way as product **6**, by using 17 U 3β-hydroxysteroid dehydrogenase and 20 U formate dehydrogenase. The reaction was complete after 25 h. The turnover number for the coenzyme was 140. The product was recovered as was product **3**, and the yield was 0.49 g (86%). The product was crystallized from ethyl acetate–acetone: mp 270–272 °C; R_f 0.51 (system 2); HPLC t_r 18 min; $[\alpha]_D$ +22°; ^{1}H NMR δ 0.98 (3 H, s, C-18 Me), 1.26 (3 H, s, C-19 Me), 0.74 (3 H, d, J = 6.3 Hz, C-21 Me).

Synthesis of 7α-Hydroxy-3,12-dioxo-5β-cholan-24-oic Acid (8).[37] Sodium dehydrocholate (0.7 g) was reduced in the same way as product **6**, by using 0.1 M glucose instead of 0.1 M formate, 5 U 7α-hydroxysteroid dehydrogenase, and 10 U glucose dehydrogenase. The reaction was complete after 9 h. The turnover number for the coenzyme was 160. The product was recovered in the same way as product **3**, and the yield was 0.61 g (91%). The product was crystallized from ethyl acetate: mp 193–195 °C; R_f 0.48 (system 2); HPLC t_r 36 min; $[\alpha]_D$ +73°; ^{1}H NMR δ 1.00 (3 H, s, C-18 Me), 1.05 (3 H, s, C-19 Me), 0.77 (3 H, d, J = 6.3 Hz, C-21 Me).

Synthesis of 7β-Hydroxy-3,12-dioxo-5β-cholan-24-oic Acid (9). Sodium dehydrocholate (0.6 g) was reduced in the same way as product **8**, by using NADPH instead of NADH, 8 U 7β-hydroxysteroid dehydrogenase, and 15 U glucose dehydrogenase. The reaction was complete after 30 h. The turnover number for the coenzyme was 140. The product was recovered in the same way as product **3**, and the yield was 0.49 g (86%). The product was crystallized from ethyl acetate: mp 187–189°C; R_f 0.47 (system 2); HPLC t_r 14 min; $[\alpha]_D$ +90°; ^{1}H NMR δ 1.01 (3 H, s, C-18 Me), 1.05 (3 H, s, C-19 Me), 0.77 (3 H, d, J = 6.3 Hz, C-21 Me); mass spectrum (positive FAB), m/z (relative intensity) 405 ($(M + H)^+$, 100), 387 (98), 369 (30), 353 (14), 351 (17), 341 (21), 323 (12), 263 (12), 219 (9), 145 (18), 121 (23), 105 (35). Anal. Calcd for $C_{24}H_{36}O_5$: C, 71.26; H, 8.97. Found: C, 70.97; H, 8.78.

Synthesis of 12α-Hydroxy-3,7-dioxo-5β-cholan-24-oic Acid (10).[38] Sodium dehydrocholate (0.6 g) was reduced in the same way as product **9**, by using 12 U 12α-hydroxysteroid dehydrogenase coimmobilized with 8 U glucose dehydrogenase. The reaction was complete after 26 h. The turnover number for the coenzyme was 140. The product was recovered as was product **2**, and the yield was 0.50 g (87%). The product was crystallized from ethyl acetate: mp 168–170 °C (lit.[18] mp 168–169 °C, from ethyl acetate); R_f 0.44 (system 2); HPLC t_r 31 min; $[\alpha]_D$ −9°; ^{1}H NMR δ 0.63 (3 H, s, C-18 Me), 1.22 (3 H, s, C-19 Me), 0.93 (3 H, d, J = 6.3 Hz, C-21 Me).

Acknowledgment. We thank the Consiglio Nazionale delle Ricerche, Rome, Progetto Finalizzato "Chimica Fine e Secondaria", and the Biomolecular Engineering Programme of the Commission of the European Communities for financial support of this work. We also thank Dr. Lucia Zetta (CNR Milano) for obtaining the NMR spectra and Prof. Annamaria Ferrari (Agricultural University, Milano) for providing *C. absonum* cells.

Registry No. **1**, 81-25-4; **2**, 2304-89-4; **3**, 911-40-0; **4**, 2458-08-4; **5**, 81-23-2; **6**, 517-33-9; **7**, 3615-35-8; **8**, 2304-91-8; **9**, 102649-81-0; **10**, 2304-92-9; EC 1.1.1.50, 9028-56-2; EC 1.1.1.159, 39361-64-3; EC 1.1.1.51, 9015-81-0; EC 1.4.1.4, 9029-11-2; EC 1.1.1.47, 9028-53-9; EC 1.1.1.176, 61642-40-8; EC 1.4.1.3, 9029-12-3; EC 1.2.1.2, 9028-85-7.

