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Inhibitory effects of cardols and related compounds on superoxide anion generation by xanthine oxidase

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ABSTRACT

5-Pentadecatrienylresorcinol, isolated from cashew nuts and commonly known as cardol ($C_{15:3}$), prevented the generation of superoxide radicals catalysed by xanthine oxidase without the inhibition of uric acid formation. The inhibition kinetics did not follow the Michelis–Menten equation, but instead followed the Hill equation. Cardol ($C_{10:0}$) also inhibited superoxide anion generation, but resorcinol and cardol ($C_{5:0}$) did not inhibit superoxide anion generation. The related compounds 3,5-dihydroxyphenyl alkanoates and alkyl 2,4-dihydroxybenzoates, had more than a C9 chain, cooperatively inhibited but alkyl 3,5-dihydroxybenzoates, regardless of their alkyl chain length, did not inhibit the superoxide anion generation. These results suggested that specific inhibitors for superoxide anion generation catalysed by xanthine oxidase consisted of an electron-rich resorcinol group and an alkyl chain having longer than C9 chain.

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1. Introduction

Xanthine oxidase (EC 1.1.3.22), a molybdenum-containing enzyme, catalyses the oxidation of hypoxanthine to xanthine and ultimately to uric acid. The accumulation of uric acid leads to hyperuricaemia and gout (Hatano et al., 1990; Nakanishi et al., 1990), therefore xanthine oxidase inhibitors may serve as therapeutic agents for these conditions. Xanthine oxidase also generates a superoxide anion, and excess superoxide anion generation leads to peroxidative damage in cells (Fong, McCay, Poyer, Keele, & Misra, 1973; McCord, 1985). Intake of xanthine oxidase inhibitors from foods may be useful to prevent postischemic injury. In our continuing investigation to understand the functions of xanthine oxidase inhibitors, the balance of hydrophilic and hydrophobic moieties of molecules is associated with the inhibitory activity (Masuoka & Kubo, 2004; Masuoka, Nihei, & Kubo, 2006; Masuoka et al., 2012). 1, 1-Diphenyl-2-*p*-picryhydrazyl (DPPH) scavenging activity of antioxidants is attributed to their conjugated endiol structures, and the activity indicates a reduction activity. Flavonoids are essentially competitive inhibitors for uric acid formation catalysed by xanthine oxidase. Some flavonoids, which have DPPH scavenging activity, are able to strongly reduce xanthine oxidase molecules to suppress superoxide anion generation (Masuoka, Matsuda, & Kubo, 2012). Finally it has become evident that the reaction of xanthine oxidase with inhibitors consisted of inhibition of uric acid formation, reduction reactions of the xanthine oxidase molecule and radical scavenging reactions. The active sites of uric acid formation catalysed by xanthine oxidase and the superoxide anion generation are different. That is, an inhibitor which binds the xanthine binding site in xanthine oxidase inhibits the uric acid formation by xanthine oxidase. An inhibitor of superoxide anion generation binds to other sites, and this binding leads to the inhibition of superoxide anion generation or reduction of the enzyme molecules to catalyse hydrogen peroxide formation. Therefore, as xanthine oxidase inhibitors are able to multifunction, it is necessary to distinguish each function and to find specific inhibitor.

Anacardic acids ($C_{15:3}$) (**1**) in Fig. 1, isolated from the cashew *Anacardium occidentale* (*Anacardiaceae*) (Kubo, Komatsu, & Ochi, 1986), had no DPPH activity and cooperatively bound to xanthine binding site to inhibit both uric acid formation and superoxide anion generation (Masuoka & Kubo, 2004). As cardol ($C_{15:3}$) (**2**), 5-[8′(*Z*),11′(*Z*),14′-pentadecatrienyl]resorcinol, isolated from the cashew, was not an inhibitor of uric acid formation but did inhibit the generation of the superoxide anion, we suggested that cardol was a specific inhibitor for superoxide anion generation catalysed by xanthine oxidase (Masuoka et al., 2012). However, it is still unclear about the specific character of cardols. In the current study, prepared cardol ($C_{10:0}$) (**3**) and ($C_{5:0}$) (**4**) and related compounds (**7**–**15**) were examined and their effects on the xanthine oxidase

