

# First evidence of hexameric and heptameric ellagitannins in plants detected by liquid chromatography/electrospray ionisation mass spectrometry

Maarit Karonen<sup>1\*</sup>, John Parker<sup>2</sup>, Anurag Agrawal<sup>3</sup> and Juha-Pekka Salminen<sup>1</sup>

<sup>1</sup>Laboratory of Organic Chemistry and Chemical Biology, Department of Chemistry, FI-20014 University of Turku, Finland

<sup>2</sup>Smithsonian Environmental Research Center, Edgewater, MD 21037, USA

<sup>3</sup>Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, NY, USA

Received 21 June 2010; Revised 10 August 2010; Accepted 24 August 2010

Ellagitannins are bioactive plant polyphenols of which more than 500 individual compounds have been identified from plants. An ellagitannin-rich fraction was isolated by Sephadex LH-20 from *Oenothera biennis* (common evening primrose) leaves and roots and analysed by high-performance liquid chromatography/diode-array detection coupled to high-resolution mass spectrometry with an electrospray ionisation interface. The high-molecular mass ellagitannins were characterised by their UV spectra, molecular masses and mass spectral fragments. In addition to the previously reported dimers and trimers, an entire series of oligomeric ellagitannins from dimers to heptamers was characterised in both roots and leaves of *O. biennis*. This is the first report of natural ellagitannins larger than pentamers. Copyright © 2010 John Wiley & Sons, Ltd.

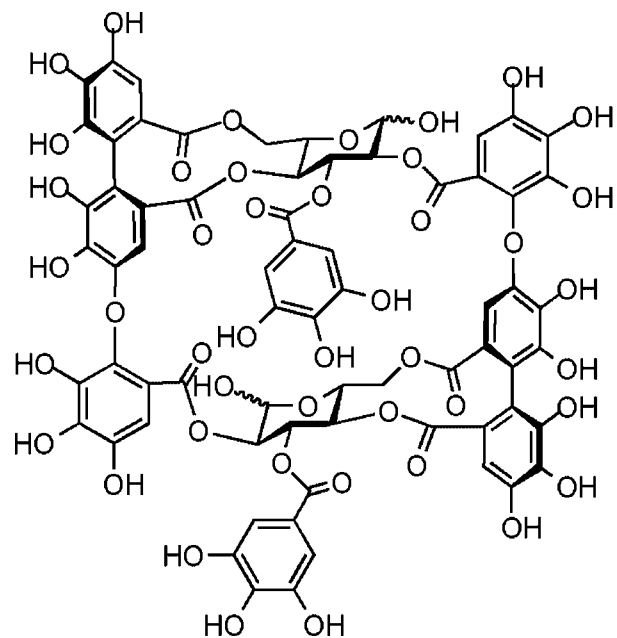
Ellagitannins are the most numerous plant phenolics among the hydrolysable tannins. They are often connected with potent biological activities, including antiviral and immunomodulatory to defence against herbivores.<sup>1–6</sup> In fact, the developments in ellagitannin chemistry over the past decades are turning ellagitannins from the most underestimated class of plant tannins into one of the most promising ones. The past 45 years have witnessed the isolation and characterisation of over 500 pure ellagitannins, many of which have been used in traditional herbal medicines. More than half of these ellagitannins are oligomers, structures ranging from dimers up to pentamers. Until now, there has been no evidence of the presence of ellagitannins larger than pentamers in any plant species.

*Oenothera biennis* (Onagraceae), common evening primrose, is a well-studied plant species with known medical applications.<sup>5</sup> Its foliage in particular has been reported to contain ellagitannins, including the primary molecules oenothetin B and oenothetin A (a macrocyclic dimer and a macrocyclic trimer, respectively; Fig. 1).<sup>1,7</sup> Oenothetin B and A have both also been isolated as main components from other *Oenothera* species.<sup>8</sup> Oenothetin B has been detected in several other plant species, including *Lythrum anceps* (striped loosestrife), *Epilobium parviflorum* (small-flowered hairy willowherb), *Cuphea hyssopifolia* (Mexican heather), *Melaleuca*

