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Biosynthetic Origin of the Tetrahydropyranyl Side Chain of Verucopeptin

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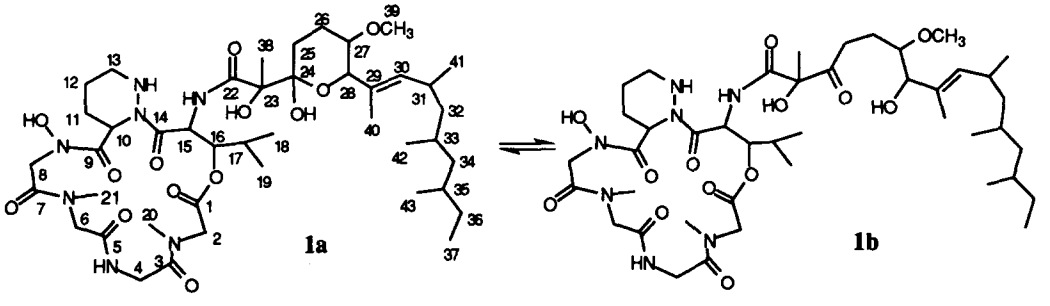
Abstract: The biosynthesis of the tetrahydropyranyl side chain of verucopeptin (**1**) in cultures of *Actinomadura verrucosospora* was investigated. Incorporation experiments using ^{13}C-labeled acetate and propionate, followed by analysis of the labeled metabolite by NMR, revealed that the tetrahydropyranyl side chain of **1** is derived from three acetate and five propionate units. A biosynthetic scheme consistent with the observed labeling patterns has been proposed. © 1997 Elsevier Science Ltd.

The antitumor metabolite verucopeptin (**1a, 1b**) has been isolated from cultures of *Actinomadura verrucosospora* and shown to exhibit specific activity against B16 melanoma.[1] It is structurally related to the antibiotics A83586C,[2] azinothricin,[3] citropeptin,[4] and variapeptin,[4] the anti-inflammatory agent L-156,602,[5] and the extracellular matrix antagonist IC101.[6] These unique natural products are structurally characterized by a polyketide moiety (tetrahydropyranyl side chain) and a 19-membered cyclodepsipeptide containing units of piperazic acid, β-hydroxyleucine and *N*-hydroxy-amino acids. We have speculated that the biosynthetic pathways leading to the formation of the polyketide chains are similar to those of the polyether[7] and macrolide[8] antibiotics and have begun investigating this hypothesis in the biosynthesis of verucopeptin. In this report we assigned all of the resonances of the ^1H NMR spectrum of **1a** and established that the polyketide side chain of verucopeptin is derived from three acetate and five propionate units.



Fermentation of *A. verrucosospora* and isolation of verucopeptin was achieved following a modified protocol from that reported in the initial discovery of the compound.[1a,9] Due to the complexity of verucopeptin, which in solution exists in a dynamic equilibrium between the hemiketal **1a** and keto **1b** (~ 3:1 ratio in $CDCl_3$ at 21 °C), its structure was originally deduced from spectroscopic analysis of a reduced analog (formed upon treatment of **1** with $NaBH_4$), and its chemical degradation products.[1b] We have analyzed the NMR data of metabolite **1** extensively by high-field ^1H, ^{13}C, DEPT, COSY, TOCSY, HMQC, HMBC and NOESY NMR experiments. Based on our data, we were able to confirm the previously reported ^{13}C chemical shifts of **1a** (Table 1)[1b] and to assign the ^1H chemical shifts for the first time (Table 1).

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Chemical Shift Assignments of Verucopeptin **1a** in $CDCl_3$

