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Solid-Phase Synthesis of Arginine-Containing Peptides and Fluorogenic Substrates Using a Side-Chain Anchoring Approach

Abdallah Hamzé, Jean Martinez, and Jean-François Hernandez*

Laboratoire des Aminoacides Peptides et Protéines, CNRS UMR 5810, Universités Montpellier I et II, Faculté de Pharmacie, 15 avenue Charles Flahault, BP 14491, 34093 Montpellier cédex 5, France

hernandez@colombes.pharma.univ-montp1.fr

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Attachment of an amino acid to a solid support by its side chain is sometimes necessary to take advantage of an α-carboxylic group available for diverse modifications, including the incorporation of a fluorophore for the preparation of fluorogenic substrates. In contrast to most other amino acids, anchoring the guanidinium group of an arginine to a resin requires the use of a supplementary linker. To avoid the usually multistep synthesis of such a linker as well as its difficult attachment to the guanidine group, we developed a simple method where the guanidine group is built on a Rink amide resin. Our strategy followed the steps of guanidine formation: (i) addition of an isothiocyanate derivative of ornithine to the amino group of a solid support, yielding Nω-linked thiocitrulline; (ii) *S*-methylation of thiourea; (iii) guanidinylation using ammonium acetate. Cleavage of the resin generated the arginine-containing compound, the amine group of the resin becoming part of the guanidine. We have demonstrated the usefulness of this method by the synthesis of a series of fluorogenic substrates for trypsin-like serine proteases, which were obtained in high yield and purity. Then, our strategy also allowed generation from the same precursor differentially substituted arginine derivatives, including Nω-methyl- and Nω-ethylarginines. The ability to prepare such analogues together with the intermediates thiocitrulline and *S*-methylisothiocitrulline from a unique precursor while the α-amine and carboxylic groups remain available for modification also makes this method a powerful tool for combinatorial solid-phase synthesis of NO synthase inhibitors.

Introduction

Solid-phase synthesis of peptides and related compounds is usually performed by linking the first amino acyl residue to the solid support through its α-carboxylic group. However, it is sometimes preferred to keep this α-carboxylic group available for modification. Modifications include on-resin head-to-tail cyclization, incorporation of a chromophore or fluorophore group, homologation, and Hofmann rearrangement. This can be accomplished by connection on the resin via the α-amino group[1] or more commonly via a side-chain group. Most of the functional groups present on amino acyl side chains can be directly attached to commercial supported linkers. This is obviously the case for the β- and γ-carboxylic groups of aspartyl and glutamyl residues, respectively.[2,3] The hydroxyl group of serine, threonine, and tyrosine can be attached to a Wang resin using active carbonate linker[4] or the Mitsunobu reaction[5] or trichloroacetimidate activation[6] or to a trityl chloride resin.[7] The latter resin has also been used for the attachment of lysine, cysteine, tryptophane by their side chain.[7] However, arginine remains a problematic residue as its guanidine group cannot be directly anchored to such supports. Although Bernhardt et al.[7] were able to load an arginine derivative on 2-chlorotrityl chloride resin via its side chain, cleavage from the resin led to a complex mixture of products. As an alternative, they attached ornithine through its δ-amino group which was guanidinylated in solution after resin cleavage. Linking a guanidine group to a resin can only be performed through the use of a supplementary handle. This extra linker derives from known arenesulfonyl protecting groups. A first example came from the use of a *p*-alkoxybenzenesulfonyl linker in the solid-phase synthesis of a guanidinium-based "Tweezer" receptor.[8] A similar linker was used by Maryanoff and co-workers

* To whom correspondence should be addressed. Phone: 33-4 67 54 86 53. Fax: 33-4 67 54 86 54.

(1) (a) Redemann, T.; Bandel, H.; Jung, G. *Mol. Diversity* **1998**, *4*, 191−197. (b) Lee, Y.; Silverman, R. B. *Synthesis* **1999**, 1495−1499. (c) Cantel, S.; Boeglin, D.; Rolland, M.; Martinez, J.; Fehrentz, J.-A. *Tetrahedron Lett.* **2003**, *44*, 4797−4799.

(2) (a) Breipohl, G.; Knolle, J.; Stüber, W. *Int. J. Pept. Protein Res.* **1990**, *35*, 281−283. (b) Albericio, F.; Van Abel, R.; Barany, G. *Int. J. Pept. Protein Res.* **1990**, *35*, 284−286. (c) Rovero, P.; Quartara, L.; Fabbri, G. *Tetrahedron Lett.* **1991**, *32*, 2639−2642. (d) Romanovskis, P.; Spatola, A. F. *J. Peptide Res.* **1998**, *52*, 356−374.

(3) In addition, Asn and Gln can be obtained by attachment of Asp and Glu side chains, respectively, to an amine resin. For instance: Medzihradszky, K. F.; Ambulos, N. P.; Khatri, A.; Osapay, G.; Remmer, H. A.; Somogyi, A.; Kates, S. A. *Lett. Pept. Sci.* **2002**, *8*, 1−12.

(4) Alsina, J.; Chiva, C.; Ortiz, M.; Rabanal, F.; Giralt, E.; Albericio, F. *Tetrahedron Lett.* **1997**, *38*, 883−886.

(5) Cabrele, C.; Langer, M.; Beck-Sickinger, A. G. *J. Org. Chem.* **1999**, *64*, 4353−4361.

(6) Yan, L. Z.; Edwards, P.; Flora, D.; Mayer, J. P. *Tetrahedron Lett.* **2004**, *45*, 923−925.

(7) Bernhardt, A.; Drewello, M.; Schutkowski, M. *J. Peptide Res.* **1997**, *50*, 143−152.

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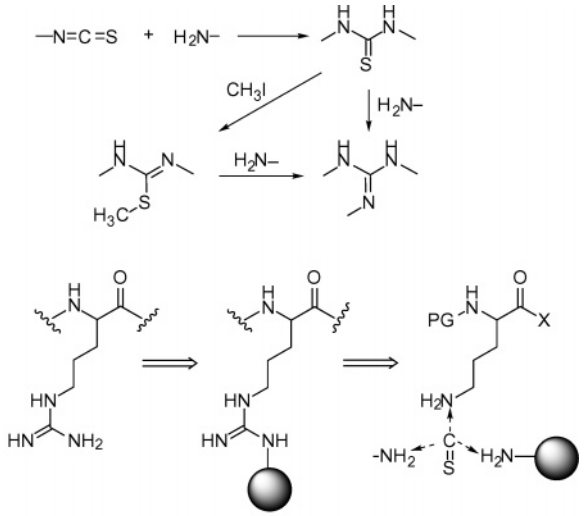
SCHEME 1[a]

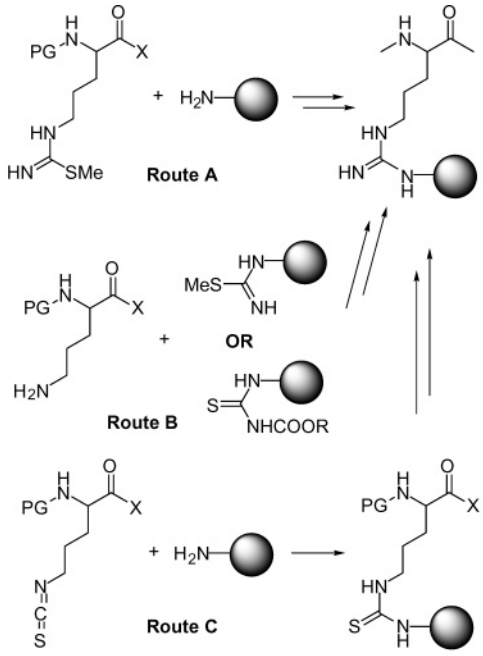


[a] H_2N–●: Rink amide resin.

for anchoring arginine.[9] However, attachment of arginine to this handle requires drastic conditions while cleavage is performed using neat liquid HF. More recently, two linkers based on TFA-labile arenesulfonyl protecting groups were reported. CMtr,[10] a modified Mtr containing a carboxylic group for attachment to the resin, is first condensed to the guanidine group of arginine but with low yield. Then, subsequent coupling of the resulting product to the resin showed significant difficulties due to the relatively low accessibility of the carboxylic acid. The second handle is derived from the Pmc protecting group and carries also a carboxylic group for attachment to the resin.[11] Synthesis of this linker needs several steps including an "on resin" chlorosulfonation. Then a large excess of an arginine derivative (5 equiv) is reacted to give the resin-bound guanidine in 79% yield. One drawback of these methods is the difficulty of condensing the guanidine group to the sulfonyl linker leading to a significant loss of the arginine derivative.

