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Aryltetralin-lignan formation in two different cell suspension cultures of *Linum album*: Deoxypodophyllotoxin 6-hydroxylase, a key enzyme for the formation of 6-methoxypodophyllotoxin

Katja Federolf, A. Wilhelm Alfermann, Elisabeth Fuss *

Institut für Entwicklungs- und Molekularbiologie der Pflanzen, Heinrich-Heine-Universität Düsseldorf, Universitätsstr. 1, 40225 Düsseldorf, Germany

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Dedicated to the memory of Professor Dr. Martin Luckner.

Abstract

Suspension cultures initiated from two different *Linum album* seedlings accumulate either podophyllotoxin (PTOX, 2.6 mg/g DW) or 6-methoxypodophyllotoxin (6MPTOX, 5.4 mg/g DW) as main lignans. Two molecules of coniferyl alcohol are dimerized to pinoresinol which is converted via several steps into deoxypodophyllotoxin (DOP) which seems to be the branching point to PTOX or 6MPTOX biosynthesis. DOP is hydroxylated at position 7 to give PTOX by deoxypodophyllotoxin 7-hydroxylase (DOP7H). In contrast, 6MPTOX biosynthesis is achieved by DOP hydroxylation at position 6 to β-peltatin by the cytochrome P450 enzyme deoxypodophyllotoxin 6-hydroxylase (DOP6H). The following methylation to β-peltatin-A-methylether is catalyzed by β-peltatin 6-*O*-methyltransferase (βP6OMT) from which 6MPTOX is formed by hydroxylation at position 7 by β-peltatin-A-methylether 7-hydroxylase (PAM7H). DOP6H and βP6OMT could be characterized in protein extracts from cell cultures of *L. flavum* and *L. nodiflorum*, respectively, and here in *L. album* for the first time. DOP7H and PAM7H activities could not yet be detected with protein extracts. Experiments of feeding DOP together with inhibitors of cytochrome P450 depending as well as dioxygenase enzymes were performed in order to shed light on the type of DOP7H and PAM7H. Growth parameters and specific activities of enzymes from the phenylpropane as well as the lignan specific biosynthetic pathway were measured during a culture period of 16 days. From the enzymes studied only the DOP6H showed a differential activity sustaining the hypothesis that this enzyme is responsible for the differential lignan accumulation in both cell lines.

Keywords: *Linum album*; Linaceae; Lignan; Podophyllotoxin; 6-Methoxypodophyllotoxin; Deoxypodophyllotoxin 6-hydroxylase; Deoxypodophyllotoxin 7-hydroxylase; β-Peltatin 6-*O*-methyltransferase; β-Peltatin-A-methylether 7-hydroxylase

1. Introduction

According to the definition by IUPAC (Moss, 2000) lignans are a class of secondary metabolites derived from two phenylpropanoid units that are linked by a C–C bond between carbon atoms 8 and 8′ of the side chain carbon atoms. Podophyllotoxin (PTOX) (**5**) is the most important aryltetralin-lignan for human health. It shows cytotoxic and antiviral activities and is used for the treatment of genital warts (*Condylomata acuminata*) caused by the human papilloma virus (Damayanthi and Lown, 1998; Imbert,

Abbreviations: ABT, 1-aminobenzotriazole; CAD, cinnamyl alcohol-dehydrogenase; C4H, cinnamic acid 4-hydroxylase; clot, clotrimazole; cyt *c*, cytochrome *c*; DOP (**1**), deoxypodophyllotoxin; DOP6H, deoxypodophyllotoxin 6-hydroxylase; DOP7H, deoxypodophyllotoxin 7-hydroxylase; 6MPTOX (**4**), 6-methoxypodophyllotoxin; NDA, tetcyclacis; PAL, phenylalanine ammonia-lyase; PAM (**3**), β-peltatin-A-methylether; PAM7H, β-peltatin-A-methylether 7-hydroxylase; βP6OMT, β-peltatin 6-*O*-methyltransferase; 2,4-PCA, 2,4-pyridinedicarboxylic acid; 2,5-PCA, 2,5-pyridinedicarboxylic acid; PTOX (**5**), podophyllotoxin; SAM, S-adenosyl-L-methionine; SAH, *S*-adenosyl-L-homocysteine; Trinex, trinexapac-ethyl (Pestanal®).

* Corresponding author. Tel.: +49 211 8114603; fax: +49 211 8111466.
E-mail address: fuss@uni-duesseldorf.de (E. Fuss).

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1998; and literature cited therein). Semisynthetic derivatives of PTOX (**5**) e.g. etoposide, teniposide or etopophos® are used in chemotherapy of cancer. Several *Linum* species accumulate PTOX (**5**) and its derivative 6-methoxypodophyllotoxin (6MPTOX) (**4**) (Weiss et al., 1975; Berlin et al., 1986; Broomhead and Dewick, 1990). In *L. album*, a perennial herbaceous plant growing in Iran and surrounding countries, a PTOX (**5**) content of 0.0005% on a fresh weight basis was observed (Weiss et al., 1975). We have established cell cultures and hairy roots of *L. album* by using plant material from different places in Iran. From 12 lines investigated during the last 10 years, two lines show almost no accumulation of PTOX (**5**) and/or 6MPTOX (**4**), two lines synthesize up to 0.35% PTOX (**5**) and eight lines accumulate up to 0.8% 6MPTOX (**4**) (Empt et al., 2000; Petersen and Alfermann, 2001; Fuss, 2003). The differences in lignan accumulation patterns may be due to the fact that the cultures were initiated from individual seeds with different genotypes collected from the wild. In addition, somaclonal variation can contribute to these differences as well (Phillips et al., 1994; Fuss, 2003; van Fürden et al., 2005). Our hairy root lines of *L. album* accumulate up to 3.5% 6MPTOX (**4**) as main lignan (will be published elsewhere). Taken together this indicates that 6MPTOX (**4**) is the main lignan in *in vitro* cultures of *L. album* and the accumulation of PTOX (**5**) as main compound is the exception. (Empt et al., 2000; Petersen and Alfermann, 2001; Fuss, 2003).

