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High-performance liquid chromatography/mass spectrometric identification of dibenzylbutyrolactone-type lignans: insights into electrospray ionization tandem mass spectrometric fragmentation of lign-7-eno-9,9′-lactones and application to the lignans of *Linum usitatissimum* L. (Common Flax)

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In continuation of our studies into the mass spectrometric detection of natural lignans and their identification in complex mixtures such as crude plant extracts, the electrospray ionization tandem mass spectrometric (ESI-MS/MS) fragmentation of $\Delta^{7,8}$-unsaturated dibenzylbutyrolactone-type lignans (lign-7-eno-9,9′-lactones) was studied in detail. It is demonstrated that the characteristic fragmentation allows unambiguous identification including distinction between constitutional isomers. These lignans containing an α,β-unsaturated lactone structure exist as equilibrium mixtures of *E*- and *Z*-isomers indistinguishable by mass spectrometry, but it is shown that chromatographic retention time can be used to distinguish between the isomeric forms. Based on these observations, re-analysis of the dichloromethane extract obtained from flowering aerial parts of *Linum usitatissimum* L. by high-performance liquid chromatography (HPLC)/ESI-MS/MS led to the identification of eighteen lignans of these types (five lignano- and one lignenolactone previously reported along with five further lignano- as well as seven lignenolactones hitherto unreported for this plant). The simultaneous identification of eighteen different lignans in the complex matrix of a crude plant extract by a single analysis demonstrates the potential of this method, which will certainly lead to new insights into the lignan composition and metabolism of different *Linum* species and many other plants.

Lignans are an important class of phenylpropanoid secondary metabolites with a widespread occurrence in green land plants.[1,2] Currently, approximately 3000 different structures of lignans (dimeric β,β′-linked phenylpropanoids) and related natural products such as neolignans and flavonolignans are known.[3] They have attracted considerable attention because of the diversity of their biological activities and potential pharmacological effects, which parallel their immense structural variation.[2]

Prompted by the need for efficient analytical tools to investigate the full chemical diversity of lignan-producing plants in terms of comparative metabolomic and chemosystematic studies, our group,[4,5] as well as several others,[6,7] have recently applied high-performance liquid chromatography/mass spectrometry (HPLC/MS) methods.

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We have reported on the application of positive ion electrospray ionization mass spectrometry (ESI-MS) which reliably and reproducibly yields characteristic fragmentation patterns that allow the identification of lignans from various classes in a single HPLC/ESI-MS/MS run.[4] It was recently demonstrated that the resolution of very complex mixtures of aryltetralin lignans, their glycosides as well as acylated derivatives, by this method can be used to obtain valuable information with respect to chemosystematics.[5]

In this report, we wish to extend the known fragmentation pathways of lignans to a further structural type, namely, lign-7-eno-9,9′-lactones whose behaviour in ESI-MS has not been studied in detail before.

Furthermore, in order to demonstrate the potential of the method, we applied this information to re-investigate the lignan pattern of an important medicinal and crop plant, *Linum usitatissimum* L. (Common Flax), in which we were able to identify, in addition to six previously known major compounds,[4] twelve other lignans, all hitherto unknown to occur in the aerial parts of this plant.

EXPERIMENTAL

Reference compounds

The structures of all compounds described in this study are shown in Fig. 1. The equilibrium mixture of *E*- and *Z*-7,8-dehydrohinokinin (savinin and isohibalactone, **15a** and **15b**, respectively) and *E*-7,8-dehydroyatein (=nemerosin = *E*-anhydropodorhizol, **11a**) were isolated from *Linum corymbulosum* [8] and from *L. usitatissimum*,[4] respectively, and characterized as described earlier. Re-investigation of **11a** by nuclear magnetic resonance (NMR) in the course of this study revealed that *Z*-anhydropodorhizol (=isochaihulactone) **11b** was present as a minor constituent (^{1}H-NMR, 600 MHz, $CDCl_3$: approx. 20%), so that equilibration had taken place in the same way as described for **15a**/**15b**.[8] The NMR data were identical with those reported[9] in all respects. Deoxypodophyllotoxin **16** was synthesized by Federolf *et al*.[10] Compounds **5**, **6**, **8**–**10** and **11a** were previously isolated in our laboratory from *Linum usitatissimum* and characterized by NMR and optical rotation studies.[4]

HPLC/ESI-MS

All analyses were performed with a Finnigan LCQ Deca XP ion trap mass spectrometer (ThermoFinnigan, Dreieich, Germany) coupled to an Agilent (Waldbronn, Germany) 1100 series HPLC system with a diode-array detector (DAD).[4,5] Separations were achieved with a Knauer (Berlin, Germany) Eurosphere RP C18 column (250 × 2 mm i.d., 5 μm) using methanol/water (containing 0.1% formic acid) for elution in a gradient from 3:7 to 7:3 in 30 min, followed by isocratic elution with 7:3 between 30 and 40 min, a further increase from 7:3 to 100:0 between 40 and 55 min, and finally isocratic elution with methanol from 55 to 65 min. The flow rate was typically 0.4 mL/min.

The following ESI-MS traces were recorded: (1) positive ions from *m*/*z* 100 to 1000, (2) wideband MS/MS of the most abundant ion from (1), (3) negative ions from *m*/*z* 100 to 1000, and (4) wideband MS/MS of the most abundant ion from (3).

