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LC/MS/NMR analysis of isomeric divanilloylquinic acids from the root bark of *Fagara zanthoxyloides* Lam.

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Abstract

Gradient HPLC coupled to DAD/UV, MS/MS and NMR has been applied to the rapid structure determination of three new isomeric divanilloylquinic acids from *Fagara zanthoxyloides* collected in Burkina Faso: 3,4-*O*-divanilloylquinic acid, 3,5-*O*-divanilloylquinic acid and 4,5-*O*-divanilloylquinic acid. Furthermore these new compounds named burkinabins A-C could play a useful role in sickle cell disease, as the active agents of *Fagara zanthoxyloïdes* are said to be unidentified aromatic compounds with carboxylic acid grouping (Adesanya, S.A., Sofowora, A., 1983. Biological standardisation of *Zanthoxylum* roots for antisickling activity. Planta Med. 48, 27–33).

Keywords: *Fagara zanthoxyloides*; Rutaceae; Divanilloylquinic acids; Burkinabins A–C; HPLC/MS/NMR; Antisickling activity

1. Introduction

Coupling techniques combining UV or mass spectrometry with chromatography are well-established laboratory tools for the analysis of complex liquid mixtures. In the last years, the arsenal of coupling techniques has reached a new dimension by the introduction of an NMR spectrometer as an HPLC detector. This technique links the highly flexible liquid chromatography separation with the enormous information content of the NMR experiment. The advantage of this methodology is that enables fast identification of compounds without isolation but just separation (Albert et al., 1999). Nevertheless, biological activity on pure compounds cannot be measured and in some cases the amount of metabolites present in the botanicals cannot be precisely estimated. We have applied this promising tool to detect three new secondary metabolites that are the main constituents of an active crude extract. Moreover thanks to these hyphenated techniques HPLC/DAD, HPLC/MS/MS and HPLC/ NMR we have been able to propose the structures of three new isomeric divanilloylquinic acids (**1**–**3**).

The root bark of *Fagara zanthoxyloides* Lam. (Rutaceae) is specifically used by local populations for its antimicrobial properties (Odebiyi and Sofowara, 1979; Chaaib et al., 2003) and to treat sickle cell anaemia, particularly in Burkina Faso (Ouattara et al., 1992). Sickle cell anaemia (SCA) is a genetic disease considered as a major public health problem in many countries, particularly in West Africa. The symptoms of this disease result from an aggregation of heamoglobins in the Hb SS erythrocytes. Many publications have already described the effects of *Fagara zanthoxyloides* extract on drepanocytic erythrocytes (Sofowora et al., 1971, 1975; Honig et al., 1975; Isaacs-Sodeye et al., 1975).

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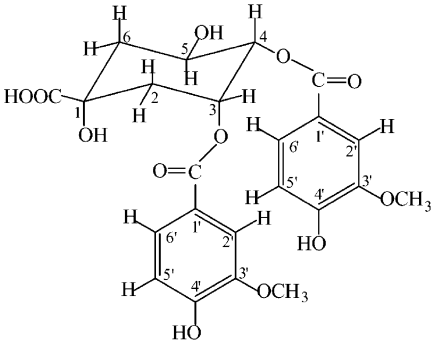
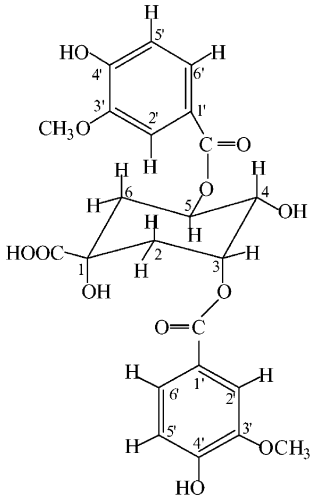
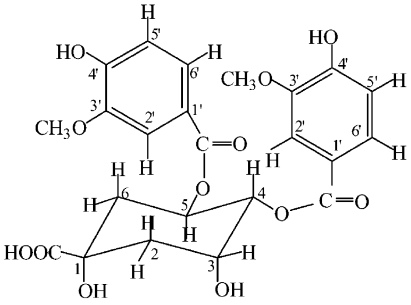
(1) Burkinabin A
3,4-*O*-divanilloylquinic acid



