amino acids appeared, as well as for ATP. Sequence was assigned, and iterative refinement continued with introduction of TLS refinement (1 TLS per chain, 4 total). It yielded R-factors around 30 and 35 for R and Rfree respectively, with small grooves in the helices. Re-definition of TLS (1 for a dimer of TMD, 1 for a dimer of NBD, 4 total) resulted in a dramatic decrease of R-factors by 3 points, and much clearer electron density features, helices with large grooves and side chain density. Unwinding of TM3 next to residue 136 was apparent, as well as helix breaks in the trans-membrane region and clear density for ATP. Some incorrect modeling of ATP became apparent with negative and positive density showing where the correct position was then defined. NCS was relaxed and correct modeling of geometry clashes was carried out in ISOLDE. Registry was built by starting to assign using initial first large density features clearly visible as refinement converged for the TMD, then using superpositions for the NBD. Registry at key locations was then probed by replacing several amino-acids, or by trying to turn helices by one amino-acid clockwise or counterclockwise and probed by refinement. Newly refined structures clearly showed positive or negative densities indicative of incorrectly modeled features, thus granting the modelling of a full amino-acid model guided by restrained-refinement. Ramachandran and
rotamers outliers were corrected, yielding a final model with $R = 26.0\%$ and $R_{\text{free}} = 32.1\%$. The final model was deposited in the Protein Data Bank under the accession code 6r72.

**Cryo-EM**

**BmrA E504A [ATP-Mg$^{2+}$, R6G] sample**

*Sample preparation.* Detergent-purified BmrA E504A mutant at 3.4 mg/mL was incubated with 0.1 mM R6G for 30 min at 4 °C, followed by addition of 5 mM ATP-Mg in the same conditions. Three microliters of this mixture were applied to cryo-EM Au-grids (Cflat 1.2/1.3 3Au) previously discharged in air for 40 s at 20 mA (PELCO easiGlow), blotted for 3 s, and plunged frozen in liquid ethane with a Vitrobot grid freezing device.

*Data acquisition*, image processing. Best grids screened with a Talos Arctica were then imaged with a Titan Krios G3 electron microscope equipped with a K2 camera and operating at 300 keV. A total of 3477 movies of 40 frames each were acquired over 2 data-collection sessions originating from the same grid in electron counting mode at 1.06 Å/pixel, 6.4 electrons/pixel/s, with a total exposure time of 6 s and combined into a single MRC stack using EPU automatic data collection control software using defocus values ranging from 1.2 to 3.2 µm. Contrast transfer function (CTF) parameters were estimated from the averaged movie with CTFFIND4 and 2170 particle images were selected manually and subjected to 2D classification in cryoSPARC v2. Automatic particle selection was performed with templates from the 2D classification. Beam induced particle motion between fractions was corrected with a new implementation of alignparts lmbfgs in cryoSPARC v2. The number of particle images were reduced to 128372 by further 2D and 3D classifications and refinements. Models were calculated ab initio and refined without the application of symmetry with cryoSPARC v2. For each data collection session automatically picked particles were cleaned with 2 rounds of 2D classification followed by a preliminary round of 3D classification to further remove obvious junk particles such as empty detergent micelles that were not eliminated during the 2D classification process. Although no discrete conformation could be isolated to high resolution, removal of additional particles improved the resolution of the final maps suggesting significant non-discrete or continuous flexing. Since these maps suggested a significant amount of small, non-discrete flexing, better resolved maps were obtained using cryoSPARC v2’s non-uniform refinement feature. An additional refinement with the application of C2 symmetry was performed that resulted in a gain of 0.3 Å in overall resolution which helped to slightly improve the interpretability of the map in the model building process. The asymmetric and C2 symmetrized maps have been deposited in the Electron Microscopy database under the accession codes EMD-4749 and EMD-12170 respectively.

*Model building and refinement.* The X-ray model was docked into a 3.9 Å C2 symmetrized cryo-EM density map and improved with iterative rounds of manual building in Coot and Isolde followed by real_space_refine in Phenix. Of Note, sharpening the C2 symmetry map using Phenix led to improved features in the trans-membrane domain, but worse in outer loops and the NBD. The final model was thus built using both sharpened and unsharpened maps. The final model was validated with MolProbity and EMringer and deposited in the Protein Data Bank under the accession code 6R81 and electron microscopy database EMDB-4749.

Two small densities were visible in the C2 symmetrized map at the locations of R6G. Re-examination of the data with no symmetry led to the identification of clearer densities in which R6G could be placed and suitably refined. Notably, both densities are not equivalent in the two halves of BmrA, suggesting that in both binding sites there is a heterogeneity/flexibility of binding, reminiscent of substrate release. Understandably, the application of C2 symmetry masked the quality of the reconstructions at these locations since these sites are not identical with respect to R6G binding. Thus, BmrA was refined in the
presence of R6G, following the same procedure as above using the asymmetric map. Final model and maps were deposited in the Protein Data Bank under the accession code 7bg4 and electron microscopy database EMD-12170. Model statistics are provided in supplementary Table S2.

*BmrA E504A [ATP-Mg\textsuperscript{2+}] sample*

**Sample preparation.** Detergent purified BmrA E504A mutant at 3.8 mg/mL was incubated with 5 mM ATP-Mg\textsuperscript{2+} for 30 min at 4 °C.

Three microliters of this mixture were applied to cryo-EM Au-grids (Cflat 1.2/1.3 3Au) previously discharged in air for 40 s at 20 mA (PELCO easiGlow), blotted for 3 s, and plunge frozen in liquid ethane with a Vitrobot grid freezing device.

