BI-32169, a Bicyclic 19-Peptide with Strong Glucagon Receptor Antagonist Activity from *Streptomyces* sp.

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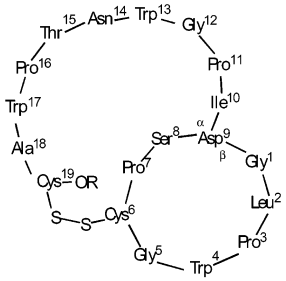
A new bicyclic 19-peptide, BI-32169 (**1**), has been isolated from the culture broth of *Streptomyces* sp. (DSM 14996). Its structure has been established by amino acid analysis, mass spectrometry, and 2D NMR analysis. BI-32169 consists exclusively of protein amino acids and is cyclized from the side chain of Asp^9 to the N-terminus of Gly^1. One disulfide bond between Cys^6 and Cys^{19} forms a bicyclic structure. BI-32169 and its methyl ester derivative (**2**) showed potent inhibitory activity against the human glucagon receptor (IC_{50} 440 and 320 nM, respectively) in a functional cell-based assay.

Glucagon, a 29 amino acid peptide hormone, is synthesized and secreted by the alpha cells of the pancreas. Glucagon binds to specific receptors mainly in the liver, where it stimulates hepatic glucose production via glycogenolysis and gluconeogenesis. According to the bihormonal hypothesis, plasma glucose levels are regulated by insulin and glucagon action. In the diabetic situation, paradoxically elevated levels of circulating glucagon contribute to hyperglycemia in addition to lacking insulin or diminished insulin action. Therefore, antagonism of glucagon action at the receptor level has the potential for a new antidiabetic therapy.[1]

In the course of our search for novel glucagon receptor antagonists we noticed that an extract of *Streptomyces* sp. (DSM 14996), contained in our natural product extract library, exhibited a strong inhibitory activity against glucagon-induced cAMP elevation. We report here on the isolation and the chemical and biological characterization of the active component, which was shown to be a new bicyclic 19-peptide (Figure 1).

Results and Discussion

The extract showing glucagon receptor antagonist activity was obtained by methanolic extraction of a dried 50 mL culture of *Streptomyces* sp. (DSM 14996) and subsequent prepurification by solid-phase extraction (SPE). Semipreparative fractionation of this extract by HPLC and subsequent testing of the fractions enabled the activity to be attributed to a main peak (t_R 16.1 min). HPLC-UV-MS analysis revealed a UV spectrum (λ_{max} 221, 281 nm) similar to that of tryptophan and a MW of 2036 amu. Since, using SciFinder (ACS), no record corresponding to these data could be found in the literature, purification of the compound was undertaken on a preparative scale. For this purpose, strain DSM 14996 was cultured on a 6 L scale in Erlenmeyer flasks containing a glucose caseinpepton medium. After 5 days, the mycelium and the culture filtrate were separated by filtration and freeze-dried. The mycelium and the dried culture medium were subsequently recombined and extracted with MeOH containing a small



1 R = H

2 R = CH_3

Figure 1. Structures of BI-32169 (**1**) and its methyl ester **2**.

percentage of DMSO. After concentration under reduced pressure, compound **1** was isolated by a combination of preparative HPLC on RP-18 with a MeCN/aqueous NH_4-OAc (pH 4) gradient and column chromatography on Sephadex LH-20 with MeOH.

After acidic hydrolysis (6 N HCl) the amino acids were identified by HPLC after AccQTag derivatization as Asp/Asn (2), Ser (1), Gly (3), Thr (1), Ala (1), Pro (4), Cys (2), Ile (1), and Leu (1). The presence of three tryptophan residues was inferred from the NMR data. The L-configuration of the amino acids was established in a separate experiment by gas chromatography of their *N*-trifluoroacetyl *n*-propyl ester derivatives on a Chirasil-Val column.[2] The molecular mass obtained by ESI TOF MS was 20 u smaller than the one expected from the amino acid composition. This suggested a bicyclic structure resulting from an internal loss of water and the presence of a disulfide bridge. Treatment of **1** with trimethylsilyldiazomethane provided the methyl ester **2** ($[M + H]^+$ *m*/*z* 2051.1), thus enabling the unambiguous assignment of the C-terminus. The whole sequence of **2** was established by NMR investigation as follows.

