Supporting Information

Protein Desulfurization and Deselenization

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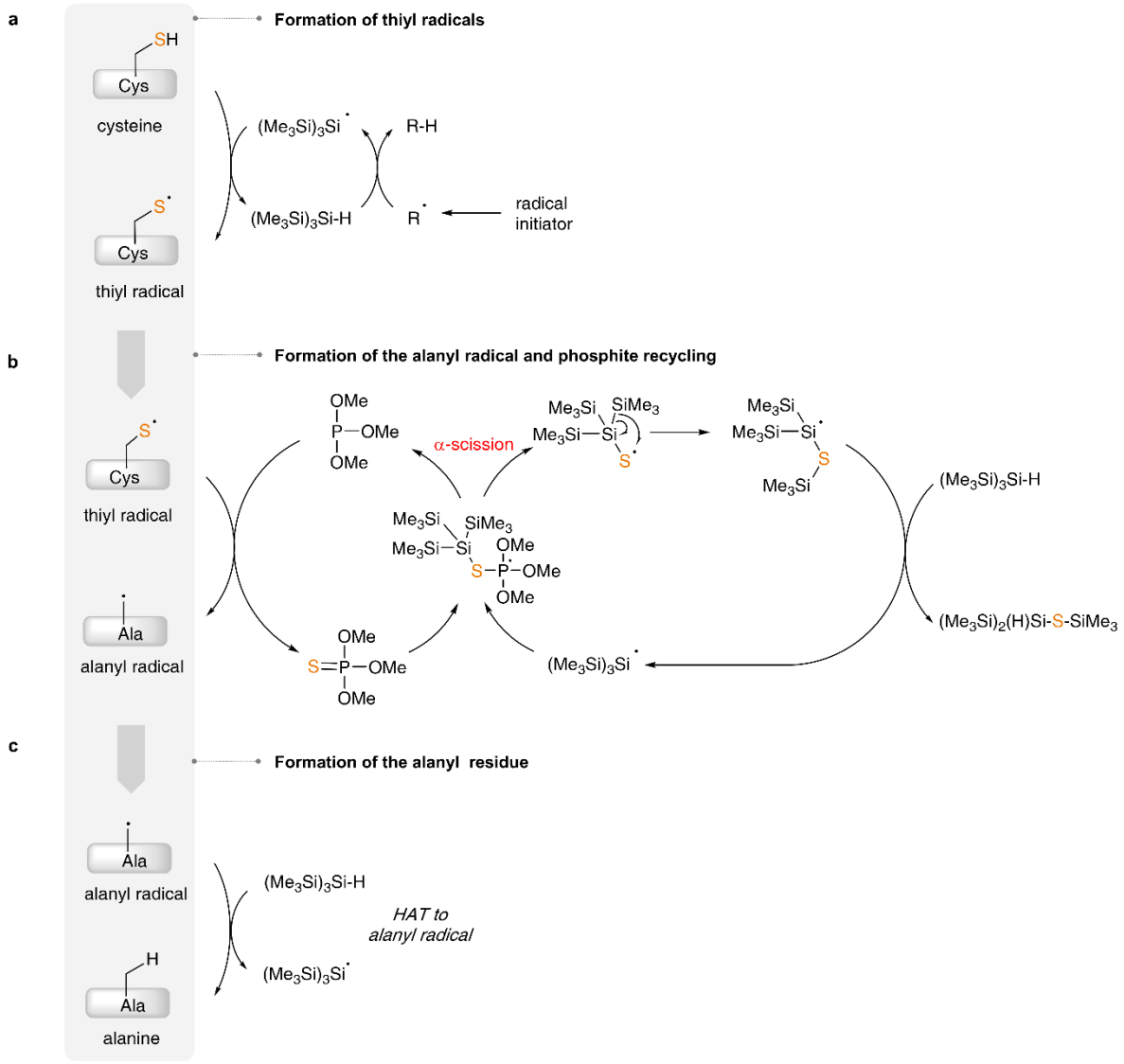
Free-radical polypeptide desulfurization by phosphorus(III) reagents

Desulfurization promoted by radical initiators

Morsy *et al.* found a way to perform peptidic thiol desulfurization using substoichiometric amounts of a phosphite (see Supplementary Figure 1).[1]

The authors proposed that *tris*(trimethylsilyl)silane participates to the initiation (formation of thiyl radicals) and to the termination (hydrogen atom transfer to alanyl radical) of the radical chain mechanism as depicted in Supplementary Figure 1a and Supplementary Figure 1c respectively. Furthermore, it was postulated that the *tris*(trimethylsilyl)silyl radical generated in the initiation and termination steps is involved in the reduction of the phosphite sulfide produced alongside the alanyl radical. Such a process enables the recycling of the active phosphite species and the propagation of the desulfurization process (Supplementary Figure 1b).

