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Butelase-1 as the Prototypical Peptide Asparaginyl Ligase and Its Applications: A Review

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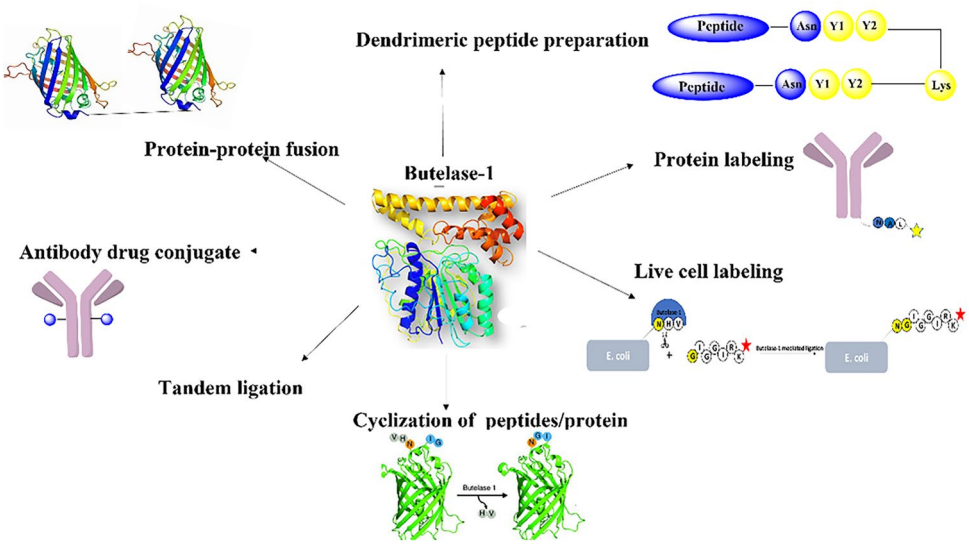
Abstract

Peptide ligation strategies are important biotechnological tools. Examples of applications using peptide ligation strategies include cyclization or pegylation of protein drugs for stability improvement, protein labeling for basic research, and preparation of protein constructs with unusual architectures such as antibody drug conjugate. Traditionally, peptide ligation is achieved by chemical methods which may need harsh conditions incompatible with biomolecules. More recently, enzymatic ligation based on the use of peptide ligases is gaining increasing attention owing to its intrinsic specificity and mild reaction conditions. Here, we review the features and applications of one particular peptide ligase, butelase-1—a prototypical and super-efficient peptide asparaginyl ligase.

Graphical Abstract



Keywords Asparaginyl endopeptidases · Peptide asparaginyl ligases · Butelase-1 · Hydrolysis · Transpeptidase · Intramolecular ligation · Intermolecular ligation · Tandem ligation

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Introduction

The rapid development of peptide- and protein-based therapeutics has benefited greatly from the advancement of recombinant DNA technology and chemical peptide synthesis. However, these two technologies have their intrinsic shortcomings. There is a size limitation for chemical peptide synthesis

(Merrifield 1963, 1985) and proteins with site-specific modifications are not accessible by the traditional recombinant technology. Combination of these two methods would be conducive to addressing either method's own limitations. To achieve this, different peptide/protein ligation strategies have been developed.

In the past decades, peptide ligation chemistries have been extensively explored by the peptide/protein chemistry community. Methods developed include prior thiol capture (Kemp et al. 1981, 1986; Kemp and Kerkman 1981), thiazolidine-mediated ligation (Liu and Tam 1994), native chemical ligation (NCL) (Dawson et al. 1994; Tam et al. 1995), thioacid capture ligation (Liu et al. 1996; Nomura et al. 2021), sugar-assisted glycopeptide ligations (Brik and Wong 2007; Payne et al. 2008), traceless Staudinger ligation (Saxon et al. 2000), peptide hydrazide ligation (Fang et al. 2011, ketoacid-hydroxylamine ligation (KAHA) (Bode et al. 2006), serine/threonine peptide ligation (Li et al. 2010). Chemical peptide ligations generally involve two steps. The first step is formation of a new covalent bond between an orthogonal pair of functionalities, bringing together two peptides (intermolecular ligation) or two ends of a same peptide (intramolecular ligation). The second step is a proximity-driven acyl transfer which results in the formation of a peptide bond at the ligation site.

Besides chemical ligation, enzymatic ligation is gaining increasing popularity. Enzymatic ligation relies on the use of peptide ligases which can recognize and modify specific peptide sequences. Peptide ligases, unlike the ubiquitous peptide bond-breaking proteases, catalyze the reverse reaction which is peptide bond-formation. They are suitable protein engineering tools because of their capabilities to work under mild, aqueous conditions and exquisite site-specificity, which is crucial for precision manufacture of peptide/protein-based drugs and antibody–drug conjugates. Despite their usefulness, the scarcity of peptide ligases has impeded their applications in the past. With the advancement of technologies such as protein engineering and bioinformatics, more peptide ligases have been discovered, which has thus spurred the development of enzymatic peptide ligation methods. Well-known examples of peptide ligase include transpeptidase sortase A (Mazmanian et al. 1999; Popp and Ploegh 2011) and subtilisin-derived subtiligase (Neet and Koshland 1966; Polgar and Bender 1967; Nakatsuka et al. 1987; Wu and Hilvert 1989; Abrahmsén et al. 1991; Chang et al. 1994). One group of peptide ligases that recently has received a lot of attention are asparaginyl endopeptidases (AEPs) which are involved int the production of ribosomally synthesized and posttranslationally modified peptides (RiPPs) (Arnison et al. 2013). Many RiPPs are head-to-tail cyclic in structure which require an enzymatic posttranslational modification from their linear precursors.

There have been quite a few excellent reviews covering AEPs and other peptide ligases, some of which mainly focus on their capabilities and applications in cyclizing peptides/proteins (Schmidt et al. 2017a, b; Schmidt et al. 2017a, b; James et al. 2018; Xu et al. 2018; Tam et al. 2020; Rehm et al. 2021; Tang and Luk 2021). In this review, we focus on one particular AEP-type ligase, butelase-1 (Nguyen et al. 2014). Butelase-1 is the prototypical AEP-like ligase and has many unique advantages including the highest catalytic efficiency among all the peptide ligases found so far and "traceless" feature. The following aspects of butelase-1 are discussed in this review: the discovery, the enzyme's feature, the structure activity relationships (SAR), and the most updated summary of applications by intramolecular ligation, intermolecular ligation, and combination with other ligation/conjugation strategies.

Characteristics of Butelase-1

Asparaginyl Endopeptidases

AEPs, also known as legumains or vacuolar processing enzymes, are cysteine endopeptidases that cleave at the C-terminal side of an Asx (Asn or Asp) residue in acidic conditions upon recognizing a specific motif (Abe et al. 1993). They are generally expressed as inactive zymogens containing a signal peptide, an N-terminal pro-domain, an active core domain, and a C-terminal pro-domain which "caps" the active site of the core domain (Fig. 1). The zymogens get auto-activated at low pH by removing the pro-domains on both N- and C-terminus to expose the substrate-binding surface of the active core domain (Zauner et al. 2018; Zhao et al. 2014). This hydrolytic activity of AEPs relies on the catalytic cysteine residue attacking the Asx-peptide bond of the substrate to from a thioester-linked acyl-enzyme intermediate which is subsequently hydrolyzed (Fig. 2).

