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β-Peltatin 6-*O*-methyltransferase from suspension cultures of *Linum nodiflorum*

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Dedicated to the memory of Professor Jeffrey B. Harborne

Abstract

S-Adenosyl-L-methionine:β-peltatin 6-*O*-methyltransferase was isolated and characterized from cell suspension cultures of *Linum nodiflorum* L. (Linaceae), a *Linum* species accumulating aryltetralin lignans such as 6-methoxypodophyllotoxin. The enzyme transfers a methyl group from S-adenosyl-L-methionine to the only free OH-group of β-peltatin in position 6 thus forming β-peltatin-A methylether. This reaction is a putative biosynthetic step in the biosynthesis of 6-methoxypodophyllotoxin from deoxypodophyllotoxin. The enzyme has a pH-optimum at pH 7.7 and a temperature optimum at 40 °C. The enzyme activity is strongly inhibited by $MnSO_4$, $FeCl_3$, $FeSO_4$ and $ZnSO_4$ as well as S-adenosyl-homocysteine. Mg^{2+} and EDTA did not influence the methylation of β-peltatin. Substrate saturation curves were obtained for S-adenosyl-methionine and β-peltatin and apparent K_m-values of 15 μM and 40 μM, respectively, were determined for these substrates. Substrate inhibition was observed for β-peltatin. No other lignan substrate tested nor caffeic acid were accepted. The suspension cell line of *Linum nodiflorum* was characterized with respect to growth, medium alterations and lignan production as well as activity of SAM:β-peltatin 6-*O*-methyltransferase. Highest specific activities of β-peltatin 6-*O*-methyltransferase were determined on day 7 of the culture period corresponding to the highest levels of 6-methoxypodophyllotoxin on days 7 to 12.

Keywords: *Linum nodiflorum*; Linaceae; Plant cell cultures; Aryltetralin lignans; β-peltatin; 6-methoxypodophyllotoxin; Lignan biosynthesis; *O*-methyltransferase

1. Introduction

Aryltetralin lignans derived from the naturally occurring podophyllotoxin **1** are important cytotoxic drugs used in cancer treatment. Currently, podophyllotoxin **1** is isolated from rhizomes of *Podophyllum hexandrum* (Podophyllaceae) growing in the Himalayas, which is still collected from the wild and therefore counts among the endangered species. Podophyllotoxin **1** is then semi-synthetically transformed to e.g. Teniposide, Etoposide and Etopophos®. These compounds act cytotoxically by binding to DNA/topoisomerase II complexes and thus inducing DNA strand breakages (Damayanthi and Lown, 1998; Imbert, 1999). This is in contrast to the microtubule-inhibiting activity of the parent compound podophyllotoxin **1** itself. Podophyllotoxin **1** and related compounds are not only present in the family Podophyllaceae, but also in e.g. Juniperaceae, Lamiaceae and Linaceae (Petersen and Alfermann, 2001). We are currently concentrating on species of the Linaceae, mainly the species *Linum album*, *L. flavum* and *L. nodiflorum* L. (Smollny et al., 1998; Empt et al., 2000; Petersen und Alfermann, 2001; Kuhlmann et al., 2002). Cell cultures of these *Linum* species accumulate considerable amounts of podophyllotoxin **1** and/or 6-methoxypodophyllotoxin **2**. Therefore, these cell cultures can serve as suitable systems for the investigation of the biosynthesis of the aryltetralin lignans.

It is generally accepted that most lignans are dimers of hydroxycinnamyl alcohol moieties, e.g. coniferyl alcohol. They can be stereospecifically coupled to (+)-pinoresinol by a system of two proteins called "pinoresinol synthase" consisting of a laccase and a non-catalytic "dirigent protein" acting together (Davin et al., 1997).

