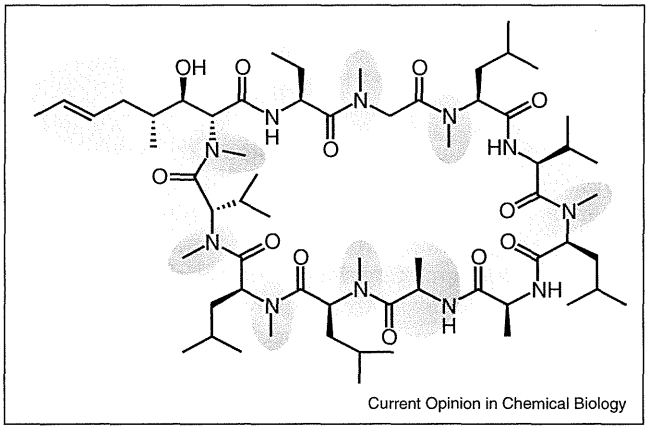
Figure 1



Structure of cyclosporin A, a nonribosomal peptide. Non-standard sidechains, *N*-methyl groups, a D-amino acid, and a macrocyclic peptide backbone are highlighted by yellow ovals, orange ovals, a blue oval, and a green doughnut, respectively.

factors IF1–3, elongation factors EF-Tu, G, and Ts) together with fMet-tRNA$^{fMet}_{CAU}$ and 70S ribosomes constituted a translation system with universally vacant elongator codon boxes. Subsequent refilling of three vacant boxes with three artificial amino acids by the addition of their aminoacyl-tRNAs resulted in successful expression of fMet-initiated tri-peptides or tetrapeptides [8]. Although this experiment was performed under single turnover conditions, it represented the first example of formatting codon boxes and reprogramming the genetic code by reconstituting the translation apparatus.

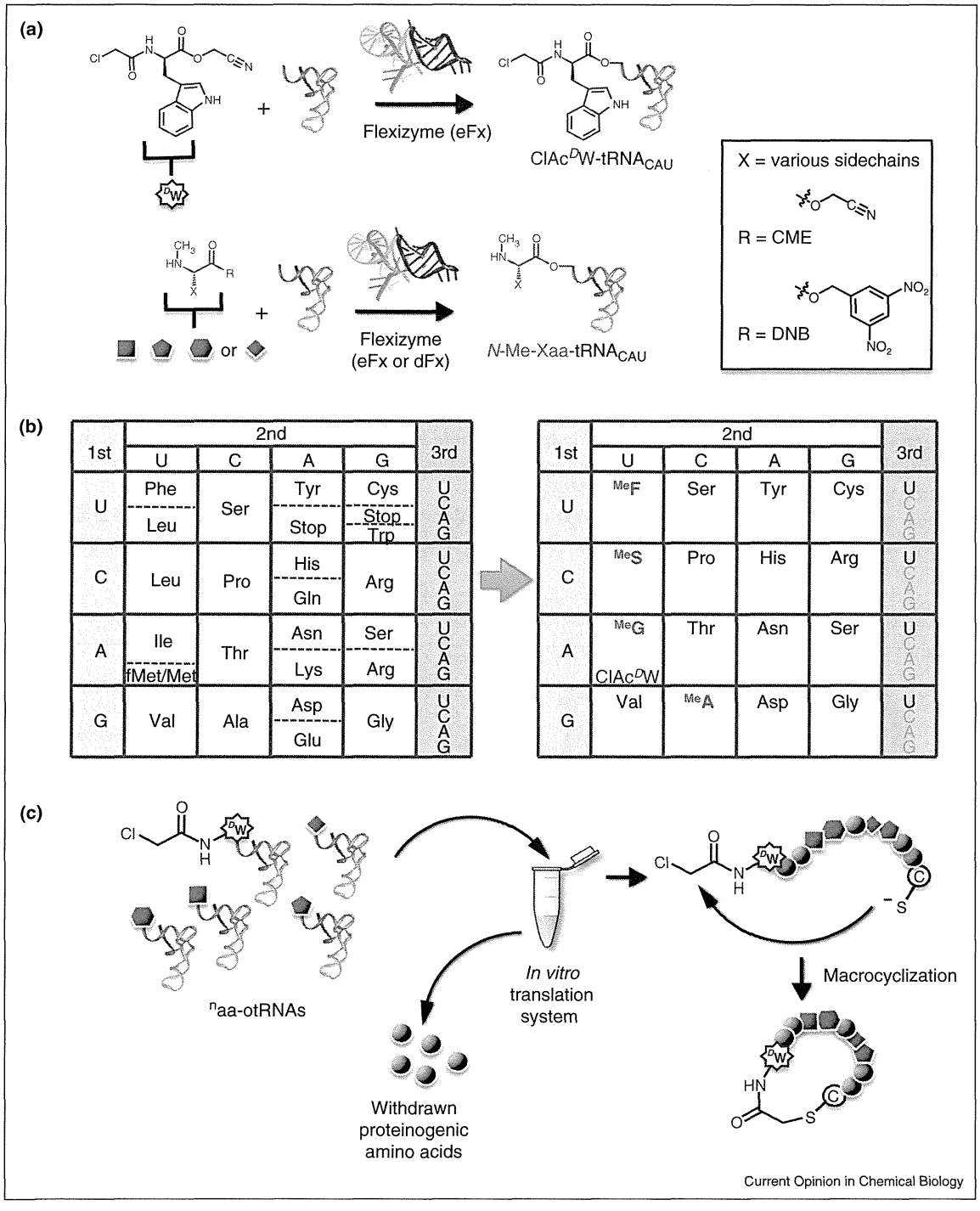
Since the Forster's demonstration, two methods, which are technically more advanced but both achieved through genetic code reprogramming, have been reported. Szostak *et al.* used a reconstituted *E. coli* translation system based on the protein synthesis using recombinant elements (PURE) system in which certain amino acids were depleted to make vacant codon boxes [10–12]. This method relied on the ability of some aminoacyl-tRNA synthetases (ARSs) to mischarge a wide array of non-standard amino acids onto cognate tRNAs under conditions lacking the cognate proteinogenic amino acids. The resulting mischarged non-standard amino acids were assigned to the vacant codon boxes by the tRNAs, thus allowing for expression of peptides containing multiple non-standard amino acids. The virtue of this method is the simplicity; the undesired amino acids were simply substituted with non-standard amino acids in the PURE system, and peptide translation could be performed. On the contrary, shortcomings also exist in this method. Since the choices of non-standard amino acids rely on the mischarging properties of ARSs, they often fall into the category of 'proteinogenic-like' non-standard amino acids. Moreover, even though a certain set of proteinogenic amino acids was not added to the PURE system, because ribosomes and ARSs are not completely proteinogenic amino acid-free (*i.e.* they carry over some amino acids into the PURE system), contaminating amino acids could compete with non-standard amino acids for incorporation. Particularly, since the proteinogenic amino acids are intrinsically much better substrates for ARSs, elongation factors, and ribosomes, their trace amount of competitive incorporation against non-standard amino acids is difficult to avoid. Therefore, the application of this method to *de novo* library synthesis of non-standard peptides must be carefully tuned in a balance of choice between non-standard amino acids and codons having the least contaminants of cognate proteinogenic amino acids.

The second method, developed by our group, also uses a reconstituted *E. coli* translation system, but the genetic code reprogramming relies on the preparation of desired non-standard aminoacyl-tRNAs by a *de novo* catalytic system based on flexizymes [13]. Flexizymes are flexible tRNA acylation ribozymes of which the prototype ribozyme was artificially selected from a random pool of RNA sequences. Reviews on the history and catalytic abilities of flexizyme can be found in recently written articles [14–17]. In brief, flexizymes are short ribonucleotides consisting of 45 or 46 nucleotides in length, and recognize specific leaving groups of amino acid esters. Since flexizymes strictly recognize neither sidechain nor amino group of given amino acids, they are able to charge a wide variety of non-proteinogenic (as well as proteinogenic) amino acids onto tRNAs. Moreover, the recognition of tRNA by flexizyme is dictated by the interaction of flexizyme's 3′-end motif with tRNA's 3′-end sequence via only 3 base pairs, that is, neither body nor anticodon sequences of tRNAs are involved. This feature grants flexizymes the ability to charge any kind of tRNA. Integration of the flexizymes with a reconstituted translation system, such as the PURE system, allowed us to devise a highly versatile peptide expression system with regards to the choice of amino acids and tRNAs. We refer to this system as the flexible *in vitro* translation (FIT) system [18].

The flexible *in vitro* translation (FIT) system

The FIT system is a translation apparatus comprised of two main groups of components. One group is made up of reconstituted *E. coli* translation components (IF1–3, EF-Tu, EF-G, EF-Ts, release factors RF2 and RF3, ribosome recycling factor RRF, methionine trans-formylase MTF and select ARSs), 70S ribosome, and desired proteinogenic amino acids [18]. By omitting the components (amino acids and ARSs) corresponding to specific codon boxes, vacant codon boxes are made available for non-proteinogenic amino acids. The other group of components is made up of the desired non-proteinogenic amino acids charged, by a flexizyme or flexizymes, onto orthogonal tRNAs (otRNAs) that correspond to the vacant codon boxes and are inert with the ARSs present in the FIT system. Their addition to the above custom-made translation apparatus complements

Figure 2



The FIT system [18,35••]. **(a)** Aminoacylation of tRNA using an activated *N*-acyl-D-Trp or an *N*-methyl amino acid along with the appropriate flexizyme. The flexizyme used depends on the activating group used [13,22••]. If the sidechain X is an aromatic group, the activating group shown in the rectangular box would be a cyanomethyl ester (CME), and the corresponding flexizyme would be enhanced-flexizyme (eFx). If the sidechain X is not an aromatic group, the activating group would be a dinitrobenzyl ester (DBE), and the corresponding flexizyme would be dinitro-flexizyme (dFx). **(b)** A reprogrammed genetic code that utilizes only NNU codons for elongation and the AUG codon for initiation by *N*-chloroacetyl (ClAc)-^{D}Trp. White boxes represent blank codon boxes for which the corresponding codon is not found in the random region of the mRNA sequence. **(c)** General scheme depicting genetically encoded macrocyclization of a non-standard peptide using the FIT system. Colored polygons represent non-standard amino acids (^{n}aa) depicted in **(a)**. Gray spheres represent proteinogenic amino acids. A 'C' within a black circle indicates the location of a cysteine.

the FIT system. A brief note that we previously referred to such a translation system as *withdrawn* PURE (*w*PURE); however, since we started developing more variations of the translation system differing from the original PURE system (*e.g.* the *c*PURE system that is specifically designed for macrocyclization between the peptide's N-terminus and C-terminus includes two N-terminal modifying enzymes), we renamed the *w*PURE system to the FIT system to reflect its versatility.

