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LOW-MOLECULAR-MASS METABOLITES OF FUNGI

I. STACHYBOTRIN FROM *Stachybotrys alternans*

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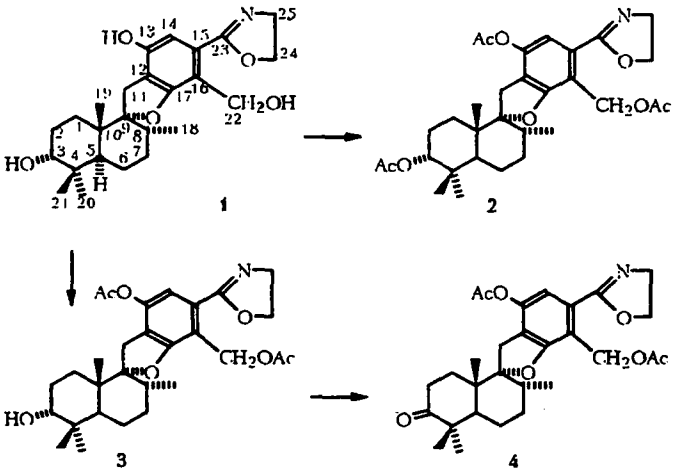
Stachybotrys alternans *produces a series of low-molecular-mass compounds. By their column chromatography we have isolated the nitrogen-containing compound stachybotrin. The structure of stachybotrin has been determined on the basis of chemical transformations together with the results of* ^1H *and* ^{13}C *NMR spectroscopy, 2M NMR* $^1H-^1H$ *and* $^1H-^{13}C$ *chemical shift correlation (COSY), correlation of long-range* $^1H-^{13}C$ *interactions (HMBC), measurements of NOEs in a rotating coordinate system (ROESY), and IR and electron-impact mass spectra.*

Stachybotrys alternans damages cellulose-containing fodders, producing mycotoxins causing toxicoses in animals [1]. The chemical nature of these mycotoxins has not been elucidated. In view of this, we have studied the low-molecular-mass metabolites of *Stachybotrys alternans*. By column chromatography of the total products of the vital activity of the organism grown under laboratory conditions we isolated the quantitatively predominant component, which we have called stachybotrin (**1**, Scheme 1). In the present paper we report the establishment of the structure of this compound.



Scheme 1

The elemental composition of stachybotrin was determined by high-resolution electron-impact mass spectrometry — $C_{25}H_{35}NO_5$. The ^{13}C NMR spectrum of the compound (**1**) under study (Table 2), containing signals at 156.88, 155.39, 135.66, 117.60, 113.22, and 101.80 ppm, showed that the stachybotrin molecule included a pentasubstituted benzene nucleus. In agreement with this, in the ^1H NMR spectrum of compound (**1**) (Table 1) there was a one-proton singlet at 7.31 ppm, assigned to a single aromatic proton.

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TABLE 1. Chemical Shifts (δ, ppm), Multiplicities, and SSCCs (J, Hz) of the Protons of Stachybotrin (1) and Its Derivatives (0-TMS)

Position of the protons	Compound			
	1	2	3	4
1α-H	2.35 td (13; 3)			
1β-H	1.13 dt (13; 3)		1.06 dt (13; 3)	
2α-H	1.70			
2β-H	1.95 tt (13; 3)			
3β-H	3.54	4.61 t (3)	3.37 t (3)	
5α-H	2.56 dd (13; 2.4)			
6α-H	1.55			
6β-H	1.42 qd (13; 3.5)			
7α-H	1.70			
7β-H	1.55			
8β-H	1.75			
11α-H	3.50 d (16.7)	3.13 d (17)	3.14 d (17)	3.16 d (17)
11β-H	3.09 d (16.7)	2.76 d (17)	2.74 d (17)	2.82 d (17)
14-H	7.31 s	7.03 s	7.01 s	7.04 s
18-CH_3	0.80 d (5.8)	0.73 d (6.5)	0.71 d (5.3)	0.73 d (6.5)
19-CH_3	0.97 s	0.91 s	0.96 s	1.12 s
20-CH_3	1.19 s	0.98 s	0.99 s	1.16 s
21-CH_3	0.88 s	0.90 s	0.85 s	1.06 s
22-H_2	4.09; 4.35 d (16.7)	4.32; 4.44 d (17)	4.37; 4.43 d (17)	4.35; 4.40 d (17)
24-H_2	3.90 m	4.29 m	4.28 m	4.30 t (5.5)
25-H_2	3.65; 3.90 m	3.82 m	3.81 m	3.82 t (5.5)
3-OAs	–	2.01 s	–	–
13-OAs	–	2.29 s	2.28 s	2.29 s
22-OAs	–	2.04 s	2.04 s	2.06 s

