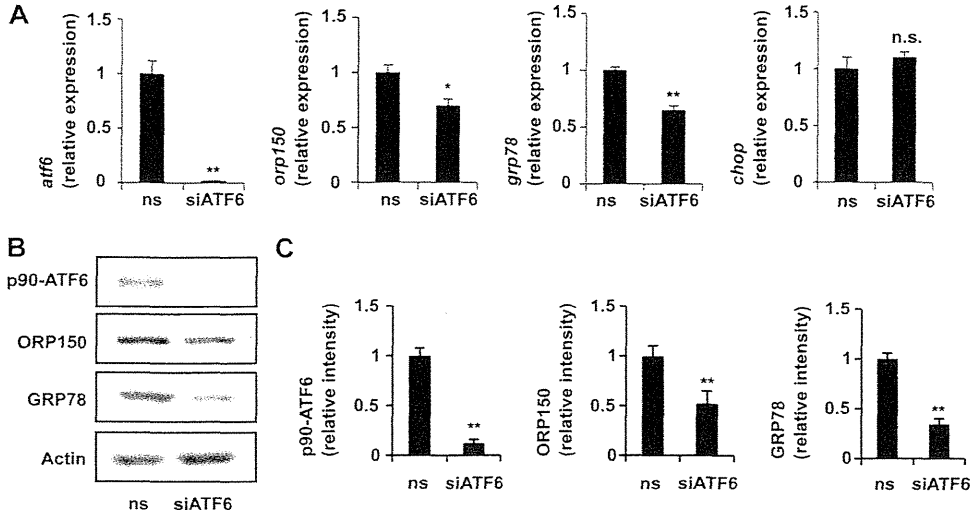

FIGURE 3. **Effect of siRNA for ATF6 on expression of ER chaperones.** AGS cells were transfected with siRNA for ATF6 (*siATF6*) or nonspecific siRNA (*ns*) and were incubated for 48 h (*A*) or 72 h (*B*). The mRNA (*A*) and protein (*B* and *C*) expression was monitored and expressed as described in the legends of Figs. 1 and 2. Values are the mean ± S.D. ($n = 3$). **, $p < 0.01$; *, $p < 0.05$; *n.s.*, not significant.

Effect of H. pylori on the Gastric Ulcerogenic Response and Expression of ER Chaperones Induced by Indomethacin in Mice—To evaluate the *in vivo* relevance of our *in vitro* observation that *H. pylori* suppress the expression of ER chaperones, we first examined the effect of oral inoculation of *H. pylori* on the expression of ER chaperones at gastric mucosa. We monitored by real-time RT-PCR analysis the mRNA expression of ER chaperones and CHOP after administration of indomethacin and/or *H. pylori* in gastric tissues. Oral inoculation of *H. pylori* to mice suppressed the background (without indomethacin administration) expression of *orp150* and *grp78* mRNAs but not *chop* mRNA (Fig. 5*A*). Indomethacin administration up-regulated the expression of *orp150, grp78*, and *chop* mRNAs, whereas the expression of *orp150* and *grp78* mRNAs but not that of *chop* mRNA was significantly suppressed by prior administration of *H. pylori* (Fig. 5*A*). Immunohistochemical analyses also demonstrated that oral inoculation with *H. pylori* decreased the levels of ORP150 and GRP78 at gastric mucosa in both the presence and absence of indomethacin administration (Fig. 5*B*). We consider that the staining of ORP150 and GRP78 in Fig. 5*B* is specific, because no positive staining was observed without a primary antibody (supplemental Fig. S2). We also performed immunoblotting analysis, and suppression of the gastric expression of ORP150 and GRP78 by inoculation with *H. pylori* in both the presence and absence of indomethacin treatment was confirmed (Fig. 5, *C* and *D*). Indomethacin-induced expression of GRP78 and CHOP was also confirmed (Fig. 5, *C* and *D*). Furthermore, we found that the gastric level of p90-ATF6 was decreased by inoculation with *H. pylori* in both the presence and absence of indomethacin treatment (Fig. 5, *C* and *D*). We also found that there is a tendency that inoculation with *H. pylori* decreases the levels of ORP150 and GRP78 in the presence of indomethacin treatment in the small intestine but not other organs (supplemental Fig. S3*A*).

The observation that inoculation with *H. pylori* reduces the gastric expression of ER chaperones suggests that this inoculation stimulates protein aggregation in ER in gastric cells. To address this idea, we examined the effect of *H. pylori* inoculation on the gastric level of connexin 43, which is known to be degraded by ER-associated degradation when it is aggregated in the ER (52, 53). As shown in supplemental Fig. S3, *B* and *C*, the gastric level of connexin 43 was decreased by *H. pylori* inoculation, suggesting that *H. pylori* stimulate aggregation of the protein in ER.

The effect of pre-administration of *H. pylori* on the development of gastric lesions after oral administration of indomethacin was examined. Administration of indomethacin produced gastric lesions, and this lesion production was significantly enhanced by pre-administration of *H. pylori* (Fig. 6*A*). Administration of *H. pylori* alone did not significantly produce gastric lesions under the conditions used (Fig. 6*A*). Histological analysis with H&E staining also supported the notion that indomethacin-produced gastric lesions are exacerbated by pre-administration of *H. pylori* (Fig. 6*B*).

As mentioned above, gastric mucosal cell death plays an important role in the formation of NSAID-induced gastric lesions. We, therefore, examined the effect of pre-administration of *H. pylori* on this process. The level of gastric mucosal cell death was determined by TUNEL assay. An increase in the number of TUNEL-positive cells was observed after indomethacin administration, and this increase was more apparent in mice pre-administered with *H. pylori* than in control mice (Fig. 6*C*). We also examined the effect of *H. pylori* on indomethacin-induced expression of ORP150, GRP78, and p90-ATF6 or apoptosis *in vitro*. Treatment of cells with *H. pylori* decreased the levels of these proteins and increased apoptotic cells in both the presence and absence of indomethacin treatment (supplemental Fig. S4, *A* and *B*). These results suggest that *H. pylori* exacerbate indomethacin-induced gastric lesion formation by stimulating indomethacin-induced gastric mucosal cell death.

The results in Figs. 5 and 6 suggest that *H. pylori* exacerbates indomethacin-induced gastric lesion formation through down-regulation of expression of ER chaperones. To test this idea using a genetic approach, the development of gastric lesions after oral administration of indomethacin was compared between heterozygous ORP150-deficient mice ($ORP150^{-/+}$) and wild-type mice ($ORP150^{+/+}$). Indomethacin-induced gastric lesions were significantly worse in heterozygous ORP150-deficient mice than in wild-type controls (Fig. 7*A*). ORP150 deficiency did not affect the background level of gastric lesions (Fig. 7*A*). Immunohistochemical analyses confirmed that the level of ORP150 in gastric mucosa was lower in heterozygous ORP150-deficient mice than wild-type mice in both the presence and absence of indomethacin administration (Fig. 7*B*). These results show that ORP150 plays an important role

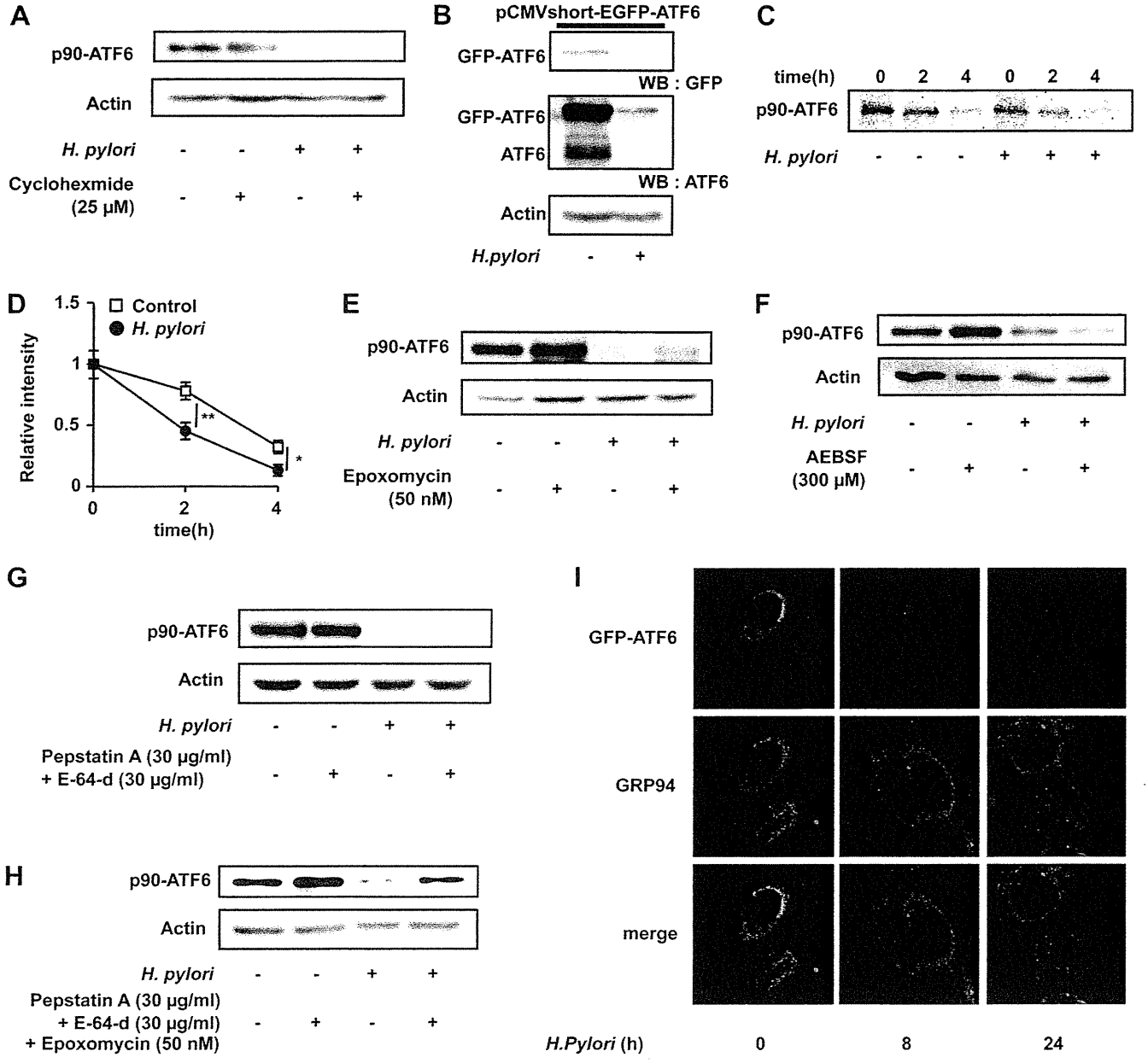

FIGURE 4. **Mechanism for the *H. pylori*-dependent decrease in the level of ATF6.** *A* and *E–H*, AGS cells were pre-incubated with or without each drug for 1 h and further incubated with or without *H. pylori* at a bacteria:cell ratio of 200:1 for 24 h in the presence (*E*, *G*, and *H*) or absence (*A* and *F*) of the same concentration of each drug as in the preincubation step. *B* and *I*, AGS cells were transfected with pCMVshort-EGFP-ATF6α (42) and co-cultured with or without *H. pylori* at a bacteria:cell ratio of 200:1 for 24 h (*B*) or indicated periods (*I*). *A*, *B*, and *E–H*, whole cell extracts were analyzed by immunoblotting (*WB*) with an antibody against GFP, ATF6, or actin. *C*, AGS cells were pulse-labeled for 30 min with [^{35}S]methionine and [^{35}S]cysteine and then chased with excess amounts of cold methionine and cysteine for the indicated periods in the absence or presence of *H. pylori* at a bacteria:cell ratio of 200:1. Labeled proteins were extracted, immunoprecipitated with antibody against ATF6, subjected to SDS-PAGE, and autoradiographed. *D*, the band intensity of p90-ATF6 was determined and expressed relative to the control. *I*, cells were fixed, stained with antibody against GRP94, and analyzed by confocal laser-scanning fluorescence microscope (magnification, 600 times). Values are the mean ± S.D. ($n = 3$). **, $p < 0.01$; *, $p < 0.05$. AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride.

in protecting gastric mucosa against indomethacin-induced lesions.

We also examined the indomethacin-induced gastric mucosal cell death in heterozygous ORP150-deficient mice. Indomethacin-induced gastric mucosal cell death was more apparent in heterozygous ORP150-deficient mice than in wild-type mice (Fig. 7*C*). These results suggest that ORP150 protects the gastric mucosa from indomethacin-induced cell death. Combining the results in Figs. 5–7, we consider that *H. pylori* exacerbates indomethacin-induced gastric lesion formation partly through down-regulation of ER chaperones and the resulting stimulation of indomethacin-induced gastric mucosal cell death.

DISCUSSION

There have been contradictory reports about whether infection with *H. pylori* increases the risk of developing NSAID-induced gastric lesions (in other words, whether eradication of *H. pylori* reduces the risk of developing NSAID-induced gastric lesions) (1–6, 54). This may be due to differences in diagnostic criteria (endpoints), standards for patient recruitment, and populations used for these studies. The most we can conclude is

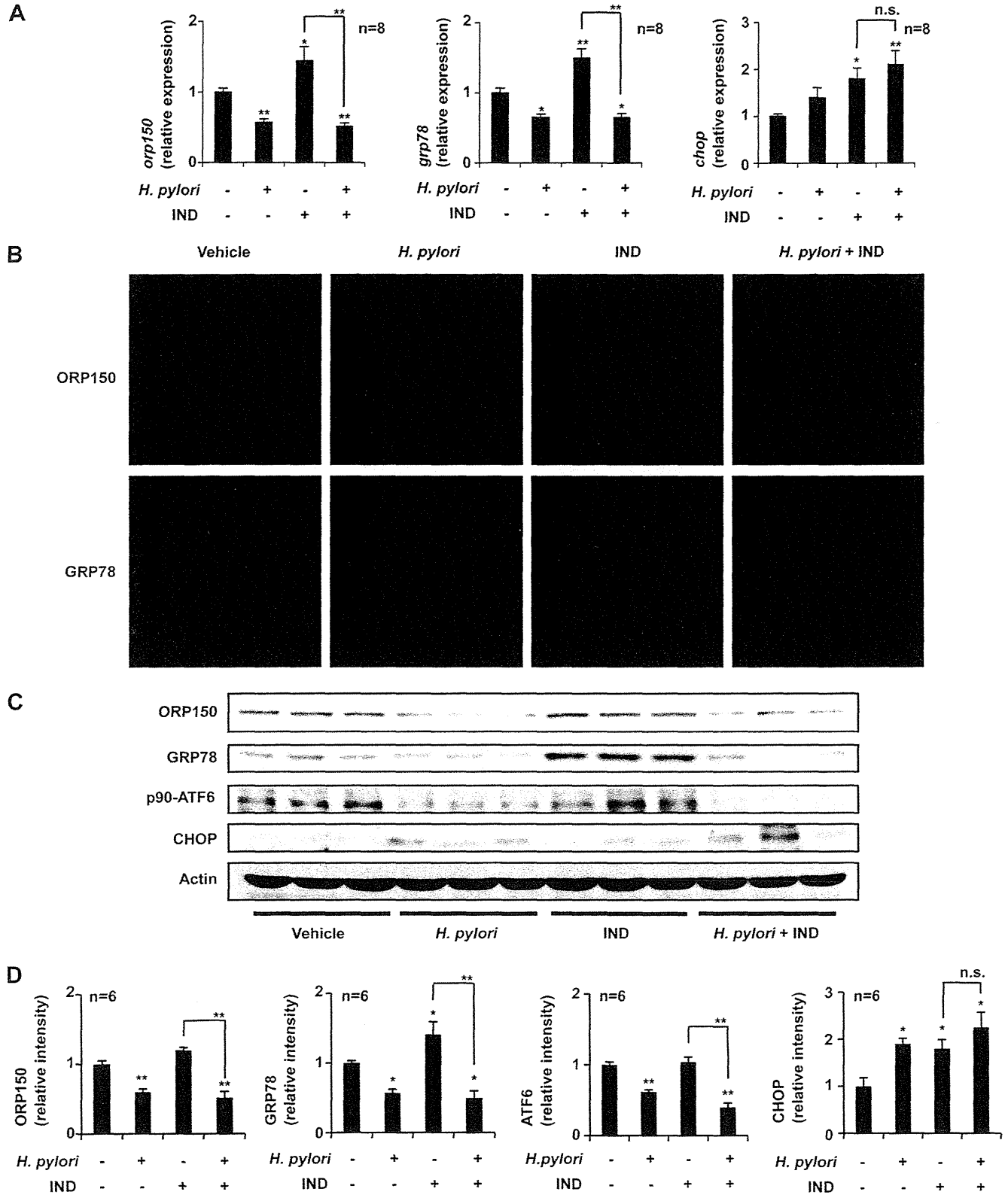

FIGURE 5. **Effect of *H. pylori* on expression of ER chaperones at gastric mucosa.** *H. pylori* were orally inoculated into mice (C57/BL6) at a dose of 2.0×10^8 *H. pylori*/animal every second day for 6 days (total 3 times). One day after the final inoculation, *H. pylori*-inoculated or control mice were orally administered 10 mg/kg of indomethacin (*IND*), and their stomachs were removed after 12 h. *A*, total RNA was extracted and subjected to real-time RT-PCR using a specific primer for each gene. Values normalized to the *gapdh* gene are expressed relative to the control sample. *B*, sections of gastric tissues were subjected to immunohistochemical analysis with an antibody against ORP150 or GRP78 and DAPI staining (magnification, 200 times). *C* and *D*, protein expression was monitored and expressed as described in the legend of Fig. 1. Values are given as the mean ± S.E. **, $p < 0.01$; *, $p < 0.05$; *n.s.*, not significant.

