Ribosomal Synthesis of *N*-Methyl Peptides

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Abstract: *N*-Methyl amino acids (*N*-Me AAs) are a common component of nonribosomal peptides (NRPs), a class of natural products from which many clinically important therapeutics are obtained. *N*-Me AAs confer peptides with increased conformational rigidity, membrane permeability, and protease resistance. Hence, these analogues are highly desirable building blocks in the ribosomal synthesis of unnatural peptide libraries, from which functional, NRP-like molecules may be identified. By supplementing a reconstituted *Escherichia coli* translation system with specifically aminoacylated total tRNA that has been chemically methylated, we have identified three *N*-Me AAs (*N*-Me Leu, *N*-Me Thr, and *N*-Me Val) that are efficiently incorporated into peptides by the ribosome. Moreover, we have demonstrated the synthesis of peptides containing up to three *N*-Me AAs, a number comparable to that found in many NRP drugs. With improved incorporation efficiency and translational fidelity, it may be possible to synthesize combinatorial libraries of peptides that contain multiple *N*-Me AAs. Such libraries could be subjected to in vitro selection methods to identify drug-like, high-affinity ligands for protein targets of interest.

Introduction

The nonribosomal peptides (NRP) represent a significant source of clinically important therapeutics, such as cyclosporin A, vancomycin, and penicillin.[1,2] These microbial secondary metabolites are typically generated by large, multimodular synthetases that add and modify residues in an ordered, template-independent fashion.[3] NRPs are frequently decorated with *N*-methylated backbones, D-amino acids, unusual side chains, and glycosylations, which increase their target affinity and resistance to proteolysis.[4] Attempts to generate novel NRPs by genetically manipulating NRP synthetases are progressing but face numerous challenges, including the large size of synthetase gene clusters and the decreased catalytic efficiency associated with unnatural module rearrangements.[5,6] An alternative strategy for the discovery of functional, NRP-like molecules involves applying ultrahigh throughput selection methods such as mRNA display to combinatorial peptide libraries generated by mRNA-directed ribosomal translation.[7,8] The chemical space sampled by such libraries can be expanded through the cotranslational incorporation of nonproteinogenic or unnatural amino acids, an approach that requires the reassignment of sense or nonsense codons to one or more unnatural residues. The ribosome has been shown to tolerate amino acid analogues containing unnatural side chains and, to a lesser extent, backbone modifications including *N*-methyl amino acids, α,α-disubstituted amino acids, D-amino acids, β-amino acids, and α-hydroxy acids. Numerous groups have shown that certain nonstandard residues can be efficiently incorporated into peptides.[9–18]

From a pharmacological perspective, *N*-methyl amino acids (*N*-Me AAs) are especially valuable as unnatural building blocks because they confer increased conformational rigidity, membrane permeability, and protease resistance on the resulting peptides.[19,20] A commonly used technique for the incorporation

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of *N*-Me AAs into peptides is amber suppression, in which an orthogonal tRNA specific for the amber stop codon (UAG) is chemoenzymatically aminoacylated and added to cell-free translation systems.[9,10] Using this strategy, Bain et al. demonstrated the efficient incorporation of *N*-Me Phe at a single site in a peptide.[21] A disadvantage of this and other nonsense suppression methods is the inability to simultaneously and independently direct the incorporation of more than two unnatural amino acids since at least one stop codon is required for the termination of translation. Reassignment of sense codons to unnatural amino acids allows for the incorporation of multiple unnatural amino acids into the same peptide.[22] Frankel et al. used chemically acylated tRNAs to insert multiple *N*-Me Phe residues at reassigned Val and Ala codons, thereby generating a protease-resistant peptide.[23] Using a similar strategy, Tan et al. incorporated *N*-Me Ala and *N*-Me Phe at a single Val codon in a tripeptide,[18] and Zhang et al. demonstrated the single incorporation of these and other *N*-alkyl amino acids using three different sense codons.[24]

