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Anthracenone ABA analogue as a potential photoaffinity reagent for ABA-binding proteins

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Abstract

An anthracenone analogue of abscisic acid (ABA) was synthesized as a potential photoaffinity reagent and tested for biological activity. Reaction between 10,10′-dimethoxy-9-anthrone with two equivalents of the lithiated dianion of *cis*-3-methylpent-2-en-4-yn-1-ol afforded an acetylenic alcohol key intermediate. Subsequent reduction of the triple bond, functional group manipulation of the side chain alcohol and deprotection of the dimethoxy protected anthrone provided anthracenone ABA analogue **7** as a potential photoaffinity reagent for ABA-binding proteins. The effect of natural ABA and the potential photoaffinity anthracenone ABA **7** on corn cell growth was determined at various concentrations. The results show that anthracenone ABA **7** is perceived as ABA-like, although producing less inhibition than ABA itself. For example, **7** at 33 μM produces approximately the same inhibition as ABA at 10 μM.

Keywords: Growth inhibition; Photoaffinity probes; Abscisic acid; Benzophenone; Anthracenone

1. Introduction

The plant hormone abscisic acid [(S)-(+)-ABA (anthracenone analogue of abscisic acid), (**1**)] regulates diverse aspects of plant growth including development and germination of seeds, transpiration, and adaptive responses to environmental stresses (Addicott & Carns, 1983; Zeevaart & Creelman, 1988; Hetherington & Quatrano, 1991; Davies & Jones, 1991). Considerable progress has been made in the identification of ABA-responsive genes, mutant characterization (Bray, 1993; Chandler & Robertson, 1994), localization of ABA perception in the cell (Gilroy & Jones, 1994) and understanding the ABA signal transduction pathway (Grill & Himmelbach, 1998; Leung & Giraudat, 1998). Advances also include cloning genes involved in ABA biosynthesis (Schwartz, Tan, Gage, Zeevaart & McCarty, 1997) and characterizing ABA catabolizing enzymes (Windsor & Zeevaart, 1997; Cutler, Squires, Loewen & Balsevich, 1997; Krochko, Abrams, Loewen, Abrams & Cutler, 1998). However, no ABA-binding proteins that interact directly with ABA have been isolated to date. One of the goals of our research is to develop useful, bioactive ABA photoaffinity reagents as tools to identify ABA-binding proteins involved in the regulation of specific ABA functions.

Photoaffinity reagents are specifically designed analogues of a ligand that under photolysis conditions form short-lived, highly reactive intermediates that will cross-link irreversibly in the ligand-binding site of the protein. Aromatic azides, diazo groups, and benzophenones (BP) are examples of photoreactive groups that have been used successfully in photoaffinity labeling (Jones, Mehadoi, Ho, Pearce & Leonard, 1984; Hooley, Beale, Smith & MacMillan, 1990; Fleming, 1995). Photoaffinity labeling requires that the ligand analogue be stable under the experimental conditions employed

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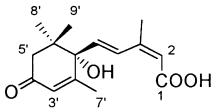
(until the point of photolysis) and biologically active in the same manner as the parent compound (i.e. fit into the same binding site). The photochemical conditions required to activate the analogue should not damage cells or denature proteins. Photoaffinity labeling has been used successfully in the identification of auxin- (Jones, 1994), fusicoccin- (Feyerabend & Weiler, 1989) and gibberellin- (Lovegrove, Barret, Beale & Hooley, 1998) binding proteins; however, there has been limited success to date in the isolation of ABA-binding proteins using these methods.