*The spectrum of compound (1) was taken in deuteropyridine and those of compounds (2-4) in deuterochloroform. The chemical shifts given without multiplicities and SSCCs were determined from the 2M NMR $^1H-^1H$ and $^1H-^{13}C$ chemical shift correlation spectra. Abbreviation: s) singlet; d) doublet; t) triplet; dd) doublet of doublets; td) triplet of doublets; dt) doublet of triplets; tt) triplet of triplets; qd) quartet of doublets; m) multiplet.

The interpretation of the ^1H and ^{13}C NMR spectra and the 2M NMR $^1H-^1H$ and $^1H-^{13}C$ chemical shift correlation spectra enabled us to construct the fragmentary structure of stachybotrin shown in Fig. 1. The alicyclic part of the molecule consists of 15 carbon atoms and resembles the skeleton of the sesquiterpenoid drimane [2].

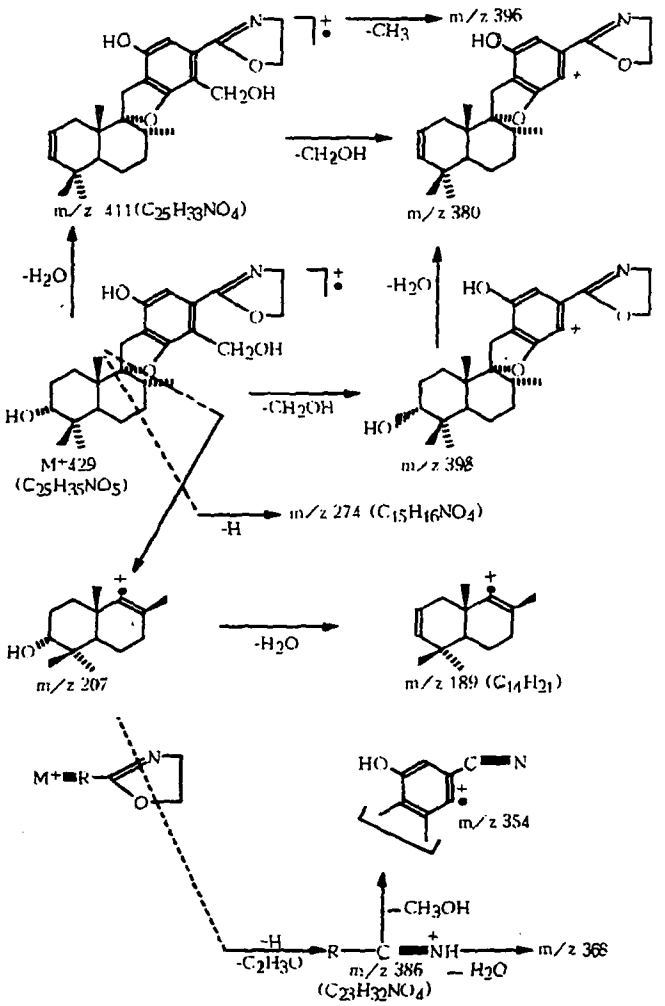
Acetylation of stachybotrin with acetic anhydride in pyridine gave the triacetate (2) The formation of the triacetate permitted the assumption that there were three hydroxy groups in the molecule of compound (1). In the PMR spectrum of the triacetate (2), the protons of one of the acetate groups resonated at 2.29 ppm, showing the phenolic nature of the corresponding hydroxy group. In actual fact, in the ^{13}C NMR spectrum a signal belonging to a phenolic carbon atom was observed at 155.39 ppm.

