A Novel Macrolactam-trisaccharide Antifungal Antibiotic

Taxonomy, Fermentation, Isolation, Physico-chemical Properties, Structure Elucidation and Biological Activity

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A novel secondary metabolite SCH 42282 (**1**), with antifungal activity was isolated from the fermentation broth of a soil actinomycete identified as a *Microtetraspora* sp. The active compound was separated from the fermentation broth by butanol extraction and purified on a silica gel column and by multi-coil counter current chromatography. The compound was identified as a novel macrolactam trisaccharide related to SCH 38518 (**4**). The structure was established by hydrolysis of the parent compound and spectroscopic studies of the acetate derivative. The compound is active against *Candida* spp. (Geometric Mean MIC's, 18 μg/ml) but less active than SCH 42729 (**3**), the disaccharide (Geometric Mean MIC's, $\geq$10.7 μg/ml) and SCH 38518 (**4**), the monosaccharide (Geometric Mean MIC's, 3.8 μg/ml).

During our search for novel antifungal antibiotics using the galactose permeation assay,[1] we isolated a series of eight novel macrolactam monosaccharides,[2~5] and one macrolactam disaccharide,[6] all from the fermentation broths of various actinomadurae. Another actinomycete, SCC 1829, produced the macrolactam SCH 38518 (**4**)[2] along with an unknown compound, SCH 42282 (**1**), identified as a novel macrolactam trisaccharide. In this report, we describe the taxonomy of the producing strain, the fermentation conditions for production of the active metabolite, as well as isolation, physico-chemical properties, structure elucidation and biological activity of the novel antifungal compound.

Materials and Methods

The Microorganism

Strain SCC 1829 was isolated from a soil sample collected in India. The soil was air dried, suspended in sterile water, serially diluted and plated on the following medium; molasses 1.0 ml, $NaNO_3$ 0.1 g, agar 15 g, distilled water 1 liter. Plates were incubated at 30°C, 50% humidity for 14 to 21 days. SCC 1829 appeared as a hard filamentous colony which was isolated to purity.

Microscopic and Macroscopic Observations

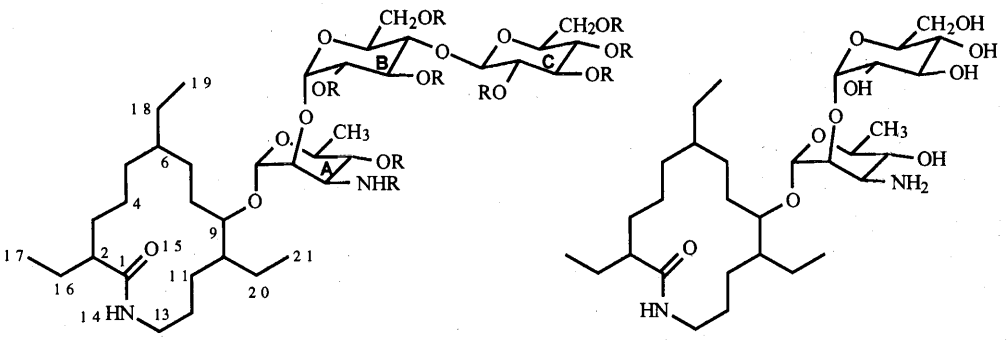
Washed cells were inoculated onto the surface of petri dishes containing either tap water agar, inorganic salts-starch agar (ISP 4),[7] or Czapek-sucrose agar,[8] incubated at 28°C for 28 days and examined weekly under a microscope (400×). If characteristic morphological structures were not formed the culture was grown on all of the media described by SHIRLING and GOTLEIB[7] in an attempt to induce aerial mycelia and spore formation.