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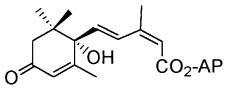

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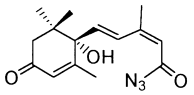

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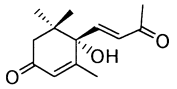

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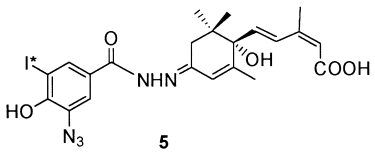

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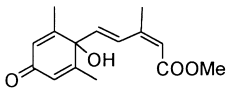

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In 1984, Hornberg and Weiler reported that binding of unmodified ABA to plasmalemma proteins occurred upon irradiation of guard-cell protoplasts (Hornberg & Weiler, 1984); however, these ABA-binding proteins were not characterized or subsequently reported. In 1995, Cornelussen and coworkers (Cornelussen, Karssen & van Loon, 1995) determined the optimal conditions for UV-induced cross-linking of ABA through its enone chromophore to anti-ABA monoclonal antibodies. They found that ABA linked to alkaline phosphatase (AP) through the C-1 carboxylic acid (**2**) could potentially be used as a photoaffinity reagent. However, such a modification would only be useful in assays where the C-1 acid was not critical for biological activity, and where the added bulk of the protein would not interfere in transport to the active site of a binding protein.

Milborrow and Willows (Willows & Milborrow, 1993) have reported the synthesis and physical properties of the acyl azido derivative of ABA **3**, which showed good biological activity in growth inhibition and stomatal closure assays. However, the azide was not stable to the experimental conditions applied and converted to compound **4** which was also found to cause stomatal closure. Recently, Beale et al. (Kohler et al., 1997) reported the synthesis of a radio-iodinated ABA hydrazone photoaffinity probe **5**. The synthesis was based on linking ABA via the C-4′ carbon to a hydrazone derivative. However, Hite et al. (Hite, Outlaw & Seavy, 1994) had reported that ABA-4′-hydrazones were unstable and that the observed biological activity was due to ABA formed by hydrolysis of the ABA-hydrazone in vivo. Although Beale and coworkers (Kohler et al., 1997) showed that compound **5** was stable in aqueous or alcoholic solvents, they did not test the stability of the molecule in plant cells and thus the usefulness of this molecule in labeling ABA-binding proteins is still in doubt.

Benzophenone-containing substrate analogues have been gaining popularity as tools for the photoaffinity labeling of proteins (Dorman & Prestwich, 1994) for a variety of reasons. They are chemically more stable than aryl azides, diazo esters, and diazirines. They can be manipulated in ambient light and can be activated at 350–360 nm, avoiding protein-damaging wavelengths. Also, they react preferentially with unreactive C–H bonds, even in the presence of water and bulk nucleophiles. Most importantly, the benzophenone photophore has multiple opportunities to react within the active site, as the reactive intermediate that is formed upon irradiation can undergo many excitation-relaxation cycles. For these reasons, we undertook to design a benzophenone containing ABA analogue to identify ABA-binding proteins.

Structure activity studies have shown the C-1 acid and C-4′ carbonyl of ABA to be important for biological activity, possibly as important sites for hydrogen bonding within the active site (Perras, Rose, Pass, Chatson, Balsevich & Abrams, 1997; Van der Meulen, Heidekamp, Jastorff, Horgan & Wang, 1993). Comparison of the relative importance of the 7′, 8′ and 9′ methyl groups of ABA in inhibiting wheat embryo germination indicated that the presence of the 7′ methyl group was essential but that the others were less crucial (Nanzyo, Oritani & Yamashita, 1977; Walker-Simmons, Rose, Shaw & Abrams, 1994). The dienoic methyl ester **6** was also found to be as active as racemic ABA acid and ABA methyl ester for inhibiting root emergence of cress seeds, showing that alteration in orientation of the 8′-methyl group did not interfere with perception (Lei, Abrams, Ewan & Gusta, 1994). With these factors in mind, we designed an ABA analogue containing a benzophenone unit within the ABA ring system in the form of an anthracenone, minimizing the steric changes to the molecule and keeping the C1 acid and C4′ carbonyl available for binding to the active site. This paper describes a short, efficient synthesis and preliminary biological activity of an achiral, anthracenone-ABA analogue as a potential photoaffinity reagent for ABA-binding proteins.

