The mechanism of sulphide oxidation by *Mortierella isabellina* NRRL 1757

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A series of aryl substituted phenyl and benzyl methyl sulphides has been oxidized to the corresponding sulphoxides by *Mortierella isabellina* NRRL 1757. The oxygen atom introduced during this enzymic oxidation is derived from the atmosphere and not from water. The rates of oxidation have been determined by uv analysis, and correlated with the Hammett p. sigma constants for the phenyl methyl sulphide series. The value of $\rho = -0.67$ so obtained is interpreted in terms of a mechanism of oxidation at sulphur involving an electrophilic attack on the sulphide sulphur by the enzymic activated iron–oxygen complex, followed by conversion of the resulting sulphur cation to sulphoxide.

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On a oxydé une série de méthylthio-benzènes et -phénylméthanes substitués en sulphoxydes correspondants à l'aide de la *Mortierella isabellina* NRRL 1757. L'atome d'oxygène introduit lors de cette oxydation enzymatique provient de l'atmosphère et non de l'eau. On a déterminé les vitesses d'oxydation par l'analyse uv et on les a relié aux constantes sigma p de Hammett, dans le cas des méthylthiobenzènes. On interprète la valeur de $\rho = -0{,}67$ ainsi obtenue en fonction d'un mécanisme d'oxydation au niveau du soufre impliquant une attaque électrophile sur le soufre du sulfure par un complexe fer-oxygène activé enzymatiquement, suivi de la transformation sur cation soufré résultant en sulphoxyde.

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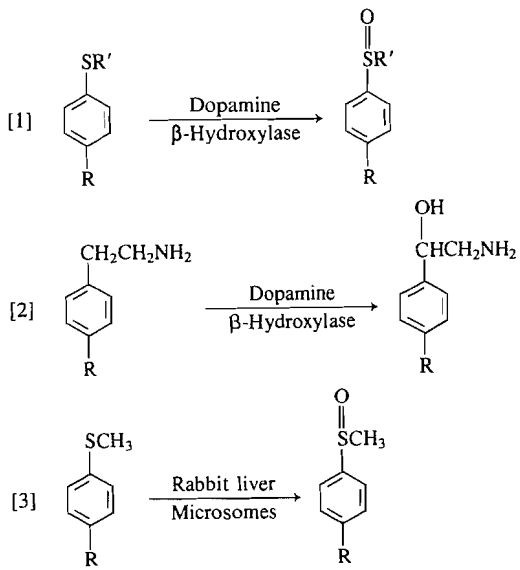
The enzymic oxidation of sulphides to sulphoxides and sulphones can be efficiently carried out by enzymes from mammalian (1–3) or microbial (4–6) sources. Early work in this area (4–6) was concerned largely with the stereospecificity of sulphoxide formation and metabolism by fungi, whereas more recent work (2, 3, 7, 8) has centered on an investigation of the mechanism of the oxidative reaction when carried out by enzymes from mammalian sources.

May *et al.* (7) found that dopamine β-hydroxlase, a copper-containing mono-oxygenase, can perform sulphoxidation of aryl substituted phenyl alkyl sulphides (eq. [1]), and that with this enzyme the reaction proceeds at rates which indicate a ρ value of −3.6 in the conventional Hammett plot. This value differs considerably from that shown by the same enzyme for β hydroxylation of substituted dopamine derivatives (eq. [2]), where a value of −0.4 was reported (7). These values were interpreted by May *et al.* (7) in terms of a mechanism of enzymic sulphoxidation similar to that of hydrogen peroxide oxidation of sulphides, where a ρ value of −1.13 was observed (9). The large difference in ρ values (−3.6 vs. −1.13) was not commented upon. However, the enzymic ρ value was obtained using only halo substituted and unsubstituted thioanisole, and because of the small range of σ values concerned should be regarded as an approximate value. The mechanistic proposal of May (Scheme 1) involves nucleophilic attack by



sulphur at an electron deficient activated oxygen intermediate. Hydroxylation, on the other hand, is proposed to proceed via neutral radical intermediates which would necessitate little charge separation in the transition state, a state of affairs consistent with the observed values.

