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Article

Long Chain Alkyl and Alkenyl Phenols from the Roots of *Ozoroa insignis*

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O fracionamento biodirecionado do extrato etanólico das raízes da espécie *Ozoroa insignis*, colhida na Guiné-Bissau, conduziu ao isolamento de uma biblioteca de 41 compostos alquil- e alquenil-fenólicos, cujas estruturas foram elucidadas por RMN ^{1}H e ^{13}C, e CGEM. A localização das insaturações nas cadeias laterais dos alquenil-fenóis foi determinada por CGEM dos respectivos derivados metiltiolados.

Bioassay-guided fractionation of the ethanolic root extract of *Ozoroa insignis*, collected in Guinea-Bissau, led to the isolation of a 41-member library of alkyl and alkenylphenols, whose structures were determined by ^{1}H and ^{13}C NMR, and GCMS. Determination of double-bond positions in the side chains of alkenylphenols were established by methylthiolation-GCMS.

Keywords: *Ozoroa insignis*, Anacardiaceae, cardanols, anacardic acid methyl esters, GCMS

Introduction

Ozoroa insignis Del. (*Heeria insignis* Del.) (Anacardiaceae) is a shrub or tree to 6.5 m high, of the soudanian savanna from Senegal to Niger and Nigeria, and across Africa to Ethiopia, Zaire and E. Africa.[1] This species has a wide range of healing properties, namely, in the treatment of diarrhea and venereal diseases,[2] tapeworm and hookworm,[3] schistosomiasis,[4,5] and kidney trouble.[6] The anthelmintic effect of root bark and leaves of *O. insignis* was evidenced *in vitro* against the worms *Schistosoma mansoni* and *Hymenolepis diminuta*,[5] whereas a bark extract displayed cytotoxic activity against Hep-G2, MDA-MB-231, and 5637 human cancer cell lines.[2] Stembark and stemwood were also investigated for *in vitro* topoisomerase inhibition.[7] In Guinea-Bissau, the infusion of roots of *O. insignis* is taken by women after childbirth to increase lactation.[8] Although other traditional uses of the roots of *O. insignis* have been reported,[1] so far, only the leaves, flowers, twigs, and bark have been submitted to a limited phytochemical investigation that led to the isolation of essential oils, anacardic acid, and ginkgolic acid.[2,6,9]

In a previous antitumor screening of medicinal plants from Guinea-Bissau,[8] we have observed that an ethanolic root extract of *O. insignis* showed moderate activity in KB, A 549 and MDA-MB cell lines, with IC_{50} values of 30.5, 22.0 and 15.5 μg mL^{-1}, respectively. In the present paper, we describe the isolation and structural determination of a series of alkyl and alkenylphenols from non-polar fractions of the ethanolic extract, whose activity was monitored by the brine shrimp assay.

Results and Discussion

The ethanol extract of *O. insignis* was fractionated according to Scheme 1, and the chromatographic fractions monitored by TLC and the brine shrimp assay.[10,11] ^{1}H and ^{13}C NMR, and GLC analysis indicated the presence of five mixtures (I-V) of long chain alkyl and alkenylphenols (Figure 1), which were further characterized by GCMS of their α,β-bis-methylthiolated derivatives. The substitution pattern of the aromatic ring was assigned on the basis of the NMR data (see experimental), including COSY, DEPT, HMQC, HMBC and NOESY spectra, which were similar to those reported for cardanols and anacardic acids.[6,12-17]

GCMS of mixture I showed peaks of $[M]^+$ at *m/z* 232, 276, 304, and 332, three peaks with the same $[M]^+$ at *m/z* 302, two peaks of $[M]^+$ at *m/z* 330, and one peak of $[M]^+$ at *m/z* 442. These data indicated the presence of cardanols **1**, **3**, and **5**, with saturated side chains (Table 1), and seven

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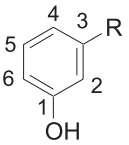
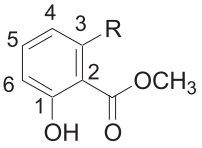


Cardanols (**1-31**)



Anacardic acid methyl esters (**32-41**)