Most AEPs are predominantly hydrolases. Some, although quite rare, prefer peptide α-amines over water molecules as incoming nucleophiles to resolve the thioester intermediate at around neutral pH, forming a new peptide

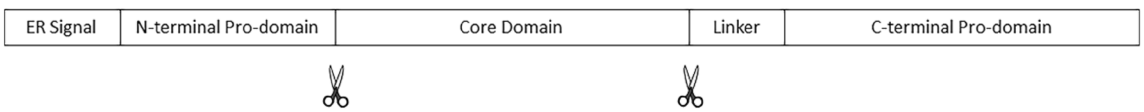

Fig. 1 General domain structure of zymogenic asparaginyl endopeptidase and its autoactivation process

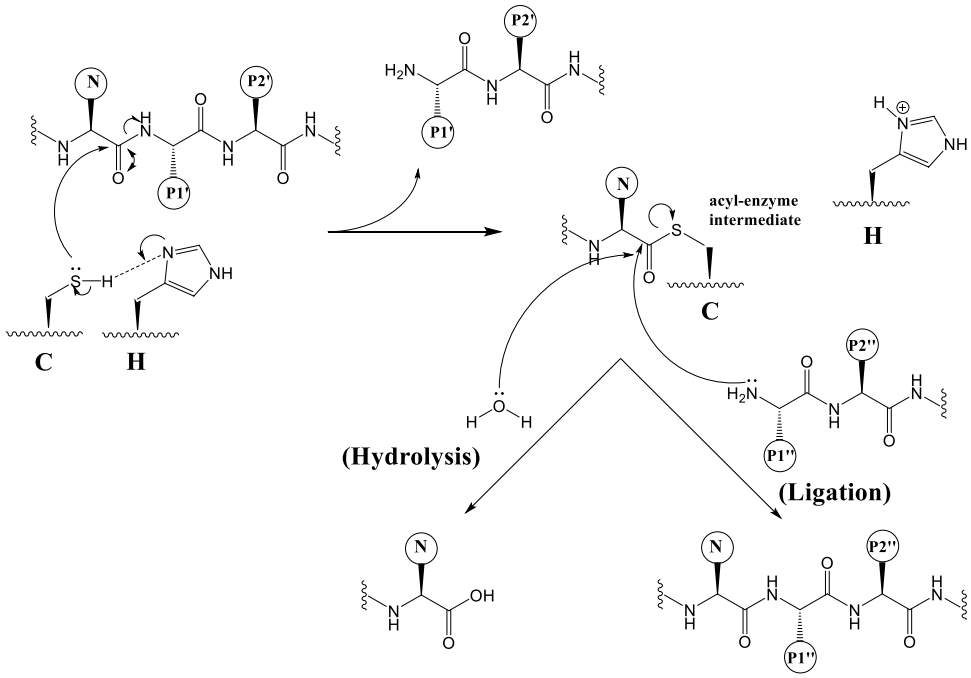

Fig. 2 Molecular mechanism of hydrolysis/ligation of AEPs/PALs

bond. To differentiate them from other AEPs and emphasize their transpeptidase activity, this unique group of AEP members are generally referred to as peptide asparaginyl ligases (PALs). Although AEPs and PALs show high similarities in structure, PALs have evolved subtle changes to reduce the hydrolytic activity as compared with the proteolytic AEPs (Hemu et al. 2019a, 2020b; Jackson et al. 2018; Yang et al. 2017). It is worth mentioning that PALs also have certain degree of hydrolytic activity at low pH, because, as a subgroup of AEPs, they also need to remove the flanking prodomains through hydrolysis for autoactivation (Hemu et al. 2019a, 2020b).

So far three key regions have been proposed to affect the catalytic activity of AEPs/PALs. The first region lies in S2 binding pocket. In the study of OaAEP1b, a PAL derived from *Oldenlandia affinis*, Yang and coworkers found that Cys247 near the catalytic site plays a "gatekeeping" role (Yang et al. 2017). This residue has been found to control access to the S2 substrate-binding pocket as mutation of this residue to larger residues decreased the ligation catalytic efficiency of OaAEP1 while its substitution with Ala increased the ligation catalytic efficiency by over 100 folds. What is interesting is that mutation of this site to glycine led to increased hydrolytic activity. Later study done by Hemu et al. (2019a) found that the "gatekeeper" residue alone might not be enough to determine the protease-ligase balance as butelase-1 and *Vy*PAL—both being very powerful ligases—have a bulky "gatekeeper" residue (Val or Ile). Moreover, VyPAL isoforms, despite having similar "gatekeeper" residues (Ile and Val), display very different enzymatic activities. Therefore, additional surrounding residues, together with the "gatekeeper" residue, are later referred to as ligase-activity determinant 1 (LAD1). The second key region, referred as ligase activity determinant 2 (LAD2), lies in the S1' pocket (A174 and P175 in *Vy*PAL1-3) (Hemu et al. 2019a). It was found that ligases favor small residue as the first residue of the dipeptide of LAD2 such as Gly-Ala/Ala-Ala/Ala-Pro. Besides the above two regions, residues in the polyproline loop and a deletion in a loop region (α5-β6 loop), together referred to as marker of ligase activity (MLA), also play an important role in determining the ligation efficiency of AEPs (Jackson et al. 2018). This finding was based on the mutagenesis study on the different isoforms of PxAEPs. Isoform with predominantly ligation activity was found to have a shortened MLA, while the hydrolytic isoform had a longer and more hydrophilic MAL. Substitution of the shorter MLA of the ligase OaAEP1b with a longer MLA from the protease OaAEP2 slowed down the reaction efficiency but not shifted the cyclization/hydrolysis balance. Taking advantage of the knowledge regarding the structure–activity relationship of AEPs, Tam's group successfully engineered a peptide ligase from the hydrolysis-preferring butelase-2 by two substitutions (Gly-Pro to Gly-Ala in LAD2 and Gly to Ala in LAD1) (Hemu et al. 2020b).

Currently, only a handful PALs have been discovered which include butelase-1 (Nguyen et al. 2014), OaAEP1b/3–4 (Harris et al. 2015, 2019), [C247A]OaAEP1 (Yang et al. 2017), HeAEP3 (Jackson et al. 2018), and VyPAL2 (Hemu et al. 2019a) (Table 1). Of all these PALs, butelase-1 is the first reported one and also exhibits the highest catalytic efficiency. Butelase-1 was first isolated from the medicinal plant *Clitoria ternatea* (butterfly pea) by Tam's

group in 2014. The name butelase is derived from the Malay name of butterfly pea, *Bu*nga *tel*ang plus lig*ase*. As a PAL, butelase-1 belongs to the C13 subfamily like all AEPs. Naturally butelase-1 is involved in the bioproduction of cyclotides which belong to a family of circular cysteine-rich plant defense peptides. In this process, butelase-1 recognizes a tripeptide motif, Asx (Asn/Asp)-His-Val, at the C-terminus of the linear precursor, cleaves the peptide bond after Asx by forming a thioester intermediate, which is subsequently attacked by the nucleophilic N-terminal amine in an S-to-N acyl transfer reaction, forming a new peptide bond. At its active site, butelase-1 has a conserved Cys and His as the catalytic dyad. Study has revealed that butelase-1 recognizes a relatively broad spectrum of peptide motifs (Nguyen et al. 2014; Nguyen et al. 2015a, b). The C-terminal tripeptide sorting sequence obeys the rule of Asx (Asn or Asp)-Xaa (any amino acid except Pro)-Yaa (bulky hydrophobic residue including Leu, Val, or Ile). As for nucleophile substrates, almost all the N-terminal natural amino acids can be recognized by butelase-1 with Pro as the sole exception. Butelase-1 has been shown as a very suitable ligase for enzymatic peptide ligations for the following reasons: (1) Butelase-1-mediated reaction is nearly traceless since it only leaves an Asx at the junction site, a feature quite desirable for preparation of naturally occurring peptides/proteins. (2) Butelase-1 can tolerate unnatural structures in the substrates which allows installations of chemical modifications for desirable features. (3) Butelase-1 exhibits the highest catalytic efficiency among all the ligases known so far.

Sources of Butelase-1

In the beginning, the butelase-1 was extracted from the pods of *Clitoria ternatea* with an isolation yield of 5 mg/kg (Nguyen et al. 2014). To improve isolation yield, Hemu et al. (2021) first profiled nine different tissues of the plant to determine their expression level of butelase-1 and found that young tissues, such as shoots, as a better source of natural butelase-1 as they have the highest butelase-1 activity. As for downstream processes, they simplified the procedure to a 3-step chromatography process comprising anion exchange flash chromatography, anion-exchange fast protein liquid chromatography, and size exclusion fast protein liquid chromatography. Other improvements include omitting sodium sulfate precipitation step and addition of polyvinylpolypyrrolidone. The cumulative effect of the above improvements is a three-fold increase in the isolation yield. For the wide application of butelase-1, the accessibility to the butelase-1-producing plant, the tedious extraction and purification are deterring elements needed to be addressed.

