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Repurposing the Pummerer Rearrangement: Determination of Methionine Sulfoxides in Peptides

Carolyn C. Woodroofe,*[a] Joseph Ivanic,[b] Sarah Monti,[c] Rodney L. Levine,[c] and Rolf E. Swenson[a]

The reversible oxidation of methionine residues in proteins has emerged as a biologically important post-translational modification. However, detection and quantitation of methionine sulfoxide in proteins is difficult. Our aim is to develop a method for specifically derivatizing methionine sulfoxide residues. We report a Pummerer rearrangement of methionine sulfoxide treated sequentially with trimethylsilyl chloride and then 2-mercaptoimidazole or pyridine-2-thiol to produce a dithioacetal product. This derivative is stable to standard mass spectrometry conditions, and its formation identified oxidized methionine residues. The scope and requirements of dithioacetal formation are reported for methionine sulfoxide and model substrates. The reaction intermediates have been investigated by computational techniques and by ^{13}C NMR spectroscopy. These provide evidence for an α-chlorinated intermediate. The derivatization allows for detection and quantitation of methionine sulfoxide in proteins by mass spectrometry and potentially by immunochemical methods.

Introduction

Oxidative modification of proteins by reactive oxygen species had long been viewed as an inevitable, negative by-product of aerobic metabolism. Over the past two decades, many investigators have established that cells produce reactive oxygen species in a controlled fashion. These reactive oxygen species mediate the reversible oxidative modification of specific proteins as an important mechanism of cellular regulation.[1] Methionine in proteins is often thought to be a generic hydrophobic residue, functionally replaceable with another hydrophobic residue such as valine or leucine. However, this frequently is not the case because of the presence of sulfur in methionine. The methionine can be oxidized to methionine sulfoxide, and all aerobic organisms contain methionine sulfoxide reductases capable of reducing the sulfoxide back to the thioether.[2,3] Moreover, the cycle also constitutes a reversible post-translational covalent modification analogous to phosphorylation that can regulate cellular metabolism.[4–6] The reversible oxidative modification of methionine enables methionine residues to provide a catalytically efficient antioxidant defense that scavenges reactive oxygen species.

Progress in identifying the proteins and pathways affected by methionine oxidation and reduction has been slow because detection and quantitation of methionine sulfoxide in specific proteins is difficult. A general antibody capable of specifically binding to any methionine sulfoxide residue would be very useful, but such antibodies cannot be raised.[7,8] Mass spectrometry would seem an ideal method for analysis, and various mass spectrometric methods have been proposed,[9] but they often suffer from artifactual sulfoxide formation during sample preparation or analysis. A method for covalently and specifically derivatizing methionine sulfoxide residues could circumvent these issues. Such a method could be used in a variety of detection techniques, including one and two dimensional separations, mass spectrometry, and fluorescence. Modified proteins or their proteolytic digests could also be enriched by affinity techniques based on the covalent modification.

The Pummerer rearrangement is a reaction specific to sulfoxides. Sulfoxides can be O-alkylated or -acylated with a strong electrophile or other acid, forming an adduct that then eliminates to give rise to a thionium ion. The thionium ion may undergo a variety of reactions: in the most common incarnation of the Pummerer rearrangement, an acetate ion or similar weak to moderate nucleophile adds to generate an α-substituted sulfide as the product, essentially transferring the oxidation from the original sulfur atom to the adjacent carbon. Attack of water yields a hemithioacetal, which can decompose

[a] Dr. C. C. Woodroofe, Dr. R. E. Swenson
Imaging Probe Development Center
National Heart, Lung and Blood Institute, National Institutes of Health
9800 Medical Center Drive, Rockville MD 20850 (USA)
E-mail: carolyn.woodroofe@nih.gov

[b] Dr. J. Ivanic
Advanced Biomedical Computational Sciences Group
Frederick National Laboratory for Cancer Research operated by
Leidos Biomedical Research, Inc.
1011 Beasley Drive, Frederick, MD 21701 (USA)

[c] Dr. S. Monti, Dr. R. L. Levine
Laboratory of Biochemistry, National Heart, Lung, and Blood Institute
National Institutes of Health
50 South Drive, Bethesda, MD 20892 (USA)

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to the corresponding aldehyde and thiol. Other nucleophiles have included carboxylic acids, alcohols, phenyl rings, indoles, phenols, anilines and amides.[10,11] Intramolecular Pummerer-like rearrangements have recently been reviewed.[12] Sulfur nucleophiles, while known, are somewhat underrepresented in comparison, with just a few examples of conversion of a sulfoxide to a dithioacetal.[13,14] We now report the application of Pummerer rearrangement using thiol nucleophiles to selectively transform oxidized methionine residues from sulfoxides into dithioacetals.

Results and Discussion

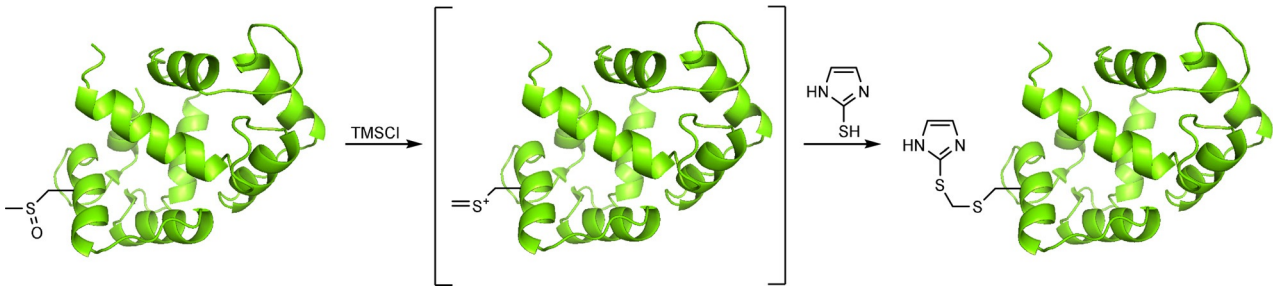
An attempt to quantitate methionine oxidation via the Pummerer rearrangement was reported in 1972 by Lunder.[15] However, we have been unable to reproduce his results, and there are no published papers that used this protocol. Classical Pummerer protocols were often carried out in acetic acid or trifluoroacetic acid with acetic anhydride or trifluoroacetic anhydride as initiators.[11] These harsh conditions usually covalently modify or degrade proteins. Trimethylchlorosilane[16] (TMSCl) was an intriguing alternative to acetic anhydride or trifluoroacetic anhydride, particularly as TMSCl has been used as a cleavage and deprotection reagent in peptide synthesis[17] and would be expected to cause minimal protein decomposition. In addition, TMSCl is often used for transient protection of sensitive groups, as it readily forms adducts with hydroxy groups or amines that are spontaneously removed during aqueous workup.[18,19] We considered that TMSCl could transiently protect alcohols, amines, or other sensitive side chains during the activation step, an effect that should simultaneously increase solubility in organic solvents. Subsequent interception of the thionium ion or other reactive intermediate[20] with an appropriate thiol could give rise to a dithioacetal adduct, as shown in Scheme 1. Dithioacetals are generally quite stable to all but forcing conditions,[21] including mass spectrometry, and a methionine-based dithioacetal should be readily differentiated from a methionine thioether by MS or other analytical methods. Such an approach could offer the ability to both monitor total sulfoxide levels and determine the regiospecificity of oxidation; that is, which specific methionine residues within the protein of interest were in the sulfoxide oxidation state.

