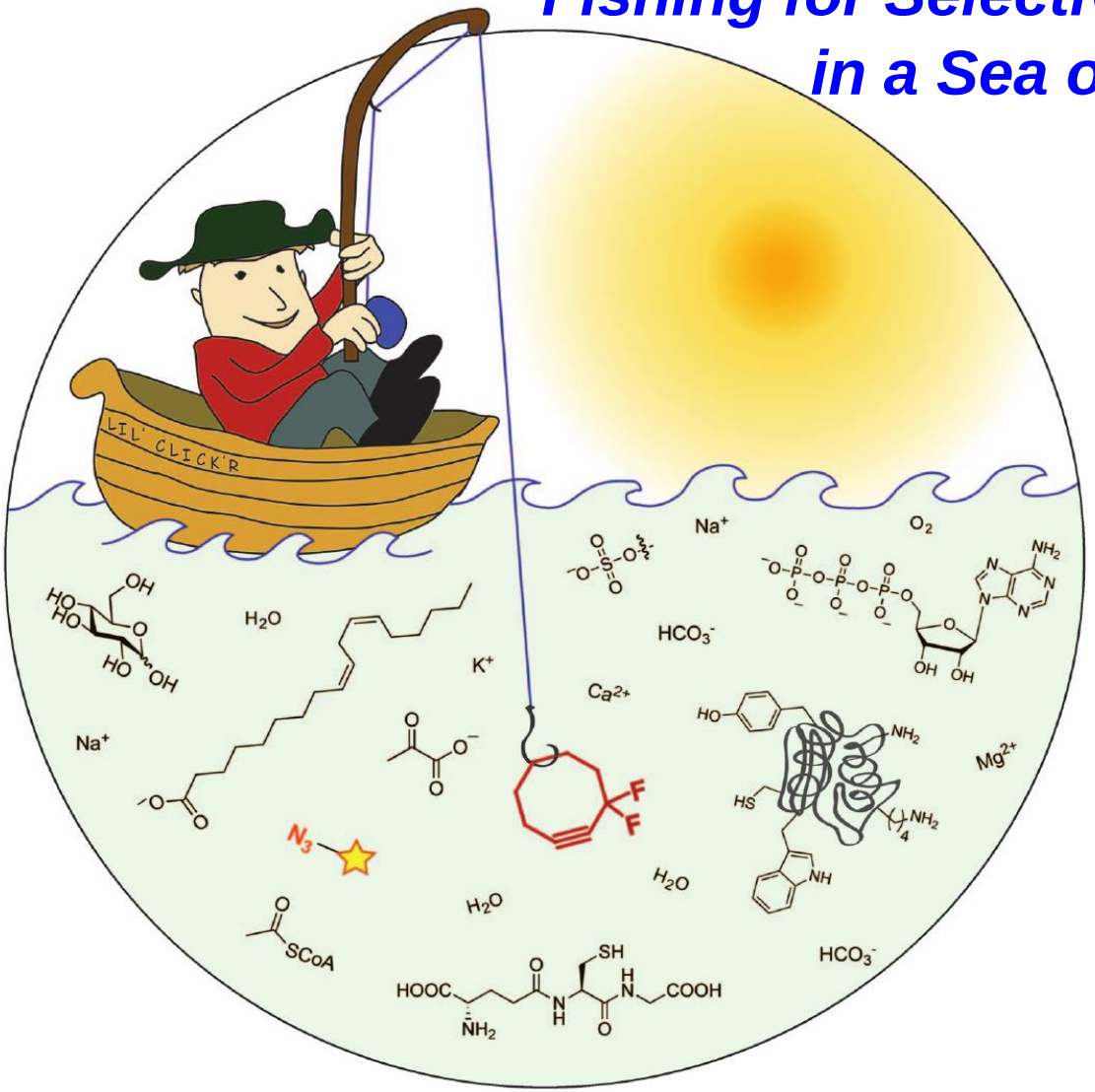
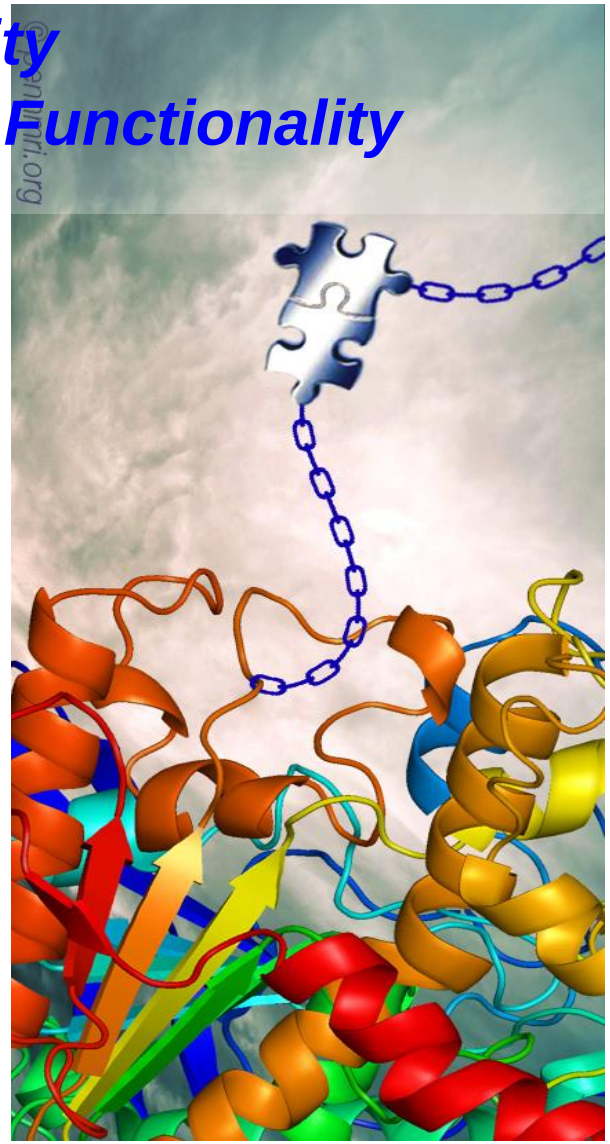
CHAPTER 6 Bioorthogonal Chemistry

Fishing for Selectivity in a Sea of Functionality





CHAPTER 6 Bioorthogonal Chemistry

The Nobel Prize in Chemistry 2022

Ill. Niklas Elmehed © Nobel Prize Outreach

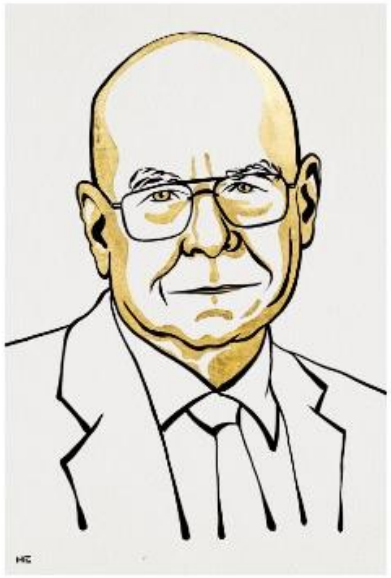
Carolyn R. Bertozzi

Prize share: 1/3

Ill. Niklas Elmehed © Nobel Prize Outreach

Morten Meldal

Prize share: 1/3



Ill. Niklas Elmehed © Nobel Prize Outreach

K. Barry Sharpless

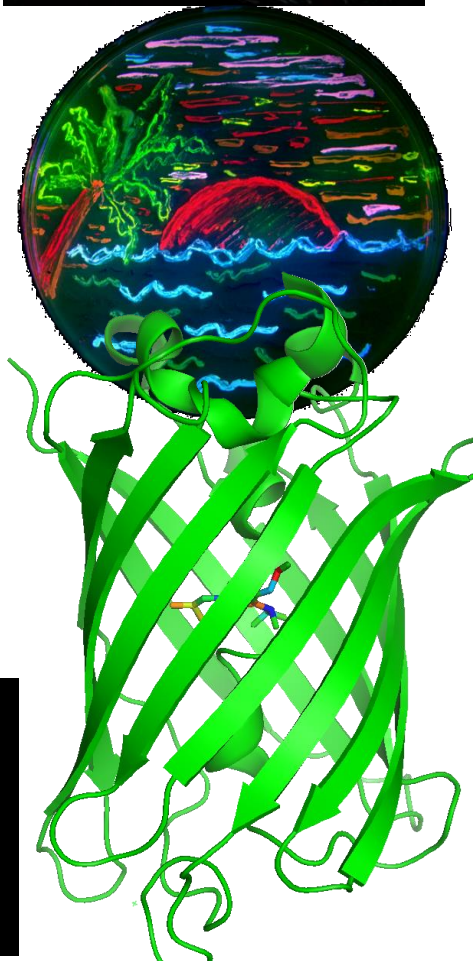
Prize share: 1/3

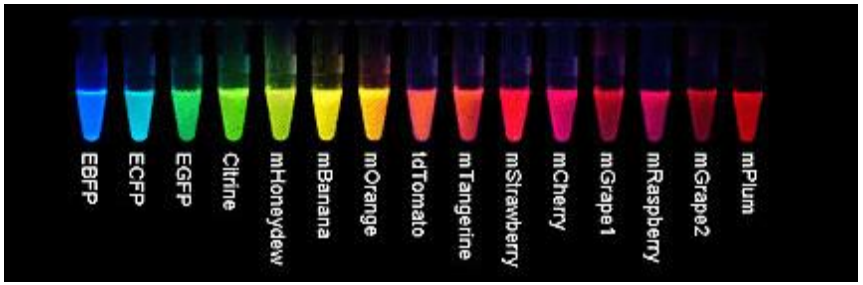
The Nobel Prize in Chemistry 2022 was awarded jointly to Carolyn R. Bertozzi, Morten Meldal and K. Barry Sharpless

"for the development of click chemistry and bioorthogonal chemistry"

Fluorescent Proteins (FPs)

