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Antimicrobial and cytotoxic agents from *Calophyllum inophyllum*

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Abstract

The study of the chemical constituents of the root bark and the nut of *Calophyllum inophyllum* has resulted in the isolation and characterization of a xanthone derivative, named inoxanthone, **3**, together with 12 known compounds: caloxanthones A, **4** and B, **5**, macluraxanthone, **6**, 1,5-dihydroxyxanthone, **7**, calophynic acid, **8**, brasiliensic acid, **9** inophylloidic acid, **10**, friedelan-3-one, **11**, calaustralin, **12**, calophyllolide, **13**, inophyllums C, **14** and E, **15**. Their structures were established on the basis of spectral evidence. Their in vitro cytotoxicity against the KB cell line and their antibacterial activity and potency against a wide range of micro organisms were evaluated.

Keywords: *Calophyllum inophyllum*; Clusiaceae; Roots; Nut; Xanthones; Triterpene; Phenylpyranocoumarins; Inoxanthone; Cytotoxicity; Antibacterial activity

1. Introduction

The dipyranocoumarins, a group of natural products isolated from several tropical plants of the genus *Calophyllum*, Clusiaceae, are characterized by chromane and chromene ring systems assembled around a phloroglucinol core (Polonsky, 1957; Kawazu et al., 1968; Gunasekera et al., 1977; Patil et al., 1993; Ishikawa, 2000). In 1992, the research group of the National Cancer Institute reported that (+)-calanolide A, **1** and inophyllum B, **2**, isolated from *Calophyllum lanigerum* Miq. and *C. inophyllum* L., respectively, showed strong activity against human immunodeficiency virus type 1 (HIV-1) (Kashman et al., 1992; Patil et al., 1993). Since then, the chemical constituents of several *Calophyllum* species have been extensively studied (Goh and Jantan, 1991; Chenera et al., 1993; Iinuma et al., 1994, 1995; Kijjoa et al., 2000; Ito et al., 2002, 2003). These studies have revealed that, besides pyranocoumarins, the genus *Calophyllum* is also a rich source of xanthones (Iinuma et al., 1994, 1995), triterpenes (Gunatilaka et al., 1984), steroids (Gunasekera and Sultanbawa, 1975), and biflavonoids (Cao et al., 1997). As part of a continuing search for bioactive metabolites from the plant family Clusiaceae, the chemical constituents of the root bark and fruit of *C. inophyllum* L., which is the only species of *Calophyllum* genus found in Cameroon, has been investigated. In this country, the aqueous extracts of the root bark and leaves are used as a cicatrisant, whereas those of the nut had analgesic properties and are also used in the treatment of wounds and herpes

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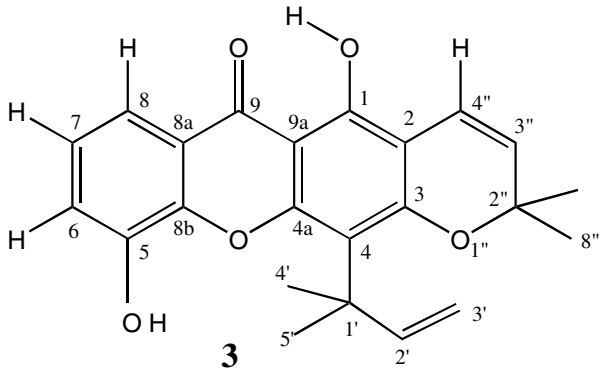
(Bruneton, 1993). The isolation, structural elucidation, and biological activity of a new xanthone derivative, inoxanthone, and nine other compounds **4**, **6**, **8**–**10**, and **12**–**15** were conducted, including evaluation for their antimicrobial and cytotoxic activities.