(36) Compound **7** was previously prepared from cholic acid by a five-step synthesis, which also involved a chromatographic purification of an intermediate, with a yield lower than 30% (lit.[21] and: Chang, F. C. *J. Org. Chem.* **1979**, *44*, 4567. Blickenstaff, R. T.; Atkinson, K.; Breaux, D.; Foster, E.; Kim, Y.; Wolf, G. C. *J. Org. Chem.* **1971**, *36*, 1271).

(37) Compound **8** was previously prepared from cholic acid by a four-step synthesis (lit.[20] and: Nicotra, F.; Ranzi, B. M. *Ann. Microbiol.* **1978**, *28*, 11). The yield was not given.

(38) Compound **10** was previously prepared from cholic acid by a six-step synthesis (lit.[18]). The yield was not given.

Roritoxins, New Macrocyclic Trichothecenes from *Myrothecium roridum*

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Received February 3, 1986

Four new macrocyclic trichothecenes (roritoxins A–D) which contain a tetrahydropyranyl ring fused to a γ-lactone or lactol ring in the macrolide side chain were isolated from a culture of *Myrothecium roridum* ATCC 20605 grown on rice substrate. Roritoxin C is unique in that it is the first reported fungal-produced trichothecene which possesses a 9,10-epoxide group. In liquid media this fungal culture produces a new antibiotic, (+)-(1*R*,6*R*)-5-(*S*)-hydroxy-4-methyl-7-oxabicyclo[4.1.0]hept-3-en-2-one.

During the past few years we have been involved in screening *Myrothecium verrucaria* and *M. roridum* isolates for the production of trichothecenes, which are a family of sesquiterpenes important as mycotoxins[1] and anticancer agents.[2]

The research group at Warner-Lambert has reported on an interesting isolate of *M. roridum*, CL-514 (ATCC 20605), which in a liquid medium produced a novel array of trichoverroids (roridin L-2[3] and trichoverritone[4]) as well as two unusual macrocyclic trichothecenes, 12′-hydroxyisoverrucarin J and isosatratoxin H.[5] We have studied a variety of fermentation conditions for this isolate and now report the isolation of a series of new antibiotics whose production depends strongly on the type of media used to grow this culture.

Results and Discussion

Initially, when *M. roridum* isolate CL-514 was grown in a sucrose/glycerol based liquid medium, conditions we

(1) (a) Bamburg, J. R.; Strong, F. M. In *Microbial Toxins*; Kadis, S., Ciegler, A., Ajl, S. J., Eds.; Academic Press: New York, 1971; Vol. VII p 207. (b) Ueno, Y., Ed. *Trichothecenes–Chemical, Biological, and Toxicological Aspects*; Halsted-Kodansha Scientific Press: New York-Tokyo, 1983. (c) Ueno, Y. *Adv. Nutr. Sci.* **1980**, *3*, 301. (d) Bamburg, J. R. *Prog. Mol. Subcell.* **1983**, *8*, 41. (e) McDougal, P. G.; Schmuff, N. R. *Fortschr. Chem. Org. Naturst.* **1985**, *47*, 153.

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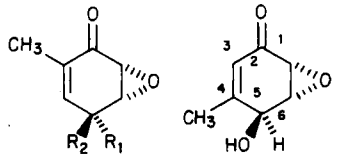
(4) Jarvis, B. B.; Vrudhula, V. M.; Pavanasasivam, G. *Tetrahedron Lett.* **1983**, *24*, 3539.

(5) Smitka, T. A.; Bunge, R. H.; Bloem, R. J.; French, J. C. *J. Antibiotics* **1984**, *37*, 823.

0022-3263/86/1951-2906$01.50/0

have found to be well-suited for trichothecene production by *Myrothecium* species,[6] we observed production of a trichothecene metabolite (ca. 10 mg/L)[7] whose spectral characteristics did not match any of those reported for known trichothecenes. However, numerous attempts to reproduce this result using a variety of conditions failed to produce this new toxin (roritoxin A, vide infra), and we observed production of only small quantities (<10 mg/L) of trichothecenes, principally roridin L-2.

Although these attempts led only to low production of trichothecenes, we did observe significant production of a material which gave an intense royal blue spot on TLC when the plate was sprayed with 4-(*p*-nitrobenzyl)-pyridinium reagent which is indicative of an alkylating agent.[8] A 3-L fermentation of this isolate in a liquid medium gave 750 mg of an oil whose spectral characteristics (IR, UV, MS, and NMR) were similar to those of (+)-epoformin (**1**)[9] and (+)-epiepoformin (**2**).[10]