The PTOX (**5**) and 6MPTOX (**4**) biosynthesis starts with the "general phenylpropanoid" pathway (Fig. 1a) where phenylalanine is deaminated by phenylalanine ammonia-lyase (PAL) to give cinnamic acid, hydroxylation by cinnamic acid 4-hydroxylase (C4H) leads to *p*-coumaric acid and after additional steps coniferyl alcohol is formed by cinnamyl alcohol-dehydrogenase (CAD) (Whetten and Sederoff, 1995; Dixon and Reddy, 2003). Two molecules of coniferyl alcohol are coupled to pinoresinol (Fig. 1b). A so called dirigent protein leads to the exclusive formation of (+)-pinoresinol in *Forsythia intermedia* (Davin et al., 1997). In contrast, both enantiomers of pinoresinol can

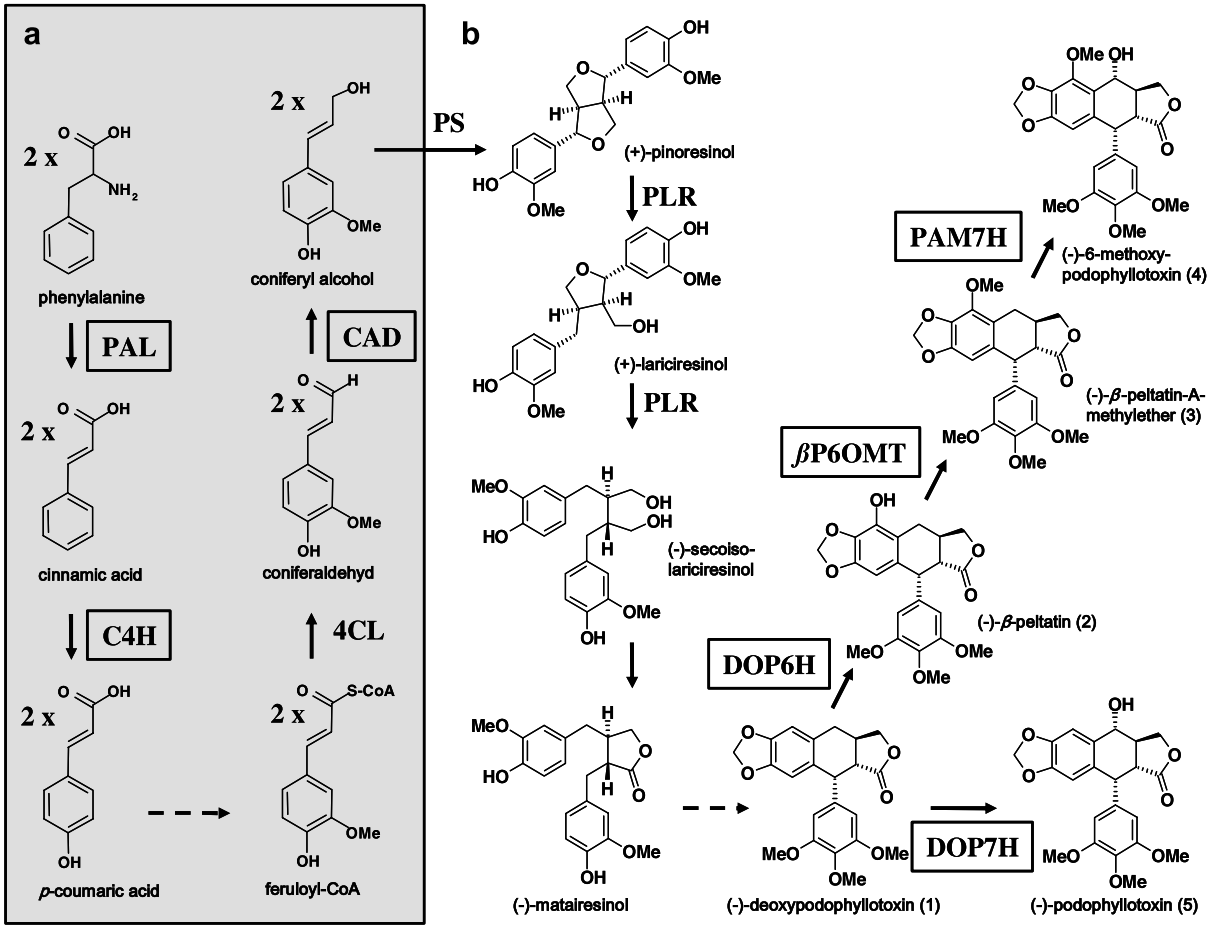

Fig. 1. Hypothetical biosynthetic pathway leading to 6MPTOX and PTOX. (a) Simplified scheme of the general phenylpropanoid pathway; (b) simplified picture of the hypothetical biosynthetic pathway to 6-methoxypodophyllotoxin (6MPTOX, **4**) and podophyllotoxin (PTOX, **5**); two molecules of coniferyl alcohol give one molecule of pinoresinol. PAL, phenylalanine ammonia-lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, hydroxycinnamate: CoA ligase; CAD, cinnamyl alcohol-dehydrogenase; PS, pinoresinol synthase; PLR, pinoresinol–lariciresinol reductase; DOP6H, deoxypodophyllotoxin 6-hydroxylase, DOP7H, deoxypodophyllotoxin 7-hydroxylase; βP6OMT, β-peltatin 6-*O*-methyltransferase; PAM7H, β-peltatin-A-methylether 7-hydroxylase.

