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Component Analysis of Protein-Bound Polysaccharide (SN-C) from *Cordyceps ophioglossoides* and Its Effects on Syngeneic Murine Tumors

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A protein-bound polysaccharide (SN-C) obtained from the culture filtrate of *Cordyceps ophioglossoides*, which has been reported to have antitumor activity, was analyzed for sugar and amino acid components. Gas chromatography revealed that the neutral sugar component of SN-C was mainly composed of glucose. By using an amino acid auto analyzer, it was shown that SN-C was composed of galactosamine as the sole aminosugar. An intraperitoneal administration of SN-C into mice inoculated intraperitoneally with syngeneic murine tumors such as MM46 mammary carcinoma or L5178Y leukemia suppressed the tumor growth and resulted in a significant prolongation of the life span. When used *in vitro*, SN-C significantly inhibited the proliferation of various murine tumor cells including P388. Futhermore, SN-C inhibited the incorporation of glucose into Meth-A tumor cells with a consequent decrease of deoxyribonucleic acid (DNA) synthesis. SN-C may be a new type of antitumor polysaccharide since this material seems to posess both direct antitumor effect and stimulating activity on the host-mediated effect.

Keywords——*Cordyceps ophioglossoides*; SN-C; protein-bound polysaccharide; antitumor activity; syngeneic tumor; membrane permeability

Introduction

Various polysaccharides having antitumor activity have been obtained from microorganisms.[1] Some of these polysaccharides, including PSK (Krestin), Lentinan and Schizophyllan, are now in clinical use. Most of these antitumor polysaccharides from microorganisms contain neutral sugars (glucan) as a main component. These polysaccharides have no direct effect against tumor cells, and their actions are believed to be exerted by the mediation of immune mechanisms of the host.

We have already reported the presence of a unique protein-bound polysaccharide in the liquid culture of *C. ophioglossoides*, which belongs to *Ascomycetes*. The polysaccharide, SN-C, consists mainly of glucose and galactosamine with a small amount of protein. SN-C showed a strong antitumor activity against murine tumors such as sarcoma 180 and Ehrlich carcinoma.[2] The tumor cells used in the previous study were a limited number of allotransplantable tumors. In the present study, the direct antitumor effect of SN-C was investigated using a wide range of syngeneic murine tumors.

Materials and Methods

Analysis of Sugar Components——To analyze neutral sugars, the sample (2 mg) was hydrolyzed with 2 ml of 1—4 N sulfuric acid at 100 °C for 6 h and then neutralized with barium carbonate. The hydrolysate was then subjected to thin layer chromatography (TLC) on cellulose using ethyl acetate : pyridine : acetic acid : water (5 : 5 : 1 : 3) as the developing solvent. The sugars were detected by using alkaline-silver nitrate reagent.[3] A portion of the hydrolysate was trimethylsilyated and applied to a gas chromatography (Shimadzu 5A, Shimadzu Ltd., Japan) which was equipped with flame ionization detector and a glass column (0.3 × 200 cm) of 3% ethylene succinate-cyanoethyl silicon polymer (ECNSS-M) at 190 °C. For the analysis of aminosugars, the sample (2 mg) was hydrolyzed with 2 ml of 4 N hydrochloric acid at 100 °C for 22 h, and centrifuged to remove the insoluble materials. The supernatant was diluted with citrate buffer and analyzed on an amino acid auto analyzer (Hitachi 835; Hitachi Ltd., Tokyo, Japan). An aliquot of the supernatant was also applied to TLC in the same manner as used for neutral sugars, and the aminosugars were identified by ninhydrin reaction.[4]

Analysis of Amino Acid Components——The sample (20 mg) was hydrolyzed with 2 ml of 6 N hydrochloric acid at 110 °C for 22 h in a sealed test tube and analyzed by an amino acid auto analyzer according to the standard method.

Preparation of Sample (SN-C)——The cultivation and the purification of SN-C were performed as described in a previous report.[2] A summary of the procedure for purification is shown in Fig. 1. For administration to animals or to cultured cells, SN-C was dissolved in 0.01 N acetic acid, neutralized with sodium hydroxide, equilibrated with glucose and sterilized by passage through a 0.45 μm Mirex filter (Millipore Co., Bedford, MA., U.S.A.).

