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# Expanding the versatility and scope of the oxime ligation: rapid bioconjugation to disulfide-rich peptides†

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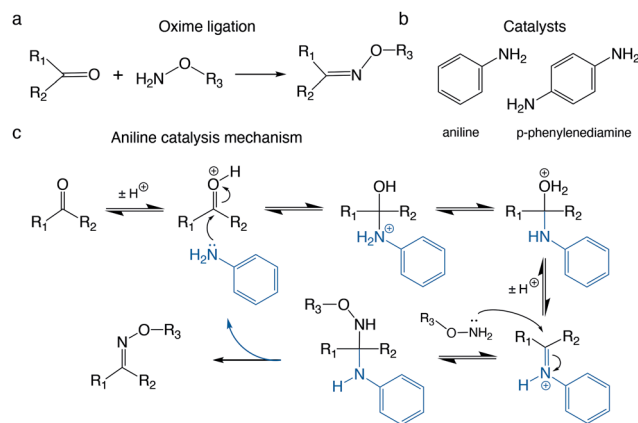
**The oxime ligation is a valuable bioorthogonal conjugation reaction but with limited compatibility with disulfide-rich peptides/proteins and time-sensitive applications. Here we overcome these limitations by introducing a strategy that supports regiospecific control, oxidative folding, production of stable aminoxy-precursors for on-demand modification, and complete ligation within 5 min.**

The oxime ligation is a useful bioorthogonal reaction between a nucleophilic aminoxy group ( $\text{H}_2\text{N}-\text{O}-\text{R}$ ) and an electrophilic carbonyl group (e.g. aldehyde/ketone) (Fig. 1a).<sup>1,2</sup> The reaction is typically carried out in aqueous media and catalysed by aniline or phenylenediamine derivatives (Fig. 1b and c).<sup>2–7</sup> It is a reliable and versatile conjugation technique due to the mild reaction conditions, the high chemoselectivity, and the hydrolytic stability of the oxime bond.<sup>8</sup> It does not require metal ion catalysts, which can cause problems with purifications and certain classes of peptides/proteins.<sup>8–11</sup> The oxime ligation has been successfully applied for the preparation of bioconjugates, including polymer-proteins,<sup>12,13</sup> peptide dendrimers,<sup>14</sup> oligonucleo-peptides,<sup>15,16</sup> glycoconjugates,<sup>17</sup> protein-protein probes,<sup>18</sup> <sup>18</sup>F-PET tracers<sup>19,20</sup> and hydrogels.<sup>3,21</sup>

The synthesis of aminoxy-peptide precursors is challenging due to the high reactivity of the aminoxy moiety. Strategies have been developed to overcome this problem, including orthogonally protected aminoxy acetic acid (Aoa) derivatives compatible with Fmoc- and Boc-SPPS (Fmoc-Aoa, Boc-Aoa, Boc<sub>2</sub>-Aoa).<sup>22</sup> Boc<sub>2</sub>-Aoa was developed to avoid *N*-overacylation when using HCTU/DIPEA.<sup>22–24</sup> Alternatively, preactivated NHS-ester, EEDQ (*N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline) or Eei-Aoa (2-(1-ethoxyethylideneaminoxy)acetic acid) can be used.<sup>25,26</sup>

While this works for linear peptides, the aminoxy group is not compatible with the oxidative folding step for producing disulfide-rich peptides or proteins (Fig. S1a, ESI†). Indeed, the oxime ligation has only been reported for two disulfide-rich peptides, namely insulin and the c-Met peptide.<sup>27,28</sup> In these studies, the ligation was carried out either before the folding step, or the aminoxy moiety was introduced after folding, circumventing the problem without solving it.<sup>27,28</sup> Neither of these two strategies is ideal since they require two purification steps (after ligation and folding), which takes time and lowers the yields. Moreover, the introduction of the aminoxy group remains restricted to the N-terminus.

The handling and storing of aminoxy-containing peptides are also difficult due to the aminoxy's high reactivity towards aldehydes and ketones, including the laboratory solvent acetone. These limitations of an otherwise powerful, efficient, and clean bioorthogonal reaction prompted us to investigate new strategies to expand the scope of the oxime ligation.



