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COMMUNICATION

A sulfoxide-based isobaric labelling reagent for accurate quantitative mass spectrometry

Michael Stadlmeier[a],+, Jana Bogena[a],+, Miriam Wallner[a],+, Martin Wühr[b] and Thomas Carell[a],*

Abstract: *Modern proteomics requires reagents for exact quantification of peptides in complex mixtures. Peptide labelling is most typically achieved with isobaric tags that consist of a balancer and a reporter part that separate in the gas phase. An ingenious distribution of stable isotopes provides multiple reagents with identical molecular weight but a different mass of the reporter groups, allowing relative quantification of multiple samples in one measurement. Current generation reagents require a high fragmentation energy for cleavage, leading to incomplete fragmentation and hence loss of signal intensity. Here we report a new isobaric labelling reagent, where the balancer and the reporter are linked by a sulfoxide group, which, based on the sulfoxide pyrolysis, leads to easy and asymmetric cleavage at low fragmentation energy. The fragmentation of our new design is significantly improved, yielding more intense complementary ion signals, allowing complementary ion cluster analysis as well.*

After the development of new genome sequencing methods that allow human genomics studies in just a few hours,[1] today, we are witnessing the emergence of novel mass spectrometry methods that enable the investigation of the complete proteomes of cells and tissues.[2-4] The proteome is defined as the collection of all proteins present in a sample and hence proteomes differ dramatically from cell type to cell type and in different tissues.[5] Proteomics data therefore provide fingerprint-type information about cellular situations and potentially existing disease states.[6] To gain deep insight into the proteome of biological systems, it is necessary to obtain quantitative information about the levels of the individual proteins in the different samples. Nowadays, this is performed with mass spectrometry. Since exact quantification of intact proteins is difficult, the proteomes need to be digested (trypsinated) to give the corresponding peptides. For quantification of these peptides, methods such as metabolic labelling,[7-8] label-free quantification[9] or isobaric labelling[10-11] are performed. Since isobaric labelling is able to reveal even small differences in peptide abundances and because many samples can be compared in one measurement, it is one of the most commonly used quantification methods.[12] A typical proteomics experiment illustrating the procedure is shown in Fig. 1A.

