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Solid phase synthesis of mono- or disubstituted arginine containing peptides from an isothiocitrulline precursor

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Abstract—A method for the solid phase synthesis of substituted arginine containing peptides starting from an isothiocitrulline precursor is described. In this procedure, a peptide containing one or more protected ornithine residue(s) was assembled on a solid support. Following selective deprotection, ornithine residue(s) was (were) converted into *S*-methyl-isothiocitrulline in three steps. Subsequent reaction with primary or secondary amines afforded mono and disubstituted arginine-containing derivatives, respectively. Using lysine instead of ornithine afforded substituted homoarginine-containing derivatives.

1. Introduction

L-Arginine is a unique cationic ion-pairing amino acid containing a guanidino group, which is involved in several interactions inducing important physiological and pathophysiological processes. For instance, arginine is the preferred residue in P1 position[1] of substrates for numerous serine proteases (trypsin, thrombin and others proteases of the coagulation cascade). It is also an essential residue in the integrin recognition sequence Arg-Gly-Asp, and as a unique amino acid it is the substrate of NO synthases (NOS) for the production of NO. Arginine-rich sequences found in some proteins are able to cross biological membranes[2] and synthetic arginine oligomers were shown to be very efficient transporters across these barriers.[3–5]

Although the guanidino group of arginine is the optimal functional group in most of the interactions stated above, the use of arginine in the design of compounds with therapeutic interest is precluded. Indeed, its strongly basic character limits oral bioavailability. Furthermore, arginine-containing peptides are prone to degradation by trypsin-like proteases shortening their in vivo half-life. Significant efforts have been focused on the design and synthesis of arginine mimetics with reduced basicity and/or higher lipophilicity.[6] Among the diverse modifications reported for the synthesis of arginine mimetics, there are *N*ω-alkyl-arginines.[7] Their incorporation into peptides or protease inhibitors was shown to confer increased binding affinity, resistance to proteases, lipophilicity (including blood–brain barrier partitioning) and selectivity.[7–11] *N*ω-alkyl-arginines (*N*ω-Me, -Et, -Pr, -allyl,...) were also shown to behave as NOS inhibitors.[12–16] Numerous proteins (for instance, nucleolin) from human cells have been found to have methylated Arg residues, the predominant form being *N*ω,*N*ω-dimethylarginine, and synthesis of fragments containing this residue is of interest.[17] Another example is the recent report of mono- and dialkylated derivatives of an arginine analogue as inhibitors of dimethylarginine dimethylamino hydrolase, an enzyme involved in the degradation of endogenous methylated arginine.[18] *N*ω-mono- and dialkylated arginine analogues are generally prepared through guanidinylation of ornithine with the corresponding substituted *S*-methyl-isothiourea salts[6,19] or 1*H*-pyrazole-*N*-alkyl-1-carboxamidines,[20–22] or from thiocitrulline.[23] They can be produced separately as protected derivatives and then introduced into a peptide sequence.[19,23] A more interesting approach is to achieve guanidinylation after assembly of ornithine-containing precursors, either in

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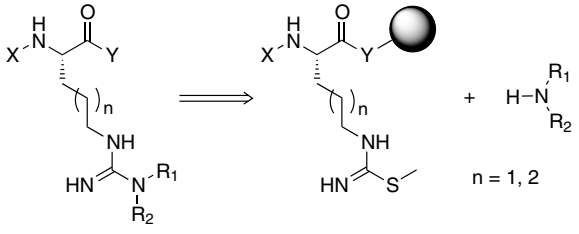
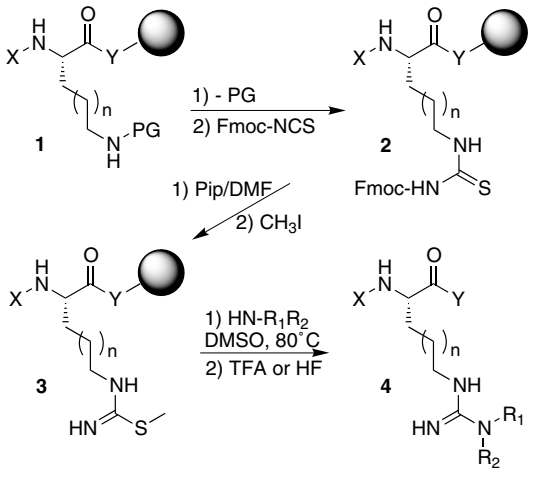
solution[24] or more interestingly on solid supports.[21] One advantage of this last method is the possibility to prepare diversely substituted derivatives in one step from a unique precursor. However, these strategies require availability and synthesis of a guanidinylating agent for each desired substitution. To avoid this limitation, we developed a simple and efficient solid-phase strategy for the synthesis of *N*ω-mono- and di-alkylated-arginine-containing compounds in which guanidinylation was performed through reaction of a S-methyl-isothiocitrulline (Itc)-containing precursor with a series of primary and secondary amines. Obviously, this method applies also to the synthesis of *N*ω-alkyl-homoarginine derivatives (Scheme 1).