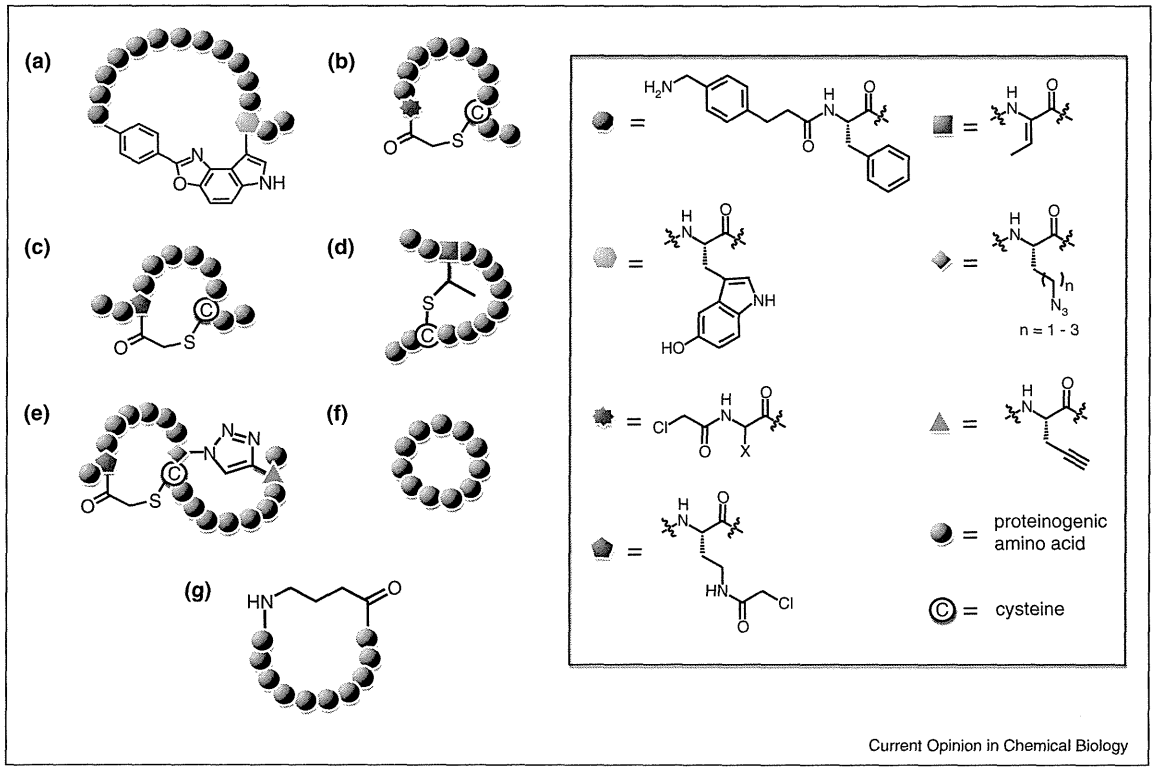
Despite the fact that otRNAs are supposedly inert with the ARSs present in the FIT system, to completely prevent the recycling of otRNAs that have been used by the translation machinery, omission of ARSs corresponding to any of the reprogrammed codons is crucial; this securely dismisses the possibility of reacylation of hydrolyzed otRNAs with contaminating proteogenic amino acids or any unexpected mischarging events. Using this technology, a variety of non-proteinogenic amino acids and α-hydroxy acids have been successfully incorporated into non-standard polypeptides and polyesters, respectively [13–20,21••,22••]. Recently, a new assortment of artificial amino acids that are chemically and biochemically valuable have been shown to be efficiently incorporated into non-standard peptides [23•,24,25••,26–28].

Reprogramming initiation and elongation

Needless to say, naturally occurring bioactive compounds have heavily influenced the inspiration for non-proteinogenic amino acid choice and design. One such non-proteinogenic amino is the D-amino acid. Unfortunately, elongation with D-amino acids has been known to occur with poor efficiency in the translation apparatus even when using the FIT system [13]. However, we have found that D-amino acid-tRNA$^{fMet}_{CAU}$ can be used for initiating translation, and while the translation efficiency is D-amino acid sidechain-dependent, *N*-acylation of the D-amino acid-tRNA$_{CAU}$ in general causes a significant increase in translation efficiency [21••] (Figure 2a). An alternative method of introducing D-amino acids is to initiate translation using an exotic peptide containing the D-amino acids [29]. It was demonstrated that a peptide bearing as many as four consecutive D-amino acids could be used to initiate translation.

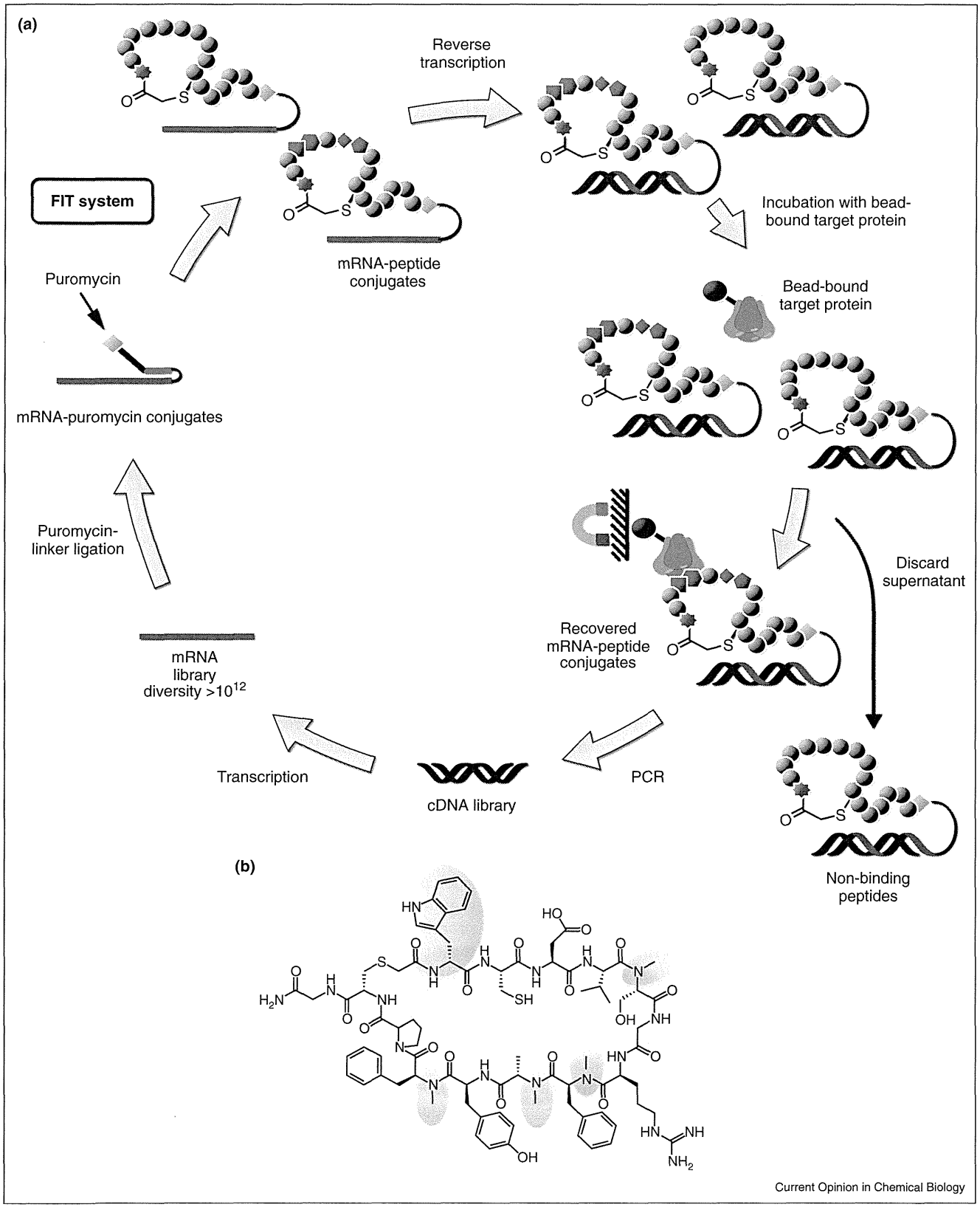
N-methylation, a deceptively small modification to amino acids, could have a tremendous impact on peptides'

Figure 3



Genetically encoded macrocyclized (exotic non-standard) peptides using the FIT system. Macrocyclization can occur via **(a)** oxidative coupling [24], **(b)** *N*-terminus-cysteine thioether bond formation [25••], inter-sidechain thioether bond formation using **(c)** S_N2 substitution [26] or **(d)** Michael addition [28], **(e)** Huisgen 1,3-dipolar cycloaddition in conjunction with thioether bond formation [27], **(f)** seamless head-to-tail polyamide backbone cyclization [37], or **(g)** backbone cyclization with an embedded γ-amino acid [38]. Colored polygons represent artificial amino acids. Gray spheres represent proteinogenic amino acids. The inset box on the right shows the chemical identities of the artificial amino acids, represented by the colored polygons, before macrocyclization. X can be any D-proteinogenic or L-proteinogenic sidechain. A 'C' within a black circle indicates the location a cysteine.

Figure 4


(a)
Reverse transcription
FIT system
Puromycin
mRNA-peptide conjugates
Incubation with bead-bound target protein
Bead-bound target protein
mRNA-puromycin conjugates
Puromycin-linker ligation
Discard supernatant
Recovered mRNA-peptide conjugates
mRNA library diversity >10^12
Transcription
cDNA library
PCR
Non-binding peptides
(b)
Current Opinion in Chemical Biology

pharmacological properties including oral availability, peptidase resistance and cell permeability [30]. It has been shown that the translation machinery is capable of utilizing *N*-methyl-amino acid-tRNAs (Figure 2a) and incorporating *N*-methyl-amino acids using genetic code reprogramming [22••,31–33,34•]. Careful reprogramming of the genetic code has resulted in a new genetic code that allows for the maximum diversity of *N*-methyl amino acids as well as proteinogenic amino acids [35••] (Figure 2b). Furthermore, these *N*-methylated peptides can be cyclized using the efficient and non-reducible thioether bond formation (*vide infra*, Figure 2c) [22••]. Expanding on the theme of incorporating *N*-substituted amino acids, the incorporation of *N*-substituted glycines (rGly) were also shown to proceed efficiently, and the resulting peptide–peptoid hybrids could, likewise, be cyclized [23•]. Nitrogen modification is not limited to the amide nitrogen (*e.g.* other recently incorporated non-standard amino acids include sidechain acylated or methylated lysines [20]).

Cyclization technologies adopted into the FIT system

In NRPSs, the thioesterase domain catalyzes linear peptide release via hydrolysis or aminolysis. Alternatively, the peptide can be released by macrocyclization. As with *N*-methylation, macrocyclization also enhances peptides' pharmacological properties. It is no surprise that many natural product peptides are macrocycles and that researchers are developing ways to introduce post-translational macrocyclization [36–43]. A unique way of genetically encoding backbone macrocyclization is by withdrawing an mRNA-encoded amino acid to promote elongation arrest and peptidyl-tRNA drop-off, which can then be utilized to promote a diketopiperazine-mediated macrocyclization [36]. This system requires the use of peptidyl deformylase (PDF) and, optionally, methionine aminopeptidase (MAP) if the initiation methionine is undesired in the final product.

The flexibility of the FIT system was demonstrated by the incorporation of an artificial amino acid at the initiation position, an internal elongation position(s), or both internal and elongation positions for the purpose of genetically encoding macrocyclization. Genetically encoded macrocyclization can take place via chemically assisted oxidative coupling or spontaneous thioether formation [24–27] (Figure 3a–c and e). Thermal isomerization of a vinylglycine residue to a dehydrobutrine residue also affords access to thioether formation through an inter-sidechain Michael addition by a nearby cysteine [28] (Figure 3d). Bicyclic structures are also accessible and can be precisely formed using thioether formation coupled with the orthogonal Huisgen 1,3-dipolar cycloaddition [27] (Figure 3e). Seamless head-to-tail polyamide backbone macrocyclization can be genetically encoded [37] (Figure 3f) with the option of inserting a novel γ-amino acid in the middle of standard α-amino acids [38] (Figure 3g). Among this abundance of FIT system-based macrocyclization methods, the *N*-terminus-to-sidechain thioether formation [25••] was the preferred method in a recently performed *in vitro* selection of non-standard peptides (*vide infra*).

The random non-standard peptide integrated discovery (RaPID) system

Several years ago, it was anticipated that the introduction of non-standard amino acids into a translation system would inevitably lead to the discovery of bioactive non-standard peptides with enhanced pharmacological properties using *in vitro* selection [44]. With the ability to translate natural product-like peptidomimetics with high efficiency and fidelity, we have the means to identify bioactive compounds using *in vitro* selection by an appropriate display method, like mRNA display [45,46]. Toward this goal, the FIT system was integrated with the mRNA display format, and referred to as the random non-standard peptide integrated discovery system [35••] (Figure 4a). To date, more than a dozen of bioactive non-standard cyclic peptides have been discovered using the RaPID system in our laboratory and some have recently been reported in literature [35••,47•]. Among them, Yamagishi *et al.* [35••] recently reported an *in vitro* selection for *N*-methylated cyclic peptide inhibitors of a formerly non-druggable enzyme, E6AP. E6AP is known to ubiquitinate p53, a protein known to promote apoptosis, and other potentially tumor-related proteins.

