Isolation, Structural Elucidation, Biological Properties, and Biosynthesis of Maleimycin, A New Bicyclic Maleimide Antibiotic Isolated from the Culture Filtrates of *Streptomyces showdoensis*†

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ABSTRACT: Maleimycin is a bicyclic maleimide antibiotic elaborated by *Streptomyces showdoensis*. The physical and chemical data show the structure for maleimycin to be 3-aza-6-hydroxybicyclo[3.3.0]oct-$\Delta^{1,5}$-ene-2,4-dione. The nuclear magnetic resonance (nmr) spectra in D_2O showed five well-defined resonances corresponding to the five nonexchangeable protons. The ^{13}C spectra consisted of seven resonances: two resonances in the carbonyl region at −41.3 and −40.7 ppm, two resonances in the aromatic or unsaturated region at −29.3 and −23.7 ppm, and one resonance at +56.3 ppm corresponding to the carbinol carbon and two saturated carbons at +95.1 and +103.9 ppm. Maleimycin is an effective inhibitor of Gram-positive, Gram-negative, and acid-fast bacteria and leukemia L-1210 cells. Maleimycin reacts more slowly with cysteine or with the sulfhydryl groups of guanosine-5′-triphosphate 8-formylhydrolase at equimolar concentrations. The biosynthesis of the maleimide ring of maleimycin differs from the biosynthesis of showdomycin even though both compounds have the maleimide ring and are synthesized at the same time by the same organism.

During a study of showdomycin, a second ultraviolet absorbing compound was observed in the culture filtrates of *Streptomyces showdoensis*. This compound has been isolated and crystallized, and its physical and biological properties have been studied in much detail. By the use of H (proton) nuclear magnetic resonance (pmr) and ^{13}C nmr (cmr), the resonances of the five nonexchangeable protons and the spectra of the seven carbons are described. It is now possible to report the structure of this new maleimide antibiotic as 3-aza-6-hydroxybicyclo[3.3.0]oct-$\Delta^{1,5}$-ene-2,4-dione. The biochemical properties and biosynthesis are also described.

Experimental Procedure

Isolation of Maleimycin. Cultures of *S. showdoensis* were grown for 42 hr as described by Elstner *et al.* (1973). The medium was filtered, passed through Dowex 50-X8 (H^+) (20–50 mesh, 3 cm × 20 cm). The pH was adjusted to 4.5 (ammonium hydroxide), adsorbed on Norit A (10 g/l.), and washed with 1 l. of water. Maleimycin was eluted batchwise with acetone–water (80:20, v/v) (three times, 500 ml each time). The eluent was concentrated under vacuum to 2 ml, spotted on four Whatman No. 3MM paper chromatograms for development in solvents A, B, and C. The solvents used were: solvent A, water–1-butanol (14:86, v/v); solvent B, 1-propanol–water (70:30, v/v); solvent C, water. The R_F values of maleimycin for solvents A, B, and C were 0.75, 0.81, and 0.85. The maleimycin eluted after development in solvent C was lyophilized and extracted with hot ethyl acetate. The ethyl acetate was removed by evaporation and the maleimycin was dissolved in hot benzene–toluene (1:1, v/v) and crystallized overnight at 2°; yield, 15 mg/l. of medium.

Physical and Chemical Properties of Maleimycin. The following properties were observed: mp 116°; ultraviolet (uv) λ_{max}^{water} 225 nm (ϵ 14,000), shoulder at 230 nm; infrared spectrum, 225, 1245, and 1095 cm^{-1}. *Anal.* Calcd for $C_7H_7NO_3$: C, 54.90; H, 4.60; N, 9.20. Found: C, 54.00; H, 4.69; N, 9.55.

