Isolation of Three High Molecular Weight Polysaccharide Preparations with Potent Immunostimulatory Activity from *Spirulina platensis*, *Aphanizomenon flos-aquae* and *Chlorella pyrenoidosa*

Nirmal Pugh[1], Samir A. Ross[1, 2], Hala N. ElSohly[2], Mahmoud A. ElSohly[2, 3], David S. Pasco*[, 1, 2]

[1] Department of Pharmacognosy, School of Pharmacy, University of Mississippi, University, Mississippi, USA
[2] National Center for Natural Products Research, School of Pharmacy, University of Mississippi, University, Mississippi, USA
[3] Department of Pharmaceutics, Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi, University, Mississippi, USA

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Abstract: This research describes the identification of three new high molecular weight polysaccharide preparations isolated from food-grade microalgae that are potent activators of human monocytes/macrophages: "Immulina" from *Spirulina platensis*, "Immunon" from *Aphanizomenon flos-aquae*, and "Immurella" from *Chlorella pyrenoidosa*. These polysaccharides are structurally complex and have estimated molecular weights above ten million daltons. All three polysaccharides are highly water soluble and comprise between 0.5% and 2.0% of microalgal dry weight. Immunostimulatory activity was measured using a transcription factor-based bioassay for nuclear factor kappa B (NF-kappa B) activation in THP-1 human monocytes/macrophages. Using this system the EC_{5a} values for these microalgal polysaccharides are between 20 and 110 ng/ml (about 10pM). THP-1 activation was confirmed by measuring immune cytokine mRNA induction using reverse transcriptase-polymerase chain reaction (RT-PCR). Each polysaccharide substantially increased mRNA levels of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). These polysaccharides are between one hundred and one thousand times more active for *in vitro* monocyte activation than polysaccharide preparations that are currently used clinically for cancer immunotherapy.

Key words: *Aphanizomenon flos-aquae* (Nostocaceae), *Spirulina platensis* (Oscillatoriaceae), *Chlorella pyrenoidosa* (Oocystaceae), polysaccharide, THP-1 monocytes, nuclear factor kappa B.

Introduction

During the last several decades there has been an increasing interest in the commercial production of food-grade microalgae for human consumption. Among the various microalgae that have been explored for their commercial potential, *Spirulina* species, *Chlorella* species and *Aphanizomenon flos-aquae* are three major types that have been successfully produced and that are in widespread use.

Studies on the consumption of food-grade microalgae have reported enhanced immune function in both animals and humans. Oral administration of *Chlorella vulgaris* has been correlated with enhanced granulocyte-macrophage progenitor cells in mice infected with *Listeria monocytogenes* (1). Dietary *Spirulina* species increases macrophage phagocytic activity in chickens (2) and exhibits chemopreventive effects in humans (3). Human consumption of *Aphanizomenon flos-aquae* has been reported to produce changes in immune cell trafficking and enhanced immune surveillance (4). The active components for all these effects have not been conclusively established.

In the present study we have identified robust macrophage stimulating activity in the crude extracts of *Spirulina platensis*, *Aphanizomenon flos-aquae*, and *Chlorella pyrenoidosa*. Our objective was to isolate and characterize the compound(s) responsible for this activity. Macrophage activation was evaluated using a luciferase reporter gene based bioassay where luciferase expression is driven by the binding of NF-kappa B. The activation of transcription factor NF-kappa B coordinates gene expression and regulates many immune and inflammatory responses in activated monocytes/macrophages (5).

Materials and Methods

Materials

Freeze-dried microalgae were purchased from the following sources: *Spirulina platensis* (Lot No. B16933, MISS accession No. 63118) from Triarco Industries, Inc. (Wayne, NJ), distributed through General Nutrition Corporation; *Aphanizomenon flos-aquae* (Lot No. 0110FA, MISS accession No. 63116) from Cell Tech (Klamath Falls, OR); and, *Chlorella pyrenoidosa* (Lot No. VP0978, MISS accession No. 63117) from Sun Chlorella (Torrance, CA). MISS accession numbers refer to voucher specimens deposited at the Pullen Herbarium (MISS), Department of Biology, The University of Mississippi, University MS 38677. Bacterial lipopolysaccharide (*E. coli*, serotype 026:B6) and polymyxin B were obtained from Sigma Chemical Co. Carrington Laboratories Inc. (Irving, TX) provided two different preparations of acemannan: *Aloe vera* mucilaginous polysaccharide (AVMP, Lot. No. 11586) and Manapol (Lot. No. 116018). Schizophyllan polysaccharide was a gift from Dr. David Williams. The polysaccharide lentinan was also a gift from Dr. Yukiko Maeda (Lot. No. 2L832). JHS Natural Products (Eugene, OR) generously provided the polysaccharide krestin (PSK).