A comparative analysis of the PMR spectra of compounds (1) and (2) showed that the one-proton triplet at 4.61 in the PMR spectrum of the triacetate (2) must relate to a proton geminal to an acetoxy group. Consequently, the corresponding hydroxy group was secondary. A confirmation of this was a signal from a secondary carbinol carbon atom at 74.83 ppm in the ^{13}C NMR spectrum of stachybotrin.

In the electron-impact mass spectrum of stachybotrin the maximum peak was that of an ion with *m/z* 398 arising on the elimination of a hydroxy radical (Scheme 2). Corresponding ions with *m/z* 440 and 438 were formed in the mass-spectrometric fragmentation of the diacetate (3) and the ketone (4), but the peaks of these ions were not the maximum ones. These facts witnessed the presence in the (1) molecule of a primary hydroxy group in the form of a hydroxymethyl function. In confirmation of this conclusion, in the ^1H NMR spectrum of compound (1) we observed the one-proton doublets of an *AB* system at 4.09 and 4.35 ppm, which correlates with a signal from a carbon atom at 48.54 ppm in the 2M NMR $^1H-^{13}C$ chemical shift correlation spectrum.

A signal at 169.09 ppm in the ^{13}C NMR spectrum of stachybotrin showed that the compound under consideration contained an N,N-disubstituted carbamoyl grouping $(>N-\overset{|}{C}=O)$ or an azomethineoxy grouping isomeric with it $(-N=\overset{|}{C}-O-)$. The choice between them will be made later. An ester group was excluded by the IR spectrum of the compound (1) under discussion, which contained an intense absorption band at 1675 cm^{-1} and also by the fact that, as shown below, stachybotrin contains another oxygen function.

Thus, of the five oxygen atoms, three participate in primary, secondary, and phenolic hydroxy groups, and the fourth in a function the carbon atom of which resonates at 169.09 ppm. The chemical shifts of two aromatic carbon atoms at 155.39 and 156.88 ppm in the ^{13}C NMR spectrum of stachybotrin showed that, in addition to the phenolic hydroxy group, another oxygen function was attached to the benzene nucleus. The observation in the same spectrum of a signal at 98.72 ppm, characteristic for a quaternary carbon atom bound to oxygen showed that the oxygen atom under consideration linked the alicyclic and aromatic parts of the molecule and, namely, the carbon atoms resonating at 156.88 and 98.72 ppm, in the form of an epoxide function.



Scheme 2

The appearance of ions with *m/z* 207 and 189 in the fragmentation of stachybotrin under electron impact showed that there was only the secondary hydroxy group in the sesquiterpene part of the molecule, while the hydroxymethyl function and that including the C-23 atom were attached to the benzene ring.

The chemical shifts of three of the substituted carbon atoms of the benzene nucleus, 113.22, 117.60, and 135.66 ppm, permitted the conclusion that these aromatic carbon atoms were linked with the substituents by carbon–carbon bonds.

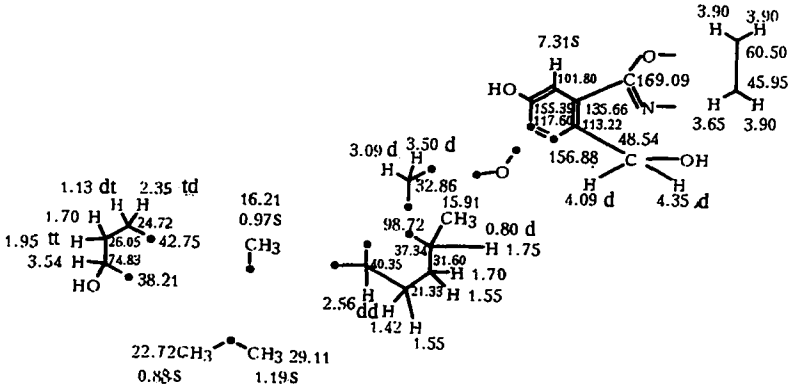


Fig. 1. Fragments of the structure of stachybotrin (1) deduced from the ^{1}H and ^{13}C NMR spectra and the 2M NMR $^{1}H-^{1}H$ and $^{1}H-^{13}C$ chemical shift correlation spectra.