1 C13:0 R = $C_{13}H_{27}$
2 C14:0 R = $C_{14}H_{29}$
3 C15:0 R = $C_{15}H_{31}$
4 C16:0 R = $C_{16}H_{33}$
5 C17:0 R = $C_{17}H_{35}$
6 C10:1 R = $(CH_2)_4CH=CH(CH_2)_3CH_3$
7 C11:1 R = $(CH_2)_9CH=CH_2$
8 C12:1 R = $(CH_2)_9CH=CHCH_3$
9 C13:1 R = $CH_2CH=CH(CH_2)_9CH_3$
10 C13:1 R = $(CH_2)_9CH=CHCH_2CH_3$
11 C13:1 R = $(CH_2)_{10}CH=CHCH_3$
12 C13:1 R = $(CH_2)_{11}CH=CH_2$
13 C14:1 R = $(CH_2)_9CH=CH(CH_2)_2CH_3$
14 C15:1 R = $(CH_2)_6CH=CH(CH_2)_6CH_3$
15 C15:1 R = $(CH_2)_9CH=CH(CH_2)_3CH_3$
16 C15:1 R = $(CH_2)_{10}CH=CH(CH_2)_2CH_3$
17 C15:1 R = $(CH_2)_{11}CH=CHCH_2CH_3$
18 C15:1 R = $(CH_2)_{13}CH=CH_2$
19 C16:1 R = $(CH_2)_9CH=CH(CH_2)_4CH_3$
20 C17:1 R = $(CH_2)_9CH=CH(CH_2)_5CH_3$
21 C17:1 R = $(CH_2)_{11}CH=CH(CH_2)_3CH_3$
22 C17:1 R = $(CH_2)_{12}CH=CH(CH_2)_2CH_3$
23 C18:1 R = $(CH_2)_9CH=CH(CH_2)_6CH_3$
24 C19:1 R = $(CH_2)_9CH=CH(CH_2)_7CH_3$
25 C20:1 R = $(CH_2)_9CH=CH(CH_2)_8CH_3$
26 C21:1 R = $(CH_2)_9CH=CH(CH_2)_9CH_3$
27 C25:1 R = $(CH_2)_{10}CH=CH(CH_2)_{12}CH_3$
28 C25:1 R = $(CH_2)_{11}CH=CH(CH_2)_{11}CH_3$
29 C25:1 R = $(CH_2)_{17}CH=CH(CH_2)_5CH_3$
30 C25:2 R = $(CH_2)_9CH=CH(CH_2)_2CH=CH(CH_2)_9CH_3$
31 C25:1 R = $(CH_2)_{18}CH=CH(CH_2)_4CH_3$
32 C15:0 R = $C_{15}H_{31}$
33 C16:0 R = $C_{16}H_{33}$
34 C17:0 R = $C_{17}H_{35}$
35 C18:0 R = $C_{18}H_{37}$
36 C22:0 R = $C_{22}H_{45}$
37 C23:0 R = $C_{23}H_{47}$
38 C24:0 R = $C_{24}H_{49}$
39 C14:1 R = $(CH_2)_5CH=CH(CH_2)_6CH_3$
40 C18:1 R = $(CH_2)_{12}CH=CH(CH_2)_3CH_3$
41 C20:1 R = $(CH_2)_5CH=CH(CH_2)_{12}CH_3$

Figure 1. Structures of alkyl and alkenylphenols from *Ozoroa insignis*.

cardanols (**6**, **16**-**18**, **21**, **22**, and **29**) with monounsaturated side chains differing in the position of the double bond. In all cases, the mass spectra displayed a base peak at *m/z* 108 due to benzylic cleavage of alkyl 3-hydroxybenzene.[15] Location of the double bond in the alkenyl side chain was deduced from mass spectral analysis of the corresponding α,β-bis-methylthiolated derivatives, which provide readily recognizable fragments on electron bombardment (Table 1; Figure 2a).[18,19] The *Z* stereochemistry of the double bond was assigned from the appearance of a low resolution multiplet in the ^{1}H NMR spectrum at δ 5.40 (2H, m), and carbons at allylic positions at δ 27.2.[17]