In a parallel investigation, Oae and co-workers (2, 3, 8) have studied the sulphoxidation of substituted phenyl methyl sulphides by a cytochrome P-450 dependent enzyme preparation from rabbit liver (eq. [3]). Their kinetic data gave a ρ^+ value of −0.16; the low magnitude of this result, together with the results of model reactions with

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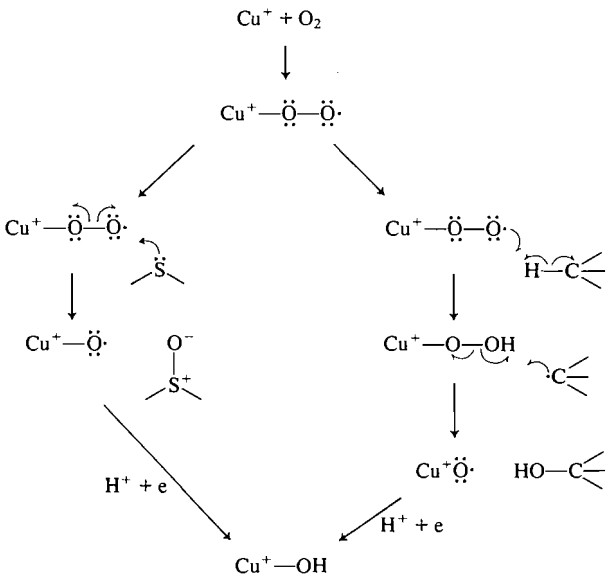
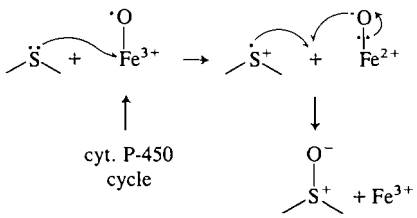


SCHEME 1. Proposed mechanism for sulphoxidation and hydroxylation by dopamine β-hydroxylase (7).

hydroxyl radical generating systems (10–12) and a study of the one-electron oxidation potentials of sulphides, led to the mechanistic proposal shown in Scheme 2, in which the rate determining step involves an activated iron–oxygen species. The correlation coefficients obtained in both the above studies are presented in Table 2.

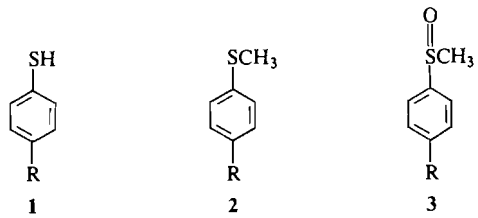


SCHEME 2. Proposed mechanism for sulphoxidation by rabbit liver microsomes (2, 3).

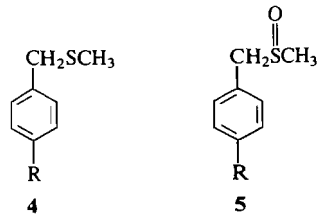
In the present study, we have investigated the sulphoxidation of a series of substituted methyl phenyl (**2**) and benzyl methyl (**4**) sulphides by the fungus *Mortierella isabellina* NRRL 1757 (eq. [4]). This fungus was chosen because it oxidizes sulphides of type **2** and **4** to the corresponding sulphoxides **3** and **5** in high yield and without any detectable further oxidation to sulphone (6); the formation of sulphone occurs to a greater or lesser extent with most of the other fungi reported to perform this reaction (13).

By performing incubations in ^{18}O labelled water and air (see Experimental) we have demonstrated that the enzyme catalysing the sulphoxidation of **2***a* shows one of the major characteristic properties of mono-oxygenases, in that the oxygen entering the substrate is derived directly from the atmosphere and not from the aqueous medium. However, other cofactor requirements for this reaction have not been investigated.

