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Nitric Oxide Release Is Not Required to Decrease the Ulcerogenic Profile of Nonsteroidal Anti-inflammatory Drugs

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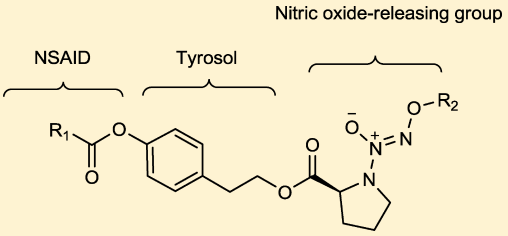
ABSTRACT: The objective of this work was to evaluate the biological properties of a new series of nitric oxide-releasing nonsteroidal anti-inflammatory drugs (NO-NSAIDs) possessing a tyrosol linker between the NSAID and the NO-releasing moiety (PROLI/NO); however, initial screening of ester intermediates without the PROLI/NO group showed the required (desirable) efficacy/safety ratio, which questioned the need for NO in the design. In this regard, NSAID ester intermediates were potent and selective COX-2 inhibitors in vitro, showed equipotent anti-inflammatory activity compared to the corresponding parent NSAID, but showed a markedly reduced gastric toxicity when administered orally. These results provide complementary evidence to challenge the currently accepted notion that hybrid NO-NSAIDs exert their cytoprotective effects by releasing NO. Results obtained in this work constitute a good body of evidence to initiate a debate about the future replacement of NSAID prodrugs for unprotected NSAIDs (possessing a free carboxylic acid group) currently in clinical use.



INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) is one of the most widely used classes of medications worldwide. NSAIDs are employed predominantly to treat pain, fever, and inflammation; however, new studies support an expanded repertoire for NSAIDs and, more importantly, their chemically modified derivatives. Expanded medical applications include the prophylactic treatment of a wide variety of human diseases such as atherosclerosis,[1] thrombosis,[2] cancer,[3,4] Alzheimer's disease,[5] and other disorders for which chronic inflammation is an etiological factor. The major pharmacological mechanism of action of NSAIDs is inhibiting production of prostaglandins (PGs) and thromboxanes (TXs), molecular species that are derived from the enzymatic transformation of arachidonic acid (AA) by cyclooxygenase (COX)-1 and COX-2 enzymes. COX-1 is generally regarded as a constitutive enzyme that is present in most tissues; it is involved in the physiological production of PGs and provides maintenance functions such as cytoprotection in the stomach. In contrast, COX-2 has been regarded as an inducible enzyme and is expressed in inflammatory cells.[6] However, recent reports have challenged these "traditional" roles for COX-1 and COX-2 enzymes, emphasizing the importance of re-evaluating their roles not only in the inflammatory process,[7] but their contribution in the underlying mechanisms of NSAID-induced side effects.

Gastrointestinal (GI) erosions and bleeding are two of the most common toxic side effects associated with the administration of NSAIDs, which have been observed even with low prophylactic doses of aspirin (81 mg/day).[8] It is estimated that approximately 50% of patients taking NSAIDs on a long-term basis develop mucosal damage in the small intestine,[9] and 2−4% of these individuals present clinically significant GI ulcers and bleeding, sometimes leading to death.[10] Consequently, the development of new anti-inflammatory drugs is still a strong clinical need, especially after the withdrawal of some selective COX-2 inhibitors such as rofecoxib and valdecoxib[11,12] and current concerns about the potential dose-dependent hypertensive effect of other NSAIDs which is not easily predicted by COX selectivity alone.[13]

The discovery that nitric oxide (NO) and hydrogen sulfide (H_2S) exert protective effects in the GI tract, modulate many aspects of GI mucosal defense, and accelerate the healing of pre-existing ulcers, led to the development of chemically modified NSAIDs possessing "donor" groups which release NO or H_2S upon metabolic activation in vivo. In this regard, the most extensively studied hybrid prodrugs are the NO-NSAIDs (**1**, Figure 1) and the first NO-releasing group employed in the design of NO-NSAIDs was the organic nitrate ($-ONO_2$),[14] which invariably necessitates the presence of a "linker" between the NO-donor group and the NSAID.

A few years ago, we proposed the use of NONOates (**2**, *N*-diazen-1-ium-1,2-diolates) as a suitable replacement to organic nitrates.[15] Three attributes distinguished NONOates from organic nitrates, namely structural diversity, dependable rates of NO-release, and rich derivatization chemistry that facilitates targeting of NO to specific target organ and/or tissue sites.[16] The first generation of NONOate-containing NSAIDs (**3**, Figure 1) were obtained using secondary amines as the source of NONOates; however, concerns about the generation of

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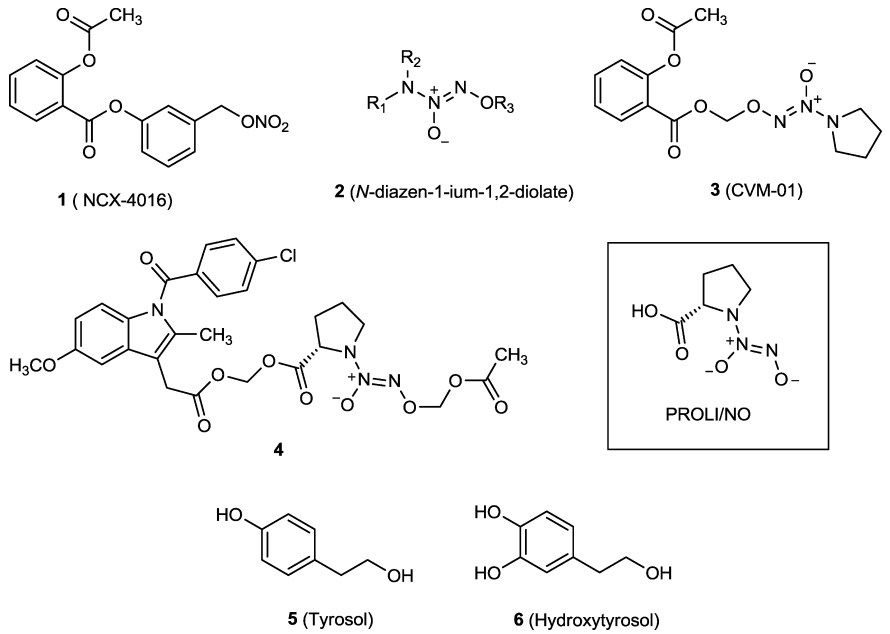


