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Biosynthesis of the dimeric ellagitannin, cornusiin E, in *Tellima grandiflora*

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Dedicated to the memory of Professor Jeffrey B. Harborne

Abstract

First evidence for the in vitro synthesis of a dimeric ellagitannin has been obtained with cell-free extracts from the weed *Tellima grandiflora* (fringe cups, Saxifragaceae). Partially purified enzyme preparations from leaves of this plant catalyzed the oxidation of 1,2,3,4,6-pentagalloyl-β-D-glucose to the monomeric ellagitannin, tellimagrandin II, followed by oxidative coupling of two units of this intermediate to yield a dimeric derivative. Chemical degradation, MALDI-TOF mass spectrometry, ^{1}H and ^{13}C nuclear magnetic resonance, and CD spectroscopy were employed to identify this enzyme reaction product as cornusiin E which is characterized by a *(S)*-valoneoyl bridge between glucose-positions 2, 4′ and 6′. This result was supported by comparison with data obtained for cornusiin E that had been isolated from leaves of intact *T. grandiflora* plants. No indication for the earlier proposed existence of rugosin D (an isomer with a 1,4′,6′-bound valoneoyl unit) in *T. grandiflora* has been obtained in this investigation.
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1. Introduction

According to a traditional definition (Freudenberg, 1920), plant tannins are classified as flavonoid-derived proanthocyanins (or condensed tannins) and hydrolyzable tannins. The latter group comprises a wide variety of polygalloyl ester derivatives of a central polyol carbohydrate moiety, typically β-D-glucopyranose, and is usually divided into the gallotannin and ellagitannin subclasses. It is now generally accepted that 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose (compound **1** in Fig. 4) represents the common and immediate precursor of both groups. For the biosynthesis of gallotannins, this pivotal intermediate is substituted with additional *meta*-depsidically linked galloyl units. On the basis of recent enzyme studies, a metabolic scheme summarizing the major routes to hexa- and heptagalloylglucoses has been presented (Fröhlich et al., 2002). Our insights into the biosynthesis of ellagitannins, in contrast, are still in their infancy, in spite of the fact that this subclass clearly predominates the rather limited number of gallotannins in Nature, spanning over 500 structurally characterized members (Feldman and Sahasrabudhe, 1999). Already decades ago, it has been postulated that suitably orientated galloyl residues of the pentagalloylglucose core should be connected by oxidative processes that form the 3,4,5,3′,4′,5′-hexahydroxydiphenol (HHDP) moieties characteristic of ellagitannins (Schmidt and Mayer, 1956), a view that was refined later by Haslam and coworkers (reviewed in detail, e.g., by Haslam 1989, 1998). Eventual subsequent oxidation steps were thought to produce a host of oligomeric derivatives by formation of intermolecular C–O and/or C–C linkages; so far, more than 150 ellagitannin dimers, trimers and tetramers have been isolated (Okuda et al., 1993).

First experimental proof of the correctness of the merely theoretical considerations on the biosynthesis of ellagitannins has been presented in a recent paper (Niemetz et al., 2001) reporting the in vitro oxidation of pentagalloylglucose (**1**) to the monomeric ellagitannin, tellimagrandin II (**2**), by enzyme extracts from leaves of

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the weed *Tellima grandiflora* (fringe cups, Saxifragaceae). Closer investigations led to the isolation of an O_2-dependent laccase-type phenol oxidase that specifically formed tellimagrandin II (Niemetz and Gross, 2003). Analysis of side-products encountered in these studies, obtained with crude enzyme preparations, revealed the existence of a higher molecular weight compound that was identified as cornusiin E (**3**), i.e. a dimeric elagitannin that apparently resulted from the oxidative condensation of two molecules of tellimagrandin II (**2**). The in vitro formation, structure identification and isolation of this compound from native *T. grandiflora* plants is reported below.

