"*O*-Acyl isopeptide method" for peptide synthesis: synthesis of forty kinds of "*O*-acyl isodipeptide unit" Boc-Ser/Thr(Fmoc-Xaa)-OH†

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The *O*-acyl isopeptide method has recently received attention as an efficient synthetic method for peptides. Herein, forty kinds of "*O*-acyl isodipeptide unit" Boc-Ser/Thr(Fmoc-Xaa)-OH (**1–40**) were effectively synthesized in two-steps without epimerization. The *O*-acyl isodipeptide units are important building blocks to enable the routine use of the *O*-acyl isopeptide method.

Introduction

Solid-phase peptide synthesis (SPPS) has been routinely used for chemical synthesis of peptides. Specifically, the development of automated SPPS has enabled rapid and convenient preparation of target peptides. However, the synthesis of "difficult sequence"-containing peptides is still a problematic area, and the peptides often have low synthetic yield and purity in SPPS.[1] Current examples of such sequences include amyloidogenic peptides and membrane peptides that are difficult to synthesize and handle in various conditions because of their high self-assembling characters. The difficult sequences are generally hydrophobic and are prone to aggregate in solvent during synthesis and purification. This aggregation is attributed to inter-/intra-molecular hydrophobic interactions and the hydrogen bond network among resin-bound peptide chains, resulting in the formation of extended secondary structures such as β-sheets.[1] To solve this problem, Mutter *et al.* developed building blocks, named "pseudo-prolines", which are dipeptide derivatives, including Ser/Thr-derived oxazolidines or Cys-derived thiazolidines.[1a] Sheppard and Johnson *et al.* also reported building blocks, 2-hydroxy-4-methoxybenzyl (Hmb), as a protecting group for the backbone amide nitrogen.[1b] These special building blocks were designed to disrupt the secondary structure formed by inter-/intra-chain interactions.

In 2003, we discovered that the presence of an *O*-acyl instead of *N*-acyl residue within the peptide backbone significantly changed the secondary structure of the native peptide. Moreover, the target peptide was subsequently generated by an *O–N* intramolecular acyl migration reaction. These findings led to the development of a novel method, called the "*O*-acyl isopeptide method",[2] for the synthesis of peptides containing difficult sequences (Fig. 1). The method has been successfully applied to efficiently synthesize difficult sequence-containing peptides such as Ac-Val-Val-Ser-Val-Val-NH_2 and Alzheimer's disease-related amyloid β peptide (Aβ) 1–42.[2a–f] Moreover, a "Click Peptide", which provides a new basis to investigate pathological functions of amyloid β peptides in Alzheimer's disease by inducible activation of its self-assembly, represents a valuable use of the isopeptide method in chemical biology-oriented research.[2g,i,j,l] Furthermore we developed a "racemization-free segment condensation" based on the *O*-acyl isopeptide method, in which an *N*-segment possessing a C-terminal *O*-acyl isopeptide structure with a urethane-protected Ser/Thr residue was employed for the segment condensation.[2k,l] Our studies indicated that modification of the peptide backbone to an ester structure at only one position within the whole peptide sequence significantly changed the unfavorable secondary structure of the native peptides, leading to improved coupling and deprotection efficacy during SPPS. Shortly after our disclosure, the *O*-acyl isopeptide method began to be utilized by several other groups, especially Mutter *et al.*,[3] Carpino *et al.*,[4] and Börner and co-workers,[5] indicating that the *O*-acyl isopeptide method is widely advantageous for peptide preparation.

However, epimerization during esterification has been a drawback in the solid-phase synthesis of *O*-acyl isopeptides. During the synthesis of *O*-acyl isopeptide H-Thr(Ac-Val-Val)-Val-Val-NH_2, epimerization at the esterified Val residue occurred during esterification using the 1,3-diisopropylcarbodiimide (DIPCDI)–*N*,*N*-dimethylaminopyridine (DMAP) method and the crude deprotected mixture contained 21% of D-Val diastereomer.[2h] Thus, to solve this problem, we designed an "*O*-acyl isodipeptide unit", Boc-Ser/Thr(Fmoc-Xaa)-OH (Fig. 2) based on the hypothesis that esterification-derived epimerization should be suppressed in solution due to the faster coupling rate as compared to that on a solid support. Along this line, we previously used Boc-Thr(Fmoc-Val)-OH for synthesizing a small difficult sequence-containing Ac-Val-Val-Thr-Val-Val-NH_2 based on the *O*-acyl isopeptide method.[2h]

In the present study, we report the synthesis of forty kinds of *O*-acyl isodipeptide unit, Boc-Ser/Thr(Fmoc-Xaa)-OH **1–40**, with all naturally coded amino acids (Fig. 2). Interestingly, we did not observe any epimerized D-derivative in each ester bond formation. Additionally, the synthesized isodipeptide units were successfully applied to synthesize a bioactive influenza A virus-related peptide containing difficult sequence. The synthesis of *O*-acyl isodipeptide units required only two simple reactions with good total

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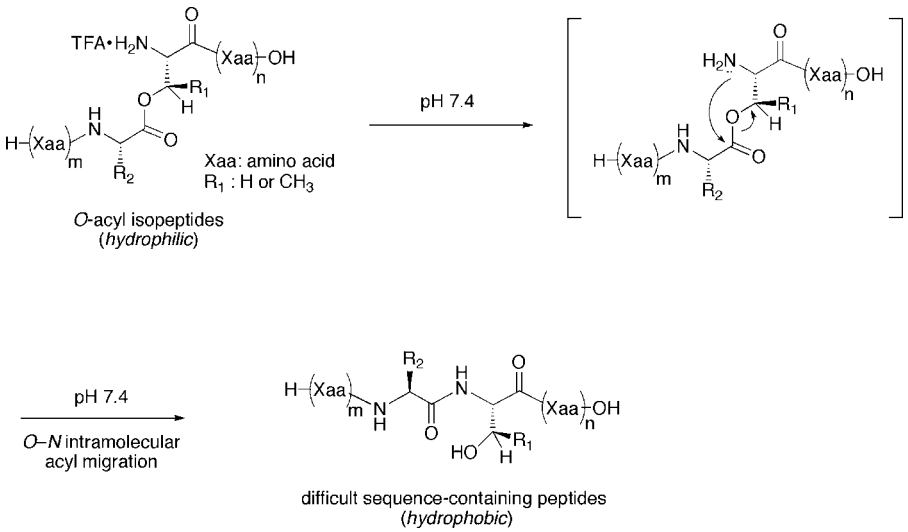
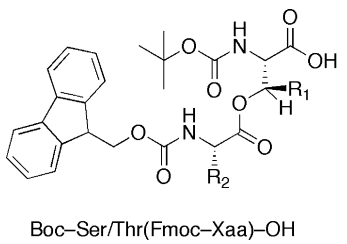


Fig. 1 The "*O*-acyl isopeptide method": synthetic method for the preparation of peptides through *O*–*N* intramolecular acyl migration reaction of *O*-acyl isopeptides.



entry	R_1	structure
1–20	H	Boc–Ser(Fmoc–Xaa)–OH
21–40	CH_3	Boc–Thr(Fmoc–Xaa)–OH

Xaa: 20 naturally occurring amino acids

Fig. 2 General structure of *O*-acyl isodipeptide units **1–40**.

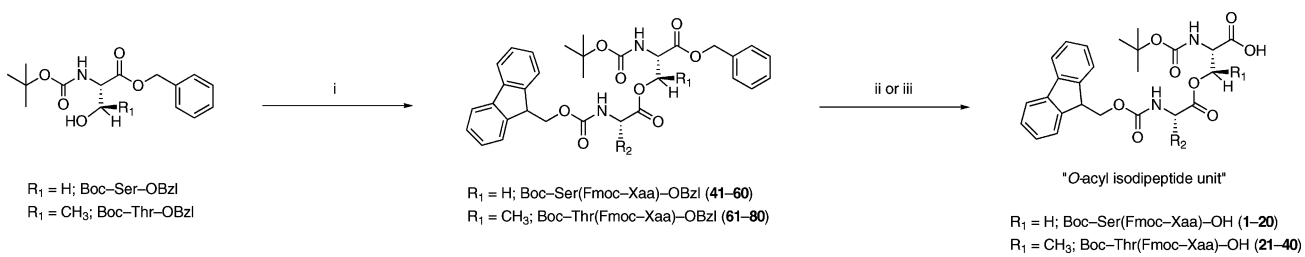
yields (except for the case of Cys). Using isodipeptide units, the epimerization-inducing esterification step on the resin could be omitted. Hence, the set of *O*-acyl isodipeptide units developed herein are be important building blocks to enable the routine use of the *O*-acyl isopeptide method.

Results and discussion

As a drawback of the *O*-acyl isopeptide method, we recently found that epimerization at the esterified amino acid residue occurred during the ester bond-forming reaction on the resin. We postulated the esterification in solution should suppress the extent of D-derivative formation due to faster coupling rate as compared to that on a solid support. Our previous synthesis of Boc-Thr(Fmoc-Val)-OH also indicated that the Val-Thr ester forming reaction in solution was sufficiently fast to avoid epimerization, while 21% of D-Val derivative was observed in the solid-phase synthesis.[2h] Hence, we envisioned synthesizing a set of *O*-acyl isodipeptide units with all naturally coded amino acids by carefully evaluating whether epimerization could be avoided in the esterification with various amino acid residues.

