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Review

Liquid chromatography with ultraviolet absorbance–mass spectrometric detection and with nuclear magnetic resonance spectroscopy: a powerful combination for the on-line structural investigation of plant metabolites

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Abstract

In order to discover new bioactive compounds from plant sources which could become new leads or new drugs, extracts should be submitted at the same time to chemical screening and to various biological or pharmacological targets. Metabolite profiling using hyphenated techniques such as LC/UV, LC/MS and more recently LC/NMR, quickly provides plenty of structural information, leading to a partial or a complete on-line de novo structure determination of the natural products of interest. As a complement to this approach, bioassays performed after LC/microfractionation of the extracts allow efficient localisation of the bioactive LC-peaks in the chromatograms. The combination of metabolite profiling and LC/bioassays provides the possibility of distinguishing between already known bioactive compounds (dereplication) and new molecules directly in crude plant extracts. Thus, the tedious isolation of compounds of low interest can be avoided and targeted isolation of new bioactive products or constituents presenting novel or unusual spectroscopic features can be undertaken. Several examples of rapid localisation of bioactive compounds, based on post-chromatographic bioautographic testing of LC/NMR microfractions and subsequent on-line identification will be illustrated. Application of hyphenated techniques for the efficient characterisation of labile constituents or constituents difficult to separate at the preparative scale will also be mentioned. The possibilities and limitations of LC/UV/NMR/MS and LC/bioassay as well as future development expected in this field will be discussed.

Keywords: Reviews; Plant metabolites; Structural investigation; Nuclear magnetic resonance spectroscopy; Mass spectrometry; Metabolite profiling

Contents

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1. Introduction

For a long time, plants have been the most important therapy available to humans. The plant kingdom is still an untapped reservoir of new molecules with potential therapeutic interest and only a relative small percentage of the 350 000 known plant species have been studied from a phytochemical or a pharmacological viewpoint [1]. Research in pharmacognosy has demonstrated that potent bioactive products can be obtained from plants. In the present drug discovery programs, natural products or compounds derived from natural products account for more than 40% of the new registered drugs [2]. A recent statistical investigation into the structural complementarity of natural and synthetic compounds also proved that the potential for new natural products is not exhausted and they still represent an important source for the lead finding process [3].

In order to discover new bioactive compounds from plant sources which could become new leads or new drugs, extracts should be submitted at the same time to chemical screening and to various biological or pharmacological targets. The chemical screening or metabolite profiling is aimed at distinguishing between already known compounds (dereplication) and new molecules directly in crude plant extracts. Thus, the tedious isolation of known compounds can be avoided and targeted isolation of constituents presenting novel or unusual spectroscopic features can be undertaken [4]. It has to be noted that metabolite profiling clearly refers here to the detection and identification of plant metabolites which is different from the metabolic profiling process which will take place in order to detect the metabolites issued from a given new lead compound.

Metabolite profiling in crude plant extracts is not an easy task to perform since natural products display a very important structural diversity. For each compound, the order of the atoms and stereochemical orientations have to be elucidated de novo in a complex manner and the compounds cannot simply be sequenced as is the case for genes or proteins. Consequently and unlike genomics and proteomics, a single analytical technique does not exist that is capable of profiling all secondary metabolites in a plant extract.

In order to develop innovative strategies for the metabolite profiling of crude plant extracts, we have taken advantage of the extraordinary development of hyphenated techniques and particularly LC/MS and LC/NMR over the last decade for studying their possible application to the on-line identification of natural products. The principle of operation of these latter techniques will not be explained in detail here since different papers including a chapter in this issue [5] have been dedicated to these powerful methodologies [6–10].

In this paper, the emphasis will be put on the strategy developed for an efficient dereplication of natural products and for the on-line identification of bioactive constituents based on the combination of LC/UV-DAD, LC/MS, LC/MS/MS and LC/NMR applied to crude plant extract screening. The role of these techniques in the structural investigation of unstable products or compounds difficult to isolate at the preparative level will also be highlighted by different examples of application.

2. Metabolite profiling: an LC-multihyphenated strategy

When compared to the classical use of UV, MS and NMR spectroscopy applied to pure natural products, ideally, the integration of all these techniques in their hyphenated forms (LC/UV, LC/MS and LC/NMR) in a single setup, with centralised acquisition of the spectroscopic data, should permit the complete spectroscopic characterisation of different metabolites in a mixture in a single analysis. Furthermore other existing hyphenated techniques such as LC/IR [11] or LC/CD [12] may also bring valuable additional information. In practice however many factors may hinder on-line detection and structure determination of an unknown plant metabolite and often only partial structure information will be obtained. These on-line data however already provide very precious information for targeting the isolation of new compounds or for the dereplication of known constituents [4,13–15].

In our approach, the LC/NMR analyses are performed independently from the LC/UV/MS runs. LC/UV/MS is used as a first dereplication step for the chemical profiling of crude plants extracts and compounds are tentatively identified based on molecular weight and fragment information with manual search in natural product libraries [16] as well as on matching in UV in-house spectral libraries. LC/NMR is mainly used in a second step for a more detailed structural investigation of compounds presenting original structural features or displaying interesting activities after LC-bioassays (Fig. 1).

The combination of all these techniques in a single setup is however possible and the creation of a ''total analysis device'' has been recently demonstrated in the case of on-line HPLC-UV(DAD)-FT-IR-NMR-MS analyses [17]. The coupling of all these different techniques (especially LC/NMR-MS) is however not an easy task to perform since operation conditions that are compatible with all of them have to be found [18,19]. The possibility of acquiring all data during a unique analysis gives the possibility to efficiently associate the set of on-line spectroscopic data to a given peak and renders the processing easier to perform.

3. Problems encountered in metabolite profiling

The factors that hamper a complete on-line identification of unknown natural products are mainly

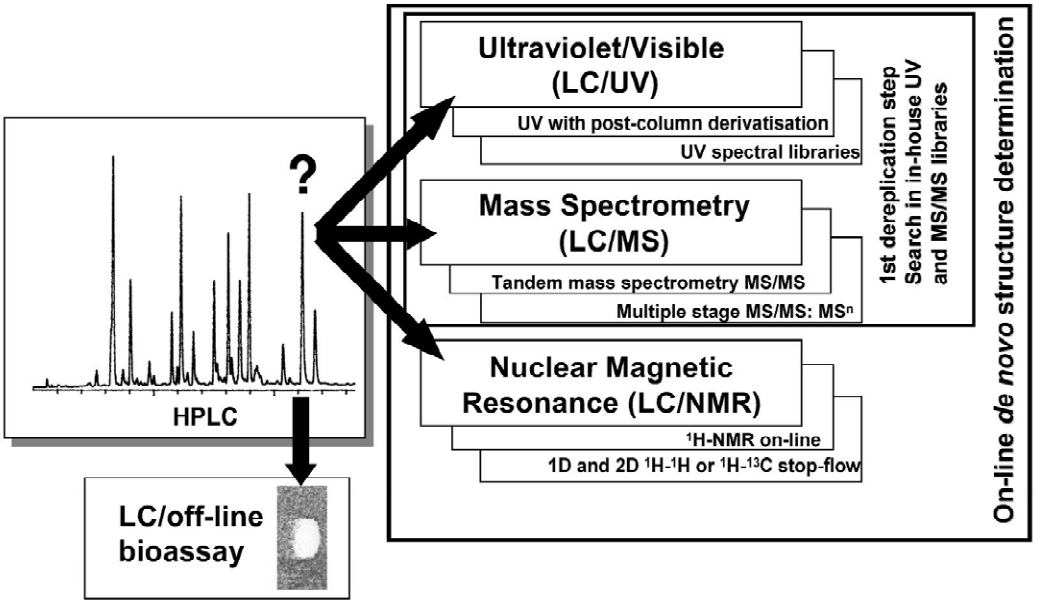

Fig. 1. Type of information that can be obtained on a given LC peak with the different LC-hyphenated techniques available. In this approach LC/NMR can be regarded as an efficient addition to LC/UV/MS for de novo structure identification of natural products on-line.

linked to the present limitations of LC/NMR. Indeed the other hyphenated techniques such as LC/UV or LC/MS provide comparable types of spectroscopic information to those recorded for pure constituents and their sensitivity does not compromise the HPLC separation.

It has to be noticed however that the use of LC/MS for the characterisation of unknown constituents is important since the response is compound-dependent and several sets of conditions have often to be tested to ascertain correct on-line molecular determination [13]. Furthermore, the atmospheric pressure ionisation techniques employed (APCI or ESI) produce a soft ionisation of the analytes. Thus intense molecular ion species are generated and only limited structural information is obtained. Complementary information can be generated by LC/MS/MS or in-source collision induced (CID) fragmentation. The CID spectra generated are however not comparable to those recorded by electron impact (EI) and this hampers direct use of the standard EI-MS natural products libraries for dereplication purposes. For performing automated dereplication procedures, specific LC/MS/MS libraries have to be built up based on standards available in a given laboratory and this greatly limits the approach.

Because of these actual limitations of LC/MS and LC/MS/MS, LC/NMR can be regarded as an invaluable additional tool for dereplication and for on-line de novo structure identification purposes. One of the main problems of this technique is its inherent *lack of sensitivity* which renders the on-flow measurements of minor products impossible and which hampers the direct observation of ^{13}C-NMR resonances even for the main constituents of a crude plant extract. The difficulty of accessing ^{13}C information, which represents an important element for natural product identification, is probably the biggest hindrance to a wider use of LC/NMR. As a consequence, many plant metabolites, especially with characteristic ^{1}H signals which are not well separated in the NMR scale, are difficult to identify at present by this technique. Compounds which exhibit very few ^{1}H signals and which have mainly quaternary carbons or hydroxyl substituents in their structure will also yield little structural information in LC/^{1}H-NMR as was demonstrated for hypericin [20].

