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Bicycloalternarenes produced by the phytopathogenic fungus *Alternaria alternata*

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Abstract

Eleven compounds, ACTG toxins A and B and nine new substances named bicycloalternarenes (BCAs), were isolated and characterized from the culture filtrate of the phytopathogenic fungus *Alternaria alternata*. Under acidic conditions these mixed terpenoids convert to pairs of tricycloaltenarenes that can be used for identification of the native compounds. We could not confirm the phytotoxic effects reported with ACTG-toxins in the past. However, BCA 2 at concentrations of 10^{-4} M strongly inhibited germination and growth of *Spirodela polyrhiza* turions.

Keywords: *Alternaria alternata*; Fungi imperfecti; Phytotoxins; Bicycloalternarenes; Tricycloalternarenes; ACTG-toxins; Structural elucidation

1. Introduction

The *Alternaria* genus belongs to the most common Deuteromycetes occurring in nature. The media of choice are living, weakened or dead plants where fungi live as pathogens, weak facultative parasites or saprophytes. *Alternaria* species produce many secondary metabolites. These compounds act not only as phytotoxins in infected plants but also have other biological activities. For example, such substances can contaminate foods and feeds and elicit mycotoxic effects. In the early 1990s, about 70 bioactive metabolites of *Alternaria,* most of which are produced by *A. alternata* (Fr.) Keissler and its pathotypes, were known (Montemurro and Visconti, 1992). An additional number of such compounds have since been described.

The chemical structures of these *Alternaria* metabolites vary a great deal. Among others are some mixed terpenoids with a distinct isoprenoid side chain, for instance zinniol (King and Schade, 1984), porritoxin (Suemitsu et al., 1992), zinnimidine and 5-(3′,3′-dimethylallyloxy)-7-methoxy-6-methylphthalide (Suemitsu et al., 1995). Three isoprenoid units are recognizable in the ACTG-toxins, compounds that are partly tricyclic and partly bicyclic in structure (Kono et al., 1986). Recently, we succeeded in the isolation and structure elucidation of 19 tricycloalternarenes (TCAs) closely related to the ACTG-toxins D and E (Liebermann et al., 1997; Nussbaum et al., 1999).

In this paper we report the isolation and structure elucidation of a further 11 compounds biosynthesized by *A. alternata*. In contrast to the related TCAs they show a bicyclic structure, hence they are designated bicycloaltemarernes.

2. Results and discussion

2.1. Isolation and structure elucidation

Isolation of BCAs, which are more hydrophilic than the TCAs, started with a methanolic extraction of the lyophilized culture filtrate of *A. alternata*. The extraction residue dissolved in water was passed through a Sephadex G-15 column, in order to separate the total BCAs from the bulk of the tentoxin/dihydrotentoxin also produced by *A. alternata*. The BCAs were isolated by preparative HPLC, after a gradient elution to acquire groups of similar compounds, the final purification was accomplished by isocratic elution. Both Sephadex

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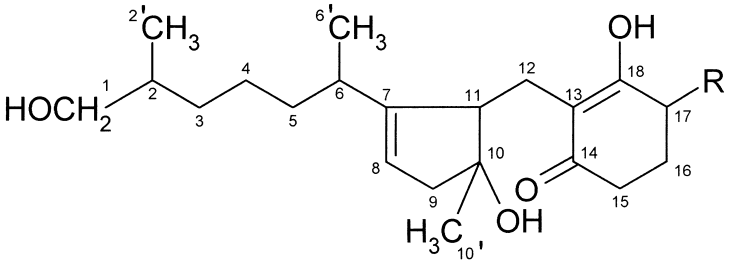
chromatography and HPLC were carried out in 0.001 N NH_3 to stabilize the BCAs in their enolate form. Otherwise, multiple peaks and variable retentions times are observed. In all we isolated 11 compounds (Table 1). The numbering of these BCAs corresponds to that of the related TCAs whose numeration has been denoted independently from their chemical structure (Liebermann et al., 1997; Nussbaum et al., 1999).

