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Bioorganic & Medicinal Chemistry Letters 14 (2004) 4343–4346
Bioorganic & Medicinal Chemistry Letters

Synthesis of an immunosuppressant SQAG9 and determination of the binding peptide by T7 phage display

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Received 24 March 2004; accepted 19 May 2004

Abstract—SQAG9, a new class of immunosuppressive sulfoquinovosylacylglycerol, and its biotinylated derivatives have been synthesized. A T7 Phage library, composed of random cDNA fragments from *Drosophila melanogaster*, displayed a possible binding peptide of 14 amino acids. The immobilized synthetic peptide on a sensor chip showed a dissociation constant of $K_D = 1.5 \times 10^{-6}$ against SQAG9 in a surface plasmon resonance experiment.

1. Introduction

Sulfoquinovosylacylglycerol (SQAG) is a C-6 sulfonic analogue of D-glucose bonded to a fatty acid mono- or diester of glycerol. We recently carried out a screen for inhibitors of DNA polymerases and identified several natural derivatives of SQDG (sulfoquinovosyldiacylglycerol) and SQMG (sulfoquinovosylmonoacylglycerol).[1–3] Since DNA polymerases are essential for DNA replication and repair, inhibition of this activity will lead to cell death. In 1997, Sahara et al. reported that SQMG effectively suppressed the growth of solid tumors derived from human lung cancer (adenocarcinoma cell line A-549) in nude mice.[4,5] Synthetic work on the α-anomers of SQDG and SQMG and their biological activities were reported elsewhere.[6–8] However, anomeric β-isomers of these compounds have not yet been found from natural sources. We evaluated the immunosuppressive effect of a synthetic β-isomer SQAG9 {3-*O*-(6-deoxy-6-sulfono-β-D-glucopyranosyl)-1,2-di-*O*-stearoylglycerol, **1**}, which was designated as β-SQDG by mixed lymphocyte reaction (MLR) and rat allogeneic skin graft. SQAG9 inhibited human MLR in a dose-dependent manner without overt cytotoxicity and prolonged rat skin allograft rejection in vivo.[9,10] In contrast, α-SQDG, α-SQMG, and β-SQMG displayed no biological activity in these tests. SQAG9 did not alter the expression of either major histocompatibility antigen complex (MHC) class I or class II molecules on the cell surface of the stimulator cells and antigen-presenting cells (Fig. 1).

Although SQAG9 inhibited the binding among allogeneic lymphocytes, the expression of known cell surface accessory and adhesion molecules, such as CD4, CD28, leukocyte function-associated antigen 1, intercellular adhesion molecule 1, and CTLA-4, was not affected by the treatment. Since neither binding proteins nor

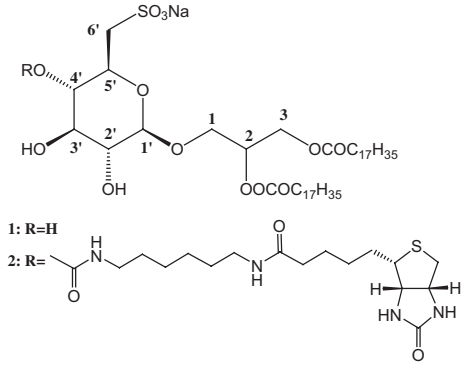

Figure 1. Structure of SQAG9 (**1**) and the biotinylated derivative (**2**).

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doi:10.1016/j.bmcl.2004.05.085

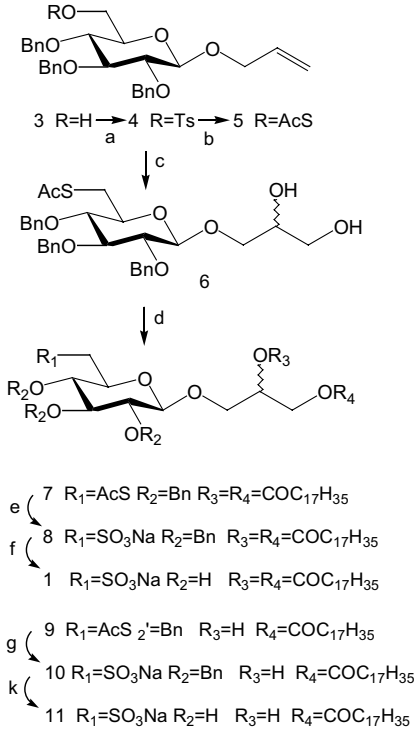
receptors of SQDG and SQMG were identified, a phage display cloning method was used to identify a peptide fragment(s) involved in the binding process. Here we report synthetic work on the biotinylated derivatives of SQAG9 (**2**) and the results of an affinity guided phage display experiment.[11,12]