2. Results and discussion

2.1. Enzyme studies

Cell-free extracts were obtained by homogenizing leaves of *T. grandiflora* in liquid N_2 and extraction of the powder with a mixture of strong Tris–HCl buffer supplemented with borate that complexes inhibitory endogenous plant phenolics. The resulting crude extracts were depleted of residual contaminants by stirring with Amberlite XAD ion-exchange resin, followed by ammonium sulfate fractionation, hydrophobic-interaction chromatography (HIC) on butyl sepharose and a final column chromatography step on hydroxyapatite (for details, see Experimental). As depicted in Fig. 1A, incubation for 30 min under standard assay conditions (45 °C, pH 5.0) of this partially purified enzyme with 1,2,3,4,6-pentagalloylglucose (**1**) as substrate afforded only one new product that was identified as the dimeric ellagitannin, cornusiin E (**3**) (cf. Section 2.2). Theoretically, such a transformation should require a monomeric ellagitannin as intermediate, most likely tellimagrandin II (**2**). This latter compound was actually detected after short-time incubation (3 min) of reaction mixtures (Fig. 1B), indicating its extremely efficient conversion to the dimeric end-product. The supposed role of tellimagrandin II (**2**) as intermediate metabolite was finally proven in a third experiment in which this compound was found to be directly converted to cornusiin E (**3**) (Fig. 1C). When [*U*-^{14}C]-labelled pentagalloylglucose was used as substrate, both reaction products were found to be radioactive. This finding was important as it provided unequivocal proof that the isolated ellagitannins had actually been synthesized in vitro. It should be emphasized that, owing to the 'tanning' properties of these compounds, i.e. their strong tendency to form complexes with proteins and carbohydrates (cf., e.g., Haslam, 1989, 1998), such investigations always bear the inherent risk that enzyme assays are contaminated with in vivo formed polyphenols.

The above postulated intermediary role of tellimagrandin II (**2**) was corroborated in a time-course experiment with [*U*-^{14}C]pentagalloylglucose (**1**). As depicted in Fig. 2, the substrate **1** was rapidly oxidized to tellimagrandin II (**2**). This product, however, could not accumulate because it was almost simultaneously transformed to cornusiin E (**3**). After ca. 3 min, net synthesis was observed only for this end product, at the expense of tellimagrandin II (**2**). The experiment sug-

