Chinese Journal of Chemistry, **2004**, *22*, 492—497 **Article**

Studies on the Physicochemical Properties, Structure and Antitumor Activity of an Oligosaccharide Homologue SnS-2 from the Root of *Scrophularia ningpoensis* Hemsl.

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An oligosaccharide homologue named SnS-2 was isolated from the root of *Scrophularia ningpoensis* Hemsl. SnS-2 was purified by means of gel-permeation chromatography and ion-exchange chromatography. Its physicochemical properties, including carbohydrate content and molecular weight were determined. The structure of SnS-2 was elucidated by chemical methods along with ^{1}H and ^{13}C NMR spectroscopy, including two-dimensional DQCOSY and H-detected ^{1}H, ^{13}C HMQC experiments. These results show that SnS-2 possesses a backbone consisting of terminal α-Gal*p*-(1→, α-Gal*p*-(1→6), α-Glc*p*-(1→6) and nonreducing end β-Fru*f*-(2→. The bioactive assay showed that it could inhibit the growth of Lewis pulmonary carcinoma implanted in mice.

Keywords *Scrophularia ningpoensis* Hemsl., physicochemical property, structure, antitumor activity, oligosaccharide homologue

Introduction

Scrophularia ningpoensis Hemsl. is a well-known traditional Chinese herbal medicine that has been found to possess the abilities of "cooling the blood, nourishing the kidney, purging the schenic fire, clearing away heat and toxic material, profiting the throat".[1] In the literature, its components were also reported including alkaloid, flavone glycoside, sterol, amino acid, fatty acid, naphtha, carotene and trace elements.[1] At present some other components, mainly small molecular compounds have been studied,[2-7] but studies on its saccharide components have not been reported so far. Here we report the isolation, purification, physicochemical properties, structural elucidation and antitumor activity of an oligosaccharide homologue SnS-2.

Experimental

Material

Scrophularia ningpoensis Hemsl. is a product of Zhejiang Province, China. DEAE-cellulose was purchased from Shanghai Heng Xin Chemical Agent Ltd. Sephadex G-50 and Sephadex G-10 were purchased from Ammersham Pharmacia Biotech.

General

Total sugar content was determined by the phenol-H_2SO_4 method.[8] A standard curve was calibrated with a mixture of galactose and glucose (2∶1, mol/mol). HPLC was performed on a Shimadzu LC-10AD instrument equipped with a TSK-G2000 SW exclusion column and using water as eluant (1.0 mL/min), and the eluate was monitored by an RI detector. Capillary electrophoresis (CE) was performed on a Waters Quanta 4000 E instrument using 0.1 mol/L boric acid-KOH buffer (pH＝10) as solvent, with detection at 254 nm. The infrared spectrum (IR) of the methylated oligosaccharide was recorded on a Bio-Rad FTS 185 spectrometer. GC-MS was conducted with a Shimadzu QP 5000 instrument. The temperature program was from 140 to 220 ℃ at 10 ℃/min. For quantitative analysis, an HP 6890 and an OV-17 capillary column (0.2 mm×30 m) were used with the above temperature program and N_2 was used as the carrier gas. Mass spectra were acquired by scanning from *m/z* 40 to 400. ESIMS was conducted with a PE PerSeptive Mariner instrument, and the data were collected in the negative-ion mode. The NMR spectra were obtained on a Varian Unity Inova 600 spectrometer equipped with a dual probe, in the FT mode at 30 ℃.

Isolation and purification of SnS-2

The root of *Scrophularia ningpoensis* Hemsl. (100 g) was shattered and the carbohydrates were extracted with 1 L of water for 24 h. The extract was centrifuged, and the residue was extracted with 500 mL of water again for 12 h. The solution was combined and concentrated to 500 mL. Acetone (2 L) was added, and the solution

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was centrifuged at 4500 r/min. The precipitate was dissolved in water, centrifuged, dialyzed with water and freeze-dried. The crude saccharide (7.07 g) was named SnP-2. After decolorized by active carbon, SnP-2 was separated on a DEAE-cellulose column (3.5 cm×20 cm, HCO_3^{-1} form) with water as eluent, and then stepwise with 0.25 and 0.5 mol/L $NaHCO_3$ solution at a flow rate of 1 mL/min. SnP-2a eluted with water and SnP-2b eluted with 0.25 mol/L $NaHCO_3$ were obtained. SnP-2a was further purified using Sephadex G-50 (1.5 cm×60 cm) eluted with 0.1 mol/L NaCl at a flow rate of 0.5 mL/min. Two saccharide fractions: SnP-2a_1 and SnP-2a_2 were collected. SnP-2a_2 was desalted by Sephadex G-10 (2 cm×30 cm), and SnS-2 was obtained by freeze drying. All separation processes were monitored by the phenol-H_2SO_4 method at 490 nm. The chemical homogeneity of SnS-2 was determined by HPLC and CE.