The reprogrammed genetic code designed by Yamagishi *et al.* was thoroughly optimized for the RaPID system. For example, to create an mRNA library free from random stop codons, only NNU codons (where N is any of the four nucleobases and U is uracil) were used in the random region of the mRNA. The threat of misincorporation of proteinogenic amino acids forced the generation of multiple variations and optimization of reprogrammed genetic codes. In addition to the incorporation rate of *N*-alkyl amino acids being lower than the incorporation rate of proteinogenic acids [48], the choice of codons and the corresponding tRNAs [49,50] can affect the efficiency and sometimes the fidelity of *N*-methyl amino acid incorporation. While the incorporation of multiple non-standard amino acid adds yet another layer of complexity [51], library construction using a reprogrammed genetic

(Figure 4 Legend) The RaPID system [35••]. **(a)** A schematic representation of single cycle of a generalized RaPID system. **(b)** CM_{11}-11, an NRP-like inhibitor of E6AP discovered using the RaPID system. Non-standard *N*-methyl groups, D-amino acid and macrocyclic peptide backbone are shown within orange ovals, a blue oval and a green doughnut, respectively.

code containing low non-standard amino acid content could result in the selection of peptides possessing high affinity, but lacking the desired non-standard amino acid [43]. The final reprogrammed genetic code designed by Yamagishi *et al.* [35••] shown in Figure 2b resulted in the highest fidelity and still retained a high *N*-methyl amino acid content. Since UGG, the codon for tryptophan, was also eliminated; peptide library synthesis was initiated using a D-tryptophan to ensure that tryptophan is represented. As previously mentioned, the reprogrammed genetic code was optimized to accommodate elongation using any of 4 different *N*-methyl amino acids and 11 different proteogenic amino acids. Four out of 16 codon boxes represent an *N*-methyl amino acid, which gives a 25% chance of incorporating an *N*-methyl amino acid at any position in the random region. The peptides isolated at the end of the selection possessed an average of four *N*-methyl residues out of eleven residues in the random region. The cyclic peptide with the highest affinity, CM_{11}-1 (K_d = 0.60 nM), bears the three notable non-canonical features found in nonribosomal peptide cyclosporin A – a D-amino acid, *N*-methylation, and a macrocyclic backbone (Figure 4b). Most importantly, *in vitro* assays show that CM_{11}-1 inhibits E6AP poly-ubiquitination of target proteins, p53 and Prx1.

Conclusions and perspectives

Standard ribosomal synthesis is limited to twenty canonical amino acids. The FIT system has addressed and diminished these limitations allowing us to produce custom natural product-like peptidomimetics. The RaPID system has firmly placed itself amidst the growing repertoire of novel techniques for the *in vitro* selection of non-standard peptides. The promiscuity of flexizyme allows us to quickly conjugate tRNAs with amino acids from an incredibly diverse repertoire of non-standard amino acids. Initiation using a non-standard amino acid has proven to be extremely robust and has allowed us to produce stable D-amino acid-containing macrocyclic peptides that have been found to be bioactive. The above example of macrocyclic *N*-methyl-peptides against E6AP has opened a new door for the discovery of non-druggable enzyme inhibitors. However, the RaPID system has no limitation for targets of choice. The RaPID system could be used not only for the discovery of enzyme inhibitors, but also disruptors of protein–protein interaction based on various different natural product-like peptidomimetic scaffolds. More examples of such *in vitro* selections are forthcoming in the near future.

Acknowledgements

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Natural Product-Like Macrocyclic *N*-Methyl-Peptide Inhibitors against a Ubiquitin Ligase Uncovered from a Ribosome-Expressed De Novo Library

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SUMMARY

Naturally occurring peptides often possess macrocyclic and *N*-methylated backbone. These features grant them structural rigidity, high affinity to targets, proteolytic resistance, and occasionally membrane permeability. Because such peptides are produced by either nonribosomal peptide synthetases or enzymatic posttranslational modifications, it is yet a formidable challenge in degenerating sequence or length and preparing libraries for screening bioactive molecules. Here, we report a new means of synthesizing a de novo library of "natural product-like" macrocyclic *N*-methyl-peptides using translation machinery under the reprogrammed genetic code, which is coupled with an in vitro display technique, referred to as RaPID (random nonstandard peptides integrated discovery) system. This system allows for rapid selection of strong binders against an arbitrarily chosen therapeutic target. Here, we have demonstrated the selection of anti-E6AP macrocyclic *N*-methyl-peptides, one of which strongly inhibits polyubiqutination of proteins such as p53.

INTRODUCTION

Peptides discovered as natural products share structural features that are not seen in ordinary polypeptides (proteins) expressed by the translation apparatus. Such "nonstandard" peptides are often macrocyclized and *N*-methylated in the backbone (Chatterjee et al., 2008); moreover, some of their side chains are modified to noncanonical functional groups or epimerized to *D*-configuration (Grünewald and Marahiel, 2006; Kohli et al., 2002; Li and Roller, 2002). These features concede critical biological and pharmacological properties, such as high affinity to binding partners, proteolytic resistance, and membrane permeability, and thus increase their specific potencies in vivo (Biron et al., 2008; Doedens et al., 2010; Driggers et al., 2008; Nestor, 2009; Sagan et al., 2004). Most of these peptides are produced by nonribosomal peptide synthetases (Grünewald and Marahiel, 2006; Kohli et al., 2002; Li and Roller, 2002) or are derived from ribosomally expressed peptides by enzymatic posttranslational modifications (Chatterjee et al., 2005; McIntosh et al., 2009; Oman and van der Donk, 2010). Despite the fact that libraries of "natural product-like" nonstandard peptides could be a very attractive source for drug discovery campaigns, the mechanistic and functional complexities of their production systems have been making it difficult for researchers to degenerate the sequences and lengths of nonstandard peptides and build their de novo library. Therefore, we have not yet witnessed a successful outcome of the generation of a highly complex "human-made" library and the discovery of novel peptide sequences against therapeutic targets.

Here, we report a unique means of synthesizing a "natural product-like" peptide library using a custom-made translation apparatus under the reprogrammed genetic code. The peptides in the library have the features of macrocyclic and *N*-methylated backbone along with a *D*-amino acid involvement in the sequences. Moreover, the library could be coupled with an in vitro display, and thus over a trillion members of nonstandard peptides can be rapidly screened against a chosen target. We referred to this platform system as RaPID (random nonstandard peptides integrated discovery) system. As a showcase of this system, we have performed selection of anti-E6AP macrocyclic *N*-methyl-peptides, and one of the abundant classes of selected peptides exhibited an inhibitory activity against E6AP-catalyzed polyubiquitination of target proteins, such as p53 and peroxiredoxin 1. Thus, the present work demonstrates a proof-of-technology and potentials of the RaPID system for the discovery of a novel class of nonstandard peptides against not only an E3 Ubquitin ligase but also previously nondruggable target families.

RESULTS AND DISCUSSION

Design of a Nonstandard Peptide Library Used in the RaPID System

The methodology of genetic code reprogramming, where arbitrary codons are reassigned from proteinogenic amino acids to nonproteinogenic amino acids, allows us to express "nonstandard" peptides using a translation apparatus (Forster et al., 2003; Josephson et al., 2005; Ohta et al., 2007; Ohta et al., 2008). To facilitate such a reprogramming, we have

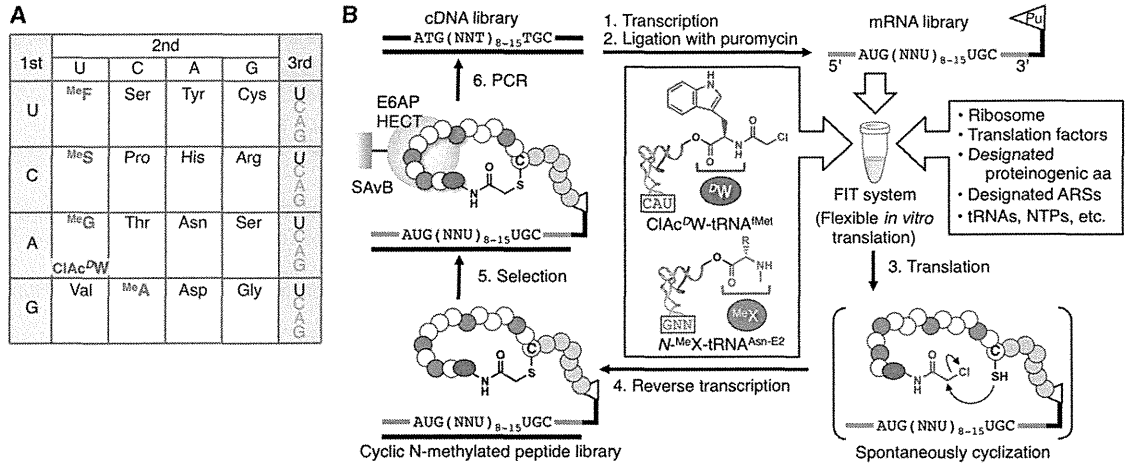


Figure 1. In Vitro Selection of Macrocyclic *N*-Methylated Peptides against E6AP HECT Domain by RaPID System

(A) The genetic code reprogrammed for this study. Four *N*-methyl-amino acids (^{Me}F, ^{Me}S, ^{Me}A, and ^{Me}G) and chloroacetyl-*D*-tryptophan (ClAc^{D}W) are shown in red and blue, respectively.

(B) Overview of the RaPID system for the selection of macrocyclic *N*-methyl-peptides. Messenger RNA libraries containing random sequence domain, $(NNU)_{8-15}$, were transcribed from the corresponding cDNA library and were conjugated with an oligonucleotide bearing a 3′-puromycin residue. The resulting mRNAs were translated by FIT system in the presence of the appropriate aminoacyl-tRNAs prepared by flexizymes. Linear peptides displayed on the individual mRNAs were spontaneously cyclized after translation, and the resulting macrocyclic peptides are displayed. The peptide libraries were then subjected to biotin-Avi-$(His)_6$-GB1-HECT immobilized on streptavidin magnetic beads (SAvB), and active species are isolated. Reverse transcription was performed after the selection in the first round and before the selection from the second round. The cDNAs on active mRNA-peptide fusion were recovered and amplified by PCR.

developed the FIT (flexible in-vitro translation) system (Goto et al., 2011). This system involves a custom-made *Escherichia coli* reconstituted cell-free translation system (Kung et al., 1977; Shimizu et al., 2001), where arbitrary amino acids and cognate aminoacyl-tRNA synthetases (ARSs) can be omitted to make the corresponding codons vacant, to which nonproteinogenic amino acids are assigned by supplementing the corresponding aminoacyl-tRNAs prepared by the flexizyme (flexible tRNA acylation ribozyme) technology (Murakami et al., 2006; Ohuchi et al., 2007). In fact, by using a customized FIT system, we were able to express macrocyclic *N*-methyl-peptides under the genetic code reprogrammed with more than four kinds of *N*-methyl-amino acids (Kawakami et al., 2008). To construct highly diverse libraries of nonstandard peptides and to effectively screen them for designated bioactivity, the FIT system was further integrated with an in vitro display method, so-called mRNA display (Nemoto et al., 1997; Roberts and Szostak, 1997). By this integration, nonstandard peptides expressed by the FIT system would be covalently ligated to the respective mRNAs via puromycin, displaying nonstandard peptides for the desired activity panning. We referred this mRNA display system integrated with FIT system to as RaPID (random nonstandard peptides integrated discovery) system.

In the present work, we assigned five nonproteinogenic amino acids, *N*-(2-chloroacetyl)-*D*-tryptophan (ClAc^{D}W), *N*-methylphenylalanine (^{Me}F), *N*-methylserine (^{Me}S), *N*-methylglycine (^{Me}G), and *N*-methylalanine (^{Me}A), to AUG, UUU, CUU, AUU, and GCU codons by the addition of ClAc^{D}W-tRNA$^{fMet}_{CAU}$, ^{Me}F-tRNA$^{Asn\text{-}E2}_{GAA}$, ^{Me}S-tRNA$^{Asn\text{-}E2}_{GAG}$, ^{Me}G-tRNA$^{Asn\text{-}E2}_{GAU}$, and ^{Me}A-tRNA$^{Asn\text{-}E2}_{GGC}$, respectively, prepared by the flexizyme technology (Figure 1A). A mRNA library was constructed to have NNU codon (N represents any of four bases, A, G, C, and U) with the mixture of repeats from eight to 15, $(NNU)_{8-15}$; thereby, *N*-methyl-amino acids would randomly appear in this region with the lengths of eight to 15 residues. In right downstream of the random region, UGC that assigns cysteine (Cys) was installed. Because all expressed peptides should have a ClAc^{D}W at the N terminus assigned by AUG start codon, the ClAc group would intramolecularly react with the sulfhydryl group of the Cys residue assigned by UGC or potentially UGU appeared in the random region, macrocyclizing their backbone via a nonreducible thioether bond (Goto et al., 2008; Kawakami et al., 2008). Following the UGC codon, three repeats of (GGC)(AGC) encoding $(Gly\text{-}Ser)_3$ followed by UAG stop codon are embedded. The 3′ common sequence would facilitate the display of macrocyclic *N*-methyl-peptides on the respective mRNA molecules via the $(Gly\text{-}Ser)_3$-puromycin linkage (Figure 1B).

