A polyketide synthase of *Plumbago indica* that catalyzes the formation of hexaketide pyrones

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Plumbago indica L. contains naphthoquinones that are derived from six acetate units. To characterize the enzyme catalyzing the first step in the biosynthesis of these metabolites, a cDNA encoding a type III polyketide synthase (PKS) was isolated from roots of *P. indica*. The translated polypeptide shared 47–60% identical residues with PKSs from other plant species. Recombinant *P. indica* PKS expressed in *Escherichia coli* accepted acetyl-CoA as starter and carried out five decarboxylative condensations with malonyl coenzyme A (-CoA). The resulting hexaketide was not folded into a naphthalene derivative. Instead, an α-pyrone, 6-(2′,4′-dihydroxy-6′-methylphenyl)-4-hydroxy-2-pyrone, was produced. In addition, formation of α-pyrones with linear keto side chains derived from three to six acetate units was observed. As phenylpyrones could not be detected in *P. indica* roots, we propose that the novel PKS is involved in the biosynthesis of naphthoquinones, and additional cofactors are probably required for the biosynthesis of these secondary metabolites *in vivo*.

Plumbago indica L. is a traditional medicinal plant from South-east Asia. The roots of *P. indica* accumulate the naphthoquinone plumbagin [1], which exhibits antimicrobial [2], insecticidal [3], antitumor [4,5], and antifertility activities [6].

Plumbagin originates from six acetate units (Fig. 1A). This was demonstrated for the first time by tracer experiments with *Drosophyllum lusitanicum* cultures and *Plumbago europaea* plants [7,8]. Only recently, Bringmann and coworkers established the biosynthetic origin of acetate-derived naphthoquinones and naphthylisoquinolines in palaeotropical lianas of the Ancistrocladaceae and Dioncophyllaceae families [9,10]. From these studies, it can be postulated that the basic skeleton of the naphthoquinones is produced by a polyketide synthase.

Abbreviations
ALS, aloesone synthase; CHR, chalcone reductase; CHS, chalcone synthase; CID, collision-induced dissociation; EI, electron impact; HKS, hexaketide synthase; HR-ESIMS, high resolution electrospray ionization mass spectrometry; OKS, octaketide synthase; PCS, pentaketide chromone synthase; THNS, 1,3,6,8-tetrahydroxynaphthalene synthase.

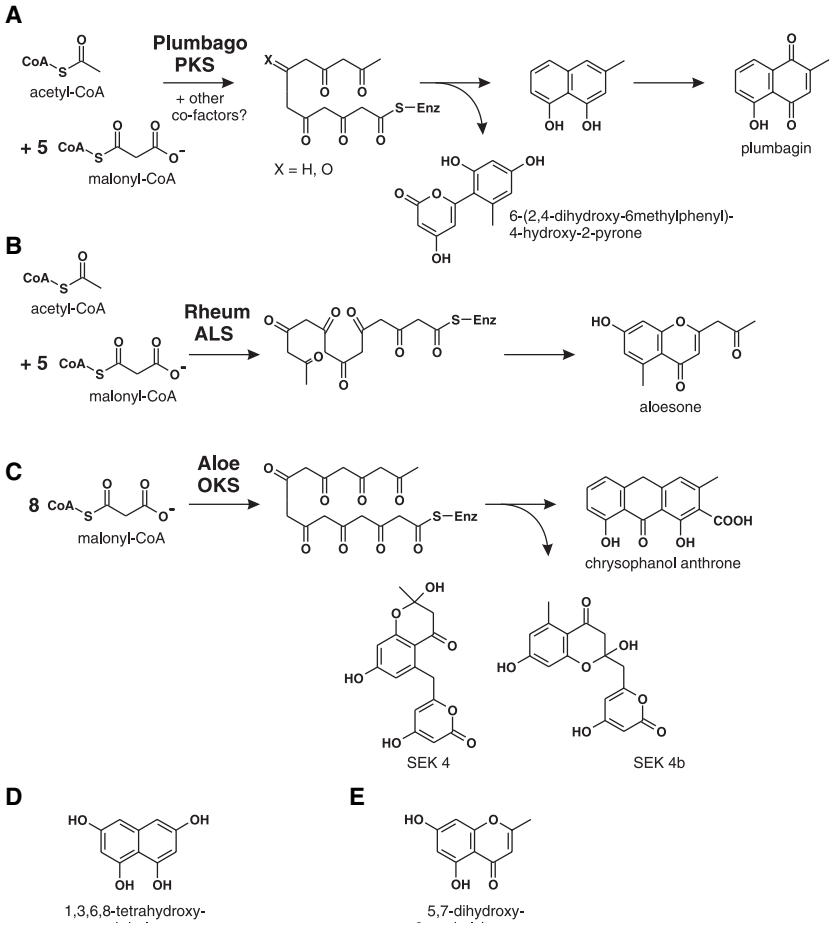


Fig. 1. Reactions and products of type III PKSs catalyzing the formation of two or three six-membered rings. (A) Putative reaction of naphthoquinone-forming PKS in *P. indica*. The biosynthesis of plumbagin includes loss of one carbon by decarboxylation and presumably involves a reduction step. (B) *Rheum palmatum* aloesone synthase (ALS), (C) *Aloe arborescens* octaketide synthase (OKS), (D) 1,3,6,8-tetrahydroxynaphthalene, and (E) 5,7-dihydroxy-2-methylchromone, the product of *A. arborescens* pentaketide synthase (PCS).

In plants, polyketides are synthesized by type III polyketide synthases (PKSs). Type III PKSs are typically homodimeric proteins with two functionally independent active site cavities [11,12]. They use thioesters of coenzyme A as substrates and catalyze up to seven decarboxylative condensation reactions followed by cyclization of the intermediate polyketide. The prototype of type III PKSs is chalcone synthase (CHS), which synthesizes naringenin chalcone, the precursor of flavonoids, by condensing three acetate units derived from malonyl coenzyme A (-CoA) with a *p*-coumaroyl-CoA starter unit. Several other type III PKSs have been isolated and characterized from plants, bacteria, and recently also from fungi [13]. They differ from CHS in starter molecule specificity, number of extensions with malonyl-CoA, or the cyclization of the intermediate polyketide. Analysis of several type III PKSs by X-ray diffraction and subsequent mutational studies has revealed structural and functional details about this class of enzymes [14–18]. A catalytic triad of three highly conserved residues, C164, H303 and N336 (numbers according to *Medicago sativa* CHS2), constitutes the core machinery of type III PKSs [19]. The architecture of the active site cavity acts as a size-based filter and determines starter molecule specificity, number of elongation cycles and the stereochemistry of the cyclization reaction [15,20].

