Supporting Information
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Potassium-Ion-Selective Fluorescent Sensors To Detect Cereulide, the Emetic Toxin of B. cereus, in Food Samples and HeLa Cells
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# Supporting Information

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1. Instrumentation and general methods

Solvents and reagents were obtained from commercial sources and used as received. Column chromatography: SiO$_2$ (40-63 μm) TLC plates coated with SiO$_2$ 60F254 were visualized by UV light. The solvents for spectroscopic studies were of spectroscopic grade and used as received. NMR spectra were recorded at 25°C using a Varian Mercury 300 MHz and Varian Unity Inova 400 MHz. Ultraviolet–visible (UV-Vis) and fluorescence spectra were recorded using a Hitachi U-3900 and an F-7000 Hitachi Fluorescence spectrophotometers, respectively. Fluorescence decay lifetimes were measured using a time-correlated single photon counting instrument (FLS980 Series, Edinburgh instruments) with a 510 nm pulsed LED (Edinburgh instruments, EPL-510) light source having a 177.4 ps IR spectra were recorded with a Nicolet Impact 400D spectrophotometer. FTIR spectra were recorded with a JASCO FT/IR-4200 fitted with a JASCO “ATR PRO ONE” ATR. High resolution Mass spectra were obtained from a Bruker Autoflex matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) using dithranol (DIT) or trans-2-[3-(4-tert-butylphenyl)-2-methyl-2-propenylidene]malononitrile (DCTB) as matrix. Time of Flight Mass Spectrometry (MS-TOF) was performed on a Bruker Maxis Impact coupled to an ultra-performance liquid chromatography device Waters Acquity (UPLC-MS-TOF). Cell Culture, Fixation and Staining: HeLa cells (human cervical carcinoma cells) were cultured under standard conditions in Dulbecco’s Modified Medium containing 10% serum containing antibiotics (all from Gibco). Cells were incubated with 18µM of the probe dissolved in DMSO until 1% DMSO in the culture medium (v/v). Cells were fixed at the indicated times in the text with 4% paraformaldehyde at the indicated times (12, 24, 48, 72, 96 and 120 h). The nucleus of fixed cells were stained with Hoechst dye (Bisbenzimide) (from Sigma-Aldrich). Confocal Microscopy Imaging: High-resolution confocal imagining was performed using a Plan Apochromatic 100x oil N.A. 1.45 objective. Confocal microscopy images were obtained with a Nikon A1R confocal microscope and were processed with the NIS-Elements Advanced Research software. All confocal cell images are pseudo-colored. The fluorescent probe was imaged using the Dicroic mirror configuration: 488/561/640 and the following sequential emission filter configuration: 500-550; 570-620; 662-737.
2. Synthesis - Schemes

This process is the same described by Biesta-Peters et al. (E. G. Biesta-Peters, M. W. Reij, R. H. Blaauw, P. H. in’t Veld, A. Rajkovic, M. Ehling-Schulz and T. Abee,: Quantification of the Emetic Toxin Cereulide in Food Products by Liquid Chromatography-Mass Spectrometry Using Synthetic Cereulide as a Standard, *Appl. Environ. Microbiol.*, 2010, *76*, 7466–7472). NMR and MS spectra are shown for the first time.
Figure S2: Synthesis of cryptand (JG103) and crown ether (JG76)
3. Synthetic procedures

3.1. Synthesis of the perylene monoimide derivatives

Synthesis of N-(1-(1-adamantyl)ethyl)perylene-3,4-dicarboxylmonoimide (I) (JG62)

Perylene tetracarboxylic dianhydride (0.50 g, 1.28 mmol), DABCO (0.72 g, 6.4 mmol), and 1-(1-adamantyl)ethylamine hydrochloride (0.31 g, 1.43 mmol) were dissolved in N-methylpyrrolidone (NMP)/water 18.5 mL/1.5 mL and stirred in a high-pressure reactor at 190 °C for 3 days. Then, the reaction mixture was poured into a 1M HCl solution in water (50 mL) and stirred for one hour. The resulting mixture was filtered under reduced pressure. The solid residue was purified by column chromatography (SiO₂, hexane to dichloromethane (DCM)), from which N-(1-(1-adamantyl)ethyl)perylene-3,4-dicarboxylmonoimide JG62 (303 mg, 49%) was obtained as an orange solid, m.p. 309–311 °C. IR (KBr, cm⁻¹) 2903-2846, 1699 (C=O), 1594, 1572, 1351, 1291, 1242, 1057, 1058, 810, 750. ¹H NMR (CDCl₃, 300 MHz) δ 8.48 (m, 2H, H Ar), 8.30 (m, 4H, H Ar), 7.83 (d, J = 7.9 Hz, 2H, H Ar), 7.56 (t, 2H, J = 7.2 Hz, H Ar), 5.10 (m, 1H, CH), 1.97 (s, 3H, 3xCH), 1.84-1.64 (m, 15H, 6xCH₂ + CH₃). ¹³C NMR (CDCl₃, 75 MHz) δ (ppm) 165.8 (C=O), 165.1 (C=O), 136.9 (C Ar), 134.5 (C Ar), 132.1 (C ArH), 131.4 (C Ar), 130.9(C Ar), 129.9 (C Ar), 129.5 (C ArH), 128.2 (C ArH), 127.2 (C ArH), 126.8 (C ArH), 123.7 (C ArH), 122.2 (C Ar), 121.3 (C Ar), 120.4 (C ArH), 58.3 (CH), 40.5 (CH₂), 38.3 (C q), 37.2 (CH₂), 30.0 (CH₂), 13.4 (CH₃). HRMS (MALDI) m/z calcd for C₃₄H₂₉NO₂ (M⁺): 483.2193; found: 483.2224.

Figure S3: ¹H NMR (CDCl₃, 300 MHz) of JG62 (I)
Figure S4: $^{13}$C NMR (CDCl$_3$, 300 MHz) of JG62 (I)

Figure S5: DEPT NMR (CDCl$_3$, 300 MHz) of JG62 (I)

Figure S6: HRMS (MALDI) of JG62 (I)

| Measured m/z | Mass   | err [ppm] | Formula    |
|--------------|--------|-----------|------------|
| 483.2224     | 483.2193 | 6.439    | C34H29NO2  |
Synthesis of N-(1-(1-adamantyl)ethyl)-8-bromoperylene-3,4-dicarboxylmonoimide (II) (JG73)

A solution of Br₂ (0.11 mL, 2.1 mmol) in DCM (2 mL) was added dropwise to a mixture of N-(1-(1-adamantyl)ethyl)perylene-3,4-dicarboxylmonoimide (JG62, 100 mg, 0.21 mmol) and Fe powder (2.3 mg, 20% mmol) in DCM (30 mL) and the resulting solution was refluxed for 3 hours. Then a saturated solution of sodium sulfite or bisulfite in water was added to the reaction solution and the mixture was extracted 3 times with DCM (100 mL) and the combined organic extracts were washed with 20 mL of water, dried (Na₂SO₄) and the solvent evaporated under reduced pressure. The solid residue was purified by column chromatography (SiO₂, DCM), from which N-(1-(1-adamantyl)ethyl)perylene-8-bromo-3,4-dicarboxylmonoimide JG73 (117 mg, 89%) was obtained as a red solid, m.p. 338–339 °C. IR (KBr, cm⁻¹) 3090, 3072, 2905, 2847, 1692 (C=O), 1658 (C=O), 1594, 1565, 1454, 1410, 1374, 1347, 1313, 1288, 1247, 1204, 1179, 1114, 1099, 1058, 1036, 1017 (C-Br), 910, 841, 822, 803, 754, 684. ¹H NMR (300 MHz, CDCl₃) δ 8.62–8.50 (m, 2H, H Ar), 8.37 (ddd, ⁴J = 27.5 Hz, ⁵J = 16.8 Hz, ⁶J = 8.0 Hz, 9H, H Ar), 8.19 (d, ⁷J = 8.2 Hz, 1H, H Ar), 7.88 (d, ⁸J = 8.1 Hz, 1H, H Ar), 7.70 (t, ⁹J = 8.0 Hz, 1H, H Ar), 5.10 (q, ¹⁰J = 7.3 Hz, 1H, H Ar), 5.00 (s, 1H, 3xCH), 1.98 (s, 3H, 1.5xCH₂), 1.80 (m, 3H, 1.5xCH₂), 1.66 (m, 12H, 4.5xCH₂ + CH₃). ¹³C NMR (100 MHz, CDCl₃) δ 165.6 (C=O), 164.9 (C=O), 155.2 (C Ar), 133.2 (C Ar), 132.2 (C Ar), 132.1 (C ArH), 131.5 (C ArH), 131.4 (C ArH), 131.0 (C ArH), 129.9 (C ArH), 129.9 (C ArH), 128.3 (C ArH), 128.3 (C ArH), 124.3 (C Ar), 123.7 (C Ar), 123.7 (C Ar), 120.9 (C ArH), 120.6 (C ArH), 120.6 (C ArH), 58.3 (CH), 40.5 (CH₂), 38.2 (C q), 37.2 (CH₂), 29.0 (CH₂), 13.3 (CH₃). HRMS (MALDI) m/z calcd for C₃₄H₂₈BrNO₂ (M⁺): 561.1298; found: 561.1287.

Figure S7: ¹H NMR (CDCl₃, 300 MHz) of JG73 (II)
Synthesis of N-(1-(1-adamantyl)ethyl)-8-pinacolylboronateperylene-3,4-dicarboxylmonoimide (III) (JG75)

Pd(dppf)Cl₂ (18.5 mg, 5% mol) and potassium acetate (131 mg, 1.34 mmol) were added to a solution of N-(1-(1-adamantyl)ethyl)perylene-8-bromo-3,4-dicarboxylmonoimide (JG73, 250 mg, 0.45 mmol) and bispinacolylboronate (170 mg, 0.67 mmol) dissolved in dry 1,4-dioxane (15 mL) under nitrogen atmosphere in a 100 mL Schlenk, and the solution was stirred at 75 °C for 24 hours. Then water was added (50 mL) and the mixture was extracted with DCM (3x100 mL), worked-up, and the residue was purified by column chromatography (SiO₂, DCM), to obtain N-(1-(1-adamantyl)ethyl)perylene-8-pinacolylboronate-3,4-dicarboxylmonoimide JG75 (111 mg, 41%) as a red layer, red fluorescent under UV light (366 nm), m.p. = 256-258 °C. IR (KBr, cm⁻¹) 2976, 2922, 2905, 2849, 1694 (C=O), 1658 (C=O), 1592-1577, 1512, 1456, 1419, 1410, 1378, 1371, 1349, 1327 (B-O), 1276, 1247, 1206, 1170, 1138, 1126 (C-B), 1060, 1051, 1041, 966, 864, 847, 811, 755, 699, 667. ¹H NMR (300 MHz, CDCl₃) δ
8.81 (d, J = 8.1 Hz, 1H, C₆H), 8.53–8.46 (m, 2H, C₆H), 8.34–8.29 (m, 4H, C₆H), 8.18 (d, J = 7.6 Hz, 1H, C₆H), 7.60 (m, 1H, C₆H), 5.11 (q, J = 7.2 Hz, 1H, CH), 1.98 (s, 3H, 3xCH), 1.85–1.81 (m, 3H, 1.5xCH₂), 1.73–1.63 (s, 12H, 4.5xCH₂ + CH₃), 1.47 (s, 12H, 4xCH₃(pinacol)). ¹³C NMR (75 MHz, CDCl₃) δ (ppm) 165.8-165.1 (C=O), 138.1 (C₆), 137.2 (C₆), 136.4 (C₆H), 133.2 (C₆), 132.3-130.8 (C₆H + C₆), 131.5 (C₆H), 129.7 (C₆H), 129.1 (C₆H), 127.8 (C₆), 127.3 (C₆), 126.5 (C₆), 123.5 (C₆), 122.5 (C₆), 121.9 (C₆), 121.6 (C₆), 120.8 (C₆H), 120.3 (C₆H), 84.4 (C₆), 58.2 (CH), 40.5 (CH₂), 38.2 (C₆), 37.2 (CH₂), 29.0 (CH₂), 25.2 (CH₃), 13.3 (CH₃). MS (MALDI, DCTB) m/z (%) 608 (25, M⁺), 609 (100, M+H⁺), 610 (40, M⁺ + 2), 611 (10, M⁺ + 3). HRMS (MALDI, DCTB) m/z calcd for C₄₀H₄₀BNO₄: 608.3081(M⁺), found: 608.3017.

Figure S10: ¹H NMR (CDCl₃, 300 MHz) of JG75 (III)

Figure S11: ¹³C NMR (CDCl₃, 300 MHz) of JG75 (III)
3.2. **Synthesis of the crown ether probe (F) (JG76)**

Synthesis of 16-(2-(2-methoxyethoxy)phenyl)-1,4,7,10,13-pentaaza-16-azacyclooctadecane (D) (JG66) (S. Ast, T. Schwarze, H. Müller, A. Sukhanov, S. Michaelis, J. Wegener, O. S. Wolfbeis, T. Körzdörfer, A. Dürkop and H.-J. Holdt: A highly K⁺-selective phenylaza-[18]crown-6-lariat-ether-based fluoroionophore and its application in the sensing of K⁺ ions with an optical sensor film and in cells, *Chem. Eur. J.*, 2013, **19**, 14911–14917).

NaH (60%, 0.8 g, 20 mmol) was added slowly, under stirring, to a solution of \(N,N\)-bis(2-hydroxyethyl)-(2-(2-methoxyethoxy)phenyl)amine (2 g, 7.8 mmol) in dry acetonitrile (70 mL), under nitrogen atmosphere, in a 250 mL Schlenk. Then tetraethyleneglycol-bis(p-toluenesulfonate) (3.97 g, 7.9 mmol) were dissolved in 30 mL of dry MeCN. The schlenk was heated to reflux and the solution of 1,17-ditosyl-3,6,9,12,15-pentaazaoctadecane was added dropwise over a period of 4 h. Then, the mixture was heated under reflux for 11 h and the resulting precipitate was filtered off. Then, the solvent was removed under reduced pressure and the residue was purified by column chromatography on silica gel (CH\(_2\)Cl\(_2\)/MeOH, 95:5) to yield 16-(2-(2-methoxyethoxy)phenyl)-1,4,7,10,13-pentaaza-16-azacyclooctadecane (1.75 g, 54%) as a brown oil. IR (KBr, cm\(^{-1}\)) 3058, 2873, 1721, 1665, 1592, 1500, 1451, 1352, 1240, 1199, 1116, 1055, 956, 844, 749. \(^1\)H NMR (CDCl\(_3\), 300 MHz) \(\delta\) 7.05 (dd, 1H, \(J = 7.5, J = 2.0\) Hz, C\(_{Ar}\)H), 6.95–6.81 (m, 3H, C\(_{Ar}\)H), 4.10 (m, 2H, CH\(_2\)), 3.73–3.53 (m, 22H, 11xC\(_{CH2}\)), 3.39-3.35 (m, 7H, 2xCH\(_2\) + CH\(_3\)). \(^13\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 152.5 (C\(_{Ar}\)), 132.5 (C\(_{Ar}\)), 127.2 (C\(_{Ar}\)H), 122.2 (2xC\(_{Ar}\)H), 115.2 (C\(_{Ar}\)H), 70.6-70.4 (CH\(_2\)), 69.5 (CH\(_2\)), 69.5 (CH\(_2\)), 68.9 (CH\(_2\)), 64.8 (CH\(_2\)), 59.2 (CH\(_3\)), 56.7 (2xCH\(_3\)). HRMS (ESI\(^{-}\)) m/z calcld for C\(_{21}\)H\(_{37}\)NO\(_7\)(M+H\(^+\)):: 414.2486; found: 414.2494.
Figure S13: $^1$H NMR (CDCl$_3$, 300 MHz) of JG66 (D)

Figure S14: $^{13}$C NMR (CDCl$_3$, 300 MHz) of JG66 (D)
Synthesis of 16-(4-bromo-2-(2-methoxyethoxy)phenyl)-1,4,7,10,13-pentaoxa-16-azacyclooctadecane (E) (JG70)

Bromine (0.2 mL, 4 mmol) in dry dichloromethane (20 mL) was added dropwise at room temperature to a solution of 16-(2-(2-methoxyethoxy)phenyl)-1,4,7,10,13-pentaoxa-16-azacyclooctadecane (1 g, 2.4 mmol) in dry dichloromethane (50 mL). After 1 hour at reflux and under stirring, the reaction was quenched and extracted with sodium sulfite solution in water (3x100 mL) and finally washed with water (100 mL). The organic layer was dried over anhydrous sodium sulfate and the solvent removed under reduced pressure to give 16-(4-bromo-2-(2-methoxyethoxy)phenyl)-1,4,7,10,13-pentaoxa-16-azacyclooctadecane (1.12 g, 95%) as a brown viscous liquid. IR (KBr, cm\(^{-1}\)) 2883, 1721, 1665, 1580, 1492, 1470, 1456, 1400, 1349, 1245 (C-O-C), 1106 (C-Br), 1048 (C-O-C), 956, 837. \(^{1}\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 7.05–6.88 (m, 3H, CH Ar), 4.11–4.06 (t, \(J\) = 4.7 Hz, 2H, CH\(_2\)), 3.78–3.54 (m, 22H, 11xCH\(_2\)), 3.42 (m, 7H, 3xCH\(_2\) + CH\(_3\)). \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 153.6 (C Ar), 138.8 (C Ar), 124.4 (C\(_{Ar}\)H), 124.3 (C\(_{Ar}\)Br), 117.0 (C\(_{Ar}\)H), 71.1-70.2 (CH\(_2\)), 69.3 (CH\(_2\)), 67.9 (CH\(_3\)), 59.2 (CH\(_3\)), 53.7 (CH\(_2\)), 53.1 (CH\(_2\)). HRMS (ESI\(^{+}\)) m/z calcd for C\(_{21}\)H\(_{37}\)BrNO\(_7\) (M+H\(^{+}\)): 492.1591; found: 492.1590.
Figure S16: $^1$H NMR (CDCl$_3$, 300 MHz) of JG70 (E)

Figure S17: $^{13}$C NMR (CDCl$_3$, 300 MHz) of JG70 (E)