**Fig. 1** The oxime ligation and commonly used catalysts. (a) Reaction of an aminoxy and an aldehyde or ketone to form an oxime bond. (b) Aniline and *p*-phenylenediamine accelerate the reaction by acting as catalysts. (c) Nucleophilic catalysis mechanism of aniline (blue) during the oxime ligation.

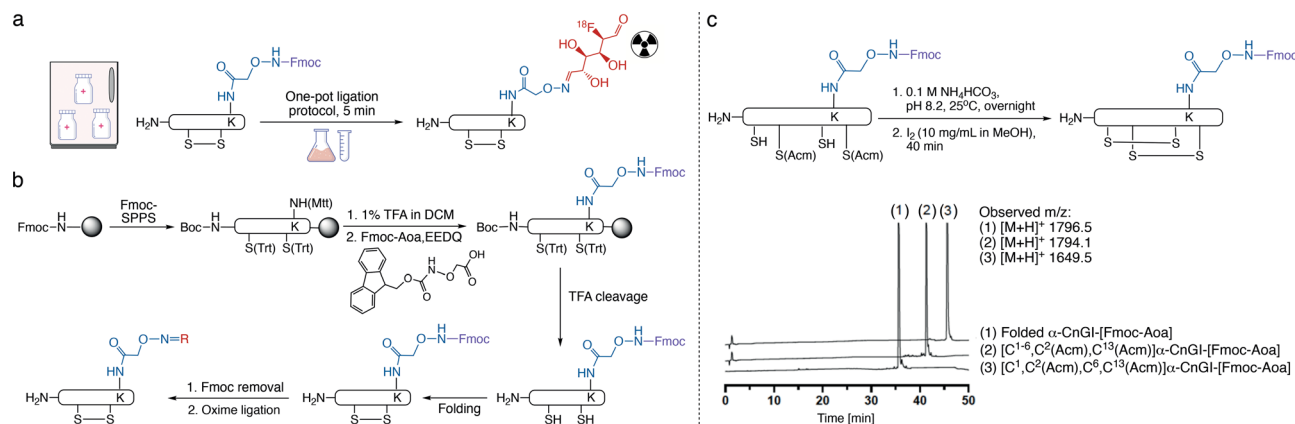
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**Fig. 2** Expanding the scope of the oxime ligation. (a) Application scheme of the stable Fmoc-aminoxy-containing precursor peptide used with the rapid one-pot ligation protocol to support on-demand and on-site preparation of radioactive tracers for clinical settings. (b) Synthetic strategy for the regiospecific introduction of Fmoc-Aoa within a peptide during Fmoc-SPPS, followed by TFA cleavage and oxidative folding of the Fmoc-aminoxy-containing peptide, leading to a fully folded Fmoc-aminoxy-containing peptide precursor compatible with long-term storage. New functional groups can be conveniently introduced *via* Fmoc-removal and standard oxime ligation protocols. EEDQ: *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline. (c) Scheme of the directed disulfide bond formation of a two-disulfide-bond-containing peptide including the Fmoc-protected aminoxy group. Analytical C<sub>18</sub>-RP-HPLC traces of the directed folding of α-conotoxin analogue α-CnGI-[Fmoc-Aoa] with observed and calculated masses. Peak (1): reduced [C<sup>1-6</sup>, C<sup>2</sup>(Acm), C<sup>6</sup>, C<sup>13</sup>(Acm)]-α-CnGI-[Fmoc-Aoa]; peak (2): partially folded [C<sup>1-6</sup>, C<sup>2</sup>(Acm), C<sup>13</sup>(Acm)]-α-CnGI-[Fmoc-Aoa]; peak (3): fully folded [C<sup>1-6</sup>, C<sup>2-13</sup>]-α-CnGI-[Fmoc-Aoa]. α-CnGI sequence: CCHPACGKYFKC\*, with \*C-terminal amide and Fmoc-Aoa on K<sup>8</sup>.