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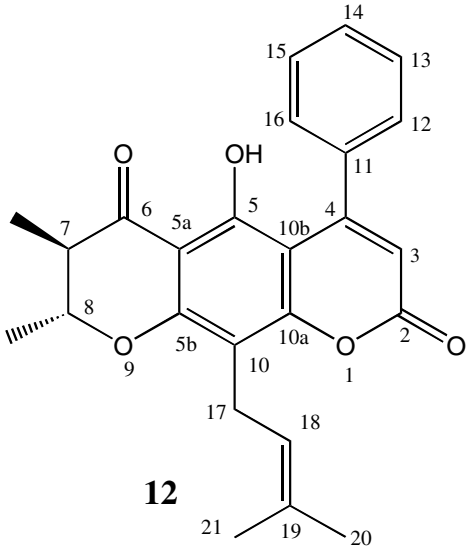

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2. Results and discussion

Bioassay-directed fractionation of the crude CH_2Cl_2–MeOH (1:1) extract of the root bark and crude CH_2Cl_2–MeOH (1:1) extract of the nut of *C. inophyllum* by flash and column chromatography afforded, respectively, several fractions containing antimicrobial and cytotoxic compounds. The active fractions from the former extract yielded, by repeated column chromatography over silica gel, a novel compound inoxanthone, **3**, together with eight known compounds, including four xanthones derivatives, caloxanthones A, **4** and B, **5**, macluraxanthone, **6** and 1,5-dihydroxyxanthone, **7** (Iinuma et al., 1994), three calophyllic acid derivatives, calophynic, **8** (Gautier et al., 1972), brasiliensic, **9** and inophylloidic, **10** acids (Stout et al., 1968), and one pentacyclic triterpene, friedelan-3-one, **11**. The active fractions from the nut extract led to the isolation of four known phenylcoumarin derivatives, including calaustralin, **12** (Breck and Stout, 1969), calophyllolide, **13** (Polonsky, 1957) and inophyllums C, **14** and E, **15** (Kawazu et al., 1968). It is important to note that brasilliensic acid and inophylloidic acid were both obtained in great amount. All of the known compounds were identified from their spectral data and their structures confirmed by comparison with published literature data.

Compound **3**, inoxanthone, m.p. 217 °C, was obtained as yellow needles and reacted positively to the Gibbs and $FeCl_3$ reagents indicating the presence of a phenolic group. The high resolution ESI-TOF mass spectrum showed a $(M + H)^+$ at *m/z* 379.1553 corresponding to a molecular formula of $C_{23}H_{22}O_5$ and implying 13 unsaturation sites. The broad-band decoupled ^{13}C NMR spectrum of **3** (Table 1) showed 21 carbon signals which were attributed by APT and HSQC techniques as four methyls, one methylene, six methines, and 12 quaternary carbons including a carbonyl ($\delta = 181.3$), five oxygenated sp^2 carbons, four sp^2, and two sp^3 carbons. The IR spectrum displayed free hydroxyl ($\nu_{max} = 3458$ cm^{-1}), chelated hydroxyl ($\nu_{max} = 3293$ cm^{-1}), conjugated carbonyl ($\nu_{max} = 1646$ cm^{-1}), and aromatic ring (1620, 1585 cm^{-1}) absorptions. These data, together which those obtained from the UV spectrum [λ (MeOH) nm 237, 249*sh*, 280*sh*, 292, 310, 340 and 376] were consistent with the presence of a xanthone skeleton (Iinuma et al., 1994, 1995). In the ^1H NMR spectrum ($CDCl_3$, Table 1) of compound **3**, analysed by ^1H–^1H COSY, an ABC spin system, formed by two double doublets at $\delta = 7.67$ (1H, *dd*, $J = 2.2$, 7.2 Hz) and $\delta = 7.22$ (1H, *dd*, $J = 2.2$, 7.2 Hz) and a triplet at $\delta = 7.19$ (1H, t, $J = 7.2$ Hz), corresponding to a 1,2,3-trisubstituted benzene ring, was observed in addition to a free hydroxyl signal at $\delta = 6.37$ and a chelated hydroxyl signal at $\delta = 13.41$. Furthermore, the ^1H and ^{13}C NMR spectra also displayed the presence of two sets of signals. The first set, comprising a six-proton singlet at $\delta = 1.51/\delta = 27.9$ and two *cis*-olefinic protons ($\delta = 5.60/\delta = 127.3$ and $\delta = 6.75/\delta = 116.0$, each, $J = 10$ Hz) was due to a dimethylchromene ring. The second set of signals, consisting of three one-proton double doublets at $\delta = 6.72/\delta = 155.8$ (1H, *dd*, $J = 10$ and 17 Hz), $\delta = 5.18/\delta = 104.0$ (1H, *dd*, $J = 1$ and 17 Hz), and $\delta = 5.06/\delta = 104.0$ (1H, *dd*, $J = 1$ and 10 Hz) and a six-proton singlet at $\delta = 1.64/\delta = 28.2$ (6H, *s*), established the presence of a 1,1-dimethylallyl substituent. A combination of the COSY and HSQC experiments permitted the assignment of all of the protonated carbons (Table 1). It remained to establish the positions of the substituents on the xanthone skeleton. In the HMBC spectrum (Fig. 1), the chelated hydroxyl group ($\delta = 13.41$) was correlated to the quaternary carbons at $\delta = 103.6$ (C-