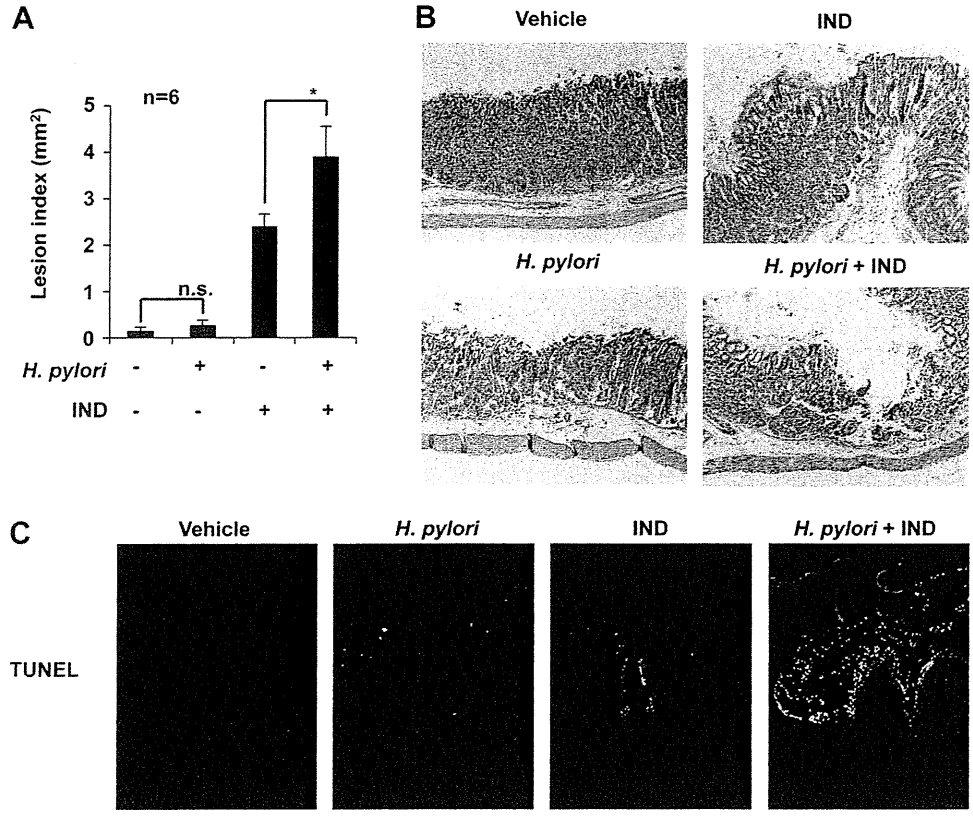

FIGURE 6. **Effect of *H. pylori* on indomethacin-induced gastric lesions and mucosal cell death.** Mice (C57/BL6) were administered *H. pylori* and indomethacin (*IND*), as described in the legend of Fig. 5. *A*, the stomach was scored for hemorrhagic damage. Values are the mean ± S.E. $*p < 0.05$; *n.s.*, not significant. *B*, sections of gastric tissues were subjected to H&E staining (magnification, 200 times). *C*, sections of gastric tissues were subjected to TUNEL assay and DAPI staining (magnification, 200 times). *n.s.*, not significant.

that under certain conditions infection with *H. pylori* increases the risk of developing NSAID-induced gastric lesions. Thus, it is important to examine the effect of *H. pylori* on factors that affect the formation of NSAID-induced gastric lesions. In this study we have focused on ER chaperones and found that co-culture of gastric cells with *H. pylori* decreases the level of ER chaperones. This is the first observation that *H. pylori* affect the expression of ER stress response-related proteins. However, although we used here the transient infection model of *H. pylori,* the infection in humans is chronic. The *H. pylori* strain used in this study does not colonize mice (data not shown), and thus, studies in the future need to be done with strains that do colonize mice.

By using real-time RT-PCR and luciferase reporter assays, we have shown that the *H. pylori*-dependent decrease in the level of ER chaperones *in vitro* is regulated at the level of transcription. Of three ER stress response-related transcription factors (ATF6, ATF4, and XBP-1), only the level of ATF6 (but not the *atf6* mRNA) was decreased by co-culture of cells with *H. pylori,* suggesting that ATF6 is involved in the *H. pylori*-dependent suppression of transcription of ER chaperone genes. Because the *H. pylori*-dependent decrease in the level of ER chaperones was observed in cells whose protein synthesis was inhibited and *H. pylori* decreased the stability of p90-ATF6, post-translational modification (protein degradation) of ATF6 would be involved in this process. Analysis with each inhibitor suggested that the proteasome-ubiquitin system rather than degradation by S1P is involved in this degradation of ATF6. The observation that the level of p50-ATF6 (the proteolytic product of S1P and S2P) did not increase after co-culture of cells with *H. pylori* further supports this notion. We also suggest that protein degradation in lysosomes is involved in this degradation of ATF6. It is known that VacA perturbs endocytic traffic at a late stage (55, 56), and as such it is possible that *H. pylori* affects the traffic of ATF6 to lysosomes and its degradation in lysosomes. Furthermore, because the suppression of *H. pylori*-dependent degradation of ATF6 by inhibitors of proteasomal and lysosomal proteases was weak, other proteases seem to be involved in this degradation.

We found that not only *H. pylori* themselves but also cell extracts of *H. pylori* suppress the expression of ER chaperones *in vitro*. However, the suppression of expression of ER chaperones by cell extracts of *H. pylori* was not as great as that induced by *H. pylori* themselves, and cell extracts of *H. pylori* did not affect the level of ATF6 so distinctly (Fig. 1, *D–G*). Furthermore, the decrease in level of ATF6 occurred more slowly that that of ORP150 and GRP78 in the presence of cell extracts of *H. pylori* (Fig. 1), suggesting that the decrease in levels of ORP150 and GRP78 observed with cell extracts of *H. pylori* is not due to the decrease in levels of ATF6. In other words, results suggest that the mechanism for the decrease in levels of ORP150 and GRP78 is different between *H. pylori* cells and cell extracts of *H. pylori*. We also compared the signal pathway for induction of apoptosis between *H. pylori* cells and cell extracts of *H. pylori*. As shown in supplemental Fig. S5, the decrease in Bax and increase in cytochrome *c* in cytosol fractions (an indicator for mitochondria-mediated apoptosis) and induction of expression of CHOP were not observed with apoptosis induced by cell extracts of *H. pylori* so apparently as that induced by *H. pylori* cells, suggesting that the signal pathway for induction of apoptosis is also different between *H. pylori* cells and cell extracts of *H. pylori*. Although siRNA for ATF6 suppressed the expression of ER chaperones, the extent of suppression was not as apparent as that seen with *H. pylori*. These results suggest that in addition to the mechanism described above (the ATF6-mediated mechanism), an ATF6-independent and as yet unknown mechanism that can be reproduced with cell extracts of *H. pylori* should also be mainly involved in the *H. pylori*-dependent suppression of expression of ER chaperones.

We have previously reported that suppression of expression of GRP78 and ORP150 by siRNA stimulated NSAID-induced

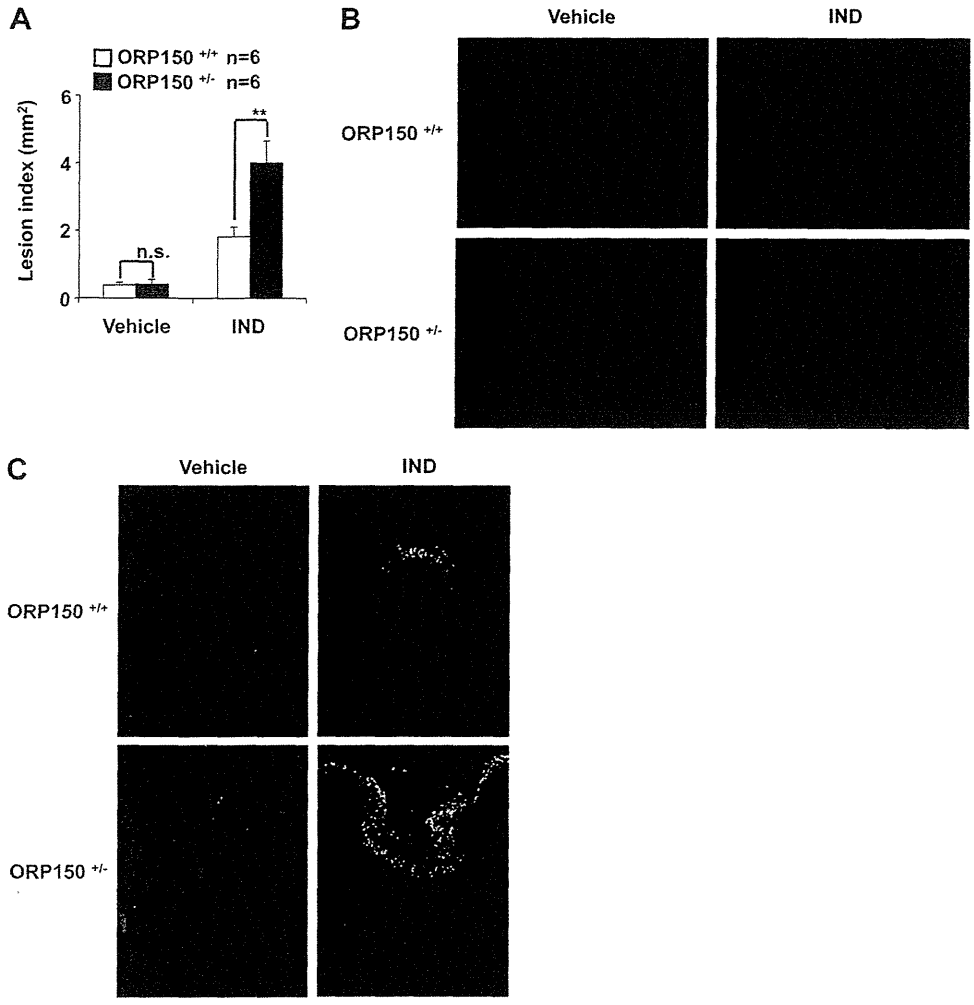

FIGURE 7. **Indomethacin-induced gastric lesions and mucosal cell death in heterozygous ORP150-deficient mice.** Heterozygous ORP150-deficient mice ($ORP150^{+/+}$) and wild-type mice ($ORP150^{+/+}$) were orally administered with 10 mg/kg of indomethacin (*IND*), and their stomachs were removed after 12 h. Gastric lesions (*A*), expression of ORP150 (*B*), and mucosal apoptosis (*C*) were assayed as described in the legends of Figs. 5 and 6. Values are mean ± S.E. **, $p < 0.01$; *, $p < 0.05$; *n.s.*, not significant. *n.s.*, not significant.

apoptosis in cultured gastric cells (19, 20). We have also suggested that NSAID-induced apoptosis at gastric mucosa plays an important role in the formation of NSAID-induced gastric lesions (16–18). These results suggest that ER chaperones play a protective role against the development of NSAID-induced gastric lesions; however, there is no direct evidence supporting this notion. In this study we have shown that heterozygous ORP150-deficient mice display phenotypes sensitive to indomethacin-induced gastric mucosal cell death and gastric lesion formation. This is the first genetic evidence that an ER chaperone is protective against NSAID-induced gastric lesion formation. This result also suggests that inducers of ER chaperones may be therapeutically beneficial against NSAID-induced gastric lesions, as is the case for heat shock proteins inducers (18, 57).

The *in vitro* observation that expression of ER chaperones is suppressed by *H. pylori* suggests that *H. pylori* would suppress the expression of GRP78 and ORP150 at the gastric mucosa and stimulate NSAID-induced gastric mucosal cell death and lesion formation. In fact, we have shown that pre-inoculation of mice with *H. pylori* not only suppresses the expression of ER chaperones but also stimulates NSAID-induced cell death and gastric lesion formation.

We also showed *in vitro* that co-culture of gastric cells with *H. pylori* up-regulates the expression of CHOP, suggesting that this up-regulation is involved in the *H. pylori*-dependent stimulation of NSAID-induced cell death. There are two possible mechanisms that could explain this up-regulation of CHOP. One is that *H. pylori* directly affects the expression of CHOP. However, because siRNA for ATF6 did not up-regulate the expression of CHOP, *H. pylori*-dependent degradation of ATF6 must not be involved. The other possibility is that this up-regulation is a result of the suppression of expression of ER chaperones, as we previously reported that suppression of expression of GRP78 and ORP150 by siRNA induces the expression of CHOP in the presence of NSAIDs (19, 20).

Although we suggest that the *H. pylori*-dependent exacerbation of indomethacin-induced gastric lesion formation is mediated by the suppression of expression of ER chaperones, various other mechanisms are likely to be involved in this exacerbation. For example, cytotoxic proteins produced by *H. pylori*, such as VacA and CagA, which are known to induce apoptosis in gastric cells (32–36), may stimulate indomethacin-induced cell death, resulting in exacerbation of indomethacin-induced gastric lesions. CagA disrupts the epithelial apical junction complex (58), which may also be involved in *H. pylori*-dependent exacerbation of indomethacin-induced gastric lesions. We believe that this animal model for *H. pylori*-dependent exacerbation of indomethacin-induced gastric lesion formation will be useful in future studies for examining the relationship between *H. pylori* and NSAIDs and their involvement in the production of gastric lesions.

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Synthesis and biological evaluation of loxoprofen derivatives

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ABSTRACT

Non-steroidal anti-inflammatory drugs (NSAIDs) achieve their anti-inflammatory actions through an inhibitory effect on cyclooxygenase (COX). Two COX subtypes, COX-1 and COX-2, are responsible for the majority of COX activity at the gastrointestinal mucosa and in tissues with inflammation, respectively. We previously suggested that both gastric mucosal cell death due to the membrane permeabilization activity of NSAIDs and COX-inhibition at the gastric mucosa are involved in NSAID-induced gastric lesions. We have also reported that loxoprofen has the lowest membrane permeabilization activity among the NSAIDs we tested. In this study, we synthesized a series of loxoprofen derivatives and examined their membrane permeabilization activities and inhibitory effects on COX-1 and COX-2. Among these derivatives, 2-{4′-hydroxy-5-[(2-oxocyclopentyl)methyl]biphenyl-2-yl}propanoate **31** has a specificity for COX-2 over COX-1. Compared to loxoprofen, oral administration of **31** to rats produced fewer gastric lesions but showed an equivalent anti-inflammatory effect. These results suggest that **31** is likely to be a therapeutically beneficial and safer NSAID.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) comprise one of the most frequently used classes of medicines in the world and account for nearly 5% of all prescribed medications.[1] NSAIDs are inhibitors of cyclooxygenase (COX), a protein essential for the synthesis of prostaglandins (PGs), which have a strong capacity to induce inflammation. However, NSAID administration is associated with gastrointestinal complications, such as gastric ulcers and bleeding. In the United States, about 16,500 people per year die as a result of NSAID-associated gastrointestinal complications.[2] Inhibition of COX by NSAIDs was thought to be fully responsible for their gastrointestinal side effects, because PGs have a strong protective effect on the gastrointestinal mucosa. In 1991, two subtypes of COX, COX-1 and COX-2, which are responsible for the majority of COX activity at the gastrointestinal mucosa and tissues with inflammation, respectively, were identified.[3,4] Thus, it is reasonable to speculate that selective COX-2 inhibitors maintain anti-inflammatory activity without gastrointestinal side effects. In fact, a greatly reduced incidence of gastroduodenal lesions has been reported for selective COX-2 inhibitors (such as celecoxib and rofecoxib).[5–7] Thus, increasing the specificity for COX-2 over COX-1 is one of the strategies that could be employed to develop safer NSAIDs. However, a recently raised issue concerning the use of selective COX-2 inhibitors is their potential risk for cardiovascular thrombotic events (see Section 3).[8,9] Because of this concern, rofecoxib and valdecoxib were withdrawn from the worldwide market.[8,10]

It is now believed that the inhibition of COX by NSAIDs is not the sole explanation for the gastrointestinal side effects of NSAIDs.[11] We previously demonstrated that NSAIDs induce necrosis and apoptosis in cultured gastric mucosal cells and in the gastric mucosa in a manner independent of COX inhibition.[12–16] We clearly showed that the primary target of NSAIDs for the induction of necrosis and apoptosis is the cytoplasmic membranes.[12,14] The following pathway has been proposed to describe the molecular mechanism governing this apoptosis.[12,17,18] Permeabilization of cytoplasmic membranes stimulates Ca^{2+} influx and increases intracellular Ca^{2+} levels, which in turn induces the endoplasmic reticulum (ER) stress response. In this response, an apoptosis-inducing transcription factor, C/EBP homologous transcription factor (CHOP), is induced, resulting in mitochondrial dysfunction and apoptosis.[13,19] Furthermore, we have suggested that both COX inhibition (decrease in the gastric level of PGE_2) and gastric mucosal cell death are required for the formation of NSAID-induced gastric lesions in vivo.[16,20] Thus, decreasing the membrane permeabilization activity of NSAIDs is another strategy that could be followed to develop safer compounds that provide the clinical effects sought.

Loxoprofen sodium (**1**, Fig. 1) has been used clinically for many years as a standard NSAID in Japan, and clinical studies have suggested that it is safer than other NSAIDs, such as

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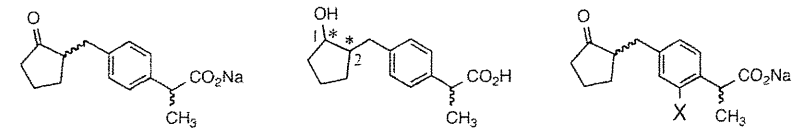


Figure 1. Structure of loxoprofen sodium and its derivatives.

indomethacin.[21,22] Loxoprofen is a pro-drug, which is converted to its active metabolite (the *trans*-alcohol form, **2**, Fig. 1) by aromatic aldehyde–ketone reductase only after absorption in the gastrointestinal tract.[23] We recently reported that loxoprofen has lower membrane permeabilization activity than other NSAIDs.[24] Therefore, synthetic modification of loxoprofen to either increase specificity for COX-2 or decrease membrane permeabilization activity is a valuable strategy to obtain safer NSAIDs.