To this end, recombinant expression has also been developed to provide a complementary source. The first reported successful recombinant expression of butelase-1 comes from James et al. (2019) by transforming a synthetic butelase-1 encoding DNA sequence to T7 Shuffle Express strain of

Table 1 Peptide asparaginyl ligases found so far

AEPs	Origin	Recognition signal	Remarks
Butelase-1 (Nguyen et al. 2014)	*Clitoria ternatea*	GI-X*n*-NHV	Butelase-1 was first isolated from plant. It is the first reported PAL and exhibits the highest catalytical efficiency among all the peptide ligases found so far
OaAEP1b, 3–5 (Harris et al. 2015, 2019)	*Oldenlandia affinis*	GL-X*n*-NGL	OaAEP1b was identified from the genomic DNA extracted from *O. affinis* leaf tissue and recombinantly expressed. It is a Glu371Val variant of OaAEP1. OaAEP3-5 were later identified and recombinantly expressed
[C247A]OaAEP1 (Yang et al. 2017)	*Oldenlandia affinis*	GL-X*n*-NGL	It was an engineered peptide asparaginyl ligase from the wildtype OaAEP1 by substituting the Cys247 to Ala247. The single residue mutation results in a drastic ligation efficiency improvement
HeAEP3 (Jackson et al. 2018)	*Hybanthus enneaspermus* F.Muell	Not reported	Using marker of ligase activity (MLA) as the searching criterion, HeAEP3 was predicted to be a ligase among the three AEPs identified from the transcriptomes of the Violaceae member. Functional study validated the prediction
VyPAL2 (Hemu et al. 2019a)	*Viola yedoensis*	GI-X*n*-NSL	It was identified from plants of Violaceae through sequence homology with butelase-1 and OaAEP1b. "Gatekeeper" residue was used as the searching criterion. Further functional study validated the ligase identity
Butelase-2 mutants (Hemu et al. 2020a, b)	*Clitoria ternatea*	GI-X*n*-NHV	Based on the LAD hypothesis, several peptide ligases were successfully engineered from an asparaginyl endopeptidase, butelase-2. These mutants have a combination of mutations in both S2 and S1' sites

E. coli. The codon-optimized DNA sequence includes an N-terminal 6-His tag, followed by a Gly-Ser linker in *lieu* of its ER signal and 462 residues of butelase-1 zymogen from Ile21 to Val482. This represents butelase-1's full open reading frame minus the ER signal. After the expressed butelase-1 was purified by metal affinity column at neutral pH, the enzyme still existed in the inactive form possessing both N-terminal and C-terminal pro-domains. During the subsequent dialysis at low pH, butelase-1 was found to be able to self-activate by removing the flanking N- and C-terminal pro-domains. The so-obtained butelase-1 was found to be able to catalyze both cyclization and cleavage when co-incubated with several model AEP substrates. After this report, three other groups (Pi et al. 2019; Hemu et al. 2021; Zhao et al. 2021) have also reported successful recombinant expression of butelase-1 using *E. coli* and *Pichia pastoris-shas*. It is worth mentioning that: (1) Recombinant butelase-1 is not as stable as the natural version which is glycosylated, providing protection against degradation. (2) The in vitro activation of butelase-1 needs to be optimized which would otherwise result in heterogeneous products with different auto-processing sites on both termini, complicating the downstream purification.

Forms of Butelase-1

Compared with enzymes in the soluble form, immobilized enzymes on solid supports have distinct features and have been used with a long history (Klibanov 1983). Tam's group explored the feasibility to immobilize two PALs, plant-derived activated butelase-1 and insect cell-expressed VyPAL2 (Hemu et al. 2020a). Taking advantage of the surface glycans and amino groups of these two PALs, the research group adopted three different immobilization strategies which included concanavalin A agarose beads (affinity towards glycans on PALs), NeutrAvidin agarose beads (affinity towards biotin-labeled PALs), and N-hydroxysuccinimide (NHS) ester functionality-bearing beads (covalent conjugation with surface amines of PALs). All these three methods were found to be capable of immobilizing the two enzymes with the sole exception that concanavalin A beads worked well only with butelase-1 but not VyPAL2. The unsuccessful application of concanavalin A beads on VyPAL2 is probably because VyPAL2, unlike butelase-1, mainly contains simple N-glycans which have low binding affinity towards concanavalin A beads. The activities of the immobilized PALs were found to correlate with the spacer length between the enzyme and the beads since it affects the mobility of the enzyme and its accessibility to substrate. All the immobilized PALs were found to well retain their activities after repetitive use and have longer shelf life compared with their soluble counterparts. For demonstration, immobilized PALs were added in high concentration to drive some difficult ligation reactions to completion which could not be achieved with soluble enzymes. Furthermore, the use of immobilized PALs was shown to simplify the downstream purification since it avoids the contamination of the product by the enzymes.

Applications of Butelase-1

Intramolecular Ligation (Cyclization)

Cyclization is an effective strategy to increase bioactivity and thermal/protease stability of peptide- and protein-based therapeutics (Antos et al. 2009). Also, large cyclic peptides have been shown to well inhibit protein–protein interactions due their large footprint. Due to the above reasons, efficient methods to prepare this type of molecules have long been sought by peptide chemists. Various peptide ligation chemistries coupled with Solid Phase Peptide Synthesis (SPPS) have been adopted to prepare cyclic peptides, such as click chemistry (Aimetti et al. 2010; Rashad 2019), oxime ligation (Roberts et al. 2004), etc. Native chemical ligation (NCL) is one popular method to head-to-tail cyclize linear peptides with an N-terminal cysteine residue without the need of side chain protections (Tulla-Puche and Barany 2004). This reaction processes through a proximity-driven S-to-N acyl transfer. Since cysteine is not in abundance in natural peptides, an additional desulfurization step might be needed following the NCL reaction. Besides chemical methods, enzymatic methods represent another appealing strategy for cyclization due to the intrinsic site-specificity of enzymes. As a natural cyclase, butelase-1 has been well demonstrated to be a reliable tool in this regard (Nguyen et al. 2014, 2015a, b, 2016a).

Bacteriocins are the largest cyclic peptide antimicrobials found in nature, with lengths ranging from 35 to 70 amino acids (Gálvez et al. 1985; Gálvez et al. 1989). Circular bacteriocins are active against a broad spectrum of bacteria. AS-48, one group of bacteriocin, is a promising alternative to the conventional chemical food preservatives because: (1) Its head-to-tail cyclization structure increases the thermostability during cooking process. (2) It is degradable by digestive proteases, avoiding potential adverse effects. (3) It exhibits high potency against a majority of food-borne pathogens. Synthesis of AS-48, however, poses challenge considering its cyclic structure and high hydrophobicity, making it prone to aggregation. Hemu and coworkers first attempted to access natural AS-48 by native chemical ligation coupled with desulfurization since bacteriocins do not possess cysteine residue. This attempt was not successful due to the hydrophobic nature of this molecule. Later butelase-1 was exploited to achieve this goal. To do this, linear precursor of AS-48 was first prepared by standard Fmoc chemistry. A bifunctional pentapeptide HVKKK was added after the Asn

on the linear precursor's C-terminus for both butelase-1 recognition and aqueous solubility enhancement. A subsequent folding step, which gave the linear precursor proper conformation for bringing both termini into proximity, was found to be essential prior to the butelase-1-mediated cyclization. The cyclization reaction completed in 1 h with a satisfactory 85% yield (Fig. 3) (Hemu et al. 2016). This synthetic AS-48 was shown to be comparable with the natural counterpart in both structure and antimicrobial activity. Using similar strategies, three other bacteriocins uberolysin, garvicin ML, and carnocyclin A were also successfully prepared.

Peptides containing D-amino acids are proteolysis resistant and weakly immunogenic but have uncompromised activity compared with their L counterparts, making them attractive as peptide-based therapeutics. Nguyen et al. (2016a; b) demonstrated that butelase-1 can well tolerate D-amino acids at P1″ and P2″ positions in intramolecular ligations. Taking advantage of this desirable feature, three linear bioactive D-peptides were successfully macrocyclized using butelase-1: sunflower trypsin inhibitor (SFTI), conotoxin MrIA, and θ-defensin. All these three peptides are entirely composed of D-amino acids except Asn residue at the ligation site which must be present for enzyme recognition. The antimicrobial activity of the prepared D-θ-defensin has been shown to be comparable with its L counterpart. It is worth mentioning that the amide bond formed between L-Asn and Gly at the ligation site might be a potential site of protease degradation.

