

Research Article

Lysine Reacts with Cholesterol Hydroperoxide to Form Secosterol Aldehydes and Lysine-Secosterol Aldehyde Adducts

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Received 21 August 2019; Revised 11 January 2020; Accepted 17 January 2020; Published 10 February 2020

Academic Editor: Silvia Persichilli

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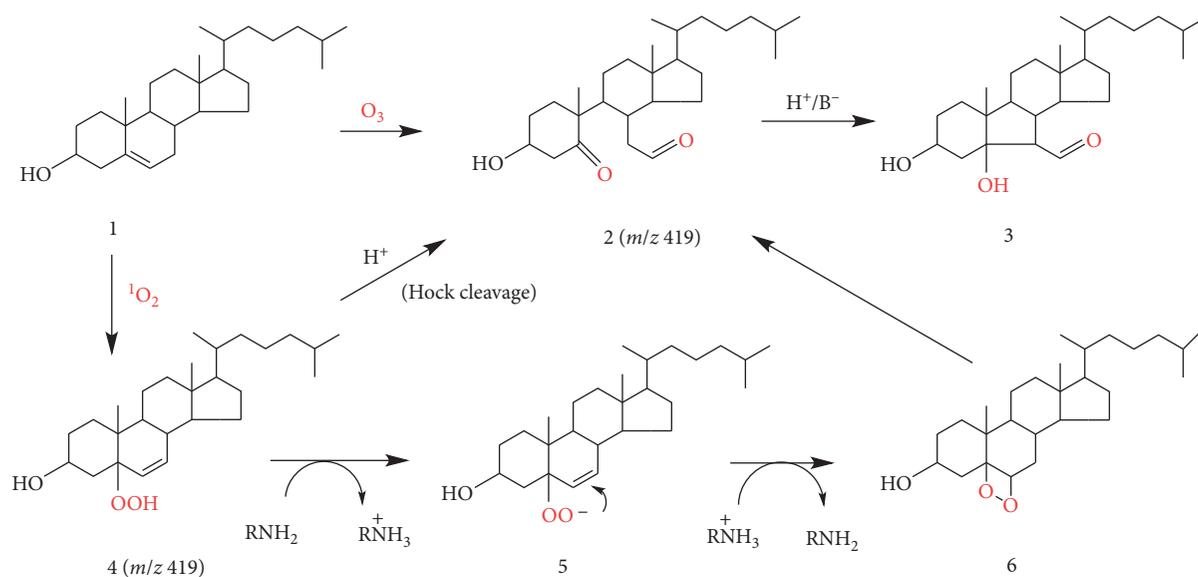
Two cholesterol secosterol aldehydes, namely, 3 β -hydroxy-5-oxo-5,6-secocholestan-6-al (secosterol A) and its aldolization product 3 β -hydroxy-5 β -hydroxy-B-norcholestan-6 β -carboxyaldehyde (secosterol B), are highly bioactive compounds which have been detected in human tissues and potentially contribute to the development of physiological dysfunctions such as atherosclerosis, Alzheimer's disease, diabetes, and cancer. They were originally considered to be exclusive products of cholesterol ozonolysis and thus to be evidence for endogenous ozone formation. However, it was recently postulated that primary amines such as lysine may catalyse their formation from cholesterol-5 α -hydroperoxide (Ch-5 α -OOH), the main product of the oxidation of cholesterol with singlet oxygen. This involves cyclization of Ch-5 α -OOH to an unstable dioxetane intermediate, which decomposes to form secosterol aldehydes with triplet carbonyl groups, whose return to the singlet state is at least partly coupled to the conversion of triplet molecular oxygen to singlet oxygen. Here, we subjected cholesterol to photosensitized oxidation, which predominantly produces Ch-5 α -OOH and minor amounts of the 6 α - and 6 β -hydroperoxides, exposed the hydroperoxide mixture to lysine in the presence of the antioxidant 2,6-ditertiary-butyl-4-hydroxytoluene (BHT), and analysed the reaction mixture by liquid chromatography-electrospray ionization-mass spectrometry. Consistent with the postulated lysine-catalysed formation of secosterol aldehydes, we detected formation of the latter and several types of their lysine adducts, including carbinolamines, Schiff's bases, and amide-type adducts. We propose that the amide type adducts, which are major biomarkers of lipid oxidation, are mainly formed by singlet oxygen-mediated oxidation of the carbinolamine adducts.

