TWO NEW TRICHOTHECENES, PD 113,325 AND PD 113,326

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Two new trichothecenes, PD 113,325 and PD 113,326, were isolated and their structures were shown to be 12′-hydroxy-2′-(*E*)-verrucarin J (**1a**) and a stereoisomer of satratoxin H (**3**).

During the course of our antitumor screening program the known trichothecenes, verrucarin A, verrucarin J, roridin E, *iso*-roridin E, and roridin H[1,2] were isolated from fermentation broths produced by several *Myrothecium* cultures. The fermentation of a particular strain of *Myrothecium roridum* produced beers that exhibited good activity against P388 lymphocytic leukemia in mice. Careful chromatography of ethyl acetate extracts of this beer on silica gel led to the isolation of three novel trichothecenes. The structure of one of these compounds, roridin L-2, has already been reported.[3] The present paper describes the isolation and structure elucidation of the remaining two trichothecenes, PD 113,325 and PD 113,326, which were shown, respectively, to be **1a**, a 12′-hydroxylated, C(2′)-C(3′) geometric isomer of verrucarin J, and a stereoisomer of satratoxin H (**3**).[4]

Preliminary Fractionation

Fermentation beer† (1,350 liters), prepared as described elsewhere[5] was mixed vigorously with 680 liters of ethyl acetate at pH 6.6 for one hour. Celite 545 (91 kg) was added and the mixture was filtered using a plate and frame filter press. The filter cake was washed with 245 liters of ethyl acetate followed by 188 liters of deionized water. The filtrate and filter cake washes were combined and allowed to stand. The lower aqueous phase was separated and mixed with 475 liters of fresh ethyl acetate. After standing, the upper organic layer was added to the first ethyl acetate extract. The combined ethyl acetate extract (1,015 liters) was concentrated *in vacuo* to approximately two liters. An upper oily layer (300 ml) that formed contained only trace amounts of trichothecenes and was discarded. The lower ethyl acetate layer was dried using anhydrous sodium sulfate and then filtered. The solvent was removed *in vacuo* to leave approximately 650 g of an oily residue.

A portion (118 g) of this product was diluted to 2 liters with dichloromethane and fractionated using a Prep LC/System 500 apparatus (Waters Associates, Inc., Milford, MA, USA) fitted with two Prep-Pak 500 silica gel cartridges. After application of the charge, the silica gel columns were eluted consecutively with 2.9 liters of CH_2Cl_2; 4.3 liters of CH_2Cl_2 - EtOAc (75: 25); 6 liters of CH_2Cl_2 - EtOAc (50: 50); and 4 liters of EtOAc. A total of 18 fractions were collected and each was analyzed by high-performance liquid chromatography (HPLC), monitored at 254 nm, using 0.39 cm (ID) × 30 cm columns. Detection of all the UV absorbing components present in the various fractions was possible by using: System-1, MeOH - H_2O (65: 35) and a μBondapak C-18 column or System-2, 1-PrOH - hexane (12: 88) and a μPorasil column. Peaks that were not (or poorly) resolved by one system were

† Many of the compounds produced by this culture are powerful skin and eye irritants. Great care should be taken when handling concentrated extracts and chromatographic fractions.

separated by the other system. These analyses showed that the CH_2Cl_2 fractions contained verrucarin A, verrucarin J (isolated as a crystalline compound) and other components, including 4-hydroxybenzaldehyde. The fractions representing the first 0.95 liter of the CH_2Cl_2 - EtOAc (75: 25) eluate contained the majority of PD 113,325, PD 113,326 and roridin E. Later 25% EtOAc fractions contained unidentified roridins. Roridin L-2 (PD 110,208) appeared in the 50% EtOAc fraction and other, more polar trichothecenes were present in the EtOAc eluate.

Isolation of PD 113,325

The fractions containing most of PD 113,325 and PD 113,326 were combined, dried over anhydrous sodium sulfate, and concentrated *in vacuo* to leave a residue (12 g). A portion (7.2 g) of this product was dissolved in 60 ml dichloromethane and further fractionated by low pressure chromatography on

Table 1. ^{13}C and ^{1}H NMR data for PD 113,325 (**1a**) and verrucarin J (**2**).