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THP-1 human monocytes were obtained from American Type Culture Collection (Rockville, MD). LucLite™ luciferase reporter gene assay kit was purchased from Packard (Downers Grove, IL). NF-kappa B plasmid construct (pBIIXLUC) was a gift from Dr. Riccardo Dalla-Favera that contains two copies of NF-kappa B motif from HIV/IgK (6). Reverse Transcriptase (RT)-PCR kits were obtained from Promega (Madison, WI) and for RNA isolation the TRI Reagent® system was used (Molecular Research Center, Inc., Cincinnati, OH). RT-PCR primers for IL-1β, TNF-α and GAPDH were purchased from Integrated DNA Technologies, Inc. (Coralville, IA).

Isolation procedure

Freeze-dried microalgae (35 g *Spirulina platensis*, 125 g *Aphanizomenon flos-aquae* and 35 g *Chlorella pyrenoidosa*) were extracted three times with 70% ethanol at 40°C, 4 hours each time. Ethanol extracts were evaporated to dryness and then solvent partitioned between water and chloroform (1 : 1), followed by further partitioning of the water layers with *n*-butanol (water : *n*-butanol, 63 : 37). The water layers from the second solvent partition was subjected to alcohol precipitation (water : methanol : ethanol, 1 : 2 : 3) at −80°C for 24 hours. Precipitatable materials were passed through an ultrafiltration device with a 100,000 molecular weight cut-off polyethersulfone membrane (Centricon Plus-20 from Millipore, Bedford, MA). The retentates were subsequently washed several times with 3% KCl (w/v) to remove impurities that adhered (probably through ionic interaction) to the large molecular weight materials.

The high molecular weight retentates were analyzed using size exclusion chromatography (SEC). The set-up consisted of a Model 600E system controller, UK6 injector, Model 600 solvent delivery system, Model 401 differential refractometer and a Model 3396A Hewlett-Packard integrator. Analyses were performed at a flow rate of 1 ml/minute using HPLC grade water and a Shodex Ohpak KB-805 SEC column (300 mm length × 8 mm ID) held at 30°C. The high molecular weight retentates from each microalgae contained predominantly one peak that eluted in the void volume: "Immulina" for *Spirulina platensis*, "Immunon" for *Aphanizomenon flos-aquae*, and "Immurella" for *Chlorella pyrenoidosa*. Estimation of the molecular weight for each peak was achieved by comparison with retention times for dextran standards (12,000, 0.1 million, 1.66 million and 5–40 million daltons).

Structural characterization

Carbohydrate content of the purified polysaccharides (Immulina, Immunon and Immurella) were estimated using a colorimetric assay based on reaction with phenol (5% w/v in water) and concentrated sulphuric acid. Absorbance was determined at 450 nm and 490 nm (7). Elemental analyses for carbon, hydrogen, nitrogen and sulfur was performed by Galbraith Laboratories, Inc. (Knoxville, TN). Glycosyl composition and glycosyl linkage analyses were performed by The University of Georgia, Complex Carbohydrate Research Center. The glycosyl composition was determined using GC-mass spectrometry analysis of the TMS-methyl glycosides. In order to identify the O-methylated sugars detected during the TMS-methyl glycoside procedure, glycosyl composition was also determined using the alditol acetate procedure (8). Glycosyl linkage analysis was performed using the Hakomori procedure (9), in combination with carboxyl-reduction in order to detect uronic acid linkages (10).

Macrophage assay

Macrophage activation was measured using a luciferase reporter gene assay in THP-1 human monocytic cells as previously described (11). This assay measures immunostimulatory activity as indicated by increased expression of an NF-kappa B-driven luciferase reporter. THP-1 cells are transiently transfected using DEAE-dextran and the pBIIXLUC reporter plasmid containing two binding sites for NF-kappa B. Activation is reported as a percentage relative to maximal activation of NF-kappa B by 10 μg/ml LPS.

RT-PCR for IL-1β, TNF-α and GAPDH

Detection of mRNAs for IL-1β and TNF-α was performed as previously described (11). In brief, total RNA was isolated from THP-1 cells using the TRI Reagent® method and RT-PCR reactions were run using kit reagents from Promega. Sequence for the primers were described in Su et al. (12). Total RNA amounts used in the reactions were not saturating.

