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Formation of Thomasidioic Acid from Dehydrosinapinic Acid Dilactone under Neutral Conditions, and a Remaining Inhibitory Activity against Peroxynitrite-Mediated Protein Nitration

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Abstract—Dehydrosinapinic acid dilactone (**1**) was converted to thomasidioic acid (**3**) and (*E*,*E*)-2,3-bis(3,5-dimethoxy-4-hydroxybenzylidene)succinic acid (**4**) via an α,β-unsaturated γ-lactone type dimer (**2**) in phosphate buffer (pH 7.4). A study of the reaction mechanism was accomplished by observing the reaction of methyl ester of **2**. The lignans (**3**, **4**) were also prevented the peroxynitrite-mediated protein nitration.

p-Coumaric acid and related compounds have some biological activities.[1–3] They are easily dimerized in oxidative conditions, and produce several kinds of products, called lignan. The lignans also exhibit some interesting activities.[4,5] Thomasidioic acid (**3**) is synthesized from sinapinic acid via dehydrosinapinic acid dilactone (**1**),[6] however, the precise mechanism of this reaction is ambiguous. Peroxynitrite, prepared from nitric oxide and super oxide anion radical in near-diffusion rate,[7,8] was considered as one of the plausible oxidants in vivo, because of the detection of 3-nitrotyrosine, a stable end-product of peroxynitrite, in lots of (model) diseases.[9] In our previous study, we described the inhibitory activity of sinapinic acid against peroxynitrite-mediated protein nitration, and isolated an adduct (**2**) by treating sinapinic acid with peroxynitrite.[10] We also observed the spontaneous conversion of **1** to **2** in phosphate buffer. In this study, we describe the stereochemistry of **2**, and the further conversion of **1** to thomasidioic acid (**3**) and (*E*,*E*)-2,3-bis(3,5-dimethoxy-4-hydroxybenzylidene)succinic acid (**4**) via **2** in phosphate buffer. We also examine the inhibitory activity of the produced lignans (**3**, **4**) against peroxynitrite-mediated protein nitration in vitro.

Determination of the Stereochemistry of 2

Previous studies suggested that the stereochemistry of **1** is characterized as shown in Scheme 1.[6,11] Based on the results that **2** was obtained from **1** under mild conditions, the stereochemistry of **2** comprising a five-membered lactone ring seemed to be the *anti*-configuration of the remains from **1**. It was sustained by the NOESY experiment of **2** that had a weak cross-peak correlation between the multiplet methine signal (δ 4.28) and the aromatic methine protons (δ 6.63). The stereochemistry was in agreement with that of α,β-unsaturated γ-lactone type lignans that were previously reported.[12,13] To determine the configuration of the tri-substituted double bond, we isomerized **2** by daylight irradiation, and compared the ^{1}H NMR spectra of the isomers.[14] The olefinic proton of the isomer obtained shifted to 7.19 ppm from 7.56 ppm, whereas the aromatic methine protons were observed 0.59 ppm downfield from those in **2**. We assumed these alterations derived from a deshielding effect of the lactone carbonyl,[15,16] and therefore determined the structure of **2** as an (*E*) configuration. In these five-membered lactone ring openings, Hart and Heller[17] described that steric resistance resulted in the selectivity. The (*E*) configuration was confirmed by the structure of **4** obtained from **2** under neutral conditions, as described in the next section.

Conversion of 1 to Thomasidioic Acid (3) via 2

As we previously described,[10] **1** was easily converted to **2** in phosphate buffer (pH 7.4). We allowed this reaction to proceed further, and found two novel peaks by HPLC analysis.[18] Structural evaluation revealed these peaks as thomasidioic acid (**3**) and (*E*,*E*)-2,3-bis(3,5-