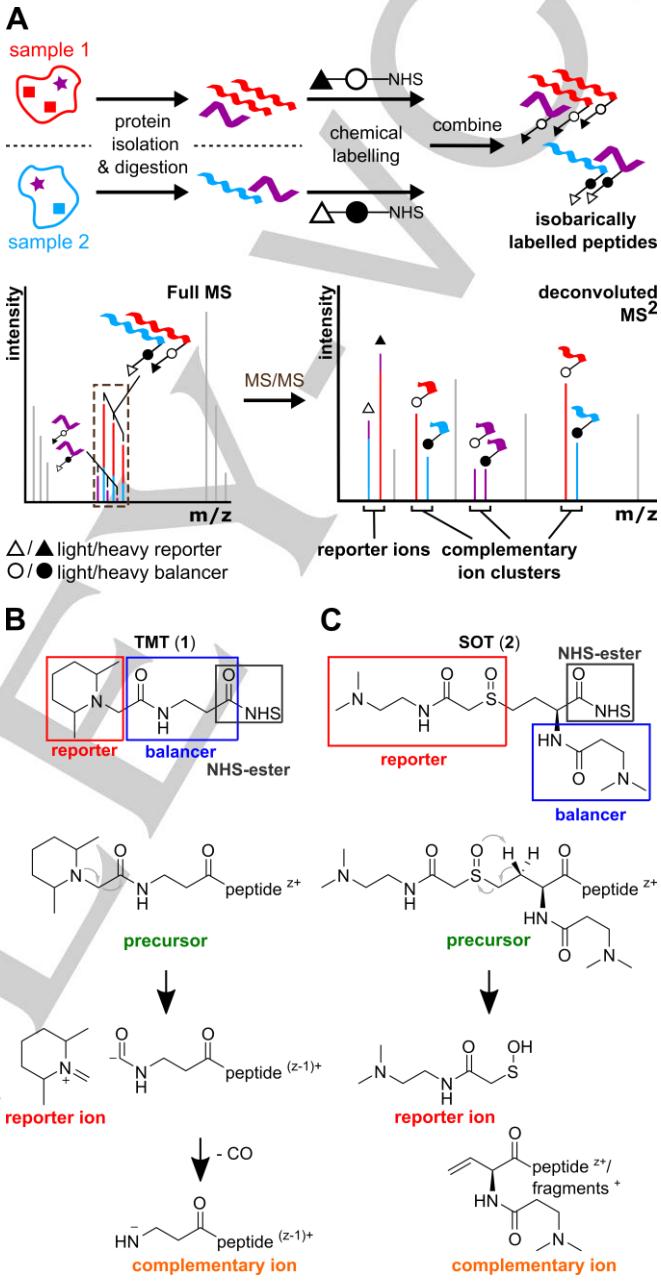

Figure 1. A) Isobaric labelling experiment for quantitative proteomics. The samples are individually labelled with isotopologues of the reagents **1** or **2** and combined for LC-MS² studies. Fragmentation in the gas phase yields reporter and complementary ions for relative quantification. The reporter ion ratio is distorted by co-isolated peptides (purple). The complementary ion clusters can be analysed without such a distortion. B) Currently used TMT (**1**) reagent. Fragmentation introduces a negative charge on the balancer, reducing the overall charge state on the complementary ions. C) SulfOxide Tag (SOT**,** **2**) reported in this study with a charge-neutral fragmentation that retains all charges on the complementary ions to facilitate fragmentation.

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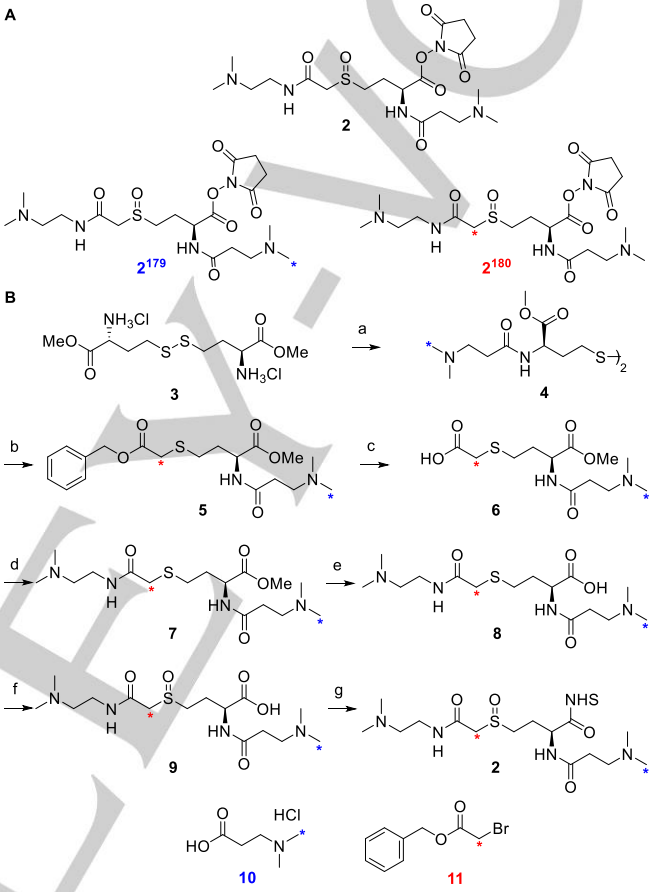
The proteomes of two samples are isolated, digested and the peptides are reacted with an isobaric labelling reagent such as the Tandem Mass Tag (TMT) reagent **1** (Fig. 1B) or the new labelling reagent **2** (Fig. 1C) reported in this study. The prepared derivatised peptide mixtures are next combined and the mixture is analysed by HPLC-MS[13] or even CE-MS.[14-15] During separation, the same peptides derived from the two samples (blue and red in Fig. 1) will feature the same retention time due to the isobaric character of the labels. They will consequently enter the mass spectrometer at the same time, leading to one indistinguishable m/z-value in the full MS-scan. This allows performing mass spectrometry-based identification by selecting a single precursor m/z for fragmentation (MS^2). Cleavage of the isobaric labels provides two now different reporter ions (Δ, Fig 1A), which allows relative quantification. The sensitivity of the methods depends on the cleavage efficiency.

