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TRICYCLOALTERNARENES PRODUCED BY *ALTERNARIA ALTERNATA* RELATED TO ACTG-TOXINS

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Key Word Index—*Alternaria alternata*; Fungi Imperfecti; phytotoxins; tricycloalternarenes; ACTG-toxins; structural elucidation.

Abstract—Ten metabolites, named tricycloalternarenes, were isolated from the culture filtrate of the phytopathogenic fungus, *Alternaria alternata*, and their structures elucidated by spectroscopic methods. Seven compounds are described for the first time. Tricycloalternarenes related to the ACTG-toxins from *A. citri* cannot be denoted as host-specific toxins. © 1997 Elsevier Science Ltd

INTRODUCTION

The genus *Alternaria* Nees ex Wallr. is well known as a source of many bioactive substances with vastly differing structures. Besides perylenes, dihydrobenzopyrones, dihydropyrones and quinones, these comprise tetramic acids, decatrienoic acids, cyclic peptides and others [1–3]. Many of these secondary metabolites are phytotoxic, acting as either host-specific or as non-specific toxins. The species, *A. alternata* [Fries] Keissler, is of particular scientific interest, because different pathotypes producing host-specific toxins exist, which are involved in phytopathogenic actions [4].

In some cases however, the opinion about the nature of such toxins must be corrected. For example, AAL-toxin produced by a pathotype of *A. alternata*, first seen as a host-specific toxin against tomatoes, must be denoted as non-specific, because of its additional actions on a number of weeds [5]. The evaluation of the ACTG-toxins first found in the tangerine pathotype of *A. alternata* also changed over the course of time; these compounds damage leaves of sensitive varieties of orange. At first the ACTG-toxins were regarded as host-specific [6–8], later this view became rather doubtful [9, 10].

Recently, we isolated two minor ACTG-toxins (D and E) from a strain of *A. alternata* that does not originate from *Citrus* species but from *Brassica sinensis* [11]. This strain has been in culture for 25 years. Several optimization procedures were carried out to yield a high content of the cyclic peptide tentoxin, a non-specific phytotoxin. The existence of ACTG-toxins in such a strain of *A. alternata* supports the opinion that these compounds are non-specific toxins. In this paper we report the isolation and structural elucidation of several novel ACTG-related compounds. In contrast to other ACTG-toxins (A–C, and F) [7] they show a tricyclic structure. Therefore, we will call them tricycloalternarenes (TCAs).

RESULTS AND DISCUSSION

Altogether, ten compounds with a TCA structure were isolated. To obtain an acceptable yield of all the compounds, two different culture media were used. In favour of TCAs **1** and **2** and TCAs **3**–**5** Richard's solution with Zn^{2+} and phosphate starvation medium were used, respectively. By continuous ether extraction of 20–30 ml of culture filtrate, and evaluation by TLC and analytical HPLC (details not shown here), TCA contents were estimated to vary between 1 and 6 $\mu g\ ml^{-1}$ of culture filtrate.

Isolation of the TCAs started with continuous ether extraction of culture filtrates of *A. alternata*. The extraction residue was suspended in hot water and filtered. Separation of the filtrate on a Sephadex-G-15 column revealed, besides tentoxin/dihydrotentoxin and other substances, TCAs **1a** and **2a** and TCAs **1b** and **2b** at V_E/V_0 3.5 and 4.4, respectively. By a preparative isocratic HPLC run in MeOH–H_2O (3:1), the four purified compounds were obtained. The residue of the hot water filtration containing the other

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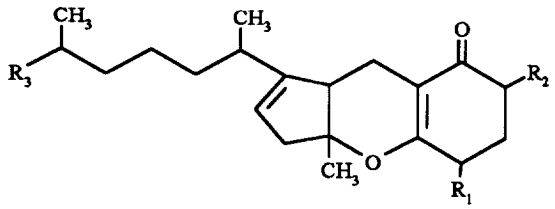
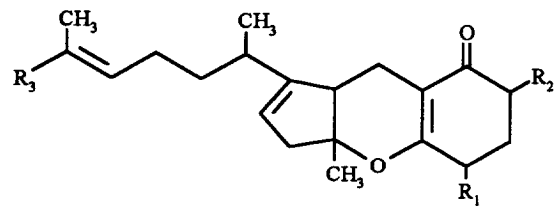


