ELSEVIER

www.elsevier.nl/locate/carres

Carbohydrate Research 328 (2000) 71–76

CARBOHYDRATE RESEARCH

Synthesis of the HSA-conjugate of the *S*-linked thiomimetic of the branched tetrasaccharide repeating unit of the immunostimulant polysaccharide, schizophyllan. Evaluation as potential immunomodulator

Yili Ding [a], Sefik S. Alkan [b,1], Gerhard Baschang [2], Jacques Defaye [a,*]

[a] *Departement de Pharmacochimie Moléculaire/Glucides, CNRS (UMR 5063) and Université Joseph Fourier-Grenoble 1, BP 138, F-38243 Meylan, France*

[b] *Allergy/Immunology Department, Pharmaceutical Research Division, Novartis Pharma Inc., CH-4002 Basel, Switzerland*

Received 18 October 1999; accepted 7 March 2000

Abstract

The conjugate with human serum albumin (HSA) of the *S*-linked thioanalogue of the branched tetrasaccharide repeating unit of the polysaccharide, schizophyllan, was synthesized from 1,2,4,6-tetra-*O*-acetyl-3-*S*-[2,4-di-*O*-acetyl-3,6-di-*S*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-3,6-dithio-β-D-glucopyranosyl]-3-thio-β-D-glucopyranose [M.O. Contour-Galcera et al., *Carbohydr. Res.*, 281 (1996) 119–128] in five steps, and its potential immunomodulatory activity was evaluated in human blood mononuclear cells. The protein glycoconjugate did not effect proliferation or production of IL-4, IL-5 and IFN-g in a significant way.

Keywords: Sulfur-linked thiotetrasaccharide conjugate; Immunostimulant activity; Schizophyllan tetrasaccharide repeating unit; *S*-Linked thioanalogue

1. Introduction

The extracellular polysaccharide, schizophyllan, produced by the fungus *Schizophyllum commune*, contains a main chain of β-(1→3)-D-glucopyranosyl units in which every third unit carries a (1→6)-branched β-D-glucopyranosyl substituent [1]. Schizophyllan has been in use since 1986 as an anticancer drug in Japan, and its clinical effectiveness was observed in patients with lung, cervical and gastric cancers. The antitumor effect is host-mediated and believed to be correlated with an activation of macrophages and T-lymphocyte cells, as well as enhancement of interferon production [2]. It has been claimed that the antitumor activity is molecular weight dependent [3] and related to the overall conformation of the polysaccharide [4]. However, there is some discrepancy in the literature on this point since it has also been reported that ultrasonic degradation of schizophyllan, in which there was a 75% decrease in molecular weight, resulted in a similar or even slightly

* Corresponding author. Tel.: +33-4-76041018; fax: +33-4-76041013.

E-mail address: j.defaye@ujf-grenoble.fr (J. Defaye).

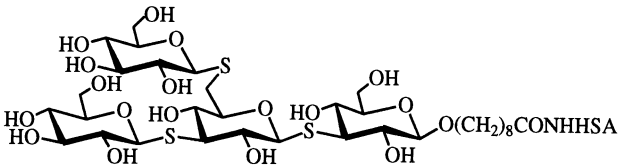
[1] Present address: Aventis Pharmaceuticals, G-317A, P.O. Box 6800, Bridgewater, NJ 08807, USA.

[2] Formerly: Department of Chemistry, Pharmaceutical Research, Ciba Ltd, CH-4002 Basel, Switzerland; present address: Bückenweg 7, CH-4126 Bettingen, Switzerland.

0008-6215/00/$ - see front matter

PII: S0008-6215(00)00082-3

better antitumor effect against Sarcoma 180 (solid tumor form) in mice [5]. It was then of interest to investigate the biological activity of simpler analogues, namely of the repetitive branched tetrasaccharide sequence. The synthesis of a bovine serum albumin (BSA) conjugate of this tetrasaccharide has already been reported [6]. We now describe the preparation of the conjugate with human serum albumin (HSA) of the branched *S*-linked thiotetrasaccharide analogue of the polysaccharide repetitive sequence **1** as an enzymatically stable mimetic [7], and its biological evaluation on human cells.



