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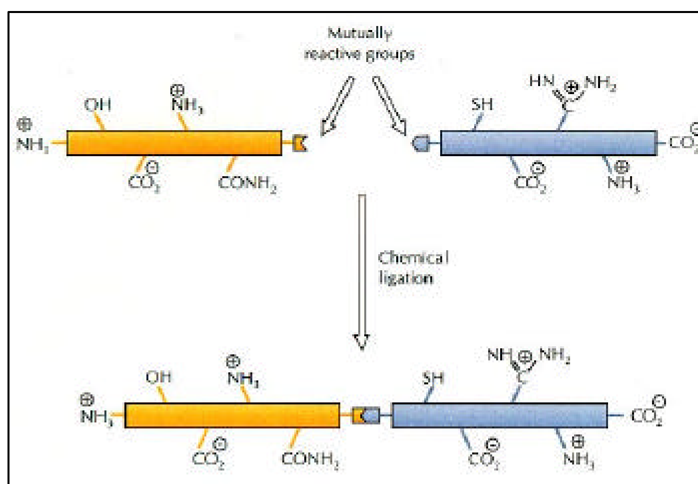
Chemoselective Ligation of Proteins and Glycoproteins

Chemoselective Ligation is a method by which two peptide fragments are coupled via mutually and uniquely reactive functional groups (**Scheme 1**).[1] The two mutually reactive groups, which are orthogonal in reactivity to side chain functionalities, allow for the use of fully unprotected peptides. Chemoselective ligation has proven successful in the formation of non-native as well as native amide linkages and has found widespread success in the total synthesis of proteins and glycoproteins.

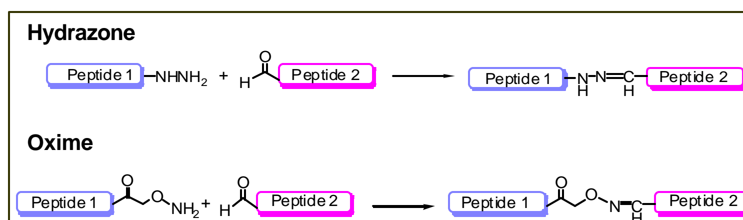


Scheme 1: Chemoselective Ligation

Protein synthesis has traditionally been accomplished through step-wise solid phase synthesis and convergent synthesis through segment condensation, both of which use protected peptide fragments. Step-wise synthesis can be accomplished via either Boc- or Fmoc-Solid Phase Peptide Synthesis (SPPS). Some drawbacks to this methodology include byproduct formation, intermolecular β-sheet aggregation, and decreased solubility of fully protected fragments. SPPS is rarely successful for the synthesis of peptides with >50 residues. Convergent syntheses of protected peptide fragments by segment condensation are inefficient, may undergo C-terminal racemization, and are poorly soluble in organic and aqueous solvents. Chemoselective ligation, which uses unprotected peptide fragments and highly reactive functional groups, increases the solubility and coupling efficiency in protein synthesis.[2]

Chemoselective ligation can be divided into two classes: non-native ligations and acyl transfer ligations. Non-native ligations take advantage of unique reactivities that are absent from protein side chains, forming hydrazone, oxime, thioester or thioether bonds. Acyl transfer reactions generally undergo prior ligation followed by intramolecular acyl transfer.[3] Some examples include Prior Thiol Capture, Pseudo-Proline Ligation, Native Chemical Ligation, and the Staudinger Ligation.

Non-Native Ligations



Scheme 2: Hydrazone and Oxime formation

Aldehydes, which have a unique electrophilic nature orthogonal to nucleophilic peptide side chains, are used in both hydrazone and oxime formations.[4-6] An aldehyde is reacted with a hydrazide or amino-oxy group in an acid catalyzed reaction to form a hydrazone or oxime, respectively (**Scheme 2**). Both hydrazides and amino-oxy groups are strong nucleophiles, preventing unwanted side reactions from occurring with weaker side chain nucleophiles.

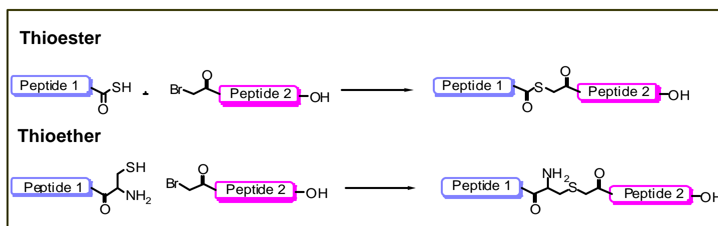
Hydrazone formation was the earliest non-native ligation technique used for protein synthesis.[5] In a proof of principle experiment by Rose and co-workers, a recombinant G-CSF protein was first site specifically

digested at Lys-Ser.[7] The two fragments were then derivatized and allowed to react to form a regiospecific hydrazone.

