Synthesis and phytotoxicity evaluation of novel 6α,7β-dihydroxyvouacapan-17β-oic acid derivatives

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The 6α,7β-dihydroxyvouacapan-17β-oic acid (**1**) isolated from *Pterodon polygalaeflorus* Benth has shown allelopathic and plant growth regulatory properties. Six novel esters of (**1**) were prepared and their effects on the radical growth of *Sorghum bicolor* L. and *Cucumis sativus* L. were evaluated.

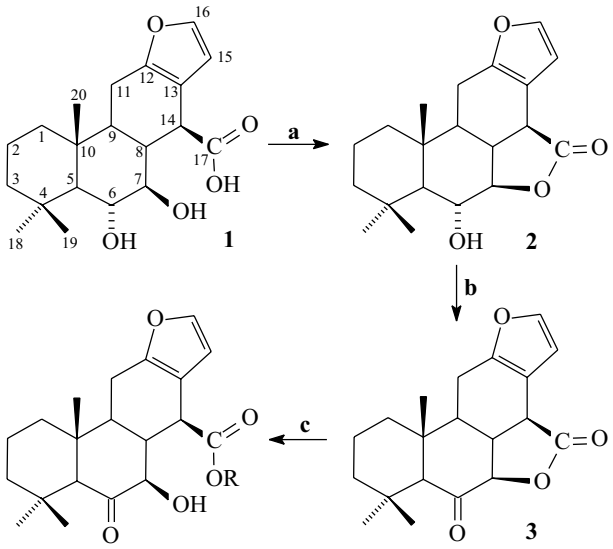
Keywords: vouacapane derivatives, furanditerpene esters, phytotoxic activity.

The natural product 6α,7β-dihydroxyvouacapan-17β-oic acid (**1**) and the lactone **2** (Scheme 1), exhibit anti-inflammatory, analgesic activities,[1] and allelopathic activity.[2] Several terpenoids are involved in plant allelopathic interactions but little is known about the effects of diterpenes on plant growth regulation.[3,4] Some derivatives of **1** have been prepared for biological evaluation.[2,5,6] Here we report the preparation of the esters **4–9** (Scheme 1), and an evaluation of their phytotoxic properties.

The acid (**1**) was isolated from the *P. polygalaeflorus* Benth fruits.[7] The δ-hydroxy-lactone **2**[5] and δ-keto-lactone **3**[8] were prepared as shown in Scheme 1. The novel compounds **4–9** were prepared in good yields (77–89 %) using catalytic amounts of NaOH. The lactone **3** is not very soluble in the alcohols. Thence the end of the reaction could be observed by TLC and by the total disappearance of the solid phase.

The esters **4–9** were characterised by elemental analysis, IR and NMR spectroscopy. The IR spectra of all the esters **4–9** showed a hydroxyl absorption between 3495 and 3465 cm^{-1} and strong C=O stretching bands in the 1735–1709 cm^{-1} range due to the ester and ketone groups. In the ^{13}C NMR spectra, the carbonyl signals were observed around δ_C 174 (ester) and δ_C 209 (ketone). The ^1H NMR signals due to the furanditerpene backbone hydrogens of the esters **4–9** are very similar to those observed in the spectrum of the parent lactone **3**.[8] It is interesting to note the multiplicity of the signal at δ_H 1.70 in the ^1H NMR spectrum of **6** which is a sextet assigned to H-2', indicating non-resolvable coupling constants (*J* 7.3 Hz)with the theoretically non-equivalent hydrogens H-1' and H-3'. Other features worth mentioning are the non-equivalence of the H-1' methylenic hydrogens revealed by the double doublets observed in the ^1H NMR spectrum of compound **9** at δ_H 4.61 and 4.71 with *Jgem* 12.2 Hz and *Jvic* 7.2 Hz with H-2'. The remaining signals were in accordance with the proposed structures.

It was previously reported that **1** at 100 ppm inhibited the root growth of *Sorghum bicolor* L. (30–40 %) and stimulated *Cucumis sativus* L. (5–10 %).[2] Recently it was shown that lactones **2** and **3** inhibited the uncoupled non-cyclic electron transport and ATP synthesis, behaving as Hill reaction inhibitors, and the carbonyl at C-6 of **3** affected the photophosphorylation.[2,8] Compounds **1–9** were evaluated *in vitro* against *S. bicolor* and *C. sativus* using a different methodology.[9] As the compounds were not soluble in the usual formulations,[9] an aqueous solution of a triblock copolymer and DMSO was used instead. Table 1 shows the radical lengths. Under these conditions, the novel compounds and the parent lactones did not significantly affect the growth of *S. bicolor*.