We recently reported that the loxoprofen derivatives 2-fluoroloxoprofen and 2-bromoloxoprofen (**4a** and **4b**, respectively, Fig. 1) have lower membrane permeabilization activity and their oral administration to rats produced fewer gastric lesions. Nevertheless, these compounds had equivalent anti-inflammatory effects compared to loxoprofen.[25] In the present study, we synthesized a series of loxoprofen derivatives and examined their membrane permeabilization activities and inhibitory effects on COX-1 and COX-2. Among these derivatives, 2-{4′-hydroxy-5-[(2-oxocyclopentyl)methyl]biphenyl-2-yl}propanoate (**31**, Scheme 3) has a specificity for COX-2 and its oral administration produced fewer gastric lesions but showed an equivalent anti-inflammatory effect, compared to loxoprofen. These results suggest that this compound could be a valuable candidate for use as a safer NSAID.

2. Chemistry

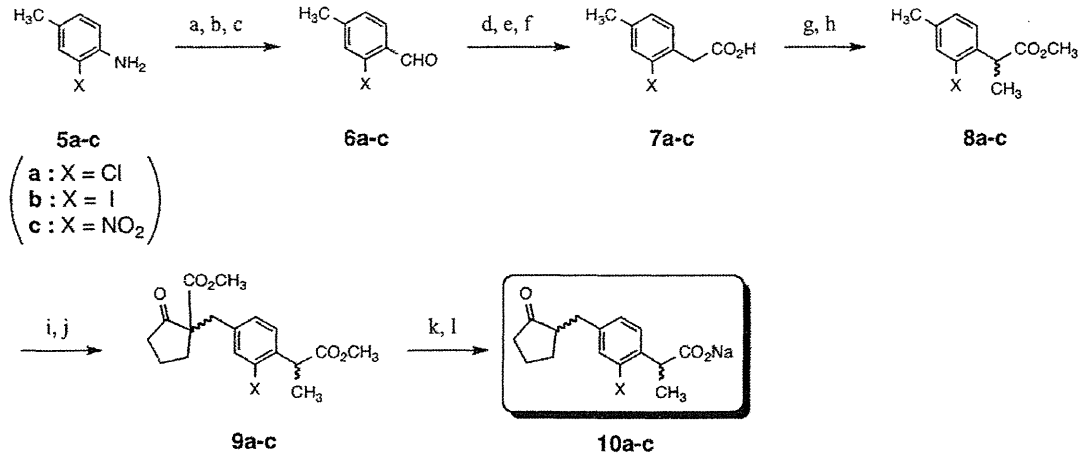
Loxoprofen derivatives with modification at the 2-position of the phenyl ring by halogens and the nitro group **10a–c** were obtained by the method described previously[25] (Scheme 1).

Loxoprofen derivatives with modification at the 3- or 2-position of the phenyl ring by a para-substituted aryl group were synthesized via the Suzuki–Miyaura cross-coupling reaction[26,27] between aryl bromide derivatives **14** or **4b** and a variety of commercially available boronic acids (Schemes 2 and 3).

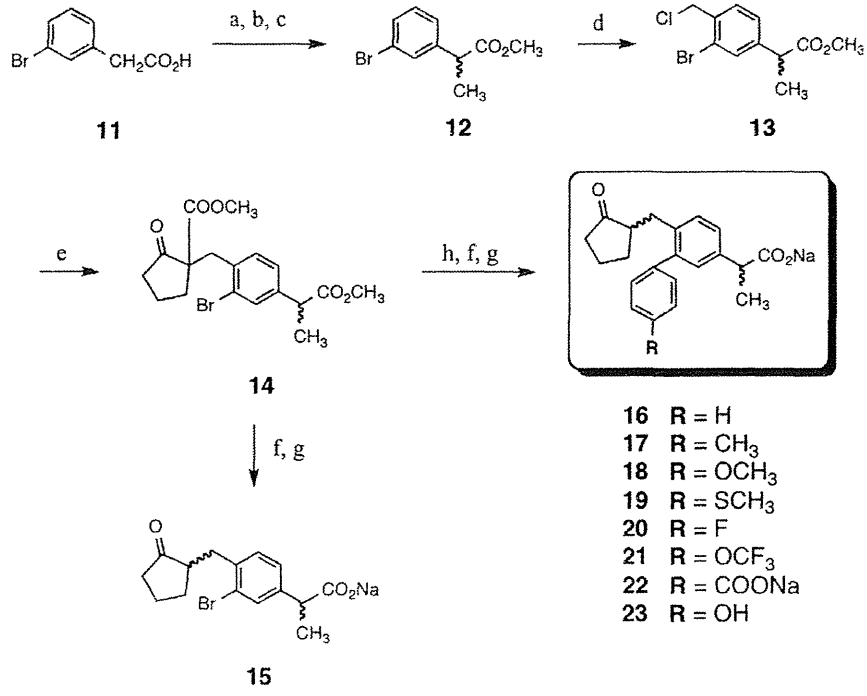
The synthetic route for target compounds **16–23** is outlined in Scheme 2. The commercially available (3-bromophenyl)acetic acid **11** was converted to the methyl 2-(3-bromophenyl)propanoate **12** by methyl esterification and α-methylation. Friedel–Crafts chloromethylation of **12** under Lewis acid conditions gave the methyl 2-[3-bromo-4-(chloromethyl)phenyl]propanoate **13**, having an active methylene group. The hetero-nuclear multiple-bond connectivity (HMBC) nuclear magnetic resonance (NMR) spectrum of **13** revealed correlations between the methylene carbon and the 5-position proton on the phenyl ring or the methylene carbon and the 2- and 6-position protons on the phenyl ring (data not shown).

Treatment of compound **13** with methyl 2-oxocyclopentanecarboxylate provided the key intermediate **14**. Compound **15** (3-bromoloxoprofen) was obtained by decarboxylation, hydrolysis and treatment with NaOH of **14**. The cross-coupling reaction between **14** and a variety of boronic acids afforded the precursors of target compounds **16–23**. Finally, the carboxylic acid group was transformed into the sodium salt by treatment with NaOH to yield target compounds **16–23**.

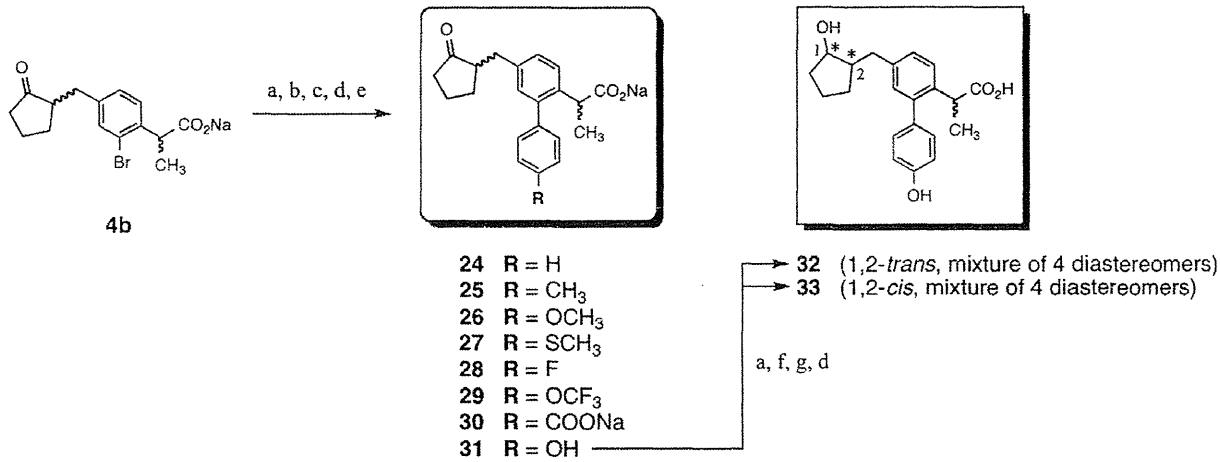
The synthetic route for target compounds **24–31** is outlined in Scheme 3. A key intermediate **4b** was prepared, as described previously.[25] The methyl ester of **4b** was prepared by treatment with methanol in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and *N*,*N*-dimethyl-4-aminopyridine (DMAP). After the cross-coupling reaction between the compound **4b** and a variety of boronic acids, the ester group was converted to a carboxylic acid group by alkaline hydrolysis,



Scheme 1. Synthesis of loxoprofen derivatives with modification at the 2-position of the phenyl ring by Cl (**10a**), I (**10b**) and NO_2 (**10c**). Reagents and conditions: (a) 3 M HCl aq, $NaNO_2$, $CuSO_4$, Na_2SO_3, AcONa, H_2O, 0 °C; (b) NH_2OH·HCl, $(HCHO)_n$, AcONa, H_2O; (c) concd HCl, reflux; (d) Me $OCH_2P(OH_3)Cl$, $C_6H_{18}KNSi_2$, toulene; (e) 3 M HCl aq, acetone, reflux; (f) PFC (2.0 mol %), H_5IO_6, acetonitrile; (g) concd HCl, CH_3OH, reflux; (h) 2.0 M LDA, CH_3I, dry THF, −70 to −40 °C; (i) NBS, AIBN, CCl_4, reflux; (j) dry Na_2CO_3, methyl 2-oxocyclopentanecarboxylate, dry acetone, reflux; (k) concd HCl, reflux; (l) 1 M NaOH aq, C_2H_5OH, reflux.



Scheme 2. Synthesis of loxoprofen derivatives with modification at the 3-position of the phenyl ring by Br (**15**) and a *para*-substituted aryl group (**16–23**). Reagents and conditions: (a) MeOH, HCl, reflux; (b) LDA, THF, −78 °C; (c) CH_3I, −78 to −50 °C; (d) $AlCl_3$, $SnCl_4$, 1,3-dioxolane, CH_3OCH_2Cl, 0 °C to rt; (e) methyl 2-oxyocyclopentanecarboxylate, K_2CO_3, acetone, reflux; (f) AcOH, HCl, reflux; (g) 1 M NaOH aq, C_2H_5OH, reflux; (h) R-⟨⟩-$B(OH)_2$, $PD(PPh_3)_4$, Na_2CO_3, THF, reflux.



Scheme 3. Synthesis of loxoprofen derivatives with modification at the 2-position of the phenyl ring by a *para*-substituted aryl group (**24–33**). Reagents and conditions: (a) 6 M HCL aq, CH_2Cl_2; (c) R-⟨⟩-$B(OH)_2$, $PD(PPh_3)_4$, Na_2CO_3, THF, reflux; (d) KOH, C_2H_5OH, H_2O, reflux; (e) 1 M NaOH, C_2H_5OH, reflux; (f) 4-DMAP, EDC, CH_2OH; (g) $NaBH_4$, C_2H_5OH.

followed by acidification. Finally, the carboxylic acid group was transformed into the sodium salt by treatment with NaOH to yield target compounds **24–31**.

The reduction products of **31**, *trans*-alcohol **32** and *cis*-alcohol **33** were prepared by treatment of the methyl ester intermediate of **31** with sodium borohydride ($NaBH_4$) followed by alkaline hydrolysis. The structures of **32** and **33** were identified based on the characteristic NMR signal of the proton on the asymmetric carbon attached to the hydroxyl group.

All target compounds were pure and stable. The final compounds were characterized by ^1H NMR, ^{13}C NMR, infrared spectroscopy (IR), high resolution mass spectra (HR-MS) and elemental analysis.

3. Results and discussion

We have employed loxoprofen sodium **1** (Fig. 1) as a lead compound to obtain NSAIDs with lower membrane permeabilization activity or higher COX-2 specificity. On this basis we synthesized a series of derivatives of **1** by modification of the phenyl ring with electron withdrawing groups such as halogens or modified phenyl

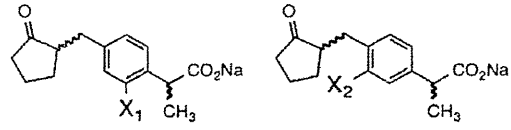
rings. We previously reported that two of the compounds, 2-fluoroloxoprofen **4a** and 2-bromoloxoprofen **4b** (Fig. 1), have lower membrane permeabilization activity than **1**.[25] In this study, we examined the membrane permeabilization activities and inhibitory effects on COX-1 and COX-2 of other derivatives to find other valuable compounds, such as those with COX-2 specificity.

We previously established an assay system for assessing the membrane permeabilization activity of NSAIDs, using calcein-loaded liposomes. Calcein fluorescence is very weak at high concentrations due to self-quenching, so the addition of membrane-permeabilizing drugs to a medium containing calcein-loaded liposomes causes an increase in fluorescence by diluting the calcein.[14] In this study, we used the EC_{50} index, defined as the concentration of each compound required for 50% release of calcein.

Table 1 shows the membrane permeabilization activities and inhibitory effects on COX-1 and COX-2 of loxoprofen derivatives with modification at the 3- or 2-position of the phenyl ring by halogens and the nitro group. The inhibitory effect on COX-1 and COX-2 is shown as the IC_{50} index, defined as the concentration of each compound required for 50% inhibition of each form of COX. Compared to **1**, 4a and **4b**, 2-chloroloxoprofen **10a** and 2-iodoloxoprofen **10b** showed higher membrane permeabilization activity, thus demonstrating that the species of halogen introduced to **1** is an important determinant of the membrane permeabilization activity. We also found that 3-bromoloxoprofen **15** has much higher membrane permeabilization activity than **4b** (Table 1), showing that the modification position on the phenyl ring is also important. Furthermore, we found that 2-nitroloxoprofen **10c** has lower membrane permeabilization activity and a lower inhibitory effect on COX-1 and COX-2 than **1** (Table 1).

The orientation of the active metabolite of **1** and interaction between the compound and amino acid residues in the active site of COX-1 or COX-2 were examined by molecular modeling and docking studies. As shown in Fig. 2, the cyclopentanone ring interacts with Y385 and S530, whereas propanoic acid interacts with R120 and Y355. All of these amino acids were reported to be important for the interaction between COXs and NSAIDs.[28–31] It is also well known that COX-2 has a side pocket[28,32] (Fig. 2). Thus, it could be predicted that introduction of a bulky functional group into the 3- or 2-position of the phenyl ring of **1** results in an increase in its specificity for COX-2 over COX-1. Therefore, we synthesized loxoprofen derivatives with modification at the 3- or 2-position of the phenyl ring by para-substituted aryl groups.

Table 1
In vitro membrane permeabilization assay and human whole blood assay for inhibition of COX-1- and COX-2-derived PG biosynthesis; loxoprofen derivatives with modification at the 3- or 2-position of the phenyl ring by halogens and the nitro group



Compounds	X_1 or X_2	EC_{50} (mM)	IC_{50} (μM)		COX-1/COX-2
		Calcein release	COX-1	COX-2	
1		800[a]	24[a]	10[a]	2.5[a]
4a	X_1 = F	>1000[a]	24[a]	14[a]	0.2[a]
4b	X_1 = Br	>1000[a]	30[a]	65[a]	0.1[a]
10a	X_1 = Cl	100	4	2	1.8
10b	X_1 = I	150	270	540	0.5
10c	X_1 = NO_2	>1000	93	49	1.9
15	X_2 = Br	<100	49	23	2.1

Calcein-loaded liposomes were incubated with each compound. The release of calcein from the liposomes was determined by measuring fluorescence intensity. Triton X-100 (10 μM) was used to establish the 100% level of membrane permeabilization. EC_{50} value (concentration of each compound required for 50% release of calcein) is shown.
The inhibitory effect of each compound on COX-1- and COX-2-derived PG biosynthesis was measured and the IC_{50} value (concentration of each compound required for 50% inhibition) and the COX-1/COX-2 ratio of IC_{50} value are shown. The values of IC_{50} were estimated from the sigmoid-like dose–response curve (4-parameter logistic curve model) drawn by the logistic-curve fitting software (ImageJ 1.43u; National Institutes of Health, USA). Mean values are presented (n = 3).
[a] Data from our previous report.[25]

Table 2 shows the membrane permeabilization activities and inhibitory effects on COX-1 and COX-2 of these derivatives, indicating the importance of the modification position of the phenyl ring (3- or 2-position) for determining membrane permeabilization activity and inhibitory effect on COX-1 and COX-2. For example, the membrane permeabilization activity and inhibitory effects on COX-1 and COX-2 of **31** were much higher than those of **23** (Table 2) and we have no clear explanation for this difference. All derivatives except **23** showed higher membrane permeabilization activity than **1**. On the other hand, none of these derivatives showed a more potent inhibitory activity on COX-1 and COX-2 than **1**. Among these derivatives, 2-{4′-hydroxy-5-[(2-oxocyclopentyl)methyl]biphenyl-2-yl}propanoate 31 showed the most potent inhibitory effect on COX-2 and the highest specificity for COX-2 over COX-1; the extent of this specificity is similar to that of celecoxib (Table 2). The combined results show that **31** is a loxoprofen derivative with higher membrane permeabilization activity, a similar inhibitory effect on COX-2, and a higher specificity for COX-2, compared to **1**. On this basis we selected this compound for further investigation (see below).

