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ISOLATION AND CHARACTERIZATION OF NATURAL PRODUCTS FROM PLANT TISSUE CULTURES OF *MAYTENUS BUCHANANII*

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Key Word Index—*Maytenus buchananii*; Celastraceae; tissue culture; polpunonic acid; sitosterol; quinone-methides; tingenone; 22β-hydroxytingenone.

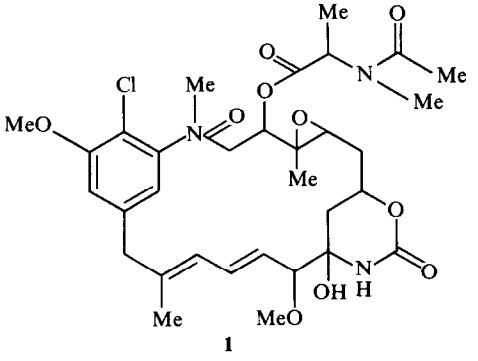
Abstract—Explants of *Maytenus buchananii* were induced to form a callus and subsequently to form suspension cultures on a wide variety of media. Culture extracts showed cytotoxic activity, but examination by TLC did not indicate the presence of maytansine. Isolation of natural products from a large scale suspension culture led to the identification of polpunonic acid, sitosterol and the cytotoxic triterpene quinone-methides, tingenone and 22β-hydroxytingenone. Possible biosynthetic relationships of these and other triterpene quinone-methides are discussed.

INTRODUCTION

Maytenus buchananii (Loes.) R. Wilezck is one of several members of the Celastraceae from which the antitumour and antileukemic macrocycle, maytansine (**1**), has been isolated [1–3]. As an alternative source of supply we have investigated the possible production of maytansine in tissue cultures of *M. buchananii*. In this initial study we have shown that this species grows very well on culture media but maytansine could not be detected in the culture extracts. However the cultures did produce the known cytotoxic compounds tingenone (**2**) and 22β-hydroxy-tingenone (**3**).

RESULTS

Since secondary metabolite production in tissue culture has been reported to be influenced by the nature and levels of auxin, cytokinins, vitamins, carbon source, mineral content of media, temperature, light, culture age and explant tissue [4–11], a large number (>200) of explants were cultured onto a wide variety of media. *M. buchananii* tissues from all sources readily formed calluses on B5, PRL-4, MS and SH [12–15] basic media, variously supplemented with indoleacetic acid (IAA), naphthyl-acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), 4-aminobenzoic acid, kinetin, 4-chlorophenoxyacetic



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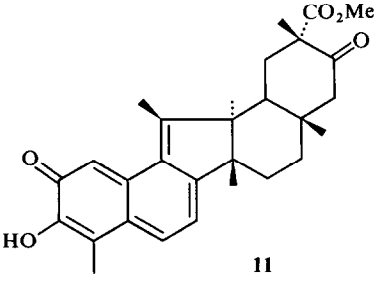

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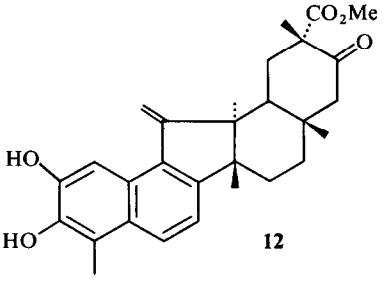

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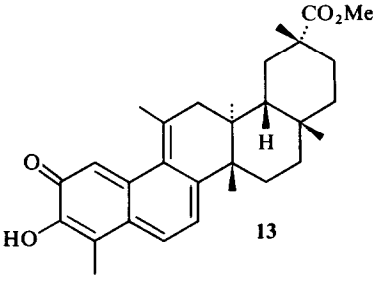

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acid, thiamine hydrochloride and coconut water. With the exception of MS medium containing 6×10^{-5} M IAA acid with 1.9×10^{-7} M kinetin, and B5 medium containing no phytohormone supplements, all media and conditions promoted rapid induction and growth of calluses. Calluses appeared to be of heterogeneous composition as indicated by green, black, yellow and orange regions. Many of the calluses bore a profusion of roots.

A number of calluses were chosen for suspension culture. Typically after 3–6 weeks, suspensions of mixed aggregate and single cells were produced. The most rapidly growing of these cultures achieved mass doubling times of approximately 3–4 days. Cell yields were in the order of 12–16 g dry wt of cells per l. of culture.