In addition to a reporter ion, a balancer-peptide conjugate, the complementary ion, is also generated from each peptide. Because the attached balancer retains the distinct isotope pattern from the isobaric labelling reagent, the complementary ions allow quantification as well.[16] This even has the advantage that co-eluting peptides (purple), which give reporter ions indistinguishable from the reporter signals of interest, cannot disturb the signal. This problem, known as ratio distortion, often hinders accurate quantification based on reporter ion analysis.[17] Again, sensitivity is determined by cleavage efficiency and this is a drawback of the contemporary reagents.[16]

Here we report the development of a new SulfOxide Tag reagent **2** (SOT, Scheme 1 A) that fragments more easily, which improves quantitation. Importantly, the reagent features two basic *tert*-amino groups, which are protonated in the gas phase. This avoids formation of neutral species during reagent cleavage and it increases the charge density, which facilitates fragmentation. The SOT reagent **2** design allows the introduction of up to eight heavy stable isotopes into the structure generating reporters with Δm/z = 1, while keeping isobaricity. This design was chosen to enable mass spectrometric quantification of nine different samples in parallel with one single measurement. The proposed structures of this higher 9-plex reagents are shown in the SI (Supporting Fig. 3).

The synthesis of reagent **2** and of two isobaric isotope derivatives ($\mathbf{2}^{179}$ and $\mathbf{2}^{180}$), which feature different reporter ion molecular weights (179 Da and 180 Da), is shown in Scheme 1B. To our knowledge, the masses of the generated reporter ions do not coincide with immonium- or other frequently observed fragment ions. The synthesis (Supporting Information) starts with the methylester of the homocystine dimer **3**, which is first converted with dimethylamino propionic acid into the bis-amide **4**. Reduction of the disulfide and alkylation of the thiol with benzyl bromoacetate furnishes the key intermediate **5**. Cleavage of the benzylester to **6** and reaction of **6** with 1,1-dimethylethylenediamine gives the bisamide **7**. Saponification of the methylester in **7** to **8**, oxidation of the sulfide to the sulfoxide **9** and conversion of the acid provides reagent **2** as the active ester. To access the isotopologues, we replaced the dimethylamino propionic acid by the similar compound **10** in which one methyl group carries a ^{13}C atom (SI) in a second synthesis. In a third synthesis, we used the ^{13}C-labelled benzyl bromoacetate **11** (SI). This gave the corresponding reagents $\mathbf{2}^{179}$ and $\mathbf{2}^{180}$ (Scheme 1A) in similar yields. The synthesis takes roughly one week, and the overall yield is ca. 12%. Storing the reagent in pure form or even in a stock solution (DMSO) is possible at -20 °C for several weeks.

To examine the fragmentation properties of SOT **2**, we digested a *HEK* lysate, containing all translated proteins into the corresponding peptides following a standard protocol (SI). The obtained complex peptide mixture (P) was divided into two portions. While one portion was reacted with reagent $\mathbf{2}^{179}$ (P-2^{179}) the other was combined with $\mathbf{2}^{180}$ to give P-2^{180} (pH = 8.5, 150 mM triethylammonium bicarbonate buffer, 1.5 mg $\mathbf{2}^{179}$ or $\mathbf{2}^{180}$, 60 min). We subsequently quenched unreacted reagent **2** with hydroxylamine. Next, the labelled mixtures {P-2^{179}+P-2^{180}} were combined in a 1:1 ratio, and the complex mixture was desalted and concentrated according to reported procedures.[18]



Scheme 1. A) Depiction of the new reagent **2** and of the isotopologues $\mathbf{2}^{179}$ and $\mathbf{2}^{180}$. B) a) 3-(Dimethylamino)propionic acid hydrochloride, NEt_3, HOBt, 60 °C, 2 h, 85%; b) Over two steps i) TCEP*HCl, $NaHCO_3$, H_2O/DMF (4:1), r.t., 10 min; ii) Benzyl bromoacetate, r.t., 2 h, 95%; c) 10% Formic acid in MeOH, 100 wt% Pd black, 40 °C, 2 h, 81%; d) *N*,*N*'-dimethylethane-1,2-diamine, DIPEA, PyBOP, DMF, 40 °C, 1 h, 73%; e) LiOH, MeOH/H_2O (2.5:1), r.t., 1 h, quant.; f) pH = 2, *m*CPBA, H_2O, r.t., 20 min, 75%; g) NHS-TFA, pyridine, DMF, r.t., 2 h, 35%. 179 Da and 180 Da are the molecular weights of the generated reporter ions.

