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Dinitroglyceryl and diazen-1-ium-1,2-diolated nitric oxide donor ester prodrugs of aspirin, indomethacin and ibuprofen: Synthesis, biological evaluation and nitric oxide release studies

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ABSTRACT

A new group of hybrid nitric oxide (NO) releasing anti-inflammatory (AI) ester prodrugs (NONO-NSAIDs) wherein a 1,3-dinitrooxy-2-propyl (**12a–c**), or O^2-acetoxymethyl-1-[2-(methyl)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate (**14a–c**), NO-donor moiety is directly attached to the carboxylic acid group of aspirin, indomethacin or ibuprofen were synthesized. NO release from the dinitrooxypropyl, or diazen-1-ium-1,2-diolate, ester prodrugs was increased substantially upon incubation in the presence of L-cysteine (**12a–c**) or rat serum (**14a–c**). The ester prodrugs (**12a–c, 14a–c**), which did not inhibit the COX-1 isozyme, exhibited modest inhibitory activity against the COX-2 isozyme. The NONO-NSAIDs **12a–c** and **14a–c** exhibited in vivo AI activity that was similar to that exhibited by the parent drug aspirin, indomethacin or ibuprofen when the same oral dose (μmol/kg) was administered. These similarities in oral potency profiles suggest these NONO-NSAIDs act as classical prodrugs that require metabolic activation by esterase-mediated hydrolysis. Hybrid NO-donor/anti-inflammatory prodrugs of this type (NONO-NSAIDs) offer a potential drug design concept targeted toward the development of anti-inflammatory drugs with reduced adverse gastrointestinal effects.

Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, indomethacin and ibuprofen are widely used for the treatment of pain, fever and inflammation. The major mechanism by which NSAIDs exert their ant-inflammatory activity, inhibition of cyclooxygenase-derived prostaglandin synthesis, is also responsible for the gastrointestinal (GI),[1–5] renal,[6–8] and hepatic[9] side effects observed in patients undergoing long-term treatment. The most common side effect is the propensity of NSAIDs to induce gastric or intestinal ulceration. Thus, the patients who use NSAIDs on a chronic basis have about three times greater relative risk for serious adverse gastrointestinal events compared to the general population of non-users.[10] A variety of approaches have been investigated to prevent and/or treat NSAID-induced gastroenteropathy. Among these diverse strategies, prodrugs wherein a nitric oxide (NO)-donating moiety is covalently attached to NSAIDs have been designed with the aim of reducing gastrointestinal toxicity by exploiting the protective effect of locally released NO on the gastric mucosa.[11] Accordingly, we showed that a novel group of hybrid NO-releasing NONO-NSAID ester prodrugs possessing a 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate or 1-(*N*,*N*-dimethylamino) diazen-1-ium-1,2-diolate moiety attached via a one-carbon spacer, or an O^2-acetoxymethyl-1-(*N*-ethyl-*N*-methylamino)diazen-1-ium-1,2-diolate moiety attached directly to the carboxylic acid group of aspirin (**1a–c**), ibuprofen (**2a–c**) and indomethacin (**3a–c**) showed approximately equipotent anti-inflammatory activity to the parent NSAID, without significant gastric toxicity when administered orally.[12,13] (see structures in Fig. 1). Studies carried out by others have shown that nitrate-based NO-NSAIDs such as NO-aspirin (**4, 5**),[10,11] NO-indomethacin (**6, 7**),[14,15] and NO-ibuprofen (**8**),[15] are gastrointestinal sparing while simultaneously suppressing prostaglandin synthesis similar to the parent drugs. As part of our ongoing research program to develop anti-inflammatory agents devoid of side effects, we now report the synthesis, in vitro COX-1/COX-2 inhibitory activity, in vivo antiinflammatory activity and nitric oxide release data for a group of ester prodrugs of aspirin, indomethacin or ibuprofen that possess alternative dinitroglyceryl (**12a–c**) or diazen-1-ium-1,2-diolate (**14a–c**) NONO-donor moieties.

A group of ester prodrugs possessing 1,3-dinitrooxy-2-propyl **12a–c** and O^2-acetoxymethyl-1-[2-(methyl)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate **14a–c** ester substituents were synthesized using the reaction sequence illustrated in Scheme 1. Accordingly, aspirin (**9a**), indomethacin (**9b**) and ibuprofen (**9c**) were converted