Experimental Animals——Female C3H/He mice (7 weeks of age, 20—25 g) were purchased from Shizuoka Laboratory Animal Center (SLC), Hamamatsu, Japan. Female DBA/2, CDF_1, BALB/c, and C57BL/6 mice (7 weeks of age, 18—25 g) were obtained from Charles River Japan, Inc., Atsugi, Japan. Mice were used for experiments at 7—8 weeks of age. Each experimental group consisted of nine mice.

Tumors——P388 leukemia, L5178Y leukemia, Meth-A fibrosarcoma, and MM46 mammary carcinoma were kindly provided by the National Cancer Center; MM2 mammary carcinoma, MM102 mammary carcinoma and MH134 hepatoma by The Research Institute for Tuberculosis and Cancer, Tohoku University; L1210 leukemia, EL4 lymphoma and P815 mastocytoma by the National Defense Medical College; IMC carcinoma by Kitasato University; YAC-1 lymphoma cell by Kyushu University. $P388D_1$ lymphoid neoplasm cell, an ATCC strain,

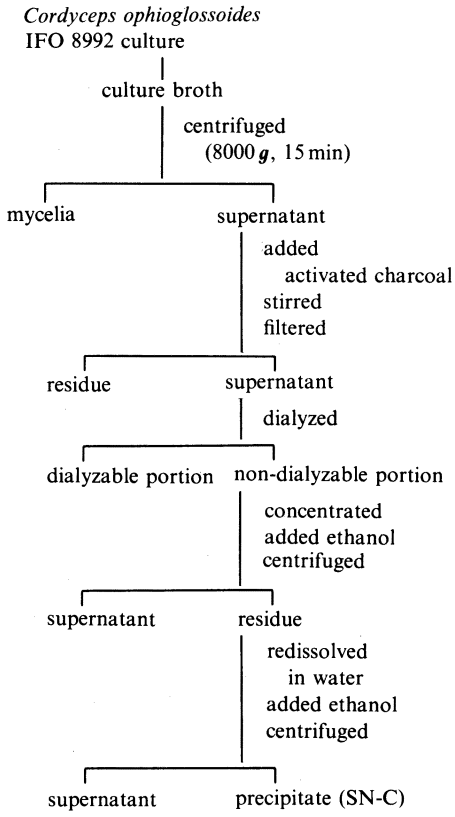

Fig. 1. Procedure for the Isolation of SN-C

TABLE I. Mice and Tumors

Tumor	Host	Cell number/mice
P388 leukemia	DBA/2, CDF_1	1×10^4, 1×10^5
$P388D_1$ leukemia	DBA/2, CDF_1	1×10^4, 1×10^5
L1210 leukemia	DBA/2, CDF_1	1×10^3, 1×10^4
L5178Y leukemia	DBA/2, CDF_1	1×10^3, 1×10^4
EL4 leukemia	C57BL/6	1×10^3, 1×10^4
MM46 mammary carcinoma	C3H/He	1×10^4, 1×10^5
MM2 mammary carcinoma	C3H/He	1×10^4, 1×10^5
MM102 mammary carcinoma	C3H/He	1×10^4, 1×10^5
IMC carcinoma	CDF_1	1×10^4, 1×10^5
MH134 hepatoma	C3H/He	1×10^5, 1×10^6
P815 mastocytoma	DBA/2	1×10^5, 1×10^6
Meth-A fibrosarcoma	BALB/c	1×10^5, 1×10^6

was purchased from Dainippon Pharmaceutical Co., Ltd., Osaka, Japan. All tumor cells except YAC-1 were maintained by serial passages in the peritoneal cavity of inbred mice. YAC-1 cells were maintained in RPMI-1640 (10% fetal calf serum (FCS)) medium.

