CYP71B15 (PAD3) Catalyzes the Final Step in Camalexin Biosynthesis

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Camalexin represents the main phytoalexin in Arabidopsis (Arabidopsis thaliana). The camalexin-deficient phytoalexin deficient 3 (pad3) mutant has been widely used to assess the biological role of camalexin, although the exact substrate of the cytochrome P450 enzyme 71B15 encoded by PAD3 remained elusive. 2-((3H)-3-yl)-4,5-dihydro-1,3-thiazole-4-carboxylic acid (dihydrocamalexic acid) was identified as likely intermediate in camalexin biosynthesis downstream of indole-3-acetaldoxime, as it accumulated in leaves of silver nitrate-induced pad3 mutant plants and it complemented the camalexin-deficient phenotype of a cyp79b2/cyp79b3 double-knockout mutant. Recombinant CYP71B15 heterologously expressed in yeast catalyzed the conversion of dihydrocamalexic acid to camalexin with preference of the (S)-enantiomer. Arabidopsis microsomes isolated from leaves of CYP71B15-overexpressing and induced wild-type plants were capable of the same reaction but not from microsomes from induced leaves of pad3 mutants. In conclusion, CYP71B15 catalyzes the final step in camalexin biosynthesis.

Camalexin (3-thiazol-2’-yl-indole), originally isolated from Camelina sativa (Browne et al., 1991), is the main phytoalexin of the model plant Arabidopsis (Arabidopsis thaliana; Tsuji et al., 1992). Its formation has been studied in detail as part of the highly sophisticated network of plant defense reactions, including hypersensitive response after interaction with incompatible pathogens (for review, see Kliebenstein, 2004; Glazebrook, 2005). Camalexin has been shown to inhibit growth of particular plant pathogens, e.g., Alternaria brassicicola or some Botrytis cinerea isolates, while others remain unaffected (Rogers et al., 1996; Thomma et al., 1999; Ferrari et al., 2003; Kliebenstein et al., 2005).

It was recently shown that camalexin is derived from indole-3-acetaldoxime (IAOx) that is synthesized from Trp by the cytochrome P450 enzymes CYP79B2 and CYP79B3 (Fig. 1; Glawischnig et al., 2004). IAOx also is an intermediate in the biosynthesis of indole glucosinolates and a precursor for the phytohormone indole-3-acetic acid. This makes IAOx a key branching point between primary and secondary metabolism. In vivo feeding experiments suggest that the thiazole ring of camalexin is derived from Cys that has reacted with a product of IAOx, e.g., indole-3-carbaldehyde (Zook and Hammerschmidt, 1997).

So far five phytoalexin deficient mutants (pad1–pad5) have been isolated in a screen for mutants with reduced camalexin content (Glazebrook and Ausubel, 1994; Glazebrook et al., 1997). Having consistently low camalexin levels independent of the inducing pathogen or abiotic treatment, pad3 has been widely used to investigate the role of camalexin in various Arabidopsis-pathogen interactions (Glazebrook and Ausubel, 1994; Thomma et al., 1999; Roetschi et al., 2001; Ferrari et al., 2003; Mert-Türk et al., 2003; Bohman et al., 2004). The corresponding PAD3 gene that was identified by positional cloning encodes for the cytochrome P450 enzyme CYP71B15 (Zhou et al., 1999). This suggested a function as camalexin biosynthetic gene (Zhou et al., 1999), or that CYP71B15 plays an indirect regulatory role, similar to MAX1, a regulatory P450 enzyme involved in flavonoid biosynthesis (Lazar and Goodman, 2006). Recently, 2-((3H)-3-yl)-4,5-dihydro-1,3-thiazole-4-carboxylic acid (dihydrocamalexic acid) was shown to accumulate in infected pad3 root cultures and was suggested as intermediate in camalexin biosynthesis (Bednarek et al., 2005).

