Molecular Umbrella Conjugate for the Ocular Delivery of siRNA

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Supporting Information

Spermidine-N_2-trifluoroacetamide. A solution was made from 1.400 g (4.06 mmol) of N_1,N_3-diboc-spermidine and 6 mL of ethyltrifluoroacetate and stirred at 45ºC for 48 h at room temperature.[1] Removal of solvent under reduced pressure afforded a crude product, which was then purified by column chromatography [25 g SiO_2, chloroform/methanol/28% aqueous NH_3, 75/25/3/ (v/v/v)] to give 0.888 g (51%) of N_1,N_3-diBoc-spermidine-N_2-trifluoracetamide having ^{1}H NMR (CD_3OD, ppm): 3.40(m, 4H); 3.06(t, 4H); 1.77(m, 2H); 1.62(m, 2H); 1.47(m, 2H); 1.42(m, 2H) and negative HR-ESI MS for $C_{19}H_{34}N_3O_5F_3$ (Na^+) Calcd: 464.234. Found: 464.236.

A solution made from 27.6 mg (0.0626 mmol) of N_1,N_3-diBoc-spermidine-N_2-trifluoracetamide, 1.5 mL of chloroform and 1.5 mL of trifluoroacetic acid was then stirred at room temperature for 4 h. Removal of solvent under reduced pressure and

drying (0.5 Torr, 23°C, 1h), followed by dissolution in 2 mL of methanol, removal of solvent under reduced pressure and drying (0.5 Torr, 23°C, 1h) gave 0.190 g (47 %) of spermidine-N_2-trifluoroacetamide having ^{1}H NMR (CD_3OD, ppm): 3.53 (t, 2H); 3.46(t, 2H); 2.95(m, 4H); 2.00(m, 2H); 1.60-1.78(m, 4H).

Bis-(N_1N_2-diBoc-L-lysyl)-L-lysine, 2. To a dispersion made from 73 mg (0.5 mmol) of L-lysine, 2 mL of anhydrous DMF and 0.516 g (4.0 mmol) of N,N-diisopropyl-N-ethylamine was added 0.444 g (1.0 mmol) of N_1,N_2-diBoc-L-Lysine -N- (O-succinimidyl ester (Sigma/Aldrich). After stirring at room temperature for 24 h, the mixture was concentrated under reduced pressure at 60°C. Chromatographic purification of the residue [40 g SiO_2, chloroform/methanol/28%aqueous NH_3, 70/18/3 (v/v/v)] afforded 0.190 g (47%) of **3** as a white solid having R_f=0.6 and ^{1}H NMR (CD_3OD, ppm): 4.40(m, 1H); 4.0(m, 2H); 3.18(t, 2H); 3.02(t, 4H); 1.30-1.90(m, 18H); 1.41(d, 36H) and negative HR-ESI MS for $C_{38}H_{70}N_6O_{12}Na^+$ Calcd: 825.494 Found: 825.496.

(N_1,N_2-Bis-L-lysyl)-L-lysine, 3. A solution made from 0.126 g (0.157 mmol) of **2**, 2.5 mL of chloroform and 2.0 mL of CF_3COOH (TFA) was stirred at room temperature for 4 h. Removal of solvent under reduced pressure at 40°C and drying (0.5 Torr, 23°C, 0.5 h), followed by dissolution in 5 mL of MeOH, removal of solvent under reduced pressure and drying (0.5 Torr, 23°C, 1 h) gave 0.063 g (100%) of **3** having ^{1}H NMR (CD_3OD, ppm): 4.40(m, 1H); 3.95(t, 1H); 3.82(t, 1H); 3.22(m, 2H); 1.42-1.90 (m, 18H).

Bis-(N_1N_2-dicholoyl-L-lysyl)-L-lysine, 4. To a stirred solution made from 0.258 g (0.632 mmol) of cholic acid and 6.0 mL of anhydrous DMF was added 84 mg (0.651

mmol) of N, N-diisopropyl-N-ethylamine, followed by addition of 0.228 g (0.635 mmol) of O-(3.4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)-N,N,N'N'-tetramethyluronium tetrafluoroborate) (DBTU). After stirring at room temperature for 2 h, a solution made from 0.0628 g (0.157 mmol) of **3**, 2 mL of anhydrous DMF and 0.200 mL (1.15 mmol) of N, N-diisopropyl-N-ethylamine was then added. After additional stirring at room temperature for 18 h, the solvent was then removed under reduced pressure at 60°C and the crude product triturated three times with 20 mL of H_2O. The residue was then freeze dried and purified by column chromatography [SiO_2, chloroform/methanol/water/28%aqueous NH_3, 65/25/4/1 (v/v/v/v)] affording 0.241 g (77%) of **4**. This compound was further purified by HPLC using an HPLC gradient that ranged from *A*/*B* 30/70 to 0/100, where *A* was composed of H_2O/EtOH/acetonitrile, 18/2/1 (v/v/v), and *B* was composed of H_2O/EtOH/acetonitrile, 1/24/2 (v/v/v). This purified product showed ^1H NMR (CD_3OD, ppm): 4.35(m, 3H); 3.92(s, 4H); 3.72 (s, 4H); 3.38(m, 4H); 3.12(m,6H); 0.87-2.35(m, 138H); 0.69(s, 12H) and negative HR-ESI MS for $C_{114}H_{190}N_6O_{20}$ ($[Na]^-$) Calcd: 1986.393. Found: 1986.397.

N_1,N_3-Bis-[bis-(N_1N_2-dicholoyl-L-lysyl)-L-lysyl)-N_2-trifluoroacetospermidine triamide. To a stirred solution made from 0.246 g (0.125 mmol) of **4** and 1.70 mL of anhydrous DMF, was added 0.022 mL (0.128 mmol) of N, N-diisopropyl-N-ethylamine, followed by addition of 0.050 g (0.143mmol) of O-(3.4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate. After stirring this mixture at room temperature for 2 h, a solution that was made from 0.0149 g (0.062 mmol) of spermidine-N_2-trifluoroacetamide, 1 mL of anhydrous DMF and 0.080 mL of

N,N-diisopropyl-N-ethylamine was directly added. Additonal stirring of the reaction mixture at room temperature for 18 h, followed by removal of solvent under reduced pressure at 60°C afforded a crude product, which was isolated by precipitation into 40 mL 1% aqueous sodium bicarbonate. This precipitate was triturated three times with 20 mL of H_2O, freeze dried, and purified by preparative thin layer chromatography [SiO_2, chloroform/methanol/water, 75/20/3 (v/v/v)] to give 0.170 g (72%) of N_1,N_3-bis-[bis-(N_1N_2-dicholoyl-L-lysyl)-L-lysyl)-N_2-trifluoroacetospermidine triamide having ^{1}H NMR (CD_3OD, ppm): 4.25(m, 6H); 3.95(s, 8H); 3.41(m, 16H); 3.15(m, 12H); 0.9-2.45(m, 282H); 0.70(s, 24H) and negative HR-ESI MS for $C_{237}H_{394}N_{15}O_{39}F_3([Na_2]^{++})$ Calcd: 2088.952 Found: 2088.948