The butelase-1-mediated cyclization could be affected by reaction time, substrate concentration, peptide length, and its sequence. By tuning these factors, cyclo-oligomers with different sizes and shapes could be obtained. Based on this, Hemu et al. (2019b) prepared cyclooligomeric antimicrobial peptides using $RLYRX_n$ as repeating unit (Fig. 4). Their antimicrobial activities were comparable with their dendrimeric counterparts, while the cyclic structure confers additional stability.

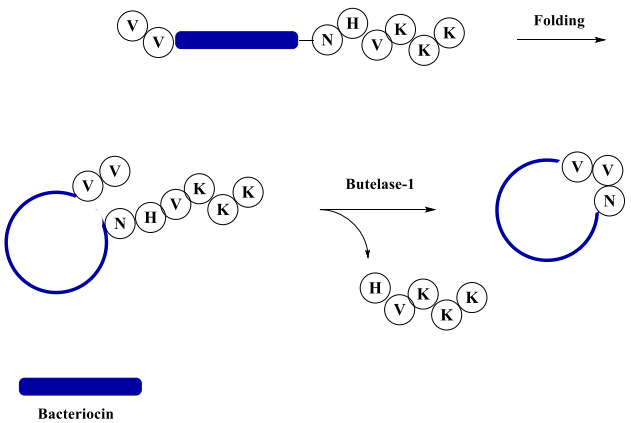

Fig. 3 Illustration of butelase-1-mediated cyclization of bacteriocin

Although AEPs can catalyze the hydrolysis of both asparaginyl and aspartyl peptide bonds in acidic conditions, almost all reported butelase-1-mediated macrocyclization are based on P1-Asn ligation. Compared with P1-Asn ligation, P1-Asp ligation by butelase-1 is more challenging. This could be explained that the binding of butelase 1 and substrate relies on the hydrogen bonding between the hydroxyl group of $^{\gamma}$COOH of P1-Asp and a key residue in the S1 pocket of the enzyme, which can only be maintained in acidic conditions; however, low pH would jeopardize the nucleophilicity of the incoming amino group. By determining the cyclization rates of a panel of model Asn- and Asp-containing linear peptides, Liu's group examined the possibility of P1-Asp ligation using butelase-1, oaAEP, and VyPAL2 (Zhang et al. 2021). Among the three PALs, VyPAL2 was found to be the most suitable for practical use in cases where the cyclization junctions do not represent optimal recognition sites by the ligase. To further improve the P1-Asp ligation by PAL, the same research group later adopted substrate engineering strategy. The asparagine mimic N^{γ}-hydroxyl-asparagine or Asn(OH) can well tackle the above mentioned dilemma (Fig. 5) (Xia et al. 2021). Unlike the $^{\gamma}$COOH of Asp, the N^{γ}-hydroxyl group remains protonated at neutral pH owing to its higher pKa, making it a good hydrogen bond donor for PAL binding. Compared with the P1-Asn peptides, the P1-Asn(OH) peptides did not show significant decreased catalytic efficiency in a model study to measure the kinetics of butelase-1-mediated peptide cyclization. After the cyclization, the Asn(OH) at the ligation site can readily be oxidized to Asp using NaIO4, making P1-Asp ligation by butelase-1 practically feasible. This concept-of-proof was well demonstrated by successful synthesis of several bioactive Asp-containing peptides (MCoTIII, kB2, SFTI, and integrin-targeting RGD peptides). Besides being used for the indirect P1-Asp butelase-1 ligation, the unnatural amino acid Asn(OH) itself can also be utilized to prepare peptidyl metalloenzymes inhibitors, owing to the strong metal-chelating capability of the hydroxamate group. In the study of β-amyloid precursor protein-derived inhibitor peptide (APP-IP), substitution of Asp6 with Asn(OH) showed inhibitory activity improvement. Proper modification of the structure coupled with butelase-1-mediated cyclization further improved activity and proteolytic stability.

Not only butelase-1 can cyclize peptides, it can also cyclize proteins.

Nguyen et al. (2015a, b) successfully prepared two cyclic protein therapeutics, interleukin-1 receptor antagonist (IL-1Ra) and human growth hormone (somatropin), from their linear precursor using butelase-1. Similar to the case of bacteriocin described above, proper conformation bringing together the N- and C-termini is essential in the production as the two proteins after heat-denaturation could not be cyclized like their natural counterparts. After