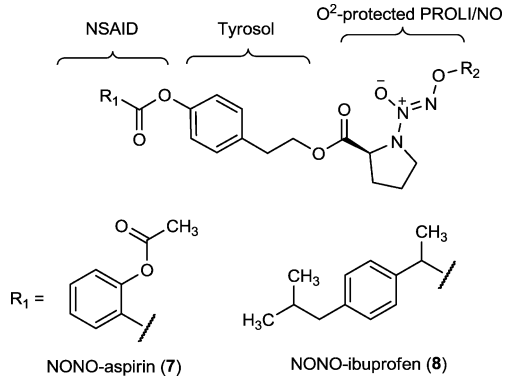
Figure 1. Chemical structures of organic nitrate-containing NO-aspirin (**1**); representative NONOate ion (**2**, where R_1 and R_2 groups represent different alkyl moieties); first-generation NONO-aspirin (**3**); second-generation NONO-indomethacin (**4**); naturally occurring phenols tyrosol (**5**) and hydroxytyrosol (**6**); and the NONOate ion obtained from L-proline (PROLI/NO).

potentially carcinogenic *N*-nitrosamines as metabolic products in vivo prompted us to modify this strategy. Subsequent NONO-NSAIDs (**4**) were designed to possess a PROLI/NO moiety (NONOate obtained from L-proline), which were synthesized and evaluated in vivo (Figure 1).[17] Nevertheless, even though NONO-NSAIDs possessing a PROLI/NO moiety were effective anti-inflammatory agents and their design addressed the safety concerns related to the generation of carcinogenic nitrosamines,[18] they released one or two equivalents of formaldehyde (HCHO) per mol of drug upon metabolism, which may be considered favorable if the goal is to kill cancer cells (chemotherapeutic), but it might be a potential safety concern in routine anti-inflammatory treatments.

As part of our ongoing research program aimed to develop new anti-inflammatory agents with a suitable efficacy/safety profile, we now propose the design and biological evaluation of new NONO-NSAIDs possessing PROLI/NO and a naturally occurring diol linker which replaced formaldehyde-generating acetal linkers ($-OCH_2O-$) used in our previous work. We considered the use of a wide variety of diols including sugars and polyphenols. However, sugar chemistry represented a challenging approach and the use of polyphenols would require a cumbersome protection−deprotection synthetic strategy, so we decided to start with a relatively simple phenol such as 4-(2-hydroxyethyl)phenol (**5**, also called tyrosol); tyrosol is structurally related to hydroxytyrosol (**6**, Figure 1), a well studied natural anti-inflammatory and antioxidant compound found in olives.[19,20]

The rationale behind the design of new NONO-NSAIDs possessing a PROLI/NO moiety as the source of NO and a simple phenol such as tyrosol as linker (Scheme 1) is based on the assumption that, upon metabolic activation, these ester prodrugs would release the anti-inflammatory NSAID, cytoprotective NO, and innocuous metabolites such as L-proline, tyrosol, and a β-D-glucopyranosyl moiety (Scheme 2) containing 0−4 acetate ester groups. We now report the synthesis, in vitro COX inhibitory activity, in vivo anti-inflammatory potency, and the unexpected low ulcerogenicity of four NSAID ester prodrugs possessing a tyrosol moiety.

Scheme 1. Design of New NONO-NSAIDs Possessing PROLI/NO and a Simple Phenol Linker (Tyrosol)[a]



[a] R_2 = 2,3,4,6-tetra-*O*-acetyl-D-glucopyranosyl protecting group.

RESULTS AND DISCUSSION

Determination of Molecular Length, Surface, and Volume. Considering that the COX-1 active site consists of a long narrow channel about 8 Å × 25 Å (total volume 316 Å^3), and the COX-2 binding site is about 25% larger (394 Å^3),[21] bulkier molecules normally fit better into the bigger active site of COX-2 and, consequently, larger compounds generally show a higher selectivity for this enzyme.[22] This is a well established concept reported in the literature which has been employed in the development of potent and selective COX-2 inhibitors by several groups.[23−25] Therefore, the first step was the determination of molecular length (Å), surface (Å^2), and volume (Å^3) of prodrugs to assess if they would possess the minimum essential structural requirements to enter the COX binding site. In this regard, after a standard energy minimization procedure using Alchemy 2000 (Version 2.0,

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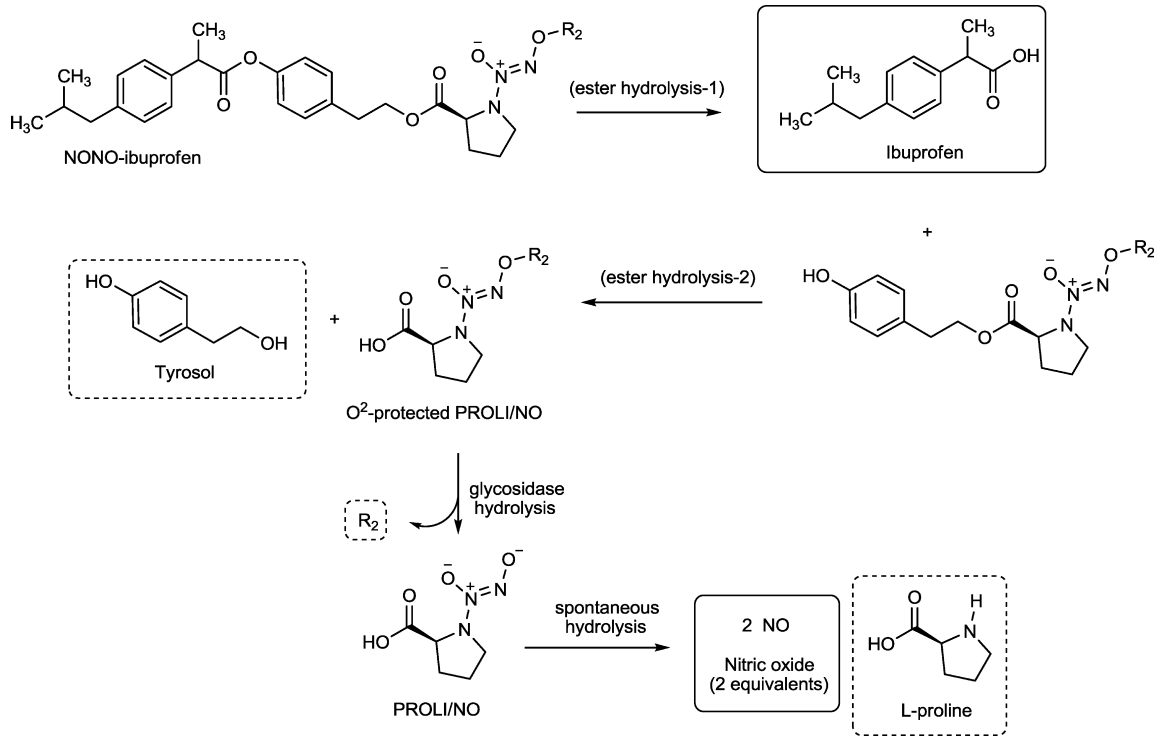
Scheme 2. Theoretical Metabolic Activation of NONO-Ibuprofen in Vivo[a]



[a]Compounds marked with a solid rectangle represent the two active components (ibuprofen and NO) released by metabolic activation; compounds marked with a broken-line rectangle represent innocuous metabolites produced after ester and glycosidase hydrolysis. R_2 represents a protecting 2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl moiety. An alternative ester hydrolysis would involve the hydrolysis of the L-proline (ester hydrolysis-1), and then the NSAID carboxylate (ester hydrolysis-2).