Three critical notes should be given for securing a high quality of the macrocyclic *N*-methyl-peptide library: (1) In this FIT system, the amino acids and their cognate ARSs, whose codons were reprogrammed (F, L, I, and A), were omitted from the translation components, minimizing the unwanted incorporation of these proteinogenic amino acids competing with the *N*-methyl-amino acids. Similarly, those unassigned by NNU codons (Q, K, E, and W) were omitted. Moreover, release factor-1 (RF1) was withdrawn to aim at halting elongation at UAG codon and thus increasing the efficiency of puromycin-peptide fusion. (2) Because four of 16 codons assigned by the NNU mRNA library encode *N*-methyl-amino acids, one *N*-methyl-amino acid residue would appear in every four residues by chance. Our previous studies suggested the thioether macrocyclization takes place cleanly in nearly quantitative manner, regardless of the length and composition of peptide

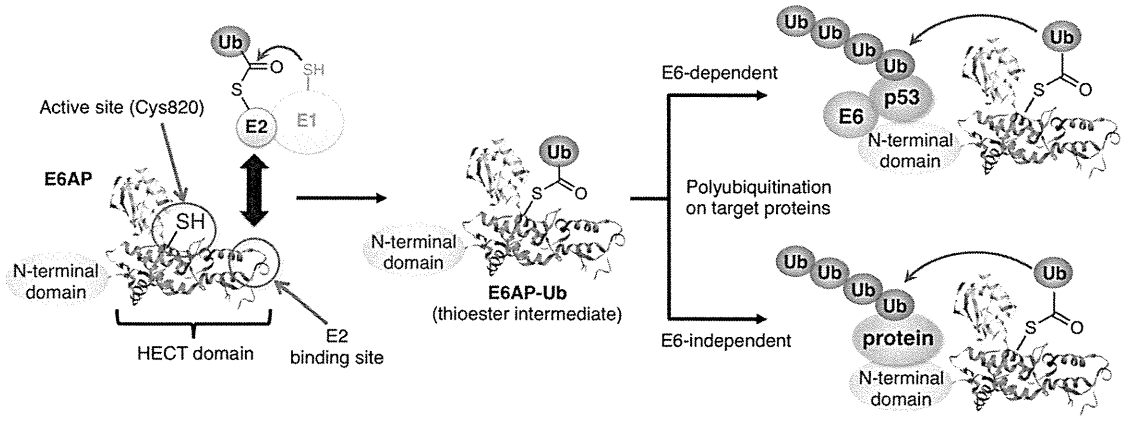


Figure 2. E6AP-Catalyzing Polyubiqutination of Target Proteins in E6-Independent and -Dependent Manners
In general, ubiquitin-activating enzyme E1 delivers an ubiquitin molecule (Ub) onto ubiquitin-conjugating enzyme E2 via a thioester linkage, and then Ub on E2 is transferred to ubiquitin ligase E3 forming a conjugate, E3-Ub. E6AP HECT (homologous to E6-associated protein [E6AP] C-terminus) domain (PDB 1C4Z), belonging to a member of E3 protein family, cooperatively catalyzes polyubiquitination on certain proteins, such as p53, with E6 (E6-dependent pathway) or directly polyubiquitinates various target proteins (E6-indendent pathway).

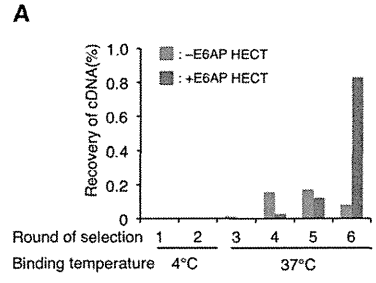
sequences (Goto et al., 2008; Kawakami et al., 2008). Moreover, because no stop codon was encoded in the $(NNU)_{8-15}$ random region, the highly reliable macrocyclic *N*-methyl-peptide library could be displayed by RaPID system. (3) The initial mRNA library consists of greater than 6×10^{12} unique members. It should be noted that the complexity of $(NNU)_{8-15}$ mRNA library should almost directly reflect to the peptide library complexity because only Ser is redundant in the genetic triplets used. Because the generally observed yield of mRNA-peptide fusion in the FIT system was 30% or more of the total input of mRNA, the diversity of the initial RaPID display library was estimated to be 10^{12} or more.

RaPID Selection against E6AP HECT Domain

The ubiquitin-proteasome system regulates the degradation of cellular proteins through enzyme cascade consisting of ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2, and ubiquitin (Ub) ligase E3 (Figure 2) (Hershko and Ciechanover, 1998; Pickart, 2001). Many families of E3 Ub ligases are known and are responsible for specific conjugation of polyubiquitins (polyUbs) to designated proteins, directing them to proteasome and thus triggering their proteolysis. Misregulation of the proteolysis of certain proteins caused by malfunction of E3 family ligases influences their downstream signal transduction processes, and therefore causes human disorders such as cancer (Hoeller et al., 2006) and neurodegeneration (Layfield et al., 2005; Rubinsztein, 2006). Thus, these E3 Ub ligases could be attractive targets for new therapeutic intervention (Eldridge and O'Brien, 2010; Hoeller and Dikic, 2009; Nalepa et al., 2006). Homologous to E6AP C-terminus (HECT) domain belongs to a family member of E3, and its N terminus domain cooperates with the human papillomavirus (HPV) E6 protein originating from the high-risk virus types 16 and 18 (Beaudenon and Huibregtse, 2008; Scheffner et al., 1993). The resulting E6AP and E6 complex provides the specific E3 ligase activity in the transfer of polyUbs onto p53 for the promotion of degradation, inhibiting p53-dependent apoptosis pathways (Figure 2). Moreover, it has been discovered that E6AP ubiquitinates some endogenous human proteins, such as HHR23A (a human homolog of the yeast DNA repair protein Rad23) (Kumar et al., 1999) and PML (promyelocytic leukemia) tumor suppressor (Louria-Hayon et al., 2009), in an E6-independent manner, suggesting that it also promotes the degradation of tumor-associated proteins. Despite advances in understanding of the molecular mechanism and structural study for HECT domains of E6AP (Huang et al., 1999) and other related enzymes (Eletr and Kuhlman, 2007; Ogunjimi et al., 2005), to the best of our knowledge, no selective inhibitor against E6AP has been yet reported by means of high-throughput screenings. Therefore, the development of inhibitors against HECT domain remains a formidable challenge. We here chose E6AP as a previously nondruggable target and performed selection of anti-E6AP peptides using RaPID system.

E6AP HECT domain was expressed as a fusion protein with an Avi-$(His)_6$-tag followed by a solubility-enhancement tag GB1 (streptococcal B1 immunoglobulin-binding domain of protein G) (Liu et al., 2009; Zhou et al., 2001) at the N terminus. This protein construct, Avi-$(His)_6$-GB1-HECT, in which Avi domain was biotinylated during the expression, was immobilized on streptavidin magnetic beads (SAvB, Figure 1B). The library was applied to the protein beads, and the bound fractions were selected at 4°C in the first and second round (Figure 3A). From the third round, GB1-immobilized SAvB was used as the negative selection to remove GB1-binding peptides, and then the positive selection was performed at 37°C to enrich the population with specific binding activity against the HECT domain. In the sixth round, we observed a significant increase in recovery rate of active fractions (Figure 3A), and therefore the resulting cDNA sequences in the pool were analyzed.

The sequence alignment of 47 clones revealed eight independent sequence families found in the pool 6 (Figure 3B). Six families, CM_{11}-1–6, are originating from the $(NNU)_{11}$ pool, whereas the remaining two families, CM_{13}-7 and CM_{13}-8, are from the $(NNU)_{13}$ pool. The most abundant sequence, CM_{11}-1, bearing four *N*-methyl residues, shares several common signatures of



B

Clone	Random region	MALDI-TOF analysis
	$Ac^{D}W$ X_1 X_2 X_3 X_4 X_5 X_6 X_7 X_8 X_9 X_{10} X_{11} X_{12} X_{13} X_{14} X_{15} C_{16}-S-$(GS)_3$	Calc. / Obs. $[M+H]^+$
CM_{11}-1 (15/47)	C D V ^{Me}S G R ^{MeF} ^{MeG} Y ^{MeF} P · · · · ·	2064.85 / 2064.68
CM_{11}-2 (1/47)	C D V ^{Me}S G R ^{MeF} ^{MeG} Y ^{MeG} P · · · · ·	1974.80 / 1974.79
CM_{11}-3 (6/47)	C D V ^{Me}S H Y ^{MeF} P Y ^{MeF} P · · · · ·	2177.86 / 2177.65
CM_{11}-4 (5/47)	C H S ^{Me}S G H ^{MeF} R Y ^{MeF} P · · · · ·	2140.87 / 2140.69
CM_{11}-5 (7/47)	C S N ^{MeA} G H ^{MeF} P Y ^{MeF} P · · · · ·	2042.79 / 2042.65
CM_{11}-6 (1/47)	C H N ^{MeA} G H ^{MeF} S Y ^{MeF} P · · · · ·	2082.81 / 2082.79
CM_{13}-7 (1/47)	C D V ^{Me}S G R ^{MeF} S Y ^{MeF} P R ^{MeA} · ·	2322.00 / 2321.47
CM_{13}-8 (11/47)	S R P V T C ^{MeG} Y R Y Y ^{MeF} ^{MeG} · ·	2471.08 / 2470.48

Figure 3. In Vitro Selection of Macrocyclic *N*-Methyl-Peptides against E6AP HECT Domain and Selected Active Species

(A) Progress of the selection. Recovery rates of cDNA from each round were estimated by recovered amounts over input amounts of cDNAs monitored by real-time PCR. The first and second rounds of selection were performed at 4°C, and the subsequent rounds were performed at 37°C. Those determined against SAvB•biotin-Avi-$(His)_6$-GB1-HECT as a positive selection are shown in blue, whereas those against SAvB•biotin-Avi-$(His)_6$-GB1 as a negative selection shown in red.

(B) Peptide sequences identified from the pool in round 6. The apparent frequency over a total number of clones and *N*-methyl amino acids of each sequence are shown in parentheses and red, respectively. The parental random region is highlighted in gray, in which a dot denotes a residue that did not appear in the random X_{12-15} region. Calculated (Calc.) and observed (Obs.) mass values ($[M+H]^+$) of each peptide expressed by FIT system are shown. See also Figure S1 and Table S1.

residues with other sequences. $^{Me}S_4$, G_5, $^{Me}F_7$, Y_9, $^{Me}F_{10}$, and P_{11} are highly conserved in CM_{11}-1–6 and CM_{13}-7, where four residues are notably the secondary amino acids. Among them, $^{Me}F_{10} \rightarrow {}^{Me}G_{10}$ as well as $^{Me}S_4 \rightarrow {}^{Me}A_4$ substitutions were found in CM_{11}-2 and CM_{11}-5/CM_{11}-6, respectively, suggesting that the role of side chain group may be less important in activity but critical to be the *N*-methyl substituent. Moreover, $^{Me}G_8$ could be substituted with P_8 observed in abundant CM_{11}-3 and CM_{11}-5, whereas another abundant CM_{11}-4 and two other independent peptides CM_{11}-6–7 have R_8 and S_8, respectively. This may suggest that X_8 prefers to be the secondary amino acid but tolerates other amino acid substitutions. Mutations occurred during the course of selection is discussed more details in the Supplemental Information (see Figure S1 available online).

It is intriguing that the selection yielded only two lengths of *N*-methyl-peptides from the $(NNU)_{8-15}$ random region, given that other lengths of peptides should coexist in the initial pool, suggesting that the three-dimensional structure arisen from these two lengths might be critical to exhibit binding activity against E6AP HECT domain (Figure 3B). Importantly, the successful outcomes clearly demonstrated that the *N*-methyl-peptide library used in this study had a reliable quality and high complexity giving the *N*-methyl residues in the random region, and the RaPID system enabled us to select active species effectively and rapidly. The observed similarity in the composition of amino acid residues, particularly the positions as well as kinds of *N*-methyl residues appeared in the sequences, also suggests that specific interactions between the selected *N*-methyl-peptides and E6AP HECT domain are very likely occurring.