Here, we present a different approach which avoids the need of a supplementary linker. This approach consists of building the guanidine moiety on the resin. Scheme 1 illustrates the well-known general synthetic sequence for guanidine synthesis via a thiourea.[12] Briefly, an isothiocyanate is first reacted with an amine resulting in the formation of thiourea. A second amine can then be coupled to the thiourea to give the guanidinium. If the thiourea is not sufficiently activated, it can be first *S*-methylated, giving an *S*-methylisothiourea. This synthesis often proceeds through carbodiimide intermediates. The second part of Scheme 1 shows the retrosynthetic pathway of our approach. It uses the amine group of a resin (Rink amide) as one of the nitrogen precursors of the guanidine group, while the two other nitrogens are provided by the δ-amino group of an ornithine residue and an ammonia equivalent. We describe here our effort in developing among all possible ways a simple and efficient protocol to obtain arginine derivatives and its application to the solid-phase synthesis of fluorogenic substrates for trypsin-like proteases.

SCHEME 2[a]



[a] PG: protecting group; H_2N–●: Rink amide resin.

Results and Discussion

Several routes were explored, and some of them are presented in Scheme 2. These routes resulted from the different strategies that can be found in the literature for the synthesis of guanidine-derived compounds, in solution or on solid supports.[12] The key step is the guanidinylation reaction. It can be performed by condensing the thiourea to an amine group with the assistance of a coupling reagent such as DIC[13] or EDC,[12a,14] or Mukaiyama's reagent,[15,14a,14f] or triphenylphosphine dichloride,[16] or heavy salts.[17] However, to be efficient,

(8) Bonnat, M.; Bradley, M.; Kilburn, J. D. *Tetrahedron Lett.* **1996**, *37*, 5409−5412.
(9) Zhong, H. M.; Greco, M. N.; Maryanoff, B. E. *J. Org. Chem.* **1997**, *62*, 9326−9330.
(10) (a) Urban, J.; Dattilo, J. W. *Abstracts of Papers*, 15th American Peptide Symposium, Nashville, TN, 14−19 June, 1997. (b) Edwards, P. D.; Mauger, R. C.; Cottrell, K. M.; Morris, F. X.; Pine, K. K.; Sylvester, M. A.; Scott, C. W.; Furlong, S. T. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2291−2294. (c) Furlong, S. T.; Mauger, R. C.; Strimpler, A. M.; Liu, Y.-P.; Morris, F. X.; Edwards, P. D. *Bioorg. Med. Chem.* **2002**, *10*, 3637−3647.
(11) Garcia, O.; Nicolas, E.; Albericio, F. *Tetrahedron Lett.* **2003**, *44*, 5319−5321.
(12) For an overview of solid-phase methods following this synthetic scheme, see: (a) Schneider, S. E.; Bishop, P. A.; Salazar, M. A.; Bishop, O. A.; Anslyn, E. V. *Tetrahedron* **1998**, *54*, 15063−15086. (b) Manimala, J. C.; Anslyn, E. V. *Eur. J. Org. Chem.* **2002**, 3909−3922.
(13) (a) Li, M.; Wilson, L. J. *Tetrahedron Lett.* **2001**, *42*, 1455−1458. (b) Li, M.; Wilson, L. J.; Portlock, D. E. *Tetrahedron Lett.* **2001**, *42*, 2273−2275.
(14) (a) Josey, J. A.; Tarlton, C. A.; Payne, C. E. *Tetrahedron Lett.* **1998**, *39*, 5899−5902. (b) Wilson, L. J.; Klopfenstein, S. R.; Li, M. *Tetrahedron Lett.* **1999**, *40*, 3999−4002. (c) Linton, B. R.; Carr, A. J.; Orner, B. P., Hamilton, A. D. *J. Org. Chem.* **2000**, *65*, 1566−1568. (d) Mamai, A.; Madalengoitia, J. S. *Org. Lett.* **2001**, *3*, 561−564. (e) Manimala, J. C.; Anslyn, E. V. *Tetrahedron Lett.* **2002**, *43*, 565−567. (f) Li, J.; Zhang, G.; Zhang, Z.; Fan, E. *J. Org. Chem.* **2003**, *68*, 1611−1614.
(15) Yong, Y. F.; Kowalski, J. A.; Lipton, M. A. *J. Org. Chem.* **1997**, *62*, 1540−1542.

thioureas have to be substituted by at least one electron-withdrawing group (carbamoyl protecting groups, benzoyl, acyl, aryl, arenesulfonyl, ...), which helps to further activate carbodiimide and alkylated thiourea intermediates toward nucleophilic attack. Otherwise, condensation can be done using the *S*-methylated derivative of thiourea, in the presence or not of heavy salts.[18,17e] An alternative pathway is to generate a carbodiimide, most commonly via tandem Staudinger and aza-Wittig reactions but also from ureas, before reacting with amine.[19] However, this latter strategy appeared less appropriate for our major goal and less straightforward than the preceding ones and was not considered.

Preliminary Results. Route A (Scheme 2) is the reaction between a deprotected Rink amide resin and a protected *S*-methylisothiocitrulline derivative, which is obtained by condensation of the δ-amine group of an ornithine with Fmoc-isothiocyanate (Fmoc-NCS),[20,14c] followed by piperidine-promoted deprotection and *S*-methylation using methyl iodide. The reaction was performed using 5 equiv of the compound at 80 °C overnight. Low attachment yield (2%) was obtained, and the expected arginine derivative was only a minor component of the cleavage mixture. Such failure was already observed for the coupling of *S*-alkylated benzyl thioureas with resin bound amines.[12a] In addition to the absence of an electron-withdrawing group on isothiourea, this low reactivity might also be due to a steric effect as the Rink resin amine group is a relatively hindered primary amine. We did not try to use a carbamoyl-protected thiourea derivative as this would have added to steric hindrance. Route B is opposite to route A. Treatment of supported thioureas with an excess of amine is more commonly described in the literature.[13,14a,18b,e] Here, the thiourea group was first formed on Rink amide resin, which was then reacted with the δ-amine function of an ornithine derivative. Formation of thiourea was achieved through reaction of Fmoc-NCS with the amine group of Rink resin.[20] Then, two pathways were tested. First, Fmoc removal, *S*-methylation, and coupling with an excess of an ornithine derivative (10 equiv) at 80 °C overnight afforded the expected compound in very low yield (2% crude). In a second assay, the Fmoc-protected thiourea was coupled with 4 equiv of Fmoc-Orn-NH_2 in the presence of EDCI, at room temperature, overnight. A peptide[21] was then assembled following classical Fmoc chemistry, and cleavage afforded the expected peptide (Ac-Val-Gly-Leu-Arg-NH_2, see below) in higher but still low yield (8% and 75% purity). As Fmoc might hinder the thiourea group, we considered using a smaller protecting group. Success has already been reported using the ethylcarbamate protecting group,[14c−e] but this group requires too drastic deprotection conditions for use in peptide synthesis. We used instead the allyloxycarbonyl (Alloc) protecting group, which was introduced by reacting Alloc-NCS with the amine function of Rink amide resin. However, synthesis of the same peptide using these conditions did not allow to get yield greater than 10%.

Route C. We then explored route C, which does not afford directly the arginine moiety but a thiocitrulline intermediate. Indeed, it involves the reaction of an isothiocyanate derivative of ornithine with the amine function of Rink amide resin, giving the grafted thiourea. The thiocitrulline (Tci) residue can then be converted into arginine in two steps (*S*-methylation then reaction with ammonium acetate), just before resin cleavage. This approach is similar to the procedure described by Li et al.[13b] for the synthesis of disubstituted guanidines. In that study, aromatic isothiocyanates had to be used to achieve subsequent guanidinylation efficiently. In the present work, using an aliphatic isothiocyanate (i.e., the ornithine derivative) might not be problematic as, in our primary objective (synthesis of arginine), guanidinylation is effected using ammonia, the least hindered amine. Route C effectively gave very satisfactory results. It has even some more advantages comparing to routes A and B. First, attachment can be easily monitored through detection of unreacted resin amine groups (Kaiser and TNBS tests). Then, there is no need for guanidine protecting group as this function is revealed at the last step before resin cleavage. Finally, route C allowed formation of substituted arginines and opened the way toward the solid-phase synthesis of a biologically important class of compounds (see below).

Scheme 3 describes the synthetic pathway, which was followed to perform a primary evaluation of route C. The ornithine isothiocyanate compound **1** was first prepared in three steps from the Fmoc-protected commercial derivative Fmoc-Orn(*N*δ-Boc)-OH. Amidation followed by Boc removal afforded Fmoc-Orn-NH_2. Conversion into the isothiocyanate **1** was performed according to the smooth conditions described by Li et al.,[22] i.e., reaction of the δ-amine function with CS_2 in the presence of triethylamine followed by the addition of hydrogen peroxide as a dehydrosulfurization reagent. This method has been used by Shioiri's group for the synthesis of a Boc-protected ornithine isothiocyanate derivative.[23] Compound **1** was obtained in an overall yield of 81%. Several conditions have been tested for the preparation of supported thiocitrulline **2** by condensation of isothiocyanate **1** with the amine function of Rink amide resin. Satisfactory results were obtained when 3 equiv of compound **1**

(16) Kilburn, J. P.; Lau, J.; Jones, R. C. F. *Tetrahedron* **2002**, *58*, 1739−1743.

