Available online at www.sciencedirect.com
ScienceDirect
Phytochemistry 68 (2007) 546–553
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

ent-Beyerane diterpenoids from the heartwood of *Excoecaria parvifolia*

Mary H. Grace [a,1], Juan A. Faraldos [b,1], Mary Ann Lila [a], Robert M. Coates [b,*]

[a] *Department of Natural Resources and Environmental Sciences, University of Illinois at Urbana–Champaign, Urbana, 1201 S Dorner Drive, Urbana, IL 61801, USA*
[b] *Department of Chemistry, University of Illinois at Urbana–Champaign, 600 South Mathews Avenue, 470 RAL, Box 40, Urbana, IL 61801, USA*

Received 17 August 2006; received in revised form 6 October 2006
Available online 29 December 2006

Abstract

Chromatographic fractionations of the toluene extract of the heartwood of *Excoecaria parvifolia* collected in Australia resulted in the isolation of 12 beyerane diterpenes (**1**–**12**), and the triterpene, lupeol. Four of the isolated diterpenoids (**5**–**7** and **12**) have unusual structures: *ent*-3-oxa-beyer-15-en-2-one, (**5**); *ent*-15,16-epoxy-2-hydroxy-19-norbeyer-1,4-dien-3-one (**6**); methyl *ent*-2,4-seco-15,16-epoxy-4-oxo-3,19-dinorbeyer-15-en-2-oate (**7**); and *ent*-2,17-dihydroxy-19-norbeyer-1,4,15-trien-3-one (**12**). The structures were established by spectroscopic analyses, NMR data comparisons with similar diterpenes, and chemical correlations. All the diterpenes are assumed to have the same absolute configuration as the co-occurring (+)-stachenol (**4**). Diosphenol **2** and nor-lactone **5** exhibited significant potency in bioassays for cytotoxic activity against leukemia cells (L1210). Plausible biosynthetic pathways are proposed to explain the origin of the diterpene metabolites.

Keywords: *Excoecaria parvifolia*; Euphorbiaceae; Heartwood; Diterpenoids; *ent*-Beyeranes; Diosphenols; L1210; Cytotoxicity

1. Introduction

The *Excoecaria* genus in the family Euphorbiaceae is comprised of about 40 species, which are distributed throughout tropical Africa, Asia, and northwestern Australia (Wills, 1973; Wiriyachitra et al., 1985). Many plants in this genus are recognized as skin irritants and tumor promoters (Erickson et al., 1995). The most widely reported species is the mangrove, *Excoecaria agallocha* L. the latex and leaves of which may cause blindness, and have long been used as fish poison and poison arrow heads (Ohigashi et al., 1974). The bark and wood of *Excoecaria* plants have been applied in Thai medicine as a remedy against flatulence (Karalai et al., 1994). In Sri Lanka, the smoke of the burning wood is used in treatment of leprosy, while the root pounded with ginger is an embrocation for swelling hands and feet (Jayaweera, 1980). *E. parvifolia* J. Muell., an endemic species of the wet–dry savannah of northern Australia also known as the Gutta percha tree, has not yet been chemically investigated (Maguire and Saenger, 2000).

In this paper, we describe the isolation and structure determination of four novel diterpenoids (**5**–**7** and **12**, Fig. 1), as well as the partial synthesis of the first three. In addition, the wide-spread triterpene alcohol lupeol (Burns et al., 2000) and the following known *ent*-diterpenoids were isolated by chromatographic means: stachenone (**1**) (Baarschers et al., 1962), nor-dienone **2**, nor-keto ester **3**, stachenol (**4**) (Chalmers et al., 1977; Munkombwe et al., 1997), dihydroxy dienone **8**, keto acid **9**, rhizophorin C (**10**), and the 6 epimer of **8** (**11**). The beyerene diosphenols **2**, **8**, and **11** were previously isolated from the heartwood of the South African species *Spirostachys africana* (syn. *E. africana*) (Munkombwe et al., 1997). The nor-keto acid **9** and its methyl ester (**3**), prepared from **9** and diazomethane, were subsequently reported in a later investigation of *Spirostachys africana* (Munkombwe et al., 1998). Keto ester **3** is herein isolated as a natural product for

* Corresponding author. Tel.: +1 217 333 4280; fax: +1 217 244 8024.
E-mail address: rmcoates@uiuc.edu (R.M. Coates).
[1] These co-authors made approximately equal contributions to the work reported.

0031-9422/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2006.11.010

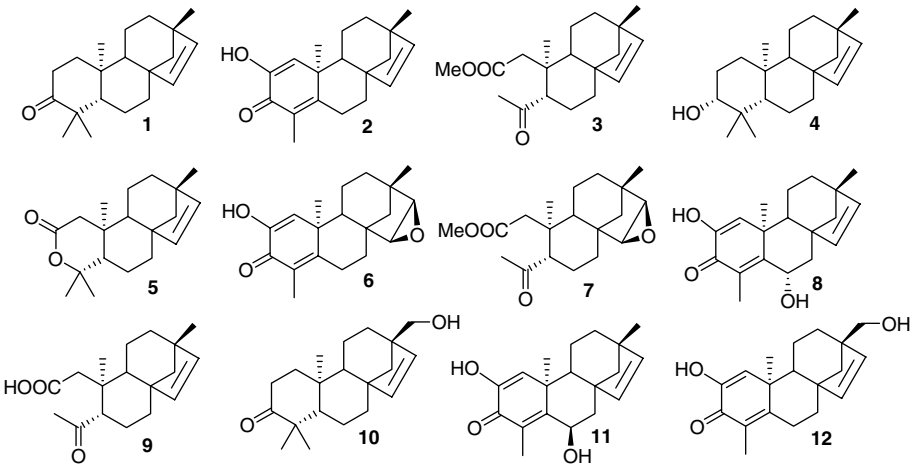


Fig. 1. *ent*-Beyerane diterpenes isolated from *Excoecaria parvifolia* in order of increasing polarity on silica gel.

the first time. 17-Hydroxystachenone (rhizophorin C, **10**) has been recently isolated from the roots of *Rhizophora mucronata* and fully characterized (Anjaneyulu et al., 2002). In addition, diterpenes (**1-12**) and the triterpene alcohol lupeol were tested for their cytotoxic activity against L1210 (mouse leukemia cell line).