1. Introduction

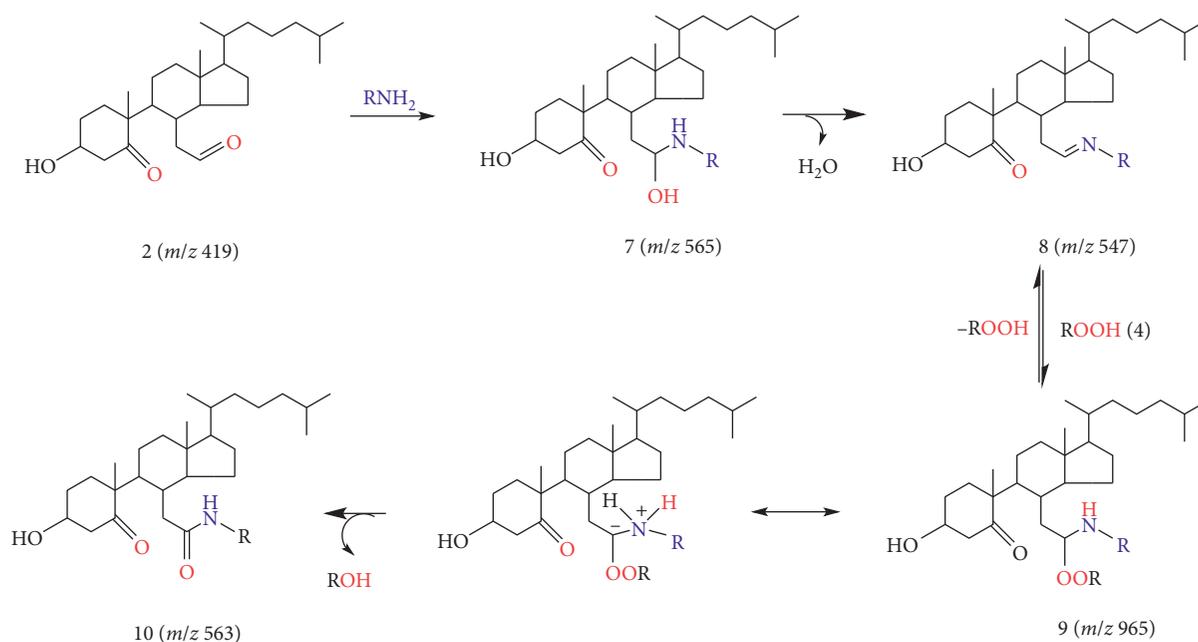
Cholesterol (**1** in Scheme 1) is an important component of animal cell membranes, but cholesterol oxidation products, formed either in food or in vivo, have been implicated as contributors to various noncommunicable diseases [1, 2]. Identification of the cholesterol ozonolysis product, 3 β -hydroxy-5-oxo-5,6-secocholestan-6-al (secosterol A, **2**) and its aldolization product 3 β -hydroxy-5 β -hydroxy-B-norcholestan-6 β -carboxyaldehyde (secosterol B, **3**) in human atherosclerotic plaque and brain tissue of Alzheimer's

disease patients was cited as evidence for endogenous ozone production in human tissues [3]. Such endogenous ozone production was suggested to involve antibody- or amino acid-catalysed oxidation of water by singlet oxygen [3–5] or more recently through multiple reactions of singlet oxygen with amino acids [6, 7].

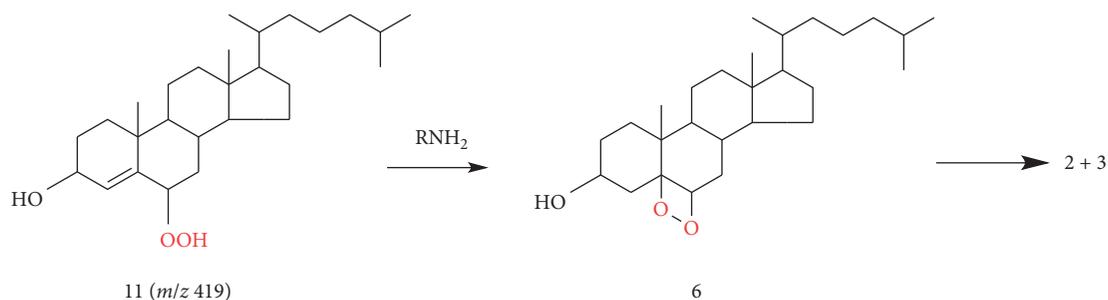
On the contrary, cholesterol-5 α -hydroperoxide **4** (the major product of the reaction of cholesterol with singlet oxygen) was found to be readily converted to secosterol **3** under acidic conditions, and it was concluded that **4** was initially converted to secosterol **2** by Hock cleavage, that the



SCHEME 1: Previously proposed pathways for formation of secosterol aldehydes 2 and 3.



SCHEME 2: Postulated reaction of secosterol aldehyde 2 with an amine to form amide adduct 10 via carbinolamine 7, Schiff's base 8, and peroxide adduct 9. The expected masses of the protonated molecular ions are given in brackets, when RNH_2 is from lysine.



SCHEME 3: Expected lysine-catalysed conversion of cholesterol-6-hydroperoxide 11 (this structure is general for both 6a- and 6b-hydroperoxides) to secosterol aldehydes 2 and 3.

