Elusive Dehydroalanine Derivatives with Enhanced Reactivity

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For the first time, a simple methodology for the chemical synthesis and use of highly reactive 4-methylenoxazol-5(4H)-ones from serine is presented. These dehydroalanine derivatives, which resemble the natural 4-methylidenimidazole-5-one (MIO) cofactor present in lyases and aminomutases, undergo rapid reaction with carbon nucleophiles such as silyl enol ethers, as well as cycloaddition reactions with diazo compounds and reactive dienes, under very mild conditions and without any need for metal catalysts or ring-strain activation, offering potential for bioconjugation.

Chemical modification of proteins is a very active field of research in current chemical biology.[1] Such post-translational modification (PTM) of proteins requires site-selective reactions with high chemoselectivity.[2] In this context, α,β-unsaturated amino acids are of special interest, because they constitute a modular platform for site-selective PTM,[3] mainly through 1,4-conjugate addition of thiols, such as those found in cysteine side chains.[4] However, controlling the stereoselectivity in reactions involving α,β-dehydroamino acids and peptides still represents a challenge for chemists. In addressing this topic, we have reported the synthesis of chiral dehydroalanine (Dha) and dehydrobutyryl (Dhb) building blocks and their application in the asymmetric synthesis of lanthionine and β-methylthionine derivatives.[5] Our first-generation chiral Dha scaffold was a versatile Michael acceptor towards nucleophilic thiols such as protected 1-thiocarbohydrates.[6]

More recently, we developed an improved version of these chiral Dha/Dhb derivatives through lactonisation of the first-generation scaffolds, yielding chiral bicyclic structures with reduced conformational flexibility and superior reactivity and dia stereoi nducing properties with thiols as nucleophiles.[7] This methodology allowed the synthesis of cell-penetrating peptides containing fluorescent δ-cysteine components.[8] Unfortunately, such Dha/Dhb scaffolds were unsuitable for introducing any other nucleophiles besides thiols, due to their limited reactivity.

On the other hand, naturally occurring Dha and Dhb have been functionalised through S-, N- and C-Michael addition, but natural reactions involving O-nucleophiles have not been discovered yet.[9] Hence, the incorporation into peptides and proteins of a highly reactive α,β-dehydroamino acid derivative capable of undergoing conjugate addition with weak nucleophiles such as carbohydrates, which would directly lead to O-glycopeptides and O-glycoproteins through site-specific chemical PTM, is still a major challenge in chemical biology.

In our continuous search for α,β-dehydroamino acid scaffolds with improved reactivity, the natural 4-methylidenimidazole-5-one (MIO) protein cofactor drew our attention.[10] The MIO motif is generated as a PTM from the Ala-Ser-Gly triad in ammonia lyases and aminomutases (Figure 1A, B) and it is responsible for their activity towards amino acids. The amino groups of aromatic α-amino acids are N-alkylated through aza-Michael addition to the MIO structure, and this promotes β-elimination to give cinnamic acid derivatives (in lyases) and subsequent isomerisation to β-amino acids (in aminomutases). The structures of the chromophores of the green fluorescent protein (GFP) and its relatives are very closely related to that of the MIO motif,[11] with the central serine residue in the triad being replaced by an aromatic residue, such as tyrosine in the case of GFP, leading to stable 4-arylenimidazol-5-ones.

To the best of our knowledge, discrete 4-methylidenimidazole-5-ones have not been prepared or isolated outside the protein context of the MIO cofactor, probably because of their very high reactivity towards nucleophiles, relative to other α,β-dehydroamino acid derivatives. In an attempt to synthesise the MIO scaffold chemically under physiological conditions, we synthesised Ac-Ala-Ser-Gly-NH₂, the minimal natural sequence leading to cyclisation in lyases and aminomutases (Figure 1C). Solid-phase peptide synthesis (SPPS) was used to obtain the linear tripeptide, with the C and N termini capped as amides, in good yield (see the Supporting Information). The desired spontaneous cyclisation of this triad, through intramolecular aminal formation followed by two consecutive dehydrations, however, could not be observed either by 1H NMR spectroscopy or by MS spectrometry after prolonged heating at 50 °C in pH 8.0 PBS buffer.