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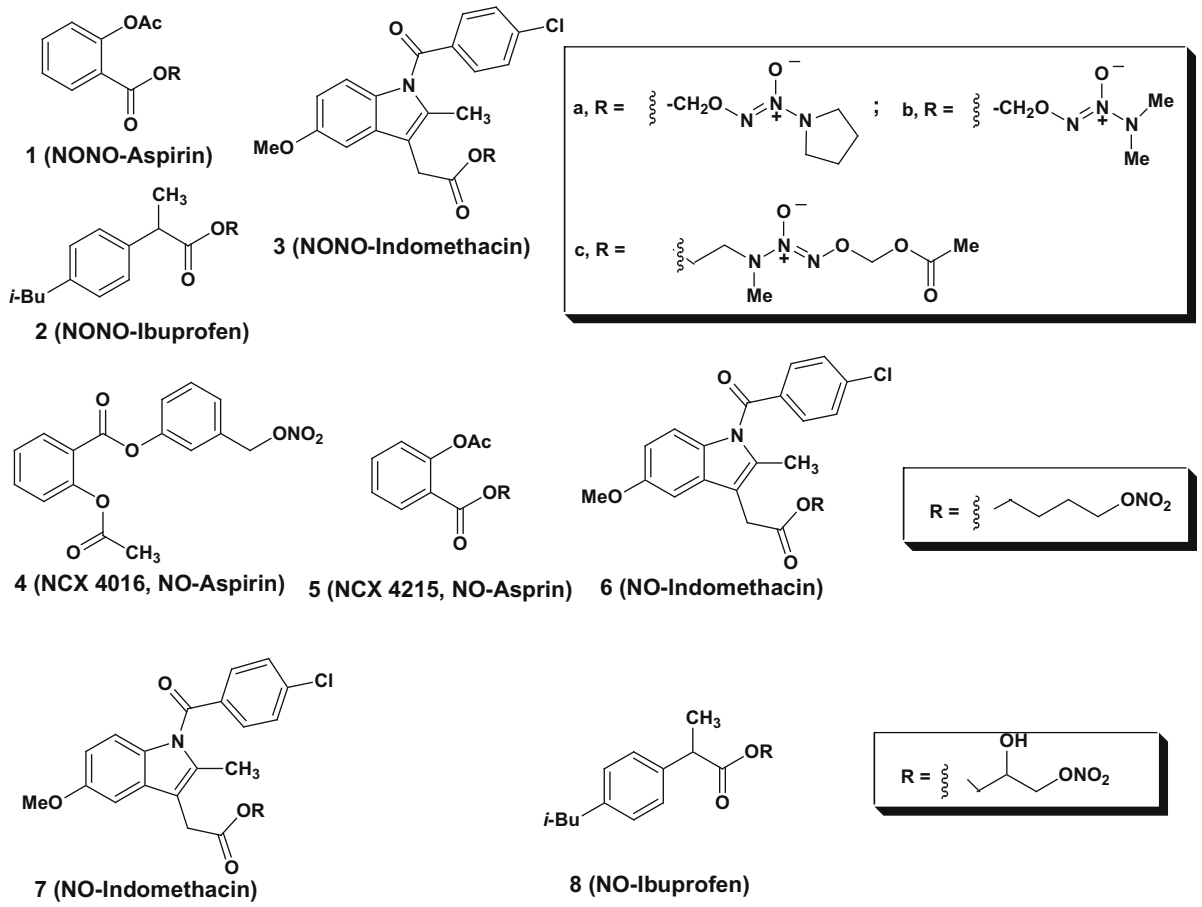
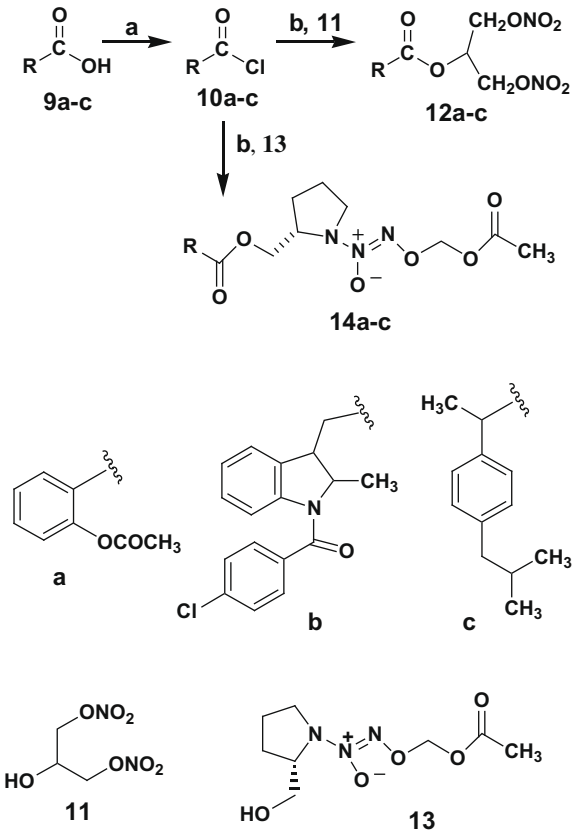


Figure 1. Chemical structures of some diazen-1-ium-1,2-diolate ester prodrugs of aspirin (**1a**–**c**), ibuprofen (**2a**–**c**) and indomethacin (**3a**–**c**), the 3-(nitrooxymethylphenyl) ester of aspirin (**4**), some nitrooxybutyl ester prodrugs of aspirin (**5**) and indomethacin (**6**), and the nitroglyceryl esters of indomethacin (**7**) and ibuprofen (**8**).

to the respective acid chloride **10a–c** upon treatment with either thionyl chloride or oxalyl chloride. Reaction of each acid chloride **10a–c** with 1,3-dinitrooxy-2-propanol (**11**) in dry THF, in the presence of the non-nucleophilic base triethylamine, furnished the respective dinitrate ester **12a–c** in 19–35% yield. The corresponding O^2-acetoxymethyl-1-[2-(methyl)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate esters **14a–c** were prepared in higher yields (51–78%) using the same method employing O^2-acetoxymethyl 1-[2-(hydroxymethyl)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate (**13**) in place of 1,3-dinitrooxy-2-propanol (**11**).