Figure S18: HRMS (MALDI) of JG70 (E)
Synthesis of \( N-(1-(1\text{-adamantyl})\text{ethyl})-8-(4-(1,4,7,10,13\text{-penta}o\text{x}a-16\text{-azacyclooctadecan-16-yl})-3-(2\text{-methoxyethoxy})\text{phen}y\text{l})\text{per}y\text{len}e-3,4\text{-dicarboxyl}m\text{onomoi}m\text{ide} \) (F) (JG76)

\[ \text{Pd(PPh}_3\text{)}_4 (10 \text{ mg, } 5\% \text{ mol}) \text{ was added to a solution of } 16-(4\text{-bromo-2-(2-methoxyethoxy)}\text{phen}y\text{l})-1,4,7,10,13\text{-penta}o\text{x}a-16\text{-azacyclooctadecane} \text{ (JG70, 100 mg, 0.16 mmol) in toluene:nBuOH (15 mL:4 mL) under nitrogen atmosphere in a 100 mL Schlenck. Then a solution of } N-(1-(1\text{-adamantyl})\text{ethyl})-8\text{-pinacol}y\text{boronateperylene-3,4\text{-dicarboxyl}monomoi}m\text{ide} \text{ (JG75, 81 mg, 0.16 mmol) in toluene:nBuOH:water (5 mL:1.5 mL:0.5 mL) was added and then Na}_2\text{CO}_3 \text{ (174 mg, 1.64 mmol) was added and the mixture was heated under reflux for 24 hours. The mixture was then poured on water (100 mL), extracted with CH}_2\text{Cl}_2 \text{ (3x100 mL), worked-up and then the residue was purified by column chromatography (SiO}_2 \text{, DCM:MeOH, 92:8 v/v) to obtain } N-(1-(1\text{-adamantyl})\text{ethyl})-8-(4-(1,4,7,10,13\text{-penta}o\text{x}a-16\text{-azacyclooctadecan-16-yl})-3-(2\text{-methoxyethoxy})\text{phen}y\text{l})\text{per}y\text{len}e-3,4\text{-dicarboxyl}m\text{onomoi}m\text{ide} \text{ JG76 (58 mg, 40%) as a purple solid, m.p. 135-136 °C. IR (KBr, cm}^{-1} \) 2955, 2924, 2848, 1738 (C=O), 1692 (C=O), 1685 (C=O), 1651, 1590, 1571, 1506, 1457, 1384, 1354, 1248, 1122. ¹H NMR (300 MHz, CDCl₃) δ 8.53 (m, 2H, CArH), 8.49-8.33 (m, 3H, C ArH), 8.02 (m, 1H, C ArH), 7.59–7.54 (m, 2H, C ArH), 7.25–6.91 (m, 4H, C ArH), 5.10 (q, \( J = 7.1 \text{ Hz} \), 1H, CH), 4.23 (m, 2H, CH₂), 3.80-3.54 (m, 24H, 12xCH₂), 3.66-3.40 (s, 5H, CH₂ + CH₃), 1.98 (m, 3H, 3xCH), 1.85–1.81 (m, 3H, 1.5xCH₂), 1.73–1.62 (m, 12H, 4.5xCH₂ + CH₃). ¹³C NMR (100 MHz, CDCl₃) δ 173.7 (C=O), 165.7 (C Ar), 165.0 (C Ar), 136.8 (C Ar), 132.6–123.2 (C Ar + CH Ar), 121.9 (C Ar), 121.0 (C Ar), 120.1 (C Ar), 116.5 (C Ar), 72.1–67.0 (CH₂), 59.3–58.2 (CH + CH₃), 40.5 (CH), 38.9 (CH₂), 38.2 (CH₃), 34.2 (CH), 32.1–29.5 (CH + CH₃). HRMS (MALDI) calcld for C₅₅H₆₃N₂O₉: 894.4455; found: 894.4572 (M⁺). HRMS (MALDI) m/z calcld for C₅₃H₆₃N₂O₈: 895.4528 (M+H⁺); found: 895.4535.

Figure S19: ¹H NMR (CDCl₃, 300 MHz) of JG76 (I)
3.3. Synthesis of the cryptand probe (M, JG103)

Synthesis of triazacrown ether (J) (JG89) (Sui, B.; Yue, X.; Tichy, M. G.; Liu, T.; Belfield, K. D.: Improved Synthesis of the Triazacryptand (TAC) and its Application in the Construction of a Fluorescent TAC-BODIPY Conjugate for K⁺ Sensing in Live Cells, Eur. J. Org. Chem. 2015, 1189–1192) (See also: H. He, Mark A. Mortellaro, Marc J. P. Leiner, Robert J. Fraatz, James K. Tusa: A Fluorescent Sensor with High Selectivity and Sensitivity for Potassium in Water, J. Am. Chem. Soc. 2003, 125, 1468-1469)
A mixture of K$_2$CO$_3$ (1.158 g, 8.4 mmol) and degassed acetonitrile (135 mL) was heated to reflux under N$_2$ atmosphere. Then a solution of N,N-bis(2-(2-amino-5-methylphenoxy)ethyl)-2-(2-methoxyethoxy)aniline (1.31 g, 2.8 mmol) and 1,2-bis(2-iodoethoxy)ethane (0.58 mL, 3.1 mmol) in degassed acetonitrile (135 mL) was added dropwise over 4 h. The resulting reaction mixture was stirred under reflux for 4 days. Then, the solvent was removed under reduced pressure and the residue was poured into water, extracted with CH$_2$Cl$_2$ and washed three times with brine. The organic layer was dried over Na$_2$SO$_4$, then filtered and the solvent evaporated. The crude product was purified by flash column chromatography (silica gel, 2.5% methanol/97.5% CH$_2$Cl$_2$ as eluent) to give the precryptand product JG89 (682 mg, 40%) as an off-white solid. $^1$H NMR (300 MHz, CDCl$_3$) δ 7.13–7.11 (m, 1H, C$_{Ar}$H), 6.93–6.88 (m, 3H, C$_{Ar}$H), 6.66–6.60 (m, 2H, C$_{Ar}$H), 6.55–6.50 (m, 4H, C$_{Ar}$H), 4.17–4.14 (m, 2H, CH$_2$), 4.04 (t, $J = 5.8$ Hz, 4H, CH$_2$), 3.85 (t, $J = 5.8$ Hz, 4H, 2xCH$_2$), 3.79–3.74 (m, 6H, 3xCH$_2$), 3.70 (s, 4H, 2xCH$_2$), 3.44 (s, 3H, CH$_3$), 3.37–3.24 (t, $J = 5$ Hz, 4H, 2xCH$_2$), 2.20 (s, 6H, 3xCH$_3$). $^{13}$C NMR (75 MHz, CDCl$_3$) δ 152.4 (C$_{Ar}$), 146.7 (C$_{Ar}$), 138.6 (C$_{Ar}$), 136.0 (C$_{Ar}$), 126.3 (C$_{Ar}$), 122.7 (C$_{Ar}$), 121.8 (C$_{Ar}$), 121.7 (C$_{Ar}$), 121.4 (C$_{Ar}$), 114.4 (C$_{Ar}$), 112.1 (C$_{Ar}$), 110.5 (C$_{Ar}$), 71.3 (CH$_2$), 70.9 (CH$_2$), 70.0 (CH$_2$), 68.0 (CH$_2$), 67.7 (CH$_2$), 59.2 (CH$_3$), 53.4 (CH$_3$), 44.1 (CH$_3$), 21.0 (CH$_3$). HRMS (ESI$^+$) m/z calc'd for C$_{33}$H$_{46}$N$_3$O$_6$: 580.3381(M+H$^+$); found 580.3384.

Figure S22: $^1$H NMR (CDCl$_3$, 300 MHz) of JG89 (J)

Figure S23: $^{13}$C NMR (CDCl$_3$, 300 MHz) of JG89 (J)
Synthesis of triazacryptand (K) (JG93) (B. Sui, X. Yue, M. G. Tichy, T. Liu and K. D. Belfield: Improved Synthesis of the Triazacryptand (TAC) and its Application in the Construction of a Fluorescent TAC-BODIPY Conjugate for $K^+$ Sensing in Live Cells, Eur. J. Org. Chem., 2015, 1189–1192)

A mixture of CaCO$_3$ (0.70 g, 7.0 mmol) and distilled water (115 mL) was heated to reflux under N$_2$ atmosphere followed by the addition of 115 mL degased 1,4-dioxane. Then, a solution of precryptand JG89 (1.36 g, 2.3 mmol) and 1,2-bis(2-iodoethoxy)ethane (0.441 mL, 2.3 mmol) in degased 1,4-dioxane (115 mL) was added dropwise over a period of 4 h. The resulting reaction mixture was stirred under reflux for 4 days. Then, after cooling to room temperature, the mixture was filtered and the filtrate was condensed to 100 mL. After that, the filtrate was extracted with CH$_2$Cl$_2$ (3 × 100 mL). The organic layer was dried over Na$_2$SO$_4$, filtered and concentrated in vacuo.

Purification by flash column chromatography using 3.0% methanol/97% CH$_2$Cl$_2$ provided triazacryptand JG93 (0.488 g, 30%) as a colorless foamy solid. $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.11 (dd, $J = 7.9$, $J = 1.6$ Hz, 1H, C$_{Ar}$H), 6.98–6.87 (m, 5H, C$_{Ar}$H), 6.64 (d, $J = 7.9$ Hz, 2H, C$_{Ar}$H), 6.58 (d, $J = 1.6$ Hz, 2H, C$_{Ar}$H), 4.15 (t, $J = 4.3$ Hz, 2H, CH$_2$), 4.07 (t, $J = 5.9$ Hz, 2H, CH$_2$), 3.80–3.68 (m, 8H, 4xCH$_2$), 3.58–3.48 (m, 18H, 9xCH$_2$), 3.41 (s, 3H, CH$_3$), 3.36–3.33 (m, 6H, 3xCH$_2$), 2.21 (s, 6H, 2xCH$_3$). $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 152.5 (C$_{Ar}$), 146.8 (C$_{Ar}$), 138.7 (C$_{Ar}$), 136.0 (C$_{Ar}$), 126.4 (C$_{Ar}$), 122.8 (C$_{Ar}$), 121.9 (C$_{Ar}$), 121.8 (C$_{Ar}$), 114.5 (C$_{Ar}$), 112.2 (C$_{Ar}$), 110.6 (C$_{Ar}$), 72.7 (CH$_2$), 72.1 (CH$_2$), 71.4 (CH$_2$), 71.0 (CH$_2$), 70.6 (CH$_2$), 70.4 (CH$_2$), 70.0 (CH$_2$), 68.1 (CH$_2$), 67.7 (CH$_2$), 62.0 (CH$_2$), 59.3 (CH$_3$), 53.3 (CH$_2$), 44.2 (CH$_2$), 21.1 (CH$_3$). HRMS (ESI$^+$) m/z calcd for C$_{39}$H$_{55}$N$_3$O$_8$: 694.4062 (M+H$^+$); found 694.4062; m/z calcd for C$_{39}$H$_{54}$N$_3$NaO$_8$: 716.3881 (M+Na$^+$); found 716.3878.
Figure S25: $^1$H NMR (CDCl$_3$, 300 MHz) of JG93 (K)

Figure S26: $^{13}$C NMR (CDCl$_3$, 300 MHz) of JG93 (K)

Figure S27: HRMS (MALDI) of JG93 (K)

**Table S2**

| Meas. m/z | m/z  | err [ppm] | mSigma | Ion Formula |
|-----------|------|-----------|--------|-------------|
| 694.4062  | 694.4062 | 0.0       | 43.5   | C$_{39}$H$_{56}$N$_3$O$_8$ |
| 716.3878  | 716.3881 | 0.4       | 63.2   | C$_{39}$H$_{55}$N$_3$NaO$_8$ |
Synthesis of bromo-triazacryptand (L) (JG101):

Bromine (0.03 mL, 0.058 mmol) in dry dichloromethane (2 mL) was added dropwise at RT to a solution of triazacryptand (JG93, 0.1 g, 0.144 mmol) in dry dichloromethane (10 mL). After 1 hour under stirring and reflux, the reaction was quenched and extracted with sodium sulfite solution in water (3x50 mL) and finally washed with water (100 mL). The organic layer was dried over anhydrous sodium sulfate and the solvent removed under reduced pressure to give bromo-triazacryptand JG101 (82 mg, 74%) as a brown foam. IR (KBr, cm⁻¹) 2923-2859, 1576, 1506, 1455, 1362, 1250, 1169, 1119, 1049, 1031, 937, 836, 812. ¹H NMR (300 MHz, CDCl₃) δ 6.99–6.97 (m, 3H, C ArH), 6.88–6.84 (m, 2H, C ArH), 6.64 (d, J = 6.5 Hz, 2H, C ArH), 6.54 (s, 2H, C ArH), 4.15–3.97 (m, 6H, 3xCH₂), 3.84–3.75 (m, 6H, 3xCH₂), 3.59–3.36 (m, 27H, 12xCH₂ + CH₃), 2.22 (s, 6H, 2xCH₃). ¹³C NMR (75 MHz, CDCl₃) δ 153.1 (C Ar), 153.0 (C Ar), 137.8 (C Ar), 137.3 (C Ar), 132.7 (C Ar), 124.5 (C Ar), 123.1 (C Ar), 122.3 (C Ar), 121.5 (C Ar), 121.3 (C Ar), 117.3 (C Ar), 114.7 (C Ar), 114.4 (C Ar), 71.2 (CH₂), 70.9 (CH₂), 69.6 (CH₂), 68.2 (CH₂), 67.1 (CH₂), 59.3 (CH₂), 53.6–52.4 (CH₂), 43.0 (CH₂), 39.0 (CH₂), 21.3 (CH₂). HRMS (ESI⁺) m/z calculated for C₉₉H₅₅BrN₃O₈ (M+H⁺): 772.3167; found 772.3174.

Figure S28: ¹H NMR (CDCl₃, 300 MHz) of JG101 (L)

Figure S29: ¹³C NMR (CDCl₃, 300 MHz) of JG101 (L)
Synthesis of triazacryptand-perylenemonoimide (M) (JG103)

Pd(PPh₃)₄ (5.6 mg, 5% mol) was added to a solution of bromo-triazacryptand JG101 (59 mg, 0.097 mmol) dissolved in Toluene:nBuOH (10 mL:3.3 mL) under nitrogen atmosphere in a 100 mL schlenck. Then, boronic ester (82 mg, 0.10 mmol) dissolved in Toluene:nBuOH (3.5 mL:1 mL) was added dropwise. Then, Na₂CO₃ (102.2 mg, 0.97 mmol) dissolved in water (3 mL) was added and the mixture was stirred under reflux for 24 hours. The reaction mixture was poured on water (30 mL) and the product was extracted with DCM (3x100 mL). After work-up, the solid residue was purified by column chromatography (Silica gel, DCM:MeOH (50:4) as eluent) from which the triazacryptand-perylenemonoimide JG103 (48 mg, 42%) was obtained as a purple solid, m.p. 193-195ºC. IR (KBr, cm⁻¹) 2955, 2922, 2856, 1736 (C=O), 1696 and 1682 (C=O), 1651, 1592, 1509, 1456, 1351, 1280, 1170, 1119, 1106, 1049, 959, 812, 750, 721, 697, 667. ¹H NMR (300 MHz, CDCl₃) δ 8.53 (m, 2H, C ArH), 8.49–8.33 (m, 3H, CArH), 8.02 (m, 1H, C ArH), 7.59–7.54 (m, 2H, C ArH), 7.25–6.91 (m, 4H, C ArH), 5.10 (q, J = 7.1 Hz, 1H, CH), 4.23-3.50 (m, 39H, 18xCH₂ + CH₃), 2.26-2.20 (m, 6H, 2xCH₃), 1.98 (m, 3H, 3xCH), 1.73–1.62 (m, 13H, 6xCH₂ + CH₃). ¹³C NMR (100 MHz, CDCl₃) δ 165.8 (Cα), 165.1 (Cα), 153.2 (Cα), 137.3 (CH₃), 132.9, 132.3, 132.2, 130.0, 129.6, 128.9, 128.5, 128.2, 127.0, 126.8, 126.7, 123.0, 123.5, 122.3, 121.3, 120.4, 120.1, 114.8, 114.4, 114.2, and 110.0 (CH Ar + CH Ar), 71.4, 71.0, 70.8, 70.5, 69.6, 68.2, and 67.3 (CH₂), 59.2 (CH₂), 58.2 (CH), 53.6–52.5 (CH₂), 40.4 (CH), 38.2 (C₂), 37.1 (CH), 31.7, 31.1, 29.2, and 29.0 (CH + CH₂), 21.3 (CH₂), 14.3, and 13.3 (CH₃). HRMS (ESI) m/z calcd for C₇₃H₅₂N₄O₁₀: 1197.5923 (M+Na⁺); found 1197.6001.
Figure S31: $^1$H NMR (CDCl$_3$, 300 MHz) of JG103 (M)

Figure S32: $^{13}$C NMR (CDCl$_3$, 300 MHz) of JG103 (M)

Figure S33: HRMS (MALDI) of JG103 (M)

| Meas.        | m/z       | m/z       | err [ppm] | Ion Formula          |
|--------------|-----------|-----------|-----------|----------------------|
| 1197.6001    | 1197.5923 | 7.8       | C73H82N4O10Na |
3.4. Synthesis of cereulide (15) (JG100)

(15) E. G. Biesta-Peters, M. W. Reij, R. H. Blaauw, P. H. in’t Veld, A. Rajkovic, M. Ehling-Schulz and T. Abee: Quantification of the Emetic Toxin Cereulide in Food Products by Liquid Chromatography-Mass Spectrometry Using Synthetic Cereulide as a Standard, Appl. Environ. Microbiol., 2010, 76, 7466–7472). NMR and MS spectra are shown for the first time.

