Published in final edited form as:

J Med Chem. 2009 August 13; 52(15): 4631–4639. doi:10.1021/jm900326c.

Activation of peroxisome proliferator-activated receptor γ (PPAR γ) by nitroalkene fatty acids: importance of nitration position and degree of unsaturation

Michael J. Gorczyński[§], Pamela K. Smitherman[‡], Taro E. Akiyama[¶], Harold B. Wood[¶], Joel P. Berger[¶], S. Bruce King[§], and Charles S. Morrow^{‡,*}

[‡]Department of Biochemistry, Wake Forest University School of Medicine, Winston-Salem, NC

[§]Department of Chemistry, Wake Forest University, Winston-Salem, NC

[¶]Merck Research Laboratories, Rahway, NJ

Abstract

Nitroalkene fatty acids are potent endogenous ligand activators of PPAR γ -dependent transcription. Previous studies with the naturally occurring regioisomers of nitrolinoleic acid revealed that the isomers are not equivalent with respect to PPAR γ activation. To gain further insight into the structure-activity relationships between nitroalkenes and PPAR γ , we examined additional naturally occurring nitroalkenes derived from oleic acid, 9-nitrooleic acid (*E*-9-NO₂-18:1 [1]) and 10-nitrooleic acid (*E*-10-NO₂-18:1 [2]), and several synthetic nitrated enoic fatty acids of variable carbon chain length, double bonds, and nitration site. At submicromolar concentrations, *E*-12-NO₂ derivatives were considerably more potent than isomers nitrated at carbons 5, 6, 9, 10 or 13; and, chain length (16 versus 18) or number of double bonds (one versus two) was of little consequence for PPAR γ activation. Interestingly, at higher concentrations (> 2 μ M) the nitrated enoic fatty acids (*E*-9-NO₂-18:1 [1], *E*-9-NO₂-16:1 [3], *E*-10-NO₂-18:1 [2], and *E*-12-NO₂-18:1 [7]) deviated significantly from the saturable pattern of PPAR γ activation observed for nitrated 1,4-dienoic fatty acids (*E*-9-NO₂-18:2, *E*-10-NO₂-18:2, *E*-12-NO₂-18:2, and *E*-13-NO₂-18:2).

INTRODUCTION

Unsaturated nitrated fatty acids are naturally occurring electrophiles implicated in the modulations of several signaling pathways: they can activate PPAR γ ^a, inhibit NF κ B, and activate ARE-dependent gene transcription by influencing the Nrf2/Keap1 pathway.¹⁻⁴ As consequence of these and perhaps other activities, the nitroalkene fatty acids can mediate vasodilation, inhibit inflammation, induce differentiation in susceptible cells, and may, via PPAR γ -dependent mechanisms, influence fat and carbohydrate homeostasis.^{2, 4-6} The nitroalkenes, which include the abundant naturally occurring nitrated derivatives of linoleic and oleic acid, are among the most potent endogenous activators of PPAR γ with significant

*To whom correspondence should be addressed at: Department of Biochemistry; Wake Forest University School of Medicine; Medical Center Boulevard; Winston-Salem, NC 27157 Telephone: 336-713-7218; Fax: 336-716-7671; Email: cmorrow@wfubmc.edu.

^aAbbreviations used: ARE, antioxidant or electrophile response element; Keap1, kelch-like ECH-associated protein 1; NF κ B, nuclear factor kappa B; *E*-9-NO₂-16:1, (*E*)-9-nitrohexadec-9-enoic acid; *E*-10-NO₂-16:1, (*E*)-9-nitrohexadec-9-enoic acid; *E*-5-NO₂-18:1, (*E*)-5-nitrooctadec-5-enoic acid; *E*-6-NO₂-18:1, (*E*)-6-nitrooctadec-5-enoic acid; *E*-9-NO₂-OA or *E*-9-NO₂-18:1, (*E*)-9-nitrooctadec-9-enoic acid; *E*-10-NO₂-OA or *E*-10-NO₂-18:1, (*E*)-10-nitrooctadec-9-enoic acid; *E*-12-NO₂-LA or *E*-12-NO₂-18:2, (9*Z*,12*E*)-12-nitrooctadeca-9,12-dienoic acid; *Z*-9/10-NO₂-OA or *Z*-9/10-NO₂-18:1, (*Z*)-9-nitrooctadec-9-enoic + (*Z*)-10-nitrooctadec-9-enoic acid mixture; *E*-12-NO₂-18:1, (*E*)-12-nitrooctadec-12-enoic acid; *E*-13-NO₂-18:1, (*E*)-13-nitrooctadec-12-enoic acid; Nrf2, NF-E2-related factor 2; PPAR, peroxisome proliferator-activated receptor; PPARE, PPAR responsive element

PPAR γ -dependent transcription observed at nanomolar concentrations.^{3, 7} Moreover, their physiological significance is suggested by publications reporting aggregate concentrations of these nitroalkenes that can approach 1-2 μ M.⁸

Recently, we examined the four naturally occurring regioisomers of nitrolinoleic acid (NO₂-LA or NO₂-18:2). While all activate PPAR γ in a concentration dependent and saturable manner, the 12-nitro derivative (*E*-12-NO₂-18:2) is by far the most potent with respect to maximum agonist activity and lowest EC₅₀.⁷ We speculated that the superiority of *E*-12-NO₂-18:2 over the other regioisomers may be related to unique interactions suggested to occur between amino acid residues within the ligand binding pocket of PPAR γ and the 12-NO₂ moiety of *E*-12-NO₂-18:2.⁹ To investigate these structure-activity relationships further, we synthesized and tested, using PPAR γ -dependent reporter gene and ligand-binding assays, the relative potencies of a number of both naturally occurring and model nitroalkene fatty acids (Figure 1).

The main goal of this study was to determine the structural features of nitroalkene fatty acids that influence their relative abilities to activate PPAR γ . The results of these studies establish the general importance, for optimal agonist activity, of nitration at the 12-position. In addition, they demonstrate, for the first time, the unique properties of nitrated enoic fatty acids towards PPAR γ activation at higher ligand concentrations that are not observed with the nitrated 1,4-dienoic fatty acids or the pharmaceutical ligand, rosiglitazone.

RESULTS

Synthesis and characterization of nitroalkene fatty acids

The nitrooleic acid isomers (**1-2**, *E*-9-NO₂-18:1 and *E*-10-NO₂-18:1) were regio and stereoselectively (*E* isomer) prepared using our previously reported procedure.¹⁰ Schemes 1-6 depict a similar synthetic strategy that produced the nitro-16:1 derivatives (**3-4**, *E*-9-NO₂-16:1 and *E*-10-NO₂-16:1) and the nitro-oleic acid isomers (**5-8**, *E*-5-NO₂-18:1, *E*-6-NO₂-18:1, *E*-12-NO₂-18:1 and *E*-13-NO₂-18:1). In general, the synthesis of these nitrated fatty acids involved four steps: a nitro aldol condensation (Henry reaction) between the appropriate nitroalkane and aldehyde, acetylation of the corresponding nitro alcohol, dehydroacetylation and hydrolysis of the methyl ester.¹⁰ The Henry reaction generally proceeded in good yield to form the nitro alcohols (**11**, **15**, **19**, **24**, **29** and **32**) and ultimately allows complete control of the position of the nitro group in the final product (Schemes 1-6).¹¹ Dehydroacetylation of the intermediate nitro alcohols gives the corresponding nitroalkene methyl esters (**12**, **16**, **20**, **25**, **30** and **33**) in moderate yield (16-58%, Schemes 1-6).¹¹ Proton NMR chemical shift analysis indicates exclusive formation of the *E* isomer as the alkene proton of *E*-nitroalkenes display a characteristic chemical shift of approximately δ 7.00 ppm.^{12, 13} Hydrolysis of the methyl esters provided the desired nitro fatty acid derivatives (**3-8**; Schemes 1-6) that were characterized by proton and carbon NMR spectroscopy, mass spectrometry and elemental analysis.¹⁰ A similar sequence: Henry reaction, dehydroacetylation followed by sodium borohydride reduction afforded a variety of nitroalkanes required for subsequent Henry reactions in good yield (for example **14**; Scheme 2).^{12, 14} Nitro selenation of eldaidic acid (*trans*-oleic acid) followed by elimination yielded a mixture (~1:1) of the *Z* nitro alkenes (**9**, *Z*-9/10-NO₂-18:1, Scheme 7) and chemical shift analysis supports the formation of the *Z* isomer as *Z*-nitroalkenes demonstrate a characteristic chemical shift of about δ 5.80 ppm.^{12, 13, 15}

