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Rhodamine analogues for molecular ruler applications

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Abstract

A series of geometrically well-defined cationic fluorophores were designed based on molecular mechanics. They contain biaryl linkers to impart rigidity preventing intramolecular folding between a conjugated biomolecule and fluorophore. All probes have absorption and emission maxima within 20 nm from Texas Red, as predicted by TDDFT calculations and validated experimentally.

Graphical Abstract

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Keywords

molecular ruler; rhodamine; ion channel; pore dilation

1. Introduction

Fluorescent probes are widely used for the detection of specific biomolecular pathways and cellular events. The objective of this study is to synthesize rigid, geometrically well-defined rhodamine derivatives (**1–4**, Fig. 1) to serve as the optical components of bioactive conjugates. A longer-term goal is to use drug-fluorophore conjugates to investigate pore dilation and the dimensions of ion channels involved in the cellular uptake of aminoglycosides, such as gentamicin (GT). This would lead to further understanding of the mechanism and potential prevention of gentamicin's serious ototoxic and nephrotoxic side effects.

Fluorescein, BODIPY and acridine dyes have been conjugated to aminoglycosides such as gentamicin and neomycin to study cellular uptake,[1, 2] HIV-RNA binding[3] and for use in fluorescent immunoassays.[4] The fluorescent conjugate of gentamicin and Texas Red (**1-GT**, Fig. 2) has been of great utility in the study of non-endocytotic aminoglycoside uptake and intracellular localization.[5, 6] Texas Red succinimidyl ester, **1** (Fig. 1) is the fluorophore used for gentamicin conjugation because it imparts amphiphilic properties, cationic charge and enables the retention of drug bioactivity.[6, 7] However, **1-GT** diameter, at first estimated to be 1.47 nm,[8] is larger than the channel pore size (1.25 nm) of the hair cell stereocilia mechanoelectrical transduction (MET) channel that it permeates. This led researchers to conclude that either the MET channel pore size has been underestimated, or that the MET channel pore temporarily dilates upon **1-GT** entry.[8] The biophysical mechanisms associated with pore dilation are poorly understood.[9]

Previously, we reported the synthesis of **2** possessing a rigid linker to address the propensity of the **1-GT** to quench in live cells.[10] Extending the length of the fluorophore as well as the rigidified linker may allow the conjugate to serve as a molecular ruler and enable to study the diameter and dilation properties of cationic channels. The synthesis of compounds **3** and **4** are described herein, as they embody systematically increased dimensions as compared to **1**, **2** and each other. They exhibit only minor changes in spectral properties compared to the parent structure **1**. [Fig. 1]

Molecular modeling was used as a tool to guide the design of the conjugatable fluorophores. Energy-minimized structures generated by molecular mechanics (MM2, SYBYL-X™ 2.0, Certara L. P.) showed that the sulfonamide group of **1** promotes folding of the structure, including interactions between the gentamicin and Texas-Red moieties. An energy-minimized structure of an unfolded conformer of **1-GT** may exhibit a maximal cross-sectional diameter of 1.35 nm (Fig. 2A).[10] Although less than the 1.47 nm value reported by others, it also shows that **1-GT** is larger than non-selective cation channels on hair cells, such as the MET channel[11] and TRPV1,[12] which has an open pore size of ~1 nm[13, 14] and increases upon agonist-induced pore dilation.[15, 16]

2. Experimental

2.1 General

All chemicals were used as received without further purification. ^1H -NMR, ^{13}C -NMR and were acquired on an ARX-400 Avance Bruker spectrometer. Chemical shifts (δ) are given in ppm. Mass spectra were obtained at the PSU Bioanalytical Mass Spectrometry Facility on a ThermoElectron LTQ-Orbitrap Discovery high resolution mass spectrometer coupled to an Accela HPLC system. Microwave synthetic procedures were performed in an InitiatorTM microwave synthesizer (Biotage). Fluorescence measurements were carried out on a Cary EclipseTM fluorescence spectrophotometer (Agilent Technologies). Absorption spectra were acquired with a Cary EclipseTM 50 Bio UV-Vis spectrophotometer.