The disulfide bond in Oxytocin, a short neurohypophyseal hormone, was replaced by a proteolytically stable oxime.[8] The two building blocks necessary for oxime formation, a serine and an aminooxy side chain, were introduced via solid phase synthesis in place of the naturally occurring cysteines. Cyclization of the peptide occurred in two steps: oxidation of the side chain serine followed by oxime formation.

Thioester and thioether formations have also been employed in chemoselective ligation (**Scheme 3**). In these ligations, a thiocarboxylate or thiol is reacted with an N-terminal bromoacetate in an S_N2 reaction. In thioester formations, unwanted side reactions with cysteine side chains are prevented because of the substantial pKa difference between the thiocarboxylate (pKa ~ 3) and the sulfhydryl (pKa ~ 8). When the reaction is run at at pH 5-6, the thioocarboxylate anion is a stronger nucleophile, reacting much faster than the neutral sulfhydryl groups.[9]

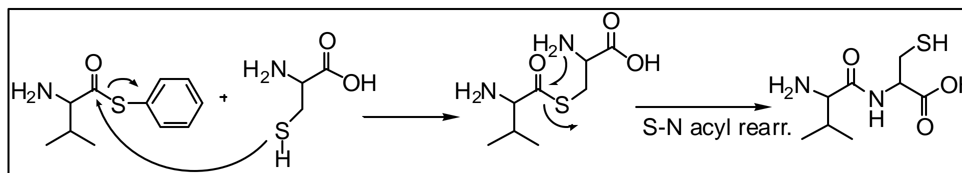
Kent and coworkers synthesized an engineered HIV-1 Protease with a thioester bond replacing Gly[51]-Gly[52] in the flap region of the enzyme.[10,11] This region was chosen for three reasons: the flap region is a flexible portion of the enzyme that is not involved in activity, glycine has no chiral information and therefore cannot undergo racemization, and the thioester bond is approximately isosteric with the Gly-Gly bond. Both peptide fragments were synthesized by Boc-SPPS and brought together in the presence of guanidinium chloride to prevent structure formation during the ligation. In another example, thioether formation was used to form a model of a heterodimeric transmembrane receptor, replacing the transmembrane portion with a coiled coil.[12] The two cytoplasmic portions of the transmembrane receptor were synthesized, each connected to an alpha-helical region. The two helical regions were linked by thioether formation.



Scheme 3: Thioester and Thioether formation

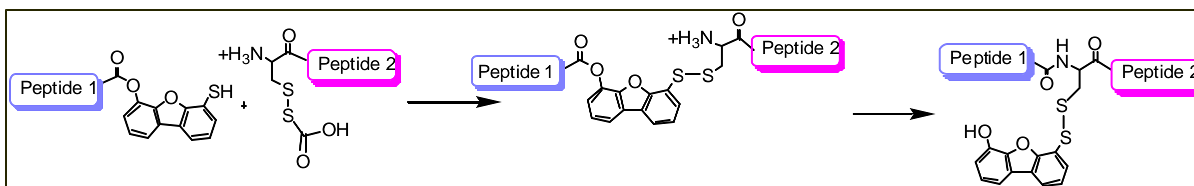
Acyl Transfer Ligations:

Chemoselective ligation has found great success in the formation of native amide bonds through acyl transfer. Wieland first reported peptide bond formation by S-N acyl transfer (**Scheme 4**).[13 14]



Scheme 4: Peptide bond formation between valine thiophenylester and cysteine

Kemp and coworkers employed O-N acyl transfer successfully in Prior Thiol Capture[15,16], an intellectual precursor to Native Chemical Ligation. Model studies showed that O-N acyl transfer could be initiated through a 12-membered ring intermediate using a dibenzofuran template. This method was applied to the synthesis of two short peptides (25 and 39 residues).[17] Intramolecular acyl transfer followed by reduction of the disulfide gives the free peptide in solution (**Scheme 5**).