C	^{1}H (δ)	^{13}C (δ)	C	^{1}H (δ)	^{13}C (δ
1		166.8	18	1.07 (d, J=6.6 Hz)	18.3
2	3.44 (d, J=17.1 Hz, Ha)	51.7	19	0.86 (d, J=6.6 Hz)	19.1
	4.65 (d, J=17.1 Hz, Hb)		20	3.11 (s, 3H)	36.7
3		170.8	21	2.91 (s, 3H)	34.7
4	3.66 (d, J=17.1 Hz, Ha)	42.4	22		176.2
	5.05 (dd, J=17.1, 6.3 Hz, Hb)		23		77.6
4-NH	7.20 (d, J=6.3 Hz, H)		24		98.4
5		170.2	25	1.82 (m, 2H)	27.2
6	3.58 (d, J=17.1 Hz, Ha)	52.5	26	1.82 (m, Ha), 2.03 (m, Hb)	24.1
	4.12 (d, J=17.1 Hz, Hb)		27	3.05 (m)	75.7[a]
7		167.1	28	4.09 (d, 9.3 Hz)	80.0[a]
8	3.89 (d, J=15.6 Hz, Ha)	51.3	29		130.1
	5.27 (d, J=15.6 Hz, Hb)		30	5.17 (d, J=9.8 Hz)	137.0
8-N-OH	9.12 (s)		31	2.55 (m)	29.7
9		172.0	32	1.04 (m), 1.20 (m)	46.1
10	5.31 (d, J=7.6 Hz)	48.5	33	1.50 (m)	27.7
11	1.86 (m, Ha), 2.19 (m, Hb)	23.9	34	1.06 (m)	45.1
12	1.45 (m, Ha), 1.57 (m, Hb)	21.3	35	1.38 (m)	31.7
13	2.65 (dd, J=13.9, 1.5 Hz, Ha)	46.9	36	1.10 (m, Ha), 1.25 (m, Hb)	30.4
	3.11 (m, Hb)		37	0.84 (t, J=7.4 Hz)	11.4
13-NH	4.89 (d, J=13.9 Hz)		38	1.40 (s)	20.6
14		171.3	39	3.28 (s)	56.8
15	6.09 (dd, J=9.6, 2.8 Hz)	46.5	40	1.65 (s)	11.4
15-NH	7.35 (d, J=9.6 Hz)		41	0.97 (d, J=6.6 Hz)	21.3
16	4.79 (dd, J=9.5, 2.8 Hz)	80.0	42	0.78 (d, J=6.4 Hz)	19.4
17	1.72 (m)	30.3	43	0.80 (d, J= 6.6 Hz)	19.2

[a] In the original report, these chemical shifts were interchanged,[1b] however, our NMR data (COSY, HMQC and HMBC), as well as our incorporation results of isotopically labeled acetate and propionate, clearly show that the resonances of C27 and C28 are at δ 75.7 and 80.0 respectively.

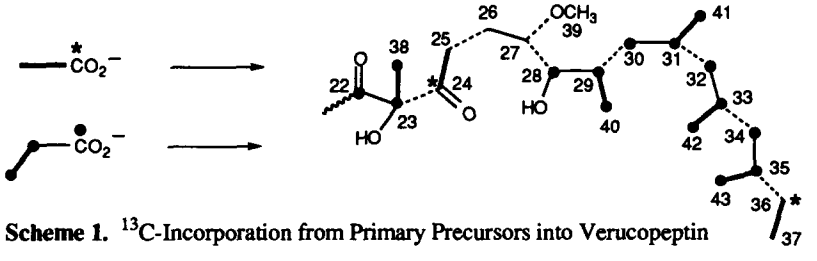
Feeding of ^{13}C-labeled precursors to cultures of *A. verrucosospora* led to the isolation of isotopically labeled verucopeptin (2-4 mg).[10] In most cases, extensive overlapping of the ^{13}C resonances, as well as low signal to noise ratio, made it impossible to clearly observe the incorporation of the labeled precursors in the 1D ^{13}C NMR spectrum. However, NMR analysis of the labeled verucopeptin samples by 1D-INADEQUATE and ^{13}C 1D-COSY (using selective excitation with a shaped pulse)[11] allowed for the unambiguous detection of the labeling patterns from each feeding experiment. ^{13}C Enrichment at C24 and C36 from the incorporation of [1-^{13}C]acetate, as well as intact incorporation of [1,2-$^{13}C_2$]acetate in C24-C25 and C36-C37 was clearly observed, but not in C26 and C27, as we had expected (Scheme 1, Table 2).[12] The addition of acetate to cultures of *A. verrucosospora* seemed to inhibit the production of verucopeptin. Thus the absence of an observable coupling between C26-C27 in the INADEQUATE NMR spectrum might have been due to the very low level of acetate incorporation at those carbons or a coupling constant of unusual magnitude.[12]

Incorporation of label into C12-C13 and C14-C15 of the depsipeptide moiety was also observed, most likely due to the *in situ* formation of labeled ornithine and β-hydroxyleucine, respectively, from acetyl-CoA. Feeding experiments using [1-^{13}C]- and [2,3-$^{13}C_2$]propionate clearly showed the incorporation of five propionate units at C22-C23-C38, C28-C29-C40, C30-C31-C41, C32-C33-C42 and C34-C35-C43 (Scheme 1, Table 2). Furthermore, the observed ^{13}C enrichment at C22, C28, C30, C32 and C34 from the incorporation of [1-^{13}C]propionate (Scheme 1, Table 2), confirmed the expected direction of polymerization during biosynthesis of the polyketide chain.