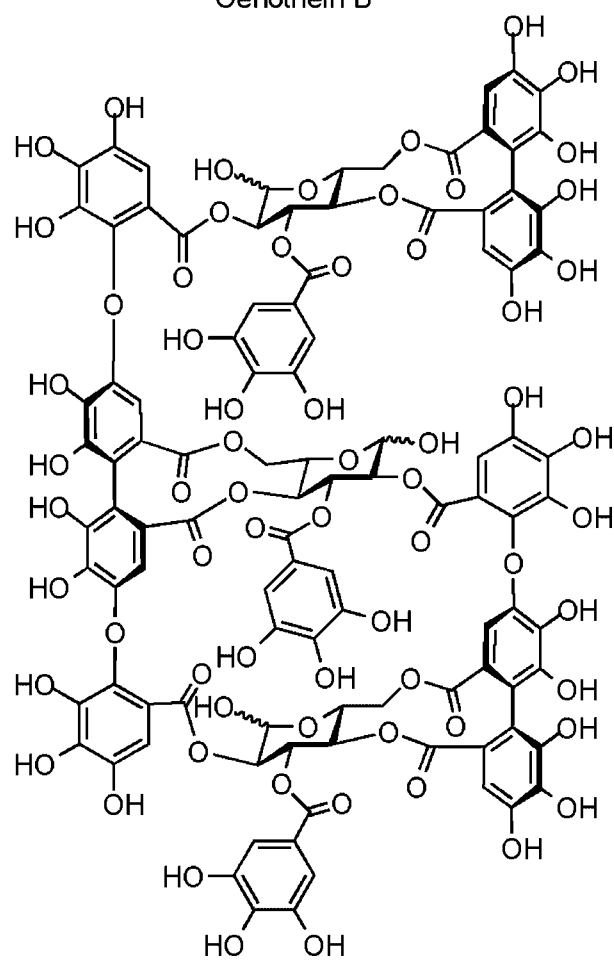
*leucadendron* (melaleuca), *Eugenia uniflora* (surinam cherry), *Eucalyptus alba* (eucalyptus), *Quercus rubra* (red oak) and *Woodfordia fruticosa* (as listed in Coca *et al.*<sup>6</sup>). We have previously isolated oenothetin B from the inflorescences of *Epilobium angustifolium* (fireweed/willow herb)<sup>2–4</sup> and used it together with other purified ellagitannins to explore the *in vitro* anti-herbivore activity of ellagitannins, i.e. their ease of oxidation at the high pH values found in guts of lepidopteran insects. Here, we focus additional attention on *O. biennis* ellagitannins for two reasons: (1) our high-performance liquid chromatography/diode-array detection mass spectrometric (HPLC-DAD-MS) analyses revealed high complexity of ellagitannins in both roots and leaves of *O. biennis*, and (2) *O. biennis* ellagitannins have been shown to possess important traits in connection with the herbivores of *O. biennis*.<sup>7,9</sup>

In this study, we utilised Sephadex LH-20 gel chromatography and high-resolution HPLC/electrospray ionisation (ESI)-MS to characterise the complex ellagitannins from leaves and roots of *O. biennis*. We report, for the first time, the presence of hexameric and heptameric ellagitannins in plants, in addition to a range of oligomers from dimers to pentamers. These findings open up new possibilities for the further exploration of even more complex ellagitannin structures in nature. Moreover, nothing is known about the bioactivities – for humans or against herbivores or pathogens – of these novel structures. Therefore, we presume that these types of developments in ellagitannin chemistry will unravel new possibilities for us to utilise plant ellagitannins or to understand the *raison d'être* for their presence in plants, e.g. chemical defense against herbivores.

\*Correspondence to: M. Karonen, Laboratory of Organic Chemistry and Chemical Biology, Department of Chemistry, FI-20014 University of Turku, Finland.  
E-mail: maarit.karonen@utu.fi



Oenothetin B



Oenothetin A

**Figure 1.** Structures of oenothetin B (dimeric ellagitannin) and oenothetin A (trimeric ellagitannin).

## EXPERIMENTAL

### Materials

The acetonitrile used was LiChrosolv<sup>®</sup> hypergrade for LC/MS (Merck KGaA, Darmstadt, Germany) and formic acid was purchased from Sigma-Aldrich (Steinheim, Germany). Water was filtered through an Elgastat UHQ-PS purification system (Elga, Kaarst, Germany).

### Sample preparation

Seeds of *Oenothera biennis* (common evening primrose) were collected from multiple populations around Tompkins County, New York (USA) and grown for approximately 60 days on an open-air rooftop patio on the Cornell University campus. After harvesting, clean freeze-dried root and shoot samples were ground to a fine powder. Separately 8.0 g of leaves and roots were extracted with 2 × 800 mL acetone/water (7:3) with a planary shaker. Extracts were concentrated into the aqueous phase by rotary evaporation at <40°C and freeze-dried: 1.9 g leaf extract and 2.1 g root extract were obtained. 800 mg of freeze-dried leaf or root extract was dissolved into 2 mL ethanol and diluted with 5 mL water. The solution was applied to a Sephadex LH-20 column and fractions were collected by elution with water, 30% and 50% aqueous MeOH, 10%, 30%, 50%, and 70% aqueous acetone. The obtained fractions were concentrated into the aqueous phase by rotary evaporation at <40°C and freeze-dried. The freeze-dried fractions were dissolved in water and filtered through 0.45 μm PTFE filters.

