Furan fatty acid as an anti-inflammatory component from the green-lipped mussel *Perna canaliculus*

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A lipid extract of *Perna canaliculus* (New Zealand green-lipped mussel) has reportedly displayed anti-inflammatory effects in animal models and in human controlled studies. However, the anti-inflammatory lipid components have not been investigated in detail due to the instability of the lipid extract, which has made the identification of the distinct active components a formidable task. Considering the instability of the active component, we carefully fractionated a lipid extract of *Perna canaliculus* (Lyprinol) and detected furan fatty acids (F-acids). These naturally but rarely detected fatty acids show potent radical-scavenging ability and are essential constituents of plants and algae. Based on these data, it has been proposed that F-acids could be potential antioxidants, which may contribute to the protective properties of fish and fish oil diets against chronic inflammatory diseases. However, to date, in vivo data to support the hypothesis have not been obtained, presumably due to the limited availability of F-acids. To confirm the in vivo anti-inflammatory effect of F-acids in comparison with that of eicosapentaenoic acid (EPA), we developed a semisynthetic preparation and examined its anti-inflammatory activity in a rat model of adjuvant-induced arthritis. Indeed, the F-acid ethyl ester exhibited more potent anti-inflammatory activity than that of the EPA ethyl ester. We report on the in vivo activity of F-acids, confirming that the lipid extract of the green-lipped mussel includes an unstable fatty acid that is more effective than EPA.

natural product | tetraalkyl-subsituted furan ring

The green-lipped mussel *Perna canaliculus* is native to the New Zealand coast and is a dietary staple of the indigenous Maori culture. It is distinguished from other bivalve species by the presence of a bright green stripe around the posterior ventral margin of the shell and its distinctive green lip, which is visible on the inside of the shell. The anti-inflammatory effects of the green-lipped mussel have been studied since the observation of the lower incidence of arthritis in coastal Maoris compared with its prevalence in European or inland Maori people. The development of a natural remedy for the management of arthritis that will not only replace nonsteroidal anti-inflammatories (NSAIDs) as the standard treatment for all types of arthritis but also treat the condition successfully, without any known side effects, is being eagerly pursued (1). Therefore, numerous studies have been published on the lipid composition of the extract (2–4), its complex mode of action (5–8), activity in animal models (9, 10), and efficacy in controlling osteoarthritis (11, 12) and moderate asthma in patients (13). However, the active component from the mussel is a lipid rich in polyunsaturated fatty acids (PUFAs) and is therefore prone to activation of degrading enzymes including phospholipases and lipoxygenases (14, 15) after cell damage, unless it is stored and transported properly. In the case of severe damage, this enzymatic reaction switches to a nonenzymatic one producing radicals (16). A freeze-dried powdered preparation of whole mussel (i.e., without shell) was first tested orally in rats, and showed either modest or no anti-inflammatory activity (carrageenan paw edema in rats), probably due to the remaining activity of degrading enzymes. The preparation process was subsequently improved, and a lipid-rich fraction (Lyprinol), a supercritical fluid [CO_2] extraction of the freeze-dried stabilized green-lipped mussel powder, was prepared. This extraction method avoids warming the biological material, and therefore prevents activation of enzymes. The lipid-rich extract was found to contain five main lipid classes including sterol esters, triglycerides, free fatty acids, sterols, and polar lipids. A detailed analysis of the fatty acid composition revealed that omega-3 PUFAs, such as eicosapentaenoic acid (EPA; 20:5) and docosahexanoic acid (22:6), were the most abundant PUFAs among the 90 fatty acid components in this extract (3, 4). Although omega-3 PUFAs are the likely source of the anti-inflammatory activity of the green-lipped mussel, via the inhibition of both the 5-lipoxygenase and cyclooxygenase arachidonate oxygenation pathways, Whitehouse and coworkers reported that the lipid-rich oil of the green-lipped mussel showed potent anti-inflammatory activity in rat paw edema assays at a concentration two orders of magnitude lower than that of fish oil containing abundant EPA (9). More recently, Howarth and coworkers also reported that the beneficial effect is unlikely to be due to omega-3 fatty acid content (10). These data indicated the presence of another active component, in addition to the omega-3 PUFAs commonly found in fish oils. Based on these reports, we hypothesized that other unstable fatty acids remained to be identified as the active components in the lipid extract.

