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NEW TRICHOVERROIDS FROM *MYROTHECIUM VERRUCARIA* ISOLATED BY HIGH SPEED COUNTERCURRENT CHROMATOGRAPHY

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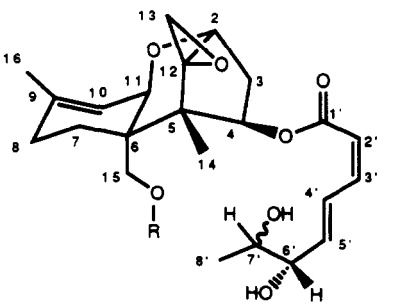
ABSTRACT.—Three new double-bond isomers of the trichothecene trichoverrins [**3**, **4a**, and **4b**] have been isolated, principally through the use of high speed countercurrent chromatography, which proved to be a powerful tool in the separation of these closely related structural isomers.

High speed countercurrent chromatography (ccc) (1) is being used more often in natural products chemistry (2). It is proving to be a very mild but powerful separation technique which is useful on both the analytical and preparative scale. Herein, we report its application in the isolation of three new trichoverroids which were part of a complex mixture obtained from a fermentation of the fungus *Myrothecium verrucaria* (ATCC 20540). These three compounds are very close structural relatives of other components of the mixture and hence difficult to separate.

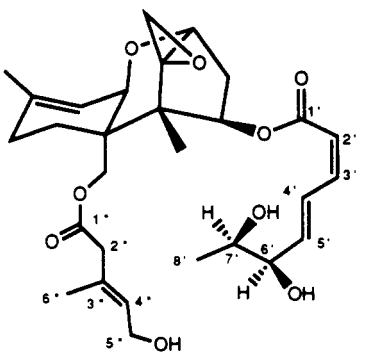
The trichoverroids are complex trichothecenes that are biosynthetic precursors to the macrocyclic trichothecenes (3). The trichoverrols **1a** and **1b** and trichoverrins **2a** and **2b** may be readily separated by reversed-phase cc, but the diastereomers, **1a/1b** and **2a/2b**, are difficult to separate from one another and were done so only by hplc or by careful preparative tlc (4). Recently, we reported the isolation and characterization of 12,13-deoxytrichoverrins A and B whose separation from the other closely related trichoverroids involved extensive use of preparative tlc and hplc (5). A slightly more polar fraction from this work was subjected to ccc, and, with far less trouble, we have isolated and characterized three new trichoverroids: trichoverrin C [**3**], 2′,3′-isotrichoverrin A [**4a**], and 2′,3′-isotrichoverrin B [**4b**].

RESULTS AND DISCUSSION

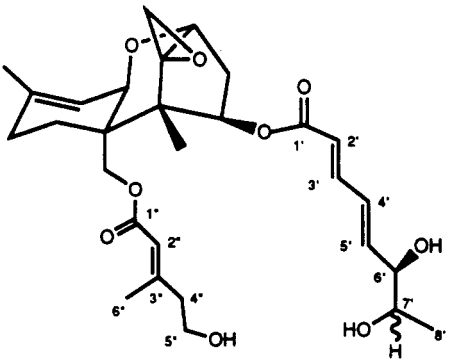
Chromatography of a crude extract of a fermentation of the fungus *M. verrucaria* gave a polar fraction rich in trichoverrins A and B. Further chromatography (mplc) gave



1a R=H, C-7′=(*S*)
1b R=H, C-7′=(*R*)
2a R=C(O)CH=CMeCH$_2$CH$_2$OH, C-7′=(*S*)
2b R=C(O)CH=CMeCH$_2$CH$_2$OH, C-7′=(*R*)



