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Structure−Activity Relationships of Pentacyclic Triterpenoids as Inhibitors of Cyclooxygenase and Lipoxygenase Enzymes

Nhu Ngoc Quynh Vo,[†] Yuhta Nomura,[†,‡] Toshiya Muranaka,[†] and Ery Odette Fukushima*[,†,§,⊥]

[†]Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan

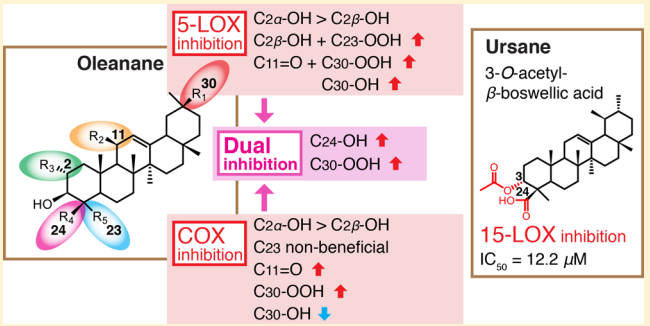
[‡]RIKEN Center for Sustainable Resource Science, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

[§]Center for Open Innovation Research and Education, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan

[⊥]Department of Biotechnology, Faculty of Life Sciences, Universidad Regional Amazónica IKIAM, Vía Muyuna Km 7, Tena, Ecuador

Supporting Information

ABSTRACT: Pentacyclic triterpenes may be active agents and provide a rich natural resource of promising compounds for drug development. The inhibitory activities of 29 natural oleanane and ursane pentacyclic triterpenes were evaluated against four major enzymes involved in the inflammatory process: 5-LOX, 15-LOX-2, COX-1, and COX-2. It was found that 3-*O*-acetyl-β-boswellic acid potently inhibited human 15-LOX-2 (IC_{50} = 12.2 ± 0.47 μM). Analysis of the structure−activity relationships revealed that the presence of a hydroxy group at position 24 was beneficial in terms of both 5-LOX and COX-1 inhibition. Notably, the introduction of a carboxylic acid group at position 30 was important for dual 5-LOX/COX inhibitory activity; furthermore, its combination with a carbonyl group at C-11 considerably increased 5-LOX inhibition. Also, the presence of an α-hydroxy group at C-2 or a carboxylic acid group at C-23 markedly suppressed the 5-LOX activity. The present findings reveal that the types and configurations of polar moieties at positions C-2, -3, -11, -24, and -30 are important structural aspects of pentacyclic triterpenes for their potential as anti-inflammatory lead compounds.



Inflammation is a complex biological response to tissue injuries in an organism caused by a variety of harmful stimuli, such as physical damage, microbial invasions, and irritants. The process often involves the release of biochemical mediators and leads to the development of diseases, including rheumatoid arthritis, osteoarthritis, multiple sclerosis, chronic asthma, and inflammatory bowel disease.[1−4]

The arachidonic acid (AA) pathway plays a pivotal role in the mechanism of inflammation. Upon inflammation-associated cell damage, AA is released from cellular membrane phospholipids via the action of phospholipase A_2 and is directly subjected to eicosanoid metabolism governed by three classes of enzymes. These include cyclooxygenases (COXs) such as COX-1 and COX-2, which produce prostanoids such as prostaglandins (PGs) and thromboxanes; lipoxygenases (LOXs) such as 5-LOX and 15-LOX, which produce leukotrienes (LTs) and hydroxy-eicosatetraenoic acids (HETEs); and cytochrome P450s (CYPs), which produce epoxyeicosatrienoic acids.[1−5] The products of the COX and LOX pathways are key bioactive lipid mediators involved in the induction of pathophysiological inflammatory conditions requiring pharmacological intervention.

COX-1 and COX-2 catalyze the conversion of AA to the hydroperoxy-endoperoxide prostaglandin G_2 by cyclooxygenase activity and subsequently to the hydroxy-endoperoxide prostaglandin H_2 by peroxidase activity, which is further metabolized to PGs, thromboxanes, and prostacyclins. COX-1 isozyme is constitutively expressed in most tissues and is involved in the production of physiological PGs that maintain homeostasis of the gastrointestinal tract and kidneys. On the other hand, the COX-2 isoform is an inducible enzyme that is nearly undetectable in most healthy tissues but highly upregulated in inflammation-related cell types in response to a variety of pro-inflammatory stimuli, such as cytokines, bacterial lipopolysaccharides, and/or growth factors.[1,6,8−10] Since COX-1 is essential for protection of the stomach lining, blocking its activity may cause gastrointestinal complications, including ulceration, bleeding, and renal insufficiency.

It has been documented that during COX-2 inhibition, AA metabolism can be shifted to other pathways, which may produce potent mediators of inflammation and/or trigger undesirable adverse effects.[7] Hence, the inhibition of multiple pathways has been suggested for the development of safer anti-inflammatory drugs. In fact, the therapeutic effects of COX

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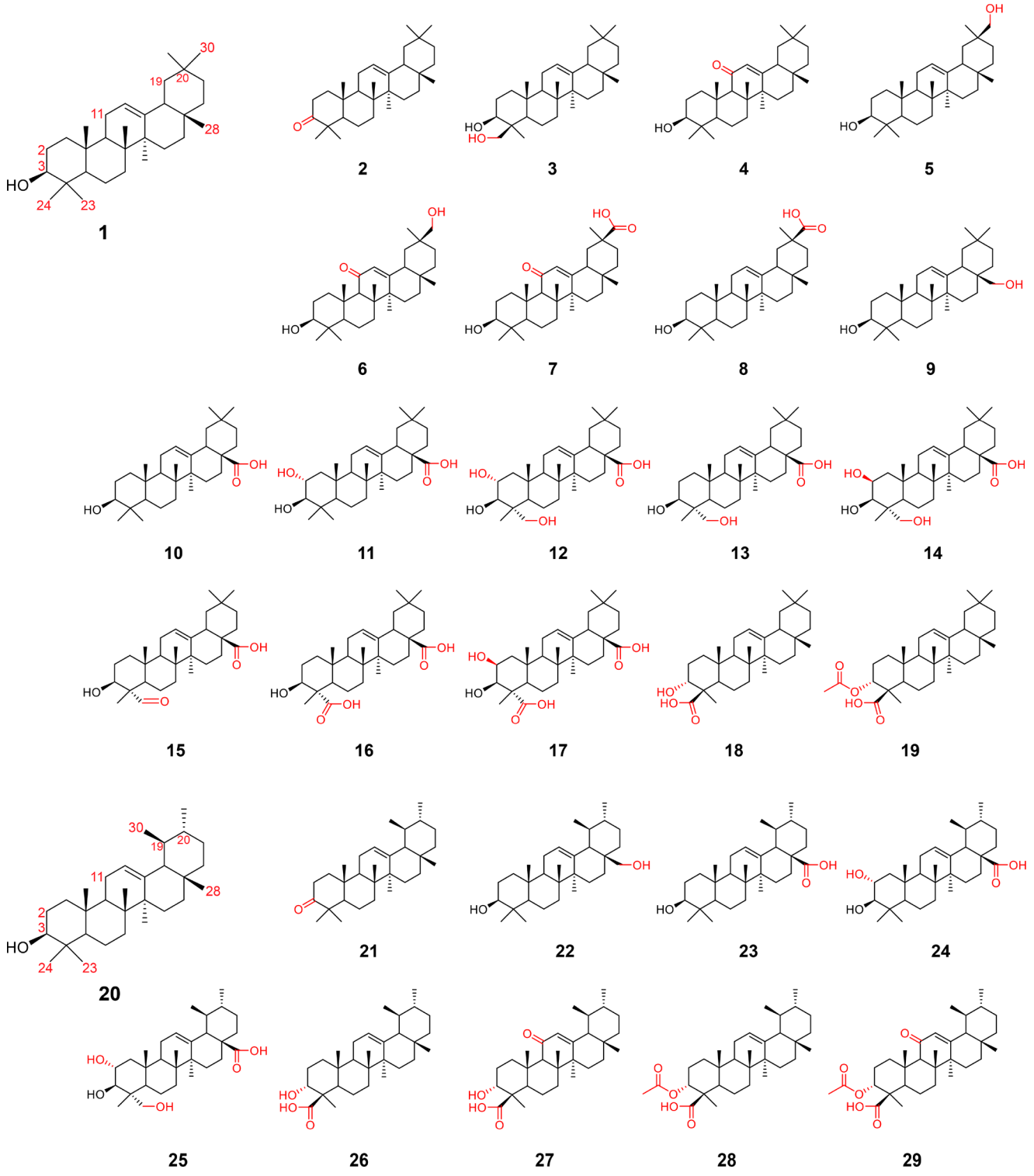

