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Supporting Info

Synthesis and evaluation of linear CuAAC-oligomerized antifreeze neo-glycopeptides

Cite this: DOI: 10.1039/x0xx00000x

S. van der Wal[a], C.J. Capicciotti[b], S. Rontogianni[a], R.N. Ben[b], R.M.J. Liskamp*[a]

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

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Materials and methods

General: All reactions were carried out at ambient temperatures unless stated otherwise. Solvents used in these synthesis procedures were supplied by BioSolve BV, (Valkenswaard, The Netherlands) and stored over molecular sieves (CH_2Cl_2 and THF on 4Å and MeCN on 3Å). Routine analysis of reaction progression and product identity was checked by thin layer chromatography (TLC) on Merck precoated silicagel $60F_{254}$ glass plates. Spots were visualized by UV light and stained by ninhydrin or sulphuric acid charring for (protected) amine- and carbohydrate moieties respectively. Column chromatography was performed using Silicycle Siliflash P60 (40-63 µm) silicagel obtained from Screening Devices (Amersfoort, The Netherlands)

^{1}H NMR spectra, ^{13}C and Attached proton test (APT) spectra were recorded on a Varian Gemini 300 spectrometer at 300 MHz and 75 MHz respectively. High pressure liquid chromatography was performed analytically on a Shimadzu automated HPLC system with a dual wavelength detector at 220 and 254 nm operating at a flow-rate of 1 ml/min. An Alltech Alltima C8 column (100 Å, 5 µm, 250 x 4.6 mm) column was used together with acetonitrile TFA buffers for elution (buffer A: 5:95 MeCN:H_2O v/v + 0.1% TFA and buffer B: 95:5 MeCN:H_2O v/v + 0.1% TFA) in a 20- or 48 minute gradient. Preparative HPLC was performed on an Applied Biosystems system using identical buffers and an Alltech Alltima C8 column (100 Å, 10 µm, 250 x 22 mm) and a gradient of 100 minutes. Electron spray mass spectrometry was performed on a Shimadzu LCMS-QP8000 single quadrupole spectrometer operating in positive mode. MALDI-TOF analysis was performed on a Kratos Axima CFR spectrometer using α-cyano-4-hydroxycinnamic acid as matrix.

(S)-2-azido-5-((tert-butoxycarbonyl)amino)pentanoic acid (2)

To a solution of H-Orn(Boc)-OH (4.73 g, 20 mmol) in MeOH (100 ml), copper sulfate solution (42 mg, 0.2 mmol) in water (100 ml) was added, followed by K_2CO_3 (6.14 g, 44 mmol). Subsequently solid imidazole-1-sulfonyl-azide hydrochloride[1] (5.13 g, 24 mmol) was added and the reaction mixture was stirred for 4 hours. The reaction mixture was concentrated to about 100 ml, acidified with 1 M $KHSO_4$ and extracted with EtOAc. The organic layer was washed with saturated sodium chloride and dried with Na_2SO_4. After filtration and concentration *in vacuo*, the residual oil was dissolved in Et_2O and dicyclohexylamine (4 ml, 20 mmol) was added drop-wise while stirring. After cooling at -20 ºC overnight the white precipitate was collected by filtration and dried *in vacuo* to obtain the dicyclohexylamine salt of (S)-2-azido-5-((tert-butoxycarbonyl)amino) pentanoic acid (8.28 g, 18.8 mmol, 94%) as a fine white powder.
The free acid for subsequent reactions and analysis was obtained by suspending the salt in EtOAc in an extraction funnel and washing with 1 M $KHSO_4$. After drying the organic layer with saturated NaCl and Na_2SO_4 the free acid was obtained as an oil in near quantitative yield.
^{1}H NMR ($CDCl_3$): δ = 10.40 (bs, 1H, COOH), 6.46 + 4.72 (two bs. 1H, NH), 3.93 (bs, 1H, α-CH), 3.15 (bs, 2H, δ-CH_2), 1.89-1.77 (m, 2H, β-CH_2), 1.63 (m, 2H, γ-CH_2), 1.44 (s, 9H, Boc-CH_3)
^{1}H NMR (CD_3OD): δ = 3.98 (m, 1H, α-CH), 3.07 (t, *J*= 6.6 Hz, 2H, δ-CH_2), 1.91-1.68 (m, 2H, β-CH_2), 1.59 (m, 2H, γ-CH_2), 1.43 (s, 9H, Boc-CH_3)
^{13}C NMR (CD_3OD) δ = 172.4, 157.1, 78.5, 61.5, 39.2, 28.3, 27.4, 25.9

