Cyclic Peptides

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N^{γ}-Hydroxyasparagine: A Multifunctional Unnatural Amino Acid That is a Good P1 Substrate of Asparaginyl Peptide Ligases

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Abstract: *Peptidyl asparaginyl ligases (PALs) are powerful tools for peptide macrocyclization. Herein, we report that a derivative of Asn, namely N^{γ}-hydroxyasparagine or Asn(OH), is an unnatural P1 substrate of PALs. By Asn(OH)-mediated cyclization, we prepared cyclic peptides as new matrix metalloproteinase 2 (MMP2) inhibitors displaying the hydroxamic acid moiety of Asn(OH) as the key pharmacophore. The most potent cyclic peptide ($K_i = 2.8 \pm 0.5$ nM) was built on the hyperstable tetracyclic scaffold of rhesus theta defensin-1. The Asn(OH) residue in the cyclized peptides can also be readily oxidized to Asp. By this approach, we synthesized several bioactive Asp-containing cyclic peptides (MCoTI-II, kB2, SFTI, and integrin-targeting RGD peptides) that are otherwise difficult targets for PAL-catalyzed cyclization owing to unfavorable kinetics of the P1-Asp substrates. This study demonstrates that substrate engineering is a useful strategy to expand the application of PAL ligation in the synthesis of therapeutic cyclic peptides.*

Macrocyclization is an effective strategy to restrict the conformations of linear peptides.[1] The large surface areas of cyclic peptides confer high binding affinity and selectivity to inhibit protein–protein interactions that are often not easily druggable using small-molecule compounds.[2] Responsible for cyclotide synthesis in plants, asparaginyl endopeptidases (AEPs) are a useful tool to cyclize synthetic peptides.[3] AEPs are cysteine endopeptidases that typically recognize and cleave peptide bonds after an Asn or Asp residue.[4] Some members of the AEP family catalyze transpeptidation at the asparaginyl peptide bonds with little hydrolase activity, which qualifies them as peptidyl asparaginyl ligases (PALs).[3b,5] Most PALs have a strong preference for P1-Asn over P1-Asp substrates. In fact, the rate of cyclization reactions for these two types of substrates can differ by 100 to 800 folds.[3b,6] Therefore, most applications of PAL-mediated cyclization reported to date have been limited to P1-Asn substrates.[5,7] Applications of PALs will be expanded if one can broaden their substrate scope.

The optimal pH for the hydrolysis of Asp/Asn–peptide bonds by AEPs is around 4.5–5.5.[4,5b,c] Structural characterization of AEP–aspartyl peptide inhibitor complexes shows that the hydroxy group of side chain $^{\gamma}$COOH of P1-Asp acts as a hydrogen bond donor to bind to a key residue in the S1 pocket of the enzyme.[8] Given the low p*K*a value of the carboxyl group, an acidic pH is required to maintain the neutral, protonated form needed for enzyme binding (Figure 1). However, at acidic pH, the N-terminal amine of an acyl-acceptor substrate is mostly protonated, making it largely unavailable in a ligation reaction. This dilemma is likely the main reason that limits the rate of PAL-catalyzed ligation at aspartyl bonds.[7h] In this study, we used the asparagine analog N^{γ}-hydroxyasparagine or Asn(OH) as the P1 amino acid of PAL substrates (Figure 1). Because the N^{γ}-hydroxy group has a higher pKa than COOH, it can remain protonated as a hydrogen bond donor at near neutral pH which favors the ligation reaction. We show that P1-Asn(OH) peptides are well recognized by butelase-1 and VyPAL2 in the cyclization reaction (Table 1, Figure S2). This unnatural Asn-

