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Note

Synthesis of mimetic peptides containing glucosamine

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ABSTRACT

A convenient synthesis of 2-amino-3,4,6-tri-*O*-benzyl-2-deoxy-β-D-glucopyranoside was described from the readily available starting material 2-acetamido-2-deoxy-D-glucose (*N*-acetyl-D-glucosamine). Herein, the coupling of different lipophilic amino acids with 2-amino-3,4,6-tri-*O*-benzyl-2-deoxy-β-D-glucose was reported via an amide linkage as useful building blocks for the synthesis of glycopeptides. Of particular interest, bioactive peptide Arg-Gly-Asp (RGD) was incorporated into the building block containing valine was also reported. The 15 examples of corresponding di-, tri- and tetra-peptides were obtained as single αanomers.

The carbohydrates of glycoproteins have been subjected to an increasing interest during the last few years because they play important roles in numerous biological processes including modification of proteins, the immune response,[1–3] cell adhesion,[4–7] inflammation, tumor metastasis,[8] and substrate–receptor recognition.[9,4,3,10] The effects of carbohydrates on biological activity and stability of glycoproteins has been studied previously.[11–13]

Carbohydrates that coat the surfaces of bacteria are capable of inducing an immune response that can recognize whole bacteria,[14–18] and some functional glycoproteins that are expressed on tumor cell surfaces have been shown to induce specific antitumor cell–antibody responses in mice and patients.[19] There is a growing interest in carbohydrate mimetic peptides as vaccines to target cell-surface polysaccharides of infectious bacteria and tumor.[20–23]

Owing to the inherent complexity of carbohydrates, glycosylation can produce enormous structural diversity in proteins and induce a variety of functional changes. Since glycosyl amino acids and glycopeptides may be of value for medicinal chemistry, another important issue is their stability toward enzymatic degradation under physiological conditions. The field of glycobiology has grown explosively, and the interest in the development of methods for the synthesis of glycoconjugates is rapidly expanding as their biological and medical roles come into focus. Recent progress in this area has been remarkable and a number of carbohydrate moieties have been covalently attached to amino acids[25] for incorporation into glycopeptide sequences. Thus, many efforts have been devoted to establish easy and efficient methods for glycopeptide synthesis.[24–26]

The synthesis of monosaccharide conjugates is more straightforward than disaccharide or higher order oligosaccharide conjugates and this should facilitate a more rapid investigation of this possibility. We were interested to develop compounds reduced in carbohydrate character (monosaccharides). Amino sugars are widely distributed in living organisms and occur as constituents of glycoproteins, glycolipids, bacterial lipopolysaccharides, and proteoglycans. 2-Amino-2-deoxy-D-glucose (glucosamine) is the most common amino sugar and is generally found as an N-acetylated and β-linked glycoside that is a natural component of glycoproteins found in connective tissues and gastrointestinal mucosal membranes. However, the ability to synthesize glucosamine in the body declines with age and this, in turn, incapacitates the generation of proteoglycan, which is known to result in senile osteoarthritis (OA).[27] Therefore, glucosamine (GlcN) salts (sulfate and chloride) represent a new generation of drugs that possess potentially chondroprotective or disease-modifying properties and were originally suggested to promote the repair of damaged cartilage. A publication by W. Bohne in 1969 showed that GlcN can be used as a single pharmacologic agent to relieve the symptoms of osteoarthritis. Glucosamine has recently received a great deal of attention from the public as a potential treatment for OA.[28] Furthermore, the multiple antioxidant activity of glucosamine was evident as it showed pronounced reducing power, and superoxide/hydroxyl radical-scavenging ability. Because it is non-toxic, it may be a desired food supplement as a potential antioxidant. Experimental evidence was presented that glucosamine possess a unique range of anti-inflammatory activities and inhibits IL-1β- and TNF-α-induced NO production in normal human articular chondrocytes. It is also a therapeutic agent for inflammatory bowel diseases.[29]

Its amide bond is very stable in buffer solution at pH 7.4 and in culture medium. Amides are, in general, important functional groups widely found in natural products, pharmaceuticals, and polymers. They may be prepared by coupling reactions between