The 60-MHz pmr spectra were determined on a Hitachi Perkin-Elmer R20A nmr spectrometer with the probe temperature at 34°. Chemical shifts were measured from an external TMA capillary. The 220-MHz spectra were obtained with a modified Varian HR-220 superconducting nmr spectrometer equipped with a frequency sweep and multinuclei capabilities. The ambient probe temperature was 17°. Chemical shifts from 220-MHz spectra are reported in parts per million downfield from internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate. The cmr spectra were obtained on a Bruker HX-90 nmr spectrometer equipped with a Nicolet 1074 Digital Equipment Corporatum-PDP-8/e computer system operating at 22.62 MHz in the Fourier transform mode. The protons were broad band coupled with decoupling frequency at 90 MHz. The ambient probe temperature was about 35°. ^{13}C chemical shifts were measured from an external C_6F_6 capillary and were converted to a C_6H_6 scale using the experimentally determined relationship, $\delta_{C_6F_6} = \delta_{C_6H_6}$ (ext) −9.9 ppm (infrared spectrophotometer). Ultraviolet (uv) spectra were recorded on a Beckman DB spectrometer and mass spectra on a Du Pont mass spectrometer, Model 210492,

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TABLE I: Carbon-13 Chemical Shifts for Showdomycin and Maleimycin.

Compound	Carbon Positions								
Showdomycin[a]	C_2	C_5	C_3	C_4	C_1'	C_2'	C_3'	C_4'	C_5'
	−54.4	−53.9	−28.5	−10.7	+41.2	+44.1	+47.6	+35.1	+57.0
Maleimycin[b]	C_4	C_2	C_5	C_1	C_6	C_7	C_8		
	−41.3	−40.7	−29.3	−23.7	+56.3	+103.9	+95.1		

[a] 135 mg/2.5 ml of H_2O, 512 transients on Varian XL-100 ^{13}C Fourier transform (25.1 MHz, 4K data points), shifts measured from CS_2, converted to C_6H_6 using the relationship $\delta_{C_6H_6} = \delta_{CS_2} - 64.1$ ppm (Pregosin and Randall, 1972). [b] 40 mg/2.5 ml of H_2O, 31,400 transients on Bruker HX-90 ^{13}C Fourier transform (22.6 MHz, 4K data points), shifts measured from a C_6F_6 capillary, converted to C_6H_6 using the experimentally determined relationship, $\delta_{C_6H_6} = \delta_{C_6F_6(ext)} - 9.9$ ppm.

by using the direct inlet probe at about 120°. Analytical data were obtained from Baron Consultants. Melting points (uncorrected) were determined on a Thomas-Hoover apparatus. Antimicrobial activity and dry weight determinations were done as described earlier (Elstner and Suhadolnik, 1971a). D-[1-^{14}C]Ribose was obtained from International Chemical and Nuclear Corp., [1-^{14}C]pyruvate was obtained from Amersham/Searle; [3-^{3}H]glutamic acid, [2-^{14}C]acetate, and [5-^{14}C]glutamate were obtained from New England Nuclear; [4-^{3}H]glutamic acid was a generous gift of Dr. J. Katz, Cedars of Lebanon Hospital, Los Angeles, Calif. [1-^{13}C]Acetate and [2-^{13}C]acetate were obtained from Merck and Co.

Results

Maleimycin is elaborated by *S. showdoensis* along with showdomycin. Both antibiotics reach a maximum production 27 hr after inoculation. Crystalline maleimycin is isolated in yields of 15 mg/l. of medium while the yield of showdomycin is 18 mg/l. (Figure 1). The structure of the bicyclic compound was determined by pmr, cmr, and mass spectrometry. The 60-MHz spectrum of maleimycin in dry Me_2SO-d_6 consisted of a complex multiplet centered at 2.7 ppm which integrates for four protons, a broad one-proton peak at 5.3 ppm, another second-order one-proton multiplet at 5.75 ppm, and a one-proton resonance at 10.8 ppm. In D_2O, the resonances at 5.3 and 10.8 ppm disappeared and were attributed to hydroxyl and ring imide protons, respectively. The 220-MHz spectrum in D_2O (Figure 2) showed a one-proton multiplet at 2.63 ppm, an AB-type pattern centered at about 3.1 ppm, a complex multiplet at 3.23 ppm which integrated for one proton, and the one-proton multiplet at 5.55 ppm. Upon strong irradiation of the downfield multiplet at 5.55 ppm, partial collapse was observed in all three upfield multiplets indicating coupling of all these proton spins.