Affinities of the nitroalkene fatty acids for the ligand binding domain of PPAR γ

A radioligand competition scintillation proximity assay was used to examine nitroalkene interactions with the ligand binding domains of PPAR γ , and α . The results are presented in Table 1 as IC₅₀ values which provide measures for the relative affinities of the various ligands

for PPARs. The previously determined IC₅₀ values for the pharmaceutical PPAR γ ligand, rosiglitazone, and the 12-NO₂ isomer of nitrolinoleic acid (*E*-12-NO₂-18:2) are shown for reference.⁷ All of the nitroalkenes are relatively high affinity ligands of PPAR γ that substantially favor interactions with PPAR γ over PPAR α . Affinities toward PPAR γ of the naturally occurring nitrated derivatives of oleic acid, *E*-9-NO₂-18:1 (**1**) and *E*-10-NO₂-18:1 (**2**), are comparable to most of the model synthetic nitroalkenes with IC₅₀ values ranging from 0.63–1.73 μ M. Exceptions are the nitroenoic fatty acids, *E*-12-NO₂-18:1 (**7**) and *E*-13-NO₂-18:1 (**8**), which bind PPAR γ with considerably greater affinity (IC₅₀ values of 0.039 μ M and 0.19 μ M, respectively).

Differential activation profiles of PPAR γ by the various nitroalkene fatty acids

In our earlier work, a major feature distinguishing *E*-12-NO₂-18:2 from its other regioisomers of nitrolinoleic acid was its ability to activate PPAR γ at low concentrations (EC₅₀ = 0.045 μ M) whereas the others, *E*-9/10-NO₂-18:2 and *E*-13-NO₂-18:2, were considerably less active at low concentrations (EC₅₀ = 0.41 μ M and 0.62 μ M, respectively). Hence, the nitroalkenes studied here were screened for PPAR γ activation at low (0.1 μ M) and high (3 μ M) concentrations. These results are summarized in Figure 2 and include, for comparison, the pharmaceutical ligand, rosiglitazone. At 3 μ M, all nitroalkenes activated PPAR γ -dependent transcription with induction levels ranging from a low of ~2.2 fold for the *Z*-9/10-NO₂-18:1 (**9**) *trans*-nitrooleic acid mixture and *E*-6-NO₂-18:1 (**6**) to a high of ~7.5 fold for the 12-nitroenoic fatty acid, *E*-12-NO₂-18:1 (**7**). More remarkably, though, were differences in PPAR γ activation between the nitroalkenes at low, 0.1 μ M, concentrations. Only rosiglitazone and the 12-nitroalkenes, *E*-12-NO₂-18:2 and *E*-12-NO₂-18:1 (**7**), supported robust induction at low concentrations; whereas, the other nitroalkenes, including the naturally occurring oleic acid derivatives, *E*-9-NO₂-18:1 and *E*-10-NO₂-18:1 (**1**, **2**), supported little or no activation at these concentrations.

To examine the concentration dependencies of nitroalkene-mediated activation of PPAR γ further, we selected five representative nitroalkene fatty acids for additional studies: the naturally occurring nitroalkenes, *E*-9-NO₂-18:1 (**1**), *E*-10-NO₂-18:1 (**2**) and *E*-12-NO₂-18:2; the 16 carbon chain analog of *E*-9-NO₂-18:1 (**1**), *E*-9-NO₂-16:1 (**3**); and the enoic fatty acid analog of *E*-12-NO₂-18:2, *E*-12-NO₂-18:1 (**7**). The results using natural nitroalkene fatty acids are shown in Figure 3. The 12-NO₂-18:2 isomer shows saturable induction of PPAR γ -dependent transcription with robust induction at nanomolar concentrations (IC₅₀ = 0.07 μ M—similar to the value reported previously⁷). In contrast, the *E*-9-NO₂-18:1 (**1**) and *E*-10-NO₂-18:1 (**2**) isomers are relatively poor activators at submicromolar concentrations. Moreover, activation does not appear to saturate over the range of concentrations used but, rather, increases steadily for both and nearly exponentially for the *E*-9-NO₂-18:1 (**1**) isomer. In Figure 4, *E*-12-NO₂-18:2 activation is compared with the synthetic model nitroalkenes. *E*-9-NO₂-16:1 (**3**) shows an activation profile similar to its 18-carbon analog, *E*-9-NO₂-18:1 (**1**): PPAR γ activation is poor at submicromolar concentrations but steadily increases up to the highest concentration used, 5 μ M. The *E*-12-NO₂-18:1 (**7**) isomer has an activation profile that is, at low concentrations, similar to that of its 1,4-dienoic fatty acid analog, *E*-12-NO₂-18:2. Induction of PPAR γ -dependent transcription is robust at nanomolar concentrations and appears to begin to saturate at ~1 μ M. However, at concentrations above 1 μ M, the *E*-12-NO₂-18:1 (**7**)-mediated activation profile deviates significantly from *E*-12-NO₂-18:2-mediated activation in that the former appears to increase exponentially from 1–5 μ M. Collectively, these results indicate that the 12-position of nitration is the most important for optimal activation of PPAR γ at low, submicromolar concentrations; and, at high concentrations, the activation of PPAR γ by the nitrated enoic fatty acids examined differs fundamentally from the activation of PPAR γ by the nitrated 1,4-dienoic fatty acids.

Because all of the nitroalkene fatty acids are strong electrophiles, it was important to assess their potential toxicities over the range of concentrations used in the PPAR γ activation studies. As shown in Figure 5, exposure of cells to the nitroalkenes at concentrations up to 1–2 μ M was associated with little or no growth inhibition or cytotoxicity. However, at 5 μ M, treatments with all five nitroalkenes were associated with significant cytotoxicities with seven day survival ranging from 60 % for *E*-12-NO₂-18:2 treated to < 5 % for *E*-9-NO₂-18:1 (**1**) treated cells.

DISCUSSION AND CONCLUSIONS

The results described herein provide further evidence that the nitroalkene fatty acid isomers are *not* equivalent with respect to activation of PPAR γ -dependent transcription. By examining a variety of naturally occurring and model synthetic nitroalkenes, we demonstrate both quantitative differences in their potencies of PPAR γ activation as well as qualitative differences in their dose-response patterns of activation. These results reveal that the site of nitration plays an important role in optimal PPAR γ activation at submicromolar concentrations. Previous work identifies the natural 12-nitrolinoleic acid, *E*-12-NO₂-18:2, as the most potent of the nitrated linoleic acid regioisomers;⁷ and, results of the present studies with the synthetic compound, *E*-12-NO₂-18:1 (**7**), verify the importance of nitration at C-12 for optimal activation of PPAR γ . The recently reported X-ray structure of the PPAR γ ligand-binding domain complexed with nitrated linoleic acid provides insight regarding the interaction of this compound with the protein.⁹ As suggested for *E*-12-NO₂-18:2, specific non-covalent interactions of the nitro group of *E*-12-NO₂-18:1 (**7**) with Glu343 may induce conformational changes particularly favorable for transcriptional activation of PPAR γ .⁷ The relatively limited activation observed with both natural (**1** and **2**) and synthetic (**5**, **6** and **8**) regioisomeric nitrated fatty acids at submicromolar concentrations further supports the importance of C-12 nitro group interactions with the protein for optimal PPAR γ activation. The 9-enoic 16-carbon nitroalkene fatty acid homologues (**3** and **4**) and the *Z*-nitroalkenes (**9**) of nitrated oleic acid (**1** and **2**) were designed and prepared to examine chain length and alkene stereochemistry on activity. While, the relatively lower level of PPAR γ activation observed at low concentrations of **1**, **2**, **5**, **6** and **9** makes structure-activity interpretations based on these compounds tentative at best; analysis of 9-enoic 16-carbon and 18-carbon nitroalkene fatty acid homologues suggests that carbon chain length—at least over this narrow range—has little effect on PPAR γ activation. Importantly, in aggregate, our results show that non-natural nitrated fatty acids, particularly if nitrated at C12, can potently activate PPAR γ and provide a structural basis for the development of new compounds designed to interact with this transcription factor.

