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Nitrated fatty acids: synthesis and measurement

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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 re-form 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

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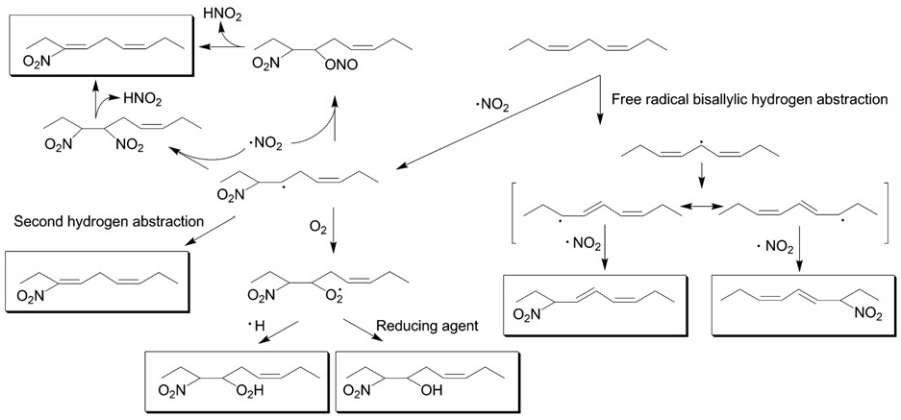

Fig. 1. Radical-induced mechanism of polyunsaturated acid nitration. Starting with a radical abstraction from the bis-allylic position, the delocalized radical may react with oxygen or nitrogen dioxide. Alternatively, direct addition of nitrogen dioxide to one double bond produces a nonstabilized radical 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.

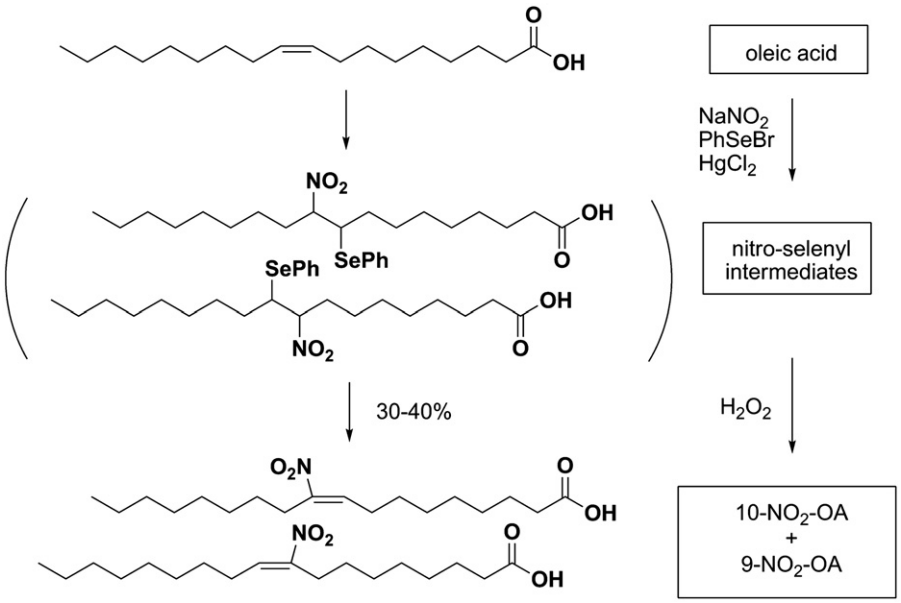

Fig. 2. Nitroselenation/nitromercuration synthesis of nitro-oleic acid. This is a two-step method of synthesizing an equimolar distribution of nitrated regioisomers. The combination of selenyl and mercurial reagents activates the double bond to nitration and are oxidized in a second step to generate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

for the proper characterization of the various regioisomers [12]. 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

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