Characterization of Isolated Anti-E6AP Macrocyclic *N*-Methyl-Peptides

To confirm whether the identified mRNA sequences of CM_{11}-1–6 and CM_{13}-7–8 correctly produced the encoding macrocyclic *N*-methyl-peptides, we constructed the respective mRNA templates with a substitution of UAG with UAA that acts as a terminator of endogenous RF2 and performed in vitro expression using the same FIT system as the selection (Figure 3B, data in the column of MALDI-TOF analysis). MALDI-TOF analysis of the crude product expressed from the respective mRNA template showed a clean peak of which molecular mass is consistent with that calculated. This observation made us confident that the selected peptides had the *N*-methylated backbone as well as the composition of residues, as we expected from the cDNA sequences. For further characterization, we decided to focus on three abundant peptides, CM_{11}-1, CM_{11}-3, and CM_{11}-5, all of which consist of a total of 14 amino acid residues. The respective *N*-methyl-peptides were chemically synthesized by standard Fmoc solid-phase chemistry where the C terminus of G_{17} (corresponding to the 14th residue) was modified with carboxyamide (Table S1).

Because the above three peptides have a cysteine residue at position 1 (C_1) in the random region adjacent to $ClAc^{D}W$, cyclization between the ClAc group with C_1 side chain designated by UGU would potentially compete with that between the ClAc group with C_{16} side chain designated by UGC. To decipher which cyclization preferentially or selectively occurred, we synthesized three peptides based on CM_{11}-1 as a representative peptide; one is CM_{11}-1 itself, and the others are $C_1 \rightarrow S_1$ mutant of CM_{11}-1 (CM_{11}-1S_1) and the corresponding to linear *N*-methyl-peptide by altering ClAc to acetyl (Ac) group, referred to as LM_{11}-1 (Table 1). Fragmentation of LM_{11}-1 by MALDI-TOF/TOF yielded peaks corresponding to linear fragments, as expected (Figure S2A). On the other hand, MALDI-TOF/TOF fragmentation of CM_{11}-1 and CM_{11}-1S_1 was much difficult than that for LM_{11}-1, and both gave similar fragmentation patterns (Figures S2B and S2C). Importantly, we were able to identify peaks corresponding to fragments containing the thioether linkage between the N-terminal acetyl group and the sulfhydryl group of C_{16} side chain in both CM_{11}-1 and CM_{11}-1S_1, but not between Ac and C_1 side chain in CM_{11}-1. These results well agree with the selective formation of the thioether linkage of Ac-S-C_{16}.

To evaluate the binding abilities of chosen peptides, CM_{11}-1, CM_{11}-3, and CM_{11}-5 (a series of these peptides are referred to as CM_{11}-peptides), we determined their kinetic and dissociation constants by means of surface plasmon resonance (SPR)

Table 1. Kinetic and Equilibrium Constants of Macrocyclic *N*-Methyl-Peptides against E6AP HECT Domain

Peptide	Sequence	k_{on} (x10^6) $M^{-1}s^{-1}$	k_{off} (x10^{-3}) s^{-1}	K_d nM
CM_{11}-1	$Ac^{D}WCDV^{Me}SGR^{Me}F^{Me}GY^{Me}FPCG$-$NH_2$ (S–C)	2.66	1.60	0.60
LM_{11}-1	$Ac^{D}WCDV^{Me}SGR^{Me}F^{Me}GY^{Me}FPCG$-$NH_2$	0.047	8.46	180
CM_{11}-1S_1	$Ac^{D}WSDV^{Me}SGR^{Me}F^{Me}GY^{Me}FPCG$-$NH_2$ (S–C)	1.33	15.5	11.7
CP_{11}-1	Ac^{D}WCDV S GR F G Y F PCG-NH_2 (S–C)	not detectable		>1000
LP_{11}-1	Ac^{D}WCDV S GR F G Y F PCG-NH_2	not detectable		>1000
CM_{11}-3	$Ac^{D}WCDV^{Me}SHY^{Me}F$ P Y ^{Me}FPCG-NH_2 (S–C)	0.257	0.320	1.24
LM_{11}-3	$Ac^{D}WCDV^{Me}SHY^{Me}F$ P $Y^{Me}FPCG$-NH_2	0.014	4.46	325
CM_{11}-5	$Ac^{D}WCSN^{Me}AGH^{Me}F$ P $Y^{Me}FPCG$-NH_2 (S–C)	2.19	2.87	1.31
LM_{11}-5	$Ac^{D}WCSN^{Me}AGH^{Me}F$ P $Y^{Me}FPCG$-NH_2	0.089	20.6	231

Data were collected by the standard SPR method using Biocore T100 and the constants were generated by the equipped data fitting program. See also Figures S2 and S3.

analysis against the E6AP Avi-$(His)_6$-GB1-HECT domain immobilized on a SAv-sensor chip (Table 1). All macrocyclic CM_{11}-peptides have values of k_{on} with a range of 0.2–3 × 10^6 $M^{-1}s^{-1}$, k_{off} with a range of 0.3–3 × 10^{-3} s^{-1}, resulting in the dissociation constants (K_d) with subnanomolar to 1 nM range. Thus, the representative CM_{11}-peptides studied here have remarkably strong affinity to E6AP HECT domain, and particularly CM_{11}-1 has the lowest K_d values among the CM_{11}-peptides. In addition to the CM_{11}-peptides, we synthesized the respective linear peptides bearing N-terminal acetyl group, referred to as LM_{11}-peptides. All LM_{11}-peptides lost affinity over 170-fold. This suggests that the macrocyclic structure closed by the Ac-S-C_{16} thioether bond in CM_{11}-peptides is crucial to exhibit high binding activity to the HECT domain.

In addition to the above peptides, we synthesized three more mutants of CM_{11}-1 to further validate the importance of the specific structure. One was the aforementioned $C_1 \rightarrow S_1$ mutant peptide, CM_{11}-1S_1, and the others are a macrocyclic peptide without N-methyl backbone but having the same side chains, CP_{11}-1, and its linear peptide, LP_{11}-1 (Table 1). CM_{11}-1S_1 had a 9-fold faster k_{off} rate than CM_{11}-1, implying that C_1 somehow contributes to slowing the dissociation from the target site but is not absolutely essential for binding activity. On the other hand, the other two mutants completely lost binding ability, indicating that *N*-methylated backbone with the N-terminal Ac-C_{16} thioether macrocycle is crucial to maintain the full binding capability of CM_{11}-1 against E6AP HECT domain.

Furthermore, we verified the binding specificity of CM_{11}-peptides against E6AP HECT domain using a different HECT-type E3 Ub ligase, Smurf2 (Ogunjimi et al., 2005). None of CM_{11}-peptides had any SPR signature of binding against Smurf2 Avi-$(His)_6$-GB1-HECT domain immobilized on a SAv-sensor chip, indicating that CM_{11}-peptides have high selectivity toward the E6AP HECT domain over Smurf2 HECT domain nor Avi-$(His)_6$-GB1-tag region (data not shown). Moreover, we investigated human plasma stability of CM_{11}-peptides compared with other control peptides (see more details in Supplemental Information and Figure S3), indicating that a CM_{11}-peptide (CM_{11}-1S_1) is very stable in plasma. Taken together, CM_{11}-peptides have remarkable binding activity and specificity to E6AP HECT domain and plasma stability.

CM_{11}-1 Inhibits Ubiquitination of Target Proteins Catalyzed by E6AP

Despite the observation of strong binding activity of CM_{11}-peptides to E6AP HECT domain, it does not necessarily mean that they are able to inhibit the E6AP ubiquitination activity. To assess whether CM_{11}-peptides have an ability to inhibit the ubiquitination activity, we chose the most active CM_{11}-1 and performed an in vitro assay to monitor ubiquitin thioester formation. It is known that E6AP or even its HECT domain alone forms the ubiquitin thioester intermediate (Beaudenon et al., 2005; Scheffner et al., 1990; Scheffner et al., 1995) in the presence of Ub, E1, and E2 in vitro (Figure 2). When an in vitro translation system from rabbit reticulocyte lysate (RRL), which contains these essential Ub-related components, was used for translating the E6AP HECT domain from the appropriate mRNA template in the presence of [^{35}S]-Met (Huibregtse et al., 1995),

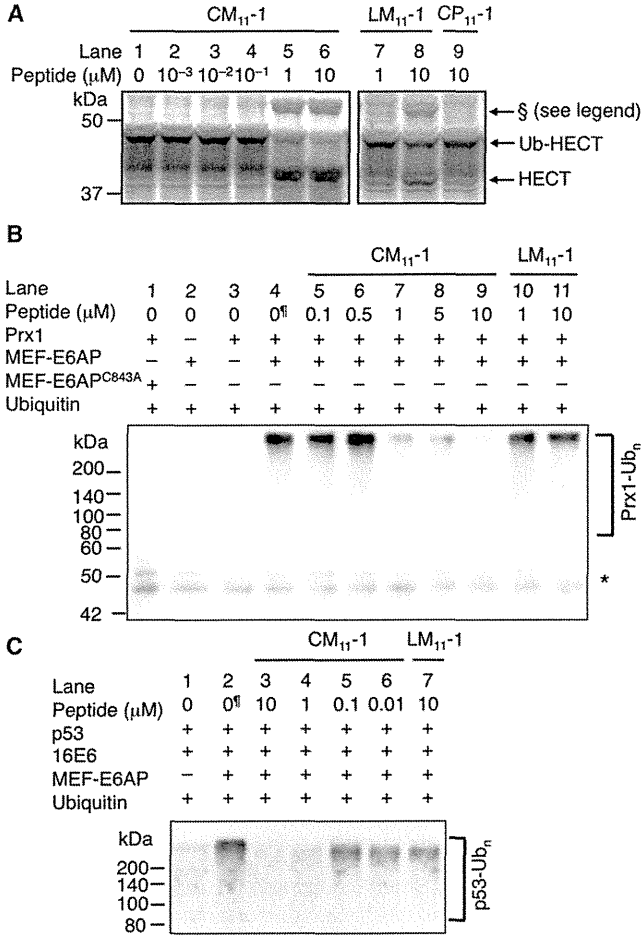


Figure 4. Inhibition of Ub-Thioester Formation with E6AP HECT Domain and Polyubiqutination of Target Proteins by CM_{11}-1 and Its Derivatives

(A) Inhibition of Ub-thioester formation with E6AP HECT domain. ^{35}S-labeled E6AP HECT (43 kDa) was translated in a rabbit reticulocyte lysate cell-free translation system. Because the translation lysate supposedly contained ubiquitin (8 kDa), E1, and E2, the expressed E6AP HECT would be endogenously converted to the ubiquitin-adduct (E6AP HECT-Ub, 51 kDa). The translation mixture was treated with various concentrations of peptides (10^{-3}–10 μM) at room temperature for 30 min. The resulting mixtures were analyzed by SDS-PAGE without DTT. §When the Ub-thioester formation on E6AP was inhibited by CM_{11}-1 (as well as 10 μM LM_{11}-1), a slower migrating band than Ub-E6AP appeared on the gel. Although the product of this band has not yet been defined, this band disappeared upon addition of a free thiol reagent such as DTT (data not shown), suggesting a possibility of dimer formation of HECT domain via a disulfide bond. Alternatively, the free cysteine in HECT domain formed a disulfide bond with other proteins containing a free cysteine residue.

(B) Inhibition of E6-independent polyubiquitination on Prx1. $(His)_6$-Prx1 was incubated with 250 nM MEF-E6AP or inactive mutant MEF-E6AP^{C843A}, $(His)_6$-E1, $(His)_6$-UbcH7, ubiquitin and peptide at 37°C for 30 min. Reaction products were immunoprecipitated with anti-Prx1 pAb and visualized by antiubiquitin mAb immunoblotting. Asterisk denotes immunoglobulin heavy chain. ¶As a negative control, DMSO used as a cosolvent for CM_{11}-1 inhibition was added.

(C) Inhibition of E6-dependent polyubiquitination on p53. p53 was incubated with 170 nM MEF-E6AP, $(His)_6$-E1, $(His)_6$-UbcH7, ubiquitin, HPV16 E6, and peptide at 37°C for 30 min. Reaction products were immunoprecipitated with anti-p53 pAb and visualized by antiubiquitin mAb immunoblotting. See also Figure S4.

a Ub-thioester intermediate of the ^{35}S-Met-labeled HECT domain (Ub-HECT) was distinguished from the parental HECT domain by means of a SDS-PAGE mobility shift assay (Figure 4A, lane 1). When CM_{11}-1 was added to this translation assay system, the migration shift was suppressed at 1 μM or higher concentrations (lanes 5 and 6). As a negative control, LM_{11}-1 and CP_{11}-1 were also tested for the same inhibition assay, in which an approximately 50% inhibition was observed at 10 μM LM_{11}-1, whereas neither 1 μM LM_{11}-1 nor 10 μM CP_{11}-1 exhibited the inhibition. Although the observed potency by CM_{11}-1 seemed not as strong as expected from the SPR data, this could be attributed to that the RRL translation system might contain endogenous label-free E6AP (Huibregtse et al., 1991; Scheffner et al., 1993) that might interact with some fractions of CM_{11}-1, resulting in a reduction of the apparent inhibitory potency. Nevertheless, this result suggests that CM_{11}-1 is able to inhibit the charge of Ub onto the HECT domain of E6AP.

