Site-specific nonenzymatic peptide S/O–glutamylation reveals the extent of substrate promiscuity in glutamate elimination domains

Alexander A. Vinogradov[1,*], Yuki Goto[1] and Hiroaki Suga[1,*]

[1]Department of Chemistry, Graduate School of Science, The University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan

*Correspondence to hsuga@chem.s.u-tokyo.ac.jp and a_vin@chem.s.u-tokyo.ac.jp

The authors declare no competing interests.

Abstract

Formation of dehydroalanine and dehydrobutyrine residues via tRNA-dependent dehydration of serine and threonine is a key post-translational modification in the biosynthesis of lanthipeptides and thiopeptides. The dehydration process involves two reactions, wherein the O–glutamyl Ser/Thr intermediate, accessed by a dedicated enzyme utilizing Glu-tRNA^{Glu} as the acyl donor, is recognized by the second enzyme, referred to as the glutamate elimination domain (ED), which catalyzes the eponymous reaction yielding a dehydroamino acid. Owing to the fact that many lanthipeptides and thiopeptides possess strong antibiotic activities, the enzymology of their biosynthesis became the focus of numerous studies, and although significant progress has recently been made, much remains to be elucidated. This is especially true for the downstream EDs, because the scope of available substrates for testing is limited to those that the upstream enzymes can furnish. Here, we report two complementary strategies for direct, nonenzymatic access to diverse ED substrates. First, we establish that a thiol-thioester exchange reaction between a Cys-containing peptide and an $^{\alpha}$thioester of glutamic acid leads an S–glutamylated intermediate which can act as a substrate for EDs. Furthermore, we show that the native O–glutamylated substrates can be accessible from S–glutamylated peptides upon a site-specific S-to-O acyl transfer reaction. Combined with flexible in vitro translation utilized for rapid peptide production, these chemistries enabled us to dissect the substrate recognition requirements of three known EDs. Our results establish that EDs are uniquely promiscuous enzymes capable of acting on substrates with arbitrary amino acid sequences, and performing retro-Michael reaction beyond the canonical glutamate elimination. To aid in the substrate recruitment process, EDs apparently engage in nonspecific hydrophobic interactions with their substrates. Altogether, our results establish the substrate scope of EDs and provide clues to their modes of action.

Introduction

Dehydroamino acids (**dhAA**s) are one of the most common nonproteinogenic elements found in ribosomally synthesized and post-translationally modified peptides (**RiPP**s).[1,2] Usually exemplified by dehydroalanine (**Dha**) and dehydrobutyrine (**Dhb**), dhAAs are prominently featured in the structures of lanthipeptides,[3] thiopeptides,[4] linaridins,[5] lipolanthines,[6] some linear azole-containing peptides (**LAP**s)[7] and other recently identified RiPP classes.[2] Additionally, due to their relatively high chemical reactivity,[8] Dha and Dhb serve as biosynthetic precursors to more elaborate post-translational modifications (**PTM**s), notably to lanthionines and labionines in lanthipeptide biosynthesis, and to backbone-embedded pyridines during the assembly of thiopeptides.

One of the most common post-translational pathways to dhAAs is tRNA-dependent dehydration of Ser/Thr.[3] This pathway involves two sequential reactions (Fig. 1a), wherein an enzyme called the glutamylation domain (**GD**) first catalyzes a transesterification reaction using Glu-tRNA^{Glu} and the side chain of Ser/Thr in the substrate peptide as the acyl donor and acceptor, respectively.[9–11] Usually, the GD recognizes and binds the N-terminal sequence of substrate (leader peptide, **LP**) to promote transesterification in its C-terminal part (core peptide, **CP**). In the second step, the enzyme referred to as the glutamate elimination domain (**ED**) utilizes the Ser(OGlu) or Thr(OGlu) intermediate to perform a retro-Michael reaction, which eliminates a molecule of glutamate and furnishes the dhAA. This biosynthetic logic serves as the defining characteristic of class I lanthipeptide biosynthesis[12] and also operates during the assembly of all known thiopeptides[13] and goadsporin, a unique dhAA-containing LAP.[7] In RiPP biosynthetic gene clusters (**BGC**s), GD and ED can be encoded as a single enzyme as in most class I lanthipeptide BGCs, or as two separate polypeptides in BGCs of thiopeptides, goadsporin and antifugal pinensins (Fig. 1b).[14]

Because class I lanthipeptides and thiopeptides account for hundreds of bioactive natural products[4,15–17] (with many more bioinformatically predicted),[18–20] the biochemistry of tRNA-dependent Ser/Thr dehydration has become the focus of numerous studies. For instance, recent reports revealed the crystal structures of several GDs and EDs,[11,21,22] investigated the selectivity of GDs for Glu-tRNA^{Glu},[21] and established basic requirements for substrate engagement.[23] Nevertheless, many details such as the catalytic mechanisms and the extent

of substrate specificity remain elusive. During biosynthesis of many RiPPs, tRNA-dependent dehydration of Ser/Thr is often contingent upon the formation of other PTMs, which obfuscates the analysis. For EDs, an additional difficulty stems from the fact that as the downstream enzyme, the range of available substrates for testing is limited to those that the corresponding GD can furnish. For example, because GDs are selective for Glu-tRNA^{Glu},[21,24,25] it remains unknown whether EDs can perform an analogous reaction with other O-acyl Ser/Thr derivatives. Moreover, in vitro reconstitution of tRNA-dependent Ser/Thr dehydration can be, at times, challenging, both because the cognate tRNA^{Glu} and GluRS need to be identified and produced, and because many GDs frequently resist heterologous overexpression.[26]

To facilitate the study of EDs, here we report two protocols (Fig. 1c) which directly afford ED substrates and thus bypass the requirement for the tRNA^{Glu}/GluRS/GD system. First, we establish that some EDs can accept a thioester substrate, Cys(SGlu), accessed via an in situ thiol-thioester exchange reaction between a Cys-containing peptide and a synthetic $^{\alpha}$thioester of glutamic acid. We refer to this sequence as “S–elimination”. Further, we show that the substrates containing the native Ser(OGlu) residue can be obtained from the Cys(SGlu) intermediates using the recently developed site selective S-to-O acyl shift chemistry (“O–elimination”). We show that these reactions can be utilized in tandem as a tool to study EDs, demonstrated here by probing basic substrate recognition requirements of three known EDs. Our results suggest that EDs utilize weak hydrophobic interactions to engage and recognize their substrates, which endows them with the ability to act on diverse peptides and to catalyze a retro-Michael reaction beyond the canonical glutamate elimination. Overall, our work illuminates the properties of EDs masked by substrate specificities of GDs, and provides a tool to facilitate their further study.

Results and discussion

Reaction development

For this study, we focused on three known EDs (Fig. 1b). NisB is a class I lanthipeptide synthase from the nisin biosynthetic pathway, which served as a model enzyme for the majority of the studies to date.[9,11,23,27,28] NisB is expressed as a single polypeptide featuring two pronounced domains (GD and ED). TbtC is a standalone ED, which operates in tandem with its partner, TbtB, during the biosynthesis of thiomuracin, a thiopeptide with a strong antibiotic activity.[24,29] LazF, the third studied ED, is another thiopeptide biosynthetic enzyme,[30] although its sequence similarity to TbtC is low (30 %). LazF is unique in the fusion of the ED to an FMN dehydrogenase, utilized for azoline to azole conversion during lactazole assembly.[31] The enzymes were heterologously expressed (S.I. 2.1) in *Escherichia coli* as soluble proteins following the established procedures.[9,24,25] For rapid production of diverse peptide substrates, we utilized the flexible in vitro translation (**FIT**) system,[32] previously deployed for in vitro biosynthesis of azoline-containing peptides,[33] goadsporin,[34] and lactazole[25] analogs. The $^{\alpha}$thioester of glutamic acid, namely *S*-$^{\alpha,L}$glutamyl 4-chlorobenzyl thiol (^{L}Glu–CBT), was chemically synthesized (S.I. 2.7) using the standard methods in two steps. The isolated compound proved stable for over a year when frozen as a solution in DMSO at –20 °C.

With all components in hand, we focused on developing the optimal reaction conditions for S–elimination. We chose LazF as the model enzyme and designed a precursor peptide bearing its cognate LP followed by an arbitrary CP (LazA^{LP}/CP1) as the substrate (Fig. 2a). In general, we opted to use the peptides containing arbitrary CP sequences because i) prior work indicated that EDs can process nonnative substrates[31,35,36] and ii) accessing the native substrates with the FIT system might be challenging (for instance, TbtBC acts on a thiomuracin CP containing 6 non-proteinogenic thiazole amino acids).[37] After a modicum of experimentation, we found that the treatment of LazA^{LP}/CP1 expressed in the FIT system with 5 μM LazF and 2 mM ^{L}Glu–CBT in HEPES buffer (pH 7.2) for 20 h at 25 °C (the "standard conditions") led to a clean formal elimination of hydrogen sulfide (Δ33.99 Da) from the substrate, as judged by LC-MS (Fig. 2a). Tandem mass spectrometry (MS/MS) of the reaction product confirmed that the elimination took place at Cys6 and resulted in an amino acid with a molecular weight of 69.01 Da, consistent with the formation of Dha6. The reaction

product could be conjugated to cysteamine, which further supported the presence of a Dha residue in the peptide (Fig. S2a). The elimination efficiency responded to changes in the enzyme concentration, and no detectable Dha formed without LazF (Fig. 2b). Similarly, no reaction occurred in the absence of ^{L}Glu–CBT or when LazA^{LP}/CP1 S6 mutant was used as a substrate (Fig. S2b). We confirmed the formation of the key Cys(SGlu) intermediate by LC-MS after incubating LazA^{LP}/CP1 with 5 mM ^{L}Glu–CBT for 3 h in the absence of the enzyme (Fig. S3). The same experiment also revealed a pronounced instability of Cys(SGlu)-containing peptides, because the Cys6(SGlu) thioester partially reverted to Cys6 during sample preparation and RP-HPLC. Likely owing to this instability, Cys(SGlu) intermediates did not accumulate under the standard conditions and were usually undetectable by LC-MS after a 20 h reaction. The lability of glutamic acid thioesters is documented,[38,39] and we confirmed by ^{1}H NMR that ^{L}Glu–CBT slowly decomposes in water at neutral pH (Fig. S4).