Figure 1. Chemical structures of the triterpenes evaluated of the oleanane (**1**–**19**) and ursane (**20**–**29**) types.

inhibitors may be limited by the production of LTs via the LOX pathway.[2,3]

LOXs are a family of non-heme iron-containing dioxygenases that catalyze the insertion of one molecule of O_2 in polyunsaturated fatty acids at different positions classified as 5- and 15-LOX to produce the 5- and 15-hydroperoxy-eicosatetraenoic acids, respectively, which are then reduced to the corresponding HETEs or transformed into LTs.[1] Of the subtypes of LOXs, 5-LOX is the key enzyme that catalyzes the biosynthesis of LTs and is assisted by factors such as Ca^{2+}, ATP, and 5-LOX activating protein (FLAP). In the majority of cells, the 5-LOX enzyme freely localizes in the cytosol and translocates to the nuclear membrane adjacent to its substrate and FLAP upon cellular activation.[11] Binding of intracellular Ca^{2+} promotes the association of the enzyme with phosphatidylcholine, which directs 5-LOX toward the membrane.[12,13] Following the membrane binding of 5-LOX

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Table 1. Inhibitory Effects of the Test Compounds with Oleanane (1−19) and Ursane (20−29) Skeletons and of the Standard Inhibitors (30 and 31) on the Activity of LOXs[a]

			human 5-LOX activity		human 15-LOX-2 activity	
compound	trivial name	skeleton	IC_{50} (μM)	remaining activity at 42 μM (%)	IC_{50} (μM)	remaining activity at 42 μM (%)
1	β-amyrin	oleanane	>40	73.3 ± 5.32	>40	97.2 ± 4.33
2	β-amyrone	oleanane	n.d.[d]	100	n.d.[d]	100
3	24-hydroxy-β-amyrin	oleanane	30.6 ± 0.91	29.8 ± 0.75	n.d.[d]	100
4	11-oxo-β-amyrin	oleanane	>40	70.4 ± 6.00	n.d.[d]	100
5	30-hydroxy-β-amyrin	oleanane	31.8 ± 0.26	34.8 ± 2.49	n.d.[d]	100
6	30-hydroxy-11-oxo-β-amyrin	oleanane	25.7 ± 1.46	25.2 ± 4.68	n.d.[d]	100
7	glycyrrhetinic acid	oleanane	24.8 ± 0.52	28.3 ± 2.06	>40	98.6 ± 6.81
8	11-deoxoglycyrrhetinic acid	oleanane	~42	49.0 ± 1.70	n.d.[d]	100
9	erythrodiol	oleanane	>40	60.7 ± 0.94	n.d.[d]	100
10	oleanolic acid	oleanane	n.d.[d]	100	n.d.[d]	100
11	maslinic acid	oleanane	>40	62.9 ± 16.35	n.d.[d]	100
12	arjunolic acid	oleanane	~42	47.7 ± 6.12	n.d.[d]	100
13	hederagenin	oleanane	>40	59.8 ± 4.88	>40	64.7 ± 1.24
14	bayogenin	oleanane	>40	71.9 ± 1.72	n.d.[d]	100
15	gypsogenin	oleanane	n.d.[d]	100	n.d.[d]	100
16	gypsogenic acid	oleanane	>40	86.0 ± 3.14	n.d.[d]	100
17	medicagenic acid	oleanane	30.4 ± 4.81	40.7 ± 7.57	n.d.[d]	100
18	α-boswellic acid	oleanane	~42	48.7 ± 8.16	n.d.[d]	100
19	3-*O*-acetyl-α-boswellic acid	oleanane	>40	93.1 ± 7.94	n.d.[d]	100
20	α-amyrin	ursane	>40	55.5 ± 3.45	n.d.[d]	100
21	α-amyrone	ursane	n.d.[d]	100	n.d.[d]	100
22	uvaol	ursane	~42	45.0 ± 1.73	>40	70.2 ± 4.01
23	ursolic acid	ursane	>40	64.0 ± 3.19	>40	63.6 ± 0.12
24	corosolic acid	ursane	>40	66.7 ± 1.75	n.d.[d]	100
25	asiatic acid	ursane	>40	61.9 ± 3.51	n.d.[d]	100
26	β-boswellic acid	ursane	>40	53.4 ± 0.63	n.d.[d]	100
27	11-keto-β-boswellic acid	ursane	28.3 ± 3.11	31.9 ± 8.74	n.d.[d]	100
28	3-*O*-acetyl-β-boswellic acid	ursane	>40	69.5 ± 0.56	12.2 ± 0.47	24.9 ± 0.93
29	3-*O*-acetyl-11-keto-β-boswellic acid	ursane	35.6 ± 3.59	41.2 ± 7.07	>40	81.7 ± 0.82
30[b]	zileuton		23.9 ± 2.07	23.3 ± 7.43		
31[c]	NDGA				13.6 ± 0.30	31.4 ± 3.10

[a]Data are expressed as means ± standard deviation (SD) for three independent experiments. [b]Zileuton, positive inhibitor against 5-LOX. [c]NDGA (nordihydroguaiaretic acid), positive inhibitor against 15-LOX-2. [d]n.d.: no inhibition detected at the tested concentrations.

enzyme, free AA is transferred to 5-LOX by FLAP for further metabolism.