Synthesis of benzyl (S)-2-hydroxy-3-methylbutanoate (1) (JG82)

Benzyl bromide (25.2 mL, 211.6 mmol) was added dropwise to a stirred mixture of (S)-2-hydroxy-3-methylbutyric acid (5 g, 42.3 mmol) and anhydrous NaHCO₃ (7.11 g, 84.6 mmol) in dry DMF (130 mL) under an atmosphere of argon. The resulting mixture was stirred at room temperature 48 hours and the mixture diluted on CH₂Cl₂ (500 mL) and washed with water (3x150 mL). The organic layer was dried (Na₂SO₄) and evaporated in vacuo to leave a yellow oil. The oil was purified by silica gel chromatography using 2% ethyl acetate – hexane as eluent to give (S)-2-hydroxy-3-methylbutyric acid benzyl ester (8.68 g, 99%) as a colorless oil. ¹H NMR (300 MHz, CDCl₃) δ 7.37 (s, 5H, (C ArH)), 5.22 (d, J = 12.2 Hz, 2H, CH₂), 4.09 (dd, J = 3.6, J = 6.2 Hz, 1H, CH), 2.69 (d, J = 6.1 Hz, 1H, OH), 2.04–2.12 (m, 1H, CH), 1.01 (d, J = 6.9 Hz, 3H, CH₃), 0.83 (d, J = 6.9 Hz, 3H, CH₃). ¹³C NMR (75 MHz, CDCl₃) δ 175.0 (C=O), 135.3 (C Ar), 128.8 (C ArH), 128.7 (C ArH), 75.1 (CH), 67.5 (CH₂), 32.3 (CH), 19.0 (CH₃), 16.0 (CH₃). HRMS (ESI⁺) m/z calcd. for C₁₂H₁₆NaO₃: 231.0997(M+Na⁺); found: 231.0989.

Figure S34: ¹H NMR (CDCl₃, 300 MHz) of JG82 (1)

Figure S35: ¹³C NMR (CDCl₃, 300 MHz) of JG82 (1)
Synthesis of benzyl (S)-2-hydroxy-4-methylpentanoate (2) (JG86)

Benzyl bromide (29.73 mL, 250 mmol) was added dropwise to a stirred mixture of L-Leucic acid (6.61 g, 42.3 mmol) and anhydrous NaHCO₃ (8.40 g, 100 mmol) in dry DMF (140 mL) under an atmosphere of argon. The resulting mixture was stirred at room temperature for 48 hours and the mixture diluted on CH₂Cl₂ (500 mL) and washed with water (3x200 mL). The organic layer was dried (Na₂SO₄) and evaporated in vacuo to leave a yellow oil. The oil was purified by silica gel chromatography using from hexane to 10% ethyl acetate–hexane as eluent to get (S)-2-hydroxy-4-methylpentanoate (11.11 g, 94%) as a colorless oil. ¹H NMR (300 MHz, CDCl₃) δ 7.37 (s, 5H, (C_ArH)), 5.21 (s, 2H, CH₂), 4.25 (dd, J = 3.6, J = 6.2 Hz, 1H, CH), 2.66 (d, J = 6.1 Hz, 1H, OH), 1.82–1.96 (m, 1H, CH), 1.55-1.60 (m, 2H, CH₂), 0.95 (d, J = 6.9 Hz, 3H, CH₃), 0.93 (d, J = 6.9 Hz, 3H, CH₃). ¹³C NMR (75 MHz, CDCl₃) δ 176.0 (C=O), 135.4 (C_Ar), 128.9 (C_ArH), 128.8 (C_ArH), 128.6 (C_ArH), 69.4 (CH), 67.5 (CH₂), 43.6 (CH₂), 24.6 (CH), 23.5 (CH₃), 21.8 (CH₃). HRMS (ESI⁺) m/z calcd. for C₁₃H₁₈NaO₃ (M+Na⁺): 245.1148; found: 245.1146.
Synthesis of Boc-(D-Ala-L-O-Val)-OBn (3) (JG88)

\[\text{N,N-dicyclohexylcarbodiimide (DCC)} \ (8.40 \text{ g, 40.7 mmol}) \text{ was added to a stirred solution of Boc-D-Ala (7.70 g, 40.7 mmol), benzyl (S)-2-hydroxy-3-methylbutanoate (8 g, 38.4 mmol), and 4-(dimethylamino)pyridine (DMAP) (938.5 mg, 7.7 mmol) in CH}_2\text{Cl}_2 (1955 \text{ mL}) \text{ at } 0^\circ\text{C. The resulting suspension was warmed until room temperature and stirred for 2 h. The suspension was filtered and concentrated on CH}_2\text{Cl}_2 (150 \text{ mL}). Purification by flash column chromatography (ethyl acetate (EtOAc)/heptane, 1:4; Rf, 0.28) afforded Boc-(D-Ala-L-O-Val)-OBn (14.576 g, 85%) as a white solid. \]  

\[\text{H NMR} (300 \text{ MHz, CDCl}_3) \delta 7.34 (s, 5H, CArH), 5.18 (dd, 2H, J = 12.1 Hz, CH₂), 5.03 (m, 1H, NH), 4.93 (d, J = 4.4 Hz, 1H, CH), 4.43 (m, 1H, CH), 2.27 (m, 1H, CH), 1.44-1.40 (m, 12H, 4xCH₃), 0.96 (dd, 6H, J = 12.8, J = 6.9 Hz, 2xCH₃). \]  

\[\text{C NMR} (75 \text{ MHz, CDCl}_3) \delta 173.0 (C=O), 169.3 (C=O), 135.5 (C_Ar), 128.8 (C_ArH), 128.6 (C_ArH), 77.4 (CH), 67.2 (CH₂), 49.6 (CH), 30.4 (CH), 28.6 (CH₃), 19.0 (CH₃), 18.9 (CH₃), 17.3 (CH₃). \]  

\[\text{HRMS (ESI⁺) m/z calcd. for C}_{20}\text{H}_{29}\text{NNaO}_6: 402.1887 (M+Na⁺); found: 402.1888.} \]
Figure S40: $^1$H NMR (CDCl$_3$, 300 MHz) of JG88 (3)

Figure S41: $^{13}$C NMR (CDCl$_3$, 300 MHz) of JG88 (3)

Figure S42: HRMS (MALDI) of JG88 (3)
Synthesis of H-(D-Ala-L-O-Val)-OBn · HCl (4) (JG88B)

HCl(g) was bubbled in a solution of Boc-(D-Ala-L-O-Val)-OBn (11 g, 28.9 mmol) in EtOAc (150 mL). The resulting mixture was stirred for 4 hours and then concentrated in vacuo, to afford H-(D-Ala-L-O-Val)-OBn · HCl (9.10 g, 99%) as a colorless very viscous oil, which was used without further purification. $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 8.92 (s, 2H, NH$_2$), 7.33 (s, 5H, C$_{Ar}$H), 5.15 (dd, 2H, $J$ = 12.1 Hz, CH$_2$), 5.03 (d, 1H, $J$ = 4.2 Hz, CH), 4.28 (m, 1H, CH), 2.22 (m, 1H, CH), 1.75 (d, 3H, CH$_3$), 0.95 (dd, 6H, $J$ = 15.5, $J$ = 6.9 Hz, 2xCH$_3$). $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 169.6 (C=O), 168.7 (C=O), 135.4 (C$_{Ar}$), 128.8, 128.7, and 128.6 (C$_{Ar}$H), 78.4 (CH), 67.5 (CH$_2$), 49.5 (CH), 30.4 (CH), 18.9 (CH$_3$), 17.2 (CH$_3$), 16.2 (CH$_3$). HRMS (ESI$^+$) m/z calcd. for C$_{15}$H$_{22}$NO$_4$: 280.1543 (M+H$^+$); found: 280.1546.

Figure S43: $^1$H NMR (CDCl$_3$, 300 MHz) of JG88B (4)

Figure S44: $^{13}$C NMR (CDCl$_3$, 300 MHz) of JG88B (4)
At 0°C, triphenylphosphine (18.59 g, 70.9 mmol) was added to a stirred solution of benzyl (S)-2-hydroxy-4-methylpentanoate (5 g, 22.5 mmol) and Boc-L-Val (4.89 g, 22.5 mmol) in dry tetrahydrofuran (THF) (190 mL). Next, diethyl azodicarboxylate (DEAD) (40 wt% in toluene, 16.01 mL, 70.9 mmol) was added dropwise. The resulting yellow solution was allowed to warm to room temperature and stirred for 2 h. Subsequently, the mixture was concentrated in vacuo, redissolved in CH₂Cl₂ (150 mL), and extracted with water. The organic phase was dried over Na₂SO₄, filtered, and concentrated in vacuo. Purification by flash column chromatography (EtOAc/heptane, 1:9; Rf, 0.25) afforded Boc-(L-Val-D-O-Leu)-OBn (8.02 g, 85%) as a colorless oil. ¹H NMR (300 MHz, CDCl₃) δ 7.34 (m, 5H, C ArH), 5.15 (s, 2H, CH₂), 5.09 (dd, 1H, J = 9.9, J = 3.7 Hz, CH), 4.98 (d, J = 9.1 Hz, 1H, NH), 4.32 (dd, 1H, J = 9.1, J = 4.6 Hz, CH), 2.19 (m, 1H, CH), 1.88-1.58 (m, 3H, CH₂-CH), 1.44 (s, 9H, 3xCH₃), 0.98-0.87 (m, 12H, 4xCH₃). ¹³C NMR (75 MHz, CDCl₃) δ 172.0 (C=O), 170.4 (C=O), 155.7 (C=O), 135.4 (C Ar), 128.8 (C ArH), 128.5 (C ArH), 79.9 (C q), 71.3 (CH), 67.3 (CH₂), 58.8 (CH), 39.8 (CH₂), 31.5 (CH), 28.5 (CH₂), 24.7 (CH), 23.3 (CH₃), 21.5 (CH₂), 19.3 (CH₃), 17.5 (CH₃). HRMS (ESI⁺) calcd. for C₂₃H₃₆NO₆: 422.2537; found: 422.2542 (M+H⁺); m/z calcd. for C₂₃H₃₅NNaO₆: 444.2357(M+Na⁺); found: 444.2362.

Figure S46: ¹H NMR (CDCl₃, 300 MHz) of JG90 (5)
Palladium on carbon (10% [wt/wt] Pd, 750 mg) was added to a stirred solution of Boc-(L-Val-D-O-Leu)-OBn (8 g, 19 mmol) in methanol (MeOH; 60 mL) at room temperature. The resulting mixture was placed under a hydrogen atmosphere and stirred vigorously for 1 h. Next, the mixture was filtered over Celite and concentrated in vacuo to afford Boc-(L-Val-D-O-Leu)-OH (5.30 g, 84%) as a colorless oil, which was used without further purification. 

1H NMR (300 MHz, CDCl₃) δ 9.75 (s, 1H, COOH), 6.46 (d, 1H, J = 8.5 Hz, NH), 5.09 (d, 1H, J = 9.1 Hz, CH), 5.02 (m, 1H, CH), 4.27-4.02 (dd, 1H, J = 9.1, J = 4.6 Hz, CH), 2.20-2.11 (m, 1H, CH), 1.84-1.60 (m, 3H, CH₂-CH), 1.39 (s, 9H, 3xCH₃), 0.95-0.85 (m, 12H, 4xCH₃). 

13C NMR (75 MHz, CDCl₃) δ 173.5 (C=O), 171.8 (C=O), 155.8 (C=O), 79.9 (C₆), 71.3 (CH), 58.6 (CH), 50.3 (CH) 39.6 (CH₂), 31.1 (CH), 28.2 (CH₃), 28.1 (CH₃), 24.5 (CH), 23.0 (CH₃), 21.1 (CH₃), 19.0 (CH₃), 17.3 (CH₃). 

HRMS (ESI⁺) m/z calcd. for C₁₆H₃₀NO₆: 332.2068; found: 332.2068 (M+H⁺); m/z calcd. for C₁₆H₂₉NNaO₆: 334.1887 (M+Na⁺); found: 354.1889.
Figure S49: $^1$H NMR (CDCl$_3$, 300 MHz) of JG90 (6)

Figure S50: $^{13}$C NMR (CDCl$_3$, 300 MHz) of JG90 (6)

Figure S51: HRMS (MALDI) of JG90 (7)
Diisopropylethylamine (DIPEA) (5.26 mL, 30.18 mmol) was added dropwise to a stirred solution of Boc-(L-Val-D-O-Leu)-OH (6) (5 g, 15.09 mmol) in dimethylformamide (DMF) (95 mL) at 0°C. Next, 1-hydroxybenzotriazole (HOBt) (2.24 g, 16.6 mmol) and N-((3-dimethylaminopropyl)-N-ethylcarbodiimide (EDCI) (2.57 g, 16.6 mmol) were added successively. Finally, a solution of H-(D-Ala-L-O-Val)-OBn · HCl (4) (5 g, 15.84 mmol) in DMF (45 mL) was added dropwise. The resulting mixture was allowed to warm to room temperature and stirred for 16 h. Next, the mixture was concentrated in vacuum to 70 mL. The solution was extracted CH₂Cl₂:H₂O (3x100 mL), dried over Na₂SO₄, filtered, and concentrated in vacuo. Purification by flash column chromatography (EtOAc/heptane, 1:3; Rf, 0.23) afforded Boc-(L-Val-D-O-Leu-D-Ala-L-O-Val)-OBn (7) (8.944 g, 96%) as a colorless oil.

1H NMR (300 MHz, CDCl₃) δ 7.32 (m, 5H, C₆ArH), 7.12 (d, 1H, , J = 6.9 Hz, NH), 5.31-5.27 (m, 1H, CH), 5.16 (q, 2H, , J = 12.2 Hz, CH₂), 5.05 (d, J = 9.1 Hz, 1H, NH), 4.88 (d, 1H, J = 4.3 Hz, CH), 4.55 (m, 1H, CH), 4.08 (m, 1H, CH), 2.22 (m, 1H, CH), 2.08 (m, 1H, CH), 1.79-1.66 (m, 3H, CH₂-CH), 1.48 (d, 3H, J = 7.3 Hz, CH₃), 1.39 (s, 9H, 3xCH₃), 0.99-0.87 (m, 18H, 6xCH₃). 13C NMR (75 MHz, CDCl₃) δ 172.1 (C=O), 171.9 (C=O), 170.0 (C=O), 156.1 (C=O), 135.5 (C₆Ar), 128.8, 128.6, and 128.5 (C₆ArH), 80.4 (C₆q), 77.4 (CH), 72.9 (CH), 67.2 (CH₂), 59.8 (CH), 48.8 (CH), 40.7 (CH₂), 30.6 (CH), 30.3 (CH), 28.5 (CH₂), 24.6 (CH), 23.4 (CH₃), 21.6 (CH₃), 19.4 (CH₃), 18.9 (CH₃), 18.4 (CH₃), 17.4 (CH₂), 17.2 (CH₃). HRMS (ESI⁺) m/z calcd. for C₃₁H₄₉N₂O₉ (M+H⁺): 593.3433; found: 593.3431; m/z calcd. for C₃₁H₄₈N₂NaO₉: 615.3258 (M+Na⁺); found: 615.3252.
Synthesis of Boc-(L-Val-D-O-Leu-D-Ala-L-O-Val)-OH (8) (JG94)

Palladium on carbon (10% [wt/wt] Pd, 0.6 g) was added to a stirred solution of Boc-(L-Val-D-O-Leu-D-Ala-L-O-Val)-OBn (3.25 g, 5.5 mmol) in methanol (60 mL) at room temperature. The resulting mixture was placed under hydrogen atmosphere and stirred vigorously for 1 h. Next, the mixture was filtered over celite and concentrated in vacuo to afford Boc-(L-Val-D-O-Leu-D-Ala-L-O-Val)-OH (2.756 g, 86%) as a colorless oil, which was used without further purification. $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 8.23 (s, 1H, COOH), 7.32 (d, 1H, $J$ = 7.2 Hz, NH), 5.30 (dd, 1H, $J$ = 8.0, $J$ = 4.8 Hz, CH), 5.13 (d, $J$ = 7.6 Hz, 1H, NH), 4.96 (d, 1H, $J$ = 3.9 Hz, CH), 4.56 (m, 1H, CH), 4.13 (m, 1H, CH), 2.28 (m, 1H, CH), 2.11 (sext, 1H, $J$ = 6.7 Hz, CH), 1.79-1.64 (m, 3H, CH$_2$-CH), 1.47 (d, 3H, $J$ = 7.1 Hz, CH$_3$), 1.41 (s, 9H, 3xCH$_3$), 1.00-0.89 (m, 18H, 6xCH$_3$). $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 172.2 (C=O), 172.1 (C=O), 171.9 (C=O), 170.6 (C=O), 156.6 (C=O), 81.0 (C$_{q}$), 77.4 (CH), 73.0 (CH), 59.7 (CH), 48.7 (CH), 40.9 (CH$_2$), 30.6 (CH), 30.3 (CH), 28.5 (CH$_3$), 24.6 (CH), 23.5 (CH$_2$), 21.5 (CH$_3$), 19.4 (CH$_3$), 19.0 (CH$_3$), 18.2 (CH$_3$), 17.1 (CH$_3$). HRMS (ESI$^+$) m/z calcd. for C$_{24}$H$_{43}$N$_2$O$_9$: 503.2963(M+H$^+$); found: 503.2964.
Figure S55: $^1$H NMR (CDCl$_3$, 300 MHz) of JG94 (8)

Figure S56: $^{13}$C NMR (CDCl$_3$, 300 MHz) of JG94 (8)

Figure S57: HRMS (MALDI) of JG94 (8)
HCl(g) was bubbled in a solution of Boc-(L-Val-D-O-Leu-D-Ala-L-O-Val)-OBn (7) (4.75 g, 8 mmol) in EtOAc (95 mL). The resulting mixture was stirred for 4 hours and then concentrated in vacuo, to afford H-(L-Val-D-O-Leu-D-Ala-L-O-Val)-OBn · HCl (9) (4.124 g, 97%) as a white solid, which was used without further purification.