Synthesis of *O*-acyl isodipeptide units

Forty kinds of the *O*-acyl isodipeptide units **1–40** (Fig. 2) were synthesized according to Scheme 1. Fmoc-amino acid was coupled to the β-hydroxyl group of Boc-Ser/Thr-OBzl[6] using *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide (EDC)–DMAP method in



Scheme 1 Synthetic scheme for the "*O*-acyl isodipeptide unit". *Reagents and conditions*: (i) Fmoc-Xaa-OH, EDC·HCl (*N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide·HCl), DMAP (*N*,*N*-dimethylaminopyridine), dry $CHCl_3$, overnight; (ii) 10% Pd/C (10 w/w%), H_2, AcOEt, overnight (for **1–7**, **10–20**, **21–27**, **30–40**); (iii) 10% Pd/C (60 w/w%), ammonium formate (10 eq.), EtOH–H_2O (95:5), 40 °C, 3 h (for **8** and **28**, iii × 2 for **9** and **29**).

dry $CHCl_3$ to obtain the corresponding Boc-Ser/Thr(Fmoc-Xaa)-OBzl (**41–80**). Although this esterification was mainly performed overnight, the reaction could be stopped after an appropriate time (maybe a few hours) with TLC monitoring. Subsequently, the *O*-Bzl esters of the fully protected dipeptides were removed using Pd/C under hydrogen atmosphere to afford the corresponding Boc-Ser/Thr(Fmoc-Xaa)-OH (**1–7**, **10–27** and **30–40**). The *O*-Bzl ester of Boc-Ser/Thr(Fmoc-Cys(Trt))-OBzl (**48** and **68**) and Boc-Ser/Thr(Fmoc-Met)-OBzl (**49** and **69**), which contain sulfur atoms, were deprotected using catalytic transfer hydrogenation (CTH)[7,8] at 40 °C with ammonium formate as a hydrogen donor to afford Boc-Ser/Thr(Fmoc-Cys(Trt))-OH (**8** and **28**) and Boc-Ser/Thr(Fmoc-Met)-OH (**9** and **29**). Boc-Ser/Thr(Fmoc-D-Xaa)-OBzl (**81–118**) were also synthesized in a similar manner described in Scheme 1.

In Tables 1 and 2, we summarize the isolated yield, extent of epimerization and some physicochemical data of Boc-Ser/Thr(Fmoc-Xaa)-OBzl (**41–80**), Boc-Ser/Thr(Fmoc-D-Xaa)-OBzl (**81–118**) and Boc-Ser/Thr(Fmoc-Xaa)-OH (**1–40**). All Boc-Ser/Thr(Fmoc-Xaa)-OBzl (**41–80**) were obtained in high yields (70–99%). Treatment of the protected isodipeptide units (**41–47**, **50–67**, **70–80**) with Pd/C–H_2 also gave the desired dipeptides in high yields of 75–99%. However, in the treatment of Cys- and Met-containing protected isodipeptide (**68** and **69**, respectively) with Pd/C–H_2, the desired compound (either **28** or **29**) was not obtained due to the catalyst poisoning[8] of the sulfur atom. We therefore adopted the catalytic transfer hydrogenation (CTH) method with ammonium formate as a hydrogen donor, which is reported as a powerful hydrogenolysis system under mild conditions,[8] for Bzl ester deprotection of **48**, **49**, **68** and **69**. In the CTH method, although a small amount of decomposition of Fmoc was observed under mass spectra analysis, each desired product was readily obtained after HPLC purification. Deprotection by the CTH method resulted in moderate yields: 58% for **8**, 30% for **28**, 70% for **9**, 73% for **29**, respectively.

Thus, the synthesis of *O*-acyl isodipeptide units generally requires only simple two reactions (Scheme 1) with moderate to excellent total yields (55%–99%). The synthesis of *O*-acyl isodipeptide units would be simpler than that of other building blocks such as pseudo-prolines[1a] and Hmb,[1b] for synthesizing difficult sequence-containing peptides.

Study of epimerization during esterification

In the synthesis of thirty-eight kinds of protected isodipeptide units **42–60** and **62–80** with various Fmoc-protected amino acids, we examined whether epimerization occurred during the esterification reaction. To evaluate this matter, we synthesized all corresponding D-derivatives **81–118**. Interestingly, no detectable epimerized product **81–118** was observed in crude **42–60** and **62–80**, even in the esterification with epimerization-favored Cys[9] and His[10] residues, indicating that epimerization did not occur in any ester bond-forming reaction with EDC–DMAP,[11] although whether epimerization had occurred could not be determined in the case of Boc-Ser(Fmoc-Met)-OBzl (**49**) and Boc-Ser(Fmoc-His(Trt))-OBzl (**55**) because separation of L-derivative and independently synthesized D-derivative was achieved by neither RP-HPLC nor chiral HPLC. Fig. 3 shows the HPLC profiles of crude Boc-Ser(Fmoc-Ser(*t*Bu))-OBzl (**46**), Boc-Thr(Fmoc-Cys(Trt))-OBzl (**68**) and Boc-Thr(Fmoc-Arg(Pmc))-OBzl (**77**). These results suggest that the coupling rate of *O*-acylation with Fmoc-Xaa-OH is commonly sufficiently fast to avoid epimerization, while products from solid-phase synthesis exhibited large amounts of undesired diastereomers.[2h]

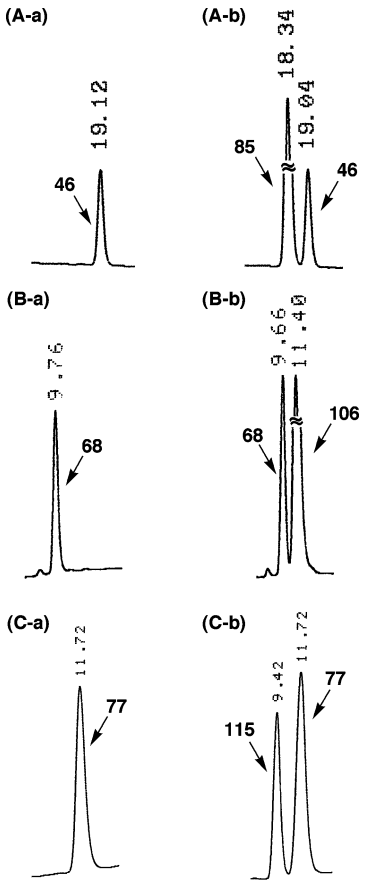


Fig. 3 Evaluation of epimerization during esterification. (A-a) crude Boc-Ser(Fmoc-Ser(*t*Bu))-OBzl (**46**); (A-b) analytical HPLC of mixture of L-Ser derivative **46** and D-Ser derivative **85**; (B-a) crude Boc-Thr(Fmoc-Cys(Trt))-OBzl (**68**); (B-b) analytical HPLC of mixture of L-Cys derivative **68** and D-Cys derivative **106**; (C-a) crude Boc-Thr(Fmoc-Arg(Pmc))-OBzl (**77**); (C-b) analytical HPLC of mixture of L-Arg derivative **77** and D-Arg derivative **115**. Analytical HPLC was performed using a C18 reverse phase column (4.6 × 150 mm; YMC Pack ODS AM302) with a binary solvent system: a linear gradient of CH_3CN (60–100% CH_3CN, 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL min^{-1} (40 °C), detected at 230 nm for separation **46** and **85**; Chiralcel® OD normal phase column (4.6 × 250 mm; Daicel Chemical Ind., Ltd, Tokyo, Japan) with an isocratic solvent system: *n*-hexane–isopropanol or *n*-hexane–ethanol at a flow rate of 0.9 mL min^{-1} (room temperature), detected at 230 nm for separation **68** and **106** (*n*-hexane : ethanol = 7 : 3), **77** and **115** (*n*-hexane : isopropanol = 7 : 3).

Applying the *O*-acyl isodipeptide unit to solid-phase peptide synthesis

The synthesized *O*-acyl isodipeptide unit, Boc-Thr(Fmoc-Phe)-OH (**38**) was applied to SPPS of influenza A virus matrix M1 58–66 (H-GILGFVFTL-OH, **119**)[12] containing a difficult sequence. In standard SPPS (see Experimental section), the difficult

Table 1 Data for **1–20**, **41–60**, **81–99** (serine series)