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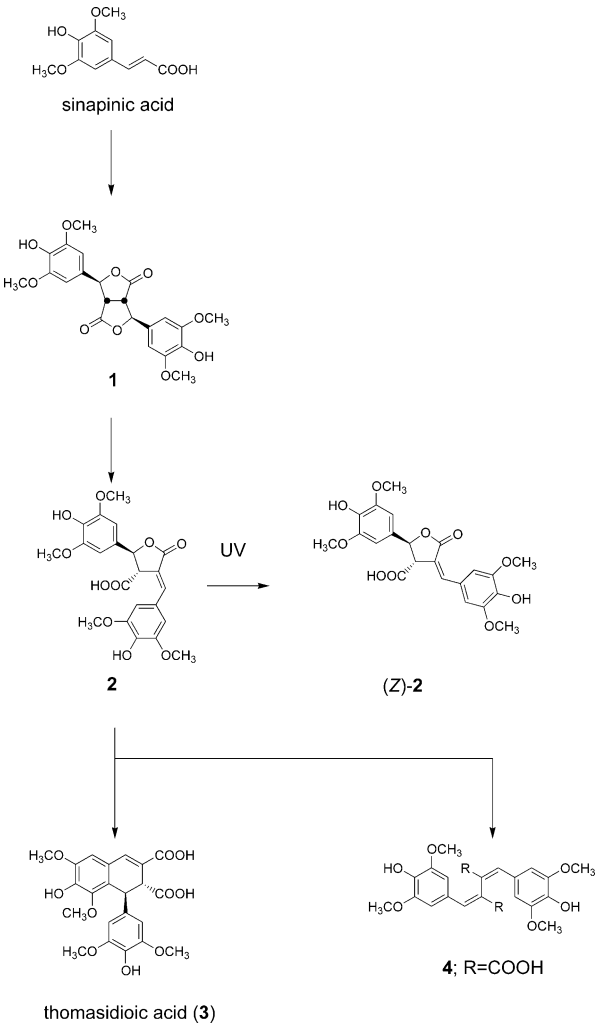
dimethoxy-4-hydroxybenzylidene)succinic acid (**4**)[19] (61%; **3**:**4** = ca. 3:2). The structure of **4**, including its configuration, was confirmed by the derivatization to dimethylether[20] by (trimethylsilyl)diazomethane treatment, followed by alkaline hydrolysis with aqueous NaOH. The production of **3** and **4** was also observed when **2** itself was treated in phosphate buffer.

Conversion of Methyl Ester of 2 to Thomasidioic Acid Monomethyl Ester

To elucidate the mechanism for the formation of thomasidioic acid (**3**) from **2**, we prepared methyl-**2**[12] from **1** in four steps. Methyl ester of **2** was also subjected to phosphate buffer; however, monomethyl ester of **4** was the predominant product, and only a trace amount of the desired thomasidioic acid ester was detected. Because we had observed that the alkaline treatment of **2** resulted in high-selectivity yields of thomasidioic acid, we then treated the methyl ester of **2** to more alkaline conditions, in a 0.1 M borate buffer (pH 9.5). As expected, the rate of thomasidioic acid ester production increased (81%; monomethyl-**3**/monomethyl-**4** = ca. 1:2). The obtained thomasidioic acid monomethyl ester was examined by HMBC spectral analysis of the two carbonyl carbons (acetone-d_6; δ_C 172.9 and 168.0), and the location of the methoxyl moiety was identified (Scheme 2).



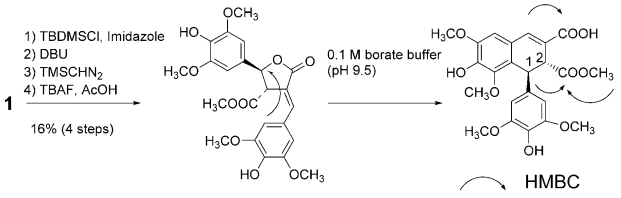
Scheme 1. Reaction pathways of sinapinic acid to thomasidioic acid (**3**) and (*E*,*E*)-2,3-bis(3,5-dimethoxy-4-hydroxybenzylidene)succinic acid (**4**) via an α,β-unsaturated γ-lactone type dimer (**2**).

Inhibitory Activity of Sinapinic Acid and its Dimers against Peroxynitrite-Mediated Protein Nitration

We previously described the inhibitory activity of sinapinic acid against peroxynitrite by an enzyme-linked immunosorbent assay (ELISA).[10] We then aimed to evaluate the inhibitory activity of the oxidative products using this ELISA method, and found that **3** and **4** had about twice the activity to that of sinapinic acid at the same molecular concentration (Fig. 1). These results suggested that *p*-coumaric acid derivatives, such as sinapinic acid, do not lose their inhibitory activity after an oxidative modification at the β position, and that lignans would act as a useful inhibitor against peroxynitrite.

Discussion

Thomasidioic acid (**3**) was originally synthesized under acidic conditions from **1**,[6] and the similar reaction was also observed in dehydroferulic acid dilactone.[21] Recently, the conversion of an α,β-unsaturated γ-lactone type dimer of ferulic acid to dihydronaphthalene type lignans in an alkaline condition has been reported.[13] However, the reaction mechanism(s) are as yet ambiguous.



Scheme 2. Conversion of methyl ester of **2** to thomasidioic acid monomethyl ester.

