NEW CHOLESTEROL BIOSYNTHESIS INHIBITORS MC-031 (*O*-DEMETHYLCHLOROTHRICIN), -032 (*O*-DEMETHYLHYDROXYCHLOROTHRICIN), -033 AND -034

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In the course of our screening for microbial compounds which possessed inhibitory activities against biosynthesis of cholesterol from mevalonate, we isolated four new compounds from the cultural broth of *Streptomyces* sp. A7361. They are structurally related to, but distinct from chlorothricin.

In a previous paper[1], we reported the isolation of new chloesterol biosynthesis inhibitors, neokadsuranic acid A and (24*Z*)-3-oxo-lanosta-8,24-dien-26-oic acid from the ethanolic extract of stems of *Kadsura heteroclita*. Subsequent efforts have led to the isolation of four new compounds, designated MC-031, -032, -033 and -034, from a cultured broth of *Streptomyces* sp. A7361. The fermentation, isolation, structure determination and biological activities are described here.

Results and Discussion

Strain

Streptomyces sp. A7361 was isolated from a soil sample collected in Ohmiya city, Saitama Prefecture in Japan. The cultural characteristics of this strain on nine media are shown in Table 1. The aerial

Table 1. Cultural characteristics of strain A7361.

Medium	Growth	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar	Moderate Cream	None	None
Glucose-asparagine agar	Moderate Cream	None	None
Glycerol-asparagine agar	Good Pale brown	Poor White	None
Inorganic salts-starch agar	Good Pale brown	Moderate Gray white	None
Tyrosine agar	Good Dark brown	None	Pale brown
Nutrient agar	Moderate Cream	None	None
Yeast extract-malt extract agar	Good Pale brown	Moderate White	None
Oatmeal agar	Good Pale brown	Good Gray white	None
Peptone-yeast extract ion agar	Moderate Dark brown	None	Dark brown

mycelium was gray or white and branched. It bears more than 10 spores in a chain. Scanning electron micrograph of strain A7361, shown in Fig. 1, indicated the spores are smooth-surfaced and $0.65\sim0.80\times0.90\sim1.25\,\mu m$ size. The results of analysis of cell wall hydrolysate established the cell wall belonged to type I. Physiological properties of strain A7361 are presented in Table 2. This strain utilize glucose, arabinose, xylose, fructose, inositol, mannitol, sucrose, rhamnose and raffinose. From the comparisons of morphological, cultural and physiological properties to the descriptions of *Streptomyces* species[2,3], strain A7361 resembled *Streptomyces griseosporeus*, but direct comparison was not carried out.

Fermentation and Isolation

Seed culture was carried out in Erlenmeyer flasks each containing 100 ml of medium consisting of oatmeal 2%, glucose 2%, NaCl 0.4%, meat extract 0.4%, Fe_2SO_4 0.04%, $MnCl_2$ 0.04% and $CaCO_3$ 0.3%. The broth was cultured for 4 days on a rotary shaker at 28°C. Two liters of the culture was transferred to a 200-liter jar fermenter containing 120 liters of the above medium and fermented for 4 days at 28°C with aeration at 120 liters per minute and agitation at 200 rpm.

Fig. 1. Scanning electron micrograph of the spores of strain A7361 on oatmeal agar.

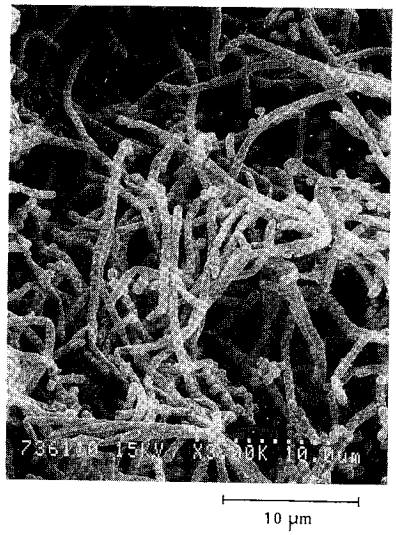


Table 2. Physiological properties of strain A7361.

Temperature range for growth	10~40°C
Optimum temperature range for growth	20~34°C
Gelatin liquefaction	Negative
Starch hydrolysis	Positive
Milk coagulation	Negative
Milk peptonization	Positive
Melanin formation	Positive

Table 3. Physico-chemical properties of MC-031, -032, -033, -034 and chlorothricin.