N_1,N_3-Bis-[bis-(N_1N_2-dicholoyl-L-lysyl)-L-lysyl)-spermidinediamide. A solution made from 170 mg (0.0411 mmol) of N_1,N_3-bis-[bis-(N_1N_2-dicholoyl-L-lysyl)-L-lysyl)-N_2-trifluoroacetospermidine triamide, 3.0 mL of CH_3OH and 0.06 mL of 1 N aqueous NaOH, was stirred at 60ºC for 48 h. The solution was then cooled to room temperature and acidified by addition of 0.120 mL of 1 N aqueous HCl. Removal of solvent under reduced pressure, followed by column chromatographic purification of the residue [SiO_2, chloroform/methanol/28% aqueous NH_3/, 75/20/3 (v/v/v)] afforded 0.103 mg (62%) of N_1,N_3-bis-[bis-(N_1N_2-dicholoyl-L-lysyl)-L-lysyl)-spermidinediamide having ^{1}H NMR (CD_3OD, ppm): 4.24(m, 6H); 3.95(s, 8H); 3.79(s, 8H); 3.37(m, 8H); 3.18(m, 16H); 2.80(m, 4H); 0.9-2.32(m, 282H); 0.70(s, 24H) and negative HR-ESI MS for $C_{235}H_{396}N_{15}O_{38}$ ([H^+Na^+] Calcd:2029.970. Found: 2029.967.

N_1,N_3-Bis-[bis-($N_1$$N_2$-dicholoyl-L-lysyl)-L-lysyl)-N_2-[3-(2-dithiopyridyl) propionyl] spermidinetriamide, 1. To a solution made from 103 mg (0.0255 mmol) N_1,N_3-bis-[bis-($N_1$$N_2$-dicholoyl-L-lysyl)-L-lysyl)-spermidinediamide, 1.0 mL of anhydrous DMF and 0.015 mL of N,N-diisopropyl-N-ethylamine, was added 0.0150 g (0.0417mmol) of *N*-[O-1,2,3-benzotriazin-4(3H)one-yl]-3,(2-pyridyldithio)propionate (BPDP).[2] After stirring for 24 h at room temperature, the product mixture was concentrated under reduced pressure at 35°C. The resulting oil was washed, sequentially, with 20 mL of 1% aqueous $NaHCO_3$ and 2 x 20 mL of H_2O. The resulting solid was purified by preparative thin layer chromatography [SiO_2, chloroform/methanol/water/, 75/20/3 (v/v/v)], to give 0.049 g (45%) of **1** having R_f=0.67 and ^{1}H NMR(CD_3OD, ppm). 8.4(d, 1H); 7.85(m, 2H); 7.25(d, 1H); 4.35(m, 6H); 7.95(s, 8H); 3.72(s, 8H); 3.38(m, 12H); 3.12(m, 16H); 3.05(t, 2H); 2.75(m, 2H); 0.85-2.43(m, 282H); 0.70(s, 24H) and negative HR-ESI MS for $C_{243}H_{402}N_{16}O_{39?}$ S_2([Na][H^{++}) Calcd: 2128.469. Found: 2128.466. This compound was further purified by gradient HPLC using H_2O/EtOH/acetonitrile *A*: 900/100/5 and *B*: 10/240/20, v/v/v, starting at *A*/*B*=50/50 to 0/100 and flow rate 2.5ml/min.

Coupling of Molecular Umbrella with siRNA. In a typical coupling reaction, 24.5 mg of a disulfide-protected siRNA passenger strand (MW 6229.17) was dissolved in 0.55 mL of PBS (0.01M phosphate buffer, 140mM NaCl, 1mM EDTA, pH 7.4). To generation the thiol form of the siRNA, 0.250 mL of water was added to this solution followed by 0.500 mL of a freshly prepared 1 M solution of dithiothreitol (DTT). After this mixture was stirred under argon for 24 h at room temperature, the excess DTT and its

oxidzed form were removed by extracting (13 times) using 2 mL of ethyl acetate. The aqueous solution was then purged with argon to remove traces of ethyl acetate and analyzed for thiol content using a standard Ellman assay.

To this solution of the aqeuous solution was then added a solution made from 15.3 mg (3.623 μmol) of **3** and 2.25 mL of methanol, followed by addition of 4μL of 0.5 M aqueous NaOH. The mixture was then stirred under argon atmosphere for 48 h at room temerature. The resulting conjugate was purified by direct injections of the product mixture (150 μL injection volumes) onto a reverse phase semi preparative column (Nova-Pak, 7.8 × 300 mm), which was maintained at 37ºC. Elution was made using a flow rate of 2.500 mL/min and an HPLC gradient that ranged from *A*/*B*=50/50 to 0/100, where *A* was 0.1 M NH_4Oac (pH 6.9)/ethanol/acetonitrile, 900/100/5 (v/v/v), and *B* was 0.1 M NH_4Oac (pH 6.9)/ethanol/acetonitrile, 10/240/20 (v/v/v). Fractions that were collected with retention times between 6.5 and 8.2 min were combined and concentrated under reduced pressure at 40°C. The resulting product was dissolved in 3.5 mL of water and dialyzed for 4 h at room temperature against 500 mL of water using a dialysis bag equipped with a Spectra/Por membrane, MWCO 1000 Da. The aqueous solution was then lyophilized to give 16.4 mg of the molecular umbrella-siRNA single strand conjugate, (43% based on umbrella **1**) as a white powder having ^1H NMR (CD_3OD/D_2O, 1/1, 37ºC, ppm): 7.90-8.40(m, 30H); 5.70-6.3(m, 27H); 4.9-5.0(m,); 3.92(s, 8H); 3.79(s, 8H); 3.3-3.6(m, 39H); 3.15(m, 16H); 2.55-2.90 (m, 6H); 0.70-2.35(m, 314H); 0.63(s, 24H). See attached data for capillary electrophoresis data. LCMS for single strand-umbrella used to prepare **1b** 10424.7 (calculated), 10433.0 (found); LCMS for single strand-umbrella used to prepare **5**: 10430.7 (calculated), 10440.0 (found).

siRNA sequences: The siRNA sequence is designed to target Sjogren syndrome antigen B (NM_031119) and is conserved across human, mouse and rat species.

