Using the ribosome to synthesize peptidomimetics
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Abstract
Peptidomimetic research is an approach to identify peptide-based drugs designed to mimic structural, conformational, and biological properties of peptides while overcoming their limitations, such as protease instability and poor cell penetration. With recent advances in ribosomal synthesis of peptides containing unnatural amino acids, this technology appears suitable for preparing large structurally diverse libraries of peptidomimetics for drug discovery screening.

Introduction and context
Biologically active peptides are attracting increasing interest as potential therapeutic agents or leads toward such agents [1]. For many applications, native peptides are not ideal due to inherent issues, including short biological half-life (rapid cleavage by proteases and/or fast clearance), poor membrane permeability that limits bioavailability and access to intracellular targets, and physical-chemical properties [2]. Research over the last several decades has focused on the discovery, design, and synthesis of novel structures that possess the desirable properties of native peptides while overcoming their liabilities [3,4]. These ‘peptidomimetics’ have originated from both chemical synthesis and natural product sources [5,6]. An emerging complementary technology providing access to amide backbone-based peptidomimetic structures is ribosomal synthesis using unnatural amino acids. The attractive potential of ribosomal synthesis is rapid and specific preparation of large structurally diverse libraries of peptidomimetics for screening against biological targets.

The history of this approach and key recent advances in the technology have been detailed in review articles [7,8] in the last year and will be treated only briefly. Twenty years ago, two groups were able to reassign stop (or nonsense) codons to non-proteinogenic amino acids, and using chemically generated misaminoacylated transfer ribonucleic acid (tRNA), they demonstrated the incorporation of unnatural amino acids into a protein and a polypeptide [9,10]. This ‘nonsense suppression’ is suitable for the incorporation of only a single type of non-proteinogenic amino acid into a peptide chain. A complementary method using nucleotide four-base codons allowed the incorporation of two or three unnatural amino acids but is still limited in its generality. Other limitations of these methods are the restricted choice of unnatural amino acids that can be incorporated and the technically challenging process of chemically or enzymatically aminoacylating tRNA.

One key technical advance in the current decade was the development of a reconstituted Escherichia coli cell-free translation system (for example, protein synthesis using recombinant elements, or PURE [11]) in which certain amino acids and aminoacyl-tRNA synthetases (aaRSs) are removed from the translation elements. Specific vacant codons thus can be created and used for unnatural amino acids of choice. Lack of competition from the natural aminoacyl-tRNA thereby improves the efficiency of sense suppression, and the potential of this approach for production of large screenable libraries of small peptidomimetics was proposed [12]. It was emphasized that such directed in vitro evolution is much faster than lead optimization by chemical synthesis and that the superior library sizes may yield higher-affinity ligands. A second major advance came with the development of more general methods for the preparation of
nonstandard aminoacyl-tRNA. One of the most promising developments was the generation of artificial RNA enzymes (ribozymes) for tRNA aminoacylation. These so-called flexizymes [13] have a broad range of substrates (both tRNAs and amino acids), thus making available a general group of aminoacylated tRNAs. Recent publications have demonstrated that the use of one or both of these technologies for genetic code reprogramming is beginning to provide access to a diverse group of peptidomimetics (see Figure 1).

**Figure 1. Schematic examples of peptidomimetic modifications achieved by ribosomal synthesis**

| Peptidomimetic modifications | Natural peptide: R1, R2, R3 are side chains of coded amino acids |
|------------------------------|---------------------------------------------------------------|
| Peptidomimetic: One or more of R1, R2, R3 comes from an unnatural amino acid |
| Peptidomimetic incorporating Meaa (a) and hydroxy acid (b) backbone modifications |
| Peptidomimetic incorporating peptidol (rGly) modifications |

Ribosomal synthesis has produced peptidomimetics with considerable structural diversity. Examples include unnatural side chains, backbone modifications such as N-methyl amino acids (Meas) and hydroxy acids, and peptoids (N-substituted glycine, or rGly). In addition, peptidomimetics incorporating such features can be cyclized. The macrocyclic rings can be formed by amino terminus-to-side chain or side chain-to-side chain cyclization, forming thioether bridges. Bicyclic peptides are synthesized using two pairs of orthogonally reactive groups to specifically locate the thioether and triazole bridges. The circled residues in the structures illustrate that a variety of ring sizes have been shown to be accessible.

Further development of the PURE system for peptide synthesis allowed simultaneous reassignment of 35 of 61 sense codons to 12 unnatural amino acids that were previously known to be substrates for aaRSs and incorporated into proteins by *E. coli* [16]. Except for azetidine carboxylic acid, a backbone modification, these amino acids contain unnatural side chains spanning a range of physicochemical properties. The alkynyl side chain of 2-amino-hex-5-ynoic acid is of note, as it can undergo a copper (I)-catalyzed [3 + 2] cycloaddition reaction with small-molecule organic azides, leading to increased post-translational structural diversity [17]. A survey of additional unnatural amino acids showed that a diverse set of more than 50 amino acids can be incorporated into peptides by ribosomal translation [18]. Most of these are side-chain analogs, but also included are α, α-disubstituted and N-methyl amino acids (Meas) and α-hydroxy acids. With this reprogrammed genetic code, up to 13 different unnatural substitutions could be incorporated into a single peptidomimetic. This system potentially can synthesize unnatural peptide libraries of $10^{14}$ unique members.

