Inhibition of Human 5-Lipoxygenase by 3-Nitro-2,4,6-trihydroxybenzamide Derivatives

Ichiro HONDA, Masato NOGUCHI, Masahiro FURUNO, Takashi MATSUMOTO,
Makoto SHIBAGAKI, Masana NOMA, Koichi YONEYAMA,*
Tetsuyoshi HAYASHI,** Hajime IWAMURA,**
Nobutaka TAKAHASHI*** and Shigeo YOSHIDA***

Life Science Research Laboratory, Japan Tobacco Inc., 6-2 Umegaoka, Midori-ku, Yokohama 227, Japan

**Weed Control Research Institute, Faculty of Agriculture, Utsunomiya University,
350 Mine-machi, Utsunomiya 321, Japan*

***Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto University,
Kitashirakawa-Oiwake-cho, Sakyo-ku, Kyoto 606, Japan*

****The Institute of Physical and Chemical Research (RIKEN) 2-1 Hirosawa,
Wako 351-01, Japan*

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The inhibitory effects of 3-nitro-2,4,6-trihydroxybenzamide derivatives on human 5-lipoxygenase (5-LO), a key enzyme in arachidonic acid cascades, were examined using 5-LO produced by *Escherichia coli*. Some of the tested compounds inhibited the conversion of arachidonic acid to 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE), and in particular the *N*-phenylbutyl derivative was about 30 times more active ($IC_{50} = 35 \mu M$) than caffeic acid ($IC_{50} = 1000 \mu M$), a known selective 5-LO inhibitor.

Isolation and characterization of biologically active substances from natural resources have been contributing to both the progress of the biosciences and the use of such substances in our daily lives. In particular, a large number of medicines in use are natural products or have been designed based on the structures of naturally occurring biologically active compounds. Natural products with biological activities have also been the target of synthetic studies, since they often have diverse and/or unique structures.

Among these natural products, compounds

containing phloroglucinol nuclei in their structures have constituted a unique class of biologically active substances. For example, grandinol (I)¹⁾ and homograndinol (II)²⁾ isolated from *Eucalyptus grandis* are strong inhibitors of photosynthesis, and chemical modification of these compounds afforded highly active photoelectron transport (PET) inhibitors such as 3-nitro-2,4,6-trihydroxybenzamide derivatives (III).^{3,4)} These compounds also show germination inhibitory¹⁾ and anti-tumor-promotion activities.⁵⁾ On the other hand, euglobals⁶⁾ and robustadiols,⁷⁾ which

also contain phloroglucinol nuclei, show anti-inflammatory and anti-malarial activities, respectively. These results indicate that compounds **III** are also expected to have anti-inflammatory and anti-malarial activities.

In 1971, aspirin and indomethacine, famous anti-inflammatory drugs, were found to inhibit cyclooxygenase,⁸⁾ which catalyzes the first step of the biosyntheses of prostaglandins (PG). Following this finding, relationships between inflammation reactions and arachidonic acid metabolites such as PGs, thromboxanes (TX), and leukotrienes (LT) were investigated.^{9,10)} LTs have been reported to be slow reacting substances of anaphylaxis (SRS-A)^{11–13)} and chemical mediators of immediate hypersensitivity reactions and inflammation.^{9–13)} As LT biosyntheses are initiated by 5-lipoxygenase (5-LO), inhibition of this enzyme is an effective target for new drugs. Caffeic acid¹⁴⁾ and its derivatives,^{15,16)} flavonoids,¹⁷⁾ BW-755C,¹⁸⁾ KF-8940,¹⁹⁾ AA-861²⁰⁾ *etc.* have reported to inhibit 5-LO.

We have previously reported the expression of human 5-LO cDNA in *Escherichia coli*²¹⁾ and established a bioassay method for 5-LO inhibitors. In this paper, inhibitory activities of compounds **III** on the 5-LO catalyzing reaction were examined using this bioassay method.

Materials and Methods

Chemicals. Syntheses and analytical data of most of the compounds listed in Table I were described in our previous papers.^{3,4)} Compounds **16**, **17**, **18**, and **19** were prepared in a similar manner. Satisfactory analytical data were obtained for all the compounds.