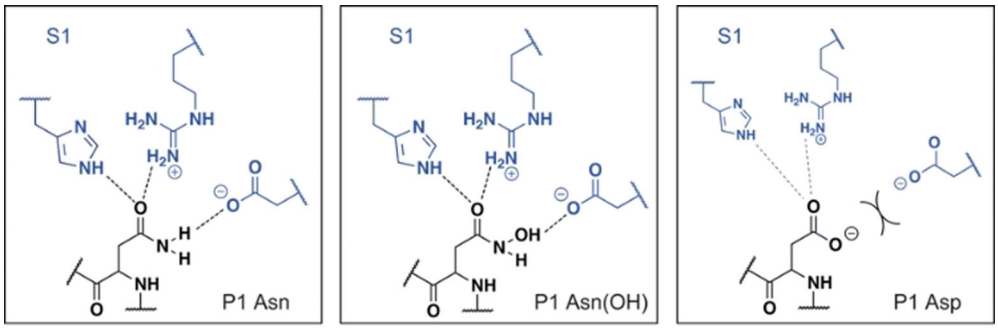

Figure 1. Modeled H-bond interactions between the S1 pocket of AEPs and the P1 amino acid of the substrates at neutral pH. Residues in the S1 pocket and P1 amino acids of the substrates are shown in blue and black, respectively.

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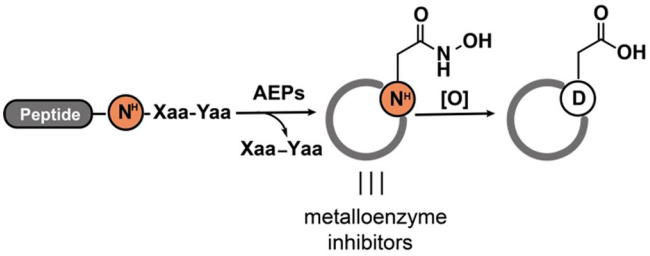
Table 1: Kinetics of peptide cyclization by butelase-1 and VyPAL2

Sequence	k_{cat} [s^{-1}]	K_m [μM]	k_{cat}/K_m [M^{-1}/s^{-1}]
AIYRRGRLYRR**N**HV **1**[a]	14.86 ± 0.34	67.5 ± 2.8	220148 ± 10429
AIYRRGRLYRR**N(OH)**HV **2**[a]	3.46 ± 0.27	98.8 ± 17.8	35020 ± 6876
AIYRRGRLYRR**D**HV **3**[a]	0.454 ± 0.02	629.7 ± 19.5	721 ± 39
AIYRRGRLYRR**N**SL **4**[b]	4.56 ± 0.24	42.5 ± 8.83	107294 ± 22996
AIYRRGRLYRR**N(OH)**SL **5**[b]	0.942 ± 0.082	119.9 ± 19.2	7857 ± 1432
AIYRRGRLYRR**D**SL **6**[b]	0.581 ± 0.09	2069 ± 407	281 ± 70

[a] Peptides for cyclization by butelase-1. [b] Peptides for cyclization by VyPAL2. The cyclization reactions were performed at 37 °C in 20 mM PBS (pH 6.5) with various substrate concentrations. Cyclization was monitored by analytical RP-HPLC.

(OH) residue can function as a good chelator for metal cations to inhibit metalloenzymes or be further converted to natural Asp in a mild oxidation reaction (Scheme 1).



Scheme 1. Cyclization of P1-Asn(OH) peptide substrates as catalyzed by AEPs and subsequent oxidation of Asn(OH) to Asp.

Compounds **1**–**6** (Table 1 and S1) were prepared by Fmoc solid-phase peptide synthesis (SPPS) and used as substrates for butelase-1 or VyPAL2-catalyzed cyclization. As expected, the P1-Asn(OH) peptides were good substrates of these PALs and exhibited a much higher binding affinity (K_m) and catalytic turnover (k_{cat}) than the native P1-Asp peptides (Table 1). The catalytic efficiency of butelase-1 on the N(OH)HV substrate **2** is only approximately 6-fold lower than that on the NHV peptide **1**, but 48-fold higher than that on the DHV peptide **3** (Table 1). Similarly, the catalytic efficiency of VyPAL2 on the N(OH)SL peptide **5** is approximately 14-fold lower than that on the NSL peptide **4**, but 28-fold higher than that on the DSL peptide **6** (Table 1). As a control, the P1-Asn-(Me) peptide **7** was not recognized by butelase-1 (Table S1 and Figure S3). Therefore, we conclude that the N^{γ}-hydroxy group of Asn(OH) is involved in H-bonding to the S1 pocket of PALs (Figure 1).

