Phytochemistry Letters 2 (2009) 196–200

Contents lists available at ScienceDirect

Phytochemistry Letters

journal homepage: www.elsevier.com/locate/phytol

In vitro hydroxylation of a norlignan: From agatharesinol to sequirin C and metasequirin C with a microsomal preparation from *Cryptomeria japonica*

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ARTICLE INFO

Article history:
Received 23 October 2008
Received in revised form 6 July 2009
Accepted 7 July 2009
Available online 16 July 2009

Keywords:
Cryptomeria japonica
Sugi
Biosynthesis
Norlignan
Heartwood extracts
Microsomal preparation
Agatharesinol
Metasequirin C
Sequirin C

ABSTRACT

In vitro hydroxylation of the norlignan agatharesinol to sequirin C and metasequirin C was demonstrated for the first time. After incubating agatharesinol with a microsomal preparation from the heartwood side of the intermediate wood of *Cryptomeria japonica* in the presence of cofactors, the aromatic ring-monohydroxylated derivatives of agatharesinol, sequirin C and metasequirin C, were formed. Although hydroxylation hardly occurred in the absence of cofactors, it could be initiated by adding NADPH or NADH, and was enhanced by further adding FAD or FMN. When microsomal preparations from the sapwood or from the sapwood side of the intermediate wood were used, hydroxylation did not occur. This *in vitro* conversion of the norlignans indicates that the hydroxylation of agatharesinol to sequirin C and metasequirin C is part of the *in vivo* biosynthetic pathway of norlignans. Another *C. japonica* norlignan, sugiresinol, which is a side chain-cyclized isomer of agatharesinol, does not seem to be accepted as a substrate, because hardly any hydroxysugireinol was formed after similar incubation with the enzyme.

1. Introduction

Norlignans are a class of secondary metabolites with C_6–C_5–C_6 carbon skeletons, which occur preferentially in the heartwood of certain species of coniferous trees. In *Cryptomeria japonica*, norlignans are considered to affect original wood color, and to cause change in color in response to damage by mechanical wounding or by biological agents (insects, fungi). Especially in these woods, norlignans that differ from those in normal wood are found (Takahasi et al., 1983; Takahasi and Ogiyama, 1985a,b, 1986), which presumably results from alteration of norlignan biosyntheses. However, the biosynthetic pathway of norlignans is not well understood.

Beginning with a feeding study on the biosynthesis of one norlignan, *cis*-hinokiresinol (Suzuki et al., 2002), *in vitro* formation of *cis*- and *trans*-hinokiresinol using an enzyme preparation was demonstrated (Suzuki et al., 2001, 2004). Recently, molecular biological characterization of hinokiresinol synthase was reported (Suzuki et al., 2007). A similar study of the biosynthesis of agatharesinol was undertaken by feeding experiments (Imai and Nomura, 2005; Imai et al., 2006a), and biosynthetic relationships between agatharesinol and *trans*-hinokiresinol in *C. japonica* have been proposed (Imai et al., 2006b).

The preferential occurrence of norlignans in tree heartwood has hindered development of research on norlignan biosynthesis, because (1) the number of living cells in wood is insufficient for extraction of biomacromolecules such as enzymes or nucleic acids, (2) the cells are surrounded by thick lignified secondary cell walls and (3) raw wood materials are very moist, which make it difficult to grind such materials for extracting biomacromolecules. Recently, using frozen wood powders of *C. japonica*, we have performed biochemical experiments including measurement of the activity of phenylalanine ammonia-lyase (PAL), which is involved in norlignan biosynthesis (Imai et al., 2005).

Norlignans from the heartwood of *C. japonica* consist exclusively of agatharesinol and its aromatic ring-monohydroxylated derivative sequirin C (Ogiyama et al.,1983). The content of both norlignans increases from the intermediate wood toward the heartwood, which is consistent with the observed elevated PAL activity in the intermediate wood (Imai et al., 2005), indicating that the intermediate wood is a likely biosynthetic site for the heartwood norlignans. Furthermore, the content of agatharesinol increases gradually from the sapwood side of the intermediate wood toward the heartwood, while that of sequirin C increases rapidly from the heartwood side of the intermediate wood toward the heartwood (Imai et al., 2005): agatharesinol formation is

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doi:10.1016/j.phytol.2009.07.002

initiated at the early stages in the changes from sapwood to heartwood, while sequirin C formation is occurring at the late stages, suggesting that the formation of sequirin C is preceded by that of agatharesinol in norlignan biosyntheses.

In the present report, we demonstrated *in vitro* hydroxylation of norlignan from agatharesinol to sequirin C and metasequirin C using a microsomal preparation from *C. japonica* intermediate wood.