**Data acquisition, image processing.** Grids were imaged with a Titan Krios electron microscope equipped with a K2 camera and operating at 300 keV. A total of 4,221 movies of 40 frames each were acquired in electron counting mode at 1.052 Å/pixel, 8.4 electrons/pixel/s, with a total exposure time of 5 s and combined into a single MRC stack using SerialEM automatic data collection control software using defocus values ranging from -0.8 to -2.2 µm. All processing was done in cryoSPARC v3.1, including motion correction and CTF estimation. Automatic particle picking (blob picker) on the first 200 micrographs followed by 2D classification and particle selection, was then fed into a template picker for the entire data set. Several rounds of 2D classification narrowed down the total amount of particles to about 1 million, which was subjected to ab-initio model generation. The number of particles were reduced to 327,764 by further 3D classifications and refinements. The best resolution was obtained using Non-Uniform refinement in cryoSPARC v3.1 using C2 symmetry. Note that the influence of the amphipathic belt on reconstruction was tested by performing particle subtraction using a mask encompassing the amphipathic belt, followed by focused refinement. A 0.2 Å decrease in overall resolution was observed as expected (FSC=0.143) but no difference was observed on the protein map quality. The maps deposited were thus without particle subtraction. The C2 symmetrized maps have been deposited in the Electron Microscopy database under the accession codes EMD-13095.

**Model building and refinement.** The X-ray model was docked into a 3.6 Å C2 symmetrized cryo-EM density map using Phenix dock\_in\_map. The model was improved with iterative rounds of manual building in Coot (0.9.4) and Isolde v1.2 followed by real\_space\_refine in Phenix. No map sharpening was performed on this map. The map quality was of higher quality than the X-ray or cryoEM map with R6G and allowed to build a BmrA model comprising the loop between TM5-6 that was missing in the previous structures. The final model was validated with MolProbity (Phenix) and Coot and deposited in the Protein Data Bank under the accession code 7ow8 and electron microscopy database EMDB-13095. Model statistics are provided in supplementary Table S2.

**Bioinformatics**

Both the X-ray (6r72) and the cryo-EM (C2, 7bg4 with and without R6G) structures span residues 10 to 589, and both miss a few residues (271-278 in the X-ray structure, 273-278 in the cryo-EM structure), corresponding to the loop region between TM5 and TM6. Complete models of dimeric WT BmrA were generated using Modeler (v9.12), for both the X-ray and the cryo-EM structures, using the structure of the ABC transporter related protein from *Novosphaeribium aromaticivorans* (PDB code ID 4mrs)\,(53) as a template for the missing residues, and the alignment generated by HHpred (54). The N-termini were capped with acetyl groups, and the missing N-terminal residues were not modeled. Both models contained ATP molecules and Mg\textsuperscript{2+} ions, as observed in both the X-ray and the cryo-EM structures. The models were then oriented using the OPM server (http://sunshine.phar.umich.edu/server.php)
and embedded into a mixed POPE/POPG bilayer (ratio 3/1) using the CHARMM-GUI membrane builder (56), and the replacement method. The systems were solvated and 150 mM KCl was added to the solution, yielding a total of ~157,000 atoms in tetragonal boxes of dimensions ~100x100x165 Å³. CHARMM-GUI web server was used also to build R6G in POPC lipid bilayer.

**Simulations without R6G.**
All simulations were run with the GROMACS (v2016.4) software package (57). The CHARMM36 force field was used for both the lipids and the protein, together with the CHARMM TIP3P water model. Non-bonded interactions were calculated with a cutoff of 1.2 Å, with a shift function on the potential to avoid discontinuities. Neighbor lists were updated using the Verlet scheme. Long-range electrostatic interactions were calculated with the Particle Mesh Ewald method (58). Bonds involving hydrogen atoms were constrained using the P-LINCS algorithm (59).
Each system was minimized by steepest descent and then equilibrated using a 6-cycle equilibration scheme, using position restraints on the protein and gradually reducing the force constant. Equilibration and production runs were performed at 303.15 K and 1 bar; the temperature was kept constant with the velocity rescale algorithm (60) and the pressure with the Parrinello-Rahman barostat (61). The integration time step was set to 2 fs. For each system, four replicates were simulated for 500 ns each. The first 200 ns of each simulation were treated as equilibration, and average quantities (average structures, inter-atomic distances, RMSD, RMSF, B factors) were computed on the remaining 300 ns. Two additional replicates were run with long equilibration steps (275 ns before 500 ns of production) to confirm the closing movement of the cavity. Due to the relatively large size and the transmembrane nature of BmrA, we expect functional motions of the transport cycle to take place on time scales much longer than the simulation time (probably 3-4 orders of magnitude longer), which are currently not accessible by all-atom molecular dynamics. Therefore, we only expect to observe relatively fast conformational changes, and changes driven by strong driving forces.

**Simulations with R6G.**

**System setup.** R6G was first extracted from the structure with BmrA E504A and then parametrized with CHARMM General force field (CGenFF) program (62) to provide the initial topology file of the R6G molecule compatible with the CHARMM36 force field. Some of the topology parameters required optimizations and verifications because of their high penalty scores. Hence, we carried out molecular dynamic simulations of the R6G molecule alone with 300 POPC molecules forming the lipid membrane for 500 ns at 303 K. The resulting values were used for carrying out three unbiased simulations of two R6G molecule incubated with BmrA and reconstituted in 276 POPE:POPG (3:1 ratio) forming a lipid membrane, ran for 1 µs at 303 K.

**Parametrization of Rhodamine.** The parametrization was carried out following the procedure previously described (62). Once the initial topology file was obtained, we carried out molecular dynamic simulations of R6G in POPC membrane for 500 ns and clustered the most probable conformations of R6G in the lipid bilayer (Fig. S18AB). In GROMACS, the *gmx cluster* program allowed clustering the most preferred conformations of R6G during the simulation time. For each conformation, we used the CGenFF program to generate the corresponding topology files, which were then averaged to create the final topology file (Fig. S18C).

**Unbiased simulations.** The system including BmrA E504A, ATP-Mg²⁺, R6G, lipids and water was minimized by steepest descent and then equilibrated using a 7-cycle equilibration scheme,
using position restraints on the protein and gradually reducing the force constant. Three replicates were simulated for 1 µs using different starting velocities.