A key technology used by the latter two groups is the PURE system, a reconstituted, in vitro translation system consisting of purified recombinant factors derived from *Escherichia coli*.[25] This system is highly suitable for the translation of peptides containing unnatural amino acids because it provides the ability to control which aminoacyl-tRNA synthetases (AARSs) and amino acids are present during translation. This makes it possible to reassign multiple sense codons to *N*-Me AAs by simply withdrawing the corresponding natural amino acids and AARSs and replacing these with chemically charged tRNAs. Moreover, instead of relying on chemoenzymatic aminoacylation methods, it is possible to prepare *N*-methyl aminoacyl-tRNA (*N*-Me AA-tRNA) by chemically transforming total tRNA that has been enzymatically precharged with the standard set of natural amino acids using AARSs. This method is appealing because it permits the simultaneous generation of multiple *N*-Me AA-tRNA species while using purified tRNAs that contain post-transcriptional base modifications. Merryman and Green developed an efficient synthesis for *N*-Me AA-tRNA involving the sequential mono-derivatization of precharged total tRNA with a bulky, photolabile *o*-nitrobenzyl group and formaldehyde under reductive amination conditions.[26] Subsequent photocleavage of the *o*-nitrobenzyl group afforded *N*-methylated, precharged total tRNA, which could be added to translation reactions to generate *N*-methyl peptides. Using this strategy, Merryman and Green demonstrated the efficient incorporation of six *N*-Me AAs (*N*-Me Ala, *N*-Me His, *N*-Me Ile, *N*-Me Leu, *N*-Me Phe, and *N*-Me Val) into dipeptides.[26] However, this method has not been used for the incorporation of multiple *N*-methyl residues into longer peptides, which is more challenging because the cumulative effects of inefficient incorporation may drastically lower peptide yield and because of the increased potential for misincorporation of competing amino acids.

In this report, we demonstrate the synthesis of peptides containing up to three *N*-Me AAs by mRNA-directed ribosomal translation. Our strategy relies on the addition of *N*-methylated, precharged total tRNA to PURE system translations in which the natural amino acids and AARSs corresponding to each *N*-Me AA-tRNA have been withdrawn. We first identify three *N*-Me AAs (*N*-Me Leu, *N*-Me Thr, and *N*-Me Val) that show efficient single incorporation, with peptide yields exceeding 50% of those for the corresponding natural amino acid and with the desired *N*-methyl peptide as the sole translation product. Next, we show that peptides containing two or three of these analogues can be translated by the ribosome with high yield and purity. In principle, our method could be used to generate combinatorial libraries of NRP-like, poly-*N*-methyl peptides that could be subjected to selection strategies such as mRNA display in order to identify high-affinity ligands for biomedically important protein targets.

Materials and Methods

Materials. All materials were purchased from Sigma, Aldrich, or New England Biolabs unless otherwise noted and used without further purification. *E. coli* total tRNA was purchased from Roche. Radiolabeled amino acids ^{35}S-Met (2600 dpm/fmol) and ^{3}H-His (84 dpm/fmol) were from Perkin-Elmer. Expression plasmids containing His$_6$-tagged ArgRS, GlnRS, IleRS, LeuRS, TrpRS, AsnRS, HisRS, and ProRS were from T. Ueda, and TyrRS and LysRS were from P. Schimmel. The expression, purification, and storage of recombinant translation factors and enzymes were performed according to a previously published protocol[25] with the following modifications: cells were lysed with B-PER reagent (Pierce), and proteins were purified using Ni-NTA resin (Qiagen). Protein concentrations were determined from the UV absorbance at 280 nm, and the extinction coefficient was calculated from the protein sequence. MetRS required storage in buffer containing 50% glycerol at −20 °C.

Ribosomes were prepared from *E. coli* strain A19. Unless otherwise noted, all steps of the purification were performed at 4 °C. Four liter cultures were grown at 37 °C to OD 0.6−0.8 and harvested by centrifugation at 5000*g* for 30 min. The pelleted cells were resuspended in 300 mL of buffer A (10 mM Tris-HCl pH 7.5, 10 mM Mg(OAc)$_2$, 100 mM NH$_4$Cl, 0.25 mM EDTA, 7 mM β-ME) and spun at 4400*g* for 15 min. The resulting pellet was transferred into a BeadBeater (Biospec) together with a roughly equal volume of precooled 0.1 mm diameter glass beads, and the cells were lysed with six 20 s pulses separated by 40 s. The bead−lysate mixture was centrifuged at 30 000*g* for 30 min, and the supernatant (∼20 mL) was layered in 5 mL aliquots onto 7.5 mL of 30% sucrose in buffer B (10 mM Tris-HCl pH 7.5, 10 mM Mg(OAc)$_2$, 500 mM NH$_4$Cl, 7 mM β-ME). Ribosomes were pelleted by centrifuging at 300 000*g* for at least 2.5 h in a Beckmann Ti70.1 rotor. Following resuspension in a total of 8 mL of buffer B, the ribosomes were once again layered onto 30% sucrose in buffer B and centrifuged. The resulting pellets were resuspended in 600 μL of buffer C (10 mM Tris-HCl pH 7.5, 10 mM Mg(OAc)$_2$, 60 mM NH$_4$Cl, 3 mM β-ME), divided into aliquots, and flash frozen in liquid nitrogen.

