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Condensation of Macrocyclic Polyketides Produced by *Penicillium* sp. DRF2 with Mercaptopyruvate Represents a New Fungal Detoxification Pathway

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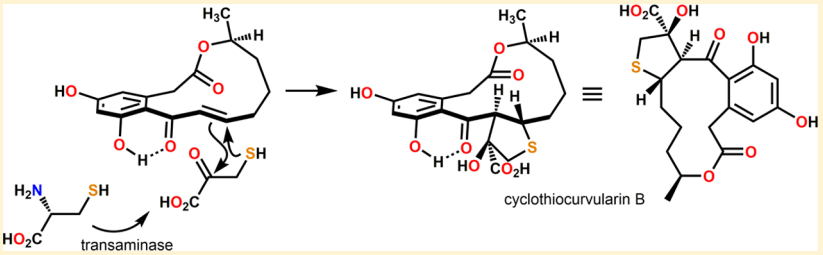
Supporting Information



ABSTRACT: Application of a refined procedure of experimental design and chemometric analysis to improve the production of curvularin-related polyketides by a marine-derived *Penicillium* sp. DRF2 resulted in the isolation and identification of cyclothiocurvularins **6**−**8** and cyclosulfoxicurvularins **10** and **11**, novel curvularins condensed with a mercaptolactate residue. Two additional new curvularins, **3** and **4**, are also reported. The structures of the sulfur-bearing curvularins were unambiguously established by analysis of spectroscopic data and by X-ray diffraction analysis. Analysis of stable isotope feeding experiments with [U−$^{13}C_3$ ^{15}N]-L-cysteine confirmed the presence of the 2-hydroxy-3-mercaptopropanoic acid residue in **6**−**8** and the oxidized sulfoxide in **10** and **11**. Cyclothiocurvularins A (**6**) and B (**7**) are formed by spontaneous reaction between 10,11-dehydrocurvularin (**2**) and mercaptopyruvate (**12**) obtained by transamination of cysteine. High ratios of [U−$^{13}C_3$ ^{15}N]-L-cysteine incorporation into cyclothiocurvularin B (**7**), the isolation of two diastereomers of cyclothiocurvularins, the lack of cytotoxicity of cyclothiocurvularin B (**7**) and its methyl ester (**8**), and the spontaneous formation of cyclothiocurvularins from 10,11-dehydrocurvularin and mercaptopyruvate provide evidence that the formation of cyclothiocurvularins may well correspond to a 10,11-dehydrocurvularin detoxification process by *Penicillium* sp. DRF2.

Macrocyclic polyketides constitute a remarkable group of secondary metabolites presenting complex structural features, potent biological activities, and essential adaptative functions for the corresponding producing organisms.[1] Fungi are notable producers of structurally diverse polyketides with a variety of structural motifs,[2−4] including 10- to 18-membered macrolides known as resorcinylic acid lactones (RALs) and dihydroxyphenylacetic acid lactones (DALs).[5] The 12-membered macrocyclic dihydroxyphenylacetic acid lactone curvularin (**1**) was first isolated from growth media of *Curvularia* fungi.[6] Curvularin (**1**) induces the formation of disordered micro-

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tubule-organizing centers in centrosomes and of barrel-shaped spindles in nuclei of sea urchin eggs.[7] Several curvularin derivatives have been isolated from fungal strains belonging to the genera *Alternaria*,[8] *Aspergillus*,[9] *Brickellia*,[10] *Cochliobolus*,[11] *Corynespora*,[12] *Curvularia*,[13] *Eupenicillium*,[14] and *Penicillium*.[15] Curvularins exhibit an array of biological activities,[16] including phytotoxic,[8a] cytotoxic,[7,9,13b,15i] nematicidal,[9] antibacterial,[13a] kinase inhibitory,[12] antifungal and antialgal,[13c] antitrypanosomal, and anti-inflammatory activities[15i] as well as inhibiting nitric-oxide synthase expression.[17] Recent investigations on the biosynthesis of curvularin-related metabolites highlighted the details of the biochemical machinery involved in the formation of this unique class of macrocyclic polyketides.[18]

Our current investigation of secondary metabolites from marine-derived fungi[19−21] led to the isolation and selection of a *Penicillium* sp. DRF2 strain obtained from the sponge *Dragmacidon reticulatum*.[22] *Penicillium* DRF2 produced a cytotoxic extract from which the known 10,11-dehydrocurvularin (**2**) and the new 12-keto-10,11-dehydrocurvularin (**3**) were isolated as the major metabolites when the fungus was grown under standard conditions. Improved production of minor metabolites detected by HPLC-UV-MS was accomplished by applying a refined version of our previously described experimental design and chemometrics analysis procedure.[19,21] We have been able to promote the fungus production, as well as to isolate and identify the new curvularin **4**, as well as the novel sulfur-bearing curvularin derivatives **6**−**8**, **10**, and **11**, which, along with **3** and **4**, are the subject of the present report.

RESULTS AND DISCUSSION

The extract obtained from the culture medium of *Penicillium* DRF2 displayed cytotoxic activity and HPLC peaks with UV absorptions at λ_{max} 222−230, 270−280, and 300−310 nm and $[M + H]^+$ ions at m/z 300−320, along with minor compounds with m/z > 400. A larger growth of the fungus in 3% malt medium under standard conditions (see SI), followed by purification of the medium extract by a series of chromatographic separations, led to the isolation of 10,11-dehydrocurvularin (**2**) and 12-keto-10,11-dehydrocurvularin (**3**). Application of a refined procedure of our previously described experimental design and chemometric analysis[19,21] (see SI), followed by solid-phase extraction of the culture medium, Sephadex LH20 column chromatography, and reversed-phase HPLC purifications, enabled us to establish growth conditions, optimize the production, and isolate minor curvularin derivatives. These include the new curvularin **4**, along with the tetrahydrothiophene-bearing metabolites **6**−**8**, **10**, and **11**.

The (+)-HRESIMS of 12-keto-10,11-dehydrocurvularin (**3**) gave a $[M + H]^+$ quasi-molecular ion at m/z 305.1050, which corresponded to the molecular formula $C_{16}H_{16}O_6$. This formula required nine degrees of unsaturation, one more than that for 10,11-dehydrocurvularin (**2**), with one additional oxygen and two hydrogens less than **2**. The NMR data of **3** were very similar to those of **2**, the only difference being that the resonances assigned to the CH_2-12 methylene (δ_H 2.38 (m)/δ_C 33.0) in **2**[13b] had been replaced by an additional α,β-unsaturated ketone resonating at δ_C 201.5 (C-12). The assignment of a ketone at C-12 was confirmed by the observation of HMBC correlations between both CH_2-13 (δ_H 2.61/δ_C 37.0) and CH_2-14 (δ_H 1.81 and 2.10/δ_C 32.0) and C-12 (δ 201.5) and between the olefinic protons assigned to the CH-10/CH-11 *E* double bond (J = 16.4 Hz) (δ_H 7.44/δ_C 139.6, δ_H 6.51/δ_C 135.0) and C-12 (δ 201.5). Since the specific rotation of **2** isolated in the current investigation ($[\alpha]^{25}_D$ −8.2 (c 10.0, MeOH)) compared favorably to that reported in the literature,[13] the absolute configuration at C-15 is assumed to be the same as that previously published. Similarly for **3**, with a specific rotation recorded as $[\alpha]^{25}_D$ −7.6 (c 0.05, MeOH), the absolute configuration at C-15 is also assumed to be *S*. Although C-12 oxygenation is unexpected since curvularins are regular polyketides,[23] 12-oxocurvularin,[24] curvulone A,[13c] and 8-β-hydroxy-7-oxocurvularin[25] also possess oxygenation at this position.