Furthermore, we found that S–elimination is unusually thiol-sensitive. The use of a less reactive (compared to ^{L}Glu–CBT) alkyl $^{\alpha}$glutamic acid thioester (^{L}Glu–ET; Fig. 2c) decreased the efficiency of the process, whereas a more reactive thiophenol-type thioester (^{L}Glu–MTP; Fig. 2c) was entirely ineffective, resulting in <2% of the Dha-containing product. Analogously, the addition of 10 mM 4-mercaptophenyl acetic acid (MPAA), a common thiol-thioester exchange catalyst extensively used for native chemical ligation,[40,41] was counterproductive. Supplementing the reaction with 10 mM CBT also inhibited S–elimination, as did increasing the concentration of ^{L}Glu–CBT beyond the optimal 2 mM.

Overall, conversion to Dha via S–elimination proceeded conspicuously slower than the analogous canonical reaction performed by LazBF. To exclude the possibility that LazF is activated by LazB, we performed S–elimination in the presence of 5 µM LazB or with 5 µM LazB, 20 µM tRNA^{Glu}, and 1 µM GluRS, and found no major difference in the reaction efficiency (Fig. 2c). This result suggests that LazF can function independently of LazB.

Next, we turned to the development of O–elimination, i.e., ED-mediated Dha formation from Ser(OGlu)-containing peptides accessed in situ from ^{L}Glu–CBT (Fig. 1c). A recent report from our laboratory[42] describes site-selective S-to-O acyl transfer to synthesize various Ser(O-acyl) peptides, including cyclodepsipeptides. Upon treating a peptide containing the reactive motif, which can be as short as Ser-Xaa-Cys, with a thioester at ambient

temperature and pH, a Cys(S-acyl) intermediate forms, which further yields Ser(O-acyl)-containing peptides upon a spontaneous S-to-O acyl transfer. We envisaged that if ^{L}Glu–CBT is used as a thioester, ED substrates bearing the native Ser(OGlu) could be prepared to complement our S–elimination conditions.

For consistency, we performed O–elimination reactions under the standard conditions without additional optimization. Upon incubation of LazA^{LP}/CP2 S6 with LazF under the standard conditions, approximately a 1: 1 mixture of S– (Δ33.99 Da) and S,O– (Δ52.00 Da) double elimination products formed, and MS/MS analysis confirmed the formation of Dha6 and Dha8 as expected (Fig. S5–S7). S–elimination and S-to-O acyl shift processes are in competition once the key Cys(SGlu) intermediate forms. If the S–elimination occurs prior to the acyl shift, the resulting Ser-Xaa-Dha peptide represents a reaction dead-end, because its Ser residue can no longer undergo modification. Although this competition somewhat limits the preparative utility of the method, analysis of the enzyme substrates preferences is still feasible (see S.I. 2.6 for details). More importantly, we found that in numerous cases where S–elimination is slow, clean formation of O–elimination products, i.e., Dha-Xaa-Cys peptides, can be achieved. For example, because TbtC is nearly incapable of catalyzing S–elimination (vide infra), the incubation of TbtA^{LP}/CP2 S6 with 5 μM TbtC under the standard conditions led mostly to the formation of Dha6-Met7-Cys8 product, as judged by LC-MS (Fig. 2d) and MS/MS analyses (Fig. S8). The reaction occurred only when both TbtC and ^{L}Glu-CBT were present, and no elimination took place for TbtA^{LP}/CP2 A6 or TbtA^{LP}/CP2 A8 mutants, indicating that the elimination occurred according to the proposed pathway (Fig. S9). In a separate experiment, we also confirmed the site-specific nature of the acyl transfer, since a substrate containing multiple Ser residues in the CP led to Dha formation only at Ser in position –2 relative to Cys (Fig. S10).

Substrate specificity of EDs

Core peptide specificity. The combination of the developed chemistry with the FIT system opens access to rapid assaying of diverse and previously inaccessible ED substrates. Here, we sought to demonstrate the utility of this platform in dissecting the substrate recognition requirements of LazF, NisB, and TbtC. EDs are known as enzymes capable of processing substrates with nonnative CPs.[31,35,36] We began by establishing the extent of this promiscuity. For each studied enzyme, we prepared six substrates with the cognate LP and a randomized

CP sequence containing Ala-Xaa-Cys or Ser-Xaa-Cys tripeptides to study S– and O–elimination reactions, respectively (Fig. 3a). The FIT system-derived peptides were incubated with the enzymes and ^{L}Glu–CBT under the standard conditions, and the outcomes were analyzed by LC-MS (see S.I. 2.6 for quantification details, Fig. S1 and S.I. 2.3 for the specifics of performing S– and O–elimination reactions with NisB). Our results indicate that all studied EDs efficiently promote O–elimination in diverse CPs, confirming the ability of the EDs to accept noncognate CPs (Fig. 3b; Fig. S11–14). Elimination of α-thioglutamate occurred consistently slower than that of glutamate in all cases, which suggests that the noncanonical S–elimination reaction enables a more sensitive analysis of substrate recognition requirements compared to the native O–elimination, and that the combination of the two allows for a more comprehensive study. α-Thioglutamate is expected to be a better leaving group (pK_a(glutamate) = 2.2; pK_a($^{\alpha}$thioglutamate, predicted in ChemAxon) = –1.3), indicating that the relative difficulty of S–elimination is related to the size of sulfur atom in Cys(SGlu), which might weaken some of the substrate-enzyme interactions important for a productive reaction. Whereas LazF and NisB efficiently executed S–elimination on randomized CPs, TbtC was nearly incapable of doing so, achieving at most 12% reaction efficiency. Nonetheless, we still conclude that TbtC is a promiscuous enzyme capable of catalyzing O–elimination in sequence-randomized CPs, which stands in contrast to the selectivity of the canonical TbtB/C-mediated dehydration toward a TbtA substrate containing six thiazole residues.[37] Thus, it appears that during thiomuracin biosynthesis, substrate discrimination is performed primarily by the partner GD, TbtB. This mode of action is also operational during the assembly of lactazole, where the GD (LazB) controls which Ser residues in the substrate undergo dehydration.[31]

To gain further insight into the nature of CP recognition by LazF, the most active of the studied enzymes, we prepared seven S–elimination substrates (LazA^{LP}/CP5–11) and investigated them under the standard reaction conditions (Fig. 3c). LC-MS analysis confirmed that LazF can process diverse substrates, including peptides containing charged amino acids around the modification site (CP10), which often impair enzymatic processing by RiPP biosynthetic enzymes.[31,43–45] For CP10, a substrate with a modest elimination efficiency, we prepared 10 single point mutants at positions 6 (–1 relative to the reactive site) and 8 (+1), in an attempt to ascertain whether the sluggish modification of LazA^{LP}/CP10

can be attributed to the local structure around the reaction site (Fig. S15). We found that the introduction of a Trp residue on either side of Cys7(SGlu) remedies S–elimination (91% elimination extent for A6W and 84% for A8W), suggesting that hydrophobic amino acids in the vicinity of the modification site might play a role in the substrate engagement process. To examine whether this hypothesis is feasible, we studied a number of S–elimination substrates based on the sequences of LazA^{LP}/CP2 A6, CP4 A4 and CP5. For each peptide, we prepared two series of mutants, obtained by either progressively truncating the CP sequence or by gradually replacing hydrophobic amino acids with hydrophilic ones (Fig. 3d,e,f). In every case, we found that LazF-mediated S–elimination proceeded more slowly for CPs depleted of hydrophobic residues, even when mutations were distal to the modification site (e.g., CP2.1 and CP5.2). Similarly, progressive truncation of CP2 A6, CP5 and to a lesser extent CP4 A4 compromised reaction yields. This effect was especially pronounced for CP5, where truncating the six C-terminal amino acids abrogated S–elimination (97% for CP5 vs. 0.3% for CP5 Δ6). For two substrates, CP2.2 and CP2 Δ9, we prepared the corresponding O–elimination peptides, CP2.2 S6 and CP2 Δ9 S3, to investigate whether a similar effect persists for O–elimination (Fig. S16). In both cases, we found that O–elimination was also hampered compared to the parent peptide (CP2 S6), albeit less than the S–elimination.

These results allow several interpretations. We argue that LazF likely utilizes “hydrophobic steering” (by analogy with the well-known electrostatic steering)[46] by engaging in several nonspecific, mostly hydrophobic interactions with the CP to facilitate the recognition process. During lactazole biosynthesis, LazF acts four times on Ser(OGlu) intermediates in different but invariably hydrophobic local environments,[31] and thus the emergence of such a mechanism to facilitate catalysis might not be unexpected. The role of hydrophobic enzyme–substrate interactions in RiPP biosynthesis is well-documented, but most studies on the topic to date have focused on the LP–enzyme interactions (vide infra). The preference for hydrophobic amino acids in substrate CPs for promiscuous biosynthetic enzymes has also been noted,[44,45,47–49] but to our knowledge, never clearly articulated. Thorough biophysical studies on LazF and other RiPP enzymes will be needed to further evaluate this idea.

