Improvement of Native Chemical Ligation by Extending from Sulfur to Selenium

2021/06/17

Literature Seminar

M1 Habazaki

Contents

Contents

The need for chemical synthesis of proteins

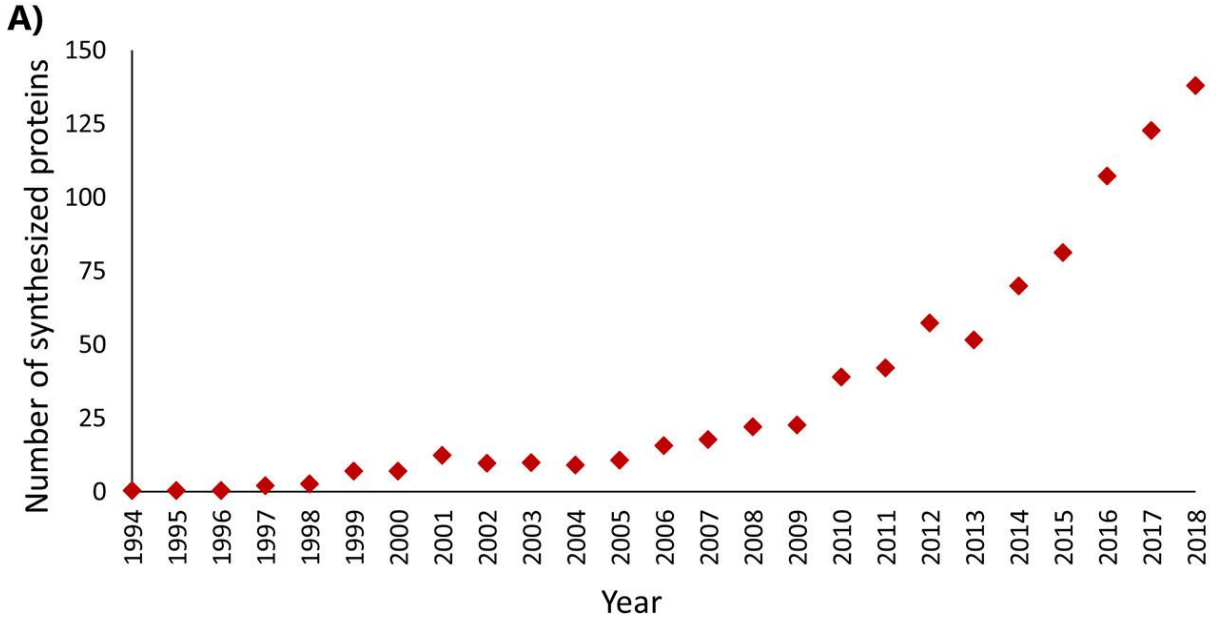
Significance of access to proteins

- Elucidation of **protein and PTM functions**
- Development of **peptide/protein drugs**

How to access to large proteins

- Biological expression
- Chemical synthesis

Specific modifications can be introduced **site-selectively** and **homogeneously**!



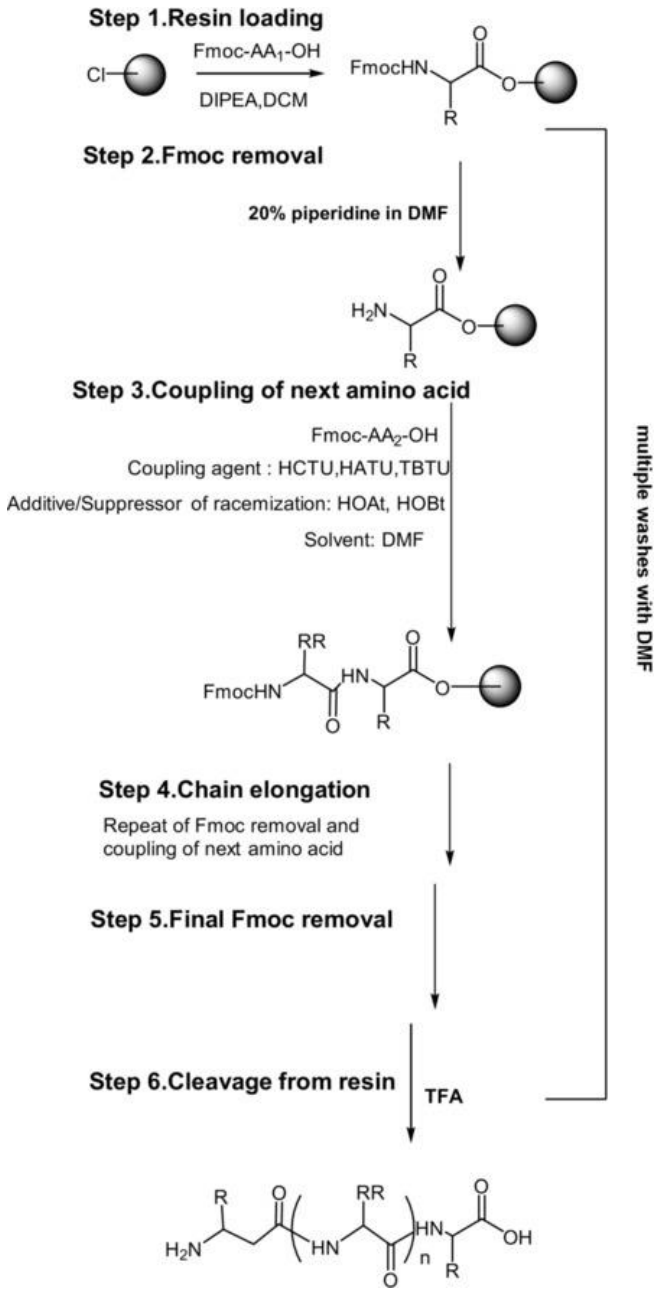
Monbaliu, Melnyk *et. al. Chem. Rev.* **2019,** *119,* 7328–7443

Payne *et. al. Nat. Rev. Chem.* **2018,** *2*, 1–17

Solid phase peptide synthesis (SPPS)

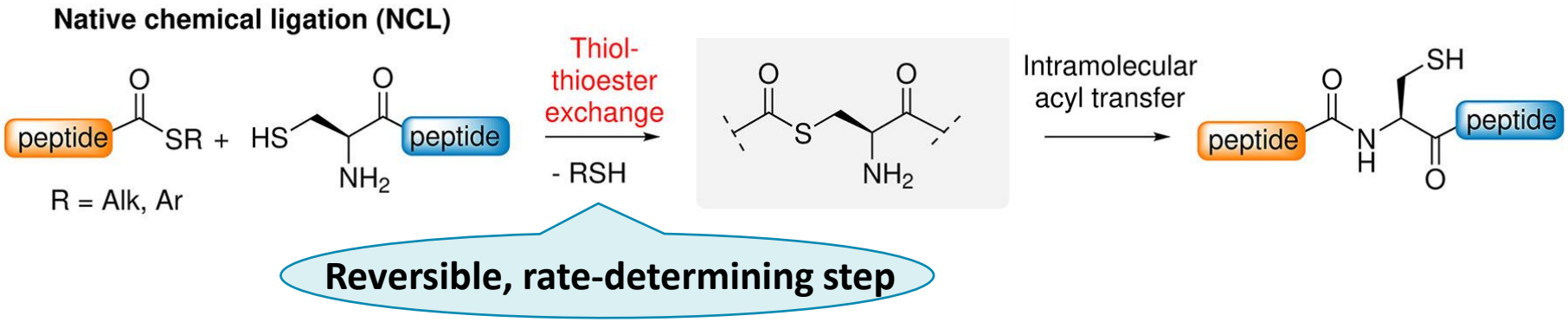
- An extremely reliable platform for the preparation of peptides

- **Longer syntheses are often plagued** by the accumulation of uncoupled sequences, unwanted side products, and epimerization. (**limited to 40-50 residues**)

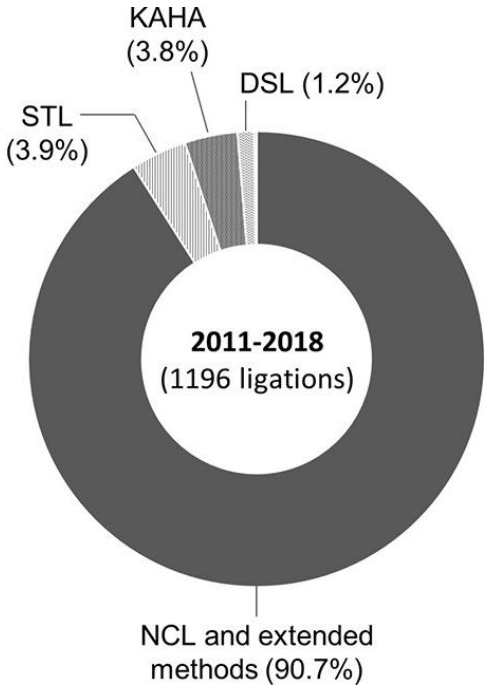


Payne *et. al. J. Pept. Sci.* **2014,** *20,* 64–77
Sarojini *et. al. Chem. Asian J.* **2019,** *14,* 1088–1097

Native Chemical Ligation (NCL)



- A pioneering method for chemical ligation of peptides



- **Without any other functional or protecting groups** than thioester!
- In **purely aqueous media** at **neutral pH**!
- Synthesis of proteins about **200-400 residues** in length!

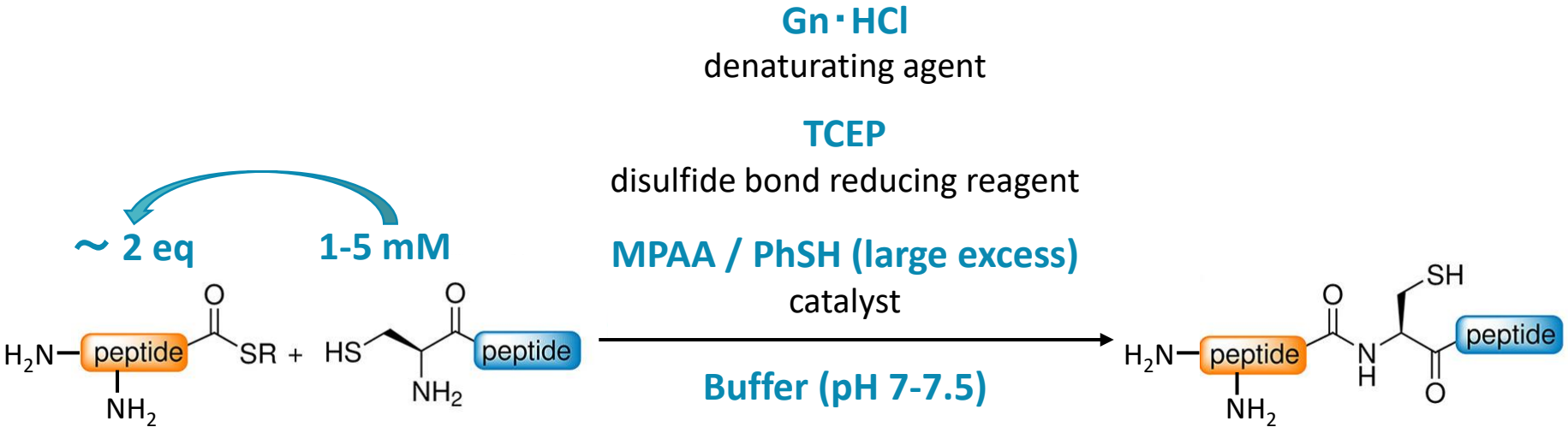
Kent *et. al. Science* **1994,** *266, 776*–779

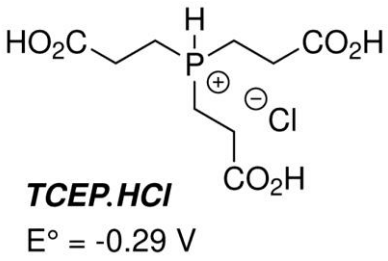
Monbaliu, Melnyk *et. al. Chem. Rev.* **2019,** *119,* 7328–7443

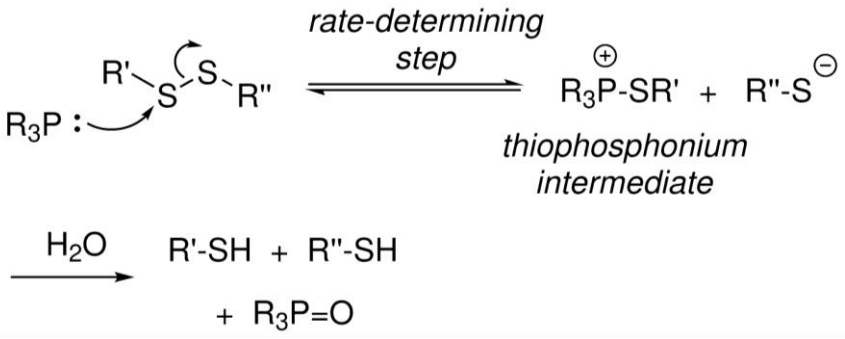
Payne *et. al. Nat. Rev. Chem.* **2018,** 2, 1–17

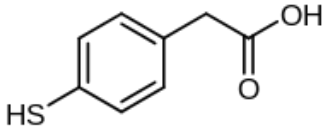
Native Chemical Ligation (NCL)

Typical reaction conditions









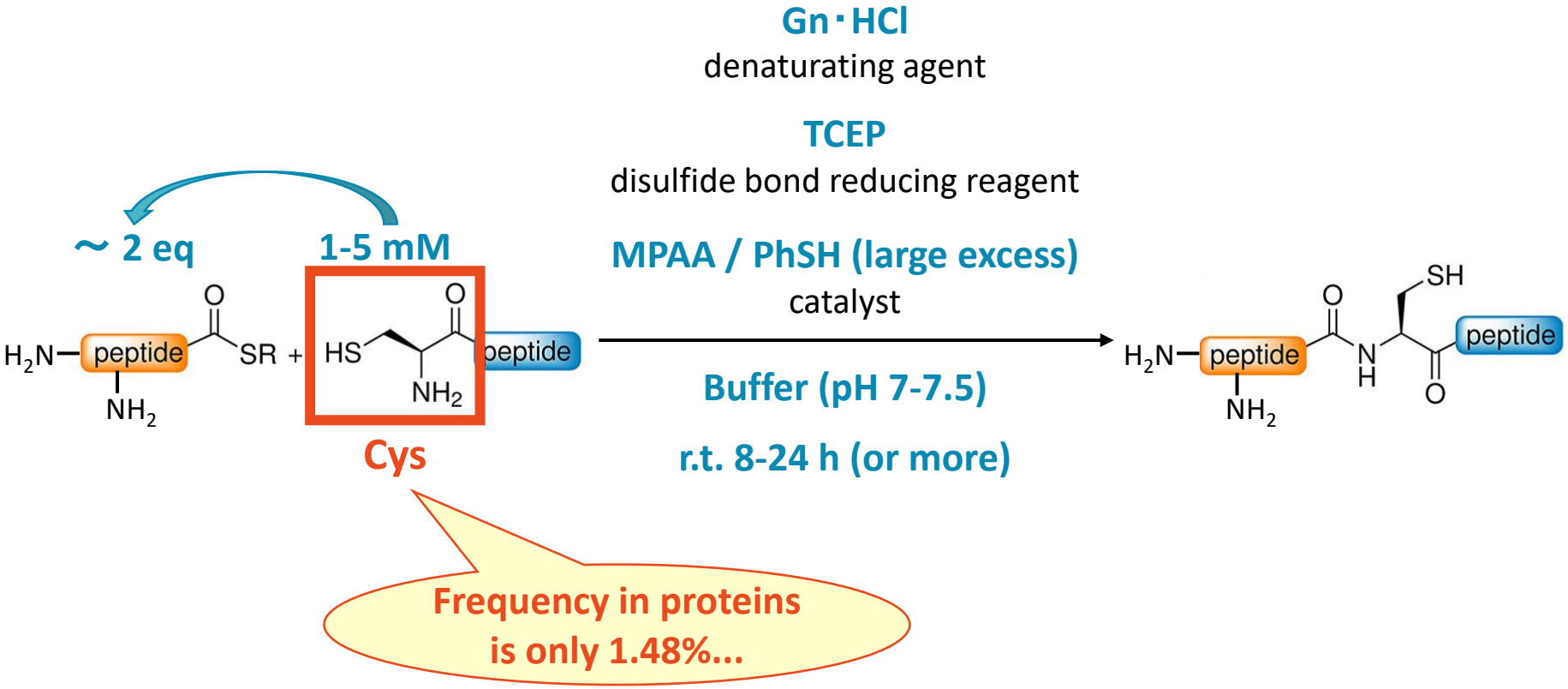
MPAA

Monbaliu, Melnyk *et. al. Chem. Rev.* **2019,** *119,* 7328–7443

Whitesides *et. al. J. Org. Chem.* **1991,** *56*, 2648–2650

Limitations of Native Chemical Ligation (1)