The four different modes were cycled through once every second. For the MS/MS spectra, the normalized collision energy was set at 35%. The capillary temperature was set at 285°C, and the source voltage was 5 kV. Simultaneously, the UV absorbance was monitored between 210 and 400 nm with the DAD.

Plant material

Aerial parts of flowering *L. usitatissimum* L. were cultivated and collected at the Botanical Garden, University of Düsseldorf, Germany, in summer 2003. A voucher specimen is kept at the Institut für Pharmazeutische Biologie und Phytochemie, Münster, Germany (voucher No TS_LU_01). The plants were freeze-dried and stored in the dark at ambient temperature until extraction.

Extraction and sample preparation

Plant material (204 g) was powdered and extracted exhaustively with CH_2Cl_2 (Soxhlet). The resulting solution was evaporated to dryness under reduced pressure yielding 13.8 g of dry extract. An aliquot representing 1.0 g dried plant material (68 mg) was reconstituted in 1.0 mL MeOH and sonicated for 15 min. The non-soluble part was removed by centrifugation. The supernatant was submitted to HPLC/MS analysis.

Physical and spectral data

The HPLC retention times and ESI-MS data for all lignans identified in this study are reported in Tables 1 and 2. Ion chromatograms and ESI-MS and ESI-MS/MS spectra of all compounds are provided as Supporting Information.

RESULTS AND DISCUSSION

Lignano-9,9′-lactones (normal, i.e. saturated dibenzylbutyrolactones), as well as various other types of lignans, were previously reported by us to show a clear and well-defined fragmentation behaviour under ESI-MS/MS.[4] It was also shown that the structural assignments based on the mass spectra were very reliable, since the major lignans detected by ESI-MS in *L. usitatissimum* and *L. bienne* were subsequently isolated and all the structures were unambiguously confirmed by NMR analyses.[4] In this previous study, two lign-7-eno-9,9′-lactones ($\Delta^{7,8}$-unsaturated dibenzylbutyrolactones) were also included, namely, savinin **15a** and *E*-anhydropodorizol **11a**, but their fragmentation was not analyzed in detail at that time. It has been reported that **15a**, the 7,8-dehydro derivative of hinokinin **5**, cannot be maintained in pure form in solution since an equilibration between the *E*- and the *Z*-configured forms is observed.[8] Reinvestigation of **15a** (isolated from *L. corymbulosum* [8]) and of **11a** (isolated from *L. usitatissimum* [4]) by HPLC/ESI-MS/MS revealed that in both cases two peaks in a ratio of approximately 8:2 are detectable, the larger eluting at lower, the smaller at higher retention time on RP 18 material. The major component in both cases represents the *E*-isomer. In the case of the isolate from *L. corymbulosum*, this isomer was identified as the dominant form in the NMR spectra.[8] In case of the isolate from *L. usitatissimum*,[4] re-analysis by NMR spectroscopy led to unambiguous identification of the major (**11a**) and minor constituent (**11b**) as the *E*- and *Z*-isomer, respectively, in comparison with the data reported for nemerosin and isochaihulactone.[9]

The ESI mass spectra of both isomers are essentially identical in each case, and the fragmentation differs conspicuously from that of the saturated congeners. Figure 2 shows the positive ion ESI-MS and ESI–MS/MS spectra for the two isomeric forms in the case of **11a** (major constituent, identified by NMR as *E*-anhydropodorhizol[4] = nemerosin[9]) and **11b**. As the most pertinent difference from the mass spectrum of the saturated lactone yatein **9** (see Fig. S3, Supporting Information), the base peak is represented by an ion at *m*/*z* 231, for which no corresponding product ion is observed in **9** and which is not explained by the common fragmentation pattern established for dibenzylbutyrolactones.[4] The same ion is also detected in **15a** and **15b** but no corresponding ion occurs in **5** (Figs. S14 and S5, respectively, Supporting Information). The mass of this ion corresponds to the loss of the trimethoxybenzene moiety from **11a**/**11b** and of a methylenedioxybenzene moiety from **15a**/**15b**. In both cases, moreover, a further abundant product ion at *m*/*z* 203 is