In this article, we demonstrate that CYP71B15, expressed heterologously in yeast, catalyzes the conversion of dihydrocamalexic acid to camalexin. The same reaction was obtained with Arabidopsis microsomes isolated from untreated 35S::CYP71B15 and induced wild-type leaves, but not from silver nitrate-induced pad3 plants. In conclusion, CYP71B15 catalyzes the final step in camalexin biosynthesis (Fig. 1).
RESULTS

Dihydrocamalexic Acid Accumulates in Induced pad3 Leaves and Complements the Camalexin-Deficient Phenotype of the cyp79b2/cyp79b3 Knockout Mutant

The level of dihydrocamalexic acid has been shown to be increased in root culture liquid of pad3 and pad5 knockout mutants (Bednarek et al., 2005). To identify potential intermediates in camalexin biosynthesis, methanolic leaf extracts from silver nitrate-treated plants were analyzed by liquid chromatography (LC)-mass spectrometry (MS) for accumulation of metabolites in the pad3 mutant. Dihydrocamalexic acid accumulated approximately 5-fold in pad3 mutant in comparison to wild-type leaves (Fig. 2), but was undetectable in control leaves (data not shown). These data are consistent with a role of dihydrocamalexic acid as a camalexin biosynthetic intermediate.

cyp79b2/cyp79b3 knockout mutants (Zhao et al., 2002) are camalexin deficient due to their inability to synthesize the intermediate IAOx (Glawischnig et al., 2004). When silver nitrate-treated cyp79b2/cyp79b3 rosette leaves were incubated in a 100 μM solution of (S)-dihydrocamalexic acid, wild-type levels (11.6 ± 4.8 μg g⁻¹) of camalexin were synthesized. This shows that dihydrocamalexic acid is a precursor for camalexin. The enantiomer (R)-dihydrocamalexic acid (100 μM), derived from nonproteinogenic d-Cys, also was converted to camalexin but to a lesser extent (4.2 ± 0.6 μg g⁻¹). Only minor camalexin formation in comparison to background level (0.1–0.2 μg g⁻¹) was observed when pad3 mutants were incubated with either enantiomer (approximately 0.5 μg g⁻¹), indicating that PAD3 (CYP71B15) is involved in the conversion of (S)-dihydrocamalexic acid to camalexin.

Dihydrocamalexic Acid Is the Substrate of CYP71B15

The enzymatic function of CYP71B15 was investigated by analysis of recombinant CYP71B15 expressed in the yeast strain WAT11, carrying the Arabidopsis cytochrome P450 reductase ATR1 (Pompon et al., 1996). When CYP71B15-expressing yeast microsomes were incubated with (S)-dihydrocamalexic acid, HPLC analysis of the ethyl acetate extract of the reaction mixture identified a product peak that comigrated with authentic camalexin and showed its typical fluorescence signal and UV absorption spectrum (Fig. 3). Gas chromatography (GC)-high-resolution MS analysis confirmed that the reaction product was camalexin based on the product molecular composition (for C11H8N2S calculated 200.04082, found 200.04113) and identical mass spectra with those of the standard. The enzymatic reaction was NADPH dependent, and an apparent Kᵣ of 26.8 ± 1.6 μM for the substrate was determined (Fig. 4A). No activity was observed in yeast transformed with the empty vector control or in boiled enzyme preparations. Similarly, (R)-dihydrocamalexic acid was converted to camalexin and an apparent Kᵣ for this substrate of 45.5 ± 5.2 μM was determined. In addition, IAOx, indole-3-carbinol, indole-3-carboxaldehyde, and indole-3-carboxylic acid were tested as substrates, but no apparent NADPH-dependent product was detected (data not shown).

Arabidopsis Microsomes Are Capable of the Same Enzymatic Conversions

To address whether this reaction is performed in planta and can be linked to a functional PAD3 gene, three genotypes were analyzed: Columbia (Col)-0 wild type, the pad3 mutant, and 35S::CYP71B15 lines. Seven independent overexpression lines were generated, all of which did not show any obvious morphological changes (data not shown). Line #1 showed a 44 ± 8.8-fold induction of the constitutive CYP71B15 expression in comparison to wild type. Twenty-four hours after silver nitrate spraying, camalexin level in 35S::CYP71B15#1 was 9.4 ± 5.9 μg g⁻¹ fresh weight (FW; n = 10) and differed not significantly from wild-type plants treated the same (7.3 ± 3.2 μg g⁻¹ FW). In untreated 35S::CYP71B15#1 plants, no reproducible camalexin formation was observed. This indicates that
in vivo CYP71B15 is not rate limiting, consistent with its role in catalyzing the last biosynthetic step. Microsomes were prepared from untreated leaves and leaves 16 h after silver nitrate spraying of Col-0, pad3, and 35S::CYP71B15#1 plants. NADPH-dependent camalexin formation from dihydrocamalexin acid was determined in the six microsomal preparations (Fig. 5). While no activity was detected in microsomes from untreated and silver nitrate-sprayed pad3 leaves, untreated wild-type microsomes showed a low dihydrocamalexin acid turnover, which was strongly enhanced after induction of the camalexin pathway by silver nitrate spraying. 35S::CYP71B15#1 microsomes showed activity without silver nitrate induction (Fig. 5). This demonstrates that Arabidopsis microsomes convert dihydrocamalexin acid to camalexin dependent on functional CYP71B15.