Structure	Yield[a] (%)	Epimerization[b]	RP-HPLC[c] t_R/min	HRMS (FAB)[d] Calcd	HRMS (FAB)[d] Found
Boc-Ser(Fmoc-Gly)-OBzl (**41**)	96	—	34.9	597.2213	597.2217
Boc-Ser(Fmoc-Gly)-OH (**1**)	91	—	29.2	507.1743	507.1735
Boc-Ser(Fmoc-Ala)-OBzl (**42**)	>99	N.D.[e]	35.6	611.2369	611.2373
Boc-Ser(Fmoc-D-Ala)-OBzl (**81**)	97	—	35.6	611.2369	611.2366
Boc-Ser(Fmoc-Ala)-OH (**2**)	89	—	29.8	521.1900	521.1907
Boc-Ser(Fmoc-Val)-OBzl (**43**)	93	N.D.	36.4	639.2682	639.2685
Boc-Ser(Fmoc-D-Val)-OBzl (**82**)	85	—	36.9	639.2682	639.2678
Boc-Ser(Fmoc-Val)-OH (**3**)	82	—	32.7	549.2213	549.2217
Boc-Ser(Fmoc-Leu)-OBzl (**44**)	>99	N.D.	38.5	653.2839	653.2844
Boc-Ser(Fmoc-D-Leu)-OBzl (**83**)	92	—	38.4	653.2839	653.2845
Boc-Ser(Fmoc-Leu)-OH (**4**)	93	—	33.2	563.2369	563.2365
Boc-Ser(Fmoc-Ile)-OBzl (**45**)	84	N.D.	38.6	653.2839	653.2835
Boc-Ser(Fmoc-D-*allo*-Ile)-OBzl (**84**)	97	—	38.5	653.2839	653.2835
Boc-Ser(Fmoc-Ile)-OH (**5**)	>99	—	33.1	563.2369	563.2373
Boc-Ser(Fmoc-Ser(*t*Bu))-OBzl (**46**)	98	N.D.	38.5	683.2945	683.2939
Boc-Ser(Fmoc-D-Ser(*t*Bu))-OBzl (**85**)	98	—	38.2	683.2945	683.2939
Boc-Ser(Fmoc-Ser(*t*Bu))-OH (**6**)	>99	—	32.5	593.2475	593.2471
Boc-Ser(Fmoc-Thr(*t*Bu))-OBzl (**47**)	>99	N.D.	39.8	697.3101	697.3096
Boc-Ser(Fmoc-D-*allo*-Thr(*t*Bu))-OBzl (**86**)	>99	—	39.3	697.3101	697.3096
Boc-Ser(Fmoc-Thr(*t*Bu))-OH (**7**)	89	—	34.4	607.2632	607.2639
Boc-Ser(Fmoc-Cys(Trt))-OBzl (**48**)	>99	N.D.	42.1	885.3186	885.3179
Boc-Ser(Fmoc-D-Cys(Trt))-OBzl (**87**)	77	—	42.1	885.3186	885.3179
Boc-Ser(Fmoc-Cys(Trt))-OH (**8**)	58	—	40.2	795.2716	795.2723
Boc-Ser(Fmoc-Met)-OBzl (**49**)	89	Not determined[f]	38.6	671.2403	671.2407
Boc-Ser(Fmoc-D-Met)-OBzl (**88**)	>99	—	37.6	671.2403	671.2410
Boc-Ser(Fmoc-Met)-OH (**9**)	70	—	32.6	581.1934	581.1927
Boc-Ser(Fmoc-Pro)-OBzl (**50**)	98	N.D.	38.5	637.2526	637.2531
Boc-Ser(Fmoc-D-Pro)-OBzl (**89**)	89	—	38.4	637.2526	637.2531
Boc-Ser(Fmoc-Pro)-OH (**10**)	>99	—	32.0	547.2056	547.2061
Boc-Ser(Fmoc-Asp(O*t*Bu))-OBzl (**51**)	>99	N.D.	38.3	711.2894	711.2889
Boc-Ser(Fmoc-D-Asp(O*t*Bu))-OBzl (**90**)	>99	—	38.2	711.2894	711.2900
Boc-Ser(Fmoc-Asp(O*t*Bu))-OH (**11**)	>99	—	33.3	621.2424	621.2420
Boc-Ser(Fmoc-Asn(Trt))-OBzl (**52**)	>99	N.D.	40.6	896.3523	896.3528
Boc-Ser(Fmoc-D-Asn(Trt))-OBzl (**91**)	>99	—	41.0	896.3523	896.3528
Boc-Ser(Fmoc-Asn(Trt))-OH (**12**)	96	—	36.9	806.3054	806.3058
Boc-Ser(Fmoc-Glu(O*t*Bu))-OBzl (**53**)	98	N.D.	38.9	725.3050	725.3044
Boc-Ser(Fmoc-D-Glu(O*t*Bu))-OBzl (**92**)	>99	—	38.7	725.3050	725.3057
Boc-Ser(Fmoc-Glu(O*t*Bu))-OH (**13**)	99	—	33.4	636.2581	636.2585
Boc-Ser(Fmoc-Gln(Trt))-OBzl (**54**)	85	N.D.	40.6	910.3680	910.3673
Boc-Ser(Fmoc-D-Gln(Trt))-OBzl (**93**)	>99	—	40.9	910.3680	910.3677
Boc-Ser(Fmoc-Gln(Trt))-OH (**14**)	89	—	36.6	820.3210	820.3204
Boc-Ser(Fmoc-His(Trt))-OBzl (**55**)	78	Not determined[f]	35.2	919.3683	919.3688
Boc-Ser(Fmoc-D-His(Trt))-OBzl (**94**)	97	—	34.7	919.3683	919.3688
Boc-Ser(Fmoc-His(Trt))-OH (**15**)	81	—	31.9	829.3213	829.3208
Boc-Ser(Fmoc-Lys(Boc))-OBzl (**56**)	>99	N.D.	38.0	768.3472	768.3468
Boc-Ser(Fmoc-D-Lys(Boc))-OBzl (**95**)	93	—	38.0	768.3472	768.3480
Boc-Ser(Fmoc-Lys(Boc))-OH (**16**)	99	—	33.1	678.3003	678.2997
Boc-Ser(Fmoc-Arg(Pmc))-OBzl (**57**)	94	N.D.	38.8	962.3986	962.3978
Boc-Ser(Fmoc-D-Arg(Pmc))-OBzl (**96**)	>99	—	38.7	962.3986	962.3978
Boc-Ser(Fmoc-Arg(Pmc))-OH (**17**)	88	—	34.5	872.3516	872.3525
Boc-Ser(Fmoc-Phe)-OBzl (**58**)	>99	N.D.	37.9	687.2682	687.2678
Boc-Ser(Fmoc-D-Phe)-OBzl (**97**)	>99	—	38.3	687.2682	687.2678
Boc-Ser(Fmoc-Phe)-OH (**18**)	>99	—	32.7	597.2213	597.2220
Boc-Ser(Fmoc-Tyr(*t*Bu))-OBzl (**59**)	91	N.D.	40.7	759.3258	759.3262
Boc-Ser(Fmoc-D-Tyr(*t*Bu))-OBzl (**98**)	>99	—	38.9	759.3258	759.3262
Boc-Ser(Fmoc-Tyr(*t*Bu))-OH (**19**)[g]	93	—	36.4	669.2788	669.2783
Boc-Ser(Fmoc-Trp(Boc))-OBzl (**60**)	>99	N.D.	41.2	826.3316	826.3321
Boc-Ser(Fmoc-D-Trp(Boc))-OBzl (**99**)	>99	—	41.3	826.3316	826.3311
Boc-Ser(Fmoc-Trp(Boc))-OH (**20**)	91	—	37.5	736.2846	736.2840

[a] Isolated yield. [b] Epimerization was evaluated by comparison with authentic D-derivatives. [c] Analytical HPLC was performed using a C18 reverse phase column (4.6 × 150 mm; YMC Pack ODS AM302) with a binary solvent system: a linear gradient of CH_3CN (0–100% CH_3CN, 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL min^{-1} (40 °C), detected at 230 nm. [d] $(M + Na)^+$. [e] Not detected. [f] In these cases, we couldn't separate L-derivative and D-derivative by HPLC. [g] Ref. 2*k*.

Table 2 Data for **21–40**, **61–80**, **100–118** (Threonine series)