The rates of oxidation of sulphides **2** and **4** were determined by monitoring the release of sulphoxides **3** and **5** into the aqueous medium by ultraviolet spectral analysis. The uv data on which these analyses were based, together with the rates so obtained, are presented in Table 1. The following control experiments were performed. Firstly, a culture of fungus grown in the absence of sulphide was monitored over a 36 h period for the production of uv absorbing compounds which might interfere with the analysis: none were found. Secondly, calibration mixtures of sulphide **2***a* and sulphoxide **3***a* of varying composition were made up, and confirmed that the uv method gave reliable estimates of sulphoxide concentration. Thirdly,



1–3 *a* R = H
b R = CH_3
c R = C_2H_5
d R = F
e R = Cl
f R = Br
g R = NO_2
h R = OCH_3



4, 5 *a* R = H
b R = CH_3
c R = NO_2

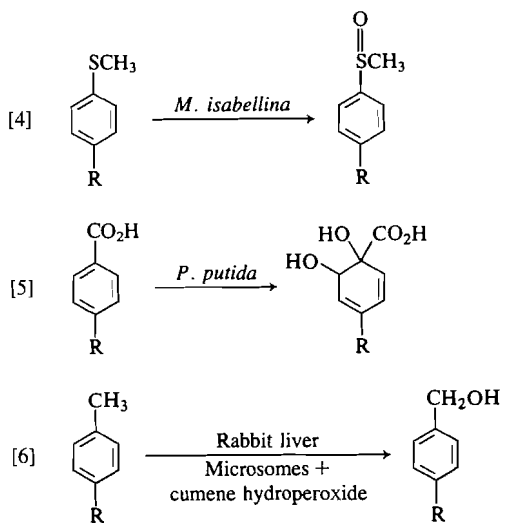
confirmation of sulphoxide formation was obtained in all cases by both the isolation and characterization of sulphoxide from the incubation medium, and by comparison of the uv trace obtained at the termination of the kinetic runs with that of an authentic sample of sulphoxide. In no case was any

TABLE 1. Ultraviolet spectral data of sulphides **2** and **4** and sulphoxides **3** and **5**, and rate constants K for the production of sulphoxides **3** and **5**

Compound	λ_{max} (nm)	log ε (nm)	K (mol s^{-1})	log k/k_H
2*a*	254	4.0(254), 3.57(237)		
2*b*	255	4.05(255), 3.65(236)		
2*c*	257	4.04(257), 3.69(235)		
2*d*	249	3.86(249), 3.51(230)		
2*e*	260	4.16(260), 3.71(242)		
2*f*	262	4.21(262), 3.79(243)		
2*g*	340	4.10(340), 3.61(248)		
2*h*	257	3.92(257), 3.78(243)		
4*a*	247	2.89(247), 2.61(260)		
4*b*	220	3.24(220), 2.79(232)		
4*c*	267	3.92(267), 3.79(278)		
3*a*	237	3.66(237), 3.35(254)	$1.37\pm0.05 \times 10^{-8}$	0
3*b*	236	3.58(236), 3.30(255)	$6.1\pm0.1 \times 10^{-9}$	−0.35
3*c*	235	3.78(235), 3.27(257)	$6.5\pm0.1 \times 10^{-9}$	−0.32
3*d*	230	3.53(230), 3.27(249)	$1.20\pm0.05 \times 10^{-8}$	−0.06
3*e*	242	3.72(242), 3.27(260)	$1.0\pm0.05 \times 10^{-8}$	−0.14
3*f*	243	4.0(243), 3.50(262)	$3.2\pm0.1 \times 10^{-9}$	−0.63
3*g*	248	4.03(248), 2.71(340)	$3.9\pm0.1 \times 10^{-9}$	−0.56
3*h*	243	4.02(243), 3.59(257)	$1.9\pm0.1 \times 10^{-8}$	+0.14
5*a*	260 (sh)	2.40(260), 2.50(247)	$2.2\pm0.2 \times 10^{-8}$	
5*b*	232	3.83(232), 3.83(220)	$2.1\pm0.2 \times 10^{-8}$	
5*c*	278	3.94(278), 3.94(267)	$2.1\pm0.2 \times 10^{-8}$	

evidence obtained for the formation of a uv absorbing compound other than the sulphoxide.

The complexity of biological reactions, especially in systems involving membrane bound enzymes and very poorly soluble substrates, as is the case in the present study, often makes the interpretation of correlation analyses such as ρ values extremely complex. In an attempt to demonstrate that the variation in rate of sulphoxide production reported herein can be directly correlated to the rate of sulphide oxidation, we have studied the oxidation of the corresponding benzyl methyl sulphides **4** by *M. isabellina*.