((N-tert-butoxycarbonyl)glycyl)-propargylamine (3a)
Propargylamine hydrochloride (4.25 g, 40 mmol) and Boc-Gly-OH (6.90 g, 40 mmol) were dissolved in CH_2Cl_2 (250 ml). The stirred mixture was cooled on ice, and diisopropylethylamine (DiPEA, 21 ml, 120 mmol) was added, followed by BOP (17.60 g, 40 mmol). After stirring for 2h at room temperature, the mixture was concentrated, redissolved in EtOAc and subsequently washed with 1 M $KHSO_4$, 5% $NaHCO_3$ and saturated NaCl. After drying with Na_2SO_4, the product was crystallized from CH_2Cl_2/ hexanes to yield colourless crystals of ((N-tert-butoxycarbonyl)glycyl)-propargylamine (6.6 g, 31 mmol, 79% overall yield).
^{1}H NMR ($CDCl_3$): δ = 6.46 (bs, 1H, amide-NH), 5.16 (bs, 1H, Boc-NH), 4.06 (dd, J_1=2.5 Hz, J_2=5.3 Hz, 2H, CH_2-propargyl), 3.81 (d, *J*=5.8 Hz, 2H, glycine-CH_2), 2.23 (t, *J*=2.5 Hz, 1H, alkyne-H), 1.45 (s, 9H, Boc-CH_3)
^{13}C NMR ($CDCl_3$): δ = 169.3, 156.1, 80.4, 79.2, 71.6, 44.3, 29.0, 28.3

Triisopropylsilyl-((N-tert-butoxycarbonyl)glycyl)-propargylamine (3b)
3-Triisopropylsilyl-propargylamine[2] (5.0 g, 23 mmol), Boc-Gly-OH

(3.83 g, 21.9 mmol) and DiPEA (11.6 ml, 21.9 mmol) were dissolved in CH_2Cl_2 (200 ml) and cooled on ice. While stirring, BOP (9.68 g, 21.9 mmol) was added. After 3 hours at room temperature the reaction mixture was concentrated *in vacuo* and redissolved in EtOAc. This was washed with 1 M $KHSO_4$, 5% $NaHCO_3$ and saturated NaCl. After drying with Na_2SO_4 and concentration *in vacuo*, Triisopropylsilyl-((N-tert-butoxycarbonyl)glycyl)-propargylamine was obtained as a yellowish oil that solidified upon standing (8.61 gram, 23 mmol, 99%)
^{1}H NMR ($CDCl_3$): δ = 6.19 (bs, 1H, amide-NH), 5.08 (m, 1H, Boc-NH), 4.11 (m, 2H, CH_2-propargyl), 3.80 (d, *J*=5.9 Hz, 2H, glycine-CH_2), 1.46 (s, 9H Boc-CH_3), 1.06 (s, 21H, TIPS)
^{13}C NMR ($CDCl_3$): δ = 169.0, 156.2, 102.4, 84.8, 80.3, 44.3, 30.2, 28.3, 18.5, 11.1