Most type III PKSs perform one, two or three extensions of a starter molecule with malonyl-CoA and catalyze the formation of only one six-membered ring. The enzyme involved in naphthoquinone biosynthesis, however, presumably catalyzes five extensions of an acetyl-CoA starter with malonyl-CoA yielding a hexaketide, which subsequently undergoes two cyclizations to a naphthalene ring (Fig. 1A). Mechanistically related reactions are performed by a small number of type III PKSs: aloesone synthase (ALS) of *Rheum palmatum* [21], 1,3,6,8-tetrahydroxynaphthalene synthase (THNS) of *Streptomyces griseus* and *Streptomyces coelicolor* [22,23], pentaketide chromone synthase (PCS) and octaketide synthase (OKS) of *Aloe arborescens* [24,25] (Fig. 1B–E). All four enzymes perform more than three condensations and two or three cyclization reactions.

The crystal structure of THNS [17] and the calculated structure of ALS [26] suggest that the preference of small starter units and the ability to catalyze more than three extensions is determined by changes in the geometry of the active site cavity relative to CHS. G256 is replaced by a bulkier residue relative to *M. sativa* CHS2 leading to a 'horizontal constriction' of the active site and the preference of smaller starter units. Moreover, smaller residues substitute T197 and, in THNS, S338 (Fig. 2), thus extending the active site vertically. Consequently, more than three condensations are possible [17]. This observation was confirmed by site-directed mutagenesis of diverse type III PKSs of plant and bacterial origin [20,24–28].

It is very likely that a polyketide synthase involved in naphthoquinone biosynthesis shares similar features with ALS and THNS. Here, we describe the isolation and characterization of such a type III PKS from roots of the naphthoquinone-producing plant *P. indica*.

Results

To isolate a PKS cDNA, RT-PCR with degenerate oligonucleotide primers based on conserved regions of plant type III PKSs was carried out using total RNA from roots of *P. indica* plantlets as template. The partial cDNA of 600 bp obtained in this experiment was used to screen a cDNA library of ~80 000 plaque forming units prepared from Poly(A)$^+$ RNA of *P. indica* roots. After three rounds of screening, the full-length cDNA clone *PinPKS* of 1622 bp was isolated. The nucleotide sequence of this cDNA clone will appear in the DDBJ/EMBL/GenBank databases with the accession number AB259100.

The translated polypeptide of 396 amino acids showed 47–60% identity with plant type III PKSs, but only 20% identity with bacterial type III PKSs, e.g. THNS of *S. coelicolor*. Highest identities were found with *Vitis vinifera* CHS. Like other members of the CHS superfamily, PinPKS contains the conserved Cys-His-Asn catalytic triad (Fig. 2). However, three other residues which play an important role in shaping the architecture of the active site cavity are changed in comparison with CHS. Instead of T197, G256 and S338 (numbers according to *M. sativa* CHS2), PinPKS contains the amino acids Ala, Leu and Thr in these positions, respectively (Fig. 2). As pointed out above, substitutions of T197 with smaller residues and G256 with bulkier amino acids may be indicative of a PKS that prefers small start units and performs more than three decarboxylative condensations with malonyl-CoA.

To further analyze the relationship between PinPKS and other type III polyketide synthases, a phylogenetic analysis was performed with plant, bacterial and fungal type III PKSs, using *Escherichia coli* β-ketoacyl synthase III as outgroup (Fig. 3). In the tree, PinPKS groups together with the plant PKSs and appears to be most closely related to *R. palmatum* ALS [21]. Other close neighbors are *A. arborescens* PCS [24] and OKS [25] and *Wachendorfia thyrsiflora* PKS [29].

Subsequently, *PinPKS* was expressed in *E. coli* BL21 (DE3) with an N-terminal hexahistidine tag, and the recombinant protein was purified by cobalt affinity chromatography. Recombinant PinPKS was incubated with acetyl-CoA as starter and [2-^{14}C]malonyl-CoA as extender unit, and the products extracted from the incubations were analyzed by thin layer chromatography (TLC). For comparison, recombinant *Cassia alata* CHS [30] was analyzed under the same assay conditions. The products of *C. alata* CHS with the non-physiological start substrate acetyl-CoA had been previously identified as 4-hydroxy-6-methyl-2-pyrone (triacetate lactone, TAL) and 6-acetonyl-4-hydroxy-2-pyrone [30]. Radio-TLC analysis revealed that several radiolabeled compounds were extracted from incubations with the two PKSs. Some products were exclusively formed by PinPKS suggesting that this PKS is functionally different from CHS (Fig. 4A). In addition, it was investigated whether PinPKS accepts acetyl-CoA as starter. The recombinant PKS was incubated with unlabelled malonyl-CoA and [1-^{14}C]acetyl-CoA. The product spectrum extracted from these assays was identical to that obtained with [2-^{14}C]malonyl-CoA and unlabelled acetyl-CoA, indicating that PinPKS can utilize acetyl-CoA as a primer unit (Fig. 4B).

To identify the products, scaled-up assays were performed with unlabeled substrates, and the compounds extracted from the assays were analyzed by HPLC/ESI-MS/MS. High resolution (HR)/ESI-MS analysis was carried out to determine the elemental composition of the enzymatically formed products. The results of the MS analyses and postulated structures of products are summarized in Table 1. For additional structural elucidation, GC/electron impact (EI)-MS analysis of the permethylated product mixture was performed (Table 2).

