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Accepted Article

Title: Elusive Dehydroalanine Derivatives with Enhanced Reactivity

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To be cited as: *ChemBioChem* 10.1002/cbic.201800758

Link to VoR: http://dx.doi.org/10.1002/cbic.201800758

A Journal of ChemPubSoc Europe

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Elusive Dehydroalanine Derivatives with Enhanced Reactivity

Carlos Aydillo+,[a,b,c] Nuria Mazo+,[a] Claudio D. Navo+,[a] and Gonzalo Jiménez-Osés*[a,d,e]

Abstract: For the first time, a simple methodology for the chemical synthesis and utilization of highly reactive 4-methylen-oxazol-5(4*H*)-ones from serine is presented. These dehydroalanine derivatives, which resemble the natural 4-methylideneimidazole-5-one (MIO) cofactor present in lyases and aminomutases, undergo rapid reaction with carbon nucleophiles such as silyl enol ethers, and cycloaddition reactions with diazo compounds and reactive dienes under very mild conditions and in the absence of metal catalysts and 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] Along these lines, α,β-unsaturated amino acids are of special interest, since they constitute a modular platform for site-selective PTM,[3] mainly through 1,4-conjugate addition of thiols such as those found in cysteine sidechains.[4] However, controlling the stereoselectivity in reactions involving α,β-dehydroamino acids and peptides still represents a challenge for chemists. In this context, we have reported the synthesis of chiral dehydroalanine (Dha) and dehydrobutyirine (Dhb) building blocks and their application in the asymmetric synthesis of lanthionine and β-methyllanthionine 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 through lactonization of the first-generation scaffolds, yielding chiral bicyclic structures with reduced conformational flexibility and superior reactivity and diastereoinducing properties with thiols as nucleophiles.[7] This methodology allowed synthesizing cell-penetrating peptides containing fluorescent D-cysteines.[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 functionalized through *S*-, *N*- and *C*-Michael reactions, but natural reactions involving *O*-nucleophiles have not been discovered yet.[9] Hence, the incorporation in peptides and proteins of a highly reactive α,β-dehydroamino acid derivative able to undergo conjugate additions 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-methyliden-imidazole-5-one (MIO) protein cofactor drew our attention.[10] MIO 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 an aza-Michael addition reaction with MIO, which promotes β-elimination to give cinnamic acid derivatives (in lyases) and subsequent isomerization to β-amino acids (in aminomutases). The structures of the chromophores of the green fluorescent and related proteins (GFP) are very related to that of MIO,[11] were the central serine of the triad is replaced by an aromatic residue such as tyrosine in GFP, leading to stable 4-arylidene-imidazole-5-ones.

To the best of our knowledge, discrete 4-methylidene-imidazole-5-ones have not been prepared or isolated outside the protein context of the MIO cofactor, likely due to their very high reactivity towards nucleophiles compared to other α,β-dehydroamino acid derivatives. In an attempt to chemically synthetize the MIO scaffold under physiological conditions, we synthesised the minimal natural sequence leading to cyclization in lyases and aminomutases, namely Ac-Ala-Ser-Gly-NH_2 (**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 Supporting Information). The desired spontaneous cyclization of this triad through intramolecular aminal formation followed by two consecutive dehydrations, could not be observed either by ^{1}H NMR spectroscopy or MS spectrometry after prolonged heating at 50 ºC in pH 8.0 PBS buffer.

Aiming to facilitate cyclization by bringing the reacting fragments closer, the stapled peptides Ac-Cys-Asp-Ser-Gly-Cys-NH_2 and Ac-Cys-Lys-Ser-Gly-Cys-NH_2 (**Figure 1d**) were likewise synthesised by performing an oxidative cleavage from the resin to form disulfide bonds between the two C- and N-terminal cysteines (see Supporting Information). The native Ala residue was mutated to Asp and Lys to solubilize the resulting peptides in water. These peptides were also heated at 50 ºC in pH 8.0 PBS buffer for many days, but no significant changes were detected by ^{1}H NMR or MS analyses. These experiments proved 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 cyclization by imposing severe confinement constraints.[12]