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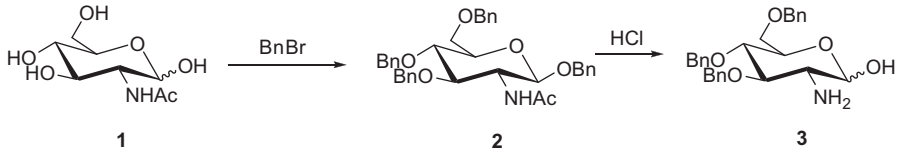
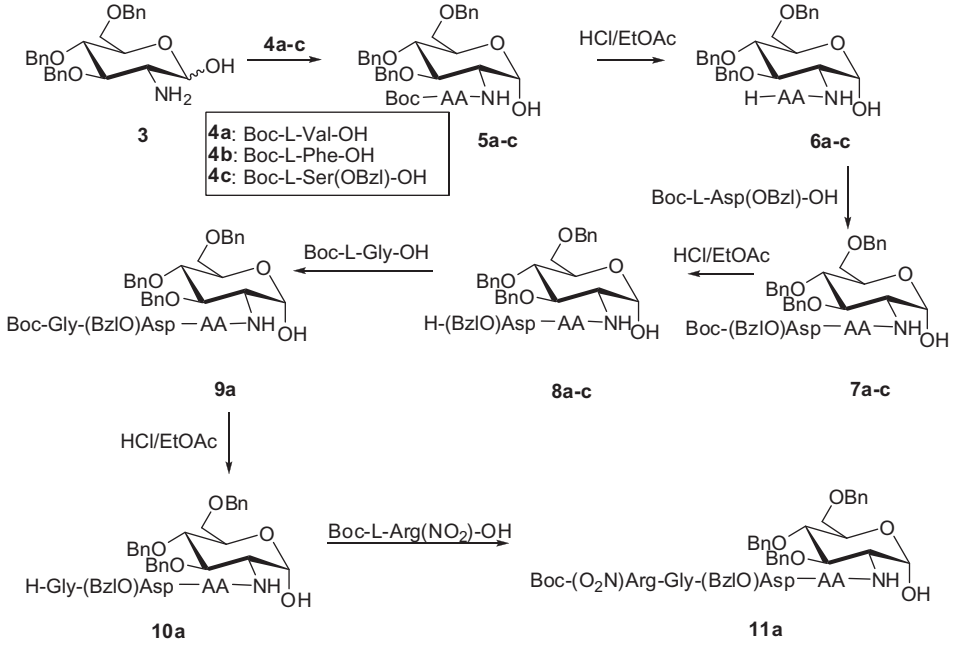
carboxylic acids and amines.[30–32] The synthetic design used for preparation of glycosyl amides was based upon a number of reasons: (1) glucosamine possess the biological activity; (2) *N*-acetylglucosamine has several potential advantages over glucosamine as a potential therapeutic anti-inflammatory agent. We report herein the synthesis of peptidomimetics in which Boc-protected amino acids were conjugated via an amide linkage to the amino sugar, D-glucosamine.[33] The resulting peptide mimetics can be incorporated in a peptide structure to develop compounds. In this context, we designed different lipophilic amino acids that were used as building units for the preparation of a series of peptide mimetics: these were D-glucosamine derivatives N-linked via valine, phenylalanine, and serine. Versatility is one of the major advantages of this stepwise procedure, which allows the synthesis of peptidomimetics with variations in the peptide part. Asparagines may be useful for the preparation of a diversity of structurally longer peptides.[34] As shown in Scheme 2 the building blocks **6a–c** were coupled with L-Boc-Asp (OBzl)-OH to furnish the asparagine conjugate dipeptides. After the protecting groups were removed in the stepwise protocol, a wider range of amino acids were explored and introduced either into the side chain or into the α-amino group of the asparagine to provide maximum diversity for peptide elongation. Furthermore, some bioactive peptides containing asparagines such as Arg-Gly-Asp (RGD) peptide were incorporated into the building blocks. Peptides related to RGD are known to contribute to various biological functions. Integrin receptors constitute a large family of proteins with structural characteristics of noncovalent heterodimeric glycoproteins formed of α and β subunits.[35–37] One important recognition site for many integrins is the arginine–glycine–asparagine tripeptide sequence. Regarding RGD-dependent integrins, $\alpha_v\beta3$ and $\alpha_v\beta5$ receptors have received increasing attention as therapeutic targets, as the integrins $\alpha_v\beta3$ and $\alpha_v\beta5$ can be expressed by both tumor cells and tumor endothelial cells.[38–40] It is speculated that drugs that inhibit the adhesive function of these integrins can inhibit tumor growth. Among selective receptor-targeting small peptides, integrinmediated RGD peptides appear as attractive candidates. Moreover, it is well known that RGD peptide can suppress platelet aggregation by blocking the interaction between platelet and fibrinogen.[41] In this study, an interest has been increased in modifying the building blocks by the addition of RGD and investigating the preparation for the tetrapeptide from the building block containing valine and RGD peptide by adopting stepwise approach. D-Glucosamine and *N*-acetyl-D-glucosamine have been reported to possess interesting biological activity,[42–45] therefore, the creation of these compounds would be useful for further biological evaluation, as well as for detailed structure–activity studies that may lead to potential drugs or provide leads for drug development.

The synthetic routes for the preparation of mimetic peptides containing D-glycosamine from *N*-acetyl-D-glucosamine (**1**) as starting material are presented in Schemes 1 and 2. The free OH groups of *N*-acetyl-D-glucosamine (**1**) were benzylated with BaO, $Ba(OH)_2{\cdot}8H_2O$ and BnBr to give benzyl 2-acetamido-1,3,4,6-tetra-*O*-benzyl-2-deoxy-β-D-glucopyranoside (**2**), which was easily obtained as the single anomer by simple crystallization from methanol in 67% yield from 1.68 mmol (0.3 g) of *N*-acetyl-D-gucosamine used. The ^{1}H NMR spectrum of **2** showed the signal for the anomeric proton at δ 5.49 as a doublet with a coupling



Scheme 1.



Scheme 2.

constant of 9.0 Hz, which was assigned as the β-anomer. It is believed that β-benzylation proceeds via neighboring group participation by an acyl group at *N*-**2** of the D-gucosamine. Problems were encountered with the removal of the acetyl group of intermediate **2** where using KOH in refluxing ethanol, NaOBu in butyl alcohol, hydrazine hydrate, hydrazine in refluxing ethanol, and aqueous methylamine, respectively, were ineffective. The acetyl group and the anomeric benzyl groups of **2** were removed by subjecting the **2** to a mixture of 3 N hydrochloric acid and terahydrofuran to give **3** as a mixture of anomers[46] (43% yield, α:β = 1:0.5) (Scheme 1).