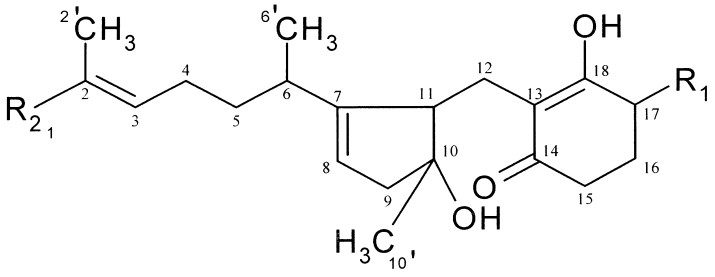
In connection with the previous isolation and structure elucidation of the TCAs in the past we already detected the existence of structurally related compounds in the culture filtrate. In comparison with the TCAs, the B-ring appeared not to be closed. In order to verify this assumption, the isolated substances were analyzed with standard spectrometric methods, partly with all compounds and partly, because of the evident close relationship, only with selected BCAs.

In ammoniacal MeOH, all BCAs show UV-maxima at 292–296 nm. However, in neutral medium BCAs take on the same absorption maxima as TCAs (262–264 nm). This is in accordance with the enolate/enol tautomerism in the nonterpenoid 1,3-diketone part of the molecule. The molecular masses of all the BCAs were revealed very well by electro spray ionization mass spectrometry (ESIMS) that show each an increase by one H_2O unit in comparison with the related TCAs. However, by EIMS, because of the rapid ring closure through the loss of water, BCAs yield only a very small molecular ion peak

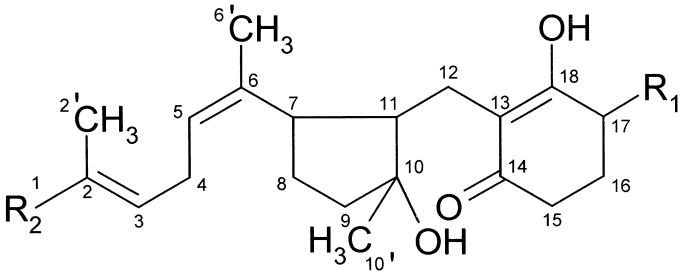
Table 1
Survey of isolated BCAs. Structure proposal with asterisk



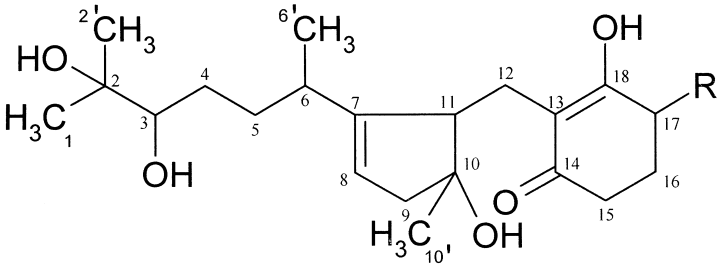
BCA **1**: R = OH
BCA **11***: R = OCH_3



BCA **2**: R_1 = OH, R_2 = CH_2OH
BCA **3**: R_1 = OH, R_2 = CH_3
BCA **4** (ACTG A): R_1 = OCH_3, R_2 = CH_3
BCA **10**: R_1 = OCH_3, R_2 = CH_2OH



BCA **5**: R_1 = OCH_3, R_2 = CH_3
BCA **8**: R_1 = OH, R_2 = CH_2OH
BCA **9** (ACTG B): R_1 = OH, R_2 = CH_3



BCA **6**: R = OH
BCA **7**: R = OCH_3

and to a great extent the same mass spectra as the corresponding TCAs (studied only with BCA **2** and **11**). The IR-spectrum of BCA **4** differs from those of TCAs by the lack of the band at about 1620 cm^{-1} indicating the absence of the enolether.