The ^{13}C spectrum consisted of seven resonances; these resonances are assigned as shown in Table I. The possibility that the maleimide ring of maleimycin was substituted at the 3 position was ruled out for several reasons. First, the lowest field resonance showed extensive coupling to all of the other protons. In showdomycin, the nonexchangeable resonance at lowest field (H_4) was coupled only to H_1'. Second, the chemical shifts of the low-field proton (Darnall *et al.*, 1967) and one vinyl carbon (Elstner *et al.*, 1973; Elstner and Suhadolnik, 1972) (Table I) are considerably different from those which have been reported for showdomycin.

The coupling of the downfield proton to the other four nonexchangeable protons strongly suggests a fused ring. Since there are two carbonyl peaks in the cmr spectrum in the same chemical-shift range as found for showdomycin (Table I) and a ring imide proton appears in the pmr spectrum in Me_2SO, a maleimide ring is present. There are one alcohol group, one nonexchangeable proton at 5.55 ppm, and four aliphatic protons in a ring fused to the maleimide ring. From these data and the empirical formula $C_7H_7NO_3$ several possible structures were proposed (Figure 3).

On the basis of chemical-shift data alone, structure **3** could be eliminated because enol functional groups usually have chemical shifts of about 15 ppm (Silverstein and Bassler, 1964) for the hydroxyl proton. Structure **4** could be eliminated due to its symmetry.

Two structures could be eliminated by analysis of the proton spin–spin coupling constants. Using the LAOCOON III program (Castellano and Bothner-By, 1964; Bothner-By *et al.*, 1965) and the chemical shifts and coupling constants summarized in Table II, a theoretical spectrum (Figure 2a) was calculated which is in reasonable agreement with the ob-

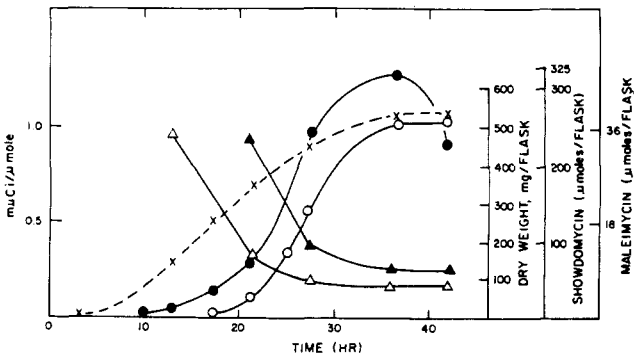

FIGURE 1: Growth of *S. showdoensis* (×---×); production of showdomycin (○—○); production of maleimycin (●—●); specific activities of showdomycin (▲—▲) and maleimycin (△—△) following the addition of [2-^{14}C]acetate.

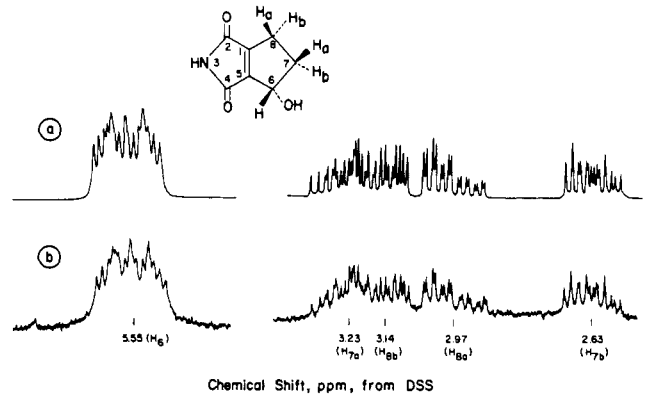

FIGURE 2: Nmr spectra for maleimycin.