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(+)-Pinoresinol is reduced via (+)-lariciresinol to (−)-secoisolariciresinol by pinoresinol/lariciresinol reductase (Dinkova-Kostova et al., 1996) and then oxidized to (−)-matairesinol by secoisolariciresinol dehydrogenase (Xia et al., 2001). The biosynthetic pathway from matairesinol to the aryltetralin lignans is not yet clarified. Feeding experiments with plant cell cultures suggested that deoxypodophyllotoxin **3** is the first aryltetralin lignan and the common precursor for the biosynthesis of both, podophyllotoxin **1** and 6-methoxypodophyllotoxin **2** (Van Uden et al., 1995, 1997). These two lignans **1**, **2** are the main lignans in the above-mentioned *Linum* species where they are stored as glucosides in the vacuole (Henges, 1999). Other experiments with *Linum flavum* and *Podophyllum peltatum* suggested a biosynthetic pathway via 7-hydroxymatairesinol to 6-methoxypodophyllotoxin **2** (Xia et al., 2000). First hints towards the enzyme hydroxylating deoxypodophyllotoxin **3** in position 7 to podophyllotoxin **1** were found by Henges (1999) in microsomal preparations from cell cultures of *Linum album*. The enzyme introducing a hydroxyl group in position 6 of deoxypodophyllotoxin **3** forming β-peltatin **4** was detected and characterized from cell cultures of *Linum flavum* and *L. nodiflorum*. This deoxypodophyllotoxin 6-hydroxylase (DOP6H) was characterized as a cytochrome P450 enzyme system (Molog et al., 2001; Kuhlmann et al., 2002). The product β-peltatin **4** is further methylated in position 6 to β-peltatin-A methylether **5** and finally hydroxylated in position 7 to 6-methoxypodophyllotoxin **2**. 6-Methoxypodophyllotoxin **2** as well as podophyllotoxin **1** in cells accumulating this lignan are safely stored in the vacuole as glucosides (Henges et al., 1999) thus preventing cytotoxic effects on the producing cells. We here describe the identification and characterization of a new enzyme in plant secondary metabolism, S-adenosyl-L-methionine:β-peltatin 6-*O*-methyltransferase (β-peltatin 6OMT), from *Linum nodiflorum* suspension cultures, the S-adenosylmethionine-dependent methyltransferase responsible for the transformation of β-peltatin **4** to β-peltatin-A methylether **5**.

2. Results and discussion

2.1. Identification and characterization of S-adenosylmethionine:β-peltatin 6-O-methyltransferase

According to precursor administration, the biosynthetic pathway from deoxypodophyllotoxin **3** to 6-methoxypodophyllotoxin **2** follows the steps (1) hydroxylation of deoxypodophyllotoxin **3** to β-peltatin **4**, (2) methylation of β-peltatin **4** to β-peltatin-A methylether **5** and (3) 7-hydroxylation of the latter to 6-methoxypodophyllotoxin **2** (Van Uden et al., 1995, 1997) (Fig. 1). Previous studies have shown the presence