Numerous studies[14−16] have shown that the 5-LOX pathway is associated with various human diseases, including cancer, cardiovascular disease, and inflammatory bowel disease. In addition, products of the 15-LOX isozyme cause inflammatory vascular diseases, atherosclerosis, epilepsy, and bronchial asthma.[1,17−19] However, 15-LOX has also been found to be anticarcinogenic.[20] 15-LOX is a pivotal enzyme in the biosynthesis of metabolically active lipoxins, which are generated during cellular interactions in response to inflammation. The production of lipoxins can counteract the inflammatory effects of LTs, whereas PGs and LTs exert complementary effects in the progression of inflammation.[1,4,10]

Based on the crucial roles of LOX pathways, the development of specific inhibitors of the 5- and 15-LOX enzymes represents a promising approach to the management of various diseases. Zileuton is the only specific 5-LOX inhibitor available on the pharmaceutical market for the treatment of asthma. Zileuton significantly and selectively blocks LT formation in nasal lavage fluids in patients challenged with allergens, but its use is restricted due to hepatic toxicity and its short half-life.[21−24] Thus, medications that inhibit both the COX-1/2 and 5-LOX pathways, as the main metabolic AA pathways, and thereby block the production of both LTs and prostanoids without affecting the lipoxin formation mediated by 15-LOX are believed to be superior to single-target drugs, since they can achieve a broad range of safer anti-inflammatory effects.

Plant-derived bioactive compounds are well-known for their wide spectrum of pharmacological activities in the prevention and treatment of human diseases. Natural compounds have great structural diversity, serving as a vital source of leads for new drug discovery. The pharmacological activities of a vast number of natural compounds have been evaluated, and potential multitarget compounds have been identified for their ability to inhibit the enzymes involved in AA cascades.[1,25,26]

In the search for potential dual COX/5-LOX inhibitors acquired from natural products, there has been an increasing interest in natural pentacyclic triterpenes, which are major constituents of many traditional herbal medicines. Previous reports have demonstrated that natural triterpenoids are powerful active compounds owing to their wide range of pharmacological properties and biological mechanisms.[27−29] The anti-inflammatory actions of pentacyclic triterpene acids such as glycyrrhetinic, boswellic, ursolic, and oleanolic acids have been described extensively.[30−32] Despite various studies

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evaluating the anti-inflammatory activities of triterpenoids originating from natural products and chemical synthesis, only a few have focused on describing the structural requirements for those activities.

The aim of the present study was to determine the inhibitory effects of natural triterpenoids of various chemical structures against the major anti-inflammatory targets of the AA cascade, to analyze the structure−activity relationships (SARs) among the different types of triterpenes, and to elucidate important structural features for drug development. The in vitro inhibitory activities were investigated of 29 natural triterpenes with oleanane (**1**−**19**) and ursane (**20**−**29**) skeletons (Figure 1) against enzymes including 5-LOX, 15-LOX, COX-1, and COX-2 and were evaluated for their potential activities in subtype inhibition and dual inhibition. The SARs obtained for all compounds tested should assist in the discovery of more potent and highly selective inhibitors.

RESULTS AND DISCUSSION

Screening of LOX Inhibitors from Natural Triterpenoids and a SAR Study. Triterpenes, a widespread class of natural product compounds and consisting of six isoprene units, are an impressive reservoir of biologically active agents. In the present study, 29 natural triterpenoids were obtained, and their inhibitory effects against human 5-LOX and 15-LOX-2 (an epithelial 15-LOX isozyme that exclusively oxygenates AA at C-15) were assayed. The bioassay results are summarized in Table 1 and Figures 2 and 3. The standard inhibitors used for each assay included zileuton (**30**), a 5-LOX inhibitor,[2] and nordihydroguaiaretic acid (NDGA) (**31**), a 15-LOX-2 inhibitor.[33]

Based on the results of the 15-LOX-2 assays, the two parent triterpenoids, β-amyrin (**1**) and α-amyrin (**20**), and their corresponding analogues (**2**−**12**, **14**−**21**, **24**−**27**) failed to inhibit the activity of human subtype 15-LOX up to 42 μM. Among the compounds tested, only 3-*O*-acetyl-β-boswellic acid (**28**) showed a strong and preferential inhibitory effect against 15-LOX-2, with an IC_{50} of 12.2 μM, which was almost equipotent to that of NDGA (**31**) (13.6 μM), whereas the derivative 3-*O*-acetyl-11-keto-β-boswellic acid (**29**) showed inferior inhibition of this enzyme subtype (IC_{50} > 40 μM; Table 1). Compound **28** exhibited a trend of 15-LOX-2 inhibition similar to that of the positive control **31** (Figure 2).

These observations suggest that the introduction of a carboxylic acid group at the C-24 site combined with an acetoxy group at C-3 resulted in increased activity toward human 15-LOX-2. However, the presence of a carbonyl group at C-11 may reduce the activity, as observed in compound **29**. Also, hederagenin (**13**), uvaol (**22**), and ursolic acid (**23**) exhibited moderate inhibition, with IC_{50} > 40 μM (Table 1). The structural character of **23** is also beneficial for its bioactivity,[34] because oleanane-type oleanolic acid (**10**), which differs from **23** only in the position of one methyl group (located at C-20 instead of C-19), was not active at any concentration tested.

Regarding the human 5-LOX assay, it is noteworthy that 24-hydroxy-β-amyrin (**3**), bearing a hydroxy group at the C-24 position, exhibited potent and selective inhibition against human 5-LOX (IC_{50} = 30.6 μM) (Table 1 and Figure 3A). This result suggests that the C-24 position is favorable for increased inhibitory activity toward 5-LOX, compared with the parent compound **1**, which had a remaining activity of 73.3% at 42 μM. No LOX inhibitory activity was detected for β-amyrone (**2**) and α-amyrone (**21**), which carry a carbonyl moiety at C-3, at the highest concentration tested. Interestingly, 30-hydroxy-β-amyrin (**5**), 30-hydroxy-11-oxo-β-amyrin (**6**), and glycyrrhetinic acid (**7**) strongly inhibited 5-LOX by 65.2%, 74.8%, and 71.7%, respectively (Table 1). The IC_{50} values of these triterpenoids were 31.8, 25.7, and 24.8 μM, respectively, which are comparable with that of zileuton (**30**) (IC_{50} = 23.9 μM) (Table 1 and Figure 3B−E). The results indicate that the modifications at the C-11 and C-30 sites were essential and led to the increased selectivity toward human 5-LOX over 15-LOX-2.

Notably, the introduction of a carbonyl group at C-11 and a hydroxy or carboxylic acid group at C-30 led to an increase in activity, whereas the presence of a C-11 carbonyl group alone, as in 11-oxo-β-amyrin (**4**), or its absence, as in 11-deoxoglycyrrhetinic acid (**8**), dramatically reduced the activity (IC_{50} > 40 μM for **4** and ~42 μM for **8**). Compound **7** has been reported previously to possess potent anti-inflammatory activity comparable with that of hydrocortisone.[35,36] To the best of our knowledge, this is the first study to report the inhibitory effects of triterpenoids **3**, **5**, and **6** against human 5-LOX.