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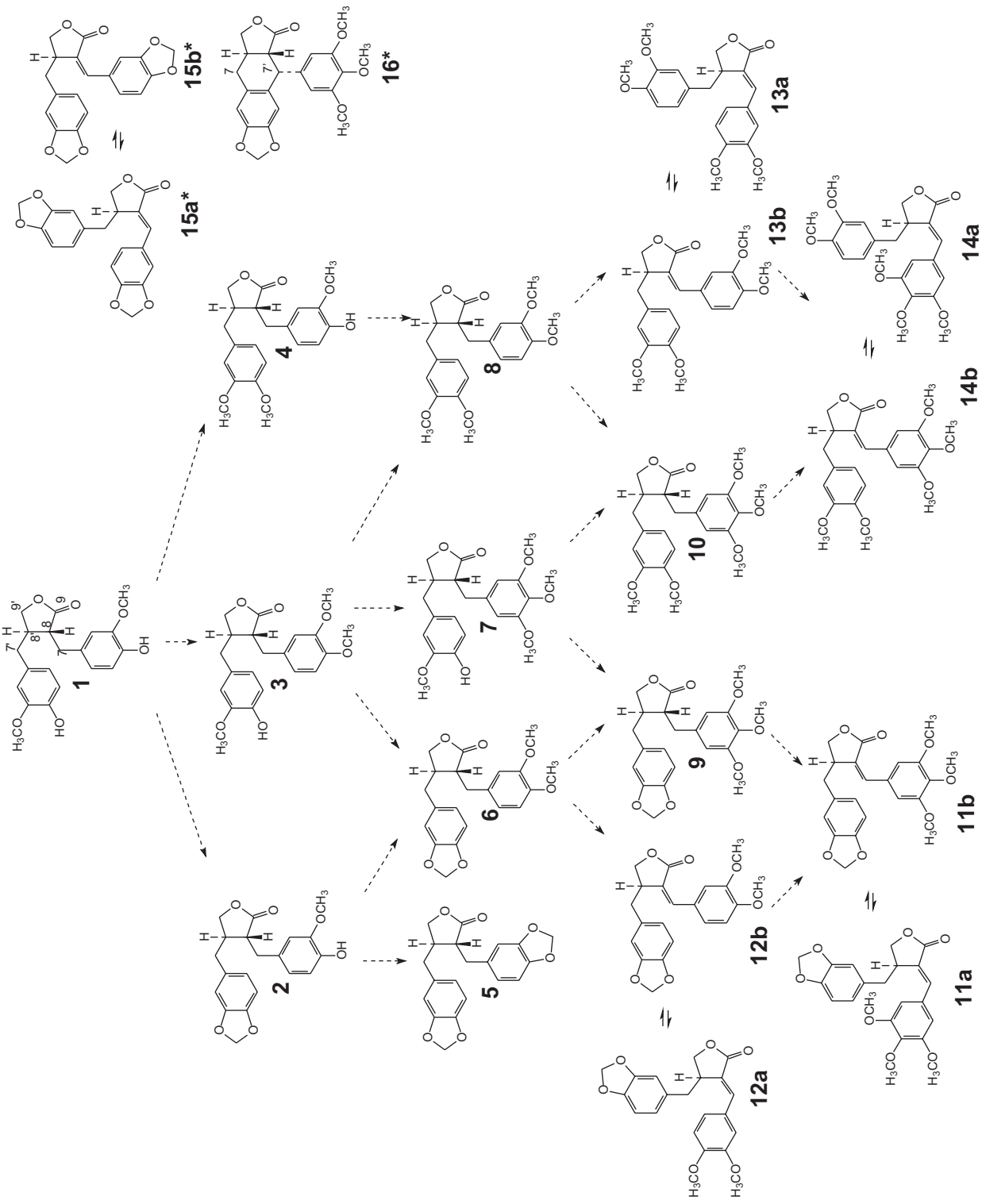

Figure 1. Structures of the lignans investigated in this study. Lignano-9,9′- (**1–10**) and lign-7-enolactones (**11a–14b**) were identified in the dichloromethane extract of flowering aerial parts of *L. usitatissimum* L. Arrows indicate likely biosynthetic interrelations. *Compounds **15a/15b** and **16** are not constituents of *L. usitatissimum*.

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Table 1. HPLC retention times and mass spectral data of the lignano-9,9′-lactones **1**–**10** (for product ion definition, see Scheme 1)

		(+)ESI-MS		MS/MS of $[M+H]^+$									
		$[M+H]^+$		$[M+H-H_2O]^+$		$[M+H-2H_2O]^+$		$[A]^+$		$[A']^+$		$[B]^+$	
Cpd.	Rt	*m/z*	int(%)	*m/z*	int(%)	*m/z*	int(%)	*m/z*	int(%)	*m/z*	int(%)	*m/z*	int(%)
1	37.25	359	(100)	341	(60)	323	(100)	137	(55)[a]	137	[a]	163	(10)
2	49.90	357	(100)	339	(65)	321	(100)	137	(35)	135	(<5)	161	(10)
3	45.14	373	(100)	355	(65)	337	(100)	151	(60)	137	(20)	163	(10)
4	45.54	373	(100)	355	(25)	337	(30)	137	(25)	151	(<5)	177	(10)
5	66.62	355	(100)	337	(80)	319	(100)	135	(30)[a]	135	[a]	161	(20)
6	58.97	371	(100)	353	(80)	335	(100)	151	(45)	135	(5)	161	(15)
7	47.20	403	(100)	385	(100)	367	(45)	181	(30)	137	(<5)	163	(10)
8	52.61	387	(100)	369	(75)	351	(40)	151	(90)[a]	151	[a]	177	(10)
9	57.19	401	(100)	383	(100)	365	(35)	181	(20)	135	(<5)	161	(15)
10	51.19	417	(100)	399	(100)	381	(35)	181	(40)	151	(<5)	177	(15)

[a] Signal intensity reported only once where $[A]^+ = [A']^+$.

observed, probably formed by the loss of 28 Da (CO) from the ion at *m/z* 231.

