Bioconjugate Chemistry

Review

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α-Oxo Aldehyde or Glyoxylyl Group Chemistry in Peptide Bioconjugation

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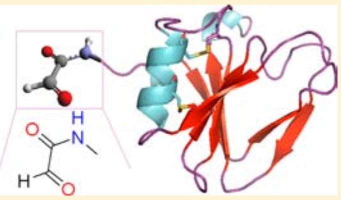
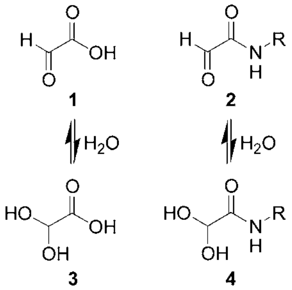
ABSTRACT: Since the 1990s, α-oxo aldehyde or glyoxylic acid chemistry has inspired a vast array of synthetic tools for tailoring peptide or protein structures, for developing peptides endowed with novel physicochemical properties or biological functions, for assembling a large diversity of bioconjugates or hybrid materials, or for designing peptide-based micro or nanosystems. This past decade, important developments have enriched the α-oxo aldehyde synthetic tool box in peptide bioconjugation chemistry and explored novel applications. The aim of this review is to give a large overview of this creative field.



A. INTRODUCTION

The structure of glyoxylic acid **1** is shown in Scheme 1. Glyoxamides **2**, i.e., amide derivatives of glyoxylic acid **1**, are particularly relevant to the field of bioconjugation chemistry. In this case, R is usually an alkyl group linking the α-carboxamido aldehyde group to the biomolecule of interest.

Scheme 1. Structures of Glyoxylic Acid 1, Glyoxamide 2, and Their Corresponding Hydrates 3 and 4, Respectively



Glyoxylyl peptides have been mentioned in the literature since the late 1950s. Their potential formation from N-terminal seryl peptide hormones or proteins during the staining of tissue sections using periodic acid and Schiff's reagent was discussed at that time.[1] During the same period, quantification of the formaldehyde formed during the periodate oxidation of peptides was shown to be an accurate method for demonstrating the presence of N-terminal seryl residues in peptides.[2] A few years later, Dixon and co-workers pioneered the use of glyoxylyl chemistry for selectively tailoring the N-terminus of peptides.[3−6]

A growing interest in glyoxylyl group chemistry began in the early 1990s with the development of various novel chemoselective ligations enabling the assembly of homogeneous macromolecular scaffolds.[7] The ease of introducing the glyoxylyl group on peptides or proteins, its good stability, and the mildness and efficiency of the ligation techniques was soon considered as a great advantage in obtaining complex synthetic or semisynthetic conjugates. Later on, the orthogonality of α-oxo aldehyde-based ligation reactions with other popular site-specific ligation methods such as the Huisgen 1,3-dipolar cycloaddition of organic azides and terminal alkynes extended the synthetic usefulness of the glyoxylyl group by allowing the design of efficient one-pot sequential ligation schemes.[8] Recently, the peculiarities of glyoxylyl group chemistry have also enabled useful developments in the field of protein total or semisynthesis.[9−12]

Some of these studies provided peptide or protein conjugates of potential therapeutic or diagnostic interest. One important application of glyoxylyl group chemistry in medical research is certainly the development of synthetic vaccines. Pioneering studies by Rose and co-workers led to the development of a potential synthetic vaccine against *Plasmodium falciparum* B with demonstrated efficacy in volunteers.[13,14] Very recently, glyoxylyl group chemistry was used to assemble a peptide-based vaccine which was able to induce neutralizing antibodies against heat-stable enterotoxin from enterotoxigenic *Escherichia coli*.[15] In the same field, the usefulness of glyoxylyl lipids for the synthesis of lipopeptide-based self-adjuvanting vaccines was illustrated by the synthesis of an HIV-derived lipopeptide cocktail.[16,17] A similar approach has been recently used for designing a vaccine able to protect the female genital tract from herpes in mouse.[18] Other medical areas have also benefited from glyoxylyl chemistry. A seminal contribution to the field is the design of chemokine RANTES analogues as anti-HIV agents.[19−23]

The beginning of the 21st century has seen the rapid development of micro- and nanotechnologies. Their application in life sciences stimulated the use of glyoxylyl chemistry for the mild and efficient immobilization of biomolecules on

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surfaces.[24,25] Today, the richness of glyoxylic acid chemistry is still a source of innovation in various fields of research. The aim of this review is to give a broad overview of the use of glyoxylic acid chemistry in the field of peptide bioconjugation.

Apart from the Introduction and Conclusion, this review is composed of five main sections (sections B to F). Section B discusses the general chemical properties of glyoxylic acid and amides such as the hydration of the aldehyde group and the formation of imine or iminium species by reaction with amines. Section C is focused on the various methods allowing the installation of the glyoxylyl group onto peptides, proteins, or surfaces. Section D describes the chemoselective ligation methods using the glyoxylyl group as a key reacting partner. Section E describes the powerfulness of glyoxylyl chemistry for synthesizing sophisticated scaffolds, by combining two or more chemoselective ligations in one-pot processes. The last main section, Section F, details how glyoxylyl group chemistry has contributed to the development of novel synthetic tools in the field of protein total or semisynthesis.

B. GLYOXYLYL GROUP: GENERAL CHEMICAL PROPERTIES

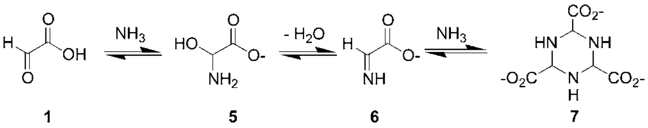
B1. Hydration. α-Keto acids are intermediates in amino acid metabolism. Glyoxylic acid **1** (Scheme 1) corresponds to the α-keto acid derivative of glycine and has a pK_a of 3.3 at 298 K.[26] Glyoxylic acid **1** equilibrates in aqueous solution with its hydrate form **3** (Scheme 1). The mechanism of hydration of glyoxylic acid **1** has been studied by density functional theory.[27] NMR studies have shown that the hydrate form **3** is by far the dominant species in aqueous solution.[28,29] The proportion of hydrate **3** is >99% at pH 0.5 and 95% at pH 6.0 according to Cooper and co-workers.[28] Glyoxylic acid thus shows an increase in the aldehyde form when the pH is raised from 0.5 to 6, in accordance with a higher hydration equilibrium constant for glyoxylic acid ($K_h = H(HO)_2CCO_2H/HCOCO_2H = 300$, 298 K) than for glyoxylate ion ($K_h = H(HO)_2CCO_2^-/HCOCO_2^- = 15.1$, 298 K).[26] Formation of the hydrate form of aldehydes or ketones is known to be favored by the presence of electron-withdrawing groups adjacent to the carbonyl group. The higher hydration equilibrium constant for glyoxylic acid compared to glyoxylate ion is probably due to the greater electron-withdrawing properties of CO_2H compared to CO_2^-. By comparison, the proportion of hydrate for pyruvic acid is much lower in the same conditions, i.e., 65% at pH 0.5 and only 8% at pH 6.0, in accordance with a hydration equilibrium constant of only 1.6 at 298 K for this α-keto acid. The hydrate form **3** of glyoxylic acid is stable and commercially available as a white crystalline solid. Dimethylacetal of methyl glyoxylate can be converted into the corresponding reactive aldehyde form by treatment with phosphorus pentoxide and distillation.[30]

Hydrate **4** is also the dominant species in aqueous solution for glyoxamides of type **2** (Scheme 1).[31] In this case, the pH of the solution is expected to have a minor impact on the hydration equilibrium constant between **4** and **2**, although this aspect has not been studied in detail. Several NMR studies have confirmed the predominance of the hydrate form **4** for glyoxylyl peptides in mildly acidic aqueous solution (pH 4.6),[32,33] i.e., at a pH often used for hydrazone, thiazolidine, or oxime ligations. The chemical shift for $-NHCOC\underline{H}(OH)_2$ proton in water is ~5.52 ppm. The ^{13}C chemical shift for the hydrated aldehyde $-NHCO\underline{C}H(OH)_2$ is ~89.6 ppm. The hydrate form **4** could be detected also by electrospray mass spectrometry (ESI-MS) and its stability probed using the collision-induced dissociation (CID) technique.[33]

The tendency of glyoxamides **2** to be significantly hydrated in aqueous solution has been exploited for designing HIV-1 protease inhibitors,[34] the hydrate form **4** being considered as a transition state analogue of the peptide bond cleavage reaction catalyzed by serine proteases.

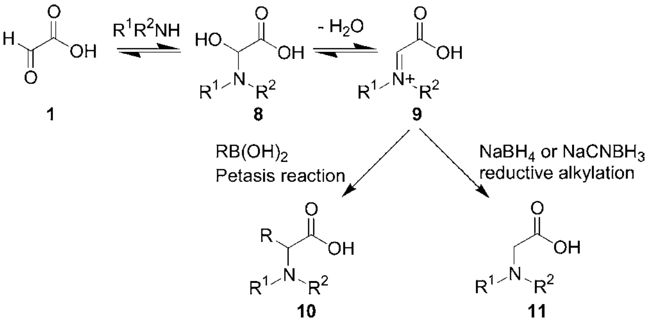
B2. Reaction with Amines. The reaction of glyoxylic acid with ammonia has been studied by Hoefnagel and co-workers (Scheme 2).[35] This study revealed the formation of carbinol adduct **5** by addition of ammonia to the aldehyde group. Carbinol **5** was not stable in aqueous solution, and spontaneously lost a molecule of water to give iminoacetate **6** and oligomers such as hexahydrotriazine derivative **7**.

Scheme 2. Reaction of Glyoxylic Acid with Ammonia[35]



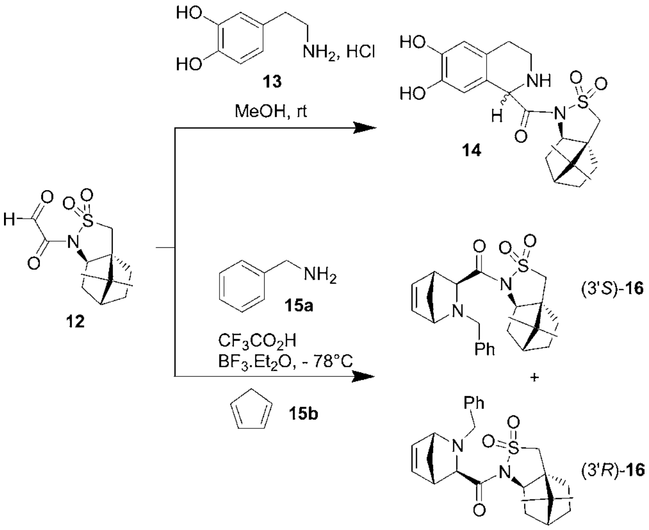
Glyoxylic acid **1** also reacts with primary or secondary amines in organic solvents or aqueous solution to give transient carbinols and iminium derivatives of type **8** and **9**, respectively (Scheme 3). Iminiums **9** are of great synthetic value, especially when the iminium moiety can be further trapped to give a stable end product. In particular, glyoxylic acid and some of its derivatives have been exploited by numerous authors for synthesizing a large diversity of α-amino acids. The field has been reviewed.[36] For example, the use of glyoxylic acid as a component in the Petasis-Borono Mannich three-component reaction has been particularly useful (**1** → **10**, for a comprehensive review, see ref 37). The reaction is also useful on the solid phase.[38] Glyoxylic acid is also an efficient partner in reductive alkylation using either $NaBH_4$ or $NaCNBH_3$ as reducing agents (**1** → **11** in Scheme 3). This reaction has been used in solution to attach the carboxymethyl group to the α- or ε-amino groups of proteins,[39,40] or for synthesizing glycine analogues.[41,42] Other useful reactions of glyoxylic acid derivatives involving iminium species as intermediates include the Pictet-Spengler (Scheme 4, **12** → **14**)[43] or the hetero-Diels–Alder reactions (Scheme 4, **12** → **16**).[44]

Scheme 3. Synthetic Applications of the Reaction of Glyoxylic Acid 1 with Amines Involving an Intermediate Iminium Species[a]



[a]The Petasis multicomponent reaction generates α-amino acids **10**.[37] The reductive alkylation of amines generates glycine analogues **11**.[41,42]

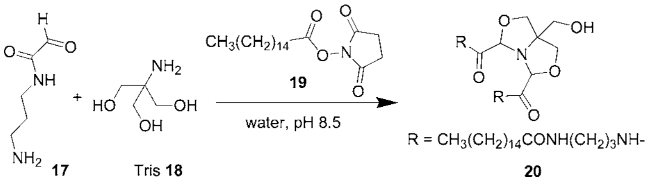
Scheme 4. Synthetic Applications of the Reaction of Glyoxylic Amides with Amines Involving an Intermediate Iminium Species[a]



[a] Illustration of the Pictet-Spengler (12 → 14)[43] or hetero-Diels-Alder (12 → 16)[44] reactions.

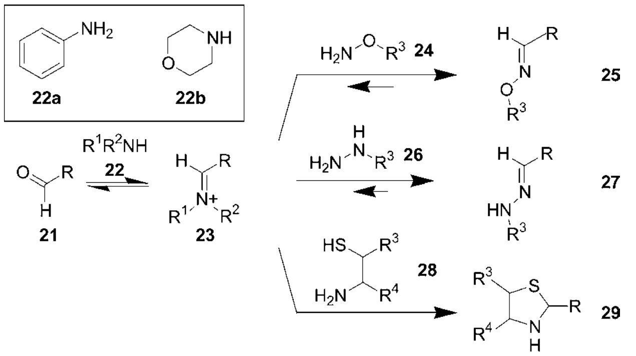
Another interesting application of the propensity of glyoxylic acid derivatives to react with amines is the one-pot synthesis of 1-aza-3,7-dioxabicyclo-(3.3.0)octane derivative **20** starting from glyoxamides such as **17** and *tris*(hydroxymethyl)aminomethane (Tris) **18** as shown in Scheme 5.[45] This reaction probably proceeds through two successive iminium and oxazolidine-forming steps. Formation of structure **20** is the result of the overall condensation of two glyoxamide molecules **17** and one Tris molecule **18**, with the concomitant elimination of two molecules of water. In addition to this sequence of reactions, the process described in Scheme 5 includes the acylation of two primary amines by palmitate *N*-hydroxysuccinimidyl ester **19**.

Scheme 5. Synthesis of 1-Aza-3,7-dioxabicyclo-(3.3.0)octane Derivative 20[45]



B3. Nucleophilic Catalysis of the Reaction of Amines with Glyoxylic Acid Derivatives. The ease of formation of transient iminium species **23** (Scheme 6) in aqueous solution from a large variety of aldehydes **21** and amines **22** is well-known and has a wide range of synthetic applications. Interestingly, the *in situ* formation of iminium species such as **23** also explains the powerfulness of some amines such as aniline **22a** or morpholine **22b** in catalyzing the formation of oximes **25**,[46,47] hydrazones **27**,[46,48,49] or thiazolidines **29**[50] from aldehydes and hydroxylamines **24**, hydrazines **26**, or β-aminothiols **28**, respectively.

Scheme 6. Catalysis of Oxime, Hydrazone, or Thiazolidine Bond Formation by Amines[46−50]



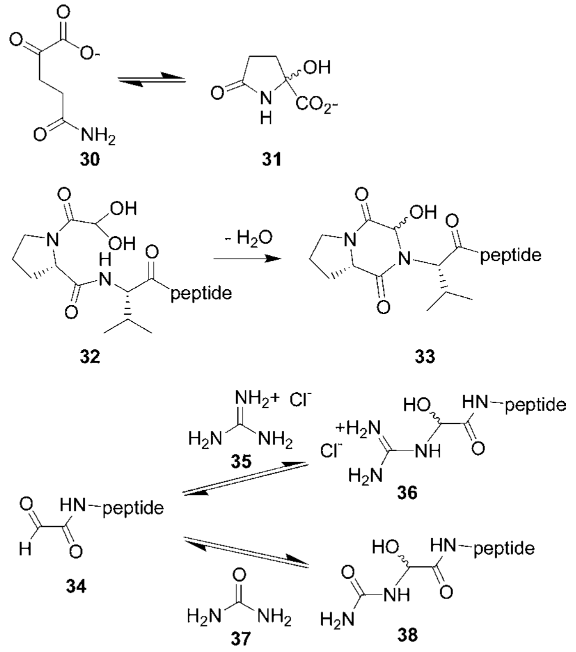
The nucleophilic catalysis of the reaction of hydroxylamines, hydrazines, or β-aminothiols with aldehydes has been studied in the early 1960s. In particular, the catalysis of semicarbazone or oxime formation by aniline **22a** and the concept of "trans-Schiffisation" has been put forward by Jencks and co-workers in 1962 using aromatic aldehydes such as benzaldehyde[46] or pyridoxal[48] as model compounds. Semicarbazone formation is susceptible to general acid catalysis. However, aniline **22a** is 10−1000 times more powerful in catalyzing semicarbazone formation than acids with similar pK_a values. This abnormal catalytic activity is due to the rapid formation of iminium intermediate **23**. Iminium formation is the rate-limiting step of the process. Once formed, the iminium intermediate **23** reacts very quickly with amine compounds **24**, **26**, and **28** according to a "trans-Schiffisation" reaction.

The equilibrium between glyoxylic acid **1** or glyoxamide **2** and iminium species is usually shifted significantly toward the aldehyde form in aqueous solution at pH below 6. On this basis, carbinols or other derivatives that could be formed intramolecularly or intermolecularly by reaction of an α-oxo aldehyde group with α- or ε-amino groups within peptides are usually not observed, either during the purification of peptide α-oxo aldehydes or during chemoselective ligations which are usually carried out at a pH close to or below 5. However, the ease of formation of transient iminium species from α-oxo aldehydes in mildly acidic solutions suggests that aniline may also catalyze chemoselective ligations involving the formation of a Schiff base derived from α-oxo aldehyde group. In fact, the potential of aniline catalysis in α-oxo hydrazone[49] or oxime ligations[47] has been realized only recently by Dawson and co-workers. Aniline catalysis of oxime or hydrazone ligations enables these reactions to be performed efficiently at neutral pH. Various recent applications illustrate the interest of aniline catalysis in hydrazone bioconjugation chemistry.[51−55]

B4. Reaction with Amides. The α-oxo carbonyl group also reacts with amides in certain circumstances. One early mention of such a reaction was made by Cooper and co-workers during an NMR study of an aqueous solution of α-ketoglutaramic acid **30** (Scheme 7).[28] This compound is in equilibrium in aqueous solution with pyrrolidone derivative **31**, the equilibrium lying predominantly toward closed form **31** whatever the pH. In accordance with the predominance of the cyclic structure **31** in aqueous solution, the rate of semicarbazone formation with α-ketoglutaramic acid **30** was only 3% of that for α-ketoglutaric acid under the same conditions, for which the keto group is not masked by cyclization.