Typical reaction conditions



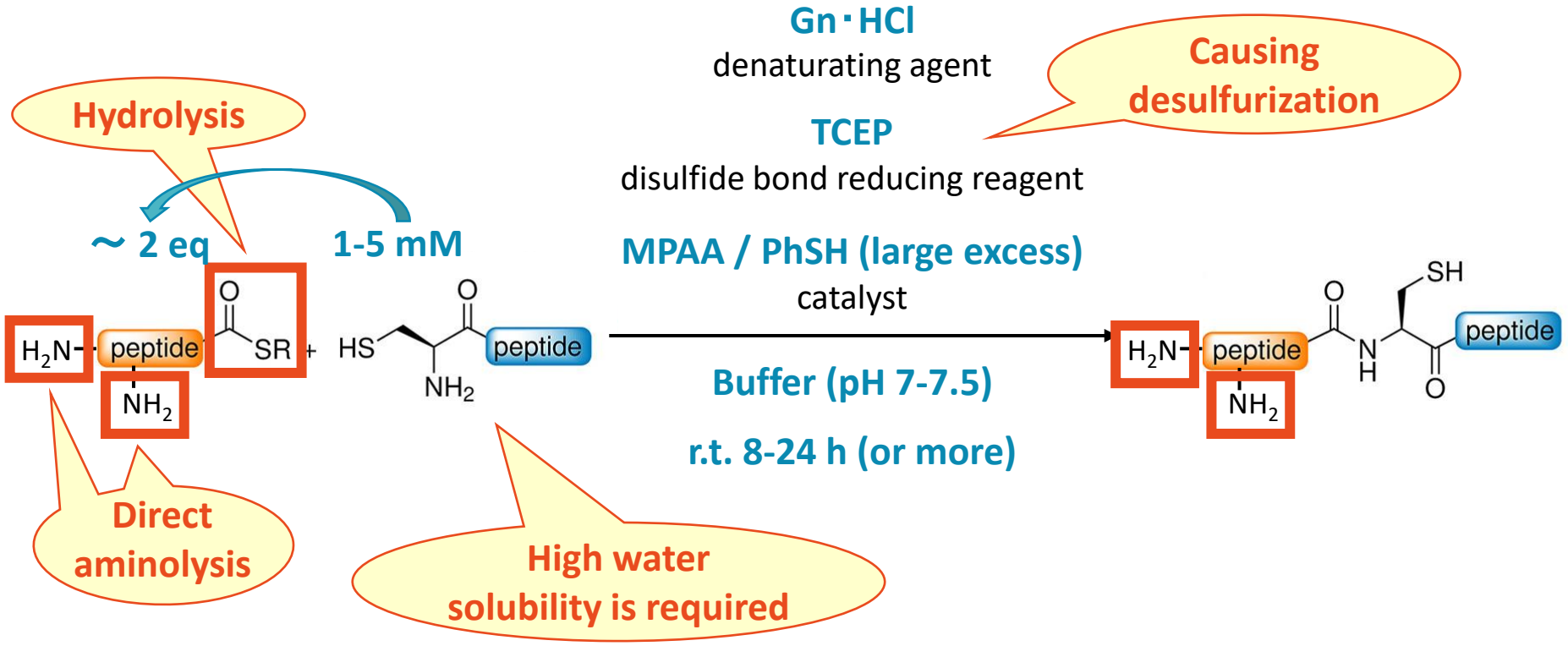
Limitation (1)

- **The need for a Cys residue** on the N terminus of one of the peptide fragments

Agouridas, Melnyk *et. al. Bioorg. Med. Chem.* **2017,** *25,* 4938–4945 Payne *et. al. Nat. Rev. Chem.* **2018,** 2, 1–17

Limitations of Native Chemical Ligation (2)

Typical reaction conditions



Limitation (2)

- **The need for relatively long reaction time** and **high concentration of peptide fragments** owing to moderate reactivity

Durek *et. al. Angew. Chem. Int. Ed.* **2011,** *50, 12042*–12045

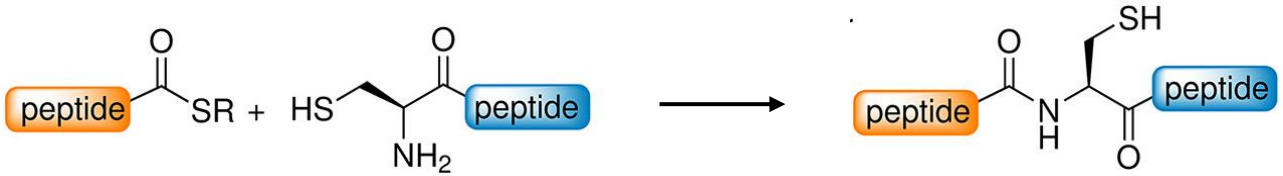
Payne *et. al. Nat. Rev. Chem.* **2018,** 2, 1–17

Extending NCL from sulfur to selenium

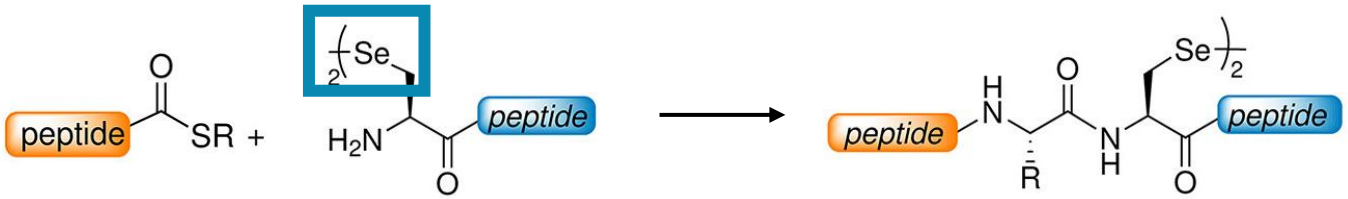
One of the most succussed ligation

- **Extended NCL using selenium (Se) instead of sulfur (S) has unexpectedly succeeded!**

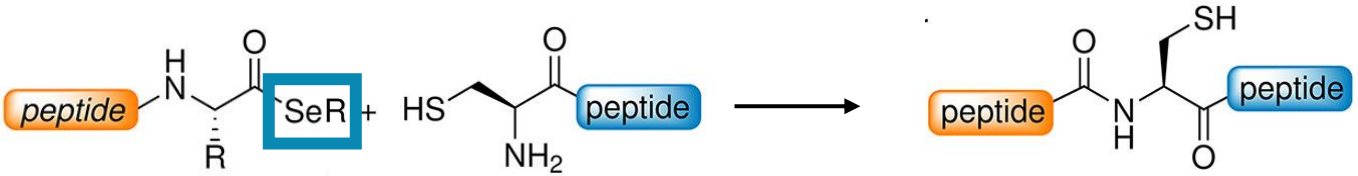
1994
NCL



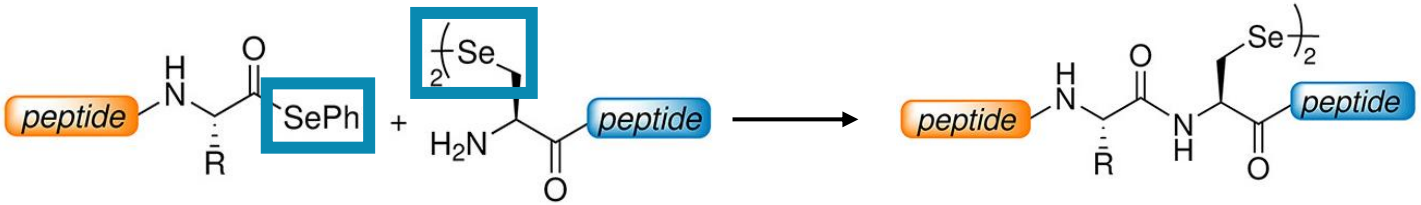
2001
NCL
@Selenocysteine



2011
NCL
@Selenoester



2015
DSL
@Selenocysteine
@Selenoester

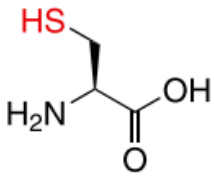


Contents

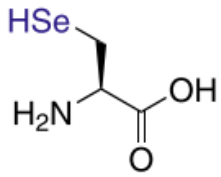
Extending NCL to selenocysteine

Selenocysteine (Sec)

- The 21st proteinogenic amino acid
- Incorporated into natural selenoproteins (25 selenoproteins in human!)

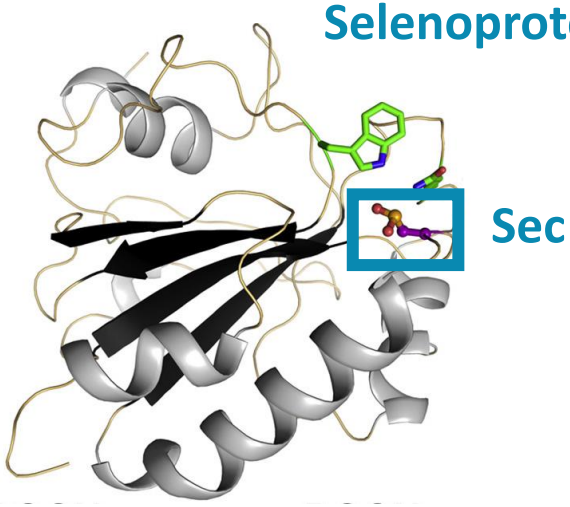


Cysteine (Cys / C)



Selenocysteine (Sec / U)

Selenoproteins



Scheerer *et. al. Biochim. Biophys. Acta, Mol. Cell. Biol. Lipids* **2018,** *1863,* 1095–1107

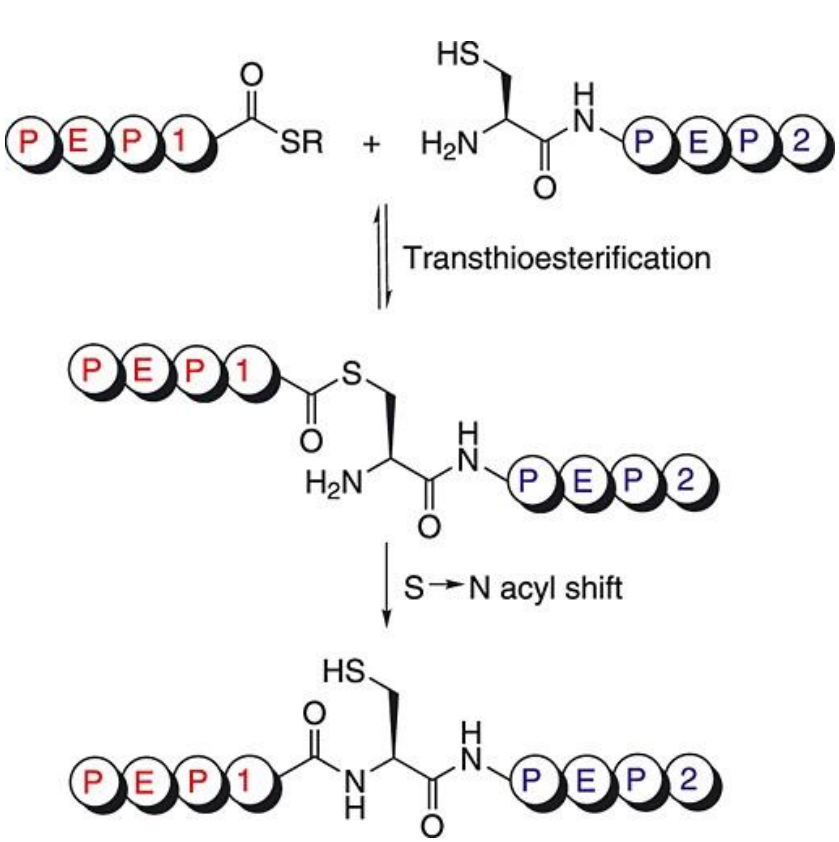
Selenoproteins plays important and unique roles and has been gaining interest!

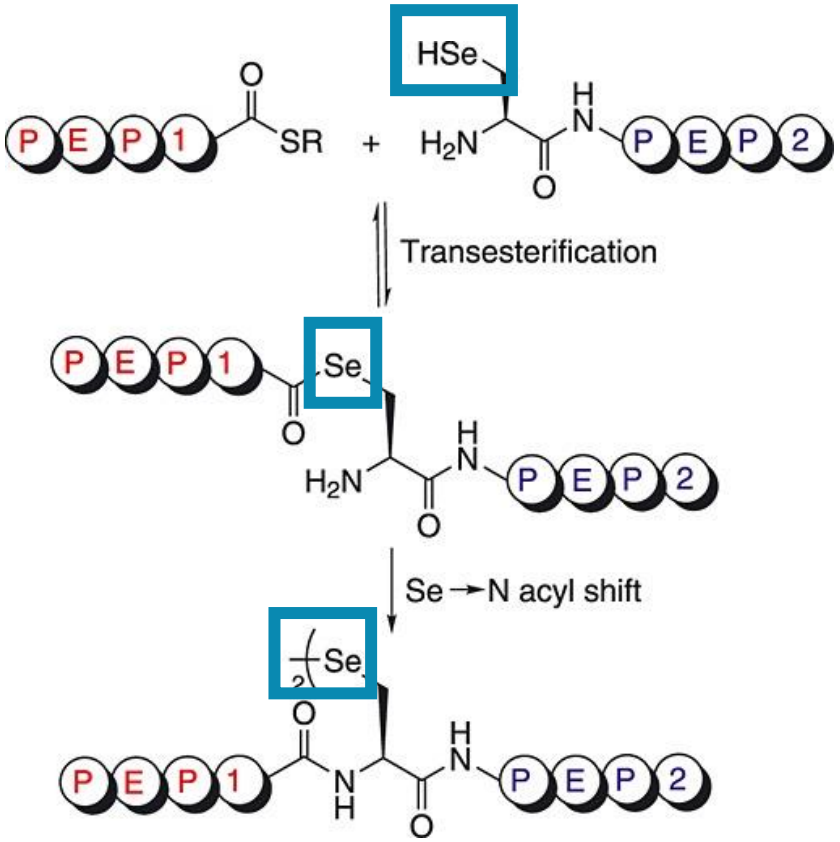
The production of selenocysteine-containing proteins **by biological expression is problematic**…

Gladyshev *et. al. Science* **2003,** *300,* 1439–1443

Payne *et. al. J. Pept. Sci.* **2014,** *20,* 64–77

Extending NCL to selenocysteine



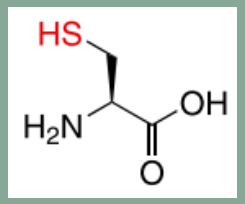
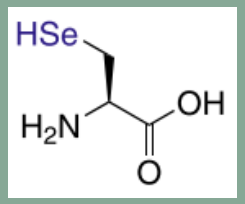


- Sec is the chalcogenic analogue of Cys

  → **Sec was competent in NCL-like transformations** with peptide thioesters!