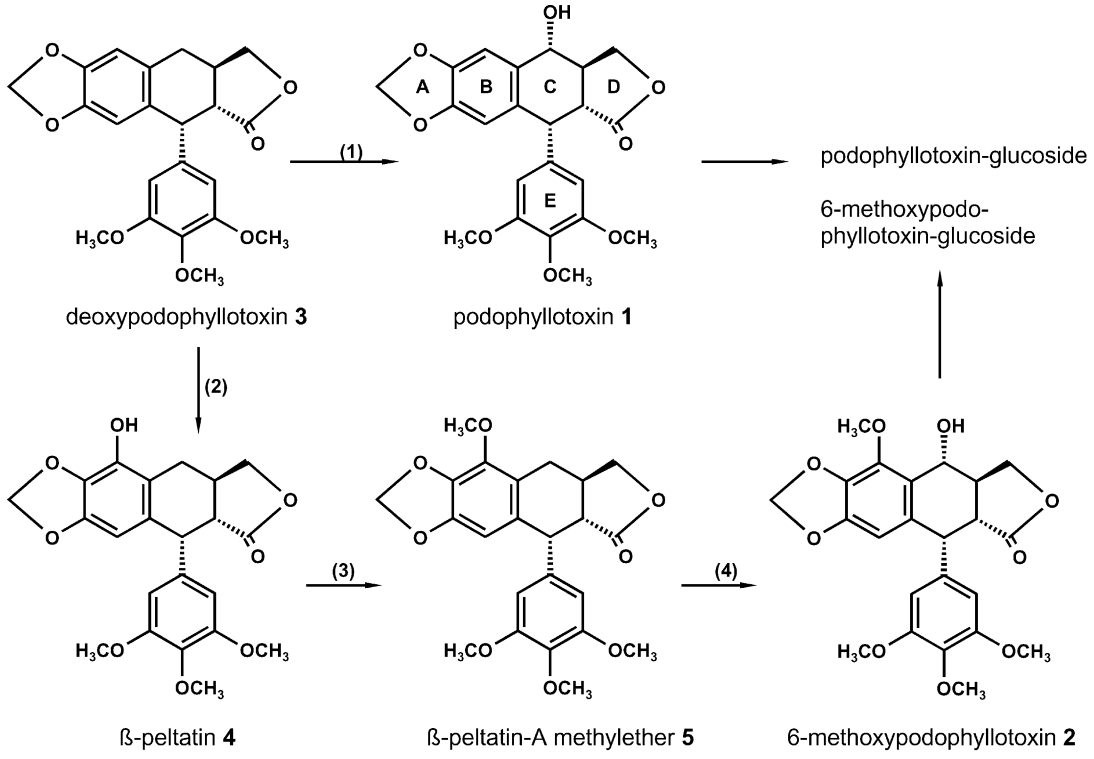

Fig. 1. Hypothetical scheme for the biosynthesis of the aryltetralin lignans podophyllotoxin **1** and 6-methoxypodophyllotoxin **2** from deoxypodophyllotoxin **3**; (1) = deoxypodophyllotoxin 7-hydroxylase, (2) = deoxypodophyllotoxin 6-hydroxylase, (3) = SAM:β-peltatin 6-*O*-methyltransferase, (4) = β-peltatin-A methylether 7-hydroxylase.

of deoxypodophyllotoxin 6-hydroxylase in cell cultures of *Linum flavum* and *L. nodiflorum* that catalyzes the formation of β-peltatin **4**, the 6-hydroxy substrate for this hence uncharacterized *O*-methyltransferase reaction (Molog et al., 2001; Kuhlmann et al., 2002). *O*-Methylation reactions in plant secondary metabolism are mostly dependent on S-adenosylmethionine (SAM) as methyl donor and therefore we incubated protein preparations from cell cultures of *Linum nodiflorum* with SAM and β-peltatin **4** and were able to demonstrate the formation of β-peltatin-A methylether **5** by HPLC analysis. The reaction product was identified by co-chromatography with authentic β-peltatin-A methylether **5** on different columns (Hypersil ODS, Hypersil HyPurity Elite C_{18}) and with different solvent systems (30–50% acetonitrile; 60% methanol). Furthermore, diode array-detection showed identical retention times and UV-spectra of the reaction product and authentic β-peltatin-A methylether **5**. The same protein preparations did not show methylation of caffeic acid to ferulic acid excluding side activity of caffeic acid *O*-methyltransferase. The enzyme is soluble and can be precipitated between 40 and 80% saturation of ammonium sulfate; this, however, often results in severe loss of enzyme activity. The enzyme activity is essentially dependent on the addition of the substrates β-peltatin **4** and SAM, but no other co-factors are needed such as for example Mg^{2+} which has no effect on the activity of β-peltatin 6OMT. Mg^{2+} markedly stimulated the activity of e.g. caffeoyl-CoA *O*-methyltransferase from *Daucus carota* (Kühnl et al., 1989) or *Petroselinum crispum* (Pakusch et al., 1989) and is required for different quercetin *O*-methyltransferases from *Chrysosplenium americanum* (Ibrahim and De Luca, 1982). Correspondingly, EDTA has no influence on the β-peltatin 6OMT activity. Other ions showed inhibiting activities: 10 mM $MnSO_4$ inhibited the methylation of β-peltatin **4** down to a residual activity of 35%. Concentrations higher than 5 mM $FeSO_4$ or $FeCl_3$ resulted in activities lower than 10% of the control. Strongest inhibition was observed for $ZnSO_4$ which completely inhibited β-peltatin 6OMT activity at 5 mM. Similar effects have been observed for other *O*-methyltransferases from plants, e.g. isoflavone 4′-*O*-methyltransferase from *Cicer arietinum* (Wengenmayer et al., 1974) or norcoclaurine 6-*O*-methyltransferase from *Coptis japonica* (Sato et al., 1994). The temperature optimum of the methylation reaction was determined to be at 40 °C with a steep decline of the activity above 45 °C. For this reason standard assays were performed at 38 °C. The pH-optimum in Tris/HCl-buffer is at pH 7.7 with half-maximal activities at pH 6.2 and pH 8.5.

