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Inhibition of Phenylalanine Ammonia-lyase by Flavonoids

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The inhibitory activity of flavonoids against L-phenylalanine ammonia-lyase [PAL; EC 4.3.1.5] was systematically investigated. Twenty-three kinds of flavonoids were tested with three PAL preparations of different origins. Quercetin(**8**) was found to be the most active among the flavonoids tested and inhibited all three PAL preparations. Isoliquiritigenin(**11**), a biosynthetic intermediate of flavonoids, also showed relatively strong inhibition of PAL. However, the chalcone deactivated PAL in a time-dependent manner, and a model reaction with L-cysteine revealed that the chalcone formed an adduct with L-cysteine, indicating that PAL is deactivated by the Michael type addition of chalcone. The results obtained in this study suggest that the inhibitory activity of flavonoids against PAL does not occur by the mechanism of end product inhibition, as was suggested by Smith *et al.*, but is merely a result of nonspecific deactivation of PAL.

Keywords——PAL; inhibition; pea; sweet potato; *Rhodotorula*; TAL; flavonoid; chalcone; SH group

Introduction

L-Phenylalanine ammonia-lyase [PAL; EC 4.3.1.5] is an enzyme catalyzing the formation of *trans*-cinnamic acid from L-phenylalanine by the elimination of ammonia. It is widely distributed among higher plants as well as fungi, including yeast.[1] Since cinnamic acid is the first secondary metabolite in the biosynthetic pathway leading to a large variety of phenolics, such as phenylpropanoids, flavonoids, lignans and lignin, the production of these phenolics has been postulated to be controlled by the regulation of PAL activity. In fact, a positive correlation between the level of PAL activity and the rate of production of phenolics has been observed in many plants. For example, the induction of chlorogenic acid formation in potato and sweet potato was preceeded PAL activity.[2] Immediately before the increased production of flavonoids in grapefruit,[3] strawberry,[4] pea[5] and tissue culture of parsley,[6] PAL activity reached high levels. Lignification in bamboo shoots was also accompanied by an increase of PAL activity.[7] On the other hand, detailed kinetic investigations have revealed that PAL shows negative homotropic cooperativity.[8] This indicates that PAL may act as a regulatory enzyme in the biosynthesis of phenolic substances. In fact, quercetin showed an inhibitory effect on PAL prepared from pea seedlings, and an end product inhibition mechanism has been suggested by Attridge *et al.*[9] Since polyphenols are known to cause non-specific denaturation of proteins,[10] it is of interest from the view point of metabolic regulation to clarify whether flavonoids actually act as regulatory substances in the biosynthesis of phenolic compounds or whether the inhibitory effect of flavonoids against PAL is merely caused by non-specific action. The present paper deals with the results of systematic studies on the effect of flavonoids upon PAL preparations derived from pea, sweet potato and yeast.

Results and Discussion

The physiological function of PAL in higher plants is quite different from that in microorganisms. In higher plants, cinnamic acid formed by the action of PAL is utilized in the

biosynthesis of various secondary metabolites, whereas PAL in microorganisms plays a role in the catabolism of phenylalanine to supply carbon and nitrogen sources.[11] PAL preparations derived from three different sources were used to investigate the inhibitory effect of flavonoids upon PAL. One of the PAL preparations was obtained from pea pods which had been treated with 3 mM cuprous chloride to induce the biosynthesis of pisatin.[12] This PAL preparation can be regarded as the enzyme responsible for flavonoid biosynthesis. The other PAL preparation of higher plant origin was obtained from sweet potato in which PAL had been induced by cutting.[2b] The main phenolic compounds produced by this treatment are chlorogenic and isochlorogenic acids, and PAL would act to supply caffeic acid.[2b] In addition to these PAL preparations of higher plant origin, yeast PAL of *Rhodotorula* possessing TAL activity was also used in the experiments.[11,13] In a previous paper we reported that the inhibitory effects of cinnamate derivatives against three kinds of PAL preparations depend markedly on the enzyme source.[14] The effects of flavonoids on the three kinds of PAL are summarized in Table I.