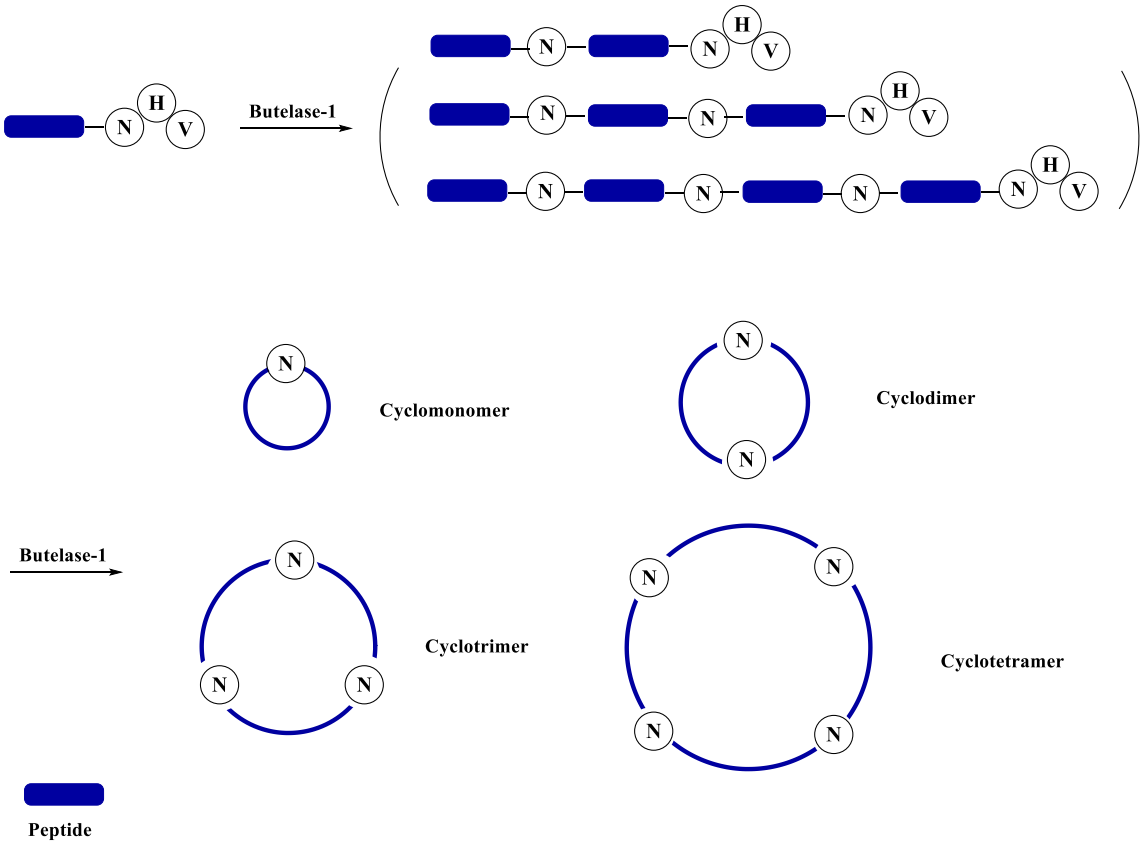

Fig. 4 One-pot cyclo-oligomerization of peptide mediated by butelase-1

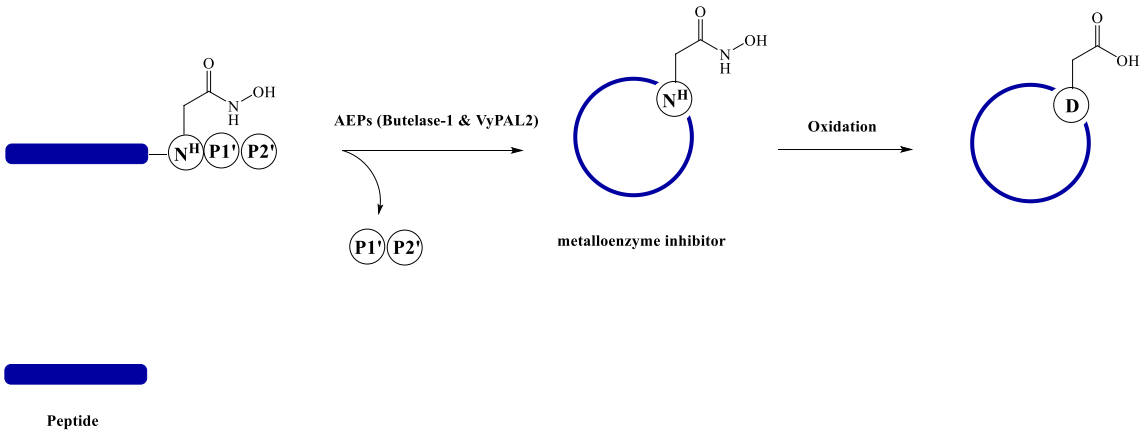

Fig. 5 Illustration of butelase-1-mediated P1-Asp cyclization. Reproduced with permission from Xia et al. (2021)

cyclization, IL-1Ra was found to have a 4 °C increase in the melting temperature (T_m). Its inhibitory activity on IL-1β-induced ICAM-1 expression was comparable with the native linear one.

Industrial enzymes with improved features could be achieved by certain enzyme engineering strategies, such as site mutagenesis and directed evolution. One strategy to boost the thermostability of industrial enzymes is through macrocyclization. The macrocyclization of industrial enzymes is challenging due to their large size and high complexity. The utility of butelase-1 in this application has been demonstrated by Tam's group (Hemu et al.

2021). Two types of common industrial enzymes, lipase and phytase, in circular form were successfully produced from their linear precursors using butelase-1. Although the melting temperature of lipase only increased 1 °C after macrocyclization, the esterase activity was found to be greatly increased. The thermostability of the prepared cyclized phytase was found to have been improved compared with its linear counterpart. The melting temperature of cyclized phytase increased 6 °C and 10 °C in salt-free and salt-containing conditions, respectively.

Increasing protein stability can also facilitate structural investigation. With the exploitation of butelase-1, Pi et al. (2019) efficiently cyclized the p53-binding domain (N-terminal domain) of murine double minute X (N-MdmX) which is an important target for cancer therapeutics. Cyclized N-MdmX showed improved thermostability and binding affinity towards its inhibitor compared with the uncyclized one as revealed by differential scanning calorimetry (DSC) and isothermal titration calorimetry (ITC), respectively. Its secondary structure was found to be comparable as measured by circular dichroism (CD). Successful crystallization was achieved using complex of cyclized N-MdmX and its inhibitor. Experiments of ^{15}N-^{1}H heteronuclear single-quantum coherence (HSQC) nuclear magnetic resonance (NMR) demonstrated that, compared with the linear form, cyclized form of MdmX could reveal more resonance peaks from flexible regions owing to the rigid conformation induced by cyclization. Additional resonance peaks of cyclized N-MdmX could be found when it formed complex with its inhibitor.

Intermolecular Ligation

Besides the capability of butelase-1 to catalyze cyclization reaction described above, its ability to catalyze peptide intermolecular ligation has also been well documented.

In the first paper on butelase-1, Nguyen et al. (2014) reported that when cyclizing kB1 peptide using butelase-1, besides the desired cyclized product, a minor side product corresponding to cyclodimerization could also be found at high substrate concentrations. This unexpected result clearly demonstrated the potential of butelase-1 in the application of peptide intermolecular ligation. It is noteworthy that this is the first reported case of butelase-1-mediated intermolecular ligation.

Unlike butelase-1-mediated macrocyclization reaction which, provided with proper substrate conformation, can proceed with high efficiency, intermolecular ligation is more challenging. For entropic reasons, the intermolecular reaction is inherently less favorable than the intermolecular reaction. In addition, the intermolecular reaction efficiency is hindered by the intrinsic reversible nature of butelase-1-mediated reaction because the ligation products are also recognizable by butelase-1 just like the starting materials.

To address this reversibility issue, Tam's group reported the use of a thioester linkage, rather than the natural asparaginyl amide bond, as the scissile bond in the butelase-1-mediated ligation (Nguyen et al. 2015a, b). This devised thioester electrophilic substrate bears a recognizable motif and generates a thioacetyl byproduct after its release, which cannot be recognized by butelase-1 like the native dipeptide (Fig. 6). In this way the reaction can only proceed forward, rendering the ligation irreversible. This efficiency-boosting strategy has been well demonstrated in two applications: N-terminal protein labeling and peptide dendrimers synthesis which are both highly demanding for ligation efficiency.

Proteins that are site-specifically modified with tags and probes are useful tools in the study of structure–activity relationship and protein–protein interactions. These materials could be prepared by chemical means which usually utilize N-terminal amino group, functionalities of cysteine or lysine, and incorporated unnatural amino acids on which

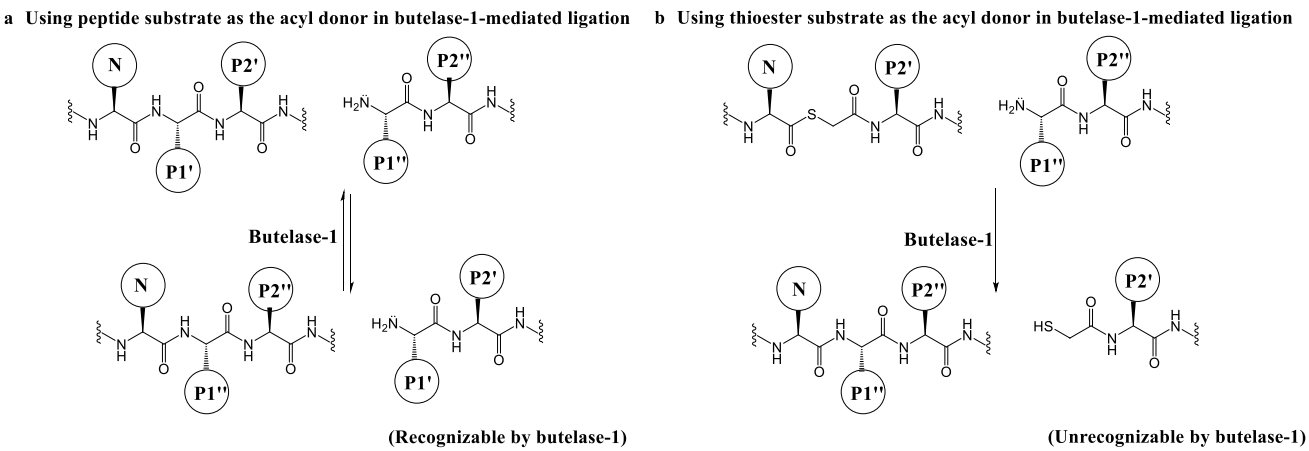

Fig. 6 Butelase-1-mediated ligation using **a** natural peptide and **b** thiodepsipeptide as substrate

orthogonal chemistries can be done (Chalker and Davis 2010; Gamblin et al. 2004; Li et al. 2011; Dawson et al. 1994; Pasunooti et al. 2009; Yang et al. 2009, 2010). Butelase-1, with its intrinsic site-specificity and high catalytic efficiency, can be a useful tool for protein labeling. Using the above described thiodepsipeptide as the labeling reagent, ubiquitin and green fluorescent protein were efficiently labeled on their N-terminus (Nguyen et al. 2015a, b).