2. Results and discussion

2.1. Isolation, structure determinations, and chemical correlations

The powdered heartwood of *E. parvifolia* was exhaustively extracted with toluene, and after solvent evaporation, the residue was fractionated by column chromatography on silica gel using a gradient of hexane–ethyl acetate as eluent. Further purification by column chromatography and/or TLC resulted in the isolation of the four new diterpenoids, **5**–**7** and **12**, and eight known *ent*-beyer-15-ene derivatives (**1**–**4** and **8**–**11**) (Fig. 1), along with the triterpene alcohol, lupeol. The known compounds were identified following analyses of ^{1}H NMR, ^{13}C NMR, and MS data, and by comparisons with reported spectroscopic data. In addition, the measured optical rotation $[\alpha]_D^{25}$ + 33.6 ($CHCl_3$, *c* 0.25) for the *ent*-beyerane alcohol stachenol (**4**) agrees with the literature $[\alpha]_D^{25}$ + 32.0 (Munkombwe et al., 1997).

Nor lactone **5**, obtained as colorless needles (m.p. 119–120 °C), possesses the molecular formula of $C_{19}H_{28}O_2$ according to its HREIMS molecular ion peak observed at *m*/*z* 288.20849. The IR spectrum displays a strong δ-lactonic absorption at 1727 cm^{-1} and a diagnostic bending frequency for a cis disubstituted double bond at 748 cm^{-1}. The ^{1}H NMR spectrum exhibits a pair of doublets at δ 5.67 and 5.53 (1H each, $J = 5.6$ Hz, H-15 and H-16), very similar to those previously assigned to the D-ring double bond of the known *ent*-beyer-15-enes, stachenone (**1**) and stachenol (**4**). In addition, sharp singlets for four angular methyls were observed at δ 0.89, 1.03, 1.35 and 1.43 ppm, the three furthest downfield of which match very well with those recently assigned to an *ent*-kaurane diterpenoid having an A-ring lactone (1.44, 1.35 and 1.16 ppm) (Kono et al., 2004). Furthermore, the positions of the three A-ring methyl signals at 0.89 (C-20 Me), 1.35 (C-19 Me) and 1.43 (C-18 Me) ppm are in excellent agreement with those previously reported for agallochin E (0.89, 1.32 and 1.43 ppm), a similar A-ring lactone diterpenoid isolated from *E. agallocha* (Anjaneyulu and Rao, 2000).

The ^{13}C NMR spectrum of the lactone component (Table 1) shows separate peaks for 19 carbons that were identified as 4 CH_3, 6 CH_2, 4 CH, and 5 quaternary carbons from a DEPT spectrum. The quaternary carbon peak assigned to C-4 bearing the two methyl groups appears at δ_c 85.9 ppm and therefore suggests an oxygen substituent

Table 1
^{13}C NMR Spectroscopic data (δ in ppm) and assignments for diterpenes **1**, **3**, **5**, **6**, and **7** (125 MHz, $CDCl_3$)

Carbon	1	3	5	6	7
1	38.1	42.0	44.9	123.7	42.3
2	34.6	172.6	171.0	144.7	172.0
3	217.9		–	181.4	–
4	47.9	212.8	85.9	126.7	212.2
5	56.0	45.1	52.4	165.7	48.2
6	20.6	20.4	20.1	26.2	19.3
7	36.9	35.8	36.1	39.6	35.1
8	48.9	49.0	48.8	45.2	46.4
9	52.1	56.6	50.1	53.3	51.3
10	37.0	39.7	36.7	43.5	39.6
11	21.2	23.3	22.7	21.2	22.8
12	33.1	33.0	32.7	33.6	31.4
13	43.9	43.9	43.9	44.4	44.0
14	61.1	60.9	60.8	35.0	38.9
15	134.7	137.4	137.8	59.9	55.9
16	137.1	134.3	133.6	55.1	54.4
17	25.0	24.9	25.2	21.6	21.3
18	26.3	31.6	33.2	10.7	31.4
19	22.1		24.9	–	–
20	14.7	17.7	15.4	22.3	18.5
OCH_3	–		–	–	56.1

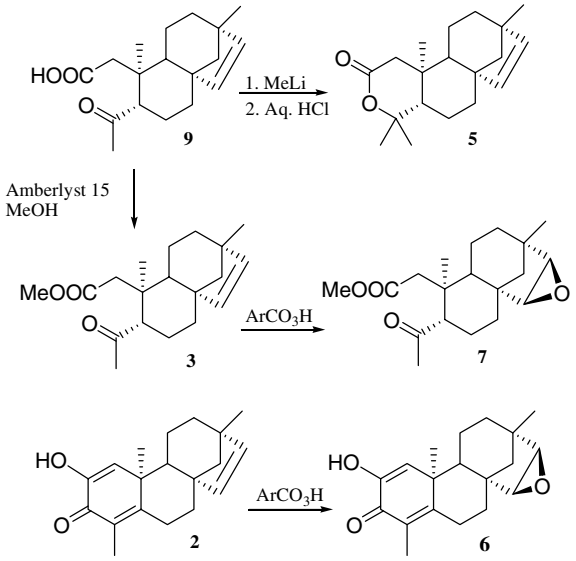
at this position. The ^{13}C NMR spectrum also shows a characteristic carbonyl peak for a six-membered ring lactone at δ_c 170.9 ppm (Pretsch et al., 1976), and vinylic carbons at 137.8 and 133.6 ppm (C-15 and C-16). In addition to these diagnostic peaks, the A-ring methyl signals at 33.2 (C-18 Me), 24.9 (C-19 Me) and 15.4 (C-20 Me) ppm are in good agreement with those published for agallochin E (32.5, 24.3 and 16.2 ppm) (Anjaneyulu and Rao, 2000). These considerations provided the basis for tentative assignment of structure **5** to this diterpene, i.e. 3-oxa-beyer-15-en-2-one.

The lactone structure **5** was confirmed by partial synthesis from the co-occurring keto acid **9** (Scheme 1). Thus, reaction of **9** with two equivalents of MeLi in THF at low temperature (Kametani et al., 1988) followed by mild aq. acidic work up afforded a δ-lactone shown to be identical with the isolated nor-diterpene. Evidently, the δ-hydroxy acid intermediate underwent spontaneous lactonization under the acidic conditions of the hydrolysis. The ^1H and ^{13}C NMR spectra and data of the synthetic and isolated lactone **5** are in total agreement, thereby verifying the structure assignment.