A $K_m$ of approximately $26.7 \pm 2.5 \mu M$ was determined for this reaction by Col-0 microsomes (Fig. 4B). For the $(R)$-enantiomer, an apparent $K_m$ of $67.7 \pm 3.6 \mu M$ was determined and the catalytic efficiency was approximately 36% in comparison to the $(S)$-enantiomer, which clearly shows the preference for the assumed natural substrate originated from conjugation with l-Cys.

**Figure 2.** LC-MS analysis of dihydrocamalexin acid in methanol extracts of rosette leaves of pad3 and wild-type plants 18 h after silver nitrate spraying. A and B, Extracted ion chromatogram ($m/z = 247$) of wild type (A) and pad3 mutants (B) leaf extract is shown. C, Dihydrocamalexin acid standard. One of three independent experiments with comparable results is presented.

**Figure 3.** Analysis of CYP71B15 activity. A, HPLC profile with fluorescence detection after enzymatic conversion of $(S)$-dihydrocamalexin acid to camalexin by microsomes from yeast expressing CYP71B15 in the presence and absence of NADPH, or from yeast vector control (with NADPH). The product identity was confirmed by UV spectroscopy (B) and electron ionization MS spectrometry (C).
DISCUSSION

We have demonstrated that CYP71B15 (PAD3) catalyzes the final step in camalexin biosynthesis. Large data sets exist on CYP71B15 induction upon infection with camalexin-inducing pathogens and abiotic treatments (Zhou et al., 1999; Glazebrook et al., 2003; Eulgem et al., 2004; Glawischnig et al., 2004; https://www.genevestigator.ethz.ch). We showed that CYP71B15 expression is induced locally, which is in accordance with its role in the biosynthesis of a phytoalexin. This expression pattern matches accumulation of camalexin and expression of other biosynthetic genes (Schuhegger et al., 2006) indicating that the whole biosynthetic pathway is colocalized and no transport of a biosynthetic intermediate occurs.

The pad3 mutant has been a valuable tool to study the effect of camalexin on pathogen growth. Being mutated in the last biosynthetic step with the precursor dihydrocamalexic acid still being synthesized, it is now clear that the enhanced susceptibilities of pad3 are the specific results of camalexin deficiency. In some cases where no enhanced susceptibility of pad3 is observed, the effect of dihydrocamalexic acid accumulation might mask the effect of camalexin deficiency. Dihydrocamalexic acid is an intermediate in camalexin biosynthesis and, in addition, is released from roots (Bednarek et al., 2005), where it possibly exhibits antimicrobial activity in the soil. This suggests that, in roots, all enzymes of the dihydrocamalexic acid-forming metabolon are tightly coregulated, but that PAD3 is regulated independently. Similar to pad3, the pad5 mutant also releases elevated amounts of dihydrocamalexic acid into the soil (Bednarek et al., 2005). In contrast to PAD3, the PAD5 protein, which remains to be identified, is unlikely to be involved in the conversion of dihydrocamalexic acid to camalexin, as yeast-expressed PAD3 (CYP71B15) is fully functional. Possible functions of PAD5 include regulation of PAD3 or integrity of a camalexin biosynthetic metabolon at the level of dihydrocamalexic acid.

PAD3 belongs to the large CYP71B family of P450 genes consisting of 37 members (Wерck-Reichhart et al., 2002). The pad3 mutant synthesizes only minor amounts of camalexin, which shows that no other expressed CYP71B enzyme efficiently catalyzes the same reaction. A search for gene expression data revealed that most CYP71B genes are not significantly expressed. Only a few were markedly induced after pathogen challenge or under abiotic stress (e.g. CYP71B6, CYP71B7, and CYP71B20; Narusaka et al., 2004; https://www.genevestigator.ethz.ch). These expressed genes showed less than 60% identity to CYP71B15 on the amino acid level. As there are a number of examples of plant P450 enzymes with higher homology catalyzing different enzymatic reactions, it is not surprising that none of these genes can complement the pad3 mutant phenotype.

