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Supporting Information

Protection by Conformationally Restricted Mobility. First Solid-Phase Synthesis of Triostin

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ABBREVIATIONS

Abbreviations used for amino acids and the designations of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* **1982**, *247*, 977–983. The following additional abbreviations are used: ACN, acetonitrile; Alloc, allyloxycarbonyl; Boc, *t*-butyloxycarbonyl; CTC, chlorotrityl chloride (Barlos) resin; DIEA, *N*,*N*-diisopropylethylamine; DIPCDI, *N*,*N*′-diisopropylcarbodiimide; DKP, diketopiperazine; DMAP, 4-(*N*,*N*-dimethylamino)pyridine; DMF, *N*,*N*-dimethylformamide; EDC·HCl, 1-[3-(dimethylaminopropyl]-3-ethylcarbodiimide; ESMS, electrospray mass spectrometry; HATU, 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo-[4,5-*b*]pyridinium hexafluorophosphate 3-oxide; HOAt, 1-hydroxy-7-azabenzotriazole(3-hydroxy-3*H*-1,2,3-triazolo-[4,5-*b*]pyridine); HPLC, high performance liquid chromatography; MS, mass spectrometry; PyBOP, benzotriazol-1-yl-oxytris (pyrrolidino) phosphonium hexafluorophosphate; TFA, trifluoroacetic acid; TIS, triisopropylsilane; Trt, trityl. Amino acid symbols denote L-configuration unless indicated otherwise. All reported solvent ratios are expressed as v/v, unless otherwise stated.

GENERAL

General methods: Protected amino acid derivatives, PyBOP, were obtained from Applied Biosystems (Framingham, MA), Bachem (Bubendorf, Switzerland), Albatross (Montreal, Canada), and NovaBiochem (Läufelfingen, Switzerland). Wang resin was obtained from Novabiochem (Läufelfingen, Switzerland). DIEA, DIPCDI, piperidine, TFA, iodomethane, and allyl chloroformate, and 2-quinoxalinecarboxylic acid were obtained from Aldrich (Milwaukee, WI), and EDC·HCl and HOAt

were from Luxembourg Industries (Tel Aviv, Israel). DMF, CH_2Cl_2, Acetonitrile (HPLC grade), methanol (HPLC grade), Dioxane, Et_2O, TBME (*t*-butyl methyl ether) and EtOAc (ethyl acetate) were obtained from SDS (Peypin, France). Trifluoromethanesulfonic acid and *N*-hidroxyacetamide methyl were obtained from Fluka (Buchs, Switzerland). All commercial reagents and solvents were used as received with the exception of DMF and CH_2Cl_2 , which were bubbled with nitrogen to remove volatile contaminants (DMF) and stored over activated 4 Å molecular sieves (Merck, Darmstadt, Germany), and THF which was distilled from sodium/benzophenone.

Solution reactions were performed in round-bottomed flasks. Organic solvent extracts were dried over anhydrous $MgSO_4$, followed by solvent removal under reduced pressure at temperatures below 40 °C.

Solid-phase syntheses were performed in polypropylene syringes (2, 5 mL) fitted with a polyethylene porous disc. Solvents and soluble reagents were removed by suction. Removal of the Fmoc group was carried out with piperidine–DMF (1:4, v/v) (1 × 1 min, 2 × 5 min). Washings between deprotection, coupling, and final deprotection steps were carried out with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min) using 5 mL solvent.g^{-1} resin for each wash. Peptide synthesis transformations and washes were performed at 25 °C.

HPLC columns (Symmetry® C18 reversed-phase analytical column, 5.0 μm × 4.6 mm × 150 mm and Symmetry® C18 reversed-phase semi-preparative column, 5.0 μm × 7.8 mm × 100 mm) were obtained from Waters (Ireland). Analytical HPLC was carried out on a Waters instrument comprising a separation module (Waters 2695), automatic injector, photodiode array detector (Waters 996), and system controller

(Millenium[32] login). UV detection was at 220 and 254 nm, and linear gradients of CH_3CN (+0.036% TFA) into H_2O (+0.045% TFA), were run at 1.0 mL·min^{-1} flow rate over 15 min. Semi-preparative HPLC was carried out on a Waters instrument comprising a separation module (Waters 1525 binary pump), automatic injector, and a dual absorbance detector (Waters 2487). UV detection was at 220 and 254 nm, and linear gradients of CH_3CN (+0.036% TFA) into H_2O (+0.045% TFA), were run at 3.0 mL·min^{-1} flow rate in the conditions specified for each case.