Chemotaxonomy

Whole-cell and cell wall preparations were analyzed for diaminopimelic acid, sugars[9,10] phospholipids,[11] and menaquinones.[12]

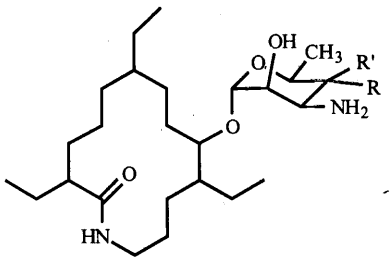
Fermentation

Fermentation studies were carried out in shake flasks. Stock cultures were maintained as frozen whole broths at −20°C in a final concentration of 10% glycerol. The inoculum medium for antifungal production contained beef extract 0.3%, tryptone 0.5%, yeast extract 0.5%, cerelose 0.1%, potato starch 2.4%, calcium carbonate 0.2% and 1.0 ml antifoam (Dow Corning Antifoam B, with 10% active emulsion, Lot # LL126337) in one liter of tap water. A 250 ml Erlenmeyer flask containing 70 ml

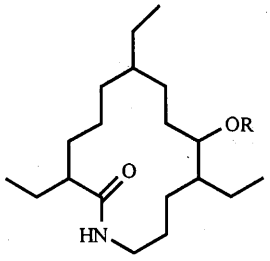


1 R=H
2 R=Ac

3



4 R=OH, R'=H
5 R=H, R'=OH

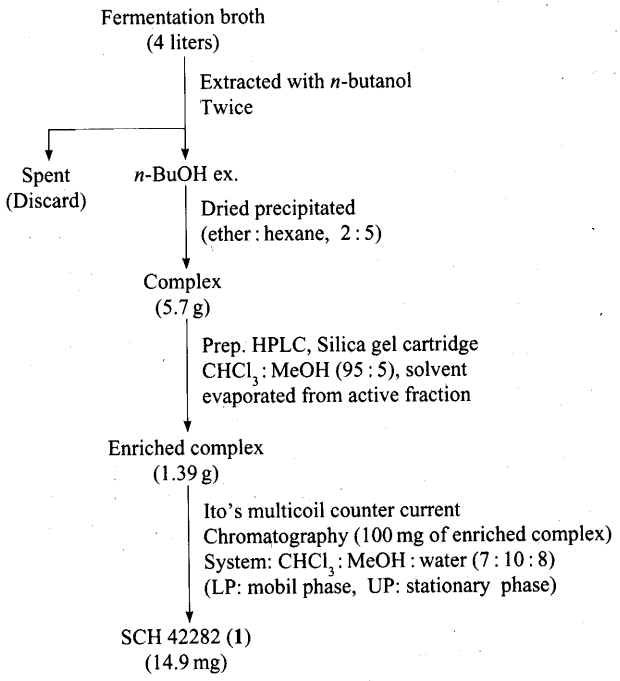


6 R=H
7 R=Ac

of this medium was inoculated with 2.0 ml of the stock culture. The flask was incubated at 30°C on a rotary shaker (New Brunswick Model # G-53, 3 tier, 2″ stroke) at 300 rpm for 66~72 hours.

Eighteen ml of this seed culture was used to inoculate a 2 liter Erlenmeyer flask containing 350 ml of the fermentation medium consisting of lactose 2.5%, molasses 0.5%, ProFlo flour 1.0%, tryptone 0.5%, Edamins 0.1%, EDTA (0.01% solution) 1 ml, boric acid (0.0035% solution) 1 ml and 0.5 ml antifoam (Dow Corning Antifoam B, with 10% active emulsion, Lot # LL126337) in one liter of tap water. The pH of the medium was adjusted to 7.8 prior to autoclaving. The fermentation was carried out at 300~320 rpm agitation on an NBS (Model # G-53, 3 tier, 2″ stroke) two tier shaker at 30°C for 120 hours. The antibiotic production along with pH and packed cell volume were monitored at regular intervals. The production of antibiotic was measured by an agar diffusion well (12 mm) assay against *Candida albicans* 406.

Fig. 1. Isolation and purification scheme for SCH 42282 (**1**).



Isolation

The culture, SCC 1829, produces one major compound, **1**, and several minor components including macrolactam monosaccharide **4**[2]. The procedure for the isolation of **1** is described in Figure 1. A 4.0 liters portion of the culture broth was extracted twice with eight liters of *n*-butanol. The organic extract was concentrated to about 2 liters, washed with water and evaporated to

dryness. The oily residue was dissolved in a minimum amount of methanol and precipitated by addition to a mixture of ether and hexane (2:5). The resulting precipitate was filtered, dried (5.7 g) and purified on a Waters Preparative 500 HPLC using a silica gel cartridge and eluting with a mixture of chloroform and methanol (95:5). Fractions containing **1**, were combined and dried to yield 1.39 g of solids enriched in **1**. Further purification was achieved by counter current chromatography on Ito's Multicoil CCC instrument. The lower phase of the mixture of chloroform, methanol and water (7:10:8) was used as the mobile phase while the upper phase served as the stationary phase. Purity of the fractions were monitored by TLC on silica gel using chloroform: toluene (8:2) solvent mixture as developing solvent. Compound **1** was visualized on TLC by bioautography against *C. albicans* 406, by spraying with water and acid spray [sulfuric acid and methanol, (1:1)].