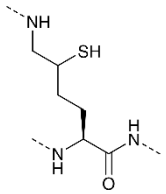
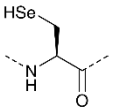
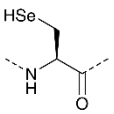
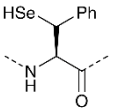
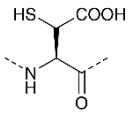
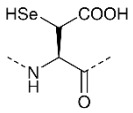
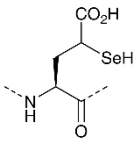


Supplementary Fig. 1. Phosphite-induced desulfurization of peptidic thiols uses tris(trimethylsilyl)silane as the stoichiometric reductant and sulfur atom sink.

NCL/dechalcogenation strategies

The search for selectivity

Supplementary Table 1. Works describing the selective dechalcogenation of mercapto or seleno AAs in the presence of other amino acid residues of the same type.

Entry	Name of protein or peptide sequence	Mercapto or seleno AA dechalcogenized		AA preserved		Origin of selectivity	Ref
		nb	AA	nb	AA		
1	SUMO	1	 Lys(δSH)	1	Cys	Lack of accessibility of the preserved Cys residue (the δ-thiolysine residue is solvent-exposed while the preserved Cys is buried within the protein)	[2] [3]
2	Grx3(1-38)(C11**U**-C14**U**-A38**C**)	2	 Sec	1	Cys	Ease of selenyl radical formation compared to thiyl radical formation	[4]
3	PHPT1(2-125)(A35**U**)	1	 Sec	3	Cys	Ease of selenyl radical formation compared to thiyl radical formation	[5]
4	Ac-LYRANG**F(βSeH)**SPGY**C**-NH_2 Ac-LYR**C**NG**F(βSeH)**SPGYS-NH_2 Ac-LYR**C**NM**F(βSeH)**SPGYS-NH_2	1	 Phe(βSH)	1	Cys	Ease of selenyl radical formation compared to thiyl radical formation	[6]
5	CXCR4(1-38)(D20**D(βSH)**)	1	 Asp(βSH)	1	Cys	Lower BDE of the C-S bond in Asp(βSH) owing to the presence of the stabilizing nearby carboxylic acid group allows selective desulfurization in the presence of Cys	[7]
6	SelK(2-94)(D61**D(βSeH)**)	1	 Asp(βSeH)	1	Sec	Due to the stabilization of the electron-deficient carbon-centered radical by the nearby carboxylate, Asp(βSeH) can be selectively deselenized in the presence of Sec.	[8]
7	H-**E(γSeH)**SP**U**YS-NH_2	1	 Glu(γSeH)	1	Cys	Due to the stabilization of the electron-deficient carbon-centered radical by the nearby carboxylate, Glu(γSeH) can be selectively deselenized in the presence of Cys.	[8]

How green are desulfurization methods?

Energy and type of reactor

The majority of NCL/desulfurization techniques described so far proceed in the temperature range 25-45 °C, which is within the limits for obtaining a green flag (0-70 °C) according to Chem21 toolkit.[9] Most of the works published so far use batch reactors (amber flag). The adaptation of peptide ligation techniques to the synthesis of peptides or proteins under microfluidic conditions is rare (green flag),[10, 11] with only one study tackling the challenge of conducting tandem NCL and Cys desulfurization in a flow system.[11]

