ISOLATION, STRUCTURE DETERMINATION AND BIOLOGICAL ACTIVITIES OF A NOVEL ANTIFUNGAL ANTIBIOTIC, S-632-C, CLOSELY RELATED TO GLUTARIMIDE ANTIBIOTICS

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A new antifungal antibiotic, S-632-C, was extracted with ethyl acetate from the culture filtrate of *Streptomyces hygroscopicus* S-632 and isolated through a combination of column and preparative thin-layer chromatographies on silica gel. The structure of S-632-C was determined by analysis of ^{1}H and ^{13}C-NMR, MS, UV and IR spectra in comparison with those of S-632-A_2 (9-methylstreptimidone). The signals were assigned on the basis of 2D NMR experiments, which involved ^{1}H-^{1}H DQF COSY, HMQC and HMBC spectral analysis. From these results, the chemical structure of S-632-C was elucidated as 6-(3,5-dimethyl-2-oxo-4,6-octadienyl)-4-carbamoylmethyl-3,4,5,6-tetrahydro-2-pyrone. The antibiotic exhibited exclusively weak *in vitro* antifungal activity against *Saccharomyces* spp. and similar cytotoxic activity against KB carcinoma cells, as compared with the glutarimide antibiotic S-632-A_2. In addition, this antibiotic had the ability to change the morphology of *ras*^{ts}-transformed NRK cells to that of normal cells, also a characteristic S-632-A_2 and B_1.

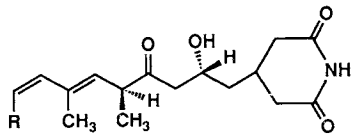
During our screening program for new antibiotics, we found that *Streptomyces hygroscopicus* S-632 produced a mixture of homogeneous antifungal antibiotics. In preceding papers[1,2], we described the isolation of four biologically active compounds, S-632-A_1 (streptimidone), -A_2 (9-methylstreptimidone), -B_1, and -B_2. S-632-B_1 and -B_2 were found to be two stereoisomers of new members of the glutarimide family of antibiotics. This paper deals with the isolation, structural determination, and biological activities of an additional component, S-632-C produced by this strain. S-632-C is a novel antifungal antibiotic structurally related to glutarimide antibiotics (Scheme 1).

Materials and Methods

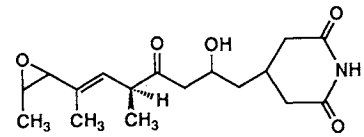
Fermentation

Fermentation conditions were essentially the same as in the previous method[1], except for use of a rotary shaker. The progress of the fermentation was monitored by growth inhibition against *Saccharomyces cerevisiae* by the conventional paper disk-agar plate method, and the fermentation process reached its maximum after 120 hours of cultivation. The ratio of the antibiotics was determined by the HPLC method described previously[2].

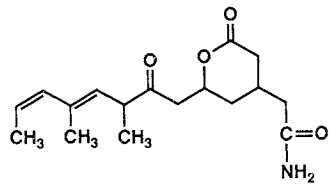
Scheme 1. Structures of S-632-A_1, A_2, B_1, B_2 and C.



S-632-A_1 (streptimidone) R=H
S-632-A_2 (9-methylstreptimidone) R=CH_3



S-632-B_1 and S-632-B_2
(isomer of 8,9-epoxy-9-methylstreptimidone)



S-632-C

Isolation

The fermentation broth was centrifuged at 2,800 rpm for 10 minutes and filtered to remove the mycelial cake. The filtered broth (11 liters) was adjusted to pH 7.0 with 0.1 N NaOH and then extracted twice with ethyl acetate. The extract was dried over anhydrous Na_2SO_4 and evaporated to dryness *in vacuo* to give 4.6 g of oily residue. The isolation of respective components was performed by a purification procedure that was an improvement over the previous method[2], as follows. The residue was applied on a silica gel column (45 × 6.0 cm, i.d.), and then eluted stepwise with a series of benzene - acetone from 9 : 1 to acetone. The active fractions containing S-632-A_2, which eluted with benzene - acetone (8 : 2), were concentrated *in vacuo* to yield 1,019 mg of crude oil. The following active fractions containing S-632-B_1 and -B_2, eluted with benzene - acetone (7 : 3), were concentrated *in vacuo* to give 324.3 mg of crude oil. The objective S-632-C was eluted with benzene - acetone (5 : 5), and the corresponding fractions were concentrated *in vacuo* to yield 177.5 mg of crude oil.

