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4-Substituted Prolines: Useful Reagents in Enantioselective Synthesis and Conformational Restraints in the Design of Bioactive Peptidomimetics

Recent Advances in Alkene Metathesis for Natural Product Synthesis—Striking Achievements Resulting from Increased Sophistication in Catalyst Design and Synthesis Strategy

ALDRICHIMICA ACTA 50 YEARS 1968 • 2017

From the Editor's Desk

The Aldrichimica Acta's GOLDEN JUBILEE YEAR

Dr. S. J. Firsan

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With this issue of the *Aldrichimica Acta*, we are pleased to introduce our readers to the fresh and vibrant new look of this venerable journal they have come to value and love. This look weds the traditional successful elements of the *Acta* with the design principles of the *Acta*'s new owner, Merck KGaA, Darmstadt, Germany. This makeover couldn't have come at a better time, as we are celebrating the *Aldrichimica Acta*'s golden jubilee year. While this look is new, I'm happy to assure our contributors and readers that the *Acta*'s aim will remain the same: providing the best value and scholarship to the scientific community in fast-changing academic, business, and technology environments. Happily, Merck KGaA, Darmstadt, Germany, has made the same commitment to the chemistry community through the *Acta* that its predecessors Sigma-Aldrich and Aldrich had made: full open access to the entire *Acta* archive, no author or page charges, no subscription fees, topical reviews by some of the best and most active researchers from around the world, top-notch editorial assistance, worldwide print distribution, and a strong presence on the Web, just to name a few. At a time when most scientific publishers have now monetized their digital archives and are charging for access, perhaps no one appreciates what the *Acta* has to offer more than our readers who work at institutions with limited library budgets and, thus, limited access to scientific journals and databases.

As we celebrate this milestone, and as I reflect back on the *Acta*'s journey since its humble beginning in 1968, I am thrilled to let some of those who have helped the *Acta* grow into a leading review journal in the field of organic chemistry express in their own words what the *Acta* means to them.

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To all the *Aldrichimica Acta* readers and contributors, I offer a big thank-you from the bottom of my heart and a promise that the *Acta* will continue to aim to be *Best in Science, Best in Business.*

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Nielsen, M. K.; Ugaz, C. R.; Li, W.; Doyle, A. G. *J. Am. Chem. Soc.* **2015**, *137*, 9571.

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Alte Apotheke im Museum der Firma Merck (watercolor and ink, 38.5 × 49.5 cm) was painted by an unknown artist in Darmstadt, Germany. It depicts original components of the Merck family's Engel-Apotheke (Engel Pharmacy), such as the pharmacy table within a niche of the company museum. Beneath a cross-ridge vault, the pharmacy is set up as was typical for the early 1800s, stocked with various vessels and grinding implements. A stuffed alligator, an Egyptian symbol against illness, hangs from the ceiling. The scene transports the viewer back in time to an era when apothecarists combined raw animal, plant, and mineral substances to create drugs. After Friedrich Jacob Merck's acquisition of the Engel Pharmacy in 1668, generations of pharmacists strove to refine the pharmaceutical craft into an independent academic discipline. Emmanuel Merck (1794–1855) is generally credited with setting the foundation for his family's pharmacy to grow into a chemical company in the first half of the 19th century. Emmanuel Merck's early research on alkaloids led him to connect with others and seek out pure products rather than raw materials.

Detail from ***Alte Apotheke im Museum der Firma Merck***. Photo courtesy of Merck KGaA, Darmstadt, Germany.

This painting is part of the collection housed at the company museum located at our Darmstadt, Germany, headquarters, where the Engel Pharmacy work tables are still on display. **www.emdgroup.com/history**.

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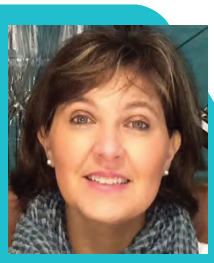
4-Substituted Prolines: Useful Reagents in Enantioselective Synthesis and Conformational Restraints in the Design of Bioactive Peptidomimetics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Keywords. 4-substituted prolines; peptide conformation; enantioselective synthesis; modified peptides; bioactive compounds.

Abstract. Among the substituted prolines, 4-substituted ones deserve particular attention. The ring conformation and the puckering preference of the prolyl ring are strongly affected by insertion of the substituent, and a great number of peptides containing these noncanonical amino acids have been investigated in the last five years. Moreover, the distance of the substituent from the functions involved in peptide chain formation causes minimal steric hindrance, thus offering the opportunity for conjugation with other chemical groups with little perturbation of the peptide chain.

Outline

1. Introduction

The unusual properties of proline (Pro), in comparison with the other amino acids, are responsible for its protective role toward proteolysis and for the specific conformation constraint it induces in proteins. For the same reasons, proline is commonly inserted into synthetic peptides to modulate the sequence conformation, thus inducing specific affinity towards biological receptors and increasing peptide strength. This unique behavior is due to its cyclic structure; but, attaching a substituent to the pyrrolidine ring may introduce an additional effect: the modulation of ring puckering and cis–trans amide equilibration.[1] Moreover, the introduction of a novel functional group into Pro may offer an opportunity to conjugate proline-containing peptides to other useful entities in the design of drugs or diagnostic agents.

It's well known that Pro usually breaks repeated structural motifs, and changes peptide direction by means of hairpin or reverse-turn folding. The lack of hydrogen-bond-donating capacity prevents Pro from participating in secondary stabilized structures, thus making it poorly compatible with α-helixes and β-sheets. In general, Pro is best located in the (i+1) and (i+2) positions of β- and γ-turns or as the terminal N-residue of an α-helix. Moreover, while for proteinogenic amino acids the trans conformation of the peptide bond is energetically favored, the lower energy gap in the isomerization of the ω-dihedral angle observed for Pro makes the cis conformation of comparable

stability (**Figure 1**, Part (a)). Even if the cis and trans isomers are essentially isoenergetic, more than 90% of prolyl amide bonds in proteins adopt the trans conformation.[2] Polyproline or Pro-rich peptide fragments can form right-handed PPI helixes when all-cis peptide bonds are present, or left-handed PPII helixes when all peptide bonds assume the trans conformation. With respect to the pyrrolidine ring, two puckering conformations are favored and are defined as exo (up) and endo (down), depending on the out-of-plane displacement of the C^{γ} atom relative to the carbonyl group, as described by the value of the χ^1 torsion angle (Figure 1, Part (b)).[1] The exo and endo ring puckers influence the trans and cis conformations of prolyl amide bonds, and, consequently, the φ, ψ, and ω main-chain torsion angles that define the backbone of a whole protein.

The introduction of substituents on the pyrrolidine ring provides an exceptional opportunity to exploit the unique properties of proline in the design of complex molecules. The choice of the functionalized position has to be carefully planned, taking into account both the desired effect on the properties of the peptide and the synthetic pathway, since substituent introduction is rarely performed on proline itself. The cis or trans configuration of the novel moiety relative to the carbonyl group would determine the conformation of the five-membered ring.

Insertion of a substituent at position 2 leads to the formation of C_{α}-tetrasubstituted amino acids and induces a dramatic restriction in the allowed torsion angles. 2-Alkylprolines and 2-spiro compounds are of great interest since they have been successfully employed in the synthesis of bioactive synthetic peptides.[3] Attaching a substituent to the pyrrolidine β carbon generates a 3-substituted Pro, and leads to a real "chimera" bearing the side chain of another proteinogenic amino acid and thus possessing characteristics from both molecules.[4] Finally, 5-aryl-substituted prolines are privileged elements in medicinal chemistry,[5] and fused azabicyclo compounds containing the 5-substituted proline scaffold are important structural constraints in several bioactive molecules.[6]

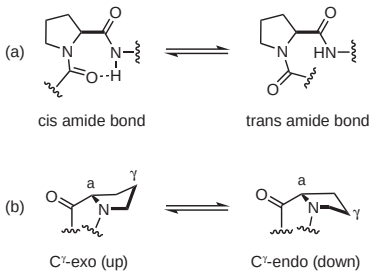


Figure 1. Proline: (a) Cis–Trans Amide Equilibration, and (b) Favored Exo or Endo Puckering of the Pyrrolidine Ring. *(Ref. 1)*

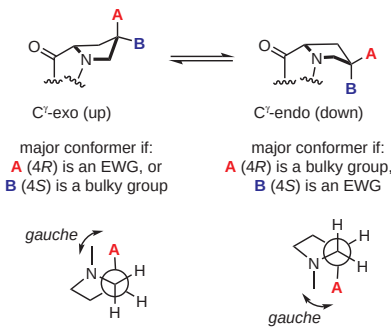


Figure 2. Preferred Conformers and Gauche Effect for 4-Substituted Prolines. *(Ref. 7)*

Among the substituted prolines, those bearing a novel moiety at position 4 deserve particular attention. The first advantage is the distance of the substituent from the functions involved in peptide chain formation, resulting in minimal steric hindrance. This offers an opportunity to exploit substitution in order to conjugate peptides to other chemical entities with only a low perturbation of the peptide chain. Moreover, the puckering preference of the prolyl ring is affected by insertion of a substituent at position 4. The presence of an electron-withdrawing group, in particular, changes the relative energies of the ring conformations and leads in some cases to a unique preferred conformation due to the gauche effect (**Figure 2**).[7]

A review of the synthesis of alkyl-substituted prolines has recently been reported,[8] and other interesting surveys have been published, describing specific aspects of these compounds, such as the physiological properties and metabolism of L-Pro analogues or the properties of hydroxyproline derivatives.[9] A more specific review of 4-[^{18}F]-labelled prolines and their applications has also been published relatively recently.[10] In the present review, we highlight the more recent studies (since 2010) of 4-substituted prolines. The synthetic aspects of these unusual amino acids will only be briefly covered, since many of them are commercially available, while major attention will be focused on their insertion into bioactive molecules to induce proper conformations or as point of attachment in conjugation. These topics deserve particular attention since they represent innovative approaches in medicinal chemistry, biomimetic chemistry, and materials chemistry. Moreover, Pro derivatives are very efficient organocatalysts, and the novel applications in this field will also be reported. The great number of papers dealing with the chemistry of 4-substituted prolines has compelled us to choose only a few selected recent examples.

Principal classes of 4-substituted prolines will be presented, each in a separate section. With respect to halogen-substituted prolines, most of the studies reported in the literature over the last five years have been about fluorine, while chlorine, bromine, and iodine have been rarely used, and, for this reason, only the compounds containing fluorine will be described.

2. 4-Hydroxyproline (4-Hyp)

2.1. General Properties and Biological Relevance

Among substituted prolines, 4-hydroxyproline (4-Hyp) is present in nature as a fundamental component of collagen proteins, the major extracellular proteins in connective tissues.[11] The biosynthesis of this amino acid takes place as a post-translational event during polypeptide chain elongation by specific prolyl 4-hydroxylases (P4Hs) that are present in the endoplasmic reticulum. The hydroxylation process, in mammalian systems, requires interaction of the enzyme with the unfolded collagen chain in the presence of molecular oxygen, 2-oxyglutarate, ferrous iron, and ascorbate, and leads to the selective formation of *trans*-4-hydroxy-L-proline (T4LHyp). The transformation occurs exclusively in specific positions, resulting in the formation of Gly-Xaa-Yaa consensus tripeptide sequences, where Pro may be found in the Xaa or Yaa position, while 4-Hyp is always found in the Yaa position. The amount of hydroxylated proline in Types I and II collagen is close to 10%, and the regular occurrence of the trimeric sequences is responsible for the triple helical regions of collagen fibrils and for

the increased stability of the protein. In collagen's structure, T4LHyp establishes hyperconjugative interactions, inducing a preference for the exo ring-puckering conformation and favoring trans amide bond formation.[6] In bacteria, T4LHyp is metabolized following different enzymatic pathways that involve the formation of *cis*-(4*S*)-D-Hyp (C3DHyp) via deprotonation of the C_α chiral carbon and racemization. Hydroxyprolines have also been detected in nonribosomal peptides, such as pneumocandins, which display antibiotic or antifungal activity. Since the origin of the hydroxylated amino acids in this context is still unclear, Hüttel and co-workers[12] recently investigated the reactivity and regioselectivity of Pro hydrolases (PHs), acting on the isolated amino acid or small congeners. All characterized members of this family have been obtained from bacteria, and biocatalysts able to synthesize three of the four possible Hyp isomers (T4LHyp, C4LHyp, and C3LHyp; this last isomer bearing the hydroxyl group at position 3) are already known. The authors studied the reaction catalyzed by GloF, a hydroxylase obtained by the pneumocandin biosynthesis cluster of the fungus *Glarea lozoyensis* ATCC 74030, with the aim of obtaining preference towards the formation of the elusive T3LHyp. Even if the simultaneous formation of T4LHyp and T3LHyp could be observed for the first time, T4LHyp ended up being the predominant isomer under all conditions tested.

2.2. Novel Syntheses of 4-Hyp

Although all the stereoisomers of 4-Hyp are commercially available, novel enzymatic or synthetic methodologies for their preparation are always of great interest. As mentioned in the preceding section, biocatalysts able to produce T4LHyp, C4LHyp, and C3LHyp are already known, but their use on a preparative scale is limited by their insolubility and low activity. Moreover, they undergo rapid denaturation in vitro, while their use in vivo involves elaborate workup processes. By producing recombinant hydroxylases and optimizing conditions, Klein and Hüttel[13] succeeded in the quantitative and selective conversion of Pro into specific hydroxylated derivatives, easily isolating the products via ion-exchange chromatography. Furthermore, to overcome the milligram-scale limit of the in vitro protocol, the in vivo reaction was also optimized in shake flask culture, leading to a significant increase in productivity.

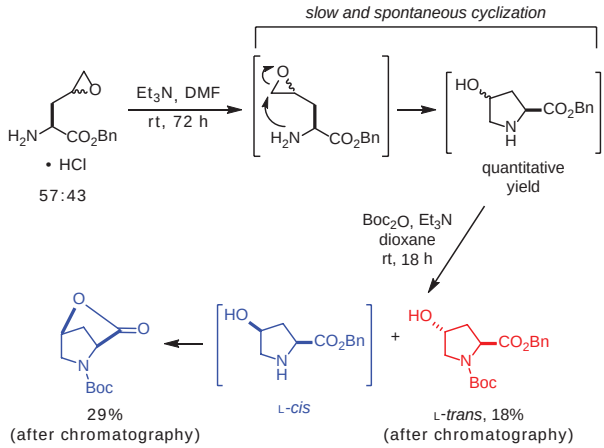
All four stereoisomers of 4-Hyp have been synthesized by Arai and co-workers starting from enzymatically resolved, enantiopure N-protected α-allylglycine benzyl ester that was treated with *m*-CPBA (**Scheme 1**).[14] The reaction afforded a diastereomeric mixture of epoxides, due to the uncontrolled formation of the new C4 stereocenter. Removal of amine protection induced the slow spontaneous intramolecular cyclization to 4-Hyp. The unexpected formation of a bicyclic lactone by reaction of the 4-hydroxyl group with the benzyl ester group was observed for *cis*-Hyp derivatives. Although stereocontrol of the epoxidation step is low, the methodology offers some advantages because all of the stereoisomers of 4-Hyp can be obtained with a simple and cheap procedure.

2.3. Applications of 4-Hyp in Bioactive Compound Synthesis

Recently, an Hyp-based class of histamine H_3 receptor antagonists that play a therapeutic role in CNS disorders has been reported in the literature. Moreover, the (2*R*,4*S*) stereoisomer, T4DHyp, is found at the core of the structures of the members of this class (**Figure 3**).[15] To address the industrial requirements of simplicity and low cost, Pippel and co-workers selected for their synthetic approach the natural and inexpensive T4LHyp as starting material, taking into account that a double-inversion strategy would have to be utilized.[15] The epimerization of C2 was accomplished by optimizing the conditions for the transformation of T4LHyp into La Rosa's lactone, a reactive intermediate, which underwent ring opening by means of the free base homopiperazine. The homopiperazine amide product reductively aminated cyclobutanone, resulting in elongation of the side chain. The last step was a Mitsunobu reaction, which caused inversion of configuration at C4 and yielded the target H_3 receptor antagonist (104 g, 57% (crude); 96 g, 53% (after chromatography)).

T4LHyp has also been employed by Hanessian's group as a building block in the synthesis of the model tetracyclic core present in calyciphylline B, a member of the family of alkaloids of the *Daphniphyllum* genus.[16] These alkaloids possess seven contiguous stereogenic carbons in their common backbone. As depicted in **Figure 4**, the Pro ring is conserved in the final structure, while nitrogen, carboxylate, and the hydroxyl moiety are used to build the tetracyclic structure through a stereocontrolled multistep synthesis.

An efficient synthesis of β-arabinofuranosylated-4-Hyp oligomers was reported in 2014 by Xie and Taylor,[17] with the aim of defining the minimal epitope of Art v 1, an allergenic glycoprotein present in the most common pollen and responsible for hay fever. A detailed study on coupling methodologies and peptide capping was performed in order to prepare the dimer, trimer, and tetramer starting from the glycosylated



Scheme 1. Synthesis of L-*cis*- and L-*trans*-4-Hyp from Epoxy Amino Acids Derived from Allylglycines. The Same Approach Was Also Employed for the Synthesis of the D-*cis* and D-*trans* Stereoisomers. *(Ref. 14)*

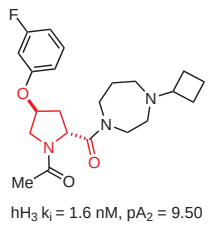
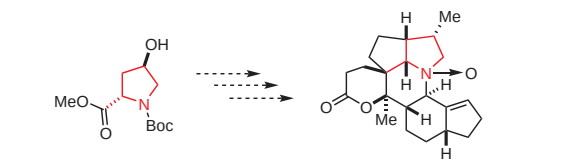


Figure 3. Selective Histamine H_3 Receptor Antagonist. *(Ref. 15)*

monomer. The trimer and tetramer were analyzed with circular dichroism, which showed the presence of a typical PPII-type helical structure that is usually observed only for longer Hyp-rich peptides. This showed that glycosylation of the 4-hydroxyl group stabilizes and promotes the formation of this ordered secondary structure.

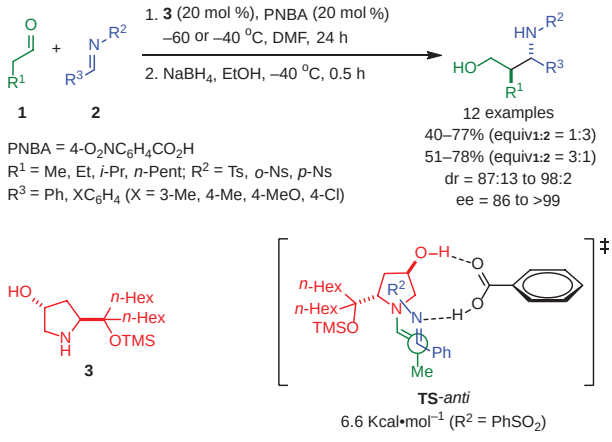
2.4. Conformational Studies on Peptides Containing 4-Hyp

The secondary structure assumed by 4-Hyp-rich peptides has been extensively investigated due to the fundamental role of these sequences in animal and plant structural proteins. The preference of 4-Hyp for a γ-exo pucker conformation, due to the gauche effect and to hyperconjugative interactions, induces the formation of trans peptide bonds, thus stabilizing left-handed PPII helixes. The rigid PPII secondary structure is also typical of Hyp-rich glycoproteins (HRGPs), in which Hyp residues are extensively O-glycosylated. A well-defined model to evaluate the stabilizing effect of Hyp and O-glycosylated-Hyp was developed by Schweizer and co-workers[18] by comparing the homooligomers Ac-$(Pro)_9$-NH_2, Ac-$(Hyp)_9$-NH_2, and Ac-[Hyp(β-D-Gal)]$_9$-NH_2. Although a decrease in the PPII typical signals in the circular dichroism spectrum was observed for the O-glycosylated peptide, the presence of contiguous sugar groups increased the stability of the secondary structure owing to the tendency to form hydrogen bonds with the peptide backbone and with the solvent.



N-Boc-4-(*R*)-hydroxy-L-proline (T4LHyp) — calyciphylline B

Figure 4. Structure of Calyciphilline B, an Alkaloid of the *Daphniphyllum* Genus. *(Ref. 16)*



Scheme 2. *anti*-Selective Mannich Reaction between Aldehydes and *N*-Sulfonylimines Catalyzed by *trans*-4-Hydroxy-L-proline Derivatives with Brønsted Acid as Cocatalyst and Representation of the Transition State, with Corresponding Single-Point Value at B3LYP Level. *(Ref. 20)*

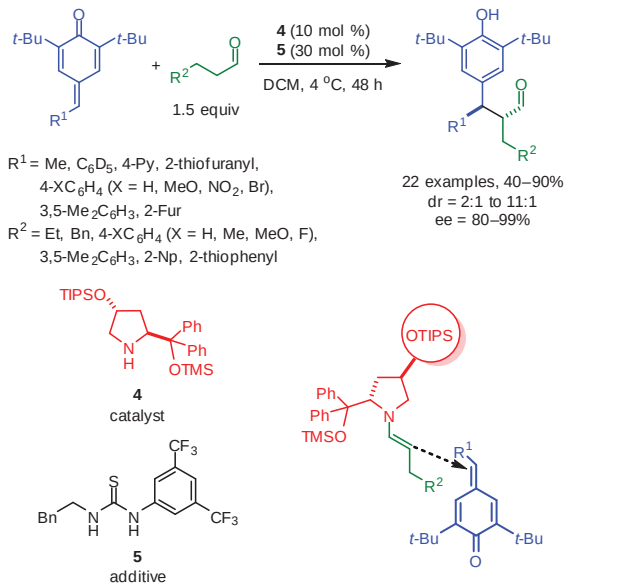
2.5. Application of 4-Hyp and Its Derivatives as Organocatalysts

In the past few years, Pro was extensively studied as an organocatalyst because of its ability to act with high stereoselectivity in aldol condensations, Mannich and Diels–Alder reactions, Michael additions, and other transformations.[19] This privileged role in organocatalysis has led, in the last fifteen years, to the development of a great number of derivatives, that have been successfully applied in asymmetric synthesis. Among them, Hyp and its reduced alcohol form deserve particular attention, since the hydroxyl group may be exploited for enhancing selectivity by introducing novel constraints into the catalytic complex, or for supporting the catalyst on a solid carrier.

The catalytic efficiency of *trans*-4-hydroxyprolinamides had been established by several research groups, but Palomo and co-workers have recently reported an *anti*-selective Mannich reaction performed in the presence of a Brønsted acid.[20] They have also demonstrated that the reaction occurs via a three-component arrangement, where the Pro nitrogen activates the aldehyde donor substrate while the 4-hydroxyl group, together with the added Brønsted acid, activate the imine acceptor component, controlling in this way the stereochemistry of the process. Among the screened catalysts, the best results were obtained with 4-hydroxy-dialkylprolinol derivatives (**Scheme 2**).

Similar, sterically demanding catalysts were successfully synthesized by Jørgensen and co-workers and applied to the enantioselective α-alkylation of aldehydes. In addition, these 4-hydroxyprolinol derivatives, possessing two bulky silyloxy moieties, afforded excellent stereocontrol in the addition of aldehydes to *p*-quinone methides to give α-diarylmethine-substituted aldehydes (**Scheme 3**).[21]

The importance of the 4-hydroxyl moiety was also demonstrated by Kelleher and co-workers in the optimization of the asymmetric Michael addition of aldehydes to nitro-olefins (**Scheme 4**).[22] The reaction of valeraldehyde with *trans*-β-nitrostyrene was performed in



Scheme 3. α-Alkylation of Aldehydes with *p*-Quinone Methides Catalyzed by *trans*-4-Hydroxy-L-proline Derivatives and Model of the Reaction Pathway. *(Ref. 21)*

the presence of a range of 4-hydroxyprolinamide catalysts, differing only in the presence or absence of an α-methyl group in the proline ring or in the steric hindrance of the amide group. The study showed that the *trans*-4-hydroxy group controls the facial stereoselectivity, and the simplest chiral prolinamide affords the best results. Additional substituents on the ring or at the amide nitrogen seem to be detrimental to the efficient control of the stereoselectivity, since small changes in the reagent and catalyst structures lead to a significant decrease of the enantiomeric excess.

Gauchot and Schmitzer sulfonylated the hydroxyl group to obtain a chiral counterion for the imidazolium cation, with the aim of exploring organocatalysis in ionic solvents such as [Bmim]NTf_2. This study afforded two efficient catalysts for the asymmetric aldol reaction (**Figure 5**, Part (a)).[23]

Since proline itself is an excellent organocatalyst for many reactions, the 4-hydroxyl group has also been considered a good handle for attaching the catalyst to a solid support.[24] By following this approach, silica nanoparticles—grafted with polymeric chains and linked to 4-Hyp via ester formation—were prepared to explore modulating the performance of the polymeric organocatalyst by ion-specific effects. This study, performed by Liu and co-workers, provided useful information about the salting-in or out effect induced by different counteranions, as well as about the stabilization of the transition state in proline-catalyzed reactions, induced by the anion polarization of hydrogen bonds (Figure 5, Part (b)).

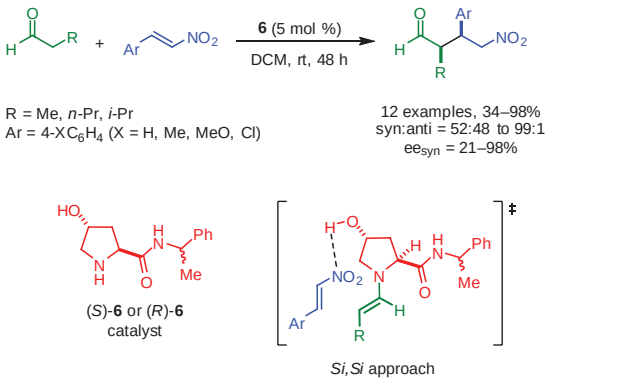
Heterogeneous catalysts with a unique architecture were prepared by Toste, Somorjai, and co-workers by immobilizing on mesoporous SiO_2 self-assembled monolayers (SAM) of chiral molecules such as Pro, T4LHyp, or diproline (Figure 5, Part (c)).[25] It is worth noting that the attachment of T4LHyp to the silica surface occurred via the carboxylic acid function. Au nanoclusters were then synthesized within the dendrimeric matrixes. The reactivity and selectivity of the catalysts were studied in the cyclopropanation reaction. For the T4LHyp containing catalyst, an increase in enantioselectivity was observed in comparison to proline, due to better packing of the SAM around the gold nanoclusters via hydrogen-bond formation. Another heterogeneous synergistic catalyst system was recently reported by Córdova and co-workers.[26] It consisted of a diamino-Pd complex on silica and a 4-hydroxyprolinol anchored to the silica surface via azide–alkyne click chemistry. This system proved to be effective in cascade Michael–carbocyclization reactions as a result of the concerted action of the two catalytic sites (Figure 5, Part (d)).

2.6. 4-Hyp as Synthetic Building Block

The reactivity of Hyp may involve the 4-hydroxyl substituent or the pyrrolidine ring. Zondlo and co-workers[7] have prepared a large number of 4-substituted prolyl amino acids, with 4*R* or 4*S* stereochemistry, starting from T4LHyp via Mitsunobu, oxidation, reduction, acylation, and substitution reactions. These residues may be inserted into peptides as other amino acid mimetics, recognition motifs, handles for spectroscopic studies (NMR, fluorescence, IR) or EWG-containing fragments capable of inducing stereoelectronic effects.

Substitution of the 4-OH group with a sulfur-containing moiety and Mitsunobu inversion were also exploited by Otaka and co-workers[27] for a previously unreported sequential coupling of peptide fragments via a 4-sulfanylproline-mediated native chemical ligation (NCL). The original NCL protocol requires two peptide segments, one possessing a thioester group and the other possessing an N-terminal cysteinyl residue. The limitation of the original protocol to peptides containing cysteine (Cys) was overcome by the authors using a Pro surrogate possessing a sulfanylated tether in position 4 of the proline ring.

Five-membered-ring systems are basic building blocks in heterocyclic chemistry, and pyrrolidines have gained particular prominence due to their presence in a wide variety of fundamental structural motifs. With



Scheme 4. Michael Addition of Valeraldehyde and *trans*-β-Nitrostyrene Catalyzed by 4-Hydroxy-L-prolinamides. *(Ref. 22)*

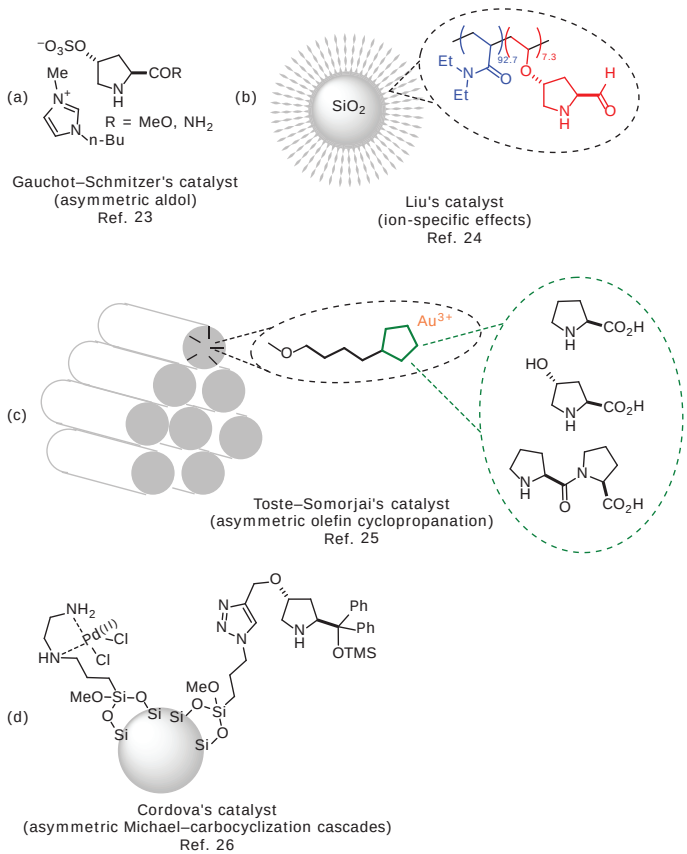
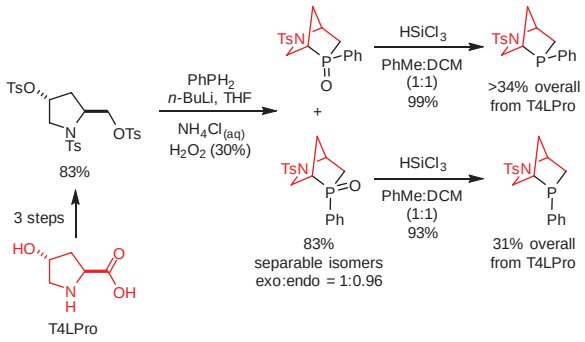
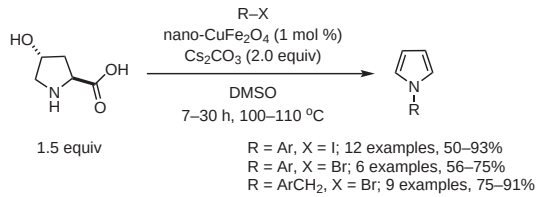


Figure 5. *trans*-4-Hydroxy-L-proline Derivatives in Heterogeneous Catalysis. *(Ref. 23–26)*

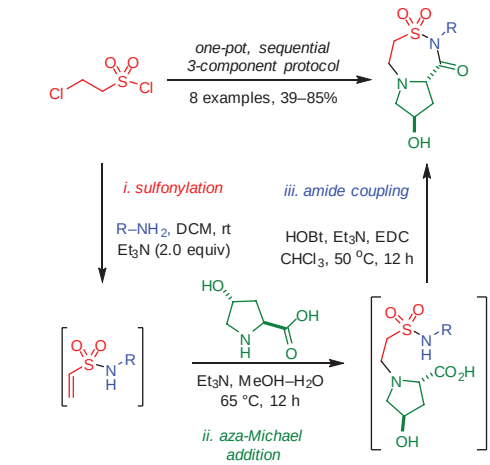
the aim of developing novel chiral phosphine catalysts to facilitate allene–imine [3 + 2] annulations leading to pyrrolines, Kwon's group designed rigid, bridged bicyclic structures using T4LPro as a source of chirality and as the origin of the catalyst pyrrolidine core (**Scheme 5**).[28] The key step of the synthesis is the bisalkylation of dilithium phenylphosphide with tritosylated hydroxyprolinol that affords *exo*- and *endo*-2-aza-5-phosphabicyclo[2.2.1]heptanes. These two diastereoisomeric phosphines are highly effective catalysts for



Scheme 5. Synthesis of Pseudoenantiomeric Bicyclic Phosphines Derived from *trans*-4-Hydroxy-L-proline. *(Ref. 28)*



eq 1 *(Ref. 30)*



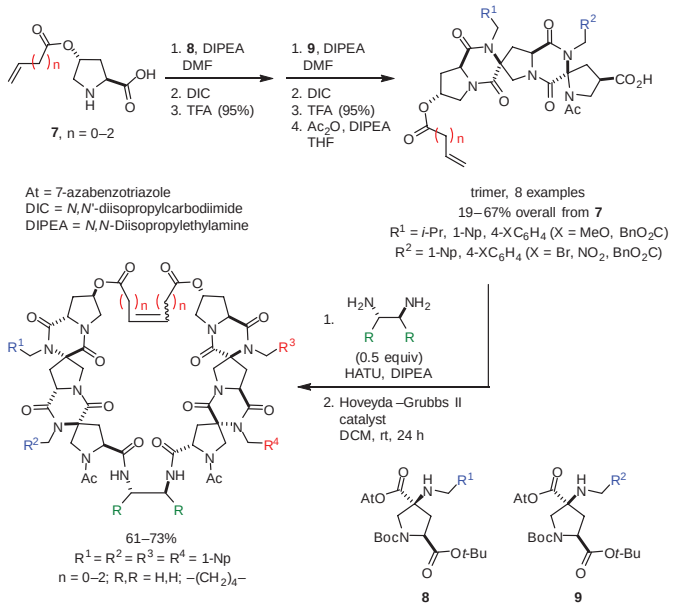
Scheme 6. Synthesis of Bicyclic Sultams via a One-Pot, Sequential, Three-Component Protocol. *(Ref. 31)*

the asymmetric synthesis of 1,2,3,5-tetrasubstituted pyrrolines, and function as pseudoenantiomers, producing the products in opposite enantiomeric forms.

A new synthesis of *N*-arylpyrroles from T4LPro and aryl iodides in the presence of CuI has recently been reported by Rao and co-workers.[29] Moderate-to-good yields were obtained, and the procedure was operationally simple and used inexpensive reagents and a readily available metal catalyst. Later, Nageswar's group optimized a ligand-free C–N cross-coupling reaction by employing recyclable and magnetically separable nanoparticles of $CuFe_2O_4$ as catalyst (**eq 1**).[30] Under these conditions, aryl iodides and bromides, as well as benzyl bromides, were reacted with T4LPro to give the corresponding aromatized 1-aryl- and 1-benzyl-1*H*-pyrroles.

A scalable, one-pot protocol for the preparation of stereochemically rich mono- and bicyclic acyl sultam libraries was recently reported by Hanson and co-workers.[31] This general methodology relies on the complementary ambiphilic pairing (CAP) of vinyl sulfonamides and unprotected amino acids. Vinyl sulfonamides may undergo both hetero-Michael additions and N-alkylations, and are ideal substrates for CAP. By using T4LPro and C4DPro, together with the enantiomers of α-methylbenzylamine-derived vinyl sulfonamides, a collection of all possible four diastereoisomers of the sultam were efficiently obtained in good yields and without any sign of racemization (**Scheme 6**).[31] The versatility of the sultam products allows further in situ transformations to be carried out as sequential 3-, 4-, and 5-component reactions.

T4LHyp is a fundamental building block in the convergent synthesis of a novel class of macrocyclic spiroligomers designed to create molecular surfaces and pockets with applications in supramolecular recognition and catalysis. Preactivated, alkylated proline derivatives, possessing a quaternary stereocenter, were synthesized by Zhao and Schafmeister in a few steps starting from T4LHyp, and were then coupled with the 4-pentenoate ester of T4LHyp (**Scheme 7**).[32] The reaction follows a complex mechanism—involving direct acylation



Scheme 7. Synthesis of Spiroligomer-Containing Macrocycles Starting from *trans*-4-(4-Pentenoyloxy)proline. *(Ref. 32)*

of the prolyl amine and an acyl-transfer coupling—that leads to the formation of hexasubstituted spiro-diketopiperazines, whose stereocenters and functional groups are determined during product design. Insertion of a diamine linker and olefin cross-metathesis led to spiroligomer-based macromolecules possessing highly preorganized structures that are suitable for binding proteins and for creating enzyme-like active sites.

Irradiation of Hyp with visible light in the presence of (diacetoxy)-iodobenzene (DIB) and iodine induces a mild, metal-free domino process involving ring opening of pyrrolidine, oxidation of the alcohol to the aldehyde, and addition of acetate ion to the iminium ion (**Scheme 8**). This particular reactivity has been exploited by Romero-Estudillo and Boto for the generation of a customizable unit for the site-selective modification of peptides with the aim of creating peptide libraries starting from a single, parent peptide.[33] The reaction, which occurs with negligible epimerization of the Hyp stereocenter at C2, generates a molecule possessing two reactive chains, which can be transformed independently or through concerted mechanisms. A reductive amination–lactamization took place with amino acids and peptides, leading to α-amino-γ-lactams (Agl, Freidinger–Veber lactams), that are very useful conformational constraints for rigidifying peptide backbones and for inducing conformations mimicking β-turns. The process afforded good results both with the free Hyp and with peptides containing one or more Hyp units in terminal or internal positions. Moreover, elaboration of the *N*-acetoxymethyl moiety permits the isolation of free amines or N-methylated units, which are also useful for the modification of peptide conformation and activity.

3. Conformational Studies of Peptides Containing 4-Aminoproline (Amp)

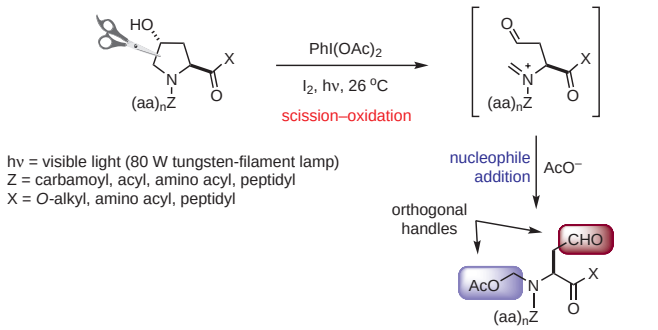
As already reported, a substituent at C4 of the Pro residue has a large influence on the puckering of the pyrrolidine ring and on the trans:cis conformer ratio of its amide bond. This effect is mainly due to steric effects, stereoelectronic gauche effects, repulsive interactions, and transannular hydrogen bonding.

Wennemers and co-workers have determined the behavior of (4*S*)-aminoproline under different pH conditions.[34] They demonstrated that (4*S*)-Amp adopts a C4-*endo* ring pucker in an acidic environment due to the formation of a transannular hydrogen bond and to the electron-withdrawing nature of the ammonium group, which induces an *endo* ring pucker. The uncharged amino group should exert a similar steric effect, and favor a C4-*exo* ring pucker without a transannular hydrogen bond. Thus varying the pH induces a flip between the two ring puckers of the pyrrolidine and the on-off switch of the transannular hydrogen bond. These observations have been confirmed by studying the effect of changes in the protonation state of (4*S*)-Amp-containing collagen on triple-helix formation. The stability of the supramolecular assembly has also been studied by switching between single- and triple-helical states of collagen (**Figure 6**). These results would help with the design of pH-sensitive, collagen-based materials that are capable of specifically releasing drugs in an acidic environment.

A new class of collagen analogues defined as "chimeric cationic collagens" was reported by Ganesh and co-workers.[35] In this case, both the X and Y residues of the collagen triad were simultaneously substituted by a combination of 4(*R*/*S*)-(OH/NH_2/NH_3^+/NHCHO)-prolyl units, and triplex stabilities were measured at different pHs. The observed unique effect of the 4-amino substituent on triplex stability allowed the critical combination of factors that dictate triplex strength to be defined (C4 puckering, intra-residue hydrogen bonding, stereoelectronic (*R*/*S*) and electronic interactions).