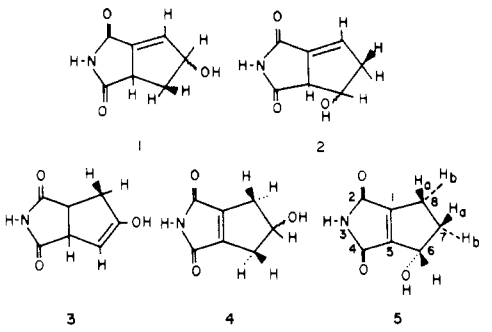

FIGURE 3: Possible structures for maleimycin.

served spectrum (Figure 2b). The estimated accuracy of the J values is ± 0.1 Hz except where noted. Two methylene groups are present (J_{8a-8b} and J_{7a-7b} in the range of coupling). Comparisons with values for other alkene rings (Garbisch, 1964; Watanabe *et al.*, 1970; Ferrier and Ponpipom, 1971) eliminate structures **1** and **2**. Maleimycin is optically active ($[\alpha]_D^{25}$ $+22.2°$, c (water) 1.18). The stereochemistry indicated for structure **5** is arbitrary and was only done for convenience in discussion of the pmr chemical shifts and coupling constants. In terms of the pmr data, the chemical shift of H_6 should be quite similar to that of the anomeric proton of showdomycin except that it should be shifted downfield by ~0.3–0.5 ppm because of the loss of the shielding effect of the 2′-hydroxyl as is observed upon comparison of deoxyribonucleosides with ribonucleosides (Gatlin and Davis, 1962). H_{7a} and H_{7b} are expected to be nonequivalent with H_{7b} shifted upfield by about 0.5 ppm because of the shielding effect of the neighboring hydroxyl group. H_{8a} and H_{8b} are also expected to be nonequivalent but to a lesser extent than H_{7a} and H_{7b} and should consist of an AB-type multiplet. The data summarized in Table II are totally consistent with known geminal constants, vicinal coupling constants predicted by the Karplus relationship, and known long-range coupling constants (Becker, 1969).

The specific assignment of the carbonyl C_2 and C_4 resonances for maleimycin in Table I is completely arbitrary since the chemical-shift difference is only 0.5 ppm. The 13-ppm upfield shift of the two carbonyl ^{13}C resonances of maleimycin compared with showdomycin is not too unexpected for at least C_2 since minor structural alternatives such as replacement of a hydrogen with an alkyl group as on C_1 can lead to shifts of this magnitude (Jones *et al.*, 1970). The same shifting of C_4 is not understood.

C_5 was assigned on the basis of its similar shift to C_3 in showdomycin (Elstner *et al.*, 1973). The difference in ^{13}C chemical shift of the upfield vinyl carbon (C_1) of the maleimide

TABLE II: Summary of the 220-MHz Proton Magnetic Resonance Data for Maleimycin.

	Coupling Constants J_{x-y} (Hz)[a]				Chem Shifts
	H_{8b}	H_{7a}	H_{7b}	H_6	(ppm)[a]
H_{8a}	−18.5	8.9	4.4	1.2	−2.97
H_{8b}		4.1	8.4	2.4	−3.14
H_{7a}			−14.5	7.6	−3.24
H_{7b}				3.2	−2.63
H_6					−5.55

[a] Estimated error ±0.2 Hz.

TABLE III: Mass Spectral Pattern of Maleimycin.

