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On-resin synthesis of novel arginine-isostere peptides bearing substituted amidine headgroups

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A methodology is presented for the facile synthesis of Arg-containing peptides modified at the guanidine headgroup as substituted amidine cores. This process allows for the iterative construction of these Arg isosteres while the peptide is being built out on the solid support, providing a high potential for diversity in substitution pattern in the resulting peptide. A series of *N*-Pmc-substituted thioamides were condensed with deprotected δ-*N* Orn-bearing peptides while attached to the solid support using Mukaiyama's reagent as coupling reagent, yielding isosteric Arg-containing analogs. Peptides were cleaved using trimethylsilyl trifluoromethanesulfonate/TFA and analyzed in their crude form in order to illustrate the amenability of this process toward production of peptide isolates in high crude purity. Arg-containing peptides having a single Arg isostere were utilized to show the general utility of this approach as well as a multiple-Arg-containing construct, illustrating the amenability of this method toward stepwise construction of differently substituted amidine headgroups within the same peptide.

Keywords: amidine; arginine; arginine analogs; solid-phase synthesis; combinatorial chemistry

Introduction

Protein–protein interactions form the basis of most enzymatic and signal transduction pathways in biological systems. Similarly, small peptides are frequently the preferred binding substrates for a diverse range of signaling cascades and hormone interactions [1]. Binding specificity in these cases is an exquisitely tuned process based primarily upon interactions between the amino acid side chains of the binding partners, normally mediated by protein tertiary structure. Of the four major types of tertiary structure-maintaining vectors in the structural stabilization of proteins, salt bridge interactions between cationic and anionic side chains provide a crucial staple in preserving active three-dimensional conformation as well as maximizing binding efficiency between partners.

Arginine is a vital and ubiquitous amino acid residue, essential to many crucial biochemical functions. The uniquely basic character of the Arg side chain guanidine headgroup keeps it protonated and positively charged at physiological pH, allowing it to participate in ion pairings and salt bridge interactions crucial to binding and recognition events in cell signaling processes. Native Arg is the preferred substrate for nitric oxide synthase (NOS) in the production of NO for various cellular and neuronal processes [2]. As a component residue in various small peptide sequences and enzyme active sites, the Arg side chain mediates critical interactions and is heavily represented in natural substrates of trypsin-like serine proteases [3] and those involved in the blood coagulation cascade. Additionally, polyarginine-containing peptides have synergistic cell membrane-permeating abilities that have been routinely utilized as a peptidyl carrier module for drug delivery [4].

Because of the high level of relevance and importance imbued upon this amino acid residue, a great deal of interest has been generated toward the maximization of its binding efficiency and salt-bridge-forming potential in chemically altered analogs. Isosteric replacement of Arg in biologically relevant peptides has been of value in improving efficiency of the substrate as well as in SAR studies of enzyme-binding pockets [5]. Moreover, carefully designed structural analogs of native Arg have provided entry into NOS inhibitory constructs [6]. The extent of Arg diversification described in the literature is immense and has been well described in various reviews [7,8]. Structural diversification of Arg, whether in its discrete form or incorporated into a peptide sequence, can be approached in one of three possible manners: (i) $^{\alpha}$N or $^{\alpha}$C diversification in order to elongate or cap these termini [9,10], (ii) alteration within the three-carbon extension of its side chain to introduce constraint [11] or structural adjustment [12], or (iii) diversification of the guanidine headgroup to alter its basicity [13] and/or form [14,15].

Structural diversification of the guanidine headgroup of Arg offers the most profitable means of maximizing binding potential or interaction efficacy because of the highest potential for stuctural diversity. One of the means of carrying this out is through the installation of an alkyl or aryl substituent at one or more of the guanidine nitrogens. There has been a fair degree of interest and research activity in this area recently, evidenced by multiple publications by our research group [16,17] as well as those of Fan [18] and Martin [19,20]. All of these research efforts were based upon a similar synthetic design: the nucleophilic attack

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of an Orn amine upon an activated *N*-arylsulfonyl-*N'*-substituted thiourea under gentle 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI)-mediated conditions to afford the corresponding substituted Arg adduct, sulfonyl-protected at its guanidine moiety. Syntheses that resulted in the formation of properly protected Arg adducts suitable for solid phase peptide synthesis [17,20] allowed for insertion of these substituted derivatives into a growing synthetic peptide.