The NMR profiles of mixtures II and III were identical to those of I. In mixture II, thirteen cardanols with monounsaturated side chains (**7**, **8**, **10**, **13**-**15**, **19**, **20**, **23**-**26,** and **31**) could be characterized from their GCMS and mass spectra fragmentation of the methylthiolated derivatives (Table 1; Figure 2a),[18,19] whereas in mixture III, in addition to five cardanols with saturated side chains (**1**-**5**), and six with monounsaturated side chains (**9**, **11**, **12**, **27**, **28**, **31**) (Table 1), one compound with a diunsaturated side-chain (**30**) was identified. In this case, the derivatization reaction leads to a cyclic methylthiolated derivative, whose mass spectrum showed two fragments at *m/z* 279 (base peak) and *m/z* 201, indicative of the presence of two double bonds separated by two methylene groups (Figure 2b, o = 2).[19]

The NMR data of mixture IV, when compared to those of I-III, showed a different substitution pattern of the aromatic ring. Three coupled aromatic protons were observed at δ 6.72 (d, *J* 7.6 Hz), 6.85 (d, *J* 8.0 Hz) and δ 7.27 (t, *J* 8.0 Hz), and, in addition to the presence of the alkyl side chain and a phenolic hydroxyl group, a carboxyl methyl ester was assigned from a HMBC correlation of a carbonyl resonance at δ_C 172.0 with a methyl group at δ 3.96. Analysis of COSY, DEPT, HMQC, HMBC and NOESY spectra, and comparison with literature data allowed to confirm a basic structure of anacardic acid methyl esters for the constituents of mixture IV.[6,13-17] Its GCMS indicated the presence of three compounds (**32**,

Table 1. Mass Spectra data of cardanols **1-31** and anacardic acid methyl esters **32-41**

Compound	$[M]^+$	$[M]^+$ of methylthiolated derivatives	$[a]^+$ (*m/z*)[a]	$[b]^+$ (*m/z*)[a]	Retention time (min)
1 ($C_{19}H_{32}O$)	276				27.99
2 ($C_{20}H_{34}O$)	290				29.72
3 ($C_{21}H_{36}O$)	304				31.64
4 ($C_{22}H_{38}O$)	318				33.32
5 ($C_{23}H_{40}O$)	332				35.70
6 ($C_{16}H_{24}O$)	232	326	209 (20)	117 (25)	19.48
7 ($C_{17}H_{26}O$)	246	340	279 (10)	61 (3)	21.62
8 ($C_{18}H_{28}O$)	260	354	279 (10)	75 (10)	23.82
9 ($C_{19}H_{30}O$)	274	368	167 (40)	202 (100)	46.19
10 ($C_{19}H_{30}O$)	274	368	279 (10)	89 (100)	29.73
11 ($C_{19}H_{30}O$)	274	368	293 (40)	75 (10)	38.56
12 ($C_{19}H_{30}O$)	274	368	307 (20)	61 (10)	45.12
13 ($C_{20}H_{32}O$)	288	382	279 (20)	103 (20)	23.45
14 ($C_{21}H_{34}O$)	302	396	237 (10)	159 (10)	35.92
15 ($C_{21}H_{34}O$)	302	396	279 (100)	117 (70)	34.31
16 ($C_{21}H_{34}O$)	302	396	293 (100)	103 (30)	37.70
17 ($C_{21}H_{34}O$)	302	396	307 (95)	89 (25)	38.84
18 ($C_{21}H_{34}O$)	302	396	335 (30)	61 (15)	39.05
19 ($C_{22}H_{36}O$)	316	410	279 (20)	131 (15)	29.67
20 ($C_{23}H_{38}O$)	330	424	279 (15)	145 (10)	31.70
21 ($C_{23}H_{38}O$)	330	424	307 (100)	117 (35)	43.85
22 ($C_{23}H_{38}O$)	330	424	321 (50)	103 (20)	44.64
23 ($C_{24}H_{40}O$)	344	438	279 (20)	159 (10)	35.92
24 ($C_{25}H_{42}O$)	358	452	279 (20)	173 (20)	37.92
25 ($C_{26}H_{44}O$)	372	466	279 (30)	187 (10)	42.16
26 ($C_{27}H_{46}O$)	386	480	279 (20)	201 (100)	35.60
27 ($C_{31}H_{54}O$)	442	536	293 (25)	243 (20)	38.52
28 ($C_{31}H_{54}O$)	442	536	307 (30)	229 (10)	45.11
29 ($C_{31}H_{54}O$)	442	536	391 (20)	145 (25)	36.92
30 ($C_{31}H_{52}O$)	440	566	279 (100)	201 (50)	37.64
31 ($C_{31}H_{54}O$)	442	536	405 (10)	131 (25)	22.85
32 ($C_{23}H_{38}O_3$)	362				11.29
33 ($C_{24}H_{40}O_3$)	376				16.20
34 ($C_{25}H_{42}O_3$)	390				18.44
35 ($C_{26}H_{44}O_3$)	404				24.07
36 ($C_{30}H_{52}O_3$)	460				27.90
37 ($C_{31}H_{54}O_3$)	474				24.10
38 ($C_{32}H_{56}O_3$)	488				36.40
39 ($C_{22}H_{34}O_3$)	346	440	281 (20)	159 (25)	14.20
40 ($C_{26}H_{42}O_3$)	402	496	379 (30)	117 (10)	39.34
41 ($C_{28}H_{46}O_3$)	430	524	281 (20)	243 (10)	30.09

