METABOLIC PRODUCTS FROM *SPICARIA DIVARICATA* NRRL 5771

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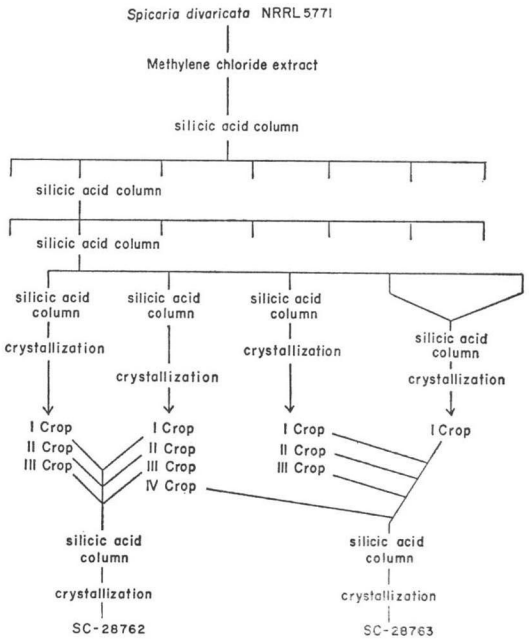
Two metabolites, SC-28762 and SC-28763, were produced by *Spicaria divaricata* NRRL 5771. The production, the isolation and purification, and the physicochemical properties of the metabolites are described. SC-28762 is 3,3′-di-(methoxycarbonylmethyl)-3,3′,4,4′-tetrahydro-9,9′,10,10′-tetrahydroxy-7,7′-dimethoxy-1,1′-dioxo-8,8′-bi-1H-naphtho[2,3-c]pyran (viriditoxin). SC-28763 is 3-(2-oxopropyl), 3′-methoxycarbonylmethyl-3,3′,4,4′-tetrahydro-9,9′,10,10′,-tetrahydroxy-7,7′-dimethoxy-1,1′-dioxo-8,8′-bi-1H-naphtho[2,3-c]pyran. SC-28763 exhibits good antimicrobial activities against anaerobic microorganisms.

In our search for pharmacologically active microbial metabolites, two chemically related metabolites, SC-28762 and SC-28763 (Fig. 1) were isolated from the culture broth of a fungus grown in the presence of sitosterol. The producing strain was isolated from the air in the laboratory and was identified as *Spicaria divaricata* THOM.[1] The type strain has been deposited in the Northern Regional Research Laboratory, Peoria, Illinois, U.S.A., and has been assigned accession number NRRL 5771.

The present paper reports on the production, isolation and purification, the physicochemical properties, and the biological properties of the metabolites. The structure of SC-28762 was determined to be viriditoxin,[2,3] 3,3′-di-(methoxycarbonylmethyl)-3,3′,4,4′-tetrahydro-9,9′10,10′-tetrahydroxy-7,7′-dimethoxy-1,1′-dioxo-8,8′-bi-1H-naphtho[2,3-c]pyran. The structure of SC-28763 was determined to be 3-(2-oxopropyl), 3′-methoxycarbonylmethyl-3,3′, 4,4′, tetrahydro-9,9′,10,10′-tetrahydroxy-7,7′-dimethoxy-1,1′-dioxo-8,8′-bi-1H-naphtho[2,3-c] pyran (Fig. 1).

Chart 1. Isolation and purification of SC-28762 and SC-28763.



Production of Metabolites

Spicaria divaricata NRRL 5771 was found to produce metabolites inhibitory to *Candida albicans* in broth containing sitosterol. The fungus was grown in shaken flasks and in a

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stirred fermentor. The fermentation medium used for both was as follows: 1.0% cottonseed meal, 5.0% glucose, 0.35% corn steep-water, and 0.035% hydrochloric acid in tap water. The pH of the medium after sterilization was 3.9±0.2. Five hundred ml shaken flasks containing 100 ml of the medium were inoculated with spores and mycelium from 2-week old cultures of *S. divaricata* grown on potato dextrose agar slant at 24°C. The flasks were incubated on a rotary shaker at 190 rpm for 3 days at 25°C until there was good growth. Then 10 mg of sitosterol in 1 ml of acetone was added to each flask. Incubation was continued for an additional 6 days. A 3,500-liter fermentor containing 2,200 liters of medium, 350 g of antifoam, and 550 g of sitosterol was inoculated with 100 liters of a 3-day old mycelial growth.