Interestingly, it was found that the aryltetralin lignan deoxypodophyllotoxin **16**, isomeric to **11a** and **11b**, yields the same base peak (Fig. 2). In this lignan class, this ion typically occurs in compounds not hydroxylated at C-7′ (corresponding to C-7 in the lignenolactones, cf. Fig. 1). It has been interpreted as resulting from the elimination of the 'pendant' aromatic moiety and was termed $[B+H]^+$ in our previous studies.[4,5] The fragmentation observed in the $\Delta^{7,8}$-unsaturated lignenolides can straightforwardly be rationalized by cyclization of the $[M+H]^+$ ion to an aryltetralin intermediate which then loses the substituted benzene moiety, leading to the same ion as observed in the spectra of native aryltetralin lignans (Fig. 2, ion $[C+H]^+$ in Scheme 1). In addition to this base peak, moreover, an ion corresponding to a benzylic/tropylium cation (termed $[A']^+$ in ref. 4, cf. Fig. 2), also observed in saturated dibenzylbutyrolactones, can be detected at *m/z* 135 in **11a**/**11b** as well as in **15a**/**15b**. Since benzylic cleavage is possible only from the saturated part of the molecule, the mass of this ion – as that of the base fragment described above – is of diagnostic value in cases where the two aromatic moieties show different substitution, such as in **11a**/**11b**. Both ions clearly rule out the presence of a positional isomer with exchanged aromatic substitution.

Based on these observations, the crude dichloromethane extract of *L. usitatissimum* (aerial parts at the flowering stage) was re-analyzed. The complexity of the lignan pattern in this extract is illustrated by the ion chromatograms depicted in Figs. 3 and 4. In addition to the lignano-9,9′-lactones **5**, **6**, **8**, **9** and **10** reported earlier,[4] and the isomeric pair **11a**/**11b**,

Table 2. HPLC retention times and mass spectral data of lign-7-eno-9,9′-lactones **11a**–**14b** from *L. usitatissimum*, and **15a** and **15b** from *L. corymbulosum*.[8] For product ion definition, see Scheme 1

	HPLC			(+)ESI-MS		MS/MS of $[M+H]^+$									
			rel. area	$[M+H]^+$		$[M+H-H_2O]^+$		$[M+H-2H_2O]^+$		$[A']^+$		$[C+H]^+$		$[C+H-28]^+$	
cpd.	Rt	rRt[a]	(E/Z)	*m/z*	int(%)	*m/z*	int(%)	*m/z*	int(%)	*m/z*	int(%)	*m/z*	int(%)	*m/z*	Int(%)
11a	62.50	1.09	5.35	399	(100)	381	(55)	363	(20)	135	(10)	231	(100)	203	(35)
11b	67.92	1.19		399	(100)	381	(40)	363	(20)	135	(15)	231	(100)	203	(40)
12a	64.21	1.09	4.21	369	(100)	351	(70)	333	(20)	135	(45)	231	(75)	203	(100)
12b	68.25	1.16		369	(100)	351	(95)	333	(15)	135	(40)	231	(55)	203	(100)
13a	56.70	1.08	3.99	385	(100)	367	(5)	349	(<5)	151	(15)	247	(100)	219	(30)
13b	64.47	1.23		385	(100)	367	(5)	349	(<5)	151	(15)	247	(100)	219	(20)
14a	56.31	1.10	5.34[b]	415	(100)	397	(5)	379	(<5)	151	(10)	247	(100)	219	(15)
14b	64.74	1.27		415	(100)[b]	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
15a	62.71[c]	1.04[c]	4.12	353	(100)	335	(60)	317	(35)	135	(50)	231	(50)	203	(100)
15b	66.48[c]	1.11[c]		353	(100)	335	(50)	317	(25)	135	(35)	231	(40)	203	(100)

[a] Relative retention time with respect to the corresponding saturated lignan.
[b] Spectrum of **14b** obtained by subtraction of overlapping signals; the ratio of peak areas is therefore possibly not reliable.
[c] Retention time (Rt) data obtained from a separate analysis performed several months later. The Rt of the corresponding saturated lignan **5** analyzed on the same day as reference was 60.15 min (cf. Fig. S15, Supporting Information).
n.a.: not available due to very low concentration.