[a] fragments a and b (relative intensities) as described in Figure 2.

35, and **36**) with saturated side chains and three others (**39**-**41**) with one *Z* double bond.[17] Mixture V was comprised of cardanol **29**, and anacardic acid methyl esters **32**-**38**, which were identified as described above.

Anacardic acids are natural products isolated mainly from the Anacardiaceae family, but they are also present in fungi, marine organisms, and insects.[20-22] These compounds are known to possess a wide range of biological properties, such as antimicrobial, antitumoral, molluscicide, antifungal, insectide, regulator of gene expression, antioxidant, lipoxygenase and uncoupling activity of oxidative phosphorylation. They inhibit the generation of superoxide radicals by xanthine oxidase, and lower the levels of serum cholesterol in rats.[2,14,21-27] 6-Oxa isosteres of anacardic acids proved to be among the most potent inhibitors of the bacterial two-component regulatory systems KinA/SpoOF and NRII/NRI, reported to date.[28] They also constitute some of the active principles of *Ginkgo biloba*,[29] with a recognized effect as inhibitors of prolyl endopeptidase and glycerol-3-phosphate dehydrogenase,[30,31] antifeedant and cytotoxic activities.[17,32,33] In this plant they occur together with cardanols (or ginkgols), whose antitumor activity has also been demonstrated.[17]

The pool of anacardic acid methyl esters and cardanols identified in the present work possess some unusual structural features. Their alkyl side chains varies from 13 to 25 carbons

