Isolation and Structure Determination of Sulfonoquinovosyl Dipalmitoyl Glyceride, a P-Selectin Receptor Inhibitor from the Alga *Dictyochloris fragrans*

Jerzy Golik,[†] Joyce K. Dickey,[†] Gordon Todderud,[‡] Deborah Lee,[‡] Julie Alford,[‡] Stella Huang,[†] Steven Klohr,[†] David Eustice,[⊥] Alejandro Aruffo,[§] and Michele L. Agler*[,†]

Bristol-Myers Squibb Pharmaceutical Research Institute, 5 Research Parkway, Wallingford, Connecticut 06492, 100 Forest Avenue, Buffalo, New York 14213, 3005 First Avenue, Seattle, Washington 98121, and Bayer Pharmaceutical, Morgan Lane, West Haven, Connecticut 06516

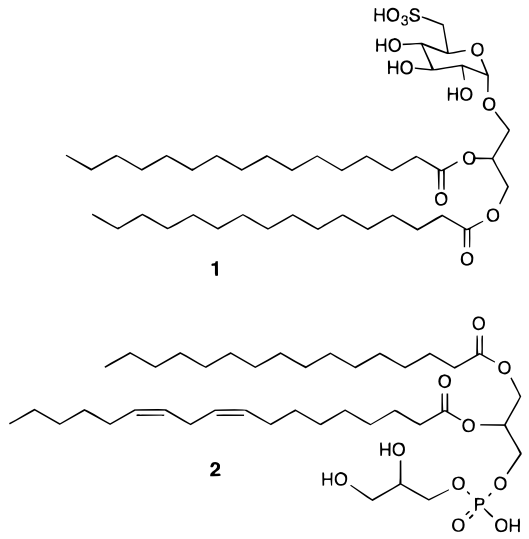
Received October 4, 1996[⊗]

Bioassay-guided fractionation of the marine alga *Dictyochloris fragrans* led to the isolation and identification of sulfonoquinovosyl dipalmitoyl glyceride (**1**). The structure of **1** was determined by a combination of spectroscopic methods. On the basis of P-selectin inhibition assays (i.e., P-selectin−IgG ELISA, cell binding assay of receptor globulin, and platelet:HL60 adhesion, it was demonstrated that **1** selectively blocks the P-selectin−ligand interaction in vitro and could be considered a lead compound for synthetic modification in order to design more potent inhibitors of cell adhesion processes that play important roles in development of inflammatory-mediated disease states.

P-selectin, a member of the selectin family of adhesion molecules, is a 140-kd glycoprotein that is located in the cytoplasmic granules of platelets and endothelial cells.[1,2] Upon initiation of an inflammatory process, it is quickly translocated to the plasma membrane where it mediates leukocyte rolling on the endothelium.[3] Several ligands with distinct affinities have been identified for P-selectin, including sialylated and sulfated fucooligosaccharides such as sialyl Lewis^X, (SLe^X),[4,5] sulfatides,[6] and several glycoproteins,[7,8] but the exact ligand structure remains to be determined. A carbohydrate recognition site has been implicated in the selectin-mediated adhesion. The discovery of novel selectin antagonists will be useful in evaluating chronic and acute inflammatory conditions and may ultimately lead to useful therapeutic antiinflammatory agents. A high-throughput screen was developed to identify inhibitors of P-selectin binding. In the course of our screening effort, crude alcohol extracts from the marine alga *Dictyochloris fragrans* Vischer ex Starr (Chlorophyceae) exhibited considerable activity in our P-selectin−IgG-based primary screen. Consequently, we developed a P-selectin-guided isolation procedure that provided two polar lipids, namely, sulfonoquinovosyl dipalmitoyl glyceride (**1**) and phosphatidylglycerol (**2**), both of which were active in the primary screen. Only **1** has shown selectivity in the secondary assays, however. Although both compounds have been previously isolated from various natural sources,[9−11] the selective inhibition of P-selectin by **1** has not been reported.

The dry alga was extracted either with MeOH or with 85% EtOH, and the crude extract was purified as described in the Experimental Section yielding two compounds bioactive in the primary assay congeners **1** and **2**.

