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Antineoplastic Agents. Part 409: Isolation and Structure of Montanastatin from a Terrestrial Actinomycete[1,‡]

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Abstract—A Montana soil actinomycete, *Streptomyces anulatus*, produced (1×10^{-2}% yield) a new cancer cell growth inhibitory cyclooctadepsipeptide named montanastatin (**1**) accompanied by the potent anticancer antibiotic valinomycin (**2**) in very high (5.1%) yields. Valinomycin but not montanastatin inhibited growth of a number of pathogenic bacteria and fungi. Interpretation of high-field (500 MHz) NMR and high-resolution FAB mass spectral data allowed assignment of the structure *cyclo*-(D-Val-L-Lac-L-Val-D-Hiv) to montanastatin. Valinomycin (**2**) was also isolated from actinomycetes cultured from a tree branch and animal feces collected in Malaysia. *Streptomyces exfoliatus*, isolated from the tree branch, was found to contain valinomycin in 1.6% yield, while the fecal isolate, *S. anulatus*, gave valinomycin in 0.9% yield.

Terrestrial Gram-positive eubacteria of the diverse actinomycetes group (including *Streptomyces*) continue to be a very productive source of potentially important new drugs for a broad spectrum[2] of medical problems including human cancer.[2a–d] As part of our biosynthetic products-based anticancer drug discovery research, we isolated *Streptomyces anulatus* from a soil sample collected (August, 1989) in the Flathead National Forest, Montana. The strain produced a human cancer cell growth inhibitory cyclodepsipeptide herein designated montanastatin (**1**), accompanied by the potent antineoplastic antibiotic valinomycin (**2**).[3] A description of the isolation, structural elucidation and initial biological evaluation of montanastatin, as well as further biological studies of valinomycin, now follows.

Gas–liquid chromatography of fatty acid methyl esters (FAME, MIDI Sherlock Microbial Identification System, Newark, DE) indicated that the Montana actinomycete was most closely related to *S. anulatus*. The *S. anulatus* isolate was cultured at 25 °C for 72 h in dextrose (0.01%), yeast extract (0.025%), peptone (0.05%), $MgSO_4$ (0.01%), and K_2HPO_4 (0.01%), with shaking. The combined ethyl acetate extract (84 g) from 925 liters of fermentation broth was partitioned between hexane and methanol/water (9/1) to yield a hexane fraction (52 g) very active (ED_{50} 0.027 μg/mL) against the P388 lymphocytic leukemia cell line (significant activity is considered to be an ED_{50} value of <10 μg/mL in this and the human cancer cell lines cited in the sequel). Further separation was guided by results from this murine leukemia evaluation technique. A methanol solution of the hexane fraction was initially separated by gel permeation chromatography on a column of Sephadex LH-20. The most active fraction from this procedure led to valinomycin (**2**) in 5.1% yield (4.3 g) following rechromatography and crystallization. One of the smaller fractions (1.1 g, P388 $ED_{50}=0.20$ μg/mL) that appeared free of valinomycin was separated by employment of a series of partition column chromatographic steps using LH-20 Sephadex and the following solvent systems: (1) hexane/toluene/methanol (3/1/1); (2) hexane/ethyl acetate/methanol (5/2/1); and (3) hexane/dichloromethane/acetone (3/2/1). When an active fraction (P388 $ED_{50}=0.32$ μg/mL) from the latter separation was treated with methanol, montanastatin (**1**) precipitated as an amorphous powder. Montanastatin (**1**) was purified by crystallization from hexane/methanol to give colorless crystals (9.1 mg, 1×10^{-2}%).

Interpretation of ^1H and ^{13}C NMR and HMQC spectra of depsipeptide **1** revealed eighteen carbon signals