For comparison, we performed the same experiment with the commercially available isotopically labelled TMT reagents **1** (duplex) according to manufacturer's recommendations to obtain the mixture {P-TMT^{126} + P-TMT^{127}}. The peptide mixtures {P-2^{179} + P-2^{180}} and {P-TMT^{126} + P-TMT^{127}} were next measured by nanoHPLC-MS^2 and the data were analysed using the MaxQuant software and a software package developed in-house (Supplementary Figure 1&2).[19] The obtained data are depicted in Fig. 2 and Fig. 3. As an example, Fig. 2A shows the cleavage of the SOT reagent **2** after reaction with the peptide $DLPEHAVLK^{2+}$ (bearing two labels) in direct comparison to

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the corresponding TMT labelled peptide in the complex mixture. We measured at a normalized fragmentation energy of 28% HCD (*higher-energy collisional dissociation*), which is ideally suited to fragment peptides for their identification. The SOT reagent **2** clearly generates higher intensity reporter ions and in addition, we observe more fragmentation, indicating that the reagent supports peptide fragmentation. Fig. 2B shows an analysis of all peptides identified in the SOT- and TMT-labelled sample and here, we see that in most MS²-spectra, the relative reporter ion intensity for the SOT-sample is as high as 70-100%, which is unprecedented. This enables easy relative quantification by determining reporter ion ratios with available software packages. In comparison, TMT-labelled peptides produce reporter ions of lower relative intensity at this HCD-energy. Fig. 2C shows the charge states of the intact labelled peptides before fragmentation (precursors). In agreement with our design, we see that the SOT reagent **2** generates labelled peptides with much higher charge states. Importantly, more than 65% of the labelled peptides have charge states equal to or above +3. This high charge density facilitates the subsequent fragmentation, which results in the formation of more complementary ions.

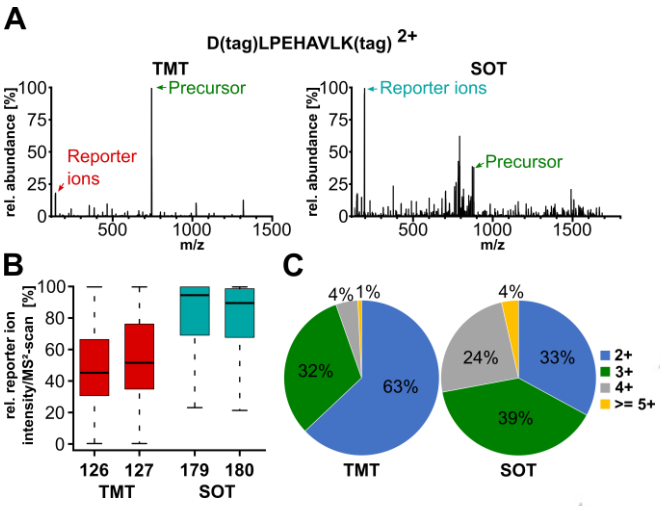

Figure 2. A) Comparison of fragmentation efficiency between TMT (**1**) and SOT (**2**) on a peptide. At a normalized collision energy of 28% HCD, the SOT-labelled peptide fragments more readily and yields high reporter ion signals. Tag = reacted reagent. B) Statistical analysis of the reporter ion relative intensities observed in the MS²-experiment. For SOT (**2**), the reporter ion exhibits an excellent visibility, facilitating reporter ion quantification. In comparison, TMT (**1**) produces reporter ions of lower intensity. C) Charge state distribution of precursor-peptides labelled with the TMT (**1**) or SOT (**2**) duplex. By using the SOT-reagent, higher charge states become more abundant, which should lead to more efficient fragmentation due to higher charge density.