(17) (a) Kim, K. S.; Qian, L. *Tetrahedron Lett.* **1993**, *34*, 7677−7680. (b) Levallet, C.; Lerpiniere, J.; Ko, S. Y. *Tetrahedron* **1997**, *53*, 5291−5304. (c) Linkletter, B. A.; Bruice, T. C. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1285−1290. (d) Barawkar, D. A.; Linkletter, B.; Bruice, T. C. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1517−1520. (e) Chang, J.; Oyelaran, O.; Esser, C. K.; Kath, G. S.; King, G. W.; Uhrig, B. G.; Konteatis, Z.; Kim, R. M.; Chapman, K. T. *Tetrahedron Lett.* **1999**, *40*, 4477−4480. (f) Le, V.-D.; Wong, C.-H. *J. Org. Chem.* **2000**, *65*, 2399−2409.

(18) (a) Kent, D. R.; Cody, W. L.; Doherty, A. *Tetrahedron Lett.* **1996**, *37*, 8711−8714. (b) Kearney, P. C.; Fernandez, M.; Flygare, J. A. *Tetrahedron Lett.* **1998**, *39*, 2663−2666. (c) Lin, P.; Ganesan, A. *Tetrahedron Lett.* **1998**, *39*, 9789−9792. (d) Székely, Z.; Zakhariev, S.; Guarnaccia, C.; Antcheva, N.; Pongor, S. *Tetrahedron Lett.* **1999**, *40*, 4439−4442. (e) Dodd, D. S.; Zhao, Y. *Tetrahedron Lett.* **2001**, *42*, 1259−1262.

(19) (a) Drewry, D. H.; Gerritz, S. W.; Linn, J. A. *Tetrahedron Lett.* **1997**, *38*, 3377−3380. (b) Hopkins, T. P.; Dener, J. M.; Boldi, A. M. *J. Comb. Chem.* **2002**, *4*, 167−174. (c) Boguszewski, P. A.; Rahman, S. S.; Ganesan, A. *J. Comb. Chem.* **2004**, *6*, 32−34.

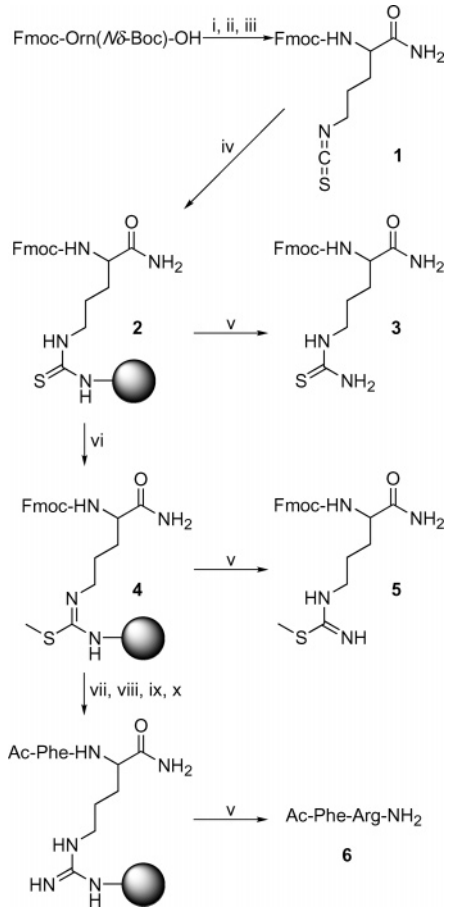
(20) Kearney, P. C.; Fernandez, M.; Flygare, J. A. *J. Org. Chem.* **1998**, *63*, 196−200.

(21) Preliminary work was done on a model peptide, Ac-Val-Gly-Leu-Arg-NH_2, derived from the substrate of Pfgp76, a *Plasmodium falciparum* protease. See ref 31.

(22) Li, G.; Tajima, H.; Ohtani, T. *J. Org. Chem.* **1997**, *62*, 4539−4540.

(23) Yokokawa, F.; Sugiyama, H.; Shioiri, T.; Katagiri, N.; Oda, O.; Ogawa, H. *Tetrahedron* **2001**, *57*, 4759−4766.

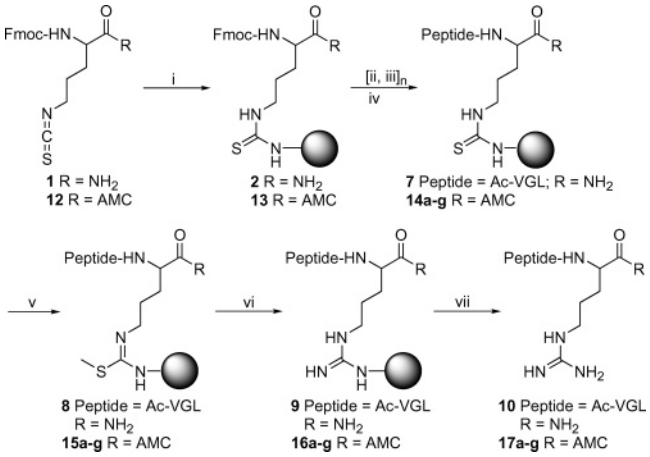
SCHEME 3[a]



[a] Reagents and conditions: (i) isobutyl chloroformate, NMM, DME, then 28% NH_4OH; (ii) TFA/DCM (50:50); (iii) CS_2, Et_3N, THF, then H_2O_2; (iv) deprotected Rink amide resin, Et_3N, THF, 60 °C then rt; (v) TFA/TIS/H_2O (95:2.5:2.5); (vi) CH_3I, DMF (3×); (vii) piperidine/DMF (20:80); (viii) Fmoc-Phe-OH, HBTU, HOBt, DIEA; (ix) CH_3COOH, HBTU, HOBt, DIEA; (x) 2 M ammonium acetate in DMSO, 80 °C.

reacted with the resin in the presence of 3 equiv of TEA at 62 °C for 1.5 h and then at room temperature overnight. THF was used because compound **1** has good solubility, while being a well-swelling solvent for the resin. Completion of the reaction was assessed by observation of negative Kaiser and TNBS tests. A portion of resin **2** was cleaved with TFA/TIS/H_2O (95/2.5/2.5) mixture affording the thiocitrulline derivative Fmoc-Tci-NH_2, **3**, in 79% yield and high purity (95%), confirming the efficiency of isothiocyanate loading on resin. Resin **2** was then methylated using methyl iodide, giving the supported *S*-methylisothiocitrulline (-Itc) **4**. Again, a portion of resin **4** was cleaved affording the *S*-methylisothiocitrulline derivative Fmoc-Itc(*S*-Me)-NH_2, **5**, in 65% yield and high purity (95%). No trace of Tci derivative **3** was detected, indicating that *S*-methylation was complete. Resin **4** was not directly converted into the supported arginine derivative because the Fmoc protecting group would probably suffer from guanidinylation conditions. Then, coupling of a chiral amino acid would allow us to determine if synthesis was performed without significant epimerization of the starting ornithine. Thus, Fmoc was removed and Fmoc-Phe-OH was coupled to the free α-amine. *N*-Terminal acetylation was then performed

SCHEME 4[a]



[a] Reagents and conditions: (i) deprotected Rink amide resin, Et_3N, THF, 60 °C then rt; (ii) piperidine/DMF (20:80); (iii) Fmoc-AA-OH, HBTU, HOBt, DIEA; (iv) CH_3COOH, HBTU, HOBt, DIEA; (v) CH_3I, DMF (3×); (vi) 2 M ammonium acetate in DMSO, 80 °C; (vii) TFA/TIS/H_2O (95:2.5:2.5).

after Fmoc deprotection. Guanidinylation of the obtained peptide-resin was achieved by reaction with a 2 M ammonium acetate solution in DMSO at 80 °C for 12 h. Cleavage of supported Ac-Phe-Arg-NH_2 provided compound **6** in 50% crude yield (90% purity). Again, no trace of the precursor Ac-Phe-Itc(*S*-Me)-NH_2 was present, indicating that guanidinylation was complete. Then, reversed-phase HPLC analysis of **6** showed only one peak, indicating that only one diastereoisomer has been formed and that no significant epimerization occurred.[24] Identification of compound **6** was realized using ESI-MS and ^1H and ^{13}C NMR. In addition, RP-HPLC analysis showed that the compound coeluted with Ac-Phe-Arg-NH_2 synthesized by standard solid-phase procedures.

We next evaluated this method for the synthesis of a longer peptide, Ac-Val-Gly-Leu-Arg-NH_2[21] (Scheme 4). Starting from resin **2**, the peptide was assembled following standard Fmoc chemistry protocols. Cleavage of a portion of resin before *N*-terminal acetylation afforded the tetrapeptide H-Val-Gly-Leu-Tci-NH_2 in 35% yield after preparative HPLC purification. Following acetylation, resin **7** was treated with methyl iodide giving the supported *S*-methylisothiocitrulline derivative **8**. Cleavage of a portion of resin **8** produced the peptide Ac-Val-Gly-Leu-Itc(*S*-Me)-NH_2 in 37% yield after purification. Finally, guanidinylation was achieved as already described. Cleavage of resin **9** gave the expected compound **10** in 52% crude yield and with good purity (85%). Again, RP-HPLC analysis showed that compound **10** coeluted with the same peptide prepared through classical solid-phase methodology.