Similarly, glyoxylic acid **1** is known to react with urea,[56] amides,[57,58] or carbamates.[57,59] These reactions were exploited for the synthesis of masked forms of glyoxylic acid, which proved to be useful tools for attaching the glyoxylyl group to

Scheme 7. Intra-[28,31] or Intermolecular[32] Reaction of α-Ketoacid or α-Oxo Aldehyde Groups with Amide, Guanidine, or Urea Derivatives



various biomolecules.[60−63] This point will be discussed in more detail in section C.

Not surprisingly, a similar reactivity was observed for glyoxamides of type **2** and was shown to induce some side reactions during the preparation of peptide α-oxo aldehydes or their reactions. The only early mention of the reactivity of the α-oxo aldehyde group toward amides in peptides was made by Rose and co-workers,[31] who showed that the glyoxylyl group, when followed by a prolyl residue such as in compound **32** (Scheme 7), cyclized spontaneously into structure **33** in aqueous solution. Cyclization involved the intramolecular nucleophilic attack of the backbone amide nitrogen of the third residue on the N-terminal aldehyde group. Structure **33** was found to be unreactive toward hydroxylamine. Efficient oximation could nevertheless be achieved by generating the N-terminal glyoxylyl prolyl peptides in the presence of the hydroxylamine compound, showing that the intermolecular oxime bond formation proceeded more rapidly than the intramolecular cyclization process. Note that a similar side-reaction was probably observed by Feibush and co-workers in a stability study of an antimicrobial peptide (pexiganan acetate) in a drug formulation.[64] The N-terminal glycine residue of pexiganan was partially oxidized into an *N*-glyoxylyl group by an unknown mechanism. The *N*-glyoxylyl peptide equilibrated with a piperazinedionyl-des-Gly$_1$-pexiganan side-product, whose structure was similar to structure **33** (Scheme 7), although the residues in position 2 and 3 were Ile and Gly.

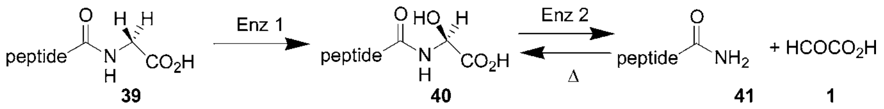
A nice ^{1}H NMR study from Barany and co-workers showed the capacity of glyoxylyl peptides to react in water at pH 4.6 with an excess of guanidinium chloride (Gdn.HCl **35**) or urea **37**.[32] A partial conversion of the glyoxylyl peptide **34** into aminal **36** was observed with Gdn.HCl **35**, which could be reversed by extensive diafiltration. The reversibility of aminal **36** formation in water at pH 4.6 permitted oxime ligation to be carried out in the presence of Gdn.HCl. However, the oximation reaction rate decreased slightly by raising the Gdn.HCl concentration from 6 to 8.5 M. On the other hand, glyoxylyl peptide **34** was completely converted into aminal **38** in the presence of 8 M urea **37**. The oximation reaction was sluggish under these conditions and was accompanied by the formation of other urea-derived byproducts.

Note that peptide derivatives of type **40** (Scheme 8) are formed during the maturation of glycine extended peptide prohormones **39**.[65,66] The first step is catalyzed by α-hydroxyglycine monooxygenase (Enz 1).[65] The second enzyme peptidyl amidoglycolate lyase (Enz 2) then catalyzes the decomposition of **40** to the peptide amide **41**, i.e., the active hormone, and glyoxylic acid **1**. Peptides **40** could also be synthesized by direct reaction of peptide amides with glyoxylic acid at elevated temperatures.[65−67] The spontaneous or enzyme-catalyzed conversion of peptidyl-α-hydroxyglycine derivatives **40** into peptide amides of type **41** in biological fluids was used for designing prodrugs of peptide amides **41**.[68] Note that the conversion of **40** into **41** is also catalyzed by various transition metal ions (Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, and Cd $^{2+}$).[69]

Some C-terminal peptide α-oxo aldehydes with the general structure Aa^1-Aa^n-NH(CH$_2$)$_3$NHCOCHO, synthesis of which will be discussed later, have been shown to decompose partially during lyophilization.[70] MS analysis indicated the formation of dimeric side-products, the structure of which was not determined. Dimer formation was never observed in solution, even on prolonged storage. Where it occurred, the degradation could be prevented by adding an excess of mannitol to the peptide solution before the lyophilization step. Mannitol probably avoids the formation of dimers by a dilution mechanism. The propensity of C-terminal peptide α-oxo aldehydes to decompose during the lyophilization step was highly dependent on the peptide sequence. However, no clear relationship between peptide composition and sensitivity to degradation was found. The fact that an acetylated polyalanine C-terminal α-oxo aldehyde peptide degraded partially during lyophilization suggested a potential reaction of the α-oxo aldehyde with peptide bonds, in line with the capacity of glyoxylic acid to react with amides as discussed above.[57,58] To the best of our knowledge, this side-reaction was never observed with N-terminal glyoxylyl peptides.

B5. Reaction with Alcohols. Glyoxylic acid **1** as other reactive aldehydes such as formaldehyde, acetaldehyde, or glyoxal has been shown to oligomerize in aqueous solution. ^{13}C NMR studies showed the formation of dimers with a hemiacetal structure.[29] Glyoxamides of type **2** (Scheme 1)

Scheme 8. Enzymatic Conversion of C-Terminally Gly-Extended Peptides into Peptide Amides (Enz1 = α-hydroxyglycine monooxygenase, Enz 2 = peptidyl amidoglycolate lyase)[65,66]



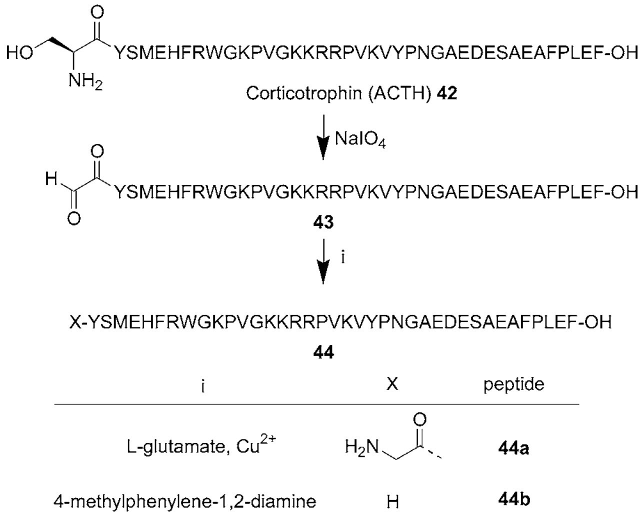
also react easily with alcohols to give hemiacetals. Thus, the analysis or purification of such compounds using chromatographic methods can lead to the formation of hemiacetal side-products in significant amounts if an alcohol such as methanol or ethanol is present in the eluent. For example, Gaertner and co-workers reported the formation of a methyl hemiacetal derivative during the ESI-MS analysis of N-terminal glyoxylyl IL-8 dissolved in methanol/water/acetic acid.[71]

C. GLYOXYLYL GROUP: SYNTHETIC METHODS

C1. Periodate Oxidation of *β*-Amino Alcohols. The capacity of periodic acid to oxidize vicinal diols is long known, and was first discovered by Léon Malaprade in 1928.[72] The field has been reviewed recently.[73] The possibility of extending this reaction to *β*-amino alcohols such as serine or threonine has been described by Nicolet and co-workers.[74] The rate of the oxidative cleavage of *β*-amino alcohols was found to be 10^2−10^4 times that of the vicinal diol analogues in sugar series.[75] The rate of periodate oxidation of *β*-amino alcohols such as 2-aminoethanol depends on the pH of the aqueous solution, the reaction rate being dramatically reduced at acidic pH due to protonation of the amino group.[76,77]

The selectivity of this reaction for *β*-amino alcohols led rapidly to its application to the determination of serine[2,78−80] or threonine[81] in protein hydrolysates or biological systems. The formation of *N*-glyoxylyl peptides by reaction of seryl peptides with periodate was mentioned in the 1950s.[82] The potential of this reaction for the synthesis of *N*-glyoxylyl peptides and their site-specific modification has been proposed by Dixon and co-workers in their pioneering studies on corticotrophin (ACTH **42**, Scheme 9).[3] In this work, the glyoxylyl group formed by periodate oxidation of N-terminal serine (**42** → **43**) was converted in a second step into a Gly residue by transamination with L-glutamate in the presence of cupric ions (**43** → **44a**). The partial oxidation of the Met[4] residue in ACTH **42** during periodate oxidation was noted. The same group later reported the removal of N-terminal residue of corticotrophin **42** by reacting glyoxylyl peptide **43** with 4-methylphenylene-1,2-diamine (**43** → **44b**).[5,83]

Scheme 9. Conversion of Ser[1] Residue of Corticotrophin 42 into *N*-Glyoxylyl Derivative 43 by Periodate Oxidation[a]



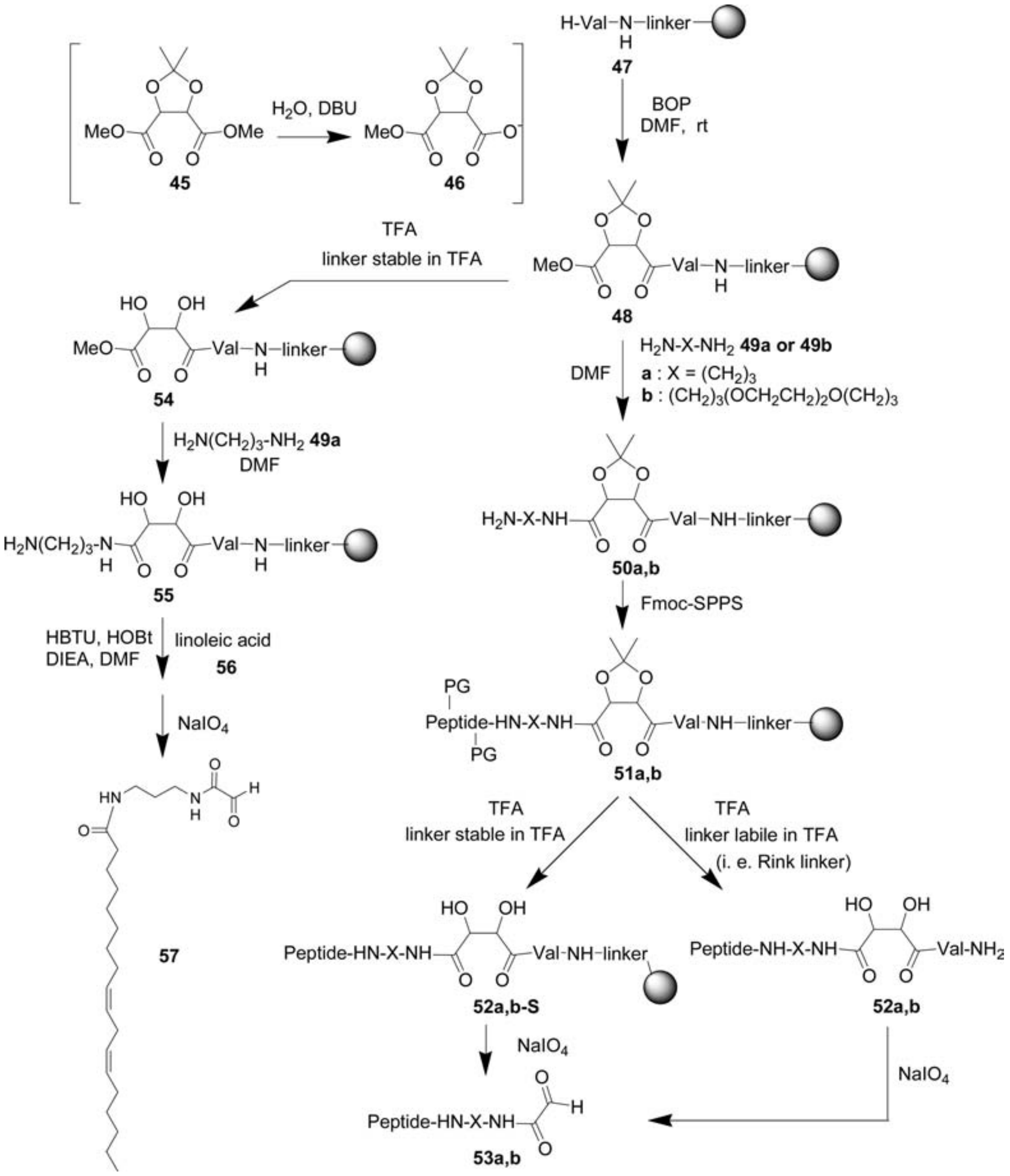
[a] *N*-Glyoxylyl peptide **43** was further transaminated (X = H-Gly, **44a**)[3] or cleaved with 4-methylphenylene-1,2-diamine (X = H, **44b**).[5]

Later on, this reaction was studied in more detail by Geoghegan and co-workers.[84] In particular, these authors used the N-terminal decapeptide derived from ACTH to optimize and characterize the periodate oxidation step. Here again, partial oxidation of a Met[4] residue was observed, especially at pH below 7. Periodate oxidation at neutral pH limited Met oxidation to acceptable levels, and left other residues such as Tyr, Trp, or His unaltered, thereby confirming Dixon's reports.[3] Although periodate oxidation of N-terminal Ser or Thr residues proceeds much more rapidly than for other internal residues, cysteine, cystine, methionine, tryptophan, tyrosine, and histidine residues can also be oxidized by periodate, depending on the experimental conditions. In line with this, periodate has been shown to cause the loss of biological activity of some proteins by modification of Tyr,[85,86] Trp,[85] Met,[86] or Cys[87] residues. Consequently, synthesis of *N*-glyoxylyl peptides by periodate oxidation of N-terminal Ser or Thr residues must be carried out by carefully controlling the pH and stoichiometry of the reaction, and by keeping the reaction time to a minimum to avoid the overoxidation of the peptide chain or of polysaccharide chains in the case of a glycoprotein. It is also recommended to quench the reaction with an excess of ethylene glycol or 2-ethanolamine and to purify the product immediately. A typical example is the periodate oxidation of ribonuclease-S protein (RNase A 21−124 glycoprotein), which required 5 min at room temperature in a 2-fold excess (∼50 μM) of periodate.[88] In this work, Dixon's group used 1,2-di-(*p*-dimethylaminophenyl)ethane-1,2-diol to quench the excess of periodate and determine spectrophotometrically the amount of oxidant consumed in the reaction. To stress again the importance of controlling the conditions of periodate oxidation, we would like to mention that even compounds devoid of sulfur containing amino acids (Met, Cys) can be degraded by periodate if prolonged reaction times and/or high reagent concentrations are used. For example, Vilaseca and co-workers reported the synthesis of a carrier molecule H-Gly_3-[Lys-(COCHO)$]_5$-Gly-OH, which was obtained by periodate oxidation of seryl precursor H-Gly_3-[Lys(H-Ser)$]_5$-Gly-OH.[89] Good yields were obtained using dilute solutions (5 μM peptide, 50 μM periodate) whatever the reaction time (5 min to 1.5 h). At 250 μM peptide concentration (2.5 mM periodate), slight degradation of the polyaldehyde molecule was observed even for short reaction times (5 min). Addition of ethylene glycol (2 equiv over periodate) after 5 min of reaction was found to prevent the overreaction of the target peptide.

Soon after the report of Geoghegan and co-workers,[84] Gaertner and co-workers reported the use of the same method for synthesizing human granulocyte colony stimulating factor (G-CSF) derived glyoxylyl peptides.[90] Periodate oxidation of Ser is now an established method for synthesizing *N*-glyoxylyl peptides and has been intensively used in bioconjugation chemistry.[7] It is probably the most frequently used technique today, even though novel nonoxidative methods have appeared recently,[60,62,91,92] which allow circumvention of the problems associated with the oxidation of sulfur-containing amino acid residues.

Modification of proteins by an *α*-oxo aldehyde group is straightforward when the protein sequence starts with a Ser or Thr residue. For example, chemokine RANTES features a Ser residue on the N-terminus. Periodate oxidation of RANTES followed by oxime bond formation with aminooxypentane

Scheme 10. Application of Isopropylidene-D-Tartaramide Linker to the Synthesis of Lipidic α-Oxo Aldehydes[115] or C-Terminal Peptide α-Oxo Aldehydes[70,109,113]



generated aminooxypentane (AOP)-RANTES, a potent inhibitor of infection of diverse cell types by HIV-1.[19−23] This RANTES analogue was also produced by total chemical synthesis.[93]

When the native sequence of the protein features residues other than Ser or Thr at the N-terminus, it is possible to mutate this position using standard recombinant techniques. A typical example is the synthesis of F(ab′)$_2$-carboxypeptidase G2 (CPG2) conjugates reported by Werlen and co-workers with the goal of developing an antibody-directed enzyme prodrug therapy.[94] CPG2 is an enzyme which has the capacity to convert nontoxic prodrugs into toxic drugs. CPG2 N-terminal Ala residue was replaced by Thr. Mutated CPG2 was oxidized with periodate in the presence of methionine. α-Oxo aldehyde CPG2 was conjugated to a F(ab′)$_2$ carbohydrazide derivative through formation of a hydrazone bond.

Several groups have used the periodate oxidation of a seryl residue for attaching the α-oxo aldehyde group to the end of Lys side-chains.[13,89,95−100] This approach allows incorporation of the α-oxo aldehyde group anywhere in the peptide sequence. This approach has proven its usefulness for the functionalization of cyclic peptide templates,[100,101] or peptide dendrimers.[13,92,102−104] In particular, the functionalization of lysine dendrimers or templates by α-oxo-aldehyde groups and the use of chemoselective ligation methods for the preparation of artificial proteins or multiple antigenic peptides (MAPs) have been pioneered by Tam and co-workers.[92,105,106] and Rose and co-workers.[13,95,103,104]

A Lys(H-Ser) residue can be introduced into peptides using standard *tert*-butyloxycarbonyl (Boc) solid phase peptide synthesis (SPPS) protocols. For example, Tam and co-workers described the synthesis of peptide H-AlaAlaValAlaLeuLeuProAlaValLeuLeuAlaLeuLeuAlaLys(H-Ser)-NH$_2$ by first coupling Boc-L-Lys(Fmoc)-OH to 4-methylbenzhydrylamine resin.[98] After the peptide elongation step, the peptidyl resin was treated with piperidine to remove the Fmoc group present on the Lys side-chain. Boc-L-Ser(Bzl)-

OH was then coupled to the ε amino group before HF deprotection and cleavage of the peptide from the resin.

