Regiospecific Hydroperoxidation of Anacardic Acid (15:2) by Soybean Lipoxygenase 1

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REGIOSPECIFIC HYDROPEROXIDATION OF ANACARDIC ACID (15:2) BY SOYBEAN LIPOXYGENASE 1[1]

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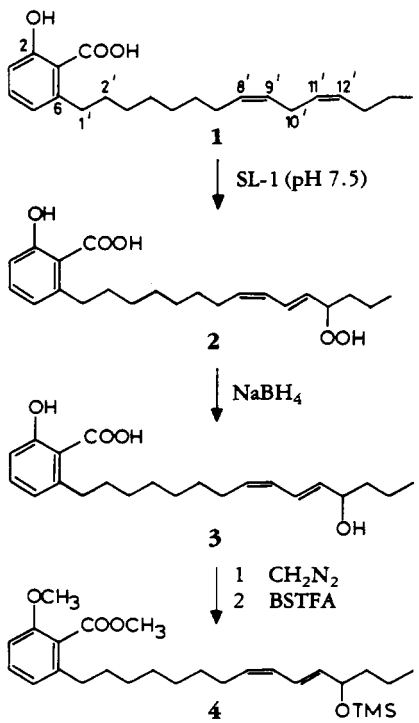
ABSTRACT.—Anacardic acid (15:2) [**1**] is rapidly dioxygenated by soybean lipoxygenase 1, yielding 2-hydroxy-6-[12′-hydroperoxy-(8′*Z*,10′*E*)-pentadecadienyl]benzoic acid [**2**] exclusively. The structure and homogeneity of the product have been determined by its reduction to the alcohol **3** and elaborate chromatographic and spectrometric investigations on the latter. Thus, the enzyme is shown to possess the unusual $\omega-4$ positional specificity towards the hitherto unreported salicyclic acid type of substrate.

Lipoxygenases are an important class of enzymes involved in the arachidonic acid cascade, leading to leukotrienes and other biologically active metabolites (1). Several of the leukotrienes are understood to account collectively for what is known as the slow-reacting substance of anaphylaxis (SRS-A), associated with hypersensitivity. While the role of lipoxygenases in plants is uncertain, they are implicated in the generation of flavor principles and allelochemicals. In view of their importance in the biological system, the lipoxygenases have attracted considerable attention in recent years (2).

While lipoxygenases are ubiquitous in nature, legume seeds have been a rich source of the enzymes and the soybean lipoxygenase 1 is among the most extensively studied enzymes (3,4). Lipoxygenases are generally known to catalyze dioxygenation (hydroperoxidation) of polyunsaturated lipids (mainly straight-chain fatty acid derivatives) that contain the (1*Z*,4*Z*)-pentadiene moiety, yielding the corresponding 1-hydroxy-(2*E*,4*Z*)-diene. Of particular interest in this context are the regio- and stereo-specificity of hydroperoxidation. Soybean lipoxygenase 1, for example, catalyzes dioxygenation of arachidonic acid at C-15 and of linoleic acid at C-13; in other words, at $\omega-6$ position from the far end of the fatty acid. We now report that the enzyme can also utilize an unusual substrate (a salicylic acid derivative), namely, anacardic acid (15:2) [**1**] with $\omega-4$ position specificity.

RESULTS AND DISCUSSION

Anacardic acids, or 2-hydroxy-6-pentadecylbenzoic acids, are the major constituents of cashew nut shells (*Anacar-*



SCHEME 1

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dium occidentale L.), of which the (8′*Z*,11′*Z*)-diene **1** accounts for 3–4%. Anacardic acids (15:1) and (15:3), which are the (8′*Z*)-pentadecenyl and (8′*Z*, 11′*Z*,14′*Z*)-pentadecatrienyl analogues, respectively, account for 5–6% each by weight of the shells (5). As a part of our program on the biological activity of the anacardic acids and in view of the close structural similarity of anacardic acids (15:2) and (15:3) to linoleic acid and linolenic acid, respectively, we have examined the possibility of their hydroperoxidation by lipoxygenase.