Table 1
^{1}H (400 MHz) and ^{13}C (100 MHz) NMR ($CDCl_3$) spectral data of inoxanthone (**3**) and ^{13}C NMR ($CDCl_3$) spectral data of calaustralin (**12**)

Carbon no.	**3** δ_C	δ_H	Carbon no.	**12** δ_C
1	156.7		1	
2	105.5		2	160.17
3	159.4		3	113.33
4	113.1		4	156.6
5	153.9		5	160.82
6	120.5	7.22 (1H, *dd*, *J* = 2.2, 7.2)[a]	5a	102.68
7	124.2	7.19 (1H, *t*, *J* = 7.2)	5b	159.43
8	116.03	7.67 (1H, *dd*, *J* = 2.2, 7.2)	6	200.5
9	181.3		7	46.28
4a	144.1		8	79.45
8a	119.6		9	
9a	103.6		10	109.11
8b	145.3		10a	160.98
1′	41.3		10b	103.92
2′	155.8	6.72 (1H, *dd*, *J* = 10, 17)	11	139.28
3′	104.0	5.18 (1H, *dd*, *J* = 1, 17)	12	127.71
	5.06 (1H, *dd*, *J* = 1, 10)	13	128.07	
4′	28.2	1.64 (3H, *s*)	14	128.7
5′	28.2	1.64 (3H, it s)	15	128.07
2″	78.4		16	127.71
3″	127.3	5.60 (1H, *d*, *J* = 10)	17	21.93
4″	116.01	6.75 (1H, *d*, *J* = 10)	18	121.55
7″	27.9	1.51 (3H, *s*)	19	133.07
8″	27.9	1.51 (3H, *s*)	20	18.00
1-OH	13.41 (1H, *s*)[b]		21	26.00
5-OH	6.38 (1H, *s*)[b]		22	10.53
			23	19.98

[a] Coupling constants (*j* in Hz) given in parentheses.
[b] Exchangeable with D_2O.

9a), 105.5 (C-2), and 156.7 (C-1). The latter resonance at $\delta = 156.7$ also gave cross peaks with one of the *cis*-olefinic protons of the chromene ring (at $\delta = 6.75$), while the other *cis*-olefinic protons at $\delta = 5.60$ was correlated with the quaternary carbon at $\delta = 105.5$ (C-2). These results demonstrated clearly that the *gem*-dimethylchromene moiety was fused in a linear manner to the aromatic ring A of xanthone skeleton bearing the chelated hydroxyl group. The positions of the α,α-*gem*-dimethylallyl group and the remaining phenolic hydroxyl group were established as follows.