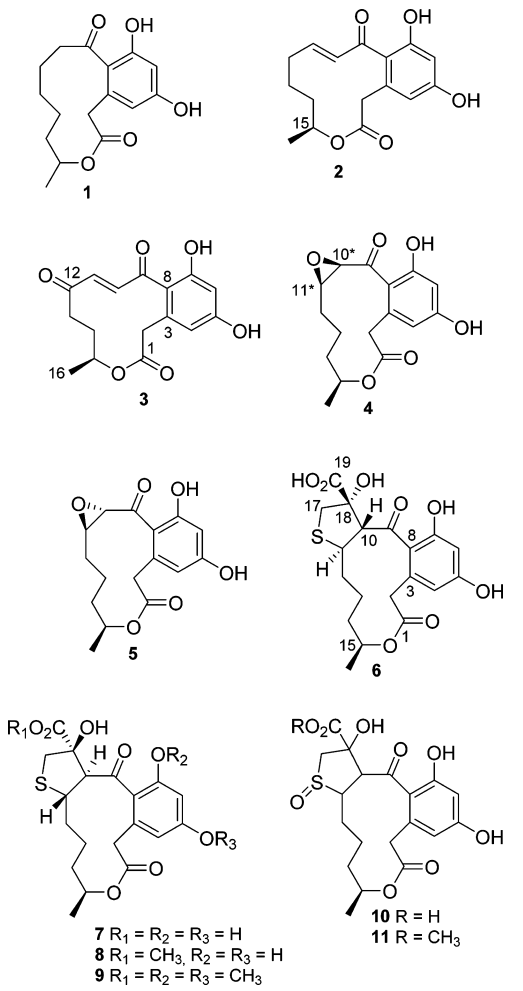

Figure 1. Structures of curvularin (**1**), 15(*S*)-10,11-dehydrocurvularin (**2**), 15(*S*)-12-keto-10,11-dehydrocurvularin (**3**), 15(*S*)-*cis*-10,11-epoxycurvularin (**4**), *trans*-10,11-epoxycurvularin (**5**), 10(*R*),11(*R*),15(*S*),18(*S*)-cyclothiocurvularin A (**6**), 10(*S*),11(*S*),15(*S*),18(*R*)-cyclothiocurvularin B (**7**), 10(*S*),11(*S*),15(*S*),18(*R*)-cyclothiocurvularin B methyl ester (**8**), 10(*S*),11(*S*),15(*S*),18(*R*)-dimethoxymethylcyclothiocurvularin B methyl ester (**9**), 15(*S*),18(*S*)-cyclosulfoxicurvularin (**10**), and 15(*S*),18(*S*)-cyclosulfoxicurvularin methyl ester (**11**).

HRESIMS analysis of *cis*-10,11-epoxycurvularin (**4**) gave a $[M + H]^+$ ion at m/z 307.11883 and a $[M + Na]^+$ ion at m/z 329.10063, appropriate for the molecular formula $C_{16}H_{18}O_6$ requiring eight degrees of unsaturation, the same as 10,11-dehydrocurvularin (**2**) with the addition of H_2O. Although analysis of the ^1H, ^{13}C, HSQC, COSY, and HMBC spectra indicated a close similarity between **4** and **3** (Table 1), the ^1H and ^{13}C resonances of the C-10/C-11 double bond were no longer observed. Differently from **2** and **3**, compound **4**

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Table 1. NMR Data (^{1}H 400 MHz, ^{13}C 100 MHz, MeOH-d_4) for Curvularins 3 and 4[a]

	12-keto-10,11-dehydrocurvularin (3)		*cis*-10,11-epoxycurvularin (4)	
position	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
1	170.7, C		173.2, C	
2	41.5, CH_2	3.71 (d, 17.2); 3.68 (d, 17.2)	39.4, CH_2	3.57 (d, 18.2); 3.08 (d, 18.4)
3	138.5, C		135.8, C	
4	113.5, CH	6.36 (d, 2.4)	111.2, CH	6.32 (d, 1.9)
5	163.5, C		162.5, C	
6	101.9, CH	6.26 (d, 2.6)	102.9, CH	6.19 (d, 2.1)
7	164.9, C		159.7, C	
8	113.9, C		120.2, C	
9	197.2, C		204.8, C	
10	139.6, CH	7.44 (d, 16.4)	62.0, CH	3.83 (d, 2.2)
11	135.0, CH	6.51 (d, 16.4)	63.8, CH	3.13 (ddd, 2.2, 3.0, 10.2)
12	201.5, C		33.7, CH_2	1.92 (m); 1.02 (m)
13	37.0, CH_2	2.61 (dd, 5.7 and 6.6)	22.2, CH_2	1.77 (m); 1.02 (m)
14	32.0, CH_2	1.81 (m); 2.10 (m)	35.1, CH_2	1.72 (m); 1.44 (m)
15	71.1, CH	4.85 (m)	75.4, CH	4.54 (m)
16	19.4, CH_3	1.17 (d, 6.8)	20.7, CH_3	1.11 (d, 6.2)

[a]The signal of the 7-OH group (δ 12.1, s) was observed in DMSO-d_6.

presented two oxymethine signals at δ_H 3.83/δ_C 62.0 (CH-10) and δ_H 3.13/δ_C 63.8 (CH-11), assigned to an epoxide group at C-10 and C-11. The small coupling constant (2.2 Hz) observed between H-10 and H-11,[15i,26] as well as the molecular formula, provided further support for this assignment. A ^{1}H NOEDiff experiment irradiating at δ 3.83 (H-10) showed a clear NOE on H-11 at δ 3.13 (Figure S11, SI), indicating a *cis* relative stereochemistry at C-10/C-11. Compound **4** corresponds to the *cis*- stereoisomer of the *trans*-stereoisomer **5**, which has been previously named 10,11-epoxycurvularin.[15i] *trans*-10,11-Epoxycurvularin (**5**) was erroneously drawn as **4**.[15i] The structure of **5** is herein correctly drawn as the *trans*-stereoisomer of 10,11-epoxycurvularin. The NMR data reported for *trans*-10,11-epoxycurvularin (**5**)[15i] are clearly different from the data we obtained for **4**. The stereochemistry at C-15 was suggested as 15(*S*) based on the configuration of **2** and **3**, produced in the same pool of polyketides by *Penicillium* sp. DRF2, but the relative configuration between the epoxide group and C-15 could not be established. Compound **4** was named 15(*S*)-*cis*-10,11-epoxycurvularin.

HRESIMS analysis of cyclothiocurvularin A (**6**) gave a $[M - H]^-$ ion at m/z 409.0948, appropriate for the molecular formula $C_{19}H_{22}O_8S$, requiring nine sites of unsaturation and differing from the molecular formula of **2** by the addition of $C_3H_4O_3S$. Analysis of the ^{1}H, ^{13}C, HSQC, COSY, and HMBC spectrum of **6** in MeOH-d_4 indicated close similarity to compound **2**. The resonances assigned to the C-10/C-11 olefin seen in **2** were also not observed in **6**. Instead, C-10 and C-11 were assigned to saturated methines at δ_H 4.50 (d, 8.5 Hz)/δ_C 64.5 and δ_H 3.68 (bt, 8.5 Hz)/δ_C 49.8, respectively. Methines CH-10 and CH-11 coupled to one another in the COSY experiment. H-11 also vicinally correlated to the methylene CH_2-12 at δ_H 1.96 and 1.50/δ_C 35.8. The additional $C_3H_4O_3S$ fragment was assigned to a 2-hydroxy-3-mercaptopropanoic acid moiety connected to CH-10 and CH-11. In the HMBC spectrum H-10 correlated to C-9 (δ_C 208.3), to the quaternary C-18 (δ_C 87.6), and to the C-19 carboxylic carbon (δ_C 176.3). H-11 also correlated with C-9 along with C-10, C-12, and C-13, while H-17a/H17b correlated with C-10, C-11, and C-18 and weakly with C-19. The aforementioned HMBC correlations confirmed the presence of a 3-hydroxytetrahydrothiophene-3-carboxylic acid structural moiety in **6** (Figure 2). The *trans*

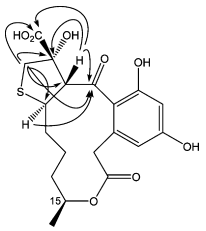

Figure 2. Key HMBC correlations observed for cyclothiocurvularin A (**6**).

cyclic ring junction between the tetrahydrothiophene ring and the macrocyclic ring was suggested by a vicinal coupling of 8.5 Hz between H-10 (δ_H 4.50) and H-11 (δ_H 3.68), indicating an *anti*-relationship. Crystallization of **6** from MeOH gave colorless block crystals that were suitable for single-crystal X-ray diffraction analysis. The absolute configuration of cyclothiocurvularin A (**6**) was thus established as 10(*R*), 11(*R*), 15(*S*), 18(*S*) on the basis of the refined Flack parameter, 0.05(5) (Figure 3).