3



4a C-7′ = (*R*)
4b C-7′ = (*S*)

two trichoverrin-containing fractions (I and II) which were subjected to ccc. Ccc of the more polar fraction gave a fraction rich in a new trichoverroid (and well separated from the trichoverrins) which was purified by preparative tlc to give trichoverrin C [**3**]. The molecular formula of **3** is $C_{27}H_{40}O_9$, as shown by hrms, and it formed a triacetate. The H-7′ protons in **3** and in the triacetate of **3** appear as five-line multiplets with $J_{6',7'} = J_{7',8'} = 6$ Hz which establishes the stereochemistry at C-6′, C-7′ as threo, the same as is found in the A-series trichoverroids: trichoverrol A [**1a**], trichoverrin A [**2a**], and 12,13-deoxytrichoverrin A (4,5). However, X-ray crystallographic analysis showed that the trichoverrol A [**1a**] obtained from this isolate, *M. verrucaria* ATCC 20540 is C-6′(*R*)/C-7′(*R*) whereas the trichoverrol A from *M. verrucaria* ATCC 24571 is C-6′(*S*)/C-7′(*S*) as was reported earlier (6). In fact, both isolates produce all four diastereomeric C-6′/C-7′ trichoverroids; isolate 24571 produces mainly the C-6′(*S*) series: C-6′(*S*)/C-7′(*S*) and C-6′(*S*)/C-7′(*R*); whereas, isolate ATCC 20540 produces mainly the C-6′(*R*) series: C-6′(*R*)/C-7′(*S*) and C-6′(*R*)/C-7′(*R*). The trichoverrins described here are the C-6′(*R*) series. The details of the resolution of the stereochemical assignments for this complex system will be presented elsewhere.

The major difference in the ^{1}H-nmr spectrum of **3** compared with that of **2a** is the presence of an AB signal for H-2′ methylene protons at 3.08 ppm ($J = 15.3$ Hz). In addition, the C-6″ vinyl methyl in **3**, which normally resonates in the trichoverrins around δ 2.2, has moved upfield to δ 1.73. There is a vinyl hydrogen (H-4″) coupled to the protons of the methylene associated with the primary alcohol (CH_2OH). This vinyl proton also shows three-bond C-H coupling to the C-6″ vinyl methyl. A series of 2D nmr correlation experiments established the structure of **3**. Trichoverrin C is the first example of a trichothecene that has the double bond in the C-15 sidechain between C-3″ and C-4″ instead of between C-2″ and C-3″. The stereochemistry of this double bond was shown to be *E* by a NOESY experiment in which the H-10 proton correlated with the C-16 proton signal but no correlation was observed between H-4″ and the vinyl methyl protons of C-6″.

An aliquot of the less polar fraction from the mplc chromatography was submitted to ccc in a hexane-$CHCl_3$-MeOH-H_2O (1:1:1:1) system and gave 2′,3′-isotrichoverrins A [**4a**] and B [**4b**], with baseline separation from the known *Z,E* isomers, **2a** and **2b**. 2′,3′-Isotrichoverrin B was the most abundant and the first eluted of the *E,E* isomers and was obtained pure from this chromatography. To purify the minor diastereomer it was necessary to perform a second ccc with hexane-EtOAc-MeOH-H_2O (1:1:1:1). In addition to the 2′,3′-isotrichoverrin A, this gave a small sample of verrol (8).

The isotrichoverrins were indeed isomeric with the trichoverrins by ms and,

TABLE 1. ^{1}H Chemical Shifts of Compounds **2a**, **2b**, **3**, **4a**, and **4b**.