We chose the commercially available Fmoc-Met[O]-OH (**1**) as our initial test substrate, as the Fmoc group is both acid-stable and presents a useful UV handle for LC analysis studies. Initial reaction of **1** with TMSCl in THF was promising, as a mass of 370 corresponding to a putative thionium intermediate was observed by LCMS. Upon treatment with 2-mercaptopyridine (**2d**, Table 1) robust signals with *m/z* 481 were observed, consistent with the desired adduct. With this preliminary result in hand, we set out to explore the scope and limitation of the reaction of **1** to form dithioacetal adducts.

An initial screen of small molecule thiols for adduct formation, with a focus on thiol-substituted heterocycles, suggested that the scope of effective nucleophiles was relatively narrow (Table 1). Most tested thiols formed adducts of **1** in very modest amounts, even when the structure was closely related to a thiol that formed the corresponding dithioacetal adduct in good yield. 4-Mercaptopyridine (**2e**) for example, yielded far less adduct than **2d**, and 2-mercaptopyrimidine (**2i**) yielded very little adduct. 2-Mercaptoimidazole (**2a**), in contrast, displayed adduct formation similar to that of **2d**. Closer analysis suggested the formation of multiple regioisomeric products for most nucleophiles, presumably owing to formation of the thionium ion from either carbon adjacent to the unsymmetrical sulfoxide (Scheme 2).

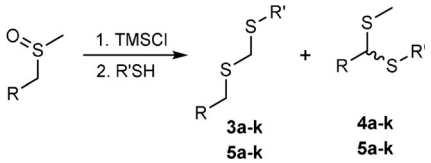
Adducts of C3 produced two distinct diastereomers (**4b–k**). The dithioacetal adducts **3a** and **4a,** formed from reaction of **1** with **2a**, eluted as a single slightly asymmetric peak in most cases, and this nucleophile was therefore used in reaction optimization screenings. While some conversion was observed with a single equivalent of **2a**, the best yields were obtained with the addition of 4 equivalents or more. Estimated conversions (LCMS) of **1** to **3**+**4** for all nucleophiles are listed in Table 1. The formation of **2a** dithioacetal adduct was also examined for simpler systems, using DMSO and methyl phenyl sulfoxide as substrates, to ensure a single product (Scheme 3). The expected adducts **5a** and **6a** were formed in 70 and 64% isolated yield after preparative HPLC. The thiol nucleophiles previously examined via HPLC analysis of their reaction with **1** were screened against a DMSO substrate in order to enable full characterization and yield analysis of the products **5a–k**. The results are shown in Table 1.

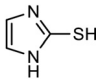
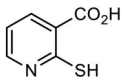
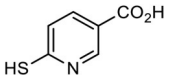
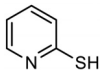
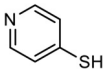
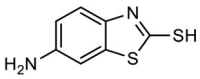
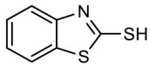
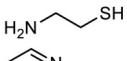
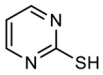
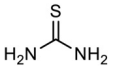
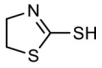
The reaction was most effective when conducted in two steps: an initial activation step in ethereal solvent with 0.2–0.33 M TMSCl followed by condensation with a thiol, typically 2-mercaptoimidazole. The initial activation appeared to be a multi-step process itself, as premature addition of the thiol nucleophile resulted in the production of significant amounts of reduced Fmoc-Met-OH (**7**), presumably due to thiol attack on a



Scheme 1. Proposed labeling approach.

Table 1. Thiol nucleophiles.[a]



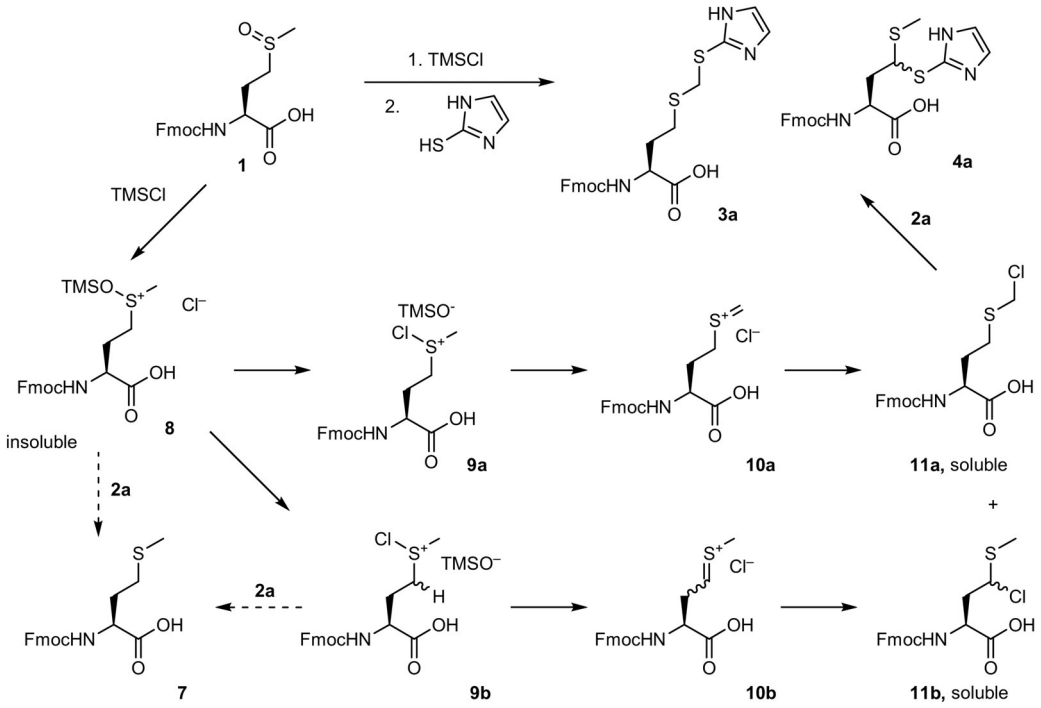
	Thiol name	Structure	Fmoc-Met adduct (**3**+**4**)[b]	DMSO adduct (**5**)[c]
2a	2-mercaptoimidazole		82	70
2b	3-carboxy-2-mercaptopyridine		6	6.1
2c	3-carboxy-6-mercaptopyridine		48	19
2d	2-mercaptopyridine		81	78
2e	4-mercaptopyridine		12	56
2f	6-amino-2-mercaptobenzothiazole		4	9
2g	2-mercaptobenzimidazole		37	42
2h	cysteamine		5.2	–
2i	2-mercaptopyrimidine		7	21
2j	thiourea		21	–
2k	thiazolidine		15	46