Stoichiometry for reagents

The Chem21 toolkit assigns a green, amber or red flag to reactions that respectively utilize sub-stoichiometric, stoichiometric or over-stoichiometric amounts of reagents and catalysts. Following this classification, most of NCL and desulfurization techniques and, therefore, most of one-pot NCL/desulfurization processes developed up to now are marked with a red flag due to their inclination to utilize a large excess of additives. New approaches are emerging to address this issue. For example, Ollivier *et al.* showed that electrostatic assistance of the NCL reaction enabled the sub-stoichiometric use of MPAA (0.1 equiv), while achieving significant rate accelerations.[12, 13] This approach allowed for a one-pot NCL/desulfurization process without intermediate MPAA extraction as the final MPAA concentration used (50 µM) was below the threshold that would inhibit desulfurization. Nevertheless, this work could not circumvent the large excess of phosphine (TCEP 172 mM), radical initiator (VA-044 17 mM) and hydrogen donor (GSH 43 mM) needed at the desulfurization stage. In another example that has already been discussed before, the desulfurization of Cys-containing peptides could be performed in high yield upon light irradiation at 365 nm while using stoichiometric amounts of the phosphine reagent and no thiol additive.[14] However, the coupling of this desulfurization method with NCL in one-pot has not been realized yet. Other promising methods for peptide desulfurization using nearly stoichiometric (tBuOOtBu 1.2 equiv./light,[15] amber flag) or sub-stoichiometric amounts (Togni-II reagent 0.1 equiv.,[16] $FeSO_4$ 0.001-0.01 equiv.,[17] green flag) of radical initiator are worth to be mentioned in that context (vide infra). These studies underscore the growing preference for more stoichiometric methods in NCL and Cys desulfurization. However, they also reveal the challenge of integrating these approaches to achieve a one-pot NCL/desulfurization process without the need for reagent excesses at any stage.

Type of purification technique used

The Chem21 toolkit has been primarily devised for accessing reactions enabling small organic molecule synthesis. In that context and logically, a green flag is given for purification methods such as filtration, crystallisation or low temperature distillation, while the resort to chromatographic methods results in the attribution of a red flag. This toolkit will certainly benefit from adaptions to take into account the special nature of peptides and proteins, especially regarding their methods of purification. Chemical protein synthesis necessitates the purification of polypeptides that are highly polar biomolecules. Various studies have been faced with the challenge of purifying large polypeptides when employing classical chromatography methods such as reversed-phase HPLC, during which significant mass losses are often observed. As already quickly discussed in this review, this limitation has spurred significant research efforts towards performing series of chemical transformations within a single reactor or on solid phase.[18, 19] Complementary, works aiming at replacing reversed-phase HPLC purification by liquid handling and simple filtrations using solid phase selective and reversible capture techniques show

great promise.[20] Some of these approaches have already integrated the assets of the NCL/desulfurization tactic.[20, 21]

Solvents, reagents and catalysts safety and toxicity

Supplementary Table 2. Solvents, reagents and catalysts safety and toxicity: flag assignment according to Chem21 toolkit rules.

Entry	Chemical name (acronym)	Chem 21 flag for safety/toxicity	GHS code: H phrase physical hazards
	Solvents		
1	Guanidine hydrochloride (Gnd·HCl)		
	NCL catalysts		
2	4-mercaptophenylacetic acid (MPAA)[22]		
3	Thiophenol[23]		H300 : Fatal if swallowed H310 : Fatal in contact with skin H330 : Fatal if inhaled H410: Very toxic to aquatic life with long lasting effects
4	4-mercaptophenol[22, 24]		
5	Methyl thioglycolate (MTG)[25]		H301 : Toxic if swallowed
6	2,2,2-Trifluoroethanethiol (TFET)[26]		H224: Extremely flammable liquid & vapor
	Radical initiators		
7	2,2'-Azobis[2-(2-imidazol in-2-yl)propane]dihydrochloride (VA-044)[27]		H411: Toxic to aquatic life with long lasting effects
8	Sodium tetraethylborate[28]		H250: Catches fire spontaneously if exposed to air
9	*t*BuOO*t*Bu[15]		H341: Suspected of causing genetic defects H412: Harmful to aquatic life with long lasting effects
10	Togni-II[16]		
11	$FeSO_4$[17]		
	P(III) reagents		
12	Tris(2-carboxyethyl)phosphine hydrochloride (TCEP)[27]		
13	Trimethylphosphite		
14	Triethylphosphite		H412 Harmful to aquatic life with long lasting effects
	Hydrogen donors		
15	2-Methyl-2-propanethiol (*t*BuSH)[27]		H411 Toxic to aquatic life with long lasting effects
16	Sodium 3-mercapto-1-propanesulfonate (MPSNa)[17]		
17	Sodium 2-mercaptoethanesulfonate (MESNa)[29]		
18	Glutathione (GSH)[30]		

