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On-line identification of the antifungal constituents of *Erythrina vogelii* by liquid chromatography with tandem mass spectrometry, ultraviolet absorbance detection and nuclear magnetic resonance spectrometry combined with liquid chromatographic micro-fractionation

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Abstract

In our continuing search for new antifungal agents of plant origin, the investigation of *Erythrina vogelii* Hook. f. (Leguminosae), a plant used in the traditional medicine of Ivory Coast to treat various infectious ailments, was undertaken. In order to rapidly identify the active principles, the crude extract was analysed by low-flow LC–^{1}H nuclear magnetic resonance spectrometry (NMR) which gave a sensitive detection of all the main peaks. LC microfractionation was performed just after LC–NMR detection and all peaks collected were submitted to antifungal bioautography assays against *Cladosporium cucumerinum*. By this means, the antifungal activity could be efficiently linked to three of the LC peaks. In order to obtain complementary on-line structural information for all peaks of interest, high-resolution LC–MS–MS together with LC–UV with post-column addition of UV shifts reagents was undertaken on the crude extract. This chemical screening strategy with integrated antifungal bioassays has permitted the on-line identification of numerous constituents and has given useful information for an efficient peak-guided isolation procedure.

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1. Introduction

In the course of the study of medicinal plants from Ivory Coast, the root bark of *Erythrina vogelii* Hook. f. (Leguminosae) was investigated. The tropical genus *Erythrina* has a significant history of folkloric use to treat various infectious diseases [1]. In a preliminary biological screening, the CH_2Cl_2 extract was found to display interesting antifungal properties against the phytopathogenic fungus *Cladosporium cucumerinum* in a direct thin-layer chromatography (TLC) bioautographic assay [2]. Based on this screening a detailed investigation of this plant was decided since antifungal agents active against *C. cucumerinum* are of agrochemical interest and

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might be of therapeutic value against other fungi [3]. As a part of our on-going investigations on the development of liquid chromatography (LC) hyphenated methods for on-line identification of natural products [4], this plant extract was also chosen to evaluate a strategy for on-line structural assignment of bioactive compounds based on combined chemical and biological LC screening methods.

For the on-line identification of the main constituents of the crude extract (''LC chemical screening''), LC–nuclear magnetic resonance spectrometry (NMR), high-resolution LC–tandem mass spectrometry (MS–MS) and LC–UV with post-column addition of UV shift reagents have been employed. These different LC hyphenated techniques have been used separately or in partial combination beforehand but in this study, the on-line structural identification process have included all these independent set of on-line spectroscopic data.

The coupling of LC–NMR is one of the most powerful methods for the separation and structural elucidation of unknown compounds in mixtures [5]. However NMR is also known to be a rather insensitive LC detection method in the on-flow mode [6]. Besides the development of new generation of flow-probes or the use of high-resolution magnets [5], several HPLC strategies for improving the quality of the on-flow LC–^{1}H-NMR spectra have been described [7]. Previously, we have experimented the use of LC–NMR with standard flow-rates (1 ml/min) by performing a series of stop-flow experiments at constant time intervals on a whole chromatogram (time-slice) over a long time period for improving sensitivity in the case of a metabolite profiling of *Gnidia involucrata* Steud.ex A.Rich. (Thymelaceae) [8]. In this paper, a sensitive low-flow LC–NMR method has been developed for a detailed analysis of the main constituents of the crude CH_2Cl_2 extract of *E. vogelii*.

The use of high-resolution LC–MS–MS in phytochemical analysis is relatively new and only few papers have demonstrated the potential of this technique for crude plant extract analysis [9]. The on-line molecular formula assignments of several peptides have been mentioned [10]. Recently we have demonstrated the superiority of Q-TOF (time-of-flight) LC–MS–MS for the analysis of various flavonoids over ion-trap LC–MS^{n} [11,12]. For this application, high-resolution Q-TOF-LC–MS–MS will be used as an efficient complement to on-flow LC–^{1}H-NMR for on-line structural investigations.

LC–UV with post-column addition of UV shift reagents has been used successfully in our laboratory for the characterisation of various polyphenols such as xanthones [13] or flavones [14]. This method gives the possibility to acquire a set of UV shifted spectra on-line by repeated injection of a given extract. As for classical UV shift reagents [15], interpretation of the shifted UV bands provides precious indications on the position of free OH groups on the polyphenolic nucleus. In the present study, these data will be used in combination with the other on-line information to ascertain the structure assignments made.

For the HPLC biological screening of the extract of *E. vogelii*, a method based on the LC microfractionation of the LC peaks separated during LC–NMR analysis has been developed. This method takes advantage of the HPLC conditions developed for the high loading necessary for LC–NMR and the sensitivity of the TLC bioautographic assay against *C. cucumerinum*.

The on-line identification of various known antifungal constituents of *E. vogeli* (''dereplication'') as well as the tentative on-line identification of putative new products from this plants by means of a combination of the different LC hyphenated techniques mentioned above are presented.