As described above, **1** is a pro-drug and the *trans*-alcohol derivative is the active metabolite. In order to test whether or not **31** maintains this characteristic, we examined the COX-inhibitory activity of the *trans*- and *cis*-alcohol forms of **31** (**32** and **33**, respectively). The *trans*-alcohol derivative of **1** (**2**, Fig. 1) showed a more potent inhibitory effect on both COX-1 and COX-2 than **1** or its *cis*-alcohol derivative (**3**, Fig. 1) (Table 2). In contrast to the case of **1**, the inhibitory effect on COX-2 was similar between **31**, 32 and **33** (Table 2). Furthermore, the inhibitory effect of **32** on COX-1 was less than that of **33** (Table 1). These results suggest that **31** does not retain the pro-drug characteristic of **1**.

We then evaluated the activity of **31** in vivo. Compound **1** (40 or 50 mg/kg) and equivalent molar amounts of **31** were orally administered to rats and the lesion index was calculated (see Section 5.5). Administration of **1** produced gastric lesions in a dose-dependent manner (Fig. 3), as described previously.[21,22] In contrast, production of gastric lesions was not detected after oral administration of **31** (Fig. 3). We also measured the gastric level of PGE_2 by enzyme immunoassay (EIA) after oral administration of these compounds. As shown in Fig. 3B, the administration of **31** decreased the level of PGE_2, albeit to an extent less than that seen with **1**. Considering our hypothesis that both a decrease in the gastric level of PGE_2 and an increase in gastric mucosal damage due to membrane permeabilization activity of NSAIDs are involved in the production of NSAID-induced gastric lesions, the lower lesion-producing activity of **31** seems to be due to its selectivity for COX-2, resulting in less activity for decreasing the gastric level of PGE_2.

Finally, we compared the anti-inflammatory effects of **31** to **1** by employing a rat carrageenan-induced footpad edema assay. As shown in Fig. 4A, the volume of edema was significantly decreased after oral administration of **1**, confirming it's previously described anti-inflammatory activity.[23,33] The effects of **31** were mostly the same as that of **1** (Fig. 4A). We also found that the level of PGE_2 associated with the footpad edema decreased after oral administration of **31** and the extent was similar to that seen with **1** (Fig. 4B). These results show that **31** has an anti-inflammatory activity equivalent to **1**. This finding may be related to the

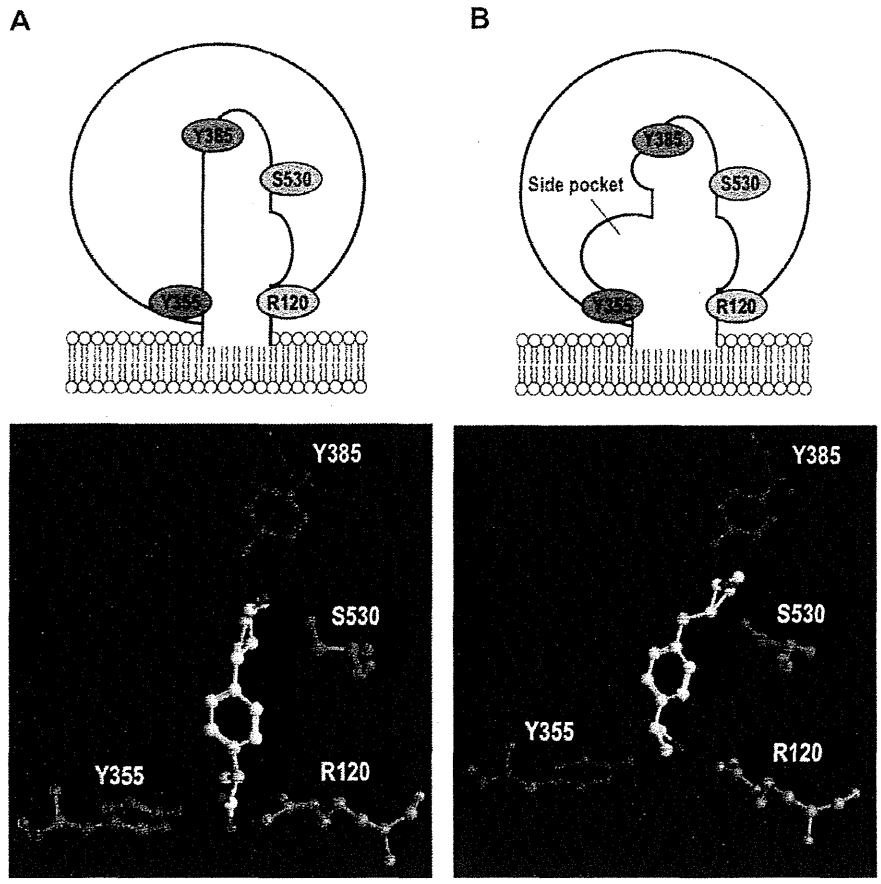


Figure 2. Potential binding mode of (*S*)-2-{4-[((1*R*, 2*S*)-2-hydroxycyclopentyl)methyl]phenyl}propanoic acid to the active site of sheep COX-1 (A) or murine COX-2 (B). Hydrogen atoms of the amino acid residues and the ligand have been removed.

in vitro observation that the inhibitory effect of **1** on COX-2 was indistinguishable from that of **31** (Table 2).

The inhibitory activity of **31** on COX-2 was much higher than that of **23** (Table 2), indicating the importance of the modification position of the phenyl ring (3- or 2-position) for determining the inhibitory effect on COX-2. Thus, we compared the interaction with COX-2 between **23** and **31** by molecular modeling and docking studies. The interaction between the cyclopentanone ring with Y385 and S530 and propanoic acid with R120 and Y355 was similar between **31** (Fig. 5B) and the active metabolite of **1** (Fig. 2B). Furthermore, the introduced phenyl ring of **31** interacts with some amino acids (H90, R513, F518 and V523) (Fig. 5B), which are reported to be located in the side pocket of COX-2.[34,35] On the other hand, molecular modeling and docking studies suggested that the interaction between the cyclopentanone ring with Y385 and S530 and propanoic acid with R120 and Y355 was not possible for **23** (Fig. 5A). As a result, lowest U_{total} index is calculated to be 59.2 and 29.5 kcal/mol for **23** and **31**, respectively; the lower lowest U_{total} index means the higher interaction of two molecules.[36]

A recently raised issue concerning the use of selective COX-2 inhibitors is their potential risk for cardiovascular thrombotic events.[8,9] This may be due to the fact that prostacyclin, a potent anti-aggregator of platelets and a vasodilator, is mainly produced by COX-2 in vascular endothelial cells, while thromboxane A_2, a potent aggregator of platelets and a vasoconstrictor, is mainly produced by COX-1 in platelets.[37–39] Because of this concern, rofecoxib and valdecoxib were withdrawn from the worldwide market.[8,10] On the other hand, it is not clear whether or not celecoxib use is a potential risk factor for cardiovascular thrombotic events. It was proposed that the weaker COX-2 specificity of celecoxib compared to rofecoxib and valdecoxib (COX-1/COX-2 ratios of IC_{50} index of celecoxib, rofecoxib and valdecoxib are 37, 141 and 270, respectively) is responsible for the relative safety of celecoxib in relation to cardiovascular thrombotic events.[40–42] From this point of view, **31** may be safer for use with respect to possible cardiovascular thrombotic events compared to rofecoxib and valdecoxib.

4. Conclusion

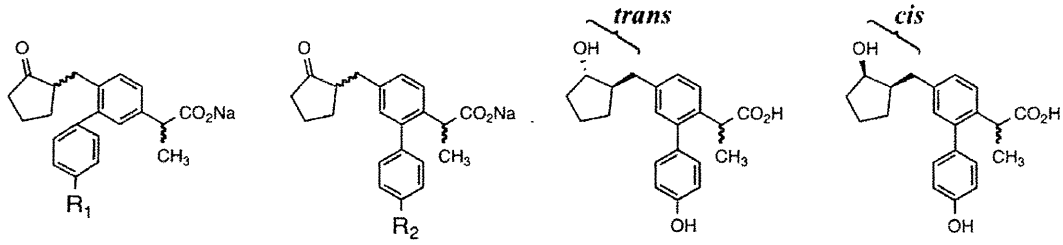
We have found that a loxoprofen derivative, **31**, administered orally to rats, produced fewer gastric lesions but provided similar anti-inflammatory effects compared to **1**. This may be due to its selectivity for COX-2, resulting in a lower propensity for the gastric level of PGE_2 to be reduced. Although **31** exhibits higher membrane permeabilization activity and does not maintain the pro-drug characteristic of **1**, we consider that it is likely to be therapeutically beneficial as a safer NSAID.

5. Experimental

5.1. Molecular modeling studies

Docking studies were performed with MOE (The Molecular Operating Environment) Version 2009.10 software (Chemical Computing Group Inc., Montreal, Canada).

Table 2
In vitro membrane permeabilization assay and human whole blood assay for inhibition of COX-1- and COX-2-derived PG biosynthesis: loxoprofen derivatives with modification at the 3-position (**16–23**) and the 2-position (**24–31**) of the phenyl ring by a para-substituted aryl group



32 **33**

Compounds	R_1 or R_2	EC_{50} (mM)	IC_{50} (μM)		COX-1/COX-2
		Calcein release	COX-1	COX-2	
1		800[a]	24[a]	10[a]	2.5[a]
2			1.3[a]	2.4[a]	0.6[a]
3			6.3[a]	12.2[a]	0.6
16	R_1 = H	<100	54	290	0.2
17	R_1 = CH_3	<100	56	420	0.1
18	R_1 = OCH_3	<100	800	>1000	–
19	R_1 = SCH_3	<10	758	>1000	–
20	R_1 = F	<100	174	36	1.0
21	R_1 = OCF_3	<10	460	72	6.4
22	R_1 = CO_2Na	200	>1000	>1000	–
23	R_1 = OH	>1000	>1000	–	–
24	R_2 = H	<100	310	70	4.4
25	R_2 = CH_3	<100	470	540	0.9
26	R_2 = OCH_3	<100	74	430	0.2
27	R_2 = SCH_3	<100	575	150	3.8
28	R_2 = F	20	174	36	4.8
29	R_2 = OCF_3	6	515	>1000	–
30	R_2 = CO_2Na	<10	>1000	76	–
31	R_2 = OH	25	326	11	31
32			650	20	33
33			47	17	2.8
Celecoxib		0.09[a]	7[b]	0.19[b]	37[b]

Experiments and data analysis were performed as described in the legend of Table 1.
[a] Data from our previous report.[25]
[b] Data from a reference.[41]

5.1.1. Construction of the ligand molecule

The ligand molecule of (*S*)-2-{4-[((1*R*,2*S*)-2-hydroxycyclopentyl)methyl]phenyl}propanoic acid was constructed using the Builder module. The geometric stereochemistry was constrained, and all carboxylic acid groups were modeled in their ionized forms.

5.1.2. Construction of the receptor protein

The crystal structures of sheep COX-1 complexed with aspirin (1PTH)[30] and murine COX-2 complexed with indomethacin (4COX)[28] were obtained from the Protein Data Bank. After removal of the ligand and water, the structure of each receptor protein was optimized with the addition of hydrogen atoms and charge to acidic amino acid residues.

5.1.3. Molecular docking of the ligand with COX-1 and COX-2

Modeling calculations were performed only for each active site of COX-1 and COX-2 using the automatic docking program (ASE Dock 2005), which includes energy minimization applied to the ligand. The ligand–receptor complexes were subjected to energy minimization to convergence using the standard conditions at MMFF94 force fields. All amino acid residues within a 4.5 Å radius around the ligand were minimized, and the best conformation of ligand corresponding to the minimum docking energy of each ligand–receptor complex was adopted.

5.2. Chemistry

All solvents and reagents were purchased from Tokyo Kasei Chemical Co. (Tokyo, Japan) and Wako Pure Chemical Industries (Tokyo, Japan), and used without further purification. Fourier transform IR spectra were recorded on a JASCO FT/IR-410 spectrophotometer using potassium bromide (KBr) pellets. ^{1}H NMR and ^{13}C NMR spectra were recorded on a JNM AL-300 spectrometer (JEOL Ltd., Tokyo, Japan) operating at 300 MHz, in a ca. 2% solution of $CDCl_3$ or CD_3OD. Coupling constant (*J*) values are estimated in hertz (Hz) and spin multiples are given as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad). Mass spectra were detected with a fast atom bombardment (FAB) mass spectrometer (JMS-700, JEOL Ltd, Tokyo, Japan). The progress of all reactions was monitored by thin-layer chromatography (TLC) with silica gel glass plates (60 F_{254}) (Merck Ltd, Tokyo, Japan), and spots were visualized with ultraviolet (UV) light (254 nm) and stained in 5% ethanolic phosphomolybdic acid. Column chromatography was performed using Silica Gel 60 N (Kanto Chemical Co., Tokyo, Japan). Elemental analysis was performed for C and H (Instrumental Analysis Center, Kumamoto University) and was within ±0.4% of the theoretical values. Loxoprofen sodium (**1**), loxoprofen-OH (**2**, **3**), and compound **4b** were synthesized as reported previously.[25]

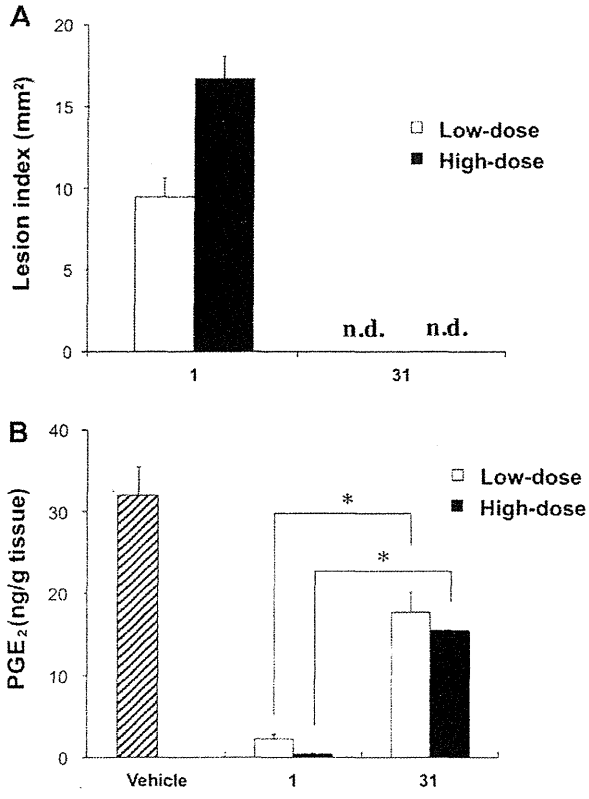


Figure 3. Production of gastric lesions and gastric PGE_2 levels in the presence of loxoprofen sodium and its derivative. Rats were orally administered a low (40 or 54 mg/kg) or high (50 or 67 mg/kg) dose of **1** or **31**, respectively, or vehicle and their stomachs were removed after 8 h. Stomachs were scored for hemorrhagic damage (A). Gastric PGE_2 level was determined by EIA (B). Values are mean ± SEM (n = 3–6). * P <0.05; n.d., not detected.

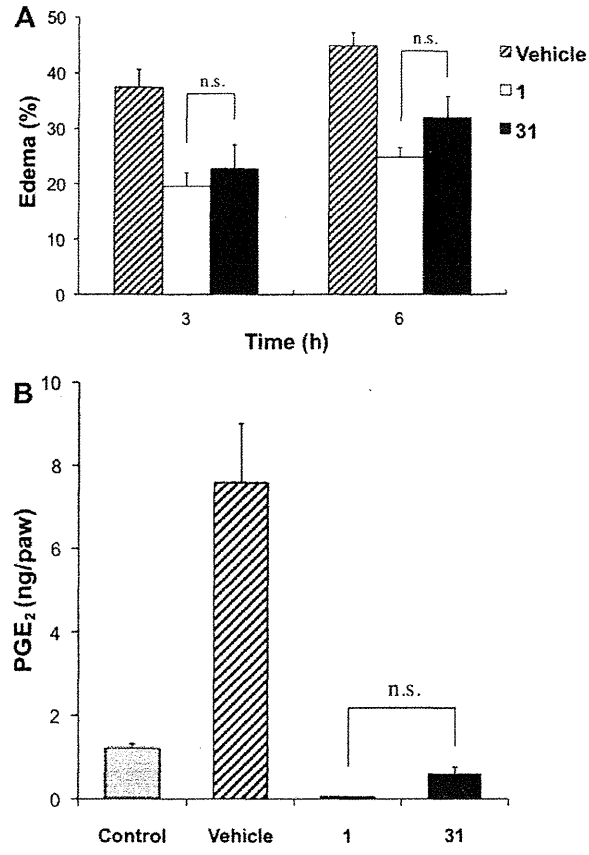


Figure 4. Anti-inflammatory activities of loxoprofen sodium and its derivative. Rats were orally administered 10 or 13 mg/kg of **1** or **31**, respectively, or vehicle and 1 h later received an intradermal injection of carrageenan (1%) into the left hindpaw. Footpad edema was measured 3 h and 6 h after the administration of carrageenan (A). The level of PGE_2 in the footpad was determined by EIA. Control rats were not treated with carrageenan (B). Values are mean ± SEM (n = 3–6). n.s., not significant.

5.2.1. Synthesis of 2-{2-halogeno (or nitro)-4-[(2-oxocyclopentyl)methyl]phenyl}propanoic acid (10a–c)

Compounds **10a–c** were synthesized from the corresponding starting materials **5a–c** by the method described previously.[25]

5.2.1.1. 2-Chloro-4-methylbenzaldehyde (6a). Yellow liquid (yield 52.0%), ^{1}H NMR ($CDCl_3$) δ: 2.34 (3H, s, Ar-CH_3), 7.66 (1H, d, J = 7.5, Ar-H5), 8.14 (1H, d, J = 7.5 Hz, Ar-H6), 8.90 (1H, s, Ar-H3), 10.34 (1H, br s, CHO). EI-MS (m/z): 154.07 (M^+).

