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## Squaric acid as a new chemoselective moiety for mass spectrometry-based metabolomics analysis of amines†

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**The investigation of microbiome-derived metabolites is important to understand metabolic interactions with their human host. New methodologies for mass spectrometric discovery of undetected metabolites with unknown bioactivity are required. Herein, we introduce squaric acid as a new chemoselective moiety for amine metabolite analysis in human fecal samples.**

The gut is one of the most important organs of human metabolism. It harbours complex interconnected communities of bacteria, fungi, viruses, and yeast, which is collectively referred to as gut microbiota.<sup>1</sup> Within the past decade, an increasing number of studies has demonstrated the complexity of the gut microbiota and their impact on human physiology. The microbiome can also be considered as an additional organ or even a “second brain” as the gut-brain axis has been identified.<sup>2,3</sup> These microbial communities are highly metabolically active and exchange metabolites with other microbes and their host.<sup>4</sup> While metagenomic analyses rapidly elucidate details on microbial species and community changes, our understanding of the metabolism in the gut is still limited. Mass spectrometric-based metabolomics is the method-of-choice for investigation of this metabolic relationship and the elucidation of affected pathways. However, the majority of these metabolites are still unknown due to the complexity of metabolic processes in this interspecies communication and limited analytical methods.<sup>5,6</sup> The detailed elucidation of the metabolic exchange between the human host and its gut microbiota has a tremendous potential for the discovery of

small molecule biomarkers of diseases and bioactive compounds.<sup>7</sup>

Amine-containing metabolites have important roles in biological processes and this class of metabolites contains the largest percentage of gut microbiota-derived metabolites.<sup>8</sup> Especially, amino acids, short peptides as well as neurotransmitters are crucial metabolites for human physiology. However, it is estimated that many metabolites still need to be discovered in human fecal samples and their chemical structures elucidated.<sup>7</sup> The identification of these metabolite classes in fecal samples is very important to provide greater insight into gut microbiota metabolism associated with human health as this sample type contains a high abundance of gut microbiota-derived metabolites. Chemical modification is a useful technique to enrich metabolites and to enhance their detection.<sup>9–11</sup> However, the available chemical derivatization methods have to overcome matrix interferences in mass spectrometric analysis. For examples, *N*-hydroxysuccinimide (NHS) is the most commonly used moiety for labelling amine-containing metabolites but it rapidly hydrolyzes under neutral conditions. Other moieties have also been reported for investigation of amine metabolites such as methyl acetimidate and dansyl chloride.<sup>9</sup>

We have recently introduced a new methodology using chemoselective probes immobilized to magnetic beads for mass spectrometric analysis of human samples.<sup>12–14</sup> To gain access to additional amine metabolites, we sought to activate our chemoselective probe with a new and more stable moiety for chemoselective amine analysis. 3,4-Diamino analogues of squaric acid (squaramides) are utilized for selective amine conjugation in diverse research fields such as materials science, medicinal chemistry and sensors.<sup>15</sup> In addition, squaric acids are used for bioconjugation of macromolecules, specific cell labelling and bioisosteric replacement due to a high selectivity for reaction with amines under slightly basic conditions. Additionally, Tietze *et al.* reported the efficient use of squaric acid diesters for sequential conjugation of two amines with different structures in 1991.<sup>16</sup> However, this chemoselective