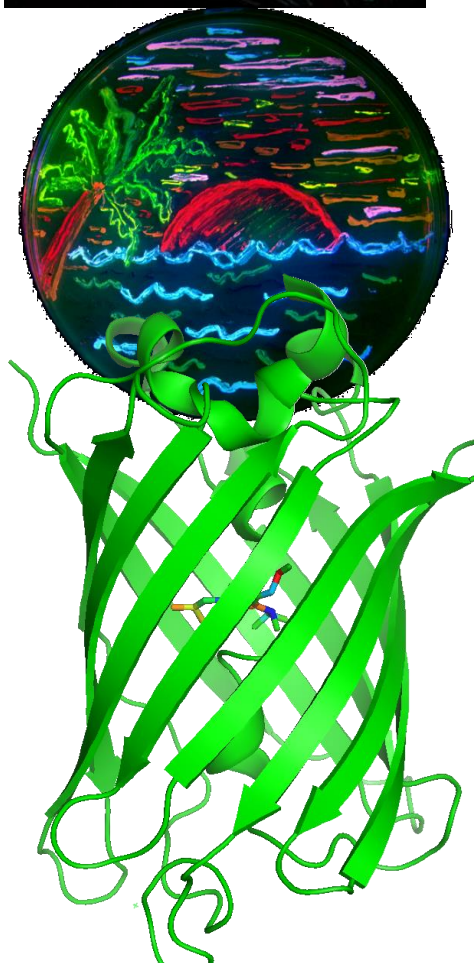
- The development of fluorescent proteins (e.g. GFP) has dramatically increased our understanding of protein dynamics and function in living systems
  - Genetic fusion of a target protein – labeling
  - Placing the gene under a promotor - probing
- Advantages of FPs
  - Simple tagging
  - Sensitive (bright fluorescence)
  - Various derivatives





Fluorescent Proteins (FPs)

- Large size
  - 238 amino acids 26.9 kDa for GFP
- Labeling with a FP requires genetic fusion
  - Their location is limited to termini
- Not applicable to other biomolecules
  - Nucleic acids, lipids, and glycans
  - Posttranslational modifications



A growing area of chemical biology strives to probe important biomolecules in living systems by using bioorthogonal chemical reactions

Angew. Chem. Int. Ed. **2009**, *48,* 6974.

Bioorthogonal Chemistry

- The term "***bioorthogonal chemistry***" refers to any chemical reaction that can occur inside of living systems without interfering with native biochemical processes
- The term was coined by Carolyn R. Bertozzi in 2003
- The concept of the bioorthogonal reaction has enabled the study of biomolecules in real time in cells without cellular toxicity

PNAS PNAS

A metabolic labeling approach toward proteomic analysis of mucin-type O-linked glycosylation

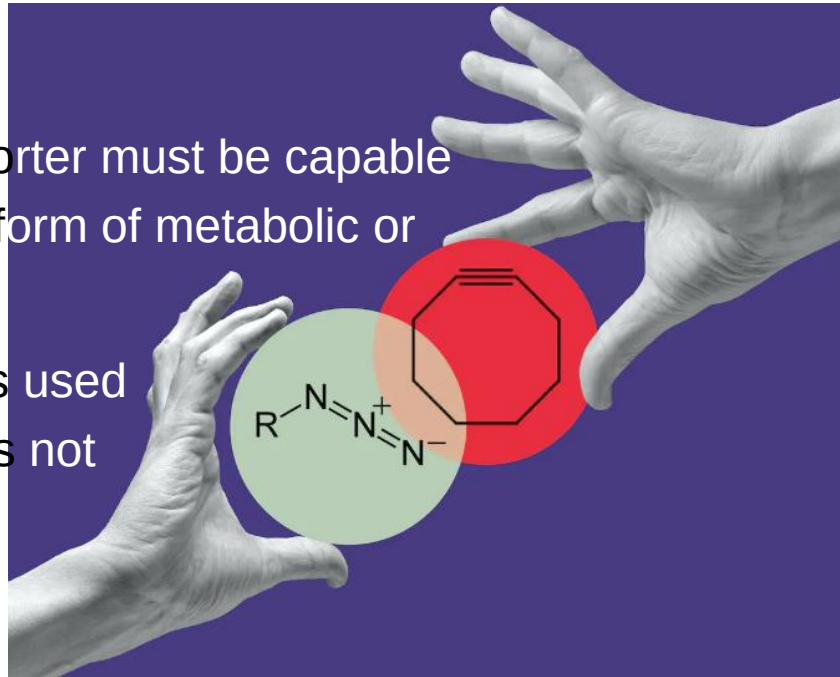
Howard C. Hang*†, Chong Yu*†, Darryl L. Kato*†, and Carolyn R. Bertozzi*†‡§¶

Mucin-type O-linked glycoproteins are involved in a variety of biological interactions in higher eukaryotes. The biosynthesis of these glycoproteins is initiated by a family of polypeptide N-acetyl-galactosaminyltransferases (ppGalNAcTs) that modify proteins in the secretory pathway. The lack of a defined consensus sequence for the ppGalNAcTs makes the prediction of mucin-type O-linked glycosylation difficult based on primary sequence alone. Herein we present a method for labeling mucin-type O-linked glycoproteins with a unique chemical tag, the azide, which permits their selective covalent modification from complex cell lysates. From a panel of synthetic derivatives, we identified an azido GalNAc analog (N-azidoacetylgalactosamine, GalNAz) that is metabolized by numerous cell types and installed on mucin-type O-linked glycoproteins by the ppGalNAcTs. The azide serves as a **bioorthogonal chemical handle** for selective modification with biochemical or biophysical probes using the **Staudinger ligation**. The approach was validated by labeling a recombinant glycoprotein that is known to possess O-linked glycans with GalNAz. In addition, GalNAz efficiently labeled mucin-type O-linked glycoproteins expressed at endogenous levels. The ability to label mucin-type O-linked glycoproteins with chemical tags should facilitate their identification by proteomic strategies.

PNAS **2003**, *100*, 14846

Requirements for Bioorthogonality

- **Selectivity**: The reaction must be selective between endogenous functional groups to avoid side reactions with biological compounds
- **Chemical & Biological inertness**: Reactive partners and resulting linkage should not possess any mode of reactivity with the native chemical functionality
- **Kinetics**: The reaction must be rapid so that covalent ligation is achieved prior to probe metabolism and clearance. (for overcoming competition and rapid response)
- **Reaction biocompatibility**: Reactions have to be non-toxic and must function in biological conditions taking into account pH, aqueous environments, and temperature
- **Accessible engineering**: The chemical reporter must be capable of incorporation into biomolecules via some form of metabolic or protein engineering
- **Minimal disturbance**: The functional groups used in application need to be small so that it does not disturb native behavior



- "**Bioorthogonal Chemistry**" Wikipedia -

◆ Staudinger Reaction

Über neue organische Phosphorverbindungen III.
Phosphinmethylenderivate und Phosphinimine

von

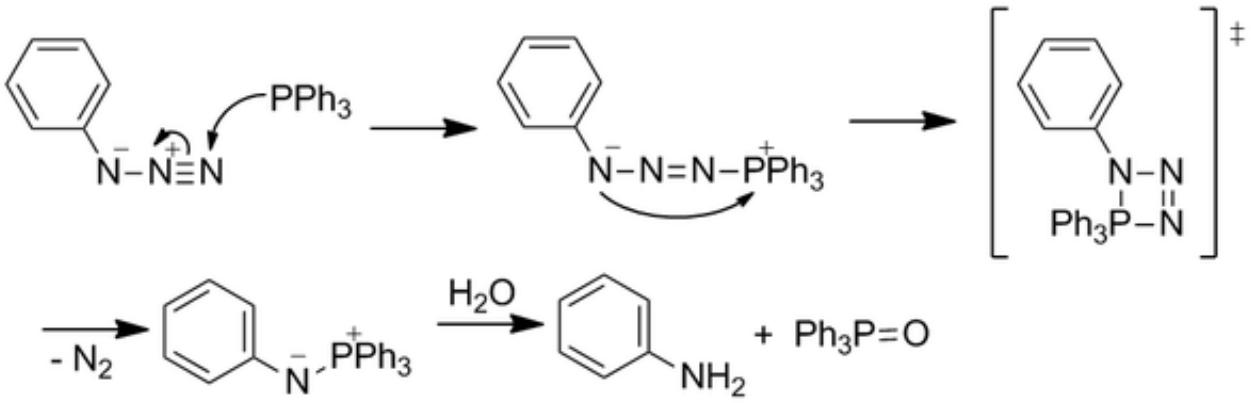
H. Staudinger und Jules Meyer.

(10. IX. 19.)

Helv. Chim. Acta **1919**, *2*, 635.

$$C_6H_5N=N\equiv N + P(C_6H_5)_3 \longrightarrow C_6H_5N=N-N=P(C_6H_5)_3 \longrightarrow C_6H_5N=P(C_6H_5)_3$$

- The Staudinger reaction is a chemical reaction of an azide with a phosphine or phosphite produces an iminophosphorane (ylide)



Staudinger Ligation

Cell Surface Engineering by a Modified Staudinger Reaction

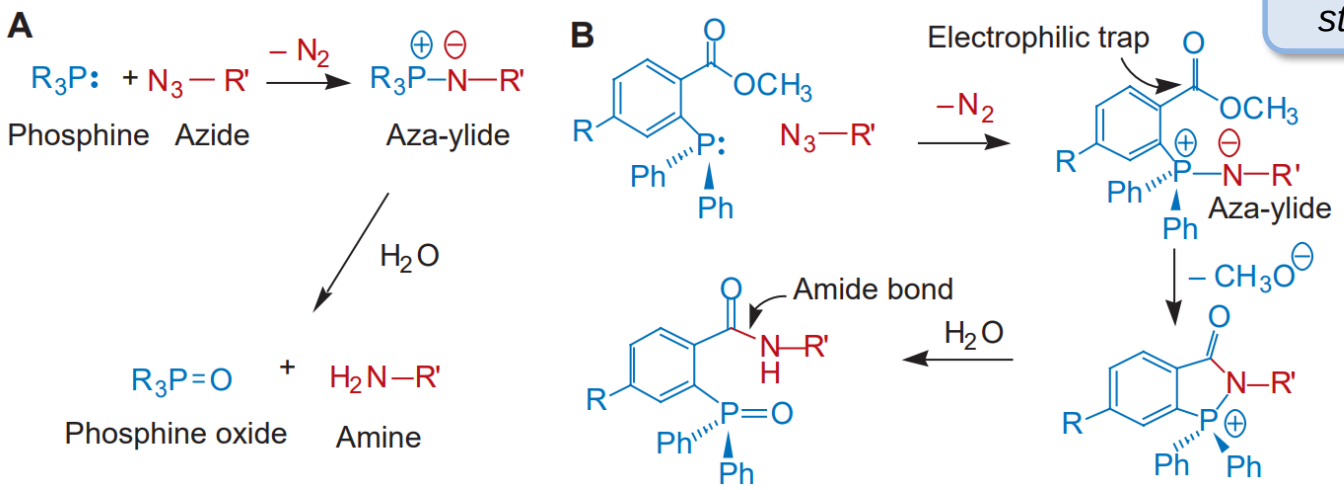
Eliana Saxon and Carolyn R. Bertozzi*

Selective chemical reactions enacted within a cellular environment can be powerful tools for elucidating biological processes or engineering novel interactions. A chemical transformation that permits the selective formation of covalent adducts among richly functionalized biopolymers within a cellular context is presented. A ligation modeled after the Staudinger reaction forms an amide bond by coupling of an azide and a specifically engineered triarylphosphine. Both reactive partners are abiotic and chemically orthogonal to native cellular components. Azides installed within cell surface glycoconjugates by metabolism of a synthetic azidosugar were reacted with a biotinylated triarylphosphine to produce stable cell-surface adducts. The tremendous selectivity of the transformation should permit its execution within a cell's interior, offering new possibilities for probing intracellular interactions.