Another problem is that the whole range of ^{1}H-NMR signals is not directly observable in classical LC/^{1}H-NMR spectra because of the need for *solvent suppression*. Indeed the signals of the analytes of interest, which reside under the solvent peak, will be suppressed together with the solvent signal. This can be a major drawback when dealing with unknown constituents. In order to detect all analyte signals, one alternative is to carry out the solvent suppression in two independent solvent systems such as MeCN–D_2O and MeOH–D_2O. Such an approach is shown for the analysis of hepatotoxic pyrrolizidine alkaloids in the crude extracts of *Senecio* species (Fig. 2) [21]. In this case, the LC/^{1}H-NMR analyses performed in both solvent systems have enabled recording of the whole range of ^{1}H-NMR signals after combination of the information found in both spectra. For example for senecionine (**1**), the signals in the spectrum recorded in MeCN–D_2O corresponding to H-14a and H-6b were hidden by the suppression of the acetonitrile signal (Fig. 2b). These resonances were however clearly visible when LC/NMR was performed in MeOH–D_2O while H-5a and H-3b were, in this case, hidden by the solvent suppression of the MeOH signal (Fig. 2a). This LC/NMR analysis was found particularly useful in addition to LC/MS to differentiate the isomeric structures of macrocyclic diesters directly in the crude extracts of *Senecio* species [21].

One way of overcoming the problem of solvent suppression is to perform the separation in fully deuterated solvents. This, however, cannot be envisaged routinely on standard HPLC columns, operated at typical flow rates of 1 ml/min, due to the cost of the deuterated solvents. Nevertheless, separations may be envisaged on micro or capillary columns [22] or by column-trapping and backflushing into the LC/NMR flow cell using an appropriate deuterated solvent [23]. Applications of the micro LC/NMR to metabolite profiling of crude plant extracts have not yet been reported to our knowledge.

4. Application of LC/UV/MS and LC/NMR in phytochemical analysis

LC/UV/MS and LC/NMR are used in a complementary manner for metabolite profiling. They often give an unambiguous assignment for known

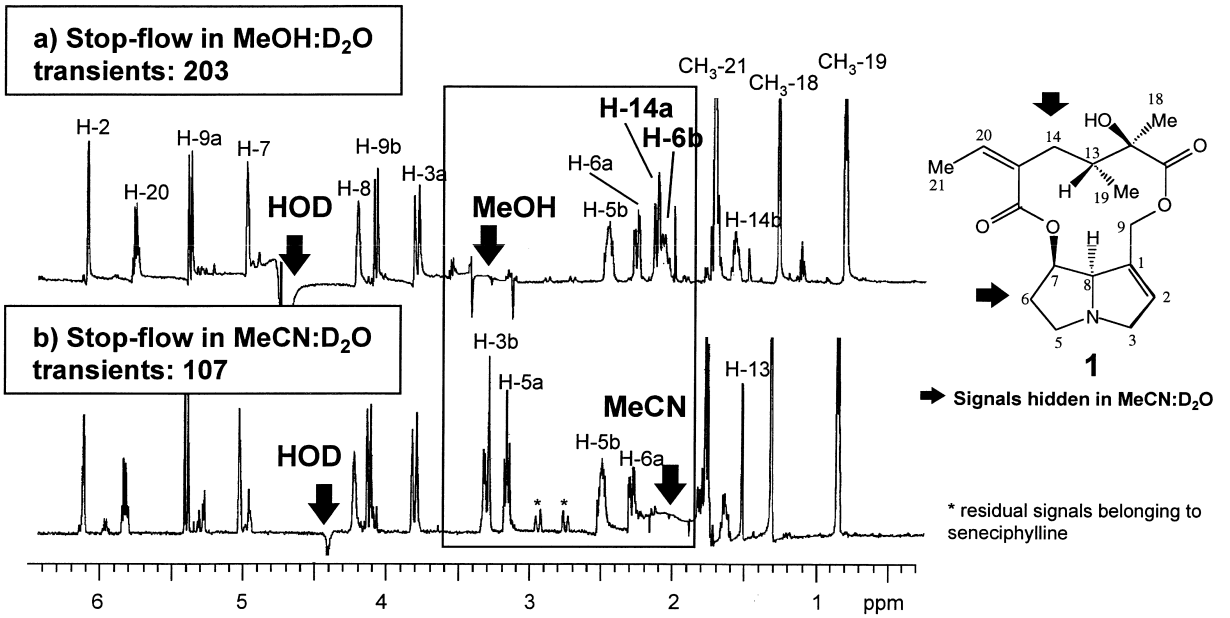

Fig. 2. Stop-flow spectra measured for senecionine (**1**) in the lipophilic extract of *Senecio vulgaris* (Asteraceae) in both MeCN–D_2O and MeOH–D_2O solvent systems for the recording of the whole range of ^1H signals without alteration by solvent suppression. HPLC conditions: injection, 3 mg; column, Symmetry C_{18} (150×3.9 mm I.D., 5 μm); (a) MeOH–D_2O gradient (10:90 to 70:30 in 30 min); (b) MeCN–D_2O gradient (5:95 to 50:50 in 20 min); 0.015 *M* NH_3; 0.8 ml/min. LC/NMR conditions: 24 scans/increment, flow cell (60 μl, 3 mm I.D.), 500 MHz (© 2003, John Wiley & Sons Limited. Reproduced with permission) [7].

natural products and permit the partial structure determination of unknown constituents. Based on these results, and LC-bioactivity assays performed in parallel, the targeted isolation of interesting compounds can be efficiently performed. The strategy followed in this case will be discussed for the dereplication of various prenylated flavones and isoflavones.

The combined use of these hyphenated techniques is also very useful for the on-line de novo structure determination of compounds in enriched mixtures not separable at the preparative scale or for the study of unstable natural products. In this case, the on-line experiments are often performed in parallel with standard in-mixture 1D and 2D NMR experiments which are deconvoluted with the help of the single LC/^{1}H-NMR spectra obtained on-line. This aspect will be shown for the structure determination of various unstable iridoid derivatives.

Finally LC/UV/MS and LC/NMR are also very efficient for the recording of spectra in crude reaction mixtures at the microgram level. An example of this application will be illustrated here with the on-line absolute structure configuration determination of natural products via their Mosher's esters.

4.1. Dereplication of flavanones and isoflavones in crude plant extracts

In our search for new antifungal agents from plant origin, we have screened numerous plant extracts for activity against a plant pathogenic fungus *Cladosporium cucumerinum* with a TLC bioautography method [24]. Several interesting antifungal flavanones and isoflavones have been evidenced by this means. This was the case in particular for two African plants *Erythrina vogelii* (Leguminosae) [25] and *Monotes engleri* (Dipterocarpaceae) [26]. The active extracts of both plants have been analysed by LC/NMR and LC/UV/MS following different strategies and the results are detailed here.

4.1.1. Dereplication of the antifungal constituents of E. vogelii *by LC/UV/MS and on-flow LC/^{1}H-NMR*

In order to rapidly identify its antifungal agents, the crude dichloromethane extract of the roots of *E. vogelii* was analysed by low-flow LC/^{1}H-NMR and by high resolution LC/Q-TOF/MS/MS together with LC/UV with post-column addition of UV shift reagents. For LC/NMR, the use of the low flow mode (0.1 ml/min) was preferred to the standard on-flow mode (1 ml/min) since it was found to give a more sensitive detection of all the main LC peaks. Indeed, it has been demonstrated that the number of acquisitions/increments can be significantly increased in low-flow mode and thus better detection limits can be achieved [27]. A LC-microfractionation was performed just after LC/NMR detection and all peaks collected were submitted to the antifungal bioautography assays against *C. cucumerinum*. By this means, antifungal activity could be efficiently linked to five of the LC peaks. De novo structure determination based on the complementary sets of on-line data obtained was performed for each LC-peak of interest.

4.1.1.1. High resolution LC/UV/APCI-Q-TOF/MS/MS analyses

LC/UV-DAD and positive ion high resolution LC/APCI-MS/MS analyses were carried out by reversed-phase HPLC with a broad MeCN–H_2O gradient on the crude dichloromethane extract of *E. vogelii*. 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 [28].

The LC/UV trace revealed the presence of a dozen 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**, **6**–**9**), three maxima were recorded at around 210, 260 and 290 (sh) nm, for the other group (**3**, **4** and **5**) these bands were present at around 210, 290 and 333 (sh) nm (for structures see Fig. 4). Most of the minor peaks in the chromatogram also presented one of these two types of chromophores.

From the high resolution LC/MS and LC/MS/MS data, the molecular formula could be assigned precisely thanks to the on-line high accuracy measurements performed on a Q-TOF instrument with post-column addition of a reference compound for lock mass. Molecular weights 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, with the exception of compound **6**, revealed the possible presence of prenyl chains. From these preliminary LC/UV/MS results, it could be concluded that the CH_2Cl_2 extract of *E. vogelii*, most probably, consisted of a combination of various prenylated isoflavanones or isoflavones.

4.1.1.2. Low-flow LC/^{1}H-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 HPLC microfractionation, was carried out. 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. A significant amount of crude extract (10 mg) was injected on-column and separation was achieved at low flow rate (0.1 ml/min). At the flow rate chosen, the number of scans per increment was increased to 256 and the limit of detection was consequently lowered. This experiment permitted the acquisition of the LC/NMR spectra of more than ten LC-peaks in the crude extract over 19 h overnight (Fig. 3).

During this LC/NMR analysis, the fractions were collected every 10 min (1 ml) and submitted to bioautography with *C. cucumerinum*. This post-chromatographic antifungal assay revealed that the fractions associated with the LC-peaks eluting at 8.4, 10.8, 11.3, 12.2 and 14.6 h in the low-flow LC/NMR experiment display distinguishable antifungal activities.