Preparation of *N*-Methyl Aminoacyl-tRNA. We followed the procedure of Merryman and Green[26] with modifications as follows. *E. coli* total tRNA was aminoacylated with 4 mM amino acid and 10−20 μM of the corresponding AARS at 37 °C for 30 min in charging buffer (30 mM HEPES-KOH pH 7.4, 15 mM MgCl$_2$, 25 mM KCl, 2 mM β-ME, 6 mM ATP, 0.09 mg/mL BSA, 0.02 unit/mL yeast inorganic pyrophosphatase). Following addition of 0.1 volumes of 3 M NaOAc pH 5.0 to the charging reaction, the aminoacylated tRNA (AA-tRNA) was extracted with a 25:24:1 mixture of phenol/chloroform/isoamyl alcohol and precipitated with ethanol at −20 °C. After centrifugation, the resulting pellet was washed twice with 70% ethanol, air-dried, and redissolved in 200 mM NaOAc pH 5.0.

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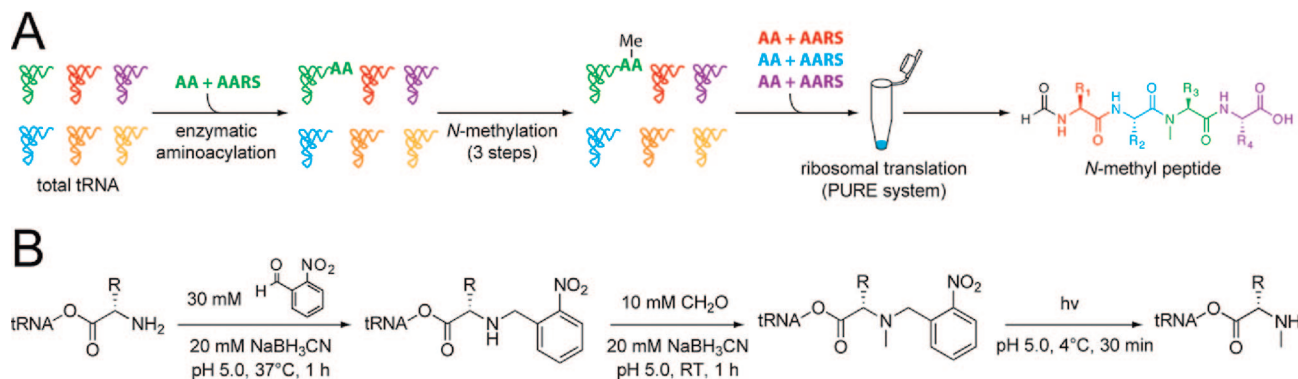
Figure 1. (A) Scheme for the ribosomal incorporation of *N*-Me AAs into peptides. Total tRNA is enzymatically precharged with a natural amino acid and chemically *N*-methylated, then added to PURE system translation reactions in place of the corresponding natural amino acid (AA) and aminoacyl-tRNA synthetase (AARS). The translation reaction contains the minimal set of other AAs and AARSs required for synthesis of the desired peptide. (B) Synthesis of *N*-methyl aminoacyl-tRNA, after Merryman and Green.[26] Aminoacylated tRNA (AA-tRNA) is monoderivatized at the α-amino group with a photolabile *o*-nitrobenzyl protecting group under reductive amination conditions. Formaldehyde is added to the same reaction, generating a bisalkylated AA-tRNA. UV irradiation of this species results in cleavage of the *o*-nitrobenzyl group, furnishing the desired *N*-monomethyl AA-tRNA.

The AA-tRNA was monoderivatized with 30 mM *o*-nitrobenzaldehyde in the presence of 20 mM sodium cyanoborohydride at 37 °C for 1 h under slightly acidic conditions (55 mM NaOAc, 33% dioxane, pH 5.0). Formaldehyde (in dioxane) was subsequently added to 10 mM, and the methylation reaction was allowed to proceed at ambient temperature for 1 h. The reaction was quenched with 0.1 volumes of 4.4 M NH$_4$OAc pH 5.0, and the resulting bisalkylated AA-tRNA was precipitated with ethanol at −20 °C. After centrifugation, the pellet was washed twice with 70% ethanol and twice with 95% ethanol, air-dried, and redissolved in 5 mM KOAc pH 5.0. Deprotection was achieved by spotting samples of bisalkylated AA-tRNA onto a Petri dish chilled on ice and subjecting the drops to UV irradiation for 30 min from a 450 W Hanovia lamp situated 10 cm above the plate. The resulting *N*-monomethylated AA-tRNA was purified by ethanol precipitation and redissolved in 5 mM KOAc pH 5.0.

