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Article

Extended Solution-phase Peptide Synthesis Strategy Using Isostearyl-Mixed Anhydride Coupling and a New C-Terminal Silyl Ester-Protecting Group for *N*-Methylated Cyclic Peptide Production

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ABSTRACT: Herein, we present a new and efficient convergent solution-phase synthetic strategy for producing peptides containing *N*-methyl amino acids. Specifically, we have synthesized a model cyclic octapeptide with two *N*-methyl amino acids, utilizing an isostearyl-mixed anhydride coupling methodology and a novel silyl ester-protecting group, cyclohexyl di-*tert*-butyl silyl (cHBS). This newly developed method uses an isostearic acid chloride (ISTA-Cl) and silylation reagent that allows coupling between N- and C-terminally unprotected amino acids with sterically hindered *N*-methyl amino acids. High yields of four dipeptide fragments are efficiently synthesized by omitting the traditional C-terminal deprotection step. The silyl ester-protecting group at the C-terminus is stable during general peptide synthesis, and is selectively cleaved by fluoride ions. This group further suppresses diketopiperazine formation during the deprotection of the N^{α}-amino-protecting group. The linear octapeptide precursor is convergently synthesized utilizing protection and selective deprotection of the silyl ester-protecting group, and the cyclic octapeptide can be obtained with high purity using this novel methodology, via a route shorter than conventional solution-phase peptide synthesis strategies.

KEYWORDS: *liquid-phase peptide synthesis, solution-phase peptide synthesis, silyl, isostearyl-mixed anhydrides, cHBS*

INTRODUCTION

Discovering candidate peptides using in vitro display libraries,[1−3] multiple peptide loop cyclization,[4,5] or cell-penetrating lipopeptides[6,7] in addition to conventional peptide drug discovery approaches, such as using endogenous peptides[8−10] or phage display,[11,12] has accelerated the pace of peptide drug discovery. The new methodologies for identifying novel peptides composed of non-canonical amino acids allow for the creation of cyclic peptide libraries with significantly greater structural diversity, compared to those previously studied.[13,14] In particular, the strategy of incorporation of *N*-methyl amino acids can be utilized to improve cell membrane permeability and protease resistance.[15] However, the synthesis of peptides containing *N*-methyl amino acids is difficult due to steric hindrance, which represents a significant obstacle in peptide drug research and development.[16−18] Solid-phase peptide synthesis (SPPS) has been widely used for long-chain peptide manufacturing and, more recently, for the synthesis of short-chain peptides.[19−21] Although SPPS is straightforward and rapid, it is not considered economically or environmentally favorable (green chemistry) due to its excessive use of reaction reagents and washing solvents.[22−24] However, convergent solution-phase (liquid-phase) peptide synthesis (LPPS) is suitable for producing short to moderate length peptide-active pharmaceutical ingredients (APIs) as it allows for the purification of intermediates by crystallization and facilitates scale-up production. LPPS also has a better green chemistry profile than SPPS as the reagent and solvent requirements can be minimized.[25] As such, we have developed a new coupling method using isostearic acid halide (ISTA-X) and silylation agents,[26] by which N- and C-terminal unprotected amino acids can be introduced without double insertion by a temporary protecting effect of the carboxyl functionality by the silyl group (Scheme 1).

Generally, prolonged reaction times caused by the low reactivity of *N*-methyl amino acids increases the risk of epimerization; however, this method allows for the rapid coupling of *N*-methyl amino acids without epimerization. It also enables N-to-C reverse elongation, which is in the opposite direction of the conventional C-to-N elongation method. Reverse elongation permits the rapid and environmentally friendly synthesis of peptide fragments for convergent LPPS in fewer steps due to elimination of the deprotection step.[25] In a previous study, we showed that tetrapeptides containing *N*-methyl amino acids could be efficiently synthesized without epimerization using this method.[26] One of the limitations of peptide fragment synthesis by convergent LPPS is C-terminal carboxy protection during peptide elongation. The protective groups must be selectively

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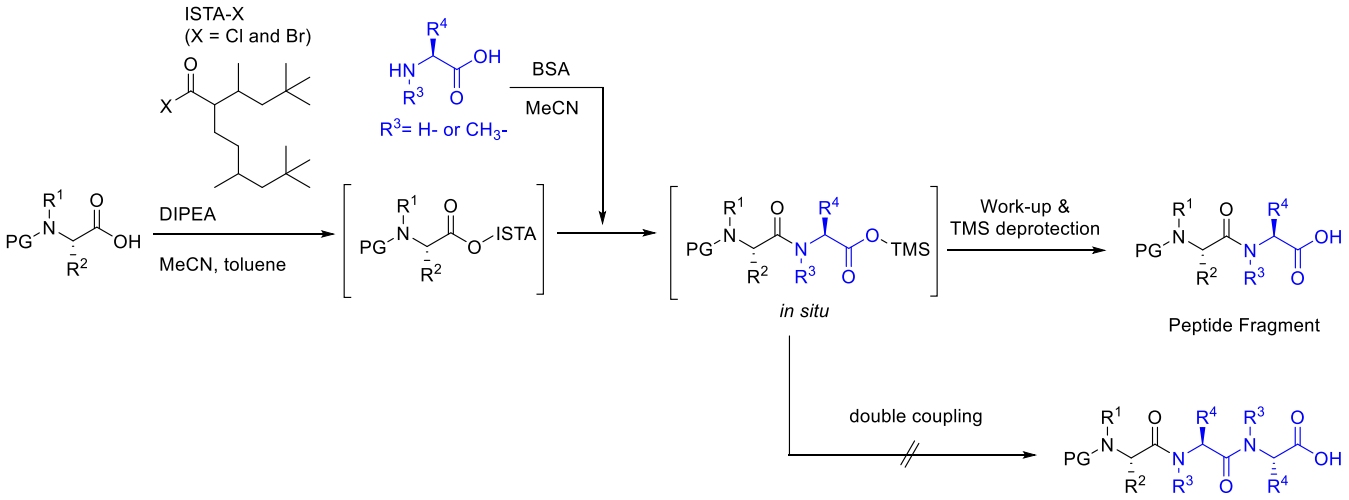
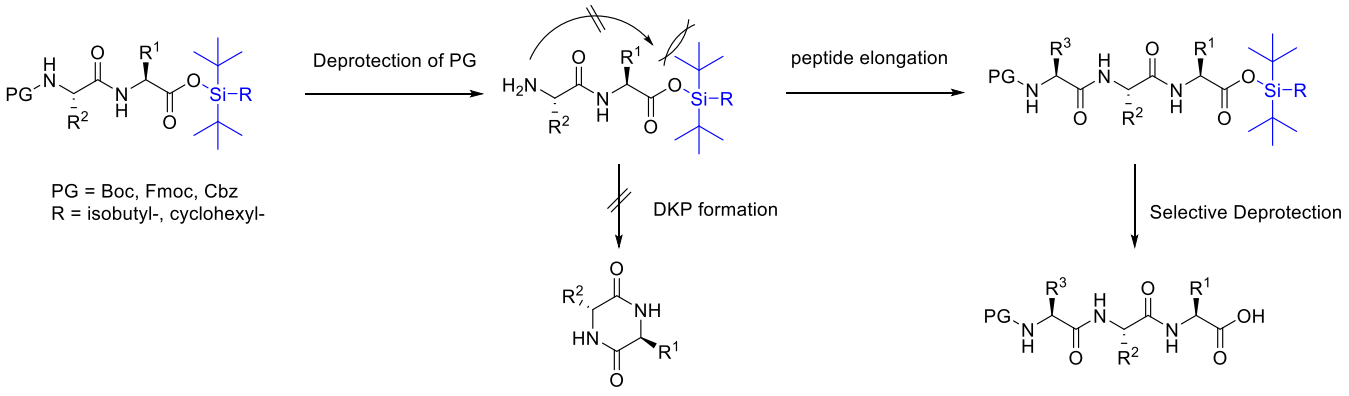
Scheme 1. Coupling Between N-Protected Amino Acids and N-, C-Terminal Unprotected Amino Acids With ISTA-X and Silylating Agents[a]