2. Results and discussion

2.1. Synthesis

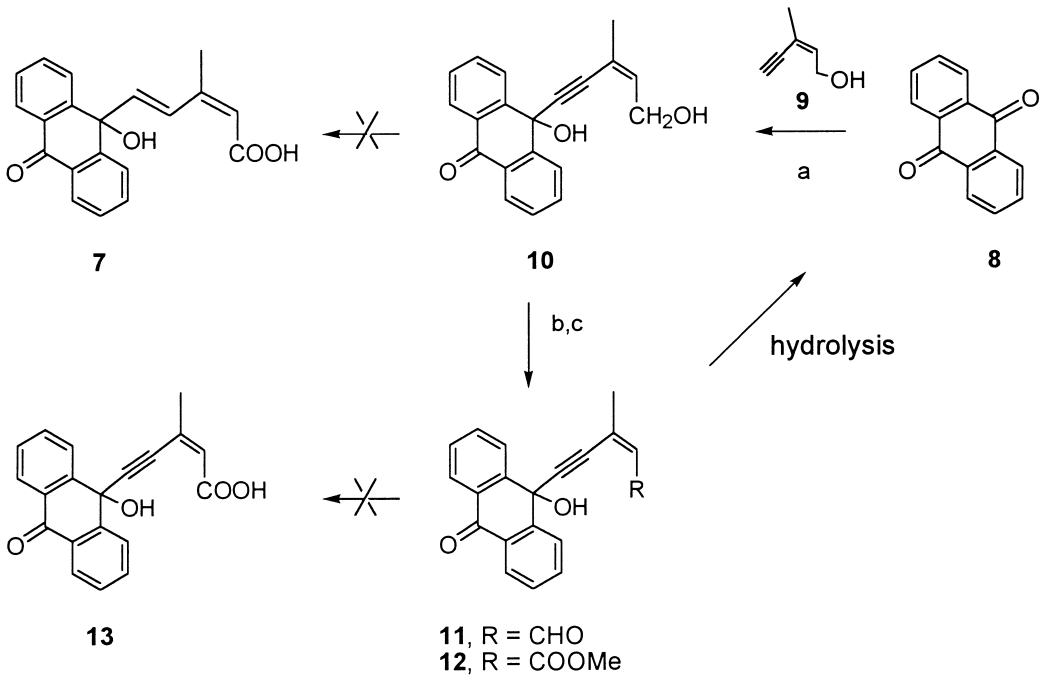
Our initial plan was to synthesize anthracenone ABA **7** from anthraquinone **8**, through direct addition of the lithium dianion of *cis*-3-methyl-2-penten-4-yn-1-ol **9**, followed by reduction of the triple bond and modification of the C-1 carbon to the free acid. Thus, reaction between anthraquinone and two equivalents of **9** at −78° afforded compound **10** in 56% yield (see Schemes 1 and 2). Unfortunately, attempts at reducing the triple bond led to decomposition of **10**. Analogues containing a triple bond at the C4–C5 position often show good ABA-like activity (Walker-Simmons, Anderberg, Rose & Abrams, 1992); therefore we attempted to synthesize acetylenic acid **13**. Thus, alcohol **10** was oxidized to aldehyde **11** in 85% yield using manganese dioxide, and then further oxidized to ester **12** in 62% yield. Unfortunately, acetylenic ester **12** was found to be unstable under conditions required for base hydrolysis and reverted back to anthraquinone. All other attempts to hydrolyze **12** to the desired acid were unsuccessful.

An alternative strategy was adopted using 10,10′-dimethoxy-9-anthrone, **14**, with introduction of the side chain, reduction of the triple bond, followed by the usual functional group manipulations and subsequent deprotection of the acetal. Employing known chemistry, anthraquinone was therefore converted to 9,10-dimethoxyanthracene via reductive methylation (Kraus & Man, 1986), ozonolysis in MeOH at −78° then gave the appropriate ozonide which on warming in CH_2Cl_2 to room temperature provided 10,10′-dimethoxy-9-anthrone **14** (Rigaudy, Chelu & Cuong, 1985).

