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Microaerobic steroid biosynthesis and the molecular fossil record of Archean life

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The power of molecular oxygen to drive many crucial biogeochemical processes, from cellular respiration to rock weathering, makes reconstructing the history of its production and accumulation a first-order question for understanding Earth’s evolution. Among the various geochemical proxies for the presence of O2 in the environment, molecular fossils offer a unique record of O2 where it was first produced and consumed by biology: in sunlit aquatic habitats. As steroid biosynthesis requires molecular oxygen, fossil steranes have been used to draw inferences about aerobic processes in the early Precambrian. However, better quantitative constraints on the O2 requirement of this biochemistry would clarify the implications of these molecular fossils for environmental conditions at the time of their production. Here we demonstrate that steroid biosynthesis is a microaerobic process, enabled by dissolved O2 concentrations in the nanomolar range. We present evidence that microaerobic marine environments (where steroid biosynthesis was possible) could have been widespread and persistent for long periods of time prior to the earliest geologic and isotopic evidence for atmospheric O2. In the late Archean, molecular oxygen likely cycled as a biogenic trace gas, much as compounds such as dimethylsulfide do today.

Oxygen photosynthesis, in which water is split to provide reducing equivalents for carbon fixation, is the source of nearly all molecular oxygen on Earth. In developing the ability to produce O2, life introduced a powerful chemical agent into the earth system and accelerated the biogenic oxidation of the surface environment. This biochemistry was apparently invented exactly once, by an ancestor of the cyanobacteria, but the specifics of how and when oxygenic photosynthesis came to be remain the subject of active investigation and debate (1–4).

The geologic record provides a minimum age for oxygenic photosynthesis. An ensemble of geological and geochemical evidence for atmospheric oxygenation, which has come to be known as the “Great Oxidation Event” (GOE), points to the period ca. 2.32–2.45 Ga as the time when O2 became a persistent and geochemically significant component of the atmosphere, and prior to which atmospheric oxygen was less than 1 ppmv (4–9). Thus 2.32 Ga stands as a minimum age by which oxygenic photosynthesis must have arisen. Recently, geochemical evidence has been presented for the transient or localized presence of O2 as much as 300 million years before the GOE (9–14), which suggests that the evolutionary origin of oxygenic photosynthesis may have significantly predated 2.32 Ga. This geologic record has given rise to divergent interpretations of the relative timing of the origin of oxygenic photosynthesis and the oxygenation of the atmosphere: Either (i) the GOE records both the evolutionary origin of oxygenic photosynthesis and resultant rapid atmospheric oxygenation, and apparent indicators of earlier O2 production have been erroneously interpreted (15, 16), or (ii) the origin of oxygenic photosynthesis predates the GOE by at least several hundred million years, with the time gap between the two events reflecting the variety of geochemical sinks and buffers that had to be overcome before O2 could accumulate in the atmosphere (17, 18).

Models of Archean atmospheric evolution have shown how a biogenic O2 flux to the atmosphere could have persisted for hundreds of millions of years without causing oxygenation to an extent that would “trip” the geologic and geochemical proxies whose signals appear at or near the GOE (19–22). So long as the O2 is accompanied by sufficient inputs of appropriate reductants (such as methane), atmospheric consumption of O2 is rapid and there is no particular threshold value of the biogenic O2 flux that forces the atmosphere to become oxygenated. The history of O2 accumulation in the atmosphere is thus sensitive to variation over time in the balance between sources (oxygenic photosynthesis) and sinks (volcanic and biogenic reduced gases) of oxidizing power to the atmosphere.

The geologic and geochemical proxies that have most clearly defined the GOE are sensitive to the involvement of O2 in weathering processes, sedimentation, and/or atmospheric chemistry and so reflect this net balance between O2 sources and sinks. These proxies include occurrences of oxidized paleosols, detrital redox-sensitive minerals, and the mass-independent fractionation of S isotopes, as well as indices of the mobility, speciation, and/or isotopic fractionation of redox-sensitive metals (7–13, 23, 24). For signals to be recorded through these processes, molecular oxygen must have already accumulated in the atmosphere to some extent and persisted there for a geologically significant period of time. These geologic and geochemical proxies are hence somewhat removed—separated by considerations of transport and source–sink balances—from the biology of O2 production.