In the LC/ESI-MS analysis, 4-hydroxy-2-pyrones derived from tri- (**1**) and tetraketides (**2**) could be identified by the characteristic fragment ion at *m/z* 125 corresponding to $[C_6H_5O_3]^-$, which indicated the presence of a pyrone ring (Table 1). Furthermore, fragment ions corresponding to $[M\text{-}H\text{-}CO_2]^-$ were detected. In the GC-MS analysis, two peaks were obtained for the methylether of TAL (**1a** and **1b**) (Table 2). The EIMS

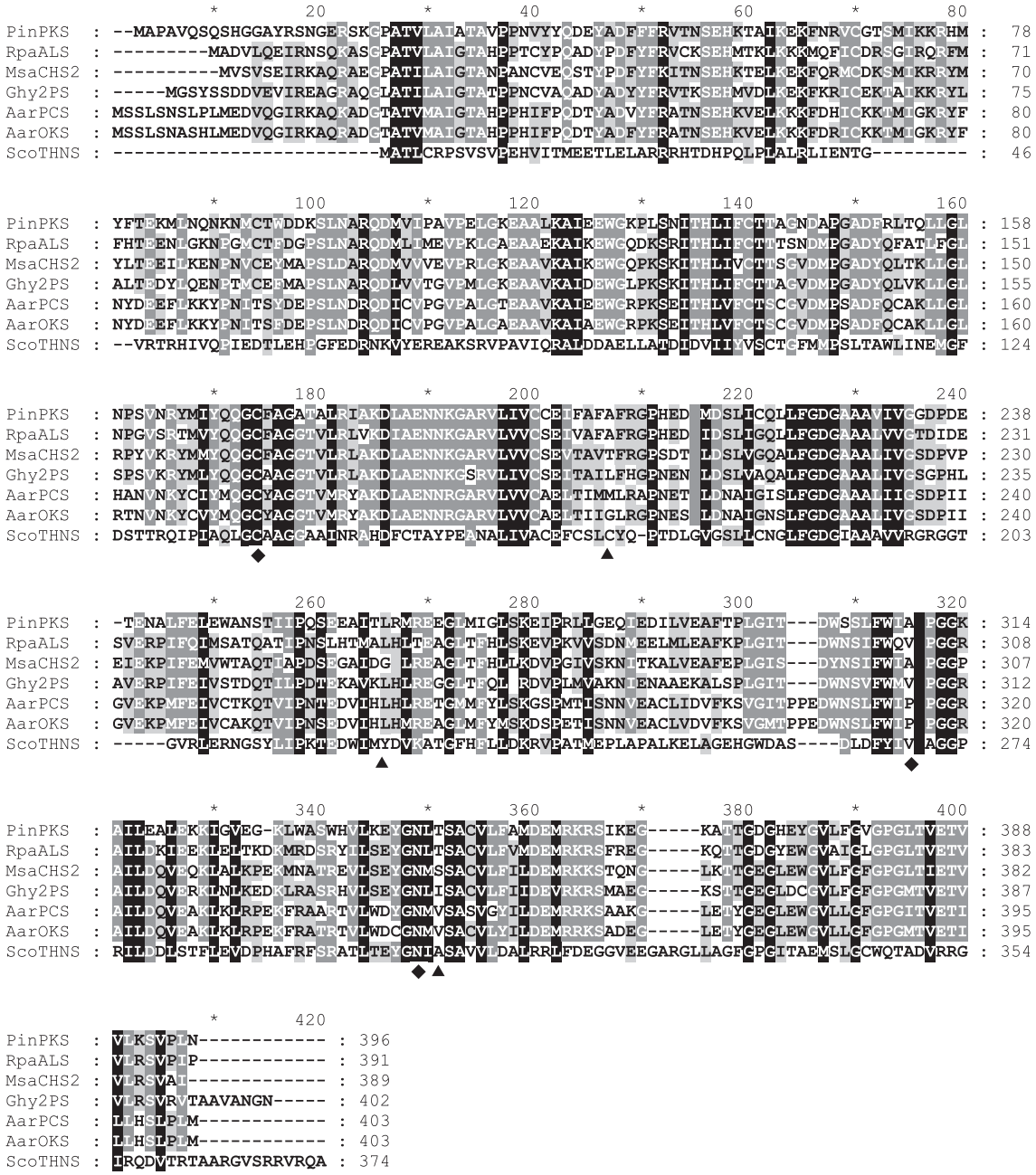


Fig. 2. Alignment of type III PKS amino acid sequences. The alignment was generated by CLUSTALW [42] and edited using GENEDOC [43]. AarPCS, *Aloe arborescens* pentaketide chromone synthase; AarOKS, *A. arborescens* octaketide synthase; Ghy2PS, *Gerbera hybrida* 2-pyrone synthase; MsaCHS2, *M. sativa* CHS2; PinPKS, *P. indica* PKS; RpaALS, *Rheum palmatum* aloesone synthase; ScoTHNS, *S. coelicolor* THNS. Residues highlighted black are 100% similar. Dark grey indicates >80% and light grey >60% similarity. The amino acids of the catalytic Cys-His-Asn triad are marked by diamonds (◆). The residues at positions 207, 266 and 351 (marked by triangles, ▲) correspond to *M. sativa* T197, G256 and S338 and have been shown to be crucial for the geometry of the active site [15,17,26].

revealed that the compound (**1b**) is 2-methoxy-6-methyl-4-pyrone derived from the enolized form of TAL.

Compound **3** could not be detected by LC/ESI-MS. In the HR-ESIMS analysis, however, **3** showed the $[M\text{-}H]^-$ peak at *m/z* 209.04505 (calculated for $C_{10}H_9O_5^-$ 209.04555), indicating that a pentaketide was formed. Further information about the pentaketide (**3**) was obtained by GC-MS analysis of the per-