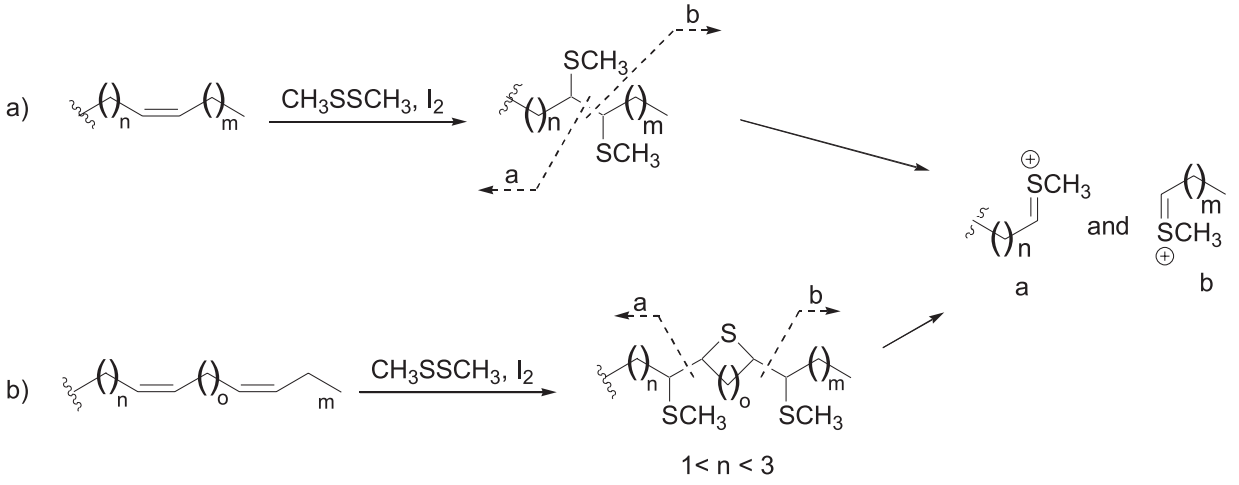


Figure 2. Mass Spectra fragmentation of methylthiolated alkenyl phenols.

in length, and monounsaturated side chains at C2, C5, C7, C10 to C14, C18 and C19 positions could be confirmed. A C25:2-$\Delta^{10,14}$ cardanol (**30**) was also identified. In alkylphenols isolated from other Anacardiaceae the length do not exceeds 17 carbons, and when only one double bond is present, the position is most frequently Δ^{8}.[28] To our best knowledge, compounds **6**-**19**, **21**-**25**, **27**-**31**, and **35**-**41** are new naturally occurring substances. Moreover, this is the first reported case of isolation of anacardic acid methyl esters.

Experimental

General experimental procedures

NMR spectra were recorded on a Bruker ARX 400 NMR spectrometer (^{1}H at 400 MHz; ^{13}C at 100.61 MHz), using $CDCl_3$ as solvent and TMS as reference. FT-IR spectra were measured on a Perkin-Elmer 157G infrared spectrophotometer. GCMS were run on a Micromass GCTOF spectrometer at 70 eV, equipped with a J&W DB-5 column (30 m × 0.25 mm; 0.25 µm film thickness), helium flow rate at 4 mL min^{-1}, splitless time 1 min, injector and detector temperatures 280 °C. Oven temperature program was set at 120 °C to 240 °C at 10 °C min^{-1}; 8 mn at 240 ºC; 240 °C to 280 °C at 2 °C min^{-1}, and 10 min at 280 °C. Celite, Macherey-Nagel Si gel (0.04-0.063 mm/230-400 mesh), and Sephadex LH-20 were used for column chromatography (CC). TLC were performed on normal phase Si gel F_{254} plates (MN 818133), and visualized with Ce_2SO_4 and phosphomolybdic acid reagents.

Plant material

Ozoroa insignis Delile (Anacardiaceae), was collected in January 1994 at Contuboel, Guinea-Bissau, and identified at the Herbarium of Botany Centre (LISC), where the voucher specimen nº 854 is deposited.

Extraction and fractionation

The air dried roots of *O. insignis* (1.65 kg) were powdered, and extracted in a Soxhlet apparatus with EtOH (5 L) at room temperature. Evaporation of the solvent under reduced pressure afforded an oily residue (83.3 g), that was submitted to fractionation according to Scheme 1.