Enzyme. To prepare the human 5-LO enzyme used in this study,²¹⁾ *E. coli* MV1184 strain, containing a pH5LOKC plasmid, was cultured at 20°C for 10 h. After harvesting the cells and subsequent buffer suspension, lysozyme treatment, sonication, and centrifugation, the supernatant was obtained as a crude 5-LO fraction. The enzyme used in the bioassay was the precipitated fraction of this crude enzyme between 30%–60% saturated ammonium sulfate solution, dialyzed in TEG buffer (50 mM Tris-HCl/2 mM EDTA/2 mM DTT/20% glycerol; pH 8.0). The protein concentration of this enzyme was 20 mg

protein/ml which was measured by the method of Bradford²²⁾ using bovine plasma γ -globulin as a standard. Activity of this enzyme was 1.8 nmol products/mg protein/10 min under our assay conditions.

Inhibition assay. The standard mixture for the 5-LO inhibition assay (total vol, 100 μ l) contained 0.1 M Tris-HCl buffer (pH=8.0), 2 mM CaCl₂, 2 mM ATP, 1 mM glutathione (reduced form), 10 μ l of the enzyme, and 10 μ l of DMSO solution containing a test compound (DMSO solution). After preincubation (30°C, 2 min), the reaction (30°C, 10 min) was started by adding 2 μ l of arachidonic acid (50 mM in ethanol). The reaction was stopped by the addition of 0.3 ml of cold methanol (–20°C) containing 0.2 nmol of 13-hydroxylinoleic acid as an internal standard, and subsequently 1 μ l of 1 N acetic acid was added. Following centrifuging, the supernatant was analyzed by HPLC on a Capcellpak C₁₈ column (4.6 ϕ $\times$ 150 mm, Shiseido) using methanol–water–acetic acid (75:25:0.01, v/v) as solvent, with detection of the absorbance at 233 nm. 5-LO inhibitory activity was measured from the peak area ratio of 5-HPETE (5-hydroperoxy-6,8,11,14-eicosate-trienoic acid) and 5-HETE (5-hydroxy-6,8,11,14-eicosate-trienoic acid) compared to the internal standard. All assays were done in triplicate and the inhibitory activity of each compound was expressed as the IC₅₀ value; that is the concentration (M) of a compound for 50% inhibition of 5-LO activity. Caffeic acid, one of the typical 5-LO inhibitors, was used as a positive control.

Results and Discussion

In this study, we used human 5-LO produced by *E. coli* as a target enzyme for the 5-LO inhibition assay. The properties of the enzyme were reported to be consistent with those of the natural one.²¹⁾ The inhibitory activities of tested compounds were estimated by the decrease of total amount of 5-HPETE and 5-HETE converted from arachidonic acid. The IC₅₀ of caffeic acid, a typical 5-LO inhibitor, was 1000 μ M in these assay conditions.

5-LO inhibitory activities of compounds **III** are shown in Table I. The compounds are classified into the following 3 categories by the structure of the substituent on the amide nitrogen atom; *N*-alkyl derivatives (**1**)–(**10**), *N*-phenyl- or *N*-phenoxyalkyl derivatives (**11**)–(**19**), and the *N*-substituted phenyl derivatives (**20**)–(**31**).

In the *N*-alkyl derivatives (**1**)–(**10**), the inhibitory activity on 5-LO increased gradually

Table I. HUMAN 5-LIPOXYGENASE INHIBITION OF COMPOUNDS **III**

No.	R	mp ^a	IC ₅₀ (μM)
1	Methyl	163	5900
2	Ethyl	149–150	3000
3	Butyl	98–100	2600
4	Hexyl	73–74	400
5	Heptyl	93–95	180
6	Octyl	84–86	160
7	Undecyl	99–100	200
8	Tridecyl	97–98	430
9	Pentadecyl	102–103	720
10	Octadecyl	105–106	1600
11	Phenyl	182–183	1000
12	Benzyl	151–152 d	1800
13	2-Phenethyl	142–143 d	460
14	3-Phenylpropyl	102–103	60
15	4-Phenylbutyl	101–102	35
16	5-Phenylpentyl	71–73	40
17	4-Phenoxybutyl	108–109	270
18	6-Phenoxyhexyl	101–102	160
19	8-Phenoxyoctyl	89–90	50
20	2-Cl-Phenyl	194–196	1400
21	3-Cl-Phenyl	199–201 d	450
22	4-Cl-Phenyl	186–188 d	300
23	2-F-Phenyl	164–166 d	4500
24	3-F-Phenyl	203–205 d	1200
25	4-F-Phenyl	199–202 d	510
26	2-CF ₃ -Phenyl	148–150 d	200
27	3-CF ₃ -Phenyl	172–175 d	370
28	4-CF ₃ -Phenyl	229–231 d	110
29	2,3-Cl ₂ -Phenyl	190–193 d	150
30	3,4-Cl ₂ -Phenyl	181–183 d	130
31	3,5-Cl ₂ -Phenyl	176–179 d	190
32	<i>N</i> -Methyl-Butyl	147–149	7100
33	<i>N</i> -Ethyl-Butyl	176–178	1900
34	<i>N</i> -Me-Phenyl	204–205 d	9200
Cont Caffeic acid			1000