The number of double bonds, one versus two, had little effect on PPAR γ activation by 12-NO₂ derivatives at concentrations at or below 1 μ M (Figure 4); however, at higher concentrations, the dose-response patterns for PPAR γ activation of all nitroenoic fatty acids examined differed substantially from the patterns observed for the nitrated 1,4-dienoic fatty acids—including the *E*-12-NO₂-18:2 studied here as well as the *E*-9/10-NO₂-18:2 and *E*-13-NO₂-18:2 isomers studied previously.⁷ While treatments with nitrated 1,4-dienoic fatty acids resulted in clearly saturable responses over the concentration range up to 5 μ M (here, Figures 3 and 4; and, previously⁷), treatments with nitroenoic fatty acids resulted in steady increases in the response profiles that, for some nitroenoic fatty acids, appeared exponential as concentrations approached 5 μ M (Figures 3 and 4). The reason(s) for these deviations from saturation dose-response observed for nitroenoic fatty acids is (are) unclear. We can speculate that at high concentrations the PPAR γ ligand binding domain may simultaneously bind two nitroenoic acid molecules—preferentially, over nitrated 1,4-dienoic fatty acids—as has been suggested by X-ray crystal structures solved for PPAR γ in complex with the oxo-fatty acid ligand, 9-(*S*)-hydroxyoctadeca-10,12-dienoic acid.¹⁶ The binding of two such ligands could substantially modify the conformation of PPAR γ and thereby its activation of transcription. Alternatively, the nitroenoic fatty acids may uniquely, or preferentially, interact with other

signaling pathways that may, then, indirectly influence PPAR γ activity. Indeed, for example, these nitroalkenes are potent electrophiles that, as a group, have the potential to influence a variety of other redox-sensitive signaling pathways and protein kinases^{5, 17-20} which may, then, secondarily modulate PPAR γ -dependent transcription via phosphorylation of PPAR γ or other ancillary transcription factors. Nevertheless, it should be recognized that the physiological significance of these deviations from saturation at high nitroalkene concentrations are uncertain, because: first, the nitroalkene fatty acids have significant cytotoxicities at concentrations above 2 μ M (Figure 5); and second, such high concentrations of nitroalkene fatty acids have yet to be reported in vivo.

EXPERIMENTAL SECTION

Chemistry—General

Heptanal, hexanal, methyl 4-oxobutanoate, tridecanal, δ -valerolactone, dodecylaldehyde, methyl 11-bromoundecanoate, cyclododecene, and 1-nitrohexane were purchased from Aldrich Chemical Company and were used as received. Melting points (mp) were determined on a Mel-Temp capillary melting point apparatus and are uncorrected. Analytical TLC was performed on silica gel plates with QF-254 indicator. Visualization was accomplished with UV light, iodine, KMnO₄, bromocresol green, and/or dinitrophenylhydrazine. Solvents for extraction and purification were technical grade and were used as received. All reactions were performed in flame-dried glassware under an inert atmosphere of dry argon. ¹H NMR and ¹³C NMR spectra were recorded in CDCl₃ using a BrukerAvance 300 MHz NMR spectrometer. Chemical shifts are given in ppm (δ); multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broadened). Mass spectra were performed by HT Laboratories (San Diego, CA). Data are reported in the form *m/z*. Purity of the compounds for biochemical assay was confirmed to be greater than 95% by elemental combustion analyses that were performed by Atlantic Microlab, Inc. (Atlanta, GA). Stock solutions of nitroalkene fatty acids were prepared with anhydrous dimethylsulfoxide and stored at -80°C.

Chemistry—Synthesis

General Method 1 - Henry Reaction: Methyl 10-hydroxy-9-nitrohexadecanoate (11)—Powdered *t*-BuOK (57 mg, 0.51 mmol) was added to a solution of nitroalkane(**10**)¹⁰ (1.32 g, 6.10 mmol) and heptanal (0.58 g, 0.71 mL, 5.09 mmol) in *t*-BuOH:THF (1:1, 5 mL) at 0 °C. After stirring at room temperature for 24 h, the solution was diluted with Et₂O (25 mL) and water (25 mL). The organic layer was washed with saturated aqueous NaHCO₃ (25 mL) and brine (25 mL). The combined aqueous layers were back-extracted with Et₂O (2 $\times$ 50 mL) and the combined organic layers were dried over MgSO₄, filtered, and concentrated in vacuo. The crude residue was purified by column chromatography (silica gel, 4:1 hexanes:EtOAc, R_f = 0.21) to give **11** as a mixture of diastereomers (1.44 g, 86%): ¹H NMR 4.47 - 4.38 (m, 1H), 4.03 - 3.96 (m, 1H), 3.90 - 3.84 (m, 1H), 3.66 (s, 3H), 2.29 (t, *J* = 7.5 Hz, 2H), 1.80 - 1.18 (m, 22H), 0.84 (m, 3H); ¹³C NMR 174.4, 93.0, 92.5, 72.5, 72.2, 51.6, 34.1, 33.8, 33.4, 32.1, 31.8, 30.6, 29.8, 29.5, 29.2, 29.1, 29.0, 28.9, 28.1, 26.0, 25.7, 25.4, 24.9, 22.7, 14.2.

General Method 2 - Dehydration: (E)-Methyl 9-nitrohexadec-9-enoate (12)—A solution of **11** (1.44 g, 4.36 mmol), DMAP (54 mg, 0.44 mmol), and Ac₂O (0.55 g, 0.51 mL, 5.45 mmol) in Et₂O (10 mL) was stirred for 6 h at room temperature and concentrated. DMAP (0.64 g, 5.23 mmol) was added to a solution of the crude nitroacetates in CH₂Cl₂ (20 mL) and the solution was stirred at room temperature for 24 h, diluted with CH₂Cl₂ (50 mL) and washed with water (50 mL), 0.1 N aqueous HCl (50 mL), and brine (50 mL). The combined aqueous layers were back-extracted with CH₂Cl₂ (2 $\times$ 50 mL) and the combined organic layers were dried over MgSO₄, filtered, and concentrated in vacuo. The crude residue was purified by

column chromatography (silica gel, 4:1 hexanes:EtOAc, R_f = 0.27) to give **12** as a yellow oil (0.61 g, 45%): ^1H NMR 7.06 (t, J = 7.8 Hz, 1H), 3.65 (s, 3H), 2.55 (t, J = 7.5 Hz, 2H), 2.28 (t, J = 7.5 Hz, 2H), 2.20 (q, J = 7.5 Hz, 2H), 1.67 - 1.20 (m, 18H), 0.88 (t, J = 6.6 Hz, 3H); ^{13}C NMR 174.3, 151.9, 136.5, 51.5, 34.1, 31.6, 29.14, 29.10, 29.08, 29.01, 28.6, 28.1, 28.0, 26.4, 25.0, 22.6, 14.1.

General Method 3 - Hydrolysis: (E)-9-Nitrohexadec-9-enoic acid (3, E-9-NO₂-16:1)—A solution of **12** (0.60 g, 1.92 mmol), 1,4-dioxane (5 mL), and 6 M aq. HCl (15 mL) was refluxed for 20 h. The solution was cooled to room temperature and extracted with EtOAc (3 × 20 mL). The combined organic layers were washed with water (30 mL) and brine (30 mL), dried over MgSO₄, filtered, and concentrated. The crude residue was purified by column chromatography (silica gel, 4:1 hexanes:EtOAc → 1:1 hexanes:EtOAc, R_f = 0.08) to give **3** as a yellow oil (0.30 g, 52%): ^1H NMR 11.12 (bs, 1H), 7.04 (t, J = 8.1 Hz, 1H), 2.56 (t, J = 7.5 Hz, 2H), 2.32 (t, J = 7.2 Hz, 2H), 2.17 (q, J = 7.2, 7.5 Hz, 2H), 1.59 - 1.51 (m, 2H), 1.50 - 1.20 (m, 16H), 0.85 (t, J = 6.9 Hz, 3H); ^{13}C NMR 180.4, 151.8, 136.5, 34.1, 32.7, 31.6, 29.0, 28.9, 28.5, 28.3, 28.0, 27.9, 27.2, 26.3, 24.6, 22.5, 14.0; ESI MS m/z 298 (M-H⁺). Anal. calcd. for C₁₆H₂₉NO₄: C, 64.18; H, 9.76; N, 4.68. Found: C, 64.36; H, 9.87; N, 4.47.

