Free Radical Biology and Medicine 59 (2013) 14–26

Contents lists available at SciVerse ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Methods in Free Radical Biology and Medicine

Nitrated fatty acids: synthesis and measurement

Steven R. Woodcock, Gustavo Bonacci, Stacy L. Gelhaus, Francisco J. Schopfer*

[a] Department of Pharmacology & Chemical Biology, University of Pittsburgh, Pittsburgh, PA 15261, USA

ARTICLE INFO

Available online 29 November 2012

Keywords:
Nitrated fatty acid
Nitration
Mass spectrometry
Organic synthesis
Quantification
Free radicals

ABSTRACT

Nitrated fatty acids are the product of nitrogen dioxide reaction with unsaturated fatty acids. The discovery of peroxynitrite and peroxidase-induced nitration of biomolecules led to the initial reports of endogenous nitrated fatty acids. These species increase during ischemia/reperfusion, but concentrations are often at or near the limits of detection. Here, we describe multiple methods for nitrated fatty acid synthesis and sample extraction from complex biological matrices and a rigorous method of qualitative and quantitative detection of nitrated fatty acids by liquid chromatography–mass spectrometry. In addition, optimized instrument conditions and caveats regarding data interpretation are discussed.

Introduction

The enzymatic and free radical-induced oxidation of unsaturated fatty acids generates bioactive molecules that participate in cell signaling [1–3]. These signaling actions include the activation of G-protein-coupled receptors [4] and alkylation of both thiol-containing small molecules and cysteine residues in proteins [3]. In addition to oxidative stress, nitrative stress is characterized by sustained nitration through the formation of the nitrogen dioxide ($^{\bullet}NO_2$) radical. The biomolecules that are targets of nitration include tyrosine residues [5], nucleic acids (guanine, cGMP, GTP) [6], and unsaturated fatty acids [7]. In particular, the nitration of unsaturated fatty acids results in the formation of electrophilic species that contain a conjugated nitroalkene moiety. The electrophilicity of nitrated fatty acids (NO_2-FA), mainly represented by nitro-oleic (NO_2-OA), nitro-linoleic (NO_2-LA), and nitro-arachidonic acids, promotes reaction with nucleophiles to generate Michael addition products [8]. The targeting of specific cysteine residues by lipid-derived electrophiles is central to modulating enzymatic activity and signaling pathways. Nitrated fatty acids have been shown to potently activate the Nrf2/Keap1 pathway, chaperone heat shock pathways, and inhibit inflammatory responses through multilevel inhibition of NF-κB [3]. These actions result in protective effects in various animal models ranging from metabolic disorders (diabetes) and atherosclerosis to sepsis and ischemia/reperfusion [3]. The data stemming from the pharmacological actions of NO_2-FA is in stark contrast to their characterization and quantification *in vivo*. This is partially due to synthetic challenges of obtaining pure regioisomers, sensitivity to alkaline conditions, the electrophilic nature of the nitrated fatty acid, and the reversible binding to cysteines, all of which result in additional challenges for accurate quantification.

Principles

Nitrated fatty acids form upon exposure of unsaturated fatty acids to nitrating species. In particular, $^{\bullet}NO_2$ plays a central role in the formation of these fatty acid nitroalkenes [9]. The type and characteristics of the precursor fatty acid define the formation of various products. Two main mechanisms have been proposed for the formation of NO_2-FA (Fig. 1). The first involves hydrogen atom abstraction from the bis-allylic carbon of a polyunsaturated fatty acid, yielding a delocalized pentadienyl radical. Various radicals may participate in this initial step including hydroxyl, peroxyl, and carbon-centered radicals derived from fatty acid oxidation and $^{\bullet}NO_2$. These steps are common to the formation of other lipid oxidation products such as isoprostanes and hydroperoxides. Whereas the formation of hydroperoxides and isoprostanes is characterized by the subsequent insertion of oxygen to form a peroxyl radical [10], NO_2-FA are generated by addition of $^{\bullet}NO_2$ to the fatty acid radical. A second, less studied, pathway initially involves the direct addition of $^{\bullet}NO_2$ to the fatty acid to form a nitroalkenyl radical. This radical can then react with oxygen to form a nitroperoxyl fatty acid, react with another $^{\bullet}NO_2$ to form unstable nitro-nitrito or dinitro compounds, or lose a hydrogen atom via abstraction by another radical (i.e., $^{\bullet}NO_2$, $^{\bullet}OH$) to reform the double bond [11]. Thus, the formation of a nitrated fatty acid can occur via multiple reaction mechanisms and its analysis involves the development of mass spectrometry tools that allow for the proper characterization of the various regioisomers [12].

* Corresponding author. Fax: +1 412 648 2999.
E-mail address: fjs2@pitt.edu (F.J. Schopfer).

0891-5849/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.freeradbiomed.2012.11.015

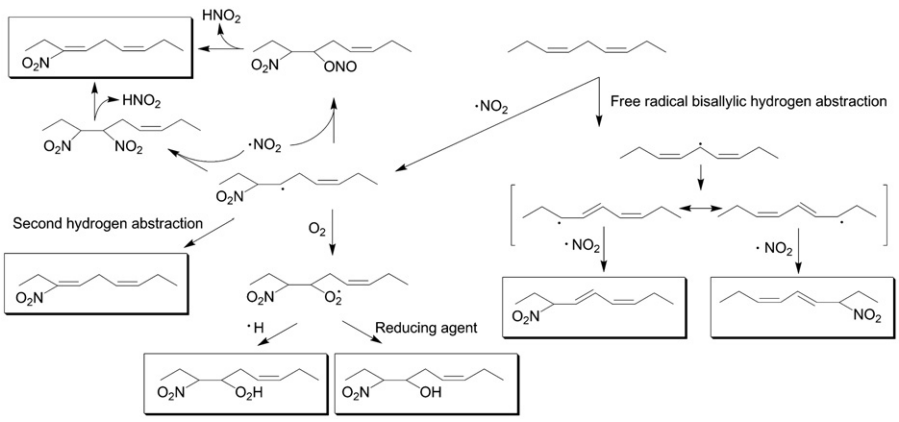

Fig. 1. Radical-induced mechanism of polyunsaturated acid nitration. Starting with a radical abstraction from the bis-allylic position, the delocalized radical may react with oxygen or nitrogen dioxide. Alternatively, direct addition of nitrogen dioxide to one double bond produces a nonstabilized radical intermediate that may react with available oxygen or nitrogen dioxide (the products of which may in turn eliminate nitrous acid and generate the double bond) or lose a second hydrogen to radical abstraction and regenerate the double bond. Radical intermediates may also abstract available bis-allylic hydrogens and propagate the radical chain reaction.

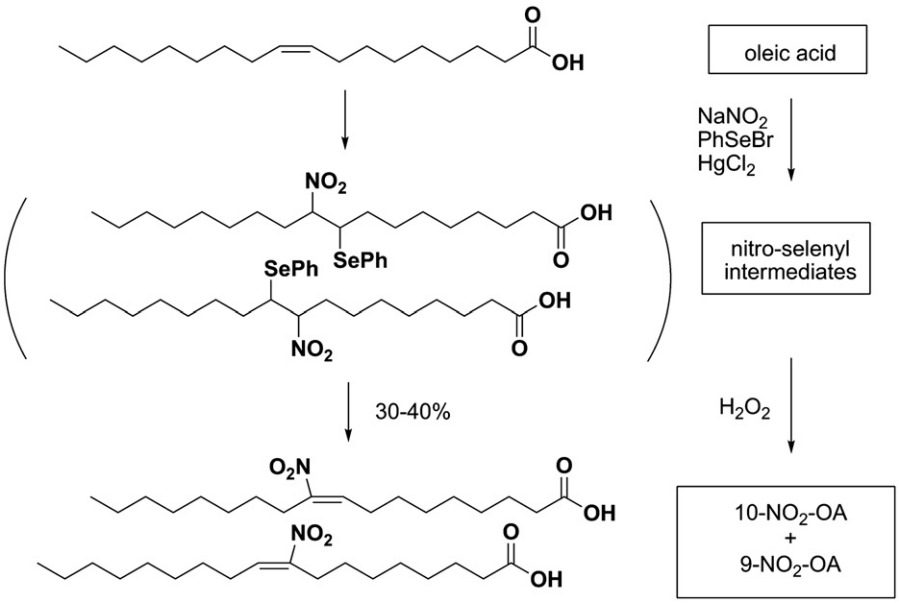

Fig. 2. Nitroselenation/nitromercuration synthesis of nitro-oleic acid. This is a two-step method of synthesizing an equimolar distribution of nitrated regioisomers. The combination of selenyl and mercurial reagents activates the double bond to nitration and are oxidized in a second step to generate the nitroalkene. This approach provides a convenient method of synthesizing NO_2-FA appropriate to many uses, particularly isotopically labeled material.

Consequently, synthetic strategies are critical for the confirmation of proposed structures derived from mass spectrometric analysis.

Pros and cons of available nitro fatty acid synthetic strategies

Many different approaches have been described for generating NO_2-FA. These methods can be separated into three groups based on specificity (product diversity) and practicality.

Nitrogen dioxide/nitronium ion

This method is based on the direct application of a nitrogen dioxide source to unsaturated fatty acids. Despite giving an array of products and by-products, these approaches are of value because the reaction mimics some biological conditions. Applying this reaction to a biological matrix results in the formation of higher concentrations of putative endogenous products; thus allowing for the initial identification, analysis, and characterization of multiple novel nitrated species that could be formed during pathophysiological conditions resulting in increased NO_2^- levels and decreased pH values, without regard to stability or subsequent reactivity. The limitation of $^{\bullet}NO_2$-induced nitration is its high reactivity and low selectivity.