We envisioned a strategy that could produce stable aminoxy precursors compatible with the folding of disulfide-rich peptides, long-term storage, and late-stage modifications. We furthermore realised that for time-sensitive applications such as the labelling with <sup>18</sup>F (109.7 min half-life) to produce PET tracers,<sup>20,29</sup> reaction conditions with vastly faster ligation kinetics are required than the currently used multi-hour protocols (Fig. 2a). <sup>18</sup>F-labelling is typically carried out *via* an <sup>18</sup>F-containing group compatible with different chemistries. Examples include activated carboxylic acids to react with free amines, maleimides to react with thiols, or alkynes/azides for click chemistry.<sup>20</sup> These approaches have their own limitations, including lack of regioselective control when multiple free amines are present, and compatibility issues with disulfide-rich peptides/proteins for using maleimides or Cu-catalysts for click chemistry.<sup>20</sup> The oxime ligation would be a powerful addition to the labelling repertoire if the reaction could be accelerated and made compatible with disulfide-rich peptides/proteins.

Thus, we first pursued regioselective on-resin incorporation of the aminoxy group into disulfide-bond-containing peptides (Fig. 2a). As a model peptide, we used the nonapeptide oxytocin (OT), which has a disulfide bond between Cys<sup>1</sup> and Cys<sup>6</sup>. We replaced Leu<sup>8</sup> with Lys(Mtt) (OTK<sup>8</sup>) as a controllable side chain handle to introduce the aminoxy group. Position 8 was selected based on previous studies demonstrating that position 8 modifications are well-tolerated in terms of bioactivity.<sup>30–32</sup> OTK<sup>8</sup> was assembled *via* Fmoc-SPPS on a Rink-amide resin, and Boc-Cys(Trt) was used as the N-terminal amino acid. The Mtt group was removed with 1% TFA in dichloromethane (DCM), and Fmoc-Aoa was coupled to the now unprotected ε-amino group of Lys<sup>8</sup> using EEDQ as the coupling reagent.

OTK<sup>8</sup>[Fmoc-Aoa] was cleaved from the resin using TFA and scavengers, with the aminoxy group remaining Fmoc-protected.

Exposure of reduced/linear OTK<sup>8</sup>[Fmoc-Aoa] to common oxidative folding conditions for 12 hours demonstrated that the Fmoc-protected aminoxy group remained intact, yielding folded OTK<sup>8</sup>[Fmoc-Aoa] in high purity (>90%) with no side product formation observed (Fig. S1b, ESI<sup>†</sup>). We then validated our strategy under conditions of directed disulfide bond formation using pairs of Cys(Acm) groups, one of the most commonly used directed folding strategies. We applied our strategy to α-CnIG (CCHPACGKYFKC\*), an α-conotoxin from the venom of *Conus consors*, with disulfide bonds between Cys<sup>1-6</sup> and Cys<sup>2-13</sup>. α-Conotoxins are an important class of venom peptides from the marine predatory cone snail that potently and selectively inhibit nicotinic acetylcholine receptors.<sup>33,34</sup> α-Conotoxins, like many other disulfide-rich peptides, are synthetically produced *via* a directed disulfide-bond formation strategy and are ideal models to demonstrate the compatibility of our aminoxy strategy with directed folding. α-CnIG was assembled by Fmoc-SPPS with the side chains of Cys<sup>1-6</sup> and Cys<sup>2-13</sup> protected with Trt and Acm, respectively. Boc-Cys<sup>1</sup>(Trt) was used as the N-terminal amino acid and Lys<sup>8</sup>(Mtt) as the aminoxy handle. After complete peptide assembly, the Mtt group was removed with 1% TFA/DCM, and Fmoc-Aoa was coupled to Lys<sup>8</sup>. Sidechain deprotection and peptide cleavage were carried out with TFA and scavengers, leaving Cys<sup>2/13</sup> and Lys<sup>8</sup>-Aoa protected with Acm and Fmoc, respectively. The first disulfide bond (Cys<sup>1-6</sup>) was formed in 0.1 M NH<sub>4</sub>HCO<sub>3</sub> at pH 8.2, followed by RP-HPLC purification. The second disulfide bond (Cys<sup>2-13</sup>) was then formed through I<sub>2</sub> oxidation, followed by another purification (Fig. 2c and Fig. S2, ESI<sup>†</sup>). Both oxidations proceeded cleanly with no side reactions, confirming the compatibility with directed disulfide bond formation.