2. Experimental

2.1. Plant material and extraction

The roots of *E. vogelii* Hook f. were collected on the road to Petit Yapo (region of Agbovelli) in the Ivory Coast, in September 1999. A voucher specimen was identified by Henri Téré from the Centre Suisse de Recherches Scientifiques (CSRS) in Abidjan, Ivory Coast, and deposited at the CSRS herbarium (voucher No. HGT 2797). The root bark (3 kg) was grounded after cryocooling with liquid nitrogen and was exhaustively extracted by maceration at room temperature with CH_2Cl_2 (2.0 l, 3×24 h), followed by MeOH (2.0 l, 3×24 h), and concen-

trated under vacuum to give 15 g CH_2Cl_2 extract and 10 g MeOH extract.

2.2. Solvents and reagents

Acetonitrile (MeCN) was HPLC-grade (Maechler, Basel, Switzerland). Water was purified on a Reinstwasser system (St-Gallen, Switzerland). Deuterated water (isotopic purity 99.8 atom % D) was obtained from Dr. Glaeser (Basel, Switzerland). Both solvents were passed through Millipore filters (water: 0.45 μm HA; MeCN: 0.50 μm FH) (Bedford, MA, USA). Trifluoroacetic acid (TFA) for spectroscopy was purchased from Fluka (Buchs, Switzerland).

2.3. LC–UV analysis

Reversed-phase HPLC of the crude CH_2Cl_2 extract was performed on a Hewlett-Packard (Waldbronn, Germany) Series 1100 photodiode array detection (DAD) liquid chromatography system. The separations were achieved on a Bondapak C_{18} prepacked column Waters (100×8 mm I.D.; 10 μm) with MeCN–water (5:95 to 100:0; 60 min). The flow-rate was 1 ml/min; the UV traces were measured at 210 and 254 nm and UV spectra (DAD) were recorded between 200 and 500 nm.

2.4. LC–UV analysis with post-column addition of UV shift reagents

The method used for post-column addition of UV shift reagents is based on a previously reported protocol [16]. For the specific analysis of the isoflavones, only two reagents were used: the weak base and $AlCl_3$ in acidic conditions. The extract was analysed under the HPLC conditions described above. For the LC–UV analysis under weak base conditions, a first pump was used for the addition of NaOAc (0.5 *M*, 0.4 ml/min) and a second for NaOH (0.01 *M*, 0.3 ml/min). The resulting pH in the eluent was 8. For the analysis with $AlCl_3$, the first pump delivered $AlCl_3$ (0.3 *M*, 0.2 ml/min), in this case TFA was added to both elution solvents (0.1%) leading to a pH of 3.5. The reaction coil was heated at 90 °C to ensure completion of the complexation reaction. Before analysing the extract, quercetin was used for testing the effect of the UV shift reagents. All the reagent solutions were filtered through a 0.50 μm filter (Millipore).

Post-column addition of the bases and of the "shift reagents", was achieved by two M-6000 pumps from Waters (Bedford, MA, USA). Neutralization of the mobile phase was done with an Upchurch mixing tee (Oak Harbor, WA, USA) while reaction with the shifts reagent was carried out in a 10-μl visco mixer from Lee (Westbrook, CO, USA) followed by a reaction coil. UV spectra were recorded on a photodiode array detector HP-1050 and data were processed on Chemstation from Hewlett-Packard (Palo Alto, CA, USA).

2.5. Q-TOF-LC–MS analysis

For the high-resolution LC–MS–MS analyses, the extracts were analysed under the same conditions as for the LC–UV analyses. Q-TOF-MS–MS experiments were conducted on a Q-TOF 2 mass spectrometer (Micromass, Manchester, UK). The atmospheric pressure chemical ionisation (APCI) conditions were as follows: corona pin voltage, 5 kV; vaporiser, 600 °C; nebuliser gas, nitrogen; cone voltage, 26 V. MS scan time 1 s+0.1 s interscan delay; MS–MS scan time 0.5 s+0.1 s interscan delay.

For accurate mass measurements a reference compound sulfadimethoxine ($[M+H]^+$: 311.0814) (Aldrich, Buchs, Switzerland) was added post-column.

2.6. Collision induced dissociation (CID) MS–MS conditions

On the Q-TOF-MS–MS scan dependent MS–MS, autoswitch experiments were performed sequentially on the three main ions recorded by LC–APCI-MS. The combination of quadrupole ion-selectivity with the full scan sensitivity of the TOF analyser allowed the on-line recording of autoswitch MS–MS experiments at five different CID energies levels (20, 25, 30, 35 and 40 eV) during a three second period. Argon was used as collision gas. For accurate mass measurements in MS–MS, the $[M+H]^+$ precursor ion or a characteristic fragment ion of the compound of interest was used as lock mass.

The energy ranges chosen (20–40 eV) covered all the range of setting where CID MS–MS was practi-

cally recordable. At low energy (20 eV), almost no fragmentation was recorded, while at high energy (40 eV), only a general loss in sensitivity without any increase in the number or the abundance of the fragment ions formed was observed.