Peptide dendrimers are a kind of synthetic bioconjugates with a central lysine core to tether identical peptides as branches. This structure was first developed by Tam to amplify the immunological signal of antigenic peptides (Posnett et al. 1988; Tam 1988). Dendrimeric structure of peptides was later extended to other applications such as diagnostics, peptide-based therapeutics, and biomaterials (Sadler and Tam 2002). Stepwise solid phase peptide synthesis (SPPS) can be adopted to prepare this type of molecules. However, when the size of dendrimer increases, the difficulty to obtain a homogeneous and chemically defined product also increases. Convergent synthetic strategy offers a quite attractive alternative in this regard. By this strategy, the dendron core and the repeated linear peptides are separately prepared with high quality and then conjugated together. Catalyzed by butelase-1, peptide dendrimers with di-, tetra-, and octabranches were efficiently synthesized using thiodepsipeptides as acyl donors for conjugation with lysine cores (Cao et al. 2016). One antimicrobial tetravalent peptide prepared by this strategy was found to have great potency and broad activity against drug-resistant strains.

Butelase-1-mediated intermolecular ligation can also be applied to live cell labeling. Conventional cell labeling employs fusion expression to exhibit reporter proteins or peptides on the cell surface. To install non-natural cargos on cells, other strategies need to be adopted. Butelase-1 is a particularly promising tool for this purpose as it has unique substrate specificity and can operate under mid conditions which are pivotal for the viability of the cells during the labeling. Bi et al. (2017) demonstrated the feasibility of this idea. To make the *E. coli* cells amenable for butelase-1-mediated modification, they were first transformed with plasmid encoding the Lpp-OmpA protein modified with a *C*-terminal NHV motif. To the surfaces of the transformed *E. coli* cells now expressing the NHV-tagged OmpA protein, butelase-1 efficiently linked fluorescein- and biotin-bearing peptides, monoglycosylated peptide and mCherry proteins within just 30 min (Fig. 7). Based on this principle, human transferrin receptor 1 (TfR1) in human cell lines were successfully labeled with a disulfide FRET probe which allows real-time monitoring of redox states in TfR1-mediated endocytosis (Bi et al. 2020).

Compatibility with Other Ligation/Conjugation Strategies

The unique rule obeyed by butelase-1 makes it possible for its combination use with other ligases, which extends its application.

Cao et al. (2015) demonstrated that dual protein labeling could be achieved by using a combination of sortase A and butelase-1. For C-terminal modification, protein thioester was first efficiently prepared using butelase-1 from the target protein with C-terminal recognition motif and glycine thioester. The prepared protein thioester acted as a substrate of subsequent chemical ligation for C-terminal protein modification (Fig. 8). This two-step strategy for C-terminal modification bears the following advantages: 1. The glycine thioester is easy and cheap to prepare so it can be added in large excess to drive the enzymatic ligation to completion. 2. Compared with the conventional intein-mediated protein thioester preparation, this method only requires co-expression of a short recognition motif on the C-terminus of the target proteins which does not affect the expression level. 3. This method leaves only a dipeptide tag -NG on the target protein, allowing minimum perturbation of protein structure. Using sortase A, an N-terminal modification was later installed on the N-terminus of the target protein with a tag containing the sorting sequence -LPTGG at its C-terminus.

Another reported research work using a combination of these two enzymes comes from Ploegh's group (Figs. 9 and 10) (Harmand et al. 2018). Taking advantage of the biorthogonality of these two ligases, two different kinds of

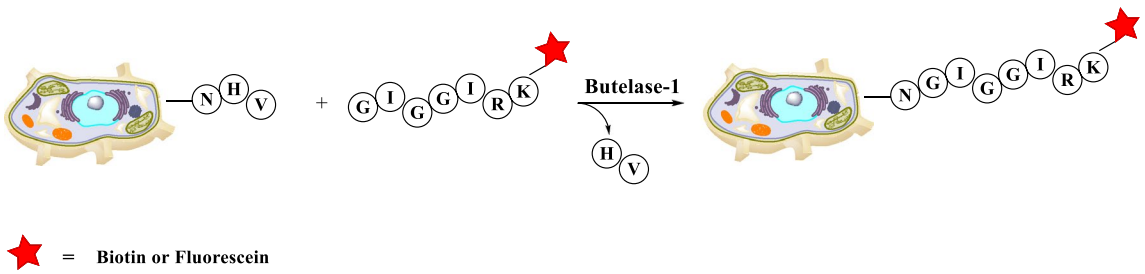

Fig. 7 Cell labeling mediated by butelase-1

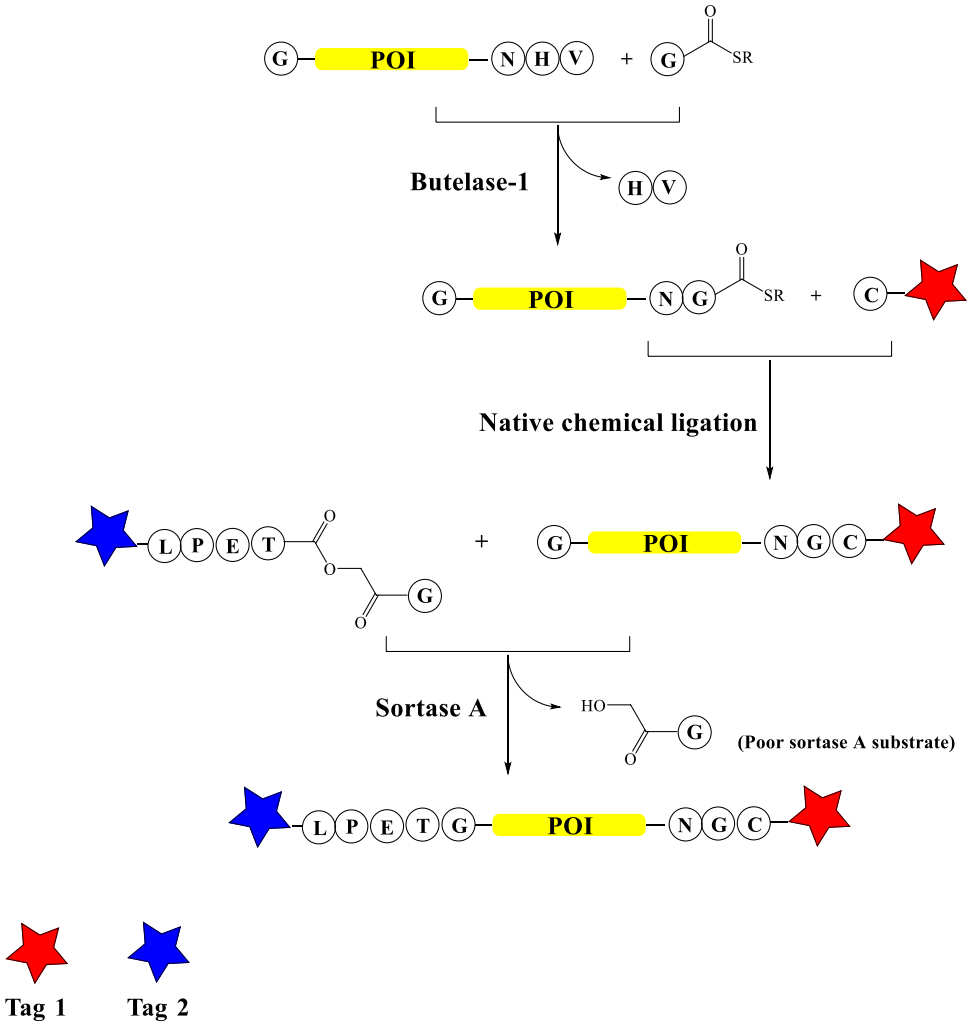

Fig. 8 Butelase-1-mediated ligation coupled with chemical ligation and sortase A-ligation for protein dual labeling

non-natural C-to-C fusion nanobodies were prepared using PEG-based and oligonucleotide-based two-headed linkers. Also, in a one-pot fashion, a full-size IgG1 was site-specifically modified by the two enzymes at the respective C-termini of its heavy chains and light chains with two different fluorescent probes.