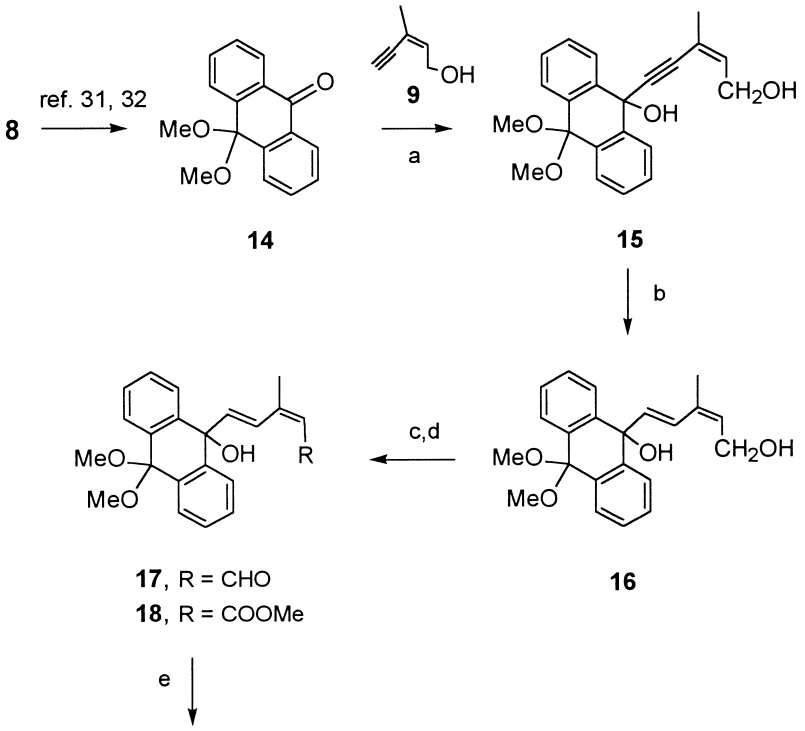
The side chain was introduced through reaction of **14** with the dianion of **9** to give acetylenic alcohol **15** in 83%. Red-Al® reduction of the carbon–carbon triple bond gave diene **16**. Oxidation with manganese dioxide gave unstable aldehyde **17** which was oxidized immediately to methyl ester **18** (57% over two steps). The acetal was removed under acidic conditions to provide the ABA methyl ester analogue **19**. Ester **19** was smoothly converted to the desired anthracenone analogue **7** using methanolic KOH.

2.2. Biological activity

ABA inhibits the growth of suspension-cultured cells of maize (variety Black Mexican Sweet) in a dose-dependent manner (Balsevich et al., 1994). We have found this culture system to be useful in structure/activity and metabolism studies for ABA and ABA analogues, and a good predictor of hormonal activity in other bioassays (Balsevich et al., 1994; Abrams, Rose, Cutler, Balsevich, Lei & Walker-Simmons, 1997; Rose, Cutler, Loewen, Hogge & Abrams, 1996). Maize cell cultures were treated as usual (Balsevich et al., 1994). The cultures were incubated on a rotary shaker for 4 days, then the cells were separated from the medium by vacuum filtration and weighed immediately. The



Scheme 1. Outline of synthesis of compound **12**. a. *n*-BuLi, and **9**, −78°, b. MnO_2, acetone, c. MnO_2, NaCN, HOAc, MeOH.



Scheme 2. Outline of synthesis of compounds **7**. a. *n*-BuLi, and **9**, −78° to room temperature, b. Red-Al, −78° to 0°, c. MnO_2, acetone, d. MnO_2, NaCN, HOAc, MeOH, e. 10% HCl, 0–10°, f. (i) 5% KOH–MeOH, 55–60°, (ii) 10% HCl.

effect of natural ABA and the potential photoaffinity anthracenone ABA **7** on cell growth was determined at various concentrations (0–25 μM) by calculating the percentage increase in fresh weight [(final weight × 100/initial weight) − 100) (see Fig. 1). Measurements were performed in triplicate and the average values were normalized to a control (untreated) value of 100%. The results show that anthracenone ABA **7** is perceived as ABA-like, although producing less inhibition than ABA itself. For example, **7** at 33 μM produces approximately the same inhibition as ABA at 10 μM.