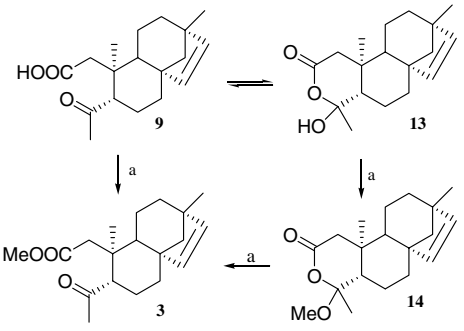
The component eluted after lactone **5** was also obtained as a white crystalline solid (m.p. 144–145° C). The HRESIMS shows *m/z* 301.1807 $[M+H]^+$ corresponding to $C_{19}H_{25}O_3$. The IR spectrum displays peaks at 3376, 1625, 1237 and 847 cm^{-1} assigned to hydroxyl, carbonyl and epoxide functionalities, respectively. The ^1H NMR spectrum is very similar to that of known diosphenol **2** (Munkombwe et al., 1997), except for the lack of signals corresponding to the olefinic protons. Instead, a new pair of doublets at a higher field δ 3.59 (1H, $J = 4.0$ Hz) and 3.16 (1H, $J = 4.0$ Hz) was observed. These NMR spectroscopic data are in good agreement with those reported for excoecarin E (*ent*-15,16-epoxybeyeran-3-one) (Konishi et al., 2000) and its 3α-hydroxy relative (Konishi et al., 2003), both having an 15,16-epoxy moiety, and recently isolated from *E. agallocha*. Careful analysis of the ^{13}C NMR spectrum of the diterpene in the present work clearly indicated that the peaks corresponding to the olefinic carbons C-15 and C-16 at 138.3 and 133.1 ppm had been replaced by two new peaks at 59.9 and 55.1 ppm, characteristic of the *ent*-15,16-epoxybeyeranes (Konishi et al., 2000). Proof for diosphenol epoxide structure **6** was obtained by epoxidation of nor-trienone **2** with *m*-chloroperoxybenzoic acid (Scheme 1). The ^1H NMR spectrum of the resulting epoxide was superimposable upon that of the isolated diterpene.

The next component in order of elution (**7**) was obtained as a clear oil that was shown by HRESIMS (*m/z* 321.2068 $[M+H]^+$) to have the molecular formula $C_{19}H_{28}O_4$. The IR spectrum shows peaks for two carbonyl groups at 1731 and 1707 cm^{-1} assigned to ester and ketone carbonyl groups and two bands characteristic of an epoxide (1209 and 849 cm^{-1}). The ^1H NMR spectroscopic data were almost identical to those previously reported for keto ester **3** (Munkombwe et al., 1998). Characteristic ^1H NMR signals for this new diterpene at 3.66 (*s*, OMe), 2.97 (*dd*, $J = 2.8$ and 12.8 Hz, H-5), 2.38 and 2.30 (AB*dd*, $J = 14.4$ Hz, CH_2CO_2Me), 2.23 (*s*, C-18 Me) and 1.03 (*s*, C-17 Me) are in excellent agreement with those reported for keto ester **3**. Notable however was the absence of the pair of doublets diagnostic for an *ent*-beyer-15-ene diterpene. Instead, a new pair of doublets at 3.42 and 3.05 ppm (1 H each, $J = 3.5$ Hz, H-15 and H-16) similar to that mentioned above for diosphenol epoxide **6**, and characteristic for the D-ring epoxide, was observed. These inferences were confirmed as before by epoxidation of keto ester **3** with *m*-chloroperoxybenzoic acid to the exo epoxide **7** (Scheme 1) which proved to be identical to the isolated diterpene **7**, i.e. methyl *ent*-2,4-seco-15,16-epoxy-4-oxo-3,19-dinorbeyer-15-en-2-oate.

The keto ester structure **3** assigned to the third component was proven by means of partial synthesis from keto acid **9** (Scheme 2). Thus, esterification using Amberlyst 15 as the acid catalyst in methanol at room temperature provided methyl ester **3** without any evidence of epimerization at C-5, even after the 5-7 days required to achieve complete conversion (Petrini et al., 1988). After 22 h, the



Scheme 1. Semisynthesis of diterpenoids **3**, **5**, **6** and **7**.



Scheme 2. Semisynthesis of tricyclic terpenoid **3**. *Reagents and conditions*: (a) Amberlyst 15, MeOH, rt.

composition of products was as follows: keto acid **9** (36%), methyl ester **3** (24%), and a new compound identified as the isomeric lactone ether **14** (28%), which was obtained as an inseparable 5:2 mixture of C-4 epimers. The third component presumably arises from methanolysis of the cyclic lactol or pseudoacid **13**, a common tautomeric form of open chain δ-keto acids (Jones, 1963). When the reaction time was increased to 48 h, no starting keto acid was detected, and the product mixture was composed of a 5:2 mixture of **14** and keto ester **3**. The expectation that the pseudo ester is less stable than the normal keto ester **3** was corroborated by submitting the former (**14**) to the action of Amberlyst 15 in MeOH. After 72 h at room temperature, keto ester **3** was formed in quantitative yield (Scheme 2).

The ^{1}H NMR spectra and data for the keto ester sample obtained by esterification were identical to those of the isolated diterpene **3**, and furthermore the data coincide with the recently reported literature values (Munkombwe et al., 1998). The ^{13}C NMR signals for the synthetic and isolated diterpenes are also identical, and most of the data agree with the literature values (Table 1). However, we observe substantially larger chemical shifts for the ester and ketone carbonyl carbons (172.6 and 212.8 ppm) than the shifts reported previously (167.8 and 157.9 ppm). The higher values obtained in this work agree well with the ^{13}C NMR spectroscopic data for the carbonyl carbons of epoxide **7** (172.0 and 212.2 ppm) and keto acid **9** (176.7 and 216.1) ppm.

The most polar diterpene isolated (**12**) was obtained as a colorless oil that was shown to possess the molecular formula $C_{19}H_{24}O_3$ according to its HREIMS molecular ion peak observed at m/z 301.1801 $[M+1]^+$. The IR spectrum displays stretching absorptions at 3586 and 1618 cm^{-1} for hydroxyl and ketone carbonyl groups, respectively. The ^{1}H NMR spectrum indicates the presence of a ring A hydroxydienone with characteristic peaks at 6.42 (OH), 6.28 (H-1), 1.99 (C-18 Me) and the C-20 angular methyl at 1.11 ppm, identical to those reported for the parent diosphenol **2** (6.42, 6.28, 1.99 and 1.11) (Munkombwe et al., 1997). Furthermore, the absence of one angular Me (C 17) singlet and the appearance of an AB doublet of doublets at 3.59 and 3.50 ppm (ABdd, $J = 10.5$ Hz) indicated that oxidation of diosphenol **2** at C-17 took place giving a 17-hydroxy derivative. These values are in good agreement with those observed for 17-hydroxystachenone (**10**) (Anjaneyulu et al., 2002). Accordingly, the novel structure **12** is assigned to this diterpene, *ent*-2,17-dihydroxy-19-norbeyer-1,4,15-trien-3-one.