MALDI-TOF and ES(+)-MS analyses of peptide samples were performed on an Applied Biosystems VoyagerDE RP, using using ACH matrix, and in a Waters Micromass ZQ spectrometer and in an Agilent Ion Trap 1100 Series LC/MSDTrap. ^{1}H NMR and ^{13}C NMR spectroscopy was performed on a Varian Mercury 400 and a Bruker Digital Avance 600. Chemical shifts (δ) are expressed in parts per million downfield from tetramethylsilyl chloride. Coupling constants are expressed in Hertz. Homonuclear and heteronuclear NMR experiments were carried out on a Bruker Digital Avance 600 spectrometer at 298 °K. TOCSY experiment was recorded with a dipsi2ph mixing sequence of 70 ms duration, 16 scans, 2048 data points in t_2 and 512 data points in t_1. 2D NOESY experiment was recorded with a mixing time of 500 ms, 2048 data points in t_2 and 1024 data points in t_1, and 40 scans. HSQC experiment was recorded with 2048 data points in t_2 and 512 data points in t_1, and 64 scans. HMBC experiment was recorded with 2048 data points in t_2 and 317 data points in t_1, 80 scans, and a spectral width of 189.861 ppm in t_1 and 11.97 ppm in t_2.

Experimental procedures for *N*Me amino acids

Alloc-*N*Me-Val-OH. Alloc-*N*Me-Val-OH was synthesized from Boc-*N*Me-Val-OH by using TFA–CH_2Cl_2 (1:1) to remove the Boc group and allyl chloroformate in dioxane to introduce the Alloc group, using standard procedures (60% yield, 90% purity). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.95 (m, 1H, CH Alloc), 5.31 (dd, 1H, CHH' Alloc, J = 17.2 Hz), 5.22 (dd, 1H, CHH' Alloc, J = 10.4 Hz), 4.64 (dt, 2H, CH_2 Alloc, J = 5.6 Hz, J = 1.6 Hz), 4.29 (dd, 1H, CH^{α}, J = 10.4 Hz), 2.95 (broad s, 3H, *N*Me), 2.26 (m, 1H, CH^{β}), 1.05 (d, 3H, CH_3, J = 6.4 Hz), 0.94 (d, 3H, CH_3, J = 6.8 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 174.3, 157.5, 132.8, 117.9, 66.9, 65.5, 31.7, 27.7, 19.9, 19.3. HPLC-ESMS: *m/z* calcd for $C_{10}H_{17}NO_4$, 215.12; found, 215.04 $[M + H]^+$.

Boc-*N*Me-Cys(Acm)-OH. This amino acid was prepared from Boc-Cys(Trt)-OH using reported procedures (E. Marcucci, N. Bayó-Puxan, J. Tulla-Puche, F. Albericio. *J. Comb. Chem.* **2008**, *10*, 69-78).

Experimental procedures for Triostin A

Qcx-D-Ser(Trt)-Ala-OH

Wang resin (200 mg, 0.93 mmol/g) was placed in a 3 mL-polypropylene syringe fitted with a polyethylene filter disc. The resin was then washed with DMF (3 × 1 min) and CH_2Cl_2 (3 × 1 min). Fmoc-Ala-OH (420 mg, 2.0 mmol) was dissolved in CH_2Cl_2–DMF (9:1) and DIPCDI (310 μL, 2.0 mmol) was added. The mixture was then added to the resin and DMAP (24 mg, 0.2 mmol) in DMF (0.5 mL) was finally added and the mixture was reacted for 14 h at 25 °C. The Fmoc-Ala-O-Wang resin was subjected to the following washings/treatments: filtration, CH_2Cl_2 (3 × 1 min), DMF (3 × 1 min), piperidine–DMF (1:4) (1 × 1 min, 2 × 5 min). Next, Fmoc-D-Ser(Trt)-OH (0.24 g, 0.42 mmol) was introduced with HATU (0.16

g, 0.42 mmol) and HOAt (57 mg, 0.42 mmol) as coupling reagent in DMF in the presence of DIEA (0.15 mL, 0.84 mmol) to the peptide resin. The mixture was stirred for 1 h and, after filtration Kaiser test indicated the completion of the coupling reaction. The peptide resin was then washed with DMF (3 × 1 min), CH_2Cl_2 (3 × 1 min), and treated with piperidine-DMF (1:4) (1 × 1 min, 2 × 5 min) to remove the Fmoc group. In order to introduce the heterocyclic moiety, the resin was treated with 2-quinoxaline carboxylic acid (49 mg, 0.28 mmol) and DIEA (100 μL, 0.56 mmol) in CH_2Cl_2 for 45 min. An aliquot of the resin was cleaved [TFA–H_2O–CH_2Cl_2 (25:5:70) (0.5 mL, 1 × 30 min)] and analyzed by HPLC-ESMS: *m/z* calcd for $C_{15}H_{16}N_4O_5$ 332.11, found 332.14 $[M + H]^+$.