[a]BSA, *N,O-bis*(trimethylsilyl)acetamide; DIPEA, *N,N*-diisopropylethylamine; ISTA-X, isostearic acid halide; MeCN, acetonitrile; PG, protecting group; and TMS, trimethylsilyl.

Scheme 2. Peptide Elongation on the C-Terminal Silyl Ester-Protecting Group[a]



[a]DKP, diketopiperazine; PG, protecting group

deprotected over N^{α}- and side chain-protecting groups. The most frequently used methyl and ethyl esters are stable under all deprotection conditions used in standard Boc, Fmoc, and Cbz chemistry; however, the amino acids that can be incorporated at the C-terminus are limited as deprotection requires basic hydrolysis, which has a high risk of epimerization.[27] Although the development of new coupling reagents has helped to suppress epimerization during fragment condensation,[28] the epimerization risk during the basic deprotection step limits the fragmentation sites that can be used in synthetic strategies for peptide synthesis by convergent LPPS. Furthermore, there is a high risk of peptide cleavage by diketopiperazine (DKP) formation when the C-terminal amino acid is proline (Pro) or an *N*-methyl amino acid.[29,30] DKP structures can form under Fmoc deprotection conditions, even with natural amino acids other than Pro. Additionally, solubility issues can be limiting during solution-phase synthesis due to decreasing solubility of the protected peptide during long peptide chain construction. The decrease in solubility induces incomplete coupling/deprotection reactions and complicates separation and purification in postreaction workup. Although SPPS and tag-assisted LPPS have been utilized to significantly improve the solubility of the protected peptides,[31,32] these efforts have not resulted in enhanced purity of the peptidic intermediate in the workup process. To address this issue, we evaluated the use of silyl ester-type carboxyl-protecting groups as a C-terminal-protecting group.

Previously, the trimethylsilyl group has been used in peptide synthesis as an aqueous labile temporary protecting group of the carboxylic acid.[33,34] However, there are no examples of C-terminal carboxylic acid protection with a silyl ester during peptide elongation, due to instability under the peptide synthesis conditions. We hypothesized that a silyl ester with bulky hydrocarbon substituents would allow for enhanced stability under peptide synthesis conditions. Additionally, we expected that the steric bulk would prevent DKP formation and the high hydrophobicity of the bulky silyl group would result in improved overall solubility for the protected peptide fragment. The silyl ester could be cleaved by a fluorine source, such as potassium fluoride without affecting other protecting groups on the peptide (Scheme 2). These effects were expected to overcome the limitations of conventional C-to-N solution-phase synthesis, and in combination with N-to-C reverse elongation could be expected to greatly improve the diversity of synthetic strategies available for peptide synthesis.

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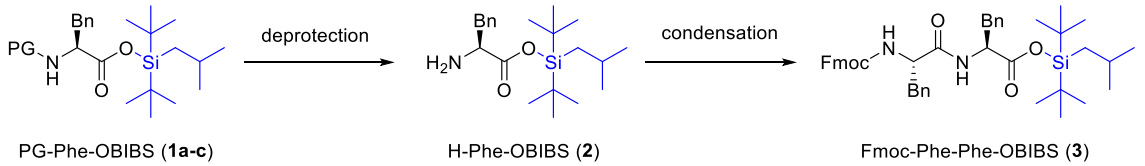
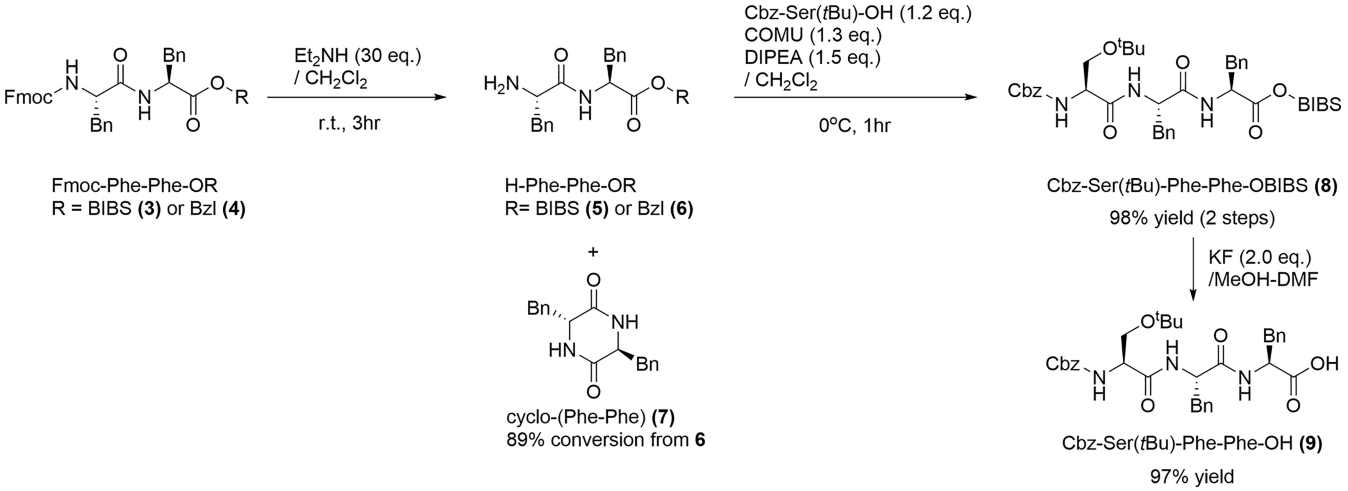
Table 1. Tests of BIBS Methodology Applied to Fmoc-, Boc-, and Cbz-Based Chemistry for the Synthesis of Dipeptide (3)[a]



		deprotection		condensation	
entry	PG-Phe-OBIBS (1)	condition	yield (%)	condition	yield (%)
1	PG = Fmoc (1a)	Et_2NH/CH_2Cl_2, room temperature, 6 h	98	Fmoc-Phe-OH (1.2 eq.) EDCI (1.3 eq.)/CH_2Cl_2, 0 °C, 3 h	95
2	PG = Boc (1b)	4 N HCl-dioxane/CH_2Cl_2, room temperature, 7 h	100		
3	PG = Cbz (1c)	Pd−C, H_2/TFE, room temperature, 24 h	100		