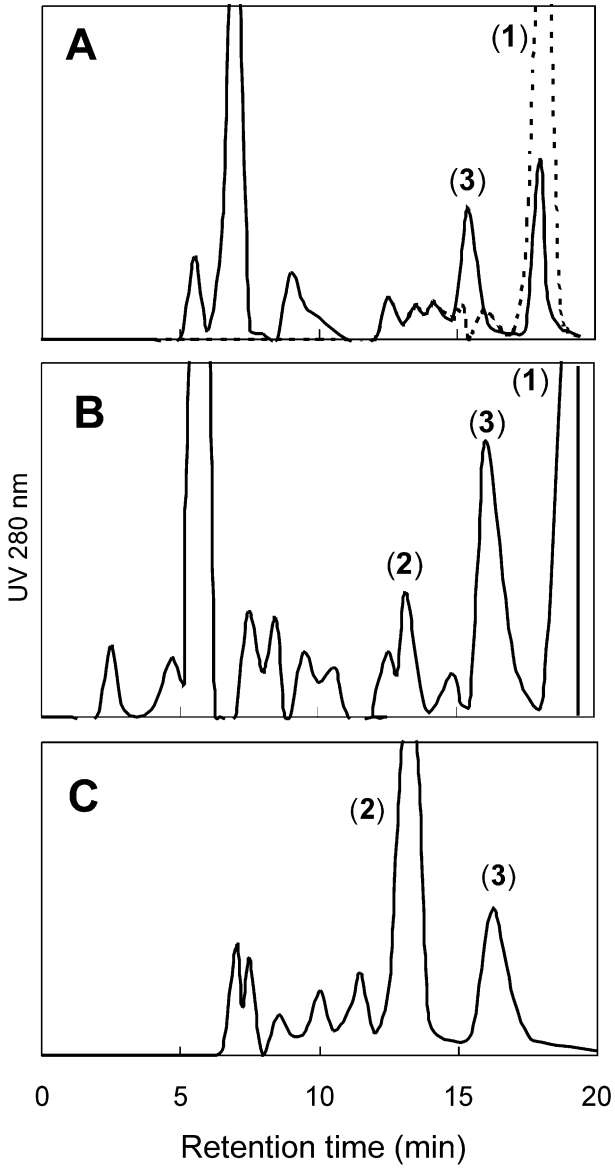

Fig. 1. HPLC analysis of enzyme reactions forming the dimeric ellagitannin, cornusiin E (**3**), with partially purified extracts from *Tellima grandiflora* leaves. (A) Oxidation of pentagalloylglucose (**1**) to cornusiin E (**3**) after incubation for 30 min at 45 °C. (B) The same reaction as in A, but analyzed in a short time experiment (3 min incubation), indicating the intermediacy of the monomeric ellagitannin, tellimagrandin II (**2**), in the biosynthesis of cornusiin E (**3**). (C) Direct dimerization (30 min, 45 °C) of tellimagrandin II (**2**) to cornusiin E (**3**). (–), Enzyme assays; (...), blank with heat-denatured enzyme. For HPLC conditions, see Experimental.

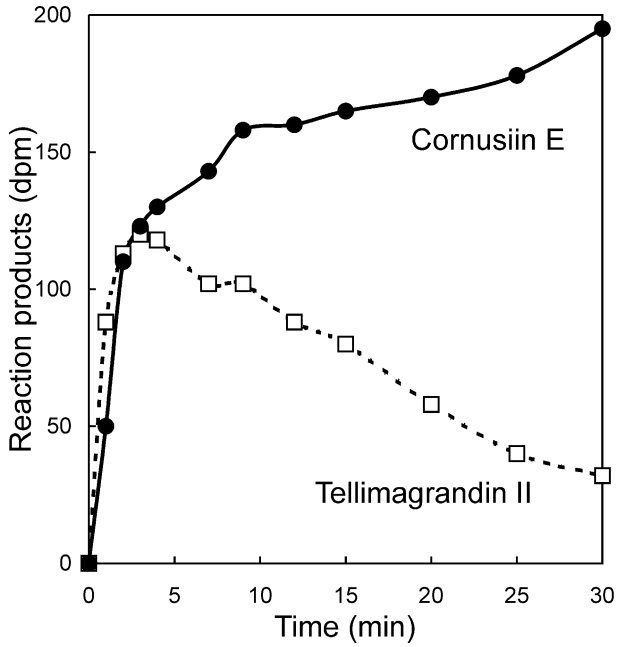

Fig. 2. Time course of the in vitro oxidation of [*U*-^{14}C]pentagalloylglucose to tellimagrandin II (**2**) (...□...) and subsequent dimerization of this intermediate to cornusiin E (**3**) (–●–).

gested a pronounced affinity of the enzyme towards tellimagrandin II (**2**). This assumption was supported by analyzing the substrate dependence of this compound which revealed an initial lag phase from 0 to ca. 4 μM tellimagrandin II (**2**), followed by normal Michaelis–Menten kinetics up to ca. 15 μM; pronounced substrate inhibition was observed at higher concentrations. Replotting the data according to Lineweaver and Burk (1934) revealed a K_m of 8 μM. For comparison, the enzyme catalyzing the first step in this sequence, i.e. oxidation of pentagalloylglucose (**1**) to tellimagrandin II (**2**), displayed a relatively high K_m of 80 μM (Niemetz and Gross, 2003).

2.2. Reaction product identification

For the unambiguous identification of the dimeric enzyme reaction product, 100 mg of pentagalloylglucose (**1**) were incubated in a scaled-up assay from which 3 mg of product with a purity of >95% could be isolated (for details, see Experimental). To facilitate NMR spectroscopy studies, greater amounts of this compound were purified from dried leaves of *T. grandiflora* (cf. Experimental); both preparations were found to be identical.

MALDI-TOF analysis displayed a molecular weight of 1874.3 (M^+) which is consistent with the expected formula $C_{82}H_{58}O_{52}$ for a dimeric derivative of tellimagrandin II (**2**). RP-HPLC of a total acid hydrolysate of the reaction product revealed gallic, ellagic and valoneic acid as phenolic constituents, while sanguisorbic acid (an isomer of valoneic acid that functions as analogous linking unit in various ellagitannins) was absent (Fig. 3). (*S*)-Configuration of both the HHDP and valoneoyl moiety was shown in the circular dichroism (CD) spectrum by a strong positive Cotton effect at 225 nm (+95.3).