Another possibility is to use the Fmoc-SPPS and an orthogonal protecting group such as methyltrityl (Mtt)[107] or allyloxycarbonyl (Aloc)[108] groups for the side chain of Lys residues. Resin selective deprotection of the ε-amino group of Lys is followed by the coupling of a protected serine derivative such as Boc-L-Ser(*t*Bu)-OH. Deprotection and cleavage of the peptide from the resin in trifluoroacetic acid generates the Lys(H-Ser) peptide which can be oxidized in solution in a subsequent step. An alternative is to incorporate Nα-Fmoc-protected lysine[101] or diaminopropionic acid (Dap)[97] derivatives featuring a Boc-L-Ser(*t*Bu) residue on their side-chains during the Fmoc SPPS.

Periodate oxidation of seryl residues is compatible with some hydrazone bonds. For example, Vilaseca and co-workers reported the synthesis of protein conjugates of defined structure by forming in a first step a hydrazone bond between a protein hydrazide and an aldehyde template featuring several Lys(H-Ser) residues.[89] After this step, seryl residues were converted into α-oxo aldehyde groups by treatment with periodate. Finally, reaction of the formed aldehyde groups with drugs possessing a hydrazide group generated homogeneous protein−drug conjugates.

Periodate oxidation of seryl residues is also compatible with *O*-alkylhydroxylamine functionality.[31,97] Formation of α-oxo aldehyde group in the presence of an *O*-alkylhydroxylamine results in the spontaneous formation of the oxime product either inter-[31] or intramolecularly.[97]

C2. Periodate Oxidation of 1,2-Diols. As discussed above, the periodate oxidation of a Ser residue is a simple method for attaching the α-oxo aldehyde group to the N-terminus of peptides. Incorporation of this functionality on or near the C-terminus is less straightforward. A solution to this problem is to incorporate a protected Lys(H-Ser) residue at the C-terminus during the peptide elongation step, as already mentioned before. An alternative based on the use of a Fmoc-SPPS compatible isopropylidene-D-tartaramide (IPT) solid support has been described as shown in Scheme 10.[7,70,109] In this work, a tartaric acid derivative was chosen as an α-oxo aldehyde surrogate since periodate oxidation of tartaric acid[110] or tartaric acid amide[111] derivatives is known to yield the corresponding α-oxo aldehydes efficiently. Dimethyl-2,3-*O*-isopropylidene-D-tartrate **45**, which is commercially available, was used to build IPT linkers as shown in Scheme 10. Tartrate derivative **46** was generated by dissolving water and DBU in a 10-fold excess of dimethyl-2,3-*O*-isopropylidene-D-tartrate **45**. Carboxylate **46** was then coupled to a valine derivatized water compatible resin such as polyethylene glycol polyacrylamide copolymer (PEGA)[112] resin using *in situ* BOP activation. The presence of a Val residue was essential to prevent succinimide formation and thus the premature cleavage of the linker in the subsequent steps. Reaction of ester resin **48** with an excess of diamine such as 1,3-diaminopropane **49a** (H_2N-X-NH_2, X = $(CH_2)_3$) or 4,7,10-trioxa-1,13-tridecanediamine **49b** (H_2N-X-NH_2, X = $(CH_2)_3(OCH_2CH_2)_2O(CH_2)_3$) generated IPT solid supports **50a,b** ready for the Fmoc SPPS. The time required for converting ester resin **48** into IPT resins **50a,b** depended on the nature of the diamine used in this step (20 min for **49a**, 45 min for **49b**). Diffusion filtered high resolution magic angle spinning NMR (DF HR-MAS NMR) was found to be a useful analytical technique for optimizing IPT linker synthesis.[70,113]

The isopropylidene group is stable in the presence of piperidine. It is removed during the final deprotection step in TFA. The use of an acid-stable bond between tartaramide moiety and PEGA resin resulted, after TFA treatment, in a deprotected peptide **52a,b-S** still attached to the solid support. Treatment of the peptidyl resin with periodate led to the simultaneous formation of a C-terminal α-oxo aldehyde group and to the detachment of the peptides **53a,b** from the resin. Oxidative cleavage of the 1,2-diol moiety occurred within few minutes in aqueous acetic acid.[70,109,113] These conditions resulted also in the concomitant oxidation of Met or Cys residues when present in the peptide sequence. Oxidation of Cys could be prevented by using *tert*-butylsulfenyl protection for the side-chain thiol group.[70] Alternatively, the use of an acid labile linker such as Rink linker permitted the isolation of soluble tartaramide peptides **52a,b**, which were further oxidized in solution with periodate. The latter strategy is preferred when the peptide features moieties sensitive to oxidants such as Met or biotin, since the oxidation step can be monitored and adapted more easily than for the solid phase reaction. A typical example is the synthesis of biotinylated peptide H-Lys(biotin)-AlaTyrValLeuAlaGly-$NH(CH_2)_3(OCH_2CH_2)_2O$-$(CH_2)_3NHCOCHO$, which required the performance of the oxidative cleavage of the tartaramide precursor at pH 6.5 in the presence of methionine.[114] A recent adaptation of tartaramide linker for the solid phase synthesis of lipidic α-oxo aldehydes involved the coupling of carboxylate **46** to a TFA resistant amine resin, followed by the removal of isopropylidene protecting group in TFA (**48** → **54**, Scheme 10).[115] Solid phase amidification with 1,3-diaminopropane **49a** was followed by the coupling of various lipidic moieties among which TFA sensitive fatty acids such as linoleic acid **56**. Finally, oxidative cleavage of the tartaramide moiety led to the detachment of the lipidic α-oxo aldehydes of type **57**. In this case, removal of the isopropylidene moiety in an early step of the synthesis avoided the exposure of sensitive lipid moieties to TFA. Tartaramide derivatives were also used successfully for the synthesis of α-oxo aldehyde functionalized one-bead-one-compound libraries,[116] oligodeoxynucleotides,[114] 4-aminoquinoline derivatives,[117] or Wang resin[118] to cite only few applications.

C3. Masked Glyoxylic Acid Equivalents. The synthesis of α-oxo aldehyde functionalized peptides or proteins by oxidative cleavage of β-amino alcohols or 1,2-diols with periodate has met with a lot of success in bioconjugation chemistry. However, one major limitation of these synthetic methods is the potential degradation of Met, Cys, and sometimes other residues during the oxidative cleavage. To overcome this problem, several groups have explored the use of protected α-oxo aldehyde derivatives which can be introduced on the peptide chain during SPPS. Glyoxamides of type **2** (Scheme 1) usually decompose in the acidic cocktails used for peptide deprotection and cleavage. Thus, the α-oxo aldehyde protecting group must be stable during the acidic resin cleavage and side-chain deprotection reactions, but easily removed in solution in a subsequent step.

One early attempt in this direction is the work of Tam and co-workers describing the synthesis of α-oxo aldehyde lysine dendrimers.[92] For this, α- and ε-amino groups of lysine dendrimers were modified with glyoxylic acid dimethyl acetal **58** (Figure 1) using standard coupling procedures. The dimethyl acetal is stable in TFA because of the stabilization effect of the adjacent carbonyl group. It was hydrolyzed in concentrated hydrochloric acid.

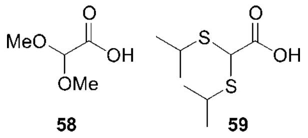
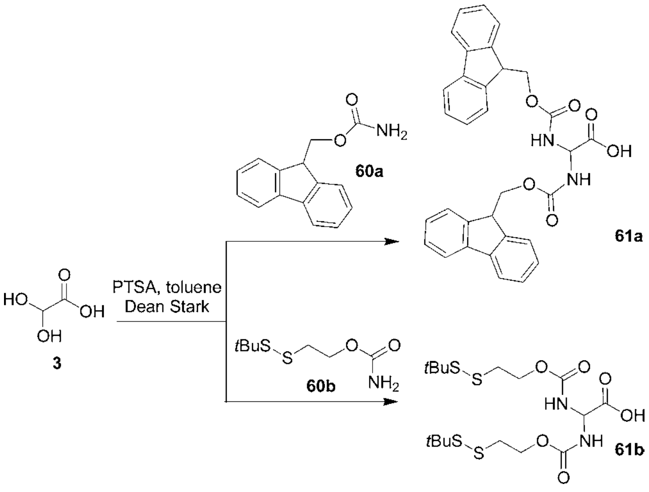


Figure 1. Dimethyl acetal **58**[92] or diisopropylthio acetal **59**[91] derivatives of glyoxylic acid.

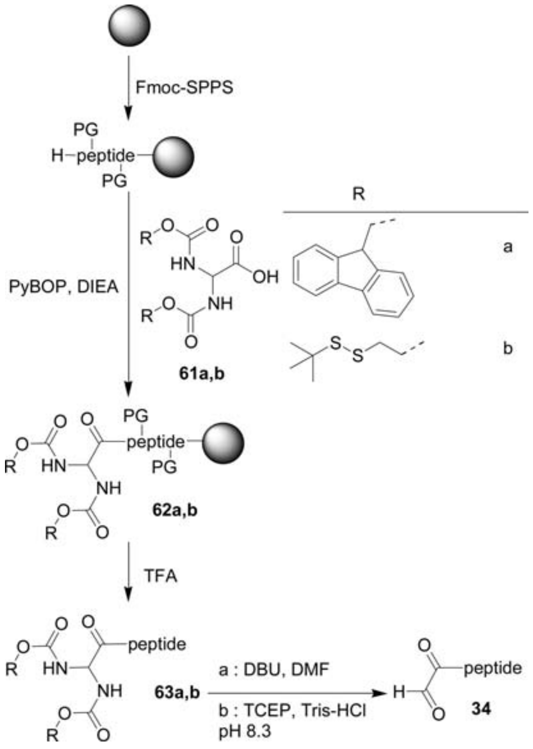
Later on, another glyoxylic acid derivative **59** (Figure 1) featuring a diisopropyl thioacetal moiety was described by Qasmi and co-workers.[91] Dithioacetal **59** was introduced in the last stage of the Fmoc-SPPS, and survived the TFA cleavage and deprotection step. Unmasking of the aldehyde group was carried out in water/acetonitrile solution in the presence of *N*-bromosuccinimide.

Scheme 11. Synthesis of α,α'-Diaminoacetic Acid Derivatives 61a[60,62] or 61b[61,63]



The harsh conditions used for deprotecting amide derivatives of dimethylacetal **58** or diisopropylthioacetal **59** stimulated the development of α,α'-diaminoacetic acid derivatives **61a,b** (Scheme 11).[60−63] Derivatives **61a,b** were easily prepared in one step by reacting glyoxylic acid monohydrate **3** with the corresponding carbamates **60a,b**. These α-oxo aldehyde surrogates were introduced after Fmoc-SPPS using standard activation procedures, and survived the peptide deprotection and cleavage step in TFA (Scheme 12). Peptides **63a,b** were purified using standard HPLC procedures. Unmasking of the glyoxylyl group was carried out in solution. Derivative **63a** was unmasked using DBU in DMF.[60,62] In this case, peptide concentration was shown to strongly influence the purity of the target α-oxo aldehyde. Indeed, high peptide concentrations (>5 mmol/L) favored the formation of an amidine side-product featuring two peptide chains linked through their N-termini. The use of a large excess of DBU (>30-fold excess) or dilution of the reaction mixture down to 1 mmol/L permitted suppression of amidine side-product formation, and enabled isolation of glyoxylyl peptides in good yield.[62]

Scheme 12. Synthesis of Glyoxylyl Peptides 34 Using Fmoc-SPPS and α,α'-Diaminoacetic Acid Derivatives 61a,b[60−62]



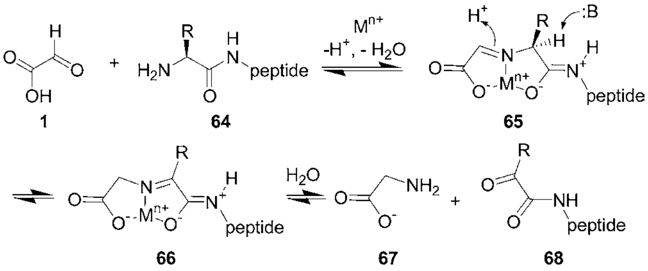
Derivative **61b** (Scheme 11) was designed to allow unmasking of the α-oxo aldehyde group in water using mild conditions.[61,63] In this case, the cleavage of the carbamate bond is triggered by the reduction of the disulfide bond by a phosphine and the subsequent intramolecular nucleophilic attack of the thiol group on the carbamate carbonyl bond. Indeed, treatment of peptide derivatives of type **63b** (Scheme 12) with *tris*(2-carboxyethyl)phosphine (TCEP) at pH 8.3 resulted in the very clean formation of the corresponding α-oxo aldehydes using nonoxidizing and very mild experimental conditions.

C4. Transamination. Studies in the 1940s showed the reversible interconversion of pyridoxal and pyridoxamine by transamination in the presence of an amino acid at a temperature above 100 °C.[119] Inhibition of the transamination reaction by citrate or ethylenediaminetetraacetate suggested a potential catalysis by traces of metal ions. Indeed, copper(II), iron (II or III), and aluminum(III) and to a lesser extent nickel(II) or cobalt(II) ions were found to catalyze the transamination reaction at 100 °C.[120] Four years later, Cennamo and co-workers applied a similar metal-catalyzed transamination reaction to peptides and isolated the corresponding 2,4-dinitrophenylhydrazones.[121] A significant advance was made by Mix and co-workers, who showed that the transamination was accelerated by pyridine due to general-base catalysis.[122] This observation was rapidly exploited by Dixon and co-workers for performing the transamination of peptides[6] or proteins[123] at room temperature using glyoxylic acid as the aldehyde donor. Later on, the reaction was improved by using acetate buffer instead of pyridine.[124,125]

The probable mechanism of the metal-catalyzed transamination reaction is shown in Scheme 13.[126] It first involves the formation of metal ion complex **65** which features an imine bond between glyoxylic acid and the α-amino group of the starting peptide **64**. Abstraction of the α-proton by a base triggers the formation of complex **66**, hydrolysis of which yields glycinate ion **67** and α-oxo aldehyde (R = H) or ketone (R ≠ H) **68**. The role of the metal is to stabilize the imine bond within **65** and to render the α-proton more acidic.

Recent investigations revealed the usefulness of pyridoxal-5-phosphate (PLP) **69** (Scheme 14) for performing the

Scheme 13. Catalysis of the Transamination between N-Terminal Residue in Peptides and Glyoxylic Acid by Metal Ions[126]



transamination reaction in water, at nearly neutral pH and in the absence of metal ion catalyst.[127] The experimental conditions are very mild and preserve the fold and binding properties of various proteins such as myoglobin,[127] green fluorescent protein,[127] or antibodies.[128] The scope and limitations of this PLP transamination reaction have been studied.[129,130] The yields for the transamination of an N-terminal glycyl residue, one of the most favorable amino acids, are in the range 40−80%. This reaction is thus a valuable tool for producing N-terminal glyoxylyl peptides or proteins.

C5. Quantification of α-Oxo Aldehydes in Solution. The previous sections show that several solution or solid-phase synthetic methods currently enable straightforward access to peptides or proteins derivatized by an α-oxo aldehyde group. The availability of analytical tools permitting the quantitative detection of α-oxo aldehydes with a good sensitivity is of prime importance for characterizing the biomolecules derivatized by this group, or for determining the concentration of the α-oxo aldehyde partner prior to a chemical reaction.

Dixon and co-workers developed a method for the quantification of glyoxylyl groups in peptides or proteins based on the detection of the corresponding 2,4-dinitrophenylhydrazone by UV spectroscopy. This method requires a standard curve for each aldehyde.[131]

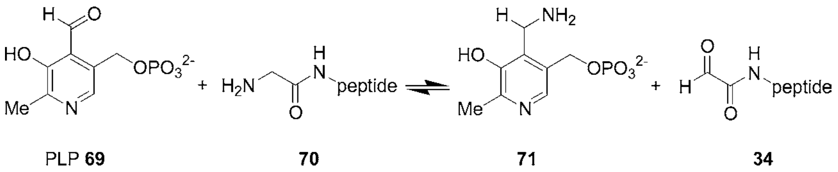
An alternative method relies on the formation of the corresponding *O*-benzyloxime by reaction with an excess of *O*-benzylhydroxylamine. The amount of *O*-benzylhydroxylamine consumed in the reaction, which is related to the concentration of the peptide α-oxo aldehyde, is quantified by HPLC by comparing the area of the *O*-benzylhydroxylamine peak before and after the derivatization step. This method is more suited for the analysis of several samples since it can be automated and does not require a standard curve for each aldehyde.[132]

C6. Preparation of α-Oxo Aldehyde Functionalized Materials. In the postgenomic area, proteomic research has stimulated the development of miniaturized high-throughput screening techniques and in particular of peptide or protein microarray techniques. These devices allow the screening of thousands of molecules while needing small quantities of reagents or biological material. Usually, microarrays are prepared on a flat substrate on which the capture molecules are applied in a controlled fashion using robotic systems. The target molecules can be immobilized on a solid substrate such as a metal (i.e., gold), a metal oxide (i.e., titanium oxide), a metalloid oxide (i.e., silicon oxide), or an organic polymer (i.e., polystyrene, polycarbonate) using different techniques. The need for controlling the orientation and density of target molecules has stimulated the development of site-specific immobilization techniques, most of which were derived from site-specific ligation methods already used for producing bioconjugates in aqueous solution. The field has been reviewed recently.[133,134] Another area of research which has benefited from site-specific ligation chemistries is the design of biomaterials and more generally of bioorganic−inorganic materials. Here again, the properties of materials can be profoundly changed by grafting biomolecules in a controlled manner.