5.2.1.2. 2-Iodo-4-methylbenzaldehyde (6b). Red-brown solid (yield 40.1%), ^{1}H NMR ($CDCl_3$) δ: 2.35 (3H, s, Ar-CH_3), 7.26 (1H, d, J = 8.1, Ar-H5), 7.89 (1H, d, J = 8.1 Hz, Ar-H6), 7.90 (1H, s, Ar-H3), 10.34 (1H, br s, CHO). EI-MS (m/z): 245.99 (M^+).

5.2.1.3. 4-Methyl-2-nitrobenzaldehyde (6c). Yellow liquid (yield 36.3%), ^{1}H NMR ($CDCl_3$) δ: 2.54 (3H, s, Ar-CH_3), 7.59 (1H, d, J = 8.4 Hz, Ar-H5), 7.87 (1H, d, J = 7.7 Hz, Ar-H6), 7.89 (1H, s, Ar-H3), 10.36 (1H, s, CHO). EI-MS (m/z): 164.99 (M^+).

5.2.1.4. 2-Chloro-4-methylphenylacetic acid (7a). White solid (yield 59.9%), ^{1}H NMR ($CDCl_3$) δ: 2.34 (3H, s, Ar-CH_3), 3.56 (2H, s, CH_2), 7.06 (1H, dd, J = 7.7, 1.8 Hz, Ar-H5), 7.17 (1H, d, J = 7.7 Hz, Ar-H6), 7.27 (1H, s, Ar-H3), 10.54 (1H, s, CO_2H). FAB-MS (m/z): 184.59 (M^+).

5.2.1.5. 2-Iodo-4-methylphenylacetic acid (7b). White solid (yield 61.3%), ^{1}H NMR ($CDCl_3$) δ: 2.29 (3H, s, Ar-CH_3), 3.76 (2H, s, CH_2), 7.21–7.70 (2H, m, Ar-H5, Ar-H6), 7.68 (1H, s, Ar-H3), 10.56 (1H, s, CO_2H). FAB-MS (m/z): 275.69 (M^+).

5.2.1.6. 4-Methyl-2-nitrophenylacetic acid (7c). White solid (yield 60.0%), ^{1}H NMR ($CDCl_3$) δ: 2.44 (3H, s, Ar-CH_3), 4.01 (2H, s, CH_2), 7.23 (1H, d, J = 7.7 Hz, Ar-H5), 7.41 (1H, d, J = 8.1 Hz), 7.95 (1H, s, Ar-H3), 10.66 (1H, s, CO_2H). FAB-MS (m/z): 196.21 (M^++H).

5.2.1.7. Methyl 2-(2-chloro-4-methylphenyl)propanoate (8a). Slightly-yellow liquid (yield: 71.4%), ^{1}H NMR ($CDCl_3$) δ: 1.48 (3H, d, J = 7.0 Hz, α-CH_3), 2.34 (3H, s, Ar-CH_3), 3.66 (3H, s, OCH_3), 3.66 (1H, q, J = 7.2 Hz, CH), 7.08 (1H, dd, J = 8.1, 1.8 Hz, Ar-H5), 7.17 (1H, d, J = 7.7 Hz, Ar-H6), 7.28 (1H, d, J = 1.8 Hz, Ar-H3). FAB-MS (m/z): 213.20 (M^++H).

5.2.1.8. Methyl 2-(2-iodo-4-methylphenyl)propanoate (8b). Colorless liquid (yield: 65.3%), ^{1}H NMR ($CDCl_3$) ^{1}H NMR ($CDCl_3$) δ: 1.44 (3H, d, J = 7.0 Hz, α-CH_3), 2.27 (3H, s, Ar-CH_3), 3.67 (3H, s, OCH_3), 4.07 (1H, q, J = 7.2 Hz, CH), 7.20–7.13 (2H, m, Ar-H5, Ar-H6), 7.69 (1H, s, Ar-H3). FAB-MS (m/z): 305.13 (M^++H).

5.2.1.9. Methyl 2-(4-methyl-2-nitrophenyl)propanoate (8c). Yellow liquid (yield: 54.3%), ^{1}H NMR ($CDCl_3$) δ: 1.58 (3H, d, J = 7.0 Hz, α-CH_3), 2.42 (3H, s, Ar-CH_3), 3.66 (3H, s, OCH_3), 4.27 (1H, q, J = 7.1 Hz, CH), 7.36–7.39 (2H, m, Ar-H5, Ar-H6), 7.74 (1H, s, Ar-H3). FAB-MS (m/z): 224.28 (M^++H).

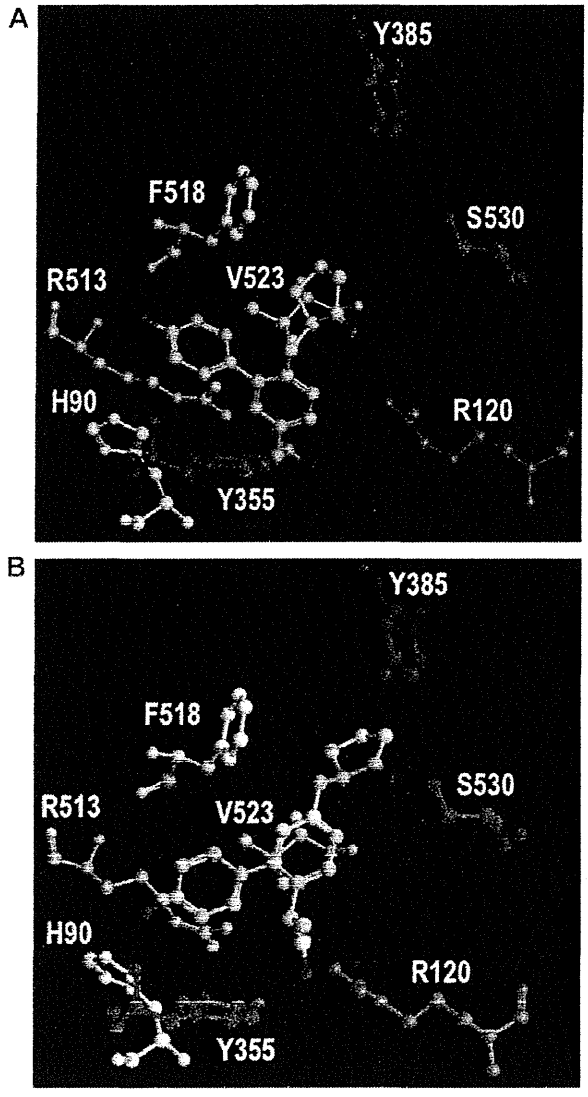


Figure 5. Potential binding mode of **23** (A) or **31** (B) to the active site of murine COX-2. Hydrogen atoms of the amino acid residues and the ligand have been removed.

5.2.1.10. Methyl 1-[3-chloro-4-(1-methoxy-1-oxopropan-2-yl)-benzyl]-2-oxocyclopentanecarboxylate (9a). Colorless liquid (yield: 54.0%), ^{1}H NMR (CD_3Cl_3) δ: 1.47 (3H, d, J = 7.3 Hz, α-CH_3), 1.69–2.14 (4H, m, H3′, H4′), 2.35–2.50 (2H, m, H5′), 3.22 (1H, d, J = 14.3 Hz, CH_2), 3.49 (1H, d, J = 14.3 Hz, CH_2), 3.66 (1H, q, J = 7.1 Hz, CH), 3.67 (3H, s, CO_2CH_3), 3.74 (3H, s, CO_2CH_3), 7.08 (1H, d, J = 8.1 Hz, Ar-H5), 7.14 (1H, d, J = 8.1 Hz, Ar-H6), 7.30 (1H, s, Ar-H3). FAB-MS (*m/z*): 353.21 (M^++H).

5.2.1.11. Methyl 1-[3-iodo-4-(1-methoxy-1-oxopropan-2-yl)-benzyl]-2-oxocyclopentanecarboxylate (9b). Colorless liquid (yield: 53.3%), ^{1}H NMR (CD_3Cl_3) δ: 1.43 (3H, d, J = 7.3 Hz, α-CH_3), 1.70–2.17 (4H, m, H3′, H4′), 2.36–2.47 (2H, m, H5′), 2.95 (1H, d, J = 13.9 Hz, CH_2), 3.17 (1H, d, J = 13.9 Hz, CH_2), 3.68 (3H, s, CO_2CH_3), 3.73 (3H, s, CO_2CH_3), 4.06 (1H, q, J = 7.1 Hz, CH), 7.10 (1H, d, J = 8.8 Hz, Ar-H5), 7.17 (1H, d, J = 8.1 Hz, Ar-H6), 7.64 (1H, s, Ar-H3). FAB-MS (*m/z*): 445.11 (M^++H).

5.2.1.12. Methyl 1-[4-(1-methoxy-1-oxopropan-2-yl)-3-nitrobenzyl]-2-oxocyclopentanecarboxylate (9c). Yellow liquid (yield: 38.6%), ^{1}H NMR (CD_3Cl_3) δ: 1.58 (3H, d, J = 7.3 Hz, α-CH_3), 1.75–2.22 (4H, m, H3′, H4′), 2.40–2.51 (2H, m, H5′), 3.05 (1H, d, J = 14.1 Hz, CH_2), 3.32 (1H, d, J = 13.9 Hz, CH_2), 3.67 (3H, s, CO_2CH_3), 3.74 (3H, s, CO_2CH_3), 4.28 (1H, q, J = 7.2 Hz, CH), 7.39 (2H, br s, Ar-H5, Ar-H6), 7.73 (1H, br s, Ar-H3). FAB-MS (*m/z*): 364.31 (M^++H).

5.2.1.13. Sodium 2-{2-chloro-4-[(2-oxocyclopentyl)methyl]-phenyl}propanoate (10a). White solid (yield: 96.0%), IR (KBr) ν: 1736 (CO_2^-), 1713 (C=O), cm^{-1}. ^{1}H NMR (CD_3OD) δ: 1.38 (3H, d, J = 7.1 Hz, α-CH_3), 1.53–2.03 (4H, m, H3′, H4′), 2.06–2.58 (4H, m, H1′, H5′, CH_2), 3.23 (1H, dd, J = 12.7, 3.2 Hz, CH_2), 3.52 (1H, q, J = 7.1 Hz, CH), 7.15 (1H, d, J = 7.9 Hz, Ar-H5), 7.21 (1H, d, J = 7.9 Hz, Ar-H6), 7.38 (1H, s, Ar-H3). ^{13}C NMR (CD_3OD) δ: 19.80 (α-CH_3), 21.44 (C5′), 30.17 (C4′), 33.69 (CH_2), 38.80 (C3′), 49.68 (CH), 50.71 (C1′), 127.43 (Ar-C5), 129.53 (Ar-C3), 131.81 (Ar-C1), 134.56 (Ar-C6), 136.34 (Ar-C2), 145.69 (Ar-C4), 182.40 (CO_2Na), 222.45 (C=O). HR-FAB-MS (*m/z*): 325.0580 (M^++Na, calcd for $C_{15}H_{16}ClNa_2O_3$: 325.0583). Anal. Calcd for $C_{15}H_{16}ClNa_2O_3{\cdot}H_2O$: C, 56.17; H, 5.66. Found: C, 56.25, H, 5.75.

5.2.1.14. Sodium 2-{2-iodo-4-[(2-oxocyclopentyl)methyl]phenyl}propanoate (10b). White solid (yield: 94.1%), IR (KBr) ν: 1733 (CO_2^-), 1715 (C=O), cm^{-1}. ^{1}H NMR (CD_3OD) δ: 1.34 (3H, d, J = 7.0 Hz, α-CH_3), 1.48–2.14 (4H, m, H3′, H4′), 2.01–2.38 (3H, m, H1′, H5′), 2.46 (1H, dd, J = 13.4, 9.0 Hz, CH_2), 2.98 (1H, dd, J = 13.0, 3.5 Hz, CH_2), 3.85 (1H, q, J = 7.1 Hz, CH), 7.12 (1H, d, J = 8.1 Hz, Ar-H5), 7.34 (1H, d, J = 8.1 Hz, Ar-H6), 7.64 (1H, s, Ar-H3). ^{13}C NMR (CD_3OD) δ: 20.04 (α-CH_3), 21.42 (C5′), 29.97 (C4′), 35.27 (CH_2), 38.95 (C3′), 51.93 (CH), 54.07 (C1′), 102.35 (Ar-C2), 128.70 (Ar-C5), 130.13 (Ar-C6), 140.61 (Ar-C4), 141.10 (Ar-C3), 146.39 (Ar-C1), 182.03 (CO_2Na), 222.54 (C=O). HR-FAB-MS (*m/z*): 416.9935 (M^++Na, calcd for $C_{15}H_{16}INa_2O_3$: 416.9940). Anal. Calcd for $C_{21}H_{21}NaO_3{\cdot}H_2O$: C, 43.71; H, 4.40. Found: C, 43.64, H, 4.22.

5.2.1.15. Sodium 2-{2-nitro-4-[(2-oxocyclopentyl)methyl]phenyl}propanoate (10c). Yellow solid (yield: 69.4%), IR (KBr) ν: 1738 (CO_2^-), 1711 (C=O), cm^{-1}. ^{1}H NMR (CD_3OD) δ: 1.39 (3H, d, J = 7.3 Hz, α-CH_3), 1.53–2.07 (4H, m, H3′, H4′), 1.94–2.38 (3H, m, H1′, H5′), 2.52 (1H, dd, J = 7.1, 3.5 Hz, CH_2), 3.02 (1H, dd, J = 13.9, 5.1 Hz, CH_2), 3.92 (1H, q, J = 7.1 Hz, CH), 7.32 (1H, d, J = 8.1 Hz, Ar-H5), 7.45 (1H, d, J = 8.1 Hz, Ar-H6), 7.55 (1H, s, Ar-H3). ^{13}C NMR (CD_3OD) δ: 19.82 (α-CH_3), 21.21 (C5′), 29.85 (C4′), 35.38 (CH_2), 38.86 (C3′), 45.17 (CH), 51.71 (C1′), 125.18 (Ar-C3), 130.85 (Ar-C1), 134.31 (Ar-C6), 137.74 (Ar-C5), 140.78 (Ar-C4), 151.04 (Ar-C2), 181.20 (CO_2Na), 222.26 (C=O). HR-FAB-MS (*m/z*): 336.0814 (M^++Na, calcd for $C_{15}H_{16}NNa_2O_5$: 336.0824). Anal. Calcd for $C_{15}H_{16}NNaO_5{\cdot}H_2O$: C, 54.38; H, 5.48; N, 4.23. Found: C, 54.36, H, 5.45, N, 4.09.

5.2.2. Synthesis of loxoprofen derivatives with modification at the 3-position of the phenyl ring (15–23)

5.2.2.1. Methyl 2-(3-bromophenyl)propanoate (12). (3-Bromophenyl)acetic acid **11** (5.0 g, 23.3 mmol) and methanol (50 mL) were refluxed for 3 h in the presence of 0.2 mL of concentrated hydrochloric acid (HCl) to give the methyl (3-bromophenyl)acetate. After neutralization with saturated $NaHCO_3$ and washing with brine, a pure product was obtained from the diethyl ether extract. This methyl acetate (4.9 g, 21.4 mmol) in dry THF (35 mL) was added dropwise to a stirred solution of 2.0 mol/L lithium diisopropylamide (LDA) (12.9 mL, 25.8 mmol) in THF/ethylbenzene/heptane at −78 °C under argon (Ar), and after 30 min, iodomethane (CH_3I) (2.0 mL, 32.2 mmol) was added slowly. The resulting solution was stirred for 5 h with the temperature changed from −78 to −40 °C, then evaporated to dryness, and extracted with CH_2Cl_2 (50 mL). Evaporation of the solvent and purification of the residue

by silica gel chromatography (*n*-hexane/AcOEt, 20:1) yielded the title compound as a colorless liquid (77.2%). ^{1}H NMR ($CDCl_3$) δ: 1.49 (3H, d, J = 7.1 Hz, α-CH_3), 3.67 (3H, s, CO_2CH_3), 3.68 (1H, q, J = 7.1 Hz, CH), 7.18 (1H, t, J = 7.5 Hz, Ar-H5), 7.23 (1H, dt, J = 7.8, 1.7 Hz, Ar-H6), 7.38 (1H, dt, J = 7.3, 1.8 Hz, Ar-H4), 7.44 (1H, st, J = 1.7 Hz, Ar-H2). FAB-MS (*m/z*): 243.02 (M^++H, calcd for $C_{10}H_{12}{}^{79}BrO_2$: 243.00).

5.2.2.2. Methyl 2-[3-bromo-4-(chloromethyl)phenyl]propanoate (13). To a suspension of aluminium(III) chloride ($AlCl_3$) (1.52 g, 11.4 mmol) in CH_2Cl_2 (10 mL), 1,3-dioxolane (1.21 mL, 17.5 mmol) was added and the mixture was stirred at 0 °C for 30 min. Tin (IV) chloride ($SnCl_4$) (2.68 mL, 14.6 mmol), **5** (1.78 g, 7.31 mmol) in CH_2Cl_2 (5 mL) and chloromethylmethyl ether (5.50 mL, 73.1 mmol) were added to the reaction mixture. After stirring at room temperature for 20 h, the mixture was poured into dilute HCl solution, and the product was extracted with CH_2Cl_2. Evaporation of the solvent and purification of the residue by silica gel chromatography (*n*-hexane/AcOEt, 10:1) yielded the title compound as a colorless liquid (50.3%). ^{1}H NMR ($CDCl_3$) δ: 1.49 (3H, d, J = 7.3 Hz, α-CH_3), 3.67 (3H, s, CO_2CH_3), 3.70 (1H, q, J = 7.1 Hz, CH), 4.67 (2H, s, CH_2), 7.26 (1H, dd, J = 7.9, 1.8 Hz, Ar-H6), 7.43 (1H, d, J = 7.9 Hz, Ar-H5), 7.53 (1H, sd, J = 1.8 Hz, Ar-H2). FAB-MS (*m/z*): 291.12 (M^++H, calcd for $C_{11}H_{13}{}^{79}BrClO_2$: 290.98).