[a] Reactions were performed at 0.25 mmol scale (Fmoc-Met(O)-OH, **1**) following the General Procedure for Dithioacetal Formation or 1 mmol scale (DMSO), following the General Procedure for Condensation of DMSO with Thiol Nucleophiles. [b] R = Fmoc-alanine; estimated percent yield of adducts are reported based on integration of the 280 nm absorbance trace between 3 min and 5 min. No correction was made for the contribution of the nucleophile to the absorbance of **3**+**4**. [c] R = H; isolated percent yield.

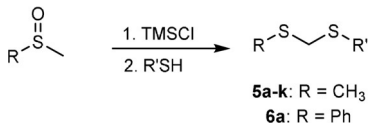
sulfur-electrophile species. However, allowing the reaction mixture to progress from a turbid and opaque mixture in the ethereal solvent to a translucent solution prior to addition of 2-mercaptoimidazole delivered consistent LCMS yields of approximately 80% dithioacetal adduct **3a** and **4a**, as judged by absorbance at 280 nm. Reactions were analyzed at 280 nm in order to maximize the Fmoc absorbance signal while minimizing the imidazole contribution to dithioacetal absorbance.

The reaction was strongly dependent on solvent, as shown in Table 2, with ethereal solvents consistently producing optimal yields. Less polar solvents such as dichloromethane or chloroform were significantly less effective, while EtOAc and acetonitrile were moderately effective. Dimethylformamide, *N*-methylpyrrolidone and dimethylacetamide yielded varying amounts of reduction to the thioether **7**. Acidic solvents such as trifluoroethanol or acetic acid tended to favor reduction to **7** over formation of **3a** and **4a**. In the case of trifluoroethanol, it is possible that the well-precedented mechanism of alcohol oxidation by activated sulfoxide dominates,[22] which would support the high efficiency of reduction to **7**. Mixed-phase systems such as 1:1 water/THF or water/CH_2Cl_2 were not effective.

A variety of activating agents were screened for effectiveness. Silyl chlorides were found to be generally effective, although increased bulk around the silane corresponded to a requirement for extended reaction times (Table 3). Indeed, TBDPSCl failed to generate any **3a** or **4a** products. Several traditional Pummerer electrophiles proved to be ineffective in the conversion into **3a** and **4a**, as acetic or trifluoroacetic anhydrides yielded no adduct (Table S1 in the Supporting Information). Interestingly, chloride appears to be required for dithioacetal formation. While it is perhaps unsurprising that the strongly reactive TMSBr does not yield a great deal of desired products,[23,24] or that the less reactive TMS-imidazole and TMS-polyphosphate are ineffective, it is telling that the addition of



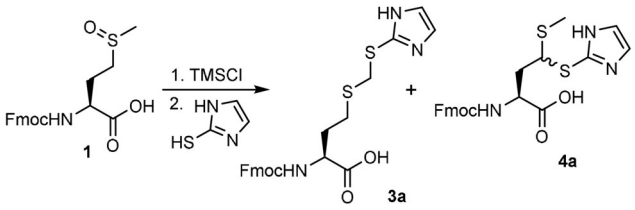
Scheme 2. Mechanism of dithioacetal formation.



Scheme 3. Model system Pummerer rearrangement.

LiCl to the TMS-polyphosphate reaction is sufficient to "rescue" this reaction and produce a significant amount of dithioacetal adduct. LiBr and LiI were not effective in this context, perhaps owing to the in situ generation of TMSBr or TMSI.[25] Of the non-silyl-based activating agents tested, only those containing chloride were even modestly effective (Table S1). The Pummerer-like reaction of thioethers with *N*-chlorosuccinimide is known,[26] and treatment of **1** with NCS followed by addition of **2a** gave rise to a moderate yield of **3a**+**4a** as well. The addition of base was not productive as smaller amounts tended to promote thioether **7** formation and larger amounts suppressed Pummerer-like reactions altogether. Lower temperature promoted the side reduction to **7**, while modestly heating the reaction to 40 or 60 °C accelerated the activation for adduct formation, with an optimal activation time of 2–4 h. Isolated yields of the product mixture **3a** and **4a** using **1** as a substrate were identical (77%) for the reaction with activation at RT (20 h) or 40 °C (3 h). The dithioacetal adduct of the terminal methyl group **3a** was isolated and characterized. However, the adducts **4a** of the internal C3 methylene were inseparable chromatographically and appeared to be less stable to prolonged exposure to aqueous TFA solution. This runs counter to other dithioacetals, which are typically stable to the mildly acidic conditions of preparative HPLC,[21] and could be largely

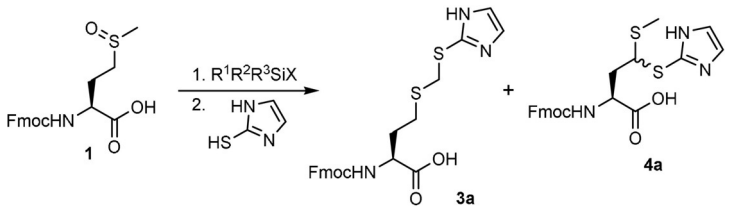
Table 2. Effect of solvent on Fmoc-methionine sulfoxide Pummerer reaction.[a]



Solvent	Dithioacetal adduct **3a**+**4a**[b]	Thioether **7**	Starting material **1**
acetic acid	17	58	–
chloroform	7	41	–
dichloromethane	6	43	–
diglyme	83	1	–
dioxane	82	3	–
dimethoxyethane	71	6	–
triglyme	71	3	–
THF	79	3	–
ethyl acetate	62	8	–
acetonitrile	43	31	–
dimethylacetamide	–	32	63
dimethylformamide	–	90	3
NMP	–	54	37
toluene	–	19	79
trifluoroethanol	–	94	–

[a] Reaction as shown in Scheme 2; percent yields of each species are based on LCMS analysis. A 1 mmol portion of TMSCl at ambient temperature was added to a suspension of 0.25 mmol Fmoc-Met(O)-OH (**1**) in 3 mL of the indicated solvent and the mixture was stirred for a minimum of 16 h, followed by the addition of **2a** and stirring for a further 24 h. [b] Adduct percent yields are reported as the sum of mixed isomers.

