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An exogenous marker: A novel approach for the characterization of oxidative stress

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Abstract—Oxidative stress (OS) and its consequences which promote alterations in biomolecules, to tissue damage and to the development of pathological conditions, continue to attract many investigators. The identification of reliable biomarker is essential for the characterization of OS and possibly for early discovery of OS-associated diseases. The aim of the present study was to offer a new concept in the development of novel probes for OS, based on the design, synthesis, and utilization of exogenous markers, as alternative to the search for endogenous markers. This article describes: (a) the synthesis of such a marker, linoleoyl tyrosine 2-deoxyguanosyl ester (LTG), constructed from three endogenous subunits: linoleic acid, tyrosine, and 2′-deoxyguanosine, representing the three major groups from which the body is composed, unsaturated fatty acids (USFA), proteins, and DNA, respectively, all bound covalently and (b) the development of analytical tools (LC/MS/MS) to enable the identification of the different LTG oxidized products formed under OS by exposure of LTG to different reactive oxygen species (ROS) such as, copper ions and hypochlorous acid.

1. Introduction

Oxidative stress (OS) is proposed to be involved in several human diseases, such as cardiovascular and neurological diseases, cancer, inflammation-related diseases, and aging.[1–3] Reactive oxygen and nitrogen species (ROS/RNS), such as superoxide ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\cdot}$), nitrogen oxide ($NO^{\cdot}$), peroxynitrite ($ONOO^-$), and hypochlorous acid (HOCl), are all products of normal metabolic pathways in humans and are formed during the destruction of invading organisms. ROS/RNS take part in signaling cascades and are involved in cellular functions, such as cell proliferation, inflammation, and adhesion. Under certain conditions, they may exert harmful effects,[4] cause unwanted collateral damage to normal neighboring cells,[5] as well as attack and damage key biomolecules, such as lipids, proteins, and DNA.[6,7] Polyunsaturated fatty acids (PUFA) are readily oxidized, which in turn generate changes in membrane density, fluidity, and permeability, thus affecting cellular functions.[8] Proteins may undergo deamination, decarboxylation, and modifications in their aromatic rings,[9] which can lead to changes in their three-dimensional structure and activity. DNA oxidation may lead to strand breaks, DNA–protein cross-linkage, sugar damage, and base modifications, such as the formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG), one of the major known markers of DNA damage (reviewed).[10,11]

The identification of reliable biomarkers, for example, modified endogenous compounds formed in cells and organs as a result of OS, is becoming essential for the characterization of OS and for the prediction of the early development of pathological conditions.[12] Modified endogenous compounds, which have been proposed as indicators of OS, include peroxide values, thiobarbituric reactive substances, isoprostanes, and oxysterols for PUFA; keto proteins, chloro- or nitro- tyrosine for proteins; and 8-oxo-guanosine for DNA.[12–14] However, most of these biomarkers, and the proposed methods for their detection, bear several limitations. Some are not specific, measuring uncharacterized products of oxidative stress, whereas the application in vivo of others is questionable.[3] Their levels are determined by a dynamic process, involving formation, accumulation, and removal, and may not be due to the oxidative process within the system, but rather to alternative processes.

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Furthermore, biomarker levels may change during their isolation and sample preparation.[15]

In the present study, a new methodology for characterizing OS in biological systems is proposed. Instead of searching for an endogenous reporter within the investigated target, a sensitive molecule was designed, synthesized, and constructed from various endogenous subunits, which were connected covalently together to form a novel probe (not present as such in organs). The designed marker of this investigation consists of the amino acid tyrosine (Ty), connected to linoleic acid (LA) forming an amide bond (*N*-linoleoyl tyrosine, [LT]), as well as to 2′-deoxyguanosine (2′-dG), through the primary hydroxyl group of the 2′-dG, and to the free tyrosine carboxylic group, forming an ester bond (Fig. 1). This molecule, linoleoyl tyrosine 2-deoxyguanosyl ester (LTG), contains the three major groups from which the body is composed: LA represents the PUFA, the Ty residue represents proteins, and the hydrophilic subunit 2′-dG represents the DNA. Each part of the synthetic marker is well known to be easily oxidized and to form specific products, depending on the type of ROS/RNS present. Here the aim was (a) to describe the synthesis of such a marker and (b) to develop the analytical tools using LC/MS/MS for the separation and identification of the different oxidized marker products, which may be formed as a result of the incubation of the marker with various types of ROS (copper ions or HOCl). Such a study is the prerequisite for the use of such a marker in cells,[16,17] organs, animals, and in ex vivo human samples.