Results and Discussion

The luciferase reporter gene bioassay for activation of NF-kappa B in human THP-1 cells was used to guide purification of the immunostimulatory polysaccharides. For all three microalgae, the same isolation procedure was used for purification. A crude extract for each microalgae was prepared by extracting the freeze-dried material with 70% ethanol. Extraction with 70% ethanol allowed for efficient separation of the active substance from the bulk of other inactive polysaccharides that would be isolated if a typical hot water extraction was employed (refer to additional details below). Crude extracts at 50 μg/ml (*Spirulina platensis*), 10 μg/ml (*Aphanizomenon flos-aquae*) and 25 μg/ml (*Chlorella pyrenoidosa*) increased NF-kappa B directed luciferase expression to levels 50% of those achieved by maximal concentrations (10 μg/ml) of LPS.

Semi-pure microalgal polysaccharides were obtained by a combination of solvent partitioning and alcohol precipitation. Final purification was accomplished by removal of all material less than 100,000 daltons using an ultrafiltration device (refer to experimental section). The high molecular weight polysaccharides were analyzed using size exclusion chromatography and were found to contain one peak: "Immulina" for *Spirulina platensis*, "Immunon" for *Aphanizomenon flos-aquae*, and "Immurella" for *Chlorella pyrenoidosa*. These polysaccharides have retention times between 5.2 and 4.8 minutes (estimated molecular weight above 10 million daltons) and are very water soluble at 10 mg/ml. By comparison, immunostimulant polysaccharides such as acemannan and β-glucans are difficult to dissolve even at low concentrations. Polysaccharides Immulina and Immurella comprise between 0.5 and 1.0% of the dry weight of *Spirulina platensis* and *Chlorella pyrenoidosa*, respectively. The percent composition of Immunon is higher and represents about 2.0% of the dry weight of *Aphanizomenon flos-aquae*.

In addition to our active polysaccharides, microalgae hot water extracts contain substantial amounts of other high molecular weight material (size exclusion chromatography, data not shown). Removal of these contaminating substances from our active polysaccharides could be difficult and time consuming. Hence, the initial extraction procedure using 70% ethanol provides an elegant method whereby the active polysaccharides can be separated from potentially interfering substances that would be present with the hot water extraction.

Fig. 1 presents a dose response for both LPS and the isolated microalgal polysaccharides. The EC_{50} (50% of maximal LPS induction) values for NF-kappa B directed luciferase expression were as follows: Immulina at 110 ng/ml, Immunon at 20 ng/ml, Immurella at 80 ng/ml, and LPS at 250 ng/ml. To confirm THP-1 macrophage activation by purified microalgal polysaccharides, mRNA levels of proinflammatory cytokines IL-1β and TNF-α were measured using RT-PCR (Fig. 2). Treatment of THP-1 cells with either LPS or microalgal polysaccharides resulted in a dramatic increase in both IL-1β mRNA (810 bp) and TNF-α mRNA (444 bp), as compared with the control. This was not the case for the mRNA of the housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH, 1000 bp) (Fig. 2).

It is possible that the observed NF-kappa B activation by Immulina, Immunon and Immurella was due to endotoxin contamination of the preparation. To address this possibility two experiments were conducted. First, polymyxin B (10 μg/ml) was added in combination with each polysaccharide (0.1 to 1 μg/ml) to observe whether there was any abrogation in NF-kappa B activation. Polymyxin B is a polycationic antibiotic known to block many of the biological effects of LPS by binding to the lipid A portion of the molecule. All three microalgal polysaccharides were insensitive to polymyxin B addition (data not shown). Addition of polymyxin B to LPS (10 μg/ml) suppressed NF-kappa B activation by 75%. The second experiment used to examine possible endotoxin-mediated effects was to look for the presence of 3β-hydroxymyristate in the glycosyl composition analysis. In sample preparations of Immulina and Immurella there were no detectable levels of 3β-hydroxymyristate. Thus, it is unlikely that the observed macrophage activation by Immulina and Immurella is due to endotoxins.

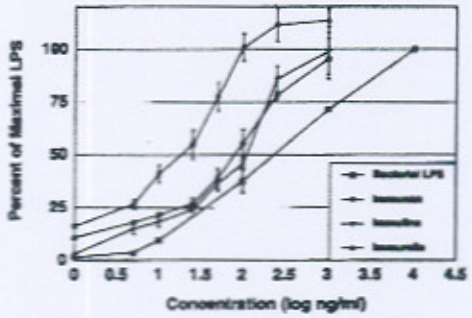

Fig. 1 Dose response for Immulina polysaccharide, Immunon polysaccharide, Immurella polysaccharide, and bacterial LPS activation of NF-kappa B in THP-1 monocytes/macrophages at 4 hours. Samples run in quadruplicate. Means ± standard deviation.