Table 1

Physicochemical properties	SC-28762	SC-28763
Melting point (0°C)	263~267 (dec)	238~242 (dec)
Elementary analysis	Found Calcd.	Found Calcd.
C	61.51 61.63	62.65 63.15
H	4.57 4.56	4.69 4.68
O	33.88 33.87	31.89 32.17
Mass spectral		
M^+	662	646
M-18	644	628
M-44	618	602
M-44-18	600	584
M-44-32	586	
M-44-18-18		566
M-44-44	574	558
M-44-44-18		540
M-44-44-32	542	
M-44-44-32-18		508
Molecular formula	$C_{34}H_{30}O_{14}$	$C_{34}H_{30}O_{13}$
Molecular weight	662.58	646.58
Ultraviolet absorption (acetonitrate)	226 nm (ε31,300) 264 nm (ε75,500) 374 nm (ε20,400) 387 nm (ε18,800)	227 nm (ε31,800) 264 nm (ε66,000) 274 nm (ε18,400) 387 nm (ε17,700)
Infrared absorption (chloroform)	3420, 1745, 1650 1585, 1265 cm^{-1}	3420, 1743, 1730, 1650 1587, 1360, 1265 cm^{-1}
Optical rotation $[\alpha]_D^{28}$ (chloroform)	−210.5	−200.5
Circular dichroism (chloroform)	$[\theta]_{307}$ 0 $[\theta]_{274}$ −781,000 $[\theta]_{264}$ 0 $[\theta]_{253}$ +583,000	$[\theta]_{304}$ 0 $[\theta]_{273}$ −698,000 $[\theta]_{262}$ 0 $[\theta]_{252}$ +453,000
Optical rotatory dispersion (chloroform)	$a=-13,571$	$a=-9,690$

Incubation was continued for an additional 7 days at 25°C with aeration (10 cfm of air) and agitation (50 rpm by stirrer). The antifungal activity of the culture broth was assayed by the agar diffusion method using *Candida albicans* ATCC 10231 as the test organism and by *in vivo*, intraperitoneal testing in mice infected with *C. albicans* ATCC 10231.

Isolation of Metabolites: SC-28762 and SC-28763

The culture broth was extracted twice with one-half volume of methylene chloride. SC-28762 and SC-28763 were recovered from the methylene chloride extract according to the scheme on Chart 1. The steps in the isolation and purification scheme were monitored using both the agar diffusion assay against *C. albicans* ATCC 10231, and by thin-layer chromatography. Thin-layer plates, 20×20 cm, were prepared from silica gel G (E.M. Laboratories, Inc., N.Y., 10523). Solvent systems used were: (A) chloroform-methanol-formic acid (90:4:6), and (B) benzene-ethanol-formic acid (90:4:6). The developed plates were visualized as blue spots after spraying with 50% sulfuric acid and then with phosphomolybdic acid solutions.

The methylene chloride extract was dissolved in benzene and absorbed on 27 kg Mallinckrodt CC-7 silicic acid. About two-fifths of the material eluted with 50% ethyl acetate-benzene and with 100% ethyl acetate was further absorbed on a second column containing 10 kg of Mallinckrodt CC-7 silicic acid. About one-fourth of the material eluted with 25~35% ethyl

Table 2. ^{1}H NMR

Proton	Chemical shift, multiplicity and coupling constants (Hz)	
	28762	28763
3-H	4.97, ddt, (6.5, 7.0 and 6.0)	4.98, m(1)
3′-H		
4a-H	2.67, dd, (6.5 and 16.0)	overlapped with others (2)
4′a-H		
4b-H	2.94, dd, (7.0 and 16.0)	overlapped with others (2)
4′b-H		
5-H	6.27, s	6.27, s
5′-H		
6-H	6.81, s	6.81, s
6′-H		
9-OH	9.72, s	9.80, s
9′-OH		
10-OH	13.75, s	13.82, s
10′-OH		
11-H_2	2.88, d, (6.0)	overlapped with others (2)
11′-H_2		
13-H_3	3.72, s	2.21, s
13′-H_3		3.72, s
14-H_3	3.79, s	3.79, s
14′-H_3		(shoulder at 3.77) (3)

(1) overlap of 2 sets of ddt
(2) overlap of 2 sets of dd+dd+d
(3) partial splitting probably due to -OCH_3 in slightly different environment

acetate-benzene was further purified on 10 kg of Mallinckrodt CC-7 silicic acid. Crystallization of several crops from the appropriate fractions, followed by further chromatography and final recrystallization from ethyl acetate to yield purified SC-28762; and from ethanol to yield SC-28763.

Physicochemical Properties of SC-28762 and SC-28763

The physicochemical properties of SC-28762 and SC-28763 are summarized in Tables 1, 2 and 3. The elemental analysis and mass spectra data indicated SC-28762 to have a molecular formula of $C_{34}H_{30}O_{14}$, and SC-28763 to have a molecular formula of $C_{34}H_{30}O_{13}$. SC-28762 was found to be similar to viridi-toxin,[2,3] a mycotoxin isolated from *Aspergillus viridi-nutans*.

