SQ 30,957, A NEW ANTIBIOTIC PRODUCED BY *PENICILLIUM FUNICULOSUM*

TAXONOMY, FERMENTATION, ISOLATION, STRUCTURE DETERMINATION, SYNTHESIS AND ANTIBACTERIAL ACTIVITY

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A new antibiotic, SQ 30,957, 4-diazo-3-methoxy-2,5-cyclohexadien-1-one, has been isolated from fermentation broths of *Penicillium funiculosum*. The structure (**1**) was deduced from its spectroscopic properties and its degradation reaction. SQ 30,957 has excellent activity against anaerobic bacteria such as *Clostridium* and *Bacteroides* and has moderate activity against aerobic bacteria. The compound has an LD_{50} of less than 17 mg/kg in mice by intraperitoneal administration.

In the course of screening for antibiotics with activity against anaerobic bacteria, we found a new natural product, 4-diazo-3-methoxy-2,5-cyclohexadien-1-one (SQ 30,957), produced by *Penicillium funiculosum*. In this paper we describe the taxonomy of the producing organism, fermentation, isolation and physico-chemical properties of the compound including its synthesis, and biological properties.

Taxonomy

P. funiculosum (ATCC 20783) was isolated from a soil sample collected in Princeton, New Jersey. Colonies of *P. funiculosum* on CZAPEK's agar grow to 5~6 cm in 14 days. Growth is azonate with a marked tendency to form aerial ropes (funiculose hyphae) interspersed with wooly (floccose) areas. The reverse color ranges from yellow to orange or red. Sporulation on this medium is scant; sporulation is good on malt agar.

Conidiophores arise perpendicularly to the funiculose hyphae. In the marginal areas, they arise directly from the substrate hyphae and terminate in biverticillate, symmetric penicilli bearing dark green to bluish conidial heads. The conidia are produced in compact and columnar chains from clusters of lanceolate phialides.

The metulae, structures bearing the phialides, are 8 to 9 μm$\times$1.7 to 2.5 μm and are arranged in a terminal whorl on branches. The latter, 1 to 3 in number, support the metulae and attach directly to the conidiophore. Conidiophore length and diameter, below the penicillus, range from 40 to 70 μm$\times$2.5 to 3.0 μm. The conidia, as well as the conidiophores, are smooth. Conidia are thick walled, ovoid to sub-globose in shape and 2.5 μm$\times$2.1 μm in size.

The exudate from *P. funiculosum* on CZAPEK's agar is colorless, but is amber on CZAPEK's steep agar and is absent on malt agar. Yellow incrustations are seen on the funiculose hyphae. Sclerotia or perithecia are absent.

The above characteristics serve to identify the organism as *Penicillium funiculosum*[1].

Fermentation

Seed cultures of *P. funiculosum* (ATCC 20783) were prepared by transferring a loopful of surface growth from an agar slant into 500-ml Erlenmeyer flasks containing 100 ml of the following medium; yeast extract 0.3%, malt extract 0.3%, Tryptone 0.5% and glucose 1.0% in distilled water. The flasks were incubated at 25°C on a rotary shaker (300 rpm, 5-cm stroke) for approximately 96 hours.

A 5% transfer was made from the seed culture flasks to 500-ml Erlenmeyer flasks containing 100-ml portions of the same medium as was used for the germination stage. The flasks were incubated at 25°C for approximately 72 hours, under the same operating conditions as described for the germinator flasks. At the end of the incubation period, the content of the flasks was pooled and filtered to remove the mycelial cake.

Isolation

SQ 30,957 was isolated from fermentation broths as outlined in Fig. 2. The antibiotic was absorbed onto Diaion HP-20 and eluted with CH_3CN - H_2O (2 : 3). Further purification was affected by chromatography on Whatman LPS-1 silica gel, eluting with CH_3CN - MeOH (4 : 1) followed by two successive chromatographies on Baker silica gel eluting with $CHCl_3$ - MeOH (9 : 1) and CH_3CN - MeOH (4 : 1). The antibiotic is photo-labile and therefore, all operations were performed in the dark.

Physico-chemical Properties of SQ 30,957

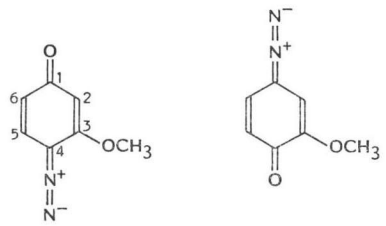
The physico-chemical properties of SQ 30,957 are listed in Table 1. The antibiotic is obtained as yellow crystals. It is a weak base as judged by paper electrophoresis. The antibiotic is unstable to light and alkaline pH (>9). The IR spectrum indicated the presence of a diazo group (2110 cm^{-1}) and a quinone (1630 and 1600 cm^{-1}). The UV spectrum indicated the presence of a *p*-quinoid rather than an *o*-quinoid[2,3] type of structure. Two possible structures, **1** and **2**, (Fig. 1) were deduced from its ^1H and ^{13}C NMR data. The molecular weight of SQ 30,957 was determined by mass spectrometry. The positive-ion fast atom bombardment (FAB)[4] mass spectrum showed peaks at *m/z* 151 $(M+H)^+$ and 259 $(M+H+thioglycerol)^+$ and the negative-ion spectrum showed a peak at *m/z* 257 $(M-H+thioglycerol)^-$ indicating a molecular weight of 150. In the CI mass spectrum, the observed peaks at *m/z* 303 and 301 in the positive and negative-ion modes respectively, have been attributed to dimerization of SQ 30,957. The empirical formula, $C_7H_6N_2O_2$, was determined from elemental analysis

Table 1. Physico-chemical properties of SQ 30,957.