The inhibitory effects of flavonoids were correlated with the structures of the flavonoids. For instance, the introduction of a hydroxyl group at C-3′ in the B-ring enhances the

TABLE I. Inhibition of PAL by Flavonoids

Compound	Source of PAL					
	Pea		Sweet potato		Yeast	
Flavanones						
Liquiritigenin (**1**)	10[a]	12[b]	3[a]	4[b]	9[a]	12[b]
Naringenin (**2**)	6	15	2	8	14	30
Eriodictyol (**3**)	—	—	48	64	11	36
Flavones						
Apigenin (**4**)	10	11	6	12	33	59
Luteolin (**5**)	—	—	53	62	67	81
Wogonin (**6**)	—	—	0	—	17	—
Flavonols						
Kaempferol (**7**)	10	24	14	34	33	57
Quercetin (**8**)	49	52	53	74	68	84
Morin (**9**)	—	—	11	30	48	67
Fisetin (**10**)	—	—	21	31	29	58
Chalcones						
Isoliquiritigenin (**11**)	27	45	26	55	52	77
Butein (**12**)	—	—	4	15	23	50
Isoflavones						
Daizein (**13**)	4	10	0	6	0	0
Genistein (**14**)	—	—	2	5	33	61
Catechins						
(+)−Catechin (**15**)	—	—	0	0	0	0
(−)−Epicatechin (**16**)	—	—	0	0	0	0
Glycosides						
Naringin (**17**)	9	10	0	2	0	0
Apiin (**18**)	4	4	0	2	0	7
Rutin (**19**)	23	26	0	0	0	0
Isoliquiritin (**20**)	10	14	0	3	0	0
Daizin (**21**)	4	3	0	0	0	0

Expressed as per cent inhibition at final concentrations of 0.5 mM (*a*) and 1.0 mM (*b*).