The (+)-HRESIMS of cyclothiocurvularin B (**7**) gave a $[M + H]^+$ ion at m/z 411.11299 and a $[M + Na]^+$ ion at m/z 433.09405, appropriate for the same molecular formula as that of **6** ($C_{19}H_{22}O_8S$). The NMR data of **7** (Table 2) were very similar to those of **6**. Only minor differences were observed in the ^{1}H and ^{13}C NMR chemical shifts assigned to **6** and **7**, suggesting that the two compounds were diastereomers (Table 2). Treatment of **7** with diazomethane generated colorless plate crystals of the dimethoxymethyl ester of **7** (**9**) that were suitable for single-crystal X-ray diffraction analysis. The result of the analysis points to an absolute configuration for **7** of 10(*S*), 11(*S*), 15(*S*), 18(*R*) (Figure 3), which as suspected is diastereomeric with **6**.

Cyclothiocurvularin B methyl ester (**8**) gave a $[M + H]^+$ ion at m/z 425.12865 and a $[M + Na]^+$ ion at m/z 447.10943 (calcd 447.10841) that were appropriate for the molecular formula $C_{20}H_{24}O_8S$, which differs from that of **6** and **7** by the addition of CH_2. Other than the presence of a methyl ester at δ_H 3.62/δ_C 53.3 (CH_3-20), the NMR data obtained for **8** (Table 3) were strikingly similar to those of **7** (Table 2). Therefore, the structure of **8** was assigned as the methyl ester of **7**.

Sulfoxides **10** and **11** were also isolated from the growth medium of *Penicillium* sp. DRF2 obtained under the same improved conditions. The HRESIMS of compound **10** gave a $[M + H]^+$ ion at m/z 427.1049 and a $[M + Na]^+$ ion at m/z 449.0855, appropriate for the molecular formula $C_{19}H_{22}O_9S$, which differs from that of **6** and **7** by the addition of an oxygen atom. The only significant differences observed in the ^{13}C spectrum of **9** when compared with **6**–**8** was that in **10** C-10 (δ_C 58.4) was more shielded than in **6**–**8** (64.0 < δ_C < 64.9), and C-11 in **10** was deshielded (δ_C 59.8) compared to **6**–**8** (49.8 < δ_C < 50.4), as was the C-17 methylene resonance in **10** (δ_C 65.5) relative to **6**–**8** (40.9 < δ_C < 41.2). Such changes,

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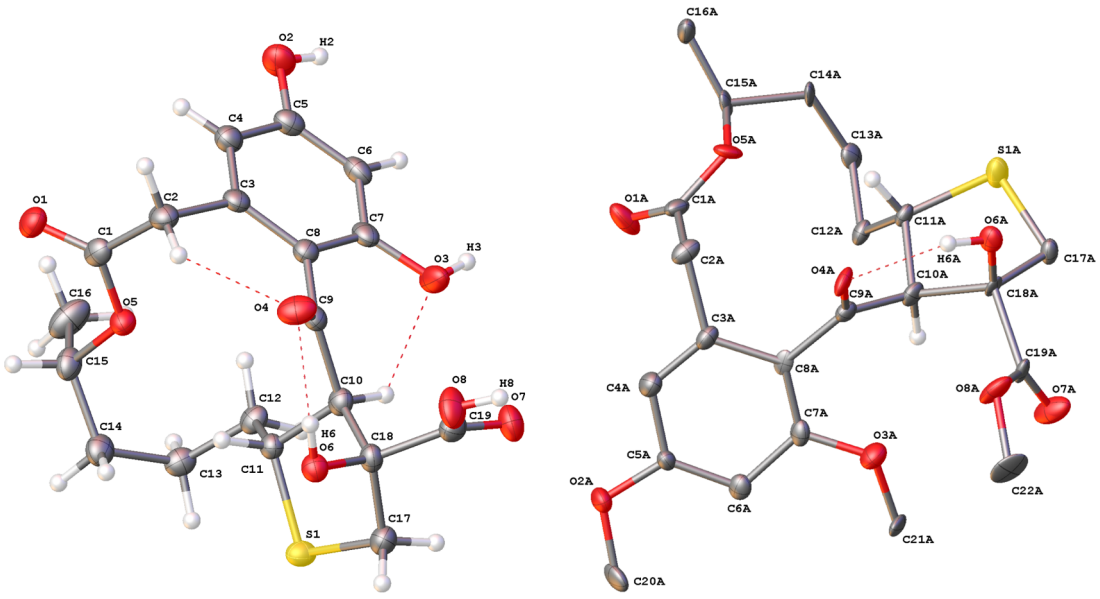

Figure 3. ORTEP diagram generated from the X-ray diffraction analysis of cyclothiocurvularin A (**6**) and of the dimethoxymethyl ether of cyclothiocurvularin B (**9**), respectively.

Table 2. NMR Data (^{1}H 400 MHz, ^{13}C 100 MHz) for Cyclothiocurvularins A (6) and B (7)

	cyclothiocurvularin A (**6**)[a]		cyclothiocurvularin B (**7**)[b]		cyclothiocurvularin B (**7**)[a]	
position	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
1	171.7, C		171.4, C		172.6, C	
2	40.7, CH_2	4.33 (d, 10.6); 3.27 (d, 10.5)	38.4, CH_2	4.07 (d, 12.1); 3.16 (d, 12.2)	38.5, CH_2	4.13 (d, 11.9); 3.12 (d, 11.9)
3	138.4, C		136.2, C		135.9, C	
4	112.5, CH	6.19 (d, 1.5)	112.0, CH	6.48 (bs)	111.6, CH	6.45 (d, 1.9)
5	163.3, C		161.6, C		162.7, C	
6	103.4, CH	6.25 (d, 1.5)	103.3, CH	6.25 (bs)	103.4, CH	6.22 (d, 1.9)
7	161.4, C		159.0, C		160.2, C	
8	120.9, C		121.8, C		121.7, C	
9	208.3, C		209.3, C		209.7, C	
10	64.5, CH	4.50 (d, 8.5)	64.2, CH	4.10 (d, 11.2)	64.4, CH	4.27 (d, 11.0)
11	49.8, CH	3.68 (bt, 8.5)	49.3, CH	3.88 (dt, 2.2, 11.3)	50.1, CH	3.93 (t, 11.5)
12	35.8, CH_2	1.96 (m); 1.50 (m)	34.6, CH_2	1.56 (m); 1.37 (m)	34.9, CH_2	1.66 (m); 1.36 (m)
13	23.4, CH_2	1.60 (m); 1.43 (m)	27.3, CH_2	1.53 (m); 1.43 (m)	27.7, CH_2	1.50 (m)
14	30.3, CH_2	1.67 (m); 1.34 (m)	31.2, CH_2	1.67 (m); 1.45 (m)	31.6, CH_2	1.73 (m); 1.42 (m)
15	73.5, CH	4.98 (m)	75.6, CH	4.64 (dq, 6.0, 10.0)	76.4, CH	4.64 (m)
16	17.7, CH_3	1.03 (d, 4.3)	21.7, CH_3	1.18 (d, 6.2)	21.7, CH_3	1.21 (d, 6.3)
17	41.2, CH_2	3.44 (d, 7.3); 2.91 (d, 7.5)	40.9, CH_2	3.43 (d, 11.4); 2.91 (d, 11.4)	40.9, CH_2	3.45 (d, 11.4); 2.92 (d, 11.4)
18	87.6, C		87.2, C		88.0, C	
19	176.3, C		174.8, C		176.6, C	

[a]MeOH-d_4. [b]MeCN-d_3.

along with the additional oxygen atom and the lack of evidence for any additional oxygen-substituted carbons, suggested the presence of a sulfoxide in the structure of **10**. In all other respects cyclosulfoxicurvularin (**10**) was the same as cyclothiocurvularin B (**7**), including the *trans*-junction between the tetrahydrothiophene moiety and the macrolactone ring, indicated by the coupling constant (J = 8.2 Hz) between H-10 and H-11 in **10**. The C-16 chemical shift suggests the same configuration as **7** and **8** at C-15. The HRESIMS of compound **11** gave a $[M - H]^-$ ion at m/z 439.1056, appropriate for the molecular formula $C_{20}H_{24}O_9S$, which differs from that of **10** by the addition of CH_2. As with **8** the presence of a methyl ester (δ_H 3.75 (s)/δ_C 53.6) was readily established, and **11** was assigned as the methyl ester of sulfoxide **10**. Although the coupling constant of 8.1 Hz between H-10 and H-11 in **10** indicates the same *trans*-relationship as observed for **6**–**8**, the absolute configurations of **10** and **11** at C-10, C-11, and C-18 and of the sulfoxide group were not assigned because we have been unable to obtain suitable crystals for X-ray diffraction analyses.