1.1. Synthesis of SQAG9

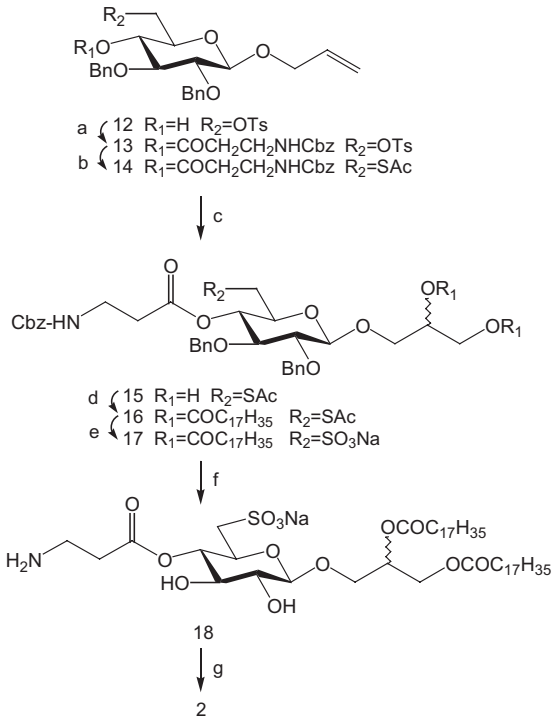
The synthetic route for the β-anomeric isomers of SQDG and SQMG are essentially the same as those of the α-anomers[6,8] (Scheme 1). Briefly, the treatment of 1-*O*-allyl-2,3,4-tri-*O*-benzyl-β-D-glucose (**3**)[13] with TsCl in the presence of DMAP in pyridine gave the 6-*O*-tosylate (**4**). Reaction with AcSK in EtOH gave **5**. Oxidation of the olefin with OsO_4 in the presence of NMO, in a solution of water and *tert*-BuOH, afforded a diastereomeric diol derivative **6** in a ratio of 1:1 determined by ^1H NMR.[14] The diol (**6**) gave the monoester (**7**) and diester (**9**) derivatives, in a ratio of 31:69, respectively, by a condensation reaction with stearic acid (2.0 equiv) in the presence of EDCI (1.2 equiv) and DMAP (catalytic amount) in CH_2Cl_2. After separating **8** and **10** by silica gel column chromatography, oxidation of the acetylthio derivative with oxone gave the sulfonic analogue of glucose. The protective group was subsequently removed, by reductive cleavage with Pd/C in an atmosphere of hydrogen, to yield **1** (SQAG9) and **11**, respectively.

1.2. Biotinylated derivatives of SQAG9

The stereochemistry at C-4 of the glucose moiety did not affect the DNA polymerase inhibitory activity in our previous structure–activity relationships (SAR) study. For the binding protein assay, we prepared a SQAG9 derivative, which was biotinylated at C4′ on the carbohydrate ring (Scheme 2). 1-*O*-Allyl-2,3-di-*O*-benzyl-6-*O*-tosyl-β-D-glucose (**12**) was prepared from readily available 1-*O*-allyl-2,3-di-*O*-benzyl-β-D-glucose by treatment with TsCl in the presence of DMAP in pyridine. The condensation reaction of **12** and Cbz–alanine in CH_2Cl_2 gave **13**, which was converted to the acetylthio derivative with AcSK in EtOH to give **14**. Oxidation of the olefin by OsO_4 in the presence of NMO, in a solution of water and *tert*-BuOH, afforded a diastereomeric diol derivative **15**. Treatment of **15** in pyridine with an excess amount of stearoyl chloride in CH_2Cl_2 gave the diester (**16**). Oxidation of the acetylthio derivative with oxone gave the sulfonic analogue of glucose (**17**). The protective group was then removed, by reductive cleavage with Pd/C in an atmosphere of hydrogen, to give **18**. Treatment of **18** with biotin 4-nitrophenyl ester, in the presence of Et_3N in DMF, gave the biotinylated derivative of SQAG9 (**2**).