	MC-031	MC-032	MC-033	MC-034	Chlorothricin
Appearance	White powder	White powder	White powder	White powder	White powder
$[\alpha]_D^{25}$	+8.8° (*c* 0.5, acetone)	+9.4° (*c* 0.5, acetone)	+1.2° (*c* 0.5, acetone)	+6.4° (*c* 0.5, acetone)	+1.5° (*c* 1.0, $CHCl_3$)
MP (°C)	208~211	199~202	285~287	267~269	206~207
Elemental analysis					
Calcd:	C 62.55	C 61.51	C 62.55	C 61.51	C 62.89
	H 6.49	H 6.38	H 6.49	H 6.38	H 6.60
	Cl 3.72	Cl 3.66	Cl 3.72	Cl 3.66	Cl 3.67
Found:	C 61.56	C 60.40	C 62.36	C 60.88	C 61.49
	H 6.68	H 6.63	H 6.38	H 6.19	H 6.72
	Cl 3.43	Cl 3.36	Cl 3.45	Cl 3.33	Cl 3.21
FAB-MS $(M-H)^-$ (*m/z*)	939	955	939	955	953
Molecular formula	$C_{49}H_{61}O_{16}Cl$	$C_{49}H_{61}O_{17}Cl$	$C_{49}H_{61}O_{16}Cl$	$C_{49}H_{61}O_{17}Cl$	$C_{50}H_{63}O_{16}Cl$
UV λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$)	221 (274), 290 (29)	220 (246), 290 (22)	221 (264), 253 (sh, 170), 287 (28)	221 (224), 254 (sh, 78), 292 (22)	227 (241), 285 (26)

Table 4. ^{1}H NMR spectral data of the sugar moiety.

	Chlorothricin[a]	Hydroxychlorothricin[a]	MC-031[a]	MC-032[a]	MC-033[b]	MC-034[c]
1″	4.65 (br d, J=9.5)	4.65 (br d, J=9.5)	4.63 (dd, J=9.5, 1.5)	4.67 (dd, J=10.0, 1.5)	4.69 (br d, J=9.5)	5.03 (dd, J=9.5, 1.5)
2″	1.83 (m), 2.48 (m)	1.70 (m), 2.47 (m)	1.83 (m), 2.48 (m)	1.82 (m), 2.51 (m)	1.55 (m), 2.17 (m)	2.22 (m), 2.74 (m)
3″	5.08 (ddd, J=13.5, 9.0, 5.0)	5.07 (m)	5.11 (m)	5.12 (m)	3.68 (m)	4.28 (m)
4″	3.38 (t, J=9.0)	3.51 (t, J=8.5)	3.41 (t, J=9.0)	3.43 (t, J=9.0)	4.60 (t, J=9.5)	5.40 (t, J=9.5)
5″	3.32 (m)	3.40 (m)	3.28 (m)	3.54 (m)	3.55 (m)	3.89 (m)
6″	1.41 (d, J=6.0)	1.40 (d, J=6.0)	1.38 (d, J=6.0)	1.40 (d, J=6.0)	1.24 (d, J=6.0)	1.60 (d, J=6.0)
1‴	4.53 (br d, J=10.0)	4.38 (d, J=7.0)	4.50 (br d, J=9.0)	4.37 (d, J=7.0)	4.52 (br d, J=9.5)	4.82 (d, J=8.0)
2‴	1.61 (m), 2.31 (m)	3.41 (m)	1.57 (m), 2.26 (m)	3.50 (m)	1.31 (m), 2.12 (m)	4.02 (dd, J=9.5, 8.0)
3‴	3.61 (m)	3.53 (dd, J=6.0, 9.0)	3.58 (m)	3.48 (dd, J=9.0, 6.5)	3.51 (m)	4.10 (dd, J=9.5, 9.0)
4‴	3.04 (t, J=8.5)	3.15 (t, J=8.5)	3.01 (t, J=9.0)	3.14 (t, J=9.0)	2.93 (t, J=9.5)	3.55 (t, J=9.5)
5‴	3.56 (m)	3.38 (m)	3.50 (m)	3.39 (m)	3.29 (m)	3.72 (m)
6‴	1.32 (d, J=6.5)	1.31 (d, J=6.0)	1.28 (d, J=6.0)	1.30 (d, J=6.0)	1.16 (d, J=6.0)	1.45 (d, J=6.5)
2′-OCH_3	3.83	3.83	—	—	—	—

[a] In $CDCl_3$.
[b] In DMSO-d_6.
[c] In pyridine-d_5.