Nitroselenation/nitromercuriation

This approach generates nitroalkenes through a nitroselenation reaction, which activates the alkene to direct nitration. These reactions require additional synthetic skills and have at least two steps, but greatly reduce the purification phase and allow for well-defined products. Although nitromercuriation [13] has not been specifically applied to fatty acids, it has been successfully used in other synthetic procedures. Nitroselenation [14] is a subsequent version of the method that is preferred for synthesis of NO_2-FA when mono- or diunsaturated fatty acids are used as substrates. In particular, this reaction has been used to generate NO_2-LA and NO_2-OA regioisomers that were obtained in equal proportions (25% of each of the four NO_2-LA isomers and 50% of each NO_2-OA) [7,15].

Full synthesis

The third approach consists of specific isomer synthesis. All of the strategies used to this end are based on the Henry nitroaldol reaction and have been used to successfully synthesize positional and stereoselective isomers of NO_2-OA and NO_2-LA, namely 9(*E*)-nitro-octadec-9-enoic acid (9(*E*)-NO_2-OA), 10(*E*)-nitro-octadec-9-enoic acid (10(*E*)-NO_2-OA) [16,17], 10, 12(*E, Z*)-nitro-octoadeca-10, 12-dienoic acid (10(*E*)-NO_2-LA) [18], and the positional analogue 12(*E*)-nitro-octadec-12-enoic acid [19]. These techniques require better synthetic skills than the previously mentioned techniques, but afford stereo- and regiospecific synthetic products that can reach upward of 99% purity. This is the main advantage over the other techniques in which single-species purification is very difficult or not possible. This approach requires protecting the carboxylic acid group during the synthetic steps. Two strategies have been successfully used for this: methyl [17] and allyl esters [16]. Methyl esters are easy to synthesize, but require either 6 M HCl reflux or an enzymatic lipase-based saponification method with unknown scalability. In contrast, allyl esters can be removed under milder conditions (i.e., formic acid and catalytic palladium) for higher yields. With regard to the selection of the base to catalyze the nitro aldol condensation, either 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) or *t*-BuOK has been successfully used. The activation/elimination step has been typically performed by acetylating the nitrohydroxy intermediates. The acetoxy groups were subsequently eliminated to form the nitroalkene double bond, using 4-dimethylaminopyridine (DMAP) or Na_2CO_3 as a mild base.

Commercial sources

Two different nitrated fatty acids are commercially available from multiple vendors. These are 9(*E*)-NO_2-OA (CAS 875685-44-2) and 10(*E*)-NO_2-OA (CAS 88127-53-1). Unfortunately, the actual cost of the products makes their use in animal models prohibitively expensive. If delivery in animal or other large-scale use is planned, in-house synthetic strategies are encouraged. No commercial sources are currently available for isotopically labeled NO_2-FA.

Synthesis of isotopically labeled standards

Various isotopically labeled standards can be synthesized and purified. The utility of the various NO_2-FA isotopes differs and their benefits and drawbacks are specifically discussed in view of their use in stable isotope dilution mass spectrometry-based quantifications and radioactive tracer experiments.

^{15}N-labeled standards

^{15}N-labeled standards are easy to obtain by acidic nitration and nitroselenation reactions. A 99% Na[^{15}N]O_2 is available from Cambridge Isotope Labs at a low cost, making it easy and affordable to replace [^{14}N]O_2^- salts. The full synthetic approach, based on the nitroaldol condensation, relies on one of the alkyl chains containing a terminal NO_2 group. Thus, if not available, a [^{15}N]O_2^- is added during the initial synthetic steps to the terminal position of the starting alkyl material using a nitroaldol or Kornblum nitration reaction [20]. The subsequent loss of material at each step requires the generation of a large amount of ^{15}N-labeled starting material, which is not economical. This makes the full synthesis of [^{15}N]O_2-labeled products less convenient compared to the other approaches used to generate isotopically labeled internal standards.

Stable isotope ^{13}C- and ^{2}H-labeled standards

Various fatty acids labeled with ^{13}C at one or all backbone carbons are commercially available, making methods that start from a ^{13}C-labeled unsaturated fatty acid very attractive. The first and most nonspecific method (nitration under acidic conditions) is not a viable option to obtain ^{13}C-labeled products. The yields are too low, purification is difficult, and the initial ^{13}C-labeled material is too expensive for this strategy to be of value. The second approach, nitroselenation, is the best option for obtaining labeled standards. The yields are high enough (~30%) to obtain pure labeled products. The third approach, nitroaldol condensation, is less practical for synthesizing and purifying ^{13}C-labeled products because it again relies on obtaining ^{13}C-labeled substrates for the nitroaldol condensation reactions, in larger quantities because of subsequent mass loss (often less than 10% overall yield). Most potential reactants either are not commercially available or are more expensive than the available $^{13}C_{18}$-labeled oleic or linoleic acids. Some ^{2}H-labeled lipids are also available, but often the deuteration is placed only on the alkenyl positions, resulting in the loss of a deuterium during synthesis.

^{3}H-labeled standards

These standards are of value for following the metabolic pathways of NO_2-FA both *in vivo* and *in vitro*. Commercially available lipids are typically ^{3}H-labeled on the double bonds. Similar to ^{13}C labeling, the use of acidic nitration is impractical when starting from ^{3}H-labeled unsaturated fatty acids. Note that the last step of the nitroselenation reaction proceeds through an oxidation/elimination that eliminates one olefinic hydrogen located at the carbon α to the NO_2 group. The loss of one ^{3}H atom reduces the effective labeling by 50% in the case of NO_2-OA and by 25% for NO_2-LA. This methodology has been previously used to radioactively follow the metabolites of NO_2-OA *in vivo* [21].

Synthetic protocols

Reagents for synthetic protocols

Note. **In general, use caution as most of the reagents in these protocols are toxic or dangerous in quantity.** Please read the associated MSDS for specific precautions. For all synthetic procedures, ensure adequate ventilation, and preferably handle chemicals in well-ventilated fume hoods. Use gloves and safety goggles during all procedures.

Method 1: nitroselenation

- Oleic acid (Nucheck Prep, Cat. No. U-46-A)
- Sodium nitrite (Sigma–Aldrich, Cat. No. 237213)
- Phenylselenyl bromide (Sigma–Aldrich, Cat. No. 243965) (CAUTION: stench, toxic, and a powerful lachrymator)
- Mercury (II) chloride (Sigma–Aldrich, Cat. No. 215465)
- Hydrogen peroxide, 30% (Sigma–Aldrich, Cat. No. 216763)
- Acetonitrile (Acroseal, Fisher, Cat. No. AC61096)

Method 2: 9-nitro-oleic acid full synthesis

- 9-Bromononanoic acid (TCI America, Cat. No. B2323)
- Allyl alcohol (Sigma–Aldrich, Cat. No. 240532)
- Silver nitrite (Sigma–Aldrich, Cat. No. 227188)
- *p*-Toluenesulfonic acid (Sigma–Aldrich, Cat. No. 402885)
- DBU (Sigma–Aldrich, Cat. No. 139009)
- Acetic anhydride (Sigma–Aldrich, Cat. No. 242845)
- DMAP (Sigma–Aldrich, Cat. No. 107700)
- Sodium carbonate (Sigma–Aldrich, Cat. No. 223484)
- Toluene (Fisher, Cat. No. AC17716)
- Palladium tetrakis(triphenylphosphine) (Strem, Cat. No. 46-2150)
- Formic acid (95%) (Sigma–Aldrich, Cat. No. F0507)

Common reagents for both methods

- Ethyl ether, anhydrous ACS (Fisher)
- Tetrahydrofuran (Acroseal, Fisher, Cat. No. AC32697)
- Ethyl acetate (Fisher, Cat. No. E145)
- Silica gel (Silicycle, F60 Silica 40–63 μm, Cat. No. R10030B)
- Sodium sulfate (Sigma–Aldrich, Cat. No. 238597)
- Celite 545 (Sigma–Aldrich, Cat. No. 22140)
- Iodine (Aldrich, Cat. No. 207772)
- Phosphomolybdic acid solution (for chromatography) (Sigma–Aldrich, Cat. No. 02553)

Prepared solutions

- Sodium hydroxide, 1 M
- Hydrochloric acid, 1 M
- Hydrochloric acid, 0.1 M
- Saturated sodium bicarbonate
- Diluted sodium bicarbonate (dilute the saturated solution to one-half concentration)
- Saturated ammonium chloride
- Saturated sodium chloride (brine)

Equipment for synthetic protocols

- Magnetic stir bar(s)
- Separatory funnel, 60 ml
- Separatory funnel, 125 ml
- Round-bottom flask, 25 ml
- Round-bottom flask, 50 ml
- Round-bottom flask, 100 ml
- Heating mantle or oil bath
- Variac heating control
- Condenser (Quark, Cat. No. QC-21)
- Rubber septa
- N_2 source (manifold or balloon)
- Ice bath
- Glass syringe (Aldrich, Cat. No. Z181455)
- Needles, 18-gauge × 10 in. (Aldrich, Cat. No. Z117102)
- Disposable syringe, 1 ml (VWR, Cat. No. 89174-490)
- Disposable syringe, 3 ml (Aldrich, Cat. No. Z248002)
- Disposable syringe, 10 ml (Aldrich, Cat. No. Z248029)
- Needles, 18-gauge × 1½ in. (Aldrich, Cat. No. Z118044)
- Dean-Stark trap (2 ml) (Quark, Cat. No. QD-43)
- Fritted glass Büchner funnel (coarse, 15–30 ml) (Quark, Cat. No. QFN-10)
- Chromatography column, 203 × 19 mm (Quark, Cat. No. QCH-3)
- Culture tubes, 13 × 100 mm (VWR, 47729-572)
- Culture tubes, 16 × 100 mm (VWR, 47729-576)
- Thin-layer chromatography (TLC) plates, analytical (silica gel, aluminum or glass back, i.e., VWR, Cat. No. EMD-5549-4 or EM-15326-1)
- TLC plates, preparatory (20 × 20 cm, silica gel, glass back, Analtech, GF 81003)
- Pipettes, $5^3/_4$ in.
- UV lamp, 254 nm (Fisher, Cat. No. 11-992-29)

General techniques

See [22,23] for general methods and descriptions of these techniques.

