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Characterization of new thioacidolysis products of sinapyl aldehyde and coniferyl aldehyde

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Abstract To elucidate the chemical structure of *p*-hydroxycinnamyl aldehyde moieties of abnormal angiosperm lignins of cinnamyl alcohol dehydrogenase down-regulated plants, sinapyl and coniferyl aldehydes were subjected to thioacidolysis, and the products were analyzed by gas chromatograph-mass spectrometer (GC-MS). The chromatograms indicated that two pairs of new isomeric compounds were released during thioacidolysis of sinapyl and coniferyl aldehydes, respectively, together with the previously found products. These products were also found in the thioacidolysis products of dehydrogenation polymer incorporating *p*-hydroxycinnamyl aldehydes. The new compounds had the novel indane structure in that the γ position of the side chain was linked to an aromatic ring. In the case of sinapyl aldehyde, these isomer compounds were the main products, which indicated different reactivities of sinapyl and coniferyl aldehydes during thioacidolysis.

Key words Thioacidolysis · Sinapyl aldehyde · Coniferyl aldehyde · CAD down-regulated plant · Altered lignin

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Introduction

The regulation of lignin content and composition of woody constituents by genetic engineering methods were undertaken to determine effective paper production. For example, suppression of enzyme activity such as cinnamyl alcohol dehydrogenase (CAD) involved in lignin biosynthesis has been conducted by an antisense RNA method. The obtained CAD-down-regulated tobacco and poplar plants formed red xylems without a significant reduction in their lignin contents.[1–5] Recently, one mutant of loblolly pine with the similar red xylem was discovered in natural trees. This pine was naturally down-regulated to 1% of the CAD activity.[6,7] Abnormal lignins of CAD-down-regulated plants and mutant plants severely depleted of CAD activity probably contain a remarkable amount of *p*-hydroxycinnamyl aldehyde structure because the reduction of *p*-hydroxycinnamyl aldehydes to the corresponding alcohols is inhibited in these plants. However, the chemical structure of cinnamyl aldehyde moieties of abnormal lignin has been poorly characterized. Thioacidolysis proceeds by cleavage of arylglycerol-β-aryl ether linkages and provides C_6-C_3 trithioethyl phenylpropane compounds as the main degradation products.[8,9]

The thioacidolysis products derived from *p*-hydroxycinnamyl aldehyde end-groups linked via β-*O*-4 linkages in normal lignin have been reported.[10,11] However, the thioacidolysis experiment of dehydrogenation polymer (DHP) incorporating sinapyl aldehyde showed that the incorporation of sinapyl aldehyde into DHP as end-groups was small. Then we performed thioacidolysis of sinapyl aldehyde, and the thioacidolysis products were analyzed by gas chromatograph-mass spectrometer (GC-MS). The chromatogram gave a small peak of the expected product, $Me_3SiOC_6H_2(OMe)_2$-$CH(SEt)$-CH_2-$CH(SEt)_2$, with relatively large amounts of two novel products presented in thioacidolysis products of DHP incorporating sinapyl aldehyde.

In this study we performed thioacidolysis of sinapyl and coniferyl aldehydes to gain a better understanding

of *p*-hydroxycinnamyl aldehyde moieties in abnormal lignins of CAD-down-regulated plants. The thioacidolysis products of sinapyl and coniferyl aldehydes were analyzed by GC-MS and nuclear magnetic resonance (NMR).

Material and methods

Instruments

Total ion chromatograms and mass spectra were recorded on a Shimadzu GCMS-QP 5000 gas chromatograph-mass spectrometer (EI 70eV, capillary column DB-1; J&W Scientific) that was 30m × 0.25mm (i.d.), with film of 1μm; carrier gas He; and column temperatures of 130°–260°C, increased at +5°C/min. The NMR spectra were obtained with Varian Unity Inova 400 (400MHz) and 500 (500MHz) FT-NMR spectrometers.