Science **2000**, *287*, 2007.

- The aza-ylide in the Staudinger reaction hydrolyzes spontaneously to yield a primary amine and the corresponding phosphine oxide in the presence of water
  → **Not bioorthogonal**

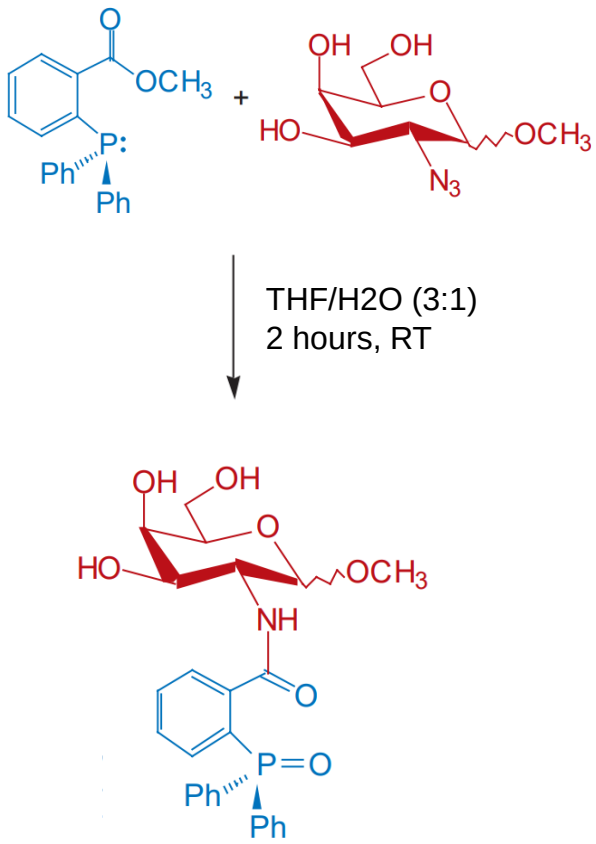
Designing a phosphine that would enable rearrangement of the unstable aza-ylide to a stable covalent adduct

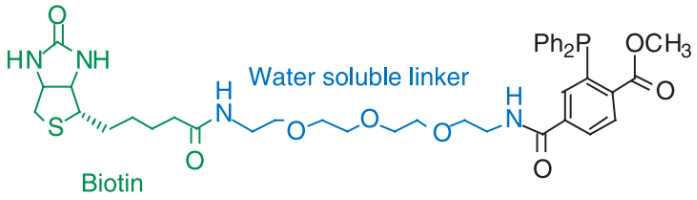


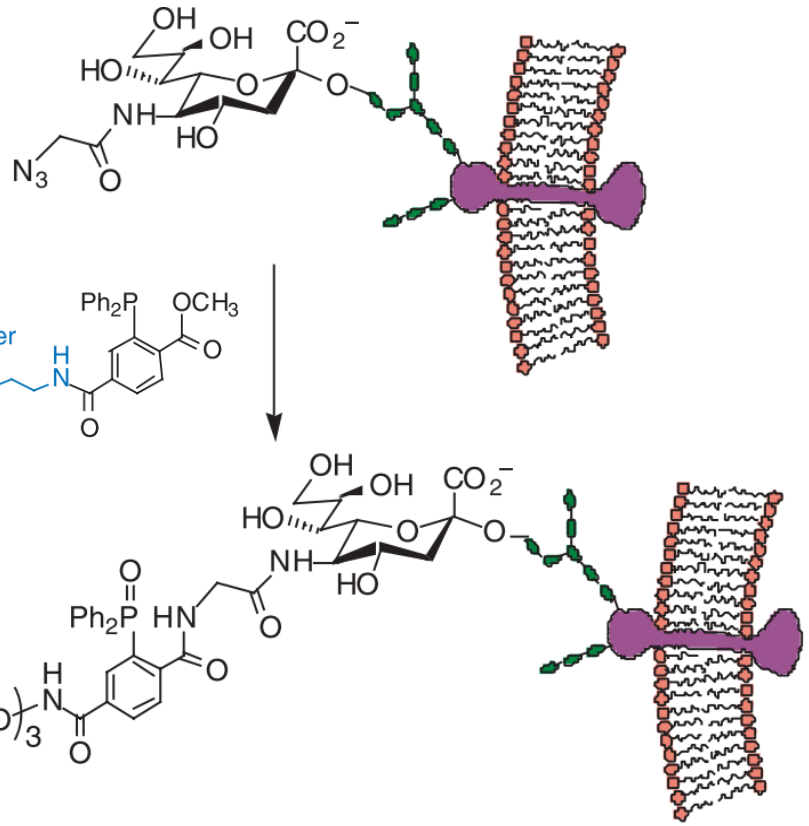
Staudinger Ligation

- Azides installed within cell surface glycoconjugates by metabolism of a synthetic azidosugar were reacted with a biotinylated triarylphosphine to produce stable cell-surface adducts

Model Reaction







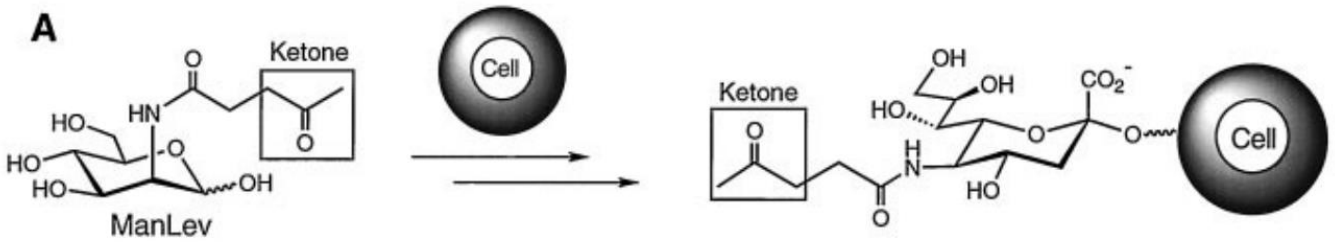
Science **2000**, *287*, 2007.

Biosynthetic incorporation of a ketone

Engineering Chemical Reactivity on Cell Surfaces Through Oligosaccharide Biosynthesis

Lara K. Mahal, Kevin J. Yarema, Carolyn R. Bertozzi*

Science **1997**, *276*, 1125.



- Sialic acids are the most abundant terminal components of oligosaccharides on mammalian glycoproteins
- The enzymes that participate in sialic acid metabolism are permissive for simple unnatural substrates

- Biosynthetic incorporation of ketone groups into cell surface–associated sialic acid

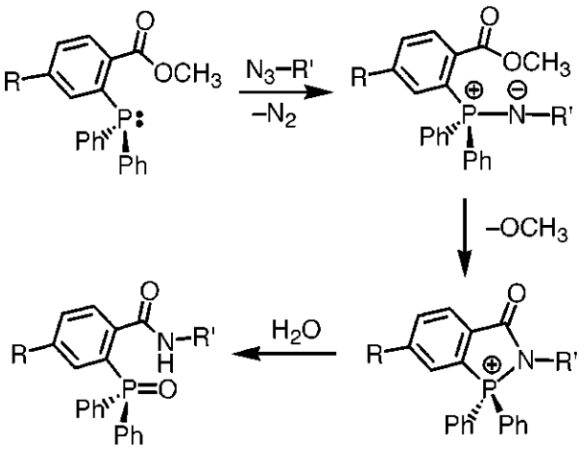
◆ Traceless Staudinger Ligation

A "Traceless" Staudinger Ligation for the Chemoselective Synthesis of Amide Bonds

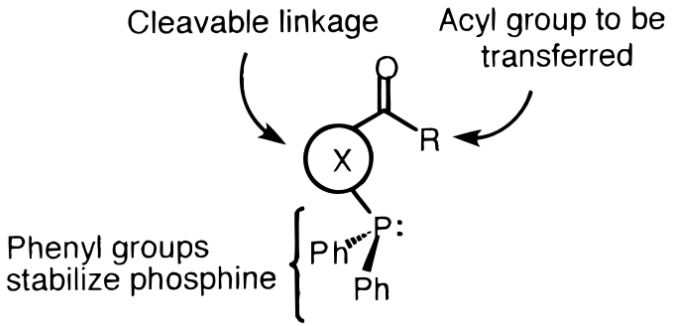
Eliana Saxon, Joshua I. Armstrong, and Carolyn R. Bertozzi*

Org. Lett. **2000**, *2*, 2141.