be found in cell cultures of *L. album* (von Heimendahl et al., 2005). (+)-Pinoresinol is reduced via (+)-lariciresinol to (−)-secoisolariciresinol by pinoresinol–lariciresinol reductase and subsequently oxidized to (−)-matairesinol (Dinkova-Kostova et al., 1996; Xia et al., 2001; Okunishi et al., 2004; von Heimendahl et al., 2005; Youn et al., 2005; Moinuddin et al., 2006). The steps leading from (−)-matairesinol to deoxypodophyllotoxin (DOP) (**1**) are not well characterized. Feeding experiments were conducted to clarify the steps leading from DOP to PTOX and 6MPTOX. DOP (**1**) fed to plants of *Podophyllum hexandrum* leads to PTOX (**5**) formation indicating that DOP is the precursor for PTOX (Jackson and Dewick, 1984). DOP (**1**) added to cell cultures of *Linum flavum* is converted to 6MPTOX (**4**) and its 6MPTOX-*β*-D-glucoside whereas PTOX (**5**) is only converted to PTOX-*β*-D-glucoside indicating that DOP and not PTOX is the precursor for 6MPTOX (van Uden et al., 1995). Therefore, DOP (**1**) might be the branching point to either PTOX (**5**) or 6MPTOX (**4**) synthesis. The hydroxylation at position 7 of DOP (**1**) to PTOX (**5**) (deoxypodophyllotoxin 7-hydroxylase, DOP7H) has still to be characterized. On the way to 6MPTOX the alternative hydroxylation at position 6 of DOP by deoxypodophyllotoxin 6-hydroxylase (DOP6H) was proven to be catalyzed by a cytochrome P450 enzyme which was partially characterized in *L. flavum* and *L. nodiflorum* (Molog et al., 2001; Kuhlmann, 2004). *β*-Peltatin (**2**) is converted to *β*-peltatin-A-methylether [PAM (**3**)] by *β*-peltatin 6-*O*-methyltransferase (*β*P6OMT). This enzyme was characterized for the first time in *L. nodiflorum* (Kranz and Petersen, 2003). The enzyme for the last hydroxylation step to form 6MPTOX (**4**) (*β*-peltatin-A-methylether 7-hydroxylase, PAM7H) is not yet known.

Here we report on the comparison of growth and secondary metabolism of two cell cultures of *L. album* accumulating either PTOX (**5**) (line PT) or 6MPTOX (**4**) (line 6M) as their main lignans. DOP6H and *β*P6OMT from *L. album* could be characterized for the first time. These data together with the biotransformation experiments give strong evidence that the absence of DOP6H is the key to channel DOP (**1**) to the formation of PTOX (**5**) instead of 6MPTOX (**4**) in *L. album*.

2. Results and discussion

2.1. Characterization of L. album cell suspension cultures

The cell lines 6M and PT were used for the experiments discussed. Line 6M accumulates up to 5.4 mg/g DW 6MPTOX (**4**) and lower amounts of PTOX (**5**) (0.05 mg/g DW). The PTOX (**5**) content in line PT is highest with 2.6 mg/g DW at day 14, versus about 0.5 mg/g DW 6MPTOX (**4**) from days 8 to 16, respectively (Fig. 2a). Fresh weight (FW) accumulation reaches a maximum value at day 7 in line 6M with 14.9 g/flask, relative to 18.2 g/flask in line PT at day 10 (Fig. 2b). DW accumulation has a maximum value of 0.9 g/flask for line 6M at days 5 and 6, and for line PT at day 7. These growth parameters are comparable with other *L. album* cell lines investigated (Smollny et al., 1998; Empt et al., 2000; Seidel et al., 2002; van Fürden et al., 2005).

The specific activities of five enzymes involved in the "general phenylpropanoid" and lignan forming pathways were compared during a cultivation period of 16 days (Figs. 2c–e). Specific PAL activity is high at the beginning of the cultivation period reaching maximal activities of 210 μkat/kg in both cultures. C4H, a cytochrome P450 enzyme, is responsible for hydroxylation of cinnamic acid to form *p*-coumaric acid. Its highest specific activity of approximately 83 μkat/kg protein is detected in line 6M at days 4 and 5, whereas the maximum in line PT is at days 0 and 1 with about 63 μkat/kg protein. CAD catalyzes the conversion of coniferaldehyde to coniferyl alcohol which is the precursor for lignans as well as lignin. The CAD activity (measured as back reaction) increases in line PT up to 176 μkat/kg protein at day 3, whereas in line 6M the maximum is reached at day 10 with approximately 248 μkat/kg protein. The late maximum of CAD in line 6M does not fit to the early maxima (days 3–6) of other enzymes involved in lignan biosynthesis and the maximum plateau of lignan accumulation starting at day 8. This can be indicative for other non-specific dehydrogenases in the protein extract used for the determination of CAD activities.

One of the known enzymes from the final steps of the lignan forming pathway, DOP6H, could be detected in *L. album* for the first time. Its specific activity reaches a maximum at day 6 with approximately 4.0 μkat/kg protein in line 6M, whereas in line PT the activity is only 0.1 μkat/kg protein. In contrast to DOP6H, specific activity of *β*P6OMT is found in both cell lines with similar maximal activities of about 14 μkat/kg protein at days 2–6 and 5 for lines 6M and PT, respectively.

Neither the growth parameters nor the specific activities of PAL, C4H, CAD and *β*P6OMT showed large differences between the cell lines examined. The most striking difference beside the differential lignan accumulation was DOP6H activity, which was almost only detectable in line 6M. The latter accumulated mainly 6MPTOX (**4**) together with trace amounts PTOX (**5**). In addition, we hardly detected any DOP6H activity even after elicitation with methyljasmonate in line X4SF which accumulates mainly PTOX (**5**) (data not shown, van Fürden et al., 2005). Therefore, the presence of DOP6H might be a key to channel the DOP for formation of either 6MPTOX (**4**) or PTOX (**5**).