4: R= -$\overset{1'}{C}H_3$ **7**: R= -$\overset{1'}{C}H_2\overset{2'}{C}H_2\overset{3'}{C}H_2\overset{4'}{C}H_3$

5: R= -$\overset{1'}{C}H_2\overset{2'}{C}H_3$ **8**: R= -$\overset{1'}{C}H_2\overset{2'}{C}H_2O\overset{3'}{C}H_3$

6: R= -$\overset{1'}{C}H_2\overset{2'}{C}H_2\overset{3'}{C}H_3$ **9**: R= -$\overset{1'}{C}H_2\overset{2'}{C}H=\overset{3'}{C}\overset{4'}{C}H_3$ ($^{4''}CH_3$)

a: Ac_2O, AcONa, THF, 45ºC

b: *i*. DMSO, $(COCl)_2$, DCM, N_2, -60 ºC; *ii*. Et_3N

c: ROH, NaOH, -10ºC

Scheme 1

Interestingly the lactone **2** inhibited the radical growth of *C. sativus* by 32 %. Among the esters, only compounds **5** and **8** showed phytotoxic activity (17 and 26 %, respectively).

In summary, we have prepared and characterised six novel derivatives of the natural product **1**. The compounds did not affect significantly the radical growth of *S. bicolor*. Compounds **1**, **3**, **4**, **7** and **9** stimulated the root growth of *C. sativus* (9–19 %), while the esters **5** and **8** and the precursor lactone **2** showed significant phytotoxic activity. The influence of the *O*-alkyl group in the activity presented by **8** will be further investigated.

Experimental

General

Melting points are uncorrected. Elemental analyses were performed on a Perkin Elmer 2400 apparatus. Infrared Spectra (KBr) were recorded on a Perkin Elmer PARAGON 1000 grating spectrometer.

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Table 1 Radical lengths* (cm) of *Sorghum bicolor* L. and *Cucumis sativus* L. after 48 h of treatment with compounds **1–9** at the concentration of 1 x 10^{-4} mol l^{-1}, at 30 °C

Treatments	Control	1	2	3	4	5	6	7	8	9
S. bicolor	7.15a	6.00a	7.21a	7.34a	6.97a	6.86a	6.50a	7.31a	7.10a	7.55a
C. sativus	8.21a	8.93b	5.61c	9.00b	9.62b	6.84c	7.73a	9.50b	6.10c	9.73b

*The measurements marked "a" do not differ from control while "b" stimulates the radical growth and "c" inhibits it (According to Scott–Knott's test at 0.05 probability level.[10])

^{1}H and ^{13}C NMR spectra ($CDCl_3$) were recorded on a Bruker DRX 400 *AVANCE* spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C). COSY, NOESY, HMQC and HMBC experiments were carried out using pulse sequences and programs provided by the manufacturer. The acid **1** was extracted from the fruits of *P. polygalaeflorus* Benth as described in the literature,[7] and the lactones **2**[5] and **3**[8] were prepared as shown in Scheme 1.

General procedure for the preparation of esters (**4–9**)
Lactone **3** (164 mg, 0.5 mmol) was slowly added to a solution of NaOH (1 mg; 0.025 mmol) in the alcohol (5 ml) at –10 °C. The mixture was stirred for 1.5 to 3.5 h, when TLC revealed the total consumption of **3**. The solution was poured onto crushed ice, and the precipitate was filtered and dried under reduced pressure. In the preparation of **4**, twice the amount of **3** (1 mmol) was employed and the reaction time was reduced to 50 min.