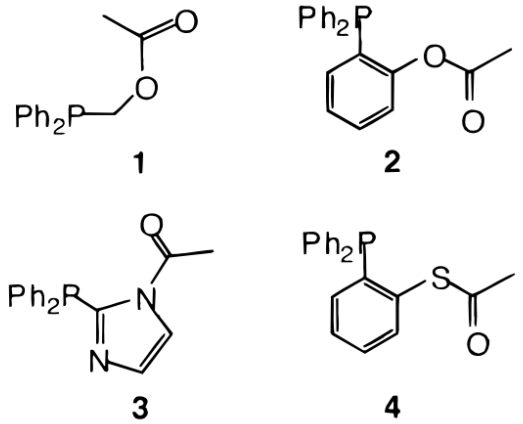
Scheme 1. The Staudinger Ligation



Scheme 2. Design of a Phosphine Reagent for the Selective Formation of Amides from Azides

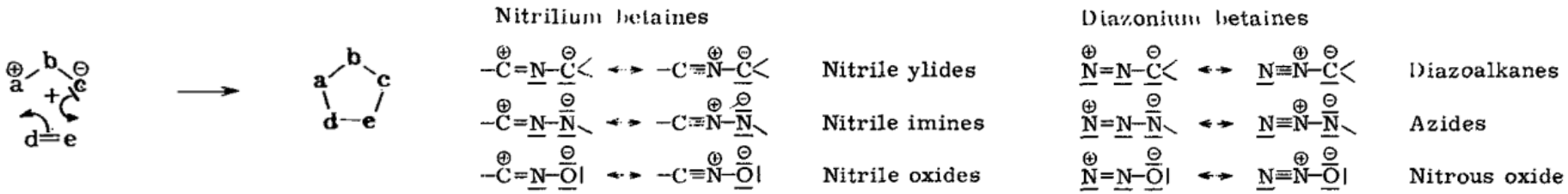


Scheme 3. Phosphines Designed To Test the Traceless Staudinger Ligation



1,3-Dipolar cycloaddition reactions

- The reaction of azides with alkenes and alkynes was first reported at the end of the 19th century (*J. Prakt. Chem.* **1893**, *48*, 94.)
- The concept of 1,3-dipolar cycloaddition reaction between azide and alkyne was initially reported by Huisgen in 1963 (*Angew. Chem. Int. Ed*. **1963**, *2*, 565.)



- 1,3-dipolar cycloaddition reaction between azide and alkyne requires high temperature, which is not compatible with living systems
- The cycloaddition of azides and alkynes to form aromatic triazole products was considered to have a great potential

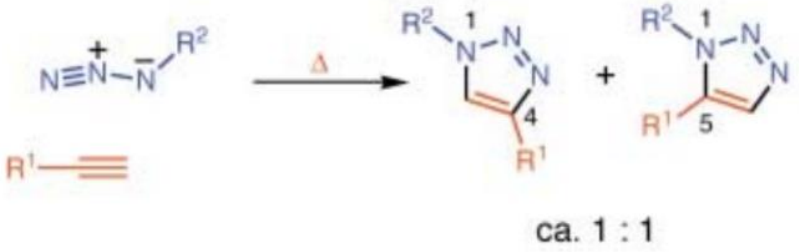
◆ Copper as a catalyst for azide-alkyne Cycloadditions

A Stepwise Huisgen Cycloaddition Process: Copper(I)-Catalyzed Regioselective "Ligation" of Azides and Terminal Alkynes**

Vsevolod V. Rostovtsev, Luke G. Green,
Valery V. Fokin,* and K. Barry Sharpless*

Angew. Chem. Int. Ed. **2002**, *41*, 2596.

- What makes azides unique for click chemistry purposes is their extraordinary stability toward H_2O, O_2, and the majority of organic synthesis conditions
- The desired triazole-forming cycloaddition may require elevated temperatures and, usually results in a mixture of the 1,4 and 1,5 regioisomers



Copper as a catalyst for azide-alkyne Cycloadditions

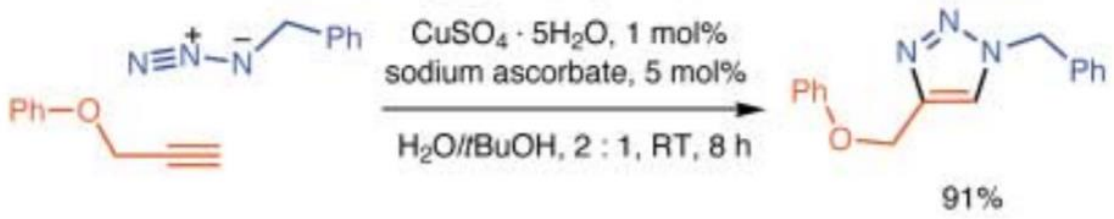
A Stepwise Huisgen Cycloaddition Process: Copper(I)-Catalyzed Regioselective "Ligation" of Azides and Terminal Alkynes**

Vsevolod V. Rostovtsev, Luke G. Green,
Valery V. Fokin,* and K. Barry Sharpless*

Angew. Chem. Int. Ed. **2002**, *41*, 2596.

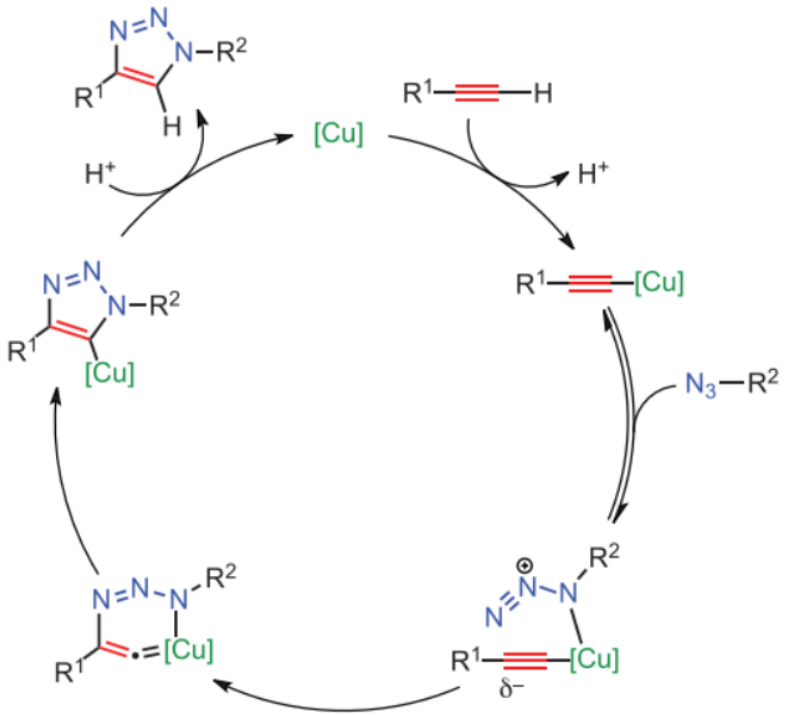
- The copper(I)-catalyzed reaction regiospecifically unites azides and terminal acetylenes to give only 1,4-disubstituted 1,2,3-triazoles
- Cu(II) as a Cu(I) source
- Ascorbic acid as a reductant

→ In situ generation: cheaper and purer



Copper(I)-catalyzed Azide-Alkyne Cycloadditions (CuAAC)

- Known as “Click Chemistry” (but not limited to CuAAC)
- Sharpless and Meldal independently discovered a dramatic rate acceleration when a Cu(I) salt was used
- The copper(I)-catalyzed cycloaddition proceeds roughly seven orders of magnitude faster than the uncatalyzed cycloaddition
- CuAAC has taken widespread attention since its debut in 2001 as a facile and robust method for the creation of covalent linkages in a variety of environments



First proposed mechanism of CuAAC

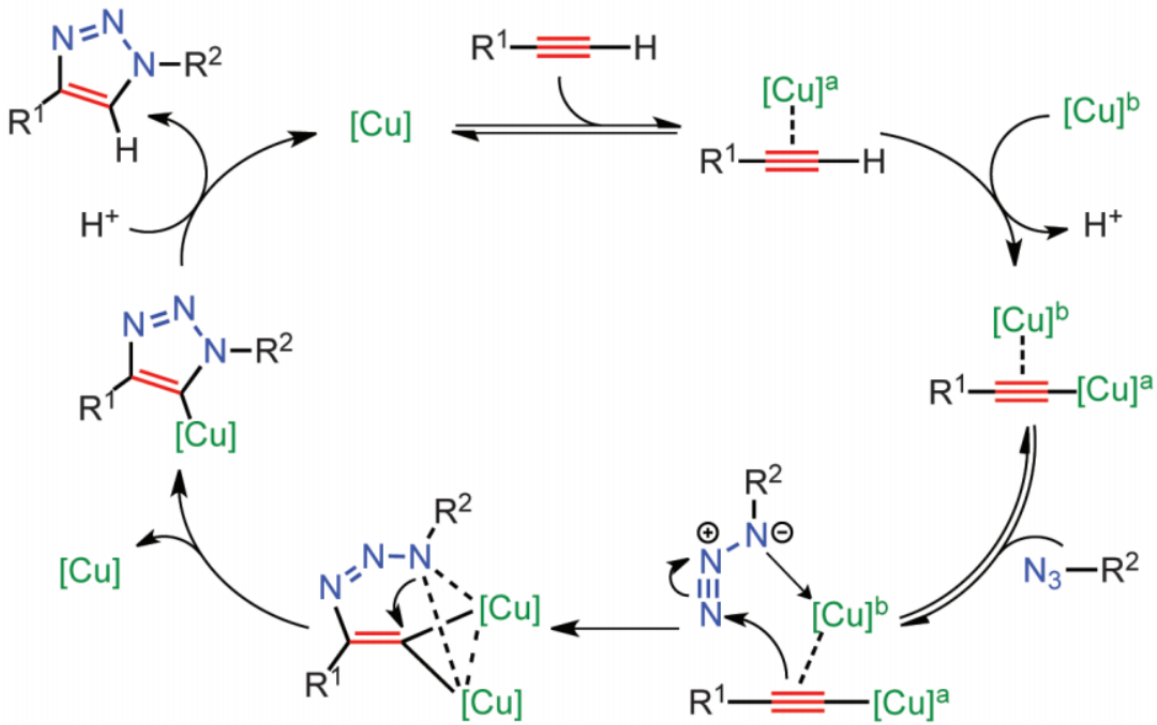
Science **2013**, *340*, 457.

Copper(I)-catalyzed Azide-Alkyne Cycloadditions (CuAAC)

Direct Evidence of a Dinuclear Copper Intermediate in Cu(I)-Catalyzed Azide-Alkyne Cycloadditions

B. T. Worrell, J. A. Malik, V. V. Fokin*

Science **2013**, *340*, 457.