Asn(OH) contains a hydroxamic acid moiety that has interesting properties,[9,10] including the ability to bind to metal ions[9a,b,10] and reactivity toward an *S*-aryl thioester for peptide ligation.[9c] A variety of hydroxamic acid-derived inhibitors of metalloenzymes have been designed as pharmacologic agents.[9b,10] Our method provides a convenient approach to prepare hydroxamic acid displaying cyclic peptides as potential inhibitors of pathogenic metalloenzymes such as matrix metalloprotease 2 (MMP2).[11] We used peptide **8,** a β-amyloid precursor protein derived inhibitor peptide (APP-IP),[12] to demonstrate the utility of this method (Figure 2). APP-IP **8** is an MMP inhibitor and is largely selective for MMP2 ($K_i \approx 60$ nM).[12a] Asp6 in APP-IP **8** mediates Zn^{2+} bonding. Simple replacement of Asp6 with Asn(OH) resulted in a 2.6-fold improvement in inhibitory activity (Figure 2b) that could be attributed to the stronger Zn^{2+}-chelating ability of the Asn(OH) hydroxamate group compared to the carboxylate in Asp.[10] Nevertheless, the linear APP-IP peptide **8** has poor proteolytic stability and its half-life in human serum is short.[12c] Cyclization is a useful strategy to improve the stability and selectivity of MMP2 inhibitory peptides.[10] Therefore, we performed head-to-tail cyclization of APP-IP peptides of various lengths using the Asn(OH) ligation method. However, the cyclized products, peptides **10**–**15**, had decreased inhibitory activities toward MMP2 (Table S3, Figure 2b), likely because simple cyclization restrained the peptides into undesirable conformations. We then grafted Ile1Ser2Tyr3, Leu8 and Asn(OH)6 from

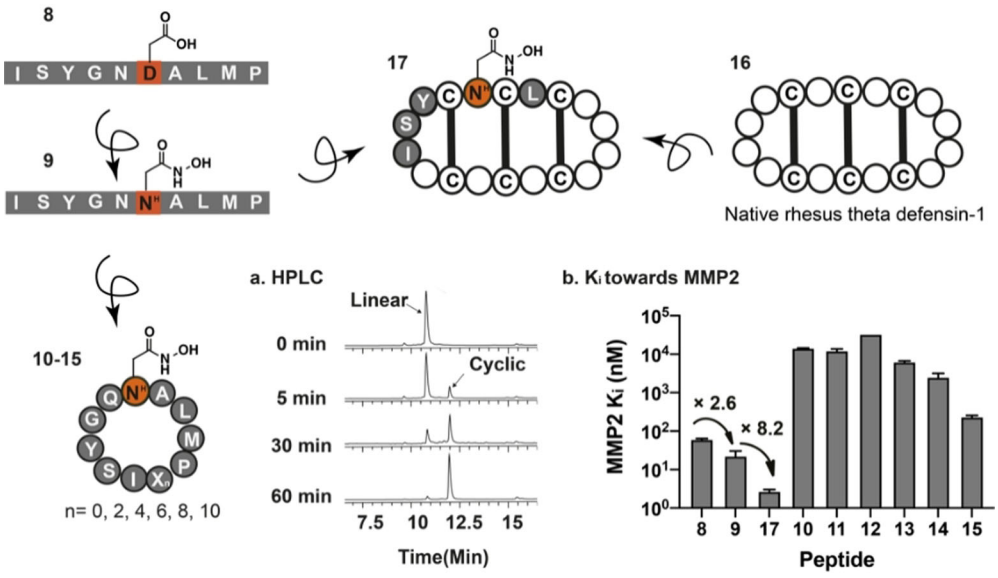


Figure 2. Design of MMP2 peptide inhibitors based on APP-IP and theta defensin. a) HPLC and ESI-MS monitoring of butelase-1-catalyzed cyclization of the linear precursor CLCRRGVCRCICTISYC**N**(OH)-AL for the preparation of **17** (reduced form). b) K_i values of peptides **8**–**17** towards MMP2.