1997, Tripos Inc.), we determined these parameters for NONO-aspirin (**7**), NONO-ibuprofen (**8**), and their corresponding ester intermediates without the PROLI/NO group (**9** and **10**, see Scheme 3).

The predicted physicochemical parameters for ester intermediates possessing a tyrosol moiety, suggested that the aspirin tyrosol ester (**9**) has a suitable molecular volume (264.7 Å^3) to fit into the active site of both COX-1 and COX-2 enzymes, whereas the bulkier ibuprofen tyrosol ester (**10**, volume = 322.6 Å^3) would probably fit better into the larger COX-2 binding site (Table 1). As expected, the corresponding NONO-aspirin (**7**, volume = 411.9 Å^3) and NONO-ibuprofen (**8**, volume = 469.6 Å^3) are considerably bulkier than their corresponding ester intermediates. Considering that molecular volume and length are the initial determinant features controlling the access to the active site of COX enzymes, it is unlikely that large NONO-NSAIDs would enter the long narrow channel, and therefore, they will require bioactivation (ester hydrolysis, see Scheme 2) to release either the tyrosol esters (**7** and **8**) or the active NSAIDs. Nevertheless, we recognized that even though tyrosol esters possessed proper volumes and lengths, they may or may not interact favorably with essential residues within the binding site of either enzyme (this would be better assessed by specific molecular modeling (docking) studies).

Chemistry. The chemical synthesis of new NONO-NSAID prodrugs possessing a tyrosol moiety involved three steps; the first one was the preparation of the corresponding ester intermediates (**7** and **8**) by reacting aspirin (**11**) or ibuprofen (**12**) acid chlorides with tyrosol (Scheme 3A). Under the experimental conditions used for these reactions (THF/TEA, at 25 °C), the phenol group reacted much faster than the aliphatic alcohol, which was in agreement with reports describing similar esterification reactions using a structurally related 3-hydroxybenzyl alcohol.[26] The second step involved the preparation of O^2-protected PROLI/NO derivatives (**16**, Scheme 3B); in this regard, it has been reported in the literature that it is not possible to obtain O^2-alkylation products by simple nucleophilic displacement (S_N2) using PROLI/NO (O^2-sodium salt of **16**) and electrophiles; therefore, the preparation of intermediate (**16**) would require an indirect route involving the synthesis of O^2-sodium PROLINOL/NO (**14**), O^2-alkylation with 1-bromo-2,3,4,6-tetraacetoxyglucose to obtain **15**, and subsequent oxidation of the alcohol group in L-prolinol to obtain the target carboxylic acid **16**.[27] The last step involved the esterification reaction between intermediates **9** or **10** with **16** to yield the corresponding NONO-aspirin (**7**) or NONO-ibuprofen (**8**, Scheme 3C). However, on the basis of the preliminary screening of intermediate esters **9** and **10**, we decided not to carry out the synthesis of NONO-NSAIDs (see Results and Discussion).

In Vitro Cyclooxygenase Inhibition Assay. We observed that esterification of ibuprofen with a tyrosol moiety increased both the inhibitory potency and COX-2 selectivity, whereas esterification of aspirin increased its potency but maintained the COX-1 selective inhibitory profile of the parent NSAID. In this regard, aspirin ester intermediate **9** showed a COX-1 IC_{50} = 0.03 μM, and COX-2 IC_{50} = 0.38 μM, which compared to aspirin (COX-1 = 0.30 μM, COX-2 IC_{50} = 2.40 μM) represents a 10-fold increase in COX-1 inhibitory activity and about 6-fold increase on that of COX-2 (Table 2). This effect was also observed for the ibuprofen ester (**10**), which showed a remarkable inhibitory potency on COX-2 (IC_{50} = 0.2 nM) and

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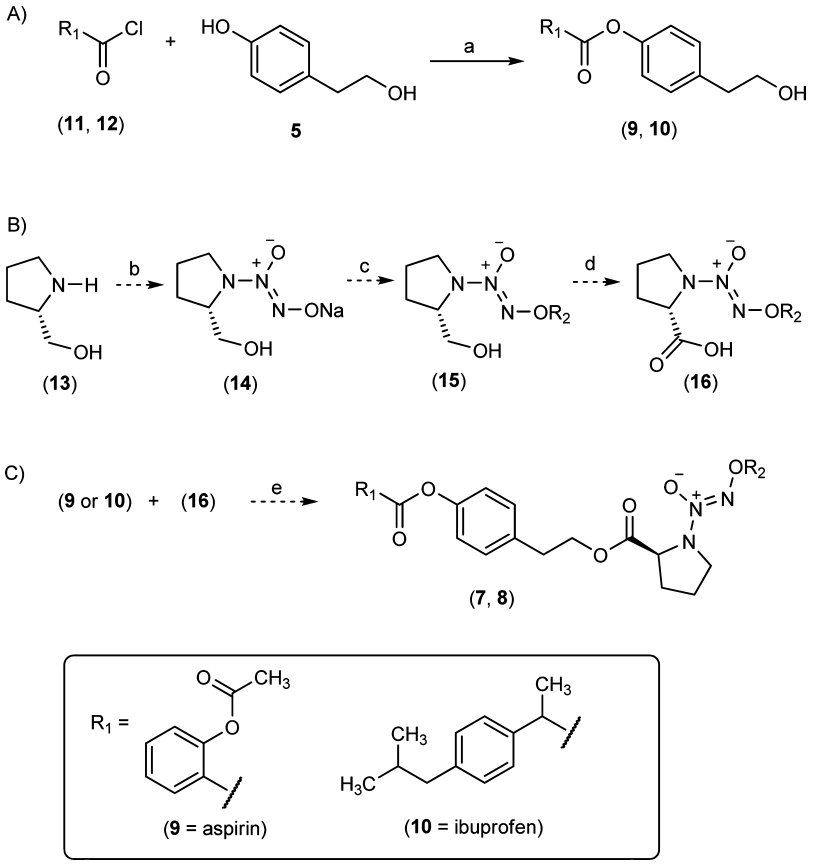
Scheme 3. Chemical Synthesis of NONO-NSAIDs Possessing PROLI/NO and a Tyrosol Linker[a]