Component analysis of SnS-2[9]

SnS-2 was hydrolyzed with 0.5 mol/L H_2SO_4 at 100 ℃ for 1 h, neutralized with $BaCO_3$, then centrifuged, filtrated, and concentrated. HPLC was carried out on a Carbohydrate Analysis Column (Waters, 3.9 mm I.D.× 30 cm) with MeCN/H_2O (82∶18, *V*∶*V*) as eluent at a flow rate of 1.0 mL/min.

Molecular weight determination of SnS-2

SnS-2 was analyzed with ESIMS. The mass spectra showed some ion peaks at *m/z* 364, 504, 527, 689. One major ion at *m/z* 527 corresponds to $[M+Na^+]$ of Gal-(1→6)-Glc-(1→2)-Fruc, and another ion at *m/z* 504 corresponds to $[M^+]$ of Gal-(1→6)-Glc-(1→2)-Fruc. Some minor peaks at *m/z* 364 and 689 correspond to $[M+Na^+]$ of Gal-(1→6)-Glc~OH, and $[M+Na^+]$ of $[Gal\text{-}(1\rightarrow6)]_2$-Glc-(1→2)-Fruc, respectively.

Carbohydrate content of SnS-2[10]

The carbohydate content of SnS-2 was determined by the phenol-H_2SO_4 method according to a standard curve with Gal and Glc in a molar ratio of 2∶1.

Methylation analysis of SnS-2

SnS-2 was methylated using the modified Hakomori method.[11] Dimsyl carbanion was generated by adding hexane-extracted NaH (25 mg) to Me_2SO (0.5 mL, vacuum distilled at 70 ℃ and stored over CaH_2 under nitrogen). After warming the Me_2SO mixture for 30 min at 40 ℃, SnS-2 (10 mg) was added and allowed to react for 3 h at 25 ℃. Iodomethane (0.8 mL) was added at ice-water during 20 min and allowed to react overnight at 25 ℃. The reaction mixture was extracted three times with $CHCl_3$. The combined $CHCl_3$ layers were extracted with water. The dried (over Na_2SO_4) $CHCl_3$ layer was evaporated. IR spectroscopy was used to determine the completeness of methylation.

The fully methylated product was hydrolyzed with 2 mol/L CF_3CO_2H (0.5 mL) at 100 ℃ for 2 h, reduced with $NaBH_4$ and acetylated by acetic anhydride. The resulting mixture of alditol acetates was analyzed by GC-MS. The chromatographic conditions were the same as those for the GC analysis.

Assay of antitumor activity of SnS-2

Assay of antitumor activity of SnS-2 was carried out by the method of Yu and Zhang.[12] C57BL/C mice (weight 20 g) were obtained from Shanghai Animal Center of the Chinese Academy of Sciences, and Lewis pulmonary carcinoma cells (5×10^6/mL) were transplanted into the toe of the mice. The test samples were dissolved in 0.9% NaCl solution and injected intra-peritoneally daily for 10 d (injection volume, 0.2 mL) after tumor had been implanted for 24 h. All mice were kept under observation for 2 weeks and then killed for final evaluation of the effects of treatment on tumor growth. Tumors were excised and weighed. The growth inhibition ratio of tumor growth was calculated by the following equation:

Inhibition ratio$=(A-B)/A\times100\%$

where *A* is the average tumor weight of the control group and *B* that of the treated group.

Results and discussion

Isolation and purification of SnS-2 from *Scrophularia ningpoensis* Hemsl.

The white solid SnS-2 was obtained according to experimental section, yielding 1.17% of the herbal medicine *Scrophularia ningpoensis* Hemsl.

Physicochemical properties of SnS-2

Chemical homogeneity of SnS-2 was examined by HPLC and CE (Figures 1 and 2).