Definitive structural proof was achieved by ^{1}H and ^{13}C NMR spectroscopy. ^{1}H NMR spectra revealed five galloyl groups, one valoneoyl group and one HHDP group. The δ values of the two glucose units, determined by COSY and TOCSY experiments, showed complete acylation of all OH groups and β-configuration of glucoses I and II (for numbering, refer to **3** in Fig. 4). The linkage between the two glucose units was determined by HMQC and HMBC experiments via the ester CO signals, showing that ring C of the valoneoyl group was connected to O-2 of glucose I, while rings A and B were bonded to O-6′ and O-4′ of glucose II, respectively. The data provided clear evidence that this compound was identical with cornusiin E (**3**) that had been isolated from fruits of *Cornus officinalis* by Hatano et al. (1989).

2.3. Concluding remarks

Our results present first evidence of the enzymatic formation of a dimeric ellagitannin, cornusiin E (**3**). As depicted in Fig. 4, the reaction involves oxidation of pentagalloylglucose (**1**), the committed precursor of both gallotannins and ellagitannins, to a monomeric ellagitannin, tellimagrandin II (**2**), which in turn undergoes dimerization to the end product. This metabolic

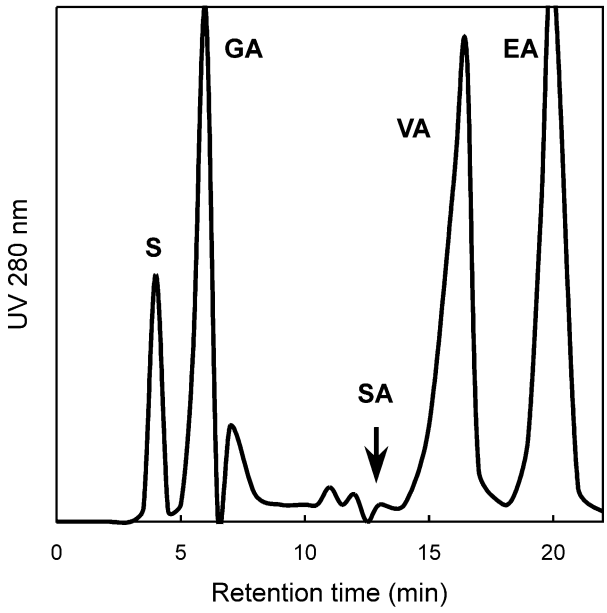

Fig. 3. HPLC analysis of phenolic components after total acid hydrolysis of the enzyme reaction product, cornusiin E (**3**). S, Solvent peak; GA, gallic acid; VA, (*S*)-valoneic acid; EA, ellagic acid. The retention time of (*S*)-sanguisorbic acid (SA), a naturally occurring isomer of valoneic acid with the galloyl residue attached in *ortho*-position to the HHDP moiety, is indicated by the arrow.