β-Peltatin 6OMT uses SAM as methyl donor. The enzyme showed Michaelis–Menten-kinetics for this substrate and was saturated at a concentration of 150 μM SAM. The apparent K_m-value was determined at 15 μM showing a high affinity for this substrate. The K_m-values reported for SAM for different plant *O*-methyltransferases vary over a wide range. Apparent K_m-values as low as 3 μM were determined for caffeate *O*-methyltransferase from *Nicotiana tabacum* (Collendavelloo et al., 1981) and *Medicago sativa* root nodules (Vance and Bryan, 1981) or 10-hydroxydihydrosanguinarine 10-*O*-methyltransferase from *Eschscholtzia californica* (De Eknamkul et al., 1992), values as high as 160 μM were reported for isoflavone 4′-*O*-methyltransferase from *Cicer arietinum* (Wengenmayer et al., 1974). S-Adenosylhomocysteine (SAH) strongly inhibited the methylation reaction in a concentration-dependent manner. Inhibition curves indicate competitive inhibition by SAH (which is a reaction product) as was already observed for other *O*-methyltransferases, for example caffeate *O*-methyltransferase from *Beta vulgaris* (Poulton and Butt, 1975) or isoflavone 4′-*O*-methyltransferase from *Cicer arietinum* (Wengenmayer et al., 1974).

The substrate specificity was determined using different potential substrates from the lignan biosynthetic pathway carrying a free OH-group (e.g. podophyllotoxin **1**, matairesinol, pinoresinol) as well as caffeic acid. A methylation reaction was only observed for β-peltatin **4**. For this substrate Michaelis–Menten-kinetics were determined with a marked substrate inhibition at concentrations higher than 100 μM β-peltatin **4** (Fig. 2). The apparent K_m-value for β-peltatin **4** was at 40 μM (Fig. 2 insert).

2.2. Characterization of a suspension cell line of Linum nodiflorum

A callus culture, and subsequently a suspension culture, was established from aseptically germinated seedlings of *Linum nodiflorum* (Konuklugil et al., 1999). The cell line used in this study consisted of relatively large brownish aggregates. This cell line was characterized for

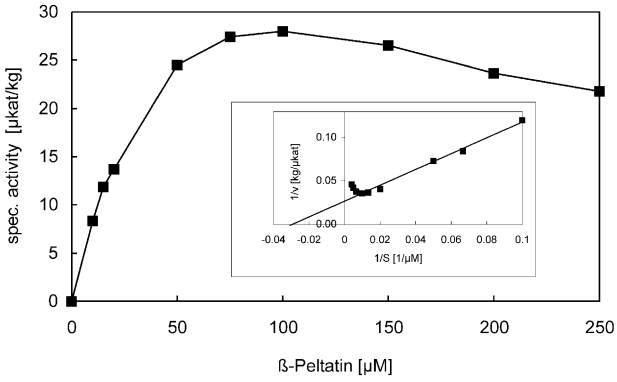

Fig. 2. Substrate saturation curve for β-peltatin **4** showing the substrate inhibition at concentrations higher than 100 μM; insert: Lineweaver–Burk-diagram for the determination of the apparent K_m-value at 40 μM.