4.1.1.3. LC/UV-DAD with post-column derivatisation

A first evaluation of the LC/NMR and LC/MS data revealed that complementary spectroscopic information was lacking for a precise localisation of

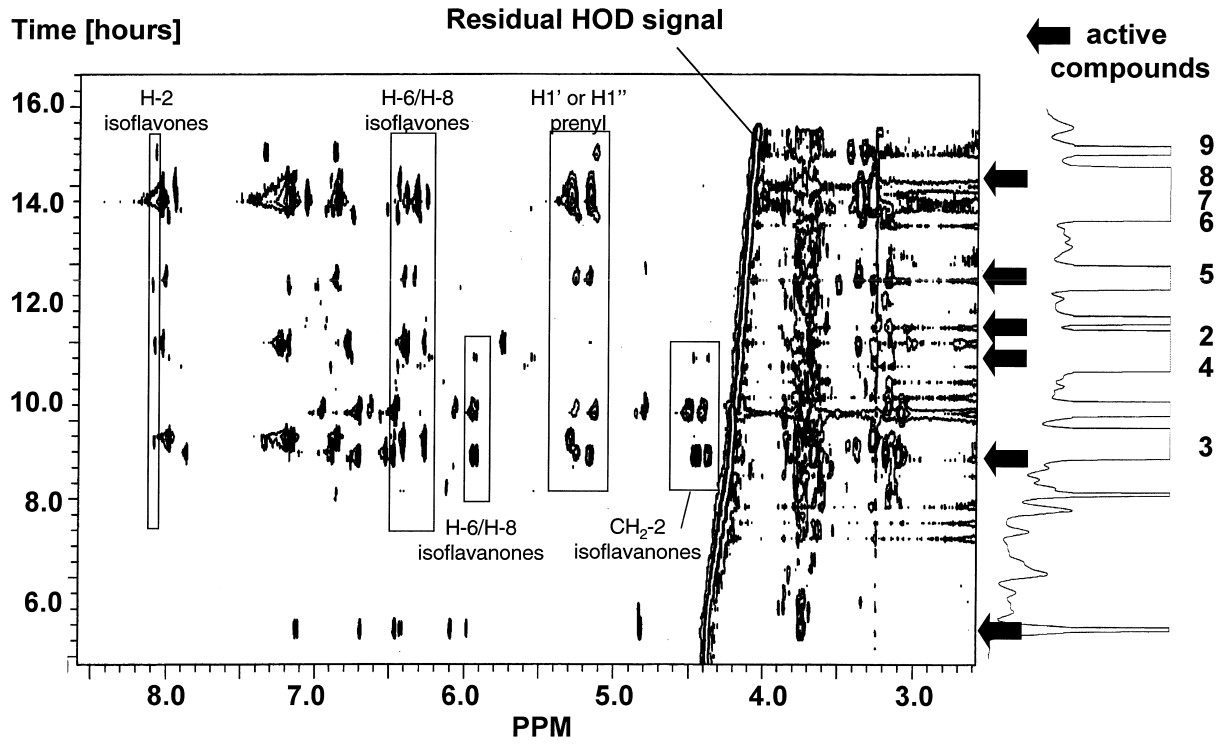

Fig. 3. On-flow LC/NMR contour plot of the crude CH_2Cl_2 extract of *E. vogelii* (Leguminosae). On this contour plot all ^{1}H-NMR resonances of the analytes are clearly visible. A sensitive detection was obtained thanks to the low flow rate used. Several characteristic regions for the resonances of isoflavanones and isoflavones are highlighted. The signals at ~4.5 ppm clearly indicate that the isoflavanones are eluting first on the chromatogram. HPLC conditions: injection, 10 mg; column, μ-Bondapak C_{18} (100×8 mm I.D., 10 μm); MeCN–D_2O gradient (5:95 to 100:0; 19 h); 0.05% TFA; 0.1 ml/min. LC/NMR conditions: 256 scans/increment, flow cell (60 μl, 3 mm I.D.), 500 MHz.

the hydroxyl groups of the different polyphenols detected. Thus, LC/UV-DAD analyses of the extract with post-column addition of UV shift reagents was performed according to a protocol already tested for flavonoids and xanthones [29,30]. The crude extract was analysed twice, once after post-column addition of a weak base and then with acidic $AlCl_3$. For each peak, the shifted DAD-UV spectra were superimposed on the original spectra and compared (see UV spectra of **2** and **4** in Fig. 5). The shifts observed were interpreted according to the rules previously established for the analysis of pure polyphenols [31]. These analyses have permitted efficient localisation of the position of the hydroxy groups on most of the compounds detected. The results are discussed in the next section together with the interpretation of the LC/^{1}H-NMR and LC/MS data.

4.1.1.4. De novo structure determination based on on-line data

The ^{1}H-NMR data extracted from the low-flow LC/NMR analyses confirmed that all the major constituents of the crude extract of *E. vogelii* were prenylated isoflavones and isoflavanones (Fig. 4). In all peaks, aromatic signals between 5.9 and 6.4 ppm were indicative of an 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 **3**, **4** and **5**, methylene protons at 4.39–4.57 ppm were characteristic for an isoflavanone nucleus while for peaks **2** and **6**–**9**, the presence of an aromatic singlet at ~8 ppm was indicative for isoflavones. This was also confirmed by the presence of two distinct types of chromophores for each group of constituents in the

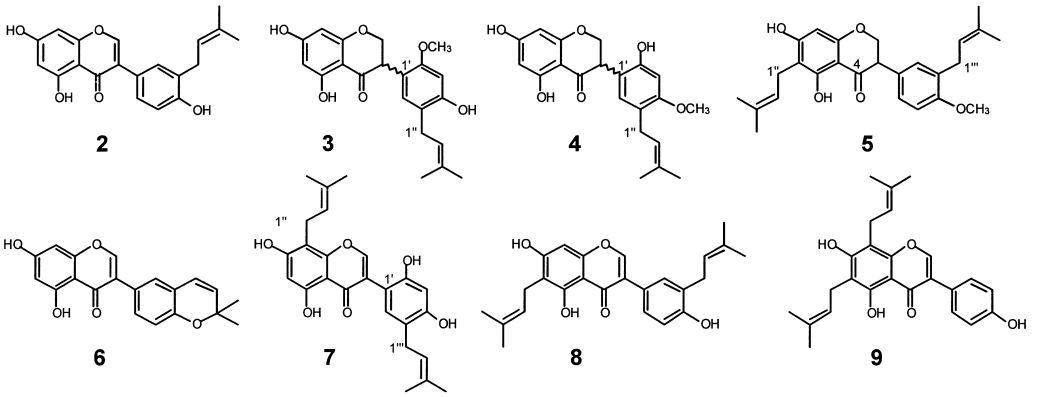

Fig. 4. Structure of the isoflavones and isoflavanones identified on-line in the crude CH_2Cl_2 extract of *E. vogelii* (Leguminosae).

UV-DAD spectra recorded (see **2** and **4** in Fig. 5). Signals of prenylated groups were detected between 3.0 and 3.3 ppm (H-1″), 5.1 and 5.3 ppm (H-2″) and 1.5 and 1.6 ppm (2 CH_3) for all compounds except **6**. These diagnostic signals were already observable on the contour plot of the on-flow LC/NMR experi-

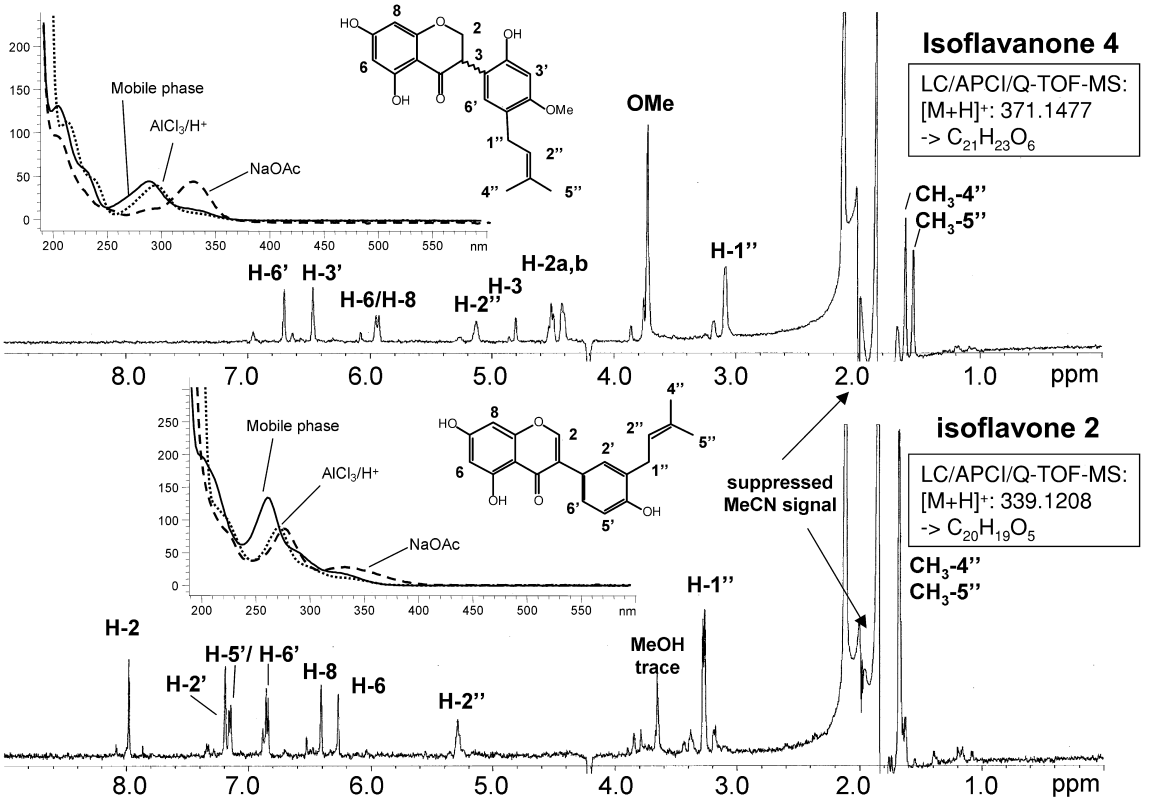

Fig. 5. On-flow LC/NMR spectra of an isoflavanone (**2**) and an isoflavone (**4**). Complementary UV-DAD and shifted UV-DAD spectra recorded on-line as well as exact molecular formula assignments based on LC/APCI-Q-TOF-MS data are also displayed. Same LC/NMR conditions as in Fig. 3.

