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# A New IL-1 Receptor Inhibitor 139A:

## Fermentation, Isolation, Physico-chemical Properties and Structure

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Interleukin (IL)-1 is known to be a cytokine which plays a major role in pathological conditions like septic shock, inflammation and auto-immune disease, hence, methods that reduce the activity of IL-1 have an impact on clinical medicine. Inhibiting the binding of IL-1 to IL-1 receptors is one of the methods. A new inhibitor of IL-1 receptor, 139A, was isolated from the fermentation broth of *Streptomyces* sp.139. It was extracted from the broth filtrate, purified by Diaion HP-20, cation exchange resin and DEAE Sephadex A-25. 139A was identified as polysaccharide, its structure was elucidated on the basis of spectral analysis, the immobilized ligand IL-1 receptor binding assay (IL-1LRBA) proved 139A can competitively inhibits the binding of IL-1 to IL-1 receptors.

Many harmful biological effects, such as fever, anorexin and hypotension, are known to be mediated by IL-1. Moreover, IL-1 is a major cytokine which take part in pathological conditions like septic shock, inflammation and auto-immune disease[1]. Consequently, there is strong interest in pharmaceutical industry to find effective ways to inhibit IL-1 action. Trials of a recombinant form of IL-1 receptor antagonist (IL-1ra) show a relatively modest anti-inflammatory effect and a possible retardation of joint damage[2]. On the other hand, the discovery of non-peptidic molecules acting like IL-1ra as specific antagonists of IL-1R, might lead to the development of orally available, less expensive alternatives to IL-1ra. STEFANIA *et al.*[3] had obtained three small molecules which exhibited activity from microbial metabolites.

In the course of our screening work to obtain IL-1R inhibitors, a compound produced by *Streptomyces* sp. 139 named as 139A was obtained and identified as a new polysaccharide. In this paper, we describe the fermentation, isolation and structural elucidation of 139A.

## Materials and Methods

### Microbial Samples for High Throughput Screening

Streptomycetes and fungal strains were isolated from soil. Streptomycetes were inoculated in 50 ml of a culture medium having the following composition: glucose 1%, starch 2%, soybean flour 2%, trytone 0.2%, beef extract 0.2%, yeast extract 0.4%, $K_2HPO_4$ 0.05%, $CaCO_3$ 0.3%. The medium for fungal strains consisted of glucose 2%, glycerol 1%, soybean flour 2%, peptone 1%, PEG6000 0.25%, sucrose 1%, $K_2HPO_4$ 0.05%, $NaNO_3$ 0.3%, $(NH_4)_2SO_4$ 0.3%. 5 ml of each culture were centrifuged to remove mycelia, and the supernatants were tested directly in the immobilized ligand IL-1 receptor binding assay (IL-1LRBA).

### Immobilized Ligand IL-1 Receptor Binding Assay

The bioactivities of every components were tested in the IL-1LRBA. The preparation of reagents and experimental procedure have been described in detail by ZHANG[4]. Briefly, flat-bottomed mocroplate wells were incubated with 100 $\mu$l of 2 $\mu$g/ml recombinant IL-1ra in PBS (0.05 M

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phosphate buffer, pH 7.3, 0.15 M NaCl) overnight at 4°C, then with 250 μl/well of 3% BSA in PBS for 8 hours at 4°C, then washed with PBS. 60 μl samples or controls were mixed with 60 μl sIL-1R I in PBSA (1% BSA in PBS). Then 100 μl of the mixtures were transferred to the IL-1ra-coated plates and incubated overnight at 4°C. Microplates were washed with Tween 20-PBS (0.1% Tween 20 in PBS) and PBS, then 100 μl/well of 1 μg/ml (diluted in PBSA) antibody (an anti-sIL-1R I monoclonal antibody) were added, incubated at 4°C for 1 hour, washed with PBS, then 100 μl/well of 1 : 500 dilution of horseradish peroxidase (HRP)-labeled anti-mouse IgG in PBSA were added and incubation for 1 hour at 4°C, washed with Tween 20-PBS and PBS, then with 100 μl/well tetramethyl-benzidine for 1 hour at room temperature. 2 M HCl were added to end the reaction.