Spin systems were assigned from the 2D TOCSY spectrum with 65 ms mixing time. Besides 15 amide protons, four more spin systems were identified with only aliphatic resonances that matched the proline pattern. The other spin systems consisted of one alanine, three glycines, one leucine, one isoleucine, one threonine, and eight AMXY spin systems, plus one isolated O-methyl group at δ 3.65. In the downfield region of the spectrum, several additional resonances could be seen: one coupled spin pair at δ 6.66/

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5.72, probably belonging to a side chain NH_2 group, and three patterns typical of the indolic side chains of tryptophan residues; otherwise no more aromatic resonances were found.

A gradient-selected COSY and double-quantum filtered COSY were used to verify the assignments within the spin systems. All protonated ^{13}C resonances were then assigned from the 2D HSQC spectrum; in cases of severe overlap the correct assignment could be deduced from the ^{13}C-HMQC-TOCSY data.

Most of the sequential connections could be detected in 2D HMBC spectra as correlations across peptide bonds between two adjacent amino acids. For this purpose, the amide carbonyl resonances had to be assigned first. In the H^N region of the HMBC, all 14 sequential $C'_i-H^N_{i+1}$ correlations (excluding the four prolines and the N-terminus) could be observed (plus one intraresidue $C'_i-H^N_i$ cross-peak of Ser[8]). For three proline H^α resonances (all except Pro[3]), a weak correlation to the preceding carbonyl was observed. The carbonyl carbons of the Asp[9] and Asn[14] side chains could be identified by correlations to the H^β and—for Asn[14]—also the side chain amide resonances. The intraresidual $C'_i-H^\alpha_i$ correlations could be unambiguously detected only for 16 residues (including the C-terminal Cys[19]), plus one partially overlapped correlation (Thr[15]). For Pro[7], the $C'_i-H^\beta_i$ peaks were available instead. In all other cases, the $C'_i-H^\beta_i$ correlations confirmed the previous intraresidual assignments.

The C-terminus could be readily identified from the very intense HMBC cross-peak of the O-methyl protons (δ 3.65) to the Cys[19] carbonyl carbon. From there, sequential assignment was possible in a stepwise fashion down to Pro[3], which showed no more correlation to a preceding carbonyl. Another two-amino-acid segment (Gly[1]-Leu[2]) could be constructed from the remaining two spin systems.

Since the longer fragment must contain the C-terminus, it was concluded that the smaller fragment had to be the N-terminal one. Indeed, in the NOESY spectrum (100 ms mixing time) unambiguous correlations were observed between the Pro[3] H^δ resonances and the H^α as well as the side chain protons of Leu[2], confirming their sequential order.

The N-terminal residue, Gly[1], clearly showed an H^N signal shift and line shape typical for an amide rather than a free amino group. In addition, the chemical shift of the Asp[9] δ-carbonyl carbon (δ 171.3) clearly corresponds to an amide or ester, not a free carboxylic acid. The suspected cyclization of the N-terminus with the Asp[9] side chain could be verified from an—although rather weak—HMBC correlation of Asp[9] $C^\delta O$ to Gly[1]-H^α, as well as from the very intense NOE correlations between Gly[1]-H^N to both H^β resonances of Asp[9]. At the same time, NOE correlations between the unique shifts from Cys[6] H^β to Cys[19] H^α and Ala[18] H^β confirmed the close spatial neighborhood of the two cysteine side chains, as expected for the existence of a disulfide bridge.

The inhibitory activity of compounds **1** and **2** against the human glucagon receptor was assessed in a BHK-21 cell line stably transfected with a plasmid construct coding for the human glucagon receptor under control of the viral CMV promoter. Production of cAMP after treatment with half-maximal concentrations of glucagon (30 pM) was inhibited by compounds **1** and **2** with an IC_{50} of 440 and 320 nM, respectively (Figure 2). Both compounds are full antagonists of glucagon action and exhibit no agonistic activity. They were found to be selective versus the human GLP-1 receptor (data not shown).