The occurrence of sulfur-bearing macrolides produced by fungi is rare. The few examples reported in the literature include pandangolide 2, bearing a 2-mercaptoacetic acid,[27] and pandangolide 4, which presents a sulfide bridge connecting two macrocyclic polyketides.[28] Pandangolide 3 contains an uncyclized 2-hydroxy-3-mercaptopropanoic acid (mercaptolactate) moiety,[28] the same sulfur-bearing residue observed in sumalarins A–C, recently isolated from culture media produced

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Table 3. NMR Data (^{1}H 400 MHz, ^{13}C 100 MHz) for Cyclothiocurvularin B (8) and Cyclosulfoxicurvularins 10 and 11

	cyclothiocurvularin B methyl ester (8)[a]		cyclosulfoxicurvularin (10)[b]		cyclosulfoxicurvularin methyl ester (11)[b]	
position	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
1	172.7, C		170.7, C		172.7, C	
2	38.7, CH_2	4.07 (d, 12.1); 3.01 (d, 12.0)	37.7, CH_2	4.10 (d, 7.9); 3.15 (d, 8.0)	38.9, CH_2	4.36 (d, 7.9); 3.12 (d, 7.9)
3	136.4, C		135.2, C		137.2, C	
4	112.2, CH	6.35 (d, 2.4)	110.9, CH	6.34 (d, 1.4)	112.6, CH	6.44 (d, 1.3)
5	163.1, C		161.4, C		161.1, C	
6	103.2, CH	6.13 (d, 2.2)	102.8, CH	6.23 (d, 1.4)	103.3, CH	6.25 (d, 1.3)
7	160.5, C		159.5, C		163.7, C	
8	121.7, C		119.7, C		120.5, C	
9	209.8, C		206.0, C		207.5, C	
10	64.9, CH	4.04 (d, 11.2)	58.4, CH	4.55 (d, 8.2)	59.8, CH	n.o.
11	50.4, CH	3.84 (dt, 2.2, 11.3)	59.8, CH	3.50 (t, 8.2)	62.3, CH	3.72 (dd, 1.2, 8.1)
12	35.6, CH_2	1.55 (m); 1.28 (m)	22.4, CH_2	1.70 (m); 1.26 (m)	24.0, CH_2	1.88 (m); 1.47 (m)
13	28.1, CH_2	1.44 (m)	24.6, CH_2	1.49 (m); 1.43 (m)	26.1, CH_2	1.68 (m); 1.55 (m)
14	31.8, CH_2	1.65 (m); 1.37 (m)	30.6, CH_2	1.75 (m); 1.52 (m)	32.0, CH_2	1.88 (m); 1.59 (m)
15	76.5, CH	4.56 (m)	74.7, CH	4.62 (m)	76.6, CH	4.74 (m)
16	22.0, CH_3	1.11 (d, 6.3)	21.6, CH_3	1.11 (d, 6.3)	21.9, CH_2	1.24 (d, 4.1)
17	41.0, CH_2	3.48 (d, 11.4); 2.80 (d, 11.5)	65.5, CH_2	3.90 (d, 9.1); 2.68 (d, 8.9)	66.2, CH_2	4.02 (d, 9.4); 3.13 (d, 9.4)
18	87.6, C		84.0, C		85.0, C	
19	174.4, C		173.0, C		173.1, C	
20	53.3, CH_3	3.62 (s)			53.6, CH_3	3.75 (s)

[a]MeCN-d_3. [b]MeOH-d_4.

by *Penicillium sumatrense*.[29] To the best of our knowledge, only a single compound has been previously reported with a mercaptolactate moiety incorporated into a tetrahydrothiophene ring: pharbitic acid, a giberellin derivative isolated from the Japanese morning-glory *Pharbitis nil*.[30] The isolation of cyclothiocurvularins and cyclosulfoxicurvularin provides additional examples of this unique class of secondary metabolites.

By feeding cultures of *Penicillium* sp. DRF2 with [U−$^{13}C_3{}^{15}N$]-L-cysteine the biogenetic origin of the mercaptolactate residue in both **6** and **7** has been unambiguously established. The ^{13}C NMR spectrum of cyclothiocurvularin B (**7**) isolated from this stable isotope feeding experiment showed >99% ^{13}C incorporation at C-17, C-18, and C-19 (Table 4 and Figure S24, SI). This incorporation ratio was calculated by integration of the ^{13}C-enriched signals of C-17, C-18, and C-19 and division of the integral values by the integral values of the ^{13}C-nonenriched signals of the same carbons. These results demonstrate that L-cysteine is the amino acid precursor of the mercaptolactate moiety in cyclothiocurvularins. The very high levels of [U−$^{13}C_3{}^{15}N$]-L-cysteine incorporation into cyclothiocurvularins suggest that, when added to *Penicillium* sp. DRF2 growth medium, labeled cysteine is completely converted into 3-mercaptopyruvate, which is subsequently condensed to the 10,11-dehydrocurvularin scaffold. Earlier studies on cysteine metabolism provided only indirect evidence that mercaptolactate is derived from cysteine through 3-mercaptopyruvate (**12**, Scheme 1).[31] It has also been demonstrated that *Aspergillus nidulans* can synthesize cysteine and methionine from inorganic sulfate.[32] Since the growth medium of *Penicillium* sp. DRF2 contains Na_2SO_4 in 2 mM concentration (SI), the availability of sulfate may stimulate the production of cyclothiocurvularins and cyclosulfoxicurvularins under the optimal growth conditions used for the production of these metabolites.

The isolation of two cyclothiocurvularin stereoisomers at C-10 and C-11, as well as the very high levels of [U−$^{13}C_3{}^{15}N$]-L-cysteine incorporation into the mercaptolactate moiety of **6** and **7**, raised the hypothesis that both **6** and **7** were products of a spontaneous reaction between 10,11-dehydrocurvularin (**2**) and mercaptopyruvate (**12**). We hypothesized that when the fungus increases the production of 10,11-dehydrocurvularin, it starts to produce 3-mercaptopyruvate, which would spontaneously react with **2** in order to diminish its concentration in the growth medium. This hypothesis was tested by performing a reaction between **2** and **12** in a buffer medium. Four transaminases were evaluated for the catalytic conversion of L-cysteine into 3-mercaptopyruvate (**12**) (SI). Conversions superior to 80% were observed. The ω-transaminase ATA-

Table 4. Incorporation Ratios (%) of [U−$^{13}C_3{}^{15}N$]-L-Cysteine into Cyclothiocurvularin B (7)

position	δ ^{13}C[a]	[U−$^{13}C_3{}^{15}N_1$]-L-cysteine
1	172.5	1.80
2	38.5	2.03
3	136.3	2.13
4	111.8	2.28
5	162.8	1.85
6	103.6	2.37
7	160.5	1.84
8	121.7	2.19
9	209.7	1.09
10	64.0	2.42
11	50.0	n.d.[b]
12	34.9	2.30
13	27.7	2.45
14	31.5	2.11
15	76.3	2.18
16	21.7	2.30
17	40.9	99.0
18	88.0	99.9
19	176.6	99.9

[a]100 MHz, 12 h acquisition time. [b]n.d.: overlapped by the solvent signal.

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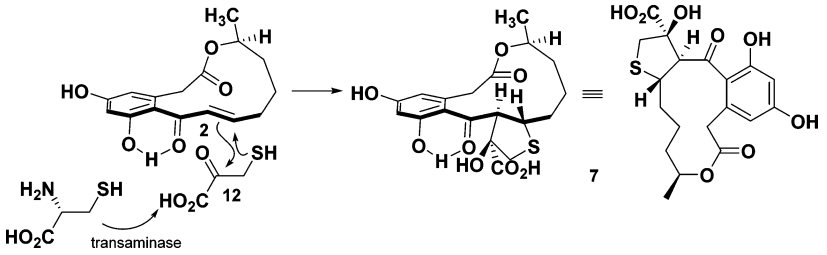
Scheme 1. Spontaneous Formation of Cyclothiocurvularin B (7) from 10,11-Dehydrocurvularin (2) and Mercaptopyruvate (12)