ment and most of the characteristic resonances are highlighted in Fig. 3. Isoflavone **2** presented the strongest zone of inhibition in the post-chromatographic antifungal bioautography assays. The high resolution LC/APCI-Q-TOF-MS spectrum of **2** exhibited a protonated molecule at m/z 339.1208 $[M+H]^+$ which indicated a molecular formula of $C_{20}H_{19}O_5$. The fragments at m/z 283.0667 $[M\text{-}56]^+$ and m/z 271.0683 $[M\text{-}68]^+$ were characteristic of losses of C_4H_7 or C_5H_9 from one prenyl moiety. From the molecular formula, it could be deduced that three hydroxyl groups were also present on the isoflavone nucleus. The LC/^{1}H-NMR spectrum showed two singlets at δ 6.26 and 6.40 attributable to two aromatic protons (H-6 and H-8). Three signals corresponding to an ABX system at δ 6.85($J=8.0$), 7.14 ($J=8.0$) and 7.19 (br s), were attributed to the B-ring aromatic protons H-5′, H-6′ and H-2′, respectively. 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 and the MS/MS experiments. The 5,7-dihydroxylation of the A-ring was deduced from the UV shifts observed upon addition of $AlCl_3$ and NaOAc [31]. 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′ (Fig. 5). In the CID MS/MS spectra obtained at a collision energy of 40 eV, 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 [28] (Fig. 5). All these data suggested that **2** was isowighteone, a known prenylated isoflavone which was dereplicated by this means.

Another compound of the extract (**4**) showed a UV spectrum and ^{1}H-NMR signals of a three-proton spin system at δ 4.39 (H-2a) 4.47 (H-2b) and 4.80 (H-3) typical of an isoflavanone nucleus. The protonated molecule at m/z 371.1477 $[M+H]^+$ suggested a molecular formula of $C_{21}H_{22}O_6$ for **4** and consequently, a substitution by 3 OH, 1 OMe and 1 prenyl was deduced. The presence of the methoxyl group was confirmed by a singlet at δ 3.66 (3H) in the LC/NMR spectrum.

As in the case of **2**, UV shifted and MS/MS data confirmed the presence of a 5,7-dihydroxylation of A-ring. The two broad singlets corresponding to H-6 and H-8 were however slightly shifted high field compared to **2** at δ 5.93 and 5.95. In the LC/^{1}H-NMR spectrum, two broad singlets at δ 6.47 and 6.70 were attributed to the aromatic protons of the B-ring (Fig. 5). The position of the prenyl unit and that of the methoxyl group could not however be precisely assigned based on ^{1}H-NMR data only since the resolution of the aromatic signals did not allow *meta* and *para* coupled protons to be distinguished from each other. 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 4′. It was not possible to ascertain fully the structure on these indications only. However, this information suggested that it was very possible that **4** was a new natural product and its targeted isolation was undertaken. Its structure was confirmed to be 5,7,2′-trihydroxy-4′-methoxy-5′-(3-methylbut-2-enyl)-isoflavanone [32].

By following the same approach, eight polyphenols were thus identified partially or totally on-line in the crude extract of *E. vogelii* [25].

4.1.2. Stop-flow LC/^{1}H-NMR with 2D correlation experiments for the structure elucidation of a flavanone of M. engleri

As for *E. vogelii*, the dichloromethane extract of *M. engleri* (Dipterocarpaceae) was investigated for its antifungal properties [26]. In this case the antifungal activity could be linked to the main constituents (**10**) detected on the HPLC/UV chromatogram (Fig. 6).

The UV spectrum of this compound was characteristic of a flavanone and its LC/TSP-MS spectrum showed a protonated molecule $[M+H]^+$ at m/z 357 which exhibited a loss of 68 Da ($[M+H\text{-}C_4H_8]^+$), due to an O-prenyl substituent (Fig. 6). Based on these data and according to the molecular weight of a flavanone skeleton, it could be deduced that **10** was most probably a flavanone with one O-prenyl unit and three hydroxyl substituents. In order to ascertain the on-line structure assignments made for this compound, a stop-flow LC/^{1}H-NMR experiment

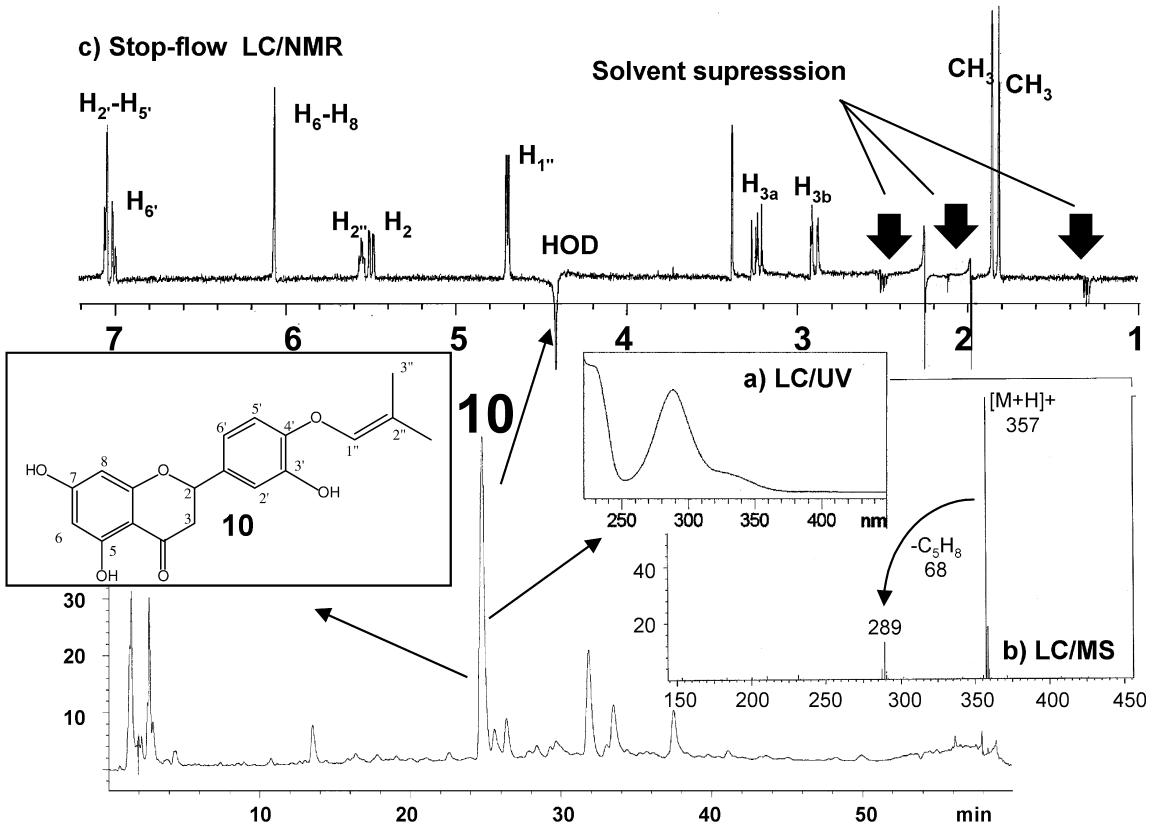

Fig. 6. LC/UV chromatogram (254 nm) of the crude CH_2Cl_2 extract of *M. engleri* (Dipterocarpaceae). Insert: (a) UV spectrum of the flavanone **10**; (b) LC/TSP-MS spectrum of **10**; (c) stop-flow LC/^{1}H-NMR of **10**. HPLC conditions: injection, 1 mg; column, Nova-Pak C_{18} (150×3.9 mm I.D., 4 μm); MeCN–D_2O gradient (20:80 to 70:30 in 50 min); 1 ml/min. LC/NMR conditions: 24 scans/increment, flow cell (60 μl, 3 mm I.D.), 500 MHz (© 2003, John Wiley & Sons Limited. Reproduced with permission) [7].