In the ^{1}H NMR spectrum of stachybotrin, another two protons of the *AB* system resonated at 3.09 and 3.50 ppm, and in the 2M NMR $^{1}H-^{13}C$ chemical shift correlation spectrum these correlated with the carbon atom resonating at 32.86 ppm (C-11). In the correlation spectrum of long-range $^{1}H-^{13}C$ interactions (HMBC) (Table 3 and Fig. 2), these protons of an isolated methylene, correlated with three aromatic carbon atoms, C-12 (117.60 ppm), C-13 (155.39 ppm), and C-17 (156.88 ppm), and three carbon atoms of the terpene part of the molecule, C-8 (37.34 ppm), C-9 (98.72 ppm), and C-10 (42.75 ppm). Consequently, this was the carbon atom of an isolated methylene (C-11) and was attached at the sesquiterpene part of the molecule to the aromatic atom resonating at 117.60 ppm (C-12) by a carbon–carbon bond. The epoxide function linking carbon atoms C-9 (98.72 ppm) and C-17 (156.88 ppm) created a spirobenzofuran system.

In the same HMBC spectrum, the signal of the H-14 aromatic proton (7.31 ppm) correlated with the C-12 and C-13 carbon atoms (117.60 and 155.39 ppm, respectively), showing the location of the proton under discussion at C-14 (101.80 ppm). An analogous conclusion followed from a comparison of the ^{1}H NMR spectra of the triacetate (2) (CD_3OD) and of compound (1) (CD_3OD). In the spectrum of triacetate (2) the signal of the aromatic proton was shifted downfield in comparison with that in stachybotrin by 0.25 ppm. Consequently, the aromatic proton and the acetoxy group in compound (2) were vicinal. The aromatic hydrogen atom, H-14, also correlated with the C-23 carbon atom (169.09 ppm). This meant that the latter was present at C-15, and the hydroxymethyl group at C-16. In agreement with this, the hydrogen atoms of the hydroxymethyl group correlated with the C-15, C-16, and C-17 carbon atoms, and also with C-23.

Thus, still unidentified were two methylene groups, the protons of which, judging from the ^{1}H NMR spectrum (CD_3OD) formed a four-spin *AA'BB'* system changing in the spectra of derivatives (2-4) into an *AA'XX'* system. In the 2M NMR $^{1}H-^{13}C$ chemical shift correlation spectrum the protons under discussion correlated with the signals of carbon atoms at 45.95 and 60.50 ppm. Taking into account the elucidated part of the molecule, it followed from the elemental composition of compound (1), $C_{25}H_{35}NO_5$, that the stachybotrin molecule must include another ring. This could be a N-acylaziridine ring including the N,N-disubstituted carbamoyl group and the unidentified methylene groups or a 2-substituted 2-oxazoline ring, isomeric with it and consisting of the same three methylene groups and an azomethineoxy grouping. The choice between the alternative rings in favor of the latter was made on the basis of the chemical shifts of the 2H-24 and 2H-25 hydrogen atoms and the corresponding carbon atoms. This conclusion was conformed by the mass-spectrometric fragmentation of stachybotrin. The appearance of an ion with *m/z* 386 ($C_{23}H_{32}NO_4$) and of two daughter ions with *m/z* 368 and 354 was due to the presence in the stachybotrin molecule of a 2-substituted 2-oxazoline.

The linkage of rings *A/B* and the conformation of the terpenoid part of the molecule of the new compound (1) were elucidated in the following way.

The signal of H-5 was observed in the ^{1}H NMR signal of stachybotrin at 2.56 ppm in the form of a doublet of doublets with the SSCCs $^{3}J_1 = 13$ and $^{3}J_2 = 2.4$ Hz (Table 1). These constants showed the axial orientation of H-5 at least with respect to ring *B*, which was possible with the *trans*-linkage of rings *A* and *B*. At the same time, the SSCCs of H-1α, H-1β, H-2β, H-3β, and H-6β in the PMR spectra of compounds (1-3) determined the conformations of rings *A* and *B* as $^{10}C_3$ and $^{8}C_5$ chairs, respectively (Fig. 3).