1

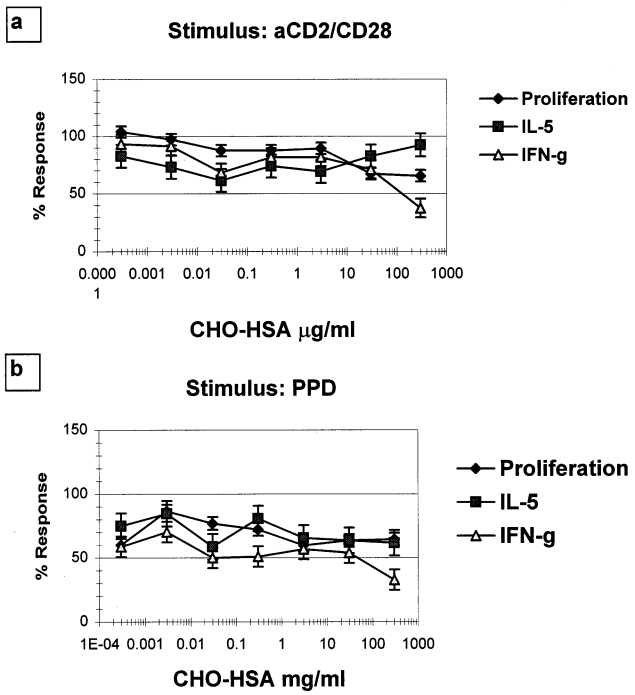
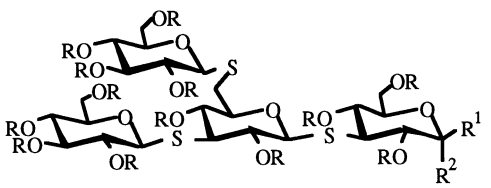


Fig. 1. The conjugate, CHO–HSA, has no effect on lymphocyte proliferation and cytokine production. PBMC from healthy donors ($0.7–1.0 \times 10^5$ cells/well) were stimulated by various means for 72 h, and supernatants were tested for cytokines by ELISA or CLIA. Cell proliferation was determined by ^{3}H-thymidine incorporation as described in Section 2: (a) anti-CD2/CD28 stimulation induced 6-fold IL-5, 80-fold IFN-g and 65-fold proliferation over background; (b) PPD stimulation induced 4-fold IL-5, 7-fold IFN-g and 6-fold proliferation over background. All samples were run in triplicate. Percent response was calculated from the difference of cytokine levels induced in the presence or absence (100%) of test compounds. The data are representative of three different experiments.

The synthesis of the target glycoconjugate followed well-established procedures. The peracetylated thiotetrasaccharide **2** [8] was converted into its glycosyl bromide **3** by reaction with 33% hydrogen bromide in acetic acid, and the product was then reacted with 8-methoxylcarbonyloctanol in the presence of silver triflate, resulting in **4** in 80% overall yield (two steps). O-Deacetylation of **4** with sodium methoxide in methanol, afforded the tetrasaccharide **5** in 80% yield, which was then converted into the hydrazide **6** in 90% yield by treatment overnight with hydrazine hydrate in ethanol. NMR spectroscopy and FABMS data were consistent with the structure 8-carbazoyloctyl β-trithiotetrasaccharide (**6**). The hydrazide was then converted into its acyl azide, which was conjugated to the carrier protein HSA, by the method of Chatterjee et al. [9], to give the thiotetrasaccharide conjugate **1**. Its MALDI mass spectrum showed a molecular ion at m/z 79,980, as compared with the corresponding signal at m/z 67,199 for native HSA, indicating that this glycoconjugate incorporated an average of 14 thiotetrasaccharide units.



