10′-DESMETHOXYSTREPTONIGRIN, A NOVEL ANALOG OF STREPTONIGRIN

W. C. LIU, M. BARBACID, M. BULGAR, J. M. CLARK†, A. R. CROSSWELL†, L. DEAN†, T. W. DOYLE†, P. B. FERNANDES, S. HUANG†, V. MANNE, D. M. PIRNIK†, J. S. WELLS† and E. MEYERS

Bristol-Myers Squibb Pharmaceutical Research Institute,
P. O. Box 4000, Princeton, New Jersey 08543-4000, U.S.A.
†P. O. Box 5100, Wallingford, Connecticut 06492-7660, U.S.A.

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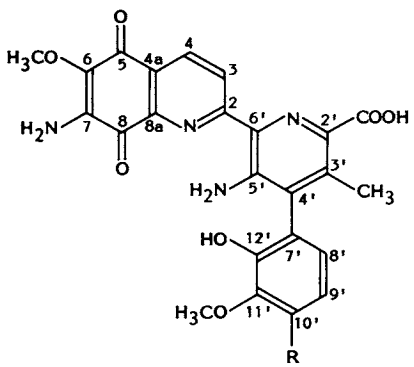
10′-Desmethoxystreptonigrin, a novel analog of streptonigrin produced by *Streptomyces albus*, was discovered in a screen for inhibitors of farnesylation of RAS p^{21} protein. The compound was isolated from the fermentation broth and its structure determined. It is markedly cytotoxic to several human tumor cell lines and also exhibits potent broad-spectrum antibacterial activity.

In the course of screening for compounds that can inhibit the farnesylation of *ras* protein, we have discovered a novel analog of streptonigrin. A description of the fermentation, isolation, structure and some biological properties of this analog, 10′-desmethoxystreptonigrin (Fig. 1), forms the subject of this report.

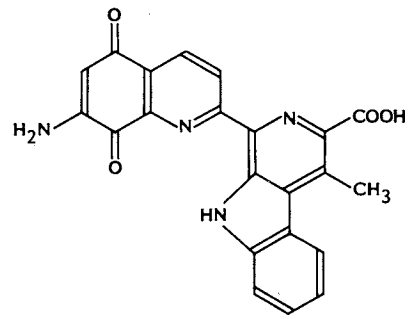
The producing organism, isolated from a soil sample collected in Yosemite National Park, California, has been identified as a strain of *Streptomyces albus*. The organism produces oyster white (ISCC-NBS white 263) aerial mycelium and smooth, spiral spore chains with a cream-colored to brown substrate mycelium (Prauser color code Coo5m). No spore formation is observed when the organism is grown on ISP media 2 or 6. On ISP medium 2, a soluble, faint rose-colored pigment is produced. No melanoid pigments are produced on ISP medium 6 or 1. When utilized as the sole carbon source for growth in ISP medium 9, glucose, mannitol, xylose, rhamnose, fructose, raffinose and salicin support growth. On the other hand, arabinose, sucrose and inositol do not. Readings were made after 7 and 14 days incubation.

From these studies, the organism was identified as a strain of *Streptomyces albus*. All the described

Fig. 1. Structures of 10′-desmethoxystreptonigrin (**1**), streptonigrin (**2**), and lavendamycin (**3**).



1 R=H
2 R=OCH_3



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characteristics are in agreement with the description given by NONOMURA.[1] A culture of *Streptomyces albus* has been deposited in the American Type Culture Collection with the accession number, ATCC 55161.

To produce 10′-desmethoxystreptonigrin, the organism, maintained on tomato paste-oatmeal agar slants (3% oatmeal plus 3% tomato paste in boiling water), was grown in a medium containing: yeast extract 0.4%, malt extract 1% and glucose 0.4%. After incubation at 25°C on a rotary shaker (280 rpm) for 72 hours, a 5% transfer was made into a medium composed of the following: Pharmamedia 2%, glucose 5% and $CaCO_3$ 0.7%.