Mass	Intensity (%)	Mass	Intensity (%)
27.00	13.70	84.09	3.81
28.00	56.11	85.10	4.79
29.00	5.19	91.00	77.38
32.00	12.53	92.00	24.48
37.00	3.50	93.00	7.55
38.00	6.98	94.09	2.33
38.00	3.45	95.10	66.96
39.00	19.78	96.00	23.98
40.00	33.38	97.10	13.30
41.00	16.11	98.00	5.60
42.00	4.29	99.00	2.74
43.00	14.25	106.00	4.52
44.00	11.10	107.00	12.72
45.00	8.15	108.00	26.60
50.00	5.74	109.10	6.05
51.00	9.10	110.10	7.58
52.00	12.80	111.00	20.66
53.00	24.55	112.10	2.57
54.00	17.54	113.10	3.00
55.00	14.82	119.00	3.86
56.00	8.72	120.00	33.81
57.00	13.53	121.10	3.57
60.00	7.03	123.10	3.83
61.00	5.26	124.00	13.46
63.00	5.48	125.00	13.27
64.00	7.05	126.10	1.97
65.00	7.17	127.10	1.78
66.00	22.74	129.10	4.19
67.00	6.36	130.90	4.98
68.00	10.44	134.00	3.86
69.10	23.59	135.00	11.03
70.00	5.45	136.00	82.19
71.00	7.69	137.10	6.19
73.00	7.79	138.00	4.76
78.00	8.31	139.10	1.71
79.00	13.96	149.10	3.31
80.00	18.14	152.10	2.16
81.00	24.98	153.00	100.00
82.00	24.36	154.00	9.00
83.10	10.75		

TABLE IV: Exact Molecular Weight and Empirical Formula for Maleimycin As Determined by High-Resolution Mass Spectrometry.

Nominal Mass	Mass		m/e
	Obsd	Calcd	
136	136.0163	136.01604	$C_7H_4O_3$[a]
124	124.03961	124.03985	$C_6H_6O_2N$
108	108.02150	108.02113	$C_6H_4O_2$
97	97.05245	97.05276	C_5H_7ON
96	96.04490	96.04493	C_5H_6ON
92	92.02645	92.02621	C_6H_4O

[a] The fragment, $C_7H_6O_2N$ (a loss of OH), is also observed but the ratio of $C_7H_4O_3$:$C_7H_6O_2N$ is 100:1.

TABLE V: Inhibition of Growth of *E. coli*, *S. aureus*, *M. phlei*, and Leukemia L-1210 Cells by Maleimycin and Showdomycin.

[Maleimycin] or [Showdomycin] (mmol)	Inhibition (mmol)[a]						Leukemia L-1210
	E. coli		*S. aureus*		*M. phlei*		
	Maleimycin	Showdomycin	Maleimycin	Showdomycin	Maleimycin	Showdomycin	Maleimycin % Inhibition
0.005							50
0.05	7		22	24			100
0.10	10	12	24	25	7	0	
0.15	13	15	25	25		0	
0.25	17	16	27				
0.30	19	18	30	00	15	0	
0.40	20	20	32		20	1	
0.60		22					
0.90		22					
1.30				25		6	

[a] Experimental procedure for the inhibition of bacteria. The inhibitor was added to 10-mm paper discs and placed on nutrient agar plates. Zones of inhibition were measured 24 hr later. The *in vitro* antitumor assays were performed by Dr. A. Bloch, Roswell Park Memorial Institute.

ring of this structure as compared to that of showdomycin (C_4) can be rationalized as being consistent with the expected downfield shift upon replacement of a vinyl proton with an alkyl group, for example, in the case of C_5 in uridine and thymidine (Jones *et al.*, 1970).

The assignment of the aliphatic carbons C_8 at +95.1 ppm and C_7 at +103.9 ppm is somewhat tenuous due to two opposing factors. Carbons β (C_7) to a vinyl carbon resonate about 17 ppm to higher field than α carbons (C_8) (Pregosin and Randall, 1972), but C_7 is α to the carbinol carbon, which is expected to resonate about 16 ppm to lower field than a β carbon (C_8). The mass spectral patterns show parent ion (*m/e* −153) with a relative intensity of 100 (Tables III and IV).