[a]Reagents and conditions: (a) THF, TEA, 25 °C, 5 h; (b) NO (40−60 psi), $NaOCH_3/CH_3OH$, ether, 25 °C, 6 h; (c) 1-bromo-2,3,4,6-tetraacetoxyglucose, 5% aq $NaHCO_3$/acetone; (d) $NaIO_4$, $RuCl_3$ (cat.), CH_3CN, EtOAc, H_2O, 25 °C, 2 h; (e) DCC, THF, TEA, 25 °C. (A) Chemical synthesis of tyrosol intermediates **9** and **10**; (B) proposed synthesis of intermediate O^2-protected PROLI/NO (R_2 = 2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl); (C) Proposed synthesis of NONO-aspirin (**7**) and NONO-ibuprofen (**8**). Reactions represented by arrows with a broken line were not actually carried out (see Results and Discussion Ulcer Index Assay).

Table 1. Prediction of Physicochemical Parameters for NONO-NSAIDs (7, 8) and the Corresponding Intermediates without the PROLI/NO Group (9 and 10)

compd	volume[a]	surface[a]	length[a]	Log *P*[b]
9	264.7	314.7	14.1	2.0 ± 0.4
7	411.9[c]	507.2[c]	17.2[c]	3.3 ± 0.6[c]
aspirin	153.7	195.9	8.1	1.2 ± 0.2
10	322.6	394.6	16.6	4.6 ± 0.2
8	469.6[c]	574.9[c]	20.7[c]	5.9 ± 0.6[c]
ibuprofen	209.5	260.4	10.1	3.7 ± 0.2

[a]Calculated using Alchemy 2000 (version 2.0, 1997, Tripos Inc.) after energy minimization; molecular volume is expressed in cubic angstroms ($Å^3$), molecular surface is expressed in squared angstroms ($Å^2$), length is expressed in angstroms (Å). [b]Negative logarithm of the distribution coefficient of each compound between n-octanol/water (theoretical value, expressed as Log *P*, calculated using ACD/ChemSketch version 12.01 freeware, 2010, ACD Laboratories Inc.) [c]Calculated for the corresponding NO-NSAIDs possessing an O^2-methyl PROLI/NO moiety.

Table 2. In Vitro COX-1/COX-2 Enzyme Inhibition, Selectivity Index, and in Vivo Anti-Inflammatory Activity Data for NSAID Tyrosol Esters (9 and 10)

compd	COX-1 IC_{50} (μM)[a]	COX-2 IC_{50} (μM)[a]	SI[b]	AI activity[c]	UI[d]
9	0.03	0.38	0.08	58	2.6 ± 1.2
aspirin	0.30[e]	2.40[e]	0.14	50[f]	57.4 ± 3.7
10	6.44	0.0002	32220	63	3.5 ± 0.8
ibuprofen	2.90[e]	1.10[e]	2.63	50[f]	45.8 ± 2.9

[a]The in vitro test compound concentration required to produce 50% inhibition of COX-1 or COX-2. The result (IC_{50}, μM) is the mean of two determinations acquired using an ovine COX-1 and human recombinant COX-2 inhibitor screening assay kit (cat. no. 560131, Cayman Chemicals Inc., Ann Arbor MI), and the deviation from the mean is <10% of the mean value. [b]Selectivity index (SI) = COX-1 IC_{50}/COX-2 IC_{50}. [c]Results are expressed as % decrease in the inflammatory response compared to control group receiving vehicle at 3 h after oral administration of the test compounds (equimolar dose to the corresponding reference drugs). [d]The average overall length (in mm) of individual ulcers in each stomach ± SEM, n = 4, 6 h after oral administration of the test compound (aspirin = 1.4 mmol/kg, ibuprofen = 1.2 mmol/kg, **9** and **10** = equimolar amounts to their corresponding parent NSAIDs). [e]Reported literature values.[15] [f]ID_{50} values reported for aspirin (0.7 mmol/kg) and ibuprofen (0.3 mmol/kg).[15]

a significantly increased selectivity (SI = 32200) compared to the parent drug ibuprofen (COX-2 IC_{50} = 1.1 μM, SI = 2.63). As it was anticipated, the calculated size and volume for the smaller aspirin ester **9** allowed it to fit into the active sites of both COX enzymes but it maintained the preferential selectivity for COX-1 of aspirin, whereas the bulkier ibuprofen ester **10** seems to fit better into the active site of the bigger COX-2 active site.

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These results are in agreement with previous reports published by other groups, describing an improved potency and COX-2 selectivity of different NSAID derivatives by "masking" the carboxylic acid present in classical anti-inflammatory drugs of this type. For example, Kalgutkar et al. reported the increased potency and improved COX-2 selectivity of 28 "normal" indomethacin esters possessing a wide variety of aliphatic and aromatic moieties,[28] and 20 "reversed" indomethacin amides and esters showing the same biological profile in vitro.[29]

In Vivo Anti-inflammatory Activity. After evaluating the in vitro inhibitory profile of tyrosol esters **9** and **10**, both NSAID prodrugs were screened in vivo using the carageenan-induced rat paw edema assay.[30] Experimental drugs were administered orally (1% methylcellulose as vehicle). Tyrosol esters **9** and **10** showed an equipotent anti-inflammatory profile compared to aspirin and ibuprofen; the administration of these prodrugs resulted in 58% and 63% decrease in the inflammatory response, respectively, when compared to control animals receiving vehicle only. Equimolar amounts of aspirin and ibuprofen produced about 50% decrease in inflammation (Table 2). It is reasonable to assume that the modest improvement in anti-inflammatory potency observed for tyrosol esters **9** and **10** may be due to the increased lipophilic character of esters (Log P = 2.0 and 4.6 respectively) compared to the parent NSAIDs (aspirin Log P = 1.2, ibuprofen Log P = 3.7). Increased lipophilicity increases cell membrane permeability (to a certain extent) and, ultimately, improves bioavailability.[31] Nevertheless, this possibility does not necessarily mean that the intact ester prodrugs reach the target tissues before they are metabolized by nonspecific esterases in blood and other organs, it would only increase their bioavailability compared to the parent NSAID. In this regard, another plausible explanation to support the increased anti-inflammatory activity of tyrosol esters involves the cleavage (via ester hydrolysis) of the prodrug before they interact with the active site of COX enzymes, releasing one equivalent of NSAID + one equivalent of tyrosol; this assumption would implicate complementary mechanisms of action, other than, or in addition to, COX inhibition, exerted by the NSAID and tyrosol. In this regard, there are several reports in the literature describing the antioxidant and anti-inflammatory properties of naturally occurring phenols such as hydroxytyrosol, tyrosol, and 4-hydroxybenzyl alcohol, three common phenols present in olive fruits.[32] Hydroxytyrosol has been reported to reduce serum TNFα in a LPS treated BALB mice model[33] and inhibits 5-LOX activity in vitro;[34] 4-hydroxybenzyl alcohol (4-HBA) released from a biodegradable polymer induced a dose-dependent down-regulation of *i*NOS and significantly reduced the expression of TNFα in RAW 264.7 cells.[35] Additionally, Lim et al. reported the anti-inflammatory effect of 4-HBA on carageenan-induced pouch model in rats by preventing the release of inflammatory mediators and reducing vascular permeability.[36] These mechanisms of anti-inflammatory activity might play a role in the improved anti-inflammatory profile of prodrugs **9** and **10** and are the subject of current research in our group.