CYP71B15 catalyzes an oxidative decarboxylation of both enantiomers of dihydrocamalexic acid. To our

CYP71B15 Expression Is Locally Induced in Leaves and Roots

To confirm that the expression pattern of CYP71B15 is in accordance with its role as a camalexin biosynthetic gene, CYP71B15p:β-glucuronidase (GUS) plants were generated. For B. cinerea and Alternaria alternata, it was shown that camalexin accumulation was induced locally at the infection sites (Kliebenstein et al., 2005; Schuhegger et al., 2006). Enhanced CYP79B2p::GUS activity was detected at the site of silver nitrate exposure or pathogen infection (Glawischnig et al., 2004; Schuhegger et al., 2006), demonstrating localized induction of the biosynthetic gene CYP79B2. The spatial distribution of CYP71B15 induction was investigated in rosette leaves of CYP71B15p::GUS reporter plants exposed to silver nitrate as abiotic camalexin inducer, and A. alternata and Pseudomonas syringae as eukaryotic and prokaryotic plant pathogens, respectively. Induction of CYP71B15 expression localized to the site of treatment was observed (Fig. 6, A–E). Roots also showed CYP71B15p::GUS activity that was enhanced by exposure to silver nitrate (Fig. 6, F–H).

Figure 4. Kinetic properties of CYP71B15. The conversion of the (S)- and (R)-enantiomer of dihydrocamalexic acid to camalexin by microsomes of yeast expressing CYP71B15 (A) and Arabidopsis (B) was determined for different substrate concentrations. Substrate turnover (pmol mg⁻¹ min⁻¹) is plotted against substrate concentration (μM). Squares, (S)-enantiomer; circles, (R)-enantiomer.
knowledge, this is the only cytochrome P450 reaction described in plants that results in simultaneous decarboxylation and introduction of a C–C– double bond. For this unusual reaction, we propose that the pentavalent oxoiron in the reaction center of CYP71B15 initially abstracts a hydrid ion from C-5 of the thiazole ring. The formed intermediate liberates carbon dioxide in a fast spontaneous process forming a C-4/C-5 carbon-carbon double bond in the thiazole ring of camalexin (Fig. 7). In this model, the rate-determining process is the CYP71B15-catalyzed hydride abstraction, and the subsequent decarboxylation is not under enzymatic control. In addition to the (S)-enantiomer, the binding pocket of CYP71B15 can accommodate “unnatural” (R)-enantiomer, although with lower relative catalytic efficiency. We expect that the mechanism is similar to the one described for isovalerate decarboxylation (Fukuda et al., 1994), and that it also resembles related desaturation or hydroxylation reactions (Buist, 2004). An alternative model includes hydroxylation at C-5 of the thiazole ring, also resulting in spontaneous formation of a double bond and decarboxylation.

The camalexin biosynthetic pathway from IAOx to dihydrocamalexic acid remains to be resolved. In analogy to indole glucosinolate biosynthesis, it has been hypothesized that the IAOx-metabolizing step is catalyzed by a cytochrome P450 enzyme (Glawischneg et al., 2004). The product of this reaction is unknown. Indole-3-carbaldehyde has been suggested to condensate with Cys in the camalexin biosynthetic pathway (Zook and Hammerschmidt, 1997). This intermediate remains speculative, as external application of indole-3-carbaldehyde did not complement the camalexin-deficient phenotype of the cyp79b2/cyp79b3 knockout mutant (E. Glawischneg, unpublished data). In bacteria,
the introduction of thiazoline rings into secondary products is catalyzed by nonribosomal peptide synthetases with Cys as substrate (for review, see Crosa and Walsh, 2002). As no nonribosomal peptide synthetase genes have been described in Arabidopsis, the challenging task remains to demonstrate to which family the enzyme belongs that condensates the indolic and Cys-related intermediate and to identify the corresponding gene(s).

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (Arabidopsis thaliana) ecotype Col-0 and pad3 plants were obtained from Lehle Seeds and the Nottingham Arabidopsis Stock Center, respectively. Plants were grown in soil mixed with sand (3:1) in a growth chamber at 12/12-h photoperiod at 80 to 100 μmol m⁻² s⁻¹, 21°C, and 40% relative humidity.

Generation and Analysis of CYP71B15-Overexpressing Plants

The CYP71B15 gene (At3g26830) was PCR amplified, sequenced, and cloned into the binary plant transformation vector pCAMBIA2300 under the control of 3SS promoter (for details, see http://www.cambia.org). Agrobacterium-mediated transformation of Arabidopsis Col-0 plants was performed using the floral-dipping method, and successful transformation was confirmed by kanamycin resistance of the seedlings and by PCR analysis. RNA extraction and cDNA synthesis has been described by Schuhegger et al. (2006). Quantitative real-time PCR was performed with the CyberGreen/Light Cyclet system (Roche) using the following primers: Actin1, gatctcggacactattttgagatgatc and gatcatctcaaggaatacttgttgacat; CYP71B15, gatctcggactatattttgacagacctactccataacctgcag; and CYP71B15-Overexpressing Plants.