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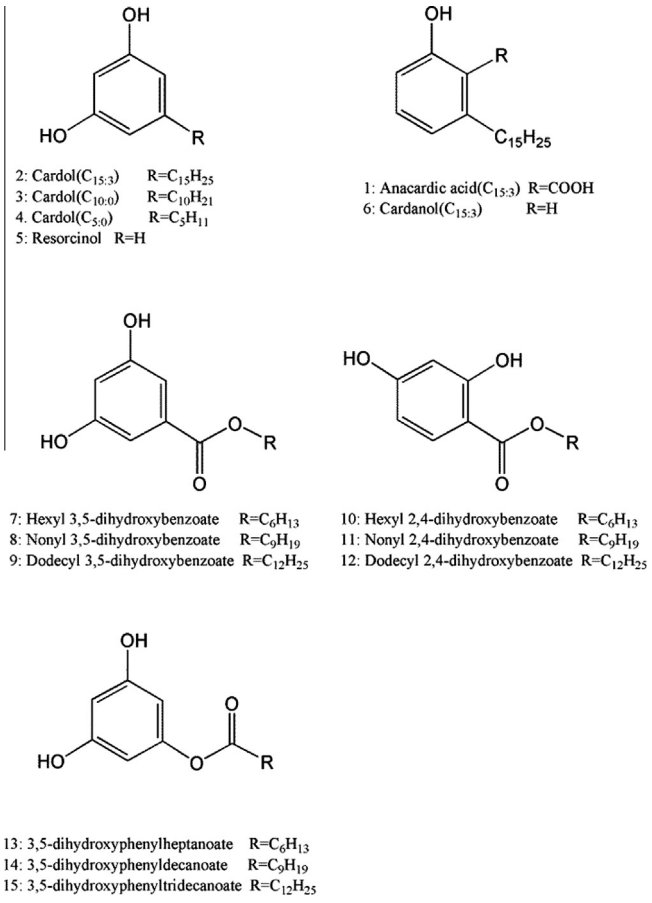

Fig. 1. Cardols and related compounds.

reaction to study the function of the hydrophobic head portion and hydrophilic tail portion of cardols.

2. Materials and methods

2.1. Chemicals

Cardol ($C_{15:3}$) (**2**), anacardic acid ($C_{15:3}$) (**1**) and cardanol ($C_{15:3}$) (**6**) used for the assay were previously isolated from the cashew nut shell oil and were re-purified by recycle HPLC (R-HPLC) using an ODS C_{18} column. Cardols (**3, 4**) possessing different alkyl side chains were synthesized from 3,5-dimethoxybenzaldehyde via a Wittig reaction. Xanthine oxidase, DPPH, EDTA and resorcinol were purchased from Sigma Chemical Co. (St. Louis, MO) and Aldrich Chemical Co. (Milwaukee, WI). Cardol related derivatives, such as alkyl 3,5-dihydroxybenzoates (**7**–**9**), alkyl 2, 4-dihydroxybenzoates (**10**–**12**) and 3,5-dihydroxyphenyl alkanoates (**13**–**15**), were synthesized as follows. First, hexyl, nonyl and dodecyl 3,5-dihydroxybenzoates (**7**–**9**) were synthesized by a two-step procedure. The corresponding dibenzyloxybenzoic acids were obtained first by a method previously reported (Nihei, Nihei, & Kubo, 2003). Then, alkyl 3,5-dihydroxybenzoates, were obtained in high yield by a two step procedure, a Mitsunobu reaction the first step, followed by hydrogenation to remove the protecting group. Second, the same alkyl (hexyl, nonyl and dodecyl) 2,4-dihydroxybenzoates (**10**–**12**) were synthesized in a similar manner using 2,4-dibenzyloxybezoic acid as a starting material. Third, 3,5-dihydroxyphenyl (heptanoate, decanoate and tridecanoate) alkanoates (**13**–**15**) were readily prepared in one step from phloroglucinol and the corresponding carboxylic acid using DCC as a coupling reagent.