TABLE 2. Chemical Shifts of the Carbon Atoms of Stachybotrin (**1**) (δ, ppm; 0-TMS; C_5D_5N)

C atom	δ, ppm	C atom	δ, ppm
1	24.72	14	101.80
2	26.05	15	135.66
3	74.83	16	113.22
4	38.21	17	156.88
5	40.85	18	15.91
6	21.33	19	16.21
7	31.60	20	29.11
8	37.34	21	22.72
9	98.72	22	48.54
10	42.75	23	169.09
11	32.86	24	60.50
12	117.60	25	45.95
13	155.39		

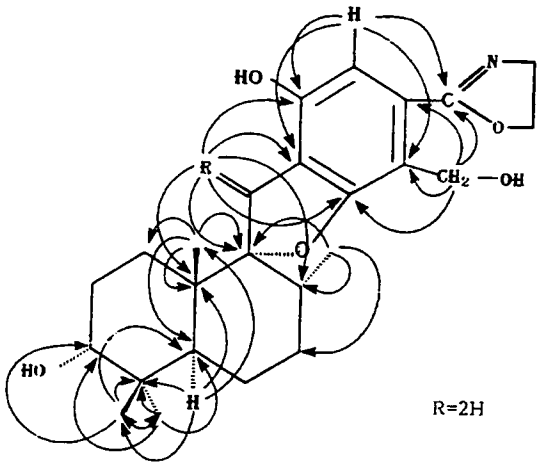


Fig. 2. HMBC spectrum of stachybotrin in deuteropyridine.

In the PMR spectra of the acetates (**2**) and (**3**) the H-3 signal was observed in the form of a triplet with the SSCC ^3J = 3 Hz at 4.61 and 3.37, respectively. These values of the H-3 chemical shifts showed that the free hydroxy group in the diacetate (**3**) was located at C-3 and, consequently, compound (**3**) was the 13,22-diacetate of stachybotrin. Ketone (**4**), obtained by the Jones oxidation of the diacetate (**3**), was the 13,22-diacetate of dehydrostachybotrin. The SSCCs of the triplets under consideration (^3J = 3 Hz) determined the β-equatorial orientation of H-3. This means that the acetoxy group at C-3 in compound (**2**) and the corresponding hydroxy groups in the molecules of stachybotrin and its diacetate (**3**) had the α-axial orientation.

These conclusions were confirmed by the ROESY spectrum of stachybotrin, which is given in Fig. 3. Moreover, the detection in the same spectrum of an NOE between the CH_3-19 and H-8 protons permitted the latter to be ascribed the β-, and the methyl group at C-8(CH_3-18) the α-, orientation.

The observation of an NOE between the antiperiplanar pairs of protons H-1α–H-2β and H-5–H-6β was a feature of the ROESY spectrum of stachybotrin and was in harmony with the established conformations of rings *A* and *B*.

Thus the experimental results presented permitted us to conclude that stachybotrin had the structure illustrated by formula (**1**).

EXPERIMENTAL

General Observations. Thin-layer chromatography (TLC) was conducted on Silufol plates. The substances were detected in TLC by spraying with a 25% ethanolic solution of molybdophosphoric acid, followed by heating at 100-110°C for 5 min.