Soybean lipoxygenase 1 showed very little activity towards the anacardic acids at pH 9.0, the optimum pH recorded for the enzymatic reaction for linoleic acid and for monohydroperoxidation of arachidonic acid. The lipoxygenase activity was monitored by observing the change in absorbance of the reaction mixture at 235 nm, where the expected product of the reaction, a conjugated diene, would absorb (3). However, at pH 7.5, the absorption rapidly increased in the case of **1**, reaching a V_{max} value 60% of that for linoleic acid, indicating that it is an effective substrate for the enzyme; the V_{max} and the K_m value (0.013 mM) were calculated as described earlier (4). The activity of the enzyme in the case of anacardic acid (15:3) was less than 2% of that of linoleic acid at this pH. As expected, the enzyme was inactive towards anacardic acid (15:1).

For the identification of the product **2** of the lipoxygenase action on **1**, the hydroperoxide was reduced to the alcohol **3** by $NaBH_4$, (Scheme 1), and the product was separated from the inorganic salts and protein by solid-phase extraction using a Sep-Pak cartridge (see Experimental). The product was eluted from the cartridge using MeOH; complete conversion of **1** to a homogenous product **3** was shown by hplc, monitored at both 235 nm and 310 nm. The product from four such experiments was combined to yield sufficient material for structural analysis. The geometry of the olefinic double bonds in **3** was established by the ^{1}H-nmr spectrum, which showed the coupling constant between H-8′ (5.47) and H-9′ (6.02) to be 11 Hz and that between H-10′ (6.58) and H-11′ (5.69) to be 15.2 Hz.

The hydroxyanacardic acid obtained above was methylated using CH_2N_2, yielding the dimethyl derivative. The methyl ether methyl ester was further derivatized by treating with bis(trimethylsilyl)trifluoroacetamide. The trimethylsilyl derivative **4** was injected into a gc-ms, and the total-ion chromatogram showed a single major peak at 21.9 min with the $[M]^+$ at *m/z* 460, $[M-43]^+$ at 417, and the base peak at *m/z* 73, which clearly indicated that of the two possible positions of hydroxylation, namely, 8′ and 12′ of the pentadecadienyl side chain, the product was the 12′-hydroxy derivative (as shown), so that a C_3H_7 moiety α to -CH=CH-CH(OTMSi)- could be cleaved off the molecular ion. The *m/z* 197 signal could rise from an allylic cleavage between C-6′ and C-7′, possibly stabilized by a 1,6-double allylic shift of the -OTMSi moiety, as in the case of the linoleic acid-lipoxygenase product, 13-hydroxy-9,11-octadecadienoic acid methyl ester trimethylsilyl derivative, whose mass spectrum shows a base peak at *m/z* 225 (6).

Thus, the product of soybean lipoxygenase action on anacardic acid (15:2) has been shown to be 2-hydroxy-6-[12′-hydroperoxy-(8′*Z*,10′*E*)-pentadecadienyl] benzoic acid [**2**]. By analogy with the lipoxygenase reaction of linoleic acid, the configuration at C-12′ is considered to be *S* (7,8). Recent reports claim that while the hydroperoxidation by soybean lipoxygenase 1 is stereospecific, the regiospecificity decreases with increased lipophilicity at the far end of the unsaturated carboxylic acid substrate (8). In the present case, although the far end is a linear alkyl chain, the reaction appeared to be regiospecific, as no other product could be detected either by hplc or gc-ms.

Several other features of the above findings are of significance. Thus, this is probably the first report of a non-fatty acid natural substrate acted upon by lipoxygenase and of its $\omega-4$ positional specificity. Characterizations of products of lipoxygenase action are of potential importance in medicine, as they are implicated in hypersensitivity and interference with prostaglandin synthesis. They are also considered to play a role in the control of insect fecundity and thus contribute to the resistance of the plants to infestation (9).