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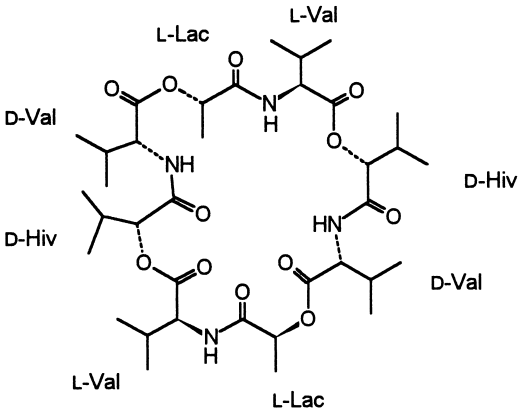
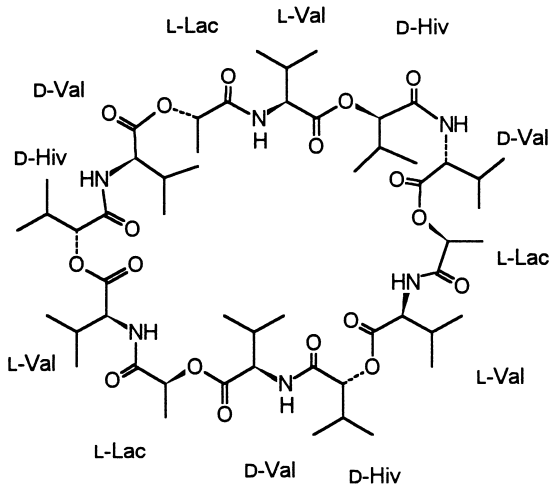


1, MONTANASTATIN



2, VALINOMYCIN

distributed as four carbonyls, seven methines, and seven methyl groups (Table 1). All the related hydrogen atoms bonded to carbon atoms were found in the ^{1}H NMR spectrum. In addition, two exchangeable proton signals were observed at δ 6.96 ppm (d, $J = 10$ Hz) and δ 6.94 (d, $J = 10$ Hz). Assignments of the cross-peaks in the COSY and HMBC spectra suggested montanastatin (**1**) was composed of four units: two valines $(CH_3)_2$-CH-CH(NH)-CO, one α-hydroxyisovaleryl unit $(CH_3)_2$-CH-CH-(O)-CO, and a lactoyl unit CH_3-CH(O)-CO. The sequence was found to be Hiv-Val-Lac-Val by interpretation of 2-D signals in the HMBC spectrum: δ 170.91/δ 6.94 for CO(Hiv)/NH(Val[a]), δ 170.38/δ 5.05 for CO(Val[a])/CH(Lac), δ 172.61/δ 6.96 for CO(Lac)/NH(Val[b]), δ 172.61/δ 4.25 for CO(Lac)/CH(Val[b]) and δ 170.98/δ 5.00 for CO(Val[b])/CH(Hiv). The HRFAB mass spectral data found for montanastatin established the molecular formula as $C_{36}H_{60}N_4O_{12}$, corresponding to a doubling of the fundamental four units. That fact, combined with an unsaturation number of nine, led to assignment of the cyclooctadepsipeptide structure, *cyclo*-(D-Hiv-D-Val-L-Lac-L-Val)$_2$ to montanastatin. The absolute configuration was assigned by analogy with that of valinomycin from analysis of the acid hydrolysate *N*-pentafluoropropionylisopropyl ester derivatives via chiral capillary chromatography.

Interestingly, valinomycin is composed of these four units in the same sequence, but tripled. Apparently, expansion of the ring from two to three base units leads to greatly increased activity against the minipanel of human cancer cell lines summarized in Table 2. As part

Table 1. ^{13}C NMR (d, ppm at 100 MHz) and ^{1}H NMR (500 MHz) assignments for montanasatin (**1**) in deuteriochloroform solution with tetramethylsilane as reference

Unit and position		^{13}C NMR	^{1}H NMR (J in Hz)	HMBC (C to H)
Hiv				
	1-CO	170.91		2-H,7-NH
	2-CH	79.25	5.00(1H, d, $J = 4.0$)	4-H,5-H
	3-CH	30.98	2.25 (1H, m)	2-H,4-H,5-H
	4-CH_3	19.11	0.96 (3H, d, $J = 6.0$)	2-H,3-H,5-H
	5-CH_3	17.08	0.95 (3H, d, $J = 7.5$)	2-H,3-H,4-H
Val[a]				
	6-CO	170.38		7-H,12-H
	7-CH	57.28	4.30 (1H, t, $J = 8.5$)	7-NH,8-H,9-H,10-H
	8-CH	29.00	2.25 (1H, m)	7-H,7-NH,9-H
	9-CH_3	19.77	0.98 (3H, d, $J = 6.5$)	7-H,8-H,10-H
	10-CH_3	18.77	0.96 (3H, d, $J = 6.0$)	7-H,9-H
	NH		6.94 (1H, d, $J = 10$)	
Lac				
	11-CO	172.61		12-H,13-H,15-H,15-NH
	12-CH	71.90	5.05 (1H, q, $J = 7$)	
	13-CH_3	18.41	1.43 (3H, d, $J = 6.5$)	12-H
Val[b]				
	14-CO	170.98		2-H,15-H
	15-CH	58.41	4.25 (1H, t, $J = 9.0$)	15-NH, 16-H, 17-H, 18-H
	16-CH	29.08	2.23 (1H, m)	15-H,17-H
	17-CH_3	19.58	0.96 (3H, d, $J = 6.0$)	15-H,18-H
	18-CH_3	18.71	0.99 (3H, d, $J = 6.5$)	15-H,17-H
	NH		6.96 (1H, d, $J = 10$)	