2.2 Synthesis

2.2.1 Synthesis of compound 6—Compound **6** was synthesized using a modified procedure reported previously.[17] Under Ar, ytterbium(III) triflate (353 mg, 0.568 mmol) was added to a solution of *m*-anisidine (1 g, 8.12 mmol) in acetone (40 mL). The reaction mixture was stirred at rt for 24 h. Acetone was removed *in vacuo* and the residue dissolved in EtOAc, washed with H₂O, brine and dried over Na₂SO₄, filtered and the solvent evaporated under vacuum. The mixture was then separated by flash column chromatography on SiO₂ using Hexane:EtOAc 8:1 as the eluent to afford a pale yellow oily product (1.32 g, 80%). ^1H NMR, ^{13}C NMR and MS data are in agreement with the literature.[17]

2.2.2 Synthesis of compound 7—Compound **7** was synthesized using a modified procedure reported previously.[17] Under Ar, compound **6** (5.55 g, 27.3 mmol), Cs₂CO₃ (4.98 g, 15.29 mmol), and MeI (6.46 mL, 103.75 mmol) were added to anhydrous DMF (15 mL) and stirred at 90 °C for 3 h. DMF was removed *in vacuo* and the residue poured into H₂O and washed with EtOAc (3 × 200 mL). The combined organic layers were washed with brine and dried over Na₂SO₄. The solvent was removed under reduced pressure and a colorless oily product (5.28 g, 89%) was obtained. ^1H NMR, ^{13}C NMR and MS data are in agreement with the literature.[17]

2.2.3 Synthesis of compound 8—Compound **8** was synthesized using a modified procedure reported previously.[17] Under Ar, compound **7** (3.05 g, 14.04 mmol) was dissolved in glacial AcOH (8.19 mL). 48% aqueous HBr (10.05 mL, 88.84 mmol) was added and the mixture heated at reflux (118 °C) for 8 h. After cooling, the solution was neutralized to about pH 5–6 with 30% aq. NaOH. The aqueous phase was extracted with CH₂Cl₂ (3 × 150 mL) and the combined organic layers were washed with brine and dried over Na₂SO₄. The solvent was evaporated under reduced pressure. The mixture was then separated by flash column chromatography on SiO₂ using Hexane:EtOAc 5:1 as the eluent to yield dark red oily product (1.54 g, 54%). ^1H NMR, ^{13}C NMR and MS data are in agreement with the literature.[17]

2.2.4 Synthesis of compound 10—In a 5 mL microwave reaction vessel, compound **8** (85 mg, 0.737 mmol) and methyl 4-(4-formylphenyl)benzoate **9** (50 mg, 0.208 mmol) were dissolved in 1 mL CH₂Cl₂. The solvent was purged using an N₂ stream. The mixture was

heated in a microwave reactor at 150 °C for 10 min. The mixture was dissolved in CH₂Cl₂:MeOH (95:5, 1 mL) and tetrachloro-1,4-benzoquinone (DDQ) (76.75 mg, 0.312 mmol) was added to the solution, and vortexed at 1000 rpm for 15 min. The solvent was purged using an N₂ stream. The mixture was separated via a short SiO₂ plug using DCM:MeOH 97:3 as the eluent to afford compound **10** as a purple solid (85.15 mg, 67%). ¹H NMR(CDCl₃) : 1.49 (12H, s), 1.80 (6H, s), 3.21 (6H, s), 3.97 (3H, s), 5.51(2H, s), 6.85 (2H, s), 6.96 (2H, s), 7.52 (2H, d, *J* = 8.4 Hz), 7.81 (2H, d, *J* = 8.6 Hz), 7.91 (2H, d, *J* = 8.4 Hz), 8.18 (2H, d, *J* = 8.6 Hz). ¹³C NMR (CDCl₃): 18.5, 29.44, 29.82, 33.51, 52.43, 59.96, 96.20, 113.79, 122.00, 123.64, 125.59, 127.28, 127.76, 129.97, 130.23, 130.51, 132.08, 132.75, 141.88, 143.95, 153.46, 154.12, 158.45, 166.91. HR ESI-MS found: [M⁺] 609.3112, molecular formula C₄₁H₄₁N₂O₃⁺, requires [M⁺] 609.3125.