Table 5. ^{13}C NMR spectral data of the sugar moiety.

	Chloro-thricin[a]	Hydroxy-chlorothricin[a]	MC-031[a]	MC-032[a]	MC-033[b]	MC-034[c]
1″	100.4	100.5	100.2	101.1	99.9	101.1
2″	36.2	36.2	36.4	36.4	38.7	41.0
3″	74.8	74.8	74.7	74.7	67.4	69.1
4″	74.0	74.0	73.5	73.5	77.8	79.2
5″	70.2	70.2	70.1	70.0	69.3	70.9
6″	17.5	17.1	17.4	17.4	17.2	18.2
1‴	101.2	103.4	101.1	103.4	100.6	105.5
2‴	38.1	74.8	38.0	74.7[d]	39.9	75.4
3‴	69.6	74.5	69.5	74.1[d]	68.4	76.0
4‴	88.4	86.9	88.2	86.6	87.0	86.7
5‴	72.1	72.2	72.3	72.4	69.5	70.7
6‴	17.9	17.6	17.7	17.5	17.8	18.2
2′-OCH_3	56.3	56.4	—	—	—	—

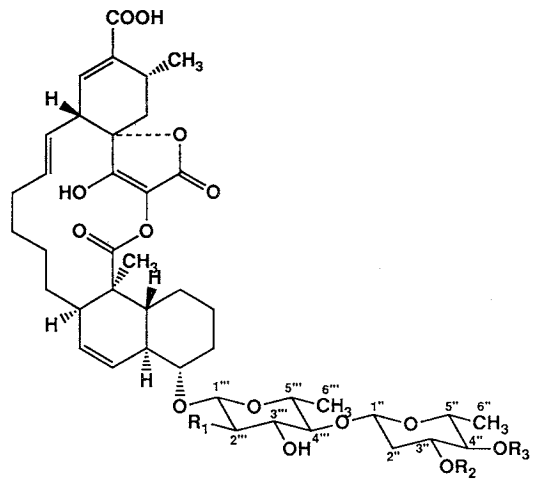
[a] In $CDCl_3$.
[b] In DMSO-d_6.
[c] In pyridine-d_5.
[d] May be interchanged.

Table 6. Inhibition of cholesterol biosynthesis by MC-031, -032, -033, -034 and chlorothricin (*in vitro*).

Sample	IC_{50} value ($\times 10^{-5}$ M)
MC-031	10.6
MC-032	10.5
MC-033	1.06
MC-034	18.3
Chlorothricin	10.5

The filtered broth (120 liters) was passed through a column of Diaion HP-20 (6 liters). After washing with water (10 liters) the antibiotics were eluted with acetone (10 liters). Mycelial cake was extracted with acetone (60 liters) and the acetone layer was combined and concentrated *in vacuo*. The residue was extracted with EtOAc and the upper layer was evaporated to dryness under reduced pressure. The brown syrup (50 g) thus obtained was applied to a silica gel column (3 liters) which was eluted with a mixture of CHC_3-MeOH (95:5) and the active fractions were concentrated to give 8.5 g of yellow solid. This solid was subjected to a column of Sephadex LH-20 column (MeOH) to afford 2.5 g of yellow powder. Further purification was carried out by reverse phase HPLC (column: ODS-5251-N, Senshu Kagaku

Fig. 2. Structures of MC-031, -032, -033, -034, chlorothricin and hydroxychlorothricin.



	R_1	R_2	R_3
MC-031	H	A	H
MC-032	OH	A	H
MC-033	H	H	A
MC-034	OH	H	A
Chlorothricin	H	B	H
Hydroxychlorothricin	OH	B	H

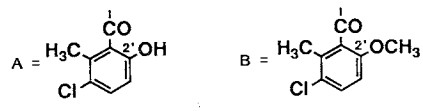


Table 7. Antimicrobial activities of MC-031, -032, -033, -034 and chlorothricin against Gram-positive and Gram-negative bacteria.