Molecular Fossils and Oxygen Constraints

Hydrocarbon molecular fossils (biomarkers) have provided some of the earliest and most direct evidence for the biological production and utilization of molecular oxygen (25–30). Molecular fossils deriving from microbial membrane lipids have been found in sedimentary deposits dating as far back as 400 million years before the GOE, including in samples with the strictest contamination controls (30). Although the challenges involved in their analysis and interpretation are considerable (31, 32), authigenic molecular fossils from Archean sedimentary rocks provide a unique record of O2 where it was first produced, accumulated, and utilized: sunlit aquatic ecosystems. As diagenetically altered yet still-identifiable biochemicals, biomarkers offer a proxy for O2 cycling that is intimately tied to biological activity. Here our goal is to refine the inferences concerning environmental oxygenation that can be made from steranes, which are molecular fossils of
steroids and have been detected in sedimentary rocks as old as 2.72 Ga.

Fossilized steroids have been especially significant as proxies for oxygenation, because their biosynthesis specifically requires molecular oxygen (Fig. 1). There are at least four steps in the biosynthesis of steroids that require \( \text{O}_2 \). The first \( \text{O}_2 \)-dependent step is also the first committed step in steroid synthesis: the epoxidation of the linear isoprenoid squalene to produce 2,3-oxidosqualene. The enzyme that cyclizes oxidosqualene to form the characteristic steroidal 6,6,6,5-ring structure cannot act on squalene, but requires squalene (3S) 2,3-epoxide (33, 34).

Subsequently, to produce the 4,14-desmethyl steroids that make up the vast majority of both membrane lipids and molecular fossils, nine more molecules of \( \text{O}_2 \) are necessary to effect three oxidative demethylation reactions. The synthesis of some steroids (e.g., cholesterol and ergosterol) requires yet more \( \text{O}_2 \) to introduce unsaturations into the carbon skeleton. These postcyclization modifications involve the chemically challenging activation of unreactive C-H bonds, in which \( \text{O}_2 \) plays an important role (35). Furthermore, these principal steps in steroid biosynthesis are conserved across some of the deepest phylogenetic divisions in the eukaryotic domain, suggesting that a version of the \( \text{O}_2 \)-dependent pathway was present in the last common ancestral lineage of all extant eukaryotes (34, 36). Although a hypothetical anaerobic route to steroids has been discussed (37), there is no evidence that such a pathway exists today or did so in the past, and operation of the aerobic steroid biosynthesis pathway remains the most plausible explanation for the presence of fossil steranes in the geologic record (34).

The presence of steranes in late Archean rocks implies that some molecular oxygen was available in marine environments that produced the organic matter preserved in those sediments, but the implied degree of environmental oxygenation remains unclear. In order to leave a fossil record, \( \text{O}_2 \) levels must have been sufficient for steroid biosynthesis. To date, constraints on the concentration of dissolved \( \text{O}_2 \) that enables steroid biosynthesis are limited to three reports: Jahnke and Klein (38) reported an apparent oxygen \( \text{K}_\text{m} \) (half-saturation content) for yeast squalene epoxidase of 4.3 \( \mu \text{M} \), and Rogers and Stewart reported apparent \( \text{O}_2 \text{K}_\text{m} \) values for cellular ergosterol contents in yeast of 0.5 \( \mu \text{M} \) (39) and “0.3 \( \mu \text{M} \) or less” (40). These reports, although suggestive of de novo steroid production, have not clearly documented the operation of the complete biosynthetic pathway under microaerobic conditions (here used to designate the range from approximately 1 nM to 1 \( \mu \text{M} \) dissolved \( \text{O}_2 \)), which is a key link to interpretation of the molecular fossil record. The experiments presented here seek to establish whether steroid biosynthesis can proceed at such low dissolved \( \text{O}_2 \) concentrations, and thereby to more quantitatively assess consistency between the molecular fossil record and other geochemical proxies for oxygenation and models of atmospheric evolution.