### HPLC/DAD-ESI-MS analysis

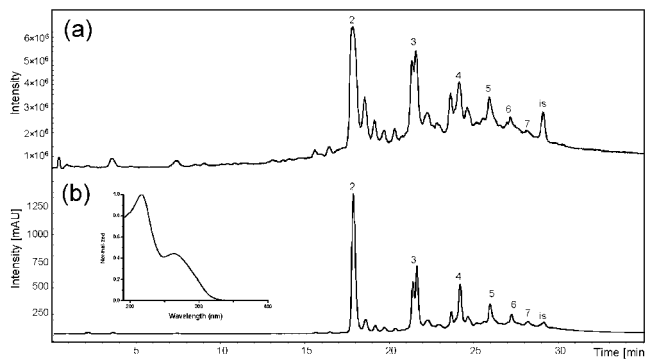
HPLC analysis was carried out using a HPLC/DAD-ESI-MS system consisting of an Agilent HPLC 1200 Series equipped with a diode-array detector (Agilent Technologies, Waldbronn, Germany) and a micrOTOF<sub>Q</sub> ESI mass spectrometer (Bruker Daltonics, Bremen, Germany). Chromatographic separations were performed using an XBridge<sup>™</sup> column (2.1 × 2100 mm, C-18, 3.5 μm; Waters, Dublin, Ireland). The binary mobile phase consisted of acetonitrile (A) and water and formic acid (99.6:0.4, v/v) (B). The elution started with 0–2 min 0% A and a linear gradient elution was performed to obtain 30% A at 33 min and 70% A at 35 min staying constant until 43 min. The flow rate was 0.3 mL/min and the injection volume was 5 μL. Chromatograms were recorded at 280 nm. The HPLC system was controlled by Hystar software (version 3.2.; Bruker BioSpin, Rheinstetten, Germany). The mass spectrometer was controlled by Compass micrOTOF control software (Bruker Daltonics) and operated in negative ion mode. The capillary voltage was maintained at +4000 V with the end plate offset at –500 V. The pressure for the nebuliser gas (N<sub>2</sub>) was set at 1.6 bar and the drying gas (N<sub>2</sub>) flow rate was 8.0 L/min and the drying gas (N<sub>2</sub>) temperature 200°C. The full scan mass ranged from *m/z* 100 up to 2000. Calibration with 5 mM sodium formate injected via a six-port valve was used at the end of the LC/MS experiment in order to provide high-accuracy mass measurements. The data were handled by Compass DataAnalysis software (version 4.0; Bruker Daltonics).

## RESULTS AND DISCUSSION

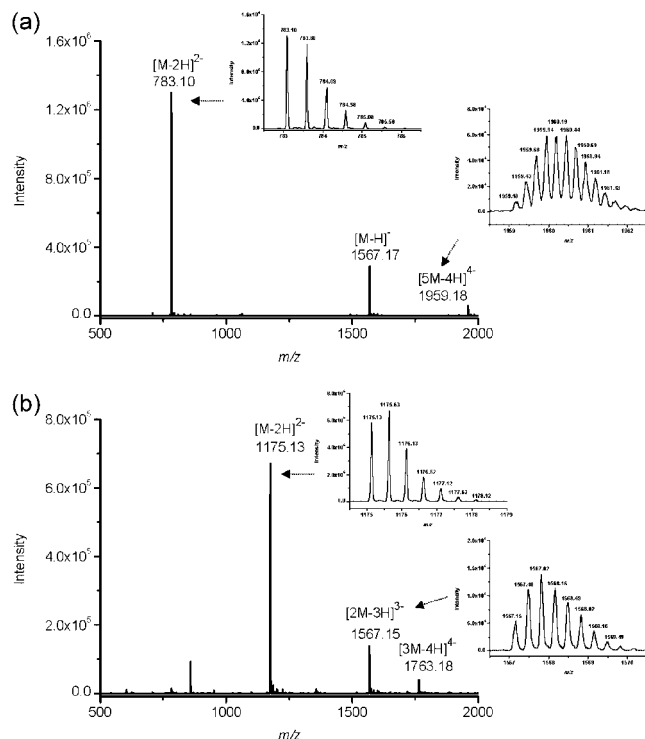
Characterisation of previously detected *Oenothera biennis* ellagitannins

On the basis of Sephadex LH-20 fractionations and subsequent HPLC-DAD-MS analyses, *O. biennis* roots and leaves were found to contain a complex mixture of galloylglucoses, ellagitannins and flavonoid glycosides; more than 50 individual phenolics were detected. Many of the compounds had been detected previously from *O. biennis* or from other plant species; the characterisation of these phenolics will be published separately (Karonen *et al.*, unpublished data). However, the high-resolution MS analyses revealed that *O. biennis* roots and leaves contain ellagitannins that have never before been detected in *O. biennis* or any other plant species. These new types of ellagitannin oligomers (hexamers and heptamers) and their biosynthetic precursors (from dimers to pentamers) will now be described in more detail.

All galloylglucoses, flavonoid glycosides and monomeric ellagitannins eluted into the first six Sephadex LH-20 fractions, while the oligomeric ellagitannin set was found primarily in the last fraction eluted with 70% aqueous acetone. Figure 2 shows the HPLC traces of the chromatographically well-separated set of ellagitannins in that fraction. Interestingly, all major peaks that eluted between 17 and 28 min had almost identical UV spectra (see Fig. 2) which is characteristic to ellagitannins having both hexahydroxydiphenoyl (HHDP) and galloyl groups attached to their glucopyranose core; UV maxima were found both in the 205–220 nm and 270–290 nm regions with only a small valley preceding the second maximum at 270–290 nm.<sup>10</sup> High-resolution ESI-MS measurements enabled the calculation of the molecular mass for each of the ellagitannins. All the ellagitannins were represented by multiply charged ions in the mass spectra. The isotopic patterns enabled accurate mass calculation to be carried out for each of the multiply charged ions and thus for the corresponding ellagitannins. Typically, the following types of ions were observed: [M-



**Figure 2.** LC/MS analysis of ellagitannin-rich fraction from common evening primrose: (a) the total ion chromatogram and (b) the HPLC-DAD profile at 280 nm and the characteristic UV spectrum of ellagitannins. The labels 2–7 on the peaks indicate the degree of oligomerisation of ellagitannins: (2) dimeric ellagitannins, (3) trimeric ellagitannins, (4) tetrameric ellagitannins, (5) pentameric ellagitannins, (6) hexameric ellagitannins and (7) heptameric ellagitannins. 'is' refers to the internal standard 6-bromo-2-naphthyl- $\beta$ -D-glucopyranoside.