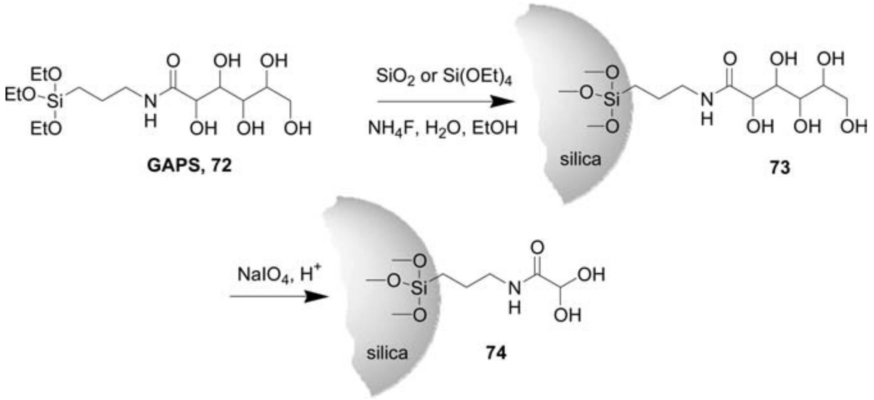
Not surprisingly, α-oxo aldehyde-based ligation chemistries have met a lot of success in these fields. There are several reasons for this. One important aspect is the development of efficient methods giving access to α-oxo aldehyde functionalized biomolecules[60−62,70,84,91,92,109,126,127] or materials,[24,135−141] and the high reactivity of this aldehyde moiety, which compensates for the usually low concentrations of ligating partners. Another advantage is the stability of the bonds formed with α-oxo aldehydes compared with other aldehydes.[25,114,142,143] Moreover, hydrazone and oxime ligations are compatible with internal Cys residues and are insensitive to air oxidation, in contrast to thiol-based ligations. Last but not least, the contamination of surfaces is minimized since hydrazone, oxime, or thiazolidine ligations proceed spontaneously in aqueous media in the absence of additives and without side-product formation, apart from one molecule of water. The discussion below is focused on the preparation and use of α-oxo aldehyde surfaces.

C6.1. Silica Substrates. One of the early attempts to adapt glyoxylyl chemistry to silicon oxide substrates was described by Lam and co-workers in 2001.[24] The goal of the study was to prepare peptide or small molecule microarrays for cell adhesion or functional assays. This required the immobilization of peptide probes by reacting N-terminal aminooxyacetyl or Cys peptides with glyoxylyl-functionalized microscope glass slide substrates. α-oxo aldehyde functionalized glass slides were prepared by coupling Fmoc-L-Ser(*t*Bu)-OH to amino-functionalized glass slides. *Tert*-butyl and Fmoc protecting groups were removed in this order by soaking the slides in TFA and piperidine in DMF, respectively. Finally, α-oxo aldehyde groups were formed by treating the slides with periodate in phosphate buffered saline (PBS). An alternative method based on the coupling of ethylene acetal protected glyoxylic acid to amine slides was described in the same paper. Deprotection of the acetal group was carried out by soaking the slides in dilute HCl. The same group described later on the preparation of α-oxo

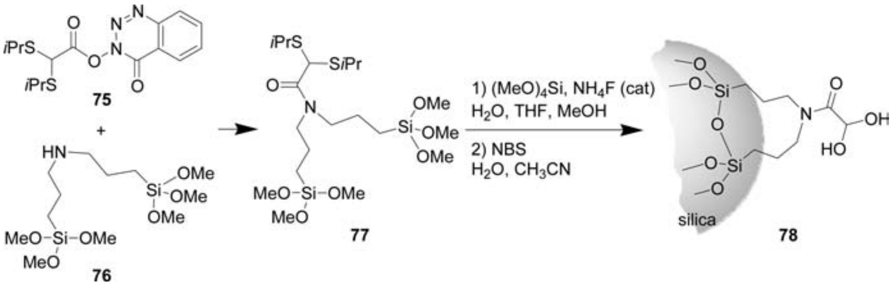
Scheme 14. Transamination using Pyridoxal-5-Phosphate 69 (PLP)[127]



Scheme 15. Synthesis of α-Oxo Aldehyde Functionalized Silicas Using GAPS 72 and Periodate Oxidation[140]



Scheme 16. Synthesis of α-Oxo Aldehyde Functionalized Silicas Using Diisopropylthio Acetal Derivative 75[147]



aldehyde functionalized glass slides by coupling acrylic acid to amine slides followed by ozonolysis of the carbon−carbon double bond.[144] Despite the optimization of α-oxo aldehyde glass slide preparation and printing procedures, the quality of the microarrays was found to be highly variable and unable to meet the criteria for high-throughput screening studies.[145] One explanation for the disappointing results observed with glyoxylyl glass slides is the potential oligomerization of glyoxamide groups on the surface, in line with the known capacity of glyoxylic acid to form acetal oligomers in solution or to react with amide groups as already discussed in section B. These reactions might be favored by the high local concentration of glyoxylyl group on the surface. Another aspect which has not been studied is the potential catalysis of these side-reactions by the silanol groups present within the silica material.

At the same period, Durand and co-workers also described an efficient method for preparing glyoxamide silicas[140] using triethoxysilylpropylgluconamide **72** (GAPS, Scheme 15) as the α-oxo aldehyde precursor. Besides 1,2-diols, periodate is known to cleave and oxidize α-hydroxy ketones and α-diketones, but not α-keto amides such as glyoxamide group.[146] On this basis, it was anticipated that the oxidative cleavage of the gluconamide chain would end up with the formation of an α-oxo aldehyde group. CP MAS ^{13}C NMR analysis of the silica **74** obtained by treating gluconamide silica **73** with periodate in aqueous acetic acid showed resonances at 87.5 ppm which are typical of the α-oxo aldehyde function in the hydrated form. Signals which could have arisen from partial oxidation of the gluconamide chain were not observed.

Subsequently, the diisopropylthio acetal derivative **59** (see Figure 1)[91] was used as the α-oxo aldehyde precursor for the synthesis of glyoxamide silicas as shown in Scheme 16.[147] First, silane **76** was coupled to activated ester **75**, i.e., the 3-hydroxy-1,2,3-benzotriazin-4(3*H*)-one ester derivative of diisopropylthio acetal derivative **59**, to give silane **77**. The latter was subsequently polymerized in the presence of water, tetramethoxysilane, and ammonium fluoride used as catalyst for the gelation procedure. The diisopropyl acetal moiety was removed subsequently using NBS in aqueous acetonitrile. The successful formation of α-oxo aldehyde groups within the silicas was verified using CP MAS ^{13}C NMR spectroscopy and other analytical techniques.

The various α-oxo aldehyde silicas prepared in this study were ligated with a model α-hydrazinoacetyl peptide Fmoc-GlyAsnAlaArgGlyArgGlyAsnGlnGlyLys($COCH_2NHNH_2$)-NH_2 (the synthesis and properties of hydrazinoacetyl peptides are discussed in more detail in section D1.5).[148] The Fmoc group enabled the determination of the ligation yield by piperidine treatment and UV quantification of the piperidine-dibenzofulvene adduct released in solution. Ligation yield was 60% for high loadings (250 μmol/g) and close to 0% for small loadings (50 μmol/g). In contrast, the ligation yields obtained by reacting semicarbazide silicas with peptide α-oxo aldehydes were high (>84%), even for the less substituted silicas. These studies show the efficiency of α-oxo aldehyde Schiff-base chemistry for linking peptides to inorganic surfaces, but also the importance of attaching the α-oxo aldehyde to the soluble peptide rather than to the silica substrate. Overall, these results are in line with the low reactivity of α-oxo aldehyde glass slides reported by Lam and co-workers.[24]

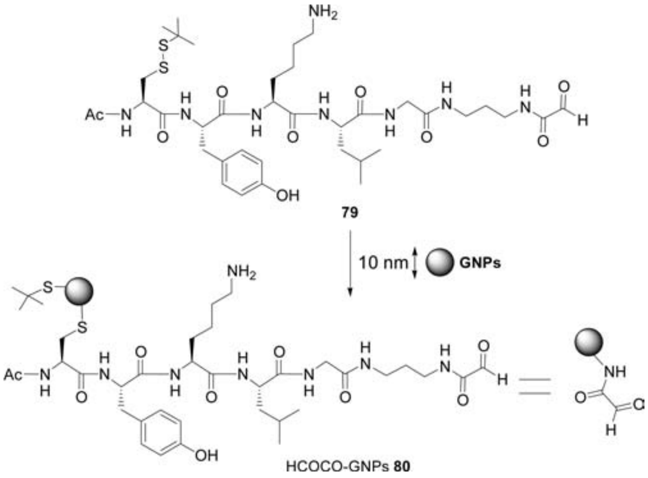
C6.2. Gold Substrates. Another key material for the design of micro or nanosystems is gold. This metal is used for the fabrication of conductive electrodes,[136,149−153] of surface plasmon resonance (SPR)-based detection systems,[154,155] of piezoelectric sensors,[156] and of nanoparticles-based materials,[155,157,158] to cite only a few applications. A great advantage of gold is its strong affinity for sulfur compounds, which provides an efficient and simple means of functionalization.[159] Indeed, thiols as well as their disulfides react efficiently with metallic gold to give monolayers. The reaction of thiols with gold proceeds through the oxidative addition of an S−H bond to the gold surface, followed by a reductive elimination of hydrogen. In the case of disulfides, the chemisorption probably proceeds through the oxidative addition of an S−S bond to the gold surface.

One of the first applications of glyoxylyl chemistry in this field was reported by Scheibler and co-workers,[160] who used regioselectively addressable functional templates (RAFT) for the synthesis of self-assembled monolayers (SAMs) on gold. For this, a bifunctional RAFT molecule presenting two alkanethiol moieties for binding to gold surface on one face, and four serine residues on the opposite face as glyoxylyl group surrogates was synthesized by a combination of solid phase and solution chemistries. The RAFT molecule was attached to gold through thioalkane linkers. Serines were oxidized with periodate in a subsequent step, and glyoxylyl groups thus formed were ligated with an aminooxyacetyl-derivatized peptide antigen. The use of a gold substrate enabled the use of diverse analytical techniques such as SPR, time-of-flight secondary-ion mass spectrometry (TOF-SIMS), and Fourier transform infrared (FTIR) spectroscopy, for characterizing the formation of the SAM, its functionalization by several consecutive surface chemical reactions, and finally the specific capture of a monoclonal antibody by surface-bound peptide antigen.

Gold nanogaps, i.e., gold nanoelectrodes separated by a gap of a few nanometers, show great promise for the electrical detection of nucleic acids[161] or proteins.[149,151] In these systems, the electrons are transferred from one nanoelectrode to the other as a consequence of a specific binding event occurring between the nanoelectrodes. For this, one of the biochemical probes used in the detection experiment is labeled with gold nanoparticles (GNPs). Insertion of the GNPs inside the nanogap facilitates the transfer of electrons by a tunneling effect. The fabrication of functional nanogaps requires controlled positioning of the capture molecules inside the nanogap in the space delimited by the metallic walls of the nanogap and the substrate. In this context, the control of the physicochemical properties of the substrate between the nanoelectrodes is of prime importance, since the presence of a specific chemical group within the nanogap can not only serve for the attachment of probe molecules, but also ensure the imbibition of the nanogap. The filling of the nanogap by the surrounding aqueous phase is essential for allowing the diffusion of target molecules and the occurrence of chemical or biochemical reactions inside the nanogap. Imbibition properties of the nanogap can be deduced from the macroscopic physicochemical properties of surfaces and liquids.[162] In contrast, probing the presence of a specific chemical group inside the nanogap is highly challenging due to the dimensions of the system.

Melnyk and co-workers designed functionalized GNPs for probing the presence of chemical groups within a nanogap using electrical detection.[136] In a proof of concept study (Scheme 17), α-oxo aldehyde derivatized GNPs **80** (HCOCO-GNPs) were synthesized by reacting bifunctional peptide **79**, featuring a N-terminal Ac-Cys(*St*Bu) residue and a C-terminal glyoxylyl group, with 10 nm gold GNPs. The diameter of the GNPs was below the gap width to allow their diffusion and insertion inside the nanogap. The thiol group of the Cys residue served for the attachment of the peptide to the GNPs.

Scheme 17. Synthesis of HCOCO-GNPs by Reaction of GNPs with Bifunctional Peptide 79[136]



The α-oxo aldehyde served as a chemical probe for the presence of semicarbazide groups inside the nanogap. Disulfide **79** reacted efficiently with the GNPs, thereby avoiding the need to reduce the Cys(*St*Bu) residue in the presence of the α-oxo aldehyde group. Indeed, the use of a reducing thiol such as dithiothreitol (DTT) or TCEP would require purifying the reduced peptide prior to reaction with the GNPs.

The principle underlying the detection of semicarbazide groups inside a nanogap using HCOCO-GNPs **80** is presented in Figure 2. HCOCO-GNPs **80** and control GNPs were printed on the nanogaps using a noncontact piezoelectric microarrayer. The chips were washed and analyzed by scanning electron microscopy. HCOCO-GNPs **80** inserted inside the nanogap, whereas different control GNPs did not (data not shown). Importantly, the presence of GNPs inside the gap resulted in large current increments with factors between 2 and 4 orders of magnitude compared to bare nanogaps, as well as in typical $I(V)$ curves. This study constitutes the first demonstration of the presence of a specific functional group in a nanogap by combining peptide-capped gold nanoparticles and electrical detection.

C6.3. Other Metallic Surfaces. Metal oxides are used in various fields of research and in particular for the design of biomaterials. The modification of the surface properties of metal oxides is often an important step in many applications. The use of phosphonic acids for the modification of metal oxide-based materials has grown significantly in the last decades, due to the ease of formation and stability of the M−O−P bond.

Phosphonic acid chemistry was used by Durand and co-workers for the preparation of metal oxide particles decorated with α-oxo aldehyde groups (Scheme 18).[141] Phosphonic acid derivative **81**, featuring a diisopropylsulfenyl acetyl moiety as α-oxo aldehyde surrogate, was successfully grafted onto TiO_2, SnO_2 or Al_2O_3 particles. Analytical data were consistent with the formation of a dense monolayer. Removal of the diisopropylsulfenyl moiety with NBS yielded α-oxo aldehyde particles **82**. The reactivity of the particles was confirmed by ligating 3-methyl-2-benzothiazolinone hydrazone (MBTH **83**) onto the surface of the particles and detecting the hydrazone formed **84** by fluorescence spectroscopy.

C6.4. Surface Plasmon Resonance Spectroscopy Chips. Surface plasmon resonance (SPR) spectroscopy is a label free

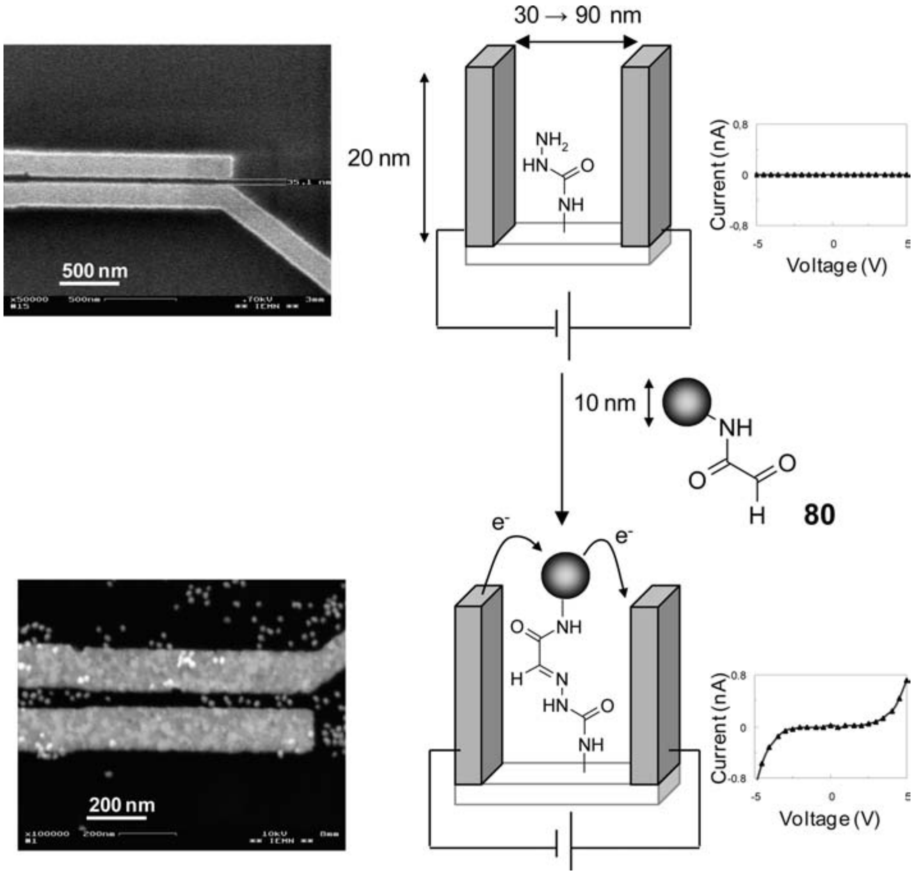
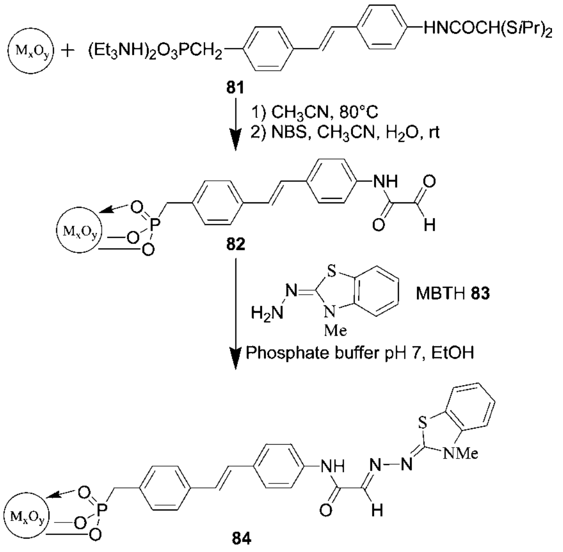


Figure 2. Principle of the selective detection of a chemical group inside a nanogap using electrical detection. Images on the left correspond to scanning electron microscopy analyses of the nanogaps.[136]

Scheme 18. Synthesis of α-Oxo Aldehyde Derivatized Metal Oxide Particles, and Hydrazone Ligation with MBTH 83[141]

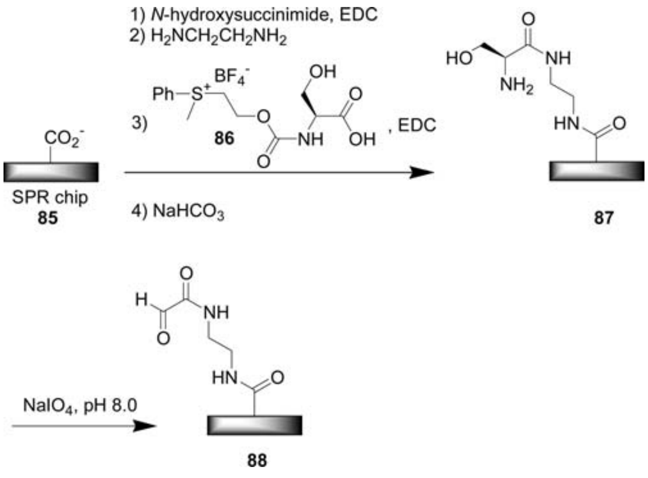


technique enabling the study of specific or nonspecific adsorption processes occurring near a thin metal film. Given the importance of SPR for studying biomolecular interactions and determining binding constants and kinetics, various methods have been developed for immobilizing biomolecules on SPR chips. In particular, the design of site-specific immobilization techniques is of prime importance for presenting a probe to the aqueous medium while retaining the accessibility of binding sites and its native fold. Not surprisingly, chemoselective chemical ligations which were first developed for the controlled assembly of large scaffolds in aqueous solution were also often applied to the immobilization of biomolecules on SPR chips.