2.2.5 Synthesis of compound 11—Compound **10** (36 mg, 0.059 mmol) was dissolved in MeOH:THF:H₂O (5:3:1, 0.9 mL) and LiOH (7.07 mg, 0.295 mmol) was added. The reaction was stirred at rt overnight. After the reaction was complete (TLC), the residue was dissolved in CH₂Cl₂:H₂O (1:1, 2 mL). 1 N HCl was slowly added until the mixture was at pH 5. The aqueous layer was extracted with CH₂Cl₂ (3 × 5 mL), and the combined organic layers were washed with brine and dried over Na₂SO₄. The solvent was evaporated under reduced pressure to afford compound **11** as a purple solid (27.43 mg, 78%). ¹H NMR(CD₃OD) : 1.52 (12H, s), 1.83 (6H, s), 3.21 (6H, s), 5.69 (2H, s), 6.86 (2H, s), 7.09 (2H, s), 7.65 (2H, d, *J* = 8.3 Hz), 7.91 (2H, d, *J* = 8.3 Hz), 8.05 (2H, d, *J* = 8.3 Hz), 8.16 (2H, d, *J* = 8.3 Hz). ¹³C NMR (CD₃OD): 18.26, 29.16, 33.44, 61.26, 96.49, 114.89, 123.14, 124.77, 126.63, 128.17, 128.75, 131.61, 133.38, 134.13, 143.18, 145.14, 154.82, 156.00, 159.86. HR ESI-MS found: [M⁺] 595.2955, molecular formula C₄₀H₃₉N₂O₃⁺, requires [M⁺] 595.2962.

2.2.6 Synthesis of compound 3—Compound **11** (20 mg, 0.033 mmol) was dissolved in 0.2 mL of anhydrous THF. *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC, 7.72 mg, 0.04 mmol) was added in one portion followed by *N*-Hydroxy succinimide (NHS) (4.64 mg, 0.04 mmol). The reaction was stirred at rt for 24 h. Another 10 equiv of EDAC (64.32 mg, 0.336 mmol) were added and the reaction stirred at rt overnight. The reaction mixture was poured into 10 mL of 50 mM pH 6 phosphate buffer and extracted with CH₂Cl₂ (3 × 15 mL). The combined organic layers were washed with brine and dried over Na₂SO₄, and the solvent evaporated under reduced pressure. The mixture was separated via a short SiO₂ plug using CH₂Cl₂:MeCN 1:1, followed by gradient elution with CH₂Cl₂:MeOH 98:2 to 95:5 to afford the target compound **3** as a purple solid (19.55 mg, 84%). ¹H NMR(CDCl₃) : 1.50 (12H, s), 1.81(6H, s), 1.95(4H, s), 3.23 (6H, s), 5.52 (2H, s), 6.86 (2H, s), 6.95 (2H, s), 8.28 (2H, d, *J* = 8.4 Hz), 7.93 (2H, d, *J* = 8.2 Hz), 7.94 (2H, d, *J* = 8.6 Hz), 8.27 (2H, d, *J* = 8.3 Hz), 8.29 (2H, d, *J* = 8.6 Hz). ¹³C NMR (CDCl₃): 18.54, 25.87, 29.46, 33.62, 60.00, 96.25, 113.79, 121.92, 123.70, 124.86, 125.57, 127.71, 127.90, 130.34, 131.50, 132.80, 141.28, 145.95, 153.48, 158.45, 161.72, 169.39. HR ESI-MS found: [M⁺] 692.3119, molecular formula C₄₄H₄₂N₃O₅⁺, requires [M⁺] 692.3129.

2.2.7 Synthesis of compound 17—*m*-Anisidine (6 g, 48.72 mmol) was dissolved in 180 mL anhydrous C₂H₂Cl₂ (DCE) followed by consecutive addition of ethyl 2-

cyclohexanonecarboxylate (9.35 mL, 58.46 mmol), NaBH(OAc)₃ (15.49 g, 73.08 mmol) and AcOH (2.79 mL, 48.72 mmol). The reaction was stirred at rt overnight and monitored by TLC. After the reaction was complete, 180 mL of saturated NaHCO₃ was poured into the mixture followed by stirring for 30 min. The reaction mixture was extracted with CH₂Cl₂ (3 × 150 mL) and the combined organic layers were dried over Na₂SO₄. The solvent was removed under reduced pressure and the residue was purified by flash column chromatography on SiO₂ using Hexane:EtOAc 15:1 as the eluent to afford compound **17** as a colorless oil (9.3 g, 69%). ¹H NMR(CDCl₃) : 1.23(3H, t, *J* = 7.1 Hz), 1.42–1.57 (3H, m), 1.64–1.75 (3H, m), 1.88–1.95 (1H, m), 1.97–2.07(1H, m), 2.84–2.88 (1H, m), 3.75–3.77 (1H, m), 3.79 (3H, s), 4.07–4.17 (2H, m), 4.4 (1H, s), 6.20 (1H, t, *J* = 2.3 Hz), 6.24–6.28 (2H, m), 7.08 (1H, t, *J* = 8.1 Hz). ¹³C NMR (CDCl₃): 14.30, 23.03, 23.34, 26.08, 29.08, 44.79, 51.62, 55.20, 60.43, 99.67, 102.52, 106.85, 130.09, 148.59, 160.97, 174.09. HR ESI-MS found: [M+H]⁺ 278.1751, molecular formula C₁₆H₂₃NO₃, requires [M+H]⁺ 278.1743.