The common rate observed for the oxidation of the different benzyl sulphides **4***a*–**4***c* confirms that the variation in rate observed in the case of the phenyl sulphides **2** is probably attributable to the electronic effect of the *para* substitutent, and not to the difference in size and/or polar nature of the groups concerned. The assumption that the rate of release of sulphoxide into the aqueous medium can be correlated with the rate of oxidation at sulphur, and is not attributable to other factors (such as membrane transport or cofactor oxidation), is based on the following arguments.

Firstly, although the rate determining step in enzymic sulphoxidation is unknown, in cytochrome P-450 dependent mono-oxygenase reactions the rate determining step is the decomposition of the cytochrome P-450 oxygen–substrate complex to products (14); and secondly, externally added sulphoxide is not taken up by the fungal mycelia, suggesting that the degree of association between sulphoxide and cell membrane is low. We therefore conclude that sulphoxide, on its release from the active site, is rapidly passed through the cell wall into the aqueous medium. This conclusion is supported by control experiments in which extraction of both the medium and fungal mycelia at various times after addition of sulphide indicates that sulphoxide is present only in the medium, and that little if any sulphoxide remains in the mycelia.

The Hammett plot obtained for sulphoxidation of the phenyl methyl sulphides **2** is presented in Fig. 1. With the exception of data for **2***b* (R = CH_3), **2***c*

TABLE 2. Hammett ρ values obtained for oxidation at sulphur and hydroxylation at carbon

Transformation	Oxidation at	Oxidizing species	ρ	Reference
eq. [1]	S	Dopamine β-hydroxylase	−3.6	7
eq. [3]	S	Rabbit liver microsomes	−0.16*	2
eq. [4]	S	*M. isabellina*	−0.67	Present work
eq. [7]	S	Rabbit liver microsomes	−0.2*	37
eq. [2]	C	Dopamine β-hydroxylase	−0.4	7
eq. [5]	C	*Pseudomonas putida*†	−1.14	15
eq. [6]	C	Rabbit liver microsomes/ cumene hydroperoxide	−1.6	16

*ρ^+ value.
†Source of a dioxygenase enzyme.

(R = C_2H_5), and **2***f* (R = Br), the points fall on a straight line with a value of −0.67 (r = 0.994). The rates observed in our work, unlike those obtained by Oae and co-workers (2), do not correlate well with σ^+ values. The deviation of the rates for alkyl substituted sulphide oxidation from this value may reflect a change in the rate limiting step of the reaction, or a degree of hydrophobic interaction between substrate and fungal membrane not present for the other substrates involved. However, this phenomenon is not apparent for the oxidation of the corresponding benzyl sulphide. A comparison of this value with other ρ values for enzymic oxidation at sulphur (2, 3, 7, 8, 37) and carbon (7, 15, 16) is presented in Table 2. The reactions concerned are outlined in eqs. [1]–[7]. With the exception of eq. [1], all the enzymic processes have been proposed, on the basis of a low negative ρ value, to involve a mechanism in which there is not a high degree of electron demand

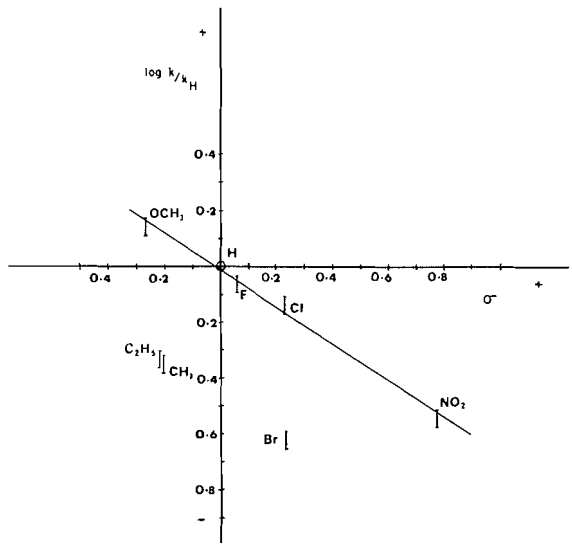
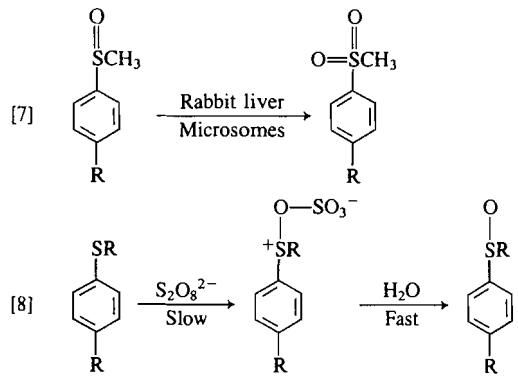


