ORIGINAL ARTICLE

A one-electron oxidation of carcinogenic non-aminoazo dye Sudan I by horseradish peroxidase

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Abstract

OBJECTIVES: The aim of the study was to examine oxidation of carcinogenic Sudan I by peroxidase and characterize the structure of its two major peroxidase-mediated metabolites. Another target of the study was to evaluate a mechanism of this oxidation.

METHODS: Thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) with ultraviolet (UV) and visible (VIS) detection was employed for the separation of Sudan I metabolites formed by peroxidase. UV/VIS-, and mass- spectroscopy as well as nuclear magnetic resonance (NMR) were used to characterize structures of two major Sudan I metabolites.

RESULTS: Peroxidase oxidizes Sudan I by a one electron oxidation to eight products. Two major Sudan I metabolites were isolated by TLC on silica gel and HPLC and structurally characterized. The major product formed during the Sudan I oxidation by peroxidase is Sudan I metabolite M_2, which corresponds to a Sudan I dimer molecule. The second major metabolite (M_1) is the product of secondary, enzyme independent reactions, being formed from the Sudan I dimer that lost the benzenediazonium moiety.

CONCLUSIONS: The data are the first report on structural characterization of Sudan I metabolites formed by its oxidation with peroxidase.

Abbreviations

APCI	- atmospheric pressure chemical ionization
BDI	- benzenediazonium ion
CYP	- cytochrome P450
ESI	- electrospray-ionization;
HPLC	- high performance liquid chromatography
HRP	- horseradish peroxidase
M	- mol/liter
NMR	- nuclear magnetic resonance
ppm	- parts per million
TLC	- thin layer chromatography
U	- units
UV	- ultraviolet
VIS	- visible
v/v	- volume in volume.

INTRODUCTION

Sudan I [1-(phenylazo)-2-naphthol, C.I. Solvent Yellow 14] was used as a food coloring in several countries [2], but it was not recommended to be safe, because it causes tumors in the liver and/or the urinary bladder in rats, mice, and rabbits, and is considered a possible carcinogen and mutagen for man [2,3,11–13]. Nevertheless, it is widely used to color materials such as hydrocarbon solvents, oils, fats, waxes, plastics, printing inks, and shoe and floor polishes [2,3]. Such a wide use of these azo dyes could result in a considerable exposure. While the metabolism of Sudan I is not understood in humans, its metabolism has been characterized in rabbits [2], where it is metabolized primarily in the liver by oxidative or reductive reactions. Oxidation of Sudan I catalyzed by cytochrome P450 (CYP) enzymes [7,11–13] and peroxidases [4–6,8–10], was investigated and helped us to resolve its carcinogenic mechanism [12]. C-Hydroxylated metabolites 4'-OH-Sudan I and 6-OH-Sudan I were found to be the major products of Sudan I oxidation *in vivo* (excreted in urine) [2], and of its oxidation by rat and human hepatic microsomal CYP *in vitro* [7,11–13]. Besides the C-hydroxylated metabolites, which are considered detoxication products, the benzenediazonium ion (BDI) formed by enzymatic splitting of the azo group of Sudan I was found to react with DNA *in vitro* [4,7,11–13] (Figure 1). The major DNA adduct formed in this reaction has been characterized and identified as the 8-(phenylazo)guanine adduct [7]. This adduct was also found in liver DNA of rats exposed to Sudan I [13]. Sudan I and its C-hydroxylated metabolites are also oxidized by peroxidases, as a consequence DNA, RNA and protein adducts are formed [4–6,8–10] (Figure 1). While CYPs were found to be responsible for the activation of Sudan I in human or animal liver, their role in the *in vivo* metabolic activation of Sudan I in the urinary bladder is limited. This organ has little or no detectable CYP, however, peroxidases are present at relatively high levels in this tissue. In the case of peroxidase, the Sudan I metabolites formed by this enzyme have not been identified as yet. Therefore, the aim of the present work was to isolate two major Sudan I peroxidase-mediated metabolites and characterize them by mass and/or NMR spectroscopy.