*Calculating the distances in simulations.* We calculated the distance between the center of mass (COM) of the two R6G molecules and the COM of the protein (along the bilayer normal vector, i.e., the z axis), using the GROMACS command *gmx distance.*

*Calculating the number of contacts between the molecules.* The number of contacts of R6G with water, protein and lipids were quantified during the simulations runs with the GROMACS command *gmx mindist,* considering a maximal distance between the respective COMs of 0.4 nm.
Fig. S1. Purification of BmrA, ATPase activity and transport assay. (A) Preparative SEC profile of detergent purified BmrA (left panel). The peak fraction was analyzed by SDS-Page (right panel). (B) ATPase activities of WT BmrA purified with DDM or DDM-cholate mixture. (C) Doxorubicin (doxo) transport activity of WT BmrA and the inactive E504A mutant.
Fig. S2. Crystallographic packing and difference between monomers in the asymmetric unit. (A) Overall crystal packing. The 4 BmrA monomers A-D of the asymmetric unit, assembled in 2 dimers, AB colored in blue, and the symmetric dimer CD in grey. Proteins are represented as cartoon, and the cell is drawn in blue. (C) TM1-2 of chain B interacts with TM5-6 of chain D, and of TM1-2 of chain C (in the back). (D) TM1-2 of chain A and chain D are free of crystal contact interactions. (E) Differences between monomers in the X-ray structure. Structures are represented in cartoon, colored in grey. Flexible regions are highlighted in green (chain B), blue (chain C) and red (chain D). Each 4 monomers of the asymmetric unit of the crystallographic structure were superposed onto chain A.
Fig. S3. X-ray 2mFo-DFc density maps of BmrA. (A) TM helices, coupling helices and ATP-Mg$^{2+}$ binding site of chain A. (B) Densities displayed at 1σ of the TM1-extracellular loop 1 for each chain.
Fig. S4. Crystallization of BmrA E504A in complex with ATP-Mg\(^{2+}\) and R6G.
Fig. S5. Image collection, 2D-3D classification, and processing workflow of cryo-EM image analysis of BmrA in complex with R6G. Micrographs from two separate data collection sessions done on the same sample and grid were processed in parallel and the best particles from each session were later combined to produce the final maps. For each session, picked particles were cleaned using 2 rounds of 2D classification followed by a 3D...
classification. Heterogeneity within the dominating outward-facing conformation was further assessed with additional rounds of 3D classification that removed an additional 35% of the outward-facing particles. Although removal of these particles improved the resolution of the dominating outward-facing conformation, discrete conformations could not be refined to high resolution suggesting that a large degree of small, non-discrete flexing was interfering with particle alignment. Due to the significant flexing amount, the final maps were refined using cryoSPARC’s non-uniform refinement feature resulting in better resolved maps. An additional refinement with the application of C2 symmetry was performed that resulted in an improvement in resolution. **Boxed:** example micrograph with particles used for 2D classification (red circles), and corresponding representative 2D class averages.
Fig. S6. Cryo-EM densities of BmrA E504A [ATP-Mg$^{2+}$, R6G]. Sharpened EM densities of the TM helices (and also unsharpened for TM1), coupling helices and the Magnesium and ATP binding sites for J196 (C1, no symmetry) and J197 (C2 symmetry map). Densities are displayed at $1.0\sigma$. 
Fig. S7. Assessment of the cryo-EM data. (A, B) Local-resolution estimation of the C1 (A) and C2 (B) density maps and their corresponding Fourier Shell Correlation (FSC). (C, D) Unsharpened C1 maps (no symmetry) of the R6G-binding sites. Densities are displayed at 2.0σ. (E-F) Molecular interactions of the two R6G in their binding sites. (G) R6G-binding site of AcrB (30).
Fig. S8. Image collection, 2D-3D classification, and processing workflow of cryo-EM image analysis of BmrA E504A [ATP-Mg\textsuperscript{2+}] condition. All particles originated from a single data collection with a straightforward data processing scheme, initiated by 2D classification, followed by 2 rounds of 3D classification and refinement. The final maps with C2 symmetry were deposited in the EMDB. A representative micrograph with particles used for 2D classification (white circle), and corresponding representative 2D class averages are displayed.
Fig. S9. 

Symmetry C2

TM1
5 sigma

TM1
3.5 sigma

TM2

TM3

TM4

TM5

TM6

Coupling Helix 1

Coupling Helix 2

Magnesium site

ATP site
Fig. S9. Cryo-EM densities of BmrA E504A [ATP-Mg$^{2+}$]. Unsharpened EM densities of the TM helices, coupling helices, and the Magnesium and ATP binding sites for the map with C2 symmetry. Densities are displayed at 5.0σ unless written differently.
**Fig. S10.**