- Starting SSB291 Passenger Strand Sequence to Prepare Cmpd 1b:

[omeA][omeC][rA][rA][omeC][omeU][rG][rA][omeC][omeU][omeU][omeU][rA][rA][omeU][rG][omeU][rA][rA][C3SSC3OH]

- SSB291 Guide Strand Sequence to Prepare Duplex Cmpd 1b:

[p][fluU][fluU]A[fluC]A[fluU][fluU]AAAG[fluU][fluC][fluU]G[fluU][fluU]G[fluUs][rUs]U

- Starting non-target Passenger Strand Sequence to Prepare Cmpd 5:

[omeU][omeG]G[omeU][omeU]AA[omeC]A[omeU][omeC][omeU][omeC]GA[omeC][omeU]AA[C3SH]

- Non-target Guide Strand Sequence to Prepare Duplex Cmpd 5:

[p][fluU][fluU]AG[fluU][fluC]GAGA[fluU]G[fluU]AAA[fluC][fluC][rAs][rUs]U

Abbreviations: p- phosphate; flu-2'Fluoro; ome-2'O-CH_3,r- ribose; s- Phosphorothioate linkage; C3SH = CH2CH2CH2SH

General Duplexing Protocol:[3] A solution of 16.91 mg (1.621 umol) of umbrella – siRNA passenger strand was dissolved in 8.5 ml of deionized water(2 mg/ml). A solution of 11.04 mg (1.621 umol) the corresposnding SSB291 guide strand was dissolved in 5.5 ml of deionized water (2 mg/ml). Each solution was analyzed by capillary electrophoresis, and the optimal ratio for duplexing determined to be 6.4 PS : 3.3 GS. A solution of 7.95 mls of PS solution and 4.13 mls of GS solution was mixed, and the resulting solution heated at 90°C for 1 minute. The solution was cooled, and was diluted with PBS and was centrifugally dialyzed 3x against PBS and 1x against deionized water

using 5K dialysis membranes.The solution was lyophilized overnight to give 19 mg of the desired duplex product **1b** as a tacky white amorphous solid.

In an identical manner to that described above, the scrambled PS and the GS was prepared 16 mg of **6**, recovered as a tacky white amorphous solid after lyophilization.

In vitro activity of umbrella-siRNA conjugates. To test the silencing activity of siRNA conjugates, HEK293T cells were plated in media (DMEM) supplemented with 10% fetal calf serum (FCS) and allowed to culture overnight (37°C, 5%CO2). On next day, the media was replaced with serum free media containing the siRNA conjugates at concentrations ranging from 10-0.0015 μM and left on cells for total of 72 hrs (37°C, 5%CO2). The SSB mRNA levels were analyzed using branched-DNA assay as per instructions by supplier (Panomics Quantigene 1.0 bDNA Kit # QG0002). The cell viability was assessed using MTS assay (Promega cat# TB245) and all the data was normalized to levels from untreated cells.

To test the intrinsic potency of siRNA conjugates by transfection with lipofectamine, HEK293T cells stably transfected with luciferase vector were plated in media (DMEM) supplemented with 10% fetal calf serum (FCS) and allowed to culture overnight (37°C, 5%CO2). This reporter cell system contains a Renilla-Firefly Dual-luciferase construct and has the SSB target sites incorporated in the 3' UTR of Renilla luciferase. Next day, the media was replaced with serum free media containing the siRNA conjugates at concentrations ranging from 10000-0.1 pM and left on cells for total of 24 hr (37°C, 5%CO2). The Firefly and Renilla protein levels were analyzed using the Dual-Glo assay from Promega as per instructions by supplier (Promega Cat#E2920). The Firefly signal

was used as a control for cell viability & Renilla/Firefly luciferase activity was used for specific mRNA knockdown. All data was normalized to levels from untreated cells.

In vivo testing of conjugates in rat eye. All procedures involving animals were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee (IACUC) of Merck Research Laboratories, West Point, PA. Male Brown Norway rats (6-8 weeks) were purchased from Charles River Laboratories. siRNAs were prepared aseptically to minimize the risk of infection. For intravitreal dosing, rats were anesthetized with ketamine/xylazine (40-90/5-10 mg/kg, IM), and 1% proparacaine hydrochloride (1-2 drops) was applied to the eye as topical anesthetic. For intravitreal injection, a pair of clean forceps was used to gently proctose and hold in place the eye, and a 30G sharp-needled syringe was used to inject 5ul of test siRNA or control vehicle into the vitreous just posterior to the limbus. On the day of sacrifice, rats were euthanized with sodium pentobarbital (150-200 mg/kg, IP). Following enucleation, vitreous, retina, and RPE/choroid were dissected and frozen. The retinal tissue was homogenized in RLT lysis buffer (Quaigen catalog # 79216) and total RNA was purified using the Qiagen Rneasy 96 kit (catalog # 74181). The total RNA was then analyzed by quantitative PCR to determine relative mRNA levels of target gene SSB with housekeeping genes like GAPDH and PPIB.

Computer Modeling. To better understand the intra-molecular interactions, classical molecular dynamics (MD) simulations were performed. The AMBER force field was selected for approximating the atomic interactions.[4] This force field is particularly

appropriate for modeling interactions between nucleic acids and small molecules especially where covalent bonds are involved.[5] For this hybrid system we also employed the AM1 bond charge corrected version for which we have extensive experience and confidence.[6] The AMBER interatomic force fields are constructed from harmonic potentials for bonds, 3-body angles and torsion angles. Non-bonded interactions are described through standard Lennard-Jones and Coulombic pair potentials. The previous published parameters were fitted by matching high-level quantum mechanical calculations results on a variety of molecules.[4] The objective in determining the values of the adjustable parameters in the force field is to reproduce the correct bond lengths, bond angles, and conformational energies of the reference molecules. The equations of motion in the present simulations were integrated using the NAMD software package.[7] All simulations were performed at constant pressure with rectangular periodic boundary conditions. The MD simulation cell was constructed with TIP3 water using LEaP within AMBER 9.[8, 9] Initially a 15/ border between the large molecule and the closest point of the MD cell was created. Sodium chloride (NaCl) was added at a physiological concentration of 150mM. In addition 41 sodium ions (Na+) were added to ensure charge neutrality. LEaP was instrumental in the creation of the necessary potential files and initial coordinates of the entire solvated umbrella-siRNA conjugate. The initial conformation of the ds nucleic acids was placed in the A-form while the umbrella assumed a more extended form to minimize any preference for the intra-molecular umbrella-siRNA interaction. This initial configuration of umbrella-siRNA conjugate, explicit water and ions was minimized before slowing heating to ~310K. After reaching

constant pressure equilibrium, a 40ns MD simulation was performed using a 0.001ps time step.