Carbon No.	PD 113,325[a]		Verrucarin J[b]	
	^{13}C	^{1}H (J)	^{13}C	^{1}H (J)
2	79.2 d	3.86 d (6)	78.8 d	3.86 d (5)
3	34.9 t	A: 2.19 dt (17, 6) B: 2.45 dd (17, 8)	34.9 t	A: ~2 m B: 2.53 m
4	75.2 d	5.98 dd (8, 4)	75.1 d	6.01 dd (8, 4)
5	49.4 s		48.6 s	
6	43.7 s		42.8 s	
7	20.7	~1.95 m	20.5 t	~2 m
8	26.9	~2.05 m	27.4 t	~2 m
9	143.3 s		140.2 s	
10	118.3 d*	5.44 dd (5, 2)	117.9 d*	5.47 d (5)
11	67.3 d	3.7 d (5)	67.1 d	3.76 d (5)
12	65.2 s		65.2 s	
13	48.1 t	A: 2.83 d (4) B: 3.14 d (4)	47.8 t	2.84 d (4) 3.15 d (4)
14	7.0 q	0.64 s	6.8 q	0.83 s
15	62.7	A: 3.7 d (12) B: 5.07 d (12)	63.1 t	A: 3.97 d (13) B: 4.44 d (13)
16	23.2	1.72 s	23.0 q	1.72 s
1′	165.3 s*		165.6 s*	
2′	116.7 d*	6.1 d (~2)	118.5 d*	5.85 d (1.5)
3′	157.4 s		156.3 s	
4′	28.1	A: 2.3 m B: ~4.14 m	40.0 t	2.50 t
5′	61.8	~4.47 m	60.2 t	A: 4.15 m (6) B: 4.47 m
6′	165.9 s*		165.8 s*	
7′	127.3 d*	5.9 d (16)	127.2 d*	6.01 d (16)
8′	139.4 d*	8.2 dd (16, 11)	138.9 d*	8.07 dd (16, 11)
9′	140.0 d*	6.65 t (11)	139.3 d*	6.63 t (11)
10′	125.0 d*	6.10 d (11)	125.3 d*	6.11 d (11)
11′	166.1 s*		165.3*	
12′	64.4 t	A: 4.20 d (16) B: 4.27 d (16)	17.0 q	2.28 d (1.5)

* In each column, ^{13}C signals within 2.3 ppm of each other may be interchanged.

[a] ^{1}H and ^{13}C spectra were measured in $CDCl_3$ at 360 MHz and 22.6 MHz, respectively. Chemical shifts are reported as ppm downfield from TMS.

[b] Data for ^{13}C NMR signals are from ref 9; ^{1}H signals are from ref 8 and 10.

500 g of 20 μm silica gel (Whatman, Inc., Clifton, New Jersey, USA). After the charge was applied, the silica gel was eluted with one liter of hexane followed by a solvent system consisting of 1-PrOH - hexane (10: 90). The eluates were analyzed *via* HPLC using System-2. The fractions containing PD 113,325 were combined and concentrated to dryness *in vacuo*. The residue (1.37 g) was crystallized from dichloromethane - cyclohexane to yield 568 mg of PD 113,325: mp>250°C[12]; MS 500 (M^+); λ_{max} in MeOH (ε) 217 nm (23,500), 262 nm (18,900); IR (CCl_4) 3640, 2980, 2920, 1745, 1660, 1270, 1210 and 1190 cm^{-1}; ^{13}C and ^1H NMR data are given in Table 1.

Anal Calcd for $C_{27}H_{32}O_9$: C 64.79, H 6.44
Found: C 65.20, H 6.55

Table 2. ^{13}C and ^1H NMR data for PD 113,326 and satratoxin H (3).

Carbon No.	PD 113,326[a]		Satratoxin H[b]	
	^{13}C	^1H (J)	^{13}C	^1H (J)
2	79.13*	3.9 m	79.1 d	3.9 m
3	34.46	A: 2.18 dt (15, 5) B: 2.45 dd (15, 8)	34.4 t	A: 2.20 dt (15, 4.5) B: 2.45 dd (15, 7.5)
4	74.16*	5.9 m	74.2 d	5.9 m
5	48.97		49.0	
6	43.36		43.4	
7	20.33	1.92 m	20.4 t	1.9 m
8	27.61	~2 m	27.6 t	2.1 m
9	140.40		140.2	
10	118.88*	5.43 d (5)	119.0 d	5.46 d (5)
11	68.23	3.59 d (5)	68.2 d	3.62 d (5)
12	65.48		65.4	
13	48.05	A: 2.83 d (4) B: 3.14 d (4)	48.0 t	A: 2.81 d (4) B: 3.12 d (4)
14	7.54	0.83 s	7.6 q	0.83 s
15	64.02	A: 4.54 d (13) B: 5.10 d (13)	64.2 t	A: 3.88 d (12) B: 4.56 d (12)
16	23.30	1.72 s	23.3 q	1.74 s
1′	165.35*		166.2	
2′	118.23*	5.80 d (2)	119.0 d	5.85 d (2)
3′	154.91		155.1	
4′	25.45	A: ~2.7 m B: ~3.78 m	25.3 dd	A: 2.6 m B: 3.74 dt (10, 3)
5′	61.05	3.9 m	60.4 t	3.9 m
6′	78.69*		81.4	
7′	133.56*	5.39 d (17)	132.2 d	6.09 d (17.5)
8′	134.15*	7.26 dd (17, 10)	134.2 d	7.36 dd (17.5, 10.5)
9′	142.35	6.52 dd (12, 10)	143.0 d	6.63 t (10.5)
10′	120.98	5.93 d (12)	120.4 d	5.91 d (10.5)
11′	166.67*		167.0	
12′	75.35*	4.22 s	73.7 d	3.97 s
13′	74.65*	4.0 q (7)	69.7 d	4.38 q (7)
14′	15.80	1.35 d (7)	15.7 q	1.16 d (7)