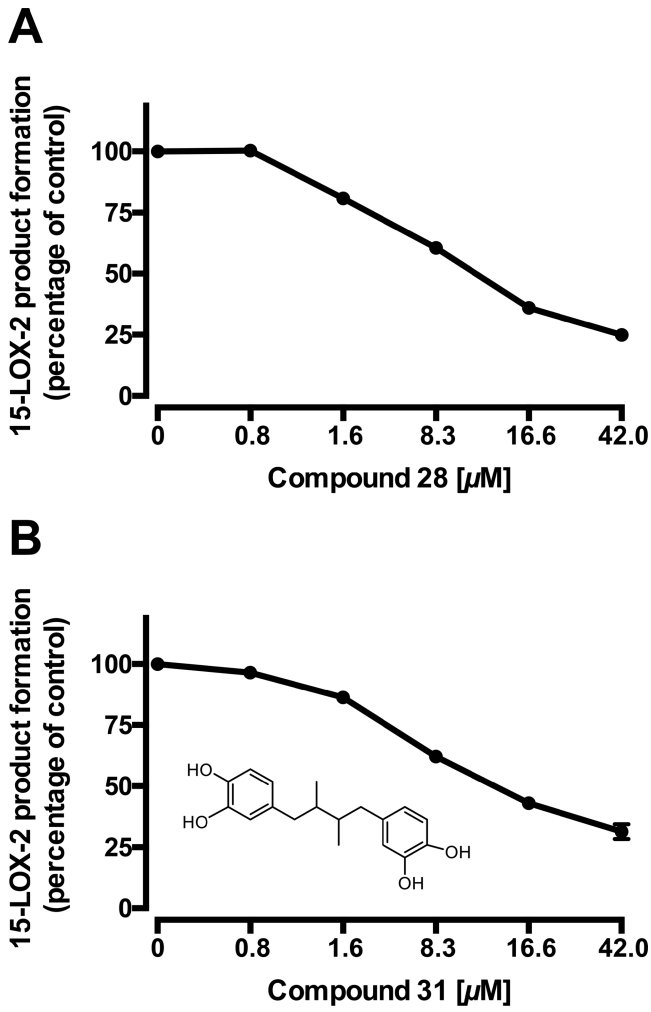

Figure 2. Concentration−response curves for the test compound and positive control: (A) 3-*O*-acetyl-β-boswellic acid (**28**) and (B) 15-LOX inhibitor NDGA (**31**) for inhibition of human 15-LOX-2 activity. Data are given as means ± SD of three independent experiments.

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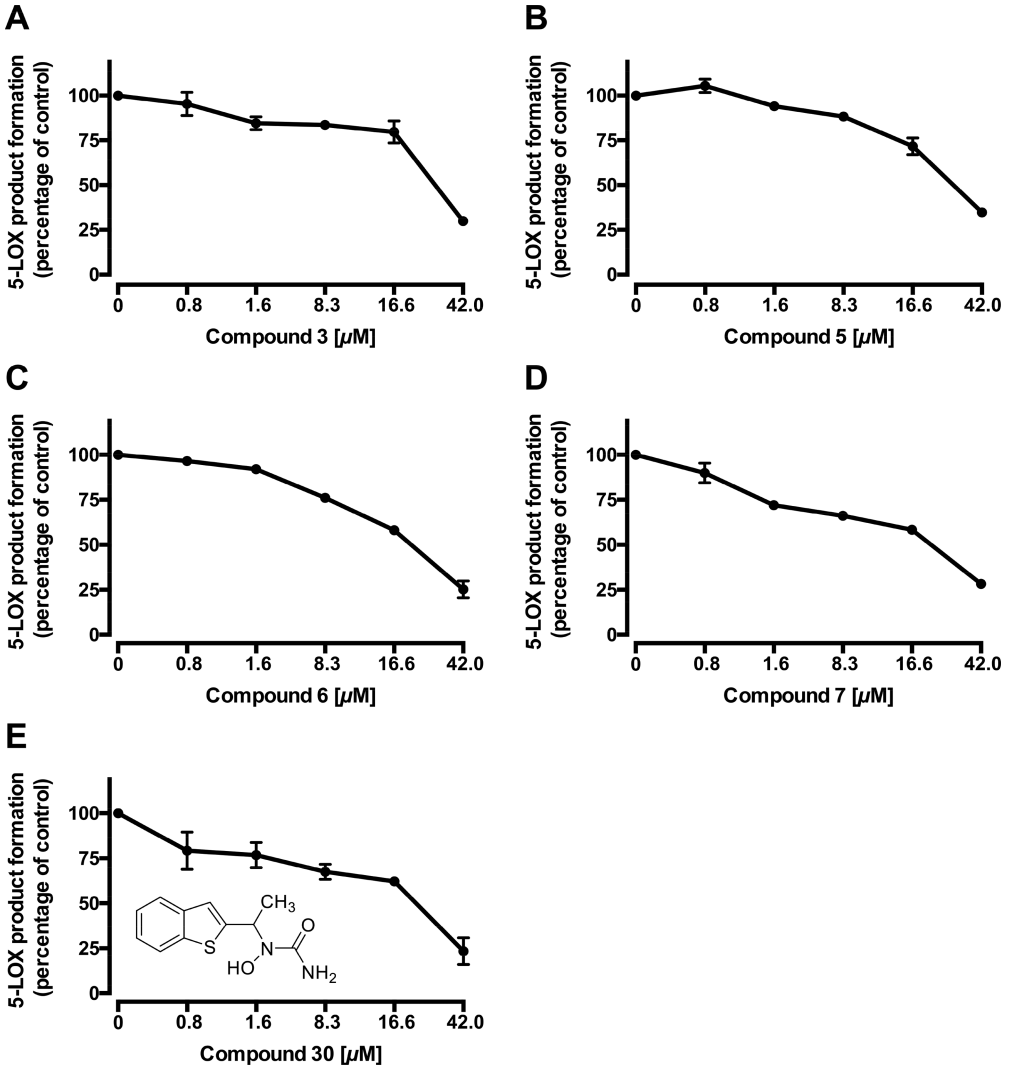

Figure 3. Concentration−response curves for test compounds and positive control: (A) 24-hydroxy-β-amyrin (**3**), (B) 30-hydroxy-β-amyrin (**5**), (C) 30-hydroxy-11-oxo-β-amyrin (**6**), (D) glycyrrhetinic acid (**7**), and (E) 5-LOX inhibitor zileuton (**30**) for inhibition of human 5-LOX activity. Data are given as means ± SD of three independent experiments.

Among the four β-configured boswellic acids, 11-keto-β-boswellic acid (**27**) and compound **29** were the most potent 5-LOX inhibitors, with IC_{50} values of 28.3 and 35.6 μM, respectively (Table 1; Figure S6B and C, Supporting Information). This result confirmed that modifications at C-24, C-11, and C-3 were advantageous for human 5-LOX inhibition. The removal of the carbonyl group at C-11, as in **28**, reduced the potency of **29**. These findings are consistent with those reported previously.[37−39] Compound **29** inhibited LTB_4 formation in a concentration-dependent manner in Ca^{2+}-ionophore-stimulated polymorphonuclear leukocytes and can therefore be proposed as a novel specific non-redox-type inhibitor of 5-LOX.[40] Furthermore, while the oleanane-type compound **10** had no effect on human 5-LOX, its analogues bearing a hydroxy group at C-2 in maslinic acid (**11**) or C-23 in **13** showed considerably improved inhibitory activity (IC_{50} > 40 μM for **11** and **13**; Table 1). This inhibitory activity was further increased in arjunolic acid (**12**) (IC_{50} ~ 42 μM), which had a combination of modifications at both C-2 and C-23.