Assessment of the cryo-EM data for BmrA E504A [ATP-Mg$^{2+}$]. Local-resolution estimation of the C1 and C2 density maps and their corresponding Fourier Shell Correlation (FSC) with resolution given for FSC value of 0.143.
Fig. S11. Correlation coefficient model to map and identification of the flexible parts of X-ray and cryo-EM structures. (A) The value of correlation coefficient model to map was calculated for each model against the corresponding density map. The results are plotted as a function of the amino acid sequence (X-ray in blue, cryo-EM unsharpened in green and sharpened in red). (B) The X-ray and the cryo-EM structures are superposed; the flexible parts are colored in red and blue for the cryo-EM and X-ray structure, respectively. These flexible parts correspond to the lowest CC values. The cartoon is represented with the thickness of the sausage corresponding to the B-factor, the higher the B-factor, the larger the sausage.
Fig. S12. Binding of compounds to BmrA probed by intrinsic fluorescence. (A) Binding of R6G (filled symbols) and cholate (empty symbols) to BmrA WT (red) or E504A mutant (blue) purified in DDM. Data were fitted with equation 1. No significant fluorescence change was observed upon cholate addition in same conditions. (B) Effect of DDM (blue) and decyl maltoside (DM, green) on empty nanodiscs (ND) and BmrA-nanodiscs complexes. BmrA E504A was purified in DDM and then reconstituted into nanodiscs to which DM or DDM were added. The same experiments were done with empty nanodiscs (ND). Data were fitted using equation 3, giving a half-maximal fluorescence increase detergent concentration, [DDM]_{50} of 106 ± 6 µM (n = 1, p < 0.0001) and 74.1 ± 7.7 µM (n = 1, p < 0.0006), and [DM]_{50} of 931 ± 23 µM (n = 2, p < 0.0001) and 850 ± 21 µM (n = 2, p < 0.0001) for the BmrA-nanodiscs complexes and empty nanodiscs, respectively. (C, D) Binding of R6G to BmrA WT-nanodiscs (C, blue), BmrA E504A-nanodiscs (D, blue) and corresponding empty nanodiscs (C or D, red). The amount of empty nanodiscs used in these experiments correspond to that of MSP1E3D1 proteins in complex with BmrA, estimated by SDS-PAGE using each purified protein as standard. Data best fitted with equation 1 for empty nanodiscs (one site saturation) and equation 4 for BmrA-nanodiscs complexes (two sites saturation). (E)
Specific ATPase activity of BmrA reconstituted into nanodiscs, in the presence of substrates. WT BmrA was purified in DDM and then reconstituted into nanodiscs as above to which the indicated concentrations of substrates were added, followed by the measure of the ATPase activity using the coupled-enzyme system. The amount of BmrA was determined by SDS-PAGE. The ATPase activity in absence of substrate was \( \sim 1.8 \mu \text{mol ATP/\text{min/mg}} \). Data correspond to 2 to 4 independent experiments.
Fig. S13. BmrA residues equivalent to those of the human ABCB1 involved in Taxol binding. Cryo-EM BmrA structure is displayed in grey in which residues in yellow correspond to those involved in taxol binding in the human ABCB1 (14).
Fig. S14. Transmembrane regions of BmrA showing an increased accessibility to deuterium upon transition to the OF state as probed by HDX-MS. (A) BmrA WT or E504A were prepared either in DDM-cholate mixture or reconstituted in nanodiscs. Data were recorded after 30 min D₂O exchange and only the transmembrane peptides with increased deuterium uptake in the ATP-bound (for E504A) or Vi-trapped (for WT) conformation as compared to the apo state are shown (salmon color, p < 0.01). The star indicates the position of the peptide 46-56 for which the deuterium uptake is plotted as a function of time. (B) BmrA sequence coverage maps. The sequence coverage was ~93% and ~86% for the E504A mutant in DDM-cholate and nanodiscs, respectively, and ~93% for the WT in nanodiscs.
**Fig. S15.**

| Initial | Run 1 | Run 2 | Run 3 | Run 4 |
|---------|-------|-------|-------|-------|
| X-ray   |       |       |       |       |
|         |       |       |       |       |
| cryo-EM |       |       |       |       |
|         |       |       |       |       |

**Fig. S15. Molecular dynamic simulation results.** X-ray and cryo-EM structures are in grey and red (TM1-2). The final structures after 500 ns of simulation resulting from the four simulation runs are in light blue and orange (TM1-2). The side view (upper panel) and the top view (lower panel), from the outside of the membrane, are shown for each simulation.
Fig. S16. Conformational changes of the TM region in the 8 simulations. Chain A is shown in pink and chain B in blue. Arrows indicate the displacement of the TM regions from the starting structures to the average structures.
Fig. S17. R6G parametrization. (A) Snapshot from POPC:R6G simulation showing the R6G inside the lipid bilayer. (B) Four different conformations of R6G and their probabilities inside the membrane during the simulation run. (C) Weighted averages of the partial charges of R6G molecule after optimization.
Fig. S18. Molecular dynamics simulations of BmrA in complex with ATP-Mg$^{2+}$ and R6G. (A) Time-evolution of the (Q52-G281-Q52’-G281’) distance for each of the 3 simulations from cryo-EM+R6G model. (B) Distance between the center of mass (COM) of R6G molecules (R6G1 and R6G2) and the COM of BmrA in the three different runs. (C) Snapshots at 300 and 900 ns. Water is colored in ice blue and lipids in green. POPG1 and POPG2 invading the drug-binding pocket are in cyan balls and sticks.
Fig. S19. Molecular dynamics simulations of BmrA in complex with ATP-Mg\(^{2+}\) and R6G. (A) Number of contacts between the COM of R6G and water molecules in the three replicates. (B) Number of contacts between R6G molecules and BmrA in the three runs. (C) Overlay of every 50-ns snapshot over 1-\(\mu\)s simulation. BmrA is seen from the periplasmic side looking down the cavity. TM1 and 2 are transparent on the left panel to better see the R6G2 molecule movement. R6G1 is displayed in black magenta wireframe and R6G2 in magenta sticks.
Table S1. X-ray statistics.

| X-ray                     | Non-corrected data | Corrected data |
|---------------------------|--------------------|----------------|
| Data collection           | P2₁                | P2₁            |
| Space group               | P2₁                | P2₁            |
| Cell dimensions           | a, b, c (Å)        | 117.8, 110.8, 155.6 |
|                           | α, β, γ (°)        | 90.93, 2.90    |
| Resolution (Å)            | 48.6-3.92 (4.3-3.95) | 80.6-3.95 (4.3-3.95) |
| Rmerge                    | 0.068 (3.7)        | (-)            |
| I/σ                       | 11.58 (1.39)       | 11.65 (1.39)   |
| Completeness (%)          | 56.5 (11.0)        | 92 (71.5)      |
| Redundancy                | 3.5 (3.4)          | (-)            |
| Ellipsoid<sup>b</sup>     | 0.851 a* + 0.525 c* | -0.36 a* + 0.933 c* |
| Refinement                |                    |                |
| Resolution (Å)            | 28.5-3.95          |                |
| No. reflections           | 20484              |                |
| R<sub>work</sub>/R<sub>free</sub> | 26.0/32.1         |                |
| No. atoms                 | 17680              |                |
| Protein                   | 17552              |                |
| Ligand/ion                | 128                |                |
| B-factors (Å<sup>2</sup>) | Protein            | 114.4          |
|                           | Ligand/ion         | 47.3           |
| R.m.s deviations         | Bond lengths (Å)   | 0.013          |
|                           | Bond angles (°)    | 1.97           |
| Ramachandran (%)          | Favored            | 92.26          |
|                           | Allowed            | 7.39           |
|                           | Outliers           | 0.35           |

<sup>a</sup> Highest resolution shell is shown in parenthesis.