2.2. DOP6H from cell suspension cultures of L. album

The cytochrome P450 enzyme DOP6H introduces a hydroxyl group into DOP (**1**) leading to *β*-peltatin (**2**) at position 6 of the aromatic ring (Fig. 1b) (Molog et al., 2001). Enzyme characterization was carried out with protein

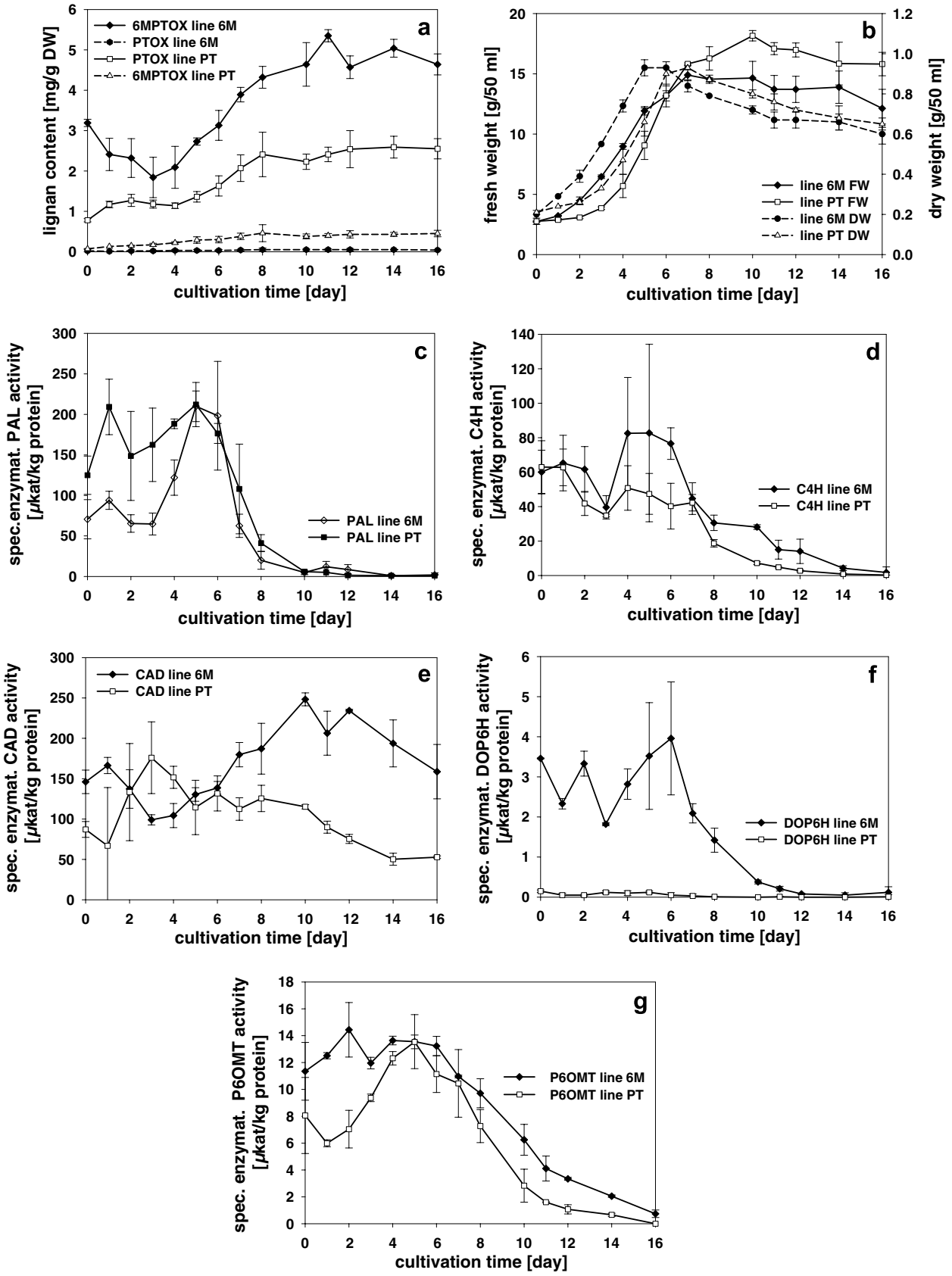

Fig. 2. Characterization of *L. album* cell suspension cultures. Time course of (a) PTOX (**5**) and 6MPTOX (**4**) accumulation; (b) fresh (FW) and dry weight (DW); (c) PAL activity; (d) C4H activity; (e) CAD activity; (f) DOP6H activity; (g) βP6OMT activity of lines 6M and PT over a cultivation period of 16 days. (Data were obtained from two independent experiments; enzyme activities were determined in triplicate in each experiment.)

extracts from line 6M because highest activity of DOP6H is found in this line.

DOP6H activity was highest between pH 7.5–8.5 and a temperature of 26–34 °C (data not shown). For oxygen it is supposed that its concentration is present at saturated quantities. The apparent K_m value for DOP (**1**) is 3 ± 1 μM and for NADPH 41 ± 14 μM (Fig. 3). Further conceivable substrates tested like β-peltatin (**2**), PAM (**3**), PTOX (**5**), yatein and matairesinol gave no hydroxylated products when incubated with the microsomal fraction confirming DOP (**1**) as the only substrate known for DOP6H up to now.

These data fit to basic characteristics of DOP6H from *L. flavum* and *L. nodiflorum* (Molog et al., 2001; Kuhlmann, 2004). The pH and temperature optima are 7.6 and 26 °C for *L. flavum* and 7.2–7.4 and 33–38 °C for *L. nodiflorum*. Apparent K_m values were 20 μM and 8.9 μM for DOP (**1**), and 36 μM and 55 μM for NADPH in *L. flavum* and *L. nodiflorum*, respectively.

The low K_m values which show high enzyme-substrate specificity are also found with other cytochrome P450 enzymes like 11 μM for tabersonine and 14 μM for NADPH for tabersonine 16-hydroxylase from *Catharanthus roseus* (St-Pierre and De Luca, 1995). However, the