*Methyl 7*β*-hydroxy-6-oxovouacapan-17*β*-oate* **(4)**: (275 mg, 77 %); white solid; m.p. 120.0–122.2 °C; Anal. Calcd. for $C_{21}H_{28}O_5$: C, 69.98; H, 7.83; Found: C, 70.06; H, 7.47; IR ν_{max}/cm^{-1} 3485, 2925, 2850, 1735, 1710, 1465, 1425, 1390, 1265, 1140, 1060, 750; ^{1}H NMR δ 7.26 (d, ^3J = 2.0 Hz, 1H, H-16), 6.17 (d, ^3J = 2.0 Hz, 1H, H-15), 3.90 (br, dd, ^3J = 10.3 and 4.3 Hz, 1H, H-7), 3.75 (s, 1H, CH_3-1'), 3.65 (d, ^3J = 4.3 Hz, 1H, OH), 3.62 (ddd, ^3J = 8.9, 2.8 and 1.5 Hz, 1H, H-14), 2.75 (ddd, ^2J = 16.6 Hz, ^3J 5.9 and 1.5 Hz, 1H, H-11β), 2.51 (ddd, ^2J = 16.6 Hz, ^3J = 11.1 and 2.8 Hz, 1H, H-11α), 2.41 (ddd, ^3J = 12.3, 10.3 and 8.9 Hz, 1H, H-8), 2.18 (s, 1H, H-5), 1.97 (ddd, ^3J = 12.3, 11.1 and 5.9 Hz, 1H, H-9), 1.80 (dtd, ^2J = 13.2 Hz, ^3J = 3.1 Hz, ^4J = 1.2 Hz, 1H, H-1β), 1.64 (qt, ^2J = 13.2 Hz, ^3J = 13.2 and 3.1 Hz, 1H, H-2α), 1.56 (dqui, ^2J = 13.2 Hz, ^3J = 3.1 Hz, 1H, H-2β), 1.40 (dtd, ^2J = 13.2 Hz, ^3J = 3.1 and 1.2 Hz, 1H, H-3β), 1.32 (s, 3H, CH_3-19), 1.22 (td, ^2J = 13.2 Hz, ^3J = 13.2 and 3.1 Hz, 1H, H-1α), 1.11 (td, 1H, ^2J = 13.2 Hz, ^3J = 13.2 and 3.1 Hz, H-3α), 0.97 (s, 3H, CH_3-18), 0.91 (s, 3H, CH_3-20); ^{13}C NMR δ 209.3 (C-6), 174.5 (C-17), 149.9 (C-12), 141.7 (C-16), 113.8 (C-13), 108.4 (C-15), 80.5 (C-7), 63.3 (C-5), 52.2 (C-1'), 48.1 (C-9), 47.2 (C-14), 46.6 (C-8), 42.8 (C-10), 42.2 (C-3), 38.6 (C-1), 32.5 (C-18), 32.3 (C-4), 22.2 (C-19), 22.0 (C-11), 18.3 (C-2), 15.1 (C-20).

*Ethyl 7*β*-hydroxy-6-oxovouacapan-17*β*-oate* **(5)**: (159 mg, 85 %); white solid; m.p. 92.1–92.7 °C; Anal. Calcd. for $C_{22}H_{30}O_5$: C, 70.56; H, 8.07; Found: C, 70.90; H, 8.01; IR ν_{max}/cm^{-1} 3490, 3475, 2975, 2925, 2855, 1730, 1500, 1460, 1430, 1365, 1300, 1155, 1020, 715; ^{1}H NMR δ 7.26 (d, ^3J = 2.0 Hz, 1H, H-16), 6.18 (d, ^3J = 2.0 Hz, 1H, H-15), 4.15–4.30 (m, 2H, CH_2-1'), 3.90 (dd, ^3J = 10.3 and 1.1 Hz, 1H, H-7), 3.59 (ddd, ^3J = 8.9, 2.9 and 1.7 Hz, 1H, H-14), 2.75 (ddd, ^2J = 16.3 Hz, ^3J = 5.8 and 1.7 Hz, 1H, H-11β), 2.50 (ddd, ^2J = 16.3 Hz, ^3J = 11.2 and 2.9 Hz, 1H, H-11α), 2.42 (ddd, ^3J = 12.2, 10.3 and 8.9 Hz, 1H, H-8), 2.17 (s, 1H, H-5), 1.96 (ddd, ^3J = 12.2, 11.2 and 5.8 Hz, 1H, H-9), 1.80 (dtd, ^2J = 13.2 Hz, ^3J = 3.2 Hz, ^4J = 0.9 Hz, 1H, H-1β), 1,64 (qt, ^2J = 13.2 Hz, ^3J = 13.2 and 3.2 Hz, 1H, H-2α), 1.55 (dqui, ^2J = 13.2 Hz, ^3J = 3.2 Hz, 1H, H-2β), 1.40 (dtd, ^2J = 13.2 Hz, ^2J = 3.2 Hz, ^4J = 0.9 Hz, 1H, H-3β), 1.32 (s, 3H, CH_3-19), 1.29 (t, ^3J = 7.2 Hz, 3H, CH_3-2'), 1.22 (td, ^2J = 13.2 Hz, ^3J = 13.2 and 3.2 Hz, 1H, H-1α), 1.10 (td, 1H, ^2J = 13.2 Hz, ^3J = 13.2 and 3.2 Hz, H-3α), 0.97 (s, 3H, CH_3-18), 0.91 (s, 3H, CH_3-20); ^{13}C NMR δ 209.4 (C-6), 174.0 (C-17), 149.9 (C-12), 141.6 (C-16), 113.9 (C-13), 108.5 (C-15), 80.7 (C-7), 63.3 (C-5), 61.0 (C-1'), 48.1 (C-9), 47.2 (C-14), 46.4 (C-8), 42.8 (C-10), 42.2 (C-3), 38.6 (C-1), 32.5 (C-18), 32.3 (C-4), 22.2 (C-19), 22.0 (C-11), 18.3 (C-2), 15.1 (C-20), 14.2 (C-2').