Raines *et. al. J. Am. Chem. Soc.* **2001**, *123,* 5140–5141
Donk *et. al. Org. Lett.* **2001**, *3,* 1331–1334
Hilvert *et. al. Helv. Chim. Acta* **2001**, *84,* 1197–1206
Payne *et. al. J. Pept. Sci.* **2014**, *20,* 64–77

Differences between Cys and Sec

		Cysteine (S) 	Selenocysteine (Se) 
Atomic radius		100 pm	115 pm
Electronegativity		2.58	2.55
Polarizability volume		2.9 Å	3.8 Å
Redox potential		-180 mV	-381 mV
pK_a		8.30	5.24
BDE	X-H	367 kJ/mol	310–315 kJ/mol
	C-X	309.3 kJ/mol*	257 kJ/mol**

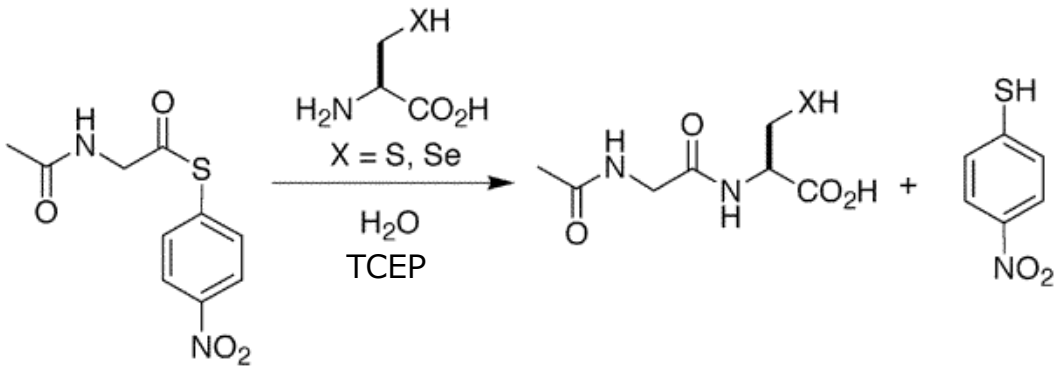
*Value of H_3C–SH, **Value of H_3C–SeH

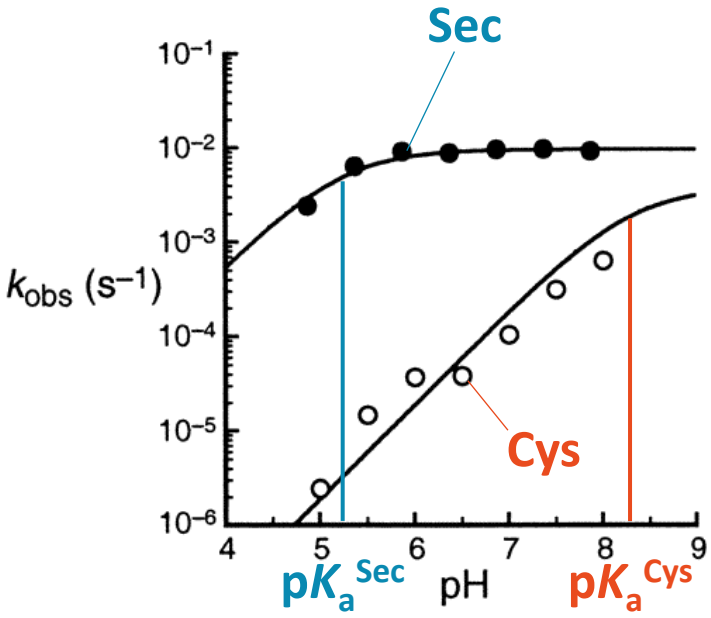
- Sec is the chalcogenic analogue of Cys

  ↔ **Sec exhibits some strikingly different physicochemical properties!**

Monbaliu, Melnyk *et. al. Chem. Rev.* **2019,** *119,* 7328–7443
Raines *et. al. J. Am. Chem. Soc.* **2001,** *123,* 5140–5141
Koppenol *et. al. J. Org. Chem.* **2010,** *75,* 6696–6699
Payne *et. al. J. Pept. Sci.* **2014,** *20,* 64–77

Reactivity of Sec compared to Cys





k_{Cys} (pH=pK_a=8.30)	k_{Sec} (pH=pK_a=5.24)	k_{Sec}/k_{Cys}
3.7 x 10^2 $M^{-1}s^{-1}$	9.5 x 10^2 $M^{-1}s^{-1}$	**2.6**

- **Selenols/selenolate** pairs are **more nucleophilic** because of their **higher polarizability.**
- **Sec selenols** are **significantly more reactive** mainly because of their **higher acidity.**

	Cys (S)	Sec (Se)
Polarizability volume	2.9 Å	**3.8 Å**
pK_a	8.30	**5.24**

Raines *et. al. J. Am. Chem. Soc.* **2001,** *123,* 5140–5141

Koppenol *et. al. J. Org. Chem.* **2010,** *75,* 6696–6699

Rabenstein *et. al. J. Am. Chem. Soc.* **1989,** *111,* 6553–6558

The rate of Sec NCL compared to Cys NCL

Sec selenols are **significantly more reactive** than Cys thiol!

Sec NCL often does **NOT proceed faster** than original Cys NCL…

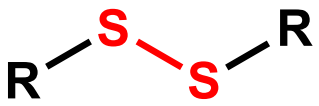
Problem ①

- The rate-determining step of ligation at Sec might be **the reduction of diselenide.**

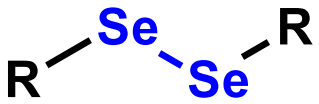
	Cys (S)	Sec (Se)
Redox potential	-180 eV	**-381 eV**

More rapidly formed and highly stable

Disulfide



Diselenide



Donk *et. al. Org. Lett.* **2001,** *3,* 1331–1334

Payne *et. al. J. Pept. Sci.* **2014,** *20,* 64–77

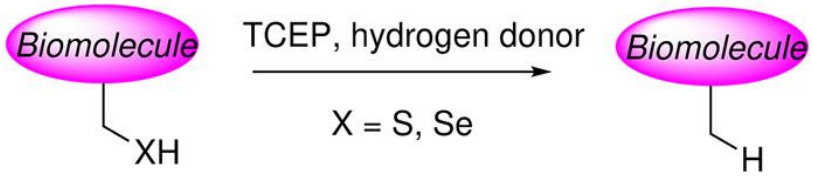
The rate of Sec NCL compared to Cys NCL

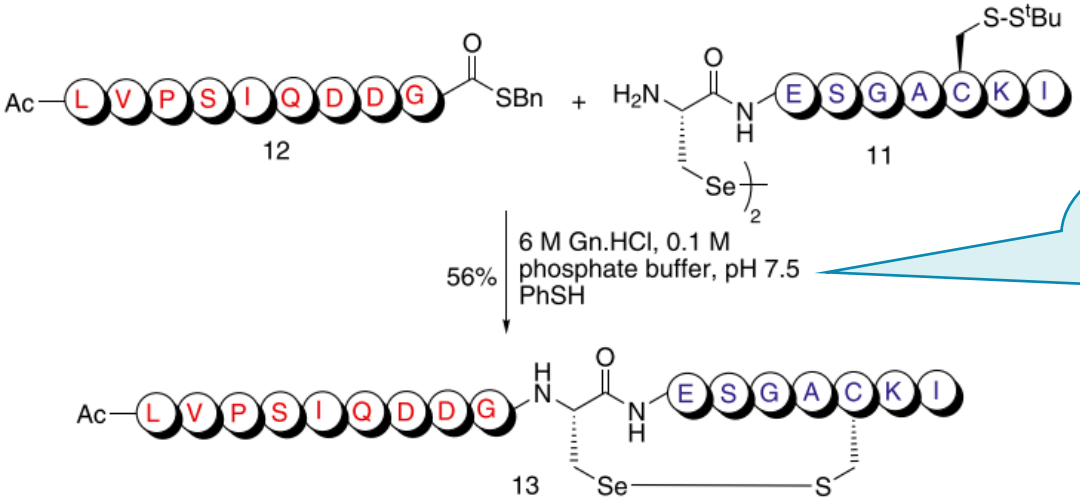
Sec selenols are **significantly more reactive** than Cys thiol!

Sec NCL often does **NOT proceed faster** than original Cys NCL…

Problem ②

- The use of TCEP as a reducing agent can induce **problematic deselenization** during ligation.





Thiol-based additive (PhSH/MPAA) acts as both a **catalyst** and as a **mild reducing agent**.

Donk *et. al. Org. Lett.* **2001,** *3,* 1331–1334

Monbaliu, Melnyk *et. al. Chem. Rev.* **2019,** *119,* 7328–7443

Payne *et. al. J. Pept. Sci.* **2014,** *20,* 64–77

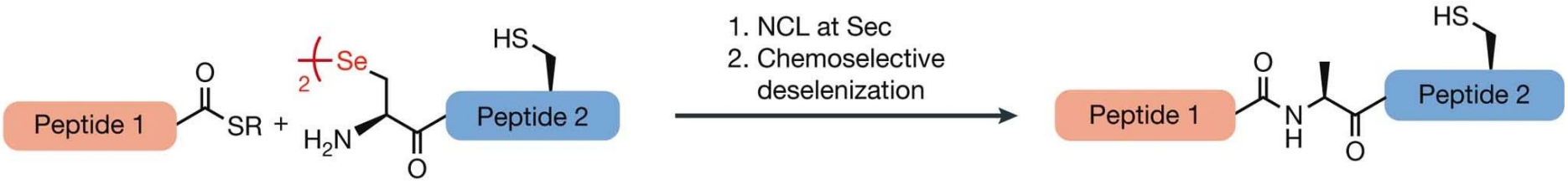
A landmark discovery in Sec NCL chemistry

Sec NCL often does **NOT proceed faster** than original Cys NCL…

Sec NCL have NOT been extensively used for the synthesis of peptides and proteins that do not possess Sec residues in the final product…

A groundbreaking discovery (2010)

- Application of the unique chemical properties of Sec to **selective cleavage of C–Se bond of Sec** in the presence of unprotected Cys after ligation reaction



Keinan, Dawson *et. al. Angew. Chem. Int. Ed.* **2010,** *122,* 7203–7207

Payne *et. al. Nat. Rev. Chem.* **2018,** 2, 1–17

Payne *et. al. J. Pept. Sci.* **2014,** *20,* 64–77

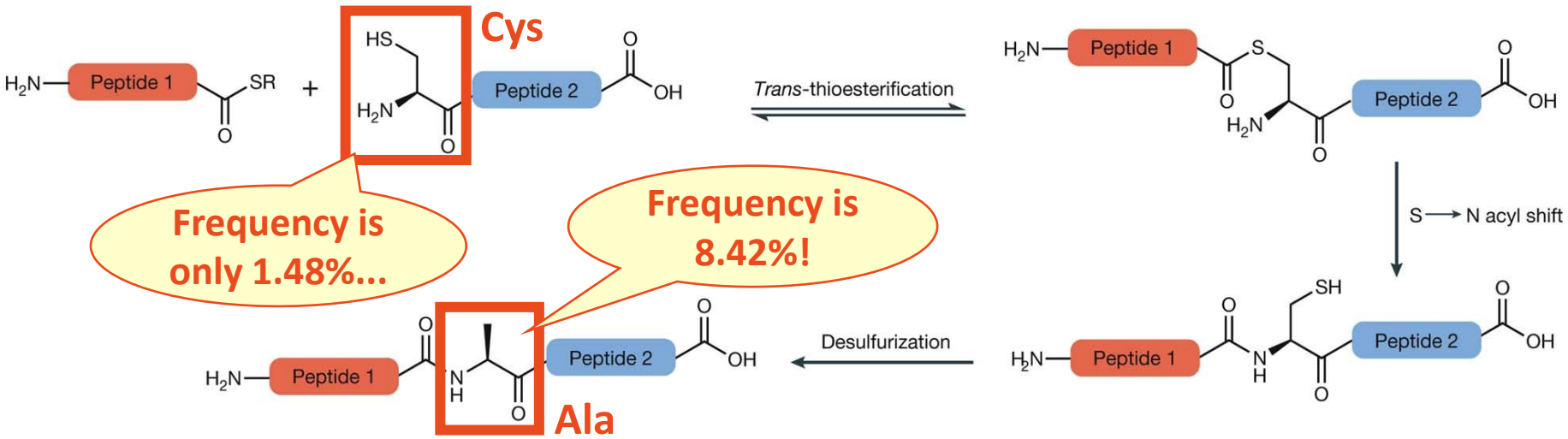
Re: Limitations of Native Chemical Ligation (1)

Limitation (1)

- **The need for a Cys residue** on the N terminus of one of the peptide fragments

Solution

- A **post-ligation desulfurization** permits the use of Cys as a surrogate for ligation sites containing a **substantially more abundant amino acid, Ala**!

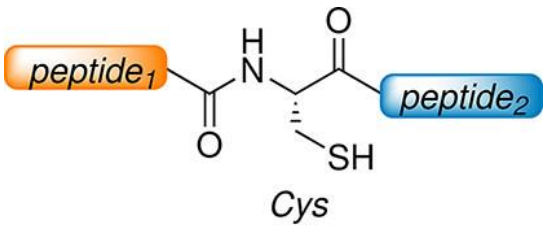


Dawson *et. al. J. Am. Chem. Soc.* **2001**, *123,* 526–533
Agouridas, Melnyk *et. al. Bioorg. Med. Chem.* **2017,** *25,* 4938–4945
Payne *et. al. Nat. Rev. Chem.* **2018,** 2, 1–17

Re: Limitations of Native Chemical Ligation (1)

Limitations of post-ligation desulfurization

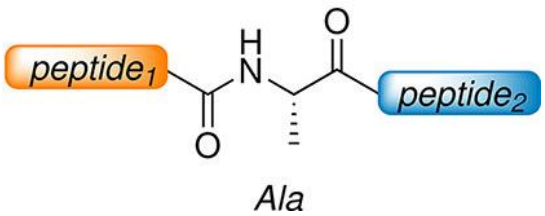
- Any conditions result in **global desulfurization of all thiols** in the protein.

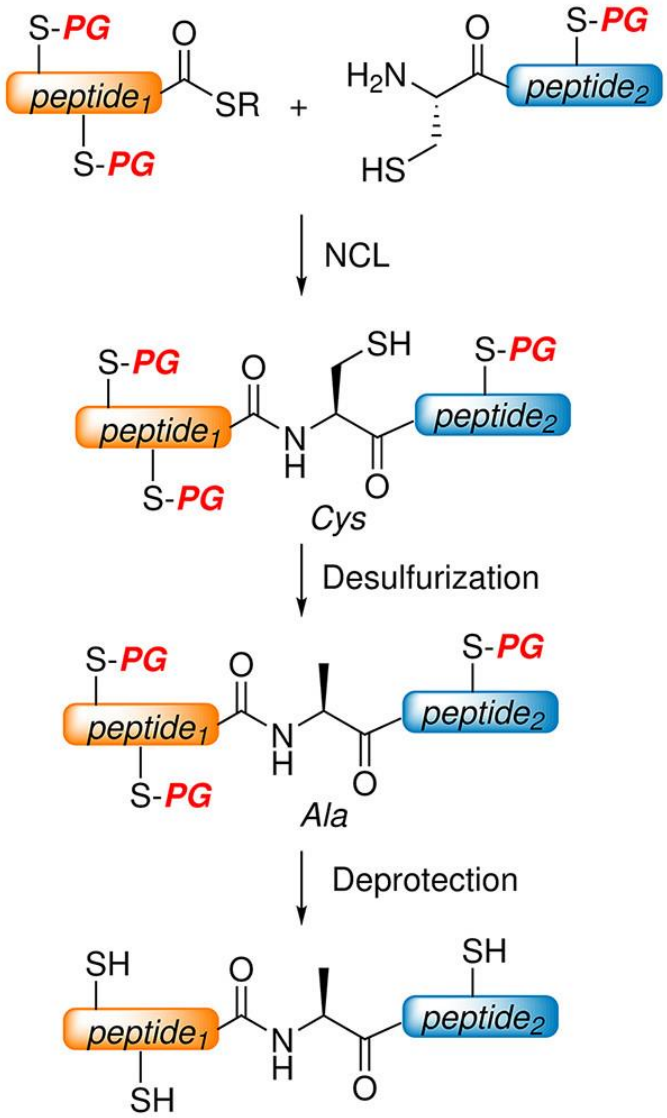


Desulfurization

Metal reagents
(Raney Ni or Pd/Al_2O_3, H_2)

Radical initiator (VA-044)**, TCEP**

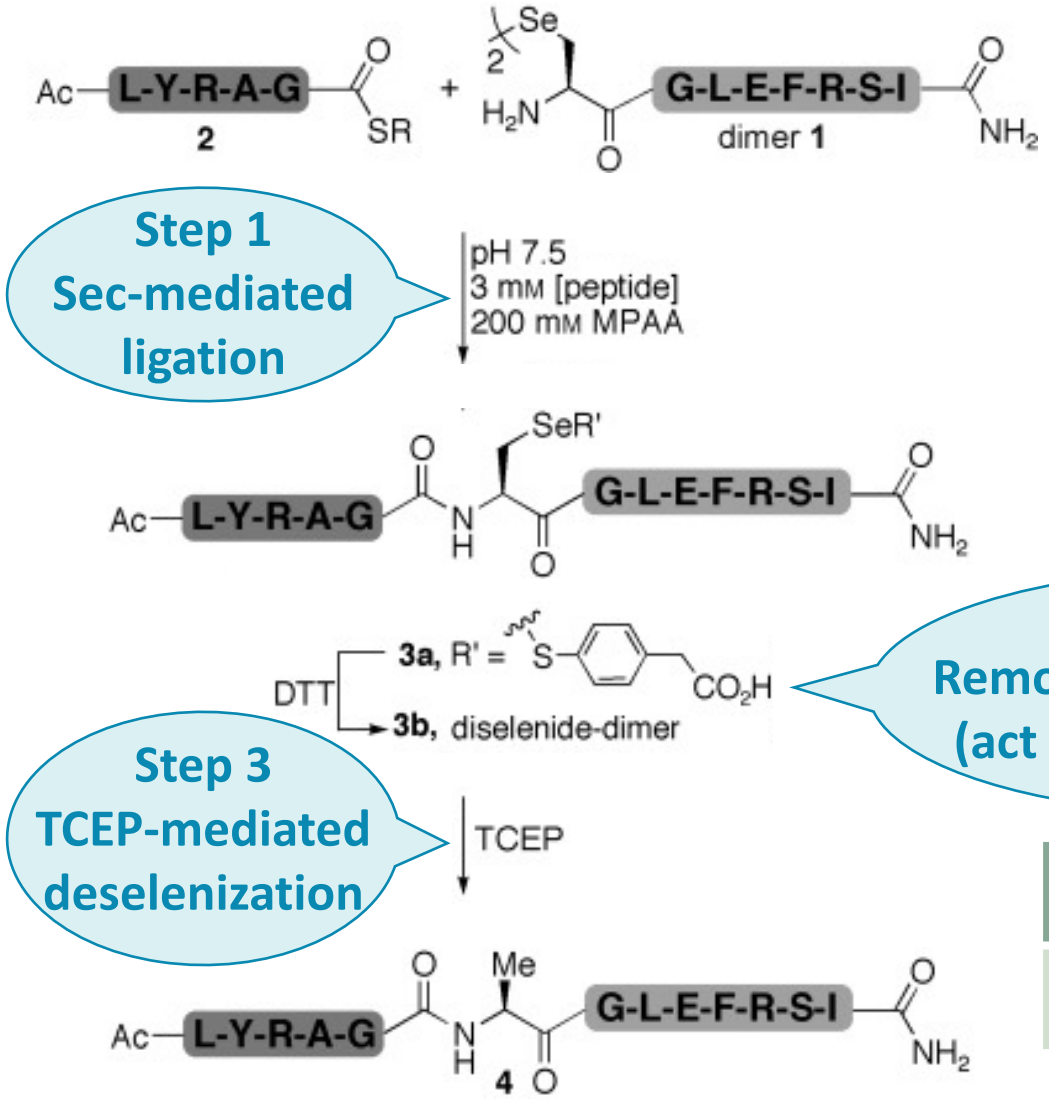




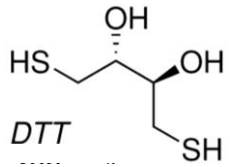
Dawson *et. al. J. Am. Chem. Soc.* **2001**, *123,* 526–533
Danishefsky *et. al. Angew. Chem. Int. Ed.* **2007**, *46*, 9248–9252