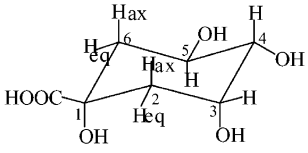
(2) Burkinabin B
3,5-*O*-divanilloylquinic acid



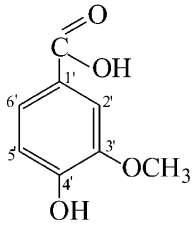
(3) Burkinabin C
4,5-*O*-divanilloylquinic acid



(4) Quinic acid



(5) Vanillic acid



In previous works, the active compounds have been identified as benzoic acid derivatives (Elujoba et al., 1977; Adesanya et al., 1983; Osoba et al., 1989), but the structures of these substances were not firmly elucidated.

By HPLC/DAD, we have looked for these supposed active constituents (benzoic acid, vanillic acid, ferulic acid) in crude aqueous and ethanolic extracts, but we didn't find them. However with this technique, we have detected substances that have the same UV spectrum as vanillic acid. Consequently, we have adapted an extraction method with pH modification to select these compounds and have found them in an ethyl acetate extract.

While the dicaffeoylquinic acids have been isolated from many sources (Pauli et al., 1998; Tolonen et al., 2002), this is the first time that vanilloylquinic acids are described.

2. Results and discussion

Starting from an hydro-ethanolic extract ($EtOH/H_2O$: 50/50) of *Fagara zanthoxyloides* root bark, sequential liquid-liquid extraction and HPLC/DAD/MS/NMR analysis of ethyl acetate extract led to the structure determination of three new isomeric products, named burkinabins A–C (**1**–**3**). They are indeed the main compounds of an active extract of a plant collected and used in Burkina Faso. Moreover they have the same UV spectrum as vanillic acid in HPLC/DAD chromatography (see Experimental). This was the first piece of information about the structural similarity of these three compounds that were partially identified with the help of LC/MS/MS. The positive ion electrospray MS of burkinabin A **(1)** showed a fragment ion at m/z 475 compatible with the protonated molecular peak with loss of water $[MH\text{-}18]^+$. The base peak at m/z 151 (100%) could be attributed to the dehydrated and protonated vanillic acid, as the fragment at m/z 307 could fit the loss of vanillic acid from ion at m/z 475. The HR ESIMS negative-ion mode data for burkinabin A yielded a molecular formula of $C_{23}H_{23}O_{12}$ (found 491.1133 ($M-H^+$) calcd 491.1190), the ion base peak at m/z 323 corresponding to the loss of vanillic acid (ion at m/z 167) from the pseudomolecular peak The ions at m/z 173 and

155 were probably formed from deprotonated quinic acid (m/z 191) and were attributed to the loss of respectively one or two molecules of water (Tolonen et al., 2002). This fragmentation is presented in Table 1.

The positive and negative ESI-MS spectra of the two other isomers (**2** and **3**) were almost identical to the spectra of **1.**

The locations of the vanilloyl groups on the quinic ring of the divanilloylquinic acid isomers could not be solved by reference to MS alone, but required NMR data. More information about structure of these compounds was obtained by the analysis of the peaks at the retention time (Rt) at about 18 min for **1**, 19.5 min for **2** and 20.5 min for **3** by stop-flow LC/NMR, including online ^{1}H and TOCSY experiments. Because of the use of deuterated solvents (D_2O) in LC/MS/NMR, the observed molecular ion was increased by one unit for each exchangeable proton replaced with deuterium, and in our case we observed a molecular ion at 494 (in negative ionisation) instead of 491. Thus we could conclude that three protons in addition to the COOH were easily exchangeable.

The NMR spectra were taken in two different solvent systems: MeCN–D_2O–TFA for the NMR detection in direct stopped-flow mode and CD_3OD after the process of solid-phase extraction (SPE) to concentrate the sample before NMR analysis. The combination of the information found in both spectra is useful to exclude the interference of solvents.