General Procedure

IR spectra were determined on a Nicolet FTIR model 10-MX instrument. Ultraviolet spectra were obtained by using a Hewlett Packard '8450 A' UV-vis spectrophotometer equipped with HP-9872B plotter. All FAB mass spectra were obtained by using a Finnigan MAT-312 mass spectrometer in a glycerol-thioglycerol matrix. NMR spectra were measured on a Varian XL-300 instrument operating at 300 and 75 MHz for ^{1}H and ^{13}C NMR respectively. ^{1}H and ^{13}C NMR spectra were recorded relative to TMS as an internal standard.

Acetylation of 1

One hundred mg of **1** was acetylated with a mixture of 1:1.5 (v/v) acetic anhydride and pyridine at 0°C for 3 hours and then left overnight at room temperature. The reaction mixture was quenched in ice and extracted with 100 ml of ethyl acetate. The organic extract was washed with dilute HCl, brine (25 ml each), dried on anhydrous Na_2SO_4 and solvent evaporated to afford the crude solid. Chromatography of this crude solid on a silica gel column and elution with a mixture of ethyl acetate, hexane and methanol (50:50:1.5), gave 35.7 mg of pure acetyl derivative (**2**).

Hydrolysis with 4 N HCl to obtain the Aglycone

One hundred mg of **1** was mixed with 5 ml 4 N HCl in a sealed tube under reflux for 4 hours, diluted with water and extracted with 200 ml ethyl acetate. The organic extract was dried over anhydrous Na_2SO_4 and the solvent was evaporated to obtain the aglycone (**6**) 28.5 mg as white solid. This was compared by TLC (solvent system: chloroform: methanol, 95:5) with the aglycone of SCH 38518.

Acetylation of the Aglycone

The aglycone **6**, (25 mg) was converted to acetate by stirring with a mixture of acetic anhydride/pyridine (1:1.5, 5 ml) at room temperature overnight, quenched in ice and extracted with 100 ml ethyl acetate. The solids obtained were purified by passing through a silica gel column and eluting with a mixture of ethyl acetate and hexane (3:7). The pure aglycone monoacetate **7**, obtained was compared by TLC (ethyl acetate: hexane: methanol 50:50:1) with the aglycone monoacetate of SCH 38518[2] by TLC.

Mild Hydrolysis

One hundred mg of **1** was partially hydrolyzed by dissolving it in 30 ml mixture of 0.1 N HCl and methanol (2:1) and stirred overnight at room temperature, methanol was removed from the reaction mixture, filtered and the solids obtained were dried and compared by TLC (toluene: methanol 8:2) and HPLC with authentic samples of SCH 38518 (**4**) and SCH 39185 (**5**).

The filtrate was freeze dried and the sugars obtained were converted to pentaacetate by stirring with a mixture (5 ml) of acetic anhydride and pyridine (1:1.5) overnight. The reaction mixture was quenched in ice and the aqueous solution was extracted with 100 ml ethyl acetate. The organic extract was washed with dilute HCl, brine and dried over anhyrous Na_2SO_4 and the solvent removed. The sugars obtained was compared (by TLC) with pentaacetates of various hexoses.

Results

Identification of SCC 1829

The producing strain, SCC 1829, is filamentous and gram positive. On agar, non-fragmenting substrate mycelia were formed. Aerial mycelia were not formed on any of the media tested nor were spores formed on the vegetative mycelia. Chemical analysis of the cell wall indicated the presence of *meso*-diaminopimelic acid, glutamic acid, alanine, glucosamine and muramic acid with traces of rhamnose (type III). Whole cell sugar analysis showed the presence of large amounts of madurose with traces of rhamnose (type B). The phospholipid pattern was PIV: major amounts of phosphatidylmethylethanolamine, minor amounts of phosphatidyl-

Fig. 2. Fermentation profile of SCC 1829.