Table 2. Comparison of valinomycin and montanastatin cell growth inhibition

Cancer cell line		Valinomycin GI_{50} μg/mL	Montanastatin GI_{50} μg/mL
Murine P388 leukemia		0.019	7.5
Human			
Ovary	OVCAR-3	1.9×10^{-4}	1.2
Brain	SF-295	3.5×10^{-4}	3.3
Renal	A-498	1.9×10^{-3}	2.0
Lung	NCI-H460	2.1×10^{-4}	1.4
Colon	KM20L2	2.7×10^{-4}	1.4
Melanoma	SK-MEL-5	2.6×10^{-4}	1.3

of another anticancer antibiotic study in 1973, one of us (GRP) initiated an anticancer study of valinomycin in collaboration with the U.S. National Cancer Institute. Owing to very strong in vivo activity against a selection of model murine cancer evaluation systems, valinomycin was advanced into preclinical development. During the present investigation, we established valinomycin (**2**) as the principal P388 cell-line-active constituent of two rainforest (Terengganu, Malaysia) actinomycetes. The actinomycetes were isolated from the branch of an unidentified tree and from animal feces. FAME analysis revealed that the plant isolate was most closely related to *S. exfoliatus*, and the fecal isolate to *S. anulatus*. To our knowledge, this is the first description of valinomycin production by *S. exfoliatus* and *S. anulatus*. The yields of valinomycin were 1.6% and 0.9%, respectively, with use of fermentation and isolation procedures analogous to those summarized above for montanastatin (**1**). At this point, it became quite clear (and confirmed by Dr. John Leet)[2c] that valinomycin (**2**) is easily extracted by hexane from aqueous methanol solution. Thus, high P388 activity exhibited by such hexane fractions from actinomycetes may signal the presence of valinomycin (**2**).

While the structure determination of montanastatin was in progress, it became useful to reexamine the X-ray crystal structure of valinomycin (**2**), which has been the subject of X-ray structure determinations by other groups,[4a,b] as well as our own.[4c] Although valinomycin is a relatively simple molecule, it has considerable conformational flexibility, even in the solid state. The uncomplexed form of valinomycin is known to exist in at least two different conformations, depending upon what solvent is used to grow the crystals. Other conformations are also observed in the solid state for crystalline complexes formed between alkali metals and valinomycin. Although crystals of montanastatin could be grown from ethyl acetate–hexane solution, they were deemed unsuitable for a definitive structure determination via X-ray diffraction because of rapid decomposition and weak intensities, which occurred during an attempted data collection. However, crude cell parameters obtained for montanastatin (a = 12.66 Å, b = 16.44 Å, c = 22.81 Å, V = 4725 Å^3), when compared to those of valinomycin (a = 14.52 Å, b = 10.32 Å, c = 23.12 Å, V = 3417), suggested that montanastatin might possibly be a slightly larger, ring-expanded version of valinomycin. This was later proven to be incorrect from NMR and mass spectral data interpretations. Instead, the montanastatin unit cell apparently consists of four molecules of the compound, whereas the valinomycin unit cell consists of two molecules per unit cell. Owing to the smaller ring size of montanastatin, as compared to valinomycin, the conformational flexibility is presumed to be more restricted. Also, whether montanastatin has the complexing properties with alkali cations analogous to valinomycin is unknown. Both the conformational and complexing aspects of this compound require further investigation.

Meanwhile preliminary biological evaluations of montanastatin (**1**) were begun. Although montanastatin and valinomycin are composed of the same peptide units, the tripling and attendent ring expansion dramatically affects biological activities. The P388 leukemia and human cancer cell line data for valinomycin and montanastatin are compared in Table 2. The antimicrobial action of montanastatin and valinomycin were also compared. In disk diffusion assays,[5] valinomycin inhibited growth of the Gram-positive bacteria *Enterococcus faecalis* [minimum inhibitory concentration (MIC) = 0.39–0.78 μg/disk], *Streptococcus pneumoniae* (MIC = 0.39–0.78 μg/disk) and *Micrococcus luteus* (MIC = 25–50 μg/disk) and the fungi *Candida albicans* (MIC = 0.39–0.78 μg/disk) and *Cryptococcus neoformans* (MIC = 50–100 μg/disk). At 100 μg/disk, montanastatin did not inhibit the growth of these microbes. Neither peptide inhibited growth of the Gram-negative bacteria *Escherichia coli*, *Enterobacter cloacae*, *Stenotrophomonas maltophilia* or *Neisseria gonorrhoeae*.