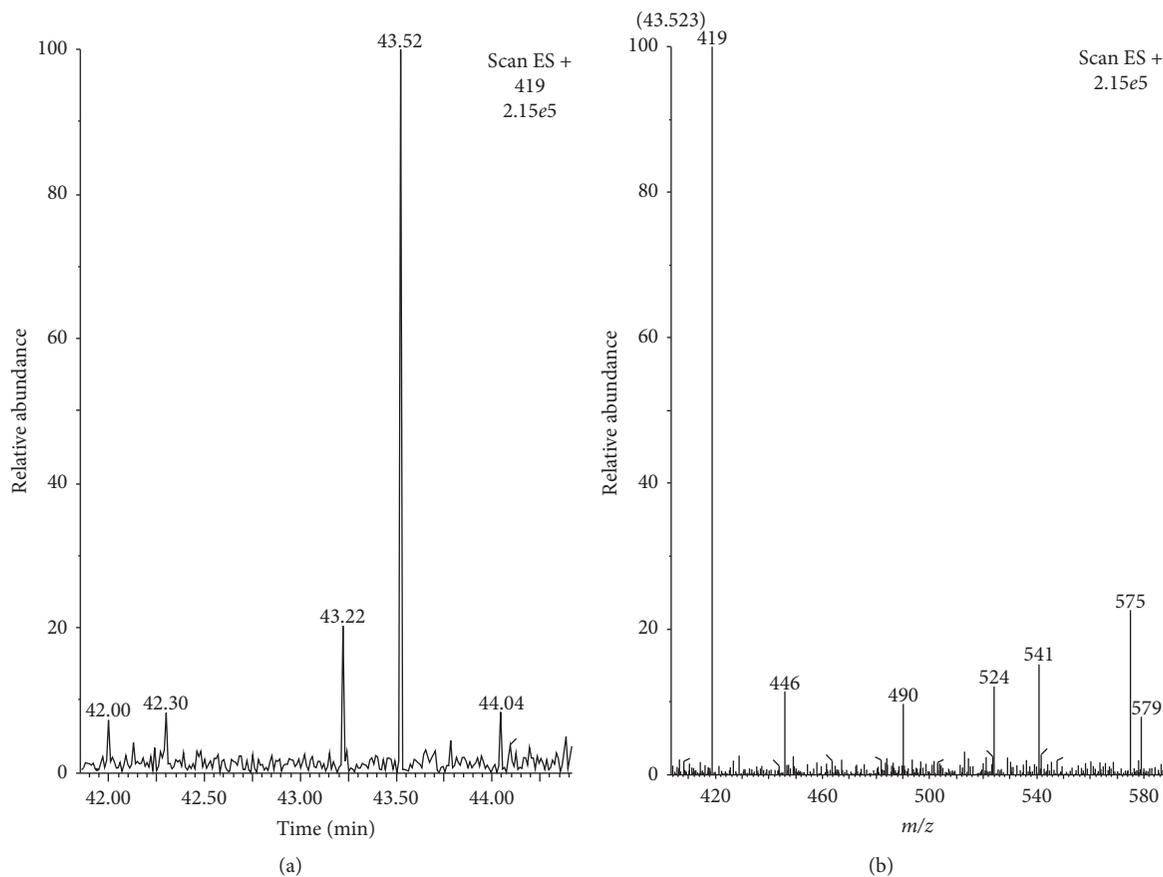


FIGURE 1: Extracted ion chromatogram (a) and the corresponding mass spectrum (b) based on the protonated molecular ion at m/z 419 that could arise from unreacted cholesterol hydroperoxide **4** or secosterol aldehydes **2** and **3**.

latter rapidly underwent aldolization to form **3** (Scheme 1), and that ozone may not therefore be necessary for the formation of cholesterol secosterol aldehydes *in vivo* [8]. However, it was later argued that since ozone largely converts cholesterol to secosterol **2**, which is also the major secosterol aldehyde in atherosclerotic plaques, endogenous ozone rather than Hock cleavage of cholesterol-5 α -hydroperoxide **4** should be important for secosterol formation *in vivo* [9]. Nevertheless, Tomono et al. [10] found that roughly equal amounts of secosterol **2** and secosterol **3** were formed by human myeloperoxidase independently of antibody involvement and suggested that in this case singlet oxygen and possibly another oxidant, but not ozone, were involved in both secosterol **2** and secosterol **3** formation. In addition, it was recently postulated that lysine (RNH₂ in Scheme 1) may catalyse the conversion of cholesterol hydroperoxide **4** to secosterol **2**, via peroxy anion **5** and dioxetane intermediate **6** (Scheme 1) [11]. This was based on an earlier report that lysine residues in proteins directly react with the 13-hydroperoxide of linoleic acid (13-LA-OOH) to form the amide-type adduct, N^c-(hexanoyl)-lysine [12] and the subsequent proposal that lysine initially catalyses decomposition of 13-LA-OOH to hexanal and 12-oxo-9, undecanoic acid, followed by reaction of lysine with hexanal to form the corresponding Schiff's base, and further reaction of the Schiff's base with a molecule of 13-LA-OOH to form the hexanoyl-lysine adduct [13]. In an analogous

manner, if lysine (RNH₂) catalyses conversion of cholesterol hydroperoxide **4** to secosterol **2** (Scheme 1), the latter should react with lysine to form an amide-type adduct. It is becoming increasingly evident that such amide-type adducts are readily formed both *in vitro* and *in vivo*, and could have major contributions to pathophysiological processes. For example, formyl lysine adducts formed by the reaction of formaldehyde with lysine residues have been detected in various types of proteins, including histone proteins, and could be involved in epigenetic modifications [14], while hexanoyl lysine and propentofylline adducts are considered as good biomarkers of lipid oxidation in food and *in vivo* [15–17]. However, the mechanism of formation of these adducts is not well understood [18]. According to the recently postulated pathway for amide-type adduct formation [13], secosterol **2** would react with lysine to successively form carbinolamine **7**, Schiff's base **8**, peroxide intermediate **9**, and amide-type adduct **10** (Scheme 2).