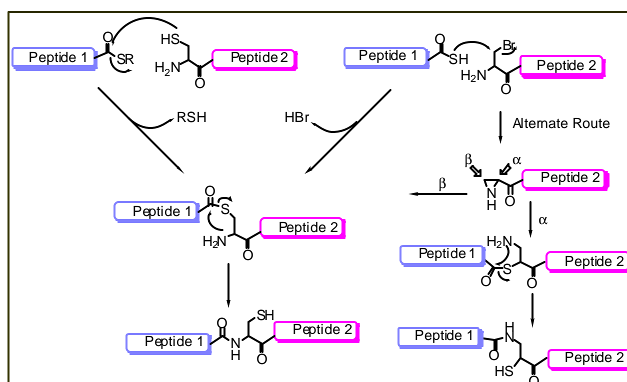


Scheme 5: Prior Thiol Capture to form short peptides

In a control experiment, the N-terminal fragment was side chain protected. This reaction showed a marked decrease in efficiency. It is hypothesized that an asparagine residue in the N-terminal fragment which is proximal to the transition state and can assist in proton transfer. Upon protection of this residue, the efficiency of proton transfer is decreased.[17]

Prior ligation strategies have also proven successful for the formation of Pseudo-Proline.[18] Tam and coworkers found that reaction of an aldehyde with a cysteine residue resulted in the formation of a thiazolidine ring which could then undergo O-N acyl transfer through a [3.3.0] fused ring intermediate to form a proline analogue. This was applied to the synthesis of the 99 residue HIV-1 protease in which Proline-39 was replaced with the thiazolidine ring. This analogue retained enzymatic activity equal to that of the wild type, implying that this non-native linkage did not affect the structure of the protein.[19]

Forty years after Wieland's original discovery, Kent and coworkers established Native Chemical Ligation (NCL), a method for the direct synthesis of a native chemical backbone via prior ligation followed by S-N acyl transfer.[20] An intermolecular thioester exchange takes place between a C-terminal thioester and an N-terminal Cysteine fragment. Intramolecular S-N rearrangement allows for the formation of a native peptide with a cysteine side chain at the ligation site.

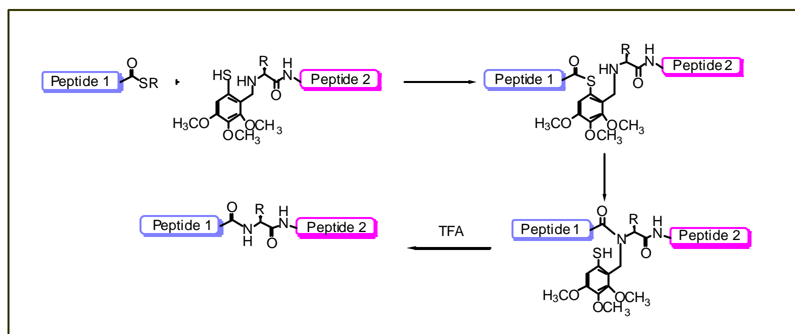


Scheme 7: Native Chemical Ligation

The first example of NCL was synthesis of a 72-aa residue protein, Human Interleukin 8, which contains four cysteine residues.[20] An excess of thiol kept the cysteine residues reduced and did not interfere with the reaction, which resulted in 60% yield after 3d. In a comparison of two methods of intermediate formation, Tam and coworkers found that Kent's thioester capture is superior to acyl initiated capture, which can undergo a side reaction via aziridine formation.[21]

Thioester exchange of the C-terminal benzyl ester with a phenyl thioester greatly increased the rate of native chemical ligation, as phenyl thioester is more reactive than benzyl ester. In this manner, a 110 residue barnase polypeptide analogue with a Lys^{49}-Cys^{49} mutation was synthesized in 4.5 hours.[22]

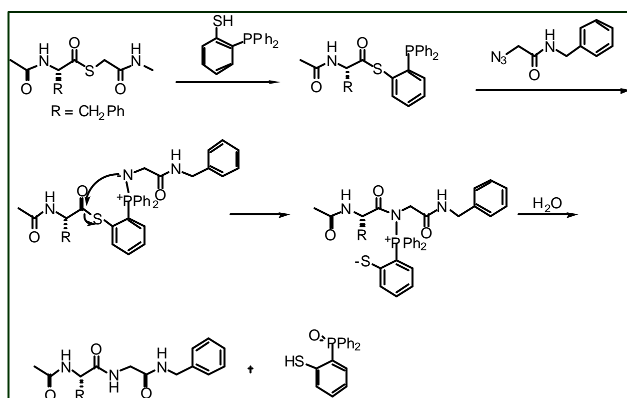
Though NCL is an efficient method for the synthesis of peptides containing cysteine residues, cysteine comprises only 1.7% of all residues in proteins. In order to extend NCL to non-cysteine containing peptides, Kent utilized a cleavable thiol ($HSCH_2CH_2O$-N)[23]. This linker was not as successful as the cysteine side chain because the resulting S-N acyl transfer occurred through a less favored 6-membered ring intermediate.