[a]Bn, benzyl; EDCl, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; PG, protecting group; and TFE, 2,2,2-trifluoroethanol.

Scheme 3. Evaluation of DKP Suppression During Fmoc Deprotection and Selective Deprotection of BIBS[a]



[a]Bn, benzyl; Bzl, benzyl; COMU, 1-[(1-(cyano-2-ethoxy-2-oxoethylidene aminooxy) dimethylamino morpholino)] uronium hexafluorophosphate; and DIPEA, *N*,*N*-diisopropylethylamine.

In this study, we reported synthetic studies of a model cyclic octapeptide using a combination of our new coupling method with silyl ester carboxylate protection. This model peptide derived from an in vitro display library had a challenging synthetic target due to the presence of both a thioether linkage from the N-terminus to a cysteine (Cys) residue, and the coupling of a sterically hindered *N*-methylleucine (MeLeu) and sarcosine (Sar) amino acid. It also contained a Pro residue that made the structure prone to DKP formation.

RESULTS AND DISCUSSION

Evaluation of Silyl-Protecting Groups in Peptide Synthesis. To evaluate the applicability of bulky silyl carbonyl-protecting groups in peptide synthesis, the stability of the di-*tert*-butyl-isobutylsilyl (BIBS) group was examined.[35] The BIBS-protecting group was introduced into the carboxyl group by employing the reagent di-*tert*-butylisobutylsilyl trifluoromethanesulfonate (BIBS-OTf) under basic conditions. The resultant BIBS-protected, N^{α}-protected phenylalanine was treated under conditions typically employed for the deprotection of Fmoc, Boc, and Cbz groups in standard peptide chemistry (Table 1). Each of the deprotection reactions proceeded quickly without cleavage of the silyl group, and the resulting compound H-Phe-OBIBS was coupled with Fmoc-Phe-OH to produce the corresponding dipeptide in high yield. Obtained Fmoc-Phe-Phe-OBIBS (3) showed high solubility in organic solvents due to the high hydrophobicity of BIBS group. The solubility of Fmoc-Phe-Phe-OBzl (4) in cyclopentyl methyl ether (CPME), toluene, and ethyl acetate was approximately 2−7 mg/mL, while that of (3) was 300−400 mg/mL. It is expected to improve solubility during peptide elongation by using silyl-protecting groups. Another type of highly bulky "super silyl" protecting group, tris(trimethylsilyl)-silyl, which was expected to be superior at preventing DKP production, was cleaved under the conditions used for deprotection of the Fmoc group (data not shown).[36] Hence, we concluded that the super silyl-protecting group was not suitable for peptide elongation syntheses.

To evaluate the preventative effect of DKP formation by bulky silyl groups, the Fmoc-deprotection reactions of (3) and (4) were both conducted. Deprotection was complete in 3 h with diethylamine treatment, and DKP formation was not observed for the C-terminal BIBS-protected dipeptide (5), while 89% of the Bzl-protected peptide (6) was converted to DKP product (7) (Figure S1). Peptide (5) was coupled with Cbz-Ser(*t*Bu)-OH to produce high-yield Cbz-Ser(*t*Bu)-Phe-Phe-OBIBS (8). Treatment with potassium fluoride resulted in selective removal of the C-terminal BIBS group without cleavage of the Cbz or *t*Bu groups. The deprotected BIBS group was easily removed by washing with *n*-heptane, and the

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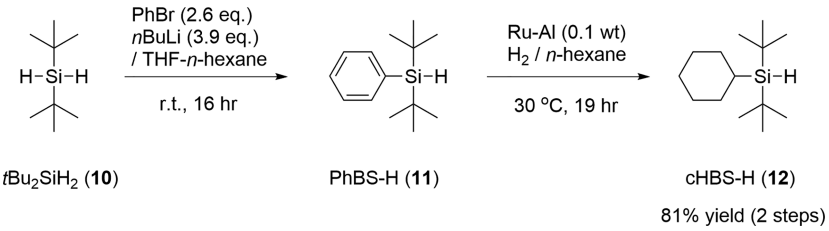
Scheme 4. Synthesis of cHBS-H[a]



[a]*n*BuLi, *n*-butyllithium; PhBr, bromobenzene; and Bzl, Ru−Al, ruthenium on alumina

Scheme 5. Overall Synthetic Scheme for the Synthesis of Cyclic Octapeptide (24)

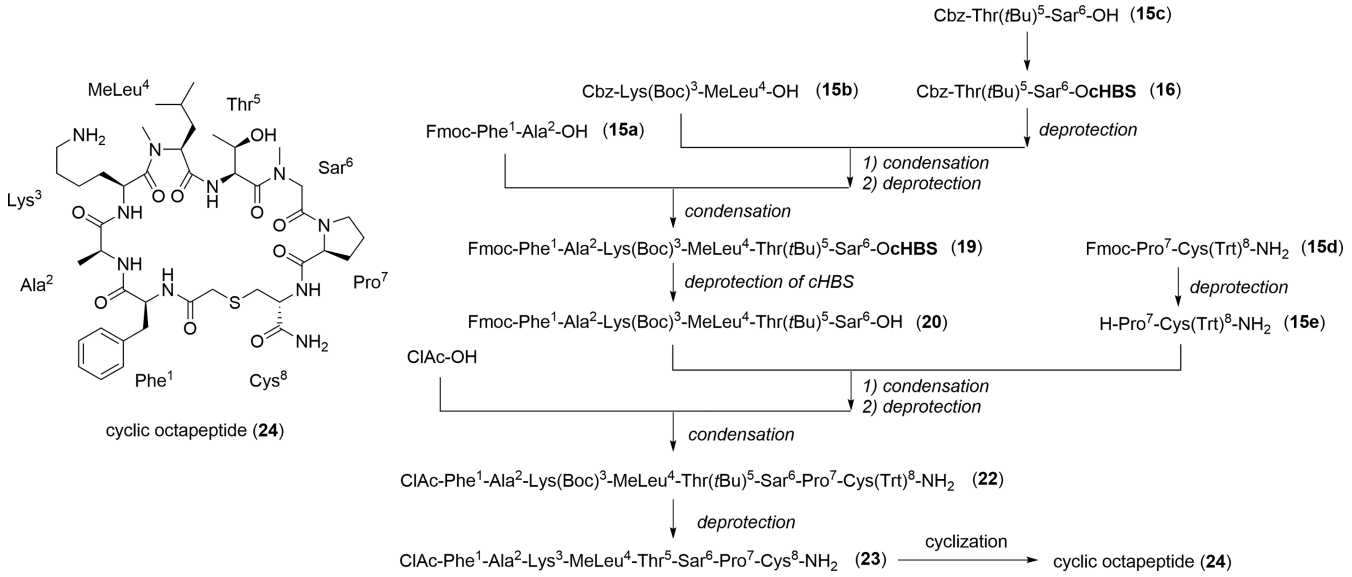
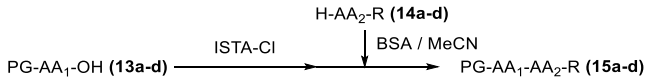