A group of new hybrid nonsteroidal anti-inflammatory prodrugs, derived from aspirin (**12a**, **14a**), indomethacin (**12b**, **14b**) and ibuprofen (**12c**, **14c**) possessing a 1,3-dinitrooxy-2-propyl, or O^2-acetoxymethyl-1-[2-(methyl)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate, NO-donor moiety were synthesized. The 1,3-dinitrooxy-2-propyl esters **12a**-c were designed (i) to have two NO-donor nitrooxy groups compared to one NO-donor moiety in the previously reported NO-NSAIDs **4-8** (see structures in Fig. 1),[10,11,14,15] and (ii) following complete release of NO from the 1,3-dinitrooxy-2-propyl group in conjunction with cleavage of the ester group would release the parent NSAID **9a–c** and the non-toxic glycerol (**11**) as illustrated in Figure 2A. The diazen-1-ium-1,2-diolates **14a–c** were designed (i) such that the NSAID (**9a–c**) carboxyl group is covalently attached directly to the alcohol substituent of the diazenium-1,2-diolate (**15**), and (ii) subsequent cleavage of the ester groups and release of NO would furnish the parent NSAID (**9a–c**) and the natural amino alcohol L-prolinol (Fig. 2B).

In vitro COX-1/COX-2 enzyme inhibition studies (Table 1) showed that none of the NONO-NSAIDs (**12a–c**, **14a–c**) inhibited the COX-1 isozyme at the highest test compound concentration used (100 μM). In contrast, this same group of compounds exhibited COX-2 selectivity (COX-2 S.I. = 13.0–166.7 range) with COX-2 inhibitory activities (IC_{50} = 0.6–9.3 μM range) that were generally lower than that of the parent drugs aspirin, indomethacin and ibuprofen showing COX-2 IC_{50} values (μM) of 2.4, 5.7 and 1.1, respectively. These COX isozyme inhibition data indicate that attachment of a NO-releasing 1,3-dinitrooxy-2-propyl, or O^2-acetoxymethyl-1-[2-(methyl)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate, moiety to the parent NSAID (**9a–c**) completely abolished COX-1 inhibitory activity and decreased COX-2 inhibitory activity. The only amino acid residue lining the COX binding site that is not identical between COX-1 and COX-2 is at position 523 where COX-2 has Val and COX-1 has Ile. This difference changes the size and shape of the NSAID binding site for COX-1 and COX-2. In this regard, the COX-2 enzyme has a second internal pocket extending off the NSAID binding site. In COX-2, the volume of the primary inhibitor binding site and the secondary pocket (394 Å^3) is about 25% larger than the COX-1 binding site (316 Å^3) due to the presence of Val523 in COX-2 relative to Ile523 in COX-1. It is plausible that these differences in size, shape and conformation of the COX isozyme binding sites may explain the differences in COX isozyme inhibition observed.[16,17] However, when the hybrid compounds **12a–c** and **14a–c** were administered orally to rats, data acquired using the carrageenan-induced rat paw edema assay to determine anti-inflammatory activity (see data in Table 1) showed that the % inhibition of anti-inflammatory activity was similar for the same oral dose (μmol/kg) of the parent drug aspirin, indomethacin or ibuprofen (**9a–c**) and the respective NONO-NSAID (**12a–c**, **14a–c**). These



Scheme 1. Reagents and conditions: (a) **9a**, **9c**: $SOCl_2$, benzene, reflux 3–6 h; **9b**: $(COCl)_2$, CH_2Cl_2, 25 °C, 12 h; (b) Et_3N, THF, 25 °C, 24–48 h.

similarities in oral potency profiles between the parent NSAIDs **9a–c**, and the respective NONO-NSAID ester, suggest that **12a–c** and **14a–c** act as classical prodrugs that require metabolic activation by esterase-mediated hydrolysis.