3. Experimental

3.1. General

Mps are uncorrected. IR spectra were recorded using KBr cells on a Perkin–Elmer Paragon 1000. Proton nuclear magnetic resonances (^{1}H-NMR) were recorded on a Bruker AM 500 MHz spectrometer. Unless otherwise stated, $CDCl_3$ was used as solvent in all NMR experiments with $CHCl_3$ as a reference. Chemical shifts (δ) and coupling constants (J) are reported as if they are first order. Low (LRMS) and high (HRMS) resolution mass spectra were recorded in either the electron impact (EI) mode, the chemical ionization (CI) mode, or fast atom bombardment (FAB) mode on a VG 70-250SEQ double-focusing hybrid spectrometer with a Digital PDP 11/73 data system. Mass spectral data are reported in mass to charge units (m/z).

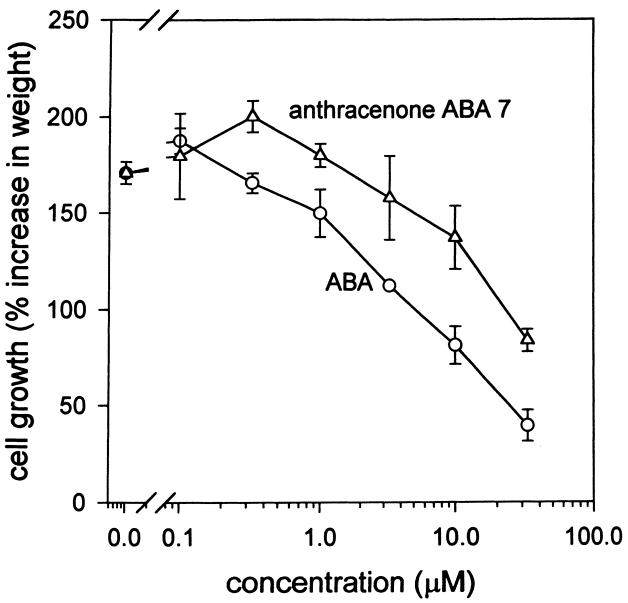


Fig. 1. The figure shows the inhibitory effects on cell growth (% weight) of various concentrations (μM) of ABA and anthracenone ABA **7**. Cell growth is expressed as the % increase in weight over the 4 days. Error bars represent the standard error of three replicates.

Flash column chromatography was performed using Merck silica gel 60 (230–400 mesh). Merck silica gel 60 F254 plates (0.2 mm) with aluminum sheet backing were used in analytical TLC. UV active materials were detected under a UV lamp. The plates were then dipped into a solution of phosphomolybdic acid and heated on a hot plate to visualize the spots.

The solvent tetrahydrofuran (THF) was dried by distillation from sodium and benzophenone. Unless otherwise indicated, all reactions were conducted under an atmosphere of dry argon.

Maintenance of corn cell cultures and growth inhibition experiments were performed as described (Balsevich et al., 1994).

3.2. (3Z)-10-Hydroxy-10-(5-hydroxy-3-methylpent-3-en-1-ynyl)-9(10H)-anthracenone **10**