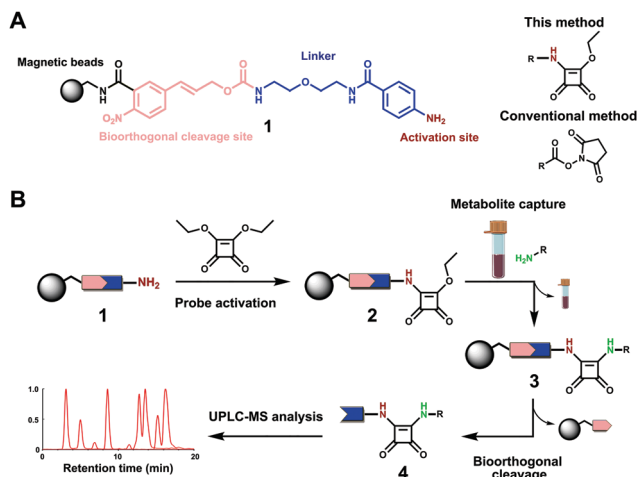
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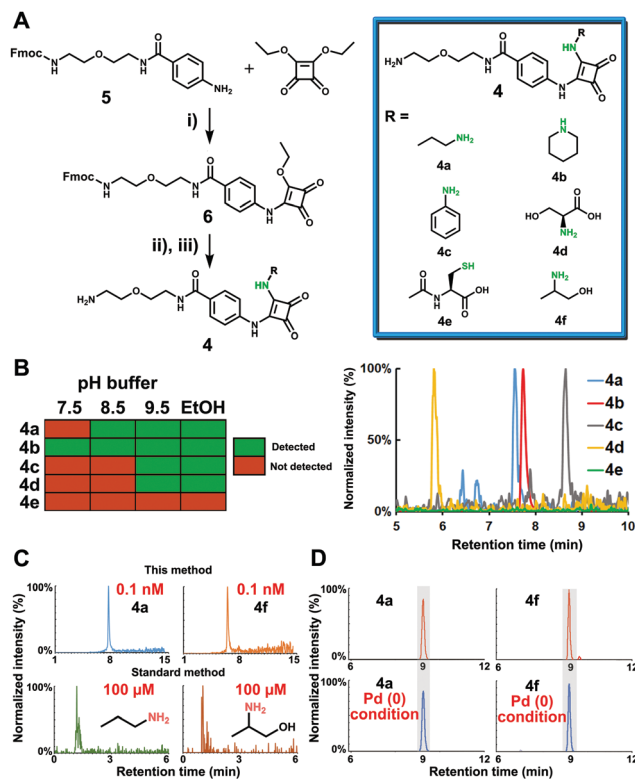


**Fig. 1** Chemoselective probe for amine-containing metabolite analysis. (A) Probe design: magnetic beads (black), bioorthogonal cleavage site (pink), linker (blue), and activation site (red). The reactive moiety for amine capturing is a squaramide ester and the conventional moiety an NHS ester. (B) Chemoselective probe treatment workflow that includes a magnetic separation step of **3** from the sample matrix after incubation.

functional group has so far not been reported for metabolomics-based investigations.

Due to these beneficial properties of the squaric acid moiety, we have activated our recently developed chemoselective probe with this moiety for analysis of amine-containing metabolites in human samples. In contrast to previous reports, we have shortened the scaffold synthesis for immobilization to amine activated beads **1** (Fig. 1A). This chemical biology tool is immobilized to magnetic beads, which allows for separation of the captured metabolites from the sample matrix. Straight-forward amidation of the squaric acid ester with magnetic beads-immobilized probe **1** is performed (Fig. 1B). Activated probe **2** can be incubated with any metabolite extract. The analytes for analysis with ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) were released from the magnetic beads under mild bioorthogonal conditions using Pd(0) catalysis (Scheme S1, ESI<sup>†</sup>).<sup>14</sup>

Before applying this new methodology to human samples, we first tested the properties of the probe activation, conjugation chemistry and stability of the released metabolite conjugates on synthesized simplified probe **6** (Fig. 2A and Scheme S2, ESI<sup>†</sup>). The conditions for synthesis of **6** from intermediate **5** with squaric acid diethylester were optimized for activation of immobilized probe **1**. Monosquaramide **6** was then tested for reactivity and chemoselectivity with five representative metabolites to cover diverse amine functionalities present in metabolites. The reaction of **6** with 1-aminopropane (primary amine), piperidine (secondary amine), aniline (aromatic amine), *L*-serine (amino acid), and *N*-acetyl-*L*-cysteine (thiol) was monitored under different reaction conditions such as pH 7.5, pH 8.5, pH 9.5 as well as 1% trimethylamine in ethanol (Fig. 2B and Fig. S1, ESI<sup>†</sup>). The results were analyzed *via* UPLC-MS analysis and confirmed that basic conditions or addition of an organic base leads to higher reactivity.<sup>17</sup> Importantly, this moiety