1, R_1=OH; R_2=H
2, R_1=H; R_2=OH

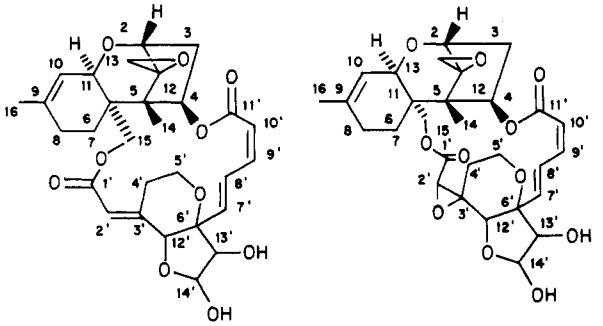
3

An analysis of the ^1H and ^{13}C NMR data established the structure of this compound as (+)-(1*R*,6*R*)-5(*S*)-hydroxy-4-methyl-7-oxabicyclo[4.1.0]hept-3-en-2-one ((+)-isoepiepoformin, **3**), whose absolute stereochemistry was determined by CD spectroscopy. The vinyl methyl protons of **3** appear farther downfield than the vinyl methyl protons of epoformin (**1**) or epiepoformin (**2**), indicating that the methyl group is placed β with respect to the carbonyl group and not α as in **1** and **2**. Decoupling experiments with **3** revealed that the methyl protons are coupled only to the vinyl proton. The proton at C-6 appears as a doublet of doublets due to coupling with protons at C-5 and C-1. The proton at C-1 appears as an eight-line pattern (ddd) due to coupling with protons at C-3, C-5, and C-6. The signal due to the proton at C-5 sharpened when the ^1H NMR spectrum was taken after H–D exchange, indicating that it is coupled to the proton of the OH group on the same carbon. The proton NMR spectrum of epoformin (**1**) shows a doublet for the C-1 proton, indicating that it is not coupled to the proton at C-5, whereas the same signal is a doublet of doublets for epiepoformin (**2**), indicating long-range coupling between H-5 and H-1. Since **3** shows the same type of coupling as in epiepoformin, the hydroxy group and epoxide are anti to each other as is the case for epiepoformin. The stereochemistry of isoepiepoformin (**3**) was established by CD spectroscopy which gave a positive cotton effect at 327 nm, indicating that the oxirane ring possesses the 1*R*,6*R* configuration just as it does in (+)-epiepoformin.[10] Since the hydroxy group at C-5 is anti to the oxirane ring, the stereochemistry at C-5 is *S*.

Earlier, we showed[6] that a New Zealand isolate of *M. roridum* (ATCC 24570) produced (–)-epoformin (major) and (–)-epiepoformin (minor) when grown in a submerged liquid culture of an infusion of brown rice. These are the first reports of *Myrothecium* producing these types of epoxycyclohexenone antibiotics.[11]

Since liquid cultures of *M. roridum* isolate CL-514 gave erratic results with respect to trichothecene production, we turned to fermentation on solid substrates. We have previously examined cultures of *Myrothecium* and *Stachybotrys* on a variety of solid substrates and found that Uncle Ben's parboiled long grain rice gave superior yields of trichothecene toxins over that observed with other white and brown rices, oats, barley, corn, and soy beans.

TLC analysis of a crude extract of *M. roridum* CL-514 grown on rice substrate indicated that under these conditions no epoformin-type compounds were produced; only traces of previously reported trichothecenes (e.g., roridin L-2) were present, but a small amount of roritoxin A (**4**) and three other trichothecenes in larger amounts were produced. Purification of the latter compounds (see Experimental Section) yielded four new macrocyclic trichothecenes: roritoxins A (**4**, 20 mg), B (**5**, 980 mg), C (**6**, 350 mg), and D (**7**, 125 mg).



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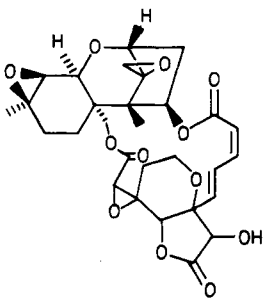

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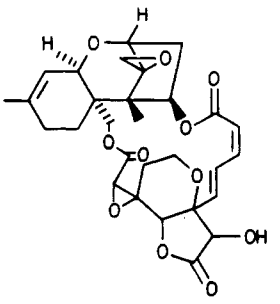

7

High-resolution mass spectra (HRMS) of **4**–**7** made it evident that these compounds were of the roridin/satratoxin class (C29) of macrocyclic trichothecenes.[12-14] However, unlike roridins A, D, and E[12] and satratoxins G and H,[14] these new compounds lack a pendent 1-hydroxyethyl group attached to the macrolide ring as shown by the lack of a methyl doublet at ca. δ 1.2 in the ^1H NMR spectra. Furthermore, the ^1H NMR spectrum of roritoxin A (**4**) is similar to that of vertisporin[15] with the principal difference being that **4** clearly has the 7′,8′,9′,10′-dienic chromophore, whereas in vertisporin this portion of the macrolide ring has only the 9′,10′-double bond. The ^1H and ^{13}C NMR spectra of roritoxins B (**5**), C (**6**), and D (**7**) show that these compounds possess a 2′,3′-epoxide group, a structural feature they share in