To a solution of *cis*-3-methylpent-2-en-4-yn-1-ol **9** (208 μl, 2.0 mmol) in THF (5 ml) under Ar at −78° was slowly added *n*-BuLi (2.5 M in hexane, 1.6 ml, 4.0 mmol). After 30 min, the solution was transferred by cannula into a suspension of anthraquinone **8** (208 mg, 1.0 mmol) in THF (5 ml) at −78°. After 1 h at −78°, the reaction mixture was quenched with saturated NH_4Cl and was extracted with ether. The combined organic extracts were washed with brine, dried (Na_2SO_4), and concentrated. Purification by flash chromatography (CH_2Cl_2 then EtOAc–hexane, 1:1) yielded 169 mg (56%) of compound **10**, mp 127–129° (hexane/EtOAc), IR λ_{max} cm^{-1}: 3408 (OH), 2214, 1654, 1601, ^{1}H-NMR spectral data : δ 8.23 (2H, *dd*, J = 7.8, 1.2 Hz), 8.05 (2H, *d*, J = 7.5 Hz), 7.48 (2H, *ddd*, J = 7.8, 7.5, 1.3 Hz), 7.52 (2H, *m*), 5.87 (1H, *m*), 4.17 (2H, *d*, J = 6.6 Hz, CH_2), 3.19 (1H, *s*, OH) 1.82 (3H, *d*, J = 1.0 Hz, CH_3) LR–EIMS *m*/*z*: 304 $[M]^+$ (4), 286 (100), HR–EIMS *m*/*z* (rel. int.): found: 304.1074, $C_{20}H_{16}O_3$ requires 304.1099.

3.3. (3Z)-10-Hydroxy-10-(3-methyl-5-oxopent-3-en-1-ynyl)-9(10H)-anthracenone **11**

Dienol **10** (331 mg, 1.02 mmol) was dissolved in acetone (50 ml) and manganese dioxide (360 mg) was added. The suspension was stirred at room temperature for 1 h and was then filtered through celite. The filtrate was concentrated to provide the crude aldehyde. Flash chromatography (EtOAc-hexane, 1:1) gave 280 mg (85%) of aldehyde **10**, mp 150–151° (hexane/EtOAc). IR λ_{max} cm^{-1}: 3391 (OH), 2240, 1681, 1599, ^{1}H-NMR spectral data: δ 9.82 (1H, *d*, J = 8.2 Hz, CHO), 8.27 (2H, *dd*, J = 8.5, 0.8 Hz), 8.05 (2H, *d*, J = 7.9 Hz), 7.75 (2H, *m*), 7.57 (2H, *m*), 6.15 (1H, *dd*, J = 8.2, 1.2 Hz), 3.00 (1H, *s*, OH), 2.06 (3H, *d*, J = 1.2 Hz, CH_3 LR–EIMS *m*/*z* (rel. int.): 302 $[M]^+$ (11), 273 (100), HR–EIMS *m*/*z* found: 302.0923, $C_{20}H_{14}O_3$ requires 302.0943.

3.4. (3Z)-10-Hydroxy-10-(4-carbomethoxy-3-methylbut-3-en-1-ynyl))-9(10H)-anthracenone **12**

To a solution of aldehyde **11** (205 mg, 0.679 mmol) in methanol (10 ml) were sequentially added MnO_2 (0.88 g), NaCN (80 mg, 1.63 mmol), and acetic acid (39 μl, 0.679 mmol). The reaction mixture was stirred at room temperature for 1.5 h and was then filtered through celite. Concentration of the filtrate gave a residue which was taken up into CH_2Cl_2 and brine. The organic layer was separated, dried (Na_2SO_4) and evaporated. Flash chromatography (EtOAc–hexane, 1:3) gave keto ester **12** (139 mg, 62%), mp 156–158° (hexane/EtOAc), IR λ_{max} cm^{-1}: 3402 (OH), 2258, 1646, 1600, ^{1}H-NMR spectral data: δ 8.26 (2H, *dd*, J = 7.8, 1.3 Hz), 8.14 (2H, *dd*, J = 7.8, 0.7 Hz), 7.72 (2H, *m*), 7.53 (2H, *m*), 6.01 (1H, *d*, J = 1.5 Hz), 3.91 (1H, *s*, OH), 3.67 (3H, *s*, COOMe), 1.94 (3H, *d*, J = 1.5 Hz, CH_3), LR–EIMS *m*/*z* (rel. int.): 332 $[M]^+$ (14), 273 (100), HR–EIMS *m*/*z* found: 332.1033, $C_{21}H_{16}O_4$ requires 332.1048.