Monbaliu, Melnyk *et. al. Chem. Rev.* **2019**, *119,* 7328–7443

Traceless ligation by chemoselective deselenization



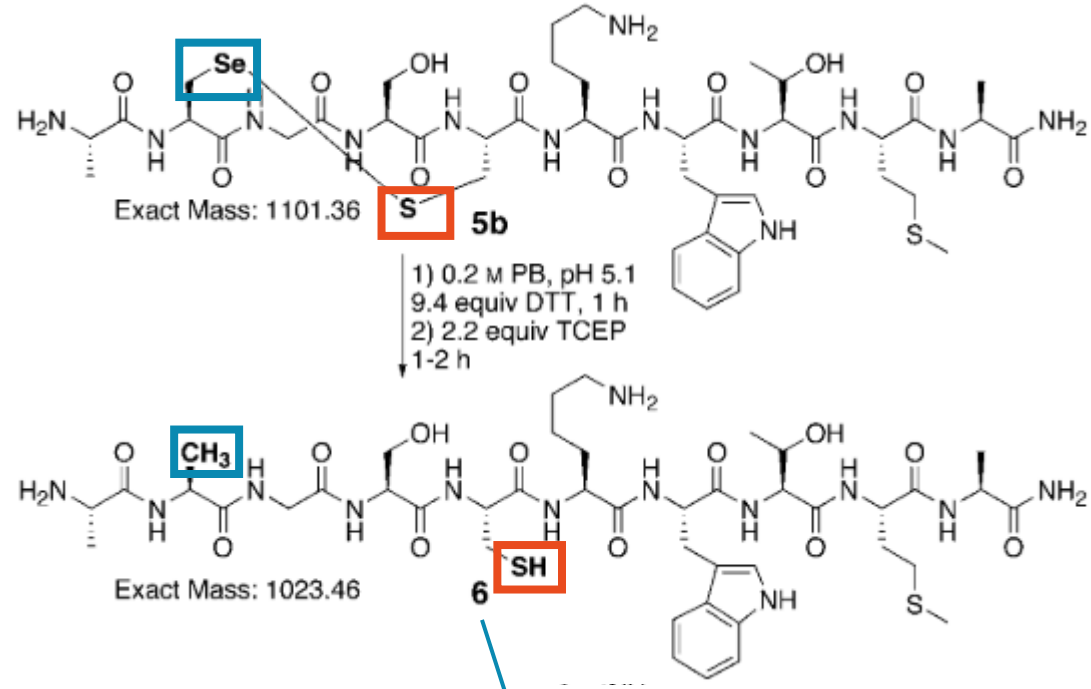
- **Traceless ligation using mild deselenization of Sec** was achieved **using TCEP without an additional radical initiator**!



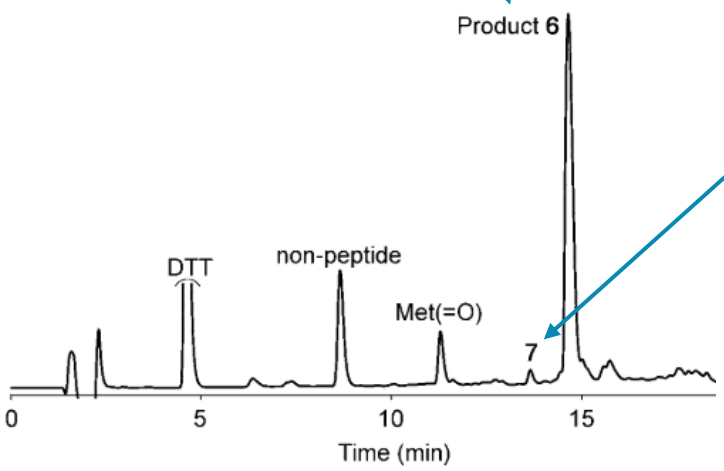
	Cys (S)	Sec (Se)
Redox potential	-180 eV	**-381 eV**

Keinan, Dawson *et. al. Angew. Chem. Int. Ed.* **2010**, *122,* 7203–7207

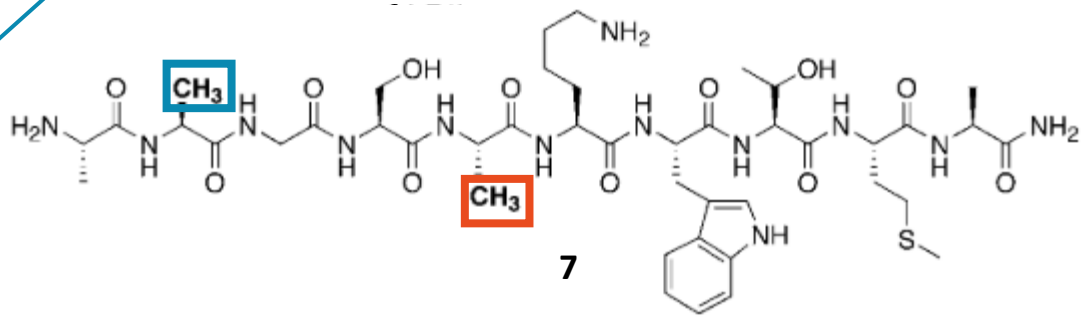
Traceless ligation by chemoselective deselenization



- **Selective cleavage of C–Se bond of Sec** in the presence of unprotected Cys was demonstrated!

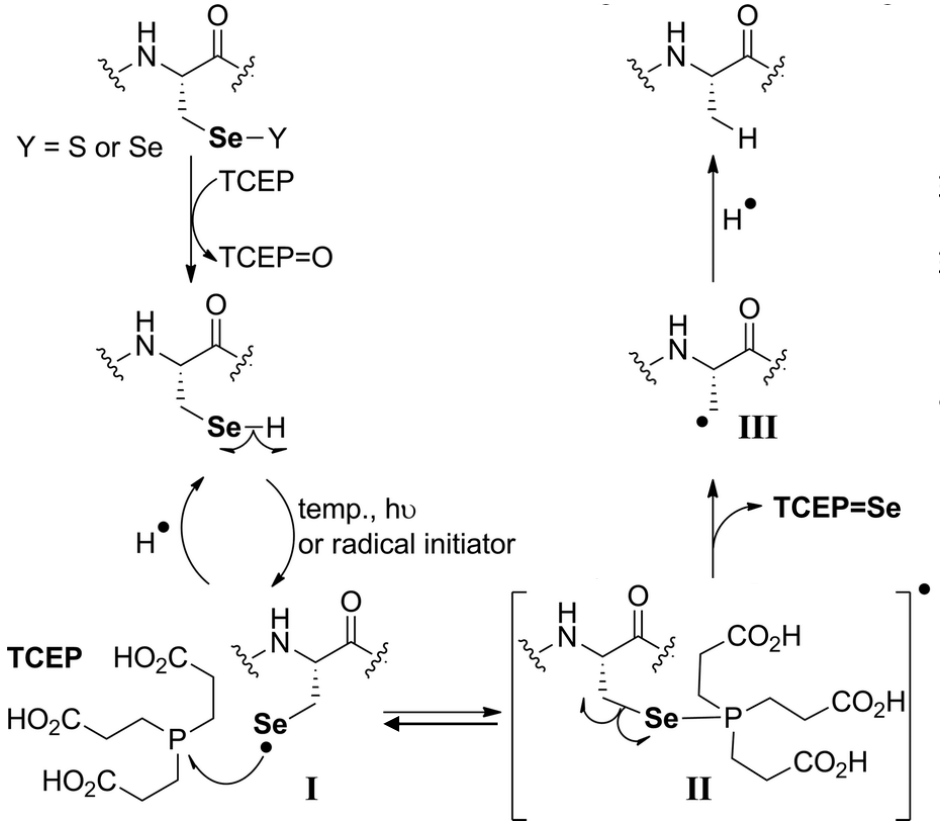


Only traces of the deselenized–desulfurized peptide!



Keinan, Dawson *et. al. Angew. Chem. Int. Ed.* **2010,** *122,* 7203–7207

Proposed mechanism of TCEP-mediated deselenization



- **The Se–H bond is much weaker than S–H**
  → Selective formation of **selenyl radical (Ⅰ)** at room temperature!
- **The C–Se bond is much weaker than C–S**
  → Faster breakdown of **phosphoranyl radical (Ⅱ)**

		Cys (S)	Sec (Se)
BDE	X-H	367 kJ/mol	310–315 kJ/mol
	C-X	309.3 kJ/mol*	257 kJ/mol**

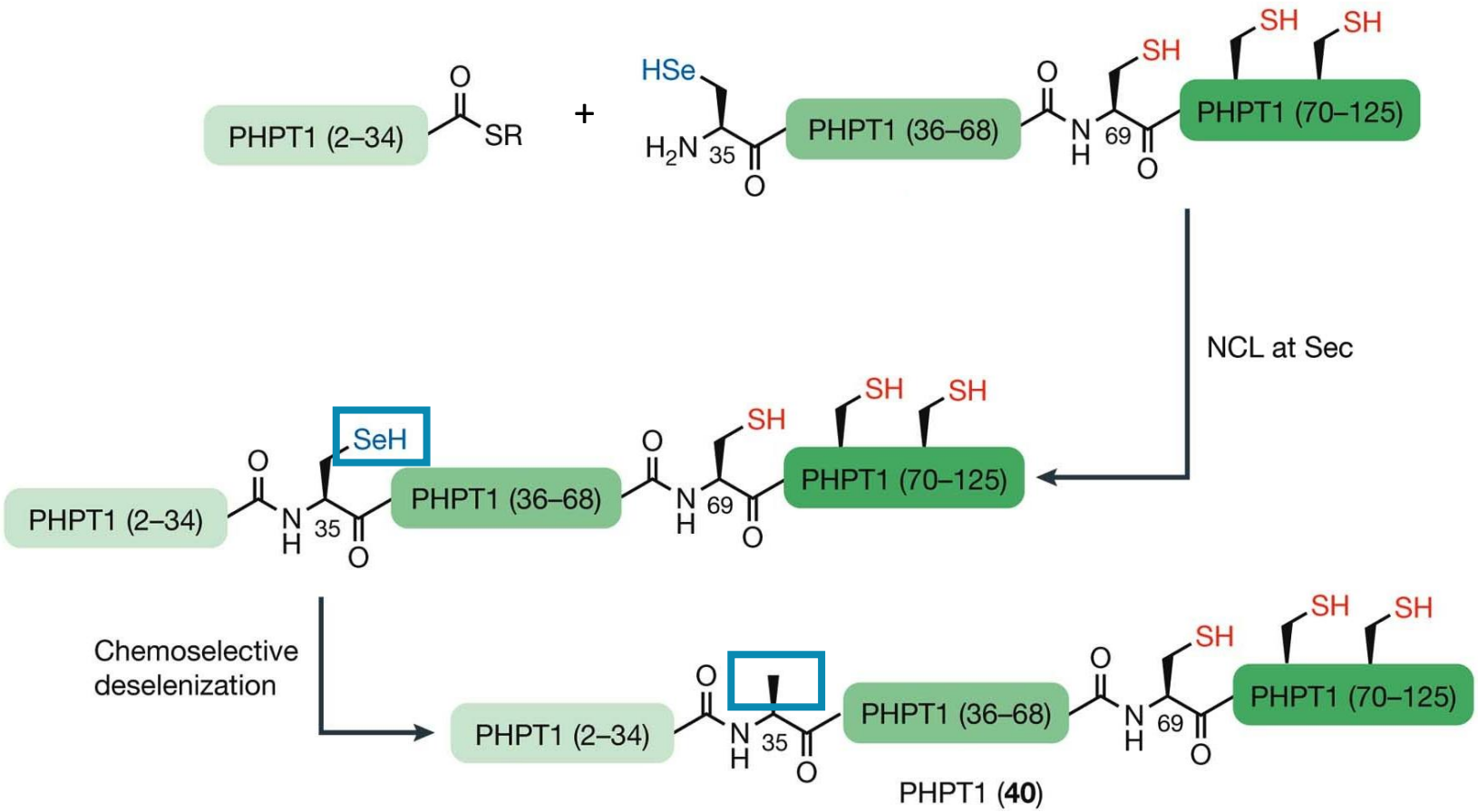
*Value of H_3C–SH, **Value of H_3C–SeH

High selectivity!

Keinan, Dawson *et. al. Angew. Chem. Int. Ed.* **2010,** *122,* 7203–7207

Metanis *et. al. Chem. Sci.* **2015**, *6,* 6207–6212

Traceless ligation by chemoselective deselenization



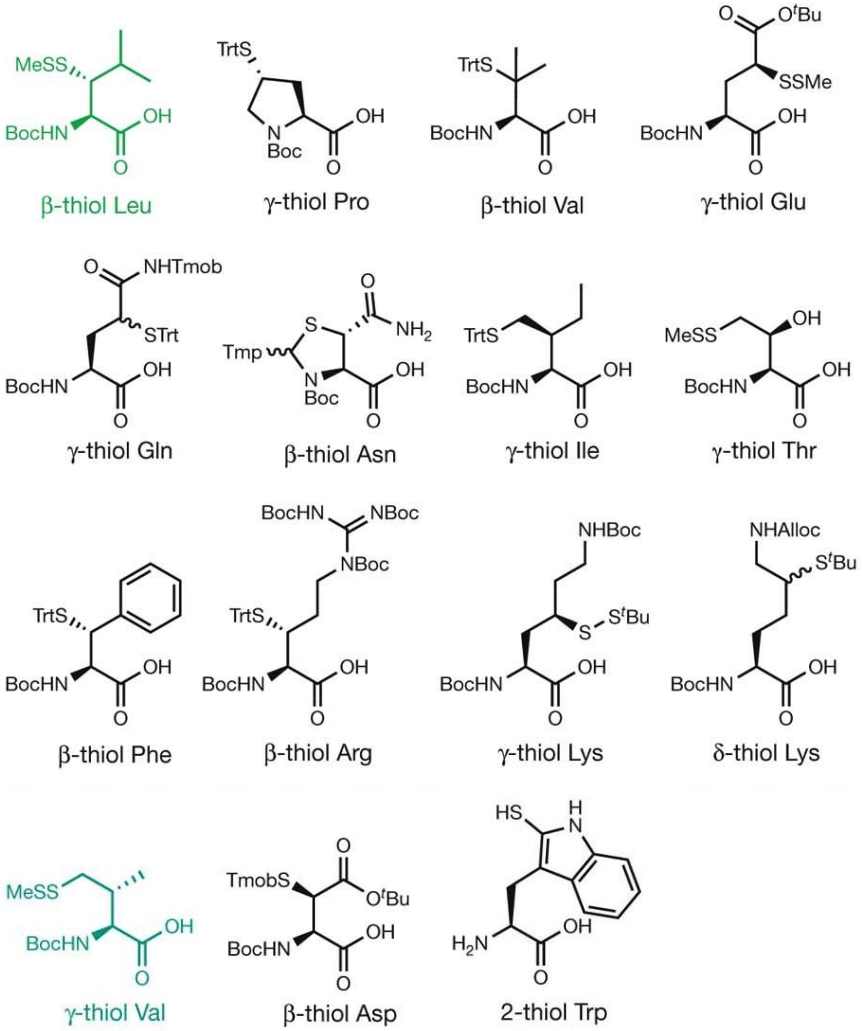
- The power of traceless Sec NCL methodology was exemplified in protein synthesis!