(S)-2-azido-5-((tert-butoxycarbonyl)amino)pentanoyl-glycyl-propargylamine (4a)
The Boc-group in **3a** (2.0 g, 9.4 mmol) was removed by 1:1 TFA/ CH_2Cl_2 for 2 hours. After concentration *in vacuo* the oil was redissolved in CH_2Cl_2 (100 ml) and **2** (4.2 g as the dicyclohexylamine salt of **2**, workup as stated at **2**, 9.4 mmol), BOP (4.2 g, 9.4 mmol) and DiPEA (5.2 ml, 30 mmol) were added. After 2 hours the reaction mixture was concentrated *in vacuo*, redissolved in EtOAc, washed with 1M $KHSO_4$, 5% $NaHCO_3$ and brine. After drying of the organic layer over Na_2SO_4 and filtration the product was crystallized from EtOAc/ hexanes and obtained as a white solid (1.8g, 5.1 mmol, 55%).
^{1}H NMR ($CDCl_3$): δ = 7.27 (s, 1H, NH) 6.70 (s, 1H, NH), 4.73 (s, 1H, Boc-NH), 4.06 (m, 3H, propargyl-CH_2 + Orn-α-CH), 3.96 (d, 2H, gly-CH_2 *J*=4.7 Hz), 3.15 (m, 2H, Orn-δ-CH_2), 2.26 (s, 1H, alkyne-H), 1.89 (m, 2H, Orn-β-CH_2), 1.62 (m, 2H, Orn-γ-CH_2), 1.44 (s, 9H, Boc-CH_3).
^{13}C NMR ($CDCl_3$): δ = 170.5, 168.4, 156.2, 79.32, 79.26, 71.6, 62.8, 42.9, 39.7, 29.11, 29.08, 28.4, 25.9

Triisopropylsilyl-((S)-2-azido-5-((tert-butoxycarbonyl)-amino)pentanoyl-glycyl)-propargylamine (4b)
This procedure was analogous to the preparation of **4a**, using the following amounts: **3b** (4.0 g, 10.8 mmol), and **2** (4.8 g as the dicyclohexylamine salt of **2**, workup as stated at **2**, 10.8 mmol), BOP (4.8 g, 10.8 mmol) and DiPEA (5.7 ml, 32.4 mmol).
Purification by column chromatography instead of crystallization was performed using 10% acetone in CH_2Cl_2 to obtain **4b** as a colourless oil (4.1 g, 8.1 mmol, 75%)
^{1}H NMR ($CDCl_3$): δ = 7.21 (s, 1H, NH), 6.29 (s, 1H, NH), 4.70 (s, 1H, Boc-NH), 4.11 (m, 2H, propargyl-CH_2), 4.03 (m, 1H, Orn-α-CH), 3.93 (d, 2H, glycine-CH_2, *J*=5.3 Hz), 3.15 (m, 2H, Orn-δ-CH_2), 1.89 (m, 2H, Orn-β-CH_2), 1.63 (m, 2H, Orn-γ-CH_2), 1.42 (s, 9H, Boc-CH_3), 1.04 (s, 21H, TIPS)
^{13}C NMR ($CDCl_3$): δ = 170.3, 167.7, 156.2, 102.1, 85.1, 79.4, 63.0, 43.0, 39.5, 30.4, 29.2, 28.4, 26.0, 18.5, 11.1

Tetraacetylated carboxymethyl galactoside-OSu ester (6)

Carboxymethyl tetraacetyl galactoside[3,4,5] (1.0 g, 2.5 mmol) and N-hydroxysuccinimide (0.3 g, 2.7 mmol) were dissolved in dry CH_2Cl_2 (50 ml) and cooled on ice. DCC (0.6 g, 2.7 mmol) was subsequently added and the reaction mixture was stirred for 16 hours at room temperature. Precipitated DCU was removed by filtration and after concentration *in vacuo* to near dryness, more precipitation occurred. After another filtration step the product was crystallized from CH_2Cl_2/ *i*PrOH to yield **6** as white fluffy needles (1.0 g, 2 mmol, 82%)
^{1}H NMR ($CDCl_3$): δ = 5.44 (bs, 1H, 4-H), 5.37 (m, 1H, 2-H), 5.20 (m, 1H, 3-H), 4.79 (dt, 1H 1-H, J_1=J_2=6.3 Hz,), 4.30-4.10 (m, 3H, 5-H+6-CH_2), 3.00 (m, 2H, 1-CH_2), 2.84 (s, 4H, 2xCH_2-Osu), 2.13 (s, 3H, acetyl-CH_3), 2.08 (s, 3H, acetyl-CH_3), 2.043 (s, 3H, acetyl-CH_3), 2.037 (s, 3H, acetyl-CH_3)
^{13}C NMR ($CDCl_3$): δ = 170.5, 170.0, 169.8, 169.6, 168.7, 165.6, 69.5, 68.6, 67.9, 67.2, 67.0, 60.9, 30.4, 25.5, 20.6