Gypsogenin (**15**) and gypsogenic acid (**16**) both lack a hydroxy group at C-2 and were inactive. Notably, the presence of a β-hydroxy group at C-2 in bayogenin (**14**) led to weaker inhibitory activity (IC_{50} > 40 μM) than that for its 2α-isomer **12**, suggesting that the steric positioning of the 2-hydroxy group also contributes to activity. The configuration at C-2 was also associated with the hemolytic activity of triterpenes in that a β-hydroxy group significantly increased hemolysis, whereas an α-isomer reduces the activity.[41] However, medicagenic acid (**17**), in which the C-23 hydroxy group of compound **14** was converted to a C-23 carboxylic acid moiety, displayed enhanced inhibitory potency against 5-LOX (IC_{50} = 30.4 μM; Table 1; Figure S6A, Supporting Information). This suggested that the introduced carboxylic acid group facilitated enzyme inhibition.

In the ursane family, triterpenes **23**−**25** exhibited nearly equal inhibitory potencies against 5-LOX (36% inhibition by **23**, 33.3% by **24**, 38.1% by **25** at 42 μM; Table 1), and their IC_{50} values exceeded 40 μM, irrespective of the presence of modifications at C-2 or C-23.

Inhibition of COX by Natural Triterpenoids and a SAR Study. The 29 triterpenoids obtained were evaluated to assess their human COX-2 and ovine COX-1 inhibition. The bioassay results are shown in Table 2. The IC_{50} values were approximately 39 nM for SC-560 (**32**), as a selective COX-1

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Table 2. Inhibitory Effects of the Test Compounds with Oleanane (1−19) and Ursane (20−29) Skeletons and of the Standard Inhibitors (32 and 33) on the Activity of COXs[a]

			ovine COX-1 activity		human COX-2 activity	
compound	trivial name	skeleton	IC_{50} (μM)	remaining activity at 42 μM (%)	IC_{50} (μM)	remaining activity at 42 μM (%)
1	β-amyrin	oleanane	n.d.[d]	100	n.d.[d]	100
2	β-amyrone	oleanane	~42	46.7 ± 7.25	n.d.[d]	100
3	24-hydroxy-β-amyrin	oleanane	>40	60.2 ± 6.17	n.d.[d]	100
4	11-oxo-β-amyrin	oleanane	>40	55.1 ± 6.14	>40	69.2 ± 2.95
5	30-hydroxy-β-amyrin	oleanane	>40	93.8 ± 6.59	>40	73.6 ± 2.85
6	30-hydroxy-11-oxo-β-amyrin	oleanane	>40	86.2 ± 4.68	>40	86.8 ± 1.97
7	glycyrrhetinic acid	oleanane	>40	64.3 ± 7.46	>40	72.3 ± 7.03
8	11-deoxoglycyrrhetinic acid	oleanane	~42	45.0 ± 5.64	>40	75.2 ± 8.96
9	erythrodiol	oleanane	n.d.[d]	100	n.d.[d]	100
10	oleanolic acid	oleanane	~42	44.0 ± 2.22	>40	89.7 ± 3.61
11	maslinic acid	oleanane	~42	45.3 ± 5.10	>40	77.5 ± 3.61
12	arjunolic acid	oleanane	>40	72.3 ± 5.97	>40	72.8 ± 8.23
13	hederagenin	oleanane	n.d.[d]	100	n.d.[d]	100
14	bayogenin	oleanane	n.d.[d]	100	>40	77.8 ± 2.79
15	gypsogenin	oleanane	n.d.[d]	100	n.d.[d]	100
16	gypsogenic acid	oleanane	n.d.[d]	100	n.d.[d]	100
17	medicagenic acid	oleanane	n.d.[d]	100	>40	71.7 ± 0.85
18	α-boswellic acid	oleanane	>40	83.0 ± 3.95	>40	85.1 ± 4.93
19	3-*O*-acetyl-α-boswellic acid	oleanane	>40	92.8 ± 5.97	>40	77.8 ± 1.86
20	α-amyrin	ursane	n.d.[d]	100	n.d.[d]	100
21	α-amyrone	ursane	n.d.[d]	100	>40	94.0 ± 2.59
22	uvaol	ursane	n.d.[d]	100	n.d.[d]	100
23	ursolic acid	ursane	>40	71.1 ± 3.95	>40	80.1 ± 8.87
24	corosolic acid	ursane	>40	77.6 ± 9.96	>40	82.3 ± 2.01
25	asiatic acid	ursane	>40	90.7 ± 4.93	>40	76.0 ± 1.40
26	β-boswellic acid	ursane	>40	69.5 ± 5.77	>40	71.1 ± 4.97
27	11-keto-β-boswellic acid	ursane	>40	65.9 ± 3.16	>40	74.4 ± 2.87
28	3-*O*-acetyl-β-boswellic acid	ursane	>40	62.2 ± 7.59	>40	69.5 ± 2.41
29	3-*O*-acetyl-11-keto-β-boswellic acid	ursane	>40	51.7 ± 3.23	>40	61.0 ± 2.07
32[b]	SC-560		0.039 ± 0.01	1.3 ± 0.51		
33[c]	CAY10452				0.045 ± 0.006	3.3 ± 0.39

[a]Data are expressed as means ± standard deviation (SD) for three independent experiments. [b]SC-560, positive inhibitor against COX-1. [c]CAY10452, positive inhibitor against COX-2. [d]n.d.: no inhibition detected at the tested concentrations.

positive inhibitor, and 45 nM for CAY10452 (**33**), as a COX-2 standard inhibitor (Table 2). These results were consistent with those reported previously.[42,43] The triterpenes tested exhibited low or moderate activity compared to that of the standard inhibitors.

An absence of COX inhibitory activity was observed in the parent triterpenes **1** and **20**. However, replacement of a hydroxy group with a carbonyl at C-3 in **2** or introduction of a hydroxy group at C-24 in **3** resulted in increased selectivity toward COX-1 inhibition (IC_{50} ~ 42 μM for **2** and >40 μM for **3**). As revealed in Table 2, compound **7**, carrying a carboxylic acid group at C-30 and a carbonyl group at C-11, moderately affected COX activity (35.7% and 27.7% inhibition against COX-1 and COX-2 at 42 μM, respectively).

Note that the removal of the carboxylic acid group at C-30 in **4** or the carbonyl group at C-11 in **8** preferentially increased the inhibitory potency toward COX-1 (44.9% inhibition by **4** and 55% by **8**). However, as compared with **7** and **8**, compounds **5** and **6** displayed weaker effects on COX-1 (6.2% inhibition by **5** and 13.8% by **6**), thus indicating that replacement of the carboxylic acid moiety with a hydroxy group at C-30 did not aid COX inhibition. Moreover, the attachment of a carboxylic acid group to C-28 or an α-hydroxy group to C-2 led to enhanced inhibition, with higher selectivity toward COX-1 rather than COX-2, as observed in oleanane-type compounds **10** (56% and 10.3% inhibition against COX-1 and COX-2, respectively) or **11** (54.7% and 22.5% against COX-1 and COX-2, respectively). Nevertheless, modifications at the C-23 position (e.g., in **12**−**17**) considerably reduced COX inhibitory activity (Table 2).