2-Hydroxy-1-nitroheptane (13)—Synthesized via General Method 1 using nitromethane (2.44 g, 2.15 mL, 40.00 mmol) and hexanal (2.00 g, 2.46 mL, 20.00 mmol). The crude residue was purified by column chromatography (silica gel, 4:1 hexanes:EtOAc, R_f = 0.30) to give **13** as an oil (3.22 g, 100%): ^1H NMR 4.46 - 4.29 (m, 3H), 2.74 (bs, 1H), 1.60 - 1.24 (m, 8H), 0.88 (t, J = 6.9 Hz, 3H).

General Method 4 - Reduction: 1-Nitroheptane (14)—A solution of **13** (3.22 g, 20.00 mmol), DMAP (0.24 g, 2.00 mmol), Ac₂O (2.55 g, 2.36 mL, 25.00 mmol), and Et₂O (40 mL) was stirred for 4 h at room temperature and concentrated. A solution of NaBH₄ (1.51 g, 40.00 mmol) in EtOH (40 mL) was added dropwise to the crude nitroacetates at 0 °C and the solution was stirred for 2.5 h at room temperature, and then acidified with 1 M aq. HCl. The mixture was extracted with Et₂O (3 × 100 mL), dried over MgSO₄, filtered, and concentrated in vacuo to give a crude residue that was purified by column chromatography (silica gel, 4:1 hexanes:EtOAc, R_f = 0.79) to give **14** as a pale yellow oil (1.76 g, 61%): ^1H NMR 4.53 (t, J = 7.2 Hz, 2H), 2.25 - 2.11 (m, 2H), 1.78 - 1.37 (m, 10H), 1.01 (t, J = 6.9 Hz, 3H).

Methyl 9-hydroxy-10-nitrohexadecanoate (15)—Synthesized via General Method 1 using 1-nitroheptane (**14**, 0.89 g, 6.10 mmol) and methyl-9-oxononanoate¹⁰ (0.95 g, 5.09 mmol). The crude residue was purified by column chromatography (silica gel, 4:1 hexanes:EtOAc, R_f = 0.21) to give **15** as a mixture of diastereomers (0.76 g, 45%): ^1H NMR 4.45 - 4.39 (m, 1H), 3.95 - 3.81 (m, 1H), 3.62 (s, 3H), 2.23 (t, J = 7.5 Hz, 2H), 1.80 - 1.18 (m, 22H), 0.82 (m, 3H); ^{13}C NMR 174.3, 93.4, 92.8, 72.7, 72.4, 51.9, 34.4, 33.9, 33.6, 31.8, 30.9, 30.1, 29.8, 29.5, 29.4, 29.1, 29.0, 28.5, 26.3, 26.1, 25.9, 25.6, 25.2, 23.1, 22.9, 14.4.

(E)-Methyl 10-nitrohexadec-9-enoate (16)—Synthesized via General Method 2 using **15** (0.76 g, 2.30 mmol). The crude residue was purified by column chromatography (silica gel, 4:1 hexanes:EtOAc, R_f = 0.27) to give **16** as a yellow oil (0.12 g, 16%): ^1H NMR 7.05 (t, J = 7.8 Hz, 1H), 3.65 (s, 3H), 2.55 (t, J = 7.5 Hz, 2H), 2.29 (t, J = 7.5 Hz, 2H), 2.20 (q, J = 7.5 Hz, 2H), 1.68 - 1.18 (m, 18H), 0.87 (t, J = 6.9 Hz, 3H); ^{13}C NMR 174.2, 152.1, 136.3, 51.5, 34.1, 31.6, 29.23, 29.08, 29.05, 28.99, 28.6, 28.1, 28.0, 26.5, 25.0, 22.6, 14.1.

(E)-10-Nitrohexadec-9-enoic acid (4, E-10-NO₂-16:1)—Synthesized via General Method 3 using **16** (0.10 g, 0.32 mmol). The crude residue was purified by column chromatography (silica gel, 4:1 hexanes:EtOAc → 1:1 hexanes:EtOAc, R_f = 0.08) to give **4** as

a yellow oil (0.09 g, 92%): ^1H NMR 10.62 (bs, 1H), 7.06 (t, $J = 8.1$ Hz, 1H), 2.56 (t, $J = 7.5$ Hz, 2H), 2.34 (t, $J = 7.2$ Hz, 2H), 2.20 (q, $J = 7.2, 7.5$ Hz, 2H), 1.68 - 1.20 (m, 18H), 0.87 (t, $J = 6.9$ Hz, 3H); ^{13}C NMR 180.7, 152.4, 136.6, 34.5, 31.9, 29.5, 29.4, 29.31, 29.27, 29.12, 28.9, 28.4, 28.3, 26.8, 25.04, 24.96, 22.9, 14.4; ESI MS m/z 298 (M-H⁻). Anal. calcd. for $\text{C}_{16}\text{H}_{29}\text{NO}_4$: C, 64.18; H, 9.76; N, 4.68. Found: C, 65.44; H, 10.02; N, 4.03.

Methyl 4-hydroxy-5-nitropentanoate (17)—Synthesized via General Method 1 using nitromethane (0.57 g, 0.50 mL, 9.32 mmol) and methyl 4-oxobutanoate (0.54 g, 4.66 mmol) to give a pale yellow oil that was used without further purification (0.58 g, 71%): ^1H NMR 4.42 - 4.30 (m, 3H), 3.66 (s, 3H), 2.51 (t, $J = 7.2$ Hz, 2H), 1.82 - 1.73 (m, 2H); ^{13}C NMR 174.0, 80.7, 67.7, 52.0, 29.8, 28.7.

Methyl 5-nitropentanoate (18)—Synthesized via General Method 4 using **17** (0.71 g, 3.98 mmol). The crude residue was purified by column chromatography (silica gel, 4:1 hexanes:EtOAc, $R_f = 0.30$) to give **18** as a yellow oil (0.33 g, 51%): ^1H NMR 4.38 (t, $J = 6.9$ Hz, 2H), 3.66 (s, 3H), 2.35 (t, $J = 7.2$ Hz, 2H), 2.07 - 1.97 (m, 2H), 1.74 - 1.64 (m, 2H); ^{13}C NMR 173.2, 75.2, 51.8, 33.0, 26.7, 21.6.

Methyl 6-hydroxy-5-nitrooctadecanoate (19)—Synthesized via General Method 1 using **18** (0.33 g, 2.04 mmol) and tridecanal (0.37 g, 0.44 mL, 1.85 mmol). The crude residue was purified by column chromatography (silica gel, 4:1 hexanes:EtOAc, $R_f = 0.07$) to give **19** as a yellow oil (0.20 g, 30%): ^1H NMR 4.45 - 4.32 (m, 1H), 4.00 (bs, 1H), 3.65 (s, 3H), 2.35 (t, $J = 7.2$ Hz, 2H), 2.14 - 1.05 (m, 26H), 0.85 (t, $J = 6.9$ Hz, 3H); ^{13}C NMR 173.4, 92.0, 72.4, 72.05, 51.8, 33.2, 33.1, 32.0, 29.72, 29.7, 29.6, 29.5, 29.41, 29.39, 27.4, 26.7, 25.7, 22.8, 21.6, 21.4, 14.2.

(E)-Methyl 5-nitrooctadec-5-enoate (20)—Synthesized via General Method 2 using **19** (0.20 g, 0.56 mmol). The crude residue was purified by column chromatography (silica gel, 4:1 hexanes:EtOAc, $R_f = 0.58$) to give **20** as a yellow oil (0.11 g, 58%): ^1H NMR 7.12 (t, $J = 7.8$ Hz, 1H), 3.66 (s, 3H), 2.63 (t, $J = 7.5$ Hz, 2H), 2.34 (t, $J = 7.2$ Hz, 2H), 2.23 (t, $J = 7.5$ Hz, 2H), 1.81 (quintet, $J = 7.2$ Hz, 2H), 1.50 - 1.43 (m, 2H), 1.42 - 1.07 (m, 18H), 0.86 (t, $J = 6.9$ Hz, 3H); ^{13}C NMR 173.4, 150.8, 137.7, 51.7, 33.1, 32.0, 29.71, 29.69, 29.6, 29.4, 28.6, 28.1, 25.7, 23.1, 22.8, 14.2.