2.2.8 Synthesis of compound 18—Under Ar, compound **17** (6 g, 21.63 mmol) was dissolved in 16 mL of anhydrous DMF followed by addition of Cs₂CO₃ (3.95 g, 12.11 mmol) and MeI (5.12 mL, 82.2 mmol). The reaction mixture was heated at 90 °C and vigorously stirred for 3 h. After the reaction was complete, DMF was removed *in vacuo* and the residue poured into H₂O (100 mL) and washed with EtOAc (3 × 150 mL). The combined organic layers were dried over Na₂SO₄ and the solvent was removed under reduced pressure to obtain a light yellow oil (5.04 g, 80%). ¹H NMR(CDCl₃) : 1.20 (3H, t, *J* = 7.1 Hz), 1.35–1.45 (1H, m), 1.53–1.55 (1H, m), 1.64–1.68 (3H, m), 1.93–2.01(2H, m), 2.39 (1H, qd, *J* = 12.3, 3.7 Hz), 2.82 (3H, s), 3.08 (1H, m), 3.75 (1H, dt, *J* = 12.0, 4.0 Hz), 3.78 (3H, s), 4.00–4.12 (2H, m), 6.27 (1H, m), 6.34 (1H, d, *J* = 8.3 Hz), 7.15 (1H, t, *J* = 8.2 Hz). ¹³C NMR (CDCl₃): 14.28, 21.45, 26.69, 26.95, 28.83, 32.45, 42.09, 55.27, 59.13, 60.20, 98.92, 100.67, 105.32, 129.95, 150.86, 160.97, 174.55. HR ESI-MS found: [M+H]⁺ 292.1907 molecular formula C₁₇H₂₅NO₃, requires [M+H]⁺ 292.1894.

2.2.9 Synthesis of compound 14—A mixture of compound **18** (5.03 g, 17.26 mmol) and KOH (2.42 g, 43.14 mmol) in 15 mL MeOH were stirred at rt for 1h. An additional 5 equiv of KOH (4.84 g) were added and stirring was continued overnight. After the reaction was complete, MeOH was removed under vacuum. The residue was poured into CH₂Cl₂ and diluted with H₂O. The solution was acidified to pH 3 via slow addition of 1 N HCl. The reaction mixture was extracted with CH₂Cl₂ (3 × 150 mL) and the combined organic layers were dried with Na₂SO₄. The solvent was removed *in vacuo* to afford a brown oil (crude yield: 4.5 g, 99%). ¹H NMR(CDCl₃) : 1.34–1.43 (2H, m), 1.34–1.56 (2H, m), 1.74–1.82 (4H, m), 2.31–2.35 (1H, m), 2.58(1H, td, *J* = 11.6, 3.8 Hz), 2.77 (3H, s), 3.58 (1H, td, *J* = 11.4, 3.6 Hz), 3.80 (3H, s), 6.44 (1H, ddd, *J* = 8.2, 2.3, 0.6 Hz), 6.54 (1H, t, *J* = 2.3 Hz), 6.60 (1H, dd, *J* = 5.3, 4.8 Hz), 7.15 (1H, t, *J* = 8.2 Hz). ¹³C NMR (CDCl₃): 25.22, 25.57, 26.76, 29.43, 31.86, 46.12, 55.39, 63.07, 103.97, 105.29, 110.12, 129.86, 151.29, 160.84, 177.78. HR ESI-MS found: [M+H]⁺ 264.1594, molecular formula C₁₅H₂₁NO₃, requires [M+H]⁺ 264.1582.