The ursane-type triterpenes **23**−**25** weakly inhibited both COX enzymes. As shown in Table 2, all tested boswellic acids reduced COX activity; compound **29** exhibited the most potent activity toward COX enzymes, as reported in previous studies.[44−46] This suggested that the combination of an acetoxy group at C-3, a carbonyl group at C-11, and a carboxylic acid group at C-24 is important for the COX-inhibitory effect.

Computational Docking of Potent Inhibitors of 15-LOX-2, 5-LOX, COX-1, and COX-2. To characterize the inhibitory behavior of potent compounds against LOX and COX enzymes from the view of ligand−enzyme interactions, molecular docking was performed using the previously reported crystal structures of 5-LOX, 15-LOX-2, COX-1, and

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COX-2 enzymes as macromolecular models. The docked structures were analyzed, and enzyme−ligand interactions were observed.

As depicted in Figure 4, the AA substrate exhibited U-shaped binding in the cavity pocket of human 15-LOX-2,

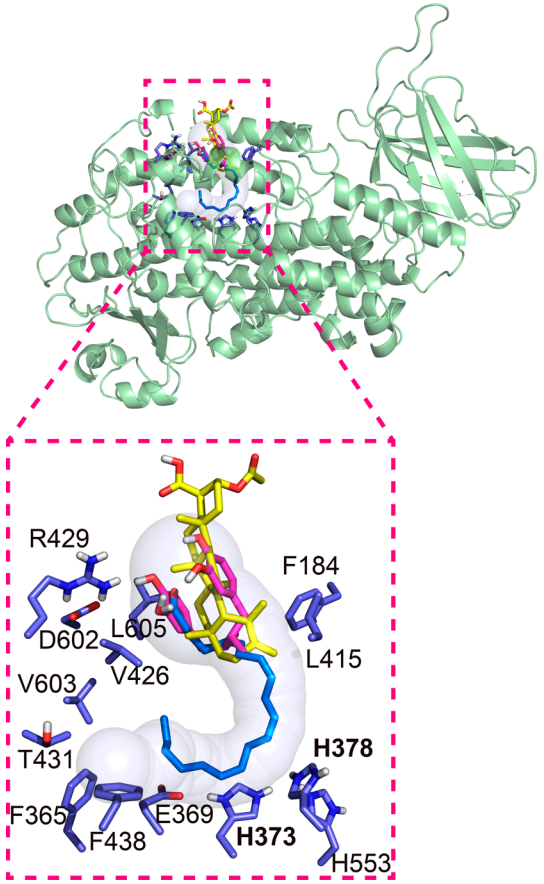

Figure 4. Molecular docking of 3-*O*-acetyl-*β*-boswellic acid (**28**), the standard inhibitor NDGA (**31**), and AA substrate into the crystal structure of human 15-LOX-2 (PDB ID: 4NRE). The upper panel shows the binding modes of compounds **28** (yellow) and **31** (magenta) and the substrate-binding cavity of 15-LOX-2 in the overview map. AA (blue stick rendering; red, oxygen) is positioned in a U-shaped conformation. The lower panel shows a close-up view of the amino acids that line the active site cavity. Shown is atomic coloring (purple, carbon; red, oxygen; blue, nitrogen), with invariant amino acids (H373 and H378) in bold font.

which is similar to the binding mode of AA to this enzyme reported previously.[47] Compound **28** was predicted to bind near the substrate-binding pocket defined by the side chains of the active-site amino acids F184, L415, H553, E369, F438, F365, T431, V603, V426, L605, D602, and R429, with the invariant amino acids being H378 and H373. There was a high binding energy between compound **28** and human 15-LOX-2 (−9.3 kcal mol^{-1}), which was comparable to that of the standard inhibitor **31** (−8.6 kcal mol^{-1}). A two-dimensional plot of compound **28** binding to 15-LOX-2 depicted hydrophobic interactions of atoms of **28** with contacting binding-site residues of the enzyme (Figure S7, Supporting Information).

In accordance with previous reports,[48,49] the human 5-LOX active site of the present 3D model comprises a bent-shaped cleft in which the substrate binding site is surrounded by active-site amino acids S171, F169, I406, A672, F177, H367, H372, L607, Q557, Q363, F359, and A603 (Figure 5A and 5B). The potent compounds **3** (binding energy of −8.5 kcal mol^{-1}), **5** (−8.3 kcal mol^{-1}), and **6** (−8.3 kcal mol^{-1}) bind to the same pocket as the standard inhibitor **30** (Figure 5C). The possible hydrogen bond of the hydroxy group at C-24 in **3** with T444 is shown. Also, compound **7** (binding energy of −8.5 kcal mol^{-1}) was predicted to position in a different cavity from the binding site of **30** and showed possible interactions with D559 (Figure 5D). The hydrophobic interactions of interacting residues and the atoms they contact were displayed in four two-dimensional plots of potent compounds **3**, **5**, **6**, and **7** binding to human 5-LOX (Figure S8, Supporting Information). Furthermore, the possible hydrogen bonds of compounds **5** and **6** with the residue D285 and of **7** with N187 and Y660 are shown (Figure S8, Supporting Information). The binding modes of compounds **17**, **27**, and **29** on the surface of 5-LOX are depicted (Figure S9, Supporting Information); the compounds interact with the enzyme at selective sites different from the substrate-binding site.

Additionally, the binding interactions between compound **29** and the catalytic amino acid residues of COX enzymes are represented (Figure S10, Supporting Information). The compound docked into the active site of COX-1. Possible interactions are formed by the acetoxy and the carbonyl group with E454 and H207, respectively, of COX-1 (Figure S10A, Supporting Information) and by the acetoxy group of **29** with Q203 of COX-2 (Figure S10B, Supporting Information), which might be favorable to enzyme binding of **29**. Also, the hydrophobic contacts of atoms of **29** with interacting residues of the COX enzymes are shown (Figure S11, Supporting Information). The results of our docking studies were consistent with those of the bioactivity assays (Figure S12, Supporting Information).

EXPERIMENTAL SECTION

General Experimental Procedures. In the 15-LOX-2 inhibition assay, visible-light spectra were measured using the Infinite 200 PRO multimode microplate reader (Tecan, Männedorf, Switzerland). For the 5-LOX inhibition assay, UV spectra were measured using the V-630 Bio UV−vis spectrophotometer (JASCO, MD, USA). In the inhibitory studies of COX-1 and COX-2, liquid chromatography−mass spectrometry (LC-MS) spectra were obtained on the Acquity UPLC/MS system (Waters, Milford, MA, USA) in the electrospray ionization negative ion mode with selected ion monitoring. Chromatographic separation was performed on a BEH C_{18} analytical column (2.1 × 150 mm, 1.7 μm; Waters) at a flow rate of 0.2 mL min^{-1}.