Solid-Phase Synthesis of Fluorogenic Substrates. To exemplify and to show the usefulness of this method, and aiming to take advantage of an available C-terminal carboxylic group of arginine, we prepared a small library of fluorogenic substrates for trypsin-like serine proteases.

(24) Co-injection of compound **6** with its diastereoisomer Ac-*D*Phe-Arg-NH_2 on analytical reverse phase HPLC showed two well-separated peaks (compound **6**: t_R(C) = 14.44 min; Ac-D-Phe-Arg-NH_2 t_R(C) = 14.02 min. See conditions and chromatograms in the Supporting Information).

This class of serine proteases is specific for basic residues and more particularly arginine. It has numerous members, which are often biologically and/or medicinally important, such as proteases of the coagulation cascade (thrombin, factor Xa, factor IXa, ...). Their detection in biological fluids, as well as the study of their role in biochemical pathways and screening of protease inhibitors of therapeutic interest, need a sensitive enzymatic assay. Substrates for serine proteases are generally composed of a fluorophore attached to the C-side of the P1[25] position through an amide bond, which is cleaved upon enzyme action releasing the fluorophore. The most commonly used fluorophore is AMC (7-amino-4-methylcoumarine).[26] These compounds are usually synthesized in solution, but their synthesis is long and gives overall poor yields. Several solid-phase strategies have been reported, including a safety catch linker approach where peptide displacement is done with a P1-AMC compound,[27] a side-chain-anchoring approach,[10b,c] and the use of a bifunctional AMC derivative containing a carboxylic group for attachment to a solid support.[28] In the former case, a P1 lysine library for thrombin was prepared and not a P1 arginine library, although arginine is preferred by this enzyme, suggesting that synthetic difficulties might have been encountered with this residue.[27] The second method has already been mentioned and needs a supplementary linker to attach a protected Arg-AMC compound.[10b,c] In the latter case,[28] the carboxy-AMC derivative ACC (7-amino-4-carbamoylmethylcoumarin), originally reported by Kanaoka et al.[29] and Besson et al.,[30] is a very convenient alternative. However, while its synthesis needs careful conditions, it was shown that loading of arginine onto the ACC-derived resin provided the lowest yield (50%) among the 21 amino acids tested, although a double coupling with 5 equiv of amino acid was performed.[31]

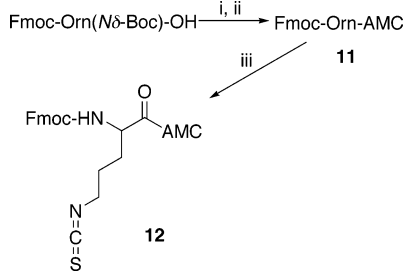
The substrates we decided to synthesize were chosen among commercial ones.[32] We were also interested in preparing a fluorogenic substrate for Pfgp76, a serine protease isolated from *Plasmodium falciparum* merozoites.[33] Some of these compounds were chosen according to the presence of trifunctional residues in order to check the stability of protecting groups toward the guanidinylation conditions (Table 1).

TABLE 1. Synthetic Fluorogenic Substrates

fluorogenic substrates	protease	yield[a] (%)
17a,[b] Ac-Val-Gly-Leu-Arg-AMC	Pfgp76	23
17b, Ac-Val-Pro-Arg-AMC[26b]	thrombin	17
17c, Ac-Ile-Glu-Gly-Arg-AMC[26b]	factor Xa	49
17d, Ac-Ala-Lys-Arg-AMC[34]	IRC serine protease	27
17e, Ac-Phe-Val-Arg-AMC[35]	thrombin	32
17f, Ac-Val-Gly-Arg-AMC[36]	trypsin	31
17g, Ac-Leu-Ser-Thr-Arg-AMC[26d]	activated C protein	37

[a] Yield of purified substrates was calculated from starting Rink amide resin. [b] Substrate **17a** was tested on Pfgp76 and was shown to behave as the same substrate prepared using classical methodology (Catherine Braun Breton, personal communication).

SCHEME 5[a]



[a] Reagents and conditions: (i) AMC, $(Boc)_2O$, pyridine, dioxane; (ii) TFA/DCM (50:50); (iii) CS_2, Et_3N, THF, then H_2O_2.

The isothiocyanate derivative of Fmoc-Orn-AMC, **12**, was first prepared as presented in Scheme 5. The key step was the coupling between Fmoc-Orn(Boc)-OH and AMC. Indeed, the aromatic amine group of AMC has low nucleophilicity. We used a method introduced by Pozdnev[37] for the coupling of carboxylic acids to arylamines and experienced by Furlong et al.[10b,c] for the preparation of an Arg-AMC compound. Thus, coupling using $(Boc)_2O$ and pyridine gave Fmoc-Orn(Boc)-AMC in 83% yield. Subsequent Boc removal afforded **11**, and isothiocyanate derivatization of the free δ-amine group was performed as already described providing **12** almost quantitatively (82% overall yield).

Synthesis of the fluorogenic substrates was performed as described for the preparation of peptide **10**. Condensation of isothiocyanate **12** with the amine group of Rink amide resin afforded Fmoc-Tci(*N*ω-Rink)-AMC resin **13**. Resin **13** was then divided in several portions. Peptide assembly followed Fmoc chemistry protocols, and the *N*-terminus was capped by acetylation. The obtained Tci-containing resins **14a**−**g** were then treated with CH_3I yielding the Itc(*S*-Me)-containing resins **15a**−**g**. Guanidinylation afforded Arg-containing resins **16a**−**g**. Acidic cleavage provided substrates **17a**−**g** in more than 60% crude yield and with purities in the range 70−85%. They were purified by preparative reversed-phase HPLC

(25) Nomenclature for substrate residues from: Schechter, I.; Berger, A. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157−162.

(26) (a) Zimmerman, M.; Yureuicz, E.; Patel, G. *Anal. Biochem.* **1976**, *70*, 258−262. (b) Morita, T.; Kato, H.; Iwanaga, S.; Takada, K.; Kimura, T. *J. Biochem. (Tokyo)* **1977**, *82*, 1495−1498. (c) Castillo, M. J.; Nakajima, K.; Zimmerman, M.; Powers, J. C. *Anal. Biochem.* **1979**, *99*, 53−64. (d) Ohno, Y.; Kato, H.; Morita, T.; Iwanaga, S.; Takada, K.; Sakakibara, S.; Stenflo, J. *J. Biochem.* **1981**, *90*, 1387−1395. (e) Caporale, L. H.; Gaber, S. S.; Kell, W.; Gotze, O. *J. Immunol.* **1981**, *126*, 1963−1965.

(27) Backes, B. J.; Harris, J. L.; Leonetti, F.; Craik, C. S.; Ellman, J. A. *Nature Biotechnol.* **2000**, *18*, 187−193.

(28) (a) Harris, J. L.; Backes, B. J.; Leonetti, F.; Mahrus, S.; Ellman, J. A.; Craik, C. S. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 7754−7759. (b) Zhu, Q.; Li, D. B.; Uttamchandani, M.; Yao, S. Q. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1033−1036.

(29) Kanaoka, Y.; Kobayashi, A.; Sato, E.; Nakayama, H.; Ueno, T.; Muno, D.; Sekine, T. *Chem. Pharm. Bull.* **1984**, *32*, 3926−3933.

(30) Besson, T.; Joseph, B.; Moreau, P.; Viaud, M.-C., Coudert, G.; Guillaumet, G. *Heterocycles* **1992**, *34*, 273−291.

(31) Maly, D. J.; Leonetti, F.; Backes, B. J.; Dauber, D. S.; Harris, J. L.; Craik, C. S.; Ellman, J. A. *J. Org. Chem.* **2002**, *67*, 910−915.

(32) The commercial substrates are blocked at their *N*-terminus by a protecting group (Boc, Z, or benzoyl). For convenience, we replaced it with an acetyl group.

(33) Roggwiller, E.; Morales Bétoulle, M. E.; Blisnick, T.; Braun Breton, C. *Mol. Biochem. Parasitol.* **1996**, *82*, 13−24.

(34) Cromlish, J. A.; Seidah, N. G.; Chretien, M. *J. Biol. Chem.* **1986**, *261*, 10850−10858.

(35) Lottenberg, R.; Christensen, U.; Jackson, C. M.; Coleman, P. L. *Methods Enzymol.* **1981**, *80*, 341−361.

(36) Kawabata, S.; Miura, T.; Morita, T.; Kato, H.; Fujikawa, K.; Iwanaga, S.; Takada, K.; Kimura, T.; Sakakibara, S. *Eur. J. Biochem.* **1988**, *172*, 17−25.