Among the prolines substituted with nitrogen at C4, 4-azido-Pro has been inserted by Bernardes's group into the structure of collagen as a chemical reporter for tagging.[36] The metabolic incorporation of this residue by foetal ovine osteoblasts, obtained by supplementation of the growth medium with *cis*-4-azido-L-proline, was followed by labelling with the fluorescent probe dibenzooctyne DIBO via azide–alkyne cycloaddition. Even if the methodology suffers from unspecific reaction of the strained alkyne DIBO with sulfhydryl-rich proteins, the strategy could be particularly useful for monitoring collagen accumulations in fibrotic diseases.

The epimerization of position 2 of N-protected *trans*-4-AMP methyl ester under basic conditions was immediately and irreversibly followed by an intramolecular cascade reaction to form a bridged lactam intermediate, which could be easily converted into various derivatives of 2,5-diazabicyclo[2.2.1]heptane (DBH).[37] This bicyclic scaffold has been utilized in structure–activity relationship studies in medicinal chemistry and as chiral ligand in asymmetric synthesis. Key



Scheme 8. Site-Selective Conversion of the *trans*-4-Hyp Unit Can Lead to Optically Pure Amino Acids, Including Valuable *N*-Alkyl Amino Acids, as Well as Homoserine Lactones and Agl Lactams. *(Ref. 33)*

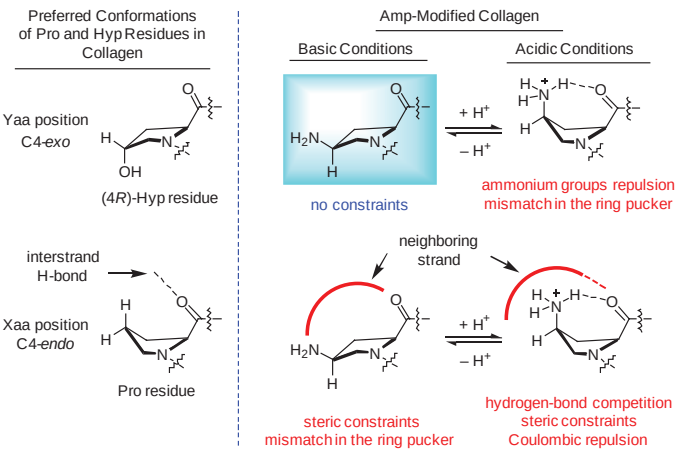


Figure 6. Effects of Introducing Amp in the Yaa or Xaa Position in Triple-Helical Collagen on the Stability of the Supramolecular Assembly under Different pH Conditions. *(Ref. 34)*

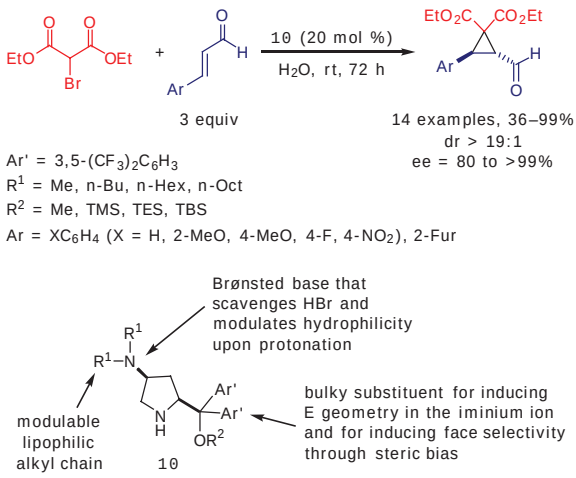
factors controlling the cascade transformation are the N-protective group being an electron-withdrawing group and promotion of the reaction by a strong base.

4-Dialkylamino-α,α-diarylprolinol ethers have been proposed by Vicario and co-workers[38] as efficient organocatalysts in the enantioselective cyclopropanation of α,β-unsaturated aldehydes. The presence of the 4-dialkylamino substituent, possessing linear alkyl chains of different lengths, is crucial for modulating the formation of micellar aggregates in disperse aqueous solutions. Moreover, the C4 amine represents an additional Brønsted basic moiety that may play a role in the activation of the substrate. The results obtained in the Michael–intramolecular α-alkylation cascade confirm that this novel class of catalyst is active in reactions in aqueous media (**eq 2**).[38]

4. 4-Fluoroprolines

4.1. General Properties

Due to the unique physicochemical properties of fluorine, such as highest electronegativity, strong lipophilicity, extremely low polarizability, and the ability to participate in a hydrogen bond, organofluorine compounds have been utilized in a range of applications in medicinal chemistry. In particular, fluorine has been found to have great potential in modulating the properties of peptides and proteins, and to increase stability and bioavailability by affecting the folding.[39] As a consequence of the introduction of substituents at C4 of the pyrrolidine ring, the energies of the conformers can be significantly altered, which results in stabilizing a different arrangement of the protein backbone. When the electron-withdrawing fluorine is incorporated at C4 of the pyrrolidine ring, the hyperconjugative interactions lead to a strong gauche preference for the β-fluoro amide moiety.[40] An exo pucker and a high trans:cis ratio are preferred when the fluorine-substituted C4 has an *R* configuration, while an endo pucker is favored with the *S* configuration of C4. The endo conformation, however, does not lead to an overall preference of cis over trans, even if it exhibits an increased probability of forming a cis peptide bond. These stereoelectronic effects can influence protein folding, thermostability, ligand binding, and catalytic activity. Therefore, investigating the effects of 4-fluoroproline is extremely important both in theoretical studies and in medicinal and diagnostic applications.



eq 2 *(Ref. 38)*

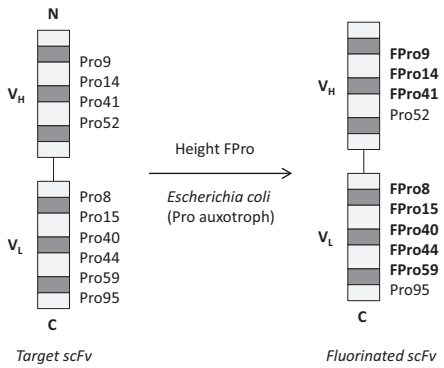


Figure 7. Approach for Improving the Stability of scFv Proteins with FPro. The Available Conserved Pro Residues in the Target scFv´s Can Be Easily Replaced with FPro by a Residue-Specific Substitution Method in the Cytoplasm of *E. coli*. *(Ref. 43)*

4.2. Effects of Fluorination on Protein Folding and Stability

The single-chain Fv protein (scFv) is the smallest functional antigen-binding unit of an antibody; it is composed of two domains, named variable heavy (V_H) and variable light (V_L), joined together by a flexible peptide linker. scFv fragments are powerful tools in radiotherapy and medicinal chemistry applications, owing to their increased rate of blood clearance and low immunogenicity as compared to the parent monoclonal antibodies.[41] However, the scFv fragments suffer from lower binding affinity and insufficient thermostability, which limit their therapeutic potential.[42] The approach proposed by Lee and co-workers to enhance the thermal stability of scFv incorporates fluorinated prolines in the target protein, in order to take advantage of the stereoelectronic changes induced by the new amino acids on the folding and thermal stability of the protein.[43] hu-MscFv, a bacterial cytoplasmic protein containing a total of eight Pro residues in the framework region (FR) affecting the folding and stability, was used as the model system (**Figure 7**).[43]

Only the replacement of Pro with (4*R*)-FPro (not the 4*S* epimer) had a positive influence on folding and activity. The thermal stability of the fluorinated protein was also improved, although only for temperatures below 50 °C. The explanation for this improved thermal stability was provided with the help of modeling studies, which showed ten new dipole interactions between fluorine and other polar groups in the protein.

Wild-type ubiquitin (wt-ub) is a small thermally and chemically stable protein of eukaryotes that regulates the cellular processes of other proteins in the body. Crespo and Rubini studied the effects of introducing 4-FPro in wt-ub, and found that (4*R*)-FPro exerts a positive effect on the stability, while (4*S*)-FPro is not incorporated into the protein.[44] The authors initially evaluated the structure of the modified enzyme by circular dichroism (CD), and discovered that its far UV-CD spectrum was practically identical, at pH 2.0 and 5.0, to that of wt-ub, suggesting that the secondary structure is not modified by incorporation of (4*R*)-FPro. The thermal stability after denaturation at acidic pH, and the equilibrium against denaturation in guanidine-induced unfolding experiments were also investigated, indicating a stabilizing effect of (4*R*)-FPro-ub. The folding–unfolding kinetics of (4*R*)-FPro-ub and wt-ub were also determined by stopped-flow analysis, which proved that (4*R*)-FPro exerts a positive effect, accelerating the folding process of the protein. This effect was attributed to the ability of the fluorine

atom to establish stabilizing interactions with the amino groups of the protein backbone, made possible by the Cγ-endo pucker and by the energetically favored trans conformation of the protein.

Lantibiotics are peptides synthesized by Gram-positive bacteria as secondary metabolites typically containing an intramolecular ring structure and the thioether cross-linked amino acids lanthionine or methyllanthionine.[45] In recent years and to date, many variants have been engineered and characterized, with the aim of obtaining compounds with activity against multidrug-resistant pathogens. Budisa, Süssmuth, and co-workers reported the possibility of obtaining lantibiotics containing noncanonical amino acids (ncAAs) by heterologous expression from *Bacillus licheniformis* in *E. coli* as host.[46] In their ribosomal peptide synthesis (RPS) of the two-component lantibiotic lichenicidin, they selected Pro and tryptophan (Trp) as the amino acids to be replaced in JM83 and ATCC 49980 auxotrophic *E. coli* strains, respectively. (4*R*)- and (4*S*)-FPro were successfully embedded at high levels into the peptide, as demonstrated by mass spectrometry, thus opening the way for obtaining new lantibiotics with improved chemical and biological properties.

Collagen mimetic peptides (CMPs) are small synthetic peptides that mimic natural collagen and that were developed as synthetic models to study the structure and folding behavior of collagen, as well as to generate new collagen-like scaffolds for use in many biomedical fields. In this context, Raines and co-workers found that replacing—in the typical Gly-Xaa-Yaa consensus tripeptide—of Hyp in the Yaa position with (4*R*)-FPro and Pro in the Xaa position with (4*S*)-FPro enhanced the collagen triple helix stability by adopting a favorable ring puckering (C^{γ}-exo for Yaa and C^{γ}-endo for Xaa), as a consequence of electronic inductive effects.[47] The same research group implemented these discoveries by investigating the annealing of some fluoroproline-based CMPs to natural collagen.[48] They synthesized Ac-[(4*S*)-FPro-(4*R*)-FPro-Gly]$_7$-(Gly-Ser)$_3$-LysOH and other CMPs as controls, and evaluated them by using fluorescently labeled peptides to establish (i) the annealing of collagen and the time-dependent retention in vitro, (ii) the annealing of collagen in vivo, and (iii) the cytotoxicity toward human dermal fibroblasts. They found that the selected peptides were effectively included into the collagen and that the annealing took place without any preheating. The annealing strategy described in this work could have useful applications in the medical field such as in the treatment of highly traumatized or slowly healing wounds.

The type 1 DNA polymerase from *Thermus aquaticus* (KlenTaq) is a single-chain polypeptide with a molecular mass of 94 kDa containing 32 Pro residues dispersed throughout the whole structure. A (4*R*)-FPro-modified peptide was expressed and purified in *E. coli*, together with wild-type KlenTaq, and extensive studies on crystallization, prolyl ring puckering, cis–trans properties, and structural noncovalent interactions were performed.[49] (4*R*)-FPro-KlenTaq was crystallized in a ternary complex with DNA primer/template duplex and 2',3'-dideoxycytidine-5'-triphosphate (ddCTP) as a chain terminator. It was observed that the crystallization of the fluorinated KlenTaq was faster than that of the wild-type enzyme and occurred under a broader range of conditions. Comparison of the two structures did not show substantial differences, and the authors reported that (4*R*)-FPro-KlenTaq establishes a new network of noncovalent interactions and tends to assume an exo puckering, while the wild-type enzyme has a high percentage of endo puckering, and this conformational change could explain the better crystallization results from the fluorinated enzyme.

Employing a similar approach and aiming to enhance the tendency to adopt the PPII helix conformation, Borgogno and Ruzza carried out the synthesis in the solid phase of analogues of the proline-rich decapeptide PPPLPPKPKF (P2) by replacing some prolines with (4*R*)-FPro and (4*R*)-FHyp.[50] CD spectroscopy revealed a higher content of PPII in the fluorinated compounds, but an unfavorable binding interaction with GST-SH3$_{m\text{-}cort}$ fusion protein, probably due to a decrease in the flexibility of the peptide. The Src homology 3 region (SH3)[51] is a peptide recognition domain of about 60 amino acids that typically interacts with Pro-rich motives, in particular regular periodic PPII helices.

The lipase from the anaerobic bacterium *Thermoanaerobacter thermohydrosulfuricus* (TTL) is widely used as biocatalyst in many industrial processes due to its thermal stability and resistance to solvents and proteases. These properties make TTL a very attractive target to study the effects of incorporation of noncanonical amino acids (ncAAs). Among TTL Pro congeners, the fluorinated ones exhibited highest catalytic activity in *tert*-butyl alcohol and isooctane used as co-solvents for lipase-based biodiesel production.[52]

Enhanced thermal and chemical stabilities were also observed by incorporation of (4*R*)-FPro into the monomeric red fluorescent protein (mRFP1), a versatile biological indicator for monitoring gene expression and protein localization, providing valuable insights on physiological processes.[53] Found in prokaryotes and eukaryotes, thioredoxins (Trx's) are protein disulfide reductases that promote the fast oxidation of two Cys thiol groups to a disulfide with concomitant transfer of two electrons and two protons in a hydrophobic environment. *E. coli* Trx, the prototype of the family, contains a *cis*-Pro76 which is important for stability and function of the enzyme, and which is located in close proximity to Cys32 and Cys35 that form the active site at the N-terminal end of α-helix 1.[54] Trx1P (a variant containing Pro76 and other prolines replaced by Ala) and fluorinated analogues Trx1P-(4*R*)-FPro and Trx1P-(4*S*)-FPro were expressed in *E. coli* and crystallized by Rubini and co-workers.[54] The three enzymes were found practically identical, and, in particular, the Pro ring pucker adopted an endo conformation to avoid unfavorable steric interactions, as demonstrated by a modeling study. The effects on thermodynamic stabilities were determined by guanidium chloride dependent unfolding–refolding equilibria by taking into account both the oxidized and reduced forms of the three enzyme variants. However, all variants showed similar catalytic activities, slightly higher than the wild-type enzyme.

Finally, an interesting CD study by Lin and Horng explored the impact of C-terminal FPro on polyproline helices of type I (PPI, all-cis amide bonds) and type II (PPII, all-trans amide bonds).[55] In particular, (4*R*)-FPro significantly stabilized the PPII conformation and reduced the rate of conversion of PPII to PPI. Thus, these stereoelectronic effects favor a C^{γ}-*exo* pucker and trans peptide bonds.

4.3. 4-Fluoroproline in Catalysis

The incorporation of fluorine at C4 of Pro changes its stereoelectronic properties, and permits control of the conformation and modulation of the reactivity, stereoselectivity, and catalysis mechanism.[56] In a study by Yun and co-workers,[57] FPro and DOPA (two ncAAs) were incorporated into the industrially important biocatalyst ω-transaminase (ω-TA) through a combination of residue-specific and site-specific incorporation methods.[58] Two variants were obtained, ω-TAdopa and ω-TAdp[(4*R*)-FP], exhibiting enhanced thermal stability and half-life. ω-TAdopa and ω-TAdp[(4*R*)-FP] were then immobilized onto soluble chitosan or polystyrene (PS) beads, and the PS-immobilized ω-TAdp[(4*R*)-FP] proved to be the most suitable catalyst for the biocatalytic synthesis of chiral amines.[57]

Coxon's group has reported the synthesis and X-ray crystal structure of two fluorinated analogues of the well-known nickel(II) Schiff base

complex of (*S*)-2-[*N*-(*N*'-benzylprolyl)amino]benzophenone (BPB) and Ala, namely L-Ala-Ni-(*S*)-F-BPB and L-Ala-Ni-(*R*)-F-BPB.[59] These complexes are artificial analogues of pyridoxal 5'-phosphate (PLP) dependent enzymes, and have been used in the asymmetric synthesis of α-amino acids as glycine (Gly) equivalents. The authors highlighted, on the basis of DFT calculations, a novel fluorine–nickel interaction that may enhance the stability of the complexes.

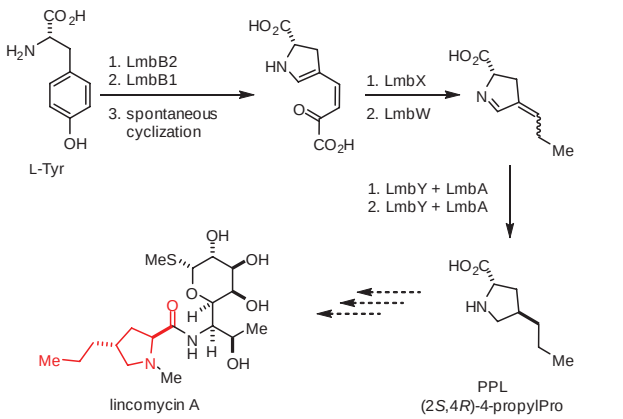
4.4. Applications of 4-Fluoroproline in Medicinal Chemistry

Many wet-adhesion proteins produced by mussels are biomaterials with high biomedical relevance, and are used as "biological glues" for bone surgery and bone regeneration. Budisa's group developed a co-translational method for incorporating FPro into a mussel Pro-rich protein Fp151, with stereoselective preference for *R* isomers. This methodology gives access to a class of biomaterials with new adhesive and mechanical properties.[60]

β-Sheet breaker peptides are fluorinated small peptides that can act against β-amyloid aggregation and fibrillogenesis in Alzheimer's disease.[61] Based on the sequence LVFFD of β-amyloid 1–42 peptide that plays an important role in β-amyloidogenesis, the peptides were obtained by substituting valine (Val) with 4,4,4-trifluoroVal or 4FPro, or substituting phenylalanine (Phe) in the 3 position with 3,4,5-trifluorophenylalanine. These modified fragments induced an α-helix structure on β-amyloid, a conformation which delays the aggregation process.

5. 4-Alkylprolines

4-Alkyl-L-proline derivatives (APD) are present in at least three groups of natural compounds: the anticancer agents pyrrolo[1,4]-benzodiazepines, the bacterial hormone hormaomycin, and the antibiotic lincomycin.[62] These compounds share a specialized biosynthetic pathway encoded by five or six homologous genes. Janata and co-workers revised the biosynthesis of APD on the lincomycin model on the basis of gene inactivation experiments and in vitro assays with recombinant enzymes.[63] The proposed pathway (**Scheme 9**) could be utilized in the preparation of biologically active lincomycin derivatives and other compounds with specific modifications of the APD moiety.



Scheme 9. Structure of Lincomycin A and Proposed Pathway for the Synthesis of Its 4-PropylPro Precursor from Tyrosine. *(Ref. 63)*

Other recent studies have dealt mainly with molecules containing 4-methyl- or 4-cyclohexylproline. 4-Methylproline (4-MePro) is a rare nonproteinogenic amino acid found in secondary metabolites from cyanobacteria and actinobacteria, and is produced from leucine (Leu) through the action of a long-chain dehydrogenase and a pyrroline-5-carboxylic acid (P5C) reductase.[64] The conformational preferences and prolyl cis–trans isomerizations of Ac-4*S*/4*R*-MePro-NHMe in water and in the gas phase were calculated using DFT with a self-consistent reaction field (SCRF) method.[65]

Recently, Sivonen and co-workers[66] developed a method for detecting bioactive peptides in cyanobacteria through biosynthetic 4-MePro, by using a combination of polymerase chain reaction (PCR) and liquid chromatography–mass spectroscopy (LC-MS) methods. They screened 116 cyanobacteria strains from 8 genera, and confirmed the presence of eleven new 4-MePro-containing nonribosomal cyclic depsipeptides, nostoweipeptins and nostopeptolides, from two *Nostoc* strains. On the other hand, spumigins J and A are 4-MePro-containing linear peptides that were extracted from the freshwater cyanobacterium *Anabaena compacta*, characterized, and examined for thrombin and cathepsin B inhibitory activities.[67]

To evaluate the effect of 4-MePro on peptide stability, other cyclic peptides have been investigated. Griselimycin (GM) is a natural cyclic peptide that was isolated from *Streptomyces* a half century ago, and developed by Rhône-Poulenc as an antituberculosis drug. Metabolic studies identified the Pro residue in the 8 position as a main site of metabolic reactions. Kling et al. reinitiated studies on GM, and increased the metabolic stability by alkylation of 8-Pro; the resulting methyl GM (MeGM) and cyclohexyl GM (CGM), obtained by total synthesis, showed improved pharmacokinetic properties.[68]

A54556 cyclic acyldepsipeptides are antibacterial natural products that have been isolated from *Streptomyces hawaiiensis*. Novel analogues of A54556, containing a 4-MePro residue, have been synthesized and evaluated against a variety of Gram-positive and Gram-negative bacteria.[69] Optimization of the macrocyclic core residues and the *N*-acyl side chain of the peptides led to the development of a lead analogue that showed potent activity against all Gram-positive strains tested.

6. Dehydroprolines

Dehydroprolines (DHPs), proline analogues possessing a carbon–carbon double bond in the pyrrolidine ring, may be used as intermediates for the preparation of functionalized prolines or may be introduced into peptides to induce conformational restraints. The specific properties depend, of course, on the position of the unsaturated bond.

Traces of 3,4-dehydroproline (3,4-DHP) have been found in Sandal leaves (*Santalum album L.*), together with *cis*- and *trans*-4-Hyp.[70] The detected amount changes depending on the season of sample collection, and it has been proven that, in this environment, the amino acid has a short half-life. This suggests a role as intermediate in the biosynthesis of the more abundant 4-Hyp or as a metabolite of the parent compounds. On the other hand, 3,4-DHP has been identified as a lead compound for the control of fire blight, a disease occurring in apples and pears, that leads to significant losses in fruit production. The disease is caused by the Gram-negative bacterium *Erwinia amylovora*. An SAR study was developed by Sarojini and co-workers, by testing the antibacterial activity of a library of heterocyclic compounds selected for their similarity to quorum-sensing signalling molecules in Gram-negative

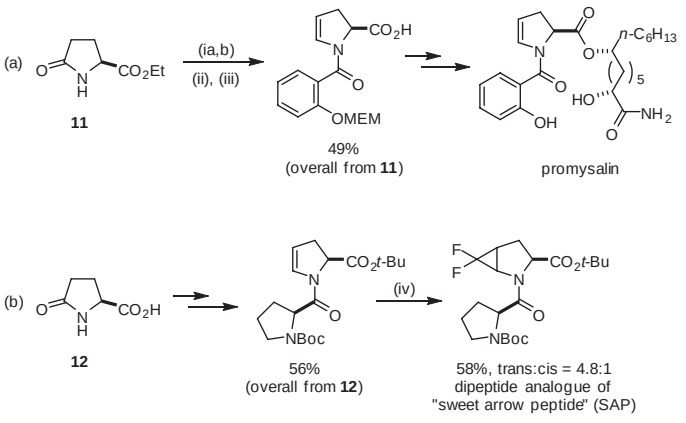
bacteria.[71] Among them, 3,4-DHP showed excellent in vitro and good in vivo inhibition of biofilm formation, as compared to streptomycin. Decreased or loss of activity was observed for pyrrolidine and other derivatives, indicating that the complete structure of 3,4-DHP is essential for bioactivity.

Although the substitution of proline with DHP in proteins or bioactive peptides has been performed in the past with the aim of modifying their chemical, physical, and biological properties, a limited number of studies on the conformational preference of 3,4-DHP have been reported. Kang and Park have performed a computational investigation on 3,4-DHP-containing dipeptide models to verify the conformational preferences and the cis–trans isomerization of this molecule in the gas phase and in chloroform solution.[72] The study suggested that 3,4-DHP is considerably less puckered than the prolyl ring, and the barriers for cis–trans isomerization in different environments are considerably different from the corresponding ones for proline. It also suggested that 3,4-DHP appears to favor a PPII-like helical conformation in a nonpolar environment.

4,5-DHP, another unsaturated proline analogue, has been found in bioactive compounds such as promysalin, an antimicrobial agent that is isolated from *Pseudomonas putida* and which exhibits a remarkable spectrum of growth inhibition activity in other *Pseudomonas*. The total synthesis of this novel antibiotic has been reported by Musso's group, who employed a straightforward approach to obtain the 4,5-DHP-containing fragment (**Scheme 10**, Part (a)).[73] Kubyshkin et al. exploited the same approach to obtain 4,5-DHP for the preparation of 4,5-difluoromethanoprolines, which can exert a substantial influence on the three-dimensional structure of proteins. The crucial step of this synthesis was the difluorocyclopropanation through the in situ generated difluorocarbene (Scheme 10, Part (b)).[74]

7. Conclusion and Outlook

In the past few years, the unique conformational properties of 4-substituted prolines and the possibility of exploiting the substituent at C-4 for further functionalization have offered new research opportunities in synthetic and medicinal chemistry, as well as in catalysis and chemical biology. Examples include interesting transformations of the pyrrolidine ring that lead to polyfunctionalized cores of bioactive products, the successful use of 4-substituted proline derivatives in enantioselective organocatalysis, and the significant influences of these ncAAs on the structures and properties of modified peptides. In particular, the commercially available 4-OH and 4-F amino acids have been successfully introduced into bioactive sequences to modulate their binding affinity to proper receptors and to enhance their physicochemical properties, providing new insights in the field of protein–protein interaction. Moreover, conjugation of peptides to nanoparticles or site-selective chemical ligation via Pro branches opens new avenues in materials science and medicinal chemistry.



(i) (a) 2-MEMC$_6$H$_4$CO$_2$H, Ghosez's reagent, CH$_2$Cl$_2$, 0 °C–rt, 1 h; (b) Et$_3$N, PhMe, 80 °C, 3 h; 82%. (ii) (a) LiBHEt$_3$, PhMe, –78 °C, 1 h; (b) TFAA, DIPEA, DMAP (cat.), –78 °C–rt, 3 h; 62%; (iii) LiOH, EtOH–H$_2$O, 0 °C–rt, 5 h; 97%; (iv) F$_2$ClCCO$_2$Na (27 equiv), diglyme, 177 °C

Scheme 10. Synthesis and Reactivity of 4,5-Dehydroproline (4,5-DHP). *(Ref. 73,74)*

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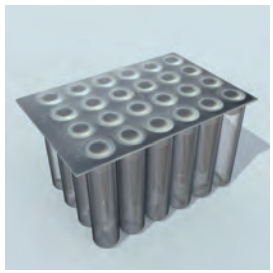

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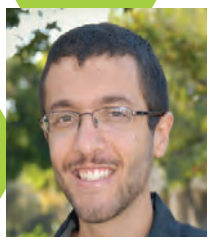
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Abstract. Every year, advances in the design of metathesis catalysts and insightful strategic applications of alkene metathesis work in concert to drive the field into new and exciting directions. From ring-closing to enyne and cross-metathesis, and from late-stage steps that directly furnish natural products to early transformations that supply starting materials, metathesis can play a role at every stage of a synthesis. This review will highlight some of the particularly innovative or surprising ways in which alkene metathesis has been implemented in natural product synthesis.

Outline

1. Introduction

The importance of alkene metathesis to synthetic chemistry cannot be overstated. The pioneering work by Chauvin, Grubbs, and Schrock on improving our mechanistic understanding of metathesis, on developing novel metathesis catalysts, and on developing metathesis applications garnered them the 2005 Nobel Prize in Chemistry.[1] For over two decades, organic chemists have harnessed the power of alkene metathesis in academic and industrial settings, and new applications continue to be reported on a regular basis. In the decade following Nicolaou's extensive review of the use of alkene metathesis in natural product synthesis,[2] the number of examples of such uses has grown at a dizzying rate. Books[3–7] and reviews[8–11] have been written to chronicle the breadth of applications of alkene metathesis. Herein, we aim to provide a structured look at a select group of alkene metathesis reactions that are employed in natural product synthesis. We discuss accomplishments from the past ten years that exemplify groundbreaking strategic applications of alkene metathesis and/or a particularly impressive reactivity or selectivity in metathesis processes. We have organized the discussion by transformation type: (i) ring-closing metathesis for normal- and medium-size rings, (ii) metathesis in the synthesis of macrocycles, (iii) tandem metatheses, (iv) ring-opening metathesis, and (v) cross-metathesis.

We note here that our purpose is only to demonstrate the power of metathesis for complex-molecule synthesis using select examples.

Furthermore, although closely related, alkyne metathesis[12] is not a prime focus of this article, and select examples are provided only to provide context. In this short review, only a small number of the myriad and incredibly versatile metathesis catalysts developed to date are showcased (**Figure 1**).

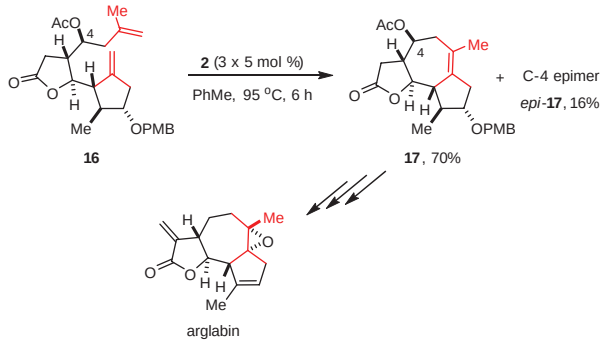
2. Ring-Closing Metathesis

By the mid-2000s, ring-closing metathesis (RCM) to form unstrained, normal-size rings was a well-established, reliable tool for synthesis. The examples below were selected because they have pushed forward the frontiers of what was thought possible in terms of reactivity and/or selectivity.

Reiser's group reported the first enantioselective synthesis of the complex sesquiterpenoid arglabin (**Scheme 1**).[13] A challenging RCM of two 1,1-disubstituted alkenes to forge a tetrasubstituted alkene within a 7-membered ring serves as a key step. A particularly direct, stereoselective synthesis of the RCM precursor, **16**, set the stage for this challenging ring closure. This metathesis required three separate charges (5 mol %) of the Grubbs second-generation catalyst[14] (**2**) and inert-gas sparging at 95 °C to successfully provide the tetrasubstituted alkene in **17**. Epoxidation of the tetrasubstituted alkene and installation of the requisite functional groups completed the synthesis of arglabin. At the time, and to this day, this RCM is striking for its efficient generation of a ring size that can often be slow to form, while simultaneously forging a tetrasubstituted alkene.

Stoltz's group was among the first to employ the RCM of alkenyl chlorides in natural product synthesis in the course of their innovative synthesis of elatol.[15] The authors built on Weinreb's earlier work which had demonstrated the general feasibility and utility of this type of RCM.[16] To access the salient chlorinated cyclohexene of elatol, the researchers utilized an RCM between two 1,1-disubstituted alkene groups in **18** (**Scheme 2**), one with two carbon substituents and one bearing an alkyl substituent and a chlorine atom. Substrate **18** underwent RCM in the presence of **3** to provide intermediate **19** containing a tetrasubstituted alkene. Following its introduction, catalyst **3** has become an important addition to the arsenal of available metathesis catalysts.[3,17]

In one of a few reported examples of stereochemical equilibration–RCM, Tang, Chen, Yang and co-workers reported the synthesis



Scheme 1. Efficient Formation by RCM of a Cycloheptene with a Tetrasubstituted Double Bond in Reiser's Enantioselective Synthesis of Arglabin. *(Ref. 13)*

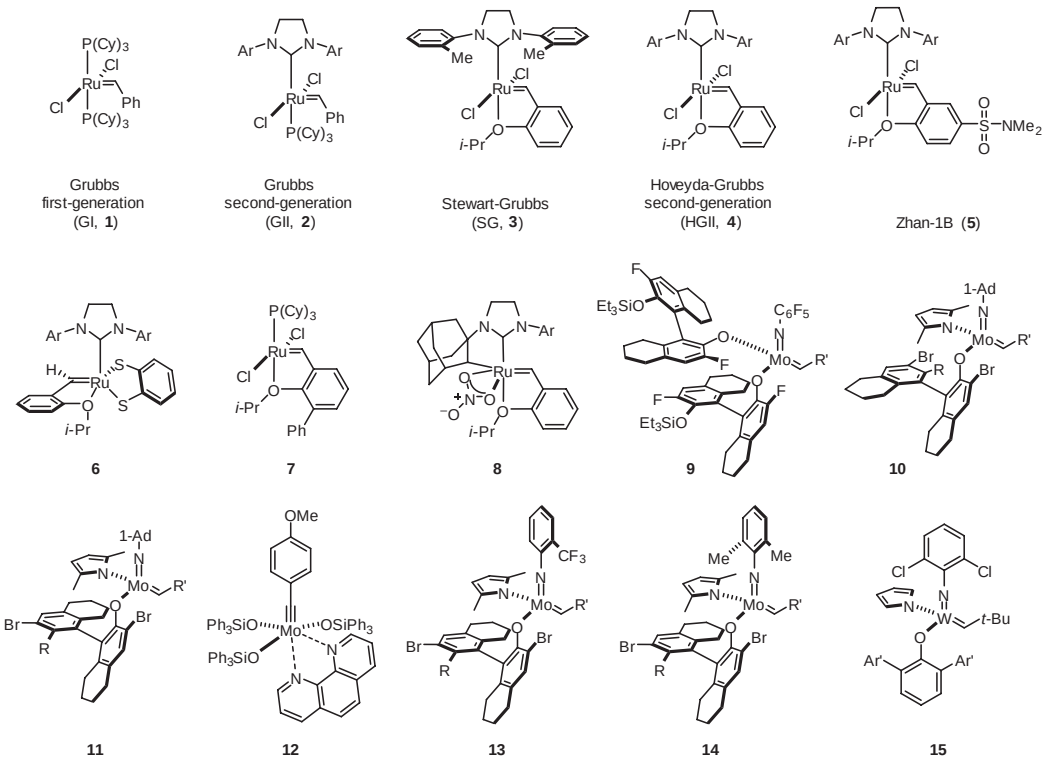
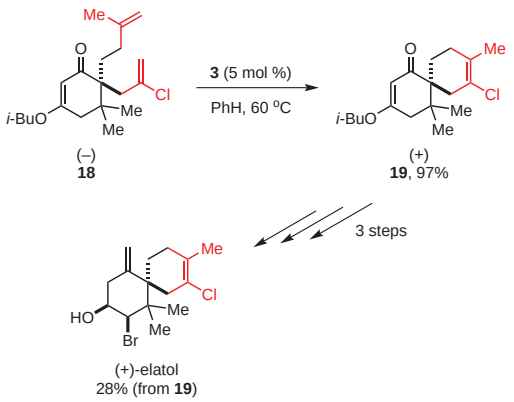


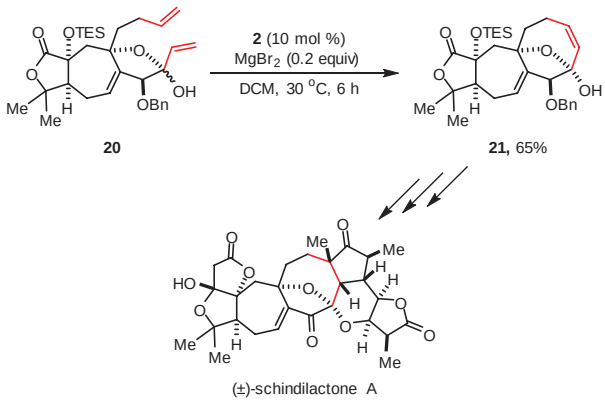
Figure 1. Structures of Metathesis Catalysts Featured in This Review.

of schindilactone A, in which that process provided the central oxabicyclo[4.2.1]nonane ring system of the target (**Scheme 3**).[18] In the presence of GII (**2**), the desired RCM gave **21**, with in situ epimerization producing a single diastereomer at the hemiketal carbon.[19] The resulting cyclooctene later participated in a Pauson–Khand reaction to annulate another one of the rings of the natural product. Other examples of stereochemical equilibration during RCM mostly involve epimerization of stereogenic centers adjacent to ketones.[20]

In their synthesis of sculponeatin N, a bioactive diterpene, Thomson and co-workers accomplished the equivalent of a butadiene–cyclopentenone Diels–Alder cycloaddition by a sequence featuring an unusual equilibrating diastereoselective RCM reaction (**Scheme 4**).[21] When **24** could not be made by the more straightforward Diels–Alder approach, sequential alkene installation converted the spiro-cyclopentenone precursor to the triene-containing RCM substrate **22**. Subjecting triene **22** to metathesis conditions with GII (**2**) afforded the desired cis-fused cyclohexene-containing **24**. The authors commented that spirocyclopentene **23** could be isolated by stopping the reaction early; however, the trans-fused cyclohexene was never observed. This particularly creative workaround to the unsuccessful cycloaddition also sets a key quaternary stereogenic center of the target.



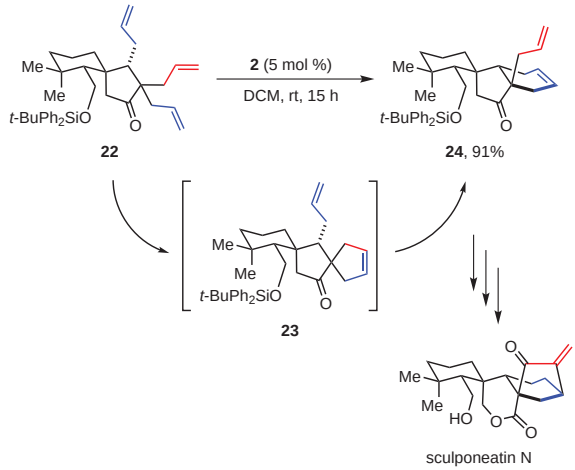
Scheme 2. First Application of the RCM of Alkenyl Chlorides in Natural Product Synthesis as Demonstrated in Stoltz's Asymmetric Total Synthesis of (+)-Elatol. *(Ref. 15)*



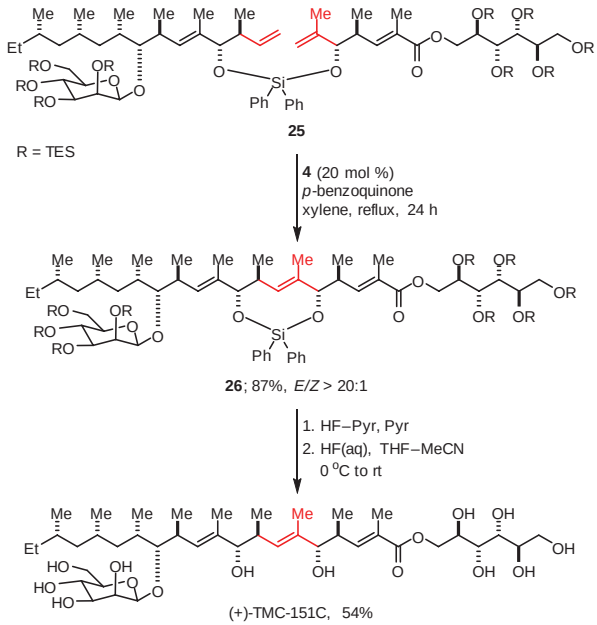
Scheme 3. Yang's Diastereoselective Synthesis of the Fully Functionalized CDE Ring System in (±)-Schindilactone A by RCM. *(Ref. 18)*

3. Tethered Ring-Closing Metathesis

Temporary tethers are enormously useful for increasing the rates of slow bimolecular reactions, and are advantageous with respect to both chemo- and stereoselectivity. In Kobayashi's total synthesis of (+)-TMC-151C, a silicon-tethered RCM reaction convergently assembled the polyketide natural product from two fragments of similar complexity in a reaction that would have been virtually impossible to achieve in a bimolecular setting (**Scheme 5**).[22] Further, the tether served to reinforce the selectivity of the alkene geometry in forming the trisubstituted alkene. The RCM reaction of **25** was effected with the Hoveyda–Grubbs second-generation catalyst (**4**),[23] and global desilylation of **26** afforded (+)-TMC-151C directly.