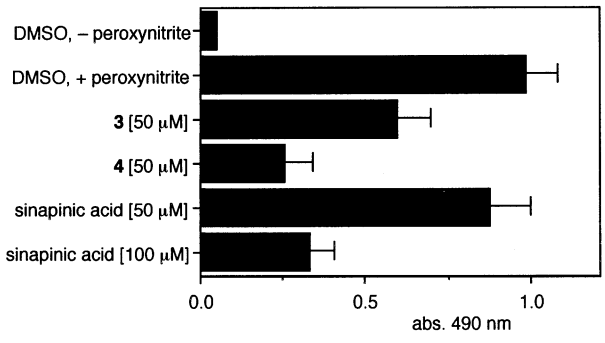
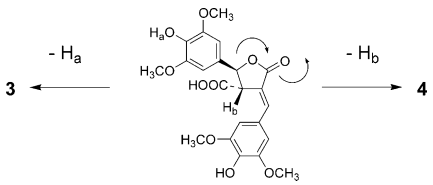


Figure 1. The inhibitory effect of sinapinic acid and the dimers on protein nitration by peroxynitrite. Protein solutions (collagen type I) that contained 50 μM (final concentration) of each sample were treated with 1.0 mM peroxynitrite. Data represent the mean ± SD of three independent measurements.



Scheme 3. Proposed mechanism for the conversion of an α,β-unsaturated γ-lactone type dimer to lignans.

Our finding that the conversion of **1** to thomasidioic acid (**3**) under neutral conditions suggests that **2** was an intermediate in this intramolecular reaction. To elucidate the mechanism of this reaction, we labeled a carboxylic acid of **2** with methyl ester. The location of the methyl moiety of the corresponding monomethyl ester of **3** was determined by HMBC spectrum. This result indicated the reaction site illustrated in Scheme 2. An S_N2 type reaction of **2** would be difficult, and the product, if ever, would be an epimeric product, which has a *cis*-configuration at the C1 and C2 positions. Then, we concluded that the mechanism involved a quinone type intermediate[22] resulting from a deprotonation of H_a, in accordance with the γ-lactone ring opening (Scheme 3). On the other hand, a deprotonation of H_b would produce **4**. Then, the esterification of **2** would increase the rate of the deprotonation of H_b, and a methyl ester of **2** would predominantly produce a methyl ester of **4** in neutral conditions. As another type of intramolecular cyclization reaction, bisbenzylidenes are reported as precursors of naphthalene type lignans;[23,24] therefore, a potential pathway that took **4** as an intermediate to thomasidioic acid (**3**) was also considered. However, **4** did not produce any reaction, even in an alkaline treatment.

We were unable to elucidate the whole mechanism of lignan production, because the methyl ester of sinapinic acid is also converted to thomasidioic acid dimethyl ester,[12,25] and other (synthetic) routes are also known to occur.[26–28] However, our study did reveal in part the mechanism that produces some lignan compounds, not only thomasidioic acid, but also bisbenzylidensuccinic acid type lignans which have (*E*) configurations.[29,30]

From the detection of 3-nitrotyrosine moiety in several diseases, peroxynitrite is receiving attention as one of the strong oxidants in vivo.[9] As we have previously described, sinapinic acid inhibited the peroxynitrite-mediated protein nitration by receiving one-electron oxidation.[10] However, the oxidative end-products (**3**, **4**) still had the phenolic moiety which might be responsible for the antioxidative activity, and retained most of the inhibitory activity, compared to the substrate, sinapinic acid, against peroxynitrite.

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14. (*Z*)-**2**: LC–MS(ESP–) *m/z*: 445[M−H]$^-$; UV $\lambda_{max}^{E\tau OH}$ nm (ε): 207.5 (59,700), 244 (17,600), 348 (17,600); IR (KBr) ν_{max} cm^{-1}: 3350, 1735, 1615; ^{1}H NMR (400 MHz, acetone-d_6) δ 7.97 (brs, 1H), 7.61 (s, 2H), 7.45 (brs, 1H), 7.19 (d, J = 1.9 Hz, 1H), 6.77 (s, 2H), 5.75 (d, J = 5.1 Hz, 1H), 4.11 (dd, J = 1.9, 5.0 Hz, 1H), 3.86 (s, 6H), 3.83 (s, 6H).
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18. (a) A solution of **1** dissolved in DMSO (10.0 mg/1.0 mL) was added to 19 mL 0.1 M phosphate buffer (pH 7.4). The reaction proceeded for 24 h at ambient temperature with gentle stirring. (b) Reactions were monitored by HPLC using a Wakosil-II 5C18HG column (i.d. 4.6×250 mm; Wako Pure Chemical) on a Shimadzu CLASS-LC10 series HPLC system equipped with a photo diode array detector (SPD-M10Avp; Shimadzu). The solvent mixture, H_2O/MeOH/trifluoroacetic acid (600:400:1), was used at a flow rate of 1.0 mL/min at 40 °C.
19. **4**: LC–MS(ESP–) *m/z*: 445 [M−H]$^-$; UV $\lambda_{max}^{E\tau OH}$ nm (ε): 240.5 (27,400), 320 (23,000); IR (KBr) ν_{max} cm^{-1}: 3345, 1685, 1610, 1515; ^{1}H NMR (400 MHz, acetone-d_6) δ 7.85 (s, 2H), 7.63 (brs, 2H), 7.01 (s, 4H), 3.76 (s, 12H).
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