TCA **1a**: $R_1 = OH$, $R_2 = H$, $R_3 = CH_2OH$

TCA **1b**: $R_1 = H$, $R_2 = OH$, $R_3 = CH_2OH$



TCA **2a**: $R_1 = OH$, $R_2 = H$, $R_3 = CH_2OH$

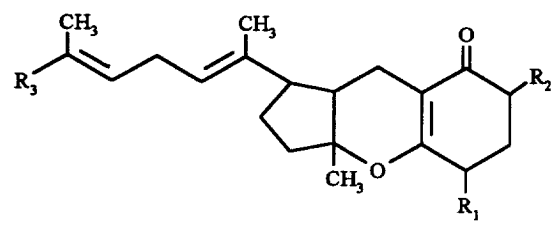
TCA **2b**: $R_1 = H$, $R_2 = OH$, $R_3 = CH_2OH$

TCA **3a**: $R_1 = OH$, $R_2 = H$, $R_3 = CH_3$

TCA **3b**: $R_1 = H$, $R_2 = OH$, $R_3 = CH_3$

TCA **4a**: $R_1 = OCH_3$, $R_2 = H$, $R_3 = CH_3$

TCA **4b**: $R_1 = H$, $R_2 = OCH_3$, $R_3 = CH_3$



TCA **5a**: $R_1 = OCH_3$, $R_2 = H$, $R_3 = CH_3$

TCA **5b**: $R_1 = H$, $R_2 = OCH_3$, $R_3 = CH_3$

TCAs were subjected to separation on a silica gel column to remove remaining cyclic peptides (with longer retention times). An additional run on a second silica gel column eluted the TCAs in the $CHCl_3$ fraction. By a final step of a preparative HPLC in MeOH–H_2O (17:3), purified TCAs **3–5** were obtained. The chromatographic data (TLC, analytical HPLC) of TCAs **1–5** are given in Table 1 arranged in order of decreasing polarity. The compounds are relatively unstable; diluted methanolic solutions at $-20°$ are necessary for long-term storage.

UV absorption of all TCAs at *ca* 265 nm can be attributed to the constant conjugation system in rings C and B (enone and oxygen). The structures of TCAs **1a** and **1b** are derived from data in Table 2 (Schemes 1 and 2). Both compounds have the molecular formula $C_{21}H_{32}O_4$ and the fragmentation patterns by EI mass spectrometry (Scheme 1). ^{13}C NMR data (Table 2) confirmed the molecular formulae of each showing 21 resonances, three of which were due to methyls, eight to methylenes, five to methines and five to quaternary carbons. The signals at δ_C 196.9 and 197.8 were each attributed to carbonyls, the fourth oxygen should be in the ring system. The structure of both compounds was deduced unambiguously from analysis of H,H-COSY, H,H-TOCSY and C,H-correlation (HMQC, HMBC). The position of the secondary hydroxyl in TCA **1b** was assigned to C-17 whose proton (δ_H 3.99, double doublet) gave a long-range cross-peak with the carbonyl in the HMBC experiment. TCA **1a** shows a similar cross-peak with the methylene (δ_H 2.56–2.21) that couples with δ_C 28.9; therefore, the hydroxyl must be situated at C-15. These structures were supported by mass spectral fragmentation. Three out of four oxygens are neighbouring, as seen in the relevant fragments (*m/z* 219 and 248). The different chromatographic behaviour of these closely related substances can be explained by a diminished polarity of TCA **1b** caused by the presence of the acyloin group.

Both TCA **2a** and **2b** have the molecular formula $C_{21}H_{30}O_4$, indicating an additional double bond in comparison with TCAs **1a** and **1b**. The change in the EI mass spectral fragmentation pattern (*m/z* 205 instead of 207 and retention of 133, 121, 107) showed

Table 1. Chromatographic data of TCAs

Solvent	TLC/R_f **1a/2a**	**1b/2b**	**3a**	**3b-5b**
I	0.25	0.32	0.41	0.55
II	0.35	0.45	0.55	0.67

Analytical HPLC/R_f(min) MeOH–H_2O (3:1)						MeOH–H_2O (17:3)			
2a	**1a**	**2b**	**1b**	**3a**	**3b**	**5a**	**5b**	**4a**	**4b**
6.20	6.93	9.57	10.48	7.22	9.81	9.89	10.46	11.35	11.90