5.2.2.3. Methyl 1-[2-bromo-4-(1-methoxy-1-oxopropan-2-yl)-benzyl]-2-oxocyclopentanecarboxylate (14). To a suspension of potassium carbonate (K_2CO_3) (1.26 g, 9.1 mmol) in acetone (20 mL), methyl 2-oxocyclopentanecarboxylate (0.64 mL, 5.1 mmol) was added and the mixture was stirred at room temperature for 30 min. A solution of **13** (1.47 g, 5.1 mmol) in acetone (5 mL) was added and the resulting mixture was refluxed for 12 h. The reaction mixture was cooled to room temperature, filtered through paper, and the filtrate was evaporated to dryness. The resulting residue was purified on silica gel chromatography (*n*-hexane/AcOEt, 7:2) to yield the title compound as a colorless oil (78.0%). ^{1}H NMR (CD_3Cl_3) δ: 1.47 (3H, d, J = 7.3 Hz, α-CH_3), 1.71–2.13 (4H, m, H3′, H4′), 2.36–2.55 (2H, m, H5′), 3.28 (1H, d, J = 14.3 Hz, CH_2), 3.51 (1H, d, J = 14.3 Hz, CH_2), 3.66 (1H, q, J = 7.3 Hz, CH), 3.67 (3H, s, CO_2CH_3), 3.74 (3H, s, CO_2CH_3), 7.12 (2H, d, J = 0.7 Hz, Ar-H5, Ar-H6), 7.49 (1H, s, Ar-H2). FAB-MS (*m/z*): 396.22 (M^++H, calcd for $C_{18}H_{21}{}^{79}BrO_5$: 396.06).

5.2.2.4. General procedure for the decarboxylation and hydrolysis by acid. To the bis-methylester intermediate **14** (ca. 5 mmol) in acetic acid (AcOH) (40 mL), concentrated HCl (80 mL) was added and the mixture was refluxed for 12 h. After cooling to room temperature, the reaction mixture was evaporated to dryness. The resulting residue was diluted with CH_2Cl_2 (50 mL), followed by addition of saturated NaHCO3 solution (50 mL). After removal of organic layer, CH_2Cl_2 (30 mL) was added, and the aqueous layer was adjusted to acidity (pH 1) with 6 M HCl. The organic layer was extracted with CH_2Cl_2, dried over anhydrous Na_2SO_4, and evaporated to dryness. The resulting precipitate was collected to yield the carboxylic acid (precursor of **15**) (92%).

5.2.2.5. General procedure for preparation of the sodium salts of compounds. To a solution of the carboxylic acid (precursor of **15**) in EtOH (30 mL), 1 M NaOH solution (1.0 equiv, ca. 2.2 mmol) was added and refluxed for 2 h. After cooling to room temperature, the resulting mixture was evaporated to dryness. The precipitated product was collected, and recrystallized with ethanol/ether to yield title compounds **15.**

*5.2.2.5.1. Sodium 2-{3-bromo-4-[(2-oxocyclopentyl)methyl]phenyl}propanoate (**15**).* ^{1}H NMR (CD_3OD) δ: 1.38 (3H, d, J = 7.3 Hz, α-CH_3), 1.53–2.02 (4H, m, H3′, H4′), 2.07–2.50 (3H, m, H1′, H5′), 2.56 (1H, dd, J = 13.9, 9.3 Hz, CH_2), 3.22 (1H, dd, J = 13.6, 4.8 Hz, CH_2), 3.52 (1H, q, J = 7.2 Hz, CH), 7.15 (1H, d, J = 7.7 Hz, Ar-H5), 7.26 (1H, d, J = 7.7 Hz, Ar-H6), 7.56 (1H, s, Ar-H3). ^{13}C NMR (CD_3OD) δ: 19.83 (α-CH_3), 21.44 (C5′), 30.15 (C4′), 36.19 (CH_2), 38.80 (C3′), 49.62 (CH), 50.75 (C1′), 125.00 (Ar-C3), 128.04 (Ar-C6), 131.78 (Ar-C5), 132.88 (Ar-C2), 138.05 (Ar-C1), 145.91 (Ar-C4), 182.38 (CO_2Na), 222.37 (C=O). HR-FAB-MS (*m/z*): 369.0089 (M^++Na, calcd for $C_{15}H_{16}{}^{79}BrNa_2O_3$: 369.0078). Anal. Calcd for $C_{15}H_{16}{}^{79}BrNaO_3{\cdot}H_2O$: C, 49.33; H, 4.97. Found: C, 49.42, H, 5.05.

5.2.2.6. General procedure for the Suzuki–Miyaura cross-coupling reaction. The intermediate **14** (1.0 equiv, ca. 0.9 mmol) and each arylboronic acid (R-PhB$(OH)_2$) (1.5 equiv) were dissolved in THF (16 mL), followed by addition of 2 M Na_2CO_3 in water (3 mL) and $Pd(PPh_3)_4$ (0.03 equiv). After refluxing overnight, the reaction mixture was cooled to room temperature, and diluted with water. The mixture was extracted with AcOEt, dried over anhydrous sodium sulfate (Na_2SO_4), and filtered. The filtrate was evaporated to dryness, and the residue was purified on silica gel chromatography (*n*-hexane/AcOEt, 7:2) to yield the biphenyl compound (bis-methylester intermediate, the precursor of **16–23**) as a yellow oil (52–85%). Decarboxylation, hydrolysis by acid and sodium salt preparation of the bis-methylester intermediate (the precursor of **16–23**) was done as described above to yield **16–23.**

*5.2.2.6.1. Sodium 2-{6-[(2-oxocyclopentyl)methyl]biphenyl-3-yl}-propanoate (**16**).* Yield: 69%, three steps. IR (KBr) ν: 1423, 1712 (CO_2^-), 1730 (C=O), cm $^{-1}$. ^{1}H NMR (CD_3OD) δ: 1.32 (3H, d, J = 7.3 Hz, α-CH_3), 1.42–2.35 (6H, m, H3′, H4′, H5′), 2.38–2.50 (1H, m, H1′), 3.05 (1H, dd, J = 14.1, 3.0 Hz, CH_2), 3.14 (1H, d, J = 12.4, 3.0 Hz, CH_2), 3.48 (1H, q, J = 7.1 Hz, CH), 7.05–7.10 (3H, s, Ar-H5, Ar-H6), 7.23 (3H, m, Ar-H2′, Ar-H4′), 7.25–7.31 (2H, m, Ar-H3′), 7.47 (1H, s, Ar-H2). ^{13}C NMR (CD_3OD) δ: 19.88 (α-CH_3), 21.39 (C5′), 30.23 (C4′), 33.32 (CH_2), 36.20 (C3′), 38.79 (CH), 51.65 (C1′), 127.80 (Ar-C5), 128.07 (Ar-C4′), 129.13 (Ar-C2′), 130.44 (Ar-C6), 130.48 (Ar-C3′), 131.78 (Ar-C2), 132.93 (Ar-C1, Ar-C3), 136.08 (Ar-C4), 138.05 (Ar-C4), 143.52 (Ar-C1′), 183.38 (CO_2Na), 222.83 (C=O). HR-FAB-MS (*m/z*): 367.1289 (M^++Na, calcd for $C_{21}H_{21}Na_2O_3$: 367.1286). Anal. Calcd for $C_{21}H_{21}NaO_3{\cdot}H_2O$: C, 76.11; H, 7.00. Found: C, 76.24, H, 7.05.

*5.2.2.6.2. Sodium 2-{4′-methyl-6-[(2-oxocyclopentyl)methyl]biphenyl-3-yl}propanoate (**17**).* Yield: 70%, three steps. IR (KBr) ν: 1420, 1711 (CO_2^-), 1733 (C=O), cm $^{-1}$. ^{1}H NMR (CD_3OD) δ: 1.30–2.16 (6H, m, H3′, H4′, H5′), 1.41 (3H, d, J = 7.3 Hz, α-CH_3), 2.31 (3H, s, Ar-CH_3), 2.34–2.24 (1H, m, H1′), 2.48 (1H, dd, J = 20.5, 12.8 Hz, CH_2), 3.16 (1H, dd, J = 24.4, 13.7 Hz, CH_2), 3.64 (1H, q, J = 7.1 Hz, CH), 7.10 (1H, d, Ar-H6), 7.16–7.18 (5H, m, Ar-H5, Ar-H2′, Ar-H3′), 7.49 (1H, s, Ar-H2). ^{13}C NMR (CD_3OD) δ: 19.22 (α-CH_3), 21.33 (C5′), 30.14 (C4′), 33.30 (Ar-CH_3), 36.32 (CH_2), 38.82 (C3′), 45.79 (CH), 50.40 (C1′), 125.23 (Ar-C5), 127.84 (Ar-C6), 129.84 (Ar-C2′), 130.15 (Ar-C3′), 132.18 (Ar-C3), 132.81 (Ar-C4′), 137.42 (Ar-C2), 140.08 (Ar-C4), 142.54 (Ar-C1), 143.55 (Ar-C1′), 178.38 (CO_2Na), 222.18 (C=O). HR-FAB-MS (*m/z*): 381.1447 (M^++Na, calcd for $C_{22}H_{23}Na_2O_3$: 381.1443). Anal. Calcd for $C_{22}H_{23}NaO_3{\cdot}H_2O$: C, 76.11; H, 7.00. Found: C, 76.24, H, 7.05.

*5.2.2.6.3. Sodium 2-{4′-methoxy-6-[(2-oxocyclopentyl)methyl]-biphenyl-3-yl}propanoate (**18**).* Yield: 75%, three steps. IR (KBr) ν: 1416, 1713 (CO_2^-), 1729 (C=O), cm $^{-1}$. ^{1}H NMR (CD_3OD) δ: 1.21–2.04 (6H, m, H3′, H4′, H5′), 1.41 (3H, d, J = 7.0 Hz, α-CH_3), 2.07–2.22 (1H, m, H1′), 2.39 (1H, dd, J = 14.5, 3.1 Hz, CH_2), 3.15 (1H, dd, J = 14.1, 5.3 Hz, CH_2), 3.57 (1H, q, J = 7.1 Hz, CH), 2.80 (3H, s, Ar-OCH_3), 6.93 (2H, d, J = 7.1 Hz, Ar-H3′), 7.15 (1H, d, J = 7.7 Hz, Ar-H6), 7.17 (1H, s, Ar-H2), 7.19 (2H, d, J = 6.2 Hz, Ar-H2′), 7.27 (1H, dd, J = 8.1, 1.8 Hz, Ar-H5). ^{13}C NMR (CD_3OD) δ: 18.96 (α-CH_3), 21.46 (C5′), 30.27 (C4′), 33.69 (CH_2), 38.56 (C3′), 46.19 (CH), 51.45 (C1′), 55.79 (Ar-OCH_3), 114.85 (Ar-C3′), 115.44 (Ar-C5), 127.34 (Ar-C6), 130.53 (Ar-C2′), 131.37 (Ar-C1′), 137.68

(Ar-C2), 140.20 (Ar-C1), 143.44 (Ar-C3), 157.64 (Ar-C4), 160.28 (Ar-C4′), 178.51 (CO_2Na), 222.83 (C=O). HR-FAB-MS (*m/z*): 397.1389 (M^++Na, calcd for $C_{22}H_{23}Na_2O_4$: 397.1392). Anal. Calcd for $C_{22}H_{23}NaO_4{\cdot}0.5H_2O$: C, 68.92; H, 6.31. Found: C, 68.88, H, 6.25.

*5.2.2.6.4. Sodium 2-{4′-(methylthio)-6-[(2-oxocyclopentyl)met]-hylbiphenyl-3-yl}propanoate (**19**).* Yield: 74%, three steps. IR (KBr) *v*: 1417, 1711 (CO_2^-), 1731 (C=O), cm $^{-1}$. ^{1}H NMR (CD_3OD) *δ*: 1.24–2.22 (7H, m, H1′, H3′, H4′, H5′), 1.41 (3H, d, *J* = 7.0 Hz, α-CH_3), 2.40 (1H, dd, *J* = 13.9, 10.3 Hz, CH_2), 2.49 (3H, s, Ar-SCH_3), 3.15 (1H, dd, *J* = 13.9, 4.4 Hz, CH_2), 3.57 (1H, q, *J* = 7.1 Hz, CH), 7.15–7.23 (4H, m, Ar-H5, Ar-H6, Ar-H3′), 7.27–7.30 (3H, m, Ar-H2, Ar-H2′). ^{13}C NMR (CD_3OD) *δ*: 15.74 (Ar-SCH_3), 19.96 (α-CH_3), 21.31 (C5′), 30.33 (C4′), 33.37 (CH_2), 38.71 (C3′), 49.91 (CH), 51.66 (C1′), 127.32 (Ar-C3′), 127.78 (Ar-C5), 130.46 (Ar-C1), 130.54 (Ar-C6), 130.94 (Ar-C2′), 136.18 (Ar-C3), 138.58 (Ar-C2), 140.23 (Ar-C1′), 142.54 (Ar-C4), 143.41 (Ar-C4′), 183.03 (CO_2Na), 222.83 (C=O). HR-FAB-MS (*m/z*): 413.1169 (M^++Na, calcd for $C_{22}H_{23}Na_2SO_3$: 413.1163). Anal. Calcd for $C_{22}H_{23}NaSO_3{\cdot}0.5H_2O$: C, 66.15; H, 6.06. Found: C, 66.28, H, 6.05.

*5.2.2.6.5. Sodium 2-{4′-fluoro-6-[(2-oxocyclopentyl)methyl]biphenyl-3-yl}propanoate (**20**).* Yield: 68%, three steps. IR (KBr) *v*: 1203 (Ar-F), 1410, 1709 (CO_2^-), 1730 (C=O), cm $^{-1}$. ^{1}H NMR (CD_3OD) *δ*: 1.23–2.23 (7H, m, H1′, H3′, H4′, H5′), 1.41 (3H, d, *J* = 7.3 Hz, α-CH_3), 2.39 (1H, dd, *J* = 14.1, 10.1 Hz, CH_2), 3.13 (1H, dd, *J* = 14.1, 4.2 Hz, CH_2), 3.58 (1H, q, *J* = 7.2 Hz, CH), 7.07–7.19 (4H, m, Ar-H5, Ar-H6, Ar-H3′), 7.25–7.31 (3H, m, Ar-H2, Ar-H2′). ^{13}C NMR (CD_3OD) *δ*: 19.95 (α-CH_3), 21.31 (C5′), 30.32 (C4′), 33.32 (CH_2), 38.68 (C3′), 49.91 (CH), 51.66 (C1′), 115.83 (d, J_{C-F} = 21.1 Hz, Ar-C3′), 127.97 (Ar-C5), 130.54 (d, J_{C-F} = 3.7 Hz, Ar-C2′), 132.19 (Ar-C6), 132.29 (Ar-C2), 132.29 (Ar-C1), 139.60 (d, J_{C-F} = 3.1 Hz, Ar-C1′), 142.06 (Ar-C3), 143.46 (Ar-C4), 164.98 (Ar-C4′), 183.02 (CO_2Na), 222.67 (C=O). HR-FAB-MS (*m/z*): 385.1199 (M^++Na, calcd for $C_{21}H_{20}FNa_2O_3$: 385.1192). Anal. Calcd for $C_{21}H_{20}FNaO_3{\cdot}H_2O$: C, 66.31; H, 5.83. Found: C, 66.28, H, 5.99.

*5.2.2.6.6. Sodium 2-{6-[(2-oxocyclopentyl)methyl]-4′-(trifluoromethoxy)biphenyl-3-yl}propanoate (**21**).* Yield: 54%, three steps. IR (KBr) *v*: 1422, 1709 (CO_2^-), 1731 (C=O), cm^{-1}. ^{1}H NMR (CD_3OD) *δ*: 1.35–2.23 (7H, m, H1′, H3′, H4′, H5′), 1.41 (3H, d, *J* = 7.3 Hz, α-CH_3), 2.41 (1H, dd, *J* = 7.1, 3.5 Hz, CH_2), 3.14 (1H, dd, *J* = 14.1, 5.7 Hz, CH_2), 3.58 (1H, q, *J* = 7.1 Hz, CH), 7.19–7.40 (7H, m, Ar-H2, Ar-H5, Ar-H6, Ar-H2′, Ar-H3′). ^{13}C NMR (CD_3OD) *δ*: 19.95 (α-CH_3), 21.30 (C5′), 30.34 (C4′), 33.24 (CH_2), 38.66 (C3′), 49.91 (CH), 51.69 (C1′), 121.70 (d, *J* = 1.2 Hz, Ar-C3′), 128.24 (Ar-C5), 129.27 (Ar-OCF_3), 130.43 (Ar-C6), 130.61 (Ar-C1′), 132.19 (Ar-C2′), 136.16 (Ar-C2), 141.64 (Ar-C1), 142.63 (Ar-C3), 143.61 (Ar-C4), 149.48 (d, *J* = 1.2 Hz, Ar-C4′), 183.02 (CO_2Na), 222.57 (C=O). HR-FAB-MS (*m/z*): 451.1112 (M^++Na, calcd for $C_{22}H_{20}F_3Na_2O_4$: 451.1109). Anal. Calcd for $C_{22}H_{20}F_3NaO_4{\cdot}H_2O$: C, 59.19; H, 4.97. Found: C, 59.22, H, 5.00.