Fermentation of *Streptomyces* sp. 139

A loopful of slant culture of *Streptomyces* sp. 139 was inoculated into one 500 ml Erlenmeyer flask containing 100 ml of the same medium used for screening process. The flask was incubated on a rotary shaker at 28°C for 48 hours. Further, 5 ml of the culture was transferred to 20 of 500 ml Erlenmeyer flask containing 100 ml of the same medium and cultured on a rotary shaker at 28°C for 96 hours.

Isolation of 139A

The filtrate obtained by filtration of the whole broth was applied to a Diaion HP-20 column. The eluate was subjected to 001×7 styrene cation exchange resin. Add 60% ethanol to the eluate to precipitation twice, centrifuge and discard the supernatant, volatilize ethanol. The resultant brownish product was dissolved in a minimum amount of water and loaded on a DEAE-Dextran A-25 column. The column was eluted with water, 0.2 M $NH_4Cl$ and 0.5 M $NH_4Cl$ and the fractions were monitored by the IL-1LRBA. The active fractions were dialyzed respectively, obtained as white solids A and B by freeze drying.

Composition Analysis

The composition analysis was performed as described[5]. In short, the sample (2 mg) was dissolved in 2 ml of TFA to hydrolysis for 1 hour at 120°C, then drying under a stream of nitrogen. Subsequently, the residue was treated with 0.5 ml of 0.5 M NaOH and $NaBH_4$ at room temperature overnight, neutralized with acetic acid, then discharged the excess $NaBH_4$ with methanol, the residue was dried with $P_2O_5$ *in vacuo* for 4 hours, then acetylated with 0.5 ml of acetic anhydride in 0.5 ml of pyridine at 100°C for 30 minutes. After cooling, discharge the excess reagent under a stream of nitrogen, then sample was analyzed by GC.

Methylation Analysis[6]

The sample (2 mg) was dissolved in 2 ml of DMSO, then fine-powdered NaOH was added and proceeded for 5 minutes. Subsequently, 2 ml of methyl iodide was added to react for 4 hours. This reaction was conducted repeatedly twice or more till no IR absorption was shown for free hydroxyl groups, then diluted with water and added chloroform to extract the methylated polysaccharide. The methylated sugar was hydrolyzed, reduced and acetylated the same as the composition analysis procedure. The resulting substance was analyzed by GC-MS.

## Results

During the screening of microbial metabolites, we found that *Streptomyces* sp. 139 showed significant and stable inhibition. The inhibitory active metabolite was purified and two compounds, 139A and B, were obtained. IL-1LRBA showed that A had strong activity and B had little activity, so we conducted chemical analysis to 139A. Physico-chemical properties of 139A are summarized in Table 1. The color reactions of 139A was negative to ninhydrin reaction and positive to phenol-sulfuric acid. It showed strong absorption at 194 nm in UV spectrum. The molecular weight of 139A was estimated to be $6.33478\times 10^5$ by Gel and the distribution width (Mw/Mn) is 20.26.

The polysaccharide 139A was composed of rhamnose, xylose, glucose, mannose, arabinose, fucose, galactose, as shown by paper chromatography and gas chromatography of the hydrolysate.

Table 1. Physico-chemical properties of 139A.

| | |
|---|---|
| Appearance | white |
| $[\alpha]_{Na}^{28}$ ($H_2O$) | +18.2° |
| Molecular weight | $6.33478\times 10^5$ |
| UV $\lambda_{max}^{H2O}$ ($\varepsilon$) | 194nm |
| Color reaction | phenol-sulfuric acid |

Table 2. Methylation analysis of 139A by gas chromatography-mass spectrometry.