Generation of CYP71B15p::GUS Plants and GUS Analysis

A 2.9-kb promoter region of CYP71B15 was PCR amplified with the primer pair gaattcgcgctcttatactgtggctatatatgttatagac/cgccatggtccttgccctgttcttgttt and cloned into pGEM-T Easy (Promega) according to the manufacturer’s instructions. The fragment was then excised and inserted into the GUS reporter vector pCAMBIA1305.1 (for details, see http://www.cambia.org). Hygromycin-resistant transformants were selected and checked for positive GUS staining. GUS staining after silver nitrate spraying or challenge with Pseudomonas syringae or Alternaria alternata, respectively, was performed as described previously (Glawischnig et al., 2004).

Synthesis of Dihydrocamalexic Acid

For (+)-(R)-enantiomer, a solution of 1H-indole-3-carbonitrile (40 mg, 0.28 mmol) in degassed MeOH (2 mL) was added to t-Cys (150 mg) dissolved in MeOH (1 mL), phosphate buffer (pH 8, 1 mL). Powdered NaHCO₃ (130 mg) was added and the reaction mixture was stirred at 77°C for 4 d under argon atmosphere. After cooling to ambient temperature, solvents were removed on a rotary evaporator. The residue was mixed with NaHCO₃ solution (8% [w/v], 10 mL) and washed with ethyl acetate (2 × 10 mL). The aqueous phase was acidified with 2 M HCl to pH 3, and extracted into ethyl acetate (3 × 10 mL). The combined organic extracts were washed with brine (10 mL), dried over Na₂SO₄, and concentrated in vacuum. The product was obtained after crystallization from a hexane-ethyl acetate mixture as a beige–rose powder (50 mg, 72%).

H NMR (CD₃OD, 400 MHz): δ = 8.40 (1H, s, indol-H-2), 8.02 (1H, s, indol-H-4), 7.62 (1H, d, indol-H-7, J = 8 Hz), 7.40 (2H, m, indol-H-5,6), 5.40 (1H, m, CH-4), 3.95 (1H, dd, J = 11 Hz, 9 Hz, CH-5a), 3.80 (1H, dd, J = 11 Hz, 8 Hz, CH-5b). Electron ionization MS: (m/z, relative intensities) 246 (M⁺, 24), 201 (M⁺-COOH, 100), 160 (23), 144 (63), 142 (68), 115 (28). [α]D₂⁰ +55 (0.2, MeOH); [α]D₂⁰ +45 (0.2, H₂O).

For (−)-(S)-enantiomer, the optical isomer (11 mg) was prepared from t-Cys hydrate hydrochloride as described above. Electron ionization MS: (m/z, relative intensities) 246 (M⁺, 24), 201 (M⁺-COOH, 100), 160 (23), 144 (63), 142 (68), 115 (28). [α]D₂⁰ +50 (0.2, MeOH); [α]D₂⁰ +45 (0.2, H₂O).

Mass Spectroscopy

Electron ionization and high-resolution mass spectra were obtained on a MassSpec 2 instrument (Micromass) in positive ion mode using 70-eV ionization energy and direct insertion probe. Peryleneoxinsine mixture was used as an internal standard. For GC-high-resolution MS, analyses were performed with a Hewlett-Packard HP6890 gas chromatograph interfaced to a MassSpec 2. Separation was achieved on a J&W Scientific DB-5 capillary column, 30 m × 0.25 mm, 0.25-μm film thickness using helium (30 mL s⁻¹) as carrier gas.

In Vivo Feeding

The camalexin-deficient cyp79b2/cyp79b3 knockout and pad3 mutants were sprayed with 5 mM silver nitrate to test complementation of the pathway with dihydrocamalexic acid. After 8 h, rosette leaves were cut at the petiole and incubated in 100 μL of 100 μM (S)- or (R)-dihydrocamalexic acid for an additional 16 h.