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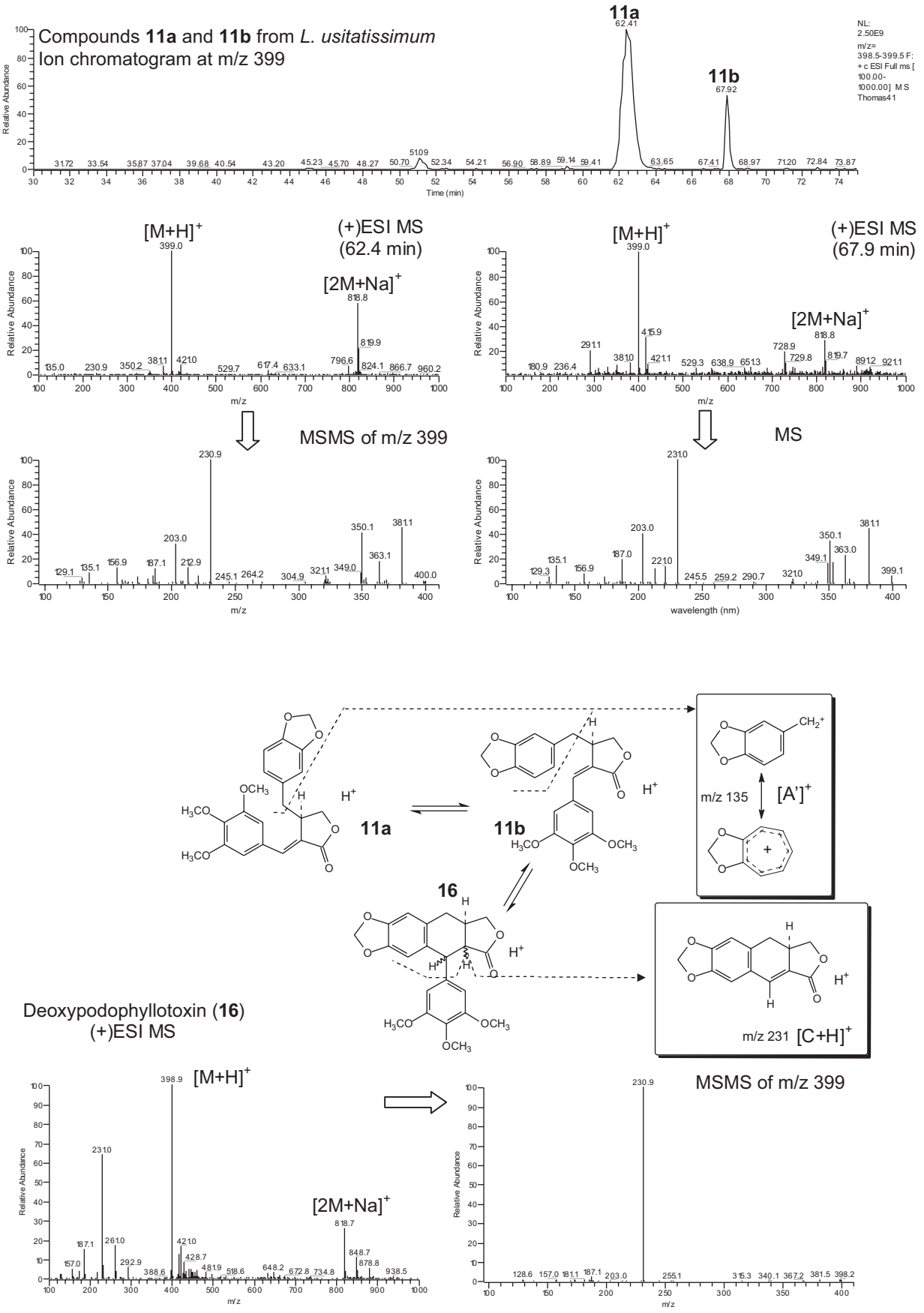

Figure 2. ESI-MS and ESI-MS/MS spectra of lign-7-eno-9,9′-lactones, represented by the isomeric pair **11a**/**11b** (top), in comparison with an isomeric aryltetralin lignan, deoxypodophyllotoxin **16** (bottom),[4] and proposed pathway of fragmentation. The other lignenolactones yielded analogous mass spectra (see Table 2 and Figs. S10–S14, Supporting Information).

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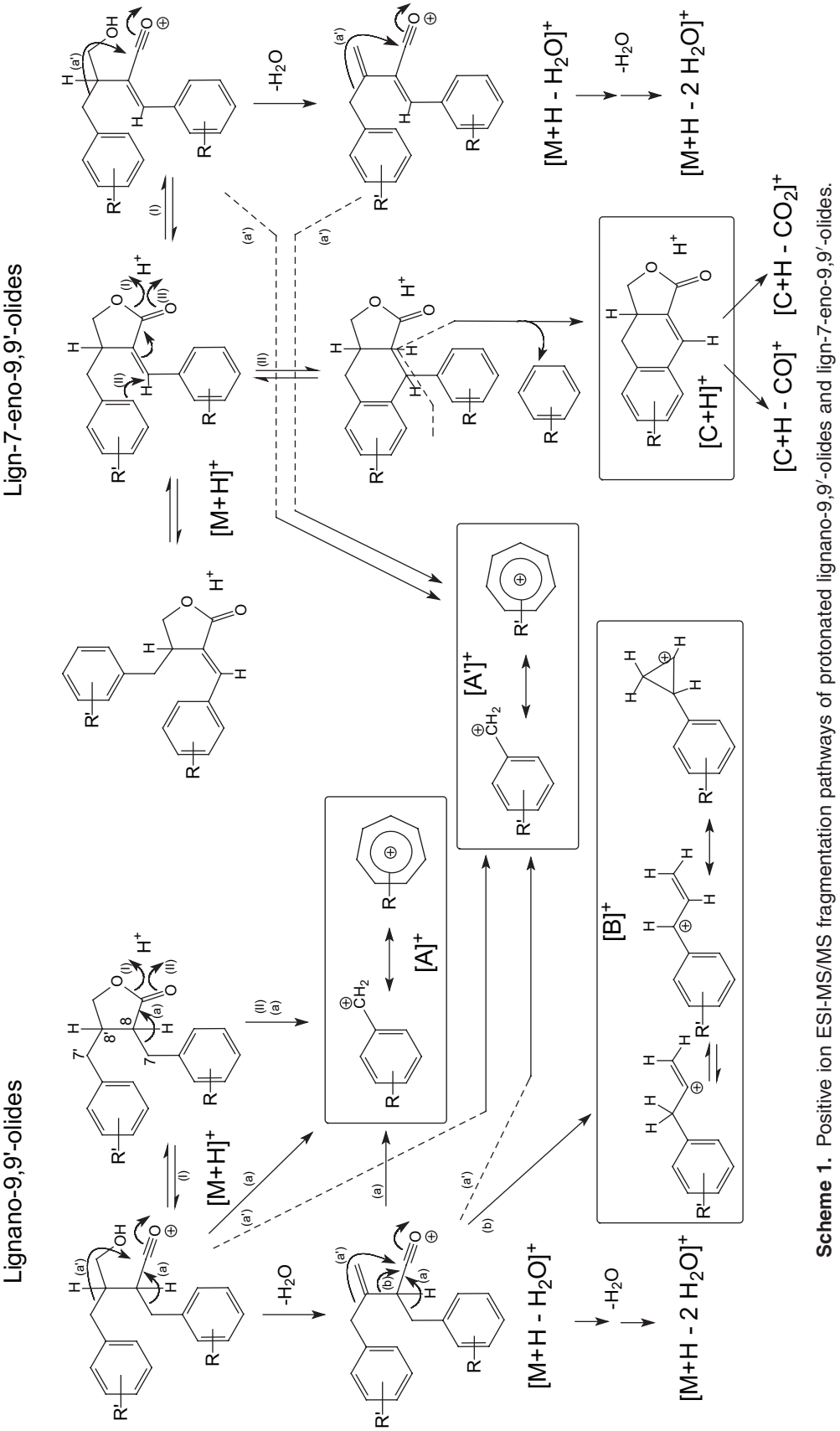