Scheme 4. Diastereoselective RCM as a Butadiene–Cyclopentenone Diels–Alder Equivalent in Thomson's Total Synthesis of Sculponeatin N. *(Ref. 21)*



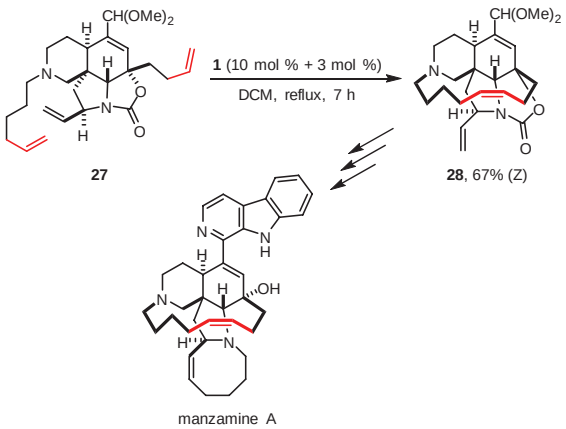
Scheme 5. Silicon-Tethered RCM in Kobayashi's Convergent Total Synthesis of (+)-TMC-151C. *(Ref. 22)*

4. Macrocycles

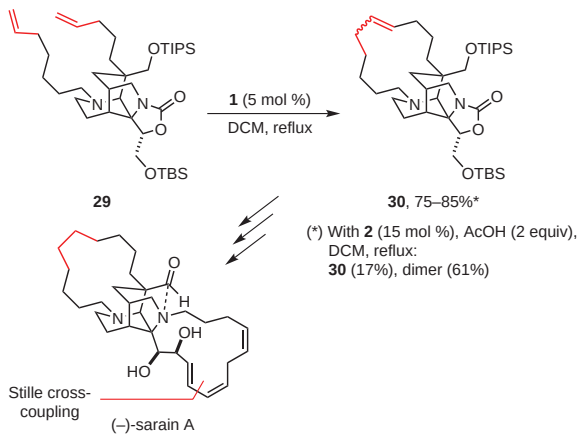
4.1. Macrocyclic Ring Closure by Metathesis

4.1.1. The Alkylpiperidine Alkaloids

A number of structurally related alkaloids, including the manzamines,[24a] sarains,[24b] haliclonacyclamines,[24c] and haliclonins,[24d] belong to the family of alkylpiperidine natural products. In addition to an alkylpiperidine subunit, these secondary metabolites usually contain one or more macrocycles. The synthesis of the macrocycle(s) of the various alkylpiperidines has been accomplished by ring-closing alkene or alkyne metathesis numerous times, by employing various strategies aimed at accomplishing the transformation selectively for the challenging *Z*-configured alkene present in many of the natural products. In 1999, Martin and co-workers were the first to utilize a macrocyclizing RCM reaction in the synthesis of an alkylpiperidine natural product (**Scheme 6**).[25] Since then, few have achieved the same level of substrate-controlled *Z*-selectivity Martin's group observed for the conversion of **27** to **28**, without the use of modern *Z*-selective metathesis catalysts. In the past decade, a number of syntheses of alkylpiperidine natural products relying on RCM reactions for macrocyclic ring-closure have been reported.



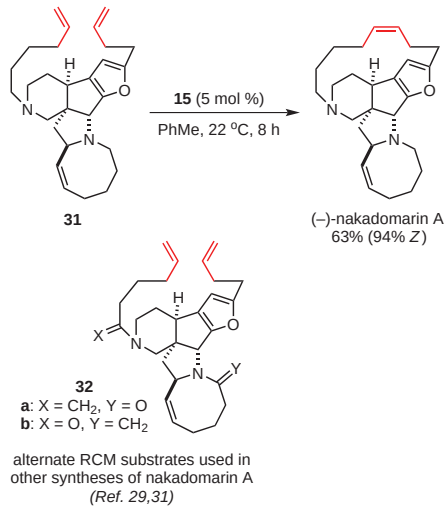
Scheme 6. Macrocycle Ring Closure by Metathesis, as Illustrated in Martin's Total Synthesis of Manzamine A, an Alkylpiperidine Alkaloid. *(Ref. 25)*



Scheme 7. Macrocycle Ring Closure by Metathesis in Overman's Total Synthesis of (–)-Sarain A. *(Ref. 26)*

Overman's group reported the first total synthesis of the incredibly complex alkaloid sarain A, which exhibits antibacterial, insecticidal, and antitumor activities. In this work, the saturated 13-membered-ring of the natural product was formed via RCM and subsequent hydrogenation (**Scheme 7**),[26] while the second, 14-membered ring of sarain A containing the skipped-triene was ultimately constructed by a Stille cross-coupling, and not by metathesis. In the RCM step to generate the saturated macrocycle, the use of catalyst **2** with **29** led to significant quantities of dimeric byproducts, in addition to some of the desired macrocyclization product, **30**. Switching to the less active, first-generation Grubbs catalyst (**1**)[27] diminished the amount of dimer formed and, after optimization, a 75–85% yield of the macrocyclization product, **30**, was obtained. It was postulated that the use of the more active catalyst **2** gave a thermodynamic ratio of products, as supported by subjection of either the isolated macrocycle or dimer to the same reaction conditions, which yielded in each case a similar mixture of macrocycle and dimer. The less active **1**, however, may have been unable to initiate metathesis on the resultant internal alkene, making the RCM step effectively irreversible, and providing only the desired RCM product.

Nishida's first-generation synthesis of nakadomarin A[28] was published in 2003, and, since then, many other groups have contributed impressive syntheses of their own. The *Z*-alkene-containing, 15-membered ring of nakadomarin A has been constructed by ring-closing alkene metathesis,[28–32] ring-closing alkyne (RCAM) metathesis and semi-reduction,[33–34] and by macrolactamization.[35] In their pioneering work, Nishida and co-workers achieved a *Z:E* ratio of 1:1.8 by using catalyst **1** to close the 15-membered ring from **32a** (**Scheme 8**). Subsequent syntheses by the groups of Kerr,[29] Dixon,[30] and Zhai,[31] using either substrate **32a**, **32b**, or **31** resulted in only slightly improved *Z:E* ratios (up to 2:1). Nilson and Funk[33] were the first to employ a two-step RCAM–semi-reduction to afford the *Z* alkene as the sole product. Dixon's group embraced this approach as well,[34] but later reported a collaborative effort with the Schrock and Hoveyda labs, making use of the recently developed *Z*-selective, tungsten-based metathesis catalyst



Scheme 8. Final Step in the Synthesis of (–)-Nakadomarin A by Catalyst-Controlled *Z*-Selective RCM. *(Ref. 36)*

15.[36] Use of this catalyst with **31** produced (–)-nakadomarin A in 63% yield and 94% *Z*-selectivity, the highest *Z*-selectivity observed in a synthesis of nakadomarin A by RCM.

Huang and co-workers reported the first asymmetric total synthesis of the alkaloid (–)-haliclonin A using catalyst **1** for RCM, followed by hydrogenation, to form the saturated macrocycle of the molecule.[37] At a later point in the synthesis, an RCAM with molybdenum benzylidyne catalyst **12**[38] closed the second, unsaturated, 15-membered ring of compound **33**, affording intermediate **34**. Partial hydrogenation installed the desired *Z*-alkene geometry (**Scheme 9**).[37] It is worth noting that the alkyne metathesis and partial hydrogenation steps were both tolerant of the unconjugated alkene, and no isomerization was noted.

4.1.2. The Ansatrienins

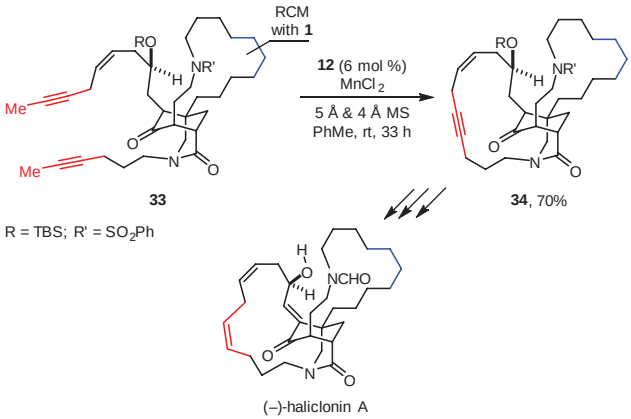
The ansatrienins,[39a-c] a subclass of the ansamycins,[39] bear an all-trans conjugated triene subunit in their macrocyclic core. Cross-coupling[40] and alkenylation[41] approaches to this triene fragment have been successfully employed in syntheses of some of the members of this class of natural products. The application of an RCM between two diene units to furnish an all-trans triene—an approach that certainly would appear to harbor risk—was central to two successful syntheses.[42–43] Hayashi and co-workers reported an asymmetric total synthesis of the anticancer drug (+)-cytotrienin A, where RCM successfully provided the macrocyclic ring containing the all-trans triene (**eq 1**).[42] Krische's group rapidly assembled a related tetraene-containing RCM substrate using their C–H functionalization methodology, and completed the synthesis of trienomycins A and F (not shown).[43]

4.1.3. Other Natural Products

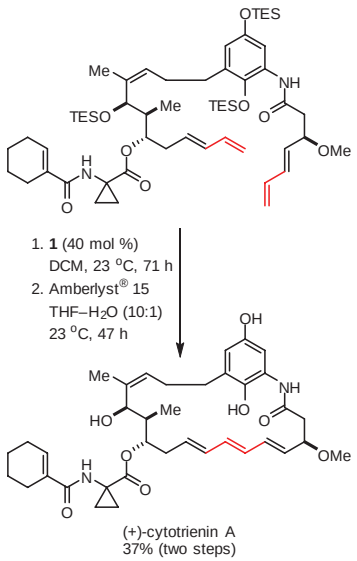
Krische's group utilized a ring-closing enyne metathesis (RCEYM) to form the macrocyclic ring in their total synthesis of 6-deoxyerythronolide B (**Scheme 10**).[44] Some of the challenges that were overcome in this synthesis include: (i) Terminal alkene isomerization from **35** was the only observed product in the absence of ethylene. (ii) Enyne metathesis proceeded at 80 °C, converting the alkyne into a terminal diene but not into the macrocycle. To overcome this second challenge, the ethylene atmosphere was replaced with nitrogen after the enyne metathesis was complete, and the reaction mixture was then heated to 110 °C, accomplishing the desired ring closure. The macrocyclic diene **36** was then converted in a few steps into 6-deoxyerythronolide B. Together with Krische's efficient methods for assembling the precursor, this RCEYM process was strategically advantageous.

In their synthesis of (+)-neopeltolide, Fuwa and co-workers utilized a chemoselective cross-metathesis (CM) to form an α,β-unsaturated ester from a terminal alkene and methyl acrylate, ultimately facilitating pyran formation by an oxa–Michael cyclization (**Scheme 11**).[45] Since the pendant styrenyl group in substrate **37** was also poised to competitively undergo an undesired RCM, the ability of the proximal hydroxyl group to form an intramolecular H….Cl interaction between the OH and the Cl in the substrate-bound catalyst was key to accomplishing the selective CM leading to **38**. When the proximal OH was protected with a BOM group, a significant amount (46–71%) of the ring-closed product was formed. Finally, RCM of intermediate **39** with catalyst **2** afforded the macrocyclic, trisubstituted alkene **40**, which was then hydrogenated to the saturated macrolactone, constituting a formal synthesis of (+)-neopeltolide. Hoveyda, Schrock, and Yu also reported the synthesis of (+)-neopeltolide, making extensive use of alkene metathesis to construct the natural product (not shown).[46]

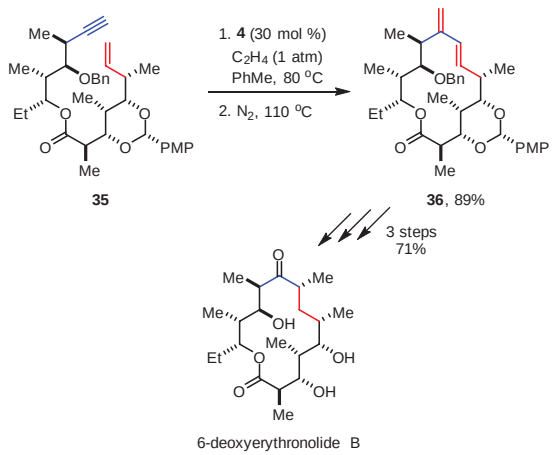
Kita and Kigoshi reported an asymmetric total synthesis of the marine macrolides mycalolides A and B, and evaluated both an RCM



Scheme 9. Ring-Closing Alkyne Metathesis (RCAM) Step in Huang's Asymmetric Total Synthesis of (–)-Haliclonin A. *(Ref. 37)*



eq 1 *(Ref. 42)*



Scheme 10. Ring-Closing Enyne Metathesis (RCEYM) in Krische's Total Synthesis of the Polyketide 6-Deoxyerythronolide B. *(Ref. 44)*

and a CM approach (**Scheme 12**).[47] In their work, the RCM strategy suffered from low selectivity for the desired alkene geometry; even after extensive optimization, it proceeded in only a 63% yield and an *E:Z* ratio of 2.7:1. The CM approach was more successful; the reaction of **41** with **42** proceeded to give **43** in 77% yield and an *E:Z* ratio of 5:1. Although adhering to the rules[48] for best-case CM substrates (Type 1 and Type 2 alkenes), this CM is remarkable for the extreme complexity of both reaction partners used in close to equimolar amounts.

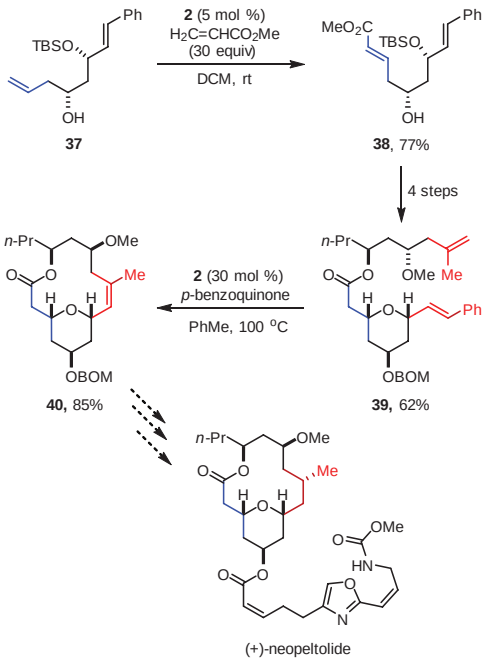
Krische and co-workers synthesized swinholide A using their asymmetric, hydrogen-mediated C–C bond-forming methodology and alkene metathesis each at multiple stages (**Scheme 13**).[49] An enantioselective, iridium-catalyzed allylation provided product **46**, which underwent CM with acrolein catalyzed by **2** to afford dihydropyran hemiacetal **47**; this product was elaborated into a key fragment for convergent coupling. Construction of a second fragment began with a diastereo- and site-selective iridium-catalyzed allylation to supply **48**, which was subjected to CM with *cis*-1,4-diacetoxy-2-butene. The resulting allylic acetate **49** underwent palladium-catalyzed Tsuji–Trost cyclization to give a *cis*-2,4-disubstituted vinyl tetrahydropyran **50**. The two fragments were elaborated and coupled, yielding the final metathesis substrate, **51**. In the presence of **4**, intermediate **51** was converted, via sequential CM–RCM, into the dimeric macrodiolide swinholide A in 25% yield, as well as via an RCM of the monomer, into the macrolide hemiswinholide in 43% yield. This final step that directly affords two natural products is striking for its efficiency in the presence of two other alkenes, a host of unprotected hydroxyl groups, and numerous other Lewis basic sites.

4.1.4. Tiacumicin B Aglycon

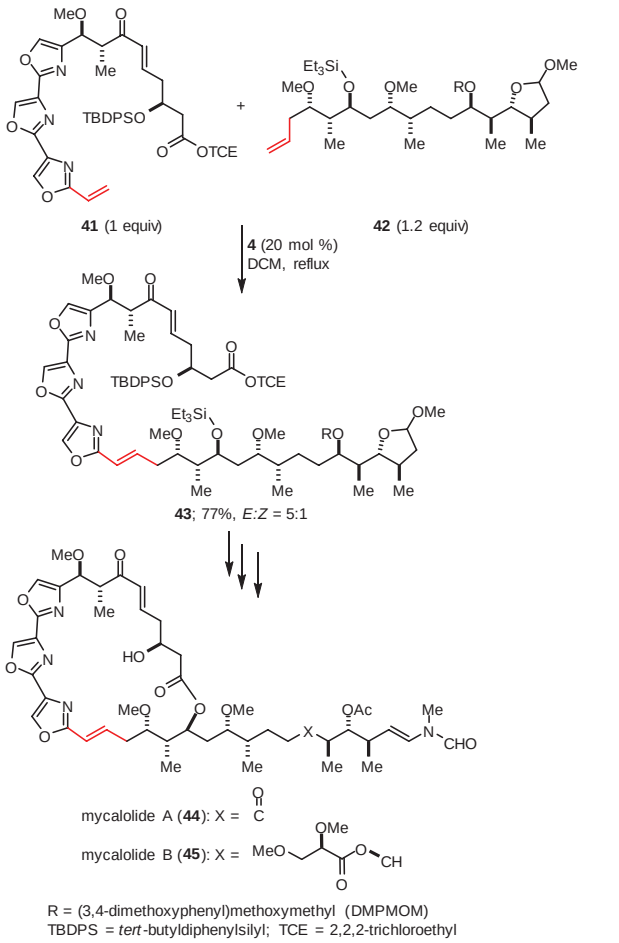
In 2015, three research groups concurrently reported syntheses of tiacumicin B, with each group utilizing alkene metathesis in their synthesis (**Scheme 14**).[50–52] Zhu and co-workers targeted a protected form of the tiacumicin B aglycon,[50] whereby ester-linked ring-closing metathesis precursor **52a** underwent the desired RCM macrocyclization in the presence of **2**, with only deprotection required to complete the synthesis. Gademann's group targeted the protected tiacumicin B aglycon and utilized a macrocyclic RCM similar to that used in Zhu's synthesis, closing the diene fragment of **52b**.[51] Gademann's substrate underwent a more efficient ring closure, reminding us of the significant effect that different protecting group strategies can have on macrocyclization by RCM (and indeed by any method). The authors also reported a procedure to isomerize the undesired *Z* alkene to the *E* alkene allowing material to be recycled. Altmann's group reported a synthesis of the tiacumicin B aglycon, in which they employed a CM to assemble the linear precursor to the natural product.[52] Complex **4** catalyzed the synthesis of tetraene fragment **53**, attaining the highest *E/Z* ratio of the three syntheses (6.7:1). Yamaguchi esterification with a vinyl boronate containing fragment and Suzuki macrocyclization completed the synthesis.

4.2. Macrocyclic Ring Closure by Other Means

Hoye and co-workers synthesized (+)-peloruside A—a cytotoxic marine macrolide that is being evaluated for use against paclitaxel-resistant cancers—by utilizing a relay RCM between the tethered alkenes to



Scheme 11. CM and RCM Key Steps in Fuwa's Concise Total Synthesis of (+)-Neopeltolide. *(Ref. 45)*

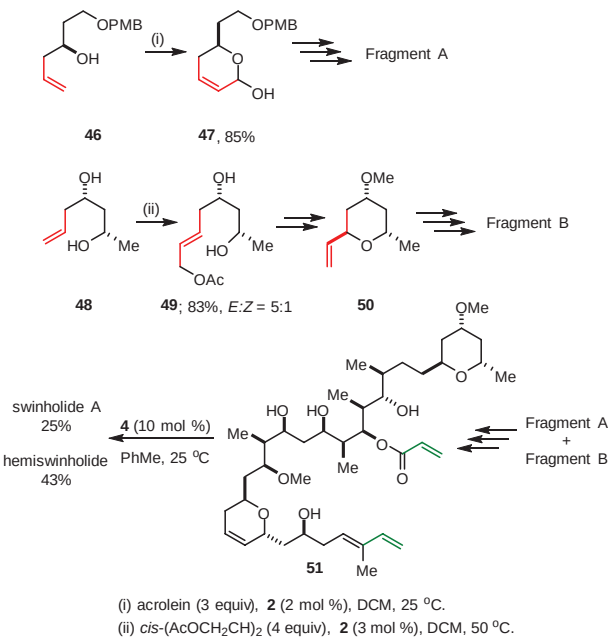


Scheme 12. CM between Two Advanced Intermediates in Kita and Kigoshi's Total Synthesis of Mycalolides A and B. *(Ref. 47)*

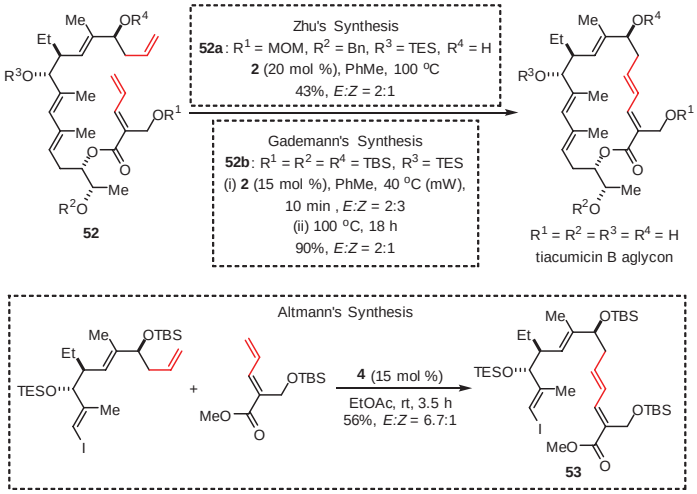
construct the trisubstituted alkene group present in the natural product (**Scheme 15**).[53] Two tethering approaches, one via a silaketal (**54**) and the other via an ester linkage (**55**), were investigated. Each tether contained an (*R*)-citronellene derived tail, initially incorporated to enable efficient enzymatic resolution of the diastereomeric mixtures of alcohols. This tail was cleaved during the metathesis step and the two routes converged to a substrate with differentially protected alcohol groups. This fragment was coupled to a polyol fragment via an aldol addition, after which a macrolactonization–deprotection sequence completed the synthesis of peloruside A.

Volchkov and Lee completed the asymmetric total synthesis of (–)-amphidinolide V by employing metatheses at multiple stages in the synthesis (**Scheme 16**).[54] RCEYM of silyl-tethered enyne **56** formed the desired silacycle **57**, which was ring-opened and coupled to provide polyene **58**. RCM of the latter compound led to an 8-membered silacycle (**59**). Subsequent elaboration, including allylic transposition, gave a fragment corresponding to roughly half of the target. Acetylenic intermediate **60** was subjected to enyne metathesis with ethylene, catalyzed by **2** to afford the salient 1,3-diene, **61**. Ultimately, fragments **59** and **61** were combined to complete the synthesis of (–)-amphidinolide V. Another member of the amphidinolide family, (–)-amphidinolide K, was synthesized by Lee and co-workers, whose work featured strategic use of enyne metathesis (not shown).[55]

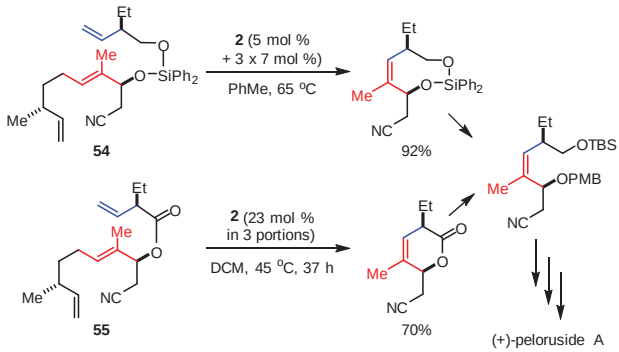
In their total synthesis of disorazole C_1, an antifungal and anticancer macrocyclic natural product, Hoveyda and co-workers employed a number of metathesis steps to tackle the construction of the C_2-symmetric dimeric macrocycle (**Scheme 17**).[56] The disorazole C_1 ring contains two conjugated triene units, within which are found four *Z*-configured carbon–carbon double bonds. The convergent, stereoselective route employed required the RCM of a *Z*-vinyl iodide tethered to a *Z*-vinylborane in the precursor **68**, which was assembled with the help of a number of *Z*-selective cross-metathesis steps. A



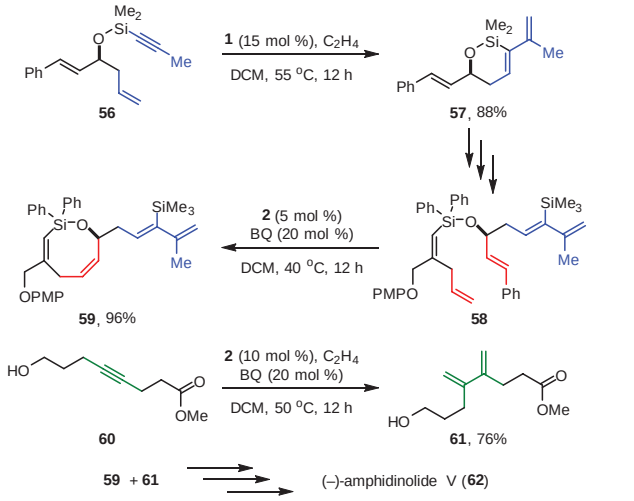
Scheme 13. CMs and CM–RCM Sequences Employed in Krische's Total Synthesis of the Actin-Binding Marine Polyketide Swinholide A. *(Ref. 49)*



Scheme 14. RCM and CM in the Synthesis of Tiacumicin B Aglycon. *(Ref. 50–52)*



Scheme 15. Relayed, Tethered RCMs in Hoye's Total Synthesis of Peloruside A. *(Ref. 53)*



Scheme 16. Enyne Metatheses and RCM in Lee's Asymmetric Total Synthesis of (–)-Amphidinolide V. *(Ref. 54)*

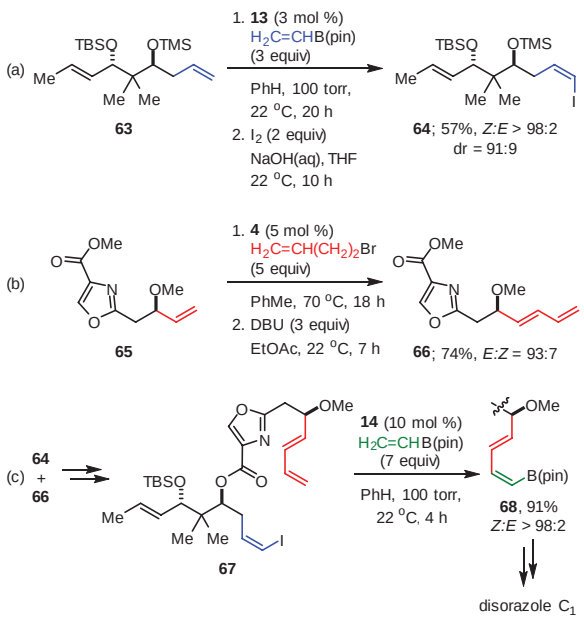
double Suzuki cross-coupling strategy served to dimerize compound **68**, affording less than 2% of the unimolecular cross-coupling product. A careful deprotection led to completion of the synthesis of disorazole C_1.

5. Tandem Metatheses

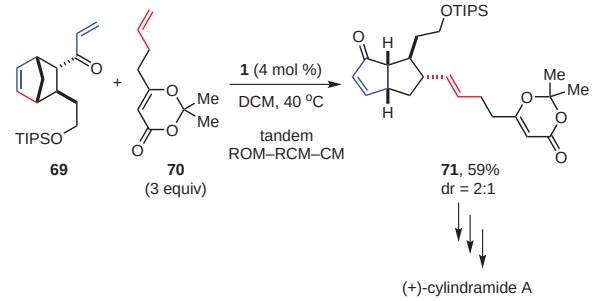
In their synthesis of (+)-cylindramide A, Hart and Phillips developed a tandem ROM–RCM–CM sequence to rearrange a bicyclo[2.2.1]-heptene into the bicyclo[3.3.0]octene core of (+)-cylindramide A (**eq 2**).[57] In the presence of GI (**1**) and the butenyl-substituted dioxinone **70**, the metathesis cascade substrate **69** was transformed into the desired bicyclo[3.3.0]octene **71** with incorporation of the butenyl-substituted dioxinone. This work showcases how the reliably predictable stereochemical relationships generated in the course of Diels–Alder reactions can be transferred to very different ring topologies via tandem metathesis chemistry.

5.1. Enyne Metatheses

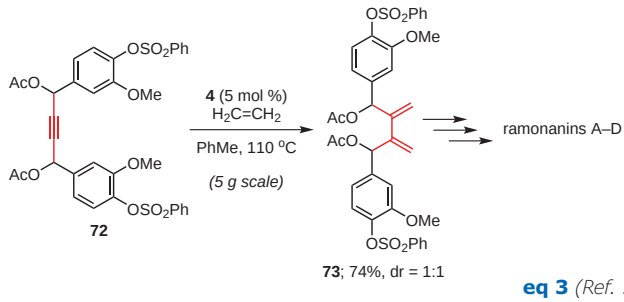
Ramonanins A–D are spirocyclic phenylpropanoid tetramers that show cytotoxic activity against lines of human breast cancer cells. In the first total synthesis of these lignan natural products, Sherburn's group. targeted a dimethylene tetrahydrofuran intermediate that could be dimerized to a mixture of the different ramonanins (**eq 3**).[58] Starting from vanillin, the authors arrived at **72**, the alkyne-bridged diacetate substrate for enyne metathesis, after four steps. Enyne metathesis with ethylene, catalyzed by **4**, furnished the diene diacetate **73**. Hydrolysis of the diacetate, tetrahydrofuran ring formation from the resultant diol, and cleavage of the benzenesulfonate protecting groups afforded the desired dimerization precursor, which was taken to generate the natural product targets by intermolecular Diels–Alder cycloadditions.



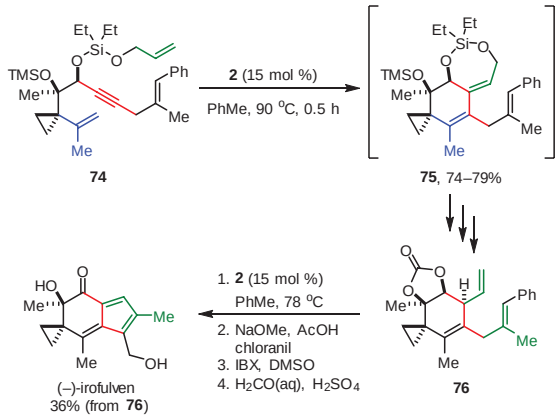
Scheme 17. *Z*-Selective CM and RCM in Hoveyda's Convergent, Diastereoselective, and Enantioselective Total Synthesis of Disorazole C_1. *(Ref. 56)*



eq 2 *(Ref. 57)*



eq 3 *(Ref. 58)*



Scheme 18. RCEYEM and RCM in Movassaghi's Enantioselective Total Synthesis of (–)-Irofulven. *(Ref. 59)*

5.2. Ene-yne-ene Metatheses

Tandem, ring-closing ene-yne-ene metathesis (RCEYEM) sequences are powerful for the construction of bicyclic ring systems and have been applied in a number of different natural product syntheses in the past decade.

Movassaghi's group reported particularly non-obvious metathesis-based approaches to the semisynthetic illudin derivatives (–)-acylfulvene and (–)-irofulven, the latter being an especially active antitumor agent against a variety of solid tumors (**Scheme 18**).[59] Silyl-tethered RCEYEM substrate **74** underwent the desired metathesis cascade catalyzed by GII (**2**) providing **75**, which was then converted into **76** via a reductive allylic transposition. The resulting intermediate **76** underwent RCM in the presence of **2** to form the cyclopentane ring of the illudins. Oxidation with DDQ or chloranil and IBX (*ortho*-iodoxybenzoic acid) furnished (–)-acylfulvene (not shown), and reaction of (–)-acylfulvene with aqueous formaldehyde provided (–)-irofulven.

Spectacular use of RCEYEM was demonstrated in the enantioselective total synthesis of three tetracyclic kempene diterpenes (**Scheme 19**).[60] Tandem RCEYEM substrate **77**, when heated in the presence of GII (**2**), gave rise to intermediate **78**, possessing the tetracyclic core of the kempenes. The key to the success of this complexity-building transformation was the substitution pattern of each of the unsaturated reaction partners, which was carefully considered so that the order of reaction was the proper one to yield the desired product outcome. Following the synthesis of **78**, protecting group exchange, ketone reduction, and acylation afforded the kempene natural products.

Yang, Li, and co-workers reported the stereoselective total syntheses of the alkaloids (–)-flueggine A and (+)-virosaine B, derived in a biomimetic fashion from (–)-norsecurinine and (+)-allonorsecurinine, which were each constructed via relay RCEYEM.[61] An *N*-Boc-protected, commercially available, D-proline-derived Weinreb amide served as the starting material to construct the RCEYEM substrate possessing a heptadienoate chain. This tethering strategy successfully controlled the direction of ring closure in the cascade process. Both diastereomers of the RCEYEM substrate could be successfully carried through the reaction sequence to furnish both (–)-norsecurinine and (+)-allonorsecurinine, which were ultimately converted into their more complex relatives.

In 2016, Smith's group reported a total synthesis of (±)-morphine that makes use of an RCEYEM sequence (**Scheme 20**).[62] In the presence of catalyst HGII (**4**), the desired RCEYEM proceeded to give the intermediate tetracycle **81**, which, after amine deprotection, underwent an intramolecular 1,6-addition forming **82**, a final reduction away from morphine.

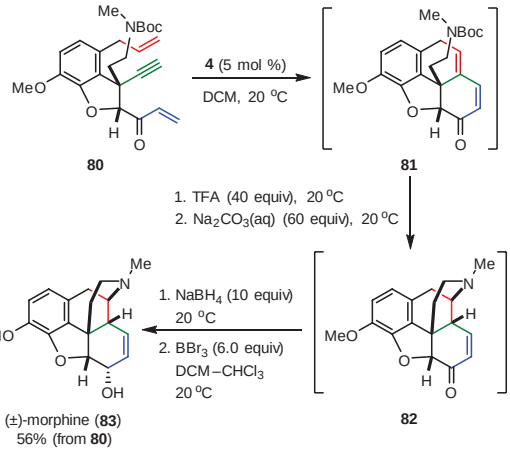
6. Ring-Opening Cross-Metathesis

A collaborative synthesis of the potent antifungal agent (±)-hippolachnin A has been disclosed by the groups of Wood and Brown. This complex natural product has an unusual structure that features six contiguous stereocenters, a quaternary center, and a congested compact core. The two groups had arrived at similar and "complementary" approaches and sought to design a collaborative synthesis playing to the strengths of each of their separate syntheses. In their combined strategy, the [2 + 2] photocycloaddition of quadricyclane and an α,β-unsaturated acid chloride ultimately forged the tricyclic ROCM precursor **84**, which underwent ethylenolysis catalyzed by **1** to give **85** (**Scheme 21**).[63] Strategically, the use of ring-opening metathesis to afford the bis-alkenyl bicycle served to introduce two two-carbon groups that would give rise to two of the four ethyl groups present in the natural product. This method of ethyl group introduction is distinct from other approaches to hippolachnin A.

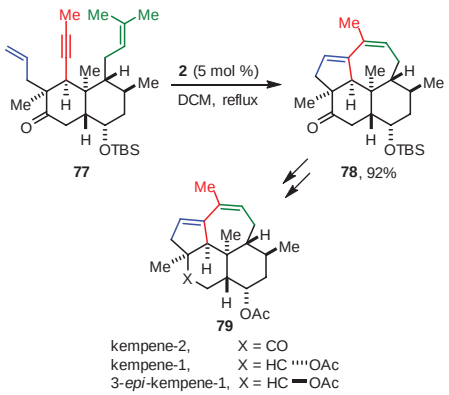
7. Cross-Metathesis

Kim and co-workers developed a procedure for the installation of a *Z*-enyne fragment, which they applied to the synthesis of (+)-3-(*Z*)-laureatin and *ent*-elatenyne (**Scheme 22**).[64] In the key CM step in the synthesis of *ent*-elatenyne, a protected enyne bearing a tethered allyl ether reacted with the terminal alkene of **87** to provide the enyne CM product **88** with high *Z*-selectivity.

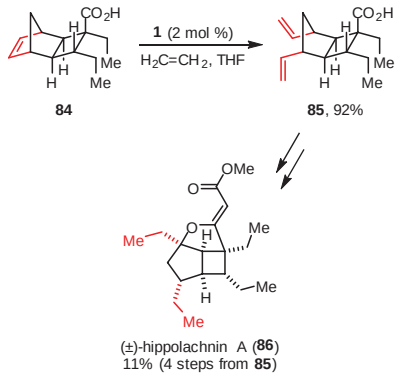
The first applications of Grubbs *Z*-selective ruthenium metathesis catalysts[65] to natural product synthesis were reported by the Grubbs group, when they prepared, in stereochemically pure form, nine lepidopteran female sex pheromones that had been approved by the EPA as insecticide alternatives (**Scheme 23**).[66] Starting from two different seed-oil derivatives, CM with a variety of terminal alkenes provided, either directly or after one step, seven different



Scheme 20. Smith's Use of RCEYEM for a Key Strategic Cyclization That Forms the Tetracyclic Morphine Core. *(Ref. 62)*



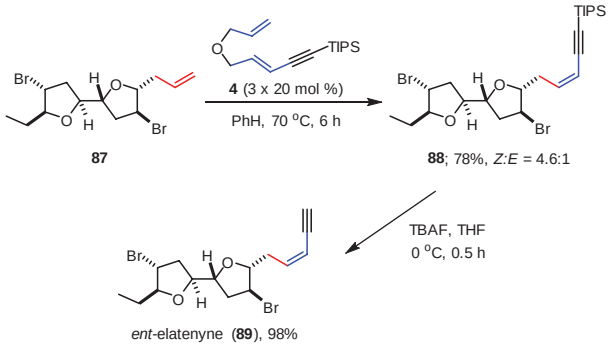
Scheme 19. Spectacular Use of RCEYEM by Schubert and Metz in the Enantioselective Total Synthesis of the Kempene Diterpenes. *(Ref. 60)*



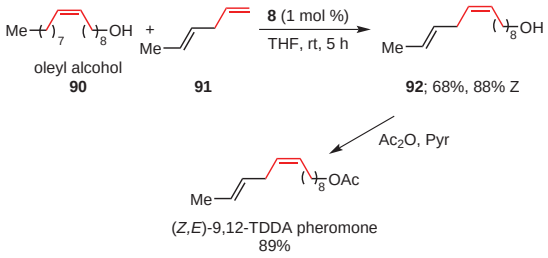
Scheme 21. ROCM in a Collaborative Total Synthesis by Wood and Brown of the Potent Antifungal Agent (±)-Hippolachnin A. *(Ref. 63)*

pheromones. The final two pheromones synthesized each required a total of four steps to complete. Though not structurally complex, these pheromones are otherwise challenging to synthesize because of the remoteness of the functional groups and necessity for control of alkene geometrical isomers. The metatheses employed each required catalyst loadings of 2 mol % or less, with Z-selectivities all greater than 75%. One CM partner, *trans*-1,4-hexadiene (**91**), underwent selective CM at the terminal position to afford **92**, not engaging the (*E*)-alkene moiety, owing to the catalyst's selectivity for *Z* alkenes.

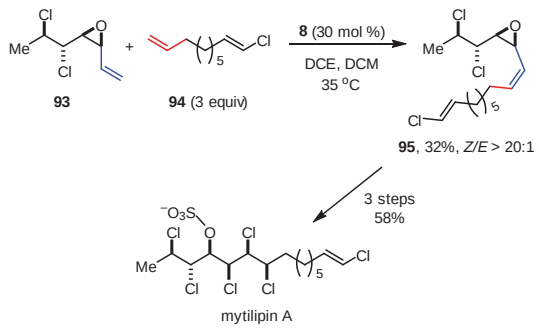
Our group's chlorosulfolipid syntheses took advantage of catalyst **8** for a highly *Z*-selective convergent cross-metathesis of two chlorinated partners (**Scheme 24**).[67] The stereoselective CM between the chlorine-containing vinyl epoxide **93** and the dienyl chloride partner **94** proceeded with very high *Z*-selectivity to give **95**, setting the stage for alkene chlorinolysis, a second dichlorination, and finally sulfation to complete a short synthesis of the chlorosulfolipid mytilipin A. The key aspect of selectivity in reaction partner **94** can be explained in part by the reduced reactivity of alkenyl chlorides and the low rates of cyclooctene formation; however, almost certainly, the key determinant of selectivity involves the recalcitrance of catalyst **8** to engage any kind of *E* alkene, thus sparing the chlorinated alkene moiety of **94** and product **95**.