Structure	Yield[a] (%)	Epimerization[b]	RP-HPLC[c] t_R/min	HRMS (FAB)[d] Calcd	HRMS (FAB)[d] Found
Boc-Thr(Fmoc-Gly)-OBzl (**61**)	99	—	35.8	611.2369	611.2375
Boc-Thr(Fmoc-Gly)-OH (**21**)	95	—	30.8	521.1900	521.1904
Boc-Thr(Fmoc-Ala)-OBzl (**62**)[f]	81	N.D.[e]	36.1	603.2706 $(M + H)^+$	603.2703
Boc-Thr(Fmoc-D-Ala)-OBzl (**100**)	86	—	36.0	625.2526	625.2522
Boc-Thr(Fmoc-Ala)-OH (**22**)[f]	98	—	30.5	535.2056	535.2051
Boc-Thr(Fmoc-Val)-OBzl (**63**)	94	N.D.	39.2	653.2839	653.2833
Boc-Thr(Fmoc-D-Val)-OBzl (**101**)	89	—	38.4	653.2839	653.2845
Boc-Thr(Fmoc-Val)-OH (**23**)[g]	92	—	33.5	563.2369	563.2373
Boc-Thr(Fmoc-Leu)-OBzl (**64**)	>99	N.D.	39.3	667.2995	667.2999
Boc-Thr(Fmoc-D-Leu)-OBzl (**102**)	96	—	39.1	667.2995	667.2991
Boc-Thr(Fmoc-Leu)-OH (**24**)	96	—	34.3	577.2526	577.2520
Boc-Thr(Fmoc-Ile)-OBzl (**65**)	92	N.D.	39.1	667.2995	667.2991
Boc-Thr(Fmoc-D-*allo*-Ile)-OBzl (**103**)	>99	—	39.0	667.2995	667.2991
Boc-Thr(Fmoc-Ile)-OH (**25**)	94	—	33.0	577.2526	577.2520
Boc-Thr(Fmoc-Ser(*t*Bu))-OBzl (**66**)	>99	N.D.	39.4	697.3101	697.3107
Boc-Thr(Fmoc-D-Ser(*t*Bu))-OBzl (**104**)	89	—	39.6	697.3101	697.3105
Boc-Thr(Fmoc-Ser(*t*Bu))-OH (**26**)	93	—	34.0	607.2632	607.2624
Boc-Thr(Fmoc-Thr(*t*Bu))-OBzl (**67**)	86	N.D.	40.3	711.3258	711.3252
Boc-Thr(Fmoc-D-*allo*-Thr(*t*Bu))-OBzl (**105**)	65	—	40.0	711.3258	711.3253
Boc-Thr(Fmoc-Thr(*t*Bu))-OH (**27**)	83	—	35.0	621.2788	621.2792
Boc-Thr(Fmoc-Cys(Trt))-OBzl (**68**)	97	N.D.	43.2	899.3342	899.3348
Boc-Thr(Fmoc-D-Cys(Trt))-OBzl (**106**)	>99	—	43.2	899.3342	899.3348
Boc-Thr(Fmoc-Cys(Trt))-OH (**28**)	30	—	40.5	809.2873	809.2867
Boc-Thr(Fmoc-Met)-OBzl (**69**)	>99	N.D.	37.2	685.2560	685.2555
Boc-Thr(Fmoc-D-Met)-OBzl (**107**)	>99	—	36.6	685.2560	685.2554
Boc-Thr(Fmoc-Met)-OH (**29**)	73	—	33.5	595.2090	595.2097
Boc-Thr(Fmoc-Pro)-OBzl (**70**)	86	N.D.	37.8	651.2682	651.2688
Boc-Thr(Fmoc-D-Pro)-OBzl (**108**)	>99	—	37.7	651.2682	651.2676
Boc-Thr(Fmoc-Pro)-OH (**30**)	96	—	31.6	561.2213	561.2220
Boc-Thr(Fmoc-Asp(O*t*Bu))-OBzl (**71**)	>99	N.D.	40.2	725.3050	725.3057
Boc-Thr(Fmoc-D-Asp(O*t*Bu))-OBzl (**109**)	>99	—	38.7	725.3050	725.3046
Boc-Thr(Fmoc-Asp(O*t*Bu))-OH (**31**)	94	—	35.1	635.2581	635.2585
Boc-Thr(Fmoc-Asn(Trt))-OBzl (**72**)	>99	N.D.	39.7	910.3680	910.3675
Boc-Thr(Fmoc-D-Asn(Trt))-OBzl (**110**)	>99	—	40.2	910.3680	910.3675
Boc-Thr(Fmoc-Asn(Trt))-OH (**32**)	78	—	36.1	820.3210	820.3205
Boc-Thr(Fmoc-Glu(O*t*Bu))-OBzl (**73**)	74	N.D.	38.7	739.3207	739.3214
Boc-Thr(Fmoc-D-Glu(O*t*Bu))-OBzl (**111**)	88	—	39.0	739.3207	739.3201
Boc-Thr(Fmoc-Glu(O*t*Bu))-OH (**33**)	90	—	33.9	649.2737	649.2728
Boc-Thr(Fmoc-Gln(Trt))-OBzl (**74**)	>99	N.D.	41.9	924.3836	924.3830
Boc-Thr(Fmoc-D-Gln(Trt))-OBzl (**112**)	>99	—	40.7	924.3836	924.3830
Boc-Thr(Fmoc-Gln(Trt))-OH (**34**)	89	—	36.9	834.3367	834.3362
Boc-Thr(Fmoc-His(Trt))-OBzl (**75**)	72	N.D.	35.3	933.3839	933.3836
Boc-Thr(Fmoc-D-His(Trt))-OBzl (**113**)	>99	—	35.3	933.3839	933.3836
Boc-Thr(Fmoc-His(Trt))-OH (**35**)	77	—	31.7	843.3370	843.3374
Boc-Thr(Fmoc-Lys(Boc))-OBzl (**76**)	98	N.D.	37.8	782.3629	782.3624
Boc-Thr(Fmoc-D-Lys(Boc))-OBzl (**114**)	>99	—	37.8	782.3629	782.3634
Boc-Thr(Fmoc-Lys(Boc))-OH (**36**)	96	—	35.1	692.3159	692.3154
Boc-Thr(Fmoc-Arg(Pmc))-OBzl (**77**)	79	N.D.	39.0	976.4142	976.4137
Boc-Thr(Fmoc-D-Arg(Pmc))-OBzl (**115**)	55	—	38.3	976.4142	976.4138
Boc-Thr(Fmoc-Arg(Pmc))-OH (**37**)	>99	—	37.0	886.3673	886.3669
Boc-Thr(Fmoc-Phe)-OBzl (**78**)	94	N.D.	39.3	679.3019 $(M + H)^+$	679.3015
Boc-Thr(Fmoc-D-Phe)-OBzl (**116**)	97	—	38.6	701.2839	701.2843
Boc-Thr(Fmoc-Phe)-OH (**38**)	96	—	33.5	611.2369	611.2375
Boc-Thr(Fmoc-Tyr(*t*Bu))-OBzl (**79**)	>99	N.D.	40.8	773.3414	773.3418
Boc-Thr(Fmoc-D-Tyr(*t*Bu))-OBzl (**117**)	>99	—	39.7	773.3414	773.3420
Boc-Thr(Fmoc-Tyr(*t*Bu))-OH (**39**)	76	—	35.0	683.2945	683.2939
Boc-Thr(Fmoc-Trp(Boc))-OBzl (**80**)	94	N.D.	40.8	840.3472	840.3476
Boc-Thr(Fmoc-D-Trp(Boc))-OBzl (**118**)	96	—	40.7	840.3472	840.3476
Boc-Thr(Fmoc-Trp(Boc))-OH (**40**)	91	—	37.0	750.3003	750.3007

[a] Isolated yield. [b] Epimerization was evaluated by comparison with authentic D-derivatives. [c] Analytical HPLC was performed using a C18 reverse phase column (4.6 × 150 mm; YMC Pack ODS AM302) with a binary solvent system: a linear gradient of CH_3CN (0–100% CH_3CN, 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL min^{-1} (40 °C), detected at 230 nm. [d] $(M + Na)^+$. [e] Not detected. [f] Ref. 4*b*. [g] Ref. 2*h*.

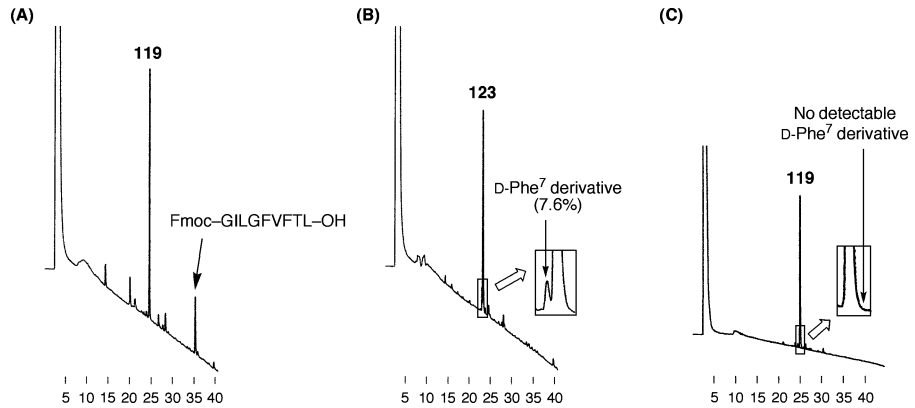


Fig. 4 HPLC profile of crude (A) peptide **119** (synthesized by standard SPPS), (B) *O*-acyl isopeptide **123** (synthesized without *O*-acyl isodipeptide unit), and (C) peptide **119** (synthesized using *O*-acyl isopeptide method with Boc-Thr(Fmoc-Phe)-OH **38**).

sequence-derived byproduct, Fmoc-GILGFVFTL-OH, was obtained after final deprotection (Fig. 4A).

On the other hand, in the *O*-acyl isopeptide method without the *O*-acyl isodipeptide unit (Scheme 2A), Fmoc-Phe-OH was coupled to Boc-Thr-Leu-resin (2-chlorotrityl resin) using the DIPCDI–DMAP method in dry CH_2Cl_2. After continuous couplings of Fmoc-amino acids and TFA treatment, *O*-acyl isopeptide **123**·TFA was obtained without any undesired residue (Fig. 4B). Hence, the protected peptide resin was efficiently synthesized with no interference from the difficult sequences. The result is comparable with our previous observation that modification of difficult sequence-containing peptide to ester structure changed the secondary structure of the peptide to that which is more favorable for Fmoc-deprotection.[2] As expected, a large amount (7.6%) of isopeptide epimer H-Thr(H-Gly-Ile-Leu-Gly-Phe-Val-D-Phe)-Leu-OH was observed in the final deprotected mixture (Fig. 4B), which was confirmed by an independent synthesis of H-Thr(H-Gly-Ile-Leu-Gly-Phe-Val-D-Phe)-Leu-OH.

In these contexts, we synthesized **119** based on the *O*-acyl isopeptide method using *O*-acyl isodipeptide unit **38** (Scheme 2B). The use of isodipeptide unit **38** omitted the epimerization-inducing esterification reaction on the resin. *O*-Acyl isodipeptide unit **38**, which readily solubilized in DMF, was coupled to H-Leu-resin (2-chlorotrityl resin) using the standard DIPCDI–HOBt method to obtain **121**. The completeness of coupling was verified by Kaiser test. After assembly of the remaining residues followed by TFA treatment, *O*-acyl isopeptide **123**·TFA was obtained. **123**·TFA was further dissolved and stirred in phosphate buffer (pH 7.4) at rt to induce quantitative *O*–*N* intramolecular acyl migration to the corresponding target peptide **119** with a half-life of approximately 1 min. As shown in Fig. 4C, HPLC analysis of crude **119** (synthesized using *O*-acyl isodipeptide unit **38**) exhibited a high purity of the desired product with no by-product derived from the difficult sequence or epimerization. The use of isodipeptide **38** did not lead to any additional side reaction. After HPLC purification, the overall yield of **119**·TFA was 73%.