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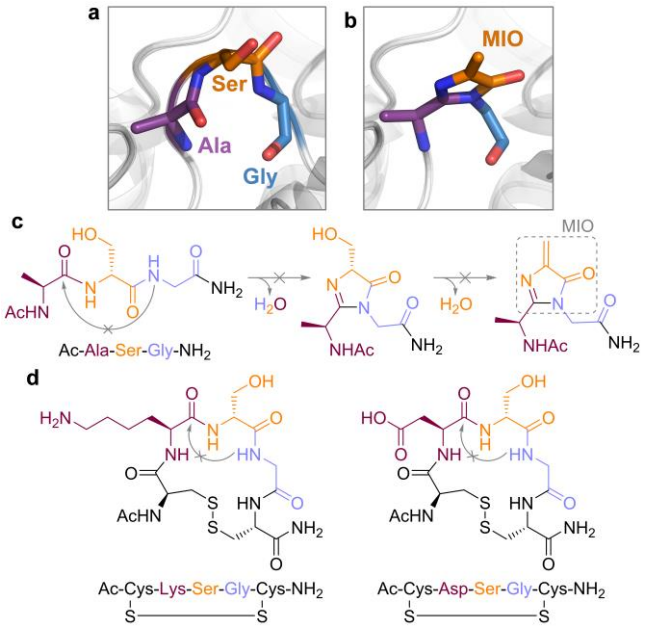


Figure 1. The Ala-Ser-Gly triad in its open (**a**) and cyclic MIO form (**b**), in histidine ammonia-lyase (HAL) from *Pseudomonas putida* (crystallographic structures; PDB codes 1GK2 and 1EB4, respectively. Minimal (**c**) and extended stapled peptides (**d**) used to attempt the chemical synthesis of MIO scaffolds; the accepted mechanism for MIO formation involving intramolecular cyclization and a double dehydration is shown for the minimal assayed peptide (**c**).

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

The possibility to generate transient 4-methylen-oxazol-5(*4H*)-ones as highly reactive Dha scaffolds for bioconjugation, encouraged us to attempt their chemical synthesis. To this aim, we first attempted the formation of the oxazol-5(*4H*)-one ring using carbodiimides as coupling reagents from *O*-protected *N*-acyl serine derivatives,[16] followed by base-promoted β-elimination. Thus, racemic *N*,*O*-dibenzoyl serine (**1**) was readily obtained after treatment of DL-serine with excess BzCl under basic aqueous conditions (unoptimized conditions, see Supporting Information). After chromatographic purification, 5(*4H*)-oxazolone ring formation was assayed using *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide (EDCI) in dichloromethane at 0 ºC, followed by an aqueous workup. Although the starting material was completely consumed, no identifiable product could be obtained. The ^{1}H NMR spectrum of the obtained material showed very broad signals likely 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 $CDCl_3$ by ^{1}H NMR (**Figure 1**) showed the fast disappearance of the starting material signals, and the rise of two narrow doublets in the 6.00–6.20 ppm region associated to methylene protons,[14] reaching a maximum conversion of 90% in 15 min. Thus, it was clear that 4-methylen-2-phenyloxazol-5(*4H*)-one **2** (abbreviated as MPO) forms immediately via DIC-promoted cyclization and subsequent β-elimination of benzoic acid, although this compound is too reactive to be isolated by a conventional workup. As expected, the lifetime of **2** depends on the solvent used in the reaction and the concentration of the sample. In non-polar solvents such as chloroform, compound **2** can be preserved in solution for at least 3 h at concentrations up to 190 mM at 25 ºC (**Figure S1**). Conversely, **2** disappears completely in acetonitrile after 15 minutes 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.