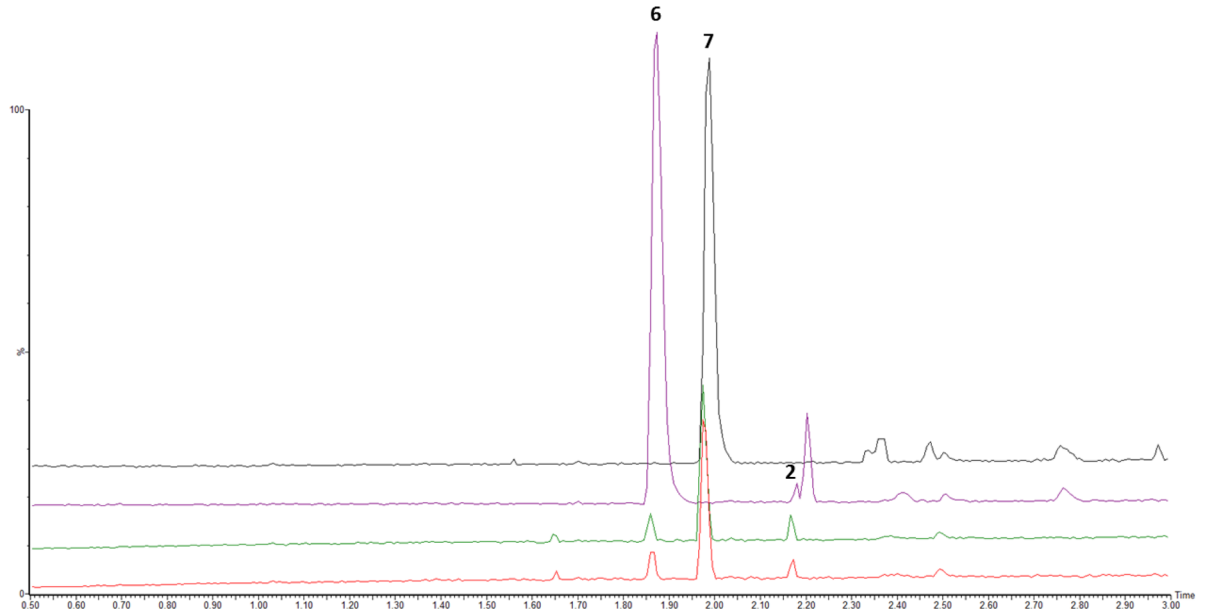


Figure 4. Spontaneous conversion of 10,11-dehydrocurvularin (**2**) to cyclothiocurvularins A (**6**) and B (**7**) in the presence of mercaptopyruvate (**12**). Black line: Cyclothiocurvularin B (**7**) standard (t_R 1.99 min). Mauve line: Cyclothiocurvularin A (**6**) standard (t_R 1.87 min). Green line: 10,11-Dehydrocurvularin (**2**) + L-cysteine + ω-ATA 113 transaminase reaction replicate 1. Red line: 10,11-Dehydrocurvularin (**2**) + L-cysteine + ω-ATA 113 transaminase reaction replicate 2. 10,11-Dehydrocurvularin (**2**) is observed at t_R 2.17 min. uPLC-qTOF analysis conditions: column: Acquity UPLC BEH C_{18} (1.7 μm, 2.1 × 50 mm); eluent: gradient of MeCN (+ 0.01% HCO_2H) in H_2O (+ 0.01% HCO_2H), starting at 10% to 100% MeCN (+ 0.01% HCO_2H) in 4 min. Detection: MS^E continuum during 5 min, m/z 250−450 molecular weight range; detection mode: ESI(+); scan time: 0.2 s; collision energy: 3 V; ramp collision energy: 20−30 V.

113 gave the best conversion of L-cysteine into mercaptopyruvate (94% in 24 h). The subsequent reaction of 10,11-dehydrocurvularin (**2**) in the presence of L-cysteine and ω-transaminase ATA-113 successfully yielded cyclothiocurvularins A (**6**) and B (**7**) in >90% conversion and in a ratio of 1:3, by comparison with authentic standards (Figure 4).

Our results unambiguously showed that, while L-cysteine requires enzymatic conversion to mercaptopyruvate (**12**), formation of cyclothiocurvularins A (**6**) and B (**7**) result from the spontaneous condensation of **12** with 10,11-dehydrocurvularin (**2**), via a Michael-type addition to the conjugated double bond followed by cyclization at the α-carbonyl group of mercaptopyruvate (Scheme 1). It is well known that **2** suffers Michael-type additions at the enone group.[8,19c] The high levels of [U−$^{13}C_3{}^{15}N$]cysteine incorporation into cyclothiocurvularin B (**7**) is thus explained by its efficient transamination to mercaptopyruvate, followed by condensation with **2**. Cyclothiocurvularin B (**7**) is the major cyclothiocurvularin isolated from the growth medium of *Penicillium* sp. DRF2, either in the presence of [U−$^{13}C_3{}^{15}N$]-cysteine or not. The overall isolation ratio of 1:12 between **6** and **7** is related to the 1:3 ratio of **6**/**7** obtained by reaction of **2** with L-cysteine in the presence of the transaminase, observed for the spontaneous formation of **6** and **7** from **2** and **12** (Figure 4). The isolation ratio of **6**/**7** from the experiments using [U−$^{13}C_3{}^{15}N$]-L-cysteine is 2:7. The ratio for the spontaneous formation of **6** and **7** from **2** and **12** is explained considering the most stable conformation of 15(*S*)-**2** (Scheme 1), which shows an upper more sterically hindered face, which does not favor the reaction between 15(*S*)-**2** and **12**. The spontaneous reaction between 15(*S*)-**2** and **12** leading to **7** is likely thermodynamically driven at the bottom face of 15(*S*)-**2**, which is sterically less hindered. Formation of **7** involves a *syn*-addition to the double bond *si*−*si* face of **2**. The resulting product from the addition at the bottom face is 10(*S*),11(*S*),15(*S*),18(*S*)-cyclothiocurvularin B (**7**). 10(*R*),11(*R*)-Cyclothiocurvularin A (**6**) is the minor compound formed by *syn*-addition of **12** at the upper face of the 10,11-dehydrocurvularin (**2**) enone double bond. The above results clearly demonstrate a chemical rather than a biochemical conversion of **2** and **12** into **6** and **7**.

The spontaneous formation of cyclothiocurvularins and cyclosulfoxicurvularin is related to the recent report that when the enone-bearing polyketide Sch-642305, produced by the endophyte *Phomopsis* sp. CMU-LMA, is added to the culture of *Aspergillus niger* ATCC 16404, the product of Sch-

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642305 condensation with mercaptolactate was isolated. The authors suggest that the resulting product is a protected form of Sch-642305, from its reaction with mercaptolactate, itself derived from cysteine.[31a] An analogous pathway is proposed for the formation of paraphaeosphaeride A by the endophytic fungus *Paraphaeosphaeria neglecta* FT462, although the authors do not indicate a protective role for the producing strain in providing paraphaeosphaeride A from paraphaeosphaeride C and either mercaptolactate or 3-mercaptopyruvate.[33] A similar Michael-type addition reaction of mercaptopyruvate (**12**) with an enone, followed by cyclization, is proposed in the pathway for the biogenesis of the mercaptolactate-bearing plant metabolite pharbitic acid.[30] The fact that 10,11-dehydrocurvularin (**2**) and cyclothiocurvularins A (**6**) and B (**7**) have the same absolute configuration at C-15 is related to the recent report that the thioesterase domain of curvularins promotes a stereospecific cyclization at C-15, leading exclusively to the 15(*S*) stereoisomer.[34]

Isolation of cyclothiocurvularins and cyclosulfoxicurvularin and the high level of cysteine incorporation have implications for the understanding of curvularin production by *Penicillium* sp. DRF2. The nonenzymatic condensation of 3-mercaptopyruvate (**12**) with 10,11-dehydrocurvularin (**2**) leading to cyclothiocurvularins **6** and **7**, possibly followed by further oxidation to cyclosulfoxicurvularin **10**, probably results from the enhanced production of **2** by the fungus under the optimal conditions herein established. High concentrations of **2** are very likely toxic to the fungus due to its intrinsic reactivity as a Michael acceptor. We propose that in order to minimize high concentrations of **2**, the fungus produces 3-mercaptopyruvate (**12**) from cysteine, to react with **2**. Thus, high concentrations of 10,11-dehydrocurvularin may well stimulate the biocatalytic formation of cyclothiocurvularins as detoxification products.

Compounds **3**, **4**, **7**, and **8** were tested against the A549 (lung), HeLa (cervix), and MCF-7 (breast) tumor cell lines, as well as against the nontumoral breast cell line MCF-10A. Compounds **3**, **4**, **7**, and **8** were essentially inactive in these assays. The uncyclized sumalarin A displayed a much more potent cytotoxicity to cancer cells[29] than compounds **6** and **7**. Therefore, the formation of cyclothiocurvularins and cyclosulfoxicurvularin clearly abolishes the cytotoxic activity of 10,11-dehydrocurvularin.