Preparation of mRNA Templates. DNA templates for the in vitro transcription of mRNAs were prepared by cloning synthetic DNA inserts into a pET12b-derived plasmid doubly digested with *Nde*I and *Bam*HI. The resulting constructs were transformed into *E. coli* Top10 cells (Invitrogen), which were plated onto ampicillin-containing medium to select successful transformants. Plasmids from these cells were isolated using a Plasmid Mini purification kit (Qiagen) and served as templates in PCR reactions with primers that corresponded to the T7 promoter and terminator sequences. After the PCR products were purified using a QIAquick PCR purification kit (Qiagen), they were added to overnight in vitro transcription reactions with T7 RNA polymerase.[27] The reactions were phenol/chloroform extracted and the transcripts subsequently precipitated with 2.5 M LiCl for 1 h on ice and 30 min at −20 °C. The pellets were resuspended in 0.3 M KCl and were precipitated with 3 volumes of ethanol at −20 °C. The resulting pellets were dissolved in water and diluted to a final concentration of 50 μM.

In Vitro Translations. Translations were performed using the PURE system, with minor modifications from previous reports.[13,25] Fifty microliter reactions contained 1 mM dithiothreitol, 20 mM creatine phosphate (Calbiochem), 30 μM 10-formyl-5,6,7,8-tetrahydrofolic acid, 1 mM ATP, 1 mM GTP, 8 μg/mL creatine kinase, 1.1 μg/mL nucleoside diphosphate kinase, 1 unit/mL yeast inorganic pyrophosphatase, 3 μg/mL myokinase, and 3.7 μM ribosomes. Since the polymix buffer[28] typically used with PURE system translations is prone to precipitation, we employed a simplified buffer consisting of 10 mM Tris-HCl, 10 mM Mg(OAc)$_2$, and 100 mM NH$_4$Cl

(adjusted to pH 7.5 at 37 °C).[14] In addition, this buffer contained a sufficiently high Mg^{2+} concentration to permit the incorporation of *N*-Me AAs into peptides by the ribosome.[26] Translation factors were present at the following concentrations: 0.2 μM MTF, 1.0 μM IF1, 0.3 μM IF2, 0.7 μM IF3, 3.2 μM EF-Tu, 0.6 μM EF-Ts, 0.5 μM EF-G, 0.3 μM RF1, 0.4 μM RF3, and 0.1 μM RRF. Only the minimal set of amino acids (100 μM each, unless otherwise indicated) and AARSs (0.1 μM MetRS, 0.3 μM LeuRS, 0.6 μM GluRS, 0.2 μM ProRS, 1.0 μM GlnRS, 1.0 μM HisRS, 0.3 μM PheRS, 1.5 μM TrpRS, 0.2 μM SerRS, 0.2 μM IleRS, 0.4 μM ThrRS, 0.6 μM AsnRS, 0.6 μM AspRS, 0.5 μM TyrRS, 0.5 μM LysRS, 0.4 μM ArgRS) required for peptide synthesis was present during translations. Radiolabeling was performed with 0.1 μM ^{35}S-Met + 10 μM Met or with 2.7 μM ^{3}H-His.

Translation reactions were supplemented with *N*-methylated, precharged total tRNA and initiated by the addition of mRNA to 1.0 μM. For single *N*-Me AA incorporations, 1.5 A$_{260}$ units of total tRNA were added, and the reaction was allowed to proceed for 60 min at 37 °C. Dual incorporations were supplemented with 1.5 A$_{260}$ units of tRNA and incubated for 30 min, while triple incorporations were provided with 3.0 A$_{260}$ units of tRNA and incubated for 45 min. For dual and triple incorporations, the total tRNA was added in two half-aliquots, at the start and midway through the reaction. Reactions were quenched with 100 μL of wash buffer (50 mM Tris-HCl, 300 mM NaCl, pH 8.0) and supplemented with 50 μL of suspended Ni-NTA agarose beads (Qiagen). After incubating for 1 h, the beads were washed twice with wash buffer and twice with deionized water and eluted with 50 μL of 1% TFA for 15 min.

Peptide yields were determined by liquid scintillation counting of ^{35}S-Met (or ^{3}H-His for *N*-Me Met). For MALDI-TOF analysis, peptides were purified and concentrated using C18 ZipTip reverse phase microchromatography columns (Millipore, Billerica, MA) and eluted with a saturated α-cyano-4-hydroxycinnamic acid solution containing 50% acetonitrile and 0.1% TFA. Mass spectra were obtained using an Applied Biosystems Voyager MALDI-TOF.