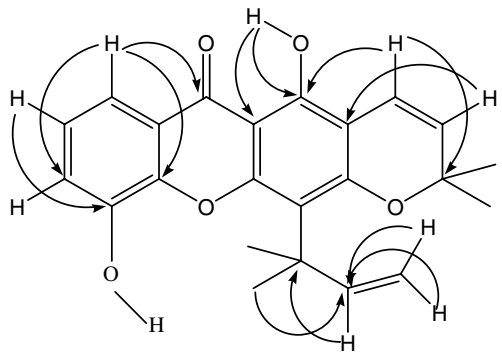

Fig. 1. HMBC correlations of **3**.

In the HMBC spectrum (Fig. 1), one of the ABC spin protons ($\delta = 7.67$) displayed cross-peaks with the carbonyl carbon [$\delta = 181.3$ (C-9)], indicating its *peri* position (H-8) whereas the two other protons belonging to the same ABC spin system [H-7 ($\delta = 7.19$, *t*, *J* = 7.2 Hz) and H-6 ($\delta = 7.22$, *dd*, *J* = 2.2, 7.2 Hz)] gave each cross peaks with an oxygenated sp^2 carbon at $\delta = 153.9$. This finding clearly indicated that the free hydroxyl group was located at C-5 position. Thus, the α,α-*gem*-dimethylallyl group was assigned to be at the C-4 position. This was further confirmed by the NOESY spectrum which showed correlated peaks between H-6 proton ($\delta = 7.22$) and free hydroxyl signal at $\delta = 6.38$. On the basis of the above results, the structure of inoxanthone, (**3**) was assigned to be 1,5-dihydroxy-4(3-dimethylpropenyl)-2″,2″-dimethylpyrano[5″,6″:2,3] xanthone.

Some of the isolated compounds were evaluated, for their cytotoxicity against human epidermoid carcinoma of the nasopharynx cell (KB) and for their antimicrobial and potency against representative Gram-(+), *Staphylococcus aureus* (ATCC6538), *Vibrio anguillarium* (ATCC19264), Gram-(−), *Escherichia coli* (ATCC8739) bacteria, and yeast, *Candida tropicalis* (ATCC 66029) organisms, in agar well diffusion assays. The results are summarized in Table 2. At the dose of 20 μg per disc, caloxanthone A, **4**, calophynic acid, **8**, brasiliensic

Table 2
Antimicrobial and cytotoxic activities of compounds **3**–**4**, **6**, **8**–**10** and **12**–**15**

Compounds	Diameter of inhibition (mm) at 20 μg/disk				KB cell IC_{50} μg/ml
	S. aureus	*V. anguillarium*	*E. coli*	*C. tropicalis*	
Caloxanthone A (**4**)	9.0	−ve	−ve	−ve	7.4
Calophynic acid (**8**)	10.0	−ve	−ve	−ve	10.5
Brasiliensic acid (**9**)	11.0	−ve	−ve	−ve	11.0
Inophylloidic acid (**10**)	9.0	−ve	−ve	−ve	9.7
Calaustralin (**12**)	11.0	−ve	−ve	−ve	42.0
Calophyllolide (**13**)	16.0	−ve	−ve	−ve	3.5
Inophyllum C (**14**)	10.0	−ve	−ve	−ve	n.t[a]
Inophyllum E (**15**)	13.0	−ve	−ve	−ve	36.1
Crude extract of root bark	13.0	−ve	−ve	−ve	n.t
Crude extract of nut	14.0	−ve	−ve	−ve	n.t
Oxacillin	30	−ve	−ve	−ve	n.t
Inoxanthone (**3**)	−ve	−ve	−ve	−ve	n.t
Macluraxanthone (**6**)	−ve	−ve	−ve	−ve	n.t

[a] Not tested.

acid, **9**, inophylloidic acid, **10**, calophyllolide, **13**, and inophyllum C, **14** and E, **15** were found to exhibit significant inhibitory activity against *S. aureus*, but not against other microorganisms. The activity of the seven compounds was less than that of the control, oxacillin, as shown in Table 2. It also appears, on the other hand, and as summarized in Table 2, that calophyllolide **13** displayed the most significant cytotoxic activity against KB cells with an IC_{50} value of 3.5 μg/ml. Other compounds, such as caloxanthone A, **4**, calophynic acid, **8**, brasiliensic acid, **9**, and inophylloidic acid **10**, which showed IC_{50} value of 7.4, 10.5, 11.0 and 9.7 μg/ml, respectively, were considered, in addition to calaustralin, **12**, and inophyllum E, **15**, as inactive. Inoxanthone, **3**, and macluraxanthone, **6**, were also found to be devoid of both cytotoxic and antimicrobial activities in vitro.