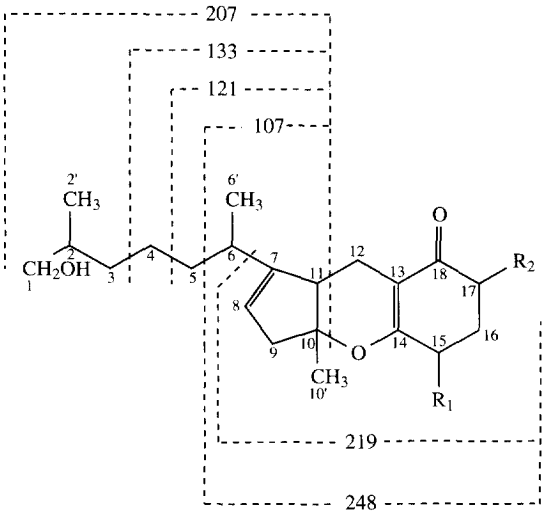
Table 2. ^1H and ^{13}C NMR data for TCAs **1a** and **1b** in $CDCl_3$

Carbon no.	**1a** δ_C	δ_H	DEPT	**1b** δ_C	δ_H	DEPT
1	68.2	3.39 (*m*)	CH_2	68.3	3.35/3.40 (*m*)	CH_2
2	35.6	1.52 (*m*)	CH	35.4	1.53 (*m*)	CH
2′	16.6	0.87 (*d*)	CH_3	16.5	0.84 (*d*)	CH_3
3	33.1	1.02/1.30 (*m*)	CH_2	33.0	0.98/1.30 (*m*)	CH_2
4	24.7	1.28/1.33 (*m*)	CH_2	24.6	1.17/1.28 (*m*)	CH_2
5	35.0	1.37/1.22 (*m*)	CH_2	35.0	1.19/1.40 (*m*)	CH_2
6	32.4	1.92 (*m*)	CH	32.4	1.97 (*m*)	CH
6′	20.2	0.92 (*d*)	CH_3	20.2	0.93 (*d*)	CH_3
7	150.4		C	150.3		C
8	119.6	5.27 (*s br*)	CH	119.8	5.29 (*s br*)	CH
9	44.9	2.58/2.42 (*m*)	CH_2	44.9	2.39/2.56 (*m*)	CH_2
10	88.8		C	88.4		C
10′	23.6	1.46 (*s*)	CH_3	23.3	1.39 (*s*)	CH_3
11	46.7	2.76 (*m*)	CH	46.3	2.72 (*m*)	CH
12	15.2	2.59 (*m*)	CH_2	15.2	2.17/2.67 (*m*)	CH_2
13	107.8		C	105.2		C
14	170.0		C	172.5		C
15	66.5	4.32 (*m*)	CH	27.8	2.35/2.47 (*m*)	CH_2
16	28.9	1.22/2.22 (*m*)	CH_2	29.5	1.70/2.30 (*m*)	CH_2
17	33.6	2.56/2.21 (*m*)	CH_2	70.9	3.99 (*dd*)	CH
18	196.9		C	197.8		C

the position of this olefin moiety in the side-chain between C-2 and C-3. Both ^{13}C and ^1H NMR data were similar to ACTG-toxins E and D [7].

In comparison with TCAs **1** and **2**, TCAs **3a** and **3b** (molecular formulae $C_{21}H_{30}O_3$) have one less oxygen, consistent with more apolar properties. The unchanging presence of the EI mass spectral fragment *m/z* 248, and the exchange of *m/z* 205 or 207 with *m/z* 189, proved that C-1 is a methyl group. NMR data (Table 3) also indicated the presence of four methyls. Two of these (each C-1 and C-2′) showed long-range cross peaks with δ_C 131.4 (C-2 in TCAs **3a** and **3b**), respectively. The allyl coupling of these methyls with δ_H 4.98 (**3a**) and 5.04 (**3b**) confirmed the position of this double bond in the side-chain. The positions of the hydroxyls on ring C were attributed in a comparable way to TCAs **1a** and **1b**. Additionally, the existence of only one proton at C-17 in TCA **3b** was proved by a ^{13}C-gated-decoupling experiment, showing the proton-coupled ^{13}C signal of the carbonyl group with δ_H 4.04 as a doublet. Further H,H-COSY and H,H-TOCSY data confirmed the presumed structures. TCA **3b** is identical with ACTG-toxin G; mass and ^1H NMR spectra of these compounds were obtained from acetate derivatives [12, 13] confirming our results.