TABLE 3. Spectrum of $^1H-^{13}C$ Long-Range Interaction Correlations (HMBC) of Stachybotrin (1) in Deuterochloroform

Proton		Carbon atom	
position	δ, ppm	position	δ, ppm
H-5	2.56	C-4	38.21
		C-10	42.75
		C-19	16.21
		C-21	22.72
2H-11	3.09; 3.50	C-8	37.34
		C-9	98.72
		C-10	42.75
		C-12	117.60
		C-13	155.39
		C-17	156.88
H-14	7.31	C-12	117.60
		C-13	155.39
		C-16	113.22
		C-23	169.09
CH_3-18	0.80	C-7	31.60
		C-8	37.34
		C-9	98.72
CH_3-19	0.97	C-1	24.72
		C-5	40.35
		C-9	98.72
		C-10	42.75
CH_3-20	1.19	C-3	74.83
		C-4	38.21
		C-5	40.35
		C-21	22.72
CH_3-21	0.88	C-3	74.83
		C-4	38.21
		C-5	40.35
		C-20	29.11
2H-22	4.09; 4.35	C-15	135.66
		C-16	113.22
		C-17	156.88
		C-23	169.09

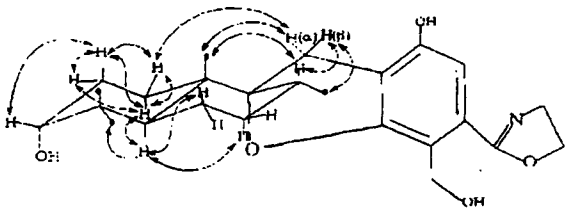


Fig. 3. Conformation of the terpene part of stachybotrin and details of the ROESY spectrum in deuteropyridine.

For column chromatography we used silica gel of the Silpearl and L types with a particle size of 50-100 μm. Silpearl was used for separating the *Stachybotrys alternans* metabolites. The purification and separation of the products of chemical transformations were conducted on column of mark L silica gel.

The following solvent systems were used: 1) benzene–methanol (10:1); 2) chloroform–methanol (100:1); and 3) chloroform–methanol (50:1).

Mass spectra and the elemental compositions of the ions were measured on a MKh-1310 instrument at an ionizing energy of 50 eV and a temperature of 100°C.

IR spectra were recorded on UR-20 and Perkin-Elmer System 2000 FT-IR spectrophotometers in KBr.

^1H and ^{13}C NMR spectra and 2M NMR $^1H-^1H$ and $^1H-^{13}C$ chemical shift correlation spectra (COSY) were taken on a Bruker AM 400 instrument. ^{13}C NMR spectra were obtained with complete decoupling of C–H interactions and J-modulation. 2M NMR long-range $^1H-^{13}C$ chemical shift correlation spectra (HMBC) and measurements of rotating-frame NOEs (ROESY) were taken on a Bruker AC 300 instrument. The spectra were recorded in deuteropyridine unless otherwise mentioned. All the spectra were taken in accordance with standard Bruker programs.

The ^1H NMR spectra of compounds (1) and (2) in deuteromethanol were obtained on a Tesla BS 567 A spectrometer (0-HMDS).

Isolation of Stachybotrin. A dry chloroform extract of strains of *Stachybotrys alternans* (12.8 g) was deposited on a column containing 300 g of Silpearl silica gel. The column was eluted successively with benzene and system 1. Elution with system 1 yielded 60 mg of stachybotrin.

Stachybotrin, $C_{25}H_{35}NO_5$, mp 202° (from MeOH), $[\alpha]_D^{24} -12.5 \pm 2°$(*c* 0.8; $CHCl_3$–MeOH, 1:1). Mass spectrum (KBr, ν, cm^{-1}): 3350-3140; 1675; 1650; 1630; 1475; 1360. Mass spectrum, *m/z* (%): M^+429 (95) [429.2483; $C_{25}H_{35}NO_5$], 411 (17.5) [411.2401; $C_{25}H_{33}NO_4$], 398 (100), 396 (11.3), 386 (16.3). [386.2319; $C_{23}H_{32}NO_4$], 380 (10) [380.2251; $C_{24}H_{30}NO_3$], 368 (2.8), 354 (2), 342 (1.9), 339 (1.8), 312 (1.9), 300 (3), 287 (3.3), 274 (10), [274.1097; $C_{15}H_{16}NO_4$], 260 (10), 256 (7.5), 242 (10), 234 (12.5), 223 (30), 221 (20), 207 (12.5), 189 (15), [189.1649, $C_{14}H_{21}$], 149 (12.5), 135 (12.5), 129 (12.5), 109 (12.5).