With the aim of facilitating cyclisation by bringing the reacting fragments closer together, the stapled peptides Asp-Ser-Gly-Cys-NH₂ and Ac-Cys-Lys-Ser-Gly-Cys-NH₂ (Fig-
were likewise synthesised by performing oxidative cleavage from the resin to form disulfide bonds between the two C- and N-terminal cysteine residues (see the Supporting Information). The native Ala residue was mutated to Asp and Lys to solubilise the resulting peptides in water. These peptides were also heated at 50°C in pH 8.0 PBS buffer for many days, but no significant changes were detectable by 1H NMR or MS analyses. These experiments demonstrated that MIO scaffolds are very difficult to obtain in vitro in the absence of the protein scaffold of the corresponding enzyme, which appears to promote cyclisation by imposing severe confinement constraints.\[12\]

Changing the lactam nitrogen atom of the MIO scaffold to an oxygen atom—thus forming a lactone—would be expected to preserve the high reactivity of the native analogue. Along these lines, 2-methyl-4-methyleneoxazol-5(4H)-one has been proposed as a key Michael acceptor intermediate in the biomimetic synthesis of N-acetyl-4-bromotryptophan en route to clavicipitic acids.\[13\] In further studies, such a highly reactive type-2 alkene was found to be formed in situ from serine and acetic anhydride and could be detected in solution by NMR spectroscopy.\[14\] However, and unlike 4-ethyliden- and especially 4-benzylidenoxazol-5(4H)-ones, which have been profusely synthesised and used in organic synthesis,\[15\] the 4-methylene analogues have remained very elusive to both synthesis and application, due to their very low stability.

The possibility of generating transient 4-methyleneoxazol-5(4H)-ones as highly reactive Dha scaffolds for bioconjugation encouraged us to attempt their chemical synthesis. To this end, we first attempted the formation of the oxazol-5(4H)-one ring from O-protected N-acyl serine derivatives by using carbodiimides as coupling reagents,\[16\] followed by base-promoted B-elimination. Thus, racemic N,O-dibenzoylserine (1) was readily obtained after treatment of dl-serine with excess BzCl under basic aqueous conditions (unoptimised conditions, Supporting Information). After chromatographic purification, 5(4H)-oxazolone ring formation by treatment with N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDCI) in dichloromethane at 0°C, followed by an aqueous workup, was attempted. Although the starting material was completely consumed, no identifiable product could be obtained. The 1H NMR spectrum of the obtained material showed very broad signals probably corresponding to a polymeric material.

The same results were obtained with N,N'-dicyclohexylcarbodiimide (DCC) and N,N'-diisopropylcarbodiimide (DIC) as carboxylic acid activators. Careful monitoring of the reaction between 1 and DIC in CDCl3 by 1H NMR (Figure 2) showed the fast disappearance of the starting material signals and the appearance of two narrow doublets in the 6.00–6.20 ppm region associated with methylene protons,\[14\] reaching its maximum level of conversion of 90% in 15 min. Thus, it was clear that 4-methylene-2-phenyloxazol-5(4H)-one (MPO, 2) is formed imme-

Figure 1. The Ala-Ser-Gly triad in A) its open form, and B) its cyclic MIO form, in histidine ammonia-lyase (HAL) from Pseudomonas putida (crystalllographic structures; PDB IDs 1GK2 and 1EB4, respectively. C) Minimal, and D) extended stapled peptides used to attempt the chemical synthesis of MIO scaffolds; the accepted mechanism for MIO formation, involving intramolecular cyclisation and a double dehydration, is shown for the minimal assayed peptide (C).
Immediately through DIC-promoted cyclisation and subsequent β-elimination of benzoic acid, although this compound is too reactive to be isolated through conventional workup. As expected, the lifetime of 2 depends on the solvent used in the reaction and the concentration of the sample. In nonpolar solvents such as chloroform, compound 2 can be preserved in solution for at least 3 h at concentrations up to 190 mM at 25 °C (Figure S1 in the Supporting Information). Conversely, in acetonitrile 2 disappears completely after 15 min at concentrations around 190 mM, although it can be preserved in solution for longer times at lower concentrations (Figure S2). Likewise, 2 is highly unstable in a 1:2 mixture of acetonitrile and water, even at low concentrations (27 mM, Figure S3), which raises concerns about its potential use for bioconjugation in physiological media.