Further purification of the crude S-632-A_2 (90.2 mg) was achieved by preparative TLC on Silica gel $60PF_{254}$ (Merck) with benzene - acetone (3 : 1) as the developing solvent, to obtain 53.8 mg of S-632-A_2. The S-632-B_1 and -B_2 (60.5 mg) were also obtained as a mixture of diastereomers (53.9 mg) by preparative TLC and separated by preparative HPLC on a Unisil Q C_{18} column (250 × 4.6 mm, i.d., Gasukuro Kogyo) with MeOH - water (50 : 50) as a mobile phase, to obtain S-632-B_1 (4.6 mg) and S-632-B_2 (1.1 mg). The crude material containing S-632-C was purified by preparative TLC on silica gel with hexane - acetone (3 : 2) as the developing solvent. The active band corresponding to Rf 0.4 was scraped off and after extraction from the gel with acetone, purified S-632-C (42.4 mg) was isolated. S-632-A_1, -A_2, -B_1, and -B_2 were members of the glutarimide family of antibiotics and we have reported their structures as corresponding to streptimidone[3], 9-methylstreptimidone[4,5], and two diastereomers of 8,9-epoxy-9-methylstreptimidone[1,2].

Instrumental Analysis

Analysis by HPLC was performed with a Hitachi L-6200 Intelligent pump system on a Unisil Q C_{18} column (250 × 4.6 mm, i.d.) at UV-210 nm with MeOH - water (50 : 50) used as a mobile phase. Optical rotation was measured in chloroform at 25°C on a Horiba SEPA-200 polarimeter.

UV and IR spectra were recorded in MeOH on a Shimadzu Double-beam Spectrophotometer UV-180 and in KBr pellets on a Perkin Elmer FT-IR Spectrometer 1760X, respectively. ^1H and ^{13}C-NMR spectra were recorded in $CDCl_3$ on JEOL GSX-400 and JEOL GX-270 FT-NMR Spectrometers, respectively. EI-Mass spectrum was obtained on a JEOL DX-303 spectrometer.

Biological Activities

The *in vitro* antimicrobial activities of components S-632-C and 9-methylstreptimidone were

determined by a serial agar dilution method in nutrient agar (Difco) for bacteria and in SABOURAUD's agar (Nissui) for filamentous fungi and yeasts. Minimum inhibitory concentration (MIC) was observed after overnight incubation 37°C and 2~3 days incubation at 27°C, respectively.

For the *in vitro* cytotoxicity assay, KB cells were maintained in EAGLE's minimal essential medium (MEM) supplemented with 10% fetal calf serum and 100 μg/ml kanamycin. The cells (2×10^4 cells/ml) were incubated for 24 hours before addition of the compounds tested. The various concentrations of test compounds were added on day 1, and the concentration of the compound required for 50% inhibition of cell growth (IC_{50}, μg/ml) was determined by plotting a semi-log of the drug concentration (μg/ml) vs. percent of the growth rate of treated cells.

Cells of the rat kidney cell line (NRK) infected with ts371 murine sarcoma virus (*ras*^{ts}NRK)[6] were cultured at either permissive temperature (33°C) or nonpermissive temperature (39°C) in DULBECCO's modified MEM supplemented with 5% heat-inactivated calf serum and 100 μg/ml kanamycin. The cells were seeded into Costar 24-well tissue culture clusters at 1.5×10^4 cells/ml/well and cultured for 1 day at 33°C in a 5% CO_2-95% air incubator. Solutions of various concentrations of test samples were added on day 1, and after incubation for 2 days the *ras*^{ts}-NRK cells were observed microscopically for evidence of morphological reversion.