was performed on the LC/peak of interest in an enriched fraction (Fig. 6). Interpretation of the signals recorded in the stop-flow LC/^{1}H-NMR confirmed that **10** was a prenylated flavanone. Two methylene protons at δ 2.9 and δ 3.2 ppm suggested the presence of a γ-dihydropyrone ring. The deshielded doublet for the methylene of the prenyl unit at δ4.7 ppm confirmed the O-connection of the prenyl unit, as deduced from the LC/MS data. These LC/^{1}H-NMR data were however not sufficient for a complete identification and the position of the prenyl unit could not be deduced. 2D-stop-flow LC/NMR correlation experiments were thus performed in complement. In the stop-flow mode indeed, ^{1}H–^{1}H correlations such as COSY or NOESY can be measured and information on ^{13}C can be extracted from ^{1}H–^{13}C correlation experiments such as HSQC or HMBC, provided that the quantity of compound is high enough (typically >100 μg for a 500-MHz instrument) [7]. The LC/GDQCOSY (gradient double quantum filtered COSY) confirmed the coupling of the different protons especially between H-2 and H-3 in the C-ring and H-1″ and H-2″ on the prenyl chain. The LC/GHSQC (gradient heteronuclear single quantum coherence) showed all C–H connectivities. In the LC/GHMBC (gradient heteronuclear multiple quantum coherence) the long-range couplings of the γ-dihydropyrone ring were visible and the position of the prenyl unit on the B-cycle could be determined. The combination of the data obtained with these two inverse ^{1}H–^{13}C correlation experiments allowed assignment of all the ^{13}C resonances of **10** on-line, directly in the LC eluent [26]. The recording of these data was however possible only

because of the very high concentration of **10** in the LC/NMR flow cell. In order to determine the exact position of the prenyl unit on the flavanone skeleton of **10**, a 2-D LC/WET-NOESY (2-D nuclear Overhauser effect) experiment was undertaken (Fig. 7). The sensitivity was good enough for the observation of clear NOE effects. However, the weak resolution of the ^{1}H-pattern of the B-cycle in the MeCN–D_2O solvent system did not permit the differentiation of H-2′ and H-5′, preventing the full structural assignment of **10** (Fig. 7a). A second stop-flow LC/NMR analysis of the active fraction was then performed in MeOH:D_2O. In this latter solvent system, the three aromatic protons of the B-cycle were observed at distinct chemical shifts. Two doublets ($J = 7.8$ Hz) at δ 6.90 and δ 6.95 were attributable to the *ortho*-coupled protons H-6′ and H-5′, and one broad singlet at δ 6.97 was due to H-2′. A second WET-NOESY was performed in this latter solvent system (Fig. 7b). First, an irradiation of H-2 gave enhancement of the two H-2′ and H-6′ aromatic protons at δ 6.97 and δ 6.90. A correlation with the H-2 and the two protons on C-3 was also shown (Fig. 7). Secondly, irradiation of H-1″ gave enhancement of the *ortho*-coupled proton at δ 6.95 (H-5′). Other NOE effects were also measured on the prenyl unit, particularly between H-1″ and H-2″, H-1″ and H-5″, as well as between H-2″ and H-4″ (Fig. 7). According to these observations, the attachment of the O-prenyl substituent at position C-4′ was confirmed and **10** could thus be identified on-line as 2,3-dihydro-5,7-dihydroxy-2-[3-hydroxy-4(3-methyl-2-butenyl)oxyphenyl]-4*H*-1-benzopyran-4-one. It was found to be a new natural product and its complete isolation was finally performed for the measurement of its physical data, as well as the determination of its absolute configuration. Pure compound **10** showed a negative optical rotation

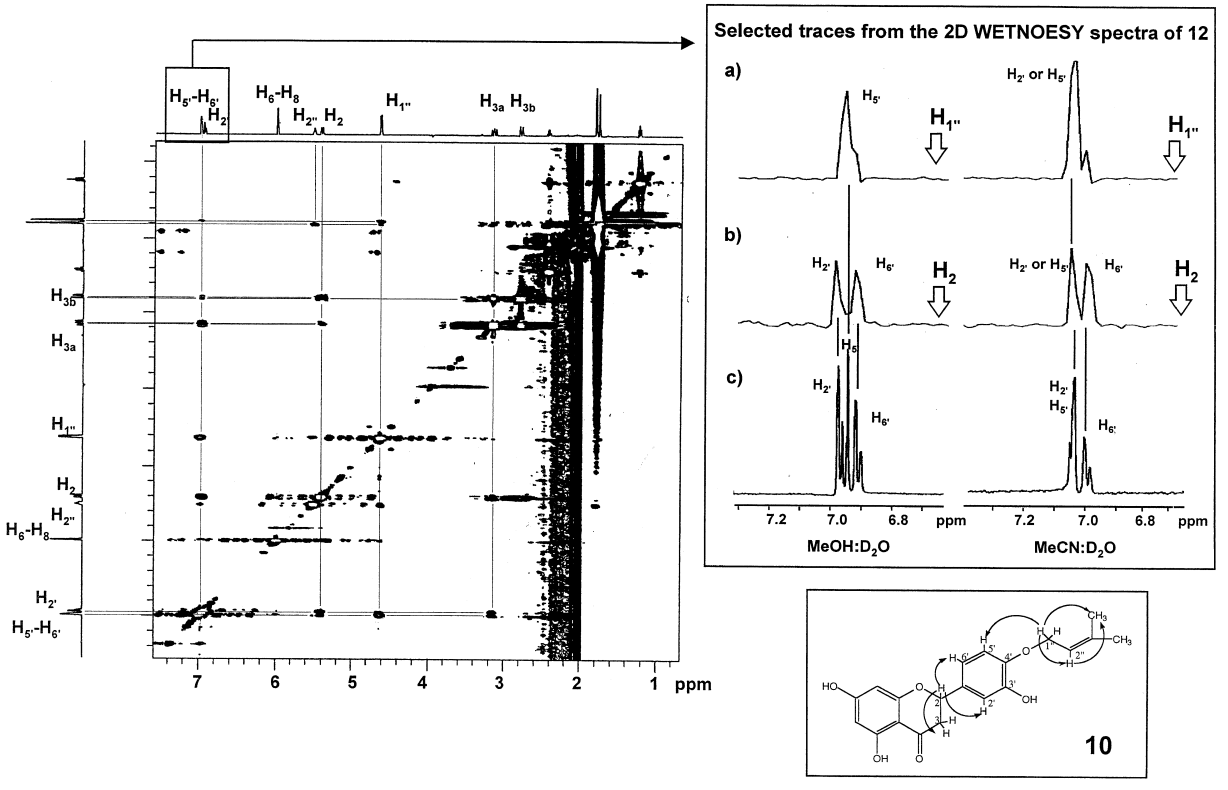

Fig. 7. Stop-flow LC/WET-NOESY spectrum of the flavanone **10**. A 1-mg sample of the enriched fraction injected, solvent MeOH–D_2O. Insert: Selected traces showing the main NOE correlations in both MeCN–D_2O and MeOH–D_2O solvent systems. LC/WET-NOESY: transients, 8; increments, 256; time, 2.5 h; quantity injected, 1 mg (© 2003, John Wiley & Sons Limited. Reproduced with permission) [7].

($[\alpha_D] = -24.7$), and the absolute configuration at carbon C-2 has been determined by CD experiments [26].

It should be noted however that absolute configuration determination is possible on-line by HPLC-CD. Bringmann and co-workers [12] have demonstrated with on-line structural investigation of an isoquinoline and a naphthylisoquinoline alkaloid from the crude extract of a Dioncophyllaceae species *Habropetalum dawei* that 2-D LC/ROESY and LC/TOCSY allows their relative configuration to be determined. The absolute configuration was determined on-line by subsequent stop-flow HPLC-CD experiments on these compounds in the extract and by empirical analysis of their CD data.

4.2. Combination of LC/NMR and LC/MS with in-mixture NMR experiments for the investigation of labile constituents

Beside their use as tools for the dereplication of natural products in crude plant extracts, LC/NMR and LC/UV/MS can also be very useful for the structural investigation of labile products in simple fraction in combination with standard in-mixture NMR measurements.

4.2.1. On-line characterisation of unstable cinnamic ester derivatives in Jamesbrittenia fodina

A preliminary metabolite profiling study performed with LC/UV-DAD and LC/ESI-MS on the methanolic extract of an African Scrophulariaceae species *Jamesbrittenia fodina* revealed the presence of a common cinnamic ester derivative verbascoside as well as other unknown compounds of the same type [33]. Based on these first chemical screening results, attention was focused on two pairs of isomeric compounds **11a/11b** and **12a/12b** displaying characteristic UV spectra for cinnamic esters and having protonated molecules at m/z 699 $[M+H]^+$ and m/z 669 $[M+H]^+$, respectively, together with intense dehydrated ions at m/z 681 and m/z 651 (Fig. 8). They could not be identified directly based on the LC/UV/MS analysis and their isolation was undertaken. The purification steps led to two enriched fractions each containing one pair of isomeric peaks. These isomers were well separated by semi-preparative HPLC, but it was noticed that the resulting fractions still contained the two isomeric peaks in the same proportions. These results suggested that these constituents were probably unstable upon drying of the fraction.

In order to obtain more information about these unstable compounds, stop-flow LC/NMR analyses of fractions **11a/11b** and **12a/12b** were performed. The LC/^{1}H-NMR spectra of compounds **12a** and **12b** showed many similarities (Fig. 9). Both isomers displayed two multiplets between δ 2.20 and δ 2.60, and a number of signals from δ 3.20 to δ 5.20 suggesting the presence of two sugars. Moreover, the presence of a methyl group at about δ 1.1 indicated that one of the sugars might be a rhamnose. Integration of the signal at δ 3.82 for **12a** showed the presence of three protons and thus suggested the presence of a methoxyl group. Seven aromatic protons were observed between δ 5.8 and δ 7.8. Six of these were attributed to a cinnamic acid moiety, the presence of which had already been suggested by the DAD-UV spectra: two signals corresponding to four *ortho*-coupled protons at δ 7.01 (2H, δ, $J = 8.8$ Hz) and δ 7.61 (2H, δ, $J = 8.2$ Hz) in **12a** and δ 6.89 (2H, δ, $J = 8.8$ Hz) and δ 7.50 (2H, δ, $J = 8.8$ Hz) in **12b** could be attributed to a 1,4-disubstituted aromatic ring. The striking difference between the spectra of **12a** and **12b** concerned the signals attributed to the cinnamoyl double bond: δ 7.72 (1H, δ, $J = 15.9$ Hz) and δ 6.42 (1H, δ, $J = 15.9$ Hz) were characteristic of a *trans* double bond in the case of **12a**, whereas in **12b**, the two corresponding doublets at δ 7.04 (1H, δ, $J = 12.6$ Hz) and δ 5.84 (1H, δ, $J = 12.6$ Hz) were typical of a *cis* double bond (Fig. 9).