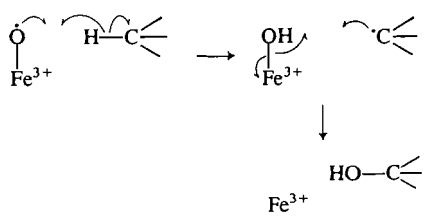
FIG. 1. The Hammett plot for sulphoxidation of phenyl methyl sulphides **2**.



Reference 39

on the substrate in the transition state. In the case of hydroxylation at saturated carbon, a carbon radical intermediate has been proposed (16) (Scheme 3), whereas in the case of oxidation at sulphur, a radical cation species is the proposed intermediate (Scheme 2).

The ρ value of −0.67 obtained in the present study differs appreciably from those obtained using peroxide oxidants (H_2O_2, −1.13 (9), isoalloxazine hydroperoxide, −1.67 (38)), and that reported by May *et al.* (7) for dopamine β hydroxylase, −3.6 (but *vide supra* and ref. 38). However, it is close to the value reported by Srinivasan *et al.* (39) for oxidation by potassium persulphate (ρ = −0.87, ρ^+ = −0.56), in which the slow step is electrophilic attack at sulphide sulphur by the persulphate ion to form an intermediate sulphur cation (eq. [8]), and



SCHEME 3. Proposed mechanism for hydroxylation at saturated carbon involving radical intermediates (16).

also the value observed for the oxidation of sulphoxides to sulphones by *m*-chloroperoxybenzoic acid (−0.54) (24). Our data therefore do not strongly support the one-electron oxidation route proposed for the corresponding mammalian enzymic reaction (2, 3, 37) (Scheme 2), but indicate a transition state in which there is greater electron demand on sulphur. This is consistent with an electrophilic attack by the activated iron–oxygen enzymic complex on sulphur, analogous to that shown in eq. [8].

The relevance of the sulphur oxidation mechanism to the mechanism of hydroxylation at saturated carbon remains to be established, however. The values of ρ obtained for the latter reaction (Table 2) may not reflect the value for a normal cytochrome P-450 dependent mono-oxygenase. Equation [2] involves a copper dependent enzyme, eq. [4] refers to a dioxygenase, and eq. [6] involves the use of cumene hydroperoxide rather than oxygen as the ultimate oxidizing species. It is therefore apparent that definitive data for the carbon hydroxylation reaction are needed so that a more meaningful comparison with sulphur oxidation can be made.

Experimental

Apparatus, materials, and methods

Melting points were determined on a Kofler heating stage and are uncorrected. Infrared spectra were recorded with a Perkin–Elmer 237B spectrometer, and ultraviolet spectra with a Hitachi Perkin–Elmer 124 or Cary 14 spectrophotometer. Proton nmr spectra were obtained at 60 MHz with a Varian A-60 or Bruker WP-60, using $CDCl_3$ as solvent and TMS as internal standard, and ^{13}C nmr at 15.18 MHz with a Bruker WP-60, using $CDCl_3$ as solvent and TMS as internal standard. Mass spectra were obtained with an AEI MS 30 interfaced to a Kratos DS 55 data system: isotopic composition was determined by repeated data accumulation over the relevant ion region and application of the appropriate corrections for natural abundances; values are accurate to ± 0.5%. Column chromatography was performed on silica gel (60–200 mesh), and thin layer chromatography on Merck silica gel 60 F-254 (0–2 mm).

Mortierella isabellina NRRL 1757 was maintained on slopes composed of 4% malt agar.

Preparation of substrates

p-Toluenethiol (**1***b*), *p*-fluorobenzenethiol (**1***d*), *p*-chlorobenzenethiol (**1***e*), *p*-bromobenzenethiol (**1***f*), *p*-nitrobenzenethiol (**1***g*), thioanisole (**2***a*), and benzyl methyl sulfide (**4***a*) were commercial samples.

p-*Ethylbenzenethiol* (**1***c*) *and* p-*methoxybenzenethiol* (**1***h*)

These were prepared from the corresponding phenol by the method of Newman and Karnes (17), and gave satisfactory analytical (bp and spectral) data.