Table 3. Silane-based Pummerer activation of Fmoc-Met(O)-OH (**1**).[a]



R^1	R^2	R^3	X	Reaction time [h]	Adduct **3a**+**4a**	Thioether **7**	Starting material **1**
Me	Me	Me	Cl	16–20	82±0.8	2±0.4	–
Me	Me	Me	Br	16	8±0.5	44±2.6	–
Me	Me	Me	imidazole	16	–	11±6.9	82±10.5
Me	Me	Me	PP[b]	16	–	56±1.8	38±2.5
Me	Me	allyl	Cl	20	77±1.6	4±1.9	–
Me	Me	vinyl	Cl	40	77±3.2	4±0.3	–
Me	Me	Et	Cl	40	73±0.6	6±4.5	–
Me	Me	*i*Pr	Cl	40	51±0.6	34±4.9	–
Me	Me	*t*Bu	Cl	64	45±6	31±6	–
Me	Me	Ph	Cl	24	75±0.1	3±0.7	–
Me	Me	Pfp	Cl	40	79±0.85	4.5±1.5	–
Me	Me	CH_2Cl	Cl	40	65±2.7	5±2.1	–
Me	Me	Cl	Cl	16	85±4.9	2±0	–
$CH_2CHSiMe_2Cl$[c]	Me	Me	Cl	20	78±3.5	3±1.4	–
Et	Et	Et	Cl	64	35±4.5	39±8.2	–
*t*Bu	Ph	Ph	Cl	64	–	55±4.1	24±5.8

[a] Activation was carried out by stirring for a minimum of 16 h at RT after addition of 1 mmol of a silyl activating agent $R^1R^2R^3SiX$ to 0.25 mmol of **1** in 3 mL of dioxane. Upon clarification of the reaction solution, **2a** was added and the reaction was stirred for an additional 24 h before sampling. Yields are based on LCMS analysis and are an average of at least two runs. Adduct yields are reported as the sum of mixed isomers. [b] Polyphosphate. [c] Entry refers to 1,2-bis(chlorodimethylsilyl)ethane.

due to the relatively electron-poor nature of **2a**, which is in resonance with a thiourea form.

We propose that the mechanism of dithioacetal formation involves a fast initial reaction of the sulfoxide **1** with TMSCl to generate the adduct **8** shown in Scheme 2, which could then either first undergo chloride exchange to form species **9** or lose TMSOH directly to form thionium ion **10**. The dependence of dithioacetal **3a**+**4a** formation on the presence of a halide ion, preferably a chloride ion, suggests that the active species is either the chloride-thionium ion pair **10** or quite possibly the α-chloro thioether **11**. The latter species has been invoked previously,[20] and Jung et al observed incorporation of a chloride ion into an aromatic ring under Pummerer rearrangement conditions.[27] Both of these examples used the highly reactive thionyl chloride.

To empirically examine the nature of the active intermediate, we carried out the reaction on **1** that had been ^{13}C-labeled at the methyl group of the side chain and monitored the reaction by ^{13}C NMR spectroscopy. The parent sulfoxide ^{13}C-enriched resonances appeared at 38.86 and 38.78 ppm presumably owing to diastereomeric oxidation of the parent **7**. Treatment with excess TMSCl in deuterated THF for 2 h at 40 °C yielded roughly equivalent new signals at 50.6, 13.5, and 14.1 ppm. This result is consistent with formation of α-chlorinated thioethers **11a**+**11b** as a statistical mixture either at the ^{13}C-enriched methyl (50.6 ppm) or at the interior C3 methylene (13.50, 14.1 ppm). A trace of presumed thioether **7** was also observed at 15.3 ppm. Subsequent condensation of the reaction with excess **2a** saw these peaks migrate to 40.2, 13.8, and 14.5 ppm, respectively; consistent with formation of one primary (**3a**) and two secondary (diastereomeric, **4a**) dithioacetals. A similar experiment was carried out using unlabeled DMSO as a substrate. The initial ^{13}C NMR showed a single signal at 42.0 ppm, which gave rise to a split signal at 53.00/52.99 ppm as well as a signal at 15.0 ppm after 20 h of TMSCl treatment. Condensation with **2a** resulted in peaks at 42.7 and 15.4 ppm. ^{13}C NMR spectra for both model systems are shown in Figure S1.

To shed light on the mechanisms and energetic profiles of the synthesis processes described above, we performed quantum chemistry calculations on the Pummerer rearrangement of DMSO using the density functional theory (DFT) method with the hybrid *meta*-GGA M11 functional. All reported results represent the M11/cc-pVTZ level of theory (see the Experimental Section for full details).

We first investigated the initial step, reaction of DMSO with TMSCl, in order to elucidate: 1) The highest energy barriers, or "bottlenecks," that must be overcome, and 2) which active species product, chloride–thionium ion pair or α-chloro thioether, is produced. Geometry optimizations and subsequent vibrational frequency calculations (to confirm true minima and transition states) were performed in gas and tetrahydrofuran (THF) solvent phases. Figure 1 illustrates the full computed energy profile for this initial activation process. Note that gas-phase structures are shown where transition states have been definitively mapped to their corresponding neighboring minima.