<sup>b</sup> Definition of ellipsoid: Data has been fitted to the ellipsoid defined by the following parameters:

Ellipsoid definition: 0.1742 0.2691 0.2191 2.0533

Diffraction limits & principal axes of ellipsoid fitted to diffraction cut-off surface:

\[
\begin{array}{ccccccc}
4.564 & 0.8858 & 0.0000 & 0.4640 & 0.851 & a^* & + 0.525 & c^* \\
3.717 & -0.0000 & 1.0000 & -0.0000 & b^* & \\
5.739 & -0.4640 & -0.0000 & 0.8858 & -0.360 & a^* & + 0.933 & c^*
\end{array}
\]

Worst diffraction limit after cut-off:

5.976 at reflection -1 1 26 in direction -0.038 \(a^*\) + 0.038 \(b^*\) + 0.999 \(c^*\)

Best diffraction limit after cut-off:

3.917 at reflection -2 28 5 in direction -0.070 \(a^*\) + 0.982 \(b^*\) + 0.175 \(c^*\)
Table S2. Cryo-EM data collection and refinement statistics.

|                      | EMDB-12170 PDB: 7bg4 | EMDB-4749 PDB: 6r81 | EMDB-13095 PDB: 7ow8 |
|----------------------|-----------------------|----------------------|----------------------|
| **Data collection and processing** |                       |                      |                      |
| Magnification        | 130000                | 130000               | 130000               |
| Voltage (kV)         | 300                   | 300                  | 300                  |
| Electron exposure (e/Å²) | 38.4                 | 38.4                 | 37.95                |
| Defocus range (μm)   | 1.2 to 3.2            | 1.2 to 3.2           | 0.8 to 2.2           |
| Pixel size (Å)       | 1.06                  | 1.06                 | 1.052                |
| Symmetry imposed     | C1                    | C2                   | C2                   |
| Initial particle images (no.) | 486404               | 486404               | 511984               |
| Final particle images (no.) | 128372              | 128372               | 327764               |
| Map resolution (Å)   | 4.2                   | 3.9                  | 3.6                  |
| FSC threshold        | 0.143                 | 0.143                | 0.143                |
| Map resolution range (Å) | 3.6 to 25            | 3.5 to 9.1           | 3.0 to 6.0           |
| **Refinement**       |                       |                      |                      |
| Initial model used (PDB code) | 6R81                 | 6R72                 | 6R72                 |
| Model resolution (Å) | 4.3                   | 4.2                  | 3.8                  |
| FSC threshold        | 0.5                   | 0.5                  | 0.5                  |
| Model resolution range (Å) | 3.6 to 7.6          | 3.6 to 7.6           | 3.5 to 3.7           |
| Map sharpening B factors (Å²) | 187, 238              | 187, 218             | 217                  |
| Model composition    |                       |                      |                      |
| Non-hydrogen atoms   | 8927                  | 8861                 | 8942                 |
| Protein residues     | 1141                  | 1141                 | 1154                 |
| Ligands              | 6                     | 4                    | 4                    |
| B factors (Å²)       |                       |                      |                      |
| Protein              | 95.8                  | 170                  | 94.6                 |
| Ligand               | 109                   | 153                  | 62.9                 |
| R.m.s. deviations    |                       |                      |                      |
| Bond lengths (Å)     | 0.004                 | 0.011                | 0.011                |
| Bond angles (°)      | 0.834                 | 1.61                 | 1.608                |
| Validation           |                       |                      |                      |
| MolProbity score     | 2.24                  | 2.33                 | 2.43                 |
| Clashscore           | 16.2                  | 14.1                 | 10.2                 |
| Poor rotamers (%)    | 0.21                  | 1.25                 | 4.22                 |
| Ramachandran plot    |                       |                      |                      |
| Favored (%)          | 90.6                  | 88.1                 | 93.6                 |
| Allowed (%)          | 9.40                  | 11.5                 | 6.3                  |
| Disallowed (%)       | 0.00                  | 0.40                 | 0.17                 |

*Note that 2 different map sharpening levels were used to aid model building.*

**Data S1. (separate file)**
Detergent quantitation.

**Data S2. (separate file)**
Thermostability assays.

**Rhodamine6G.itp (separate file)**
R6G parametrization

**ForcefieldR6G.itp (separate file)**
*In silico* system (BmrA, ATP-Mg²⁺, R6G, POPG, POPE, water, KCl) parametrization file.
REFERENCES AND NOTES

1. O. Jardetzky, Simple allosteric model for membrane pumps. *Nature* **211**, 969–970 (1966).

2. K. Linton, C. Higgins, Structure and function of ABC transporters: The ATP switch provides flexible control. *Pflügers Arch.* **453**, 555–567 (2007).

3. K. P. Locher, Mechanistic diversity in ATP-binding cassette (ABC) transporters. *Nat. Struct. Mol. Biol.* **23**, 487–493 (2016).

4. Y. Kim, J. Chen, Molecular structure of human P-glycoprotein in the ATP-bound, outward-facing conformation. *Science* **359**, 915–919 (2018).

5. R. J. Dawson, K. P. Locher, Structure of a bacterial multidrug ABC transporter. *Nature* **443**, 180–185 (2006).

6. A. Ward, C. L. Reyes, J. Yu, C. B. Roth, G. Chang, Flexibility in the ABC transporter MsbA: Alternating access with a twist. *Proc. Natl. Acad. Sci.* **104**, 19005–19010 (2007).