Tentative ^{13}C NMR assignments for beyerane diterpenes **5**, **6** and **7** (Table 1) were made by comparison with those for similar compounds and by DEPT spectra. Thus, the ^{13}C NMR signals of lactone **5** were assigned by comparisons with those for similar diterpenes with a ring A lactone such as agallochin E (Anjaneyulu and Rao, 2000) and an *ent*-kaurane diterpenoid lactone recently isolated from rice leaves (Kono et al., 2004). Assignments for the B, C and D ring carbons were made by careful comparisons with the reported ^{13}C NMR spectroscopic data and assignments for stachenol (**4**) (Chalmers et al., 1977 and Munkombwe et al., 1997). Comparison of the ^{13}C NMR signals for compounds **6** and **7** with those previously established for diosphenol **2** and methyl ester **3** (Munkombwe et al., 1997 and Munkombwe et al., 1998) provided the grounds for assignments. The epoxy moiety of the ring D was assigned by comparisons with the relevant parts of other ring D epoxy diterpenoids such as excoecarin E (Konishi et al., 2000).

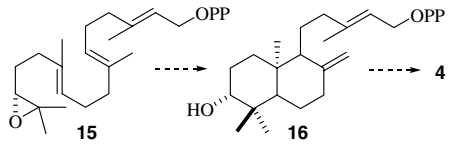
2.2. Proposals for biosynthetic pathways to E. diterpenes

It seems reasonable to propose that the all of diterpenes isolated from *E. parvifolia* are derived from stachenol (**4**) and stachenone (**1**) by oxidative reactions occurring on rings A and D. Since the absolute configuration of (+)-stachenol has been determined by optical rotatory dispersion measurements and chemical correlations (Baarschers et al., 1962; Jefferies et al., 1973), all of the compounds isolated in the present work are assumed to belong to the ent family of diterpenes.

Ring A oxidized derivatives of polycyclic diterpenes occur widely in the heartwood and oleoresin of plants (Connolly and Hill, 1991; Buckingham, 1994), and the pattern of *ent*-beyerane metabolites observed in this work is similar to those previously noted (Baarschers et al., 1962; Bakker et al., 1972; Munkombwe et al., 1998). In the following discussion, we consider briefly plausible biosynthetic pathways to these compounds.

The biosynthesis of *ent*-beyerene, and related tetracyclic diterpene hydrocarbons, is proposed to occur in two stages by reactions similar to those on the pathway to *ent*-kaurene: proton-initiated cyclization of (*E*,*E*,*E*)-geranylgeranyl diphosphate (GGPP) to *ent*-copalyl diphosphate (CPP) and then conversion to beyerene by vinyl bridging cyclization of an *ent*-pimarenyl carbocation intermediate (West, 1983; MacMillan and Beale, 1999; Davis and Croteau, 2000). The frequent occurrence of this hydrocarbon in plants is consistent with a scheme requiring enzymatic hydroxylation at C3 to form stachenol. However, the common presence of the 3α-hydroxyl functionality in beyerane and kaurane diterpenes suggests the alternative possibility that the substrate for the cyclase might be (*R*)-14,15-epoxy GGPP (**15**), which is known to be efficiently cyclized to the 3α-hydroxy derivative of *ent*-kaurene by a crude enzyme extract having kaurene synthase activity (Coates et al., 1976). In fact, a recent publication concerning phenalinolactone biosynthesis cited genetic evidence indicating that these 3α-hydroxy isoagathane (isocopalane) diterpene derivatives arise by cyclization of epoxyGGPP (Dürr et al., 2006). A biosynthetic pathway to stachenol via cyclizations of epoxyGGPP and 3α-hydroxy CPP (**16**) is shown in Scheme 3.

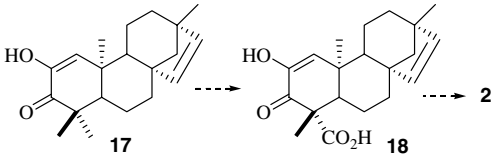
The common hydroxylation of the C19 methyl groups and conversion to the corresponding carboxylic acids affords a plausible rationale for the biosynthesis of 19-nor-diterpenes by decarboxylation at the level of the β-keto acids. Similar oxidation of the known beyerane diosphenol



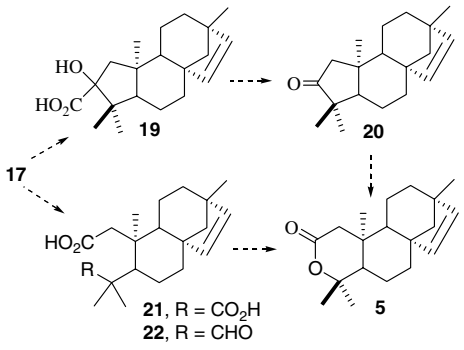
Scheme 3.

17 (Baarschers et al., 1962) to β-keto acid **18** followed by oxidative decarboxylation constitutes a reasonable pathway to hydroxydienone **2** as shown in Scheme 4. Alternatively, **2** might arise more directly by retro-aldol fragmentation of the related β-keto alcohol intermediate with loss of formaldehyde.

The A ring lactone established for norbeyerane **5** is rather unusual, albeit precedented in naturally occurring diterpenes (Anjaneyulu and Rao, 2000; Kono et al., 2004). One biosynthetic scheme to create the lactone would involve a ring contraction by a benzilic acid rearrangement of diosphenol **17** in its 2,3-dione form to α-hydroxy acid **19** followed by oxidative cleavage to A-nor beyeranone **20** and subsequent enzymatic Bayer-Villiger oxidation to insert the endocyclic oxygen (Scheme 5). Precedent exists for the first two steps in the occurrence of related A-nor metabolites derived from manoyl oxide (Grant and Carman, 1962; Grant et al., 1969) as well as an A-nor beyeran-3,12-dione (Sakai and Nakagawa, 1988). A-Nor beyerenes **19** and **20** are in fact known compounds prepared from diosphenol **17** by analogous chemical reactions (Baarschers et al., 1962). The oxidation of menthone and camphor to the respective ring-enlarged lactones in the catabolism of these monoterpene ketones in plants provides relevant analogy for the biological equivalent of the Bayer-Villiger oxidation (Croteau, 1987).



Scheme 4.



Scheme 5.

Two alternative biosynthetic pathways to nor-lactone **5** should be mentioned. Oxidative cleavage of the A ring of disphenol **17** to the known 2,3-seco diacid **21** (Munkombwe et al., 1998) followed by oxidative decarboxylation of the C3 carboxyl group with lactone formation would give rise to **5** (Scheme 5). Another possibility would be oxidative deformylation of the corresponding aldehyde acid **22**, in analogy with the transformations that occur to form the A-ring bridged lactones in gibberellins GA_9 and GA_{20} (MacMillan, 1997; Hedden et al., 2002).