Air-sensitive reactions

- Cut off the large end of a 3 ml plastic syringe.
- Attach and seal a balloon filled with N_2 or Ar to the open end.
- Top with an 18-gauge needle to provide a constant source of low-pressure inert atmosphere in the absence of a nitrogen or argon line system.

Drying glassware

- Glassware for reactions, including glass syringes and needles for solvent transfers, should be oven-dried and cooled under dry nitrogen before using.

TLC

- Spot solutions of products or reaction mixtures along with a co-spot of starting material for comparison.
- Elute with the given solvent mixture.
- Allyl esters can be easily visualized by using an iodine chamber.
- Nitroalkenes can be easily visualized with a UV lamp at 254 nm.
- Other impurities or reagents may be visualized with a phosphomolybdic acid dip.

Nitroselenation protocol for NO_2-OA (1 g scale) (Fig. 2) [7]

Synthesis

- Oven-dry a 100 ml round-bottom flask containing a stir bar (see Drying glassware).
- Cool to room temperature under nitrogen (see Air-sensitive reactions).
- Add 12 ml tetrahydrofuran to the flask via a glass syringe.
- Add one freshly opened 1.00 g ampoule of oleic acid (3.5 mmol) to the flask.
- Rinse in with 9 ml acetonitrile (ACN) via glass syringe.
- Stir and cool the flask in an ice bath for 20 min.
- Add 1.15 g mercury chloride (4.2 mmol).
- Stir cold for 10 min.
- Add 0.93 g phenylselenyl bromide (3.9 mmol). CAUTION: phenylselenyl bromide is a powerful lachrymator. Weigh and transfer rapidly with adequate ventilation.
- Stir cold for 10 min.
- Add 0.49 g sodium nitrite (7.1 mmol).
- Rinse in with 3 ml remaining ACN to the flask.
- Stir the flask under nitrogen, in the ice bath, at approximately 0 °C for 4 h.
- The solution should gradually develop cloudiness and a yellow color. (*Note:* This reaction is difficult to follow by TLC.)
- Layer 5 ml silica gel (bottom) and 5 ml Celite (top) in a 30 ml fritted glass Büchner funnel and cover with a filter paper.
- Filter the yellow cloudy suspension containing the nitroselenyl intermediate through the fritted glass funnel and collect in a 100 ml round-bottom flask.
- Wash the flask and silica twice with 5 ml portions of tetrahydrofuran and combine the filtrate and washings.
- Remove the solvents by rotary evaporation. CAUTION: nitrogen dioxide vapors, use adequate ventilation.
- Redissolve the residual oil in 12 ml tetrahydrofuran.
- Transfer to a clean 50 ml round-bottom flask containing a stir bar.
- Cool the solution in an ice bath. (*Note:* No nitrogen inlet necessary for this step.)
- Add aqueous hydrogen peroxide (30% aqueous, 4.0 ml, 35 mmol) to the stirred solution dropwise over 10 min.
- Stir the resulting solution cold for 1 h.

- Add 10 ml deionized water and 10 ml diethyl ether to quench and partition, stir 5 min.

Aqueous workup

- Transfer the mixture to a 60 ml separatory funnel.
- Separate and set aside the (top) product-containing organic layer.
- Extract the aqueous phase 3 × with 10 ml portions of ether.
- Combine the organic layers and replace in separatory funnel. The aqueous layer may be discarded.
- Wash 3 × with 10 ml water. These and subsequent aqueous layers may also be discarded.
- Wash 2 × with 5 ml diluted sodium bicarbonate.
- Wash 3 × with 5 ml water.
- Wash 2 × with 5 ml 0.1 M hydrochloric acid.
- Wash 3 × with 5 ml water.
- Wash 2 × with 5 ml brine.
- Transfer the organic layer to an Erlenmeyer flask.
- Add 2–4 g of anhydrous sodium sulfate to dry solution, let stand 20 min.
- Filter through a plug of Celite and silica gel in a fritted glass funnel.
- Collect the crude product in a 100 ml round-bottom flask.
- Remove the solvents from the collected solution by rotary evaporation.
- Purify the crude product by chromatography.
- TLC (5% MeOH/$CHCl_3$) R_f = 0.58. (*Note:* Product formed only after hydrogen peroxide step.)
- UV–Vis: the product NO_2-OA or 9-NO_2-OA can be identified by UV–Vis, λ_{max} = 257 (MeOH), ε = 7000 M^{-1} cm^{-1}.
- ^{1}H NMR: the product has two diagnostic triplet signals centered at δ7.06 ppm corresponding to the nitroalkene proton. This may appear as a quadruplet in some instruments.

Purification method: chromatography column [24]

This method is used for separating approximately 1 g of free fatty acid.

- Suspend 50–60 ml of silica gel in hexanes.
- Fill a flash chromatography column with hexanes.
- Slurry the silica gel in hexanes into the column.
- Pack the column with shaking and air- or nitrogen-line pressure.
- Top off silica gel with a layer of sand.
- Preelute the column with 100 ml 0.5% acetic acid/hexanes (v/v).
- Dissolve the crude product in a minimal amount of 0.5% acetic acid/hexanes solvent mixture.
- Apply the crude product solution to the sand at the top of the column.
- Elute with air- or nitrogen-line pressure and solvent steps of increasing polarity:
  - 0.5% acetic acid/hexanes (v/v), 100 ml,
  - 0.5% acetic acid + 1% ether (v/v), 100 ml,
  - 0.5% acetic acid + 2% ether (v/v), 100 ml,
  - 0.5% acetic acid + 5% ether (v/v), 200 ml.
- Collect the runoff in 13 × 100 mm or 16 × 100 mm culture tubes until well after the product elutes.
- The typical elution order is:
  - diphenyl diselenide (strong yellow band, UV active, solidifies upon evaporation),
  - unreacted oleic acid,
  - nitrated lipid (pale yellow oil),
  - oxidized side products (waxy yellow).
- Recycle the column runoff until the first yellow band elutes.
- Check collected fractions by TLC (strong UV activity at 254 nm).
- Combine the fractions containing the majority of nitrated lipid product and remove the solvent by rotary evaporation.
- Dry the final product under high vacuum for 2–3 h to remove residual solvents.
- Repurify the column runoff if necessary in a second cycle of column chromatography, using similar conditions. A second chromatography column is the easiest way to ensure pure material.
- The theoretical yield is 1.16 g (3.5 mmol); the typical yield is 0.25–0.30 g.

Suggestions

This method is used with other purification steps by changing the solvent mixtures used. Esterified fatty acids (such as allyl esters) are more easily purified by column chromatography. The product is an equimolar combination of 9-NO_2-OA and 10-NO_2-OA isomers. Most applications have not shown a difference in reactivity and cell signaling between the isomers. Positional isomers may be separable by preparatory liquid chromatography (LC). The purification can be performed alternatively with an MPLC system (such as a Sorbtech EZ Flash, Yamazen Smart Flash, or Teledyne Isco CombiFlash system) or by dividing the compound into portions and purifying with preparatory TLC.

Notes on isotopic standard synthesis

This method is preferred for synthesizing isotopic standards, i.e., NO_2-[^{13}C]LA and NO_2-[^{13}C]OA. Starting materials (100 mg of [$^{13}C_{18}$]LA or [$^{13}C_{18}$]OA) are commercially available. To synthesize these lipids on an appropriate scale, the preceding method should be modified as follows: (a) divide reagent quantities by 10 to account for 0.1 g starting material; (b) divide solvent amounts by 4; (c) divide aqueous wash amounts by 2; and (d) purify the product by preparatory TLC plate, performed twice to remove impurities.

Protocol for 9-NO_2-OA (9-nitro-oleic acid) synthesis (0.25 g scale) (Fig. 3) [16]

Note that each intermediate product is stable and can be stored, and the synthesis can be continued at a later time.

Synthesis of 9-bromononanoic acid, allyl ester [25]

- Place 1.00 g bromononanoic acid (4.2 mmol) in a 50 ml round-bottom flask
- For TLC of bromononanoic acid (ethyl acetate:hexanes, 1:3): Rf=0.24.
- Add 3 ml allyl alcohol.
- Add 20 ml toluene.
- Add approximately 5 mg of *p*-toluenesulfonic acid.
- Connect the flask to a condenser and Dean-stark trap under nitrogen.
- Heat to near-reflux at 95–100 °C for 16 h.
- Cool to room temperature.
- Concentrate the solution by rotary evaporation.
- Blow a gentle stream of nitrogen over the product until odorless.
- Redissolve the concentrates in 30 ml ether and transfer to a 60 ml separatory funnel.
- Extract the organic layer twice with 1 M NaOH solution.
- Combine and back-extract the aqueous fractions twice with 10 ml ether.
- Combine the ether layers.
- Wash once with 10 ml water.
- Wash once with 10 ml brine.
- Transfer the organic layer to an Erlenmeyer flask.

- Add 2–3 g of anhydrous sodium sulfate to dry the organic layer and let stand 20 min.
- Filter the solution through a fritted glass funnel containing a layer of Celite and silica covered by a filter.
- Remove the solvents by rotary evaporation to yield crude 9-bromononanoic acid, allyl ester.
- The theoretical yield is 1.17 g; the typical yield is 1.08 g.
- TLC (ethyl acetate:hexanes, 1:3) R_f = 0.58. The product is easily visible with iodine.
- ^{1}H NMR: the product has signals at δ5.92, δ5.31, δ5.23, and δ4.53 ppm corresponding to the allyl group protons.
- IR: the broad –CO_2H signal at 2500–3300 cm^{-1} has disappeared.