Results

Single Incorporation of *N*-Me AAs. To identify the *N*-Me AAs that are efficient substrates for peptide synthesis, we assayed the incorporation of each *N*-Me AA at a single site in a short peptide containing an N-terminal His$_6$ (or for *N*-Me His, FLAG) tag. Total tRNA that was enzymatically precharged with a natural amino acid and *N*-methylated by the method of Merryman and Green was added to PURE system translation reactions lacking the natural amino acid and the corresponding aminoacyl-tRNA synthetase (AARS) (Figure 1), but containing

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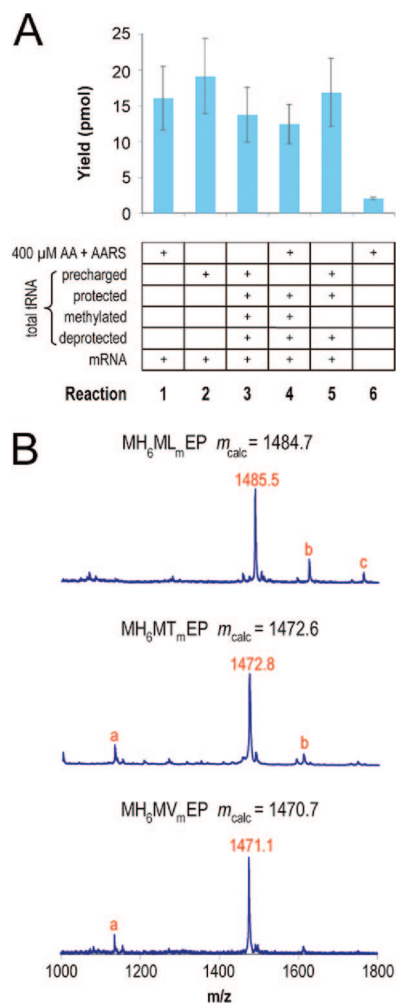
Figure 2. Incorporation of single *N*-Me AAs into peptides. (A) Average yields for successful translations producing peptides MH$_6$MXEP, where X is Leu, Thr, or Val or *N*-Me Leu, Thr, or Val. Error bars correspond to ±1 standard deviation. Translations performed with total tRNA that was precharged with natural Leu, Val, or Thr and methylated (reaction 3) yielded on average 74% as much peptide as those supplied with the corresponding unmodified precharged total tRNA (reaction 2). (B) MALDI-TOF MS analysis of the peptide products from translations with *N*-Me Leu (L$_m$), *N*-Me Thr (T$_m$), and *N*-Me Val (V$_m$). Each analogue was incorporated with high efficiency. Minor peaks resulted from truncation products (a = MH$_6$M) or were consistent with sodium trifluoroacetate adducts of the protonated *N*-methyl peptide (b = +137 Da, c = +137 Da × 2). The latter signals were observed in all reactions with each template.

the minimal set of all other amino acids and AARSs required for synthesis of the peptide. To assess the fidelity and efficiency of *N*-Me AA incorporation, peptide products of translation reactions were analyzed by MALDI-TOF MS and their yields quantified by scintillation counting of ^{35}S-Met or ^{3}H-His activity. Efficient incorporation—where the *N*-methyl peptide was the sole translation product detected by MALDI-TOF MS analysis— occurred only for *N*-Me Leu, *N*-Me Thr, and *N*-Me Val (Figure 2B and Table 1). For these three *N*-Me AAs, translation reactions supplemented with *N*-methylated, precharged total tRNA yielded on average 74% as much peptide as reactions provided with the corresponding unmodified precharged total tRNA (Figure 2A, compare reactions 3 and 2). To control for tRNA damage caused by methylation and by addition and photocleavage of the *o*-nitrobenzyl protecting group during the preparation of *N*-Me AA-tRNA, translations were performed with uncharged total tRNA that had been methylated (reaction

Table 1. Efficiency of *N*-Me AA Incorporation

analogue	efficient incorporation	with misincorporation[a]	with truncation[b]	no incorporation
N-Me Ala			x	
N-Me Arg		His	x	
N-Me Asn		His	x	
N-Me Asp			x	
N-Me Cys			x	
N-Me Gln		His	x	
N-Me Glu			x	
N-Me Gly			x	
N-Me His		Gln		
N-Me Ile				x
N-Me Leu	x			
N-Me Lys				x
N-Me Met				x
N-Me Phe			x	
N-Me Ser			x	
N-Me Thr	x			
N-Me Trp				x
N-Me Tyr		His	x	
N-Me Val	x			

^{a} Of competing natural amino acids present in the translation reaction. ^{b} Premature termination of translation was observed before the *N*-Me AA codon.

4) as well as with precharged, unmethylated total tRNA that had been protected and deprotected (reaction 5). The relative yields of these reactions (82 and 88% of reactions 1 and 2, respectively) suggested that tRNA damage had only minor effects on peptide yield (Figure 2A).