2.2. Preparation of sample solution

Compounds were dissolved in dimethyl sulfoxide (DMSO), to a concentration of 10 mM, which was used for the experiments.

2.3. Assay of uric acid generated by xanthine oxidase

The xanthine oxidase (EC 1.1.3.22, Grade IV) used for the bioassay was purchased from Sigma Chemical Co. The reaction mixture consisted of 2.76 ml of 40 mM sodium carbonate buffer containing 0.1 mM EDTA (pH 10.0), 0.06 ml of 10 mM xanthine and 0.06 ml of the sample solution (dissolved in DMSO). The reaction was started by the addition of 0.12 ml of xanthine oxidase (0.04 units), and the absorbance at 293 nm was recorded for 60 s. A control experiment carried out by replacing the sample solution with the same amount of DMSO. The reaction rate was calculated from the proportional increase in absorbance.

2.4. Assay of superoxide anion generated by xanthine oxidase

In the reaction, the superoxide anion generated by the enzyme reduces nitroblue tetrazolium to a blue formazan. The absorbance of the formazan produced was determined at 560 nm. The reaction mixture consisted of 2.7 ml of 40 mM sodium carbonate buffer containing 0.1 mM EDTA (pH 10.0), 0.06 ml of 10 mM xanthine and 0.03 ml of 0.5% bovine serum albumin, 0.03 ml of 2.5 mM nitroblue tetrazolium and 0.06 ml of the sample solution (dissolved in DMSO). To the mixture at 25 °C, 0.12 ml of xanthine oxidase (0.04 units) was added, and the absorbance at 560 nm was recorded for 60 s (by formation of blue formazan) (Masuoka & Kubo, 2004). A control experiment was carried out by replacing the sample solution with the same amount of DMSO.

2.5. Radical scavenging activity on DPPH

First, 1 ml of 100 mM acetate buffer (pH 5.5), 1.87 ml of ethanol and 0.1 ml of ethanolic solution of 3 mM DPPH were put into a test tube. Then, 0.03 ml of the sample solution (dissolved in DMSO) was added to the tube and incubated at 25 °C for 20 min. The absorbance at 517 nm (DPPH, $\varepsilon = 8.32 \times 10^3$) was recorded. As a control, 0.03 ml of DMSO was added to the tube. The scavenging activity was calculated from the decrease in absorbance and expressed as the number of scavenged DPPH molecules per each sample molecule.

2.6. Radical scavenging activity for the O_2^- generated by the PMS-NADH system

The superoxide anion was generated nonenzymatically with a PMS-NADH system. The reaction mixture (final volume was 3.0 ml) containing 25 μM NBT, 150 μg of BSA, 78 μM NADH and 0.06 ml of sample solution in 40 mM sodium carbonate buffer containing 0.1 mM EDTA (pH 10.0) was prepared and incubated at 25 °C for 3 min. Then, 0.03 ml of 155 μM PMS was added to start the reaction and the absorbance at 560 nm was recorded for 60 s (Nishikimi, Rao, & Yagi, 1972). As the control, 0.06 ml of DMSO was used. The reaction rate was calculated from the proportional increase of absorbance, and the scavenging activity of the sample was expressed as the inhibition percentage.

2.7. Assay and data analysis

Each assay was performed at least in triplicate in separate experiments, and the analysis was performed using Sigma plot 2001 (SPSS Inc., Chicago, IL). The inhibition mode and kinetic parameters were analysed with Enzyme Kinetics Module 1.1 (SPSS

Inc.) equipped with Sigma Plot 2001. When the sigmoidal inhibition was observed with inhibitors, the inhibition rates ($iv_i = v_{i=0} - v_i$) were calculated and analysed using the Hill equation: $iv_i = v_{i=0}[I]^n/(K_i + [I]^n)$; where the inhibition rates are indicated as iv_i and calculated difference between enzymatic reaction rate at no inhibitor ($v_{i=0}$) and the rates (v_i) at various concentrations of inhibitor.$iv_i = v_{i=0}[I]^n/(K_i + [I]^n)$; $[I]$ indicates concentrations of the inhibitor; n is a slope factor of the sigmoidal curve (iv_i); $v_{i=0}$ is enzymatic reaction rate with no inhibitor and K_i is a constant. When K_i is equal to $[I]^n$, iv_i becomes $v_{i=0}/2$. As a result, K_i indicates IC_{50}.