2 R = Ac, R^1 = OAc, R^2 = H
3 R = Ac, R^1 = H, R^2 = Br
4 R = Ac, R^2 = H, R^1 = $O(CH_2)_8CO_2Me$
5 R = R^2 = H, R^1 = $O(CH_2)_8CO_2Me$
6 R = R^2 = H, R^1 = $O(CH_2)_8CO_2NHNH_2$

The results of the immunostimulation tests for compound **1** are reported in Fig. 1. This glycoconjugate does not effect proliferation or production of IL-4, IL-5 and IFN-g in a significant way. The conjugate inhibited IFN-g by 50% only at very high doses (300 μg/mL), indicating that this thiotetrasaccharide conjugate was inactive. As can be seen in Figs. 2 and 3, two classical immunosuppressive drugs, cyclosporin A and dexamethasone, inhibited the proliferation and the cytokine production with expected IC_{50} values in the same test. In

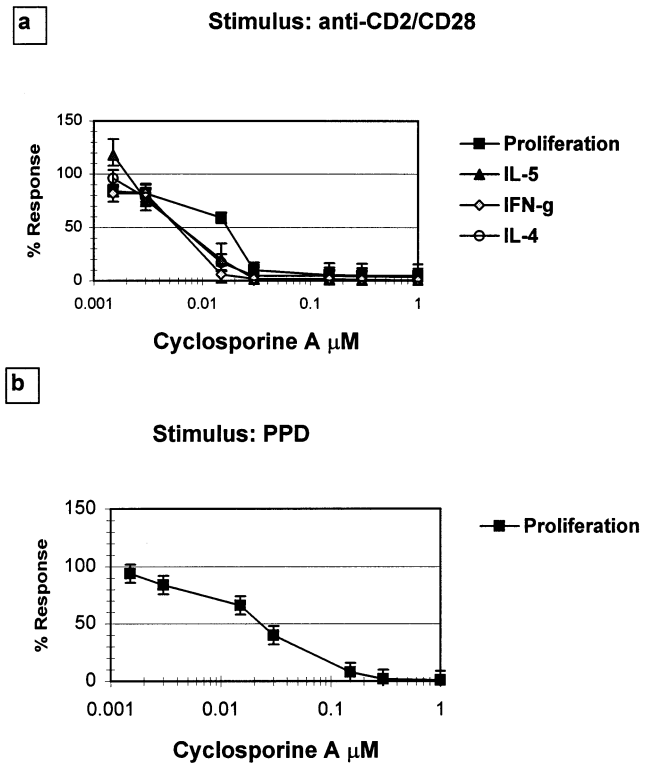


Fig. 2. Cyclosporine A, used as control, is a potent inhibitor of lymphocyte proliferation and cytokine production. PBMC were stimulated for 72 h and cell proliferation and cytokines levels were determined as described in the legend to Fig. 1. (a) Anti CD2/CD28 stimulation induced 30-fold IL-4, 50 fold IL-5, 118-fold IFN-g and 30-fold proliferation over background; (b) PPD stimulation induced 226-fold proliferation over background (cytokines levels were not determined). All samples were run in triplicate. Percent response was calculated from the difference of cytokine levels induced in the presence or absence (100%) of test compounds. The data are representative of two experiments.

addition, using the same assays, we have observed that certain compounds enhanced cytokine levels significantly (data not shown), indicating that our assay system is capable of detecting immunomodulatory activities. This absence of immunomodulatory activity with the conjugate **1** might then be in further support of the hypothesis that the immunostimulant activity of schizophyllan is correlated with the molecular weight and helical structure of the polysaccharide [4], although it would have been of interest to compare in parallel biological tests the corresponding *O*-linked tetrasaccharide–protein conjugate [6].

2. Experimental

Materials and methods.—TLC was performed on Silica Gel 60-F_{524} (E. Merck) with detection by charring with 5% H_2SO_4; column chromatography was performed on Silica Gel 60 (E. Merck, 40–63 mesh). ^1H NMR spectra were recorded at 300 MHz (Bruker AM 300) or 360 MHz (Bruker AM 360) on solutions in $CDCl_3$ or CD_3OD; ^{13}C NMR spectra were recorded at 75 or 90 MHz on the same instruments. The FAB mass spectra were obtained on a Kratos AEI MS9 instrument; the MALDI mass spectra were recorded on HP G2020A (LD-TOP) instrument using gentisic acid as the matrix. Elemental analyses were carried out on a Carlo Erba EAI108. HSA was obtained from Novartis.