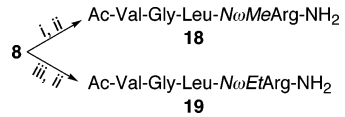
(37) Pozdnev, V. F. *Int. J. Peptide Protein Res.* **1994**, *44*, 36−48.

(Table 1). All substrates were obtained in satisfactory yields whether or not multifunctional residues (Lys, Glu, Ser, Thr) were present, and no secondary product arising from MeI treatment and/or guanidinylation was identified. In the case of glutamyl residue, we found in a different project that benzyl protection cannot be used for the γ-carboxylic group (Note: such protection is used in Boc chemistry; in this case, cyclohexyl ester might be a safe alternative). Indeed, the ester was quantitatively converted to the corresponding amide residue (i.e., glutaminyl residue) during the guanidinylation step. Here (compound **17c**), the *tert*-butyl protecting group prevented this secondary reaction.

MeI treatment might be detrimental to some other trifunctional residues (Met, Cys, His, ...). It should be resolved by performing this step immediately after condensation of the isothiocyanate derivative and before peptide assembly, as for the synthesis of compound **6** (Scheme 3). Otherwise, it is important to choose the right protecting group. We found that this method was also compatible with the presence of arginine (as Pbf-protected derivative) as nona-arginine was prepared without observable methylation by MeI (C. Gandreuil and J.-F. Hernandez, results not shown). Then, cysteine should be used as its *S*-trityl-protected derivative and histidine, with *N-im*-Boc protection, as these protecting groups were found to prevent alkylation of sulfur atom and imidazole by MeI.[38,39] One uncompatible residue is methionine, which would be methylated by MeI on its sulfur atom giving an onium salt. However, methionine can be replaced by the isosteric norleucine residue, a frequent choice because of its susceptibility to oxidation. If indispensable, it should be introduced as its sulfoxide derivative, which can be easily reduced during resin cleavage.[40]

Peptides Containing a Monosubstituted Arginine. Replacement of arginine residues by guanidine-alkylated derivatives is useful to increase stability of peptides toward digestion by trypsin-like proteases, to enhance selectivity, or to reduce guanidine basicity while increasing hydrophobicity.[18d,41] Our method allowed us to consider the preparation of such derivatives on the solid support when peptide assembly is completed, from the Itc(*S*-methyl)-containing resin by reacting with an alkylamine during the guanidinylation step. By this approach it was possible to synthesize arginine-containing peptides as well as Nω-alkylated analogues in one step from a unique precursor. Despite a poorly activated and probably hindered *S*-methylisothiourea, we were able to prepare the simple *N*ω-methyl (compound **18**) and *N*ω-ethyl (compound **19**) derivatives of peptide **10** from resin **8** (Scheme 6). Guanidinylation using methylamine followed the conditions already described (2 M methylamine in DMSO for 12 h) and afforded **18** after resin cleavage in 35% crude yield (>90% pure). By contrast, guanidinylation with ethylamine was less efficient using these conditions with less than 15% conversion. A drastic increase (>80% conversion) was obtained using higher amine concentration (2.5 M) and increasing time (17 h). Peptide **19** was obtained in 56% crude yield (approximately 80% pure) under these conditions.

SCHEME 6[a]



[a] Reagents and conditions: (i) 2 M $MeNH_2$,HCl, 2 M NMM in DMSO, 80 °C, 12 h; (ii) TFA/TIS/H_2O (95:2.5:2.5); (iii) 2.5 M $EtNH_2$·HCl, 2.5 M NMM, in DMSO, 80 °C, 17 h.

Conclusion

A side-chain-anchoring strategy for the synthesis of arginine-containing compounds has been developed that avoids the synthetic effort necessary when using a supplementary spacer for linking the guanidine group of arginine to the resin. All synthesized compounds were produced in satisfactory yield and purity, suggesting that this method should be of general use. Another advantage is the possibility to obtain from the same precursor (i.e., the supported Itc(*S*-Me) derivative) arginine-containing as well as *N*ω-alkylated arginine-containing compounds, allowing to easy investigation of the effects of such variations on biological properties. Then, it is noteworthy that thiocitrulline, *S*-methylisothiocitrulline, and *N*ω-methyl- and -ethylarginine derivatives, here prepared from a unique precursor, are well-known inhibitors of nitric oxide synthases (NOS).[42] It appeared that this method would be of great interest for the synthesis of libraries of NOS inhibitors and would help for the discovery of isoform selective inhibitors.[43,1b] Indeed, as shown in Scheme 7, our strategy allows combination of variability at three levels, the lateral chain, the α-amine, and more particularly, carboxylic functions. The use of this method in the synthesis of NOS inhibitors will be reported soon.

(38) A dimethoxytrityl (analogous to trityl) protecting group prevents sulfur alkylation by MeI in excess, see: Chiba, J.-y.; Tanaka, K.; Ohshiro, Y.; Miyake, R.; Hiraoka, S.; Shiro, M.; Shionoya, M. *J. Org. Chem*. **2003**, *68*, 331−338.

(39) A Boc protecting group prevents imidazole alkylation by MeI in excess, see: Zou, Y.; Fahmi, N. E.; Vialas, C.; Miller, G. M.; Hecht, S. M. *J. Am. Chem. Soc*. **2002**, *124*, 9476−9488.

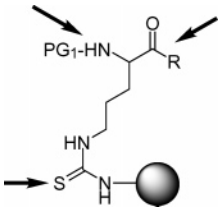
(40) Among others, a convenient method of Met(O) reduction effected during resin cleavage was reported by: Taboada, L.; Nicolas, E.; Giralt, E. *Tetrahedron Lett*. **2001**, *42*, 1891−1893.

(41) (a) Peterlin-Masic, L.; Kikelj, D. *Tetrahedron* **2001**, *57*, 7073−7105. (b) Kennedy, K. J.; Lundquist, J. T., IV; Simandan, T. L.; Kokko, K. P.; Beeson, C. C.; Dix, T. A. *J. Peptide Res.* **2000**, *55*, 348−358. (c) Kokko, K. P.; Arrigoni, C. E.; Dix, T. A. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1947−1950.

(42) (a) Kilbourn, R. G.; Gross, S. S.; Adams, J.; Jubran, A.; Griffith, O. W.; Levi, R.; Lodato, R. F. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 3629−3632. (b) Narayanan, K.; Griffith, O. W. *J. Med. Chem.* **1994**, *37*, 885−887. (c) Furfine, E. S.; Harmon, M. F.; Paith, J. E.; Knowles, R. G.; Salter, M.; Kiff, R. J.; Duffy, C.; Hazelwood, R.; Oplinger, J. A.; Garvey, E. P. *J. Biol. Chem.* **1994**, *269*, 26677−26683. (d) Moore, W. M.; Webber, R. K.; Fok, K. F.; Jerome, G. M.; Kornmeier, C. M.; Tjoeng, F. S.; Currie, M. G. *Bioorg. Med. Chem.* **1996**, *4*, 1559−1564.

(43) For instance, dipeptides containing arginine or thiocitrulline analogues were shown to be selective for one of the three NOS isoforms. See: (a) Silverman, R. B.; Huang, H.; Marletta, M. A.; Martasek, P. *J. Med. Chem.* **1997**, *40*, 2813−2817. (b) Kobayashi, N.; Higuchi, T.; Urano, Y.; Kikuchi, K.; Hirobe, M.; Nagano, T. *Biol. Pharm. Bull.* **1999**, *22*, 936−940. (c) Huang, H.; Martasek, P.; Roman, L. J.; Masters, B. S. S.; Silverman, R. B. *J. Med. Chem.* **1999**, *42*, 3147−3153. (d) Huang, H.; Martasek, P.; Roman, L. J.; Silverman, R. B. *J. Med. Chem.* **2000**, *43*, 2938−2945. (e) Park, J. M.; Higuchi, T.; Kikuchi, K.; Urano, Y.; Hori, H.; Nishino, T.; Aoki, J.; Inoue, K.; Nagano, T. *Br. J. Pharmacol.* **2001**, *132*, 1876−1882. (f) Hah, J.-M.; Martasek, P.; Roman, L. J.; Silverman, R. B. *J. Med. Chem.* **2003**, *46*, 1661−1669. (g) Flinspach, M. L.; Li, H.; Jamal, J.; Yang, W.; Huang, H.; Hah, J.-M.; Gomez-Vidal, J. A.; Litzinger, E. A.; Silverman, R. B.; Poulos, T. L. *Nature Struct. Mol. Biol.* **2004**, *11*, 54−59.

SCHEME 7[a]



[a] PG = protecting group; R = NH_2 or O-PG_2.