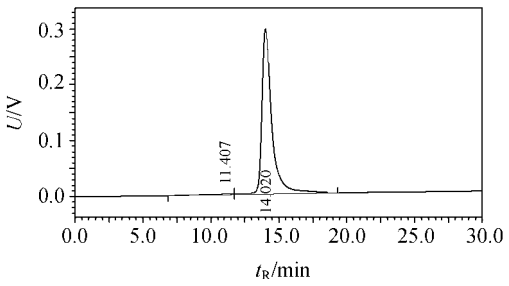


Figure 1 HPLC of SnS-2. Column: TSK-G2000SW; flow rate: 1.0 mL/min; detection: RI; eluent: H_2O.

Figure 2 CE of SnS-2. Voltage: 20 kV; UV: 254 nm; eluent: 100 mmol/L H_3BO_3-KOH; pH=10.0.

Compositional analysis showed that it was composed of Gal, Glc and Fru in a molar ratio of 2.25∶1∶1. The molecular weight distribution of SnS-2 was determined to be from 364 to 689 Da by ESIMS (Electrospray-ionization mass spectrometry) as shown in Figure 3.

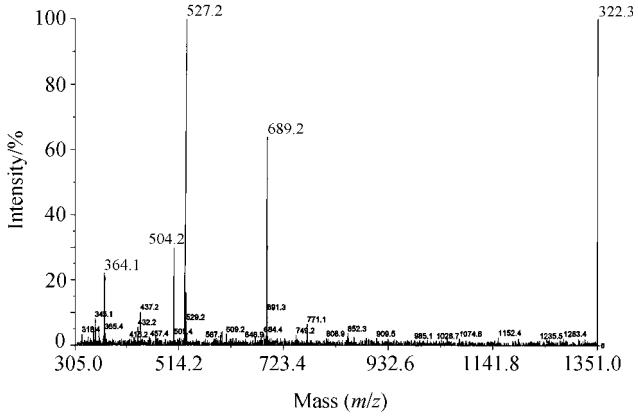


Figure 3 ESI MS of SnS-2.

Linkage analysis of SnS-2 by methylation

SnS-2 was completely methylated by a modified Hakomori method.[11] The per-*O*-methylated sample was completely hydrolyzed with 2 mol/L CF_3COOH, reduced with $NaBH_4$, and the alditol product was then acetylated. The partially methylated and acetylated sugar residues were submitted to GC and GC-MS analyses. As shown in Table 1, four peaks for SnS-2 (Figure 4) were identified by their retention time and mass spectra. The integrated peak areas were corrected by using the effective-carbon response method,[13] and according to the standard spectra, the four peaks were 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylgalactitol, and 2,5-di-*O*-acetyl-1,3,4,6-tetra-*O*-methylfructitol, respectively. The trace amount of Fru suggested that it was damaged in hydrolysis. So it was proved that SnS-2 possesses a backbone consisting of terminal α-Gal*p*-(1→, α-Gal*p*-(1→6), α-Glc*p*-(1→6) and nonreducing end β-Fru*f*-(2→.

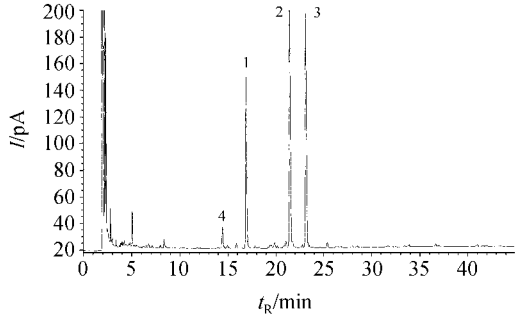


Figure 4 Gas chromatogram of the products derived by methylated derivatives of SnS-2. 1. 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, 2. 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol, 3. 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylgalactitol, 4. 2,5-di-*O*-acetyl-1,3,4,6-tetra-*O*-methylfructitol.