Leader peptide specificity. Next, we examined the specificity of EDs toward cognate LPs. In addition to the aforementioned LazA^{LP}/CP2, NisA^{LP}/CP2, and TbtA^{LP}/CP2 constructs, we

prepared GodA^{LP}/CP2 (*godA* encodes goadsporin precursor peptide, and LazF can replace the endogenous GodG during goadsporin biosynthesis[34]), random^{LP}/CP2 (a random 35-mer sequence positioned as LP), and a leaderless peptide, CP2 (Fig. 4a and b). The substrates were incubated with the enzymes and ^{L}Glu–CBT under the standard conditions and the outcomes were analyzed by LC-MS. Consistent with the results above, LazF was the most active of the studied enzymes, as it efficiently catalyzed O–elimination for every tested substrate, including the leaderless peptide (no LP; Fig. 4b). Nevertheless, the LP sequence played a role in facilitating substrate recognition, as the substitution of LazA^{LP} with a noncognate sequence in S–elimination compromised reaction yield in every case. At the same time, any N-terminal sequence was preferred to the leaderless peptide. Analogously, for TbtC, the use of O–elimination substrates equipped with noncognate LPs decreased the conversion to Dha (S–elimination was not performed due to low efficiency). In general, LazA^{LP} and GodA^{LP}, i.e. the peptides with some sequence similarity to TbtA^{LP}, were better substrates than the peptides bearing no resemblance (NisA^{LP} or no LP). NisB also preferred the cognate LP but still accepted noncognate and leaderless substrates in O–elimination. Because GD and ED are fused in NisB, our results clearly demonstrate the functional differences between the domains. Canonical tRNA-dependent elimination by NisB is strictly LP-dependent.[11,27] NisB is known to utilize its RiPP recognition element (RRE),[50] a small PqqD-like domain grafted inside the GD, to bind NisA^{LP}, mostly via interacting with the $^{-18}$FNLD^{-15} box in the LP.[11,27] Moreover, prior studies established that upon binding to the RRE of NisB, NisA is processed by both the GD and ED without translocating between the reactions.[51] Our results indicate that for the ED-mediated reaction, the RRE/LP interaction is dispensable, whereas dehydration via the canonical tRNA-dependent pathway proceeded only for NisA^{LP}/CP2 S6, pointing to the functional difference between the domains. Collectively, these results indicate that the enzyme/LP interaction although not absolutely essential, serves to facilitate glutamate elimination by EDs, regardless of the presence of RRE.

Because LazF displayed some preference for the native LP (S–elimination data, Fig. 4b), we sought to explore the nature of this interaction in more detail. For the following studies, we utilized several variants of LazA^{LP}/CP1 (Fig. 4c). First, we prepared five peptides bearing a progressively truncated LazA^{LP} sequence and studied them under the standard conditions.

We found that removing up to 13 N-terminal amino acids (LazA$^{LP[-24 \rightarrow -1]}$) had a minimal impact on processing by LazF, but the peptides with more than 18 truncated residues afforded almost no Dha (~5% elimination extent; similar to leaderless substrates), which suggests that residues –24 to –19 are critical for the LP/enzyme interaction. Accordingly, we conducted an Ala-scanning mutagenesis of LazA$^{LP[-27 \rightarrow -1]}$/CP1 for positions –24 to –13, and studied the fitness of the resulting peptides in S–elimination (Fig. 4c). The experiment pointed to an extended $^{-22}$LDLxxL^{-17} sequence, or more narrowly to $^{-20}$LxxL^{-17} double Leu motif as the primary enzyme-interacting motif. At the same time, bio-layer interferometry assays showed no measurable binding affinity between full length LazA^{LP} and LazF (K_D > 20 μM).

These results, combined with the fact that LazF also modified noncognate substrates lacking the LxxL motif (for example, random^{LP}/CP2; Fig. 4b), again suggest a mode of action, wherein LazF associates with LazA^{LP} by making several weak, likely nonspecific contacts. Such hydrophobic interactions between the enzyme and substrate LP are a common theme in RiPP biosynthesis.[52–60] For instance, a recent study[61] described how RRE-bearing enzymes utilize steric complementarity to pack 2–3 LP residues into the hydrophobic pockets on the protein surface, enabling promiscuous LP binding. It is tempting to speculate that LazF might recruit its substrates in a similar manner, even though it lacks an annotatable RRE domain. Indeed, pyridine synthases are homologous (both structurally and in terms of their primary sequence) to EDs, and a co-crystal structure[62] between RRE-less TbtD (pyridine synthase from thiomuracin biosynthesis) and TbtA^{LP} confirmed the predominantly hydrophobic nature of the interaction.

Curiously, the identified LazF recognition motif in LazA^{LP} (LDLxxL) is also frequently found in the LPs of lipolanthine and class III/IV lanthipeptide precursor peptides.[63] A recent report[64] established that the θxxθ (θ: hydrophobic amino acid) motif in lipolanthine precursor peptides forms an amphipathic α-helix indispensable for enzymatic processing. MicKC, the type III lanthipeptide synthase in question, also lacks an RRE. As such, hydrophobic substrate recruitment by RiPP biosynthetic enzymes seems to represent a general phenomenon, regardless of the affinity of the interaction (NisB binds to NisA with 1 μM affinity,[23] whereas LazF does not show strong binding to LazA^{LP}), and regardless of the

presence of particular structural elements in the enzyme, as both RRE-containing and RRE-less enzymes can engage in this behavior.

Specificity toward glutamate. In the final series of experiments, we studied the specificity of EDs toward Ser(OGlu). To this end, we prepared CBT $^{\alpha}$thioesters of 10 ^{L}glutamic acid analogs and studied them with the substrates bearing cognate LPs and CP2 sequences as described above (Fig. 5a). Because GDs are selective for Glu-tRNA^{Glu},[21,24,25] it remains unknown whether EDs can catalyze a more general retro-Michael reaction on Ser(O-acyl) peptides. A cocrystal structure of NisB with a Dap(NHGlu) substrate analog[22] indicates that the side chain of Glu is scaffolded in the enzyme active site (Fig. 5b). Tyr739, Tyr776 and Tyr820 form a small pocket for the side chain, inside which Arg784 and Arg786 make close contacts with the side-chain carboxylate, and the α-amino group is located within 3.5 Å of Glu823. Our results (Fig. 5c) indicate that these interactions are not absolutely required for productive elimination. In general, S–elimination was highly susceptible to structural perturbations for both LazF and NisB, and any appreciable reaction took place only when ^{L}Glu(OMe)-CBT was used as the thioester. O–elimination reactions proved more informative in examining other thioesters. Although the ability of the Glu side chain carboxylate to act as a hydrogen bond acceptor when interacting with Arg784 and Arg786 promotes the reaction (compare ^{L}Glu, ^{L}Gln and ^{L}Glu(OMe) vs. the rest), this interaction is not absolutely required for either LazF or NisB, with the former being more tolerant of structural perturbations in the substrate. Accordingly, the use of Gly-CBT, which lacks the side chain, and β-branched ^{L}Val-CBT still led to over 50% Dha formation for LazF. The nearly identical reaction efficiency observed for ^{L}Val, ^{L}Met, Gly, ^{L}Ala and ^{L}Asp(OMe) thioesters suggests that the substrate can be oriented in the active site without occupying the “side-chain pocket”, because the ^{L}Val and ^{L}Asp(OMe) side chains are likely too bulky to be accommodated. The α-amino group proved to be more important for all three enzymes, as is evident from the low reaction yields obtained for ^{L}Lac-CBT, N-Ac-^{L}Glu-CBT, and ΔNH_2-Glu-CBT. In view of these results, it is somewhat puzzling that even though Glu823 in NisB is most likely responsible for the recognition of the $^{\alpha}NH_2$ group, NisB E823A mutant is still a functional enzyme.[9] Perhaps, the role of the amino group is confined to decreasing the pK_a of the leaving carboxylate (pK_a(^{L}Ala–OH) = 2.34; pK_a(^{L}Lac–OH) = 3.86).

Overall, the enzymes had similar glutamate recognition profiles. An alignment of NisB, LazF and TbtC primary sequences (Fig. S17a) indicates that despite low overall similarity, the active sites of the enzymes are conserved, and Phyre2 protein structure modelling[65] for LazF and TbtC (Fig. S17b) results in folds analogous to NisB, including the arrangement of the active site residues. As such, similar specificities for the Ser(O-acyl) moiety might be expected. Nevertheless, several minor differences were discernible. For instance, whereas LazF was more sensitive toward acetylation of $^{\alpha}NH_2$ (compare N-Ac-^{L}Glu-CBT vs. $^{\alpha}NH_2$-unblocked substrates), NisB responded more strongly to the absence of the side-chain carboxylate.

All tRNA-dependent Ser/Thr dehydration pathways characterized to date invariably utilize glutamate as the acyl donor,[3] which raises the question of glutamate's significance in the process. Our results by no means provide a definitive answer but indicate that glutamate recognition is not particularly critical, at least for EDs. Therefore, if there is some special significance to the use of glutamic acid, it must be explained by the enzymology of GDs.

In summary, here we described the chemistry to access ED substrates without the need for the partner GDs and associated GluRS/tRNA pairs. The established S– and O–elimination protocols complement each other, and when used in combination, enable facile and comprehensive profiling of ED substrate recognition requirements. Our results indicate that of the studied enzymes, LazF appears to be most active toward structurally diverse substrates. The enzyme does not have a single essential substrate recognition requirement. LazF can efficiently process a unique breadth of substrates, including the peptides containing arbitrary LP and CP sequences, as well as structurally diverse Ser(O-acyl) intermediates. Substrate engagement appears to be facilitated by hydrophobic steering, i.e., multiple weak, mostly hydrophobic interactions between the peptide and the enzyme. Both LP and CP regions contribute to the recruitment by the enzyme, but some of these interactions are dispensable; for example, O–elimination proceeds on leaderless peptides. To a variable degree similar promiscuity manifests in NisB and TbtC, suggesting that this mode of action might a general phenomenon in ED catalysis. We believe that further insights into the origins of such a perplexing substrate promiscuity might be achieved by utilizing the S– and O–elimination protocols in combination with dedicated biophysical techniques. LazF

might be an excellent model RiPP biosynthetic enzyme for such studies due to its particularly broad substrate scope.