Appearance	Yellow crystals
IR ($CHCl_3$) cm^{-1}	2110, 1630, 1600
UV nm ($E^{1\%}_{1cm}$) (MeOH, 0.01 M NaOH in MeOH)	337 (730), 255 (110)
UV nm ($E^{1\%}_{1cm}$) (0.01 M HCl in MeOH)	300 (430), 233 (190)
^1H NMR ($CDCl_3$) δ[a]	3.88 (s, 3H), 5.90 (d, 1H, J=1.6 Hz), 6.18 (dd, 1H, J=9.5, 1.5 Hz), 7.18 (d, 1H, J=10 Hz)
^{13}C NMR ($CDCl_3$) δ[b]	56.1, 69.7, 104.0, 123.7, 126.4, 160.9, 183.3
Molecular weight[c]	150
Molecular formula[d]	$C_7H_6N_2O_2$

[a] ppm downfield from TMS, using TMS (0.0 ppm) as an internal standard.
[b] ppm downfield from TMS, using $CDCl_3$ (77.0 ppm) as an internal standard.
[c] Molecular weight was determined by FAB and CI mass spectrometry.
[d] Molecular formula was determined by microanalysis.

Fig. 1. Possible structures of SQ 30,957.



SQ 30,957 (**1**) **2**

Table 2. Antibacterial (aerobic) activity of SQ 30,957.

Organism	MIC (μg/ml)
Staphylococcus aureus SC1276	12.5
S. aureus SC2399	12.5
S. aureus SC2400	12.5
Streptococcus agalactiae SC9287	6.3
Enterococcus faecalis SC9011	6.3
Escherichia coli SC8294	3.1
E. coli SC10896	6.3
E. coli SC10909	3.1
Klebsiella aerogenes SC10440	12.5
K. pneumoniae SC9527	25.0
Proteus mirabilis SC3855	3.1
Salmonella typhosa SC1195	1.6
Shigella sonnei SC8449	1.6
Enterobacter cloacae SC8236	50.0
Pseudomonas aeruginosa SC8333	25.0

MICs were determined at 37°C by agar dilution; with inoculum of 10^4 colony forming units.

Table 3. Antibacterial (anaerobic) activity of SQ 30,957.

Organism	MIC (μg/ml)
Bacteroides thetaiotaomicron SC9005	0.4
B. thetaiotaomicron SC10278	0.4
B. fragilis SC9844	0.4
B. fragilis SC10277	0.4
B. fragilis SC11085	0.2
Clostridium histolyticum SC8572	0.1
C. perfringens SC11256	0.2
C. septicum SC1780	<0.05
C. difficile SC11251	0.4
Haemophilus vaginalis SC8568	0.2
H. vaginalis SC9640	0.4
Fusobacterium necrophorum SC10338	<0.05
Peptostreptococcus anaerobius SC11263	<0.05

MICs were determined at 37°C by agar dilution; with inoculum of 10^5 colony forming units.

data. Calculated values for $C_7H_6N_2O_2 \cdot \frac{1}{2}H_2O$ are: C 52.83, H 4.43, N 17.60; those found are: C 52.39, H 4.13, N 16.91.

Reduction of SQ 30,957 with hypophosphorus acid[5] gave *m*-methoxyphenol, establishing structure **1** rather than **2** (Fig. 1). To synthesize the antibiotic, *m*-methoxyphenol was oxidized with FREMY's salt to 2-methoxy-*p*-benzoquinone which then gave, on treatment with *p*-toluenesulfonylhydrazide[5], SQ 30,957 as one of several products. The spectroscopic data (UV, IR, ^{1}H and ^{13}C NMR) were identical to those for the natural product.

Biological Properties

The activity of SQ 30,957 against aerobic and anaerobic bacteria is given in Tables 2 and 3. SQ 30,957 has excellent anti-anaerobic activity and moderate activity against aerobes. When tested in mice by intraperitoneal administration the compound was found to have an LD_{50} of less than 17 mg/kg.

Experimental

NMR spectra were determined on Jeol GX 400 and FX 270 spectrometers. IR spectra were recorded on a Perkin-Elmer model 1420 spectrometer. UV-visible spectra were recorded on a Shimadzu UV-260 spectrophotometer. Mass spectra were determined on a VG Analytical model ZAB 1F spectrometer. Isolation was monitored by agar diffusion assay on *Escherichia coli* SGB4 and by TLC on silica gel ($CHCl_3$ - MeOH, 9: 1, Rf 0.25).