(E)-5-Nitrooctadec-5-enoic acid (5, E-5-NO₂-18:1)—Synthesized via General Method 3 using **20** (0.11 g, 0.33 mmol). The crude residue was purified by column chromatography (silica gel, 4:1 hexanes:EtOAc → 1:1 hexanes:EtOAc, $R_f = 0.14$) to give **5** as a white solid (0.056 g, 52% (63% brsm)): ^1H NMR 7.16 (t, $J = 8.1$ Hz, 1H), 2.67 (t, $J = 7.5$ Hz, 2H), 2.42 (t, $J = 7.2$ Hz, 2H), 2.23 (q, $J = 7.5$ Hz, 2H), 1.85 (quintet, $J = 7.2$ Hz, 2H), 1.52 - 1.10 (m, 20H), 0.88 (t, $J = 6.9$ Hz, 3H); ^{13}C NMR 179.3, 150.7, 137.9, 33.1, 32.0, 29.77, 29.75, 29.72, 29.6, 29.5, 28.6, 28.2, 25.7, 22.9, 22.8, 14.2; ESI MS m/z 326 (M-H⁻). Anal. calcd. for $\text{C}_{18}\text{H}_{33}\text{NO}_4$: C, 66.02; H, 10.16; N, 4.28. Found: C, 67.06; H, 10.29; N, 3.79.

Methyl 5-oxopentanoate (21)—Synthesized via a literature procedure in two steps from δ -valerolactone (50 mmol). The crude residue was purified by column chromatography (silica gel, 2:3 hexanes:EtOAc, $R_f = 0.67$) to give **21** as a colorless oil (5.86 g, 66%). ^1H and ^{13}C NMR matched the reported values.

1-Nitrotridecan-1-ol (22)—Synthesized via General Method 1 using nitromethane (1.33 g, 1.17 mL, 21.7 mmol) and dodecylaldehyde (2.00 g, 10.85 mmol). The crude residue was purified by column chromatography (silica gel, 4:1 hexanes:EtOAc, $R_f = 0.39$) to give **22** as a white solid (2.16 g, 81%): mp 26 °C; ^1H NMR 4.44 - 4.21 (m, 3H), 2.77 (bd, $J = 4.5$ Hz, 1H),

1.52 - 1.16 (m, 20H), 0.86 (t, $J = 6.9$ Hz, 3H); ^{13}C NMR 80.8, 68.8, 33.9, 32.0, 29.7, 29.6, 29.5, 29.4, 25.3, 22.8, 14.2.

1-Nitrotridecane (23)—Synthesized via General Method 4 using **22** (2.16 g, 8.83 mmol). The crude residue was purified by column chromatography (silica gel, 4:1 hexanes:EtOAc, $R_f = 0.69$) to give **23** as a pale yellow oil (1.62 g, 80%): ^1H NMR 4.42 (t, $J = 7.2$ Hz, 2H), 1.54 - 1.21 (m, 22H), 0.86 (t, $J = 6.9$ Hz, 3H); ^{13}C NMR 76.1, 32.3, 30.0, 29.9, 29.7, 29.5, 29.3, 28.9, 28.1, 27.8, 26.6, 23.1, 14.5.

Methyl 5-hydroxy-6-nitrooctadecanoate (24)—Synthesized via General Method 1 using **23** (0.71 g, 3.12 mmol) and **21** (0.34 g, 2.60 mmol). The crude residue was purified by column chromatography (silica gel, 4:1 hexanes:EtOAc, $R_f = 0.19$) to give **24** as a pale yellow oil as a mixture of diastereomers (0.40 g, 43%): ^1H NMR 4.47 - 4.36 (m, 1H), 4.13 - 3.86 (m, 1H), 3.66 (s, 3H), 2.36 (t, $J = 7.5$ Hz, 2H), 1.89 - 1.13 (m, 26H), 0.86 (t, $J = 6.9$ Hz, 3H); ^{13}C NMR 174.0, 93.1, 92.5, 72.0, 71.8, 51.8, 33.5, 33.4, 32.9, 32.6, 32.0, 30.5, 29.7, 29.5, 29.4, 29.3, 29.0, 28.4, 28.1, 26.1, 25.8, 22.8, 21.5, 20.9, 20.6, 14.2.

(E)-Methyl 6-nitrooctadec-5-enoate (25)—Synthesized via General Method 2 using **24** (0.36 g, 1.01 mmol). The crude residue was purified by column chromatography (silica gel, 4:1 hexanes:EtOAc, $R_f = 0.43$) to give **25** as a pale yellow oil (0.13 g, 36%): ^1H NMR 7.02 (t, $J = 7.8$ Hz, 1H), 3.66 (s, 3H), 2.55 (t, $J = 7.2$ Hz, 2H), 2.36 (t, $J = 7.2$ Hz, 2H), 2.27 (q, $J = 7.5$ Hz, 2H), 1.82 (quintet, 7.2, 7.5 Hz, 2H), 1.50 - 1.14 (m, 20H), 0.86 (t, $J = 6.9$ Hz, 3H); ^{13}C NMR 173.3, 152.7, 134.8, 51.8, 33.3, 32.0, 29.7, 29.6, 29.44, 29.36, 28.0, 27.3, 26.5, 23.8, 22.8, 14.2.

(E)-6-Nitrooctadec-5-enoic acid (6, E-6-NO₂-18:1)—Synthesized via General Method 3 using **25** (0.13 g, 0.38 mmol). The crude residue was purified by column chromatography (silica gel, 4:1 hexanes:EtOAc → 1:1 hexanes:EtOAc, $R_f = 0.14$) to give **6** as a pale yellow oil (0.06 g, 49% (98% brsm)): ^1H NMR 10.84 (bs, 1H), 7.03 (t, $J = 7.5$ Hz, 1H), 2.57 (t, $J = 7.2$ Hz, 2H), 2.43 (t, $J = 7.2$ Hz, 2H), 2.30 (q, $J = 7.5$ Hz, 2H), 1.83 (quintet, 7.2 Hz, 2H), 1.49 - 1.22 (m, 20H), 0.87 (t, $J = 6.9$ Hz, 3H); ^{13}C NMR 179.0, 152.9, 134.5, 32.0, 29.8, 29.6, 29.5, 29.4, 28.1, 27.3, 26.5, 23.6, 22.8, 14.2; ESI MS m/z 326 (M-H⁻). Anal. calcd. for C₁₈H₃₃NO₄: C, 66.02; H, 10.16; N, 4.28. Found: C, 65.60; H, 10.22; N, 3.68

Methyl 11-oxoundecanoate (26)—Synthesized via a literature procedure from methyl 11-bromoundecanoate (10.00 g, 35.81 mmol) to give **26** as a yellow oil (4.10 g, 53%): ^1H NMR 9.66 (t, $J = 1.8$ Hz, 1H), 3.56 (s, 3H), 2.32 (td, $J = 7.2$ Hz, 1.8 Hz, 2H), 2.20 (t, $J = 7.5$ Hz, 2H), 1.57 - 1.45 (m, 4H), 1.33 - 1.27 (m, 10H); ^{13}C NMR 202.6, 174.1, 51.3, 43.8, 33.9, 29.28, 29.19, 29.11, 29.07, 29.03, 29.00, 22.0.