*5.2.2.6.7. Sodium 5′-(1-carboxylatoethyl)-2′-[(2-oxocyclopentyl)]-methyl]biphenyl-4-carboxylate (**22**).* Yield: 81%, three steps. IR (KBr) *v*: 1424, 1690, 1720 (CO_2^-), 1728 (C=O), cm^{-1}. ^{1}H NMR (CD_3OD) *δ*: 1.18–2.17 (7H, m, H1′, H3′, H4′, H5′), 1.38 (3H, d, *J* = 7.1 Hz, α-CH_3), 2.38 (1H, dd, *J* = 14.5, 10.1 Hz, CH_2), 3.11 (1H, dd, *J* = 14.1, 5.1 Hz, CH_2), 3.55 (1H, q, *J* = 7.0 Hz, CH), 7.15–7.28 (5H, m, Ar-H2, Ar-H5, Ar-H6, Ar-H2′), 7.95 (2H, dd, *J* = 6.5, 1.9 Hz, Ar-H3′). ^{13}C NMR (CD_3OD) *δ*: 19.02 (α-CH_3), 21.30 (C5′), 30.39 (C4′), 33.32 (CH_2), 38.58 (C3′), 46.16 (CH), 51.61 (C1′), 128.17 (Ar-C5), 130.06 (Ar-C6), 130.59 (Ar-C2′), 130.62 (Ar-C3′), 130.75 (Ar-C2), 131.19 (Ar-C1), 137.46 (Ar-C3), 140.53 (Ar-C4), 142.70 (Ar-C4′), 147.85 (Ar-C1′), 169.66 (Ar-CO_2Na), 178.18 (CO_2Na), 222.35 (C=O). HR-FAB-MS (*m/z*): 433.1002 (M^++Na, calcd for $C_{22}H_{20}Na_3O_5$: 433.1004). Anal. Calcd for $C_{22}H_{20}Na_2O_5{\cdot}2H_2O$: C, 59.19; H, 5.42. Found: C, 59.31, H, 5.27.

*5.2.2.6.8. Sodium 2-{4′-hydroxy-6-[(2-oxocyclopentyl)methyl]]-biphenyl-3-yl}propanoate (**23**).* Yield: 47%, three steps. IR (KBr) *v*: 1316 (Ar-OH), 1422, 1714 (CO_2^-), 1733 (C=O), cm^{-1}. ^{1}H NMR (CD_3OD) *δ*: 1.36–2.23 (7H, m, H1′, H3′, H4′, H5′), 1.40 (3H, d, *J* = 6.6 Hz, α-CH_3), 2.40 (1H, dd, *J* = 13.9, 10.3 Hz, CH_2), 3.15 (1H, dd, *J* = 13.9, 4.4 Hz, CH_2), 3.56 (1H, q, *J* = 6.8 Hz, CH), 6.80 (2H, dd, *J* = 6.6, 2.2 Hz, Ar-H3′), 7.17–7.07 (4H, m, Ar-H2, Ar-H6, Ar-H2′), 7.24 (1H, dd, *J* = 7.7, 1.8 Hz, Ar-H5). ^{13}C NMR (CD_3OD) *δ*: 19.97 (α-CH_3), 21.30 (C5′), 30.27 (C4′), 33.45 (CH_2), 38.77 (C3′), 50.00 (CH), 51.61 (C1′), 115.90 (Ar-C3′), 127.34 (Ar-C5), 130.43 (Ar-C6), 130.66 (Ar-C1′), 131.45 (Ar-C2′), 134.68 (Ar-C2), 136.29 (Ar-C1), 143.09 (Ar-C3), 143.28 (Ar-C4), 157.41 (Ar-C4′), 183.24 (CO_2Na), 223.09 (C=O). HR-FAB-MS (*m/z*): 360.1332 (M^++Na, calcd for $C_{21}H_{21}Na_2O_4$: 360.1338). Anal. Calcd for $C_{21}H_{21}NaO_4{\cdot}H_2O$: C, 66.66; H, 6.13. Found: C, 66.58, H, 6.11.

5.2.3. Synthesis of loxoprofen derivatives with modification at the 2-position of the phenyl ring by para-substituted aryl group (24–31)

A carboxy group of 2-{2-bromo-4-[(2-oxocyclopentyl)methyl]phenyl}propanoic acid was methyl esterificated to give methyl 2-{2-bromo-4-[(2-oxocyclopentyl)methyl]phenyl}propanoate (see below), which was then reacted with corresponding arylboronic acid under the conditions of Suzuki–Miyaura coupling reaction, as described above. The resulting biphenyl compounds were hydrolyzed by base (see below), and converted to the sodium salt by the same procedure described above.

5.2.3.1. Methyl ester protection of the carboxy group of 2-{2-bromo-4-[(2-oxocyclopentyl)methyl]phenyl}propanoic acid. To 2-{2-bromo-4-[(2-oxocyclopentyl)methyl]phenyl}propanoic acid (1.5 equiv, ca. 3.3 mmol) in CH_2Cl_2 (30 mL) and methanol (2 equiv, ca. 4.4 mmol), DMAP (1 equiv, ca. 2.2 mmol) and EDC (2 equiv, ca. 4.4 mmol) were added, followed by stirring for 15 min at room temperature. The reaction mixture was poured into cold water, and the resulting solution was extracted with CH_2Cl_2. Evaporation of the solvent and purification of the residue by silica gel chromatography (*n*-hexane/AcOEt, 3:1) yielded methyl 2-{2-bromo-4-[(2-oxocyclopentyl)methyl]phenyl}propanoate as a colorless oil (92%).

5.2.3.2. General procedure for alkaline hydrolysis. To the methylester intermediate (biphenyl compound from **4b**) (ca. 5 mmol) in ethanol (20 mL), 0.063 mM aqueous solution of KOH (5 mL) was added and refluxed for 2 h. After cooling to room temperature, the reaction mixture was evaporated to dryness. The resulting residue was diluted with CH_2Cl_2 (50 mL) and saturated $NaHCO_3$ solution (50 mL) was added. The organic layer was removed, CH_2Cl_2 (30 mL) was added, and the aqueous layer was adjusted to acidity (pH 1) with 6 M HCl. The organic layer was extracted with CH_2Cl_2, dried over anhydrous Na_2SO_4, and evaporated to dryness. The resulting precipitate was collected to yield the precursor of **24–31** (90–94%).

5.2.3.3. Sodium 2-{5-[(2-oxocyclopentyl)methyl]biphenyl-2-yl}propanoate (24). Yield: 74%, three steps. IR (KBr) *v*: 1422, 1713 (CO_2^-), 1731 (C=O), cm^{-1}. ^{1}H NMR (CD_3OD) *δ*: 1.23 (3H, dd, *J* = 7.3, 1.5 Hz, α-CH_3), 1.56–2.44 (7H, m, H1′, H3′, H4′, H5′), 2.53 (1H, dd, *J* = 13.6, 9.2 Hz, CH_2), 3.05 (1H, d, *J* = 13.7, 4.2 Hz, CH_2), 3.71 (1H, q, *J* = 7.2 Hz, CH), 6.94 (1H, s, Ar-H3), 7.10 (1H, d, *J* = 8.1 Hz, Ar-H5), 7.32–7.39 (5H, m, Ar-H2′, Ar-H3′, Ar-H4′), 7.46 (1H, d, *J* = 8.1 Hz, Ar-H6). ^{13}C NMR (CD_3OD) *δ*: 21.28 (α-CH_3), 21.47 (C5′), 30.10 (C4′), 36.08 (CH_2), 39.10 (C3′), 45.71 (CH), 52.13 (C1′), 127.72 (Ar-C1), 128.67 (Ar-C4′), 128.99 (Ar-C2′), 129.04 (Ar-C3′), 130.67 (Ar-C6), 131.29 (Ar-C3), 138.38 (Ar-C2), 141.56 (Ar-C4), 143.12 (Ar-C4), 143.49 (Ar-C1′), 183.50 (CO_2Na), 223.14 (C=O). HR-FAB-MS (*m/z*): 367.1291 (M^++Na, calcd for

$C_{21}H_{21}Na_2O_3$: 367.1286). Anal. Calcd for $C_{21}H_{21}NaO_3 \cdot 0.5H_2O$: C, 71.22; H, 6.36. Found: C, 71.37, H, 6.27.

5.2.3.4. Sodium 2-{4′-methyl-5-[(2-oxocyclopentyl)methyl]]biphenyl-2-yl}propanoate (25). Yield: 77%, three steps. IR (KBr) *ν*: 1420, 1712 (CO_2^-), 1733 (C=O), cm $^{-1}$. ^{1}H NMR (CD_3OD) *δ*: 1.21 (3H, dd, *J* = 7.3, 1.5 Hz, α-CH_3), 1.54–2.44 (7H, m, H1′, H3′, H4′, H5′), 2.36 (3H, s, Ar-CH_3), 2.51 (1H, dd, *J* = 13.4, 9.3 Hz, CH_2), 3.04 (1H, dd, *J* = 13.7, 9.3 Hz, CH_2), 3.72 (1H, q, *J* = 7.1 Hz, CH), 6.92 (1H, t, *J* = 1.8 Hz, Ar-H3), 7.08 (1H, dt, *J* = 8.1, 1.8 Hz, Ar-H5), 7.18 (2H, d, *J* = 7.7 Hz, Ar-H3′), 7.26 (2H, d, *J* = 7.7 Hz, Ar-H2′), 7.44 (1H, d, *J* = 8.1 Hz, Ar-H6). ^{13}C NMR (CD_3OD) *δ*: 21.19 (α-CH_3), 21.47 (C5′), 21.44 (C4′), 30.08 (Ar-CH_3), 36.07 (CH_2), 39.08 (C3′), 45.70 (CH), 52.12 (C1′), 128.61 (Ar-C1), 128.80 (Ar-C5), 129.58 (Ar-C2′), 130.53 (Ar-C3′), 131.31 (Ar-C6), 137.33 (Ar-C3), 138.29 (Ar-C4′), 140.52 (Ar-C2), 141.64 (Ar-C1′), 143.09 (Ar-C4), 183.58 (CO_2Na), 223.09 (C=O). HR-FAB-MS (*m/z*): 381.1439 (M^++Na, calcd for $C_{22}H_{23}Na_2O_3$: 381.1443). Anal. Calcd for $C_{22}H_{23}NaO_3 \cdot 0.5H_2O$: C, 72.03; H, 6.66. Found: C, 71.92, H, 6.58.

5.2.3.5. Sodium 2-{4′-methoxy-5-[(2-oxocyclopentyl)methyl]biphenyl-2-yl}propanoate (26). Yield: 70%, three steps. IR (KBr) *ν*: 1416, 1711 (CO_2^-), 1732 (C=O), cm^{-1}. ^{1}H NMR (CD_3OD) *δ*: 1.22 (3H, dd, *J* = 7.0, 1.5 Hz, α-CH_3), 1.53–2.40 (7H, m, H1′, H3′, H4′, H5′), 2.51 (1H, dd, *J* = 13.6, 9.5 Hz, CH_2), 3.04 (1H, dd, *J* = 13.4, 3.8 Hz, CH_2), 3.73 (1H, q, *J* = 7.0 Hz, CH), 3.81 (3H, s, Ar-OCH_3), 6.92–6.95 (3H, m, Ar-H3, Ar-H3′), 7.07 (1H, d, *J* = 8.1 Hz, Ar-H5), 7.31 (2H, d, *J* = 8.4 Hz, Ar-H2′), 7.43 (1H, d, *J* = 8.1 Hz, Ar-H6). ^{13}C NMR (CD_3OD) *δ*: 21.23 (α-CH_3), 21.45 (C5′), 30.08 (C4′), 36.08 (CH_2), 39.09 (C3′), 45.70 (CH), 52.03 (C1′), 55.72 (Ar-OCH_3), 114.43 (Ar-C2′), 128.60 (Ar-C1), 128.72 (Ar-C5), 131.44 (Ar-C6), 131.70 (Ar-C3′), 135.81 (Ar-C3), 138.29 (Ar-C4′), 141.73 (Ar-C2), 142.78 (Ar-C1′), 160.07 (Ar-C4), 183.60 (CO_2Na), 223.10 (C=O). HR-FAB-MS (*m/z*): 397.1399 (M^++Na, calcd for $C_{22}H_{23}Na_2O_4$: 397.1392). Anal. Calcd for $C_{22}H_{23}NaO_4 \cdot H_2O$: C, 67.22; H, 6.38. Found: C, 67.33, H, 6.42.

5.2.3.6. Sodium 2-(4′-(methylthio)-5-((2-oxocyclopentyl)methyl)biphenyl-2-yl)propanoate (27). Yield: 60%, three steps. IR (KBr) *ν*: 1417, 1712 (CO_2^-), 1730 (C=O), cm $^{-1}$. ^{1}H NMR (CD_3OD) *δ*: 1.23 (3H, dd, *J* = 7.1, 1.3 Hz, α-CH_3), 1.55–2.45 (7H, m, H1′, H3′, H4′, H5′), 2.49 (3H, s, Ar-SCH_3), 2.52 (1H, dd, *J* = 13.9, 9.2 Hz, CH_2), 3.05 (1H, dd, *J* = 13.6, 4.0 Hz, CH_2), 3.70 (1H, q, *J* = 7.2 Hz, CH), 6.94 (1H, t, *J* = 1.8 Hz, Ar-H3), 7.10 (1H, dt, *J* = 8.1, 2.2 Hz, Ar-H5), 7.31 (4H, dd, *J* = 14.5, 8.6 Hz, Ar-H2′, Ar-H3′), 7.45 (1H, d, *J* = 8.1 Hz, Ar-H6). ^{13}C NMR (CD_3OD) *δ*: 15.91 (Ar-SCH_3), 21.21 (α-CH_3), 21.45 (C5′), 30.09 (C4′), 36.06 (CH_2), 39.06 (C3′), 45.73 (CH), 52.12 (C1′), 127.36 (Ar-C3′), 128.70 (Ar-C1), 128.98 (Ar-C5), 129.05 (Ar-C6), 131.17 (Ar-C2′), 138.33 (Ar-C3), 138.44 (Ar-C2), 140.32 (Ar-C1′), 141.61 (Ar-C4), 142.49 (Ar-C4′), 183.42 (CO_2Na), 223.01 (C=O). HR-FAB-MS (*m/z*): 413.1165 (M^++Na, calcd for $C_{22}H_{23}Na_2SO_3$: 413.1163). Anal. Calcd for $C_{22}H_{23}NaSO_3 \cdot H_2O$: C, 64.54; H, 6.10. Found: C, 64.69, H, 6.17.

5.2.3.7. Sodium 2-{4′-fluoro-5-[(2-oxocyclopentyl)methyl]biphenyl-2-yl}propanoate (28). Yield: 64%, three steps. IR (KBr) *ν*: 1204 (Ar-F), 1414, 1710 (CO_2^-), 1730 (C=O), cm^{-1}. ^{1}H NMR (CD_3OD) *δ*: 1.22 (3H, dd, *J* = 7.3, 1.1 Hz, α-CH_3), 1.55–2.41 (7H, m, H1′, H3′, H4′, H5′), 2.52 (1H, dd, *J* = 13.6, 9.2 Hz, CH_2), 3.05 (1H, dd, *J* = 13.6, 4.0 Hz, CH_2), 3.64 (1H, q, *J* = 7.2 Hz, CH), 6.93 (1H, t, *J* = 1.8 Hz, Ar-H3), 7.13–7.07 (3H, m, Ar-H5, Ar-H3′), 7.38–7.46 (3H, m, Ar-H6, Ar-H2′). ^{13}C NMR (CD_3OD) *δ*: 21.08 (α-CH_3), 21.45 (C5′), 30.09 (C4′), 36.04 (CH_2), 39.06 (C3′), 45.73 (CH), 52.09 (C1′), 115.60 (d, J_{C-F} = 21.1 Hz, Ar-C3′), 128.68 (Ar-C1), 129.19 (Ar-C5), 131.32 (Ar-C6), 132.46 (d, J_{C-F} = 8.1 Hz, Ar-C2′), 138.50 (Ar-C3), 139.57 (d, J_{C-F} = 3.7 Hz, Ar-C1′), 141.64 (Ar-C2), 142.02 (Ar-C4), 142.49 (d, J_{C-F} = 1.9 Hz, Ar-C4′), 183.28 (CO_2Na), 222.98 (C=O). HR-FAB-MS (*m/z*): 385.1188 (M^++Na, calcd for $C_{21}H_{20}FNa_2O_3$: 385.1192). Anal. Calcd for $C_{21}H_{20}FNaO_3 \cdot H_2O$: C, 66.31; H, 5.83. Found: C, 66.44, H, 5.76.

5.2.3.8. Sodium 2-{5-[(2-oxocyclopentyl)methyl]-4′-(trifluoromethoxy)biphenyl-2-yl}propanoate (29). Yield: 56%, three steps. IR (KBr) *ν*: 1421, 1709 (CO_2^-), 1731 (C=O), cm $^{-1}$. ^{1}H NMR (CD_3OD) *δ*: 1.25 (3H, dd, *J* = 7.0, 1.1 Hz, α-CH_3), 1.51–2.45 (7H, m, H1′, H3′, H4′, H5′), 2.53 (1H, dd, *J* = 13.6, 9.5 Hz, CH_2), 3.05 (1H, dd, *J* = 13.6, 4.0 Hz, CH_2), 3.62 (1H, q, *J* = 7.2 Hz, CH), 6.95 (1H, t, *J* = 2.2 Hz, Ar-H3), 7.13 (1H, dt, *J* = 8.1, 2.2 Hz, Ar-H3′), 7.28 (2H, dd, *J* = 8.8, 0.7 Hz, Ar-H5,), 7.46–7.51 (3H, m, Ar-H6, Ar-H2′). ^{13}C NMR (CD_3OD) *δ*: 21.12 (α-CH_3), 21.44 (C5′), 30.08 (C4′), 35.99 (CH_2), 39.04 (C3′), 47.75 (CH), 52.04 (C1′), 121.51 (Ar-C3′), 128.77 (Ar-C1), 129.46 (Ar-C5), 131.17 (Ar-OCF_3), 131.23 (Ar-C6), 132.37 (Ar-C2′), 138.62 (Ar-C1′), 141.52 (Ar-C3), 141.57 (Ar-C2), 142.58 (Ar-C4),, 149.45 (Ar-C4′), 183.28 (CO_2Na), 222.98 (C=O). HR-FAB-MS (*m/z*): 451.1107 (M^++Na, calcd for $C_{22}H_{20}F_3Na_2O_4$: 451.1109). Anal. Calcd for $C_{22}H_{20}F_3NaO_4 \cdot 0.5H_2O$: C, 60.41; H, 4.84. Found: C, 60.34, H, 4.98.