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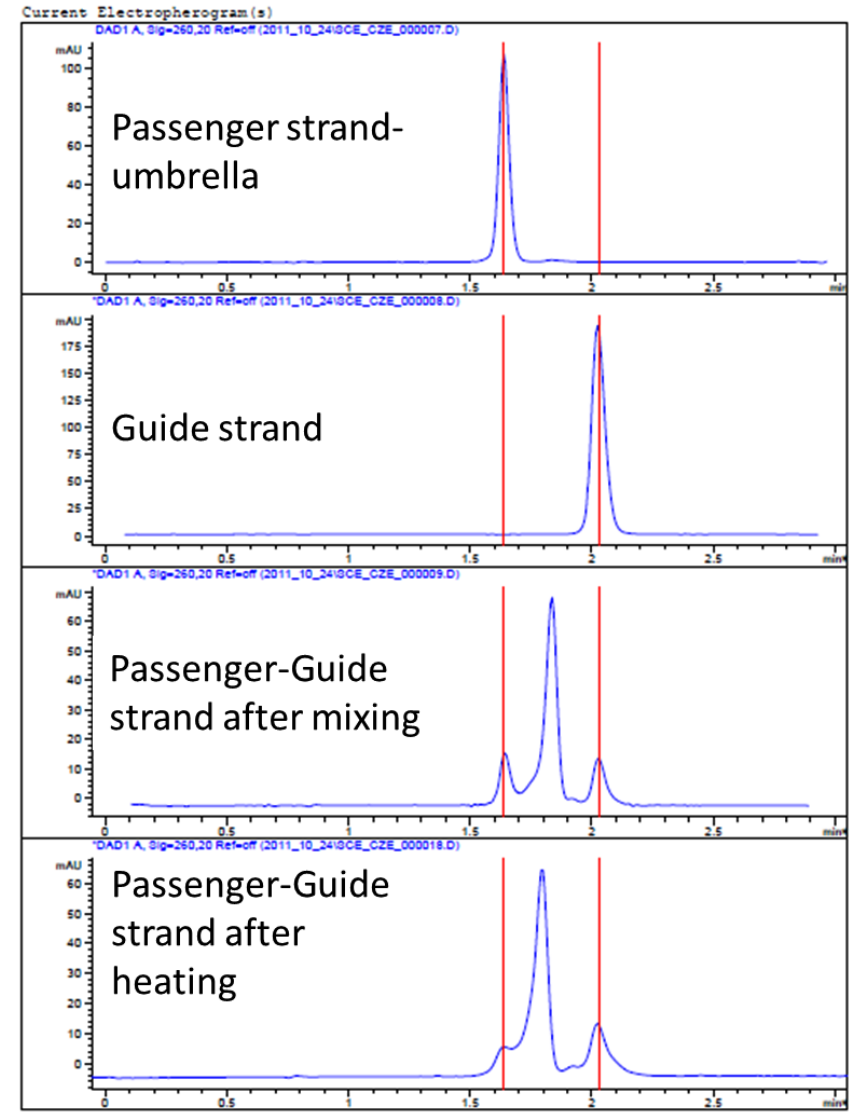
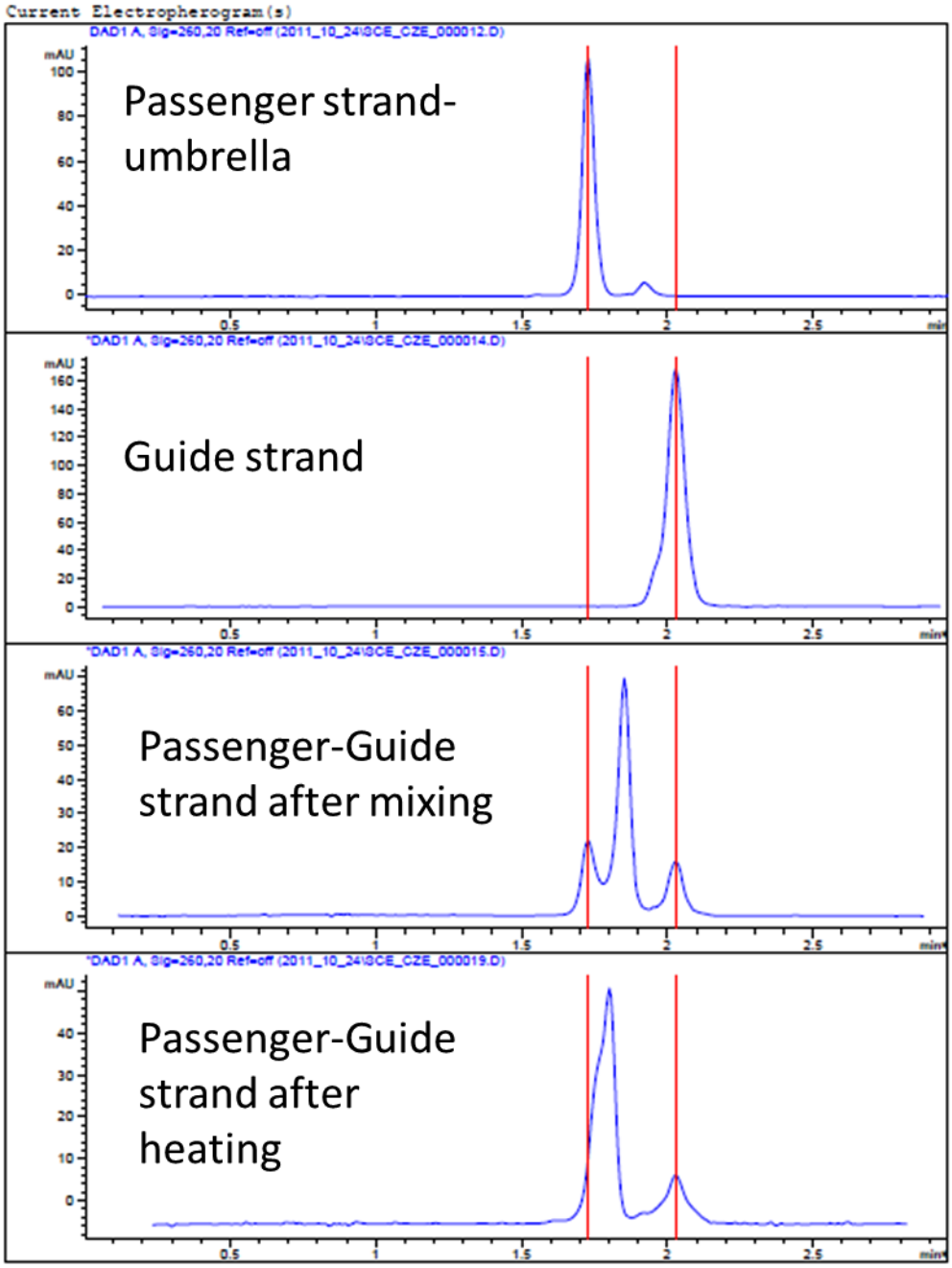
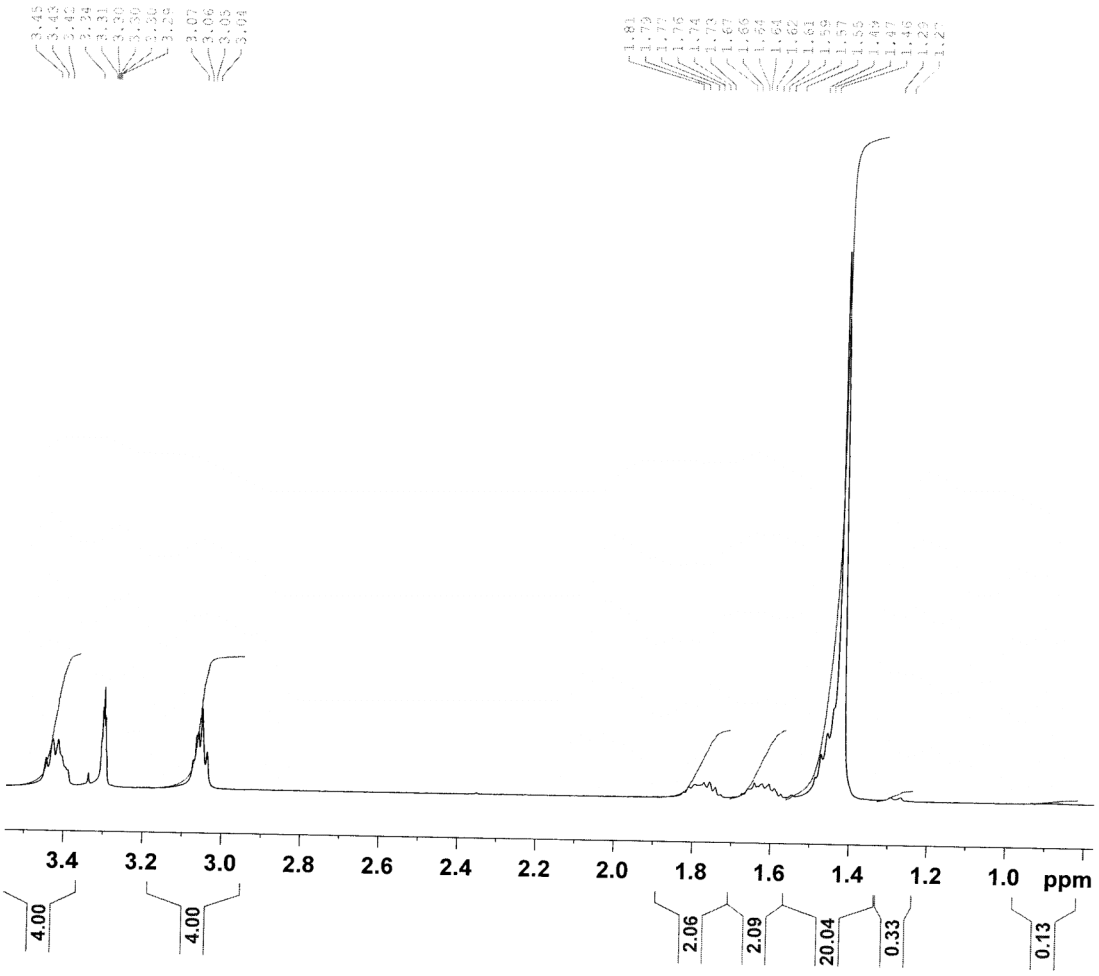
Cmpd 1b Capillary Electrophoresis