The fraction containing **11a** and **11b** was submitted to the same analysis and similar deductions could be made. The difference between **11a/11b** and **12a/12b** was related to the presence of one additional methoxyl in the case of **11a/11b.**

The LC/NMR spectra thus suggested that the two fractions consisted of mixtures of *cis*- and *trans*-cinnamoyl glycoside derivatives. Based on these LC/^{1}H-NMR spectra it was not possible, however, to deduce the complete structure of both pairs of constituents because information on the core nucleus of these molecules was lacking. The mixtures of compounds **11a/11b** and **12a/12b** were thus submitted to classical 1D and 2D NMR analysis re-

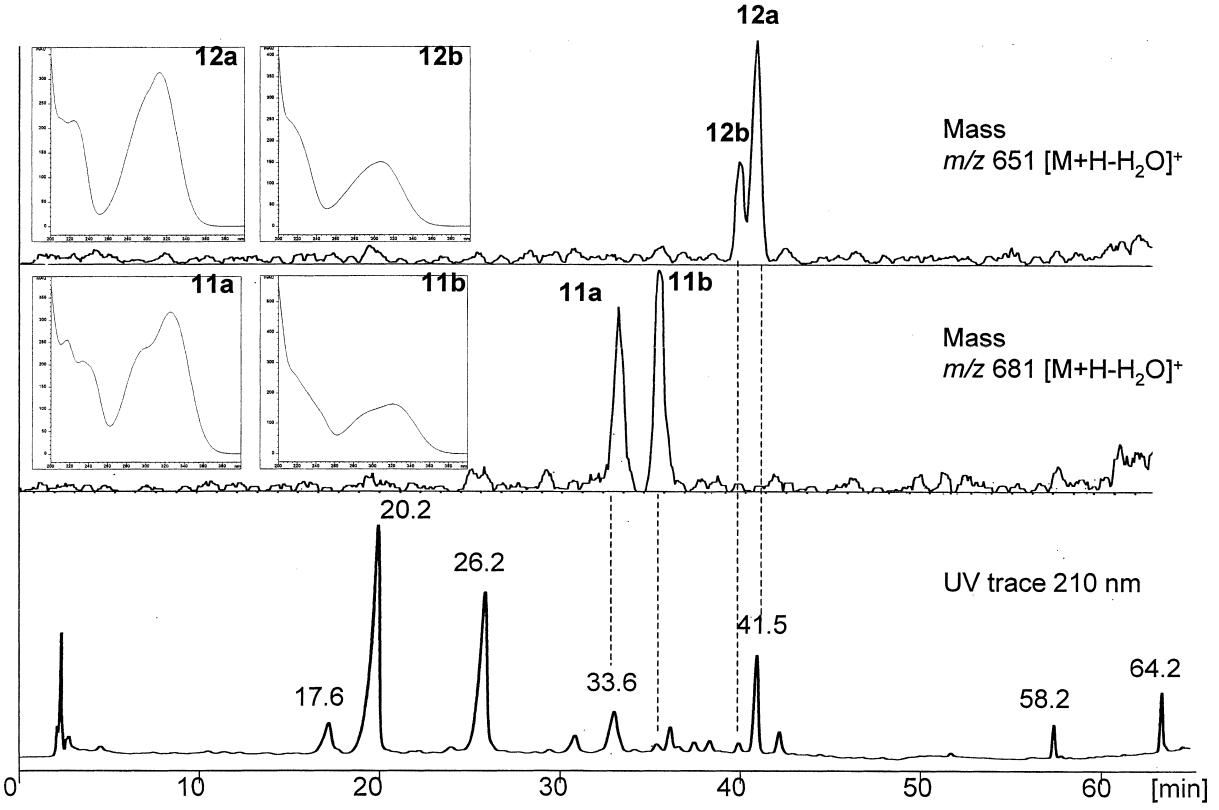

Fig. 8. LC/UV/ESI-MS of the methanol extract of *Jamesbrittenia fodina* (Scrophulariaceae). HPLC conditions: injection, 50 μg; column, Symmetry C_{18} (250×3.9 mm I.D., 5 μm); MeOH–H_2O gradient (28:72 to 46:54 in 30 min, 46:54 to 62:38 in 16 min, 62:38 to 100:0 in 14 min); 1 ml/min. UV spectra were recorded between 200 and 400 nm (© 2003, John Wiley & Sons Limited. Reproduced with permission) [33].

corded in CD_3OD. Interpretation of the 2D NMR in-mixture experiments (gCOSY, gHMBC and gHSQC) permitted the core nucleus of these molecules to be assigned to catalpol and the HMBC correlation proved the attachment positions of the sugars and cinnamoyl moieties [33].

Based on the on-line and in-mixture experiments the structures of **12a** and **12b** were established to be *trans* and *cis* forms of 6-*O*-[4-*O*-(4-methoxycinnamoyl)-α-L-rhamnopyranoside]catalpol, and **11a** and **11b** were identified as *trans* and *cis* forms of 6-*O*-[4-*O*-(3,4-dimethoxycinnamoyl)-α-L-rhamnopyranoside]catalpol, respectively. The *trans* forms of these compounds have been described in other members of the Scrophulariaceae and Buddlejaceae families but there has been no mention of the *cis* forms. We have evidenced the same type of instability using a similar approach for *p*-coumaric esters of dimeric secoiridoid glycosides in a Panamaean Gentianaceae species *Lisianthius seemannii* [34]. This chemical instability can probably be explained by a light-induced *cis*/*trans* isomerisation as this was recently postulated for *p*-coumaric acid derivatives [35].

Other iridoids in the same plant revealed the existence of an additional type of instability related to transacylation reaction. The same approach based on stop-flow LC/^{1}H-NMR and in-mixture 2D NMR has enabled their unambiguous determination [36].

4.2.2. Study of epimerisation reactions

In our on-going search for antifungal compounds from higher plants, the most promising lead compound has been discovered in the root bark of a tree

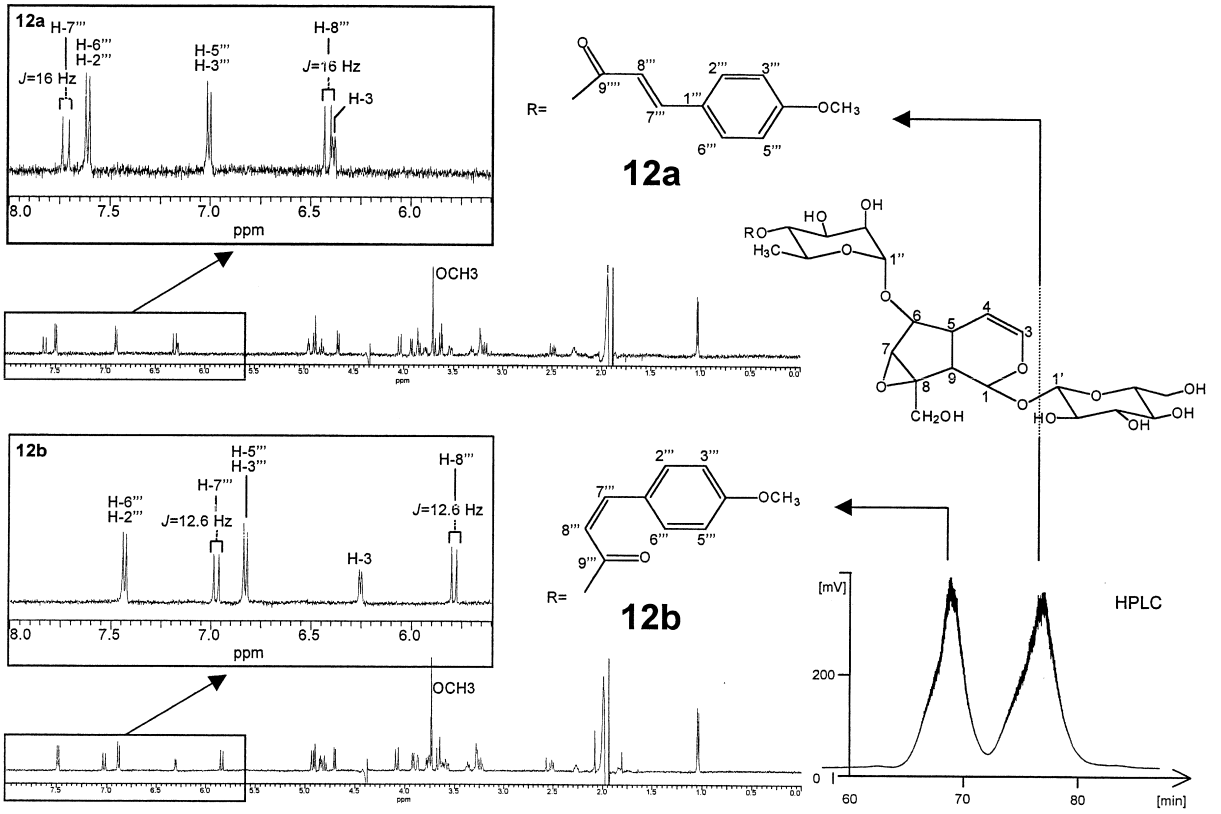

Fig. 9. Stop-flow LC/NMR spectra of the unstable catalpol derivatives **12a** and **12b** (in MeCN–D_2O). LC/NMR conditions: 256 scans, flow cell (60 μl, 3 mm I.D.), 500 MHz (© 2003, John Wiley & Sons Limited. Reproduced with permission) [33].

from Zimbabwe, *Bobgunnia madagascariensis* (Fabaceae), previously known as *Swartzia madagascariensis* [37]. Its root bark indeed contained pale yellow metabolites that strongly inhibited growth of both *Candida albicans* and *C. cucumerinum* in the TLC bioautography assays. Various compounds of the dichloromethane extract were responsible for this activity. After a preliminary dereplication by LC/UV and LC/MS which indicated that it was very possible that the main active compound of this extract was a new natural product, a targeted isolation was carried out. As suspected from the on-line data, a new type of natural product, a ''quinone methide'' diterpene with a cassane skeleton was isolated from this plant [37]. Two other antifungal compounds of this extract, **13** and **14**, could however not be isolated at the preparative scale. These compounds were probably two isomers which were interconverting in solution. In order to determine the structure of these compounds, on-flow and stop-flow LC/NMR experiments were performed on the enriched fraction containing both isomers [38]. The on-flow LC/NMR analysis demonstrated that almost no difference between the signals of **13** and **14** was detected. Indeed as shown on the 2-D plot in Fig. 10, all the signals in the high field region of the NMR dimension (1.0–4.5 ppm) were common for both compounds proving their important similarity. However, the signals detected in the 5.5–7.0-ppm region differ significantly between these two compounds; two singlets appeared at 5.9 and 6.5 ppm for **13** while they were shifted to 5.8 and 6.7 ppm in the case of **14**.