**Fig. 2** Method validation. (A) Synthetic scheme for conjugated metabolites and six representative examples. Conjugated 1-aminopropane (**4a**), piperidine (**4b**), aniline (**4c**), *L*-serine (**4d**), *N*-acetyl-*L*-cysteine (**4e**), and 2-amino-1-propanol (**4f**). Condition: (i) triethylamine, ethanol, 5 h, 25 °C; (ii) trimethylamine, ethanol, 16 h, 55 °C. (iii) Piperidine, DMF, 5 h, 25 °C. (B) Reactivity condition analysis for five metabolites. Colour-code green: detected; red: not detected. Representative extracted ion chromatograms for **4a–e** for ethanol conditions. (C) Extracted ion chromatograms (EICs) for LOD comparison of **4a** and 1-aminopropane as well as **4f** and 2-amino-1-propanol. (D) Stability of **4a** and **4f** for treatment with Pd(OAc)<sub>2</sub>, PPh<sub>3</sub>, dimethylbarbituric acid in THF, 16 h, 25 °C. Intensities were normalized to 100% for each EIC separately.

only reacted with amine-containing molecules and not with the thiol nucleophile *N*-acetyl-*L*-cysteine demonstrating the chemoselectivity for amines under the tested conditions. Based on these successful results, we synthesized metabolite conjugates **4a–e** to determine the limit of detection (LOD). The lowest LOD value was determined to be 80 pM for the conjugate of 1-aminopropane **4a** in positive MS ionization mode, which corresponds to 400 amol. As our method is based on magnetic beads separation from the sample matrix prior to cleavage and UPLC-MS analysis, this high mass spectrometric sensitivity determined by the LOD measurements for metabolite conjugates is similar to captured metabolites from human samples due to reconstitution to the same solution. The LOD for unconjugated 1-aminopropane was determined to be 100 μM, which demonstrates a mass spectrometric sensitivity increase by a factor of 1.25 million for **4a**. The LOD for the other tested metabolites ranged from 100 pM to 1.0 μM (Fig. 2C and Table 1, Table S1, ESI<sup>†</sup>).

Next, we determined the stability of squaramides of the general structure **4** under the cleavage conditions for the



Table 1 LOD experiments

Concentration	Signal/Noise				
	4a	4b	4c	4d	4f
100 $\mu$ M	1285.81	1181.49	123.09	43.39	886.35
10 $\mu$ M	1056.63	1101.21	42.98	37.25	586.38
1.0 $\mu$ M	629.76	812.36	14.41	10.39	283.95
100 nM	102.18	182.47	4.43	1.02	67.36
10 nM	34.84	60.43	ND	ND	21.89
1.0 nM	23.71	46.83	ND	ND	13.75
100 pM	11.85	20.78	ND	ND	6.78
80 pM	3.68	ND	ND	ND	1.28
50 pM	ND	ND	ND	ND	ND

bioorthogonal moiety *p*-nitrocinnamylloxycarbonyl (Noc) that is the key part of the probe design. The stability under cleavage conditions using Pd(OAc)<sub>2</sub>, PPh<sub>3</sub> and dimethylbarbituric acid was analyzed to eliminate the possibility of undesired decomposition of the captured metabolites in human samples. All conjugates were found unaltered even after 16 hours of treatment confirming the stability of **4** throughout our entire sample preparation procedure (Fig. 2D and Fig. S2, ESI<sup>†</sup>).

Upon method optimization, we treated human fecal samples collected from three different pancreatic cancer patients with probe **2**. The analysis of this sample type is crucial as only a small percentage of metabolites present has been discovered and a high number of metabolites are derived from microbiome metabolism.<sup>8</sup> The new method in this study can be applied for the analysis of known and the discovery of yet unknown metabolites. The mass spectrometric and metabolomics-based discovery of biomarkers or disease modulators in human fecal samples has so far been limited partly due to the lack of advanced analytical tools.