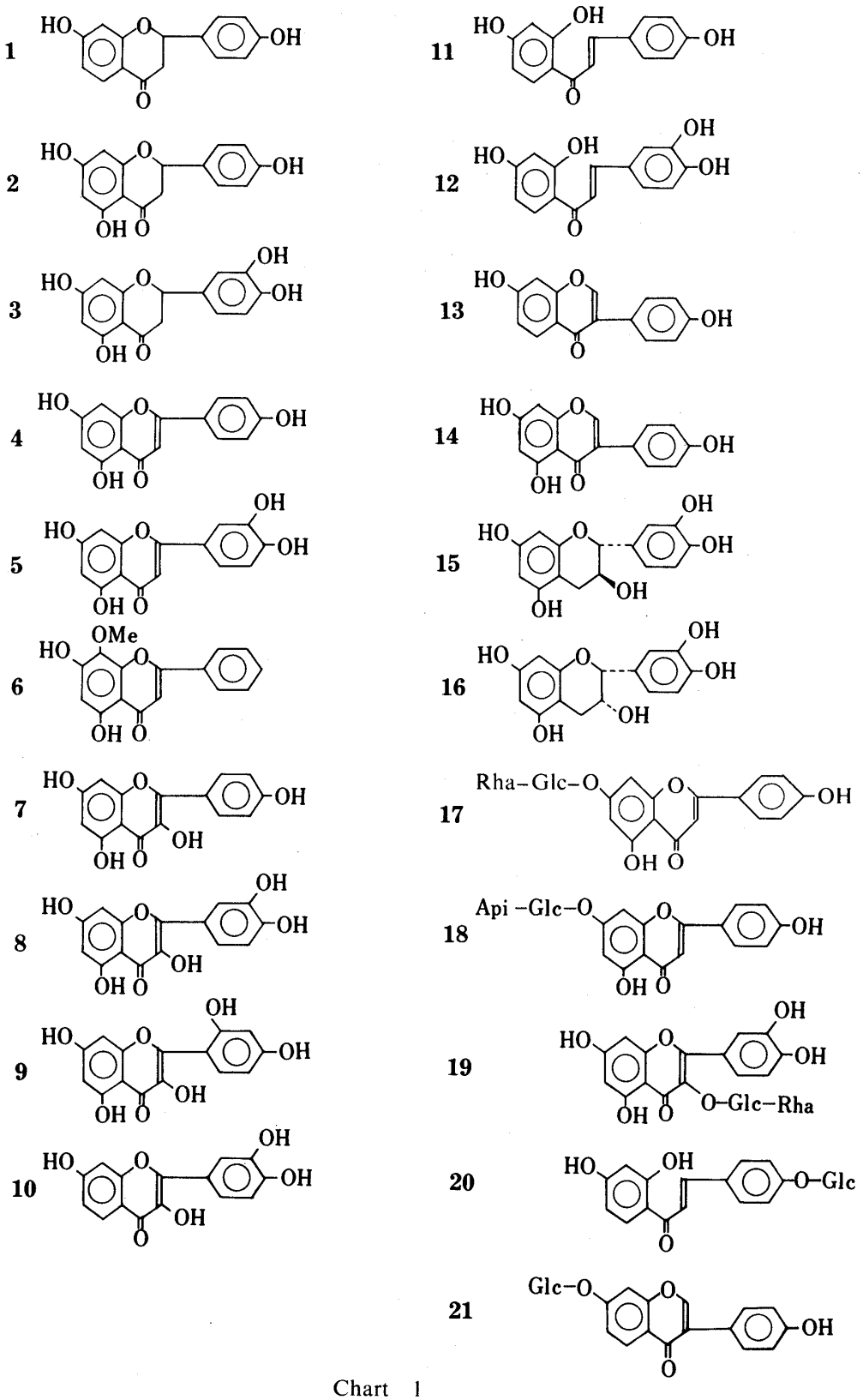
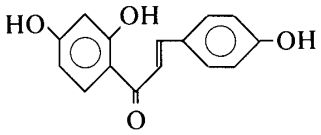
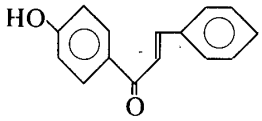
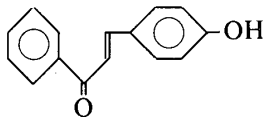
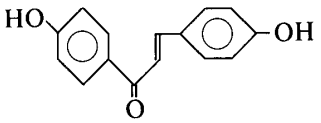
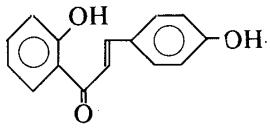
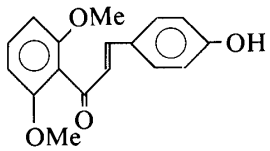
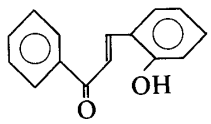
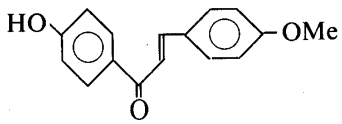
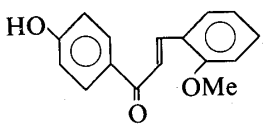


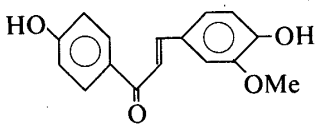
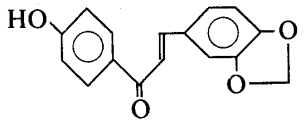
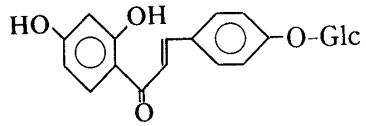
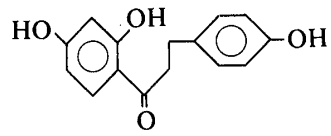
Chart 1

inhibitory effect of flavonoids. Similarly, flavonoids with hydroxyl groups at C-5 and C-7 in the A-ring showed higher activity than those with a hydroxyl group only at C-7. As catechin (**15**) and epicatechin (**16**) showed no inhibitory effect, the presence of a carbonyl group at C-4 seems to be essential for a high inhibitory activity. Comparison of the inhibitory activities of flavanones, flavones and flavonols showed that their activities are partly due to the double