A stop-flow LC/NMR analysis performed on compound **14** also demonstrated very clearly the interconversion of both constituents in solution. The

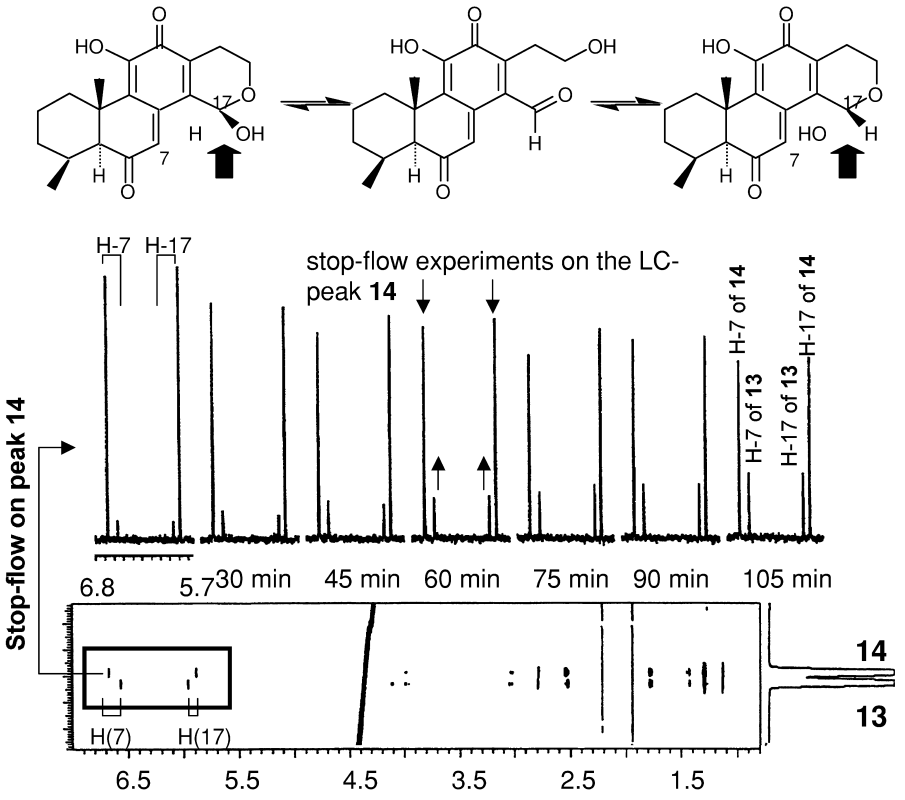

Fig. 10. Bidimensional LC/^{1}H-NMR chromatogram of the fraction containing **13**/**14** from *Bobgunnia madagascariensis* (Leguminosae). The signals of both products were identical except for those found in the 5.7–6.8-ppm region. Stop-flow LC/NMR spectra (expansion of the 5.7–6.8-ppm region) performed on peak **14** after different delays. HPLC: injection, 300 μg; column, NovaPak C_{18} (250×4.6 mm I.D., 4 μm); MeCN–D_2O gradient (25:75 to 55:45 in 45 min); 1 ml/min. LC/NMR conditions: 24 scans/increment, flow cell (60 μl, 3 mm I.D.), 500 MHz (© 2003, John Wiley & Sons Limited. Reproduced with permission) [7].

LC separation was stopped exactly when peak **14** was eluting in the LC/NMR cell. The first stop-flow LC/NMR spectrum of this peak obtained after 15 min of stabilisation showed that **14** was indeed the major constituent in this peak but already small signals of **13** were observable (Fig. 10). A comparison of the integration of the signals of **13** and **14** indicated that **14** was present with more than 93% in the LC/NMR flow cell after 15 min. This stop-flow experiment was maintained for 2 h and spectra were recorded every 15 min. As displayed on the expansion of the stop-flow spectra in Fig. 10, the signals of compound **13** were increasing while those of **14** were decreasing with time. The ratio of **13** reached one third of that of **14** after 105 min proving that indeed these two compounds were interconverted in solution. The sum of the integrals of H-7 and H-17 of **13** and **14** remains constant during the all experiment demonstrating also that no other compound was formed.

As in the case of the unstable iridoids of *J. fodina*, the structure of **13** and **14** was finally determined based on standard 1-D and 2-D NMR correlation experiments performed in-mixture taking into account the attribution of individual signals of **13** and **14** based on LC/^{1}H-NMR spectra.

A careful study of the HMBC, DQCOSY and NOE correlations allowed the attribution of an hemiacetal function (–CH_2–CH_2–O–C*HOH–) between C-13 and C-14. The presence of this hemiacetal group also explained the equilibrium which was observed between **13** and **14**. Indeed, a new

asymmetric center was present at C-17 and the instability of **13** and **14** in solution was due to the epimerisation of this chiral position. The diastereoisomers **13** and **14** could thus be interconverted by ring opening which yielded an hydroxyaldehydic form followed by a new cyclisation (Fig. 10). **13** and **14** were found to be new natural products and both isomers had important antifungal properties [38].

As shown in these last examples, LC/NMR is not only applicable as an addition to metabolite profiling, but it is also a powerful tool for the study of compounds which cannot be not isolated or are not stable in a pure form.

4.3. On-line absolute configuration determination

Determination of the absolute configuration remains a challenging task in the structure elucidation of natural products. For on-line determination, LC/CD may provide interesting information and different studies have proven its efficacy as mentioned for isoquinoline alkaloids [12].

Classically chemical methods, such as Mosher's ester synthesis, have been frequently used for the characterisation of various isolated natural products bearing secondary alcohol functions [39]. In this case, the H^1-NMR spectra of (*R*)- and (*S*)-2-methoxy-2-phenyl-(trifluoromethyl) acetic acid (MTPA) ester derivatives of the analytes are compared. The difference in the chemical shifts of the (*S*)-MTPA and (*R*)-MTPA diastereoisomers ($\Delta\delta_H = \delta_S - \delta_R$) indicates whether the alcohol function is (*R*) or (*S*) based on established conformational models [40].

Until recently Mosher's ester synthesis was only used in combination with standard NMR. The recent study of crude reaction mixtures by LC/UV/MS and LC/NMR has proved to be a rapid and sensitive method for absolute configuration determination. This on-line method can be applied to restricted amount of sample or fraction difficult to obtain in a pure form and the absolute configuration can be determined on few micrograms of natural products.

This technique was applied in particular for the determination of the absolute configuration in two α-pyrones **15** and **16** isolated from the root bark of *Ravensara crassifolia* (Lauraceae) [41].

Two aliquots of 50 μg of α-pyrone **15** were esterified with (*R*)- and (*S*)-MTPA. Then 5% of the residual reaction mixture (10 μl) was analysed by LC/ESI-MS under isocratic LC conditions in order to verify the completion of the esterification reaction. The remaining reaction mixture (95%) was then submitted to stop-flow LC/NMR analysis in the same LC isocratic conditions. A fast clean-up of the sample was thus obtained and the LC/^{1}H-NMR spectra of the Mosher's esters were recorded in the stop-flow mode. A preliminary on-flow run demonstrates well the type of clean-up which can be obtained by LC/NMR (Fig. 11). Pyridine, excess of MTPA and traces of starting material eluted rapidly while the ester derivatives eluted between 8 and 12 min. The use of isocratic conditions was found to be essential since precise comparison of the chemical shifts has to be made and changes in solvent composition would hinder such an on-line approach.

Comparison of LC/^{1}H-NMR spectra of the (*R*)-MTPA **15a**, (*S*)-MTPA **15b** derivatives showed significant chemical shift differences for the protons near the MTPA ester (Fig. 12). These diagnostic shifts were used for determination of the absolute configuration. Suppression of the MeCN solvent resonance (δ 2.00) hampered a clear assignment of the signals recorded between δ 1.60 and 2.20. For an unambiguous chemical shift attribution of each proton for both Mosher's esters **15a** and **15b**, ^{1}H–^{1}H WET COSY spectra were recorded. With these 2D correlation experiments, precise chemical shift assignments for H-1′, H-3′ and H-4′ were made possible. Differences of all chemical shifts of (*S*)-MTPA **15a** and of (*R*)-MTPA **15b** were calculated using the formula ($\Delta\delta_H = \delta_S - \delta_R$). Positive δ_H values were recorded for the α-pyrone moiety (C-1′), which was placed on the right side of the Mosher model. Negative δ_H values of C-3′/C-5′ indicated that the alkyl chain had to be placed on the left side of the model (Fig. 12). Finally, the proton geminal to the esterified hydroxyl group was placed in the opposite side of the plane and the absolute configuration at C-2′ was determined using the priority rule of Cahn-Ingold-Prelog. This arrangement indicated a sinistrorse rotation and confirmed the *S* absolute configuration for the asymmetric center C-2′ (Fig. 12).