2. Synthesis[25]

The general procedure employed for synthesizing *N*ω-alkyl-arginine- or homoarginine-containing compounds (**4**, $n = 1, 2$) is described in Scheme 2. The ornithine- or lysine-containing compound (**1**, $n = 1, 2$) was first assembled on a solid support. The ornithine or lysine protecting group, orthogonal to other side-chain protections and to the resin handle was removed. When a Fmoc strategy was followed, we used either Mtt or Alloc protection for the side chain amino group of ornithine or lysine. Mtt was eliminated using a 2% solution of TFA in DCM by two minute cycles.[26] When an Alloc group was used, it was released using a mixture of $Pd[PPh_3]_4$ (0.3 equiv) and phenylsilane (24 equiv) in DCM. In case of a Boc strategy, we used a Fmoc protection for the side-chain amino function of ornithine or lysine, which was removed by a 20% piperidine solution in DMF. Reaction of the free side-chain amine with Fmoc–isothiocyanate (Fmoc–NCS)[27–29] afforded the polymer-bound protected thiocitrulline- or homothiocitrulline-containing compound **2**. Completion of the reaction was established by a negative TNBS test for free amines. After Fmoc removal, the resin maintained a negative response to TNBS indicating that carbamate formation did not occur during the reaction between Fmoc–NCS and the side-chain amino group.[30] The *S*-methyl-isothiocitrulline or -homoisothiocitrulline derivative **3** ($n = 1, 2$) was then generated after treatment with methyl iodide (0.2 M in DMF, 1 h) at room temperature (three times). Finally, resin **3** was separated in several portions, which were reacted with different amines (2 M in dry DMSO[31]) by heating in a sealed tube for 16 h affording after TFA (Fmoc chemistry) or HF (Boc chemistry) treatment, *N*ω-alkylated arginine- or homoarginine-containing analogues (**4**, $n = 1, 2$).



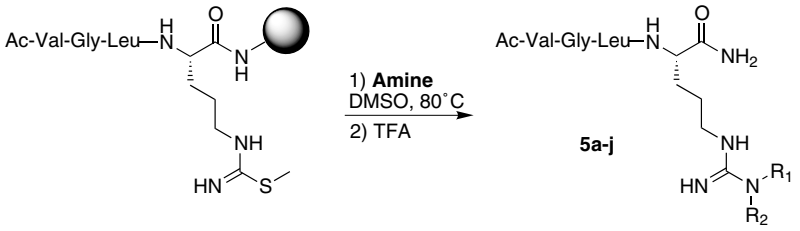
Scheme 1.



Scheme 2. General synthetic scheme for the preparation of substituted arginine- or homoarginine-containing peptides. PG = protecting group; $n = 1, 2$. X, Y = peptide segments.

As a first example, we present the synthesis of a series of tetrapeptides derived from a substrate (positions P4–P1, Val-Gly-Leu-Arg) of Pfgp76, a *Plasmodium falciparum* serine protease isolated from merozoites (Table 1).[32] The peptide Ac-Val-Gly-Leu-Orn(*N*δ-Mtt) was first built on a Rink amide resin using Fmoc chemistry and HBTU as coupling agent. The ornithine residue was then converted into thiocitrulline as described above. Cleavage of a small portion of resin at this stage followed by HPLC and mass spectrometry analyses of the crude showed that the expected compound was obtained in more than 90% purity and without significant presence of unreacted ornithine-containing peptide (i.e., Ac-Val-Gly-Leu-Orn-NH_2). The *S*-methyl-Itc derivative was generated as described above and cleavage of a small portion afforded the expected compound in more than 90% purity. Indeed, analyses showed that *S*-methylation was complete and no compound bearing an undesired extra-methylation was significantly formed. The *S*-methyl-Itc derivative was then reacted with a series of primary and secondary amines (acyclic or cyclic). Cleavage from the resin using a mixture of TFA/TIS/H_2O (95:2.5:2.5) released the expected compounds **5a**–**j** (Table 1).[33] It was interesting to also obtain from the same precursor, the arginine-containing peptide **5a** using ammonium acetate. By reverse phase HPLC and ^1H NMR analyses, **5a** was shown to be identical to an authentic sample synthesized following standard procedures. Yields of crude peptides were generally above 65% with an average purity of 80% (yields above 40% after purification by preparative reverse phase HPLC, Table 1) with the exception of **5e** (R_1 = isobutyl), which was produced with lower purity. Reverse phase HPLC and mass spectrometry analyses of crude peptides showed that the *S*-methyl-Itc intermediate was completely consumed during guanidinylation. The lower purity observed for compound **5e** was mainly due to the significant appearance of a side product with a mass of 485.4 corresponding to the conversion of *S*-methyl-Itc to citrulline. Although dry DMSO and redistilled isobutylamine were used, this side product could