2. Results

2.1. In vitro formation of sequirin C and metasequirin C

Fig. 1a shows typical partial total ion current (TIC) gas chromatography–mass spectrometry (GC–MS) chromatograms of ethyl acetate (EtOAc) extracts of the enzyme reaction mixture obtained using the substrate agatharesinol and the intact microsomal preparation from the heartwood side of the intermediate wood in the presence of both NADH and FAD cofactors. Compounds **1** and **2**, for which retention times and mass fragmentation patterns were identical to those of authentic sequirin C and metasequirin C (Fig. 1c–f), respectively, were detected. Neither compound was formed using a microsomal preparation denatured by boiling as a control (Fig. 1b). Because authentic sequirin C and metasequirin C trimethylsilylated (TMS) derivatives produce a base ion at *m*/*z* 457, which is characteristic of the structures of sequirin C and metasequirin C (these norlignans scarcely generate the molecular ion of *m*/*z* 662) (Fig. 1d and f), the enzyme reaction products were analyzed by selected ion monitoring (SIM) GC–MS using the *m*/*z* 457 ion (Fig. 1a and b; the SIM chromatograms using the *m*/*z* 384 ion are also shown for detecting the internal standard 4,3′-dihydroxychalcone). Peaks of sequirin C and metasequirin C were apparent on the SIM GC–MS chromatogram of the product obtained using the substrate agatharesinol, the intact microsomal preparation and the coenzymes (Fig. 1a), while these peaks were hardly seen in the control (Fig. 1b). Enzyme assays were performed using agatharesinol that consisted of unlabeled agatharesinol, and ^{13}C-labeled agatharesinol labeled on one or both of the aromatic rings as indicated by generation of a base ion at *m*/*z* 369 [457–89(OTMS) + 1(H)] together with *m*/*z* 375 (369 + 6) and 381 (369 + 12) ions by GC–MS, respectively (Imai et al., 2006a). These ions with increased mass numbers are not generated from native agatharesinol at all. Sequirin C and metasequirin C formed after the enzyme reaction generated *m*/*z* 457 ion together with *m*/*z* 463 (457 + 6) and 469 (457 + 12) ions. The +6 ions are generated from the products sequirin C or metasequirin ^{13}C-labeled at either aromatic ring and the +12 ions from these compounds labeled at both aromatic rings, which must be formed from the corresponding substrate ^{13}C-labeled agatharesinol. The labeling ratios of unlabeled to either aromatic ring-labeled to both aromatic rings labeled of the substrate agatharesinol, the products sequirin C, and the product metasequirin C were 100 to 8.1 to 18.2, 100 to 6.4 to 16.1, and 100 to 7.7 to 18.4, respectively, as evaluated by determining the GC peak areas of the selected ions. Thus, the labeling ratios were largely conserved between the substrate and the products, ensuring enzymatic hydroxylation from the substrate agatharesinol to the products sequirin C and metasequirin C.

Enzyme assays were performed under various reaction conditions (Table 1). Hydroxylation could occur in the absence of exogenous cofactors at less than 7% of the rate in the presence of both exogenous NADH and FAD probably using endogenous cofactors, but could be initiated by adding NADPH or NADH, and

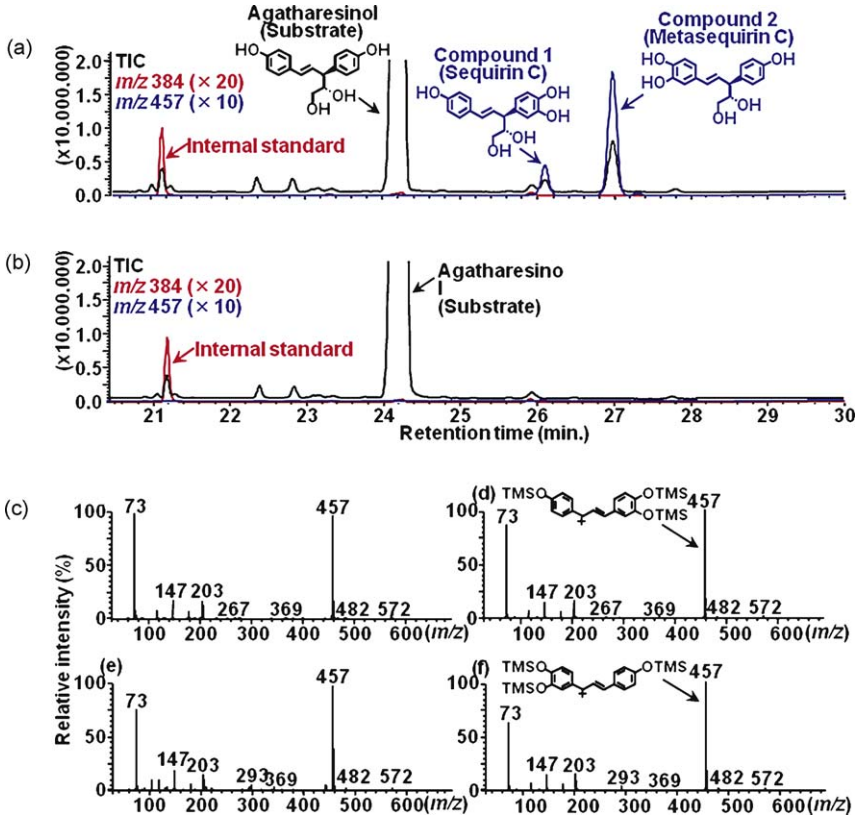

Fig. 1. GC–MS analyses of ethyl acetate extracts of the enzyme reaction mixtures. (a) Total ion current (TIC) GC–MS chromatogram and selected ion monitoring (SIM) GC–MS chromatograms of the extract obtained using an intact microsomal preparation from the heartwood side of the intermediate wood. (b) TIC GC–MS chromatogram and SIM GC–MS chromatograms of the extract obtained using a microsomal preparation denatured by boiling. (c–f) GC–MS spectra of compound 1, authentic sequirin C, compound 2 and authentic metasequirin C (TMS derivatives), respectively. The enzyme reactions were performed in the presence of both NADH and FAD.