MATERIAL AND METHODS

Incubation mixtures contained the following in a final volume of 70 ml: 10 mM sodium phosphate buffer (pH 8.4), 0.5 μM horseradish peroxidase (HRP), 100 μM Sudan I dissolved in methanol and 200 μM hydrogen peroxide. After incubation (37 °C, 20 min) the mixtures were extracted with ethyl acetate (2 × 15 ml). The extracts were evaporated, dissolved in a methanol, and separated by TLC or HPLC. Silica-gel TLC plates were developed in hexane-diethyl ether (1:3, v/v). Spots of Sudan I metabolites M_1 and M_2 with relative mobility of 0.214 and 0.193, respectively, were extracted with methanol. Alternatively, the products were separated by HPLC on a Tessek Separon Hema S 1000 (8.0 × 250 mm) C-18 column. Gradient elution (75–100% methanol) with a flow rate of 0.3–1.5 ml/min was used. Sudan I metabolites were detected at 215, 254, 333 and 480 nm. Two product peaks with r.t. of 32 and 46 min were collected and analyzed by mass spectroscopy. Spectra were measured using Esquire 3000 Bruker Daltonics [atmospheric pressure chemical ionization (APCI), electrospray-ionization (ESI) – positive and negative ionization][1]. The mass spectra were internally calibrated using background ions with known elemental composition.

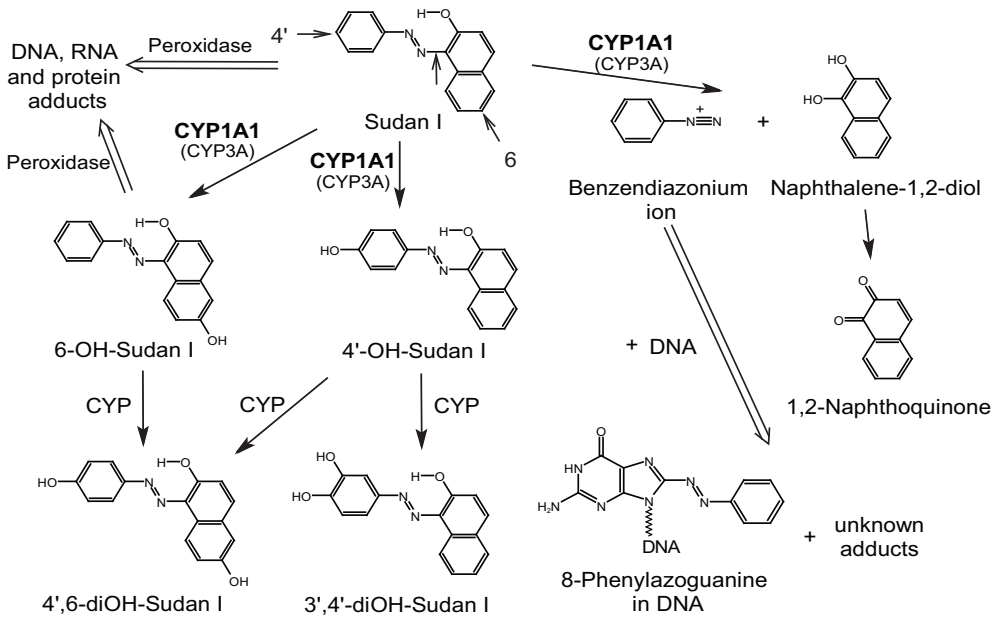

Figure 1. Scheme of Sudan I metabolism

NMR spectra of the metabolite M_1 (δ, ppm; *J*, Hz) were measured on a Bruker Avance II-600 instrument (600.1 MHz for ^1H and 150.9 MHz for ^{13}C) in hexadeuterated dimethyl sulfoxide and referenced to the solvent signal (δ 2.50 and 39.7, respectively). ^{13}C NMR (DMSO, 150.92MHz): 83.65 (C-1′); 108.15 (C-3); 121.83 (C-8); 122.61 (C-2′′); 123.03 (C-3′); 125.71 (C-5); 126.60 (C-4′′); 129.03 (C-3′′); 129.20 (C-5′); 129.51 (C-4a); 129.98 (C-1); 130.02 (C-8′); 130.88 (C-6′); 131.05 (C-4′a); 131.57 (C-7′); 131.65 (C-8a); 131.92 (C-6); 132.95 (C-7); 135.25 (C-8′a); 142.84 (C-1′′); 147.18 (C-4′); 149.09 (C-2); 183.96 (C-4); 191.46 (C-2′). ^1H NMR (DMSO, 600.13MHz): 6.17 s, 1 H (H-3); 6.30 d, 1 H, *J*(3′,2′) = 10.0 (H-3′); 6.91 m, 2 H (H-2′′); 7.15 m, 1 H (H-4′′); 7.23 m, 2 H (H-3′′); 7.52 – 7.62 m, 4 H (H-6, H-5′, H-6′ and H-7′); 7.73 m, 1 H (H-7); 7.76 d, 1 H, *J*(4′,3′) = 10.0 (H-4′); 7.86 m, 1 H (H-8′); 8.03 m, 1 H (H-5); 8.13 m, 1 H (H-8).