Other *N*-Me AAs were incorporated less efficiently, resulting in termination of translation before the *N*-Me AA codon or in the misincorporation of a natural amino acid, usually from a tRNA with a near-cognate anticodon, in place of the *N*-Me AA (Table 1). We were able to detect truncated peptides resulting from termination either prior to or after the site of *N*-Me AA incorporation due to the presence of an N-terminal affinity tag in all translation templates. Inefficiencies in *N*-Me AA incorporation could be explained in several ways. First, the *N*-Me AA might have poor peptidyl acceptor activity within the ribosome. Second, binding of the *N*-Me AA-tRNA to elongation factor Tu (EF-Tu) might be compromised, leading to both increased susceptibility to hydrolysis and inefficient delivery of the *N*-Me AA-tRNA to the ribosome.[29] Third, the *N*-Me AA-tRNA might be deacylated by the proofreading activity of noncognate synthetases present in the translation reaction; in particular, ProRS might recognize *N*-Me AAs through the secondary amine that these share with Pro. However, this explanation is unlikely because tRNAs contain unique identity elements that discriminate against recognition by noncognate AARSs.[30,31] Finally, the *N*-Me AA-tRNA could be rapidly consumed at the start of the reaction if the initial abundance of the amino-acid-specific tRNA is too low to sustain translation.[32] The diminishing *N*-Me AA-tRNA concentration would cause the ribosome to stall at the *N*-Me AA codon, allowing for binding of a competing, near-cognate AA-tRNA or dissociation of the ribosomal subunits from the mRNA. Indeed, in a 1 h translation experiment with one of the less efficiently incorpo-

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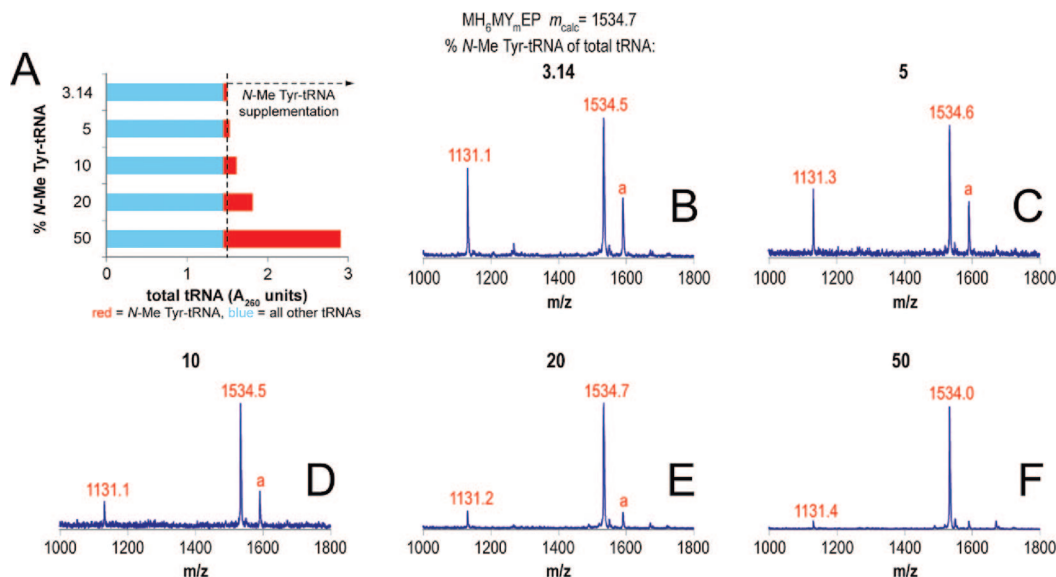
Figure 3. Increasing the relative abundance of Tyr-specific tRNAs improves *N*-Me Tyr incorporation efficiency. (A) Schematic depicting the supplementation of translations with precharged, *N*-methylated Tyr-specific tRNA to increase the relative abundance of *N*-Me Tyr-tRNA. (B) MALDI-TOF mass spectrum showing products from a translation supplied with Tyr-precharged, *N*-methylated total tRNA only; (C−F) mass spectra showing products obtained when *N*-Me Tyr-tRNA was added to the indicated percentage of total tRNA. As the fraction of *N*-Me Tyr-tRNA in total tRNA was increased, the ratio of the intensity of the desired *N*-methyl peptide peak to that of the truncation product (MH$_6$M) peak (m_{calcd} = 1131.3) became larger, indicating more efficient incorporation of *N*-Me Tyr. The origin of the minor signal a (m_{obs} = 1591.4) is unknown, although it may represent a nonpeptide artifact.

rated *N*-Me AAs, *N*-Me Asn, the desired *N*-methyl peptide was initially the sole product, but truncation and misincorporation products appeared after 30 min and became increasingly prevalent as the reaction progressed, indicating that the *N*-Me Asn-tRNA had become significantly depleted by this time in the reaction (data not shown).[33]