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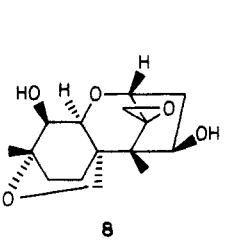
Table I. ^{13}C NMR Data (ppm) for Roritoxins A and B and Roritoxins C and D Acetates

position	roritoxin A	roritoxin B	roritoxin C acetate	roritoxin D acetate
2	79.2	78.4[a]	78.8[a]	79.1[a]
3	34.6	33.9	34.2	34.3
4	74.4	73.9	73.1	73.2
5	49.1	48.7	49.0	49.3
6	43.5	42.8	42.9	43.3
7	20.4	19.2	17.4	20.4
8	27.7	26.8	26.2	27.4
9	140.2	138.8	57.4	140.5
10	119.0	118.9	57.7[b]	118.5
11	68.1	66.6	68.2	67.8
12	65.4	65.3	64.8	65.3
13	48.0	47.3	48.0	48.0
14	7.5	7.7	8.0	7.9
15	65.3	64.2	64.7	65.2
16	23.2	22.8	22.2	22.2
1′	165.5[b]	167.3[b]	166.8[c]	166.7[b]
2′	120.8	56.4	57.2[b]	57.3
3′	151.3	62.0	60.7	60.4
4′	25.7	22.8	22.3	23.3
5′	64.0	60.1	61.4	61.3
6′	81.8	82.2	80.6	80.5
7′	133.4	131.2	132.5	132.5
8′	129.0	129.7	127.3	127.0
9′	141.4	143.3	142.2	142.0
10′	121.9	120.1	122.0	122.1
11′	166.9[b]	166.5[b]	166.6[c]	166.4[b]
12′	81.8[a]	81.9	79.5[a]	79.4[a]
13′	81.0[a]	78.1[a]	74.1	74.0
14′	96.7	100.4	169.7	169.6
$OC(O)CH_3$			20.4	20.0
$OC(O)CH_3$			168.6	168.8

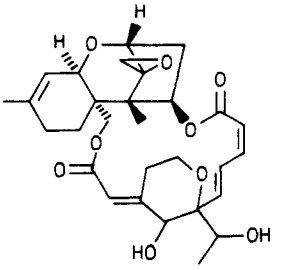
[a-c] Assignments for those signals of close chemical shift values may be interchanged within each column.

common with certain baccharinoids,[16] roridin D,[12] satratoxin G,[14] and the myrotoxins.[17] Roritoxins A, B, and D give verrucarol upon hydrolysis, whereas roritoxin C gives the ring-opened derivative 8, a known compound,[18] upon base hydrolysis. Roritoxins A and B form diacetates, and roritoxins C and D form monoacetates. Roritoxin A shows a strong carbonyl stretch in the IR spectrum at ca. 1710 cm^{-1}, and roritoxin B shows two carbonyl bands at ca. 1710 and 1750 cm^{-1}. Roritoxins C and D have three carbonyl bands which in the IR appear at 1710, 1750, 1810 cm^{-1}; the latter band at 1810 cm^{-1} is characteristic of the carbonyl stretching frequency in a γ-lactone ring system.[19] Upon oxidation with silver carbonate on Celite,[20] roritoxin B (5) was converted to roritoxin D (7), and 7 upon epoxidation with *m*-chloroperoxybenzoic acid gave roritoxin C (6). The above information combined with results obtained from proton decoupling experiments and detailed comparisons of the ^1H and ^{13}C NMR data (Tables I and II) for 4–7 with those data for the related macrocyclic trichothecenes[14,15] established the structures presented above.

The CD curves for satratoxin H (9) and roritoxin A (4) are very similar: for 9, $\Delta\epsilon \times 10^{-5}$ L mol^{-1} cm^{-1} = +6.2 (228 nm), −0.2 (255 nm), and +0.3 (273 nm); for 4, $\Delta\epsilon \times 10^{-5}$ L mol^{-1} cm^{-1} = +6.3 (231 nm), −0.5 (260 nm), and +0.3 (277 nm). Satratoxin G (10), and roritoxins B (5), C (6), and D (7) all show only one positive maximum at ca. 254 nm.