Table 2. Synthesis of Dipeptide Fragments With Isostearyl-Mixed Anhydrides



entry	PG-AA$_1$-OH	H-AA$_2$-R	PG-AA$_1$-AA$_2$-R	method[a]	time (h)[b]	yield (%)[c]	diastereomer ratio (% d.r.)
1	Fmoc-Phe-OH (**13a**)	H-Ala-OH (**14a**)	Fmoc-Phe-Ala-OH (**15a**)	A	2	91	>99.5 : 0.5
2	Cbz-Lys(Boc)-OH (**13b**)	H-MeLeu-OH (**14b**)	Cbz-Lys(Boc)-MeLeu-OH (**15b**)	A	15	100	>99.5 : 0.5
3	Cbz-Thr(*t*Bu)-OH (**13c**)	H-Sar-OH (**14c**)	Cbz-Thr(*t*Bu)-Sar-OH (**15c**)	A	3	99	99.4 : 0.6
4	Fmoc-Pro-OH (**13d**)	H-Cys(Trt)-NH$_2$ (**14d**)	Fmoc-Pro-Cys(Trt)-NH$_2$ (**15d**)[d]	B	2	101	>99.5 : 0.5

[a]Method: (A) reaction equivalent ratios are **13a−c** (1.0 equiv) and **14a−c** (1.2 equiv). (B) Reaction equivalent ratios are **13d** (1.2 equiv) and **14d** (1.0 equiv). [b]Reaction time after adding silylated **14a−d**. [c]Isolated yield without column chromatography. [d]The Fmoc group of **15d** was deprotected with piperidine in methylene chloride to yield H-Pro-Cys(Trt)-NH$_2$ (**15e**).

corresponding C-terminal free tripeptide (**9**) was obtained in high purity without column purification (Scheme 3).

Synthesis Strategy. For our synthetic studies of cyclic octapeptides, the cyclohexyl di-*tert*-butyl silyl (cHBS) group showed similar stability under acidic and basic conditions to the BIBS group and was chosen as a selectively cleavable C-terminal-protecting group. cHBS was more suitable for large-scale manufacturing than BIBS as the necessary starting materials are more readily accessible. cHBS-H (**12**) was synthesized by a two-step reaction with phenyllithium addition to di-*tert*-butylsilane (**10**) and hydrogenation of the phenyl group (Scheme 4) because one-pot synthetic procedure with di-*tert*-butyldichlorosilane, lithium, and cyclohexene was reported in low yield and Grignard reaction or hydrosilylation did not proceed in preliminary studies.[37]

We formulated a synthetic plan for the model cyclic octapeptide (Scheme 5) comprising the following steps: (1) four dipeptides (**15a−15d**), including *N*-methyl amino acid dipeptides, were synthesized using isostearyl-mixed anhydrides; (2) the Sar carboxy group of the third dipeptide (**15c**) was protected with cHBS; (3) hexapeptide (**19**) was constructed by the sequential condensation of (**15b**) and (**15a**) with the cHBS-protected dipeptide (**16**), and (**15e**) was introduced after removal of the cHBS group; (4) full-length protected peptide (**22**) was constructed by introducing a chloroacetyl group into the N-terminus; and (5) deprotection of the side chain-protecting group and cyclization produced the target peptide (**24**).

In this process, (**15e**) was introduced after the construction of the hexapeptide (**20**) because epimerization during the