Furthermore, the NMR spectra of commercial samples of quinic acid (**4**) and vanillic acid (**5**) were recorded for comparison purposes.

The first aspect to be discussed is the full assignment of the quinic acid moiety. The proton NMR spectrum of free quinic acid (**4**) in CD_3OD showed exactly the same chemical shift values (see Table 2) as those published (Pauli et al., 1998). It should be noted that different numbering systems have been used in literature; in this work we have adopted the IUPAC numbering system as described elsewhere (Pauli et al. 1998; Tolonen et al., 2002).

Generally, in the quinic derivatives, the hydroxymethine protons H3, H4 and H5 are well separated. Moreover, each hydroxymethine proton exhibits a different signal from the two others. The proton 4 is always represented by a doublet of doublet and the proton 3 can be differentiated from the proton 5 by its half-width value ($J_{wh/2}$) which is small for the proton 3 (about 10 Hz) while that is larger in the case of the proton 5 (about 28 Hz) (Nishimura et al., 1984). In contrast, signals of the methylene protons H2 and H6 are blurred because of multiple spin coupling but they can be assigned thanks to TOSCY spectra. In the latter case, LC/SPE/NMR gave us more information. For the three new compounds, LC/^{1}H NMR spectra revealed signals for seven protons of the quinic acid moiety, which were limited to the spectral region between 2.12 and 5.81 ppm (Table 2).

Comparison of different protons chemical shifts of free quinic acid (**4**) and those of divanilloylquinic acids (**1**, **2** and **3**) is important to establish the sites of esteric linkages. Indeed each substituted hydroxymethine induces paramagnetic shifts whose magnitude decreases with distance from substituted position (see **bold data** Table 3). This can be confirmed by calculation (Rumbero-Sanchez et al., 1991). Thus ^{1}H chemical shifts affecting the neighbouring protons of the ester bond are an excellent source of structural information (Pauli et al., 1998; Tolonen et al., 2002). In the new compounds, each vanilloyl group forms an ester bond with one hydroxyl group of quinic acid, deshielding the geminal proton, so that the signal of this proton is significantly moved downfield. The positions of the two vanilloyl groups could be solved with the help of chemical shifts of protons 3, 4 and 5 of the hydroxymethine groups. Indeed, if only one of the signals of these protons moves significantly downfield, then the other vanilloyl group must be attached to the hydroxyl group of carbon 1 of quinic acid. In the spectrum of compound **1,** the signals of H-3 ($J_{wh/2} = 10$ Hz) and H-4 (*dd*) are shifted 1.73 and 1.80 ppm downfield in comparison with the spectrum of quinic acid, indicating the presence of vanilloyl groups in positions 3 and 4. The protons H-5 ($J_{wh/2} = 28$ Hz) is deshielded downfield, but the magnitude of the shift of this proton located in position *ortho* of the ester bonding is weaker that those of the geminal protons (see Table 2). Accordingly, burkinabin A (**1**) is identified as 3,4-*O*-divanilloylquinic acid. In the spectrum of burkinabin B (**2**) the signals of H-3 and H5 are shifted 1.53 ppm downfield, indicating that the structure of compound is 3,5-*O*-divanilloylquinic acid. In the spectrum of burkinabin B, the H-4 is shifted 0.79 ppm downfield, indicating an additive effect of the two esters in *ortho*. The chemical shifts of the other protons of the quinic part are in agreement with this structure (see Table 3). Similarly, burkinabin C (**3**) could be identified as 4,5-*O*-divanilloylquinic acid considering of the 1.80 ppm downfield shift of the H-5 signal and the 1.90 ppm downfield shift of the H-4 signal (Table 3).