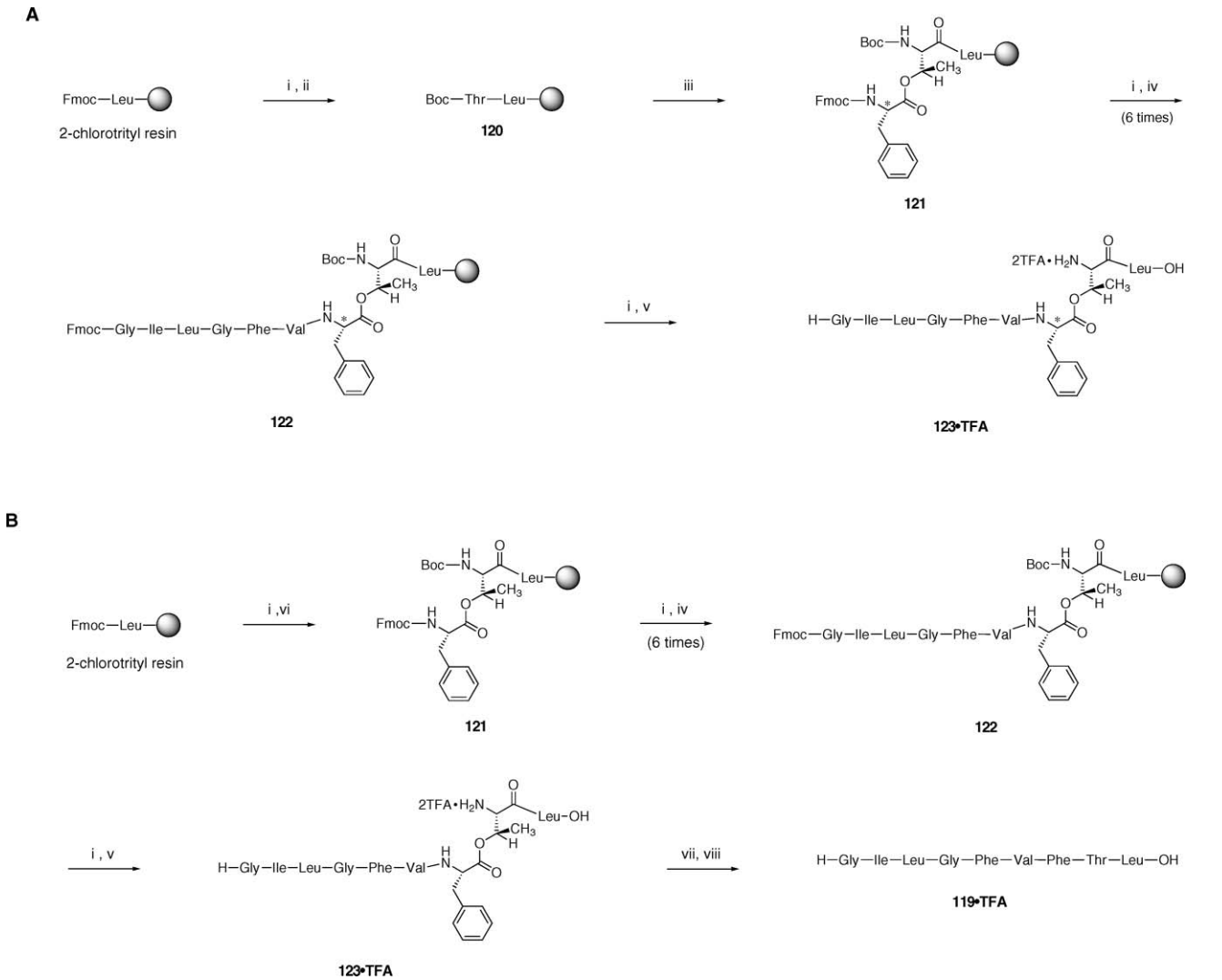
Conclusion

Forty kinds of "*O*-acyl isodipeptide unit" Boc-Ser/Thr(Fmoc-Xaa)-OH **1–40** with all naturally occurring amino acids were synthesized in two-steps without epimerization (29–99%), starting from Boc-Ser/Thr-OBzl. Interestingly, we did not observe any epimerization in all ester bond-forming reactions between Fmoc-Xaa-OH and Boc-Ser/Thr-OBzl. Additionally, the synthesized isodipeptide unit was successfully applied in synthesizing bioactive influenza A virus-related peptide with difficult sequences. Analysis of the crude peptide revealed high purity of the product with no by-product derived from the difficult sequence and epimerization. Moreover, the use of isodipeptide did not lead to any additional side reaction. Hence, using isodipeptide units, the epimerization-inducing esterification on the resin could be omitted. A set of *O*-acyl isodipeptide units developed herein would be important building blocks to enable the routine use of the *O*-acyl isopeptide method.

Experimental

General procedures

Fmoc-amino acid side-chain protections were selected as follows: *t*Bu (Asp, Glu, Ser, Thr, Tyr), Boc (Lys), Pmc (Arg), Trt (Cys, Asn, Gln, His). All protected amino acids and resins were purchased from Calbiochem-Novabiochem Japan Ltd. (Tokyo). Other chemicals were purchased from commercial suppliers, Wako Pure Chemical Ind., Ltd. (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), and Aldrich Chemical Co., Inc. (Milwaukee, WI) and were used without further purification. Analytical thin-layer chromatography (TLC) was performed using Merck 105715 silica gel 60 F_{254} precoated plate (0.25 thickness). Visualization of the chromatogram was by UV illumination (254 nm), phosphomolybdic acid, and ninhydrin, as appropriate. Merck 107734 silica gel 60 (70–230 mesh) was used for column chromatography. Analytical HPLC was performed using a C18 reverse phase column (4.6 × 150 mm; YMC Pack ODS AM302) with a binary solvent system: a linear gradient of CH_3CN in 0.1% aqueous TFA at a flow rate of 0.9 mL min^{-1} (temperature: 40 °C), detected at 230 nm. Preparative HPLC was carried out on a C18 reverse phase column (20 × 250 mm; YMC Pack ODS SH343-5) with a binary solvent system: a linear gradient of CH_3CN in 0.1% aqueous TFA at a flow rate of 5.0 mL min^{-1} (temperature: 40 °C), detected at 230 nm. To separate diastereomers, chiral HPLC analysis was performed with JASCO HPLC systems consisting of following: pump, 880-PU; detector, 875-UV, measured at 230 nm; column, Chiralcel®



Scheme 2 Synthesis of (A) *O*-acyl isopeptide of influenza A virus matrix M1 58–66 (**123**) by the *O*-acyl isopeptide method without *O*-acyl isodipeptide unit, (B) influenza A virus matrix M1 58–66 (**119**) by the *O*-acyl isopeptide method with *O*-acyl isodipeptide unit (**38**). *Reagents and conditions*: (i) 20% piperidine–DMF, 20 min; (ii) Boc-Thr-OH (2.5 eq.), DIPCDI (1,3-diisopropylcarbodiimide, 2.5 eq.), HOBt (1-hydroxybenzotriazole, 2.5 eq.), DMF, 2 h; (iii) Fmoc-Phe-OH (3 eq.), DIPCDI (1,3-diisopropylcarbodiimide, 3 eq.), DMAP (*N*,*N*-dimethylaminopyridine, 0.2 eq.), dry CH_2Cl_2, 16 h (×2); (iv) Fmoc-Xaa-OH (2.5 eq.), DIPCDI (2.5 eq.), HOBt (2.5 eq.), DMF, 2 h; (v) TFA-*m*-cresol–thioanisole–H_2O (92.5 : 2.5 : 2.5 : 2.5), 90 min; (vi) Boc-Thr(Fmoc-Phe)-OH (**38**, 2.5 eq.), DIPCDI (2.5 eq.), HOBt (2.5 eq.), DMF, 2 h (vii) phosphate buffer, pH 7.4, 25 °C; (viii) HPLC purification.

OD normal phase column (4.6 × 250 mm; Daicel Chemical Ind., Ltd, Tokyo, Japan); mobile phase, *n*-hexane–isopropanol or *n*-hexane–ethanol; flow rate, 0.9 mL min^{-1} (room temperature). Solvents used for HPLC were of HPLC grade. NMR spectra were recorded on a JEOL 300 MHz instrument, or a 400 MHz Varian UNITY INOVA 400NB spectrometer, and calibrated using tetramethylsilane (TMS) as an internal reference. MALDI-TOF MASS spectra were recorded on Voyager DE-RP using α-cyano-4-hydroxycinnamic acid as a matrix. FAB-MS was performed on a JEOL JMS-SX102A spectrometer equipped with the JMA-DA7000 data system.

Solid phase peptide synthesis

In general, the peptide chains were assembled by sequential coupling of activated N^{α}-Fmoc-amino acid (2.5 eq.) in DMF (1.5–2 mL) in the presence of 1,3-diisopropylcarbodiimide (DIPCDI, 2.5 eq.) and 1-hydroxybenzotriazole (HOBt, 2.5 eq.) with a reaction time of 2 h at room temperature. The resins were then washed with DMF (1.5 mL, ×5) and the completeness of each coupling was verified by the Kaiser test. N^{α}-Fmoc deprotection was carried out by treatment with piperidine (20% *v*/*v* in DMF) (2 mL, 1 min × 1 and 20 min × 1), followed by washing with DMF (1.5 mL, ×10) and chloroform (1.5 mL, ×5). After complete elongation of the peptide chains, the peptide resins were washed with methanol (1.5 mL, ×5) and dried for at least 2 h *in vacuo*. The peptides were then cleaved from the resin with TFA in the presence of thioanisole, *m*-cresol and distilled water (92.5 : 2.5 : 2.5 : 2.5) for 90 min at room temperature, concentrated *in vacuo*, and precipitated with diethyl ether (4–8 mL) at 0 °C followed by centrifugation at 4000 rpm for 5 min (×3). The resultant peptides were dissolved or suspended in water and lyophilized for at least 12 h.

Boc-Ser(Fmoc-Gly)-OBzl (41). EDC·HCl (0.53 g, 2.76 mmol) was added to a stirring solution of *N*-(*tert*-butoxycarbonyl)-L-serine benzyl ester (Boc-Ser-OBzl) (0.68 g, 2.30 mmol), *N*-(9*H*-fluoren-9-ylmethoxycarbonyl)-L-glycine (Fmoc-Gly-OH, 0.82 g, 2.76 mmol), and DMAP (56.3 mg, 0.46 mmol) in dry $CHCl_3$ (60 mL) at 0 °C. The mixture was slowly warmed to rt over 2 h, stirred additionally overnight, diluted with AcOEt, and washed successively with water, 1 N HCl, water, saturated $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4 and the solvent was removed *in vacuo*. The resulting oil was purified by silica gel column chromatography (AcOEt : hexane 1 : 3) to yield Boc-Ser(Fmoc-Gly)-OBzl (1.26 g, 2.20 mmol, 96%). HPLC analysis at 230 nm: purity was higher than 95% (retention time (t_R) = 34.9 min); ^{1}H NMR (CD_3OD, 300 MHz) δ 7.78 (d, J = 7.3 Hz, 2H), 7.65 (d, J = 7.3 Hz, 2H), 7.43–7.25 (m, 9H), 5.20 (d, J = 12.4 Hz, 1H), 5.12 (d, J = 12.4 Hz, 1H), 4.54–4.31 (m, 5H), 4.26–4.18 (m, 1H), 3.90–3.73 (m, 2H), 1.40 (s, 9H); HRMS (FAB): calcd. for $C_{32}H_{34}N_2O_8Na$ (M + Na)$^+$: 597.2213, found: 597.2217.