7. A. B. Ward, P. Szewczyk, V. Grimard, C. W. Lee, L. Martinez, R. Doshi, A. Caya, M. Villaluz, E. Pardon, C. Cregger, D. J. Swartz, P. G. Falsion, I. L. Urbatsch, C. Govaerts, J. Steyaert, G. Chang, Structures of P-glycoprotein reveal its conformational flexibility and an epitope on the nucleotide-binding domain. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 13386–13391 (2013).

8. H. G. Choudhury, Z. Tong, I. Mathavan, Y. Li, S. Iwata, S. Zirah, S. Rebuffat, H. W. van Veen, K. Beis, Structure of an antibacterial peptide ATP-binding cassette transporter in a novel outward occluded state. *Proc. Natl. Acad. Sci. U.S.A.* **111**, 9145–9150 (2014).

9. A. Blees, D. Januliene, T. Hofmann, N. Koller, C. Schmidt, S. Trowitzsch, A. Moeller, R. Tampé, Structure of the human MHC-I peptide-loading complex. *Nature* **551**, 525–528 (2017).

10. Z. L. Johnson, J. Chen, Structural basis of substrate recognition by the multidrug resistance protein MRP1. *Cell* **168**, 1075–1085.e9 (2017).

11. K. Bountra, G. Hagelueken, H. G. Choudhury, V. Corradi, K. el Omari, A. Wagner, I. Mathavan, S. Zirah, W. Yuan Wahlgren, D. P. Tieleman, O. Schiemann, S. Rebuffat, K. Beis, Structural basis for antibacterial peptide self-immunity by the bacterial ABC transporter McjD. *EMBO J.* **36**, 3062–3079 (2017).

12. H. Göddeke, M. H. Timachi, C. A. J. Hutter, L. Galazzo, M. A. Seeger, M. Karttunen, E. Bordignon, L. V. Schäfer, Atomistic mechanism of large-scale conformational transition in a heterodimeric ABC exporter. *J. Am. Chem. Soc.* **140**, 4543–4551 (2018).

13. S. Hofmann, D. Januliene, A. R. Mehdipour, C. Thomas, E. Stefan, S. Brüchert, B. T. Kuhn, E. R. Geertsma, G. Hummer, R. Tampé, A. Moeller, Conformation space of a heterodimeric ABC exporter under turnover conditions. *Nature* **571**, 580–583 (2019).

14. A. Alam, J. Kowal, E. Broude, I. Roninson, K. P. Locher, Structural insight into substrate and inhibitor discrimination by human P-glycoprotein. *Science* **363**, 753–756 (2019).
15. A. Kodan, T. Yamaguchi, T. Nakatsu, K. Matsuoka, Y. Kimura, K. Ueda, H. Kato, Inward- and outward-facing X-ray crystal structures of homodimeric P-glycoprotein CmABCB1. *Nat. Commun.* **10**, 88 (2019).

16. W. Mi, Y. Li, S. H. Yoon, R. K. Ernst, T. Walz, M. Liao, Structural basis of MsbA-mediated lipopolysaccharide transport. *Nature* **549**, 233–237 (2017).

17. O. Lewinson, C. Orelle, M. A. Seeger, Structures of ABC transporters: Handle with care. *FEBS Lett.* **594**, 3799–3814 (2020).

18. R. L. Juliano, V. Ling, A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim. Biophys. Acta* **455**, 152–162 (1976).

19. Z. L. Johnson, J. Chen, ATP binding enables substrate release from multidrug resistance protein 1. *Cell* **172**, 81–89.e10 (2018).

20. R. X. Gu, V. Corradi, G. Singh, H. G. Choudhury, K. Beis, D. P. Tieleman, Conformational changes of the antibacterial peptide ATP binding cassette transporter McjD revealed by molecular dynamics simulations. *Biochemistry* **54**, 5989–5998 (2015).

21. C. Thomas, R. Tampé, Structural and mechanistic principles of ABC transporters. *Annu. Rev. Biochem.* **89**, 605–636 (2020).

22. C. Thomas, S. G. Aller, K. Beis, E. P. Carpenter, G. Chang, L. Chen, E. Dassa, M. Dean, F. Duong van Hoa, D. Ekiert, R. Ford, R. Gaudet, X. Gong, I. B. Holland, Y. Huang, D. K. Kahne, H. Kato, V. Koronakis, C. M. Koth, Y. Lee, O. Lewinson, R. Lill, E. Martinova, S. Murakami, H. W. Pinkett, B. Poolman, D. Rosenbaum, B. Sarkadi, L. Schmitt, E. Schneider, Y. Shi, S.L. Shyng, D. J. Slotboom, E. Tajkhorshid, D. P. Tieleman, K. Ueda, A. Váradi, P.C. Wen, N. Yan, P. Zhang, H. Zheng, J. Zimmer, R. Tampé, Structural and functional diversity calls for a new classification of ABC transporters. *FEBS Lett.* **594**, 3767–3775 (2020).

23. E. Steinfels, C. Orelle, J.R. Fantino, O. Dalmas, J.L. Rigaud, F. Denizot, A. di Pietro, J.M. Jault, Characterization of YvcC (BmrA), a multidrug ABC transporter constitutively expressed in *Bacillus subtilis*. *Biochemistry* **43**, 7491–7502 (2004).

24. H. Krügel, A. Licht, G. Biedermann, A. Petzold, J. Lassak, Y. Hupfer, B. Schlott, C. Hertweck, M. Platzer, S. Brantl, H.P. Saluz, Cervimycin C resistance in *Bacillus subtilis* is due to a promoter up-mutation and increased mRNA stability of the constitutive ABC-transporter gene bmrA. *FEMS Microbiol. Lett.* **313**, 155–163 (2010).