We then have further pursued testing inhibition of E6AP-catalyzed polyubiquitination on target proteins. Peroxiredoxin 1 (Prx1) is an endogenous substrate of E6AP in human cells, and its polyubiquitination occurs independently from the presence of E6 (Nasu et al., 2010). To monitor the inhibitory action of CM_{11}-1 against E6AP, we used an in vitro-reconstituted Prx1 polyubiquitination assay system, in which purified His-tagged Prx1 was incubated with E6AP tagged with MEF (Myc-TEV protease site-flag) and ubiquitin in the presence of purified His-tagged E1 and E2 (UbcH7). The resulting polyubiquitinated Prx1 (Prx1-Ub_n) and free Prx1 were immunoprecipitated by anti-Prx1 polyclonal antibodies and were immunoblotted by an anti-Ub monoclonal antibody to visualize in SDS-PAGE (Figure 4B, lanes 1–9). As negative controls, LM_{11}-1 and CP_{11}-1 were also included in this examination. Clearly, polyubiquitination of Prx1 was inhibited by CM_{11}-1 in a dose-dependent manner (lanes 5–9), where an approximately 1 μM of CM_{11}-1 nearly shut down the E6AP activity. On the other hand, neither LM_{11}-1 (lanes 10 and 11) nor CP_{11}-1 (data not shown) was able to inhibit polyubiquitination. We also tested Prx1-polyubiquitination inhibition by CM_{11}-1S_1, showing a weaker inhibitory activity than CM_{11}-1 (Figure S4); the result seemed consistent with the K_d values for both peptides observed in SPR experiments.

Finally, we examined the inhibitory activity of CM_{11}-1 against polyubiquitination on an E6-dependent substrate, p53, using a reconstituted p53 polyubiquitination assay system. Immunoprecipitation of poly- and nonubiquitinated p53 using anti-p53 pAb followed by immunobolotting using anti-Ub mAb enabled us to detect the polyUb-p53 on SDS-PAGE (Figure 4C). Again, 1 μM CM_{11}-1 was able to inhibit polyubiquitination of p53 in a dose-dependent manner (lanes 3–6), whereas the control peptide, LM_{11}-1, was not (lane 7). The result shows that CM_{11}-1 acts as an E6AP inhibitor that prevents polyubiquitination of Prx1 and p53 in E6-independent and E6-dependent manner, respectively. The trend of K_d values of CM_{11}-1 and its mutant peptides against E6AP HECT domain determined by the SPR experiments well reflected to their observed inhibitory behaviors against ubiquitination of target substrates (Table 1 and Figure 4). Because the present assay method allowed us to detect polyubiquitination instead of monoubiquitination of the substrate proteins catalyzed by an excess amount of E6AP (greater than

two orders of magnitude) over the inherent K_d value of CM_{11}-1, the observed inhibitory potency of CM_{11}-1 was only qualitatively assessed. Most importantly, CM_{11}-1 was capable of inhibiting Ub ligase activity of E6AP even though it was simply selected by binding to E6AP.

SIGNIFICANCE

Here, we have demonstrated RaPID selection of "natural product-like" peptides consisting of thioether-macrocyclic and N-methylated backbone. The selection against E6AP HECT domain has yielded such desired peptides with remarkable binding abilities, falling in a range of K_d values from a subnanomolar to a single-digit nanomolar. One of the representative peptides, CM_{11}-1, chosen for further studies has displayed inhibitory activity against E6AP-catalyzing polyubiquitination on the target proteins, Prx1 and p53. The present work provides the proof-of-technology of RaPID system that enables for the discovery of potent inhibitors against a previously nondruggable ubiquitin ligase, thus opening a wide range of possibilities in the discovery of inhibitors against other ubiquitin ligase families. Most importantly, the natural product-like macrocyclic *N*-methyl-peptides have larger interaction surfaces compared with small organic molecules, as well as elevated stability under physiological conditions compared with ordinary peptides; therefore, they would provide tremendous potentials for the development of drug leads that disrupt not only enzyme activities but also protein-protein interactions.

EXPERIMENTAL PROCEDURES

In Vitro Translation and Selection

Translation of the first round selection was performed using 100 pmol mRNA-puromycin (initial complexity is 6×10^{13}) and 150 μl of translation mixture in the presence of 3750 pmol of ClAc^{D}W-tRNA$^{fMet}_{CAU}$, ^{Me}G-tRNA$^{Asn\text{-}E2}_{GAU}$, ^{Me}A-tRNA$^{Asn\text{-}E2}_{GGC}$, ^{Me}S-tRNA$^{Asn\text{-}E2}_{GAG}$, and ^{Me}F-tRNA$^{Asn\text{-}E2}_{GAA}$ (25 μM each), at 37°C for 30 min. Subsequently, the translation mixture was incubated at room temperature for 12 min to conjugate the translated peptide with the corresponding mRNA-puromycin. This solution was incubation with 15 μl of 200 mM EDTA (pH 8.0) at 37°C for 30 min in order to dissociate ribosomes from mRNA-peptide complexes. For the first-round selection, 11 μl of E6AP HECT immobilized streptavidin magnetic beads (Dynabeads M-280, Invitrogen) was used at a concentration of 200 nM target protein, and mixed with the solution of mRNA-displayed *N*-methyl-peptides. The binding reaction was performed at 4°C for 30 min with rotation. After supernatant was removed, the bead was washed with 300 μl of cold wash buffer (100 mM Tris-HCl [pH 7.5], 300 mM NaCl, 0.05% [v/v] tween 20). To the bead was added 40 μl of RT reaction buffer (50 mM Tris-HCl [pH 8.3], 75 mM KCl, 3 mM $MgCl_2$, 10 μM DTT, 0.5 mM dNTPs, 2 μM CGS3an13.R39) containing 200 units of M-MLV reverse transcriptase (Promega) and 8 units of RNase inhibitor (Promega), and reverse transcribed at 42°C for 60 min with rotation. The selected cDNA was eluted with 360 μl of PCR buffer (10 mM Tris-HCl [pH 7.5], 50 mM KCl, 0.1% [v/v] Triton X-100, 2.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.25 μM T7g10M.F48, 0.25 μM CGS3an13.R39) at 95°C for 5 min. After addition of *Taq* DNA polymerase to the eluate, the mixture was used for PCR amplification. The amplified DNA was purified by the extraction with phenol/chloroform and ethanol purification and was used for the next round of selection. Since the second round, 10 μl scale of transcription and 7.5 μl of ligation with puromycin linker were carried out. The resulting mRNA-puromycin of the second round was translated using 5 μl of the translation mixture in the presence of each 25 μM acyl-tRNAs at 37°C for 30 min, followed by incubation at room temperature for 12 min. After incubation with 1 μl of 100 mM EDTA (pH 8.0) at 37°C for 30 min, the reverse transcription of the mRNA-displayed peptides was performed by RT reaction buffer in the presence of M-MLV reverse transcriptase without RNase H activity (Promega), at 42°C for 60 min with rotation. After quenching the reaction with 1 μl of 100 mM EDTA (pH 8.0), the solution was neutralized with 1.1 μl of 0.2 M HCl. The complexes with cDNA- and mRNA-displayed peptides were subjected to 2.4 μl of the magnetic bead without target and were incubated at 4°C for 30 min for negative selection at once. Subsequently, the supernatant was mixed with 0.8 μl of the magnetic bead with E6AP HECT and was incubated with 4°C for 30 min for positive selection, followed by thrice washing with 10 μl of cold wash buffer. After addition of 100 μl of PCR buffer to the bead, the cDNA were eluted at 95°C for 5 min and amplified by *Taq* DNA polymerase. In the third round and all subsequent rounds, the all experiments were performed by a half of the reaction scale of the second round. Moreover, negative selection was performed at 4°C for 20 min at three times in the third and fourth round, and at nine times in the fifth and sixth round. On the other hand, positive selections after second round were performed by mixing with the complexes of cDNA and mRNA-displayed peptides and 200 nM E6AP HECT (not immobilized on streptavidin magnetic bead) at 37°C for 30 min, followed by pull down by streptavidin magnetic bead at 37°C for 5 min with rotation. After thrice washing with 5 μl of wash buffer at room temperature, the selected cDNA was eluted with 100 μl of PCR buffer at 95°C for 5 min. After addition of *Taq* DNA polymerase to the eluate, the mixture was used for PCR amplification. To monitor the convergence of the selection process, real-time PCR (RT-PCR) was used to quantify the amounts of input and output DNA in every round. For input cDNA, 0.25 μl aliquot of the RT mixture was diluted with 150 μl of a dilution solution (10 mM Tris-HCl [pH 8.0] and 300 mM NaCl), and 1 μl of the diluted cDNA was mixed with 19 μl of PCR buffer containing SYBR Green I (Molecular Probe) and *Taq* DNA polymerase. For output cDNA, 1 μl aliquots of the eluates from the beads of positive and negative selections were mixed with 10 μl of PCR buffer containing SYBR Green I and *Taq* DNA polymerase. The reverse transcribed $(NNU)_{10}$ mRNA mixture was serial-diluted and used for the templates as standards.

MALDI-TOF Analysis of Translated Clone Peptides

To identify the expressed cyclic *N*-methylated peptides, a 5 μl scale translation reaction was performed using FIT system with 40 nM of clone DNA, 25 μM each of ClAc^{D}W-tRNA$^{fMet}_{CAU}$, ^{Me}G-tRNA$^{Asn\text{-}E2}_{GAU}$, ^{Me}A-tRNA$^{Asn\text{-}E2}_{GGC}$, ^{Me}S-tRNA$^{Asn\text{-}E2}_{GAG}$, and ^{Me}F-tRNA$^{Asn\text{-}E2}_{GAA}$ at 37°C for 30 min. After quenching with 0.2% TFA, the crude peptide mixture was desalted with C-Tip (C18 desalting SPE, Nikkyo technos) and eluted with 80% acetonitrile and 0.5% acetic acid solution saturated with the matrix α-cyano-4-hydroxycinnamic acid (Bruker Daltonics). MALDI-TOF analysis was performed using an Autoflex TOF/TOF (Bruker Daltonics) and peptide calibration standard II (Bruker Daltonics) as external standards.

SPR Analysis of Peptides

The interaction between E6AP HECT and peptides was assessed using a BIACORE T100 instrument (GE Healthcare) equipped with research-grade streptavidin sensor chip at 25°C. Biotinylated E6AP HECT was immobilized to a surface density of approximate 1,500 response units (RU) using standard immobilization protocols (GE Healthcare). HBS-EP+ (10 mM HEPES [pH 7.4], 150 mM NaCl, 3 mM EDTA, and 0.05% [v/v] surfactant P20) containing 1.0% (v/v) DMSO was used as the running buffer for all experiments. Peptide binding was tested by injecting varying concentrations (0.3 nM to 1,000 nM) at a flow rate of 30 μl min^{-1} and measured by single cycle kinetics method. Raw data were analyzed by the BIACORE T100 evaluation software 2.01 and fitted to the standard 1:1 interaction model.

In Vitro Ubiquitin Transfer Assay by ^{35}S-Labeled E6AP HECT

E6AP HECT cDNA was subcloned into pTNT vector (Promega) at XhoI and SalI sites. ^{35}S-Methionine-labeled E6AP HECT was synthesized in vitro by TNT T7 coupled rabbit reticulocyte lysate system (Promega) at 30°C for 90 min, by following the standard procedure (Huibregtse et al., 1995). After translation, 0.5 μl of peptides in 10% (v/v) DMSO with 10× concentrations shown in Figure 4A were added to the translation reaction mixture (4.5 μL). The resulting mixture was incubated at room temperature at 30 min and

quenched with 2 × SDS-polyacrylamide gel laemli sample buffer (125 mM Tris-HCl [pH 6.8], SDS 4%, glycerol 20%, 0.002% bromophenol blue) in the absence of dithiothreitol. Samples were subjected to SDS-PAGE on a 10% polyacrylamide gel.