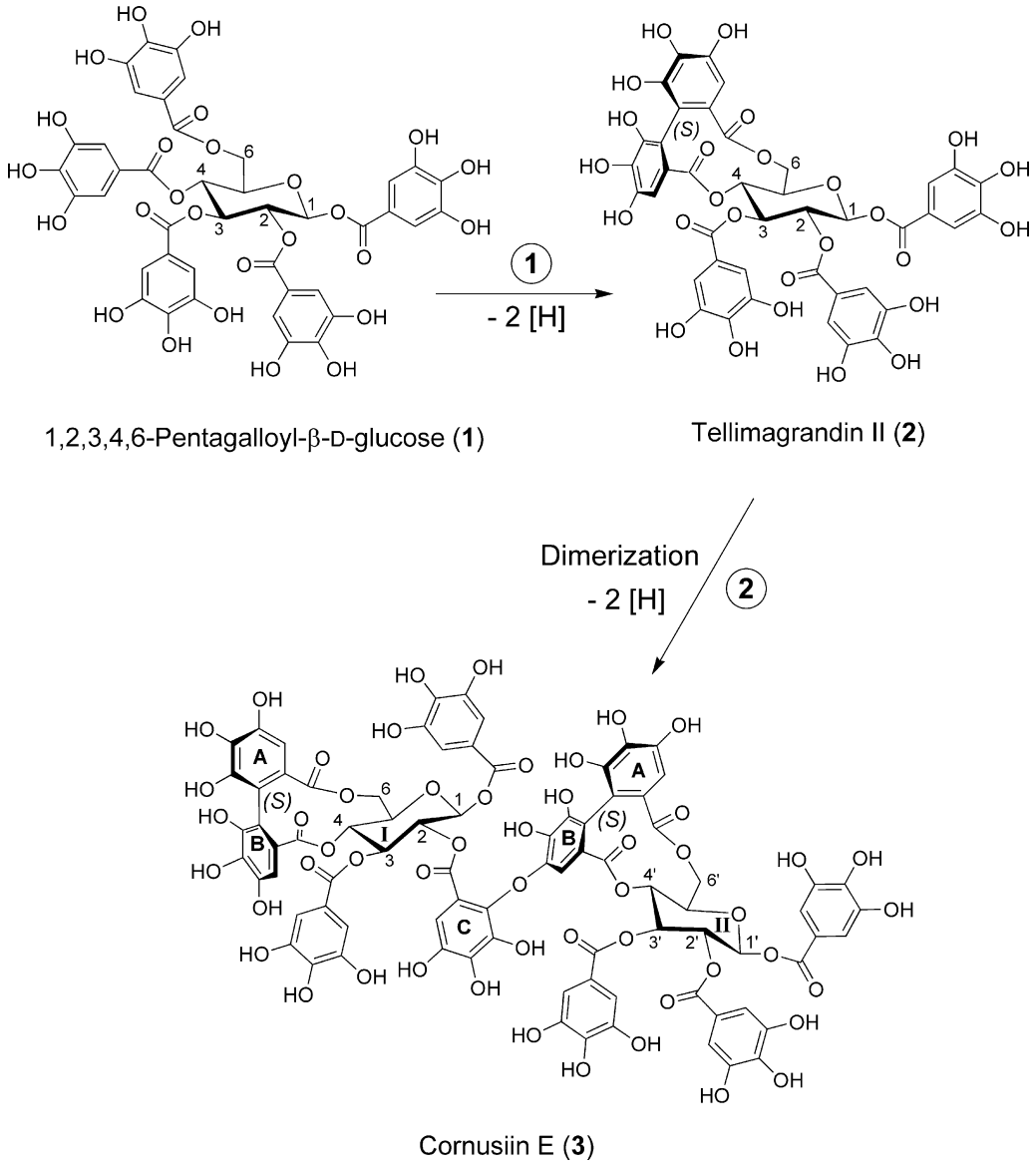

Fig. 4. Pathway from 1,2,3,4,6-pentagalloylglucose (**1**) to the dimeric ellagitannin, cornusiin E (**2**), in *Tellima grandiflora*. In reaction ①, pentagalloylglucose (**1**) is oxidized to the monomeric ellagitannin, tellimagrandin II (**2**) (Niemetz et al., 2001; Niemetz and Gross, 2003). This intermediate undergoes oxidative dimerization to yield cornusiin E (**3**) (reaction ②). Labelling A–C, I, II in (**3**) designates groups relevant to NMR analyses (cf. Section 3.5).

sequence is particularly fascinating because of its strict product specificity—no formation of alternative linkages as realized in other plants has been observed in this investigation. Step 1 of the pathway, i.e. the formation of tellimagrandin II (**2**), has been recently found to be specifically catalyzed by a laccase-type phenol oxidase (Niemetz and Gross, 2003). However, it is still unknown to date whether the transitions reported here depend on a single, rather unspecific enzyme, or whether two individual enzymes are involved. Current activities in our laboratory are devoted to this important question.

In an earlier broad survey on the taxonomic distribution of hydrolyzable tannins, based on paper chromatography as principal analytical tool, two then unidentified dimeric tannins (T_{2A}, T_{2B}) had been detected as prominent constituents of *T. grandiflora* leaves (Haddock et al., 1982). Their structures were later interpreted as rugosins D (T_{2B}) and E (T_{2A}), respectively (Haslam, 1989). These two compounds have been isolated by Hatano et al. (1990) from flowers of *Rosa rugosa* and were identified as dimers originating from two tellimagrandin II units (rugosin D) or from a combination of tellimagrandin I and II (rugosin E), both being connected by a 1,4′,6′-valoneoyl bridge. The results of our studies, based on enzyme studies and phytochemical analyses, do not lend any support to the existence of rugosin-type ellagitannin dimers in *T. grandiflora*. Instead, it must be concluded that meta-

bolic activities in this plant are specifically directed towards the biogenesis of cornusiin-type (i.e. 2,4′,6′-linked) ellagitannin dimers.