Results and Discussion

We fractionated the lipid extract of the green-lipped mussel and used a rat model of adjuvant-induced arthritis to identify the active components. After separation into each lipid class by silica gel chromatography, anti-inflammatory activities were usually found in the cholesterol ester and free fatty acid fractions. Because the common components for these fractions are apparently fatty acids, we focused on the further analysis of the fatty acid components. This estimation agrees with Macrides and coworkers' report that the free fatty acid fraction, and to a lesser extent the triglyceride fraction, were the only lipid classes to exhibit strong inhibition of COX isoforms (8). However, a precise separation of the free fatty acid fraction is usually difficult for minor fatty acids in the presence of an abundance of PUFAs. Moreover, further fractionation based on the guidance of the

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PHARMACOLOGY

bioassay was a formidable task, due to the concomitant decomposition of the active compounds.

There is a large body of evidence demonstrating a role for reactive oxygen species and oxidant stress in the pathogenesis of chronic inflammation such as rheumatoid arthritis (17–19). The high susceptibility of the cellular membrane to oxidation triggers lipid peroxidation and concomitant radical chain reactions, resulting in severe progression of symptoms (20). To suppress lipid peroxidation, there are many lipophilic antioxidants, such as vitamins A and E, astaxanthin as a natural product, and probucol as an antioxidant drugs (21). On the other hand, if an antioxidative fatty acid can be embedded into the lipid component, then its antioxidative effect would be more promising. Spiteller hypothesized that furan fatty acids (F-acids), which were first isolated from the northern pike in 1974 (22), are such naturally occurring antioxidative fatty acids that ameliorate the oxidative risk of the cellular membrane (23, 24). Even though F-acid concentration is usually low, these fatty acids are widely distributed in plants and aquatic organisms (25–36). The unique structural feature of F-acids is the tetraalkyl-substituted furan ring, which is a rarely observed skeleton in natural products. This electron-rich furan ring is susceptible to oxidation, and the subsequent ring opening generates dioxoene, which is highly reactive and further converted to other compounds (23, 30). Actually, F-acids readily decompose during prolonged exposure to silica gel or in neat oil. Thus, natural products bearing the tetraalkyl-substituted furan ring are relatively unstable, which is a possible reason why the tetraalkyl-substituted furan ring has rarely been found in nature. Recently, a macrolactone bearing a tetraalkyl-substituted furan ring was identified as the aggregation pheromone of leaf beetle (37) and, to the best our knowledge, this is the only example of a tetraalkyl furan ring in a natural product except for F-acids. Moreover, the activity of the green-lipped mussel is known to disappear in aqueous solution. This instability in water reasonably agrees with the features of F-acids, because F-acids were also missing after homogenization in aqueous solution (38). Considering these findings, we suspected the involvement of F-acids in the anti-inflammatory activity of the green-lipped mussel. In 2002, Sinclair and coworkers reported a detailed GC-MS analysis of the fatty acid composition of the green-lipped mussel (3). Their research focused on defining the similarities and differences in the lipid compositions between the New Zealand green-lipped mussel and the Tasmanian blue mussel. However, no fatty acids unique to the New Zealand green-lipped mussel were found. Considering the instability of F-acids in aqueous solution and on silica gel, previous studies using preparative TLC to separate the lipid classes would have allowed the F-acids to decompose before GC-MS analysis, even though over 90 component fatty acids were detected. Therefore, we expected that the F-acids might still be detected.