**Figure 3.** Mass spectra of (a) dimeric (1568 Da) and (b) trimeric (2352 Da) ellagitannins. The isotopic patterns are emphasised for the main peaks.

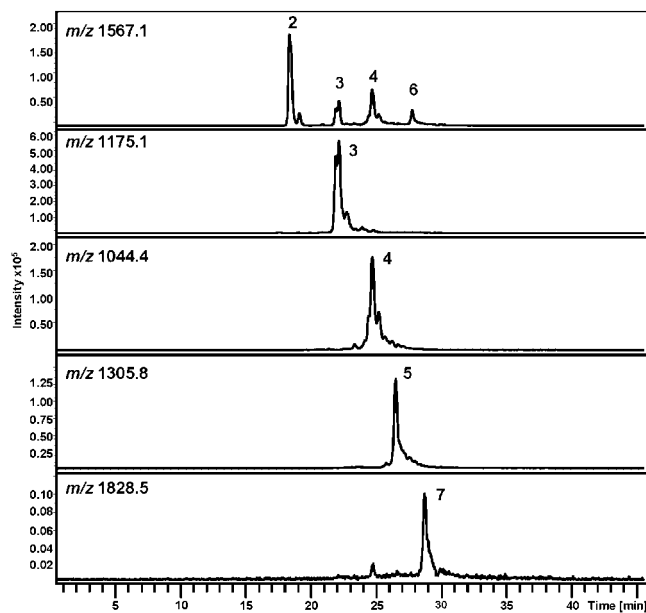
H]<sup>-</sup>, [M-2H]<sup>2-</sup>, [M-3H]<sup>3-</sup>, [2M-3H]<sup>3-</sup>, [M-4H]<sup>4-</sup>, [3M-4H]<sup>4-</sup> and [5M-4H]<sup>4-</sup>.

The main compound in the ellagitannin fraction was the dimeric oenotherin B (1568.17 Da; Fig. 3(a)). Its retention time ((2) in Fig. 2), and UV and mass spectral data (Fig. 3(a)) fitted well with our previous work where oenotherin B was also characterised by NMR.<sup>2-4</sup> This ellagitannin has also previously been reported from *O. biennis*.<sup>1</sup> It is a macrocyclic dimer formed by dimerisation of two tellimagrandin I monomers (786 Da) via two *m*-DOG-type linkages,<sup>5</sup> each linkage decreasing the molecular mass of the oligomer by 2 Da. Thus the dimer may be thought to consist of two 784 Da units. This tellimagrandin I monomer, which is attached via two *m*-DOG-type bonds, will be referred to later as Tl<sub>2</sub>DOG.

Interestingly, it was exactly this additional building block of 784 Da that was used to decorate the oenotherin B core to build up all the remaining oligomers reported in this study. This means that to construct the biosynthetically subsequent trimer ((3) in Fig. 2) from oenotherin B, the most probable monomer to be added to the oenotherin B core is again tellimagrandin I. This was also supported by the identical UV spectra of the dimer and trimer; ETs that share the same HHDP to galloyl ratio (here 1:2) have similar UV spectra.<sup>10</sup> However, since the trimer had a molecular mass of 2352.28 Da ([M-2H]<sup>2-</sup> = 1175.13; see Fig. 3(b)), it could be concluded that the additional monomer was linked to the dimer only by one DOG-type linkage (1568 Da + 786 Da - 2 Da = 2352 Da), just as in oenotherin A (Fig. 1). Indeed, Yoshida *et al.* also found oenotherin A as the main trimer in the foliage of *O. biennis*.<sup>1</sup> Henceforth, the monomeric tellimagrandin I, which is attached to the ellagitannin oligomer via one DOG-

type linkage only, will be referred as  $\text{TI}_{\text{DOG}}$ . Another theoretical possibility would have been to add a tetragalloylglucose (788 Da) unit to the oenothetin B core by two C-O-C linkages ( $1568 \text{ Da} + 788 \text{ Da} - 4 \text{ Da} = 2352 \text{ Da}$ ). However, this would have changed the HHDP to galloyl ratio of the whole ET molecule from 1:2 (dimer) to 1:4 (trimer) and this would have been evidenced by a clearly deeper valley preceding the UV maximum at 270–290 nm;<sup>10</sup> this was not observed.