{[Qcx–D-Ser(&)–Ala–OH][Alloc-*N*Me-Val)(&)]}

Removal of the trityl group was accomplished by washings with TFA–TIS–CH_2Cl_2 (2:2.5:95.5) until a colourless filtrate was obtained. The peptide-resin was then washed with CH_2Cl_2 (3 × 1 min) and Alloc-*N*Me-Val-OH (0.3 g, 1.4 mmol) was introduced by reaction with DIPCDI (0.22 mL, 1.4 mmol) and DMAP (17 mg, 0.14 mmol) in DMF–CH_2Cl_2 (1:9) for 14 h at 25 °C. An aliquot of the resin was cleaved [TFA–H_2O–CH_2Cl_2 (25:5:70) (0.5 mL, 1 × 30 min)] and analyzed by analytical HPLC (linear gradient from 0 to 100% ACN for 15 min, 1 mL/min): t_R = 10.97 min, 90.3% purity; and by HPLC-ESMS: *m/z* calcd for $C_{25}H_{31}N_5O_8$ 529.22; found, 529.33 $[M + H]^+$.

{[Qcx–D-Ser(&)–Ala–OH][Boc–*N*Me-Cys(Acm)-*N*Me-Val)(&)]}

Next, to remove the Alloc group, the peptide-resin was treated with $Pd(PPh_3)_4$ (16 mg, 14 μmol) and $PhSiH_3$ (0.17 mL, 1.4 mmol) dissolved in CH_2Cl_2 (3 × 15 min) under Ar, and was then washed with CH_2Cl_2 (3 × 1 min). The process was repeated three times and Boc-*N*Me-Cys(Acm)-OH (0.21 g, 0.7 mmol) was introduced with HATU (0.27 mg, 0.7 mmol), HOAt

(95.2 mg, 0.7 mmol), and DIEA (0.25 mL, 1.4 mmol) as coupling system for 1 h. The coupling was repeated once with using PyAOP (0.36 g, 0.7 mmol), HOAt (95.2 mg, 0.7 mmol), and DIEA (0.25 mL, 1.4 mmol). An aliquot of the resin was cleaved with TFA–H_2O–CH_2Cl_2 (25:5:70) (0.5 mL, 1 × 30 min). The compound was analyzed by analytical HPLC (linear gradient from 5 to 40% ACN for 30 min, 1 mL/min): t_R = 18.5 min, 85.4% purity; and by HPLC-ESMS: *m/z* calcd for $C_{33}H_{47}N_7O_{10}S$ 733.31; found, 633.38 $[M\text{-Boc} + H]^+$.

{[Qcx–D-Ser(&1)–Ala–*N*Me-Cys(&2)–*N*MeVal&3][Qcx–D-Ser(&3)–Ala–*N*Me-Cys(&2)–*N*Me-Val &1]}

Formation of the intermolecular disulfide bridge was obtained by treatment of the resin-bound tetrapeptide with I_2 (0.18 g, 0.7 mmol, 0.1 M) in DMF (2 × 10 min). The resin was repeatedly washed with CH_2Cl_2 (10 × 1 min), DMF (10 × 1 min), and CH_2Cl_2 (10 × 1 min). The dimerized peptide was then cleaved from the resin by treatment with TFA–H_2O–CH_2Cl_2 (25:5:70) (10 mL, 3 × 30 min), and the mixture evaporated with N_2, and lyophilized. An aliquot of the resin was cleaved with TFA–H_2O–CH_2Cl_2 (25:5:70) (0.5 mL, 1 × 30 min). The compound was analyzed by analytical HPLC (linear gradient from 0 to 100% ACN for 15 min, 1 mL/min): t_R = 7.35 min, 72.4% purity; and by HPLC-ESMS: *m/z* calcd for $C_{60}H_{82}N_{12}O_{18}S_2$ 1322.53; found, 1122.03 $[M - 2Boc's + H]^+$.

Trostin:{[Qcx–D-Ser(&1)–Ala–*N*Me-Cys(&2)–*N*Me-Val&3][Qcx–D-Ser(&3)–Ala–*N*Me-Cys(&2)–*N*Me-Val&1]}

The dimer (50.7 mg, 39 μmol) was dissolved in CH_2Cl_2 and added to a solution of HOAt (16.0 mg, 0.12 mmol) in DMF–CH_2Cl_2 (1:9), and the mixture was stirred for 30 min. Further addition of PyBOP (61.0 mg, 0.12 mmol) and DIEA (21 μL) to adjust to pH 8.0 allowed the cyclization