Synthesis of 9-nitrononanoic acid, allyl ester [20]

- Oven-dry a 50 ml round-bottom flask containing a stir bar.
- Cool to room temperature under nitrogen.
- Add 0.89 g (5.8 mmol) silver nitrite.
- Add 30 ml ether.
- Purge the suspension with nitrogen for 10 min (see Air-sensitive reactions).
- Add 1.07 g of 9-bromononanoic acid, allyl ester (3.9 mmol) to the stirred solution.
- Stopper the flask with a glass stopper.
- Cover the flask with aluminum foil to protect from light.
- Stir at room temperature for 7 days.
- Check reaction progress by TLC.
- Filter the suspension through a plug of silica and Celite.
- Remove the solvents by rotary evaporation.
- Purify the crude material via flash chromatography (50–60 ml silica gel, ethyl acetate/hexanes, 0–5%) to obtain 9-nitrononanoic acid, allyl ester as the second fraction.
- The theoretical yield is 0.94 g; the typical yield is 0.66 g.
- TLC (ethyl acetate:hexanes, 1:3) R_f = 0.50.
- ^{1}H NMR: the product has a diagnostic triplet signal centered at δ4.33 ppm corresponding to the nitroalkyl methylene protons. The bromoalkyl methylene triplet at δ3.40 ppm has disappeared.
- IR: the –NO_2 signals are at 1553 and 1378 cm^{-1}.

Synthesis of 10-hydroxy-9-nitro-octadecanoic acid, allyl ester [26]

- Oven-dry a 25 ml round-bottom flask containing a stir bar.
- Cool the flask to room temperature under nitrogen.
- Add 0.32 g nitrononanoic acid, allyl ester (1.3 mmol).
- Add 0.27 ml nonyl aldehyde (0.22 g, 1.6 mmol).
- Cool the flask in an ice bath.
- Add 1 to 2 drops of DBU (0.05 ml) to the stirred solution.
- Stir the neat solution cold, 1 h.
- Remove the ice bath and allow warming to room temperature.
- Stir the solution for 2 days at room temperature.
- Check reaction progress by TLC.
- Add 10 ml ether and 10 ml 1 M HCl to partition the solution.
- Stir 30 min.
- Transfer the layers to a separatory funnel and remove the organic layer.
- Extract the aqueous layer 3 × with 10 ml ether.
- Combine the organic layers.
- Wash the combined organic layers once with 10 ml water and once with 10 ml brine.
- Transfer the organic layer to an Erlenmeyer flask.
- Dry over 2–4 g sodium sulfate for 20 min.
- Filter through a plug of silica and Celite.
- Remove the solvent by rotary evaporation.
- Purify by column chromatography (30–40 ml silica gel, ethyl acetate/hexanes, 0–10%) to isolate 10-hydroxy-9-nitro-octadecanoic acid, allyl ester as a colorless oil.
- The theoretical yield is 0.51 g; the typical yield is 0.41 g.
- TLC (ethyl acetate:hexanes, 1:3) R_f =0.38. (Note that the product is more polar than the starting materials and is a mixture of two diastereomers that will typically show up as two distinct spots on TLC.)
- ^{1}H NMR: the product has a methyl signal at δ0.85 ppm, and two broad diagnostic signals centered at δ3.95 and δ3.83 ppm corresponding to both diastereomers of the (–CH-OH) protons. The δ4.33 ppm triplet should be replaced by a broad peak at δ4.39 ppm.

Synthesis of 10-acetoxy-9-nitro-octadecanoic acid, allyl ester [27]

- Oven-dry a 50 ml round-bottom flask containing a stir bar.
- Cool to room temperature under nitrogen.
- Add 0.41 g (1.1 mmol) hydroxynitro intermediate.
- Add 3–4 ml acetic anhydride.
- Add approximately 5 mg *p*-toluenesulfonic acid.
- Stir the solution under nitrogen for 16 h at room temperature.
- Remove the excess anhydride with a gentle stream of nitrogen.
- Redissolve the residue in 10 ml ether.
- Filter the solution through a plug of Celite and silica.
- Remove solvents by rotary evaporation to isolate crude 10-acetoxy-9-nitro-octadecanoic acid, allyl ester as an oil.
- The theoretical yield is 0.45 g; the typical yield is 0.39 g.
- TLC (ethyl acetate:hexanes, 1:3) R_f = 0.51.
- ^{1}H NMR: the product has diagnostic signals centered in the δ5.35–5.15 and δ4.60–4.55 ppm regions obscured by the allyl alkene signals. Total integration for each region is 3H. Acetyl methyl singlets appear at δ2.09 and δ2.05 ppm.
- IR: the broad 3400 cm^{-1} –OH signal is lost.

Synthesis of 9-NO_2-OA allyl ester [28]

- Oven-dry a 50 ml round-bottom flask containing a stir bar.
- Cool to room temperature under nitrogen.
- Add 0.39 g (0.91 mmol) acetoxynitro intermediate.
- Add 25–30 ml toluene.
- Add 0.10 g (0.94 mmol) sodium carbonate.
- Connect the flask to a condenser and a Dean-stark trap under nitrogen.
- Reflux solution with vigorous stirring for azeotropic removal of water, 24 h at 110–120 °C.
- Cool the solution to room temperature.
- Check reaction progress by TLC.
- Continue heating if insufficient product formation.
- Partition with 10 ml 1 M HCl and 10 ml ether.
- Stir 30 min.
- Transfer to 125 ml separatory funnel.
- Separate the aqueous layer.
- Extract the aqueous layer three times with 10 ml ether.
- Combine the organic layers.
- Wash once with 20 ml water and then 20 ml brine.
- Transfer the organic layer to an Erlenmeyer flask.
- Dry over 2–4 g sodium sulfate for 20 min.
- Filter through a plug of silica and Celite.
- Remove the solvent by rotary evaporation.
- Isolate the 9-nitro-oleic acid, allyl ester by column chromatography (25–30 ml silica gel, ethyl acetate/hexanes, 0–5%). The product is a pale yellow oil at room temperature.
- The theoretical yield is 0.34 g; the typical yield is 0.23 g.

- TLC (ethyl acetate:hexanes, 1:3) $R_f = 0.60$. The product is significantly more active by UV at 254 nm.
- ^{1}H NMR: the product has a diagnostic triplet signal at δ7.08 ppm corresponding to the nitroalkene proton.

Synthesis of 9-nitro-oleic acid [29]

- Oven-dry a 50 ml round-bottom flask containing a stir bar.
- Cool to room temperature under nitrogen.
- Add 0.28 g (0.76 mmol) nitro-oleic acid, allyl ester.
- Add 20–25 ml tetrahydrofuran.
- Sparge the solution with nitrogen via a needle for 5 min.
- Add 0.4 ml formic acid (~8 mmol).
- Add 40 mg (35 μmol) dry palladium tetrakis (triphenylphosphine).
- Sparge the solution with nitrogen for 10 min.
- Seal the flask under a condenser.
- Reflux at 95–100 °C under nitrogen for 16 h.
- Cool the solution to room temperature.
- Filter the solution through a plug of silica and Celite in a fritted glass funnel.
- Remove the solvent by rotary evaporation.
- Remove residual formic acid with a stream of nitrogen.
- Purify the 9-nitro-oleic acid as a pale yellow oil by column chromatography (20–25 ml silica gel 0.5% HOAc, 0–5% ether/hexanes; scaled down from the method under Purification method: chromatography column).
- The theoretical yield is 0.25 g; the typical yield is 0.23 g.
- TLC (5% MeOH/$CHCl_3$) $R_f = 0.58$. UV λ_{max} (MeOH) 257 nm.
- ^{1}H NMR: the product has a single triplet signal at δ7.08 ppm corresponding to the nitroalkene proton similar to the ester, but without the signals at δ6–4 ppm corresponding to the allyl group protons (see Nitroselenation protocol for NO_2-OA for additional analysis).

Suggestions

The final product is a single regioisomer, 9-NO_2-OA. This procedure can be easily adapted to produce other desired esters, such as methyl ester 9-NO_2-OA, by changing the alcohol used under the Section "Synthesis of 9-bromononanoic acid, allyl ester". Esterified fatty acids (such as allyl esters) are more easily purified by column chromatography than free fatty acids. Users without access to NMR can easily find commercial analysis services. In the intermediate purification steps, refer to the general procedure under the Section "Purification method: chromatography column" and change the amount of silica and the solvent mixtures used to those given at each product step. Typically a product is eluted with a solvent mixture (0, 1, 2, 5, 10% ethyl acetate or ether, up to the stated concentration) two to three times the volume of silica gel used, followed by a similar amount of the next more polar solvent mixture, until the product begins to elute. As stated before, the purifications can be performed alternatively with a commercially available MPLC system or by dividing the intermediate products into portions and purifying with preparatory TLC.

Measurement of nitrated fatty acids by mass spectrometry

Principles

Mass spectrometry has played a central role in the determination of nitrated biomolecules. One of the best characterized nitrated biomolecules is nitrotyrosine [30]. Unlike the single, well-defined product of tyrosine nitration, the multiplicity of substrates and reaction mechanisms involved in the nitration of fatty acids results in a diversity of products. Nitrotyrosine is chemically stable in aqueous solutions, organic solvents, a wide range of pH values, and biological environments in which it may undergo a very slow metabolic degradation. In contrast, NO_2-FAs are highly reactive electrophiles that rapidly and reversibly adduct to cysteines and histidines. In addition, rapid metabolic consumption of NO_2-FAs occurs through β-oxidation and double-bond saturation. Thus, the development of rigorous methods for proper elucidation of the various isomers of NO_2-FAs found *in vivo* is necessary. The two main techniques used for the detection and quantification of NO_2-FAs are gas chromatography (GC) or LC coupled to mass spectrometry (MS). GC-based methods are lengthy and require several derivatization steps during sample preparation that not only are tedious, but promote the degradation and modification of nitrated metabolites. Thus, LC–MS-based methods are preferential as they have the advantage of rapid sample preparation and are less prone to artifact generation during the sample workup. At this point it is important to consider that matrix interference is an important issue for both analytical procedures. GC–MS always requires intensive sample cleanup, whereas LC–MS is more forgiving, but may require additional preparation steps depending on matrix complexity.