Determination of Antitumor Activity——Increase in Life Span: Graded numbers (10—10^8) of each tumor cell line were inoculated into the peritoneal cavity of nine inbred or F_1 mice, and the minimal number of tumor cells required for the death of all mice (minimal lethal dose, MLD) was calculated. To see the effect of SN-C on the survival of mice, 10 and 100 MLD of tumor cells were transplanted into the peritoneal cavity of nine inbred or F_1 mice. The combinations of individual tumor, the host strain and the number of cells used in the experiments are listed in Table 1. Two hours after tumor transplantation, 12.5, 25.0 or 50.0 mg/kg of SN-C were administered into the peritoneal cavity of mice, followed by intraperitoneal administration of the same dose for 9 consecutive days from the day after. In addition, to examine the effect against p388, L1210 and IMC tumors, each line of tumor cells (10^5 or 10^6/mouse) was transplanted into the peritoneal cavity of nine DBA/2 or CDF_1 mice. SN-C (100 mg/kg) was administered intraperitoneally every day for 10 d, starting 24 h after tumor implantation. The mortality of mice was observed for 60 d after tumor transplantation. By comparing the median survival time (MST) with that of the control group given the vehicle (0.01 M sodium acetate solution, adjusted to pH 7.2 and equilibrated with glucose), the increase in life span (ILS) was calculated according to the following formula; ILS $(\%) = (T/C - 1) \times 100$, where T is the MST of the SN-C-administered group and C is the MST of the vehicle-control group.

Direct Cytotoxic Effect: Tumor cells including P388, $P388D_1$, L5178Y, Meth-A, EL4, MM46, IMC and YAC-1 were prepared to a concentration of 1×10^4/ml in RPMI-1640 (10% FCS), and SN-C was added to give final concentrations of 50, 100, or 200 μg/ml. Then the cells were cultured at 37 °C in a 5% CO_2 incubator. After 96 h, the number of viable cells was counted by the trypan blue dye exclusion method and compared with the number of viable cells in the vehicle control.

Effect on the Cell Membrane Permeability and the Synthesis of Nucleic Acid——The glucose permeability through the cell membrane was determined by using the incorporation of methyl-[^{3}H]glucose (NEN Research Products, Boston, MA., U.S.A.; sp. act., 80.0 Ci/nmol) as an index. The measurement was performed according to the method of Tsuru *et al.*[5] Meth-A cells were prepared to a concentration of 5×10^4/ml in RPMI-1640 (10% FCS) and cultured at 37 °C in a 5% CO_2 incubator for 24 h. Then SN-C was added to give final concentrations of 0—200 μg/ml. Immediately after and 23 h after SN-C addition, methyl-[^{3}H]glucose was added to give a concentration of 5 μCi/ml and the cells were incubated for 1 h. Then, the culture medium was removed, and the cells were washed three times with cold phosphate-buffered saline (PBS) and lysed in 0.1 N sodium hydroxide. Radioactivity was measured with a liquid scintillation counter.

Deoxyribonucleic acid (DNA) synthesis of the cells was determined by using the incorporation of methyl-[^{3}H]thymidine (NEN Research Products, Boston, MA., U.S.A.; sp. act., 15.1 Ci/nmol) as an index. Meth-A cells were prepared to a concentration of 5×10^4/ml in RPMI-1640 (10% FCS) and cultured at 37 °C in a 5% CO_2 incubator for 24 h. Then SN-C was added to give concentrations of 0—200 μg/ml. Methyl-[^{3}H]thymidine was added to give 2.5 μCi/ml 21 h after SN-C addition, and the cells were incubated for 3 h. Cells were harvested and DNA synthesis was measured.

Results

Constituents

SN-C contained 75.2% hexose, 17.3% aminosugar and 5.5% protein. The molecular

weight of SN-C was estimated to be 700000 by gel filtration on Toyopearl HW-55 (fine).[2] SN-C is insoluble in water, dilute hydrochloric acid and dilute sulfuric acid, but soluble in organic acids such as 0.01—0.1 N acetic acid, citric acid, or lactic acid. SN-C was relatively resistant to acid in comparison with other polysaccharides. Generally, neutral polysaccharides are hydrolyzed to monosaccharides by treatment with 1 N H_2SO_4 at 100 °C for 6 h. However, many oligosaccharides still remained in the hydrolysate of SN-C under the above conditions, and treatment with 4 N H_2SO_4 at 100 °C for 8 h was necessary for complete hydrolysis. The result of gas liquid chromatographic (GLC) analysis of the resultant hydrolyzed trimethylsilyl (TMS) derivatives thus obtained is shown in Fig. 2. The neutral sugar component of SN-C was mainly composed of glucose and contained small amounts of galactose and mannose. The best result in terms of the quantitative determination of aminosugar was obtained when SN-C was hydrolyzed at 4 N HCl at 100 °C for 22 h. The result of aminosugar analysis by an amino acid auto analyzer is given in Fig. 3. It was clearly indicated that SN-C contained galactosamine as the sole aminosugar. No acidic sugar such as uronic acid was detected. These component sugars were also detected by TLC. The amino acid composition of SN-C was also analyzed according to the standard method of protein hydrolysis. The result of the amino acid analysis is shown in Table II. SN-C contained no phosphorus or sulfur in the elementary analysis.