Materials for the Activity Assays. In total, 24 triterpenes (**1**, **2**, **7**, **9**−**29**) (Figure 1) were purchased as pure compounds (≥95%). Compounds **1**, **9**−**11**, **13**, **18**−**20**, **22**−**23**, **25**−**29** were from Extrasynthese (Genay, France); **2** and **21** were from BOC Sciences (Shirley, NY, USA); **7** was from Tokyo Chemical Industry (Tokyo, Japan); **12** was from Sigma-Aldrich (St. Louis, MO, USA); **15** was from Carbosynth (Compton, Berkshire, UK); **16** was from Santa Cruz Biotechnology (Santa Cruz, CA, USA); **14** and **17** were from Apin Chemicals Limited (Milton, Abingdon, UK); **24** was from Wako Pure Chemical Industries (Osaka, Japan). Five triterpenes (**3**−**6**, **8**) (Figure 1) were synthesized from either *β*-amyrin (**1**) or glycyrrhetinic acid (**7**), as described previously.[50,51] The standard inhibitors included zileuton (**30**), a 5-LOX inhibitor; NDGA (**31**), a 15-LOX-2 inhibitor; SC-560 (**32**), a COX-1 inhibitor; and CAY10452 (celecoxib analogue) (**33**), a COX-2 inhibitor. Human recombinant 5-LOX and 15-LOX-2, ovine recombinant COX-1, zileuton, NDGA, SC-560, CAY10452, AA, PGE_2, PGD_2, d_4-PGD_2, and d_4-PGE_2 (labeled with deuterium atoms at positions 3, 3′, 4, and 4′) were

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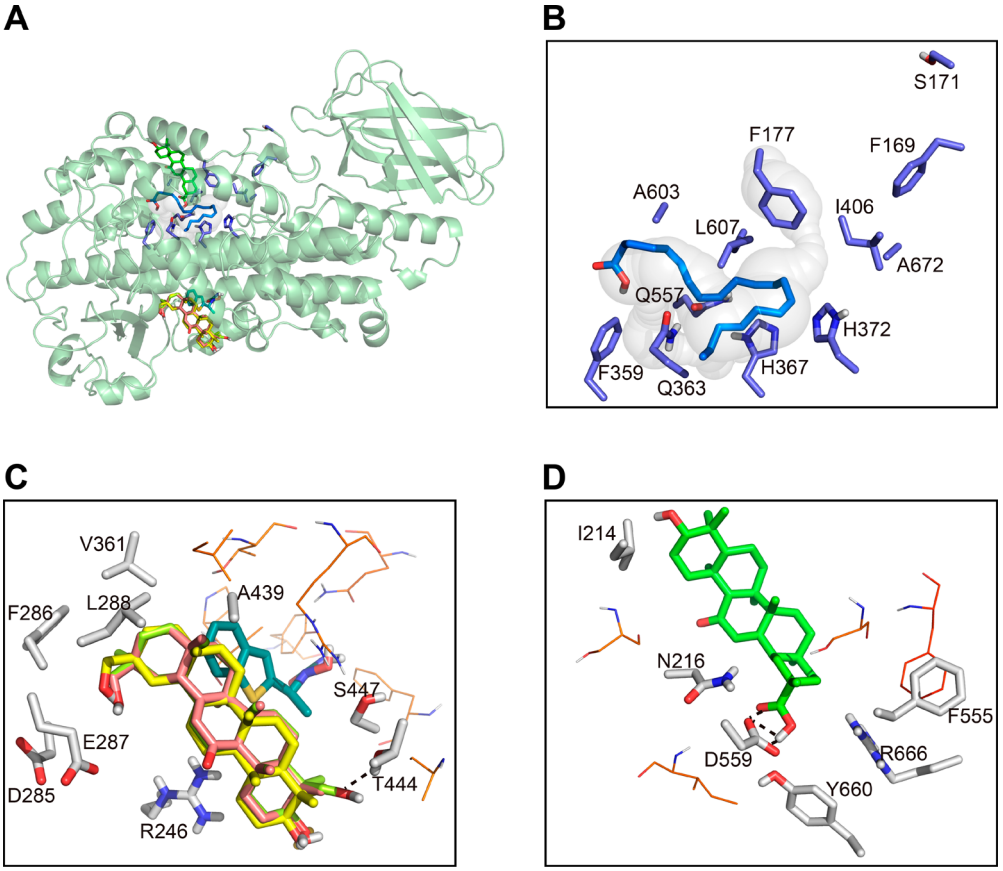

Figure 5. Molecular docking of 24-hydroxy-β-amyrin (**3**), 30-hydroxy-β-amyrin (**5**), 30-hydroxy-11-oxo-β-amyrin (**6**), glycyrrhetinic acid (**7**), the standard inhibitor zileuton (**30**), and AA substrate into the crystal structure of human 5-LOX (PDB codes: 3O8Y and 3V92). (A) Binding models of compounds **3** (lime), **5** (yellow), **6** (orange), **7** (green), and **30** (deep teal) and the binding cavity of AA (blue stick rendering; red, oxygen) in the overview map. (B) Close-up view of active-site amino acids. The side chains of amino acid residues are shown in atomic coloring (purple, carbon; red, oxygen; blue, nitrogen). (C) Close-up view of the binding modes for compounds **3** (lime), **5** (yellow), **6** (orange), and **30** (deep teal). The interacting residues are in white. Possible hydrogen bond of **3** with T444 is indicated by a black dotted line. (D) Close-up view of the binding mode for compound **7** (green), with interacting residues displayed in white. Possible hydrogen bonds of **7** with D559 are shown by black dotted lines.

purchased from Cayman Chemical (Ann Arbor, MI, USA). Human recombinant COX-2, xylenol orange, calcium chloride, L-α-phosphatidylcholine (type II-S), ATP disodium salt, and hematin were purchased from Sigma-Aldrich. All organic solvents were of analytical grade and were purchased from Kishida Chemical (Osaka, Japan). Ferrous sulfate, sulfuric acid, and formic acid were supplied by Wako Pure Chemical Industries (Osaka, Japan). Purified water was prepared using the Milli-Q purification system (Millipore, Billerica, MA, USA).

Stock solutions (2 mM) were freshly obtained by dissolving each triterpene in absolute methanol and then further diluting to the appropriate concentrations with 50 mM Tris-HCl buffer pH 7.4 for the LOX inhibition assay or 100 mM Tris-HCl buffer pH 8.0 for the COX inhibition assay (final concentration: 0.8−42 μM). For each of the inhibition assays, compounds were initially screened at a single inhibitor concentration of 42 μM. Those triterpenes displaying greater than 60% inhibition at 42 μM were subjected to full IC_{50} determinations using various inhibitor concentrations. The final concentration of methanol used was lower than 2% v/v and did not affect the activity of the assays.

