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Novel Photoreactive Cinnamic Acid Analogues to Elucidate Phenylalanine Ammonia-lyase

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Abstract—4-[3-(Trifluoromethyl) diazirinyl] cinnamic acid derivatives were synthesized to elucidate properties of phenylalanine ammonia-lyase (PAL). 2-Methoxy and 2-biotinylated alkoxy compounds have inhibitory activity on the formation of phenylalanine from cinnamic acid. Specific photolabeling of the enzyme was detected using biotinylated derivatives without the use of radioisotopes. The results indicated that the 4-[3-(trifluoromethyl) diazirinyl] skeleton will be a suitable photoreactive compound to elucidate regulation of phenylpropanoid biosynthesis. © 2000 Elsevier Science Ltd. All rights reserved.

Phenylalanine ammonia-lyase (PAL) is an important enzyme that converts L-phenylalanine into *trans*-cinnamic acid, which in turn is the precursor of various phenylpropanoids in plants.[1,2] The reverse reaction, the PAL-catalyzed enantioselective addition of ammonia to *trans*-cinnamic acid, can be used for the synthesis of L-phenylalanine.[3,4] PAL is potentially a key regulatory enzyme, because it catalyzes the initial common step in the biosynthesis of phenylpropanoids. PAL activity is absent in mammalian cells. Therefore the introduction of PAL into mammalian cells to overcome phenylketoneurea, could be of great benefit. It could alleviate associated hyperphenylalaninemia and mental retardation, by reducing the L-phenylalanine level in urine.[5,6]

On the basis of chemical modification experiments on PAL over three decades ago, it was proposed that a dehydroalanine residue could play an important role as an electrophilic group that was required for activity.[7,8] Recently a serine residue, a precursor of dehydroalanine, has been identified by mutational studies as being important for activity.[9] A similar reaction mechanism, in which dehydroalanine could play a crucial role for activity, has been suggested for the homologous enzyme histidine ammonia-lyase.[10] Isotopic studies of these two ammonia-lyases revealed that their rate-limiting step slightly differed.[11–13] This could indicate that the environment of the active site is not identical in these two enzymes.

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There have been few papers that have reported on the coordination of the enzyme–substrate complex at the PAL active site. Photoaffinity labeling is one of the most useful methods to obtain information about binding sites. This is because labeling depends on the formation of the enzyme–ligand complex and therefore upon the location of residues near the active center. Photoaffinity labeling with various chemical precursors, aryldiazirine, arylazide and benzophenone, has been used for the determination of ligand–protein interactions.[14–18] Comparative studies of these three photophors suggested that 3-(trifluoromethyl) aryldiazirine is the most promising.[19–21] But the complicated synthesis of the diazirinyl three membered ring has resulted in fewer applications of it in biomolecular studies than for other photophors.

Recently, we found a versatile approach for the post-functionalization of the phenyldiazirine photophor using the Friedel–Crafts reaction as a key step.[15] This enabled us to introduce 3-phenyl-3-trifluoromethyldiazirine photophor as a part of the phenylpropanoid skeleton via carbon–carbon linkage. In this paper, we describe the synthesis of such novel diazirinyl cinnamic acid derivatives. We have also evaluated their properties in reactions with PAL from *Rhodotorulia glutinis*.

Three *ortho*-substituted photoreactive benzaldehyde derivatives **1**–**3**[22] were treated with triphenylcarboethoxymethylenephosphorane to generate a mixture (7:3) of (*E*)- and (*Z*)-ethyl cinnamates[23] **4**–**6** with yields of 80, 88 and 75%, respectively. The hydrolysis of these ethyl cinnamates with methanolic sodium hydroxide

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generated a complicated mixture. By replacing the solvent with ethanol, cinnamic acid derivatives **7**–**9**[24] were obtained in good yields (~90%). The diazirine photophor was stable under these reaction conditions. Thus we have developed a very simple and convenient route for systematic synthesis of photoreactive phenylpropanoid analogues and their biotinyl derivatives starting from easily available aldehydes **1**–**3** (Fig. 1).