2.3. Bioassay experiments

The 12 isolated diterpenes **1**–**12** were tested against L1210 (mouse leukemia cell line) but only compounds **2** and **5** showed significant cytotoxic activity (100% growth inhibition at 30 and 10 μg/mL respectively). All other diterpenoids displayed mild activity resulting in 100% growth inhibition at a concentration range of 60–80 μg/mL. The triterpenoid lupeol was completely inactive at the highest concentration.

2.4. Conclusion

The structures of the 12 beyerane diterpenes isolated from *E. parvifolia* heartwood from northwestern Australia were elucidated by spectroscopic analyses and correlations, and were verified in some cases by semisyntheses. These beyerane metabolites are thought to arise by various oxidative reactions occurring on rings A and D of stachenol **4**, and all are therefore related in absolute configuration to the *ent*-beyerane family of diterpenes. Biosynthetic proposals are presented to rationalize the A-ring oxy functionality by initial enzymatic cyclization of epoxyGGPP to stachenol, by β-keto acid decarboxylations, and by enzymatic Bayer-Villiger oxidation or oxidative decarboxylation to form the rare A-ring lactone **5**. Nor diterpenes **2** and **5** having tetracyclic structures, polar oxygen functionalities localized in the A rings, and ring D double bonds exhibited significant cytotoxicity in L1210 mouse leukemia cell bioassays.

3. Experimental

3.1. General aspects

Fractions from column chromatography and the progress of reactions described were analyzed by TLC using silica gel 60 F254 250 μm precoated-plates and visualized with anisaldehyde spray reagent on heating at 105 °C (Stahl, 1969). See Grace et al., 2006, Supplementary Information for additional information.

3.2. Plant material

E. parvifolia heartwood was supplied and identified by Dr. Eugene Dimitriadis, Xyloaustralis, 44 Hamono RD.,

Neerim, Vic. 3831, Australia. The plant was collected in northwestern Australia in 2003.

3.3. Extraction and isolation

The dried, milled heartwood (50 g) was extracted at room temperature by soaking overnight in toluene (3 × 1 L). Filtration and evaporation of the solvent by rotary evaporation (40 °C) gave a brownish oily residue (4.5 g). This toluene extract (4.3 g) was loaded onto a silica gel column (50 × 2.5 cm) and was eluted first with hexane and then with EtOAc–hexane mixtures of increasing polarity (1–50%), while collecting 38 × 100-mL fractions. According to TLC analyses, fractions of similar composition were pooled together, evaporated to dryness, and kept in the freezer for further fractionation. A combination of fractions showing similar spots provided six main fractions: 1–9 (1.28 g, 0–10% EtOAc in hexane), 10–14 (0.443 g, 15% EtOAc in hexane), 15–19 (0.314 g, 15% EtOAc in hexane), 20–23 (0.371 g, 20% EtOAc in hexane), 24–31 (0.545 g, 25–30% EtOAc in hexane), and 32–38 (0.248 g, 40–50% EtOAc in hexane), respectively. These combined fractions were separated further on silica gel columns as described above with varying solvent ratios.

Combined fractions 1–9 (1.28 g) afforded **1** (285 mg, eluted with 2% EtOAc in hexane), **2** (406 mg, eluted with 3% EtOAc in hexane), and **3** (30.8 mg, eluted with 3% EtOAc in hexane and purified further by preparative TLC; benzene–EtOAc 9:1). Column chromatography of fractions 10–14 (0.443 g) furnished an additional amount of **2** (115.0 mg), lupeol (5.0 mg, eluted with 6% EtOAc in hexane) and **4** (20.0 mg, eluted with 6% EtOAc in hexane). The latter two compounds were purified by TLC using 2% EtOAc in hexane.

Column chromatography of fractions 15–19 (0.314 g) followed by repeated TLC ($CHCl_3$–MeOH 19:1, multiple developments) afforded diterpenes **5** (1.9 mg), **6** (1.1 mg), **7** (1.2. mg) and **8** (11.0 mg). Fractions 20–23 (0.371 g) afforded an additional amount of **8** (20.0 mg eluted with 15% EtOAc in hexane) and large amounts of keto acid **9** (270 mg, eluted with 20% EtOAc in hexane). Fractions 24–31 (0.545 g) provided a further quantity of **9** (150.0 mg), in addition to three minor compounds (**10**, **11** and **12**). Purification by repeated preparative TLC (CH_2Cl_2) afforded **10** (1.8 mg), **11**, (0.9 mg), and **12** (0.7 mg), respectively.

3.4. Known diterpenoids

The physical properties and spectra determined for the known diterpenes isolated, and the sources of comparison data from the literature are presented below. Physical and/or spectral data are given when the data are not available in the literature.

Stachenone (**1**): colorless oil (Lit.; needles, m.p. 35–36.5 ° C), IR and ^{1}H NMR (Baarschers et al., 1962; Hanson, 1970); ^{13}C NMR (Table 1). EI MS *m/z* (rel. int.): 286 $[M]^+$ (100), 271 (10), 159 (25), 147 (40), 135 (87). *Diosphenol* **2**: colorless crystals, m.p. 121–123 °C (lit.; 125–126 °C); IR, ^{1}H NMR, ^{13}C NMR and MS (Munkombwe et al., 1997). *4-Keto ester* (**3**): see below. *Stachenol* (**4**): white solid, $[\alpha]_D^{25}$ + 33.6 ($CHCl_3$, *c* 0.25) (Lit.; +32.0, $CHCl_3$), ^{1}H NMR, ^{13}C NMR and MS (Munkombwe et al., 1997). *6-Hydroxy diosphenol* **8**: white solid, ^{1}H NMR, ^{13}C NMR and MS (Munkombwe et al., 1997). *4-Keto acid* (**9**): colorless oil, IR, ^{1}H NMR, ^{13}C NMR and MS (Munkombwe et al., 1998). *Rhizophorin C* (**10**): white solid, HREIMS *m/z* 303.2325 (calc. for $C_{20}H_{31}O_2$ 303.2324) $[M + 1]^+$; IR, ^{1}H NMR, ^{13}C NMR and MS (Anjaneyulu et al., 2002). *6-Hydroxy diosphenol* **11**: clear oil, IR, ^{1}H NMR, ^{13}C NMR and MS (Munkombwe et al., 1998).