APP-IP peptide **9** into rhesus theta defensin-1 (RTD-1) **16** (Figure 2) to form Asn(OH)-RTD1 **17**. RTD-1 has a highly compact, tetracyclic Cys-ladder structure.[13] Asn(OH)-RTD1 **17** was successfully cyclized at the Asn(OH) residue by incubating the linear substrate with butelase-1 (Figure 2a, Figure S4A). RP-HPLC monitoring indicated that the reaction was nearly completed in 1 h. After enzymatic cyclization, the cyclic product (reduced form) was oxidatively refolded into the mature form by incubation in 0.1 M Tris·HCl buffer (pH 8.5, 2 M urea) at 4 °C for 8 h. Enzyme inhibition results showed that the folded backbone-cyclic Asn(OH)-RTD1 peptide **17** had a K_i value of (2.8 ± 0.5) nM toward MMP2 (Figure 2b, Figure S4), representing an 8.2-fold improvement over the linear Asn(OH)-APP-IP peptide **9**.

NMR results showed that peptide **17** has the same 3D structure as RTD-1 **16** (PDB:1HVZ).[13] The Tyr3 and Asn-(OH)5 residues in peptide **17** are constrained in a β-sheet structure with the two side chains pointing parallel in the same direction (Figure 3, Figure S5), similar to the orientation

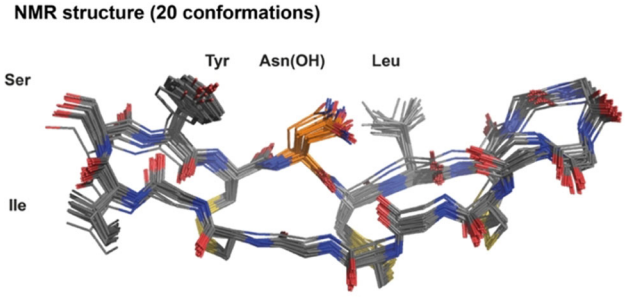


Figure 3. Superposed 20 low-energy structures of peptide **17** (PDB: 7F32; *N*-OH not shown) highlighting the spatial disposition of the side chains of residues.

adopted by Tyr3 and Asp6 of APP-IP **8** in binding to MMP2.[12b] This preorganized conformation of peptide **17** may have contributed to its improved inhibitory activity. For comparison studies, Asp-RTD1 **18** was obtained by oxidation of Asn(OH)-RTD1 **17** with $NaIO_4$[14] (1 equiv) in 20 mM PBS (pH 7.2) at 0 °C, which quickly converted Asn(OH) to Asp in 5–10 min. Previously, Pentelute and co-workers showed that $NaNO_2$ oxidation can also be used to convert Asn(OH) to Asp.[9c] Asn-RTD1 **19** was prepared in the same manner as Asn(OH)-RTD1 **17** by cyclization at the Asn residue using butelase-1. Although peptides **17, 18,** and **19** have the same conformation and nearly the same sequences (Figure S6, Table S4), MMP2 inhibition assay shows that Asn(OH)-RTD1 **17** has a 2.3- and 13-fold improvement in binding affinity over Asp-RTD1 **18** and Asn-RTD-1 **19**, respectively (Table S4, Figure S4). This result further corroborates that Asn(OH) is an excellent pharmacophore for metalloenzyme inhibition.[10] All three peptides are more potent MMP2 inhibitors than the native RTD-1 **16** (Table S4).

Similar to APP-IP **8**, Asn(OH)-RTD1 **17** is also selective for MMP2 over MMP9 (ca. 1,000-fold difference in K_i) (Table S4). The stability of the linear APP-IP and the cyclic Asn(OH)-RTD1 were examined by incubating the peptides in human serum at 37 °C. The cyclic peptide **17** remained intact after 16 h, while over 50 % of the linear APP-IP **8** was degraded within 32 min (Figure S7). We also did not observe the loss of the N^{γ}-hydroxy group on Asn(OH), which is different from what observed by the Heinis group on their bicyclic peptide.[10] This N^{γ}-hydroxy group may be stabilized by the highly constrained conformation of the tetracyclic peptide **17**. The high selectivity and stability of the Asn(OH)-RTD-1 peptide **17** thus makes it a useful pharmacological tool to study the physiological and pathological roles of MMP2.