The treatment of fecal samples with **2** was performed according to our previously reported procedures with minor modification.<sup>13</sup> In brief, metabolites from fecal samples were extracted using a specialized lysing matrix-based homogenizer and the samples were reconstituted in ethanol. Probe **2** was next incubated with metabolite extracts to form stable squaramides with amine metabolites as well as a control sample with unconjugated magnetic beads (Fig. 3).<sup>13</sup> After separation from the sample matrix, we treated the captured metabolites **3** with the bioorthogonal cleavage conditions to release the metabolite conjugates for subsequent UPLC–MS analysis. In parallel, unmodified beads were also incubated and treated with identical cleavage conditions as control for the bioinformatic analysis. The data was processed in R using the XCMS metabolomics framework. The data analysis identified more than 3000 significantly altered features after removal of features following these criteria: (i) *m/z* values smaller than the ammonium conjugate (< 319.1401 Da), (ii) less abundant in the metabolite extract compared to the control sample, (iii) not significantly altered features (*p*-values > 0.05), (iv) features that eluted before 1.5 min. The filtering process was followed by the comparison of the *m/z* values from the candidate features with the Human Metabolome Database (HMDB) to determine their tentative chemical structures.<sup>18</sup>

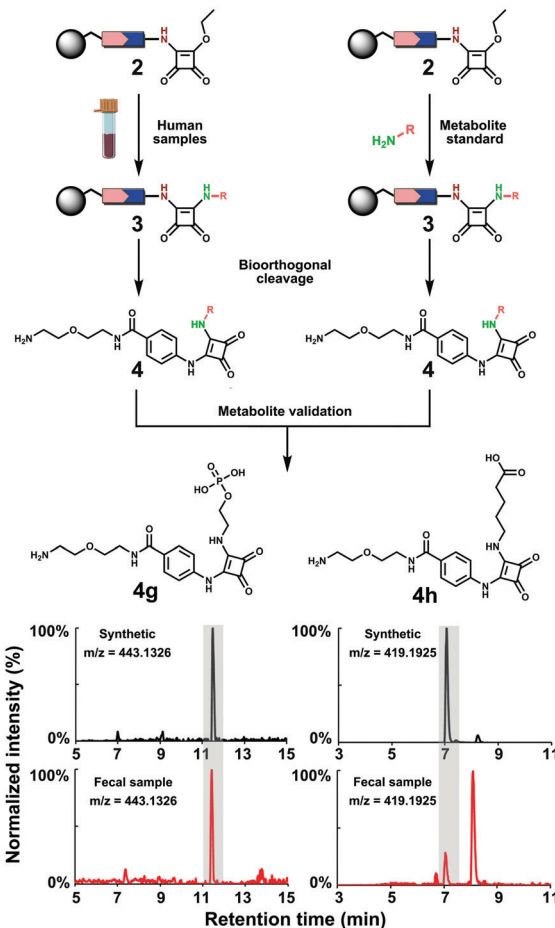


Fig. 3 Metabolite validation and library construction. Workflow for co-injection experiments with conjugated *O*-phosphoryl-ethanolamine (**4g**) and 5-aminovaleric acid (**4h**) as representative examples.

This analysis resulted in 165 metabolites that were annotated with a chemical structure with a 10 ppm accuracy (Fig. S3–S6, ESI<sup>†</sup>). This number exceeds conventional metabolomics investigations as well as other amine derivatization methods.<sup>19,20</sup> For determination of the metabolite structure at the highest confidence level, a synthetic reference metabolite is required to distinguish between regioisomers.<sup>21</sup> This level of structure validation is the current bottleneck of metabolomics research. We have constructed a metabolite conjugate library directly from conjugated probe **2** and treatment of a series of amine metabolites from our in-house metabolite library (Fig. 3). Combinatorial treatment of standard metabolites at low quantity (less than 0.1 mg) yielded a metabolite conjugate library of 92 metabolites of the general structure **4** (Table S2, ESI<sup>†</sup>). This library includes diverse classes of amine metabolites: microbial metabolites, small aliphatic amines, amino acids and their modifications as well as nucleoside metabolites.