The rate of NO release from diazen-1-ium-1,2-diolates can be decreased by chemical modification involving the attachment of alkyl substituents to the O^2-position.[18] Thus, O^2-substituted-diazen-1-ium-1,2-diolates are stable compounds that hydrolyze slowly even in acidic solution.[19] The percent of NO released from the hybrid O^2-acetoxymethyl-1-[2-(methyl)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate ester prodrugs (**14a–c**) upon incubation in phosphate-buffered-saline (PBS at pH 7.4), varied over a narrow range (4.4–7.9%) which is indicative of slow NO release.[20] In contrast, the effect of non-specific esterases present in rat serum on the NO release properties of NONO-NSAIDs **14a–c** was substantially higher (50.6–80.7% range). These data indicate the non-specific serum esterases present in rat serum cleave these hybrid prodrug esters more effectively than PBS at pH 7.4. From a mechanistic perspective, the hybrid ester prodrugs **14a–c** cannot release NO prior to cleavage of the terminal O^2-acetoxymethyl ester group. This requirement is consistent with the observation that O^2-sodium 1-[2-(hydroxymethyl)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate (**15**), which does not possess an ester group that requires prior ester cleavage, releases 84.5 and 84.8% of the theoretical maximal release of two molecules of NO/molecule of the parent NO donor in PBS and serum, respectively. Comparative studies showed that the extent of NO release upon incubation of the hybrid 1,3-dinitrooxy-2-propyl ester prodrugs (**12a–c**) in PBS at a pH of 7.4 was lower (3.4–3.8% range) than the corresponding O^2-acetoxymethyl-1-[2-(methyl)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate ester prodrugs (**14a–c**, 4.4–7.9% range). The percentage of NO released from the 1,3-dinitrooxy-2-propyl compounds **12a–c** was about twofold higher in the presence of 5 mM L-cysteine (6.8–7.8% range) than in the absence of L-cysteine. This latter observation is consistent with reports that NO release from organic nitrates is facilitated by thiols.[21,22] The lower NO release from the nitrate esters **12a–c** is attributed to the fact that production of NO from nitrate esters requires a demanding three-electron reduction (redox activation).[23] Theoretical metabolic activation pathways for the release of the parent NSAID **9a-c**, and the release of NO, from 1,3-dinitrooxy-2-propyl esters (**12a–c**, Path A) and the O^2-acetoxymethyl-1-[2-(methyl)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate esters (**14a–c**, Path B) are illustrated in Figure 2.

In conclusion, a group of hybrid NO releasing ester prodrugs possessing a 1,3-dinitrooxy-2-propyl (**12a–c**), or O^2-acetoxymethyl-1-[2-(methyl)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate (**14a–c**), moiety attached directly to the carboxylic acid group of aspirin, indomethacin and ibuprofen were synthesized[24] for evalu-

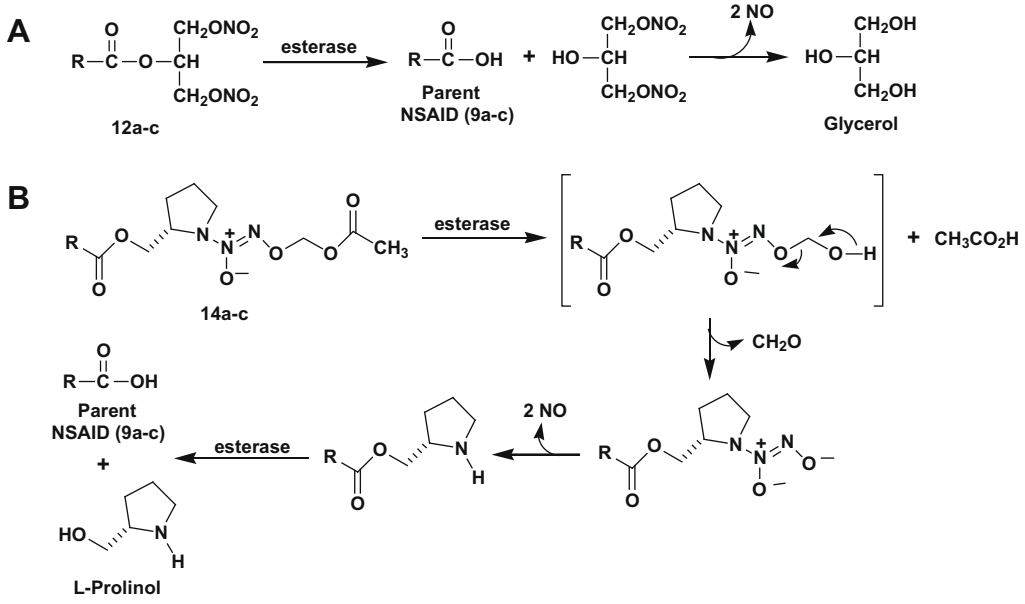
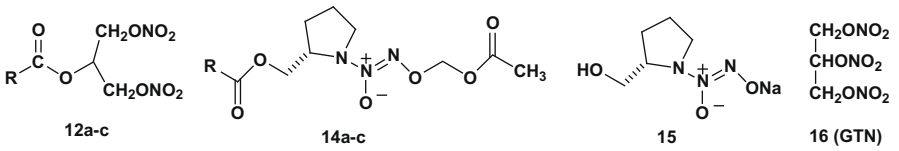


Figure 2. Theoretical metabolic activation (esterase hydrolysis) and nitric oxide release from the 1,3-dinitrooxy-2-propyl esters (**12a–c**, Path A), and the O^2-acetoxymethyl-1-[2-(methyl)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate esters (**14a–c**, Path B). The sequence of esterase cleavage and nitric oxide release may also occur in the reverse order.