The molecular weights and elemental compositions of **1** ($C_{41}H_{78}O_{12}S$) and **2** ($C_{40}H_{75}O_{10}P$) were determined by low- and high-resolution FABMS using positive and negative ionization modes. Their structures were readily elucidated by analysis of the NMR data. Based on the ^{1}H (500 MHz) and ^{13}C (125 MHz) spectra, including homonuclear decoupling, NOE difference, COSY, HETCOR, and DEPT experiments, all chemical shifts were assigned. In addition, ^{31}P (121.5 MHz) NMR of **2** confirmed the presence of the phosphate group linked to two glycerol residues. These NMR data are fully consistent with proposed structures for **1** and **2**. The chirality of the glycerol units remains to be determined. In our investigation, the amount of the material was not sufficient to complete this study.



Bioassay-guided fractionation of the algal extract was initially followed using the P-selectin−IgG ELISA assay. Sulfatides, used as the starting point in the assay, have been shown to inhibit the P-selectin adhesion in human PMN[6] and U937[8] cells as well as having in vivo protective effects in a variety of antiinflammatory animal models.[12−14] Table 1 shows that both **1** and **2** inhibited P-selectin binding to sulfatides in a dose-dependent manner, with IC_{50} values of 5 and 1 μM, respectively. As a control, phosphatidylethanolamine was tested and found to be inactive.

* To whom correspondence should be addressed. Phone: (203) 284-7506. FAX: (203) 284-6417. E-mail: Agler#m#_michele_l#d#@msmail.bms.com.
[†] Bristol-Myers Squibb, Wallingford.
[‡] Bristol-Myers Squibb, Buffalo.
[§] Bristol-Myers Squibb, Seattle.
[⊥] Bayer Pharmaceutical.
[⊗] Abstract published in *Advance ACS Abstracts,* March 15, 1997.

S0163-3864(96)00676-3 CCC: $14.00

Table 1. P-Selectin−IgG ELISA Assay

	IC_{50} (μM)[a]
compound **1**	5
compound **2**	1
phosphatidylethanolamine	NA[b]

[a] IC_{50} values were determined from competition ELISA as described in Experimental Section and represent the concentration of the compound that caused a 50% reduction in P-selectin binding to the immobilized sulfatide. [b] NA = not active.

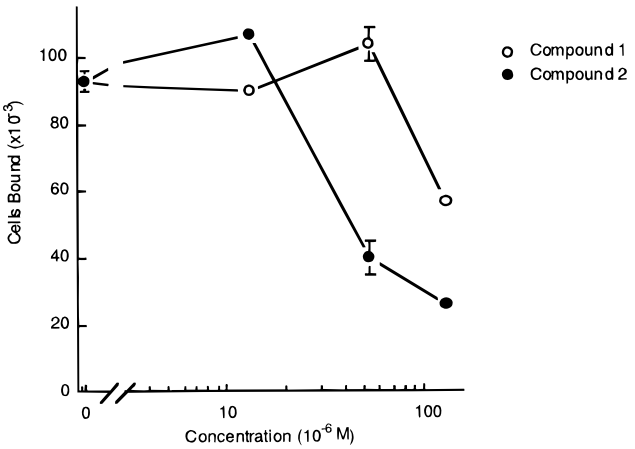


Figure 1. Inhibition of HL60 cell adhesion to immobilized P-selectin Rg by compound **1** and compound **2**.

Table 2. P-Selectin-Dependent Platelet:HL60 Cell Adhesion (Inhibition of binding by isolated natural products)

conditions	%HL60 cells containing bound platelets	% inhibition of binding
activated platelets (apl)	57.7	
apl + EDTA	14.6	
compound **1**	47.3	24
compound **2**	65.4	0

When these compounds were tested in the HL60:P-selectin Rg assay (Figure 1), only compound **1** was active, with an IC_{50} value of 40 μM. Compounds **1** and **2** were further evaluated for their ability to inhibit the P-selectin dependent binding of activated platelets to HL60 cells by flow cytometry (Table 2). Compound **1** showed 24% inhibition of binding, whereas **2** was inactive.