While the current supply of montanastatin did not allow a useful antiviral evaluation, the effect of valinomycin on the replication of vesicular stomatitis virus (VSV) in Vero cells was examined. The addition of 10 μM valinomycin through the first three hours of infection resulted in a 90% decrease in viral titer 12 h post infection. Higher concentrations of valinomycin resulted in even greater reduction in viral titer. Specific infectivities of partially purified ^{35}S-methionine-labeled VSV produced in the presence and absence of valinomycin were similar. Thus, the reduction in VSV titer in the presence of valinomycin was probably due to decreased virus particle production, as opposed to release of noninfectious particles.

As determined by ^{35}S-methionine labeling followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and fluorography, the single envelope glycoprotein (G) and the four other polypeptides of VSV were synthesized in the presence of valinomycin. The G protein,

however, was not fully processed in valinomycin-treated cells. In pulse chase experiments, G protein oligosaccharides made in the presence of valinomycin remained sensitive to endo-β-*N*-acetylglucosaminidase H (endo H) (data not shown), an enzyme that cleaves high-mannose oligosaccharides but not fully processed complex oligosaccharides.[6] In the presence of valinomycin, most of the oligosaccharides in G protein were not converted to the mature, complex form required for transport of VSV G protein to the cell surface and its further incorporation into budding particles.[7,8] When montanastatin becomes readily available by synthesis, its effects on viral glycoprotein processing will be determined.

Experimental

General methods

Prior to use, all solvents for chromatographic procedures were redistilled. The Sephadex® LH-20 (25–100 μm) employed for gel permeation and partition chromatography was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. The silica gel GF Uniplates for TLC were from Analtech Inc., Newark, DE. The TLC plates were developed with ceric sulfate-conc. sulfuric acid followed by heating at approximately 200 °C. The ^{1}H- and ^{13}C NMR spectra were recorded on a Varian Unity 500 spectrometer. Melting points are uncorrected. The X-ray data were obtained using an Enraf-Nonius CAD4 diffractionater.

Extraction and solvent partitioning

The 925-L fermentation broth of *S. anulatus* (Montana) was extracted (5×) with ethyl acetate to give a brown syrupy residue (84.2 g, P388 ED_{50} 0.035 μg/mL). The ethyl acetate extract was dissolved in methanol/water (9/1) and filtered to remove insoluble material (1.3 g). Next, the solution was partitioned between hexane and methanol/water (9/1), then diluted to methanol/water (3/2) and extracted with dichloromethane. The methanol was removed and the water portion was extracted with ethyl acetate. Results of the P388 evaluation of these fractions indicated that the hexane extract (52 g) accounted for most of the activity (ED_{50} = 0.027 μg/mL), and that fraction was used for the subsequent isolation studies.

The broth plus mycelium obtained following fermentation of the Malaysian *S. exfoliatus* was filtered. The broth was extracted with dichloromethane. The dichloromethane extract was concentrated and filtered to give a solid (9.9 g, P388 ED_{50} = 0.022 μg/mL) and an oil (12.5 g, P388 ED_{50} = 0.18 μg/mL). The broth was further extracted with ethyl acetate and this extract, when concentrated and filtered, gave a solid (6.5 g, P388 ED_{50} = 0.18 μg/mL) and an oil (60.9 g, P388 ED_{50} = 0.38 μg/mL). The mycelium was extracted first with ethyl acetate to give a solid (9.2 g, P388 ED_{50} = 0.14 μg/mL) followed by dichloromethane to give a solid (6.0 g, P388 ED_{50} = 0.034 μg/mL). The combined dichloromethane fractions from the broth and the mycelium were dissolved in methanol and filtered to remove insoluble material (0.98 g). The methanol solution was used for subsequent isolation studies.