Table 1. Preparation of compounds **5a–j** using Fmoc strategy



Compound	Amine[a]	R_1	R_2	Mass[b]	HPLC t_R[c] (min)	Yield[d]
5a	Ammonium acetate	H	H	485.4	1.19	45
5b	Methylamine	Me	H	499.4	1.18	54
5c	Propylamine	Pr	H	527.4	1.38	48
5d	Methoxyethylamine	MeOEt	H	543.4	1.28	40
5e	Isobutylamine	*i*Bu	H	541.4	1.55	25
5f	Dimethylamine	Me	Me	513.4	1.25	55
5g	Diethylamine	Et	Et	541.4	1.45	50
5h	Piperidine	$-(CH_2)_5-$		553.4	1.40	39
5i	Morpholine	$-(CH_2)_2-O-(CH_2)_2-$		555.3	1.26	41
5j	*N*-Methyl-piperazine	$-(CH_2)_2-N(Me)-(CH_2)_2-$		568.4	1.10	40

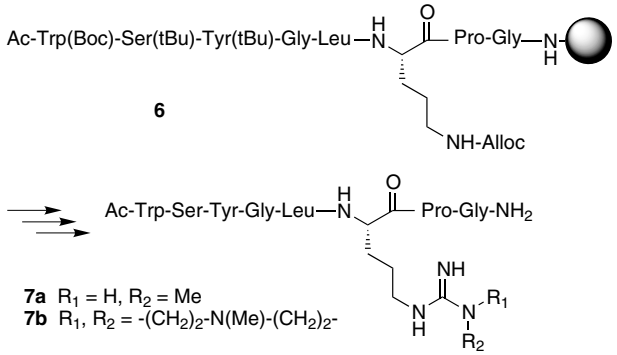
[a] Amine used for guanidinylation.
[b] $M+H^+$, measured by electrospray mass spectrometry.
[c] Reverse phase HPLC analyses run on a Chromolith SpeedRod C18 column (0.46 × 5 cm); gradient from A to B in 5 min, 3 mL/min flow rate (A: H_2O, 0.1% TFA; B: CH_3CN, 0.1% TFA).
[d] Overall % yield of HPLC purified compounds calculated from the loading of the starting resin.

not be eliminated, arising from the presence of remaining traces of water, which competed with the poorly reactive isobutylamine for reacting with the carbodiimide intermediate.

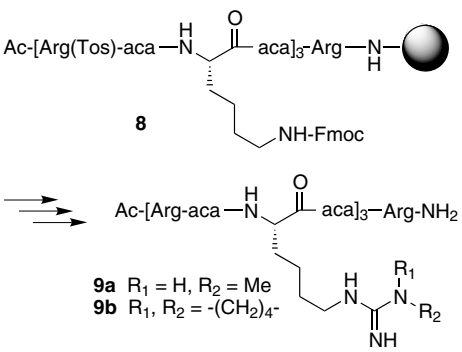
This strategy was also used for the synthesis of analogues of a GnRH fragment, where the arginine residue has been replaced by various *N*ω-alkylated residues (Scheme 3). The supported protected peptide **6** was first assembled on a Rink amide resin using Fmoc chemistry. The *S*-methyl-Itc derivative was then prepared as described before and reacted with methylamine and *N*-methyl-piperazine, affording compounds **7a–b** in 49% and 40% yield after reverse-phase HPLC purification, respectively.[34]