Triisopropylsilyl-((S)-2-azido-5-((peracetyl-1-carboxymethyl galactopyranoside)-amino)pentanoyl-glycyl)-propargylamine (5b)
4b (280 mg, 0.55 mmol) was Boc-deprotected in 1:1 TFA/ CH_2Cl_2 for two hours. After concentration *in vacuo* the oil was redissolved in CH_2Cl_2 (20 ml) and DiPEA (0.26 ml, 1.5 mmol) and **6** (195 mg, 0.5 mmol) were added. The reaction mixture was allowed to stir for 2 hours after which it was concentrated *in vacuo* and redissolved in EtOAc and subsequently washed with 1 M $KHSO_4$, 5% $NaHCO_3$ and saturated NaCl. The organic layer was dried with Na_2SO_4 and after evaporation crude **5b** was obtained.
Column chromatography using 4% MeOH in CH_2Cl_2 afforded **5b** as a colourless oil. (327 mg, 0.42 mmol, 84%)
^{1}H NMR ($CDCl_3$): δ = 7.37 (t, 1H, NH, *J*=5.1 Hz), 6.65 (m, 2x1H, NH), 5.41 (bs, 1H 4-H glycoside), 5.28 (m, 1H, 2-H glycoside), 5.18 (m, 1H, 3-H glycoside), 4.70 (m, 1H, 1-H glycoside), 4.3-4.1 (m, 5H, 5-H, 6-CH_2 glycoside, propargyl-CH_2), 4.06 (m, 1H, Orn-α-CH), 3.96 (m, 2H, glycine-CH_2), 3.30 (m, 2H, Orn-δ-CH_2), 2.69-2.42 (m, 2H, 1-CH_2 glycoside), 2.13 (s, 3H, acetyl-CH_3), 2.08 (s, 3H, acetyl-CH_3), 2.06 (s, 3H, acetyl-CH_3), 2.05 (s, 3H, acetyl-CH_3), 1.88 (m, 2H, Orn-β-CH_2), 1.66 (m, 2H, Orn-γ-CH_2), 1.06 (s, 21H, TIPS)
^{13}C NMR ($CDCl_3$): δ = 170.6, 170.2, 170.0, 169.9, 169.7, 169.6, 167.8, 102.2, 84.9, 69.2, 69.0, 67.9, 67.8, 67.0, 62.9, 61.2, 42.7, 38.5, 34.3, 30.4, 29.1, 25.3, 20.7, 18.5, 11.0