Scheme 1. Positive ion ESI-MS/MS fragmentation pathways of protonated lignano-9,9′-olides and lign-7-eno-9,9′-olides.

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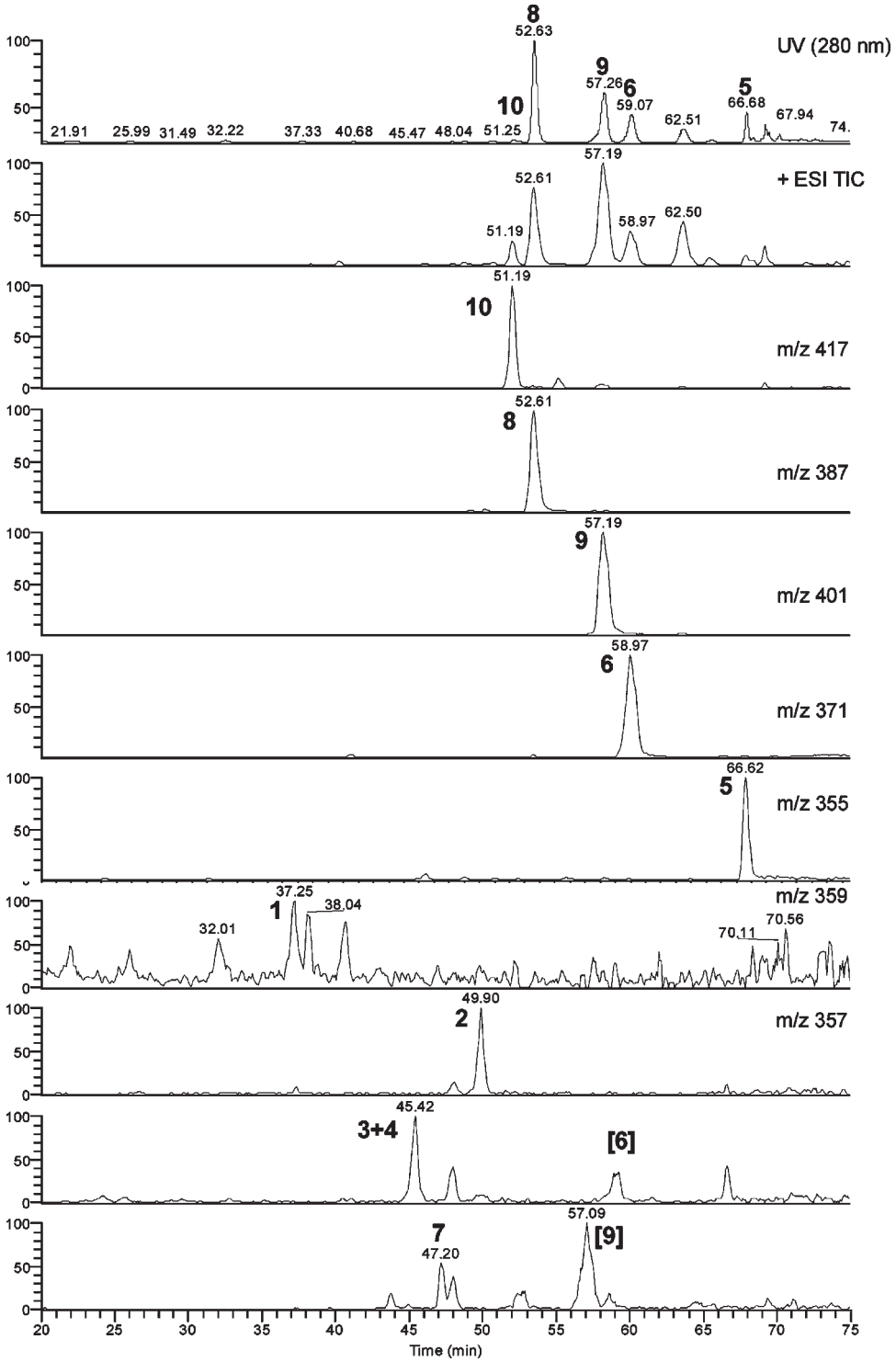