The underlying base structure of the guanidino functionality itself can similarly be altered to exist as a structural mimic of the native architecture. Although there are examples of Arg mimics whose guanidine headgroup takes a completely different isoform such as that of a borate functionality [21] or a sulfamoyl-type structure [22], the amidine core motif has emerged as highly representative of alternative guanidine architecture in a number of Arg isosteres reported in the literature. In particular, the acetamidine moiety is quite prevalent as a guanidinyl isoform as stuctural analogs bearing this basic motif have been found to act as very effective inhibitors of NOS [23–25].

Although the significant number of synthetic routes into discrete amidines have been reviewed elsewhere [26,27], amidine-based isologs of Arg have been prepared as discrete compounds through a variety of different means, including various iterations of standard Pinner syntheses of nitrile substrates [28,29], condensation of alkyl acetimidates with the δ-amine of appropriately functionalized Orn precursors [30,31], and treatment of Orn δ-thioamides with ammonia [24]. Overwhelmingly, the final product in these syntheses has been the discrete Arg isologs themselves, without their incorporation into larger peptides. Indeed, the vast majority of synthetic research in this arena has involved the construction of discrete NOS inhibitory Arg substrates. As such, this research effort signifies the first time in which Arg isosteres bearing functionalized amidine architecture have been incorporated into peptides constructed on the solid support and represents a major step forward in the iterative construction of widely diversified Arg isolog-containing peptides.

Our prior efforts in this area involved practical approaches toward the construction of Arg-containing peptides bearing diversification at the guanidine nitrogen [17]. This methodology was shown to be very effective toward the iterative synthesis of guanidinyl-substituted Arg peptides in a stepwise manner while on the solid support. In this procedure, a resin-bound Orn residue was Mtt deprotected while on the solid support followed by guanidinylation via treatment with an excess of *N*-Pmc-*N'*-substituted thiourea transfer agent under EDCI-mediated conditions to afford the Pmc-protected Arg properly substituted at the guanidine core (Figure 1(A)). We found this synthetic route to be extremely convenient and highly desirable because of its high potential for diversification and its amenability toward a completely solid-supported synthesis. This specific guanidinylation methodology was expanded in a subsequent publication to include solution syntheses of substituted *amidines* in which the scope and the limitations of the method were carefully explored [32]. This analogous synthetic route utilized *N*-Pmc-substituted thioamides rather than thioureas as the amidinylation agents. The primary salient feature which emerged from this effort was the necessity to apply a different thiophile (Mukaiyama's reagent) to mediate the smooth conversion to amidine core structure on the model systems studied. It was perhaps logical that our curiosities led us to apply this methodology to the solid support, particularly as a tool to bring about the iterative formation of amidine-functionalized guanidine cores on Arg-containing peptide systems (Figure 1(B)).

In this effort, we report a systematic assay of known thiophiles identified in our previous report to be effective at mediating amidine formation between an amine and an *N*-Pmc thioamide transfer agent as to their effectiveness when applied to a δ-amino group of a resin-bound Orn as the amine partner in the amidinylation process. We then apply these optimized conditions toward the condensation of an array of differently substituted *N*-Pmc thioamide transfer agents and an Orn-bearing test peptide in order to explore the scope and the limitations of the solid-supported reaction. Finally, the utility of this methodology is highlighted in the solid-phase iterative construction of a peptide bearing three Arg residues, all differentially functionalized as their corresponding substituted amidine headgroups. Similarly to our previous work describing the stepwise solid-supported synthesis of a substituted Arg-containing peptide [17], we believe that this is the first description of the iterative formation of Arg-containing peptides bearing amidine functionalization as the point of diversification.

Materials and Methods

Materials

Pmc chloride was purchased from Omegachem (Saint-Romuald, QC, Canada). Fmoc-Leu-Wang resin (0.47 mmol/g) and Fmoc-Orn (Boc)-OH were purchased from Novabiochem (San Diego, CA, USA). PEG-PS resin (0.21 mmol/g) was purchased from Peptides