Metanis *et. al. Angew. Chem. Int. Ed.* **2016,** *55,* 992–995

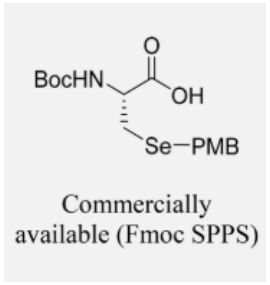
Payne *et. al. Nat. Rev. Chem.* **2018,** 2, 1–17

Traceless ligation by chemoselective deselenization

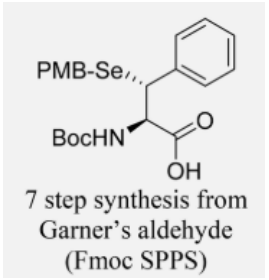
Thiol-derived amino acids



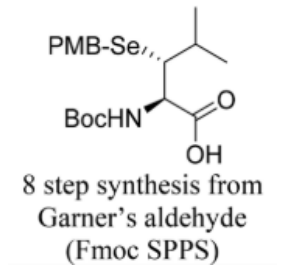
Selenol-derived amino acids



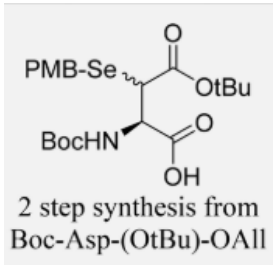
Ala / Ser



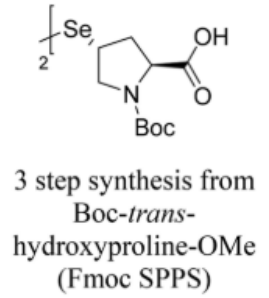
Phe



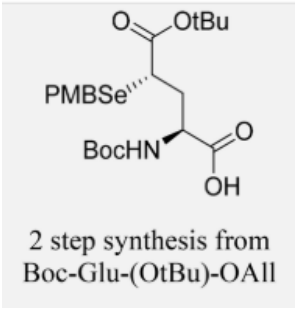
Leu



Asp



Pro

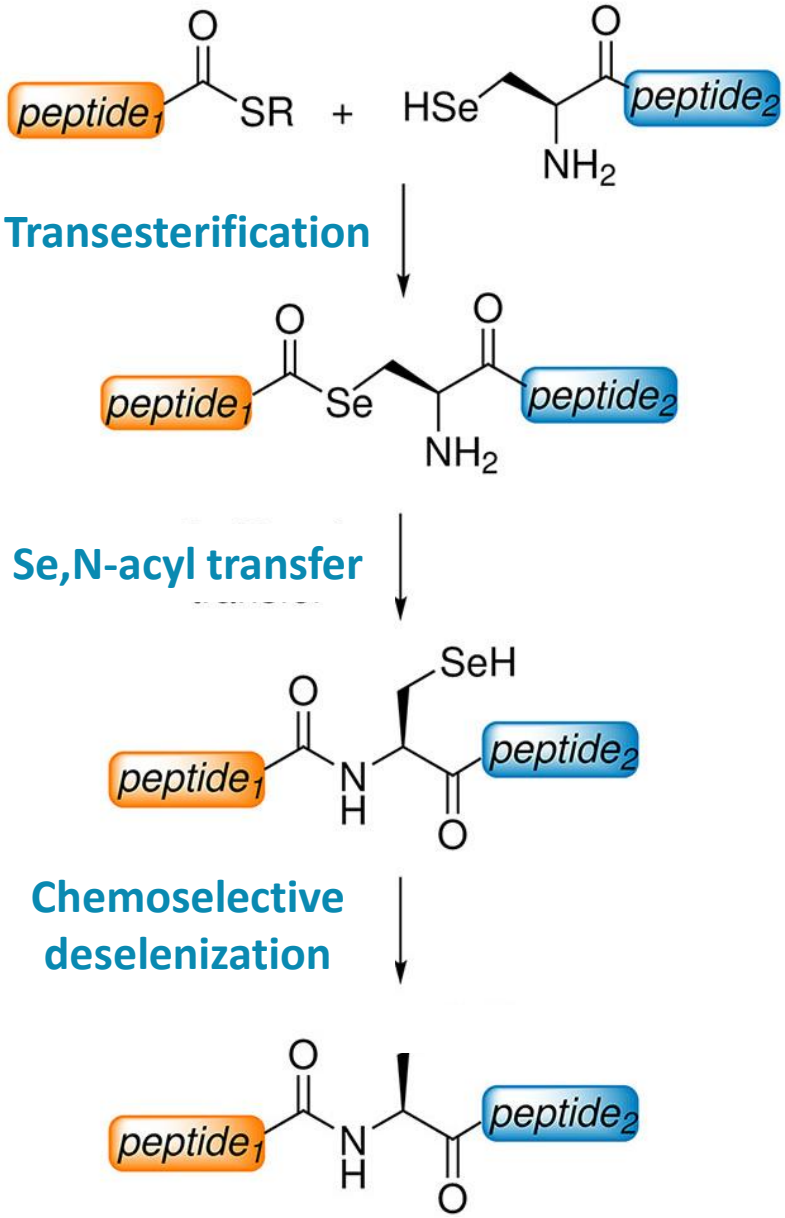


Glu

Payne *et. al. Nat. Rev. Chem.* **2018,** 2, 1–17

Monbaliu, Melnyk *et. al. Chem. Rev.* **2019,** *119,* 7328–7443

Short Summary



- Selenium was first introduced to NCL as **selenocysteine (Sec)** to synthesize selenoprotein.
- Despite the **enhanced nucleophilicity of selenols**, the reaction rate of **Sec NCL is not faster** due to the weak reductive power of aryl thiols.
- The benefit of Sec NCL was expanded by the demonstration of **traceless ligation by chemoselective deselenization**.

Limitation (1) → **Solved!**

- **The need for a Cys residue** on the N terminus of one of the peptide fragments

Contents

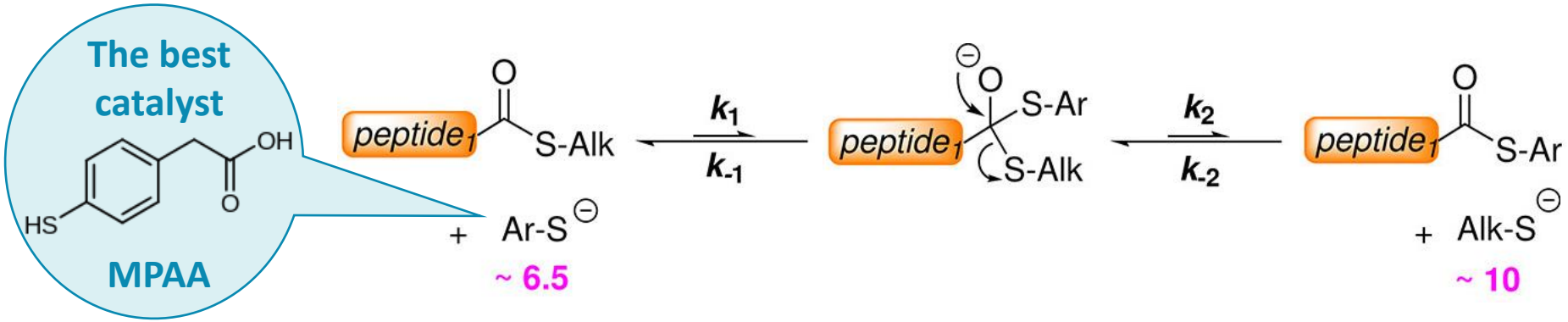
Re: Limitations of Native Chemical Ligation (2)

Limitation (2)

- **The need for relatively long reaction time** and **high concentration of peptide fragments** owing to moderate reactivity

Solution

- **Converting the starting peptide alkyl thioester into a more powerful acyl donor** by performing the NCL in the presence of thiol additives as nucleophilic catalysts!



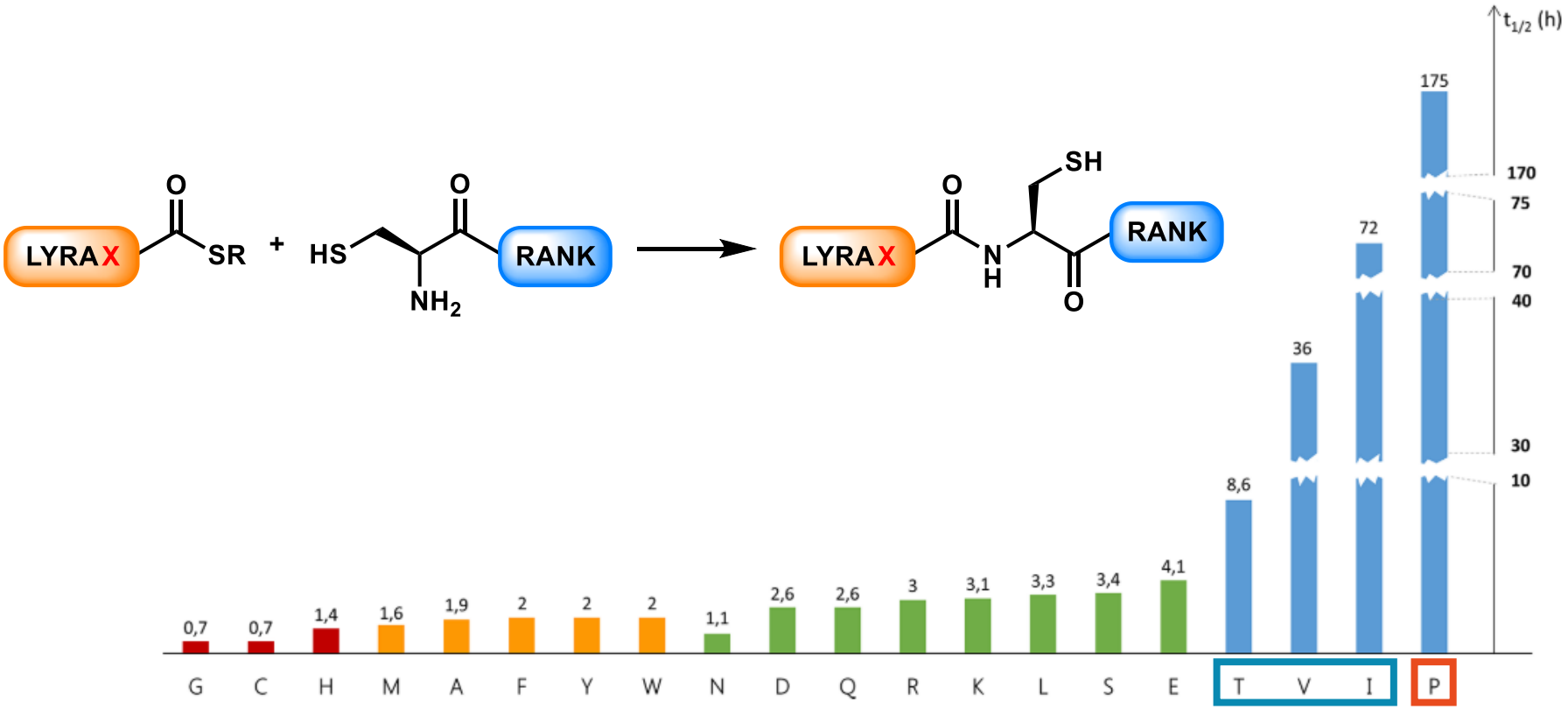
Dawson, Kent *et. al. J. Am. Chem. Soc.* **1997**, *119*, 4325–4329
Kent *et. al. J. Am. Chem. Soc.* **2006**, *128*, 6640–6646

Monbaliu, Melnyk *et. al. Chem. Rev.* **2019**, *119*, 7328–7443

Re: Limitations of Native Chemical Ligation (2)

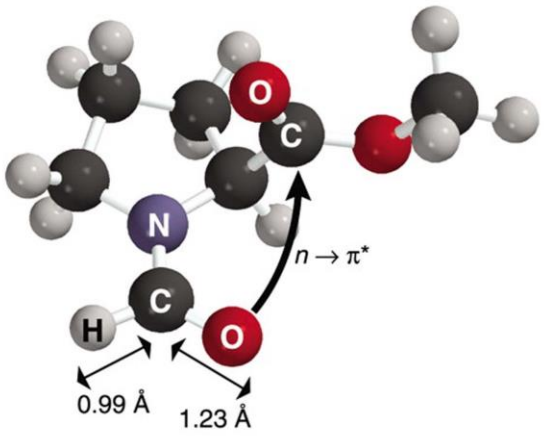
Limitation (2)

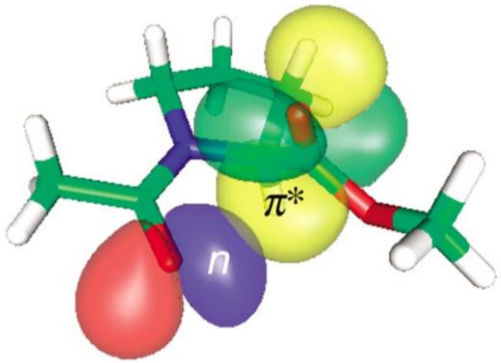
- Peptide thioesters bearing **sterically** or **electronically** **hindered amino acids at the C terminus suffer from sluggish reaction rates** even with thiol catalysts.



Monbaliu, Melnyk *et. al. Chem. Rev.* **2019,** *119,* 7328–7443
Dawson *et. al. Proc. Natl. Acad. Sci. USA* **1999,** *96,* 10068–10073

Quite low reactivity of peptidyl prolyl thioester

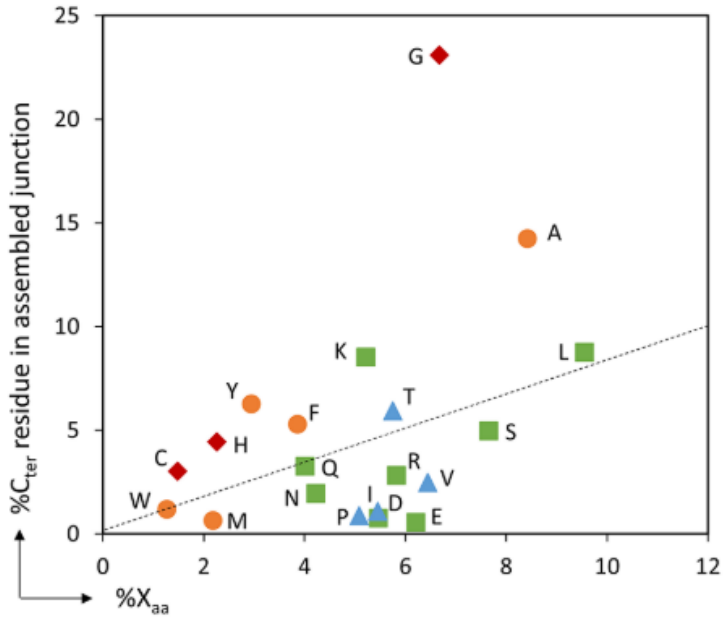




- An **n→π* electronic donation into the carbonyl carbon** leads to reduced electrophilicity of the prolyl thioesters

  → **Pro–Cys junctions are synthetically intractable…**

Reaction rate

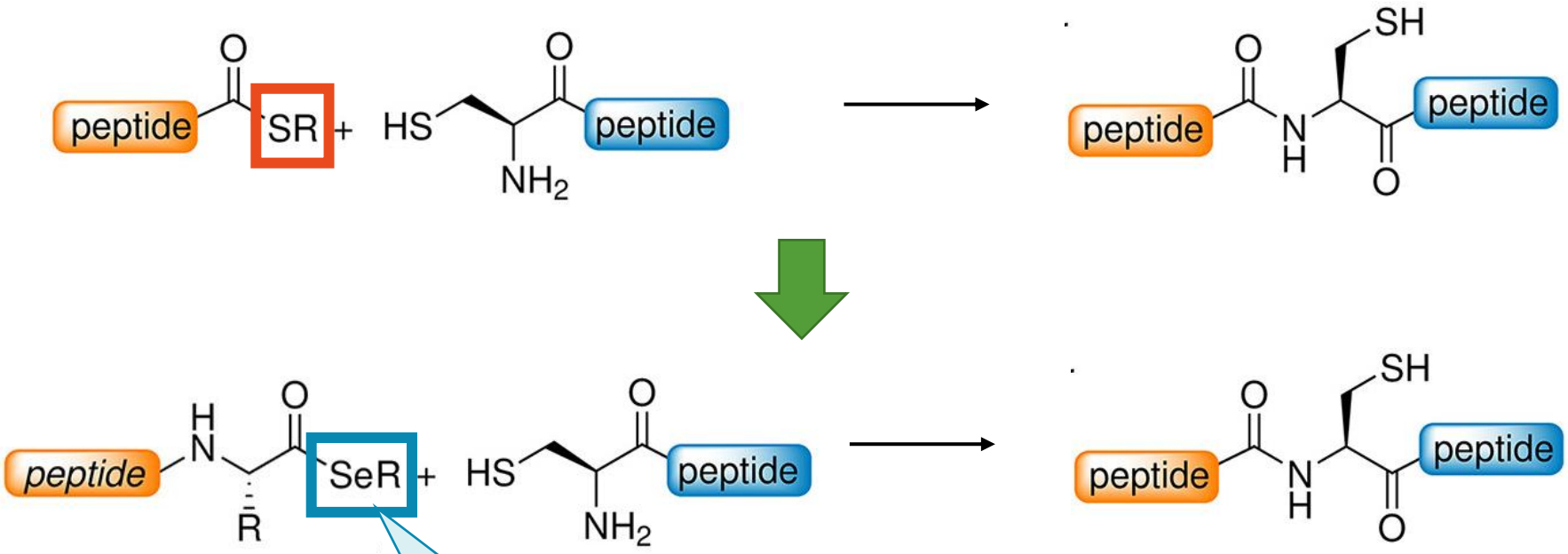


Raines *et. al. Protein Sci.* **2003,** *12,* 1188–1194
Agouridas, Melnyk *et. al. Bioorg. Med. Chem.* **2017,** *25,* 4938–4945
Monbaliu, Melnyk *et. al. Chem. Rev.* **2019,** *119,* 7328–7443