Experimental Section

Abbreviations. Standard abbreviations are as follows: Ac, acetyl; AcOEt, ethyl acetate; Alloc, allyloxycarbonyl; AMC, 7-amino-4-methylcoumarine; Boc, *tert*-butyloxycarbonyl; $(Boc)_2O$, di-*tert*-butyl pyrocarbonate); CMtr, 4-carboxymethyloxy-2,3,6-trimethylbenzenesulfonyl; DIEA, diisopropylethylamine; DME, dimethoxyethane; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; EDCI, *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide, hydrochloride; ESI-MS, electron spray ionization mass spectrometry; FAB, fast atom bombardment; Fmoc, 9-fluorenylmethyloxycarbonyl; HBTU, *N*-[(1*H*-benzotriazol-1-yloxy)-(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; HRMS, high-resolution mass spectrometry; Itc, isothiocitrulline; NMM, *N*-methylmorpholine; Pmc, 2,2,7,7,8-pentamethylchromane-6-sulfonyl; RP-HPLC, reversed-phase high-performance liquid chromatography; Tci, thiocitrulline; TEA, triethylamine; TFA, trifluoroacetic acid; THF, tetrahydrofurane; TIS, triisopropylsilane; TNBS, 2,4,6-trinitrobenzene-1-sulfonic acid.

Solid-Phase Peptide Synthesis. Peptide chain assembly was accomplished stepwise using an Fmoc/tBu strategy. Coupling of amino acids (3 equiv) was carried out using HBTU/HOBt as the coupling agent in the presence of DIEA (3−4 equiv). A 20% piperidine solution in DMF was used for Fmoc removal. Monitoring of the coupling and deprotection steps was performed using the Kaiser test or TNBS test. *N*-Terminal acetylation was mediated through coupling of acetic acid (4 equiv) using HBTU. Final cleavage of peptides from the resin and side-chain deprotection were performed with TFA/TIS/H_2O (95:2.5:2.5, 10 mL/g resin) at room temperature for 2 h.

(2*S*)-2-*N*-(9-Fluorenylmethyloxycarbonyl)amino-5-isothiocyanatopentanamide (Fmoc-L-Orn(N^{δ}=C=S)-NH_2) (1). To a stirred solution of Fmoc-L-Orn(Boc)-OH (2.0 g, 4.4 mmol) in DME (10 mL) were added at −10 °C *N*-methylmorpholine (0.48 mL, 4.4 mmol) and isobutyl chloroformate (0.60 mL, 4.4 mmol). After 10 min, a 28% aqueous ammonia solution (1.3 mL) was added to the mixture. After 8 min, the mixture was diluted with ethyl acetate (50 mL) and washed with 1 M aqueous $KHSO_4$, H_2O, saturated aqueous $NaHCO_3$, and brine. The organic layer was then dried over $MgSO_4$ and concentrated in vacuo to yield Fmoc-L-Orn(Boc)-NH_2 as a white powder (1.98 g, 99%): R_f 0.5 (AcOEt); *m/z* (ES+) 454.3 $[M + H]^+$, 398.1 $[M - tBu + H]^+$; ^1H NMR (400 MHz, $CDCl_3$) δ 7.78 (d, J = 7.5 Hz, 2H), 7.65 (d, J = 7.2 Hz, 2H), 7.40 (t, J = 7.4 Hz, 2H), 7.30 (t, J = 7.5 Hz, 2H), 6.60 (s, 1H), 5.75 (s, 1H), 5.50 (s, 1H), 4.80−4.65 (m, 1H), 4.52−4.35 (m, 2H), 4.20 (t, J = 6.8 Hz, 1H), 3.50−3.35 (m, 2H), 1.80−1.60 (m, 4H), 1.42 (s, 9H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.8, 156.8, 156.5, 144.8, 144.7, 141.6, 128.5, 127.9, 126.2, 121.0, 78.3, 66.5, 55.1, 47.6, 30.2, 29.2, 27.1.

The preceding compound (0.9 g, 1.98 mmol) was treated with TFA/DCM (50:50) at room temperature for 30 min. After this period, the solvents were removed under reduced pressure, and the residue was taken up in hexane/diethyl ether. Fmoc-L-Orn-NH_2 precipitated as a white solid, which was collected by filtration (0.93 g, 100%): *m/z* (ES+) 354.3 $[M + H]^+$; ^1H NMR (400 MHz, DMSO-d_6) δ 7.92 (d, J = 7.5 Hz, 2H), 7.72 (d, J = 7.2 Hz, 2H), 7.40 (t, J = 7.8, 2H), 7.35 (t, J = 7.4 Hz, 2H), 7.10 (s, 1H), 4.35−4.20 (m, 3H), 3.95 (t, J = 5.0 Hz, 1H), 3.70−3.40 (m, 2H), 1.75−1.50 (m, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.4, 156.9, 144.7, 144.7, 141.6, 128.5, 127.9, 126.2, 121.0, 66.5, 54.7, 47.5, 29.7, 24.6.

To a solution of the preceding compound (2.65 g, 5.66 mmol) in THF (25 mL) were added TEA (2.79 mL, 19.8 mmol) and CS_2 (3.0 mL, 49.5 mmol) at 0 °C. The reaction was stirred for 45 min at this temperature, and 6.75 mL (59.4 mmol) of H_2O_2 was added dropwise. The mixture was then diluted with diethyl ether (60 mL). The organic layer was washed with 1 M aqueous $KHSO_4$, H_2O, and brine and dried over $MgSO_4$. Concentration in vacuo afforded **1**, which was recrystallized from AcOEt/hexane (5:5). White powder (1.83 g, 82%): mp 107−109 °C; R_f 0.58 (AcOEt/hexane, 5:5); *m/z* (ES+) 396.1 $[M + H]^+$; HRMS (FAB) calcd for $C_{21}H_{22}N_3O_3S$ 396.1382 $[M + H]^+$, found 396.1400; HPLC t_R(A) 2.92 min; IR ν_{max} cm^{-1} 3305, 3193, 2950, 2188, 2102, 1690, 1656, 1530, 1446, 1372, 1252, 1083, 757, 740; ^1H NMR (400 MHz, $CDCl_3$) δ 7.75 (d, J = 7.4 Hz, 2H), 7.58 (d, J = 7.4 Hz, 2H), 7.40 (t, J = 7.4 Hz, 2H), 7.30 (t, J = 7.3 Hz, 2H), 6.10 (s, 1H), 5.65 (s, 1H), 5.55 (d, J = 7.6 Hz, 1H), 4.55−4.35 (m, 2H), 4.20 (t, J = 6.5 Hz, 1H), 3.60−3.50 (m, 2H), 2.10−1.65 (m, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.4, 157.0, 144.6, 141.6, 128.5, 127.9, 126.2, 121.0, 66.8, 54.6, 47.6, 45.3, 29.9, 26.9.

Resin Fmoc-Tci(*N*ω-Rink)-NH_2 (2). A 1.8 g portion of Rink amide resin (0.7 mmol/g substitution) was placed in a vessel for manual peptide synthesis. The resin was swollen through the addition of DMF and treated with a solution of 20% piperidine in DMF (5 min then 15 min) to remove the Fmoc protecting group. After washing with DMF and THF, a solution of compound **1** (1.5 g, 3.8 mmol, 3 equiv) and TEA (0.55 mL, 3.1 equiv) in THF (25 mL) was added. The suspension was stirred at 62 °C for 1.5 h and at room temperature overnight. Completion of the reaction was assessed using the TNBS test. The resin was then filtered and washed with DCM (once), DMF (3×), and DCM (3×), dried, and separated in several portions.

Fmoc-Tci-NH_2 (3). A portion of resin **2** (550 mg) was cleaved with TFA/TIS/H_2O (95:2.5:2.5, 5.5 mL) to yield 110 mg (79% yield, approximately 95% pure according to RP-HPLC analysis using two sets of conditions) of **3** (lyophilized white solid): *m/z* (ES+) 413.1 $[M + H]^+$; HRMS (FAB) calcd for $C_{21}H_{25}N_4O_3S$ 413.1647 $[M + H]^+$, found 413.1652; HPLC t_R(A) 2.34 min; t_R(B) 21.36 min; ^1H NMR (400 MHz, DMSO-d_6) δ 7.90 (d, J = 7.5 Hz, 2H), 7.70 (d, J = 7.2 Hz, 2H), 7.40 (t, J = 6.7 Hz, 2H), 7.25 (t, J = 7.0 Hz, 2H), 7.00 (s, 1H), 6.85 (s, 1H), 4.38−4.15 (m, 1H), 4.18−4.10 (m, 2H), 4.00−3.80 (m, 1H), 3.42−3.22 (m, 2H), 1.75−1.50 (m, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.7, 156.8, 144.8, 144.7, 141.6, 128.5, 128.0, 126.2, 121.0, 66.5, 55.1, 47.6, 44.5, 41.6, 30.3, 26.6.