Using our constructed in-house conjugated metabolite library, we have confirmed 39 metabolites at the highest confidence level by UPLC–MS validation experiments (Fig. 4). Most of these validated metabolites have been reported to be linked to development of diseases (Table S3, ESI<sup>†</sup>). For example,



HMDB ID	Metabolites	Monoisotopic mass	Probe*[M+H] <sup>+</sup>	RT (min)	18M	21F	6M
HMDB0000051	Ammonia	17.0265	319.1401	5.72	Green	Green	Green
HMDB0000184	Methylamine	31.0422	333.1558	6.15	Green	Green	Green
HMDB0000087	Dimethylamine	45.0578	347.1714	6.22	Green	Green	Green
HMDB0004101	3-Aminopropanitrile	70.0531	372.1667	5.21	Green	Green	Green
HMDB0034301	Piperidine	85.0891	387.2027	7.73	Green	Green	Green
HMDB0001414	1,4-Diaminobutane	88.1000	390.2136	5.22	Green	Green	Green
HMDB0001310	Alanine	89.0476	391.1612	6.79	Green	Green	Green
HMDB0002322	1,5-Diaminopentane	102.1156	404.2292	5.58	Green	Green	Green
HMDB0000452	L- $\alpha$ -aminobutyric acid	103.0633	405.1769	7.43	Green	Green	Green
HMDB0000112	$\gamma$ -Aminobutyric acid	103.0633	405.1769	6.61	Green	Green	Green
HMDB0000965	Hypotaurine	109.0197	411.1333	5.18	Green	Green	Green
HMDB0000883	L-Valine	117.0789	419.1925	8.08	Green	Green	Green
HMDB0003355	5-Aminovaleric acid	117.0789	419.1925	7.05	Green	Green	Green
HMDB0000167	L-Threonine	119.0600	421.1736	6.38	Green	Green	Green
HMDB0002017	1-Phenylethylamine	121.0891	423.2027	9.13	Green	Green	Green
HMDB0012275	2-Phenethylamine	121.0891	423.2027	4.77	Green	Green	Green
HMDB0002894	5-Methylcytosine	125.0589	427.1725	5.51	Green	Green	Green
HMDB0000070	Pipecolic acid	129.0789	431.1925	7.56	Green	Green	Green
HMDB0000725	L-Hydroxyproline	131.0582	433.1718	5.38	Green	Green	Green
HMDB0000172	L-Isoleucine	131.0946	433.2082	8.82	Green	Green	Green
HMDB0000742	DL-Homocysteine	135.0354	437.1490	7.21	Green	Green	Green
HMDB0001392	4-Aminobenzoic acid	137.0476	439.1612	11.05	Green	Green	Green
HMDB0000224	O-Phosphoryl-ethanolamine	141.0190	443.1326	11.49	Green	Green	Green
HMDB0002284	N-(5-Aminopentyl) acetamide	144.1262	446.2398	7.21	Green	Green	Green
HMDB0000182	L-Lysine	146.1055	448.2191	6.88	Green	Green	Green
HMDB0000641	L-Glutamine	146.1400	448.2536	7.50	Green	Green	Green
HMDB0034323	S-Allyl-L-cysteine	161.0511	463.1647	8.57	Green	Green	Green
HMDB0000450	DL-5-Hydroxylysine	162.1004	464.2140	9.80	Green	Green	Green
HMDB0002757	L-Cysteic acid	169.0044	471.1180	10.78	Green	Green	Green
HMDB0028854	Glycylvaline	174.1004	476.2140	7.47	Green	Green	Green
HMDB0000446	N- $\alpha$ -Acetyl-L-lysine	188.1160	490.2296	10.24	Green	Green	Green
HMDB0000206	N- $\epsilon$ -Acetyl-L-lysine	188.1161	490.2297	6.81	Green	Green	Green
HMDB0000472	L-5-Hydroxytryptophan	220.0847	522.1983	9.87	Green	Green	Green
HMDB0000033	L-Carnosine	226.1066	528.2202	8.65	Green	Green	Green
HMDB0001940	Triamterene	253.1075	555.2211	11.35	Green	Green	Green
HMDB0000982	5-Methyltyridine	257.1012	559.2148	8.49	Green	Green	Green
HMDB0000235	Thiamine	265.1123	567.2259	9.37	Green	Green	Green
HMDB0000085	2'-Deoxyguanosine	267.1000	569.2136	8.97	Green	Green	Green
HMDB0000133	Guanosine	283.0916	585.2052	8.93	Green	Green	Green



Fig. 4 Metabolite validation in human fecal samples. List and colour-code for validated amine metabolites (green: detected; red: not detected). Microbiota-derived metabolites (blue) and newly detected metabolites (yellow).