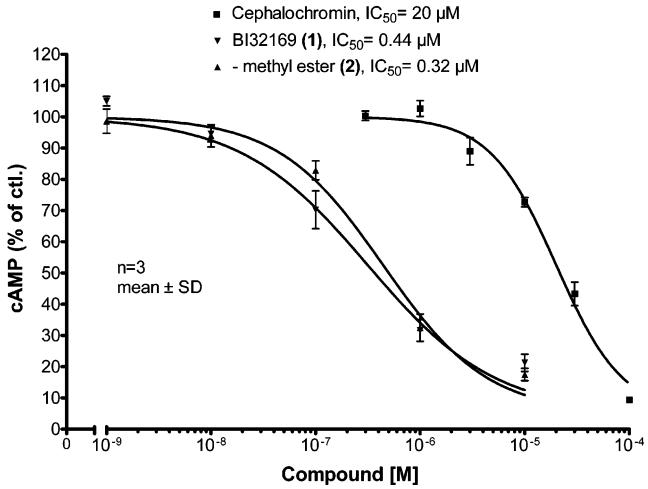

Figure 2. Inhibition of glucagon-induced cAMP elevation.

Several publications disclose peptidic and nonpeptidic glucagon receptor antagonists.[1,3] Among natural products, cephalochromin has been described as a glucagon receptor antagonist.[4] Therefore we used it as a reference compound. It inhibits glucagon-stimulated cAMP elevation with an IC_{50} of 20 μM.

BI-32169 is a new natural product belonging to a small group of structurally related cyclic peptides containing only protein amino acids which have been reported from various *Streptomyces* strains. RP 71955 (aborycin),[5−7] RES 701,[8,9] MS-271 (siamycin I),[10,11] and siamycin II[11] exhibit some sequence analogies with **1** and possess also a nine-membered ring formed by an amide linkage between the β-COOH function of Asp[9] and the N-terminal amino acid (glycine or cysteine). Two further peptides, anantin[12] and propeptin,[13] possess the same internal linkage with an eight- and a nine-membered ring, respectively.

Experimental Section

General Experimental Procedures. NMR spectra were recorded at 298 K on an Avance DRX600 spectrometer (Bruker, Karlsruhe) operating at a basic ^1H frequency of 600.13 MHz, using tetramethylsilane (TMS) for referencing. For all 2D experiments (H,H-DQF-COSY, H,H-TQF-COSY, H,H-TOCSY, H,H-ROESY, H,H-NOESY, H,C-HSQC, H,C-HSQC-TOCSY, H,C-HMBC) standard pulse sequences contained in the software release XWINNMR 2.6 PL5 (Bruker) were employed. Data processing and evaluation were performed with the same software package.

HPLC-UV-MS: Waters Alliance 2795 system equipped with a Symmetry C-18 (5 μm, 3.9 × 150 mm) column; MeCN/aqueous NH_4OAc (pH 4), 5:95 to 95:5 in 28.6 min; 1 mL/min; temperature 35 °C (system 1). UV detection was performed with a Waters 996 photodiode array detector. ESIMS spectra were obtained on a LCT time-of-flight mass spectrometer (Micromass Waters) in the positive ion mode. For accurate mass determination, erythromycin (*m*/*z* 734.4690) was used as lock mass. IR spectra: ThermoNicolet ATR-FTIR Avatar 370 system. UV spectra: Perkin-Elmer Lambda 2S. $[\alpha]_D$: Jasco P-1050 polarimeter.

Organism. *Streptomyces* sp. DSM 14996 was isolated from a soil sample collected in 1998 at Los Gigante, Teneriffa, Spain. The strain has been deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Braunschweig, Germany. Taxonomical characterization has been carried out at the DSMZ. The strain was assigned to the genus *Streptomyces* because of its morphological appearance and characteristic chemotaxonomic features, such as LL-diaminopimelic acid in the peptidoglycan and the typical pattern of iso/anteiso-branched fatty acids. Spore chains are well-developed long spirals. Aerial mycelium is gray, substrate

Table 1. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR Data of **2** in DMSO-d_6