Both TCAs **4a** and **b** had $[M]^+$ *m/z* 344 (EI mass spectrum), corresponding with the molecular formula $C_{22}H_{32}O_3$. The presence of ions at *m/z* 262 instead of 248 led us to presume the presence of methoxyl groups instead of hydroxyls in the ring system in both compounds. This was supported by signals for methyl



Scheme. 1.

singlets at δ_H 3.44. That the structure of TCAs **4a** and **4b** included the attachment of methoxyl groups was deduced unambiguously from analysis of H,H-COSY, H,H-TOCSY and long-range C,H-correlation data (Table 4).

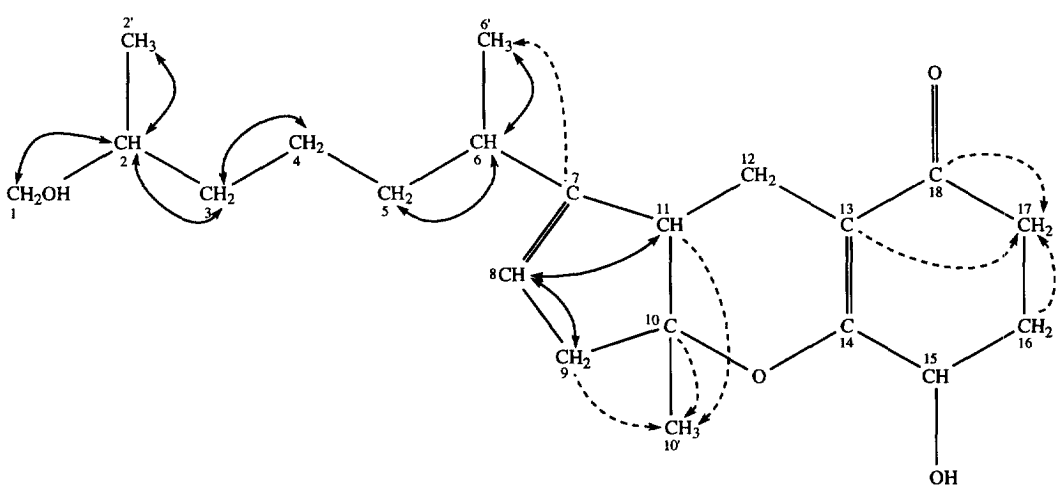
TCAs **5a** and **b** had molecular formulae identical to TCAs **4a** and **b**, but showed a modified mass spectral fragmentation pattern. The presence of the fragment *m/z* 235 instead of 262 could be due to a shift of the double bond from ring A to the side-chain at the position between C-5 and C-6 (*m/z* 133 was unchanged) and fragmentation between C-6 and C-7. NMR data (Table 5) supported by the mentioned homo- and hetero-nuclear correlations proved the supposed structures. Additionally, the position of the proton at C-11 in TCA **5a** was verified by a selected TOCSY experiment. NOESY data from both compounds suggested in each case a *cis* configuration of both the methyl C-1 and the proton at C-3 and the methyl C-6′ and the proton at C-5.

TCAs **2a**, **2b** and **3a** have already been isolated as ACTG 'minor toxins' from *A. citri* (=*A. alternata*, tangerine pathotype). The discovery of many such compounds in a strain of *A. alternata* originating from *B. sinensis* can be taken as further proof that ACTG toxins/tricycloalternarenes are non-specific toxins. It is, conceivable that TCAs function as consecutive metabolites of the more bioactive compounds with an opened B-ring, isolated partly as host-specific ACTG 'major toxins' [7]. Therefore, in future we will search for such substances in our strain. In addition, we will also investigate additional bioactivities of TCAs. TCAs **3a**, **3b**, **4a**, **4b** and **5b** at 5–200 μg ml^{-1} did not cause any symptoms in a bioassay with leaves of the original host plant, *B. sinensis*.