Extending NCL to selenoester

Strategy

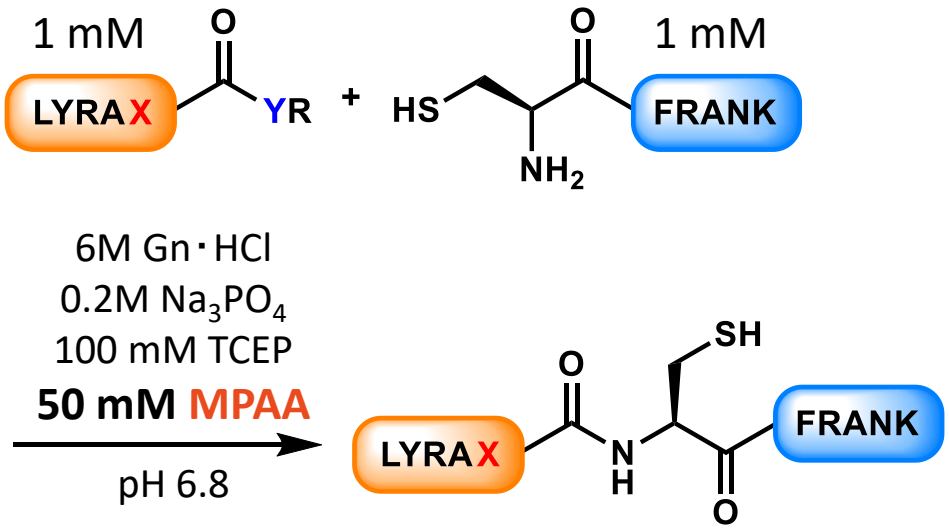
- **Thioesters can be advantageously substituted by selenoesters** in NCL reactions due to **higher reactivity of selenoesters** toward Cys peptides!

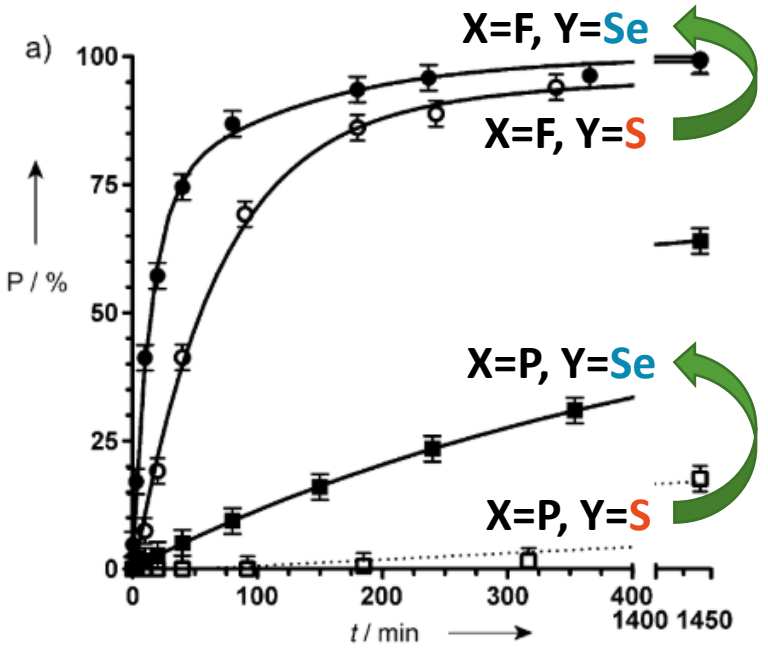


	Cys (S)	Sec (Se)
Polarizability volume	2.9 Å	**3.8 Å**

Durek *et. al. Angew. Chem. Int. Ed.* **2011,** *50,* 12042–12045 Monbaliu, Melnyk *et. al. Chem. Rev.* **2019,** *119,* 7328–7443

Rapid ligation at interactable site using selenoester



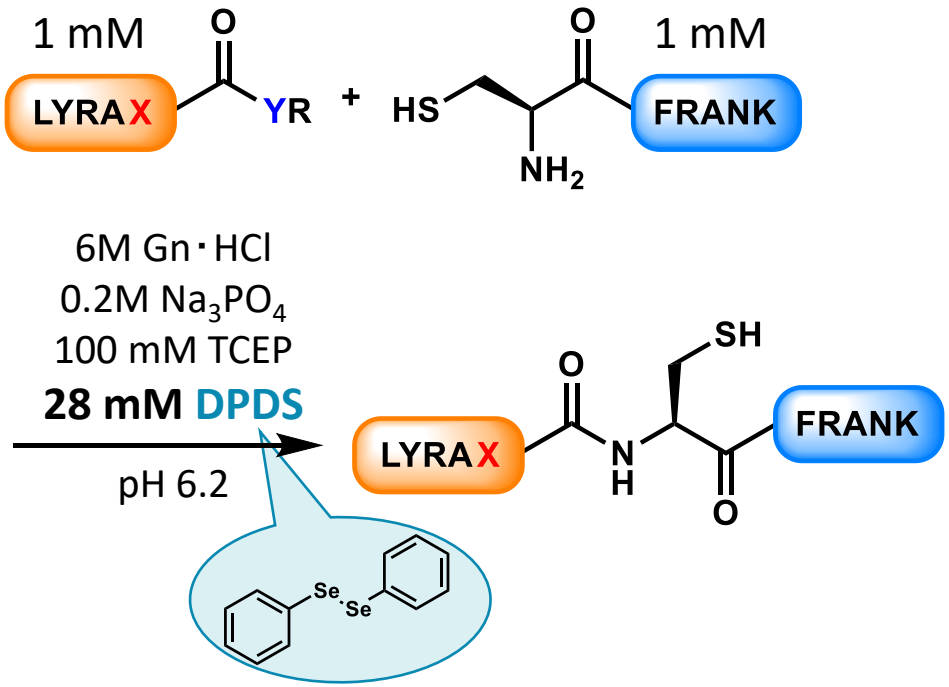


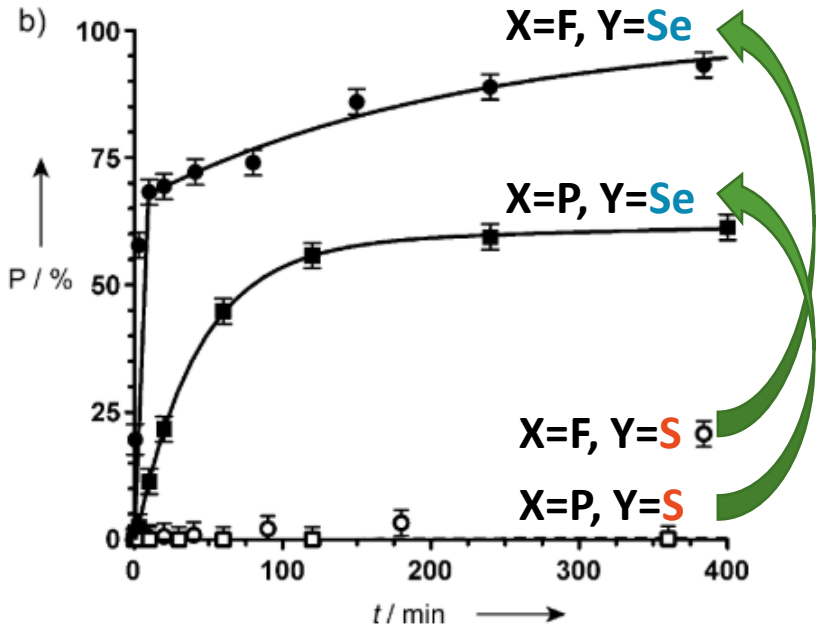
- **Peptide selenoesters reacted significantly faster** than the corresponding thioesters.
- The rate-determining step is likely to be trans-thioesterification of the rapidly formed MPAA-derived thioester with Cys…

X	Y	k [$M^{-1}s^{-1}$]	Factor
P	S	0.00057 ± 0.00066	1
F	S	0.437 ± 0.032	766
P	Se	0.019 ± 0.002	33
F	Se	0.827 ± 0.039	1450

Durek *et. al. Angew. Chem. Int. Ed.* **2011,** *50,* 12042–12045

Rapid ligation at interactable site using selenoester



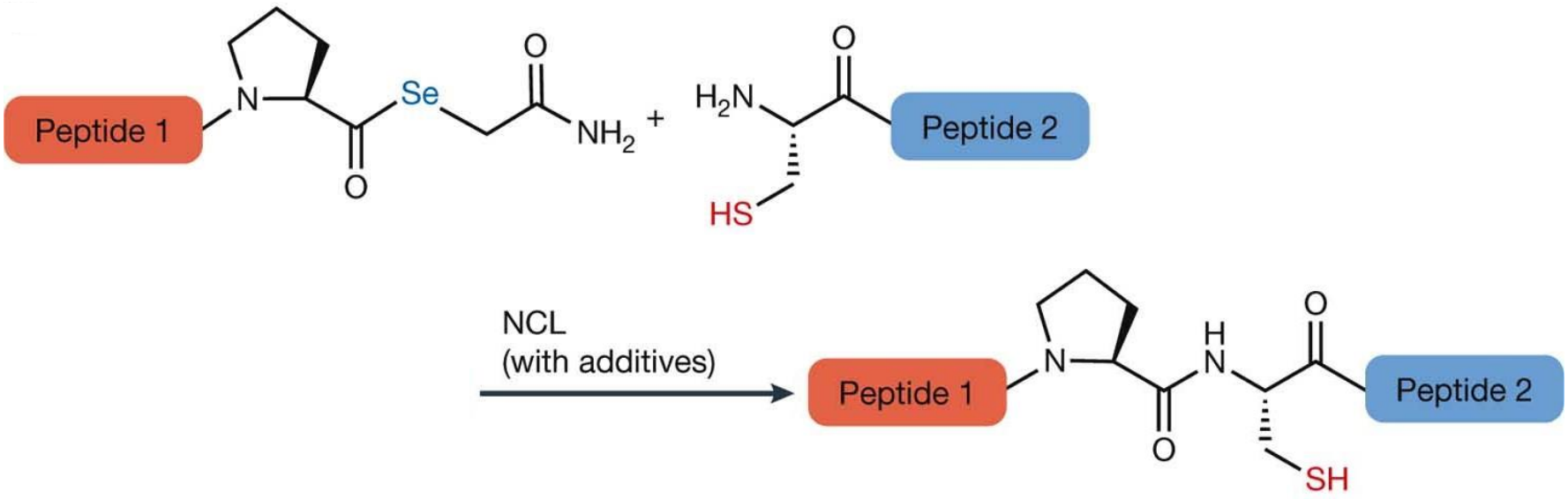


X	Y	*k* [$M^{-1}s^{-1}$]	Factor
P	S	n.d.	n.d.
F	S	n.d.	n.d.
P	Se	0.198 ± 0.007	**347**
F	Se	7.7 ± 0.1	13500

- Peptide selenoesters rapidly and quantitatively trans-selenoesterified with PhSeH.
- **Ligation at prolyl selenoester (with selenol additive) were nearly 350 times faster than traditional NCL (with thiol additive)**!

Durek *et. al. Angew. Chem. Int. Ed.* **2011,** *50,* 12042–12045

Short Summary



- **Selenoester** was introduce to NCL due to **higher reactivity** toward Cys peptides.
- Selenoester even allowed for **rapid ligation even at proline** (up to 350-fold faster reaction).

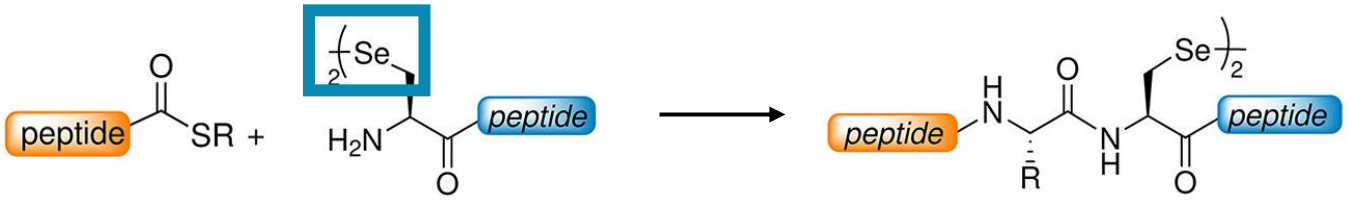
Limitation (2) → **Solved!**

- Peptide thioesters bearing **sterically or electronically hindered amino acids at the C terminus suffer from sluggish reaction rates** even with thiol catalysts.

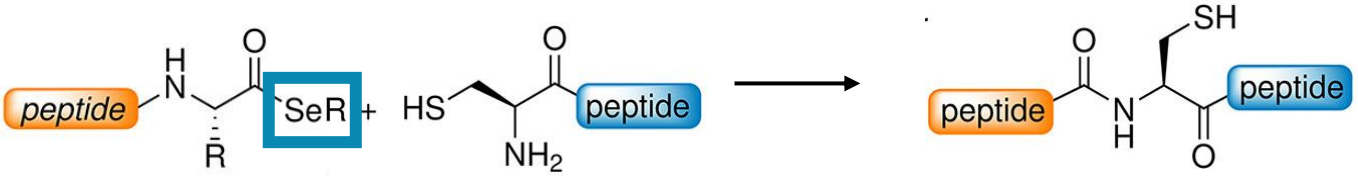
Contents

Extending NCL to diselenide-selenoester ligation (DSL)

2001
NCL
@Selenocysteine



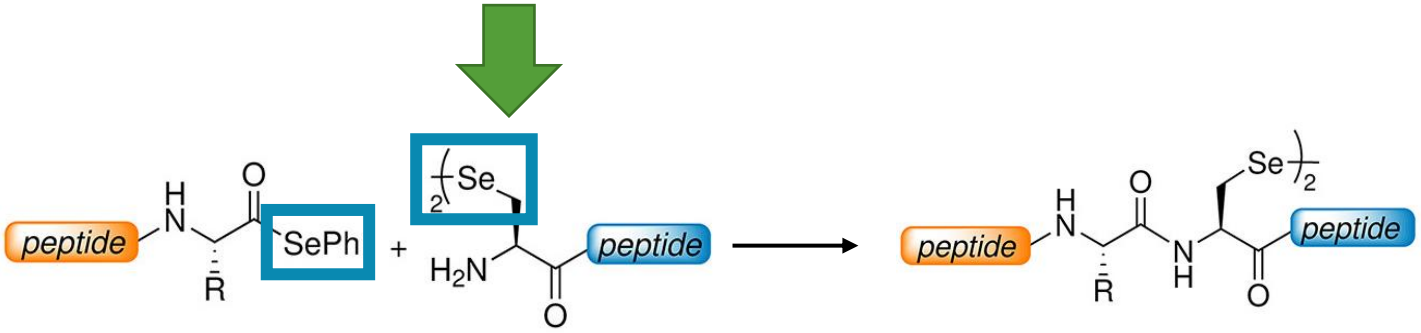
2011
NCL
@Selenoester



Concept

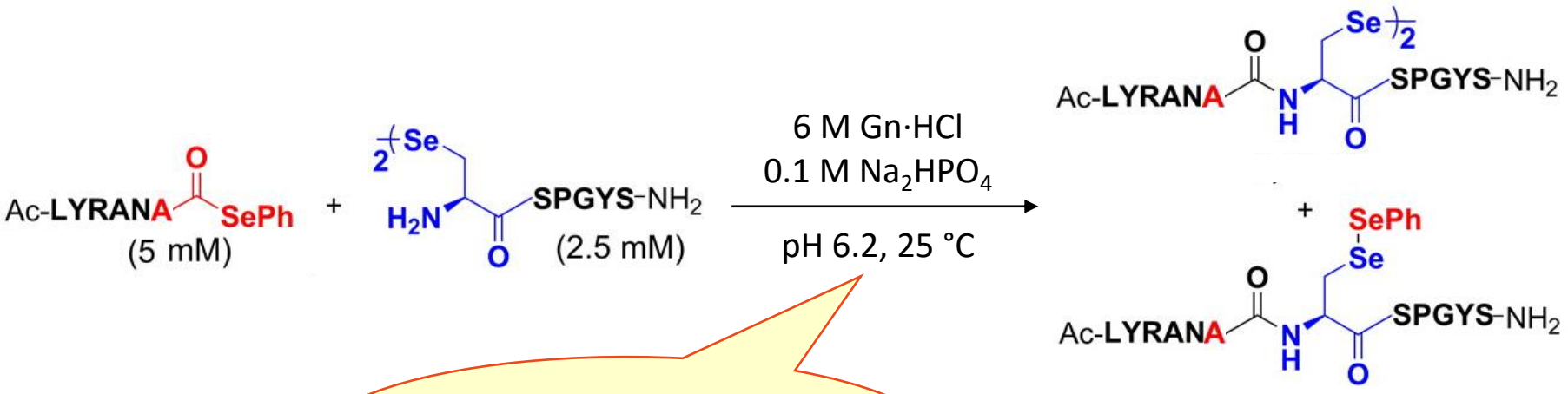
- If **the increased nucleophilicity of Sec** could be effectively harnessed and combined with **the enhanced electrophilicity of a selenoester**, the ligation rate should increase.