2. Results and discussion

2.1. The synthesis of linoleoyl tyrosine 2-deoxyguanosyl ester (LTG)

LTG was synthesized from LT[16] by a conventional esterification method (as described in Section 3) and purified by reverse phase chromatography on HPLC. The structure of LTG (**1**) (Fig. 1) was in agreement with analytical data gathered from ^{1}H-NMR (Section 3), LC/MS, LC/MS/MS, and UV–vis spectra as shown in Figure 2, and from the known structure of LT.[16] Fragmentation of the molecular ion at *m*/*z* 691.6 (M−1) revealed the fragments of the four major subunits, LT with an *m*/*z* of 442.5, a fragment corresponding to the 2′-dG with an *m*/*z* of 266.4, a fragment containing the tyrosine alone without the linoleic acid with an *m*/*z* of 180, and a fragment containing the guanine ring without the 2′-deoxyribose, with an *m*/*z* of 150 (Fig. 2a). This fragmentation enabled the detection of an alteration, as well as the precise site of change in the marker, resulting from its oxidation. Oxidation of the LT subunits, either of the tyrosine, the linoleic acid or of both, will result in the disappearance of the fragment with the mass ion of 442.5 *m*/*z* and the formation of a new molecule with a mass of a higher value. In addition, it will be possible to discern if the change occurred on the tyrosine, on the linoleic acid or on both, due to the fragment of 180 *m*/*z* (Ty). Similarly, oxidation of the 2′-dG will result in the replacement of the fragment, 266.4 *m*/*z*, with a new fragment of a different mass. Since an additional fragment at an *m*/*z* of 150 is present, which corresponds to the 2′-deoxyribose, it will be possible to determine if the change took place on the nucleotide (guanine) or on the carbohydrate residue. Additional fragmentation of the above major ions (MS/MS) may, in certain instances, enable the identification of the specific carbon that was oxidized.

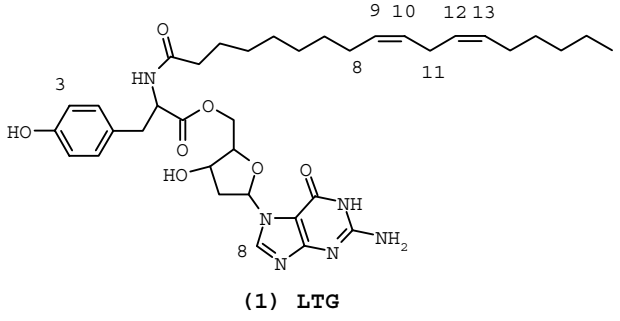

Figure 1. The structure of the exogenous designed marker, LTG, and its potentially modified positions.