Ulcer Index Assay. To evaluate the potential (unwanted) ulcerogenic side-effects of tyrosol prodrugs, we conducted an acute toxicity assay by administering aspirin (1.4 mmol/kg), ibuprofen (1.2 mmol/kg), **9**, or **10** (equimolar doses, po) to rats. All drugs were suspended in 1% methylcellulose solution. Surprisingly, aspirin tyrosol and ibuprofen tyrosol prodrugs were considerably less ulcerogenic (UI = 2.6 and 3.5, respectively) than the parent NSAIDs (aspirin UI = 57.4, ibuprofen UI = 45.8; Table 2). This observation was not in accordance with our original hypothesis because prodrugs **9** and **10** were supposed to be intermediate compounds to which the NO-releasing moiety was to be linked. In other words, the two compounds that were expected to be devoid of ulcerogenic side effects were the corresponding NONO-NSAIDs **7** and **8** (not synthesized), possessing a PROLI/NO moiety, and not prodrugs **9** and **10**. These results suggested that the NO-releasing moiety was not essential to counteract the ulcerogenic effects of aspirin or ibuprofen, and it changed our approach because it seemed that the synthesis of NONO-NSAIDs **7** and **8** was not required (Scheme 3B,C).

One plausible explanation for the lack of acute gastric toxicity of compound **9**, despite its higher selectivity for COX-1 enzyme, could be its increased lipophilic character. Ester **9** has a Log P = 2.0, whereas aspirin Log P = 1.2. As it was discussed for the modest increase in anti-inflammatory activity observed with tyrosol esters, we hypothesize that prodrugs are being absorbed intact and at a higher rate than their corresponding acid counterparts; faster rates of absroption might decrease the time at which prodrugs are available in gastric tissues to exert local toxicity. This observation is supported by previous reports describing significantly higher bioavailability for lipophilic sulindac sulfide amides compared to sulindac in mice.[37]

Evidence collected with prodrugs **9** and **10** suggested that NO-releasing moieties are not essential to protect the gastric mucosal layer from the ulcerogenic effects of an acute dose of aspirin or ibuprofen. The implications of these observations are considerable because it is possible to obtain simple ester COX inhibitors with significant anti-inflammatory activity in vivo but devoid of ulcerogenic side-effects without incorporating donors of bioactive mediators. This statement supports the discussion and conclusions described by Halen et al. in a recent review paper.[31]

In 1994, Wallace et al. proposed the design of NO-releasing NSAIDs as a promising approach to decrease the severity and the relatively high incidence of gastric ulcers associated with the long-term administration of NSAIDs.[38] This approach was based on the well documented bioregulatory properties of NO in the stomach (gastroprotection and modulation of GI mucosal defense), and it is still considered valid today. The design of hybrid NO-NSAIDs continues to be described in the literature as a suitable strategy to protect the gastric mucosa from detrimental effects of NSAIDs.[31,39]

To obtain additional/complementary evidence in support of this hypothesis, we synthesized and screened the corresponding tyrosol ester of indomethacin (**18**, Scheme 4). Indomethacin is a potent anti-inflammatory agent, but it is associated with a high incidence of gastric toxicity, and unlike aspirin or ibuprofen, indomethacin is available to patients by prescription only. In this regard, esterification of indomethacin with a tyrosol moiety did not change its inhibitory potency on COX-2 because prodrug **18** showed a similar COX-2 IC_{50} value to that obtained with indomethacin (4.6 and 5.7 μM, respectively, see Table 3); however, prodrug **18** showed a markedly reduced inhibitory potency on COX-1 (IC_{50} > 100 μM) compared to indomethacin (IC_{50} = 0.1 μM). This shift in COX selectivity was similar to that obtained for the ibuprofen tyrosol prodrug (**10**). When administered orally to rats, compound **18** showed an improved anti-inflammatory activity in vivo (61% decrease in the inflammatory response), which represents a 1.6-fold increase in potency compared to indomethacin (38% inhibition

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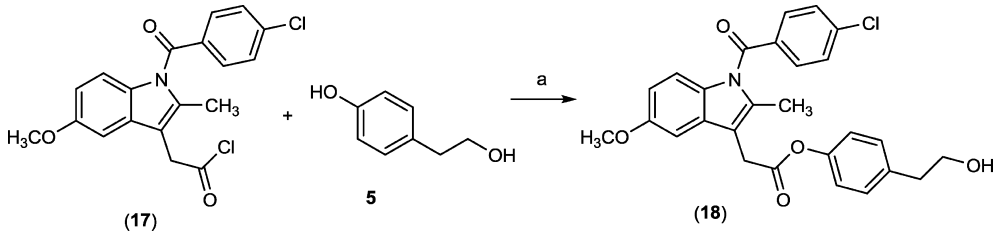
Scheme 4. Chemical Synthesis of Indomethacin Tyrosol Ester (18)[a]



[a]Reagents and conditions: (a) THF, TEA, 25 °C, 5 h.