Facile conversion of Asn(OH) to Asp makes Asp cyclization more attainable through PAL ligation, albeit indirectly. To show the general applications of this methodology, we further performed cyclization of P1-Asn(OH) peptides of 6–34 amino acids, followed by $NaIO_4$-mediated oxidation of Asn(OH) to Asp (Table 2). The first peptide prepared was MCoTI-II. Isolated from *Momordica cochinchinensis* seeds, MCoTI-II is a potent trypsin inhibitor.[15] Cyclization of MCoTI-II has been achieved using various methods.[15b,16–19] Linear MCoTI-II-N(OH)IV **20** was prepared by SPPS and oxidatively folded. Backbone cyclization of acyclic, folded MCoTI-II-N(OH)IV **20** was done by incubation with butelase-1 (0.01 equiv) for 3 h at 37 °C (Figure S8a,b). After enzymatic cyclization, the unnatural Asn(OH) residue was then converted to Asp in 10 min by $NaIO_4$ oxidation at 0 °C (Figure 4). To study the folding states,

Table 2: Peptide cyclization at the P1 site (residue in bold) and oxidation of Asn(OH) to Asp.[a]

Peptide	Sequence	Cyclization time [h]	Yield [%]	Oxidation time [min]	Yield [%]
MCoTI-II-(OH)IV **20**	GGVCPKILKKCRRDSDCPGACICRGNGYCGSGS**N(OH)**IV[b]	3	95	10	>95
MCoTI-II-DIV **21**[c]	GGVCPKILKKCRRDSDCPGACICRGNGYCGSGS**D**IV[b]	3	ND	NA	NA
kB2-N(OH)IV **22**	GLPVCGETCFGGTCNTPGCSCTWPICTR**N(OH)**IV[d]	1	80	10	>95
kB2-DIV **23**	GLPVCGETCFGGTCNTPGCSCTWPICTR**D**IV[d]	2	ND	NA	NA
SFTI-N(OH)IV **24**	GRCTKSIPPICFP**N(OH)**IV[b]	3	85	10	>95
SFTI-DIV **25**	GRCTKSIPPICFP**D**IV[b]	3	ND	NA	NA
RGD-N(OH)HV **26**	FLaRGN**(OH)**HV[e]	1	85	10	>95
RGD-defensin-N(OH)SL **27**	ACRCLCRRGDCRCICRG**N(OH)**SL[d]	1	95	15	>95

[a] Cyclization reactions were performed at 37 °C with 400 μM of the peptide substrate and butelase-1 or VyPAL2 (0.01 equiv) in 20 mM PBS (pH 6.5) for 1–3 h. Peptide **27** was cyclized by VyPAL2, and the other peptides were cyclized by butelase-1. Asn(OH) oxidation was performed in 20 mM PBS (pH 7.4) at 0 °C, with $NaIO_4$ (1 equiv). [b] Backbone cyclization was conducted after oxidative refolding. [c] Cyclization was also conducted at pH 4.5 or 6.5 at room temperature for overnight, and no cyclic product was observed. [d] Backbone cyclization was conducted before oxidative refolding. [e] Cyclic dimer [FLaRGN(OH)-FLaRGN(OH)] was formed. ND = not detectable; NA = not applicable.

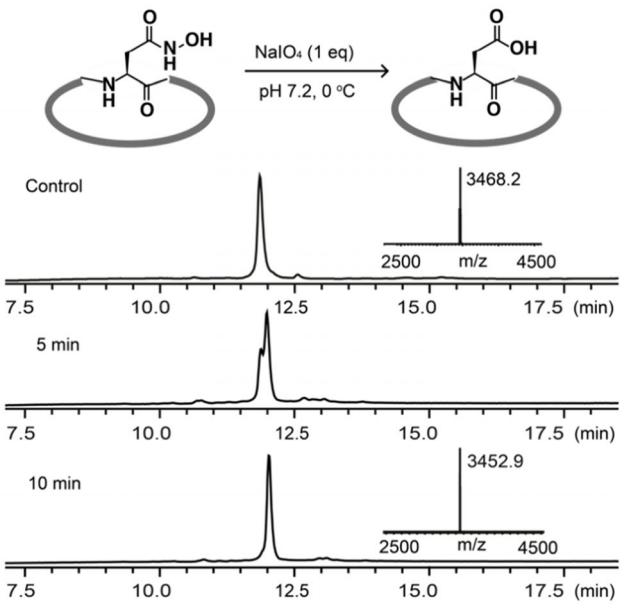


Figure 4. Conversion of Asn(OH) into Asp by $NaIO_4$-mediated oxidation. RP-HPLC and ESI-MS monitoring of the oxidation reaction of Asn(OH)-MCoTI-II.