Figure 3. HPLC/ESI-MS analysis of lignano-9,9′-lactones in *L. usitatissimum*. Traces from top to bottom: UV/DAD, +ESI TIC, single ion traces at the mentioned $[M+H]^+$. Numbers in brackets: Signals caused by product ions of other compounds. For mass spectra, see Figs. S1–S9, Supporting Information.

the *E*- and *Z*-isomers of 7,8-unsaturated derivatives of bursehernin **6** (kaerophyllin[11] and isokaerophyllin,[12] **12a** and **12b**), and of the matairesinol dimethyl ether **8** (*E*- and *Z*-7,8-dehydromatairesinol dimethyl ether,[13,14] **13a** and **13b**), as well as the *E*-isomer of the unsaturated derivative of thujaplicatin trimethyl ether **10** (*E*-7,8-dehydrothujaplicatin trimethyl ether **14a**), were identified from their mass spectra and retention behaviour (for ion chromatograms and spectra, see Figs. S10, S12, S13, Supporting Information; data in Table 2). Furthermore, a second very small (and strongly overlapped) peak containing an ion at *m*/*z* 415 (corresponding to $[M+H]^+$ of **14a**), and eluting at higher retention time

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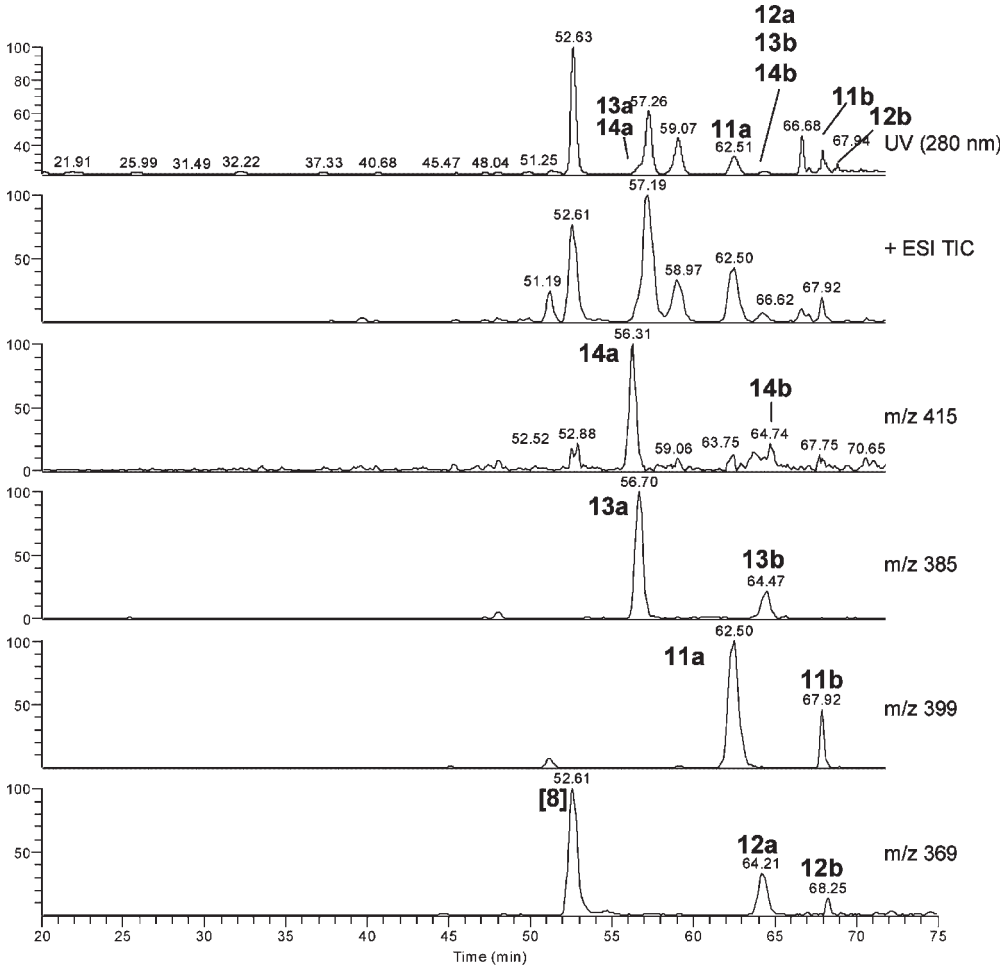

Figure 4. HPLC/ESI-MS analysis of lign-7-enolactones in *L. usitatissimum*. Traces from top to bottom: UV/DAD, +ESI TIC, single ion traces at the mentioned $[M+H]^+$. Numbers in brackets: Signals caused by product ions of other compounds. For mass spectra, see Figs. S10–S13, Supporting Information.

than **14a**, was detected (Fig. S13, Supporting Information), which was of too low abundance to yield an unambiguous MS/MS spectrum. This ion – although not finally confirmed by MS/MS – should represent the *Z*-isomer, **14b**, and this assignment is also supported by its retention time in comparison with **14a** and **10** (see Tables 1 and 2).