2015
DSL
@Selenocysteine
@Selenoester



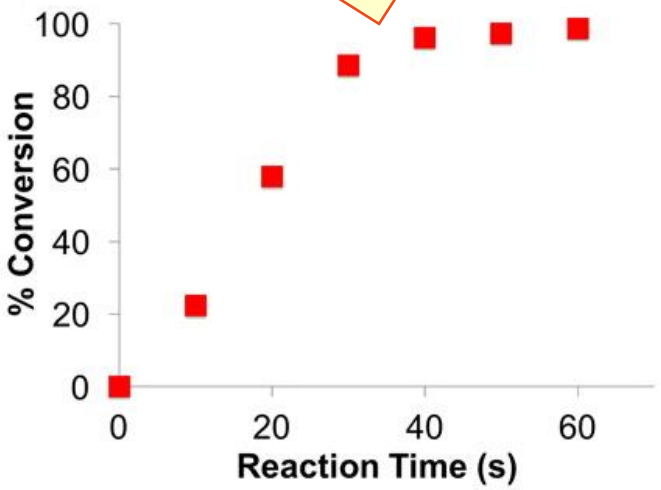
Payne *et. al. J. Am. Chem. Soc.* **2015**, *137,* 14011–14014

Rapid additive-free diselenide-selenoester ligation (DSL)



Additive-free ligation!
(without any reducing reagent!)

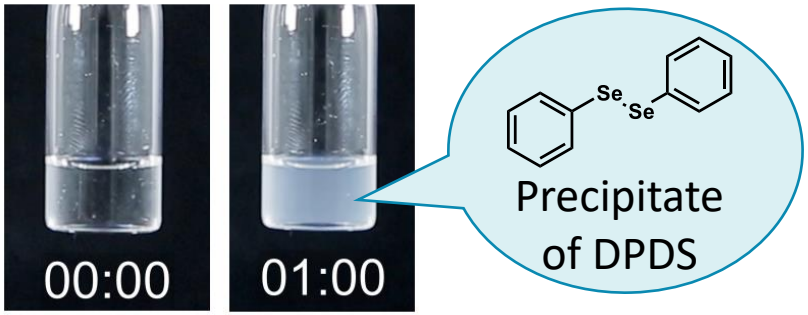
Ligation completes within 60 sec!



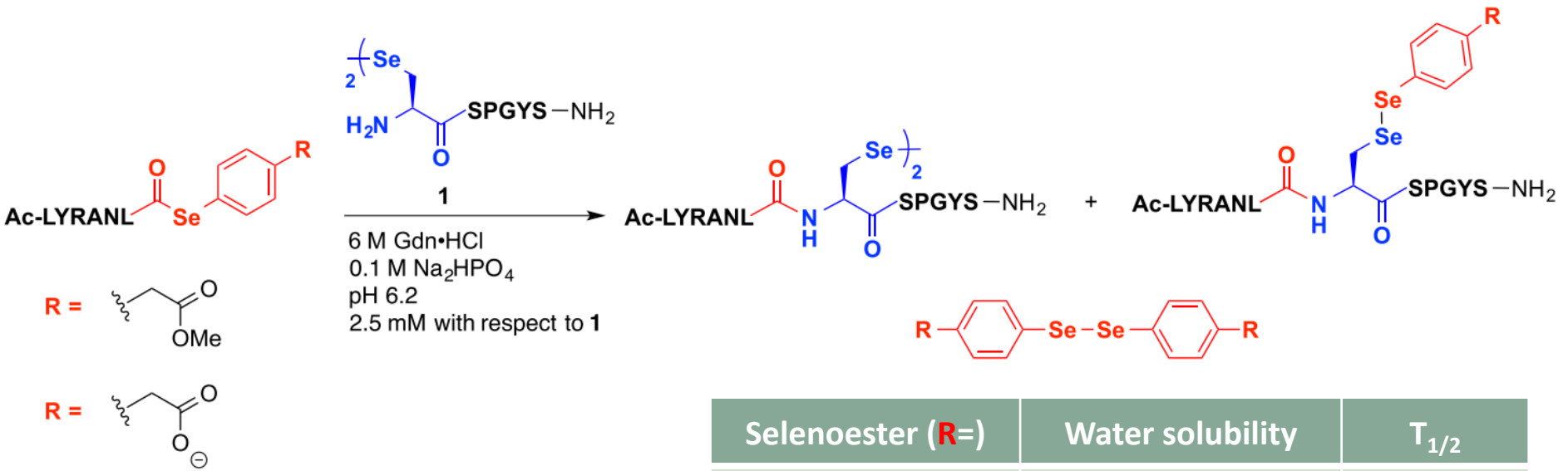
- **A control experiment** involving only diselenide peptide dimer and peptide phenylselenoester in denaturing buffer represents **unprecedented reactivity!**

Payne *et. al. J. Am. Chem. Soc.* **2015,** *137,* 14011–14014

Insights into dramatic reactivity of DSL



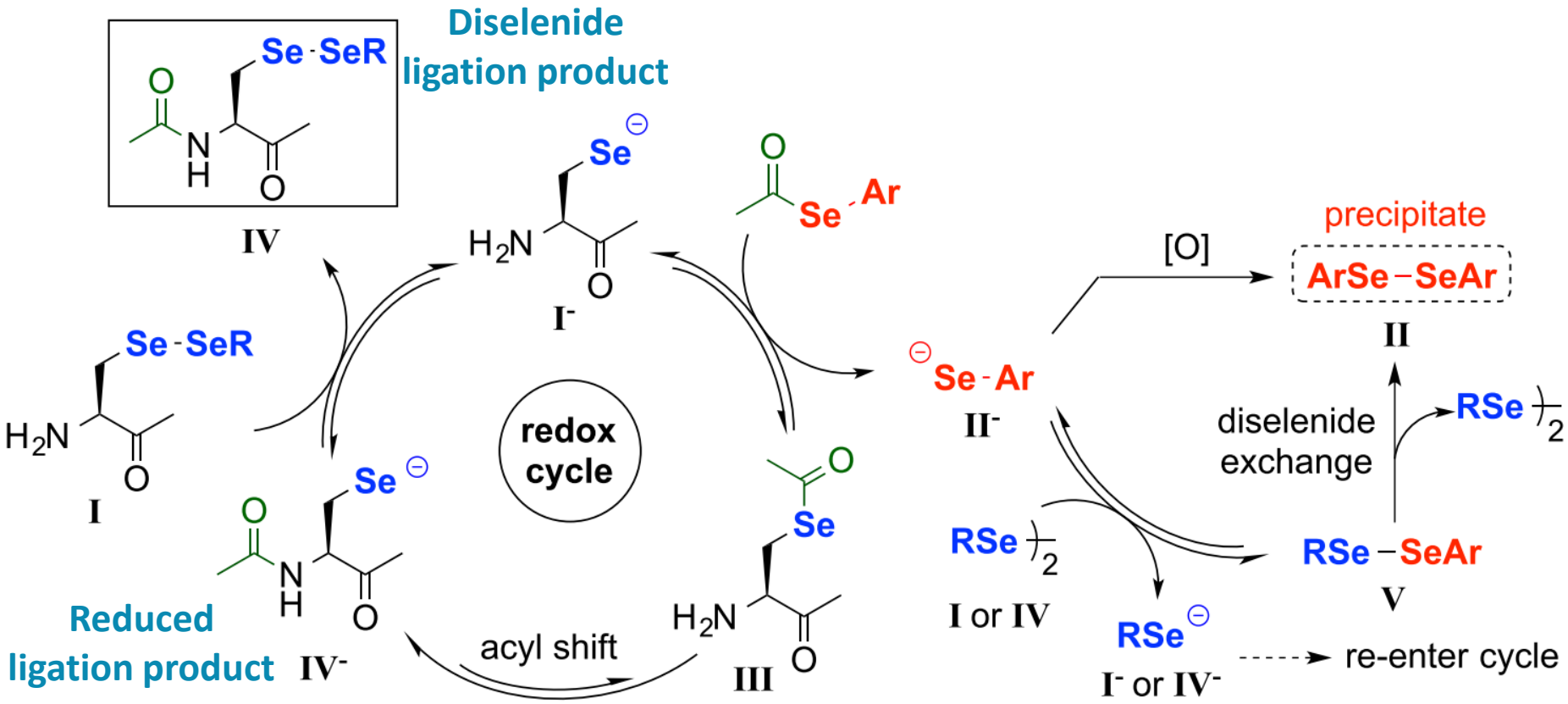
- An important factor for the dramatic ligation rate seems to be **removal of DPDS from solution by precipitation.**



Selenoester (R=)	Water solubility	$T_{1/2}$
H	Insoluble	24 s
CH_2COOMe	Sparingly soluble	48 s
CH_2COO^-	**soluble**	**> 1h**

Payne *et. al. J. Am. Chem. Soc.* **2015,** *137,* 14011–14014

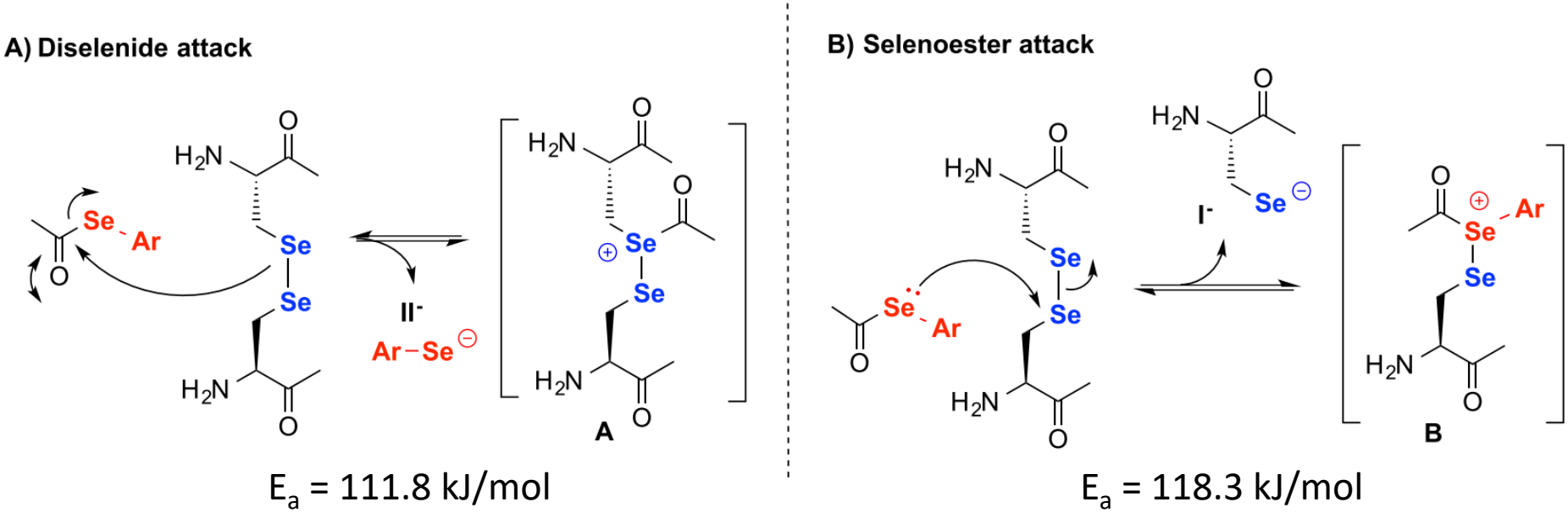
Proposed mechanism; Ligation redox cycle



- **Native chemical ligation-type mechanism** is proposed.
- The reaction is an **equilibrium process**, whereby the forward reaction is promoted by **removal of DPDS from solution by precipitation**.

Payne *et. al. J. Am. Chem. Soc.* **2015,** *137,* 14011–14014

Proposed mechanism; “Initiation” event



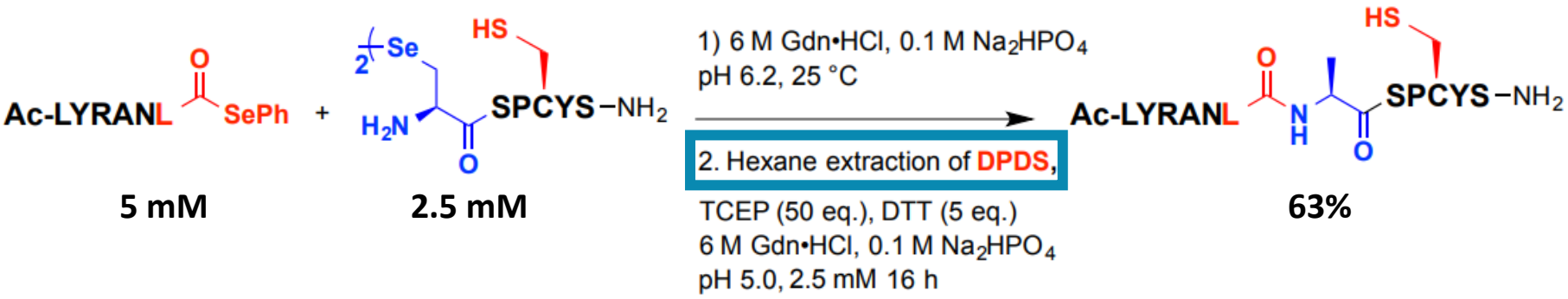
E_a = 111.8 kJ/mol

E_a = 118.3 kJ/mol

- In the absence of additives or external reductants, the propagation cycle requires initiation through an internal reduction event to generate any of the intermediates.
- **The exact mechanism of “Initiation” event has not yet been elucidated.**

Payne *et. al. J. Am. Chem. Soc.* **2015,** *137,* 14011–14014

One-pot ligation-deselenization



Problem of aryl thiol additives

- The radical quenching activity of aryl thiols prohibits in situ radical dechalcogenation.

  → **Purification and lyophilization steps must be carried out…**

Merit of additive-free DSL

- The insoluble DPDS (= radical scavenger) can be removed by hexane extraction.

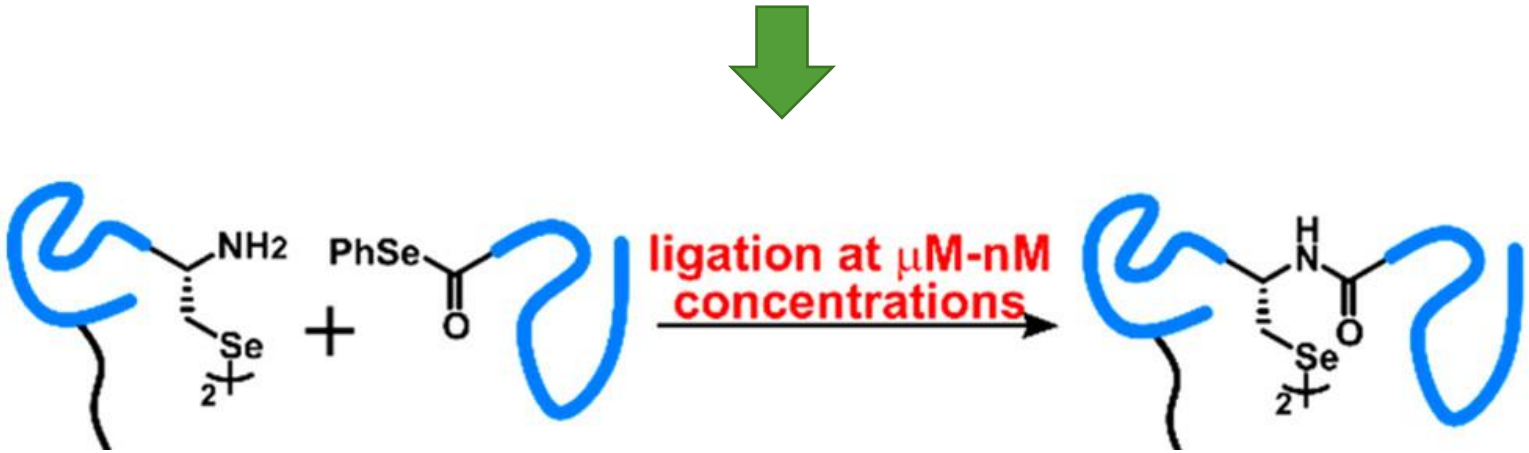
  → **One-pot ligation-deselenization can be conducted!**

Payne *et. al. J. Am. Chem. Soc.* **2015,** *137,* 14011–14014

Peptide ligation at high dilution

Limitation (2)

- **The need for high concentration of peptide fragments** remained to make challenging synthesis of lipidated polypeptides and integral membrane proteins.