EXPERIMENTAL'

GENERAL EXPERIMENTAL PROCEDURES.—Soybean lipoxygenase 1, isolated from soybeans, purified as described (3), and containing 20 mg protein/ml, with a specific activity of 120 units/min/mg protein, was used in the work. Uv spectra were recorded on a Shimadzu 2100-spectrophotometer, ir spectra on a Mattson Galaxy 4020 FTIR instrument as KBr discs, and ^{1}H-nmr spectra on a Bruker 400 MHz spectrometer using $CDCl_3$ as the solvent and TMS as internal standard. Gc-ms was carried out on a Hewlett-Packard 59770C gas chromatograph-mass selective detector system using HP-1 capillary column (0.2 mm i.d. × 12 m) and He at 1 ml/min as the carrier gas. The injection port and transfer line were maintained at 220°, and the column temperature was programmed from 150° to 300° at a rate of 5°/min.

ISOLATION OF ANACARDIC ACID (15:2).—Crude anacardic acid mixture was isolated from raw cashew nut shells by extraction with pentane and separation of the extract concentrate on a Si gel column essentially as described earlier (10). From the mixture of anacardic acids thus obtained, pure **1** was obtained by preparative hplc using a Whatman ODS-3 column (9.4 mm × 25 cm) and MeCN-H_2O-HOAc (66:33:1) at a flow rate of 6.75 ml/min and collecting the fraction eluting at 7.5 min. The MeCN was removed from the eluent under reduced pressure and the aqueous solution lyophilized.

HYDROPEROXIDATION OF **1** BY SOYBEAN LIPOXYGENASE 1.—Anacardic acid (15:2) (5.1 mg in 1 ml EtOH) was added to 150 ml of 0.2 M phosphate buffer (pH 7.5) maintained at 10–15°. To this was added the lipoxygenase (10 units). After 2 min, $NaBH_4$ (25 mg), followed by HOAc (1 ml), was added to the reaction mixture. The reaction mixture was then passed through a Sep-Pak cartridge (octadecyl-bonded silica-packed cartridge from Waters Associates, MA). After the cartridge was washed with H_2O (10 ml), the product was eluted using MeOH (2 ml). Hplc of the product on Waters Novapak C18 (4.6 mm × 15 cm) using MeCN-H_2O-HOAc (66:33:1), monitored at 235 nm and 310 nm, showed a single peak at t_R 1.33 min and no peak at 5.33 min, corresponding to **1**. The product from four such experiments was combined and the solvent evaporated to yield **3**: λ max (MeOH) 234 and 308 nm (ε = 30,000 and 3000); ν max (KBr) 3417, 2926, 1655, 973, 930 cm^{-1}; $[\alpha]_D$ −47.23° (*c* = 0.65% in $CDCl_3$, 22°), ^{1}H nmr (δ) 11.51 (1H, s, COO*H*), 7.31 (1H, dd, *J* = 7.2 and 7.5 Hz, H-4), 6.83 (1H, dd, *J* = 1.1 and 7.2 Hz, H-3), 6.72 (1H, dd, *J* = 1.1 and 7.5 Hz, H-5), 6.58 (1H, dd, *J* = 11 and 15.2 Hz, H-10′), 6.02 (1H, t, possibly overlapped dd, *J* = 11 Hz, H-9′), 5.69 (1H, dd, *J* = 6.2 and 15.2 Hz, H-11′), 5.47 (1H, m, H-8′), 4.34 (q, possibly overlapped td, *J* = 6.2 Hz, H-12′), 2.92 (2H, m, H-1′), 2.19 (2H, m, H-7′), 1.50 (14H, m) and 0.95 (3H, t, *J* = 7.3 Hz, H-15′).

METHYLATION AND TRIMETHYLSILYLATION OF **3**.—Compound **3** (10 mg) in Et_2O (1 ml) was treated with freshly prepared ethereal CH_2N_2 (from 0.5 g nitrosomethylurea and 2.0 ml 50% aqueous NaOH). After 1 h, the solvent was evaporated and the residue dissolved in EtOAc (0.2 ml) and treated with bis-(trimethylsilyl)trifluoroacetamide (Fluka, 0.1 ml) at 60° for 1.5 h. The product was directly injected into the gc-ms.

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