Brine shrimp assay

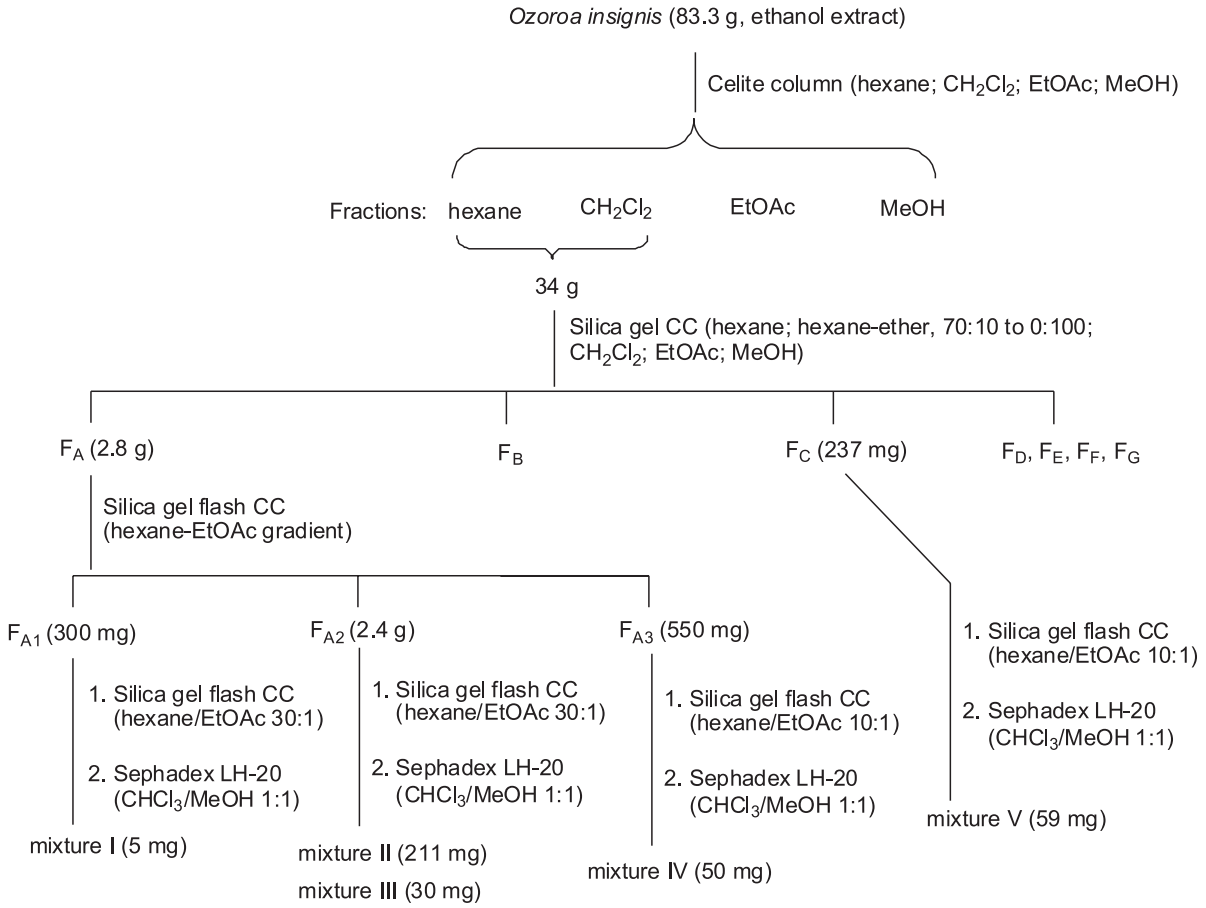
The brine shrimp (*Artemia salina*) lethality assay was carried out according to a described method.[10,11]

*Mixture I (**1**, **3**, **5**, **6**, **16**-**18**, **21**, **22**, **29**)*

IR (NaCl) ν_{max}/cm^{-1}: 2919, 2850, 2360, 1587, 1455, 1259, 1151, 1066. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.89 (t, 7.1, Me), 1.29 (bs), 1.65 (m, H-2'), 2.55 (t, 6.8, H-1'), 5.40 (m), 6.63 (d, 6.0, H-6), 6.66 (s, H-2), 6.75 (d, 7.2, H-4) and 7.13 (ddd, 7.6, 7.6, 2.5, H-5). ^{13}C NMR $CDCl_3$, 100.61 MHz): δ 14.1 (Me), 22.7, 27.2, 29.3, 29.5, 29.6, 29.7, 30.4, 31.3, 32.0, 35.8 (C-1'), 112.5 (C-6), 115.3 (C-2), 121.0 (C-4), 129.4 (C-5), 129.9, 145.0 (C-3), 155.4 (C-1). For MS data see Table 1.

*Mixture II (**7, 8, 10, 13-15, 19, 20, 23-26, 31**)*

IR (NaCl) ν_{max}/cm^{-1}: 2919, 2850, 2360, 1587, 1455, 1259, 1151, 1066. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.89 (t, 7.1, Me), 1.29 (bs), 1.65 (m, H-2'), 2.55 (t, 6.8, H-1'), 5.36 (m), 6.63 (d, 6.0, H-6), 6.66 (s, H-2), 6.75 (d, 7.2, H-4) and 7.13 (ddd, 7.6, 7.6, 2.5, H-5). ^{13}C NMR ($CDCl_3$,



Scheme 1. Fractionation of *Ozoroa insignis* extract.