The influence of the serine protecting groups on the reaction outcome was then tested. With N-benzoyl-O-benzyl-dl-serine, the fast formation of the oxazolone ring was also observed upon treatment with DIC. However, notably slower β-elimination of benzyl alcohol was observed, with mixtures of the target compound 2 and its cyclic precursor in variable ratios being produced, because 2 decomposes over time. N-Acetyl-O-benzyl-dl-serine was tested with analogous results. Thus, N,O-dibenzoyl-dl-serine (1) was selected as the most convenient starting material for the rest of the study.

Generation of 2 in situ from 1 in the presence of equimolecular amounts of DIC with subsequent addition of sulfur, nitrogen and oxygen nucleophiles led to fast alkene decomposition. In no case was the desired conjugate addition reaction observed. With basic nucleophiles such as primary and secondary amines or with thiolates or alkoxides generated in situ in the presence of bases such as N,N-diisopropylethylamine or sodium hydride, 2 was completely degraded, probably through anionic polymerisation pathways. On the other hand, protonated thiols and alcohols were not reactive enough to undergo conjugate addition during the lifetime of 2 at various concentrations.

We then moved to testing different reactions for which basic conditions are not required (Scheme 1). Mukaiyama–Michael conjugate addition with silyl enol ethers typically requires a Lewis acid in order to take place. However, after generation of 2 in situ, (1-methoxy-2-methylprop-1-en-1-yl)oxy(trimethyl)silane (MTDA) was added in the absence of any catalyst. To our delight, the desired reaction was complete in about 3 min, leading to adduct 3 in moderate yields after chromatographic purification. For comparison, the corresponding reaction with acyclic methyl 2-acetamidoacrylate (MAA) takes 17 h in the presence of methylaluminoxane as a Lewis acid, affording similar reaction yields.

1,3-Dipolar cycloaddition reactions were then evaluated. Addition of diazomethane to freshly generated 2 directly yielded the spirocyclic cyclopropane 4 in 10 min. The common pyrazo-
line intermediate, ring contraction of which through N₂ extrusion normally requires thermal or photochemical activation, such as with analogous (2/E)-4-benzylidene-2-phenyloxazol-5(4H)-ones, was not observed. Compound 4 could be fully characterised by X-ray diffraction of monocryals (Figure S6).

Treatment with ethyl diazoacetate (EDA) produced similar results, leading to a mixture of racemic cyclopropanes 5a and 5b in close to 1:1 ratio. Compound 5b could also be characterised by X-ray diffraction analysis (Figure S7). MPO (2) clearly showed greater reactivity than related acyclic dehydroamino acid analogues such as methyl 2-acetamidoacrylate towards cyclopropanation with diazo compounds (Figures S4 and S5), which normally requires transition-metal catalysts such as rhodium or palladium to generate reactive metal carbene species. On the other hand, uncatalysed 1,3-dipolar cycloadditions with ethyl diazoacetate have been reported only with highly activated substrates bearing nitro (α-carboxethoxy-1-nitrostyrenes and α-halo-α-nitroalkenes) and nitrile groups (arylidene malononitrile and arylidene ethyl cyanoacetate), although at very slow reaction rates (2–5 days needed). Conversely, compound 2 (generated in situ) completely reacts with diazo compounds in a few minutes. The second-order rate constant for the reaction between 2 and EDA in CDCl₃ at 25 °C was determined by ¹H NMR spectroscopy to be $k_2 = 3.9 \times 10^{-3} \text{ M}^{-1} \text{s}^{-1}$ (Figure S4), which is comparable to the rate constants found for 1,3-dipolar cycloadditions of strain-promoted alkynes. Recently, Raines and co-workers have described selective reactions between diazoacetamides and dehydroalanine residues under biocompatible conditions, as well as the manipulation of stereoelectronic effects of diazo compounds to increase their reactivity and selectivity for bioorthogonal applications.