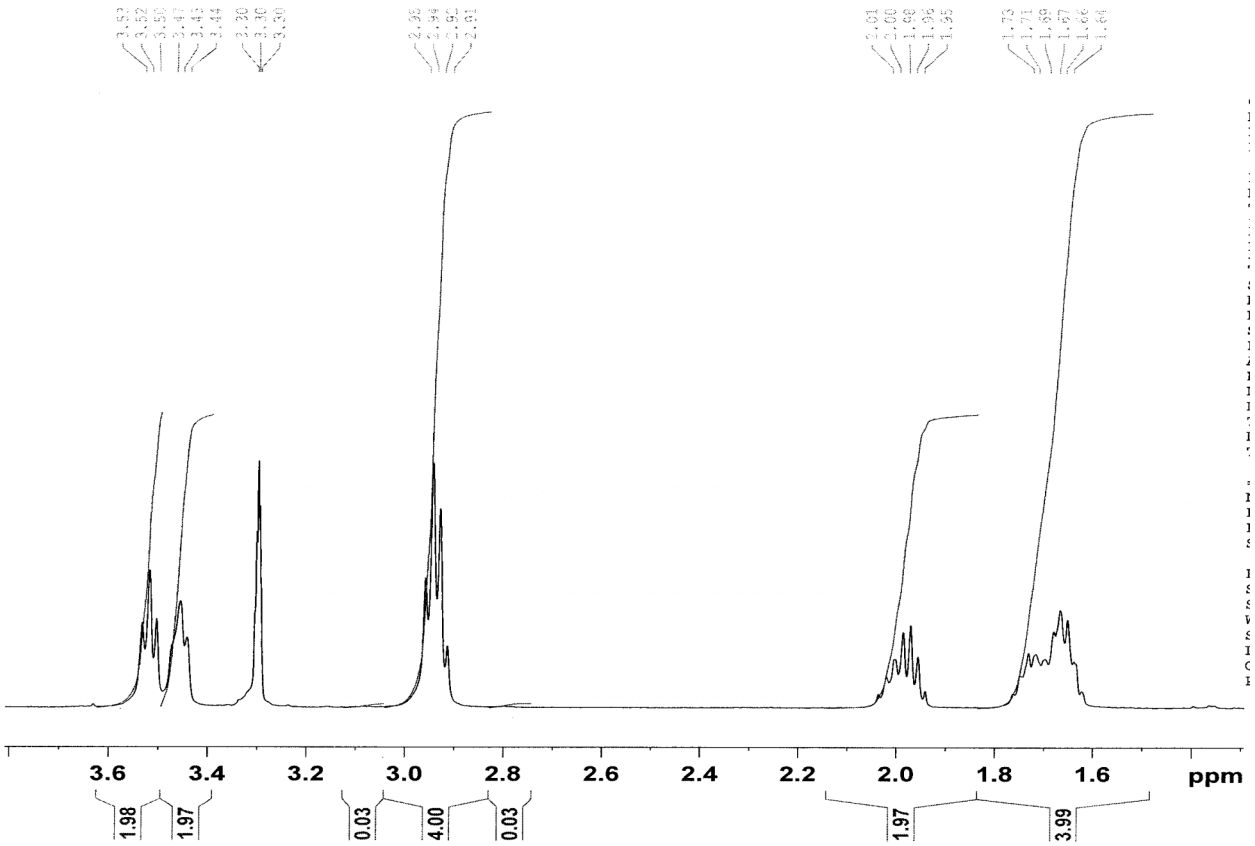


Cmpd 5 Capillary Electrophoresis





^{1}H NMR Spectrum of N_1N_3-diBoc-spermidine-N2-trifluoracetamide



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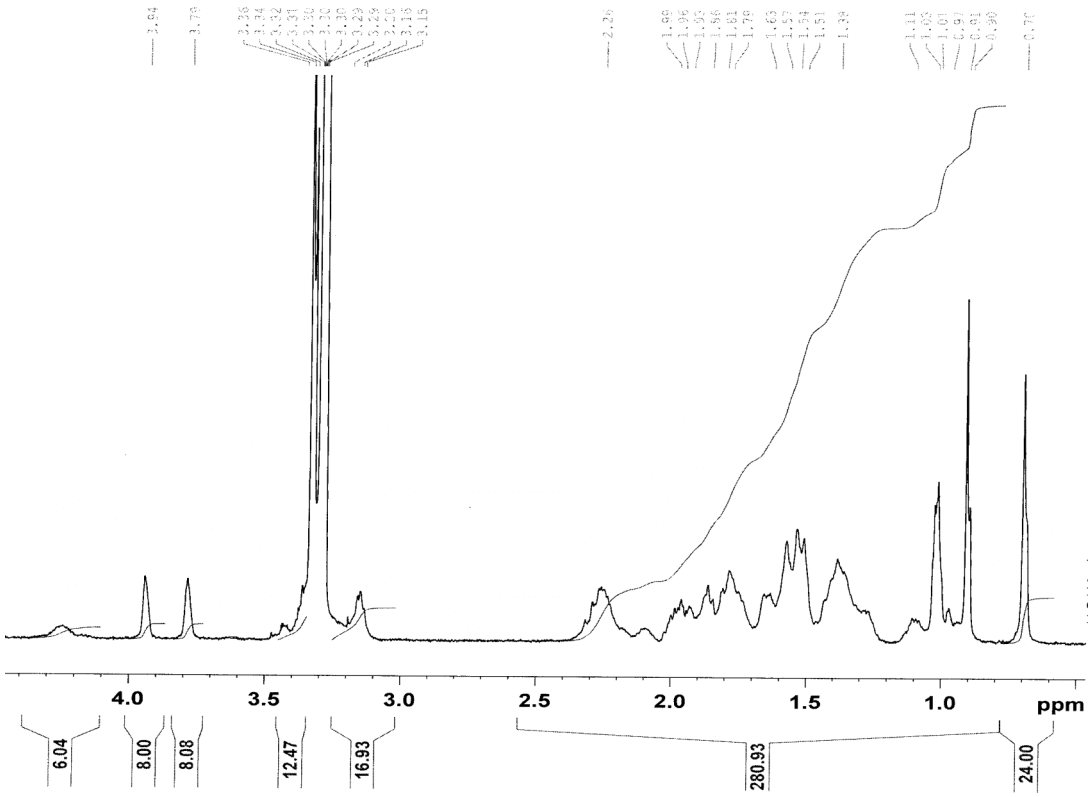