VyPAL2 was recently identified as another plant legumain from the *Viola Yedoensis* family (Hemu et al. 2019a). Although VyPAL2 and butelase-1 are both asparaginyl transpeptidase, they are quite different in substrate specificity. For example, the favored tripeptide motif NHV by butelase-1 is not preferred by VyPAL2 and a Phe at the P2″ position is well recognized by VyPAL2 but not by butelase-1. The noticeable differences in substrate specificities could be utilized in tandem ligation for dual protein labeling. Using this strategy, Liu's group prepared a dual modified EGFR-targeting affibody with both bioimaging and cytotoxic functionalities (Fig. 11) (Wang et al. 2021). To exploit the orthogonality of these two enzymes in ligation reaction, the model affibody was genetically fused with an N-terminal GF dipeptide and a C-terminal NHV tripeptide tag. It is worth mentioning that using these two enzymes, the protection-free tandem ligation could be performed in both N-to-C and C-to-N directions, albeit small amount of by-product was found in the second step (butelase-1-mediated ligation) of the C-to-N manner. This dual PAL-based strategy was further utilized to produce a cyclic affibody-drug conjugate. To achieve this, a trifunctional peptide was first chemically prepared with a VyPAL2 recognized N-terminal GF dipeptide, a butelase-1 recognized C-terminal NHV tripeptide, and an aminooxy group for oxime ligation. The model affibody was recombinantly expressed with an N-terminal CG dipeptide motif and a C-terminal NGL motif. An interesting finding was that during protein expression, the N-terminal cysteine was protected as thiazolidine by the cell metabolite glyoxylic acid. This acted as a natural protecting group in the PAL-mediated ligation. The C-terminus of affibody was first ligated with the synthetic peptide catalyzed by VyPAL2. Due to the orthogonality of the NHV tag towards VyPAL2 and the

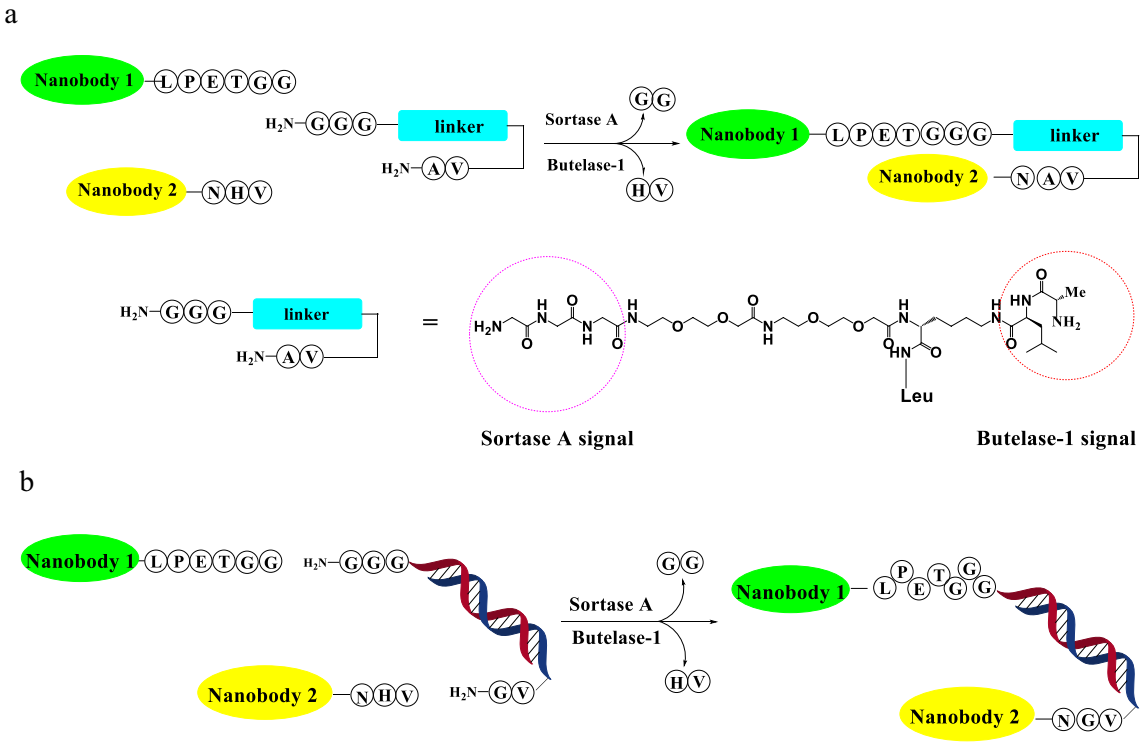

Fig. 9 Combination of butelase-1 and sortase A to produce C-to-C fusion nanobodies using a) PEG-based synthetic linker and b) double stranded oligonucleotide-based linker. Reproduced with permission from Harmand et al. (2018)

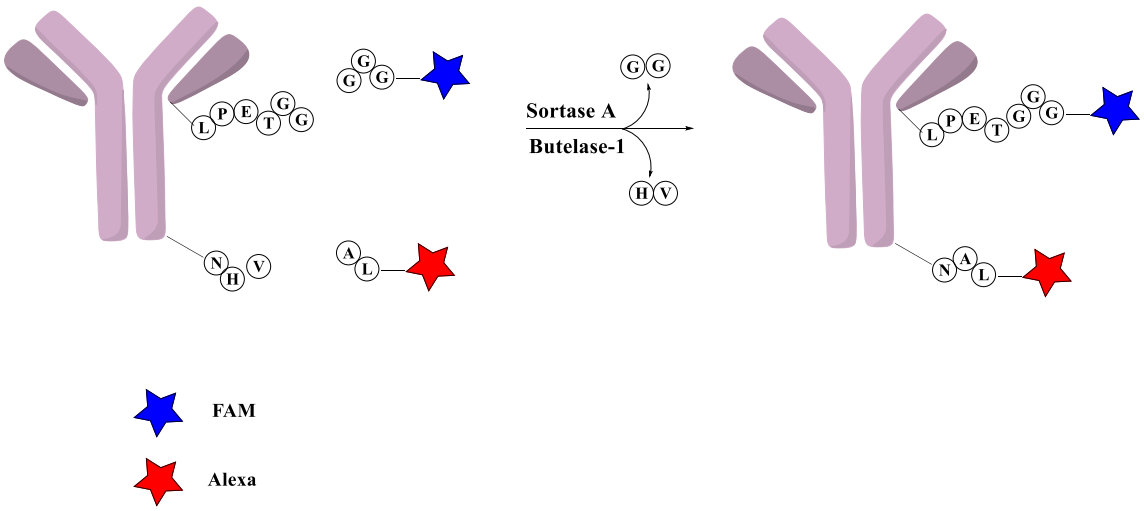

Fig. 10 Combination of butealse-1 and sortase A to achieve one-pot dual C-terminal labeling of antibody. Reproduced with permission from Harmand et al. (2018)

N-terminal protecting group on the affibody, the reaction proceeded cleanly. After the unmasking of the N-terminal cysteine, the N-terminus and C-terminus of the affibody were joined together by butelase-1, making the protein cyclized. To install the drug onto the cyclized protein, an oxime ligation was performed between the aminooxy functionality on the protein and the ketone functionality on the small-molecule drug doxorubicin. This cycloprotein-drug conjugate was shown to have better aqueous solubility compared with the free small-molecule drug, uncompromised binding affinity, and enhanced cytotoxicity.