The same type of LC/NMR analyses were performed on other α-pyrones isolated in the same plant and their absolute configuration was successfully determined [41]. The same approach enabled the

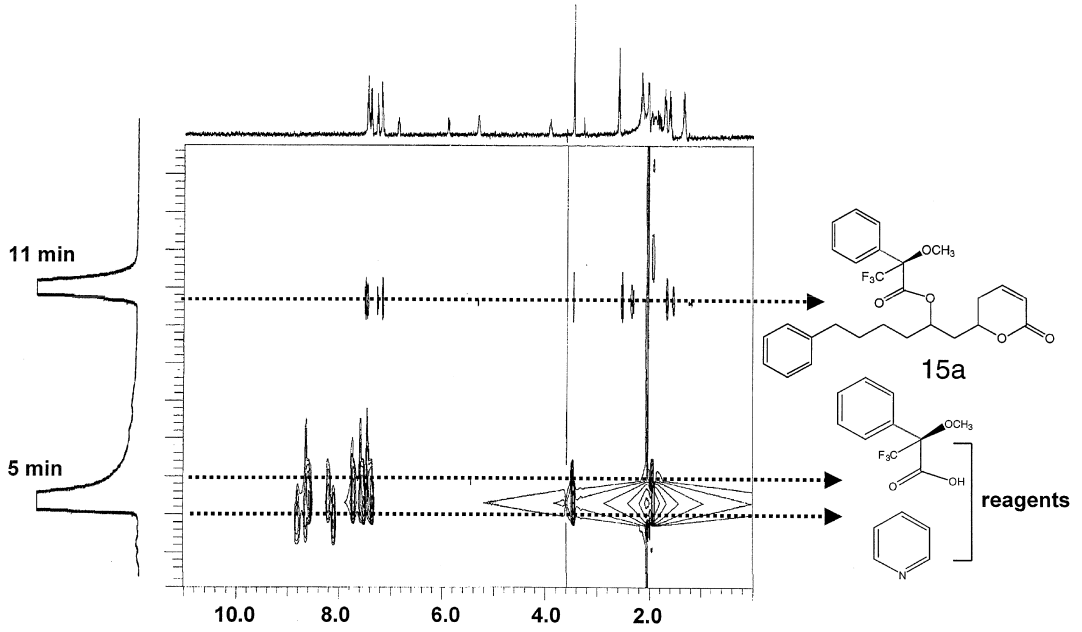

Fig. 11. On-flow LC/NMR contour plot obtained after injection of the crude reaction mixture of the (*R*)-MTPA Mosher's ester **15a**. HPLC: injection, 250 μg; column, μ-BondaPak C_{18} (100×8 mm I.D., 10 μm); MeCN–D_2O (80:20, 35 min); 0.8 ml/min. LC/NMR conditions: 32 scans/increment, flow cell (60 μl, 3 mm I.D.), 500 MHz.

absolute configuration determination of a tetrahydrophenanthrene from *Heliotropium ovalifolium* [42].

5. Conclusion

LC-hyphenated techniques are playing an increasingly important role as a strategic tool to support phytochemical investigations. Indeed, these techniques provide a great deal of preliminary information about the content and nature of constituents of crude plant extracts. This is very useful when large numbers of samples have to be processed because unnecessary isolation of known compounds is avoided. Once the novelty or utility of a given constituent is established, it is then important to process the plant extracts in the usual manner, to obtain samples for full structure elucidation and biological or pharmacological testing. Furthermore, as shown for the investigation of antifungal agents, LC-microfractionation in combination with TLC bioautography permits the localisation the LC-peaks responsible for a given activity; the dereplication process can then be focused mainly on these bioactive peaks.

The combination of LC/UV and LC/MS information can be very helpful in a first step of dereplication, especially when this information is combined with botanical and chemotaxonomical considerations for cross search in natural product databases. This approach is however limited by the unavailability of general LC/MS and LC/MS/MS libraries of spectra. For the on-line de novo structure determination of natural products, LC/NMR plays a key role and allows the recording of precious complementary on-line structure information when LC/UV/MS data are insufficient for unambiguous peak identification. Applications of this new LC detection method are still scarce but without doubt LC/NMR will become more and more widely used in this field. LC/NMR has proven to be very effective in obtaining 1-D spectra on both flowing and non-flowing samples, as well as stop-flow 2-D spectra. However, compared with UV or MS, NMR remains a rather insensitive detection method and the need for solvent suppression has restricted the observable NMR range. Improvements in sensitivity are expected in the years

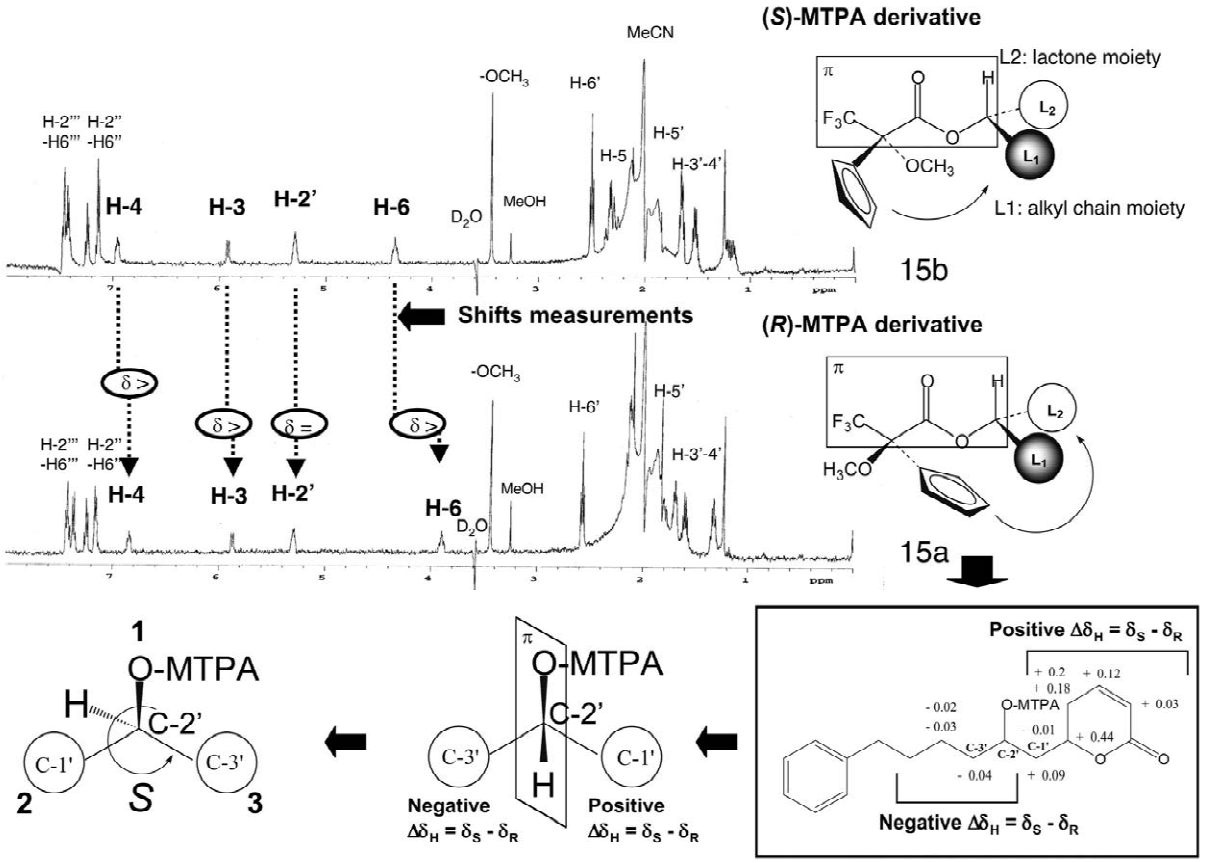

Fig. 12. Stop-flow LC/NMR spectra of the Mosher's esters derivatives **15a** and **15b** (50 μg injected; NT = 128 acquisitions each) and determination of the absolute configuration with the $\Delta\delta_H$ values ($\Delta\delta_H = \delta_S - \delta_R$) of compound **15** using the Mosher method. Same LC/NMR conditions as in Fig. 11 (© 2003, John Wiley & Sons Limited. Reproduced with permission) [41].

to come but the main effort for the efficient use of LC/NMR in phytochemical analysis with the actual technology has to be made on the chromatographic side where strategies involving efficient pre-concentration, high loading, stop-flow, time slicing or low-flow procedures have to be developed. The inability of LC/NMR to provide ^{13}C-NMR spectra with reasonable amounts of sample is still an important limitation of this technique for natural product analysis and for full on-line identification of unknowns. However LC/^{1}H-NMR information may be sufficient for the on-line identification of previously known constituents or for studying derivatives of a known core skeleton which are likely to be present in a given extract.

As shown in the examples presented, LC/NMR also represents an invaluable tool for the investigation of unstable compounds or natural products difficult to isolate at the preparative scale or for absolute configuration determination via derivatisation reactions with minute amount of compounds.

Development of multihyphenated systems are expected in the near future and several groups have now demonstrated the feasibility of such ''total organic analysis devices''. The metabolite profiling of extracts with various hyphenated techniques generates a huge amount of information. In order to rationalise this approach and use it efficiently with a high sample throughput, the next challenge will be to find a way to centralise all these data for rapid pattern recognition with reference to standard compounds. In particular LC/UV and LC/MS/MS or LC/MS^n libraries of spectra have to be built up and shared among natural product chemists. LC/NMR

analysis should be used as a complement for studying in more detail compounds which are not matching in these databases. Such a rational approach will be also of great help for metabolomic studies, a new field of study in plant chemistry, which consists of a comprehensive analysis in which all metabolites of a biological system are identified and quantified [43]. The characterisation of a complete metabolome represents indeed a challenging task since unlike genomics and proteomics, a single analytical technique does not exist that is capable of profiling all the low molecular weight metabolites of a given plant.

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