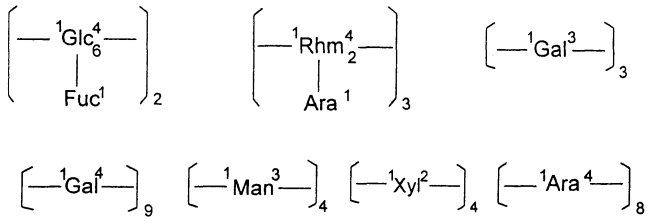
| No. | Methylated sugar | Mode of linkage | molar ratio | Relative retention time | |
|---|---|---|---|---|---|
| | | | | Test value | Refference |
| 1 | 2,3,4,5-tetra-O-methyl-arabinose | Ara(1- | 3 | 0.43 | 0.41 |
| 2 | 2,3,4,5-tetra-O-methyl-fucose | Fuc(1- | 2 | 0.58 | 0.58 |
| 3 | 2,3,5-tri-O-methyl-arabinose | -4)Ara(1- | 8 | 1.08 | 1.07 |
| 4 | 3,4,5-tri-O-methyl-xylose | -2)Xyl(1- | 2 | 1.19 | 1.19 |
| 5 | acetyl -3,5-di-O-methyl-rhamnose | -2,4)Rhm(1- | 3 | 1.69 | 1.67 |
| 6 | 2,4,5,6-tetra-O-methyl-mannose | -3)Man(1- | 4 | 1.89 | 1.90 |
| 7 | 2,4,5,6-tetra-O-methyl-glactose | -3)Gal(1- | 3 | 2.04 | 2.03 |
| 8 | 2,3,5,6-tetra-O-methyl-glactose | -4)Gal(1- | 9 | 2.23 | 2.22 |
| 9 | 2,3,5-tri-O-methyl-glucose | -4,6)Glc(1- | 2 | 4.48 | 4.50 |

139A was methylated by the method of XU *et al.* Hydrolysis of the methylated ethers is listed in Table 2. The methylated suger was identified by comparing their retention time in gas chromatography and their mass spectra. The comparision indicated the composition of the polysaccharide 139A and the molor ratio of each monosaccharides. Because the composition is so complex, we can only obtain the information of the mode of the linkage and deduce the structure fractions. The methylation analysis indicated the presence 2,3,4,5-tetra-*O*-methyl-fucose and 2,3,5-tri-*O*-methyl-glucose in the molar ratio of 1 : 1, it suggested that this polysaccharide has the fractions (6.5%) which have fucose as end-groups and backbone of (1→4)-linked glucan, every glucosyl reside has a branching point of (1→6)-linked fucosyl residue. By the presence 3,5-di-*O*-methyl-rhamnose and 2,3,4,5-tetra-*O*-methyl-arabinose in the molar ratio of 1 : 1, there is fractions (9.7%) which have arabinose as end-groups and backbone of (1→4)-linked rhamnan, every glucosyl reside has a branching point of (1→2)-linked arabinosyl residue. Moreover, the polysaccharide has the fractions $(\rightarrow 3Gal1\rightarrow)_3$ (9.7%), $(\rightarrow 4Gal1\rightarrow)_9$ (29.0%), $(\rightarrow 3Man1\rightarrow)_4$ (12.9%), $(\rightarrow 2Xyl1\rightarrow)_2$ (6.5%), $(\rightarrow 4Ara1\rightarrow)_8$ (25.8%). The formulas of the fractions which compose the basic structure of 139A was listed in Fig. 1.

## Discussion

IL-1LRBA is a rapid, non-radioactive and cell-free method for screening IL-1 Receptor Antagonists, especially effective during the high throughput screening. In the operation, the crude extracts are directly test, at the same time, we also test some components extracted by organic

Fig. 1. The formula of the fractions which compose the basic structure of 139A.



solvent, but it hard to make ideal result, even use DMSO to help dissolution. We think this method is more applicable for water-soluble compounds.

As EVANS stated[7], the multiple protein-protein contacts over a large three-dimensional space, it is not easily inhibited by small molecules. On the other hand, larger structures are also more likely to be highly immunogenic causing undesired side-effects. As a polysaccharide, 139A does not have this contradiction. It is deduced that 139A took effects by being metabolized and becoming its oligosaccharides form.

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