15 days with respect to growth, alteration of the medium composition, lignan accumulation and activity of β-peltatin 6OMT. Growth was measured as fresh (FW) and dry weight (DW) accumulation (Fig. 3A). Fresh weight increased with a slight lag phase from the beginning of the culture period until day 10 and declined afterwards. Dry weight increases linearly from day 0 to 7 and then decreases slowly. As alterations in the culture medium, the sugar content, the pH and the conductivity were measured (Fig. 3B). Sugar consumption is seen by the decrease of the refractive index; the sugar content is very low from day 7 onwards and reaches a minimum value at day 9. Similarly ion concentrations as determined by conductivity measurements decreased until day 9. The changes observed after day 9 are due to cell lysis and cell death. The declining growth and death of the *Linum* cells is clearly correlated to the depletion of sugar from the medium since the stationary phase of fresh weight accumulation starts at day 7. The pH of the culture medium increases nearly constantly from pH 4.8 at the beginning of the culture period to about pH 7.6.

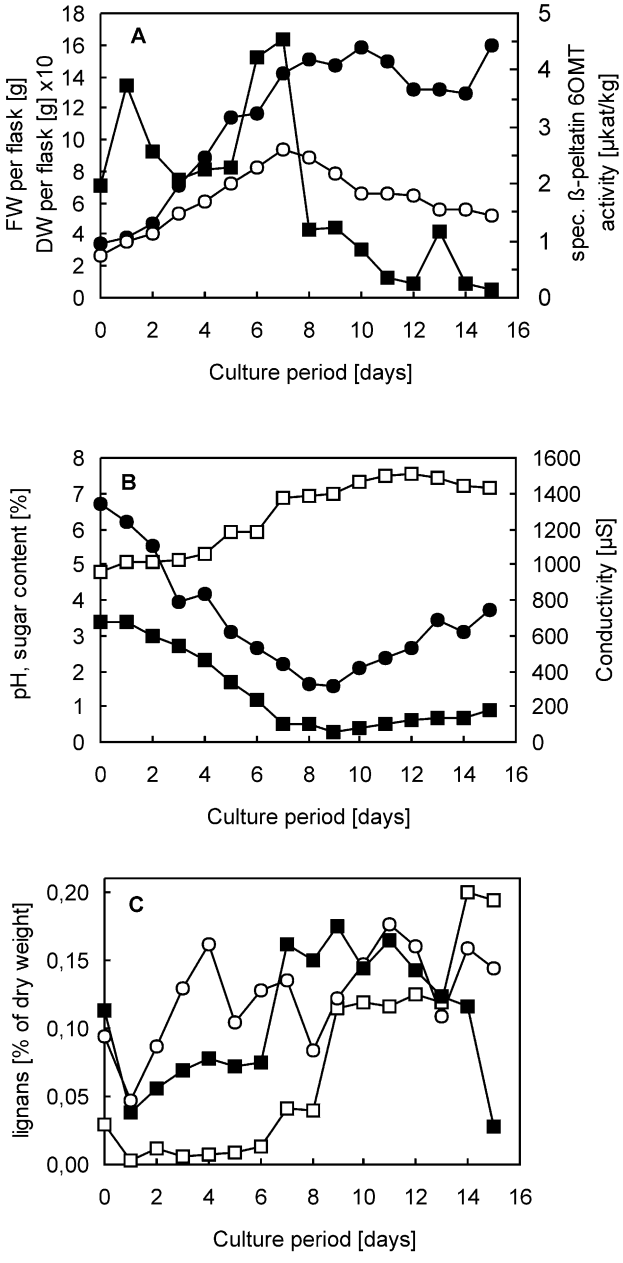

Fig. 3. Characterization of a suspension culture of *Linum nodiflorum*. A: Growth measured as fresh (FW, ●) and dry weight (DW, ○) accumulation and specific activity of β-peltatin 6OMT (■); B: Sugar content (■), pH (□) and conductivity (●) in the culture medium; C: Lignan content in the cells determined as aglyca: deoxypodophyllotoxin **3** (○), β-peltatin **4** (□) and 6-methoxypodophyllotoxin **2** (■); podophyllotoxin **1** and β-peltatin-A methylether **5** were not found in the cell extracts.