Scheme 8: Cleavable Thiol Linker

In an extension of this work, Alewood[24] and Dawson[25] found that mercaptobenzyl templates could be used as the cleavable thiol, allowing for acyl transfer via the more favored 5-membered ring transition state. A derivative of the original template can be cleaved under mild conditions to give the native peptide backbone. The 62 amino acid SH3 domain of α-spectrin was synthesized using a variety of ligation sites including Lys-Gly, Gly-Gly, and Gly-Ala. (**Scheme 8**).[26] An attempt at ligation between Ala-Ala was unsuccessful. Therefore, the authors propose that glycine must be in one of the two ligation positions.

The Staudinger Ligation is another chemoselective ligation technique that circumvents the use of cysteine residues for ligation. The Staudinger Reaction, first reported by Staudinger in 1919, involves the electrophilic addition of an azide to a trialkyl phosphine to form an iminophosphorane intermediate.[27,28] This can then undergo dissociation under aqueous conditions to form a free amine and phosphine oxide. Vilarrasa and coworkers found that the iminophosphorane intermediate formed can participate in acyl transfer.[29]



Scheme 10: Staudinger Ligation

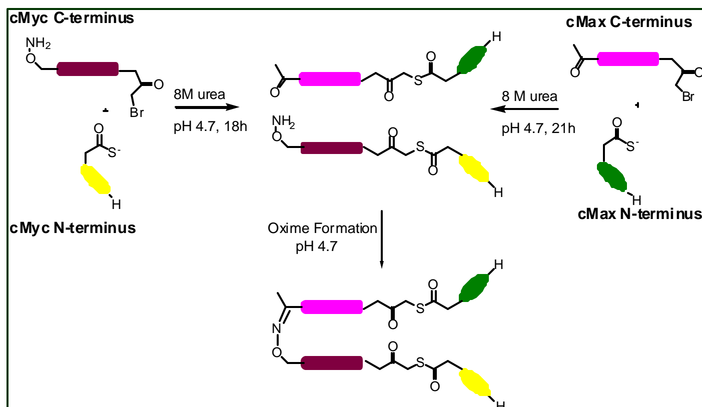
The Staudinger Ligation was first employed by Bertozzi and co-workers in cell surface engineering.[30] This original example resulted in a covalently attached phosphine after acyl transfer. Bertozzi also designed a 'Traceless' Staudinger Ligation involving the reaction between a phosphine acyl donor and an azido-nucleoside.[31] Concurrently, Nilsson et al. used a cleavable phosphinothiol for peptide formation between a thioester and an azide, resulting in dipeptide formation in 35% yield (**Scheme 10**).[32]

Raines and co-workers improved the efficiency of the Staudinger Ligation by employing a 5-membered ring transition state instead of the original 6-membered ring. This was accomplished by replacing the *o*-phenyl of the original phosphinothiol with a methylene group. This new phosphinothiol increased reaction yields to between 80-100% for the formation of di- and tri-peptides. It is proposed that this improved phosphinothiol can form a ten membered ring hydrogen bond. In this form, the iminophosphorane nitrogen is brought to within 3Å of the carbonyl, allowing for acyl transfer to take place.[33]

Tandem Ligation

Extending Chemoselective ligation to the synthesis of larger protein complexes can be accomplished through two or more compatible orthogonal ligations. The total chemical synthesis of a covalently bound 172-aa transcriptional activator protein, was accomplished by three ligations.[34] This protein, cMyc-cMax is known to form stable non-covalent heterodimers which can bind to DNA. However, biophysical studies of this interaction are impeded by cMax homodimer formation. Formation of the heterodimer by

covalent ligation prevents homodimer formation. The N- and C-terminal portions of cMyc and cMax were first coupled by thioester formation. The two fragments were then brought together by oxime formation to give the heterodimer. Unnatural linkages were placed in areas of the protein that were flexible and would not affect DNA binding. The synthesized protein showed similar binding specificity to the WT homodimer, implying that these unnatural linkages did not affect heterodimer function.