* The signals for C-6′, 12′ and 13′ of PD 113,326 are not explicitly assigned. Other signals within 1.5 ppm of each other may be interchanged.

[a] ^1H and ^{13}C spectra were measured in $CDCl_3$ at 360 MHz and 22.6 MHz, respectively. Chemical shifts are reported as ppm downfield from TMS.

[b] Data are from ref 4 and 11.

Isolation of PD 113,326

The fractions from the above 20 μm silica gel column containing PD 113,326 were combined and concentrated to dryness. The residue (1.09 g) was dissolved in 3 ml 50% aqueous acetonitrile and divided into four 270-mg aliquots. Each aliquot was chromatographed on 52 g of 40 μm C_{18}-silica gel (Analytichem International, Harbor City, California, USA) using acetonitrile - water (40: 60) as the eluent. The fractions found to contain primarily PD 113,326 by HPLC System-2, described above, were combined, concentrated *in vacuo* to remove the acetonitrile, and extracted three times with ethyl acetate. The ethyl acetate extracts were combined, dried over anhydrous sodium sulfate, and concentrated to dryness. The residue (229 mg) was rechromatographed over 50 g of C_{18}-silica gel using methanol - water (60: 40) as the eluent. The fractions containing PD 113,326 were combined, concentrated, and extracted with ethyl acetate. The extracts were combined, dried over sodium sulfate and concentrated to afford a residue (200 mg) which was crystallized from dichloromethane - cyclohexane to yield 139 mg of PD 113,326: mp 168~171°C; MS 528 (M^+); λ_{max} in MeOH (ε) 227 nm (19,500) with a pronounced inflection at 255 nm (12,500); IR (CCl_4) 3560, 3440, 2975, 2930, 1730, 1660, 1595, 1195, 1165, 1145, 1080 and 970 cm^{-1}; ^{13}C and ^1H NMR data are given in Table 2.

Table 3. Antitumor activity of PD 113,325 and PD 113,326 against P388 lymphocytic leukemia in mice.

Total dose[a] (mg/kg)	T/C×100[b]	
	PD 113,325	PD 113,326
10	—	198
5	129	177
2.5	129	144
1.25	123	125

[a] ip doses given on days 1 and 5 for PD 113,325; ip doses given on days 1 through 5 for PD 113,326.
[b] Percent increase of survival time of treated mice compared to controls.

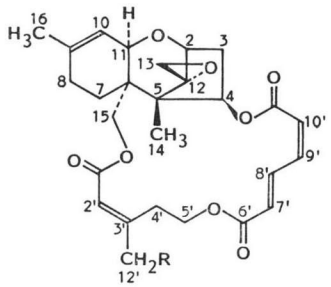
Anal Calcd for $C_{29}H_{36}O_9$: C 65.91, H 6.81
Found: C 66.42, H 7.34

PD 113,325 and PD 113,326 are highly cytotoxic to L1210 cells; the IC_{50} value for each compound is approximately 0.001 μg/ml. The *in vivo* activity of these compounds against P388 lymphocytic leukemia is shown in Table 3.

Structure Determination

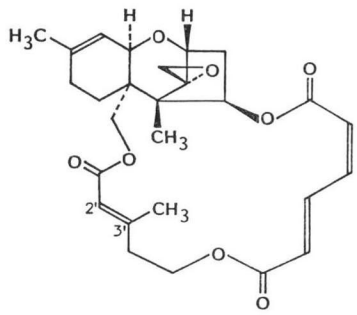
PD 113,325 (**1a**)