We found that oenothetin B and A both express characteristic elution patterns in HPLC traces. First, it is known that such monomeric ellagitannins that have ungalloylated anomeric centres spontaneously form  $\alpha$ - and  $\beta$ -glucose mixtures in aqueous solutions via opening of the glucopyranose core. These isomers are then revealed by two peaks in HPLC/MS, most often closely at a 50:50 ratio. Tellimagrandin I is one such monomer. In theory, oenothetin B (tellimagrandin I dimer) could form four isomers depending on the configuration of glucoses:  $\beta - \beta$ ,  $\beta - \alpha$ ,  $\alpha - \beta$  or  $\alpha - \alpha$ . However, our data with purified oenothetin B shows that it rather exists as a 95:5 mixture of two of the isomers only. The minor of these isomers could also be seen in the extracted ion chromatograms in Fig. 4, eluting directly after the main isomer (2) at 18.7 min (both isomers had identical UV and MS data). It is likely that the rigid macrocyclic structure (two C-O-C linkages between the monomers) of oenothetin B hinders the spontaneous opening of the two glucopyranose cores and equilibration of the anomeric mixtures in aqueous solution. Interestingly, the trimeric oenothetin A showed two major isomers with

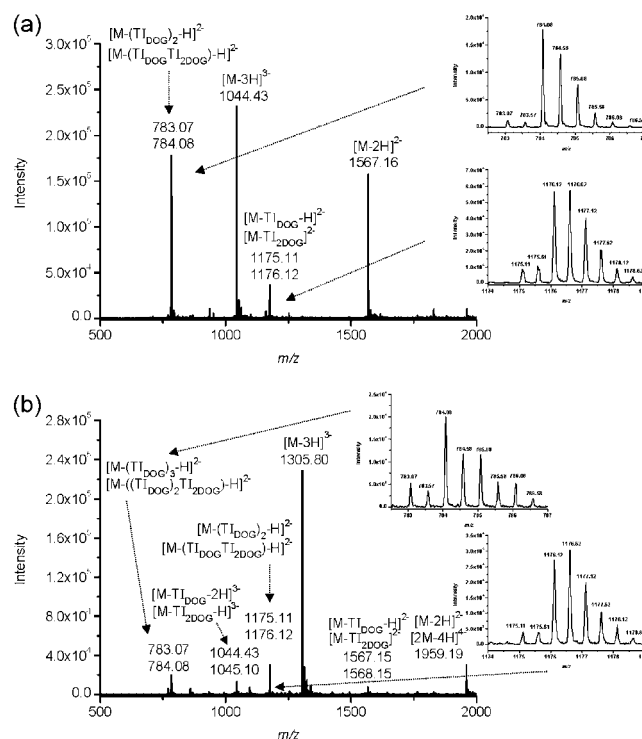


**Figure 4.** Extracted ion chromatograms (EICs) for oligomeric ellagitannins. The labels 2–7 on the peaks indicate the degree of oligomerisation of ellagitannins and also refer to Fig. 2. The EICs were obtained by using  $m/z 1567.1 \pm 0.1$  for the dimer,  $m/z 1175.1 \pm 0.1$  for the trimer,  $m/z 1044.4 \pm 0.1$  for the tetramer,  $m/z 1305.8 \pm 0.1$  for the pentamer, and  $m/z 1828.5 \pm 0.1$  for the heptamer. Note that the  $m/z 1567.1 \pm 0.1$  shows also the  $[\text{M}-3\text{H}]^{3-}$  ion of the hexamer, as well as the fragments of the trimer and tetramer.

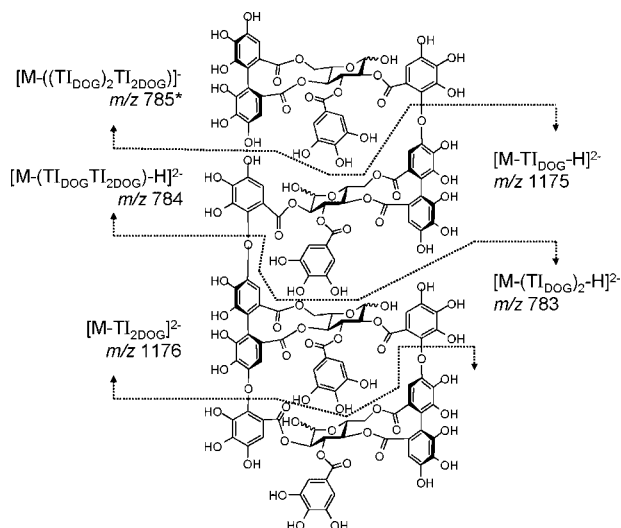
HPLC/MS analysis. These were in an approximately 50:50 ratio, indicating spontaneous opening of one of the three glucopyranose cores. This was possible for the trimer, since the third tellimagrandin I unit remains comparatively flexible as it is not attached to the oenothetin B core via two, but only one C-O-C linkage (Fig. 1). The fact that these two major HPLC peaks belong to oenothetin A was further confirmed by analysing 114 leaf and 114 root samples of *O. biennis* by HPLC-DAD (Parker *et al.*, unpublished data). The oenothetin A isomers were quantified separately from both sample sets and were found to correlate strongly with each other ( $r = 0.9965$  and  $r = 0.9950$ ). These exceedingly tight correlations both in leaf and root samples are indicative that the two HPLC peaks are isomers of the same molecule, as it is likely to be impossible to achieve such tight correlations of two different molecules across 114 individual plants spanning both root and shoot tissues.