*Propyl 7*β*-hydroxy-6-oxovouacapan-17*β*-oate* **(6)**: (171 mg, 88 %); white solid; m.p. 84.6–86.2 °C; Anal. Calcd. for $C_{23}H_{32}O_5$: C, 71.11; H, 8.30; Found: C, 71.11; H, 8.27; IR ν_{max}/cm^{-1} 3495, 2925, 1720, 1495, 1450, 1385, 1285, 1260, 1170, 1050, 950, 720; ^{1}H NMR δ 7.25 (d, ^3J = 1.8 Hz, 1H, H-16), 6.18 (d, ^3J = 1.8 Hz, 1H, H-15), 4.09–4.15 (m, 2H, CH_2-1'), 3.90 (dd, ^3J = 10.3 and 4.4 Hz, 1H, H-7), 3.62 (d, ^3J = 4.4 Hz, 1H, OH), 3.60 (ddd, ^3J = 8.8, 2.9 and 1.3 Hz, 1H, H-14), 2.75 (ddd, ^2J = 16.3 Hz, ^3J = 5.8 and 1.3 Hz, 1H, H-11β), 2.50 (ddd, ^2J = 16.3 Hz, ^3J = 11.1 and 2.9 Hz, 1H, H-11α), 2.43 (ddd, 1H, ^3J = 12.3, 10.3 and 8.8 Hz, H-8), 2,10 (s, 1H, H-5), 1.96 (ddd, ^3J = 12.3, 11.1 and 5.8 Hz, 1H, H-9), 1.80 (dtd, ^2J = 12.7 Hz, ^3J = 3.2 Hz, ^4J = 0.9 Hz, 1H, H-1β), 1.70 (sxt, ^3J = 7.3 Hz, 2H, CH_2-2'), 1.51–1.68 (m, 2H, H-2α and H-2β), 1.40 (dtd, ^2J = 13.4 Hz, ^3J = 3.2 Hz, ^4J = 0.9 Hz, 1H, H-3β), 1.32 (s, 3H, CH_3-19), 1.22 (td, ^2J = 12.7 Hz, ^3J = 12.7 and 4.5 Hz, 1H, H-1α), 1.11 (td, ^2J = 13.4 Hz, ^3J = 13.4 and 4.0 Hz, 1H, H-3α), 0.97 (t, ^3J = 7.3 Hz, 3H, CH_3-3'), 0.97 (s, 3H, CH_3-18), 0.91 (s, 3H, CH_3-20); ^{13}C NMR δ 209.4 (C-6), 174.1 (C-17), 149.9 (C-12), 141.6 (C-16), 114.0 (C-13), 108.5 (C-15), 80.6 (C-7), 66.7 (C-1'), 63.3 (C-5), 48.1 (C-9), 47.2 (C-14), 46.4 (C-8), 42.8 (C-10), 42.2 (C-3), 38.6 (C-1), 32.5 (C-18), 32.3 (C-4), 22.2 (C-19), 22.0 (C-11 and C-2'), 18.3 (C-2), 15.1 (C-20), 10.4 (C-3').