Scheme 22. Relay RCM-CM in Burton and Kim's Total Synthesis of *ent*-Elatenyne. *(Ref. 64)*



Scheme 23. *Z*-Selective CM in Grubbs's Total Synthesis of Stereochemically Pure Insect Sex Pheromones. *(Ref. 66)*



Scheme 24. *Z*-Selective CM in Vanderwal's Direct Synthesis of Mytilipin A. *(Ref. 67)*

8. Conclusion

Over the past two decades, alkene metathesis has become an essential component of the synthetic chemist's toolbox. The syntheses presented in this review, and the many more which could not be discussed, are evidence of both the objective utility of alkene metathesis as well as the widespread adoption of metathesis as a go-to, reliable reaction in synthetic planning. The featured syntheses also serve to highlight some of the advances made in the field; catalyst and reaction design have overcome supposed limitations of reactivity or selectivity, and implementation in complex settings has illuminated new, non-obvious, and increasingly clever strategies to make use of alkene metathesis. We anticipate continued developments along both of these lines in the coming years.

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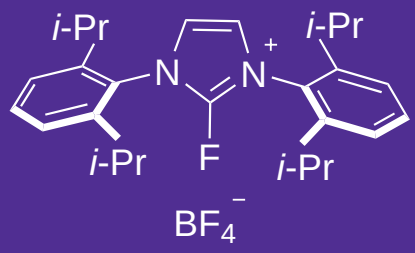

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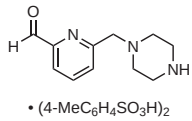
MacDonald, J. I.; Munch, H. K.; Moore, T.; Francis, M. B. *Nat. Chem. Biol*. **2015**, *11*, 326.



• $(4\text{-MeC}_6\text{H}_4\text{SO}_3\text{H})_2$

808571
(InChI Key: QDGVEYQPPPITKT-UHFFFAOYSA-N)

808571	6-(1-Piperazinylmethyl)-2-pyridinecarboxaldehyde bistosylate salt	100 mg

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Udit Batra, Ph.D.
CEO, MilliporeSigma

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ABOUT OUR COVER

Nymphenburg Palace, Munich (oil on canvas, 68.4 × 119.8 cm) was painted ca. 1761 by the renowned urban landscape painter Bernardo Bellotto (1722–1780). Born and raised in Venice, Italy, Bernardo apprenticed, starting at the age of 13, with his maternal uncle, Antonio Canaletto, another famed Venetian vedutista, or cityscape artist. Bellotto proved to be a very talented student and, early in his career, a faithful imitator of his uncle's painting methods and style. Bellotto spent his early twenties travelling to, and painting, various Italian cities and sites. At age 25, he left Venice permanently, and spent the rest of his life successfully executing commissioned work in the service of royal courts in Saxony and Poland. The result was an impressive, accurate record* of several cities and palaces of central Europe.

Detail from ***Nymphenburg Palace, Munich***. Photo courtesy National Gallery of Art, Washington, DC.

This panoramic view of Nymphenburg Palace, is part of this record and beautifully illustrates Bellotto's exacting style of painting urban landscapes that are not only grand, but also rich in detail about the inhabitants and their daily lives. Even though Bellotto was also an able etcher and draftsman, nevertheless the realism and precision exhibited in this and many of his other compositions are so extraordinary that they beg the question of whether Bellotto's initial sketches for these compositions were produced with the help of a camera obscura, a device well known at the time and already in use by other artists.

This painting is part of the Samuel H. Kress Collection at the National Gallery of Art, Washington, DC.

** Bellotto's cityscapes are so realistic and detailed that they proved invaluable almost 200 years later. To find out more, visit* ***SigmaAldrich.com/acta502***

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Disproportionate Impact of Named Reactions on Chemical Biology


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Keywords. chemistry tools; imaging studies; bioconjugation; small molecules; organic chemistry; synthesis.

Abstract. Despite their long, rich history in organic chemistry, named reactions have only recently been applied to chemical biology. This review highlights twelve named reactions that have been applied with great success in chemical biology studies.

Outline

1. Introduction

In 1822, Serullas unintentionally synthesized iodoform and potassium formate from iodine, potassium metal, and ethanol.[1] What he observed was the disproportionation of iodine in the presence of potassium ethoxide generated in situ to yield ethyl hypoiodite. This species rapidly loses hydroiodic acid to generate acetaldehyde, and sequential exhaustive iodination and ethanolysis ultimately yield the observed products. Nearly 50 years later, Lieben conducted the first comprehensive study to determine the scope of this reaction.[1] This work laid the foundation for the industrial production of various haloforms, as well as the development of a robust method to identify methyl ketone functionalities.[2] In recognition of Lieben's contributions, this transformation became known as the Lieben haloform reaction, one of the earliest named reactions in organic chemistry. Other notable examples would soon follow, typically paying homage to the inventor or scientist responsible for advancing the reaction. This reaction naming system has provided chemists at all levels with a convenient frame of reference when discussing a given chemical transformation.

With an ever-growing repertoire of chemical reactions at their disposal, organic chemists set forth to conquer some of the most challenging synthetic targets nature has to offer, doing so with remarkable creativity. Notably, pioneers such as Robert Woodward and E. J. Corey were awarded Nobel Prizes in 1965 and 1990, respectively, for their contributions to the synthesis of complex organic molecules. Other disciplines, including the pharmaceutical and biomedical sciences, have also harnessed the power of synthetic chemistry to access an impressive assortment of molecules. Likewise, synthetic chemistry has allowed scientists working at the interface of chemistry and biology to study complex biological systems with molecular precision through the development of novel chemical tools. However, named reactions merely have served as a means to an end in a majority of these cases. Thus, the aim of this review article is to survey specific named reactions that have been integrated directly into the mode of action of a chemical tool. Since many of these transformations were originally intended for chemical synthesis, careful tuning of reactivity, chemoselectivity, and biocompatibility are essential considerations when developing any tool for chemical biology. As such, we begin by outlining key criteria necessary for successful adaptation of a named reaction for biological applications. Next, we draw attention to recent examples in the literature that feature a named reaction belonging to one of three general categories: (i) pericyclic reactions, (ii) rearrangement

reactions, or (iii) metal-mediated reactions (**Figure 1**). These reactions have been selected because they offer a combination of novelty, unique chemical reactivity, and prospect for future advances. In each instance, we provide a brief historical account leading to the discovery of the reaction and offer insight into the motivation for its development. Furthermore, we discuss the specific challenges each investigator had to overcome and key biological applications. Finally, we conclude by providing a commentary on the outlook for this exciting and rapidly expanding research area.

2. Named Reaction Selection Criteria

Although there is a vast assortment of molecules used in chemical biology investigations, the examples we discuss below are broadly defined as either analyte-specific probes for molecular imaging or selective bioconjugation tools and techniques for biomolecule (e.g., protein) modification.

When developing imaging probes, the primary objective is to detect an analyte of interest in its native biological environment with minimal perturbation, typically within a live cell or animal. One strategy is to employ a named reaction that utilizes the analyte of interest as a key reagent in the transformation. In particular, the reaction must produce a discernable change in the analytical readout such as an enhancement in the fluorescence signal. Design strategies commonly employed to achieve this include chemically masking a key functional group required for emission (e.g., internal charge transfer, ICT) or tuning fluorescence via the photoinduced electron-transfer (PeT) mechanism. The specifics of these design strategies are beyond the scope of this review and have been reviewed elsewhere.[3] On the other hand, the motivations for bioconjugation include tracking, isolating, and augmenting biomolecules. However, any modification should preserve the intrinsic properties of the biomolecule (e.g., stability) since perturbation may result in undesirable consequences such as loss of function.[4] Likewise, it is essential that the reaction conditions be mild to prevent damaging the biomolecule through denaturation processes. Regardless of the application, the contemplated reaction must be (i) biocompatible, (ii) chemoselective or bioorthogonal, and (iii) fast.

A skilled chemist can exclude nearly any undesired component when performing a reaction in vitro. For instance, reagents can be rigorously dried and solvents degassed for moisture- and oxygen-sensitive reactions, respectively. However, this is challenging, if not impossible, in a biological context. As such, any chemical tool or reaction partner must be competent in an aqueous environment. This prerequisite may limit the use of many reactions requiring reagents that are not intrinsically soluble in water or those that are sensitive to hydrolysis. However, as detailed in the following sections, numerous creative approaches can be employed to overcome these constraints.

Chemoselectivity or bioorthogonality is another important criterion, especially for analyte-specific probes, where it is important to unambiguously demonstrate that a signal change is due to detection of the intended target and not from competing species with similar functional groups. Thus, it is highly desirable to begin with a reaction that is intrinsically chemoselective. Incidentally, for a reaction lacking specificity, it is possible to fine-tune its reactivity for a particular application. For example, decreasing the electrophilicity of a tool will favor reactivity with only the most nucleophilic analyte. Similarly, installation of a bulky chemical group can provide steric hindrance to exclude reactivity with larger, off-target molecules. Indeed, many other approaches are available to achieve excellent chemoselectivity. With regard to bioconjugation, careful selection of reactions that utilize abiotic functional groups (e.g., azide and alkyne groups) is important since this decreases the likelihood of cross-reactivity with physiological processes.

Finally, a named reaction should proceed with permissible kinetics at physiological temperatures. Analyte-specific probes typically require a reaction that is sufficiently fast to intercept the target before it is depleted by normal cellular mechanisms. This is often the case with highly reactive metabolites and signaling molecules such as nitroxyl (HNO), which is present only momentarily and at low concentrations.[5] In the same way, since bioconjugation is employed to identify and track rapid and dynamic cellular processes, a large rate constant is also essential.

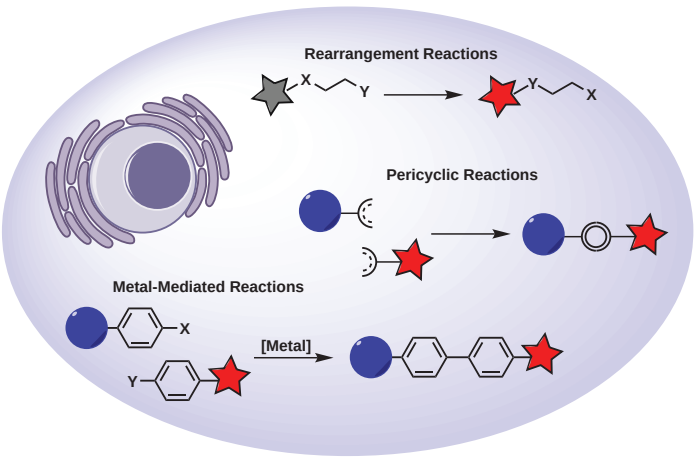


Figure 1. Overview of Reaction Classes Employed in Chemical Tools for Biological Studies.

3. Pericyclic Reactions

Pericyclic reactions are those that proceed through a concerted mechanism and are characterized by a cyclic transition state.[6] These reactions, both unimolecular (e.g., sigmatropic rearrangements) and bimolecular (e.g., intermolecular cycloadditions), are appealing to the synthetic chemist due to their ability to rapidly generate multiple new stereocenters. Nature is also believed to use such reactions to generate complex products, but the prevalence of common reactive partners, such as *s-cis*-dienes, is very low.[7] Thus, pericyclic reactions are attractive to chemical biologists particularly for their potential of affording excellent chemoselectivity and bioorthogonality. In the following subsections, we feature named pericyclic reactions that demonstrate these characteristics in their applications as analyte-specific probes and bioconjugation techniques.

3.1. 2-Aza-Cope Rearrangement

In 1950, Horowitz and Geissman reported that treating α-allylbenzylamine (**1**) with formic acid and formaldehyde at 90–100 °C resulted in the formation of benzaldehyde and trialkylamine **2**, rather than the anticipated α-allylbenzyldimethylamine product (**eq 1**, Part (a)).[8] By excluding formic acid, it was determined that formaldehyde mediates C–N bond cleavage through sequential [3,3]-sigmatropic rearrangement and hydrolysis. Owing to its similarity to the Cope rearrangement, this transformation became known as the 2-aza-Cope rearrangement. However, unlike the Cope rearrangement, which was first reported to proceed at 150–160 °C,[9] the 2-aza-Cope rearrangement can take place under milder conditions (even at room

temperature), especially when accelerated by a positive charge.[10] Because formaldehyde plays an integral role in lowering the activation barrier through iminium ion formation, our group postulated that the 2-aza-Cope rearrangement could be employed to develop a fluorescent probe, **FP1** (eq 1, Part (b)), for visualizing endogenous formaldehyde in living systems.[11] Formaldehyde has attracted significant interest due to its potential role in mediating long-term memory formation and aberrant accumulation in tumors. However, prior to the development of **FP1**, methods of formaldehyde detection involved indirect and invasive protocols, which typically led to the destruction of the biological sample being analyzed. In contrast, formaldehyde readily condenses with the secondary amine of **FP1** under physiological conditions to yield an iminium intermediate. This in turn undergoes a facile 2-aza-Cope rearrangement and hydrolysis to cleave a pendant 4-nitrobenzylamine, which had been installed to quench the fluorescence of the probe. With **FP1** in hand, we successfully imaged formaldehyde non-invasively in multiple cell lines using confocal microscopy and corroborated the results with flow cytometry. At the same time, Chang reported the development of **FAP1**, a spiro-amine-based formaldehyde probe that also utilizes the 2-aza-Cope rearrangement.[12] More recently, Chang developed **FAP488**, which features a modified formaldehyde-selective trigger based on β-elimination of the ketone intermediate.[13] Notably, the Thorpe–Ingold effect was exploited in this example to accelerate the sigmatropic rearrangement.

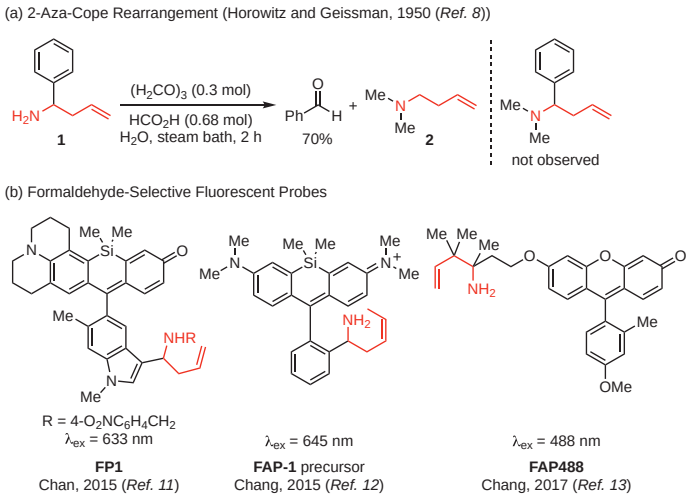
3.2. Alkyne–Azide Huisgen Cycloaddition

Although the alkyne–azide Huisgen cycloaddition is widely recognized today as the gold standard of click chemistry, this was not always the case. In 1910, Dimroth and Fester reported the formation of 1,2,3-triazole products after acetylene and hydrazoic acid or phenyl azide were heated at 100 °C in closed reaction vessels for 70 hours (**Scheme 1**, Part (a)).[14a] The reaction went unappreciated for some 50 years until Huisgen demonstrated its general utility using various 1,3-dipoles and dipolarophiles.[14b] Even then, these studies were performed in organic solvents, and the reaction rates were slow near physiological temperatures.[15] In 2002, Meldal[16] and Fokin and Sharpless[17] independently reported that Cu(I) could accelerate the cycloaddition using Cu(I) salts and Cu(II) salts under reducing conditions, respectively. In both cases, Cu(I) acetylides generated in situ from terminal alkyne precursors could readily react with organic azides at room temperature. Within a year, this reaction was employed for the fluorescent labeling of the functionalized cowpea mosaic virus (CPMV),[18] and for activity-based protein profiling where proteins in the proteasome can be enriched and identified.[19]

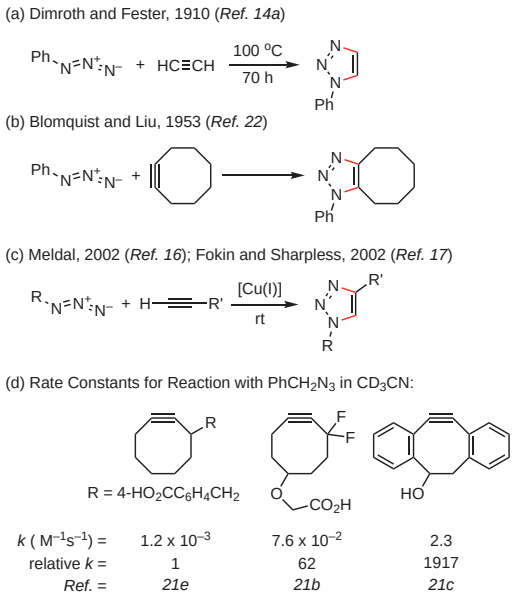
Unfortunately, the use of redox active metal ions such as Cu(I) in biological systems can generate toxic reactive oxygen species through Fenton-like chemistry.[20] To address these concerns, Bertozzi and others developed a series of substituted cyclooctyne reagents (e.g., 3,3-difluorinated cyclooctynes) that were optimized for a Cu(I)-free, strain-promoted variant of the Huisgen cycloaddition reaction.[21] This work was based on an earlier report by Blomquist and Liu that phenyl azide reacts violently with cyclooctyne to afford the corresponding 1,2,3-triazole.[22] Indeed, DIFO (3,3-difluorocyclooctyn-6-oxylacetic acid) and its congeners exhibited rate constants suitable for bioconjugation applications,[21] many of which are highlighted in another review article.[23] Notably, both strain-promoted and Cu(I)-catalyzed methods remain popular in facilitating biological advances that include discovering electrophilic modifications on human proteins,[24] identifying proteins secreted by virulent bacteria,[25] and illuminating the selectivity in the *S*-acylation of proteins.[26]

3.3. Ozonolysis

The reaction between ozone (1,3-dipole) and an alkene (dipolarophile) is another example of a Huisgen 1,3-dipolar cyclcoaddition that has recently been utilized in chemical biology. Harries published a detailed investigation in 1905 on the reactivity of ozone with unsaturated compounds, noting that, under aqueous conditions, the rapidly formed ozonide intermediate decomposes to yield two carbonyl species and hydrogen peroxide (**Scheme 2**).[27] This reactivity has been exploited in water purification processes motivating the development of first-generation probes to quantify ozone levels.[28] Using these probes, ozone was recently shown to be generated in human neutrophils[29]



eq 1



Scheme 1. Huisgen Azide–Alkyne Cycloaddition and Improvements in the Kinetics of Bioconjugation.

and atherosclerotic arteries.[30] However, these early examples reported significant cross-reactivity with singlet oxygen ($^1O_2$).[31] Koide addressed this limitation by developing **3**, a probe which features a homoallyl group appended to a dichlorofluorescein dye platform.[31] Selectivity was achieved because only ozone reacts with the trigger through a pathway first disclosed by Criegee.[32] In particular, a [3 + 2] cycloaddition sets in motion a series of rearrangements that generate two carbonyl species through oxidative cleavage of the alkene precursor. The resulting β-ether aldehyde, **4**, eliminates acrolein and releases compound **5**, which is highly fluorescent. In contrast, reaction of **3** with $^1O_2$ yields an uncleaved, non-fluorescent product, **6**. Using probe **3**, Koide successfully detected ozone in air samples and in BEAS-2B human bronchial epithelial cells.[31]

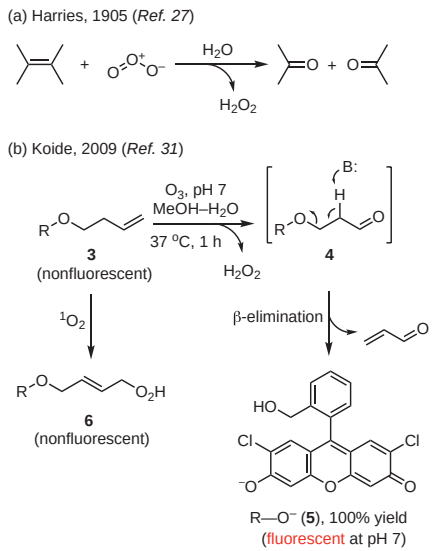
3.4. Diels–Alder Reaction

In 1928, Diels and Alder published the first in a series of articles on the [4 + 2]-cycloaddition reaction between electron-rich dienes and electron-poor dienophiles (**Scheme 3**, Part (a)).[33] This general reaction class would eventually bear their names in recognition of their seminal contributions. A major advance came in 1980 when Breslow disclosed a Diels–Alder cycloaddition that proceeded 700-times faster in water than in a nonpolar hydrocarbon solvent.[34] Owing to its compatibility with aqueous media and the relatively low abundance of *s-cis*-dienes in biological systems, the Diels–Alder reaction became one of the earliest transformations to be applied for bioconjugation. For instance, it was demonstrated that dienes could be site-selectively incorporated into RNA to enable subsequent modification with dienophiles such as maleimide via the Diels–Alder reaction (Scheme 3, Part (b)).[35]

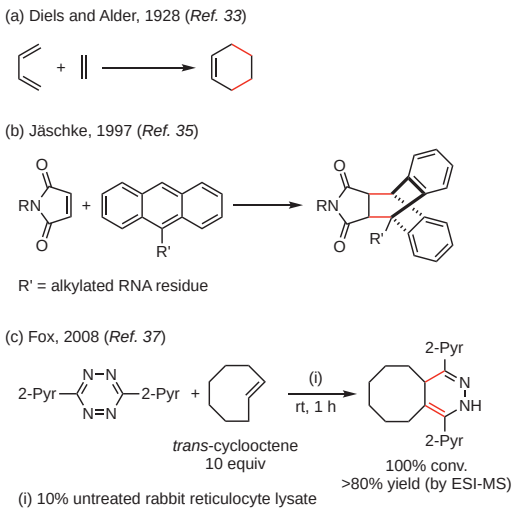
Unfortunately, maleimides are also known to react with thiol nucleophiles such as those found in protein cysteine residues.[36] This limitation prompted Fox to explore the *s*-tetrazine-based, inverse-electron-demand Diels–Alder reaction as a possible alternative (Scheme 3, Part (c)).[37] Electron-poor *s*-tetrazines were found to react with electron-rich or strained alkenes in a [4 + 2]-cycloaddition manner. Subsequently, a rapid retro-[4 + 2]-cycloaddition releases N_2, resulting in the irreversible formation of a dihydropyridazine. In addition to improved bioorthogonality, the rate of this inverse-electron-demand Diels–Alder reaction (minutes)[38] was found to be faster than that of the Diels–Alder reaction (hours).[35,39] The kinetic efficiency in combination with the cell-permeability of the *s*-tetrazine-appended dyes made this an appealing tool for intracellular studies. For instance, when microtubule-bound, *trans*-cyclooctene-modified Taxol® was reacted with tetrazine-BODIPY FL, a robust fluorescence turn-on response could be observed owing to turnover of the fluorescence-quenching monoaryl-*s*-tetrazine moiety.[40b] In addition, the inverse-electron-demand Diels–Alder reaction was employed to couple the functional components of a proteolysis-targeting chimera (PROTAC) in live cells in order to enable degradation of BRD4 and ERK1/2.[41] This was necessary because PROTACs are generally cell-impermeable. Lastly, active-site lysine residues of various protein kinases were modified with a *trans*-cyclooctene carbamate moiety that could be cleaved through an inverse-electron-demand Diels–Alder reaction. Specifically, upon introduction of a tetrazine additive, the dihydropyridazine intermediate that is generated rapidly eliminates to restore enzymatic activity.[42]

4. Rearrangement Reactions

By definition, a rearrangement reaction describes the intramolecular shift from one constitutional isomer to another. This intramolecular event can be initiated by an external stimulus such as an increase in temperature or chemical activation. In the eyes of a chemist, the thermodynamic isomer formed will typically offer new reactivity and/or stability. For the chemical biologist, rearrangement reactions resulting in more stable compounds are ideal for bioconjugation reactions that rely on the formation of new, stable linkers. On the other hand, rearrangement reactions that generate new reactivity are ideal for analyte-specific probes as these can be tailored to directly or indirectly affect an analyzable output. In the following section, we highlight recent examples of both stability-enhancing and reactivity-changing rearrangement reactions.



Scheme 2. Original Ozonolysis Research and Its Application in Ozone-Selective Probes.



Scheme 3. Diels–Alder Cycloaddition and Its Applications in Bioconjugation.

4.1. Smiles Rearrangement

One of the oldest rearrangement reactions was first described by Henriques[43] and Hinsberg,[44] who observed that, following addition of alkaline ferricyanide to bis(2-hydroxynaphth-1-yl) sulfide and bis-(2-hydroxynaphth-1-yl) sulfone, respectively, new rearranged products were generated. However, it was not until 1930 that these reactions, as well as their key Meisenheimer complex intermediates, were fully characterized by Smiles (**Scheme 4**, Part (a)).[45] What Henriques and Hinsberg had observed was an intramolecular S_NAr that would become known as the Smiles rearrangement. In 1958, Burchfield reported that the Smiles rearrangement was occurring when cysteine was reacted with 1-fluoro-2,4-dinitrobenzene.[46] During this reaction, an initial product is formed that has an absorbance maximum in the 340–345 nm range; however, as the reaction proceeds a new product with an absorbance maximum at 360 nm forms. This is consistent with a mechanism in which the thiol initially displaces the fluoride nucleofuge followed by an intramolecular S_NAr with the free amine.

A clever biological application of the sulfur-to-nitrogen Smiles rearrangement was demonstrated in a study initiated to understand the biosynthetic pathway that is responsible for producing mycothiol, a protective thiol of actinobacteria.[47] It was proposed that along this pathway, MshB catalyzes the hydrolytic cleavage of the acetamide in GlcNAc-Ins, a precursor to mycothiol.[48] However, there were no reliable methods to monitor the activity of this enzyme colorimetrically, prompting the development of GlcNAc-SDNP.[47] In particular, in the presence of active MshB, the acetamido group of GlcNAc-SDNP is hydrolyzed to afford primary amine **7**, which is primed for a subsequent Smiles rearrangement. Favoring the aniline product over the sulfide, the Smiles rearrangement generates free thiol **8**, which can then react in vitro with Ellman's Reagent, yielding disulfide **9** and thiol **10**. Free thiol **10** absorbs strongly at 412 nm, allowing reliable colorimetric detection.

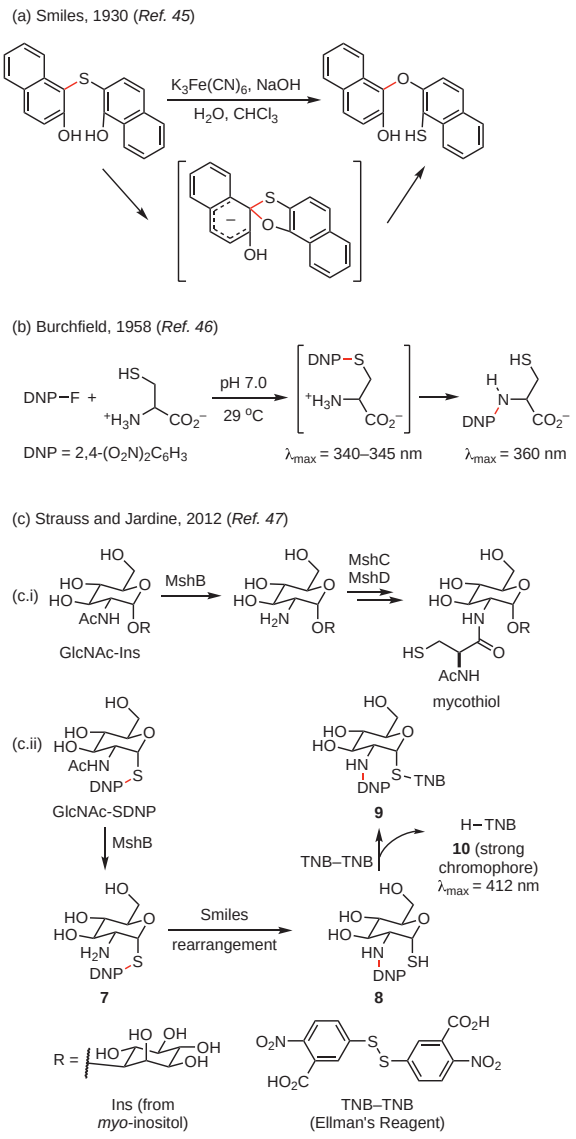
4.2. Pictet–Spengler Reaction

Although numerous approaches exist for installing aldehyde and ketone functional groups into a protein, methods to utilize them for subsequent bioconjugation have suffered from poor chemical stability. For example, condensation with hydrazine and hydroxylamine affords hydrolyzable hydrazone and oxime linkers, respectively.[49] Fukuzawa and Tachibana[50] sought to overcome this through the use of a rearrangement reaction pioneered by Pictet and Spengler[51] in 1911 (**Scheme 5**, Parts (a) and (b)). Specifically, tryptamine **11** condenses with aldehyde moieties to form iminium intermediates that undergo intramolecular nucleophilic attack and rearrangement to afford the stable, fused-ring products **12**. To improve upon the slow kinetics,[52] Bertozzi made two important improvements with **13** that would render the Pictet–Spengler reaction more biocompatible.[49] First, the amine linker was shifted from the C-3 to the C-2 position to reduce steric hindrance at the nucleophilic site; this resulted in a rearrangement-free iso-Pictet–Spengler mechanism to produce **14** (Scheme 5, Part (c)). Additionally, increasing the rate of iminium ion formation through the alpha-effect was implemented by modifying the pendent amino group into an aminooxy functionality. In combination, the rate of the reaction was increased by 3 orders of magnitude. The low pH required for efficient condensation was a final constraint that was addressed through substitution of the aminooxy group with hydrazine.[53]

Of note, the Pictet–Spengler reaction has not been limited to ligation chemistry. Another breakthrough came in the realization by Fukumura that an N-terminus tryptophan could perform a Pictet–Spengler reaction with ^{11}C-labeled formaldehyde, allowing the radiolabeling of short oligopeptides for PET imaging.[54]

4.3. Staudinger–Bertozzi Ligation

In 1919, Staudinger reported that the combination of phenyl azide and triphenylphosphine resulted in a violent reaction (**Scheme 6**, Part (a)).[55] Although it was proposed that a phosphazide intermediate was involved, N_2 evolution was so rapid that only the final iminophosphorane **15** could be isolated. Supplemental water reacted with the electrophilic phosphorus in **15** leading to hydrolysis of the P=N bond to generate an equivalent of phosphine oxide and aniline. On the other hand, reaction of iminophosphorane **15** with anhydrous benzoic acid resulted in the formation of secondary amide **16** (Scheme 6, Part (b)).[56] Requiring five days and elevated temperatures, the reaction of **15** with benzoic acid is significantly slower than the corresponding reaction with water.



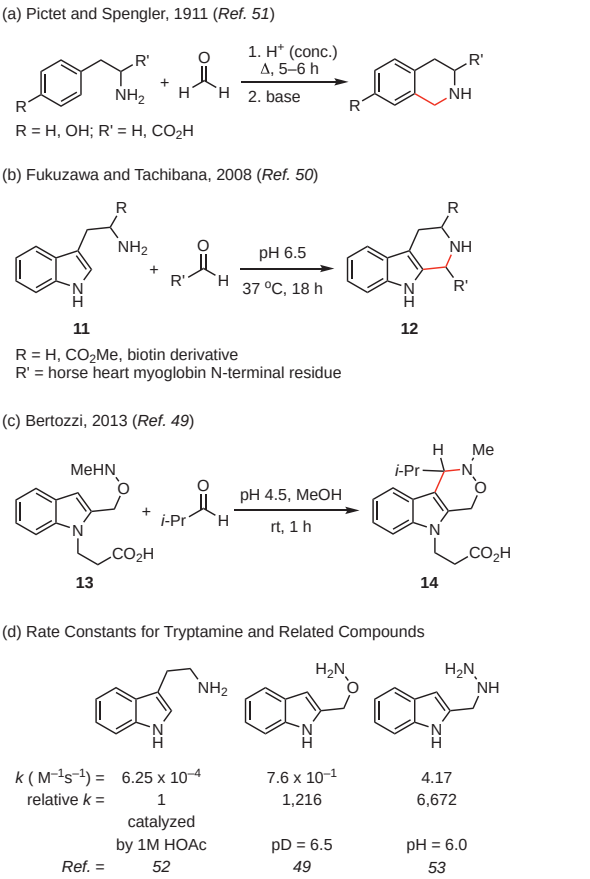
Scheme 4. The Smiles Rearrangement and Developments toward Its Use in Enzymatic Assays.

Bertozzi hypothesized that a carefully designed phosphine with an intramolecular electrophile would capture the iminophosphorane nucleophile before hydrolysis could occur. Indeed, introduction of a methyl ester group at the ortho position of one of the benzene rings in **17** proved sufficient, and, upon reaction with azide, resulted in a stable amide **18** after intramolecular rearrangement (Scheme 6, Part (c)).[57] In its first demonstration, the Staudinger–Bertozzi ligation, as it is now known, enabled the incorporation of a biotin marker only on cells displaying azido sugars on the cell surface.[57] Later, peracetylated *N*-α-azidoacetylmannosamine, an unnatural sugar, was administered to live mice for one week, resulting in glycan modification in the heart, liver, and kidney.[58] Subsequent injection of a FLAG® peptide appended with a reactive phosphine handle enabled the first in vivo application of the Staudinger–Bertozzi ligation. In addition to sugars, successful ligations have been performed on DNA[59] and proteins[60] under aqueous conditions.

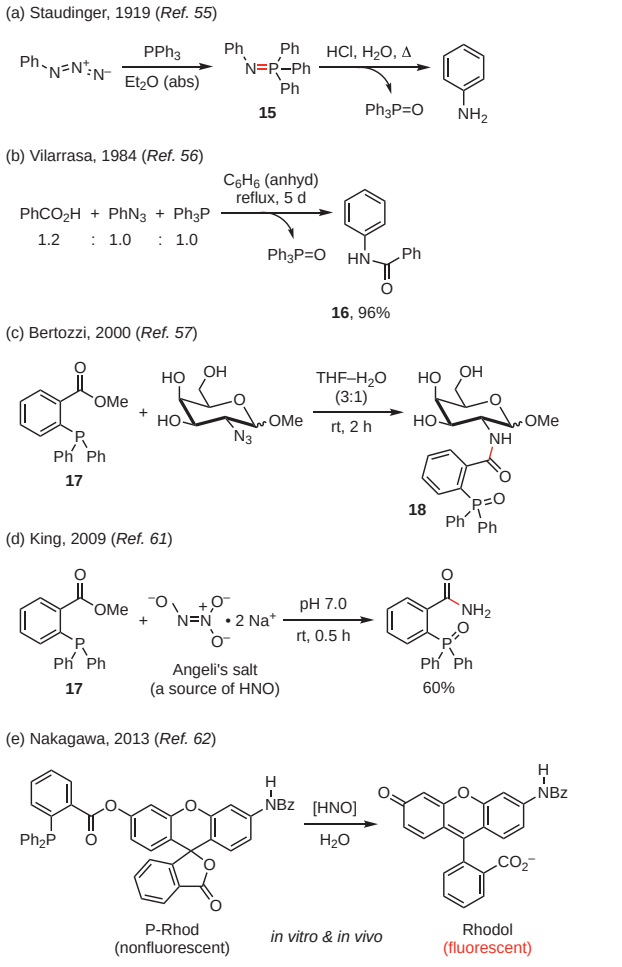
It is worth noting that the Staudinger–Bertozzi ligation does not proceed exclusively with azides. In fact, King demonstrated that HNO, a transient signaling molecule, also reacts with phosphines through a similar mechanism (Scheme 6, Part (d)).[61] Since methods to non-invasively detect HNO are limited, Nakagawa proposed to exploit its reactivity with phosphines to develop imaging probes specific for HNO detection. This led to the development of P-Rhod, a rhodol-based fluorescent probe that was used to image HNO produced upon treatment of A549 cells with Angeli's salt, an HNO donor molecule (Scheme 6, Part (e)).[62] Since this work, a number of other investigators have utilized this trigger to develop a variety of other imaging probes.[63] Unfortunately, the detection of naturally occurring HNO has remained elusive to date, presumably due to inefficient reaction kinetics.

4.4. Baeyer–Villiger Oxidation

In 1899, Baeyer and Villiger were exploring the reactivity of Caro's acid (peroxymonosulfuric acid, $HOS(=O)_2OOH$) with cyclic ketones when they discovered that ring-expanded lactones were being produced (**Scheme 7**, Part (a)).[64] Subsequent studies would establish that other peroxy compounds could also promote this transformation, now known as the Baeyer–Villiger oxidation, in a manner strongly correlated with the p*K*a value of the peroxy compound.[65a] Of note, while oxidation of ketones with H_2O_2 (p*K*a = 11.7)[65b] does occur, the reaction kinetics are generally sluggish compared to Caro's acid (p*K*a = 0.4).[65c] In contrast, 1,2-diketones such as benzil are rapidly oxidized by H_2O_2 in basic aqueous MeOH solutions to afford two benzoic acid products (Scheme 7, Part (b)).[66]

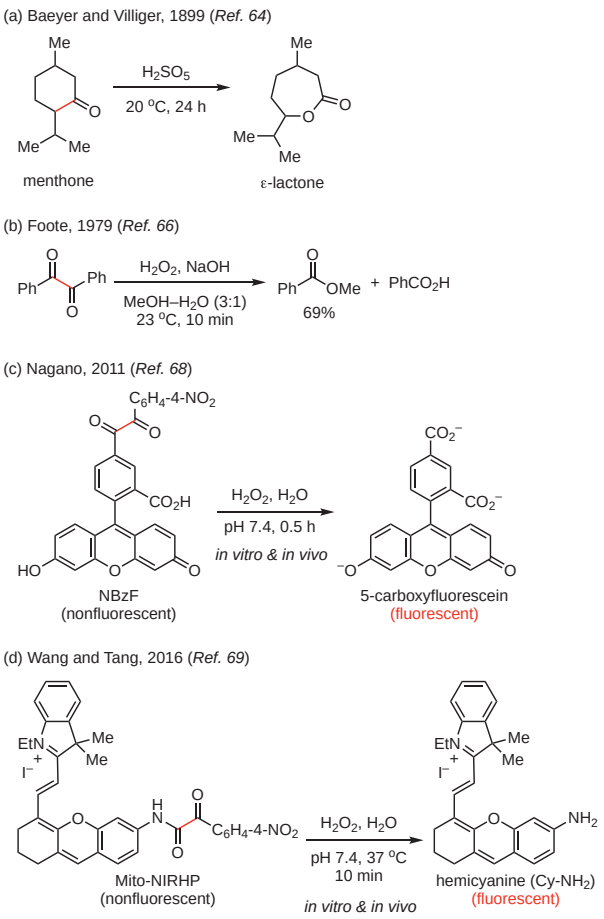


Scheme 5. The Pictet–Spengler Reaction and Its Development for Efficient Bioconjugation.



Scheme 6. The Staudinger Reaction and Subsequent Staudinger–Bertozzi Ligation.

Owing to its involvement in a large variety of physiological processes ranging from the immune response to cell signaling,[67] it is essential to develop chemical tools to detect H_2O_2 within biological samples. In this regard, Nagano designed NBzF, a fluorescein analogue equipped with an electron-deficient 4-nitrobenzil moiety to sense H_2O_2 (Scheme 7, Part (c)).[68] This probe design utilizes the 4-nitrobenzil group to quench the fluorescence of NBzF via the d-PeT (donor-excited photoinduced electron transfer) mechanism. After addition of H_2O_2, the 4-nitrobenzil moiety is oxidized to a hydrolytically unstable anhydride intermediate that spontaneously decomposes to afford 5-carboxyfluorescein, which is accompanied by a dramatic 150-fold turn-on response. The impressive selectivity for H_2O_2 and exquisite sensitivity enabled fluorescence imaging of endogenously produced H_2O_2 in multiple cell lines. In addition to the benzil moiety, the α-ketoamide functionality was recently utilized to develop Mito-NIRHP, a near-infrared, mitochondrially targeted H_2O_2-responsive probe (Scheme 7, Part (d)).[69] The α-ketoamide group functions by masking a key amino functionality crucial for fluorescence. Upon Baeyer–Villiger oxidative cleavage, the latent fluorophore is released with a concomitant increase in fluorescence. Wang and Tang successfully employed Mito-NIRHP to visualize H_2O_2 produced during ischemia-reperfusion injury in live cells and in an animal model.[69]



Scheme 7. The Baeyer–Villiger Oxidation and Its Applications in the Design of H_2O_2-Responsive Fluorescent Probes.