We then evaluated the long-term stability by monitoring OTK<sup>8</sup>[Fmoc-Aoa] dissolved in 0.1% TFA or 50% ACN/0.1% TFA over three months at 4 °C or as a lyophilised powder, confirming the high stability of the Fmoc-aminoxy-containing precursors (Fig. S3, ESI†).

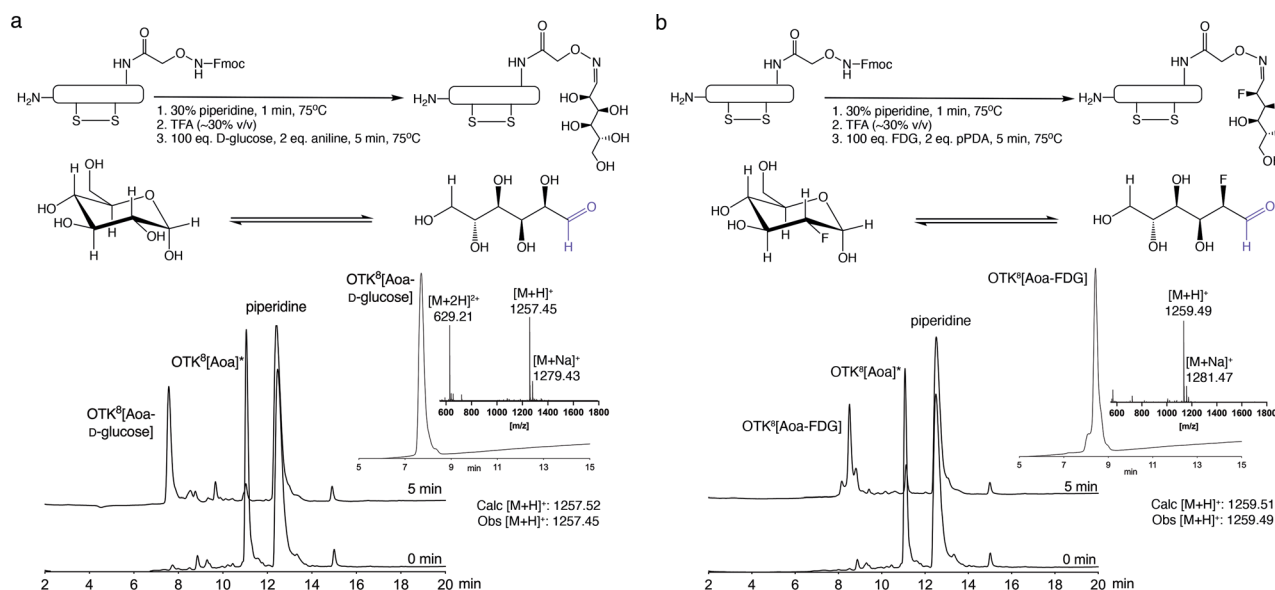
This strategy therefore overcomes the first set of limitations, supporting regiospecific incorporation of the aminoxy moiety, compatibility with oxidative folding protocols and disulfide-rich peptides/proteins, and the preparation of stable Fmoc-aminoxy-containing precursors for long-term storage and late-stage modifications. The latter should be particularly useful for preparing peptide radiotracers as theranostics, a rapidly expanding field.<sup>35,36</sup>

Next, we aimed at accelerating the reaction kinetics. The oxime ligation with peptides and proteins is typically carried out over several hours in aqueous media at pH 4–5 with either aniline or *p*-phenylenediamine (pPDA) as catalysts (μM reactants, 10 mM catalysts).<sup>1,37,38</sup> While these ligation times are acceptable for the majority of applications, they are too slow for radiochemistry applications that use <sup>18</sup>F, which ideally requires complete ligation within minutes due to its short half-life (109.7 min). Ligation kinetics are driven by the concentration of the reactants, with solubility a recognised bottleneck. We therefore investigated the ligation kinetics in different solvent systems, including H<sub>2</sub>O, 80% acetonitrile (ACN), 80% ethanol (EtOH), and DMF, using purified OTK<sup>8</sup>[Fmoc-Aoa] along with benzaldehyde, acetophenone or D-glucose as our model reactants, and pPDA as the catalyst.