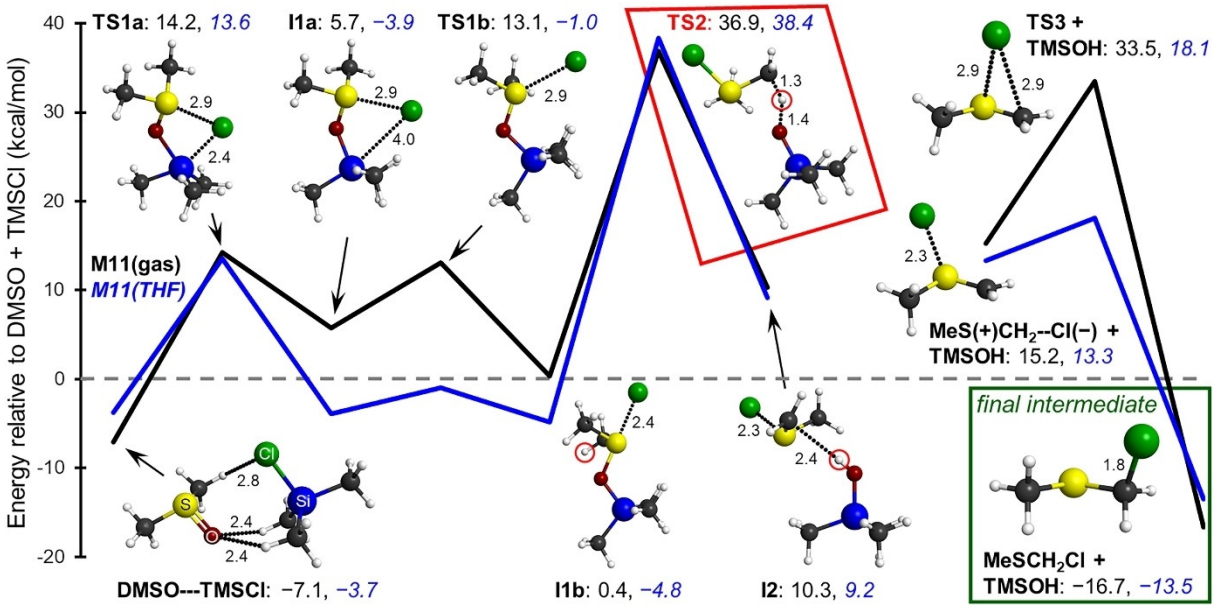


Figure 1. Computed reaction mechanism and energy profiles describing the initial activation process leading to formation of α-chlorinated thioether: DMSO + TMSCl → $MeSCH_2Cl$ + TMSOH. Calculations were performed using the M11/cc-pVTZ level of theory in gas (black lines and text, structures shown) and THF solvent (blue lines and italic text) phases. All energies (kcal mol^{-1}) are relative to separated reactants DMSO + TMSCl. The main points to note are: 1) The pathway to thioether formation progresses through four distinct transition states and three intermediates, 2) the proton transfer step is predicted to be rate-limiting, having computed barriers of 37 (gas) and 38 (THF) kcal mol^{-1}, and 3) as shown at far right, upon formation of TMSOH and the chloride-thionium ion pair MeS(+)CH_2–Cl(−), the isolated latter species should convert into the much more stable α-chlorinated thioether $MeSCH_2Cl$. Selected bond lengths [Å] are also shown.

The activation process is observed to proceed through three intermediates, and thus four distinct transition states. The final energetically most stable product is the α-chloro thioether. This computed mechanism affirms that shown in Scheme 2; however, important details are garnered from the quantum chemistry results. One of these is that the rate-limiting step, or bottle-neck, is the proton transfer, shown as TS2 in Figure 1, with computed barriers of 37 (gas) and 38 (THF) kcal mol^{-1} (relative to separated reactants DMSO and TMSCl). The best experimental thioacetal yields were obtained with aprotic ethereal solvents (Table 2) which cannot participate directly in proton exchange. While ethereal solvents may participate in hydrogen bonding, thereby slightly lowering the energy of the proton transfer transition state, stabilization of minima is also likely. The slight increase in energy barrier (one kcal mol^{-1}) when going from gas to THF-solvent phase suggests that the hydrogen bonding capabilities of the solvent do not appreciably facilitate the proton transfer step.

Thus, the only real way to go from TMSO-S(+)Me_2 to TMSOH + MeS(+)=CH_2 is via intramolecular proton transfer, as exemplified by TS2. We also find that for the portion of the activation process leading to TMSOH–MeS(+)=CH_2 (I2), solvent effects (THF) are negligible for four of the stationary points, including the rate-limiting proton transfer step, but stabilize the other species (I1a, TS1b, I1b) by 5 to 12 kcal mol^{-1}.

The other key point is that upon formation of the chloride-thionium ion pair, conversion into the substantially more stable α-chloro thioether ($MeSCH_2Cl$) should readily occur. The computed gas-phase barrier of 34 kcal mol^{-1} is lowered to 18 kcal mol^{-1} when modeled in THF solvent. This step is illustrated at far right of Figure 1 where TMSOH was considered a spectator species. We speculate that if the proton transfer energy barrier can be overcome, then formation of α-chloro thioether ($MeSCH_2Cl$) product is almost certain. Because the separated products TMSOH + $MeSCH_2Cl$ are computed to be more stable than the separated reactants by 17 (gas) and 14 (THF) kcal mol^{-1}, the complete exothermic activation process is highly unlikely to be reversible because the backward proton transfer energy barrier is over 50 kcal mol^{-1}. These quantum chemistry results nicely account for the experimental observations, particularly the slowness of reaction and irreversibility. They also indicate that the α-chloro thioether is produced, rather than ion pair, in line with the ^{13}C NMR results.

Next, we determined reaction energies in THF solvent for the final step, formation of thioacetals: RSH + $MeSCH_2Cl$ → $RSCH_2SMe$ + HCl. We investigated the four thiols 2-mercaptoimidazole (**2a**), 2-mercaptopyridine (**2d**), 4-mercaptopyridine (**2e**), and 2-mercaptopyrimidine (**2i**). Full potential energy surface (PES) scans were performed using the cc-pVDZ basis sets to discover lowest-energy structures for separated reactants and products. Their geometries were then optimized and vibrational frequencies computed using the cc-pVTZ basis sets. The results are shown in Figure 2 where electronic reaction energies (ΔE), lying between −6.5 and −5.3 kcal mol^{-1}, indicate favorable thioacetal formation. However, free energies of reaction (ΔG_{298}) are less exothermic, with products computed to be only slightly more stable than reactants by 3.8 to 2.4 kcal mol^{-1}. There are many complex factors that can shift these reaction energies up/down by a few kcal mol^{-1}, and thus cause experimental yields to vary significantly, as seen in Table 1.