Furthermore, a wider range of amino acids and monosaccharide derivatives **3** were explored to synthesize various glycosylated Boc amino acid building blocks. Initially, the pure mixture of anomers **3** and different lipophilic amino acids valine, phenyalanine and serine were reacted by standard peptide coupling methods (DDC, HOBt, and *N*-methylmolpholine) in solution phase. 1-Hydroxybenzotriazole (HOBt) was used to activate Boc-protected amino acids and dicyclohexylcarbodiimide (DCC) was used as the coupling reagent in tetrahydrofuran (THF). The products obtained were purified by silica gel column chromatography to afford **5a**–**c** as single anomers in 95%, 92% and 65% yield, respectively.[47] The assignment of the configuration was performed by ^{1}H NMR spectroscopy. The ^{1}H NMR spectra of **5a**–**c** showed signals for their anomeric protons as doublets with coupling constants of 3.0–3.5 Hz. In all cases, a noteworthy point is that the αanomers are the sole products, which can be attributed to the anomeric effect. Subsequently, removal of the Boc group with hydrogen chloride in ethyl acetate led to a set of amines **6a**–**c** which were used as building blocks for the facile preparation of longer peptides. We successfully prepared di-, tri- and tetra-peptides. Removal of the Boc group and subsequent coupling with the L-Boc-Asp (OBzl)-OH gave, sequentially, the protected forms of the dipeptides **7a**–**c** in 74%, 55% and 53% yield, respectively. ^{1}H NMR spectra indicated that **7a**–**c** were αanomers. Similarly, the iterative removal of the Boc group, together with the coupling reagents DCC/HOBt for peptide elongation, can be achieved. The coupling reaction of amine **8a** with Boc-protected glycine afforded only one α anomer tripeptide **9a** in 71% yield. Finally, we synthesized the tetrapeptide **11a** from the building block containing valine and the biological activity of a RGD peptide. Similarly, N-deprotection was performed and subsequent coupling the *N*-terminus free tripeptide **10a** and C-terminus free L-Boc-Arg (NO_2)-OH provided **11a** in 33% yield as a pure α anomer (Scheme 2). The Structures of mimetic peptides containing glucosamine are shown in Table 1.

Table 1
Structures of mimetic peptides containing 2-amino-2-deoxy-D-glucose (glucosamine)

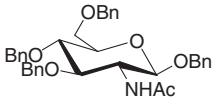
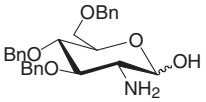
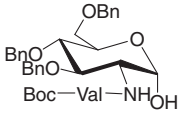
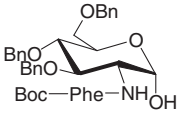
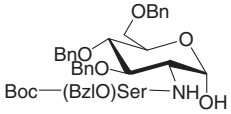
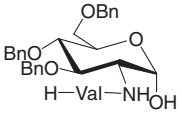
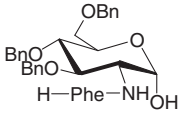
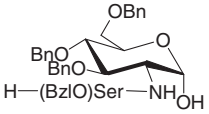
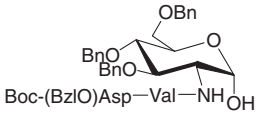
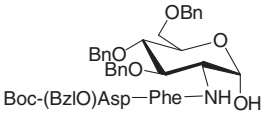
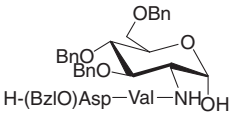
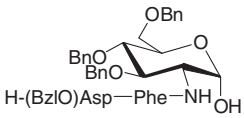
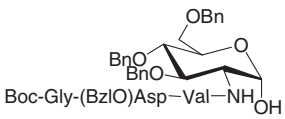
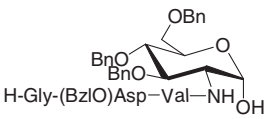
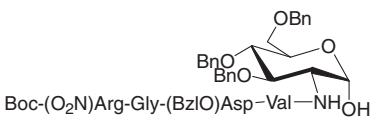
No	Chemical structure	No	Chemical structure
2		**3**	
5a		**5b**	
5c		**6a**	
6b		**6c**	
7a		**7b**	
7c		**8a**	
8b		**8c**	
9a		**10a**	
11a			

Table 2
Influenza virus neuraminidase inhibition for compounds **5a–c**, **7a–c**, **9a**, and **11a**

Compound	Concentration (μg/mL)	Inhibition ratio (%)
5a	40	39.72
5b	40	42.72
5c	40	42.28
7a	40	49.62
7b	40	41.55
7c	40	24.72
9a	40	44.16
11a	40	49.24
Zanamivir	0.0004	96.12