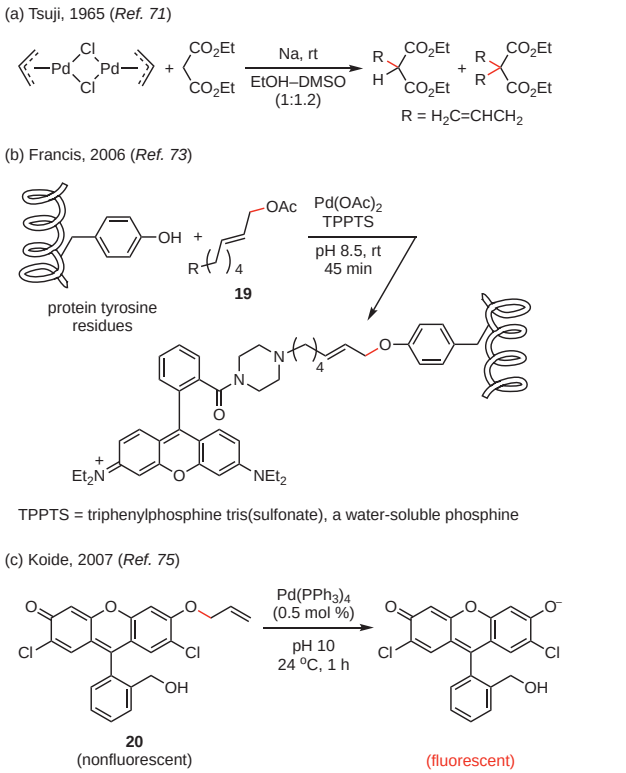
5. Metal-Mediated Reactions

Recently, there has been growing interest in utilizing second and third row transition metals such as ruthenium and palladium to develop chemical biology tools. This represents an exciting avenue for chemical biologists to develop highly bioorthogonal reagents, since these elements are not found naturally in biological systems. If the intrinsic toxicity of such heavy metals can be controlled, the introduction of new metal-mediated reactions with unique reactivities will undoubtedly lead to exciting biological discoveries. In the following subsections, we highlight recent examples involving palladium- and ruthenium-mediated transformations.

5.1. Tsuji–Trost Reaction

In 1965, Tsuji reported the reaction of diethyl malonate carbanion, a soft carbon nucleophile, with a cyclooctadiene-palladium chloride complex.[70] Depending on the workup conditions, either a cyclopropyl product was formed through one-carbon addition to one of the carbon–carbon double bonds, or a mono-substituted, conjugated cyclooctadiene product through double-bond migration. Shortly thereafter, Tsuji published similar chemistry with π-allylpalladium chloride; however, only the allylated product was observed (**Scheme 8**, Part (a)).[71] In this seminal work, pre-forming the π-allylpalladium indicates that the reaction begins with substitution at the metal center followed by reductive elimination and dissociation of the allylated nucleophile to release Pd(0).

Soon after discovery of this reaction, Trost and others made key advances by expanding the substrate scope and enabling a catalytic

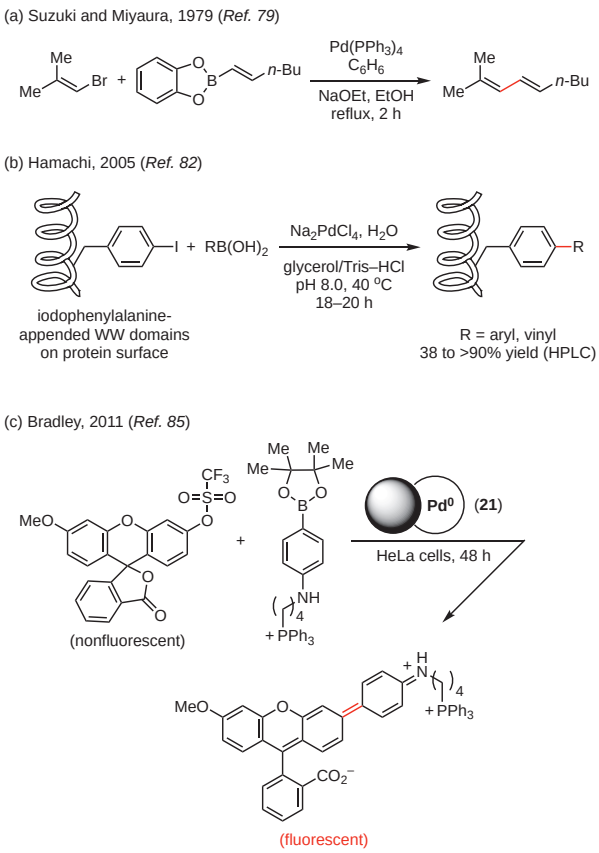


Scheme 8. The Tsuji–Trost Allylation and Its Applications in Bioconjugation and Pd(0) Sensing.

variant.[72] Exploiting the reactivity of phenols, Francis developed a bioconjugation method that is selective for tyrosine residues (Scheme 8, Part (b)).[73] To accomplish this, stoichiometric allyl donor **19**, catalytic $Pd(OAc)_2$, and a water-soluble phosphine (TPPTS) were added to a solution of chymotrypsinogen A, achieving a 50–65% labeling efficiency of the protein. The most impressive aspect of this work was the observation that neither lysine nor cysteine reacted under the labeling conditions. Unfortunately, this chemistry was limited to only ex vivo applications, and no significant improvements in the Tsuji–Trost bioconjugation have been reported since.

In synthetic chemistry, one of the most common uses of the Tsuji–Trost reaction is to selectively deprotect O- and N-allylated species under mild conditions.[74] Probes that can selectively visualize palladium are desirable in order to study its toxicity to biological systems, as well as to monitor palladium-based chemotherapeutics. In 2007, Koide utilized the reactivity of the Tsuji–Trost reaction to develop **20**, a fluorescent probe for the detection of Pd(0) through an O-deallylation mechanism (Scheme 8, Part (c)).[75] However, due to the requirements of Pd(0) and high limit of detection, the utility of this and other similar probes were never demonstrated in a biological system.[75,76]

Due to these limitations, subsequent generations of probes began to employ a propargyl protecting group rather than an allyl moiety.[77] In particular, various oxidation states including Pd(0), Pd(II), and Pd(IV) can all mediate the cleavage of the propargyl group to unmask the latent fluorophore. Similar chemistry was used to enable the activation of proteins by addition of palladium to cells, in particular restoring bacterial phosphothreonine lyase activity via depropargylation of a protected active site residue.[78]



Scheme 9. The Suzuki–Miyaura Coupling and Its Applications in Protein Tagging.

5.2. Suzuki–Miyaura Coupling

The Pd-catalyzed coupling of organoboranes with organic halides first introduced by Suzuki and Miyaura is mechanistically similar to other organometallic coupling reactions (**Scheme 9**, Part (a)).[79] However, this coupling, now named after Suzuki and Miyaura, is generally a safer alternative to other reactions such as the Stille coupling, which uses toxic organostannane reagents. In addition to excellent compatibility with water and oxygen, organoboranes and organic halides do not naturally occur in most biological systems. As such, the application of the Suzuki–Miyaura coupling in chemical biology was inevitable.

The first example of a Suzuki–Miyaura coupling that occurred primarily in aqueous solution came in 1990 with the introduction of sulfonated phosphines.[80] This new class of ligands enabled the coupling of aryl and vinyl boronic acids with aryl halides, including an iodonucleoside, albeit at 80 °C. Despite this demonstration of aqueous compatibility, the earliest examples involving unnatural amino acids were performed in organic solvents, also at 80–90 °C.[81] Transitioning to Na_2PdCl_4, a water soluble, phosphine-free catalyst, the Suzuki–Miyaura coupling was successfully performed on a protein under aqueous conditions for the first time in 2005 (Scheme 9, Part (b)).[82] Unfortunately, even when mixed at 40 °C with organic co-solvents, reaction times were still long, taking approximately 18 hours. A 2-aminopyrimidine-based, water-soluble catalyst developed by Davis set a new standard a few years later, reacting in buffered water at 37 °C with yields >90% for couplings of bromo and iodo derivatives of phenylalanine and tyrosine with phenylboronic acid.[83] With this catalyst, synthetic post-translational modifications could be made to surface-expressed *p*-iodophenylalanine (*p*IPhe) in maltose-binding protein. Importantly, the same catalyst could be used in living cells to successfully append either a fluorophore or sugar to *p*IPhe-containing membrane proteins to enable direct visualization of the cell and modification of the glycocalyx, respectively.[84] In these studies, cell impermeability prevented the demonstration of intracellular modifications. To overcome this, Bradley deviated from traditional small ligands in favor of a cell-permeable Pd(0) nanoparticle, **21** (Scheme 9, Part (c)).[85] This stable Pd(0) species successfully catalyzed the coupling of an aryl triflate with an organoborane to produce a fluorescent species within HeLa cells. Future directions include intracellular coupling of biologically relevant partners. Potential catalysts for these new bioconjugations include the aforementioned Pd(0) nanoparticle, as well as the very recently developed artificial Suzukiases.[86]

5.3. Grubbs Reaction (Olefin Metathesis)

The 2005 Nobel Prize was awarded to Chauvin, Grubbs, and Schrock for their pioneering research on olefin metathesis.[87a] In particular, Grubbs was recognized for the development of Ru(II) carbenoid complexes that exhibit good functional-group tolerance.[87b] Utilizing this coupling reaction, unnatural amino acids were some of the first molecules modified for biological studies (**Scheme 10**, Part (a)).[88] Another application was to improve the stability of peptide-based pharmaceuticals by generating rigid, stable linkers within and across secondary peptide structures.[89] Although this peptide chemistry was performed in degassed organic solvents under anhydrous conditions, improvements in catalyst design have allowed for improved reactivity, water solubility, and biocompatibility.[87b,90] With these new catalysts,

aqueous ring-closing metathesis (RCM) and ring-opening metathesis (ROM) polymerizations were readily demonstrated, but robust cross-metathesis (CM) remained elusive. Working with nontraditional CM partners, Davis discovered that *S*-allyl-cysteines, and more so *Se*-allyl-selenocysteines, reacted much more readily than the *O*-allyl species (Scheme 10, Part (b)).[91] This improved reactivity was attributed to the softness of selenium and sulfur compared to oxygen. This softness better facilitates pre-coordination to the ruthenium catalyst and allows CM between proteins and various allyl handles. The future of the Grubbs reaction as a competitive chemical biology tool will depend on continued improvements of the reactivity and stability of the ruthenium catalyst. Additionally, continuing to improve the catalyst-controlled *E*- and *Z*-selectivity is important as the final isomer can effect noticeable changes in the bioactivity of the unnaturally linked peptide.[92]

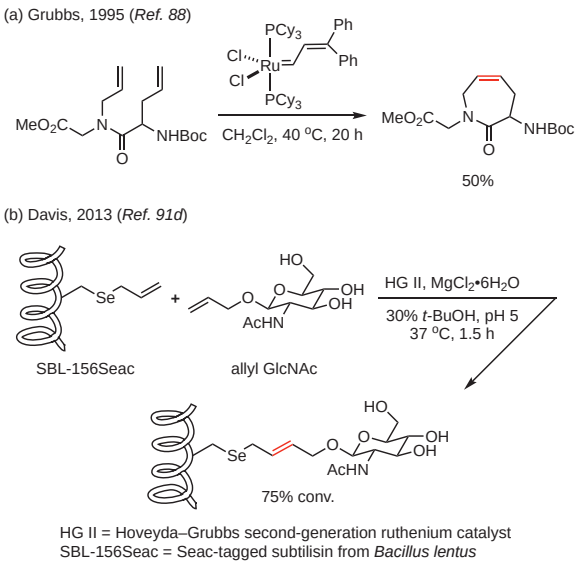
5.4. Oxidative Heck Coupling

In the early 1970s, Mizoroki and Heck independently discovered that mono- or unsubstituted olefins could be coupled to iodobenzene in the presence of base and catalytic $Pd(OAc)_2$.[93] Shortly thereafter, Heck would briefly report the first example of oxidative Heck coupling between methyl acrylate and 1-hexenylboronic acid (**22**), but this required stoichiometric amounts of $Pd(OAc)_2$ (**Scheme 11**, Part (a)).[94] Further work by Uemura enabled the use of catalytic palladium[95] and, almost a decade later, work by Jung made possible the use of O_2 as oxidant[96] and base-free couplings (Scheme 11, Part (b)).[97] With these developments, the oxidative Heck reaction became highly desirable for bioconjugation, namely because it enabled addition of substrates to unfunctionalized olefins. Using the water-soluble catalyst Pd-BIAN, Dekker was the first to successfully perform this bioconjugation in 2014 (Scheme 11, Part (c)).[98] After expressing the R16C mutant of 4-oxalocrotonate tautomerase, in which a single terminal alkene exists, addition of $Pd(OAc)_2$, BIAN, and a fluorescent boronic acid in a 6:1 buffer–DMF solution led to successful fluorescent labeling. Shortly thereafter, Dekker disclosed a fully aqueous variant involving lysine acylation post-translational modification.[99] Notably, the oxidative Heck coupling has been extremely useful for the identification of proteins that have been post-translationally modified by protein methyltransferase.[100] Using the oxidative Heck coupling, a biotin analogue can be appended to proteins allylated by an allyl-SAM (SAM = *S*-adenosyl-L-methionine) analogue for later isolation via an avidin column.

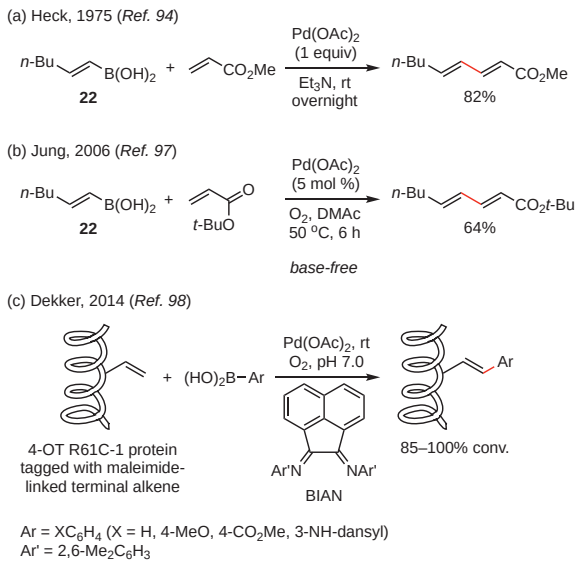
6. Conclusion and Outlook

The adaptation of named reactions for various applications in chemical biology is a rapidly emerging research area. The aim is to develop effective chemical tools with extraordinary chemoselectivity and biocompatibility for deciphering unanswered biological questions, as well as to discover new biological phenomena. Indeed, by utilizing the collective knowledge of countless organic chemists, the process of developing a new chemical tool can be greatly accelerated. However, to do so, one must have a keen eye for identifying reactions that are poised to meet the criteria detailed above in Section 2. For instance, reactions that already operate efficiently in water are favored over those that do not. Similarly, reactions exhibiting kinetics within the desirable range for a specific application will require less elaborate chemical tuning. Moreover, reactions that feature abiotic chemical moieties will suffer less from potential cross-reactivity with endogenous molecules found in the complex biological milieu. Although this is not always possible, researchers new to this area can take comfort that chemical ingenuity can overcome essentially any barrier. For instance, chemical tools that undergo reactions not compatible with aqueous media can be encapsulated within some material such as a conjugated polymer nanoparticle that offers an organic-solvent-like interior. This approach can also accelerate reaction rates by increasing the effective concentration of reactants and limit cross-reactivity by excluding the entry of competing species.

In this review article, we have highlighted select named reactions employed thus far in the development of analyte-specific imaging



Scheme 10. Application of the Grubbs Olefin Metathesis Reaction to Peptide Stabilization and Bioconjugation.



Scheme 11. The Oxidative Heck Coupling and Its Applications in Bioconjugation.

probes, as well as chemical tools and approaches for bioconjugation. However, it is noteworthy to point out that many chemical reactions, named or unnamed, may be appropriate to use. An exciting direction is to employ reactions to selectively cleave a stable chemical bond.[101] This strategy can be exploited to develop reagents (i.e., donors) that can be triggered to release a specific analyte (e.g., metal ion) to perturb normal cellular processes. Likewise, cleavage reactions can be used to deliver drug molecules with precision control. Another area that we anticipate will experience tremendous growth is the application of reversible reactions. Chemical tools based on these transformations could provide investigators a means to study dynamic processes. Lastly, to gain further spatiotemporal control, tools based on photochemically initiated reactions will allow for on-demand responses using light.[102]

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Bioconjugation—A Versatile and Essential Research Tool

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Abstract. Bioconjugation is now firmly established as an important component of successful research in many different fields. The ability to link one molecule to another or to a surface frequently forms the basis for the detection, assay, or targeting and tracking of biomolecules. Basic research, materials science, electronics, clinical diagnostics, and even drug development benefit from the science of bioconjugation.

Outline

1. Introduction

While bioconjugation is now well recognized as an indispensable tool in several areas of research, its methods can often be complicated. Moreover, the choice of the best reagent for a particular conjugation application can be imprecise and time-consuming, especially for someone who is relatively unfamiliar with the methodology. The optimal reagents and conditions for preparing a new bioconjugate can be determined by considering a number of factors, such as a reagent's chemical and physical properties, the functional groups it targets for coupling, its length, its molecular size, its water solubility, its cleavability, and precedents for its application in a given context. While a number of excellent treatments of bioconjugation have been published,[1] this review aims to bring up to date the state of the art, and simplify it enough to make it accessible to the widest audience possible.

2. Crosslinkers

2.1. Homobifunctional Crosslinkers

One of the most fundamental aspects of crosslinker design is whether the reagent is homobifunctional or heterobifunctional. The overwhelming majority of bioconjugate reagents are bifunctional, with two reactive groups usually located at the outer ends of an organic spacer. In a homobifunctional compound, the two reactive groups are identical, whereas in a heterobifunctional compound they are different.

Homobifunctional compounds will react at both ends with the same target functional group, thus forming a covalent crosslink between two molecules using the same type of bond. The most common type of homobifunctional reagent is one that reacts with amines, whereby it links together two different molecules each containing one or more amino groups. This means that proteins containing α-amines as well as lysine ε-amino groups can be conjugated in a single step using a homobifunctional, amine-reactive crosslinker. Some of the more popular homobifunctional, amine-reactive crosslinkers include the bis-imidoester compound dimethyl pimelimidate (DMP), the bis-NHS ester compounds disuccinimidyl tartrate (DST) and disuccinimidyl suberate (DSS), bis(sulfosuccinimidyl) suberate (BS^3), and ethylene glycol bis(sulfosuccinimidyl succinate) (sulfo-EGS) (**Figure 1**). When retention of the positive charge on proteins is desired, DMP is reacted with amino groups to form a positively charged amidine bond. The NHS ester and sulfo-NHS ester compounds react with amino groups to form amide bonds, which are more stable than amidine bonds. These reagents have been utilized extensively to capture and study interacting proteins in cells and other biological samples.

Other homobifunctional crosslinkers, with maleimide groups at each end, react with thiol groups to form stable thioether bonds. These compounds can link two biomolecules containing cysteine side-chain thiols, or, in some cases, they can even bridge the gap between two thiols created by disulfide reduction of a protein. Some examples of these reagents include bis(maleimido)ethane (BMOE), dithio-bis(maleimido)ethane (DTME), 1,8-bis(maleimido)diethylene glycol [$BM(PEG)_2$], and 1,11-bis(maleimido)triethylene glycol [$BM(PEG)_3$] (**Figure 2**, Part (a)).

Another type of homobifunctional, thiol-reactive crosslinker is 1,4-bis[3-(2-pyridyldithio)propionamido]butane, which, instead of maleimide groups, has a pyridyldithiol group on each end (Figure 2, Part (b)). This reactive group couples with thiols to form a reversible disulfide linkage, which can be reduced with DTT or another reducing agent to cleave the crosslink after it has formed. In some applications, the ability to reductively cleave the linkage is important for activity of the conjugate. For example, pyridyldithiol coupling has been utilized to form antibody–drug conjugates (ADC) for targeting tumor cells. The resultant dithiol linkage between the antibody and the drug gets cleaved after the bioconjugate is internalized by the cancer cell, thus releasing the toxic drug to kill the tumor.

The main shortcoming of homobifunctional crosslinkers is that they can easily polymerize proteins or other molecules containing multiple target functional groups. Such multiple coupling reactions with each protein being conjugated can lead the crosslinking molecules to act as bridges for oligomerizing proteins and creating large-molecular-weight complexes. This can be a problem when needing highly defined and reproducible conjugates between two proteins, since it would be difficult to limit such polymerizations simply by adjusting the molar ratios of the reactants or by controlling the reaction time.

On the other hand, the two most important applications of homobifunctional crosslinkers, and in which they perform very well, are: (i) the capture of interacting proteins by rapidly reacting with them while they are bound together, and (ii) the modification or activation of particles and surfaces. When using homobifunctional reagents to study protein interactions, it is important to limit their concentration so that only those proteins truly interacting are crosslinked and bound together, but not other proteins in the cellular environment nearby. Often, homobifunctional NHS esters are chosen for this purpose, because the interacting proteins usually contain amino groups capable of reacting with the NHS ester, and the reaction rate is fast enough to capture most interactions, except for highly transient ones. This approach has been employed successfully for decades to discover many of the key intracellular protein interactions.[2]

In addition, the use of homobifunctional compounds to modify and activate insoluble matrices such as particles and surfaces is highly valuable and very controllable. In this case, a solid support covered with functional groups (such as amines) can be reacted with an excess of a homobifunctional crosslinker (such as a bis-NHS ester) to create a monolayer of tethers, each of which terminating in a reactive group projecting from the surface and free to be coupled with another molecule, such as a protein. With particles, the use of a large excess of crosslinker during the initial modification reaction prevents particle-to-particle polymerization or undesirable linkages between functional groups on the same particle. In this respect, homobifunctional crosslinkers can function as building blocks to create linker arms and reactive groups on particles and surfaces of all types. After the first modification reaction of a surface or particle, any excess reagent is then simply washed away before using the other reactive end of the attached crosslinker to couple and immobilize a protein or another affinity molecule. The choice of the crosslinker's spacer or cross-bridge also regulates the resultant surface properties of the solid phase after the reaction. For example, a long, hydrophilic spacer (e.g., one containing polyethylene glycol (PEG) units) can provide increased biocompatibility and dramatically reduce nonspecific binding to a particle or surface.

Homobifunctional crosslinkers have also been utilized to create protein–protein conjugates. Some of the very first applications of these crosslinkers were in the formation of antibody–enzyme conjugates for use in immunoassays.[3] Early examples of these reactions used bis-imidoester or bis-aldehyde linkers such as DMP or glutaraldehyde. However, the drawback of forming antibody or protein conjugates with homobifunctional reagents is that each protein typically has more than one group that can react with either end of the crosslinker. Thus, as the reaction progresses, protein oligomers usually form, as multiple linkers on each protein cause polymerization. Very large complexes can result from this reaction, some of which actually precipitate from solution as clumpy or cloudy masses within the solution.[4]

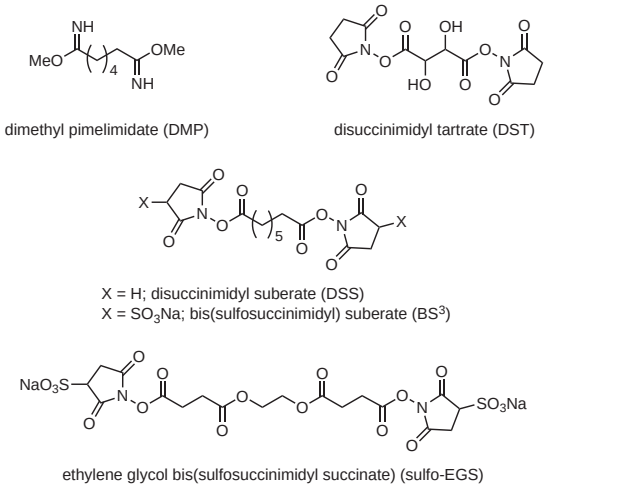


Figure 1. Popular, Amine-Reactive Homobifunctional Crosslinkers.

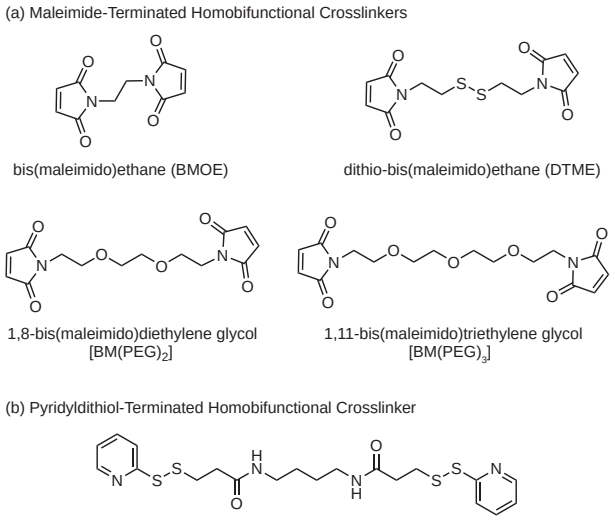


Figure 2. Popular, Thiol-Reactive Homobifunctional Crosslinkers.

2.2. Heterobifunctional Crosslinkers

Heterobifunctional reagents have major advantages over homobifunctional ones when forming bioconjugates, since one reactive end group would couple with only a specific functional group, while the other reactive end group would react with a different functional group. The discrete nature of this process makes two-step reactions possible, which has the ability to limit undesirable reactions, such as polymerizations during conjugate formation, and thus restrict the size of the resultant complex. In the case of proteins, the first reaction leads to an activated protein intermediate. This activated protein is then mixed with a second protein designed to contain one or more functional groups that were not present on the first protein. Under the right conditions, the remaining reactive, free end group on the crosslinker-modified first protein reacts with the functional targets on the second protein and forms a covalent crosslink or tether between the two proteins, thus creating the final bioconjugate. This is particularly important when the desired result is to create a known molar ratio of the two proteins in the final complex, and do so reproducibly. For instance, in the production of antibody–enzyme conjugates for immunoassays, it is desirable to modify each antibody molecule with multiple enzyme molecules in order to maximize the substrate signal produced by each conjugate. This type of reaction can be carried out repeatedly with a great deal of precision, leading to reproducible detection reagents for the most critical of immunoassay tests, such as in assays designed for clinical diagnostics.[5]

Some of the more popular choices in heterobifunctional crosslinkers include reagents with an amine-reactive NHS ester on one end and a thiol-reactive maleimide group on the other end. Examples include SMCC [4-(*N*-maleimidomethyl)cyclohexane-1-carboxylic acid *N*-hydroxysuccinimide ester] and sulfo-SMCC [4-(*N*-maleimidomethyl)cyclohexane-1-carboxylic acid 3-sulfo-*N*-hydroxysuccinimide ester sodium salt], which have been in use since the 1970s.[6] SMCC is a hydrophobic reagent that contains a cyclohexane ring in its cross-bridge, whereas sulfo-SMCC has a nearly identical structure and reactivity, but also has a sulfonate group on its NHS ester ring (**Figure 3**, Part (a)). This negatively charged sulfonate provides enough polarity to make the compound initially water soluble. The negative charge also permits the labeling of cell surface proteins and avoids penetration of the cell membrane and labeling of intracellular proteins. Other amine-reactive and thiol-reactive heterobifunctionals contain a bromoacetyl, iodoacetyl (e.g., SIAB), or a pyridyldithiol group (e.g., SPDP) instead of a maleimide group (Figure 3, Part (b)).

Another important class of amine-reactive and thiol-reactive crosslinkers is the one incorporating a hydrophilic polyethylene glycol (PEG) based cross-bridge. Such reagents often are extremely water soluble, and can provide increased solubility of the conjugates formed from them. Heterobifunctional PEG-based compounds can be used to impart biocompatibility to surfaces or particles before coupling an antibody or an affinity ligand. Examples of these compounds include maleimide-PEG_{12}-succinimidyl ester (and other PEG chain lengths in this reagent family), dibenzocyclooctyne-PEG_4-maleimide (an azide-reactive and thiol-reactive compound) (Figure 3, Part (c)), and PEG_{12}-SPDP (which is the PEG-based counterpart to the hydrophobic SPDP reagent).

2.3. Functional Groups Targeted

The most reactive functional groups in biomolecules are associated with the heteroatoms N, O, and S, which are nucleophilic due to an unshared pair of electrons and can spontaneously react with the compatible and electrophilic active groups on crosslinkers and modification reagents. In many cases, the nucleophilic functional groups in biomolecules are free and accessible, but in some instances they have to be created to allow reactivity and coupling to take place. There are several specialized reagents available that facilitate the creation of an appropriate functional group for bioconjugation if the desired one is not available. An example of this type of modification reagent is 2-iminothiolane (Traut's reagent; 2-IT),[7] which is a thiolation agent that reacts with an amine to create a free thiol at the end of a short tether. This free thiol can then be utilized for conjugation with thiol-reactive reagents.

Naturally occurring functional groups on biomolecules may consist of any combination of amines, thiols, hydroxyls, carboxylates, aldehydes, organic phosphates, and reactive hydrogens on certain activated carbon atoms. Amines, thiols, and hydroxyls are the main nucleophilic groups, and, under the right conditions, they react directly with the electrophilic reactive groups present on many bioconjugation reagents. In contrast, functional groups consisting of carboxylates, aldehydes, organic phosphates, and reactive hydrogen sites require special activation agents or secondary coupling agents before they will form covalent bonds with another functional group.

2.3.1. Amines

Amines are present in the overwhelming majority of proteins, and, for this reason, they are the number one functional group targeted for bioconjugation. Amines can be found at the N-terminal α-amino group of each polypeptide chain within a protein, provided it is not post-translationally modified and blocked (e.g., through acetylation). Moreover, the most abundant sites for amine conjugation are at lysine side-chain ε-amines that often occur several times within a polypeptide sequence. Large proteins or antibodies can contain dozens of lysine amines on their surfaces, many of which are unmodified and sufficiently accessible within the folded structure of a polypeptide for easy targeting

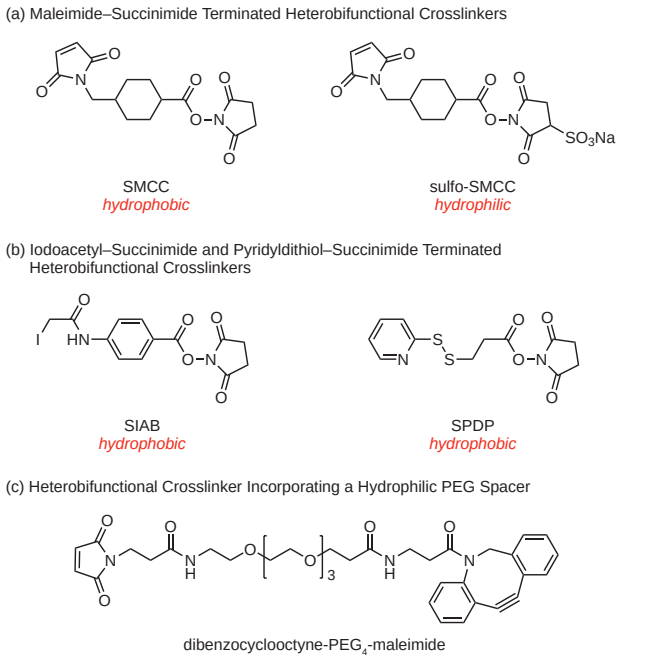


Figure 3. Popular, Amine- and Thiol-Reactive Heterobifunctional Crosslinkers.

and bioconjugation. The products of these conjugation reactions can form very stable amide bonds (e.g., acylation of the amine with an NHS ester or sulfo-NHS ester) or secondary amine bonds (alkylation of the amine), resulting in stable conjugates that are useful in a large variety of applications.

Another important reaction that amines undergo is rapid coupling to an activated phenyl azide group, which is present in many photoreactive crosslinkers. An example of this type of compound is 4-azidophenacyl bromide [4-$N_3C_6H_4C(=O)CH_2Br$], a thiol-reactive and photoreactive reagent that can be used to study protein interactions. Photoactivation of the phenyl azide group with UV light creates an extremely reactive nitrene, which undergoes ring expansion to a dehydroazepine. This reactive group then conjugates with any amine within its reach, forming a secondary amine linkage to a seven-membered ring.[8]

2.3.2. Thiols

Thiols are very good targets for bioconjugation, but since they are often found as disulfide-linked cysteine residues buried within the 3-D structure of a protein, they are not always accessible for conjugation without prior reduction. In some cases, the reduction of a disulfide has to be combined with denaturation conditions in order to unfold and open up the inner structure of the protein. In other cases, the protein subunits may be bonded together through disulfide linkages, and their reduction may result in breakdown of the quaternary structure and loss of activity or, at least, may result in major conformational changes of the protein. Even though disulfide reduction is not always possible without loss of protein structural integrity, antibodies are the one important class of proteins that have been successfully targeted for conjugation by limited reduction of the disulfide linkages without loss of antigen-binding activity.

By employing this strategy, antibody–drug conjugates (ADCs) have been produced after mild reduction of heavy-light chain disulfides to reveal just a few thiols in the structure of the monoclonal antibody cAC10. The free thiols were then successfully conjugated to a cytotoxic drug molecule.[9] In addition, antibodies have been immobilized on solid supports through thiols, and labeled with biotin or fluorescent dyes using thiol reactions.[10–12] The use of disulfide reduction of antibodies as a strategy for bioconjugation has one important benefit in that it keeps any linked proteins or other molecules away from the binding sites at the ends of the Fab regions, and thus does not block them or inhibit the docking of antigens.

2.3.3. Hydroxyls

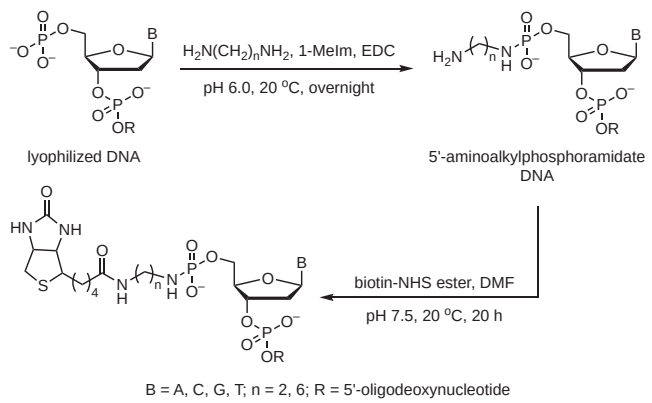
Hydroxyl groups can be found at serine, threonine, and tyrosine residues in proteins as well as on sugars and carbohydrates. They are also present on certain phospholipids and on glycans and glycoproteins, which often decorate cells, proteins, and lipids. Hydroxyl groups are nucleophilic; however, their relative nucleophilicity is less than that of amines and thiols, and thus they are less reactive. In aqueous solution, a hydroxyl group has about the same degree of nucleophilicity as that of the oxygen atom in water, and, since water is in much higher concentration than any OH groups on biomolecules, hydrolysis becomes the predominant reaction, which severely limits the successful conjugation of the hydroxyl group. In addition, if there are any other more nucleophilic groups present in a molecule that contains one or more hydroxyls, then a crosslinker has a much greater chance of reacting with those more reactive functional groups than conjugating to an OH group. An exception to this rule is the case where certain nucleophilic groups in a protein are, in a 3-D structural sense, nearby in sequence, or adjacent, to a hydroxyl-containing residue. If the reaction of the electrophilic crosslinker with such neighboring nucleophiles is transient or reversible—as in the case of an NHS ester reacting with a histidine imidazole group or temporarily hydrogen bonding with an arginine guanidino group—then labeling of a neighboring OH group in serine, threonine, or tyrosine can take place through secondary transfer from the histidine or arginine.[13,14]

Some hydroxyl groups in biomolecules can also be used to create reactive sites for bioconjugation with another molecule. In particular, if there are adjacent hydroxyl groups (1,2-diols), they can be oxidized to form reactive groups. This is especially useful when no other functional group is available for targeting. This approach can also be employed if a hydroxyl group is situated next to an amine, such as at an N-terminal serine residue in peptides, where the α-amino group is next to the serine side-chain hydroxyl and is thus susceptible to oxidation.

Diols are usually found on certain sugars, carbohydrates, glycans, and on the 3' ribose group of RNA. In these cases, one or more reactive aldehyde groups can be generated through periodate oxidation of the diol, which cleaves the carbon–carbon bond between the hydroxyl groups, and oxidizes them to aldehyde groups. Aldehydes created in this manner undergo reductive amination with amines.[15] The aldehydes can also react with hydrazide- or aminooxy-containing crosslinkers to form hydrazone or oxime bonds, respectively (see Section 5).[16–21]

2.3.4. Carboxylates and Organic Phosphates

Carboxylates and organic phosphates are two functional groups very commonly found in biomolecules. Unfortunately, these two groups are not very nucleophilic, and thus do not spontaneously react with the electrophilic reactive groups on crosslinkers or modification reagents. They are also similar in that carbodiimides can activate each to a reactive ester, which can then be utilized to acylate amines on other molecules to form an amide bond (from the carboxylate) or a phosphoramidate linkage (from the organic phosphate). The majority of all conjugation reactions of carboxylates or organic phosphates on biomolecules are accomplished using the water-soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide [EDC, $EtN{=}C{=}N(CH_2)_3NMe_2$] (**Scheme 1**).[22] Moreover, when activating organic phosphate groups with EDC, imidazole is typically added to the reaction to form the intermediate phosphorylimidazolide, which then more effectively couples to amino groups to form phosphoramidate bonds.[22–24]



Scheme 1. Labeling of the 5'-Terminus of Synthetic Oligodeoxyribonucleotides with a Biotinyl Residue. *(Ref. 22)*

2.4. Crosslinker Length

The dimensions or overall linear length of the target molecule before and after conjugation should be considered when choosing a crosslinker or modification reagent for the conjugation reaction. The spacer arm or cross-bridge of the reagent mainly determines the molecular length of the resulting compound. The length of a crosslinking reagent can be determined by use of certain molecular modeling software programs. The crosslinker is initially drawn as linearly as possible using a chemical drawing program and then saved as a three-dimensional model using the protein data bank file format (.pdb) or in a similar format that preserves the coordinates of each atom. Once the saved molecule is opened in a molecular modeling program, it is displayed in three-dimensional space. Its energy is then minimized to orient the structure as realistically as possible based on its bond angles and bond lengths. Many modeling programs then permit the measurement of the distance between any two atoms in the structure in angstroms or nanometers. If the length of a crosslinking reagent is given by the supplier, it usually refers to the approximate maximal molecular distance between the atoms of the crosslinker that are directly linked to the two molecules being conjugated. Although the molecular length of crosslinkers in real-world applications will reflect the vibrations, bending, and folding of such molecules as they undergo molecular dynamic changes in solution,[25] the relative calculated linear lengths of different crosslinkers can be used to estimate distances between crosslinked proteins. Crosslinkers of different sizes thus become molecular rulers for measuring the distances between functional groups in biomolecules. The use of crosslinkers to perform protein structural modeling in this way can lead to the discovery of the conformation of individual proteins, and can determine the orientation of protein complexes formed from multiple interacting proteins.[26] Even the binding domains within proteins have been analyzed using crosslinkers as molecular rulers.[27]

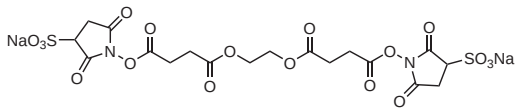
It is possible to eliminate any spacer group between two crosslinked molecules. For instance, a carbodiimide such as EDC is an activating agent that forms an intermediate reactive ester from a carboxylic acid. The reaction of this ester with an amine would result in an amide bond, which is an important step in forming a direct link between two molecules.[28] Short cross-bridges (<5 Å) can be formed from near-neighbor functional groups, or can span very short distances between two biomolecules. Some short-length reagents have even been utilized to covalently crosslink two thiols formed from disulfide reduction. A third arm on this type of linker can enable this bridge to be used to conjugate another molecule to the original site of the disulfide bond within a protein.[29] This type of specialized, short trifunctional crosslinker can help maintain the original conformation of an antibody or protein even after a critical disulfide bond has been broken. It can also create a site of modification away from binding sites or active centers on proteins and enzymes. Crosslinkers of moderate length (5–20 Å) have been employed to produce almost every type of bioconjugate complex imaginable. This includes creating linkages between two proteins to form a defined conjugate, capturing protein–protein interactions, and coupling biomolecules to particles or surfaces. Reagents in this size range offer many options for reactive groups and spacer arms in addition to being hydrophobic or hydrophilic, and cleavable or noncleavable.