Table 1
In vitro COX-1 and COX-2 inhibition, percent (%) nitric oxide release, and anti-inflammatory (AI) data for dinitroglyceryl esters (**12a–c**), diazeniumdiolate esters (**14a–c**), O^2-sodium 1-[2-(hydroxymethyl)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate (**15**), glycerol trinitrate (GTN, **16**) and the parent NSAIDs, aspirin, indomethacin and ibuprofen



Compound	IC_{50} (μM)[a]		COX-2 S.I.[b]	% NO released[c]			AI activity % inhibition (umol/kg)[g]
	COX-1	COX-2		PBS[d]	Serum[e]	L-Cysteine[f]	
12a	>100	7.7	> 13	3.6	—	7.8	30.5 (357)
12b	> 100	0.6	> 166	3.8	—	7.4	42.2 (11.7)
12c	> 100	1.7	> 58	3.4	—	6.8	59.1 (327)
14a	> 100	2.2	> 45	7.4	50.6	—	23.7 (357)
14b	> 100	9.3	> 10	7.9	80.7	—	53.6 (11.7)
14c	> 100	6.4	> 15	4.4	79.5	—	57.1 (327)
15	—	—	—	84.5	84.8	—	—
16 (GTN)	—	—	—	2.8	—	10.1	—
Aspirin	0.3	2.4[h]	2.4	—	—	—	50 (714)
Indomethacin	0.1	5.7[h]	0.02	—	—	—	50 (11.7)
Ibuprofen	2.9	1.1[h]	2.4	—	—	—	50 (327)

[a] The in vitro test compound concentration required to produce 50% inhibition of ovine COX-1 or human recombinant COX-2. The result (IC_{50}, μM) is the mean of two determinations acquired using the enzyme immuno assay kit (Catalog No. 560131, Cayman Chemicals Inc., Ann Arbor, MI, USA) and the deviation from the mean is <10% of the mean value.

[b] In vitro COX-2 selectivity index (COX-1 IC_{50}/COX-2 IC_{50}).

[c] Percent of nitric oxide released based on a theoretical maximum release of (i) 2 mol of NO/mol of the dinitroglyceryl test compounds (**12a–c**), (ii) 2 mol of NO/mol of the diazen-1-ium-1,2-diolate test compounds (**14a–c**) and (iii) 3 mol of NO/mol of glycerol trinitrate (**16**). The result is the mean value of three measurements ($n = 3$) where variation from the mean% value was ⩽0.2%.

[d] A solution of the test compound (2.4 mL of a 1.0×10^{-2} mM solution in phosphate buffer at pH 7.4, was incubated at 37 °C for 1.5 h.

[e] A solution of the test compound (2.4 mL of a 1.0×10^{-2} mM solution in phosphate buffer at pH 7.4 to which 90 μL rat serum had been added), was incubated at 37 °C for 1.5 h.

[f] A solution of the test compound (2.4 mL of a 1.0×10^{-2} mM solution in phosphate buffer at pH 7.4 which contained 5.0 mM L-cysteine), was incubated at 37 °C for 1.5 h.

[g] Inhibitory activity in a carrageenan-induced rat paw edema assay. The results are expressed as the% inhibition of inflammation at 3 h after oral administration of the test compound at the specified dose (μmol/kg).

[h] Data acquired using ovine COX-2 (Catalog No. 56101, Cayman Chemicals Inc.).

ation as COX-1/COX-2 isozyme inhibitors,[25] NO donors,[26] and as anti-inflammatory agents.[27] Structure–activity and biological stability studies showed that (i) the dinitroglyceryl (**12a–c**), and diazen-1-ium-1,2-diolate (**14a–c**), ester prodrugs do not inhibit COX-1 but that they exhibit weak COX-2 inhibitory activity, (ii) all prodrugs **12a–c** and **14a–c** show approximately equipotent in vivo anti-inflammatory activity to the parent drugs, (iii) both classes of prodrugs (**12a–c**, **14a–c**) are relatively stable in phosphate-buffered saline at pH 7.4 where NO release is in the 3.4–7.9% range, and specifically (iv) the O^2-acetoxymethyl-1-[2-(methyl)pyrrolidin-1-yl]diazen-1-ium-1,2-diolates (**14a–c**) undergo extensive ester cleavage by rat serum esterase(s) that is followed by a high release of NO in the 50.6–80.7% range, (v) L-cysteine moderately enhances the release of NO (6.8–7.8% range) from the dinitroglyceryl esters (**12a–c**) that requires a metabolically demanding three-electron reduction for the release of NO, and (vi) hybrid ester NO donor prodrugs constitute a useful concept for the rational design of NONO-NSAIDs that are devoid of adverse ulcerogenic and/or cardiovascular side effects.

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24. Experimental procedures and spectral data for compounds **10a–c**, **12a–c**, **14a–c**.*General:* Melting points were determined on a Thomas–Hoover capillary apparatus and are uncorrected. Unless otherwise noted, infrared (IR) spectra

were recorded as films on NaCl plates using a Nicolet 550 Series II Magna FT-IR spectrometer. ^{1}H NMR spectra were measured on a Bruker AM-300 spectrometer in $CDCl_3$, DMSO-d_6, or $CDCl_3$ + DMSO-d_6 with TMS as the internal standard. Microanalyses were performed for C, H, N (MicroAnalytical Service Laboratory, Department of Chemistry, University of Alberta). Nominal mass, positive polarity, electrospray, spectra were acquired using a Water's Micromass ZQ 4000 mass spectrometer. Silica gel column chromatography was performed using Merck Silica Gel 60 ASTM (70–230 mesh). 1,3-Dinitrooxy-2-propanol (**11**)[22] and O^2-acetoxymethyl 1-[2-(hydroxymethyl)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate (**13**)[28] were prepared according to literature procedures. All other reagents, purchased from the Aldrich Chemical Company (Milwaukee, WI), were used without further purification. The in vivo anti-inflammatory assay was carried out using a protocol approved by the Health Sciences Animal Welfare Committee at the University of Alberta.