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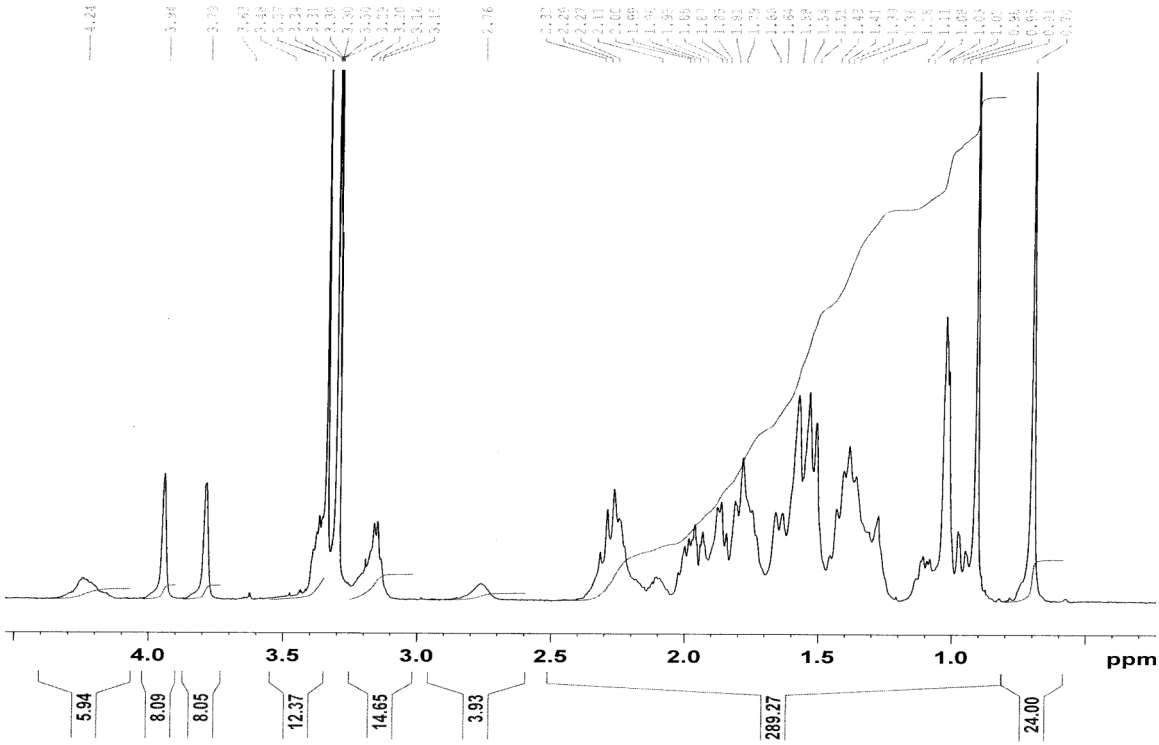

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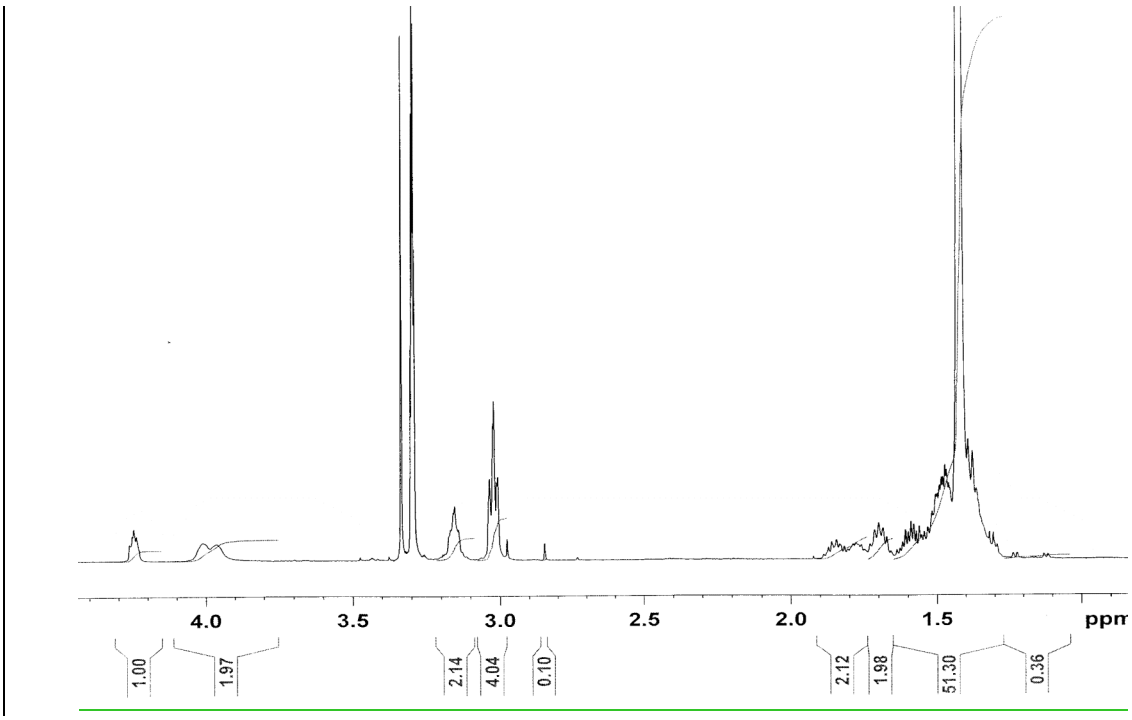