3. Results

3.1. Inhibitory activity of uric acid formation

Cardols and resorcinol (**2**–**5**) did not inhibit the uric acid formation catalysed by xanthine oxidase (Table 1). However, after 3 min of pre-incubation, cardol ($C_{15:3}$) inhibited uric acid formation, which followed the Michaelis–Menten equation. The inhibition kinetics were analysed using Lineweaver–Burk plots (Fig. 2) and indicated that cardol ($C_{15:3}$) was a non-competitive and weak inhibitor of uric acid formation. Most of the other resorcinol compounds did not inhibit the formation of uric acid catalysed by xanthine oxidase. Only dodecyl 2, 4-dihydroxybenzoate (**12**) non-competitively inhibited the formation.

3.2. Inhibitory activity of superoxide anion generation

Cardols ($C_{15:3}$) (**2**) and ($C_{10:0}$) (**3**) suppressed superoxide anion generation but cardol ($C_{5:0}$) (**4**) and resorcinol (**5**) did not. The results obtained for cardol ($C_{15:3}$) (**2**) are shown in Fig. 3. The inhibition was not detected at concentrations less than 80 μM, and a strong inhibition was observed at greater than 150 μM. It should be noted that the inhibition of superoxide anion generation by xanthine oxidase did not follow Michaelis–Menten equation, but followed the Hill equation (sigmoidal, $n = 5.2 \pm 0.2$). The IC_{50} of superoxide anion generation by cardol (**2**) was 115 ± 10 μM. The inhibition curve for cardol was not affected by the xanthine concentration (Fig. 3). In brief, it was suggested that the antioxidant activity of the cardols was not due to radical scavenging by the superoxide scavenging activity but inhibition of superoxide anion generation by xanthine oxidase. Interestingly, resorcinol and cardol ($C_{5:0}$) (**4**) did not inhibit superoxide anion generation at concentrations up to 200 μM, and it was suggested that the 5-alkyl side chain, more than C9, played an important role in eliciting the activity. It was found that cardols ($C_{15:3}$ & $C_{10:0}$) (**2, 3**) inhibited superoxide anion generation by binding cooperatively to the enzyme. To compare inhibition by cardols with other compounds having a resorcinol moiety, alkyl dihydroxybenzoates and dihydroxyphenyl alkanoates were examined. Nonyl 2,4-dihydroxybenzoate (**11**), dodecyl 2,4-dihydroxybenzoate (**12**), 3,5-dihydroxyphenyl decanoate (**14**) and 3,5-dihydroxyphenyl tridecanoate (**15**) sigmoidally inhibited superoxide anion generation (Table 2). All these compounds have an alkyl group (more than C9), and the inhibition followed the Hill equation. However, the alkyl chain alone was not enough to elicit the inhibitory activity since cardanol (**6**) and alkyl (>C9) 3, 5-dihydroxybenzoate (**8, 9**) did not exhibit noticeable inhibitory activity.

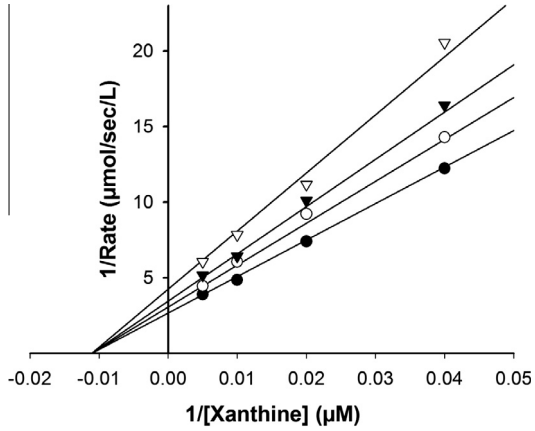

Fig. 2. Lineweaver–Burk plots of uric acid formation rates from xanthine after xanthine oxidase was preincubated with cardol ($C_{15:3}$) (**2**) for 3 min. Each cardol concentration was as follows: ●: 0 μM, ○: 50 μM, ▼: 100 μM, ▽: 200 μM.