### Tetrameric and pentameric ellagitannins previously undetected in *O. biennis*

In addition to ellagitannin dimers and trimers, several larger oligomers were detected in the *O. biennis* root and leaf fractions for the first time. Previously, ellagitannin tetramers and pentamers have been detected in various other plants, such as tetramers trapanin B from *Trapa japonica*, hirtellin Q<sub>1</sub> from *Reaumuria hirtella*, and lambertianin D from *Rubus lambertianus*, and pentamers melastoflorins A–D from *Monochaetum multiflorum*.<sup>11–13</sup> However, the high-resolution MS analyses revealed that *O. biennis* also contains ellagitannin tetramers and pentamers (Fig. 5); these eluted in the



**Figure 5.** Mass spectra of (a) tetrameric (3136 Da) and (b) pentameric (3920 Da) ellagitannins. The isotopic patterns are emphasised for the main fragment ions showing the more feasible fragmentation of  $\text{TI}_{2\text{DOG}}$  units in than of  $\text{TI}_{\text{DOG}}$  units.



**Figure 6.** The ESI-MS fragmentation of the ellagitannin tetramer. The abbreviation  $\text{TI}_{\text{DOG}}$  refers to the monomeric unit of tellimagrandin I linked to the oligomer via one DOG-type linkage and  $\text{TI}_{2\text{DOG}}$  to tellimagrandin I attached to the oligomer via two DOG-type linkages. \*The signal is hidden by the signal of the  $[M-(\text{TI}_{\text{DOG}}\text{TI}_{2\text{DOG}}-\text{H})]^{2-}$  ion.

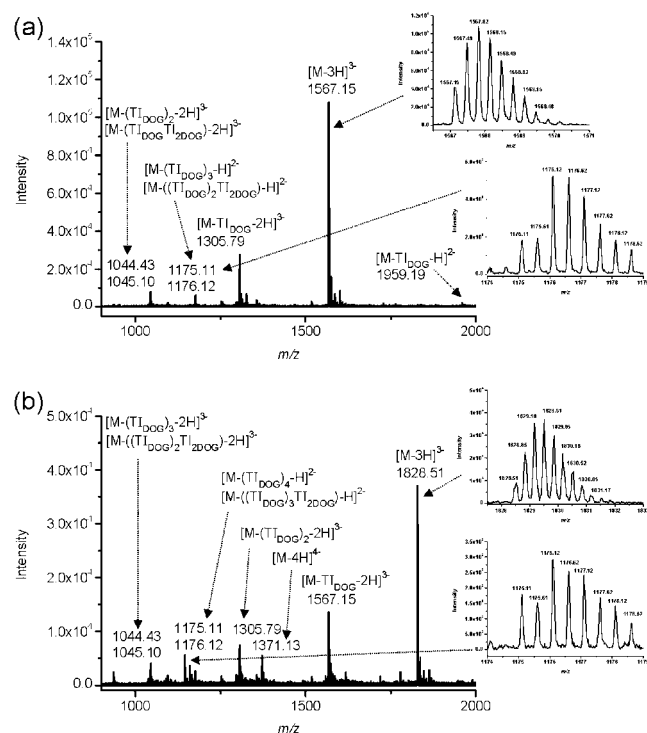
order of increasing degree of oligomerisation ((4) and (5) in Figs. 2(b) and 4). The tetramer had a molecular mass of 3136.31 Da as shown by  $m/z$  values 1044.43 ( $[M-3\text{H}]^{3-}$ ) and 1567.16 ( $[M-2\text{H}]^{2-}$ ; see Fig. 5(a)). Again, it could be concluded that the additional monomer ( $\text{TI}_{\text{DOG}}$ ) was linked to the trimer by one DOG-type linkage only ( $2352 \text{ Da} + 786 \text{ Da} - 2 \text{ Da} = 3136 \text{ Da}$ ; Fig. 6). Thus the tetramer structure could be regarded as a chain of three  $\text{TI}_{\text{DOG}}$  and one  $\text{TI}_{2\text{DOG}}$  units, i.e.  $(\text{TI}_{\text{DOG}})_3\text{TI}_{2\text{DOG}}$ . This was confirmed by the cleavage of one ( $\text{TI}_{\text{DOG}}$  or  $\text{TI}_{2\text{DOG}}$ ) or two ( $(\text{TI}_{\text{DOG}})_2$  or  $\text{TI}_{\text{DOG}}\text{TI}_{2\text{DOG}}$ ) of the terminal units of the TI chain. The fragmentations produced the following ions:  $[M-\text{TI}_{\text{DOG}}-\text{H}]^{2-}$  at  $m/z$  1175.11,  $[M-\text{TI}_{2\text{DOG}}]^{2-}$  at  $m/z$  1176.12,  $[M-(\text{TI}_{\text{DOG}})_2-\text{H}]^{2-}$  at  $m/z$  783.07 and  $[M-(\text{TI}_{\text{DOG}}\text{TI}_{2\text{DOG}}-\text{H})]^{2-}$  at  $m/z$  784.08 (Fig. 6). Interestingly, the fragmentations that involved the  $\text{TI}_{2\text{DOG}}$  unit were more feasible than the ones involving only  $\text{TI}_{\text{DOG}}$  units (see the inserts in Fig. 5(a):  $m/z$  784.08 vs. 783.07 and  $m/z$  1176.12 vs. 1175.11).