Reagents

Coniferyl aldehyde was synthesized by oxidation of isoeugenol methoxymethyl ether to coniferyl aldehyde methoxymethyl ether with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone and subsequent demethoxymethylation of the latter compound with H_2SO_4 to coniferyl aldehyde.[12] Sinapyl aldehyde (Aldrich) and other reagents were commercially available.

Preparation of β-*O*-4-linked dimer of coniferyl aldehydes

Preparation of β-*O*-4-linked dimer of coniferyl aldehydes was achieved by horseradish peroxidase (188 purpurogallin number/mg; Tokyo Chemical Industry) and 1% H_2O_2 according to the method of Connors et al.[13,14] The β-*O*-4-linked dimer of coniferyl aldehydes was isolated from the extract by column chromatography (solvent: ethyl acetate/hexane, 2:1, v/v) and subsequent silica gel thin-layer chromatography (TLC) (solvent: chloroform/methanol, 95:5, v/v). The compounds were identified by ^{1}H-NMR and mass spectra.

Preparation of DHP

Sinapyl alcohol (100mg), coniferyl alcohol (50mg), sinapyl aldehyde (100mg), and coniferyl aldehyde (50mg) (2:1:2:1, w/w) were dissolved in 1ml of acetone. The acetone solution was added into 50ml of 0.07M phosphate buffer (pH 6.4) with stirring (substrate solution). Separately, 50ml of an equimolar H_2O_2 solution (0.1%) was prepared. The substrate and H_2O_2 solution were added very slowly with stirring into the horseradish peroxidase (3mg in 50ml of the same buffer) via two silicone tubes by a minipump at room temperature for 24h. All flasks were covered with aluminum foil to avoid oxidation of the reaction mixture by light. After 24h, 2mg of peroxidase and 25ml of H_2O_2 solution were added in the same way, and the reaction was continued for another 12h with stirring. The precipitated DHP was collected by centrifugation (7000rpm, 20min). The crude DHP was suspended and washed in water and centrifuged again. This procedure was repeated twice, and the DHP was dried over P_2O_5 in vacuo. The crude DHP was dissolved or suspended in 10ml of dichloroethane/ethanol (2:1, v/v), and the solution was dispersed into 500ml of ether with stirring. The precipitate DHP was collected by centrifugation (3500rpm, 5min), dried over P_2O_5 in vacuo, and used for subsequent experiments (yield 229.3mg).

Thioacidolysis of sinapyl aldehyde and coniferyl aldehyde

Sinapyl aldehyde (2.5mg) and coniferyl aldehyde (2.5mg) were respectively subjected to thioacidolysis in a mixed solution (8ml) of 0.2M BF_3 etherate, ethanethiol, and dioxane (1:4:35, v/v) using a glass tube fitted with a Teflon-lined screw cap at 100°C (dry block bath) for 4h with occasional shaking. The reaction mixture and a few milliliters of water to rinse the reaction tube were poured on dichloromethane together with 0.5mg of internal standard (tetracosane). The aqueous layer of the reaction mixture was adjusted to pH 4 with 0.4M sodium hydrogen carbonate, and the mixture was extracted three times with dichloromethane (50ml). The combined dichloromethane extracts were dried over anhydrous sodium sulfate, and the solvent was evaporated in vacuo at 40°C. The thioacidolysis product was redissolved in 1ml of dichloromethane, and 10μl of the solution was silylated with hexamethyldisilazane/trimethylchlorosilane/pyridine (2:1:10, v/v) (TMSI-H; GL Science) (100μl) for GC-MS analysis.

Isolation of thioacidolysis products of sinapyl aldehyde and coniferyl aldehyde

Sinapyl aldehyde (25mg) and coniferyl aldehyde (25mg) were subjected to thioacidolysis in the same way as mentioned above. The thioacidolysis products were isolated by TLC (solvent: ethyl acetate/hexane 1:2 and 1:3, v/v). The isolated products were characterized by NMR and mass spectra.

Thioacidolysis of β-*O*-4-linked dimer of coniferyl aldehydes and DHP

The β-*O*-4-linked dimer of coniferyl aldehydes (2mg) and DHP (2.5mg) were subjected to thioacidolysis in the same way as mentioned above. The thioacidolysis product was silylated with TMSI-H for GC-MS analysis.

