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A Facile Method for Separation of the Cryptic Methionine Sulfoxide Diastereomers, Structural Assignment and DFT Analysis

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Dedicated to Roald Hoffmann, in admiration and friendship

Abstract: *Methionine (Met) oxidation is an important biological redox node, with hundreds if not thousands of protein targets. The process yields methionine oxide (MetO). It renders the sulfur chiral, producing two distinct, diastereomerically related products. Despite the biological significance of Met oxidation, a reliable protocol to separate the resultant MetO diastereomers is currently lacking. This hampers our ability to make peptides and proteins that contain stereochemically defined MetO to then study their structural and functional properties. We have developed a facile method that uses supercritical CO_2 chromatography and allows obtaining both diastereomers in purities exceeding 99%. ^1H NMR spectra were correlated with X-ray structural information. The stereochemical interconversion barrier at sulfur was calculated as 45.2 kcal/mol, highlighting the remarkable stereochemical stability of MetO sulfur chirality. Our protocol should open the road to synthesis and study of a wide variety of stereochemically defined MetO-containing proteins and peptides.*

Methionine (Met) sulfur oxidation is an important biomolecular redox process that yields methionine sulfoxide (MetO) as the reaction product and can, in principle, affect any Met-containing protein.[1] Because most proteins contain at least one methionine residue, Met oxidation has a vast range of potential biomolecular targets.[2] Biologically important examples of the Met → MetO reaction include regulation of cellular function,[3] protection of proteins from oxidative damage by providing sites that can scavenge ROS,[4] changes in gene transcription in response to oxidative stress,[5] as well as oxidative damage of long-lived proteins upon aging.[6]

Upon oxidation of Met to MetO, a stereogenic sulfur center is formed, and the reaction yields two diastereomers ((*S*,*S*) and (*S*,*R*) with the naturally occurring (*S*)-Met) as a consequence of that. The sulfur chirality of MetO is stable with regard to stereochemical interconversion, and living systems have evolved enzymes, termed reductases, which are capable of stereospecifically reducing the MetO diastereomers back to Met.[7] The impact of MetO sulfur chirality on peptide properties is strongly suggested through studies with the chemically similar system, β,β-dimethylmethionine oxide, in which the stereoisomers are much easier to separate due to higher sidechain rigidity than that of MetO.[8]

Whereas the biological high significance of MetO sulfur chirality is clearly established,[1]-[7] chemical biology efforts to study consequences of Met → MetO have thus far been commonly performed with mixtures of diastereomers.[9] This is presumably due to the challenges in obtaining diastereomerically pure MetO. A recent study that investigated the repair of oxidized proteins of the bacterial envelope did use the diastereomerically resolved MetO diastereomers, using a fractional crystallization method that was developed in 1947.[10a,b] Fractional crystallization of diastereomers that differ in as little as the orientation of a single methyl group may be challenging to perform reproducibly and reliably, which is the presumed reason why this method is not routinely used in the field. A method to obtain diastereomerically enriched MetO (d.e. ~95-98%) through asymmetric oxidation has also been developed.[10c]

Here, we report a novel supercritical CO_2 ($scCO_2$) purification method, through which gram quantities of diastereomerically pure MetO (>99 % purity) can be reliably and readily obtained. The access to diastereomerically pure (*S*,*S*) and (*S*,*R*)-MetO in turn enabled us to correlate NMR with absolute stereochemical information that we obtained using X-Ray crystal structure analysis. Activation barrier for the interconversion of the two MetO diastereomers was calculated using quantum chemical (DFT) means.

Separation of the MetO diastereomers proved to be exceptionally challenging, and our initial efforts that used standard HPLC-based purification methods or fractional crystallization approaches were unsuccessful. However, we were able to achieve baseline separation of the two Fmoc-MetO diastereomers by employing a purification protocol that uses supercritical CO_2. Following separation on a Chiralpak IC column,[11] the diastereomers were then recrystallized (Fraction 1: DCM/Ether; fraction 2: iPrOH/Ether), affording gram quantities of the two Fmoc-MetO diastereomers in purities exceeding 99 % (Figures S1-S3). The method represents a significant advance compared with the fractional crystallization. In particular, the purity of the (*S*,*R*)-Fmoc-MetO diastereomer obtained by our method is substantially higher than that achieved by fractional crystallization in the past, where the other diastereomer is still clearly observed by ^1H NMR.[10a]

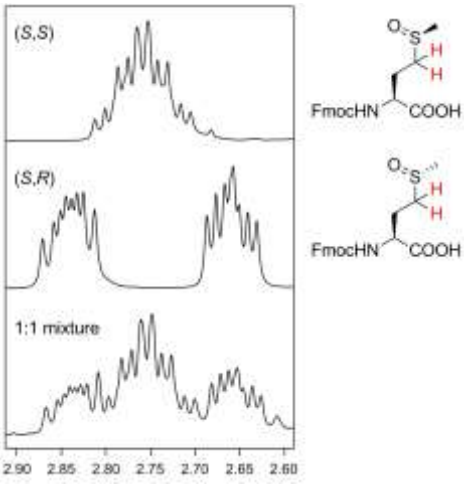

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Figure 1. 1H NMR spectra of the diagnostic region of Fmoc-MetO, with protons diagnostic for the two diastereomers indicated in red.