15-LOX-2 and 5-LOX Inhibition Assays. Inhibition of human recombinant 15-LOX-2 activity was performed using a FOX assay, as described previously[3,S2] with some modifications. Briefly, 50 μL of human 15-LOX-2 (135.8 nM final concentration) was preincubated with 20 μL of test compound or standard inhibitor (various final concentrations) in 50 mM Tris-HCl buffer (pH 7.4) for 5 min at 25 °C in the dark. The control sample contained 50 μL of human 15-LOX-2 and 20 μL of Tris-HCl buffer in the absence of inhibitor comprising an equivalent concentration of methanol. This premixture was then mixed with 50 μL of AA (50 μM final concentration) in 50 mM Tris-HCl buffer (pH 7.4) to start the reaction at 25 °C for 20 min in the dark. The reaction mixture (120 μL total volume) was terminated by the addition of 100 μL of freshly prepared FOX reagent. The Fe^{3+}/xylenol orange complex was developed at 25 °C for 30 min, and then the absorbance was measured at 570 nm in the Infinite 200 PRO multimode microplate reader (Tecan). The blue color of the Fe^{3+} complex was observed at a final concentration of 135.8 nM human 15-LOX-2 in the absence of inhibitor (Figure S1, Supporting Information). The blank consisted of the enzyme LOX during incubation, and the AA substrate was added after addition of the FOX reagent. The percentage of LOX inhibition in the presence of test compounds and standard inhibitors was calculated as follows: $[(A_C - A_B) - (A_T - A_B)]/(A_C - A_B) \times 100\%$, where A_C is the absorbance of the control, A_B is that of the blank, and A_T is that of the test sample.

In the 5-LOX inhibition assay, the kinetics of conjugated diene (5-hydroperoxyeicosatetraenoic acid and 5-HETE) production was measured by the increase in absorbance at 234 nm using an extinction coefficient of 23 000 M^{-1} cm^{-1}, as adopted from Chan et al.[2] with minor modifications. The control was 50 μL of Tris-HCl buffer containing an equivalent concentration of methanol without inhibitor. The reaction was monitored at 25 °C for 5 min using the V-630 Bio UV−vis spectrophotometer (JASCO). The reaction rates were determined from the linear portion of the activity curve, and the percentage inhibition was calculated as follows: $[(ROR_C - ROR_T)/$

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$ROR_C] \times 100\%$, where ROR_C is the initial reaction rate of the control and ROR_T is that of the test sample.

COX-1 and COX-2 Inhibition Assays. The inhibitory effects of test compounds on ovine recombinant COX-1 and human recombinant COX-2 were evaluated further based on LC-MS analysis to measure the formation of PGE_2. The method followed was that reported by Cao et al.[9,53] with some modifications. While the COX-1 enzyme used in the present study is not of human origin, ovine COX-1 is generally used for inhibition studies to evaluate the effects of active compounds on the activity of purified COX-1.[54,55] In brief, 160 μL of 100 mM Tris-HCl buffer (pH 8.0) and 10 μL of 100 μM hematin (cofactor) were mixed at room temperature. Next, 10 μL of approximately 0.2 μg of either COX-1 or COX-2 in 100 mM Tris-HCl buffer (pH 8.0) was added, followed by incubation at room temperature for 5 min. The test compound or standard inhibitor (10 μL; various final concentrations) in 100 mM Tris-HCl buffer (pH 8.0) was added to the enzyme solution and preincubated for 10 min at 37 °C. The negative control comprised 10 μL of the same solvent instead of the inhibitor. The COX reaction was initiated by adding 10 μL of AA in Tris-HCl buffer (pH 8.0) to give a final concentration of 5 μM for 2 min at 37 °C, and 20 μL of 2.0 M HCl was further added to stop the reaction. A 10 μL aliquot of the internal standards d_4-PGD_2 and d_4-PGE_2 (1 μM solution in methanol) was added, and the samples were kept for 45 min. PGD_2, PGE_2, and their internal standards were extracted twice from each sample using 800 μL of hexane/ethyl acetate (1:1, v/v). After centrifugation at 4400*g* for 5 min (4 °C), the upper organic layers were removed, concentrated by evaporation to dryness, and reconstituted in 500 μL of methanol for LC-MS analysis.

The formation of PGE_2 in each sample was measured as the peak area by LC-MS, since the PGE_2 concentration always exceeded that of PGD_2 (Figures S4 and S5, Supporting Information). The percentage inhibition of COX by each test compound and standard inhibitor was determined by comparison with the amount of PGE_2 produced in the negative controls. The levels of PGD_2 were also monitored in proportion to those of PGE_2.

LC-MS Analysis. The reaction products were detected with the Acquity UPLC/MS system (Waters) by electrospray ionization (ESI) in the negative ion mode with selected ion monitoring (Method S1, Supporting Information). During LC-MS, the chromatographic peaks corresponding to PGE_2 and PGD_2 were separated by retention times of 5.732 and 5.978 min, respectively, and there was no overlap between the two peaks (Figures S2 and S3, Supporting Information). The data acquisitions were accomplished using MassLynx v. 4.1 (Waters).

Data Analysis. Results obtained from three independent experiments were expressed as means ± standard deviation (SD). The initial reaction rates of the control and test samples were calculated using Prism 6.0h software (GraphPad Software, Inc., San Diego, CA, USA). The IC_{50} value representing the sample concentration (μM) that reduces enzyme activity by 50% was determined from the inhibition curves.

Molecular Docking Studies. Docking was performed to investigate enzyme−ligand interactions. The enzyme structures of human 5-LOX and 15-LOX-2, ovine COX-1, and human COX-2 were obtained from the Protein Data Bank (ID: 3O8Y[48] and 3V92,[49] 4NRE,[47] 4O1Z,[56] and 5F1A,[57] respectively). The entire docking process was performed using AutoDock Vina 1.1.2 software[58] with PyRx-0.8 interface.[59] The input enzyme structures were prepared for docking, including deleting alternative conformations and other unwanted ligands. The 3D conformations of input ligands were provided from PubChem (http://pubchem.ncbi.nlm.nih.gov/), and the geometries of the ligand structures were refined to minimize their free energy state using Avogadro 1.2.0 software.[60] Docking results were analyzed, and the binding energy of the docked structure was calculated. The enzyme−ligand complexes with the highest docking scores taken from the docking results were obtained, and enzyme−ligand interactions were visualized using PyMOL 1.8 (Schrödinger, LLC, New York, NY, USA). The possible catalytic site tunnels were calculated and displayed using CAVER 3.0.1 PyMOL plugin.[61] Hydrogen bond lengths were measured, and hydrophobic contacts were depicted in two-dimensional interaction patterns using LigPlot+ software.[62]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jnatprod.9b00538.

Color development from yellow to blue (left to right) in the presence of the Fe^{3+}/xylenol orange complex; LC-MS chromatograms and electrospray ionization of AA, PGE_2 and PGD_2, and d_4-PGE_2 and d_4-PGD_2; LC-MS data of PGE_2 and PGD_2; concentration−response curves for **17**, **27**, and **29** for 5-LOX inhibition; LigPlot+ diagrams for the binding sites of human 15-LOX-2 with **28** and of human 5-LOX with **3**, **5**, **6**, or **7**; molecular docking of **17**, **27**, **29**, **30**, and AA into human 5-LOX and of **29** into ovine COX-1 and human COX-2; LigPlot + diagrams for the binding sites of ovine COX-1 and human COX-2 with **28**; correlation plots of affinities and activities of ligands; method S1 (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +593-62-999-160/+593-63-700-040 ext. 304. E-mail: ery.fukushima@ikiam.edu.ec.

ORCID

Nhu Ngoc Quynh Vo: 0000-0002-7522-9761
Yuhta Nomura: 0000-0002-4954-4653
Toshiya Muranaka: 0000-0003-1058-2473
Ery Odette Fukushima: 0000-0003-4099-4292

Notes

The authors declare no competing financial interest.

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