((S)-2-azido-5-((peracetyl-1-carboxymethyl galactopyranoside)-amino)pentanoyl-glycyl)-propargylamine (5a)
5b (160 mg, 0.2 mmol) was dissolved in anhydrous THF (25 ml). Phenol (96 mg, 1.0 mmol) and TBAF.3H_2O (323 mg, 1.0 mmol) were added and the reaction mixture was stirred for 2h. The reaction mixture was diluted with EtOAc and washed with H_2O. After drying the organic layer with Na_2SO_4 and concentration *in vacuo*, the compound was purified through a small plug of silica using 4% MeOH in CH_2Cl_2 to yield **5a** as a white foam (113 mg, 0.18 mmol, 90%)
^{1}H NMR ($CDCl_3$): δ =7.35 (bt, 1H, NH, *J*=~5 Hz), 6.87 (bt, 1H, NH, *J*=~5 Hz), 6.62 (bt, 1H, NH, *J*=~5 Hz), 5.42 (bs, 1H, 4-H glycoside), 5.27 (m, 1H, 2-H glycoside), 5.19 (m, 1H 3-H glycoside), 4.70 (m, 1H, 1-H glycoside), 4.3-4.0 (m, 6H, 5-H, 6-CH_2 glycoside, propargyl-CH_2, Orn-α-CH), 3.97 (d, 2H, glycine-CH_2, *J*=5.1 Hz), 3.31 (m, 2H, Orn-δ-CH_2), 2.7-2.4 (m, 2H, 1-CH_2 glycoside), 2.29 (s, 1H, alkyne-CH), 2.13 (s, 3H, acetyl-CH_3), 2.08 (s, 3H, acetyl-CH_3), 2.06 (s,2x3H, acetyl-CH_3), 1.91 (m, 2H, Orn-β-CH_2), 1.66 (m, 2H, Orn-γ-CH_2)
^{13}C NMR ($CDCl_3$): δ = 170.7, 170.3, 170.01, 169.96, 169.8, 169.7, 168.2, 79.2, 71.8, 69.3, 69.0, 67.9, 67.8, 67.0, 62.8, 61.2, 42.8, 38.5, 34.3, 29.2, 29.1, 25.1, 20.7, 20.6,

Conditions for copper click mediated oligomerization
Azido/alkyne-monomer **5a** (100 mg, 160 µmol) was dissolved in a DiPEA/DMF solution (400 µl, 300 mM) in a 1.5 ml plastic reaction tube. A freshly prepared aqueous solution of TCEP (200 mM, 200 µl), was added to the monomer and shaken for 2 hours after which nitrogen evolution had ceased. Polymerization was then initiated by adding an aqueous copper sulfate solution (240 mM, 100 µl) followed by a freshly prepared aqueous sodium ascorbate solution (720 mM, 100 µl), added while vortexing. After 2 h the reaction mixture was diluted with acetonitrile/ water (1:2 v/v, 0.1% TFA added) and preparative HPLC was performed to separate oligomers by size. After lyophilisation the oligomers **7a**, **8a** and **9a** were

obtained as white fluffy solids.

MALDI-TOF:
Trimer 7a (6 mg), calculated *M* for $C_{78}H_{110}N_{16}O_{36}$ 1846.7, found $[M+H]^+$ 1848.4, $[M+Na]^+$ 1870.5, $[M+K]^+$ 1886.7, $[M\text{-acetyl}+Na]^+$ 1828.3.
Tetramer 8a (5.5 mg), calculated *M* for $C_{104}H_{146}N_{22}O_{48}$ 2471.0, found $[M+Na]^+$ 2494.4, $[M+K]^+$ 2510.4 $[M\text{-acetyl}+Na]^+$ 2452.4.

Pentamer 9a (4 mg), calculated *M* for $C_{130}H_{182}N_{28}O_{60}$ 3095.2, found $[M+Na]^+$ 3118.0, $[M+K]^+$ 3133.9, $[M\text{-acetyl}+Na]^+$ 3075.9.
Higher oligomers (>7mer, 30 mg), found $[8mer+Na]^+$ 4990.8, $[9mer+Na]^+$ 5615.8, $[10mer+Na]^+$ 6239.2, higher oligomers did not ionize well enough to be distinguishable from the baseline noise.

Synthesis of reference compound OGG-Gal (1) and the C-terminal carboxamide analog 11
Fmoc-Orn(Boc)-OSu (5.0 g, 9.1 mmol) (this N-hydroxysuccinimide ester of the commercially available protected amino acid was synthesized using the methodology described for compound **6** with 88% yield) was dissolved in THF (150 ml). To this solution, a solution of H-Gly-Gly-OH (1.3 g, 10 mmol) and $NaHCO_3$ (1.68 g, 20 mmol) in water (30 ml) were added. The reaction mixture was stirred for 16 hours, after which the THF was mostly removed *in vacuo* and the mixture was diluted with EtOAc. Washing with 1M $KHSO_4$ and drying gave the crude Fmoc-Orn(Boc)-Gly-Gly-OH, which was further purified by crystallization from CH_2Cl_2/hexanes to give a white solid (4.6 gram, 8.1 mmol) which was used directly for the next reaction.