Analogously to the conversion of hydroperoxide **4** to secosterol aldehydes **2** and **3** (Scheme 1), Cholesterol-6 (α and β)-hydroperoxides **11** formed as minor products of photosensitized cholesterol oxidation [19] are expected to undergo lysine-catalyzed cyclization to dioxetane **6** and thus also afford secosterol aldehydes **2** and **3** (Scheme 3).

Here, we tested such hypothesized lysine-mediated conversion of cholesterol hydroperoxides to secosterol

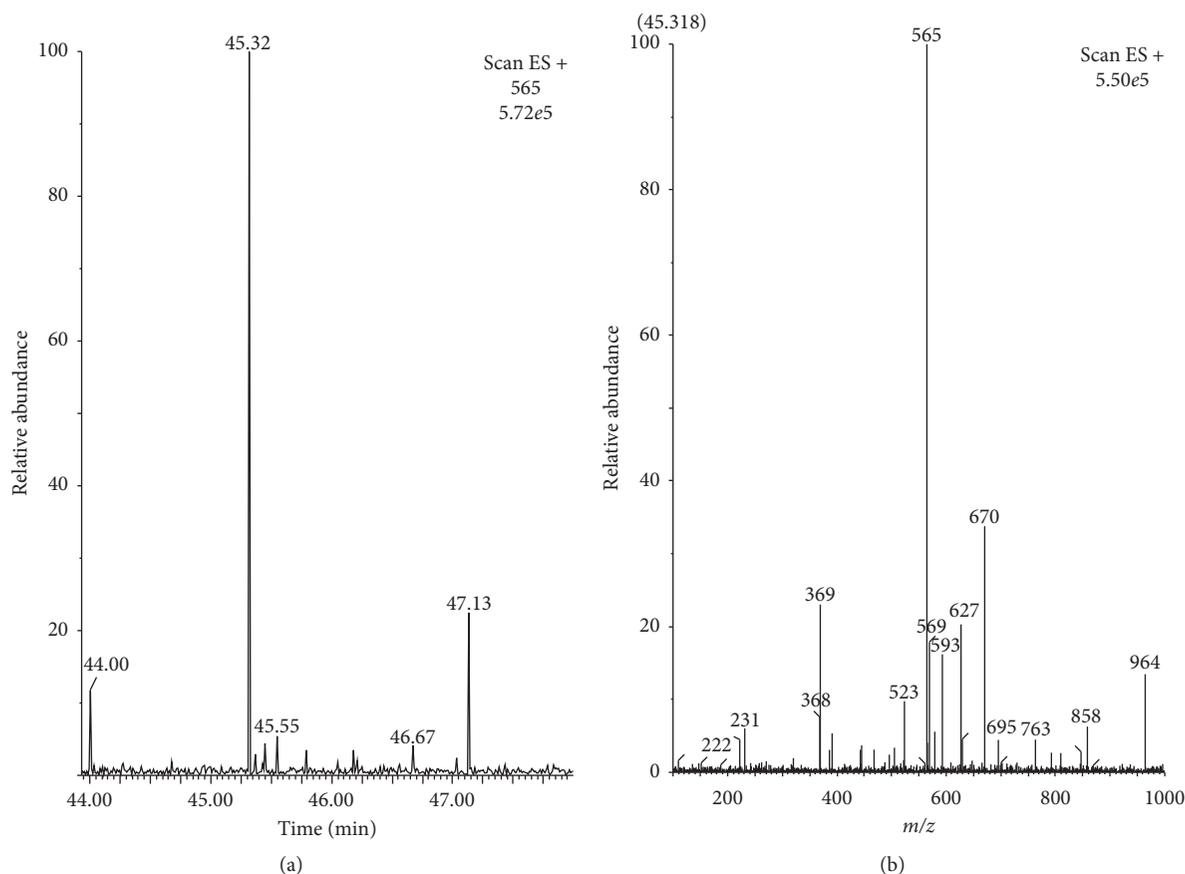


FIGURE 2: Extracted ion chromatogram (a) and the corresponding mass spectrum (b) based on the protonated molecular ion at m/z 565 that could arise from carbinolamine **7** or the corresponding carbinolamine from secosterol aldehyde **3**.

aldehydes and formation of adducts such as **7–10**. Cholesterol was subjected to photosensitized oxidation in the presence of methylene blue, and the hydroperoxide mixture thus obtained (Ch-5 α -OOH and expected small amounts of Ch-6 α -OOH and Ch-6 β -OOH) was then reacted with lysine, in the presence of the antioxidant 2,6-ditertiary-butyl-4-hydroxytoluene (BHT), to limit free radical reactions. The reaction products were analysed by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS), which readily gives molecular ion peaks. Molecular ions attributable to the secosterol aldehydes as well as adducts **7**, **8**, and **10**, but not **9**, were detected. Thus, the lysine-mediated decomposition of cholesterol hydroperoxides to form secosterol aldehydes and the facile adduction of the latter to lysine were demonstrated. An alternative mechanism for formation of amide-type adduct **10**, involving singlet oxygen-mediated oxidation of carbinolamine **7**, is proposed.