The fermentation was allowed to proceed for 120 hours, with the same conditions that were described for the germinators. At the end of this fermentation period, the mycelical growth was separated from the fermentation broth by filtration. The pH of the broth filtrate was adjusted to about 3 by the addition of 1 N HCl and the acidified broth was extracted twice with ethyl acetate, first with 0.5 volume and then with 0.25 volume. The organic extracts were combined and the pool was then extracted twice with 0.5 volume of 5% calcium carbonate. The pooled aqueous layers were slowly adjusted to a pH of about 3 by the addition of concentrated HCl. Caution was exercised during the acidification step to prevent excessive and rapid foaming. The acidified solution was extracted twice with 0.5 volume of ethyl acetate and the resulting organic extracts were pooled and concentrated *in vacuo* to a dry residue. This residue was dissolved in methanol with gentle warming and charged onto a DEAE cellulose column packed in methanol. The column was washed liberally with methanol to remove impurities after which the column was developed with a solvent mixture consisting of 1% acetic acid in methanol, with a flow rate of 1 ml per minute. The active fractions, detected by conventional paper disc-agar diffusion assay against *Staphylococcus aureus* FDA 209P, were combined and concentrated to dryness, resulting in crystals. These were recrystallized from acetone, giving pure, crystalline 10′-desmethoxystreptonigrin as blackish-red needles. The electrophoretic mobility of 10′-desmethoxystreptonigrin on paper relative to vitamin B_{12} (0.0) and *p*-nitrobenzenesulfonate anion (1.0) when using a buffer consisting of 0.05 M sodium carbonate and 0.05 M sodium bicarbonate balanced to pH 9.2 is 0.34; is 0.38 when using a buffer composed of 0.05 M KH_2PO_4 and 0.05 M K_2HPO_4 balanced to pH 1.0 and is 0.08 with a buffer of 0.05 M KH_2PO_4 balanced to pH 4.5.

10′-Desmethoxystreptonigrin has the infrared spectrum in KBr as shown in Table 1, and the UV spectrum (in methanol) shows two distinct peaks, with absorption (molar extinction coefficient) values of 247 nm (34,800) and 379 nm (14,900). The corresponding values in acidified methanol were 247 nm (41,600) and 379 nm (17,400). In alkaline methanol, the values were 246 nm (43,600) and 380 nm (16,100). Accurate mass measurement of the $(M+H)^+$ ion in the fast atom bombardment mass spectrum yielded *m/z* 477.1398 (theory 477.1410 for $C_{24}H_{21}N_4O_7$). The proton and carbon NMR

Table 1. IR absorption maxima in KBr of 10′-desmethoxystreptonigrin.

Max (cm^{-1})	Relative intensity	Max (cm^{-1})	Relative intensity
3388	39.8	1234	37.7
3372	40.5	1214	47.3
3356	39.9	1184	65.7
3272	42.8	1166	63.4
3010	70.2	1096	65.5
2942	68.3	1074	56.6
2840	73.4	1038	62.1
1738	32.2	1008	63.0
1684	59.2	920	79.8
1632	53.7	876	84.5
1602	19.0	830	85.8
1586	22.4	808	86.5
1564	41.3	790	81.3
1546	49.6	746	63.8
1480	55.2	712	78.5
1442	55.2	686	84.3
1400	57.3	658	84.7
1376	64.4	582	79.5
1344	29.0	556	85.7
1276	57.4	522	85.5

spectra are summarized in Table 2. Assignments were made unequivocally using HMQC and HMBC experiments. Comparison of the spectral data of **1** with those of streptonigrin (**2**) and lavendamycin (**3**)[2] led readily to the structural assignment shown. The compound **1** might represent an intermediate in the biosynthesis of streptonigrin.[3]

Table 2. NMR data of 10′-desmethoxystreptonigrin in DMSO-d_6.

C position	C (ppm)[a]	H (ppm)[b]	C-H Long range coupling (Hz)
C-2	159.88		8.35 (7.5 Hz)
C-3	126.02	9.00	
C-4	133.44	8.35	
C-4a	126.79		9.00 (7.2 Hz)
C-5	175.98		8.35 (3.5 Hz)
C-6	135.83		6.88 (3.8 Hz), 3.81
C-7	141.60		6.88
C-8	180.33		6.88 (6.5 Hz)
C-8a	144.19		8.35 (5.7 Hz)
C-2′[c]	134.65		2.16 (3.8 Hz)
C-3′[c]	135.78		2.16 (5.8 Hz)
C-4′	134.12		2.16, 6.61
C-5′	145.30		7.4
C-6′	129.69		7.4 (4.0 Hz)
			9.00 (1.3 Hz)
C-7′	121.47		8.72, 6.95
C-8′	143.84		8.72, 7.06, 6.61
C-9′	148.36		6.95, 3.86, 8.72
C-10′	112.00	7.06	6.61 (9.4 Hz)
C-11′	120.24	6.95	7.06
C-12′	121.67	6.61	7.06 (8.0 Hz)
6-OCH_3	59.77	3.86	
7-NH_2		6.88	
2′-COOH	167.03		2.16
3′-CH_3	16.93	2.16	
5′-NH_2		7.4	
9′-OCH	55.79	3.81	
2′-COO*H*		12.3	
8′-OH		8.72	

[a] Measured at 125 MHz on a Bruker 500 AM instrument.
[b] Measured at 500 MHz on a Bruker 500 AM instrument.
[c] Assignments may be reversed.