The immediate application of NMR spectroscopy to elucidate the BCA structure is not useful, because the keto-enol tautomerism in the nonterpenoid part of the molecules and/or aggregation phenomena due to hydroxyl groups lead to both ^{1}H- and ^{13}C-signals that are broadened. Table 2 shows these data for BCA **4** in comparison with that of the related compound TCA **4b**. A conversion of BCA **4** with trichlorous acetylic isocyanate gave rise to three proton signals with chemical shifts far downfield. Besides the signal at δ_H 11.15, which orginated from the hydroxyl at C-10, two signals at δ_H 10.22 and 9.59, each half of a full proton signal, confirmed the keto-enol tautomerism between C-14 and C-18.

In order to obtain interpretable NMR spectra the BCAs were converted to the related TCAs. Scheme 1 shows the mechanism of the conversion carried out by acidification with HCI (pH < 3). Due to the keto-enol tautomerism of the 1,3-diketone structure in the six-membered ring of the BCAs and because of the free rotation around C-12/C-13 two isomeric TCAs orginate from each BCA. ^{1}H- and ^{13}C-spectra including DEPT, ^{1}H,^{1}H–COSY, ^{1}H,^{1}H–TOCSY and ^{13}C,^{1}H–correlation (HMQC, HMBC) of the corresponding TCAs were recorded and compared with the data in literature (Kono et al., 1986; Liebermann et al., 1997; Nussbaum et al., 1999). As a rule both TCAs were measured, but the structure elucidation of BCA **8** was carried out only by using the corresponding TCA a type, BCAs **9** and **10** only by the TCA b types.

Table 2
^{1}H and ^{13}C data for BCA **4** in deuterated dioxan in comparison with TCA **4b** in $CDCl_3$ (data in parentheses, lit. Liebermann et al., 1997). n.d., not determined due to signal broadening

Carbon no.	δ_C	δ_H	DEPT
1	25.8 (25.5)	1.64 s (1.60 s)	CH_3
2	131.5 (131.2)		C
2′	17.4 (17.5)	1.58 s (1.52 s)	CH_3
3	125.6 (124.6)	5.10 m (4.99m)	CH
4	26.6 (25.9)	n.d. (1.83 m)	CH_2
5	35.6 (34.8)	n.d. (1.23/1.42 m)	CH_2
6	32.9 (32.5)	n.d. (l.96m)	CH
6′	19.7 (19.9)	1.13 d (0.88 d)	CH_3
7	152.6 (150.3)		C
8	118.0 (119.7)	5.l7s br (5.24s br)	CH
9	48.2 (44.8)	n.d. (2.35 / 2.53 m)	CH_2
10	80.4 (87.8)		C
10′	28.3 (23.6)	1.19 s (1.36 s)	CH_3
11	54.7 (46.4)	n.d. (2.67 m)	CH
12	20.3 (15.4)	n.d. (2.18/2.58m)	CH_2
13	113.0 (106.5)		C
14	n.d. (170.9)		C
15	n.d. (26.9)	n.d. (2.28 / 2.42 m)	CH_2
16	27.0 (27.0)	n.d. (2.11 / 1.87m)	CH_2
17	80.0 (79.4)	3.64 dd br (3.59 dd)	CH
17′	57.4 (57.9)	3.32 s (3.44 s)	CH_3
18	n.d. (195.5)		C

