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The occurrence of 2-hydroxy-6-methoxybenzoic acid methyl ester in *Securidaca longepedunculata* Fresen root bark

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As part of our ongoing search for natural fumigants from Senegalese plants, we have investigated *Securidaca longepedunculata* root barks and demonstrated that 2-hydroxy-benzoic acid methyl ester (methyl salicylate, **I**) is responsible of their biocide effect against stored grain insects. A second unknown apparented product, **II** has been systematically observed in all analyzed samples. The present paper describes the identification of this molecule. The analytical investigations including GCMS, GLC and ^{1}H-NMR spectrometry led to the conclusion that **II** corresponds to the 2-hydroxy-6-methoxybenzoic acid methyl ester.
Keywords. *Securidaca longepedunculata* Fresen, root bark, 2-hydroxy-6-methoxybenzoic acid methyl ester.

Présence de 2-hydroxy-6-methoxybenzoate de méthyle dans l'écorce des racines de *Securidaca longepedunculata* Fresen. Dans le cadre de recherches de fumigants naturels à partir de plantes sénégalaises, nous avons démontré que les écorces de racines de *Securidaca longepedunculata* possèdent un effet biocide dû à la présence de salicylate (2-hydroxybenzoate) de méthyle (**I**). Un second produit (**II**) apparenté à **I** a été systématiquement observé dans tous les échantillons analysés. Cet article décrit l'identification de **II** grâce à l'usage combiné de diverses techniques spectrométriques et chromatographiques (CGSM, CPG et spectrométrie ^{1}H-RMN). Les investigations ont conduit à l'identification du 2-hydroxy-6-methoxybenzoate de méthyle.
Mots-clés. *Securidaca longepedunculata* Fresen, écorce racinaire, 2-hydroxy-6-methoxybenzoate de methyle.

1. INTRODUCTION

In tropical areas, insect infestation of stored grain causes weight and quality losses leading to a significant reduction of commercial value and seed germination. To compensate these damages, several strategies including mostly the use of synthetic insecticides have been developed. Due to increasing resistance of insects to one or more pesticide(s) and also for economic reasons, it seems that "biological insecticides" from natural origin could play a role – at least locally – in pest control.

Indeed, the survey of selected Senegalese plants revealed that some of them are traditionally used for this purpose (Seck,1994). In previous works, we have reported the biological activity of *Boscia* and *Cassia* species to protect cowpea (*Vigna unguiculata* L.) seeds against several stored-grain insect pests (Seck *et al*., 1993; Liénard *et al.*, 1993; Baulard, 1999). Some investigations were also performed on *Securidaca longepedunculata* Fresen root barks which exhibit a fumigant effect against the cowpea weevil, *Callosobruchus maculatus* Fabricius (Coleoptera: bruchidae). Head-space sampling together with GCMS analyses (Seck,1994) have indicated that 2-hydroxy-benzoic acid methyl ester (**I**) better known as methyl salicylate is the bio-active molecule (CL_{50} = 13 ppm). Nevertheless the use of this plant seems limited for reasons of availability of roots.

On a chemical point of view, the composition of *S. longepedunculata* roots is characterized by the occurrence of methyl salicylate (**I**) and a saponin which contains

presenegenine (Cmelik, Ley,1984; Delaude, 1992). Two minor bitter principles: β-D-(3,4-disinapoyl)-fructofuranosyl-α-D-(6-sinapoyl)-glucopyranoside and β-D-(3-sinapoyl)-fructofuranosyl-α-D-(6-sinapoyl)-glucopyranoside have been reported in bark extracts (De Tommasi *et al.*, 1993). Seed oil of *S. longepe - dunculata* contains high levels of conjugated hydroxydienoic fatty acids and acetotriacylglycerols (Smith *et al.*, 1969).A pharmacological screening of *S. longepedunculata* root extracts has recently been published by Olajide *et al.* (1998). The present paper describes the study of a methylsalicylate apparented molecule (**II**) which has been systematically detected.

2. MATERIALS AND METHODS

2.1. Plant material and extraction procedure

S. longepedunculata roots were collected in Nioro in the Rip Department (Senegal) from five localities representing different types of soil. The samples were kept at -20°C until use. The barks were peeled off, grinded and extracted by shaking with analytical grade chloroform for three hours.

2.2. Analytical conditions for the identification procedure

The mass spectra were recorder on a Hewlett-Packard 5989 apparatus (EI 70 eV, Source T° = 200°C, scanned mass range: 25-500 amu) coupled to a HP5890 Serie II chromatograph fitted with a "cold on column" injector.