2.2.10 Synthesis of compound 13—A mixture of compound **14** (0.5 g, 1.9 mmol) and polyphosphoric acid (6.3 g) was heated to 120 °C for 20 min. The reaction was cooled to rt

and the solution neutralized to pH 7–8 with 15% aq. NaOH. The reaction mixture was extracted with EtOAc and the combined organic layers were dried over Na₂SO₄ and the solvent removed *in vacuo*. The residue was purified by column chromatography on SiO₂ using Hexane:EtOAc 8:1 as the eluent to compound **13** as a yellow solid. (75.58 mg, 16%). ¹H NMR(CDCl₃): 1.15–1.34 (3H, m), 1.42–1.51 (1H, m), 1.82–1.85 (1H, m), 1.89–1.91(1H, m), 2.22–2.29 (1H, m), 2.34–2.38 (1H, m), 2.42–2.47 (1H, m), 2.90 (3H, s), 3.06 (1H, ddd, *J* = 13.1, 11.3, 4.1 Hz), 3.84 (3H, s), 6.25 (1H, d, *J* = 2.2 Hz), 6.36 (1H, dd, *J* = 8.7, 2.3 Hz), 7.89 (1H, d, *J* = 8.7 Hz). ¹³C NMR (CDCl₃): 24.76, 24.78, 24.87, 31.62, 32.79, 50.36, 55.43, 62.12, 97.92, 104.37, 114.69, 130.34, 154.85, 165.49, 194.33. HR ESI-MS found: [M+H]⁺ 246.1489, molecular formula C₁₅H₁₉NO₂, requires [M+H]⁺ 246.1482.

2.2.11 Synthesis of compound 19—Under Ar, to a solution of compound **13** (1 g, 4.08 mmol) in 12.5 mL of anhydrous Et₂O was added LiAlH₄ (464 mg, 12.23 mmol) portion-wise slowly. Once evolution of H₂ ceased, 12.5 mL dry toluene was added and the reaction heated at reflux (120 °C) for 2h. The mixture was cooled to 0 °C and followed by dropwise consecutive additions of 0.5 mL of H₂O, 0.5 mL of 15% NaOH and 1.5 mL of H₂O. The resultant precipitate was removed by vacuum filtration, washed with 60 mL CH₂Cl₂. The filtrate was concentrated *in vacuo* to afford compound **19** as a light yellow solid (929 mg, 99%). ¹H NMR (CDCl₃): 1.07–1.21 (2H, m), 1.24–1.41 (2H, m), 1.51–1.61 (1H, m), 1.71–1.75 (1H, m), 1.83–1.88 (2H, m), 2.26–2.30 (1H, m), 2.37–2.45 (1H, m), 2.37–2.45 (1H, m), 2.52 (1H, dd, *J* = 15.1, 4.2 Hz), 2.68 (1H, td, *J* = 10.6, 3.6 Hz), 2.9 (3H, s), 3.77 (3H, s), 6.17 (1H, dd, *J* = 8.1, 2.4 Hz), 6.22 (1H, d, *J* = 2.4 Hz), 6.81(1H, d, *J* = 8.0 Hz). ¹³C NMR (CDCl₃): 25.30, 25.84, 32.44, 33.03, 34.84, 35.99, 39.28, 55.34, 63.47, 99.44, 100.73, 116.63, 128.59, 148.47, 159.30. HR ESI-MS found: [M+H]⁺ 232.1696, molecular formula C₁₅H₂₁NO, requires [M+H]⁺ 232.1684.

2.2.12 Synthesis of compound 12—Under Ar, compound **19** (1 g, 4.32 mmol) was dissolved in glacial AcOH (2.5 mL), 48% aq. HBr (3.03 mL, 26.8 mmol) was added and the mixture heated at reflux (118 °C) overnight. After cooling, the solution was neutralized to pH 5–6 with 30% aq. NaOH. The aqueous phase was extracted with CH₂Cl₂ (3 × 100 mL) and the combined organic layers were washed with brine and dry over Na₂SO₄. The solvent was removed under reduced pressure to afford compound **12** as a brown solid (938 mg, 100%). ¹H NMR(CDCl₃): 1.06–1.20 (2H, m), 1.23–1.41 (2H, m), 1.50–1.60 (1H, m), 1.71–1.74 (1H, m), 1.83–1.87 (2H, m), 2.25–2.29 (1H, m), 2.36–2.42 (1H, m), 2.50 (1H, dd, *J* = 15.1, 4.2 Hz), 2.68 (1H, td, *J* = 10.6, 3.6 Hz), 2.87 (3H, s), 6.06 (1H, dd, *J* = 7.9, 2.4 Hz), 6.14 (1H, d, *J* = 2.3 Hz), 6.74 (1H, d, *J* = 7.8 Hz). ¹³C NMR (CDCl₃): 25.30, 25.83, 32.40, 33.02, 34.83, 35.87, 39.27, 63.38, 99.83, 102.71, 116.35, 128.83, 148.64, 154.99. HR ESI-MS found: [M+H]⁺ 218.1539, molecular formula C₁₄H₁₉NO, requires [M+H]⁺ 218.1522.