Based on the results from these three assays, it can be concluded that sulfonoquinovosyl dipalmitoyl glyceride (**1**) is able to block P-selectin adhesion to its ligand in vitro. Recently, it has been reported that glycyrrhizin, a natural product from licorice, blocked selectin binding to SLe^X.[15] In vivo antiinflammatory activity was increased by substitituting fucose for the 2-glucuronic acid residues in glycyrrhizin.[15] A correlation of the in vivo with the in vitro data using novel sialyl $Lewis^X$ conjugates was demonstrated for selection-mediated adhesion by looking at the effect of endotoxins on leukocyte adherence to the endothelium.[16] Studies aimed at synthetic modifications of **1**, in order to design a more potent antagonist, would benefit further evaluation. Development of a selective inhibitor of P-selectin binding that can interfere in vivo will be a useful tool in further understanding the cell adhesion process and would have therapeutic potential in a variety of inflammatory-mediated disease states.

Experimental Section

General Experimental Procedures. The FABMS was obtained on a Finnigan TSQ-70 mass spectrometer in the positive-ion and negative-ion modes using a glycerol matrix with NaI and KI additions. High resolution measurements were taken on Kratos MS-50 instrument using CsI/glycerol as an external standard. NMR spectra were recorded in CD_3OD (99.8% of deuterium, Cambridge Isotope Laboratories) solution on Bruker AM-500 spectrometer. ^{1}H- and ^{13}C-NMR spectra were measured at 500 MHz and 125 MHz, respectively. ^{31}P-NMR spectra were recorded on Bruker AM-300 instrument at 121.5 MHz. Standard Bruker pulse sequences were used for ^{1}H−^{1}H COSY, ^{1}H−^{13}C HETCOR, DEPT, HMBC, and NOE-difference experiments. TLC was conducted on Kieselgel 60 F_{254} (Merck) 25−40 mm for flash columns and 20- × 20- × 0.5-cm plates with preloading zone. The spots were detected by spraying with H_2O for preparative workup (hydrophobic zones) or by dipping in 20% NH_4HSO_4 solution and heating at 200 °C for analytical scale.

Plant Material. The dry crushed alga *D. fragran* (24 g) and its 85% EtOH extract, 0.6 g, were provided by Bio-Technical Resources, Manitowoc, WI. All samples were stored dry, under nitrogen, at −20 °C.

Extraction and Isolation. The dried alga (23 g) was extracted with MeOH (750 mL) at room temperature, and the solvent was evaporated to dryness under reduced pressure at 25−30 °C, providing 14 g of the residue. A 1-g portion of the residue was subjected to a standard Kupchan partition protocol[17] using the following sequence of solvents: hexane, CCl_4, and $CHCl_3$. The $CHCl_3$ fraction (49 mg) was further subjected to flash chromatography (10 mL of dry Si gel, i.d. = 2.0 cm), with the following step gradient: 20 mL $CHCl_3$−MeOH (10:0.5, v/v), 20 mL $CHCl_3$−MeOH (10:1, v/v), 20 mL $CHCl_3$−MeOH (10:2, v/v), and 150 mL $CHCl_3$−MeOH−H_2O (120:45:8, v/v). Fractions of 7−9 mL were collected, providing 2.4 mg of the active material localized in fractions 7 and 8. The final purification was achieved on a preparative TLC plate (20 × 20 × 0.05 cm) in $CHCl_3$−MeOH (2:1, v/v). Two active compounds were obtained in sub-milligram quantities. The procedure was scaled up to 10 g of the crude extract, yielding 2.1 mg of compound **1** and 1.8 mg of compound **2**.

General Bioassay Procedures. The construction of soluble fusion proteins of the extracellular domain of P-selectin has been described.[6] This protein was purified from the medium of transfected COS cells. The protein contains the human lectin domain, EGF domain, and two complement repeats of the selectin fused to the hinge, CH1 and CH2 domains of human IgG1.