Extensive TLC analysis of both the crude extracts and after preparative TLC concentration of the appropriate R_f band against authentic maytansine did not reveal the presence of the target compound in any cultures. The TLC assay was shown by use of standard solutions to have a detection limit of 10 ng of maytansine. However, bioassay by the KB cell assay* showed some of the crude extracts to contain cytotoxic components ($ED_{50} < 5\ \mu g/ml$). This compares with an ED_{50} value of 0.2 μg/ml for crude *plant* extracts containing maytansine (see Table 1). In order to determine the nature of the cytotoxic components of the culture extracts, one suspension culture was scaled up to a 10 l. volume to provide sufficient material for natural product isolation.

For isolation, the crude alcohol extract of the cells was partitioned between ethyl acetate and water, and then petrol and 10% aqueous methanol. Sitosterol was the major component of the petrol fraction. Column chromatography followed by preparative TLC, of the aqueous methanol phase yielded polpunonic acid (**4**), tingenone (**2**) and 22β-hydroxytingenone (**3**) in yields of 0.043, 0.005 and 0.005% of dry wt of cells, respectively. The acid **4** was characterized as its methyl ester (**5**) and identified by direct comparison with authentic samples. The two orange pigments tingenone and 22β-hydroxytingenone were identified by their MS and ^{1}H NMR spectra. In particular, the ^{1}H NMR spectra showed signals at δ 6.40 (*d*), 6.55 (*d*) and 7.05 (*dd*), typical of the triterpene quinone-methides [16]. The molecular ion in the MS of 22β-hydroxytingenone (**3**) occurred at *m/e* 436, 16 amu higher than that in the spectrum of tingenone (**2**), indicating the presence of an extra oxygen atom. This was confirmed by the presence of a ^{1}H NMR signal at δ 4.56 for a proton on carbon bearing a hydroxyl group. The absence of the doublet (δ 2.92, $J = 14$ Hz) occurring in the ^{1}H NMR spectrum of tingenone (shown to be the 22β-proton [17]) showed the hydroxyl group to be at C-22. The identity of tingenone was confirmed by direct comparison (TLC and ^{1}H NMR) with authentic material and that of 22β-hydroxytingenone by comparison with published ^{1}H NMR [17, 18]. Tingenone and 22β-hydroxytingenone gave KB assay ED_{50} values of 0.27 and 2.5 μg/ml, respectively, and these may account for the KB activity of the crude culture extracts.

Table 1. The ED_{50} of maytansine and related compounds and extracts

Compound or extract	ED_{50}*
Maytansine†	2×10^{-5}
Crude plant extracts containing maytansine†	2×10^{-1}
Crude extracts from tissue culture MYT lel	3.8
Tingenone	2.7×10^{-1}
22β-Hydroxytingenone	2.5

* ED_{50} is expressed in μg/ml and is the calculated effective concentration which inhibits growth of 50% of control growth. Assay was done using KB cells (human epidermoid carcinoma of the nasopharynx).

† Data from Dr. M. Suffness, National Cancer Institute.

* KB assays were performed by Dr. Mildred Broome as described in (1972) *Cancer Chemother. Rep. Part* 3, **3**, 1.