Finally, in vitro chemoenzymatic Dha installation might be leveraged in a bioengineering context. In particular, LazF-mediated O–elimination on substrates with noncognate LPs proceeds especially cleanly, resulting in the formation of Dha-Xaa-Cys-containing peptides. The reaction is usually complete considerably faster than our standard conditions call for (Fig. S18), resulting in little to no thiol-Dha conjugation products, which makes it a mild and chemoselective method for converting Ser to Dha in peptidic combinatorial libraries. Additionally, LazF is a soluble, well-behaved protein with good expression yields (>15 mg/L culture; unoptimized), and ^{L}Glu-CBT is easily synthetically accessible. dhAAs are one of the most common PTMs in RiPPs[2] and nonribosomal peptides,[66] and thus, we believe that this chemistry may be useful in a variety of bioengineering applications.[25,35,67–71]

Acknowledgements

We thank Prof. Wilfred van der Donk and Prof. Hiroyasu Onaka for the generous gifts of *pRSFDuet-1/His6-nisB-nisA* and *pET-26b-lazF* expression plasmids, respectively. We are also grateful to Dr. Masanobu Nagano and Dr. Yuchen Zhang for stimulating scientific discussions. This work was supported by KAKENHI (JP20K15407 to A.V.; JP16H06444 to H.S. and Y.G.; JP20H05618 to H.S.; JP17H04762, JP19H01014, JP19K22243, JP20H02866 to Y.G.) from the Japan Society for the Promotion of Science.

References

(1) Arnison, P. G.; Bibb, M. J.; Bierbaum, G.; Bowers, A. A.; Bugni, T. S.; Bulaj, G.; Camarero, J. A.; Campopiano, D. J.; Challis, G. L.; Clardy, J.; Cotter, P. D.; Craik, D. J.; Dawson, M.; Dittmann, E.; Donadio, S.; Dorrestein, P. C.; Entian, K.; Gruber, C. W.; Fischbach, M. A.; Garavelli, J. S.; Ulf, G.; Haft, D. H.; Hemscheidt, T. K.; Hertweck, C.; Hill, C.; Horswill, A. R.; Jaspars, M.; Kelly, W. L.; Klinman, J. P.; Kuipers, O. P.; Link, A. J.; Liu, W.; Marahiel, M. A.; Nair, S. K.; Mitchell, D. A.; Moll, G. N.; Moore, B. S.; Rolf, M.; Nes, I. F.; Norris, G. E.; Olivera, B. M.; Onaka, H.; Patchett, M. L.; Piel, J.; Reaney, M. J. T.; Ross, R. P.; Sahl, H.; Schmidt, E. W.; Selsted, M. E.; Severinov, K.; Shen, B.; Sivonen, K.; Smith, L.; Stein, T.; Tagg, J. R.; Tang, G.; Truman, A. W.; Vederas, J. C.; Walsh, C. T.; Walton, J. D.; Wenzel, S. C.; Willey, J. M.; van der Donk, W. A. Ribosomally Synthesized and Post-Translationally Modified Peptide Natural Products: Overview and Recommendations for a Universal Nomenclature. *Nat. Prod. Rep.* **2013**, *30*, 108–160.

(2) Montalbán-López, M.; Scott, T. A.; Ramesh, S.; Rahman, I. R.; van Heel, A. J.; Viel, J. H.; Bandarian, V.; Dittmann, E.; Genilloud, O.; Goto, Y.; Grande Burgos, M. J.; Hill, C.; Kim, S.;

Koehnke, J.; Latham, J. A.; Link, A. J.; Martínez, B.; Nair, S. K.; Nicolet, Y.; Rebuffat, S.; Sahl, H.-G.; Sareen, D.; Schmidt, E. W.; Schmitt, L.; Severinov, K.; Süssmuth, R. D.; Truman, A. W.; Wang, H.; Weng, J.-K.; van Wezel, G. P.; Zhang, Q.; Zhong, J.; Piel, J.; Mitchell, D. A.; Kuipers, O. P.; van der Donk, W. A. New Developments in RiPP Discovery, Enzymology and Engineering. *Nat. Prod. Rep.* **2021**, *38*, 130-239.

(3) Repka, L. M.; Chekan, J. R.; Nair, S. K.; van der Donk, W. A. Mechanistic Understanding of Lanthipeptide Biosynthetic Enzymes. *Chem. Rev.* **2017**, *117*, 5457–5520.

(4) Vinogradov, A. A.; Suga, H. Introduction to Thiopeptides: Biological Activity, Biosynthesis, and Strategies for Functional Reprogramming. *Cell Chem. Biol.* **2020**, *27*, 1032–1051.

(5) Ma, S.; Zhang, Q. Linaridin Natural Products. *Nat. Prod. Rep.* **2020**, *37*, 1152–1163.

(6) Wiebach, V.; Mainz, A.; Siegert, M. A. J.; Jungmann, N. A.; Lesquame, G.; Tirat, S.; Dreux-Zigha, A.; Aszodi, J.; Le Beller, D.; Süssmuth, R. D. The Anti-Staphylococcal Lipolanthines Are Ribosomally Synthesized Lipopeptides. *Nat. Chem. Biol.* **2018**, *14*, 652–654.

(7) Onaka, H.; Nakaho, M.; Hayashi, K.; Igarashi, Y.; Furumai, T. Cloning and Characterization of the Goadsporin Biosynthetic Gene Cluster from Streptomyces Sp. TP-A0584. *Microbiology* **2005**, *151*, 3923–3933.

(8) Bogart, J. W.; Bowers, A. A. Dehydroamino Acids: Chemical Multi-Tools for Late-Stage Diversification. *Org. Biomol. Chem.* **2019**, *17*, 3653–3669.

(9) Garg, N.; Salazar-Ocampo, L. M. A.; van der Donk, W. A. In Vitro Activity of the Nisin Dehydratase NisB. *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110*, 7258–7263.

(10) Ozaki, T.; Kurokawa, Y.; Hayashi, S.; Oku, N.; Asamizu, S.; Igarashi, Y.; Onaka, H. Insights into the Biosynthesis of Dehydroalaninesin Goadsporin. *ChemBioChem* **2016**, *17*, 218–223.

(11) Ortega, M. A.; Hao, Y.; Zhang, Q.; Walker, M. C.; van der Donk, W. A.; Nair, S. K. Structure and Mechanism of the tRNA-Dependent Lantibiotic Dehydratase NisB. *Nature* **2015**, *517*, 509–512.

(12) Zhang, Q.; Yu, Y.; Velasquez, J. E.; van der Donk, W. A. Evolution of Lanthipeptide Synthetases. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 18361–18366.

(13) Shen, X.; Mustafa, M.; Chen, Y.; Cao, Y.; Gao, J. Natural Thiopeptides as a Privileged Scaffold for Drug Discovery and Therapeutic Development. *Med. Chem. Res.* **2019**, *28*, 1063–1098.

(14) Mohr, K. I.; Volz, C.; Jansen, R.; Wray, V.; Hoffmann, J.; Bernecker, S.; Wink, J.; Gerth, K.; Stadler, M.; Müller, R. Pinensins: The First Antifungal Lantibiotics. *Angew. Chem. Int. Ed.* **2015**, *54*, 11254–11258.

(15) Bagley, M. C.; Dale, J. W.; Merritt, E. A.; Xiong, X. Thiopeptide Antibiotics. *Chem. Rev.* **2005**, *105*, 685–714.

(16) Ongey, E. L.; Neubauer, P. Lanthipeptides: Chemical Synthesis versus in Vivo Biosynthesis as Tools for Pharmaceutical Production. *Microb. Cell Fact.* **2016**, *15*, 97.

(17) McAuliffe, O.; Ross, R. P.; Hill, C. Lantibiotics: Structure, Biosynthesis and Mode of Action. *FEMS Microbiol. Rev.* **2001**, *25*, 285–308.

(18) Schwalen, C. J.; Hudson, G. A.; Kille, B.; Mitchell, D. A. Bioinformatic Expansion and

Discovery of Thiopeptide Antibiotics. *J. Am. Chem. Soc.* **2018**, *140*, 9494–9501.

(19) Walker, M. C.; Eslami, S. M.; Hetrick, K. J.; Ackenhusen, S. E.; Mitchell, D. A.; van der Donk, W. A. Precursor Peptide-Targeted Mining of More than One Hundred Thousand Genomes Expands the Lanthipeptide Natural Product Family. *BMC Genomics* **2020**, *21*, 387.

(20) Zhang, Q.; Doroghazi, J. R.; Zhao, X.; Walker, M. C.; van der Donk, W. A. Expanded Natural Product Diversity Revealed by Analysis of Lanthipeptide-like Gene Clusters in Actinobacteria. *Appl. Environ. Microbiol.* **2015**, *81*, 4339–4350.

(21) Ortega, M. A.; Hao, Y.; Walker, M. C.; Donadio, S.; Sosio, M.; Nair, S. K.; van der Donk, W. A. Structure and tRNA Specificity of MibB, a Lantibiotic Dehydratase from Actinobacteria Involved in NAI-107 Biosynthesis. *Cell Chem. Biol.* **2016**, *23*, 370–380.