However, the separation of F-acids from other fatty acids is usually difficult and requires special workup procedures. F-acids, especially those with two β-methyl substituents, are quite susceptible to the peroxy radicals produced during lipid peroxidation, and are no longer detectable after homogenization in aqueous media. Thus, F-acids were found only occasionally, when a tissue rich in F-acids was homogenized in organic solvents. Additionally, to circumvent the autoxidation of the tetraalkyl-substituted furan ring on the surface of silica gel (30, 39), careful manipulations are required, especially in the case of lower quantities from natural sources. Previous reports used the method of Folch et al. (40) or Bligh and Dyer (41) to extract total lipids, which were further separated by silica gel into cholesteryl ester, triglycerides, and phospholipid fractions (25–35). These esters were transesterified with a methanolic sodium methoxide solution to form fatty acid methyl esters and then were investigated by GC-MS. Furthermore, Glass and coworkers enriched the F-acid methyl esters and separated them from PUFAs by hydrogenation of the methyl ester fraction, leaving the F-acids unchanged (22, 26, 27). The obtained saturated fatty acid methyl esters were removed by precipitation as urea complexes (42). It should be noted that the hydrogenation method of Glass is important for separation by silica gel chromatography, because F-acids could be separated due to different retention times in respect to the saturated acids. According to previous reports, we attempted to develop an isolation procedure based on a modification of Glass et al.'s protocol (26). To detect F-acids selectively, we tried to minimize the period during which the oil extract was exposed to the silica gel as much as possible. First, Lyprinol was treated with Na/MeOH to yield fatty acid methyl esters. The remaining free fatty acids were completely methylated with diazomethane. The resultant mixture was separated by silica gel flash chromatography to afford the PUFA methyl ester fractions. Because it was anticipated that the F-acid methyl esters would be eluted along with the PUFA methyl esters from the silica gel, the unsaturated fatty acid methyl esters were selectively transformed to saturated fatty acid methyl esters by hydrogenolysis with H_2 and Pd-C, to detect the F-acid methyl esters selectively (26). Finally, the mixture was separated by silica gel flash chromatography again, to afford the fractions containing the F-acid methyl esters. A GC-MS analysis revealed that these fractions predominantly contained F_4 and F_6 (Fig. 1*A* and Fig. S1) as well as other minor F-acids identified

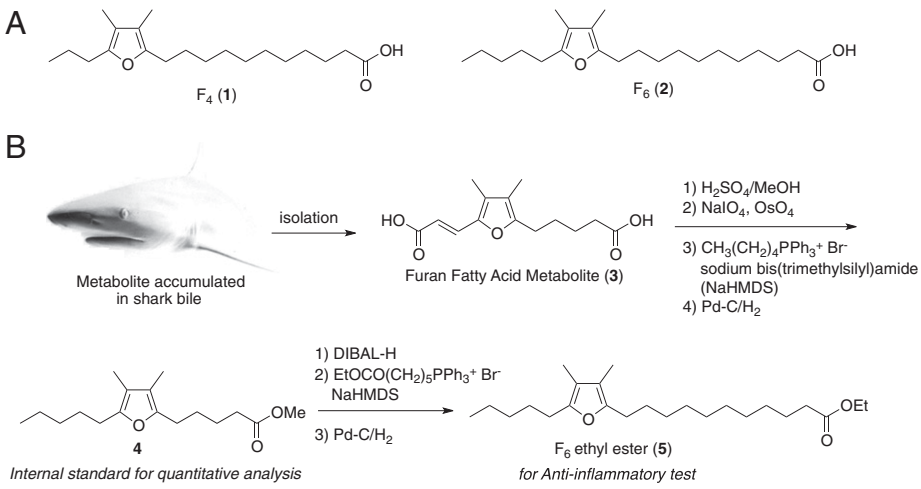

Fig. 1. (*A*) Structures of the representative furan fatty acids detected in the green-lipped mussel. (*B*) Partial synthesis of furan fatty acid F_6.

as F_2, F_3, and F_5. The predominant components, F_4 and F_6, were the most common F-acids in other organisms.