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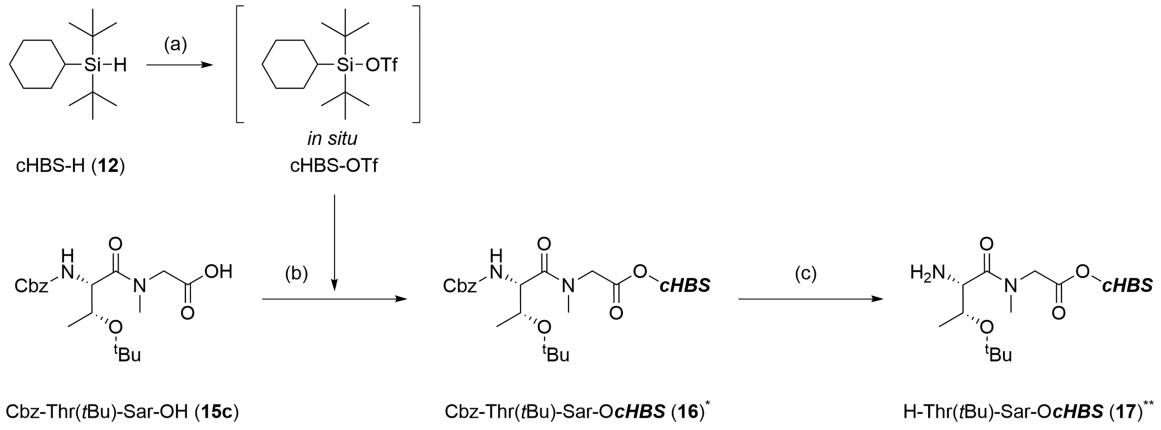
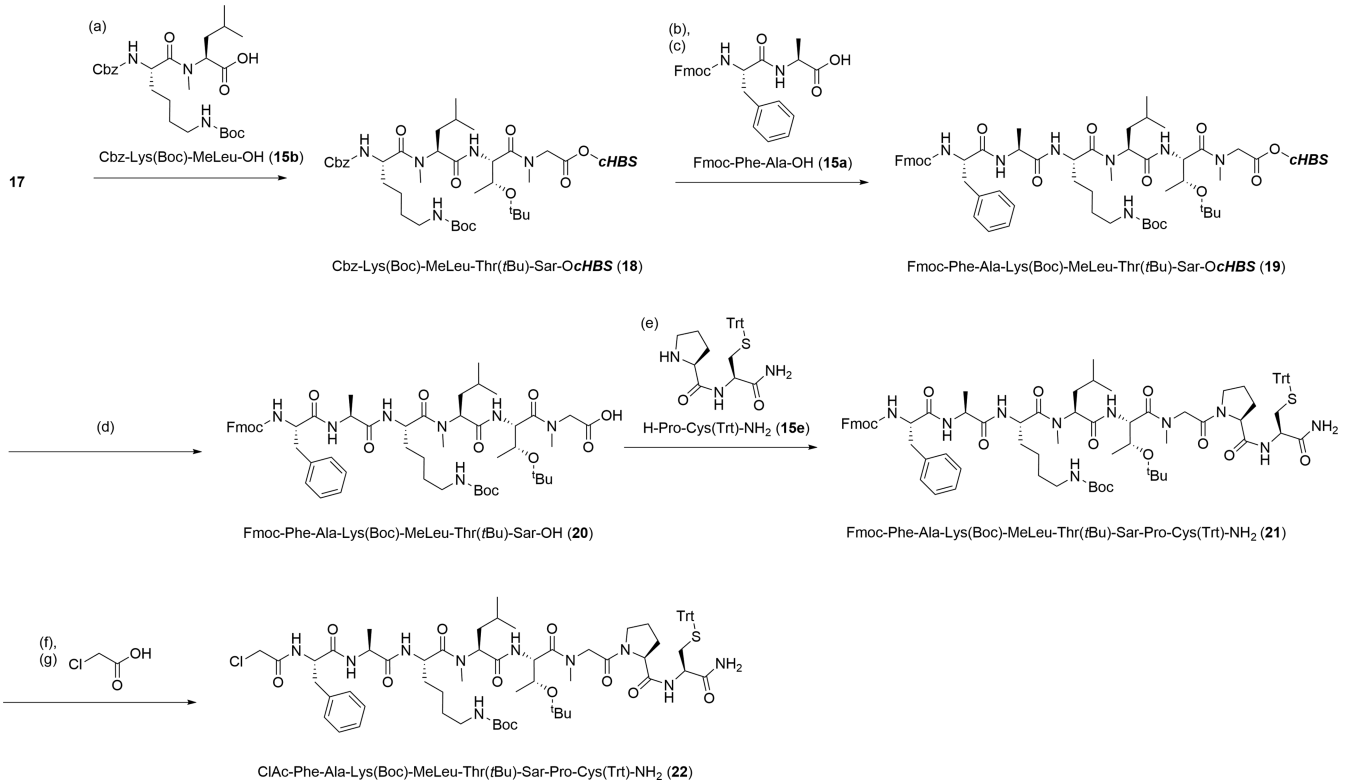
Scheme 6. Synthesis of H-Thr(*t*Bu)-Sar-OcHBS (17)[a]



[a]Reagents and conditions: (a) trifluoromethanesulfonic acid (1.2 equiv)/PhMe, 0 °C; (b) DIPEA (1.9 equiv)/CPME, 0 °C; and (c) 10% Pd−C (0.2 wt), H_2/TFE, 40 °C. *16 was used for the next reaction without isolation. The yield including the residue of cHBS was 132.9%, and the HPLC purity at 210 nm was 96.9%. **17 was used for the next reaction without isolation. The yield from 15C was 130.4%, and the HPLC purity at 210 nm was 95.1% (Figure S2).

Scheme 7. Construction of the Linear Octapeptide (22) Using Dipeptide Fragment Condensation[a]



[a]Reagents and conditions: (a) **15b** (1.3 equiv), DIPEA (6.0 equiv), oxyma (3.0 equiv), COMU (1.5 equiv)/CH_2Cl_2, −20 °C; (b) 10% Pd−C (0.2 wt), H_2/TFE, 40 °C; (c) **15a** (1.3 equiv), NMM (1.4 equiv), IBCF (1.2 equiv)/THF, −10 °C; (d) KF (2.0 equiv)/THF−MeOH, 0 °C; (e) **15e** (1.1 equiv), ISTA−Cl (1.2 equiv), DIPEA (1.2 equiv)/THF−MeCN, 0 °C; and (f) Et_2NH (30 equiv)/CH_2Cl_2, r.t.; (g) DIC (1.2 equiv), ClAcOH (2.4 equiv)/CH_2Cl_2, r.t.

condensation between fragments (**20**) and (**15e**) could be avoided by the presence of an achiral Sar at the C-terminus of (**20**). The use of H-Thr(*t*Bu)-Sar-OH was not considered an appropriate fragment for condensation by our isostearyl-mixed anhydride conditions because rapid conversion to DKP was observed during removal of the Cbz group of (**15c**) in preliminary experiments (see Supporting Information). The progress of the reaction and byproduct formation, including epimerization during dipeptide formation and fragment condensation, were analyzed by HPLC, LC−MS, and SFC. Authentic samples incorporating the corresponding D-amino acid enantiomer at each position were produced using solid-phase synthesis to determine the extent of epimerization. All epimerized standard samples could be separated from the target peptide standard during HPLC analysis.

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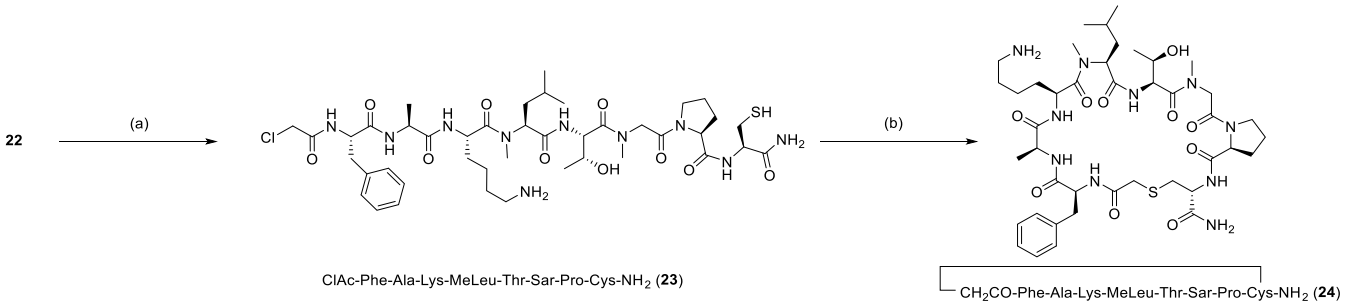
Scheme 8. Synthesis of a Cyclic Octapeptide (24)[a]



[a]Reagents and conditions: (a) TFA: *m*-cresol: thioanisole: H_2O/TIS/DTT = 80:5:5:5:2.5:2.5 v/v/v/v/v/w, r.t.; (b) 100 mM NH_3HCO_3 aq/MeCN (1:1), peptide concentration: 5 g/L, r.t.