8



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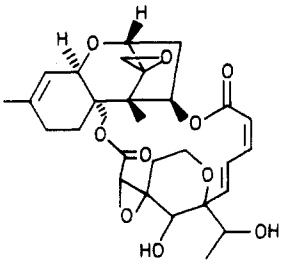
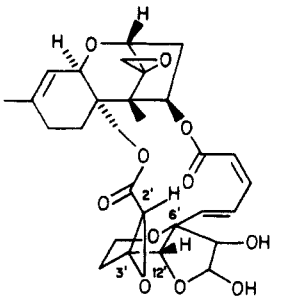


10

The configurations of the satratoxins at C2′, C3′, C6′, C12′, and C13′ and the configurations of the roritoxins at these same centers as well as at C14′ have not been established unequivocally. Recently we reported the myrotoxin class of macrocyclic trichothecenes,[17] and since that report, we have elucidated the structures of additional congeners in this series which contain the tetrahydropyranyl ring as part of the macrolide side chain.[21] The structures of several of these new compounds have been made secure by X-ray diffraction analysis.[21] By a comparison of the ^1H and ^{13}C NMR data for these compounds with the NMR data for satratoxins F, G, and H and roritoxins A–D, we conclude that the absolute stereochemistry at C2′, C3′, C6′, and C12′ in satratoxins F and G and roritoxins B, C, and D are as illustrated below for roritoxin B. The 2′,3′-double bond in satratoxin H and roritoxin A is *E* and the configurations at C6′ and C12′ in these compounds are the same as in roritoxin B.



roritoxin B

The roritoxins are acutely cytotoxic. Against L1210 leukemia cells, the roritoxins exhibit the following ID_{50}'s (μg/mL): 4 (0.0011), 5 (0.0017), 6 (0.0042), and 7 (0.0021). When tested in mice against P388 leukemia (i.p./i.p.), roritoxin B was toxic at 80 μg/kg, which makes this compound the most toxic of the reported trichothecenes.[22] Myrotoxin B[17] also exhibits toxicity in this range with an $LD_{50} \simeq 100$ μg/kg when tested against B16 melanoma (i.p./i.p.) in mice.

M. roridum CL-514 is an extraordinary isolate with respect to the wide variety of trichothecenes that it is able

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Table II. ^{1}H NMR Data (ppm) for Roritoxins A–D[a]

position	roritoxin A (4)[b]	roritoxin B (5)[c]	roritoxin C (6)[d]	roritoxin D (7)[e]
2	3.57 (5.1)	3.84 (4.8)	3.93 (4.8)	3.84 (4.6)
3α	2.46 (8.4, 15.4)	2.45 (8.5, 15.0)	~2.4	2.45 (8.6, 15.2)
4	~5.9	~5.9	~6.1	~5.8
7	~1.9	~1.9	~1.9	~2.0
8	~1.9	~1.9	~1.9	~2.0
10	5.45 (4.8)	5.41 (5.5)	3.06 (5.0)	5.41 (5.0)
11	3.85 (4.8)	3.57 (5.5)	3.56 (5.0)	3.57 (5.0)
13A	3.14 (3.9)	3.14 (3.9)	3.14 (3.8)	3.14 (3.9)
13B	2.83 (3.9)	2.81 (3.9)	2.81 (3.8)	2.81 (3.9)
14	0.80 s	0.81 s	0.82 s	0.82 s
15A	4.50 (12.4)	4.02 (12.0)	4.41 (12.6)	4.32 (12.5)
15B	~3.9	3.94 (12.0)	4.32 (12.6)	4.20 (12.5)
16	1.73 s	1.71 s	1.55 s	1.71 s
2′	~5.9	3.31 s	3.36 s	3.36 s
4′	~2.2	~2.2	~2.4	~2.0
5′	~3.9	~3.9	~3.9	~4.0
7′	6.06 (16.6)	5.87 (16.4)	5.82 (16.0)	5.88 (16.4)
8′	7.34 (10.6, 16.6)	7.01 (8.3, 16.4)	7.10 (8.0, 16.0)	7.10 (8.2, 16.4)
9′	6.60 (10.6, 10.6)	6.67 (8.3, 12.2)	6.68 (8.0, 11.0)	6.67 (8.2, 11.1)
10′	5.98 (10.6)	5.96 (12.2)	5.97 (11.0)	6.01 (11.1)
12′	4.48 s	4.25 s	4.24 s	3.87 s
13′	~5.9	5.22 (5.0)	5.40 s	5.27 s
14′	5.22 (5.0)	5.93 (5.0)		

[a] Chemical shifts are listed as ppm downfield from tetramethylsilane used as an internal standard. Coupling constants, in Hz, are included in parentheses. [b] For diacetate: acetate methyls 2.03 and 2.14; H-13′ 5.37 (5.0); H-14′ 6.28 (5.0). [c] For diacetate: acetate methyls 2.05 and 2.16; H-13′ 5.39 (5.0); H-14′ 6.34 (5.0). [d] For acetate: acetate methyl 2.25; H-4 5.86 (5.2, 8.0); H-12′ 3.92 (s); H-13′ 5.60 (s). [e] For acetate: acetate methyl 2.25; H-4 5.90 (5.3, 8.1); H-12′ 3.94 (s); H-13′ 5.60 (s).