CONCLUSION

HPLC-UV-MS screening and refinement of our previously reported experimental design and chemometric analysis to improve the production of minor metabolites of fungal strains[21] enabled us to obtain good production of cyclothiocurvularins A (**6**) and B (**7**) and cyclosulfoxicurvularin (**9**) and, in so doing, allowed us to fully characterize and investigate the biogenesis and biological activities of these unusual metabolites. To the best of our knowledge, cyclothiocurvularins A (**6**) and B (**7**) and cyclosulfoxicurvularin (**9**) are the first members of such macrolides bearing the very rare mercaptolactate residue embedded in a tetrahydrothiophene or in a tetrahydrothiophene-1-oxide cyclic moiety. The nonenzymatic formation of compounds **6**, **7**, and **9** from mercaptopyruvate and 10,11-dehydrocurvularin (**2**) probably results from the overproduction of 10,11-dehydrocurvularin (**2**). In high concentrations, compound **2** may well be toxic to the producer strain due to its intrinsic cytotoxicity as a Michael addition acceptor, leading to an efficient conversion of cysteine to mercaptopyruvate before its spontaneous condensation with the 10,11-dehydrocurvularin enone group. Thus, cyclothiocurvularins **6** and **7**, as well as cyclosulfoxicurvularin **9**, are very likely detoxification products produced under conditions of fungal stress. Our findings provide significant insights into the metabolic regulation of fungal secondary metabolite production in culture conditions.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured in MeOH on a PerkinElmer 341 MC polarimeter at 20 °C or on a Jasco P-2000 polarimeter at 25 °C. NMR spectra were recorded on a Bruker AvanceIII 9.4 T instrument, operating at 400.35 MHz for ^{1}H and 100.10 MHz for ^{13}C channels, respectively, or in a Bruker AvanceIII 14.1 T instrument, operating at 600.23 MHz for ^{1}H and 150.94 MHz for ^{13}C, respectively, with a 5 mm cryoprobe. All NMR spectra were obtained at 25 °C using tetramethylsilane as an internal reference. Solvents used for extraction and column chromatography were glass distilled prior to use. HPLC-grade solvents were used without further purification in HPLC separations. TLC analyses were performed with precoated TLC sheets of Si gel on polyester, eluting with different mixtures of MeOH in CH_2Cl_2. Plates were observed under a UV lamp (λ_{max} 254 and 365 nm). Semipreparative HPLC separations were performed with a Waters quaternary pump 600 and double beam UV detector 2487 monitored by a Waters Millenium 32. HPLC-LRESIMS analyses were performed using a Waters Alliance 2695 coupled online with a Waters 2996 photodiode array detector, followed by a Micromass ZQ2000 MS detector with an electrospray interface. The photodiode array scanned the samples at λ_{max} 200−400 nm. The MS detector was optimized to the following conditions: capillary voltage, 3.00 kV; sample cone voltage, 30.0 V; source block temperature, 100 °C; desolvation temperature, 350 °C, operating in electrospray positive and negative mode, detection range, 200−800 Da with total ion count extracting acquisition. The cone and desolvation gas flow were 50 and 350 L/h, respectively, with a Nitrogen Peak Scientific N110DR nitrogen source. Data acquisition and processing were performed using Empower 2.0. HPLC-HRMS analyses were recorded on a Micromass Q-tof Micro, in ES^+ mode, using the following experimental conditions: capillary voltage, 3.0 kV; sample cone, 42.0 V; sample was infused at 10 μL/min in 1:1 MeOH/H_2O.

Isolation and Identification of *Penicillium* sp. DRF2. *Penicillium* sp. DRF2 was isolated from the sponge *Dragmacidon reticulatum*, collected in São Sebastião, SP, Brazil, in September 2005, by scuba diving, at depths between 5 and 10 m. Exact GPS positioning collecting points are 23°45′32″ south; 45°15′8″ west and 23°47′759″ south; 45°09′380″ west. The strain isolation procedure is described in detail in the SI. After 1 week of growth, fungal strains were exhaustively purified before preservation using Castellani's method.[35,36] *Penicillium* sp. DRF2 was identified by molecular analyses, as described in the SI.

Fungal Growth, Extraction, and Isolation of Compounds 3, 4, and 6−8, 10 and 11. A small-scale growth and metabolic production experiment under standard conditions was performed starting with inocula of previously grown *Penicillium* sp. DRF2 during 7 days. Inocula were transferred into eight 500 mL Schott flasks containing 250 mL of growth medium each (malt 2% in 1 L of sterilized ASW), for growth in still mode at 25 °C during 30 days. At the end of growth, 250 mL of EtOAc was added to each Schott flask. Each flask containing the mixture EtOAc + growth medium was blended and shaken overnight. The blended mixture was filtered through a Celite pad, transferred to a separatory funnel, and subjected to liquid−liquid partitioning. The organic phase was evaporated to dryness, to give 354 mg of the EtOAc crude extract.

The EtOAc extract was partitioned between hexane and 95:5 MeOH/H_2O. The polar fraction (244 mg) was triturated with 50:50 MeOH/H_2O. The soluble portion (165 mg) was subjected to a solid-phase extraction on a C_{18} reversed-phase cartridge (2 g) using H_2O/MeOH 75:25 (50 mL), 50:50 (50 mL), 25:75 (50 mL) and 100% MeOH (50 mL), to give fractions DRF2-F1 (36.4 mg), DRF2-F2 (94.7 mg), DRF2-F3 (25.4 mg), and DRF2-F4 (2.3 mg). Fractions

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DRF2-F2 and DRF2-F3 were pooled after TLC analysis (CH_2Cl_2/MeOH, 9:1, sprayed with phosphomolybdic acid reagent heated at 100 °C during 2 min). Fraction DRF2-F2/F3 (120.1 mg) was separated by HPLC, using a reversed-phase C_{18} CSC-Inertsil ODS2 column (250 × 94 mm, 150 Å, 5 μm), to give 8.5 mg of 10,11-dehydrocurvularin (**2**)[11] and 1.5 mg of 12-keto-10,11-dehydrocurvularin (**3**).

A larger scale growth and metabolic production by *Penicillium* sp. DRF2 was performed under improved conditions defined by a refined version of our experimental design and chemometrics analysis[21] (described in detail in the SI). Two optimized growth conditions have been established. Growth condition #11 (GC#11): 10% of salts concentration of the artificial seawater (ASW) recipe,[21] 80% of nutrients concentration of the standard M3 medium preparation,[36] temperature of growth at 15 °C, under shaking during 35 days. Growth condition #17 (GC#17): 10% of salts concentration of the artificial seawater (ASW) recipe, 80% of nutrients concentration of the standard M3 medium preparation, temperature of growth at 15 °C, in still mode during 35 days. For each of the two optimized growth conditions, 2 L of M3 culture medium was prepared and inoculated with *Penicillium* sp. DRF2. At the end of the growth period, the medium of each growth experiment was separately processed. After filtration through a Celite pad, the growth media of both growth experiments were separately subjected to a solid-phase extraction using a C_{18} reversed-phase silica gel column (10 g) eluted with a gradient of MeOH in H_2O. Fractions F1 to F4 (F1 = 75%:25% H_2O/MeOH, discarded; F2 = 50:50 H_2O/MeOH; F3 = 25:75 H_2O/MeOH; F4 = 100% MeOH, discarded) were dried *in vacuo* followed by HPLC-UV-MS analysis. The growth experiment using GC#11 yielded 987 mg of F2 and 66 mg of F3, while the growth experiment using GC#17 yielded 1.1 g of F2 and 224 mg of F3. Analysis by HPLC-UV-MS of fractions F2 and F3 obtained from both growth experiments showed good similarity and were pooled to give 2.377 g of a fraction named E1117-23. This fraction was subjected to a complex separation procedure (described in detail in the SI), to give 0.6 mg of **3**, 2.6 mg of **4**, 4.4 of **6**, 60 mg of **7**, 3.4 mg of **8**, 2.9 mg of **10**, and 0.3 mg of **11**. In order to obtain crystals of **6** suitable for X-ray diffraction analysis, a portion of **6** was further purified by C_{18} reversed-phase HPLC using an InertSustain, 5 μm, 25 × 1.0 cm column, with 7:3 (0.05% TFA/H_2O)/MeCN as eluent, and **6** then recrystallized by slow evaporation from MeOH.