1H NMR (300 MHz, CDCl₃) δ 8.64 (s, 2H, NH₂), 7.78 (d, 1H, J = 6.9 Hz, NH), 7.32 (m, 5H, C ArH), 5.38-5.34 (m, 1H, CH), 5.16 (q, 2H, J = 12.2 Hz, CH₂), 4.89 (d, 1H, J = 4.5 Hz, CH), 4.58 (m, 1H, CH), 4.15 (m, 1H, CH), 2.47 (m, 1H, CH), 2.22 (m, 1H, CH), 1.85-1.69 (m, 3H, CH₂=CH), 1.51 (d, 3H, J = 7.2 Hz, CH₃), 0.95-0.88 (m, 18H, 6xCH₃). 13C NMR (75 MHz, CDCl₃) δ 172.1 (C=O), 169.6 (C=O), 169.3 (C=O), 168.0 (C=O), 135.3 (C₆H₅), 128.7 (C₆H₅), 128.5 (C₆H₅), 128.4 (C₆H₅), 77.4 (CH), 74.5 (CH), 67.1 (CH₂), 59.0 (CH), 48.8 (CH), 40.9 (CH₂), 30.3 (CH), 30.1 (CH), 24.4 (CH), 23.1 (CH₃), 21.7 (CH₃), 18.8 (CH₃), 18.5 (CH₃), 17.3 (CH₃), 17.1 (CH₃). HRMS (ES⁺) m/z calcd. for C₂₆H₄₁N₂O₇: 493.2908 (M+H⁺); found: 493.2906.

Figure S58: 1H NMR (CDCl₃, 300 MHz) of JG95 (9)

Figure S59: 13C NMR (CDCl₃, 300 MHz) of JG95 (9)
DIPEA (5.26 mL, 30.18 mmol) was added dropwise to a stirred solution Boc-(L-Val-D-O-Leu-D-Ala-L-O-Val)-OH (8) (2.75 g, 5.48 mmol) in CH₂Cl₂ (125 mL) at room temperature, followed by addition to a solution of H-(L-Val-D-O-Leu-D-Ala-L-O-Val)-OBn · HCl (9) (2.9 g, 5.5 mmol) in CH₂Cl₂ (60 mL). Finally, PyBop [benzotriazol-1-yl]tripyrrolidinophosphonium (2.2 g, 5.9 mmol) was added, and the mixture was stirred for 1 h. Next, the solution was concentrated and extracted CH₂Cl₂:H₂O (3x150 mL), dried over Na₂SO₄, filtered, and concentrated in vacuo. Purification by flash column chromatography (EtOAc/heptane, 1:2; Rf, 0.23) afforded Boc-(L-Val-D-O-Leu-D-Ala-L-O-Val-L-Val-D-O-Leu-D-Ala-L-O-Val)-OBn (10) (3.637 g, 68%) as a white solid. 

**1H NMR (300 MHz, CDCl₃)**

\[ \delta \]

- 7.81 (d, 1H, J = 6.1 Hz, NH),
- 7.70 (d, 1H, J = 8.0 Hz, NH),
- 7.67 (d, 1H, J = 7.2 Hz, NH),
- 7.32 (m, 5H, CArH),
- 5.38-5.35 (m, 1H, CH),
- 5.20-5.09 (m, 4H, CH₂ + CH + NH),
- 5.00 (d, J = 5.9 Hz, 1H, CH),
- 4.84 (d, 1H, J = 4.3 Hz, CH),
- 4.55 (m, 1H, CH),
- 4.38 (t, J = 7.8 Hz, 1H, CH),
- 4.08 (m, 1H, CH),
- 3.87 (m, 1H, CH),
- 3.82 (m, 1H, CH),
- 3.47 (m, 1H, CH),
- 2.98 (m, 1H, CH),
- 2.82-2.30 (m, 3H, 3xCH),
- 2.00 (m, 1H, CH),
- 1.82-1.67 (m, 6H, CH₂(CH₂-CH₂)),
- 1.50-1.42 (m, 15H, 5xCH₃),
- 1.05-0.88 (m, 36H, 12xCH₃).

**13C NMR (75 MHz, CDCl₃)**

\[ \delta \]

- 172.5 (C=O),
- 172.0 (C=O),
- 171.8 (C=O),
- 170.8 (C=O),
- 170.5 (C=O),
- 170.2 (C=O),
- 1169.2 (C=O),
- 156.6 (C=O),
- 134.1 (C₆H₅),
- 128.6 (C₆H₅),
- 128.4 (C₆H₄),
- 128.3 (C₆H₄),
- 81.0 (C₆H₄),
- 78.8 (CH₃),
- 72.8 (CH₃),
- 72.5 (CH₃),
- 66.8 (CH₂),
- 60.5 (CH₂),
- 58.9 (CH₂),
- 48.4 (CH₃),
- 40.5 (CH₃),
- 30.2 (CH₃),
- 29.9 (CH₃),
- 28.4 (CH₂),
- 24.3 (CH₃),
- 23.5 (CH₃),
- 23.3 (CH₃),
- 21.2 (CH₃),
- 20.9 (CH₃),
- 19.6 (CH₃),
- 19.1 (CH₃),
- 18.8 (CH₃),
- 17.7 (CH₃),
- 17.1 (CH₃),
- 16.6 (CH₃). 

HRMS (ESI⁺) m/z calcd. for C₅₀H₈₀N₄NaO₁₅: 999.5512 (M+Na⁺); found: 999.5571.
Figure S61: $^1$H NMR (CDCl$_3$, 300 MHz) of JG96 (10)

Figure S62: $^{13}$C NMR (CDCl$_3$, 300 MHz) of JG96 (10)

Figure S63: HRMS (MALDI) of JG96 (10)

| Meas. m/z | m/z    | err [ppm] | mSigma | Ion Formula |
|-----------|--------|-----------|--------|-------------|
| 999.5512  | 999.5512 | -5.9     | 201.6  | C50H80N4NaO15 |

Intens. x10^5

m/z
Synthesis of Boc-(L-Val-D-O-Leu-D-Ala-L-O-Val-L-Val-D-O-Leu-D-Ala-L-O-Val)-OH (11) (JG97)

Palladium on carbon (10% [wt/wt] Pd, 0.6 g) was added to a stirred solution of Boc-(L-Val-D-O-Leu-D-Ala-L-O-Val-L-Val-D-O-Leu-D-Ala-L-O-Val)-OBn (10) (3.34 g, 3.4 mmol) in MeOH (50 mL) at room temperature. The resulting mixture was placed under a hydrogen atmosphere and stirred vigorously for 1 h. Next, the mixture was filtered over Celite and concentrated in vacuo to obtain Boc-(L-Val-D-O-Leu-D-Ala-L-O-Val-L-Val-D-O-Leu-D-Ala-LO-Val)-OH (11) (3.09 g, 90%) as a white solid, which was used without further purification. 

1H NMR (300 MHz, CDCl3) δ 7.82 (d, 1H, J = 7.4 Hz, NH), 7.74 (d, 1H, J = 6.0 Hz, NH), 7.66 (d, 1H, J = 7.0 Hz, NH), 5.31-5.23 (m, 2H, 2xCH), 5.10 (d, J = 6.4 Hz, 1H, CH), 4.99 (m, 2H, 2xCH), 4.53 (m, 1H, CH), 4.26-4.16 (m, 2H, 2xCH), 4.08 (m, 1H, CH), 2.34-2.26 (m, 3H, 3xCH), 2.02 (m, 1H, CH), 1.80-1.71 (m, 6H, 2x(CH2-CH), 1.52-1.42 (m, 15H, 5xCH3), 1.04-0.89 (m, 36H, 12xCH3). 13C NMR (75 MHz, CDCl3) δ 172.5 (C=O), 171.9 (C=O), 171.6 (C=O), 171.5 (C=O), 171.0 (C=O), 170.9 (C=O), 170.5 (C=O), 156.6 (C=O), 80.8 (C3), 78.8 (CH), 72.7 (CH), 60.2 (CH), 49.4 (CH), 48.2 (CH), 40.9 (CH3), 40.4 (CH3), 30.2 (CH), 30.1 (CH), 29.9 (CH), 28.2 (CH3), 24.3 (CH), 24.2 (CH), 23.3 (CH3), 23.1 (CH3), 21.0 (CH3), 20.8 (CH3), 19.4 (CH3), 19.3 (CH3), 19.0 (CH3), 18.9 (CH3), 18.7 (CH3), 17.1 (CH3), 16.9 (CH3), 16.5 (CH3), 16.4 (CH3). HRMS (ESI+) m/z calcd. for C12H75N4O15: 887.5223 (M+H+); found: 887.5256.
Synthesis of Boc-(L-Val-D-O-Leu-D-Ala-L-O-Val-L-Val-D-O-Leu-D-Ala-L-O-Val-L-Val-D-O-Leu-D-Ala-L-O-Val)-OBn (12)(JG98)

DIPEA (329 µl, 1.9 mmol) was added dropwise to a stirred solution of H-(L-Val-D-O-Leu-D-Ala-L-O-Val)-OBn · HCl (9) (500 mg, 0.95 mmol) in CH₂Cl₂ (23 mL) at room temperature, followed by addition to a solution of Boc-(L-Val-D-O-Leu-D-Ala-L-O-Val-L-Val-D-O-Leu-D-Ala-L-O-Val)-OH (11) (800 mg, 0.9 mmol) in CH₂Cl₂ (12 mL). Then, (benzotriazol-1-yloxy)tripyrrolidinophosphonium (PyBop) (372 mg, 1.9 mmol) was added, and the mixture was stirred for 2 h. Then, the solution was concentrated and extracted CH₂Cl₂:H₂O (3x50 mL), dried over Na₂SO₄, filtered, and concentrated in vacuo. Purification by flash column chromatography (EtOAc/hexane, 2:6; Rf, 0.26) afforded Boc-(L-Val-D-O-Leu-D-Ala-L-O-Val-L-Val-D-O-Leu-D-Ala-L-O-Val)-OBn (12) (0.865 g, 71%) as a white solid. ¹H NMR (300 MHz, CDCl₃) δ 8.29 (d, 1H, J = 6.2 Hz, NH), 8.04 (d, 1H, J = 6.0 Hz, NH), 7.85 (m, 1H, NH), 7.74 (d, 1H, J = 7.4 Hz, NH), 7.29 (m, 5H, C ArH), 5.35 (m, 1H, NH), 5.27 (m, 1H, CH), 5.16-5.01 (m, 5H, 5xCH), 4.92 (d, 1H, J = 3.2 Hz, CH₂), 4.77 (d, 1H, J = 4.6 Hz, CH₂), 4.63 (m, 1H, CH), 4.50 (m, 1H, CH), 4.32 (m, 1H, CH), 4.01 (m, 2H, 2xCH), 3.80 (m, 1H, CH), 2.40-2.16 (m, 5H, 3xCH + CH₂), 1.99-1.59 (m, 10H, 4xCH₂ + 2xCH), 0.83-0.63 (m, 18xCH₃). ¹³C NMR (75 MHz, CDCl₃) δ 172.8 (C=O), 172.7 (C=O), 172.7 (C=O), 172.1 (C=O), 171.0 (C=O), 171.0 (C=O), 170.8 (C=O), 170.7 (C=O), 170.6 (C=O), 169.9 (C=O), 169.5 (C=O), 156.8 (C=O), 128.7 (C₂H), 128.4 (C₂H), 128.4 (C₂H), 81.1 (C₆), 79.4 (CH), 79.2 (CH), 77.4 (CH), 73.0 (CH), 72.6 (CH), 66.9 (CH₂), 60.7 (CH), 58.8 (CH), 58.4 (CH), 49.9 (CH), 49.6 (CH), 48.3 (CH), 41.5 (CH₂), 41.2 (CH₂), 40.8 (CH₂), 30.7 (CH), 30.4 (CH), 30.3 (CH), 30.0 (CH), 28.5 (CH₃), 24.6 (CH), 24.4 (CH), 23.4 (CH₃), 23.3 (CH₃), 21.4 (CH₃), 21.0 (CH₃), 20.9 (CH₃), 19.4 (CH₃), 19.3 (CH₃), 19.2 (CH₃), 18.8 (CH₃), 18.0 (CH₃), 16.8 (CH₃), 16.7 (CH₃), 16.5 (CH₃). HRMS (MALDI, DCTB) m/z calcd. for C₁₉₁H₁₁₂N₁₀O₂₁: 1383.7773 (M+Na⁺); found: 1383.8135; m/z calcd. for C₁₁₉H₇₅N₄O₁₅: 1399.7512 (M+K⁺); found: 1399.7996.

Figure S66: HRMS (MALDI) of JG97 (11)
Figure S67: $^1$H NMR (CDCl$_3$, 300 MHz) of JG98 (12)

Figure S68: $^{13}$C NMR (CDCl$_3$, 300 MHz) of JG98 (12)

Figure S69: HRMS (MALDI) of JG98 (12)
Synthesis of Boc-(L-Val-D-O-Leu-D-Ala-L-O-Val-L-Val-D-O-Leu-D-Ala-L-O-Val-L-Val-D-O-Leu-D-Ala-L-O-Val)-OH (13) (JG99)

Palladium on carbon (10% wt/wt Pd, 0.25 g) was added to a stirred solution of Boc-(L-Val-D-O-Leu-D-Ala-L-O-Val-L-Val-D-O-Leu-D-Ala-L-O-Val-L-Val-D-O-Leu-D-Ala-L-O-Val)-OBn (12) (0.860 g, 0.63 mmol) in MeOH (7 mL) at room temperature. The resulting mixture was placed under hydrogen atmosphere and stirred vigorously for 1 hour. Then, the mixture was filtered over Celite and concentrated in vacuo to obtain Boc-(L-Val-D-O-Leu-D-Ala-L-O-Val-L-Val-D-O-Leu-D-Ala-L-O-Val-L-Val-D-O-Leu-D-Ala-L-O-Val)-OH (13) (790 mg, 98%) as a white solid.

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 8.25 (d, 1H, $J$ = 6.2 Hz, NH), 8.04 (d, 1H, $J$ = 6.0 Hz, NH), 7.84 (m, 3H, NH), 5.30 (m, 1H, CH), 5.26 (m, 1H, CH), 5.10-4.88 (m, 5H, CH), 4.49 (t, $J$ = 7.2 Hz, 1H, CH), 4.40 (t, $J$ = 7.2 Hz, 1H, CH), 4.30 (t, $J$ = 7.2 Hz, 1H, CH), 4.07 (m, 2H, CH), 3.86 (t, $J$ = 7.2, 1H, CH), 2.34-2.24 (m, 5H, 3xCH + CH$_2$), 2.02-1.57 (m, 10H, 4xCH + 3xCH$_2$), 1.46-1.39 (m, 18H, 6xCH$_3$), 1.00-0.84 (m, 54H, 18xCH$_3$). 13C NMR (75 MHz, CDCl$_3$) $\delta$ 172.6 (C=O), 172.4 (C=O), 171.5 (C=O), 171.3 (C=O), 170.2 (C=O), 170.6 (C=O), 170.5 (C=O), 170.1 (C=O), 156.7 (C=O), 80.9 (C$_3$), 79.2 (CH), 79.1 (CH), 73.1 (CH), 73.0 (CH), 72.7 (CH), 60.3 (CH), 58.7 (CH), 58.5 (CH), 53.5 (CH), 49.6 (CH), 49.4 (CH), 48.3 (CH), 41.0 (CH$_2$), 40.6 (CH$_2$), 30.2 (CH), 30.0 (CH), 29.8 (CH), 28.3 (CH$_3$), 24.4 (CH), 24.3 (CH), 23.4 (CH$_3$), 23.2 (CH$_3$), 23.2 (CH$_3$), 21.1 (CH), 20.9 (CH$_3$), 20.8 (CH$_3$), 19.3 (CH$_3$), 19.2 (CH$_3$), 19.1 (CH$_3$), 19.0 (CH$_3$), 18.9 (CH$_3$), 18.8 (CH$_3$), 17.2 (CH$_3$), 17.0 (CH$_3$), 16.6 (CH$_3$), 16.5 (CH$_3$), 16.4 (CH$_3$), 16.4 (CH$_3$), 16.4 (CH$_3$), 16.4 (CH$_3$), 16.4 (CH$_3$), 16.4 (CH$_3$), 16.4 (CH$_3$), 16.4 (CH$_3$), 16.4 (CH$_3$). HRMS (ESI$^+$) m/z calcld. for C$_{62}$H$_{106}$N$_6$NaO$_{21}$: 1293.7303 (M+Na$^+$); found: 1293.7323.

Figure S70: $^1$H NMR (CDCl$_3$, 300 MHz) of JG99 (13)
Figure S71: $^{13}$C NMR (CDCl$_3$, 300 MHz) of JG99 (13)

Figure S72: HRMS (MALDI) of JG99 (13)

| Meas. m/z | m/z    | err [ppm] | mSigma | Ion Formula          |
|-----------|--------|-----------|--------|----------------------|
| 1293.7323 | 1293.7303 | -1.5     | 46.8   | C62H106N6NaO21       |

Synthesis of H-(L-Val-D-O-Leu-D-Ala-L-O-Val-L-Val-D-O-Leu-D-Ala-L-O-Val-L-Val-D-O-Leu-D-Ala-L-O-Val)-OH · HCl (14) (JG99B)

HCl(g) was bubbled in a solution of Boc-(L-Val-D-O-Leu-D-Ala-L-O-Val-L-Val-D-O-Leu-D-Ala-L-O-Val-L-Val-D-O-Leu-D-Ala-L-O-Val)-OH (13) (0.79 g, 0.62 mmol) in EtOAc (15 mL). The resulting mixture was stirred for 4 hours and then concentrated in vacuo, to afford H-(L-Val-D-O-Leu-D-Ala-L-O-Val-L-Val-D-O-Leu-D-Ala-L-O-Val-L-Val-D-O-Leu-D-Ala-L-O-Val)-OH·HCl (14) (0.75 mg, 100%) as a white solid. $^1$H NMR (300 MHz,
CD₃OD δ: 8.74 (d, 1H, J = 6.7 Hz, NH), 8.49 (d, 1H, J = 6.0 Hz, NH), 8.38 (m, 1H, NH), 5.16 (m, 3H, 3xCH), 4.90 (m, 2H, 3xCH), 4.81 (d, J = 4.2 Hz, 1H, 2xCH), 4.56 (m, 2H, 2xCH), 4.44 (m, 3H, 3xCH), 4.07 (m, 1H, CH), 4.15 (d, J = 4.3 Hz, 1H, CH), 2.40-2.18 (m, 6H, 2xCH + 2xCH₂), 1.87-1.68 (m, 10H, 4xCH + 3xCH₂), 1.46 (d, J = 7.1 Hz, 9H, 3xCH₃), 1.11 (t, J = 5.2 Hz, 6H, 2xCH₃), 1.02-0.94 (m, 48H, 16xCH₃). ¹³C NMR (75 MHz, CD₃OD) δ 173.1 (C=O), 173.0 (C=O), 172.8 (C=O), 171.3 (C=O), 172.7 (C=O), 172.5 (C=O), 172.1 (C=O), 172.0 (C=O), 171.9 (C=O), 171.6 (C=O), 169.7 (C=O), 79.9 (CH), 78.5 (CH), 75.2 (CH), 73.8 (CH), 73.6 (CH), 59.4 (CH), 42.0 (CH₂), 41.8 (CH₂), 31.6 (CH), 31.5 (CH), 31.2 (CH), 30.9 (CH), 25.6 (CH), 25.5 (CH), 25.4 (CH), 23.7 (CH₃), 23.6 (CH₃), 21.6 (CH₃), 21.5 (CH₃), 21.4 (CH₃), 19.7 (CH₃), 19.3 (CH₃), 19.1 (CH₃), 18.8 (CH₃), 18.5 (CH₃), 18.2 (CH₃), 18.0 (CH₃), 17.7 (CH₃), 17.6 (CH₃), 17.3 (CH₃). HRMS (ESI⁺) m/z calcd. for C₅₇H₉₉N₆O₁₉: 1171.6960 (M+H⁺); found: 1171.6939.