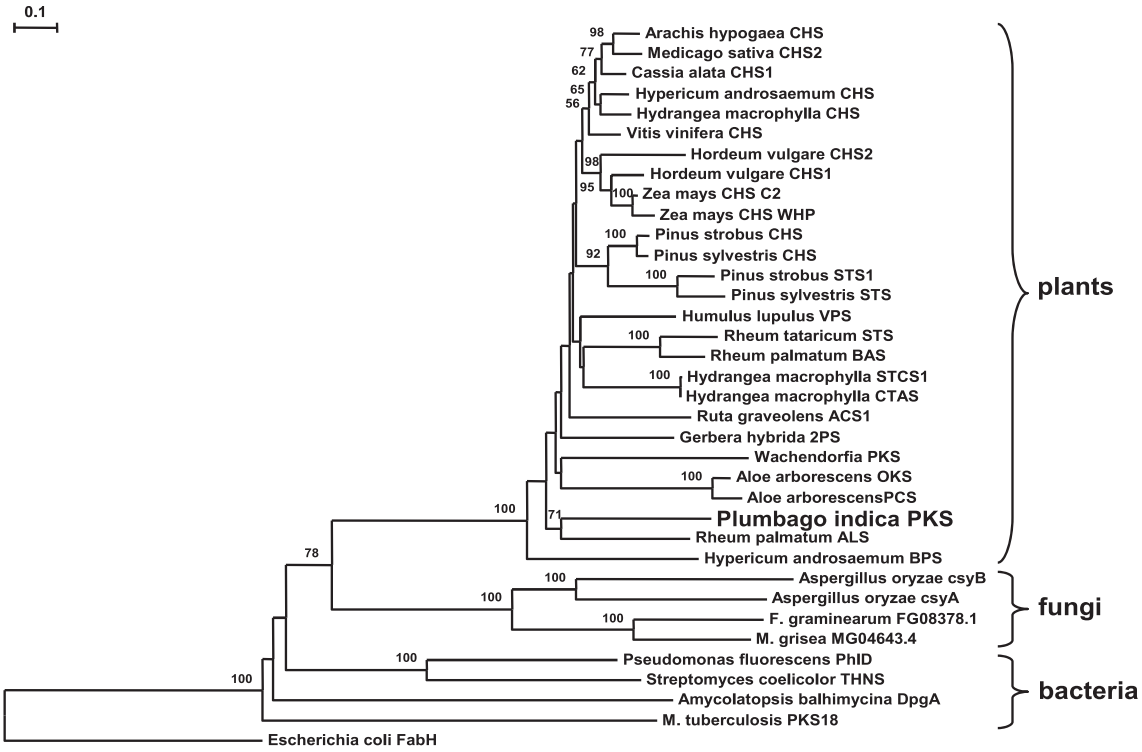


Fig. 3. Relationship of *P. indica* PKS with plant, fungal and bacterial type III PKSs. The tree was constructed with protein sequences of type III PKSs from plants, fungi, and bacteria. *E. coli* β-ketoacyl synthase III (FabH) was used as outgroup. Alignments were performed with CLUSTALW [42], and cladistic analysis was carried out using TREECON [44]. The tree was constructed using the neighbor-joining algorithm, and bootstrap values higher than 50 are shown. Abbreviations: 2PS, 2-pyrone synthase; ACS, acridone synthase; ALS, aloesone synthase; BAS, benzalacetone synthase; BPS, benzophenone synthase; CHS, chalcone synthase; CTAS; *p*-coumaroyl triacetic acid lactone synthase; OKS, octaketide synthase; PCS, pentaketide chromone synthase; STCS, stilbenecarboxylate synthase; STS, stilbene synthase; VPS, valerophenone synthase; THNS, 1,3,6,8-tetrahydroxynaphthalene synthase. GenBank accession numbers: *Aloe arborescens* OKS (AAT48709), *Aloe arborescens* PCS (AAX35541), *Amycolatopsis balhimycina* DpgA (CAC48378), *Arachis hypogaea* CHS (AAO32821), *Aspergillus oryzae* csyA (BAD97390), *Aspergillus oryzae* csyB (BAD97391), *Cassia alata* CHS1 (AAM00230), *Escherichia coli* FabH (1EBL_B), *Fusarium graminearum* FG08378.1 (XP_388554), *Gerbera hybrida* 2PS (P48391), *Hordeum vulgare* CHS1 (CAA41250), *Hordeum vulgare* CHS2 (CAA70435), *Humulus lupulus* VPS (BAA29039), *Hydrangea macrophylla* CHS (BAA32732), *Hydrangea macrophylla* CTAS (BAA32733), *Hydrangea macrophylla* STCS1 (AAN76182), *Hypericum androsaemum* CHS (AAG30295), *Hypericum androsaemum* BPS (AAL79808), *Magnaporthe grisea* MG04643.4 (XP_362198), *M. sativa* CHS2 (P30074), *Mycobacterium tuberculosis* PKS18 (AAK45681), *Pinus strobus* CHS (CAA06077), *Pinus strobus* STS1 (CAA87012), *Pinus sylvestris* CHS (CAA43166), *Pinus sylvestris* STS (AAB24341), *P. indica* PKS (AB259100), *Pseudomonas fluorescens* PhlD (AAB48106), *Rheum palmatum* ALS (AAS87170), *Rheum palmatum* BAS (AAK82824), *Rheum tataricum* STS (AAP13782), *Ruta graveolens* ACS1 (S60241), *S. coelicolor* THNS (NP_625495), *Vitis vinifera* CHS (BAA31259), *Wachendorfia thyrsiflora* PKS (AAY51378), *Zea mays* CHS C2 (AAW56964), *Zea mays* CHS WHP (CAA42763).

methylated enzymatic products. The EI mass spectrum of the permethyated compound (3-Me) shows a molecular ion at *m/z* 252 indicating three methylations (Table 2). The characteristic fragment ions at *m/z* 125 and 139 seem to be hints for a pyrone substructure. Therefore, it is postulated that compound **3** is a pentaketide possessing a 4-hydroxy-6-(2′,4′-dioxo-pentyl)-pyrone structure (Table 1).

Compound **4** is derived from a hexaketide, as indicated by its elemental composition $C_{12}H_9O_5^-$ ([M-H]$^-$ at *m/z* 233.04498, calculated for $C_{12}H_9O_5^-$ 233.04555) obtained by HR-ESIMS. The collision-induced dissociation (CID)-MS spectrum displayed key ions at *m/z* 189 [M-H-CO_2]$^-$, 165 [M-H-C_3O_2]$^-$ and 147 [M-H-CO_2-CH_2CO]$^-$. Analysis of the permethylated enzymatic products revealed a peak at *m/z* 276, indicating that the unknown hexaketide had been methylated in three positions (Table 2). These data suggested that compound **4** is identical with a hexaketide reported from an *A. arborescens* OKS mutant [25]. The OKS G207T mutant produced 6-(2′,4′-dihydroxy-6′-methylphenyl)-4-hydroxy-2-pyrone, which corresponds to the demethylated aglycone of aloenin A. To prepare a standard, aloenin A was deglucosylated and its agly-