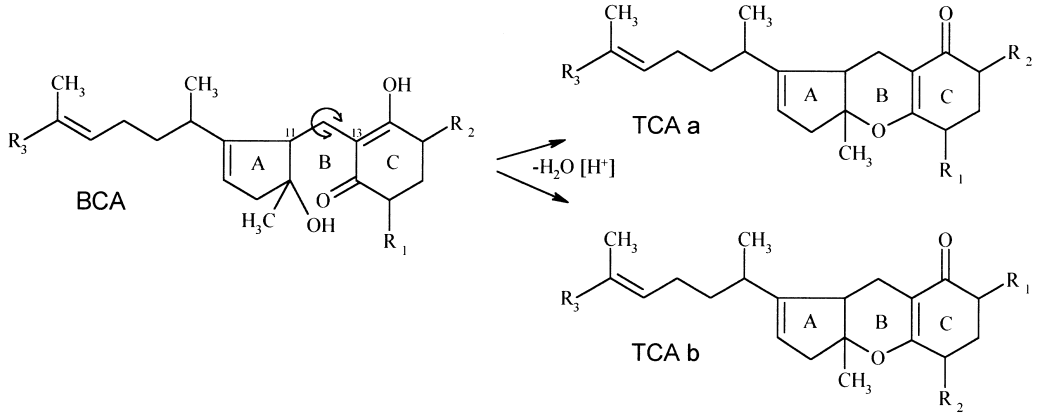
Additional proofs of the structure come from the comparison of the MS-spectra of TCAs converted from BCAs and that of literature (see above). Because the quantities of TCAs **11a** and **11b** were too small for NMR, the structure of the parent compound BCA **11** was only suggested according to EIMS data and chromatographical behaviour of the corresponding TCAs. Thus the BCA **11** structure is only tentative.

The following amounts (mg l^{-1}) could be isolated from a well-producing culture: BCA **6** (53), BCA **2** (38), BCA **7** (23), BCA **1** (18), remaining BCAs (5–8). The BCAs produced by *A. alternata* and secreted into the culture filtrate are the native compounds rather than the TCAs, based on following facts. In the untreated culture filtrate (pH 7.5–8) the content of the total BCAs is 10- to 20-fold higher (Liebermann et al., submitted) than that of the TCAs (Liebermann et al., 1997). Acidification of the culture filtrate causes a considerable increase of the TCAs at the expense of the BCAs (Nussbaum et al., 1999). The conversion of purified BCAs to the pairs of TCAs is achieved in some minutes, but a re-conversion to the BCAs needs strongly alkaline conditions for several days and is mostly incomplete. In the biosynthesis of these compounds it is most likely that BCA structures are formed first (Liebermann et al., submitted).

2.2. Bioassays

We tested the BCAs **2**, **6**, and ACTG-toxin A (≡BCA **3**) as well as the ACTG-toxins E and D (≡TCAs **2a** and **2b**) both in a leaf puncture assay and a cutting assay, positive results arise as spreading dark lesions and wilting/necrosis effects, respectively. Plant material from *Citrus reticulata* var. Dancy was used. This is a host plant of the tangerine pathotype of *A. alternata* which produces the ACTG-toxins (Kono et al., 1986). Gardner et al. (1986) reported that ACTG-toxins were host-selectively active in both assays. We could not confirm these results, both assays were absolutely negative showing no toxic influence even at 1000 μ ml^{-1}. This discrepancy can be explained by the fact that Gardner et al. used only a partially purified preparation in the tests. Probably, the effects were initiated by the coisolated ACT-toxins found some time later in the same leaf puncture assay as host-specific toxins of the tangerine pathotype of *A. alternata* (Kohmoto et al., 1993). By all means BCAs and TCAs (and with that the ACTG-toxins) cannot be denoted as host-specific phytotoxins.

No biological effects at all were recorded in bioassays with several TCAs and BCA **2** against bacteria (gram negative, gram positive) and phytopathogenic fungi (details not shown here). However, in bioassays with



Scheme 1. Formation of two isometric TCAs from a parent BCA. Due to the keto-enol tautomerism of the 1,3-diketone structure in the six-membered ring of the BCAs and because of the free rotation two isomeric TCAs orginate from each BCA. Those TCAs, which carry the oxygen substituent in γ-position to the keto oxygen were named with the letter a.

turions (buds of aquatic vascular plants) of *Spirodela polyrhiza* BCA **2** strongly inhibited the leaf growth at 10^{-4} M, but at 10^{-5} and 10^{-6} M we found a slight stimulation. Germination of "dark" turions were completely inhibited at 10^{-4} M. 50–60% inhibited at 10^{-5} M, and slightly stimulated at 10^{-6} M and 10^{-7} M. Moreover, a typical so called "double ear germinations" took place at a concentration at 10^{-5} M. These effects are similar to those of jasmonic and methyl jasmonic acid in the turion bioassay. But further bioassays (hypertrophy test on potato discs, influence on the jasmonate induced protein 23) to confirm a jasmonate-like effect were negative (details not shown here).