Figure S73: ¹H NMR (CDCl₃, 300 MHz) of JG99B (14)

Figure S74: ¹³C NMR (CDCl₃, 300 MHz) of JG99B (14)
Synthesis of Cereulide (15) (JG100)

DIPEA (0.35 mL, 1.062 mmol) was added to dry DMF (350 mL) at room temperature, followed by PyBop (146 mg, 0.39 mmol). Then, a solution of H-(L-Val-D-O-Leu-D-Ala-L-O-Val-L-Val-D-O-Leu-D-Ala-L-O-Val-L-Val-D-O-Leu-D-Ala-L-O-Val)-OH·HCl (14) was dissolved in DMF (40 mL) and slowly added (2-3 hours) to the previous solution; afterwards the mixture was stirred for 16 h. Next, the mixture was evaporated and concentrated in vacuo. Purification by flash column chromatography (EtOAc/heptane, 1:3; Rf, 0.28) afforded cereulide JG100 (284 mg, 70%) as a white solid. $^1$H NMR (300 MHz, CD$_3$OD) $\delta$ 7.76 (d, 6H, $J = 6.9$ Hz, NH), 5.29 (dd, 3H, $J = 7.7$ Hz, $J = 5.1$ Hz, CH), 4.97 (d, 3H, $J = 6.9$ Hz, CH), 4.34 (m, 3H, CH), 4.07 (dd, 3H, $J = 9.6$ Hz, $J = 7.5$ Hz, CH), 2.32-2.24 (m, 6H, 2xCH + 2xCH$_2$), 1.76-1.61 (m, 9H, 3x(CH$_2$-CH)), 1.42 (d, $J = 7.1$ Hz, 9H, 3xCH$_3$), 1.03 (d, $J = 6.6$ Hz, 9H, 3xCH$_3$), 0.96-0.85 (m, 45H, 15xCH$_3$). $^{13}$C NMR (75 MHz, CD$_3$OD) $\delta$ 172.1 (C=O), 171.7 (C=O), 171.2 (C=O), 170.7 (C=O), 79.9 (CH), 78.9 (CH), 72.9 (CH), 59.6 (CH), 49.0 (CH), 40.8 (CH$_2$), 30.7 (CH), 28.9 (CH), 24.6 (CH), 23.6 (CH), 21.4 (CH), 19.5 (CH$_3$), 18.8 (CH$_3$), 17.1 (CH$_3$), 16.0 (CH$_3$). HRMS (MALDI, DCTB) m/z calcd. for C$_{57}$H$_{99}$N$_9$NaO$_{19}$: 1175.6673 (M+Na$^+$); found: 1175.6681.
Figure S76: $^1$H NMR (CDCl$_3$, 300 MHz) of JG100 (15)

Figure S77: $^{13}$C NMR (CDCl$_3$, 300 MHz) of JG100 (16)
NMR spectra were identical to the reported for cereulide: (a) Makarasen, A.; Yoza, K.; Isobe, M.: Higher Structure of Cereulide, an Emetic Toxin from Bacillus cereus, and Special Comparison with Valinomycin, an Antibiotic from Streptomyces fulvissimus, *Chem. Asian J.* 2009, 4, 688–698. (b) Isobe, M.; Ishikawa, T.; Suwan, S.; Agata, N.; Ohta, M.: Synthesis and Activity of Cereulide, a Cyclic Dodecadepsipeptide Ionophore as Emetic Toxin from Bacillus cereus, *Bioorg. Med. Chem. Lett.* 1995, 5, 2855-2858
4. Qualitative analysis

4.1. Solvatochromism

1.1. Experimental conditions:

For each compound, a mother solution in chloroform was prepared. From this solution, an aliquot was evaporated in different vials, one for each solvent that was measured. Once evaporated, the residue was redissolved in the appropriate solvent, under bath sonication if it didn’t solve by stirring. Then, the absorbance and the fluorescence were measured.

In order to choose the best solvent for further experiments, it was necessary to fulfill several conditions:

- The solubility of the probe should be high. To prepare a solution at least $10^{-4}$ M.
- The solvent should be miscible with water.
- The final fluorescence should not be negligible.
- At least, 3 times increase in fluorescence is required for a good quantification.

Once the solvatochromic measurements were performed, the best solvent was selected.

1.2. Solvachromism of JG76:

In a concentration of 50 µM, absorbance and fluorescence of the probe JG76 were measured.

**Solvents studied, from left to right by polarity order, Snyder index:**

1. Water  2. MeOH  3. DMSO  
4. DMF  5. MeCN  6. Acetone  
7. EtOH  8. AcOEt  9. THF  
10. CHCl₃  11. CH₂Cl₂  12. Toluene  
13. Et₂O  14. Hexane  15. Cyclohexane

Qualitative tests were done by taking pictures of the samples in different solvents, under visible light and UV light, 366 nm:

![Figure S80. JG76 solutions in different solvents under visible light and under UV light.](image)

Due to the interest of the action of K⁺ cation in detection, KCl (excess) was added to the solution and the change in response was studied:

![Figure S81. JG76 solutions + KCl in different solvents under visible light and under UV light.](image)
Although 366 nm is not the best excitation wavelength to study the change in fluorescence of our probe, it may be seen how the fluorescence increases in alcoholic solution, which was further studied in the quantitative analysis. Moreover, the main drawback of K⁺ probes is the usual Na⁺ interference that could increase the fluorescence. To check that, we added to the solution NaCl (excess) and afterwards KCl, checking the changes:

**JG76 + NaCl (excess):**

![Image](S82.png)

**Figure S82.** JG76 solutions + NaCl in different solvents under visible light and under UV light.

**JG76 + NaCl (excess) + KCl (excess):**

![Image](S83.png)

**Figure S83.** JG76 solutions + NaCl + KCl in different solvents under visible light and under UV light.

It shows that the solution is selective to K⁺, even in presence of Na⁺, which doesn’t increase the fluorescence nor avoid K⁺ to increase it. This was confirmed in subsequent analysis. The absorption and fluorescent emission of the samples were measured too, obtaining the results shown. First, UV-Visible spectra were registered:

**Figure S84.** JG76 solutions 50 µM, absorption (left) and normalized absorption spectra (right) in different solvents.

In the spectra, JG76 showed similar absorbance in most solvents. The solubility is lower in toluene, Et₂O and DMF; and for water, hexane and cyclohexane the probe is insoluble. Due to the potential possibilities of increase the fluorescence in presence of K⁺, the tests were repeated after adding KCl (excess):
Figure S85. JG76 solutions 50 µM + KCl (excess), absorption (left) and normalized absorption spectra (right) in different solvents.

The absorbance is similar before and after the addition of KCl. However, the maximum of absorbance wavelength changes depending on the solvent:

| Solvent      | Without K⁺ A<sub>max</sub> λ (nm) | With K⁺ A<sub>max</sub> λ (nm) |
|--------------|----------------------------------|-------------------------------|
| DMSO         | 520                              | 520                           |
| DMF          | 517                              | -----                         |
| Toluene      | 513                              | 512                           |
| THF          | 510                              | 512                           |
| Chloroform   | 510                              | 504                           |
| AcOEt        | 509                              | 512                           |
| MeOH         | 508                              | 503                           |
| EtOH         | 507                              | 503                           |
| Diethylether | 507                              | -----                         |
| Acetone      | 506                              | 512                           |
| DCM          | 506                              | 512                           |
| MeCN         | 501                              | 497                           |

Table S1. JG76 solutions 50 µM maximum of absorption wavelength before and after adding potassium.

The variation on the maximum of absorption is not significant, changing no more than 5 nm. Furthermore, the band of absorption is quite wide.

Fluorescence results:

Taking 507 nm as the excitation wavelength:

Figure S86. JG76 solutions 50µM, fluorescent emission (left) and normalized fluorescent emission spectra (right) in different solvents. (λ<sub>exc</sub>=507nm)
Fluorescent emission intensity is high for dichloromethane, chloroform and acetonitrile, low for acetone and very low for the rest of the solvents.

**After KCl addition:**

![Graph showing emission intensity and wavelength for different solvents](image)

**Figure S87.** JG76 solutions 50 µM + KCl (excess), fluorescent emission (left) and normalized fluorescent emission spectra (right) in different solvents. ($\lambda_{exc}=507\text{nm}$)

Then, the emission intensity and the wavelength were studied:

| Solvent   | $\phi/\phi_0$ | Without $K^+$ | With $K^+$ |
|-----------|---------------|---------------|------------|
| MeOH      | 4.7           | 574           | 573        |
| DMSO      | 2.3           | ----          | 571        |
| DMF       | 8.1           | ----          | 570        |
| MeCN      | 0.7           | 563           | 564        |
| Acetone   | 3.4           | 571           | 571        |
| EtOH      | 4.5           | 563           | 563        |
| AcOEt     | 1.2           | 559           | 555        |
| THF       | 2.3           | 555           | 550        |
| Chloroform| 0.9           | 560           | 559        |
| DCM       | 1.3           | 559           | 559        |
| Toluene   | 0.3           | 553           | ----       |
| Et$_2$O   | 1.0           | ----          | ----       |
| Cyclohexane| 1.2         | ----          | ----       |

**Table S2** and **Figure S88.** JG76 solutions 50 µM (left) fluorescent increase and change in maximum of emission table. Right, graphic increase in fluorescent emission maximum.

**Results:**

- The fluorescence decreases in toluene and acetonitrile.
- In DMSO, there is an increase in fluorescence, but the initial fluorescence is very low, almost zero, and the final fluorescence is also low, but not so much. Therefore, in spite of the fluorescence increase, it isn’t a good solvent because of the low intensity of emission.
- In DMF, the increase in emission seems to be very high but it is because of the increase of the solubility in the presence of potassium; moreover, the fluorescence is too low.
- The fluorescence increase is very high for methanol, ethanol and acetone. These are the best options to make the study of the potential applications as K$^+$ detector.
- The changes in the wavelengths of emission are very small or there is no significant change.
1.3. JG103 solvatochromism:

In a concentration of 20 µM we measured the absorption and fluorescence of the probe JG103.

**Solvents studied, from left to right by polarity order, Snyder polarity index:**

1. Water  
2. MeOH  
3. DMSO  
4. DMF  
5. MeCN  
6. Acetone  
7. EtOH  
8. AcOEt  
9. THF  
10. BnOH  
11. CHCl₃  
12. CH₂Cl₂  
13. Toluene  
14. Et₂O  
15. Hexane  
16. Cyclohexane

First, qualitative tests were done by taking pictures of the samples in the different solvents, under visible light and an UV light of 366 nm:

![Figure S89. JG103 solutions in different solvents under visible light and under UV light.](image)

Due to the interest of the K⁺ cation in the detection, K(CF₃SO₃) (excess) was added to the solution and the change in response was studied:

![Figure S90. JG103 + K(CF₃SO₃) solutions in different solvents under visible light and under UV light.](image)

Although 366 nm is not the best excitation wavelength to study the change in fluorescence, it can be seen how it increases in MeCN, acetone and THF. The main issue of the test is the solubility and the fluorescent response in the different solvents, the probe is only soluble in DMSO, DMF, BnOH, THF, CHCl₃ and CH₂Cl₂. After the addition of K⁺, the solubility increases in acetonitrile and acetone, in which the fluorescence increases too.

The absorption and fluorescent emission of the samples was measured too, obtaining the results shown. First, **Uv-Visible spectra** were registered:
Figure S91. JG103 solutions 20 µM, absorption spectra (left) and normalized absorption spectra (right) in different solvents.

The graphics after adding K(CF₃SO₃) (excess) are shown.

Figure S92. JG103 solutions 20 µM + K(CF₃SO₃) (excess), absorption spectra (left) and normalized absorption spectra (right) in different solvents.

In these spectra it can be seen:
- Before adding K(CF₃SO₃), the maximum of absorption is around 510 nm for all solvents in which it is soluble, and barely changes after the addition.
- After adding K(CF₃SO₃), the maximum of absorption in acetone and acetonitrile is found around 485 nm.

Fluorescent results at 500 nm as excitation wavelength:

Figure S93. JG103 solutions 20 µM, fluorescent emission spectra (left) and normalized fluorescent emission spectra (right) in different solvents. (λₑₓc = 500 nm)
The initial fluorescence is almost zero with the exception of toluene, DCM, CHCl₃, THF and BnOH solutions.

Figure S94. JG103 solutions 20 µM + K(CF₃SO₃), fluorescent emission spectra (left) and normalized fluorescent emission spectra (right) in different solvents. (λₑₓ𝑐 = 500 nm)

Then, the emission intensity and the wavelength were studied (λₑₓ𝑐 = 500 nm) at the maximum of emission:

| Solvent   | Em.int. (au) | Em.int.K⁺ (au) |
|-----------|------------|---------------|
| Water     | ----       | 24.6          |
| MeOH      | 6.1        | 33.1          |
| DMSO      | 1.5        | 1.2           |
| DMF       | 2.5        | 0.7           |
| MeCN      | 76.3       | 1.1           |
| BnOH      | 2.3        | 1.9           |
| Acetone   | 38.4       | 1.0           |
| EtOH      | 5.2        | 1.1           |

Table S3 and Figure S95. JG103 solutions 20 µM, emission increase of the different solvents, table (left) and graph (right) (λₑₓ𝑐 = 500nm)

Results:
- The maximum of emission is found between 555-570 nm before adding K⁺, except for BnOH in which it is 575 nm. The maximum of emission after adding potassium barely changes.
- The increase in emission is very high in MeCN, acetone, EtOAc and THF but it isn't soluble without K⁺.
- In DMSO and DMF the emission is too low.
- In water, MeOH, EtOH, Et₂O, hexane and cyclohexane the probe is insoluble.

So, there are only two possibilities, quantitatively the only option should be measuring in BnOH with and without potassium cations. Qualitatively or with excess of potassium it can be studied in acetonitrile and acetone too, having the best results in final fluorescence.

4.2. JG76 and JG103 ions test

For JG76, the tests were done in ethanol (EtOH), which have the better results in solvatochromism and it is miscible with water.

For JG103, due to the results in solvatochromism, the chosen solvent was benzylic alcohol (BnOH), because the solubility after and before adding potassium. Besides, it was partially studied in acetonitrile too, which is totally miscible with water although it is not soluble without adding K⁺ (or other species that increase fluorescence at the same time too).

The tests done are:
- Test in ions presence, JG76 and JG103.
  - Test with cations.
  - Test with anions.
  - Test with alcalis and other species.
  - Test with reductive, oxidant, acids and other species.
- The change in fluorescence in mixtures
  - Mixtures BnOH:Water and MeCN:Water.
  - BnOH:EtOH mixture response.

2.1. General conditions:
- The compound was dissolved, 50 µM and the cations were dissolved in water, 5×10^{-3} M.
- 10 microliters of the cations solutions were added to 0.5 mL of the solution (2 equivalents) and the change in color and fluorescence was studied.
- For these compounds, that absorb and emit in the visible spectrum it appears to be a change of colour. Actually, it is an increase in fluorescence but the maximum of absorption is around 500 – 507 nm and constant, so what is seen are the changes of the fluorescence in the visible region, in which we don’t need a UV lamp; but allows to see the effect more clearly.

2.2. Tests with JG76:

It was studied in ethanol solution and the ions were added solved in water.

a) Cations test 1:

Cations: Nothing - Water - Ag^{+} - Ni^{2+} - Sn^{2+} - Cd^{2+} - Zn^{2+} - Pb^{2+} - Cu^{2+} - Fe^{3+} - Sc^{3+} - Al^{3+} - Hg^{2+} - Au^{3+} - Co^{2+} - Pd^{2+}

The counterions are non coordinant species like CF_{3}SO_{3}^{-} and ClO_{4}^{-}.

Figure S96. JG76 in ethanol (50 µM), in presence of different cations (100 µM) under visible light and under UV light.

- The cations Ag^{+} Ni^{2+} Cd^{2+} Co^{2+} show no changes in fluorescence.
- The cations Sn^{2+} Zn^{2+} Cu^{2+} Fe^{3+} Sc^{3+} Al^{3+} Hg^{2+} Au^{3+} Pd^{2+} show an increase in fluorescence in yellow.
- The cation Pb^{2+} shows an increase in fluorescence in orange.

b) Cations test 2:

The next test was done due to the interest of studying the fluorescence in presence of alkaline and alkaline earth cations.

Ions sequence: Nothing – Water- Li^{+} - Na^{+} - K^{+} - Rb^{+} - Cs^{+} - Mg^{2+} - Ca^{2+} - Sr^{2+} - Ba^{2+} - NH_{4}^{+}

Counterions: ClO_{4}^{-} , CO_{3}^{2-} (Cs^{+}), NO_{3}^{-} (Rb^{+})
Figure S97. JG76 in ethanol (50 µM), in the presence of different cations (100 µM) under visible light and under UV light.

- The cations K⁺, Be⁺ and Ba²⁺ show an increase in fluorescence in orange.
- There is a very slight increase in the presence of NH₄⁺.

c) Anions test:

Anions sequence: Nothing - Water - F⁻ - Cl⁻ - Br⁻ - I⁻ - BzO⁻ - NO₃⁻ - H₂PO₄⁻ - HSO₄⁻ - AcO⁻ - CN⁻ - SCN⁻

The counterions are non-coordinant cations of Bu₄N⁺

Figure S98. JG76 in ethanol (50 µM), in the presence of different anions (100 µM) under visible light and under UV light.