Scheme 11: cMyc-cMax Heterodimer formation

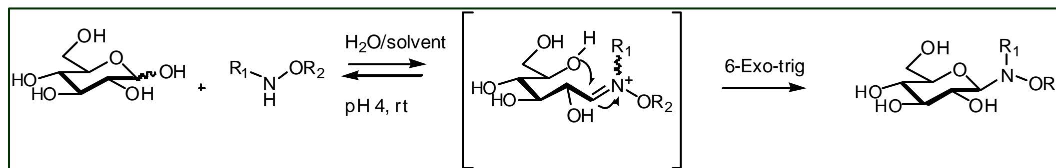
Another example of tandem ligation is in the synthesis of MP-1α and MIP-1β, members of the CC-chemokine protein family which have been shown to inhibit HIV-1 pathogenesis. Because these proteins contain multiple proline and cysteine residues, they have been used to test the conformational influence of tandem ligation reactions. In both cases, the non-native linkages formed did not disrupt biological activity.[35]

Glycoprotein Synthesis:

Chemoselective ligation has also been successful in the synthesis of glycoproteins. In nature, glycoproteins exist as a variety of glycoforms, making it difficult to isolate one specific glycoform for biological studies.[36] Total synthesis of these compounds gives access to isolated glycoforms that have been glycosolyated regioselectively.[9,36]

Proteins are generally glycosylated at a serine or threonine, forming O-linked glycoproteins, or at an asparagines, forming N-linked glycoproteins. Both the regio- and stereoselectivity of glycosylation is determined by the enzyme used. Chemoselective ligation of glycoproteins uses unnatural ligation techniques such as hydrazone and oxime to link sugars to proteins.

In an example of O-linked oxime formation, the anomeric position of maltotriose was reacted with an amino-oxy derivatized somatostatin analogue. This ligation resulted in three species in solution: the β-cyclic sugar, and the E- and Z- open chain isomers.[37] To overcome this equilibration between open and closed chain isomers, the nitrogen of the amino-oxy functionality can be alkylated (**Scheme 12**). Addition of a methyl group resulted in only one species in solution.[38] Carrasco et al. has also synthesized an N'-amino-oxy amino acid for SPPS, which upon cleavage reveals a free amino-oxy functionality within the peptide chain. This can then react with the desired sugar to form an oxime.[39]

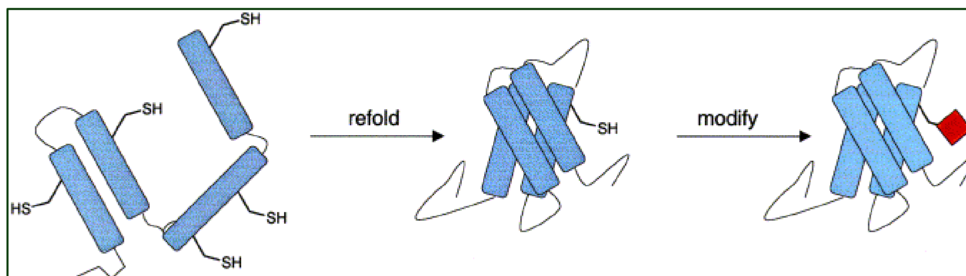


Scheme 12: Glycosylation of amino-oxy peptide

Bertozzi and coworkers have studied the effects of unnatural peptide-sugar linkages on biological activity. Drosocin, an antimicrobial agent with glycosylation dependent activity, was synthesized with an unnatural oxime linkage. Interestingly, this unnatural analogue was 4 times more potent than the unglycosylated Drosocin peptide and showed similar activity to the natural glycosolated species.[40] This

glycopeptide synthesis also used an electrophilic peptide and a nuclephilic sugar as opposed to the previous work which relied on electrophilic sugar moieties. Drocosin was linked to chitobiose in a similar manner.[41] Glycoslyation of protein-sugar conjugates can also be performed. Both C-3 thioethers and C-6 oxime ligations have been used to link two sugars together.[42]

In an interesting application of chemoselective ligation, the peptide hormone Erythropoietin was synthesized. The natural Asn glycosylation site was first modified to a cysteine. The protein was then allowed to fold into its native conformation, forming two disulfide bonds. Once folded, the free cysteine was reacted with a sugar containing an iodoacetyl group at the anomeric position (**Scheme 13**). The four native cysteine residues were not glycosylated under these conditions.[43]