25. C. Orelle, O. Dalmas, P. Gros, A. Di Pietro, J. M. Jault, The conserved glutamate residue adjacent to the Walker-B motif is the catalytic base for ATP hydrolysis in the ATP-binding cassette transporter BmrA. *J. Biol. Chem.* **278**, 47002–47008 (2003).

26. H. W. van Veen, K. Venema, H. Bolhuis, I. Oussenko, J. Kok, B. Poolman, A. J. Driessen, W. N. Konings, Multidrug resistance mediated by a bacterial homolog of the human multidrug transporter MDR1. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 10668–10672 (1996).
27. S. Nim, L. G. Lobato, A. Moreno, V. Chaptal, M. K. Rawal, P. Falson, R. Prasad, Atomic modelling and systematic mutagenesis identify residues in multiple drug binding sites that are essential for drug resistance in the major *Candida* transporter Cdr1. *Biochim. Biophys. Acta* **1858**, 2858–2870 (2016).

28. R. Ernst, P. Kueppers, C. M. Klein, T. Schwarzmueller, K. Kuchler, L. Schmitt, A mutation of the H-loop selectively affects rhodamine transport by the yeast multidrug ABC transporter Pdr5. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 5069–5074 (2008).

29. M. Ahmed, L. Lyass, P. N. Markham, S. S. Taylor, N. Vázquez-Laslop, A. A. Neyfakh, Two highly similar multidrug transporters of *Bacillus subtilis* whose expression is differentially regulated. *J. Bacteriol.* **177**, 3904–3910 (1995).

30. H. Sjuts, A. V. Vargiu, S. M. Kwasny, S. T. Nguyen, H.S. Kim, X. Ding, A. R. Ornik, P. Ruggerone, T. L. Bowlin, H. Nikaido, K. M. Pos, T. J. Opperman, Molecular basis for inhibition of AcrB multidrug efflux pump by novel and powerful pyranopyridine derivatives. *Proc. Natl. Acad. Sci. U.S.A.* **113**, 3509–3514 (2016).

31. C. Orelle, F. Gubellini, A. Durand, S. Marco, D. Lévy, P. Gros, A. di Pietro, J.M. Jault, Conformational change induced by ATP binding in the multidrug ATP-binding cassette transporter BmrA. *Biochemistry* **47**, 2404–2412 (2008).

32. S. G. Aller, J. Yu, A. Ward, Y. Weng, S. Chittaboina, R. Zhuo, P. M. Harrell, Y. T. Trinh, Q. Zhang, I. L. Urbatsch, G. Chang, Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. *Science* **323**, 1718–1722 (2009).

33. V. Chaptal, F. Delolme, A. Kilburg, S. Magnard, C. Montigny, M. Picard, C. Prier, L. Monticelli, O. Bornert, M. Agez, S. Ravaud, C. Orelle, R. Wagner, A. Jawhari, I. Broutin, E. Pebay-Peyroula, J.M. Jault, H. R. Kaback, M. le Maire, P. Falson, Quantification of detergents complexed with membrane proteins. *Sci. Rep.* **7**, 41751 (2017).

34. K. A. Nguyen, M. Peuchmaur, S. Magnard, R. Haudecoeur, C. Boyère, S. Mounien, I. Benammar, V. Zampieri, S. Igonet, V. Chaptal, A. Jawhari, A. Boumendjel, P. Falson, Glycosyl-substituted dicarboxylates as detergents for the extraction, overstabilization, and crystallization of membrane proteins. *Angew. Chem. Int. Ed. Engl.* **57**, 2948–2952 (2018).

35. D. Lacabanne, C. Orelle, L. Lecoq, B. Kunert, C. Chuilon, T. Wiegand, S. Ravaud, J.M. Jault, B. H. Meier, A. Böckmann, Flexible-to-rigid transition is central for substrate transport in the ABC transporter BmrA from *Bacillus subtilis*. *Commun. Biol.* **2**, 149 (2019).

36. F. J. Alvarez, C. Orelle, A. L. Davidson, Functional reconstitution of an ABC transporter in nanodiscs for use in electron paramagnetic resonance spectroscopy. *J. Am. Chem. Soc.* **132**, 9513–9515 (2010).

37. S. Shukla, V. Rai, D. Banerjee, R. Prasad, Characterization of Cdr1p, a major multidrug efflux protein of *Candida albicans*: Purified protein is amenable to intrinsic fluorescence analysis. *Biochemistry* **45**, 2425–2435 (2006).
38. C. A. J. Hutter, M. H. Timachi, L. M. Hürlimann, I. Zimmermann, P. Egloff, H. Göddeke, S. Kucher, S. Štefanić, M. Karttunen, L. V. Schäfer, E. Bordignon, M. A. Seeger, The extracellular gate shapes the energy profile of an ABC exporter. *Nat. Commun.* **10**, 2260 (2019).

39. S. Mehmood, C. Domene, E. Forest, J. M. Jault, Dynamics of a bacterial multidrug ABC transporter in the inward- and outward-facing conformations. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 10832–10836 (2012).

40. P. C. Wen, B. Verhalen, S. Wilkens, H. S. McHaourab, E. Tajkhorshid, On the origin of large flexibility of P-glycoprotein in the inward-facing state. *J. Biol. Chem.* **288**, 19211–19220 (2013).

41. K. Barth, S. Hank, P. E. Spindler, T. F. Prisner, R. Tampé, B. Joseph, Conformational coupling and trans-inhibition in the human antigen transporter ortholog TmrAB resolved with dipolar EPR spectroscopy. *J. Am. Chem. Soc.* **140**, 4527–4533 (2018).

42. M. H. Timachi, C. A.J. Hutter, M. Hohl, T. Assafa, S. Böhm, A. Mittal, M. A. Seeger, E. Bordignon, Exploring conformational equilibria of a heterodimeric ABC transporter. *eLife* **6**, e20236 (2017).