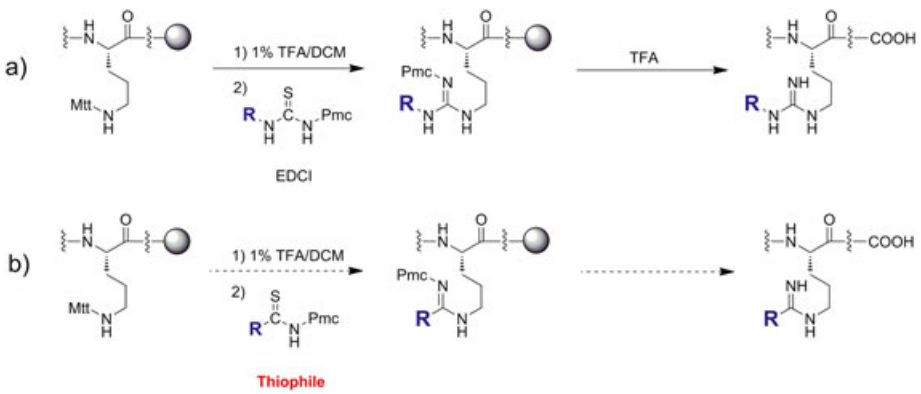


Figure 1. (A) Previously studied synthetic route toward synthesis of on-resin Arg analogs from Orn precursors. (B) Similar proposed synthesis into analogous amidine-based isologs of Arg from Orn precursors.

International (Louisville, KY, USA). 2-(7-Aza-1H-benzotriazole-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate HATU was purchased from Oakwood Products (Jackson Hole, WY, USA). All other standard Fmoc amino acids and *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) were purchased from RS Synthesis (Louisville, KY, USA). DMF, HPLC-grade acetonitrile, and TFA were purchased from Fisher Scientific (Pittsburgh, PA, USA). Mukaiyama's reagent, trimethylsilyl trifluoromethanesulfonate (TMSOTf), and all other reagents were purchased from Sigma-Aldrich (Milwaukee, WI, USA).

Peptide Syntheses

All peptides were manually synthesized on Wang resin (0.51 mmol/g) on a 40 μmol scale via Fmoc protocol. Double coupling using 1 : 3 HATU/HBTU activation was employed for peptide elongation. A typical single coupling procedure is as follows: 20% piperidine/DMF (2 × 10 min); DMF wash (6 × 30 s); 3 equiv Fmoc amino acid and 1 : 3 HATU/HBTU in 0.2 M NMM/DMF (2 × 30 min); and DMF washes (3 × 30 s). Following the last coupling and subsequent Fmoc deprotection, the α-*N*-terminal amine was Boc protected via incubation of the peptide resin with Boc_2O (10 equiv) in 5 ml 0.1 M NMM in 1 : 1 DMF/DCM for 40 min.

High Performance Liquid Chromatography

HPLC analysis was carried out on a Shimadzu analytical HPLC system with LC-10AD pumps, SPD-10A UV-vis detector, and SCL-10A controller using a Symmetry® C_{18} −5 μm column from Waters (4.6 × 150 mm). Aqueous and organic phases were 0.1% TFA in water (buffer A) and 0.1% TFA in HPLC-grade acetonitrile (buffer B), respectively. Beginning with 100% buffer A, a 1.4 ml/min gradient elution increase of 1% buffer B/min for 50 min was used for all peptide chromatograms. Peptides were detected at both 214 and 254 nm. Preparative HPLC purification was carried out on a Shimadzu preparatory HPLC system utilizing LC-8A pumps, an SPD-10A UV-vis detector, and an SCL-10A controller. A Waters Symmetry Prep C18 preparatory column (7 μm pore size, 1900 × 150 mm) was utilized in these separations. Beginning with 100% buffer A, a 17 ml/min gradient elution increase of 1% buffer B/min for 50 min was used for all preparative chromatograms.

Mass Spectrometry

MALDI–TOF MS spectra were obtained on a Voyager DE Pro instrument under positive ionization and in reflectron mode. All samples were run using a matrix of 10 mg/ml 2,5-dihydroxybenzoic acid, vacuum-dried from a solution of 1 : 1 H_2O/ACN buffered to 0.1% TFA.

Synthesis of *N*-Pmc-*N*′-Substituted Thioamide Transfer Agents

All *N*-Pmc-substituted thioamides **2a–2i** were prepared according to a previously established procedure [32].