Proton	Compound				
	Trichoverrin A [**2a**]	Trichoverrin B [**2b**]	Trichoverrin C [**3**]	Isotrichoverrin A [**4a**]	Isotrichoverrin B [**4b**]
H-2	3.86	3.87	3.84	3.86	3.86
H_a-3	2.01	2.04	ca 2.0	2.00	2.00
H_b-3	2.58	2.58	2.55	2.57	2.57
H-4	6.21	6.23	5.85	6.04	6.03
H_a-7	1.58	1.58	1.65	1.66	1.66
H_b-7	2.11	2.12	ca 2.0	2.08	2.09
H_a-8	1.97	2.20	ca 2.0	1.98	1.96
H_b-8	2.00	2.00	ca 2.0	2.05	2.03
H-10	5.49	5.50	5.53	5.48	5.48
H-11	3.99	4.01	3.80	3.90	3.91
H_a-13	2.85	2.86	2.83	2.84	2.85
H_b-13	3.17	3.18	3.15	3.15	3.16
H-14	0.82	0.82	0.78	0.83	0.83
H_a-15	4.17	4.12	4.09	4.15	4.15
H_b-15	4.17	4.12	4.17	4.15	4.15
H-16	1.72	1.73	1.70	1.73	1.73
H-2′	5.70	5.70	5.69	5.94	5.93
H-3′	6.61	6.64	6.61	7.27	7.28
H-4′	7.57	7.56	7.57	6.45	6.42
H-5′	6.09	6.26	6.05	6.12	6.16
H-6′	4.05	4.26	4.02	4.00	4.23
H-7′	3.70	3.92	3.67	3.69	3.93
H-8′	1.21	1.16	1.19	1.22	1.16
H-2″	5.87	5.88	3.06, 3.10	5.83	5.83
H_a-4″	2.42	2.42	5.53	2.42	2.42
H_b-4″	2.42	2.42	—	2.42	2.42
H_a-5″	3.85	3.87	4.1	3.80	3.81
H_b-5″	3.79	3.80	4.1	3.84	3.84
H-6″	2.20	2.20	1.73	2.21	2.21

perhaps a little surprisingly, all four compounds had identical uv spectra. Each isotrichoverrin was more levorotatory than the corresponding trichoverrin, and each had an extra band in the olefinic stretch region of the infrared. Whereas trichoverrins A and B have two strong bands at 1600 and 1644 cm^{-1} in $CDCl_3$ solution, the isotrichoverrins have a strong band at 1644 and two medium intensity bands at 1600 and 1618 cm^{-1}. The structures of the isotrichoverrins were deduced principally by nmr spectral analysis. The assigned ^{1}H and ^{13}C chemical shifts are presented in Tables 1 and 2, respectively. These assignments are supported by extensive 1D and 2D correlation experiments. Characteristic of the *Z* to *E* isomerization is the change in H-2′–H-3′ coupling from 11.3 Hz in the normal series to 15.4 Hz in the iso series (Table 3). Although the 3′ and 4′ protons give rise to the lowest field signals in the spectra of these four compounds, the relative positions are reversed in the spectra of the iso series compared to those in the spectra of the trichoverrins (Table 1). Comparison of chemical shifts of 6′, 7′, and 8′ and the somewhat poorly defined $J_{6'-7'}$ coupling serve to define the stereochemistry at 7′ and hence position individual compounds in either the A or B series. The differences are perhaps somewhat less marked in the ^{13}C-nmr spectra (Table 2), but are nonetheless entirely internally consistent with the structural assignments. We believe that these isotrichoverrins are the first known naturally occurring

TABLE 2. ^{13}C Chemical Shifts of Compounds **2a**, **2b**, **3**, **4a**, and **4b**.