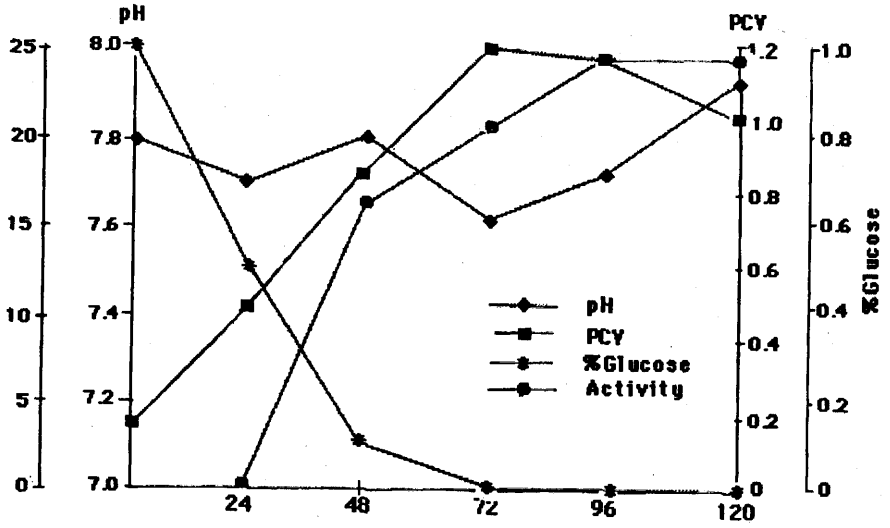
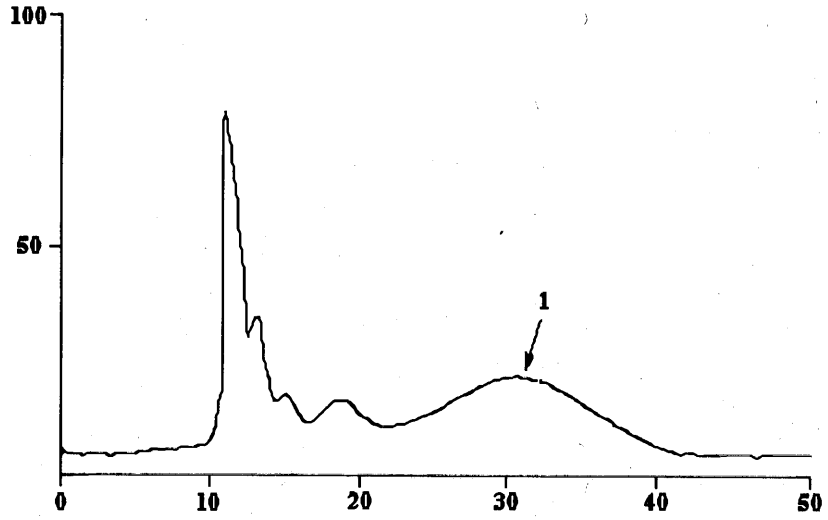


Fig. 3. Elution profile of SCH 42282 (**1**) from Ito's counter current instrument.



Instrument: Ito's counter current chromatogram
System: Chloroform : methanol : water (7 : 10 : 8)
(Lower phase: Mobile phase, 800 rpm)
Flow rate: 6 ml/minute

ethanolamine, and an unknown glucosamine-containing phospholipid (GlcNU) were the characteristic components. Phosphatidylinositol-mannoside, phosphatidylinoitol and diphosphatidylglycerol were also present. The major menaquinone was MK9(H4). Based on the chemical characteristics of SCC 1829, the culture is identified as a species of *Microtetraspora*.[13]

Fermentation

A typical time course study profile of a 4 liter fermentation is shown in Fig. 2. The sugar level in the fermentation medium depletes as the cells grow and then levels off as the antibiotic production starts. The packed cell volume (PCV) which indicates cell growth continues to increase until the production of antifungal reaches to its optimum and then levels at the end of the fermentation. The antifungal production starts between 24 and 48 hours and peaks at 100 hours of fermentation.

Isolation

SCH 42282 (**1**) is soluble in butanol and can be separated from the broth by butanol extraction. Four

Table 1. Physico-chemical properties of the acetate (**2**).