Removal of the Boc-group from the ornithine residue (3.0 gram, 5.3 mmol) was performed with 1:1 TFA/CH_2Cl_2 for 2 hours after which the reaction mixture was concentrated *in vacuo* and coevaporated with toluene to remove residual TFA. The residue was suspended in THF/H_2O (1:1 v/v, 100 ml) and the pH was adjusted to 8.5 with DiPEA. Carbohydrate derivative **6** (2.3 g, 4.8 mmol), dissolved in THF was added and the pH was kept at 8 for 3 hours. The now homogeneous reaction mixture was acidified with 1M $KHSO_4$ and extracted with EtOAc. The combined organic layers were dried on Na_2SO_4, concentrated *in vacuo*, and the residue was recrystallized from CH_2Cl_2 (with a few drops of MeOH) by addition of Et_2O. Fmoc-Orn(carboxymethyl-Gal)-Gly-Gly-OH was obtained as a white powder (2.8 gram, 3.3 mmol, 61% over the combined three steps). ^{1}H-NMR was comparable to previously published characterization.[5]
Solid phase chemistry was performed as previously described on a preloaded Fmoc-Gly-wang resin (to obtain compound **1**) or Fmoc-Rink resin (to obtain compound **11**) to yield the tetramer,[5] which after deprotection (see below) was purified by preparative HPLC, using 0.1% TFA in water as buffer A to give retention and subsequently lyophilized to yield compound **1** and **11** as white fluffy solids.

CuAAC coupling of ethyl azidoacetate
Tetramer **8a** (6.5 mg, 2.5 µmol) and ethyl azidoacetate (1 mg, 7.5 µmol, 3 equiv.) were dissolved in THF (400 µl). To this solution copper(II)sulfate pentahydrate (0.2 mg, 0.8 µmol, 0.33 equiv. in 100 µl water) was added followed by sodium ascorbate (1 mg, 5 µmol 2 equiv. in 100 µl water). The reaction mixture was stirred for 6 hours, diluted with EtOAc, and washed with $Na_2S_2O_3$ solution. The resulting crude glycopeptide was deacetylated (see below) and purified with preparative HPLC using 0.1% TFA in water as buffer A.

Typical Procedure for deacetylation
Acetylated glycopeptide oligomer was dissolved in dry MeOH (500 µl/ 5 mg oligomer) and a catalytic amount of fresly prepared sodium methoxide was added. After 3 hours of reaction time water (500 µl/ 5 mg oligomer) was added to solubilize the formed precipitate and incubated for another 30 minutes. Dowex marathon C ion exchange resin (acid form and washed beforehand with methanol) was added to neutralize the reaction mixture. The mixture was filtered and lyophilized to afford the deacetylated oligomers in 2.5-4 mg amounts and the polymer in ~20 mg. Residual salts could be removed by reversed-phase solid phase extraction, washing with water and eluting the compound with gradual increments of MeCN up to 10% v/v MeCN in water.

ESI-MS after purification:
Deprotected triazole based tetramer (8b): calculated *M* for $C_{72}H_{114}N_{22}O_{32}$ 1798.80, found $[M+2H]^{2+}$ 900.75
Deprotected triazole based tetramer-acid (10): Calculated *M* for $C_{74}H_{117}N_{25}O_{34}$ 1899.82, found $[M+2H]^{2+}$ 950.65
Deprotected OGG-Gal (1): calculated *M* for $C_{70}H_{117}N_{17}O_{38}$ 1803.8, found $[M+2H]^{2+}$ 902.70
Deprotected carboxamide OGG-Gal (11): calculated *M* for $C_{68}H_{115}N_{17}O_{36}$ 1745.77, found $[M+2H]^{2+}$ 873.70