Both RGD and glucosamine have biological functions. We had this in mind when trying to decipher the possible biological role of the synthesized compounds **5a–c**, **7a–c**, **9a**, and **11a**, which were screened for their antioxidant activity and antitumor activity in our initial research. The results obtained exhibited undesired biological activity. These derivatives do not have a biological function similar to that of glucosamine and RGD in either antioxidant activity and antitumor activity. Benzylation of *N*-acetylglucosamine led to the disappearance of its antioxidant activity. It implied that the free hydroxyl group of *N*-acetyl-D-glucosamine was necessary for the antioxidant activity. The biological activity of these derivatives was further investigated. The anti-influenza virus neuraminidase (NA) activities were evaluated and the biological data are summarized in Table 2. A standard fluorimetric assay was used to measure influenza virus NA activity .The substrate MUNANA was cleaved by NA to yield a fluorescent product, which can be quantified. The reaction mixture containing the test compounds and NA enzyme from A/PR/8/34 (H1N1) in 32.5 mM MES buffer with 4 mM calcium chloride (pH 6.5) was incubated for 1 h at 37 °C. After incubation, the reaction was terminated by the addition of 34 mM NaOH. The fluorescence was quantified at an excitation wavelength of 360 nm and emission wavelength of 450 nm. The inhibition ratio (%) was the ratio of the decrease of the fluorescence intensity of reaction mixture containing the tested compounds to the fluorescence intensity of reaction mixture containing virus but no inhibitor. The assay results showed that all tested compounds possessed a weak NA inhibitory effect at the concentration of 40 μg/mL compared with the effect of the positive compound zanamivir. The tested compounds showed anti-influenza virus activity ranging from 24.72% to 49.62%. It was observed that **5a–c** as building blocks showed 39.72%, 42.72% and 42.28% inhibition, respectively, demonstrating that an amide bond linkage to a monosaccharide (D-glucosamine) was necessary for achieving potency against influenza virus. The compounds active against virus can be derived from scaffolds containing D-glucosamine monosaccharide.

To understand the contribution of the asparagines in dipeptides to the anti-influenza virus activities, **5a–c** were used as reference compounds, and the data indicated that when compared to **5a**, **7a** was more potent (49.62%), and when compared to **5b**, **7b** was equally active (41.55%), and when compared to **5c**, **7c** exhibited lower activity (24.72%). This comparison demonstrated that the asparagines in the dipeptides had a significant contribution to the activity. The dipeptides containing building blocks might allow the asparagines to assume different conformations that render the different activities observed for the analogues. The asparagine in **7a** may be the active conformation. The subsequent peptide chain elongation resulting tripeptide **9a** and tetrapeptide **11a** retained the potency of analogue **7a**. Structural changes do not affect biological activity. Compounds **9a** and **11a** showed 44.26%, and 49.24% inhibition, respectively, which is equivalent to that of **7a**. Further investigation is required on the biological activity of these compounds in order to rationalize these observations. This study provided useful information for the further design of novel potent agents.

In summary, we have developed an effective procedure for the preparation a series of peptide mimetics containing D-glucosamine via valine, phenyalanine and serine derivatives as building blocks which can be directly used for constructing parallel and combinatorial glycopeptide libraries as well as the synthesis of various new materials and peptide-based drug candidates. We synthesized the tetrapeptide from the building block containing valine and bioactive RGD peptide by adopting stepwise solution-phase approach. We expect that the incorporation of a building block in a bioactive peptide may induce intensifying effects. All tested compounds exhibited a potent neuraminidase inhibitory effect. Therefore, it was concluded that peptide mimetics as building blocks might be essential for activity and were judged to be the most attractive analogues for further optimization.

1. Experimental

1.1. General procedures

Unless otherwise stated, all reactions were carried out under a nitrogen atmosphere (1 bar). The agents used in this work were purchased from Sigma–Aldrich Chemical Co. (USA). Optical rotations were determined with a Schmidt–Haensch Polartromic D instrument (Germany). IR spectra were recorded with an Avatar 330 (Nicolet, USA) spectrometer.

^{1}H and ^{13}C NMR spectra were recorded at 300 MHz on a VXR-300 instrument or at 500 MHz on a Bruker AM-500 instrument in $CDCl_3$ or in DMSO-d_6 with Me_4Si as the internal standard. Chemical shifts are expressed in δ-units (ppm). Chromatography was performed on Qingdao silica gel H (Qingdao of China). The purities of the intermediates and the products were confirmed by TLC (E. Merck silica gel plates of type 60 F_{254}, 0.25-mm layer thickness, Germany) and HPLC (Waters, C_{18} column 4.6 × 150 mm, USA). Mass spectra (MS) were acquired on a Quattro Micro ZQ2000, Waters, USA instrument. High-resolution mass spectra were recorded with a JEOL JMS-T100LC AccuTOF mass spectrometer.