3.5. (3Z)-10-Hydroxy-9,9-dimethoxy-10-(5-hydroxy-3-methylpent-3-en-1-ynyl)-anthracene **15**

To a solution of *cis*-3-methylpent-2-en-4-yn-1-ol 9 (161 μl, 1.54 mmol) in THF (5 ml) under Ar at −78° was slowly added *n*-BuLi (2.5 M in hexane, 1.23 ml, 3.09 mmol). After 30 min, 10,10′-dimethoxy-9-anthrone **14** (196 mg, 0.77 mmol) in THF (5 ml) was added. The reaction was then allowed to warm to room temperature and stirred for 1.5 h. After cooling to 0°, the reaction mixture was quenched with saturated NH_4Cl and was extracted with ether. The combined organic extracts were washed with brine, dried (Na_2SO_4), and concentrated. Flash chromatography (EtOAc–Hexane, 1:1) gave 223 mg (83%) of compound **15**. IR λ_{max} cm^{-1}: 3391 (OH), 2247, 1634, 1604, ^{1}H-NMR spectral data: δ 8.05 (2H, *m*), 7.74 (2H, *m*), 7.48 (4H, *m*), 5.91 (1H, *m*), 4.25 (2H, *dd*, J = 6.0, 5.9 Hz, CH_2), 3.09 (3H, *s*, OMe), 3.04 (3H, *s*, OMe), 1.91 (3H, *d*, J = 1.2 Hz, CH_3), LR–EIMS *m*/*z* (rel. int.): 332 $[M\text{-}H_2O]^+$ (17), 301 (83), 224 (100), HR–EIMS *m*/*z* found: 332.1397, $C_{22}H_{22}O_4$ requires 332.1412 $[M\text{-}H_2O]^+$.

3.6. (1E,3Z)-10-Hydroxy-9,9-dimethoxy-10-(5-hydroxy-3-methyl-1,3-pentadienyl)-anthracene **16**

A solution of alkyne **15** (117 mg, 0.334 mmol) in THF (5 mL) was cooled to −78° under Ar. Sodium bis(2-methoxy)aluminum hydride (Red-Al®) (3.4 M in toluene, 201 μl, 0.669 mmol) was slowly added and the

mixture was warmed to 0° for 1 h. The reaction mixture was carefully quenched with a few drops of water and diluted with ether. The organic layer was separated, washed with brine, dried (Na_2SO_4) and concentrated. Flash chromatography (EtOAc–hexane, 1:1) gave 104 mg (88%) of the unstable dienol **16**. IR λ_{max} cm^{-1}: 3362. (OH), 1654, 1602 (C=C), ^{1}H-NMR: δ 7.75 (4H, *m*), 7.43 (4H, *m*), 6.64 (1H, *d*, J = 15.6 Hz, H-4), 5.85 (1H, *d*, J = 15.6 Hz, H-5), 5.51 (1H, *m*), 4.21 (2H, *d*, J = 7.1 Hz), 3.12 (3H, *s*, OMe), 3.04 (3H, *s*, OMe), 1.72 (3H, *d*, J = 0.8 Hz, CH_3).

3.7. (1E,3Z)-10-Hydroxy-9,9-dimethoxy-10-(4-carbomethoxy-3-methyl-1,3-butadienyl)-anthracene ***18***

Dienol **16** (97 mg, 0.276 mmol) was dissolved in acetone (7 ml) and manganese dioxide (360 mg) was added. The suspension was stirred at room temperature for 1 h and was then filtered through celite. The filtrate was concentrated to provide the crude aldehyde **17** (77 mg) used immediately in the next oxidation step.