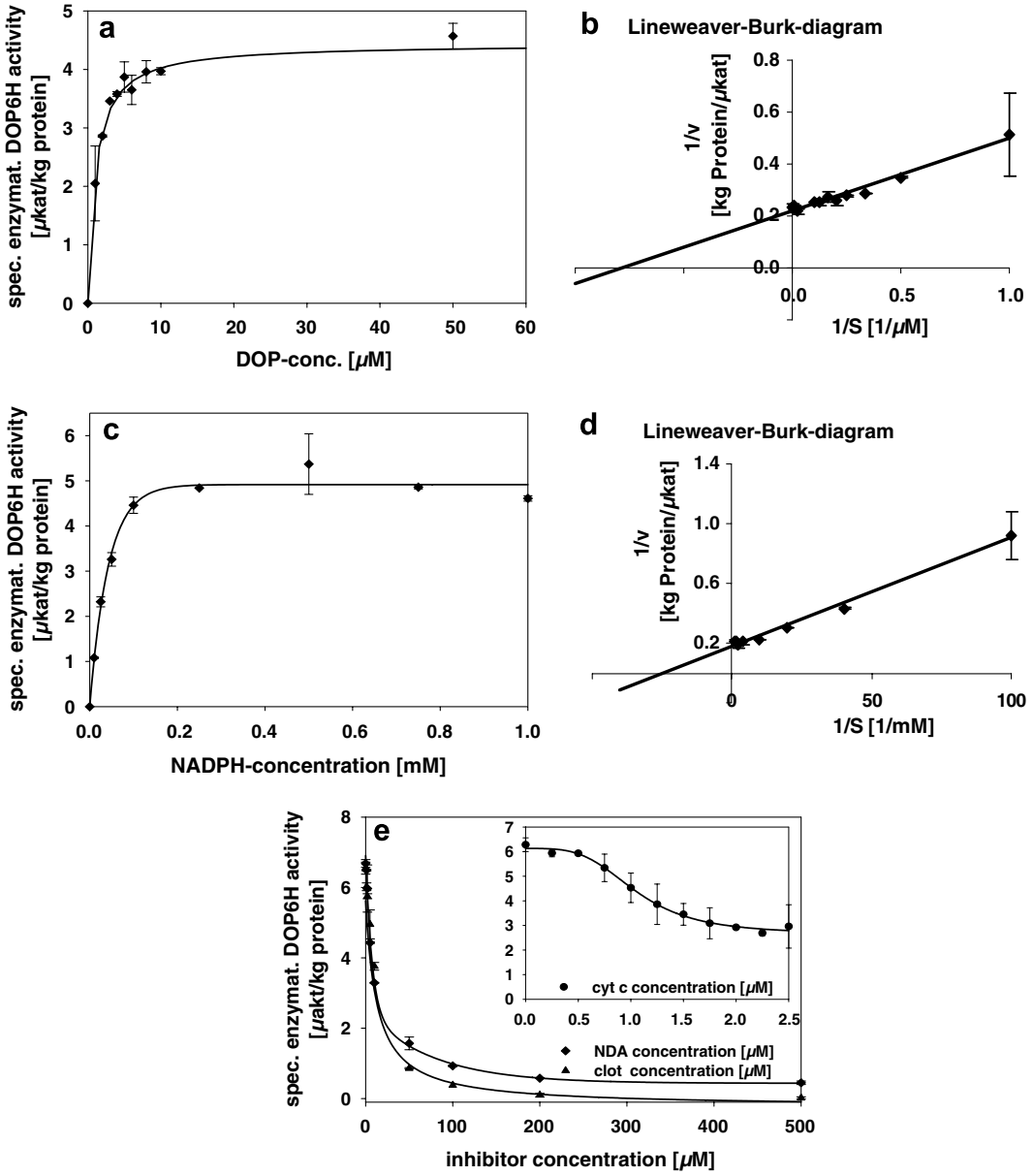

Fig. 3. DOP6H from cell suspension cultures of *L. album*. (a) Substrate saturation curve for DOP (**1**); (b) Lineweaver–Burk-diagram; apparent K_m value for DOP 3 ± 1 μM. (c) Co-substrate saturation curve for NADPH; (d) Lineweaver–Burk-diagram; apparent K_m value for NADPH 41 ± 14 μM. (The K_m values and the standard deviations are calculated upon five independent enzyme preparations. Each assay is carried out in duplicate. A representative curve based on one enzyme preparation is shown.) (e) Inhibition of DOP6H *in vitro* by Clot, NDA and cytochrome *c*. (Experiments are carried out in triplicate with each assay in duplicate. Representative data from one experiment are shown.)

range of K_m values is variable. 7-Deoxyloganin 7-hydroxylase from *Lonicera japonica* shows values of 170 μM for 7-deoxyloganin and 18 μM for NADPH (Katano et al., 2001).

Inhibition of cytochrome P450 enzymes can be observed in cell-free studies using cytochrome P450 inhibitors e.g. tetcyclacis (NDA), 1-aminobenzotriazole (ABT), clotrimazole (clot) and cytochrome *c* (cyt *c*) (Ortiz de Montellano and Matthews, 1981; Rademacher et al., 1987; Karp et al., 1990; Krochko et al., 1998). 50% inhibition of DOP6H from *L. album* is reached by adding 1.1×10^{-5} M clot, 1.1×10^{-5} M NDA or 1.6×10^{-6} M cyt *c*. Up to 500 μM ABT showed no significant inhibition (Fig. 3c). These values are comparable to concentrations necessary to inhibit other cytochrome P450 enzymes. 10^{-5}–10^{-6} M NDA leads to 50% inhibition of C4H from pea apices and of 5-*O*-(4-coumaroyl) shikimate 3-hydroxylase from parsley suspension cultures (Rademacher et al., 1987). 10^{-4} M NDA is required for 50% inhibition of tabersonine 16-hydroxylase from *Catharanthus roseus* (St-Pierre and De Luca, 1995) and 10^{-6} M can inhibit the (+)-abscisic acid 8'-hydroxylase from *Zea mays* (Krochko et al., 1998). C4H from *Helianthus tuberosus* is inhibited 50% by 10^{-4}–5×10^{-5} M ABT (Reichhart et al., 1982). Clot decreases tabersonine 16-hydroxylase activity from *Catharanthus roseus* (50% with 5×10^{-5} M) (St-Pierre and De Luca, 1995). Oxidized cyt *c* blocks cytochrome P450 reactions by siphoning off electrons from NADPH involving NADPH:Cyt P450 reductase (Krochko et al., 1998). 10^{-6} M is required to reach 50% inhibition for tabersonine 16-hydroxylase from *Catharanthus roseus* (St-Pierre and De Luca, 1995). In the case of DOP6H from *L. flavum* 2.5×10^{-6} M was necessary for a 90% inhibition (Molog et al., 2001).

2.3. βP6OMT from cell suspension cultures of L. album

βP6OMT converts β-peltatin (**2**) into PAM (**3**) via *O*-methylation at position 6 (Fig. 1b) (Kranz and Petersen, 2003). This soluble enzyme could be detected and characterized in both cell lines examined.