1.2. Introducing 3 into L-Val, L-Phe, and L-Ser

1.2.1. Preparation of 5a

HOBt (90 mg, 0.667 mmol) and DCC (151 mg, 0.734 mmol) were added to a solution of Boc-Val-OH 4a (145 mg, 0.668 mmol) in anhyd THF (10 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 0.5 h. The solution of **3** (300 mg, 0.668 mmol) in anhydrous THF (5 mL) was added and adjusted to pH 9 with *N*-methylmorpholine. The reaction mixture obtained was keep at 0 °C for 2 h and at room temperature for 24 h. Precipitated DCU was removed by filtration. The filtrate was evaporated under reduced pressure and the residue was dissolved in EtOAc (50 mL). The solution was washed successively with satd aq $NaHCO_3$, 5% $KHSO_4$ and satd aq NaCl and the organic phase was separated and dried over Na_2SO_4. After filtration and evaporation of the solvent under reduced pressure, purification by chromatography (40:1 CH_2Cl_2–MeOH) provided the product **5a** (410 mg, 0.633 mmol, 95%). $[\alpha]_D^{25}$ +53.0 (*c* 0.1, $CHCl_3$); IR (KBr, neat): 3417, 3328, 1691, 1655 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.38–7.08 (15H, m, Ar-H), 6.17 (1H,d, $J_{2,\ NH}$ 9.0 Hz, N–H), 5.27 (1H,d, $J_{1,\ 2}$ 3.0 Hz, H-1), 5.09 (1H, d, $J_{NH,\ \alpha}$ 8.0 Hz, N–H), 4.86–4.52 (6H, m, $PhCH_2$), 4.24 (1H, dt, $J_{2,\ 3}$ 10.0 Hz, $J_{1,\ 2}$ 3.0 Hz, H-2), 4.10 (1H, m, H-5), 3.88 (1H, t, $J_{2,\ 3} = J_{3,\ 4}$ = 10.0 Hz, H-3), 3.81 (2H, m, H-6a, H-6b), 3.73 (1H, dd, $J_{\alpha,\ \beta}$ 12.5 Hz, $J_{NH,\ \alpha}$ 8.0 Hz, H-α), 3.66 (1H, t, $J_{3,\ 4} = J_{4,\ 5}$ = 10.0 Hz, H-4), 2.03 (1H, m, CH), 1.47 (9H, s, CH_3), 0.88 (3H, d, *J* 7.0 Hz, CH_3), 0.85 (3H, d, *J* 7.0 Hz, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$): δ 172.1,

156.1, 138.3, 138.0, 137.9, 128.7, 128.5, 128.4, 128.3, 128.2, 128.0, 127.9, 127.8, 127.7, 127.6, 91.7, 80.0, 79.5, 78.4, 74.8, 74.7, 73.5, 70.9, 68.8, 60.3, 53.4, 30.8, 28.3, 19.5, 17.6; ESIMS: *m*/*z* 671(M+Na), 649 (M+1); HRMS calcd for: ($C_{27}H_{48}N_6O_{12}$ + Na), *m*/*z* (671.32279); found, *m*/*z* (671.32268).

1.2.2. Preparation of 5b

Compound **5b** was prepared by a method similar to the preparation of **5a** using **3** (140 mg, 0.312 mmol), and Boc-Phe-OH **4b** (83 mg, 0.312 mmol), HOBT (42 mg, 0.312 mmol), and DCC (71 mg, 0.343 mmol)**.** The residue was purified by silica gel column chromatography (40:1 CH_2Cl_2–MeOH) to provide the product **5b** (200 mg, 0.287 mmol, 92%). $[\alpha]_D^{25}$ +25.6 (*c* 0.1, $CHCl_3$); IR (KBr, neat): 3427, 3336, 1693, 1654,698 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 7.36–7.14 (20H, m, Ar-H), 6.32 (1H, d, $J_{2,\ NH}$ = 9.0 Hz, N–H), 5.20 (1H, d, $J_{1,\ 2}$ 3.5 Hz, H-1), 4.96 (1H, d, $J_{NH,\ \alpha}$ 8.0 Hz, N–H), 4.81–4.47 (6H, m, $PhCH_2$), 4.28 (1H, q, J_1 7.0 Hz, H-α), 4.20 (1H, td, $J_{2,\ 3}$ 10.0 Hz, $J_{1,\ 2}$ 3.5 Hz, H-2), 4.08 (1H, m, H-5), 3.81 (1H, t, $J_{2,\ 3} = J_{3,\ 4}$ = 10.0 Hz, H-3), 3.76 (2H, m, H-6a, H-6b), 3.67 (1H, t, $J_{3,\ 4} = J_{4,\ 5}$ = 10.0 Hz, H-4), 3.08 (1H, dd, J_1 14.0 Hz, J_2 6.0 Hz, CH_2), 2.99(1H, dd, J_1 14.0 Hz, J_2 7.0 Hz, CH_2), 1.37 (9H,s, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.7, 155.5, 138.3, 138.0, 137.9, 136.6, 136.3, 129.3, 128.6, 128.4, 128.1, 127.9, 127.7, 126.9, 91.7, 80.4, 79.6, 78.2, 74.8, 74.7, 73.5, 70.9, 68.8, 53.6, 49.4, 33.8, 28.2; ESIMS: *m*/*z* 719 (M+Na), 697 (M+1); HRMS: calcd for ($C_{30}H_{52}N_2O_{16}$ + Na), *m*/*z* (719.32155); found, *m*/*z* (719.32131).