Extremely long spacer arms, spanning distances >20 Å in length and sometimes even >100 Å, allow for greater distances between crosslinked molecules. Included in this set of bioconjugation reagents are some of the discrete and polymeric polyethylene glycol (PEG) containing compounds, which can have repeating ethylene oxide units in their spacer arms of even longer than PEG_{24}. A long linker arm also creates a longer span between the point of modification on a protein and the detection molecule or affinity tag that is attached to the other end of the linker. Furthermore, this often provides greater access to other binding molecules, as in the case of streptavidin binding to a long, PEG-based biotin tag on an antibody. When the conjugation reaction is carried out in aqueous medium, it is crucial to use hydrophilic spacers, because long hydrophobic cross-bridges would only get buried in hydrophobic pockets in proteins or cause unacceptable nonspecific binding or aggregation in conjugates. By contrast, long PEG-based crosslinkers and modification reagents maintain extreme hydrophilicity and very low nonspecific binding. In fact, modification with long PEG-based reagents actually improves the hydrophilicity and dramatically decreases the tendency of the conjugate to aggregate, as compared to the corresponding antibodies or proteins before being modified.[30]

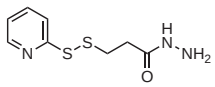
2.5. Cleavable vs Noncleavable Crosslinkers

If interacting biomolecules that have been captured by crosslinking subsequently need to be isolated and analyzed, it is important for the spacer arm of the crosslinker to be cleavable (**Figure 4**). For example, a purified protein is modified with a heterobifunctional crosslinker through its amine-reactive end, while the other end of the linker contains a photoreactive group that is capable of covalently linking to any nearby proteins after being exposed to UV light. The modified protein containing the photoreactive group is mixed with a biological sample to fish out any potential binding partners. After an incubation period, the sample is photolyzed with UV light and the photoreactive end of the modifying linker is then able to crosslink to any unknown interacting proteins within molecular reach. The interaction complex is then isolated using an immobilized antibody against the initial bait protein or through other known affinity interactions. After purification, the unknown interacting proteins can then be isolated away from the bait protein for individual analysis. It makes it easier to determine what proteins have bound to the bait protein if the photo-crosslinks can be reversed, and the unknown proteins can be obtained in purified form away from any covalent conjugates.[31]

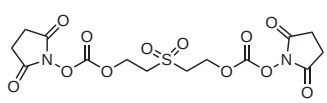
Another important application of cleavable linkers is in the use of label transfer reagents for the study of interacting proteins. In this instance, a trifunctional crosslinker is utilized that contains a spontaneously reactive (i.e., an NHS ester) component on one arm, a photoreactive group on a second arm, and a label, that is either detectable (e.g., fluorescent dye) or has an affinity tag (e.g., biotin) at its end, on the third arm. The linker



ethylene glycol bis(sulfosuccinimidyl succinate) (sulfo-EGS)
(ester groups cleavable with H_2NOH)



3-(2-pyridyldithio)propanoyl hydrazide (PDPH)
(disulfide cleavable with DTT, 2-MEA, BME, or TCEP)



bis[2-(*N*-succinimidyloxycarbonyloxy)ethyl] sulfone (BSOCOES)
(sulfone cleavable with TRIS at pH 11)

Figure 4. Crosslinkers with Cleavable Spacer Arms.

to the spontaneously thermoreactive group is also designed to have a chemically cleavable site within its cross-bridge to permit subsequent release of the bait protein. The bait protein is first labeled with the trifunctional reagent through the thermoreactive group and then allowed to interact with proteins in a sample. After an incubation period, the sample is exposed to UV light to crosslink any interacting proteins. If the trifunctional crosslinker has a biotin handle, then the entire complex can be purified using an immobilized streptavidin support. Once the non-interacting components are washed off the support, the cleavable linker is broken and the bait protein is removed. What is left on the support is any of the interacting proteins that were captured during the photoreaction process. After the cleavage reaction has taken place, the arm containing the label is effectively transferred to the interacting proteins from the initially labeled bait protein. This is the reason these crosslinkers are called label transfer reagents.

2.5.1. Cleavable Disulfides

Crosslinkers and modification reagents containing a disulfide group within their spacer arms are some the most common cleavable linkers, and allow for the easy reversal of a conjugate through disulfide reduction. An example of this type of cleavable crosslinker is 3-(2-pyridyldithio)propionyl hydrazide (PDPH), which contains a thiol-reactive pyridyldithiol group at one end and an aldehyde-reactive hydrazide group at the other end. The pyridyldithiol group couples with thiols in proteins or other molecules to form a disulfide bond. Incubation of the conjugate with a disulfide reducing agent such as dithiothreitol (DTT), 2-mercaptoethylamine (2-MEA), β-mercaptoethanol (BME), or tris-carboxyethyl phosphine (TCEP) can be utilized to cleave the disulfide crosslink.[32]

2.5.2. Cleavable Esters

Some crosslinkers contain relatively stable ester bonds within their cross-bridges, which can be cleaved only with a strong base such as hydroxylamine (H_2NOH). For instance, sulfo-EGS (see Figures 1 and 4) contains two esters within its spacer formed by esterification of the carboxylates of succinic acid with the hydroxyls of ethylene glycol. Both ester bonds are completely cleaved with 0.1 M hydroxylamine, when the incubation is carried out at 37 °C and pH 8.5 for at least 3 hours.[33]

2.5.3. Cleavable Sulfones

An example of this type of cleavable reagent is bis[2-(*N*-succinimidyloxycarbonyloxy)ethyl] sulfone (BSOCOES), which is a homobifunctional compound with a central sulfone group surrounded at both ends by amine-reactive NHS carbonate groups. After BSOCOES crosslinks proteins in a sample—perhaps to capture interacting proteins—and the conjugates isolated from the rest of the proteins, then any interacting proteins can be purified from the bait protein by cleavage of the sulfone (0.1 M TRIS, pH 11, 37° C, 2 h).[34] In certain circumstances, denaturing conditions can also be used to disrupt any strong protein interactions and permit isolation of the interacting proteins.

2.6. Hydrophobic vs Hydrophilic Crosslinkers

Many of the early reagents for crosslinking and modification employed aliphatic or aromatic moieties, which imparted a generally hydrophobic nature to the reagents. Even with the advent of sulfo-NHS esters, which possess a strong negative charge and water solubility, their underlying base chemical structure remains quite hydrophobic. In some applications, reagent hydrophobicity can be an advantage, especially when an application involves a needed penetration of cell membranes. Hydrophobic reagents without any strongly polar groups will be able to quickly pass through membranes and crosslink or label internal cell proteins. However, those otherwise hydrophobic compounds that contain one or more negatively charged sulfo-NHS groups will still be restricted to reacting with the proteins on the outer membrane surfaces of cells due to their negative charge. The ability to switch between cell surface labeling and internal cellular labeling just by choosing charged or uncharged reagents is one benefit of using hydrophobic crosslinkers. For instance, the homobifunctional compound DSS has two uncharged NHS esters, is very hydrophobic, and, therefore, can crosslink interacting proteins within cells. By contrast, the similar compound BS^3 has two negatively charged sulfo-NHS esters and will be restricted to labeling outer membrane proteins. The two compounds are otherwise identical in their cross-bridge structure and reactivity.

In recent years, relatively short and discrete polyethylene glycol (PEG) spacers have resulted in new crosslinking and modification reagents that are extremely water soluble without the use of additional charged groups. The advantages of using such reagents are many: They can enhance the performance of a bioconjugate by increasing sensitivity in assays, enhancing signal-to-noise ratios, decreasing nonspecific binding, lowering off-target effects, and improving the stability of conjugates by decreasing aggregation. For example, using non-PEG hydrophobic reagents in the modification of antibodies often results in aggregation or precipitation in aqueous solution with potential loss of antigen binding activity.[35] Using PEG-based reagents instead doesn't cause aggregation or precipitation of antibodies or proteins, and dramatically increases the overall hydrophilicity and water solubility of antibodies and proteins modified by them. The use of hydrophilic bioconjugation reagents also results in greater biocompatibility. In contrast to conjugates or proteins crosslinked with hydrophobic reagents, those modified with PEG-based reagents will result in lower off-target effects in vivo, and will significantly reduce backgrounds in assays.[36]

Microparticles or nanoparticles that have a tendency to clump and aggregate in aqueous solution can be modified with PEG-based linkers to eliminate particle interactions and prevent aggregation. Creating a lawn or monolayer of PEG modifications on a particle or surface also can reduce nonspecific binding of biomolecules during assay or capture of target molecules. PEG-based reagents can nearly eliminate background due to nonspecific binding events and enhance signal in assays, because of less fouling of nonspecifically bound proteins at the surfaces.[37]

3. Conjugation of Amino Groups with Reactive Groups

Only a few of the many types of reactive esters that have been developed for use in bioconjugation have become reagents of choice for forming amide bonds with amine-containing molecules. The most popular of these active esters facilitate reactions that can easily occur in either organic solvents or in aqueous media at or near physiological pH. In the majority of such cases, proteins and other sensitive biomolecules can be quickly modified with reactive esters without loss of activity.

3.1. *N*-Hydroxysuccinimide (NHS) Esters

The electron-withdrawing ability of the two carbonyl oxygens in the *N*-hydroxysuccinimide ring makes the active ester—formed from NHS and a carboxylate—unstable enough to be readily displaced (primarily in an S_N2-type mechanism) upon attack by a primary amine nucleophile on the biomolecule to be crosslinked. An example of this reaction is illustrated for laminin β1 and laminin γ1 (**Scheme 2**).[13] The best amine nucleophile for the reaction with an NHS ester is one that

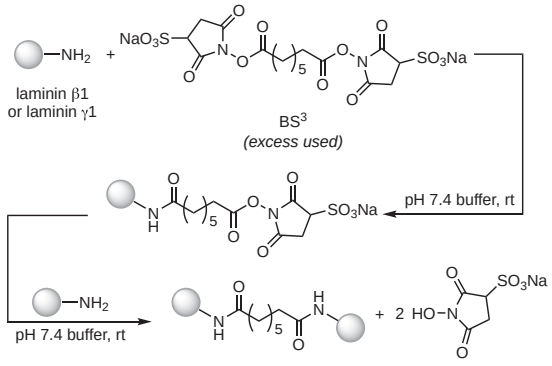
has its unshared pair of electrons fully exposed for interaction with the partial positive charge on the carbonyl group of the ester. Moreover, a primary amine that is in an unprotonated state in aqueous solution will make the best nucleophile for participation in the substitution reaction, and thus the most favorable conditions for conjugation are at pH values slightly above (and, optimally for many, at least one pH unit above) the pK_a of the amine groups being targeted for modification.

This dependence of reactivity on the protonation state of an amine-containing compound in solution, or of an amine group as part of a complex protein, is not as absolute as it might appear at first. For instance, lysine dissolved in an aqueous solution has a pK_a for its α-amine of approximately 8.9 and for its side-chain ε-amine of about 10.5. These pK_a values might lead one to believe that an NHS ester would not efficiently react with the α-amine of lysine until the pH exceeds pH 8.9, and with the ε-amine until the pH is above 10.5. In reality, the crosslinking reactions do occur over a broad pH range, even at pH values much lower than the pK_a of the amine. The pK_a values for lysine represent the pH values at which exactly 50% of the α- or ε-amine groups are protonated. Since the protonated and unprotonated forms are in equilibrium, even at pH units above or below the pK_a, there will still be enough protonated or unprotonated form present for the reaction to take place. Complicating matters further, lysine amines in proteins can have different pK_a values than they do when lysine is in solution. This is due to the intricate folded structure of protein chains that can cause micro-environmental changes to the protonation state or pK_a of an ionizable amino acid residue. For this reason, the pH titration curve of a protein is often considerably more complex than might be suggested by the titration curve of each independent amino acid residue. This is particularly the case when two ionizable residues can interact in the three-dimensional structure of a protein, perhaps forming salt bridges. Such interacting residues may have protonation states at a given pH far different than their solution-phase pK_a values would indicate.[38]

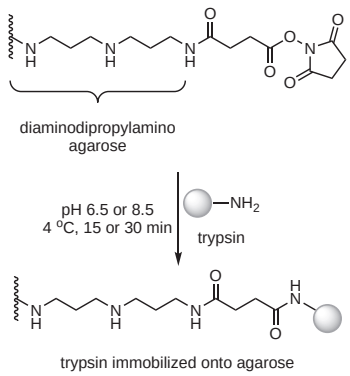
Isom et al. have reported that the ε-amine of lysine can undergo large shifts in its pK_a value, depending on where the side chain is located within the 3-D structure of staphylococcal nuclease.[39] After creating 25 mutants of the nuclease with lysine at different positions, the authors found pK_a values ranging from 5.3 to 10.4. They also discovered that, when the lysine residue was somewhat buried and therefore around more hydrophobic regions in the polypeptide chains, its pK_a was significantly depressed. This means that reactions with NHS esters may occur with high efficiency at physiological pH values, even if lysine in solution displays a much more alkaline pK_a. Since the population of protonated, [BH^+], and unprotonated, [B], lysine ε-amine groups is always in equilibrium at a given pH, the existence of even a small percentage of unprotonated amine will cause reaction with NHS esters and result in amide bond formation, as well as a shift in the [B]/[BH^+] equilibrium toward generating more [B] and more reaction, even if the pH is well below the pK_a of the amine. For these reasons, the reactivity of amino groups in proteins toward acylation with NHS esters occurs over a much broader pH range and at far more acidic values than their individual pK_a values might imply. In practice, reactions can be carried out with high efficiency and in good yields with ε-amino groups down to about pH 7, and with N-terminal α-amino groups down to about pH 5–6.

The amino group reactivity within proteins is also dependent upon *where* in the 3-D structure it resides. Leavell et al. probed the NHS ester reactivity toward primary amines in ubiquitin using mass spectrometry.[40] They found that the N-terminal amine on methionine, along with several other lysine ε-amines, were highly accessible and highly reactive, while several other amines were moderately reactive. However, they also discovered that there was a group of lysine ε-amines that were not reactive, and they correlated these residues to certain locations within the crystal structure of the protein that were involved with strong hydrogen-bonding interactions with neighboring amino acids. The results showed that the number of crosslinks observed in ubiquitin and their locations clearly followed the relative reactivity of the amines within the protein molecule. These observations are similar to the findings of Gibson and co-workers that certain lysine ε-amines in bovine cytochrome c were highly reactive in forming intraprotein crosslinks, while others were not reactive at all.[41]

An early study by Cuatrecasas and Parikh on the use of NHS ester reactive groups for the immobilization of proteins onto agarose chromatography supports revealed that proteins with aliphatic or aromatic amino groups could be immobilized through stable amide bonds over a pH range of 6–9 (**eq 1**).[42] The authors found that the rate of the crosslinking reaction over a given time frame and its yield were greater at pH 8.6 than at pH 6.3, but that the stability of the NHS ester groups toward hydrolysis was much greater at the lower pH. In addition, when the concentration of the amine-containing ligand was increased at both the low and high pH conditions, the lower pH reaction resulted in greater coupling yield than the higher pH reaction. The major reason for this result was a consequence of the rate of NHS ester hydrolysis in different pH environments.



Scheme 2. Example of the Crosslinking Reaction of *N*-Hydroxysuccinimide (NHS) Esters with Amines. *(Ref. 13)*



eq 1 *(Ref. 42)*

3.1.1. Hydrolysis of NHS Esters

The main competing reaction with the acylation of amines with NHS esters in aqueous medium is hydrolysis of the ester with water. Even though the oxygen in water is less nucleophilic than the nitrogen atom in the amine, water is typically far more abundant in concentration than the amine-containing molecule targeted for conjugation. These two competing reactions, crosslinking and hydrolysis, occur at varying rates at every pH value in aqueous solution, and it is finding the optimal balance between them that results in the best yield of amide bond formation. Hydrolysis of an NHS ester can occur at any pH, but the reaction rate generally increases as the environment becomes more alkaline. After only 40 min in aqueous solution at pH 8.6, over 80% of the NHS ester of agarose is hydrolyzed, whereas at pH 6.3 only about 23% is hydrolyzed over the same period (**Figure 5**).[42]

The stability of NHS esters toward hydrolysis in aqueous solution also depends upon the type of bioconjugation reagent containing the ester group. Both the overall hydrophobicity of the reagent and the relative hydrophobicity of the part of the reagent immediately adjacent to the ester can influence its hydrolysis rate. Thus, an NHS ester that is a part of an aliphatic, water-insoluble crosslinker often has a half-life in aqueous solution that is much longer than that of a similar reagent formed using water-soluble components. Even for extremely water-soluble crosslinkers, a hydrophobic moiety adjacent to the active ester group can significantly extend the latter's half-life. In this regard, PEG-based NHS esters are more hydrolytically stable when there is a longer aliphatic component immediately next to the ester but before the hydrophilic PEG chain. The presence of a single methylene group between the PEG chain and the carbonyl group of the NHS ester results in a highly reactive reagent, with a hydrolysis half-life at pH 8 and 25 °C of only $t_{1/2}$ = 0.75 min (hydrolyzes so fast it is almost unusable in aqueous solutions). Introducing one additional methylene group into the bridge between the PEG chain and the ester decreases the hydrolysis rate and increases the half-life to $t_{1/2}$ = 16.5 min, while inserting three methylene groups into the bridge decreases the hydrolysis rate further and increases the half-life to $t_{1/2}$ = 23 min (**Figure 6**).[43]

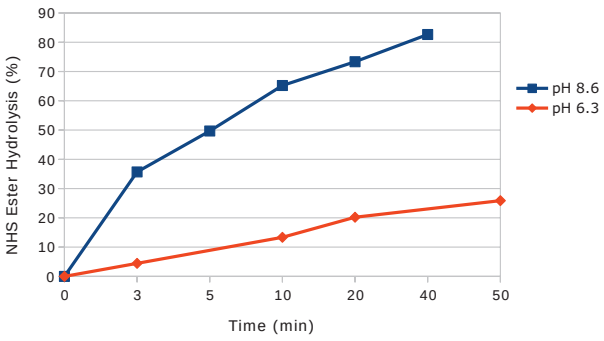
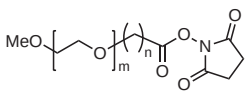


Figure 5. NHS Ester Hydrolysis at Different pH Values. *(Ref. 42)*



Abbr.	n	$t_{½}$, min
SCM-PEG	1	0.75
SPA-PEG	2	16.5
SBA-PEG	3	23

NHS ester hydrolysis half-life at pH 8 and 25 °C.

Figure 6. Effect of the Increasingly Hydrophobic Environment Adjacent to the NHS Ester Group on the Hydrolysis Rate of the Latter. *(Ref. 43)*

For many reactions in aqueous buffers, an NHS ester containing compound will react with an amine with good yield and reasonable efficiency at a pH range of 7.0–7.5. While raising the pH to more alkaline conditions will accelerate the reaction, it would also greatly accelerate the hydrolysis process and possibly negate any advantage that the higher pH condition may create. However, for some hydrophobic NHS ester compounds, such as large fluorescent dyes, the labeling of protein amines at a pH around neutral would result in unacceptably slow reactivity and poor coupling yields. Even most fluorescent dyes with sulfonates or other hydrophilic groups attached to them to make them more water soluble are best reacted at a pH of about 8.5 to obtain the best modification efficiency on antibodies and other proteins.

3.1.2. NHS Ester Reactivity toward Amino Acids in Proteins

The reactivity of NHS esters toward amino acids in proteins is often thought to be solely directed toward the N-terminal α-amines as well as the ε-amino groups of lysine residues. However, NHS esters have also been reported to react with other amino acid side-chain groups. In one study, Zenobi and co-workers carried out epitope mapping on the bovine prion protein to locate the exact positions of coupling when using homobifunctional NHS ester crosslinkers.[44] The authors found that, when crosslinking a monoclonal antibody–prion protein complex with disuccinimidyl suberate (DSS) or disuccinimidyl glutarate (DSG), both serine and tyrosine hydroxyl groups were sites of crosslinks in addition to the primary amino groups. In another report, laminin β1 and laminin γ1 N-terminal recombinant constructs were studied by crosslinking using BS^3 and BS^2G [bis(sulfosuccinimidyl) glutarate]. Kalkhof and Sinz found that up to 20% of the linkages formed were with hydroxyl-containing residues.[13] Mass spectrometric analysis of the modified peptides showed that 12.5% of these linkages to hydroxyl groups were with serine, 4.3% with tyrosine, and 3% with threonine residues.

A systematic study of DSS reactivity utilized synthetic peptides to determine the nucleophilic residues most likely to be modified.[14] The peptides were protected at their N-terminal to prevent α-amines from reacting, and each contained one or two reactive residues in their sequence. These residues were derived from the hydrophilic amino acids lysine (ε-amino), serine (primary OH), threonine (secondary OH), tyrosine (aromatic OH), arginine (guanidino group), and histidine (imidazole group). For peptides containing only a single amino acid that could potentially couple with an NHS ester (Lys, Arg, His, Tyr, Thr, or Ser), conjugation occurred only to the peptide containing a lysine ε-amino group.[14] None of the other amino acids could couple when they occurred alone within the peptide sequence. The authors assumed that histidine likely reacted with the NHS ester, but that its rapid hydrolysis prevented them from observing the labeled product. However, when they placed two potentially reactive amino acids close together in the peptide sequence, then amino acids other than lysine were observed to couple. For instance, the presence of a His close to a Ser or Tyr residue resulted in the labeling of the OH group on these residues. The likely path for this reaction is through initial formation of an acyl imidazole with the His side-chain nitrogen, and, since such

an intermediate is highly reactive, the acyl group migrates to the neighboring hydroxyl group of the Ser or Tyr residue, before the acyl imidazolide is hydrolyzed by water. When Ser or Tyr appeared alone in the peptide sequence without a neighboring His, no modification was observed, because of the competing NHS ester hydrolysis, which takes place before the NHS ester has a chance to react with the hydroxyl groups. It is worth noting that, in the complex folded structure of a protein, His does not have to be close in sequence to a hydroxyl-containing amino acid, just close enough in the 3-D structure of the folded protein to allow acyl transfer.

Other groups have reported similar modifications to Ser, Thr, or Tyr in the course of biotinylating proteins with NHS ester biotinylation reagents.[45–50] For example, several NHS ester containing biotinylation reagents were tested for their amino acid reactivity in the decapeptide [D-Lys-6-labeled] gonadotropin-releasing hormone.[50] While the majority of biotinylated peptide was modified once at the D-Lys-6 side-chain ε-amino group, a significant amount was di-biotinylated and occurred through amine reaction with D-Lys-6 and O-acylation at Ser-4 or Tyr-5. The researchers also found a novel conjugation event with the Arg-8 guanidino group, which is typically unreactive toward NHS esters, because the guanidino group is nearly always protonated in aqueous solution and therefore not nucleophilic.

The effect of neighboring arginine on the ability to acylate Ser, Thr, or Tyr hydroxyl groups in proteins or peptides with NHS esters DSS or BS^3 has also been investigated.[51] Arginine may affect such reactions, most likely due to its ability to stabilize structural intermediates through charge interactions or hydrogen bonding. This study found that unblocked Arg residues next to the hydroxyl-containing amino acids caused an increase in the modification of the hydroxyl groups by the NHS ester, while peptides with blocked guanidino groups did not have their hydroxyl residues modified except in very small amounts. The proposed mechanism for the reaction has the crosslinker first reacting with an amino group using one end of the homobifunctional compound. The carbonyl group of the NHS ester at the other end of the linker then forms a hydrogen bond with the guanidino group of Arg. This association traps the crosslinker immediately next to the hydroxyl-containing amino acid, and allows it to esterify the OH group.

Since NHS ester crosslinkers and modification reagents are capable of reacting with a number of protein functional groups, the exact location of each coupling reaction within a peptide or complex protein may not be entirely predictable just through knowledge of its sequence, because hydrogen bonding and complex polypeptide chain folding can bring residues together and enhance or inhibit their reactivity. Therefore, if bioactivity is compromised after reaction with an NHS ester reagent, it may not be solely due to the modification of α-amines or lysine ε-amines, but could also be due to O-acylation at hydroxyl-containing amino acids or even, in rare cases, modification at exposed cysteine thiols or arginine guanidino groups. Mass spectrometric analysis may ultimately be the best method for determining which amino acid residues end up being modified with NHS ester reagents.

3.2. Sulfo-NHS Esters

Sulfo-NHS esters have nearly identical properties to NHS esters, but are particularly useful in aqueous solution. The first two members of this class of crosslinkers were synthesized by Staros.[52] The sulfonic acid group is deprotonated under all pH conditions useful for coupling with amines, and its resulting strongly polar character often will be enough to drive an entire, otherwise hydrophobic, crosslinker into solution in aqueous buffers. For example, the hydrophobic NHS ester DSS must first be solubilized in a water-miscible organic solvent such as DMF, DMSO, or DMAC before adding it to an aqueous reaction medium, whereas the structurally very similar, but hydrophilic sulfo-NHS ester BS^3 can be added directly to the aqueous medium. When a hydrophobic NHS ester crosslinker is predissolved in organic solvent and an aliquot added to an aqueous reaction medium, a cloudy micro-precipitate typically ensues. In contrast, with a sulfo-NHS ester—even when predissolved in an organic solvent to prevent prior hydrolysis of the ester—a micro-precipitate is not formed and, thus, the crosslinker has much better access to water-soluble biomolecules in the still-homogeneous reaction medium. Similarly, the water solubility of sulfo-SMCC results in a more efficient conjugation reaction—as compared to that of the hydrophobic SMCC—with a higher yield of amine modification during the initial amide-bond-forming step of the two-step conjugation process. It is worth mentioning that a sulfo-NHS ester containing compound may display initial water solubility due to the presence of the sulfonate group, but, once that leaving group is lost in the amine-modification step, the rest of the crosslinker (depending on its structure) may no longer possess its beneficial solubilizing effects.

Crosslinkers or modification agents containing a sulfo-NHS ester group are generally membrane impermeable and, thus, their reactions with amines would be restricted to the outer membrane proteins on the exterior cell surface. This can be a great advantage when studying protein interactions on cell surfaces through crosslinking, or when the isolation of membrane proteins using immobilized streptavidin is desired. However, when labeling a cell surface, a sulfo-NHS ester present on a PEG-based crosslinker or modification reagent may not always be cell impermeable despite the negative charge on the sulfonate. This is because the ethylene oxide units are both water soluble and organic soluble and, thus, may have a tendency to penetrate cell-membrane structures even though the reagent displays extreme hydrophilicity and water solubility. For this reason, the concentration of the reagent in the cell suspension may have to be adjusted downward to minimize or prevent intracellular labeling.

The reactions of NHS esters (or sulfo-NHS esters) with biomolecules are typically carried out in aqueous buffers containing perhaps salts and a number of other additives. Since buffers and additives are usually present in much higher concentrations than the proteins or biomolecules being modified, it is important that they not contain substances that compete with the protein amines for the ester of the crosslinker. Such interfering substances include nitrogenous buffers (e.g., TRIS and imidazole), and amino acids in buffers, such as glycine, which is often added to control pH. They also include any salt additive containing an ammonium ion such as ammonium sulfate—which is commonly used in fractionating proteins from cells and tissues or for stabilizing proteins—ammonium bicarbonate, ammonium citrate, ammonium acetate, and any other salt containing the NH_4^+ ion. Moreover, other functional groups that can react with NHS esters and lead to interfering side reactions are: (i) Azides, which are often found as preservatives in solutions. (ii) Urea, which is often used to extract proteins from cells and tissues or to re-solubilize proteins from inclusion bodies. Urea can break down into unstable carbamic acid, which then decomposes into CO_2 and ammonia. (iii) DTT or other types of thiol-containing reducing agent, which are commonly used to promote protein stability or prevent oxidation. All of these small-molecule interfering substances can be removed from extracts or solutions by simple dialysis or size-exclusion chromatography.

Another category of potentially interfering substances is other proteins or blocking agents that contain amine groups or other nucleophiles. Antibodies in particular are often supplied with BSA or some other protein-blocking agent, or even as crude antisera, all

of which may improve their stability in storage and prevent their aggregation, but will also cause them to compete with NHS ester reactions. However, antibodies can be purified away from other proteins by affinity chromatography on immobilized protein A or protein G, or by using an immobilized antigen.

3.3. Fluorophenyl Esters

Esters derived from fluorinated phenols are growing in popularity for conjugation applications.[53–58] These reactive esters are derived primarily from three different types of phenol: pentafluorophenyl (PFP), tetrafluorophenyl (TFP), and sulfo-tetrafluorophenyl (STP). PFP esters and TFP esters are uncharged and hydrophobic and much more so than NHS esters (**Figure 7**). For this reason, they often are more hydrolytically stable in aqueous buffers than NHS esters, and typically give higher yields of amide bonds than the corresponding NHS esters at a given reaction pH. An STP ester has greater hydrophilicity than its PFP and TFP counterparts, but less so than its sulfo-NHS ester analogue.

PFP esters and TFP esters are perhaps the most commonly used fluorophenyl esters in bioconjugation and in organic synthesis, and, similarly to NHS esters, they undergo nucleophilic substitution at a slightly basic pH. Fluorophenyl esters have been utilized extensively as amine-reactive groups for linking fluorescent dyes to antibodies to make probes for cells and tissues,[53] for coupling bifunctional metal chelates to antibodies to make radiolabeled probes for imaging,[54–56] for the conjugation of drugs to monoclonal antibodies to make ADCs,[57] and for immobilizing molecules onto surfaces.[58]

The presence of a TFP ester group on a PEG chain, which is extremely hydrophilic, provides increased stability toward hydrolysis of the ester group than when an NHS ester is present. As with their NHS ester counterparts, TFP esters can undergo several side reactions, including hydrolysis, reaction with OH groups in proteins and other biological molecules, and formation of inactive cyclic NHS derivatives when the TFP ester is located at the end of a succinic acid spacer (**Scheme 3**).[57] Medley and co-workers undertook a detailed study of the rate of TFP ester hydrolysis over a pH range of 3 to 9 and a temperature range of 10 to 30 ºC.[57] The authors reported that the TFP ester was most stable at pH 5–7, was also least reactive toward amines in this pH range, and that the reaction rate increased with increasing temperature over all pH conditions studied. Thus, the coupling reactions of TFP esters with amines are best carried out at ambient temperatures and pH conditions that are slightly alkaline (pH 7–8.5).

3.4. EDC (EDAC)

A carbodiimide is considered a zero-length crosslinker, because it creates a direct link between a carboxylic acid and an amine without an intervening spacer.[28,59] The water-soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC or EDAC) is one of the most commonly used linking agents for the immobilization of proteins, antibodies, and other amine-containing molecules onto carboxylated microparticles, nanoparticles, or other nanomaterials,[60–62] and for the coupling of affinity ligands onto surfaces.[63] Because EDC is hygroscopic and susceptible to hydrolysis, it should be kept dry until needed. Gilles et al. examined the rate of hydrolysis of EDC in aqueous solution at various pH values, and found that the half-life of EDC ($t_{1/2}$) decreases with a decrease in pH: 37 h (pH 7), 20 h (pH 6), and 3.9 h (pH 5).[64]

For a conjugation reaction in an aqueous medium, EDC initially reacts with a carboxylate group to form an intermediate *O*-acylisourea. This is another type of active ester that can react with an amine to form an amide bond.[65] The *O*-acylisourea intermediate can also undergo a number of side reactions, such as hydrolysis back to the unreactive carboxylate and an inactive isourea,[28] reaction with a neighboring carboxylate group to form an anhydride, or an irreversible rearrangement into an inactive *N*-acylisourea, which may cause nonspecific binding in some applications. The adverse effects of these side reactions can be mitigated by employing EDC/NHS or EDC/sulfo-NHS combinations. When a conjugation reaction is carried out in the presence of EDC and NHS or sulfo-NHS, the intermediate *O*-acylisourea would then react with the OH group of these molecules and form a more hydrolytically stable intermediate NHS or sulfo-NHS ester. An example of the power of this approach was demonstrated in the coupling of radiolabeled glycine to keyhole limpet (*Megathura crenulata*) hemocyanin (KLH) or to bovine serum albumin as a model system for the linking of peptide haptens to carrier proteins (**Scheme 4**).[66] The reaction yield (defined as the percent of total glycine added that was precipitated with the protein, after correcting for background) increased from 0.9% with EDC alone to 21% using EDC/sulfo-NHS (for hemocyanin) and from 1.9% to 38% (for bovine serum albumin). The coupling of peptides containing a free α-amine and lysine ε-amine to carrier proteins can become so efficient with the use of the EDC/sulfo-NHS system that nearly 100% of the peptide hapten can become conjugated to the carrier protein. In addition to improving the yields of such coupling reactions, the negatively charged sulfo-NHS can create a negative-charge repulsion between particles during their activation, thus inhibiting particle aggregation during the coupling reaction. This is extremely important with particles that have a base substrate possessing highly adsorptive or hydrophobic properties.

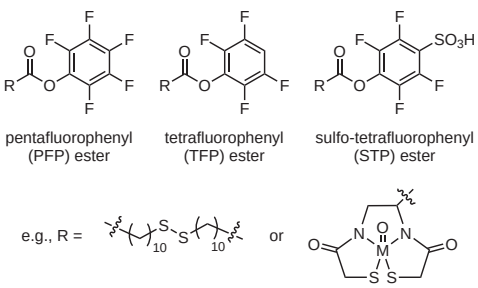
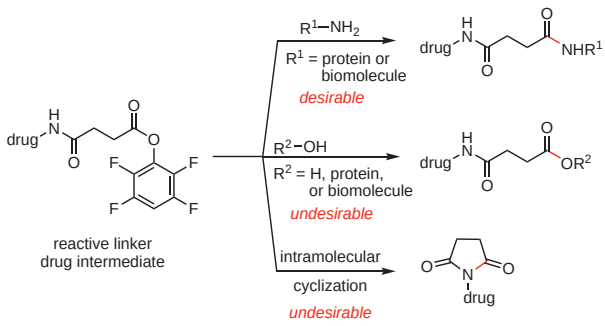


Figure 7. Popular, Reactive Fluorophenyl Esters.



Scheme 3. Typical Desirable and Undesirable Reactions of Reactive Tetrafluorophenyl (TFP) Esters. *(Ref. 57)*

4. Conjugation of Thiols with Reactive Groups

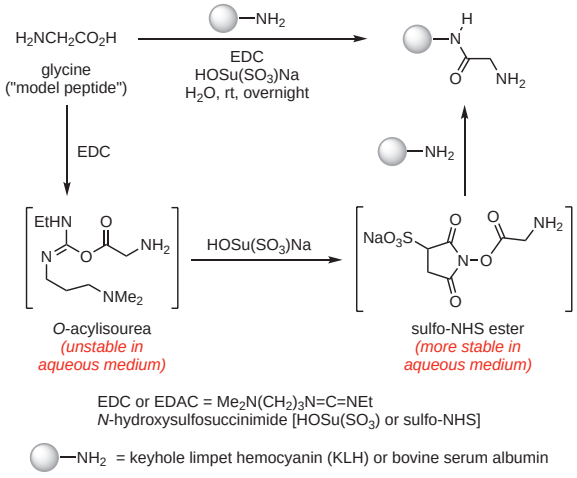
4.1. Maleimides

Reactive maleimide groups are the most popular choice for conjugation with thiols, leading to thioether bonds, which are reasonably stable linkages that cannot be reductively cleaved. However, the reaction of maleimides with thiols or amines is pH dependent. When the pH is around 7, coupling of maleimides with thiols is about 1000 times faster than their coupling with amines, which results in excellent chemoselectivity toward cysteine thiols in proteins.[67] At a much higher pH, the maleimide group becomes more reactive toward amines and thus loses its chemoselectivity.[68] The principal side reaction of maleimides in aqueous solution is hydrolysis of the ring imide to an open maleamic acid form consisting of an amide and a terminal carboxylate group with consequent loss of thiol coupling ability to the internal double bond. The rate of this hydrolysis increases with increasing pH and is dependent on the nature of other nearby structural elements present in the crosslinker. One of the most stable crosslinkers in this regard is SMCC (see Figure 3). The maleimide end of SMCC (and sulfo-SMCC) is unusually stable due to the presence of an adjacent cyclohexane ring, which probably limits access by water to the imide bond and, thus, water's ability to hydrolyze it. A study of the coupling reaction rate, as well as the hydrolysis rate, of the model maleimide, *N*-ethylmaleimide (NEM), with glutathione as a model peptide reports that the thiol coupling reaction takes place very rapidly and goes to maximal yield in only 3 minutes at pH 7 or 1 min at pH 8 (**Scheme 5**).[69] The study also found that the hydrolysis rates were much slower and similarly increased with an increase in pH. It is worth noting that hydrolysis of the maleimide group can also occur after the coupling of a thiol has taken place, and leads to two possible succinamic acid thioether isomers, depending on which side of the ring opens up relative to the position of the thioether. These isomers may result in different activities of a conjugate when used in detection applications or for in vivo therapeutic and diagnostic purposes. Moreover, ring opening after thioether bond formation yields a new, negatively charged carboxylate in the cross-bridge, which may result in new electrostatic interactions with biomolecules and unexpected nonspecific binding or off-target effects. To reduce this potential heterogeneity around the thioether bond, methods have been developed to purposely hydrolyze the imide after thiol coupling.[70] After such treatment, the thioether bond region will not undergo any further chemical changes. This strategy was applied by Lyon et al. toward stabilizing antibody–drug conjugates by creating self-hydrolyzing maleimides.[71] Maleimides in antibody–drug conjugates (ADCs) can be unstable in plasma or in circulation in vivo due to elimination of the maleimide–thiol bond through a retro-Michael process. This reaction happens if the cross-bridge is held in close proximity to a lysine amino group such as within the pocket of serum albumin. The retro-Michael reaction results in the amino group of lysine displacing the thioether linkage to the succinimide ring, and thus breaking apart the conjugate. The self-hydrolyzing maleimide was created by incorporating a basic amino group adjacent to the maleimide through the use of diaminopropionic acid.[71] ADC stabilization through accelerated hydrolysis of maleimide bonds was also accomplished through the use of *N*-arylmaleimides, such as *N*-phenyl and *N*-fluorophenyl, which were found by Christie et al. to have an inherently rapid hydrolysis rate relative to their *N*-alkyl counterparts.[72]

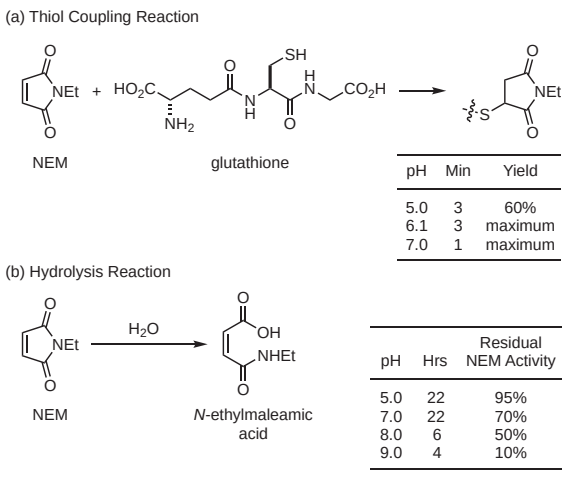
Other undesirable reactions that interfere in the coupling of thiols to maleimides involve thiol-containing reducing agents such as DTT, BME, and 2-MEA, as well as tris(2-carboxyethyl)phosphine (TCEP), a reagent that contains no thiol groups.[73,74] Glutathione (GSH)—present in cells in millimolar concentrations[75] that are orders of magnitude greater than the concentrations of most thiols in proteins from cell lysis or tissue extraction—can compete with solubilized proteins for coupling with maleimides, and should be removed by dialysis or size exclusion chromatography prior to attempting the coupling reaction. Finally, thiol coupling reactions with maleimides should not be attempted in the presence of any group VI oxometallates, such as molybdate and chromate, since these catalyze the rapid hydrolysis of the maleimide ring imide bond and inactivate the double bond toward subsequent coupling with thiols.[70]

4.2. Cyanoethynyls (3-Arylpropiolonitriles; APNs)

A new class of thiol-specific conjugation reagents consisting of a thiol-reactive 3-arylpropiolonitrile (APN) group, with good stability in aqueous media, has recently been described.[76,77] The APN group



Scheme 4. Dramatic Effect of Adding *N*-Hydroxysulfosuccinimide on the Yield of the Coupling of Glycine to Hemocyanin with EDC (Refer to the Discussion on Page 52). *(Ref. 66)*



pH	Min	Yield
5.0	3	60%
6.1	3	maximum
7.0	1	maximum

pH	Hrs	Residual NEM Activity
5.0	22	95%
7.0	22	70%
8.0	6	50%
9.0	4	10%

Scheme 5. The Coupling Rates of *N*-Ethylmaleimide (NEM) with Glutathione at Different pH Values, and the Hydrolysis Rates of NEM at the Same pH Values. *(Ref. 69)*

allows the targeted coupling of thiols in biomolecules, and results in stable thioether linkages without the risk of subsequent side reactions that can occur with maleimides. This mode of coupling is illustrated by the use of the heterobifunctional crosslinker CBTF to prepare antibody conjugates that are remarkably stable in plasma (**Scheme 6**).[77] First, the STP end of CBTF reacts with amine-containing molecules or proteins to provide intermediates containing the thiol-reactive APN group, which then undergoes coupling to a thiol group in another molecule or protein to provide the desired conjugate. This inherent stability of APN-based reagents and derived conjugates holds promise for better performance in many biological applications than the maleimide-based counterparts.