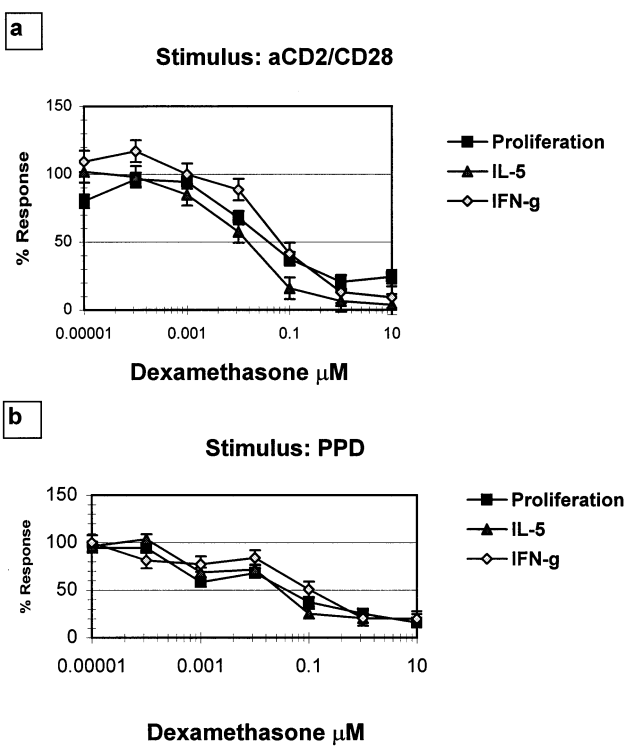


Fig. 3. Dexamethasone, used as a control glucocorticoid, is a potent inhibitor of lymphocyte proliferation and cytokine production. PBMC were stimulated for 72 h and cell proliferation and cytokine levels were determined as described in the legend to Fig. 1. (a) Anti-CD2/CD28 stimulation induced 5-fold IL-5, 80-fold IFN-g and 65-fold proliferation over background; (b) PPD stimulation induced 4-fold IL-5, 7-fold IFN-g, 6-fold proliferation over background. All samples were run in triplicate. Percent response was calculated from the difference of cytokine levels induced in the presence or absence (100%) of test compounds. The data are representative of two experiments.

2,4,6-Tri-O-*acetyl-3*-S-*[2,4-di*-O-*acetyl-3,6-di*-S-*(2,3,4,6-tetra*-O-*acetyl*-β-D-*glucopyranosyl)-3,6-dithio*-β-D-*glucopyranosyl]-3-thio*-α-D-*glucopyranosyl bromide* (**3**).—To a solution of 1,2,4,6-tetra-*O*-acetyl-3-*S*-[2,4-di-*O*-acetyl-3,6-di-*S*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-3,6-dithio-β-D-glucopyranosyl]-3-thio-β-D-glucopyranose [8] (**2**, 100 mg, 0.077 mmol) in dry CH_2Cl_2 (2 mL) was added 33% HBr in AcOH (0.5 mL) at 0 °C. The solution was stirred at 0 °C for 2 h. Toluene (10 mL) was added, and the solution was concentrated under reduced pressure to give **3** as a syrup. This compound was used directly for the next step without further purification.