1.2.3. Preparation of 5c

Compound **5c** was prepared by a method similar to the preparation of **5a** using **3** (400 mg, 0.891 mmol), Boc-Ser (OBzl)-OH **4c** (263 mg, 0.891 mmol), HOBT (120 mg, 0.891 mmol), and DCC (202 mg, 0.980 mmol). The residue was purified by silica gel column chromatography (40:1 CH_2Cl_2–MeOH) to provide the product **5c** (420 mg, 0.579 mmol, 65%). $[\alpha]_D^{25}$ +36.9 (*c* 0.1, $CHCl_3$); IR (KBr, neat): 3328, 1695, 1655 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 7.36–7.14 (20H, m, Ar-H), 6.79 (1H, d, $J_{2,\ NH}$ 9.5 Hz, N–H), 5.45 (1H, d, $J_{NH,\ \alpha}$ 9.0 Hz, N–H), 5.21 (1H, d, $J_{1,\ 2}$ 3.0 Hz, H-1), 4.87–4.42 (6H, m, $PhCH_2$), 4.29 (2H, dt, $J_{2,\ 3}$ 10.0 Hz, $J_{1,\ 2}$ 3.0 Hz, H-2), 4.23 (1H, m, H-α), 4.11 (1H, m, H-5), 3.856–3.622 (5H, m, H-3, H-4, H6a, H6b, CH_2), 3.51 (1H, dd, J_1 9.5 Hz, J_2 6.5 Hz, CH_2), 1.44 (9H, s, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$): δ 172.9, 170.3, 156.9, 155.5, 138.4, 138.1, 138.0, 137.9, 137.4, 128.5, 128.4, 128.3, 127.9, 127.8, 127.7, 127.6, 127.5, 92.1, 80.3, 78.2, 74.9, 74.8, 73.5, 73.4, 73.2, 70.9, 69.7, 69.0, 53.6, 49.2, 28.3; ESIMS: *m*/*z* 749 (M+Na), 727 (M+1); HRMS: calcd for ($C_{31}H_{54}N_2O_{17}$ + Na), *m*/*z* (749.33202); found, *m*/*z* (749.33120).

1.3. Deprotection of 5a–c

1.3.1. Preparation of 6a

A solution of **5a** (246 mg, 0.380 mmol) in HCl in EtOAc (8 mL, 4 mol/L) was stirred at 0 °C for 3 h. The solvent was evaporated and the residue was dissolved in EtOAc (20 mL) and again evaporated to dryness. The resulting solid was used immediately in the coupling reaction of the next step without additional purification.

1.3.2. Preparation of 6b

Compound **6b** was prepared by a method similar to the preparation of **6a** using **5b** (170 mg, 0.244 mmol). The resulting solid was used immediately in the coupling reaction of the next step without additional purification.

1.3.3. Preparation of 6c

Compound **6c** was prepared by a method similar to the preparation of **6a** using **5c** (270 mg, 0.372 mmol). The resulting solid was used immediately in the coupling reaction of the next step without additional purification.

1.4. Introducing 3 into L-Val-Asp, L-Phe-Asp, and L-Ser-Asp

1.4.1. Preparation of dipeptide derivative 7a

Compound **7a** was prepared by a method similar to the preparation of **5a** using **6a** and Boc-L-Asp (OBzl)-OH (123 mg, 0.380 mmol), HOBT (51 mg, 0.380 mmol), and DCC (86 mg, 0.418 mmol). The residue was purified by silica gel column chromatography (40:1 CH_2Cl_2–MeOH) to provide the product **7a** (240 mg, 0.281 mmol, 74%). $[\alpha]_D^{25}$ +21.4 (*c* 0.1, $CHCl_3$); IR (KBr, neat): 3435, 3311, 1699, 1645 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 7.36–7.11 (20H, m, Ar-H), 7.04 (1H, d, $J_{NH,\ \alpha}$ 8.7 Hz, N–H), 6.58 (1H, d, $J_{2,\ NH}$ 9.0 Hz, N–H), 5.70 (1H, d, $J_{NH,\ \alpha}$ 8.1 Hz, N–H), 5.16 (1H, d, $J_{1,\ 2}$ 3.3 Hz, H-1), 5.10 (2H, s, $PhCH_2$), 4.85–4.48 (7H, m, $PhCH_2$, H-α), 4.24 (2H, m, H-α, H-2), 4.08 (1H, m, H-5), 3.91 (1H, t, J_1 9.0 Hz, H-3), 3.80–3.71 (2H, m, H6a, H6b), 3.67 (1H, t, J_1 9.0 Hz, H-4), 2.98 (1H, dd, J_1 16.8 Hz, J_2 4.5 Hz, CH_2), 2.82 (1H, dd, J_1 16.8 Hz, J_2 6.3 Hz, CH_2), 2.14 (H, m, CH), 1.44 (9H, s, CH_3), 0.92 (3H, d, *J* 6.6 Hz, CH_3), 0.84 (3H, d, *J* 6.6 Hz,CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.6, 171.1, 170.9, 155.6, 138.5, 138.2, 138.0, 135.3, 135.2, 128.6, 128.5, 128.4, 128.3, 128.2, 127.9, 127.8, 127.7, 127.6, 127.5, 127.4, 91.6, 80.9, 79.5, 78.5, 74.8, 74.7, 73.4, 70.7, 68.7, 67.0, 58.7, 53.6, 51.1, 35.7, 30.1, 28.3, 19.5, 17.6; ESIMS: *m*/*z* 876 (M+Na), 854 (M+1); HRMS: calcd for ($C_{43}H_{59}N_5O_{13}$ + Na), *m*/*z* (876.40070); found, *m*/*z* (876.40063).