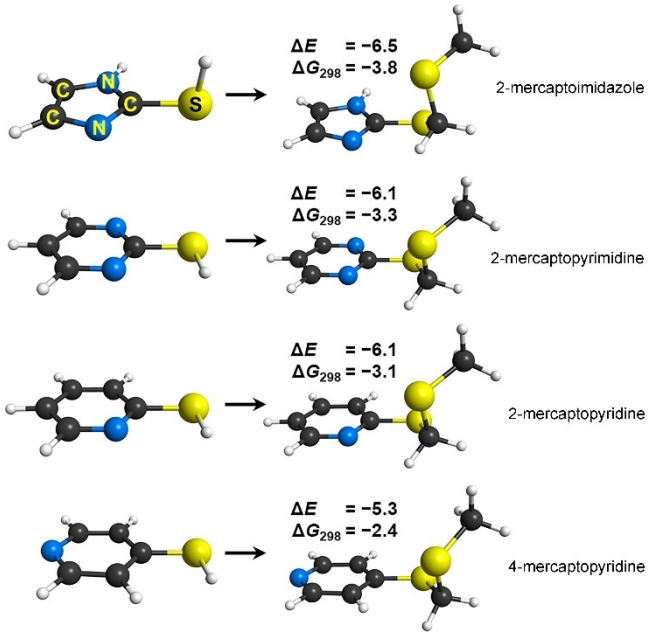


Figure 2. Computed structures and energetics for conversions of four thiols to dithioacetals: $RSH + MeSCH_2Cl \rightarrow RSCH_2SMe + HCl$. The M11(THF)/cc-pVTZ level of theory was used, electronic (ΔE) and free (ΔG_{298}) energies of reaction are given in kcal mol^{-1}.

These factors include reaction conditions, explicit solvent interactions, and reactant-reactant/product-product complex formations. As such, all we can really surmise from the computed results is that thioacetal formation is barely exothermic and that actual experimental yields may vary due to other factors that are difficult to model with kcal mol^{-1} accuracy.

Having investigated the optimal conditions for dithioacetal formation in small molecules, we turned our attention to using the optimized method for labeling methionine sulfoxide residues in peptides. Conversion of sulfoxides to dithioacetals offers a stable mass tag to differentiate oxidized methionine residues definitively from the thioether state. We chose the peptide met-enkephalin (H-Tyr-Gly-Gly-Phe-Met-OH, **12**) for our study. The peptide was oxidized with $NaIO_4$ to the sulfoxide form **13**. The sulfoxide-containing peptide **13** was then activated for a minimum of two days in 10% (*v*/*v*) TMSCl in dioxane, followed by addition of solid 2-mercaptoimidazole(**2a**) and stirring for at least 24 h. In the case of the oxidized peptide, more than 70% of peptide-associated absorbance at 220 nm corresponded to a mass of 672, consistent with the expected dithioacetal adduct **14a** + **15a**. Approximately 17% of the signal corresponded to reduction to methionine thioether form **12**. In a control reaction using the parent peptide, >90% of 220 nm absorbance corresponded to a mass of 574 consistent with the starting peptide **12** (Figure S2). Approximately 6% of peptide absorbance corresponded to dithioacetal adducts **14a** + **15a**, presumably formed after air oxidation of **12** to **13**. The use of an inert atmosphere was found to be useful in suppressing background dithioacetal formation. The requirement for ethereal solvents may not be compatible with the solubility of some proteins. However, prior fragmentation with trypsin, pepsin, or other proteases commonly employed in protein chemistry may enable Pummerer-based dithioacetal formation of the resulting fragments. In addition, TMSCl is expected to transiently protect many polar groups, such that minimal initial solubility may be sufficient to ultimately enable reaction. Although total conversion of the peptide will be less than indicated by the absorbance percentage, owing to contribution of the thioimidazole substituent at 220 nm, our protocol presents clearly differentiable results between otherwise-identical peptides containing either sulfoxide or thioether functionality.

Conclusion

We have demonstrated that the Pummerer rearrangement can be used to covalently label oxidized methionine side chains with good efficiency. We have described the use of silyl chlorides, especially TMSCl, to convert alkyl sulfoxides into intermediate α-chlorinated thioethers, which subsequently undergo chlorine displacement by thiol nucleophiles. The efficacy of a variety of thiol nucleophiles has been investigated, and 2-mercaptoimidazole and 2-mercaptopyridine proved to be most effective. Ethereal solvents were shown to be optimal for dithioacetal formation. The α-chlorinated intermediates were investigated by computation and by ^{13}C NMR spectroscopy. Finally, we have demonstrated the use of our optimized conditions to selectively label a peptide containing an oxidized methionine residue while the corresponding unoxidized peptide remained essentially unlabeled. It should be possible to raise an antibody specific for the dithioacetal derivative. Such an antibody could be used to detect and quantitate methionine sulfoxide in proteins by immunochemical methods or for affinity purification of derivatized peptides prior to mass spectrometric sequencing. We anticipate that this approach can be used to identify peptides with oxidized methionine residues by tagging them with a thiol nucleophile functionalized with mass or other reporter groups.

Experimental Section

General: Reagents were purchased from commercial sources and used without further purification. Fmoc-methionine ^{13}C-methyl was purchased from Cambridge Isotope Laboratories. Met-Enkephalin was obtained from Chempep Inc. NMR spectra were obtained on a 400 MHz Varian NMR and processed using MestReNova software. LCMS data for small molecules were acquired on an Agilent Technologies 1290 Infinity HPLC system using a 6130 quadrupole LC/MS detector and a Poroshell 120 SB-C18 2.7 um column (4.6× 50 mm). Peptides were analyzed using an Agilent 1200 series HPLC system with an LC/MSC Trap XCT detector and a Zorbax 300SB-C18 3.5 um column (4.6×50 mm). Preparative HPLC chromatography was performed on a Shimadzu system using a 30 mm× 150 mm Zorbax SB C18 column (Agilent) or a 19 mm×150 mm Xbridge C4 column (Waters). Flash chromatography was performed on a Teledyne Isco Combiflash R_f+ instrument using hexane/ethyl acetate gradients. HRMS data were acquired on a Waters XEVO G2-XS QTOF running MassLynx version 4.1.

Quantum chemistry: The computations in this study were executed using the GAMESS[28,29] package and molecular structures were

illustrated using MacMolPlt.[30] We used the density functional theory (DFT)[31] method with the hybrid *meta*-GGA M11 functional.[32] Calculations were performed in gas and tetrahydrofuran (THF) solvent phases, and the latter was accomplished using the Polarizable Continuum Model (PCM)[33–36] (with a high density of tesserae: NTSALL = 960 in $TECAV). Geometries were optimized (maximum Cartesian gradient $< 10^{-4}$ Hartree/Bohr) using analytic gradients and Hessians were computed seminumerically (double differences) using analytic gradients. The cc-pVDZ basis sets[36,37] were used to probe potential energy surfaces for locations of lowest-energy conformations and transition state structures (having exactly one imaginary frequency). Refined optimized geometries and subsequent Hessians were computed using the cc-pVTZ basis sets[36,37] and minimum energy paths connecting transition states to corresponding reactant/product minima were determined using the second-order intrinsic reaction coordinate (IRC) method of Gonzalez and Schlegel.[38] All data presented and discussed in the manuscript represent the M11/cc-pVTZ level of theory. Structures and energies (electronic (E) and free (G_{298})) of all stationary points described are given in the Supporting Information.