43. F. Husada, K. Bountra, K. Tassis, M. Boer, M. Romano, S. Rebuffat, K. Beis, T. Cordes, Conformational dynamics of the ABC transporter McjD seen by single-molecule FRET. *EMBO J.* **37**, e100056 (2018).

44. V. Debruycker, A. Hutchin, M. Masureel, E. Ficici, C. Martens, P. Legrand, R. A. Stein, H. S. Mchaourab, J. D. Faraldo-Gómez, H. Remaut, C. Govaerts, An embedded lipid in the multidrug transporter LmrP suggests a mechanism for polyspecificity. *Nat. Struct. Mol. Biol.* **27**, 829–835 (2020).

45. M. O. Jensen, D. W. Borhani, K. Lindorff-Larsen, P. Maragakis, V. Jogini, M. P. Eastwood, R. O. Dror, D. E. Shaw, Principles of conduction and hydrophobic gating in K+ channels. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 5833–5838 (2010).

46. P. P. Borbat, K. Surendhran, M. Bortolus, P. Zou, J. H. Freed, H. S. Mchaourab, Conformational motion of the ABC transporter MsbA induced by ATP hydrolysis. *PLoS Biol.* **5**, e271 (2007).

47. M. Yang, N. Livnat Levanon, B. Acar, B. Aykac Fas, G. Masrati, J. Rose, N. Ben-Tal, T. Haliloglu, Y. Zhao, O. Lewinson, Single-molecule probing of the conformational homogeneity of the ABC transporter BtuCD. *Nat. Chem. Biol.* **14**, 715–722 (2018).

48. B. Wiseman, A. Kilburg, V. Chaptal, G. C. Reyes-Mejia, J. Sarwan, P. Falson, J.M. Jault, Stubborn contaminants: Influence of detergents on the purity of the multidrug ABC transporter BmrA. *PLOS ONE* **9**, e114864 (2014).

49. E. Heftmann, S. T. Ko, R. D. Bennett, Response of steroids to sulfuric acid in thin-layer chromatography. *J. Chromatogr.* **21**, 490–494 (1966).

50. C. M. Hebling, C. R. Morgan, D. W. Stafford, J. W. Jorgenson, K. D. Rand, J. R. Engen, Conformational analysis of membrane proteins in phospholipid bilayer nanodiscs by hydrogen exchange mass spectrometry. *Anal. Chem.* **82**, 5415–5419 (2010).
51. A. M. Lau, J. Claesen, K. Hansen, A. Politis, Deuteros 2.0: Peptide-level significance testing of data from hydrogen deuterium exchange mass spectrometry. *Bioinformatics* **37**, 270–272 (2021).

52. Y. Perez-Riverol, A. Csordas, J. Bai, M. Bernal-Llinares, S. Hewapathirana, D. J. Kundu, A. Inuganti, J. Griss, G. Mayer, M. Eisenacher, E. Pérez, J. Uszkoreit, J. Pfeuffer, T. Sachsenberg, Ş. Yılmaz, S. Tiwary, J. Cox, E. Audain, M. Walzer, A. F. Jamuczak, T. Ternent, A. Brazma, J. A. Vizcaíno, The PRIDE database and related tools and resources in 2019: Improving support for quantification data. *Nucleic Acids Res.* **47**, D442–D450 (2019).

53. J. Y. Lee, J. G. Yang, D. Zhitnitsky, O. Lewinson, D. C. Rees, Structural basis for heavy metal detoxification by an Atm1-type ABC exporter. *Science* **343**, 1133–1136 (2014).

54. L. Zimmermann, A. Stephens, S.Z. Nam, D. Rau, J. Kübler, M. Lozajic, F. Gabler, J. Söding, A. N. Lupas, V. Alva, A completely reimplemented MPI bioinformatics toolkit with a new HHpred server at its core. *J. Mol. Biol.* **430**, 2237–2243 (2018).

55. M. A. Lomize, I. D. Pogozheva, H. Joo, H. I. Mosberg, A. L. Lomize, OPM database and PPM web server: Resources for positioning of proteins in membranes. *Nucleic Acids Res.* **40**, D370–D376 (2012).

56. J. Lee, X. Cheng, J. M. Swails, M. S. Yeom, P. K. Eastman, J. A. Lemkul, S. Wei, J. Buckner, J. C. Jeong, Y. Qi, S. Jo, V. S. Pande, D. A. Case, C. L. Brooks III, A. D. MacKerell Jr, J. B. Klauda, W. Im, CHARMM-GUI input generator for NAMD, GROMACS, AMBER, OpenMM, and CHARMM/OpenMM simulations using the CHARMM36 additive force field. *J. Chem. Theory Comput.* **12**, 405–413 (2016).

57. B. Hess, C. Kutzner, D. van der Spoel, E. Lindahl, GROMACS 4: Algorithms for highly efficient, load-balanced, and scalable molecular simulation. *J. Chem. Theory Comput.* **4**, 435–447 (2008).

58. U. Essmann, L. Perera, M. L. Berkowitz, T. Darden, H. Lee, L. G. Pedersen, A smooth particle mesh Ewald method. *J. Chem. Phys.* **103**, 8577–8593 (1995).

59. S. Páll, B. Hess, A flexible algorithm for calculating pair interactions on SIMD architectures. *Comput. Phys. Commun.* **184**, 2641–2650 (2013).

60. G. Bussi, D. Donadio, M. Parrinello, Canonical sampling through velocity rescaling. *J. Chem. Phys.* **126**, 014101 (2007).

61. M. Parrinello, A. Rahman, Polymorphic transitions in single crystals: A new molecular dynamics method. *J. Appl. Phys.* **52**, 7182–7190 (1981).

62. K. Vanommeslaeghe, E. Hatcher, C. Acharya, S. Kundu, S. Zhong, J. Shim, E. Darian, O. Guvench, P. Lopes, I. Vorobyov, Mackerell AD Jr, CHARMM general force field: A force field for drug-like molecules compatible with the CHARMM all-atom additive biological force fields. *J. Comput. Chem.* **31**, 671–690 (2010).