Critical elements

The Critical Elements parameter in the Chem21 toolkit analyses whether the chemical process utilizes elements that are anticipated to be in short supply in the near future. [31, 32] The aim is to incite chemists to minimize or preferably avoid the use of red flagged elements whose supply is not guaranteed over 50 years. The remaining years until depletion of known reserves for a given element is always a matter of debate and the numbers need to be regularly updated to take into account the discovery of novel resources, the changes in the rate of consumption and the development of recycling techniques among other parameters. Chem21 toolkit uses the estimations reported by Hunt et al. in 2013.[33] Supplementary Table lists common solvents and reagents used for performing NCL/desulfurization according to Chem21 critical element criteria. Most of these are carbon, oxygen and nitrogen-based organic compounds, eventually in association with chlorine (TCEP·HCl, Gnd·HCl) or sodium (MESNa, MPSNa) elements. These elements will remain available in large quantities beyond 500 years (green flag). Sulfur and phosphorus elements are characterized by a remaining supply between 50 and 500 years. Of these two elements, phosphorus is of concern with potential threats of limitation due to socio-economic and environmental issues, although existing reserves are substantial.[34] Using Hunt's scale of critical elements, thiols and P(III) reagents are therefore amber flagged. The same for lithium, boron and iodine elements that enter into the composition of some radical initiators discussed above.

Supplementary Table 3. Critical Element flag assessment for some chemicals discussed in this review. European Medecines Agency Permitted Daily Exposure (PDE) values for Ru, Ir, Mn, Co, Ni are indicated.

Entry	Chemical	Element	Critical element flag	Class / Oral PDE (µg/day)[a,b]
1	Thiol	Sulfur		
2	Phosphine/Phosphite	Phosphorus		
3	Lithium tetraethylborate[28]	Lithium, Boron		
4	Sodium borohydride[35]	Boron		
5	$Ru(bpy)_3Cl_2$[36]	Ruthenium		2B / 100
6	Pd/Al_2O_3	Palladium		2B / 100
7	$[Ir(dF(CF_3)ppy)_2(dtb\text{-}bpy)]PF_6$[37]	Iridium		2B / 100
8	$MnCl_2$,[38] $Mn(OAc)_3$[39]	Manganese		na
9	$CoCl_2$[38]	Cobalt [c]		2A / 50
10	Raney Nickel	Nickel		2A / 200
11	$FeSO_4$[17]	Iron		na / 15000[d]

[a] Class 1 elements, including arsenic (As), cadmium (Cd), mercury (Hg), and lead (Pb), are known human toxicants. Class 2 elements are route-dependent human toxicants. Within Class 2, Class 2A elements such as cobalt (Co), nickel (Ni), and vanadium (V) are toxic and require systematic risk assessment. On the other hand, Class 2B elements, such as silver (Ag), gold (Au), iridium (Ir), osmium (Os), palladium (Pd), platinum (Pt), rhodium (Rh), ruthenium (Ru), selenium (Se), and thallium (Tl), are also toxic but do not necessitate systematic risk assessment. Class 3 elements, including barium (Ba), chromium (Cr), copper (Cu), lithium (Li), molybdenum (Mo), antimony (Sb), and tin (Sn), have relatively low toxicities with oral permitted daily exposures (PDEs) exceeding 500 µg/day. Class 2B and 3 elements do not require risk assessment unless intentionally added during the manufacture of any component of the drug product. [b] Data taken from European Medecines Agency report EMA/CHMP/ICH/353369/2013.[40] [c] A recent study estimates that cobalt supply shortage appears inevitable in the short- to medium-term (2028-2033).[41] [d] According to the US Pharmacopeia (USP).[42]

One immediate concern highlighted by the examination of the periodic table of the elements as depicted by Hunt *et al.* is the significant number of elements marked with a red flag. Many metals are at high risk of shortage over the next decades and this causes a serious threat on the use of metal catalysis for synthetic purposes.[43] Most of the metals that are known to catalyse peptide desulfurization resort on amber (cobalt) or red (ruthenium, iridium, manganese) flagged metals. Another aspect discussed by the authors of the Chem21 toolkit article is the stringent control of metal content within peptide APIs. When metal catalysis is employed, the purification level must be adjusted based on the toxicity of the metal. Therefore, the utilization of toxic metals has the potential to negatively affect several metrics of a chemical process. One metal that is not on Hunt's red flagged element list is iron. Iron stands out as an earth-abundant metal. The European Union considers iron as a metal of low inherent toxicity for which no Permitted Daily Exposure (PDE) is defined.[40] According to the US Pharmacopeia (USP), the PDE for iron is 15 mg/day,[42] while the lowest-observed-adverse-effect level (LOAEL) for iron set by the Institute of Medicine for the United States and Canada is 70 mg/day.[44] Therefore, iron is an excellent candidate for developing catalytic processes and has effectively been integrated into the field of small organic molecule synthesis.[45, 46] Recent reports exploiting iron catalysis for amino acid synthesis, peptide synthesis or post-assembly peptide modification show that the field will certainly benefit from the unique reactivity of this metal toward less environmentally impactful production processes.[17, 47, 48]