Fmoc-Itc(*S*-Me)-NH_2 (5). Another portion of resin **2** (350 mg) was treated with CH_3I (0.2 M solution in DMF, 30 mL) at room temperature for 1 h and washed with DMF. This treatment and subsequent washings were repeated twice. The obtained resin **4** was then washed with DCM and dried. Cleavage afforded 60 mg (65% yield, approximately 95% pure according to RP-HPLC analysis using two sets of conditions) of compound **5**: *m/z* (ES+) 426.9 $[M + H]^+$; HRMS (FAB) calcd for $C_{22}H_{27}N_4O_3S$ 427.1804 $[M + H]^+$, found 427.1818; HPLC t_R(A) 2.25 min; t_R(B) 20.15 min; ^1H NMR (400 MHz, DMSO-d_6) δ 9.10 (s, 2H), 7.90 (d, J = 7.5 Hz, 2H), 7.70 (t, J = 6.6 Hz, 2H), 7.50 (t, J = 7.3 Hz, 2H), 7.32 (t, J = 7.4 Hz, 2H), 7.10 (s, 1H), 4.35−4.25 (m, 1H), 4.23−4.15 (m, 2H), 3.90 (t, J = 5.1 Hz, 1H), 3.35−3.25 (m, 2H), 2.58 (s, 3H), 1.75−1.42 (m, 4H).

Ac-Phe-Arg-NH_2 (6). A 400 mg (0.21 mmol) portion of resin **4** was treated with piperidine/DMF (20:80) (5 min then 15 min) to remove the Fmoc protecting group. After washing with DMF, DCM, and DMF, Fmoc-L-Phe-OH (3 equiv) was coupled to the resin as described above. *N*-Terminal acetylation was performed following Fmoc removal as already described, yielding Ac-Phe-Itc(*S*-Me, *N*ω-Rink)-NH_2 resin. The resin was swollen in DMSO, and guanidinylation was performed by treatment with a 2 M ammonium acetate solution in DMSO (10 mL) in a sealed vessel. The suspension was stirred at

80 °C for 12 h. The resin was then washed with DMSO, DMF, MeOH, and DCM, dried, and cleaved with TFA/TIS/H_2O (95:2.5:2.5, 5 mL) as described above to give compound **6** (52 mg, 50% yield, 85% pure according to RP-HPLC analysis): *m/z* (ES+) 363.2 $[M + H]^+$; HRMS (FAB) calcd for $C_{17}H_{27}N_6O_3$ 363.2145 $[M + H]^+$, found 363.2146; HPLC t_R(A) 1.23 min; t_R(C) 14.55 min; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.41 (d, J = 8.0 Hz, 1H), 8.30 (d, J = 8.0 Hz, 1H), 7.80 (t, J = 5.5 Hz, 1H), 7.55−7.45 (m, 2H), 7.35 (s, 1H), 7.35−7.30 (m, 2H), 7.20−6.90 (br, 3H), 4.80−4.68 (m, 1H), 4.48−4.35 (m, 1H), 3.42−3.35 (m, 2H), 3.30−3.20 (dd, J = 4.4 Hz, 13.8 Hz, 1H), 3.00−2.90 (dd, J = 13.8 Hz, 1H), 2.00 (s, 3H), 1.90−1.60 (m, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.5, 172.8, 170.8, 158.0, 139.3, 130.4, 129.3, 127.5, 55.7, 53.3, 38.5, 30.4, 26.3, 23.7.

Ac-Val-Gly-Leu-Arg-NH_2 (10). Resin **2** was prepared starting from 2.1 g (0.7 mmol/g substitution) of Rink amide resin as already described. Following Fmoc removal, peptide assembly was accomplished as described above, affording peptide-resin **7**. Tci was then *S*-methylated as described for resin **4** using 20 mL of a 0.2 M CH_3I solution in DMF (3×). A portion (130 mg, 75 μmol) of the obtained resin **8** was guanidinylated using a 2 M ammonium acetate solution in DMSO as described for compound **6**. Resin **9** was finally cleaved affording **10** in 39% crude yield (17 mg, approximately 85% pure according to RP-HPLC analysis): *m/z* (ES+) 485.4 $[M + H]^+$; HRMS (FAB) calcd for $C_{21}H_{41}N_8O_5$ 485.3200 $[M + H]^+$, found 485.3189; HPLC t_R(A) 1.28 min; t_R(B) 10.46 min; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.20 (t, J = 5.8 Hz, 1H), 7.85 (d, J = 7.8 Hz, 1H), 7.75 (d, J = 8.1 Hz, 1H), 7.70 (d, J = 7.9 Hz, 1H), 7.10 (s, 1H), 6.90 (s, 1H), 4.20−4.15 (m, 1H), 4.10−4.00 (m, 1H), 3.90 (t, 1H), 3.65−3.50 (q, J = 6.0 Hz, 2H), 3.00−2.85 (m, 2H), 1.85−1.75 (m, 1H), 1.72 (s, 3H), 1.60−1.50 (m, 1H), 1.45−1.20 (m, 6H), 0.75−0.65 (m, 12H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.1, 172.7 (2C), 170.8, 169.8, 157.6, 59.5, 52.9, 52.0, 42.9, 30.7, 29.8, 26.0, 24.9, 24.0, 23.3, 22.3, 20.0, 19.2 (2C).

Fmoc-L-Orn-AMC (11). Fmoc-L-Orn(Boc)-OH (5.29 g, 11.6 mmol), pyridine (0.9 mL), and $(Boc)_2O$ (3.12 g, 14.3 mmol) were dissolved in dioxane (45 mL). AMC (2.51 g, 14.3 mmol) was then added, and the mixture was stirred for 24 h at room temperature. The gel-like mixture was diluted in AcOEt (200 mL) and washed with 1 M aqueous $KHSO_4$, H_2O, saturated aqueous $NaHCO_3$, H_2O, and brine. The organic layer was then dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by silica gel column chromatography (AcOEt/hexane, 5:5, then 100% AcOEt) to give Fmoc-L-Orn(Boc)-AMC as a slightly yellow solid (5.84 g, 83%): R_f 0.22 (AcOEt/hexane, 5:5); *m/z* (ES+) 612.44 $[M + H]^+$, 556.12 $[M - tBu + H]^+$; HPLC t_R(A) 3.58 min; ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.80 (d, J = 7.4 Hz, 2H), 7.70 (s, 1H), 7.62 (d, J = 7.2 Hz, 2H), 7.60−7.50 (m, 2H), 7.40 (t, J = 7.2 Hz, 2H), 7.32 (t, J = 7.3 Hz, 2H), 6.20 (s, 1H), 5.78 (d, 1H), 4.80−4.60 (m, 1H), 4.50−4.35 (m, 2H), 4.20 (t, J = 6.8 Hz, 1H), 3.60−3.00 (m, 2H), 2.40 (s, 3H), 1.90−1.55 (m, 4H), 1.49 (s, 9H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.8, 160.9, 157.0, 156.5, 154.5, 153.9, 144.7, 144.6, 143.1, 141.6, 128.5, 127.9, 126.8, 126.2, 121.0, 116.1, 116.0, 113.2, 106.6, 78.3, 66.6, 56.3, 47.5, 30.0, 29.1, 27.2, 18.8.

The preceding Boc-protected compound (5.8 g, 9.5 mmol) was dissolved in TFA/DCM (50:50, 56 mL), and the mixture was stirred for 35 min at room temperature. After this period, the solvents were removed under reduced pressure, and the resulting residue was taken up in diethyl ether. Compound **11** precipitated as a yellow powder which was collected by filtration (6.42 g, 100%): *m/z* (ES+) 512.1 $[M + H]^+$; HRMS (FAB) calcd for $C_{30}H_{30}N_3O_5$ 512.2185 $[M + H]^+$, found 512.2191; HPLC t_R(A) 2.61 min; ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.90 (d, J = 7.5 Hz, 2 H), 7.85 (s, 1 H), 7.80 (d, J = 7.4 Hz, 2 H), 7.70−7.60 (m, 2H), 7.50−7.40 (m, 2 H), 7.40 (t, 2 H), 6.30 (s, 1 H), 4.40−4.30 (m, 3H), 4.25−4.15 (m, 1H), 2.90−2.70 (m, 2H), 2.40 (s, 3H), 1.90−1.50 (m, 4H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 172.4, 160.9, 157.0, 154.5, 154.0, 144.7, 144.6, 143.0, 141.6, 128.5, 127.9, 126.9, 126.2, 121.0, 116.2, 116.0, 113.3, 106.6, 66.6, 55.9, 47.5, 29.4, 24.7, 18.8.