amino acid		^{1}H	*J*(Hz)	^{13}C	amino acid		^{1}H	*J*(Hz)	^{13}C
Gly[1]	1			168.4 (s)	Pro[11]	1			171.2 (s)
	2	4.34	[a]	42.4 (t)		2	4.27	[a]	60.8 (d)
		3.36	[a]			3	2.14	[a]	29.0 (t)
	NH	8.80	(t, not resolved)				1.59	[a]	
Leu[2]	1			172.9 (s)		4	1.73	[a]	25.3 (t)
	2	4.86	[a]	48.5 (d)		5	3.31	[a]	46.1 (t)
	3	1.95	[a]	39.5 (t)			3.08	[a]	
		0.99	(m)		Gly[12]	1			169.9 (s)
	4	1.83	[a]	23.7 (d)		2	4.05	[a]	42.6 (t)
	5	0.85	(d, 6.3)	20.6 (q)			3.33	[a]	
	6	0.90	(d, overlapped)	23.3 (q)		NH	7.51	(t, 6.0)	
	NH	8.99	(d, 8.8)		Trp[13]	1			171.0 (s)
Pro[3]	1			170.7 (s)		2	4.64	[a]	51.8 (d)
	2	4.07	(dd, not resolved)	60.6 (d)		3	3.23	(d, not resolved)	24.3 (t)
	3	1.85	[a]	28.2 (t)			2.51	[a]	
		1.48	[a]			4			110.9 (s)
	4	1.78	[a]	24.4 (t)		5	6.89	(d, not resolved)	121.6 (d)
		1.72	[a]			$NH_{ind.}$	10.76	(s, broad)	
	5	3.55	[a]	46.6 (t)		6			135.7 (s)
		3.36	[a]			7			127.2 (s)
Trp[4]	1			170.0 (s)		8	7.08	(d, 8.1)	117.6 (d)
	2	4.90	[a]	51.6 (d)		9	6.85	(t, 7.5)	117.9 (d)
	3	3.03	(dd, not resolved)	28.7 (t)		10	7.01	[a]	120.9 (d)
		2.97				11	7.26	(d, 8.1)	111.2 (d)
	4			108.9 (s)		NH	7.86	(d, 9.8)	
	5	7.05	(d, not resolved)	123.7 (d)	Asn[14]	1			172.1 (s)
	NH_{ind}	10.84	(s, broad)			2	5.08	(t, not resolved)	50.1 (d)
	6			136.0 (s)		3	1.90	[a]	39.7 (t)
	7			127.6 (s)		1.55		[a]	
	8	7.56	(d, 8.4)	118.1 (d)		4			170.1(s)
	9	6.97	(t, 7.4)	118.2 (d)		NH_2	6.66	(s, broad)	
	10	7.05	[a]	120.9 (d)			5.72	(s, broad)	
	11	7.32	(d, 8.1)	111.4 (d)		NH	8.61	(d, 9.3)	
	NH	6.20	(d, 9.4)		Thr[15]	1			172.2 (s)
Gly[5]	1			167.6 (s)		2	5.02	(t, 6.5)	55.3 (d)
	2	3.90	[a]	39.8 (t)		3	4.65	[a]	66.8 (d)
		3.33	[a]			4	1.11	(d, 6.3)	17.9 (q)
	NH	8.74	(d, 8.9)			OH	not observed		
Cys[6]	1			167.1 (s)		NH	8.86	(d, 5.8)	
	2	4.86	[a]	52.7 (d)	Pro[16]	1			173.2 (s)
	3	3.34	[a]	41.4 (t)		2	4.41	(dd, not resolved)	61.8 (d)
		2.19	[a]			3	2.13	[a]	29.0 (t)
	NH	7.19	(d 9.9)				1.45	[a]	
Pro[7]	1			171.9 (s)		4	1.71	[a]	24.2 (t)
	2	4.67	[a]	56.3 (d)			1.58	[a]	
	3	2.14	[a]	29.7 (t)		5	3.88	[a]	48.5 (t)
		1.80	[a]				3.62	[a]	
	4	1.97	[a]	25.0 (t)	Trp[17]	1			170.2 (s)
		1.88	[a]			2	4.19	(m)	55.8 (d)
	5	3.54	[a]	47.4 (t)		3	3.35	[a]	26.2 (t)
		3.50	[a]				2.97	[a]	
Ser[8]	1			170.3 (s)		4			110.5 (s)
	2	5.39	(m, not resolved)	55.8 (d)		5	7.23	(d, not resolved)	123.4 (d)
	3	3.84	(m)	65.3 (t)		$NH_{ind.}$	10.98	(s, broad)	
		3.45	[a]			6			136.2 (s)
	OH	4.59	(t, 5.8)			7			126.6 (s)
	NH	6.09	(d, 9.0)			8	7.58	(d, 8.3)	118.0 (d)
Asp[9]	1			172.6 (s)		9	7.02	[a]	118.5 (d)
	2	4.72	[a]	47.0 (d)		10	7.12	(t, 7.5)	121.2 (d)
	3	3.49	[a]	36.2 (t)		11	7.38	(d, 8.1)	111.6 (d)
		2.46	[a]			NH	7.93	(d, 5.3)	
	4			171.3 (s)	Ala[18]	1			172.6 (s)
	NH	7.69	(d, 10.4)			2	4.29	[a]	48.5 (d)
Ile[10]	1			170.6 (s)		3	1.26	(d, 7.4)	16.8 (q)
	2	3.67	[a]	64.0 (d)		NH	7.26	(d, 8.1)	
	3	1.83	[a]	32.7 (d)	Cys[19]	1			171.0 (s)
	4	1.71	[a]	27.2 (t)		2	4.55	(m)	49.2 (d)
		1.17	(m)			3	3.11	[a]	37.0 (t)
	5	0.88	[a]	10.4 (q)			2.80	(t, 13.0)	
	6	0.80	(d, 6.7)	15.1 (q)		NH	7.17	(d, 6.9)	
	NH	8.29	(s, broad)			OCH_3	3.65	(s)	52.3 (q)