The biological properties of cinnamic acid derivatives **7**–**9** were investigated as substrates for the reverse reaction of PAL. The condition for L-phenylalanine formation[3,25] did not generate L-phenylalanine analogues from **7**–**9**. But compounds **8** and **9** inhibited L-phenylalanine formation in the presence of *trans*-cinnamic acid, whereas compound **7** did not. This inhibition was concentration dependent. The phenylalanine determination was performed by the method of Renard et al.[25] Dixon plots revealed that **8** and **9** are competitive inhibitors for PAL and K_i values were determined as 0.24±0.05 and 0.52±0.03 mM, respectively (Fig. 2).

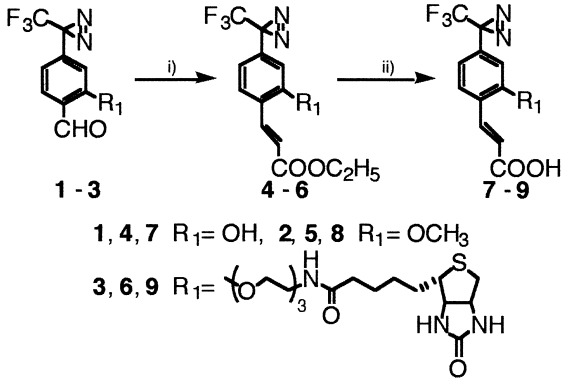

Figure 1. Synthesis of 4-[3-(trifluoromethyl)diazirinyl] cinnamic acid derivatives. (i) $Ph_3P{=}CHCOOC_2H_5$, benzene, room temperature, 2 h; (ii) 1 N NaOH, ethanol, room temperature, overnight.

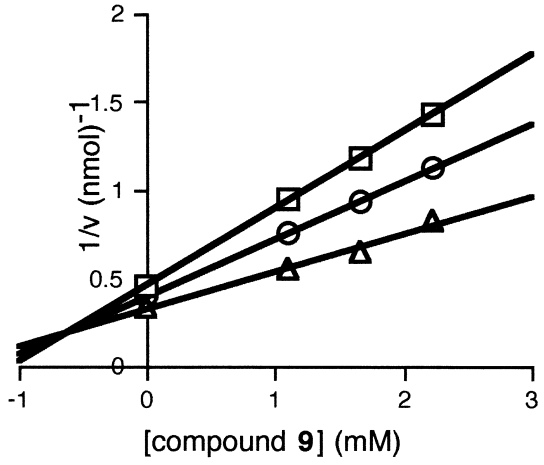

Figure 2. Inhibition by compound **9** for phenylalanine formation at different concentrations of cinnamic acid. △ 3 mM, ○ 2 mM, □ 1 mM. The assay mixture contained various concentrations of **9**, *trans*-cinnamic acid and enzyme in 9 M NH_4OH pH 10.5. It was incubated at 30 °C for 30 min. The values were determined in triplicate at each concentration.

Previous systematic studies indicated that alkoxy substitution at the *ortho* position and alkyl substitution at the *para* position of cinnamic acid caused less inhibition of PAL from *Rhodotorulia glutinis*.[26] Furthermore, *ortho*-hydroxy cinnamic acid was one of the strongest inhibitors. But our hydroxy compound **7** contains 3-(trifluoromethyl)diazirinyl group at the 4-position of cinnamic acid. It seems that 3-(trifluoromethyl)diazirinyl group on a benzene ring alters substrate specificity for PAL. It is reported that *para*-nitro substituted L-phenylalanine is a good substrate for PAL from parsley.[27] The 3-(trifluoromethyl)diazirinyl group should have similar characteristics as a nitro-group.

Compound **7** is a precursor for radiolabeled **8** by methylation, as has been established.[28] The combination of photoaffinity labeling and avidin-biotin technology (photoaffinity biotinylation) would also be a powerful tool to investigate the interaction of biomolecules and substrates. We have reported that photoaffinity biotinylation is a useful non-radioisotopic method to detect and retrieve the labeled components from a complicated mixture.[29–31] PAL (85 pmol) and the same amount of compound **9** were incubated at room temperature for 10 min in 9 M NH_4OH (pH 10.5) or 0.1 M Tris (pH 8.5) for phenylalanine formation or degradation, respectively. As indicated in the legend to Figure 3, *trans*-cinnamic acid and L-phenylalanine were included for competitive inhibition. All mixtures were irradiated with black light (15 W) for 10 min at 0 °C. The samples were directly subjected to electrophoresis by SDS-PAGE, and the protein bands were transferred onto PVDF [poly(vinylidene difluoride)] membrane. The blotted membranes were treated as previously described for the detection of biotinylated protein.[29] Chemiluminescence was detected at 78 kDa, corresponding with the PAL subunit,[32] in both the synthesis and degradation reactions (Fig. 3, lane 1 and 3).