Maximal βP6OMT activity is reached at a temperature of 50 °C (data not shown). Nevertheless, to avoid losses of water by evaporation standard assays were performed at 37 °C. The pH-optimum of the enzyme from line 6M is between pH 7.5 and 8.0, versus pH 7.0 for line PT. An apparent K_m of 20 ± 8 μM and 6 ± 1 μM for β-peltatin (**2**) was found for line 6M and PT, respectively (Fig. 4). Co-substrate saturation for *S*-adenosyl-L-methionine (SAM) shows an apparent K_m of 37 ± 14 μM for line 6M and 15 ± 4 μM for line PT. SAM concentrations higher than 200 μM in the assay lead to an inhibition by the *S*-adenosyl-L-homocysteine (SAH) formed. SAH was added to the standard assay in variable concentrations, 50% inhibition was observed when assays were incubated with 158 ± 38 μM in line 6M and 117 ± 69 μM in line PT (Fig. 4c). Therefore, a maximum of 200 μM SAM was used for determination of K_m values. The basic characteristics of βP6OMT from *L. nodiflorum* are represented with pH and temperature optima of 7.8 and 40 °C. Apparent K_m values were 42 μM for β-peltatin (**2**) and 11 μM for SAM. 50% inhibition was observed with 100–120 μM SAH (Kranz, 2004). K_m and inhibition values given are relative values only comparable with other crude extracts since OMTs are enzymes with partly bi- or multifunctional substrate specificity (Gauthier et al., 1998; Frick and Kutchan, 1999; Schröder et al., 2002). Therefore, we might have measured formation of PAM (**3**) not only by βP6OMT but also by other OMTs.

2.4. Biotransformation experiments

Since all biochemical approaches to detect 7-hydroxylation of DOP (**1**) or PAM (**3**) failed, we conducted biotransformation experiments using cell lines PT and 6M incubated with DOP (**1**) together with inhibitors of cytochrome P450 and dioxygenase enzymes, in order to shed some light on the nature of the enzymes involved in 7-hydroxylation.

DOP (**1**) incubated with *Podophyllum hexandrum* and *L. flavum* cell cultures is converted to PTOX (**5**) and 6MPTOX (**4**), respectively (van Uden et al., 1995). As prerequisite for our inhibitor studies, addition of 2.5×10^{-4} M DOP (**1**) to *L. album* cell line 6M at day 5 results in a 20-fold increase of β-peltatin (**2**) and a 700 fold increase of PAM (**3**) content from 0.002 to 1.40 (±0.106) mg/g DW. No change in 6MPTOX (**4**) content is observed. Accumulation of PAM (**3**) instead of 6MPTOX (**4**) might occur because PAM7H is already saturated with the PAM (**3**) present in the culture without additional PAM (**3**) delivery by feeding of DOP (**1**), or 6MPTOX formation is not complete before the time point of harvest at day 7. PTOX (**5**) accumulation increases after feeding of DOP (**1**) about 3-fold from 0.025 (±0) mg/g DW in the control up to 0.074 (±0.01) mg/g DW (Fig. 5). Feeding of DOP (**1**) to *L. album* line PT results in a 3.6 fold increase of PTOX (**5**) accumulation from 2.4 (±0.4) in the control up to 8.6 (±1.3) mg/g DW, whereas β-peltatin (**2**) and 6MPTOX (**4**) contents do not significantly increase (data not shown).

Cytochrome P450 inhibitors NDA, clot and ABT and dioxygenase inhibitors 2,4-pyridinedicarboxylic acid (2,4-PCA), 2,5-pyridinedicarboxylic acid (2,5-PCA) and trinexapac-ethyl (Pestanal®) (trinex) are added at day 4 with DOP (**1**) administration on day 5 (Fig. 5). The solvents used for DOP (**1**) administration and inhibitor addition are partly detrimental for lignan accumulation, e.g. accumulation of 6MPTOX (**4**) is lowered about 33% after addition of MeOH and DMSO. Some inhibitors used are detrimental to the growth (maximum inhibition by 10^{-4} M clot was about 30%) which was also described by Morgan and Shanks (1999) with hairy roots of *Catharanthus roseus*. Rademacher et al. (1987) and Zhang et al. (2002) explain this result with the possible interference of