reaction to start, which was stirred for 4 h. Then, the solvent was evaporated and the crude bicycle was redissolved in CH_2Cl_2 (40 mL), and washed with 5% aqueous $NaHCO_3$ (3 × 50 mL), dried ($MgSO_4$), and evaporated to obtain 83 mg of the crude triostin. An aliquot was analyzed by analytical HPLC (linear gradient from 30 to 90% ACN for 15 min, 1 mL/min): t_R = 10.44 min, 51% purity. The two conformers were purified by semi-preparative reversed HPLC. Conditions: linear gradient (35% to 65%) of CH_3CN (0.036% TFA) into H_2O (0.045%) over 35 min, with a flow rate of 3.0 mL/min (t_R = 14.95 min & 16.79 min) to obtain 17.2 mg (17% overall yield) of the pure triostins. ^1H NMR ($CDCl_3$, 600 MHz): most polar conformer δ 9.65 (s, 1H, CH_{Ar}), 8.60 (d, 1H, *J* = 8.4 Hz, NH Ser), 8.20 (d, 1H, *J* = 7.8 Hz, CH_{Ar}), 8.15 (d, 1H, *J* = 7.8 Hz, CH_{Ar}), 7.88 (m, 2H, CH_{Ar}), 6.39 (d, 1H, NH Ala), 5.55 (t, 1H, *J* = 7.2 Hz, CH^{α} Cys), 5.27 (m, 1H, CH^{α} Ala), 4.94 (m, 1H, CH^{α} Ser), 4.50 (m, 1H, CH^{β} Ser), 4.28 (t, 1H, *J* = 10.8 Hz, CH^{β} Ser), 4.18 (d, 1H, *J* = 9.6 Hz, CH^{α} Val), 3.02 (m, 1H, CH^{β} Cys), 3.01 (s, 3H, *N*Me Cys), 2.95 (s, 3H, *N*Me Val), 2.84 (dd, 1H, *J* = 8.4 Hz, *J* = 13.2 Hz, CH^{β} Cys), 2.28 (m, 1H, CH^{β} Val), 1.44 (d, 3H, , *J* = 6.6 Hz, CH_3 Ala), 1.05 (d, 3H, *J* = 7.2 Hz, CH_3 Val), 0.97 (d, 3H, *J* = 6.6 Hz, CH_3 Val); least polar conformer δ 9.67 (s, 1H, CH_{Ar}), 8.61 (d, 1H, *J* = 8.4 Hz, CH_{Ar}), 8.20 (d, 1H, J = 7.8 Hz, CH_{Ar}), 8.15 (d, 1H, J = 7.8 Hz, CH_{Ar}), 7.89 (m, 2H, CH_{Ar}), 6.45 (d, 1H, *J* = 7.8 Hz, NH Ala), (t, 1H, *J* = 6.6 Hz, CH^{α} Cys), 5.28 (m, 1H, CH^{α} Ala), 4.98 (m, 1H, CH^{α} Ser), 4.53 (m, 1H, CH^{β} Ser), 4.26 (t, 1H, *J* = 10.2 Hz, CH^{β} Ser), 4.18 (d, 1H, *J* = 10.2 Hz, CH^{α} Val), 3.25 (dd, 1H, *J* = 6.6 Hz, *J* = 13.8 Hz, CH^{β} Cys), 3.03 (s, 3H, *N*Me Cys), 2.95 (s, 3H, *N*Me Val), 2.91 (m, 1H, CH^{β} Cys), 2.27 (m, 1H, CH^{β} Val), 1.41 (d, 3H, *J* = 6.6 Hz, CH_3 Ala), 1.05 (d, 3H, *J* = 6.6 Hz, CH_3 Val), 0.97 (d, 3H, *J* = 6.6 Hz, CH_3 Val). ^{13}C NMR ($CDCl_3$, 150 MHz) most polar conformer: δ 173.0 (CO Ala), 169.5 (CO Cys), 169.2 (CO Val), 168.9 (CO Ser), 163.3 (CO Qxc), 144.1 (C_{Ar}), 143.5 (CH_{Ar}), 142.5 (C_{Ar}), 140.4 (C_{Ar}), 132.1 (CH_{Ar}), 131.1 (CH_{Ar}), 129.8 (CH_{Ar}), 129.5 (CH_{Ar}), 63.9 (CH^{α} Val), 63.8 (CH_2^{β} Ser),