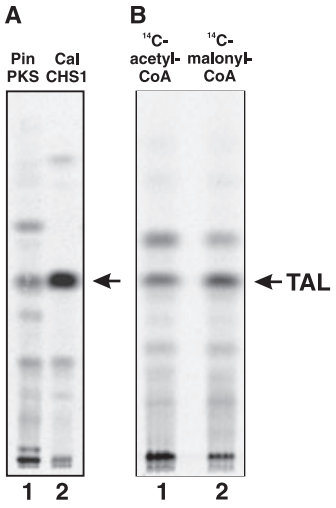


Fig. 4. Radio-TLC analysis of products extracted from PKS assays. (A) Comparison of products obtained with (1) PinPKS and (2) *C. alata* CHS1. (B) PinPKS was incubated with (1) unlabelled acetyl-CoA and [2-^{14}C]malonyl-CoA or (2) with [1-^{14}C]acetyl-CoA and unlabelled malonyl-CoA, and the same pattern of products was observed. The compound tentatively identified as triacetate lactone (TAL) (product 1 in Table 1) is marked by an arrow.

cone, 6-(2′,4′-dihydroxy-6′-methylphenyl)-4-methoxy-2-pyrone, was permethylated and analyzed by GC-MS. Both the GC retention time and the EI mass spectrum of the so obtained compound were identical with those of the methylated enzymatic hexaketide (4-Me). Therefore, the structure of compound **4** was determined to be 6-(2′,4′-dihydroxy-6′-methylphenyl)-4-hydroxy-2-pyrone (Table 1).

The putative hexaketide pyrone (**5**) with the $[M\text{-}H]^-$ ion at *m/z* 251 showed a characteristic fragmentation pattern in the CID spectrum. In addition to the key ion *m/z* 125 ($[C_6H_5O_3]^-$, pyrone moiety), the fragment ions *m/z* 207 $[M\text{-}H\text{-}CO_2]^-$, 165 $[M\text{-}H\text{-}CO_2\text{-}CH_2CO]^-$ and 123 $[M\text{-}H\text{-}CO_2\text{–}2xCH_2CO]^-$ were detected (Table 1). An $[M\text{-}H]^-$ peak at *m/z* 251.05575 (calculated for $C_{12}H_{11}O_6^-$ 251.05611) was observed in the HR-ESIMS, and we suggest that the compound possesses a 4-hydroxy-6-(2′,4′,6′-trioxo-heptyl)-pyrone structure (Table 1).

Moreover, it was examined whether recombinant PinPKS accepts larger starter or extender CoA esters. Several aromatic and short-chain aliphatic acyl CoA starters were tested together with malonyl-CoA as the extender unit. PinPKS accepted all offered starter units

Table 1. LC-MS analysis of enzymatically formed products: 20 eV collision-induced dissociation (CID) mass spectra obtained from the $[M\text{-}H]^-$ ions by LC/ESI-MS (Rt_{HPLC} = HPLC retention time) and elemental composition determined by HR/ESI-MS.

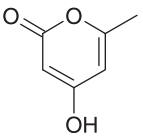
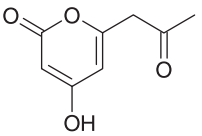
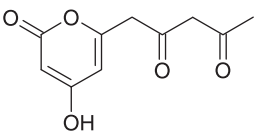
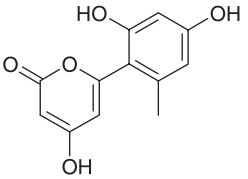
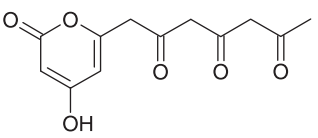
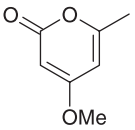
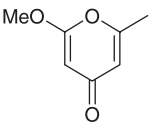
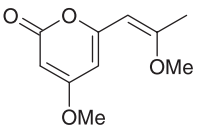
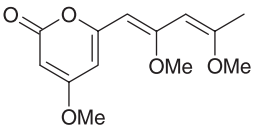
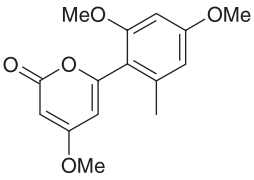
Product	Rt_{HPLC} (min)	$[M\text{-}H]^-$ (*m/z*)	Ions in the 20 eV negative ion CID mass spectra *m/z* (relative intensity)	HR-MS elemental composition	Proposed structure
1 (TAL)	4.46	125	125 (42), 83 (5), 81 (100), 41 (4)	Not determined	
2	4.03	167	167 (60), 125 (100), 123 (35), 99 (6), 83 (12), 81 (14), 75 (2), 65 (8), 57 (3)	Not determined	
3		209		*m/z* 209.04505 $[M\text{-}H]^-$ (calculated for $C_{10}H_9O_5^-$ 209.04555)	
4	9.22	233	233 (51), 191 (12), 189 (100), 165 (51), 147 (35), 145 (2), 121 (1)	*m/z* 233.04498 $[M\text{-}H]^-$ (calculated for $C_{12}H_9O_5^-$ 233.04555)	
5	22.70	251	251 (63), 209 (3), 207 (37), 183 (1), 165 (7), 141 (1), 125 (100), 123 (4), 83 (2)	*m/z* 251.05575 $[M\text{-}H]^-$ (calculated for $C_{12}H_{11}O_6^-$ 251.05611)	

Table 2. GC-MS analysis of permethylated products of PinPKS.