100.61 MHz): δ 14.1 (Me), 22.7, 27.2, 29.3, 29.5, 29.6, 29.7, 30.4, 31.3, 32.0, 35.8 (C-1'), 112.5 (C-6), 115.3 (C-2), 121.0 (C-4), 129.4 (C-5), 129.9 and 130.9, 145.0 (C-3), 155.4 (C-1). For MS data see Table 1.

*Mixture III (**1-5, 9, 11, 12, 27, 28, 30, 31**)*

IR (NaCl) ν_{max}/cm^{-1}: 2919, 2850, 2360, 1587, 1455, 1259, 1151, 1066. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.93 (t, 7.1, Me), 1.31 (bs), 1.62 (m, H-2'), 2.58 (t, 6.9, H-1'), 5.41 (m), 6.69 (dd, 7.6, 2.5, H-6), 6.71 (s, H-2), 6.78 (d, 7.6, H-4) and 7.16 (ddd, 7.6, 7.6, 2.5, H-5). ^{13}C NMR ($CDCl_3$, 100.61 MHz): δ 14.1 (Me), 22.7, 27.2, 29.4, 29.5, 29.7, 31.3, 31.9, 35.9 (C-1'), 112.6 (C-6), 115.4 (C-2), 120.9 (C-4), 129.3 (C-5), 129.9, 144.9 (C-3), 155.4 (C-1). For MS data see Table 1.

*Mixture IV (**32, 35, 36, 39-41**)*

IR (NaCl) ν_{max}/cm^{-1}: 3064, 2917, 2852, 1665, 1607, 1578, 1449, 1315, 1250, 1205. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.89 (t, 6.4, Me), 1.26 (bs), 1.53 (m, H-2'), 2.58 (t, 7.7, H-1'), 3.96 (s, OMe), 5.37 (m), 6.72 (d, 7.6, H-4), 6.85 (d, 8.0, H-6) and 7.27 (t, 8.0, H-5). ^{13}C NMR ($CDCl_3$, 100.61 MHz): δ 14.1 (Me), 22.7, 27.2, 29.4, 29.5, 29.7, 29.9, 32.0, 32.1, 36.4 (C-1'), 52.1 (OMe), 111.9 (C-2), 115.6 (C-6), 122.4 (C-4), 129.9, 134.2 (C-5), 146.2 (C-3), 162.6 (C-1), 172.0 (CO). For MS data see Table 1.

*Mixture V (**29, 32-38**)*

IR (NaCl) ν_{max}/cm^{-1}: 3064, 2917, 2852, 1665, 1607, 1578, 1449, 1315, 1250, 1205. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.89 (t, 6.4, Me), 1.27 (bs), 1.52 (m, H-2'), 2.88 (t, 7.8, H-1'), 3.95 (s), 5.36 (m), 6.72 (d, 7.2, H-4), 6.84 (d, 8.4, H-6) and 7.28 (t, 8.0, H-5). ^{13}C NMR ($CDCl_3$, 100.61 MHz): δ 14.1 (Me), 22.7, 27.2, 29.4, 29.5, 29.7, 29.9, 32.0, 32.1, 36.6 (C-1'), 52.0 (OMe), 111.8 (C-2), 115.6 (C-6), 122.4 (C-4), 129.82, 129.84, 134.1 (C-5), 146.2 (C-3), 162.6 (C-1), 172.0 (CO). For MS data see Table 1.

Methylthiolation of mixtures I-V

A 200-500 ng portion (0.4-1.0 nmol) of mixtures I-V was solubilized in 50 µL of DMDS, and 50 µL of carbon disulfide, and 300 µg (1.18 µmol) of iodine were added.

The reaction mixture was kept at 60 °C for 40 h in small-volume sealed vials. The reaction was quenched with aqueous $Na_2S_2O_3$ (3×10^{-4} mol L^{-1}). The organic phase was extracted and evaporated to dryness under a nitrogen stream. The residue was dissolved in hexane and analyzed by GCMS.

Acknowledgments

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