To examine the anti-inflammatory activity, a sufficient amount of pure material was required. Although several groups previously reported the synthesis of F-acid (43–49), the synthetic route required many steps. To prepare these fatty acids easily, a shark metabolite was selected as the starting material, because sharks are commercially available for consumption in the northeast area of Japan. We found that furan dicarboxylic acid **1**, a structural metabolite of F-acids (50), was highly accumulated in shark bile (*ca.* 10% dry weight). This metabolite is relatively stable, due to the presence of an electron-withdrawing group conjugated to the tetraalkyl furan ring. Moreover, one half of the side chains have an olefinic bond, which is versatile for chemical modification. Taking advantage of the double bond, cleavage by Lemieux–Johnson oxidation generated the conjugated aldehyde, in which the alkyl side chain was selectively elongated by a Wittig reaction. After hydrogenolysis, the methyl ester terminal was transformed to the aldehyde by diisobutylaluminium hydride (DIBAL) reduction. A second Wittig reaction with the resulting aldehyde elongated the C_6 ester side chain, and subsequent hydrogenolysis yielded the F_6 ethyl ester **3** (Fig. 1*B* and Fig. S2). Concomitantly, because the intermediate **2** with a shorter carboxylic side chain had not been reported from a natural source, it was used as the internal standard of the quantitative analysis.

It was reported that F-acids were especially accumulated by salmonid fishes (22, 26). Liver and testes lipids contain high concentrations of F-acids, which vary seasonally, depending on spawning (27, 32–35). We isolated authentic F-acid methyl esters from the testes of salmon captured at Hokkaido, Japan, in September 2007. Lyophilized testes were extracted with methanol and then fractionated by the same procedure as the isolation scheme that yielded the fraction containing both the F_4 and F_6 methyl esters. We confirmed that the ionization ratios of these two major F-acid methyl esters were identical, and therefore the peak areas for these two methyl esters were expected to correspond to each quantity. With authentic material in hand, a quantitative analysis of the F-acid in Lyprinol was implemented by GC-MS. After the addition of an internal standard to Lyprinol, according to a similar procedure as the isolation scheme, the lipid extract was methylated and separated to yield the fatty acid methyl ester fraction before the GC-MS analysis. Consequently, the mean values for the F_4 and F_6 methyl esters were 1.87 mg/g and 2.17 mg/g, respectively (Table S1).

Besides the in vitro antioxidative activity of F-acids, other biological activities, particularly in vivo, and the role in the host organism remained to be investigated. Watanabe, Okada, and coworkers reported that F-acids exhibited potent radical-scavenging activity due to the electron-rich furan ring (51). The potency of F-acids as scavengers of, for example, hydroxyl radicals is further exemplified by the findings that they suppress hemolysis better than other singlet oxygen quenchers such as dimethylfuran, α-tocopherol, ascorbic acid, and uric acid (52). One tetraalkyl furan ring can trap two radicals efficiently, which is a very effective feature, as pointed out by Spiteller (23). Lipid peroxidation, which is the initial event of inflammation or oxidative stress, would be significantly inhibited by these natural fatty acids. Considering these in vitro activities, F-acids may show potent anti-inflammatory activity in vivo. However, the in vivo activity of F-acids has not been previously reported, probably due to the difficulty in obtaining the materials either in nature or by chemical synthesis. Taking advantage of the semisynthetic route from the furan dicarboxylic acid, the shark metabolite of F-acid, we tried to examine the anti-inflammatory activity of the F_6 ethyl ester in comparison with that of the EPA ethyl ester.

The anti-inflammatory effect of Lyprinol was previously examined in various assay systems, including a rat model of adjuvant-induced arthritis (9) and a mouse model of bowel disease (10) in vivo and leukotriene biosynthesis by human polymorphonuclear leukocytes (7) and cyclooxygenase inhibition (8) in vitro. Among them, we used the rat model with adjuvant-induced arthritis, because the potent anti-inflammatory activity of Lyprinol had previously been assessed in this model after oral administration. Whitehouse and coworkers reported that rear paw swelling in the rat model was almost completely inhibited at a dose of 20 mg/kg when administered orally for 16 d (9). According to their procedure, female Wistar rats were sensitized by injecting *Mycobacterium butyricum* with squalene into the right hind limb. At day 10 after arthritis induction, test materials were administered orally for 5 d. The suppressive effect was determined based on the increase in the volume of the rear left paw between the beginning and the end of dosing. In our assay, Lyprinol significantly suppressed the rear paw swelling at a dose