Lignans were isolated from the cells and quantified as the respective aglyca (Fig. 3C). Podophyllotoxin **1** and β-peltatin-A methylether **5** were not detected in these cell extracts. Deoxypodophyllotoxin **3**, the putative common precursor of podophyllotoxin **1** and 6-methoxypodophyllotoxin **2**, is present in the cells in quite high levels (0.08 to 0.18% of the cell dry weight) throughout the culture period. β-Peltatin **4** is only found in the second half of the culture period at higher levels. An increase is seen from day 6 onwards. Low levels of 6-methoxypodophyllotoxin **2** can be determined until day 6 of the culture period. Then the levels increase and show values around 0.15% of the cell dry weight from days 7 to 12 after which a strong decrease is observed which might be caused by degradation but is most probably due to cell lysis. Aryltetralin lignans could be extracted and determined from used culture medium (data not shown). In a different cell line of *Linum nodiflorum*, up to 1.7% 6-methoxypodophyllotoxin **2** in the cell dry weight were measured on day 9 of the culture period (Kuhlmann et al., 2002). The specific activity of β-peltatin 6OMT (Fig. 3A) shows a short increase just after inoculation of the cell culture and then the activity decreases until day 3. A strong increase in specific activity is observed until day 7, and then decreases until the end of the culture period. This correlates with the accumulation of 6-methoxypodophyllotoxin **2**. Attempts to enhance the activity of β-peltatin 6OMT by addition of 50 and 100 µM methyl jasmonate to the cultures were not successful. This correlates with earlier findings that methyl jasmonate as well as other well-known elicitors did not enhance the accumulation of aryltetralin lignans in *Linum* cell cultures (data not shown).

3. Conclusion

Suspension cultures of *Linum nodiflorum* L. (Linaceae) accumulate 6-methoxypodophyllotoxin(-glucoside) as one of the main cytotoxic lignans. The hypothetical biosynthetic pathway goes from deoxypodophyllotoxin **3** which is hydroxylated in position 6 by a cytochrome

P450 monooxygenase (deoxypodophyllotoxin 6-hydroxylase) to β-peltatin **4**. β-Peltatin **4** is then methylated to β-peltatin-A methylether **5** by SAM:β-peltatin 6-*O*-methyltransferase, now detected and characterized from cell cultures of *Linum nodiflorum*. The highest activity of this enzyme was found on day 7 of the culture period and is followed by the accumulation of 6-methoxypodophyllotoxin(-glucoside) at days 7 to 12 in the *Linum* cells. These findings support the suggested hypothetical biosynthetic pathway from deoxypodophyllotoxin **3** via β-peltatin **4** and β-peltatin-A methylether **5** to 6-methoxypodophyllotoxin **2**.

4. Experimental

4.1. Plant material

Callus cultures of *Linum nodiflorum* were established from seeds provided by the Institut für Pflanzengenetik und Kulturpflanzenforschung (Gatersleben, Germany). Cell suspension cultures of *Linum nodiflorum* were derived from these callus cultures by transferring 5 g callus cells to 50 ml medium in a 250 ml Erlenmeyer flask (Konuklugil et al., 1999). Standard medium for callus and suspension cultures was MS-medium (Murashige and Skoog, 1962) containing 30 g l^{-1} sucrose and 0.4 mg l^{-1} naphthalene acetic acid. Suspension cultures are grown in continuous light with shaking at 125 rpm. The suspension cultures are subcultivated every 7 days by transferring 5 g wet cells with a perforated spoon into 50 ml fresh medium.