References

1. R. M. I. Morsy, G. Samala, A. Jalan, M. E. Kopach, N. M. Venneti and J. L. Stockdill, *Chem. Sci.*, 2023, **14**, 9016-9023.
2. P. P. Geurink, F. El Oualid, A. Jonker, D. S. Hameed and H. Ovaa, *ChemBioChem*, 2012, **13**, 293-297.
3. J. Bouchenna, M. Sénéchal, H. Drobecq, J. Vicogne and O. Melnyk, *Bioconjugate Chem.*, 2019, **30**, 2967-2973.
4. N. Metanis, E. Keinan and P. E. Dawson, *Angew. Chem. Int. Ed.*, 2010, **49**, 7049-7053.
5. P. S. Reddy, S. Dery and N. Metanis, *Angew. Chem. Int. Ed.*, 2015, **55**, 992-995.
6. L. R. Malins and R. J. Payne, *Org. Lett.*, 2012, **14**, 3142-3145.
7. R. E. Thompson, B. Chan, L. Radom, K. A. Jolliffe and R. J. Payne, *Angew. Chem. Int. Ed.*, 2013, **52**, 9723-9727.
8. N. J. Mitchell, J. Sayers, S. S. Kulkarni, D. Clayton, A. M. Goldys, J. Ripoll-Rozada, P. J. Barbosa Pereira, B. Chan, L. Radom and R. J. Payne, *Chem.*, 2017, **2**, 703-715.
9. C. R. McElroy, A. Constantinou, L. C. Jones, L. Summerton and J. H. Clark, *Green Chem.*, 2015, **17**, 3111-3121.
10. N. Ollivier, T. Toupy, R. C. Hartkoorn, R. Desmet, J.-C. M. Monbaliu and O. Melnyk, *Nat. Commun.*, 2018, **9**, 2847.
11. T. S. Chisholm, D. Clayton, L. J. Dowman, J. Sayers and R. J. Payne, *J. Am. Chem. Soc.*, 2018, **29**, 9020–9024.
12. N. Ollivier, E. Roy, R. Desmet, V. Agouridas, V. Diemer and O. Melnyk, *Org. Lett.*, 2023, **25**, 2696-2700.
13. N. Ollivier, M. Sénéchal, R. Desmet, B. Snella, V. Agouridas and O. Melnyk, *Nat. Commun.*, 2022, **13**, 6667.
14. N. M. Venneti, G. Samala, R. M. I. Morsy, L. G. Mendoza, A. Isidro-Llobet, J. K. Tom, S. Mukherjee, M. E. Kopach and J. L. Stockdill, *J. Am. Chem. Soc.*, 2023, **145**, 1053–1061.
15. W. Qiu, S. Shi, R. Li, X. Lin, L. Rao and Z. Sun, *Chin. J. Chem.*, 2021, **39**, 1255-1258.
16. J. Zhang, H. Liu, S. Teng, Z. Liao, L. Meng, Q. Wan and S. Dong, *Chem. Commun.*, 2023, **59**, 6513-6516.