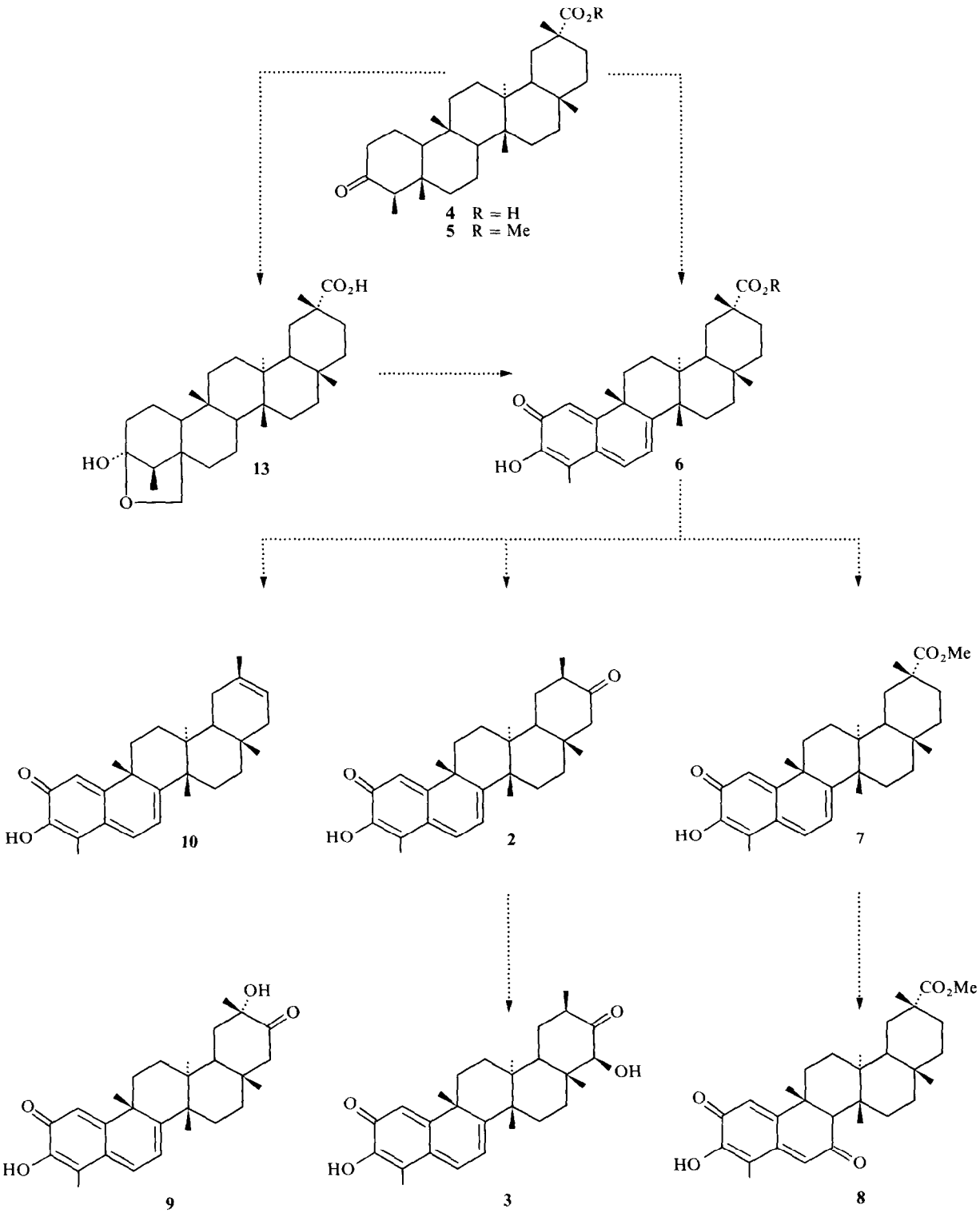
Partial purification of the more polar components of the culture extract as their methyl esters yielded a complex mixture of triterpene esters which gave six peaks on GLC as their TMSi derivatives. Separation of this mixture could not be achieved by preparative TLC on silica gel. Due to the limited amount of material, no further attempts to assign complete structures were undertaken.

DISCUSSION

The presence of tingenone and 22β-hydroxytingenone in tissue cultures of *M. buchananii* is not surprising as triterpene quinone-methides of this type have previously been isolated from the following members of the Celastraceae: *Celastrus scandens* [19] [celastrol (**6**)], *Tripterygium wilfordii* [20] (celastrol), *Pristimera indica* [21] [pristimerin (**7**)], *Maytenus chuchuhuasca* [22] (pristimerin and tingenone), *Maytenus dispermus* [23] [pristimerin and dispermoquinone (**8**)], *Euonymus tingens* [17, 24, 25] [tingenone, 22β-hydroxytingenone and 20-hydroxytingenone (**9**)], *Maytenus* sp. [18] (22β-hydroxytingenone), *Maytenus ilicifolia* [26] (pristimerin and tingenone), *Plenckia polpunea* [27] (tingenone), *Catha cassinoides* [16] [celastrol, tingenone, pristimerin and iguesterin (**10**)], *Salacia macrosperma* [28] [pristimerin tingenone, 20-hydroxytingenone and salacia quinone-methide (**11**)] and *Schaefferia cuneifolia* [29] (pristimerin and tingenone).

Abraham *et al.* [30, 31] have reported the isolation of maytenonic acid from *Maytenus senegalensis*. They identified this compound as a 3-ketofriedelane bearing a carboxyl group at C-20. The stereochemistry at C-20 was not defined. It seems likely, on chemotaxonomic grounds, that maytenonic acid is the same as polpunonic acid (i.e. 20α-COOH). The co-occurrence of polpunonic acid and tingenone in *Pleuckia polpunea* [27] and also in the *M. buchananii* tissue cultures suggests that these compounds are biogenetically related. Viswanathan [32] has reported the isolation of salaspermic acid (**13**) as well as pristimerin, tingenone and hydroxytingenone from *Salacia macrosperma*. Again the co-occurrence of these compounds suggests a common biogenesis. Possible biosynthetic relationships between polypunonic acid, salaspermic acid and the triterpene quinonemethides are summarized in Scheme 1.