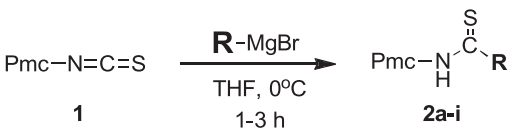
General Solid-Phase Amidinylation Protocol

Resin-bound peptide of 40 μmol containing a δ-*N*-Mtt Orn residue is swelled in DCM followed by sequential treatments with 1% TFA/DCM (5 min each) until all traces of yellow color is absent from supernatant. Resin is then washed with DCM (2 × 2 ml), DMF (2 × 2 ml), 0.4 M NMM/DMF (2 × 2 ml), and DMF (2 × 2 ml). In a separate reaction vessel, *N*-Pmc-*N*′-substituted thioamide transfer agent and Mukaiyama's reagent (4 equiv ea. with regard to resin loading) are dissolved in 2 ml 1 : 1 DCM/DMF buffered to 0.2 M NMM and allowed to incubate for 5 min. This solution is then applied to the resin, and the slurry is allowed to agitate gently for 4 h. Following the incubation period, the resin is washed thoroughly with DMF and DCM. Cleavage of peptides from their resins was accomplished through treatment of the resin with 94 : 5 TFA/TMSOTf for 2 h. Following filtration of the resin, the cleavage supernatant was evaporated to one-tenth its original volume by blowing air over it, followed by precipitation of the crude peptide by the addition of cold anhydrous ether.

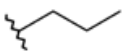
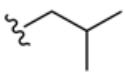
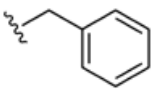
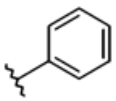
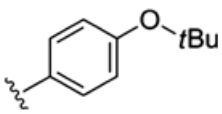
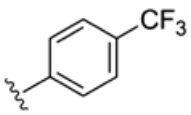
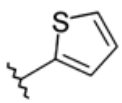
Results and Discussion

Our retrofitted approach toward carrying out this solution-phase chemistry on the solid support utilized the identical *N*-Pmc substituted thioamides as in our previous report but with a resin-bound Orn side-chain playing the role of the amine partner. As such, an assortment of *N*-Pmc thioamides was constructed as previously described [32] to explore the scope and limitations of this methodology (Table 1), using a variation on the method of Walter [33], which we had further optimized. Briefly, Pmc isothiocyanate **1** was treated with a variety of Grignard reagents to afford a series of *N*-Pmc-substituted thioamides **2a–2i**. As illustrated in Table 1, a variety of substitution patterns were chosen, which encompassed a diversity of alkyl and aryl frameworks.

Table 1. Substitution pattern on *N*-Pmc thioamides 2 used in this study.



1 **2a-i**

Entry	R
2a	
2b	
2c	
2d	
2e	
2f	
2g	
2h	
2i	

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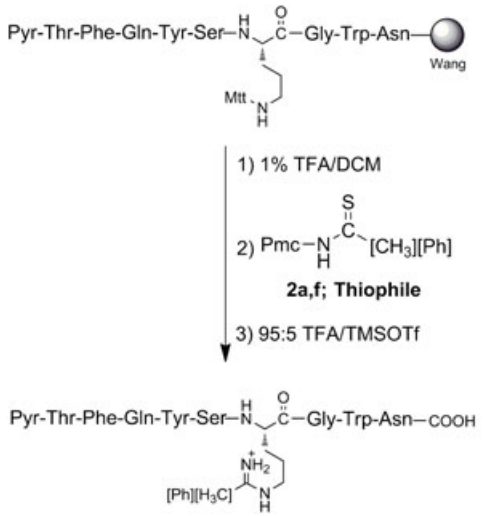
As we were attempting to synthesize amidines in analogy to our previous reports [32], we needed to carefully reevaluate our synthetic strategy in order to ensure its applicability to conditions of SPPS. As such, we decided to assay the solid phase applicability of this approach over multiple thiophile candidates in order to optimize the reaction conditions toward SPPS. In our previous work, we had found that the EDCI thiophile, which had worked so efficiently at mediating analogous guanidinylation reactions, was virtually ineffective when applied to analogous amidinylation protocol. We subsequently found that HATU, PyBOP, and 2-chloro-1-methyl-pyridinium iodide (Mukaiyama's reagent) acted as superb thiophile mediators of this reaction in solution. As such, a comprehensive assay was designed in which these four candidates were tested as to their ability to carry out solid-phase amidinylation between a peptide test sequence bearing an Orn amino side chain and two *N*-Pmc-substituted thioamides, bearing representative alkyl and aryl groups (Figure 2).