Characterization

NO_2-FA can be generated by either $^{\bullet}NO_2$ addition to olefins present in unsaturated fatty acids followed by hydrogen abstraction and re-formation of the double bond or through hydrogen abstraction, radical rearrangement, and a termination reaction between the carbon-centered radical and an additional $^{\bullet}NO_2$ radical (Fig. 1). The main difference between the species formed through these mechanisms is that the addition reaction leads to electrophilic products (also termed conjugated nitroalkenes, α, β-unsaturated nitroalkenes, or vinyl nitro groups) and the hydrogen abstraction reaction leads to nonelectrophilic species (also termed nonconjugated nitroalkenes, allylic nitro groups, nitroalkane alkenes). This distinction is of relevance because the electrophilic nature of endogenously formed NO_2-FA modulates its biological signaling, half-life, and metabolism. Moreover, conjugated nitroalkenes can be rapidly converted into nitroalkanes or nonconjugated nitroalkenes by reduction, catalyzed by a yet unidentified NADPH-dependent enzymatic activity. Thus, the differences between nitrated products and their electrophilic nature are important when characterizing and quantifying biological samples. Furthermore, the electrophilicity of the NO_2-FA will greatly affect stability, sample handling and determination of the concentration of these species.

Extraction

There are many considerations to take into account when choosing a method to process any sample from a biological matrix and the same is true for the extraction of NO_2-FA. The relevant factors include analyte concentration in the biological sample, amount of sample, pH, solvent, concentration of NO_2^- in the sample, and potential interest in detecting metabolic products of the NO_2-FA. Strategies include ACN precipitation, biphasic organic extraction, and solid-phase extraction. Analysis of endogenous content of NO_2-FA usually requires approaches that include concentrating and purification steps to reduce ionization dampening and maximize sensitivity when measuring by LC–MS. Thus, the preferred techniques are solid-phase extraction and solvent-based extractions. Acetonitrile precipitation is a very convenient technique for determining the plasma concentration of NO_2-FA and their metabolites in animals exposed to exogenously administered NO_2-FA (either by gavage, ip injection, iv injection, or osmotic minipump delivery) using as little as 5 μl of sample. Acetonitrile or methanol precipitation can be used

followed by solid-phase extraction to detect endogenous levels of NO_2-FA. This combination technique has also been effectively used to detect eicosanoids, isoprostanes, and docosanoids [31]. One of the advantages of ACN precipitation is that it allows for the recovery of many of the nitro-containing fatty acid metabolites (including carnitine and CoA thioesters, β-oxidation products, nitroalkene reduction products, and nucleophilic amino acid additions). Although these products can be obtained by solid-phase extraction, additional attention has to be given to the conditions used to equilibrate the column and elute the compounds to ensure their proper recovery. Organic extraction offers the highest recovery of NO_2-FA from samples, but many of the metabolites will be lost because of their increased hydrophilicity compared to the parent NO_2-FA.

Protocols

Reagents and materials

- ACN (Burdick and Jackson, HPLC grade, Cat. No. 015-4)
- Water (Burdick and Jackson, HPLC grade, Cat. No. 365-4)
- Methanol (Burdick and Jackson, HPLC grade, Cat. No. 230-4)
- 1.5 ml Eppendorf tubes (Fisherbrand, Cat. No. 05 408 129)
- 2 ml screw-top glass vials (Sun Sri, Cat. No. 200 252, or Fisher, Cat. No. 22313377)
- Screw polypropylene/PTFE/silicone caps for 2-ml glass vials (Sun Sri, Cat. No. 500 062, or Fisher, Cat. No. 14823306)
- 100 μl conical glass inserts for glass vials (Sun Sri, Fisher, Cat. No. 22035113)
- C18 disposable solid-phase extraction columns
- Sample dryer:
  - Analytical nitrogen evaporator, N EVAP 112 from Organomation Associates, Berlin, MA, USA
  - Solid-phase extraction vacuum manifolds, Supelco Visiprep
  - Vacuum dry evaporation systems, RapidVap by Labconco Corp
- Transfer pipettes, Samco Scientific Model 202
- 16 × 100 mm borosilicate tubes (VWR Cat. No. 47729-576)
- 13 × 100 mm borosilicate tubes (VWR Cat. No. 47729-572)
- 13 × 100 mm silanized borosilicate tubes (Kimble Chase Cat. No. 73500-13100).
- A standard mix of NO_2-FA (1 mM)
- A 100 nM internal standard mix (NO_2-[$^{13}C_{18}$]OA and NO_2-[$^{13}C_{18}$]LA) in MeOH
- Cold ACN (−20 °C)
- Eppendorf adjustable volume mechanical micropipettes, volumes 0.5–10, 2–20, 10–100, and 100–1000 μl.

Instruments

- Mass spectrometer, 4000 QTrap (Applied Biosystems)
- LC20 with autosampler and rack changer (Shimadzu)

Mass spectrometer settings

Tables 1 and 2 show the parameters and settings of the mass spectrometers and HPLCs used to determine nitrated fatty acids.

Method 1: quantification of small plasma volumes

This protocol is adequate to measure the plasma concentration of NO_2-FA from treated animals.

Preparation of a standard curve for NO_2-LA and NO_2-OA quantification

Starting from the 1 mM standard mix of NO_2-OA and NO_2-LA stock solutions are prepared (Table 3).

Final concentrations of NO_2-OA and NO_2-LA in the plasma samples that will be used to build the standard curve are 1000, 200, 40, 8, 2, 0.4, 0.1, 0.02, and 0 nM (Table 4). Determinations based on LC–MS data usually have a dynamic range of 3 orders of magnitude, which can be extended on the upper end using less sensitive multiple reaction monitoring (MRM) transitions [32]. The proposed standard curve spans over 5 orders of magnitude to accommodate quantifications using instruments with higher or lower sensitivity. Endogenous levels will generally not interfere

Table 1
HPLC and MS conditions for characterization and quantification of NO_2-FA.

Parameter	Value	
	Nitrated fatty acid quantification method	Nitrated fatty acid characterization method
Injection volume	20.00 μl (range 10–40 μl)	20.00 μl (range 10–40 μl)
Total flow	0.7500 ml/min	0.2500 ml/min
Rinsing volume	300 μl	300 μl
Rinsing speed	35 μl/s	35 μl/s
Sampling speed	15.0 μl/s	15.0 μl/s
Rinse dip time	3 s	3 s
Rinse mode	Before and after aspiration	Before and after aspiration
Cooler enabled	Cooler temperature 4 °C	Cooler temperature 4 °C
Pump method	Rinse pump and port between analyses	Rinse pump and port between analyses
Solvent A	Water + 0.1% acetic acid	Water + 0.1% acetic acid
Solvent B	ACN + 0.1% acetic acid	ACN + 0.1% acetic acid
Declustering potential	−75	−75
Entrance potential	−10	−10
Collision energy	−35	−35.00 to follow NO_2^-; −17 to −23 to follow specific chain-breaking fragments
Collision exit potential	−15	−15
Column	Phenomenex Gemini (C18, 2 × 20-mm cartridges)	Phenomenex Luna (C18, 2 × 150 mm, 3 μm)
Flow rate	750 μl/min	250 μl/min
Curtain gas	40	30
Auxiliary gas 1	60	55
Auxiliary gas 2	50	50
Ionization voltage	−4500 V	−4500 V
Source temperature	550 °C	500 °C
Collision-activated dissociation gas	4	4

ACN, acetonitrile.

with this detection method, because their levels under noninflammatory conditions are below the limit of detection (LOD) for this method.

Procedure

Using a micropipette add 4 µl of a mixture of NO_2-[$^{13}C_{18}$]OA and NO_2-[$^{13}C_{18}$]LA internal standards to each Eppendorf (final concentration 9.1 nM), vortex, leave for 5 min in ice, and add 160 µl cold ACN, vortex again, and spin down at 14,000 *g* for 10 min at 4 °C. Transfer supernatant into insert-containing glass vials and inject 10 µl into the LC–MS.

Considerations

This protocol is adequate to measure the plasma concentration of NO_2-FA from treated animals. The levels of endogenous NO_2-FA found in free form (not forming addition products with nucleophiles) in plasma are between 0.5 and 1.5 nM and require extensive workup and are therefore below the limit of detection with this method.

Table 2
HPLC conditions for characterization (long method) and quantification (short method) of NO_2-FA.

Time	A (%)	B (%)
Long method		
0.5	95	5
8.5	65	35
47	0	100
53	0	100
54	95	5
60	95	5
Short method		
0.5	65	35
4	0	100
5.5	0	100
5.6	65	35
7	95	5

Solvent A is water + 0.1% acetic acid and solvent B is ACN + 0.1% acetic acid.

Table 3
Standard curve for plasma: preparation of methanol stock solutions.

Initial NO_2-FA (µM)	Dilution	Volume (µl)	Final methanol volume (ml)	Final NO_2-FA stock (nM)
1000	1/50	20	1	20,000
20	1/25	40	1	800
0.8	1/20	50	1	40
0.04	1/20	50	1	2

Method 2: quantification of urine samples

Standard curve for a mix of NO_2-LA and NO_2-OA in urine

Starting from a 1 mM stock solution of a NO_2-OA and NO_2-LA mix prepare the dilutions shown in Table 5 in 1 ml methanol.

Prepare the dilutions of the NO_2-FA mix in urine as shown in Table 6.

Using a micropipette add 10 µl of a mix of NO_2-[$^{13}C_{18}$]OA and NO_2-[$^{13}C_{18}$]LA internal standards to each Eppendorf (final concentration 0.99 nM), vortex, leave for 5 min, and add 166 µl of methanol (final 15% methanol). Vortex again and let equilibrate for 10 min.