2.2. The identification of copper ion-induced LTG oxidation (Ox-LTG)

The next step was the development of analytical tools for the characterization and identification of the type of alterations, which may occur to LTG (Ox-LTG) after its exposure to different ROS. As an inducer of OS, copper ions were used. LTG was incubated with $CuSO_4$ (20 μM) for 4 h, after which four different types of Ox-LTG were detected by LC/MS/MS, with 8-oxo-dG (**2**) being the major product (16.5% of Ox-LTG/total LTG), which was eluted at 6.7 min, as outlined in M&M and Table 1. The molecular ion with an *m*/*z* of 707.6 (LTG [M−1]+16 mass units) was fragmented into three major peaks with *m*/*z* of 442.7 (LT), 282, and 180 (Ty). The presence of a fragment at the *m*/*z* of 442.7 provides evidence that the marker alteration was neither on linoleic acid, nor on the tyrosine subunits (LT). The second peak at the *m*/*z* of 180 (free tyrosine) further clarified that no change occurred to the tyrosine subunit. The peak at the *m*/*z* of 266 (2′-dG) disappeared, and a new peak at the *m*/*z* of 282, equivalent to 2′-dG+16 (oxygen), was formed. An injection to the MS of a standard of 2′-dG and 8-oxo-dG (synthesized from 2′-dG) provided the same data, a peak of 266 *m*/*z* was converted to 282 *m*/*z*.

The second major Ox-LTG formed during copper ion-induced LTG oxidation (12.0% Ox-LTG/total LTG) was a molecule of epoxidized linoleic acid, epoxy-LTG (**3**), as shown in Table 1. This new substance had the same molecular ion mass (707.6 *m*/*z*) as did the 8-oxo-dG, but with a different elution retention time (3.5 min, as compared to 6.7 min, respectively) and a different fragmentation in the MS. The presence of a fragment at 266 indicated that the 2′-dG was not modified. However, the fragment, 442 *m*/*z* (LT), disappeared, and a new fragment, 458.3 (442+16) *m*/*z*, was formed. The fragment of 180 *m*/*z* (Ty) was still present, implying that the oxidation occurred on the linoleic acid

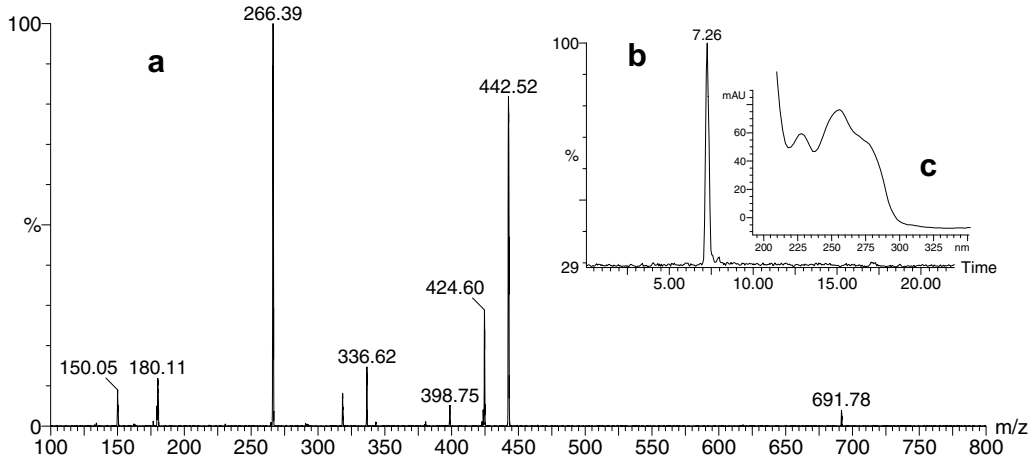

Figure 2. Spectra of LTG. (a) The LC/MS spectrum of LTG with a molecular ion of *m/z* 691.8 *m/z* (M−1) and fragments of 442.7 (linoleoyl tyrosine moiety), 266 (2′-deoxy guanosyl residue), 180 (tyrosine subunit), and *m/z* 150 (2′-deoxyribose). (b) Purified LTG separated on an RP18 column by HPLC, using AcCN:H_2O as eluents (peak at retention time of 7.26 min), with a UV/vis spectrum of λ_{max} at 228 and 256 nm (c).