*12-Keto-10,11-dehydrocurvularin (**3**):* colorless, glassy solid; $[\alpha]^{25}_D$ −7.6 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 212 (4.8), 298 (3.8), 340 (3.0) nm; ^{1}H NMR and ^{13}C NMR (Table 1); (+)-HRESIMS *m*/*z* 305.1050 $[M + H]^+$ (calcd for $C_{16}H_{17}O_6$, 305.10196).

*cis-10,11-Epoxycurvularin (**4**):* colorless, glassy solid; $[\alpha]^{25}_D$ +110.3 (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ε) 220 (4.0), 237 (3.7), 273 (3.5), 300 (3.0) nm; ^{1}H NMR and ^{13}C NMR (Table 1); (+)-HRESIMS *m*/*z* 307.11883 $[M + H]^+$ (calcd for $C_{16}H_{19}O_6$, 307.11761) and *m*/*z* 329.10063 $[M + Na]^+$ (calcd for $C_{16}H_{18}O_6Na$, 329.09956).

*Cyclothiocurvularin A (**6**):* colorless block crystals; mp 193−196 °C; $[\alpha]^{25}_D$ −18.2 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) λ_{max} 205 (4.16), 275 (3.71), 304 (3.60) nm; ^{1}H NMR and ^{13}C NMR (Table 2); (−)-HRESIMS *m*/*z* 409.0948 $[M - H]^-$ (calcd for $C_{19}H_{21}O_8S$, 409.0957).

*Cyclothiocurvularin B (**7**):* colorless, glassy solid; $[\alpha]^{25}_D$ −14.0 (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.20), 276 (3.70), 306 (3.60) nm; ^{1}H NMR and ^{13}C NMR (Table 2); (+)-HRESIMS *m*/*z* 411.11299 $[M + H]^+$ (calcd for $C_{19}H_{23}O_8S$, 411.11081) and *m*/*z* 433.09405 $[M + Na]^+$ (calcd for $C_{19}H_{22}O_8Na$, 433.09276).

*Cyclothiocurvularin methyl ester (**8**):* colorless, glassy solid; $[\alpha]^{25}_D$ −22.8 (*c* 0.17, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.20), 276 (3.70), 306 (3.60) nm; ^{1}H NMR and ^{13}C NMR (Table 3); (+)-HRESIMS *m*/*z* 425.12865 $[M + H]^+$ (calcd for $C_{20}H_{25}O_8S$, 425.12646) and *m*/*z* 447.10943 $[M + Na]^+$ (calcd for $C_{20}H_{24}O_8SNa$, 447.10841).

*Cyclosulfoxicurvularin (**10**):* colorless, glassy solid; $[\alpha]^{25}_D$ −52.0 (*c* 0.105, MeOH); UV (MeOH) λ_{max} (log ε) 210 (4.10), 277 (3.68), 302 (3.62) nm; ^{1}H NMR and ^{13}C NMR (Table 3); (+)-HRESIMS *m*/*z* 427.1049 $[M + H]^+$ (calcd for $C_{19}H_{23}O_9S$ 427.10573) and *m*/*z* 449.0855 $[M + Na]^+$ (calcd for $C_{19}H_{22}O_9SNa$, 449.08767).

*Cyclosulfoxicurvularin methyl ester (**11**):* colorless, glassy solid; $[\alpha]_D$ −52.6 (*c* 0.015, MeOH); UV (MeOH) λ_{max} (log ε) 209 (4.10), 277 (3.68), 303 (3.60) nm; ^{1}H NMR and ^{13}C NMR (Table 3); (−)-HRESIMS *m*/*z* 439.1056 $[M - H]^-$ (calcd for $C_{20}H_{23}O_9S$, 439.1063).

*X-ray crystal data for **6**:* $C_{39}H_{55}O_{22}S_2$, fw 942.66, orthorhombic, crystal size 0.10 × 0.12 × 0.35 mm^3, space group $P2_12_12_1$, *a* = 10.7952(7) Å, *b* = 17.2819(11) Å, *c* = 23.5916(13) Å, *V* = 4401.3(5) Å^3, *Z* = 4, *T* = 100(2) K, D_{calcd} = 1.483 g/cm^3, μ = 2.06 cm^{-1}, *F*(000) = 1999.0, reflections collected 30 225 (Mo Kα radiation, 2.92° ≤ 2θ ≤ 50.81°), independent reflections 8055 (R_{int} = 0.050, R_{sigma} = 0.068), final *R* indexes for $I \geq 2\sigma(I)$, R_1 = 0.041, wR_2 = 0.094, final *R* indexes for all data R_1 = 0.056, wR_2 = 0.106, the goodness-of-fit on F^2 is 1.02, the Flack parameter[37] *x* is 0.05(5). Crystallographic data for **6** have been deposited in the Cambridge Crystallographic Data Center (CCDC) as deposit no. CCDC 1472488. The data can be obtained free of charge from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/getstructures.

*X-ray crystal data for **7**:* $C_{22}H_{28}O_8S$, fw 452.50, tetragonal, crystal size 0.03 × 0.12 × 0.14 mm^3, space group $P4_3$, *a* = 10.019(3) Å, *b* = 10.019(3) Å, *c* = 42.875(12) Å, *V* = 4304(3) Å^3, *Z* = 4, *T* = 90(2) K, D_{calcd} = 1.397 g/cm^3, μ = 1.97 cm^{-1}, *F*(000) = 1920.0, reflections collected 26 253 (Mo Kα radiation, 1.90° ≤ 2θ ≤ 48.6°), independent reflections 6912 (R_{int} = 0.098, R_{sigma} = 0.115), final *R* indexes for $I \geq 2\sigma(I)$, R_1 = 0.117, wR_2 = 0.304, final *R* indexes for all data R_1 = 0.112, wR_2 = 0.300, the goodness-of-fit on F^2 is 1.05, the Flack parameter[37] *x* is 0.04(7). Crystallographic data for **7** have been deposited in the Cambridge Crystallographic Data Center (CCDC) as deposit no. CCDC 1472487. The data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/getstructures.

Preparation of Dimethoxycyclothiocurvularin B Methyl Ester (9). Cyclothiocurvularin B (**7**) (2 mg) was treated with diazomethane that was generated *in situ* by the addition of 0.7 mL of 2.0 M trimethylsilyldiazomethane in hexanes to 0.7 mL of anhydrous MeOH in 1.0 mL of C_6H_6. The reaction mixture was left stirring for 16 h at rt. After evaporation of the reagents the sample was purified by HPLC using a C_{18} reversed-phase InertSustain column (5 μm, 25 × 0.46 cm), with 3:2 MeCN/H_2O as eluent, to yield 1.3 mg of dimethoxycyclothiocurvularin B methyl ester (**9**).

*Dimethoxycyclothiocurvularin B methyl ester (**9**):* colorless plate crystals; mp 176−180 °C; UV (MeOH) λ_{max} 219, 244, 284, 309 nm; ^{1}H NMR (600 MHz, DMSO-d_6) 8.53 (s); 5.99 (s); 5.23 (s); 5.14 (s); 4.99 (bt, 5.3 Hz); 4.02 (s); 3.31 (s); 2.68 (m); 2.13−2.03 (m), 1.90 (bd); 1.87 (bd); 1.76 (m); 1.50 (s); 1.30 (m); 1.11 (m); 1.00 (s); 0.95 (s); 0.70 (d, 7.0 Hz); ^{13}C NMR (150 MHz, DMSO-d_6) 198.2, 143.6, 140.6, 136.1, 132.8, 121.6, 115.1, 64.1, 62.4, 41.9, 40.5, 39.8, 37.6, 37.5, 32.6, 32.1, 30.9, 23.3, 21.4, 16.3, 16.2, 13.9; (+)-HRESIMS *m*/*z* 475.1400 $[M + Na]^+$ (calcd for $C_{22}H_{28}O_8SNa$, 475.1403).