Scheme 1. Syntheses of SQAG9 (**1**) and its monoester derivative (**11**). Reagents and conditions: (a) TsCl, pyridine, DMAP, rt (89.8%); (b) AcSK, EtOH, reflux (86.1%); (c) OsO_4, NMO, *tert*-BuOH, water, rt (74.7%); (d) $C_{17}H_{35}COOH$, EDCI, DMAP, CH_2Cl_2, rt, 8 (91.0%, diester 30.9%; monoester 69.1%); (e) oxone, AcOK, AcOH (83.4%); (f) H_2, Pd/C, EtOH, rt (52.6%); (g) oxone, AcOK, AcOH (85.9%); (h) H_2, Pd/C, EtOH, rt (80.9%).



Scheme 2. Synthesis of the biotinylated derivative of SQAG9 (**2**). Reagents and conditions: (a) Cbz-β-Ala, EDCI, DMAP, pyridine, CH_2Cl_2, rt (89%); (b) AcSK, DMF, 80 °C (81%); (c) OsO_4, NMO, *tert*-BuOH, water, rt (72%); (d) $C_{17}H_{35}COCl$, pyridine, CH_2Cl_2, rt (quant); (e) oxone, AcOK, AcOH, rt (45%); (f) H_2, Pd/C, EtOH, $CHCl_3$, rt (16%); (g) biotin 4-nitrophenyl ester, Et_3N, DMF, rt (98%).

1.3. Selection and sequence analysis of high affinity phage

A biotinylated derivative of SQAG9 (**2**) was immobilized on a streptavidine-coated well, and then incubated with a T7 phage library composed of cDNA fragment inserts from *Drosophila melanogaster*. False positives were removed by a series of washes using different buffers. A mixture of phage particles, apparently exhibiting affinity to biotinylated SQAG9, was then subjected to a further four rounds of screening. The phage titer of the washed and eluted solutions was checked at each round. The cDNA inserts from recovered phage were amplified by the polymerase chain reaction (PCR) and analyzed by agarose gel electrophoresis. After the fifth round of screening, the selected phage DNA encoded the 14 amino acid sequence, NSRMRVRNATTYNS. No other specific peptides were detected in this experiment.

1.4. Surface plasmon resonance (SPR) analysis

A surface plasmon resonance (SPR) biosensor chip was employed to analyze the interaction between SQAG9 (**1**) and a synthetic peptide, NSRMRVRNATTYNS. Four different concentrations of 1 (29.4, 23.5, 17.6, and 7.4 μM) were used for the binding analysis to a conjugated peptide on a CM5 sensor chip. SQAG9 bound to the peptide with a high rate of association and a low rate of dissociation. The kinetic constant for the interaction was determined from the SPR association and dissociation curves obtained by varying the concentration of **1**. The determined K_D value of 1.5×10^{-6} indicated that SQAG9 tightly bound the peptide. Comparison of the peptide sequence against the SwissProt protein database indicates homology to a small inducible cytokine. A full analysis of the binding between SQAG9 and the chemokine and its immunosuppressant activity will be published elsewhere (Fig. 2).