Results and Discussion

Physico-chemical Properties and Structure Determination of S-632-C

Physico-chemical properties of S-632-C in comparison with those of S-632-A_2 are summarized in Table 1. This component was distinguished from S-632-A_2 by HPLC and TLC mobilities. It was soluble in methanol, ethyl acetate, acetone and chloroform, but insoluble in hexane and water. It showed positive color reactions with H_2SO_4, $KMnO_4$, ninhydrin reagents and iodine vapor, and a negative reaction with DRAGENDORF's reagent. The UV spectrum of S-632-C exhibited an absorption maxima, at 234 nm, very close to one of S-632-A_2, 231.5 nm, which corresponds to a conjugated diene system as is present in S-632-A_2 (9-methylstreptimidone). As shown in the IR spectrum (Fig. 1), characteristic IR absorption at

Table 1. Physico-chemical properties of S-632-C and S-632-A_2 (9-methylstreptimidone).

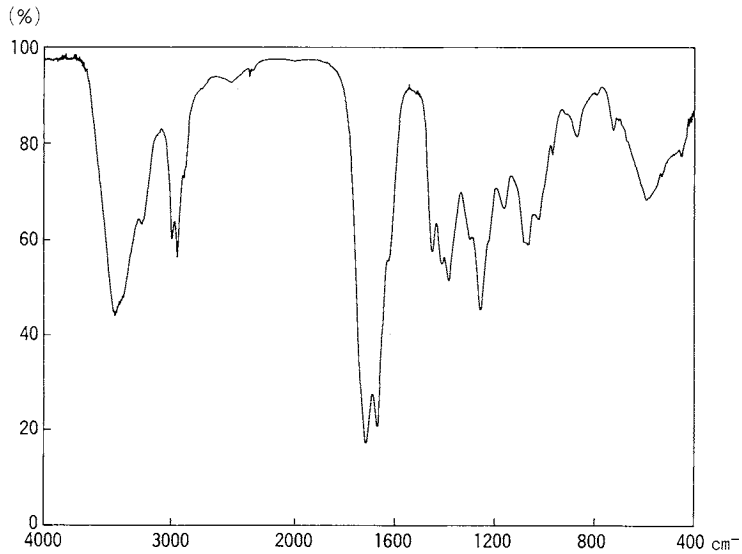
	S-632-C	S-632-A_2[a]
Appearance	Pale yellowish oil	Pale yellowish oil
$[\alpha]_D^{25}$	+35° (*c* 0.17, $CHCl_3$)	+105° (*c* 0.1, $CHCl_3$)
Molecular formula	$C_{17}H_{25}NO_4$	$C_{17}H_{25}NO_4$
Elemental analysis	—	C 65.88 (66.42)
Found (calcd)		H 8.60 (8.20)
		N 4.38 (4.56)
HREI-MS (*m/z*)		
Found (Calcd)	307.1813 (307.1784)	—
EI-MS (*m/z*)	307 (M^+), 225, 198, 180, 156, 109, 67	307 (M^+), 225, 198, 180, 152, 109, 96
UV λ_{max}^{MeOH} nm (ε)	234 (13,300), 281 (1,600)	231.5 (15,350), 283.0 (1,260), 291.0 (1,230)
IR ν_{max}^{KBr} cm^{-1}	3425, 1710, 1670, 1255	3475, 3225, 1725, 1710, 1650, 720
TLC Rf value[b]		
Benzene-acetone (1:1)	0.35	0.62
$CHCl_3$-acetone (4:1)	0.10	0.31
HPLC Rt (minutes)[c]	23.0	28.3

[a] These data were obtained from refs J. Am. Chem. Soc. 81: 500 (1959) and J. Antibiotics 27: 206 (1974), except TLC and HPLC data.