Carbon	Compound				
	Trichoverrin A [**2a**]	Trichoverrin B [**2b**]	Trichoverrin C [**3**]	Isotrichoverrin A [**4a**]	Isotrichoverrin B [**4b**]
C-2	79.1	79.1	79.0	79.2	79.2
C-3	36.8	36.9	36.7	36.8	36.8
C-4	75.0	75.0	75.2	75.4	75.4
C-5	48.6	48.6	49.0	48.8	48.9
C-6	42.9	42.9	43.2	43.1	43.1
C-7	21.8	21.9	21.2	21.6	21.6
C-8	27.8	27.8	27.9	28.0	28.0
C-9	140.4	140.4	140.7	140.7	140.7
C-10	118.5	118.5	118.3	118.5	118.5
C-11	66.6	66.6	66.6	66.8	66.8
C-12	65.8	65.8	65.7	65.4	65.5
C-13	48.2	48.2	48.0	48.0	48.0
C-14	6.7	6.7	6.9	6.7	6.7
C-15	63.4	63.5	63.9	63.1	63.1
C-16	23.2	23.2	23.2	23.2	23.2
C-1′	166.0	166.0	166.0	166.1	166.6
C-2′	118.1	118.0	118.4	122.0	121.8
C-3′	143.8	143.9	143.7	143.9	144.0
C-4′	127.1	127.5	127.8	129.7	129.9
C-5′	142.2	141.1	142.2	141.1	140.1
C-6′	76.1	75.3	77.0	77.1	75.4
C-7′	70.6	70.2	70.5	70.6	70.2
C-8′	18.8	17.9	18.9	19.0	17.6
C-1″	165.9	165.9	171.0	165.8	165.9
C-2″	116.9	116.9	44.8	117.1	117.1
C-3″	157.0	157.0	131.8	157.0	157.0
C-4″	43.6	43.5	128.3	43.8	43.8
C-5″	59.6	59.6	59.2	59.8	59.8
C-6″	19.1	19.1	16.8	19.1	19.0

trichothecenes in which the diene of the ester at C-4 is in the *E,E* configuration. Roush and Blizzard (9,10) prepared the *E,E* isomers of the macrocyclic trichothecenes, verrucarin B and verrucarin J, in the course of their syntheses of the natural *Z,E* analogues.

It should be pointed out that not only did high speed ccc allow for the isolation of three new trichoverroids, it also effected a good separation of trichoverrins A and B on a multimilligram scale in a chromatography performed in only a few hours.

TABLE 3. Selected First-Order Proton-Proton Couplings (Hz).

H_x-H_y	Compound				
	Trichoverrin A [**2a**]	Trichoverrin B [**2b**]	Trichoverrin C [**3**]	Isotrichoverrin A [**4a**]	Isotrichoverrin B [**4b**]
2′-3′	11.3	11.3	11.3	15.4	15.4
3′-4′	11.3	11.3	11.3	11.2	11.4
4′-5′	15.6	15.6	15.5	15.2	15.3
5′-6′	5.0	5.3	5.7	6.1	6.2
6′-7′	ca. 6	ca. 4	ca. 6	ca. 6	3.7

EXPERIMENTAL

GENERL EXPERIMENTAL PROCEDURES.—Ir spectra were determined on either a Nicolet 5DXC FT or 60SX FT instrument. Nmr spectra were obtained in $CDCl_3$ on a Bruker AMX-500 or a General Electric GN500 spectrometer using either δ 0.00 signal of TMS or the δ 7.24 signal of $CDCl_3$ as an internal standard. ^{1}H-nmr signals were assigned by homonuclear (^{1}H-^{1}H) COSY 45, heteronuclear (^{1}H-^{13}C) COSY, and long-range heteronuclear (^{1}H-^{13}C) shift correlation (HETCOR) carried out in the inverse detection mode (11). ^{13}C-nmr signals were assigned by the above techniques as well as INEPT or DEPT and by comparison of chemical shift data with those in the literature. Hrms data were collected on a VG 707OE mass spectrometer using dcims probe by eims (70 eV) mode or on a Kratos MS-50 using fabms in the positive ion mode. Tlc was performed on precoated tlc plates of Si gel 60F-254. Visualization was done by viewing the developed plates under short wavelength uv light or by spraying with vanillin spray [40 g/liter vanillin in EtOH-H_2SO_4 (1:4)]. Preparative tlc was achieved on the Model 7942 Chromatotron (Harrison Research Laboratories). The Chromatotron plates (1 mm) were prepared according to the instructions in the manual using E. Merck Si gel. The Ito Multilayered Coil Planet Centrifuge was purchased from P.C. Inc., Potomac, MD. The coil had a volume of approximately 325 ml.