In addition to the lignans mentioned so far, several further lignano-9,9′-lactones were detected in minor amounts. Since (−)-matairesinol **1** is the probable precursor for all dibenzylbutyrolactones as well as further downstream lignans,[2] an ion chromatogram was extracted at *m/z* 359, corresponding to the expected $[M+H]^+$ ion of this lignan, and a very small peak was detected. MS/MS analysis of this ion led to a product ion spectrum (see Fig. S6, Supporting Information) in full agreement with the general ESI fragmentation pathway for dibenzylbutyrolactone lignans previously described[4] and identical with that of authentic (−)-matairesinol **1**. Furthermore, the retention time was identical with that of the authentic substance.

The search for possible biosynthetic intermediates between the major lignans led to the detection of compounds **2** (pluviatolide, $[M+H]^+$ at *m/z* 357[4]), **3** and **4** (matairesinol-4- and -4′-methylether = buplerol[15] and arctigenin,[16] respectively, $[M+H]^+$ at *m/z* 373) and **7** (thujaplicatin-3,4-dimethylether = hernanol,[17] $[M+H]^+$ at *m/z* 403); for data, see Table 1, for spectra and $[M+H]^+$ single ion traces, see Figs. S7, S8, S9, Supporting Information.

In all cases, the presence of constitutional isomers with exchanged positions of the aromatic moieties could unambiguously be ruled out due to the presence of the very characteristic product ions $[A]^+$, $[A']^+$ and $[B]^+$ in the MS/MS spectra (see Scheme 1). The diagnostic value of these ions was reported previously.[4] In short, $[A]^+$ is generally observed with higher abundance than $[A']^+$ and, in addition, $[B]^+$ clearly shows the substitution at the aromatic part constituted by C-1′–C-6′.

It is not possible, from the MS data, to exclude the presence of stereoisomers, but based on our previous finding that the major lignans isolated from this extract (**5**, **6**, **8**, **9**, **10**, **11a**/**11b**) showed the same absolute configuration (8R, 8′R[4]), it is very likely that the minor constituents reported here also possess this same configuration at C-8 and C-8′.

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CONCLUSIONS

The ESI-MS/MS fragmentation pathways of dibenzylbutyrolactone lignans can be summarized as shown in Scheme 1. The depicted pathways show that the $[M+H]^+$ ions in both lignan subclasses show analogous breakdown pathways with the loss of two water molecules. A possible structure for the $[M+H-2H_2O]^+$ ion in the saturated lignanolactones has recently been proposed by Eklund *et al.*,[6] who, however, did not mention the other diagnostically valuable product ions. The second point of commonality between lignano- and lignenolactones is the formation of the $[A']^+$ ion, formed by benzylic cleavage of the C-7′–C-8′ bond. The analogous product ion $[A]^+$, formed by cleavage between C-7 and C-8, however, is only detected in the lignanolactones. In all cases where $[A]^+$ and $[A']^+$ differ, the former is observed at higher abundance than the latter. Furthermore, the ion $[B]^+$, formed from $[M+H-H_2O]^+$ by cleavage of the bond between C-8 and C-8′, is observed with significant abundance only in the saturated lactones, allowing their unambiguous identification in combination with the $[A]^+$ and $[A']^+$ ions. On the other hand, the lignenolactones are most prominently characterized by the very abundant $[C+H]^+$ ion resulting from loss of the 'lower' aromatic moiety (C-1–C-6) after cyclization to an aryltetralin intermediate. Thus, the information on this ion, in combination with that from $[A']^+$, also allows the straightforward assignment of aromatic substitution in this case.

Hence, unambiguous identification of lign-7-eno-9,9′-lactones, as well as of the saturated lignano-9,9′-lactones, is possible by means of HPLC/ESI-MS/MS analysis. The fragmentation pathways now established and summarized in Scheme 1 can be applied to the identification of a wide range of such compounds, even in very complex mixtures such as the extract analyzed here. Lignans of this type are extremely widespread in higher plants and a vast number of different structures are known.[2,3] However, most of the current data were obtained by means of identification/structural elucidation after isolation which, naturally, covers only the 'tip of the iceberg', i.e. in many cases only such constituents were identified that were present in comparatively high concentration. Much information on accompanying minor compounds, e.g. with respect to potential biosynthetic intermediates and the full chemical diversity, still waits to be uncovered.

Thus, the example of *L. usitatissimum*, an important medicinal and crop plant,[18] shows that the lignan pattern identified in this species, when considered as a whole, can be arranged in a continuous biosynthetic pathway scheme (symbolized by arrows in Fig. 1) of reticulate rather than linear appearance. Studies into the nature of such metabolic networks in lignan biosynthesis can now be conducted on the basis of the present results, in combination with our previous reports on other lignan classes.[4,5] It will be very interesting, for example, to investigate changes in lignan metabolism over time during plant development, or the variation between populations and cultivars, as well as between different species, taking into account – rather than only the few major constituents – the full pattern of metabolites detectable by HPLC/ESI-MS/MS. Such applications are in progress and the results will be communicated in due course.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

Acknowledgements

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