The ellagitannin pentamer ((5) in Fig. 2) with molecular mass 3920 Da was identified by its multiply charged ions:  $[M-3\text{H}]^{3-}$  at  $m/z$  1305.80 and  $[M-2\text{H}]^{2-}$  at  $m/z$  1959.19 (Fig. 5(b)). Once again the UV and mass spectral data showed that the additional monomer must be linked to the tetramer only by one DOG-type linkage ( $3136 \text{ Da} + 786 \text{ Da} - 2 \text{ Da} = 3920 \text{ Da}$ ). The main fragmentation mechanisms were similar to those of the tetramer and were supported by the ions at  $m/z$  1567.15 ( $[M-\text{TI}_{\text{DOG}}-\text{H}]^{2-}$ ),  $m/z$  1044.43 ( $[M-\text{TI}_{\text{DOG}}-2\text{H}]^{3-}$ ),  $m/z$  1568.15 ( $[M-\text{TI}_{2\text{DOG}}-\text{H}]^{2-}$ ),  $m/z$  1045.10 ( $[M-\text{TI}_{2\text{DOG}}-2\text{H}]^{3-}$ ),  $m/z$  1175.11 ( $[M-(\text{TI}_{\text{DOG}})_2-\text{H}]^{2-}$ ) and  $m/z$  1176.12 ( $[M-(\text{TI}_{\text{DOG}}\text{TI}_{2\text{DOG}}-\text{H})]^{2-}$ ) (Fig. 5(b)). In addition, a cleavage of three TI units was detected at  $m/z$  783.07 ( $[M-(\text{TI}_{\text{DOG}})_3-\text{H}]^{2-}$ ) and at  $m/z$  784.08 ( $[M-(\text{TI}_{\text{DOG}})_2\text{TI}_{2\text{DOG}}-\text{H}]^{2-}$ ). Again, the fragmentations that involved the  $\text{TI}_{2\text{DOG}}$  unit were more feasible than the ones involving only  $\text{TI}_{\text{DOG}}$  units (see the inserts in Fig. 5(b):  $m/z$  784.08 vs. 783.07 and  $m/z$  1176.12 vs. 1175.11).

## Hexameric and heptameric ellagitannins previously undetected in any plant species

In addition to the above-mentioned ellagitannin tetramers and pentamers that have been found for the first time in *O. biennis*, we were able to characterise some oligomeric ellagitannins that have not previously been detected in *O. biennis* or any other plant species. This was possible by the combination of Sephadex LH-20 gel chromatography, liquid chromatography and high-resolution mass spectrometry. The new ellagitannins included at least hexamers (6) and heptamers (7) (Fig. 2) since we found only traces of  $m/z$  values corresponding to higher oligomers, e.g. octamers and nonamers. Nevertheless, all the characterised ellagitannin oligomers eluted in reversed-phase HPLC/MS in the order of increasing degree of oligomerisation (Figs. 2 and 4); octamers and nonamers also fulfilled this pattern as their MS traces were found at a retention time (Rt) of 29.9 min.

The UV spectra of hexamers and heptamers were similar to those of the ellagitannin dimers, trimers, tetramers and pentamers indicating that they all have the same HHDP to galloyl ratio. Indeed, the molecular mass 4704 Da ( $[M-3\text{H}]^{3-}$  at  $m/z$  1567.15; Fig. 7(a)) for the hexamer and 5488 Da ( $[M-3\text{H}]^{3-}$  at  $m/z$  1828.51 and  $[M-4\text{H}]^{4-}$  at  $m/z$  1371.13; Fig. 7(b)) for the heptamer revealed that these oligomers were synthesised from the pentamer by adding one ( $3920 \text{ Da} + 786 \text{ Da} - 2 \text{ Da} = 4707 \text{ Da}$ ) and two ( $3920 \text{ Da} + 2 \text{ Da} \times 786 \text{ Da} - 2 \text{ Da} \times 2 \text{ Da} = 5488 \text{ Da}$ )  $\text{TI}_{\text{DOG}}$  units, respectively. Again, characteristic mass spectral fragment ions corresponding to the cleavage of one or more TI units verified this observation (Fig. 7). For hexamers, the fragmentations yielded the ions at  $m/z$  1959.19 ( $[M-\text{TI}_{\text{DOG}}-$



**Figure 7.** Mass spectra of (a) hexameric (4704 Da) and (b) heptameric (5488 Da) ellagitannins. The isotopic patterns are emphasised for the main multiply charged ions and for the ions showing the more feasible fragmentation of  $\text{TI}_{2\text{DOG}}$  units than of  $\text{TI}_{\text{DOG}}$  units.