Isolation of SQ 30,957

At harvest, the cells from a 10-liter fermentation were separated by filtration. The filtrate was stirred with 600 ml of Diaion HP-20 (coarse grade) at room temperature for 1.5 hours. The resin was filtered, packed into a 5×50 cm column, and washed with 2 liters of H_2O followed by 1 liter of CH_3CN -

Fig. 2. Isolation of SQ 30,957.

Broth filtrate

↓ sorbtion onto Diaion HP-20 (coarse grade) and elution with CH_3CN - H_2O (2 : 3)

↓ concentration to 1/8th the volume and extraction with $CHCl_3$ - MeOH (4 : 1)

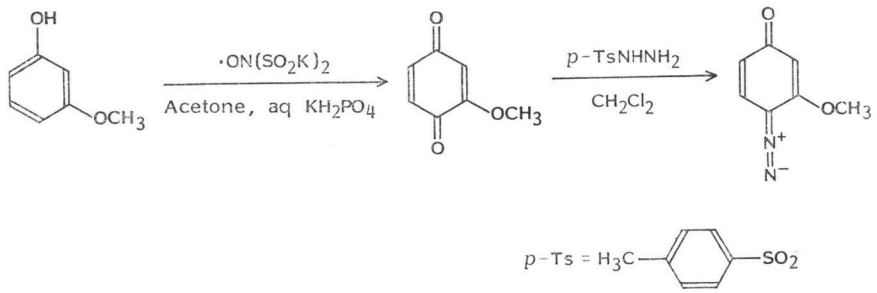
↓ chromatography on Whatman LPS-1 silica gel, eluting with CH_3CN - MeOH (4 : 1)

↓ chromatography on Baker silica gel eluting with $CHCl_3$ - MeOH (9 : 1)

↓ chromatography on Baker silica gel eluting with CH_3CN - MeOH (4 : 1)

SQ 30,957

Fig. 3. Synthesis of SQ 30,957.



H_2O (9 : 1). The antibiotic was then eluted with CH_3CN - H_2O (2 : 3), and the effluent (900 ml) concentrated to 100 ml *in vacuo*. The pH of the resulting solution was adjusted to 5 with 1 N HCl and the solution was extracted with five 100-ml portions of $CHCl_3$ - MeOH (4 : 1). The organic extract was concentrated to dryness *in vacuo* to give 376 mg of a brown oil. This was chromatographed on a 1.5×17 cm column of Whatman LPS-1 silica gel eluting with CH_3CN - MeOH (4 : 1). The antibiotic eluted between 57 and 73 ml. Concentration of the active effluent gave 27 mg of an oil that was chromatographed on a Baker silica gel column (1×12 cm) eluting with $CHCl_3$ - MeOH (9 : 1). Active fractions were combined and concentrated to give 11 mg residue which was chromatographed on a Baker silica gel column (1×6 cm) eluting with CH_3CN - MeOH (4 : 1), to give 9.4 mg of SQ 30,957 as yellow crystals.

Synthesis of SQ 30,957

A solution of potassium nitrosodisulfonate (0.75 g) and monobasic potassium phosphate (0.75 g) in H_2O (37.5 ml) was added in one portion to a solution of *m*-methoxyphenol (109 mg, 0.9 mmol) in acetone (12.5 ml) and stirred vigorously for 10 minutes. Additional potassium nitrosodisulfonate (0.75 g) was added to the reaction mixture in two portions at 30-minute intervals, and stirring continued for 2 hours. Acetone was removed under reduced pressure and the aq suspension was extracted with four 10-ml portions of CH_2Cl_2. The combined organic layer was washed with 0.01 N NaOH (10 ml) followed by H_2O (10 ml), dried over anhydrous $MgSO_4$, filtered, and the solvents were removed under reduced pressure to give 2-methoxy-*p*-benzoquinone (146 mg).

An ice-cold solution of 2-methoxy-*p*-benzoquinone (69 mg, 0.5 mmol) in CH_2Cl_2 (2 ml) was added to an ice-cold solution of *p*-toluenesulfonylhydrazide (102 mg, 0.55 mmol) in CH_2Cl_2 (2 ml) and stirred at 0°C for 1 hour. An additional 50 mg (0.27 mmol) of *p*-toluenesulfonylhydrazide was added to the reaction mixture and the mixture was stirred for 2 hours. The solvents were removed under reduced pressure and the residue was chromatographed on a silica gel column (2.5×18 cm) eluting with $CHCl_3$ - MeOH (9 : 1). Fractions containing SQ 30,957 were combined and the solvents were removed under reduced pressure to give the title compound (26.7 mg, 35% yield) as a yellow solid.

The spectroscopic data (^{1}H and ^{13}C NMR, IR, UV and mass spectrum) were identical to those of SQ 30,957.

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