Synthesis

General procedure for dithioacetal formation: TMSCl 127 μL (108 mg, 1 mmol) was added to a stirred solution of sulfoxide (0.25 mmol) in 3 mL of dioxane. The reaction was stirred for at least 24 h at RT, at which point the thiol nucleophile (1.04 mmol) was added. The reaction was stirred for an additional 24 h at RT. Neutralization with 1 M triethylammonium bicarbonate solution was followed by either preparative HPLC or extraction and flash chromatography purification to afford the pure material.

S-(((1*H*-imidazol-2-yl)thio)methyl)-*N*-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-L-homocysteine (3 a): White amorphous solid. ^{1}H NMR (CD_3OD): $\delta = 7.80$ (dd, $J = 7.6$, 3.1 Hz, 2H), 7.67 (t, $J = 7.6$ Hz, 2H), 7.62 (d, $J = 2.7$ Hz, 2H), 7.39 (t, $J = 7.4$ Hz, 2H), 7.35–7.26 (m, 2H), 4.46–4.27 (m, 5H), 4.24 (d, $J = 7.2$ Hz, 1H), 3.37–3.27 (m, 2H), 2.84 (s, 1H), 2.76 (q, $J = 6.8$, 6.1 Hz, 1H), 2.22–2.14 (m, 1H), 1.99 ppm (s, 1H); ^{13}C NMR{^{1}H} ($CD_3CN + D_2O$): $\delta = 174.6$, 157.6, 144.5, 141.8, 138.8, 128.5, 127.9, 125.9, 122.2, 120.7, 67.3, 53.4, 47.6, 39.8, 31.2, 28.1 ppm; HRMS: calcd for $C_{23}H_{24}N_3O_4S_2^+$: 470.1208; found 470.1206.

General procedure for condensation of DMSO with thiol nucleophiles: TMSCl 127 μL (108 mg, 1 mmol) was added to a stirred solution of dimethyl sulfoxide (19.5 mg, 17.7 μL, 0.25 mmol) in 3 mL of dioxane. The reaction was stirred for 24 h at RT, and the thiol nucleophile **2** (1.04 mmol) was then added. The reaction was stirred for an additional 24 h at RT. Neutralization with 1 M triethylammonium bicarbonate solution was followed by either preparative HPLC or extraction and flash chromatography purification to afford the pure material.

2-(((Methylthio)methyl)thio)-1*H*-imidazole (5 a): 28.2 mg white solid, 70%; ^{1}H NMR (CD_3OD): $\delta = 7.62$ (s, 2H); 4.33 (s, 2H); 2.27 ppm (s, 3H); ^{13}C{^{1}H} NMR (CD_3OD): $\delta = 140.3$, 123.2, 43.2, 15.2 ppm; HRMS (ESI/QTOF) m/z: calcd for $C_5H_9N_2S_2$: 161.0207 $[M+H]^+$; found 161.0207.

2-(((Methylthio)methyl)thio)nicotinic acid (5 b): 3.2 mg white solid, 6.1%; ^{1}H NMR (CD_3CN): $\delta = 8.54$ (ddd, $J = 4.8$, 1.9, 0.6 Hz, 1H), 8.23 (ddd, $J = 7.8$, 1.8, 0.5 Hz, 1H), 7.18 (dd, $J = 7.8$, 4.8 Hz, 1H), 4.25 (s, 2H), 2.15 ppm (s, 3H); ^{13}C{^{1}H} NMR (CD_3CN): $\delta = 168.3$, 161.3, 153.1, 140.5, 124.9, 120.3, 35.8, 15.9 ppm; HRMS (ESI/QTOF) m/z: calcd for $C_8H_{10}NO_2S_2$ 216.0153 $[M+H]^+$; found 216.0152.

6-(((methylthio)methyl)thio)nicotinic acid (5 c): 10.3 mg white solid, 19%; ^{1}H NMR (CD_3CN): $\delta = 8.96$ (dd, $J = 2.2$, 0.9 Hz, 1H), 8.08 (dd, $J = 8.4$, 2.2 Hz, 1H), 7.35 (dd, $J = 8.4$, 0.9 Hz, 1H), 4.39 (s, 2H), 2.21 ppm (s, 3H); ^{13}C{^{1}H} NMR (CD_3CN): $\delta = 166.6$, 164.7, 151.6, 138.1, 123.3, 122.6, 36.1, 15.7 ppm; HRMS (ESI/QTOF) m/z: calcd for $C_8H_{10}NO_2S_2$: 216.0153 $[M+H]^+$; found 216.0152.

2-(((Methylthio)methyl)thio)pyridine (5 d):[39] Colorless liquid, 33.3 mg, 78%; ^{1}H NMR ($CDCl_3$): $\delta = 8.46$ (ddd, $J = 4.9$, 1.9, 1.0 Hz, 1H), 7.51 (ddd, $J = 8.1$, 7.4, 1.9 Hz, 1H), 7.22 (dt, $J = 8.0$, 1.0 Hz, 1H), 7.02 (ddd, $J = 7.3$, 4.9, 1.1 Hz, 1H), 4.37 (s, 2H), 2.25 ppm (s, 3H); ^{13}C{^{1}H} NMR ($CDCl_3$): $\delta = 157.9$, 149.7, 136.3, 122.8, 120.1, 35.9, 15.7 ppm; HRMS (ESI/QTOF) m/z: calcd for $C_7H_{10}NS_2$: 172.0255 $[M+H]^+$; found 172.0254.

4-(((Methylthio)methyl)thio)pyridine (5 e): Colorless liquid, 23.9 mg, 56%; ^{1}H NMR ($CDCl_3$): $\delta = 8.45$ (s, 2H), 7.25–7.14 (m, 2H), 4.09 (s, 2H), 2.27 ppm (s, 3H); ^{13}C{^{1}H} NMR (CD_3CN): $\delta = 161.0$; 142.4, 123.6; 37.0, 15.7 ppm; HRMS (ESI/QTOF) m/z: calcd for $C_7H_{10}NS_2$: 172.0255 $[M+H]^+$; found 172.0253.