3. Experimental

3.1. General experimental procedures

Melting points were determined on a Büchi apparatus and are uncorrected. Silica gel 230–400 mesh (Merck) and silica gel 70–230 mesh (Merck) were used for flash and column chromatography, respectively, while precoated aluminium sheets silica gel 60 $F_{254\ nm}$ (Merck) were used for TLC. Spots were visualized by UV ($\lambda_{254\ nm}$) and 10% CeII–H_2SO_4. IR spectra were measured on a JASCO FT-IR-300 spectrometer in a KBr pellet. UV spectra were recorded on a Kontron Uvikon 932 spectrophotometer. Optical rotations were determined on a Perkin–Elmer polarimeter. One- and two-dimensional NMR spectra were recorded on a Bruker instrument equipped with a 5 mm ^1H and ^{13}C NMR probe operating at 400 and 100 MHz, respectively, with TMS as internal standard. Chemical shifts are reported in δ value in ppm using the solvent as reference. Mass spectra were performed on a APCI Qstar pulsar mass spectrometer.

3.2. Plant material

Fruits and root bark of *C. inophyllum* were collected near the beach at Kribi, South Province of Cameroon, in December 2002 and April 2003, respectively, by M. Nana, botanist at the National Herbarium, Yaounde, Cameroon, where voucher specimens documenting the collections are deposited under No. 32189/SRF/Cam.

3.3. Extraction and isolation

Fruits were slightly crushed to obtain the shell and nuts. The pulverized, air-dried nuts (850 g) were extracted by maceration at room temperature in a mixture of CH_2Cl_2–MeOH (1:1) for 24 h, yielding, after evaporation under reduced pressure an oily yellow extract (250 g). A portion of this oil (200 g) was subjected to column chromatography over silica gel packed in *n*-hexanes and eluted with *n*-hexanes–EtOAc mixtures of increasing polarity. A total of 117 fractions of ca. 400 ml each were collected and regrouped on the basis of TLC analysis to afford six major fractions (S_1–S_6): S_1 ($F_{1–10}$); S_2 ($F_{11–18}$); S_3 ($F_{19–37}$); S_4 ($F_{38–55}$); S_5 ($F_{56–79}$) and S_6 ($F_{80–117}$). Fraction S_2 (43.4 g), eluted with *n*-hexanes–EtOAc (19:1) was chromatographed on a silica gel column packed in *n*-hexanes. Gradient elution was effected with *n*-hexanes–EtOAc mixtures. A total of 110 fractions of ca. 150 ml each were collected and combined on the basis of TLC. Fractions 19–29, eluted with *n*-hexanes–EtOAc (19:1) showed one spot on TLC. They were combined and evaporated to yield a solid which was further recrystallised in MeOH to give callophyllolide,

13, as white platelets (800 mg). From fractions 65–76, eluted with *n*-hexanes–EtOAc (9:1), a solid precipitated which was further recrystallised from *n*-hexanes–EtOAc to afford calaustralin, **12**, as white crystals (300 mg). From fractions 77–87, eluted with *n*-hexanes–EtOAc (17:3), were obtained inophyllum C, **14** (25 mg) and inophyllum E, **15** (300 mg) as colourless crystals, respectively.