First, we analysed the Fmoc removal kinetics using 30% (v/v) piperidine in these four solvents (Fig. S4, ESI†). Fmoc removal occurred within seconds in all tested solvents, and no racemisation was observed. The move to more organic solvents allowed higher ligand concentrations, which substantially

accelerated reaction kinetics, with ligations of OTK<sup>8</sup>[Aoa] with benzaldehyde and acetophenone completed in seconds compared to the slower and incomplete ligations in water (Fig. S5, ESI†). The reaction rate of D-glucose, however, even though faster than in water, was still not fast enough for rapid radiochemistry applications. The slower reaction kinetics are due to the energetic equilibrium between the cyclic and linear aldehyde conformation of D-glucose, with the cyclic conformation energetically favoured, but only the linear aldehyde conformation able to react with the aminoxy group (Fig. S5, ESI†).<sup>39,40</sup> To overcome this problem, we increased the excess of D-glucose from 1 eq. to 100 eq. with 2 eq. pPDA as the catalyst in anhydrous DMF and the temperature to 75 °C. This improved the reaction rate but also induced unexpected dimerization of the peptide conjugate. This was resolved by replacing pPDA (two free amines) with aniline (single amine), resulting in clean ligation of OTK<sup>8</sup>[Aoa] with D-glucose within 5 min (compared to >1 h before optimisation). With the ligation reaction optimised, the next step was to develop a rapid one-pot Fmoc-removal, labelling and purification protocol to support time-sensitive applications. We dissolved OTK<sup>8</sup>[Fmoc-Aoa] in pre-heated 30% piperidine/anhydrous DMF (20 mM) for 1 min at 75 °C. The reaction was quenched with neat TFA (~30% v/v) followed by the addition of pre-heated aniline (2 eq.) and D-glucose (100 eq.) in anhydrous DMF. The reaction was quenched after 5 min with acetone. Using this one-pot protocol >95% of OTK<sup>8</sup>[Aoa] reacted with D-glucose within 5 min (Fig. 3a). Fast C<sub>18</sub>-RP-HPLC purification (5–35% B in 15 min) yielded OTK<sup>8</sup>[Aoa-D-glucose] in high purity (>95%) (Fig. 3a).

To apply our protocol to clinically more relevant material, we performed the ligation reaction also with fluorodeoxyglucose (FDG), a clinically used and readily available <sup>18</sup>F source for PET



**Fig. 3** One-pot Fmoc-removal and oxime ligation of OTK<sup>8</sup>[Fmoc-Aoa] with D-glucose (a) or FDG (b). The Fmoc group of OTK<sup>8</sup>[Fmoc-Aoa] was removed with 30% piperidine (1 min at 75 °C) and the reaction quenched with TFA (~30% v/v). Aniline (2 eq.) and D-glucose (100 eq.) or pPDA (2 eq.) and FDG (100 eq.) were added, and the reaction mixed for 5 min at 75 °C. The reaction process was monitored by analytical C<sub>18</sub> RP-HPLC and LC-MS. \*, excess OTK<sup>8</sup>[Aoa] was quenched with acetone. Insets: Analytical C<sub>18</sub>-RP-HPLC traces and MS data of purified OTK<sup>8</sup>[Aoa-D-glucose] and OTK<sup>8</sup>[Aoa-FDG].



imaging.<sup>20</sup> Facile introduction of <sup>18</sup>F-DG into peptides *via* oxime ligation represents a new and highly efficient way of producing PET tracers that play an increasingly important role in oncology and other applications. Interestingly, the reaction conditions optimised for D-glucose were not sufficient for FDG ligation (>1 h for complete ligation). We however resolved this by changing the aniline catalyst back to pPDA and did not observe any dimerization as with D-glucose.

The final FDG labelling protocol entailed OTK<sup>8</sup>[Fmoc-Aoa] dissolution (20 mM) in pre-heated (75 °C) 30% piperidine/anhydrous DMF to remove the Fmoc group (1 min), which was quenched with neat TFA (~30%, v/v). pPDA (2 eq.) and FDG (100 eq.) in anhydrous DMF were added, and the ligation reaction was quenched after 5 min with acetone (10% v/v). Analytical RP-HPLC and MS analysis confirmed complete product conversion within 5 min, and RP-HPLC purification yielded OTK<sup>8</sup>[Aoa-FDG] in >95% purity (Fig. 3b).