- There are no significant changes. A little increase when we add excess of acidic anions like HSO₄⁻.

d) Quantitative fluorescent response

Some of the species with different behavior were selected, and the absorbance and fluorescence were measured. The variation in absorbance is not significant, changes are most likely due to the dilution of the probe.

Figure S99. JG76 in ethanol (50 µM), in presence of different cations (100 µM) absorbance and fluorescence (λexc = 500nm).

In the table below, the maximum of emission of cations and the wavelength shifts are shown.
|         | Wavelength (nm) | $\phi/\phi_0$ |         | Wavelength (nm) | $\phi/\phi_0$ |
|---------|----------------|--------------|---------|----------------|--------------|
| HCl     | 570            | 1.9          | KClO$_4$ | 573            | 2.6          |
| KClO$_4$| 573            | 2.6          | Pb(ClO$_4$)$_2$ | 568          | 7.1          |
| Ba(NO$_3$)$_2$ | 575          | 4.1          | Fe(ClO$_4$)$_3$ | 564          | 7.9          |
| Be(NO$_3$)$_2$ | 565          | 4.9          | Sn(CF$_3$SO$_3$)$_2$ | 564         | 8.1          |

**Table S4 and Figure S100.** JG76 in ethanol (50 µM), in the presence of different cations (100 µM) under visible light and under UV light, shows an increase in maximum emission intensity (left) and a change in its wavelength of emission (right).

There are some important differences in the intensity and wavelength of emission.

- The higher wavelengths are in the case of Ba$^{2+}$ and K$^+$, and the only ones in which they don’t decrease.
- The solubility of the salt is an important factor, for example, for K$^+$ the intensity is higher when the solubility of the salt is higher, such as KCF$_3$SO$_3$.
- The emission is higher when the species are more acidic.

### 2.3. Tests with JG103

Due to the solubility of the probe, the tests were done in MeCN and BnOH, in order to compare the behavior and results.

**Test in MeCN,**

In this solvent, the probe is insoluble until the potassium cation, or some lewis acids, are added. The probe was dissolved in 50 µM concentration.

a) Test in the presence of ions (excess)

Ions sequence: Ag$^+$ - Sn$^{2+}$ - Pb$^{2+}$ - Cu$^{2+}$ - Fe$^{3+}$ - Al$^{3+}$ - Hg$^{2+}$ - Au$^{3+}$ - Na$^+$ - K$^+$ - Cs$^+$ - Ca$^{2+}$ - Ba$^{2+}$ - NH$_4^+$

Counterions: ClO$_4^-$, CF$_3$SO$_3^-$ and Cl$^-$ for Au$^{3+}$
The solubility increases in presence of Lewis acids, like Fe$^{3+}$, Sn$^{2+}$ or Al$^{3+}$.

Besides, there is an increase in fluorescence in presence of Pb$^{2+}$ and K$^+$, and less with Ba$^{2+}$. Very similar response to the probe JG76.

**Tests in BnOH:**

The compound JG103 was dissolved in BnOH, $5 \times 10^{-5}$ M and the cations were dissolved in water, $5 \times 10^{-3}$M. 20 microliters of the cations solutions were added to 0.5 mL of the JG103 solution and we studied the change in color and fluorescence.

a) Test with cations:

Cations sequence: Water - Ag$^+$ - Ni$^{2+}$ - Sn$^{2+}$ - Cd$^{2+}$ - Zn$^{2+}$ - Pb$^{2+}$ - Cu$^{2+}$ - Fe$^{3+}$ - Sc$^{3+}$ - Al$^{3+}$ - Hg$^{2+}$ - Au$^{3+}$ - Co$^{2+}$ - Pd$^{2+}$ - Au$^{3+}$

Counterions: ClO$_4^-$, CF$_3$SO$_3^-$ and Cl$^-$ for Au$^{3+}$ and Pd$^{2+}$

**Figure S102.** JG76 in ethanol (50 µM), in the presence of different cations (200 µM) under visible light and under UV light.

- On the one side, there is a slight increase of fluorescence in orange in the presence of Lewis acids like Sn$^{2+}$, Fe$^{3+}$, Sc$^{3+}$ and Al$^{3+}$.

- On the other side, there is an increase in of fluorescence in orange in the presence of Hg$^{2+}$ and Pb$^{2+}$ too, which is higher, comparing with the acidic cations.

b) Test with cations and other species:

Ions sequence: Nothing - Na$^+$ - K$^+$ - Rb$^+$ - Cs$^+$ - Be$^{2+}$ - Mg$^{2+}$ - Ca$^{2+}$ - Sr$^{2+}$ - Ba$^{2+}$ - NH$_4^+$

Counterions: ClO$_4^-$, CO$_3^-$ (for Cs$^+$), NO$_3^-$ (for Be$^{2+}$, Rb$^+$)
Figure S103. JG103 in benzylic alcohol (50 µM), in the presence of different cations (100 µM) under visible light and under UV light.

- The fluorescence increases noticeably with Ba$^{2+}$ and very slightly with Be$^{2+}$ and K$^+$. 

c) Test with anions.

Anions sequence: Nothing - F$^-$ - Cl$^-$ - Br$^-$ - I$^-$ - BzO$^-$ - NO$_3^-$ - H$_2$PO$_4^-$ - HSO$_4^-$ - AcO$^-$ - CN$^-$ - SCN$^-$

Figure S104. JG103 in benzylic alcohol (50 µM), in presence of different anions (100 µM) under visible light and under UV light.

There is no significant change.

General results of the presence of different species:

Figure S105. JG103 in benzylic alcohol (50 µM), in the presence of different species (100 µM) emission spectra (left) and the increase of the emission (right), $\lambda_{exc} = 500$ nm, $\lambda_{em} = 571$ nm.

Conclusions:

There are several groups of cations that increase the fluorescence:

- Acids and acidic cations like Au$^{3+}$ > Sn$^{2+}$ > Be$^{2+}$ > NH$_4^+$. 
- Ions that are coordinated due to the affinity of the complex, Hg$^{2+}$ > Pb$^{2+}$ > Ba$^{2+}$ > K$^+$. 

S56
4.3. pH controlled response test

First, the proportion of water that allows to dissolve the probe was tested, moreover, in case of probe JG103, the mixture with ethanol was studied to try to decrease the amount of benzylic alcohol and the possibility to increase the quantity of water in the mixture.

3.1. pH response of JG76:

For JG76 the maximum was 30 %, at least to allow a concentration superior to 20 µM and to be seen by the naked eye.

The compound JG76 was dissolved in EtOH, 2.5×10⁻⁵ M and the solvent was 70 % EtOH, 30 % H₂O, buffer solution, 20 mM of HEPES. (The work pH for HEPES buffer is between 6.8 to 8.2)

The buffers pH are 2.8 4.8 6.5 7 7.5 8 9.2.

![Figure S106. JG76 in ethanol (20 µM), in a mixture with a buffer solution of different pH, 20 mM of HEPES at different pH. Under visible and 366 nm UV light. (λexc=500 nm).](image)

In conclusion, the probe is very sensitive to the pH of the solution, especially far from the work pH of the buffer. A buffer solution, with pH higher than 7, is enough to avoid the pH effect in the fluorescence, if it is lower the pH will affect the signal significantly, but there are changes in presence of other species like potassium cations too; it can be seen in the response to oxone in which increase more than in the acid media in presence of both acids and potassium cations.

3.2. Ions response with JG76 at controlled pH

Due to the previous results, it is necessary to check the behavior of the probe in buffer solution and in the presence of cations.

- The compound JG76 was first dissolved in EtOH, from which a final solution was JG76 20 µM in 70 % EtOH - 30 % H₂O (v/v) buffer solution, 20 mM of HEPES.
- The buffer pH is 7
- First, a picture was taken by adding 5 equivalents of different species:

Sequence: Nothing – Water – Zn(ClO₄)₂ – K(CF₃SO₃) - Fe(ClO₄)₃ - Sn(ClO₄)₂ - Ba(ClO₄)₂ - Pb(ClO₄)₂ - Be(NO₃)₂ - Cu(ClO₄)₂ – Al(ClO₄)₃
**Figure S107.** JG76 in ethanol (20 µM), in a mixture with a buffer solution of different pH, 20 mM of HEPES, pH 7. Adding different cations $10^{-4}$ M. Under visible and 366 nm UV light.

**Figure S108.** JG76 in ethanol (20 µM), in a mixture with a buffer solution of different pH, 20 mM of HEPES, pH 7. Adding different cations $10^{-4}$ M. Fluorescence spectra (left) and increase in total emission (right). $\lambda_{\text{exc}} = 500\text{nm}$.

- In the presence of K$^+$ the fluorescence increases in orange.
- The cation Ba$^{2+}$ increases the fluorescence in orange, but more intensely than K$^+$.
- Pb$^{2+}$ increases the fluorescence but in yellow, moreover the Rayleigh peak increases a lot, probably because of the percentage of water, that causes a precipitation process too.
- The controlled pH allows to distinguish clearly between Lewis acid increase, which is avoided, and other causes of increase.

In conclusion, the emission increases:

- Around 30% in the presence of acid cations, and the maximum remains at a wavelength of emission below to 571 nm.
- Around 230% in the presence of K$^+$, and the maximum is between 572.5-573.5 nm.
- Around 450% in the presence of Ba$^{2+}$, and the maximum is around 577 nm.
- Around 600% in the presence of Pb$^+$, and the maximum is between 572.5-573.5 nm.

**3.3 Water and pH response of JG103:**

a) Acetonitrile solutions:

i. Water percent response:
The probe is soluble until concentrations superior to 50 % of water. The fluorescence is very low when there is water in the solvent.

**pH response:**

The sample is slightly more soluble in acid media, more soluble when the pH is lower. The fluorescence increases when the pH is lower and even more when there is the presence of potassium cations.

**Benzylic alcohol solutions:**

Due to the results in the solvatochromism, a chosen solvent was BnOH, which is miscible with EtOH and slightly miscible with water, and the fluorescence and solubility are higher than in EtOH. The concentration of the probe is always 50 µM.

**Change in fluorescence, mixtures BnOH:EtOH.**
Figure S112. JG103 in benzylic alcohol (50 µM) excess, mixture with ethanol (percent of BnOH from 10 to 100 %). Change in fluorescence intensity. Without K(CF₃SO₃) (up) and with excess of K(CF₃SO₃) (down). λ<sub>exc</sub> = 500 nm λ<sub>em</sub> = 570 nm.

So the fluorescence in EtOH decreases linearly until 6 times the fluorescence in BnOH.

ii. Mixtures BnOH-Water.

Figure S113. JG103 in acetonitrile (50 µM), up, and + K(CF₃SO₃) excess, down. Mixture with deionized water (% of water). Under visible and 366 nm UV light. Water percent: 0-10-25-35-50-60-78-80-90 (up). Maximum of emission from 0 to 10 % of water (right).

- The mixture between BnOH and H₂O is only possible when the percentage of water is 10 % or less
- The fluorescence decreases 3 times with 10 % of water.

iii. pH response test.

50 µl of buffer over 450 µl of BnOH.

Figure S114. JG103 benzylic alcohol (50 µM), in a mixture with a buffer solution of different pH, 5 mM of HEPES at different pH. Under visible and 366 nm UV light. λ<sub>exc</sub>=500 nm λ<sub>em</sub>=570 nm.

- At pH between the working limits of HEPES, or more basic, buffer makes the fluorescence stable.
- The presence of water decreases the fluorescence highly.
5. Calculation of binding constants

Due to the characteristics of the probes only JG76 was fully studied, because of the low solubility of JG103. For example, although JG103 can be dissolved in benzylic alcohol, when it was studied, the constants obtained were not related directly with the real concentration. There are many factors that lead to this conclusion, such as:

- In a Job’s plot study the quantity of potassium is too low to be associated with a real process of complexation.
- An analysis of the complexation constants leads to different results if the K⁺ is added to a JG103 solution or if JG103 solution is added over K⁻ solution.

Because of that, on one side, from JG103 was studied only the limit of detection in benzylic alcohol solution and the quantum yield in benzylic alcohol and acetonitrile.

On the other side, the probe JG76 has these characteristics in absolute EtOH solution:

- The emission intensity is sensitive to acid cations if there is no buffer in the media.
- The wavelength of emission allows to distinguish between effects from acids or K⁺, Ba²⁺ and Pb²⁺. But, it wouldn’t be possible when there are some of these species at the same time or it would require more complex processes like principal components analysis.
- The EtOH solution is useful for the introduction of the probe into cells and comparison with other protic solvents like water.
- The species that are competitive for the K⁺ complexation, valinomycin and cereulide, aren’t soluble in water media, at least in high percentages of water. Besides, there are widely studied in alcoholic solutions.

Knowing these characteristics of the probe, it was decided to develop a study in EtOH solution:

- Work concentration.
- Job’s Plot.
- Quantum yield, with and without K⁺ presence.
- Fluorescence lifetime decay.
- Limit of detection of K⁺.
- Limit of detection of valinomycin and cereulide.
- Thermodynamic complexation constants; JG76, valinomycin and cereulide comparison.

5.1. Work concentration of JG76

Before doing any calculation in solution, checking the behavior of the probe is necessary in order to avoid possible misleading in the determination of some characteristic values of the probe.

To do so, some solutions of the probe were prepared in ethanol, then the absorbance and the fluorescence was checked.

Absorbance at 500 nm:

| Conc (µM) | Absorbance | Conc (µM) | Absorbance |
|----------|------------|-----------|------------|
| 6.5      | 0.182      | 2         | 0.046      |
| 4.8      | 0.126      | 1.6       | 0.044      |
| 3.6      | 0.09       | 1.2       | 0.026      |
| 2.8      | 0.06       | 0.8       | 0.006      |

Table S5 & Figure S115. Absorbance at different concentrations of JG76

\[ y = 0.0289x - 0.0108 \]
\[ R^2 = 0.988 \]
Fluorescence ($\lambda_{\text{exc}} = 500 \text{ nm}, \lambda_{\text{em}} = 573 \text{ nm})$:

| Conc (µM) | Em. Int. 573 nm (a.u.) |
|-----------|------------------------|
| 6.5       | 140.7                  |
| 4.8       | 98.49                  |
| 3.6       | 66.28                  |
| 2.8       | 52.96                  |
| 2         | 34.8                   |
| 1.6       | 28.21                  |
| 1.2       | 34.47                  |
| 0.8       | 33.14                  |

Table S6 & Figure S116. Fluorescent emission at different concentrations of JG76

In conclusion, the absorbance and fluorescence change linearly between $1.6 \cdot 10^{-6}$ to $6.5 \cdot 10^{-6}$ M. The chosen concentration for the tests was between 2 µM to 6 µM.

### 5.2. JG76 JOB’S PLOT, stoichiometric determination of the complex

Characteristics of the measures:
- A group of solutions with a molar fraction between 0-1 of $\text{K}^+$
- The concentrations of potassium between 6.25 to 25 µM when the deviation of linearity is minimized.
- The fluorescence was measured with $\lambda_{\text{exc}} = 500 \text{ nm}$ and $\lambda_{\text{em}} = 571 \text{ nm}$.
- The molar fraction was represented versus the peak of emission minus the emission when $X_{\text{K}^+} = 0$ multiplied per the molar fraction.

A Job’s Plot analysis was done in order to calculate the stoichiometry of the complex. For the analysis, it is represented $X_{\text{JG76}} (F_0-F)$ versus $X_{\text{JG76}}$. The stoichiometry of the complex can be easily deduced due to the maximum position at $X_{\text{JG76}} = 0.5$, which means that the complex stoichiometry is 1:1.

Figure S117. Job’s Plot of JG76 with $\text{K}^+$, fluorescence analysis in EtOH (left) and EtOH and BnOH comparison (right)

The plot was represented several times obtaining always the maximum centered in 0.5, with the shape showed, which means that the complex probe-$\text{K}^+$ is 1:1.
5.3. **Quantum Yields**

Quantum yield was determined by its general equation:

$$\Phi = \frac{\Phi_R n^2 A_R F}{n_R^2 A F_R}$$

Where

- $\Phi$ is the quantum yield.
- $n$ represents the refractive index of the solvent.
  - $n$(EtOH) = 1.362
  - $n$(BnOH) = 1.540
  - $n$(MeCN) = 1.344
- $A$ is the absorbance.
- $F$ is the fluorescence.
- $R$ means that the parameter is associated to a reference sample.

**Calculations in probe JG76:**

**Parameters:**

- The chosen reference was Rodhamine 6G.
- The integral was done between 480 – 700 nm and the Rayleigh signal was deconvoluted.

![Graph](image1)

**Figure S118.** Absorbance of JG76-JG76+K⁺-Rhodamine 6G (left), fluorescence of JG76-JG76+K⁺-Rhodamine 6G with $\lambda_{exc}=515$ nm (right); solved in EtOH.

The process was repeated three times to obtain the media and a confidence interval:

**Probe:**

- Media: 0.04327
- $s$: 0.00197
- Result: $\Phi = 0.043 \pm 2.92 \times \left( \frac{0.00197}{\sqrt{3}} \right) = 0.043 \pm 0.003$

**Probe + K⁺:**

- Media: 0.1724
- $s$: 0.00312
- Result: $\Phi = 0.1724 \pm 2.92 \times \left( \frac{0.00312}{\sqrt{3}} \right) = 0.172 \pm 0.005$

Keeping in mind that the error associated to the method is at least 1%, higher than the experimental results. So:

- $\Phi_{JG76 \ (EtOH)} = 0.04 \pm 0.01$
- $\Phi_{JG76+K^+ \ (EtOH)} = 0.17 \pm 0.01$
- $\Phi/\Phi_0 = 4$

**Calculations in probe JG103:**

S63
The solubility of the probe JG103 is very low in most solvents, and the change in fluorescence signal is quite different, although the nature of the fluorophore is the same (perylenemonoimide). The main reason is likely to be a change in aggregation. Because of that, the quantum yield is studied in BnOH, in which seems to be soluble, and in acetonitrile, with excess of potassium cation.