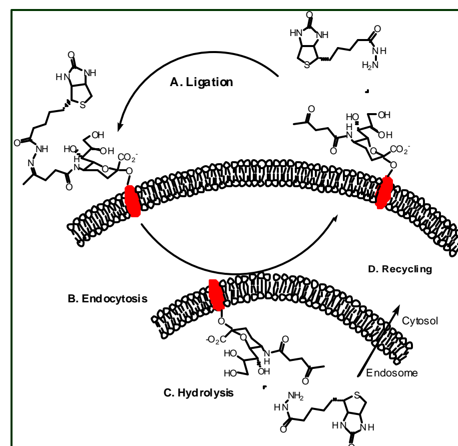


Scheme 13: Site specific glycosylation of Erythropoietin

Cell Surface Engineering

Engineering a cellular surface with an orthogonal functional group allows for site specific ligation of molecules to cell surfaces. In an early example of this, Tolvanen et al. first oxidized the cell surface with galactose oxidase or periodate to give regioselective aldehyde formation.[44] Mannosylhydrazine was then reacted with the cell surface to form hydrazone conjugates at the oxidized positions.

Bertozzi and coworkers also applied chemoselective ligation to cell surface engineering, using hydrazone formation and the Staudinger Ligation. In the case of hydrazone formation, An unnatural derivative of N-acetyl-mannosamine with a ketone functionality was added to cells and subsequently converted to its corresponding sialic acid. The cell then incorporated these sialic acid derivatives onto the cell surface. This ketone was then reacted with hydrazides, showing that this methodology is successful for selective drug delivery.[45] Further studies showed that this method can deliver similar numbers of molecules as antibodies.[46]



Scheme 14: Cell Surface Engineering

Summary

Chemoselective Ligation reactions can be employed for the total synthesis of a variety of proteins and protein conjugates. A distinct advantage of this method is that large proteins can be formed from smaller, easily synthesized fragments. Selective placement of non-native ligation sites has proven successful in the formation of biologically active protein analogues. This has provided the opportunity to synthesize proteins that were previously unavailable for biological study. Recent advances using acyl transfer reactions for ligation have proven very successful in the formation of native peptide backbones. Though many of these ligation techniques still have disadvantages such as the necessity for specific amino acid residues, this offers a method by which native proteins can be synthesized. Further studies of chemoselective ligation techniques are necessary to increase the scope of these ligations to include a

larger variety of amino acid residues. Glycoprotein synthesis via ligation techniques has provided an opportunity for the study of single glycoforms, though many of these syntheses are not stereoselective, and rely on non-native linkages. Also, utilization of ligation on cell surfaces could provide a novel and innovative method of drug delivery. In conclusion, chemoselective ligation can be used to provide access to a variety of proteins that are inaccessible by traditional protein synthesis, giving such high specificity that these reactions can even be performed in the presence of cells.