Synthesis of Dipeptide Units. Four dipeptide fragments were synthesized using the isostearyl-mixed anhydride methodology (Table 2). ISTA-Cl was reacted with (**13a**−**13d**) at 0 °C for 2−3 h, followed by coupling with the corresponding silylated amino acid (**14a**−**14d**). The silylation showed not only a temporary protective effect but also enhanced the solubility of the amino acids in organic solvents. This property was also effective at improving the solubility of the C-terminal amidated cysteine derivative (**14d**) in which the reaction did not proceed without silylation due to its low solubility. All reactions were completed within 2−15 h and quenched by adding 10% $KHSO_4$. Impurities were removed during the washing and extraction steps of this method. The rate of epimerization was evaluated by comparison with authentic samples of the corresponding diastereomers synthesized separately, using supercritical fluid chromatography (SFC) with a chiral column. We found that all target dipeptides were produced in high yield and in high purity without column purification of the final products.

Fragment Condensation. Dipeptide Cbz-Thr(*t*Bu)-Sar-OcHBS (**16**) was obtained through the introduction of cHBS-H (**12**) into Cbz-Thr(*t*Bu)-Sar-OH (**15c**) following the method described by Yamamoto et al. (Scheme 6).[36] Although rapid conversion of H-Thr(*t*Bu)-Sar-OH to DKP was observed in our preliminary study, no DKP formation was observed when the Cbz group was removed from (**16**) (Figure S2). The significant suppression of DKP formation for Sar, which is prone to DKP products, indicated the superior DKP-suppressive effect of the cHBS group.

The N-terminal chloroacetylated linear octapeptide (**22**) was constructed by fragment condensation of four dipeptides and chloroacetic acid (Scheme 7). Fragment condensation of (**15b**) and (**17**) yielded Cbz-Lys(Boc)-MeLeu-Thr(*t*Bu)-Sar-OcHBS (**18**). Slow condensation rates due to the high steric hindrance of MeLeu and Thr(*t*Bu) tended to cause epimerization, which was suppressed to below 0.5% by using 1-[(1-(cyano-2-ethoxy-2-oxoethylideneaminooxy) dimethylaminomorpholino)] uronium hexafluorophosphate (COMU) as a condensing agent and the addition of ethyl cyano(hydroxyimino)acetate (Oxyma). After Cbz deprotection, the corresponding tetrapeptide was coupled with Fmoc-Phe-Ala-OH (**15a**). We used conventional mixed anhydrides with IBCF as a coupling reagent due to the fast activation and a short reaction time. The coupling between the dipeptide (**15a**), which has Ala at the C-terminus and the tetrapeptide, which has the Lys at N-terminus proceeded quickly with good enantiopurity. Next, the cHBS group of the hexapeptide (**19**) was selectively removed by potassium fluoride treatment and the resulting compound (**20**) was coupled with H-Pro-Cys(Trt)-NH_2 (**15e**) using ISTA-Cl. The condensation was completed quickly within 2 h. The Oxyma-based condensation agent has higher thermal stability than triazole-based reagents; however, concerns remain about HCN gas generation.[38] Unlike Oxyma and chloroformate, ISTA-Cl is a less-hazardous coupling agent with high thermal stability that does not generate HCN or CO_2 gas.[26] The introduction of the chloroacetyl group was performed by adding chloroacetic anhydride, which was activated by *N*,*N*′-diisopropylcarbodiimide (DIC) in situ. This afforded the full length linear octapeptide (**22**) with side chains protected in good HPLC purity (83.2%).

The linear precursor peptide (**23**) was produced by removing the side chain protection of (**22**) with a TFA-scavenger cocktail (Scheme 8). Cyclization by thioether bond formation was performed in aqueous solution, which produced a crude product (**24**). Major impurities included cyclic tetrapeptide (**25**) and cyclic decapeptide (**26**), derived from the remaining dipeptide (**15a**) produced during the synthesis of (**21**).[39] These impurities were easily removed by HPLC purification to yield a purified peptide with a purity higher than 99% (Figure S3) in 73.5% yield from dipeptide (**15c**) to the final purified product (**24**) (TFA salt).

CONCLUSIONS

Convergent solution-phase peptide synthesis is an excellent strategy for manufacturing short- to medium-length peptides; however, conventional methodologies possess many limitations, including unidirectional elongation from only the C-terminus to N-terminus, which requires care in avoiding the risk of C-terminal epimerization. Frequently used C-terminal-protecting groups including methyl and ethyl esters have a high risk of side reactions during deprotection and limit the options for C-terminal amino acids that can be employed. Repeated coupling in the N-terminus to the C-terminus direction without an intermediate deprotection step using isostearyl-mixed anhydrides allows for the efficient synthesis of C-terminus-free peptide fragments. The silyl ester carboxy-protecting group was suitable for this methodology and orthogonal to typical peptide synthesis methods, enabling the synthesis of N-protected peptide fragments through selective deprotection without side reactions. In this study, we have reported the efficient synthesis of a highly challenging cyclic octapeptide containing two *N*-methyl amino acids, using a combination of two new methods for the synthesis of peptide

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fragments without the requirement of column purification in the intermediate steps. We believe that these methods could greatly enhance the flexibility of solution-phase peptide synthesis strategies and help accelerate future research in peptide manufacturing.

EXPERIMENTAL SECTION

General Information. All reactions for substrate preparation were performed in standard, dry glassware fitted with rubber septa. Reactions were monitored by HPLC or LC−MS. ^{1}H and ^{13}C NMR spectra were collected with a 600 MHz spectrometer using the deuterated solvent as an internal deuterium reference. High-resolution mass spectrometry (HRMS) was performed on an electrospray ionization-mass spectrometer. Solid-phase syntheses of reference peptides were conducted by Fmoc SPPS that produced Fmoc cleavage with 20% piperidine/NMP and an amino acid condensation reaction using DIC/oxyma on an automated peptide synthesizer. Protected peptide resins were treated with HFIP−DCM (3:7) to obtain side chain-protected peptides and a TFA scavenger cocktail to produce deprotected peptides, which were then analyzed by HPLC without purification and used as reference compounds. Amino acids were purchased from Watanabe Chemical Industries, Tokyo Chemical Industry, GL Biochem, and Merck. ISTA-Cl was synthesized as previously described.[26] Di-*tert*-butylsilane and Di-*tert*-butylisobutyl trifluoromethanesulfonate were purchased from Gelest. Other reagents and solvents were purchased from Kanto Chemical, FUJIFILM Wako Pure Chemical, Tokyo Chemical Industry, and Watanabe Chemical Industries.