[a] Multiplicity of signal unclear due to overlapping.

mycelium yellowish-grayish on oatmeal agar. Melanin is produced. D-Glucose, L-arabinose, D-xylose, L-rhamnose, raffinose, D-mannitol, inositol, D-fructose, and sucrose are utilized. Phylogenetic data were based on the comparison of the partial sequences of the 16S rRNA gene, which were determined by direct sequencing of the PCR-amplified 16S rDNA. The sequence of DSM 14966 was compared with all available *Streptomyces* sequences. The nearest phylogenetic neighbors of strain DSM 14996 were found to be *S. prasinosporus* DSM 40506^{T} and *S. coeruleofuscus* DSM 40144^{T}, showing sequence similarity values of 99.3% and 99.1%, respectively. Combining the phylogenetic data with morphological data[14] and physiological tests[15] did not enable a conclusive taxonomical assignment of strain DSM 14996 at the species level.

Screening Extract. Three milliliters of H_2O and 10 mL of MeOH were added to a freeze-dried 50 mL culture of *Streptomyces* sp. (DSM 14996). The mixture was stirred overnight. After filtration, the crude extract was purified on a Rapid Trace SPE workstation (Zymark) on an SDB 200 mg cartridge (Mallinckrodt Baker). The cartridge was washed with H_2O (5 mL) and subsequently eluted with MeOH (4 mL) followed by acidic acetone (4 mL). The MeOH and acetone fractions were combined, evaporated to dryness, and redissolved in 1.4 mL of DMSO. The solution was stored in our extract library and used for screening.

To localize the bioactivity, the extract was separated on a semipreparative scale on a Waters Alliance 2690 HPLC system equipped with a Symmetry C-18 (5 μm, 3.9 × 150 mm) column; the gradient was as described above (system 1); 30 μL of extract was injected. Detection was performed with a Waters 996 photodiode array detector. Fractions were collected at 1 min intervals into a 96-deepwell plate, evaporated to dryness, and redissolved in DMSO (30 μL) for biological testing.

Scale-up Fermentation. *Streptomyces* sp. DSM 14996 was cultivated on a 6 L scale in 1 L Erlenmeyer flasks each containing 250 mL of a medium consisting of glucose (2%), caseinpeptone (0.4%), yeast extract (0.05%), meat extract (0.4%), NaCl (0.25%), and $CaCO_3$ (0.3%). Prior to sterilization of the medium, the pH was adjusted to 7.2 with 8 N NaOH. Each flask was inoculated with 20 mL of a 48 h old preculture [medium: soybean meal (1.5%), glucose (1.5%), NaCl (0.5%), $CaCO_3$ (0.1%), KH_2PO_4 (0.03%), pH = 6.9]. The fermentation was carried out on a rotary shaker (160 rpm) for 120 h at 28 °C.

Isolation of 1. The mycelium and the culture filtrate were separated by filtration. After freeze-drying, both fractions were recombined and extracted at room temperature twice with a mixture of MeOH (2 and 1 L) and DMSO (2 × 5 mL). The extract was concentrated under reduced pressure to a final volume of about 40 mL. The part of the extract that precipitated during this process was separated by centrifugation, redissolved with DMSO (3 mL) and MeOH (6 mL), and combined with the extract solution.