$\text{H}]^{2-}$ ,  $m/z$  1305.79 ( $[\text{M}-\text{TI}_{\text{DOG}}-2\text{H}]^{3-}$ ),  $m/z$  1044.43 ( $[\text{M}-(\text{TI}_{\text{DOG}})_2-2\text{H}]^{3-}$ ),  $m/z$  1045.10 ( $[\text{M}-(\text{TI}_{\text{DOG}}\text{TI}_{2\text{DOG}})-2\text{H}]^{3-}$ ),  $m/z$  1175.11 ( $[\text{M}-(\text{TI}_{\text{DOG}})_3-\text{H}]^{2-}$ ) and  $m/z$  1176.12 ( $[\text{M}-(\text{TI}_{\text{DOG}})_2-\text{TI}_{2\text{DOG}}-\text{H}]^{2-}$ ). The more feasible fragmentation of  $\text{TI}_{2\text{DOG}}$  units than of  $\text{TI}_{\text{DOG}}$  units was not as clear as with tetramers and pentamers, but was still detectable, as shown in Fig. 7(a) ( $m/z$  1176.12 vs.  $m/z$  1175.11). For heptamers, the fragmentations yielded the ions at  $m/z$  1567.15 ( $[\text{M}-\text{TI}_{\text{DOG}}-2\text{H}]^{3-}$ ),  $m/z$  1305.79 ( $[\text{M}-(\text{TI}_{\text{DOG}})_2-2\text{H}]^{3-}$ ),  $m/z$  1044.43 ( $[\text{M}-(\text{TI}_{\text{DOG}})_3-2\text{H}]^{3-}$ ),  $m/z$  1045.10 ( $[\text{M}-(\text{TI}_{\text{DOG}})_2\text{TI}_{2\text{DOG}}-2\text{H}]^{3-}$ ),  $m/z$  1175.11 ( $[\text{M}-(\text{TI}_{\text{DOG}})_4-\text{H}]^{2-}$ ) and  $m/z$  1176.12 ( $[\text{M}-(\text{TI}_{\text{DOG}})_3\text{TI}_{2\text{DOG}}-\text{H}]^{2-}$ ). The more feasible fragmentation of  $\text{TI}_{2\text{DOG}}$  units than of  $\text{TI}_{\text{DOG}}$  units was demonstrated, as shown in Fig. 7(b) ( $m/z$  1176.12 vs.  $m/z$  1175.11).

## CONCLUSIONS

This study shows the first evidence of hexameric and heptameric ellagitannins in the plant kingdom. The high-resolution HPLC/ESI-MS analysis allowed the molecular mass determination of an entire series of oligomeric ellagitannins from dimers to heptamers. The molecular masses together with characteristic fragmentations showed that the ellagitannin oligomers consisted of tellimagrandin I units only. This was further supported by all the reported oligomers having similar UV spectra. Earlier studies of *Oenothera biennis* ellagitannins have shown that only monomers, dimers and trimers can be quantified by HPLC-DAD from the non-fractionated extracts.<sup>7</sup> The now reported tetra- to heptamers are found in crude extracts in low concentrations and are chromatographically overlapped by other phenolics. In the future, to clarify the role of these complex ellagitannins in *O. biennis* – or in other plant species candidates – they need to be quantified by triple quadrupole or ion trap instruments using for example ion trace analysis<sup>14</sup> or, even better, by multiple reaction monitoring that utilises the observed fragmentation patterns of the oligomers.

## Acknowledgements

We would like to thank Ms Piia Saarinen for technical assistance with the sample preparation and Ms Terhi Sundman and Dr Olli Martiskainen for the assistance with HPLC/ESI-MS analysis. This work was financially supported by the Academy of Finland (Grant No. 119659) and the US National Science Foundation (DEB-0950231).

## REFERENCES

1. Yoshida T, Chou T, Matsuda M, Yasuhara T, Yazaki K, Hatano T, Nitta A, Okuda T. *Chem. Pharm. Bull.* 1991; **39**: 1157.
2. Barbehenn RV, Jones CP, Hagerman AE, Karonen M, Salminen J-P. *J. Chem. Ecol.* 2006; **32**: 2253.
3. Barbehenn RV, Jones CP, Karonen M, Salminen J-P. *J. Chem. Ecol.* 2006; **32**: 2235.
4. Moilanen J, Salminen J-P. *Chemoecology* 2008; **18**: 73.
5. Okuda T, Yoshida T, Hatano T, Ito H. Ellagitannins renewed the concept of tannins. In *Chemistry and Biology of Ellagitannins: An Underestimated Class of Bioactive Plant Polyphenols*, Quideau S (ed). World Scientific: Singapore, 2009.
6. Coca A, Feldman KS, Lawlor MD. Immunomodulatory ellagitannin chemistry. In *Chemistry and Biology of Ellagitannins: An Underestimated Class of Bioactive Plant Polyphenols*, Quideau S (ed). World Scientific: Singapore, 2009.
7. Johnson MTJ, Agrawal AA, Maron JL, Salminen J-P. *J. Evol. Biol.* 2009; **22**: 1295.
8. Hatano T, Okonogi A, Yazaki K, Okuda T. *Chem. Pharm. Bull.* 1990; **38**: 2707.
9. Parker JD, Salminen J-P, Agrawal AA. *Ecol. Lett.* 2010; **13**: 553.
10. Salminen J-P, Ossipov V, Lojonen J, Haukioja E, Pihlaja K. *J. Chromatogr. A* 1999; **864**: 283.
11. Yoshida T, Ito H, Hipolito I. *Phytochemistry* 2005; **66**: 1972.
12. Hatano T, Yasuhara T, Matsuda M, Yazaki K, Yoshida T, Okuda T. *J. Chem. Soc. Perkin Trans. 1* 1990; **10**: 2735.
13. Ahmed AF, Yoshida T, Okuda T. *Chem. Pharm. Bull.* 1994; **42**: 246.
14. Salminen J-P, Ossipov V, Haukioja E, Pihlaja K. *Phytochemistry* 2001; **57**: 15.