to produce. Under suitable conditions, it produces not only the usual array of roridins, verrucarins, and trichoverroids[23] but also produces satratoxins and roritoxins, compound types previously isolated from different fungi, e.g., *Stachybotrys*[14] and *Verticimonosporium*,[15] respectively. Furthermore, it should be noted that roritoxin C (6) is unique in that is is the only fungal trichothecene metabolite reported in which the 9,10-double bond in the A ring is epoxidized. Previously, this substitution pattern had been observed only in the plant-derived baccharinoids[16,24] whose high in vivo antileukemic activity (cf. that of non-A-ring oxygenated roridins and verrucarins) could be traced, in part, to the presence of a 9β,10β-epoxide group.[25]

Experimental Section

General Methods. Infrared (IR) spectra were determined in chloroform on a Perkin-Elmer Model 183 spectrophotometer. Ultraviolet (UV) spectra were determined on a Perkin-Elmer Model 552 spectrophotometer. Optical rotations were determined on a Perkin Elmer Model 241 automatic polarimeter. Circular dichroism (CD) spectra were determined in methanol on a JASCO J-500C spectropolarimeter. Nuclear magnetic resonance (NMR) spectra were determined in deuteriochloroform on an IBM SY-200 MHz or a Bruker AM-400 MHz spectrometer. The ^{13}C NMR signals were assigned by using INEPT[26] and by comparison of chemical shifts reported in the literature. Mass spectra were determined on a VG 7070 EQ mass spectrometer in the negative ion mode with ammonia as the reagent gas and calibrated against PFK. Melting points were determined on a Fisher-Johns hot stage melting point apparatus and are uncorrected.

Thin layer chromatography was performed on glass plates precoated with E. Merck silica gel 60-F-254 (layer thickness 0.2 mm). Visualization usually involved viewing developed plates under short wavelength UV light (Mineralight UVS-12) and later by spraying with 4-(*p*-nitrobenzyl)pyridine reagent (NBP).[8] Flash chromatography was done under standard conditions.[27] The chromatotron (Harrison Research Laboratories) used for preparative TLC was Model 7924. The plates were either 1, 2, or 4 mm thickness depending on the amount of sample being loaded and were prepared by using E. Merck silica gel on glass circular disks according to the instructions given in the chromatotron manual.

Isolation of Roritoxins from *M. roridum* CL-514 (ATCC 20605) Grown on Uncle Ben's Converted Rice. Ten 1-L Erlenmeyer flasks each containing 200 g of a sterile rice medium prepared according to the method of Lee and Mirocha[28] were inoculated with pieces of Sabouraud-dextrose agar on which the CL-514 isolate was grown. The flasks were kept on a lab bench at room temperature and hand shaken daily until it was apparent that all rice grains were covered with the fungus. At the end of 3 weeks the rice culture was extracted with methanol (200 mL/flask, 5 times each) in a sonicator. The methanol extracts were combined, washed with hexane (2 × 5 L), and concentrated in vacuo to give ca. 3 L of an aqueous solution which was extracted with ethyl acetate (5 × 3 L). The ethyl acetate was dried (Na_2SO_4), and solvent was removed by rotary evaporation to give 30 g of brown gum. The crude extract was subjected to filtration chromatography (100 g of flash grade silica gel) with methanol in dichloromethane as the eluting solvent. Fractions obtained with 1–5% methanol in dichloromethane were combined to give 15 g of yellow material. This fraction was subjected to flash chromatography with a step gradient of isopropyl alcohol in dichloromethane as eluting solvent to obtain five fractions. Fractions 2, 3, and 4 obtained with 3–10% isopropyl alcohol were shown to contain trichothecenes by TLC analysis. These were subjected to further purification.

Purification of Fraction 2. Fraction 2 (1.2 g) was triturated with 100 mL of dichloromethane and filtered. The white solid precipitate was recrystallized from methanol to give 100 mg of roritoxin D (7). The mother liquor from the recrystallization and the filtrate from the previous step were combined and purified on the chromatotron (4 mm silica plate, eluting solvent 0–3% methanol in dichloromethane). An additional 25 mg of roritoxin D was obtained, and fractions which were collected after the pure roritoxin D band were added to fraction 3 for further purification.

Purification of Fraction 3. Fraction 3 (2.5 g) was triturated with 200 mL of dichloromethane and filtered. The dichloromethane insoluble white solid obtained was recrystallized from methanol to yield 300 mg of crystals which were found to be a mixture of roritoxins C (16) and D (7). The filtrate of the dichloromethane solution and the mother liquor of the recrystallization were combined and purified on the chromatotron, (4 mm silica plate, 0–5% methanol in dichloromethane). The roritoxin C containing fraction (70 mg) was recrystallized from ethyl acetate to yield 50 mg of pure roritoxin C (6). The fractions which, by TLC analysis, contained trichothecenes more polar than roritoxin C were combined with fraction 4.