(22) Bothwell, I. R.; Cogan, D. P.; Kim, T.; Reinhardt, C. J.; van der Donk, W. A.; Nair, S. K. Characterization of Glutamyl-tRNA–Dependent Dehydratases Using Nonreactive Substrate Mimics. *Proc. Natl. Acad. Sci. U. S. A.* **2019**, *116*, 17245–17250.

(23) Mavaro, A.; Abts, A.; Bakkes, P. J.; Moll, G. N.; Driessen, A. J. M.; Smits, S. H. J.; Schmitt, L. Substrate Recognition and Specificity of the NisB Protein, the Lantibiotic Dehydratase Involved in Nisin Biosynthesis. *J. Biol. Chem.* **2011**, *286*, 30552–30560.

(24) Hudson, G. A.; Zhang, Z.; Tietz, J. I.; Mitchell, D. A.; van der Donk, W. A. In Vitro Biosynthesis of the Core Scaffold of the Thiopeptide Thiomuracin. *J. Am. Chem. Soc.* **2015**, *137*, 16012−16015.

(25) Vinogradov, A. A.; Shimomura, M.; Goto, Y.; Sugai, Y.; Suga, H.; Onaka, H. Minimal Lactazole Scaffold for in Vitro Thiopeptide Bioengineering. *Nat. Commun.* **2020**, *11*, 2272.

(26) Fleming, S. R.; Bartges, T. E.; Vinogradov, A. A.; Kirkpatrick, C. L.; Goto, Y.; Suga, H.; Hicks, L. M.; Bowers, A. A. Flexizyme-Enabled Benchtop Biosynthesis of Thiopeptides. *J. Am. Chem. Soc.* **2019**, *141*, 758–762.

(27) Plat, A.; Kluskens, L. D.; Kuipers, A.; Rink, R.; Moll, G. N. Requirements of the Engineered Leader Peptide of Nisin for Inducing Modification, Export, and Cleavage. *Appl. Environ. Microbiol.* **2011**, *77*, 604–611.

(28) Khusainov, R.; van Heel, A. J.; Lubelski, J.; Moll, G. N.; Kuipers, O. P. Identification of Essential Amino Acid Residues in the Nisin Dehydratase NisB. *Front. Microbiol.* **2015**, *6*, 102.

(29) Morris, R. P.; Leeds, J. A.; Naegeli, H. U.; Oberer, L.; Memmert, K.; Weber, E.; Lamarche, M. J.; Parker, C. N.; Burrer, N.; Esterow, S.; Hein, A. E.; Schmitt, E. K.; Krastel, P. Ribosomally Synthesized Thiopeptide Antibiotics Targeting Elongation Factor Tu. *J. Am. Chem. Soc.* **2009**, *131*, 5946–5955.

(30) Hayashi, S.; Ozaki, T.; Asamizu, S.; Ikeda, H.; Omura, S.; Oku, N.; Igarashi, Y.; Tomoda, H.; Onaka, H. Genome Mining Reveals a Minimum Gene Set for the Biosynthesis of 32-Membered Macrocyclic Thiopeptides Lactazoles. *Chem. Biol.* **2014**, *21*, 679–688.

(31) Vinogradov, A. A.; Shimomura, M.; Kano, N.; Goto, Y.; Onaka, H.; Suga, H. Promiscuous Enzymes Cooperate at the Substrate Level En Route to Lactazole A. *J. Am. Chem. Soc.* **2020**, *142*, 13886−13897.

(32) Goto, Y.; Katoh, T.; Suga, H. Flexizymes for Genetic Code Reprogramming. *Nat. Protoc.* **2011**, *6*, 779–790.

(33) Goto, Y.; Ito, Y.; Kato, Y.; Tsunoda, S.; Suga, H. One-Pot Synthesis of Azoline-Containing Peptides in a Cell-Free Translation System Integrated with a Posttranslational Cyclodehydratase. *Chem. Biol.* **2014**, *21*, 766–774.

(34) Ozaki, T.; Yamashita, K.; Goto, Y.; Shimomura, M.; Hayashi, S.; Asamizu, S.; Sugai, Y.; Ikeda, H.; Suga, H.; Onaka, H. Dissection of Goadsporin Biosynthesis by in Vitro Reconstitution Leading to Designer Analogues Expressed in Vivo. *Nat. Commun.* **2017**, *8*, 14207.

(35) Montalbán-López, M.; van Heel, A. J.; Kuipers, O. P. Employing the Promiscuity of Lantibiotic Biosynthetic Machineries to Produce Novel Antimicrobials. *FEMS Microbiol. Rev.* **2017**, *41*, 5–18.

(36) Vinogradov, A. A.; Nagai, E.; Chang, J. S.; Narumi, K.; Onaka, H.; Goto, Y.; Suga, H. Accurate Broadcasting of Substrate Fitness for Lactazole Biosynthetic Pathway from Reactivity-Profiling mRNA Display. *J. Am. Chem. Soc.* **2020**, *142*, 20329–20334.

(37) Zhang, Z.; Hudson, G. A.; Mahanta, N.; Tietz, J. I.; van der Donk, W. A.; Mitchell, D. A. Biosynthetic Timing and Substrate Specificity for the Thiopeptide Thiomuracin. *J. Am. Chem. Soc.* **2016**, *138*, 15511–15514.

(38) Strieker, M.; Nolan, E. M.; Walsh, C. T.; Marahiel, M. A. Stereospecific Synthesis of Threo- and Erythro-β-Hydroxyglutamic Acid During Kutzneride Biosynthesis. *J. Am. Chem. Soc.* **2009**, *131*, 13523–13530.

(39) Nanda, J.; Rubinov, B.; Ivnitski, D.; Mukherjee, R.; Shtelman, E.; Motro, Y.; Miller, Y.; Wagner, N.; Cohen-Luria, R.; Ashkenasy, G. Emergence of Native Peptide Sequences in Prebiotic Replication Networks. *Nat. Commun.* **2017**, *8*, 434.

(40) Agouridas, V.; El Mahdi, O.; Diemer, V.; Cargoët, M.; Monbaliu, J. C. M.; Melnyk, O. Native Chemical Ligation and Extended Methods: Mechanisms, Catalysis, Scope, and Limitations. *Chem. Rev.* **2019**, *119*, 7328–7443.

(41) Johnson, E. C. B.; Kent, S. B. H. Insights into the Mechanism and Catalysis of the Native Chemical Ligation Reaction. *J. Am. Chem. Soc.* **2006**, *128*, 6640–6646.

(42) Nagano, M.; Huang, Y.; Obexer, R.; Suga, H. One-Pot In Vitro Ribosomal Synthesis of Macrocyclic Depsipeptides. *J. Am. Chem. Soc.* **2021**, *143*, 4741–4750.

(43) Ruffner, D. E.; Schmidt, E. W.; Heemstra, J. R. Assessing the Combinatorial Potential of the RiPP Cyanobactin Tru Pathway. *ACS Synth. Biol.* **2015**, *4*, 482–492.

(44) Young, T. S.; Dorrestein, P. C.; Walsh, C. T. Codon Randomization for Rapid Exploration of Chemical Space in Thiopeptide Antibiotic Variants. *Chem. Biol.* **2012**, *19*, 1600–1610.

(45) Zhang, F.; Kelly, W. L. Saturation Mutagenesis of TsrA Ala4 Unveils a Highly Mutable Residue of Thiostrepton A. *ACS Chem. Biol.* **2015**, *10*, 998–1009.

(46) Wade, R. C.; Gabdoulline, R. R.; Lüdemann, S. K.; Lounnas, V. Electrostatic Steering and Ionic Tethering in Enzyme-Ligand Binding: Insights from Simulations. *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95*, 5942– 5949.

(47) Zhang, F.; Li, C.; Kelly, W. L. Thiostrepton Variants Containing a Contracted Quinaldic Acid Macrocycle Result from Mutagenesis of the Second Residue. *ACS Chem. Biol.* **2016**, *11*, 415–424.

(48) Si, Y.; Kretsch, A. M.; Daigh, L. M.; Burk, M. J.; Mitchell, D. A. Cell-Free Biosynthesis to Evaluate Lasso Peptide Formation and Enzyme-Substrate Tolerance. *J. Am. Chem. Soc.* **2021**, *143*, 5917–15927.

(49) Duan, P.; Zheng, Q.; Lin, Z.; Wang, S.; Chen, D.; Liu, W. Molecular Engineering of Thiostrepton: Via Single “Base”-Based Mutagenesis to Generate Side Ring-Derived Variants. *Org. Chem. Front.* **2016**, *3*, 1254–1258.

(50) Burkhart, B. J.; Hudson, G. A.; Dunbar, K. L.; Mitchell, D. A. A Prevalent Peptide-Binding Domain Guides Ribosomal Natural Product Biosynthesis. *Nat. Chem. Biol.* **2015**, *11*, 564–570.

(51) Repka, L. M.; Hetrick, K. J.; Chee, S. H.; van der Donk, W. A. Characterization of Leader Peptide Binding during Catalysis by the Nisin Dehydratase NisB. *J. Am. Chem. Soc.* **2018**, *140*, 4200–4203.

(52) Koehnke, J.; Mann, G.; Bent, A. F.; Ludewig, H.; Shirran, S.; Botting, C.; Lebl, T.; Houssen, W. E.; Jaspars, M.; Naismith, J. H. Structural Analysis of Leader Peptide Binding Enables Leader-Free Cyanobactin Processing. *Nat. Chem. Biol.* **2015**, *11*, 558–563.