Nice and co-workers have developed a practical method for the site-specific immobilization of N-terminal cysteinyl peptides on carboxymethyldextran-derivatized SPR chips using thiazolidine ligation (Scheme 19).[163] For this, surface exposed carboxyl groups were coupled to 1,2-ethylenediamine. Free primary amino groups were then coupled to water-soluble 2-(phenyl-(methyl)sulfonio)ethyloxycarbonyl (PMS)-protected serine **86**.[164] Removal of the PMS group in aqueous sodium hydrogenocarbonate generated a free serine residue which was oxidatively cleaved with sodium periodate to give a glyoxamide sensor chip **88**. This substrate permitted the efficient immobilization of a 15 amino-acid N-terminal Cys peptide epitope derived from epidermal growth factor receptor (EGFR). The binding affinity of the site-specifically immobilized EGFR epitope for a monoclonal antibody was significantly higher compared to other thiol or amine non-site-specific coupling chemistries.

C6.5. Lipidic Colloids. Lipidic colloids such as liposomes are increasingly used for studying biological processes occurring on cellular membranes. For example, peptido- or proteoliposomes have been used for reconstituting *in vitro* several aspects of intracytoplasmic membrane trafficking. Lipidic colloids are also used as vectors for the transport of active compounds *in vitro* as well as *in vivo*. The use of these objects usually requires

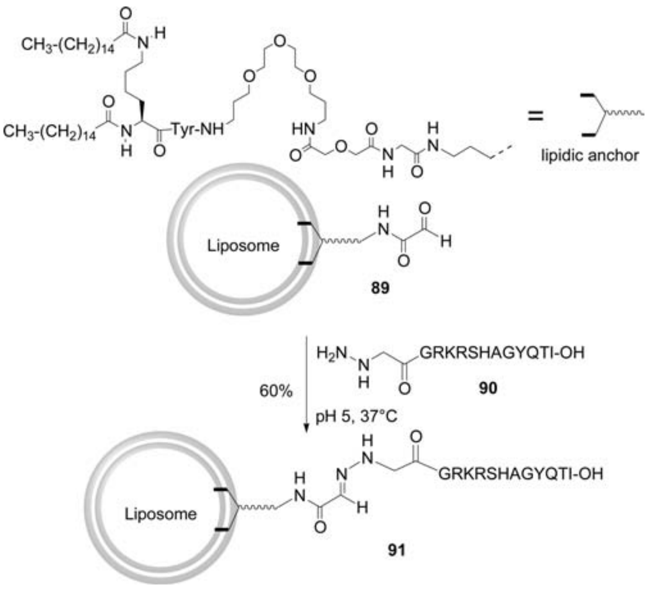
Scheme 19. Preparation of Glyoxylyl-Functionalized SPR Sensor Chips Using Water-Soluble 2-(Phenyl(methyl)sulfonio)ethyloxycarbonyl (PMS)-Protected Serine 86[163]



adapting their surface properties for controlling their half-life in biological fluids or for targeting specific cells or organs. The components to be presented on the lipidic colloids can be modified by lipid chains to allow their insertion into artificial membranes. This strategy can be problematic because lipid conjugates are often difficult to synthesize and to purify. In this context, the ligation of the components to preformed lipidic colloids presenting a specific chemical group on their outer surface appears to be an interesting alternative which has been investigated by several groups.

One early attempt to decorate liposomes with α-oxo aldehyde groups used lipidic anchor **89** as shown in Scheme 20.[165] Lipidic anchor **89** was modified by two palmitoyl groups for allowing stable insertion into liposomes. Hydrazone ligation of α-hydrazino acetyl peptide **90** with the liposome/**89** preparation proceeded efficiently and generated peptidoliposomes **91**. The peptide sequence used in this study is derived from the cytoplasmic domain of transmembrane lysosomal-associated membrane protein (LAMP). Incubation of liposomes **91** with pig brain cytosol permitted the specific recruitment of AP-3 coat complex, which is known to participate in membrane trafficking by interacting with the cytoplasmic domain of LAMP.[166]

Scheme 20. α-Oxo Aldehyde Anchor 89 Was Inserted in Liposomes during Their Preparation and Used for Grafting LAMP-Derived α-Hydrazinoacetyl Peptide 90 through Formation of a Hydrazone Bond[165]

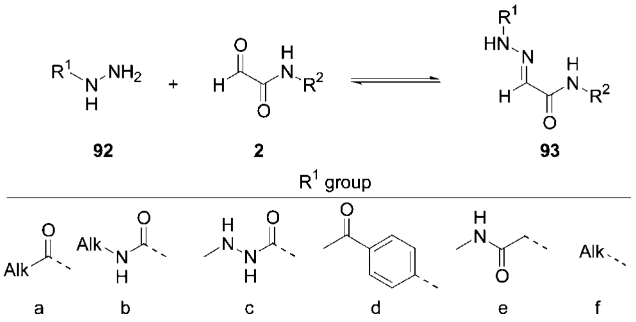


α-Oxo aldehyde and hydrazone chemistries have also been used for the modification of multilamellar vesicles obtained by shearing of lipid lamellar phases. These lipidic particles can encapsulate DNA.[167] They have proved efficient as vectors for the transport of active compounds *in vitro* as well as *in vivo*. Hydrazone ligation was shown to be a simple and efficient method for grafting specific ligands onto these lipidic particles. In particular, mannose mimetics were used to improve the uptake of multilamellar vesicles by dendritic cells.[168] In another application, an RGD peptide was used to promote the adhesion of the lipidic particles to endothelial cells.[142,169]

D. GLYOXYLYL GROUP-BASED LIGATION METHODS

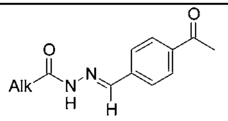
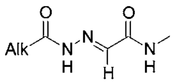
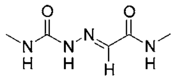
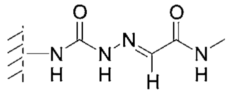
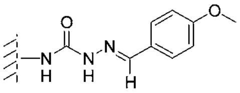
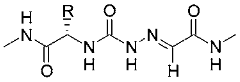
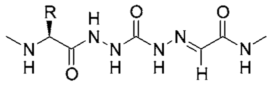
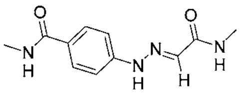
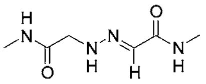
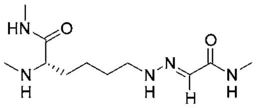
D1. Hydrazone Ligation. Ligation by hydrazone or semicarbazone chemistry is perhaps the oldest method used for synthesizing conjugates (Scheme 21). For example, Girard's reagents, which were originally designed for extracting steroid ketones or aldehydes from complex samples in the 1930s,[170] were recently used as selective chemical probes for detecting and quantifying keto or aldehyde groups within proteins.[171]

Scheme 21. Different Types of α-Oxo Hydrazone or Semicarbazone Ligations Discussed in This Review



The mechanism of hydrazone formation has been studied in detail by Jencks's and Sayer's groups.[172−174] The reaction proceeds through the formation of a carbinolamine, which dehydrates into a hydrazone. At low pH, the rate-limiting step is a hydronium ion-catalyzed formation of the carbinolamine. At intermediate pH (pH 4−6), the rate-limiting step is an uncatalyzed or solvent-catalyzed addition of the free nucleophile to the carbonyl group. At higher (neutral) pH, the rate-limiting step for Schiff base formation is the acid-catalyzed dehydration of the carbinol amine.[172,174] Hydrazone reactions are typically fastest at approximately pH 5, i.e., the experimental conditions for which the rate-limiting step is an uncatalyzed addition of the free nucleophile to the carbonyl group. With hydrazine derivatives of type **92** (Scheme 21) possessing an acidic NH hydrogen such as acetylhydrazide, the rate of the uncatalyzed addition of the nucleophile to the aldehyde is significantly higher than expected due to the stabilization of the developing negative charge on the carbonyl by internal protonation. Clearly, carbinol amine and hydrazone

Table 1. Properties of Hydrazones Derived from Hydrazines 92 (Scheme 21)

entry	type of hydrazone	structure	stability	remarks	ref
1	Benzaldehyde hydrazone		Hydrolysis after 18 h: pH 4.2: 41 %; pH 5.0: 38 %; pH 6.0: 10 %; pH 8.0: 1 %		178
2	**93a**		Decomposition after 24 h: pH 3: 32 %; pH 5 and 7: 0 %; pH 9: 26 %	× 20 reaction rate in 50 % DMSO. Ligation or exchange catalysed by aniline	92 49
3	**93b**		No hydrolysis after 24 h at 65°C and pH 7.5		114
4	**93b**	 peptide microarrays	No variation in signal strength for the serodetection of antibodies after storing chips 12 months at 37°C and 60% relative humidity		143, 185
5	Benzaldehyde semicarbazone	 desoxyoligonucleotide microarrays	40 % of hydrolysis after 24 h at 65°C and pH 7.5		192
6	**93b**		Stable at pH 7.4	Ligation at pH 8.0	194
7	**93c**		Partial hydrolysis and exchange of F(ab')2-CPG2 conjugate at pH 7.4 and 37°C after 24 h (not quantified)	Ligation at pH 4.6	94
8	**93d**		Stable at pH 6-8 after 48 h at 37°C	Ligation at pH 5.0	105
9	**93e**		Good stability at pH 2 (HPLC purification)	Ligation in salt-free *t*BuOH/water mixtures	16, 17, 207
10	**93f**		Good stability at pH 2 (HPLC purification)	Formation of a 1,3,4-oxadiazolidine side-product	206, 210

formation are affected by the structure of the aldehyde and amine nucleophile so that determination of the optimal pH for ligation for each given pair of reactants is worth considering.

Various hydrazine derivatives have been used for hydrazone ligation (**92a–f**, Scheme 21). Hydrazides of type **92a** are derived from aliphatic carboxylic acids. Such hydrazides have been used for several decades for modifying glycoproteins, on which aldehyde groups can be created by reaction with periodate or enzymatic oxidation.[175−177] For example, Itaya and co-workers have reported the labeling of galactose oxidase treated erythrocytes with methionine sulfone hydrazide.[176] Interestingly, hydrazone formation on the cell surface was significantly accelerated in the presence of Mn^{2+}. To the best of our knowledge, such a catalytic effect has not been exploited very much since. Lam and co-workers were the first to describe the use of carboxylic acid hydrazide and carboxybenzaldehyde modified proteins for synthesizing protein−protein conjugates (Table 1, entry 1).[178] The stability of the resulting hydrazone was modest at pH 4−5, since about 40% of the hydrazone hydrolyzed after 18 h at 25 °C. The authors also observed a hydrazide exchange in the presence of an excess of acetylhydrazide. Reduction of the hydrazone bond with sodium cyanoborohydride permitted stabilization of the conjugates. Recently, carboxybenzaldehyde hydrazone chemistry was used for attaching benzoic acid hydrazide-modified peptides to Cowpea mosaic virus nanoparticles,[52] or for attaching proteins to 6-hydrazinopyridine-derivatized biosensor surfaces.[53] The reaction of 6-hydrazinopyridyl-derivatives with aromatic aldehydes was catalyzed by the addition of aniline.[51]

D1.1. Alkylhydrazides. Reaction of alkylhydrazides of type **92a** with *α*-oxo aldehyde-derivatized peptides, proteins, or dendrimers was also found to be a useful ligation method (Scheme 21).[179] In aqueous solution, the equilibrium between carboxylic acid hydrazide **92a** and a glyoxamide derivative of type **2** usually favors the formation of the hydrazone **93a**. Typically, at 1 mM for both reactants, the fraction of hydrazone **93a** is about 90%. However, the fact that the equilibrium kinetics are slow has hampered the application of hydrazone ligation to dynamic covalent chemistry (DCC). Dawson and co-workers have shown that the equilibrium kinetics for *α*-oxo hydrazone formation and hydrolysis are catalyzed by aniline (see Scheme 6). Likewise, aniline catalyzes the hydrazide exchange reaction and should facilitate the application of this ligation reaction to DCC.[49]

α-Oxo hydrazone ligation with alkylhydrazides has been used in a vast array of applications. For example, Tam and co-workers reacted peptide hydrazides with α-oxo aldehyde derivatized lysine dendrimers for assembling multiple antigenic peptides (MAPs).[92] Interestingly, the resulting α-oxo hydrazone bond was found to be stable in water at pH 5–7. Some decomposition was observed at pH 3 or 9 (Table 1, entry 2). These data, which can be compared to the stabilities reported Lam and co-workers for the *N*-acetylhydrazone of *p*-carboxybenzaldehyde (see Table 1, entry 1), show the interest of using α-oxo aldehydes for obtaining hydrazone conjugates with increased stability. Vilaseca and co-workers have ligated 4-desacetyl vincaleukoblastine C3 hydrazide to α-oxo aldehyde lysine-based carrier molecules with the aim of preparing cytotoxic antitumor conjugates.[89] Up to five 4-desacetyl vincaleukoblastine C3 hydrazide molecules were attached to the carrier which was found to be stable at neutral pH for at least 24 h, but hydrolyzed partially at pH 2 in less than 5 h.

Chelius's group has reported an interesting application of α-oxo hydrazone ligation in proteomic research.[180] In this work, N-terminal seryl or threonyl peptides present in tryptic digests were converted into N-terminal glyoxylyl peptides by oxidation with periodate, and subsequently ligated with a biotin hydrazide derivative. The labeled peptides were separated from the complex mixtures by affinity purification with streptavidin prior to liquid chromatography mass spectrometry analysis. A recent application of α-oxo hydrazone ligation for the site-specific modification of RANTES was also reported by Gaertner and co-workers.[181] RANTES features a threonine residue on its N-terminus, which was easily converted into a N-terminal glyoxylyl group by oxidation with periodate. Glyoxylyl-RANTES was reacted with an excess of an alkylhydrazide modifier. The hydrazone was reduced in the presence of $NaCNBH_3$ prior to HPLC purification.

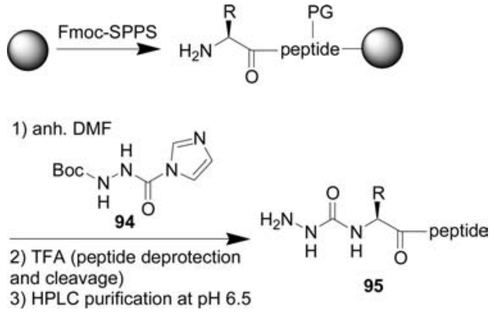
Hydrazone ligation of alkylhydrazides with α-oxo aldehyde derivatives was also applied to immobilizing peptides to surfaces. For example, hydrazone ligation of an N-terminal glyoxylyl peptide derived from parathyroid hormone to an alkylhydrazide-derivatized poly(lactic-*co*-glycolic acid) (PLGA) polymer permitted an improvement in the biocompatibility of this polymeric material.[182] In another application, an N-terminal glyoxylyl peptide ligand of laminin receptor was ligated to liposomes decorated with poly(ethylene glycol) hydrazide chains, in an attempt to improve its circulating longevity.[183]

The modest stability of α-oxo hydrazone ligation derived from alkylhydrazides at mildly acidic pH has often been regarded as a limitation. On the other hand, the acid lability of some acylhydrazones can be advantageously exploited for the design of pH-sensitive scaffolds. A recent application of this kind has been reported by Tung's group, who used the α-oxo hydrazone bond as a probe for the presence of an acidic environment (pH 4.5 or 6.5).[184] No hydrolysis was observed at pH 7.4.

D1.2. Semicarbazides. The chemistry of semicarbazides was also examined in the field (hydrazine derivatives of type **92b** in Scheme 21). In particular, α-oxo semicarbazone ligation was used for the chemoselective immobilization of α-oxo aldehyde peptides,[25,114,135,137,139,147,149,185−188] peptide cobalt−carbonyl complexes,[189] oligodeoxynucleotides,[114,190] or nanoparticle probes[136] on semicarbazide-derivatized surfaces such as silicon,[186,189] silica,[25,114,143,147,185] silicate,[187] titanium oxide,[137] diamond,[191] or polycarbonate.[135,139,188] The α-oxo semicarbazone bond was stable in solution during 24 h at pH 7.5 and 65 °C (Table 1, entry 3). In line with this, peptide microarrays fabricated by printing α-oxo aldehyde peptide probes on semicarbazide microscope glass slides were stable for at least 12 months in an accelerated aging study (Table 1, entry 4). Podyminogin and co-workers also reported the fabrication of DNA microarrays by printing benzaldehyde-derivatized oligonucleotides on semicarbazide microscope glass slides.[192] In contrast to α-oxo semicarbazone (Table 1, entry 3), hydrolysis of benzaldehyde semicarbazone bond was significant after 24 h at pH 7.5 and 65 °C (Table 1, entry 5). These data again highlight the large difference in stability between Schiff bases prepared from α-oxo aldehydes and benzaldehydes.