RESULTS

Peroxidase in the presence of hydrogen peroxide is able to oxidize Sudan I. Depending on time of incubation, the absorption spectrum of the reaction mixture containing Sudan I, peroxidase and H_2O_2 was significantly changed (Figure 2). During the reaction, the absorption maximum at 480 nm (due to Sudan I) decreases, whiles the absorbance at about 340 nm increases slightly (Figure 2). In the former studies, we identified that the products formed during peroxidase-mediated Sudan I oxidation includes BDI and C-hydroxy derivatives [6-OH-Sudan I and 4',6-di(OH)-Sudan I] [4]. But major metabolites are unstable (sensitive to light and elevated temperature) [4,6,8] and their structures have not been elucidated as yet. In the present paper we use two separation procedures (TLC and HPLC) to obtain individual Sudan I metabolites in purity sufficient for their characterization. Using TLC several Sudan I metabolites were separated, two of them, M_1 and M_2, were the major ones (Figure 3). HPLC utilizing a Tessek Separon Hema S 1000 C-18 column was originally developed here and used for further purification of the two major Sudan I oxidation products (M_1 and M_2, Figure 4). These products were characterized by their UV/VIS (data not shown) and mass spectra (Figure 5). Sudan I metabolite M_1 exhibits absorption peak between 430 and 480 nm. Metabolite M_2 exhibits absorption maximum at 480 nm, which is typical for the whole molecule of Sudan I (see Figure 2).

The metabolite M_2 was analyzed by mass spectroscopy, both in positive and negative mode using low-resolution ion trap instrument. M_2 provided singly charged molecular adduct $[M+H]^+$ at m/z 495.1 (Figure 5) and deprotonated molecule $[M-H]^-$ at m/z 493.0 (Figure 5). Therefore, nominal mass of the molecule is 494. The fragment peak at *m/z* 495 in the metabolite M_2 (Figure 5) indicates the ion composed of two Sudan I molecules. Moreover, fragmentation peaks at *m/z* 159, 172, 247, 389 (391) and 417 (Figure 5) seem to correspond to decomposition of the Sudan I dimer molecule of the structure shown in Figure 6. Further fragmentation experiments were not performed because of the sample instability. The metabolite M_2 reacts spontaneously (without enzymatic reaction) to the metabolite M_1. This feature was the reason that M_2 could not be isolated in quantities sufficient for its NMR characterization.

The metabolite M_1 provided singly charged molecular adduct $[M+H]^+$ at m/z 405.1 (Figure 5), the calculated elemental composition $C_{26}H_{17}N_2O_3$ was in agreement with the assumed structure shown in Figure 6. In ^1H NMR spectrum we observed one singlet at 6.17 ppm, two doublets with coupling constant 10.0 Hz, a set of signals of N-substituted aniline and two sets of signals of *ortho*-disubstituted benzene derivatives. No exchangeable protons were detected by addition of a drop of D_2O to the sample. The most interesting signals in ^{13}C NMR spectra were two carbonyl carbons (at 191.5 and 184.0 ppm) and a qurternary carbon atom at 83.7 ppm. The structure of M_1 was elucidated from 2D NMR spectra (H,H-COSY, H,C-HSQC a H,C-HMBC). Two naphthalene fragments and one N-substituted aniline were recognized in the molecule. By combination of 1D and 2D NMR techniques ^1H and ^{13}C chemical shifts were completely assigned. The compound M_1 is a spiro compound with one chiral center (quarternary carbon 1′, Figure 6). The metabolite M_1 should be a racemic mixture of both enantiomers. This was approved by adding of a chiral NMR shift reagent (–)-2,2,2-trifluoro-1-(9-anthryl)-ethanol (the signals split into two sets corresponding to the two antipods).