(53) Grove, T. L.; Himes, P. M.; Hwang, S.; Yumerefendi, H.; Bonanno, B.; Kuhlman, B.; Almo, S. C.; Bowers, A. A. Structural Insights into Thioether Bond Formation in the Biosynthesis of Sactipeptides. *J. Am. Chem. Soc.* **2017**, *139*, 11734–11744.

(54) Oman, T. J.; van der Donk, W. A. Follow the Leader: The Use of Leader Peptides to Guide Natural Product Biosynthesis. *Nat. Chem. Biol.* **2010**, *6*, 9–18.

(55) Ongpipattanakul, C.; Nair, S. K. Molecular Basis for Autocatalytic Backbone N-Methylation in RiPP Natural Product Biosynthesis. *ACS Chem. Biol.* **2018**, *13*, 2989–2999.

(56) Sardar, D.; Pierce, E.; McIntosh, J. A.; Schmidt, E. W. Recognition Sequences and Substrate Evolution in Cyanobactin Biosynthesis. *ACS Synth. Biol.* **2015**, *4*, 167–176.

(57) Song, H.; Van Der Velden, N. S.; Shiran, S. L.; Bleiziffer, P.; Zach, C.; Sieber, R.; Imani, A. S.; Krausbeck, F.; Aebi, M.; Freeman, M. F.; Riniker, S.; Künzler, M.; Naismith, J. H. A Molecular Mechanism for the Enzymatic Methylation of Nitrogen Atoms within Peptide Bonds. *Sci. Adv.* **2018**, *4*, eaat2720.

(58) Dong, S.-H.; Liu, A.; Mahanta, N.; Mitchell, D. A.; Nair, S. K. Mechanistic Basis for Ribosomal Peptide Backbone Modifications. *ACS Cent. Sci.* **2019**, *5*, 842–851.

(59) Ghilarov, D.; Stevenson, C. E. M.; Travin, D. Y.; Piskunova, J.; Serebryakova, M.; Maxwell, A.; Lawson, D. M.; Severinov, K. Architecture of Microcin B17 Synthetase: An Octameric Protein Complex Converting a Ribosomally Synthesized Peptide into a DNA Gyrase Poison. *Mol. Cell* **2019**, *73*, 749-762.e5.

(60) Sumida, T.; Dubiley, S.; Wilcox, B.; Severinov, K.; Tagami, S. Structural Basis of Leader Peptide Recognition in Lasso Peptide Biosynthesis Pathway. *ACS Chem. Biol.* **2019**, *14*, 1619–1627.

(61) Chekan, J. R.; Ongpipattanakul, C.; Nair, S. K. Steric Complementarity Directs Sequence Promiscuous Leader Binding in RiPP Biosynthesis. *Proc. Natl. Acad. Sci. U. S. A.* **2019**, *116*, 24049–24055.

(62) Cogan, D. P.; Hudson, G. A.; Zhang, Z.; Pogorelov, T. V; van der Donk, W. A.; Mitchell, D. A.; Nair, S. K. Structural Insights into Enzymatic [4+2] Aza- Cycloaddition in Thiopeptide Antibiotic

Biosynthesis. *Proc. Natl. Acad. Sci. U. S. A.* **2017**, *114*, 12928–12933.

(63) Hegemann, J. D.; Süssmuth, R. D. Matters of Class: Coming of Age of Class III and IV Lanthipeptides. *RSC Chem. Biol.* **2020**, *1*, 110–127.

(64) Wiebach, V.; Mainz, A.; Schnegotzki, R.; Siegert, M. A. J.; Hügelland, M.; Pliszka, N.; Süssmuth, R. D. An Amphipathic Alpha-Helix Guides Maturation of the Ribosomally-Synthesized Lipolanthines. *Angew. Chem. Int. Ed.* **2020**, *59*, 16777–16785.

(65) Kelley, L. A.; Mezulis, S.; Yates, C. M.; Wass, M. N.; Sternberg, M. J. The Phyre2 Web Portal for Protein Modeling, Prediction and Analysis. *Nat. Protoc.* **2015**, *10*, 845–858.

(66) Süssmuth, R. D.; Mainz, A. Nonribosomal Peptide Synthesis — Principles and Prospects. *Angew. Chem., Int. Ed.* **2017**, *56*, 3770–3821.

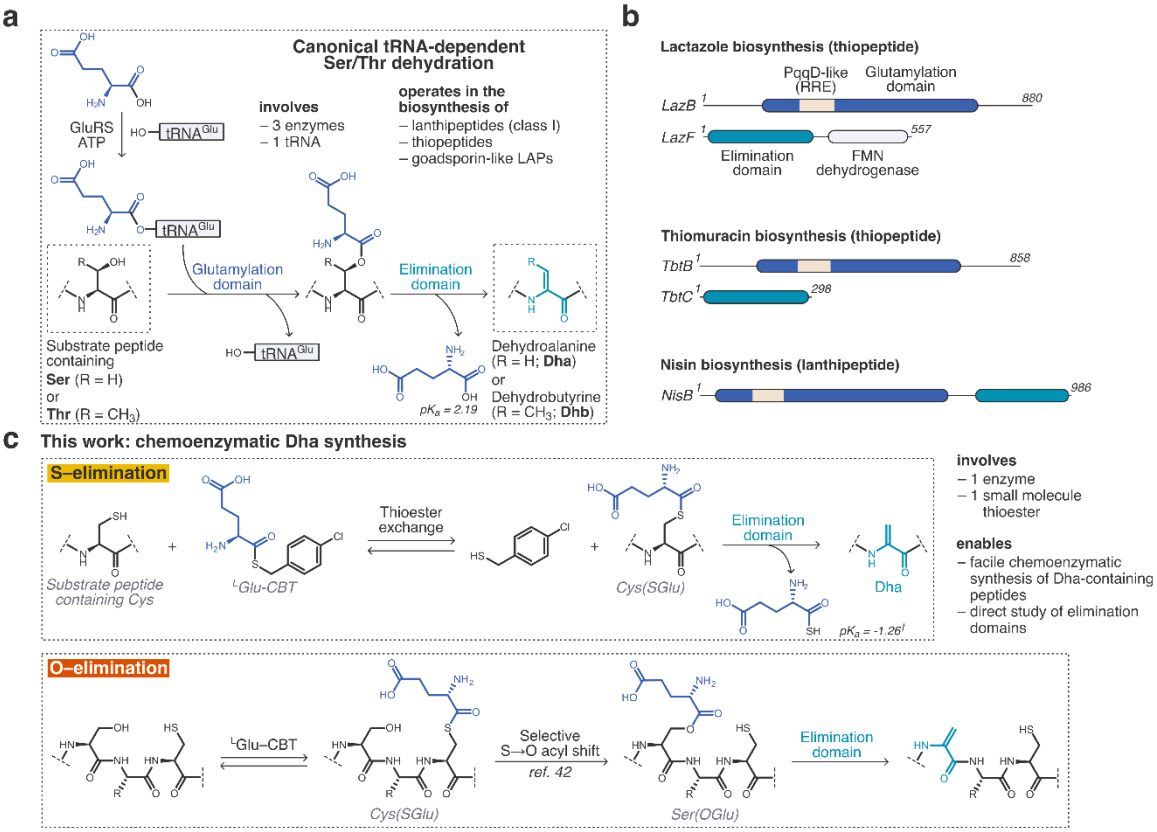
(67) Schmitt, S.; Montalbán-López, M.; Peterhoff, D.; Deng, J.; Wagner, R.; Held, M.; Kuipers, O. P.; Panke, S. Analysis of Modular Bioengineered Antimicrobial Lanthipeptides at Nanoliter Scale. *Nat. Chem. Biol.* **2019**, *15*, 437–443.

(68) Yang, X.; Lennard, K. R.; He, C.; Walker, M. C.; Ball, A. T.; Doigneaux, C.; Tavassoli, A.; van der Donk, W. A. A Lanthipeptide Library Used to Identify a Protein-Protein Interaction Inhibitor. *Nat. Chem. Biol.* **2018**, *14*, 375–380.

(69) Urban, J. H.; Moosmeier, M. A.; Aumüller, T.; Thein, M.; Bosma, T.; Rink, R.; Groth, K.; Zulley, M.; Siegers, K.; Tissot, K.; Moll, G. N.; Prassler, J. Phage Display and Selection of Lanthipeptides on the Carboxy-Terminus of the Gene-3 Minor Coat Protein. *Nat. Commun.* **2017**, *8*, 1500.

(70) Hetrick, K. J.; Walker, M. C.; van der Donk, W. A. Development and Application of Yeast and Phage Display of Diverse Lanthipeptides. *ACS Cent. Sci.* **2018**, *4*, 458–467.

(71) Hegemann, J. D.; Bobeica, S. C.; Walker, M. C.; Bothwell, I. R.; van der Donk, W. A. Assessing the Flexibility of the Prochlorosin 2.8 Scaffold for Bioengineering Applications. *ACS Synth. Biol.* **2019**, *8*, 1204–1214.



586

587 **Figure 1.** The concept of the study. a) The canonical pathway to Dha/Dhb formation via tRNA-
588 dependent dehydration of Ser/Thr residues. The pathway, which involves 3 enzymes and 1 tRNA,
589 operates during biosynthesis of many biologically active RiPP natural products, including all
590 thiopeptides and class I lantibiotics. b) Domain organization of Ser/Thr dehydratases from lactazole,
591 thiomuracin and nisin biosynthetic gene clusters. c) The generalized schemes for S– and O–
592 elimination reactions. This chemistry obviates the use of the GluRS/tRNA/GD system for the ED
593 substrate preparation, allowing for direct assaying of EDs. † The pKa value for α-thioglutamate was
594 predicted in ChemAxon (Chemicalize tool).