2.7. LC–NMR analysis

A Varian Unity Inova 500 MHz NMR instrument equipped with a $^{1}H[^{13}C]$ pulse field gradient indirect detection microflow LC–NMR probe (flow cell 60 μl; 3 mm I.D.) was used. Reversed-phase HPLC of the compounds was carried out on a Varian (Palo Alto, CA, USA) modular HPLC system, comprising a Varian 9012 pump, a Valco injection valve and a Varian 9050 UV detector. The separation was performed by using a Bondapak C_{18} prepacked column Waters (100×8 mm I.D.; 10 μm) with MeCN–$^{2}H_2O$ (5:95 to 100:0; 19 h). In order to achieve a satisfactory on-flow LC–NMR detection the amount of extract was significantly increased in comparison to that used for the LC–UV–MS analyses. The flow-rate was reduced to 0.1 ml/min and the on-flow run consisted of 120 increments of 256 transients each. The total analysis time for this experiment was 19 h. During LC–NMR the UV traces were measured at 210 nm with a dual path UV detector (Varian) for monitoring the chromatographic separation. References of the solvent signals were set at δ 2.10 for acetonitrile. Solvent suppression was performed on-line by the use of the WET sequence [17]. During gradient elution the shape of the selective pulses was automatically calculated on the fly based on a scout scan recorded before each increment.

Computer simulations were performed with the ACD NMR predictor software (Advanced Chemistry Development, Ontario, Canada).

2.8. LC microfractionation

Collection of the different peaks after the LC–NMR analysis was performed on a Gilson collector (FC204). Fractions were collected every 10 min (1 ml) in Eppendorf tubes. After collection, all fractions were evaporated to dryness on a speedvac system (RCT 90, Jouan). The content of each fraction was suspended in 50 μl of MeCN and 40 μl was used for the antifungal bioassay.

2.9. TLC bioautography

TLC bioautography was performed with *C. cucumerinum* according to a known protocol [2,3]. For detecting the antifungal activity in the extract, 100 μg of the extract was eluted on a TLC plate with CH_2Cl_2–MeOH (95:5) as solvent system. In the microfractionation experiment, 40 μl of each fraction (80%) was spotted on a TLC plate every 1.5 cm without elution. A total of 109 fractions resulting from the separation of 10 mg of extract on column were surveyed. Miconazole 10 μl (0.1 μg) was used as a reference compound.

3. Results and discussion

The CH_2Cl_2 extract of *E. vogelii* was firstly submitted to a standard TLC bioautography with *C. cucumerinum* for a rapid evaluation of its antifungal activity. The TLC revealed the presence of two spots presenting intense zone of fungal growth inhibition.

3.1. Preliminary LC–UV and high-resolution LC–APCI-Q-TOF-MS–MS analyses

In order to obtain a preliminary idea on the composition of the crude extract, a combined LC–UV and positive ion high-resolution LC–APCI-MS–MS analysis was carried out by reversed-phase HPLC with a broad MeCN–H_2O gradient. The LC–MS–MS analyses were performed on a Q-TOF instrument. In order to obtain in a single run, molecular and fragment information, scan dependent MS–MS autoswitch experiments were performed sequentially on the three main ions recorded by LC–APCI-MS at four different energy levels [12].

The LC–UV trace revealed the presence of a dozen of major peaks. These different constituents shared rather similar types of UV spectra with two main absorption bands of decreasing intensity. For a first group of peaks (**2**, **3** and **4**), three maxima were recorded at ca. 210, 260 and 290 (sh) nm, for the other group (**1**, **5**–**8**) these bands were present at ca. 210, 290 and 333 (sh) nm. Most of the minor peaks in the chromatogram presented also one of these two types of chromophores.

Table 1
High-resolution LC–APCI-Q-TOF-MS–MS data

Compound	$[M+H]^+$ measured	$[M+H]^+$ calculated	ppm difference	Elemental composition[c]	LC–MS data				
					Fragments[b]				
					$[M+H-56]^+$	$[M+H-68]^+$	$[A_1]^+$	$[B_3]^+$	Other
1	339.1208	339.1232[a]	−7.2	$C_{20}H_{19}O_5$	283.0667	271.0683	153.0450	–	311.0814, 227.0793, 215.0805, 199.0841, 111.0125, 69.0705
2	371.1503	371.1495[a]	2.3	$C_{21}H_{23}O_6$	315.0929	303.0916	–	–	205.1283, 163.0802, 179.0393, 189.0582, 151.0435, 137.0644, 123.0488
3	371.1477	371.1495[a]	−4.8	$C_{21}H_{23}O_6$	315.0890	303.0870	–	–	285.0793, 205.1282, 189.0584, 179.0363, 177.0588, 151.0419, 137.0622
4	423.2177	423.2171[a]	1.3	$C_{26}H_{31}O_5$	–	–	–	149.0424	349.1152, 337.1145, 311.1268, 299.1395, 279.1123, 239.1824, 195.1213
5	337.1067	337.1076[a]	−2.7	$C_{20}H_{17}O_5$	–	–	153.0421	–	319.1446, 295.1094, 283.0938, 267.1096, 211.1130, 185.0932, 183.1099
6	423.1831	423.1808[a]	5.5	$C_{25}H_{27}O_6$	–	303.0865	–	–	311.0980, 299.0970, 281.0892, 241.0889, 229.0887, 166.0495, 165.0450, 147.0791
7	407.1869	407.1858[a]	2.6	$C_{25}H_{27}O_5$	351.1378	–	–	149.0423	339.1438, 283.0712, 267.0885, 241.0716, 165.0335, 157.0459, 131.0621
8	407.1870	407.1858[a]	2.8	$C_{25}H_{27}O_5$	351.1703	–	–	149.0432	367.1541, 337.1091, 299.0912, 239.1483, 207.1600, 195.1218, 179.0935, 171.1488, 151.0970

$[B_3-15]^+$.