Procedure

Condition the C18 solid-phase extraction columns for sample preparation by adding 3 ml of MeOH followed by 5 ml of H_2O:MeOH (85:15) to each 500 mg C18 column, letting each conditioning step elute under atmospheric pressure.

- Next add 1 ml of urine sample. Avoid letting the column go dry before adding sample. Special care has to be taken to avoid drying the column in all steps before analyte elution, to ensure a consistent chromatographic elution profile.
- Once the sample has passed through the column, wash with 5 ml of H_2O:MeOH (85:15) under vacuum.
- Let the column dry under vacuum for 15–20 min. This drying step is intended to minimize the amount of water contained in the eluent, to allow for a faster drying and reconstitution of the sample.
- Discard the wash fractions.
- Add new silanized tubes and elute the analyte from the column using 3 ml methanol. *Note.* Silanized tubes are used to help reduce the binding of fatty acids to silica present in the glass during the drying and subsequent loss of analyte. If silanized tubes are not available, common glass tubes can be used.
- Dry the collected effluent using a vacuum dry evaporation system or under nitrogen gas using an analytical nitrogen evaporator
- Resuspend the sample in a small volume (200 µl) of methanol.
- Vortex and add to 100 µl insert-containing glass vial. *Note.* The 100 µl volume inserts hold 200 µl.
- Inject 10 µl into the LC–MS.

Table 5
Standard curve for urine: preparation of methanol stock solutions.

Initial NO_2-FA (µM)	Dilution	Volume (µl)	Final methanol volume (ml)	Final NO_2-FA stock (µM)
1000	1/20	50	1	50
50	1/25	40	1	2
2	1/20	50	1	0.1
0.1	1/20	50	1	0.005

Table 4
Standard curve in plasma: preparation of eight plasma stock solutions. Two stocks from each standard.

Solution no.	Plasma (µl)	NO_2-FA stock (nM)	NO_2-FA (µl)	Dilution	Procedure for final plasma dilutions	Final conc. (nM)
1	60	20,000	3	1/20	Transfer 40 µl into an Eppendorf	1000
2				1/5	Transfer 8 µl of solution #1 into an Eppendorf containing 32 µl of plasma	200
3	60	800	3	1/20	Transfer 40 µl into an Eppendorf	40
4				1/5	Transfer 8 µl of solution #3 into an Eppendorf containing 32 µl of plasma	8
5	60	40	3	1/20	Transfer 40 µl into an Eppendorf	2
6				1/5	Transfer 8 µl of solution #5 into an Eppendorf containing 32 µl of plasma	0.4
7	60	2	3	1/20	Transfer 40 µl into an Eppendorf	0.1
8				1/5	Transfer 8 µl of solution #7 into an Eppendorf containing 32 µl of plasma	0.02

Table 6
Standard curve in urine: preparation of eight urine stock solutions. Two stocks from each standard.

Solution no.	Urine (ml)	NO_2-FA stock (μM)	NO_2-FA (μl)	Dilution	Final urine dilutions	Final conc. (nM)
1	1.3	50	26	1/50	Transfer 1000 μl into an Eppendorf	1000
2				1/5	Transfer 200 μl of solution #1 into an Eppendorf containing 800 μl of urine	200
3	1.3	2	26	1/50	Transfer 1000 μl into an Eppendorf	40
4				1/5	Transfer 200 μl of solution #3 into an Eppendorf containing 800 μl of urine	8
5	1.3	0.1	26	1/50	Transfer 1000 μl into an Eppendorf	2
6				1/5	Transfer 200 μl of solution #5 into an Eppendorf containing 800 μl of urine	0.4
7	1.3	0.005	26	1/50	Transfer 1000 μl into an Eppendorf	0.1
8				1/5	Transfer 200 μl of solution #7 into an Eppendorf containing 800 μl of urine	0.02

After adding the corresponding NO_2-FA acid stock, vortex and incubate for 15 min to allow for analyte equilibration.

Considerations

This protocol is adequate to measure the NO_2-FA concentration in urine of human or animal origin. The mean concentration of free nitrated linoleic acid isomers in urine (first void of the day) from healthy human volunteers is 9.2 nM (9.9 pmol/mg creatinine). The urinary values display a significant dispersion with concentrations typically ranging from 0.7 to 57 nM. Standard curves are important to determine several important parameters (e.g., stability, linearity of response, reproducibility of response, LOQ, LOD, matrix interferences). The proper procedure includes performing the standard curve in urine. This allows for proper determination of parameters including LOD, LOQ, and the relative standard deviation values for each of the standard concentrations. These parameters can be calculated from a standard curve performed in methanol; however, the values will be meaningless when quantifying the biological sample. It is important to establish quality controls when measuring samples (using a low, medium, and high concentration of spiked analyte or at least a low and high one). This ensures that determinations are properly performed the day of measurement. The analyte and the internal standard need to be incubated for 15–30 min with the sample on ice to allow for proper distribution and reaction equilibrium in the matrix to better mimic endogenous distribution and free levels.

Chromatographic and mass spectrometric considerations

Recovery and stability in matrix analysis

For a new biological matrix, it is always important to know the percentage recovery and the stability of the analyte of interest. NO_2-FAs containing a nitroalkene group are electrophilic and will rapidly react with nucleophilic amino acid residues, such as cysteine and histidine. One can account for this reactivity by using spiked plasma samples to monitor the loss of free NO_2-FA over time. These samples can be directly compared to methanol spiked with standard, which would provide the greatest intensity upon LC–MS analysis.

- Spike 198 μl plasma with 2 μl NO_2-FA standards (20 μM solution) to give a 200 nM solution.
- Vortex and at 0, 10, 20, and 30 min transfer 40 μl into Eppendorf tubes.
- Immediately add 4 μl of IS
- Vortex and add 160 μl of ACN.
- Vortex samples and spin them down.
- Remove the supernatant.

In addition, comparisons can also be made between the analyte spiked in methanol and plasma previously acidified to pH 5. Acidification of the plasma will inhibit Michael addition reactions. To prepare these samples, repeat the steps listed above after acidifying the plasma. A comparison of the spiked methanol, plasma, and acidified plasma will provide an indication of the loss of NO_2-FA signal intensity due to covalent adduction, nonspecific binding, and matrix suppression of the signal.

NO_2-FA detection in positive-ion mode

NO_2-FA can be detected as Li^+ or Na^+ adducts. Depending on the concentration of Li^+ or Na^+ ions used, they will be preferentially detected as single or double adducts. The associated cation (Li^+ or Na^+) is usually infused postcolumn to maintain a consistent chromatographic profile and to be comparable to runs performed in negative-ion mode. For Li^+, the optimal concentration infused postcolumn to detect singly charged positive ions is between 20 and 35 μM. This greatly favors single adducts over double adducts, making this method 100 to 1000 times more sensitive than the original reported use of 1–5 mM Li^+ addition for polyunsaturated fatty acid characterization [33]. The advantage of positive-ion mode detection of nitroalkenes is based on the NO_2-FA heterolytic chain fragmentation upon low energy collision-induced dissociation (CID), thus generating almost exclusively moieties containing an aldehyde and a nitrosamine. These well-defined fragmentation pathways are very helpful for structural elucidation when new, unidentified products are observed and to confirm already established analytes. At higher energies the charged or neutral loss of NO_2^- or HNO_2 is respectively observed.

NO_2-FA detection in negative ion mode

NO_2-FAs are detected as $[M-H]^-$. The advantage of the negative-ion mode is its sensitivity and the specific fragmentation of molecules containing nitroalkenes and nitroalkanes that lead to the $[NO_2]^-$ and $[NO_2FA\text{-}HNO_2]^-$ product ions. MRM, multiple reaction monitoring, denotes a specific parent mass fragmentation that is used to monitor product ions in a triple-stage-quadrupole mass spectrometer. The following transitions are used for NO_2-FA analysis:

- The transitions below are used for highest sensitivity:
  $[NO_2\text{-FA-H}]^- \rightarrow [NO_2]^-$ (326.3 → 46 and 324.3 → 46 for NO_2-OA and NO_2-LA, respectively).
- In most triple quadrupoles, the MRM following the formation of 46 *m/z* anion will be the most sensitive. Owing to instrument design, Thermo Fisher triple quadrupoles are better suited to stabilizing and transmitting higher *m/z* ions. Thus, the detection of the anion resulting from neutral loss of HNO_2 (47 amu) is preferred. Fragments involving the loss of NO_2^- or HNO_2 usually require high collision energies (35 eV). If the collision energy is set