2.2.13 Synthesis of compound 20—In a 2 mL microwave reaction vessel, compound **12** (108 mg, 0.499 mmol) and methyl 4-(4-formylphenyl)benzoate (60 mg, 0.25 mmol) were dissolved in 1 mL CH₂Cl₂. The solvent was purged using an N₂ stream. The reaction mixture was heated in microwave reactor at 150 °C for 10 min. The mixture was dissolved in CH₂Cl₂:MeOH (95:5, 0.5 mL) followed by addition of DDQ (92.1 mg, 0.375 mmol) and vortexed at 1000 rpm for 15 min. The solvent was removed using an N₂ stream. The mixture

was separated via a short SiO₂ plug using CH₂Cl₂:MeCN 1:1, followed by CH₂Cl₂:MeOH 97:3 as the eluent to give a dark purple solid (67.3 mg, 42%). ¹H NMR(DMSO) : 1.11–1.47 (12H, m), 1.66–1.67 (2H, m), 1.77–1.85 (4H, m), 2.34–2.47 (4H, m), 2.66 (2H, d, *J* = 14.2 Hz), 3.21 (6H, s), 3.91(3H, s), 6.93 (2H, s), 6.99 (2H, s), 7.58–7.62 (2H, m), 7.99 (2H, dd, *J* = 8.6, 2.1 Hz), 8.05 (2H, dd, *J* = 8.5, 2.5 Hz), 8.11 (2H, dd, *J* = 8.5, 1.1 Hz). ¹³C NMR (DMSO): 24.70, 24.77, 30.67, 31.36, 33.47, 35.60, 38.16, 52.27, 63.81, 96.28, 112.75, 126.50, 127.17, 127.35, 129.05, 129.97, 140.07, 143.49, 154.90, 156.48, 156.51, 165.99. HR ESI-MS found: [M⁺] 637.3425, molecular formula C₄₃H₄₅N₂O₃⁺, requires [M⁺] 637.3381.

2.2.14 Synthesis of compound 21—Compound **20** (67 mg, 0.105 mmol) was dissolved in MeOH:THF:H₂O (5:3:1, 2.7 mL) and LiOH (12.58 mg, 0.525 mmol) was added. The reaction was stirred at rt overnight. After the reaction was complete, the residue was dissolved in CH₂Cl₂:H₂O (1:1, 2 mL), and 1 N HCl was slowly added to reach pH 5. The aqueous layer was extracted with CH₂Cl₂ (3 × 5 mL), and the combined organic layers were dried over Na₂SO₄. The solvent was removed *in vacuo* to furnish a purple solid (59.42 mg, 91%). ¹H NMR(CD₃OD) : 1.14–1.48 (12H, m), 1.66–1.69 (3H, m), 1.80–1.86 (3H, m), 2.34–2.48 (4H, m), 2.67 (2H, d, *J* = 13.6 Hz), 3.21 (6H, s), 6.9 (2H, s), 7.0 (2H, s), 7.6 (2H, d, *J* = 7.6 Hz), 7.96 (2H, d, *J* = 8.3 Hz), 8.05 (2H, d, *J* = 8.2 Hz), 8.10 (2H, d, *J* = 8.4 Hz). ¹³C NMR (CD₃OD): 24.71, 24.77, 30.68, 31.36, 33.48, 35.61, 38.17, 63.81, 96.27, 112.79, 126.52, 127.00, 127.31, 130.11, 131.86, 140.32, 143.00, 154.30, 154.92, 156.53, 167.09. HR ESI-MS found: [M⁺] 623.3268, molecular formula C₄₂H₄₃N₂O₃⁺, requires [M⁺] 623.3287.