4.3. Iodo- and Bromoacetyls

Another type of thiol-reactive group is haloacetyl, in particular bromo- and iodoacetyls, which can form stable thioether linkages. While this substitution reaction can take place with a number of nucleophilic groups in a pH dependent way, it is most specific for sulfhydryl groups in proteins when carried out at a slightly alkaline pH.[78,79] Haloacetyl compounds are very hydrolytically stable in aqueous environments under normal conditions. However, iodine can be lost due to light-catalyzed degradation or exposure to a reducing agent. Therefore, reagents containing haloacetyl groups should be handled in subdued light and reducing agents carefully removed before attempting the reaction with thiols. SIAB is an example of a heterobifunctional crosslinker containing an amine-reactive NHS ester at one end and an iodoacetyl group at the other, separated by a short *para*-aminobenzoate spacer (see Figure 3). Sulfo-SIAB is a sulfonate analogue of SIAB with similar reactivity, but is water soluble and cell membrane impermeable.

4.4. Pyridyldithiols

The pyridyldithiol group has been used for decades to form reversible conjugate linkages to sulfhydryl groups in proteins or other molecules.[80,81] This takes place through a disulfide interchange reaction, whereby a free cysteine SH group displaces the pyridine-2-thiol leaving group and forms a new mixed disulfide with the crosslinking or modification reagent. The new disulfide bond can then be cleaved, allowing the rupture of conjugate crosslinks in vitro or in vivo. One advantage of this approach is providing intracellular cleavability for biologics. For example, the pyridyldithiol group could be used to form an antibody–drug conjugate (ADC) for tumor targeting. After docking on the cell surface and internalization, the disulfide bond between the antibody and the drug is then cleaved in the reductive environment within the cell, releasing the toxic drug to kill the tumor. **Figure 8** shows some of the more popular pyridyldithiol-containing reagents, both hydrophobic and hydrophilic.

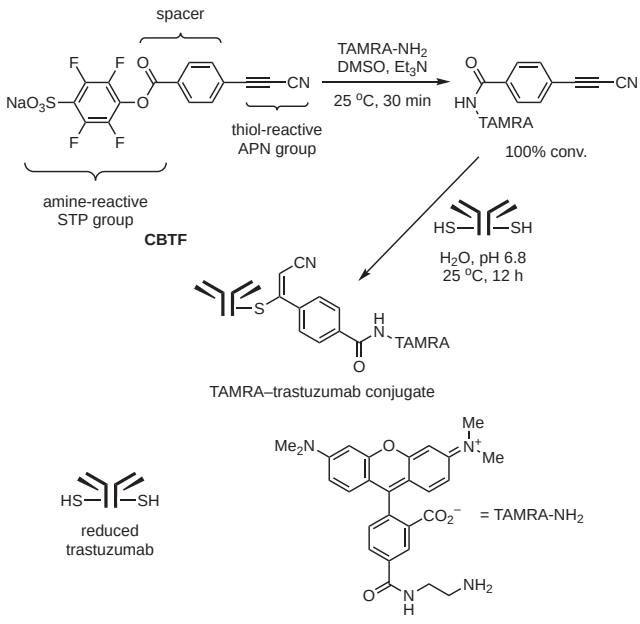
5. Conjugation of Carbonyl Groups

Aldehydes, derived from carbohydrates in biomolecules either by ring-opening of the carbohydrate or by oxidation of diols, may be specifically conjugated using hydrazide, hydrazine, or aminooxy functional groups, leading to the spontaneous formation of hydrazone or oxime linkages. Another route to conjugations using these reactive groups is to pair crosslinkers containing a hydrazide, hydrazine, or aminooxy group with crosslinkers containing an aldehyde. The opposite end of each of these crosslinkers can contain another reactive group that can be used to modify biomolecules so that one biomolecule is derivatized to contain an aldehyde and the other one is derivatized to contain a hydrazide, hydrazine, or aminooxy group. Mixing these two modified molecules together then spontaneously forms the desired bioconjugate through hydrazone or oxime linkages.

Since most biological macromolecules do not normally contain aldehydes or ketones—or hydrazide, hydrazine, or aminooxy groups—crosslinkers containing these reactive groups can find each other in complex solutions and conjugate together with excellent efficiency. Hence these complementary groups can be considered bioorthogonal. Hydrazide or aminooxy reagents can also target ketone groups in unnatural amino acids or sugars., as in the cell-surface labeling of glycoproteins and glycolipids.[82,83] The study of protein glycoengineering has been enabled by use of aminooxy- and aldehyde-based reagents,[84] and glycoconjugates can be analyzed in western blots using hydrazide-based probing.[85]

5.1. Hydrazides and Hydrazines

The hydrazone linkage, formed from the reaction of hydrazide with an aldehyde or ketone in aqueous medium, is much more stable than the corresponding Schiff base formed between an aldehyde and an amine. Nevertheless, hydrazone linkages may still hydrolyze and leach



Scheme 6. Application of Thiol-Reactive 3-Arylpropiolonitriles (APNs) to the Preparation of Remarkably Stable Antibody Conjugates. *(Ref. 77)*

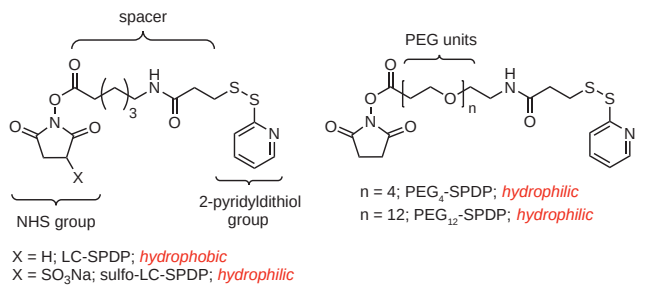


Figure 8. Popular Pyridyldithiol Crosslinking Agents.

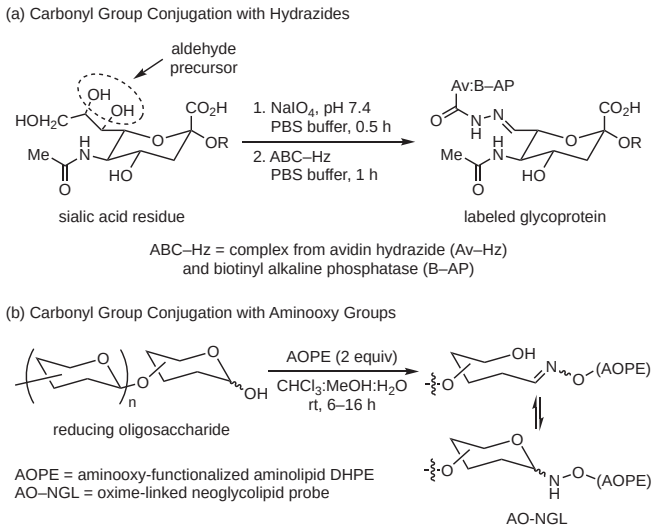
off conjugated molecules over time. To stabilize the linkage further, it may be converted into a hydrolytically stable *N*-alkylhydrazino bond by reduction with sodium cyanoborohydride. Some examples of hydrazide crosslinking reagents include the homobifunctional adipic acid dihydrazide [$(H_2NNHC(=O)CH_2CH_2)_2$] and the shorter and water-soluble carbohydrazide [$(H_2NNH)_2C=O$].

A bis-hydrazide reagent can modify a protein at points of glycosylation on the protein after aldehyde groups are generated by mild periodate oxidation of the carbohydrate groups on the protein. A bis-hydrazide reagent can also react with carboxylate groups on a protein in the presence of a carbodiimide such as EDC. Hydrazide-modified avidin prepared in this manner has been employed to detect glycoproteins on cell surfaces after periodate oxidation (**Scheme 7**, Part (a)).[86–88] Adipic acid dihydrazide has been utilized to create hydrazide functionalities on surfaces, particles, or polymers that contain aldehyde groups, as in the preparation of hydrazide-activated supports for the coupling of aldehyde-containing affinity ligands.[89,90]

5.2. Aminooxy Groups

The aminooxy or alkoxyamine ($RONH_2$) group exhibits a reactivity similar to that of hydrazides in their reactions with ketones or aldehydes, leading to the formation of oximes. However, aminooxy groups react more rapidly with ketones than do amines or hydrazides, allowing ketones in biomolecules to be effectively conjugated. Moreover, the oxime bond is far more stable in aqueous solution than the corresponding Schiff base or hydrazone, does not undergo hydrolysis under normal conditions, and rarely requires reduction (as the hydrazone linkage does in some cases) to be stable.

Aminooxy groups on crosslinking or modification reagents can be used to specifically link to aldehyde groups on other reagents or macromolecules (Scheme 7, Part (b)).[21,91–93] This bioorthogonal reaction can be highly chemoselective even in the presence of other proteins and biomolecules, provided keto-group-bearing small-molecule metabolites such as pyruvate are removed from biological samples by dialysis or size exclusion chromatography prior to the aminooxy reaction. Once the aldehyde group becomes available—through periodate oxidation of glycosylated proteins or through cleavage of glycans from glycoproteins revealing their reducing ends—it can be targeted for coupling away from active sites or binding regions in proteins or antibodies. The reaction of an aminooxy group with aldehydes or ketones can be catalyzed by arylamines, such as aniline.[18–20,94] When added in excess, aniline would condense rapidly with the aldehyde or ketone. The intermediate imine formed would then undergo protonation at moderately acidic pH more rapidly than the oxygen of the aldehyde or ketone. Thus, it is this protonated Schiff base that reacts with the aminooxy group to form the final oxime linkage.[95]



Scheme 7. Carbonyl Group Conjugation with (a) Hydrazides and (b) Aminooxy Groups. *(Ref. 21,88)*

6. References

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About the Author

Greg T. Hermanson is President of Greg T. Hermanson, Inc., a bioscience consulting company, and the Chief Technology Officer and Principal at Aurora Microarray Solutions, Inc. Greg has over three decades of experience in the development of life science products for research and diagnostics, with broad expertise in protein chemistry, assay development, immobilization, and bioconjugation. He is the author of two bestselling textbooks: *Bioconjugate Techniques*, now in its third edition, is an extensive manual on the methods of bioconjugation, and *Immobilized Affinity Ligand Techniques*, which is a manual on the covalent attachment of affinity ligands to solid supports and affinity chromatography. Greg's significant impact on the bioscience fields is attested to by nearly 27,500 citations to his publications and patents, with one of his publications being in the top 50 cited references of all time.

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Palladium-Catalyzed *meta*- and *para*-C–H Bond Functionalizations Assisted by a Directing Group

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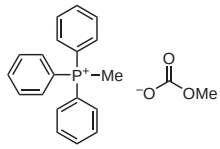
Cattelan, L.; Noè, M.; Selva, M.; Demitri, N.; Perosa, A. *ChemSusChem* **2015**, *8*, 3963.



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ABOUT OUR COVER

City Hall at Thorn (oil on canvas, 50.7 × 80 cm) was painted in 1848 by Johann Philipp Eduard Gaertner (1801–1877), a prominent 19th-century vedutista of mostly Berliner cityscapes. He was born, raised, and spent most of his life in Berlin. His art training consisted of an apprenticeship, in his teens, at the Royal Porcelain Factory and lessons at the Academy of Arts (Berlin). This was supplemented by a two-year stint with the landscapist Jean-Victor Bertin in Paris. His work-related travels took him to Russia, Austria, Bohemia, and many parts of Prussia. As happens with many presently famous artists, his reputation and output waned in his later years and his oeuvre was almost forgotten until the latter part of the twentieth century.

Detail from ***City Hall at Thorn***. Photo courtesy National Gallery of Art, Washington, DC.

Most of his early paintings, and the ones he is best known for, were commissioned by royal patrons in Prussia and Russia. His many cityscapes of Berlin chronicled the rapidly changing urban landscape of the city. Gaertner's meticulously executed urban scenes, as in the present painting,* underscore his exacting style and attention to detail, including the effects of light and shadow, and is suggestive of his possible use of a camera obscura to create his sketches.

This painting is a gift of Ethel Gaertner Pyne to the National Gallery of Art, Washington, DC.

* *Gaertner's cityscapes are often so accurate, they are almost photographic. To find out more, visit* ***SigmaAldrich.com/acta503***

Palladium-Catalyzed *meta*- and *para*-C–H Bond Functionalizations Assisted by a Directing Group


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Keywords. remote C–H functionalization; *meta*-selectivity; *para*-selectivity; chelation-assisted; palladium catalysis.

Abstract. Direct and site-selective functionalization of unactivated C–H bonds is becoming a powerful strategy of organic synthesis in academia and industry. While *ortho*-selective C–H functionalizations are well-established, chelation-assisted remote *meta*- and *para*-C–H bond functionalizations remain a noteworthy challenge. This article reviews significant and very recent advances in the palladium-catalyzed, directed *meta*- and *para*-C–H functionalizations of aromatic compounds, and covers the literature from 2015 to mid-2017.

Outline

1. Introduction

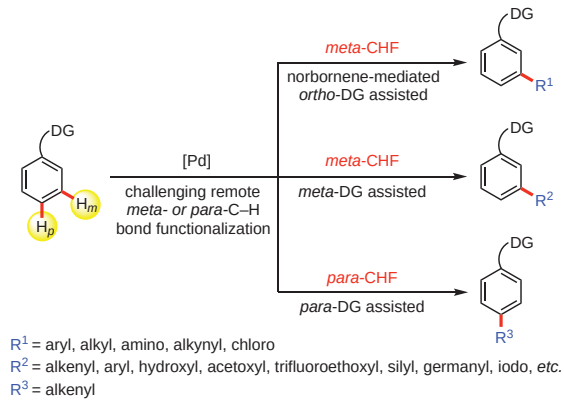
The site-selective functionalization of aromatic compounds has significant applications in natural product synthesis and in the chemistry of materials, pharmaceuticals, and polymers.[1–3] In this regard, the efficient *ortho*-, *meta*-, or *para*-selective C–H functionalization (CHF) of aromatic compounds is of paramount importance and an outstanding challenge.[4–6] The last decades have witnessed the rapid and extensive development of the transition-metal-catalyzed, *ortho*-selective CHF of arenes with the assistance of various directing groups (DGs).[7–10] In contrast, only a small number of reports exist on the *meta*-selective CHF of arenes.[11–14] One major reason for this dearth of reports is that common DGs are generally unable to assist the metal in reaching the ring meta position due to the strain of the corresponding metallacycle intermediate. Moreover, while several protocols of the *para*-selective CHF of arenes have been disclosed, they still suffer from limitations in substrate scope and poor selectivity. Since these protocols often rely on electronic and steric factors to direct the selectivity, the *para*-selective activation of common arenes remains extremely challenging.[15]

Palladium salts are playing an increasingly important role in *meta*- and *para*-C–H activation strategies that are based on directing groups,[13–15] and these strategies include three major types (**Scheme 1**). The first is the very recently developed norbornene-mediated, *meta*-C–H functionalizations assisted by an *ortho*-directing group. The second is *meta*-CHFs of arenes assisted by *meta*-directing groups. The third type uses a novel template-based approach for *para*-CHFs. Because a number of recent reviews have dealt with this general topic,[6,12–15] we will limit our discussion to the literature covering palladium-catalyzed, directing-group-assisted *meta*- and *para*-CHFs of aromatic compounds from 2015 to mid-2017.

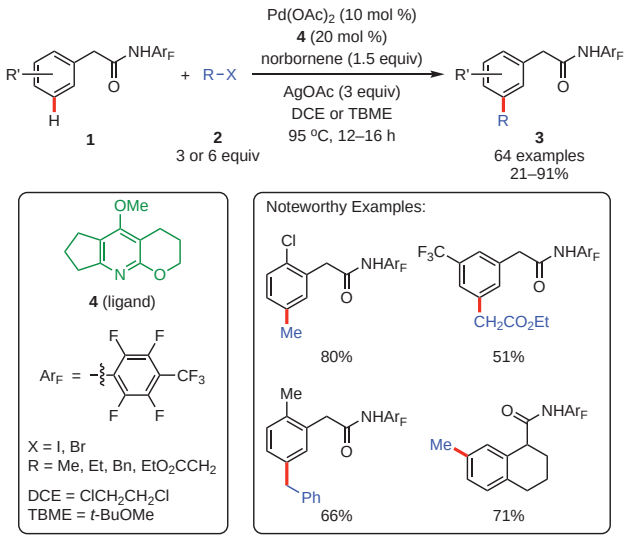
2. Norbornene-Mediated *meta*-C–H Functionalization Assisted by *ortho*-Directing Groups (DGs)

2.1. Arylation and Alkylation

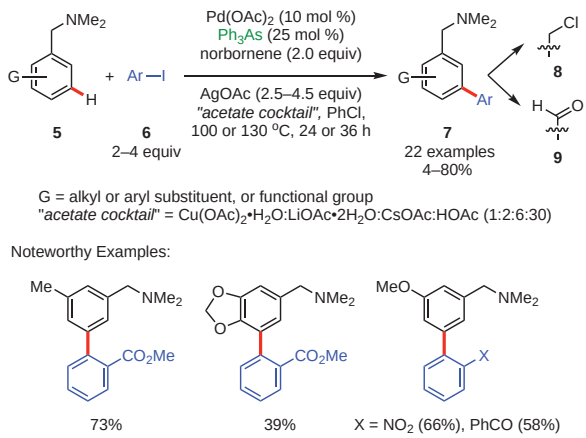
The unique reactivity of norbornene in palladium-catalyzed functionalization reactions was disclosed in 1997 by Catellani and co-workers in the regioselective synthesis of *o,o'*-disubstituted vinylarenes from aryl iodides.[16,17] This unique reactivity is the result of the differences in the reactivity of the palladium(0), palladium(II),



Scheme 1. Three Major Types of Directing-Group-Assisted *meta*- or *para*-C–H Bond Functionalization.



eq 1 *(Ref. 18)*



Scheme 2. Norbornene-Mediated *meta*-C–H Arylation of Benzylamines. *(Ref. 19)*

and palladium(IV) species formed in the catalytic sequence with norbornene acting as a transient mediator.

Inspired by Catellani's reports, Yu and co-workers developed the first norbornene-mediated catalytic *meta*-C–H activation using simple and common *ortho*-directing groups.[18] Catalyzed by palladium acetate, mediated by norbornene, and promoted with electron-rich pyridine- or quinoline-based ligands, phenylacetic acid derivatives **1** were selectively alkylated or arylated at the *meta*-position with electrophiles **2** including methyl iodide, ethyl iodoacetate, and various organohalides (**eq 1**).[18]

In the proposed catalytic cycle of this transformation, initial *ortho*-C–H activation leads to an *ortho*-palladacycle, which undergoes a norbornene-mediated, Catellani-type carbopalladation to form a five-membered palladacycle. Reaction of this palladacycle with the coupling partners, **2**, forms the new *meta*-C–C bond. Subsequent β-elimination of norbornene followed by proto-demetallation forms the desired products, **3**, and regenerates the palladium catalyst.

Simultaneously, Dong and co-workers reported a norbornene-mediated palladium(II)-catalyzed *meta*-C–H arylation of tertiary amines **5** with commercially available Ph_3As as the ligand (**Scheme 2**).[19] Interestingly, the "acetate cocktail" additive—consisting of $LiOAc·2H_2O$, CsOAc, and $Cu(OAc)_2·H_2O$ (2:6:1) in acetic acid—led to improvements in the reaction rate and yield. Under the optimized reaction conditions, a range of benzylamines, **5**, reacted regioselectively with *ortho*-substituted aryl iodides, **6**, to form *meta*-aryl-substituted products **7**. Products **7** could be easily converted by established procedures into the corresponding benzyl chlorides or aldehydes, which are synthetic precursors of a number of valuable targets.

Some of the limitations of the norbornene-mediated *meta*-CHFs—ineffective coupling with alkyl iodides containing a β-hydrogen and compatibility only with aryl iodides possessing *ortho*-coordinating groups—prompted Yu and co-workers to develop 2-carbomethoxynorbornene (NBE-CO_2Me, **10**) as a more effective transient mediator in the presence of a quinoline-based ligand, **11** (**eq 2**).[20] These conditions suppress the reductive elimination side reaction and promote unprecedented *meta*-alkylation of phenyl acetamides with a wide range of alkyl iodides, as well as *meta*-arylation with aryl iodides lacking ortho substituents.

In 2016, Zhao, Shi, and co-workers reported an approach for achieving the palladium–norbornene catalyzed, selective *meta*-C–H arylation of β-arylethylamine derivatives by taking advantage of an oxalyl amide directing group (**eq 3**).[21] This *meta*-arylation not only tolerates a wide range of electron-donating and electron-withdrawing substituents, but also proceeds well with thiophene derivatives. Notably, this was the first report of a norbornene-mediated, palladium(II)-catalyzed *meta*-CHF assisted by an N,O-bidentate directing group.

Yu's group has demonstrated the versatility of 3-acetamido-2-hydroxypyridine and 3-acetamido-5-trifluoromethyl-2-hydroxypyridine as ligands in promoting the *meta*-C–H arylation of protected anilines, heterocyclic aromatic amines, phenols, and 2-benzyl heterocycles using norbornene as a transient mediator.[22] These ligand scaffolds enable the reaction of a wide range of substrates and coupling partners. These two ligands permitted, for the first time, the *meta*-C–H arylation with heterocyclic aryl iodides as coupling partners, a transformation that can potentially be utilized in drug discovery research. The same research group also reported the first example of a silver-free, gram-scale protocol for this catalytic reaction (**eq 4**).[22]

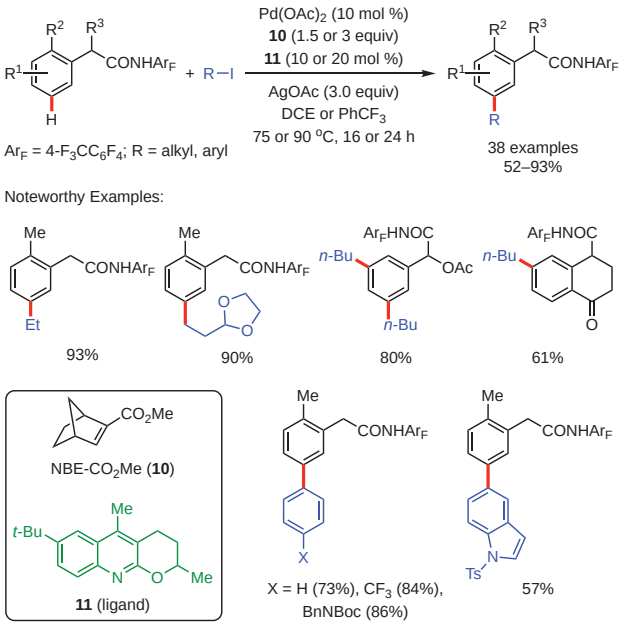
An effective, Pd(II)-catalyzed *meta*-C–H arylation of nosyl-protected phenethylamines, benzylamines, and 2-arylanilines has very recently been disclosed (**eq 5**).[23] This practical and attractive protocol

utilizes 4-acylpyridine as a preferred ligand, a common protecting group (4-nitrophenylsulfonyl or Ns) as directing group, and, for the first time, a catalytic amount of 2-norbornene in most cases. The reaction is compatible with a diverse range of aryl iodides, including heteroaryl iodides, and tolerates aryl bromides bearing *ortho*-coordinating groups.

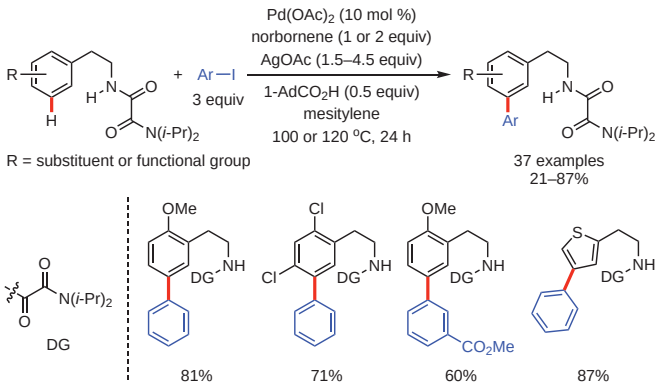
Ling et al. disclosed very recently the first interannular palladium-catalyzed *meta*-selective C–H arylation of biaryl compounds using a 2-trifluoroacetamide as the directing group (**eq 6**).[24] Remarkably, the active catalyst was isolated and shown by X-ray diffraction to be a dimeric palladacycle intermediate consisting of two cyclopalladated trifluoroacetamino biaryl units linked through the trifluoroacetamide. It is worth noting that the interannular *meta*-arylated product from this reaction can be elaborated further, affording various biaryl-2-amine derivatives through *ipso*-alkynylation or directed interannular *ortho*-C–H functionalization of the proximal C–H bond.

The *meta*-C–H arylation of phenylacetic acids could also be achieved, since the weak coordination of the carboxylic acid group results in the Catellani reaction of aryl iodides outcompeting the *ortho*-C–H palladation of phenylacetic acid (**eq 7**, Part (a)).[25] Under the optimized conditions, the reaction was compatible with both electron-rich and electron-deficient substrates as coupling partners and was amenable to scaling up. A similar Pd(II)-**10** catalysis was successfully applied to the *meta*-C–H arylation and alkylation of benzylsulfonamides using isoquinoline as ligand (eq 7, Part (b)).[26] This transformation had a broad substrate scope and excellent functional-group tolerance. Its good compatibility with both heteroaryl iodides and alkyl iodides gives it an advantage over other *meta*-C–H functionalization protocols, and the sulfonamide functional group's broad applications render it highly useful in the synthesis of *meta*-substituted sulfonamides and sulfonate esters, as well as styrene derivatives.

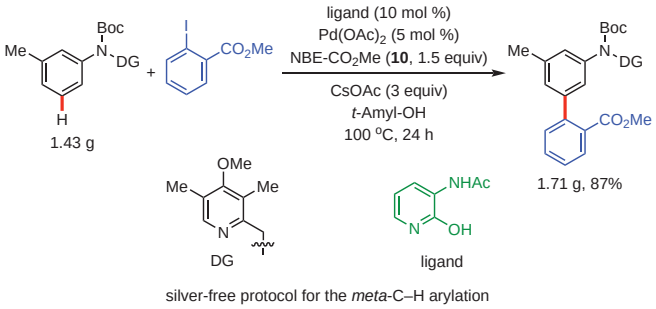
Benzylamines are distinctive structural motifs in many natural products and pharmaceuticals, and a practical and general *meta*-CHF of benzylamines would be helpful in their further elaboration. However,



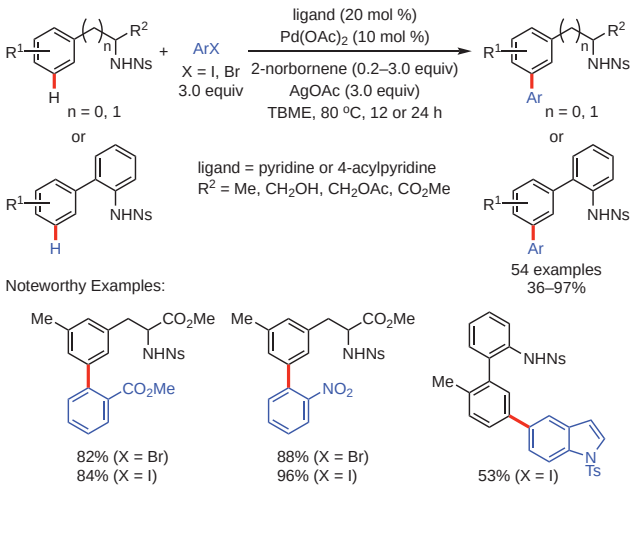
eq 2 *(Ref. 20)*



eq 3 *(Ref. 21)*



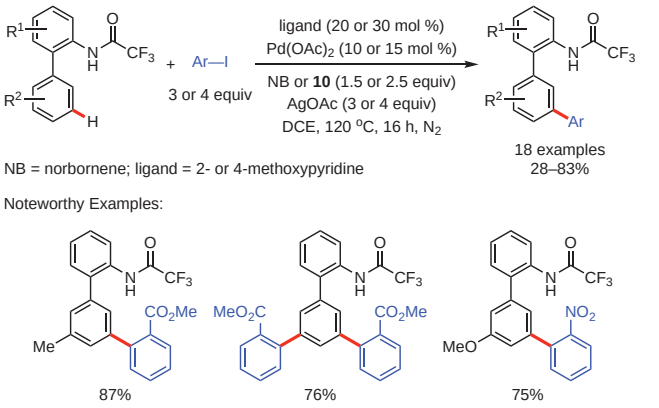
eq 4 *(Ref. 22)*



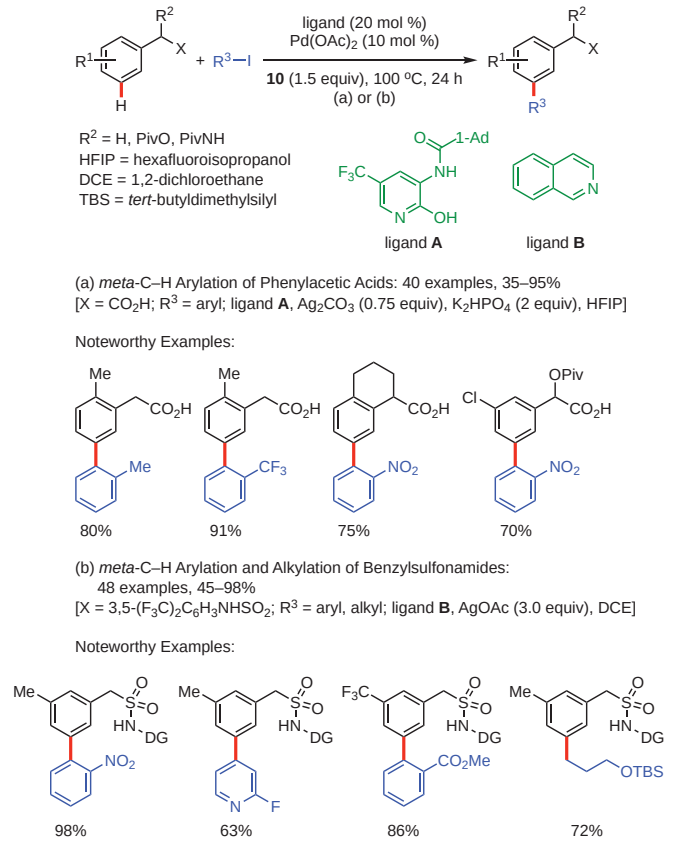
eq 5 *(Ref. 23)*

the reported norbornene-mediated *meta*-CHF of benzylamines has been limited to those aryl iodide coupling partners containing *ortho*-coordinating groups. Yu and co-workers overcame this limitation by using 2-hydroxy-3-trifluoromethylpyridine as ligand and NBE-CO_2Me (**10**) as the transient mediator.[27] The reaction, which is scalable and compatible with a variety of heterocycle-containing substrates and coupling partners, has been used to form a key intermediate in the synthesis of an analogue of the inhibitor RPR128515 (**Scheme 3**).[27]

Very Recently, Li and Ferreira disclosed a *meta*-C–H arylation of benzylic alcohols with an *ortho*-selective, quinoline-based acetal directing scaffold by using the NBE-CO_2Me mediated process.[28] The reaction provided the desired biaryl products in moderate-to-high yields, and exhibited a considerable scope and functional group compatibility. The use of an amino acid based ligand (TFA-Gly-OH) for the transformation is distinct from similar functionalization reactions in which an N-heterocycle is employed as the ligand. Moreover, the cleavage and recovery of the scaffold could be realized in good yields under mild reaction conditions.



eq 6 *(Ref. 24)*



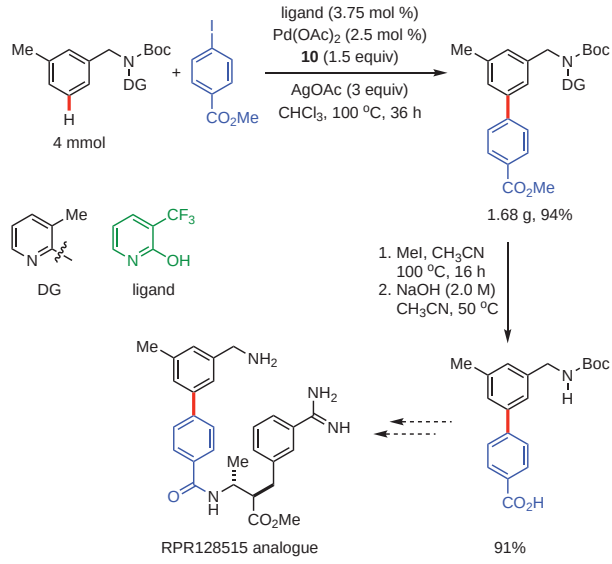
eq 7 *(Ref. 25,26)*

2.2. Amination

The aforementioned norbornene-mediated *meta*-CHFs were limited to alkylations or arylations using alkyl or aryl iodides. In 2016, Yu and co-workers expanded the scope of the reaction to include the *meta*-C–H amination of anilines and phenols with *N*-benzoyloxyamines as the aminating reagents.[29] Both ligand **A** (see eq 7, Part (a)) and NBE-CO_2Me (**10**) were crucial to the success of this transformation. Under the optimal reaction conditions, a variety of substrates were compatible with the reaction, including coupling partners containing other heteroatoms. Moreover, the Boc-protecting group and the pyridine directing group in the products can be removed simultaneously to provide the corresponding free aniline, as in the case of **12**, which is a key intermediate in the synthesis of a BRAF inhibitor (**Scheme 4**).[29]

2.3. Alkynylation

In recent decades, the Sonogashira coupling reaction has become one of the most significant methods for synthesizing arylalkynes, which are important structural motifs in many pharmaceuticals and natural products. However, up until 2016, there had been no reports of alkynylations employing Catellani's norbornene-mediated relay process, when Wang et al. disclosed an NBE-CO_2Me mediated *meta*-C–H alkynylation of anilines (**eq 8**).[29] This *meta*-C–H alkynylation is noteworthy for being compatible with indoline and indazole-containing amines; however, only alkynyl bromides protected with bulky silyl groups afforded *meta*-alkynylated products in acceptable yields.



Scheme 3. Application of the *meta*-C–H Arylation of Benzylamines to the Synthesis of RPR128515 Analogues. *(Ref. 27)*

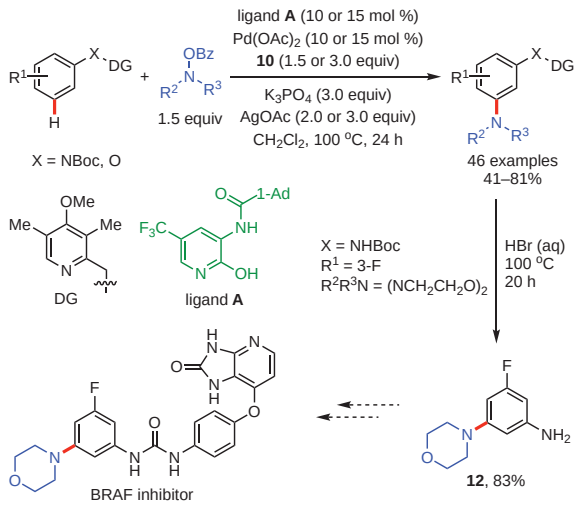
2.4. Chlorination

Given that the versatile reactivity of aromatic halides enables the installation of diverse structural motifs to meet the demands of synthetic applications, the development of the C–H halogenation of arenes would be not only useful but also essential. Shi et al. reported the first example of NBE-CO_2Me mediated *meta*-C–H chlorination of anilines using 2,6-diisopropylphenylchlorosulfate as the chlorinating reagent (**eq 9**).[30] This *meta*-chlorination reaction is promoted by new pyridone-based ligands, displays outstanding functional group tolerance, and leads to high yields of the chlorinated products. Moreover, this *meta*-C–H chlorination was successfully extended to phenols[30] and benzylamines[27] by employing slightly modified conditions.

3. *meta*-C–H Functionalization Assisted by *meta*-Directing Groups

3.1. Nitrile-Based, DG-Assisted *meta*-C–H Functionalization

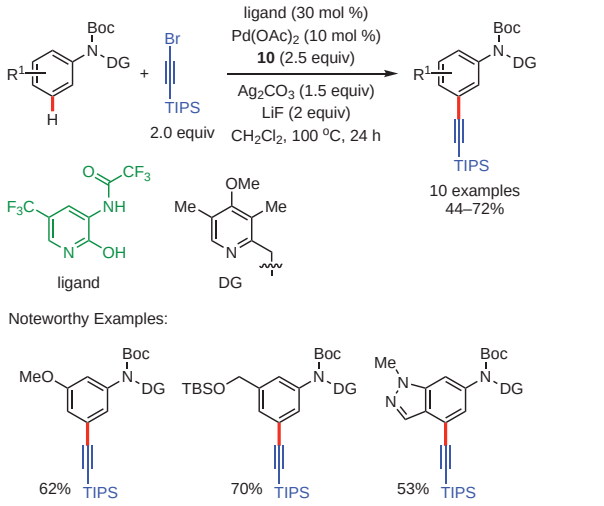
In directed C–H activations, chelation-assisted transformations have been powerful in performing a variety of selective *ortho*-C–H functionalizations.[7–10] However, extending the methodology to perform *meta*-C–H functionalizations has been difficult, since the formation of a cyclophane-like, pre-transition state is energetically and conformationally demanding. In 2012, a significant breakthrough in this respect was achieved by Yu and co-workers, who employed two types of U-shaped, nitrile-based directing groups that resulted in the remote *meta*-C–H olefination of toluene derivatives, hydrocinnamic acids, and 2-biphenylcarboxylic acids.[31] The rational design of the template was predicated on the weak coordination between Pd(II) and the nitrile group in an "end-on" fashion, which was believed to withstand the strain involved in the formation of the cyclophane-like pre-transition state in the *meta*-C–H activation event. This was the first report of a *meta*-C–H activation, via a 12-membered metallacyclic pre-transition state, that is assisted by a cleavable, nitrile-containing *meta*-directing group. It inspired the discovery of diverse *meta*-C–H functionalizations of (hetero)arenes by similar approaches that are discussed below.