Methyl 11-hydroxy-12-nitrododecanoate (27)—Synthesized via General Method 1 using nitromethane (1.11 g, 0.98 mL, 18.32 mmol) and **26** (1.96 g, 9.16 mmol). The crude residue was purified by column chromatography (silica gel, 4:1 hexanes:EtOAc, $R_f = 0.23$) to give **27** as a white solid (2.24 g, 89%): mp 36 - 38 °C; ^1H NMR 4.45 - 4.22 (m, 3H), 3.65 (s, 3H), 2.76 (bs, 1H), 2.28 (t, $J = 7.5$ Hz, 2H), 1.64 - 1.18 (m, 16H); ^{13}C NMR 174.5, 80.8, 68.8, 51.6, 34.2, 33.9, 29.8, 29.4, 29.3, 29.2, 25.2, 25.0, 14.3

Methyl 12-nitrododecanoate (28)—Synthesized via General Method 4 using **27** (2.24 g, 8.15 mmol). The crude residue was purified by column chromatography (silica gel, 4:1 hexanes:EtOAc, $R_f = 0.41$) to give **28** as a colorless oil (1.35 g, 64%): ^1H NMR 4.32 (t, $J = 6.9$ Hz, 2H), 3.60 (s, 3H), 2.23 (t, $J = 7.5$ Hz, 2H), 1.98 - 1.89 (m, 2H), 1.60 - 1.50 (m, 2H), 1.38 - 1.15 (m, 14H); ^{13}C NMR 174.6, 76.1, 51.7, 34.3, 29.7, 29.6, 29.5, 29.2, 27.7, 26.6, 25.3.

Methyl 13-hydroxy-12-nitrooctadecanoate (29)—Synthesized via General Method 1 using **28** (1.35 g, 5.20 mmol) and hexanal (0.44 g, 0.54 mL, 4.33 mmol). The crude residue was purified by column chromatography (silica gel, 4:1 hexanes:EtOAc, R_f = 0.24) to give **29** as a mixture of diastereomers (1.27 g, 82%): ^1H NMR 4.48 - 4.39 (m, 1H), 4.04 - 3.80 (m, 1H), 3.65 (s, 3H), 2.29 (t, J = 7.2 Hz, 2H), 1.85 - 1.13 (m, 26H), 0.88 (t, J = 6.3 Hz, 3H); ^{13}C NMR 174.5, 93.0, 92.5, 72.5, 72.2, 51.6, 34.2, 33.7, 33.3, 31.6, 31.6, 30.6, 29.4, 29.3, 29.2, 29.1, 29.02, 28.92, 28.1, 27.5, 26.3, 26.1, 25.8, 25.4, 25.0, 22.6, 14.1.

(E)-Methyl 12-Nitrooctadec-12-enoate (30)—Synthesized via General Method 2 using **29** (1.27 g, 3.54 mmol). The crude residue was purified by column chromatography (silica gel, 4:1 hexanes:EtOAc, R_f = 0.53) to give **30** as a yellow oil (0.50 g, 42%): ^1H NMR 7.07 (t, J = 8.1 Hz, 1H), 3.65 (s, 3H), 2.56 (t, J = 7.5 Hz, 2H), 2.39 (t, J = 7.5 Hz, 2H), 2.20 (q, J = 7.5 Hz, 2H), 1.64 - 1.53 (m, 2H), 1.52 - 1.40 (m, 4H), 1.36 - 1.18 (m, 16H), 0.86 (t, J = 6.9 Hz, 3H); ^{13}C NMR 174.4, 152.0, 136.5, 51.5, 34.2, 31.6, 29.52, 29.47, 29.33, 29.23, 28.32, 28.08, 28.02, 26.5, 25.1, 22.5, 14.0.

(E)-12-Nitrooctadec-12-enoic acid (7, E-12-NO₂-18:1)—Synthesized via General Method 3 using **30** (0.50 g, 1.47 mmol). The crude residue was purified by column chromatography (silica gel, 4:1 hexanes:EtOAc → 1:1 hexanes:EtOAc, R_f = 0.11) to give **7** as a yellow oil (0.22 g, 46% (72% brsm)): ^1H NMR 11.24 (bs, 1H), 7.06 (t, J = 8.1 Hz, 1H), 2.56 (t, J = 7.5 Hz, 2H), 2.33 (t, J = 7.2 Hz, 2H), 2.18 (q, J = 7.2, 7.5 Hz, 2H), 1.66 - 1.17 (m, 22H), 0.87 (t, J = 6.9 Hz, 3H); ^{13}C NMR 180.5, 152.0, 136.5, 34.2, 31.6, 29.48, 29.42, 29.30, 29.28, 29.10, 28.3, 28.04, 27.97, 26.4, 24.7, 22.5, 14.0; ESI MS m/z 326 (M-H⁺). Anal. calcd. for C₁₈H₃₃NO₄: C, 66.02; H, 10.16; N, 4.28. Found: C, 65.97; H, 10.28; N, 4.16.

Methyl 12-oxododecanoate (31)—Ozone was bubbled into a mixture of cyclododecene (5.00 g, 5.75 mL, 30.06 mmol) and anhydrous Na₂CO₃ (0.82 g, 7.74 mmol) in CH₂Cl₂ (90 mL) and methanol (18 mL) at -78 °C until a faint blue color appeared. Argon was then bubbled into the mixture until the blue color was discharged. The cooling bath was removed and the mixture slowly warmed to room temperature. After filtration, benzene (30 mL) was added and the mixture concentrated to ~20 mL with the resulting viscous liquid being diluted with CH₂Cl₂ (80 mL). After cooling to 0 °C, anhydrous Et₃N (4.50 g, 6.20 mL, 44.48 mmol) and anhydrous Ac₂O (8.55 g, 7.90 mL, 83.73 mmol) were sequentially added dropwise and the mixture was stirred at 0 °C for 0.5 h and then at room temperature overnight. The organic phase was washed with 0.1 M aqueous HCl (2 × 60 mL), 10% aqueous NaOH (2 × 60 mL), water (60 mL), and dried over MgSO₄. The solution was filtered and concentrated in vacuo to give **31** as a oil (4.78 g, 70%): R_f = 0.44 (silica gel, 4:1 hexanes:EtOAc); ^1H NMR 9.74 (t, J = 1.8 Hz, 1H), 3.65 (s, 3H), 2.40 (td, J = 7.2, 1.8 Hz, 2H), 2.28 (td, J = 7.5 Hz, 1.5 Hz, 2H), 1.68 - 1.55 (m, 4H), 1.37 - 1.20 (m, 12H).

Methyl 12-hydroxy-13-nitrooctadecanoate (32)—Synthesized via General Method 1 using 1-nitrohexane (1.05 g, 1.12 mL, 8.00 mmol) and **31** (0.91 g, 4.00 mmol). The crude residue was purified by column chromatography (silica gel, 4:1 hexanes:EtOAc, R_f = 0.15) to give **32** as a mixture of diastereomers (1.18 g, 82%): ^1H NMR 4.44 - 4.36 (m, 1H), 3.96 - 3.93 (m, 1H), 3.86 - 3.81 (m, 1H), 3.62 (s, 3H), 2.26 (t, J = 7.5 Hz, 2H), 1.80 - 1.17 (m, 26H), 0.84 (t, J = 6.9 Hz, 3H); ^{13}C NMR 174.5, 93.1, 92.6, 72.4, 72.1, 51.5, 34.1, 33.6, 33.3, 31.2, 31.1, 30.9, 30.4, 29.5, 29.4, 29.3, 29.2, 29.1, 28.2, 25.7, 25.6, 25.4, 25.3, 25.0, 22.3, 13.9.

(E)-Methyl 13-Nitrooctadec-12-enoate (33)—Synthesized via General Method 2 using **32** (1.18 g, 3.29 mmol). The crude residue was purified by column chromatography (silica gel, 4:1 hexanes:EtOAc, R_f = 0.46) to give **33** as a yellow oil (0.53 g, 47%): ^1H NMR 7.07 (t, J = 7.8 Hz, 1H), 3.66 (s, 3H), 2.56 (t, J = 7.5 Hz, 2H), 2.29 (t, J = 7.5 Hz, 2H), 2.20 (q, J = 7.5 Hz,

2H), 1.67 - 1.18 (m, 22H), 0.87 (t, $J = 6.9$ Hz, 3H); ^{13}C NMR 174.4, 152.0, 136.5, 51.6, 34.2, 31.5, 29.52, 29.48, 29.43, 29.34, 29.24, 28.6, 28.1, 27.7, 26.4, 25.1, 22.5, 14.1.