P-Selectin−IgG ELISA. Falcon Probind 96-well plates were incubated with 0.1 μg of sulfatides in 50 mL of MeOH and allowed to air dry overnight at room temperture. The plates were preblocked by incubation with HNC buffer (20 mM Hepes, 100 mM NaCl, 1 mM $CaCl_2$ pH 8.0) containing 5% BSA for 1.5 h. The P-selectin fusion protein was preincubated with anti-human IgG−HRPO conjugate (Fisher Scientific) to a final concentration of 100 ng/mL and 1:10 000 dilution, respectively, in HNC buffer containing 1% BSA for 30 min at 4 °C. Subsequently, the plates were washed with the HNC buffer, and 10 μL of inhibitors in 10% DMSO

were added along with 90 μL of the P-selectin−HRPO complex and incubated at 37 °C for 45 min. After incubation, the plates were washed 3 times with the HNC buffer in a Bio-Tek plate washer, and the chromogenic substrate TMB (3,3′,5,5′-Tetramethyl-benzidine) was added to the plates. The reaction was stopped in the linear range with 3 N H_2SO_4, and an endpoint measurement was taken at 450/650 nm in a Thermomax plate reader (Molecular Devices).

Platelet:HL60 Adhesion Assay. Binding of activated platelets to HL60 cells: Human blood was drawn into citrate-containing vacutainer tubes layered over 1-step platelets (Nycoprep 1.063 from Accurate Chemical) and centrifuged at 350 × *g* for 20 min. The platelet layer was collected and washed in 2 volumes of THEB buffer (Tyrode's salts, 5 mM HEPES, 10 mM EDTA, and 0.2% BSA) by centrifugation at 1200 × *g* for 10 min. The platelet pellet was resuspended in THEB buffer and rested for 1 h at room temperature, then fluorescently labeled by incubation with 10 mM of calcein acetoxymethyl ester (Molecular Probes) at 37 °C for 15 min. The labeled platelets were pelleted and resuspended in THB (same buffer as THEB except without EDTA) and activated with thrombin (2 units/mL) at 10^8 cells/mL for 10 min at 37 °C. HL60 cells and platelets were fixed in 1% formalin for 30 min. The HL60 cells were then added to the platelets and incubated at a ratio of 5 platelets per HL60 cell in the presence of the indicated concentration of inhibitor for 30 min. The adhesion was determined on a FACScan flow cytometer, by analyzing the forward scatter and fluorescent intensity of the mixed population. The platelets, which appear at lower forward scatter values, were excluded by gating on only the higher forward scatter HL60 cells present. The HL60 cells with high fluorescent intensity contain bound platelets. The percentage of HL60 cells in fluorescent and nonfluorescent populations was determined and plotted as a function of inhibitor concentration.

Cell-Binding Assay of Receptor Globulin (RG). The assay for cell binding to immobilized selectin receptor globulin was performed as described.[8] Briefly, the wells of a 96-well dish (Corning) were coated overnight with anti-human Fc antibody diluted into 50 mM of Tris pH 9.1 buffer, blocked with 1% nonfat dry milk in DPBS, and allowed to bind selectin Rg. Cells were labeled with 10 mM of calcein for 30 min at 3 × 10^7 cells/mL at room temperature. The blocked Rg-bound wells were rinsed twice, and labeled cells were added for 30 min at room temperature. Unbound cells were removed by aspiration, and the wells were washed three times. Fluorescence in each well was determined using a Millipore Cytofluor fluorescent plate reader. Inhibitors were prepared by dissolution to a final concentration of 20 mg/mL in DMSO, diluted in DPBS to 2 mg/mL and briefly sonicated prior to use. When inhibitors were tested, the Rg-coated wells were preincubated at room temperature for 15 min with the inhibitor; and 200 000 cells were added to yield the final indicated inhibitor concentration in 160 μL of DPBS. In each analysis, a vehicle control was utilized to assess the role of DMSO, which did not have a significant effect on binding or membrane integrity at the maximum final concentration of 0.5 percent by volume.

Acknowledgment. The authors wish to thank Mr. Montagu Cadiz of the Analytical Department for mass spectrometry measurements; Dr. I. Bursuker for cytoxicity testing; the Natural Products dereplication group and Drs. J. O'Sullivan and K. Kodukula for critical reading and editing the manuscript.

Supporting Information Available: Details of the isolation and structure elucidation of compounds **1** and **2** (3 pages). Ordering information is given on any current masthead page.

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NP9606761