So far we have no clear idea about the exact biological significance of BCAs and TCAs. They probably function as nonspecific phytotoxins together with other compounds. Perhaps these mixed terpenoids with a great variety of related structures can serve as a pool for the biosynthesis of other bioactive compounds.

3. Experimental

3.1. General

The strain and cultivation of *A. alternata* were described elsewhere (Nussbaum et al., 1999). Bioassays were performed as described: leaf puncture assay (Kohmoto et al., 1979), cutting assay (Gardner et al., 1986), turion assay (Appenroth et al., 1996).

Separation on Sephadex G15 is described in Liebermann et al. (1988). BCAs were eluted in a V_E/V_0-range of 1.30–1.95. Preparative HPLC of BCAs was carried out with MeOH–H_2O(0.001 N NH_3) using a linear gradient: 0–5 min 10–20% MeOH, 5–80 min 20–65% MeOH, 80–85 min 65–90% MeOH, conditions: C-l8 Polymer column (Astec), 300×21.5 mm, 9 μm, guard column 50×7.6 mm, 13 μm, flow rate 5 ml/min. Further purification was done on the same column system: BCAs **6** and **7** with MeOH–H_2O(0.001 N NH_3) (1:4), BCAs **1**, **2**, **8**, **10**, and **11** with (2:3), BCAs **3**, **4**, **5**, and **9** with (1:1). Analytical HPLC of BCAs was carried out with MeOH–H_2O(0.001 N NH_3) using also a linear gradient: 0–38 min 10–50% MeOH. 38–45 min 50%, 45–50 min 50–90%, conditions: C-8 Polymer column (Astec), 250×4.6 mm, 5 μm, guard column 10×4.6 mm, 5 μm, flow rate 0.6 ml/mm. Detection in both cases: 294 nm.

NMR measurements were performed on a BRUKER DRX 400 spectrometer using a 5 mm probe head with a z-gradient (^{1}H 400 MHz, ^{13}C 100 MHz). Data were acquired with BRUKER standard pulse programmes and processed using the BRUKER XWIN-NMR software. EIMS were recorded at 70 eV, ESIMS: temperature of the ion source 65°C, flow injection, sample in MeOH–H_2O (99:1) with or without NH_4Ac, scan mode 100–2000 Da/e.

TLC was done on Merck RP-18 F_{254}, solvent: MeOH–H_2O(0.01 N NH_3) (9:1), Detection: UV-quenching and anisic aldehyde spraying reagent.

Conversion to TCAs was done by evaporation of methanolic HC1 solutions of BCAs. Separation of TCA pairs by HPLC and all data for their identification were described recently (Liebermann et al., 1997; Nussbaum et al., 1999).

3.2. Physico-chemical properties

3.2.1. Bicycloalternarene *1*

Analytical HPLC (ret. time): 32.58 min, TLC (R_f): 0.33. UV λ_{max} (MeOH) nm: 292. ESI$^+$MS, *m/z* (rel.int.): 389 ([M + Na^+], 100), 367 ([M + H^+], 18), 349 ([M–H_2O + H^+], 54), 331 ([M–2H_2O + H^+], 2). ESI$^-$MS, *m/z* (rel.int.): 365 ([M–H^+], 100). After conversion to TCA **1a** and **1b**, both ^{1}H- and ^{13}C-NMR as well as EIMS and chromatographical data were in agreement with lit. (Liebermann et al., 1997).