bond between C-2 and C-3 and the hydroxyl groups at C-3. In conclusion, quercetin (**8**) possesses the strongest activity among all the flavonoids tested on the three PAL preparations. None of the glycosides tested had any significant effect on PAL. Sweet potato PAL was especially sensitive to the flavonoids possessing hydroxyl groups at C-3′ and C-4′ in the B ring, such as eriodictyol (**3**). This result may be related to the fact that sweet potato produces chlorogenic acid as the main phenolic on cutting and the PAL preparation was strongly inhibited by caffeic acid.[14] It has been reported that PAL prepared from pea seedlings,[9] tobacco leaves[1c] and gerkin hypocotyls[15] was inhibited by flavonoids such as quercetin (**8**) and kaempferol (**7**). Particular attention has been paid to these results because of their implications for end product inhibition.[9] However, the results in Table I show that the same flavonoids inhibited pea, sweet potato and yeast PAL. It is clear that flavonoids inhibit PAL irrespective of the enzyme source.

TABLE II. Inhibition of PAL by Chalcones

	Compound	Source of PAL	
		Sweet potato	Yeast
11		50	64
22		16	19
23		24	24
24		45	61
25		36	38
26		12	14
27		54	31
28		20	34
29		12	26

	Compound	Source of PAL	
		Sweet potato	Yeast
30		19	25
31		29	40
32		10	2
33		11	19

Expressed as per cent inhibition.
Final concentration was 1.0 mM.

Similar results were obtained with chalcones(**11**, **12**,**13**—**24**). The chalcones are a C_6–C_3–C_6 compounds first formed in the biosynthesis of flavonoids[16] and therefore are candidates for regulatory substances. As isoliquiritigenin (**11**), which is a natural chalcone acting as an intermediate in flavonoid biosynthesis, was found to be the strongest inhibitor among chalcones so far tested, it might act as a regulatory compound in flavonoid biosynthesis. Investigation with synthetic chalcones, however, revealed that 4,4′-dihydroxychalcone(**24**) was as active as isoliquiritigenin(**11**). Moreover, the inhibition of PAL by chalcones was observed in three kinds of PAL preparation irrespective of the enzyme source. The loss of inhibitory activity in dihydrochalcone(**33**) suggested that the presence of the double bond is essential for the inhibitory activity of chalcones. When isoliquiritigenin(**11**) was preincubated with PAL, the enzyme activity decreased in a time dependent manner (Fig. 1), indicating that chalcone reacted with PAL in an irreversible fashion to form a covalent bond. In order to confirm this, loss of enzyme activity on preincubation with isoliquiritigenin (**11**) was investigated by the dilution method. The enzyme was preincubated with isoliquiritigenin (**11**) (1.0 mM), then the enzyme solution was diluted 10 times with the same buffer to eliminate the effect of the inhibitor and residual enzyme activity was measured by the usual assay method. Two lines with different slopes in Fig. 2 indicate that the reaction follows pseudo-first order kinetis in good accord with the model of William and Koshland,[17] in which more than two reaction sites are present in the enzyme. In order to obtain further evidence regarding the formation of a covalent bond, 4,4′-dihydroxychalcone (**24**) was reacted with L-cysteine at pH 8.5 for 20 min. A reaction product formed showed ninhydrin positive and chalcone like colour reactions on thin-layer chromatography (TLC). The proton nuclear magnetic resonance (^{1}H-NMR) spectrum revealed that the adduct (**26**) is formed by the Michael addition of L-cysteine to the chalcone. When 4,4′-dihydroxychalcone (**24**) was incubated with L-histidine, L-lysine and L-serine, no adduct formation was observed. This model reaction supports the view that deactivation of PAL is caused by the reaction of the chalcone with thiol groups of PAL.