Table 3. In Vitro COX-1/COX-2 Enzyme Inhibition, in Vivo Anti-inflammatory Activity, and Ulcer Index Data for Indomethacin Tyrosol Ester (9)

compd	COX-1 IC_{50} (μM)[a]	COX-2 IC_{50} (μM)[a]	AI activity[b]	UI[c]
18	>100	4.6	61.3	9.6 ± 2.5
Indomethacin	0.1[d]	5.7[d]	38.3[d]	34.4 ± 4.2[d]

[a]The in vitro test compound concentration required to produce 50% inhibition of COX-1 or COX-2. The result (IC_{50}, μM) is the mean of two determinations acquired using an ovine COX-1 and human recombinant COX-2 inhibitor screening assay kit (cat. no. 560131, Cayman Chemicals Inc., Ann Arbor MI), and the deviation from the mean is <10% of the mean value. [b]Results are expressed as % decrease in the inflammatory response compared to control group receiving vehicle at 3 h after oral administration of the test compound (equimolar dose to indomethacin [0.01 mmol/kg]). [c]The average overall length (in mm) of individual ulcers in each stomach ± SEM, n = 4, 6 h after oral administration of the test compound (0.08 mmol/kg). [d]Reported literature values.[15]

at equimolar dose). Nevertheless, indomethacin tyrosol prodrug (**18**) was significantly less ulcerogenic (UI = 9.6) in rats when administered orally (0.08 mmol/kg) compared to indomethacin at an equimolar dose (UI = 34.4, Table 3).

The biological evaluation of compounds **9**, **10**, and **18** showed a common trend. All prodrugs inhibited cyclooxygenase activity in vitro; ibuprofen (**10**) and indomethacin (**18**) prodrugs showed selective inhibition of COX-2 isoform; all prodrugs showed comparable anti-inflammatory profile in vivo compared to their corresponding parent NSAID but exerted a considerably lower ulcerogenic effect. This provides complementary evidence to bring into question the cytoprotective mechanisms of action exerted by NO-donors present in hybrid NO-NSAIDs.

In a recent publication, Chattopadhyay et al. conducted a head-to-head comparison of two NO-NSAIDs, namely **1** (NCX-4016, possessing an organic nitrate) and **3** (CVM-01, possessing a NONOate group).[40] The main difference between these two compounds (Figure 1) is the amount of NO released from them; unlike organic nitrates, NONOates release two equivalents of NO per mol of drug and do not require metabolic activation (3-electron reduction) to release it. The assumption that **3** would release twice as much NO as compared to **1** led to the expectation that NONOate-containing drugs would show an improved biological profile and lower degree of ulcerogenicity; however, there was no statistically significant difference between groups of animals receiving these two drugs. Both NO-NSAIDs were equipotent analgesic and anti-inflammatory agents (they reduced stomach PGE_2 levels to the same extent), and they were devoid of major gastric side effects. In this regard, Chattopadhyay's group concluded that "it appears that gastroprotection provided by both hybrid prodrugs was not entirely dependent on the amount of NO released from them". This observation suggested that either NO was not involved in reducing the ulcerogenic profile of NO-NSAIDs, or the required threshold of NO to obtain gastroprotection is obtained with only one mol of NO/mol of drug. Results provided in our current work offer additional evidence to assume that NO might not be required at all.

Experimental prodrugs (**9**, **10**, **18**) tested in vivo did not possess a nitric oxide-releasing moiety, and in spite of this, all showed a significantly reduced ulcerogenic profile compared to their parent NSAID counterparts. In this regard, we provide complementary evidence to support the observation that esterification may be good enough to minimize the ulcerogenic toxicity of NSAIDs.

Several other groups have reported similar results, even though they did not correlate their findings to the presence (or absence) of NO-releasing groups, for example, nonulcerogenic indomethacin amide derivatives,[41] aminoalkyl esters of ibuprofen and naproxen,[42,43] sulindac sulfide amides,[37] and D-galactose esters of ketorolac.[44] In a recent publication, Jiang et al. reported a series of new conjugates of aspirin having one "phenolic acid antioxidant" group (*p*-coumaric acid, ferulic acid, or caffeic acid) connected through a diol linker to the carboxylic acid group present in aspirin. These prodrugs showed considerable anti-inflammatory activity (croton oil-induced mice-ear swelling) without significant GI side-effects;[45] one of the diol linkers used by Jiang's group was tyrosol, which is the protecting group we used in this work to form NSAID esters, meaning that it is probably not essential to have the second (additional) phenolic acid antioxidants linked to tyrosol to maintain the anti-inflammatory profile of the corresponding NSAID or to decrease its ulcerogenic effects.

It is worth mentioning that the improved anti-inflammatory profile exerted by NO-NSAIDs constitutes only one of the several potential applications for these molecules, and the presence of NO-releasing moieties may have a significant effect in other conditions (for example, the use of NO-aspirin for the prophylactic treatment of cardiovascular disorders and cancer[2]). Therefore, the scope of the current work is only limited to discussing the role of simple ester groups in decreasing the *gastric toxicity* of NSAIDs while maintaining their anti-inflammatory efficacy in vivo. In this regard, our work makes an important distinction between the potential applications of NO-NSAIDs and NSAID esters. If the intended application is to treat pain and inflammation only (as it is currently the case for most patients using NSAIDs), the use of simple NSAID esters might be good enough to decrease the severity and high incidence of gastric ulcers; on the other hand, if the intended application of NSAIDs is prophylactic or the patient is considered at high

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risk of developing thrombotic events, the use of NO-NSAIDs (particularly NO-aspirins) would offer additional advantages compared to the use of original NSAIDs. In this regard, NO-releasing moieties may counteract the cardiovascular toxicity of selective COX-2 inhibitors (particularly COXIBs) and the release of NO from NO-aspirins would potentiate the antithrombotic effect of aspirin. Furthermore, another interesting setting on which simple esters could be compared to NO-NSAIDs in future experiments is on chronic animal models designed to measure the effect of drugs on healing of pre-existing ulcers.[46] Our study used relatively high oral doses of NSAIDs rather than the administration of lower amounts over a long period of time, which would provide data regarding the effects of NSAID ester prodrugs when administered on a long-term basis. This constitutes one of the current topics of research in our group.

To provide a possible explanation to the observation that simple NSAID esters exert similar efficacy/safety ratios than those observed with NO-NSAIDs, we hypothesize that after oral administration, most of the NSAID ester prodrug (regardless if it has a NO-donor or not) is absorbed mostly intact and at a relatively faster rate than the corresponding free NSAID. Once the ester is absorbed it may interact with and inhibit COX enzymes, either as the tyrosol ester (active in vitro) or as the parent NSAID. This will depend on the rate, extent, and location of ester hydrolysis in vivo. Future research work in this area will need to address gaps in knowledge related to the rate, extent, and site of cleavage of esterified NSAIDs, and such analysis should also include the investigation of the impact of NO on NSAID-induced ulcerogenicity.