5.2.3.9. Sodium 2′-(1-carboxylatoethyl)-5′-[(2-oxocyclopentyl)-methyl]biphenyl-4-carboxylate (30). Yield: 74%, three steps. IR (KBr) *ν*: 1420, 1689, 1712 (CO_2^-), 1727 (C=O), cm $^{-1}$. ^{1}H NMR (CD_3OD) *δ*: 1.22 (3H, dd, *J* = 7.1, 1.6 Hz, α-CH_3), 1.33–2.42 (7H, m, H1′, H3′, H4′, H5′), 2.53 (1H, dd, *J* = 13.6, 9.2 Hz, CH_2), 3.06 (1H, dd, *J* = 13.6, 4.0 Hz, CH_2), 3.73 (1H, q, *J* = 7.1 Hz, CH), 6.96 (1H, st, *J* = 1.6 Hz, Ar-H3), 7.11 (1H, dt, *J* = 8.1, 1.8 Hz, Ar-H5), 7.40 (2H, d, *J* = 8.4 Hz, Ar-H2′), 7.44 (1H, d, *J* = 8.1 Hz, Ar-H6), 7.99 (2H, d, *J* = 8.4 Hz, Ar-H3′). ^{13}C NMR (CD_3OD) *δ*: 20.00 (α-CH_3), 21.45 (C5′), 30.11 (C4′), 36.06 (CH_2), 39.05 (C3′), 45.55 (CH), 52.11 (C1′), 128.64 (Ar-C1), 129.18 (Ar-C5′), 130.01 (Ar-C2′), 130.10 (Ar-C3′), 131.23 (Ar-C6), 137.37 (Ar-C3), 138.52 (Ar-C2), 141.47 (Ar-C4′), 142.81 (Ar-C4), 145.50 (Ar-C1′), 175.36 (Ar-CO_2Na), 183.17 (CO_2Na), 223.02 (C=O). HR-FAB-MS (*m/z*): 433.1001 (M^++Na, calcd for $C_{22}H_{20}Na_3O_5$: 433.1004). Anal. Calcd for $C_{22}H_{20}Na_2O_5 \cdot H_2O$: C, 61.68; H, 5.18. Found: C, 61.54, H, 5.06.

5.2.3.10. Sodium 2-{4′-hydroxy-5-[(2-oxocyclopentyl)methyl]-biphenyl-2-yl}propanoate (31). Yield: 50%, three steps. IR (KBr) *ν*: 1318 (Ar-OH), 1421, 1710 (CO_2^-), 1731 (C=O), cm^{-1}. ^{1}H NMR (CD_3OD) *δ*: 1.22 (3H, dd, *J* = 7.3, 1.5 Hz, α-CH_3), 1.52–2.42 (7H, m, H1′, H3′, H4′, H5′), 2.50 (1H, d, *J* = 13.9 Hz, CH_2), 3.02 (1H, d, *J* = 13.6 Hz, CH_2), 3.75 (1H, q, *J* = 7.2 Hz, CH), 6.80 (2H, d, *J* = 8.4 Hz, Ar-H3′), 6.92 (1H, s, Ar-H3), 7.05 (1H, d, *J* = 8.1 Hz, Ar-H5), 7.21 (2H, d, *J* = 8.4 Hz, Ar-H2′), 7.42 (1H, d, *J* = 8.1 Hz, Ar-H6). ^{13}C NMR (CD_3OD) *δ*: 21.32 (α-CH_3), 30.10 (C5′), 36.10 (C4′), 39.09 (CH_2), 45.72 (C3′), 52.20 (CH), 58.31 (C1′), 116.15 (Ar-C3′), 128.40 (Ar-C1), 128.54 (Ar-C5), 131.51 (Ar-C6), 131.66 (Ar-C2′), 134.03 (Ar-C1′), 138.19 (Ar-C3), 141.76 (Ar-C2), 143.24 (Ar-C4), 158.47 (Ar-C4′), 183.81 (CO_2Na), 219.16 (C=O). HR-FAB-MS (*m/z*): 361.1414 (M^++H, calcd for $C_{21}H_{22}NaO_4$: 361.1416). Anal. Calcd for $C_{21}H_{21}NaO_4 \cdot H_2O$: C, 68.28; H, 6.00. Found: C, 68.30, H, 6.09.

5.2.4. *Synthesis of the alcohol derivative of* 31 (32, 33)

A methyl ester intermediate derived from **31** was reduced by $NaBH_4$ (see below) and alkaline hydrolyzed.

5.2.4.1. Reduction of methyl ester intermediate derived from 31 with $NaBH_4$. To a stirred solution of methyl ester intermediate derived from **31** (1 equiv, ca. 1.8 mmol) in EtOH, $NaBH_4$ (1.3 equiv, ca. 2.4 mmol) was added, stirred for 1 h at room temperature, quenched by the addition of a few ice chips, and the resulting solution was extracted with CH_2Cl_2. The extracts were dried over anhydrous Na_2SO_4 and filtrated. The filtrate was evaporated to

dryness, and the mixture was separated into *cis*-alcohol and *trans*-alcohol as two kinds of colorless oil by silica gel chromatography (*n*-hexane/AcOEt, 7:2) (93–95%).

5.2.4.2. (±)-2-{4′-Hydroxy-5-[(trans-2-hydroxycyclopentyl)methyl]biphenyl-2-yl}propanoic acid (32). Yield: 82%, three steps. IR (KBr) *ν*: 1321 (Ar-OH), 1421, 1714 (CO_2^-), 1733 (C=O), 3466 (OH), cm^{-1}. ^1H NMR (CD_3OD) *δ*: 1.27 (3H, d, *J* = 7.1 Hz, α-CH_3), 1.20–1.94 (6H, m, H3′, H4′, H5′), 1.95–2.06 (1H, m, H1′), 2.38 (1H, dd, *J* = 13.6, 9.2 Hz, CH_2), 2.85 (1H, dd, *J* = 13.6, 5.7 Hz, CH_2), 3.78–3.88 (2H, m, CH, H2′), 6.83 (2H, d, *J* = 8.4 Hz, Ar-H3′), 7.00 (1H, s, Ar-H3), 7.11–7.16 (3H, m, Ar-H5, Ar-H2′), 7.27 (1H, d, *J* = 8.1 Hz, Ar-H6), 10.57 (1H, br s, CO_2H). ^{13}C NMR (CD_3OD) *δ*: 19.72 (α-CH_3), 22.37 (C4′), 30.30 (C5′), 34.64 (CH_2), 40.11 (C3′), 42.04 (CH), 50.67 (C1′), 78.83 (C2′), 115.94 (Ar-C3′), 127.66 (Ar-C1), 128.98 (Ar-C5), 131.55 (Ar-C6), 131.88 (Ar-C3), 133.97 (Ar-C1′), 137.99 (Ar-C2′), 140.95 (Ar-C2), 143.03 (Ar-C4), 157.73 (Ar-C4′), 178.94 (CO_2H). HR-FAB-MS (*m/z*): 340.1677 (M^+, calcd for $C_{21}H_{24}O_4$: 340.1675). Anal. Calcd for $C_{21}H_{24}O_4 \cdot 0.25H_2O$: C, 73.13; H, 7.16. Found: C, 72.91, H, 7.30.

5.2.4.3. (±)-2-{4′-Hydroxy-5-[(*cis*-2-hydroxycyclopentyl)methyl]-biphenyl-2-yl}propanoic acid (33). Yield: 80%, three steps. IR (KBr) *ν*: 1318 (Ar-OH), 1420, 1714 (CO_2^-), 1731 (C=O), 3466 (OH), cm^{-1}. ^1H NMR (CD_3OD) *δ*: 1.27 (3H, d, *J* = 7.0 Hz, α-CH_3), 1.44–1.87 (6H, m, H3′, H4′, H5′), 1.90–2.02 (1H, m, H1′), 2.56 (1H, dd, *J* = 13.4, 8.2 Hz, CH_2), 2.87 (1H, dd, *J* = 6.8, 3.4 Hz, CH_2), 3.85 (1H, q, *J* = 7.1 Hz, CH), 4.04 (1H, br s, H2′), 6.80 (2H, d, *J* = 8.4 Hz, Ar-H3′), 7.04 (1H, s, Ar-H3), 7.15 (3H, d, *J* = 8.4 Hz, Ar-H5, Ar-H2′), 7.26 (1H, d, *J* = 8.1 Hz, Ar-H6), 10.56 (1H, br s, CO_2H). ^{13}C NMR (CD_3OD) *δ*: 19.37 (α-CH_3), 22.56 (C4′), 29.62 (C5′), 35.41 (CH_2), 36.07 (C3′), 42.03 (CH), 50.67 (C1′), 75.18 (C2′), 115.92 (Ar-C3′), 127.60 (Ar-C1), 128.89 (Ar-C5), 131.56 (Ar-C6), 131.79 (Ar-C3), 134.07 (Ar-C1′), 137.77 (Ar-C2′), 141.84 (Ar-C2), 142.98 (Ar-C4), 157.69 (Ar-C4′), 178.99 (CO_2H). HR-FAB-MS (*m/z*): 340.1678 (M^+, calcd for $C_{21}H_{24}O_4$: 340.1675). Anal. Calcd for $C_{21}H_{24}O_4 \cdot 0.5H_2O$: C, 72.18; H, 7.21. Found: C, 72.35, H, 7.29.

5.3. Membrane permeability assay

Permeabilization of calcein-loaded liposomes was assayed as described previously,[14] with some modifications. Liposomes were prepared using the reversed-phase evaporation method. Egg phosphatidylcholine (PC) (10 μmol, 7.7 mg) was dissolved in chloroform/methanol (1:2, v/v), dried, dissolved in 1.5 mL of diethyl ether, and added to 1 mL of 100 mM calcein/NaOH (pH 7.4). The mixture was then sonicated to obtain a homogenous emulsion. The diethyl ether solvent was removed and the resulting suspension of liposomes was centrifuged and washed twice with fresh buffer A (10 mM phosphate buffer (Na_2HPO_4–NaH_2PO_4) (pH 6.8) containing 150 mM NaCl) to remove untrapped calcein. The final liposome precipitate was re-suspended in 5 mL buffer A. A 30 μL aliquot of this suspension was diluted with buffer A to 20 mL, and 12 or 400 μL of this diluted suspension was then incubated at 30 °C for 10 min in the presence of the compound under investigation. Control experiments were performed after addition of the same volume of water. The release of calcein from liposomes was determined by measuring the fluorescence intensity at 520 nm (excitation at 490 nm).

5.4. Human whole blood COX assay

This assay was performed as decribed[43] with some modifications. Fresh blood was collected in tubes (Protein Lobinding tube, Eppendorf Co., Ltd, Tokyo, Japan) by venipuncture from healthy volunteers who had no apparent inflammatory conditions and had not taken any NSAIDs for least 7 days prior to blood collection.

5.4.1. COX-1 assay

Aliquots of blood (500 μL) were incubated with 2 μL of test compound for 24 h at 37 °C, then centrifuged to obtain plasma. Aliquots (100 μL) of plasma were mixed with 400 μL methanol and centrifuged. The amount of TXB_2 in the supernatant was determined using an EIA kit (Cayman, Ann Arbor, MI) according to the manufacturer's protocol.

5.4.2. COX-2 assay

Blood samples (500 μL) were incubated with 100 μg/mL lipopolysaccharide (Sigma–Aldrich Japan Inc., Tokyo, Japan) for 24 h at 37 °C after addition of 2 μL of test compound, then centrifuged to obtain plasma. Aliquots (100 μL) of plasma were mixed with 400 μL methanol and centrifuged. The amount of PGE_2 in the supernatant was determined using an EIA kit (Cayman, Ann Arbor, MI) according to the manufacturer's protocol.

5.5. Gastric damage assay and determination of gastric level of PGE_2

Wistar rats (6 weeks old, 180–200 g, male) were obtained from Kyudo Co., Ltd (Kumamoto, Japan). The experiments and procedures described here were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health (Bethesda, MD), and were approved by the Animal Care Committee of Kumamoto University.

The gastric ulcerogenic response was examined as described previously,[20] with some modifications. Rats fasted for 18 h were orally administered NSAIDs. Eight hours later, the animals were sacrificed, after which their stomachs were removed and the areas of gastric mucosal lesions were measured by an observer unaware of the treatment they had received. Calculation of the scores involved measuring the area of all the lesions in square millimeters and summing the values to give an overall gastric lesion index. The gastric PGE_2 level was determined by EIA according to the manufacturer's instructions.

5.6. Carrageenan-induced rat paw edema

This assay was carried out as described previously.[44] Rats were orally administered NSAIDs and 1 h later received a 100 μL intradermal injection of carrageenan (1%) into the left hindpaw. Paw volume was measured using a plethysmometer, which measures water displacement when the paw is submerged in a water cell. The percentage difference in volume between both paws was shown as edema (%). The PGE_2 level in the paw was determined by EIA according to the manufacturer's instructions.

5.7. Statistical analysis

All values are expressed as the mean ± SEM. The Tukey test or the Student's *t*-test for unpaired results was used to evaluate differences between more than three groups or between two groups, respectively. Differences were considered to be significant for values of $P < 0.05$.

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Supplementary data

Supplementary data (NMR spectra of final compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.04.050.

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Protective effect of β-(1,3 → 1,6)-D-glucan against irritant-induced gastric lesions

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Abstract

β-(1,3)-D-Glucan with β-(1,6) branches has been reported to have various pharmacological activities, such as anti-tumour and anti-infection activities, which result from its immunomodulating effects. Gastric lesions result from an imbalance between aggressive and defensive factors. In the present study, we examined the effect of β-(1,3)-D-glucan with β-(1,6) branches isolated from *Aureobasidium pullulans* on the gastric ulcerogenic response in mice. Oral administration of β-glucan ameliorated gastric lesions induced by ethanol (EtOH) or HCl. This administration of β-glucan also suppressed EtOH-induced inflammatory responses, such as infiltration of neutrophils and expression of pro-inflammatory cytokines, chemokines and cell adhesion molecules (CAM) at the gastric mucosa. Of the various defensive factors, the levels of heat shock protein (HSP) 70 and mucin but not PGE_2 were increased by the administration of β-glucan. β-Glucan-dependent induction of the expression of HSP70 and mucin proteins and suppression of the expression of pro-inflammatory cytokines, chemokines and CAM were also observed in cultured cells *in vitro*. The results of the present study suggest that β-glucan protects the gastric mucosa from the formation of irritant-induced lesions by increasing the levels of defensive factors, such as HSP70 and mucin.

Key words: **β-(1,3 → 1,6)-D-Glucan: Gastric lesions: Heat shock protein 70: Mucin: Ethanol**

The balance between aggressive and defensive factors determines whether gastric lesions develop, with either a relative increase in aggressive factors or a relative decrease in defensive factors resulting in lesions. The gastric mucosa is challenged by a variety of both endogenous and exogenous irritants (aggressive factors), including ethanol (EtOH), gastric acid, pepsin, reactive oxygen species and non-steroidal anti-inflammatory drugs[1]. In order to protect the gastric mucosa against these aggressive factors, a complex defence system, which includes the production of surface mucus (gastric mucin) and bicarbonate and the regulation of gastric mucosal blood flow, has evolved. PG, in particular PGE_2, enhance these protective mechanisms, and are therefore thought to be a major gastric mucosal defensive factor[2].

Recently, heat shock proteins (HSP) have also attracted considerable attention as another important defensive factor. When cells are exposed to stressors, HSP are induced in a manner that is dependent on the transcription factor heat shock factor 1 (HSF1), and this cellular up-regulation, in particular the up-regulation of HSP70, provides resistance to such stressors[3–6]. We have recently reported that HSF1-null mice or transgenic mice expressing HSP70 show sensitive or resistant phenotypes, respectively, to irritant-induced gastric lesions[7,8], suggesting that HSP, especially HSP70, play an important role in the protection of the gastric mucosa against irritant-induced gastric lesions.

Chemicals that decrease the level of aggressive factors or increase the level of defensive factors are beneficial for protecting the gastric mucosa against the formation of irritant-induced lesions. Acid-control drugs, such as histamine-2 receptor antagonists and proton pump inhibitors, and anti-*Helicobacter pylori* drugs belong to the former group, while drugs that induce the production of gastric mucins, PG and HSP belong to the latter. These chemicals (anti-ulcer drugs) decrease the incidence of surgery for the treatment of gastric ulcers, resulting in a good quality of life for patients with this disease. However, in order to decrease healthcare costs and to prevent the development of gastric ulcers, it is important to find health foods and supplements (complementary and alternative medicines) that can reduce the level of aggressive factors or increase the level of defensive factors.

Abbreviations: CAM, cell adhesion molecule; EtOH, ethanol; HSF1, heat shock factor 1; HSP, heat shock protein; LMW β-glucan, low-molecular-weight β-(1,3 → 1,6)-D-glucan; MPO, myeloperoxidase.

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