4.2. Preparation of protein extracts

Suspension cells (7-day old) of *Linum nodiflorum* were separated from the medium by suction filtration. After addition of 1 ml buffer (0.1 M Tris/HCl, 1 mM dithiothreitol, pH 7.5; for the determination of the pH-optimum: 0.01 M Tris/HCl, 1 mM dithiothreitol, pH 7.5) and 0.1 g Polyclar 10 per 1 g cell fresh weight, the cells were homogenized (3×30 s) using an Ultraturrax (Janke and Kunkel, Freiburg im Breisgau, Germany) with intermediate cooling on ice. After centrifugation (4 °C, 12 000 *g*, 20 min) the supernatant was used as crude enzyme preparation. For most assays (except culture characterization) the protein fraction precipitating between 40 and 80% saturation of ammonium sulfate was used. Precipitated protein was sedimented (48,000 *g*, 4 °C, 20 min), redissolved in 0.1 M Tris/HCl, 1 mM dithiothreitol, pH 7.5 and desalted by gel filtration on PD-10 columns (Amersham Biosciences, Freiburg im Breisgau, Germany) using the same buffer. Protein concentrations were determined according to Bradford (1976) using bovine serum albumin (1 mg ml^{-1}) as standard.

4.3. Assay for β-peltatin 6-O-methyltransferase

The assays contained 150 μM β-peltatin **4**, 200 μM S-adenosyl-L-methionine and approximately 150 μg protein in a total volume of 250 μl 0.1 mM Tris/HCl (pH 7.5). They were incubated at 38 °C for 2 to 3 h (1 h for the determination of K_m-values). The reaction was stopped by adding 6 N HCl (50 μl). The assays were extracted with EtOAc (3×500 μl). The solvent was evaporated under vacuum and the residue resuspended in 50% aq. MeOH (50 μl) and analyzed by HPLC. The HPLC system consisted of a HyPurity Elite C_{18} column, 50% aq. MeCN as mobile phase at a flow of 1.25 ml/min. The detection wavelength was 280 nm. 0.1 mM β-peltatin-A methylether **5** was used as standard.

4.4. Inhibition of β-peltatin 6-O-methyltransferase

For the tests for inhibitors the standard assay was modified by adding 0 to 10 mM of $MgSO_4$, $MgCl_2$, $MnSO_4$, $ZnSO_4$, $FeSO_4$ and $FeCl_3$. S-Adenosyl-homocysteine as an inhibitor was added to the assays in a concentration range from 0 to 400 μM.

4.5. Characterization of suspension cultures of Linum nodiflorum

Suspension cultures of *L. nodiflorum* were inoculated as described above and cultivated under standard conditions for 16 days. Every day one to three flasks were harvested by suction filtration and the fresh weight recorded. Fresh cells (5 g) were frozen at −20 °C and lyophilized for 48 h for the determination of the dry weight and for lignan extraction. The remaining fresh cells were frozen in liquid nitrogen and stored at −20 °C before extraction for enzyme assays as described above. In the cell-free medium, the pH and conductivity were measured with appropriate electrodes. The sugar content was determined by measuring the refractive index.

Lignans were extracted from powdered lyophilized cells (200 mg) with MeOH (2 ml). The mixture was further homogenized in an ultrasonic bath (2×30 s) with intermediate cooling on ice and then diluted with H_2O (8 ml; adjusted to pH 5 with H_3PO_4). After addition of 1 mg β-glucosidase (from almonds, >1000 U mg^{-1}; Roth, Germany) the mixture was incubated at 35 °C for 3 h. Then CH_2Cl_2 (10 ml) was added and mixed vigorously for 20 min. After centrifugation the CH_2Cl_2 phase was collected and evaporated. The residue was dissolved in MeOH (3×500 μl) and lignan composition was analyzed by HPLC as described above with 40% and 50% aq. MeCN as mobile phase using appropriate pure lignans as standards.

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