2. Materials and Methods

2.1. Reagents. Cholesterol, L-lysine, ethanolamine, formic acid, methylene blue, hexane, 2,4-dinitrophenylhydrazine, ethyl acetate, 2-propanol, and anhydrous sodium sulphate were purchased from Sigma-Aldrich.

2.2. Photosensitized Oxidation of Cholesterol. Cholesterol (2 g) was dissolved in 10 ml of hexane containing 0.27 mM methylene blue and irradiated at 10°C with ultraviolet light of 366 nm (Funa UV, Light Model SL-800G), from a distance of 2.5 cm for 1 hour. The cholesterol hydroperoxides were purified by column chromatography on silica gel eluted with hexane/ethyl acetate (95:5). The fractions containing the hydroperoxide were confirmed by coloration with potassium iodide. After drying over anhydrous sodium sulfate, the solvent was evaporated in vacuo to obtain the cholesterol hydroperoxides (0.9 g), which was dissolved in 2-propanol containing a trace of BHT.

2.3. Reaction of Cholesterol Hydroperoxides with Lysine. L-Lysine was added at 100 μ M to vials containing cholesterol hydroperoxide in 2-propanol (100 μ M) and incubated for 1 hour at 37°C. This mixture was filtered through a micron filter, after which a 20 μ l aliquot was drawn and analyzed by LC-ESI-MS.

2.4. Derivatization of Unreacted Carbonyls with DNPH. After reacting lysine with cholesterol hydroperoxides for 1 hour, 10 mM 2,4-DNPH in 0.1% formic acid was added to the reaction mixture, and the reaction mixture was incubated at 37°C for a further 1 hour prior to analysis by LC-ESI-MS.

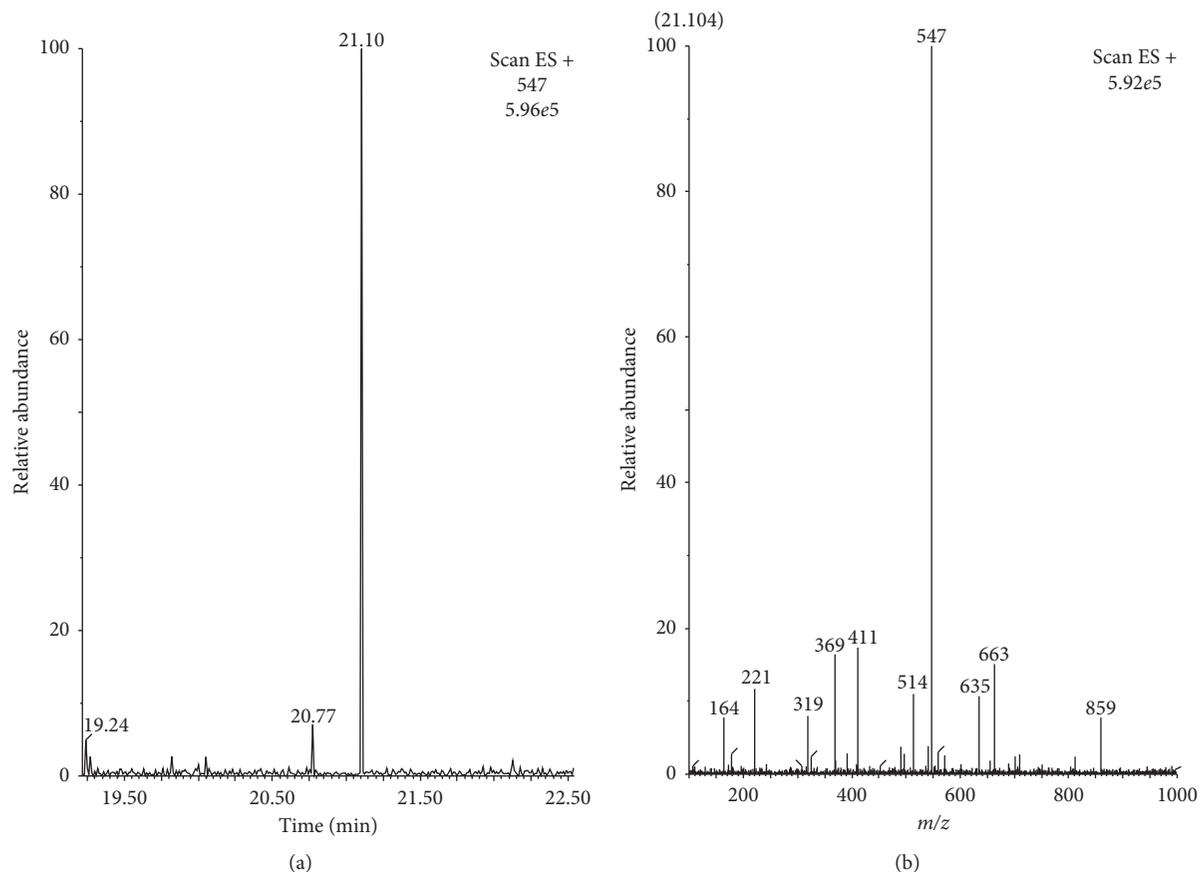


FIGURE 3: Extracted ion chromatogram (a) and the corresponding mass spectrum (b) based on the protonated molecular ion at m/z 547 that could arise from Schiff's base **8** and/or the corresponding Schiff's base derived from secosterol aldehyde **3**.