Administration of Unlabeled and of Stable-Isotope-Labeled Substrates to Cultures of *Penicillium* sp. DRF2. In order to verify the highest concentration of cysteine tolerated by the fungus *Penicillium* sp. DRF2 for the [U−$^{13}C_3$,^{15}N]-L-cysteine administration experiments, as well as a possible alteration in the cyclothiocurvularin production in the presence of L-Cys, a series of duplicate growth experiments were performed with unlabeled L-Cys. These growth experiments were performed in a total volume of 100 mL of culture media each, distributed in two Schott flasks inoculated with 5 × 10^6 spores of *Penicillium* sp. DRF2, followed by incubation in the GC#17 medium. Unlabeled L-Cys was added in six growth experiments at final concentrations of 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 mg/mL, at the sixth day of microbial growth. After 20 days of incubation, the cultures were harvested and filtered through Celite. The liquid media of each growth experiment was subjected to a solid-phase extraction using a C_{18} reversed-phase silica gel column (2 g) eluted with a gradient of MeOH in H_2O. Fractions F1 to F4 (F1 = 100% H_2O, discarded; F2 = 75:25 H_2O/MeOH, discarded; F3 = 25:75 H_2O/MeOH; F4 = 100% MeOH, discarded) were dried *in vacuo* followed by HPLC-UV-MS analysis

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using a reversed-phase C_{18} column (X-Terra, 250 × 4.6 mm, 5 μm) eluted with a MeOH/H_2O 0.1% formic acid gradient.

Small-scale [U−$^{13}C_3{}^{15}N$]-L-cysteine feeding experiments were performed in a total volume of 150 mL of culture media distributed in three Schott flasks with 50 mL each. These Schott flasks were inoculated with 5 × 10^6 spores of *Penicillium* sp. DRF2, followed by incubation in GC#17 medium (SI). [U−$^{13}C_3{}^{15}N$]-L-Cysteine was added at a final concentration of 2 mM, on the sixth day of fungal growth. Cultures were harvested after 20 days of growth and filtered through Celite. The growth media was then subjected to a solid-phase extraction using a C_{18} reversed-phase silica gel column (10 g) eluted with a gradient of MeOH in H_2O. Fractions F1 to F4 (F1 = 75%:25% H_2O/MeOH, discarded; F2 = 50:50 H_2O/MeOH; F3 = 25:75 H_2O/MeOH; F4 = 100% MeOH, discarded) were dried *in vacuo* followed by HPLC-UV-MS analysis using a reversed-phase C_{18} analytical column (X-Terra, 250 × 4.6 mm, 5 μm) eluted with a MeOH/H_2O 0.1% formic acid gradient.

Large-scale [U−$^{13}C_3{}^{15}N$]-L-cysteine feeding experiments were performed using a total volume of 0.5 L of culture media distributed in five Schott flasks with 100 mL each, inoculated with 10^7 spores of *Penicillium* sp. DRF2, followed by incubation in GC#17 medium using the same concentration of [U−$^{13}C_3{}^{15}N$]-L-cysteine. Cultures were harvested after 20 days of growth and filtered through Celite. At the end of incubation, the liquid media were subjected to a solid-phase extraction using a C_{18} reversed-phase silica gel column (10 g) eluted with a gradient of MeOH in H_2O. Fractions F1 to F4 (F1 = 75%:25% H_2O/MeOH, discarded; F2 = 50:50 H_2O/MeOH; F3 = 25:75 H_2O/MeOH; F4 = 100% MeOH, discarded) were dried *in vacuo* followed by HPLC-UV-MS analysis. A total amount of 265.9 mg of the crude F2 fraction was obtained. The F2 fraction was purified by HPLC-UV using a reversed-phase C_8 preparative column (Inertsil C8-4, 250 × 14 mm, 5 μm) eluted with 66% H_2O and 34% MeCN. This separation yielded four new fractions, of which F2-D corresponded to compound **2** (1.0 mg). Fraction F2-A was purified by HPLC using a reversed-phase C_{18} semipreparative column (Inertsil ODS2, 250 × 9.4 mm, 5 μm) eluted with 50% H_2O, 33% MeOH, and 17% MeCN. Compounds **6** (2.7 mg) and **7** (9.3 mg) were obtained from the [U−$^{13}C_3{}^{15}N$]-L-cysteine incorporation experiment. Incorporation ratios have been calculated as previously described.[38]

*[^{13}C-17,^{13}C-18,^{13}C-19]-Cyclothiocurvularin B (**7**):* colorless, glassy solid; ^{13}C NMR (100 MHz, MeOH-d_4) δ 176.0 (d, 60.4, C-19), 87.4 (dd, 60.6 and 38.0), 40.8 (dd, 38.1 and 2.0, C-17); (+)-HRESIMS *m*/*z* 414.1113 $[M + H]^+$ (calcd for $C_{16}{}^{13}C_3H_{23}O_8S$ 411.3989). The measured HRMS value results from the 99.9% incorporation ratio of [U−^{13}C,^{15}N]-L-cysteine into cyclothiocurvularin B.

Conversion of 10,11-Dehydrocurvularin (2) into Cyclothiocurvularins A (6) and B (7). A mixture of triethanolamine hydrochloride (0.1 g, 100 mmol/L), acetone (75 μL, 100 mmol/L), and pyridoxalphosphate (1.3 mg, 1 mmol/L) was added to H_2O (4 mL). The pH of the mixture was adjusted to 7.5 with NaOH (5 mol/L), and the volume was adjusted to 5 mL with H_2O. Then, 450 μL of this mixture was transferred to a 2 mL Eppendorf flask containing 2 mg of transaminase ATA-113 [from Codex ATA screening kit (ATASK 201000P, lot no. N13012) produced by Codexis, Inc. (Redwood City, CA, USA)]. L-Cysteine hydrochloride (50 μL, 70 mmol/L in H_2O) and 10,11-dehydrocurvularin (**2**, 7 mmol/L in DMSO, 50 μL) were separately added to the reaction mixture, which was kept at 30 °C and 200 rpm for 48 h. Reaction controls in the absence of enzyme were also developed in order to evaluate the substrate stability in the reaction mixture. The enzymatic reaction was quenched with MeOH (500 μL) and centrifuged (× rpm, × min). The reaction supernatant was diluted in MeOH (100×) and analyzed by UPLC-qTOF. UPLC-qTOF analysis conditions: column, Acquity UPLC BEH C_{18} (1.7 μm, 2.1 × 50 mm); eluent, gradient of MeCN (+ 0.01% HCO_2H) in H_2O (+ 0.01% HCO_2H), starting at 10% to 100% MeCN (+ 0.01% HCO_2H) in 4 min. Detection: MS^E continuum during 5 min, *m*/*z* 250−450 molecular weight range; detection mode, ESI(+); scan time, 0.2 s.; collision energy, 3 V; ramp collision energy, 20−30 V.

Cytotoxicity Assays. Breast cell lines MCF-10A and MCF-7 were purchased from National Cancer Institute Cell Bank, Rio de Janeiro, Brazil. The cervical cancer derived HeLa and human alveolar basal epithelial cell line A549 were a kind donation from Laboratory of Cytogenetics and Mutagenesis, Medical School, Ribeirão Preto, SP, Brazil. All cell lines were expanded and cultured in complete medium composed of DMEM + HAM F10 (1:1, v/v) (Sigma-Aldrich) supplemented with 10% fetal calf serum (Sigma, St Louis, MO, USA) and 1% penicillin/streptomycin stabilized solution (Sigma-Aldrich) in cell culture flasks of 25 mm^2 (TPP, Swizerland). Cells were incubated at 37 °C and 5% CO_2 in a humidified atmosphere. Experiments were performed between the third and eighth passage. The IC_{50} was determined with XTT metabolic using the Cell Proliferation Kit II (Roche) following the manufacturer's instructions. Briefly, cells were trypsinized and cultured at 1 × 10^4 cells/well in a 96-well plate. After 24 h cells were treated with **3**, **4**, **7**, and **8** (7.8, 15.6, 31.2, 62.5, 125, 250, 500, and 1000 μM) for an additional 24 h, after which the culture medium was removed and cells were incubated with DMEM medium without phenol red plus XTT reagent for 4 h. Absorbance was measured at 492 nm with a reference at 620 nm, using a microplate reader (Sunrise, Tecan, Männdorf, Switzerland). The IC_{50} values were calculated using GraphPad Prism 5.0. Doxorrubicin was used as positive control for A549 (lung cancer, 0.19 μM), HeLa (cervical cancer, 0.14 μM), MCF-7 (breast cancer, 0.12 μM), and MCF10A (normal breast cells, 0.4 μM).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.6b00295.

Procedures of chemical and biological screening, experimental design, and chemometric analysis, conversion of L-cysteine into mercaptopyruvate, ^{1}H and ^{13}C NMR data of compounds **3**, **4**, **6**−**11**, and ^{13}C-labeled **7**. (PDF)

Experimental details for the X-ray diffraction analysis for compounds **6** and **9**. (PDF)

Crystallographic data (CIF)

Crystallographic data (CIF)

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Notes

The authors declare no competing financial interest.

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