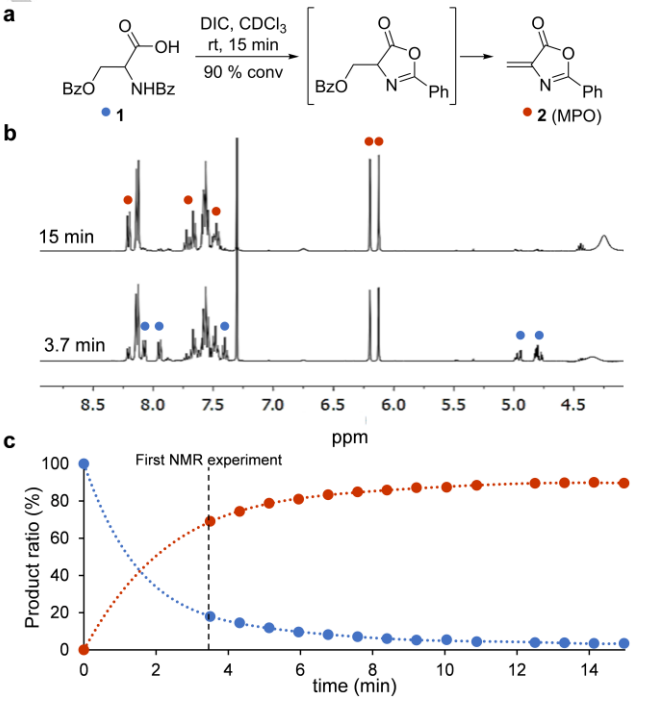


Figure 1. (**a**) Synthesis of 4-methylen-2-phenyloxazol-5(*4H*)-one (MPO, **2**) from a conveniently protected serine (**1**). (**b**) ^{1}H NMR spectra in $CDCl_3$ (27 mM for **1**) showing the formation of compound **2** from starting material **1** at 3.7 and 15 min after the addition of DIC. (**c**) Consumption of starting material **1** (in blue) and formation of compound **2** (in red) monitored by ^{1}H NMR in $CDCl_3$ (27 mM for **1**). The initial time (t=0) represents the moment when DIC was added. rt: room temperature.

The influence of the protecting groups of serine 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, a notably slower β-elimination of benzyl alcohol was observed, producing mixtures of the target compound **2** and its cyclic precursor in variable ratios, since **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 more convenient starting material for the rest of the study.

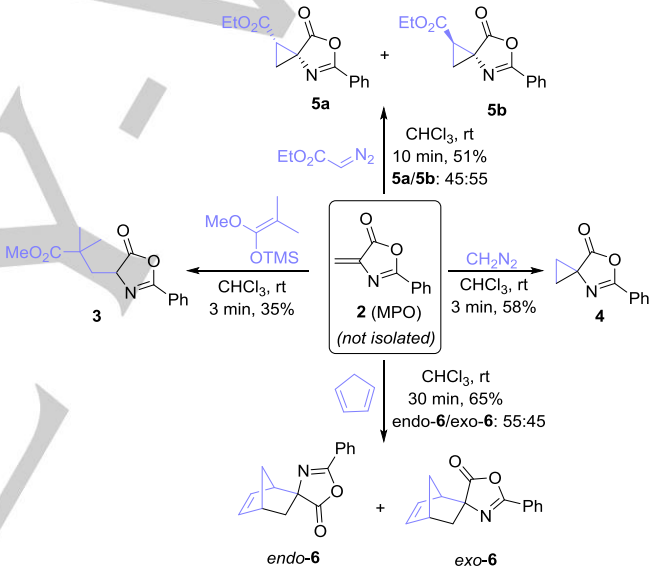
In situ generation of **2** from **1** in the presence of equimolecular amounts of DIC, and subsequent addition of sulfur, nitrogen and oxygen nucleophiles led to fast alkene decomposition. In all cases, the desired conjugate addition reaction was not observed. With basic nucleophiles such as primary and secondary amines, and thiolates and alkoxides generated *in situ* in the presence of bases such as *N*,*N*-diisopropylethylamine or sodium hydride, **2** was completely degraded, likely through anionic polymerization pathways. On the other hand, protonated thiols and alcohols were not reactive enough to undergo conjugate addition during **2** lifetime at various concentrations.