While the reporter ion intensity allows measuring the relative ratios between peptides present in P-2^{180} versus P-2^{179}, it is desirable to quantify with the complementary ion clusters generated by the balancer-peptide conjugates.[16] Because these balancer-conjugates fragment further in the mass spectrometer, a large number of complementary ion clusters are formed, which can all be used for quantification. This provides higher data density and it reduces ratio distortion, because the complementary ions are sequence specific and are therefore distinguishable, unlike the reporter ions which are the same for all peptides. In our experiment, we observe that the SOT reagent **2** has ideal properties for such a complementary ion cluster analysis. When we studied the label-containing peptide fragment ions, we saw that 58% of these fragments still contained the intact reagent **2**, while 42% have lost the reporter group yielding complementary ion clusters (Fig. 3A).

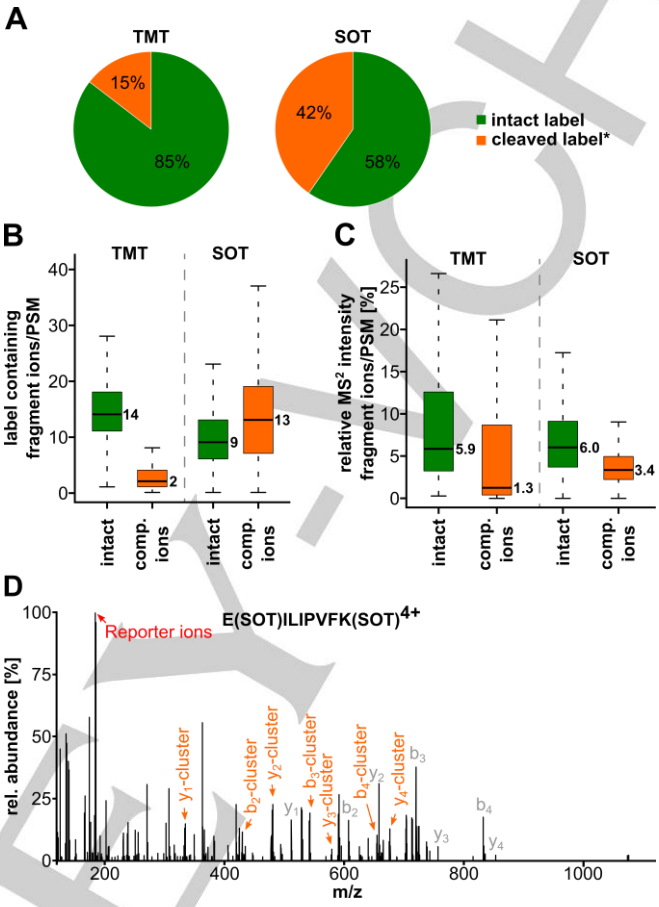

Figure 3. A) Ratio of all identified fragment ions containing either the intact label (reporter + balancer, green) or which show loss of the reporter ions, leading to the formation of complementary ion clusters (cleaved label, orange). *Because one intact label produces two cleaved labels in case of SOT (**2**) only, the amount of cleaved label containing fragments was divided by two to allow comparison with the TMT data. B) Statistical analysis of the quantity of labelled fragment ions per peptide spectral match (PSM). SOT-labelled peptides show a drastic increase in the number of complementary ions per spectrum compared to TMT-labelled peptides, resulting in 6–7 complementary ion clusters on average. C) Statistical analysis of the relative MS²-scan intensities of the labelled fragment ions per PSM. The median intensity of the complementary ions is elevated for SOT-labelled peptides when compared to the use of TMT. D) Example MS²-spectrum of the labelled peptide EILIPVFK^{4+}, depicting the reporter ions (red), seven complementary ion clusters (orange) and some fragment ions used for identification (grey).

This balanced ratio allows the parallel identification of the (intact) peptides with standard database search algorithms and quantification *via* the abundantly formed complementary ion clusters. In case of TMT, the amount of fragmentation is significantly lower and only 15% of all labelled fragment ions are cleaved. This holds true even when considering that the TMT-duplex complementary ions are indistinguishable, because they lose CO (containing the isotope marker) during fragmentation. Fig. 3B shows that the SOT reagent **2** creates on average 13 peptide-balancer fragments from every precursor for later quantification, which corresponds to 6–7 complementary ion clusters. For SOT-labelled peptides, there are not only more complementary ions compared to TMT-labelled peptides,

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but their median relative intensity in the respective MS²-spectra is also higher (Fig. 3C). An example spectrum is displayed in Fig. 3D. The signals for the reporter ions (red) and of seven complementary ion clusters (orange) are clearly visible with high relative intensities. For identification of the peptide, multiple high intensity ions are available (grey). Although the number of acquired MS^2-scans was comparable for the SOT- and TMT-samples (42169 for SOT, 46062 for TMT), the peptide identification rate was lower for SOT-labelled peptides (20% for SOT, 35% for TMT).