PMR spectrum (100 MHz, CD_3OD, δ, ppm, J, Hz): 0.70 (CH_3-18, d, ^3J=7 Hz), 0.85; 0.93; 1.01 (3 × CH_3, s), 2.79; 3.20 (2H-11, d, ^2J=18 Hz), 3.70 (4H-24/25, m, AA'BB' system), 4.35 and 4.55 (2H-22, d, ^2J=18 Hz), 6.65 (H-14, c). For the ^1H and ^{13}C NMR spectra, see Tables 1 and 2.

Stachybotrin 3,13,22-Triacetate (2) from (1). Stachybotrin (7.8 g) was treated with 0.5 ml of dry pyridine and 0.25 ml of acetic anhydride. The reaction mixture was stirred and was left at room temperature for 5 days. The solvent was evaporated off and the residue was chromatographed on a column with elution by system 2. This gave 6.7 mg of the noncrystalline triacetate (2), $C_{31}H_{41}NO_8$. IR spectrum (KBr, ν, cm^{-1}): 1770; 1743; 1726; 1700; 1616; 1459; 1373. Mass spectrum, *m/z* (%): M^+555 (15), 513 (3.75), 495 (100), 480 (10), 470 (5), 453 (37.5), 438 (10), 436 (10), 422 (5), 410 (10), 380 (5), 357 (6.25), 343 (6.25), 316 (12.5), 305 (30), 274 (8.75), 263 (20), 242 (10), 200 (45), 189 (23.8).

PMR spectrum (100 MHz, CD_3OD, δ, ppm, J, Hz): 0.68 (CH_3-18, d, ^3J=6 Hz), 0.85; 0.90; 1.00 (3 × CH_3, s), 1.94; 2.00; 2.26 (3 × CH_3COO, s), 2.78; 3.18 (2H-11, d, ^2J=18 Hz), 6.90 (H-14, s). For the PMR spectrum, see also Table 1.

Stachybotrin 13,22-Diacetate (3) from (1). Stachybotrin (20 mg) was acetylated with 0.5 ml of acetic anhydride in 1 ml of absolute pyridine at room temperature for 1 h. After the solvent had been evaporated off, the residue was chromatographed on a column with elution by system 3. This led to the isolation of 22 mg of the amorphous diacetate (3), $C_{29}H_{39}NO_7$. IR spectrum (KBr, ν, cm^{-1}): 3466; 1769; 1744; 1691; 1614; 1461; 1418; 1387; 1370. Mass spectrum, *m/z* (%): M^+513 (100), 495 (39.5), 480 (6.6), 471 (21.1), 453 (23.6), 440 (19.7), 428 (11.8), 410 (11.8), 398 (9.2), 316 (11.8), 305 (19.7), 242 (11.8), 189 (19.7), 175 (11.8), 135 (19.7), 119 (14.4), 107 (15.7), 95 (17.1), 81 (18.4), 69 (35.5), 55 (38.1). For the PMR spectrum, see also Table 1.

3-Dehydrostachybotrin 13,22-Diacetate (4) from (3). A solution of 14 mg of the diacetate (3) in 1 ml of acetone was cooled to −5°C. Then 2 drops of the Jones reagent [3] was added to the solution, and it was stirred at the same temperature for 10 min. The reaction was stopped by the addition of a few drops of methanol. After the usual work-up and evaporation of the solvents, the reaction product was chromatographed on a column with elution by system 2, giving 8 mg of the amorphous ketone (4), $C_{29}H_{37}NO_7$. IR spectrum (KBr, ν, cm^{-1}): 1765; 1745; 1696; 1615; 1460; 1417; 1386; 1369. Mass spectrum, *m/z* (%): M^+511 (50), 469 (25), 451 (5.3), 438 (9.2), 425 (13.1), 409 (5.3), 396 (7.8), 383 (6.9), 320 (6.9), 302 (2.6), 284 (5.3), 256 (19.7), 129 (15.7), 107 (15.7), 97 (36.8), 91 (18.4), 83 (27.6), 73 (42.1), 69 (65.8), 55 (100). For the PMR spectrum, see Table 1.

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