(E)-13-Nitrooctadec-12-enoic acid (8, 13-NO₂OA)—Synthesized via General Method 3 using **33** (0.53 g, 1.54 mmol). The crude residue was purified by column chromatography (silica gel, 4:1 hexanes:EtOAc $\rightarrow$ 1:1 hexanes:EtOAc, $R_f = 0.11$) to give **8** as a yellow oil (0.15 g, 30% (61% brsm)): ^1H NMR 11.34 (bs, 1H), 7.07 (t, $J = 8.1$ Hz, 1H), 2.56 (t, $J = 7.5$ Hz, 2H), 2.34 (t, $J = 7.2$ Hz, 2H), 2.20 (q, $J = 7.2, 7.5$ Hz, 2H), 1.68 - 1.20 (m, 22H), 0.87 (t, $J = 6.9$ Hz, 3H); ^{13}C NMR 180.5, 152.3, 136.8, 34.5, 31.8, 29.80, 29.75, 29.72, 29.61, 29.43, 28.9, 28.4, 28.0, 26.7, 25.1, 22.8, 14.4; ESI MS m/z 326 (M-H⁻). Anal. calcd. for C₁₈H₃₃NO₄: C, 66.02; H, 10.16; N, 4.28. Found: C, 65.48; H, 10.14; N, 3.99.

(Z)-9 and 10-Nitrooctadec-9-enoic acid (9, Z-9/10-NO₂-18:1 or Z-9/10-NO₂-OA)—Synthesized from elaidic acid (Aldrich, 0.282 g, 1.0 mmol) using a previously published non-selective nitro-selenation-elimination sequence.¹⁵ The crude residue was purified by column chromatography (silica gel, 70:30:1 hexanes:Et₂OAc:AcOH, $R_f = 0.48$) to give **9** as a yellow oil (mixture of regioisomers, 0.125 g, 38%): ^1H NMR 11.11 (bs, 1H), 5.65 (m, 1H), 2.49 (t, $J = 7.2$ Hz, 2H), 2.34 (t, $J = 7.5$ Hz, 4H), 1.64 - 1.26 (m, 22H), 0.87 (t, $J = 6.3$ Hz, 3H); ^{13}C NMR 180.5, 151.1, (131.9, 131.5), (40.0, 39.9), (32.7, 32.6), (31.8, 31.7), (29.4-28.1, multiple peaks), (27.2, 27.1), (24.6, 24.5), 22.6, 14.1.

Nitroalkene-PPAR γ interactions: Binding assays

Relative binding affinities of test ligands for PPAR were determined by a radioligand competition scintillation proximity assay using purified recombinant GST-hPPAR receptor, ²³ the radioligand [³H₂]nTZD3, and varying concentrations of unlabeled test ligands (nitroalkene fatty acids) as described previously.^{7, 24} IC₅₀ values were calculated from the average of duplicate data points obtained at each ligand concentration.

Nitroalkene-PPAR γ interactions: Activation of PPAR γ -dependent transcription

Reporter gene analysis of ligand-dependent PPAR γ transcription activation was accomplished as described previously.⁷ Briefly, the studies used the MCF7/RTO/PPARG1-4' cell line which expresses a doxycycline-repressible human PPAR γ transgene. MCF7/RTO/PPARG1-4' cells, grown in the absence of doxycycline, were plated in replicate twelve-well tissue culture plates at a density of 4×10^4 cells/well. Twenty-four hr later, cells were co-transfected with 0.4 μg of the PPARE-containing firefly luciferase reporter gene, pPPREx3-TK-LUC²⁵, and 20 pg control CMV-*Renilla* luciferase reporter gene, pGL4.75 (Promega, Madison, WI) using Superfect reagent (Qiagen, Valencia, CA) according to the manufacturer's recommendations. Twenty-four hr after the start of transfections, medium was replaced with medium containing vehicle (control) or varying concentrations of inducing agent (nitroalkene fatty acids or rosiglitazone, Cayman, Ann Arbor, MI). Cells were harvested after 24 hr continuous exposure to vehicle or inducing agent. Luciferase assays were accomplished using the Dual Luciferase Assay System (Promega, Madison, WI) and values were corrected for variations in transfection efficiencies and non-specific induction as described previously.²⁶ All incubations, including transfections and inductions, were done in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum.

Cytotoxicity of nitroalkene fatty acids

Cytotoxicity was determined using the sulforhodamine B microtiter plate assay as described.²⁷ Cells were plated at a density of 300 cells/well in 96-well plates. Twenty-four hours later, the cells were exposed to varying concentrations of nitroalkene fatty acids or vehicle control

for 24 h in Dulbecco's modified Eagle medium containing 10% fetal calf serum. The medium was subsequently replaced and the plates were incubated 7 days, then fixed and stained.

ACKNOWLEDGEMENT

This work was supported by NIH grants CA70338 (CSM) and HL62198 (SBK).

REFERENCES

1. Baker PRS, Lin Y, Schopfer FJ, Woodcock SR, Groeger AL, Batthyany C, Sweeney S, Long MH, Iles KE, Baker LMS, Branchaud BP, Chen YE, Freeman BA. Fatty Acid Transduction of Nitric Oxide Signaling: Multiple nitrated unsaturated fatty acid derivatives exist in human blood and urine and serve as endogenous peroxisome proliferator-activated receptor ligands. *J. Biol. Chem* 2005;280:42464–42475. [PubMed: 16227625]
2. Cui T, Schopfer FJ, Zhang J, Chen K, Ichikawa T, Baker PRS, Batthyany C, Chacko BK, Feng X, Patel RP, Agarwal A, Freeman BA, Chen YE. Nitrated Fatty Acids: Endogenous Anti-inflammatory Signaling Mediators. *J. Biol. Chem* 2006;281:35686–35698. [PubMed: 16887803]
3. Schopfer FJ, Lin Y, Baker PRS, Cui T, Garcia-Barrio M, Zhang J, Chen K, Chen YE, Freeman BA. Nitrolinoleic acid: An endogenous peroxisome proliferator-activated receptor {gamma} ligand. *Proc. Natl. Acad. Sci. (USA)* 2005;102:2340–2345. [PubMed: 15701701]
4. Villacorta L, Zhang J, Garcia-Barrio MT, Chen X.-l. Freeman BA, Chen YE, Cui T. Nitro-linoleic acid inhibits vascular smooth muscle cell proliferation via the Keap1/Nrf2 signaling pathway. *Am J Physiol Heart Circ Physiol* 2007;293:H770–776. [PubMed: 17468336]
5. Freeman BA, Baker PRS, Schopfer FJ, Woodcock SR, Napolitano A, d'Ischia M. Nitro-fatty Acid Formation and Signaling. *J. Biol. Chem* 2008;283:15515–15519. [PubMed: 18285326]
6. Lima ES, Bonini MG, Augusto O, Barbeiro HV, Souza HP, Abdalla DS. Nitrated lipids decompose to nitric oxide and lipid radicals and cause vasorelaxation. *Free Radic Biol Med* 2005;39:532–539. [PubMed: 16043024]
7. Alexander R, Wright M, Gorczyński M, Smitherman P, Akiyama T, Wood H, Berger J, King S, Morrow C. Differential potencies of naturally occurring regioisomers of nitrolinoleic acid in PPARgamma activation. *Biochemistry* 2009;48:492–498. [PubMed: 19105608]
8. Baker PRS, Schopfer FJ, Sweeney S, Freeman BA. Red cell membrane and plasma linoleic acid nitration products: Synthesis, clinical identification, and quantitation. *Proc. Natl. Acad. Sci. (USA)* 2004;101:11577–11582. [PubMed: 15273286]
9. Li Y, Zhang J, Schopfer FJ, Martynowski D, Garcia-Barrio MT, Kovach A, Suino-Powell K, Baker PR, Freeman BA, Chen YE, Xu HE. Molecular recognition of nitrated fatty acids by PPAR gamma. *Nat Struct Mol Biol* 2008;15:865–867. [PubMed: 18604218]
10. Gorczyński M, Huang J, King S. Regio- and stereospecific syntheses and nitric oxide donor properties of (E)-9- and (E)-10-nitrooctadec-9-enoic acids. *Org Lett* 2006;8:2305–2308. [PubMed: 16706512]
11. Denmark SE, Kesler BS, Moon YC. Intermolecular and Intramolecular [4+2] Cycloadditions of Nitroalkenes with Olefins -2-Nitrostyrenes. *Journal of Organic Chemistry* 1992;57:4912–4924.
12. Denmark SE, Gomez L. Tandem double intramolecular [4+2]/[3+2] cycloadditions of nitroalkenes. The fused/bridged mode. *Journal of Organic Chemistry* 2003;68:8015–8024. [PubMed: 14535778]
13. Lucet D, Heyse P, Gissot A, Le Gall T, Mioskowski C. Synthesis of the diastereomers of dethiobiotin using the conjugate addition of 4-phenyloxazolidin-2-one to a nitroalkene. *European Journal of Organic Chemistry* 2000:3575–3579.
14. Wollenberg RH, Miller SJ. Nitroalkane Synthesis -Convenient Method for Aldehyde Reductive Nitromethylation. *Tetrahedron Letters* 1978:3219–3222.
15. Alexander RL, Bates DJ, Wright MW, King SB, Morrow CS. Modulation of Nitrated Lipid Signaling by Multidrug Resistance Protein 1 (MRP1): Glutathione Conjugation and MRP1-Mediated Efflux Inhibit Nitrolinoleic Acid-Induced, PPARgamma-Dependent Transcription Activation. *Biochemistry* 2006;45:7889–7896. [PubMed: 16784241]