Table 1. Oxidized LTG product

Product	Molecular mass-1 (*m/z*)	Selected daughter ions (*m/z*)	Retention time (min)	% oxidized LTG/ total LTG	
LTG (**1**)	691.6	691.6; 442.5; 266.0; 180.0	7.11		
Oxidation with Cu^{2+}ions (20μM)					
8-oxoLTG (**2**)	707.6	707.5; 442.4; 282.0; 180.0	6.70	16.5	
Epoxy-LTG (**3**)	707.6	707.2; 458.2; 266.0; 180.0	3.50	12.0	
Hydroperoxy-LTG (**4**)	723.6	723.1; 474.5; 266.0; 180.0	2.00	8.0	
Epoxy-hydroperoxy-LTG (**5**)	739.6	739.7; 490.3; 266.0; 180.0	1.23	2.0	
Oxidation with HOCl (0.2 mM+0.4 mM)				0.4 mM	0.2 mM
Chloro-LTG, (**6**)	726.1	476.5; 266.0; 214.5, 149.3	7.68	24.5	10.0
Chloro-epoxy-LTG (**7**)	742.0	492.5; 266.0; 214.5; 150	4.59	6.0	0.3
Chlorohydrine-LTG (**8**)	744.0	494.7; 266.0; 180.0; 150.0	4.43	1.1	0.1
Chlorohydrine-epoxy-LTG (**9**)	760.0	510.6; 492.1; 266.0; 179.9	8.1	3.5	0.4
8-oxoLTG (**2**)				0.6	0.6
Epoxy-LTG (**3**)				3.0	1.0
Hydroperoxy-LTG (**4**)				5	0.4
LTG in HBSS					
8-oxoLTG (**2**)				0.5	
Epoxy-LTG (**3**)				0.7	

Exposure of the LTG marker to copper ions (20 μM) for 4 h resulted in the formation of a molecule, that was predominantly oxidized on the 2-deoxyguanosyl residue, with the formation of 8-oxo-deoxyguanosine. Exposure of LTG to 0.2 or 0.4 mM HOCl resulted in a molecule in which mostly the tyrosine was oxidized, and in which linoleoyl 3-chloro-tyrosine 2′-deoxyguanosyl ester was the major product formed.

moiety. Injection of a standard of synthetic linoleic acid epoxide[18] gave the same fragmentation.

The third oxidized marker obtained contained a molecule with hydroperoxide on the linoleic acid, hydroperoxide-LTG (**4**) (8.0% of Ox-LTG/total LTG), as well as a molecule with both hydroperoxide and epoxide (**5**) on the linoleic acid (2% of Ox-LTG/total LTG). With similar considerations in mind, as mentioned above (e.g., the increased mass of the molecular ions of LTG [691, M−1], and the observation that the increased mass occurred in the LT subunits, but not on the 2′-dG, nor on the tyrosine [the fragments 266 and 180 *m/z* were still present]), it was possible to come to conclusions and draw the above structures.

For further structure elucidation, the above results were compared with our previous findings, according to which, each of these subunits and their oxidized products had been injected separately to the MS.[18] In this former study, in which we had aimed to clarify if there is a difference in the oxidation of tyrosine, linoleic acid or cholesterol, when alone or together, after exposure to increasing levels of copper ions,[18] results similar to those above were obtained. Linoleic acid or cholesterol were preferentially oxidized, and not tyrosine. These results imply that tyrosine is stable under copper ion-induced oxidation in vitro.

2.3. The identification of hypochlorous acid (HOCl) induced LTG oxidation

HOCl is a potent toxic oxidant, formed by the enzyme myeloperoxidase (MPO) during the reaction of H_2O_2 in the presence of chloride ions (Cl^-), and present in neutrophils and monocytes. HOCl reacts with