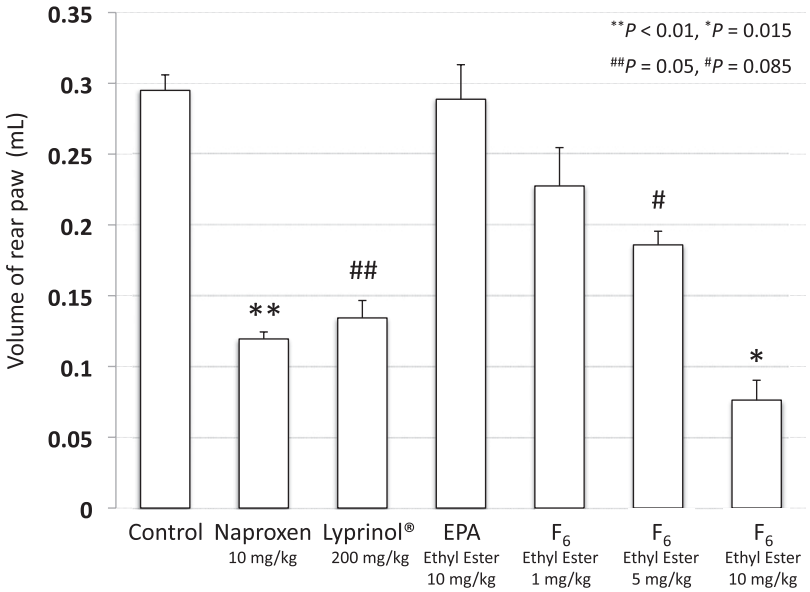

Fig. 2. Inhibition of adjuvant-induced arthritis in Wistar rats. The ordinate is the increase in the volume of the rear left paw between the beginning (day 10) and the end of dosing (day 15). Results are means ± SEM (n = 7–18).

of 200 mg/kg. Similar effects were detected by the oral administration of 5 mg/kg of a commercially available NSAID, naproxen, which were comparable to those of F_6 ethyl ester (between 5 and 10 mg/kg). These data revealed that F-acids were apparently one of the anti-inflammatory components of Lyprinol. Next, to examine the activity differences between PUFAs and F-acids, the assay was performed with both EPA and F_6 ethyl esters. The administration of the EPA ethyl ester did not induce significant inhibition of the rear paw swelling, even at a dose of 10 mg/kg. Furthermore, the whole-body symptoms, such as red sniff and front paw, were not cured (Fig. S3). In contrast, the suppression of paw swelling by the administration of F_6 ethyl ester was observed dose-dependently. The administration of 10 mg/kg of F_6 ethyl ester showed 74% suppression of paw swelling, which was a more potent effect than that of EPA at the same dose (Fig. 2).

We have thus revealed the potent anti-inflammatory activity of F-acids in an animal model and the presence of F-acids in the lipid extract of the New Zealand green-lipped mussel. Because fish and filter feeders, such as shellfish, would lack any enzymes to generate F-acids, the contents of F-acids are probably dependent on either the consumption of the real producer, such as algae (53), or the production by intestinal bacteria (54, 55). The New Zealand green-lipped mussel is specially regarded among the other bivalves by the New Zealand coastline, suggesting that the habitat of the mussel might be crucial for the higher concentration of F-acids exerting anti-inflammatory activity. Boselli and coworkers examined the contents of F-acids in several Adriatic fishes (56), and reported that the F_6 and EPA contents were positively correlated. Furthermore, the European pilchard had the highest F-acid content (30 mg/100 g fillet) among the five species studied in their report. In general, many aquatic organisms in our diets are expected to contain F-acids to some extent, but their efficient ingestion is probably limited, due to their susceptibility to processing. The New Zealand green-lipped mussel might be a preferred food for the efficient ingestion of F-acids, by consuming raw mussels or a stabilized oil extract.

In summary, this report demonstrates that F-acids are a minor component of the fatty acids in the lipid extract of the New Zealand green-lipped mussel and exhibit more potent anti-inflammatory activity than that of EPA. Although F-acids have been overshadowed by the more abundant omega-3 PUFAs in marine oils, these studies shed light on F-acids as potential anti-inflammatory agents and pave the way for more detailed examinations of the anti-inflammatory efficacy of the New Zealand green-lipped mussel as well as other food ingredients containing F-acids.