A single-Arg-containing corazonin test peptide sequence [34] was chosen as a representative model system upon which to carry out the thiophile assay. Initially prepared on Wang resin as its Mtt-protected Orn analog, Orn δ-*N* Mtt deprotection was carried out via treatment of the resin with 1% TFA/DCM. Following this, separate aliquots of the resin were incubated for 4 h with a fourfold excess of two *N*-Pmc-substituted thioamides along with a different thiophile reagent noted earlier. Methyl-substituted **2a** and phenyl-substituted **2f** were chosen as representative thioamides because of their minimal substitution pattern, offering the least steric hindrance toward condensation and allowing the process to be mediated solely by choice of thiophile while minimizing other potential mitigating steric factors. Final deprotection/cleavage of the test peptide was carried out using a 5% TMSOTf/TFA cocktail. We had earlier discovered that, in contrast to the facile removal of Pmc protection from a guanidine framework using TFA and minimal scavengers, similar Pmc deprotection of analogous amidine systems could never be driven to completion [32]. There was therefore an absolute requirement for TMSOTf as an added active component to the deprotection mixture. Treatment of the peptide resins with this cocktail for 2 h allowed for isolation of the crude Corazonin analogs, which were submitted to HPLC and MALDI analyses.

It was found that solid-supported amidinylation efficiency tracked similarly to that of previously studied solution-based condensations. EDCI was found to be completely ineffective as a condensation reagent and was immediately excluded as a candidate as determined by its complete inability to mediate any on-resin amidinylation. PyBOP, while offering acceptable amidinylation abilities for alkyl-substituted thioamide **2a**, was found to be completely ineffective at mediating the condensation of phenyl-substituted thioamide **2f** as shown by recovery of unreacted Orn-corazonin peptide. This limitation was observed previously in solution-phase amidinylations, and for this reason PyBOP was similarly disregarded as a thiophile candidtate. Gratifyingly, both HATU and Mukaiyama's reagent mediated both alkyl and aryl amidinylations completely and efficiently [32]. Although these two candidates emerged as optimal thiophiles for peptide-based amidinylations, Mukaiyama's reagent was chosen as the most favorable candidate for further study because of the known propensity of HATU to act as its own undesired guanidinylation 'capping' agent when used in excess over resin-bound primary amines [35].

Having identified the optimal thiophile for further study of amidinylation reactions on the solid support, the scope and limitations of the methodology were then probed on a different test peptide system. An angiotensin-based peptide sequence [36] bearing a single Mtt-protected Orn residue at the 2-position was utilized to probe the on-resin amidinylation with all thioamides given in Table 1 (Figure 3). The Mtt deprotection and amidinylation reaction were carried out identically as before using Mukaiyama's reagent as thiophile, and by following peptide cleavage, the crude peptides were analyzed by analytical HPLC and MALDI.

The results of the amidinylation substituent survey offered some interesting insight into the utility of this methodology when applied to the solid phase. Overall, it was evident that the majority of the amidinylations were successful, and these examples afforded crude isolates which exhibited reasonable yield and purity in their crude form, each showing a major peak attributable to the desired peptide product (see chromatograms, Figure 3). The amidinylation profile illustrated a wide tolerance for substitution pattern on the thioamide partner, allowing a



Thiophile	Amidinylation Effectiveness	
	2a (CH_3)	**2f** (Ph)
EDCI	None	None
PyBOP	Partial	None
HATU	Complete	Complete
Mukaiyama Rgt.	Complete	Complete

Figure 2. Selective amidination assay on a resin-bound corazonin (ZTFQYSRGWN) peptide template to afford acetamidyl and benzamidyl Arg analog from an Orn-substituted precursor. HATU and Mukaiyama's reagent were found to be the most effective thiophiles in these initial on-resin test systems, with Mukaiyama's reagent ultimately selected as the most optimal candidate.