■ CONCLUSIONS

Esterification of aspirin, ibuprofen, and indomethacin with tyrosol yields potent and selective COX-2 inhibitors in vitro, equivalent or slightly improved anti-inflammatory activity in vivo, and significantly decreased gastric ulcerogenicity. Even though this design is similar to others reported earlier, our work offers essential evidence suggesting that NO-releasing groups are not required to decrease the ulcerogenic profile of classical NSAIDs, regardless if they are available over-the-counter (aspirin, ibuprofen) or by prescription only (indomethacin). Results obtained in this work, along with those reported previously by other groups, constitute a good body of evidence to question why despite the reliable efficacy/safety profile of simple NSAID prodrugs, there is not a single NSAID oral prodrug commercially available in North America. In this regard, the vast majority of NSAID esters commercially available are used topically (i.e., methyl salicylate). Therefore, we believe it is essential to re-evaluate the potential use of new and/or existing NSAID prodrugs as a safer alternative to the use of classical (unprotected) NSAIDs and to start a debate about the future replacement of NSAID prodrugs for unprotected NSAIDs currently in clinical use. The significance of this statement is evident considering that (i) NSAIDs are one of the most highly used drugs worldwide, and (ii) the relatively high incidence of gastrointestinal side effects associated with their long-term use.

■ EXPERIMENTAL SECTION

Determination of Molecular Length, Surface, and Volume. Test compounds were drawn using BioDraw Ultra (version 11.0, Cambridge Soft USA); the structures were copied and pasted to Alchemy 2000 (version 2.0, 1997, Tripos Inc.) and their steric energies were minimized. Immediately after energy minimization, their molecular properties were calculated [molecular volume was expressed in cubic angstroms (Å^3), molecular surface was expressed in squared angstroms (Å^2), and length was expressed in angstroms (Å)]; molecular lengths were calculated by measuring the linear distance of two selected atoms in the molecule with the highest separation between them. Log *P* values (negative logarithm of the distribution coefficient of drugs between *n*-octanol/water) were calculated using ACD/ChemSketch version 12.01 (2010) freeware, ACD Laboratories Inc.

Chemistry. Melting points were determined with an Electrothermal Mel-Temp melting point apparatus (Dubuque, IA, USA) and are uncorrected. Infrared (IR) spectra were recorded as films (chloroform solutions or neat compounds) on NaCl plates using a Nicolet 550 series II Magna FTIR spectrometer. ^{1}H and ^{13}C NMR spectra were measured on a Bruker AM-300 spectrometer with TMS as internal standard, where coupling constants (*J*) are estimated in hertz (Hz). Mass spectra (MS) were recorded on a Water's micromass ZQ 4000 mass spectrometer using the ESI mode. Microanalyses were within ±0.4% of theoretical values for all elements listed (Faculty of Chemistry, University of Alberta). Compounds **9**, **10**, and **18** showed a single spot on RediSep silica gel glass plates (UV_{254}, 0.2 mm) using a high, medium, and low polarity solvent mixture, and no residue reminded after combustion, indicating a purity higher than 95%. Column chromatography was performed on a CombiFlash Retrieve system using RediSep Rf silica gel (40−60 μM) cartridges. Indomethacin acid chloride (**17**),[47] ibuprofen acid chloride (**12**, racemic),[47] and **1**[48] were synthesized according to reported literature procedures. Acetylsalicyloyl chloride (**11**) was obtained from TCI America (Portland, OR); all other reagents were purchased from Aldrich Chemical Company (Milwaukee, WI) and were used without further purification. The in vivo anti-inflammatory and ulcer index assays were carried out using protocols approved by the Health Sciences Animal Welfare Committee at the University of Alberta.

*4-(2-Hydroxyethyl)phenyl Acetylsalicyloate (**9**).* A solution of tyrosol (1.4 g, 7.2 mmol) and TEA (0.2 g, 1.9 mmol) in dry THF (10 mL) was stirred for 10 min under nitrogen atmosphere before adding (dropwise) a solution of acetylsalicyloyl chloride (1.0 g, 7.2 mmol) previously dissolved in dry THF (5 mL). This reaction mixture was stirred 25 °C for 5 h; all solids (triethylammonium chloride) were filtered out and the solvent was evaporated under vacuum. The residue was purified by silica gel column chromatography using EtOAc/hexane (2:8) as eluent to give (**9**) as white solid (1.1 g, 50.6% yield); mp: 66−69 °C. IR (NaCl) 3370 (OH), 2936 (C−H aromatic), 2867 (C−H aliphatic), 1740 (CO) cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.51 (bs, 1H, O*H*), 2.31 (s, 3H, CH_3), 2.89 (t, *J* = 6.7 Hz, 2H, PhCH_2), 3.88 (t, *J* = 6.7 Hz, 2H, CH_2OH), 7.12 (d, *J* = 8.5 Hz, 2H, phenyl H-2, H-6), 7.17 (dd, *J* = 7.9 Hz, 1.2 Hz, salicyloate H-3), 7.28 (d, *J* = 8.5 Hz, 2H, phenyl H-3, H-5), 7.39 (td, *J* = 7.3, 1.2 Hz, salicyloate H-5), 7.64 (td, *J* = 7.3, 1.8 Hz, salicyloate H-4), 8.22 (dd, *J* = 7.3, 1.2 Hz, salicyloate H-6). ^{13}C NMR (300 MHz, $CDCl_3$) δ = 21.0, 38.6, 63.5, 115.4, 121.7, 122.5, 124.0, 126.1, 130.1, 132.2, 134.5, 136.4, 149.1, 151.1, 163.0. MS: 301.0 [M + 1]$^+$. Anal. Calcd for $C_{17}H_{16}O_5$: C, 67.99; H, 5.37. Found C, 67.74; H, 5.15.