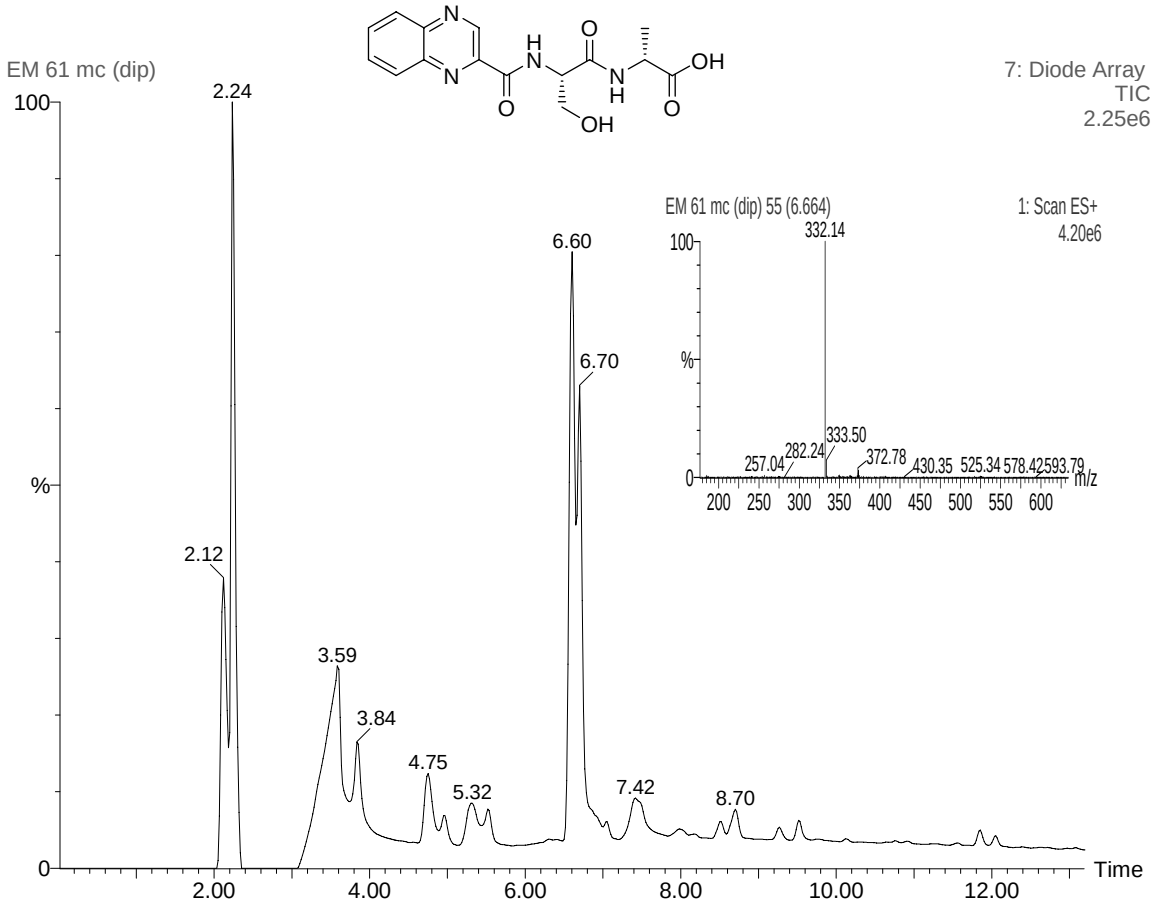
53.4 (CH^{α} Cys), 51.4 (CH^{α} Ser), 44.1 (CH^{α} Ala), 30.5 (NCH_3 Cys), 30.4 (NCH_3 Val), 28.9 (CH^{β} Val), 27.6 (CH_2^{β} Cys), 19.8 (CH_3 Val), 19.2 (CH_3 Val), 17.9 (CH_3 Ala); least polar conformer: δ 172.5 (CO Ala), 168.9 (CO Cys), 168.8 (CO Val), 168.7 (CO Ser), 163.0 (CO Qxc), 143.9 (C_{Ar}), 143.6 (CH_{Ar}), 142.1 (C_{Ar}), 140.1 (C_{Ar}), 132.0 (CH_{Ar}), 131.1 (CH_{Ar}), 129.8 (CH_{Ar}), 129.5 (CH_{Ar}), 64.1 (CH^{α} Val), 63.8 (CH_2^{β} Ser), 52.2 (CH^{α} Cys), 51.1 (CH^{α} Ser), 43.9 (CH^{α} Ala), 37.3 (CH_2^{β} Cys), 30.8 (NCH_3 Cys), 30.2 (NCH_3 Val), 28.6 (CH^{β} Val), 19.6 (CH_3 Val), 18.9 (CH_3 Val), 17.6 (CH_3 Ala).
HPLC-ESMS: *m/z* calcd for $C_{50}H_{62}N_{12}O_{12}S_2$ 1086.41; found, 1086.18 $[M + H]^+$. MALDI-TOF: found, (most polar conformer), 1088.5439 $[M + H]^+$; (least polar conformer), 1088.5807 $[M + H]^+$. HR-ESMS: *m/z* calcd for $C_{50}H_{63}N_{12}O_{12}S_2$ 1087.4124 $[M + H]^+$, found (most polar conformer), 1087.4146; (least polar conformer), 1087.4155 $[M + H]^+$.