Life Span-Prolonging Effect of SN-C in Mice with Ascitic Type Tumors

The effects of SN-C on the life span of mice inoculated with each tumor are given in Table III. In the experiment using mice inoculated with 100 MLD of tumor cells, significant effects were observed against L5178Y, Meth-A, MM2, IMC, EL4 and P388D_1. In all cases, the increase in life span (ILS) was over 200% and most of the treated mice recovered completely. Against P388, MM46 and MM102, ILS ranged from 27.6 to 200% and SN-C was judged to be effective; however, SN-C was ineffective against L1210, P815 and MH134. In the experiment using mice inoculated with 10^5 or 10^6 IMC, L1210 or P388 cells, ILS was over 200% against IMC, but SN-C was ineffective against L1210 and P388.

Inhibitory Effect of SN-C on Cultured Cell Proliferation

The direct effect of SN-C on the proliferation of each cultured cell line is shown in Fig. 4. In the preliminary experiment, no direct antitumor effect was observed 24 h after addition of SN-C but a direct effect became evident after 96 h. Accordingly, the inhibitory effect of SN-C

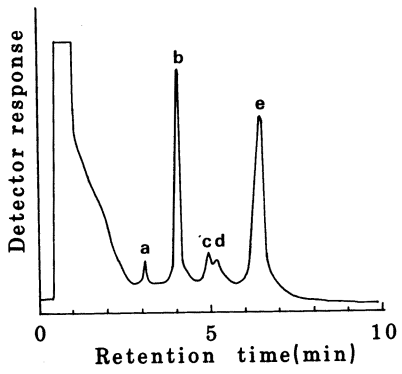

Fig. 2. GLC Trace of the Hydrolysate of SN-C (as Trimethylsilyl Derivatives)

SN-C was hydrolyzed with 4 N sulfuric acid at 100 °C for 8 h. Peak a, TMS–mannose; peaks b, e, TMS–glucose; peaks c, d, TMS–galactose; column, 3% ECNSS-M; temperature, 190 °C.

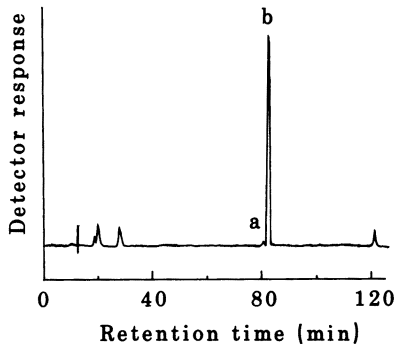

Fig. 3. Analysis of Component Aminosugar of SN-C on an Amino Acid Auto Analyzer

SN-C was hydrolyzed with 4 N hydrochloric acid at 100 °C for 22 h.
Peak a, glucosamine; peak b, galactosamine.

TABLE II. Amino Acid Composition (%) of Protein Moiety of SN-C

Amino acid	(%)	Amino acid	(%)
Aspartic acid	14.9	Leucine	4.2
Threonine	8.8	Tyrosine	1.7
Serine	8.1	Phenylalanine	2.9
Glutamic acid	26.3	Lysine	4.0
Proline	5.6	Histidine	Trace
Glycine	4.2	Arginine	2.5
Alanine	4.6	Methionine	0.5
Valine	5.6	Isoleucine	4.1

Amino acid components were measured with an amino acid auto analyzer.