17. R. Desmet, C. Boidin-Wichlacz, R. Mhidia, A. Tasiemski, V. Agouridas and O. Melnyk, *Angew. Chem. Int. Ed.*, 2023, **62**, e202302648.
18. V. Agouridas, V. Diemer and O. Melnyk, *Curr. Opin. Chem. Biol.*, 2020, **58**, 1-9.
19. L. Raibaut, O. El Mahdi and O. Melnyk, *Top. Curr. Chem.*, 2015, **363**, 103-154.
20. S. F. Loibl, Z. Harpaz, R. Zitterbart and O. Seitz, *Chem. Sci.*, 2016, **7**, 6753-6759.
21. M. Jbara, M. Seenaiah and A. Brik, *Chem. Commun.*, 2014, **50**, 12534-12537.
22. E. C. Johnson and S. B. H. Kent, *J. Am. Chem. Soc.*, 2006, **128**, 6640-6646.
23. P. E. Dawson, M. J. Churchill, M. R. Ghadiri and S. B. H. Kent, *J. Am. Chem. Soc.*, 1997, **119**, 4325-4329.
24. J. B. Blanco-Canosa, B. Nardone, F. Albericio and P. E. Dawson, *J. Am. Chem. Soc.*, 2015, **137**, 7197-7209.
25. Y.-C. Huang, C.-C. Chen , S. Gao, Y.-H. Wang, H. Xiao, F. Wang, C.-L. Tian and Y.-M. Li, *Chem. Eur. J.*, 2016, **22**, 7623-7628.
26. R. E. Thompson, X. Liu, N. Alonso-Garcia, P. J. Pereira, K. A. Jolliffe and R. J. Payne, *J. Am. Chem. Soc.*, 2014, **136**, 8161-8164.
27. Q. Wan and S. J. Danishefsky, *Angew. Chem. Int. Ed.*, 2007, **46**, 9248-9252.
28. Z. Sun, W. Ma, Y. Cao, T. Wei, X. Mo, H. Y. Chow, Y. Tan, C. H. P. Cheung, J. Liu, H. K. Lee, E. C. M. Tse, H. Liu and X. Li, *Chem*, 2022, **8**, 2542-2557.
29. P. Siman, O. Blatt, T. Moyal, T. Danieli, M. Lebendiker, H. A. Lashuel, A. Friedler and A. Brik, *ChemBioChem*, 2011, **12**, 1097-1104.
30. C. Haase, H. Rohde and O. Seitz, *Angew. Chem. Int. Ed.* , 2008, **47**, 6807-6810.
31. A. J. Hunt, T. J. Farmer and J. H. Clark, in *Element Recovery and Sustainability*, ed. A. Hunt, The Royal Society of Chemistry, 2013, DOI: 10.1039/9781849737340-00001, pp. 1-28.
32. N. Supanchaiyamat and A. J. Hunt, *ChemSusChem*, 2019, **12**, 397-403.
33. A. J. Hunt, T. J. Farmer and J. H. Clark, ed. A. J. Hunt, Royal Society of Chemistry, London, 2013, pp. 1-28.
34. C. Alewell, B. Ringeval, C. Ballabio, D. A. Robinson, P. Panagos and P. Borrelli, *Nat. Commun.*, 2020, **11**, 4546.
35. K. Jin, T. Li, H. Y. Chow, H. Liu and X. Li, *Angew. Chem. Int. Ed.*, 2017, **56**, 14607-14611.
36. X.-F. Gao, J.-J. Du, Z. Liu and J. Guo, *Org. Lett.*, 2016, **18**, 1166-1169.
37. M. Lee, S. Neukirchen, C. Cabrele and O. Reiser, *J. Pept. Sci.*, 2017, **23**, 556-562.
38. M. L. Hamm, D. Nikolic, R. B. van Breemen and J. A. Piccirilli, *J. Am. Chem. Soc.*, 2000, **122**, 12069-12078.
39. R. Griffiths, F. Smith, J. Long, H. Williams, R. Layfield and N. Mitchell, *Angew. Chem. Int. Ed.*, 2020, **59**, 23659-23667.
40. *International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Q3D on elemental impurities - Step 5 - Revision 2*, 2022.
41. A. Zeng, W. Chen, K. D. Rasmussen, X. Zhu, M. Lundhaug, D. B. Müller, J. Tan, J. K. Keiding, L. Liu, T. Dai, A. Wang and G. Liu, *Nat. Commun.*, 2022, **13**, 1341.
42. S. I. Korfali, T. Hawi and M. Mroueh, *Chem. Cent. J.*, 2013, **7**, 10.
43. A. J. Hunt and T. J. Farmer, in *Sustainable Catalysis: With Non-endangered Metals, Part 1*, ed. M. North, The Royal Society of Chemistry, 2015, DOI: 10.1039/9781782622116-00001, pp. 1-14.
44. A. D. Gernand, *Ann. New York Acad. Sci.*, 2019, **1444**, 22-34.
45. A. Fürstner, *ACS Cent. Sci.*, 2016, **2**, 778-789.
46. I. Bauer and H.-J. Knölker, *Chem. Rev.*, 2015, **115**, 3170-3387.
47. T. J. Osberger, D. C. Rogness, J. T. Kohrt, A. F. Stepan and M. C. White, *Nature*, 2016, **537**, 214-219.
48. C. Sydow, F. Sauer, A. F. Siegle and O. Trapp, *ChemSystemsChem*, 2023, **5**, e202200034.