Air-dried powdered root bark (3 kg) of *C. inophyllum* was extracted at room temperature with a mixture of MeOH–CH_2Cl_2 (1:1) and evaporated under reduced pressure to afford brown viscous residue (500 g). A portion of this crude extract (300 g) was fractionated by flash column chromatography over silica gel (230–400 mesh), eluted successively with cyclohexane–EtOAc (9:1), cyclohexane–EtOAc (4:1), cyclohexane–EtOAc (1:1), and EtOAc to yield four main fractions labelled B_1, B_2, B_3 and B_4, respectively. Fraction B_1 (6.0 g), eluted with cyclohexane–EtOAc (9:1), was repeatedly subjected to silica gel column chromatography using increasing concentrations of EtOAc in cyclohexane as eluent to give inoxanthone, **3** (500 mg), and friedelan-3-one, **11** (80 mg). Fraction B_2 (15 g), eluted with cyclohexane–EtOAc (4:1), was rechromatographed over silica gel column chromatography eluted with cyclohexane containing increasing amounts of EtOAc. Fractions of ca. 150 ml, each were collected and monitored by TLC. Fractions containing a single compound were pooled appropriately, while fractions containing mixtures were further subjected to repeated CC followed by preparative TLC using a solvent system of cyclohexane–acetone (7:3). The pure major compounds macluraxanthone, **6** (400 mg), brasiliensic acid, **9** (16 g), inophylloidic acid, **10** (14 g), 1,5-dihydroxyxanthone, **7** (150 mg) were obtained directly from the column, while compounds **4** (30 mg) and **5** (20 mg) were isolated after preparative TLC.

3.4. Bioassays

3.4.1. Antimicrobial assay

The extracts and purified active principles from *C. inophyllum* were tested against the microorganisms, *S. aureus* (ATCC6538), *V. angillarium* (ATCC19264), *E. coli* (ATCC8739), and *C. tropicalis* (ATCC66029). The qualitative antimicrobial assay employed was the classic agar disc dilution procedure using Mueller Hinton agar (Wilkins and Chalgren, 1976). Paper discs were impregnated with 20 μl of a DMSO solution of each sample (1 mg/ml) and allowed to evaporate at room temperature. Oxacillin (20 μl of 1 mg/ml solution) was used as the positive control. The plates were incubated at 37 °C for 18 h and the diameter of the zone of inhibition around the disc measured and recorded at the end of the incubation period.

3.4.2. Cytotoxicity assay

Cytotoxicity of the crude extracts, fractions, and purified compounds against human epidermoid carcinoma of the nasopharynx cancer cell line (KB) was evaluated using the protocol described in the literature (Likhitwitayawuid et al., 1993).

3.5. Inoxanthone, **3**

Yellow needles (cyclohexane–EtOAc), m.p. 217 °C. HRESI–TOFMS *m/z* $[M + H]^+$ 379.1553 (calcd. 379.1544 for $C_{23}H_{23}O_5$). IR ν(cm^{-1}, KBr): 3458, 3293, 2960, 2920, 1646, 1620, 1585. UV λ (nm, MeOH) (logε): 237 (4.35), 249sh, 280sh, 292 (5.65), 310sh, 340sh, 376 (3.63). ^{1}H NMR (400 MHz, $CDCl_3$), ^{13}C NMR (100 MHz, $CDCl_3$), see Table 1.

3.6. Caloxanthone A, **4**

Yellow needles (cyclohexane–EtOAc), m.p. 240 °C [lit. 238–240 °C (Iinuma et al., 1994)]. HRESI–TOFMS *m/z* $[M + H]^+$ 395.1491 (calcd. 395.1493 for $C_{23}H_{23}O_6$). The IR, UV, ^{1}H and ^{13}C NMR data matched well with the literature data (Iinuma et al., 1994).

3.7. Caloxanthone B, **5**

Yellow needles (cyclohexane–EtOAc), m.p. 162 °C [lit. 160.5 °C (Iinuma et al., 1994)]. HRESI–TOFMS *m/z* $[M + H]^+$ 411.1799 (calcd. 411.1805 for $C_{24}H_{27}O_6$). the IR, UV ^{1}H and ^{13}C NMR data matched well with the literature data (Iinuma et al., 1994).