TABLE III. Antitumor Effect of SN-C on Various Tumors

Tumor	Cell number /mouse	Host ILS (%) in mice given SN-C at dose of (mg/kg)			
		12.5	25.0	50.0	100.0
P388	1×10^5 [a] DBA/2	37 (1/9)[c]	40 (1/9)	41 (1/9)	—
	1×10^6 [b]	—	—	—	35 (0/9)
L1210	1×10^4 [a] DBA/2	13 (0/9)	14 (0/9)	6 (0/9)	—
	1×10^5 [b]	—	—	—	10 (0/9)
L5178Y	1×10^4 [a] DBA/2	>200 (5/9)	>200 (7/9)	>200 (7/9)	—
Meth-A	1×10^6 [a] BALB/c	>200 (5/9)	>200 (6/9)	>200 (7/9)	—
MM2	1×10^5 [a] C3H/He	>200 (5/9)	>200 (5/9)	>200 (7/9)	—
MM46	1×10^5 [a] C3H/He	60 (2/9)	60 (2/9)	>200 (5/9)	—
MM102	1×10^5 [a] C3H/He	27 (1/9)	79 (2/9)	62 (2/9)	—
IMC	1×10^5 [a] CDF_1	>200 (5/9)	>200 (7/9)	>200 (7/9)	—
	1×10^6 [b]	—	—	—	>200 (7/9)
$P388D_1$	1×10^5 [a] DBA/2	>200 (6/9)	>200 (7/9)	>200 (7/9)	—
EL4	1×10^4 [a] DBA/2	>200 (6/9)	>200 (6/9)	>200 (7/9)	—
P815	1×10^6 [a] DBA/2	6 (0/9)	10 (0/9)	0 (0/9)	—
MH134	1×10^6 [a] DBA/2	4 (0/9)	2 (0/9)	5 (0/9)	—

a) SN-C was given intraperitoneally 10 times at 2 h intervals after tumor inoculation. *b*) SN-C was given intraperitoneally for 10 consecutive days after tumor inoculation. *c*) No. of survivors/No. tested.

on tumor cell proliferation was determined after cultivation for 96 h in the presence of SN-C. At doses of 50—200 μg/ml, SN-C inhibited the proliferation of various cultured cells. The inhibitory effect was much stronger against P388, EL4 and $P388D_1$ than against L5178Y, Meth-A, MM46, IMC and YAC-1. SN-C inhibited the proliferation of tumor cells in a dose-dependent manner.

Inhibitory Effect of SN-C on Glucose Incorporation by Meth-A Cells

As shown in Fig. 5, the incorporation of methyl-[^{3}H]glucose by Meth-A cells was inhibited 1 h after the administration of SN-C at the concentrations of 50—200 μg/ml, and the effect was observed for 24 h. The inhibitory effect was dependent upon the concentration of SN-C.

Inhibitory Effect of SN-C on DNA Synthesis of Meth-A Cells

As shown in Fig. 6, the incorporation of methyl-[^{3}H]-thymidine by Meth-A cells was inhibited dose-dependently 24 h after the administration of SN-C at 50—200 μg/ml. Since SN-C does not show any direct inhibitory effect on the viability of tumor cells in 24 h as stated

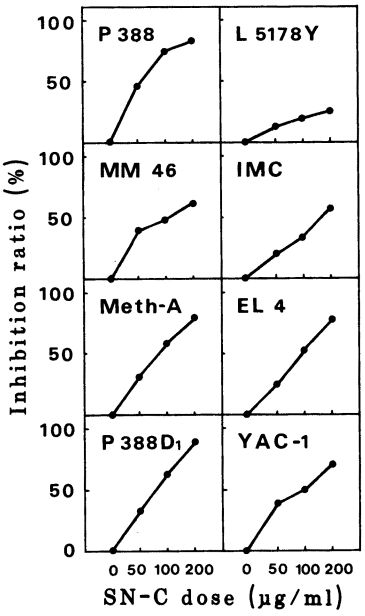

Fig. 4. Inhibition of Various Tumor Cells *in Vitro* by SN-C

Tumor cells (1×10^4 cell/ml) were cultured in RPMI-1640 (10% FCS), and SN-C was added to give the final concentration of 50, 100 or 200 μg/ml. The cells were cultured for 96 h and the number of viable cells was counted by the trypan blue dye exclusion method.