[a] Lock mass sulfadimethoxine: m/z 311.0814.

[b] Lock mass protonated molecule recalculated according to elemental composition.

[c] Elemental composition corresponding to the $[M+H]^+$ ions, one proton has to be deduced for calculation of the molecular formula of the different constituents.

From the high-resolution LC–MS and LC–MS–MS data, it could be noticed that these different constituents displayed protonated molecules $[M+H]^+$ in the range of 339 to 423 Da and that two pairs of isomers (**1** and **3**: 371 Da; **7** and **8**: 423 Da) were observed. For all these constituents, the molecular formula could be assigned precisely thanks to the on-line high-accuracy measurements performed on the Q-TOF instrument with post-column addition of a reference compound for lock mass. Molecular masses were obtained in all cases with a precision of less than 5 ppm. Presence of fragments due to losses of 56 and 69 Da in the LC–MS–MS spectra of all constituents, at the exception of compound **5**, revealed the possible presence of prenyl chains [18,19] (Table 1).

From this preliminary LC–UV–MS screening, it could be concluded that the CH_2Cl_2 extract of *E. vogelii*, most probably, consisted in a combination of various prenylated isoflavanones or isoflavones. This information was in good agreement with previous papers which reported the existence of this type of constituents in other species of the genus *Erythrina* [20].

3.2. Low-flow LC–NMR with microfractionation

In order to obtain more information and to tentatively identify the compounds responsible for the antifungal activity of the extract, an on-flow LC–^{1}H-NMR analysis of the extract followed by LC microfractionation was carried out. The HPLC conditions were optimised in order to achieve the highest loading possible for a sensitive on-flow LC–^{1}H-NMR detection and for the collection of significant amounts of the LC peaks for the antifungal assays (Fig. 1). A C_{18} radial compression column having an important internal diameter (8 mm) was preferred to classical analytical columns because of its high loading capacity and an important amount of crude extract (10 mg) was injected on-column. For obtaining similar LC resolution using a standard (4 mm I.D.) column the maximum amount loaded was found to be only of ca. 2 mg. The radial compression column was also preferred to classical column because of its ability to adapt the compression level in the low flow-rate operation mode. A satisfactory LC resolution of the main peaks was obtained at 1 ml/min flow-rate. For the LC–NMR analysis, water was replaced by deuterated water and the HPLC conditions were changed in order to achieve the same separation over 19 h (Fig. 1b) with a low flow-rate (0.1 ml/min). This low-flow experiment was needed in order to improve the sensitivity of the on-flow LC–NMR detection. Indeed in on-flow mode, LC–NMR spectra are acquired continuously during the separation and are stored as a set of scans as discrete increments and a compromise between the number of scans per increment and the LC resolution has to be made [6]. At standard HPLC flow-rate (1 ml/min), usually between 16 to 32 scans per increment are performed according to the res-

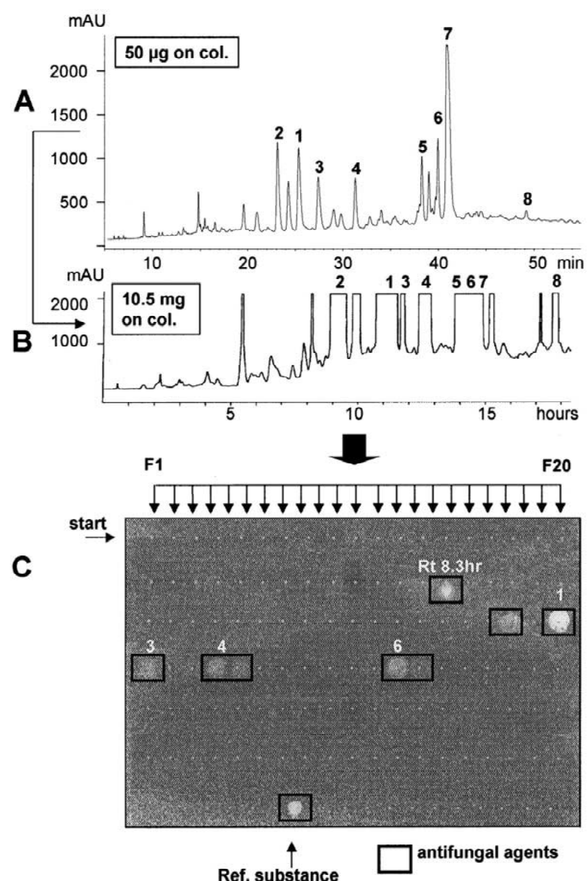

Fig. 1. (A) LC–UV analysis of the crude CH_2Cl_2 extract of *E. vogelii* under typical analytical conditions (50 μg on column). (B) LC–UV 210 nm trace recorded during LC–NMR analysis (10 mg) on-column. (C) TLC bioautography with *C. cucumerinum* of the microfractionation collected after LC–NMR analysis.

olution to achieve. With the low flow-rate chosen (0.1 ml/min), the number of scans per increment was increased to 256 and a the limit of detection was thus consequently lowered. For the acquisition of the LC–NMR spectra, a fast solvent suppression called WET was run before each set of spectra. The pulse shape for solvent suppression was calculated on the fly and automatically adapted to the changes in gradient composition. This experiment permitted the acquisition of the LC–NMR spectra of more than 10 LC peaks in the crude extract over 19 h overnight. The LC resolution was not affected by the operation in the stop-flow mode compared to the standard on-flow conditions (1 ml/min).