Materials and Methods

Isolation of Furan Fatty Acids from Lyprinol. The lipid extract of the green-lipped mussel (Lyprinol; 2.98 g) was dissolved in CH_2Cl_2 (80 mL). The resulting pale yellow solution was hydrogenated with Pd-C (80 mg) under an H_2 atmosphere (25). After 3 h of stirring, the catalyst was removed by filtration. To the colorless solution was added freshly prepared diazomethane in diethyl ether until the yellow color was retained. The methylated solution was concentrated in vacuo, and the residue was purified by silica gel flash chromatography to afford the fraction containing F_4 methyl ester (0.8 mg/g; methyl-12,15-epoxy-13,14-dimethyloctadeca-12,14-dienoate) and F_6 methyl ester (1.2 mg/g; methyl-12,15-epoxy-13,14-dimethyleicosa-12,14-dienoate). Alternatively, isolation from total fatty acids in Lyprinol was performed after methanolysis with sodium methoxide as described in the following methods.

Isolation of Furan Fatty Acids from Salmon Testicle. The salmon were captured in the Yambetsu River in Hokkaido, Japan, in late September 2007. Freeze-dried testicle (5 g) was dissolved in $CHCl_3$ (10 mL) and MeOH (10 mL). To the solution was added freshly prepared sodium methoxide (1 M, 10 mL). After stirring for 20 min, the mixture was treated with 1 M HCl (100 mL) and extracted with $CHCl_3$. The organic layer was washed with brine and dried over Na_2SO_4. The resulting solution was further methylated with a diethyl ether solution of CH_2N_2, until all free fatty acids were completely methylated. The resulting solution was concentrated in vacuo and purified by silica gel flash chromatography (3.0 × 10 cm), eluted with hexane, hexane:diethyl ether (99:1), hexane:diethyl ether (98:2), and hexane:diethyl ether (97:3). This solvent system separates the saturated and unsaturated fatty acid methyl esters. To remove the concomitant unsaturated fatty acid methyl esters, the fraction was hydrogenated with Pd-C (50 mg) under an H_2 atmosphere for 5 min, and then purified again by silica gel flash chromatography to yield the fractions containing F_4 and F_6 methyl esters (12 mg).

Quantitative Analysis of Furan Fatty Acids. Lipid extract of the New Zealand green-lipped mussel (Lyprinol; 2.0 g) was dissolved in MeOH:$CHCl_3$ (1:1, 20 mL). The mixture was treated with freshly prepared 1 M sodium methoxide (10 mL), and the resulting solution was partitioned between 1 M HCl and $CHCl_3$. The organic layer was washed with saturated aqueous NaCl, dried over Na_2SO_4, and filtered to yield a pale yellow solution. Subsequently, the remaining free fatty acids were completely methylated by a treatment with an ethereal solution of CH_2N_2. After concentration in vacuo, the resulting brown oil was purified by silica gel flash chromatography. The furan fatty acid methyl esters were eluted with the unsaturated fatty acid methyl ester fractions, which were combined and subjected to GC-MS analysis. GC-MS analysis was performed with a JEOL JMS-SUN200 mass spectrometer coupled to an Agilent 6890N gas chromatograph. The gas chromatograph oven temperature was increased from 120 °C to 240 °C at 24 °C/min. The peak areas of ion peaks derived from the total-ion chromatogram were subjected to quantitative analysis.

Assay for Arthritis Inhibition. Arthritis was induced in rats by the injection of heat-killed *M. butyricum* (Difco) suspended in squalene (1.1 mg per rat) into the right hind paw of female Wistar rats (57). The swelling of the paw (volume) was measured daily during the experiment using a plethysmometer (MK-101P; Muromachi Kikai). The injection was given on day 1 and resulted in the onset of chronic inflammation on day 9. The therapeutic regime started on day 10 and was continued until day 14. The inhibitory activity against arthritic inflammation was measured based on the inhibition of swelling of the untreated left hind paws during days 10–15 after administration of the adjuvant.

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