Peptide backbone modifications such as Meas and cyclization in peptidomimetic structures are useful for improving protease stability and membrane permeability and introducing conformational constraint [2]. A chemical reductive alkylation approach to overcoming limitations of scale in chemical aminoacylation preparation of misacylated tRNAs for ribosomal synthesis of Mea-containing peptides was developed [19]. With this chemistry, the ribosomal synthesis of peptides containing up to three Meas was demonstrated [20]. Of the Meas surveyed, N-methyl leucine (MeLeu), N-methyl threonine (MeThr), and N-methyl valine (MeVal) were most efficiently incorporated into peptides. An alternative approach to a more efficient preparation of N-methyl aminoacylated tRNA uses chemically synthesized N-methyl aminoacyl adenosine monophosphate (AMP) as the substrate for aaRSs [21]. Oligomerization of MePhe assigned to repeat consecutively two, five, or 10 times was demonstrated in an *in vitro* display system [22].
Another report expanded the study of amino acid backbone specificity of the E. coli translation machinery [23]. For the incorporation of alanine (Ala) and phenylalanine (Phe) analogs into the second position of a tripeptide, Meaas and hydroxy acids gave good yields, α, α-disubstituted amino acids incorporated poorly, and β- and δ-amino acids were not detectably incorporated. Further investigations showed that proper pairing of Meaas and tRNAs can be important for efficient expression, but the more bulky N-butyl-Ala and -Phe were not incorporated [24].

A very recent refinement in the technology for ribosomal synthesis of Mea containing peptides combines the PURE system, in which both selected amino acids and aaRSs are withdrawn (wPURE) to vacate certain codons, with flexizymes for charging unnatural amino acids onto desired tRNAs [25]. With this system, the N-methyl analogs of 11 of 19 natural plus four unnatural amino acids could be incorporated, and the preferences were somewhat different from those of previous studies. Peptides containing multiple Meaas were prepared.

In a significant advance, it was demonstrated that cyclic peptides incorporating Meaas could be synthesized by assigning an Nα-(α-chloroacetyl) amino acid to the initiation codon and a cysteine (Cys) to an elongation codon for a sequence [26]. Under the conditions of the experiment, the thiol of Cys displaces the α-chloro in an intramolecular macrocyclization. Examples containing 27-atom rings with three or four Meaas or proline [25] plus examples with 15-, 21-, 36-, and 45-membered rings with natural amino acids [26] suggest considerable potential generality for this approach. Furthermore, the ribosomal synthesis of a library of 160 distinct peptides having 21-membered macrocycles was achieved. The macrocycle introduces an additional conformational constraint into the peptidomimetic, and the ability to synthesize libraries of such compounds for screening in biological assays is attractive.

The cyclization in these cases involves side chain-to-side chain variant has also been developed with the novel amino acid Nγ-(2-chloroacetyl)-α, γ-diaminobutyric acid (Cab) [27]. This amino acid was efficiently incorporated using the wPURE and flexizyme technologies, and cyclization with Cys occurred under the experimental conditions giving examples with 20-, 23-, 26-, and 35-atom rings.

Further extension of this methodology has resulted in the ribosomal synthesis of bicyclic peptides involving two pairs of amino acids with orthogonal reactivity [28]. The pairs of amino acids are Cys and Cab and azidohomoalanine (Aha) and propargyl glycine (Pgl), respectively. As in previous cases, the first macrocyclic ring results from the formation of the thioether linkage under the translation reaction conditions. Copper (I) is then used to catalyze [3 + 2] cycloaddition between the azide and alkyne side chains to form a triazole as the second bridge [17]. The preparation of several bicyclic peptidomimetic structures of different ring sizes serves to illustrate the versatility of the methodology.

Very recently, ribosomal synthesis of polypeptoids (poly-N-substituted glycines, or poly-rGlys) [29] and peptoid-peptide hybrids was reported [30]. A group of 25 rGlys containing a variety of groups was shown to be incorporated, although bulkiness near the α-amino group and charged substituents do not work well. Consecutive rGlys could be incorporated, and cyclization strategies as previously discussed were also successful for peptoid-peptide hybrid sequences.

This technology has been extended recently to the ribosomal synthesis of polyesters [31] and to translation initiation with d-amino acids [32]. The latter advance allows for capping the amino terminus of a peptide sequence with a d-amino acid or acyl-d-amino acid.

Future directions
Recent studies have shown the potential for generating libraries of peptidomimetic structures of significant structural diversity using ribosomal synthesis. Testing of such libraries in high-throughput screens offers the potential for discovery of leads to novel therapeutic agents. This approach may be complementary to chemical synthesis of such structures in terms of ligand diversity, speed, and cost.

Certain questions remain to be addressed. How broad is the chemical structural space that can be accessed by these methods? How useful will such peptidomimetic structures be as leads to biological tools and therapeutic agents? Will the purity and integrity of the individual structures in libraries from ribosomal synthesis be sufficient? Can promising lead structures be readily amplified in scale for follow-up studies? Given the speed at which this field is developing and the creative approaches being applied, the answers to these questions should be forthcoming in the near future.

Abbreviations
aaRS, aminoacyl-transfer ribonuclease synthetase; Aha, azidohomoalanine; Ala, alanine; AMP, adenosine monophosphate; Cab, Nγ-(2-chloroacetyl)-α, γ-diaminobutyric acid; Cys, cysteine; Meaa, N-methyl amino...
acid; MeLeu, N-methyl leucine; MePhe, N-methyl-l-phenylalanine; MeThr, N-methyl threonine; MeVal, N-methyl valine; Phe, phenylalanine; PURE, protein synthesis using recombinant elements; Pgl, propargyl glycine; rGly, N-substituted glycine; tRNA, transfer ribonucleic acid; wPURE, withdrawn protein synthesis using recombinant elements.

Competing interests

The author declares that he has no competing interests.

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