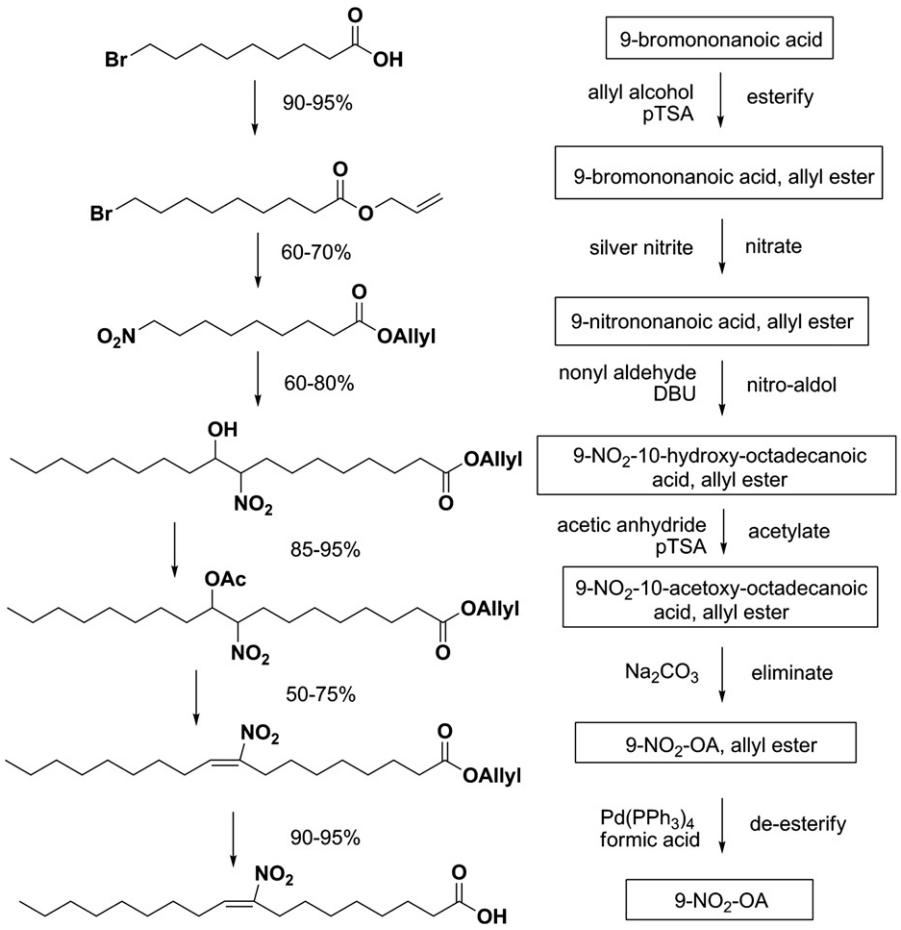

Fig. 3. Full synthesis of 9-NO_2-OA. An available bromoalkyl acid is transformed over six steps to the desired nitroalkene product. Each step produces clean, single regioisomer products, but the length of the procedure and associated loss of material (only 12–36% expected overall yield) limit the practicality of the approach.

lower (17–25 eV) fragments that contain structural information about the lipid backbone can be detected [12].

- Nitroalkanes: these molecules have no conjugated nitroalkene and fragment only through losses of NO_2^- or HNO_2 [12].
- NO_2-OA: in addition to the NO_2^--related losses, fragments occur almost exclusively through forming an aldehyde and a nitrosamine (similar to positive-ion mode). These fragments allow for the correct identification of NO_2-OA positional isomers [12]. A product ion of 168.1 *m/z* is observed for 9-NO_2-OA and an ion of 169.1 *m/z* for the 10-NO_2-OA isomer.
- The presence of an additional double bond (such as in NO_2-LA) generates, in addition to the previously described fragments of NO_2-OA, product ions derived from cyclization reactions. A configuration with an additional double bond one or two carbons away from the nitroalkene promotes cyclization reactions that facilitate formation of 5- or 6-atom heterocycles [12].
- Vicinal NO_2-OH-FA (nitrohydroxy fatty acids). These molecules originate from NO_2-FA hydration reactions in aqueous environments. They are easy to characterize, because CID fragments result in a specific fragmentation pattern that clearly indicates the positions of NO_2 and OH groups in the molecule [34].

HPLC solvent selection:

Basic solvents may result in increased degradation and/or hydration of nitroalkenes, have higher sensitivity in the negative-ion mode, and have impaired isomer separation compared to acidic solvent systems. Basic solvents used include (A) H_2O + 0.1% ammonium hydroxide and (B) ACN + 0.1% ammonium hydroxide.

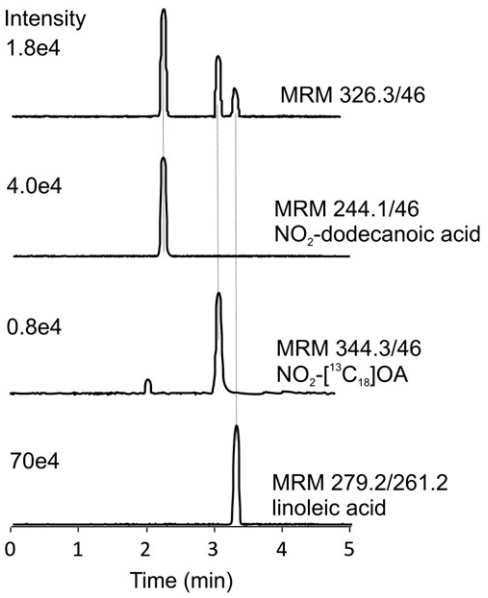

Fig. 4. Typical chromatogram obtained from the plasma of mice injected intravenously with NO_2-OA and extracted with ACN that shows the three characteristic peaks (following the 326.2 → 46 MRM) that are obtained. The first peak corresponds to an unidentified noncovalent adduct of *m/z* 82. This peak corresponds to nitrododecanoic acid (NO_2-12:0), which can be specifically followed at 244.1 → 46. The second peak coelutes with the internal standard (342.3 → 46) and is the true NO_2-OA. The third peak corresponds to a noncovalent adduct of linoleic acid, as coelution with linoleic acid (279.2 → 261.2) is shown.

Acidic solvents are slightly less sensitive, but offer better chromatographic resolution and separation of isomers. MeOH and ACN provide good separation of NO_2-FA, although ACN is most commonly used. Solvents such as formic acid and acetic acid can be used, but acetic acid is a weaker acid, resulting in less ionization dampening and in a more sensitive method compared to formic acid when working in negative-ion mode. Solvents used include (A) H_2O + 0.1% acetic acid and (B) ACN + 0.1% acetic acid.

Standard curve

It is preferable that the standard curve (plotting standard analyte area/IS area as a function of standard analyte concentration) be performed the same day as the unknown measurements. However, if this is not possible, it is important to use quality controls the day of the measurement. For this, biological samples will be spiked with a low, medium, and high concentration of analyte. The concentration of the spiked sample should be selected from the range of values normally detected in those biological samples (2–200 nM). In this regard, the range should include a low concentration reflecting the lowest values detected (a value that should be above the LOQ), a concentration close to the median, and a concentration reflecting the highest concentration detected in the biological samples. Because the matrix contains measurable levels of endogenous NO_2-FA, the intercept of the *y* axis will be higher than 0. The endogenous level of the sample used as matrix can be calculated as the value of the *y*-intercept times the internal standard concentration divided by the slope.

Quantification

Peaks are quantified using the area under the curve. Chromatographic methods can be shortened and many MRM transitions can be simultaneously monitored in a single analysis. It is important to verify that at least 8–10 data points have been determined for each peak to ensure an accurate determination of the area under the curve.

Ionization dampening

Many metabolites of NO_2-FA are present in urine. Because internal standards may not be available for all the various species, it is important to understand how the matrix and solvent compositions affect the sensitivity of the mass spectrometer during the chromatographic run. To investigate these effects infuse a 100 nM solution of NO_2-LA at 5 μl/min into a T-connected postcolumn to the mass spectrometer and solvent line from the LC. Perform a run using matrix (i.e., urine, plasma extraction) or solvent. Analyze the instrument response (peak intensity) at various times to determine if dampening is an issue at the elution time of the analytes of interest.

Characterization

NO_2-FAs are separated using a C18 reversed-phase column (2 × 150 mm, 3 μm; Phenomenex, Torrance, CA, USA) at a 250 μl/min flow rate. NO_2-FAs are eluted from the column and detected using the conditions shown in Table 1. For positive-ion mode, a lithium acetate solution (10 mM) is infused post-column at a 1 μl/min flow rate to reach a final concentration of 40 μM.

Artifacts common to NO_2-FA determinations by MS–MS

- Following the formation of an ion with 46 *m/z* can lead to artifacts if further characterization is not performed and internal standards are not used. The origin of these false positives does not stem from a single mechanism and a variety of artifacts are caused by different issues. Most importantly, false positives are caused by various noncovalent adducts. These adducts can be caused by NO_2^- ion pairing with molecules that do not contain a nitro group or by other ions pairing with molecules that have a nitro group (Fig. 4).
- Noncovalent NO_2^- adducts are commonly observed with unsaturated fatty acids, particularly linoleic and linolenic acid. They have longer retention times compared to nitro fatty acids upon C18 chromatographic separation. They are observed after the neutral loss of HNO_2 and the formation of the product ion NO_2^-. Peaks stemming from these NO_2^- adducts are easily misinterpreted as NO_2-FA in the absence of internal standards. These species display correct high accuracy mass determinations, but do not present chain-breaking specific fragmentation ions other than NO_2^- and neutral loss of HNO_2.
- NO_2-FA can be detected in positive-ion mode as Li^+ or Na^+ adducts. Additionally, in negative-ion mode an undefined adduct (+82 *m/z)* is observed (Fig. 4). This adduct is prominent for some β-oxidation metabolic products of NO_2-FA. In particular, nitrododecenoic (NO_2-12:1) and nitrododecanoic (NO_2-12:0) acids are most prominently detected as these adducts.
- If the concentration of a NO_2-FA in the sample is sufficient, specific transitions having sensitivity between 1 and 10%, compared to the more sensitive, but less specific loss of NO_2^-, should be followed. These transitions are not to be used for quantification purposes, which usually require a 20:1 signal-to-noise ratio, but for the identification purposes that require them only to be above LOD (usually a 3:1 signal-to-noise ratio).
- If possible, the mass of the parent ion should always be confirmed when studying an unknown nitro-containing molecule without internal standards. This ensures that the selected ion is less likely to be an ion pair.

Summary

Our methods employ a combination of synthetic approaches and high-resolution analytical techniques to identify and quantify nitrated lipids in biological samples. These methods are appropriate for detecting previously described NO_2-FAs, but can also detect a range of other nitrated and nonnitrated electrophilic lipids produced biologically. These approaches demonstrate the synergy between synthetic techniques and powerful analytical methods. Optimizing both components has allowed for improved structural characterization, greater confidence in structural assignments, and the avoidance of artifacts in obtaining reliable quantification.