*3.2.2. Bicycloalternarene **2***

Analytical HPLC (ret. time): 28.44 min, TLC (R_f): 0.39. UV λ_{max} (MeOH) nm (log ε): 292 (4.33). ESI^+MS [with NH_4Ac], *m/z* (rel.int.): 387 ([$M+Na^+$], 100), 365 ([$M+H^+$], 18), 347 ([$M–H_2O+H^+$], 9), 329 ([$M–2H_2O+H^+$], 18). EIMS, *m/z* (rel.int.): 364 ($C_{21}H_{32}O_5$ [M^+], 1), 346.21359 ($C_{21}H_{30}0_4$, 19), 328.20279 ($C_{21}H_{28}O_3$, 29), 287.16290 ($C_{18}H_{23}0_3$, 23), 261 (8), 248.14250 ($C_{15}H_{20}0_3$, 50), 219 (21), 205.15919 ($C_{14}H_{21}O$, 38), 187.14819 ($C_{14}H_{19}$, 69), 145 (50), 121 (52), 107 (100). After conversion to TCA **2a** and **2b**, both ^{1}H- and ^{13}C-NMR data are similar to lit. (Kono et al., 1986). EIMS and chromatographical data were in agreement with (Liebermann et al., 1997).

*3.2.3. Bicycloalternarene **3** (ACTG-toxin A)*

Analytical HPLC (ret. time): 42.96 min, TLC (R_f): 0.18. UV λ_{max} (MeOH) nm: 293. After conversion to TCA **3a** and **3b**, both ^{1}H- and ^{13}C-NMR as well as EIMS and chromatographical data were in agreement with lit. (Liebermann et al., 1997).

*3.2.4. Bicycloalternarene **4***

Analytical HPLC (ret. time): 43.13 min TLC (R_f): 0.16. UV λ_{max} (MeOH) nm (log ε): 296 (4.23). ESI^+MS, *m/z* (rel.int.): 385 ([$M+Na^+$], 61), 363 ([$M+H^+$], 84), 345 ([$M–H_2O+H^+$], 100), 327 ([$M–2H_2O+H^+$], 6). ESI^-MS, *m/z* (rel.int.): 361 ([$M\text{-}H^+$], 100). IR: ν[cm^{-1}] = 465, 867, 925, 991, 1083, 1118, 1208, 1309, 1403, 1451, 1497, 1590, 2861, 2924, 3050, 3391. ORD (23°, *c* 0.66, MeOH): $[\alpha]_{589}$ +114.3°, $[\alpha]_{546}$ +138.3°, $[\alpha]_{495}$ +186.5°, $[\alpha]_{436}$ +288,7°. After conversion to TCA **4a** and **4b**, both ^{1}H- and ^{13}C-NMR as well as EIMS and chromatographical data were in agreement with lit. (Liebermann et al., 1997).

*3.2.5. Bicycloalternarene **5***

Analytical HPLC (ret. time): 38.69 min TLC (R_f): 0.22. UV λ_{max} (MeOH) nm: 295. ESI^+MS, *m/z* (rel.int.): 385 ([$M+Na^+$], 68), 363 ([$M+H^+$], 90), 345 ([MH_2O+H^+], 100), 327 ([$M–2H_2O+H^+$], 9). ESI^-MS, *m/z* (rel.int.): 361 ([$M–H^+$], 100). After conversion to TCA **5a** and **5b**, both ^{1}H- and ^{13}C-NMR as well as EIMS and chromatographical data were in agreement with lit. (Liebermann et al., 1997).

*3.2.6. Bicycloalternarene **6***

Analytical HPLC (ret. time): 15.40 min, TLC (R_f): 0.60. UV λ_{max} (MeOH) nm: 292. ESI^+MS [with NH_4Ac], *m/z* (rel.int.): 405 ([$M+Na^+$], 100), 383 ([$M+H^+$], 53), 365 ([$M–H_2O+H^+$], 30), 347 ([$M–2H_2O+H^+$], 15). ESI^-MS, *m/z* (rel.int.): 381 ([$M\text{-}H^+$], 100). After conversion to TCA **6a** and **6b**, both ^{1}H- and ^{13}C-NMR as well as EIMS and chromatographical data were in agreement with lit. (Nussbaum et al., 1999).