References

(1) Muir, T. W. *Structure* **1995**, A Chemical Approach to the Construction of Multimeric Protein Assemblies, *3*, 649-652.
(2) Borgia, J. A.; Fields, G. B. *Trends Biotechnol.* **2000**, Chemical synthesis of proteins, *18*, 243-251.
(3) Coltart, D. M. *Tetrahedron* **2000**, Peptide Segment Coupling by Prior Ligation and Proximity-Induced Intramolecular Acyl Transfer, *56*, 3449-3491.
(4) Sayer, J. M., Peskin, M. Jencks, W.P. *J. Am. Chem. Soc.* **1973**, Imine-Forming Elimination Reactions I. General Base and Acid Catalysis and Influence of the Nitrogen Substituent on Rates and Equilibria for Carbinolamine Dehydration, *95*, 4277-4287.
(5) King, T. P. Z., S.W. Lam, T. *Biochemistry* **1986**, Preparation of Protein Conjugates via Intermolecular Hydrazone Linkage, *25*, 5774-5779.
(6) Rose, K. *J. Am. Chem. Soc.* **1994**, Facile Synthesis of Homogeneous Artificial Proteins, *116*, 30-33.
(7) Gaertner, H. F. R., K. Cotton, R. Timms, D. Camble, R. Offord, R.E. *Bioconjugate Chem.* **1992**, Construction of Protein Analogues by Site-Specific Condensation of Unprotected Fragments, *3*, 262-268.
(8) Wahl, F.; Mutter, M. *Tetrahedron Lett.* **1996**, Analogues of oxytocin with an oxime bridge using chemoselectively addressable building blocks, *37*, 6861-6864.
(9) Lemieux, G. A.; Bertozzi, C. R. *Trends Biotechnol.* **1998**, Chemoselective ligation reactions with proteins, oligosaccharides and cells, *16*, 506-513.
(10) Schnolzer, M.; Kent, S. B. H. *Science* **1992**, Constructing Proteins by Dovetailing Unprotected Synthetic Peptides - Backbone-Engineered Hiv Protease, *256*, 221-225.
(11) Baca, M.; Muir, T. W.; Schnolzer, M.; Kent, S. B. H. *J. Am. Chem. Soc.* **1995**, Chemical Ligation of Cysteine-Containing Peptides - Synthesis of a 22-Kda Tethered Dimer of Hiv-1 Protease, *117*, 1881-1887.
(12) Muir, T. W.; Williams, M. J.; Ginsberg, M. H.; Kent, S. B. H. *Biochemistry* **1994**, Design and Chemical Synthesis of a Neoprotein Structural Model for the Cytoplasmic Domain of a Multisubunit Cell-Surface Receptor - Integrin Alpha(Iib)Beta(3) (Platelet Gpiib-Iiia), *33*, 7701-7708.
(13) Wieland, T. B., E. Bauer, L. Lang, H.U. Lau, H *Liebigs Ann. Chem.* **1953***583*, 129-149.
(14) Aimoto, S. *Current Organic Chemistry* **2001**, Contemporary Methods for Peptide and Protein Synthesis, *5*, 45-87.
(15) Kemp, D. S. *Biopolymers* **1981***20*, 1793.
(16) Fotouhi, N. G., N.G. Kemp, D.S.1989 *J. Org. Chem.* **1989**, Peptide Synthesis by Prior Thiol Capture. 6. Rates of the Disulfide Bond Forming Capture Reaction and Demonstration of the Overall Strategy by Synthesis of the C-Terminal 29-Peptide Sequence of BPTI, *54*, 2803-2817.
(17) Kemp, D. S.; Carey, R. I. *J. Org. Chem.* **1993**, Synthesis of a 39-Peptide and a 25-Peptide by Thiol Capture Ligations - Observation of a 40-Fold Rate Acceleration of the Intramolecular O,N-Acyl-Transfer Reaction between Peptide- Fragments Bearing Only Cysteine Protective Groups, *58*, 2216-2222.
(18) Liu, C. F.; Tam, J. P. *Proc. Natl. Acad. Sci. U. S. A.* **1994**, Peptide Segment Ligation Strategy without Use of Protecting Groups, *91*, 6584-6588.
(19) Liu, C. F.; Rao, C.; Tam, J. P. *J. Am. Chem. Soc.* **1996**, Orthogonal ligation of unprotected peptide segments through pseudoproline formation for the synthesis of HIV-1 protease, *118*, 307-312.
(20) Dawson, P. E.; Muir, T. W.; Clarklewis, I.; Kent, S. B. H. *Science* **1994**, Synthesis of Proteins by Native Chemical Ligation, *266*, 776-779.
(21) Tam, J. P.; Lu, Y. A.; Liu, C. F.; Shao, J. *Proc. Natl. Acad. Sci. U. S. A.* **1995**, Peptide synthesis using unprotected peptides through orthogonal coupling methods, *92*, 12485-12489.