During this LC–NMR analysis, the fractions were collected every 10 min (1 ml) in Eppendorf tubes, and a total of 109 fractions were obtained. The content of each tube was removed by centrifugal evaporation and 90% of the total amount was deposited on a TLC plate which was submitted to bioautography with *C. cucumerinum*. This post-chromatographic antifungal assay revealed that the fractions associated with the LC peaks eluting at 8.4 (unknown), 10.8 (**1**), 11.3 (**3**), 12.5 (**4**) and 14.6 (**6**) h in the low-flow LC–NMR experiment display distinguishable antifungal activities (Fig. 1c).

3.3. LC–UV with post-column derivatisation

A first evaluation of the LC–NMR and LC–MS data revealed that spectroscopic information was lacking for a precise localization of the hydroxyl groups of the different polyphenols detected. Thus, complementary LC–UV analyses of the extract with post-column addition of UV shift reagents was performed according to a protocol already tested for flavonoids or xanthones [13,16]. The crude extract was analyzed twice, once after post-column addition of a weak base and then with acidic $AlCl_3$. For each peak, the shifted UV spectra were superimposed to the original spectra and compared (Table 2 and Fig. 3). The shifts observed were interpreted according to the rules previously established for the analysis of pure polyphenols [15]. These analyses provided useful complementary information for the on-line identification of the peaks of interest and this will be discussed below.

3.4. Interpretation of the on-line data

The ^{1}H-NMR data extracted from the low-flow LC–NMR analyses of the crude extract confirmed that all the majors constituents of the crude extract of *E. vogelii* were prenylated isoflavones and isoflavanones. In all peaks, aromatic signals between 5.9 and 6.4 ppm were indicative of a A-ring oxygenated in positions 5 and 7. This was in good agreement with chemotaxonomical data which indicated a 5,7,4′ oxygenation for biosynthetic reasons. For compounds **2**–**4**, methylene protons at 4.39–4.57 ppm were characteristic for an isoflavanone nucleus while for peaks **1** and **5**–**8**, the presence of an aromatic singlet at ca. 8 ppm was indicative for isoflavones (Fig. 2). This was also confirmed by the presence of two distinct types of chromophores for each group of constituents in the UV spectra recorded (Table 2). Presence of one prenylated group was detectable by signals between 3.0 and 3.3 ppm (H-1″), 5.1–5.3 ppm (H-2″) and 1.5–1.6 ppm ($2CH_3$) for all compounds except **5**. In the case of **4** and **6**–**8**, the splitting of these latter signals revealed the presence of a second prenyl group (Table 3). These diagnostic signals were already observable on the contour plot of the on-flow LC–NMR experiment and most of the characteristic resonances are highlighted in Fig. 2. This two-dimensional (2D) plot

Table 2
LC–UV–DAD data before and after post-column addition of the UV shift reagents

Compound	LC–UV–DAD post-column derivation (reagents)					
	MeOH		NaOAc		$AlCl_3$	
	Band I	Band II	Band I	Band II	Band I	Band II
Compound 1	290 (sh)	260	330 (+40 nm)	275 (+15 nm)	290 (sh)	267 (+6 nm)
Compound 2	335 (sh)	290	375 (+40 nm)	345 (+55 nm)	335 (sh)	298 (+8 nm)
Compound 3	335 (sh)	292	–	340 (+50 nm)	335 (sh)	307 (+15 nm)