*Butyl 7*β*-hydroxy-6-oxovouacapan-17*β*-oate* **(7)**: (177 mg, 88 %); white solid; m.p. 94.2–96.6 °C; Anal. Calcd. for $C_{24}H_{34}O_5$: C, 71.61; H, 8.51; Found: C, 71.32; H, 8.49; IR ν_{max}/cm^{-1} 3495, 2910, 2830, 1715, 1455, 1380, 1360, 1265, 1170, 1055, 950, 730; ^{1}H NMR δ 7.25 (d, ^3J = 1.6 Hz, 1H, H-16), 6.17 (d, ^3J = 1.6 Hz, 1H, H-15), 4.11–4.21 (m, 2H, CH_2-1'), 3.90 (dd, ^3J = 10.4 and 4.0 Hz, 1H, H-7), 3.61 (d, ^3J = 4.0 Hz, 1H, OH), 3.57–3.60 (m, 1H, H-14), 2.75 (dd, ^2J = 13.8 Hz, ^3J = 5.6 Hz, 1H, H-11β), 2.49–2.54 (m, 1H, H-11α), 2.39–2.47 (m, 1H, H-8), 2.17 (s, 1H, H-5), 1.96 (td, ^3J = 11.4 and 5.6 Hz, 1H, H-9), 1.77–1.82 (m, 1H, H-1β), 1.61–1.69 (m, 3H, H-2α and CH_2-2'), 1.52–1.60 (m, 1H, H-2β), 1.34–1.45 (m, 3H, H-3β and CH_2-3'), 1.32 (s, 3H, CH_3-19), 1.22 (td, ^2J = 13.0 Hz, ^3J = 13.0 and 4.2 Hz, 1H, H-1α), 1.11 (td, ^2J = 13.0 Hz, ^3J = 13.0 and 3.7 Hz, 1H, H-3α), 0.97 (s, 3H, CH_3-18), 0.94 (t, ^3J = 7.4 Hz, 3H, CH_3-4'), 0.91 (s, 3H, CH_3-20); ^{13}C NMR δ 209.4 (C-6), 174.1 (C-17), 149.9 (C-12), 141.6 (C-16), 114.0 (C-13), 108.5 (C-15), 80.7 (C-7), 64.9 (C-1'), 63.3 (C-5), 48.2 (C-9), 47.2 (C-14), 46.4 (C-8), 42.8 (C-10), 42.2 (C-3), 38.7 (C-1), 32.5 (C-18), 32.3 (C-4), 30.6 (C-2'), 22.2 (C-19), 22.0 (C-11), 19.2 (C-3'), 18.4 (C-2), 15.1 (C-20), 13.7 (C-4').

*2-Methoxyethyl 7*β*-hydroxy-6-oxovouacapan-17*β*-oate* **(8)**: (161 mg, 80 %); white solid; m.p. 93.4–94.0 °C; Anal. Calcd. for $C_{23}H_{32}O_6$: C, 68.29; H, 7.97; Found: C, 68.00; H, 7.99; IR ν_{max}/cm^{-1} 3465, 2923, 2840, 1732, 1709, 1649, 1509, 1286, 1263, 1169, 1129, 1036, 727; ^{1}H NMR δ 7.25 (d, ^3J = 2.0 Hz, 1H, H-16), 6.22 (d, ^3J = 2.0 Hz, 1H, H-15), 4.22–4.42 (m, 1H, H-1'a), 4.09–4.15 (m, 1H, H-1'b), 3.90 (dd, ^3J = 10.3 and 4.4 Hz, 1H, H-7), 3.54–3.68 (m, 4H, H-14, OH and CH_2-2'), 3.39 (s, 3H, CH_3-3'), 2.75 (ddd, ^2J = 16.3 Hz, ^3J = 5.9 and 1.4 Hz, 1H, H-11β), 2.50 (ddd, ^2J = 16.3 Hz, ^3J = 11.1 and 2.9 Hz, 1H, H-11α), 2.43 (ddd, ^3J = 12.3, 10.4 and 8.9 Hz, 1H, H-8), 2.18 (s, 1H, H-5), 1.97 (ddd, ^3J = 12.3, 11.1 and 5.9 Hz, 1H, H-9), 1.80 (dtd, ^2J = 13.3 Hz, ^3J = 3.2 Hz, ^4J = 1.1 Hz, 1H, H-1β), 1.64 (qt, ^2J = 13.3 Hz, ^3J = 13.3 and 3.2 Hz, 1H, H-2α), 1.55 (dqui, ^2J = 13.3 Hz, ^3J = 3.2 Hz, 1H, H-2β), 1.40 (dtd, ^2J = 13.3 Hz, ^3J = 3.2 Hz, ^4J = 1.1 Hz, 1H, H-3β), 1.32 (s, 3H, CH_3-19), 1.22 (td, ^2J = 13.3 Hz, ^3J = 13.3 and 3.2 Hz, 1H, H-1α), 1.11 (td, ^2J = 13.3 Hz, ^3J = 13.3 and 3.2 Hz, 1H, H-3α), 0.97 (s, 3H, CH_3-18), 0.91 (s, 3H, CH_3-20); ^{13}C NMR δ 209.3 (C-6), 174.0 (C-17), 149.9 (C-12), 141.6 (C-16), 113.8 (C-13), 108.6 (C-15), 80.6 (C-7), 70.4 (C-2'), 63.8 (C-1'), 63.5 (C-5), 58.8 (C-3'), 48.1 (C-9), 47.0 (C-14), 46.5 (C-8), 42.8 (C-10), 42.2 (C-3), 38.6 (C-1), 32.5 (C-18), 32.3 (C-4), 22.2 (C-19), 22.0 (C-11), 18.3 (C-2), 15.1 (C-20).