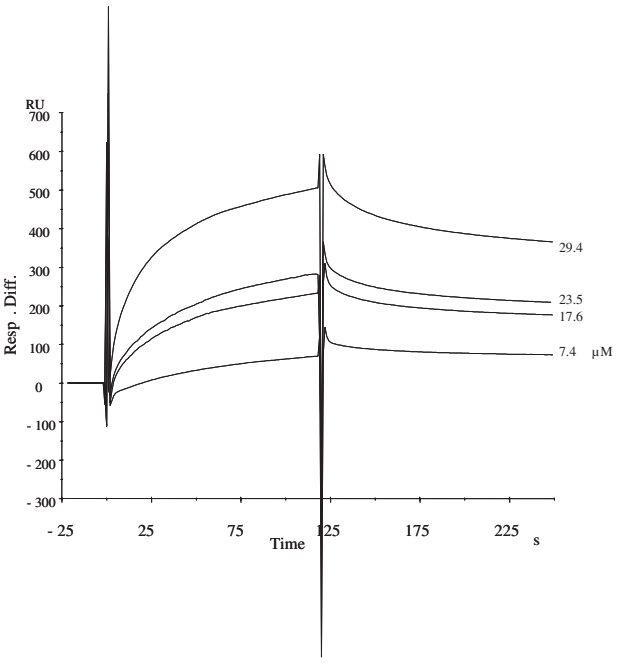


Figure 2. SPR analysis of the binding of SQAG9 to the immobilized peptide on a CM5 sensor chip.

2. Experimental

2.1. NMR and MS data

Synthetic compounds were characterized by NMR and ESIMS. Mass spectral data was collected on an ABI Q-Star, operated in negative ion mode, using a glycerol matrix. NMR data was obtained on a BRUKER DRX-400 (400 MHz for ^{1}H) in either CD_3OD or a mixture of deuterium solvents. Chemical shifts were expressed as δ ppm from TMS as internal standard. Optical rotation values were determined on a JASCO polarimeter P1010.

2.2. Compound 1

[3-*O*-(6-Deoxy-6-sulfono-β-D-glucopyranosyl)-1-*O*-stearoyl-glycerol sodium salt]: $[\alpha]_D^{20}$ +0.04 (*c* 1.03, MeOH); ^{1}H NMR (400 MHz, CD_3OD) δ 4.30–4.28 (m, 1H, H1′), 4.10–3.56 (m, 6H, H5′, H1a, H1b, H2, H3a, and H3b), 3.37–3.31 (m, 2H, H3 and H6a), 3.21–3.16 (m, 1H, H2′), 3.11–3.05 (m, 1H, H4′), 2.94–2.88 (m, 1H, H6′), 2.30–2.32 (m, 2H, $COCH_2$), 1.62–1.58 (m, 2H, $COCH_2CH_2$), 1.28 (br, 28H, $-CH_2-$), 0.91–0.87 (m, 3H, Me); HRMS calcd for $C_{27}H_{51}O_{11}S$ $(M-Na)^-$ 583.3157, found 583.3186.

2.3. Compound 11

[3-*O*-(6-Deoxy-6-sulfono-β-D-glucopyranosyl)-1,2-di-*O*-stearoyl-glycerol sodium salt]: $[\alpha]_D^{20}$ −0.44 (*c* 0.98, $CHCl_3$–MeOH–H_2O, 70:30:4); ^{1}H NMR (400 MHz, $CDCl_3$+CD_3OD+D_2O) δ 5.31–5.25 (m, 1H, H2), 4.45–4.38 (m, 1H, H3a), 4.34–4.32 (m, 1H, H1′), 4.26–4.16 (m, 1H, H3b), 4.05–4.00 (m, 1H, H1a), 3.78–3.72 (m, 2H, H1b and H5′), 3.46–3.40 (m, 1H, H3′), 3.35–3.31 (m, 1H, H6′a), 3.29–3.24 (m, 2H, H2′ and H4′), 3.11–3.06 (m, 1H, H6′), 2.37–2.27 (m, 4H, $COCH_2$), 1.64–1.58 (m, 4H, $COCH_2CH_2$), 1.28 (br, 56H, $-CH_2-$), 0.91–0.87 (m, 6H, Me); HRMS calcd for $C_{45}H_{85}O_{12}S$ $(M-Na)^-$ 849.5767, found 849.5844.