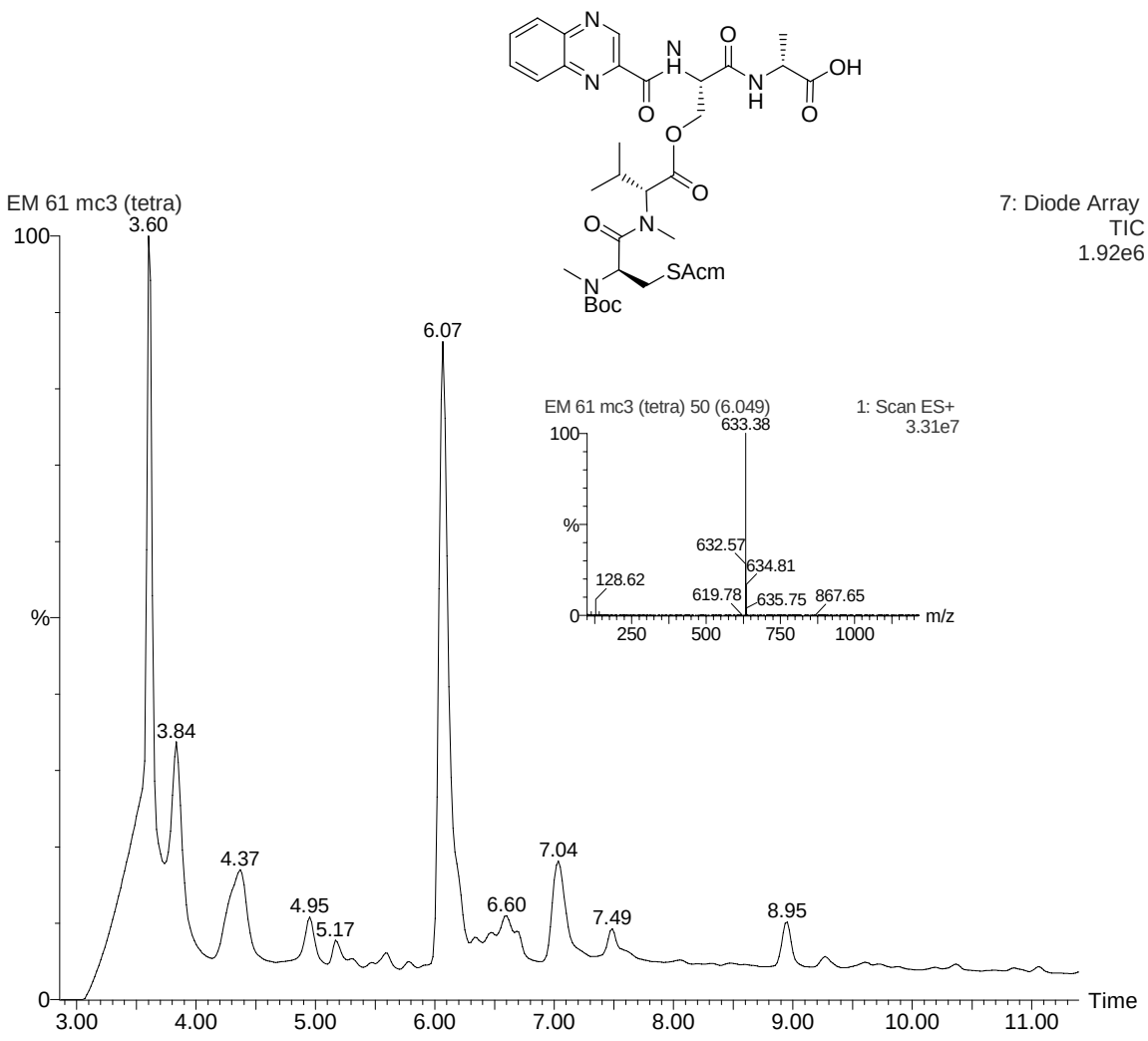
Cell growth inhibition assay

A colorimetric assay using sulforhodamine B (SRB) was adapted to perform a quantitative measurement of cell growth and viability, following a previously described method. The cells were seeded in 96-well microtiter plates at 5×10^3 cells/well in aliquots of 195 μL of RPMI medium and were left to grow in drug-free medium for 18 h to allow attachment to the plate surface. Samples were then added in aliquots of 5 μL (dissolved in DMSO–H_2O, 3:7). After 72 h exposure, the antitumor effect was measured by the SRB methodology: cells were fixed by adding 50 μL of cold 50% (wt/vol) trichloroacetic acid (TCA) and were incubated for 60 min at 4 ˚C. Plates were washed with deionized H_2O, and dried, and 100 μL of SRB solution (0.4% wt/vol in 1% acetic acid) was added to each microtiter well and incubated for 10 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air-dried, and the bound stain was dissolved with Tris buffer. Optical densities were read on an automated spectrophotometer plate reader at a

single wavelength of 490 nm. Data analyses were automatically generated by LIMS implementation. Assays were done in a dose-response manner at 10 different concentrations (from 10 μg/mL with 1:2.5 dilutions to 0.0026 μg/mL). Although concentrations were adjusted in mg/mL, GI_{50} values were calculated in molarity. All assays were run in triplicate, and the curves were automatically adjusted with 30 points by non-linear regression using "Prism 3.03" (GraphPad) software.