unsaturated lipid double bonds, such as linoleic acid or cholesterol, to yield lipid chlorohydrins.[18] Moreover, it reacts with LDL,[19] preferentially with the amino acids of the LDL, such as tryptophan, lysine, methionine, cysteine, and tyrosine, and less with the unsaturated fatty acids or cholesterol in the LDL, since they demand a higher concentration of HOCl and a longer incubation time.[20] To further investigate the type of alterations, which may occur to our designed probe, LTG, was incubated with HOCl (at 0.2 or 0.4 mM concentrations) in HBSS solution. The remaining unreacted marker and its oxidized derivatives were then extracted and analyzed in the LC/MS/MS, as previously described. Seven different Ox-LTGs resulted and were identified. The ones that were predominantly formed were mono-chlorinated tyrosine subunits (**6**), eluted at 7.7 min (10% or 24.5% of LT(Cl)G/total LTG with 0.2 or 0.4 mM HOCl concentrations, respectively). The identification of the LT(Cl)G was based on an *m*/*z* of 726, with an adjacent mass of 728 *m*/*z*, and a ratio of about 3:1, indicating the presence of chlorine atoms (3:1, isotopic ratio) and the daughters, with *m*/*z* of 266. This showing that there was no reaction on the 2′-dG moiety. The fragments, 442.7 and 180 *m*/*z* (LT and Ty, respectively), were absent, but new masses were present, with *m*/*z* of 476.5 [442.7(−1)+35.5(Cl)] and 214.5 (Ty (−1)+35.5(Cl) mass units). Additional minor oxidized products were detected when HOCl was the inducer. They were similar to the type of products obtained when copper ions were the inducers, for example, epoxidation of the linoleic acid (**3**) (eluting time 3.5 min, 1% or 3% epoxy-LTG/total LTG at 0.2 or 0.4 mM, respectively), oxidation of the 2′-dG to 8-oxo-dG (**2**), and formation of hydroperoxide on the linoleic acid, HOO-LTG (**4**).

Since oxidation of 2′-dG into 8-oxo-dG is known to occur also spontaneously, such as during workouts, we also conducted a control experiment in which the marker, LTG, was incubated in HBSS solution under similar conditions, as with the samples treated with HOCl. Indeed, a level of 0.5% ± 0.2% of 8-oxo-dG/total LTG was detected, in addition to 0.4% ± 0.1% epoxidation of the linoleic acid (epoxy-LTG/total LTG), which probably occurred during workout. If no inducer was used, 8-oxo-dG and epoxy-LTG were the only two products that could be discerned. These results imply, that under the above in vitro conditions, the 2′-dG subunit of the LTG is stable in the presence of HOCl and that most of the 8-oxo-dG observed, with HOCl as the inducer, were formed during the workout. These results are in contrast to the reaction of LTG with copper ions, in which case, 8-oxo-dG was the predominant molecule formed. Additional minor oxidation products of the marker included molecules with epoxidation of the linoleic acid together with chlorination of the tyrosine (**7**), based on its mass, 742 *m*/*z*, and its daughter fragments with *m*/*z* of 266 (free 2′-dG), 214.5 (Ty+Cl), and 492.5 [TL (442−1)+Cl+16].

In order to identify the exact position of the linoleic acid double bond epoxidation, further fragmentation of the mass, 492.5 *m*/*z*, was necessary (MS/MS). Two additional oxidized products of the HOCl induction were HOCl (**8**) bound to linoleic acid (eluted at 4.4 min) with 0.1% HOCL–LTG/total LTG, a mass of 744 and a daughter mass of 494.7 *m*/*z* (LT (442)+HOCl), in the presence of fragments with *m*/*z* of 266 (free 2′-dG) and 180 (Ty). Finally, based on similar considerations, a product containing epoxidation of the linoleic acid together with HOCl was also identified (eluted at 8.1 min) (**9**). These results demonstrate, that as opposed to incubation of LTG with copper ions, incubation of LTG with HOCl as the inducer causes the reaction with LTG to proceed preferentially on the tyrosine aromatic ring, yielding substituted chloro-tyrosine. This is in agreement with our previous findings, in which the reaction of HOCl with tyrosine alone or in a mixture of tyrosine with linoleic acid and cholesterol yielded preferentially 3-Cl-tyrosine and 3,5-diCl-tyrosine, suggesting that tyrosine is more susceptible to HOCl, than are linoleic acid or cholesterol. These results are also in accordance with others, showing that the LDL's-lipid components do not compete as effectively as the LDL protein[21] does for HOCl.