Methylated product[a]	Number of methylations	Rt_{GC} (min)	Ions in the 70 eV EI mass spectra *m/z* (relative intensity)	Proposed structure of methylated compound
1a-Me	1	11.16	140 ($[M]^+$, 70), 125 ($[M\text{-}Me]^+$, 66), 112 ($[M\text{-}CO]^+$, 100), 83 (20), 69 ($[M\text{-}Me\text{-}2CO]^+$, 88), 59 (32), 57 (23), 53 (21)	
1b-Me	1	11.63	140 ($[M]^+$, 73), 112 ($[M\text{-}CO]^+$, 10), 100 (8), 97 ($[M\text{-}CO\text{-}Me]^+$, 45), 69 ($[M\text{-}2CO\text{-}Me]^+$, 100), 67 (11)	
2-Me	2	16.90	196 ($[M]^+$, 100), 168 ($[M\text{-}CO]^+$, 66), 153 ($[M\text{-}CO\text{-}Me]^+$, 34), 139 (13), 125 (70), 113 (14), 99 (21), 87 (16), 53 (11)	
3-Me	3	19.86	252 ($[M]^+$, 12), 237 ($[M\text{-}Me]^+$, 7), 209 ($[M\text{-}Me\text{-}CO]^+$, 6), 193 ($[M\text{-}Me\text{-}CO_2]^+$, 8), 163 (8), 153 (57), 140 (49), 125 (100), 112 (19), 111 (20), 98 (30), 95 (17), 85 (31), 83 (19), 71 (41), 69 (64), 55 (38)	
4-Me	3	22.57	276 ($[M]^+$, 100), 248 ($[M\text{-}CO]^+$, 54), 233 ($[M\text{-}CO\text{-}Me]^+$, 43), 217 ($[M\text{-}CO\text{-}OMe]^+$, 14), 205 ($[M\text{-}2CO\text{-}Me]^+$, 60), 191 (14), 190 (15), 189 (21), 179 (33), 178 (36), 162 (11), 125 (14), 124 (20), 91 (15), 77 (16), 69 (30), 59 (11)	

[a]A permethylated compound derived from **5** could not be detected.

and produced multiple products of various lengths. With *n*-butyryl-CoA as starter, pyrones derived from tri-, tetra-, penta- and hexaketides were detected. In addition to the pyrones with linear keto side chains, 6-(2′,4′-dihydroxy-6′-propylphenyl)-4-hydroxy-2-pyrone, the hexaketide corresponding to compound **4**, could be identified. When PinPKS was incubated with isovaleryl-CoA, hexanoyl-CoA, or benzoyl-CoA, pyrones with linear keto side chains arising from two, three and four extensions were detected. From the large starter CoA esters cinnamoyl-CoA and *p*-coumaroyl-CoA, only tri- and tetraketide pyrones were produced. Hence, PinPKS performs fewer extensions when larger primer units are offered. However, in all incubations, pyrones derived from an acetate starter were found, indicating that malonyl-CoA can serve as primer unit if it is decarboxylated to acetyl-CoA. For example, the triketide TAL (compound **1**) and one of the hexaketides (compounds **4** or **5**) were present in all product mixtures. In incubations with acetyl-CoA as starter CoA ester and methylmalonyl-CoA as alternative extender unit, tri- and tetraketide lactones similar to compounds **1** and **2** were produced. Interestingly, methylmalonyl-CoA did not only serve as extender, but also as starter unit, presumably after decaboxylation to propionyl-CoA.

Recombinant PinPKS from *P. indica* roots was identified as hexaketide synthase (HKS). The products **1**, **2**, **3** and **5** can be classified as derailment products of type III PKSs. Compound **4** is a precursor of the secondary metabolite aloenin A. However, phenylpyrones related to aloenin A have never been isolated from *P. indica*. To re-investigate whether these metabolites are produced in *P. indica*, root tissue was lyophilized, extracted with ethanol and analyzed by LC-ESI-MS or GCMS. Aloenin A, its aglycone or the PinPKS product 6-(2′,4′-dihydroxy-6′-methylphenyl)-4-hydroxy-2-pyrone could not be detected in ethanolic root extracts of *P. indica*.

Discussion

PinPKS produces 4-hydroxy-2-pyrones from tri- to hexaketides. Tri- and tetraacetate lactones (**1** and **2**) are often synthesized by recombinant PKSs when they are incubated with acetyl-CoA as start substrate

[28,30,31]. Pentaketide pyrones such as **3**, however, have never been reported from any type III PKS. Two other pentaketides are known from previous studies. THNS produces 1,3,6,8-tetrahydroxynaphthalene from five units malonyl-CoA [22,23], and *A. arborescens* PCS folds its pentaketide intermediate into a chromone [24] (Fig. 1D,E).

Recombinant PinPKS is the first unmodified type III PKS that produces 6-(2′,4′-dihydroxy-6′-methylphenyl)-4-hydroxy-2-pyrone (**4**). In addition, a hexaketide pyrone (**5**) with a triketo side chain was extracted from enzymatic incubations. The linear hexaketide presumably represents a precursor of the phenylpyrone **4** prior to cyclization of the aromatic ring. A hexaketide pyrone very similar to **5** is synthesized by THNS with octanoyl-CoA as starter and malonyl-CoA as extender unit [32]. The ability of THNS to accept this long starter and perform five extension reactions may be explained by the presence of an additional pocket in the floor of the active site cavity [17]. This tunnel can accommodate starter substrates much longer than the physiological substrate malonyl-CoA, and there is still sufficient space in the active site cavity to allow five condensations with malonyl-CoA. A similar tunnel was also discovered in the crystal structure of *Mycobacterium tuberculosis* PKS18 [18] and in the calculated structure of *R. palmatum* ALS [26]. In *M. tuberculosis* PKS18, the tunnel accommodates the long chain fatty acid starter the recombinant enzyme is utilizing. In *R. palmatum* ALS, the residue corresponding to T197 in *M. sativa* CHS2 is replaced by the smaller residue alanine. This not only increases the size of the active site cavity, as described above, it also opens access to a new tunnel which would be otherwise buried. PinPKS contains the T197A substitution, too, and it is tempting to speculate that the enzyme possesses a tunnel adjacent to its active site cavity that affords longer polyketide chains.

PinPKS catalyzes the formation of at least five pyrones *in vitro* from acetyl-CoA and malonyl-CoA, but none of these compounds has ever been isolated from *P. indica*. Our own investigations revealed that the hexaketide phenylpyrone **4** and methylated or glucosylated derivatives are not produced in *P. indica* roots, although PinPKS is expressed in this tissue. Plumbagin, which is derived from six acetate units, or a related naphthalene derivative were not synthesized by the recombinant enzyme. Like most other type III PKSs, recombinant PinPKS shows a broad substrate acceptance and produces pyrones of various length with different aliphatic and aromatic starter CoA esters. With methylamonyl-CoA as extender, however, only tri- and tetraketides are formed. This may be explained by increased size of the extender unit that does not allow more than three condensations. Although numerous pyrone products were synthesized with alternative starter and extender units, none of these compounds has ever been described as a constituent of *P. indica*.