3. Experimental

3.1. Plant material

Young leaves (2–4 months old) from greenhouse grown *T. grandiflora* (Pursh) Lindley (fringe cups, Saxifragacea) plants were washed with dist. H_2O and either immediately used as enzyme source, or frozen in liquid N_2 and stored at −20 °C in evacuated plastic bags where they could be kept for more than 6 months without apparent loss of enzyme activity.

3.2. Chemicals

[*U*-^{14}C]Pentagalloylglucose (**1**) was prepared by photoassimilation of $^{14}CO_2$ with leaves of *Rhus typhina* (staghorn sumac) and isolated in >99% purity by Sephadex LH-20 chromatography and preparative reversed-phase HPLC (Rausch and Gross, 1996); unlabelled pentagalloylglucose (**1**) was isolated from dried leaves of *R. typhina* by the same techniques. Tellimagrandin II (**2**) was prepared enzymatically (Niemetz and Gross, 2003). Rugosin D (Hatano et al., 1990) and Sanguiin H-6 (Okuda et al., 1992) were gifts of Prof. T. Yoshida (Okayama). Valoneic and sanguisorbic acid, respectively, were obtained from these compounds by hydrolyzing ca. 0.5 mg of each compound in 200 µl 6 N HCl at 100 °C for 4 h. After adjusting the pH to 5.0, 50 µl aliquots were analyzed by isocratic RP-HPLC on Reprosil NE (5 µm; 250×4 mm i.d.; solvent 0.01% aq. TFA/EtOH/EtOAc = 10:2:1, by vol.; flow rate 0.5 ml min^{-1}). Cornusiin E (**3**) was hydrolyzed identically.

3.3. Cornusiin E (*3*) from native plant material

Cornusiin E (**3**) was isolated from *T. grandiflora* leaves that had been dried overnight at 30 °C, followed by powdering in an ultracentrifugal mill (Retsch KG, Haan, Germany) in liquid N_2 and extracting with 70% aq. acetone on a gyrotory shaker at room temp. for 3 days. After removing the acetone by rotary evaporation, the aqueous phase was washed 5-times with petroleum benzene (40–60 °C) and lyophilized. A 200 mg aliquot of the solid residue was dissolved in 5 ml H_2O and subjected to semi-preparative isocratic RP-HPLC (modified from Yoshida et al., 1991) on Kromasil (5 µm, 250×20 mm i.d.; solvent: 0.01% aq. TFA/EtOH/EtOAc = 10:2:1; flow rate 12 ml min^{-1}; detection UV 280 nm). Relevant fractions were depleted of acid and organic solvent by rotary evaporation and lyophilized to yield 50 mg of >95% pure product.

3.4. Enzyme preparation and assay

Enzyme from leaves of *T. grandiflora* was extracted and partially purified according to Niemetz and Gross (2003).The enzyme fraction eluting with 0.6 M ammonium sulfate from the butyl-sepharose column was subjected to a final purification step on a hydroxyapatite column from which it was recovered with 0.3 M Na phosphate buffer. Standard enzyme activity assays, containing 12.5 µg (42 Bq) pentagalloylglucose (**1**) and enzyme in 50 µl acetate buffer (50 mM, pH 5.0), were incubated at 45 °C for 30 min, stopped by heating (100 °C, 5 min), and analyzed by isocratic RP-18 HPLC on Reprosil NE (cf. Section 3.2). Acid-denatured enzyme was used as a control. Radioactivity was determined by fractionation of eluates and subsequent liquid-scintillation counting.