Table 1 GC-MS data of methylation analysis of SnS-2

GC peak	Methylated sugars	Mode of linkage	Relative molar ratio
1	1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol	α-Galp-(1→	1
2	1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylgluctitol	→ 6)α-Glcp-(1→	1
3	1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylgalactitol	→ 6)α-Galp-(1→	1
4	2,5-di-*O*-acetyl-1,3,4,6-tetra-*O*-methylfructitol	β-Fru*f*-(2→	trace

NMR spectroscopy of SnS-2

The 1D ^1H and ^{13}C NMR spectra of SnS-2 (Figures 5 and 6) showed a simple signal pattern in terms of peak intensities in the anomeric proton and carbon region. Assignments of the ^1H chemical shifts of SnS-2 as shown in Table 2 were performed by means of 2D DQCOSY and 2D HMQC experiments. Starting points for the interpretation of the spectra were the H-1 signals of residues II—IV. In the DQCOSY spectra of SnS-2 (Figure 7), the H-1^{II} (δ 5.456) has a correlation with δ 3.588, so the chemical shift of H-2^{II} is δ 3.588. By the same method, the H-3, 4, 5, 6^{II} could be assigned, respectively, $\delta_{H\text{-}3}$ 3.783, $\delta_{H\text{-}4}$ 4.079, $\delta_{H\text{-}5}$ 3.568 and $\delta_{H\text{-}6}$ 3.767. The 2D ^{13}C-^1H HMQC spectra of SnS-2 (Figure 8) allowed the assignment of the ^{13}C chemical shifts.[14] The 2D ^{13}C-^1H HMQC spectra were ^1H-^{13}C directly coupled. From the chemical shift of ^1H, the chemical shift of ^{13}C could be obtained. The resonance signals of I, III and IV were assigned in the same way.

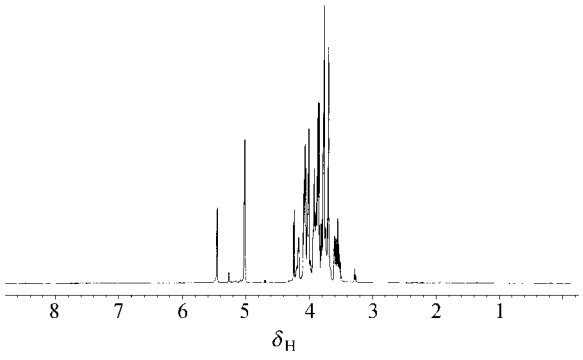


Figure 5 ^1H NMR (600 MHz) spectrum of SnS-2, recorded in D_2O at 30 ℃.

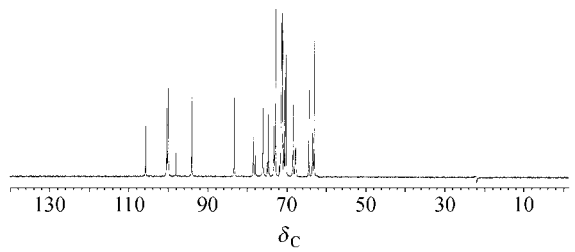


Figure 6 ^{13}C NMR (150 MHz) spectrum of SnS-2 , recorded in D_2O at 30 ℃.

Table 2 Chemical shift assignments (δ) for the ^{1}H and ^{13}C NMR spectra of SnS-2 [a]

Residue	Proton	δ_H	Carbon	δ_C	Residue	Proton	δ_H	Carbon	δ_C
I	H-1	3.629	C-1	64.128	III	H-1	5.018	C-1	100.044
	H-2	—	C-2	106.474		H-2	3.849	C-2	70.276
	H-3	4.185	C-3	79.055		H-3	3.923	C-3	71.243
	H-4	3.943	C-4	84.013		H-4	4.071	C-4	71.347
	H-5	4.002	C-5	76.689		H-5	4.023	C-5	72.970
	H-6	3.792	C-6	65.126		H-6	3.767	C-6	68.497
II	H-1	5.456	C-1	94.094	IV	H-1	5.018	C-1	100.370
	H-2	3.588	C-2	72.970		H-2	3.865	C-2	70.423
	H-3	3.783	C-3	74.723		H-3	3.923	C-3	71.243
	H-4	4.079	C-4	73.281		H-4	4.086	C-4	71.347
	H-5	4.020	C-5	71.502		H-5	4.032	C-5	72.970
	H-6	3.724	C-6	67.909		H-6	3.777	C-6	64.446

[a] Recorded in D_2O at 30 ℃ for ^{1}H and ^{13}C NMR spectra.