2.5. Analysis of Products by LC-ESI-MS. LC-MS analysis was performed on a Waters 2790 separations module connected to a Micromass Quattro Ultima MS equipped with an electrospray ionization interface (Micromass UK Ltd., Floats Road, Wythenshawe, Manchester, UK). Separation was achieved using the Supelco column (150 mm * 4.6 mm * 5 μ m) (Supelco Analytical, North Harrison Road, Bellefonte, USA) eluted with acetonitrile: water (70:30) at flow rate of 0.1 ml–0.3 ml/minute. MS data were taken in the positive ion mode. The data were collected and analyzed by masslynx 4.1 software (Waters, USA).

3. Results and Discussion

The total ion chromatogram (TIC) obtained upon LC-ESI-MS analysis of the reaction mixtures of lysine and cholesterol hydroperoxide indicated the formation of many products. Therefore, we relied on extracted ion chromatograms for detection of the secosterol aldehydes and their adducts. Figures 1–4 show such chromatograms and matching mass spectra for some of the expected products shown in Scheme 2. These include results for detection of ions attributable to secosterol aldehyde **2** (m/z 419) (Figure 1), carbinolamine **7** (m/z 565.19) (Figure 2), Schiff's base **8** (m/z 547.19) (Figure 3), and amide adduct **10** (m/z 563.19) (Figure 4). Analogous adducts were

obtained when ethanolamine rather than lysine was reacted with cholesterol hydroperoxide (not shown). Carbinolamines are often considered to be too unstable for detection, and detection of carbinolamine **7** (Figure 2) was therefore not quite anticipated. Nevertheless, there are previous reports on the observation of such species by H NMR and mass spectrometry [20]. 2,4-Dinitrophenyl hydrazine (DNPH), which has a weight of 198.14 g/mol, is a widely used derivatization agent for aldehydes, and in this study, we found adducts consistent with the reaction of DNPH with secosterol aldehydes to form both the corresponding carbinolamine (m/z 617) and the Schiff's base (m/z 599) (not shown).

It is noted that Figures 2–4 could also belong to analogous adducts formed from secosterol aldehyde **3**, since lysine is known to catalyse Aldol condensation [21] and thus could have promoted some conversion of secosterol **2** to secosterol **3**. A key difference between these two compounds is that secosterol **2** has an additional carbonyl group at C-5, which is lacking in secosterol **3**. Hence, secosterol **2**, but not **3**, can react with two lysine molecules to form an adduct having two carbinolamine moieties, such as dicarbinolamine **12** (Scheme 4). Thus, detection of an ion peak at m/z 711, attributable to the latter (Figure 5), specifically supports the formation of secosterol **2**. An analogous dicarbinolamine containing

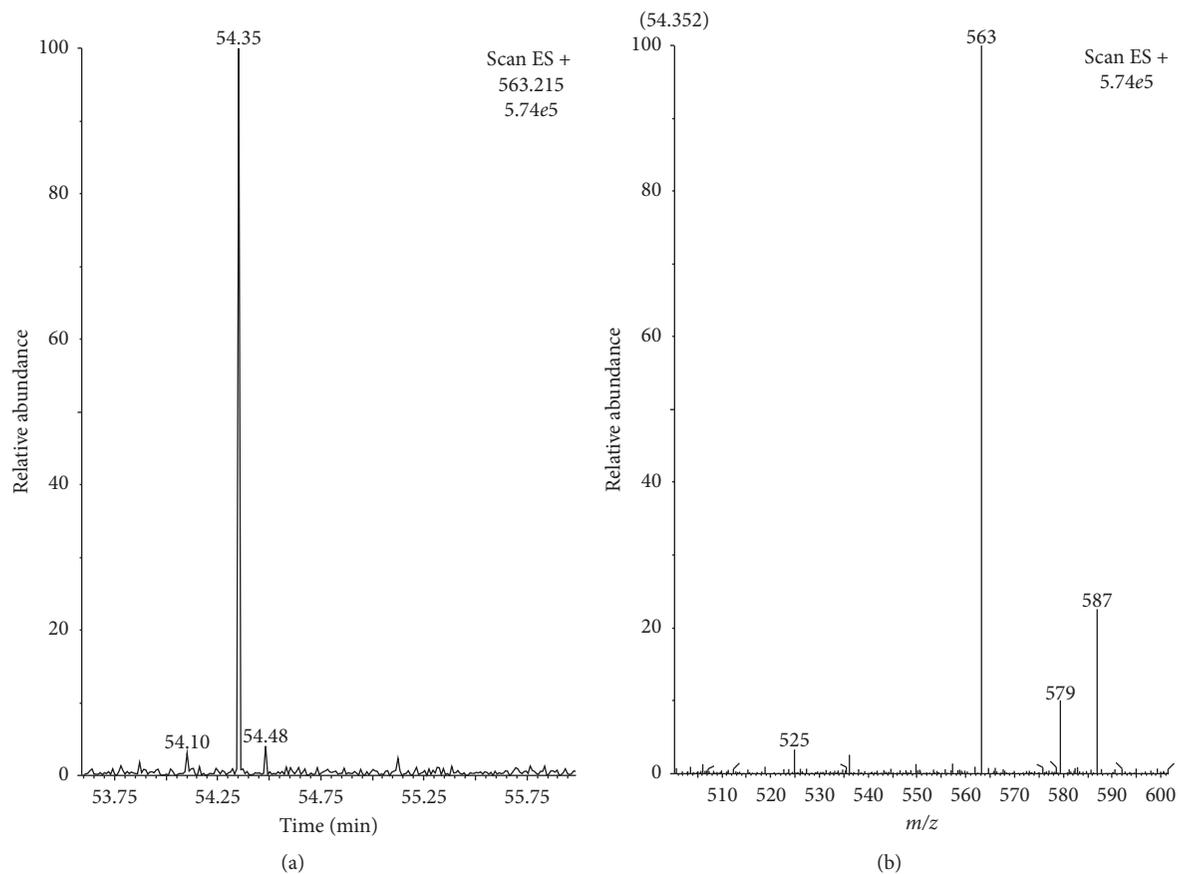
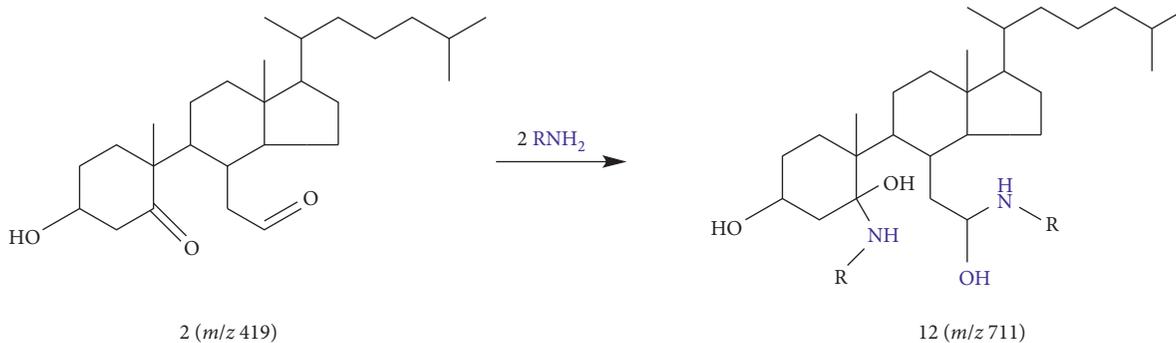