The kinetics of inhibition by quercetin (**8**) and luteolin (**5**) were investigated by using sweeet potato and yeast PAL, and the flavonoids tested were found to be "mixed type" inhibitors, as was found in PAL preparations from pea and gerkin seedlings. This was presented as one line of evidence for end product inhibition.[9,14] However, when quercetin (**8**) was preincubated

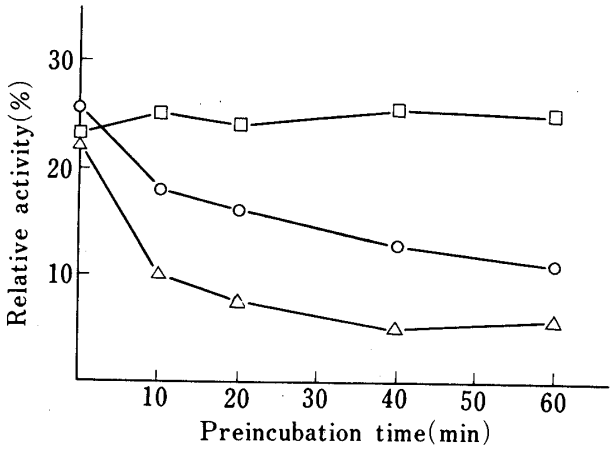


Fig. 1. Time-dependent Deactivation of PAL on Preincubation with Quercetin (**8**), △——△, Isoliquiritigenin (**11**) ○——○ or Cinnamic Acid □——□ at a Concentration of 1 mM

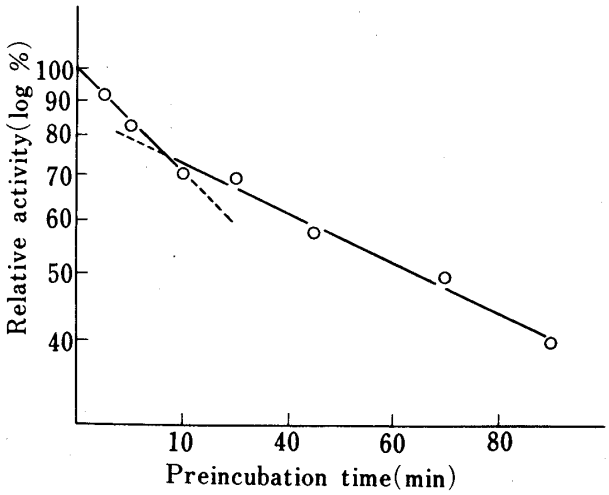


Fig. 2. Time-dependent Loss of PAL Activity on Treatment with 1 mM Isoliquiritigenin (**11**)

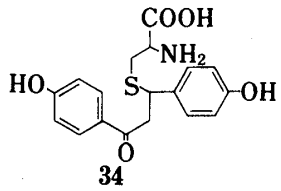


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Chart 2

with PAL, PAL was deactivated in a time dependent manner (Fig. 1). This is in contrast to *trans*-cinnamic acid, which is a competitive inhibitor of PAL and caused no deactivation in a preincubation experiment, as shown in Fig. 1. On the other hand, flavonoids are known to inhibit various enzymes. For example, flavones and flavonols with 3′, 4′ and 7 hydroxyl groups are reported to be potent inhibitors of bovine pancreatic ribonuclease[18] and bovine hyaluronidase.[19] Further, the aglycones showed higher inhibitory activity than glycosides in almost all the examples so far reported. These results are very similar to those for PAL. Furthermore, in this study, PAL preparations from three different sources were equally affected by flavonoids. The results so far obtained indicate that the inhibitory action of flavonoids against PAL is not due to a regulatory function of flavonoids as was suggested by Smith *et al.*,[9,14] but merely to a non-specific inhibitory action.

Experimental

^{1}H-NMR spectra were measured on a JEOL JNM-PS 100 spectrometer. Radioactivity was measured with an Aloka LSC-670 scintillation counter. Protein was determined by the method of Lowry *et al.*[20] Amino acids were purchased from Tokyo Kasei Co. The samples of flavonoids including chalcones were kind gifts of Dr. A. Komamine of the Department of Botany, University of Tokyo and Dr. T. Saitoh of the Faculty of Pharmaceutical Sciences, Teikyo University.