[b] TLC: Silica gel $60F_{254}$ (Merck, 5715).

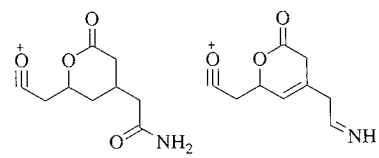
[c] HPLC conditions; Column: Unisil Q C_{18} (250 × 4.6 mm, i.d., Gasukuro Kogyo), Mobile phase: MeOH-water (50:50), Flow rate: 1.0 ml/minute, Detection: UV 210 nm.

Fig. 1. IR spectrum of S-632-C.



3425, 1670, 1710 cm^{-1} indicated the presence of carbonyl and amide/imide groups.

The molecular formula of S-632-C was determined to be $C_{17}H_{25}NO_4$ (MW 307) by high resolution electron impact mass spectrometry (HREI-MS), which was the same as that of 9-methylstreptimidone. The EI-MS of S-632-C gave the same molecular ion peak (M^+) at *m/z* 307, along with abundant fragment ion peaks at *m/z* 198 ($C_9H_{12}NO_4$), *m/z* 180 ($C_9H_{10}NO_3$) and *m/z* 109 (C_8H_{13}). It showed a fragment pattern almost identical to that commonly observed for the glutarimide group antibiotics, as reported previously[2]. Therefore, S-632-C was deduced to be a structural isomer of 9-methylstreptimidone.



m/z: 198 *m/z*: 180 *m/z*: 109

The structure was elucidated from the ^{1}H-NMR, ^{13}C-NMR and ^{1}H-^{1}H DQF COSY spectral analysis, and from the results of the HMBC and HMQC experiments. The ^{1}H-NMR spectrum of S-632-C showed fifteen signals due to three methyl, four methylene, six methine and two NH protons. The ^{13}C-NMR spectrum exhibited seventeen signals consisting of three methyl, four methylene, three methine, four olefinic and three carbonyl carbons. The correlation between protons and carbons revealed by the HMQC spectrum of S-632-C is presented in Table 2, where it is compared with that for S-632-A_2. It was, however, impossible to assign exactly the two carbonyl signals at 171.3 and 172.7 ppm in the ^{13}C-NMR spectrum of S-632-C. Thus, we performed a deuterium exchange experiment using the method of NEWMARK *et al.*[7] to distinguish the two carbonyl signals. The latter signal was observed as two peaks and one diminutive shoulder, and the C-3′ methylene signal was split into two peaks, due to the isotope effect by deuterium exchange of carbamoyl group in CD_3OD-CD_3OH (1 : 1) solution, while the former signal remained. Therefore, we clarified that the former carbonyl signal was due to an ester carbonyl group and the latter carbonyl signal was due to a carbamoyl carbonyl group.

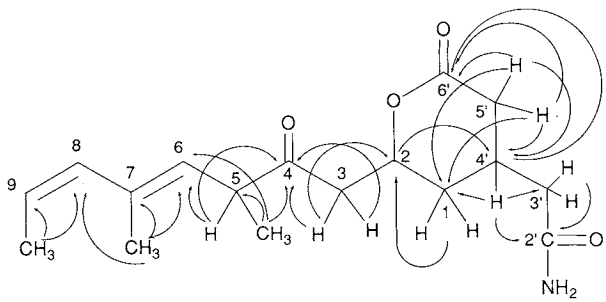
In the ^{1}H-^{1}H DQF COSY spectrum, ^{1}H-^{1}H coupling correlation were observed among 5-CH_3, 5-H,

Table 2. Comparison of NMR spectral data of S-632-C and -A_2.