FIGURE 4: Extracted ion chromatogram (a) and the corresponding mass spectrum (b) based on the molecular ion at m/z 563 that could arise from amide adduct **10** or from the corresponding amide adduct from secosterol aldehyde **3**.



SCHEME 4: Reaction of secosterol aldehyde **2** with two molecules of lysine (RNH_2) to form dicarbinolamine **12**.

one molecule of lysine and one molecule of DNPH was also obtained at m/z 763 (not shown).

The conversion of aldehydes to carbinolamines and Schiff's bases, such as the conversion of secosterol **2** to compounds **7** and **8**, respectively (Scheme 2), is well known. Although a molecular ion attributable to amide-type adduct **10** was observed at m/z 563.19 (Figure 4), none was observed for peroxide **9**. Hence, no direct evidence for the involvement of the latter in formation of **10**, according to Scheme 2, was obtained.

Kato et al. [12] found that the reaction of tertiary butyl hydroperoxide with hexanal and lysine did not produce the

amide-type adduct, N^ϵ -(hexanoyl) lysine, and suggested that aldehyde and hydroperoxide are not directly involved in amide-type adduct formation. Based on this, it was further postulated that linoleic acid hydroperoxide may be converted to a triplet ketone whose reaction with lysine produces the hexanoyl-lysine adduct [18]. On the contrary, Trezl et al. [22] found that the reaction of formaldehyde or acetaldehyde with hydrogen peroxide in the presence of lysine led to the formation of triplet state formaldehyde or acetaldehyde, singlet oxygen, and the corresponding amide-type adducts, namely, formyl lysine and acetyl lysine. However, they did not propose the mechanism for the