UV (MeOH) λ_{max} nm	End absorption
IR (KBr) ν_{max} cm^{-1}	3320, 2930, 1750, 1655, 1540, 1460, 1375, 1230, 1050 cm^{-1}
FAB-MS	1159 $(M+H)^+$, 848, 457
^1H NMR($CDCl_3$)	5.54 (b, 1H), 5.4 (m, 2H), 5.0~5.15 (m, 4H), 4.92 (dd, J=4, 12 Hz, 1H), 4.68 (d, J=1 Hz, 1H), 4.62 (d, J=6.5 Hz, 1H), 4.56 (dd, J=1.5, 9 Hz, 1H), 3.9~4.2 (m, 6H), 3.5~3.8 (m, 5H), 3.08 (d, J=15 Hz, 1H), 3.00 (db, J=15 Hz, 1H), 2.05 (s, 3H), 2.00 (s, 6H), 1.96 (s, 3H), 1.94 (s, 3H), 1.92 (s, 3H), 0.9~1.6 (several CH and CH_2), 1.08 (d, J=7 Hz, 3H), 0.82 (t, J=7 Hz, 3H), 0.77 (t, J=7 Hz, 3H), 0.74 (t, J=7 Hz, 3H)

liters of fermentation broth gave 5.7 g of active crude material. The active fractions obtained after preparative HPLC showed mixture of **1**, **4** and minor amounts of other macrolactoms. The profile of elution of macrolactams obtained by Counter Current Chromatography (CCC) is shown in Fig. 3. SCH 42282 elutes as a broad band after three column volumes of elution through the CCC coil. One hundred mg of enriched solids yielded 14.9 mg of **1** (80% purity).

SCH 42282 is basic in nature, showed a positive ninhydrin test, and an end absorption in the UV spectrum. The IR spectrum indicated the presence of an amide functionality (1655, 1540 cm^{-1}). The physicochemical properties and lipid nature showed a resemblance to other macrolactams.[2~5] This compound, like other macrolactams, behaves as a lipid and can be detected on a silica gel plate by water spray. The FAB mass spectrum showed a molecular ion peak at 781 $(M+H)^+$, 162 mass units higher than macrolactam disaccharide **3**[6] and a fragment ion peak at 457, which is due to the SCH 38518 moiety. High resolution mass measurements revealed the molecular formula to be $C_{37}H_{69}N_2O_{15}$ (Obsd. 781.4651 calcd for $C_{37}H_{69}N_2O_{15}$, 781.4697). The molecular formula of **1** is $C_6H_{10}O_5$ higher than that of SCH 42729 (**3**). These data suggested that the compound contained one hexose sugar more than **3**. However because of the unstable nature of the compound, further structural studies were accomplished by evaluation of the spectral data of the stable acetyl derivative.

The SCH 42282 purified over CCC was acetylated with a mixture of acetic anhydride and pyridine to obtain an acetyl derivative (**2**). The spectroscopic properties of **2** are shown in Table 1. The FAB mass spectrum of this compound showed a molecular ion peak at 1159 $(M+H)^+$, revealing it to be the nonaacetyl derivative. ^1H NMR showed three primary methyls at δ 0.8~0.9, and a secondary methyl at δ 1.08 and revealed the pattern is similar to that in macrolactam monosaccharides (**4** or **5**) with two additional sugars. ^{13}C NMR of **2** along with triacetate of SCH 38518 and SCH 39185 are shown in Table 2. 2D (^1H-^1H) correlation spectrum showed that the sugar B is attached to sugar A at C-2 and that the sugar A is attached to the aglycone at C-9. 2D (^1H-^1H) correlation spectrum also showed that the sugar C was attached to sugar B and not to sugar A. The attachment of sugar C was established to be at C-4 of sugar B. Comparison of the ^{13}C chemical shift data of the acetates of **1**, **3** and **4** confirmed the presence of macrolactam ring and three sugars in the molecule.

Aglycone **6**, obtained from hydrolysis of **1** with 4 N HCl on TLC comparison with the aglycone of **4** indicated that they are identical. The aglycone was further acetylated with acetic anhydride/pyridine to yield a monoacetate (**7**) which on further comparison (TLC and spectral data) with aglycone monoacetate of SCH 38518[2] confirmed its structure.