the neurotransmitter  $\gamma$ -aminobutyric acid (GABA) has been associated with development of Alzheimer's disease.<sup>22</sup> Additionally, it has recently been identified that GABA is produced by human Intestinal *Bacteroides* spp. to counter acid stress.<sup>23</sup> A representative example for the efficient metabolite validation procedure is the conjugate of O-phosphoryl-ethanolamine (**4g**). O-Phosphoryl-ethanolamine is a part of sphingomyelin and exists in all living species ranging from bacteria to humans.<sup>24</sup> Furthermore, we have validated the three amines 3-aminopropanitrile, 5-methylcytosine and L-cysteic acid that are newly detected in fecal samples, which we believe stems from the enhanced mass spectrometric sensitivity of our method.

Furthermore, we have detected 12 microbiota-derived metabolites in these fecal samples that can be considered a direct readout for gut microbiota metabolism and interaction with the human host. For example, 5-aminovaleric acid (**4h**) has been associated with colorectal cancer.<sup>25</sup> Other microbial metabolites of importance are the volatile metabolite ammonium, the two diamines 1,4-diaminobutane and 1,5-diaminopentane, as well as 4-aminobenzoic acid. The latter compound cannot be produced by humans due to the lack of the enzymes. Additionally, our method allows to successfully map metabolic pathways from the microbiome in these fecal samples e.g. the valine, leucine and isoleucine pathway. Within this pathway, threonine is an essential amino acid that is produced by microbes through the conversion of glycine from serine.<sup>26</sup> Isoleucine is one of the essential branched-chain amino acids (BCAA) that is produced through bacterial metabolism in the human body.<sup>27</sup> Parallel analysis of

these microbial metabolites utilizing a single method has a high potential to monitor microbial bioactivity in human samples.

## Conclusions

This study represents the first application of squaric acid for chemoselective analysis of amine metabolites in metabolomics-based studies. The high stability of the formed conjugates of squaric acid with metabolites and the enhanced ionization properties of these conjugates led to successful capture and analysis of amine-containing metabolites in human fecal samples. The highly improved LOD at pM concentrations yielded three previously undetected metabolites in this sample type as well as a series of microbiota-derived metabolites. Furthermore, we have designed a reliable and straightforward method to synthesize a 93 amine-containing metabolite library for efficient validation of metabolites with coverage of different metabolite classes. This method can now be utilized for monitoring amine metabolites in any biological sample.

## Ethical statement

Patient fecal samples were obtained in accordance with the World Medical Association Declaration of Helsinki and all patients gave written informed consent. Approval for the study was obtained from the ethical committee at Karolinska Institute Hospital (Ethical approval number: Dnr 2017/290-31). Fecal samples were collected using routine clinical collection protocols and all patient codes have been removed in this publication. All samples were stored at  $-80^{\circ}\text{C}$ .

## Conflicts of interest

There are no conflicts to declare.

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## References

- 1 T. S. B. Schmidt, J. Raes and P. Bork, *Cell*, 2018, **172**, 1198–1215.
- 2 R. Mehrian-Shai, J. K. V. Reichardt, C. C. Harris and A. Toren, *Trends Cancer*, 2019, **5**, 200–207.
- 3 E. Sherwin, S. R. Bordenstein, J. L. Quinn, T. G. Dinan and J. F. Cryan, *Science*, 2019, 366.
- 4 N. Koppel, V. Maini Rekdal and E. P. Balskus, *Science*, 2017, 356.
- 5 R. R. da Silva, P. C. Dorrestein and R. A. Quinn, *Proc. Natl. Acad. Sci. U. S. A.*, 2015, **112**, 12549–12550.
- 6 Y. Qiao, H. Hayashi and S. Chong Teo, *Chem. – Asian J.*, 2020, **15**, 2117–2128.