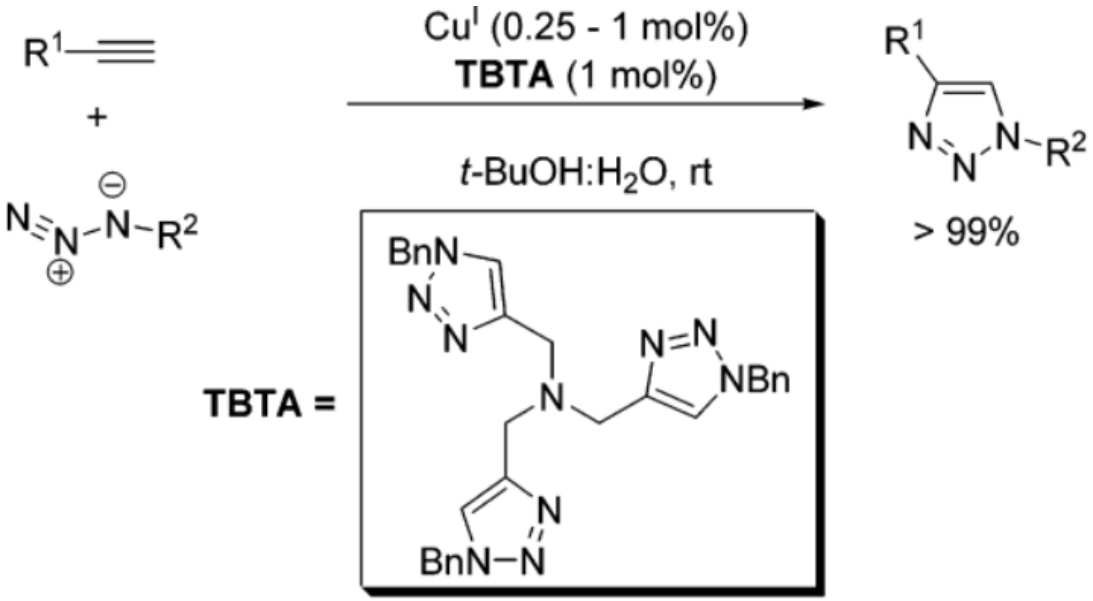
- Monomeric copper acetylide complexes are not reactive toward organic azides unless an exogenous copper catalyst is added

Ligand effect on CuAAC

- Polytriazolylamines are powerful stabilizing ligands for copper(I), protecting it from oxidation, while enhancing its catalytic activity.

Polytriazoles as Copper(I)-Stabilizing Ligands in Catalysis

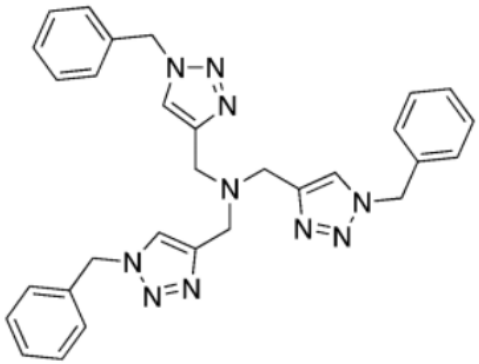
Timothy R. Chan, Robert Hilgraf, K. Barry Sharpless, and Valery V. Fokin*



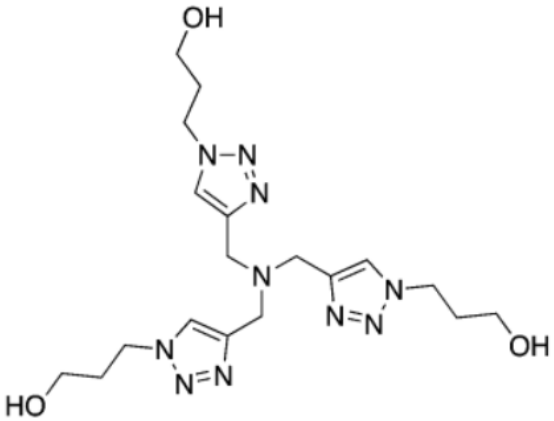
Org. Lett. **2004**, *6*, 2853.

Ligand effect on CuAAC

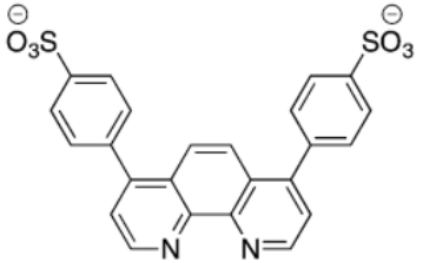
- Various ligands are available for CuAAC
- BTTES and BTTAA are popular with better water solubility and catalysis



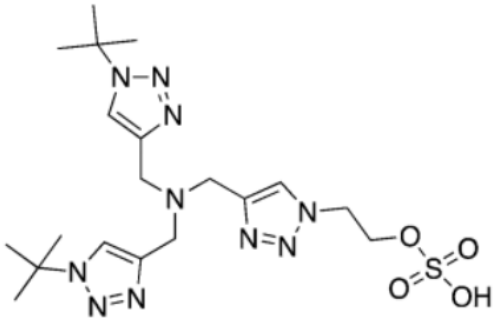
TBTA



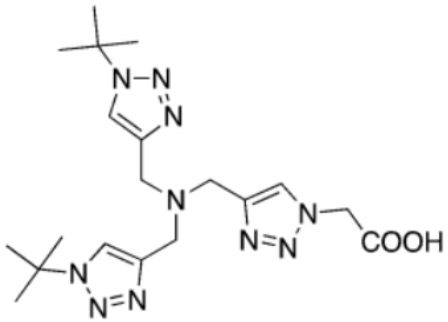
THPTA



BPS

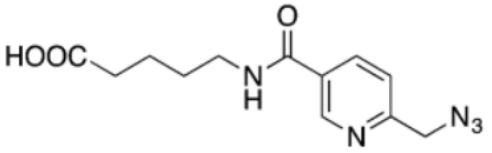


BTTES



BTTAA

azide substrates containing auxiliary ligands



picolyl azide

Chem. Commun. **2013**, *49,* 11007.

Bioconjugation using CuAAC

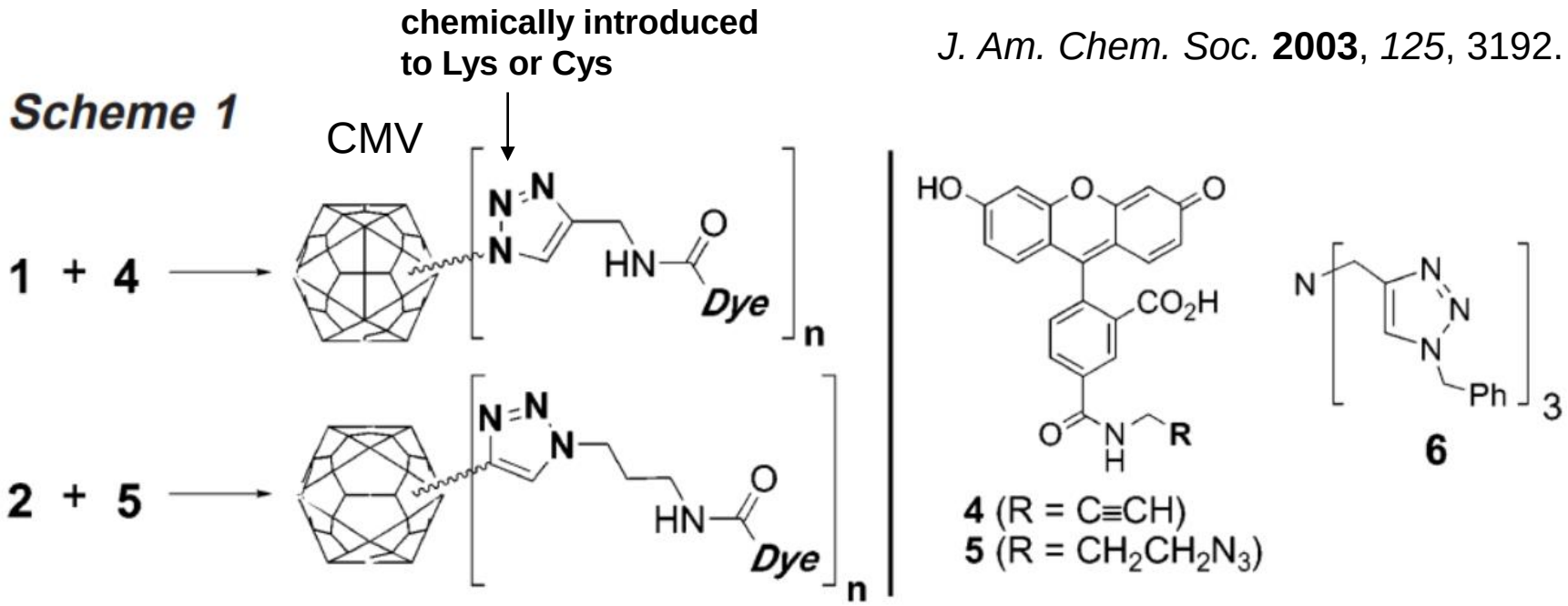
- The first report of CuAAC as a bioconjugation strategy was demonstrated by Finn and co-workers through the attachment of dyes to cowpea mosaic virus

Bioconjugation by Copper(I)-Catalyzed Azide-Alkyne [3 + 2] Cycloaddition

Qian Wang, Timothy R. Chan, Robert Hilgraf, Valery V. Fokin,* K. Barry Sharpless,* and M. G. Finn*

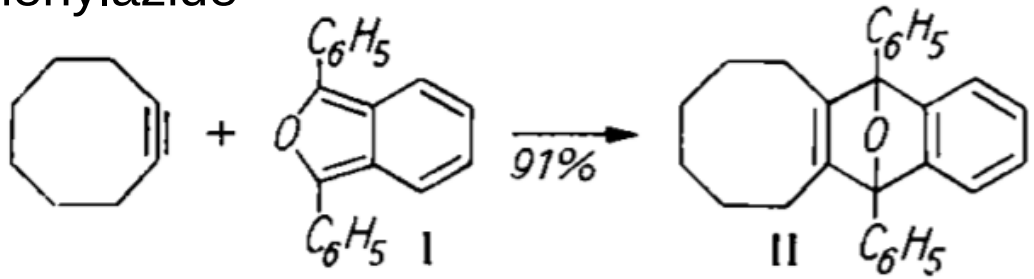
Departments of Chemistry and Molecular Biology and The Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037

J. Am. Chem. Soc. **2003**, *125*, 3192.



Ring strain and reactivity

- The cytotoxicity of Cu(I) required alternative methods
- To improve upon the biocompatibility of the azide–alkyne cycloaddition, activation of alkynes by ring strain was considered
- The roots of the strain-promoted azide cycloadditions precede the Huisgen era and date back to when Alder and Stein discovered that dicyclopentadiene reacted considerably faster than cyclopentadiene in reactions with azides
- Studies on strained alkenes and alkynes continued through the 1960s, and during this time, Wittig and Krebs reported that cyclooctyne, the smallest stable cycloalkyne, reacted “like an explosion” when combined with phenylazide



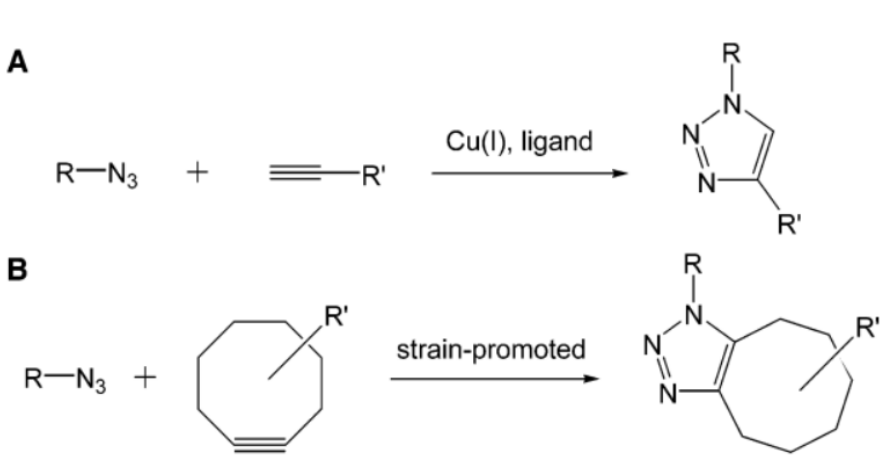
Chem. Ber. **1961**, *94*, 3260.