To directly assay the \( \text{O}_2 \) requirements of steroid biosynthesis, we adopted an isotopic labeling strategy. Yeast (\textit{Saccharomyces cerevisiae}) was chosen as a test organism because it is a facultatively anaerobic eukaryote, able to grow at any \( \text{O}_2 \) concentration in an appropriate medium. Although \textit{S. cerevisiae} is likely only distantly related to, and certainly incompletely representative of, eukaryotic life of the late Archean, the antiquity and conservation of the core of steroid biosynthesis across the eukaryotic domain make it a useful model. Yeast cells were grown in a defined minimal medium containing \( ^{13}\text{C}\)-glucose (99 atom\%) and unlabeled ergosterol (i.e., sterol with \( ^{13}\text{C} \) at the natural abundance of 1.1\%). Because \( \text{O}_2 \) is essential for steroid biosynthesis, the cells are obligately auxotrophic for steroids under anaerobic conditions and take up the supplied (unlabeled) ergosterol. When \( \text{O}_2 \) is supplied to the culture, however, steroids can be made de novo from carbon substrates and thereby acquire the \( ^{13}\text{C} \) label from glucose. We grew cells at three different dissolved \( \text{O}_2 \) concentrations: 6.5 \( \mu \text{M} \), 0.6 \( \mu \text{M} \), and 7 nM, as well as anaerobically (<0.7 nM); \( \text{O}_2 \) levels were controlled by bubbling with defined gas mixtures (5.030 ± 1.00, 473.0 ± 9.5, 5.32 ± 0.27, and <0.5 ppmv \( \text{O}_2 \) in \( \text{N}_2 \), respectively). By analyzing the cellular lipids using mass spectrometry, we could examine the incorporation of \( ^{13}\text{C} \) into specific compounds, including steroids, at the various \( \text{O}_2 \) levels.

Results

The Oxygen Requirements of Steroid Biosynthesis. The incorporation of the \( ^{13}\text{C} \) label from glucose produces a distinct shift in the appearance of lipid mass spectra (Fig. 2). Compounds that carry

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**Fig. 1.** A summary of the steroid biosynthetic pathway in yeast, showing the flow of carbon from glucose to ergosterol. The first oxygen-requiring step is the epoxidation of squalene; subsequently, \( \text{O}_2 \) is also required for the removal of three methyl groups from the steroidal ring system and the introduction of two unsaturations in order to produce the functional membrane lipid. In these experiments, cells can either synthesize steroids de novo (and hence become labeled with \( ^{13}\text{C} \)) or take up unlabeled ergosterol from the medium. Ultimately, steroid lipids may be incorporated into sedimentary organic matter and, after diagenetic alteration, observed as sterane molecular fossils.
abundant $^{13}$C show broad, multisisotopologue peaks in their mass spectra that are shifted to higher $m/z$ values relative to the natural abundance isotopic patterns of unlabeled compounds. These distinct spectral features provide an unambiguous signal of de novo lipid biosynthesis.

We found that the carbon-13 label is incorporated into squalene—the last steroid biosynthetic intermediate that can be produced in the absence of O$_2$—under all conditions (Fig. 3). Under anaerobic conditions (<0.7 nM O$_2$), no lanosterol is detected, and only unlabeled ergosterol (taken up from the medium) is present. This is the pattern expected if steroid synthesis is not operative and is consistent with steroid biosynthesis requiring molecular oxygen. Once O$_2$ is provided to the culture by bubbling, however, the observed labeling pattern changes. In the presence of O$_2$, lanosterol is synthesized and bears the $^{13}$C label, and labeled ergosterol is also present, demonstrating de novo steroid production. Steroid biosynthesis occurred at each of the dissolved labeled ergosterol is also present, demonstrating de novo steroid biosynthesis.

The lowest O$_2$ level yet observed to enable steroid production indicates that a modern organism can utilize such low concentrations of O$_2$—even at 7 nM (Fig. 3)—resulting from incorporation of $^{13}$C from labeled glucose, which produces multisisotopologue ion clusters shifted to masses higher than the unlabeled compound. This is the pattern expected if steroid synthesis is not operative and is consistent with steroid biosynthesis requiring molecular oxygen. Once O$_2$ is provided to the culture by bubbling, however, the observed labeling pattern changes. In the presence of O$_2$, lanosterol is synthesized and bears the $^{13}$C label, and labeled ergosterol is also present, demonstrating de novo steroid production. Steroid biosynthesis occurred at each of the dissolved labeled ergosterol is also present, demonstrating de novo steroid biosynthesis.