Purification of Fraction 4. Fraction 4 (2.8 g) was crystallized from 95% ethanol to yield 750 mg of roritoxin B (5). The mother liquor was purified on the chromatotron (4 mm silica plate; 0–7% methanol in dichloromethane as eluting solvent). The roritoxin B containing fraction (260 mg) was recrystallized from ethyl acetate to give 230 mg of roritoxin B (5). From a more polar fraction of this chromatography, 20 mg of roritoxin A (4) was isolated.

Roritoxin A (4): mp 220–225 °C; $[\alpha]^{25}_D$ +16° (*c* 0.51, $CHCl_3$); UV λ_{max} (MeOH) 231 nm (log ε 4.35), 260 nm (log ε 4.7); MS, *m/e* 542.2145 (M^- calcd 542.2151). See Tables I and II for NMR data.

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Roritoxin B (5): mp 262–265 °C dec; $[\alpha]^{25}_D$ +2.6° (*c* 0.60, $CHCl_3$); UV λ_{max} (MeOH) 254 nm (log ϵ = 4.99); MS, *m/e* 558.2106 (M^- calcd 558.2139). See Tables I and II for NMR data.

Roritoxin C (6): mp 288–290 °C dec; $[\alpha]^{25}_D$ +8.9° (*c* 0.40, $CHCl_3$); UV λ_{max} (MeOH) 254 nm (log ϵ = 4.97); MS, *m/e* 572.1955 (M^- calcd 572.1974). See Table II for NMR data.

Roritoxin D (7): mp 294–297 °C dec; $[\alpha]^{25}_D$ +30.0° (*c* 0.10, $CHCl_3$); UV λ_{max} (MeOH) 253 nm (log ϵ = 4.98); MS, *m/e* 556.1940 (M^- calcd 556.1980). See Table II for NMR data.

Acetylation of Roritoxins A and B. Roritoxin B (0.050 g) was dissolved in dichloromethane (2.5 mL). Triethylamine (1.0 mL) was added to this solution dropwise, followed by acetic anhydride (0.90 mL). A small crystal of 4-(dimethylamino)-pyridine (DMAP) was added to the mixture. At the end of 2 h the reaction mixture was poured into water and extracted with CH_2Cl_2. The organic layer was washed with 5% HCl, dried (Na_2SO_4), and concentrated in vacuo. The residue was purified on the chromatotron (1-mm silica gel plate, eluting solvent 10–30% ethyl acetate in hexane) to obtain 20 mg of roritoxin B diacetate and 18 mg of roritoxin B monoacetate. In the same manner, roritoxin A (0.010 g) gave 8 mg of roritoxin A diacetate (see Table II for ^1H NMR data).

Acetylation of Roritoxins C and D. A 100-mg portion of the 300-mg crystalline mixture of roritoxins C and D was subjected to acetylation in the same manner as described for roritoxin B. At the end of 1 h the reaction was stopped, and the reaction mixture was purified on the chromatotron (1-mm silica gel plate, 5–15% ethyl acetate in hexane as eluting solvent) to obtain 55 mg of roritoxin D acetate and 25 mg of roritoxin C acetate (see Tables I and II for NMR data).

Conversion of Roritoxin B (5) to Roritoxin D (7). A suspension of silver carbonate–Celite (70 mg) (Alfa) in 30 mL of a toluene solution of roritoxin B (30 mg) was heated under reflux, with stirring, for 2 h. The reaction mixture was filtered and the insoluble material was washed with warm CH_2Cl_2. The filtrate and wash were combined and concentrated in vacuo, and the residue was purified on the chromatotron (1-mm silica gel plate, 0–5% methanol in dichloromethane as eluting solvent). The major product obtained was roritoxin D (7) (22 mg) as shown by proton NMR and IR spectroscopy.

Conversion of Roritoxin D (7) to Roritoxin C (6). To a solution of 10 mg of **6** in 5 mL of chloroform was added 5 mg of *m*-chloroperoxybenzoic acid. After 30 h, the mixture was diluted to 10 mL with dichloromethane, washed once with 10 mL of saturated sodium bicarbonate, dried (Na_2SO_4), and concentrated in vacuo. Preparative TLC on the chromatotron (1-mm silica gel plate, 0–50% ethyl acetate in dichloromethane as eluent) yielded 5 mg of **6**.