In Vitro Polyubiquitination Assay for Prx1 and p53

The plasmid pGEM p53 was used for in vitro translation (Werness et al., 1990). In vitro translation was performed using TNT T7 coupled rabbit reticulocyte lysate system. Recombinant baculovirus for HPV16 E6 was produced using the BaculoGold system (PharMingen) as described previously (Shirakura et al., 2007). Hi5 cells (Invitrogen) were infected with the recombinant baculovirus to produce HPV16 E6 protein. HPV16 E6 Protein was partially purified by anion-exchange chromatography as previously described (Huibregtse et al., 1993). In vitro polyubiquitination assays for Prx1 were performed essentially as described previously (Nasu et al., 2010). Hi5 cells were infected with recombinant baculoviruses AcMEF-E6AP and Ac MEF-E6AP^{C843A} to produce MEF-E6AP and MEF-E6AP^{C843A}, respectively (Shirakura et al., 2007). MEF-E6AP and MEF-E6AP^{C843A} were purified on anti-FLAG M2 agarose beads (Sigma) according to the manufacturer's instructions. Assays were done in 40 μl volumes containing 20 mM Tris (pH 7.6), 50 mM NaCl, 5 mM $MgCl_2$, 100 μM DTT, 5 mM ATP, 250 nM MEF-E6AP or inactive mutant MEF-E6AP^{C843A}, 62.5 nM $(His)_6$-E1, 1.1 μM $(His)_6$-UbcH7, 25 μM ubiquitin, 8 μM $(His)_6$-Prx1 and peptide, and incubation at 37°C for 30 min. Reactions were performed at 37°C for 30 min. The ubiquitination reaction was terminated by freezing the samples with liquid nitrogen. To dissociate proteins, 1% SDS was added to lysates, which were then heated at 90°C for 15 min. The samples were diluted 10-fold with a dissociation dilution buffer containing 1% NP-40, 0.5% deoxycholate, 120 mM NaCl, 50 mM HEPES, 1 mM EDTA, and complete protease inhibitor cocktail (Roche). Samples were immunoprecipitated with anti-Prx1 PAb and analysis by immunoblotting with antiubiquitin mouse monoclonal antibody (anti-Ubi-1, Millipore) to detect ubiquitinated Prx1. In vitro polyubiquitination assays for p53 were performed essentially as described previously (Nakagawa and Huibregtse, 2000). Assays were done in 75 μl volumes containing 25 mM Tris-HCl (pH 8.0), 125 mM NaCl, 2 mM $MgCl_2$, 50 μM DTT, 5 μM ubiquitin, 2 mM ATP, 170 nM MEF-E6AP, 33 nM $(His)_6$-E1, 0.6 μM $(His)_6$-UbcH7, 2 μl of partially purified HPV16 E6, and 5 μl of in vitro translated p53. Peptides inhibitors were added to the samples as indicated. The reaction mixtures were incubated at 37°C for 30 min. The ubiquitination reaction was terminated by freezing the samples with liquid nitrogen. To dissociate proteins, 1% SDS was added to lysates, which were then heated and diluted as described above. Samples were immunoprecipitated with anti-p53 rabbit polyclonal antibody (FL393, Santa Cruz), followed by immunoblotting with antiubiquitin mouse monoclonal antibody (anti-Ubi-1, Millipore) to detect ubiquitinated p53.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, one table, and supplemental Experimental Procedures and may be found with this article online at doi:10.1016/j.chembiol.2011.09.013.

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Ribosomal Synthesis of Backbone-Macrocyclic Peptides Containing γ-Amino Acids

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Pepstatin, discovered in 1970 as an extraordinarily potent inhibitor of pepsin, is a classical example of a γ-amino acid-containing natural product.[1] It has been proven that the statine residue, (3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid, mimics the tetrahedral transition-state structure of peptide-bond hydrolysis.[2] This knowledge led to the development of specific inhibitors of clinically relevant aspartic proteases, including renin and HIV proteases.[3] More recently, a new class of γ-amino acid-containing natural products has been discovered that is active against a non-protease enzyme. Spiruchostatin A, which was isolated from *Pseudomonas* sp., is a representative example; it has a macrocyclic structure containing a D-valine-derived (3*S*,4*R*)-statine and acts as a histone deacetylase inhibitor.[4] Clearly, the statine residue in this molecule does not act as a transition-state analogue but rather serves as a critical component of the macrocyclic scaffold. Moreover, didemnins B, isolated from the genus *Tridemnum* as an agent against kidney and epithelial ovarian cancer, has a γ-amino acid, isostatine, and also has a macrocyclic moiety.[5] These examples imply that statine and probably other γ-amino acids in a macrocyclic scaffold can be versatile structural elements for constructing bioactive peptides. Despite the attractiveness of γ-amino acid-containing peptides, their availability still relies on the traditional methodologies, that is, the serendipitous discovery of bioactive γ-amino acid-containing peptides from secondary-metabolite sources or by chemical synthesis from small libraries. We here report a new methodology involving genetic code reprogramming to ribosomally express backbone-cyclized peptides containing γ-amino acids (Figure 1 A) that potentially gives us more diverse libraries of γ-amino acid-containing peptides with a greater ease.

Since the translation apparatus allows for the facile construction of a peptide library with vast diversity, ribosomal synthesis of drug-like peptides can be a powerful tool for discovering novel bioactive compounds.[6] However, despite a number of reports describing the incorporation of various nonstandard amino acids into nascent peptide chains by using a conventional strategy such as nonsense or frame-shift suppression,[7] to the best of our knowledge, a successful demonstration of ribosomal incorporation of γ-amino acids has never appeared in literature. There are two reasons for this failure: 1) γ-Aminoacyl-tRNA is not stable under near-neutral aqueous conditions as the γ-amino group tends to intramolecularly attack the acyl group on the 3′ terminus of tRNA; this results in self-deacylation. 2) Even if some fractions of γ-aminoacyl-tRNA can be brought into the ribosome A site, the ribosome very likely fails to promote the peptidyl-transfer reaction as its catalytic environment is incompatible with bond formation between the γ-amino group and the acyl group on the P site tRNA.[8] As a result, no successful example of expressing any peptide containing γ-amino acids has been reported.

We have recently developed two new methods of generating unique peptides by using a custom-made, reconstituted, cell-free translation system integrated with flexizymes (flexible tRNA aminoacylation ribozymes)[9] and referred to as FIT (flexible in-vitro translation) system.[10] Using this system, we reprogrammed an initiation event in translation in which a FIT system lacking methionine was supplemented with a $tRNA^{fMet}_{CAU}$ charged with short exotic peptides by flexizyme; thereby, peptides containing unusual auxiliary residues at the N terminus were expressed.[11] The second method was ribosomal synthesis of backbone-cyclic peptides in a FIT system in which a codon was assigned to glycolic acid (^{HO}G). Expression of linear peptides bearing cystidyl-prolidyl-glycolate (C-P-^{HO}G) resulted in self-rearrangement[12] into peptides with a C-terminal diketopiperadine (dkp) thioester upon cleavage of the ester bond between P and ^{HO}G. The presence of two recombinant enzymes, peptide deformylase (PDF) and methionine aminopeptidase (MAP), in the FIT system generates an N-terminal free amino group that spontaneously reacts with the intramolecular dkp-thioester to afford the backbone-cyclized peptides.[13]

Although direct acylation of γ-amino acid onto tRNA would suffer from self-deacylation through intramolecular cyclization, acylation of a dipeptide consisting of γ- and α-amino acids onto tRNA could avoid such an undesired side reaction. To test whether this approach would yield stable peptidyl-tRNAs, we

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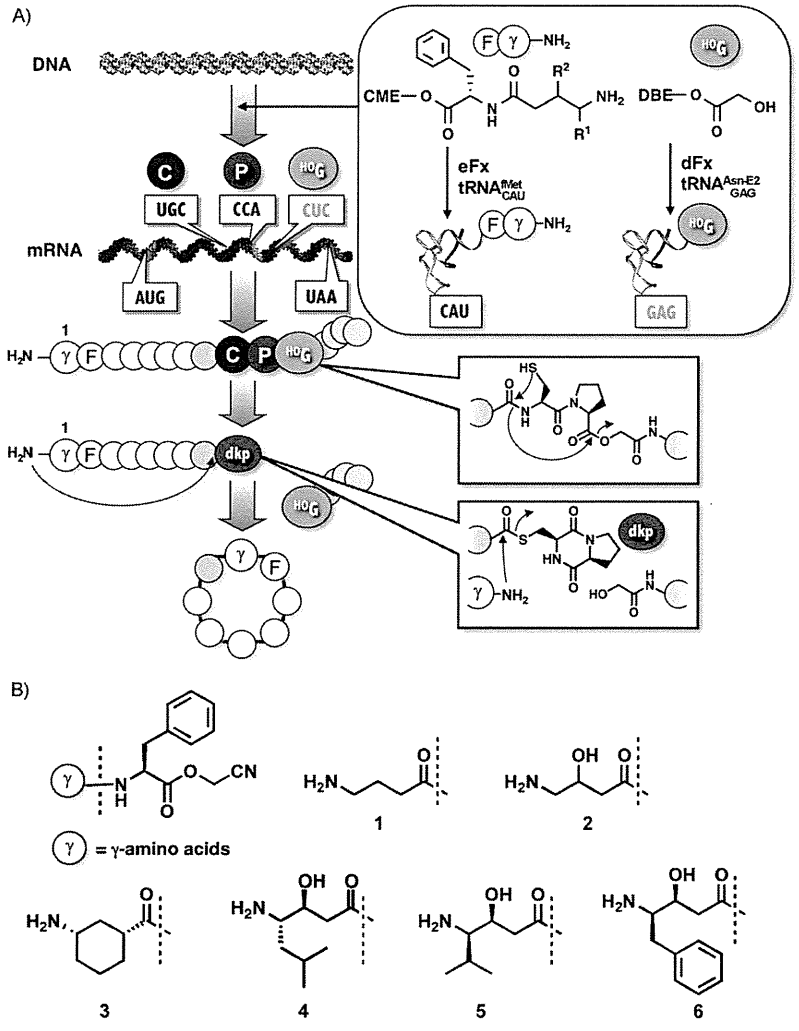

Figure 1. A) Outline of the ribosomal synthesis of cyclic γ-peptides. A linear precursor peptide containing a γ-amino acid at the N terminus and a C-P-^{HO}G motif downstream is expressed in a FIT system that lacks M and L and supplied with γ-aa-F-tRNA$^{fMet}_{CAU}$ and ^{HO}G-tRNA$^{AsnE2}_{GAG}$. Self-rearrangement of the C-P-^{HO}G segment to form dkp-thioester and the subsequent intramolecular addition of the N-terminal γ-amino group to the C-terminal thioester results in a backbone-cyclized peptide containing the γ-amino acid. B) γ-aa-F-CMEs used in this study. Each γ-amino acid (**1**: 4-aminobutyric acid, **2**: (*R/S*)-4-amino-3-hydroxy butyric acid, **3**: (3-aminocyclohexane)carboxylic acid, **4**: (3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid, **5**: (3*S*,4*R*)-4-amino-3-hydroxy-5-methylhexanoic acid, **6**: (3*S*,4*R*)-4-amino-3-hydroxy-5-phenylpentanoic acid) is chemically coupled with F-CME to obtain the corresponding dipeptide, and its tRNA aminoacylation is performed with flexizyme.

chose six γ-amino acids, including 4-aminobutanoic acid, statine, and its analogues, and condensed them with phenylalanine cyanomethyl ester (F-CME) to generate the corresponding γ-amino-dipeptide-CMEs **1**–**6** (Figure 1B). All the γ-amino-dipeptides were charged onto the acceptor analogue of tRNA, microhelix RNA, by a flexizyme (eFx), and the resulting γ-amino-dipeptidyl-RNAs were confirmed by acid-PAGE (Figure S1 in the Supporting Information). Moreover, when a model mRNA is expressed in a methionine-deficient FIT system containing γ-amino-dipeptide-tRNA$^{fMet}_{CAU}$ (Figure S2A), the γ-amino-dipeptides were successfully assigned as a reprogrammed initiator; this indicated that the designated model peptides bearing N-terminal γ-amino acids were generated with satisfactorily high expression profiles (Figure S2B) as well as the correct molecular mass (determined by MALDI-TOF MS; Figure S2C). These results clearly demonstrated that reprogramming the initiation event with short peptides containing γ-amino acids is a versatile and efficient method of ribosomally expressing polypeptides bearing γ-amino acids at the N terminus.