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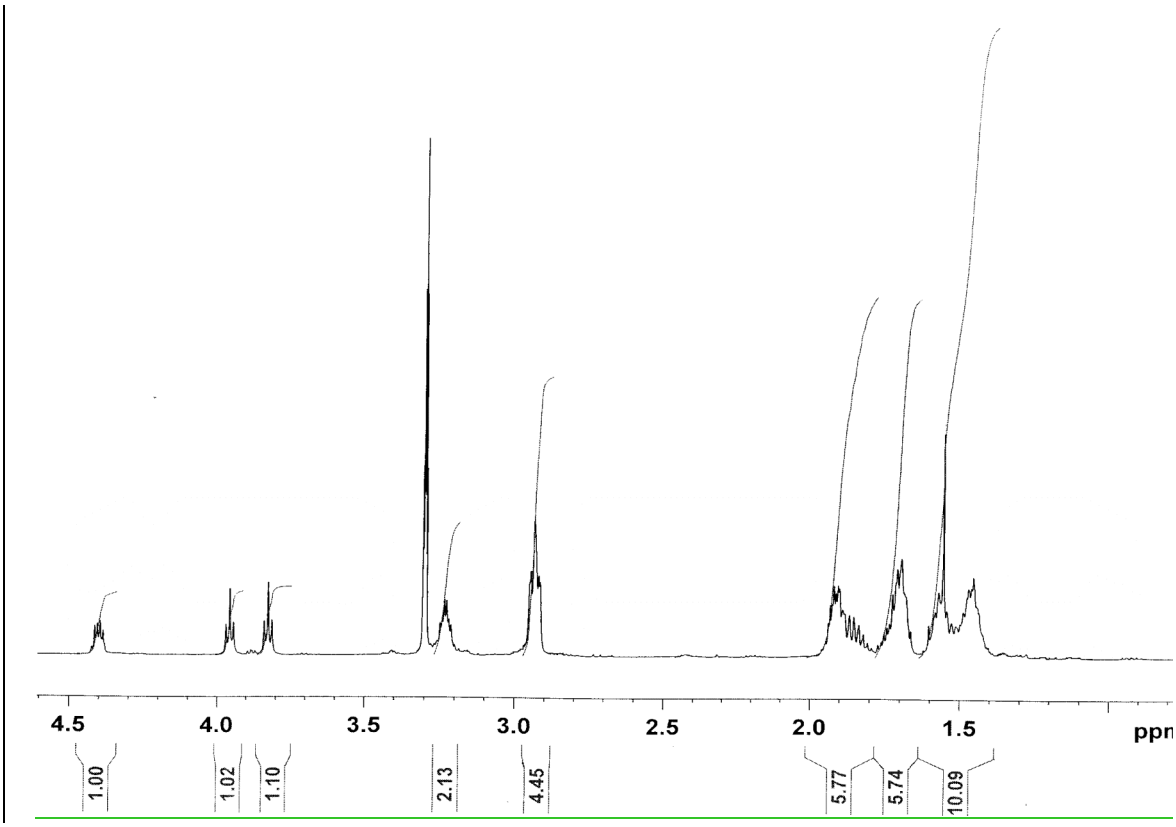

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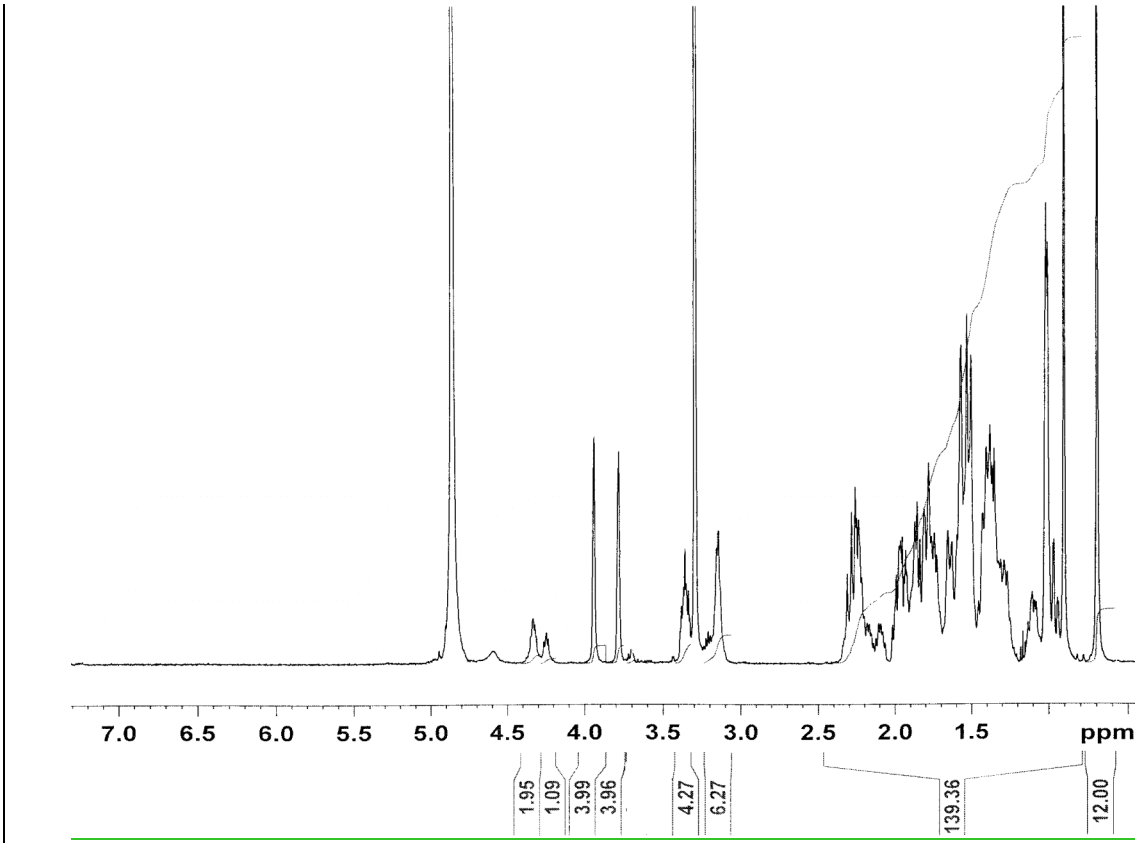