Table 1
The exact masses of ions formed from (**1**) with negative electrospray ionisation

Measured mass (Da)	Calculated mass (Da)	Molecular formula
491.1133	491.119	$C_{23}H_{23}O_{12}$
323.0668	323.0767	$C_{15}H_{15}O_8$
173.05	173.045	$C_7H_9O_5$
167.0377	167.0344	$C_8H_7O_4$

For the other part of the molecule, as for quinic part, we have compared the ^{1}H NMR spectra of the three HPLC peaks (which were acquired in the stop-flow mode resonance), with the spectrum of vanillic acid (**5**). We observed six aromatic protons and two methoxy groups corresponding to two vanilloyl substructures. 2D-selective TOSCY experiments led to the assignments of the individual vanilloyl subunits (see Table 2). The shifts of H6′ protons of aromatic rings are more in agreement with values calculated for vanillic acid than in the case of its isomer, isovanillic acid (Dyke et al., 1978). However our proposal—compatible with the common occurrence of vanillic acid in plants—still has to be proved by further NOESY and/or HMBC experiments on isolated compounds.

The different values of the aromatic moieties of the burkinabins indicate a greater interaction between the two aromatic residues in burkinabins A and C (**1** and **3**) than in burkinabin B (**2**). That can be explained by the fact that in **1** and **3**, the two ester bonds between vanilloyl group and hydroxymethine of quinic acid were adjacent, while they were well separated in burkinabin B (**2**).

In summary, we have described a new phytochemical investigation of the African tree *Fagara zanthoxyloides* using hyphenation triad HPLC/DAD, HPLC/MS/MS and LC/NMR. LC/DAD/MS is firstly used to identify the molecular weight and to get fragment information on the three new phenolic products that have a UV spectrum similar to that of vanillic acid. As these molecules appeared to be original, LC/DAD/MS/NMR was later used for a more detailed investigation.

The structures of the new compounds, named burkinabins A, B and C (**1**, **2** and **3**), were elucidated thanks to this powerful hyphenated method, and so without any need for isolation.

Concurrently with the above structural work, antisickling bioassays were carried out (Table 4). The ethyl acetate extract (with burkinabins as main compounds) had antisickling activity at the same dose level of ca 0.25 mg/ml as the reference compound

Table 2
Proton NMR chemical shift values for the quinic moiety and the vanilloyl moiety in the burkinabins A-C (**1**, **2** and **3**), in CD_3OD after SPE: comparison with quinic acid (**4**) and vanillic acid (**5**) in CD_3OD

Quinic acid moiety Compound	H2ax	H2eq	H3	H4	H5	H6ax	H6eq
4	2.05	2.02	4.08 ($J_{wh/2}$ = 10.5)	3.38 ($J_{wh/2}$ = 13.6)	3.99 ($J_{wh/2}$ = 26)	1.85	2.12
1	2.48	2.20	5.81 ($J_{wh/2}$ = 10)	5.18 ($J_{wh/2}$ = 15.7)	4.52 ($J_{wh/2}$ = 28.5)	2.12	2.30
2	2.42	2.32	5.61 ($J_{wh/2}$ = 10.8)	4.17 ($J_{wh/2}$ = 16.3)	5.52 ($J_{wh/2}$ = 28.5)	2.34	2.44
3	2.42	2.18	4.49 ($J_{wh/2}$ = 11)	5.28 ($J_{wh/2}$ = 15.7)	5.79 ($J_{wh/2}$ = 27.6)	2.38	2.40
[a]**1, 2, 3, 4**	*dd*	*ddd*	*dt*	*dd*	*Ddd*	*Dd*	*ddd*

Vanillic moiety Compound	H2′	H5′	H6′	OCH_3
5	7.56	6.94	7.55	3.88
1	7.34	6.87	7.49	3.55
	7.53	6.96	7.58	3.82
2	7.69	6.98	7.68	3.93
	7.6	6.98	7.61	3.93
3	7.44	6.89	7.5	3.82
	7.52	6.91	7.58	3.83
[b]				
1,2,3	(*d*, 1.53)	(*d*, 8.24)	(*dd*, 8.24;1.53)	*(s)*

[a] Multiplicities are in italics (the signals of protons 2 and 6 are sometimes blurred). Half-width values ($J_{wh/2}$) in Hz are in parentheses.
[b] Multiplicities and coupling constants in Hz are in parentheses.