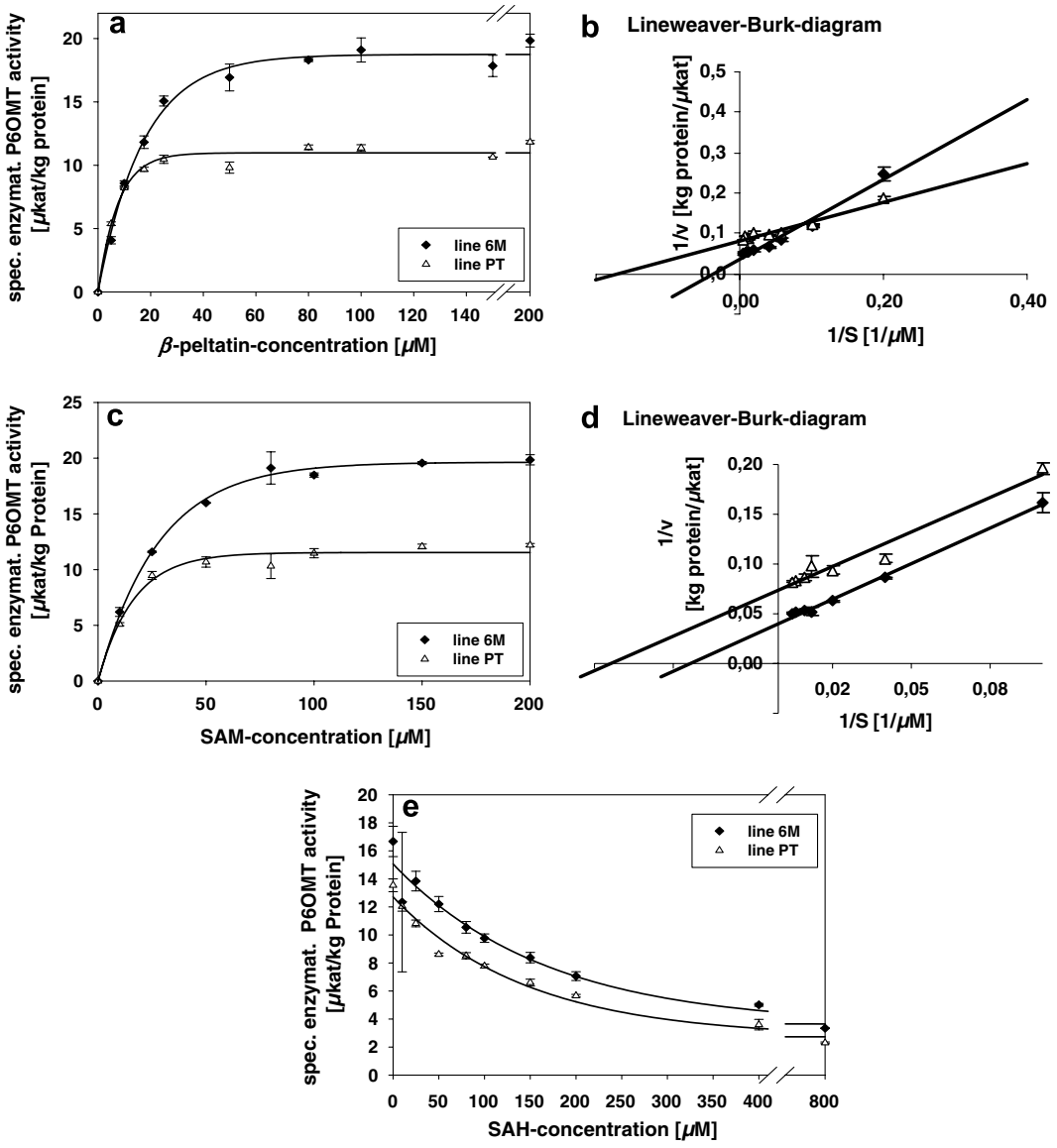

Fig. 4. βP6OMT from cell suspension cultures of *L. album*. (a) Substrate saturation curve for β-peltatin; (b) Lineweaver–Burk-diagram; apparent K_m value: line 6M 20 ± 8 μM, line PT 6 ± 1 μM; (c) Co-substrate saturation curve for SAM; (d) Lineweaver–Burk-diagram; apparent K_m value: line 6M 37 ± 14 μM, line PT 15 ± 4 μM. (The K_m values and standard deviations are calculated from three independent enzyme preparations; each assay was carried out in duplicate. A representative curve based on one enzyme preparation is shown.) (e) Inhibition experiments of βP6OMT with variable *S*-adenosyl-homocysteine (SAH) concentrations. 50% co-product inhibition is reached at 158 ± 38 μM SAH in line 6M and 117 ± 69 μM SAH in line PT. (Inhibition values are determined graphically by using three independent enzyme preparations; each assay is carried out in duplicate. Representative data from one experiment are shown.)

these inhibitors with hormone biosynthesis (e.g. gibberellins).

Inhibitor concentrations around 10^{-4} M have no influence on PTOX (**5**) formation in line PT. Higher concentrations of inhibitors lead to cell death, shown by 2,3,5-triphenyl-tetrazolium chloride viability assay (Towill and Mazur, 1974; data not shown).

As control the same experiments are conducted with line 6M. DOP6H activity, respectively the formation of β-peltatin (**2**) is inhibited by 10^{-4} M of the cytochrome P450 inhibitors NDA (by 91%), clot (by 82%) and ABT (by 73%) showing that cytochrome P450 enzymes can be inhibited by such concentrations. The dioxygenase inhibitors 2,5-PCA and trinex had no significant influence on DOP6H. Addition of 2,4-PCA leads to a slight increase in β-peltatin (**2**) and PAM (**3**) formation (Fig. 5). Formation of PTOX (**5**) is increased 14.4 fold by NDA, 3.5-fold by clot and 5.3-fold by ABT. The dioxygenase inhibitors used do not influence PTOX (**5**) accumulation.

Hydroxylation of DOP (**1**) and PAM (**3**) at position 7 might be performed by the same enzyme. If no or less PAM (**3**) is present, because DOP6H activity is missing (line PT) or reduced (line 6M with cytochrome P450 inhibitors), DOP (**1**) is converted into PTOX (**5**). Since the DOP6H could be efficiently inhibited by cytochrome P450 inhibitors, whereas PTOX (**5**) accumulation increased

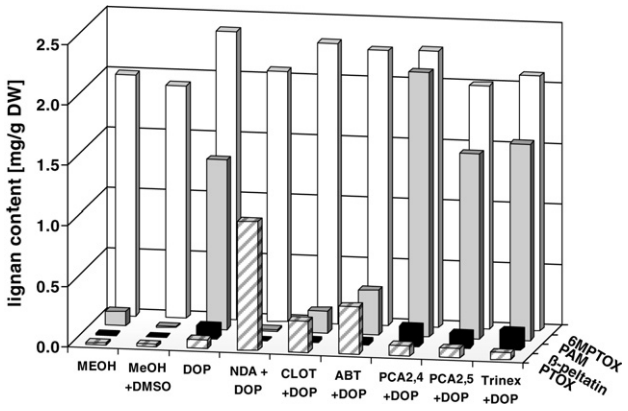

Fig. 5. Influence of NDA (in DMSO), ABT (in MeOH), clot (in MeOH), 2,4-PCA (in DMSO), 2,5-PCA (in DMSO) or trinex (in MeOH) on the content of β-peltatin (**2**), PAM (**3**), 6MPTOX (**4**) and PTOX (**5**) in cells of line 6M. The inhibitors were added at a final concentration of 10^{-4} M at day 4. 2.5×10^{-4} M DOP (**1**) (in MeOH) was administered at day 5. The lignan content was determined after harvest at day 7.

under the same conditions, we conclude that the hydroxylation at position 7 is not a cytochrome P450 enzyme. The DOP7H/PAM7H might be a dioxygenase or peroxidase, but further experiments are needed.