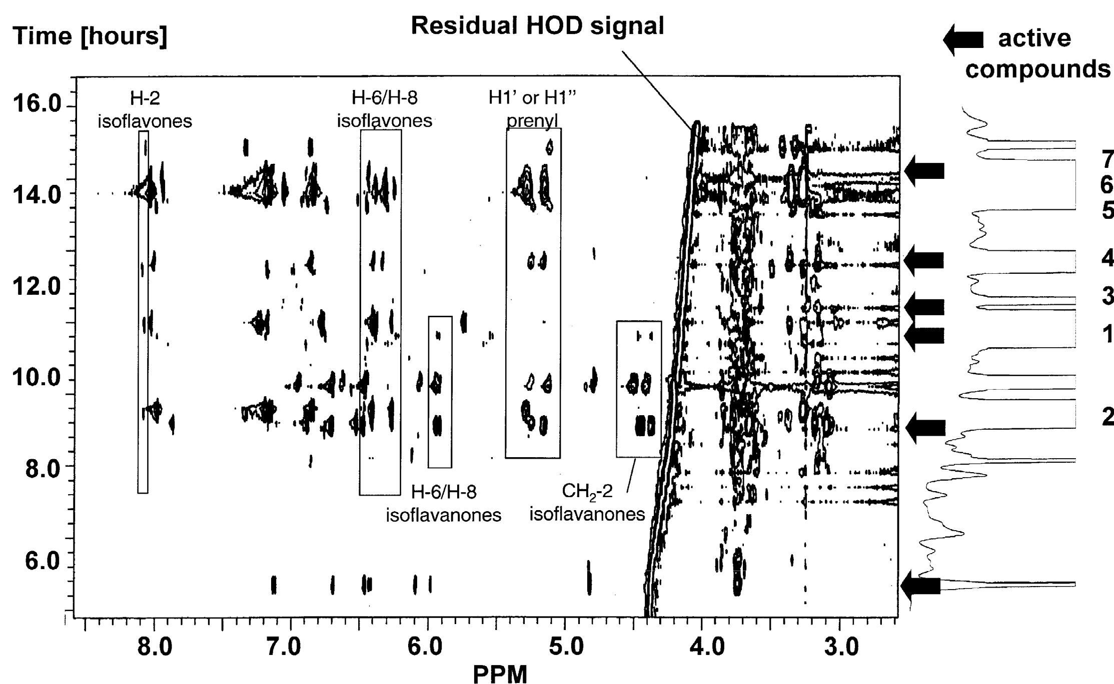

Fig. 2. On-flow LC–NMR contour plot of the crude CH_2Cl_2 extract of *E. vogelii*. On this 2D plot all ^{1}H-NMR resonances of the analytes appeared as dots. Several characteristic region for the resonances of isoflavanones and isoflavones are highlighted. The signals at ca. 4.5 ppm clearly indicate that the isoflavanones are eluting first on the chromatogram.

Table 3
LC–^{1}H-NMR data

Position	LC–^{1}H-NMR (MeCN–^2H_2O) (δ)		
	Compound 1	Compound 2	Compound 3
H-2	7.97, s	4.39–4.47, m	4.39–4.47, m
H-3	–	4.90, s	4.80, s
H-5	–	–	–
H-6	6.26, s	5.92, s	5.93, s
H-7		–	–
H-8	6.40, s	5.94, s	5.95, s
H-2′	7.19, broad s	3.72, s (OCH_3)–	–
H-3′	–	6.42, s	6.47, s
H-4′	–		3.66, s (–OCH_3)
H-5′	6.83, d, J=8 Hz	–	–
H-6′	7.19, d, J=8 Hz	6.70, s	6.70, s
H-1″	3.26, d, J=7 Hz	3.11, d, J=7 Hz	3.08, d, J=7 Hz
H-2″	5.29, broad s	5.17, broad s	5.16, broad s
H-4″	1.67, s (CH_3)	1.55, s (CH_3)	1.58, s (CH_3)
H-5″	1.62, s (CH_3)	1.62, s (CH_3)	1.64, s (CH_3)
H-1‴	–	–	–
H-2‴	–	–	–
H-4‴	–	–	–
H-5‴	–	–	–

represents the usual way of processing on-flow LC–NMR data, one dimension represents the NMR ppm scale and the other the time scale. All cross peaks on this plot represent signal resonances of the different constituents [6].

The isoflavone **1** presented the strongest zone of inhibition in the post-chromatographic antifungal bioautography assays (Fig. 1). The high-resolution LC–APCI-Q-TOF-MS spectrum of **1** exhibited a molecular ion at *m/z* 339.1208 $[M+H]^+$ which indicated a molecular formula of $C_{20}H_{19}O_5$ (Fig. 4a). The fragments at *m/z* 283.0667 $[M-56]^+$ and *m/z* 271.0683 $[M-68]^+$ were characteristic of losses of C_4H_7 or C_5H_9 from one prenyl moiety (Fig. 4b). From the molecular formula, it could be deduced that three hydroxyl groups were also present on the isoflavone nucleus (Fig. 3 and Table 3). The LC–^{1}H-NMR spectrum showed two singlets at δ 6.26 and 6.40 attributable to two aromatic protons at H-6 and H-8. Three signals corresponding to an ABX system at δ 6.85 (d, J=8.0), 7.14 (d, J=8.0), and 7.19 (br s), were attributed to the aromatic protons at H-5′, H-6′ and H-2′, respectively, in the B-ring. Finally, a singlet at δ 7.9 was assigned to H-2, a proton characteristic for the isoflavone nucleus. The position of the three hydroxyl groups and the prenyl unit was deduced from the complementary UV shifted spectra observed upon addition of $AlCl_3$ and NaOAc [15]. The bathochromic shift of band II with $AlCl_3$ was due to the formation of a complex between the OH in position 5 and the carbonyl in 4. On the other hand, the shift of bands I and II upon addition of the weak base suggested the presence of OH in the most acidic positions 7 and 4′. In the CID MS–MS spectra, a fragment at *m/z* 153.0450 $[A_1]^+$ resulting from a typical RDA cleavage confirmed the presence of two hydroxyl groups on the A-ring [12] (Fig. 4c). All these data suggested that **1** was isowighteone, a prenylated isoflavone already isolated in *Cajanus cajan* (L.) Druce (Fabaceae) [21] and known for its antifungal activity. As demonstrated the on-line data obtained have permitted its efficient dereplication.