Table 3
Substituent chemical shifts induced by esterification of vanillic acid esters **1**–**3** calculated for the protons of quinic acid in CD_3OD

Compound	H2ax	H2eq	H3	H4	H5	H6ax	H6eq
1	+0.43	+0.18	**+1.73**	**+1.80**	+0.53	+0.27	+0.18
2	+0.37	+0.30	**+1.53**	+0.79	**+1.53**	+0.49	+0.32
3	+0.37	+0.16	+0.41	**+1.90**	**+1.80**	+0.53	+0.28

Table 4
In vitro antisickling activity of ethyl acetate extract. The activity was expressed as mean values (SEM) of the percentage of sickle red cells just after deoxygenation with PBS as control and cromolyn sodium as reference

Conc (mg/ml)	Ethyl acetate extract $n=4$ for each conc. (% of sickle cells)	Cromolyn sodium $n=5$ for each conc (% of sickle cells)
0.00	66.6 (0.8)	67.1 (1.8)
0.125	41.0 (1.3)	27.9 (0.9)
0.25	26.3 (1.0)	20.5 (1.5)
0.50	20.1 (0.8)	18.8 (1.2)
1.00	14.7 (1.1)	17.3 (0.8)
2.00	13.1 (1.1)	16.0 (0.7)

n = Number of experiments

disodium cromoglygate (Fall et al., 1998). In the future we intend to perform other tests on isolated burkinabins to confirm their role in the antisickling activity of the ethyl acetate fraction.

Nevertheless our preliminary structural results provide a basis for further investigation. The purification of these three metabolites by preparative methods as well as that of minor derivatives with the same chromophore is in progress. The complete spectroscopic data of all these compounds as well the results of their potential antisickling activity will be published in due course.

3. Experimental

3.1. General

LC/DAD spectra were recorded with an HP G1311A quaternary pump (with HP 1100 Series vacuum degasser) equipped with a diode-array detector Hewlett Packard model 1040 M- series 2.

The Q-TOF LC/MS/MS experiments were performed with a Waters 2690 Alliance Model (serial number: E00SM4) for the LC using Mass Lynx NT 3.5 as operating system. This instrument is mainly dedicated to the generation of product ion spectra recorded by scanning CID generated daughter ions issued from pre-selected specific quasi-molecular ionic species.

NMR spectra were recorded on a Bruker LC/UV/MS/NMR Avance 500 MHz spectrometer (see Section 3.5).

3.2. Plant material

The roots of *Fagara zanthoxyloides* were collected in Burkina Faso (area of Bobo Dioulasso) by one of us (B.O.). They were authenticated by the Botany Department Staff of the "Institut de Recherche en Sciences de la Santé" (IRSS) of Burkina Faso and voucher specimens Nr 2001/0504 were deposited in the Herbarium of the IRSS and in the Laboratory of Pharmacognosy of the University of Liège. The roots were washed, chopped into small pieces, dried under a ventilated entrance hall and then powdered.

3.3. Extraction and isolation

The powdered roots of *Fagara zanthoxyloides* (50 g) were macerated with 100 ml of ethanol–H_2O (50/50) and then percolated with 400 ml of the same solvent at room temperature. The extract was concentrated under reduced pressure until 100 ml. The aqueous solution was basified to pH 8 with Na_2CO_3 and repeatedly extracted with ether. The aqueous solution was then acidified with 4% HOAc. The resulting acidic (pH 3) solution was repeatedly extracted by EtOAc. The solutions were dried over Na_2SO_4 and concentrated to give crude extract containing **1**, **2** and **3**.

3.4. Chromatographic separations

LC/UV/DAD and LC/MS/MS were performed using Hypersil ODS (C-18) columns from Alltech (5 μm, 4.6×250 mm). The mobile phases used were (A) H_2O with 0.05% of TFA and (B) CH_3CN. Binary gradient was programmed as follows: 0 min 90% A, 30 min 60% A and 50 min 90% A. The flow rate was set to 1.0 ml / min. UV detection was performed at 265 nm.