8-Methoxycarbonyloctyl 2,4,6-tri-O-*acetyl-3*-S-*[2,4-di*-O-*acetyl-3,6-di*-S-*(2,3,4,6-tetra*-O-*acetyl*-β-D-*glucopyranosyl)-3,6-dithio*-β-D-*glucopyranosyl]-3-thio*-β-D-*glucopyranoside* (**4**).—To a solution of **3** in dry CH_2Cl_2 (2 mL) containing 4 Å powdered molecular sieves (400 mg), was added methyl 8-hydroxyoctanoate (30 mg, 0.15 mmol). After stirring for 30 min at rt, this mixture was cooled to −10 °C and AgOTf (26 mg, 0.1 mmol) was added. Stirring was continued for another 2 h at this temperature under argon, and the reaction mixture was filtered through Celite and concentrated. The residue was purified on a silica gel column with 1:2 hexane–EtOAc, affording the desired tetrasaccharide **4** as a syrup (88 mg, 80%). ^{1}H NMR (360 MHz, $CDCl_3$): δ 4.58 (d, 1 H, *J* 10.4 Hz, H-1^{IV}), 4.54 (d, 1 H, *J* 10.1 Hz, H-1^{III}), 4.51 (d, 1 H, *J* 9.9 Hz, H-1^{II}), 4.37 (d, 1 H, *J* 7.6 Hz, H-1^{I}), 3.65 (s, 3 H, OCH_3), 2.27 (t, 2 H, *J* 7.5 Hz, COCH_2); ^{13}C NMR (300 MHz, $CDCl_3$): δ 101.83, 85.83, 84.11, 83.17 (C-1^{I}, C-1^{II}, C-1^{III} and C-1^{IV}), 79.92, 75.92, 75.57, 74.39, 73.20, 73.63, 73.61, 71.90, 70.01, 69.91, 69.62, 68.24, 68.06, 66.87 (C-2^{I}, C-2^{II}, C-2^{III}, C-2^{IV}, C-3^{I}, C-3^{II}, C-4^{I}, C-4^{II}, C-4^{III}, C-4^{IV}, C-5^{I}, C-5^{II}, C-5^{III}, and C-5^{IV}), 62.43, 61.88, 61.79 (C-6^{I}, C-6^{III} and C-6^{IV}), 51.35, 49.65 (C-3^{III} and C-3^{IV}), 33.45 (C-6^{II}); FABMS: *m*/*z* 1453 [M + Na]$^+$. Anal. Calcd for $C_{60}H_{86}O_{33}S_3$: C, 50.35%; H, 6.01. Found: C, 49.79, H, 5.72.

8-Methoxycarbonyloctyl 3-S-*[3,6-di*-S-*(β*-D-*glucopyranosyl)-3,6-dithio*-β-D-*glucopyranosyl]-3-thio*-β-D-*glucopranoside* (**5**).—Methanolic NaOMe (1 M, 0.5 mL) was added to a solution of **3** (60 mg, 0.042 mmol) in dry MeOH (2 mL). After stirring at 0 °C for 24 h, the solution was demineralized with Dowex 50 (H^+) resin. The resin was removed by filtration, the solvent was evaporated, and the residue was purified on a silica gel column with 5:1 $CHCl_3$–MeOH to afford **5** as a syrup (33 mg, 90%). ^{1}H NMR (360 MHz, $CDCl_3$): δ 4.72 (d, 1 H, *J* 9.5 Hz, H-1^{IV}), 4.60 (d, 1 H, *J* 9.7 Hz, H-1^{III}), 4.59 (d, 1 H, *J* 9.8 Hz, H-1^{II}), 4.30 (d, 1 H, *J* 7.5 Hz, H-1^{I}), 3.64 (s, 3 H, OCH_3), 2.31 (t, 2H, *J* 7.8 Hz, COCH_2); ^{13}C NMR (300 MHz, $CDCl_3$): δ 105.44, 87.89, 87.89, 86.52 (C-1^{I}, C-1^{II}, C-1^{III}, C-1^{IV}), 83.82, 82.00, 81.72, 80.27, 79.44, 79.44, 74.69, 74.25, 74.69, 74.25 (C-2^{I}, C-2^{II}, C-2^{III}, C-2^{IV}, C-3^{I}, C-3^{II}, C-4^{I}, C-4^{II}, C-4^{III}, C-4^{IV}, C-5^{I}, C-5^{II}, C-5^{III} and C-5^{IV}), 63.00, 63.00, 62.72 (C-6^{I}, C-6^{III} and C-6^{IV}), 58.12, 56.44 (C-3^{III} and C-3^{IV}), 33.24 (C-6^{II}); FABMS: *m*/*z* 907 [M + Na]$^+$.