3.5. Methyl ent-2,4-seco-4-oxo-3,19-dinorbeyer-15-en-2-oate (*3*)

A suspension of natural 4-keto acid **9** (32.0 mg, 0.11 mmol) and Amberlyst 15 (75.0 mg) in 2.5 mL of MeOH was stirred at room temperature for 1 week. Solids were filtered, and the filtrate was evaporated with a stream of N_2. Purification by silica gel chromatography using 10% EtOAc/hexane as eluent gave 20.0 mg (60 % yield) of methyl ester **3**. Similarly, keto acid **9** (5.3 mg, 0.018 mmol) was allowed to react for 48 h with MeOH (1.5 mL) in the presence of Amberlyst 15 (10.0 mg) to give, after preparative TLC (10% EtOAc/hexane), methyl ester **3** (1.4 mg, 25%) and the isomeric lactone ether **14** (3.5 mg, 63%) as a 5:2 mixture of C-4 epimers. Data for compound **14**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.69 (*d*, $J = 6.0$ Hz, H-15, major isomer), 5.67 (*d*, $J = 6.0$ Hz, H-15, minor isomer), 5.53 (*d*, $J = 6.0$ Hz, H-16, minor isomer), 5.51 (*d*, $J = 6.0$ Hz, H-16, major isomer), 3.37 (*s*, OCH_3, minor isomer), 3.36 (*s*, OCH_3, major isomer), 2.62 (*d*, $J = 17.0$ Hz, H-1′, minor isomer), 2.61 (*d*, $J = 17.0$ Hz, H-1′, major isomer), 1.99 (*dd*, $J = 17.0$ 1. Hz, H-1, minor isomer), 1.97 (*dd*, $J = 17.0$, 1.0 Hz, H-1, major isomer), 1.56 (*s*, Me-18, major isomer), 1.48 (*s*, Me-18, minor isomer), 1.02 (*s*, Me-17, minor isomer), 1.01 (*s*, Me-17, major isomer), 0.93 (*d*, $J = 1$ Hz, Me-20, major isomer), 0.85 (*d*, $J = 1$ Hz, Me-20, minor isomer).

A suspension of pseudo ester **14** (4.1 mg, 0.013 mmol) and Amberlyst 15 (10.0 mg) in 1.5 mL of MeOH was stirred at room temperature for 72. h. Work up as described above gave 4.0 mg (quant.) of compound **3**: colorless oil $[\alpha]_D$ +3.5 ($CHCl_3$, *c* 1.5) (Lit., −2.6, $CHCl_3$, *c* 0.0232); IR, ^{1}H NMR, and MS (Munkombwe et al., 1998); ^{13}C NMR (Table 1).

3.6. ent-3-Oxa-beyer-15-en-2-one (*5*)

White crystals; m.p. 119–120° C; $[\alpha]_D^{25}$ − 19.7 ($CHCl_3$, *c* 0.8); UV (CH_3OH) λ_{max} nm ($\log\varepsilon$) 261 (2.24) nm; IR ν_{max} ($CHCl_3$) 2924, 2850, 1727 (CO), 748 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 5.67 (*d*, $J = 6.0$ Hz, H-15), 5.53 (*d*, $J = 6.0$ Hz, H-16), 2.61 (*d*, $J = 16.5$ Hz, H-1′), 1.89 (*dd*, $J = 16.5$, 0.5 Hz, H-1), 1.72 (*dt*, $J = 13.5$, 2.5 Hz, 1H),

1.54 (*d*, $J = 12.5$, 1H), 1.42 (*s*, Me-18 or Me-19), 135 (*s*, Me-19 or Me-18), 1.06 (*d*, $J = 9.5$ Hz, H-14a), 1.02 (*s*, Me-17). 0.88 (*d*, $J = 1$ Hz, Me-20); ^{13}C NMR: see Table 1; HREIMS *m/z* 288.20849 (calc. for $C_{19}H_{28}O_2$ 288.2089); EIMS *m/z* (rel. int.): 288 $[M]^+$ (44), 273 (12), 220 (16), 205 (48), 188 (100), 159 (30), 133 (40), 105 (28), 84 (50).

To a well-stirred solution of natural keto acid **9** (31.3 mg, 0.11 mmol, 85 % pure by ^{1}H NMR analysis) in 2 mL of THF was added dropwise MeLi (1.6 M in Et_2O, 142 μL, 0.23 mmol, 2.1 equiv.) at −50 °C under an atmosphere of N_2. After the addition, the resulting solution was stirred at −10 ° C for 1 h and then at room temperature for 2 h. The reaction was quenched by addition of 1 mL of 10% aq. HCl. The two-phase mixture was stirred at room temperature for 30 min and then extracted with EtOAc (3 × 2 mL). Purification by flash chromatography on silica gel using a gradient of 10–20% EtOAc/hexane gave pure compound **5** (10.8 mg, 35 % yield) as a white crystalline solid. The ^{1}H and ^{13}C NMR spectra were identical with those previously obtained for the natural lactone.

3.7. ent-15,16-Epoxy-2-hydroxy-19-norbeyer-1,4-dien-3-one (*6*)

To a well-stirred solution of natural diosphenol **9** (8.8 mg, 0.03 mmol) in 2 mL of dry CH_2Cl_2 was added 3-chloroperoxybenzoic acid (mCPBA, 77% peroxy content, 34.1 mg, 0.152 mmol) at 0 ° C. After 2.5 h, H_2O (3 mL) was added, and the resulting mixture extracted with Et_2O (3 × 2 mL). The ethereal extracts were washed with 5% NaOH (3 × 1 mL) and dried (Na_2SO_4). Purification by preparative TLC on silica gel using CH_2Cl_2 as the developing solvent gave compound **6** (9.3 mg, quant.) as a white solid: m.p. 144–145 °C; $[\alpha]_D^{25}$ − 6.5 ($CHCl_3$, *c* 0.5); UV (CH_3OH) λ_{max} (log ε) 261 (3.34) nm; IR ν_{max} ($CHCl_3$) 3376 (OH), 2945, 2868, 1625 (CO), 1237 and 847 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 6.43 (*s*, OH), 6.26 (*s*, H-1), 3.59 (*d*, $J = 3.0$ Hz, H-15), 3.16 (*d*, $J = 3.0$ Hz, H-16), 2.86 (*dt*, $J = 13.5$, 3.5 Hz, 1H). 2.46 (*ddd*, $J = 14.5$, 13.5, 3.5 Hz, 1H), 2.11 (*ddd*, $J = 13.5$, 4.5, 3.5 Hz, 1H), 2.00 (*d*, $J = 1.0$ Hz, Me-18), 1.86 (*ddd* $J = 13.5$, 12.5, 3.5 Hz, 1H), 1.79 (*m*, 1H), 1.73 (*m*, 1H), 1.39 (*ddd*, J =13.5, 12.5, 5.0 Hz, 1H),1.32 (*dd*, $J = 13.5$, 3.5 Hz, 1H), 1.27 (*s*, Me-20), 1.24 (*dd*, $J = 11.0$, 3.0 Hz, H-14e), 1.06 (*s*, Me-17), 0.49 (*d*, $J = 11.0$ Hz, H-14a); ^{13}C NMR: see Table 1; HRESIMS *m/z* 301.1807 (calc. for $C_{19}H_{25}O_3$ 301.1804) $[M + 1]^+$; ESIMS *m/z* (rel. int.): 301 $[M + 1]^+$ (30), 283 (8), 151 (100). The ^{1}H NMR spectrum was identical with that previously obtained for the natural epoxide.