The last presented illustration of this method was the synthesis of analogues of an arginine-rich transporter, Ac-(Arg-aca)$_6$-Arg-NH$_2$ (Scheme 4).[35] In this example, three out of seven arginines were replaced by *N*ω-alkylated analogues using lysine instead of ornithine as starting material.[36] The polymer-bound peptide **8** was built on an MBHA resin using Boc chemistry. Guanidinylation of the three lysyl residues was then achieved simultaneously as already described. Specifically, formation of the thioureas (homothiocitrulline residues) was achieved using 3 equiv of Fmoc–NCS per lysyl residue. Subsequent removal of the Fmoc group and treatment with methyl iodide afforded the *S*-methyl-homoItc precursor, which was reacted with methylamine or pyrrolidine. HF cleavage in the presence of 10% anisole as a scavenger released the expected peptides **9a–b** in 60% (75% pure[37]) and 55% (90% pure, Fig. 1) crude yield, respectively.[38]

The last two steps of guanidinylation of the synthetic sequence (i.e. treatment with methyl iodide and amines) were found to be compatible with the protected



Scheme 3. Synthesis of analogues of a GnRH fragment (Ac-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH$_2$) containing an *N*ω-alkylated arginine.



Scheme 4. Synthesis of analogues of an Arg-rich transporter containing three *N*ω-alkylated homoarginines. aca = 6-aminocaproyl.

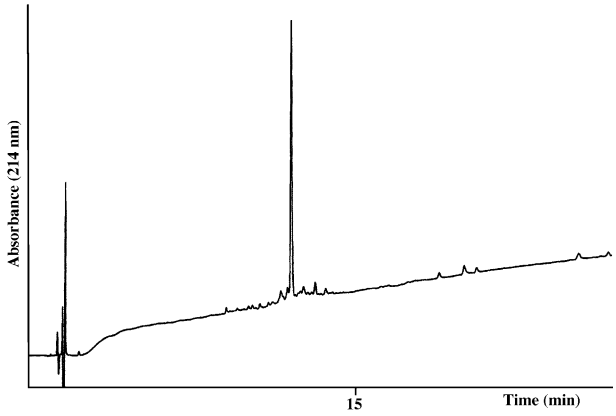


Figure 1. Reverse phase HPLC profile of crude compound **9b**.[39]

tri-functional amino acids (Arg(Tos), Trp(Boc), Ser(*t*Bu) and Tyr(*t*Bu)). We also checked that protected residues such as Arg(Pbf), Glu(O*t*Bu), Lys(Boc), Thr(*t*Bu), Cys(Trt) were not modified in these reaction conditions. However, the use of methionine is problematic since it probably would be *S*-methylated by methyl iodide. In methionine containing sequences, the methionine residue should be replaced by the isosteric norleucine residue as it is often done, given its propensity to oxidation. If required, methionine can be introduced within the sequence as its sulfoxide derivative, which can be easily reduced later on.

3. Conclusion

Although this strategy was not readily suitable for some amines (i.e., primary amines with long alkyl chains or hindered secondary amines[40]), it allowed to produce the unsubstituted arginine-containing peptide as well as a large diversity of *N*ω-alkyl arginine analogues from a unique *S*-methyl-isothiocitrulline precursor. This easily obtained diversity is useful to study the structure–activity relationships of arginine-containing peptides.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2005.08.120.