*3.2.7. Bicycloalternarene **7***

Analytical HPLC (ret. time): 17.94 min, TLC (R_f): 0.55. UV λ_{max} (MeOH) nm: 295. ESI^+MS, *m/z* (rel.int.): 419 ([$M+Na^+$], 100), 397 ([$M+H^+$], 51), 379 ([MH_2O+H^+], 37), 361 ([$M–2H_2O+H^+$], 13). ESI^-MS, *m/z* (rel.int.): 395 ([$M\text{-}H^+$], 100). After conversion to TCA **7a** and **7b**, both ^{1}H- and ^{13}C-NMR as well as EIMS and chromatographical data were in agreement with lit. (Nussbaum et al., 1999).

*3.2.8. Bicycloalternarene **8***

Analytical HPLC (ret. time): 24.07 min, TLC (R_f): 0.40. UV λ_{max} (MeOH) nm: 293. ESI^+MS, *m/z* (rel.int.): 387 ([$M+Na^+$], 100), 365 ([$M+H^+$], 2). ESI^-MS, *m/z* (rel.int.): 363 ([$M–H^+$], 100). After conversion to TCA **8a**, both ^{1}H- and ^{13}C-NMR as well as EIMS and chromatographical data were in agreement with lit. (Nussbaum et al., 1999).

*3.2.9. Bicycloalternarene **9** (ACTG-toxin B)*

Analytical HPLC (ret. time): 42.96 min, TLC (R_f): 0.18. UV λ_{max} (MeOH) nm: 295. After conversion to TCA **9b**, both ^{1}H- and ^{13}C-NMR as well as EIMS and chromatographical data were in agreement with lit. (Nussbaum et al., 1999).

*3.2.10. Bicycloalternarene **10***

Analytical HPLC (ret. time): 29.74 min, TLC (R_f): 0.35. UV λ_{max} (MeOH) nm: 294. ESI^+MS, *m/z* (rel.int.): 401 ([$M+Na^+$], 100), 379 ([$M+H^+$], 9), 343 ([$M–2H_2O+H^+$], 7). ESI^-MS, *m/z* (rel.int.): 377 ([$M\text{-}H^+$], 100). After conversion to TCA **10b**, both ^{1}H- and ^{13}C-NMR as well as chromatographical data were in agreement with lit. (Nussbaum et al., 1999).

*3.2.11. Bicycloalternarene **11***

Analytical HPLC (ret. time): 32.42 min. TLC (R_f): 0.30. UV λ_{max} (MeOH) nm: 292. ESI^+MS, *m/z* (rel.int.): 403 ([$M+Na^+$], 100), 381 ([$M+H^+$], 44), 363 ([MH_2O+H^+], 71), 345 ([$M–2H_2O+H^+$], 1). ESI^-MS, *m/z* (rel.int.): 379 ([$M\text{-}H^+$], 100). EIMS *m/z* (rel.int.): 380.25879 ($C_{22}H_{36}0_5$, [M^+], 6), 362.24571 ($C_{22}H_{34}0_4$, 50), 344 (6), 332.23361 ($C_{21}H_{32}O_3$, 25), 262.15838 ($C_{16}H_{22}0_3$, 27), 251 (13), 235 (19), 233 (13), 225 (10), 220 (25), 207.17460 ($C_{14}H_{23}O$, 71), 177 (8), 155 (10), 133 (25), 121 (43), 108 (100), 107 (99); exact mass calcd for $C_{22}H_{36}O_5$ 380.25628 found 380.25879. After conversion to TCA **11**a and **11**b, EIMS and chromatographical data were in agreement with lit. (Nussbaum et al., 1999).

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