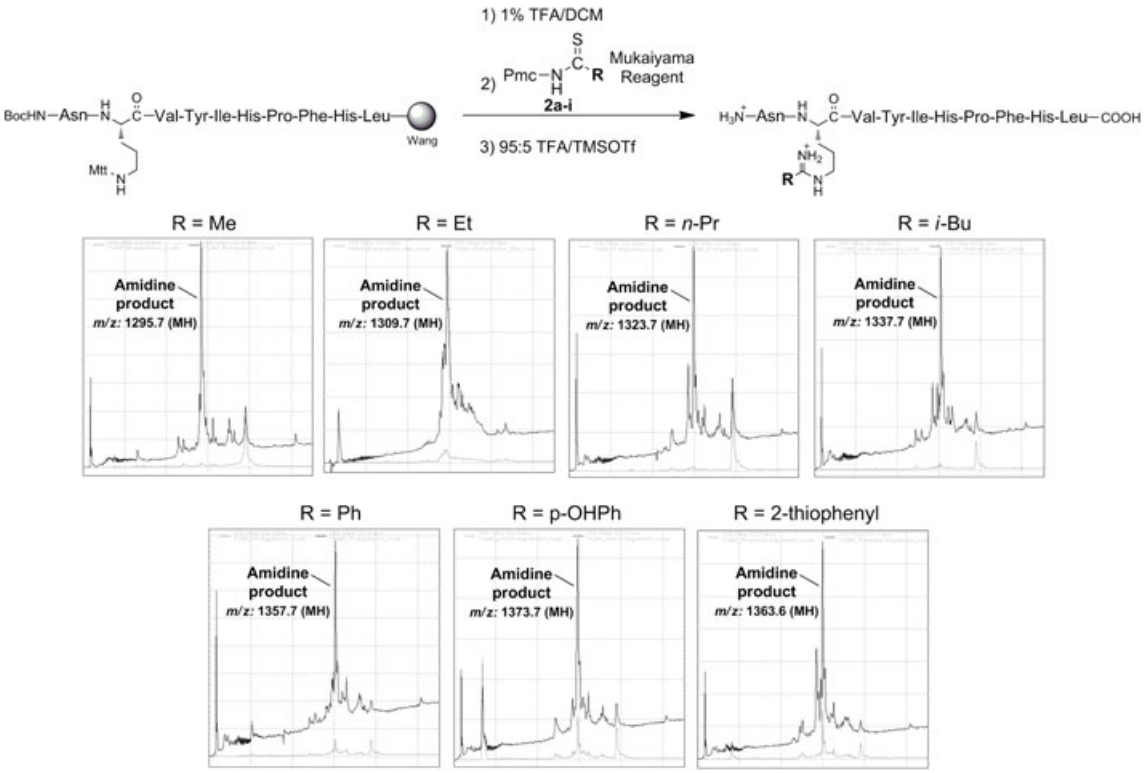


Figure 3. Amidination series on NRVYIHPFHL angiotensin template using Mukaiyama's reagent as the optimized choice of thiophile. A representative series of alkyl-, aryl-, and heterocycle-substituted amidine systems were prepared as Arg headgroup architectural mimics. Crude HPLC chromatograms of each diversified peptide isolate illustrates the generally good purity of peptide product from this methodology.

diverse range of alkyl, aryl, and heteroaryl architecture to be co-opted into the final products.

Perhaps more revealing than the solid-phase amidinylation reactions that were successful, the instances in which the condensations were ineffective offered important information as to the limitations of the process and provided the first examples of instances in which the solid-phase reaction results differed in scope from the previously studied solution phase condensations. Benzyl-substituted thioamide **2e** gave none of the corresponding amidinylated product, affording only the Orn precursor peptide upon cleavage. This was indicative of a reaction pathway which precluded amidinylation. It was clear that an alternate mechanistic route was being undertaken as evidenced by the reaction solution taking on a deep purple coloration immediately following addition of Mukaiyama's reagent to the dissolved **2e** thioamide. None of the other amidinylation reactions took on this coloration at any time during their incubation period. This result was perhaps not unexpected as the identical observations were recorded