Several recombinant type III PKSs are known to synthesize metabolites that do not occur in their plant of origin. The recently isolated PKS from *Wachendorfia thyrsiflora*, for example, produces benzalacetones and pyrones from di- and triketides, respectively, but it does not synthesize phenylphenalenones which are accumulated in the plant [29]. *A. arborescens* OKS catalyzes the formation of an octaketide that would be required for the biosynthesis of anthraquinones [25] (Fig. 1C). However, cyclization of the octaketide intermediate yields two polyketides which do not occur in plants, SEK 4 and SEK 4b (Fig. 1C). These compounds were first isolated from the minimal type II PKS of the 16-carbon actinorhodin [33,34]. In type II polyketide biosynthesis, aromatases and cyclases are required for the correct folding of the polyketide intermediate into polycyclic aromatic systems. These enzymes interact with the minimal type II PKS and stabilize the highly reactive poly β-keto chain. Abe *et al.* [25] suggested that 'tailoring enzymes' are required for the folding of anthraquinones from the octaketide intermediate provided by OKS. Although proteins similar to the cyclases and aromatases of type II PKSs biosynthesis have never been isolated from plants, it is possible that a cyclase-like cofactor is required for plumbagin formation.

The failure of PinPKS to produce naphthoquinones could also be due to other missing accessory proteins, e.g. a ketoreductase. In the course of plumbagin biosynthesis, the oxygen of the third acetate unit of the hexaketide has to be removed. If ketoreduction occurs during the elongation of the polyketide, the possibility for nonspecific cyclizations will be reduced. Also, for the biosynthesis of actinorhodin and enterocin-wailupemycins, aromatic polyketides synthesized by type II PKSs, it was postulated that ketoreduction happens prior to cyclization [35,36]. The only polyketide reductase of plant origin known so far is chalcone reductase (CHR), and it shares little similarity with the bacterial polyketide reductases. CHR catalyzes the formation of 6′-deoxychalcones in coaction with CHS. Precursor studies suggested that the reduction of the keto group occurs before the formation of the aromatic ring [37]. Recent investigations based on the three-dimensional structure of CHR indicated that the substrate of CHR is the nonaromatized coumaroyl-trione [38] rather than the intermediate coenzyme A-linked polyketide. An enzyme similar to CHR was proposed for the biosynthesis of stilbenecarboxylates in *Hydrangea macrophylla*, because these metabolites

appear to be derived from a reduced tetraketide [12,39]. It is therefore possible that the formation of plumbagin also requires a polyketide reductase which interacts with PinPKS to produce a correctly folded naphthalene ring system.

In conclusion, PinPKS is a new member of the superfamily of type III polyketide synthases. Since the longest polyketides produced by PinPKS are hexaketides, the enzyme should be termed hexaketide synthase (HKS). It remains to be established whether the HKS provides the backbone of acetate-derived naphthoquinones *in vivo*. The recombinant enzyme catalyzes the condensation of three to six acetate units *in vitro*, but it does not perform the two cyclization reactions required for a naphthalene ring system. Although only one hexaketide-forming PKS was isolated from *P. indica*, is difficult to rule out completely the existence of additional PKSs that catalyze the formation of naphthalenes. Moreover, it is still possible that the HKS described herein is involved in an as yet unknown pathway. However, the exclusive formation of pyrone-type derailment products by the recombinant enzyme suggests that an important aspect is missing under *in vitro* conditions. In recent years, several type III PKSs were isolated that do not perform the reactions *in vitro* that they are supposed to catalyze in plants [25,29,39]. There is emerging evidence that accessory proteins like ketoreductases and cyclases may play a more important role in the biosynthesis of plant polyketides than previously believed. Consequently, *P. indica* HKS may be a good model to study interactions with such cofactors.

Experimental procedures

Chemicals

Acetyl-CoA and malonyl-CoA were purchased from Sigma (Munich, Germany). [1-^{14}C]Acetyl-CoA (4 mCi·mmol^{-1}) and [2-^{14}C]malonyl-CoA (55 mCi·mmol^{-1}) were purchased from Biotrend Chemikalien (Cologne, Germany). Authentic aloenin A was obtained from Phytoplan GmbH (Heidelberg, Germany).

Plant material

P. indica plants were grown at the greenhouse of the Leibniz Institute of Plant Biochemistry, Halle, Germany, at 24 °C with 18 h of light and 50% humidity.

Isolation of a partial PKS cDNA by RT-PCR

Total RNA was isolated from roots of *P. indica* according to the method of Salzman *et al.* [40] and reverse-transcribed using a cDNA synthesis kit (Invitrogen, Karlsruhe, Germany). The obtained first-strand cDNA was used as a template for PCR amplification with degenerate primers that correspond to conserved regions of plant PKSs [31,41]. PCR was carried out as described previously [30,31]. A PCR product of approximately the desired size (575–600 bp) was gel purified, ligated into pGEM-T Easy (Promega) and sequenced.

Construction and screening of a cDNA library

Poly(A)$^+$ RNA was isolated from total RNA of *P. indica* roots using the Oligotex^{TM} Kit (Qiagen, Hilden, Germany), and synthesis of first- and second-strand cDNA was carried out according to the instructions of the ZAP-cDNA Synthesis Kit (Stratagene, Heidelberg, Germany). The cDNA was extended with *Eco*RI and *Xho*I adaptors and ligated into the Uni-ZAP XR vector (Stratagene). The phagemids were packaged with a Gigapack III Gold Extract (Stratagene). The cDNA fragment *Pin1* that had been obtained by RT-PCR was ^{32}P-labeled and used to screen the cDNA library of 80 000 recombinant phages by plaque hydridization. After three rounds of screening, one positive clone (*PinPKS*) was identified. The insert of *PinPKS* in pBluescript SK (–) was isolated by *in vivo* excision using the ExAssist helper phage (Stratagene), and the nucleotide sequence of *PinPKS* was determined on both strands.