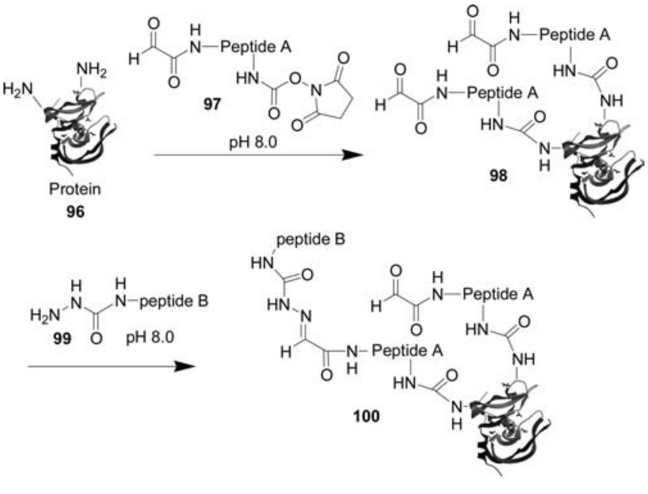
To the best of our knowledge, ligation between semicarbazide and glyoxylyl peptides has not been used so much for the synthesis of conjugates. One reason for this can be the lack of methods giving access to semicarbazide peptides such as **95**, i.e., peptides featuring a hydrazinocarbonyl group on their N-terminus (Scheme 22). It was shown recently that a hydrazinocarbonyl group can be easily attached to the N-terminus of peptides after the Fmoc-SPPS elongation step by using *tert*-butylcarbazate derivative **94**.[193,194] Semicarbazide peptide **95** survived the TFA cleavage and deprotection step but hydrolyzed rapidly during analytical or preparative RP-HPLC at pH 2. In contrast, the use of an eluent system buffered at pH 6.5 permitted prevention of semicarbazide bond hydrolysis and isolation of semicarbazide peptides **95** in good yield.

Scheme 22. Fmoc-SPPS Synthesis of N-Terminal Semicarbazide Peptides 95



Another interesting observation is the capacity of the semicarbazide group of peptide **95** to react with an α-oxo aldehyde moiety at pH 8 (Table 1, entry 6). This property was exploited for designing a one-pot procedure permitting the synthesis of peptide−protein conjugates based on a carbonylation-semicarbazone bond forming reaction sequence (Scheme 23).[194] For this, bifunctional peptide A **97** featuring an α-oxo aldehyde group and an *N*-succinimidylcarbonyl group was reacted at pH 8.0 with surface-exposed amino groups of a protein to form stable urea bonds. This resulted in the grafting of peptide A molecules onto the surface of the protein. N-Terminal semicarbazide peptide B **99** was then added to the mixture to give conjugate **100**. The number of peptide A and B chains attached to the protein can be adjusted by varying the stoichiometry of both reactions. This method was validated by the rapid synthesis of a biologically active Shiga toxin B-subunit conjugate.

Scheme 23. One-Pot Synthesis of Peptide Protein Conjugates Can Be Performed by Combining *N*-Succinimidyl Carbamate Chemistry and α-Oxo Semicarbazone Ligation[a]



[a]Both reactions were carried out at pH 8.0.[194] For clarity, this scheme shows as an example the grafting of two peptide A chains and of one peptide B chain.

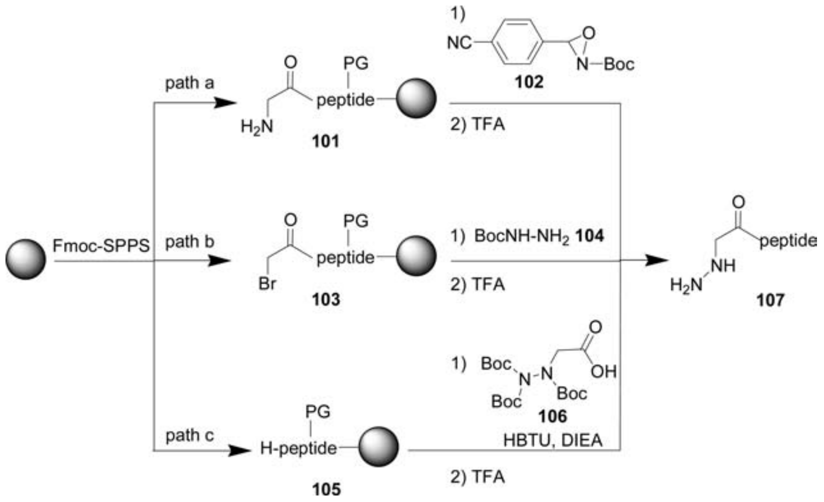
D1.3. Carbohydrazides. Reaction of carbohydrazide derivatives of type **92c** (Scheme 21) with α-oxo aldehydes and other aldehydes was studied by Rose and co-workers during their pioneering studies on the preparation of protein conjugates using protein carbohydrazides (Table 1, entry 7).[89,90,94,195−197] Protein carbohydrazides, which feature a carbohydrazide group attached to the C-terminus of the protein, were produced by enzyme-assisted reverse proteolysis of proteins in the presence of an excess of carbohydrazide. This method permitted the semisynthesis of various carbohydrazide proteins derived for example from des-Ala^{B30}-insulin[89,104,195] or from an F(ab′)$_2$ fragment.[94,197] Hydrazone ligation with α-oxo aldehydes is highly efficient and permitted the synthesis of large scaffolds such as insulin octahydrazone (47 605 Da)[104] or a F(ab′)$_2$-carboxypeptidase G2 conjugate (147 kDa).[94]

In another application, Geoghegan and co-workers reported an efficient synthesis of double fluorescent enzyme substrates of renin or collagenase using hydrazone ligation of a 5-carboxytetramethylrhodamine-labeled N-terminal glyoxylyl peptide with a carbohydrazide derivative of Lucifer Yellow.[198]

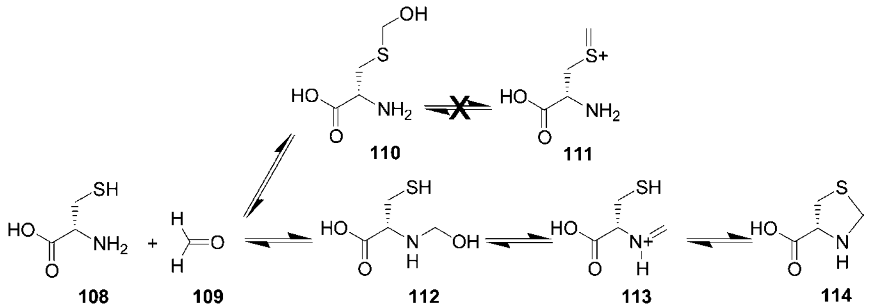
D1.4. Arylhydrazines. The reaction between arylhydrazines of type **92d** (Scheme 21) and α-oxo aldehydes was studied by Tam and co-workers.[105] Peptides derivatized on the N-terminus by a 4-hydrazinobenzoyl group were prepared by coupling Boc-NHNH-C_6H_4−CO_2H to the peptidyl resin. Ligation between N^{α}-4-hydrazinobenzoyl peptide and a tetrameric α-oxo aldehyde MAP core matrix proceeded rapidly and efficiently at pH 5. The tetrameric hydrazone was stable at 37 °C at pH 6−8 for up to 48 h (Table 1, entry 8).

D1.5. Hydrazinoacetamides. The usefulness of α-hydrazinoacetamides of type **92e** (Scheme 21) for hydrazone ligation with peptide α-oxo aldehydes has been studied in detail by Melnyk and co-workers. Fmoc SPPS is the method of choice for the peptide elongation step because some degradation of the N−N bond was observed in HF.[199] A α-hydrazinoacetyl group can be introduced into peptides after SPPS by solid phase *N*-electrophilic amination[199−202] of an N-terminal glycine residue[199,203] with *N*-Boc-3-(4-cyanophenyl)oxaziridine **102** (BCPO, path a, Scheme 24). The usefulness of this reagent for the synthesis of hydrazine derivatives in solution was originally described by Collet and co-workers.[204] α-Hydrazinoacetyl peptides **107** can also be synthesized by solid phase nucleophilic substitution of bromine atom of bromoacetyl group by *tert*-butylcarbazate BocNHNH$_2$ **104** (Scheme 24, path b).[205,206] However, the more practical method for introducing the α-hydrazinoacetyl group during Fmoc-SPPS is to use *N,N,N′-tris*(Boc)hydrazinoacetic acid **106**, i.e., (Boc)$_2$NN(Boc)CH_2CO_2H, which is commercially available. It can be coupled to α- or ε-amino groups using standard activation procedures (Scheme 24, path c).[148] Note that mono- and diprotected derivatives of α-hydrazinoacetic acid such as BocNHNHCH_2CO_2H or BocNHN(Boc)CH_2CO_2H can lead to variable amounts of oligomeric side-products depending on the activation procedure, and therefore must be avoided.[148]

Scheme 24. Access to α-Hydrazinoacetyl Peptides 107 by *N*-Electrophilic Amination of an N-Terminal Glycine Residue with BCPO 102,[199,203] by Nucleophilic Displacement of Bromine Atom of Bromoacetyl Group by BocNHNH$_2$ 104,[205,206] or by Coupling (Boc)$_2$NN(Boc)CH_2CO_2H 106 to a Peptidyl Resin[148]



Scheme 25. Mechanism of Thiazolidine Formation between L-Cysteine and Formaldehyde[50]



The hydrazinoacetyl group is highly reactive toward α-oxo aldehydes and enables ligations to be carried out in the absence of buffer in *tert*-butanol/water mixtures.[16,17,207] The great interest of a high yielding salt-free hydrazone ligation is the possibility of skipping the purification or desalting step which inevitably leads to loss of material. The final product is obtained following a simple lyophilization step. The procedure is of particular value for synthesizing lipopeptides, whose purification by HPLC is known to be problematic due to their amphiphilic properties,[16] or mixtures of lipopeptides as potential synthetic vaccines.[17] Another significant advantage of hydrazone ligation in a salt-free medium is the possibility of easily combining hydrazone ligation with another chemoselective ligation in a sequential manner.[207] Indeed, if a salt-free hydrazone ligation is carried out in the first step of the assembly process, a second ligation can be carried out subsequently in the buffer of choice with no need to separate the intermediate hydrazone product, or to adjust the pH or composition of the mixture. This aspect will be discussed in more detail later in the section dedicated to one-pot sequential ligation methods.

Hydrazones obtained by reaction of α-hydrazinoacetyl peptides **107** with α-oxo aldehydes have a good stability at pH 2, thereby allowing HPLC purification of the ligated products using standard HPLC separation procedures (Table 1, entry 9).[207]

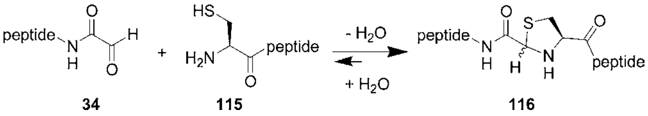
To conclude this section on α-hydrazinoacetyl peptides, it is important to mention that the chemical properties of hydrazino groups of type **92e** are critically dependent on the presence of a carboxamido group in close proximity to the N−N bond. The pK_a of α-hydrazinoacetyl group is 6.5,[203] i.e., about 1.5 pK_a units less than the pK_a of a glycyl residue[208] or of *N*-ethylhydrazine.[209] For comparison, reaction of *N*-alkylhydrazine derivatives of the type **92f** (Scheme 21) such as ε-amino lysine[199−202] containing peptides (Table 1, entry 10) with peptide α-oxo aldehydes gave mixtures of the expected hydrazone together with significant amounts of a 1,3,4-oxadiazolidine side-product, the proportion of which was highly dependent on pH and peptide composition.[210]

D2. Thiazolidine Ligation. The facile reaction of cysteine with various aldehydes to give stable thiazolidine products has long been known. Schubert studied the reaction of methyl or phenylglyoxal with various thiol compounds in 1935.[211] Those presenting a free amino group in close proximity to the thiol such as cysteine were found to give stable adducts with elimination of one mole of water. The products formed in this reaction were rapidly identified as being thiazolidines.[212,213]

The mechanism of thiazolidine formation between cysteine and formaldehyde has been studied in detail (Scheme 25).[50] The fast formation of hemithioacetal **110** has been observed in a broad pH range. However, dehydration of hemithioacetal **110** into sulfonium ion intermediate **111** is unlikely to occur to a significant extent. Thus, although hemithioacetal **110** is formed more rapidly than carbinol amine **112**, dehydration of the latter and the subsequent intramolecular cyclization of iminium intermediate **113** into thiazolidine product **114** is believed to be the main mechanistic pathway. The rate-determining step for thiazolidine formation is the formation of carbinol amine intermediate **112** at acidic pH, whereas dehydration of carbinol amine **112** is rate-limiting at basic pH. The transition between these two rate-determining steps occurs at about pH 6, thereby explaining the bell-shaped relationship between the rate of thiazolidine formation and pH.

The related thiazolidine ligation of a peptide α-oxo aldehyde with a N-terminal cysteinyl peptide is shown in Scheme 26.

Scheme 26. Thiazolidine Ligation between a Peptide α-Oxo Aldehyde and a Cysteinyl Peptide[92,102,105]



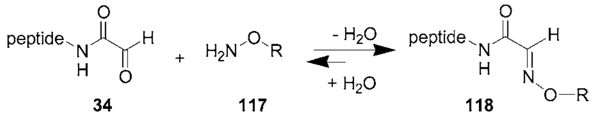
Note that this reaction results in the formation of an asymmetric center within the thiazolidine ring. The mechanism of this reaction has not been studied in detail. For aldehydes, the reactive form toward nucleophilic attack is the unhydrated form. In the previous reaction of L-cysteine with formaldehyde, dehydration of formaldehyde was not rate-determining.[50] As discussed at the beginning of this review, the hydrate form of α-oxo aldehyde group predominates in aqueous solution. The importance of α-oxo aldehyde dehydration to the rate of thiazolidine ligation remains to be established.

Thiazolidine ligation proceeds efficiently in water at pH 4−6. The reaction is accelerated when *N*-methylpyrrolidone (NMP) or DMF is used as cosolvent, or by raising the temperature of the reaction mixture.[105] Formylation side-products have been observed sometimes with DMF as cosolvent.[102] Thiazolidine ligation is compatible with the use of Gdn.HCl.[98,214]

The potential of thiazolidine ligation for the site-specific ligation of unprotected peptide segments was realized only in the mid 1990s with the seminal work of Tam and co-workers, who exploited this reaction as a site-specific capture step for designing a pseudonative amide bond forming reaction (pseudoproline ligation[215,216]) or for assembling complex scaffolds such as MAP dendrimers.[92,102,105] This chemistry was also used by Tam's group for synthesizing end-to-side-chain cyclic peptides, starting from a peptide precursor featuring a N-terminal Cys residue and an α-oxo aldehyde group on a lysine side-chain.[217] Thiazolidine ligation involving α-oxo aldehydes has proved to be a useful ligation method for the site-specific labeling of a single-chain antibody,[218] of mouse epidermal growth factor,[219] and for assembling various structures such as cell-permeable peptides,[98] template-assisted synthetic protein scaffolds (TASP),[214] or various peptide-based conjugates such as peptide–lipid conjugates.[220]

D3. Oxime Ligation. Reaction of an aldehyde or ketone group with a hydroxylamine results in the formation of an aldoxime or ketoxime compound, respectively, and in the elimination of one molecule of water. The application of this reaction to glyoxylyl peptides is described in Scheme 27. The potential of this reaction for the chemoselective synthesis of large scaffolds was pioneered by Rose's group in the mid 1990s,[95] which reported in a seminal paper the synthesis of a 20 kDa scaffold assembled by oxime ligation of a N-terminal aminoxyacetyl peptide to an octavalent glyoxylyl lysine template.

Scheme 27. Oxime Bond Formation with α-Oxo Aldehydes[92,95,104]



Soon after this discovery, Tam's[92] and Rose's[104] groups independently examined the effects of pH and other parameters on the rate and yield of oxime bond formation and stability.

Tam's group examined the ligation of a 20-amino-acid N-terminal aminoxyacetyl peptide to a tetravalent glyoxylyl MAP core and found that increasing the pH from 4.2 to 5.7 led to an increase of the reaction rate, with the concomitant increase of side-product formation.[92] The optimal pH for oxime ligation thus appeared to be around 5.0. Addition of an organic cosolvent such as DMF (50%) or heating the reaction mixture from 22 to 37 °C increased the rate of oxime ligation. With regard to the stability of the oxime bond, the tetravalent construct was stable for 24 h at pH 3, 5, or 7 but hydrolyzed partially (21%) into a three-branched dendrimer at pH 9.

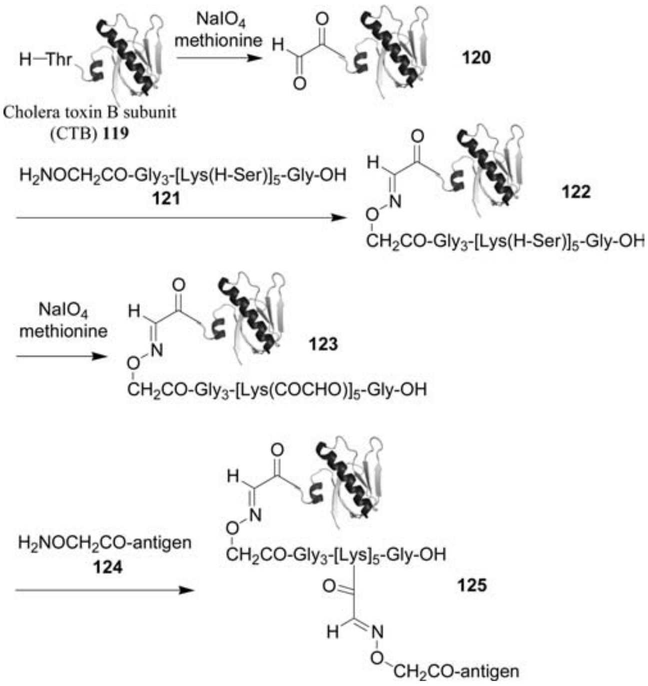
Rose's group examined the effect of pH on the rate of oxime ligation using small model peptides or an hexaglyoxylyl polylysine template and a 12-amino-acid N-terminal aminoxyacetyl peptide.[104] The study with small model peptides showed a significant decrease in reaction rate with increasing pH (3.0, 4.6, and 5.3), while no reaction was observed at pH 7.0. The same trend was observed with the hexaglyoxylyl polylysine template, in contradiction with Tam's studies. Rose's group also examined the stability of the oxime bond at different pH values. The oxime bond was stable for 65 h at pH 7.0, for 20 h at pH 8.0, or for 2 h at pH 9.0, but significant decomposition was observed after 65 h at pH 9.0 or 10.

Note that, as discussed in detail earlier in this review, oxime ligation of peptide α-oxo aldehydes with hydroxylamine derivatives can be catalyzed by aniline, thereby now enabling oxime ligations to be performed efficiently at neutral pH.[46−48] Aniline has been shown also to catalyze oxime ligation of a peptide α-oxo aldehyde to an aminoxyacetyl-functionalized SPR chip.[221]

Since its discovery, oxime ligation involving peptide α-oxo aldehydes has been used in a myriad of applications. For example, glyoxylyl chemistry[63,190,222−224] and oxime ligation have been extensively used for the synthesis of oligonucleotide–peptide conjugates,[223−227] or for attaching oligonucleotides[228] or peptides[229] to various surfaces.