Having successfully incorporated γ-amino acids into the expressed peptides, we envisioned that this γ-amino-dipeptide initiation approach could be integrated with C-terminal dkp-thioester formation to promote the head-to-tail backbone cyclization (Figure 1A). Because no terminus in the final cyclic peptide is generated in this strategy, the γ-amino acid is embedded in the middle of sequence, that is, a defect of the γ-amino-dipeptide initiation approach, in which the position of the γ-amino acid is limited to the N terminus, can be overcome. We first designed an mRNA with the AUG initiation and CUC leucine elongation codons reprogrammed with γ-amino-dipeptides and ^{HO}G, respectively, to synthesize a model cyclic peptide containing a γ-amino acid (Figure 2A). With the above reprogrammed genetic code supported by a FIT system lacking M and L and supplied with **1**-F-tRNA$^{fMet}_{CAU}$ and ^{HO}G-tRNA$^{AsnE2}_{GAG}$, the peptide bearing γ-amino acid **1** at the N terminus and a C-P-^{HO}G segment in the downstream region was synthesized. After incubation at 37 °C for 12 h to facilitate the self-rearrangement of C-P-^{HO}G to dkp-thioester and subsequent macrocyclization, the peak corresponding to the expected backbone-cyclic peptide (**7**–**1**, Figure 2B) along with a minor peak with the molecular weight of a linear peptide generated by hydrolysis of the thioester intermediate (**8**–**1**, Figure 2C) was detected in the MALDI-TOF mass spectrum (Figure 2D). Tandem mass spectrometry of **7**–**1** proved the formation of an amide bond between the N-terminal γ-amino acid and the glycine at the ninth position (Fig-

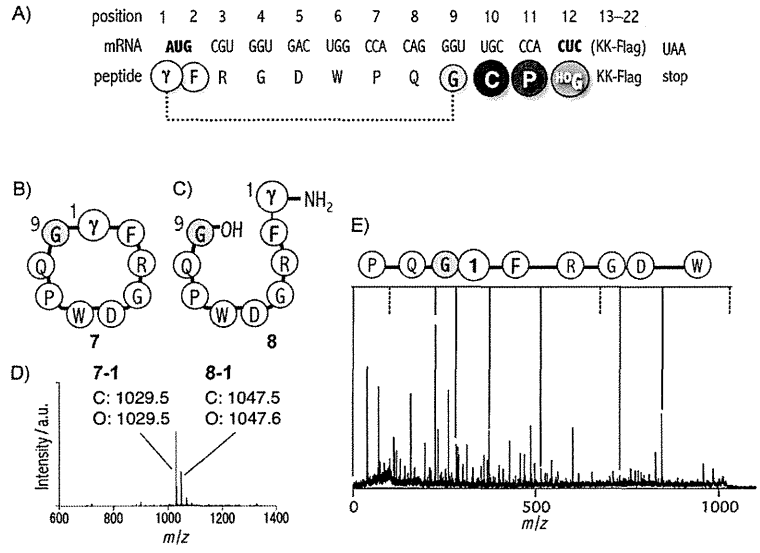

Figure 2. Ribosomal synthesis of backbone-cyclic peptides containing γ-amino acids by thioesterification. A) Sequences of the mRNA and the expressed linear precursor peptide. AUG and CUC codons are reassigned with γ-aa-F (γ-aa = **1**–**6**) and ^{HO}G, respectively. The KK-Flag in parenthesis indicates the RNA sequence encoding this peptide (KK-DYKDDDDK). *The dotted line shows the ring-closing pattern after macrocyclization.* B) *Schematic structure* of the objective γ-amino acid-containing cyclic peptide (**7**). "γ" indicates various γ-amino acid residues (**1**–**6**). The residue numbering is given. C) Schematic structure of the linear hydrolysate of the dkp-thioester intermediate (**8**). D) Mass spectrum of the reaction product from reprogrammed translation with **1**-F initiator. The detected peaks corresponding to the cyclic product (**7**–**1**) and hydrolysis product (**8**–**1**) were labeled in the spectrum. The calculated mass (C) and observed mass (O) for singly charged species, $[M+H]^+$, are also shown in the spectrum. E) *Tandem mass spectrum of* **1** *containing cyclic peptide* (**7**–**1**). Peaks corresponding to a set of assigned sequential fragment ions, generated by cleavage of two amide bonds, are shown.

ure 2E). These data demonstrate ribosomal synthesis of backbone-cyclic peptide containing a γ-amino acid. When we used dipeptides bearing relatively small γ-amino acids (**1** and **2**) as reprogrammed initiators, the objective macrocyclic peptides were generated as the main products (Figure S3A); however, in the case of more sterically demanding γ-amino acids (**3**–**6**), a peak corresponding to the linear peptide in which the peptide bond between G9 and C10 was cleaved (originating from the hydrolysis of dkp-thioester) dominated the mass spectra (Figure S3B–E); in particular when **5**- or **6**-F-tRNA$^{fMet}_{CAU}$ was used, no desired backbone-macrocyclic peptide was observed. This was a serious limitation of the present strategy.

To increase the level of production of backbone macrocyclization, we embedded cysteine residues in the peptide sequence (Figure 3A). We expected that their sulfhydryl side chains could rapidly form cyclic thioester intermediates by intramolecular trans-thioesterification, thereby promoting the subsequent attack of N-terminal γ-amino group on the thioester, possibly with a decreased level of the competing hydrolysis (Figure S4). An mRNA (Figure 3C) was designed based on a scaffold of bicyclic sunflower trypsin inhibitor-1 (SFTI-1)[14] and expressed under the same reprogrammed genetic code in the presence of various γ-amino-dipeptidyl-tRNA$^{fMet}_{CAU}$. The resulting translation product was incubated for 16 h to promote backbone macrocyclization in situ. In all cases, we observed a peak corresponding the desired backbone-macrocyclic peptide **9**, but the level of competing hydrolysis product **10** depended on the γ-amino acids (Figure 3D). It should be noted, however, that the consistent observation of the backbone-macrocyclic peptides throughout the γ-amino acid kinds indicates the reliability of this strategy.

In summary, we have developed a method to express peptides bearing γ-amino acid at the N terminus by reprogramming the genetic code of initiation using various dipeptide initiators. To the best of our knowledge, this is the first demonstration of ribosomal synthesis of γ-amino acid-containing polypeptides. Moreover, this methodology was coupled with the in situ generation of dkp-thioester at the C terminus of the peptide to yield backbone-macrocyclic peptides containing various γ-amino acids at an internal position. Given the previous report that the *reprogrammed initiation* event accepts short peptides of a wide variety of nonstandard structures, such as D-, β-, *N*-methyl, and α-disubstituted amino acids, as well as aminobenzoic acids,[11c] this methodology should be applicable to the ribosomal synthesis of backbone-macrocyclic peptides containing diverse nonstandard amino acids, thereby enabling us to construct exotic macrocyclic peptide libraries and explore new chemical space for novel functions.

Experimental Section

Flexizyme acyl-tRNAs were prepared by aminoacylation according to the following procedure. For γ-aa-F-tRNA$^{fMet}_{CAU}$, tRNA$^{fMet}_{CAU}$ (6.25 μL, 40 μM) in Tris-HCl (0.1 M, pH 8.0) was heated at 95 °C for 1 min, then cooled to room temperature over 5 min. $MgCl_2$ (2.5 μL, 3 M) and eFx (1.25 μL, 200 μM) were added to the solution, which was incubated at room temperature for 5 min. γ-aa-F-CMEs in DMSO (2.5 μL, 25 mM each) were then added to the mixture, which was incubated on ice for 2 h. ^{HO}G-tRNA$^{AsnE2}_{GAG}$ was synthesized as previously reported.[13] Each acylation reaction was quenched by the addition of sodium acetate (40 μL, 0.6 M, pH 5.0), and the acyl-tRNA was recovered by ethanol precipitation. The pellet was rinsed twice with 70% ethanol with sodium acetate (0.1 M, pH 5.0) and once with 70% ethanol, then dried and stored at −80 °C. The acyl-tRNAs were dissolved in sodium acetate (0.5 μL, 1 mM) just before being added to the translation mixture.

For reprogrammed translation initiation with γ-amino acids, translation mixture was prepared as previously reported.[10,11c,13] Translation was carried out in a reaction mixture containing Thr, Tyr, Lys,

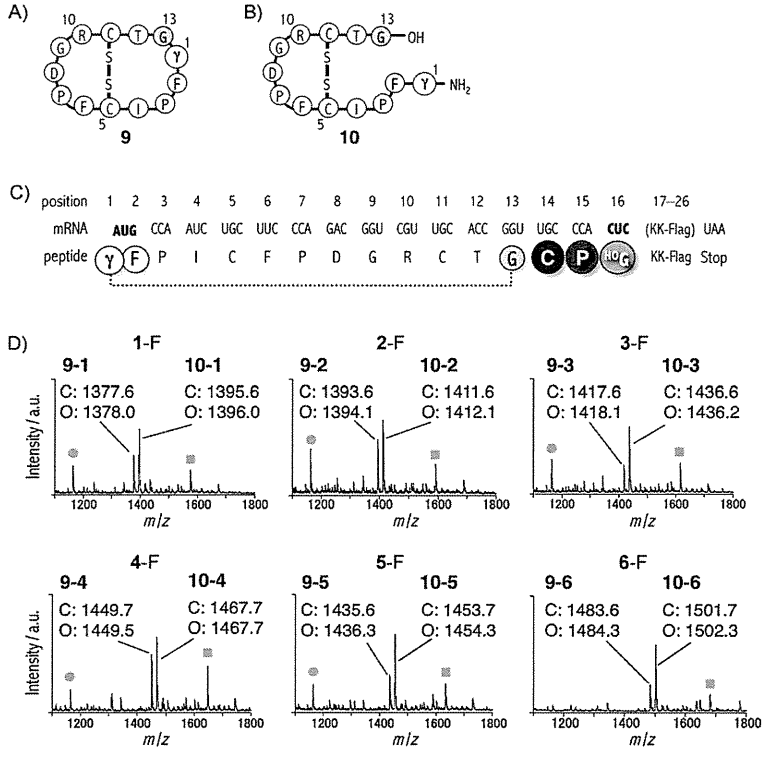

Figure 3. Ribosomal synthesis of bicyclic peptides containing γ-amino acids by thioesterification. A) Schematic structure of the bicyclic peptides closed by a backbone peptide bond and a disulfide bond (**9**). "γ" indicates various γ-amino acid residues (**1**–**6**). Residue numbering is shown. B) Schematic structure of the hydrolysate of dkp-thioester intermediate (**10**). C) Sequences of the mRNA and the expressed linear precursor peptide. AUG and CUC codons are reassigned with γ-aa-F (γ-aa = **1**–**6**) and ^{HO}G, respectively. The KK-Flag in parenthesis indicates the RNA sequence encoding this peptide (KK-DYKDDDDK). The dotted line shows the ring-closing pattern after macrocyclization. D) Mass spectra of the reaction product from reprogrammed translation with γ-aa-F dipeptide initiators. The detected peaks corresponding to the cyclic product (**9**-γ-aa) and hydrolysis product (**10**-γ-aa) are labeled in the spectra (γ-aa = **1**–**6**). The calculated (C) and observed (O) masses of singly charged species, $[M+H]^+$, are also shown in the spectra. The peaks corresponding to a translation side product initiated with Pro at the third position and a side product generated by addition of a cysteine onto the thioester intermediate are shown by gray circles and squares, respectively.

and Asp (200 μM each), various γaa-F-tRNA$^{fMet}_{CAU}$ molecules (50 μM) and DNA template1 (40 nM). The translation mixture (5 μL total volume) was incubated at 37 °C for 1 h. The resulting translation product was analyzed by Tricine-SDS PAGE or MALDI-TOF MS in a previously reported manner.[9–11]

For the ribosomal synthesis of cyclic peptides containing γ-amino acids, translation was carried out in a reaction mixture containing minimum required amino acids (200 μM each), γ-aa-F-tRNA$^{fMet}_{CAU}$ (100 μM), ^{HO}G-tRNA$^{AsnE2}_{GAG}$ (100 μM), and DNA template2 (40 nM). The mixture (10 μL) was incubated at 37 °C for 16 h to facilitate the translation reaction and macrocyclization. The reaction was quenched by adding 1% TFA (10 μL). The product was desalted on a C18 SPE column (T300-C18, Nikkyo Technos) and eluted with 80% acetonitrile/0.5% acetic acid (1.2 μL) saturated with (*R*)-cyano-4-hydroxycinnamic acid. The mass was measured by MALDI-TOF MS (autoflex TOF/TOF, Bruker) using the linear mode and externally calibrated with peptide calibration standard II (Bruker). Tandem mass spectrometry was also performed by using autoflex TOF/TOF in the lift mode.

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