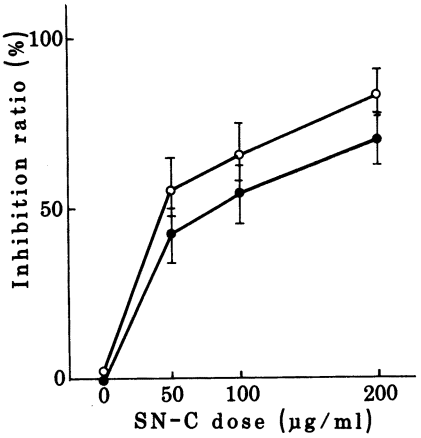

Fig. 5. Inhibitory Effect of SN-C on Glucose Incorporation by Meth-A Cells

Meth-A cells were prepared to a concentration of 5×10^4/ml in RPMI-1640 (10% FCS) medium. SN-C was added to give the final concentration of 0—200 μg/ml.
○, after 1 h; ●, after 24 h.

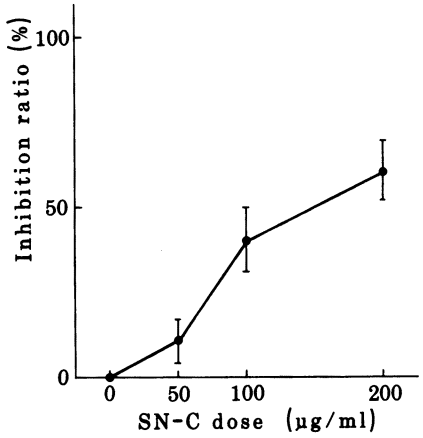

Fig. 6. Inhibitory Effect of SN-C on DNA Synthesis of Meth-A Cells

above, it can be argued that the inhibitory effect on DNA synthesis is not merely a reflection of a decreased number of viable cells in the well to which SN-C was added.

Discussion

Various polysaccharides from microorganisms have been shown to exert antitumor activity against solid tumors including sarcoma 180 or Meth-A when given by intravenous, intraperitoneal or oral administration. On the other hand, against ascitic tumors such as Ehrlich carcinoma, P388 or EL4, their effects are usually very weak even in the case of

intraperitoneal administration. It is believed that these antitumor polysaccharides exert antitumor activity by mediation of the host immune systems and do not cause direct damage to tumor cells.[6]

We have reported that a protein-bound polysaccharide (SN-C) from *C. ophioglossoides* has both host-mediated and direct effects against Ehrlich carcinoma and sarcoma 180.[2] In the present study, we investigated the antitumor spectrum of SN-C *in vivo* and *in vitro*. An inhibitory effect was observed on the *in vivo* growth of Meth-A, MM2, IMC and P388D$_1$ tumors, resulting in a significant prolongation of the survival time of mice implanted with these tumors. This effect was as strong as that observed in the previous study against Ehrlich carcinoma, an allotransplantable tumors. In contrast, such an inhibitory effect was not observed on the *in vivo* growth of L1210 or P388 tumors. The MST of control mice inoculated with L1210 or P388 was 10.5 and 13.5 d respectively, while that with the former group of tumors ranged between 15.0 and 27.5 d. The administration of SN-C seemed to be effective, especially against tumors which grow relatively slowly *in vivo*.

In the studies *in vitro*, SN-C was shown to have an inhibitory effect on the proliferation of various tumor cells. Furthermore, SN-C seemed to alter the membrane permeability of tumor cells, since the inhibition of glucose uptake by the cells was observed as early as 1 h after the addition of SN-C. It was reported that the surface of the tumor cell membrane is more negatively charged due to the influence of sialic acid than that of normal cells.[7] SN-C contains aminosugar, and may adhere to the membrane surface of tumor cells more readily than to the normal cell surface. One of the mechanisms for antitumor activities of SN-C may be that SN-C decreases the permeability of glucose through the tumor cell membrane, resulting in the inhibition of DNA synthesis. Such an effect is not seen with other polysaccharides.

Among several polysaccharides showing antitumor activity, the glucan components are reported to be probably responsible for the host-mediated effect.[1] Recent studies on the chemical structure of SN-C revealed that the polysaccharide component of SN-C is composed of β(1—3)-D-glucan and α(1—4)-galactosaminoglycan. The direct effect of SN-C as observed in this study might be attributable to the galactosaminoglycan moiety of SN-C. The β(1—3)-D-glucan moiety of SN-C is probably responsible for the host-mediated effect as suggested in the previous paper. It is now possible to dissociate SN-C into two separate moieties, glucan and galactosaminoglycan. The biological properties of each moiety will be described in a separate paper.

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