3.5. Hyphenated analysis (LC/UV/MS/^{1}H NMR)

Ethyl acetate extract containing **1**, **2** and **3** was dissolved (100 mg/ml) in methanol and 5 μl was injected into Agilent 1100 Series HPLC system coupled to diode array detector (Agilent 1100), NMR detector (Bruker, Avance 500 MHz) equipped with an LC-NMR probe with a 60 μl flow-cell (active volume), and ion-trap mass spectrometer (Bruker, Esquire 3000 series). Column was Hypersil ODS C18 with column oven temperature set at 35 °C. Separation was achieved using 0.05% TFA in deuterated water as solvent system with a flow rate of 1.0 ml/min and linear gradient from 7 to 70% acetonitrile over a 45 min period. The proportions are mentioned for each burkinabin (see Sections 3.7, 3.8 and 3.9). The basic interface was Bruker, the stop flow unit (BSFU-O). Data were acquired using HyStar PP Version 2.1 software (Bruker, Germany). The ^{1}H-NMR spectra were obtained in stopped-flow mode. WET solvent suppression and related sequences were used to suppress the peaks of CH_3CN, its C-13 satellites, and the residual HOD in D_2O.

3.6. LC/SPE/NMR

Solid phase extraction (SPE) is done thanks to Bruker Spark Prospekt II system used after the column. The selection of the three peaks for trapping is detected by

DAD values and negative MS (m/z 491). For this operation, the solvents used are nondeuterated because their cost is very high, and moreover with D_2O, there would be exchange with –OH, –NH, in mass spectroscopy. After peak trapping, the cartridges are dried with nitrogen to remove all residual solvents. CD_3OD is used to flush the peak on the cartridge into the NMR; this creates a very sharp elution band (about 40 μl eluting volume).

3.7. Burkinabin A (**1**),

Peak at the retention time about 18 min in the hyphenated system LC/NMR [with the proportions of solvent A (68.2) and B (31.8)].

LC/DAD/UV: λ_{max} 221 (100%), 264 (62.4%) and 295 (35.9%).

LC/MS/MS, HR-ESI-MS: m/z 491.1133 $[M^{-1}]^-$, (calcd for $C_{23}H_{23}O_{12,}$ 491.1190).

NMR values: see Table 2

3.8. Burkinabin B (**2**)

Peak at the retention time about 19.5 min in the hyphenated system [with the proportion of solvents A (65.7) and B (34.3)].

LC/DAD/UV: λ_{max} 221 (100%), 264 (62.4%) and 295 (35.9%).

LC/MS/MS, HR-ESI-MS: m/z 491.1181 $[M^{-1}]^-$, (calcd for $C_{23}H_{23}O_{12,}$ 491.1190).

NMR values: see Table 2

3.9. Burkinabin C (**3**)

Peak at the retention time about 20.5 min in the hyphenated system [with the proportion of solvents A (64.2) and B (35.8)].

LC/DAD/UV: λ_{max} 221 (100%), 264 (62.4%) and 295 (35.9%).

LC/MS/MS, HR-ESI-MS: m/z 491.1181 $[M^{-1}]^-$, (calcd for $C_{23}H_{23}O_{12,}$ 491.1190).

NMR values: see Table 2

3.10. Antisickling assays

3.10.1. Patient selection

For the antisickling test, patients with severe SCA were followed at the "Hôpital Universitaire Brugmann" (Brussels/Belgium). The protocol of the study was approved by the Hospital Ethics Committee. Oral informed consent was obtained from patients and their legal guardians.

3.10.2. Blood collection, erythrocytes preparation, sickle cells imaging and counting

The bioassay was based on sickle cells counting, before and just after deoxygenation, in blood samples taken from patients with severe sickle cell anaemia and pre-incubated with the drugs to be tested (100 μl of the ethyl acetate solution were added with 100 μl of erythrocyte suspensions in a phosphate-buffered saline (PBS, pH 7.4)).

The device used in the bioassay was derived from earlier described apparatus and protocols (Fall et al, 1998). Concentrations of the ethyl acetate solution ranged from 2.00 mg/ml to 0.125 mg/ml and was tested in four fold.

Cromolyn sodium was used as antisickling reference and PBS as control. At 0.25 mg/ml ethyl acetate extract activity was similar that of cromolyn sodium at 0.25 μmol/ml corresponding to 0.128 mg/ml instead of 0.128 μg/ml as erroneously mentioned in reference (Fall et al., 1998).

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