Reddy *et al.* [28] have reported the isolation of salacia quinone-methide from *Salacia macrosperma*. They have suggested structure **11** for this new quinone-methide on spectroscopic evidence. The structure proposed appears


CO2R
O
4 R = H
5 R = Me
CO2H
HO
O
13
CO2R
O
HO
6
O
HO
10
O
O
HO
2
CO2Me
O
HO
7
OH
O
O
HO
9
O
O
OH
O
HO
3
CO2Me
O
HO
O
8

Scheme 1.

unlikely as it would exist in the more stable aromatic form (**12**)! In fact a very recent paper on new quinone-methides has also expressed doubt as to the correctness of structure **11** and has suggested that it might be identical with the compound pristimerinene (**13**) isolated from *Prionostemma aspera* [33].

TLC analysis indicated that most of the cultures grown produced tingenone and 22β-hydroxytingenone. No attempt was made to maximize the yield of these compounds. The failure to detect maytansine in the cultures may be due to several reasons: (a) the fast growth rate observed may not favour secondary metabolite production [34]; (b) there is some debate as to whether maytansine is a plant product or is produced by a microorganism either totally or in a symbiotic relationship with the plant [35]; (c) there are many instances where callus and suspension cultures have failed to produce the secondary metabolites present in the parent plant. The reason for this is not known ([36] and refs. cited therein).

EXPERIMENTAL

Plant material. Maytenus buchananii (Loes.) R. Wilczek plants and seeds (seed lot PR 49836) were provided by the National Institutes of Health, Bethesda, Maryland.

Callus initiation. Callus cultures were started from stem and leaf explants and from the radicle, cotyledon, hypocotyl and plumule tissue of aseptically germinated seeds. Solid media were formulated with 0.9% Difco Bacto Agar and cultures were maintained at 28° in the dark or at 22° under subdued ('coolwhite' fluorescent) lighting. The basic media (B5, PRL4, MS and SH) [11–14] were supplemented with various combinations of IAA (0 or 0.6–6.0 $\times 10^{-5}$ M), NAA (0 or 10^{-5} M), 2,4-D (0 or 0.2–9.0 $\times 10^{-6}$ M), *p*-aminobenzoic acid (0 or 7×10^{-7} M), kinetin (0 or 2.0–5.0 $\times 10^{-7}$ M), *p*-chlorophenoxyacetic acid (0 or 1×10^{-5} M), thiamine HCl (3.0–5.0 $\times 10^{-5}$ M) and coconut water (0 or 10%). Calluses were routinely transferred at 4 week intervals and assayed by TLC and/or KB cell bioassay at time of transfer.

Suspension cultures. Fragments of callus were suspended in 100 ml volumes of the appropriate liquid medium and shaken, placed at 100 rpm, at 28° in the dark or at 22° under subdued room lighting. Suspension cultures were routinely transferred and analysed at 3–4 week intervals.

Natural product analysis. The cultures were freeze-dried and extracted with MeOH. TLC against authentic maytansine was carried out on Si gel with 4% MeOH in $CHCl_3$. Plates were visualized with 5% ammonium molybdate in 10% H_2SO_4 spray followed by heating at 110°.

Scale-up for natural product isolation. This culture originated from a stem explant which callused on solid PRL4 medium containing 10% coconut water and 9.7×10^{-6} M, 2,4-D at 28° in the dark. When 2 months old, the callus was transferred to a 100 ml suspension culture in PRL4 medium containing 10% coconut water and 12×10^{-5} IAA. After 3 transfers using the same medium, sixteen 600 ml cultures were inoculated, grown for 1 month and then harvested.

Isolation of natural products. The culture medium was extracted with EtOAc. The freeze-dried cells (186 g) were pulverized and extracted with 95% EtOH at room temp. overnight. After filtration the residue was further extracted with boiling EtOH for 5 min. The combined filtrates were evapd and the residue partitioned between EtOAc and H_2O. Recovery from the combined EtOAc, extracts of the cells and medium, followed by partition between petrol and 10% aq. MeOH yielded fractions A (petrol soluble) and B (aq. MeOH soluble).