8-Carbazoyloctyl 3-S-*[3,6-di*-S-*(β*-D-*glucopyranosyl)-3,6-dithio*-β-D-*glucopyranosyl]-3-thio*-α-D-*glucopyranoside* (**6**).—To a solution of **5** (23 mg, 0.026 mmol) in 95% EtOH (2 mL) was added hydrazine (0.5 mL), and the solution was stirred at rt for 24 h. After concentration, the residue was purified on a silica gel column with 1:1 CH_2Cl_2–MeOH, yielding **6** as a syrup (20 mg, 90%). ^{1}H NMR (360 MHz, $CDCl_3$): δ 4.73 (d, 1 H, *J* 9.6 Hz, H-1^{IV}), 4.62 (d, 1 H, *J* 9.6 Hz, H-1^{III}), 4.60 (d, 1 H, *J* 9.7 Hz, H-1^{II}), 4.31 (d, 1 H, *J* 7.6 Hz, H-1^{I}), 2.14 (t, 2 H, *J* 7.5 Hz, COCH_2); ^{13}C NMR (300 MHz, $CDCl_3$): δ 105.45, 87.91, 87.91, 86.51 (C-1^{I}, C-1^{II}, C-1^{III} and C-1^{IV}), 83.79, 82.01, 81.74, 80.28, 79.44, 79.44, 74.66, 74.24, 74.24, 72.01, 71.51, 71.31, 71.31, 70.80 (C-2^{I}, C-2^{II}, C-2^{III}, C-2^{IV}, C-3^{I}, C-3^{II}, C-4^{I}, C-4^{II}, C-4^{III}, C-4^{IV}, C-5^{I}, C-5^{II}, C-5^{III} and C-5^{IV}), 62.99, 62.99, 62.71 (C-6^{I}, C-6^{III} and C-6^{IV}), 58.12, 56.44 (C-3^{III} and C-3^{IV}), 33.26 (C-6^{II}); FABMS: *m*/*z* 907 [M + Na]$^+$.

3-S-*[3,6-Di*-S-*(β*-D-*glucopyranosyl)-3,6-dithio*-β-D-*glucopyranosyl]-3-thio*-β-D-*glucopyranosyl-(1→8)-oxyoctyl-HSA* (**1**).—To a stirred solution of **6** (8 mg, 0.009 mmol) in dry DMF (0.2 mL) at −30 °C, was added aq 1.3 M HCl in dioxane (60 μL) and *tert*-butyl nitrite in DMF (1:10, w/v, 50 μL). After stirring for 10 min at −30 °C, this mixture was

then added dropwise to a solution of HSA (15 mg) in 0.1 M $Na_2B_4O_7$ at 0 °C. This mixture was then stirred at rt for 4 h, and dialyzed for 4 h with a membrane that retained proteins of molecular weight 12,000 or higher. Removal of water by lyophilization gave the thiotetrasaccharide conjugate **1** (11.4 mg) as a white powder. MALDIMS: *m/z* 79980.

Biological evaluation for **1**.—(1) Antigen (PPD)-induced T-cell proliferation and cytokine production [10,11]: peripheral blood mononuclear cells (PBMC) were prepared from heparinized venous blood (10 U heparin/mL, liquemine, Roche) by the standard Ficoll–Hypaque (Pharmacia) method. The cells were washed 2–3 times with phosphate-buffered saline (PBS) and resuspended in RPMI 1640 supplemented with 10% fetal calf serum (all from Gibco). Cell concentration was adjusted to $0.7–1 \times 10^6$ cells/mL. After adding 1.0 mg/mL PPD (purified protein derivative of *M. tuberculosis*, Serum Inst., Kopenhagen) the cells (150 mL per well) were distributed in 96-well flat-bottom plates (Costar) which already contained 50 mL/well of test compounds. Stimulated cultures were pulsed after 4–5 days (37 °C, 7% CO_2 in air) with 1.0 mCi ^{3}H-thymidine/well (Amersham: spec. act, 5 Ci/mmol) in 10 mL RPMI 1640. After an additional 20 h, the cells were harvested with a 96-well harvester on glass fibre filters (LKB). Results are expressed as the mean cpm of triplicate wells in the presence and absence of drugs. Standard deviations were generally below 15%. Supernatants from parallel triplicate cultures were taken at certain time points and tested for cytokine activity.