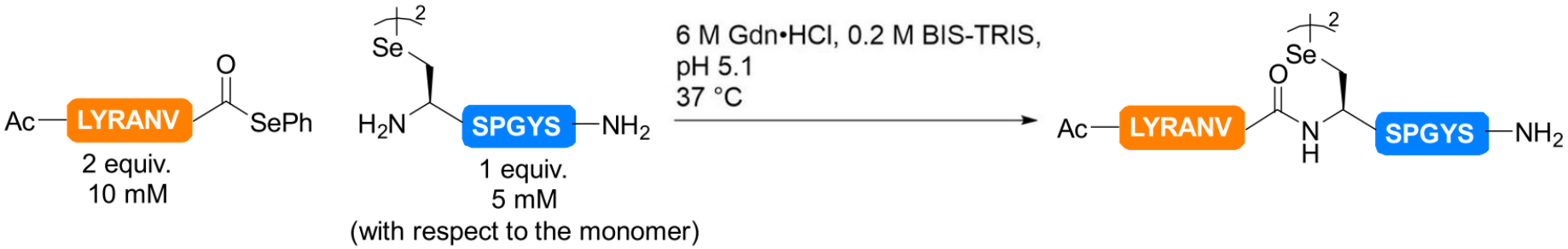


Solution using DSL

- **DSL at extremely low concentrations** has been achieved and provides application in the synthesis of a large range of **hydrophobic and lipidated targets**!

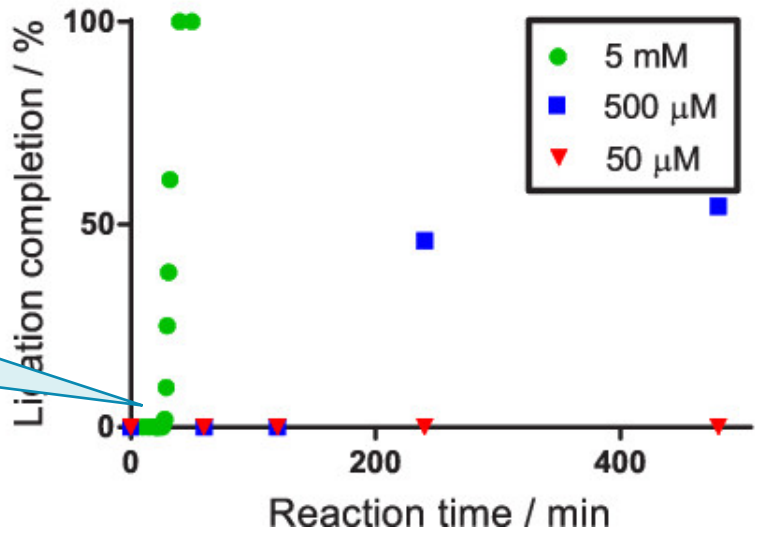
Payne *et. al. J. Am. Chem. Soc.* **2020**, *142,* 1090–1100

Concentration Limit of additive-free DSL



	Cys (S)	Sec (Se)
pK_a	8.30	**5.24**

Ligation occurs following the **"initiation period"**.



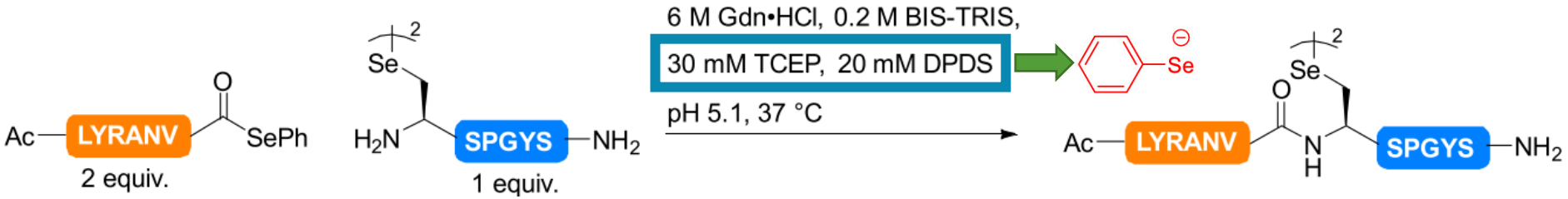
- As the concentration of the peptide decreases, **the "initiation period" becomes longer**.
- **No ligation product was observed at 50 µM** (= the reaction did not initiate)…

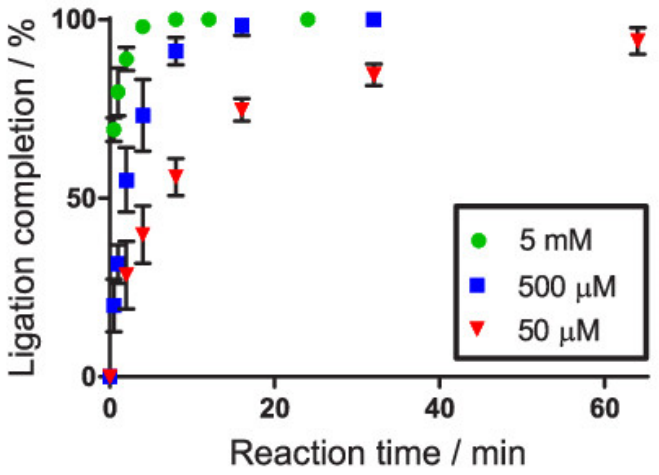
Payne *et. al. J. Am. Chem. Soc.* **2020,** *142,* 1090–1100

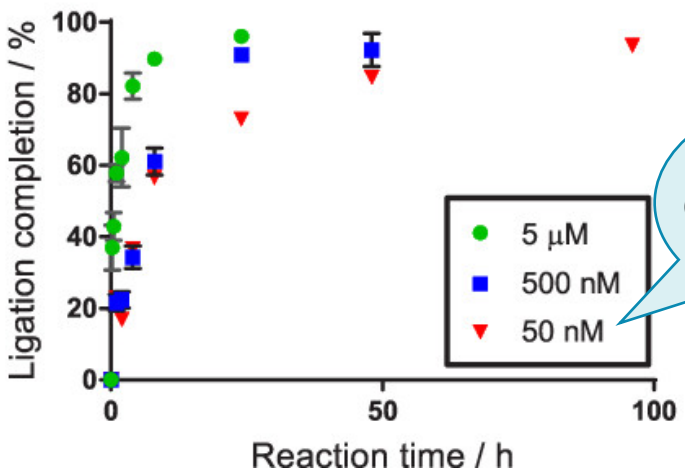
Development of the Reductive DSL

Hypothesis

- The **"initiation step"** of DSL is proposed to proceed **through a bimolecular reaction.**
  → **Phenylselenoate is needed at high concentrations** to enable highly diluted ligation.
- TCEP can be used with **DPDS as a radical scavenger** to suppress the deselenization.



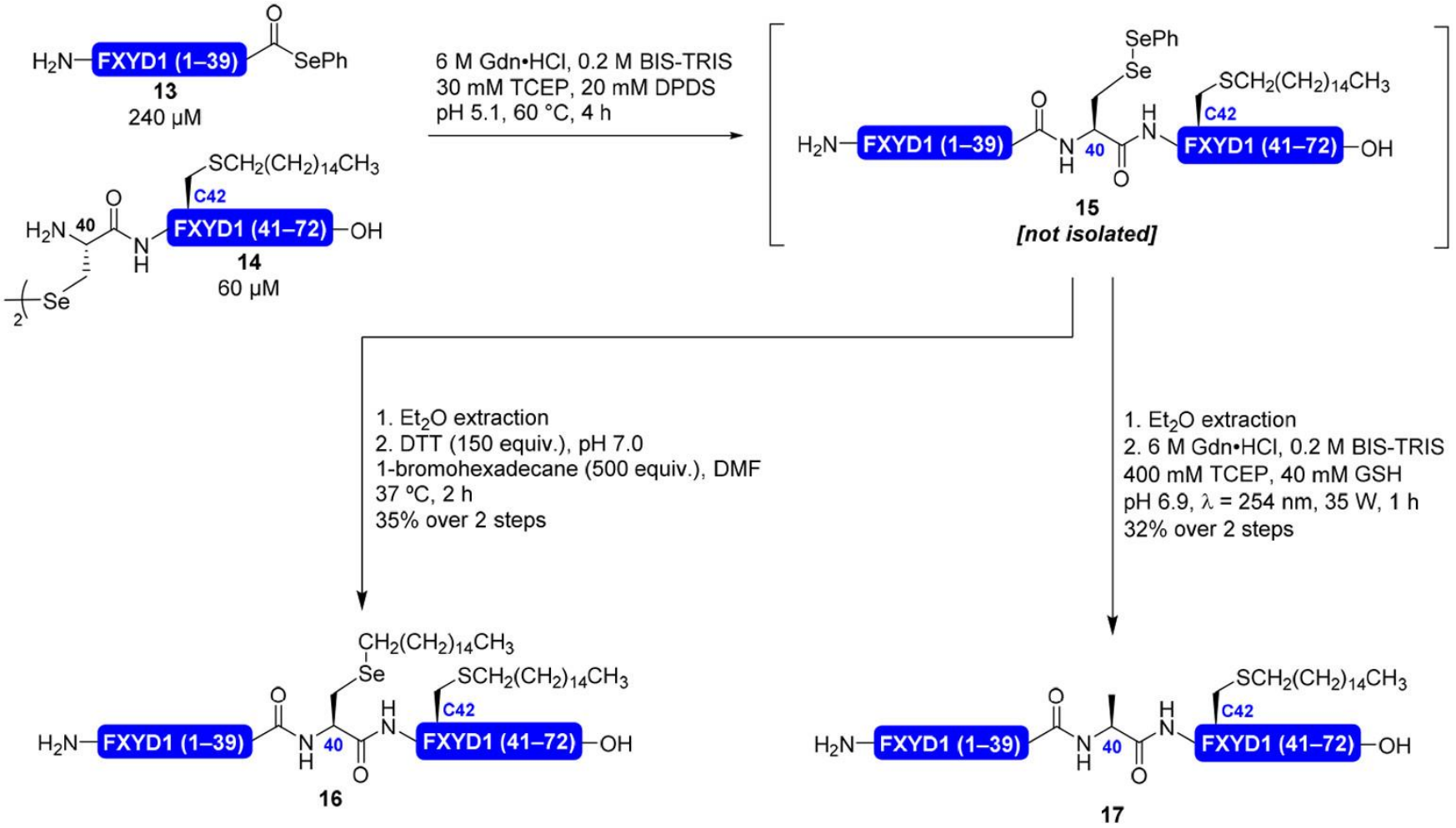




The lowest concentration at to date!

Payne *et. al. J. Am. Chem. Soc.* **2020,** *142,* 1090–1100

Synthesis of FXYD1 by reductive DSL at high dilution

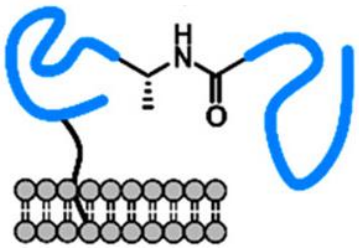


- **The power of high-dilution DSL technology was exemplified** through the high-yielding synthesis of stable analogues of the membrane protein FXYD1.

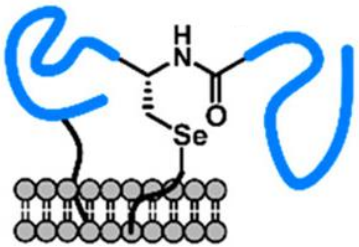
Payne *et. al. J. Am. Chem. Soc.* **2020,** *142,* 1090–1100 Fuller *et. al. J. Biol. Chem.* **2011,** *286,* 36020–36031

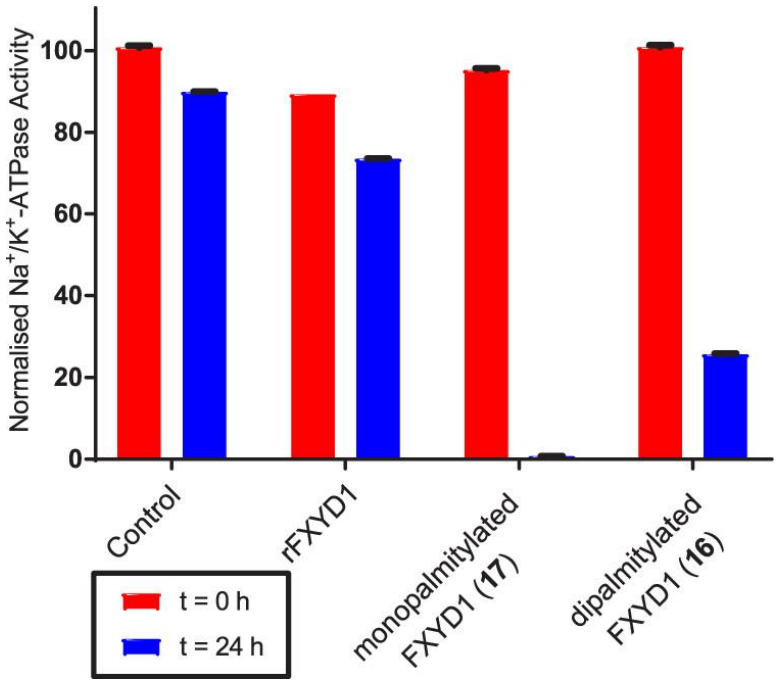
Assay of the effect of palmitoylation using synthesized FXYD1

Monopalmitylated FXYD1



Dipalmitylated FXYD1





- Mono/di-palmitylated FXYD1 displayed inhibition of Na^+/K^+-ATPase activity.
- Interestingly, dipalmitylated FXYD1 was less effective than the monopalmitylated variant.
- The inhibitory activity observed in this study will aid in understanding how post-translational palmitoylation of FXYD1 regulates the Na^+/K^+ pump!

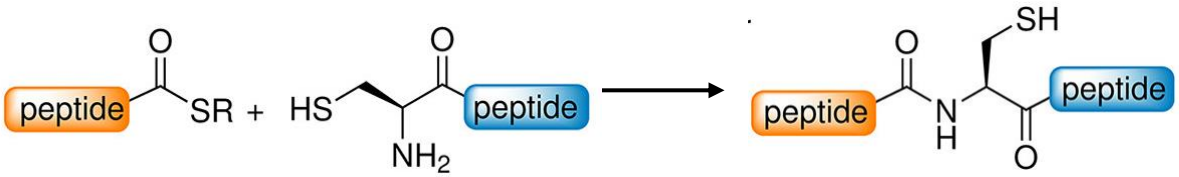
Payne *et. al. J. Am. Chem. Soc.* **2020,** *142,* 1090–1100

Fuller *et. al. J. Biol. Chem.* **2011,** *286,* 36020–36031

Contents

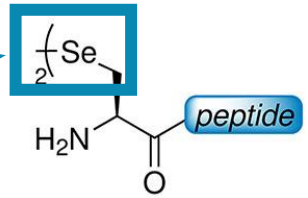
Summary & Perspective

Native Chemical Ligation (NCL)

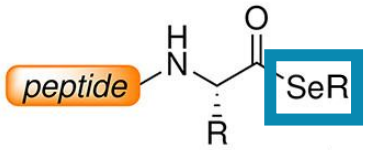


Selenocysteine

Extending the **N-terminal residue** of ligation site!

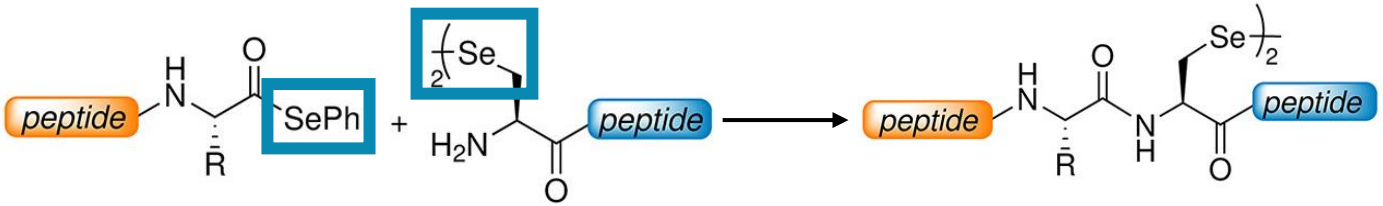


Selenoester



Extending the **C-terminal residue** of ligation site!

Diselenide-Selenoester Ligation (DSL)



The unprecedented reactivity of DSL is opening up new possibilities for protein chemical synthesis!