Organisms	MIC (μg/ml)				
	MC-031	MC-032	MC-033	MC-034	Chlorothricin
Micrococcus luteus ATCC 9341	50	>100	100	>100	100
Bacillus subtilis ATCC 6633	25	50	25	50	25
Staphylococcus aureus 209P-JC	50	>100	50	100	25
S. aureus Smith 4	>100	>100	>100	>100	>100
Enterococcus faecalis CSJ1212	>100	>100	>100	>100	>100
Escherichia coli K-12	>100	>100	>100	>100	>100
Klebsiella pneumoniae PCI-602	>100	>100	>100	>100	>100

Co., Japan, solvent: 80% MeOH (+0.1% H_3PO_4), detection UV 220 nm). Four active fractions with Rt 7.4, 10.0, 10.6 and 14.4 minutes were obtained at a flow rate of 10 ml/minute. After evaporation of the eluates, MC-031 (530 mg), MC-032 (420 mg), MC-033 (340 mg) and MC-034 (100 mg) were obtained as white powder.

Table 8. Inhibition of the growth of HL-60 cells MC-031, -032, -033, -034 and chlorothricin (*in vitro*).

Sample	IC_{50} value ($\times 10^{-5}$ M)
MC-031	053
MC-032	2.62
MC-033	1.06
MC-034	2.62
Chlorothricin	1.05

Physico-chemical Properties and Structure

Their physico-chemical properties (Table 3) indicated that these compounds were structurally similar to chlorothricin[4] which was co-produced. The molecular formulae were deduced by a combination of elemental analyses and negative FAB-MS.

The ^{1}H NMR spectra of MC-031 and MC-032 are very similar to those of chlorothricin and hydroxychlorothricin[5], respectively, however they lacked the signals arising from the aromatic methoxy group (Tables 4 and 5). Furthermore, the MWs of MC-031 and MC-032 were 14 mmu less than either chlorothricin or hydroxychlorothricin, respectively. So the structures of MC-031 and MC-032 were determined as shown in Fig. 2. MC-033 and MC-034 were isomers of MC-031 and MC-032, respectively, and their ^{1}H NMR spectra were similar to those of the corresponding isomer except for the signals assigned to the terminal sugar moiety where there were changes in the chemical shifts of 3″-H and 4″-H. The signal assigned to 3″-H was about 0.8~1.4 ppm upfield from its position in spectra of MC-031 and MC-032 while 4″-H resonated about 1.2~2.0 ppm downfield. This observation together with the fact 4″-H is coupled to both vicinal protons with coupling constants of 9.5 Hz indicated the point of attachment of the benzoyl group as C-4″ and tht 4″-H was in the axial orientation. The location of the benzoyl group was confirmed by a ^{13}C-^{1}H long-range coupling between 4″-H and an ester carbonyl carbon in the HMBC spectra. Based on these analyses the structures of MC-033 and MC-034 were assigned as shown in Fig. 2.

Biological Properties

Inhibitory activities against cholesterol biosynthesis from mevalonate were shown in Table 6. Among the five compounds, MC-033 was the most active. The MICs of these compounds assayed by agar dilution method are shown in Table 7. They were weakly antimicrobial against Gram-positive bacteria and inhibited the growth of HL-60 cells (Table 8).

Experimental

General Methods

MP's were determined on a Yanagimoto micro melting point apparatus and were uncorrected. NMR spectra were measured on a Jeol GX-400 spectrometer. UV spectra were recorded on a Hitachi 220A spetrophotometer and optical rotations were measured on a Jasco DIP-360 polarimeter.

Inhibition of Cholesterol Biosynthesis from Mevalonate

Inhibition of cholesterol biosynthesis was measured according to the previous report[6]; [2-^{14}C]-mevalonic acid (1 mM, 0.5 μCi/mol), rat liver homogenate (10,000 $\times g$ sup), co-enzyme solution which contained nicotinamide (30 mM), $MgCl_2$ (5 mM), ATP (30 mM), glucose (30 mM), NADP (10 mM) and potassium phosphate (100 mM, pH 7.6), and test sample were incubated 3 hours at 37°C. To stop the reaction, 10% KOH in methoanol was added and the solution was extracted with petroleum ether after heating for 1 hour at 70°C. To the petroleum layer a 0.25% digitonin solution was added to precipitate the sterol fractions. The radio-activities of biosynthesized sterols was measured with a liquid scintillation counter.

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