Detection of IFN-g in culture supernatants by ELISA *and* CLIA. Coating buffer or PBS (phosphate buffered saline, pH 7.4, 100 μL) containing 5 mg/mL mAb (43–11) was distributed into the wells of microtiter plates (Dynatech) and left overnight at 4 °C. The solution was removed by flicking, and the plates were washed six times with washing buffer (washing buffer plus 1% BSA and 0.01% merthiolate) and incubated for 1 h at rt. ELISA buffer was removed, and the plates were washed six times with washing buffer. Then, mAb (45–15) coupled to biotin was diluted appropriately (usually 1 mg mAb/mL) in ELISA buffer and distributed (50 μL/well), followed by 50 μL/well of IFNg standard or test samples. The plates were then incubated at rt for 2 h. The plates were washed and incubated with 100 μL/well extravidin–peroxidase conjugate (Sigma 2 mg/mL, diluted 1/1000 in ELISA buffer) for 1 h at rt. After washing, 150 μL/well of substrate solution containing 1.5 mg OPD/mL buffer (add 1/1000 vol water before use) was distributed in each well. The intensity of color was determined by a computerized Titertech multiscan ELISA reader (Biorad) at 490/630 nm. The amount of IFN-g present in the samples was calculated by using a standard curve. For each experiment, an IFN-g standard (Biogen or Kyowa Hakko) consisting of at least seven dilutions (0.002–10 ng/mL IFN-g) was used. These samples were freshly prepared from a stock solution (100 mg/mL), which was stored at −80 °C. All samples were run in triplicate. For the measurement of other human cytokines, the following mAb pairs were used in the CLIA (chemiluminecent immunoassay, all modified from ELISA): 3H4/8F12 for IL-4; 4H8/5A5 for IL-5. First, mAbs were immobilized (5 μg/mL, 100 μL/well), and then mAbs were labeled with acridinium ester and used at 0.2–0.5 μg/mL final concentrations, as described previously [10].

(2) Antibody (CD2/CD28)-induced T-cell proliferation and cytokine production. Normal human PBMC were prepared as above and stimulated with a cocktail of soluble anti-CD2 and anti-CD28 (4B2 and 6G4 all from Holland Biotechnology, CLB, each 1 μg/mL) mAbs for 72 h, and cytokine levels were determined by CLIA as described [10,11]. Inhibition of cytokine production was calculated from the difference of cytokine levels induced in the presence or absence of test compounds.

References

[1] (a) S. Kikumoto, T. Miyajima, S. Yoshizumi, S. Fujimoto, K. Kimura, *Nippon Nogei Kagaku Kaishi*, 44 (1970) 337–342; *Chem. Abstr.*, 74 (1971) 61776g. (b) S. Kikumoto, T. Miyajima, K. Kimura, S. Okubo, N. Komatsu, *Nippon Nogei Kagaku Kaishi*, 45 (1971) 162–168; *Chem. Abstr.*, 76 (1972) 123125w.

[2] I. Azuma, in I. Azuma, G. Jollès (Eds.), *Immunostimulants: Now and Tomorrow*, Springer, Berlin, 1987, pp. 41–56.
[3] A. Misaki, M. Kakuta, Fungal (1→3)-β-D-glucans: chemistry and antitumor activity, in Z.J. Witczak, K.A. Nieforth (Eds.), *Carbohydrates in Drug Research*, Marcel Dekker, New York, 1997, pp. 655–689.
[4] T. Norisuye, *Makromol. Chem. Suppl.*, 14 (1985) 105–118.
[5] K. Tabata, W. Ito, T. Kojima, S. Kawabata, A. Misaki, *Carbohydr. Res.*, 89 (1981) 121–135.
[6] (a) M. Kaltenbach, H. Kunz, *Eur. Carbohydr. Symp. 7th*, Cracow, 1993, abstract A 151. (b) H. Kunz, M. Kaltenbach, unpublished results, personal communication. (c) K. Takeo, K. Nagayoshi, K. Nishimura, S. Kitamura, *J. Carbohydr. Chem.*, 13 (1994) 1159–1177.
[7] J. Defaye, J. Gelas, in Atta-ur-Rahman (Ed.), *Studies in Natural Product Chemistry*, vol. 8, Elsevier, Amsterdam, 1991, pp. 315–357.
[8] M.O. Contour-Galcera, Y. Ding, C. Ortiz-Mellet, J. Defaye, *Carbohydr. Res.*, 281 (1996) 119–128.
[9] D. Chatterjee, S.N. Cho, P.J. Brennan, G.O. Aspinall, *Carbohydr. Res.*, 156 (1986) 39–56.
[10] S.S. Alkan, *J. Immunoassay*, 15 (1994) 217–238.
[11] C. Akdis, H. Towbin, P. Libsig, J. Motz, S.S. Alkan, *J. Immunol. Methods*, 182 (1995) 251–261.