Oxime ligation has also been used for the assembly of homogeneous MAPs, dendrimers (for the synthesis of an insulin octaoxime dendrimer of 48 kDa, see ref 104) or of branched multivalent scaffolds.[92,95,104,230] Very often, these synthetic constructs have been evaluated as potential synthetic vaccines.[13,15,103,231−234] For example, Rose's group reported a method for the synthesis of well-defined immunogens using two sequential serine oxidative cleavage/oximation reactions as shown in Scheme 28.[234] In this work, the N-terminal threonine residue of Cholera toxin B subunit (CTB, **119**) was converted into glyoxylyl-CTB **120** using sodium periodate in the presence of methionine. Oxime ligation of glyoxylyl-CTB **120** with aminooxyacetyl linker **121** yielded CTB intermediate **122**, which was again oxidized with the sodium periodate/methionine mixture to provide pentavalent glyoxylyl CTB derivative **123**. Finally, oxime ligation with aminoxyacetyl functionalized antigen **124** generated hexaoxime construct **125**, which was isolated by gel filtration. A variant of this approach using an aminoacetaldehyde functionality for the first ligation step, formed by hydrolysis of an aminoacetaldehyde dimethylacetal precursor, has been described by Delmas and coworkers.[232]

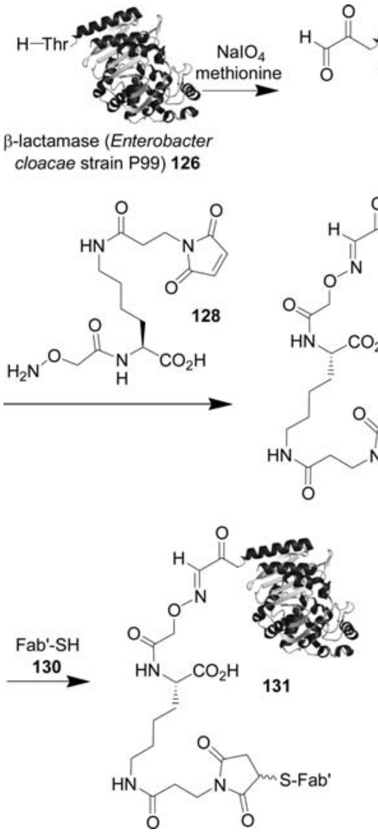
Scheme 28. Synthesis of Cholera Toxin B Subunit (CTB)-Derived Immunogens Using Two Sequential Serine Oxidative Cleavage/Oximation Reactions[234]



In another application, α-oxo oxime ligation was used to attach a VEGF-R2 binding peptide to the surface of multilamellar vesicles, as a potential way to target the vesicles to angiogenic cells.[235] For this, a hydroxylamine derivative of cholesterol was synthesized and incorporated into the multilamellar vesicles. Incubation of the functionalized vesicles with the soluble N-terminal glyoxylyl peptide at pH 5.5 led to the attachment of the peptide to the surface of the vesicles.

Glyoxylyl chemistry and oxime ligation have also been used for the synthesis of homogeneous Fab′-enzyme conjugates of potential therapeutic interest. For example, the N-terminal threonine residue of β-lactamase from *Enterobacter cloacae* strain P99 **126** (Scheme 29) was oxidized with sodium periodate to give glyoxylyl β-lactamase **127**.[236] Oxime bond formation with bifunctional linker **128** featuring aminoxyacetyl and maleimide groups generated maleimide-functionalized β-lactamase intermediate **129**, which was finally conjugated to a Fab′-SH segment **130** using thiol-maleimide chemistry to give conjugate **131**.

Scheme 29. Synthesis of an Fab′-β-Lactamase Conjugate 131 by Combining Oxime and Thiol-Maleimide Chemistries[236]



Another interesting application of the oximation reaction involving α-oxo aldehydes is the synthesis of cyclic peptides by intramolecular oxime bond formation as shown in Scheme 30.[97,237] For example, Mutter's group synthesized cyclic oxytocin analogues featuring an oxime bridge instead of a disulfide bridge as in the native peptide.[97] In these studies, the N-terminal serine residue of peptide **132** was oxidized in the presence of a free aminoxyacetyl group. The glyoxylyl intermediate **133** evolved spontaneously into cyclic product **134**. The alternative cyclization mode with the aminooxyacetyl group on the N-terminus and the glyoxylyl group on the lysine side-chain was also examined.

Various studies have also exploited the ease of synthesis of N-terminal glyoxylyl proteins and oxime ligation for the site-specific introduction of modifications on the N-terminus of proteins. For example, Gaertner and co-workers described the modification of the amino terminus of IL-8 or G-CSF by various polyethylene glycol chains with the aim of improving their pharmacokinetics.[71] More generally, α-oxo aldehyde chemistry has frequently been used for the site-specific conjugation of polymers to peptides or proteins. The field has been reviewed recently.[238,239] In another application, the semisynthesis of potent human immunodeficiency virus-type 1 (HIV-1) entry inhibitor aminooxypentane (AOP)-RANTES[19−21,240−242] and of other RANTES derivatives[181] constitutes a seminal contribution to the field of protein engineering using α-oxo aldehyde and site-specific oxime conjugation chemistries.

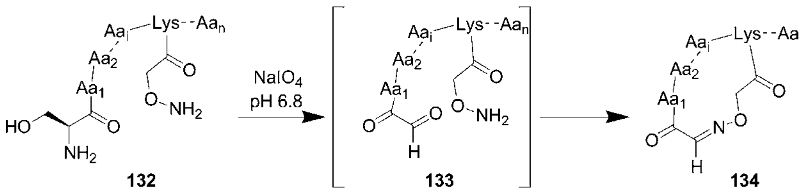
Finally, α-oxo oxime ligation has been used extensively by Mutter and co-workers[96] and more recently by Dumy and co-workers for synthesizing template assembled synthetic proteins (TASP, also called regioselectively addressable functionalized template RAFT) as potential functional mimics of proteins or as scaffolds for the simultaneous presentation of multiple binding or functional moieties (ligation of divalent aminooxy TASP to *bis* glyoxylyl peptide for loop formation,[96] ligation of tetravalent glyoxylyl TASP to 4 mono or oligosaccharide aminoxy ligands,[108,243,244] ligation of tetravalent aminooxy TASPs to 4 glyoxylyl cyclic ArgGlyAsp peptide[245,246] to cite a few applications). The field has been reviewed recently.[101]

Dumy and co-workers have also developed methods to selectively decorate the two faces of TASP scaffold using two sequential α-oxo oxime forming reactions (Scheme 31).[100] For example, a TASP scaffold **135** derivatized by four seryl residues on one face as α-oxo aldehyde precursor and two aminooxyacetyl groups on the other face was first ligated with CHOCO-GlyGly(LysLeuAlaLysLeuAlaLys)$_2$-OH peptide **136** to give dioxime product **137**. Seryl residues were then converted to glyoxylyl groups with sodium periodate to give tetraaldehyde compound **138**. Finally, oxime ligation with an aminooxyacetyl-derivatized cyclic ArgGlyAsp peptide **139** generated the target hexaoxime construct **140**.

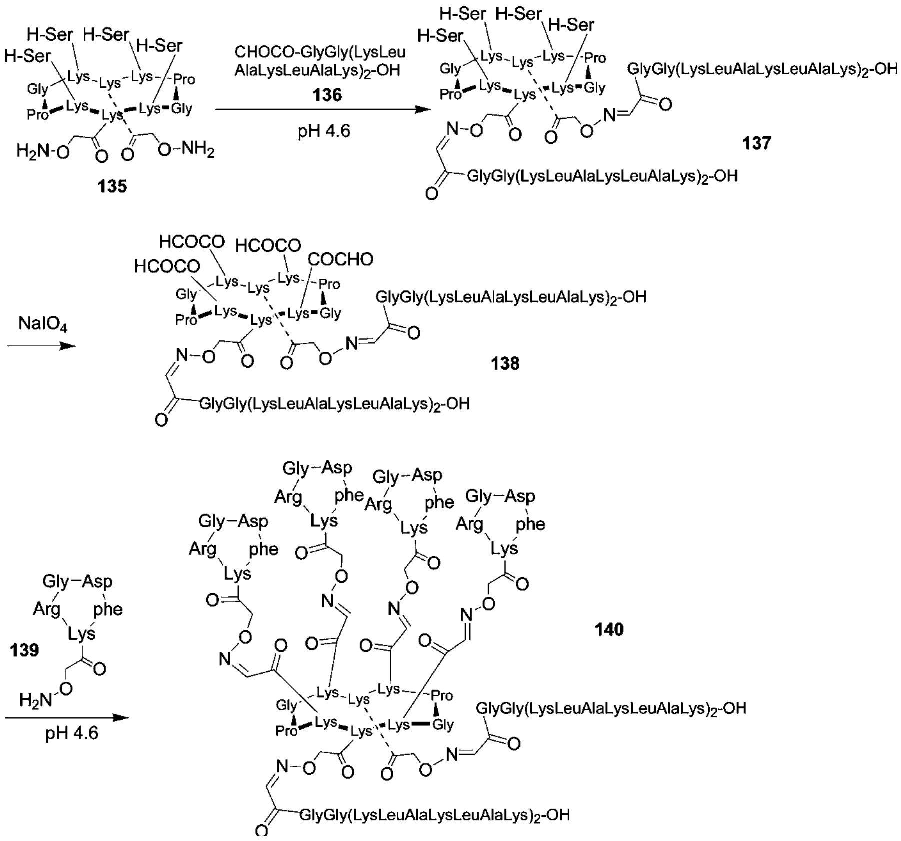
E. ONE-POT SEQUENTIAL LIGATIONS

Site-specific ligations can be combined sequentially in certain circumstances, thereby giving access to complex and homogeneous scaffolds. The field has been reviewed recently.[247] The example discussed in the previous section and illustrated in Scheme 31 illustrates the powerfulness of such methods. The combination of only two site-specific ligations potentially gives access to a vast array of conjugates in which different motifs can be clustered or associated, thereby enabling tailoring for novel biological or physicochemical properties. Very often, sequential

Scheme 30. Synthesis of Cyclic Peptides by Intramolecular Oxime Ligation[97,237]



Scheme 31. Two Sequential α-Oxo Oxime Ligations Enable the Selective Functionalization of the Two Faces of RAFT Scaffolds (phe is for D-Phe)[100]



ligations require an intermediate isolation step, because the efficiency of the first ligation step or the experimental conditions used for it are not compatible with the second ligation step. The need for rapid access to complex synthetic conjugates in good yield has stimulated the design of one-pot sequential ligation techniques, which are the focus of this section.

Various sequential ligation schemes have been described to date, but only very few are one-pot processes. Kent's group has pioneered the field by assembling a 20 kDa Max homodimer mimetic using a one-pot thioester and ketoxime ligations process.[248] Not surprisingly, the powerfulness of α-oxo aldehyde based chemoselective ligations has stimulated various developments in this area.

One of the first applications of glyoxylyl group chemistry for the one-pot chemoselective assembly of complex scaffolds was disclosed by Melnyk and co-workers in 2000 (Scheme 32).[205,206] Lysine-based dendrimer **141** featuring an α-oxo aldehyde group on the C-terminus and 8 chloroacetyl groups on its periphery was synthesized on a solid phase using the dimethyl-2,3-*O*-isopropylidene-D-tartrate-based linker and polyethylene glycol-dimethylacrylamide copolymer (PEGA) resin as discussed earlier in this review.[70,109] The assembly was carried out by first ligating chloroacetyl groups with the mercapto-sugar derivative **142** at pH 8.5. After 24 h, the pH of the reaction mixture was adjusted to 5.2 to allow the second α-oxo hydrazone ligation step with α-hydrazinoacetyl modified HA epitope ProLysTyrValLysGlnAsnThrLeuLysLeuAlaThr **143**[148,203] to proceed. The target glycopeptide dendrimer **144** was purified by HPLC (51% yield) thanks to the stability of the hydrazone bond in the acidic water/acetonitrile eluent (pH 2).

The reverse sequence of reactions, i.e., α-oxo hydrazone/thioether ligations, also proved satisfactory for tetravalent chloroacetyl/α-hydrazinoacetyl-functionalized dendrimers as shown in Scheme 33.[207] In this case, the first α-oxo hydrazone bond was formed efficiently in a salt-free *t*BuOH/water mixture.[16,17] Thioether ligation was subsequently triggered by the addition of mercapto-sugar derivative **142** and potassium carbonate.

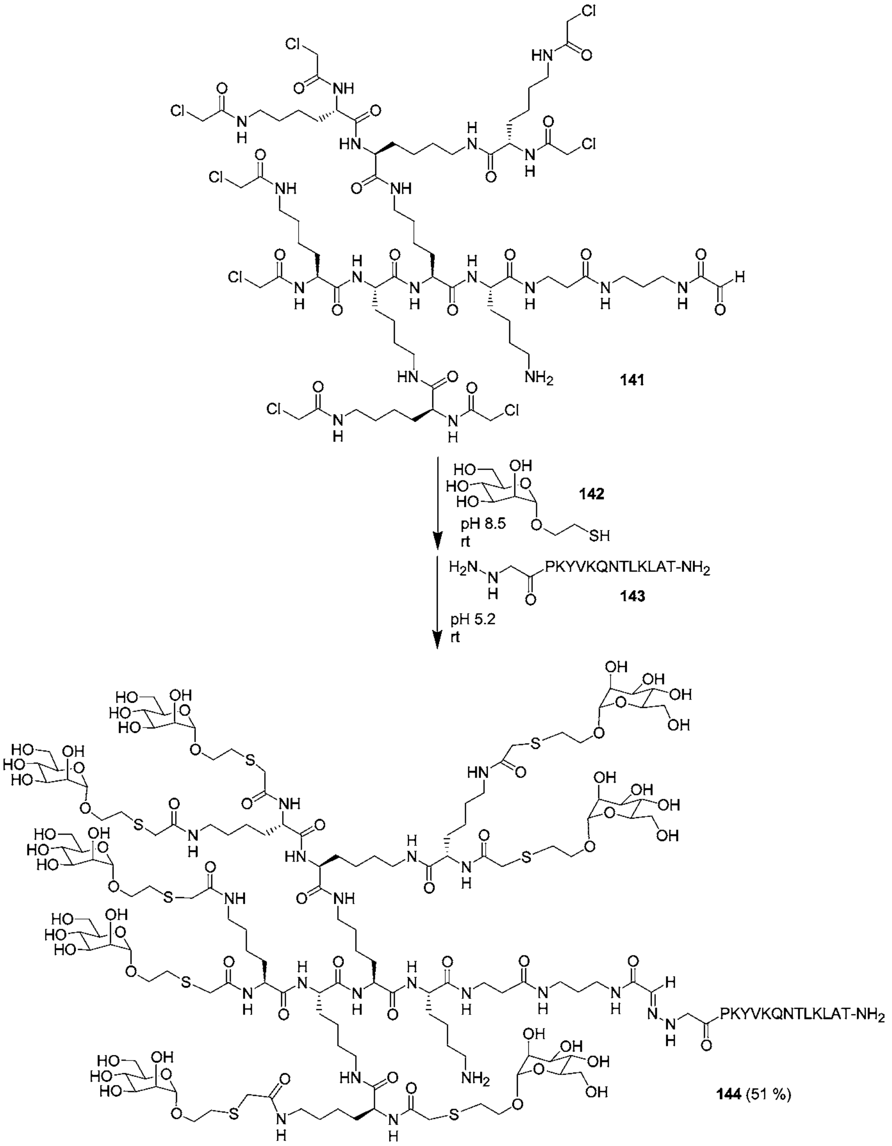
More recently, Dumy and co-workers designed one-pot sequential assembly methods enabling the synthesis of sophisticated TASP conjugates. The method described in Scheme 34 combines α-oxo oxime ligation and the copper(I)-catalyzed 1,3-dipolar cycloaddition of organic azides and terminal alkynes (CuAAC).[8] The first α-oxo oxime ligation was carried out in acidic solvent starting from TASP molecule **148**, thereby enabling the one-pot deprotection of aminooxyacetyl moieties and oxime bond formation to give scaffold **151**. The pH was then adjusted to 7 to allow the CuAAC reaction to proceed. The methodology was used later on for synthesizing fluorescently labeled TASP molecules decorated with carbohydrate and cyclic peptide ArgGlyAsp motifs.[249]

Later on, the concept was extended to a one-pot triple orthogonal sequential chemoselective ligation process based on an α-oxo oxime/thioether/CuAAC reaction sequence.[250]

F. GLYOXYLYL GROUP CHEMISTRY AND SEMI OR TOTAL PROTEIN SYNTHESIS

Chemical synthesis is now established as a complement and potential alternative to the use of recombinant techniques for producing small proteins. Interestingly, chemical synthesis

Scheme 32. One-Pot Assembly of Lysine-Based Cluster Mannosides Using Thioether and α-Oxo Hydrazone Chemoselective Ligation Reactions[a]



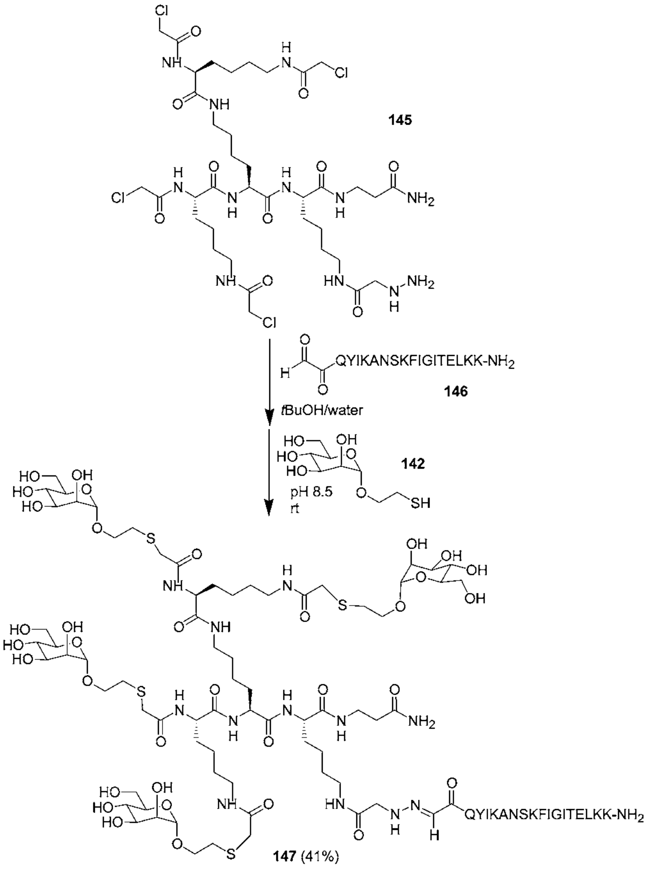
[a]PKYVKQNTLKLAT is the one-letter code for ProLysTyrValLysGlnAsnThrLeuLysLeuAlaThr.[205,206]

allows the introduction at specific sites of various natural or non-natural modifications that are often difficult to achieve using recombinant techniques.[251] Total chemical synthesis of proteins can be carried out by joining unprotected peptide segments using native peptide ligation methods.[252,253] An alternative is to use protected or minimally protected peptide segments which are linked together using amide bond forming reactions. Glyoxylic acid chemistry has permitted the design of novel routes to functionalized peptide segments useful for both types of synthetic approaches.