3.8. Macluraxanthone, **6**

Yellow needles (cyclohexane–EtOAc), m.p. 171 °C [lit. 170–172 °C (Iinuma et al., 1994)]. HRESI–TOFMS *m/z* $[M + H]^+$ 395.1492 (calcd. 395.1493 for $C_{23}H_{23}O_6$). The IR, UV ^{1}H and ^{13}C NMR spectral data identical to the literature values (Iinuma et al., 1994).

3.9. Dihydroxyxanthone, **7**

Yellow amorphous solid (cyclohexane–EtOAc), HRESI–TOFMS *m/z* $[M + H]^+$ 229.0496 (calcd. 229.0500 for $C_{13}H_9O_4$). The IR, UV ^{1}H and ^{13}C NMR data matched well with the literature data (Iinuma et al., 1994).

3.10. Calophynic acid, **8**

Yellow sticky oil (cyclohexane–EtOAc), $[\alpha]_D^{20} = -266^\circ$ (*c* 0.1, $CHCl_3$). (HRESI–TOFMS) *m/z* $[M + H]^+$ 561.3210 (calcd. 561.3213 for $C_{35}H_{44}O_6$). The IR, UV, ^{1}H and ^{13}C NMR data (100 MHz, $CDCl_3$) matched well with the literature data (Polonsky et al., 1972).

3.11. Brasiliensic acid, **9**

Greenish gum (cyclohexane–EtOAc), HRESI–TOFMS *m/z* $[M + H]^+$ 527.3361 (calcd. 527.3369 for $C_{32}H_{47}O_6$). The IR, UV, ^1H and ^{13}C NMR data matched well with the literature data (Stout et al., 1968).

3.12. Inophylloidic acid, **10**

Yellow gum (cyclohexane–EtOAc), HRESI–TOFMS *m/z* $[M + H]^+$ 527.3361(calcd. 527.3369 for $C_{32}H_{47}O_6$). The IR, UV, ^1H and ^{13}C NMR data matched well with the literature data (Stout et al., 1968).

3.13. Calaustralin, **12**

White, crystals (*n*-hexane–EtOAc), m.p. 193–195 °C [lit. 190 °C (Breck and Stout, 1969)]. HRESI–TOFMS *m/z* $[M + H]^+$ 405.1698 (calcd. 405.1700 for $C_{25}H_{25}O_5$). The IR, UV, and ^1H NMR data matched well with the literature data (Stout et al., 1968). For the ^{13}C NMR spectral data, see Table 1.

3.14. Calophyllolide, **13**

White crystals (*n*-hexane–EtOAc), m.p. 155 °C [lit. 158 °C (Polonsky, 1957)]). HRESI–TOFMS *m/z* $[M + H]^+$ 417.1697 (calcd. 417.1700 for $C_{26}H_{25}O_5$). The IR, UV, ^1H and ^{13}C NMR data matched well with the literature data (Polonsky, 1957; Patil et al., 1993).

3.15. Inophyllum C, **14**

Colourless crystals (*n*-hexane–EtOAc), m.p. 190 °C [lit. 188–191 °C (Kawazu et al., 1968)], $[\alpha]_D^{20^\circ} = +13^\circ$ (*c* 1.1, $CHCl_3$). HRESI–TOFMS *m/z* $[M + H]^+$ 403.1541 (calcd. 403.1544 for $C_{25}H_{23}O_5$). The IR, UV, ^1H and ^{13}C NMR data matched well with the literature data (Kawazu et al., 1968; Patil et al., 1993).

3.16. Inophyllum E, **15**

Colourless crystals (*n*-hexane–EtOAc), m.p. 150 °C [lit. 149–151 °C (Kawazu et al., 1968)], $[\alpha]_D^{20^\circ} = +70^\circ$ (*c* 1.2, $CHCl_3$). HRESI–TOFMS *m/z* $[M + H]^+$ 403.1541 (calcd. 403.1544 for $C_{25}H_{23}O_5$). The IR, UV, ^1H and ^{13}C NMR data matched well with the literature data (Kawazu et al., 1968; Patil et al., 1993).

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