To investigate whether our new reagent reduces the ratio distortion effect, we performed an experiment in which a 1:1 labelled mixture of HEK-lysate served as a background. Into this background, a labelled bovine serum albumin (BSA) digest was added in a 4:1 ratio in a low quantity (Fig. 4A). This ensures that only a small amount of BSA peptides gets selected for isolation and fragmentation in a large background of 1:1 labelled human peptides. This should give a large ratio distortion. As observed previously in similar datasets with strong distortion,[16] the normalized median reporter ion ratio for the BSA peptides is 1.15 in case of the SOT-sample and 1.11 in case of the TMT-sample, showing the massive distortion of the ratio towards 1:1 (Fig. 4B). We next studied the same SOT-dataset regarding the complementary ion clusters of the BSA peptides.

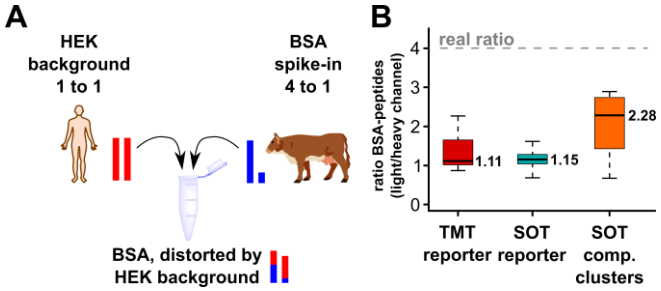

Figure 4. A) Experiment to show quantification of a protein with strong ratio distortion. HEK-lysate was labelled and mixed in a 1 to 1 ratio to serve as a background. Bovine serum albumin (BSA) was labelled and a 4 to 1 ratio was added into the background. Reporter ion intensities of BSA-peptides are highly distorted in the sample. B) Quantification results for measured BSA-peptides. Median reporter ion intensities of both TMT- and SOT-samples show a high distortion, leading to the impression of a nearly 1 to 1 ratio. Complementary ion cluster analysis results in a higher and therefore improved value of the BSA peptide ratio. Thus, SOT complementary ion cluster analysis reduces ratio distortion.

In 17 quantified peptides, 155 complementary ion clusters were detected and used for quantification. Analyzing their intensities provided a normalized median ratio of 2.3:1. The determined ratio is by a factor of 2 closer to the expected 4:1 ratio, showing the advantage of SOT complementary cluster analysis (SOT^c).

In summary, we report the design of a new isobaric labelling reagent that allows the efficient parallel formation of reporter ions and complementary ion clusters for peptide quantification. The reagent helps to reduce quantification errors caused by ratio distortion. Particularly attractive is the efficient formation of complementary ion clusters that results from fragmentations of the tag and the peptide backbone. These ion-clusters enable multiple quantification events per spectrum. Furthermore, due to the peptide-specific fragmentation, we expect the resulting quantifications to be even more robust against interference than when using the intact peptide complimentary ion cluster. This enables more accurate determination of relative peptide and hence protein abundances even in complex samples and might be particularly attractive for a targeted multiplexed approach.[20] The most important properties of the SOT reagent **2** are that reporter ions and complementary ion clusters are formed in parallel and that the introduction of multiple *tert*-amino groups generates the expected higher charge states which results in better peptide fragmentation. A current drawback is that the SOT reagent leads to identification of fewer peptides. The lower number is certainly caused by the currently available software, which is not yet optimized for the SOT reagent. In addition, the reagent increases the charge state, which could be another limiting factor. Software optimization and synthesis of a reagent that lacks the tertiary amine, can solve these problems. Research in this direction is ongoing.[21]

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Keywords: Peptide derivatization reagent, sulfoxide pyrolysis, isobaric labelling, quantitative mass spectrometry, complementary ion clusters, proteomics

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[21] For a sample of the new reagent **2** and the in house software package used to analyze the data please visit our homepage at www.carellgroup.de and contact us