Boc-Ser(Fmoc-Gly)-OH (1). Pd/C (115 mg) was added to a stirring solution of Boc-Ser(Fmoc-Gly)-OBzl (1.15 g, 2.00 mmol) in AcOEt (50 mL), and the reaction mixture was vigorously stirred under a hydrogen atmosphere overnight. The catalyst was filtered off through Celite. The solvent was removed *in vacuo* and the crude product was filtered *via* silica gel, at first with AcOEt : hexane 1 : 2 and then the final product was washed out by methanol to give pure Boc-Ser(Fmoc-Gly)-OH (0.89 g, 1.83 mmol, 91%). HPLC analysis at 230 nm: purity was 95% (t_R = 29.2 min); ^{1}H NMR (CD_3OD, 300 MHz) δ 7.78 (d, J = 7.3 Hz, 2H), 7.66 (d, J = 7.4 Hz, 2H), 7.42–7.26 (m, 4H), 4.55–4.46 (m, 1H), 4.41–4.19 (m, 5H), 3.94–3.81 (m, 2H), 1.42 (s, 9H); HRMS (FAB): calcd. for $C_{25}H_{28}N_2O_8Na$ (M + Na)$^+$: 507.1743, found: 507.1735.

Boc-Ser(Fmoc-Ala)-OBzl (42). EDC·HCl (77.9 mg, 0.41 mmol) was added to a stirring solution of *N*-(*tert*-butoxycarbonyl)-L-serine benzyl ester (Boc-Ser-OBzl) (50 mg, 0.17 mmol), *N*-(9*H*-fluoren-9-ylmethoxycarbonyl)-L-alanine (Fmoc-Ala-OH, 126.5 mg, 0.41 mmol), and DMAP (2.1 mg, 0.017 mmol) in dry $CHCl_3$ (3 mL) at 0 °C. The mixture was slowly warmed to rt over 2 h, stirred additionally overnight, diluted with AcOEt, and washed successively with water, 1 N HCl, water, saturated $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4 and the solvent was removed *in vacuo*. The resulting oil was purified by silica gel column chromatography ($CHCl_3$: methanol 90 : 1) to yield Boc-Ser(Fmoc-Ala)-OBzl (101.7 mg, 0.17 mmol, >99%). Epimerization during the synthesis was not detected, confirmed by comparison with independently synthesized D-alanine derivative **81**. HPLC analysis at 230 nm: purity was 88% (t_R = 35.6 min); ^{1}H NMR (CD_3OD, 300 MHz) δ 7.78 (d, J = 7.5 Hz, 2H), 7.66 (t, J = 7.1 Hz, 2H), 7.40–7.27 (m, 9H), 5.19–5.07 (m, 2H), 4.52–4.45 (m, 2H), 4.42–4.28 (m, 3H), 4.23–4.08 (m, 2H), 1.47–1.32 (m, 12H); HRMS (FAB): calcd. for $C_{33}H_{36}N_2O_8Na$ (M + Na)$^+$: 611.2369, found: 611.2373.

Boc-Ser(Fmoc-D-Ala)-OBzl (81). **81** was synthesized in a similar manner to **42**. Yield: 97%; HPLC analysis at 230 nm: purity was 95% (t_R = 35.6 min); ^{1}H NMR (CD_3OD, 300 MHz) δ 7.78 (d, J = 7.5 Hz, 2H), 7.67–7.64 (m, 2H), 7.40–7.27 (m, 9H), 5.20 (d, J = 12.2 Hz, 1H), 5.12 (d, J = 12.2 Hz, 1H), 4.52–4.44 (m, 2H), 4.43–4.26 (m, 3H), 4.24–4.12 (m, 2H), 1.45–1.26 (m, 12H); HRMS (FAB): calcd. for $C_{33}H_{36}N_2O_8Na$ (M + Na)$^+$: 611.2369, found: 611.2366.

Boc-Ser(Fmoc-Ala)-OH (2). **2** was synthesized in a similar manner to **1**. Yield: 89%; HPLC analysis at 230 nm: purity was 92% (t_R = 29.8 min); ^{1}H NMR (CD_3OD, 300 MHz) δ 7.78 (d, J = 7.3 Hz, 2H), 7.67 (t, J = 6.1 Hz, 2H), 7.40–7.28 (m, 4H), 4.58–4.47 (m, 1H), 4.40–4.33 (m, 2H), 4.30–4.19 (m, 4H), 1.44–1.37 (m, 12H); HRMS (FAB): calcd. for $C_{26}H_{30}N_2O_8Na$ (M + Na)$^+$: 521.1900, found: 521.1907.

Boc-Ser(Fmoc-Leu)-OBzl (44). EDC·HCl (0.19 g, 0.97 mmol) was added to a stirring solution of *N*-(*tert*-butoxycarbonyl)-L-serine benzyl ester (Boc-Ser-OBzl) (0.24 g, 0.81 mmol), *N*-(9*H*-fluoren-9-ylmethoxycarbonyl)-L-leucine (Fmoc-Leu-OH, 0.34 g, 0.97 mmol), and DMAP (9.9 mg, 0.081 mmol) in dry $CHCl_3$ (10 mL) at 0 °C. The mixture was slowly warmed to rt over 2 h, stirred additionally overnight, diluted with AcOEt, and washed successively with water, 1 N HCl, water, saturated $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4 and the solvent was removed *in vacuo*. The resulting oil was purified by silica gel column chromatography (AcOEt : hexane 1 : 4) to yield Boc-Ser(Fmoc-Leu)-OBzl (0.51 g, 0.81 mmol, >99%). Epimerization during the synthesis was not detected, confirmed by comparison with independently synthesized D-leucine derivative **83**. HPLC analysis at 230 nm: purity was 98% (t_R = 38.5 min); ^{1}H NMR (CD_3OD, 300 MHz) δ 7.78 (d, J = 7.5 Hz, 2 H), 7.68–7.63 (m, 2 H), 7.40–7.26 (m, 9 H), 5.18–5.07 (m, 2 H), 4.49–4.35 (m, 5 H), 4.23–4.16 (m, 2 H), 1.69–1.53 (m, 3 H), 1.40 (s, 9 H), 0.93 (d, J = 6.4 Hz, 3 H), 0.90 (d, J = 6.4 Hz, 3 H); HRMS (FAB): calcd. for $C_{36}H_{42}N_2O_8Na$ (M + Na)$^+$: 653.2839, found: 653.2845.

Boc-Ser(Fmoc-D-Leu)-OBzl (83). **83** was synthesized in a similar manner to **44**. Yield: 95%; HPLC analysis at 230 nm: purity was 93% (t_R = 38.4 min); ^{1}H NMR (CD_3OD, 300 MHz) δ 7.78 (d, J = 7.4 Hz, 2 H), 7.67–7.61 (m, 2 H), 7.40–7.27 (m, 9 H), 5.19 (d, J = 12.3 Hz, 1 H), 5.13 (d, J = 12.3 Hz, 1 H), 4.49–4.35 (m, 5 H), 4.23–4.15 (m, 2 H), 1.70–1.51 (m, 3 H), 1.39 (s, 9 H), 0.92 (d, J = 6.6 Hz, 3 H), 0.89 (d, J = 6.6 Hz, 3 H); HRMS (FAB): calcd. for $C_{36}H_{42}N_2O_8Na$ (M + Na)$^+$: 653.2839, found: 653.2844.

Boc-Ser(Fmoc-Leu)-OH (4). **4** was synthesized in a similar manner to **1**. Yield: 93%; HPLC analysis at 230 nm: purity was higher than 95% (t_R = 33.2 min); ^{1}H NMR (CD_3OD, 300 MHz) δ 7.78 (d, J = 7.4 Hz, 2 H), 7.70–7.65 (m, 2 H), 7.40–7.28 (m, 4 H), 4.53–4.49 (m, 1 H), 4.37–4.20 (m, 6 H), 1.73–1.57 (m, 3 H), 1.40 (s, 9 H), 0.95 (d, J = 6.2 Hz, 3 H), 0.91 (d, J = 6.2 Hz, 3 H); HRMS (FAB): calcd. for $C_{29}H_{36}N_2O_8Na$ (M + Na)$^+$: 563.2369, found: 563.2365.

Boc-Ser(Fmoc-Ser(*t*Bu))-OBzl (46). **46** was synthesized in a similar manner to **44**. Epimerization during the synthesis was not detected, confirmed by comparison with independently synthesized D-serine derivative **85**. Yield: 98%; HPLC analysis at 230 nm: purity was higher than 95% (t_R = 38.5 min); ^{1}H NMR (CD_3OD, 300 MHz) δ 7.79 (d, J = 7.7 Hz, 2H), 7.69–7.64 (m, 2H), 7.41–7.27 (m, 9H), 5.20 (d, J = 12.5 Hz, 1H), 5.12 (d, J = 12.3 Hz, 1H), 4.53–4.21 (m, 7H), 3.73–3.68 (m, 1H), 3.61–3.56 (m, 1H), 1.40 (s, 9 H), 1.17 (s, 9H); HRMS (FAB): calcd. for $C_{37}H_{44}N_2O_9Na$ (M + Na)$^+$: 683.2945, found: 683.2939.