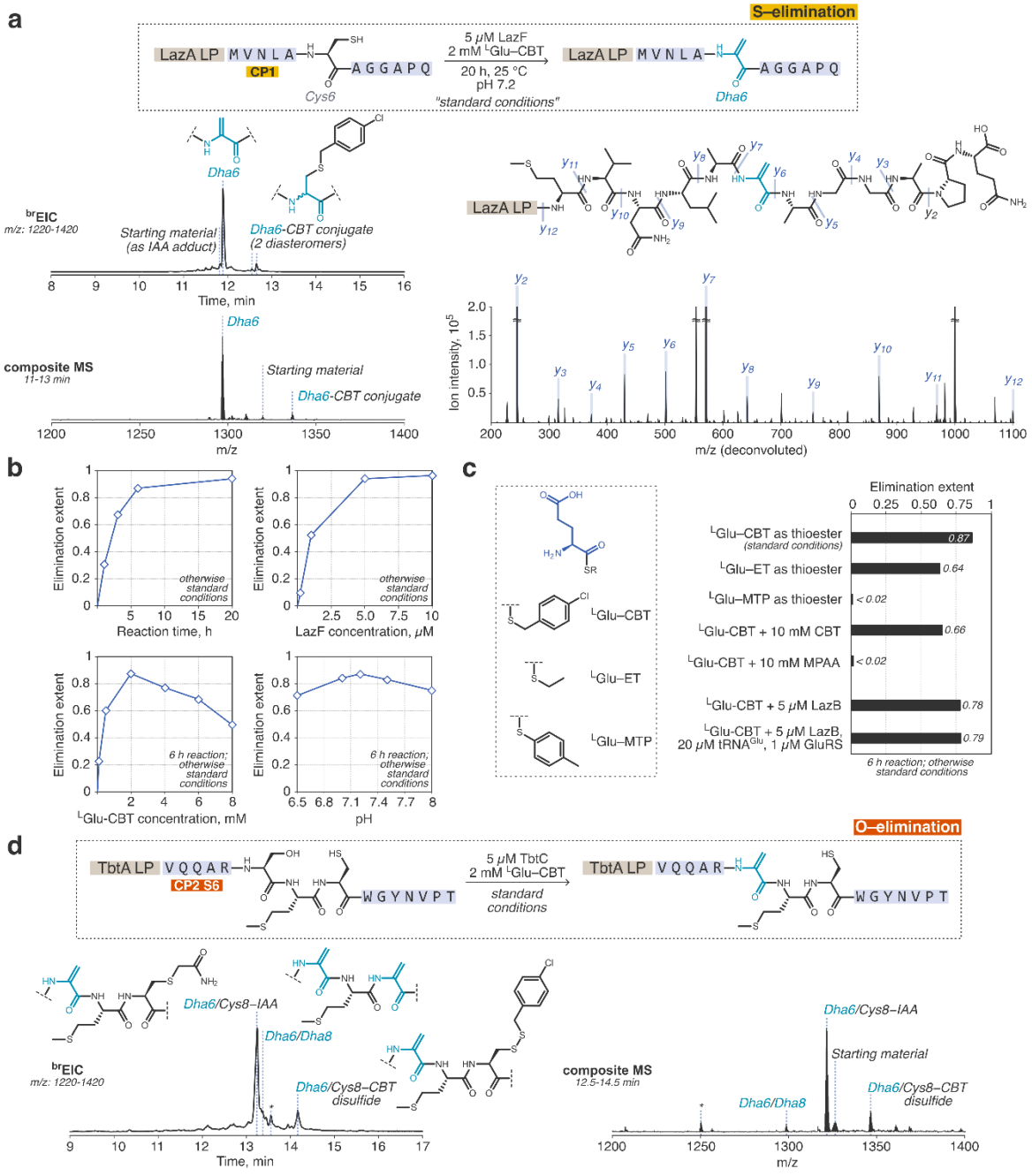


Figure 2. Development of the optimal S– and O–elimination conditions. a) Under the standard conditions (LazF as the enzyme), S–elimination proceeds to cleanly furnish Dha-containing peptides. The peptide LazA^{LP}/CP1 was in vitro translated using the FIT system, incubated with LazF and ^{L}Glu–CBT under the standard conditions, and the outcomes were analyzed by LC-MS as described in S.I. 2.4–2.6. A ^{br}EIC chromatogram (see S.I. 2.6 for details) and a composite MS spectrum integrated over substrate-derived peaks showing the overall product distribution are displayed. The zoomed-in section of a charge-deconvoluted CID fragmentation spectrum for the product assigned as Dha6 is also shown. The spectral inset shows the low molecular weight region with relevant y-ion assignments.

Fragmentation annotations show a good match with the expected product structure, confirming the structural assignment. b,c) Development of the standard reaction conditions. Reactions were performed and analyzed as described in panel a). Combined, these data suggest the use of the standard conditions for performing S–elimination with LazF. d) Under the standard conditions TbtC catalyzes O–elimination resulting in a clean Ser to Dha conversion for substrates containing a Ser-Xaa-Cys motif. The reaction was performed and analyzed as described in panel a), with $TbtA^{LP}$/CP2 S6 as the substrate and TbtC as the enzyme. The peak labelled with an asterisk (*) corresponds to a translation-derived truncation in $TbtA^{LP}$/CP2 S6.

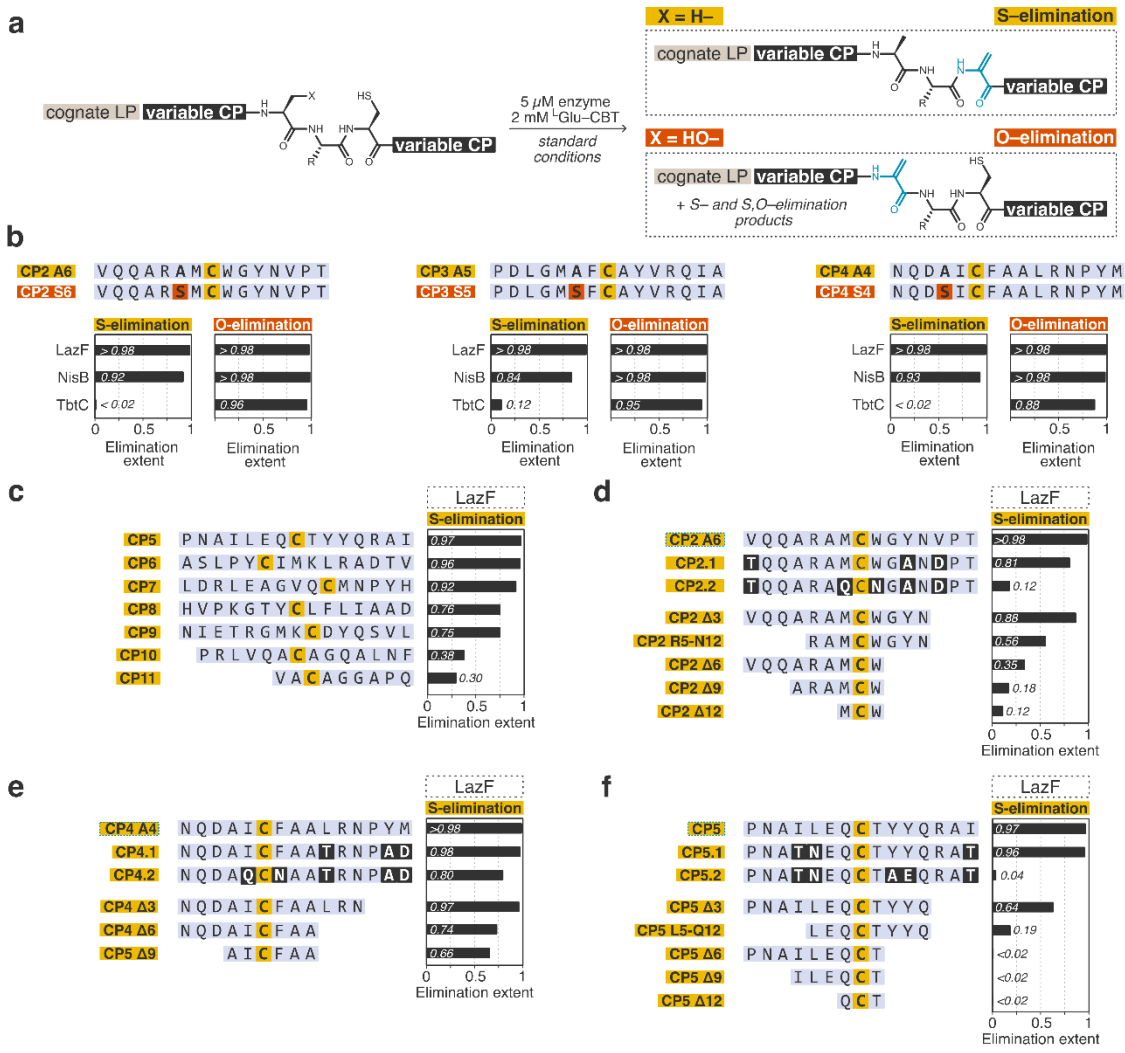
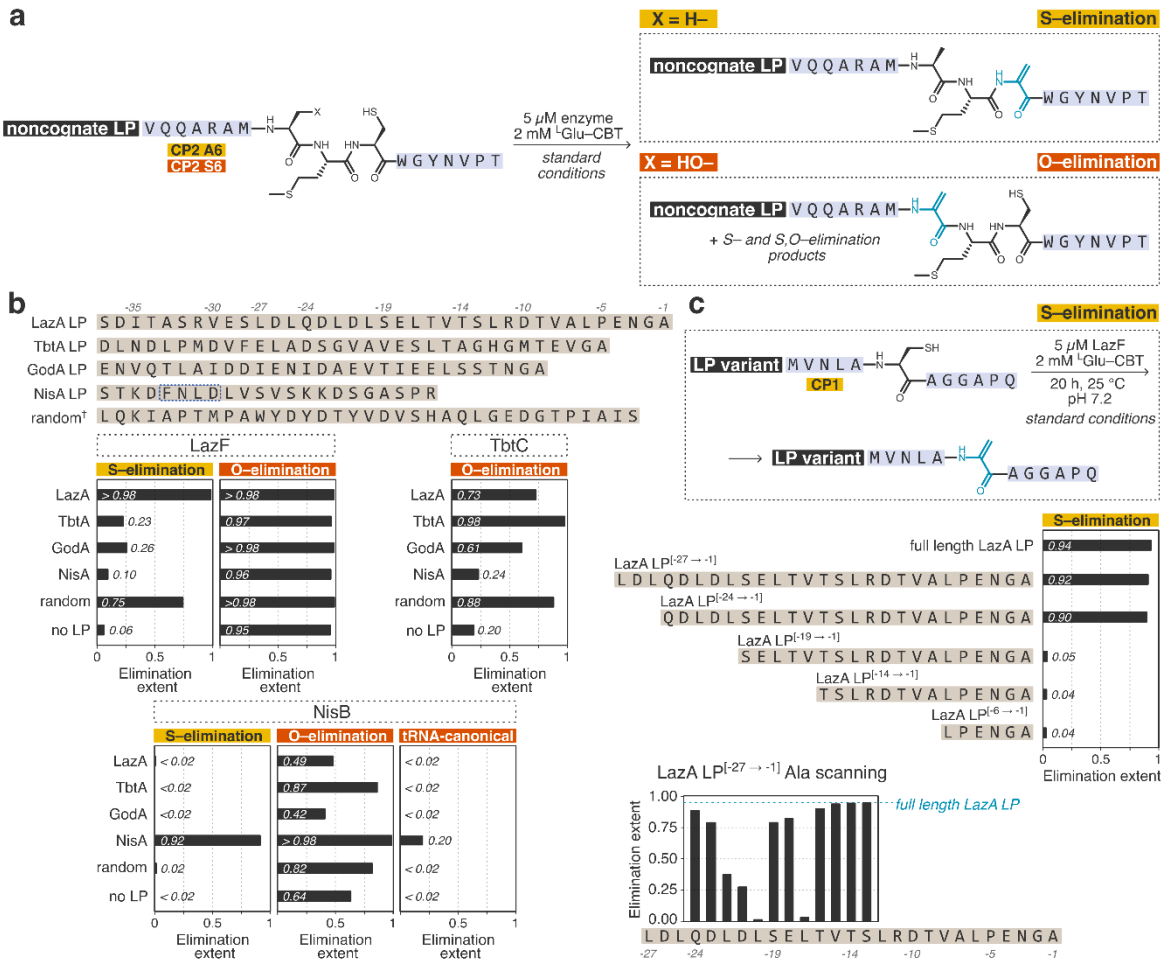