We then moved to test different reactions for which basic conditions are not required (**Scheme 1**). Mukaiyama-Michael conjugate addition with silyl enol ethers typically requires a Lewis acid to take place.[17] However, after generating **2** *in situ*, methyl trimethylsilyl dimethylketene acetal (MTDA) was added in the absence of any catalyst. To our delight, the desired reaction was completed in about 3 min leading to adduct **3** in moderate yields after chromatographic purification. For comparison, the same reaction with acyclic methyl 2-acetamidoacrylate (MAA) takes 17 h in the presence of methyl aluminoxane as a Lewis acid to achieve similar reaction yields.[18]

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 pyrazoline intermediate whose ring-contraction via N_2 extrusion normally requires thermal or photochemical activation, such as with analogous (*Z*/*E*)-4-benzyliden-2-phenyloxazol-5(*4H*)-ones,[19] was not observed. Compound **4** could be fully characterized by X-ray diffraction of monocrystals (**Figure S6**).

The reaction with ethyl diazoacetate (EDA) produced similar results, leading to a mixture of racemic cyclopropanes **5a** and **5b** in a nearly 1:1 ratio. Compound **5b** could also be characterized by X-ray diffraction analysis (**Figure S7**). MPO **2** clearly showed a greater reactivity than related acyclic dehydroamino acid analogues such as methyl 2-acetamidoacrylate towards cyclopropanation with diazo compounds (**Figures S4** and **S5**), which normally require transition metal catalysts such as rhodium or palladium to generate reactive metal carbene species.[20] On the other hand, uncatalyzed 1,3-dipolar cycloadditions with ethyl diazoacetate have been reported only with highly activated substrates bearing nitro (α-carbethoxy-1-nitrostyrenes and α-halo-α-nitroalkenes)[21] and nitrile groups (arylidene–malononitrile and arylidene–ethyl cyanoacetate),[22] although at very slow reaction rates (2-5 days needed). Conversely, *in situ* generated compound **2** completely reacts with diazo compounds in a few minutes. The second-order rate constant for the reaction between **2** and EDA in $CDCl_3$ at 25 ºC was determined to be k_2 = $3.9 \cdot 10^{-3}$ M^{-1} s^{-1} by ^1H NMR spectroscopy (**Figure S4**), which is comparable to those found for the 1,3-dipolar cycloadditions of strain-promoted alkynes.[23] Recently, Raines and co-workers have described the selective reaction of diazoacetamides with dehydroalanine residues in biocompatible conditions,[23] as well as the manipulation of steroelectronic effects of diazocompounds to increase their reactivity and selectivity for bioorthogonal applications.[24]

Finally, uncatalyzed Diels-Alder cycloadditions with MPO **2** were tested under the same conditions using various dienes such as cyclohexadiene, cyclopentadiene, 2,3-dimethoxy-1,3-butadiene, 2,3-dimethyl-1,3-butadiene and 3,6-di-2-pyridyl-1,2,4,5-tetrazine –the latter being commonly used for protein labelling via metal-free strain-promoted inverse electronic demand Diels-Alder reactions. Of note, cyclopentadiene was reactive enough to cleanly react 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 uncatalyzed and non-strain-promoted reaction with **2** proceeds much faster at room temperature than with its acyclic analogue MAA, which requires prolonged heating at around 100 ºC for 5 h or the presence of a metal catalyst such as $TiCl_4$.[25]



Scheme 1. Fast reactions of MPO with different reagents under the same mild conditions and without an extra activation.

The superior reactivity (i.e. electrophilicity) of cyclic MPO **2** compared to acyclic analogues such as methyl 2-acetamidoacrylate can be rationalized by the large stabilization of the LUMO of the former by 1.2 eV due to extensive conjugation along the five-membered lactone and phenyl rings. This translates into quite lower activation free energies ($\Delta G^{\ddagger}$) from **2** with respect to those from MAA, such as those calculated quantum mechanically for the cycloaddition reactions with diazomethane, methyl diazoacetate and cyclopentadiene (**Figures 3 and S9-S12**). Regarding 1,3-dipolar cycloaddition reactions with diazo compounds, a stepwise zwitterion-mediated cyclopropanation mechanism with spontaneous nitrogen release has been recently proposed[26] as an altenative pathway to the commonly accepted asynchronous concerted cycloaddition followed by nitrogen extrusion from the pyrazoline intermediates. In fact, such stepwise mechanism was calculated to be

significantly favored for compound **2**, likely due to its ability to delocalize the negative charge generated upon the Michael-type addition of diazo compounds. Considering 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) compared to the retro-cycloaddition reaction from the same intermediate, and given that the energy barrier for the stepwise zwitterionic cyclopropanation reaction between compound **2** and methyl diazoacetate is only ~2 kcal mol^{-1} above the concerted transition state (**Figure S10**), such process is likely to take place to some extent under the assayed experimental conditions.