The fermentation broth (117 gal) of the Malaysian *S. anulatus* was extracted with hexane (4×) to give a solid (38.6 g, P388 ED_{50} = 0.046 μg/mL) and with ethyl acetate (4×) to give a solid (34.1 g, P388 ED_{50} = 0.015 μg/mL). The ethyl acetate extract was dissolved in methanol/water (9/1) and filtered to remove the insoluble material (2.44 g). The solution was partitioned between hexane and 9/1 methanol/water, and then the diluted methanol/water (3/2) was extracted with dichloromethane. Results of the P388 evaluation of these fractions indicated that the hexane extract (17.9 g) accounted for the most activity (ED_{50} = 0.004 μg/mL), and that fraction was used for the subsequent isolation studies.

Isolation of montanastatin (1) and valinomycin (2)

The hexane fraction of the Montana *S. anulatus* was dissolved in methanol, filtered and added to a column of Sephadex LH-20 (10×130 cm) in methanol to yield nine fractions.

The third fraction (1.19 g, ED_{50} = 0.20 μg/mL) was separated by employment of partition column chromatography on Sephadex LH-20 in the sequence: (1) hexane/toluene/methanol (3/1/1); (2) hexane/ethyl acetate/methanol (5/2/1); and (3) hexane/dichloromethane/acetone (3/2/1). The second fraction from the last column chromatogram (126 mg, ED_{50} = 0.32 μg/mL) afforded montanastatin (**1**) as colorless crystals (9.1 mg from hexane/methanol): mp 215∼217 °C; $[\alpha]_D^{24}$ +10° (*c* 0.07, $CHCl_3$); NMR (refer to Table 1); and HRFAB, calcd for $C_{36}H_{60}N_4O_{12}$; $(M+H)^+$ 741.4286. Found, 741.4288. The first two fractions from the original gel permeation chromatogram in methanol were combined (5.6 g, P388 ED_{50} ∼0.03 μg/mL) and rechromatographed on a column of Sephadex LH-20 in hexane/toluene/methanol (3/1/1) to give four fractions. Valinomycin (3.13 g, ED_{50} = 0.019 μg/mL) was crystallized from a hexane–methanol solution of the second fraction: mp 189–190 °C; $[\alpha]_D^{25}$ +16° (*c* 0.34, $CHCl_3$). Additional valinomycin was isolated from other fractions to give a total yield (5.1% based on the 84.2 g fraction) of 4.3 g.

Valinomycin (**2**) was isolated from the Malaysian *S. exfoliatus* as follows. The methanol solution obtained from the combined dichloromethane extracts was added to a column of Sephadex LH-20 (5×100 cm) in methanol to yield twelve fractions. The second fraction which contained most of the activity (22 g, P388 ED_{50} = 0.026 μg/mL) was rechromatographed on a column of Sephadex LH-20 in dichloromethane/methanol (3/2) to give six fractions, the first five of which all showed good activity and were combined and chromatographed on a Sephadex LH-20 column in hexane/toluene/methanol (3/1/1) to give ten fractions. Valinomycin crystallized from the first fraction (0.38 g, P388 ED_{50} = 0.029 μg/mL). Additional valinomycin

(0.746 g) was isolated from the remaining active fractions following chromatography on Sephadex LH-20 in hexane/methanol/isopropyl alcohol (8/1/1) and finally on Sephadex LH-20 in hexane/toluene/acetone (1/4/4). Valinomycin crystallized upon standing (0.68 g) from the hexane extracts which had been concentrated to a yellow oil (8.72 g, P388 $ED_{50} = 0.16\,\mu g/mL$) to give a total yield (1.6% based on the combined weight of the initial fractions, 105 g) of 1.73 g. The Malaysian *S. anulatus* also yielded valinomycin (**2**) as follows. The hexane extract was dissolved in methanol and separated using a series of Sephadex LH-20 gel permeation and partition chromatographic procedures with (1) methanol, (2) hexane/toluene/methanol (3/1/1), (3) hexane/isopropyl alcohol/methanol (8/1/1), and (4) hexane/dichloromethane/ethyl acetate (8/1/1) as solvent. The final column chromatography was on silica gel using a gradient elution of dichloromethane (100%) to ethyl acetate (100%) to give eleven fractions. Valinomycin crystallized from fractions seven, eight and nine to give a total yield (0.9%, based on combined weight of 72.7 g) of 0.67 g.

Absolute structure of montanastatin

Both montanastatin (**1**) and valinomycin (**2**) were treated in the same way to transform them into the corresponding *N*-pentafluoropropionyl isopropyl esters.[9] Their gas chromotograms (GC) were obtained on a Chiral Val III FSOT capillary column under the same conditions.[10] The sample from montanastatin showed the same GC signals as those of valinomycin by either separated or mixed injections.

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