**Parameters:**
- The chosen patron was Rhodamine 6G.
- The solvent is BnOH.
- The absorbance spectra were measured with a maximum inferior to 0.1.
- The integral is between 480 – 700 nm for the Rhodamine patron and 515-700 nm for JG103 measures.

![Absorbance and Fluorescence](image)

*Figure S119. Absorbance of JG103-JG103+K+-Rhodamine 6G (left), fluorescence of JG103-JG103+K+-Rhodamine 6G with λex=515 nm (right) solved in BnOH.*

The process was repeated three times to obtain the media and a confidence interval:

**Probe in BnOH:**
- Media: 0.009364
- s: 0.000635
- Result: \( \Phi = 0.094 \pm 2.92 \times \left( \frac{0.000635}{\sqrt{3}} \right) = 0.0094 \pm 0.0011 \)

**Probe in BnOH + K⁺:**
- Media: 0.048619
- s: 0.003338
- Result: \( \Phi = 0.0482 \pm 2.92 \times \left( \frac{0.00334}{\sqrt{3}} \right) = 0.048 \pm 0.006 \)

Keeping in mind that the error associated to the method is 1 %, higher than the experimental results. So:
- \( \Phi_{JG103} \text{ (BnOH)} = 0.01 \pm 0.01 \)
- \( \Phi_{JG103+K⁺} \text{ (BnOH)} = 0.05 \pm 0.01 \)
- \( \Phi/\Phi_0 \text{ (BnOH)} = 5.2 \)

By the same method, the quantum yield may be calculated changing the solvent and/or the analyte: Other results:
- \( \Phi_{JG103+Oxone} \text{ (BnOH)} = 0.13 \pm 0.01 \)
- \( \Phi_{JG103+K⁺} \text{ (MeCN)} = 0.14 \pm 0.01 \)

The results lead to the conclusion that in case of the benzylic alcohol there is a secondary process that decreases the solubility and the quantum yield until a high amount of potassium cation and/or the right media is chosen. That is probably associated to an aggregation process typical from perylene based structures.

Besides, it is very interesting the coincidence between the maximum quantum yield of JG76 and JG103 which means that probably the probe is affected by the same processes.

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5.4. Fluorescence lifetime decays

Fluorescence decay lifetimes were measured using a time-correlated single photon counting instrument (FLS980 Series, Edinburgh instruments) with a 510 nm pulsed LED (Edinburgh instruments, EPL-510) light source having a 177.4 ps. Decays were recorded at 510 nm for each probe each probe. The fluorescence decay lifetime of the probes was analyzed using the software FAST 3.4.2 (Edinburgh instruments). The software uses an iterative re-convolution of the source time profile of the instrument, IRF; or an iterative tail fitting of the sample by avoiding the overlapping between IRF and the lifetime of the sample, which is necessary when the lifetime is low. The theoretical fluorescence decay is modeled as a sum of exponentials

\[
F(t) = \sum A_i e^{-\frac{t}{\tau_i}}
\]

where \(A_i\) is the weighted amplitude (fractional value between 0 and 1) and \(\tau_i\) is the lifetime of the i-th fluorescent component. IRF is recorded from a scattering sample that does not fluoresce (in this case LUDOX dispersion). Both F(t) and IRF are recorded at a sampling rate adapted to the repetition rate of the pulsed source in order to avoid non-linear effects in the acquisition. The convolution of F(t) is fitted to the experimental decay curve, I(t), by iterative change of the amplitude and lifetime parameters, using the least-squares method to optimize the fitting parameters. The quality of the fit is determined by ensuring that the \(\chi^2\) statistical parameter is between 1 and 1.3 there is no deviation or tendencies in the residues. The probes JG76, JG103 and JG121 were tested in the studied solvents and in presence of potassium. Calculating the lifetime decays:

![Graphs showing fluorescence decay lifetimes of JG76, JG103, and JG121 in different solvents.](image)
The conclusions of the analysis were:

- Both JG76 and JG103 have lifetime decays between 3-4.5 ns.
- In the same solvents, the quantum yield is very similar between JG103 and JG76, due to the perylene structure responsible of fluorescence.
- A possible change in lifetime was checked too. Measuring with and without potassium cations wasn’t observed any change.

### 5.5. Limits of Detection (LoD) in EtOH solution

The limits of detection were done in EtOH solution with probe JG76, which have better solubility and reproducibility in its measures.

The LODs of valinomycin and cereulide are studied in a solution of probe and potassium, adding valinomycin or cereulide. These results are only valid when the proportions of probe and potassium are around the range studied, if not, it might change:

- If [JG76]/K⁺ increases the necessary concentration of valinomycin or cereulide will be higher in order to be detected.
- If [JG76]/K⁺ is too low the concentration of cereulide or valinomycin detected will be higher too, because it won’t be detected until the free potassium cation is very low.

#### 1.1. Limit of detection of K⁺

In a solution 5·10⁻⁶ M of JG76, the concentration of K(CF₃SO₃) was gradually increased and the fluorescence spectra were registered.

---

**Figure S120.** Lifetime decay, JG76 solved in EtOH, sample, IRF and fitting (up); JG76 in Acetone (middle left), JG103 BnOH (middle right) and JG103 acetone (down), fitting.

| Probe  | Solvent | Lifetime (ns) | X²  |
|--------|---------|---------------|-----|
| JG76   | EtOH    | 3.581         | 1.138 |
| JG103  | BnOH    | 3.221         | 1.192 |
| JG76   | Acetone | 3.952         | 1.075 |
| JG103  | Acetone | 4.215         | 1.099 |

**Table S7.** Lifetime decay, JG76 and JG103 in different solvents

---

**Figure S121.** Absorbance and fluorescence spectra of a titration with JG76 5 µM in EtOH, increasing concentration from 0.01 to 0.2 µM.
Figure S122. Linear regression of a titration of JG76 5 µM with K⁺ in EtOH, increasing concentration from 0.01 to 0.2 µM and studying the increase in the fluorescent emission.

The integral was adjusted to a minimum squares linear regression. Then, with the program “R”, the limit of detection associated to the linear regression was calculated. In order to obtain a reliable limit, the values of false positive and false negative were fixed as equal or inferior to 5%. The obtained limit of detection for K⁺ was 0.06 µM.

1.2. Limit of detection of Valinomycin

In a solution 5 µM of JG76, the concentration K(CF₃SO₃) was increased until 0.75 equivalents. Then, the concentration of valinomycin was increased in several additions, and the fluorescence spectrum was registered.

Figure S123. Regression of a titration with valinomycin of JG76 5 µM and K⁺ 3.75 µM in EtOH studying the decreasing fluorescent emission.

The detection limit with a probability of false positive and false negative inferior to 5% is 0.54 µM.

1.3. Limit of detection of Cereulide:

In a solution 2 µM of JG76, the concentration K(CF₃SO₃) was gradually increased until 0.75 equivalent. Then, the concentration of valinomycin was increased in several additions, and the fluorescence spectrum was registered.
Figure S124. Regression of a titration with cereulide of JG76 2 µM and K⁺ 1.5 µM in EtOH studying the decreasing fluorescent emission.

The detection limit with a probability of false positive and false negative inferior to 5% is 0.21 µM.

5.6. Equilibrium constants

5.6.1. Measuring parameters and method:

Equilibrium constants were measured in EtOH solution by increasing the concentration of the probe in presence of the analyte, after checking that it was the method with more repetibility in the results.

First, the complex JG76-K⁺ was studied by preparing a solution 2 µM of K(CF₃SO₃) and increasing the concentration of JG76 (0 to 10 µM) without changing the concentration of K⁺ in solution. Moreover, it was checked the other way around, by adding K⁺ to JG76, and the same results were obtained.

Second, for studying the constants Valinomycin-K⁺ and Cereulide-K⁺, the method is more complicated as is explained in subsequent explanations but the final conditions were by doing a titration with JG76 (0 to 20 µM) while the concentration of K⁺ and Val or Cer are constants and 2 and 20 µM respectively.

By introducing the parameters [JG76], [K⁺] and [Cereulide] or [Valinomycin] obtained in the titration to the equations of the equilibrium the constant was obtained.

The software used for adjusting was Origin 9.0.

For the constants calculation by CD it was used the reported method.

5.6.2. Equilibrium constant of the complex JG76-K⁺

The complexation reaction can be stated by the next scheme:

\[ S + K \rightleftharpoons SK \]

By taking into account the mass balance and the fluorescence:

\[ C_S = [S] + [SK] \] (1)

\[ C_K = [K] + [SK] \] (2)

\[ I_F = f_S[S] + f_{SK}[SK] \] (3)

Where \( C_S, C_K, I_F, f_S \) and \( f_{SK} \) are the total concentrations of the probe (\( C_s \)) and K⁺ (\( C_k \)), the intensity of fluorescence and the proportional fluorescence factors of the probe and the complex SK, respectively. The rest are the concentrations of the species on the equilibrium. Resolving the concentration of the probe in the equilibrium on the equation (1) and by substitution on the equation (3), allows to get the following equation:

\[ I_F = f_S C_S + (f_{SK} - f_S)[SK] \] (4)

Starting by the definition of the equilibrium constant:
Solving the previous equation, the [SK] and replacing on equation (4), the next equation is obtained (6):

$$I_F = f_S C_S + \frac{f_{SK} - f_S}{2} \left[ C_S + C_K + \frac{1}{K_1} - \left( C_S + C_K + \frac{1}{K_1} \right)^2 - 4C_S C_K \right]$$

Fitting the fluorescence by nonlinear least square regression, starting in an initial value of $K_1$, $f_S$ and $f_{SK}$ the next results are obtained:

![Fluorescence Emission Graph]

**Figure S125.** Fitted fluorescent emission of a 2 µM solution of K(CF$_3$SO$_3$) solution and JG76 in EtOH (left) and EtOH and BnOH compared (right)

The fitting calculation of the complexation constants was repeated 3 times for each solvent. Besides, the results were compared doing a titration of K$^+$ with JG76 and JG76 with K$^+$. The same results were obtained:

- **Ethanol solution:**
  - $K(JG76+K^+) = (2.2 \pm 0.2) \times 10^6$ M$^{-1}$
  - Log $K(JG76+K^+) = 6.34 \pm 0.04$

- **Benzylic alcohol solution:**
  - $K(JG76+K^+) = (1.3 \pm 0.1) \times 10^6$ M$^{-1}$
  - Log $K(JG76+K^+) = 6.11 \pm 0.03$

### 5.6.3. Valinomycin and Cereulide equilibrium constant calculation:

Basically, the conditions for the tests are the same than in fluorescent studies of the interaction between JG76+K$^+$. Now it is necessary to include a new equilibrium:

$$S + K \leftrightarrow SK$$

$$V + K \leftrightarrow VK$$

Mass and fluorescence balance:

$$C_S = [S] + [SK]$$  \hspace{1cm} (7)

$$C_K = [K] + [VK] + [SK]$$  \hspace{1cm} (8)

$$C_V = [V] + [VK]$$  \hspace{1cm} (9)

$$I_F = f_S C_S + f_{SK} C_K$$  \hspace{1cm} (10)

Where $C_S$, $C_K$, $C_V$, $I_F$, $f_S$ y $f_{SK}$ are, concentrations of probe, K and valinomycin, the fluorescence intensity (only depends on the probe and the complex probe-potassium) and the factors of proportion between probe and SK complex. The rest are the concentration of the species on the equilibrium. By taking into account $K_1$ and $K_2$ and the equations (7) and (9):

$$K_1 = \frac{[SK]}{[S][K]} = \frac{[SK]}{(C_S-[SK])(C_K-[SK]-[VK])}$$  \hspace{1cm} (11)
\[ K_2 = \frac{[VK]}{[V][K]} = \frac{[VK]}{(C_V-[VK])(C_K-[SK]-[VK])} \]  \hspace{1cm} (12)

**a) First option, solving the equation:**

By solving (11), the concentration \([VK]\), in (12):

\[
[SK]^3(K_2 - K_1^2) + [SK]^2 \left( (1 + K_1(C_K + 2C_S))K_1 - (1 + K_1(C_z + C_K - C_V))K_2 \right) - [SK]K_1C_5 \left( 1 + K_1(2C_K + C_S) + K_2(C_V - C_K) \right) + (K_1C_5)^2C_K = 0
\]

This third grade equation was solved by the next way:

Being the equation (14):

\[ ax^3 + bx^2 + cx + d = 0 \]  \hspace{1cm} (14)

Dividing between “a” and replacing \(x = z-b/3a\), \(z^3+pz+q=0\) (Tschirnhaus transformation) is obtained; where \(p\) and \(q\) are calculated as:

\[ p = \frac{3ac-b^2}{3a^2} \]  \hspace{1cm} (15)

\[ q = \frac{2b^2-9abc+27a^2d}{27a^3} \]  \hspace{1cm} (16)

To know the number of real roots, the discriminant \(\Delta\) is calculated as

\[ \Delta = -4p^3 - 27q^2 \]  \hspace{1cm} (17)

With these data, further calculations for the constant were done, but in the end:

- The equation turns out to be too much complicated, many parameters to adjust.
- The results of adjusting to the equation are more dependent of the initial values than the variation of them.

It was decided that, in order to make a more accurate calculation of the constant it is possible to make some approximations to simplify the equation.

**b) Simplification based on a system Valinomycin + Potassium cation, and titration with JG76:**

The experiment started with a solution in ethanol of Valinomycin (V) and Potassium cations (K), so there is complex (VK) in the equilibrium. When the probe (S) is added, it forms a complex with free K⁺ creating the new complex (SK) and replacing the previous complex (VK). In conclusion, the concentration of VK decreases, whereas the concentration of V increases. Then, a possible approximation can be done:

\[ CV - [VK] \approx CV \]

And this simplification is more realistic when the initial proportion V/K is as higher as possible.

The new equation obtained, from 11 and 12 is:

\[ [SK] = \frac{\left( C_S + C_K + \frac{1+K_2C_V}{K_1} \right) - \sqrt[3]{\left( C_S + C_K + \frac{1+K_2C_V}{K_1} \right)^2 - 4C_SC_K}}{2} \]  \hspace{1cm} (18)

First, the test was repeated several times with different initial proportions of V:K, a proportion 1:1, 1:0.25 and 1:0.1. It was checked that the results are slightly different, but having the best adjust when the proportions are 1:0.1 in which the approximation is more valid.

The next graphs were obtained by changing between cereulide/valinomycin and EtOH/BnOH:
Figure S126. Fitted fluorescent titration emissions with JG76 of a 2 µM solution of K(CF₃SO₃) and 20 µM of valinomycin and cereulide solution in EtOH (A and C) and BnOH (B and D), respectively.

The complexation equilibrium constants, after doing the average of three titrations, turn out to be:

- In EtOH solution:
  - K(Val EtOH) = (9.4 ± 0.2) x 10⁵ M⁻¹, log K(Val EtOH) = 5.97 ± 0.01
  - K(Cer EtOH) = (9.7 ± 0.2) x 10⁵ M⁻¹, log K(Cer EtOH) = 5.99 ± 0.01

- In BnOH solutions:
  - K(Val BnOH) = (9.6 ± 0.2) x 10⁴ M⁻¹, log K(Val EtOH) = 4.98 ± 0.01
  - K(Cer BnOH) = (10.3 ± 0.2) x 10⁴ M⁻¹, log K(Val EtOH) = 5.01 ± 0.01

Moreover, with the data obtained it can be represented, in the same graph, the amount of the reagents and the proportion of complex that we have during the titration:

- With the values of K₂ and K₁, the concentration of [SK]ₘₐₜ may be determined.
- With these data, the concentration of the species was calculated by these equations:

\[
[S]_{eq} = C_S - [SK]_{eq} \quad (19)
\]

\[
[VK]_{eq} = \frac{C_V + C_K - [SK]_{eq} + \frac{1}{K_2} \sqrt{(C_V + C_K - [SK]_{eq} + \frac{1}{K_2})^2 - 4C_V(C_K - [SK]_{eq})}}{2} \quad (20)
\]
\[ [V]_{eq} = C_{V} - [VK]_{eq} \quad (21) \]
\[ [K]_{eq} = C_{K} - [SK]_{eq} - [VK]_{eq} \quad (22) \]

Figure S127. Concentration in the equilibrium in EtOH solution. Left, V-VK-K-SK equilibrium. Right, comparison of the concentration in equilibrium of the complexes of cereulide and valinomycin.

5.6.4. Equilibrium constant Valinomycin-K⁺ calculated by circular dichroism:

Following the standard method explained in bibliography, the calculation of the equilibrium constant of valinomycin can be done by circular dichroism. Starting in a concentration of 0.3 mM of valinomycin the equivalents of potassium are gradually increased until 3.75 equivalents.

Figure S128. Circular dichroism of valinomycin 3·10⁻⁴ M solution in ethanol, increasing the quantity of potassium from 0 to 3.75 equivalents.

The concentration of valinomycin was chosen because of the optimal concentration to follow changes on the dichroism signal. Although the results are apparently the same explained in bibliography it turned out that checking the fitting at different wavelengths the constant seems to change depending on its value.

Adjusting between 230 nm-250 nm:
Figure S129. Molar ellipticity of a 0.3 mM solution in ethanol of valinomycin increasing the quantity of potassium from 0 to 3.75 equivalents at different wavelengths.

The results can be represented by the same equation used for fluorescence.

| λ, nm | $10^5K, M^{-1}$ | Log K |
|-------|----------------|-------|
| 230   | 10.3           | 6.01  |
| 232   | 8.62           | 5.94  |
| 234   | 8.07           | 5.91  |
| 238   | 6.55           | 5.82  |
| 242   | 5.88           | 5.77  |
| 246   | 6.33           | 5.80  |
| 250   | 8.51           | 5.93  |

Table S8. Equilibrium constant calculated at different wavelengths.

Although in bibliography the equilibrium constant given was calculated at 238 nm, and taken as independent from other factors, it turned out to be slightly dependent from the wavelength at which it was calculated, obtaining the same values of bibliography at the same wavelength. This characteristic was observed when the complexation occurs in DNA structures (different binding sites), but it is not the case. So, in this case, is understood as consequence of the own method being the real value between these results.

The complexation order is around $0.6 - 1.0 \cdot 10^6 M^{-1}$ or Log K between 5.8 to 6.0.