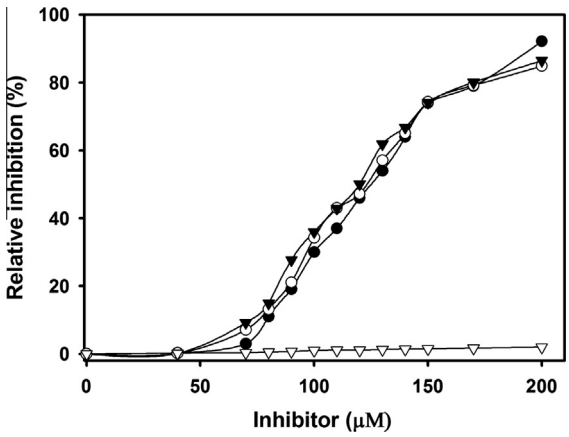

Fig. 3. Effects of cardol ($C_{15:3}$) (**2**) and resorcinol (**5**) on the generation of superoxide anion by xanthine oxidase. Closed circle (50 μM xanthine), opened circle (200 μM xanthine) and closed triangle (500 μM xanthine) indicated inhibition of superoxide anion generation by cardol ($C_{15:3}$). Open triangle indicated the inhibition by resorcinol.

Table 1
Inhibition of uric acid formation by cardols and related compounds.

Compounds tested	(μM)		
	IC_{50}	K_I	K_{IS}
Cardol ($C_{15:3}$) (2)	370 ± 30[a]	370 ± 30[a]	370 ± 30[a]
Cardol ($C_{10:0}$) (3)	No inhibition[b]	–	–
Cardol ($C_{5:0}$) (4)	No inhibition[c]	–	–
Resorcinol (5)	No inhibition[d]	–	–
Anacardic acid ($C_{15:3}$) (1)	162 ± 10[e]	–	–
Salicylic acid	No inhibition	–	–
Cardanol ($C_{15:3}$) (6)	No inhibition	–	–
Hexyl 3,5-dihydroxybenzoate (7)	No inhibition	–	–
Nonyl 3,5-dihydroxybenzoate (8)	No inhibition	–	–
Dodecyl 3,5-dihydroxybenzoate (9)	No inhibition	–	–
Hexyl 2,4-dihydroxybenzoate (10)	No inhibition	–	–
Nonyl 2,4-dihydroxybenzoate (11)	No inhibition	–	–
Dodecyl 2,4-dihydroxybenzoate (12)	189 ± 17	189 ± 17	189 ± 17
3,5-Dihydroxyphenylheptanoate (13)	No inhibition	–	–
3,5-Dihydroxyphenyldecanoate (14)	No inhibition	–	–
3,5-Dihydroxyphenyltridecanoate (15)	No inhibition	–	–

[a] Preincubation for 3 min.
[b] Preincubation for 3 min. 23.4 ± 3.2 % inhibition at 200 μM.
[c] Preincubation for 3 min. 9.1 ± 0.9 % Inhibition at 200 μM.
[d] 1.0 ± 0.1 % inhibition at 200 μM.
[e] Inhibition type was sigmoidal inhibition, and Hill constant was 1.7 ± 0.2.

Table 2
Inhibition of superoxide anion generation by cardols and related compounds.