	S-632-C		S-632-A_2 (9-methylstreptimidone)[a]	
	^{13}C (67.5 MHz) ppm	^1H (400 MHz) ppm (J, Hz)	^{13}C (25 MHz) ppm	^1H (400 MHz) ppm (J, Hz)
1	32.5 (t)	1.84 (t, J=6.7)	40.9 (t)	1.34 (ddd, J=14.0, 8.4, 2.0), 1.61 (ddd, J=14.0, 10.4, 4.8)
2	72.9 (d)	4.88 (dq, J=6.7, 5.5)	64.8 (d)	4.12 (m)
3	45.3 (t)	2.66 (dd, J=17.6, 6.7), 3.08 (dd, J=17.6, 5.5)	47.3 (t)	2.57 (dd, J=18.0, 3.2), 2.64 (dd, J=18.0, 8.2)
4	208.1 (s)		212.5 (s)	
5	46.9 (d)	3.44 (dq, J=9.7, 7.0)	46.9 (d)	3.44 (dq, J=9.6, 6.8)
5-CH_3	16.1 (q)	1.18 (d, J=7.0)	14.7 (q)	1.18 (d, J=6.8)
6	127.7 (d)	5.19 (br d, J=9.7)	127.9 (d)	5.17 (dm, J=9.6)
7	135.9 (s)		135.7 (s)	
7-CH_3	17.3 (q)	1.85 (d, J=1.5)	16.2 (q)	1.85 (dd, J=1.5, 0.7)
8	132.7 (d)	5.82 (br d, J=11.7)	132.7 (d)	5.81 (dm, J=11.7)
9	125.3 (d)	5.50 (dq, J=11.7, 7.1)	125.3 (d)	5.50 (dq, J=11.7, 7.2)
9-CH_3	14.7 (q)	1.78 (dd, J=7.1, 1.8)	17.3 (q)	1.78 (dd, J=7.2, 1.5)
2′	172.7 (s)		172.7 (s)	
3′	40.6 (t)	2.34 (d, J=7.1)	38.4 (t)	2.32 (m), 2.76 (m)
4′	26.0 (d)	2.61 (m)	27.1 (d)	2.48 (m)
5′	35.0 (t)	2.33 (dd, J=16.0, 8.0), 2.67 (dd, J=16.0, 6.0)	37.1 (t)	2.32 (m), 2.76 (m)
6′	171.3 (s)		172.6 (s)	

[a] These data were measured in this study.

Fig. 2. Long-range coupling observed by HMBC experiment.



6-H, 7-CH_3, 8-H, 9-H and 9-CH_3, and 3′-H, 5′-H, 4′-H, 1-H, 2-H and 3-H. In the HMBC spectrum, long-range ^1H-^{13}C coupling was observed between 1-H and 2-C; 2-H and 4′-C; 3-H and 2-C, 4-C; 5-CH_3 and 4-C, 5-C, 6-C; 5-H and 4-C, 6-C; 7-CH_3 and 6-C, 7-C, 8-C; 9-CH_3 and 8-C, 9-C; 3′-H and 2′-C; 4′-H and 1-C, 2′-C, 3′-C, 6′-C; 5′-H and 1-C, 4′-C, 6′-C (Fig. 2). The degree of unsaturation of S-632-C was estimated as six from its molecular formula. The structure of this antibiotic must contain five double bonds and one ring structure. Accordingly, the chemical structure of S-632-C was determined to be 6-(3,5-dimethyl-2-oxo-4,6-octadienyl)-4-carbamoyl methyl-3,4,5,6-tetrahydro-2-pyrone, which leads to the structure as shown in Scheme 1.