References and notes

1. Nomenclature of substrate residues from: Schechter, I.; Berger, A. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157–162.
2. Futaki, S.; Goto, S.; Sugiura, Y. *J. Mol. Recognit.* **2003**, *16*, 260–264.
3. Mitchell, D. J.; Kim, D. T.; Steinman, L.; Fathman, C. G.; Rothbard, J. B. *J. Pept. Res.* **2000**, *56*, 318–325.
4. Wender, P. A.; Mitchell, D. J.; Pattabiraman, K.; Pelkey, E. T.; Steinman, L.; Rothbard, J. B. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 13003–13008.
5. Futaki, S.; Suzuki, T.; Ohashi, W.; Yagami, T.; Tanaka, S.; Ueda, K.; Sugiura, Y. *J. Biol. Chem.* **2001**, *276*, 5836–5840.
6. For a review, see Peterlin-Masic, L.; Kikelj, D. *Tetrahedron* **2001**, *57*, 7073–7105.
7. Kennedy, K. J.; Lundquist, J. T., IV; Simandan, T. L.; Kokko, K. P.; Beeson, C. C.; Dix, T. A. *J. Pept. Res.* **2000**, *55*, 348–358.
8. Kennedy, K. J.; Orwig, K. S.; Dix, T. A.; Christopher, J.; Jaffa, A. A. *J. Pept. Res.* **2002**, *59*, 139–148.
9. Lundquist, J. T., IV; Büllesbach, E. E.; Golden, P. L.; Dix, T. A. *J. Pept. Res.* **2002**, *59*, 55–61.
10. Kokko, K. P.; Kyle Hadden, M.; Orwig, K. S.; Mazella, J.; Dix, T. A. *J. Med. Chem.* **2003**, *46*, 4141–4148.
11. Kokko, K. P.; Arrigoni, C. E.; Dix, T. A. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1947–1950.
12. Sakuma, I.; Stuehr, D. J.; Gross, S. S.; Nathan, C.; Levi, R. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 8664–8667.
13. Olken, N. M.; Marletta, M. A. *J. Med. Chem.* **1992**, *35*, 1137–1144.
14. Olken, N. M.; Marletta, M. A. *Biochemistry* **1993**, *32*, 9677–9685.
15. 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.
16. Zhang, H. Q.; Fast, W.; Marletta, M. A.; Martasek, P.; Silverman, R. B. *J. Med. Chem.* **1997**, *40*, 3869–3870.
17. Zahariev, S.; Guarnaccia, C.; Zanuttin, F.; Pintar, A.; Esposito, G.; Maravic, G.; Krust, B.; Hovanessian, A. G.; Pongor, S. *J. Pept. Sci.* **2005**, *11*, 17–28.
18. Rossiter, S.; Smith, C. L.; Malaki, M.; Nandi, M.; Gill, H.; Leiper, J. M.; Vallance, P.; Selwood, D. L. *J. Med. Chem.* **2005**, *48*, 4670–4678.
19. Kennedy, K. J.; Simandan, T. L.; Dix, T. A. *Synth. Commun.* **1998**, *28*, 741–746.
20. Bernatowicz, M. S.; Matsueda, G. R. *Synth. Commun.* **1993**, *23*, 675–676.
21. Lee, Y.; Silverman, R. B. *Synthesis* **1999**, 1495–1499.
22. Lee, Y.; Martasek, P.; Roman, L. J.; Silverman, R. B. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2771–2774.
23. Székely, Z.; Zakhariev, S.; Guarnaccia, C.; Antcheva, N.; Pongor, S. *Tetrahedron Lett.* **1999**, *40*, 4439–4442.
24. Rothbard, J. B.; Jessop, T. C.; Lewis, R. S.; Murray, B. A.; Wender, P. A. *J. Am. Chem. Soc.* **2004**, *126*, 9506–9507.
25. Abbreviations of some reagents and protecting groups: aca, 6-aminocaproyl; Alloc, allyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; *t*Bu, *tert*-butyl; Fmoc, fluorenyl-methyloxycarbonyl; HBTU, *N*-[(1*H*-benzotriazol-1-yloxy)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate; Itc, isothiocitrulline; Mtt, 4-methyltrityl; $Pd[PPh_3]_4$, tetrakis(triphenylphosphine)palladium(0); Pip, piperidine; TFA, trifluoroacetic acid; TIS, triisopropylsilane; TNBS, 2,4,6-trinitrobenzene sulfonic acid; Tos, tosyl; Trt, trityl.
26. Completion of deprotection can be assessed by reverse phase HPLC detection of released Mtt; 5–10 two minute cycles were generally sufficient.
27. Kearney, P. C.; Fernandez, M.; Flygare, J. A. *Tetrahedron Lett.* **1998**, *39*, 2663–2666.
28. Kearney, P. C.; Fernandez, M.; Flygare, J. A. *J. Org. Chem.* **1998**, *63*, 196–200.