Compounds tested	IC_{50} (μM)	Hill constant	Inhibition type
Cardol ($C_{15:3}$) (2)	115 ± 10	5.2 ± 0.2	Sigmoidal
Cardol ($C_{10:0}$) (3)	106 ± 8	5.1 ± 0.2	Sigmoidal
Cardol ($C_{5:0}$) (4)	No inhibition[a]	–	–
Resorcinol (5)	No inhibition	–	–
Anacardic acid ($C_{10:3}$) (1)	51.3 ± 1.5	4.2 ± 0.5	Sigmoidal
Salicylic acid	No inhibition	–	–
Cardanol ($C_{15:3}$) (6)	No inhibition	–	–
Hexyl 3,5-dihydroxybenzoate (7)	No inhibition	–	–
Nonyl 3,5-dihydroxybenzoate (8)	No inhibition	–	–
Dodecyl 3,5-dihydroxybenzoate (9)	No inhibition	–	–
Hexyl 2,4-dihydroxybenzoate (10)	No inhibition	–	–
Nonyl 2,4-dihydroxybenzoate (11)	118 ± 1	3.7 ± 0.1	Sigmoidal
Dodecyl 2,4-dihydroxybenzoate (12)	128 ± 35	10.3 ± 3.6	Sigmoidal
3,5-Dihydroxyphenylheptanoate (13)	No inhibition	–	–
3,5-Dihydroxyphenyldecanoate (14)	121 ± 4	1.9 ± 0.4	Sigmoidal
3,5-Dihydroxyphenyltridecanoate (15)	75.3 ± 5.0	5.5 ± 1.8	Sigmoidal

[a] 18.5 ± 1.3 % inhibition at 400 μM.

3.3. DPPH scavenging activities

The DPPH scavenging activities of cardols (**1**–**3**), resorcinol (**4**), anacardic acid (**5**), cardanol (**6**), salicylic acid, alkyl 3,5-dihydroxybenzoates (**7**–**9**), alkyl 2,4-dihydroxybenzoates (**10**–**12**) and 3,5-dihydroxyphenyl alkanoates (**13**–**15**) were examined at least in triplicate. The scavenging activity from all these compounds was less than 0.03 molecules of DPPH/one molecule of each compound.

3.4. Scavenging activity of superoxide anion generated with a PMS-NADH system

The rate of generation of the superoxide anion by the addition of cardols (**2**–**4**) and resorcinol (**5**) were diminished dose-dependently. The scavenging activity of resorcinol and cardol ($C_{5:0}$) (**4**) were 10 ± 4 % and 13 ± 3 % at 200 μM, respectively. The cardols scavenging activity ($C_{10:0}$) (**3**) and ($C_{15:3}$) (**2**) were 73 ± 5 % and 75 ± 7 % at 200 μM, respectively. These scavenging activities were low compared to the inhibitory rates of superoxide anion generated by xanthine oxidase (Table 2).

4. Discussion

Cardol (**2**) is isolated from many edible plants, such as cashew (*A. occidentale*) (*Anacardiaceae*), pistachio (*Pistacia vera*), macadamia (*Macademia ternifolia*) and mango (*Mangifera indica*) (Cojocaru et al., 1986) and is also known to inhibit various enzymes, such as glycerol-3-phosphate dehydrogenase, tyrosinase (Kubo, Kinst-Hori, & Yokokawa, 1994), lipoxygenases (Shobha, Ramadoss, & Ravindranath, 1994), aldose reductase, cycloxygenases (Grazzini et al., 1991) and prostaglandin synthase (Bhattacharya, Mukhopadhyay, Mohan Rao, Bagchi, & Ray, 1987; Kubo et al., 1987). Since resorcinol has little or no effect on these enzymes, the hydrophobic alkenyl side chain in cardols is undoubtedly associated with the enzyme inhibitory activity. In addition, the number of double bonds in the side chain is not directly associated with the enzyme inhibitory activity (Kubo et al., 1994; Shobha et al., 1994), suggesting that the interaction of the double bond with a specific amino acid residue of the enzymes is unlikely. Cardol (**2**) is a unique xanthine oxidase inhibitor without pro-oxidant effects (Kamal-Eldin, Pouru, Eliasson, & Åman, 2000). The resorcinol lipid was also reported to inhibit the generation of the superoxide anion catalysed by xanthine oxidase (Trevisan et al., 2006). The head and tail structure of these compounds suggests that optimisation of xanthine oxidase inhibitory activity is possible via a synthetic approach. For example, effective xanthine oxidase inhibitors can be designed by selecting specific head portions first. Once the head portion is selected, the inhibitory activity can be maximised by the selection of the appropriate tail portions. Based on this concept, alkyl gallates were found to be potent xanthine oxidase inhibitors (Masuoka et al., 2006). Hence, to examine cardol ($C_{15:3}$) (**2**) having a resorcinol as the head portion, 5-alkylresorcinols (**3, 4**), alkyl 3,5-dihydroxybenzoates (**7**–**9**), alkyl 2,4-dihydroxybenzoates (**10**–**12**) and 3,5-dihydroxyphenyl alkanoates (**13**–**15**) were selected and synthesized.