Boc-Ser(Fmoc-D-Ser(*t*Bu))-OBzl (85). **85** was synthesized in a similar manner to **44**. Yield: 98%; HPLC analysis at 230 nm: purity was higher than 95% (t_R = 38.2 min); ^{1}H NMR (CD_3OD, 300 MHz) δ 7.79 (d, J = 7.4 Hz, 2H), 7.68–7.64 (m, 2H), 7.40–7.27 (m, 9H), 5.22–5.12 (m, 2H), 4.51–4.06 (m, 7H), 3.71–3.67 (m, 1H), 3.61–3.57 (m, 1H), 1.40 (s, 9 H), 1.15 (s, 9H); HRMS (FAB): calcd. for $C_{37}H_{44}N_2O_9Na$ $(M + Na)^+$: 683.2945, found: 683.2939.

Boc-Ser(Fmoc-Ser(*t*Bu)-OH (6). **6** was synthesized in a similar manner to **1**. Yield: >99%; HPLC analysis at 230 nm: purity was higher than 95% (t_R = 32.5 min); ^{1}H NMR (CD_3OD, 300 MHz) δ 7.79 (d, J = 7.5 Hz, 2H), 7.69–7.66 (m, 2H), 7.40–7.28 (m, 4H), 4.54–4.21 (m, 7H), 3.81–3.76 (m, 1H), 3.65–3.60 (m, 1H), 1.42 (s, 9H), 1.19 (s, 9H); HRMS (FAB): calcd. for $C_{30}H_{38}N_2O_9Na$ $(M + Na)^+$: 593.2475, found: 593.2471.

Boc-Ser(Fmoc-Cys(Trt))-OBzl (48). **48** was synthesized in a similar manner to **42**. Epimerization during the synthesis was not detected, confirmed by comparison with independently synthesized D-cysteine derivative **87**. Yield: >99%; HPLC analysis at 230 nm: purity was higher than 95% (t_R = 42.1 min); ^{1}H NMR (CD_3OD, 300 MHz) δ 7.81–7.73 (m, 2H), 7.70–7.60 (m, 2H), 7.43–7.13 (m, 24H), 5.12–5.01 (m, 2H), 4.45–4.17 (m, 5H), 4.14–4.02 (m, 1H), 3.91–3.79 (m, 1H), 2.70–2.44 (m, 2H), 1.38 (s, 9H); HRMS (FAB): calcd. for $C_{52}H_{50}N_2O_8SNa$ $(M + Na)^+$: 885.3186, found: 885.3179.

Boc-Ser(Fmoc-D-Cys(Trt))-OBzl (87). **87** was synthesized in a similar manner to **42**. Yield: 77%; HPLC analysis at 230 nm: purity was higher than 95% (t_R = 42.1 min); ^{1}H NMR (CD_3OD, 300 MHz) δ 7.81–7.73 (m, 2H), 7.69–7.60 (m, 2H), 7.42–7.13 (m, 24H), 5.09–4.98 (m, 2H), 4.45–4.16 (m, 6H), 3.82–3.71 (m, 1H), 2.74–2.51 (m, 2H), 1.38 (s, 9H); HRMS (FAB): calcd. for $C_{52}H_{50}N_2O_8SNa$ $(M + Na)^+$: 885.3186, found: 885.3179.

Boc-Ser(Fmoc-Cys(Trt))-OH (8). **8** could not be synthesized in the same manner to **1** because the sulfur atom at the cysteine resisted the deprotection of the *O*-benzyl group. Thus we adopted catalytic transfer hydrogenation (CTH) to deprotect the benzyl group. Pd/C (6 mg) was added to the stirring solution of the Boc-Ser(Fmoc-Cys(Trt))-OBzl (10 mg, 0.012 mmol, **48**) in EtOH (1.0 mL) including H_2O (100 μL) in the presence of ammonium formate (7 mg) as hydrogen donor, and the reaction mixture was vigorously stirred for 3 h under H_2 atmosphere at 40 °C. Once the catalyst was filtered off using 0.46 μm filter unit, Pd/C (6 mg) and ammonium formate (7 mg) were added again, and the reaction mixture was vigorously stirred for 3 h under H_2 atmosphere at 40 °C. Then, the Pd/C was filtered off using 0.46 μm filter unit, then, next portions of Pd/C (6 mg) and ammonium formate (7 mg) were added, and the reaction mixture was vigorously stirred for 3 h under H_2 atmosphere at 40 °C. Again catalyst was filtered off through Celite. The solvent was removed *in vacuo* and the crude compound dissolved in DMSO, filtered using 0.46 μm filter unit, and immediately injected into preparative HPLC with a 0.1% aqueous TFA–CH_3CN system. The desired fractions were collected and immediately lyophilized, affording pure Boc-Ser(Fmoc-Cys(Trt))-OH (4.5 mg, 0.0058 mmol, 58%). HPLC analysis at 230 nm: purity was higher than 95% (t_R = 40.2 min); ^{1}H NMR (CD_3OD, 300 MHz) δ 7.79 (d, J = 7.4 Hz, 2H), 7.73–7.63 (m, 2H), 7.46–7.18 (m, 18H), 4.49–4.16 (m, 6H), 3.97–3.84 (m, 1H), 2.76–2.65 (m, 1H), 2.64–2.52 (m, 1H), 1.39 (s, 9H); HRMS (FAB): calcd. for $C_{45}H_{44}N_2O_8SNa$ $(M + Na)^+$: 795.2716, found: 795.2723.

Boc-Thr(Fmoc-Cys(Trt))-OBzl (68). **68** was synthesized in a similar manner to **103**. Epimerization during the synthesis was not detected, confirmed by comparison with independently synthesized D-cysteine derivative **106**. Yield: 97%; HPLC analysis at 230 nm: purity was higher than 95% (t_R = 43.2 min); ^{1}H NMR (CD_3OD, 300 MHz) δ 7.78 (d, J = 7.7 Hz, 2 H), 7.67–7.63 (m, 2 H), 7.39–7.18 (m, 24 H), 5.42–5.31 (m, 1 H), 5.04 (d, J = 12.4 Hz, 1 H), 4.97 (d, J = 12.4 Hz, 1 H), 4.37–4.20 (m, 4 H), 3.90–3.85 (m, 1 H), 2.67–2.56 (m, 1 H), 2.51–2.41 (m, 1 H), 1.43 (s, 9 H), 1.17 (d, J = 6.2 Hz, 3 H); HRMS (FAB): calcd. for $C_{53}H_{52}N_2O_8SNa$ $(M + Na)^+$: 899.3342, found: 899.3348.

Boc-Thr(Fmoc-D-Cys(Trt))-OBzl (106). **106** was synthesized in a similar manner to **103**. Yield: >99%; HPLC analysis at 230 nm: purity was higher than 95% (t_R = 43.2 min); ^{1}H NMR (CD_3OD, 300 MHz) δ 7.78 (d, J = 7.4 Hz, 2 H), 7.68–7.61 (m, 2 H), 7.38–7.19 (m, 24 H), 5.41–5.30 (m, 1 H), 5.02 (d, J = 12.2 Hz, 1 H), 4.92 (d, J = 12.2 Hz, 1 H), 4.35–4.32 (m, 3 H), 4.24–4.20 (m, 1 H), 3.83–3.79 (m, 1 H), 2.65–2.48 (m, 2 H), 1.42 (s, 9 H), 1.19 (d, J = 6.2 Hz, 3 H); HRMS (FAB): calcd. for $C_{53}H_{52}N_2O_8SNa$ $(M + Na)^+$: 899.3342, found: 899.3348.

Boc-Thr(Fmoc-Cys(Trt))-OH (28). **28** could not be synthesized in the same manner to **21** because the sulfur atom at the cysteine resisted the deprotection of the *O*-benzyl group. Thus we adopted catalytic transfer hydrogenation (CTH) to deprotect the benzyl group. Pd/C (6 mg) was added to a stirring solution of the Boc-Thr(Fmoc-Cys(Trt))-OBzl (10 mg, 0.011 mmol, **68**) in EtOH (1.0 mL) including H_2O (100 μL) in the presence of ammonium formate (7 mg) as hydrogen donor, and the reaction mixture was vigorously stirred for 3 h under H_2 atmosphere at 40 °C. Once the catalyst was filtered off using 0.46 μm filter unit, Pd/C (6 mg) and ammonium formate (7 mg) were added again, and the reaction mixture was vigorously stirred for 3 h under H_2 atmosphere at 40 °C. Then, the catalyst was filtered off through Celite. The solvent was removed *in vacuo* and the crude compound dissolved in DMSO, filtered using 0.46 μm filter unit, and immediately injected into preparative HPLC with a 0.1% aqueous TFA–CH_3CN system. The desired fractions were collected and immediately lyophilized, affording pure Boc-Thr(Fmoc-Cys(Trt))-OH (2.7 mg, 0.003 mmol, 30%). HPLC analysis at 230 nm: purity was higher than 95% (t_R = 40.5 min); ^{1}H NMR (CD_3OD, 300 MHz) δ 7.78 (d, J = 7.4 Hz, 2 H), 7.70–7.64 (m, 2 H), 7.40–7.20 (m, 19 H), 5.37–5.28 (m, 1 H), 4.37–4.28 (m, 2 H), 4.27–4.20 (m, 2 H), 3.97–3.88 (m, 1 H), 2.73–2.60 (m, 1 H), 2.54–2.42 (m, 1 H), 1.44 (s, 9 H), 1.18 (d, J = 5.5 Hz, 3 H); HRMS (FAB): calcd. for $C_{46}H_{46}N_2O_8SNa$ $(M + Na)^+$: 809.2873, found: 809.2867.