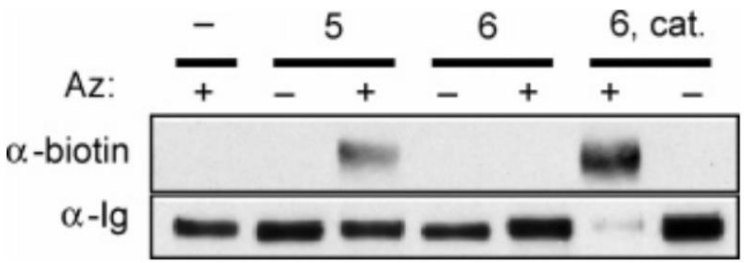
Strain-Promoted Azide-Alkyne Cycloadditions (SPAAC)

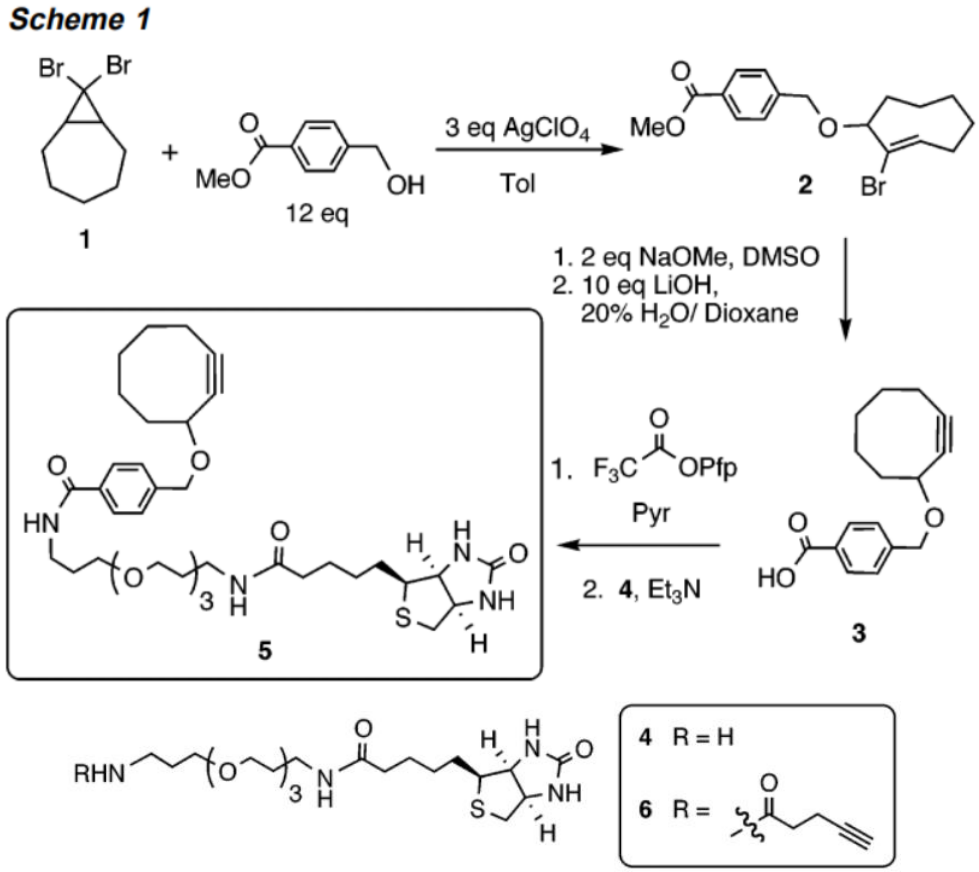
A Strain-Promoted [3 + 2] Azide–Alkyne Cycloaddition for Covalent Modification of Biomolecules in Living Systems

Nicholas J. Agard, Jennifer A. Prescher, and Carolyn R. Bertozzi*

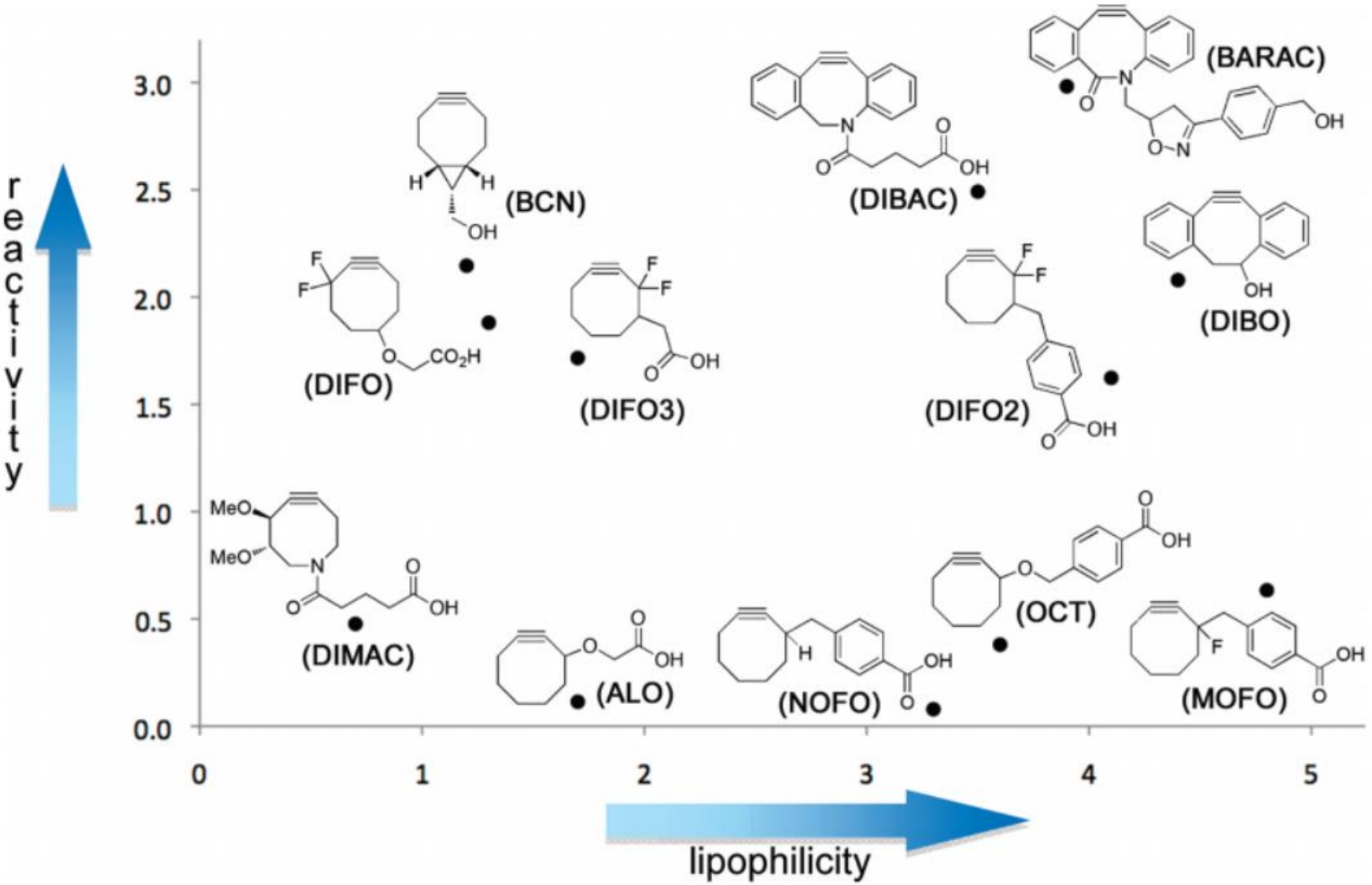
J. Am. Chem. Soc. **2004**, *126*, 15046.





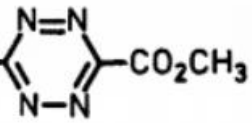


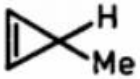
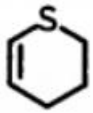
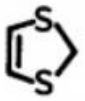
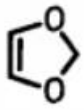
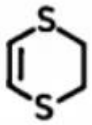
Strained Alkynes



Acc. Chem. Res. **2011**, *44*, 805.