(22) Dawson, P. E.; Churchill, M. J.; Ghadiri, M. R.; Kent, S. B. H. *J. Am. Chem. Soc.* **1997**, Modulation of reactivity in native chemical ligation through the use of thiol additives, *119*, 4325-4329.
(23) Canne, L. E.; Bark, S. J.; Kent, S. B. H. *J. Am. Chem. Soc.* **1996**, Extending the applicability of native chemical ligation, *118*, 5891-5896.
(24) Miranda, L. P. M., W.D.F. Smythe, M.L. Alewood, P.F. *J. Org. Chem.* **2000**, An activated O-N Acyl Transfer Auxiliary: Efficient Amide Backbone Substitution of Hindered, "Difficult" Peptides, *65*, 5460-5468.
(25) Offer, J. D., P.E. *Org. Lett.* **2000**, N-alpha-2-mercaptobenzylamine-assisted Chemical Ligation, *2*, 23-26.
(26) Offer, J. B., C.N.C. Dawson, P.E. *J. Am. Chem. Soc.* **2002**, Extending Synthetic Access to Proteins with a Removable Acyl Transfer Auxiliary, *124*, 4642-4646.
(27) Staudinger, H. M., J. *Helvetica Chimica Acta* **1919**2, 635.
(28) Golobolov, Y. G. K., L.F. *Tetrahedron* **1992**, Recent Advances in the Staudinger Reaction, *48*, 1353-1406.
(29) Garcia, J. U., F. Vilarrasa, J *Tetrahedron Lett.* **1984**, New Synthetic "Tricks". Triphenylphosphine mediated amide formation from carboxylic acids and azides, *25*, 4841-4844.
(30) Saxon, E.; Bertozzi, C. R. *Science* **2000**, Cell surface engineering by a modified Staudinger reaction, *287*, 2007-2010.
(31) Saxon, E.; Armstrong, J. I.; Bertozzi, C. R. *Org. Lett.* **2000**, A "traceless" Staudinger ligation for the chemoselective synthesis of amide bonds, *2*, 2141-2143.
(32) Nilsson, B. L.; Kiessling, L. L.; Raines, R. T. *Org. Lett.* **2000**, Staudinger ligation: A peptide from a thioester and azide, *2*, 1939-1941.
(33) Nilsson, B. L. K., L.L. Raines, R.T. *Org. Lett.* **2001**, High-Yielding Staudinger Ligation of a Phosphinothioester and Azide to form a Peptide, *3*, 9-12.
(34) Canne, L. E.; Ferredamare, A. R.; Burley, S. K.; Kent, S. B. H. *J. Am. Chem. Soc.* **1995**, Total Chemical Synthesis of a Unique Transcription Factor- Related Protein - Cmyc-Max, *117*, 2998-3007.
(35) Tam, J. P.; Yu, Q. T.; Yang, J. L. *J. Am. Chem. Soc.* **2001**, Tandem ligation of unprotected peptides through thiaprolyl and cysteinyl bonds in water, *123*, 2487-2494.
(36) Davis, B. G.; Jones, J. B. *Synlett* **1999**, Glycoprotein synthesis: From glycobiological tools to tailor- made catalysts, 1495-1507.
(37) Cervigni, S. E.; Dumy, P.; Mutter, M. *Angew. Chem.-Int. Edit. Engl.* **1996**, Synthesis of glycopeptides and lipopeptides by chemoselective ligation, *35*, 1230-1232.
(38) Peri, F. D., P. Mutter, M. *Tetrahedron* **1998**, Chemo- and Stereoselective Glycosylation of Hydroxylamino derivatives: A Versatile Approach to Glycoconjugates, *54*, 12269-12278.
(39) Carrasco, M. R. N., M.J. Burnell, D.R. MacLaren, M.D. Hengel, S.M. *Tetrahedron Lett.* **2002**, Synthesis of neoglycopeptides by chemoselective reaction with peptides containing a novel N'-alpha-aminooxy amino acid, *43*, 5727-5729.
(40) Marcaurelle, L. A. R., E.C. Bertozzi, C.R. *Tetrahedron Lett.* **1998**, Synthesis of an Oxime-linked neoglycopeptide with glycosylation-dependent activity similar to its native counterpart, *39*. 8417-8420.
(41) Rodriguez, E. C.; Marcaurelle, L. A.; Bertozzi, C. R. *J. Org. Chem.* **1998**, Aminooxy-, hydrazide-, and thiosemicarbazide-functionalized saccharides: Versatile reagents for glycoconjugate synthesis, *63*, 7134-7135.
(42) Marcaurelle, L. A.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2001**, Chemoselective elaboration of O-linked glycopeptide mimetics by alkylation of 3-ThioGalNAc, *123*, 1587-1595.
(43) Macmillan, D. B., R.M. Sage, K.A. Fern, D. Flitsch, S.L. *Chem. Biol.* **2001**, Selective in vitro glycosylation of recombinant proteins: semi-synthesis of novel homogeneous glycoforms of human erythropoietin, *8*, 133-145.
(44) Tolvanen, M., Gahmberg, C.G. *J. Biol. Chem.* **1986**, *In Vitro* Attachment of Mono- and Oligosaccharides to Surface Glycoconjugates of Intact Cells, *261*, 9546-9551.
(45) Mahal, L. K.; Yarema, K. J.; Bertozzi, C. R. *Science* **1997**, Engineering chemical reactivity on cell surfaces through oligosaccharide biosynthesis, *276*, 1125-1128.
(46) Nauman, D. A.; Bertozzi, C. R. *Biochim. Biophys. Acta-Gen. Subj.* **2001**, Kinetic parameters for small-molecule drug delivery by covalent cell surface targeting, *1568*, 147-154.