Aspirin acid chloride (**10a**): Aspirin (0.72 g, 4 mmol) was added to a solution of thionyl chloride (0.952 g, 8 mmol) in benzene (100 mL) and the mixture was refluxed for 6 h. The solvent and excess thionyl chloride were removed under reduced pressure. Upon cooling, the residue crystallized as white crystals (0.73 g, 92%); mp 45–47 °C; ^{1}H NMR ($CDCl_3$) δ 2.27 (s, 3H, CH_3), 7.07 (dd, J = 8.0, 1.9 Hz, 1H, phenyl H-3), 7.31 (ddd, J = 8.1, 8.0, 1.9 Hz, 1H, phenyl H-5), 7.59 (ddd, J = 8.1, 8.0, 1.9 Hz, 1H, phenyl H-4), 8.15 (dd, J = 8.0, 1.9 Hz, 1H, phenyl H-6).

Indomethacin acid chloride (**10b**): Oxalyl chloride (0.6 mL, 6.7 mmol) was added drop wise to a solution of indomethacin (2.0 g, 5.6 mmol) in dry CH_2Cl_2 (20 mL) under argon at 25 °C. The reaction mixture was stirred at 25 °C for 12 h, and the solvent was removed in vacuo. The crude product was washed with hexane (3 × 25 mL) and dried under vacuum to give **10b** (2.05 g, 95% yield) as a pale gray solid; mp 125–127 °C; ^{1}H NMR ($CDCl_3$) δ 2.42 (s, 3H, CH_3), 3.85 (s, 3H, OCH_3), 4.18 (s, 2H, CH_2), 6.70 (dd, J = 9.1, 2.4 Hz, 1H, indolyl H-6), 6.84 (d, J = 9.1 Hz, 1H, indolyl H-7), 6.89 (d, J = 2.4 Hz, 1H, indolyl H-4), 7.50 (dd, J = 6.9, 1.8 Hz, 2H, benzoyl H-3, H-5), 7.68 (dd, J = 6.9, 1.8 Hz, 2H, benzoyl H-2, H-6).

General procedure for the synthesis of the dinitrate esters (**12a–c**) and diazen-1-ium-1,2-diolate esters (**14a–c**): The respective acid chloride **10a–c** (1 mmol), either 1,3-dinitrooxy-2-propanol (**11**, 1 mmol) or O^2-acetoxymethyl 1-[2-(hydroxymethyl)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate (**13**, 1 mmol), and triethylamine (1 mmol) were dissolved in dry tetrahydrofuran (15 mL), and the resulting mixture was stirred at 25 °C for 24 h (**12a**, **12b, 14a**), or for 48 h (**12c, 14b, 14c**). The precipitate (triethylammonium chloride) was filtered off and the solvent was removed in vacuo. The residue was dissolved in dichloromethane, washed with water, the organic layer was dried (Na_2SO_4), and the solvent was removed in vacuo. The product was purified by silica gel column chromatography using EtOAc/hexane (1:3, v/v) as eluent for compounds **12a**, **12b**, **14c**, EtOAc/hexane (1:10, v/v) for compound **12c**, and EtOAc/hexane (1:1, v/v) for compounds **14a**, **14b**. Physical and spectral data for **12a–c** and **14a–c** are listed below.

Ibuprofen acid chloride (**10c**): Ibuprofen (2.0 g, 9.7 mmol) was dissolved in thionyl chloride (5 mL) and the mixture was refluxed for 3 h. Removal of the thionyl chloride under vacuum, yielded **10c** as a yellow liquid (2.07 g, 95%); ^{1}H NMR ($CDCl_3$) δ 0.92 [d, J = 6.7 Hz, 6H, CH(CH_3)$_2$], 1.60 (d, J = 7.3 Hz, 3H, $CHCH_3$), 1.87 (m, 1H, $CH(CH_3)_2$), 2.49 (d, J = 7.3 Hz, 2H, CH_2), 3.77 (q, J = 7.3 Hz, 1H, $CHCH_3$), 7.15-7.22 (m, 4H, phenyl hydrogens).

1,3-Dinitrooxy-2-propyl 2-acetoxybenzoate (**12a**): Yield, 23%; white crystals; mp 92–94 °C; IR (film) 2963 (C–H aromatic), 2904 (C–H aliphatic), 1755, 1685 (CO_2), 1633, 1287 (ONO_2) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.83 (s, 3H, CH_3), 4.63 (m, 4H, CH_2ONO_2), 4.67 (m, 1H, COOCH), 7.05 (dd, J = 8.5, 1.9 Hz, 1H, phenyl H-3), 7.22 (ddd, J = 8.5, 8.0, 1.9 Hz, 1H, phenyl H-5), 7.63 (ddd, J = 8.5, 8.0, 1.9 Hz, 1H, phenyl H-4), 8.05 (dd, J = 8.0, 1.9 Hz, 1H, phenyl H-6); MS 345.06 (M+1), 366.87 (M+Na). Anal. Calcd for $C_{12}H_{12}N_2O_{10}$: C, 41.87; H, 3.51; N, 8.14. Found: C, 41.87; H, 3.57; N, 7.76.