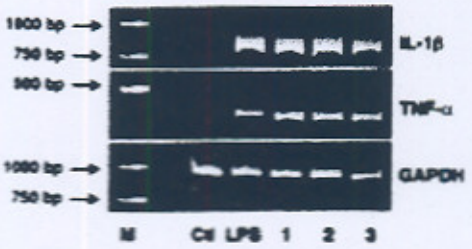

Fig. 2 Microalgal polysaccharides Immulina, Immunon and Immurella enhance proinflammatory cytokine mRNA production. RT-PCR results for IL-1β mRNA, TNF-α mRNA and GAPDH mRNA in THP-1 cells at 2 hours: (M) PCR marker, control, bacterial LPS at 10 μg/ml, (1) Immunon polysaccharide at 0.5 μg/ml, (2) Immulina polysaccharide at 0.5 μg/ml, and (3) Immurella polysaccharide at 0.5 μg/ml.

However, in two different sample preparations of Immunon, small amounts of 3β-hydroxymyristate (0.6% of total peak area) were detected. In order to determine how much "endotoxin-like" material was present, six samples of *Aphanizomenon flos-aquae* were analyzed using the Limulus amebocyte lysate (LAL) assay (analysis performed by BioWhittaker, Walkersville, MD). The amount of LAL positive material detected using this assay represented 0.002% of microalgal dry weight. By comparison, the percent composition of Immunon is about 1000 times greater (2.0% of microalgal dry weight). This means that at the concentration required to produce maximum NF-kappa B activation by Immunon (100 ng/ml), the total amount of potential LAL positive material present would be 100 pg/ml. This concentration of endotoxin would not be detectable using our THP-1 assay system. Therefore, the stimulatory effect of Immunon on macrophage activation is not due to endotoxin contamination.

Using a colorimetric assay (7) with phenol-sulphuric acid at 450 nm and 490 nm, the carbohydrate content of each isolated microalgal polysaccharide was estimated to be between 90% and 100%. This further supports the view that these compounds are predominantly polysaccharides. Treatment of Immunon and Immulina preparations with either heat (100°C for 30 minutes) or one of the following enzymes (0.1 mg/ml at 37°C for 1 hour): DNase 1, RNase A, trypsin, proteinase K, papain and α-chymotrypsin did not alter their EC_{50} values for macrophage activation. The activity of Immurella was not influenced by most of these enzymes, but it was reduced by 50% with heat treatment and by 25% with proteinase K. This suggests that although the biological activity of Immunon and Immulina is not due to nucleic acid or proteins, Immurella may contain either a protein contaminant or a peptide component to its structure that contributes to its activity. Coomassie blue based protein determinations indicate 2% protein for Immurella. Enough material was available for Immunon that elemental analysis was also performed and was found to contain the following elements: 49.1% carbon, 40.8% oxygen, 7.62% hydrogen, 2.46% nitrogen and trace amounts of sulfur.

Glycosyl composition and glycosyl linkage analysis for each polysaccharide is summarized in Tables 1 and 2. Due to the high volatility of terminal residues, especially deoxyhexoses and pentoses, reported values for these components in Table 2 may be lower than the actual levels. Based on their glycosyl

compositions, glycosyl linkages and molecular weights, all three microalgal polysaccharides are new compounds that have not been previously reported. Interestingly, all three polysaccharides contain high levels of both methylated carbohydrate residues and deoxyhexoses (e.g., rhamnose and fucose) which may explain their extractability with 70% ethanol. Due to the complex nature of these polysaccharides having a variety of glycosyl linkages (refer to Table 2), the anomeric configurations for each linkage have not yet been determined.

Neither the chemical structures nor the macrophage stimulating activity of our microalgal polysaccharides have been reported in the scientific or patent literature. Various other compounds have however been isolated from the microalgae studied in this paper. From *Spirulina* and *Chlorella* species a number of polysaccharides have been characterized for their antitumor, antiviral and immunostimulating activity (13), (14), (15), (16). In contrast, no such compounds have been isolated from *Aphanizomenon flos-aquae* showing any biological activity.