EXPERIMENTAL

General. The strain and cultivation of *A. alternaria* were as reported elsewhere [14]. The phosphate-deficiency medium was supplemented with 3.7 g NaOAc l^{-1} culture soln. Modified Richard's soln [11] was improved with 10 mg $ZnSO_4 \cdot 7H_2O$ l^{-1}.

Isolation procedures up to the run on Sephadex-G-15 were as reported in refs [15, 16]. Purification on a silica gel (Merck Kieselgel 60) column (30 × 2 cm, 25 ml h^{-1}) was carried out at first using EtOAc–MeOH–H_2O (100:4:1). Positive TCA frs were collected and evapd, the residue obtained were sepd on a second column by elution with 400 ml each of CH_2Cl_2 and $CHCl_3$ (stabilized with *ca* 1% EtOH). Prep. HPLC was performed on Hibar LiChrosorb RP-18 (250 × 25 mm, 7 μm, guard column 30 × 4 mm, 7 μm), detection: 260 nm, flow rate: 5 ml min^{-1}. TLC was done on Merck Kieselgel 60 F_{254}, using solvent I: toluene–EtOAc–HCO_2H (5:4:1), II: EtOAc–Me_2CO–*n*-hexane (2:1:1), detection: UV-quenching. Analytical HPLC was carried out on Hibar LiChrosorb RP-18 (250 × 4 mm, 5 μm, guard column 30 × 4 mm, 7 μm), detection: 260 nm, flow rate: 0.8 ml min^{-1}. EIMS were recorded at 75 eV. NMR measurements were performed using a 5 mm probe head with a z-gradient (^{1}H 400 MHz, ^{13}C 100 MHz).



Scheme. 2.

Table 3. ^{1}H and ^{13}C NMR data for TCAs **3a** and **3b** in $CDCl_3$

Carbon no.	**3a** δ_C	δ_H	DEPT	**3b** δ_C	δ_H	DEPT
1	25.6	1.61 (*s*)	CH_3	25.7	1.68 (*s*)	CH_3
2	131.4		C	131.4		C
2′	17.6	1.51 (*s*)	CH_3	17.6	1.58 (*s*)	CH_3
3	124.7	4.98 (*m*)	CH	124.4	5.04 (*m*)	CH
4	25.8	1.81 (*m*)	CH_2	25.9	1.90 (*m*)	CH_2
5	34.9	1.44/1.20 (*m*)	CH_2	34.9	1.49/1.30 (*m*)	CH_2
6	32.2	1.93 (*m*)	CH	32.3	2.05 (*m*)	CH
6′	20.0	0.89 (*d*)	CH_3	20.1	0.98 (*d*)	CH_3
7	150.3		C	150.2		C
8	119.7	5.28 (*s br*)	CH	119.8	5.34 (*s br*)	CH
9	44.8	2.43/2.58 (*m*)	CH_2	44.9	2.61/2.44 (*m*)	CH_2
10	88.8		C	88.3		C
10′	23.8	1.45 (*s*)	CH_3	23.3	1.44 (*s*)	CH_3
11	46.6	2.74 (*m*)	CH	46.2	2.75 (*m*)	CH
12	15.1	2.23/2.55 (*m*)	CH_2	15.4	2.69/2.24 (*m*)	CH_2
13	107.9		C	105.1		C
14	169.8		C	172.2		C
15	66.4	4.29 (*m*)	CH	27.8	2.50/2.38 (*m*)	CH_2
16	28.8	1.93/2.17 (*m*)	CH_2	29.4	2.32/1.75 (*m*)	CH_2
17	33.5	2.25/2.53 (*m*)	CH_2	70.9	4.04 (*dd*)	CH
18	196.7		C	197.6		C