Results

Thioacidolysis products of sinapyl aldehyde

Four compounds (1), (2), (3), and (4) were produced in the thioacidolysis of sinapyl aldehyde (Figs. 1, 2). Yields of

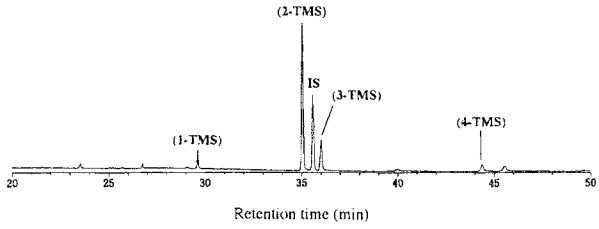

Fig. 1. Total ion chromatogram of thioacidolysis products formed from sinapyl aldehyde. *IS*, internal standard (tetracosane); *TMS*, trimethylsilane

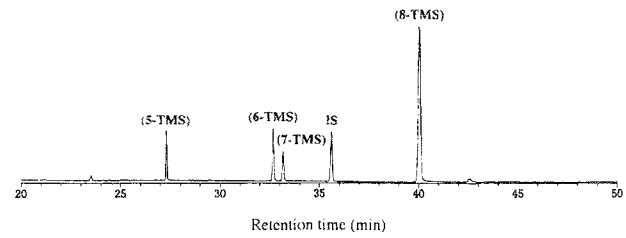

Fig. 3. Total ion chromatogram of thioacidolysis products formed from coniferyl aldehyde

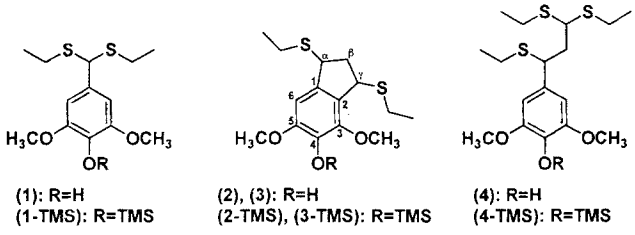

Fig. 2. Chemical structures of thioacidolysis products of sinapyl aldehyde

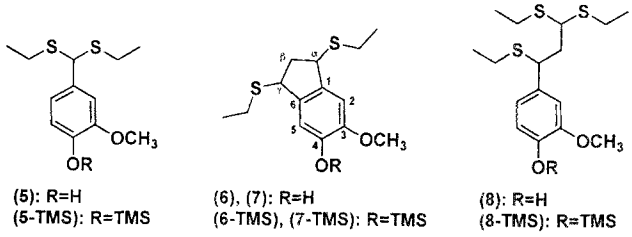

Fig. 4. Chemical structures of thioacidolysis products of coniferyl aldehyde

these compounds were 3.3%, 31.7%, 8.3%, and 1.7%, respectively. Compounds (1), (2), and (4) were isolated and analyzed by NMR and mass spectra. A novel compound (2) was determined by NMR and mass spectra, and the chemical structure was estimated to be 1,3-bis-thioethyl-4,6-dimethoxy-5-hydroxyindane. We considered compounds (2) and (3) to be isomers because these compounds have the same fragment ion peaks and almost the same fragment patterns in the mass spectra. Compounds (1) and (4) were identified by comparison of analytical data.[10]