From *Chlorella* species a number of polysaccharides have been identified that possess biological activity. In U.S. Patent 4,533,548 an acidic polysaccharide was isolated from *Chlorella pyrenoidosa* that exhibits antitumor and antiviral activity (13). The glycosyl composition for this polysaccharide was mostly rhamnose, with minor amounts of galactose, arabinose, glucose and glucuronic acid. This glycosyl composition is distinctly different from Immurella which contains arabinose, galactose and rhamnose as the major components. Another polysaccharide, isolated from marine *Chlorella minutissima*, reported in U.S. Patent 4,831,020 appears to have tumor growth-inhibiting effects. However, no molecular weight or glycosyl composition was reported (14).

From *Spirulina* species several different types of polysaccharides have been isolated that exhibit biological activity. For example, the sulfated polysaccharide calcium spirulan exhibits antiviral properties and is composed of rhamnose (52.3%), 3-*O*-methylrhamnose (32.5%), 2,3-di-*O*-methylrhamnose (4.4%), 3-*O*-methylxylose (4.8%), trace amounts of other sugars and sulfate (15). The molecular weight of calcium spirulan (74,600 daltons) is about 100 times less than Immulina (above 10 million daltons).

In U.S. Patent 5,585,365 an antiviral polysaccharide was isolated using hot water extraction from *Spirulina* species with a molecular weight between 250,000 and 300,000 datlons (16). This polysaccharide is composed of rhamnose, glucose, fructose, ribose, galactose, xylose, mannose, glucuronic acid and galacturonic acid. Both the glycosyl composition and molecular weight of Immulina is different than this polysaccharide.

Pharmaceutical development of Immulina, Immunon and Immurella as immunostimulants may reveal a significant poten-

Table 1 Glycosyl composition for isolated polysaccharides from *Spirulina platensis* (Immulina), *Aphanizomenon flos-aquae* (Immunon) and *Chlorella pyrenoidosa* (Immurella). Data obtained from one experiment

Immulina Polysaccharide		Immunon Polysaccharide		Immurella Polysaccharide	
Glycosyl Residue	Mole %	Glycosyl Residue	Mole %	Glycosyl Residue	Mole %
Rhamnose	35.4	Mannose	16.0	Arabinose	31.6
Glucuronic acid	9.7	Glucose	13.1	Galactose	26.8
Fucose	7.7	4-Me-Mannose	11.2	Rhamnose	12.4
Galactose	7.1	Rhamnose	10.3	Glucose	5.4
2-Me-Rhamnose	5.9	2-Me-Rhamnose	8.1	3-Me-Arabinose	3.0
Xylose	5.5	Galactose	8.0	3-Me-Mannose	2.5
3-Me-Rhamnose	4.2	Fucose	7.0	Xylose	2.4
3-Me-Xylose	4.2	*N*-Acetyl-galactosamine	7.0	4-Me-Arabinose	2.4
4-Me-Rhamnose	3.9	*N*-Acetyl-glucosamine	5.8	Mannose	2.3
Glucose	3.6	Xylose	4.8	Ribose	1.9
Mannose	2.4	2-Me-Fucose	3.1	2,4-di-Me-Arabinose	1.3
Galacturonic acid	2.0	3-Me-Galactose	2.6	3-Me-Galactose	1.2
3-Me-Galactose	2.0	3-Me-Arabinose	1.8	3-Me-Xylose	0.9
Arabinose	1.8	Arabinose	1.6	3-Me-Rhamnose	0.9
amino sugar	1.5	2,3-diMe-Arabinose	1.2	3,5-diMe-hexose	0.9
2,3-diMe-Fucose	1.2			6-Me-Galactose	0.7
N-Acetyl-glucosamine	0.9			Glycerol	0.5
2-Me-Glucose	0.5			2-keto-3-deoxy-Octulosonic acid	0.5
Glycerol	0.4			2,3,6-triMe-Mannose	0.4
				3,6-diMe-Mannose	0.4
				2,3-diMe-Mannose	0.4
				2-Me-Galactose	0.4
				N-Acetyl-galactosamine	0.3
				N-Acetyl-glucosamine	0.3
				amino sugar	0.3

Note: Methyl groups are represented by "Me".