Mild hydrolysis of **1** by 0.1 N HCl and methanol (2 : 1) yielded a monosaccharide and sugars in the aqueous solution. The monosaccharide was found to be identical with SCH 38518, indicating the amino sugar to be mycosamine. The sugars were converted to their pentaacetates and both were identified to be glucose pentaacetates on comparison with an authentic sample. The sugar linkages with mycosamine and glucopyranoses were determined to be α-, α- and β-, respectively, from their ^{13}C chemical shifts of anomeric carbons (94.95, 95.76 and 102.37 ppm respectively) and ^1H-^1H spin coupling constants of the anomeric protons [4.56 (dd, J=9, 1.5 Hz, 1H), 4.62 (d, J=6.5 Hz, 1H), 4.68 (d, J=1 Hz, 1H) respectively]. Thus the structure was established as **1**.

Table 2. ^{13}C Chemical shift of acetate derivatives of SCH 42282 (**1**), SCH 42729 (**3**), SCH 38518 (**4**) and SCH 39185 (**5**).

Carbon	Acetates of			
	1	**3**	**4**	**5**
$-CH_3$	8.90	9.10	8.91	8.83
	12.34	12.46	12.31	12.30
	12.40	12.66	12.41	12.43
	20.94	20.79	20.90	20.85
$>CH_2$	22.04	22.49	22.69	22.24
	24.87	23.12	23.09	22.32
	24.91	25.06	24.92	24.99
	25.13	25.27	25.09	25.04
	26.64	26.77	26.59	26.55
	26.98	27.25	27.11	27.06
	28.38	28.23	28.36	28.16
	32.12	31.78	31.69	31.72
	33.55	33.73	33.52	33.53
	38.81	38.66	38.69	38.69
>CH–	38.37	38.35	38.53	38.48
	40.92	40.89	40.91	40.70
	50.87	50.89	50.74	50.77
	77.30	77.93	78.75	78.35
>CO	176.10	175.85	176.02	176.02
Sugar A	94.95	94.06	94.28	95.02
	72.91	73.25	73.14	70.33
	48.22	55.15	48.50	45.06
	70.61	72.43	72.35	70.12
	66.23	67.00	66.66	65.50
	17.53	17.76	17.52	16.64
Sugar B	95.76	89.97		
	71.16	74.35		
	72.56	72.89		
	69.02	71.09		
	66.87	68.63		
	61.18	61.96		
Sugar C	102.37			
	71.20			
	70.77			
	69.57			
	68.55			
	62.06			
$COCH_3$	20.64	20.79	21.06	21.18
	20.64	20.79	21.64	21.40
	20.64	20.79	23.31	22.89
	20.71	20.79		
	20.71	21.20		
	20.88	21.66		
	20.94			
	21.08			
	21.79			
$COCH_3$	169.05	169.36	169.76	169.52
	170.15	169.69	170.03	170.80
	170.15	169.99	171.59	170.80
	170.34	170.15		
	170.35	170.20		
	170.57	170.61		
	170.66			
	171.04			
	171.08			

Table 3. *In vitro* activities of SCH 42282 (**1**) and SCH 38518 against various fungi.

Organism (No. of strains)	Medium	Geometric mean MIC's (μg/ml)		
		1 SCH 42282	**3** SCH 42729	**4** SCH 38518
Candida (12)	SDB[a]	18.0	≥10.7	3.8
	EMEM[b]	50.8	32.0	17.3
Dermatophytes (7)	SDB[c]	≥78.0	≥64.0	≥64.0
Aspergillus (2)	SDB[c]	≥128.0	≥128.0	≥512.0

[a] Sabouraud dextrose broth, pH 5.7, 48 hours.
[b] Eagles minimum essential medium, pH 7.0, 48 hours.
[c] Sabouraud dextrose broth, pH 5.7, 72 hours.

Biological Activity

The *in vitro* antifungal activity of **1**, **3** and **4** against various yeasts and dermatophytes in Sabouraud dextrose broth (SDB) and Eagles minimum essential media (EMEM) are shown in Table 3. All are active against *Candida* spp. They exhibit better activity in SDB (3~4 fold) than in EMEM. In general, it can be seen that the addition of sugar units to the parent resulted in lower potency. SCH 38518, the monosaccharide, was the most active compound followed by SCH 42729, the disaccharide, with SCH 42282, the trisaccharide being least active.

Acknowledgments

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