2-(((Methylthio)methyl)thio)benzo[*d*]thiazol-6-amine (5 f): Yellowish solid, 5.4 mg, 9%; ^{1}H NMR ($CDCl_3$): $\delta = 7.68$ (dt, $J = 8.7$, 0.5 Hz, 1H), 7.03 (dt, $J = 2.4$, 0.5 Hz, 1H), 6.79 (ddd, $J = 8.7$, 2.3, 0.4 Hz, 1H), 4.42 (s, 2H), 3.79 (s, 2H), 2.29 ppm (s, 3H); ^{13}C{^{1}H} NMR ($CDCl_3$): $\delta =$ 160.3, 146.8, 144.2, 137.5, 122.6, 115.5, 105.7, 39.9, 15.9 ppm; HRMS (ESI/QTOF) m/z: calcd for $C_9H_{11}N_2S_3$: 243.0084 $[M+H]^+$; found 243.0082.

2-(((Methylthio)methyl)thio)-1*H*-benzo[*d*]imidazole (5 g): White solid, 22 mg, 42%; ^{1}H NMR (CD_3OD): $\delta = 7.48$ (br s, 2H); 7.18–7.23 (m, 2H); 4.40 (s, 2H); 2.25 ppm (s, 3H); ^{13}C{^{1}H} NMR (CD_3OD): $\delta =$ 150.5, 124.1, 123.7, 111.0, 39.8, 15.4 ppm; HRMS (ESI/QTOF) m/z: calcd for $C_9H_{11}N_2S_2$: 211.0364 $[M+H]^+$; found 211.0364.

2-(((Methylthio)methyl)thio)pyrimidine (5 i): White solid, 9.0 mg, 21%; ^{1}H NMR (CD_3CN): $\delta = 8.56$ (d, 2H, $J = 4.9$); 7.12(t, 1H, $J = 4.9$); 4.33 (s, 2H); 2.21 ppm (s, 3H); ^{13}C{^{1}H} NMR (CD_3OD): $\delta = 172.6$, 158.9, 118.5, 37.3, 15.6 ppm; HRMS (ESI/QTOF) m/z: calcd for $C_6H_9N_2S_2$: 173.0207 $[M+H]^+$; found 173.0200.

2-(((Methylthio)methyl)thio)-4,5-dihydrothiazole (5 k): Colorless liquid, 20.6 mg, 46%; ^{1}H NMR ($CDCl_3$): $\delta = 4.36$–4.14 (m, 4H), 3.41 (t, $J = 8.0$ Hz, 2H), 2.24 ppm (s, 3H); ^{13}C{^{1}H} NMR ($CDCl_3$): $\delta = 165.1$, 64.4, 38.2, 35.7, 15.9 ppm; HRMS (ESI/QTOF) m/z: calcd for $C_5H_{10}NS_3$: 179.9975 $[M+H]^+$; found 179.9981.

2-(((Phenylthio)methyl)thio)-1*H*-imidazole (6 a): TMSCl 127 μL (108 mg, 1 mmol) was added to a stirred solution of methyl phenyl sulfoxide (35 mg, 0.25 mmol) in 3 mL of dioxane. The reaction was stirred for 24 h at RT, then 2-mercaptoimidazole (104 mg, 1.04 mmol) was added. The reaction was stirred for an additional 24 h at RT. Neutralization with 1 M triethylammonium bicarbonate solution was followed by preparative HPLC (5% MeCN 2 min, then ramp to 50% MeCN over 13 min), concentration of the appropriate fractions, and lyophilization to afford 30 mg (54%) of the pure material as a white solid. ^{1}H NMR (CD_3CN): $\delta = 7.43$–7.46 (m, 2H); 7.28–7.27 (m, 5H); 4.70 ppm (s, 2H); ^{13}C{^{1}H} NMR (CD_3OD): $\delta =$ 140.0, 134.3, 132.3, 130.7, 129.2, 123.4, 42.4 ppm; HRMS (ESI/QTOF) m/z: calcd for $C_{10}H_{11}N_2S_2$: 223.0364 $[M+H]^+$; found 223.0369.

[^{13}C]Methyl Fmoc-methionine sulfoxide: Sodium periodate (7.1 mg, 1.2 equiv) was added to a stirred solution of ^{13}C-methyl Fmoc-methionine (10.3 mg, 27.7 μmol) in 4 mL of 50% aqueous MeOH, and the reaction mixture was stirred for 16 h at RT. Saturated ammonium chloride solution was added and the aqueous layer was extracted with 3×4 mL CH_2Cl_2. The combined organic layers

were dried over Na_2SO_4 and evaporated to yield ^{13}C-methyl Fmoc-methionine oxide (white solid, 10.7 mg, quantitative).

^{13}C NMR experiment

[^{13}C]Methyl Fmoc-methionine oxide (10.7 mg, 27.7 μmol) was dissolved in [D_8]THF (1 mL), and the ^{13}C NMR spectrum was acquired. TMSCl (50 μL, 13.9 equiv) was added, and the reaction mixture was heated at 40 °C. After 2 h, the reaction mixture was cooled, and the ^{13}C NMR spectrum was acquired. After returning the NMR sample to the reaction, mercaptoimidazole (39.8 mg, 14 equiv) was added and the reaction was stirred at RT for 24 h. The ^{13}C NMR spectrum of the reaction was then acquired once again.

Met-enkephalin sulfoxide (13): Met-Enkephalin (**12**, 20 mg, 35 μmol) was dissolved in 1 mL of deionized water, and sodium periodate (9.0 mg, 42 μmol) was added. The reaction mixture was stirred overnight, then neutralized with AcOH. The desired oxidized peptide was purified by preparative HPLC on a Waters C4 column using a gradient of 10→45 % MeCN (0.05 % TFA) in water (0.05 % TFA); 13.3 mg of pure material was obtained after lyophilization.

Pummerer reaction of met-enkephalin: Met-enkephalin sulfoxide (**13**, 0.6 mg, 1 μmol) and met-enkephalin (**12**, 0.6 mg, 1 μmol) were each suspended in 0.1 mL of a solution of TMSCl in dioxane (10 % *v/v*) that had been degassed by passing Ar through for 30 s. The suspension was stirred for two days at RT, at which point 2 mg of 2-mercaptoimidazole were added to each vial. The reaction was stirred further, and samples were withdrawn and diluted in H_2O/MeCN prior to LCMS analysis. Integration of the 220 nm trace was performed between 4 and 6 min, and peaks were assigned based on mass analysis.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: oxidoreductases · protein modifications · Pummerer rearrangement · reaction mechanism · sulfoxides

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