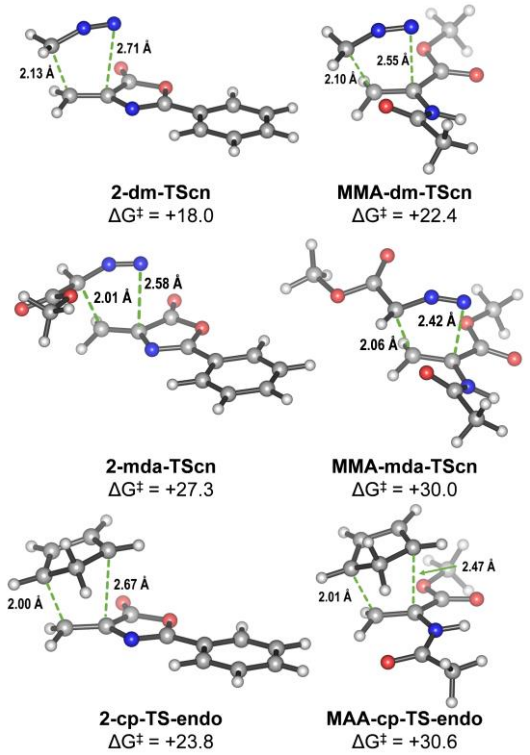


Figure 3. Lowest energy transition structures calculated with PCM($CHCl_3$)/M06-2X/6-31+g(d,p) for the concerted cycloaddition reactions between cyclic (**2**, left) and acyclic (MAA, right) dehydroalanine derivatives with diazomethane (top), methyl diazoacetate (middle) and cyclopentadiene (bottom). Activation free energies (ΔG‡) are in kcal mol^{-1}. The stepwise zwitterionic 1,3-dipolar reaction mechanisms are described in **Figures S9-S12**.

In summary, we have developed a simple methodology for the synthesis of highly elusive 4-methylen-oxazol-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 be quickly reacted *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 of ring strain activation. Given the growing interest of diazo groups as new chemical reporters for bioorthogonal labelling of biomolecules[27] and versatile tools for chemical biology[28] we believe our methodology poses 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 options 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) is introduced in a round bottomed flask and 10 mL of $CHCl_3$ is added. The heterogenous mixture is stirred at room temperature and *N*,*N*'-diisopropylcarbodiimide (DIC, 0.3 mmol) is then added. The reaction mixture dissolves immediately. Then, the reagent of interest (0.3 mmol) is added and the mixture stirred for 3-30 min at room temperature. After consumption of the starting material, the reaction mixture is transferred to an extraction funnel and the organic layer is washed with 2 x 5 mL of saturated $NaHCO_3$ solution. Organic layers are combined, dried over anhydrous Na_2SO_4 and the solvent evaporated. The reaction crude is purified by VLC (hexane/AcOEt 100:0 to 80:20 gradient).

Acknowledgements

We thank funding from MINECO (projects CTQ2015-70524-R and RYC-2013-14706 to G.J.O. and C.D.N.) and Universidad de La Rioja (fellowship to N.M.).

Keywords: dehydroalanine • 4-methylen-oxazol-5(*4H*)-ones • 4-methylideneimidazole-5-one • cycloadditions • diazo compounds

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Layout 2:

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Elusive Dehydroalanine Derivatives with Enhanced Reactivity

For the first time, a highly reactive and elusive dehydroalanine scaffold has been prepared, and quickly reacted i*n situ* with a variety of reagents without any type of extra activation, paving the way for bioconjugation applications.