Compound (2): ^{1}H-NMR($CDCl_3$): δ 1.29 (6H, t, J = 7.3, ethyl CH_3), 2.47 (1H, ddd, J = 7.1, 8.9, 13.3, $C_\beta H$), 2.61 (2H, q, J = 7.3, ethyl CH_2), 2.63 (2H, q, J = 7.3, ethyl CH_2), 2,67 (1H, ddd, J = 1.4, 6.9, 13.3, $C_\beta H$), 3.90 (3H, s, OCH_3), 4.00 (3H, s, OCH_3), 4.46 (1H, dd, J = 6.9, 8.9, $C_\alpha H$), 4.49 (1H, dd, J = 1.4, 7.1, $C_\gamma H$), 5.53 (1H, s, OH), 6.71 (1H, s, $C_6 H$); ^{13}C-NMR($CDCl_3$): δ 14.7 (ethyl CH_3), 15.1 (ethyl CH_3), 24.4 (ethyl CH_2), 26.0 (ethyl CH_2), 44.2 (C_β), 45.0 (C_γ), 47.5 (C_α), 56.4 (OCH_3), 60.7 (OCH_3), 102.6 (C_6), 127.7 (C_2), 134.5 (C_1), 137.6 (C_4), 143.0 (C_3 or C_5), 148.3 (C_3 or C_5); MS (2-TMS) m/z (%): 386 (M^+, 1.7), 326 (12.4), 325 (41.9), 324 (47.0), 295 (11.1), 265 (49.9), 264 (38.9), 263 (100.0), 234 (25.8), 233 (26.3), 219 (5.4), 218 (6.0), 203 (10.2), 190 (8.9), 73 (95.7).

Compound (3): MS (3-TMS) m/z (%): 386 (M^+, 2.1), 326 (13.0), 325 (48.4), 324 (34.3), 295 (9.8), 265 (51.1), 264 (42.0), 263 (96.6), 234 (25.7), 233 (34.6), 219 (5.9), 218 (5.8), 203 (10.8), 190 (8.5), 73 (100.0).

Thioacidolysis products of coniferyl aldehyde

Four compounds (5), (6), (7), and (8) were produced in the thioacidolysis of coniferyl aldehyde (Figs. 3, 4). Yields of these compounds were 8.9%, 12.3%, 8.2%, and 46.6%, respectively. We considered compounds (6) and (7) as isomers because they have the same fragment ion peaks and almost the same fragment patterns. Compounds (6) and (7) could not be separated from each other, and they were characterized by NMR and mass spectra as a mixture. Compounds (6) and (7) were determined to have structures corresponding to those of compounds (2) and (3), respectively, because the spectral patterns were similar. Compound (8) was isolated and identified by NMR and mass spectra.[10] The structure of compound (5) was confirmed by mass spectrometry.[10]

Compound (6): ^{1}H-NMR($CDCl_3$): δ 1.25–1.31 (6H, ethyl CH_3), 2.48–2.64 (2H, $C_\beta H$), 2.48–2.64 (4H, ethyl CH_2), 3.90 (3H, OCH_3), 4.35–4.38 (1H, $C_\alpha H$ or $C_\gamma H$), 4.38–4.42 (1H, $C_\alpha H$ or $C_\gamma H$), 5.62 (1H, OH), 6.86–6.94 (2H, $C_2 H$ and $C_5 H$); MS (6-TMS) m/z (%): 356 (M^+, 3.1), 296 (11.4), 295 (39.5), 294 (44.4), 265 (17.8), 235 (66.6), 234 (42.4), 233 (100.0), 219 (2.9), 205 (12.0), 204 (34.5), 203 (31.9), 189 (3.3), 173 (12.9), 161 (2.5), 73 (99.0).

Compound (7): ^{1}H-NMR($CDCl_3$): δ 1.25–1.31 (6H, ethyl CH_3), 2.18–2.25 (1H, $C_\beta H$), 2.48–2.64 (4H, ethyl CH_2), 3.04–3.11 (1H, $C_\beta H$), 3.90 (3H, OCH_3), 4.16–4.20 (1H, $C_\alpha H$ or $C_\gamma H$), 4.19–4.23 (1H, $C_\alpha H$ or $C_\gamma H$), 5.62 (1H, OH), 6.86–6.94 (2H, $C_2 H$ and $C_5 H$); MS (7-TMS) m/z (%): 356 (M^+, 4.8), 296 (13.1), 295 (52.2), 294 (29.0), 265 (15.4), 235 (76.5), 234 (47.6), 233 (100.0), 219 (3.5), 205 (13.9), 204 (41.2), 203 (32.1), 189 (3.5), 173 (11.7), 161 (2.9), 73 (96.6).