(The reported method from bibliography can be found in: M. C. Rose and R. W. Henkens: Stability of sodium and potassium complexes of valinomycin, *Biochim. Biophys. Acta* 1974, **372**, 426–435)
5.7. Summary

- Work concentration.

Variations of [JG76] from 2 to 6 µM follow a linear regression in absorbance and fluorescence response in EtOH.
- Job’s Plot.

The complex probe – potassium is 1:1.
- Quantum yield.

\[ \Phi_{JG76} \text{ (EtOH)} = 0.04 \pm 0.01, \quad \Phi_{JG76 + K^+} \text{ (EtOH)} = 0.17 \pm 0.01 \]

For JG103, it is only as high totally solved in some solvents like acetone.
\[ \Phi_{JG121} \text{ (EtOH)} = 0.43 \pm 0.02 \]
- Fluorescence lifetime decay.

\[ \tau \text{ (JG76 in EtOH)} = 3.58 \text{ ns. A value which is not very different in the rest of solvents, or from JG103 results.} \]
\[ \tau \text{ (JG121 in EtOH)} = 2.91 \text{ ns} \]
- Limits of detection with JG76 in EtOH.

- K⁺ = 0.06 µM.
- Valinomycin = 0.54 µM.
- Cereulide = 0.21 µM.
- Thermodinamic complexation constants in EtOH

- Log K (JG76-K⁺) = 6.34 ± 0.04 (fluorescence)
- Log K (Val-K⁺) = 5.97 ± 0.01 (fluorescence)
- Log K (Val-K⁺) = 5.8 - 6.0 (CD)
- Log K (Cer-K⁺) = 5.99 ± 0.01 (fluorescence)

6. Concentration of cereulide in rice samples

The idea was to measure the concentration of cereulide by fluorescence. To fulfill this objective, a method was suggested in which there is competition for potassium between cereulide and a developed probe.

- Conditions:

The chosen probe was JG76, this molecule is very sensitive and selective to potassium cations, with a complexation constant even higher than cereulide (in EtOH solution), \( 2.2 \cdot 10^6 \text{ M}^{-1} \) against \( 0.9 \cdot 10^{-6} \text{ M}^{-1} \).

The cereulide extracts were provided as rice extracts. The method of extraction is further detailed in part 8 and the samples were received dissolved in acetonitrile with a concentration calculated from HPLC analysis.

The idea was to measure the change in fluorescence in presence of:

- A constant concentration of probe.
- A constant concentration of potassium.
- The sample of cereulide, concentration unknown, increasing it by titration. They were provided in acetonitrile in concentrations 0.2 to 3.5 µM.

- Measuring directly from extracted samples:
First of all, the easiest way to measure would be making a solution of the samples and studying the effect of varying cereulide concentration. This direct method leads to high fluorescent results without response for potassium cations or cereulide. To explain this fact, several reasons were suggested:

- The cereulide samples contain an unknown concentration of potassium or species that act as lewis acid. The complexation constant of the probe is very high so the maximum of fluorescence is reached at a low concentration of potassium cation. Because of that, high initial concentrations of potassium or some lewis acids lead to imprecisions in the determination of cereulide.

- The cereulide samples have matrix contribution, that interferes in the measures; therefore, there is a background fluorescence from the matrix, which may be easily eliminated by substracting the fluorescence when there is no probe JG76.

It may be other unknown ways to interfere in the measures, such as species that interfere in the signal directly; in that case the matrix would need to be completely remove before measuring. All these issues were tested and solved, when possible.

c) Eliminating the excess of potassium in solution:

Due to the higher solubility of cereulide in organic solvents, the solution was extracted by liquid-liquid extraction (DCM-Water); evaporated and redissolved in EtOH, avoiding the interference of any water soluble ions before the titration.

To check if the cereulide remains solved after the extraction, the final solution in EtOH solution was measured by HPLC to compare results to the previous to the extraction verifying its presence.

The HPLC method of calculation was:

- Studying the elution time of synthetic cereulide.
- Measuring the intensity of a sample of extracted cereulide.
- Making a calibration with synthetic cereulide at different concentrations.

![Figure S132. Left, Synthetic cereulide; Right, extracted sample.](image)

In our case, the concentration of the cereulide samples was found around 1.2 µM for the rice samples.

d) Measuring the fluorescence of extracted samples:

After the extraction, the variation of the signal of emission was done similarly to a regular standard addition analysis:

- If the quantity of sample that we have is enough, an amount of samples that allows us to make a regression by changing the concentration of cereulide.
- If the quantity of sample is very low, like in this case, the quantity is increased in the same solution, but the process must be repeated several times to be trustworthy.
- The samples were measured with a λ<sub>exc</sub> = 500 nm and a λ<sub>em</sub> = 571 nm

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This analysis leads to this kind of graphs:

**Figure S133.** Rice sample and Reference Fluorescent emission with increasing quantities of cereulide. The concentrations were \([JG76] = 2 \text{ µM} \) and \([K^+] = 0.75 \text{ µM}\).

It shows a simultaneous comparison and results with cereulide samples and EtOH in which synthetic cereulide is added. Sample and reference were done several times the same day in order to minimize the experimental error.

e) Interpretation of the results for a representative experiment:

Before obtaining any conclusions from fluorescence, one of the most important factors that has influence over the results is the matrix of the rice sample rice. This matrix is supposed to be a mixture of proteins that affect the measures.

The only possibility to make reliable fluorescence quantification, or at least an approximation of the presence of cereulide in the samples, is to check how the emission of the probe is affected by the matrix when the concentration of probe (K\(^+\) constant) and the concentration of potassium (probe constant) changes. To do so, the fluorescence of the matrix was measured without adding JG76 and after adding different concentrations of JG76, to discard interferences matrix-probe.

**Figure S134.** Comparison between ethanol and matrix solutions increasing JG76 concentration in ethanol rice samples and pure ethanol solution.

This test allows us to compare that changes in fluorescence are because there is a background fluorescence, which can be calculated when \([JG76] = 0\), and it’s barely affected by \([JG76]\) at the work concentration.

f) Influence of the matrix in real cereulide titrations
From the previously explained titration of extracted sample of cereulide, the data may be processed by subtracting the background to the results of measuring real rice samples.

- In case of that rice sample, the sample has a background fluorescence of 77 au.

Knowing that the first points have deviation due to the quantity of cereulide, the initial value of fluorescence, by comparing with the reference sample, shows an approximation of the cereulide quantity in the sample:

So, if there is no initial cereulide (the same than in the reference), the curves should be coincident. The result of repeating three times the test was $1.0 \pm 0.2 \mu M$ for rice samples in comparison to the concentration obtained by mass spectrometry, $1.2 \mu M$.

g) Fast processing of the data:
Once the method is validated the next step is making it faster and useful for measuring. Th method may be simplified in five steps:

- The samples were extracted, the same way than for mass spectrometry analysis.
- It is dissolved in ethanol and measured the fluorescence of background.
- The probe JG76 and K\textsuperscript{+} are added (prefixed concentration) to another sample, and the fluorescence intensity is taken.
- The intensity of the background is substrated to the intensity of JG76 sample.
- If the value obtained is not significantly different from the one of synthetic cereulide, it means that the quantity is very low or 0, if it is very different further analysis would be necessary.
  - To obtain a concentration value from fluorescence, it is calculated a linear regression around the result by adding synthetic cereulide or valinomycin, which constant in solution is the same for this purpose.
  - Mass spectrometry analysis, if a high precision determination is required.

It must be taken into account that in this case cereulide is measured indirectly and by fluorescence. The method is more sensitive than ICP analysis to interferents so the conditions should be controlled and the results carefully evaluated.
7. Protocols for production, detection and quantification of cereulide produced by *Bacillus cereus* reference strain F4810/72

7.1 Immunocromatographic cereulide detection test

The positive control *B. cereus* strain used was strain F4810/72, a well-known cereulide producer [1]. The negative control *B. cereus* strain used was the strain ATCC 14579, a known cereulide non-producer [2]. 19 wild-type strains from LBDB collection were tested for cereulide production. Of the 19 wild-type *B. cereus* strains in which the gene *cesA* was detected by qPCR, only 4 had positive results in the immunocromatographic test. These 4 strains were selected to determine the LOD of the GLISA Singlepath® Emetic test, in parallel with the positive and negative control strains. To determine the LOD, several serial dilutions were performed. The results of the tests can be observed in Table 1. All tests were performed in triplicate.

| DILUTION | STRAINS       | F4810/72 + Ctrl | J3  | J271 | J322 | J323 | ATCC 14579 - Ctrl |
|----------|---------------|-----------------|-----|------|------|------|------------------|
| 1:2      |               | +++             | +++ | +++  | +++  | +++  | -                 |
| 1:5      |               | +++             | +++ | +++  | +++  | +++  | -                 |
| 1:10     |               | +++             | +++ | +++  | +++  | +++  | -                 |
| 1:100    |               | ++              | ++  | ++   | ++   | ++   | -                 |
| 1:200    |               | +               | +   | +    | +    | +    | -                 |
| 1:500    |               | -               | -   | -    | -    | -    | -                 |

Table S9 – Results of limit of detection test for immunocromatographic GLISA Singlepath® Emetic Tox Mrk assay

Results showed consistency between the controls and the wild-type strains. We observed positive results up to the 1:200 dilution for the F4810/72 strain. According to the data available [3, 4] this dilution corresponds roughly to an amount of 1.5 ng of CES per µL of culture medium (1500 ng/mL). The dilution 1:500 showed negative results for this strain. We observed that the Limit of Detection (LOD) in these experimental conditions was settled by the 1:200 dilution. Concerning the easiness of use the test is very simple to use and understand. Nevertheless, it requires at least 48 hours to have a final result. The test response time is short (between 5 to 30 minutes), but the overall workflow is quite slow, requiring at least two cultural steps (one in isolation medium – 24 hours; and another in the specific test growth medium – 24 hours). This test doesn’t have any electronic reader and because of that real time linkage with data bases cannot be made directly.

7.2 Testing and optimization of an easy to use cereulide extraction protocol from boiled rice food matrix

Cereulide extraction from food matrices is quite challenging, because the cereulide molecule is highly hydrophobic and easily lost during the process, by adsorption to plasticware and/or in transferring steps. To overcome these circumstances, several extraction protocols were tested. We concluded that protocols involving solvents other than acetonitrile, evaporation steps and/or filtration steps gave unfavourable results due to inconsistent extraction, need of resuspension of final product in acetonitrile and/or loss of an unknown amount of cereulide. Research has been done that allowed to understand the effects of different extraction conditions and factors affecting levels of production of CES by strain F4810/72 of *B. cereus*: Optimal temperature for cereulide production is between 20ºC and 30ºC. For practical purposes the experiments were conducted at 25ºC; oxygen levels above 4.5% increase the production of the toxin, and for this reason all incubations were done in ventilated incubators [4, 5-9]. This method uses acetonitrile as a solvent which is convenient as this is the solvent used in our HPLC-MS equipment. We
introduced some minor changes in the procedure, to optimize it for higher extraction of cereulide and to our laboratory’s equipment.

7.2.1. Cereulide extraction from solid culture medium

- Transfer 30ml of acetonitrile to 50ml falcon tubes and tare them using a laboratory scale.
- Using a sterile plastic loop, collect all the cellular mass from each petri dish and transfer it to the falcon tubes.
  - Add 30 mL of acetonitrile per 2.5 g of cellular mass. If the cellular mass is under or above this value, adjust the volume of acetonitrile used proportionally.
- Place the tubes horizontally in an orbital agitator overnight. Ensure tubes are well strapped and capped to avoid spillage. The agitator should be placed in a chemical safety cabinet.
- Remove the falcon tubes from the agitator and vortex them for 10 seconds.
- Centrifuge the tubes at 4000 G for 15 minutes.
- Using a sterile glass pipette, carefully transfer the supernatant to a glass test tube. Avoid disturbing the pellet.
- Store the extracts at 5 ºC.

7.2.2 Cereulide extraction from food matrix

- Homogenize the 60 g boiled rice portions in the stomacher for 80 seconds.
- Transfer 2.5 g boiled rice portions to 50 mL falcon tubes. This step can be scaled up if more volume of cereulide extract is necessary.
- Add 30 mL of acetonitrile to each falcon tube.
- Keeping the tubes lid partially unscrewed, place them for 15 minutes in the hot bath at 99 ºC.
- Place the tubes horizontally in an orbital agitator overnight. Ensure tubes are well strapped and capped to avoid spillage. The agitator should be placed in a chemical safety cabinet.
- Remove the falcon tubes from the agitator and vortex them for 10 seconds.
- Centrifuge the tubes at 4000 G for 15 minutes.
- Using a sterile glass pipette, carefully transfer the supernatant to a glass test tube. Avoid disturbing the pellet.
- Store the extracts at 5 ºC.

If the technique is done according to the described steps, a clear cereulide extract is obtained, without spores, as can be observed in Figure 192. The cereulide yield varies, but in our experiments cereulide on above 2000 ng/mL for the BHI-agar extracts and 300 ng/mL for the food matrix extracts were obtained.
Figure S136 – Cereulide extracts were placed in PCA culture medium before (left) and after (right) centrifugation to evaluate the presence of bacterial spores. As it is clearly visible, no spores were detected after centrifugation

7.3 Characterization of cereulide production in rice, using the standard strain F4810/72

The experiment was undertaken at 25°C, with oxygen levels over 4.5%.

7.3.1 Preparation and inoculation of food matrix;

- Boil 1 Kg of white rice in 4 L of water.
- Autoclave the rice at 121 °C for 5 minutes.
- Divide rice in 60 g portions and dispense into stomacher bags.
- Add 40 mL of distilled water to each rice portion.
- Homogenize the portions in the stomacher for 80 seconds.
- Inoculate each portion with a culture dilution that allows for an amount of 300 CFU per gram of rice.
- Homogenize the portions in the stomacher for 80 seconds.
- Incubate the samples during 72 h at 25 °C.
- Every 2 hours until 12 h of growth, then at 24 h, 48 h and 72 h of growth, take two portions of 10 g of rice and follow the procedures in 7.3.2 and 7.3.4.
- At 24 h, 48 h and 72 h of growth, take two portions of 10 g of rice and follow the procedures in 7.3.4.

7.3.2 Construction of bacterial growth curve

- Mix the 10 g portion of boiled rice with 90 mL of sterile BPW.
- Homogenize the mixture in the stomacher for 80 seconds.
- Transfer 1 mL of homogenized to 9 mL of BPW (-1 dilution).
- Make the remaining dilutions until dilution -6 to final volumes of 1000 µL in BPW.
- Plate dilutions in PCA solid medium in triplicate and incubate for 24 h.
- Count the colonies and plot on the graphic.
7.3.3 Construction of sporulating curve

- Mix the 10 g portion of boiled rice with 90 mL of sterile BPW.
- Homogenize the mixture in the stomacher for 80 seconds.
- Transfer 1 mL of the mixture to 9 mL of BPW (-1 dilution).
- Place the mixture in a hot water bath at 99°C for 5 minutes to destroy the vegetative forms.
- Centrifuge the mixture at 4000RCF for 15 minutes.
- Discard the supernatant and resuspend the pellet in 100 µL of PBS.
- Place the suspension in the dry bath at 70 °C for 15 minutes to reactivate the spores.
- Centrifuge the mixture at maximum speed for 10 minutes.
- Discard the supernatant and resuspend the pellet in 1000 µL of BHI culture medium with β-alanin at 10 mmol. Vortex mix and wait 30 minutes.
- Make the remaining dilutions until dilution -6 to final volumes of 1000 µL in BHI with β-alanin at 10 mmol.
- Plate dilutions in PCA solid medium in triplicate and incubate for 24 h.
- Count the colonies and plot on the graphic.

7.3.4 Cereulide extraction from food matrix

- Homogenize the 10 g portion in the stomacher for 80 seconds;
- Transfer 2.5 g boiled rice portions to 50 mL Falcon® tubes. This step can be scaled up if more volume of cereulide extract is necessary;
- Add 30 mL of acetonitrile to each Falcon® tube;
- Keeping the tubes lid partially unscrewed, place them for 15 minutes in the hot bath at 99 ºC;
- Place the tubes horizontally in an orbital agitator overnight.
- Remove the Falcon® tubes from the agitator and vortex them for 10 seconds;
- Centrifuge the tubes at 4000 G for 15 minutes;
  o All centrifugations were done using sealed centrifugation cups and opening of cups after centrifugation must always be done inside a chemical and biological safety cabinet;
  o This step is fundamental to deposit the organic fraction but also to ensure that bacterial spores will not be present in the extracts;
- Using a sterile glass pipette, carefully transfer the supernatant to a glass test tube. Avoid disturbing the pellet.
- Proceed to LC-MS analysis.
- Plot the concentrations obtained on the graphic.

The results obtained allowed the construction of a bacterial growth curve visible in Figure 193. It is visible that exponential growth phase begins around 2 h and ends around 24 h. From 24 h of incubation until 72 h the number of CFU per gram of boiled rice remained fairly constant, around 1x10⁸ CFU/g, indicating that the bacterial population has reached the stationary phase of growth. The HPLC-MS data allowed us to start detecting cereulide around 12 h of growth. The quantity of cereulide detected increases exponentially until 72 h, reaching values around 3x10³ ng/g. The sporulation data obtained at 24 h, 48 h and 72 h can be observed in Figure 194. The spore count was maximum at 72 h, at 2.5x10⁶ CFU per gram of rice.
Figure S137 – Growth and cereulide production data from B. cereus strain F4810/72 using boiled rice as a food matrix culture medium. Bacterial growth curve (blue), indicating CFU per gram of boiled rice, and cereulide detection curve (red), indicating ng of cereulide per gram of boiled rice measured by HPLC-MS.

Figure S138 – Sporulation data from B. cereus strain F4810/72 using boiled rice as a food matrix culture medium. Red bars represent spore count at 24, 48 and 72 hours of bacterial growth.

The growth curve obtained is a typical growth curve, demonstrating that the boiled rice food matrix is a favourable culture medium for proliferation of B. cereus. The cereulide production begins at the end of the exponential phase and continues well into stationary phase. The spore count was high at 72 h of growth. All these observation are in accordance with previous studies. They allow us to conclude that cereulide extraction is best done at 72 h, because is the time point in our study, where we obtain higher values of CES.

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