Since showdomycin and maleimycin have the maleimide ring, it was of interest to compare their biological properties (Table V). Maleimycin is 20 times more inhibitory against *Microbacterium phlei* than is showdomycin. At equimolar concentrations, showdomycin reacts three times more rapidly with cysteine than does maleimycin. Since showdomycin reacts with the sulfhydryl groups of enzymes (Roy-Burman *et al.*, 1968; Tsai *et al.*, 1970; Elstner and Suhadolnik, 1971b), a comparison of the alkylation of the sulfhydryl groups of guanosine-5′-triphosphate 8-formylhydrolase with maleimycin and showdomycin (Table VI) shows that this enzyme is inhibited 65% at 1×10^{-3} M by showdomycin, while 5×10^{-3} M maleimycin is required for the same inhibition. The inhibition of maleimycin and showdomycin is reversed by cysteine or glutathione. It had been established that C_2–C_5 of glutamate and α-ketoglutarate serve as the asymmetric four-carbon unit for C_2–C_5 of the maleimide ring of showdomycin (Elstner and Suhadolnik, 1972). Similarly, ^{14}C-labeled maleimycin was isolated following the addition of either [2-^{14}C]acetate or [5-^{14}C]glutamate (Table VII). The location of the ^{14}C in maleimycin has not been established. Tritium from [2-^{3}H]acetate and [4-^{3}H]glutamate is not incorporated into showdomycin. However, tritium was incorporated into maleimycin (Table VII). These findings indicate that C_2 of acetate or C_4 of glutamate must retain at least one hydrogen and contributes to the biosynthesis of either C_7 or C_8 of maleimycin. Tritium from [3-^{3}H]glutamate is not incorporated into either showdomycin or maleimycin. This suggests that glutamate is deaminated to α-ketoglutarate. The hydrogen on C_3 of α-ketoglutarate is acidic and would exchange with water. Two experiments eliminate the possibility that showdomycin may be the precursor for maleimycin or that C_1, C_2, and C_3 of ribose serve as the three-carbon fragment for C_6, C_7, and C_8 of the maleimide ring of maleimycin. First, there is no ^{14}C in maleimycin from the D-[1-^{14}C]ribose experiment; second, ^{3}H from [2-^{3}H]acetate and [4-^{3}H]glutamate is incorporated into either C_7 or C_8 of maleimycin. The lack of incorporation of [1-^{14}C]pyruvate eliminates pyruvate as a C_3 unit for either the maleimide ring or C_6, C_7, or C_8 of maleimycin.

Experiments were also done following the incorporation of ^{13}C from [1-^{13}C]acetate and [2-^{13}C]acetate. All of the ^{13}C from [1-^{13}C]acetate is incorporated into C_5 of the maleimide ring of showdomycin (Elstner and Suhadolnik, 1972; Elstner *et al.*, 1973). These findings were expected since the carboxyl carbon of acetate becomes C_5 of glutamate which then becomes C_5 of showdomycin. Therefore, it was expected

TABLE VI: Inhibition of Guanosine-5′-triphosphate 8-Formylhydrolase[a] by Maleimycin and Showdomycin.

Amount of Maleimycin or Showdomycin Added (M)	Formic Acid Formed (nmol)	Inhibition	
		Showdomycin (%)	Maleimycin (%)
None	16.0	0	0
5×10^{-4}	12.0		25
1×10^{-3}	9.0	65	44
2×10^{-3}	8.0		50
5×10^{-3}	6		65

[a] The enzyme was prepared from *S. rimosus* and its activity was determined as described (Elstner and Suhadolnik, 1971b). Incubations were made with enzyme solution (~10 mg of protein), 10^{-3} M EDTA–0.1 M Tris (pH 8.0), and 100 nmol of [8-^{14}C]GTP (0.75 μCi/μmol) in 1 ml for 2.5 hr at 38°.