3.5. In vitro synthesis and analysis of cornusiin E (*3*)

For the large-scale preparation of cornusiin E, *T. grandiflora* leaves (150 g) were homogenized, extracted and treated with Amberlite XAD ion-exchange resin according to Niemetz and Gross (2003). The pellet (800 mg protein) of the subsequent 30–90% ammonium sulfate precipitation step was dissolved in acetate buffer (50 mM, pH 5.0), desalted on Sephadex G-25 (Pharmacia; 30×1.6 cm i.d.) and diluted to 350 ml with buffer for use in a scaled-up enzyme assay mixture containing 100 mg pentagalloylglucose (**1**) as substrate. After incubation for 60 min at 45 °C, the reaction was stopped by heat-denaturing the enzyme. The supernatant of the centrifuged mixture was brought to pH 4.0 with 1 N HOAc and extracted 10 times with EtOAc. After removing the organic solvent by rotary evaporation, the residue was dissolved in 10 ml H_2O and subjected in two portions to semi-preparative gradient RP-18 HPLC on Kromasil (5 µm, 250×20 mm i.d.; gradient: solvent A = 0.01% aq. TFA, B = acetonitrile; 0–1 min 5% B, 1–2 min 5–18% B, then isocratic at 18% B; flow rate 22 ml/min). Relevant fractions were immediately depleted of organic solvent and TFA in vacuo, lyophilized and subjected to further isocratic RP-18 HPLC on the same column (solvent 0.01% aq. TFA/EtOH/EtOAc = 10:2:1; by vol.; flow rate 12 ml min^{-1}). The product-containing fractions were neutralized, depleted of solvent and lyophylised, affording 3 mg material of >95% purity as determined by analytical HPLC (for the isolation of cornusiin E (**3**) from native plant material, see Section 3.3).

3.5.1. Analysis of (*3*)

MALDI-TOF MS: the compound was dissolved in MeOH, and a 40% solution of isopropanol, imidazole and piperidine (1:1:1) in MeOH was added for ESI spectrometry (Finnigan MAT TSQ 700); *m*/*z* 936.5

(M^{2+}), 1874.3 (M^{+}). CD (MeOH): 225 nm (+95.2), 237 (sh), 260 (−13.3), 281 (+26.5), 315 (−3.6). ^{1}H and ^{13}C NMR spectra were recorded in acetone-d_6 on a Bruker DEX 500 MHz spectrometer; δ ppm (TMS): 7.17, 7.14 (s, 2H, 1,3-galloyl glc_I); 7.13, 6.99, 6.86 (s, 2H, 1′,2′,3′-galloyl glc_{II}); 6.72, 6.31, 6.85 (s, 1H, valoneoyl H-A,B,C); 6.59, 6.53 (s, 1H, HHDP-A,B); 5.69 (glc_I H-1, $J_{1H,2H}$ = 8.1 Hz), 5.55 (glc_I H-2, $J_{2H,3H}$ = 9.5 Hz), 5.53 (glc_I H-3, $J_{3H,4H}$ = 9.8 Hz), 5.11 (glc_I H-4, $J_{4H,5H}$ = 9.8 Hz), 4.46 (glc_I H-5, $J_{5H,6H}$ = 7.9 Hz), 5.16 (glc_I H-6, $J_{5H,6'H}$ = < 1Hz), 3.76 (glc_I H-6′, $J_{6H,6'H}$ = 13.3Hz); 6.22 (glc_{II} H-1, $J_{1H,2H}$ = 8.4 Hz), 5.58 (glc_{II} H-2, $J_{2H,3H}$ = 9.5 Hz), 5.68 (glc_{II} H-3, $J_{3H,4H}$ = 9.8 Hz), 5.15 (glc_{II} H-1, $J_{4H,5H}$ = 10.1 Hz), 4.81 (glc_{II} H-5, $J_{5H,6H}$ = 10.1 Hz), 5.60 (glc_{II} H-6, $J_{5H,6'H}$ = < 1 Hz), 3.96 (glc_{II} H-6′, $J_{6H,6'H}$ = 13.1 Hz). These data were fully consistent with those of Hatano et al. (1989). In addition, ^{13}C shifts of CO carbonyls were determined: 167.54 (val_A), 166.92 (val_B), 164.92 (val_C); 167.21 ($HHDP_A$), 166.83 ($HHDP_B$); 164.40 (gal glc_I O-1), 165.58 (gal glc_I O-3), 164.98 (gal glc_{II} O-1), 164.62 (gal glc_{II} O-2), 164.92 (gal glc_{II} O-3).

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