Enzyme Preparation——Pea PAL: Pea pods (1 kg) were immersed in 3.0 mM $CuCl_2$ solution for 17 h then homogenized with 1.1 l of 0.1 M borate buffer (pH 8.5), containing 2-mercaptoethanol (0.8 ml/l), 1 mM EDTA, 50 mM potassium isoascorbate and 100 g of Polyclar AT in a Waring blender for 1 min. From this homogenate, pea PAL was partially purified according to the method for sweet potato PAL purification reported by Tanaka and Uritani[21] involving ammonium sulfate fractionation (30—60%), column chromatographies on L-phenylalanyl Sepharose 4B and phosphocellulose. The overall purification was 20 fold. Specific activity was 5 mU/mg protein at 40°C.

Sweet Potato PAL: Sweet potato PAL was extracted and purified according to the method of Tanaka and Uritani[21] by ammonium sulfate fractionation (35—55%), column chromatographies on L-phenylalanyl Sepharose 4B, phosphocellulose and Sepharose 6B. Overall purification was 140 fold. Specific activity was 270 mU/mg protein at 40°C.

Yeast PAL: Yeast PAL (*Rhodotorula glutinis*; Cat No. 0810) was purchased from PAL Biochemicals Inc., Milwaukee, USA.

Enzyme Aassay——PAL activity was determined by the method of Minamikawa and Uritani.[22] The reaction mixture (1.0 ml) contained $[\alpha]_D^{18}$ L-[U-^{14}C]-phenylalanine (1.0 μM, 0.05 μCi), Tris-HCl buffer (pH 8.5,

50 mM), inhibitor solution (100 μl) in ethylene glycol monomethyl ether and enzyme solution. The mixture was incubated at 40°C for 20 min. The reaction was terminated by the addition of 100 μl of 6 N HCl and 100 μl of 2.0×10^{-2} M unlabelled *trans*-cinnamic acid solution as a carrier. The reaction mixture was then extracted with 2 ml of toluene. A 1 ml aliquot of the toluene layer was transferred to a counting vial and the radioactivity was determined with a liquid scintillation counter. Preincubation experiments with cinnamic acid, quercetin (**8**) and isoliquiritigenin (**11**) were carried out under the condition of the usual enzyme assay (sweet potato PAL) except that the reaction mixtures were preincubated in the presence of the inhibitors (1 mM) for various times prior to the addition of the substrate.

Time Dependent Loss of Activity on Preincubation with Isoliquiritigenin (11)——Sweet potato PAL (4 μg protein) was preincubated in 0.05 M Tris-HCl buffer (pH 8.5) in the presence of isoliquiritigenin (**11**) (1 mM) for various incubation periods. After the preincubation, the reaction mixture was diluted 10 times with the same buffer. The reaction was initiated by the addition of L-[U-^{14}C]-phenylalanine (1.1×10^5 dpm; 1 mM) and carried out for 20 min at 40°C. The reaction mixtures were worked up as described for the enzyme assay.

Reaction of 4′,4′-Dihydroxychalcone (24) with L-Cysteine——A solution of 4,4′-dihydroxychalcone (**24**) (252 mg) in EtOH (3 ml) was added to a solution of L-cysteine (121 mg) in 50 mM Tris-HCl buffer, pH 8.5 (3 ml). After 20 min incubation at 40°C, the precipitates were collected and purified by reprecipitation from EtOH to give a white powder, mp 163—164°C. UV λ_{max}^{MeOH} nm(log ϵ): 283 (4.15). ^{1}H-NMR (0.1 N DCl) δ: 2.84 (m, α-CH_2), 3.30 (br t, J=7 Hz, 3″-CH_2), 4.06 (dd, J=7, 4.7 Hz, β-CH), 4.30 (br t, J=7 Hz, 2″-CH), 6.69 (d, J=9 Hz, 3,5,3′,5′-arom.H), 7.10 (d, J=9 Hz 2,6-arom.H), 7.54 (d, J=9 Hz 2′,6′-arom.H).

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