1.4.2. Preparation of dipeptide derivative 7b

Compound **7b** was prepared by a method similar to the preparation of **5a** using **6b** and Boc-L-Asp (OBzl)-OH (79 mg, 0.244 mmol), HOBT (33 mg, 0.244 mmol), and DCC (55 mg, 0.268 mmol). The residue was purified by silica gel column chromatography (40:1 CH_2Cl_2–MeOH) to provide the product **7b** (120 mg, 0.133 mmol, 55%). $[\alpha]_D^{25}$ +13.7 (*c* 0.1, $CHCl_3$); IR (KBr, neat): 3401, 3315, 3064, 1735, 1647, 1697, 1519 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 7.37–7.03 (25H, m, Ar-H), 6.85 (1H, d, $J_{NH,\ \alpha}$ 8.1 Hz, N–H), 6.47 (1H, d, $J_{2,\ NH}$ 8.7 Hz, N–H), 5.47 (1H, d, $J_{NH,\ \alpha}$ 8.7 Hz, N–H), 5.17 (1H, d, $J_{1,\ 2}$ 3.3 Hz, H-1), 5.10 (2H, t, *J* 4.0 Hz, $PhCH_2$), 4.81–4.48 (7H, m, $PhCH_2$, H-α), 4.41 (1H, m, H-α), 4.19 (1H, dt, J_1 9.6 Hz, J_2 = 3.3 Hz, H-2), 4.03 (1H, m, H-5), 3.85 (1H, t, *J* 9.6 Hz, H-3), 3.74–3.61 (3H, m, H6a, H6b, H-4), 3.12 (1H, dd, J_1 14.1 Hz, J_2 6.0 Hz, CH_2), 3.03 (1H, dd, J_1 14.1 Hz, J_2 6.9 Hz, CH_2), 2.91 (1H, dd, J_1 17.1 Hz, J_2 4.8 Hz, CH_2), 2.75 (1H, dd, J_1 17.1 Hz, J_2 6.3 Hz, CH_2), 1.41 (9H, s, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.5, 170.8, 170.7, 155.5, 138.6, 138.2, 138.0, 136.5, 135.2, 129.3, 128.7, 128.6, 128.5, 128.4, 128.3, 127.9, 127.8, 127.6, 127.5, 126.9, 91.6, 81.1, 79.7, 78.3, 74.8, 74.7, 73.4, 70.7, 68.7, 67.1, 54.1, 53.9, 51.0, 36.7, 35.8, 28.2; ESIMS: *m*/*z* 924 (M+Na), 902 (M+1); HRMS: calcd for ($C_{37}H_{59}N_9O_{17}$ + Na), *m*/*z* (924.39266); found, *m*/*z* (924.39006).

1.4.3. Preparation of dipeptide derivative 7c

Compound **7c** was prepared by a method similar to the preparation of **5a** using **6c** and Boc-L-Asp (OBzl)-OH (120 mg, 0.372 mmol), HOBT (50 mg, 0.372 mmol), and DCC (84 mg, 0.409 mmol). The residue was purified by silica gel column chromatography (40:1 CH_2Cl_2–MeOH) to provide the product **7b** (184 mg, 0.197 mmol, 53%). $[\alpha]_D^{25}$ +24.1 (*c* 0.1, $CHCl_3$); IR (KBr, neat): 3334, 1733, 1699, 1643 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 7.38–7.13 (25H, m, Ar-H), 6.89 (1H, d, $J_{NH,\ \alpha}$ 9.5 Hz, N–H), 5.56 (1H, d, $J_{NH,\ \alpha}$ 7.5 Hz, N–H), 5.17 (1H, d, $J_{2,\ NH}$ 10.5 Hz, N–H), 5.16 (1H, t, $J_{1,\ 2}$ 3.0 Hz, H-1), 5.12 (2H, dd, J_1 22.0 Hz, J_2 12.0 Hz, $PhCH_2$), 4.85–4.34 (8H, m, $PhCH_2$, H-α_1, H-α_2), 4.28 (1H, dt, J_1 9.5 Hz, J_2 3.0 Hz, H-2), 4.08(1H, m, H-5), 3.90 (1H, t, *J* 9.5 Hz, H-3),

3.81–3.58 (4H, m, H-4, H-6a, H-6b, CH_2), 3.53 (1H, dd, J_1 9.5 Hz, J_2 6.0 Hz, CH_2), 3.01 (1H, dd, J 17.0 Hz, J_2 4.5 Hz, CH_2), 2.82 (1H, dd, J_1 17.0 Hz, J_2 5.0 Hz, CH_2), 1.45 (9H, s, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.5, 171.3, 170.8, 169.6, 155.5, 138.6, 138.2, 137.9, 137.5, 137.4, 135.3, 135.1, 129.8, 128.6, 128.4, 128.3, 128.2, 127.9, 127.8, 127.7, 127.5, 92.0, 80.9, 80.2, 78.3, 74.9, 74.8, 73.4, 73.3, 70.8, 69.1, 68.9, 67.0, 53.8, 53.1, 51.1, 36.1, 28.3; ESIMS: m/z 954 (M+Na), 932 (M+1); HRMS: calcd for ($C_{43}H_{61}N_7O_{16}$ + Na), m/z (954.40725); found, m/z (954.40714).

1.5. Deprotection of tripeptide derivatives

1.5.1. Preparation of 8a

Compound **8a** was prepared by a method similar to the preparation of **6a** using **7a** (160 mg, 0.188 mmol). The resulting solid was used immediately in the coupling reaction of the next step without additional purification.