Thioacidolysis products of β-*O*-4-linked dimer of coniferyl aldehydes and DHP

Four compounds were produced during thioacidolysis of β-*O*-4-linked dimer of coniferyl aldehydes (Fig. 5). Three products (6), (7), and (8) were the same to those of the coniferyl aldehyde derived by thioacidolysis, but product (9) did not exist.

A total ion chromatogram of the thioacidolysis products of the DHP is shown in Fig. 6. Products (2-TMS)-(4-TMS), (6-TMS)-(8-TMS), and (9-TMS), formed from *p*-hydroxycinnamyl aldehydes, were produced with the main thioacidolysis monomers (G, S) derived from cleavage of arylglycerol-β-aryl ether linkages.

Discussion

It was reported that compounds (4) and (8) were formed during the thioacidolysis of sinapyl aldehyde and coniferyl aldehyde end-groups, respectively, and that C_6-C_1 compounds originated in benzaldehyde end-groups of milled wood lignin.[10,11] In the present study, compounds (1) and (5) were found to be derived directly from sinapyl and coniferyl aldehydes. Tentative structures for compounds (2), (3), (6), and (7) were given in the previous papers.[15,16] In the present study it was confirmed by various NMR techniques that in the structures of compounds (2), (3), (6), and (7) the γ position of the side chain was linked to an aromatic ring resulting in the cyclic structure shown in Figs. 2 and 4. Thioethyl groups are introduced at both α and γ positions, and the difference in the linkage position of thioethyl groups then gives geometric isomers (*cis-trans* isomers), and enantiomers and conformational isomers exist for each isomer.

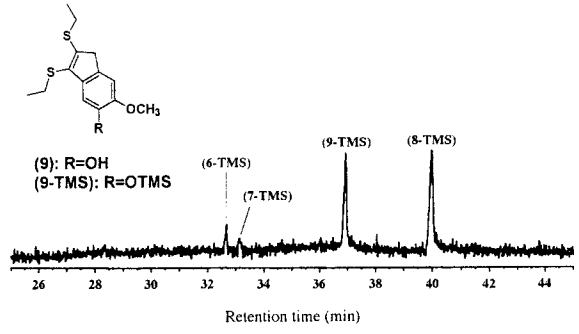

Fig. 5. Total ion chromatogram of thioacidolysis products formed from the β-*O*-4-linked dimer of coniferyl aldehydes

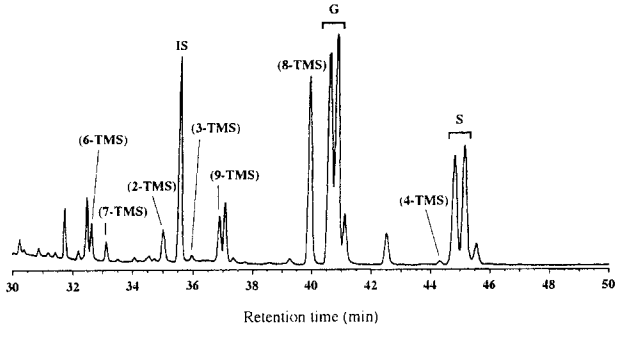

Fig. 6. Total ion chromatogram of thioacidolysis products formed from DHP incorporating *p*-hydroxycinnamyl aldehydes. *G*, $TMSOC_6C_3OMe$-CHSEt-CHSEt-CH_2SEt; *S*, $TMSOC_6C_2(OMe)_2$-CHSEt-CHSEt-CH_2SEt

The possible formation mechanisms of thioacidolysis products of sinapyl aldehyde (2)–(4) and of coniferyl aldehyde (6)–(8) are shown in Fig. 7. The key reaction of thioacidolysis is the substitution of a hydroxyl group at the benzylic (C_α) position with the thioethyl group and the resulting elimination of the β-etherated phenol moiety by the nucleophilic attack of the thioethyl group at the α position.[10] In the case of *p*-hydroxycinnamyl aldehydes, at first the BF_3-coordinated aldehyde group is attacked by ethanethiol to form an unstable cation A; then cation A reacts with another ethanethiol. The intermediate B is an important compound. Compound (4) or (8) is formed by attaching a third ethanethiol to compound B. On the other hand, if C—C bond formation occurs between C_γ and the aromatic ring of compound B, they convert to compounds (2)/(3) or (6)/(7).