CD spectroscopy
CD spectra were recorded on a JASCO J-815 spectrometer using a 1 cm cuvet in a Peltier type cell holder operating at 4 °C. A 260-190 nm window was scanned at 50 nm/min with a data pitch of 0.2 nm and an integration time of 1 second. 10 accumulations of these measurements were recorded for each spectrum. The AFGP concentration used was 50 µM in milli-Q water (freshly made) and water blanks were subtracted. The concentrations used probably correspond to non-aggregated single molecules in solution as was measured for AFGP-8.[6] SELCON-3 analysis, included in the *CDPro* package and used as described using datasets IBasis 5 (SP37A) and IBasis 10 (SMP56), which contain 37 soluble and 43 soluble+13 membrane proteins respectively, was performed.[7,8] The CONTINLL and CDSSTR tools yielded less reliable results in terms of solutions.

IBASIS 5	A-helix	B-sht	Turn	PPII	Unrd.
OGG-Gal carboxylic acid **1**	0.000	0.335	0.099	0.123	0.444
OGG-Gal carboxamide **11**	0.088	0.258	0.046	0.058	0.546
Triazole alkyne **8b**	0.087	0.258	0.046	0.058	0.549
Triazole acid **10**	0.086	0.258	0.046	0.058	0.549

IBASIS 10	H(r)	H(d)	S(r)	S(d)	Turn	Unrd.
1	0.000	0.029	0.290	0.195	0.227	0.252
11	0.006	0.087	0.295	0.160	0.107	0.337
8b	0.006	0.085	0.297	0.161	0.108	0.338
10	0.006	0.085	0.301	0.162	0.109	0.340

Thermal Hysteresis (TH) assay

Nanoliter osmometry was performed using a Clifton nanoliter osmometer (Clifton Technical Physics, Hartford, NY).[9] All measurements were performed in a phosphate buffered saline (PBS) solution. Ice crystal morphology was observed through a Leitz compound microscope equipped with an Olympus 20× (infinity-corrected) objective, a Leitz Periplan 32× photo eyepiece, and a Hitachi KPM2U CCD camera connected to a Toshiba MV13K1 TV/VCR system. Still images were captured directly using a Nikon CoolPix digital camera.

Ice Recrystallization Inhibition (IRI) assay

Sample analysis for IRI activity was performed using the "splat cooling" method as previously described[10] In this method, the analyte was dissolved in a phosphate buffered saline (PBS) solution and a 10 μL droplet of this solution was dropped from a micropipette through a two meter high plastic tube (10 cm in diameter) onto a block of polished aluminum precooled to approximately -80 °C. The droplet froze instantly on the polished aluminum block and was approximately 1 cm in diameter and 20 μm thick. This wafer was then carefully removed from the surface of the block and transferred to a cryostage held at -6.4 °C for annealing. After a period of 30 min, the wafer was photographed between crossed polarizing filters using a digital camera (Nikon CoolPix 5000) fitted to the microscope. A total of three images were taken from each wafer. During flash freezing, ice crystals spontaneously nucleated from the supercooled solution. These initial crystals were relatively homogeneous in size and quite small. During the annealing cycle, recrystallization occurred, resulting in a dramatic increase in ice crystal size. A quantitative measure of the difference in recrystallization inhibition of two compounds is the difference in the ice crystal size distribution. Image analysis of the ice wafers was performed using a novel domain recognition software (DRS) program.[11] This processing employed the Microsoft Windows Graphical User Interface to allow a user to visually demarcate and store the vertices of ice domains in a digital micrograph. The data was then used to calculate the domain areas. All data was plotted and analyzed using Microsoft Excel. The mean grain (or ice crystal) size (MGS) of the sample was compared to the MGS of the control PBS solution for that same day of testing. IRI activity is reported as the percentage of the MGS (% MGS) relative to the PBS control, and the % MGS for each sample was plotted along with its standard error of the mean. Small percentages represent a small MGS, which is indicative of more potent IRI activity.

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