In summary, we developed a new strategy for regioselective control of the oxime ligation and compatibility with disulfide-rich peptides/proteins. The Fmoc-protected aminooxy group was stable to standard and directed oxidative folding conditions and Fmoc-aminooxy-containing precursors can be kept in long-term storage for on-demand bioconjugation and labelling. This is highly useful for preparing radiotracers, where adding the radionuclide is the final and time-sensitive step before injecting the tracer into patients. Our rapid one-pot labelling protocol enabled efficient <sup>18</sup>F introduction into peptides/proteins within minutes, highlighting the application potential of this approach for (pre)clinical PET imaging.

Taken together, these new strategy and protocols considerably expand the versatility and scope of the oxime ligation, rendering it now an even more powerful tool that can be used with other bioconjugation reactions such as click chemistry, native chemical ligation, or the Staudinger reaction.<sup>41</sup> This expands the chemical biology toolbox and creates new opportunities for late-stage modifications, radiotracer development, and biomaterial engineering.

A. H. and N. B. E. contributed equally to this work and conducted the experiments; M. M. conceived the idea and supervised the project; A. H., N. B. E. and M. M. wrote the manuscript.

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## Conflicts of interest

There are no conflicts to declare.

## References

- K. Rose, *J. Am. Chem. Soc.*, 1994, **116**, 30–33.
- S. M. Agten, P. E. Dawson and T. M. Hackeng, *J. Pept. Sci.*, 2016, **22**, 271–279.
- G. N. Grover, J. Lam, T. H. Nguyen, T. Segura and H. D. Maynard, *Biomacromolecules*, 2012, **13**, 3013–3017.
- M. Rashidian, M. M. Mahmoodi, R. Shah, J. K. Dozier, C. R. Wagner and M. D. Distefano, *Bioconjugate Chem.*, 2013, **24**, 333–342.
- J. Kalia and R. T. Raines, *Angew. Chem., Int. Ed.*, 2008, **47**, 7523–7526.
- A. Dirksen, T. M. Hackeng and P. E. Dawson, *Angew. Chem., Int. Ed.*, 2006, **118**, 7743–7746.
- D. K. Kölmel and E. T. Kool, *Chem. Rev.*, 2017, **117**, 10358–10376.
- W. Tang and M. L. Becker, *Chem. Soc. Rev.*, 2014, **43**, 7013–7039.
- S. Li, H. Cai, J. He, H. Chen, S. Lam, T. Cai, Z. Zhu, S. J. Bark and C. Cai, *Bioconjugate Chem.*, 2016, **27**, 2315–2322.
- N. W. Nairn, P. A. Bariola, T. J. Graddis, M. P. VanBrunt, A. Wang, G. Li and K. Grabstein, *Bioconjugate Chem.*, 2015, **26**, 2070–2075.
- S. A. Fisher, A. E. G. Baker and M. S. Shoichet, *J. Am. Chem. Soc.*, 2017, **139**, 7416–7427.
- T. L. Schlick, Z. B. Ding, E. W. Kovacs and M. B. Francis, *J. Am. Chem. Soc.*, 2005, **127**, 3718–3723.
- K. J. Mackenzie and M. B. Francis, *J. Am. Chem. Soc.*, 2013, 245.
- J. C. Breger, M. Muttenthaler, J. B. Delehanty, D. A. Thompson, E. Oh, K. Susumu, J. R. Deschamps, G. P. Anderson, L. D. Field, S. A. Walper, P. E. Dawson and I. L. Medintz, *Nanoscale*, 2017, **9**, 10447–10464.