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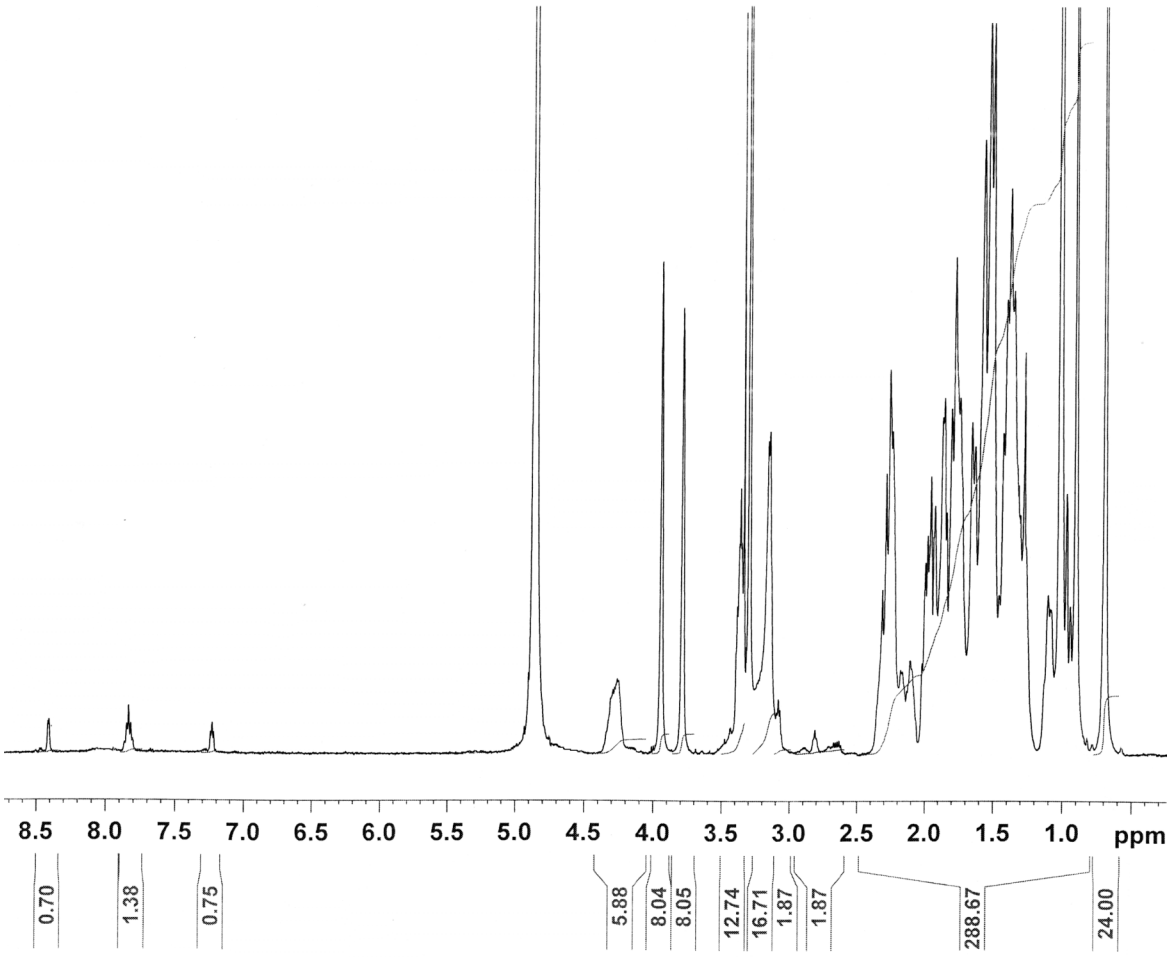
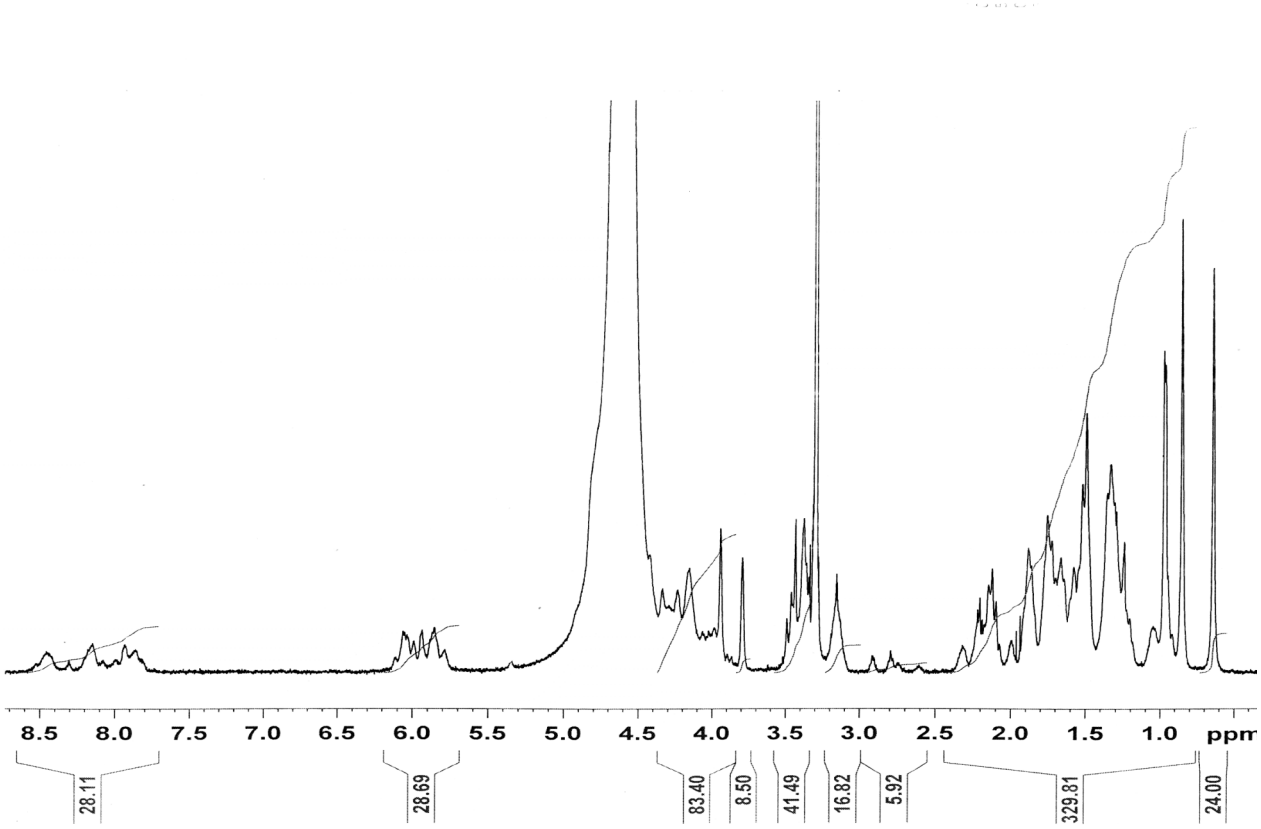


^{1}H NMR Spectrum of 1



^{1}H NMR Spectrum of 1a

Tolerability of compounds assessed by ophthalmic examination (n=8 rat eyes)

	Iris resistance to dilation incidence	Other anterior segment findings	Lens opacity incidence (size, grade)	Vitreous haze/opacity incidence (grade)	Vitreous cells (grade)	Vitreous hemorrhage incidence	Retinal vascular dilation/tortuosity incidence(grade)	Retinal edema/detachment (size, grade)
vehicle	-	-	-	-	-	-	-	-
1b	2/8 (vslt-slt)	-	-	5/8 (vslt)	2/8 (slt)			
5	-	-	1/8 (posterior capsule)	8/8 (vslt)	-			

vslt = very slight
slt = slight
= no findings

Computer Modeling: Selected Images of Conjugate 1b.