HPLC-ESI of intermediates

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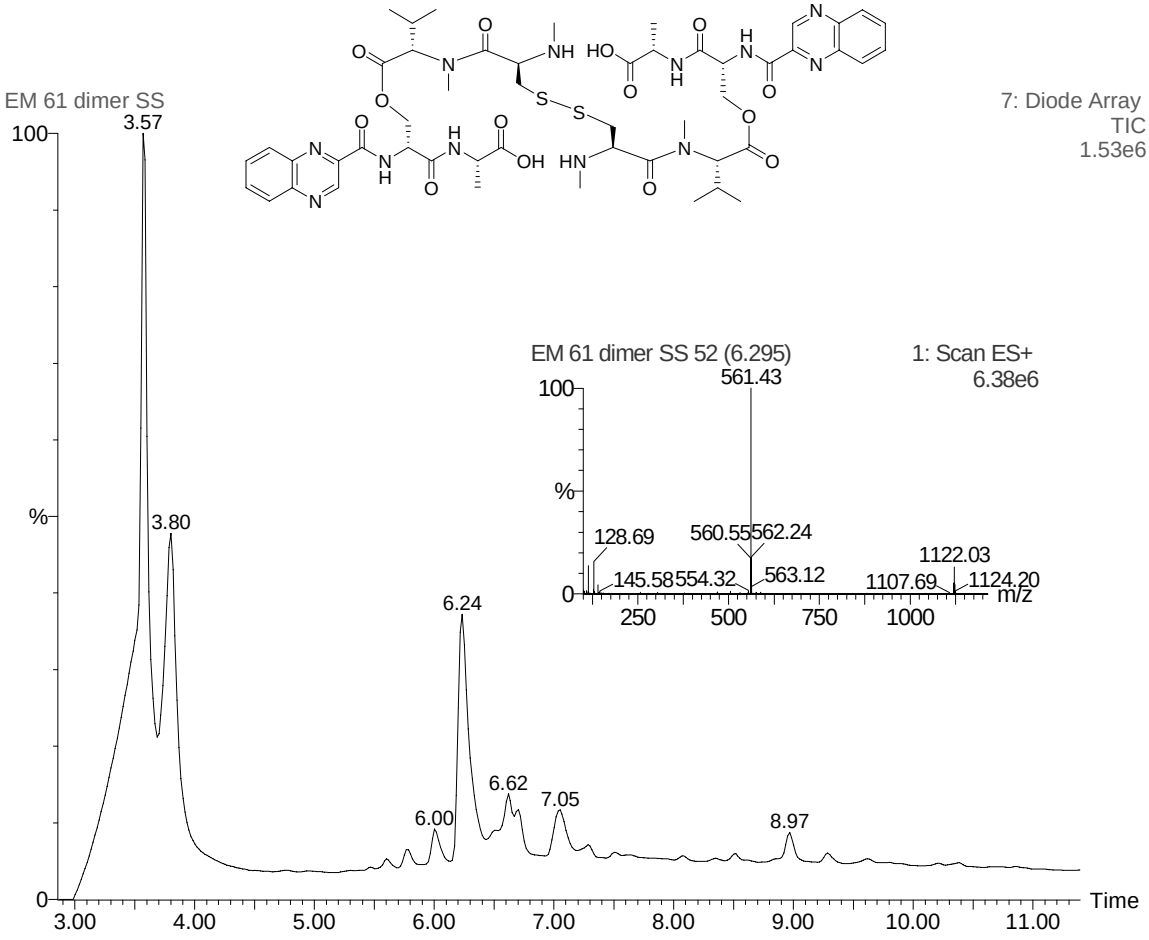
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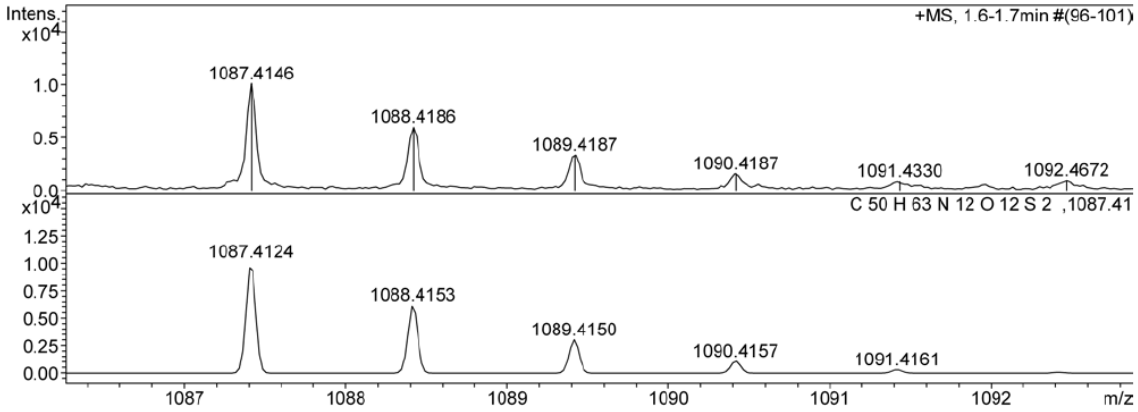
HR-ESI of Triostins

Most polar conformer:



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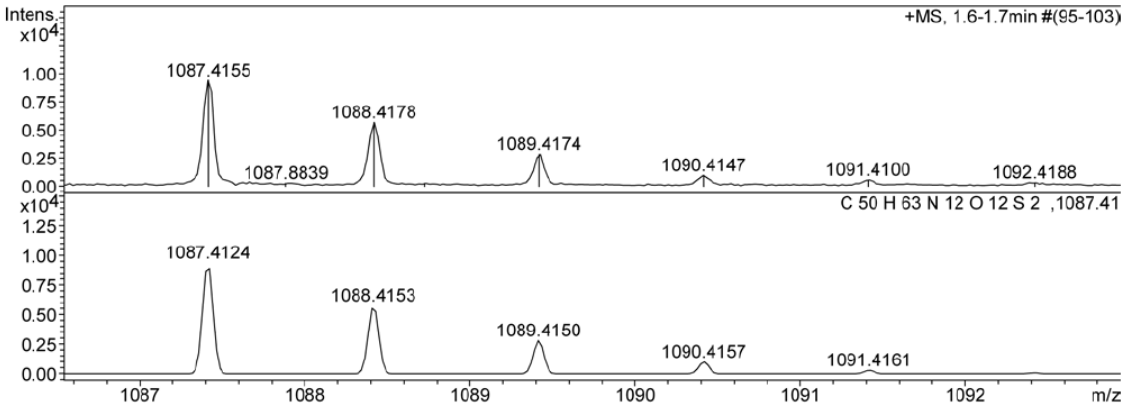
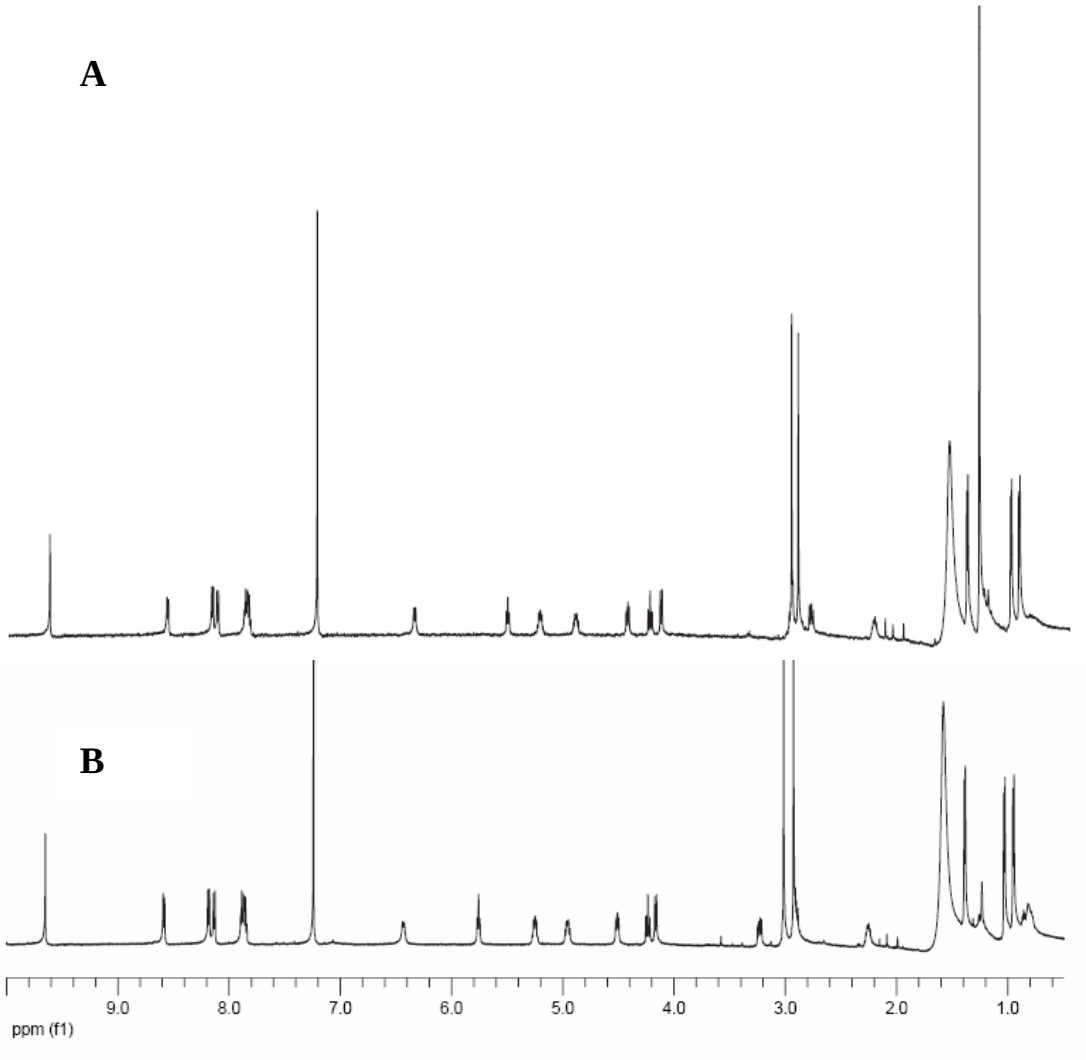


^{1}H NMR Spectra



^{1}H NMR spectra (600 MHz, $CDCl_3$) of A) most polar conformer; and B) least polar conformer