Elemental analysis, mass spectral and ^{13}C NMR data for PD 113,325 establish $C_{27}H_{32}O_9$ as the molecular formula. Mild alkaline hydrolysis[6] of PD 113,325 yielded verrucarol (**4**) as shown by chromatographic and spectral comparisons with an authentic sample. These data indicate that PD 113,325 is a verrucarin analog. Although PD 113,325 is isomeric with verrucarin B[6] and 2′-dehydroverrucarin A,[7] spectral data differentiate it from these metabolites. The UV absorption maxima at 217 nm and 262 nm exhibited by PD 113,325 indicate the presence of α,β-unsaturated ester and muconic acid moieties as found in verrucarin J (**2**).[8] Indeed, the NMR data presented in Table 1 clearly show that PD 113,325 is a monohydroxylated derivative of verrucarin J. In contrast to the many similarities in the NMR spectra of PD 113,325 and verrucarin J, distinctive spectral differences are the absence in PD 113,325 of a 12′-methyl proton signal at 2.3 ppm and the presence of an AB pattern near 4.25 ppm which is shifted to 4.63 ppm upon acetylation. The expected difference in the ^{13}C NMR of PD 113,325 resulting from the replacement of a 12′-methyl group (17 ppm) by a hydroxymethyl group (64 ppm) is also observed.

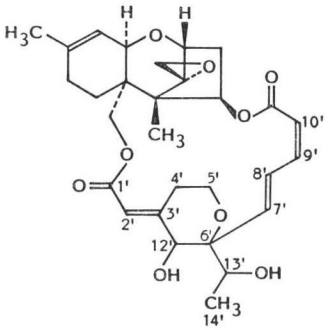


1a PD 113,325 R = OH

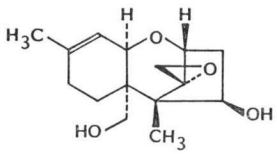
1b 2'-(*Z*)-Verrucarin J R = H



2 Verrucarin J



3 Satratoxin H



4 Verrucarol

In macrocyclic trichothecenes containing a C(2′)-C(3′) double bond, the shielding of protons, especially on C-15, 4′ and 5′, is strongly dependent on the configuration of the C(2′)-C(3′) double bond. ROUSH and BLIZZARD[10] have reported ^{1}H NMR data for both verrucarin J (**2**) and 2′-(*Z*)-verrucarin J (**1b**). Compound **1b** and PD 113,325 display nearly identical chemical shifts for H-15, H-4′ and H-5′ including the C-14 methyl singlet which appears unusually upfield at 0.64 ppm. Further, when a solution of PD 113,325-acetate in $CDCl_3$ is irradiated at δ 4.63 (the 12′ protons), a 20% enhancement of the H-2′ signal at 5.96 ppm is observed. This NOE shows that the C(2′)-C(3′) double bond of PD 113,325 has an *E*-configuration and thus does not share the same relative stereochemistry found in verrucarin J (**2**). Based on the above information, structure **1a** is assigned to PD 113,325.

PD 113,326

Elemental analysis, mass spectral and ^{13}C NMR data for PD 113,326 establish a molecular formula of $C_{29}H_{36}O_9$. NMR data for PD 113,326, shown in Table 2, show that this compound is very similar to roridin-type trichothecenes but does not contain a C-12′ methyl group. Instead, C(12′) of PD 113,326 bears an oxygen atom and a single proton as found in satratoxin H (**3**)[4] which also possesses UV absorption properties[12] similar to those of PD 113,326. Placement of a hydroxyl group at C(12′) is established by the 25% NOE enhancement of the H(12′) singlet at 4.22 ppm upon irradiation of H(2′) at 5.83 ppm. Two exchangeable protons detected in the ^{1}H NMR spectrum of PD 113,326 taken in $CDCl_3$ show the presence of two hydroxyl groups. Addition of trichloroacetylisocyanide shifts the H-13′ quartet originally at 4.0 ppm to 5.5 ppm and the H-12′ singlet to 5.4 ppm. The spectral com-

parisons shown in Table 2 plus homonuclear spin decoupling experiments support the view that PD 113,326 is a stereoisomer of satratoxin H (**3**). Significant differences exist between the ^{13}C signals at C-5′ and 13′, and the ^{1}H signals of H-15, 7′, 12′, 13′ and 14′ of PD 113,326 and satratoxin H. These differences are attributed to stereoisomerism that exists between these two compounds at one or more of the 6′, 12′ and 13′ carbon positions.

Discussion

The particular strain of *Myrothecium roridum* used in the present study appears to readily hydroxylate trichothecene analogs at allylic positions. The recent isolation of 4′-hydroxyroridin H[13] is another example of such a hydroxylation. In addition to our previous isolation of roridin L-2[3] and the compounds described here, JARVIS *et al.* have shown that our culture also produces additional 12′ and 16-hydroxylated trichothecenes, which include: trichoverritone A,[14] 16-hydroxytrichodermadienediols A and B,[13] and 16-hydroxyroridin L-2.[14]

Acknowledgments

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