General Procedure for Synthesizing Dipeptide Fragments. A solution of PG-AA-OH (1 equiv for **13a−c**, 1.2 equiv for **13d**) in MeCN was stirred at 0 °C and DIPEA (2.0 equiv, relative to **13a−d**), 50% ISTA-Cl in PhMe (1.1 equiv, relative to **13a−d**) was added. The mixture was stirred at 0 °C. Separately, H-AA-OH (**14a−c**) (1.2 equiv) or H-AA-NH_2 (**14d**) (1 equiv) and *N*,*O*-*bis*(trimethylsilyl)acetamide (BSA, 2 equiv for **14a** and **14d**, 1.3 equiv for **14b** and **14c**, relative to **14a−d**) was mixed in MeCN at 60 °C, and the mixture was stirred at 60 °C until the solution became clear. A solution of silylated **14a−d** was added to an activated **13a−d** at 0 °C, and the mixture was stirred for 2−15 h at 0 °C. Next, 10% $KHSO_4$ was added to the reaction solution, washed thrice with *n*-heptane, and extracted with AcOiPr. The obtained organic layer was washed with 10% NaCl and concentrated to produce the desired dipeptide (**15a−d**). Characterization data for **15a−d** are listed in Supporting Information.

*Cyclohexyl di-tert-butylsilane (**12**).* Bromobenzene (5.7 g, 36 mmol) in THF was added to 1.55 M *n*-BuLi (35 mL, 54 mmol) in *n*-hexane and stirred for 2 min at 0 °C, followed by removal from the ice bath. The mixture was stirred for 6 h at room temperature. Subsequently, di-*tert*-butyl silane (2.0 g, 14 mmol) in THF was added and incubated with stirring for 15 h at room temperature. The reaction mixture was washed with 2 M HCl and 5% NaCl before the organic layer was concentrated and filtered with the addition of *n*-hexane and silica gel. The filtrate was concentrated and crude **11** was dissolved in 25 mL *n*-hexane, followed by the addition of Ru−Al (5%, 0.61 g). This mixture was then stirred under 0.8 MPa H_2 gas for 19 h at 30 °C. The solution was filtered through silica gel and concentrated to produce crude **12** (5.9 g, 81% yield over 2 steps) as a colorless liquid. The unpurified **12** was used in the next reaction and a portion was purified by column chromatography to confirm the structure.

*Cyclohexyl di-tert-butylsilane (**12**).* ^{1}H NMR ($CDCl_3$ with 0.05% v/v TMS, 600 MHz): δ 3.19 (s, 1H), 1.82−1.85 (m, 2H), 1.71−1.75 (m, 3H), 1.43−1.50 (m, 2H), 1.20−1.26 (m, 3H), 1.05 (s, 18H), 0.97−1.02 (m, 1H); ^{13}C NMR (600 MHz, $CDCl_3$): δ 30.7, 29.9, 29.1, 27.2, 25.2, 20.2; IR (ATR, cm^{-1}): 2963, 2925, 2855, 2082, 1471, 1446, 1388, 1364, 1102, 1040, 1013, 1005, 934, 884, 848, 823, 807, 799, 783.

*ClAc-Phe-Ala-Lys(Boc)-MeLeu-Thr(tBu)-Sar-Pro-Cys(Trt)-NH_2 (**22**).* To a mixture of 6.8 g unpurified **12** (quantitated purity: 43%, quantified based on the purified **12**, 13.0 mmol) and TfOH (2.3 g, 15.3 mmol) at 0 °C in PhMe, **15c** (3.9 g, 10.3 mmol) in CPME and DIPEA (2.6 g, 20.1 mmol) were added. The mixture was stirred for 2 h at 0 °C, and washed with 5% $NaHCO_3$, 10% $KHSO_4$, and 10% NaCl. The organic layer was concentrated and dissolved in *n*-heptane, washed with 50% MeCN−H_2O and 10% NaCl, and concentrated to yield crude **16** (8.0 g), to which 10% Pd−C (1.2 g) was added in TFE and stirred under a H_2 gas atmosphere at 40 °C for 1.5 h. Pd−C was removed and concentrated to yield crude **17** (6.1 g). Next, **15b** (6.6 g, 13 mmol), COMU (6.4 g, 14.9 mmol), Oxyma (4.3 g, 30.3 mmol), and DIPEA (7.8 g, 60.4 mmol) were added to the crude **17** (6.1 g) in CH_2Cl_2 at −20 °C. After stirring for 3 h at −20 °C, TFE was added and stirred for an additional 1 h at −20 °C before the solution was washed with 10% $KHSO_4$, 5% NH_3, and H_2O and concentrated to obtain the brown oil of crude **18** (13.9 g). Next, 10% Pd−C (1.9 g) was suspended in the TFE solution of crude **18** (13.9 g) under a H_2 gas atmosphere at 40 °C for 2.5 h. Pd−C was removed and concentrated to yield H-Lys(Boc)-MeLeu-Thr(*t*Bu)-Sar-OcHBS (**18b**, 12.7 g). Furthermore, **15a** (6.0 g, 13.1 mmol) was stirred with NMM (1.4 g, 13.8 mmol) and IBCF (1.6 g, 11.7 mmol) in THF at −10 °C for 5 min and the mixture was added to a **18b** (12.7 g) solution in THF and stirred for 1 h at −10 °C. AcOiPr was added to the mixture and stirred for 1 h at −10 °C, after which the organic layer was washed with 10% $KHSO_4$, 5% $NaHCO_3$, and 10% NaCl and concentrated. IPE was added to the concentrated solution to form precipitated crude **19** (15.3 g). Next, crude **19** (15.1 g, set as 10.2 mmol) was dissolved in a mixture of THF−MeOH (2:1) and KF (1.2 g, 20.7 mmol) was added; the mixture was stirred for 2 h at 0 °C. Subsequently, $CHCl_3$ was added and the mixture was washed with 10% $KHSO_4$ and 10% NaCl before IPE was added to obtain the precipitated crude **20** (10.2 g) product. Next, crude **20** (9.4 g, assumed as 9.4 mmol) in MeCN was mixed with DIPEA (1.4 g, 10.8 mmol) and 50% ISTA-Cl in PhMe (6.4 mL, 10.4 mmol) at 0 °C and stirred for 6 h before **15e** (4.7 g, 10.2 mmol) in THF was added to the solution and stirred for an additional 2 h at 0 °C. The mixture was diluted in AcOiPr and washed with 10% $KHSO_4$, 5% $NaHCO_3$, and 10% NaCl before it was concentrated. IPE was added to the remaining residue and the precipitated solid **21** (12.7 g) was collected. A mixture of **21** (12.0 g, assumed as 8.9 mmol) in CH_2Cl_2 was prepared with Et_2NH (17.8 g, 243 mmol) at room temperature and stirred for 3 h. The mixture was washed with H_2O, 10% NH_4Cl, and 5% $NaHCO_3$. Separately, ClAcOH (1.8 g, 19.0 mmol) was mixed with DIC (1.2 g, 9.5 mmol) in $CHCl_2$ for 10 min at room temperature before being added to the solution. The mixture was then stirred for 0.5 h at room temperature, followed by washing with 10% $KHSO_4$ and H_2O. The organic layer was concentrated, IPE was added to precipitate the product, and the resulting pale red solid was