*4-(2-Hydroxyethyl)phenyl 2-(4-isobutylphenyl)propanoate (**10**).* A solution of tyrosol (1.5, 11.1 mmol) and TEA (3.3 g, 13.3 mmol) in dry THF (15 mL) was stirred for 10 min under nitrogen atmosphere before adding (dropwise) a solution of ibuprofen acid chloride (1b, 2.5 g, 11.1 mmol) in dry THF (10 mL). This reaction mixture was stirred at 25 °C for 5 h; all solids (triethylammonium chloride) were filtered out and the solvent was evaporated under vacuum. The residue was purified by silica gel column chromatography using EtOAc/hexane (2:8) as eluent to give (**10**) as white solid (1.9 g, 53.2% yield); mp: 47−50 °C. IR (NaCl) 3383 (OH), 2955 (C−H aromatic), 2867 (C−H aliphatic), 1740 (CO) cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 0.91 (d, *J* = 6.6 Hz, 6H, CH(CH_3)$_2$), 1.60 (d, *J* = 7.3 Hz, 3H, PhCHCH_3), 1.85 (nonet, *J* = 6.7 Hz, 1H, CH_2CH(CH_3)$_2$), 2.47 (d, *J* = 7.3 Hz, 2H, PhCH_2), 2.83 (t, *J* = 6.7 Hz, 2H, PhCH_2), 3.82 (t, *J* = 6.7 Hz, 2H, CH_2OH), 3.93 (q, *J* = 7.3 Hz, 1H, PhC*H*), 6.93 (d, *J* = 8.5 Hz, 2H, phenyl H-2, H-6), 7.14 (d, *J* = 7.9 Hz, 2H, tyrosol phenyl H-2, H-6), 7.18 (d, *J* = 8.5 Hz, 2H, phenyl H-3, H-5), 7.29 (d, *J* = 7.9 Hz, 2H, tyrosol phenyl H-3, H-5). ^{13}C NMR (300 MHz, $CDCl_3$) δ = 18.5,

22.3, 30.1, 38.5, 45.0, 45.2, 63.5, 121.2, 121.4, 127.1, 129.2, 129.3, 129.4, 129.8, 135.9, 137.1, 140.7, 149.4, 173.3. MS 327 $[M + 1]^+$. Anal. Calcd for $C_{21}H_{26}O_3$: C, 77.27; H, 8.03. Found C, 77.33; H, 8.07.

*4-(2-Hydroxyethyl)phenyl 2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetate (**18**).* A solution of tyrosol (0.18 g, 1.3 mmol) and TEA (0.16 g, 1.5 mmol) in dry THF (10 mL) was stirred under nitrogen atmosphere at −80 °C for 10 min before adding (dropwise) a solution of indomethacin acid chloride (**17**, 0.50 g, 1.3 mmol) in THF (5 mL). This reaction mixture was stirred at −80 °C for 5 h; the precipitated salts (triethylammonium chloride) were filtered out and the solvent was evaporated under vacuum. The residue was purified by silica gel column chromatography using EtOAc/hexane (3:7) as eluent to give **18** as a dark-green viscous liquid (0.12 g, 19.46% yield). IR (NaCl): 3458 (OH), 2999 (C−H aromatic), 2904 (C−H aliphatic). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 2.29 (s, 3H, CH_3), 2.81 (t, J = 6.7 Hz, 2H, PhCH_2), 3.62 (s, 2H, CH_2CO_2), 3.82 (s, 3H, OCH_3), 4.27 (t, J = 6.7 Hz, 2H, CH_2OH), 6.64 (dd, J = 9.1 Hz, 2.4 Hz, 2H, indolyl H-6), 6.65 (d, J = 8.5 Hz, 2H, phenyl H-2, H-6), 6.84 (d, J = 9.1 Hz, 1H, indolyl H-7), 6.91 (d, J = 8.5 Hz, 2H, phenyl H-3, H-5), 6.94 (d, J = 2.4 Hz, 1H, indolyl H-4), 7.43 (dd, J = 8.5 Hz, 2.4 Hz, 2H, benzoyl H-3, H-5), 7.63 (dd, J = 8.5 Hz, 2.4 Hz, 2H, benzoyl H-2, H-6). ^{13}C NMR (300 MHz, $CDCl_3$) δ = 13.3, 30.4, 34.1, 55.7, 65.5, 101.4, 111.6, 112.5, 114.9, 115.3, 129.1, 129.6, 129.8, 130.6, 130.8, 131.1, 133.9, 135.7, 139.2, 154.1, 156.0, 168.3, 170.7. MS: 477 $[M + 1]^+$. Anal. Calcd for $C_{27}H_{24}ClNO_5$: C, 67.85; H, 5.06; N, 2.93. Found C, 67.61; H, 5.10; N, 2.62.

In Vitro Cyclooxygenase Inhibition Assay. Experimental compounds **9**, **10**, and **18** were evaluated for their ability to inhibit human recombinant COX-2 and ovine COX-1 using a cyclooxygenase inhibitor screening assay kit (catalogue no. 560131, Cayman Chemical, Ann Arbor, MI, USA) following the procedure suggested by the manufacturer.

In Vivo Anti-inflammatory Assay. NSAID prodrugs (**9**, **10**, and **18**) were evaluated using the carrageenan-induced rat foot paw edema model reported previously.[30] All experimental compounds were suspended in 1.2 mL of 1% methylcellulose solution and administered orally by gavage at the following doses: aspirin and aspirin derivative (**9**) = 0.71 mmol/kg; ibuprofen and ibuprofen derivative (**10**) = 0.32 mmol/kg; indomethacin and indomethacin derivative (**18**) = 0.01 mmol/kg; animals in the control group received an equivalent volume of 1% methylcellulose solution. This assay was carried out using protocols approved by the Health Sciences Animal Welfare Committee at the University of Alberta.

Ulcer Index Assay. The ability to produce gastric damage was evaluated according to a reported procedure.[49] Ulcerogenic activity was evaluated after oral administration of aspirin (1.4 mmol/kg), ibuprofen (1.4 mmol/kg), indomethacin (0.08 mmol/kg), or an equivalent amount of the correspondent test compounds (**9**, **10**, or **18**). All drugs were suspended and administered in 1.2 mL of a 1% methylcellulose solution. Control rats received oral administration of vehicle (1.2 mL of 1.0% methylcellulose solution). Food, but not water, was removed 24 h before administration of test compounds. Six hours after oral administration of the drug, rats were euthanized in a CO_2 chamber and their stomachs were removed, cut out along the greater curvature of the stomach, gently rinsed with water, and placed on ice. The number and the length of ulcers were determined using a magnifier lense. The severity of the gastric lesion was measured along its greatest length (1 mm = rating of 1, 1−2 mm = rating of 2, >2 mm = rating according to their length in mm). The "ulcer index" (UI) for each test compound was calculated by adding the total length (L, in mm) of individual ulcers in each stomach and averaging over the number of animals in each group (n = 4): UI = $(L_1 + L_2 + L_3 + L_4)/4$.

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ABBREVIATION USED

AA, arachidonic acid; COX, cyclooxygenase enzyme; GI, gastrointestinal; NO, nitric oxide; NONOate, *N*-diazen-1-ium-1,2-diolate; NSAID, nonsteroidal anti-inflammatory drug; NO-NSAID, nitric oxide-releasing nonsteroidal anti-inflammatory drug; PG, prostaglandin; Tx, thromboxane; PROLI/NO, NONOate ion derived from L-proline; SI, selectivity index; UI, ulcer index

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