3.8. Methyl ent-2,4-seco-15,16-epoxy-4-oxo-3,19-dinorbeyer-15-en-2-oate (*7*)

Natural methyl ester **3** (6.7 mg, 0.02 mmol) was epoxidized as previously described for **6** to afford epoxide **7** (6.2 mg, 88%) as a clear oil: $[\alpha]_D^{25}$ − 11.3 ($CHCl_3$, *c* 0.4); UV (CH_3OH) λ_{max} (log ε) 208 (2.46), 260 (0.67), 322 (0.17) nm; IR ν_{max} ($CHCl_3$) 2950, 2869, 1731 (ester CO), 1707 (ketone CO) 1181 and 849 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.66 (*s*, OMe), 3.42 (*d*, $J = 3.2$ Hz, H-15), 3.01 (*d*, $J = 3.2$ Hz, H-16), 2.97 (*dd*, $J = 12.0$, 3.2 Hz, H-5), 2.38 (*d*, $J = 14.4$ Hz, H-1′), 2.30 (*d*, $J = 14.4$ Hz, H-1′), 2.23 (*s*, Me-18), 1.90–1.23 (*m*, 9 H), 1.18 (*dd*, $J = 10.8$, 2.4 Hz, H-14e), 1.14 (*s*, Me-20), 1.03 (*s*, Me-17), 0.58 (*d*, $J = 10.8$ Hz, H-14a); ^{13}C NMR: see Table 1; HRESIMS *m/z* 321.2068 (calc. for $C_{19}H_{29}O_4$ 321.2066) [M + 1]; ESIMS *m/z* (rel. int.): 321 $[M + 1]^+$ (80), 303 (9), 289 (100), 271 (8), 217 (5). The ^{1}H NMR spectrum was identical with that previously obtained for the natural epoxide.

3.9. ent-2,17-Dihydroxy-19-norbeyer-1,4,15-trien-3-one (*12*)

Colorless oil: $[\alpha]_D^{25}$ − 2.9 ($CHCl_3$, *c* 0.09); UV (CH_3OH) λ_{max} (log ε) 262 (2.45) nm; IR ν_{max} ($CHCl_3$) 3586 (OH), 2950, 2883, 1618 (CO) 1021 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 6.42 (*s*, OH), 6.28 (*s*, H-1), 6.03 (*d*, $J = 6.0$ Hz, H-15), 5.78 (*d*, $J = 6.0$ Hz, H-16), 3.59 (*d*, $J = 10.5$ Hz, H-17′), 3.50 (*d*, $J = 10.5$ Hz, H-17), 2.81 (*dt*, $J = 13.0$, 3.5 Hz, 1H). 2.37 (*ddd*, $J = 14.5$, 13.5, 3.5 Hz, 1H), 2.30 (*m*, 1H), 1.99 (*s*, C-18 Me), 1.90 (*dt*, $J = 13.0$, 4.5 Hz, 1H), 1.76 (*dd* $J = 10.0$, 3.0 Hz, H-14e), 1.12 (*s*, Me-20), 0.97 (*d*, $J = 10.0$ Hz, H-14a); HRESIMS *m/z* 301.1801 (calc. for $C_{19}H_{25}O_3$ 301.1804) [M + 1]; ESIMS *m/z* (rel. int.): 301 $[M + 1]^+$ (10), 151 (100).

3.10. In vitro L1210 mouse leukemia assay

The assays were performed according to Grace et al., 2006. The only difference was that the tested concentrations were 80, 60, 40, 20, 10 and 1.0 μg/mL.

Acknowledgements

We gratefully acknowledge the support of Fogarty International Center of the NIH under U01 TW006674 for the International Cooperative Biodiversity Groups (MAL) and the NIH through research Grant GM 13956 (RMC). The authors thank Mr. Paul Ceroke, University of Illinois at Urbana–Champaign, for donating the heartwood specimen for this research, and Dr. Eugene Dimitriadis for collecting and identifying the wood sample.

References

Anjaneyulu, A.S.R., Anjaneyulu, V., Rao, V.L., 2002. New beyerane and isopimarane diterpenoids from *Rhizophora mucronata*. J. Asian Nat. Prod. Res. 4, 53–61.

Anjaneyulu, A.S.R., Rao, V.L., 2000. Five diterpenoids (agallochins A–E) from the mangrove plant *Excoecaria agallocha* Linn. Phytochemistry 55, 891–901.

Baarschers, W.H., Horn, D.H.S., Johnson, L.F., 1962. The structure of some diterpenes from tambooti wood *Spirostachys africana*. J. Chem. Soc., 4046–4055.

Bakker, H.J., Ghisalberti, E.L., Jefferries, P.R., 1972. Biosynthesis of diterpenes in *Beyeria leschenaultia*. Phytochemistry 11, 2221–2231.

Buckingham, J., 1994 (Exec. Ed.). Dictionary of Natural Products. Chapman & Hall, London.

Burns, D., Reynolds, W.F., Buchanan, G., Reese, P., Enriquez, R., 2000. Assignment of ^{1}H and ^{13}C NMR spectra and investigation of hindered side-chain rotation in lupeol derivatives. Magn. Reson. Chem 38, 488–493.

Chalmers, A.A., Gorst-Allman, C.P., Piacenza, L.P.L., 1977. Carbon-13 NMR spectra of stachenol and related derivatives. Tetrahedron Lett. 18, 1665–1668.

Coates, R.M., Conradi, R.A., Ley, D.A., Akeson, A., Harada, J., Lee, S.C., West, C.A., 1976. Enzymatic cyclization of (*R*,*S*)-14,15-oxidogeranylgeranyl pyrophosphate to 3a- and 3b-hydroxykaurene. J. Am. Chem. Soc. 98, 4659–4661.