Substituted thioanisoles; **2**b–**2**h

These were made by alkylation of the corresponding thiophenols **1***b*–**1***h* by the method of Clark and Miller (18) and possessed physical properties similar to those reported (19, 20).

Substituted phenyl methyl sulphoxides; **3**a–**3**h

These were prepared from the corresponding sulphides **2** using sodium metaperiodate as oxidant (21), and exhibited spectral and physical properties consistent with those reported (22–27).

Substituted benzyl methyl sulphides; **4**b, **4**c

Compound **4***b* was prepared by reaction of the corresponding substituted benzyl chloride with methanethiol using the procedure described (28), and gave satisfactory analytical data (29, 30). *p*-Nitrobenzyl methyl sulphide (**4***c*) (31) was prepared from *p*-nitrobenzyl mercaptan (32) by alkylation with methyl iodide (18).

Substituted benzyl methyl sulphoxides; **5**a–**5**c

These were prepared from the corresponding sulphides **4** using sodium metaperiodate as oxidant (21), and exhibited spectral and physical properties consistent with those reported (29, 33, 34).

Incubations with Mortierella isabellina *NRRL 1757*

The fungus was grown in a medium composed of glucose (40 g), soybean meal (5 g), yeast extract (5 g), sodium chloride (5 g), and dibasic potassium phosphate (5 g) per litre of distilled water (35). One-litre Erlenmeyer flasks containing 150 mL of the above medium were sterilized, allowed to cool, and then inoculated from a growing slope of *M. isabellina*. The flasks were then placed on a 1 in. rotary shaker (150 rpm) at 25°C for 3 days. After this time, the fungus was removed by filtration and resuspended in distilled water (130 mL in each 1-L Erlenmeyer flask). For preparative studies, sulphide (100 mg in 1.15 mL ethanol) was added to each flask and incubation continued as above for 24–36 h. After this time, the fungus was removed by filtration and the filtrate extracted with chloroform. The extract was dried and evaporated, and then subjected to tlc and spectral analysis. Incubations of sulphides **2** and **4** gave the corresponding sulphoxides **3** and **5** as the only identifiable products.

Measurement of kinetic data

For kinetic studies, the mycelia from two flasks were combined and resuspended in 130 mL of distilled water in a single 1-L Erlenmeyer flask. Sulphide (100 mg in 1.15 mL ethanol) was added to each flask at time zero, and the flask returned to the rotary shaker at 25°C. Samples (1 mL) were removed by filtration at various time intervals, and diluted to 25 mL with spectroscopic grade methanol. This solution was then used for uv spectral analysis. The relative proportions of sulphide and sulphoxide were determined by the standard technique of two-component analysis (36), using the wavelengths and extinction coefficients listed in Table 1. The increase in sulphoxide concentration with time gave the rate constants reported for release of sulphoxide into the medium.

Incubation in ^{18}O*-labelled water*

Fungus from one 1-L flask, grown as described above, was placed into 100 mL of $H_2{}^{18}O$ (1.5% enriched) in a 500 mL Erlenmeyer flask, and methyl phenyl sulphide (**2***a*) (100 mg) in ethanol (1 mL) added to the flask. The sulphoxide **3***a* isolated after a 36 h incubation was examined by mass spectrometry and found to contain no higher level of ^{18}O then a sample produced in a comparable experiment using unlabelled water.

Incubation under an ^{18}O *labelled atmosphere*

Fungus from one 1-L flask, grown as described above, was placed in a side arm attached to 500 mL Erlenmeyer flask. The main body of the flask contained 100 mL water and 100 mg methyl phenyl sulphide (**2***a*). Degassing was carried out by alternate freezing, pumping, and thawing. The flask was then filled with an ^{18}O enriched atmosphere (nominally 90% ^{18}O), and sealed. The fungus was added from the side arm, and the sulphoxide **3***a* isolated after a 36 h incubation examined by mass spectrometry: isotopic content, ^{18}O, 80%; ^{16}O, 20%.

Acknowledgements

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