Metabolite Profiling

Leaf material (200 mg) was harvested 18 or 24 h after silver nitrate spraying and frozen in liquid nitrogen. The samples were kept at −80°C until processing. For extraction, the leaves were ground in liquid nitrogen, 1 mL of 50% aqueous MeOH (v/v) was added, and the samples were centrifuged for 15 min at 20,000g. The pellets were re-extracted with 600 μL of 50% MeOH, centrifuged again, and the supernatants were combined. The solvent was removed using a Speed-Vac and the residue was redissolved in 80% aqueous MeOH (1 μL per 5 mg initial fresh weight). The solutions were filtered through a 0.22-μm filter (Millipore) and LC-MS was performed as done by Glawischnig et al. (2004). For specific monitoring of dihydrocamalexic acid, extracted ion chromatograms (m/z = 247) were analyzed.

Yeast Expression, Arabidopsis Microsomes, and Product Analysis

Using recombinant PCR and the primer sets actggatccatggctgttttcctctc-ttctcgtc/ctacactgtcataaatgttgcagatcttttgattgagat and gatctacaaaggact-gatcctggactatattttgacagacctactccataacctgcag, corresponding to exon 1 and 2, respectively, on Arabidopsis ecotype Col-0 genomic DNA, a PCR fragment of the coding region of CYP71B15 was obtained and cloned into...
the binary vector pYDF80 (Pompon et al., 1996), using the 5′ and 3′ restriction sites BamHI and EcoRI, respectively. The insertion was sequenced to exclude PCR errors. Transformation of the yeast Saccharomyces cerevisiae WAT11, yeast growth, and microscopic preparations were performed according to the method of Pompon et al. (1996).

Arabidopsis microsomes were prepared using a modified protocol from Du et al. (1995). Approximately 1.2 g of rosette leaves untreated or treated with 5 mM AgNO3 were ground with sand and Polyclar AT in ice-cold extraction buffer (100 mM ascorbate, 1 mM EDTA, 5 mM dithiothreitol, 100 mM Tris, 20% [w/v] Suc, 20% [v/v] glycerol, pH 7.5), 20 mM g-1 FW, and centrifuged twice 15,000g for 10 min. The supernatant was centrifuged at 200,000g for 30 min. Then the pellet was resuspended in 50 mM KPO4, 1 mM dithiothreitol, 20% [v/v] glycerol; centrifuged at 200,000g for 40 min and resuspended in 1.5 mL of the same buffer.

Enzyme tests with yeast and Arabidopsis microsomes were performed for 30 min at 25°C in 200 mL of 50 mM Tris pH 7.5, 1 mM NADPH, 20 µg microsomal protein, and 200 µM substrate (variable concentration for Km determination), extracted twice with 1 vol ofethyl acetate, which was then evaporated under reduced pressure. The pellet was resuspended in ethanol and analyzed for camalexin by HPLC. Samples were analyzed by reverse-phase HPLC (LiChrosphere reduced pressure. The pellet was redissolved in ethanol and analyzed for protein, and 200 µL substrate (variable concentration for Km), extracted twice with 1 vol ofethyl acetate, which was then evaporated under reduced pressure. The pellet was resuspended in ethanol and analyzed for camalexin by HPLC. Samples were analyzed by reverse-phase HPLC (LiChrosphere 250-4, RP-18, 5 µM [Merck]; 1 mL min-1; MeOH/water [1:1; v/v] for 2 min, followed by a 10-min linear gradient to 100% MeOH, followed by 3 min 100% MeOH). The peak at 10.6 min was identified as camalexin by comparison with authentic standard with respect to retention time and UV spectrum (photodiode array detector; Dionex) and quantified using a Shimadzu F-10AXL fluorescence detector (318 nm excitation, 370 nm emission) and by UV-absorption at 318 nm. To confirm the identity of the product, the corresponding HPLC peak was collected, the MeOH was evaporated, and the remaining water phase was extracted with ethyl acetate and analyzed by GC-MS.

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**LITERATURE CITED**


Du L, Lykkefeldt J, Olsen CE, Halkier BA (1995) Involvement of cytochrome P450 in oxime production in glucosinolate biosynthesis as demonstrated by an in vitro microsomal enzyme system isolated from jasmonic acid-induced seedlings of Sinapis alba L. Proc Natl Acad Sci USA 92: 12505–12509


CORRECTIONS


The stereochemical nomenclature of the dihydrocamalexic acid enantiomers are listed incorrectly throughout this article. The correct listing for the dihydrocamalexic acid prepared from natural l-cysteine is (4R)-dihydrocamalexic acid and the acid prepared from d-cysteine is (4S)-dihydrocamalexic acid.