Table 2. Relative ^{13}C Enrichment[b] and $J_{C\text{-}C}$ Constants in the NMR of **1a** after Incorporation of ^{13}C-Labeled Precursors (125 MHz, $CDCl_3$)

C	AcONa [1-^{13}C]	AcONa [1,2-$^{13}C_2$] $^1J_{C\text{-}C}$, Hz	$EtCO_2Na$ [1-^{13}C]	$EtCO_2Na$ [2,3-$^{13}C_2$] $^1J_{C\text{-}C}$, Hz	C	AcONa [1-^{13}C]	AcONa [1,2-$^{13}C_2$] $^1J_{C\text{-}C}$, Hz	$EtCO_2Na$ [1-^{13}C]	$EtCO_2Na$ [2,3-$^{13}C_2$] $^1J_{C\text{-}C}$, Hz
22	1.0		3.0		33	1.0		1.0	35.0
23	1.0		1.0	40.0	34	1.0		3.5	
24	3.9	45.8	1.0		35	1.0		1.0	35.5
25	1.0	45.8	1.0		36	3.6	35.0	1.0	
26	1.0		1.0		37	1.0	35.0	1.0	
27	1.0		1.0		38	1.0		1.0	40.0
28	1.0		2.1		39	1.0		1.0	
29	1.0		1.0	44.2	40	1.0		1.0	44.0
30	1.0		2.8		41	1.0		1.0	34.3
31	1.0		1.0	34.6	42	1.0		1.0	35.0
32	1.0		3.6		43	1.0		1.0	35.2

[b] Relative ^{13}C enrichments were measured from ^{13}C inverse gated NMR experimentes. The values given are the ratios of relative intensity (areas under the signal after deconvolution using Lorentzian fit) of ^{13}C resonance in enriched sample/relative intensity of same ^{13}C resonance at natural abundance. ^{13}C enrichment of C39 is not expected from either [1-^{13}C]acetate or [1-^{13}C]propionate, thus it was used as the standard.



Scheme 1. ^{13}C-Incorporation from Primary Precursors into Verucopeptin

Based on the above results, the biosynthesis of the tetrahydropyranyl side chain of verucopeptin is most likely catalyzed by a modular polyketide synthase enzyme (PKS) complex, analogous to those associated with the biosynthesis of macrolide antibiotics.[13] The biosynthesis of the cyclic depsipeptide moiety of verucopeptin is most likely catalysed by a thiotemplate-directed peptide synthetase system which might be in direct interaction with the PKS complex. Evidence for or against this hypothesis is being sought in experiments currently in progress in our laboratory.

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9. Fermentation of *A. verrucosospora* and production of verucopeptin (**1**): Stock cultures were maintained at 4 °C in slant tubes on solid medium (1% tryptone, 0.6% yeast extract and 1.5% agar). New agar plates were grown at 28 °C for 3-4 weeks (until cultures had sporulated) and used to inoculate 100 mL of liquid medium (2% glycerol, 1% soybean meal, 0.5% $CaCO_3$, pH=7.0). This liquid culture was grown at 28 °C in an incubator shaker at 200 rpm for 5 days and then used to inoculate fresh liquid medium (~40 mL of seed culture in 1 L of fresh medium) for the production of verucopeptin. The large scale fermentation broth was incubated for 9 days under the same conditions and subsequently centrifuged at 10,000 rpm using a Sorvall RC-5B refrigerated centrifuge equipped with a GSA rotor. The supernatant was extracted with EtOAc (2x500 mL) and the organic layer was evaporated to dryness to give ~170 mg of a crude mixture of metabolites. This mixture was purified by C_{18} reversed phase flash column chromatography using a solvent system from MeOH:H_2O (1:1) to pure MeOH; pure metabolite **1** (5-10 mg/L) eluted from the column in 90% aqueous MeOH and was isolated as an amorphous white power after evaporation of the solvent. ^1H and ^{13}C NMR data are shown in Table 1.

10. Isotopically labeled precursors (total of 100 mg per 500 mL broth) were fed every 12 h over a period of 2 days, starting on day 5 of the fermentation period. Cultures were harvested 48 h after the last feeding and the labeled verucopeptin was isolated.

11. Due to the very small amounts of labeled metabolite **1** isolated after each feeding experiment and the large number of overlapping resonances, ^{13}C-^{13}C coupling could not be easily observed in the 1D ^{13}C NMR spectrum of verucopeptin (3:1 mixture of **1a**:**1b**). Coupling patterns and $J_{C\text{-}C}$ values were obtained from 1D-INADEQUATE NMR experiments. In cases where the signal to noise ratio in the INADEQUATE NMR spectrum did not allow coupling to be unambigiously observed, a series of 1D-COSY ^{13}C NMR experiments were carried out using optimized delay values ($1/2J$) between the selective excitation pulse and the mixing pulse for each set of carbons.

12. Although it is also possible that the C26-C27 unit may be derived from glycolate, we have not yet observed incorporation of label from [2,2-$^2H_2$]glycolic acid.

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