^a d shows decomposition temperature.

with the lengthening of the alkyl chain. The maximum activity was associated with C₇–C₁₁ alkyl groups (5 times more active than caffeic acid), and further lengthening of the alkyl group decreased the activity. Increases of activity with the alkyl chain lengthening were

Table II. 5-LO INHIBITORY ACTIVITY OF COMPOUNDS RELATED TO **III**

No.	X	Y	IC ₅₀ (μM)
35	H	H (Phloroglucinol)	<10,000
36	NO ₂	H	4,300
37	H	CO ₂ H	4,300
38	NO ₂	CO ₂ H	2,500
39	H	CONHC ₆ H ₁₃	700
40	H	CONHC ₁₀ H ₂₁	200

also observed with both in the *N*-phenylalkyl and *N*-phenoxyalkyl derivatives (**11**)–(**19**), but decreases as mentioned above were not observed within these derivatives probably due to the insufficiency of their alkyl chain lengths. It should be noted that the phenyl and phenoxy groups attached to the end of the *N*-alkyl chain seemed to enhance the 5-LO inhibitory activity. In particular, 4-phenylbutyl (**15**), 5-phenylpentyl (**16**), and 8-phenoxyoctyl (**19**) derivatives were 20–30 times more active than caffeic acid. In the *N*-substituted phenyl derivatives (**20**)–(**30**), the introduction of *para*-substituents enhanced the activity, and the compounds having a lipophilic *para*-substituent such as Cl (**22**) or CF₃ (**28**) showed strong activity. In addition, the dichloro (**29**)–(**31**) and 2-CF₃ (**26**) derivatives were 5–10 times more active than caffeic acid. These results indicate that a rather bulkier lipophilic substituent on the amide nitrogen atom of compounds **III** are requisite for high activity. This tendency has also been reported in caffeic acid derivatives.¹⁶⁾ The 4-phenylbutyl derivative (**15**), which was the most active among the tested compounds, was only a little less active than the caffeic acid derivatives^{15,16)} represented by TMK-777, but it was more active than the others such as KF-8940¹⁹⁾ and AA-861.²⁰⁾ Therefore, 3-nitro-2,4,6-trihydroxybenzamide derivatives can be listed as novel potent 5-LO inhibitors.

And as shown in Table II, the chemicals related to compounds **III** (synthetic starting materials and intermediates), phloroglucinol (**35**), nitrophloroglucinol (**36**), phloroglucine-carboxylic acid (**37**), and 3-nitrophloroglucine-carboxylic acid (**38**) were less active than compounds **III** that have proper bulky lipophilic substituents. These results indicate the importance of lipophilicity of the molecules for the 5-LO inhibition.

In the photosynthetic electron transport (PET) inhibition by compounds **III**, lipophilicity of the molecules played a major role as in the case of 5-LO inhibition. In contrast, a nitro function which seemed necessary for high PET inhibitory activity is not always required for 5-LO inhibition (**39** and **40**). Furthermore in PET inhibition, a free amide hydrogen atom appeared to be indispensable for high activity, while the hydrogen atom may not be needed for the 5-LO inhibition; *N*-methyl-*N*-alkyl derivatives (**32** and **34**) were slightly less active than the corresponding *N*-alkyl derivatives (**3** and **11**), but *N*-ethylation of *N*-butyl derivative did not affect the activity (**33** vs. **3**). In PET inhibition, these *N,N*-dialkyl derivatives (**32**, **33**, and **34**) were 10–100 times less active than the corresponding *N*-monoalkyl derivatives.^{4,23)} Consequently, the structural requirements of compounds **III** for the 5-LO inhibition seemed to be different from those for PET inhibitions.

In conclusion, we have discovered novel potent inhibitors of human 5-lipoxygenase. Among the tested compounds, *N*-octyl (**6**), *N*-(4-phenylbutyl) (**15**), and *N*-(8-phenoxyoctyl) (**19**) derivatives were the most active compounds.

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