DISCUSSION

Sudan I is an azo dye containing a free hydroxyl group in its molecule. Many phenolic compounds can serve as substrates for peroxidases, being oxidized to phenoxyl radicals, which will undergo secondary reactions in dependence on their individual free radicals chemistries. This mechanism was also found for Sudan I as a substrate of peroxidase previously [5,6,8] and in the present work; peroxidase oxidizes this carcinogen, giving rise to an oxygen-centered radical (naphthoxyl radical) [6,8]. Plant peroxidase, HRP, was used in these studies. Both mammalian and plant peroxidases catalyze oxidation of many substrates with similar mechanisms, having analogous arrangement of the active sites [4]. Hence, HRP is an acceptable model for the mammalian enzymes such as cyclooxygenases, which are expressed in the urinary bladder, the target tissue for the Sudan I carcinogenicity [2,4,8–10]. From the results found in the present study we can suggest the proposed mechanism of one-electron Sudan I oxidation by peroxidase. First, peroxidase forms a Sudan I radical found previously [5] by homolytic OH bond cleavage. This radical reacts with another Sudan I molecule to form aliphatic azo compound, the metabolite M_2.

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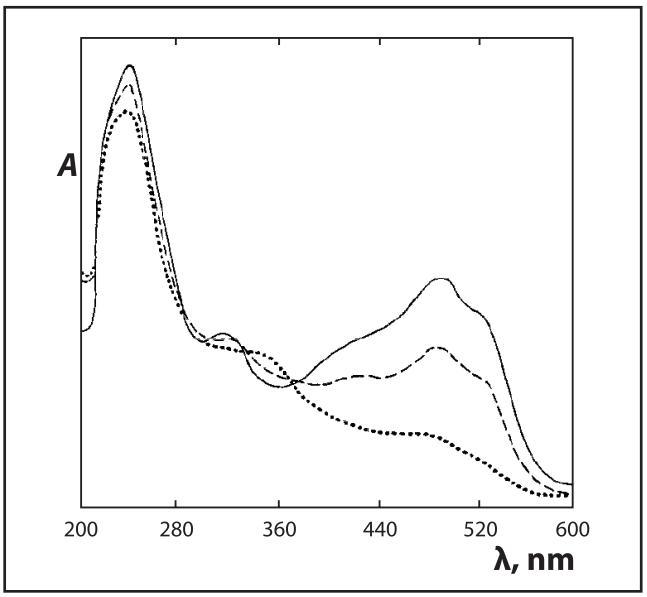

Figure 2. Oxidation of Sudan I by the peroxidase/H_2O_2 system. The samples (1 ml) contained 50 mM Na phospahte buffer pH 8.4, 0.2 mg horseradish peroxidase, 0.15 mM Sudan I, and 0.5 mM H_2O_2. The spectra were recorded sequentially at 0 min (—), 2.5 min (– – –) and 10 min (• • •).

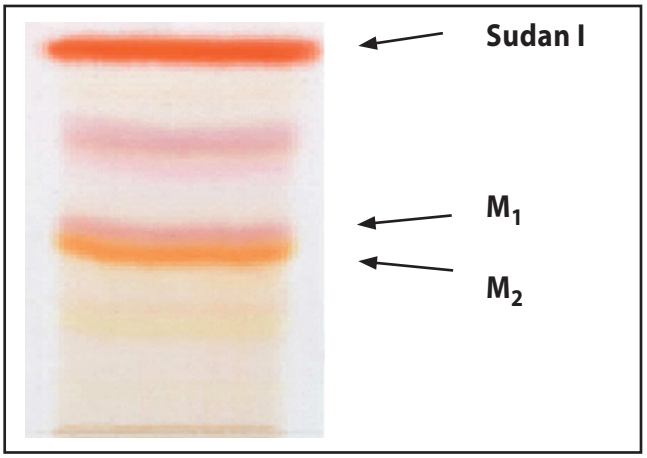

Figure 3. TLC of Sudan I metabolites formed by peroxidase (the major metabolites are assigned as M_1 and M_2)