The isoflavanone (**2**) showed a molecular ion at *m/z* 371.1503 $[M+H]^+$ which suggested a molecular

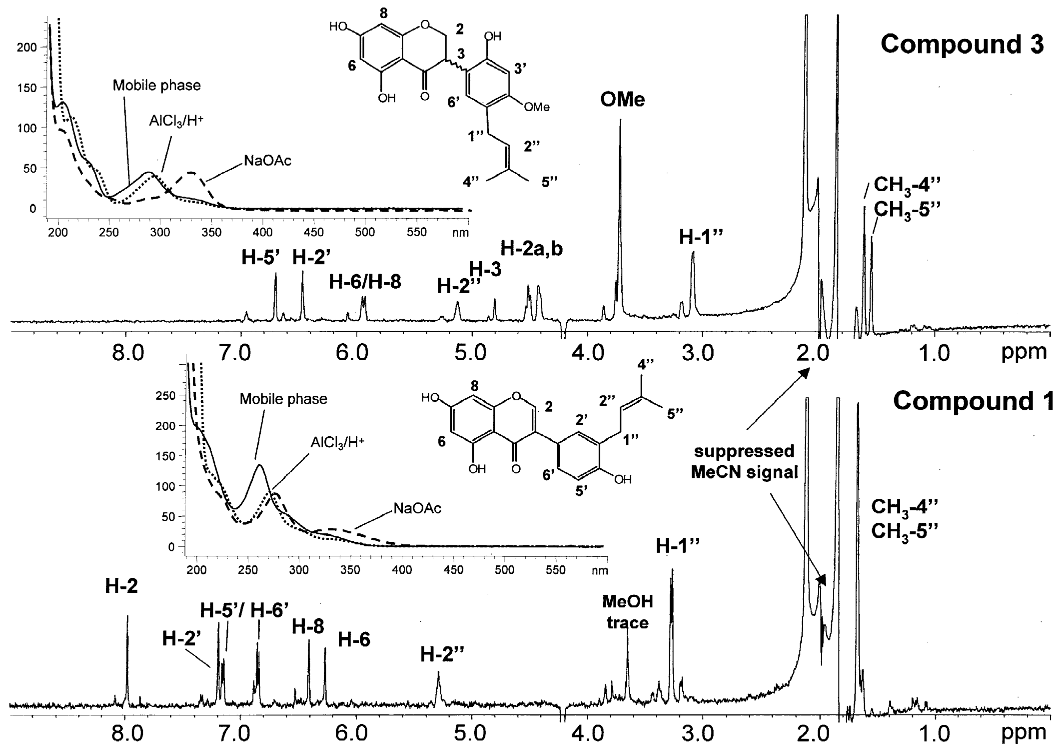

Fig. 3. On-flow LC–NMR spectra of an isoflavanone and an isoflavone and UV and shifted UV spectra recorded on-line.

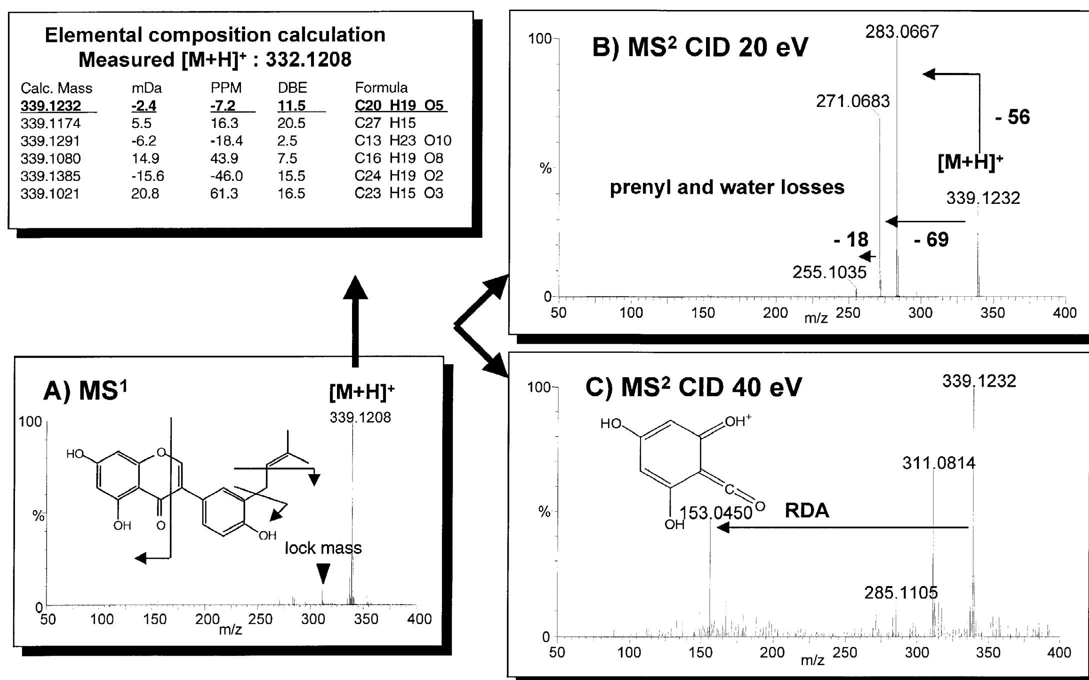

Fig. 4. LC–APCI-Q-TOF-MS–MS spectra recorded on-line for compound **1**. (A) The MS spectrum extracted from the survey scan allow a precise molecular formula assignment. (B) MS–MS spectra of the $[M+H]^+$ ion automatically selected by a scan dependent experiment (CID 20 eV). (C) Same MS–MS spectra recorded with a CID of 40 eV.