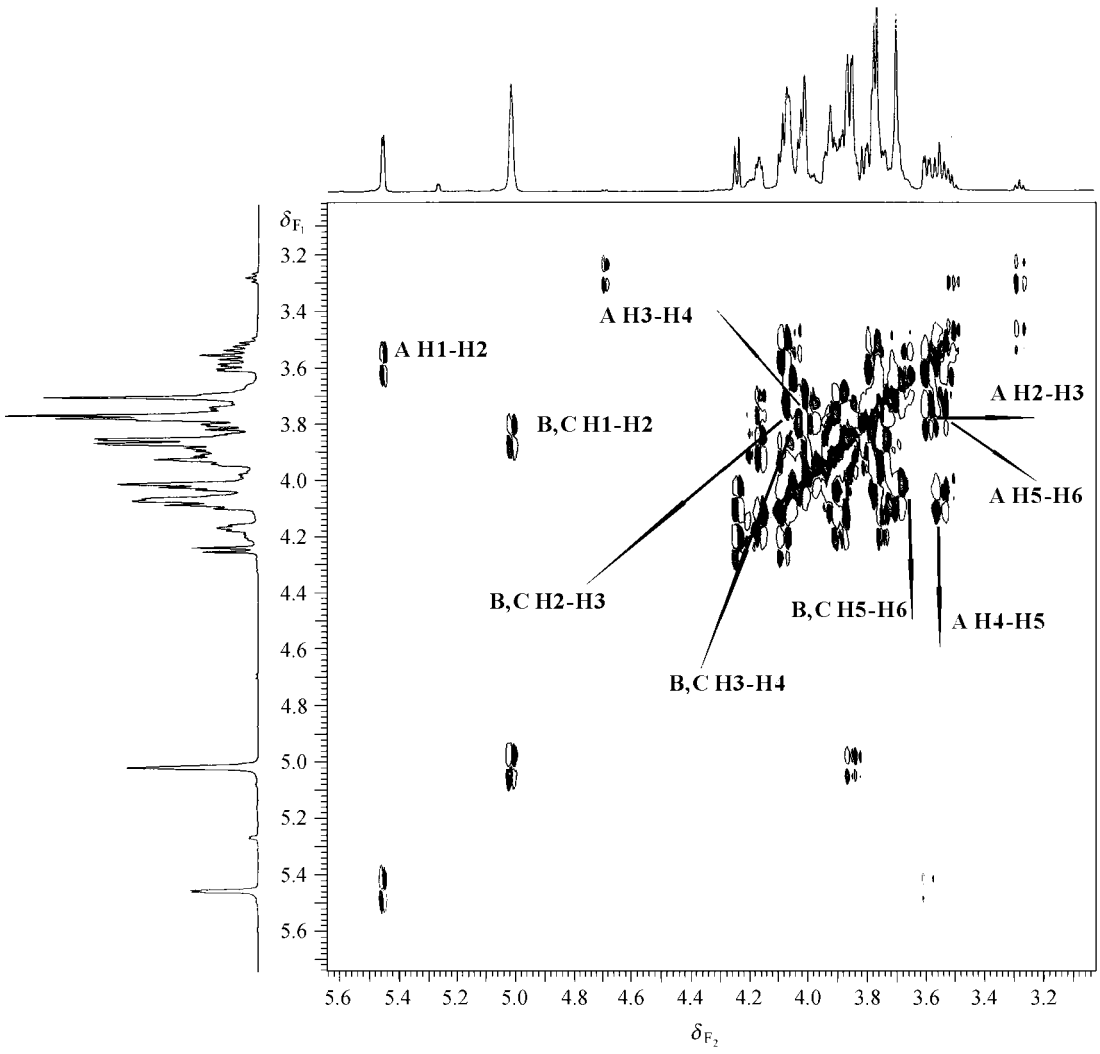


Figure 7 2D DQCOSY spectrum (600 MHz) of SnS-2, recorded in D_2O at 30 ℃.