We interpret these results as a useful, perhaps even conservative, lower bound for the O$_2$ requirement for steroid synthesis. Although yeast can serve only as a partial model for the unknown diversity of eukaryotic organisms in the Archean ocean, the fact that a modern organism can utilize such low concentrations of O$_2$—even at 7 nM (Fig. 3)—resulting from incorporation of $^{13}$C from labeled glucose, which produces multisisotopologue ion clusters shifted to masses higher than the unlabeled compound. This is the pattern expected if steroid synthesis is not operative and is consistent with steroid biosynthesis requiring molecular oxygen. Once O$_2$ is provided to the culture by bubbling, however, the observed labeling pattern changes. In the presence of O$_2$, lanosterol is synthesized and bears the $^{13}$C label, and labeled ergosterol is also present, demonstrating de novo steroid production. Steroid biosynthesis occurred at each of the dissolved labeled ergosterol is also present, demonstrating de novo steroid biosynthesis.
Our calculations show that, to provide the O$_2$ outgassing fluxes described in these models, the average concentration in ocean regions contributing to the sea-to-air O$_2$ flux would be at least 40 nM, which is well above our experimental determination of the concentration required for steroid biosynthesis. Even if the total flux were far below the lower limit considered by Zahnle et al. (22), dissolved O$_2$ concentrations in higher-productivity hot spots (“oxygen oases”), would likely have been high enough to allow steroids to be made. Such hot spots probably included phototrophic microbial mats, which could have had daytime O$_2$ concentrations as high as 450–650 μM within the mat layers (46). The presence of microaerobic regions of the euphotic ocean by the late Archean, and their persistence over long periods of time, is consistent with geologic and geochemical evidence for an anoxic atmosphere at that time.

Discussion
We have demonstrated that steroid biosynthesis is enabled by dissolved O$_2$ concentrations in the nanomolar range and that the presence of such O$_2$ levels in the surface ocean long before 2.4 Ga is consistent with models of Archean atmospheric evolution. The molecular fossil record can thus be reconciled with geochemical proxies for the oxygenation state of the Archean surface environment, which together point to an atmosphere with <1 ppmv O$_2$, deep ocean waters that were often iron-rich (and therefore anaerobic), but a surface ocean that was, at least at some times and places, microaerobic. Regions of the surface ocean may have been some of the first environments on Earth to become oxygenated, and multiple lines of evidence (10–14, 29, 30) suggest that they did so hundreds of millions of years

Fig. 3. Mass spectra of lipids from microaerobic growth experiments. Compounds are indicated (top); dissolved O$_2$ concentrations in the various growth conditions are indicated (Left). Spectra are labeled by detection of unlabeled (natural isotopic abundance; 98.9% $^{12}$C) and/or isotopically labeled (multiple-$^{13}$C) versions of each lipid in each condition; ND, not detected. The anaerobic condition was in equilibrium with an atmosphere of <0.5 ppmv O$_2$, i.e., <0.7 nM dissolved O$_2$. Data are representative of at least two independent biological replicates for each culture condition.

Fig. 4. The modeled average dissolved oxygen concentration in oxygenated regions of the Archean surface ocean, as a function of the sea-to-air O$_2$ flux and the proportion of the Earth’s surface contributing to that flux. The larger the outgassing flux, and the smaller the fraction of the earth’s surface it comes from, the more oxygenated surface waters in that region must be. Yellow contours indicate the dissolved oxygen concentrations of the steroid biosynthesis experiments (7 nM, 0.6 μM, and 6.5 μM) and the detection limit of the oxygen microelectrode (0.1 μM). Red bars indicate the range of O$_2$ fluxes in the Archean atmospheric evolution models of Pavlov et al. (21) (PBK01) and Zahnle et al. (22) (ZCC06).
before O$_2$ began to accumulate in the atmosphere to any appreciable extent.

The exceptionally low O$_2$ levels that enable steroid synthesis also highlight the close connection between steroid biochemistry and O$_2$ sensing, metabolism, and defense (47,48). Studies of industrial fermentations have documented that, during aeration pulses, the first aerobic biochemistry to become active is steroid synthesis, even prior to respiration (49). In the experiments described here, de novo steroid synthesis occurred at nanomolar O$_2$ despite an abundant supply of exogenous steroids in the growth medium. It appears that steroid production is one of the most O$_2$-responsive biochemical systems, which is consistent with its emergence in microaerobic aquatic settings, well before the oxygenation of the atmosphere.