Finally, uncatalysed Diels–Alder cycloadditions with MPO (2) were tested under the same conditions with various dienes such as cyclohexa-1,3-diene, cyclopentadiene, 2,3-dimethoxybuta-1,3-diene, 2,3-dimethylbuta-1,3-diene and 3,6-bispyridino-2-y1,2,4,5-tetratetrazen, the last of which is commonly used for protein labelling through metal-free strain-promoted inverse-electronic-demand Diels–Alder cycloaddition. Notably, cyclopentadiene was reactive enough to react cleanly with freshly generated 2 to afford racemates endo-6 and exo-6 in a 55:45 ratio. The structure of adduct exo-6 was confirmed by X-ray diffraction analysis (Figure S8). Again, this uncatalysed and non-strain-promoted reaction with 2 proceeds much more rapidly at room temperature than that with its acyclic analogue MAA, which requires prolonged heating at around 100 °C for 5 h or the presence of a metal catalyst such as TiCl₄.

The superior reactivity (i.e., electrophilicity) of cyclic MPO (2) relative to acyclic analogues such as methyl 2-acetamidoacrylate can be explained in terms of the large stabilisation of the LUMO in the former case, by 1.2 eV, due to extensive conjugation over the five-membered lactone and phenyl rings. This translates into significantly lower activation free energies ($\Delta G^*$) from 2 than in the case of MAA, such as those calculated quantum mechanically for cycloaddition with diazomethane, methyl diazoacetate and cyclopentadiene (Figures 3 and S9–S12). With regard to 1,3-dipolar cycloaddition with diazo compounds, a stepwise zwitterion-mediated cyclopropanation mechanism with spontaneous nitrogen release has recently been proposed as an alternative pathway to the commonly accepted asynchronous concerted cycloaddition and subsequent nitrogen extrusion from the pyrazoline intermediates. In fact, such a stepwise mechanism was calculated to be significantly favoured for compound 2, probably due to its ability to delocalise the negative charge generated upon the Michael-type addition of diazo compounds. In view of the large activation energy required for the cleavage of the pyrazoline intermediates (transition structures and intermediates could be calculated only in the triplet excited state), relative to the retro-cycloaddition reaction from the same intermediate, and of the fact that the energy barrier for the stepwise zwitterionic cyclopropanation reaction between compound 2 and methyl diazoacetate is only $\approx 2 \text{kcalmol}^{-1}$ above the concerted transition state (Figure S10), such a process is likely to take place to some extent under the assayed experimental conditions.

In summary, we have developed a simple methodology for the synthesis of highly elusive 4-methylenoxazol-5(4H)-ones with aryl or alkyl groups at the 2-position, depending on the
amine protection of the starting serine derivative. Such cyclic dehydroalanine derivatives are highly electrophilic and can quickly react in situ with silyl enol ethers, diazo compounds and dienes under very mild conditions at room temperature, and in the absence of metal catalysts without the need for ring strain activation. In view of the growing interest in diazo groups as new chemical reporters for bioorthogonal labelling of biomolecules\(^2\) and versatile tools for chemical biology\(^2\) we believe that our methodology offers potential for bioconjugation and post-translational modification of proteins under controlled physiological conditions, although the low stability of MPO derivatives in water currently limits their biological scope. This possibility, together with the scope to extend our methodology to serine residues within a peptide context to chemically install MIO-type modifications, are currently being evaluated in our laboratory.

**Experimental Section**

General procedure for sequential one-pot synthesis of 2 and subsequent reaction with different reagents: N,O-Dibenzoylserine (1, 0.3 mmol) was introduced into a round-bottomed flask, and CHCl\(_3\) (10 mL) was added. The heterogeneous mixture was stirred at room temperature, and DIC (0.3 mmol) was then added. The reaction mixture dissolved immediately. The reagent of interest (0.3 mmol) was then added, and the mixture was stirred for 3–30 min at room temperature. After consumption of the starting material, the reaction mixture was transferred to an extraction funnel, and the organic layer was washed with saturated NaHCO\(_3\) solution (2×5 mL). The organic layers were combined and dried over anhydrous Na\(_2\)SO\(_4\), and the solvent was evaporated. The crude reaction product was purified by vacuum liquid chromatography (VLC, hexane/AcOEt 100:1 to 80:20 gradient).

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**Conflict of Interest**

*The authors declare no conflict of interest.*

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