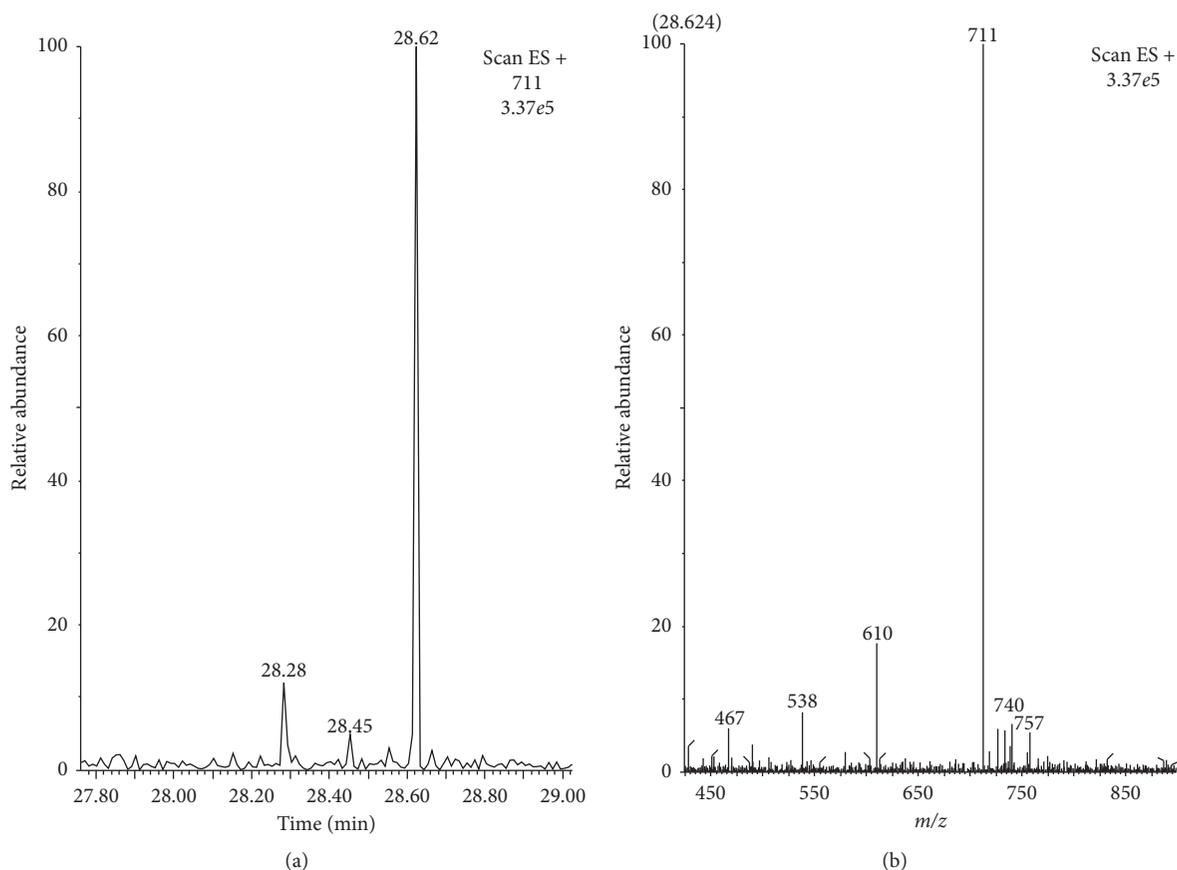
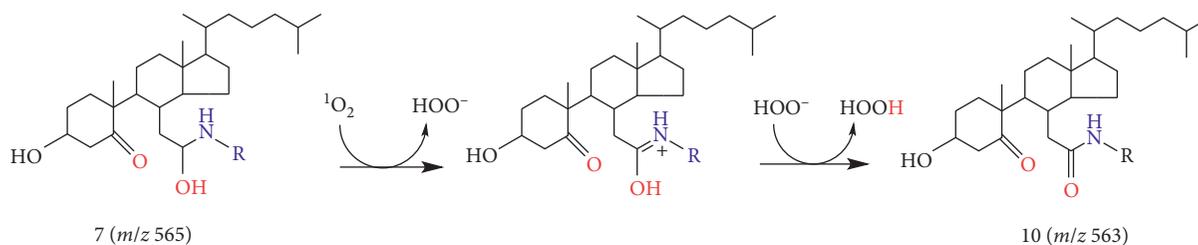


FIGURE 5: Extracted ion chromatogram (a) and the corresponding mass spectrum (b) based on the molecular ion at m/z 711 that could arise from dicarbinolamine **12**.



SCHEME 5: Proposed singlet oxygen-mediated conversion of carbinolamine **7** to amide-type adduct **10**.

reaction of the triplet formaldehyde or acetaldehyde with lysine to generate the amide-type adducts. Interestingly, it has been reported that singlet oxygen readily oxidizes amines to imines [23]. As suggested in Scheme 5, an analogous oxidation of carbinolamine **7** by singlet oxygen may well explain the formation of amide type adduct **10**. This is further consistent with the formation of dioxetane intermediate **6** in Scheme 1 because dioxetane decomposition produces triplet state carbonyls (one of the carbonyl groups in secosterol **2** formed by the lysine-mediated pathway in Scheme 1 may be in the triplet state), which transfer some of their energy to molecular oxygen to form singlet oxygen [13, 24]. Such a mechanism is also in agreement with the results of Kato et al. [12] that the reaction of tert-butyl hydroperoxide with hexanal and lysine did not

produce N^ϵ -(hexanoyl)lysine because no triplet carbonyls may be formed in that system, since tert-butyl hydroperoxide cannot cyclize into a dioxetane, unlike the unsaturated linoleic acid or cholesterol hydroperoxides.

4. Conclusion

We found that lysine reacts directly with cholesterol hydroperoxides to form secosterol aldehydes and various lysine-secosterol aldehyde adducts including amide-type adducts. The results are consistent with the lysine-catalysed cyclization of cholesterol hydroperoxides into dioxetanes as precursors of the secosterol aldehydes and singlet oxygen-mediated oxidation of carbinolamines as a major source of the amide-type adducts.

Data Availability

The data used to support the findings of this study are included within the article. Other data are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This work was partly funded through a grant from The World Academy of Sciences for the Advancement of Science in Developing Countries (TWAS) (14-075RG/CHE/AF/ACI; UNESCOFR: 324028586) to A. N. Onyango and funds provided by Kenya Industrial Research and Development Institute to G. W. Wanjala. The Department of Chemistry, JKUAT, is acknowledged for access to the LC-MS equipment.

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