We may conclude that the exposure of the designed probe to different ROS yielded different products, depending on the type of ROS present and depending on the reaction mixture and conditions. The use of LTG constructed from LA, Ty, and 2′-dG may enable us to distinguish each of them and their oxidized products from the endogenous LA, Ty, and 2′-dG, and to determine the time and the kinetics of their formation. The proposed concept, in which an exogenous probe is constructed from the main endogenous OS susceptible molecules, yielding products specific to the type of ROS/RNS, may be useful for the identification and characterization of OS, and the pathological diseases associated with it. Recently, in a preliminary experiment in our group, such probes were utilized in cells that were exposed to OS,[16] and to astrocyte and Alzheimer-related diseases.[17] In addition, OS was characterized in blood of diabetic patients versus healthy subjects, and the effect of the supplementation of pomegranate juice on the oxido/redox balance in the blood of these diabetic patients was also explored (unpublished data).

3. Materials and methods

Chemicals and reagents were purchased from Sigma. Solvents used were of HPLC grade. LT was synthesized, its structure and the structure of all other oxidized LT (Ox-LT) were elucidated and described in details in Szuchman et al.[16] 2′-deoxyguanosine(dG) was purchased from Sigma.

3.1. Synthesis of linoleoyl tyrosine 2′-deoxyguanosyl ester (LTG)

tert-Butyldimethylsilyloxy linoleoyl tyrosine: a solution of imidazol (140 mg, 2.1 mmol in 1 ml dry dimethyl formamide (DMF) was added dropwise to a solution of LT (90 mg, 0.2 mmol in 2 ml dry DMF) at 0 °C. After the mixture was stirred for 10 min, a solution of *tert*-

butyldimethylsilyl chloride (TBDMSCl) (220 mg, 1.5 mmol in 2 ml dry DMF) was added to it dropwise, followed by incubation overnight under nitrogen at room temperature. Afterwards, 5 ml of an aqueous solution of 10% K_2CO_3 was added to the mixture, which was stirred for 30 min. Following the addition of a solution of 40% H_3PO_4 to achieve pH 3.0, the mixture was extracted with three portions (20 ml each) of diethyl ether. The collected organic solvent was evaporated to dryness, and the residue purified by flash chromatography (silica, methanol:dichloromethane 10:90), affording a pure compound (HPLC) of *tert*-butyldimethylsilyloxy linoleoyl tyrosine (105 mg, 0.19 mmol) with a 94% yield, and an *m*/*z* of 557 (M−1)) in the MS, using electrospray negative ions (ES^-) and an IR (neat) ν_{max} of 3390, 2956, 1716, 1651, 1510 and 1455 cm^{-1}.

3.2. Esterification with 2′-deoxyguanosine(dG)[22]

dG (100 mg, 0.35 mmol), 1-hydroxybenzotriazole (70 mg, 0.5 mmol), and triethylamine (100 μl, 1.36 mmol) were dissolved in 5 ml DMF in a N_2 atmosphere. To the solution were added *tert*-butyldimethylsilyloxy tyrosine linoleate (150 mg, 0.27 mmol) dissolved in 2 ml tetrahydrofuran (THF) and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) (300 μl, 2.2 mmol). The resulting solution was stirred at 50 °C overnight under a N_2 atmosphere. Tetrabutylammonium fluoride hydrate (TBAF) (500 μl of 1 M solution in THF) was added to cleave the *tert*-butyldimethylsilyl protecting group, and the resulting solution was stirred at room temperature for 30 min. After the addition of $NaHCO_3$ (20 ml of a 5% solution in water), the product was extracted by three portions (30 ml each) of ethyl acetate. The organic solvent was evaporated to dryness, and the residue was purified by a preparative HPLC column (reverse phase column with acetonitrile:DDW as eluents) to yield 40 mg of LTG (22% yield). Following the injection of the pure compound (Fig. 1) into the LC/MS, a spectrum of the product (Fig. 2a) with a molecular ion of 691.6 *m*/*z* (ES^-) and fragmentations of 442.7 *m*/*z*; 266.4, 180.2, and 150.1 *m*/*z* (Fig. 2a), with the characteristic UV–vis spectrum (Fig. 2c), was obtained.