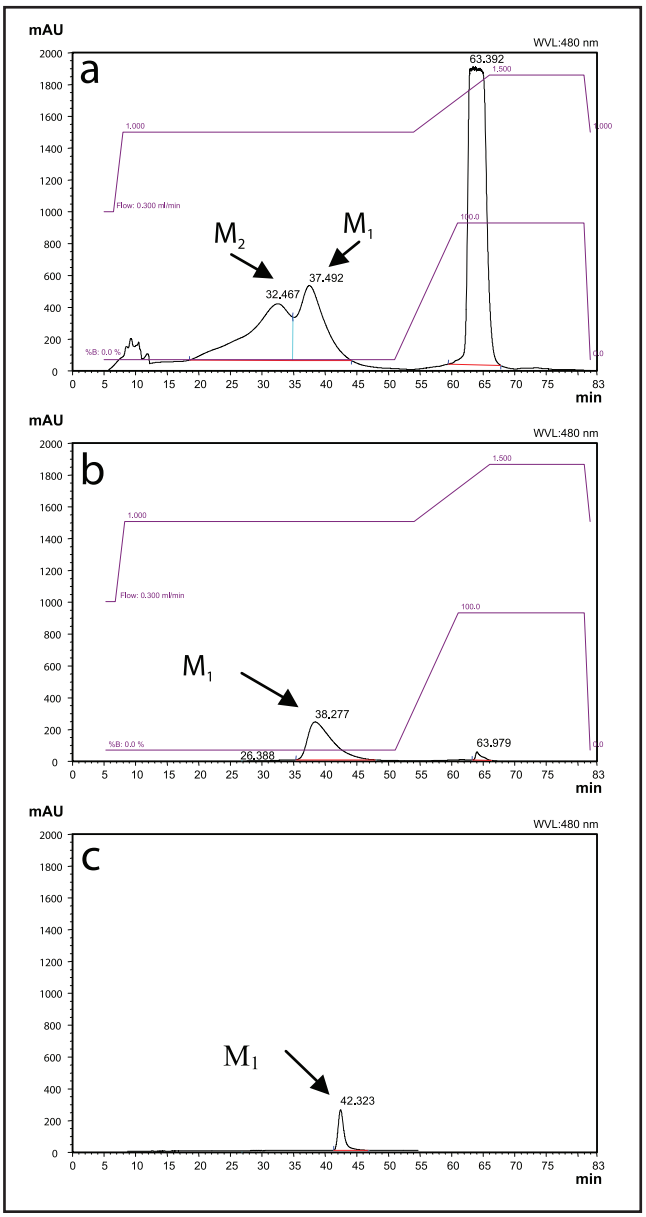

Figure 4. HPLC of Sudan I metabolites formed by peroxidase (a) and their HPLC re-chromatography (b, c)

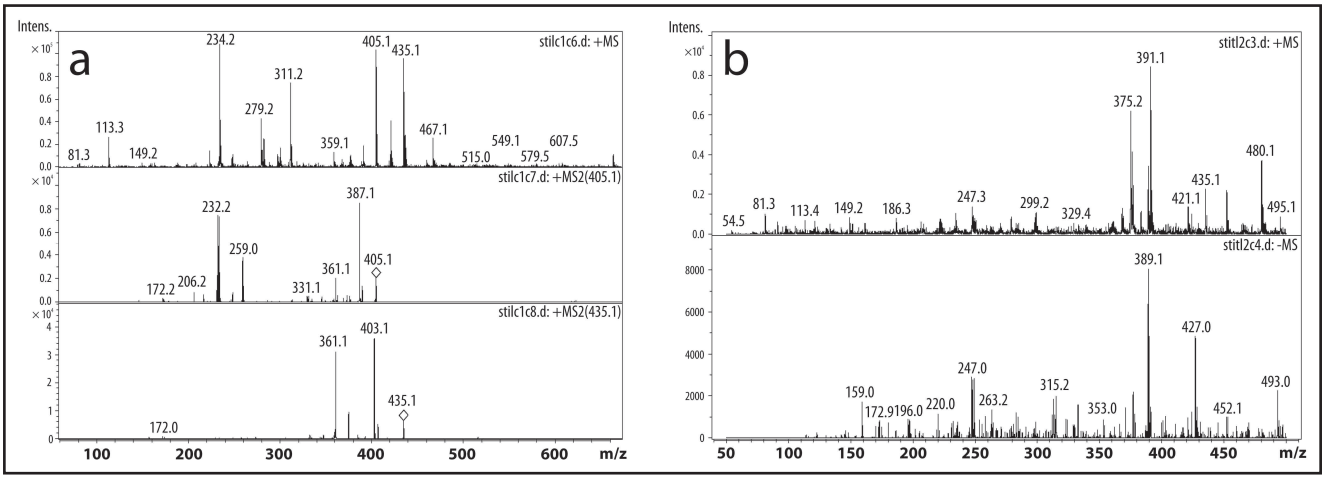

Figure 5. Mass spectra (APCI) of Sudan I metabolites M_1 (a) and M_2 (b)