1,3-Dinitrooxy-2-propyl 2-[1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl]acetate (**12b**): Yield, 35%; yellow gum; IR (film) 2962 (C–H aromatic), 2931 (C–H aliphatic), 1751 (CO_2), 1647 (CO), 1615, 1285 (ONO_2) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.39 (s, 3H, CH_3), 3.73 (s, 2H, CH_2CO_2), 3.85 (s, 3H, OCH_3), 4.56 (dd, J = 12.4, 4.4 Hz, 2H, CHH′ONO_2), 4.76 (dd, J = 12.4, 4.4 Hz, 2H, CHH′ONO_2), 5.39 (m, 1H, CO_2CH), 6.68 (dd, J = 8.5, 2.4 Hz, 1H, indolyl H-6), 6.85 (d, J = 8.5 Hz, 1H, indolyl H-7), 6.91 (d, J = 2.4 Hz, 1H, indolyl H-4), 7.47 (dd, J = 6.7, 1.9 Hz, 2H, benzoyl H-3, H-5), 7.68 (dd, J = 6.7, 1.9 Hz, 2H, benzoyl H-2, H-6); MS 544.07 (M+Na). Anal. Calcd for $C_{22}H_{20}ClN_3O_{10}$: C, 50.63; H, 3.86; N, 8.05. Found: C, 50.81; H, 4.08; N, 8.11.

1,3-Dinitrooxy-2-propyl 2-(4-(isobutyl)phenyl)propionate (**12c**): Yield, 19%; pale yellow oil; IR (film) 2959 (C–H aromatic), 2870 (C–H aliphatic), 1753 (CO_2), 1656, 1280 (ONO_2) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.89 [d, J = 6.7 Hz, 6H, CH(CH_3)$_2$], 1.51 (d, J = 7.3 Hz, 3H, $CHCH_3$), 1.85 [m, 1H, $CH(CH_3)_2$], 2.45 (d, J = 7.3 Hz, 2H, CH_2), 3.73 (q, J = 7.3 Hz, 1H, $CHCH_3$), 4.46 (dd, J = 12.8, 5.8 Hz, 1H, CHH′ONO_2), 4.55 (dd, J = 12.8, 5.8 Hz, 1H, CHH′ONO_2), 4.61 (dd, J = 12.4, 4.0 Hz, 1H, CHH′ONO_2), 4.73 (dd, J = 12.4, 4.0 Hz, 1H, CHH′ONO_2), 5.35 (m, 1H, CO_2CH), 7.10 (d, J = 8.0 Hz, 2H, phenyl H-3, H-5), 7.16 (d, J = 8.0 Hz, 2H, phenyl H-2, H-6); ^{13}C NMR ($CDCl_3$) δ 18.1, 22.3, 30.2, 44.9, 45.0, 66.4, 69.3, 69.4, 126.9, 129.5, 136.5, 141.0, 173.5; MS 371.02 (M+1), 393.1 (M+Na); Anal. Calcd for $C_{16}H_{22}N_2O_8$: C, 51.89; H, 5.99; N, 7.56. Found: C, 52.40; H, 5.99; N, 7.21.

O^2-Acetoxymethyl 1-[2-(acetylsalicyloyloxymethyl)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate (**14a**): Yield, 51%; white gum; $[\alpha]_D^{21.0}$ −47.2 (1.0200, $CHCl_3$); IR (film) 2964 (C–H aromatic), 2881 (C–H aliphatic), 1755, 1728 (CO_2), 1225, 1077 (N═N–O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.95−2.12 (m, 4H, pyrrolidin-1-yl H-3, H-4), 2.10 (s, 3H, $COCH_3$), 2.35 (s, 3H, $COCH_3$), 3.57 (m, 1H, pyrrolidin-1-yl H-5), 3.60 (m, 1H, pyrrolidin-1-yl H′-5), 4.42–4.46 (m, 3H, pyrrolidin-1-yl H-2, $COOCH_2$), 5.72 (d, J = 6.6 Hz, 1H, –OCHH′OAc), 5.76 (d, J = 6.6, 1H, OCHH′OAc), 7.11 (dd, J = 7.9, 1.2 Hz, 1H, phenyl H-3), 7.33 (ddd, J = 8.0, 7.9, 1.2 Hz, 1H, phenyl H-5), 7.58 (ddd, J = 8.0, 7.9, 1.2 Hz, 1H, phenyl H-4), 8.00 (dd, J = 8.0, 1.2 Hz, 1H, phenyl H-6); MS 396.14 (M+1), 417.94 (M+Na). Anal. Calcd for $C_{17}H_{21}N_3O_8$: C, 51.64; H, 5.35; N, 10.63. Found: C, 51.73; H, 5.58; N, 10.29.