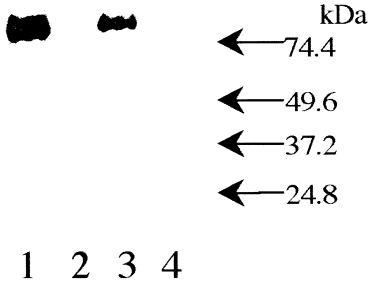

Figure 3. Photoaffinity labeling of PAL with compound **9** in phenylalanine formation (Tris pH 8.3, lane 1 and 2) and degradation (ammonium hydroxide pH 10.5, lane 3 and 4). Details of labeling conditions are described in the text. L-Phenylalanine and *trans*-cinnamic acid (0.1 M) were included as competitive inhibitors (lane 2 and 4, respectively). Each sample (1 μg) was subjected to SDS-PAGE (10% acrylamide gel) and transferred to PVDF membrane. The membrane was blocked for 1 h in 5% skim milk in 0.1% Tween 20, phosphate-buffered saline pH 7.4 (T-PBS), washed twice with T-PBS for 5 min and immersed in streptavidin–peroxidase conjugate (1:1500 dilution of the manufacturer's solution) in T-PBS for 1 h. After washing six times with T-PBS for 5 min, the membrane was immersed in chemiluminescence detection reagents for 1 min and exposed to film for 0.5 min.

This labeling was inhibited by the presence of phenylalanine (lane 2) and cinnamic acid (lane 4). The results indicate that the photoreactive cinnamic acid derivatives competed with natural substrates, suggesting that 4-[3-(trifluoromethyl)diazirinyl] aryl diazirine would fit to the substrate binding site of PAL.

This paper describes for the first time that the 4-[3-(trifluoromethyl)]diazirinyl substituted cinnamic acid derivatives works as a competitive inhibitor and photoreactive reagent for PAL. These results should promote the use of 4-[3-(trifluoromethyl)] aryldiazirinyl based photoaffinity labeling in studies of phenylpropanoid biosynthesis.

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24. Compound **7** HR-FABMS calcd for $C_{11}H_8F_3N_2O_3$ ($[M+H]^+$) 273.0487, found 273.0495; ^{1}H NMR ($CDCl_3$) δ 7.95 (1H, d, $J=16.2$ Hz), 7.63 (1H, d, $J=8.2$ Hz), 6.76 (1H, s), 6.68 (1H, d, $J=8.2$ Hz), 6.65 (1H, d, $J=16.2$ Hz). Compound **8** HR-FABMS calcd for $C_{12}H_{10}F_3N_2O_3$ ($[M+H]^+$) 287.0644, found 287.0665; ^{1}H NMR ($CDCl_3$) δ 8.01 (1H, d, $J=16.2$ Hz), 7.53 (1H, d, $J=8.2$ Hz), 6.81 (1H, d, $J=8.2$ Hz), 6.62 (1H, s), 6.55 (1H, d, $J=16.2$ Hz), 3.84 (3H, s). Compound **9** HR-FABMS calcd for $C_{27}H_{35}F_3N_5O_7S$ ($[M+H]^+$) 630.2209, found 630.2222; ^{1}H NMR ($CDCl_3$) δ 7.94 (1H, d, $J=16.2$ Hz), 7.50 (1H, d, $J=8.9$ Hz), 6.81 (1H, d, $J=8.9$ Hz), 6.62 (1H, s), 6.63 (1H, d, $J=16.2$ Hz), 4.53 (1H, m), 4.36 (1H, m), 4.19 (2H, m), 3.99 (2H, m), 3.75 (2H, m), 3.64 (2H, m), 3.57 (2H, m), 3.44 (2H, m), 3.16 (1H, m), 2.92 (1H, m), 2.75 (1H, m), 2.23 (2H, m), 1.70 (4H, m), 1.45 (2H, m).
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