2.2.15 Synthesis of compound 4—Compound **21** (8 mg, 0.013 mmol) was dissolved in 0.2 mL of anhydrous THF. EDAC (3.69 mg, 0.019 mmol) was added in one portion followed by the addition of NHS (1.77 mg, 0.015 mmol). The reaction was stirred at rt for 4 h. Another 5 equiv EDAC (12.3 mg, 0.063 mmol) were added and the reaction stirred at rt overnight. The reaction mixture was poured into 5 mL of 50 mM pH 6 phosphate buffer, and extracted with CH₂Cl₂ (3 × 5 mL). The combined organic layers were washed with brine and dried over Na₂SO₄ and the solvent evaporated *in vacuo*. The mixture was separated via a short SiO₂ plug using CH₂Cl₂:MeCN 1:1 then DCM:MeOH 96:4 to afford the target compound **4** as a purple solid (7.68 mg, 83%). ¹H NMR(CDCl₃): 1.13–1.32 (8H, m), 1.36–1.42 (2H, m), 1.47–1.57(2H, m), 1.74 (2H, d, *J* = 12.3 Hz), 1.84 (2H, d, *J* = 12.3 Hz), 1.92 (2H, d, *J* = 13.1 Hz), 2.42–2.49 (4H, m), 2.60 (2H, dd, *J* = 15.2, 2.6 Hz), 2.93 (4H, s), 3.12–3.18 (2H, m), 3.24 (6H, s), 6.82 (2H, s), 6.94 (2H, s), 7.49 (2H, d, *J* = 8.2 Hz), 7.84 (2H, d, *J* = 8.5 Hz), 7.88 (2H, d, *J* = 8.3 Hz), 8.26 (2H, d, *J* = 8.5 Hz). ¹³C NMR (CDCl₃): 25.16, 25.25, 25.84, 29.76, 31.51, 32.04, 34.63, 36.20, 38.85, 64.80, 96.96, 113.59, 124.70, 126.83, 126.85, 126.91, 127.66, 127.87, 127.91, 127.95, 130.16, 130.26, 130.36, 131.46, 132.68, 141.10, 146.13, 154.62, 155.56, 157.24, 157.26, 161.70, 169.39. HR ESI-MS found: [M⁺] 720.3426, molecular formula C₄₆H₄₆N₃O₅⁺, requires [M⁺] 720.3432.

3. Results and Discussion

3.1 Molecular Simulations

Molecular simulations of the gentamicin conjugate of **2** (Fig. 2B) reveal a cross-sectional diameter of 1.44 nm. This diameter can be increased to 1.56 nm by adding four methyl groups to the edges of the fluorophore scaffold (Fig. 2C). Insertion of two additional fused six-membered aliphatic rings to the fluorophore framework furnishes a conjugate with a cross-sectional diameter of 1.85 nm (Figure 2D) that contains an inherent degree of flexibility due to cis-trans isomerism of the octahydroacridine moiety.[18] The design of these probes will thus allow them to be used as molecular rulers, to help enable determination of the maximum diameter of dilated aminoglycoside-permeant cation channels. [Fig. 2]

Time-dependent density functional theory (TDDFT) calculations of the excitation energies were used to determine the relative impact of the proposed structural changes over the spectral properties. The calculations were performed using Gaussian 09[19] and involved the B3LYP hybrid functional and the 6-31G(d,p) basis set. The calculated absorption spectra shows blue shifts of 6 nm for **2** and 13 nm for **4** while **3** exhibits a 25 nm red shift (Fig. 3).

3.2 Synthesis

3.2.1 Synthesis of compound 3—Compound **3** was synthesized in six steps starting from 3-methoxyaniline (Scheme 1). Compound **8** was prepared via modified literature procedures by Belov *et al.*[20] Trimethyl-dihydroquinoline amine **6** was obtained in 80% yield by the condensation of *m*-anisidine with acetone using Yb(OTf)₃ as the catalyst. Further alkylation of **6** with MeI in the presence of Cs₂CO₃ gave **7** in 89% yield. Nucleophilic dealkylation of intermediate **7** with HBr and AcOH afforded **8** in 54% yield. Initial attempts to prepare compound **10** involved the synthesis of a bromo-substituted rhodamine,[17] followed by a Suzuki coupling reaction with the corresponding boronic acid.[21] Suzuki coupling afforded complex product mixtures (ESI). An alternative approach using microwave irradiation via direct condensation of **8** and **9** followed by oxidation with *p*-chloranil successfully afforded **10** in 67 % yield. Hydrolysis of **10** was conducted under basic conditions using LiOH in a mixture of MeOH:THF:H₂O (5:3:1) to give compound **11** in 78%. The activated succinimidyl ester **3** was obtained in 84% yield using *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide (EDAC) as the coupling reagent.