- 7 A. Milshteyn, D. A. Colosimo and S. F. Brady, *Cell Host Microbe*, 2018, **23**, 725–736.
- 8 G. V. Sridharan, K. Choi, C. Klemashevich, C. Wu, D. Prabakaran, L. B. Pan, S. Steinmeyer, C. Mueller, M. Yousofshahi, R. C. Alaniz, K. Lee and A. Jayaraman, *Nat. Commun.*, 2014, **5**, 5492.
- 9 Y. Iwasaki, Y. Nakano, K. Mochizuki, M. Nomoto, Y. Takahashi, R. Ito, K. Saito and H. Nakazawa, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, 2011, **879**, 1159–1165.
- 10 C. C. Hughes, *Nat. Prod. Rep.*, 2021, DOI: 10.1039/d0np00034e.
- 11 E. E. Carlson and B. F. Cravatt, *J. Am. Chem. Soc.*, 2007, **129**, 15780–15782.
- 12 W. Lin, L. P. Conway, A. Block, G. Sommi, M. Vujasinovic, J. M. Lohr and D. Globisch, *Analyst*, 2020, **145**, 3822–3831.
- 13 L. P. Conway, N. Garg, W. Lin, M. Vujasinovic, J. M. Lohr and D. Globisch, *Chem. Commun.*, 2019, **55**, 9080–9083.
- 14 N. Garg, L. P. Conway, C. Ballet, M. S. P. Correia, F. K. S. Olsson, M. Vujasinovic, J. M. Lohr and D. Globisch, *Angew. Chem., Int. Ed.*, 2018, **57**, 13805–13809.
- 15 F. R. Wurm and H. A. Klok, *Chem. Soc. Rev.*, 2013, **42**, 8220–8236.
- 16 L. F. Tietze, C. Schröter, S. Gabius, U. Brinck, A. Goerlach-Graw and H. J. Gabius, *Bioconjugate Chem.*, 1991, **2**, 148–153.
- 17 M. Ximenis, E. Bustelo, A. G. Algarra, M. Vega, C. Rotger, M. G. Basallote and A. Costa, *J. Org. Chem.*, 2017, **82**, 2160–2170.
- 18 D. S. Wishart, Y. D. Feunang, A. Marcu, A. C. Guo, K. Liang, R. Vázquez-Fresno, T. Sajed, D. Johnson, C. Li, N. Karu, Z. Sayeeda, E. Lo, N. Assempour, M. Berjanskii, S. Singhal, D. Arndt, Y. Liang, H. Badran, J. Grant, A. Serra-Cayuela, Y. Liu, R. Mandal, V. Neveu, A. Pon, C. Knox, M. Wilson, C. Manach and A. Scalbert, *Nucleic Acids Res.*, 2018, **46**, D608–D617.
- 19 K. Guo, C. Ji and L. Li, *Anal. Chem.*, 2007, **79**, 8631–8638.
- 20 A. Lkhagva, C. C. Shen, Y. S. Leung and H. C. Tai, *J. Chromatogr. A*, 2020, **1610**, 460536.
- 21 C. Ballet, M. S. P. Correia, L. P. Conway, T. L. Locher, L. C. Lehmann, N. Garg, M. Vujasinovic, S. Deindl, J. M. Lohr and D. Globisch, *Chem. Sci.*, 2018, **9**, 6233–6239.
- 22 A. N. Fonteh, R. J. Harrington, A. Tsai, P. Liao and M. G. Harrington, *Amino Acids*, 2007, **32**, 213–224.
- 23 N. Otaru, K. Ye, D. Mujezinovic, L. Berchtold, F. Constancias, F. A. Cornejo, A. Krzystek, T. de Wouters, C. Braegger, C. Lacroix and B. Pugin, *Front. Microbiol.*, 2021, **12**, 656895.
- 24 D. Schiroli, L. Ronda and A. Peracchi, *FEBS J.*, 2015, **282**, 183–199.
- 25 D. G. Brown, S. Rao, T. L. Weir, J. O'Malia, M. Bazan, R. J. Brown and E. P. Ryan, *Cancer Metab.*, 2016, **4**, 11.
- 26 Z. X. Cheng, C. Guo, Z. G. Chen, T. C. Yang, J. Y. Zhang, J. Wang, J. X. Zhu, D. Li, T. T. Zhang, H. Li, B. Peng and X. X. Peng, *Nat. Commun.*, 2019, **10**, 3325.
- 27 C. Nie, T. He, W. Zhang, G. Zhang and X. Ma, *Int. J. Mol. Sci.*, 2018, **19**.