The operating conditions were as follows:

- Column: HP-5MS (28 × 0.25 mm; df = 0.25 μm) from Hewlett-Packard;
- Carrier gas: helium 0.7 ml/min;
- Temperature programme: 50°C to 140°C at 20°C/min. then to 280°C at 5°C/min.

The measurements of chromatographic retention data were performed on two columns of different polarities:

- Apolar stationary phase: Optima-1 (30 m × 0.32 mm; df = 0.35 μm) from Macherey-Nagel; temperature programme: 60 to 250°C at 3°C/min.; apparatus: Carlo Erba Mega 5160 ("on column" injector, FID at 280°C) ;
- Polar stationary phase: CPWAX-52CB (50 m × 0.25 mm; df= 0.2 μm) from Chrompack; temperature programme: 50 to 240°C at 3°C/min.; apparatus: Hewlett-Packard 5880a ("on column" injector, FID at 250°C).

2-hydroxy 3-, 4 and 5- methoxybenzoic acid were purchased from Aldrich. The methyl esters were synthetized in diethyl ether using diazomethane as reagent. The resulting derivatives have been injected in the aforementioned conditions.

A crude chloroformic extract was subjected to several liquid chromatographies using silicagel G60 70–230 mesh (Macherey-Nagel) and n-hexane/diethyl ether mixtures as eluents. Fractions were collected with a LKB helirac 2212 collector and tested for purity. ^{1}H-NMR spectra of II were recorded on a Varian Unity 600 spectrometer ($CDCI_3$, 600 Mhz).

2.3. Quantitative analyses of I and II

One gram samples of root barks were extracted for three hours with 5 ml chloroform containing 2 mg vanillin (4-hydroxy-3-methoxy benzaldehyde) as internal standard. The molecules of interest were quantified on a CPWAX-52CB column (25 m × 0.32 m; df = 0.2 μm) in the conditions specified in 2.2.

3. RESULTS AND DISCUSSION

The total ion current (TIC) of a chloroformic extract of *S. longepedunculata* root bark is presented in **figure 1**. As shown, beside **I** and **II** only a few chromatographic peaks have been observed, the majority of them representing traces. The fact that **II** could correspond to a methoxy derivative of the methyl salicylate is supported by the analysis of its characteristic mass fragments, (**Table 1**):

- the difference of 30 amu between **I** and **II** suggests an additional methoxy-function on the aromatic ring;
- the small peak at m/z=167 correspond to the loss of a methyl group
- according to MacLafferty (1969), the m/z = 150 fragment (base peak) has been attributed to an "ortho effect" whereas the fragments 122 and 107 have been assigned to the loss of a carbonyl side-chain and a methyl group from the m/z = 150 parent ion.

The methyl esters of three available possible isomers (2-hydroxy-, 3-, 4- and 5- methoxybenzoic acids) have been prepared and analyzed by GCMS. The recorded spectra have shown that the fragment intensities of these molecules are not comparable to that of the natural product. Therefore the occurrence of the 6- methoxy isomer in *S. longepedunculata* root bark has been considered. The GLC analyses of the 3 references and **II** on capillary columns of different polarities have been carried out. From the calculated