Table 4. ^{1}H and ^{13}C NMR data for TCAs **4a** and **4b** in $CDCl_3$

Carbon no.	**4a** δ_C	δ_H	DEPT	**4b** δ_C	δ_H	DEPT
1	25.7	1.62 (*s*)	CH_3	25.5	1.60 (*s*)	CH_3
2	131.3		C	131.2		C
2′	17.6	1.52 (*s*)	CH_3	17.5	1.52 (*s*)	CH_3
3	124.5	5.00 (*m*)	CH	124.6	4.99 (*m*)	CH
4	25.9	1.84 (*m*)	CH_2	25.9	1.83 (*m*)	CH_2
5	34.9	1.26/1.45 (*m*)	CH_2	34.8	1.23/1.42 (*m*)	CH_2
6	32.3	1.96 (*m*)	CH	32.5	1.96 (*m*)	CH
6′	20.2	0.91 (*d*)	CH_3	19.9	0.88 (*d*)	CH_3
7	150.3		C	150.3		C
8	119.8	5.28 (*s br*)	CH	119.7	5.24 (*s br*)	CH
9	44.9	2.40/2.60 (*dq*)	CH_2	44.8	2.35/2.53 (*m*)	CH_2
10	88.0		C	87.8		C
10′	23.7	1.45 (*s*)	CH_3	23.6	1.36 (*s*)	CH_3
11	46.5	2.72 (*m*)	CH	46.4	2.67 (*m*)	CH
12	15.3	2.22/2.57 (*m*)	CH_2	15.4	2.18/2.58 (*m*)	CH_2
13	108.8		C	106.5		C
14	169.1		C	170.9		C
15	74.9	3.73 (*t*)	CH	26.9	2.28/2.42 (*m*)	CH_2
15′	58.1	3.44 (*s*)	CH_3			
16	26.9	1.91/2.06 (*m*)	CH_2	27.0	2.11/1.87 (*m*)	CH_2
17	32.2	2.22/2.61 (*m*)	CH_2	79.4	3.59 (*dd*)	CH
17′				57.9	3.44 (*s*)	CH_3
18	197.4		C	195.5		C

Table 5. ^1H and ^{13}C NMR data for TCAs **5a** and **5b** in $CDCl_3$

Carbon no.	**5a** δ_C	δ_H	DEPT	**5b** δ_C	δ_H	DEPT
1	25.6	1.62 (*d*)	CH_3	25.6	1.60 (*s*)	CH_3
2	131.3		C	131.2		C
2′	17.6	1.51 (*s*)	CH_3	17.5	1.50 (*s*)	CH_3
3	123.2	4.91 (*m*)	CH	123.2	4.92 (*m*)	CH
4	26.4	2.47 (*m*)	CH_2	26.4	2.57/2.42 (*m*)	CH_2
5	127.0	5.19 (*m*)	CH	127.1	5.17 (*m*)	CH
6	133.9		C	133.8		C
6′	18.2	1.58 (*d*)	CH_3	18.2	1.56 (*m*)	CH_3
7	41.6	2.73 (*m*)	CH	41.5	2.75 (*m*)	CH
8	25.4	1.77 (*m*)	CH_2	25.3	1.55/1.75 (*m*)	CH_2
9	37.8	2.09/1.79 (*m*)	CH_2	37.9	1.72/2.02 (*m*)	CH_2
10	87.0		C	86.9		C
10′	22.8	1.30 (*s*)	CH_3	22.6	1.22 (*s*)	CH_3
11	43.3	1.86 (*m*)	CH	43.2	1.84 (*m*)	CH
12	15.9	2.06/2.21 (*m*)	CH_2	15.9	1.98/2.26 (*m*)	CH_2
13	108.6		C	106.3		C
14	166.5		C	168.3		C
15	74.7	3.79 (*m*)	CH	27.1	2.48/2.36 (*m*)	CH_2
15′	58.1	3.46 (*s*)	CH_3			
16	26.9	2.14/1.97 (*m*)	CH_2	27.0	1.92/2.14 (*m*)	CH_2
17	32.2	2.26/2.65 (*t*)	CH_2	79.6	3.63 (*dd*)	CH
17′				58.0	3.46 (*s*)	CH_3
18	198.0		C	196.1		C

Tricycloalternarene **1a**. UV λ_{max}^{EtOH} nm (log ε): 265 (3.81). ^1H and ^{13}C NMR: Table 2. EIMS *m/z* (rel. int): 348 $[M]^+$ (45), 318 (5), 248 (18), 219 (13), 207 (100), 147 (12), 133 (14), 121 (30), 107 (92); exact mass calcd for $C_{21}H_{32}O_4$ 348.2301 found 348.2323.