Isolation of Isoepiepoformin (3) from *M. roridum* CL-514 (ATCC 20605). Spores of CL-514 grown on Sabouraud-dextrose agar were transferred to 300 mL of a sterile medium which contained 20.0 g of glucose, 2.0 g each of malt extract, yeast extract, and peptone, 1.0 g each of KH_2PO_4 and NH_4Cl, and 0.5 g of $MgSO_4 \cdot 7H_2O$ per 1 L of distilled water. The 2-L Erlenmeyer flask containing 300 mL of inoculated medium was placed on a gyratory shaker (150 rpm) and incubated at 28 °C for 60 h. At the end of this time, the inoculum was transferred into 3 L of another sterile medium (3 × 1 L per 4-L Erlenmeyer flask) which contained 40.0 g of sucrose, 10.0 g of glycerol, 5.0 g of NaCl, 3.0 g of K_2HPO_4, 1.0 g of $NH_4H_2PO_4$, and 0.2 g of $MgSO_4 \cdot 7H_2O$ per 1 L of distilled water. The culture was again incubated at 28 °C on a gyratory shaker (150 rpm). At the end of 4 days, the mycelium was removed by filtration through cheese cloth. The filtrate was extracted with 3 × 3 L of ethyl acetate, and the mycelium was extracted with 3 × 1 L of methanol in a sonicator. The methanol extracts were combined and washed with hexane and concentrated and the aqueous solution was extracted with ethyl acetate (3 × 1 L). The ethyl acetate extracts were all combined and concentrated in vacuo to give 5 g of a red oil. The crude extract obtained was subjected to filtration chromatography (25 g of flash grade silica gel) with dichloromethane (1 L), 5% methanol in dichloromethane (2 L), and methanol (500 mL) as eluents. The fraction eluted with 5% methanol in dichloromethane upon TLC analysis gave a spot which turned deep blue in color when sprayed with NBP reagent.[8] This fraction (2.4 g) which was in the form of a red oil was loaded onto a 4-mm thick silica gel plate in two portions and subjected to purification on the chromatotron. At the start, dichloromethane was used as eluting solvent. This was followed by 1%, 3%, and 5% methanol in dichloromethane and 100% methanol. Upon TLC analysis, the 1–3% methanol in dichloromethane fractions were found to contain the epoformin-type compound. This pale red oily fraction (≃1 g) was further purified on the chromatotron (4-mm silica gel plate, eluting solvent 1–3% methanol in dichloromethane) to afford 750 mg of isoepiepoformin (**3**) as a pale yellow oil: $[\alpha]^{25}_D$ +36.4° (*c* 0.50 chloroform); UV λ_{max} (MeOH) 230 nm (log ϵ = 3.94); CD [θ] +3240 (*c* 0.10, methanol); MS, *m/e* 140.0459 (M^- calcd 140.0472); ^1H NMR δ 2.03 (3 H, d, J = 1.3 Hz, methyl), 3.38 (1 H, ddd, J = 0.7, 3.0, and 3.3 Hz, H-1), 3.75 (1 H, dd, J = 1.3 and 3.3 Hz, H-6), 4.43 (after H–D exchange) (1 H, dd, J = 0.7 and 1.3 Hz, H-5), and 5.75 (1 H, m, H-3); ^{13}C NMR δ 21.4 (Me), 52.2 (C-1), 56.9 (C-6), 66.6 (C-5), 124.1 (C-3), 156.0 (C-4), and 193.3 (C-2).

Acknowledgment. We thank Dr. James C. French for providing us with isolate CL-514 and C. D. Pinter of Warner-Lambert Co., Ann Arbor, MI, for the cytotoxicity data. In vivo toxicity in mice was provided by the National Institutes of Health (P388) and Dr. T. C. Doyle of Bristol Laboratories, Syracuse, NY (B16). We thank Dr. Stephen Missler, USAMRIID, Ft. Detrick, Frederick, MD, for the HRMS data. This work was supported by the National Institutes of Health (Grant No. CA 25967). NSF Grant DMB-84-20175 provided funds for the purchase of the Bruker AM-400 NMR spectrometer.

Registry No. **3**, 102852-47-1; **4**, 102852-48-2; **5**, 102852-49-3; **6**, 102852-50-6; **7**, 102852-51-7.

β-Methyleneglutamic Acid and β-Methyleneglutamine

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Received December 30, 1985

The novel β,γ-unsaturated amino acids β-methyleneglutamic acid (I) and β-methyleneglutamine (II) are readily prepared by addition of the protected aminomalonates III and VIII to the allenes ethyl buta-2,3-dienoate (IV) and cyanoallene (VII) followed by acid hydrolysis. Byproducts of the reaction are 4-amino-3-methylbut-2-enoic acid hydrochloride (VI) and 4-amino-3-methylbut-2-enamide hydrochloride (X).

β,γ-Unsaturated amino acids[1,2] constitute a useful class of enzyme inhibitors. In this paper, we describe highly efficient syntheses of two new candidate inhibitors: β-methylene-D,L-glutamic acid (I) and β-methylene-D,L-

0022-3263/86/1951-2910$01.50/0