Boc-Thr(Fmoc-Arg(Pmc))-OBzl (77). **77** was synthesized in a similar manner to **103**. Epimerization during the synthesis was not detected, confirmed by comparison with independently synthesized D-arginine derivative **115**. Yield: 79%; HPLC analysis at 230 nm: purity was 94% (t_R = 39.0 min); ^{1}H NMR (CD_3OD, 300 MHz) δ 7.78 (d, J = 7.5 Hz, 2H), 7.65–7.60 (m, 2H), 7.39–7.24 (m, 8H), 5.44–5.41 (m, 1H), 5.10 (d, J = 12.2 Hz, 1H), 5.01 (d, J = 12.2 Hz, 1H), 4.39–4.34 (m, 3H), 4.20 (t, J = 6.6 Hz, 1H), 4.06–4.03 (m, 1H), 3.16–3.11 (m, 1H), 2.62 (t, J = 6.8 Hz, 2H), 2.57 (s, 3H), 2.55 (s, 3H), 2.08 (s, 3H), 1.78 (t, J = 6.8 Hz, 2H), 1.72–1.66

(m, 1H), 1.60–1.46 (m, 3H), 1.42 (s, 9H), 1.27 (s, 6H), 1.21 (d, $J = 6.4$ Hz, 3H); HRMS (FAB): calcd. for $C_{51}H_{63}N_5O_{11}SNa$ (M + Na)$^+$: 976.4142, found: 976.4137.

Boc-Thr(Fmoc-D-Arg(Pmc))-OBzl (115). **115** was synthesized in a similar manner to **62**. Yield: 55%; HPLC analysis at 230 nm: purity was 96% ($t_R = 38.3$ min); ^{1}H NMR (CD_3OD, 300 MHz) δ 7.78 (d, $J = 7.7$ Hz, 2H), 7.65 (t, $J = 6.4$ Hz, 2H), 7.39–7.26 (m, 9H), 5.41–5.38 (m, 1H), 5.12 (d, $J = 12.4$ Hz, 1H), 5.03 (d, $J = 12.4$ Hz, 1H), 4.41–4.35 (m, 3H), 4.21 (t, $J = 6.8$ Hz, 1H), 4.05–4.01 (m, 1H), 3.13–3.09 (m, 2H), 2.62 (t, $J = 6.8$ Hz, 2H), 2.57 (s, 3H), 2.55 (s, 3H), 2.07 (s, 3H), 1.77 (t, $J = 6.8$ Hz, 2H), 1.69–1.62 (m, 1H), 1.50–1.44 (m, 3H), 1.40 (s, 9H), 1.28–1.21 (m, 9H); HRMS (FAB): calcd. for $C_{51}H_{63}N_5O_{11}SNa$ (M + Na)$^+$: 976.4142, found: 976.4138.

Boc-Thr(Fmoc-Arg(Pmc))-OH (37). **37** was synthesized in a similar manner to **21**. Yield: >99%; HPLC analysis at 230 nm: purity was 91% ($t_R = 37.0$ min); ^{1}H NMR (CD_3OD, 300 MHz) δ 7.78 (d, $J = 7.4$ Hz, 2H), 7.66 (t, $J = 7.6$ Hz, 2H), 7.40–7.27 (m, 4H), 5.41–5.38 (m, 1H), 4.44–4.10 (m, 5H), 3.21–3.12 (m, 2H), 2.63 (t, $J = 6.9$ Hz, 2H), 2.57 (s, 3H), 2.55 (s, 3H), 2.08 (s, 3H), 1.79 (t, $J = 6.9$ Hz, 2H), 1.72–1.68 (m, 1H), 1.62–1.45 (m, 3H), 1.41 (s, 9H), 1.38 (s, 6H), 1.21 (d, $J = 5.3$ Hz, 3H); HRMS (FAB): calcd. for $C_{44}H_{57}N_5O_{11}SNa$ (M + Na)$^+$: 886.3673, found: 886.3669.

Except for **1**, **2**, **4**, **6**, **8**, **28**, **37**, **41**, **42**, **44**, **46**, **48**, **68**, **77**, **81**, **83**, **85**, **87**, **106**, **115**, experimental procedures and chemical data of all *O*-acyl isodipeptide units and their intermediates shown in Tables 1 and 2 (**3**, **5**, **7**, **9–27**, **29–36**, **38–40**, **43**, **45**, **47**, **49–67**, **69–76**, **78–80**, **82**, **84**, **86**, **88–105**, **107–114**, **116–118**) are described in the electronic supplementary information (ESI).†

Synthesis of influenza A virus matrix M1 58–66 (119) by SPPS. Chlorotrityl chloride resin (200 mg, 0.3 mmol) and Fmoc-Leu-OH (265.1 mg, 0.75 mmol) were mixed in a manual solid-phase reactor under an argon atmosphere and stirred for 2.5 h in the presence of DIPEA (130.6 μL, 0.75 mmol) in 1,2-dichloroethane (1.5 mL). After washing with DMF (1.5 mL, ×5), capping was performed with MeOH (200 μL) in the presence of DIPEA (52.5 μL, 0.3 mmol) in DMF for 20 min. After washing with DMF (×5), DMF–H_2O (1 : 1, ×5), $CHCl_3$ (×2) and MeOH (×2) followed by drying *in vacuo*, the loading ratio (0.18 mmol) was determined photometrically from the amount of Fmoc chromophore liberated upon treatment with 50% piperidine–DMF for 30 min at 37 °C. Sequential Fmoc-protected amino acids (0.45 mmol) were manually coupled in the presence of DIPCDI (70.2 μL, 0.44 mmol) and HOBt (68.4 mg, 0.45 mmol) for 2 h in DMF (1.5 mL) after removal of each Fmoc group by 20% piperidine–DMF for 20 min (resin: 346.7 mg). The resulting protected peptide-resin (346.7 mg) was treated with TFA (6.7 mL)–*m*-cresol (173.3 μL)–thioanisole (173.3 μL)–H_2O (173.3 μL) for 90 min, concentrated *in vacuo*, washed with diethyl ether, centrifuged, suspended with water, and lyophilized to give the crude peptide **119** (161.1 mg). This crude peptide (20 mg) was dissolved in DMSO, filtered using 0.46 μm filter unit, and immediately injected into preparative HPLC with a 0.1% aqueous TFA–CH_3CN system. The desired fractions were collected and immediately lyophilized, affording the desired peptide **119**·TFA as a white amorphous powder (12.6 mg, 53%). TOF-MS: calcd. for $C_{49}H_{76}N_9O_{11}$ (M + H)$^+$: 967.17, found: 968.55; HPLC analysis at 230 nm: purity was higher than 95%.

Synthesis of H-Thr(H-Gly-Ile-Leu-Gly-Phe-Val-Phe)-Leu-OH (123) by the *O*-acyl isopeptide method without *O*-acyl isodipeptide unit (*O*-acyl isopeptide of influenza A virus matrix M1 58–66). Fmoc-Leu-resin was prepared in the same manner described above (2-chrolotrityl resin, 200 mg, 0.19 mmol). After deprotection of the Fmoc group by 20% piperidine in DMF, Boc-Thr-OH (103.6 mg, 0.47 mmol) was coupled using DIPCDI (74.1 μL, 0.47 mmol) and HOBt (72.1 mg, 0.47 mmol). Subsequent coupling with Fmoc-Phe-OH (219.7 mg, 0.57 mmol) to the β-hydroxyl group of Thr was performed using the DIPCDI (88.8 μL, 0.56 mmol)–DMAP (4.6 mg, 0.038 mmol) method in dry CH_2Cl_2 (1.5 mL) for 16 h (×2). Subsequent amino acid residues were coupled after removing each Fmoc group using 20% piperidine for 20 min (resin: 269.2 mg). The resulting protected peptide-resin (142.7 mg) was treated with TFA (2.8 mL)–thioanisole (71.3 μL)–*m*-cresol (71.3 μL)–distilled water (71.3 μL) for 90 min at rt, concentration *in vacuo*, Et_2O wash, centrifugation, suspension in water, and lyophilization to give the crude *O*-acyl isopeptide **123**·TFA (108.6 mg). TOF-MS: calcd. for $C_{49}H_{75}N_9O_{11}Na$ (M + Na)$^+$: 989.17, found: 989.20; HPLC analysis at 230 nm: purity was 93%.

Synthesis of influenza A virus matrix M1 58–66 (119) by the *O*-acyl isopeptide method with *O*-acyl isodipeptide unit

Fmoc-Leu-resin was prepared in the same manner described above (2-chlorotrityl chloride resin, 70 mg, 0.11 mmol). After deprotection of the Fmoc group by 20% piperidine in DMF, Boc-Thr(Fmoc-Phe)-OH (72.1 mg, 0.15 mmol, **38**) was coupled using DIPCDI (23.1 μL, 0.15 mmol) and HOBt (22.6 mg, 0.15 mmol). Subsequent amino acid residues were coupled after removing each Fmoc group using 20% piperidine for 20 min (resin: 113.8 mg). The resulting protected isopeptide-resin treated with TFA (2.1 mL)–thioanisole (56.9 μL)–*m*-cresol (56.9 μL)–H_2O (56.9 μL) (92.5 : 2.5 : 2.5 : 2.5) for 90 min at rt, concentrated *in vacuo*, washed with Et_2O, centrifuged, suspended with water, and lyophilized to give a crude isopeptide **123**·TFA (51.1 mg). This isopeptide (5.5 mg) was dissolved in phosphate buffer and stirred overnight at rt and lyophilized to give the crude **119**. This crude peptide was dissolved in DMSO, filtered using 0.46 μm filter unit, and immediately injected into preparative HPLC with a 0.1% aqueous TFA–CH_3CN system. The desired fractions were collected and immediately lyophilized, affording the desired peptide **119**·TFA as a white amorphous powder (5.0 mg, 73%). TOF-MS: calcd. for $C_{49}H_{75}N_9O_{11}Na$ (M + Na)$^+$: 989.17, found: 989.26; HPLC analysis at 230 nm: purity was 94%.

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