This metabolite is probable structure shown in Figure 6, but due to its instability the structure could not be confirmed by NMR spectroscopy. However, the structure of M_2 evaluated from mass spectroscopy was further confirmed after the structural identification of the metabolite M_1, which is the product of M_2 conversion. Aliphatic azo compounds are instable and they are easily subjected to homolytic cleavage of C-N bond by formation of two radicals. The metabolite M_2 is cleaved to the benzenediazonium ion, whose formation was identified previously [4]. The residual molecule of Sudan I dimer (the metabolite M_2) without benzenediazonium reacts intramolecularly and, after oxidation with hydrogen peroxide or oxygen (present in the reaction mixture), forms the metabolite M_1 (Figure 6). The results of the present and previous studies [4–6,8–10] suggest that one-electron oxidation products (radicals) are the primary intermediates in the peroxidase-mediated oxidation of Sudan I. The fate of the primary free radical depends on the environment in which it exists. We found that the Sudan I reactive free radicals: (i) form additional products [present paper]; (ii) react with other compounds of potential physiological interest (reaction with NADH, ascorbate) [9,10], (iii) react with SH groups of glutathione (reducing Sudan I radicals with the formation of a thiyl radical) [10] and (iv) react with macromolecules (DNA, RNA, proteins) to form potentially toxic adducts *in vitro* and *in vivo*[4,5,8–10]. Structure elucidation of the Sudan I-(deoxy)guanosine-adducts found in DNA [10] and RNA [5,9] is under way in our laboratory.

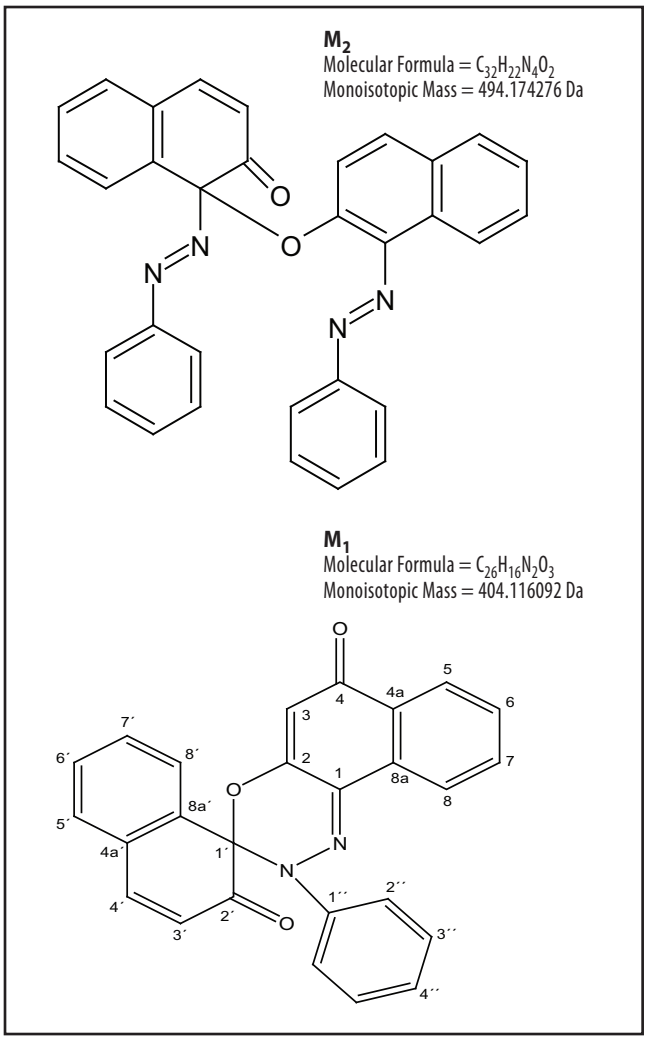

Figure 6. Sudan I metabolites M_2 and M_1

ACKNOWLEDGMENT

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