(2*S*)-*N*-(4-Methyl-2-oxo-2*H*-benzopyran-7-yl)-2-[*N*-(9-fluorenylmethyloxycarbonyl)amino]-5-isothiocyanatopentanamide (Fmoc-L-Orn(N^δ=C=S)-AMC) (12). To a solution of compound **11** (6.38 g, 10.2 mmol) in THF (50 mL) were added TEA (5.03 mL, 35.7 mmol) and CS_2 (5.4 mL, 89.4 mmol) at 0 °C. The reaction was stirred for 45 min at this temperature, and 12.2 mL (107.2 mmol) of H_2O_2 was added dropwise. The mixture was then diluted with diethyl ether (100 mL). The organic layer was washed with 1 M aqueous $KHSO_4$ (2×), H_2O (1×), and brine (1×) and dried over $MgSO_4$. Concentration in vacuo afforded **12**, which was recrystallized from AcOEt/hexane (5:5). White powder (5.59 g, 98.8%): mp 156−158 °C; R_f 0.26 (AcOEt/hexane, 5:5); *m/z* (ES+) 554.2 $[M + H]^+$; HRMS (FAB) calcd for $C_{31}H_{28}N_3O_5S$ 554.1750 $[M + H]^+$, found 554.1735; HPLC t_R(A) 3.45 min; ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.90 (d, J = 7.5 Hz, 2 H), 7.85 (s, 1 H), 7.80 (d, J = 7.4 Hz, 2 H), 7.75−7.70 (m, 2H), 7.50−7.40 (m, 2 H), 7.40 (t, J = 7.4 Hz, 2H), 6.25 (s, 1 H), 4.40−4.30 (m, 3H), 4.21 (t, J = 6.5 Hz, 1H), 3.65 (t, J = 5.9 Hz, 2H), 2.40 (s, 3H), 1.90−1.50 (m, 4H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 172.3, 160.9, 157.0, 154.5, 153.9, 144.7, 144.6, 143.0, 141.6, 128.5, 127.9, 126.8, 126.2, 121.0, 116.2, 116.1, 113.3, 106.6, 66.6, 55.8, 47.5, 45.3, 29.6, 26.9, 18.8.

Resin Fmoc-Tci(*N*ω-Rink)-AMC (13). Compound **12** (5.56 g, 10 mmol, 3 equiv) was dissolved in THF (75 mL) in the presence of TEA (1.46 mL, 3.1 equiv) and was reacted with deprotected Rink amide resin (4.78 g, 0.7 mmol/g substitution) to afford resin **13** as described for resin **2**. Completion of the reaction was assessed using the TNBS test. The dried resin was separated in several portions, each one being used for the synthesis of a fluorogenic substrate.

General Procedure for the Synthesis of Fluorogenic Substrates 17a−g. Resin **13** was deprotected and peptide assembly was accomplished as described, affording thiocitrulline-containing peptide-resins **14a−g**. *S*-Methylation of Tci was then performed as described for resin **4** using 20 mL of a 0.2 M CH_3I solution in DMF, giving *S*-methylisothiocitrulline-containing peptide-resins **15a−g**. Guanidinylation was carried out as already described, using 10 mL of a 2 M ammonium acetate solution in DMSO, providing arginine-containing peptide-resins **16a−g**. Finally, deprotection and removal of the peptides from the resins were performed as described, yielding the crude substrates **17a−g**, which were purified by preparative RP-HPLC. Details for compound **17a** are given as an example.

Ac-Val-Gly-Leu-Arg-AMC (17a). Starting from 530 mg of resin **13**, 52 mg (23%) of purified compound **17a** (>95% pure according to RP-HPLC analysis) was obtained: *m/z* (ES+) 643.5 $[M + H]^+$; HRMS (FAB) calcd for $C_{31}H_{47}N_8O_7$ 643.3568 $[M + H]^+$, found 643.3525; HPLC t_R(A) 2.0 min; t_R(B) 17.94 min; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.30 (t, 1H), 8.25 (d, J = 7.1 Hz, 1H), 8.00 (d, 1H), 7.90 (d, J = 7.0 Hz, 1H), 7.80 (s, 1H), 7.72 (d, 1H), 7.60 (t, J = 5.6 Hz, 1H), 7.50 (d, 1H), 7.40−6.80 (br, 3H), 6.30 (s, 1H), 4.50−4.30 (m, 2H), 4.05 (t, J = 7.4 Hz, 1H), 3.80−3.65 (m, 2H), 3.20−3.10 (m, 2H), 2.40 (s, 3H), 2.00−1.90 (m, 1H), 1.90 (s, 3H), 1.85−1.75 (m, 1H), 1.75−1.40 (m, 6H), 0.95−0.75 (m, 12H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.1, 172.7, 171.9, 170.7, 169.8, 160.9, 157.5, 154.5, 154.0, 142.9, 126.9, 116.1, 116.0, 113.3, 106.6, 59.4, 54.2, 51.9, 42.9, 30.7, 29.7, 26.1, 24.9, 24.0, 23.3, 22.3, 20.0, 19.2, 18.9.

Ac-Val-Gly-Leu-Arg(*N*ω-Me)-NH_2 (18). A portion (500 mg) of the Itc(*S*-Me)-containing peptide-resin **8** was treated with 20 mL of a 2 M methylamine, HCl solution in DMSO containing 2 M *N*-methylmorpholine. The suspension was stirred in a sealed tube at 80 °C for 12 h. The resin was then washed with DCM (3×), DMF (3×), and DCM (3×), dried, and cleaved as already described, affording peptide **18** in 35% crude yield (62 mg, approximately 90% pure according to RP-HPLC analysis): *m/z* (ES+) 499.3 $[M + H]^+$; HRMS (FAB) calcd for $C_{22}H_{43}N_8O_5$ 499.3356 $[M + H]^+$, found 499.3373; HPLC t_R(A)

1.39 min; t_R(B) 10.75 min; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.10 (t, J = 5.6 Hz, 1H), 7.80 (d, J = 7.8 Hz, 1H), 7.70 (d, J = 8.1 Hz, 1H, 7.65 (d, J = 7.9 Hz, 1H), 7.40−7.10 (m, 3H), 7.00 (s, 1H), 6.80 (s, 1H), 4.15−4.00 (m, 1H), 4.00−3.90 (m, 1H), 3.80 (t, J = 7.3 Hz, 1H), 3.60−3.40 (m, 2H), 3.00−2.80 (m, 2H), 2.30 (s, 3H), 1.80−1.70 (m, 1H), 1.65 (s, 3H), 1.60−1.40 (m, 1H), 1.35−1.15 (m, 6H), 0.80−0.50 (m, 12H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.1, 172.7, 170.8, 169.8, 157.1, 59.5, 52.9, 52.0, 42.9, 30.7, 29.8, 28.8, 26.0, 24.9, 24.0, 23.3, 22.3, 20.0, 19.2.

Ac-Val-Gly-Leu-Arg(*N*ω-Et)-NH$_2$ (19). Compound **19** was prepared from 500 mg of resin **8** as described for compound **18**, by treatment with a 2.5 M ethylamine, HCl solution in DMSO containing 2.5 M *N*-methylmorpholine at 80 °C for 17 h. Resin cleavage afforded 103 mg (56% crude yield, approximately 83% pure according to RP-HPLC analysis) of compound **19**: *m/z* (ES+) 513.4 $[M + H]^+$; HRMS (FAB) calcd for $C_{23}H_{45}N_8O_5$ 513.3513 $[M + H]^+$, found 513.3510; HPLC t_R(A) 1.45 min; t_R(B) 11.14 min; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.30 (t, J = 6.0 Hz, 1H), 8.05 (d, J = 7.7 Hz, 1H), 7.95 (d, J = 8.1 Hz, 1H), 7.85 (d, J = 7.9 Hz, 1H), 7.40−7.30 (m, 3H), 7.25 (s, 1H), 7.10 (s, 1H), 4.40−4.30 (m, 1H), 4.28−4.15 (m, 1H), 4.10 (t, J = 7.3 Hz, 1H), 3.85−3.70 (m, 2H), 3.25−3.10 (m, 4H), 2.10−2.00 (m, 1H), 1.95 (s, 3H), 1.80−1.70 (m, 1H), 1.65−1.45 (m, 6H), 1.15 (t, J = 7.2 Hz, 3H), 1.00−0.85 (m, 12H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.1, 172.7, 170.8, 169.7, 156.2, 59.5, 52.9, 52.0, 42.9, 36.7, 30.7, 29.9, 28.8, 26.0, 24.9, 24.0, 23.3, 22.3, 20.0, 19.2, 15.1.

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Supporting Information Available: General Experimental Paragraph. Experimental details and physicochemical characteristics for the fluorogenic substrates, compounds **17b**−**g**. Copies of analytical reversed-phase HPLC traces of compounds **3**, **5**, **6** (including coelution profile with its diastereoisomer Ac-D-Phe-Arg-NH$_2$), **10**, **17a**−**g**, **18**, and **19**. Copies of ^{1}H NMR spectra of Fmoc-L-Orn(Boc)-NH$_2$, Fmoc-L-Orn-NH$_2$, **1**, **3**, **5**, **6**, **10**, Fmoc-L-Orn(Boc)-AMC, **11**, **12**, **17a**−**g**, **18**, and **19**. Copies of ^{13}C NMR spectra of Fmoc-L-Orn(Boc)-NH$_2$, Fmoc-L-Orn-NH$_2$, **1**, **3**, **6**, **10**, Fmoc-L-Orn(Boc)-AMC, **11**, **12**, **17a**−**g**, **18**, and **19**. This material is available free of charge via the Internet at http://pubs.acs.org.

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