3. Conclusion

DOP (**1**) is supposed to be the precursor for the biosynthesis of PTOX (**5**) and 6MPTOX (**4**). It can be hydroxylated either at position 7 to give PTOX by deoxypodophyllotoxin 7-hydroxylase (DOP7H) or at position 6 to β-peltatin by the cytochrome P450 dependent deoxypodophyllotoxin 6-hydroxylase (DOP6H). β-Peltatin (**2**) can be methylated to PAM (**3**) by β-peltatin 6-*O*-methyltransferase (βP6OMT) from which 6MPTOX (**4**) is formed by hydroxylation at position 7 by a until now hypothetical PAM7H.

Two cell lines of *L. album* can be distinguished by their lignan accumulation patterns. Whereas line PT accumulates PTOX (**5**) as main lignan, 6MPTOX (**4**) is the dominant compound in line 6M. Beside this the major difference in these cell lines was DOP6H activity which is almost only detectable in the 6MPTOX (**4**) producing line 6M. This indicates that PTOX (**5**) only accumulates if DOP6H activity is missing. Further evidence for this hypothesis comes from feeding experiments. DOP (**1**) administered to the culture medium leads to the formation of PTOX (**5**) in line PT. The addition of DOP (**1**), however, to line 6M gave only PTOX (**5**) if the DOP6H was blocked by inhibitors for cytochrome P450 monooxygenases. Since PTOX (**5**) formation by DOP7H is not blocked by such inhibitors this hydroxylase is probably not a cytochrome P450 monooxygenase. In the presence of DOP6H PAM (**3**) is formed and can serve as substrate for the 7-hydroxylation by PAM7H. In this case PTOX (**5**) is almost not formed. If DOP6H is absent DOP (**1**) could accumulate instead of PAM (**3**). In that case PAM7H can hydroxylate DOP (**1**) instead of PAM (**3**) at position 7 to PTOX (**5**). This means that the hydroxylations at position 7 of PAM (**3**) to 6MPTOX (**4**) or DOP (**1**) to PTOX (**5**) might be catalysed by the same enzyme, the PAM7H and not two different enzymes, PAM7H and DOP7H, respectively. Further experiments will be necessary to proof this assumption.

4. Experimental

4.1. Plant material, characterization of cell suspension cultures

Callus cultures of *L. album* Kotschy ex. Boiss. were initiated from single germinated seeds collected in Iran (Smollny et al., 1998). From these callus lines cell suspension cultures were initiated. They were maintained, subcultured and their growth and lignan formation were measured as described by Empt et al. (2000). Proteins were extracted as published (Molog et al., 2001; van Fürden et al., 2005).

4.2. Enzyme assays

PAL and CAD activities were measured photometrically as described by Zimmermann and Hahlbrock (1975) and Wyrambik and Grisebach (1975), respectively. βP6OMT standard assays were modified according to Kranz and Petersen (2003) and consisted of 80 μM β-peltatin (**2**), 80 μM SAM and 100 μg protein in a total volume of 250 μl 0.1 M Tris/HCl (pH 7.5, 1 mM dithiothreitol, 1 mM sodium diethyldithiocarbaminate). The assays were incubated at 37 °C for 30 min, stopped by adding 25 μl 6 N HCl and extracted with EtOAc (3 × 500 μl). The dried residues were redissolved in MeOH (150 μl) and subjected to HPLC. DOP6H and C4H assays were modified according to Molog et al. (2001). They contained 30 μM DOP and 100 μM cinnamic acid, respectively, 500 μM NADPH and 100 μg protein in a total volume of 100 μl 0.1 M Tris/HCl (pH 7.5, 1 mM dithiothreitol, 1 mM sodium diethyldithiocarbaminate). For determination of K_m and inhibition values DOP6H standard assays consisted of 50 μM DOP (**1**), 500 μM NADPH and 338 μg protein in a total volume of 500 μl 0.1 M Tris/HCl (pH 7.5, 1 mM dithiothreitol, 1 mM sodium diethyldithiocarbaminate). The assays were incubated at 30 °C for 20 min, stopped by addition of 25 and 50 μl 6 N HCl, respectively, and extracted with EtOAc (3 × 500 μl). The dried residues were redissolved in MeOH (150 μl) and subjected to HPLC.

For DOP6H *in vitro* inhibition experiments, 0–500 μM NDA (in DMSO), clot (in MeOH) or 0–2.5 μM cyt *c* [in 0,1 M Tris/HCl (pH 7.5, 1 mM dithiothreitol, 1 mM sodium diethyldithiocarbaminate)] were added to the standard assay in variable concentrations. For βP6OMT

in vitro inhibition experiments, 0–800 μM SAH (in H_2O) was added to the standard assays.

4.3. In vivo inhibition experiments

2.5 g cells were cultivated under standard conditions (Empt et al., 2000), but 25 ml instead of 50 ml medium is used in 100 ml instead of 300 ml Erlenmeyer flasks. The inhibitors NDA (in DMSO), ABT (in MeOH), clot (in MeOH), 2,4-PCA (in DMSO), 2,5-PCA (in DMSO) or trinex (in MeOH) were added to a final concentration of 10^{-4} M at day 4. 2.5×10^{-4} M DOP (**1**) (in MeOH) was added to the cell suspension cultures at day 5. After harvesting (day 7) the lignan contents were determined from freeze dried cells.

4.4. HPLC analysis of lignans, βP6OMT and DOP6H assays

Lignan samples and enzyme assays were analyzed on a Grom-Sil 120 ODS 5 column (particle size 5 μm, 4.6×250 mm; pre-column: 5 μm, 4.6×40 mm) by elution with an $H_2O + 0.01\%$ H_3PO_4 (A)/CH_3CN (B) gradient as follows: start conditions 60.0% A/40.0% B flow 0.8 ml min^{-1}; linear gradient to 33.0% A/67.0% B flow 1.0 ml min^{-1} in 17 min; linear gradient to start conditions 60.0% A/40.0% B flow 1.0 ml min^{-1} in 1 min; start conditions 60.0% A/40.0% B flow 0.8 ml min^{-1} for 6 min.

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