Consistent with previous work,[10a] ^{1}H NMR experiments, conducted with the ~1:1 mixture of the two Fmoc-MetO diastereomers, revealed that the γ-CH_2 group of the MetO sidechain was well-differentiated between the two diastereomers (Figure 1, bottom), and could therefore be used as the diagnostic region to study such mixtures (Figure 1B, bottom). The two separated Fmoc-MetO diastereomers were determined to be diastereomerically pure using this set of ^{1}H NMR signals (Figure 1, top and middle), and the ^{1}H NMR resonances unambiguously assigned by ^{1}H,^{1}H-COSY NMR spectroscopy (Figures S4-S6). The diastereotopic γ-CH_2 protons are well-dispersed in the (*S*,*R*) diastereomer (Figure 1, middle) and exhibit the expected ddd coupling pattern as a consequence of coupling with three chemically inequivalent protons. In the (*S*,*S*) diastereomer, the two γ-CH_2 protons are overlapped (Figure 1, top), leading to a higher complexity peakshape due to partial overlap of two signals with ddd coupling pattern.

X-Ray quality single crystals of the Fmoc-MetO diastereomer that eluted in fraction 2 in the $scCO_2$ separation procedure (Figure 1, top and Figure S3) were grown by recrystallization from isopropanol with heating at reflux and subsequent slow cooling to room temperature. Crystallographic structural analysis allowed the compound to be unambiguously assigned as the (*S*,*S*) diastereomer of Fmoc-MetO (Figure 2), which is consistent with the previously reported assignment.[10a]

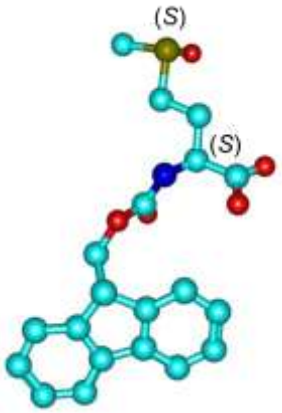

Figure 2. Crystal structure of the (*S*,*S*)-Fmoc-MetO diastereomer that eluted as fraction 2 in $scCO_2$ separation.

To gain quantitative insights into the interconversion barrier associated with the stereochemical interconversion at the chiral MetO sulfur, DFT calculations were conducted. The crystallographically obtained (*S*,*S*)-Fmoc-MetO was fully geometry-optimized (Gaussian 09, M062X/6-311++G**, see SI for further details). Sulfur chirality was subsequently inverted by adjusting the angles associated with the corresponding oxygen orientation accordingly. The resultant structural guess was then fully re-optimized, yielding the (*S*,*R*)-Fmoc-MetO diastereomer. The two Fmoc-MetO diastereomers were found to be nearly isoenergetic, with a marginal preference of 0.5 kcal/mol for the (*S*,*R*) diastereomer. Transition state for the chirality interconversion of the MetO sulfur was subsequently located using the QST3 transition state search procedure, and found to be 45.2 kcal/mol uphill of the (*S*,*R*) diastereomer, which is consistent with the high stereochemical stability of the MetO diastereomers, and is also in good agreement with the inversion barrier of 42.5 kcal/mol, reported by Mislow and co-workers for stereochemical interconversion at pyramidal sulfur in chemically related systems.[12]

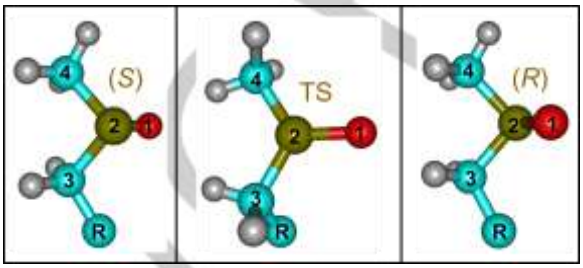

Figure 3. The DFT-optimized partial structures of (*S*,*S*)-Fmoc-MetO (left), the transition state (TS) for stereochemical interconversion at sulfur (middle) and (*S*,*R*)-Fmoc-MetO (right).