formula of $C_{21}H_{22}O_6$ and consequently, a substitution by 3 OH, 1 OMe and 1 prenyl was deduced. The presence of the OMe was confirmed by a singlet at δ 3.72 (3H) in the LC–NMR spectrum. As for **1**, a 5,7-dihydroxylation of the A-ring was deduced. The two broad singlets corresponding to H-6 and H-8 were however slightly shifted high field compared to **1** at δ 5.92 and 5.94 (Table 3). For the B-ring, the presence of an OH in 4′ was deduced by the shift of band I after addition of NaOAc. In the LC–^{1}H-NMR spectrum, two broad singlets at δ 6.42 and 6.70 were attributed to the aromatic protons of the B-ring. The position of the prenyl unit and the methoxyl group could not be precisely assigned based on ^{1}H-NMR data only since the resolution of the aromatic signals did not allow to distinguish between *meta* or *para* coupled protons. These two substituents could be either in 2′ and 5′ or 3′ and 6′. A computer simulation indicated that the best matching for H-3′ (δ calc. 6.40) and H-6′ (δ calc. 6.64) was found for a B-ring substitution by a prenyl in 5′ and a methoxyl in 2′. This information suggested that **2** had great chances to be a new natural product even if its complete on-line identification was not possible. Its targeted isolation was undertaken and the structure was confirmed to be 5,7,4′-trihydroxy-2′-methoxy-5′-(3-methylbut-2-enyl)-isoflavanone [22].

Compound **3** was found to be an isomer of **2** with similar UV, ^{1}H-NMR and MS–MS spectra. The only striking difference between **2** and **3** was that for **3** the UV band I was not changed upon addition of the weak base indicating probably a methoxylation in 4′ (Table 2). As for **2**, this compound was presumably a new natural product and its complete structure elucidation was confirmed after targeted isolation [22].

Based on similar interpretation of the independent set of spectroscopic data recorded compounds **4**–**8** were fully or partially identified on-line. Compounds **4** and **6**–**8** were bearing two prenyl chains. **7** was found to be the known isoflavone isolupalbigenin [23] and its isomer **8** was identified as 6,8-diprenylgenestein [24,25]. **4** and **6** were partially identified on-line and their possible structures were found

to be potentially new. Their targeted isolation was undertaken for complete characterisation [26].

In the case of **5**, the ^{1}H-NMR spectra showed the presence of two doublets with coupling constants of 10 Hz (δ 5.65 and δ 6.40) and the methyl groups of the prenyl unit were shifted to δ 1.38 and 1.42 in comparison with **1**. This on-line information suggested that for **5** the prenyl unit at 3′ cyclised to form a 2,2-dimethylchromene moiety, its structure was established as isoderone [27].

4. Conclusion

The method presented in this paper has the advantage to combine information from independent spectroscopic data from complementary hyphenated techniques and integrates at the same time biological information. This allows complete or partial identification of polyphenols such as isoflavones or related compounds to be performed on-line and gives, with good confidence, precious information on the presence of putative new natural products.

In the strategy presented, a complete hyphenation of all techniques has not been used while other authors have demonstrated that this is technically possible by the creation of a ‘‘total analysis device’’ consisting of on-line LC–UV–Fourier transform (FT)-IR-NMR–MS [28]. In the example presented, LC–UV and LC–MS–MS were acquired at the same time while LC–NMR and LC–microfractionation were performed in a second analysis. The advantage of these separate analyses is that the loading of the sample can be optimised for each technique and that no problem of D–H exchange caused by the use ^2H_2O for LC–NMR occur during MS detection. The drawback of this approach is however that problems of LC peak correlation between the LC–UV–MS and LC–NMR run might arise, especially if minor peaks have to be considered. In order to overcome this problem, 10% of each LC microfraction used for the bioassay were kept for post-chromatographic LC–UV–MS analysis in case of doubt in the attribution of the LC peaks.

The LC microfractionation, following the LC–NMR analysis, has permitted a precise localisation of the bioactive peaks on the LC chromatogram. It should be noted however that the detection of the antifungal activity is only indicative and it did not take into account the amount of sample deposited. A precise estimation of the biological activity would require the isolation of the compounds and the measurement at different concentration levels in dilution assays. A rough estimation of the strength of the activity with the proposed method might be envisioned by using the absolute quantification possibilities of LC–NMR [29]. Further development of the actual method, including post column addition of a standard for absolute quantification and integration of direct MS measurements with H/D back exchange are foreseen.

This integrated bioassay and chemical screening strategy has permitted the dereplication of four known constituents and the partial on-line identification of four new natural products, which were confirmed after efficient peak-guided isolation.

With the development expected in the field of LC–NMR and LC–bioassays, especially in terms of sensitivity automation and miniaturization, impressive improvements of the methods are foreseen. The main challenges will remain however those related to the structural identification of natural products since, unlike for GC–MS, valid spectroscopic databases are not available at present.

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