Figure 3. Core peptide specificity study. a) The design of the study. Peptide substrates equipped with a cognate LP sequence and a randomized CP containing either Ala-Xaa-Cys (for S–elimination) or Ser-Xaa-Cys (for O–elimination) were in vitro translated using the FIT system, and incubated with the appropriate enzyme and LGlu–CBT under the standard conditions. Reaction outcomes were analyzed by LC-MS as described in S.I. 2.4–2.6, and summarized as the elimination extent values reported here. b) Summary of S– and O–elimination reactions performed for LazF, NisB and TbtC. c–f) Summary of S–elimination studies for LazF. The data suggest that nonspecific hydrophobic contacts between LazF and the CP aid in the substrate recognition and/or catalysis.



621

622 **Figure 4**. Leader peptide specificity study. a) The design of the study. Peptide substrates equipped
623 with CP2 A6 (for S–elimination) or CP2 S6 (for O–elimination) and noncognate LP sequences were
624 in vitro translated using the FIT system, and incubated with the appropriate enzyme and ^{L}Glu–CBT
625 under the standard conditions. Reaction outcomes were analyzed by LC-MS as described in S.I. 2.4–
626 2.6, and summarized as the elimination extent values reported here. b) Primary amino acid
627 sequences of studied LP variants. For NisA, the FNLD box described in the text is highlighted in blue.
628 Also shown are the results of the specificity study for LazF, NisB and TbtC. c) Identification of amino
629 acids in LazA^{LP} critical for the recruitment by LazF. Reactions were performed and analyzed as in
630 panel a). The data point to the $^{-22}$LDLxxL^{-17} sequence as the primary recognition motif in LazA^{LP}. Note
631 that this motif is not required by LazF for O–elimination reactions (data in panel b)). † See S.I. 2.2 for
632 the choice of sequence.

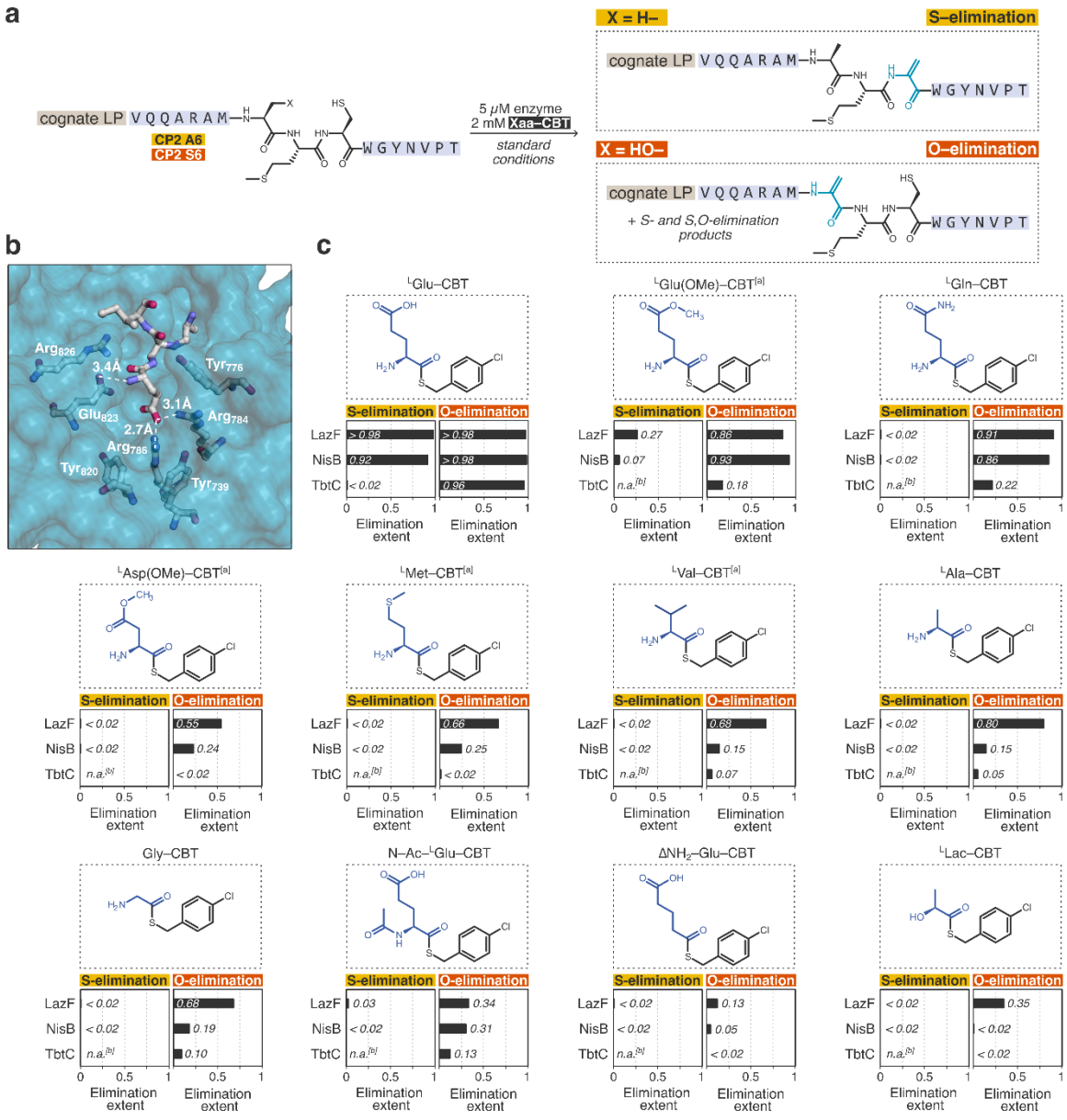


Figure 5. Specificity of EDs toward various Cys(S-acyl) and Ser(O-acyl) substrates. Peptide substrates equipped with a cognate LP sequence and either CP2 A6 (for S–elimination) or CP2 S6 (for O–elimination) were in vitro translated using the FIT system, and incubated with the appropriate enzyme and various thioesters under the standard conditions. Reaction outcomes were analyzed by LC-MS as described in S.I. 2.4–2.6, and summarized as the elimination extent values reported here. b) Cocrystal structure between NisB and Dap(NHGlu), a noneliminable substrate analog (PDB 6M7Y). A zoomed-in view of the NisB ED active site bound to Dap(NHGlu) is displayed. Residues participating in the substrate recognition are highlighted together with prominent substrate/enzyme interactions. c) Summary of S– and O–elimination reactions performed with LGlu–CBT and 10 of its analogs. These results indicate that the interactions highlighted in panel b) are not absolutely required

644 for productive elimination. [a]: The thioester was not soluble to 2 mM, and was used as a saturated
645 solution in reaction buffer. [b]: The reaction was not performed.