Steroid biosynthesis is possible at nanomolar dissolved O$_2$ concentrations, but biosynthesis is only the necessary first step in producing the molecular fossil record seen in late Archean rocks, which also reflects ecological and diagenetic influences. Here we have assumed that levels of O$_2$ sufficient for steroid biosynthesis could also be adequate to leave a range of preserved molecular fossils, but it is conceivable that the variety of stereoisomers seen in late Archean sediments, and their sustained presence throughout the time period (30), points to an even higher level of environmental oxygenation. With little clear sense of the biodiversity or ecology of these early eukaryotic communities, or of the efficiency of biomarker preservation over such long time scales, it is difficult at present to move beyond the baseline constraints imposed by biosynthesis. Conversely, it could also be argued that the O$_2$ used to synthesize steroids in the Archean ocean was actually produced intracellularly by the eukaryotes themselves and that their presence in sedimentary organic matter reflects only the intracellular availability of O$_2$ rather than broader oxygenation of the marine water column. Certain modern freshwater ciliates maintain intracellular O$_2$ while living just below an oxic–anoxic transition zone by sequestering still-active chloroplasts from algal prey, which provide an O$_2$ supply for aerobic biochemistry (50). But although solely intracellular O$_2$ is a conceivable explanation for steroid production in isolation, all eukaryotic photosynthesis ultimately derives from cyanobacteria, through either endosymbiosis or kleptoplasty. So the presence of photosynthetic eukaryotes of any sort would mean that oxygenic cyanobacteria had already arisen and that oxygenation of aquatic habitats was under way to some extent.

In the early Precambrian, O$_2$ was a biogenic trace gas that was likely very rapidly cycled between dynamic sources and sinks (18). The biogeochemical distribution of O$_2$ in the Archean ocean probably resembled (in general terms) that of biogenic trace gases in the modern ocean, such as dimethyl sulfide (DMS). In fact, the parallels between the proposed cycling of Archean O$_2$ and that of modern DMS are striking: Both have biological sources and sinks in the upper water column, very low atmospheric concentrations and atmospheric lifetimes of hours to days (51,52). The distribution of O$_2$ in the Archean ocean was probably geographically heterogeneous and seasonally variable, just as that of DMS is today; a recent modeling study (53) has explored spatial and temporal variability in the extremely low (approximately $10^{-8}$–$10^{-4}$ ppmv) atmospheric O$_2$ levels potentially generated by photochemical reactions prior to the advent of oxygenic photosynthesis. In the cases of both Archean O$_2$ and modern DMS, some degree of supersaturation of the surface ocean is persistent over long time scales and biologically relevant, enabling uptake and utilization of the trace gas by a number of metabolic pathways.

Accurate reconstruction of the history of O$_2$ in the atmosphere and oceans and evolution of the organisms and biochemistry responsible for its production are central to understanding the reciprocal interactions between life and environments. In considering where and when various aerobic biochemical processes may have occurred, the past O$_2$ content of aquatic environments cannot be inferred from atmospheric O$_2$ levels alone, nor should its concentration be assumed to have been homogeneous. Biologically sustained, nanomolar-scale deviations from gas-exchange equilibria—which are invisible to many geochemical proxies for environmental oxygenation—can enable O$_2$-dependent metabolism, as respiration (44) and steroid biosynthesis exemplify. Recent work (53) has even suggested that, depending on the CO$_2$ partial pressure of the Archean atmosphere, abiotic photochemical production of O$_2$ and H$_2$O$_2$ might have resulted in dissolved oxygen concentrations from $10^{-15}$ to possibly as high as $10^{-10}$ molar in surface waters. Exploration of biochemistry in this subnanomolar dissolved O$_2$ regime, although experimentally challenging, is likely to yield further insights into the evolutionary relationships between aerobic metabolisms, oxygenic photosynthesis, and the progressive oxygenation of the surface environment.