Fermentation procedure with *M. verrucaria* CL-72 (ATCC 20540) was carried out in a manner similar to that described previously (4). A lower R_f fraction (10 g), which was rich in trichoverrins A and B, was subjected to medium pressure cc (Whatman 13–24 μ SiO_2, 2–8% MeOH/CH_2Cl_2) to give two fractions (5.8 g of less polar I and 2.8 g of more polar II) further enriched in the trichoverrins.

ISOLATION OF 2,3-ISOTRICHOVERRINS A [**4a**] AND B [**4b**].—A 470-mg portion of Fraction I was divided in two portions and chromatographed on an Ito Multi-Layered Coil Planet Centrifuge (CPC) [solvent system: hexane-$CHCl_3$-MeOH-H_2O (1:1:1:1), at 800 rpm, flow of 2.5 ml/min, mobile upper aqueous phase, fractions monitored by tlc (8% MeOH/CH_2Cl_2)]. Like fractions from both runs were pooled to give F1 (13.3 mg of pure **4b**), F2 (8.2 mg of a mixture of trichoverrins), F3 [9.2 mg pure **2b** (C-6′*R*)], F4 [14.5 mg pure **2a** (C-6′*R*)], and F5 [21.2 mg of nearly pure **2a** (C-6′*R*)], which was further chromatographed to analytical purity on a CPC as above except that CCl_4 was substituted for hexane. F2 was further chromatographed as above (solvent system hexane/EtOAc/MeOH/H_2O) to give 2 mg of **4a** and 1 mg of verrol (9).

Compound **4a**.—An oil: ir ($CDCl_3$) ν max cm^{-1} 3680, 3610 (OH), 1705 vs (C=O), 1644, 1618, 1600 (C=C); uv λ max nm (ε) 216 (22,000), 258 (21,000); $[\alpha]^{23}D$ −20.6 (c = 0.24, MeOH); fabms *m/z* $[M+Na]^+$ 555, dcims *m/z* $[M+NH_4]^+$ 550.

Compound **4b**.—An oil: ir ($CDCl_3$) ν max cm^{-1} 3620, 3500 br (OH), 1705 vs (C=O), 1644, 1618, 1600 (C=C); uv λ max nm (ε) 217 (23,000), 258 (22,000); $[\alpha]^{23}D$ −38.5 (c = 0.39, MeOH) fabms *m/z* $[M+Na]^+$ 555, dcims *m/z* $[M+NH_4]^+$ 550.

ISOLATION OF TRICHOVERRIN C [**3**].—Fraction II (250 mg) was subjected to ccc on a high speed countercurrent chromatograph PTR model 1000-ccc (Phara-Tech Co., Baltimore, MD) [solvent system: hexane-$CHCl_3$-MeOH-H_2O (1:1:1:1), 260 ml column, 1150 rpm, flow of 3.2 ml/min, mobile upper aqueous phase, monitored at 280 nm, loaded in 2 ml of organic phase] to give five fractions: trichoverrin C, mixture of trichoverrins B and C, trichoverrin B, mixture of trichoverrins A and B, and trichoverrin A. Fraction 1 (80 mg) was further purified by preparative tlc [EtOAc-hexane (4:1)] to give 30 mg of **3**, an oil: ir ($CHCl_3$) ν max cm^{-1} 3500 (OH), 1730, 1705 (C=O); uv λ max (EtOH) nm (ε) 259 (22,000); hreims *m/z* $[M]^+$ 532.2671, calcd 532.2672 for $C_{29}H_{40}O_9$.

Upon acetylation of **3** (5 mg in 0.5 ml each of Ac_2O and pyridine), a triacetate of **3** was obtained: hreims *m/z* $[M]^+$ 658.2933, calcd 658.2989 for $C_{35}H_{46}O_{12}$; the signals in the ^{1}H-nmr spectrum for H-5″, H-6′, and H-7′ shifted to δ 4.60 (2H, d, J = 6.8 Hz, H-5″), 5.45 (1H, m, H-6′) 5.06 (1H, dq, J's = 6.3 Hz), respectively.

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