*3-Methylbut-2-enyl 7*β*-hydroxy-6-oxovouacapan-17*β*-oate* **(9)**: (184 mg, 89 %); yellowish solid; m.p. = 81.0–84.2 °C; Anal. Calcd. for $C_{25}H_{34}O_5$: C, 72.44; H, 8.27; Found: C, 72.15; H, 8.32; IR ν_{max}/cm^{-1} 3493, 2924, 2830, 1716, 1461, 1391, 1267, 1173, 957, 725; ^{1}H NMR δ 7.25 (d, ^3J = 1.7 Hz, 1H, H-16), 6.17 (d, ^3J = 1.7 Hz, 1H, H-15), 5.29–5.41 (m, 1H, H-2'), 4.71 (dd, ^2J = 12.2 Hz, ^3J = 7.2 Hz, 1H, H-1'a), 4.61 (dd, ^2J = 12.2 Hz, ^3J = 7.2 Hz, 1H, H-1'b), 3.91 (br, d, ^3J = 9.4 Hz, 1H, H-7), 3.60 (br, d, ^3J = 8.8 Hz, 1H, H-14), 3.58–3.66 (m, 1H, OH), 2.76 (dd, ^2J = 16.3 Hz, ^3J = 5.8 Hz, 1H, H-11β), 2.49–2.54 (m, 1H, H-11α), 2.38–2.46 (m, 1H, H-8), 2.17 (s, 1H, H-5), 1.96 (td, 1H, ^3J = 14.4 and 5.8 Hz, H-9), 1.76–1.82 (m, 1H, H-1β), 1.76 (s, 3H, CH_3-4''), 1.72 (s, 3H, CH_3-4'), 1.69–1.75 (m, 1H, H-2α), 1.50–1.68 (m, 1H, H-2β), 1.36–1.42 (m, 1H, H-3β), 1.32 (s, 1H, CH_3-19), 1.22 (td, 1H, ^2J = 13.0 Hz, ^3J = 13.0 and 4.3 Hz, H-1α), 1.11 (td, 1H, ^2J = 13.0 Hz, ^3J = 13.0 and 3.6 Hz, H-3α), 0.97 (s, 3H, CH_3-18), 0.91 (s, 3H, CH_3-20); ^{13}C NMR δ 209.4 (C-6), 174.1 (C-17), 149.9 (C-12), 141.6 (C-16), 138.9 (C-3'), 118.7 (C-2'), 113.9 (C-13), 108.5 (C-15), 80.7 (C-7), 63.3 (C-5), 62.1 (C-1'), 48.1 (C-

9), 47.1 (C-14), 46.5 (C-8), 42.8 (C-10), 42.2 (C-3), 38.6 (C-1), 32.5 (C-18), 32.3 (C-4), 25.7 (C-4'), 22.2 (C-19), 22.0 (C-11), 18.3 (C-2), 18.1 (C-4''), 15.1 (C-20).

Biological assay

Compounds **1**–**9** were dissolved in DMSO (250 μl) and 10 ml of the triblock copolymer [$(PEO)_x(PPO)_y(PEO)_z$ *MM* 5,800 g mol^{-1}] 10 %, and distilled water was added up to 300 ml, in order to prepare 10^{-4} mol l^{-1} solutions. The control (DMSO and the copolymer) was prepared following the same procedure. Washed sand (800 g) saturated with 100 ml of the test solution was added to rectangular plates containing four compartments. Pre-germinated seeds of *Sorghum bicolor* L. or *Cucumis sativus* L. were added to the upper one-third part of each compartment (five seeds/compartment). The plates were sealed with plastic film and were incubated at an inclination of 75° and at 30 °C for 48 hours. All treatments were replicated four times in a completely randomised design. The radical lengths were measured and the data were analysed using the Scott-Knott's test at 0.05 probability level.[10]

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