Tricycloalternarene **1b**. UV λ_{max}^{EtOH} nm (log ε): 264 (3.94). ^1H and ^{13}C NMR: Table 2. EIMS *m/z* (rel. int): 348 $[M]^+$ (49), 318 (6), 248 (19), 219 (9), 207 (100), 147 (9), 133 (18), 121 (30), 107 (76); exact mass calcd for $C_{21}H_{32}O_4$ 348.2301 found 348.2320.

Tricycloalternarene **2a**. UV λ_{max}^{EtOH} nm (log ε): 265 (4.07). ^1H and ^{13}C NMR: identical with ref. [7]. EIMS *m/z* (rel. int): 346 $[M]^+$ (100), 328 (32), 318 (18), 248 (29), 219 (18), 205 (49), 187 (54), 147 (24), 133 (18), 121 (35), 107 (66); exact mass calcd for $C_{21}H_{30}O_4$ 346.2144 found 346.2164.

Tricycloalternarene **2b**. UV λ_{max}^{EtOH} nm (log ε): 264 (4.04). ^1H and ^{13}C NMR: identical with ref. [7]. EIMS *m/z* (rel. int): 346 $[M]^+$ (86), 328 (71), 318 (16), 300 (17), 248 (32), 219 (20), 205 (72), 187 (100), 147 (31), 133 (24), 121 (40), 107 (64); exact mass calcd for $C_{21}H_{30}O_4$ 346.2144 found 346.2158.

Tricycloalternarene **3a**. UV λ_{max}^{EtOH} nm (log ε): 262 (4.09). ^1H and ^{13}C NMR: Table 3. EIMS *m/z* (rel. int): 330 $[M]^+$ (79), 248 (22), 219 (14), 189 (58), 147 (38), 133 (30), 121 (42), 107 (100); exact mass calcd for $C_{21}H_{30}O_3$ 330.2195 found 330.2184.

Tricycloalternarene **3b**. UV λ_{max}^{EtOH} nm (log ε): 264 (4.14). ^1H and ^{13}C NMR: Table 3. EIMS *m/z* (rel. int): 330 $[M]^+$ (75), 248 (68), 219 (14), 189 (64), 147 (27), 133 (31), 121 (39), 107 (100); exact mass calcd for $C_{21}H_{30}O_3$ 330.2195 found 330.2207.

Tricycloalternarene **4a**. UV λ_{max}^{EtOH} nm (log ε): 265 (4.14). ^1H and ^{13}C NMR: Table 4. EIMS *m/z* (rel. int): 344 $[M]^+$ (100), 262 (39), 230 (12), 189 (49), 147 (26), 133 (25), 121 (32), 108 (86), 107 (74); exact mass calcd for $C_{22}H_{32}O_3$ 344.2351 found 344.2352.

Tricycloalternarene **4b**. UV λ_{max}^{EtOH} nm (log ε): 266 (4.16). ^1H and ^{13}C NMR: Table 4. EIMS *m/z* (rel. int): 344 $[M]^+$ (86), 314 (32), 262 (52), 232 (82), 189 (63), 147 (37), 133 (30), 121 (36), 108 (100), 107 (94); exact mass calcd for $C_{22}H_{32}O_3$ 344.2351 found 344.2355.

Tricycloalternarene **5a**. UV λ_{max}^{EtOH} nm (log ε): 264 (4.11). ^1H and ^{13}C NMR: Table 5. EIMS *m/z* (rel. int): 344 $[M]^+$ (66), 235 (37), 189 (100), 147 (34), 133 (20), 119 (25), 105 (54); exact mass calcd for $C_{22}H_{32}O_3$ 344.2351 found 344.2345.

Tricycloalternarene **5b**. UV λ_{max}^{EtOH} nm (log ε): 264 (4.12). ^1H and ^{13}C NMR: Table 5. EIMS *m/z* (rel. int): 344 $[M]^+$ (83), 235 (39), 189 (100), 147 (31), 133 (22), 119 (22), 105 (47); exact mass calcd for $C_{22}H_{32}O_3$ 344.2351 found 344.2350.

A bioassay was performed with *Brassica sinensis* (Nippon F1 hybrid), greenhouse-grown plants with 5 whorls. TCAs in H_2O–EtOH (9:1) were injected (5 μl) into the secondary leaf veins. Evaluation for phytotoxic symptoms was carried out after 72 hr.

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