The solution was purified in 1.8 mL portions by preparative HPLC on a C-18 Nova-Pack column (Waters, 6 μm, 2.5 × 10 cm i.d.) equipped with a precolumn (2.5 × 1.0 cm i.d.) to afford 222 mg of crude **1**. Elution was carried out with a gradient of 5−51% MeCN in 1 mM aqueous NH_4OAc (pH 4) in 16 min; flow rate was 20 mL/min; UV detection was at 220 nm. Final purification was performed by gel filtration: a 100 mg portion of the crude product was dissolved in a mixture of DMSO (3 mL) and MeOH (1 mL) and chromatographed on a Sephadex LH-20 column (Pharmacia, 2.5 × 70 cm i.d.) with MeOH (1.0 mL/min) as eluent to provide 54 mg of pure **1**.

Methylation of 1. Trimethylsilyldiazomethane (2 N solution in hexane, 0.048 mL, 0.096 mmol) was dissolved in MeCN/MeOH, 9:1 (0.85 mL), and added to a solution of **1** (26.7 mg, 0.0131 mmol) in DMSO (0.85 mL). After 20 h stirring at room temperature, another portion of the trimethylsilyldiazomethane hexane solution (0.048 mL) was added to the reaction mixture. The reaction was stirred for a further 20 h and checked by HPLC-MS, revealing that 75% of the acid had been converted to its methyl ester. The crude product was purified by CC on Sephadex LH-20 with MeOH to afford 12.6 mg of pure **2**.

Amino Acid Analysis. The peptide (ca. 40 μg) was hydrolyzed for 24 h at 110 °C with 6 N HCl. The amino acids were analyzed using the AccQTag method (Waters). The sample was derivatized with the AccQTag reagent in AccQTag borate buffer for 10 min at 55 °C. Amino acid analysis was performed on a Waters AccQTag (4 μm; 3.9 × 150 mm) column using fluorescence detection at 395 nm.

The L-configuration of amino acids was determined in a separate experiment as previously described.[2] The amino acids were identified after acidic hydrolysis as *n*-propyl esters by gas chromatography on a Chirasil-Val column.

Glucagon Receptor Antagonism. The cDNA coding for the human glucagon receptor was amplified by PCR from human liver mRNA using sequence-specific oligonucleotides and subsequently cloned into expression vector pcDNA3.1 (Invitrogene). Baby hamster kidney cells (BHK-21(C-13) cells (ATCC)) were transfected with the expression construct for the human glucagon receptor, and a stable transfected cell clone was isolated after selection with G-418 (Gibco).

Confluent BHK cells transfected with the cloned human glucagon receptor were preincubated in 24-well plates in KRBH buffer (10 mM Hepes, pH 7.4; 134 mM NaCl; 3.5 mM KCl; 1.2 mM KH_2PO_4; 0.5 mM $MgSO_4$; 1.5 mM $CaCl_2$; 5 mM $NaHCO_3$; 0.1% BSA; 11 mM glucose; 0.8 mM IBMX) with various concentrations of compounds for 10 min at 37 °C. Glucagon (30 pM, Wherl) was added for a further 5 min. The reactions were terminated by addition of 250 μL of 1 M HCl. cAMP was measured by using an RIA kit (NEN).

Compound 1: white, amorphous powder; $C_{95}H_{125}N_{23}O_{24}S_2$; $[\alpha]^{20}_D$ 6° (*c* 0.2, DMF); UV (MeOH) λ_{max} (log ϵ) 221 (5.07), 282 (4.28), 290 (4.23) nm; IR ν_{max} 3285, 3072, 2959, 2924, 2875, 1634, 1524, 1449, 1230, 1247 cm^{-1}; ESIMS (positive ion mode) *m*/*z* 2037.1 ($[M + H]^+$), 1018.953 ($[M + 2H]^{2+}$, calc 1018.943); HPLC (system 1) t_R 16.1 min.

Compound 2: white, amorphous powder; $C_{96}H_{127}N_{23}O_{24}S_2$; IR ν_{max} 3285, 3060, 2959, 2930, 2875, 1739, 1633, 1521, 1438, 1231 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; ESIMS (positive ion mode) *m*/*z* 2051.1 ($[M + H]^+$), 1029.969 ($[M + 2H]^{2+}$, calc 1029.951); HPLC (system 1) t_R 16.0 min.

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Supporting Information Available: ^1H and ^{13}C NMR spectra of compounds **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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