O^2-Acetoxymethyl 1-{2-[2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetoxymethyl]pyrrolidin-1-yl}diazen-1-ium-1,2-diolate (**14b**): Yield, 78%; yellow gum; $[\alpha]_D^{21.0}$ −37.0 (1.0300, $CHCl_3$); IR (film) 2979 (C–H aromatic), 2882 (C–H aliphatic), 1733, 1717 (CO_2), 1684 (CO), 1225, 1075 (N═N–O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.65−2.04 (m, 4H, pyrrolidin-1-yl H-3, H-4), 2.11 (s, 3H, $COCH_3$), 2.39 (s, 3H, CH_3), 3.49-3.54 (m, 2H, pyrrolidin-1-yl H-5), 3.70 (s, 2H, CH_2), 3.83 (s, 3H, OCH_3), 4.19-4.33 (m, 3H, pyrrolidin-1-yl H-2, $COOCH_2$), 5.73 (d, J = 6.6 Hz, 1H, –OCHH′OAc), 5.76 (d, J = 6.6, 1H, OCHH′OAc), 6.67 (dd, J = 8.5, 2.4 Hz, 1H, indolyl H-6), 6.89 (d, J = 8.5 Hz, 1H, indolyl H-7), 6.96 (d, J = 2.4 Hz, 1H, indolyl H-4), 7.48 (dd, J = 6.7, 1.9 Hz, 2H, benzoyl H-3, H-5), 7.67 (dd, J = 6.7, 1.9 Hz, 2H, benzoyl H-2, H-6); MS 594.96 (M+Na), 596.97 (M+2+Na). Anal. Calcd for $C_{27}H_{29}ClN_4O_8$: C, 56.60; H, 5.10; N, 9.76. Found: C, 56.45; H, 5.12; N, 9.60.

O^2-Acetoxymethyl 1-{2-[2-(4-isobutylphenyl)propionoyloxymethyl]pyrrolidin-1-yl}diazen-1-ium-1,2-diolate (**14c**): Yield, 69%; pale yellow oil; $[\alpha]_D^{21.0}$ −65.9 (1.0300, $CHCl_3$); IR (film) 2961 (C–H aromatic), 2870 (C–H aliphatic), 1734, 1717 (CO_2), 1222, 1073 (N═N–O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.89 [d, = 6.7 Hz, 6H, CH(CH_3)$_2$], 1.50 (d, J = 7.3 Hz, 3H, $CHCH_3$), 1.53−1.88 [m, 5H, pyrrolidin-1-yl H-3, H-4, $CH(CH_3)_2$], 2.11 (s, 3H, $COCH_3$), 2.45 (d, J = 7.3 Hz, 2H, CH_2), 3.46–3.48 (m, 2H, pyrrolidin-1-yl H-5), 3.71 (q, J = 7.3 Hz, 3H, $CHCH_3$), 4.20–4.28 (m, 3H, pyrrolidin-1-yl H-2, CO_2CH_2), 5.73 (d, J = 6.6 Hz, 1H, –OCHH′OAc), 5.77 (d, J = 6.6, 1H, OCHH′OAc), 7.09 (d, J = 8.0 Hz, 2H, phenyl H-3, H-5), 7.19 (d, J = 8.0 Hz, 2H, phenyl H-2, H-6); MS 422.23 (M+1), 444.08 (M+Na); Anal. Calcd for $C_{21}H_{31}N_3O_6 \cdot 1/9\ H_2O$: C, 59.56; H, 7.43; N, 9.92. Found: C, 59.92; H, 7.20; N, 9.53.

25. *Cyclooxygenase inhibition assays*: The ability of the test compounds listed in Table 1 to inhibit ovine COX-1 and human recombinant COX-2 (IC_{50} value, μM) was determined using an enzyme immuno assay (EIA) kit (Catalog No. 560131, Cayman Chemical, Ann Arbor, MI, USA) according to our previously reported method (Rao, P. N. P.; Amini, M.; Li, H.; Habeeb, A.; Knaus, E. E. *J. Med. Chem.* **2003**, *46*, 4872).
26. *Nitric oxide release assays*: In vitro nitric oxide release, upon incubation of the test compound at 37 °C for 1.5 h with either 2.4 mL of a 1.0×10^{-2} mM solution in phosphate buffer at pH 7.4, or with 2.4 mL of a 1.0×10^{-2} mM solution in phosphate buffer at pH 7.4 to which 90 μL rat serum had been added, was determined by quantification of nitrite produced by the reaction of nitric oxide with oxygen and water using the Griess reaction. Nitric oxide release data were acquired for test compounds (**12a–c**, **14a–c**, **15**–**16**) using the reported procedures (Velázquez, C.; Vo, D.; Knaus, E. E. *Drug Dev. Res.* **2003**, *60*, 204).
27. *In vivo anti-inflammatory assay*: The test compounds **12a–c**, **14a–c**, and the reference drugs aspirin, indomethacin and ibuprofen were evaluated using the in vivo carrageenan-induced rat foot paw edema model reported previously (Winter, C. A.; Risley, E. A.; Nuss, G. W. *Proc. Soc. Exp. Biol. Med.* **1962**, *111*, 544).
28. Velázquez, C. A.; Chen, Q.-H.; Citro, M. L.; Keefer, L. K.; Knaus, E. E. *J. Med. Chem.* **2008**, *51*, 1954.