In the assay for the farnesylation of *ras* oncogene p21 protein described by MANNE *et al.*,[4] 10′-desmethoxystreptonigrin was found to have an IC_{50} value of 2.1×10^{-5} M. Streptonigrin, on the other hand, demonstrated 3-fold less activity, with an IC_{50} value of 6.6×10^{-5} M. Interestingly, the analog although more active was approximately 5-fold less toxic than streptonigrin, having an LD_{50} of 8.8 mg/kg, when injected intraperitoneally in Swiss-Webster mice. Streptonigrin, under identical conditions, had an LD_{50} of 1.8 mg/kg. The analog was less toxic than streptonigrin to the growth of mouse bone marrow cells (Table 3).

10′-Desmethoxystreptonigrin also exhibited marked cytotoxicity *in vitro* to human tumor cell

Table 3. Bone marrow toxicity of 10′-desmethoxystreptonigrin.

		cfu/2.05 × 10^4cells
Streptonigrin	1.0 μg/ml	0
	0.1 μg/ml	0
10′-Desmethoxy-	1.0 μg/ml	0
streptonigrin	0.1 μg/ml	95
None		237

Nucleated cells from femur marrow cavities of female Swiss-Webster mice were cultured in McCoy 5A medium containing 25 mM HEPES buffer with L-glutamine, 20% fetal bovine serum, 10,000 units of benzylpenicillin, 10 mg/ml of streptomycin with 5% L-cell conditioned medium. After 1 day at 37°C under 6% CO_2 the medium was removed by aspiration and the cells washed with buffered saline. The attached cells were stained with WRIGHT's strain and counted.

Table 4. *In vitro* cytotoxicity of 10′-desmethoxystreptonigrin (IC_{50}, μg/ml).

Compound	Cell lines				
	HCT116	HCT116/VP35	HCT116/VM46	A2780	A2780/DDP
10′-Desmethoxystreptonigrin	0.004	0.003	0.001	0.001	0.010
Etoposide	0.69	8.8	3.2	0.057	0.38
Teniposide	0.112	ND	0.404	ND	ND
Diamminedichloroplatinum	ND	ND	ND	0.722	5.0

HCT116, human colon cells; HCT116/VP35, human colon cells resistant to etoposide; HCT116/VM46, human colon cells resistant to teniposide; A2780, human ovarian cells; A2780/DDP, human ovarian cells resistant to diamminedichloroplatinum.

lines when analyzed by the microtiter method of CATINO *et al.*,[5] incorporating the dye modification of SCUDIERO *et al.*[6] It was much more potent than etoposide in its ability to inhibit growth of two human, etoposide-sensitive cell lines (172 and 57-fold more potent, respectively). In addition, it was not cross-resistant with etoposide on human colon cell lines resistant to etoposide or teniposide. However, the streptonigrin analog and etoposide were 10 and 6.6-fold less potent, respectively, on human-ovarian cells resistant to diamminedichloroplatinum than on human ovarian cells sensitive to this agent. The data on cytotoxicity to the various cell lines are shown in Table 4. 10′-Desmethoxystreptonigrin was also evaluated *in vivo* in the P388 leukemia model, showing toxicity at doses of 16 and 8 mg/kg, and produced no antitumor activity at the non-toxic doses of 4 and 2 mg/kg.

Table 5. Antibacterial spectrum *in vitro*.

Organism	MIC (μg/ml)
Staphylococcus aureus SC1276	0.4
S. aureus SC2400	0.2
Streptococcus faecalis SC9011	1.6
S. faecalis SC9610	1.6
Micrococcus luteus SC2495	0.4
Escherichia coli SC8294	3.1
E. coli SC10909	<0.05
Klebsiella pneumoniae SC10440	3.1
Proteus vulgaris SC9416	0.4
Salmonella typhosa SC1195	1.6
Shigella sonnei SC8449	1.6
Enterobacter aerogenes SC10078	25
Serratia marcescens SC9783	6.3

SC denotes the number of the culture in the Bristol-Myers Squibb Culture Collection.

10′-Desmethoxystreptonigrin is also a broad-spectrum, antibacterial antibiotic (Table 5), but has no significant anticandidal activity (data not shown).

Addendum in Proof

After the completion of our studies, we became aware of a patent (Jpn. Kokai 83980 ('91) Apr. 9, 1991) covering 10′-desmethoxystreptonigrin that had been granted to Sankyo Co., Ltd.

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