3.2.2 Synthesis of compound 4—Retrosynthetic analysis of the target compound **4** was envisioned as occurring via the condensation of **12** and **9** (Fig. 4), using similar reaction conditions as described above for compound **10**. An Initial attempt to synthesize **13** via nickel-catalyzed decarboxylative cycloaddition of isatoic anhydride with cyclohexene (ESI) led to complex mixtures with the product not detectable by mass spectrometry.[22] An alternative strategy envisioned C-C bond formation via an intramolecular Friedel-Crafts acylation of **14** followed by reduction to afford **12**. Compound **14** would be derived from reductive amination via the reaction of aniline **15** and β -ketoester **16**.

As shown in Scheme 2, the reductive amination of **15** and **16** with NaBH(OAc)₃ afforded product **17** in 69% yield.[23] Alkylation of **17** with MeI gave the corresponding *N*-methylated product **18** in 80% yield. Compound **18** was hydrolyzed under basic conditions to afford **14** in 99% yield. Cyclization of **14** in polyphosphoric acid (PPA) led to hydroacridinone **13** in 16% yield via intramolecular Friedel-Crafts acylation.[24] Reduction of **13** using LiAlH₄ under reflux conditions gave the corresponding acridine **19** in 90% yield.[25] The target intermediate **12** was successfully obtained in 99% yield by nucleophilic dealkylation of the methoxy ether using HBr and AcOH. The target compound **4** was synthesized in three steps using a similar strategy as described above for **3**, which involved the condensation of **12** and **9** in 49% yield. Basic hydrolysis of the methyl ester **20** gave **21** in 99% yield. The activated succinimidyl ester **4** was obtained in 85%.

3.3 Spectral Properties

Spectroscopic evaluation shows that compounds **3** and **4** have relatively similar absorption and emission wavelengths as compared to the parent compound **1**. Compound **3** bearing two extra conjugated double bonds exhibits a 10 nm red shift as compared to **1**. Compound **4** shows a 20 nm blue shift (Figs. 5 and 6). In addition, Table 1 shows that the structural features of compound **2**, **3** and **4** have a low impact on the relative quantum yields (ϕ) using **1** as the reference standard. [Fig. 5]

4. Conclusion

In summary, the computed-assisted systematic design of rhodamines via molecular mechanics resulted in the synthesis of modified structures with extended dimensions perpendicular to a rigid linker bearing an amine-reactive group for biomolecule conjugation. Two rhodamine probes, **3** and **4** were synthesized in six and nine steps, with an overall yield of 13% and 3%, respectively. Spectroscopic evaluation of the probes shows relatively small shifts (< 20 nm in fluorescence emission) as compared to **1**, and the trend in the absorption spectra are in agreement with TDDFT calculations. Conjugation of probe **3** and **4** to aminoglycosides (Scheme 3) to study channel properties including the mechanisms of pore dilation and drug uptake is currently ongoing and will be published in the near future. [Scheme 3]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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- Extended rigid rhodamines were designed via molecular simulations
- The trend of spectral properties of rhodamines agreed with TDDFT calculations
- Amine-reactive probes for bioconjugation were synthesized
- To be used as molecular ruler to study pore dilation in cationic channels

Fig. 1.
Texas Red succinimidyl ester **1**, inflexible linker probe **2** and target fluorophores **3** and **4**.

Fig. 2.

Optimized geometries of GT conjugate analogues of TR. (A) **1-GT**; commercial Texas Red conjugated to GT. (B) **2-GT**; the SO_3^- and SO_2 groups are removed and the alkyl chain is replaced by an inflexible *p,p'*-biphenylene linker. (C) **3-GT**; the inflexible linker is kept and four methyl groups are added at both ends of the fluorophore scaffold. (D) **4-GT**; the inflexible linker is kept and two six-membered aliphatic fused rings are added. Atom color codes: C=grey, H=white, N=blue, O=red, S=yellow.

Fig. 3.
TDDFT calculated spectra of compounds **1**, **2**, **3** and **4**.

Fig. 4.
Retrosynthetic analysis of **4**.

Fig. 5. Absorption spectra of 1.25 μ M solutions in MeOH of compounds **1** – **4**.

Fig. 6.
Normalized fluorescence emission spectra of 1.25 μM solutions in MeOH of compounds **1** – **4**.

Scheme 1.
Synthesis of compound **3**.

Scheme 2.
Synthesis of compound **4**.

Scheme 3.

Synthesis of conjugates **3-GT** and **4-GT** upon reaction of **3** and **4** with gentamicin.

Table 1Spectral properties of compound **1** – **4**.

Compound	$\lambda_{ex}/\lambda_{em}$ (nm)	Relative quantum yield (ϕ)
1	580/600	1
2	575/600	0.71
3	590/615	0.83
4	560/590	1.1