Improving *N*-Me AA Incorporation Efficiency by Increasing Cognate tRNA Abundance. The *N*-Me AAs that showed the most efficient single incorporation (*N*-Me Leu, *N*-Me Thr, *N*-Me Val) have abundant cognate tRNAs (14.00, 4.12, and 7.91% of total tRNA, respectively).[32] Therefore, we asked whether the incorporation efficiency of poorly incorporated *N*-Me AAs might be enhanced by increasing the relative abundance of their cognate tRNAs, especially if these initially represent a low proportion of total tRNA. This strategy was used to improve the incorporation efficiency of *N*-Me Tyr, which has low-abundance cognate tRNAs (3.14% of total tRNA)[32] and which showed both misincorporation and truncation in our single-incorporation screen. Tyr-specific tRNA purified from *E. coli* and therefore containing all of its natural post-transcriptional base modifications is commercially available, allowing for the facile generation of *N*-Me Tyr-tRNA using Merryman and Green's method. Translation reactions containing a constant amount of total tRNA were titrated with increasing amounts of *N*-Me Tyr-tRNA, so that the relative abundance of *N*-Me Tyr-tRNA equaled or exceeded the relative *N*-Me AA-tRNA abundances for efficiently incorporating *N*-Me AAs (Figure 3A). MALDI-TOF MS analysis of the peptide products from these translations showed that increasing the amount of added *N*-Me Tyr-tRNA increased the ratio of the *N*-methyl peptide signal to the truncation product signal, suggesting that formation of the truncation product was attenuated (Figure 3B−F). A high initial abundance of *N*-Me Tyr-tRNA would ensure that the concentra-

tion of this species remained sufficiently high over the course of the translation reaction to allow for efficient delivery to the ribosome by EF-Tu. Total peptide yields at all *N*-Me Tyr-tRNA concentrations were roughly equal; although the ratio of full-length peptide to truncation product increased, the total amount of peptide product remained constant (data not shown). Surprisingly, supplementing translations with *N*-Me Tyr-tRNA only improved *N*-Me Tyr incorporation efficiency when *N*-Me Tyr-tRNA^{Tyr} I was used; an identical titration experiment performed with *N*-Me Tyr-tRNA^{Tyr} II resulted in little attenuation of truncation product formation (data not shown). Although both isoacceptors possess the same anticodon (GUA), it is possible that *N*-Me Tyr-tRNA^{Tyr} I may be bound with a higher affinity by EF-Tu and delivered to the ribosome more efficiently than *N*-Me Tyr-tRNA^{Tyr} II.

Multiple Incorporation of *N*-Me AAs. In order to demonstrate the ribosomal synthesis of peptides containing multiple *N*-methyl backbone modifications, we attempted to incorporate combinations of *N*-Me Leu, *N*-Me Thr, and *N*-Me Val into the same peptide. For multiple incorporations, total tRNA was simultaneously precharged with multiple natural amino acids prior to *N*-methylation and addition to translation reactions. We expected that truncations and misincorporations would be more likely to occur in these experiments than in single *N*-Me AA incorporations because of the simultaneous depletion of multiple *N*-Me AA-tRNA species. Therefore, the total tRNA was added in two half-aliquots—at the start and midway through the translation—to ensure that the *N*-Me AA-tRNAs would be present at sufficiently high levels over the course of the reaction. Pairwise incorporation of *N*-Me Leu, *N*-Me Thr, and *N*-Me Val was highly efficient; reactions supplemented with *N*-Me AA-tRNA yielded 7−14 pmol of peptide per 50 μL translation, or 23−68% as much peptide as those supplied with the corresponding unmodified precharged total tRNA. Moreover, only the bis-*N*-methyl peptide was produced in each case, with misincorporation and truncation products generating relatively minor MALDI-MS

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(33) Rapid depletion of *N*-Me Asn-tRNA was likely due to the low abundance of the Asn-specific tRNA, which accounts for 1.85% of total tRNA, in *E. coli* (ref 32).