F.1. Introduction to Native Chemical Ligation. To date, only a few native peptide ligation methods have been described.[254−258] Basically, all these reactions proceed through a chemoselective capture step which brings into close proximity the C-terminus of one segment and the N-terminus of the other. This intermediate then spontaneously rearranges to give a native peptide bond between the two segments. Today, the field of total protein synthesis is dominated by Native Chemical Ligation (NCL, Scheme 35), which was reported by Kent's group in 1994.[252,254,259] NCL is based on the reaction of a C-terminal peptide thioester **153** with an N-terminal Cys peptide **115**. This reaction proceeds through a thioester-linked intermediate **154** formed in the capture step by thiol−thioester exchange. The peptide acyl chain spontaneously migrates intramolecularly from sulfur to nitrogen to yield a native peptide bond. Expressed protein ligation (EPL) is the extension of NCL to peptide thioesters produced using recombinant techniques.[260]

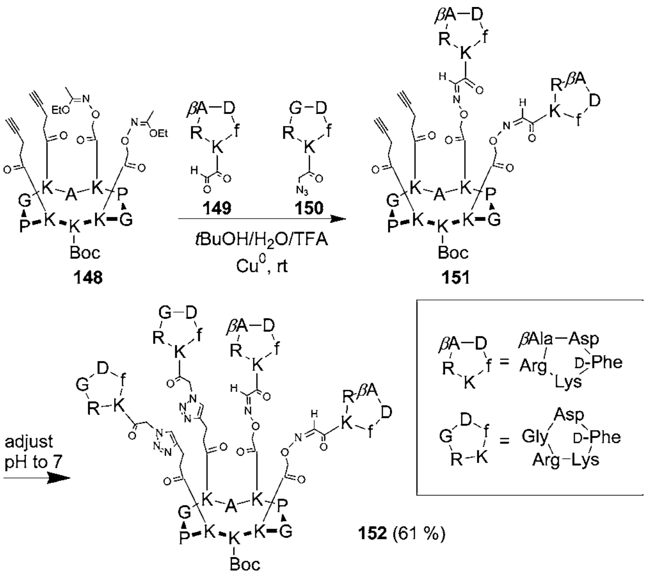
NCL also works well with a large variety of β- or γ-amino thiols or selenols derived from proteinogenic amino acids, either by modification of the α-amino group with a thiol auxiliary or by incorporation of a mercapto or seleno group into the amino acid side-chain. The goal is to overcome the problem associated with the low frequency of cysteine residues in proteins. In particular, a lot of work has been done to extend NCL to the formation of X-Gly peptide bonds, since Gly is a frequently occurring amino acid in proteins.[261] This is one

Scheme 33. One-Pot Assembly of Lysine-Based Cluster Mannosides Using α-Oxo Hydrazone and Thioether Chemoselective Ligation Reactions[a]



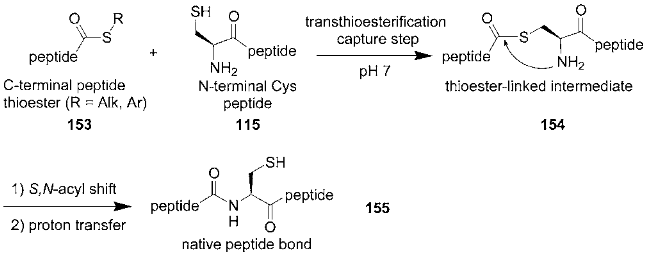
[a]The first step was carried out in a salt-free tBuOH/water mixture. QYIKANSKFIGITELKK is the one-letter code for GlnTyrIleLysAlaAsnSerLysPheIleGlyIleThrGluLeuLysLys.[207]

Scheme 34. One-Pot Assembly of TASP Molecules Using a Sequential α-Oxo Oxime/CuAAC Sequence[8]



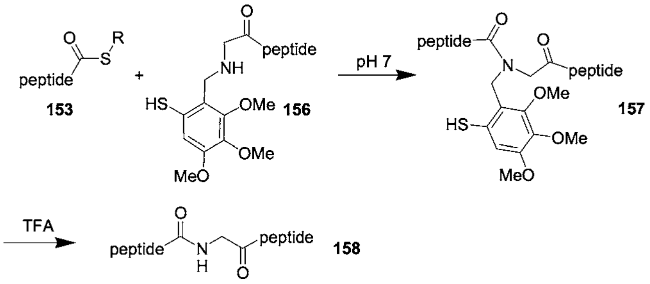
definitely interesting application of glyoxylyl chemistry in the field of total protein synthesis.

Scheme 35. Native Chemical Ligation (NCL)[252,254]



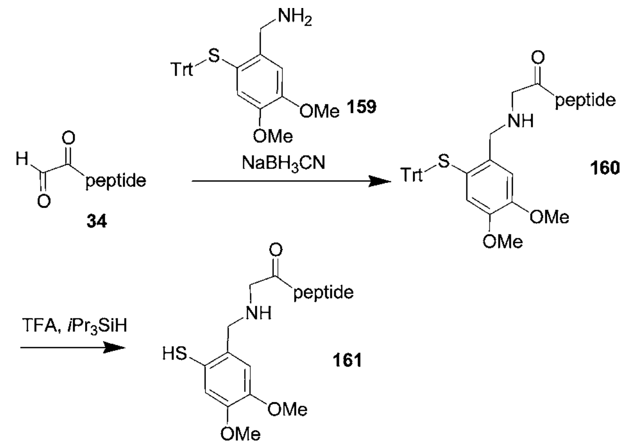
F.2. Glyoxylyl Peptides and NCL at X-Gly Sites. Ligation at X-Gly sites can be carried out by reacting a C-terminal peptide thioester with another peptide segment featuring a removable N^{α}-(2-mercaptobenzyl) auxiliary on the glycine α-amino group. The principle of the method is illustrated in Scheme 36 with the 4,5,6-trimethoxy-2-mercaptobenzyl auxiliary developed by Dawson's group.[261] Intermediate **157** formed in the NCL process was further deprotected in TFA to generate peptide **158** with a native X-Gly junction. A 4,5-dimethoxy-2-mercaptobenzyl group is equally effective but requires HF[261] or a mixture of trifluoromethanesulfonic acid (TFMSA)/TFA[10] for its removal.

Scheme 36. Ligation at X-Gly Junction Using NCL and 4,5,6-Trimethoxy-2-mercaptobenzyl Auxiliary[261]



Aimoto's group described an elegant route to N^{α}-4,5-dimethoxy-2-mercaptobenzyl glycyl peptides **161** (Scheme 37) based on the reductive amination of N-terminal glyoxylyl

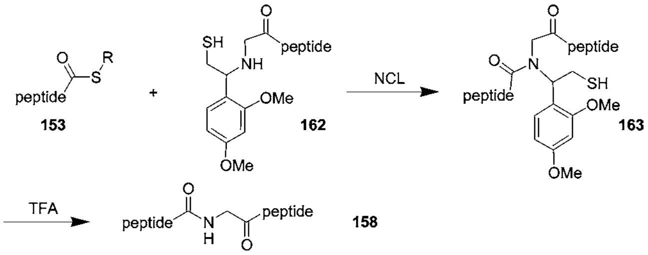
Scheme 37. Synthesis of N^{α}-4,5-Dimethoxy-2-mercaptobenzyl glycyl Peptides 161 Using the Reductive Amination of N-Terminal Glyoxylyl Peptides in the Presence of 4,5-Dimethoxy-2-(triphenylmethylthio)benzylamine 159[10]



peptides in the presence of 4,5-dimethoxy-2-(triphenylmethylthio)benzylamine **159**. This step was followed by removal of the trityl group with TFA to give peptide segment **161**, ready for ligation.[10] The great interest of using N-terminal glyoxylyl peptides as a source of peptide segments of type **161** is the possibility of using peptides or proteins of biological origin for assembling large proteins. Indeed, as described at the beginning of this review, large N-terminal serine or threonine peptides can be produced using recombinant techniques and further converted into N-terminal glyoxylyl peptides by periodate oxidation or transamination.

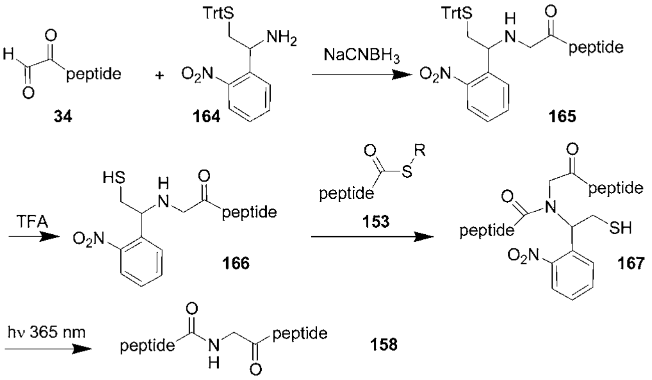
An alternative for the ligation at X-Gly sites is to use N^{α}-(1-phenyl-2-mercaptoethyl)-derived auxiliaries as shown in Scheme 38.[262−264] The method is illustrated with the use of N^{α}-(1-(2′,4′-dimethoxyphenyl)-2-mercaptoethyl) auxiliary designed by Kent's group, which can be removed in TFA after the ligation step.[262]

Scheme 38. Use of N^{α}-(1-Phenyl-2-mercaptoethyl)-Derived Auxiliaries for Ligation at X-Gly Sites Using NCL[262]



Usually, the N^{α}-(1-phenyl-2-mercaptoethyl)-derived auxiliaries are added to the peptide chain during SPPS, after the peptide elongation step. As an alternative to the development of solid phase approaches, Aimoto's group designed an access to peptides derivatized by an N^{α}-(1-(2′-nitrophenyl)-2-mercaptoethyl) auxiliary starting from N-terminal glyoxylyl peptides as shown in Scheme 39.[9] Here again, the advantage of the approach is the possibility of using N-terminal seryl or threonyl peptides of biological origin for synthesizing large polypeptides. N^{α}-(1-(2′-Nitrophenyl)-2-mercaptoethyl) group was installed by reductive amination of the N-terminal glyoxylyl peptide **34** in the presence of 1-(2′-nitrophenyl)-2-(triphenylmethylthio)ethylamine **164** and sodium cyanoborohydride. The trityl group was then removed with TFA to give N^{α}-(1-(2′-nitrophenyl)-2-mercaptoethyl) glycyl peptide **166**. The auxiliary was removed after the ligation step by UV irradiation at 365 nm.

Scheme 39. Introduction of N^{α}-(1-(2′-Nitrophenyl)-2-mercaptoethyl) Group onto Peptides by Reductive Amination of N-Terminal Glyoxylyl Peptides[9]

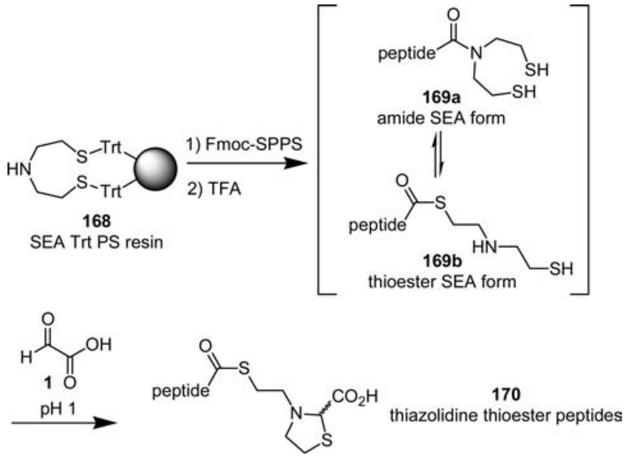


F.3. Glyoxylic Acid Chemistry for the Synthesis of Peptide Thioesters.

The previous sections have highlighted the potential of glyoxylic acid chemistry for the elaboration of thiol auxiliaries, which allow the extension of the NCL reaction to the formation of X-Gly junctions. This section is now focused on the use of glyoxylic acid chemistry for peptide thioester synthesis.

Peptide thioesters are useful starting materials in a large array of synthetic applications, one of which is the total synthesis of proteins using NCL as already discussed before. Not surprisingly, their synthesis using Fmoc SPPS, the most popular method for peptide synthesis today, has stimulated a large amount of work during the past decade.[265] Thioesters are often unstable in the presence of piperidine or other reagents used for removing Fmoc group during SPPS. Thus, peptide thioesters are frequently prepared by introducing or generating the thioester moiety in the last stage of the synthesis, after the solid phase peptide elongation step. In particular, C-terminal *N,S*-acyl shift systems have attracted a lot of attention recently in this field owing to their capacity to generate C-terminal peptide thioesters by intramolecular acyl migration from nitrogen to sulfur.[253,266] The *Bis*(2-sulfanylethyl)amido (SEA) group is one of the newest *N,S*-acyl shift units (Scheme 40).[258,267] SEA peptides **169a,b** are easily synthesized by Fmoc-SPPS starting from *bis*(2-sulfanylethyl)amino trityl polystyrene resin **168**, which is commercially available.

Scheme 40. *Bis*(2-sulfanylethyl)amido Peptides 169 and Thiazolidine Ligation with Glyoxylic Acid Give Access to Thiazolidine Thioester Peptides 170[12]



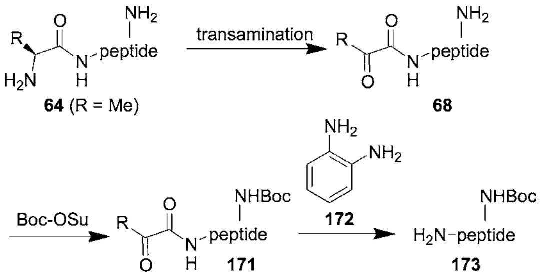
The SEA peptide amide form **169a** equilibrates in aqueous solution with SEA peptide thioester form **169b**.[12,258,268,269] Formation of thioester **169b**, which is detected only below pH 4 by HPLC, is driven by the protonation of the amino group within the thiol handle. Typically, thioester **169b** is the major species below pH ∼ 2−3. However, it is possible to displace the equilibrium between amide and thioester forms of SEA group toward a stable thioester form by chemoselectively blocking the *β*-aminothiol moiety within **169b** through the formation of a thiazolidine ring in the presence of glyoxylic acid.[12] The method permitted the obtention of thiazolidine thioester peptides in good yield and without racemization of the C-terminal residue. Moreover, thiazolidine thioester peptides proved to be significantly more reactive in NCL than typical peptide alkylthioesters, probably due to intramolecular acid or base catalysis by the thiazolidine thiol handle.

F.4. Glyoxylyl Group as a Protective Group in Protein Synthesis by the Thioester Method. The discovery of stepwise solid phase peptide synthesis (SPPS) by B. Merrifield in 1963[270] has led to the widespread use of synthetic peptides in chemistry, biology, medicine, and material sciences. However, the inherent limitations of SPPS in obtaining large (>50 amino acids) homogeneous peptides in good yield has stimulated the development of protein synthetic methods based on the assembly of short peptide segments. The use of deprotected peptide segments and of native peptide ligation methods such as NCL is a solution to this problem. Alternatively, other strategies rely on the coupling of fully or minimally protected peptide segments. In particular, the so-called thioester method which makes use of partially protected C-terminal peptide thioester segments has met with a lot of success.[271] According to this method, the thioester segment is converted to an active ester by silver ion activation in the presence of an additive such as *N*-hydroxysuccinimide, and subsequently reacted with the α-amino group of another peptide segment. All the other amino groups must be temporarily protected, usually by a Boc group, to avoid their participation in the amide bond forming reaction and the formation of side-products. The recent synthesis of the 48-amino-acid residue nonribosomal peptide polytheonamide B illustrates the usefulness of this segment coupling approach.[272]

One elegant application of glyoxylyl acid chemistry to this field is the synthesis of partially protected peptide segments featuring protected ε-amino groups but a free α-amino group starting from polypeptides of biological origin. Such partially protected peptide segments, which can be coupled by the thioester method to partially protected C-terminal peptide thioesters produced by chemical synthesis, give potential access to large polypeptides or proteins.

The method developed by Aimoto's group[11] makes use of N-terminal glyoxylyl or α-ketoacyl peptides **68** (Scheme 41).

Scheme 41. N-Terminal Glyoxylyl or α-Ketoacyl Moieties Can Serve As Temporarily Protecting Groups of α-Amino Group of Peptide Segments[11]



These peptide derivatives can be produced from free polypeptides **64** by transamination as discussed earlier in this review (see Scheme 13), for example, by using the copper(II) or nickel(II)-glyoxylic acid transamination method developed by Dixon and co-workers.[5,6,123] The glyoxylyl or α-ketoacyl group serves as temporarily protecting group of the peptide α-amino group during the protection of lysine side-chains. Then, treatment of peptide **171** by *o*-phenylenediamine[83] **172** allows removal of the N-terminal glyoxylyl group, thereby unmasking the α-amino group for subsequent peptide segment coupling. In practice, the method was illustrated using an N-terminal alanine residue (Scheme 41, R = Me).

G. CONCLUSION

The unique features of glyoxylic acid chemistry have stimulated a large number of synthetic developments in solution or on solid phase, efficient and useful chemoselective ligations, and a vast array of applications in different fields of research. Several glyoxylyl group forming reactions and glyoxylyl group-based chemoselective ligations, which are now major items in the peptide chemist's and biochemist's toolbox, enable the synthesis of highly sophisticated scaffolds or the assembly of complex systems when combined with other ligation reactions or synthetic tools. This chemistry will certainly continue to inspire novel developments in connection with important fields of research such as the design of nanosystems or total protein synthesis.

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Notes

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