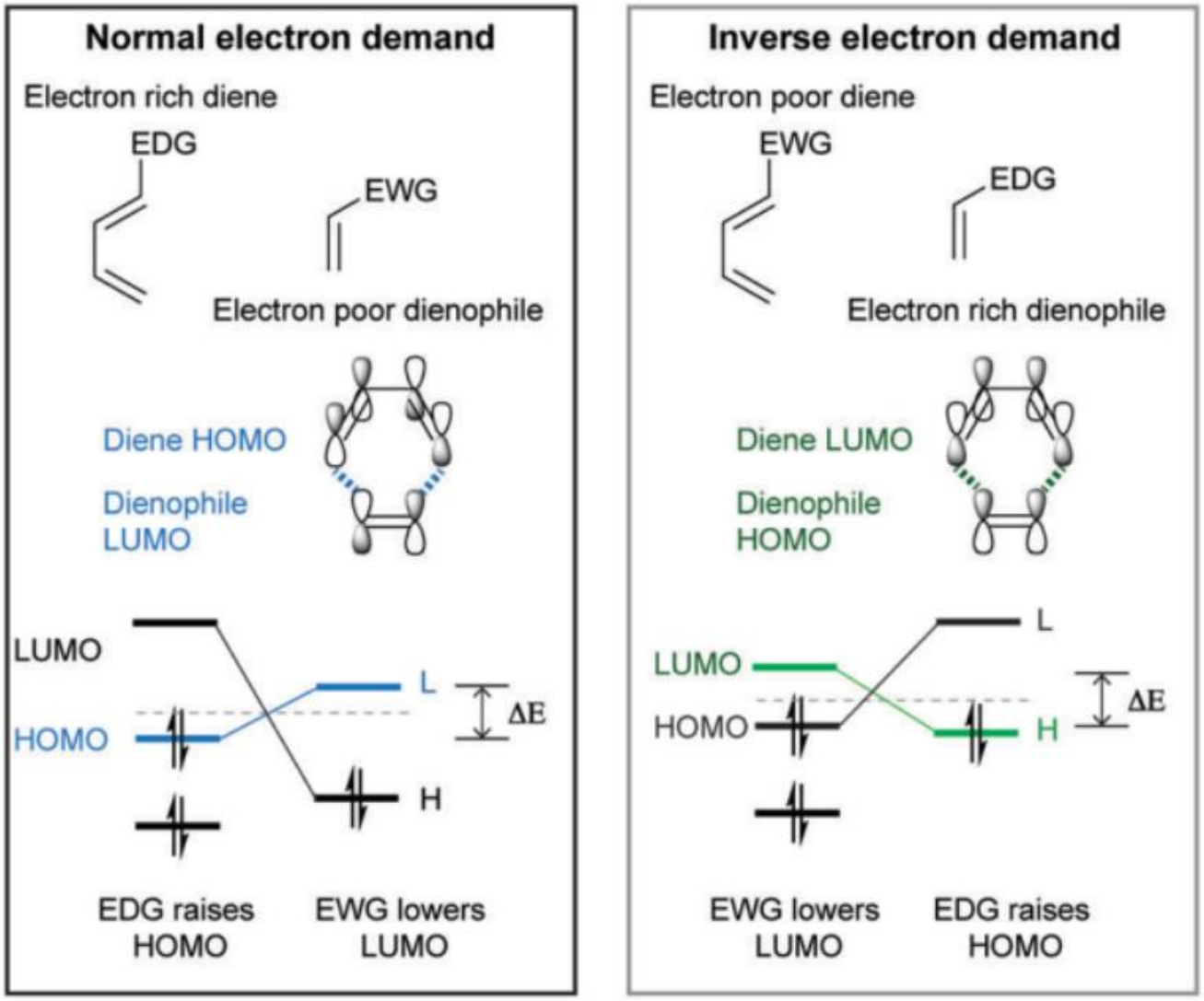
Inverse Electron Demand Diels-Alder reaction

Tab. 2: 1  $10^5 \cdot k_2$ (l/mol · s), 20°C, Dioxan

					
44 800 000	329 000	23 500	86.6	8 370	cis: 3 380 tr.: 1 270 000 000
					
54 900 000	4.86	104 000			2 970 000
					
7 770	6 310	136 000	769	36.5	1 000 000
					
	35.5	13 200	67.7	3.35 (40°C)	192 000

J. Sauer et al. *Tetrahedron Lett*. **1990**, *31,* 6851.

◆ Inverse Electron Demand Diels-Alder reaction



Frontier orbitals in normal- and inverse-electron-demand DA reactions.

Chem. Soc. Rev. **2017**, *46,* 4895.

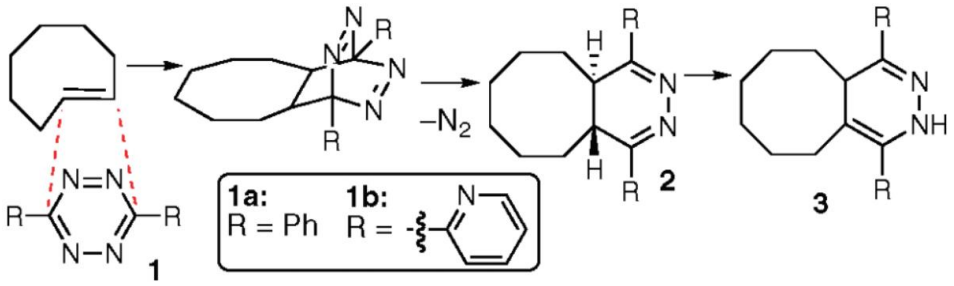
◆ Inverse Electron Demand Diels-Alder reaction

Tetrazine Ligation: Fast Bioconjugation Based on Inverse-Electron-Demand Diels–Alder Reactivity

Melissa L. Blackman, Maksim Royzen, and Joseph M. Fox*

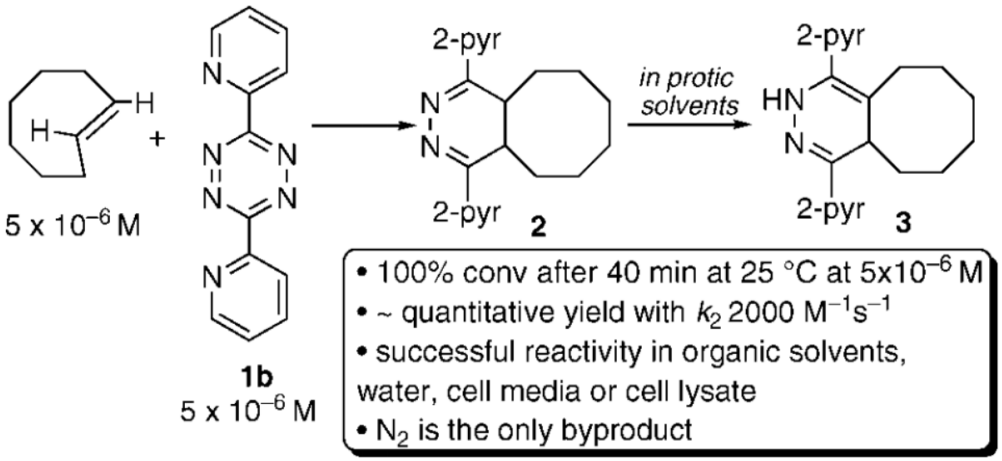
J. Am. Chem. Soc. **2008**, *130*, 13518.

Scheme 1. Diels–Alder Reactions of Tetrazines with *trans*-Cyclooctene



The reaction proceeds with very fast rates and tolerates a broad range of biological functionality

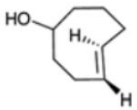
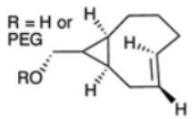
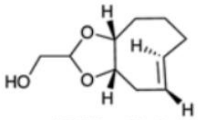
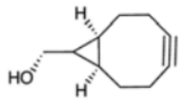
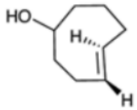
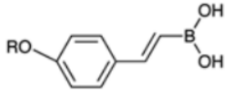
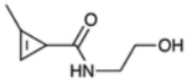
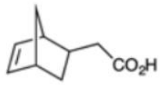
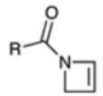
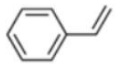
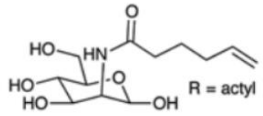
Scheme 2. Fast Reactivity at Low Micromolar Concentrations



This fast reactivity enables protein modification at low concentration

Inverse Electron Demand Diels-Alder reaction

Reactivity

		Second-order rate constant ($M^{-1}s^{-1}$)		
		Organic solvent	Mixture (Org:Water)	Aqueous solvent
a	 TCO + **Tz 8/Tz 9**	1140 [Ref 1]	2000 (90:10) [Ref 1]	22600 (eq iso) [Ref 2] 80200 (ax iso)
b	 s-TCO + **Tz 8/Tz 9**	22000 [Ref 3]		3300000 [Ref 2]
c	 d-TCO + **Tz 9**		167000 (55:45) [Ref 2]	366000 (syn iso) [Ref 2] 318000 (anti iso)
d	 BCN + **Tz 8/Tz 10**	45 [Ref 4]	1245 (55:45) [Ref 5]	
e	 TCO + **Tz 11**		7.5 (50:50) [Ref 6]	
f	 VBA + **Tz 8**			27 [Ref 7]
g	 1-methyl-3-R Cyclopropene + **TZ 8**		2.3 (50:50) [Ref 8]	
h	 Norbornene + **Tz 12**			1.9 [Ref 9]
i	 N-acylazetine + **Tz 13**		0.39 (12:88) [Ref 10]	
j	 Styrene + **Tz 8**		0.05 (50:50) [Ref 11]	
k	 ManNHxl + **Tz 14**			0.021 [Ref 12]

Examples of strained and unstrained dienophiles for IEDDA reactions and their corresponding reaction rates.

Chem. Soc. Rev. **2017**, *46*, 4895.

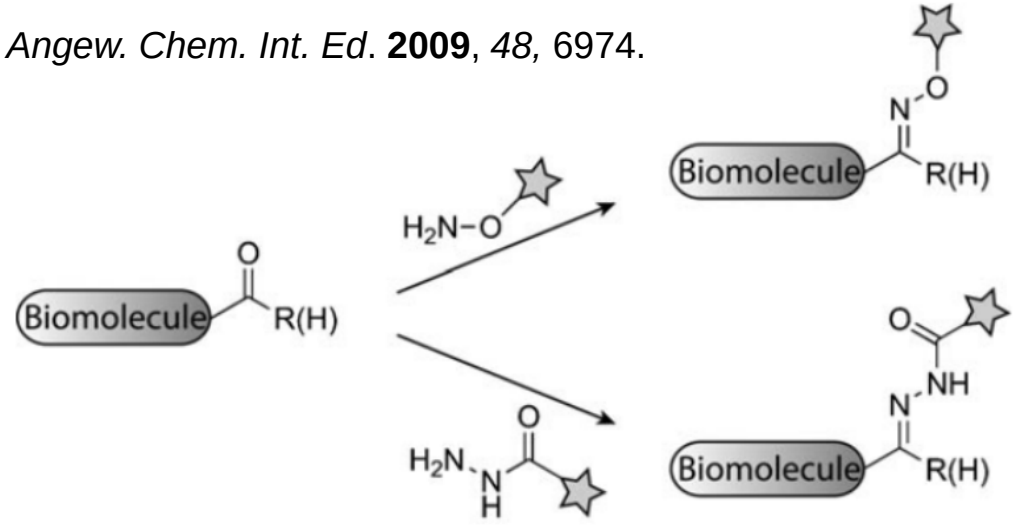
Aldehydes/Ketones as a bioorthogonal group