^{1}H-NMR: $\delta(CD_3OD)$: 7.75 (1H, d, $J = 4.50$ Hz); 6.90 (2H, dd, $J = 7.2$, and 3.90 Hz); 6.56 (2H, dd, $J = 8.4$ and 2.70 Hz); 6.13 (1H, t, $J = 6.6$ Hz); 5.24 (4H, m); 4.44 (1H, t-br, $J = 9.0$ Hz); 4.23 (1H, m); 3.99 (1H, m); 3.13 (2H, t, $J = 5.1$ Hz), 3.02 (3H, q, $J = 7.2$ Hz); 2.67 (3H, t, $J = 5.1$ Hz); 2.32 (2H, t, $J = 7.5$); 1.93 (6H, m); 1.54 (2H, m); 1.19 (14H, m), 0.78 (3H, t, $J = 6.6$ Hz).

3.3. Analysis of oxidized LTG products (Ox-LTG) by LC/MS/MS

The LC/MS was equipped with an HPLC (Waters 2790) that had a Waters photodiode array detector (model 996) connected to MS (Micromass Quattro Ultima MS, UK). The HPLC column was a 3.5 μm C18 ODS XTerra (Waters, MA) 4.6 * 20 mm. The eluents were a gradient of solution A (acetonitrile) and solution B (DDW) as follows: beginning with 40% A; followed by a change to 60% A for 2 min; and finally to 80% A for 10 min. Afterwards, the column was washed with a solution of 98% A for an additional 5 min. MS/MS analysis of the oxidized products was performed in scan and daughter modes, using ES^-. The source temperature of the MS was set at 150 °C, with a cone gas flow of 22 l/h and a desolvation gas flow of 600 l/h. Peak spectra were monitored between 30 and 800 *m*/*z*. Collision-induced dissociation MS was performed, with a collision energy of 30–35 eV and 3–3.5 kV capillary voltage. Multiple-reaction-monitoring (MRM) was performed under the same conditions as used to predict the Ox-LTG percent. A calibration curve of the LTG marker was run in each analysis.

3.4. Copper ion-induced oxidation of LTG

The LTG marker (30 μl from a stock solution of 1000 ppm dissolved in ethanol) was dissolved in 1 ml water to which $CuSO_4$ (20 μM) was added. The mixture was stirred for 4 h at 37 °C, followed by extraction with three portions of ethyl acetate (2 ml each). The collected organic layer was evaporated under vacuum to dryness, and the residue was dissolved in ethanol (300 μl) for further analysis in LC/MS/MS.

3.5. HOCl-induced oxidation of LTG

The LTG probe (40 μM) was dissolved in a 1 ml HBSS solution (28 μl of LTG stock solution in ethanol −1000 ppm) and incubated with HOCl, either at a concentration of 0.2 or 0.4 mM (14 or 28 μl of HOCl from stock solution of 14 mM NaOCl dissolved in HBSS). The original Aldrich Stock of NaOCl was diluted with 0.1 mM NaOH, and the concentration was determined spectrophotometrically ($\varepsilon_{292} = 350\ M^{-1}cm^{-1}$). The mixture was stirred for 0.5 h at 37 °C, followed by extraction with three portions (2 ml each) of ethyl acetate. The organic layer collected was evaporated under vacuum, and the residue was dissolved in 300 μl of ethanol for further analysis in the LC/MS/MS.

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