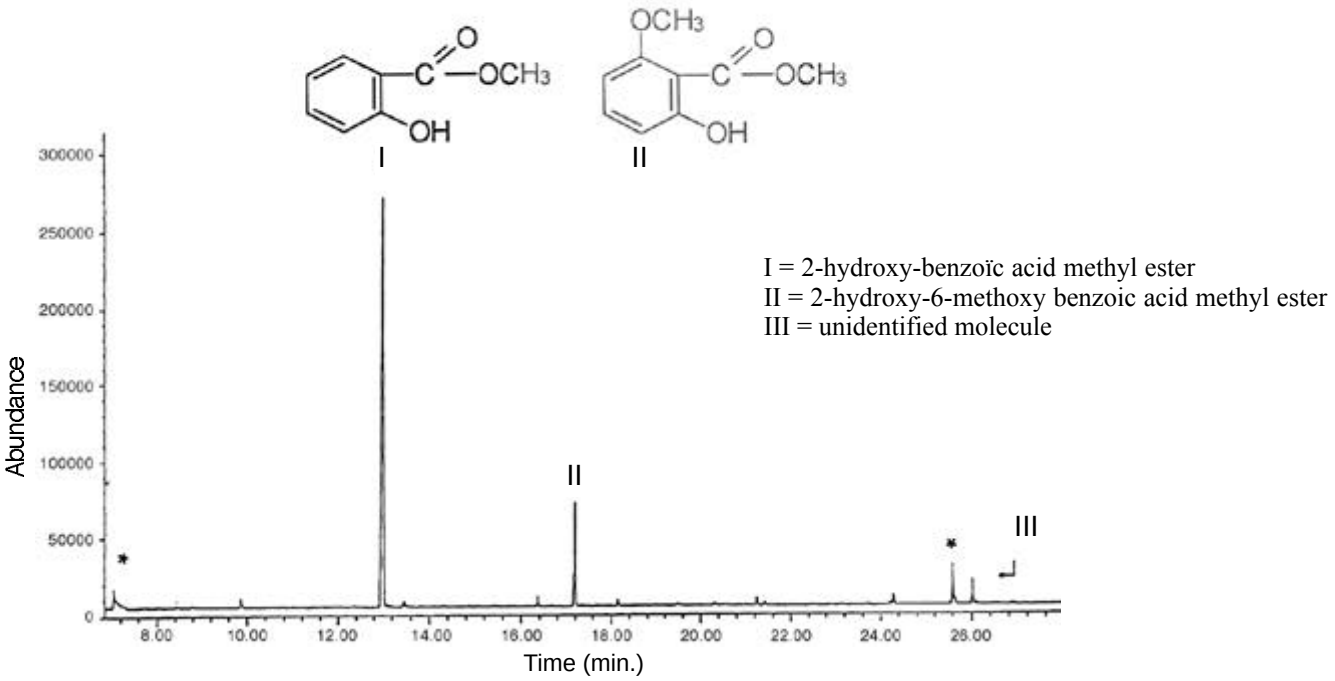

Figure 1. Total ion current of a chloroformic extract of *S. longepedunculata* root bark. The asterisk-labelled peaks correspond respectively to styrene and a phthalate, (contaminants from the plastic containers used to store the roots) — *Courant ionique total d'un extrait chloroformique de l'écorce de racines de* S. longepedunculata. *Les pics marqués d'un astérisque correspondent respectivement au styrène et à un phtalate (contaminants provenant des flacons en plastique utilisés pour le stockage des échantillons).*

Table1. Characteristic data for the identification of 2-hydroxy-6-methoxy benzoic acid methyl ester from *S. longepedunculata* root bark — *Données caractéristiques pour l'identification du 2-hydroxy-6-méthoxybenzoate de méthyle à partir d'écorces racinaires de* S. longepedunculata.

Mass spectrometric data : m/z (intensities)
M+ =182(40), 150 (Base peak), 136 (7), 122 (48), 107 (77), 93 (5), 79 (11), 65 (12), 63 (9), 51 (15).

^{1}H NMR chemical shifts (ppm)		
Proton	**Observed**	**Calculated**
CH_3	3.81 singulet	
CH_3	3.90 singulet	
H-3	6.35 quadruplet	6.47
H-5	6.55 quadruplet	6.51
H-4	7.27 triplet	7.24
OH	11.39 singulet	

Gas Chromatography (retention parameters)				
	Polar Column		**Apolar Column**	
Derivative [a]	Rt (min)	Kovats indices	Rt (min)	Kovats indices
3-CH_3O	43.65	2240	30.09	1418
4-CH_3O	42.55	2203	30.45	1428
5-CH_3O	41.12	2160	29.38	1402
II	**43.01**	**2220**	**29.47**	**1404**

[a] = 3-, 4- and 5- CH_3O correspond to the three reference isomers.

Kovats indices gathered in **table 1** it was also evident that the natural ester **II** do not co-elute with the three references and that the hypothesis of a 6-methoxy isomer was validated. The final proof has been obtained by ^{1}H-NMR spectrometry. For that purpose, several column chromatographic purifications have been undertaken and afforded 3 mg of a purified fraction (GLC purity > 98%).

The observed chemical shifts recorded by ^{1}H NMR spectrometry have corroborated the first hypotheses. Moreover, the values assigned to the H-3, H-4 and H-5 protons were found in line with the theoretical shifts which take into account the contribution of the different functional groups (Günther, 1994).

In conclusion, the GCMS, GLC and ^{1}H-NMR data have led to the unambiguous identification of the 2-hydroxy-6-methoxy benzoic acid methyl ester. The free acid form of **II** has been reported in *Gloriosa superba* L. tubers and bulbs of *Colchicum* spp.

Quantitative determinations of the two salicylic acid related products (**I** and **II**) have been undertaken with vanillin as internal standard. The repeatability tested for **I** on a selected sample has been judged acceptable (5.8 + 0.3 mg/g). The analyses of methyl-salicylate and its 6-methoxy derivative in 12 different samples have shown a quite large variation of the two molecules in *S. longepedunculata* root barks. Indeed their concentration varied from 3.3 to 11.4 mg/g and 0.2 to 1.0 mg/g respectively.

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