- Ketones and aldehydes react with amine nucleophiles that are enhanced by the α-effect.
- Prototypical examples are aminooxy and hydrazide compounds, which form oxime and hydrazone linkages, respectively, under physiological conditions
- While biological nucleophiles—amines, thiols, and alcohols—also react with ketones and aldehydes, the equilibrium in water generally favors the carbonyl compound
- Accordingly, ketones and aldehydes have a rich history in the field of protein modification (bioorthogonal functional group)
- These carbonyl compounds have not been widely employed for labeling biomolecules inside cells or within live organisms, in part because of competition with endogenous aldehdyes and ketones, including those in glucose and pyruvate

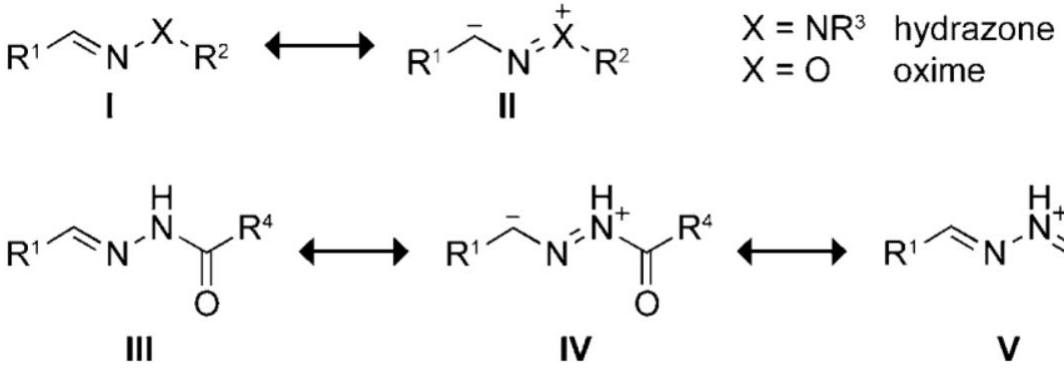
Angew. Chem. Int. Ed. **2009**, *48,* 6974.

Aldehydes/Ketones as a reactive group

Angew. Chem. Int. Ed. **2009**, *48,* 6974.



Scheme 8. Bioorthogonal reactions of aldehydes/ketones. Aldehydes and ketones can condense with aminooxy compounds (top) or hydrazide compounds (bottom) to form stable oxime or hydrazone linkages, respectively.

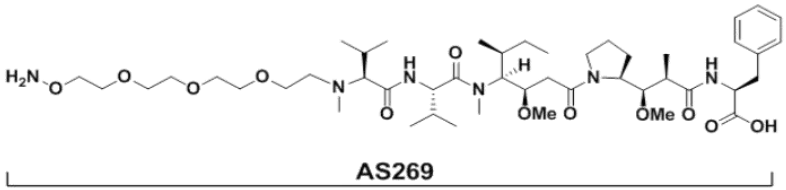


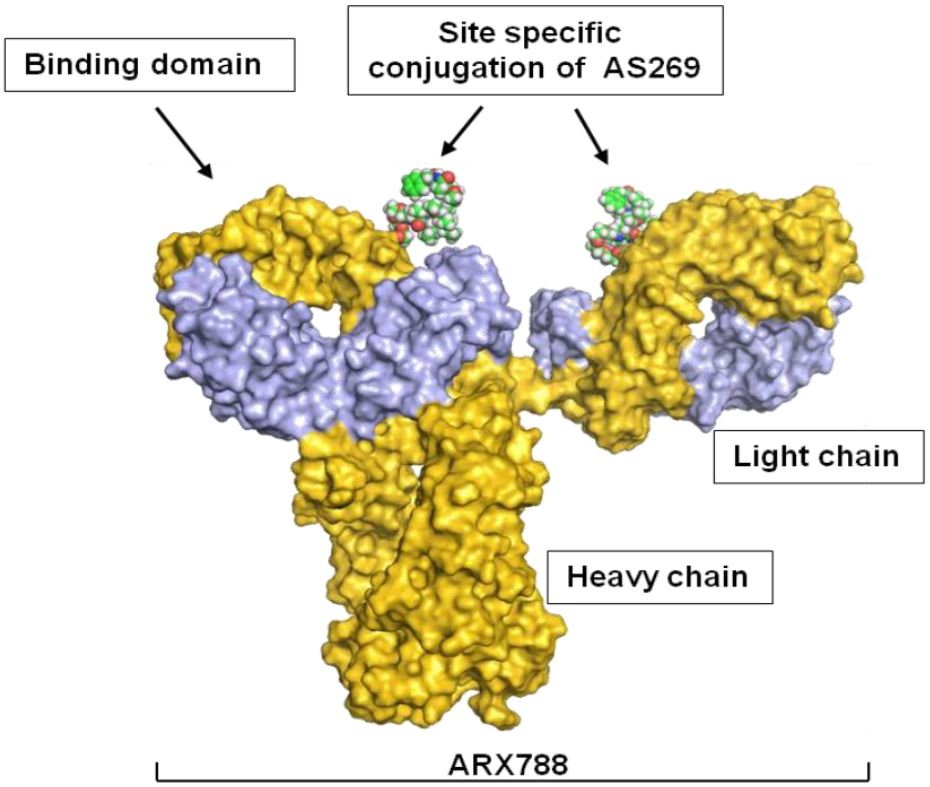
Scheme 1. Major resonance forms of conjugates.

- The reaction requires a acidic condition: pH 4~6
- The use of aniline as a catalyst accelerated the reaction under neutral conditions

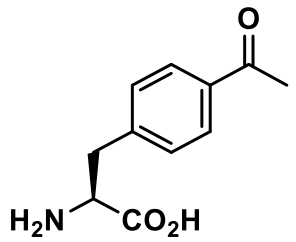
Angew. Chem. Int. Ed. **2008**, *47*, 7523.

Applications





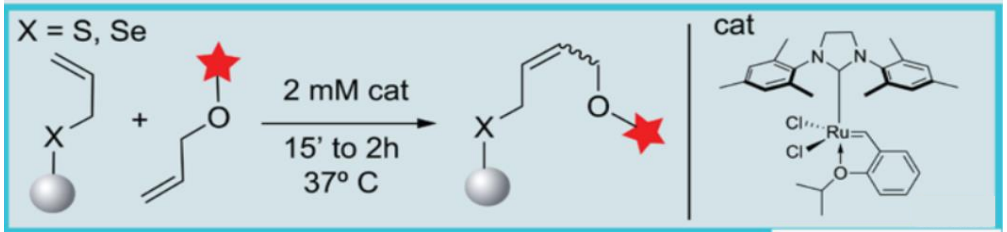
- ARX-788 is anti-HER2 ADC using the GCE technique for drug conjugation.
- ARX-788 is currently in phase 1 & 3 clinical trials
- Amberstatin (AS269) used as a payload is a microtubule inhibitor developed by Ambrx
- Amberstatin is conjugated to anti-HER2 by the ketone-alkoxyamine chemistry (DAR = 1.9)



p-Acetylphenylalanine

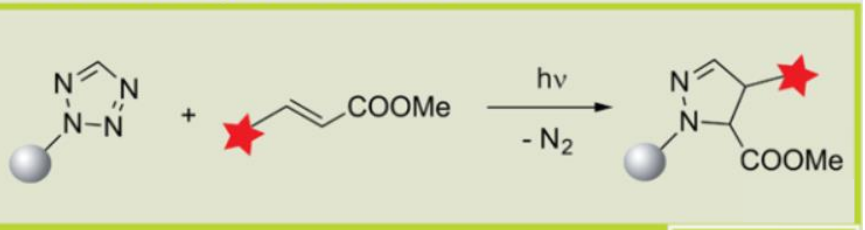
◆ Other bioorthogonal reactions

Cross-metathesis



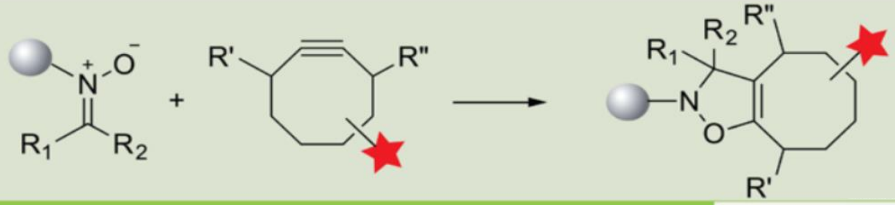
in vitro — k ~ 0.3 $M^{-1}s^{-1}$

Photo-Click cycloadditions



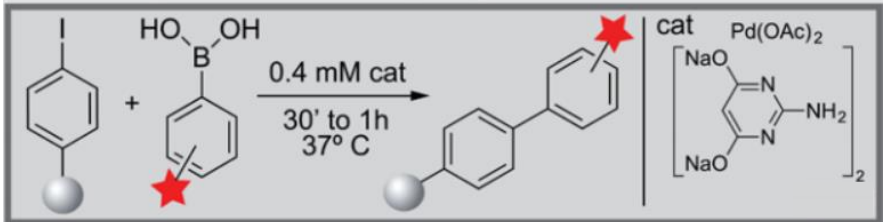
in vivo, fluorescent product, UV-inducible — k < 60 $M^{-1}s^{-1}$

SPANC (strain promoted alkyne-nitrone cycloadditions)



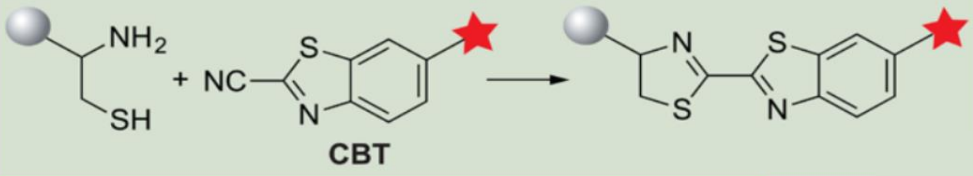
mainly *in vitro*, nitrones are prone to hydrolysis — k < 30 $M^{-1}s^{-1}$

Pd-catalyzed cross coupling



on bacterial cell surface, rate constants not reported

1,2-Aminothiol-CBT condensations



mainly *in vitro* — 2-cyanobenzothiazole (CBT) — k ~ 10 $M^{-1}s^{-1}$

ACS Chem. Biol. **2014**, *9*, 16.