Materials and Methods

Culture Conditions and Sampling. S. cerevisiae (strain D273-108, ATCC) cells were grown in a defined minimal medium containing 4 g/L uniformly labeled $^{12}$CO$_2$-glucose (99% $^{13}$C, Cambridge Isotope), supplemented with 10 mg/L ergosterol and 0.5 mL/L Tween 80, with 1 mL/L FG-10 added as an antifoaming agent. Experiments were performed in an anaerobic chamber (slightly positive with an atmosphere of 5% H$_2$, 15% CO, 80% N$_2$ and <1 ppmv O$_2$). For each growth experiment, 6 mL of late log-phase culture (OD$_{450}$ 0.8) was inoculated into 300 mL of media in a bubbler bottle with the headspace exhausted to external vacuum. Dissolved oxygen concentration in the medium was controlled by vigorous bubbling with either N$_2$ (oxygen-free grade, <0.5 ppmv O$_2$; Airgas) for anaerobic growth or one of three O$_2$:N$_2$ mixtures (certified O$_2$ contents of 3.52 ± 0.07, 473.6 ± 5.5, and 5,030 ± 100 ppmv, Airgas) for microaerobic growth. As described below, bubbling with these gas mixtures produced four dissolved O$_2$ levels: <0.7 nM ("anaerobic"), 7 nM, 0.6 μM, and 6.5 μM. Exponential growth rates in the four conditions were 0.24, 0.25, 0.28, and 0.29 h$^{-1}$, respectively. Cells were grown with bubbling and stirring for 12–16 h, until an OD$_{450}$ of 0.2 was reached; we observed that at higher cell densities, metabolic utilization of O$_2$ drew down the dissolved oxygen concentration below that expected from Henry’s law. The cells were then harvested by vacuum filtration inside the anaerobic chamber onto a precombusted GF/F filter. Filters were placed into glass centrifuge tubes filled with 19 mL of Bligh-Dyer (54) extraction solvent (10:5:4 chloroform:methanol:water) that had been preequilibrated with the anaerobic atmosphere and incubated in the anaerobic chamber overnight to ensure complete enzyme deactivation prior to oxygen exposure.

Dissolved O$_2$ Measurements. The O$_2$ concentration in the cultures during microaerobic growth was measured by two methods: a colorimetric assay based on Rhodazine-D (Chemetrics) and a STOX (Switchable Trace Oxygen) microelectrode with a polarizable front guard (55) (Unisense). In our experiments, the detection limits for the two methods were 300 nM for the Rhodazine-D assay and 100 nM for the STOX electrode. For the anaerobic (N$_2$-bubbled, <1 ppmv O$_2$) growth conditions and the lowest O$_2$ level tested (5.32 ppmv O$_2$), the dissolved oxygen concentrations were below the detection limit of both methods and were calculated from Henry’s law at <0.7 and 7 nM, respectively. Limiting the cultures to a maximum OD$_{450}$ of 0.2 kept the rate of biological O$_2$ utilization below the rate at which oxygen could be supplied by bubbling. The stability of the saturation levels for the two higher dissolved O$_2$ levels tested—0.6 and 6.5 μM, also consistent with Henry’s law for saturation with the 473 and 5,030 ppmv O$_2$ mixtures—during growth of the culture was verified with the STOX electrode.

Lipid Analysis. Tubes containing filters and solvent were removed from the anaerobic chamber and lipids extracted twice by ultrasonication for 20 min, with cell debris pelleted by centrifugation (5 min at 1,000 × g) between extracts. The two extracts (19 mL each) were pooled, and 10 mL water and 10 mL chloroform added to induce phase separation. After separation overnight at ~20°C, the lower phase was concentrated under N$_2$. Extracts were filtered over silica gel and lipids eluted with 8:2 dichloromethane:ethyl acetate. Lipid extracts were evaporated to dryness to determine yield and 1 mg of lipid reacted with 40 μL N,O-bis(trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane and 40 μL pyridine at 70°C for 60 min to produce trimethylsilyl derivatives. Derivatized lipid extracts were analyzed by gas chromatography-mass spectrometry on a 3890B GC/5975C MSD system (Agilent). The derivatized extract (1 μL) was injected onto a DB-1 or DB-5 column (0.250 mm ID, 0.25-μm film, 30-m length; Agilent), with He carrier flow at 1 mL/min. Analytes were ionized by electron impact at 70 eV.
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