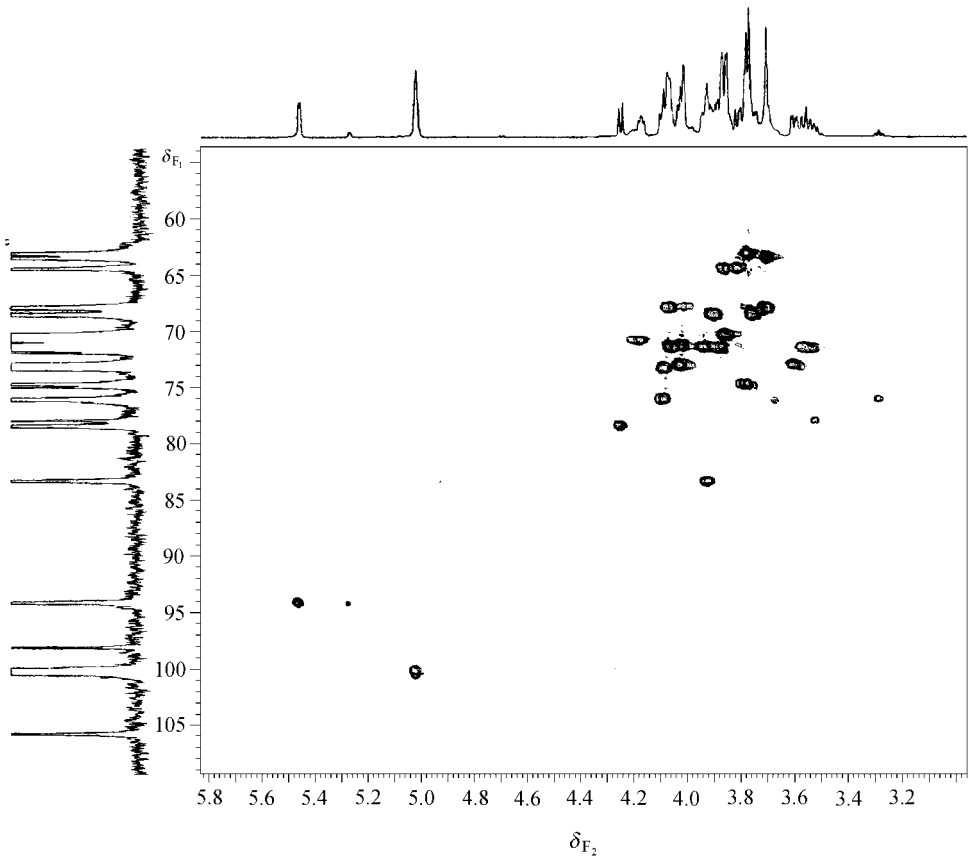
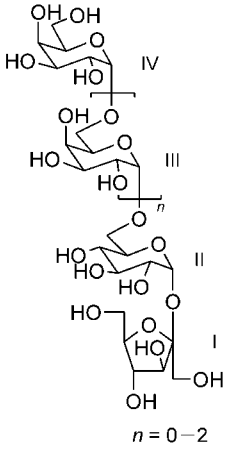


Figure 8 2D ^{13}C-^{1}H undecoupled HMQC spectrum (600 MHz) of SnS-2, recorded in D_2O at 30 ℃.

On the basis of NMR spectroscopy and the methylation data of SnS-2, it was demonstrated that residue I is a 2-substituted *β*-Fruc*f*, residue II is a 6-substituted *α*-Glc*p*, residue III is a 6-substituted Gal*p* and residue IV is a terminal *α*-Gal*p*. According to ESI-MS of SnS-2, it was composed of a disaccharide, a trisaccharide, a tetrasaccharide, and a pentasaccharide. So the structure of SnS-2 can be illustrated as:



Bioactivity of SnS-2

Bioactivity assay of SnS-2 showed that it could inhibit growth of Lewis carcinoma implanted in mice. The results are listed in Table 3.

Table 3 Effect of SnS-2 on the inhibition of Lewis pulmonary carcinoma growth in mice

Sample[a]	Administration method	No. of mice	Dose/ (mg/kg×days)	Tumor weight ($X\pm$SD)	Inhibition ratio/%
SnS-2	i.p.	8	200×10	0.481±0.07	49.90[b]
CTX	i.p.	8	30×10	0.010±0.02	99.89[b]
Control	i.p.	16	—	0.960±0.15	—
SnS-2	i.p.	8	100×10	0.610±0.06	41.90[b]
CTX	i.p.	8	30×10	0.124±0.02	88.09[b]
Control	i.p.	16	—	1.05±0.16	—

[a] Control: 0.9% NaCl solution. CTX: cyclophosphamide. [b] $P<0.01$, significantly different from SnS-2 with control.

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