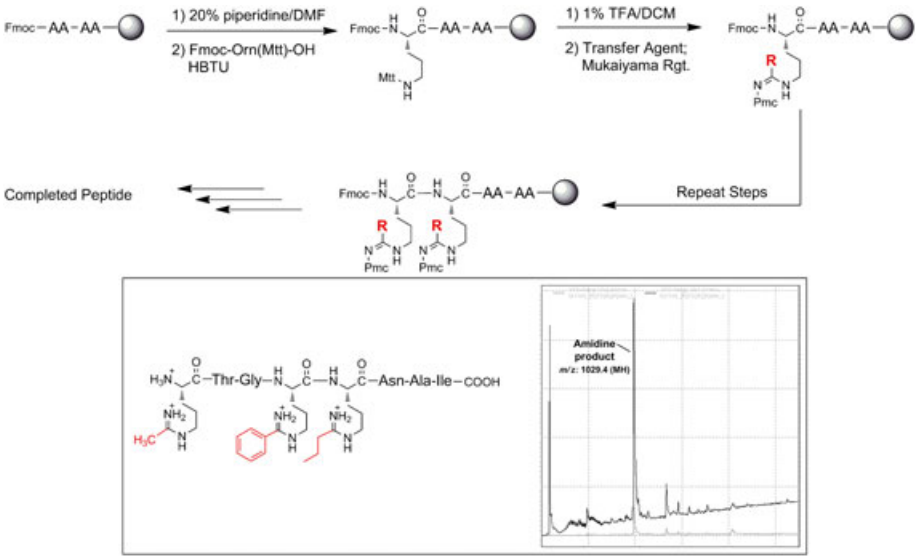


Figure 4. Amidine-diversified cGMP-dependent PKG inhibitor (RTGRRNAI) analog illustrating the potential for iterative installation of amidine diversity while peptide is being built on solid support.

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in previously reported solution phase amidinylation attempts using **2e** as the thioamide condensation partner [32]. Indeed, it was shown that this coloration phenomenon was independent of the presence or absence of added amine partner to the mixture, which would explain the unreactivity of the resin-bound Orn amine and its subsequent isolation as the lone identifiable product upon peptide cleavage and workup.

The failure of thioamide **2e** to condense with the resin-bound Orn did illustrate an important limitation to the general methodology. Another limitation was highlighted by the failure of thioamide **2h** to fully condense with the test peptide amine to yield the expected *p*-trifluoromethylphenyl-substituted amidinyl Arg angiotensin. Analytical HPLC of the crude peptide mixture showed two approximately equal peaks, one of which corresponded to the desired peptide but another corresponded to an unknown M-75 Da component following careful MALDI analysis of the two peaks. Because the two components' retention times were less than 0.5 min apart, purification of the desired product would have been virtually impossible. Further, one of the highlights of this methodology is its ability to produce crude peptide isolates in which the desired product is of reasonable purity. As a result of the failure of thioamides **2e** and **2h** to produce acceptable pure product profiles, these were excluded from entry into Figure 3.

Having shown the ability of this amidinylation methodology to be applied directly toward the construction of Arg isosteres on the solid support, we desired to illustrate the applicability of this process toward the construction of multiple Arg-containing peptides. Moreover, we desired to carry out a model synthesis using a stepwise approach similarly to the iterative installation of substituted Arg residues into model peptides as previously described by us [17]. As a final testament to the utility of this methodology, a cGMP-dependent PKG inhibitor peptide template bearing multiple Arg residues was chosen [5] to carry out the iterative installation of sequential amidinations (Figure 4). The separate introduction of three different amidine transfer agents (**2a**, **2c**, and **2f**) to Orn side chains as the peptide was being built out on the solid support was achieved in high crude purity. Orthogonally disposed Fmoc/Mtt-protected Orn residues were coupled and selectively side chain deprotected as previously described. Amidinylation was then carried out in a routine fashion before continuing the amide backbone extension of the peptide. This iterative approach was ultimately repeated twice more within the sequence to afford the triply amidinylated cGMP-dependent PKG inhibitor analog in $>$90% purity.

Conclusion

Similar to our previously published solution amidinylation accounts, the results of the initial thiophile assay on solid-supported Orn δ-amine partners upheld the previous discovery that, although EDCI may be the coupling agent of choice for *guanidinylation* reactions of resin-bound amines and thiourea transfer agents, it was a very poor choice for the analogous amidination reactions of amines and thioamide transfer agents. It was shown that three thiophiles (PyBOP, HATU, and Mukaiyama's reagent) outperformed EDCI in the mediation of these solution-condensation reactions. It was further discovered that only Mukaiyama's reagent and HATU were able to afford complete amidination between most thioamide transfer agents studied and a resin-bound amine.

The optimized conditions allowed for the facile and routine condensation of a wide diversity of *N*-Pmc thioamides with the resin-bound Orn side chain amine on various model peptide systems. It was found that this methodology tolerates a wide spectrum of thioamide architecture, giving rise to many different alkyl-substituted, aryl-substituted, and heterocycle-substituted Arg isosteres bearing amidine core headgroups. This process is further amenable to the sequential construction of multiple Arg-containing isosteric systems through the ability to build out the peptide chain concurrent with the installation of amidine headgroups in a sequential iterative fashion. Given the ease with which these amidination reactions are able to be carried out on-resin, this methodology has high synthetic potential with prospective applications toward highly diversified peptide library systems and finely tuned Arg-containing peptide targets.

Acknowledgements

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