Sulfur planarization that occurs upon transition from the (*S*,*S*) to the (*S*,*R*) diastereomer (and *vice versa*) was calculated to produce only a marginal elongation of the sulfur-oxygen bond distance from 1.51 Å in the two minima to 1.54 Å in the transition state (Table 1). Because changes in bond length do not always have to correlate with changes in bond strength, Wiberg bond index analysis was conducted. This analysis yields quantitative measures of bond strength, and we have been able to put the method to advantage in studying organic and organometallic bonding in the past.[13] Wiberg bond index calculations revealed that the associated changes in bond strength are minor between the two minima (WBI = 1.22 for both (*S*,*S*)- and (*S*,*R*)-MetO) and the transition state (WBI = 1.19), indicating a marginal weakening of the S=O bond in the transition state. As expected, sulfur planarization leads to an increase of the bond angles 1-2-3 and 1-2-4 from ~106 ° in the two minima to ~120 ° in the transition state. The dihedral angles 1-2-3-4 of (*S*,*S*)- and (*S*,*R*)-MetO are near-identical in magnitude, but have opposite signs, as expected for a local mirror image topology. Noteworthy were the changes in the $\Delta_{HOMO-LUMO}$ from 0.2569/0.2571 a.u. in (*S*,*S*)-MetO and (*S*,*R*)-MetO, respectively, to 0.2110 a.u. in the transition state, reflective of the pronounced electronic changes associated with the stereochemical interconversion at sulfur, and further confirming the predominantly electronic nature of the unusually high interconversion barrier.

Table 1. Key geometric parameters associated with the stereochemical interconversion at the stereogenic sulfur of MetO. The two Fmco-MetO diastereomers are denoted as (*S*,*S*) and (*S*,*R*), respectively, and the transition state is denoted as TS. All distances are listed in [Å]; all angles and dihedral angles are listed in [°]. Atom numbering scheme used is as shown in Figure 3. Wiberg Bond Index of the S=O bond ($WBI_{S=O}$) was obtained from a natural population analysis. All calculations were performed at the M062X/6-311++G** level of theory. $\Delta_{HOMO-LUMO}$ in a.u.

	1-2	1-2-3	1-2-4	1-2-3-4	$WBI_{S=O}$	$\Delta_{HOMO-LUMO}$
(*S*,*S*)	1.51	105.8	106.5	-109.2	1.22	0.2569
TS	1.54	120.9	121.6	-179.5	1.19	0.2110
(*S*,*R*)	1.51	105.6	106.3	108.95	1.22	0.2571

To better understand the nature of changes in electron structure between a minimum (S,S) and the transition state, the frontier orbital analysis was performed. The orbital relevant for the transition was HOMO-1 in the ground state and HOMO in the transition state (Figure 4). The re-hybridization of the sulfur lone pair in (ground state) to a p-type orbital (transition state) has an associated change in energy from -0.2928 a.u. to -0.2324 a.u., which corresponds to 37.9 kcal/mol, which is 83.85 % of the activation barrier calculated for the interconversion (45.2 kcalmol).

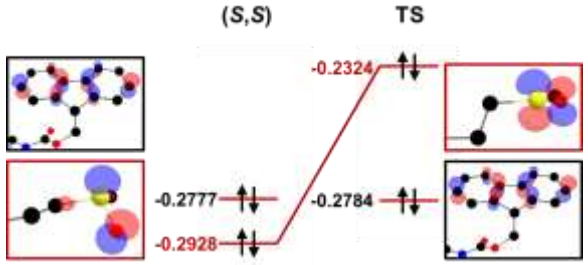

Figure 4. HOMO and HOMO-1 analysis for the (*S*,*S*)-MetO diastereomer (left) and the transition state (right).

The results of the present study should facilitate incorporation of stereochemically defined, *i.e.*, diastereomerically pure, MetO into any protein and peptide, which was challenging until now due to deficiency in separation methodology. The preparative procedure reported here should be easy to adopt by academic and industrial laboratories alike, and may have significant impact on the field studying methionine oxidation, which is an important node of cellular biomolecular redox chemistry. The preparative protocol may be also of interest for materials scientists to explore MetO sulfur chirality as a structural control element that is both extremely subtle and remarkably stereochemically stable.

The chemical fundamental property of stereochemical stability of certain sulfur centres was noted as early as 1900 by Smiles.[14] There is now, over a century later, emerging evidence that MetO sulfur stereochemistry can play pivotal roles in biological regulatory processes, as exemplified by assembly and disassembly of actin.[15] More research and better chemical tools are needed to advance our understanding of the methionine redox proteome.

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Keywords: Methionine oxidation • Super-critical CO2 separation • Absolute structural assignment • Sulfur chirality

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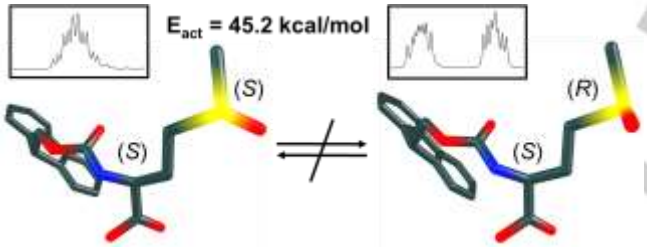
The two elusive methionine oxide diastereomers have been separated by supercritical CO_2 chromatography and their absolute stereochemistry assigned by correlation of ^1H NMR and X-ray analysis. DFT analysis revealed an unusually high interconversion barrier of 45.2 kcal/mol. The protocol should open the road to the synthesis and structure-function studies of hundreds proteins and peptides that contain diastereomerically pure MetO.



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