16. Itoh T, Fairall L, Amin K, Inaba Y, Szanto A, Balint BL, Nagy L, Yamamoto K, Schwabe JW. Structural basis for the activation of PPAR γ by oxidized fatty acids. *Nat Struct Mol Biol* 2008;15:924–931. [PubMed: 19172745]
17. Chen C, Kong A. Dietary chemopreventive compounds and ARE/EpRE signaling. *Free Radic Biol Med* 2004;36:1505–1516. [PubMed: 15182853]
18. Xiao D, Choi S, Lee Y, Singh S. Role of mitogen-activated protein kinases in phenethyl isothiocyanate-induced apoptosis in human prostate cancer cells. *Mol Carcinog* 2005;43:130–140. [PubMed: 15880419]
19. Zhang H, Liu H, Iles KE, Liu R-M, Postlethwait EM, Laperche Y, Forman HJ. 4-Hydroxynonenal Induces Rat { γ }-Glutamyl Transpeptidase through Mitogen-Activated Protein Kinase-Mediated Electrophile Response Element/Nuclear Factor Erythroid 2-Related Factor 2 Signaling. *Am. J. Respir. Cell Mol. Biol* 2006;34:174–181. [PubMed: 16195535]
20. Zhang Y, Gordon GB. A strategy for cancer prevention: Stimulation of the Nrf2-ARE signaling pathway. *Mol. Cancer Ther* 2004;3:885–893. [PubMed: 15252150]
21. Enholm E, Joshi A, Wright D. Photocurable hard and porous biomaterials from ROMP precursors cross-linked with diyl radicals. *Bioorg Med Chem Lett* 2005;15:5262–5265. [PubMed: 16203146]
22. Ravi S, Padmanabhan D, Mamdapur VR. Macrocyclic musk compounds: synthetic approaches to key intermediates for exaltolide, exaltone, and dilactones. *J. Indian Inst. Sci* 2001;81:299–312.
23. Elbrecht A, Chen Y, Adams A, Berger J, Griffin P, Klatt T, Zhang B, Menke J, Zhou G, Smith RG, Moller DE. L-764406 Is a Partial Agonist of Human Peroxisome Proliferator-activated Receptor γ . The Role of Cys313 in ligand binding. *J. Biol. Chem* 1999;274:7913–7922. [PubMed: 10075686]
24. Berger JP, Petro AE, Macnaul KL, Kelly LJ, Zhang BB, Richards K, Elbrecht A, Johnson BA, Zhou G, Doebber TW, Biswas C, Parikh M, Sharma N, Tanen MR, Thompson GM, Ventre J, Adams AD, Mosley R, Surwit RS, Moller DE. Distinct Properties and Advantages of a Novel Peroxisome Proliferator-Activated Protein { γ } Selective Modulator. *Mol Endocrinol* 2003;17:662–676. [PubMed: 12554792]
25. Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM, Evans RM. 15- Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR γ . *Cell* 1995;83:803–812. [PubMed: 8521497]
26. Sibhatu MB, Smitherman PK, Townsend AJ, Morrow CS. Expression of MRP1 and GSTP1-1 modulate the acute cellular response to treatment with the chemopreventive isothiocyanate, sulforaphane. *Carcinogenesis* 2008;29:807–815. [PubMed: 18204073]
27. Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S, Boyd MR. New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst* 1990;82:1107–1112. [PubMed: 2359136]

Figure 1.
 Structures and abbreviations used for the nitroalkene fatty acids.

Figure 2.

Activation of PPAR γ -dependent transcription by nitroalkene fatty acids.

The bar graphs show the PPAR γ -dependent induction of PPRE-containing reporter gene expression by nitroalkene fatty acids in comparison with induction by rosiglitazone (RSG) or vehicle (control). Inductions were accomplished with 0.1 μ M ligand (open bars) or 3 μ M ligand (shaded dark bars) as described in Experimental Section. Fold induction is defined as: (corrected PPRE-reporter activity + inducing agent)/(corrected PPRE-reporter activity - inducing agent). Bars represent the mean values of triplicate determinations $\pm$ 1 sd.

Figure 3.

Dose-response analysis of PPAR γ -dependent transcription induced by naturally occurring nitroalkene fatty acids.

Concentration dependent induction of PPAR γ -mediated PPRE-reporter gene expression was determined as described in Experimental Section for the 12-nitro derivative of linoleic acid, *E*-12-NO₂-18:2 (closed circles), and the 9- and 10-nitro derivative of oleic acid, *E*-9-NO₂-18:1 (1)(open circles) and *E*-10-NO₂-18:1 (2)(closed squares). Data points represent the mean values of triplicate determinations $\pm$ 1 sd. The dose-response curve for *E*-12-NO₂-18:2 was fitted to the equation described previously from which an EC₅₀ value = 0.07 μ M was determined.⁷

Figure 4.

Dose-response analysis of PPAR γ -dependent transcription induced by model synthetic nitroenoic fatty acids.

Concentration dependent induction of PPAR γ -mediated PPRE-reporter gene expression was determined as described in Experimental Section and Figure 3 for the model nitroenoic fatty acids *E*-12-NO₂-18:1 (**7**)(closed triangles) and *E*-9-NO₂-16:1 (**3**)(open triangles). The 12-nitro derivative of linoelic acid, *E*-12-NO₂-18:2 (closed circles), was included for comparison. Data points represent the mean values of triplicate determinations $\pm$ 1 sd.

Figure 5.

Cytotoxicity of nitroalkene fatty acids.

The MCF7/RTO/PPARG1-4' cells used for PPAR γ -dependent reporter gene activation (Figures 2-4) were treated with varying concentration of nitroalkenes or vehicle control and cytotoxicities were assessed as described in Experimental Section. Nitroalkene fatty acids used were 12-NO₂-18:2 (closed circles), 10-NO₂-18:1 (**2**) (open circles), 12-NO₂-18:1 (**7**) (x's), 9-NO₂-16:1 (**3**) (open squares), and 9-NO₂-18:1 (**1**) (closed squares). Data points represent the mean values of eight replicate determinations $\pm$ 1 s.d.

SCHEME 1.

SCHEME 2.

SCHEME 3.

SCHEME 4.

SCHEME 5.

SCHEME 6.

Table 1

Scintillation proximity competitive binding assay of ligand-PPAR interactions.

Compound	PPAR IC ₅₀ (μM)	
	gamma	alpha
Rosiglitazone *	0.25	>15
<i>E</i> -12-NO ₂ -18:2 *	0.41	9.6
<i>E</i> -9-NO ₂ -18:1 (1)	0.98	>15
<i>E</i> -10-NO ₂ -18:1 (2)	1.61	>15
<i>Z</i> -9/10-NO ₂ -18:1 (9)	1.73	10.8
<i>E</i> -9-NO ₂ -16:1 (3)	0.83	>15
<i>E</i> -10-NO ₂ -16:1 (4)	0.63	>15
<i>E</i> -5-NO ₂ -18:1 (5)	1.68	>15
<i>E</i> -6-NO ₂ -18:1 (6)	1.72	>15
<i>E</i> -12-NO ₂ -18:1 (7)	0.039	1.6
<i>E</i> -13-NO ₂ -18:1 (8)	0.19	1.1

* IC₅₀ values for rosiglitazone and *E*-12-NO₂-18:2 (12-NO₂-LA) were reported previously ⁷ and are included here for comparison.