2.4. Compound 2 (Biotinylated derivative of SQAG9)

$[\alpha]_D^{20}$ −0.38 (*c* 0.34, $CHCl_3$–MeOH–H_2O, 70:30:4); ^{1}H NMR (400 MHz, $CDCl_3$+CD_3OD) δ 5.34–5.26 (m, 1H, H2), 4.77–4.44 (overlapping solvent peak, 1H, H4′), 4.55–4.52 (m, 1H, H8‴), 4.48–4.42 (m, 1H, H3a), 4.38–4.35 (m, 2H, H1′ and H7‴), 4.21–4.16 (m, 1H, H3b), 4.07–4.03 (m, 1H, H1a), 3.97–3.92 (m, 1H, H5′), 3.82–3.75 (m, 1H, H1b), 3.62–3.57 (m, 1H, H3′), 3.52–3.45 (m, 2H, H3″a and H3″b), 3.38–3.31 (overlapping solvent peak, 1H, H2′), 3.23–3.18 (m, 1H, H6‴), 3.09–3.01 (m, 2H, H6′a and H6′b), 2.97–2.93 (m, 1H, H9′a), 2.66–2.72 (m, 1H, H9‴b), 2.66–2.63 (m, 2H, H2″a and H2″b), 2.37–2.31 (m, 4H, $COCH_2$), 2.26–2.23 (m, 2H, H2‴),

1.78–1.60 (m, 6H, $COCH_2CH_2$, H3‴a, H3‴b, H4‴a, and H4‴b), 1.48–1.40 (m, 2H, H3‴a and H3‴b), 1.28 (br, 56H, $-CH_2-$), 0.91–0.88 (m, 6H, Me).

2.5. Construction of T7 phage library from *D. melanogaster*

Poly(A)+RNA, random primers, 5′-methylated dCTP, T4 DNA polymerase, *Eco*RI//*Hind*III linkers, *Eco*RI, *Hind*III, T7Select10-3b vector, and T7 packaging extracts were used.[15] Aliquots (80 μg) of total RNA, extracted from *D. melanogaster* Kc cells, were used to construct the cDNA library. Oligotex-dt30 <super> was used for a second round of isolation, with minimal loss of material, to produce poly(A)+RNA suitable for random primed cDNA synthesis. cDNA synthesis was primed with 4 μg of poly(A)+RNA using random primers. 5′-Methylated dCTP was then incorporated into both strands, without extraction or precipitation between the first and second strand synthesis. The cDNA was then treated with T4 DNA polymerase to generate flush ends and ligated with directional *Eco*RI/*Hind*III linkers. Following linker ligation, the cDNA was digested sequentially with *Hind*III and *Eco*RI, then inserted into *Eco*RI/*Hind*III digested T7Select10-3b vector arms. The cDNA was cloned into the *Eco*RI/*Hind*III sites of the T7 phage 10-3B vector and packaged into phage.[16] The titer of this library was 1.6×10^{10} pfu/mL.

2.6. Biopanning and DNA sequence analysis

Following five rounds of selection, 47 plaques were randomly picked from LB plates and each dissolved in phage extraction buffer (washing: 100 mM Tris–HCl, pH 8.0, eluting: 1.5 M NaI). The candidate clones were amplified and used to check their affinity to the biotinylated derivative of SQAG9 (**2**). In order to disrupt the phages, the extract was heated at 65 °C for 10 min. After amplifying the phage DNA, the fragments were purified with ExoSAP-IT and EtOH precipitated. The PCR fragments were sequenced on an ABI Prism3100 Genetic Analyzer. Based on the sequence results, the amino acid sequence displayed on the T7 phage capsid was determined.

2.7. SPR Analysis

Binding analysis between SQAG9 and the peptide was performed using a Biosensor Biacore 3000. The synthetic peptide (332 μg/mL, 170 μL) in coupling buffer (10 mM Na_2CO_3–$NaHCO_3$, pH 8.5) was injected over a CM5 sensor chip at 10 μL/min to capture the protein on the carboxymethyl dextran matrix of the chip by using amine coupling. The surface was activated by injecting a solution containing 0.2 M EDC and 50 mM NHS for 14 min. The peptide was injected and the surface was then blocked by injecting 1 M ethanolamine at pH 8.5 for 14 min. This reaction immobilized about 1500 response units (RU) of the peptide. Binding analysis of SQAG9 was performed in running buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% Surfactant P 20, 8% DMSO) at a flow rate of 20 μL/min at 25 °C. BIAEVALUATION 3.1 software was used to determine the kinetic parameters.

Acknowledgements

Grant of this work was partially supported by Uehara Memorial Life Science Foundation, Toyo Suisan Co. Ltd, and Grant-in-Aid of JSPS.

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