Biological Activities

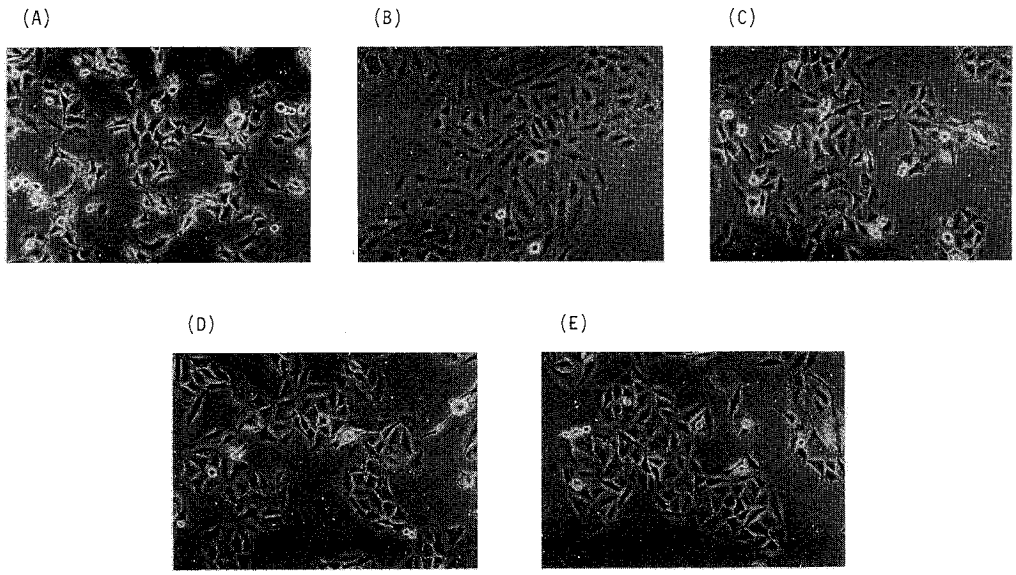
The antifungal spectrum of S-632-C is shown in Table 3, in comparison with that of S-632-A_2. S-632-C displayed specific activity against *Saccharomyces* spp., although its activity was less than that of S-632-A_2. It was devoid of any significant activity against other fungi and bacteria.

Table 3. Antifungal spectrum of S-632-C and of -A_2 (9-methylstreptimidone).

Test organism	MIC (μg/ml)		Test organism	MIC (μg/ml)	
	S-632-C	S-632-A_2		S-632-C	S-632-A_2
Candida albicans IFO 1060	>100	>100	*S. cerevisiae* TP-1	50	3.13
C. albicans IFO 1594	>100	>100	*Pichia farinosa* IFO 0193	>100	>100
C. guillirmondii IFO 0838	>100	>100	*Cryptococcus neoformans* KF-33	>100	50
C. krusei IFO 0011	>100	100	*Aspergillus niger* IFO 6341	>100	>100
C. lusitaniae IAM 12189	>100	50	*Trichophyton mentagrophytes* IFO 5466	>100	>100
Saccharomyces cerevisiae IFO 0304	25	1.56			
S. cerevisiae Kyokai No. 7	50	3.13	*T. rubrum* IFO 5467	>100	>100

Fig. 3. Conversion of the transformed morphology of *ras*^{ts}NRK cells treated by S-632-A_2, -B_1 and -C.

The cells were cultured at 33°C (A) or 39°C (B) without any test materials, and cultured at 33°C with 2 μg/ml of S-632-A_2 (C), 0.1 μg/ml of S-632-B_1 (D), and 2 μg/ml of S-632-C (E).



The IC_{50} values of S-632-A_2 and C were the same, 0.2 μg/ml, when KB cells were exposed to these agents for 3 days. Among the compounds isolated so far, S-632-B_1 has the highest cytotoxic activity, as shown in a previous report[1].

Furthermore, in screening inhibitors for inhibition of oncogene functions, we found that S-632-B_1 converted the transformed morphology of *ras*^{ts}NRK cells[6] back to the normal cell morphology at 33°C, the permissive temperature, at a concentration (0.1 μg/ml) that inhibited the cell growth only partially (Fig. 3D). Some glutarimide antibiotics, such as acetoxycycloheximide, cycloheximide[8] and epiderstatin[9,10] have also been reported to alter the morphology of *ras*-transformed cells. Since S-632-C, a weaker inhibitor of the growth of *ras*^{ts}NRK cells, also displayed this activity (Fig. 3E), the glutarimide ring may not be an essential structure to cause this morphological reversion.

Acknowledgments

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