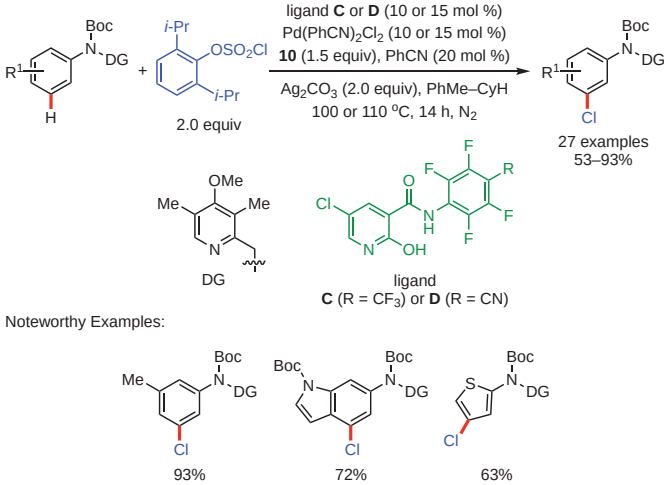
Scheme 4. *meta*-C–H Amination of Anilines and Phenols and Its Application in the Synthesis of a BRAF Inhibitor. *(Ref. 29)*

3.1.1. Olefination

Deng and Yu have developed a protocol for template-assisted *meta*-C–H olefination of phenylacetamides by using *N*-formyl-protected glycine (For-Gly-OH) as ligand and KH_2PO_4 as additive, both of which were crucial for the success of the reaction (**eq 10**).[32] A series of *meta*-olefinated phenylacetamides, some of which not readily available, were synthesized through this protocol. This report demonstrated that the benzonitrile directing group could accommodate different ring sizes of the macropalladacycle in the activation of the *meta*-C–H bonds of arenes. It should be noted that, in an earlier report, Maiti's group had also disclosed another nitrile-based directing group for the *meta*-C–H olefination of phenylacetic acids.[33] The first step in the proposed catalytic cycle involves *meta*-C–H activation of the substrate with Pd(II) through coordination with the CN group, generating a macropalladacycle. Coordination of the macropalladacycle with



eq 8 *(Ref. 29)*



eq 9 *(Ref. 30)*

the olefin results in a complex, which undergoes 1,2-migratory insertion followed by β-hydride elimination to release the *meta*-C–H functionalized product and a palladium hydride. Reductive elimination converts the palladium hydride into Pd(0) that is then reoxidized by the silver salt to Pd(II), which re-enters the catalytic cycle.

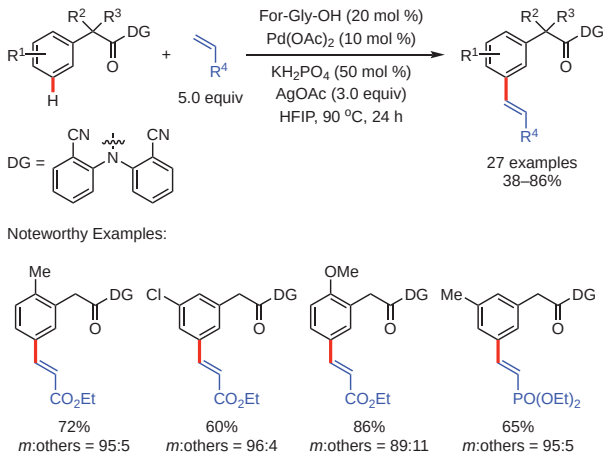
In the same year, Maiti and co-workers introduced a novel strategy for *meta*-selective mono-, di-, and sequential hetero-di-olefinations of benzylsulfonyl ester derivatives using 2-hydroxybenzonitrile as the directing group (**eq 11**).[34] This work is of great significance for the synthesis of divinylbenzene derivatives, which are an important class of molecular building blocks. The sulfonyl tether is helpful in achieving high *meta*-selectivity even for unbiased substrates. Moreover, the directing group is commercially available, simple to synthesize, and easy to remove.

Subsequently, our group reported a remote and selective *ortho*- and *meta*-C–H olefination of phenylethylamines—a key structural feature in a number of important drugs—through a regiodivergent functionalization protocol, which is promoted by a novel and simple 2-cyanobenzoyl group as the directing group. Tertiary amides undergo efficient *meta*-C–H olefination in the presence of $Pd(OAc)_2$ and Ac-Gly-OH under a nitrogen atmosphere (**eq 12**).[35] To demonstrate the potential of this approach for building complexity in a concise manner in the synthesis of highly substituted arenes, tetra-substituted phenylethylamide **13** was generated in 4 steps and 52% overall yield by sequential *ortho*- and *meta*-CHFs.

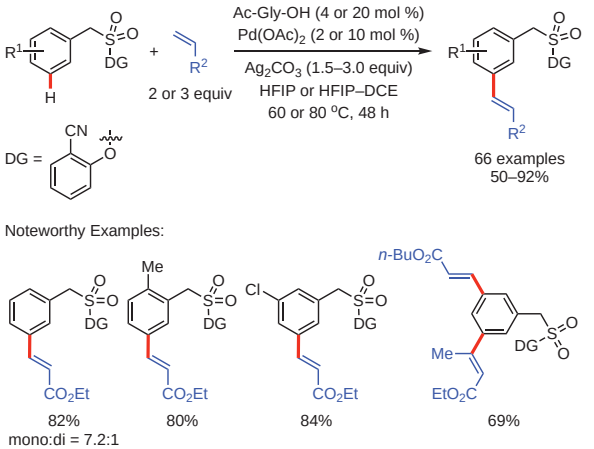
In early 2016, Maiti and co-workers reported the *meta*-selective mono-olefination and bis-olefination of benzylsilane derivatives by using 2-hydroxy-5-methoxybenzonitrile as the directing group.[36] Significantly, *meta*-olefinated toluene, benzaldehyde, and benzyl alcohol were obtained from the initial *meta*-olefination products under suitable desilylation conditions.

Although the chelation-assisted *meta*-CHF of electron-rich arenes had been reported,[11–14] the *meta*-C–H activation of electron-poor arenes such as benzoic acids or their derivatives via transition-metal catalysis had remained unsuccessful. In 2016, our group disclosed a palladium–catalyzed, *meta*-C–H olefination of electron-poor benzoic acid derivatives with a nosyl-protected 2-cyanophenylethylamine directing group (**eq 13**).[37] Notably, the new protocol introduced the environmentally benign molecular oxygen as the terminal oxidant in the presence of a catalytic amount of $Cu(OAc)_2$, which replaced the costly silver salt oxidants required in all previous chelation-assisted *meta*-C–H olefinations. Interestingly, a good ratio of mono over di-olefination was achieved by using For-Gly-OH instead of Ac-Gly-OH in the presence of the inorganic base KH_2PO_4. Moreover, the sulfonamide directing group, $2\text{-}NCC_6H_4(CH_2)_2NHNs$, can be prepared on a large scale from inexpensive starting materials, and can be easily cleaved under very mild conditions and recycled.

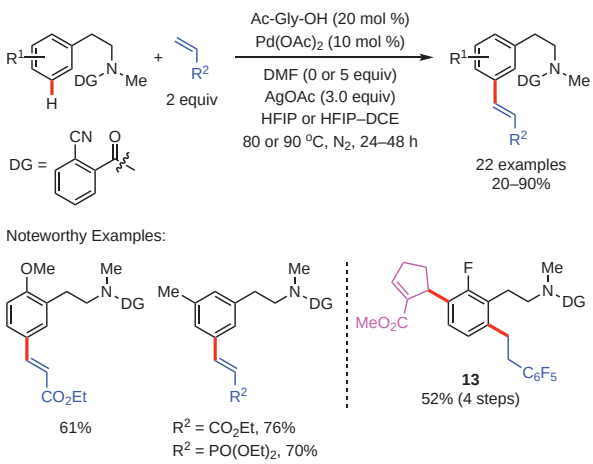
The first example of a room temperature *meta*-C–H olefination of arenes was disclosed by Maiti's group. The reaction is catalyzed by Pd(II), and a benzylic phosphonate ester is employed as the directing group (**eq 14**).[38] Remarkably, complete selectivity for the mono-olefinated arene was achieved, without any diolefinated product being observed. Moreover, the homo- and hetero-diolefinations at the *meta*-positions were achieved by raising the temperature to 80 °C in the presence of Ac-Gly-OH instead of Ac-Phe-OH ligand. Furthermore, the $-P(O)(OR)_2$ tether linkage could be readily transformed into alkene derivatives through a modified Horner–Wadsworth–Emmons reaction.



eq 10 *(Ref. 32)*



eq 11 *(Ref. 34)*



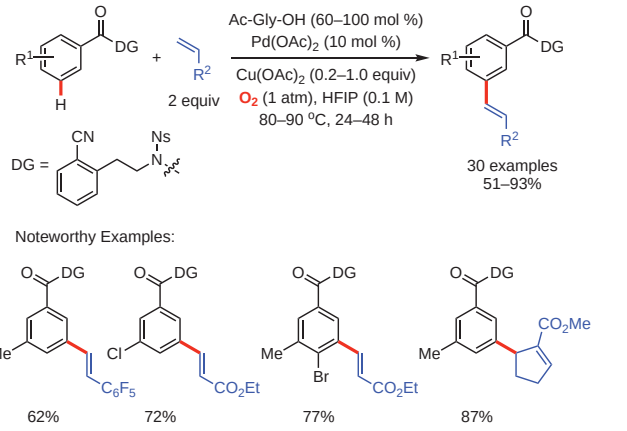
eq 12 *(Ref. 35)*

Maiti's group exploited further this template-assisted strategy in the palladium-catalyzed, selective *meta*-C–H olefination of 2-phenylethanesulfonic acid and 3-phenylpropanoic acid derivatives.[39] Sequential hetero- and homo-diolefinations were also achieved by using mono-olefinated 2-arylpropanoic acids as substrates, resulting in bis-*meta*-olefinated products. Mass spectrometric studies suggested the formation of a 12-membered cyclophane-like palladacycle in the reaction. Moreover, the cleavage and recovery of the directing template was successfully accomplished through a base-mediated hydrolysis process.

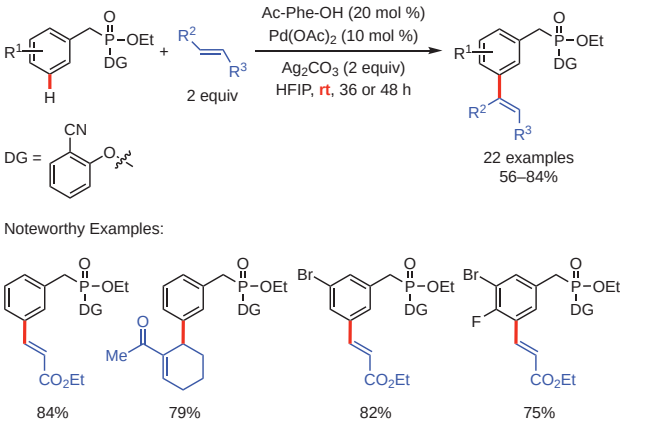
The same laboratory disclosed the distal C–H olefination of biphenylbenzoic acid and phenol derivatives with high regioselectivity (**eq 15**).[40] Some advantages of this approach are ease of scale-up and ease of cleavage and recovery of the directing group; one limitation, however, is that heterocyclic or appended naphthalene substrates could not be used. It should be mentioned that similar C–H olefinations of biphenyl derivatives had also been achieved previously by Yu's group.[31]

Carbon dioxide (CO_2) is an ideal reagent for carbamate synthesis owing to it being nontoxic, readily available, stable, and inexpensive.[41–44] In 2017, our group was the first to incorporate CO_2 into a novel, nitrile-containing directing group, **14**, for the purpose of *meta*-C–H activation of anilines (**Scheme 5**, Part (a)).[45] Under the optimal reaction conditions, a broad range of aniline derivatives were efficiently olefinated at the *meta*-position with $Pd(OAc)_2$ in good-to-excellent yields (Scheme 5, Part (b)). Moreover, the directing group could be easily cleaved under mild conditions, which would be advantageous in late-stage C–H functionalizations of complex molecules.

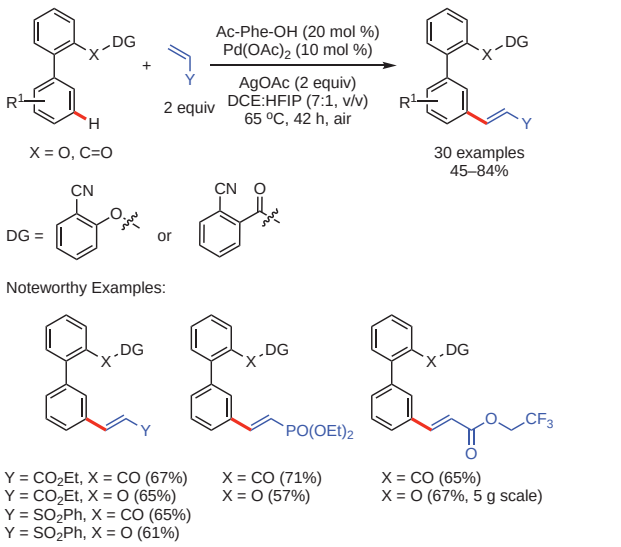
Very recently, Jin, Xu, and co-workers reported a highly regioselective and Pd-catalyzed remote *meta*-C–H olefination of distal arene-tethered alcohols assisted by a salicylonitrile template.[46] A variety of substrates, including 2-phenylethyl and 3-phenylpropyl alcohols and their long-chain homologues, were compatible with this



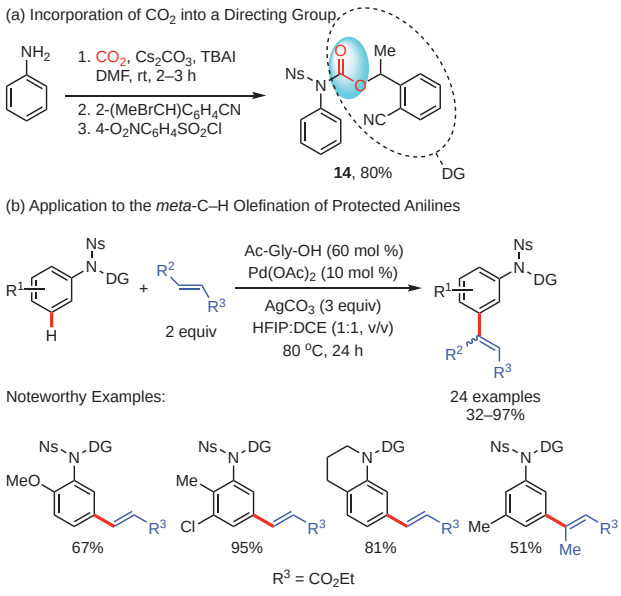
eq 13 *(Ref. 37)*



eq 14 *(Ref. 38)*



eq 15 *(Ref. 40)*



Scheme 5. (a) Incorporation of CO_2 into a Directing Group. (b) *meta*-C–H Olefination of Aniline Carbamates. *(Ref. 45)*

new protocol. It was suggested that both the C–N–Ag angle and gauche conformation of the phenyl ether in the macrocyclic transition state were the dominant factors for *meta*-selectivity, and this hypothesis was corroborated by a detailed computational study. The utility of this template in directing remote C–H activations was demonstrated in the case of two dipeptide substrates (**Scheme 6**).[46]

Simultaneously, Yu, Houk, and co-workers designed a conformationally flexible template for the *meta*-C–H olefination of benzoic acid derivatives guided by computational studies.[47] The newly designed template revealed that when the distance between the target C−H bond and the functional handle of the native substrate decreases, the templates can be lengthened to achieve *meta*-C–H selectivity. This work demonstrated that the combination of synthetic and computational chemistry might promote the development of novel templates for remotely and selectively activating C–H bonds. Interestingly, the new template used here is longer by one atom in the linkage when compared to the one disclosed by Li and co-workers for benzoic acid derivatives in an earlier report.[37]

3.1.2. Oxygenation

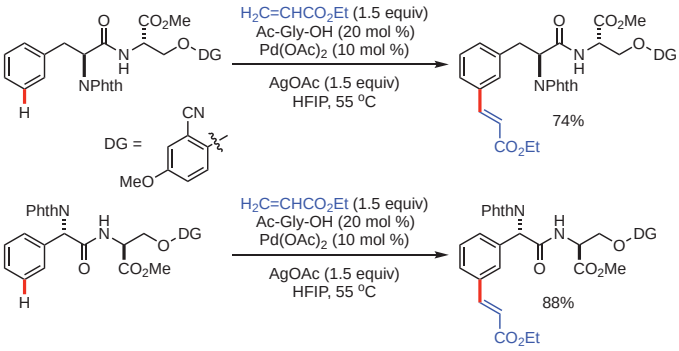
Another type of C–C bond forming reaction using a nitrile-based directing group is *meta*-C–H arylation,[48,49] which was published before 2015 and will not be covered here. The ability of the palladium-catalyzed, *meta*-C–H activation—assisted by a nitrile-based directing-group—to form carbon–heteroatom bonds was also explored and demonstrated by acetoxylation, hydroxylation, and the sole example of trifluoroethoxylation.[37,38,45,50–52] The new protocol proceeds via a Pd(II)/Pd(IV) redox cycle rather than the Pd(II)/Pd(0) catalytic cycle in other *meta*-C–H olefinations. The first remote *meta*-selective acetoxylation of aniline and benzylamine derivatives was achieved by Yu and co-workers in 2014.[50]

Subsequently, our group disclosed the *meta*-acetoxylation of electron-deficient benzoic acid derivatives by using the nosyl-protected 2-(2'-cyanophenyl)ethylamine as the directing group (**eq 16**).[37] Remarkably, the directing group in the resulting products could be easily cleaved under mild basic conditions to form several different and useful *meta*-functionalized entities containing a benzoic acid core.

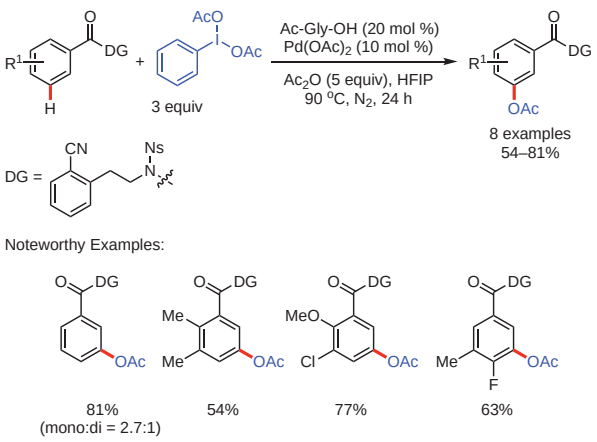
The palladium-catalyzed *meta*-C–H acetoxylation of benzylsulfonyl scaffolds using $PhI(OAc)_2$ as the acetoxylating reagent and Boc-Ala-OH as ligand has been reported.[51] Interestingly, a change of $PhI(OAc)_2$ to $PhI(TFA)_2$, with For-Gly-OH as ligand, led to *meta*-C–H hydroxylation instead of acetoxylation (**Scheme 7**).[51] This is likely due to the easier hydrolysis of the trifluoroacetate than the acetate intermediate under the reaction conditions. The synthetic utility of this approach was illustrated by concise syntheses of a resveratrol precursor and a phase II quinone reductase activity inducer.

The same laboratory successfully extended this methodology to the *meta*-C–H acetoxylation and hydroxylation of benzylic phosphonates by employing the same conditions and reagents.[38] The facile removal of the phophonate tether permitted the synthesis of 1,3- and 1,3,5-trialkenylarenes in good-to-high yields.

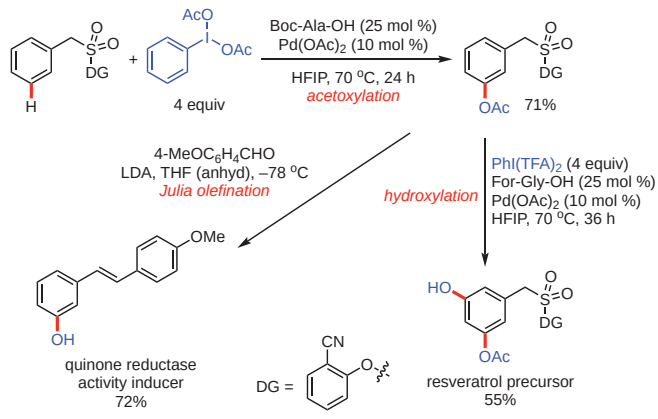
As discussed previously, our group incorporated CO_2 into a novel nitrile-containing directing group and achieved the successful *meta*-C–H olefination of anilines. We extended this methodology to the *meta*-C–H acetoxylation of unsubstituted and mono- and disubstituted protected anilines with $PhI(OAc)_2$ to afford the *meta*-acetoxylation products in moderate-to-good yields.[45] Moreover, very recently, our group successfully accomplished the first direct *meta*-C–H trifluoroethoxylation of an *N*-sulfonylbenzamide en route to a formal synthesis of flecainide, a drug that is used to prevent and treat tachyarrhythmias (**Scheme 8**).[52] This approach should be important in medicinal chemistry and drug discovery, since it is potentially



Scheme 6. Application of Jin and Xu's Remote *meta*-C–H Activation of Distal Arene-Tethered Alcohols. *(Ref. 46)*



eq 16 *(Ref. 37)*



Scheme 7. Application of Maiti's *meta*-C–H Acetoxylation and Hydroxylation of Benzylsulfonyl Scaffolds. *(Ref. 51)*

applicable to the synthesis of flecainide derivatives bearing other substituents on the benzene ring, which is difficult to achieve by the known methods.

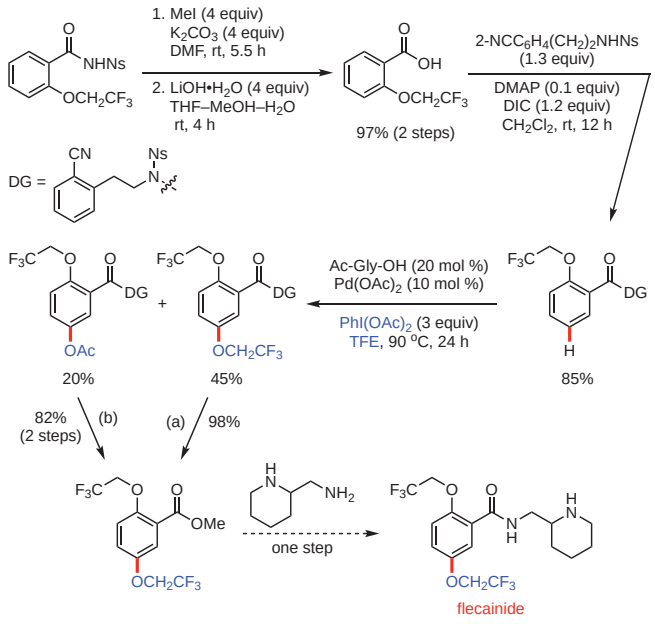
3.1.3. Silylation and Germanylation

Since silicon functional groups can be easily converted into versatile functionality,[53–56] a selective one-step *meta*-C–H silylation reaction was long overdue. Maiti and co-workers disclosed the first report of such a transformation in 2017, employing a pair of polysubstituted benzonitriles as directing groups and hexamethyldisilane as the silylating reagent (**eq 17**).[57] Benzylsulfonate esters and other arylalkanesulfonate esters were compatible with this protocol by overcoming the large strain energy associated with formation of a metallacycle intermediate possessing a greater-than-11-membered ring. In addition, *meta*-germanylation of benzylsulfonates was also achieved in moderate yields and with excellent selectivity in spite of the higher reducing property of the digermanium reagent and lower stability of the C−Ge bond. The synthetic utility of this new *meta*-C–H silylation was demonstrated in a formal synthesis of TAC101, a potential drug candidate for the treatment of lung cancer.

3.2. *meta*-C–H Functionalization Assisted by an N-Heterocycle-Containing Directing Group

In an effort to amplify on existing remote *meta*-C–H functionalizations, Yu and co-workers developed a new directing group in which the weakly coordinating nitrile group was replaced with a pyridyl group—a strongly coordinating *ortho*-directing group. Through molecular design based on distance and geometry, this group was engineered to direct the *meta*-C–H olefination and iodination of benzyl and phenylethyl alcohols.[58] This new protocol demonstrated that the pyridyl group not only maintained the U-shaped conformation, but also mimicked the end-on coordination, which paved the way for developing various unexplored *meta*-C–H transformations. In this way, a new *meta*-C–H iodination reaction was realized using 2-fluoropyridine as the directing group and DIH (1,3-diiodo-5,5-dimethylhydantoin) as the iodination reagent, affording aryl iodides in good yields (**eq 18**).[58] Such aryl iodides can serve as reaction partners in a wide range of carbon–carbon and carbon–heteroatom bond-forming reactions such as the Heck and Suzuki cross-coupling reactions. This *meta*-iodination reaction had not been possible using nitrile-based directing groups.

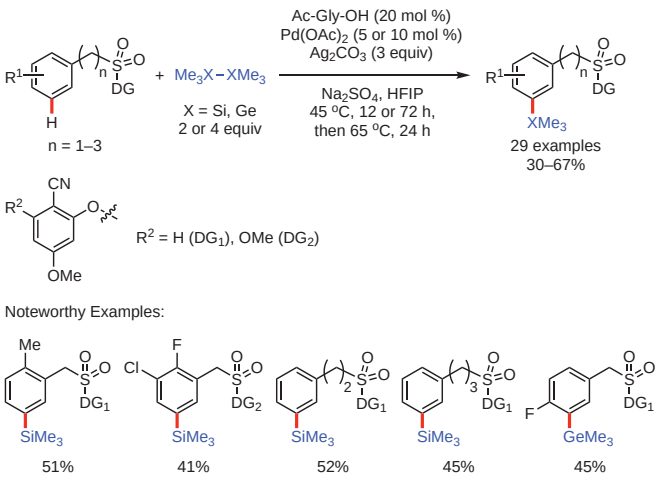
A novel, biphenylpyrimidine-based directing template has very recently been employed to effect the selective, palladium-catalyzed *meta*-C–H alkylation and alkenylation with allylic alcohols. Benzylsulfonyl, benzylphosphonate, phenethylcarbonyl, and phenethylsulfonyl ester scaffolds were successfully functionalized at the meta position, leading to the formation of β-aryl aldehydes and ketones (**eq 19**).[59] It is worth noting that the nitrile-group-directed *meta*-C–H activation had not been reported with an allyl alcohol. Additionally, this protocol permitted for



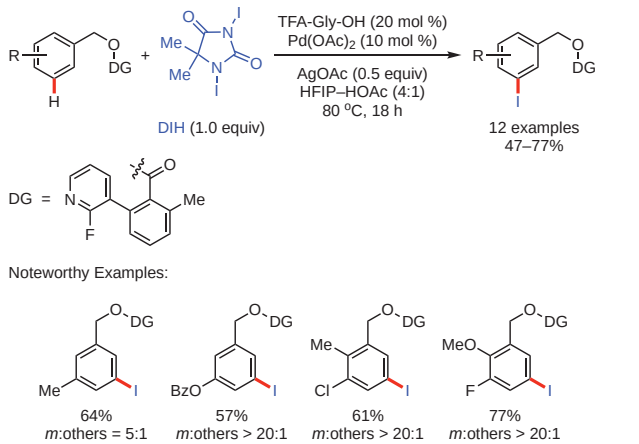
(a) K_2CO_3, MeOH. (b) (i) K_2CO_3, MeOH; (ii) CF_3CH_2I, K_2CO_3, DMF, 100 °C

DIC = *N*,*N*′-diisopropylcarbodiimide. DMAP = 4-(dimethylamino)pyridine.
TFE = 2,2,2-trifluoroethanol

Scheme 8. The First Reported *meta*-C–H Trifluoroethoxylation of an Arene en Route to a Formal Synthesis of the Antiarrhythmic Agent, Felcainide. *(Ref. 52)*



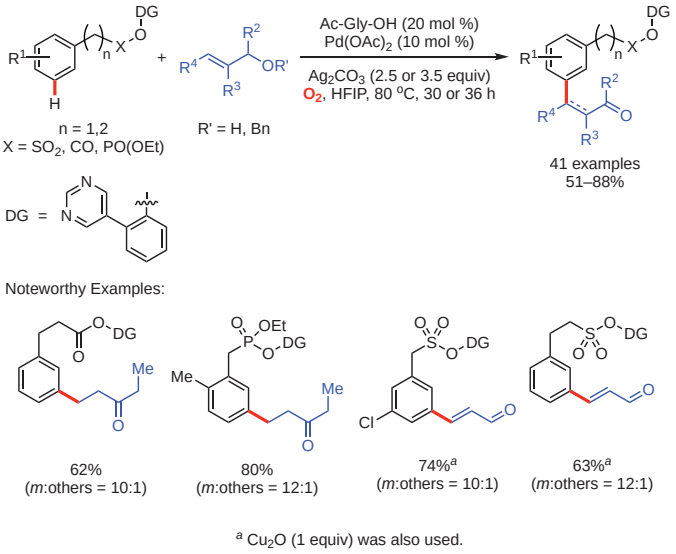
eq 17 *(Ref. 57)*



eq 18 *(Ref. 58)*

the first time the generation of α,β-unsaturated aldehydes by in situ removal of the benzyl group from the benzyl-protected allyl alcohol alkenylating partner.

Although the U-shaped-template-assisted *meta*-C–H bond activation through a macrocyclic cyclophane transition state has been improved by expanding its scope and efficiency, it is still limited by the need for an appropriate functional group to covalently attach the template to the substrate and by the incompatibility of most heterocyclic substrates with this approach. To overcome these limitations, Yu and co-workers reported very recently a remote *meta*-C–H olefination of 3-phenylpyridines by using a *catalytic amount* of a well-designed bifunctional template in the presence of both $Pd(OAc)_2$ and $Cu(OAc)_2$ *without attaching the template to the substrates* (**eq 20**).[60] This catalytic pyridine-containing sulfonamide template with two metal coordination centers binds the heterocyclic substrates via a reversible coordination instead of a covalent linkage. In this way, a stoichiometric amount of a covalently attached template was not required for this challenging *meta*-C–H olefination of pyridine derivatives.

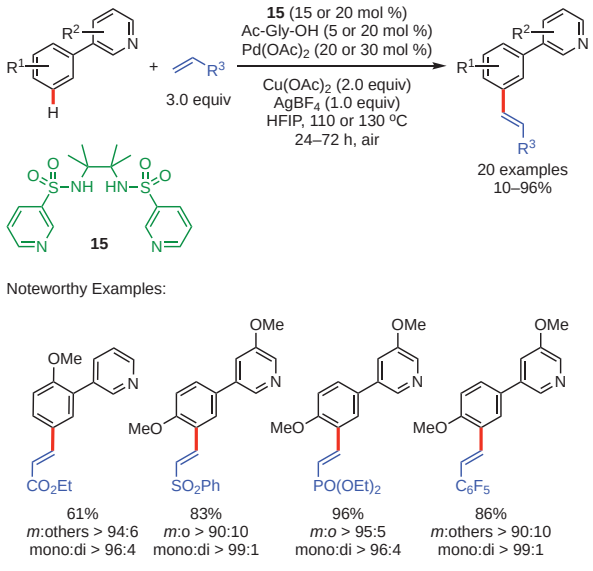


eq 19 *(Ref. 59)*

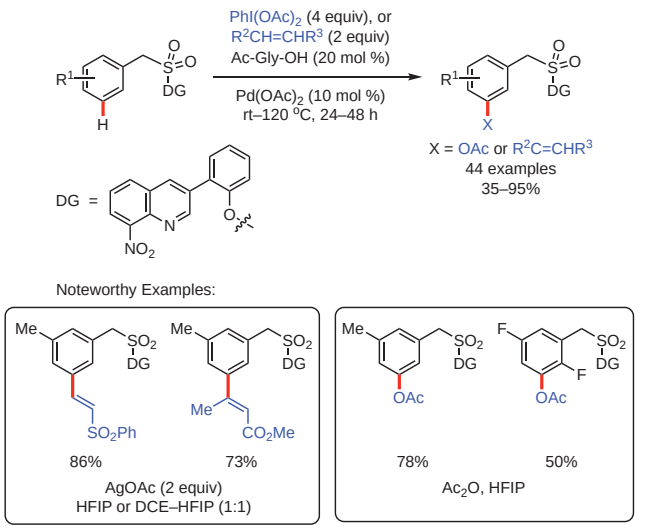
Although σ-coordination of a heteroatom-containing directing group has been extensively utilized in directed *ortho*-C–H functionalizations and aliphatic C–H activations,[61,62] selective *meta*-C–H functionalizations using strong σ-coordination has remained a great challenge. In this regard, Maiti and co-workers have introduced 8-nitroquinoline as the directing group that forms a stable palladacycle leading to selective *meta*-olefination and *meta*-acetoxylation of sulfonates (**eq 21**).[63] This palladium-catalyzed, sulfonate-linked, and 8-nitroquinoline-based *meta*-C–H functionalization mimicks the traditional *ortho*-C–H functionalization through strong σ-coordination. The *meta*-olefinated and acetoxylated arenes thus produced were further elaborated into a variety of valuable compounds some of which are difficult to synthesize by other methods. The versatility and attractiveness of this *meta*-olefination protocol was demonstrated in a high-yield (86%), gram-scale synthesis.

4. Remote *para*-C–H Functionalization Assisted by a *para*-Directing Group

Several successful outcomes have been achieved in the selective *para*-C–H activation over the past few years.[15] However, the traditional strategies for *para*-C–H functionalization are governed either by electronic factors or steric demands and suffer from lower yields, narrow substrate scopes, and poor regioselectivity. While the directing-group–assisted and palladium-catalyzed *meta*-C–H bond functionalizations have become a class of relatively mature transformations, few reports exist on the selective functionalization of the *para*-position that utilizes a template strategy. Thus, achieving the *para*-C–H functionalization with good selectivity and yields has remained problematic and extremely challenging.



eq 20 *(Ref. 60)*



eq 21 *(Ref. 63)*

In 2015, Maiti and co-workers published the first report on the remote *para*-C–H functionalization of arenes using a novel, systematically engineered, silicon-containing, and biphenyl-based template that directs the remote functionalization by forming a D-shaped assembly.[64] Moreover, *ortho*- and/or *meta*-C–H activation would be highly disfavored owing to the rigidity of the biphenyl moiety. The utilization of a di-isopropyl-substituted silyl center as the tether was essential to the *para*-selective functionalization, since the presence of two sterically hindered isopropyl groups at silicon leads to a "Thorpe–Ingold" effect, which allows the coordinating nitrile group to get closer to the *para*-C–H bond by a domino-like "steric push" (**eq 22**).[64]

This new protocol is compatible with a broad range of olefinic partners and arene substrates possessing either electron-donating or electron-withdrawing groups, and affords *para*-olefinated products in satisfactory yields and good-to-excellent *para*-selectivity. This template-assisted strategy was extended to the regioselective *para*-acetoxylation of toluene derivatives in moderate yields. Moreover, the cyanobiphenylol directing group can be easily cleaved from the products and recovered with either tetrabutylammonium fluoride (TBAF) or *para*-toluenesulfonic acid (PTSA).

In 2016, Maiti and and co-workers revisited their work on the palladium-catalyzed *para*-C–H functionalization by switching the positions of the benzylic methylene and phenolic oxygen atom in the substrate and directing group, respectively, resulting in a modified template for the *para*-C–H olefination of phenol derivatives (**eq 23**).[65] This protocol tolerated not only a wide range of electron-donating and electron-withdrawing substituents, but also a diverse array of olefins including esters, aldehydes, amides, ketones, phosphonates, sulfonyls, and olefins with long-chain alkyl substituents. The usefulness of this *para*-functionalization reaction was demonstrated by applying it to the synthesis of various phenol-based natural products and complex molecules—such as drupanin, artepillin, plicatin B, ferulic acid, dehydrozingerone, fenchyl 4-hydroxycinnamate, and *para*-coumaric acid—in good-to-excellent yields.

5. Conclusion and Outlook

The past few years have witnessed rapid developments in palladium-catalyzed *meta*- and *para*-C–H bond functionalizations assisted by a chelating directing group. Strikingly, the innovative palladium(II)–norbornene relay approach has evolved rapidly to effect a series of unprecedented *meta*-C–H bond transformations by employing novel ligand scaffolds and modified transient mediators (2-carbomethoxynorbornene). New types of "end-on" coordinating templates have been engineered to expand the scope of *meta*-C–H bond functionalization reactions, and *para*-C–H bond transformations have been accomplished by designing novel D-shaped templates. These improvements will open up new avenues for expediting access to specialized molecules. Despite these significant advances, there remains a strong need to expand the types of transformation possible as well as the substrate scopes of existing methods. For application on a large scale, significant efforts are still required to improve the current protocols, such as by lowering the catalyst loading and simplifying the template. Finally, a more promising and efficient way to achieve the desired regioselectivity would be to use a catalytic amount of a detached directing group, or to install the directing functionality on a suitable ligand that is also capable of promoting the reactivity of metal catalysts required for activating the target C–H bond.

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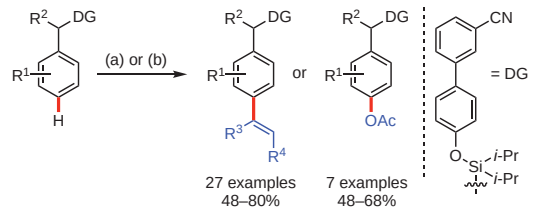
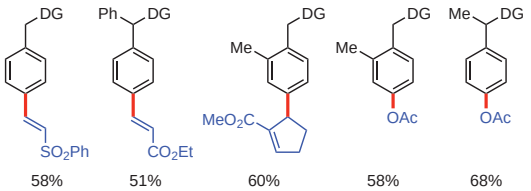
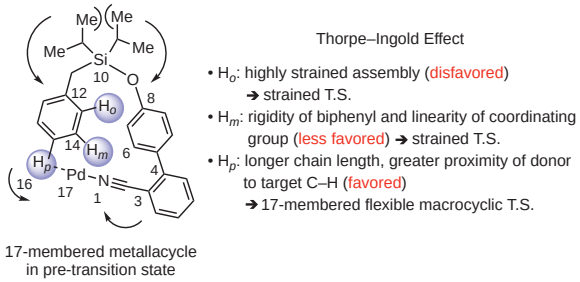
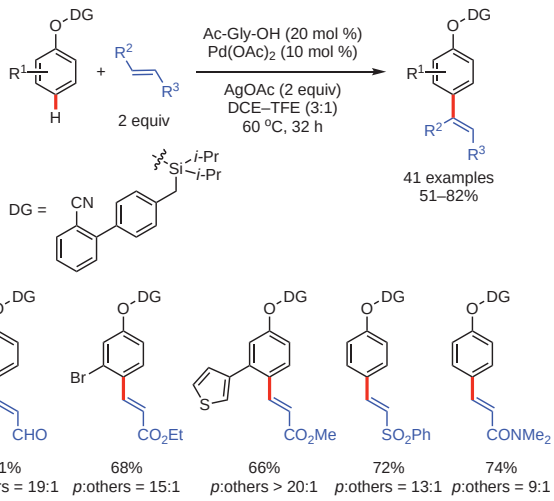


(a) $R^3HC{=}CHR^4$ (2 equiv), Ac-Phe-OH (20 mol %), $Pd(OAc)_2$ (10 mol %), AgOAc (3 equiv), HFIP, 90 °C, 36 h.
(b) $PhI(OAc)_2$ (2 equiv), Piv-Ala-OH (30 mol %), $Pd(OAc)_2$ (15 mol %), HFIP, 70 °C, 24 h.





eq 22 *(Ref. 64)*



eq 23 *(Ref. 65)*

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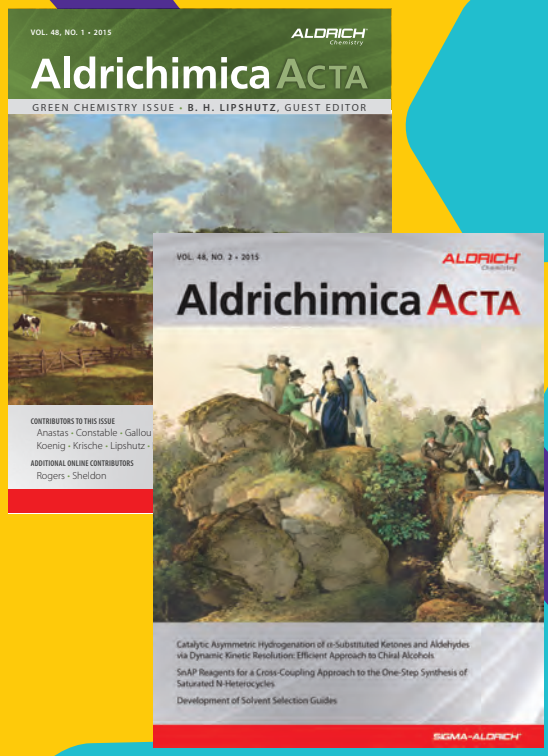

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