- N. Venkatesan and B. H. Kim, *Chem. Rev.*, 2006, **106**, 3712–3761.
- Y. Singh, P. Murat and E. Defrancq, *Chem. Soc. Rev.*, 2010, **39**, 2054–2070.
- S. E. Cervigni, P. Dumy and M. Mutter, *Angew. Chem., Int. Ed. Engl.*, 1996, **35**, 1230–1232.
- S. M. Agten, R. R. Koenen, H. Ippel, V. Eckardt, P. von Hundelshausen, K. H. Mayo, C. Weber and T. M. Hackeng, *Angew. Chem., Int. Ed.*, 2016, **55**, 14963–14966.
- A. M. Senisik, C. Ichedef, A. Y. Kilcar, E. Ucar, K. Ari, D. Goksoy, Y. Parlak, B. E. S. Bilgin and S. Teksoz, *J. Radioanal. Nucl. Chem.*, 2018, **316**, 457–463.
- X. G. Li, M. Haaparanta and O. Solin, *J. Fluor. Chem.*, 2012, **143**, 49–56.
- J. G. Hardy, P. Lin and C. E. Schmidt, *J. Biomater. Sci., Polym. Ed.*, 2015, **26**, 143–161.
- Y. Yang, in *Side reactions in peptide synthesis*, ed. Y. Yang, Academic Press, Oxford, 2016, pp. 235–256.
- I. P. Decostaire, D. Lelièvre, H. Zhang and A. F. Delmas, *Tetrahedron Lett.*, 2006, **47**, 7057–7060.
- J. Brask and K. J. Jensen, *J. Pept. Sci.*, 2000, **6**, 290–299.
- V. Dulery, O. Renaudet and P. Dumy, *Tetrahedron*, 2007, **63**, 11952–11958.
- J. Brask and K. J. Jensen, *J. Pept. Sci.*, 2000, **6**, 290–299.
- K. Thalluri, B. B. Kou, V. Gelfanov, J. P. Mayer, F. Liu and R. D. DiMarchi, *Org. Lett.*, 2017, **19**, 706–709.
- R. B. Peter Brian Iveson, B. Indrevall and G. Getvoldsen, *US Pat.*, US20130149241A1, 2011.
- E. Miele, G. P. Spinelli, F. Tomao, A. Zullo, F. De Marinis, G. Pasciuti, L. Rossi, F. Zoratto and S. Tomao, *J. Exp. Clin. Cancer Res.*, 2008, **27**, 52.
- B. Chini, B. Mouillac, Y. Ala, M. N. Balestre, S. Trumpp-Kallmeyer, J. Hoflack, J. Elands, M. Hibert, M. Manning and S. Jard, *et al.*, *EMBO J.*, 1995, **14**, 2176–2182.
- B. Chini, B. Mouillac, Y. Ala, M. N. Balestre, N. Cotte, S. Trumpp-Kallmeyer, J. Hoflack, J. Elands, M. Hibert and M. Manning, *et al.*, *Adv. Exp. Med. Biol.*, 1995, **395**, 321–328.
- B. Mouillac, B. Chini, M. N. Balestre, S. Jard, C. Barberis, M. Manning, E. Tribollet, S. Trumpp-Kallmeyer, J. Hoflack and J. Elands, *et al.*, *Adv. Exp. Med. Biol.*, 1995, **395**, 301–310.
- A. H. Jin, M. Muttenthaler, S. Dutertre, S. W. A. Himaya, Q. Kaas, D. J. Craik, R. J. Lewis and P. F. Alewood, *Chem. Rev.*, 2019, **119**, 11510–11549.
- K. B. Akondi, M. Muttenthaler, S. Dutertre, Q. Kaas, D. J. Craik, R. J. Lewis and P. F. Alewood, *Chem. Rev.*, 2014, **114**, 5815–5847.
- M. Muttenthaler, G. F. King, D. J. Adams and P. F. Alewood, *Nat. Rev. Drug Discovery*, 2021, **20**, 309–325.
- M. Fani, H. Maecke and S. Okarvi, *Theranostics*, 2012, **2**, 481.
- I. E. Decostaire, D. Lelièvre, V. Aucagne and A. F. Delmas, *Org. Biomol. Chem.*, 2014, **12**, 5536–5543.
- M. Wendeler, L. Grinberg, X. Wang, P. E. Dawson and M. Baca, *Bioconjugate Chem.*, 2014, **25**, 93–101.
- P. Finch and Z. Merchant, *J. Chem. Soc., Perkin Trans. 1*, 1975, 1682–1686.
- A. Mostad, *Acta Chem. Scand., Ser. B*, 1978, **32**, 733–742.
- D. M. Patterson, L. A. Nazarova and J. A. Prescher, *ACS Chem. Biol.*, 2014, **9**, 592–605.