During thioacidolysis of sinapyl aldehyde, the amount of compounds (2-TMS) and (3-TMS) were found to be much higher than that of compound (4-TMS), thereby concluding that the former two compounds were major products (Fig. 1). On the other hand, the amounts of compounds (6-TMS) and (7-TMS) were much lower than that of compound (8-TMS) during thioacidolysis of coniferyl aldehyde (Fig. 3). Similar results were obtained during thioacidolysis of DHP containing *p*-hydroxycinnamyl aldehydes (Fig. 6). This indicates different reactivities of sinapyl aldehyde and coniferyl aldehyde during thioacidolysis. It is thought that compounds (2) and (3) are formed readily by thioacidolysis of

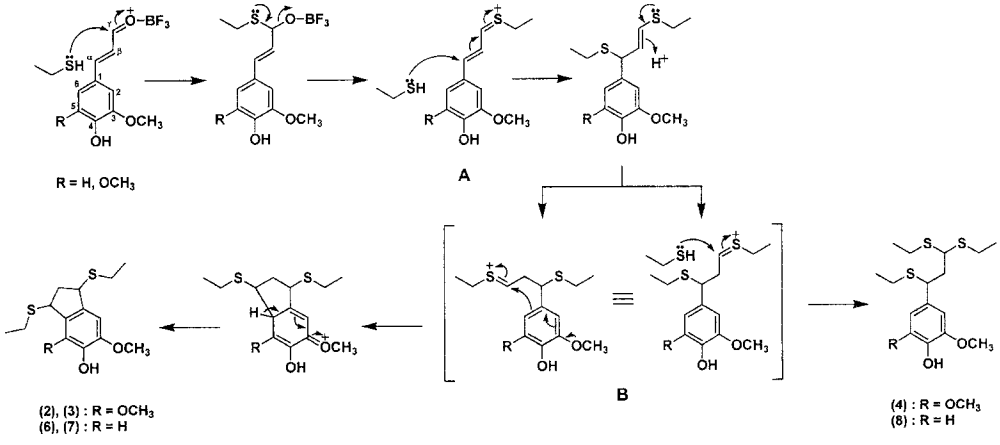

Fig. 7. Formation mechanisms of thioacidolysis products derived from sinapyl and coniferyl aldehydes

sinapyl aldehyde because the electron density of the aromatic ring on sinapyl aldehyde is dense compared with that on coniferyl aldehyde.

We investigated the thioacidolysis products formed from not only *p*-hydroxycinnamyl aldehydes but also dimeric and polymeric models. Novel compounds were also seen among the thioacidolysis products of *β-O*-4-linked dimer of coniferyl aldehydes and DHP. Recently, Lapierre et al.[17] reported other thioacidolysis products, indene derivatives, as marker compounds for CAD-deficient plants formed from sinapyl aldehyde *β-O*-4-ether model compounds. These products also existed mainly in thioacidolysis products of CAD-deficient poplar and its milled wood lignin.[17,18] We concluded that product (9) was the same as products derived from 4-*O*-*β*-end-coniferyl aldehyde groups.[16] Consequently, the compounds reported by Lapierre et al. and in this article derived from cinnamyl aldehyde structure are of considerable interest when trying to elucidate the abnormal lignins of CAD-down-regulated plants, especially that of angiosperm plants. Li et al.[19] recently reported the isolation of a novel aspen gene encoding sinapyl alcohol dehydrogenase (SAD), which is phylogenetically distinct from aspen CAD. They suggested that SAD function is essential to the biosynthesis of syringyl monolignol in angiosperms, and SAD is required for the biosynthesis of syringyl lignin in angiosperms. Therefore, the above-mentioned compounds are important for elucidating abnormal lignins in CAD- or SAD-deficient angiosperms.

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