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collected to obtain crude **22** (12.2 g, quantitative yield from **15c**).

*ClAc-Phe-Ala-Lys(Boc)-MeLeu-Thr(tBu)-Sar-Pro-Cys(Trt)-NH_2 (**22**).* ESI-MS (monoisotopic): $[M + H]^+$ 1337.6777 (calculated for $C_{70}H_{98}C_{l1}N_{10}O_{12}S_1$, 1337.6775).

*(&)CH_2CO-Phe-Ala-Lys-MeLeu-Thr-Sar-Pro-Cys(&)-NH_2 (**24**).* To a crude sample of **22** (10.0 g, set as 7.3 mmol), a mixture of TFA (80.0 mL), *m*-cresol (5.0 mL), thioanisole (5.0 mL), H_2O (5.0 mL), TIPS (2.5 mL), and DTT (2.5 g) at 0 °C was added, and the resulting mixture was stirred for 90 min at room temperature. IPE was added to the reaction solution and the resulting precipitate was collected by filtration to obtain crude **23** (7.4 g, quantitative yield). A crude sample of **23** (502 mg) was dissolved in 100 mL 100 mM aqueous NH_4HCO_3−MeCN (1:1) and stirred for 1 h at room temperature. The mixture was concentrated and lyophilized to produce crude **24** (522 mg) as a white amorphous solid. Next, 20.0 mg of crude **24** sample was applied to the preparative HPLC, and a linear density gradient elution (60 min) was conducted with eluent ratios A/B: 80:20−78:22 using a Triart Prep C18−S (10 μm, 10.0 × 250 mm). Eluent A: 0.1% TFA in water; eluent B: 0.1% TFA containing MeCN. The fractions containing the product were collected and lyophilized to produce 11.7 mg white powder **24** (70.4% yield from crude **22**).

*(&)CH_2CO-Phe-Ala-Lys-MeLeu-Thr-Sar-Pro-Cys(&)-NH_2 (**24**).* ESI-MS (monoisotopic): $[M + H]^+$ 903.4744 (calculated for $C_{42}H_{67}N_{10}O_{10}S_1$, 903.4762), elution time on RP-HPLC: 19.6 min. Elution conditions: an XBridge C18 column (2.5 μm, 100 × 2.1 mm), linear density-gradient elution with eluents A/B: 90/10 (0−2 min), 70/30−5/95 (2−15 min), and 5/95 (15−25 min), 0.1% TFA in water as eluent A and 0.1% TFA containing MeCN as eluent B, flow rate: 1 mL/min, and column oven temperature: 40 °C.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.oprd.1c00078.

Synthetic procedure and characterization data of the BIBS group;, characterization data of dipeptide fragments (**15a−d**); the results and experimental procedures of deprotection of **15c** and additional figures; HPLC profile of Fmoc deprotection reaction of **3**; HPLC profile of Cbz deprotection reaction of **16**; HPLC profile of crude and purified **24**; and copies of the ^1H and ^{13}C NMR spectra (PDF).

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Notes

The authors declare no competing financial interest.

Abbreviations used for amino acids and designation of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in IUPAC-IUB Commission on Biochemical Nomenclature.[40] Amino acid symbols denote the L-configuration unless indicated otherwise. Abbreviated nomenclature for cyclic peptides followed the rules of the application by Albericio et al.[41] "&" indicated the bridging points in the cyclic peptide.

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■ ABBREVIATIONS

APIs, active pharmaceutical ingredients; BIBS, di-*tert*-butylisobutylsilyl; BIBS-OTf, di-*tert*-butylisobutylsilyl trifluoromethanesulfonate; Bn, benzyl; Boc, *tert*-butoxycarbonyl; BSA, *N,O-bis*(trimethylsilyl)acetamide; Bzl, benzyl; Cbz, benzyloxycarbonyl; cHBS, cyclohexyl di-*tert*-butylsilyl; cHBS-H, cyclohexyl di-*tert*-butylsilane; ClAcOH, chloroacetic acid; COMU, 1-[(1-(cyano-2-ethoxy-2-oxoethyl idene aminooxy) dimethylamino morpholino)] uronium hexafluorophosphate; CPME, cyclopentyl methyl ether; DIC, *N,N′*-diisopropylcarbodiimide; DIPEA, *N,N*-diisopropylethylamine; DKP, diketopiperazine; DTT, *DL*-dithiothreitol; EDCl, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; Et_2NH, diethylamine; Fmoc, 9-fluorenylmethyloxycarbonyl; HPLC, high performance liquid chromatography; IBCF, isobutyl chloroformate; ISTA-Br, isostearic acid bromide; ISTA-Cl, isostearic acid chloride; ISTA-X, isostearic acid halide; LC−MS, liquid chromatography-mass spectrometry; LPPS, solution-phase (liquid-phase) peptide synthesis; MeCN, acetonitrile; MeLeu, *N*-methylleucine; MeOH, methanol; nBuLi, *n*-butyllithium; NMM, *N*-methylmorpholine; Oxyma, ethyl cyanohydroxyiminoacetate; Pd−C, palladium on carbon; PG, protecting group; PhBr, bromobenzene; PhBS-H, di-*tert*-butyl(phenyl)silane; PhMe,

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toluene; Ru−Al, ruthenium on alumina; r.t, room temperature; Sar, sarcosine; SFC, supercritical fluid chromatography; SPPS, solid-phase peptide synthesis; *t*Bu, *tert*-butyl; tBu$_2$SiH$_2$, di-*tert*-butylsilane; TFA, trifluoroacetic acid; TFE, 2,2,2-Trifluoroethanol; TfOH, trifluoromethanesulfonic acid; THF, tetrahydrofuran; TIS, triisopropylsilane; TMS, trimethylsilyl; Trt, triphenylmethyl

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