Connolly, J.D., Hill, R.A., 1991. In: Dictionary of Terpenoids Di- and Higher Terpenoids, vol. 2. Chapman & Hall, London.

Croteau, R., 1987. Biosynthesis and catabolism of monoterpenoids. Chem. Rev. 87, 929–954.

Davis, E.M., Croteau, R., 2000. Cyclization enzymes in the biosynthesis of monoterpenes, sesquiterpenes, and diterpenes. Top. Curr. Chem. 209, 53–95.

Dürr, C., Schnell, H.-J., Luzhetskyy, A., Murillo, R., Weber, M., Welzel, K., Vente, A., Bechthold, A., 2006. Biosynthesis of the terpene phenalinolactone in *Streptomyces* sp. Tü6071: analysis of the gene cluster and generation of derivatives. Chem. Biol. 13, 365–377.

Erickson, K.L., Beutler, J.A., Cardellina 2nd, J.H., McMahon, J.B., Newman, D.J., Boyd, M.R., 1995. A novel phorbol ester from *Excoecaria agallocha*. J. Nat. Prod. 58, 769–772.

Grace, M.H., Jin, Y., Wilson, G.R., Coates, R.M., 2006. Structures, biogenetic relationships, and cytotoxicity of pimarane-derived diterpenes from *Petalostigma pubescens*. Phytochemistry 67, 1708–1715.

Grant, P.K., Carman, R.M., 1962. Colensenone. J. Chem. Soc., 3740–3746.

Grant, P.K., Huntrakul, C., Robertson, J.M., 1969. The diterpenes of *Dacrydium colensoi*. VIII. Sandaracopimaradien-19-ol and 2a-carboxycolensen-2b-ol. Aust. J. Chem. 22, 1265–1270.

Hanson, J.R., 1970. Chemistry of the tetracyclic diterpenoids. X. Beyerene 2 and 3-alcohols. Tetrahedron 26, 2711–2715.

Hedden, P., Phillips, A.L., Rojas, M.C., Carrera, E., Tudzynski, B., 2002. Gibberellin biosynthesis in plants and fungi: a case of convergent evolution?. J. Plant Growth Regul. 20 319–331.

Jayaweera, D.M.A., 1980. Medicinal plants used in Ceylon. Natl. Sci. Council Sri Lanka 2, 214–215.

Jefferies, P.R., Rosich, R.S., White, D.E., 1973. Absolute configuration of beyerol. Tetrahedron Lett. 14, 1793–1799.

Jones, P.R., 1963. Ring-chain tautomerism. Chem. Rev. 63, 461–487.

Kametani, T., Toya, T., Ueda, K., Tsubuki, M., Honda, T., 1988. A novel synthesis of cyclobutane monoterpenes, (±)-grandisol and (±)-lineatin. J. Chem. Soc. Perkin Trans. I, 2433–2438.

Karalai, C., Wiriyachitra, P., Opferkuch, H.J., Hecker, E., 1994. Medicinal plants of Euphoribiaceae occurring and utilized in Thailand. II. Cryptic and free skin irritants of the daphnane and tigliane types in latex of *Excoecaria agallocha*. Planta Med. 60, 351–355.

Konishi, T., Konoshima, T., Fujiwara, Y., Kiyosawa, S., 2000. Excoecarins D, E and K from *Excoecaria agallocha*. J. Nat. Prod. 63, 344–346.

Konishi, T., Yamazoe, K., Konoshima, T., Fujiwara, Y., 2003. Secolabdane type diterpenoids from *Excoecaria agallocha*. Phytochemistry 64, 835–840.

Kono, K., Kojima, A., Nagai, R., Watanabe, M., Kawashima, T., Onizawa, T., Teraoka, T., Watanab, M., Koshino, H., Uzawa, J., Suzuki, Y., Sakurai, A., 2004. Antibacterial diterpenoids and their fatty acid conjugates from rice leaves. Phytochemistry 65, 1291–1298.

Maguire, T.L., Saenger, P., 2000. The taxonomic relationships within the genus Excoecaria L. (Euphorbiaceae) based on leaf morphology and rDNA sequence data. Wetlands Ecol. Manage. 8, 19–28.

MacMillan, J., 1997. The biosynthesis of gibberellin plant hormones. Nat. Prod. Rep. 14, 221–243.

MacMillan, J., Beale, M.H., 1999. In: Cane, D.E. (Ed.), Isoprenoids Including Carotenoids and Steroids. In: Barton, D., Nakanishi, K., Meth-Cohn, O. (Eds.), . In: Comprehensive Natural Products Chemistry, vol. 2. Elsevier, Amsterdam, pp. 217–243.

Munkombwe, N.M., Maswabi, T., Hughes, N.A., 1997. Diosphenols from *Spirostachys africana*. Phytochemistry 45, 1217–1220.

Munkombwe, N.M., Hughes, N.A., Duri, Z.J., 1998. Acid metabolites from *Spirostachys africana*. Phytochemistry 47, 1653–1655.

Ohigashi, H., Katsumata, H., Kawazu, K., Koshimizu, K., Mitsui, T., 1974. Piscicidal constituent of *Excoecaria agallocha*. Agric. Biol. Chem. 38, 1093–1095.

Petrini, M., Ballini, R., Marcantoni, E., 1988. Amberlyst 15: a practical, mild and selective catalyst for methyl esterification of carboxylic acids. Synth. Commun. 18, 847–853.

Pretsch, E., Clerc, T., Seibl, J., Simon, W., 1976. Tabellen zur Strukturaufklärung Organischer Verbindungen mit Spektroskopischen Methoden. Springer-Verlag, Berlin, pp. C175.

Sakai, T., Nakagawa, Y., 1988. Diterpenic stress metabolites from cassava roots. Phytochemistry 27, 3769–3779.

Stahl, E., 1969. Thin Layer Chromatography, 2nd ed. Springer-Verlag, Berlin, pp. 857.

West, C.A., 1983. In: Porter, J.W., Spurgeon, S.L. (Eds.), . In: Biosynthesis of Isoprenoid Compounds, vol. 1. Wiley, New York (Chapter 7).

Wills, J.C., 1973. A Dictionary of the Flowering Plants and Ferns, eighth ed. Cambridge University Press, Cambridge, pp. 450.

Wiriyachitra, P., Hajiwangoh, H., Boonton, P., Adolf, W., Opferkuch, H.J., Hecker, E., 1985. Investigations of medicinal plants of Euphorbiaceae and Thymelaeaceae occurring and used in Thailand; II. Cryptic irritants of the diterpene ester type from three Excoecaria species. Planta Med. 5, 368–371.