Functional expression in *E. coli*

To express *PinPKS* functionally in *E. coli*, the open reading frame (ORF) was cloned into the vector pET-14b (Novagen, San Diego, CA, USA), which allows expression of recombinant proteins with an N-terminal hexahistidine tag. *Nde*I and *Bam*HI sites were engineered in the ORF by PCR using 5′-ATT TTT CAT ATG GCA CCA GCA GTT CAA-3′ (*Nde*I site is underlined) as the forward primer and 5′-TTT AAA GGA TCC TTA GTT AAG CGG CAC ACT-3′ (*Bam*HI site is underlined) as the reverse primer. PCR was carried out with *PinPKS* cDNA in pBluescript SK (–) as template and the enzyme *Pfu* polymerase (Promega, Madison, WI, USA). The 1.2 kb PCR product was digested with *Nde*I and *Bam*HI, gel-purified and ligated into the *Nde*I/*Bam*HI digested vector pET-14b. The sequence of the resulting construct *Pin*PKS:pET-14b was confirmed, and the expression plasmid was transformed into *E. coli* BL21 (DE3) cells (Stratagene).

E. coli harboring *Pin*PKS:pET-14b was grown at 37 °C in LB medium containing 50 μg·mL^{-1} ampicillin until Abs_{600} ~0.8. Protein expression was induced with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside, and the culture was grown 16 h at 28 °C. *E. coli* cells were harvested, and recombinant PinPKS was purified by following procedures essentially as described previously [30,31].

Enzyme assay

The standard reaction mixture contained 100 mM Hepes or 100 mM potassium phosphate buffer (pH 7.0), 30 μM acetyl-CoA, 70 μM [2-^{14}C]malonyl-CoA (70 000 dpm), and 5 μg recombinant protein in a 100 μL reaction. In some incubations, 30 μM [1-^{14}C]acetyl-CoA (270 000 dpm) and unlabelled malonyl-CoA were used. The assay mixture was incubated for 1 h at 30 °C, stopped by addition of 10 μL 6 N HCl and extracted twice with 200 μL ethyl acetate. The organic phase was dried, and products were separated by TLC on silica gel with ethylacetate–methanol–H_2O 100 : 16.5 : 13.5. ^{14}C-labelled products were visualized by phosphoimaging.

Product identification by LC-MS

To identify the enzymatically formed products, scaled-up reactions were used containing 50 μg recombinant protein, 300 μM acetyl-CoA, and 700 μM malonyl-CoA in a total volume of 400 μL. After 1-h incubation at 30 °C, the mixtures were acidified with 6 M HCl and extracted twice with 800 μL ethylacetate. The combined organic phases were evaporated to dryness and the residue was dissolved in methanol. Products of at least three 400 μL reactions were combined and subjected to LC-MS analysis.

Negative ion ESI mass spectra were obtained with a Finnigan MAT TSQ 7000 instrument coupled with a Surveyor MicroLC system equipped with a RP18-column (5 μm, 1 × 100 mm, SepServ, Berlin, Germany), as described previously [30,31].

High resolution MS analysis

The high resolution negative ion ESI mass spectra were obtained from a Bruker Apex III Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (Bruker Daltonics, Billerica, MA, USA) equipped with an Infinity™ cell, a 7.0 Tesla superconducting magnet (Bruker, Karlsruhe, Germany), an RF-only hexapole ion guide and an external electrospray ion source (off axis spray, voltages: endplate, 3.700 V; capillary, 4.400 V; capillary exit, −70 V; skimmer 1, −10 V; skimmer 2, −8 V) (Agilent, Palo Alto, CA, USA). Nitrogen was used as drying gas at 150 °C. The sample solutions were introduced continuously via a syringe pump with a flow rate of 120 μL·h^{-1}. All data were acquired with 256 K data points and zero filled to 1024 K by averaging 32 scans.

Product identification by GC-MS

For GC-MS analysis the enzymatic products of upscaled assays or aloenin aglycone were dissolved in methanol and permethylated for 15 min with diazomethane. Subsequently, the solvent was evaporated to dryness. GC-MS measurements were performed on a Voyager/Trace GC 2000 (Thermo Quest CE Instruments, Austin, TX, USA) under the following conditions: 70 eV EI, source temperature 200 °C; column DB-5MS (30 m × 0.25 mm, 0.25 μm film thickness) (J & W Scientific, Cologne, Germany); injection temperature 250 °C, interface temperature 300 °C; carrier gas He, flow rate 1.0 mL·min^{-1}, constant flow mode; splitless injection, column temperature program: 60 °C for 1 min, then raised to 300 °C at a rate of 15 °C·min^{-1} and then held on 300 °C for 10 min.

Preparation of 6-(2′,4′-dimethoxy-6′-methylphenyl)-4-methoxy-2-pyrone

Aloenin A (17 mg; Phytoplan GmbH) was refluxed for 1 h in 80% methanol and 3% hydrochloric acid. Methanol was removed under reduced pressure, and the water phase was extracted three times with ethyl acetate. The combined organic phases were re-extracted twice with water, and then ethyl acetate was removed *in vacuo*. This yielded 6.4 mg of partially pure aloenin aglycone containing trace amounts of the 4-demethylated compound.

Negative ion ESI-CIDMS (20 eV) of 6-(2′,4′-dihydroxy-6′-methylphenyl)-4-methoxy-2-pyrone [retention time (RT) = 11.24 min; *m/z* (relative intensity, %)]: *m/z* 247 ([M-H]$^-$, 28), 232 (6), 215 ([M-H-MeOH]$^-$, 37), 203 ([M-H-CO_2]$^-$, 29), 188 ([M-H-CO_2-Me]$^-$, 44), 171 ([M-H-CO_2-MeOH]$^-$, 100), 123 (4).

EI-MS (70 eV) of permethylated 6-(2′,4′-dimethoxy-6′-methylphenyl)-4-methoxy-2-pyrone [RT = 22.35 min; *m/z* (relative intensity, %)]: *m/z* 276 ([M]$^+$, 100), 248 ([M-CO]$^+$, 49), 233 ([M-CO-Me]$^+$, 41), 217 ([M-CO-OMe]$^+$, 13), 205 ([M-2CO-Me]$^+$, 52), 191 (12), 190 (13), 189 (19), 179 (30), 178 (31), 162 (10), 125 (13), 124 (15), 91 (11), 77 (12), 69 (26), 59 (12).

Analysis of *P. indica* roots

Freeze-dried roots of *P. indica* plants (0.25 g) were refluxed with 10 mL ethanol for 1 h. The liquid extract was filtered and the solvent was evaporated with nitrogen gas. The dried sample was analyzed directly by LC-ESI-MS or permethylated by diazomethane and then subjected to GC-MS analysis.

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