CD spectra for the linear MCOTI-II, acyclic MCoTI-II (folded), and cyclic MCoTI-II (folded) were collected (Figure S8c). The spectra for acyclic and cyclic MCoTI-II (both folded) are comparable to the reported CD spectrum.[20,21]

Butelase-1 cyclized P1-Asn(OH) peptides (**20**, kB2 **22**,[22] and SFTI **24**[23]) within 1–3 h at 37 °C despite a sterically hindered Asn(OH)-Ile bond (Table 2). In contrast, direct cyclization of the corresponding P1-Asp peptides **21**, **23** and **25** by butelase-1 was extremely inefficient as cyclic products were not detectable after 3–24 h (Figures S9–S11). This is most likely because 1) Asp is a poor P1-amino acid, 2) Ile is a bulky P1′ residue and 3) the N-terminal motifs in **21** and **25** (GG- and GR-) are unfavorable nucleophilic substrates of butelase-1. Nevertheless, all these problems were overcome by the higher reactivity of P1-Asn(OH) during cyclization.

We also prepared cyclic peptides containing RGD (for integrin targeting)[24] using Asp as the cyclization site (Table 2). The RGD-theta-defensin linear precursor **27** was cyclized in 1 h by VyPAL2 (Figures S12 and S17). Interestingly, FLaRGN(OH)HV **26** was converted to a cyclic dimer in 1 h, likely because the high angle strain prevented the formation of the small six-residue ring, similar to a previous finding by Hemu et al.[25]

Next, trypsin inhibition assays were performed (Table S5 and Figure S21). The K_i values of the synthetic MCoTI-II and SFTI toward trypsin were 0.18 and 0.44 nM, respectively, which are consistent with reported values.[16,26] Therefore, both cyclic trypsin inhibitor peptides prepared by our method have the expected biological activities.

In conclusion, we have used an unnatural amino acid, Asn(OH), as the P1 substrate for PAL-catalyzed peptide cyclization. The P1-Asn(OH) residue is an excellent mimic of P1-Asn in PAL substrates, and the P1-Asn(OH) peptides exhibit a much higher affinity and turnover rate than the P1-Asp peptides. A wide range of cyclic peptides were prepared to demonstrate the utility of our method for P1-Asn(OH)-enabled cyclization and oxidative Asn(OH)-to-Asp conversion. The structure and bioactivity of these peptides were confirmed by CD spectroscopy, NMR and enzyme inhibition assays. More importantly, we demonstrate that cyclization of Asn(OH)-containing peptides is well-suited for generating inhibitors that have high potency and selectivity toward MMP2 as well as proteolytic stability. Taken together, the engineered Asn(OH) serves three functions: 1) mediator of PAL-catalyzed ligation and a site of peptide cyclization, 2) a chelator for metal ions to inhibit metalloenzymes, and 3) an Asp precursor. Our study shows that substrate engineering can expand the substrate scope of PALs and increase the therapeutic value of the cyclized peptides.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: amino acids · cyclic peptides · enzymes · inhibitors · macrocyclization

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Communications

Cyclic Peptides

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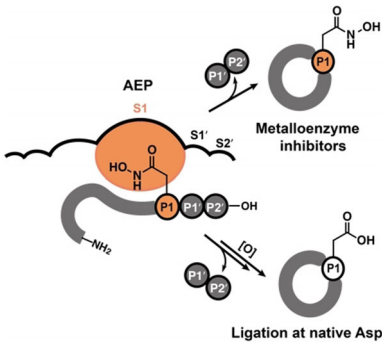
N^{γ}-Hydroxyasparagine: A Multifunctional Unnatural Amino Acid That is a Good P1 Substrate of Asparaginyl Peptide Ligases



An unnatural amino acid, Asn(OH), mimics P1-Asn in the substrates of asparaginyl ligases, enabling efficient cyclization of P1-Asn(OH) peptides. The hydroxamic acid functionality in Asn(OH) is a metal-ion chelator for metalloenzyme inhibition, and Asn(OH) can be converted into native Asp by periodate oxidation (see scheme).