In order to examine the ability of the xanthine binding site in the enzyme, formation of uric acid was measured because xanthine oxidase, a molybdenum-containing enzyme, is known to convert xanthine to uric acid. This enzyme-catalysed reaction proceeds via transfer of an oxygen atom to xanthine from the molybdenum centre. Cardols (**2**–**4**) did not inhibit this oxygen-atom-transfer reaction up to 400 μM. Dodecyl 2, 4-dihydroxybenzoate (**12**) weakly inhibited uric acid formation catalysed by xanthine oxidase but other synthesized compounds showed no inhibition up to 200 μM (Table 1). To further examine the binding ability of xanthine oxidase, cardols (**2**–**4**) were preincubated with the enzyme for 3 min. The weak inhibition by cardol (**2**) was observed and non-competitive for xanthine (Fig. 2). These results indicated that most compounds which have resorcinol as the head portion did not bind to the xanthine binding site in the enzyme.

Compounds (**1**–**15**) have no DPPH scavenging activity, which is associated with a reduction of the enzyme molecules. It indicated that these compounds did not affect the generation of the superoxide anion by enzyme modulation (Masuoka et al., 2012). Superoxide anion scavenging rates of cardol related compounds (**2, 3**) were low compared to the inhibitory rates of the superoxide anion generated by xanthine oxidase (Table 2). This indicated that these cardols inhibited superoxide anion generation catalysed by xanthine oxidase. 3,5-Dihydroxyphenyl decanoate (**14**), 3,5-dihydroxyphenyl tridecanoate (**15**), nonyl 2,4-dihydroxybenzoate (**11**) and dodecyl 2,4-dihydroxybenzoate (**12**) also inhibited superoxide generation (Table 2). The inhibition type was sigmoidal and non-competitive for xanthine in all cases. As X-ray crystallographic analysis of bovine xanthine oxidase indicates that the substrate, NAD^+, of xanthine dehydrogenase is partially blocked to access of the FAD binding site (Enroth et al., 2000), it is deduced that these inhibitors are also blocked to bind the FAD site. For the first binding to the enzyme, a alkyl chain longer than C9 was necessary. When the inhibitors first bind to the sites in the xanthine oxidase subunits, the enzyme may cause conformational changes of the solvent channel in the enzyme (Kuwabara et al., 2003) to lower the flow rate and to enhance hydrogen peroxide formation. Cardols (**2, 3**), 3,5-dihydroxyphenyl decanoate (**14**), 3,5-dihydroxyphenyl

tridecanoate (**15**), nonyl 2,4-dihydroxybenzoate (**11**) and dodecyl 2,4-dihydroxybenzoate (**12**) cooperatively inhibited superoxide anion generation but nonyl 3,5-dihydroxybenzoate (**8**), and dodecyl 3,5-dihydroxybenzoate (**9**) did not. The former have electron donating groups, and the latter have electron withdrawing (-COOR). This suggested that electron donating groups in the connected moieties between the head and tail portions have the ability to bind the allosteric sites with a π–π interaction but the electron withdrawing one does not. Therefore, we deduced that specific inhibitors for superoxide anion generation catalysed by xanthine oxidase consisted of the structures having electron-rich resorcinol group and an alkyl chain having longer than C9 chain.

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