TABLE VII: Comparison of Incorporation of ^{3}H and ^{14}C from [2-^{14}C,2-^{3}H]Acetate, [5-^{14}C,4-^{3}H]Glutamate, D-[1-^{14}C]Ribose, [3-^{3}H]Glutamate, and [1-^{14}C]Pyruvate into Maleimycin and Showdomycin.[a]

Compd Incorp	Sp Act. (μCi/μmol)		Ratio	Amount Added (μCi)		Maleimycin Isolated (nCi/μmol)		Ratio $^{3}H/^{14}C$ (nCi/ μmol)	Showdomycin Isolated (nCi/μmol)		Ratio $^{3}H/^{14}C$ (nCi/ μmol)
	^{3}H	^{14}C	$^{3}H/^{14}C$	^{3}H	^{14}C	^{3}H	^{14}C		^{3}H	^{14}C	
[2-^{14}C,2-^{3}H]Acetate	10	2.8	3.6	71	9.1	5.6	2.4	2.4	0	1.95	0
[5-^{14}C,4-^{3}H]Glutamate	80	15	5.3	10	0.8	0.19	0.17	1.1	0	0.15	0
D-[1-^{14}C]Ribose		51			2.7		0			8.9	
L-[3-^{3}H]Glutamate	0.01			50					0	0.02	
[1-^{14}C]Pyruvate		0.004			25		0		0		

[a] The labeled compounds were added to 10–18 flasks 18 hr after inoculation. Showdomycin was isolated 34 hr after inoculation. The yield of showdomycin was 6 mg/flask: the yield of maleimycin was 5 mg/flask.

that all of the ^{13}C from [1-^{13}C]acetate would reside in C_4 of the maleimide ring of maleimycin. Experimentally, all of the ^{13}C from [1-^{13}C]acetate resided in C_6 and none in C_4 (Table VIII). Similarly, all of the ^{13}C from [2-^{13}C]acetate resides in C_2, C_3, and C_4 of showdomycin (Elstner *et al.*, 1973). These findings are in agreement with the notion that ^{13}C from [2-^{13}C]acetate is located in C_2, C_3, and C_4 of glutamate which are equivalent to C_2, C_3, and C_4 of the maleimide ring of showdomycin. It was expected that ^{13}C from [2-^{13}C]acetate would reside exclusively in C_2, C_1, and C_5 of the maleimide ring of maleimycin. Experimentally, ^{13}C from [2-^{13}C]acetate was found in all carbons except C_6 of maleimycin. These ^{13}C data plus the incorporation of tritium from [2-^{3}H]acetate and [4-^{3}H]glutamate indicate that the biosynthesis of the maleimide ring of maleimycin differs from the biosynthesis of the maleimide ring of showdomycin. The culture characteristics of *S. showdoensis* following mutation and the loss of maleimycin production are shown in Table IX.

Discussion

The maleimide ring is a well-established inhibitor of sulfhydryl groups in proteins. Showdomycin (Nishimura *et al.*, 1964) and pencolide (Birkinshaw *et al.*, 1963) are the only two known naturally occurring compounds that possess the maleimide ring. Showdomycin is an inhibitor of bacteria and cancer cells (for a review, see Suhadolnik, 1970). While studying the biosynthesis of showdomycin by *S. showdoensis*, an additional compound was observed on paper chromatograms. The isolation, structural elucidation, biological properties, and biosynthesis of this compound are reported here. Based on the uv, infrared (ir), pmr, cmr, and mass spectral data, the structure is reported as the bicyclic maleimide, 3-aza-6-hydroxybicyclo[3.3.0]oct-$\Delta^{1,5}$-ene-2,4-dione. The common name is maleimycin. The pmr spectra contained sharp, well-defined resonances corresponding to the five nonexchangeable protons. The cmr spectra showed the seven carbons of maleimycin. The ^{13}C spectra of the maleimide carbons of maleimycin are very similar to the ^{13}C spectrum for the carbons in the maleimide ring of showdomycin.