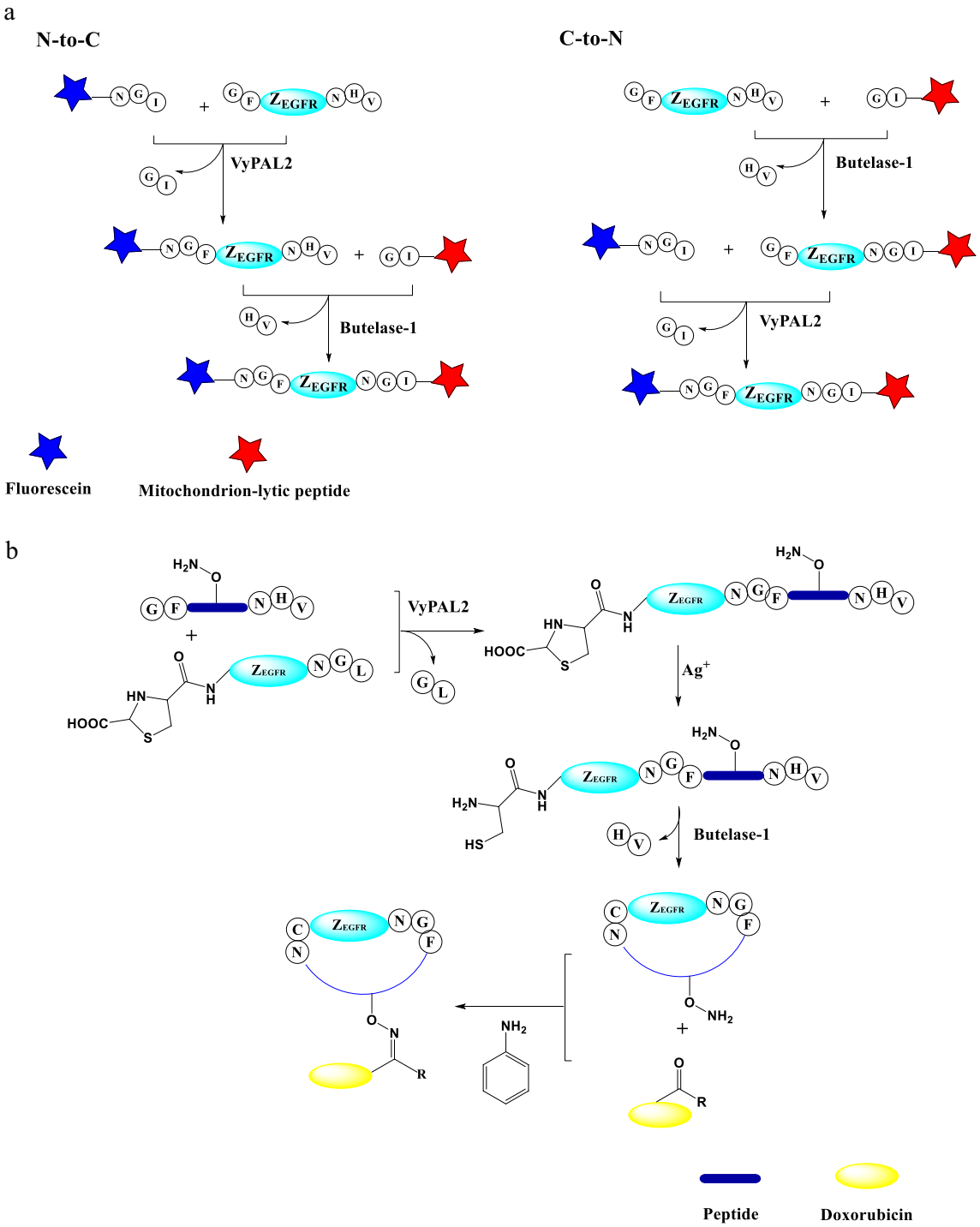

Fig. 11 Combination of butelase-1 and VyPAL2 to prepare **a** dual modified EGFR-targeting affibody in both N-to-C and C-to-N directions and **b** cycloprotein-drug conjugate. Reproduced with permission from Wang et al. (2021)

Cyclization of proteins confers stability. Site-specific introduction of functionalities to such cyclization-stabilized proteins can further improve their physicochemical and pharmacological properties. Combining amber codon suppression technology and butelase-1-mediated macrocyclization technologoy, Bi et al. (2020) prepared several cyclic murine dihydrofolate reductase (mDHFR) mutants with different unnatural amino acids at position 44 (Fig. 12).

One butelase-1-cyclized mDHFR mutant incorporated with ε-propargyloxycarbonyl (or praoc)-lysine was successfully functionalized with biotin and cell-permeable peptide (CPP) using click chemistry. The biotinylated circular mDHFR mutant was found to have comparable enzymatic activity with its linear counterpart at room temperature. However, under thermal stress such as 55 °C, the circular form retained most of its enzymatic activity whereas the linear form could not. The biotin functionality was subsequently utilized to immobilize the circular protein to streptavidin-functionalized agarose beads, which is an effective strategy to recycle enzyme and simply the enzyme-mediated reaction. To study the cellular uptake of mDHFR, HA tag was fused with the protein. The HA tag allows detection under fluorescence microscopy using a fluorescein-labelled anti-HA antibody. Since the fluorescence cannot be observed once the uptaken HA-fused protein is degraded within the cell, the intensity of the fluorescence could indicate the stability of the proteins in cells. It was found that CPP was essential to deliver mDHFR into the cells, be the structure linear or circular. Indicated by the fluorescence signal after protein removal from cell culture, circular CPP-conjugated mDHFR was found to retain in cells for longer time than its linear form. This result demonstrates the potential of intracellular targeting CPP-conjugated cyclic protein as a disease treatment.

Conclusions

Peptide ligation together with chemical peptide synthesis and recombinant protein expression are powerful tools in biotechnology. Chemical peptide ligation has been well developed. Enzymatic peptide ligation is emerging as a very attractive strategy for protein engineering because of its site-specificity, capability to work under aqueous and mild conditions and compatibility with biological systems such as live cells.

Unlike the ubiquitous peptide-breaking proteases, peptide bond-forming ligases are rare. Among the handful peptide ligases discovered so far, butelase-1, a member of the AEP family, draws particular attention owing to its high catalytic efficiency and features of clean and traceless ligation. Since its discovery in 2014, it has been used in many different applications, which include cyclization of peptides/proteins, cyclo-oligomerization of peptides, N- and C-terminal protein labeling, cell-labeling, antibody–drug conjugation, and combination use with other ligation strategies.

Further development in this field would benefit from a deeper understanding of the structure and function of butelase-1 as to what differentiates it from the other proteases in the AEP family and what makes its catalytic efficiency higher than the other PALs. This will allow systematic identification of new peptide ligases from nature, rational engineering of AEPs into PALs, and even manipulation of both substrate specificity and catalytic efficiency. Besides this,

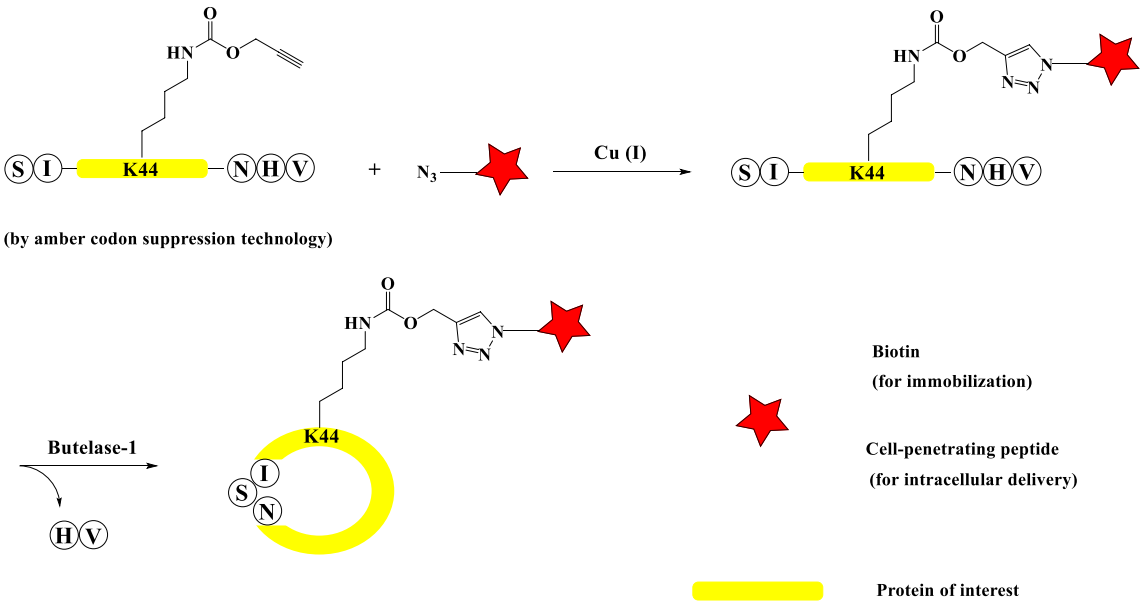

Fig. 12 Scheme for preparation of cyclic biotin- and CPP-conjugated mDHFR for site-specific immobilization and intracellular delivery, respectively

the recombinant expression of butelase-1 needs to be further optimized for its potential industrial application.

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Declarations

Conflict of interest All authors declared that they have no conflict of interest.

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