2 N-Me AAs
$m_{calc} = 1472.7$

3 N-Me AAs
$m_{calc} = 1486.8$
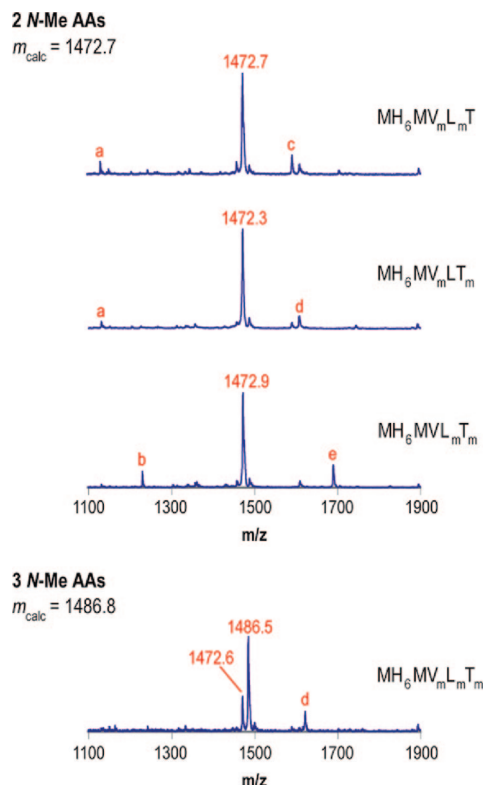
Figure 4. Translation of peptides containing multiple *N*-Me AAs. MALDI-TOF analysis of the peptide products from translation reactions designed to result in the incorporation of two or three residues of *N*-Me Leu (L_m), *N*-Me Thr (T_m), and *N*-Me Val (V_m). The desired peptide is the major product in each case. For the peptide containing three *N*-Me AAs, the smaller signal 14 Da less than the expected mass may be due to the incorporation of unmethylated AA-tRNA resulting from nonquantitative methylation of the precharged total tRNA. Other minor signals were consistent with truncation products (a = MH$_6$M; b = MH$_6$MV), possible nonpeptide artifacts (e, m_{obs} = 1690.7), and adducts of the protonated desired peptide (c = +119 Da, sodium trifluoroacetate minus water; d = +137 Da, sodium trifluoroacetate).

signals (Figure 4). Triple incorporation of *N*-Me Leu, *N*-Me Thr, and *N*-Me Val was also successful, yielding 5 pmol of peptide per 50 μL translation. Importantly, MALDI-MS analysis of the reaction products revealed the presence of the tris-*N*-methyl peptide as the major peak, with a low abundance of misincorporation or truncation products (Figure 4). Therefore, our data show that up to three *N*-Me AAs can be incorporated into a peptide efficiently and faithfully.

Discussion

In this study, we have demonstrated the ribosomal synthesis of peptides containing multiple *N*-methyl backbone modifications. Our approach relies on supplementing PURE system translations with total tRNA in which one, two, or three sets of isoaccepting tRNAs have been specifically aminoacylated and chemically methylated. We have identified three *N*-Me AAs (*N*-Me Leu, *N*-Me Thr, *N*-Me Val) that are efficiently incorporated into peptides. Our results are largely consistent with those of Merryman and Green, who observed efficient incorporation of *N*-Me Leu and *N*-Me Val and modest incorporation of *N*-Me Thr into dipeptides.[26] Moreover, we have shown that further

increases in the number of efficiently incorporated *N*-Me AAs might be achieved by supplementing translation reactions with isolated, amino-acid-specific tRNAs that have been enzymatically charged and *N*-methylated; this approach was effective in the case of *N*-Me Tyr. Alternatively, it may be possible to optimize the incorporation efficiency of other *N*-Me AAs through the judicious choice of particular codons for each analogue so as to minimize misincorporation due to the presence of tRNAs with near-cognate anticodons. Last, we have shown that combinations of two or three *N*-Me Leu, *N*-Me Val, and *N*-Me Thr residues can be successfully incorporated with good yield and fidelity to generate bis- and tris-*N*-methyl peptides, respectively.

While this paper was being revised, a paper by Kawakami et al. appeared which demonstrated the translation of linear and cyclic peptides containing multiple *N*-methyl amino acids using the PURE system and ribozyme-mediated tRNA aminoacylation.[34] In cases where the tRNA was efficiently aminoacylated, most but not all *N*-Me AAs could be incorporated into peptides with good yield. *N*-Me Ile, *N*-Me Val, and *N*-Me Asn were inaccessible due to poor aminoacylation efficiency, while *N*-Me Leu and *N*-Me AAs with charged side chains were efficiently charged but showed low levels of incorporation into peptides. Remarkably, *N*-Me Leu and *N*-Me Val were two of the three analogues for which we observed the most efficient incorporation with our system. Therefore, our approaches are highly complementary and together would enable the incorporation into peptides of almost all *N*-methyl residues.

Efficient incorporation of several *N*-Me AAs into the same peptide suggests that the synthesis of combinatorial libraries of peptides that contain multiple *N*-methyl residues may be possible. Indeed, libraries containing as many as 10^{14} unique peptides consisting of unnatural amino acids may be generated in a format compatible with mRNA display using the PURE system.[13,35] Two key criteria for the use of any unnatural amino acid in the synthesis of such libraries are high incorporation efficiency and fidelity, both of which are satisfied by *N*-Me Leu, *N*-Me Val, and *N*-Me Thr. Therefore, large libraries of peptides containing combinations of these and possibly other *N*-Me AAs could be constructed and subjected to in vitro selection methods such as mRNA display, which could yield potential peptide drug candidates with improved biostability and bioavailability.

Supporting Information Available: The mRNA sequences (Table S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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