Maleimycin inhibits *Escherichia coli*, *Staphylococcus aureus*, *Microbacterium phlei*, and leukemia L-1210 cells. The inhibition by maleimycin is greater toward acid-fast bacteria than it is toward Gram-positive and Gram-negative bacteria. To establish if the difference between showdomycin and maleimycin in alkylation of the sulfhydryl groups of guanosine-5′-triphosphate 8-formylhydrolase could be attributed to a selective reactivity of sulfhydryl groups, maleimycin and showdomycin were allowed to react with cysteine. Showdomycin reacted three times more rapidly. The slower reactivity of maleimycin might allow a more selective alkylation of proteins. It was originally assumed that the biosynthesis of the maleimide ring of showdomycin and maleimycin would use the same precursor. The data show that the maleimide rings of showdomycin and maleimycin form by different pathways. This conclusion is based on the following four observations. First, while the carboxyl carbon of [1-^{13}C]acetate resides exclusively in the carbonyl carbon (C_5) of showdomycin, ^{13}C

TABLE VIII: Enrichment of the Carbon Atoms of Maleimycin Following the Incorporation of [1-^{13}C]Acetate and [2-^{13}C]-Acetate by *S. showdoensis*.

Compound Added	^{13}C Enrichment Factor at Various Carbon Atoms of Maleimycin[a]						
	C_1	C_2	C_4	C_5	C_6	C_7	C_8
[1-^{13}C]Acetate	0	0	0	0	2	0	0
[2-^{13}C]Acetate	2	4	2	4	0	2	2

[a] Based on the intensity increase of the various carbon peaks.

TABLE IX: Observations on the Colony Characteristics of *S. showdoensis* Following the Loss of Production of Maleimycin.

Characteristic	Original Strain[a]	Mutated Strain[a]
Colony formation	Normal	Small, soft
Aerial mycelium	Yes	No
Substrate mycelium	Long branches	Small, primitive branches
Melanin production	Strong	None
Maleimycin production[b]	15–35 mg/l.	Negligible
Showdomycin production[b]	50–100 mg/l.	300–400 mg/l.

[a] Observed by maintaining culture straws frozen for no longer than 1 month. [b] Yield based on the crystalline compound.

from [1-^{13}C]acetate is only found in the carbinol carbon (C_6) of maleimycin. Second, ^{14}C, but not ^{3}H, from acetate and glutamate is incorporated into the maleimide ring of showdomycin. With maleimycin, both ^{14}C and ^{3}H were incorporated. These data show that the C–H bond of the methyl carbon of [2-^{3}H]acetate or the methylene carbon of [4-^{3}H]glutamate is incorporated intact into either C_7 or C_8 of maleimycin. Third, in experiments where maleimycin and showdomycin were isolated following the incorporation of ^{13}C from [2-^{13}C]acetate, C_2 of [2-^{13}C]acetate is found in all four carbons of the maleimide ring of maleimycin (Table VIII), but ^{13}C is located in only three carbons of the maleimide ring of showdomycin (Elstner *et al.*, 1973). Fourth, the production of maleimycin, but not showdomycin, by *S. showdoensis* is very easily lost either by storage of cultures of agar slants at −20° or by transfers in liquid culture. These four observations prove that the biosynthesis of the maleimide ring for both compounds is different. C_7 and C_8 of maleimycin utilize C_2 of acetate, but not [1-^{14}C]pyruvate or ribose. We are currently studying the mechanism by which the methyl group of acetate and the ^{3}H from [2-^{3}H]acetate and [4-^{3}H]glutamate can be incorporated into maleimycin.

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