Acknowledgments

We thank Bruce Freeman for insightful comments and helpful discussion. This work was supported by National Institutes of Health Grants R01 AT006822-01 (to F.J.S.) and R01-HL058115, R01-HL64937, and P01-HL103455 (to Bruce Freeman). F.J.S. declares a financial interest in Complexa, Inc.

References

[1] Brown, H. A.; Marnett, L. J. Introduction to lipid biochemistry, metabolism, and signaling. *Chem. Rev.* **111:**5817–5820; 2011.
[2] Groeger, A. L.; Cipollina, C.; Cole, M. P.; Woodcock, S. R.; Bonacci, G.; Rudolph, T. K.; Rudolph, V.; Freeman, B. A.; Schopfer, F. J. Cyclooxygenase-2 generates anti-inflammatory mediators from omega-3 fatty acids. *Nat. Chem. Biol.* **6:**433–441; 2010.
[3] Schopfer, F. J.; Cipollina, C.; Freeman, B. A. Formation and signaling actions of electrophilic lipids. *Chem. Rev.* **111:**5997–6021; 2011.
[4] Serhan, C. N.; Krishnamoorthy, S.; Recchiuti, A.; Chiang, N. Novel anti-inflammatory–pro-resolving mediators and their receptors. *Curr. Top. Med. Chem.* **11:**629–647; 2011.

[5] Schopfer, F. J.; Baker, P. R. S.; Freeman, B. A. NO-dependent protein nitration: a cell signaling event or an oxidative inflammatory response? *Trends Biochem. Sci.* **28:**646–654; 2003.
[6] Sawa, T.; Zaki, M. H.; Okamoto, T.; Akuta, T.; Tokutomi, Y.; Kim-Mitsuyama, S.; Ihara, H.; Kobayashi, A.; Yamamoto, M.; Fujii, S.; Arimoto, H.; Akaike, T. Protein S-guanylation by the biological signal 8-nitroguanosine 3′,5′-cyclic monophosphate. *Nat. Chem. Biol.* **3:**727–735; 2007.
[7] Baker, P. R. S.; Schopfer, F. J.; Sweeney, S.; Freeman, B. A. Red cell membrane and plasma linoleic acid nitration products: synthesis, clinical identification, and quantitation. *Proc. Natl. Acad. Sci. USA* **101:**11577–11582; 2004.
[8] Batthyany, C.; Schopfer, F. J.; Baker, P. R. S.; Durán, R.; Baker, L. M. S.; Huang, Y.; Cerveñansky, C.; Branchaud, B. P.; Freeman, B. A. Reversible post-translational modification of proteins by nitrated fatty acids in vivo. *J. Biol. Chem.* **281:**20450–20463; 2006.
[9] O'Donnell, V. B.; Eiserich, J. P.; Chumley, P. H.; Jablonsky, M. J.; Krishna, N. R.; Kirk, M.; Barnes, S.; Darley-Usmar, V. M.; Freeman, B. A. Nitration of unsaturated fatty acids by nitric oxide-derived reactive nitrogen species peroxynitrite, nitrous acid, nitrogen dioxide, and nitronium ion. *Chem. Res. Toxicol.* **12:**83–92; 1999.
[10] Yin, H.; Xu, L.; Porter, N. A. Free radical lipid peroxidation: mechanisms and analysis. *Chem. Rev.* **111:**5944–5972; 2011.
[11] d'Ischia, M.; Napolitano, A.; Manini, P.; Panzella, L. Secondary targets of nitrite-derived reactive nitrogen species: nitrosation/nitration pathways, antioxidant defense mechanisms and toxicological implications. *Chem. Res. Toxicol.* **24:**2071–2092; 2011.
[12] Bonacci, G.; Asciutto, E. K.; Woodcock, S. R.; Salvatore, S. R.; Freeman, B. A.; Schopfer, F. J. Gas-phase fragmentation analysis of nitro-fatty acids. *J. Am. Soc. Mass Spectrom* **22:**1534–1551; 2011.
[13] Corey, E. J.; Estreicher, H. New synthesis of conjugated nitro cyclo olefins, unusually versatile synthetic intermediates. *J. Am. Chem. Soc.* **100:**6294–6295; 1978.
[14] Hayama, T.; Tomoda, S.; Takeuchi, Y.; Nomura, Y. Synthesis of conjugated nitroalkenes via nitroselenenylation of alkenes. *Tetrahedron Lett.* **23:**4733–4734; 1982.
[15] Alexander, R. L.; Bates, D. J. P.; Wright, M. W.; King, S. B.; Morrow, C. S. Modulation of nitrated lipid signaling by multidrug resistance protein 1 (MRP1): glutathione conjugation and MRP1-mediated efflux inhibit nitrolinoleic acid-induced, PPAR gamma-dependent transcription activation. *Biochemistry* **45:**7889–7896; 2006.
[16] Woodcock, S. R.; Marwitz, A. J. V.; Bruno, P.; Branchaud, B. P. Synthesis of nitrolipids: all four possible diastereomers of nitrooleic acids: (E)- and (Z)-, 9- and 10-nitro-octadec-9-enoic acids. *Org. Lett.* **8:**3931–3934; 2006.
[17] Gorczynski, M. J.; Huang, J. M.; King, S. B. Regio- and stereospecific syntheses and nitric oxide donor properties of (E)-9- and (E)-10-nitrooctadec-9-enoic acids. *Org. Lett* **8:**2305–2308; 2006.
[18] Dunny, E.; Evans, P. Stereocontrolled synthesis of the PPAR-gamma agonist 10-nitrolinoleic acid. *J. Org. Chem.* **75:**5334–5336; 2010.
[19] Zanoni, G.; Bendjeddou, M. V. L; Porta, A.; Bruno, P.; Vidari, G. Improved synthesis of (E)-12-nitrooctadec-12-enoic acid, a potent PPAR gamma activator: development of a ''buffer-free'' enzymatic method for hydrolysis of methyl esters. *J. Org. Chem.* **75:**8311–8314; 2010.
[20] Kornblum, N.; Ungnade, H. E. 1-Nitroöctane. *Org. Synth.* **4:**724; 1963.
[21] Rudolph, V.; Schopfer, F. J.; Khoo, N. K. H.; Rudolph, T. K.; Cole, M. P.; Woodcock, S. R.; Bonacci, G.; Groeger, A. L.; Golin-Bisello, F.; Chen, C. S.; Baker, P. R. S.; Freeman, B. A. Nitro-fatty acid metabolome: saturation, desaturation, β-oxidation, and protein adduction. *J. Biol. Chem.* **284:**1461–1473; 2009.
[22] Vogel, A. I.; Furniss, B. S., Eds. Vogel's Textbook of Practical Organic Chemistry. 1989.
[23] Pavia, D. L., Lampman, G. M., Kriz, G. S., Engel, R. G., editors. *Brooks/Cole Laboratory Series for Organic Chemistry*. Pacific Grove, CA: Brooks Cole; 2010.
[24] Still, W. C.; Kahn, M.; Mitra, A. Rapid chromatographic technique for preparative separations with moderate resolution. *J. Org. Chem.* **43:**2923–2925; 1978.
[25] Waldmann, H.; Kunz, H. The allyl group as mildly and selectively removable carboxy-protecting group for the synthesis of labile 0-glycopeptides. *Liebigs Ann. Chem* **10:**1712–1725; 1983.
[26] Ono, N.; Maruyama, K. Preparation of pyrroles having long alkyl chains from nitroalkenes. *B Chem. Soc. Jpn* **61:**4470–4472; 1988.
[27] Soengas, R. G.; Estevez, J. C.; Estevez, R. J. Transformation of D-glucose into 1D-3-deoxy-3-hydroxymethyl-myo-inositol by stereocontrolled intramolecular Henry reaction. *Org. Lett.* **5:**4457–4459; 2003.
[28] Myers, A. G.; Barbay, J. K.; Zhong, B. Y. Asymmetric synthesis of chiral organofluorine compounds: use of nonracemic fluoroiodoacetic acid as a practical electrophile and its application to the synthesis of monofluoro hydroxyethylene dipeptide isosteres within a novel series of HIV protease inhibitors. *J. Am. Chem. Soc* **123:**7207–7219; 2001.
[29] Hey, H.; Arpe, H. J. Removal of allyl groups by formic-acid catalyzed by (triphenylphosphane)palladium. *Angew. Chem. Int. Ed.* **12:**928–929; 1973.
[30] Radi, R. Nitric oxide, oxidants, and protein tyrosine nitration. *Proc. Natl. Acad. Sci. USA* **101:**4003–4008; 2004.
[31] Farias, S. E.; Basselin, M.; Chang, L.; Heidenreich, K. A.; Rapoport, S. I.; Murphy, R. C. Formation of eicosanoids, E-2/D-2 isoprostanes, and docosanoids following decapitation-induced ischemia, measured in high-energy-microwaved rat brain. *J. Lipid Res.* **49:**1990–2000; 2008.
[32] Yuan, L.; Zhang, D.; Jemal, M.; Aubry, A. F. Systematic evaluation of the root cause of non-linearity in liquid chromatography/tandem mass spectrometry bioanalytical assays and strategy to predict and extend the linear standard curve range. *Rapid Commun. Mass Spectrom* **26:**1465–1474; 2012.
[33] Hsu, F. F.; Turk, J. Elucidation of the double-bond position of long-chain unsaturated fatty acids by multiple-stage linear ion-trap mass spectrometry with electrospray ionization. *J. Am. Soc. Mass Spectrom* **19:**1673–1680; 2008.
[34] Baker, P. R. S.; Lin, Y.; Schopfer, F. J.; Woodcock, S. R.; Groeger, A. L.; Batthyany, C.; Sweeney, S.; Long, M. H.; Iles, K. E.; Baker, L. M. S.; Branchaud, B. P.; Chen, Y. E.; Freeman, B. A. 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.* **280:**42464–42475; 2005.