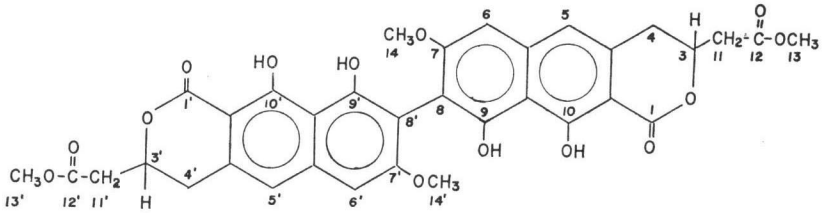
Table 3. ^{13}C NMR

Carbon number	28762	28763
1	169.0	169.1
3	75.5	76.0
4	32.8	33.1
4a	138.7[a]	138.9[a]
5	113.7	113.9
5a	107.7[b]	107.9[b]
6	97.9	98.5
7	158.5	158.5
8	not assigned	not assigned
9	160.6[c]	160.7[c]
9a	109.7[b]	109.7[b]
10	162.3[c]	162.8[c]
10a	131.5[a]	131.7[a]
11	39.3	48.0
11′	39.3	39.7
12	170.2	190.5
12′	170.2	170.5
13	51.8	30.9
13′	51.8	52.4
14	56.0	56.3

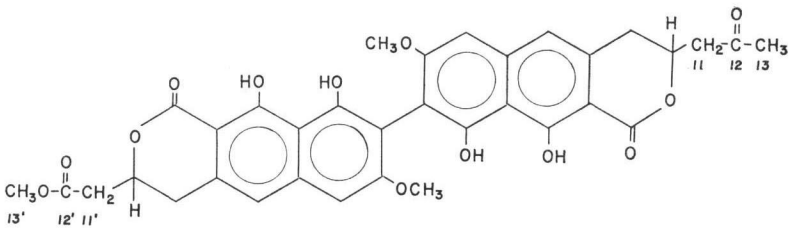
[a,b,c] Values in each vertical column may be reversed. Solvent, deuterated chloroform. Trimethyl silane standard.

A comparison of the physicochemical properties of the two metabolites indicated that SC-28763 differs from SC-28762 by only one oxygen atom (Table 1). Their mass spectral fragmentation patterns are quite similar; both exhibit similar ultraviolet spectra; and the infrared spectra are quite similar, except for additional shoulders at 1730 and 1360 cm^{-1} for SC-28763. The proton NMR of SC-28763 exhibits an additional chemical shift at δ 2.21 (3-H), suggestive of methyl ketone. The chemical

Fig. 1.



SC-28762



SC-28763

shift at δ 3.72 (3-H) assigned to the methyl protons in the methoxycarbonyl group was one-half as intense as it was in SC-28762 (6-H). All these data are suggestive that SC-28763 could be assigned the structure shown in Fig. 1. This structure for SC-28763 differs from that for SC-28762, in that one of the methoxycarbonyl groups has been replaced by a methylcarbonyl group. This structural assignment also is consistent with the observed complex splitting patterns of the C-4, C-4′, C-11 and C-11′ methylene protons and the C-3 and C-3′ carbinol protons of SC-28763 (Table 2). The ^{13}C NMR assignments are in agreement with this structure (Table 3). The additional chemical shifts at 30.9, 190.5 and 48.0 ppm of SC-28763 are assigned the methyl carbon, ketone carbonyl carbon, and the methylene carbon at C-13, C-12 and C-11, respectively.

The optical properties of these two compounds were compared with vioxanthin.[4] Although the ORD curves of both compounds are mirror images of that published for vioxanthin, the large negative molecular amplitude cannot be associated with the chirality at C-3 and C-3′ of the lactone rings. This chirality is probably associated with biaryl conformations with hindered rotations.[5]

Biological Properties of SC-28762 and SC-28763

The antimicrobial properties of SC-28762 and SC-28763 are summarized in Table 4. SC-28763 exhibits moderate antimicrobial activities against the two strains of *C. albicans*, the

Table 4. Antimicrobial spectrum

	Minimum inhibitory concentration (μg/ml)	
	SC-28762	SC-28763
Staphylococcus aureus ATCC 6538	1,000	50
Salmonella paratyphi A	1,000	100
Pseudomonas aeruginosa ATCC 9027	>1,000	500
Clostridium perfringens ATCC 13124	1,000	1
Candida albicans ATCC 10231	>1,000	50
Candida albicans M41-106	>1,000	75
Trichophyton mentagrophytes ATCC 10270	>1,000	100
Microsporum gypseum	>1,000	100
Verticillium albo-atrum	>1,100	50
Trichomonas vaginalis ATCC 30001	50	1

plant pathogen, *Verticillium albo-atrun*, and the bacterium *Staphylococcus aureus* ATCC 6538. SC-28763 exhibits good antimicrobial activities against the anaerobic microorganisms, *Clostridium perfringens* ATCC 13124 and *Trichomonas vaginalis* ATCC 30001.

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