Fraction A was chromatographed on a column of Si gel. Elution with C_6H_6 gave initially fractions containing long-chain hydrocarbons and fatty acids, as indicated by their NMR spectra (*br. s* at δ 1.26 as only major signal), admixed with small amounts of unidentified minor components. Further elution with C_6H_6 yielded crude sitosterol (451 mg) which on crystallization from MeOH gave 325 mg (0.175% dry wt), mp 138–140° (lit. [37], mp 140°).

Fraction B was further fractionated, on a Si gel column eluted with $CHCl_3$ containing increasing amounts of MeOH, into fractions B1 (334 mg, $CHCl_3$), B2 (289 mg, 1–6% MeOH) and B3 (1.3 g, 6–100% MeOH).

Fraction B1, after repetitive prep. TLC on Si gel using 2% MeOH in $CHCl_3$ gave polpunonic acid (**4**, 80 mg, 0.043%), identical (TLC) with an authentic sample. Treatment with CH_2N_2 gave the ester (**5**), mp 227–230° (from MeOH) (lit. [27] mp 226–227°). Found: M^+ 470.3747, Calc. for $C_{31}H_{50}O_3$: M^+ 470.3760. ^{1}H NMR (100 MHz, $CDCl_3$): δ 0.72 (3H, *s*, Me), 0.85 (3H, *d*, $J = 7$ Hz, Me), 0.88 (6H, *s*, 2 × Me), 0.89 (3H, *s*, Me), 1.10 (3H, *s*, Me), 1.20 (3H, *s*, Me), 3.67 (3H, *s*, CO_2Me). MS *m/e* (rel. int.): 470 (1.6) M^+, 455 (5), 273 (13), 264 (6), 249 (7), 231 (10), 189 (19), 169 (40), 163 (32), 137 (34), 121 (34), 109 (100). Direct comparison with an authentic sample (^{1}H NMR, GLC and TLC) confirmed the identity.

Fraction B2, on prep. TLC on Si gel using C_6H_6–$CHCl_3$–EtOAc (2:1:1) yielded tingenone (**2**, 10 mg, 0.005%) identified by its NMR and MS and by comparison with an authentic sample. ^{1}H NMR (100 MHz, $CDCl_3$): δ 0.98 (3H, *s*, Me), 1.00 (3H, *d*, $J = 6$ Hz, Me), 1.02 (3H, *s*, Me), 1.35 (3H, *s*, Me), 1.51 (3H, *s*, Me), 2.23 (3H, *s*, Me), 2.92 (1H, *d*, $J = 14$ Hz, C-22), 6.39 (1H, *d*, $J = 7$ Hz, C-7), 6.56 (1H, *d*, $J = 2$ Hz, C-1), 7.05 (1H, *dd*, $J = 7$ Hz, 2, C-6). MS *m/e* (rel. int.): 420 (66) M^+, 406 (23), 405 (30), 278 (28), 253 (36), 241 (100), 239 (30), 227 (51), 214 (34), 201 (66), 188 (43), 187 (38), 165 (38) and 22β-hydroxytingenone (**3**, 10.5 mg, 0.005%) identified by its ^{1}H NMR and MS. ^{1}H NMR (100 MHz, $CDCl_3$): δ 0.87 (3H, *s*, Me), 0.99 (3H, *s*, Me), 1.08 (3H, *d*, $J = 6$ Hz, Me), 1.37 (3H, *s*, Me), 1.52 (3H, *s*, Me), 2.23 (3H, *s*, Me), 4.56 (1H, *s*, C-22), 6.40 (1H, *d*, $J = 7$ Hz, C-7), 6.55 (1H, *d*, $J = 2$ Hz, C-1), 7.06 (1H, *dd*, $J = 7$ Hz, 2, C-6). MS *m/e* (rel. int.): 436 (100) M^+, 422 (33), 421 (33), 420 (28), 278 (28), 267 (21), 253 (59), 241 (94), 239 (35), 227 (61), 213 (29), 202 (47), 201 (61), 189 (35), 188 (40), 187 (38).

Fraction B3 was methylated with CH_2N_2 in the usual way. Column chromatography on Si gel using C_6H_6–$CHCl_3$–EtOAc mixtures yielded a crude mixture of triterpene esters (212 mg). Multi-elution prep. TLC in the above solvents gave a mixture (61 mg) containing six compounds as shown by GLC of their TMSi derivatives. Retention times (1% OU-210, 240°, N_2 20 ml/min) 6.27, 7.01, 8.72, 9.69, 16.17, 18.48 min.

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