To a solution of the crude aldehyde in methanol (5 ml) were sequentially added MnO_2 (257 mg), NaCN (23.3 mg, 0.473 mmol), and acetic acid (12.6 μl, 0.22 mmol). The reaction mixture was stirred at room temperature for 1.5 h and was then filtered through celite. Concentration of the filtrate gave a residue which was taken up into CH_2Cl_2 and brine. The organic layer was separated, dried (Na_2SO_4) and evaporated. Flash chromatography (EtOAc–hexane, 1:4) gave 60 mg (57% over 2 steps) of ester **18**, mp 51–52° (hexane/EtOAc), IR λ_{max} cm^{-1}: 3449 (OH), 1716 (C=O), 1664 (C=C), ^{1}H-NMR: δ 7.91 (1H, *d*, J = 16.0 Hz, H-4), 7.82 (2H, *m*), 7.73 (2H, *m*), 7.44 (4H, *m*), 6.11 (1H, *d*, J = 16.0 Hz, H-5), 5.63 (1H, brs), 3.67 (3H, *s*, COOMe), 3.13 (3H, *s*, OMe), 3.04 (3H, *s*, OMe), 1.81 (3H, *d*, J = 1.2 Hz, CH_3), LR–FABMS *m/z* (rel. int.): 363 $[M+1\text{-}H_2O]^+$ (10), 285 (100), HR–FABMS *m/z* found: 363.1582, $C_{23}H_{23}O_4$ requires 363.1596 $[M+1\text{-}H_2O]^+$.

3.8. (1E,3Z)-10-Hydroxy-10-(4-carbomethoxy-3-methyl-1,3-butadienyl)-9(10H)-anthracenone ***19***

10% HCl (8 drops) was added to an ice–cold solution of ester **18** (54 mg, 0.142 mmol) in THF (3 ml). The mixture was stirred between 0 and 10° for 2 h. Ether was added and the solution was washed with 10% $NaHCO_3$, brine, and was dried (Na_2SO_4). Flash chromatography (EtOAc–hexane, 1:4) gave 33 mg (70%) of ester **19**, mp 196–198° (hexane/EtOAc), IR λ_{max} cm^{-1}: 3438 (OH), 1647, 1629, 1600, ^{1}H-NMR spectral data: δ 8.27 (2H, *m*), 7.99 (1H, *d*, J = 15.6 Hz, H-4), 7.83 (2H, *m*), 7.66 (2H, *m*), 7.50 (2H, *m*), 5.99 (1H, *d*, J = 15.6 Hz, H-5), 5.67 (1H, *brs*), 3.68 (3H, *s*, COOMe), 1.82 (3H, *d*, J = 1.0 Hz, CH_3), LR–FABMS *m/z* (rel. int.): 335 $[M+1]^+$ (4), 285 (100), HR–FABMS *m/z* found: 335.1264, $C_{21}H_{19}O_4$ requires 335.1283 $[M+1]^+$.

3.9. (1E,3Z)-10-Hydroxy-10-(4-carboxy-3-methyl-1,3-butadienyl)-9(10H)-anthracenone 7

A mixture of ester **19** (26.8 mg, 0.08 mol) in methanol (4 ml) and 5% KOH (4 ml) was stirred at 55–60° for 1.5 h. The solvents were removed and water was added. Any unreacted ester was removed by extraction into EtOAc. The aqueous layer was then acidified to pH 3 with 10% HCl and extracted with EtOAc (×3). The organic extracts were combined, dried ($NaSO_4$), and concentrated to provide 19.6 mg (76%) of carboxylic acid **7**, mp 215–218° (hexane/EtOAc), IR λ_{max} cm^{-1}: 3406 (OH), 1651, 1629, 1598, ^{1}H-NMR spectral data ($(CD_3)_2CO$): δ 8.17 (2H, *dd*, J = 7.8, 1.2 Hz), 8.02 (1H, *d*, J = 15.7 Hz, H-4), 7.90 (2H, *brd*, J = 7.5 Hz), 7.72 (2H, *m*), 7.53 (2H, *m*), 6.20 (1H, *d*, J = 15.7 Hz, H-5), 5.70 (1H, *brs*), 1.85 (3H, *d*, J = 1.3 Hz, CH_3), LR–EIMS *m/z* (rel. int.): 320 $[M]^+$ (3), 302 $[M\text{-}18]^+$ (64), 210 (100), HR–EIMS *m/z* found: 320.1036, $C_{20}H_{16}O_4$ requires 320.1049.

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