1.6. Introducing 3 into L-Val-Asp-Gly

Compound **9a** was prepared by a method similar to the preparation of **5a** using **8a** and Boc-L-Gly-OH (33 mg, 0.188 mmol), HOBT (25 mg, 0.188 mmol), and DCC (43 mg, 0.207 mmol). The residue was purified by silica gel column chromatography (40:1 CH_2Cl_2–MeOH) to provide the product **9a** (121 mg, 0.133 mmol, 71%). $[\alpha]_D^{25}$ +10.4 (c 0.1, $CHCl_3$); IR (KBr, neat): 3436, 3282, 1718, 1639 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.82 (1H, d, $J_{NH,\ \alpha}$ 7.8 Hz, N–H), 7.76 (1H, d, $J_{NH,\ \alpha}$ 7.8 Hz, N–H), 7.44–7.14 (20H, m, Ar-H), 6.64 (1H, br s, N–H), 5.45 (1H, br s, N–H), 5.18 (1H, d, $J_{1,\ 2}$ 3.3 Hz, H-1), 5.10 (2H, m, $PhCH_2$), 4.89–4.49 (7H, m, $PhCH_2$, H-α_1), 4.24 (2H, m, H-2, H-α_2), 4.10 (1H, m, H-5), 3.99–3.60 (6H, m, H-3, H-4, H-6a, H-6b, α-CH_2), 2.99 (1H, m), 2.98 (1H, dd, J_1 16.8 Hz, J_2 7.2 Hz, CH_2), 2.82 (1H, dd, J_1 16.8 Hz, J_2 6.0 Hz, CH_2), 2.18 (1H, m, CH), 1.45 (9H, s, CH_3), 0.83 (3H, d, J 6.6 Hz,CH_3), 0.89 (3H, d, J 6.6 Hz,CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.4, 171.2, 170.5, 156.4, 138.5, 138.2, 138.1, 135.2, 128.6, 128.3, 127.8, 127.7, 127.6, 127.5, 126.8, 111.3, 91.5, 80.9, 79.6, 78.5, 74.8, 73.4, 70.8, 68.9, 67.1, 60.0, 53.9, 50.8, 50.1, 35.4, 29.5, 28.3, 19.5, 17.6; ESIMS: m/z 933 (M+Na); HRMS: calcd for ($C_{38}H_{66}N_6O_{19}$ + Na), m/z (933.42804); found, m/z (933.42609).

1.7. Deprotection of the tripeptide derivative

Compound **10a** was prepared by a method similar to the preparation of **6a** using **9a** (100 mg, 0.1094 mmol). The resulting solid was used immediately in the coupling reaction in the next step without purification.

1.8. Introducing 3 into L-Val-Asp-Gly-Arg

Compound **11a** was prepared by a method similar to the preparation of **5a** using **10a**, Boc-L-Arg (NO_2)-OH (35 mg, 0.1094 mmol), HOBT (15 mg, 0.1094 mmol), and DCC (25 mg, 0.120 mmol). The residue was purified by silica gel column chromatography (40:1 CH_2Cl_2–MeOH) to provide the product **11a** (40 mg, 0.0361 mmol, 33%). $[\alpha]_D^{25}$ +39.4 (c 0.1, $CHCl_3$); ^{1}H NMR (300 MHz, DMSO): δ 8.44 (1H, br s, N–H), 8.27 (1H, d, $J_{NH,\ \alpha}$ 7.8 Hz, N–H), 8.16 (1H, d, $J_{NH,\ \alpha}$ 7.8 Hz, N–H), 8.06–7.81(2H, br s, N–H), 7.62 (1H, d, $J_{2,\ NH}$ 9.6 Hz, N–H), 7.33–7.14 (20H, m, Ar-H), 6.93 (1H, d, $J_{NH,\ \alpha}$ 7.2 Hz, N–H), 6.76 (1H, d, J 4.2 Hz, N–H), 5.06 (2H, m, $PhCH_2$), 4.92 (1H, t, $J_{1,\ 2}$ 3.6 Hz, H-1), 4.76–4.39 (7H, m, $PhCH_2$, H-α_1), 4.31 (1H, dd, J_1 9.0 Hz, J_2 5.5 Hz, H-α_2), 4.19–3.40 (9H, m, H-2, H-3, H-4, H-5, H-6a, H-6b, H-α_3, H-α_4), 3.12 (2H, m, CH_2), 2.82 (1H, dd, J_1 16.5 Hz, J_2 6.0 Hz, CH_2), 2.62 (1H, dd, J_1 16.5 Hz, J_2 8.1 Hz, CH_2), 1.94 (1H, m, CH), 1.50 (4H, m, $-CH_2-CH_2-$), 1.37 (9H, s, CH_3), 0.75 (3H, d, J 6.6 Hz, CH_3), 0.89 (3H, d, J 6.6 Hz, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 172.7, 171.3, 170.5, 170.3, 169.4, 159.8, 155.9, 139.2, 138.8, 136.5, 128.8, 128.7, 128.6, 128.4, 128.3, 128.1, 127.9, 127.7, 91.3, 79.4, 79.2, 78.7, 74.5, 74.1, 72.8, 70.2, 69.6, 66.6, 66.2, 57.9, 54.4, 53.9, 49.8, 42.4, 36.3, 31.4, 29.6, 28.7, 25.3, 19.8, 17.7; ESIMS: m/z 1134 (M+Na), 1112 (M); HRMS: calcd for ($C_{55}H_{77}N_5O_{19}$ + Na), m/z (1134.51104); found, m/z (1134.51073).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2011.04.010.

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