29. Linton, B. R.; Carr, A. J.; Orner, B. P.; Hamilton, A. D. *J. Org. Chem.* **2000**, *65*, 1566–1568.
30. Undesired Fmoc protection could arise from attack of the side-chain amino group at the less electrophilic carbonyl of Fmoc–NCS, see Ref. 28.
31. Dry DMSO has to be used to avoid the production of more or less significant amounts of citrulline, depending on the amine reactivity.
32. Roggwiller, E.; Morales Bétoulle, M. E.; Blisnick, T.; Braun Breton, C. *Mol. Biochem. Parasitol.* **1996**, *82*, 13–24.
33. Characterization of selected compounds. Compound **5e**: *m/z* (ES+), 541.4 $[M+H]^+$; HRMS (FAB) calcd for $C_{25}H_{49}N_8O_5$ 541.3826 $[M+H]^+$, found 541.3842; HPLC t_R: 14.38 min;[39] ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.27 (m, 1H), 7.95 (d, $J = 7.6$ Hz, 1H), 7.86 (d, $J = 8.1$ Hz, 1H), 7.81 (d, $J = 7.9$ Hz, 1H), 7.51–7.27 (m, 3H), 7.17 (s, 1H), 7.03 (s, 1H), 4.25 (m, 1H), 4.14 (m, 1H), 4.00 (m, 1H), 3.68 (m, 2H), 3.10 (m, 2H), 2.92 (m, 2H), 2.05–1.25 (m, 9H+3H), 1.05–0.56 (m, 18H). Compound **5j**: *m/z* (ES+), 568.4 $[M+H]^+$, 284.9 $[M+2H]^{2+}/2$; HRMS (FAB) calcd for $C_{26}H_{50}N_9O_5$ 568.3935 $[M+H]^+$, found 568.3939; HPLC t_R: 9.93 min;[39] ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.28 (m, 1H), 7.98–7.90 (m, 4H), 7.83 (d, $J = 7.9$ Hz, 1H), 7.19 (s, 1H), 7.05 (s, 1H), 4.28 (m, 1H), 4.16 (m, 1H), 4.02 (m, 1H), 3.70 (m, 2H), 3.58–2.95 (m, 10H), 2.83 (s, 3H), 2.05–1.14 (m, 8H+3H), 0.93–0.62 (m, 12H).
34. Compound **7a**: *m/z* (ES+), 990.3 $[M+H]^+$, 495.6 $[M+2H]^{2+}/2$; HPLC t_R: 15.71 min;[39] ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.27–8.04 (m, 4H), 7.90 (d, $J = 7.6$ Hz, 1H), 7.83 (d, $J = 7.9$ Hz, 1H), 7.61 (d, $J = 7.8$ Hz, 1H), 7.41–6.93 (m, 11H), 6.64 (d, $J = 8.2$ Hz, 2H), 4.55–4.25 (m, 6H), 3.79–3.49 (m, 12H), 3.12–2.91 (m, 4H), 2.73 (d, $J = 4.3$ Hz, 3H), 2.23–1.25 (m, 9H+3H), 0.83 (m, 6H). Compound **7b**: *m/z* (ES+), 530.2 $[M+2H]^{2+}/2$; HPLC t_R: 15.03 min.[39]
35. Rothbard, J. B.; Kreider, E.; VanDeusen, C. L.; Wright, L.; Wylie, B. L.; Wender, P. A. *J. Med. Chem.* **2002**, *45*, 3612–3618.
36. Substituting arginine with homoarginine has no adverse effect on transporter efficiency. See Ref. 3.
37. Purity of the peptides was assessed by reverse-phase HPLC analysis using two sets of conditions.
38. Compound **9a**: *m/z* (ES+), 958.5 $[M+2H]^{2+}/2$, 639.5 $[M+3H]^{3+}/3$, 480.0 $[M+4H]^{4+}/4$, 384.2 $[M+5H]^{5+}/5$ (major), 320.5 $[M+6H]^{6+}/6$; HPLC t_R: 10.53 min;[39] ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.15–7.65 (m, 16H), 7.63–6.76 (m, 24H), 4.18 (m, 7H), 3.22–2.84 (m, 26H), 2.72 (d, $J = 4.6$ Hz, 3H), 2.10 (m, 12H), 1.84 (s, 3H), 1.76–0.73 (m, 70H). Compound **9b**: *m/z* (ES+), 1018.9 $[M+2H]^{2+}/2$, 679.6 $[M+3H]^{3+}/3$, 510.0 $[M+4H]^{4+}/4$, 408.3 $[M+5H]^{5+}/5$ (major), 340.4 $[M+6H]^{6+}/6$; HPLC t_R: 12.18 min;[39] ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.15–7.57 (m,14H), 7.55–6.71 (m, 23H), 4.14 (m, 7H), 3.27 (m, 12H), 3.16–2.75 (m, 26H), 2.07 (m, 12H), 1.86 (m, 12H), 1.81 (s, 3H), 1.71–0.85 (m, 70H).
39. Chromatographic conditions: C18 column, Waters Delta Pak 5 μ, 3.9×150 mm. Solvents A, 0.1% TFA/H_2O; B, 0.1% TFA/CH_3CN. Gradient, 5–60% B in A in 30 min. Flow rate, 1 mL/min.
40. For instance, use of diisopropylamine produced the expected compound in low yield (17% after purification).