Table 2 Glycosyl linkage analysis for isolated polysaccharides from *Spirulina platensis* (Immulina), *Aphanizomenon flos-aquae* (Immunon) and *Chlorella pyrenoidosa* (Immurella). Data obtained from one experiment

Immulina Polysaccharide		Immunon Polysaccharide		Immurella Polysaccharide	
Glycosyl Linkage	% total area	Glycosyl Linkage	% total area	Glycosyl Linkage	% total area
3-Rha + T-GlcA	25.8	2-Man + 3-Man	13.4	T-Galactose (*f*)	12.2
4-Galactose	7.8	4-Rha + T-Man	10.6	2-Glucose	9.2
4-Glucuronic acid	7.3	2-Rhamnose	7.6	6-Galactose (*p*)	8.6
3,4-Glucuronic acid	6.9	T-Rhamnose	7.5	2,3-Rhamnose	8.4
2-Rhamnose	5.7	3-Rhamnose	6.9	T-Glucose	5.9
3-Fucose	5.1	2-Glucose	5.3	T-Arabinose (*f*)	5.5
2,3-Rhamnose	4.9	2-Galactose	4.8	2-Arabinose (*f*)	5.4
T-Xylose (*p*)	4.8	2-Fucose	4.7	3,6-Galactose	5.1
4,6-Galactose	4.3	3,4-Fucose	4.5	2,3,6-Galactose	4.9
T-Rhamnose	4.2	4-Glucose	4.4	T-Man + 3-Rha + 4-Rha	3.7
3,4-Fucose	3.1	3-Xylose	4.4	2,3-Arabinose (*f*)	3.3
3,4-Galacturonic Acid	2.4	4-Fuc + T-Gal	4.3	T-Arabinose (*p*)	2.8
2-Man + 3-Man	2.2	T-Xylose	3.2	6-Gal (*f*)	2.6
4-Fucose	2.2	unidentified	2.7	3-Hexose (*f*)	2.4
T-Fucose	2.2	T-Fucose	2.5	3-Galactose	2.3
3,4-Rhamnose	2.1	4-Mannose	2.2	2-pento (*f*)	2.1
2-Glucose	1.5	2-Arabinose (*p*)	2.1	4-Glc/2,4-Ara(*p*)/2,5-Ara(*f*)	2.1
2,3-Mannose	1.4	4-Galactose	2.1	T-Xylose (*p*)	1.8
3-Glucose	1.2	2,3,6-Galactose	2.1	4,6-Galactose	1.9
3-Galactose	1.1	3-Galactose	1.4	4-Galactose	1.9
4-Mannose	1.0	3,5-Ara(*f*)/3,4-Ara(*p*)	1.3	3,4-Galactose	1.7
6-Mannose	0.8	2,6-Glucose	1.2	T-Galactose (*p*)	1.4
2,6-Glc + 4,6-Glc	0.8	6-Mannose	0.6	3-pentose (*f*)	1.3
3-Xylose	0.7			3,4-Rhamnose	1.1
4-Xylose	0.6			2-Mannose + 3-Mannose	1.1
				3-Arabinose (*f*)	1.0
				2,6-Glucose	0.5

Note: All glycosyl linkages are also 1-linked unless otherwise specified. Glycosyl abbreviations represent the following: "Man" for mannose, "Rha" for rhamnose, "Glc" for glucose, "Fuc" for fucose, "Ara" for arabinose, "Gal" for galactose, "GlcA" for glucuronic acid, "T" for terminal linkage, "*f*" for furanose, and "*p*" for pyranose. Presence of two or three glycosyl units indicates co-elution of components during analysis.

tial for immunotherapy. These polysaccharides have superior macrophage stimulatory activity compared with clinically used polysaccharide preparations. There are three major fungal polysaccharide immunostimulants in clinical use for a variety of human cancers: schizophyllan, lentinan and krestin (17). These pharmaceuticals are used primarily in Japan either alone or in combination with chemotherapy and/or radiotherapy. Another polysaccharide, acemannan (Carra Vet®, isolated from *Aloe vera*), is licensed by the United States Department of Agriculture for the treatment of fibrosarcoma in dogs and cats (18). In our macrophage bioassay these four commercial polysaccharide immunostimulants (schizophyllan, lentinan, krestin and acemannan) were at least one thousand times less active than our microalgal polysaccharides (data not shown). These results agree with *in vitro* studies demonstrating that these clinically used polysaccharides have weak/modest effects on macrophage function (18), (19), (20). Successful development of these microalgal polysaccharides would add to the arsenal of available agents for immunotherapy in the treatment of cancer and infectious diseases.

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Dr. David S. Pasco

National Center for Natural Products Research
Research Institute of Pharmaceutical Sciences
School of Pharmacy
The University of Mississippi
University
MS 38677
USA
E-mail: dpasco@olemiss.edu
Fax: +(662) 915–7062
Tel.: +(662) 915–7130