Tetrahedron 65 (2009) 8269–8276

Contents lists available at ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

The urea-dipeptides show stronger H-bonding propensity to nucleate β-sheetlike assembly than natural sequence

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ARTICLE INFO

Article history:
Received 27 April 2009
Received in revised form 20 July 2009
Accepted 22 July 2009
Available online 25 July 2009

ABSTRACT

In this article, we report the distinct solution behavior of a set of urea-dipeptides to that of natural sequence. The urea-dipeptides adopt β-folding conformations and form into β-sheetlike assembly in chloroform. Most surprisedly, the urea-dipeptides tend to form interpeptide H-bonding interactions even at a concentration of as low as 0.1 mM, while the natural sequence shows H-bonding propensity at a concentration of about 7 mM, indicating that the urea-dipeptides show much stronger H-bonding propensity to nucleate formation of β-sheetlike assembly than the natural sequence. CD spectra reveal that the investigated urea-dipeptides have two negative CD bands, respectively, around 217 nm and 224 nm, supporting the β-folding conformations and in turn formation of β-sheetlike assembly. The β-sheetlike assembly is also confirmed by the XRD reflections, which give two typical *d*-spacings of 12.7 and 4.8 Å, respectively, corresponding to stacking periodicity of the β-sheets and the spacing between peptide backbones running orthogonal to the β-sheet axis.

1. Introduction

H-bonds play a vital role in forming protein secondary structures such as α-helices, β-sheets, and turn structures. They are also a central feature in protein β-sheet interactions and protein–protein interactions. However, natural short peptides generally do not participate in specific H-bonds, due to the high flexibility of peptide main chain and weak interstrand H-bonding interactions between the peptide amides.

Compared with the amide, the urea unit possesses elegant hydrogen-bond-forming capacity between –C═O and –NH units. Moreover, it may provide one more H-bond, and thus may cause additional affinity in molecular recognition. Based on these considerations, the urea has been widely used as a hydrogen-bonding functionality either in supramolecular constructions,[1–7] potential inhibitors for the epoxide hydrolase,[8] mimicking β-sheets,[9] or peptide backbone mimetics, for examples, the *N*,*N*′-linked linear-[10–16] and cyclo-oligoureas developed by replacing the amide with urea unit.[17] Several reports have shown that the linear-oligoureas bearing proteinogenic side-chains can form stable helical structures in solution and solid states,[11–14] and the cyclo-oligoureas can assemble into nanotubes.[17]

Alternatively, nature also selects the urea moiety as a H-bonding functionality, for examples, the naturally occurring linear tetrapeptides and cyclic peptides, such as GE 20372 A and B,[18] and (*S*)-α- and (*R*)-β-MAPI,[19–22] monzamide A and B,[23] Schizopeptin 791,[24] brunsvicamides (BVA) A–C,[25] and psymbamide.[26] These naturally occurring compounds all contain an unusual urea-dipeptide segment, in which two amino acid residues are anti-parallel linked at the amine terminals to form a urea unit.[20] Inspired by these, a set of peptidomimetics, which bear a urea-dipeptide segment were developed[20,21,27,28] and were shown to be efficient protease inhibitors.[18–22,27–30]

Similar to the interesting structural features and in turn the highly strong and stable intermolecular H-bonds of imide-dipeptides,[31] which have been created by us, the urea-dipeptide segment also introduces the same chemical structural characters unmatched in the natural sequence (Scheme 1), including (i) self-pairing H-bonds; (ii) a peptide polindron sequence; (iii) topochemical symmetry (*C*2 symmetry); (iv) different orientation of the two side-chains. Expectedly, all these structural characters may also show positive or negative influences on the H-bonding-forming capacity and recognition affinity, and thus may result in a different H-bonding pattern to the natural sequence, as do the imide-dipeptides observed by us.[31] However, what is the difference and how the distinct structural characters influence on H-bonding

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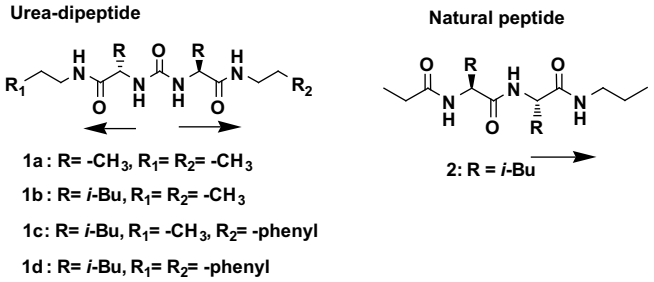
doi:10.1016/j.tet.2009.07.048



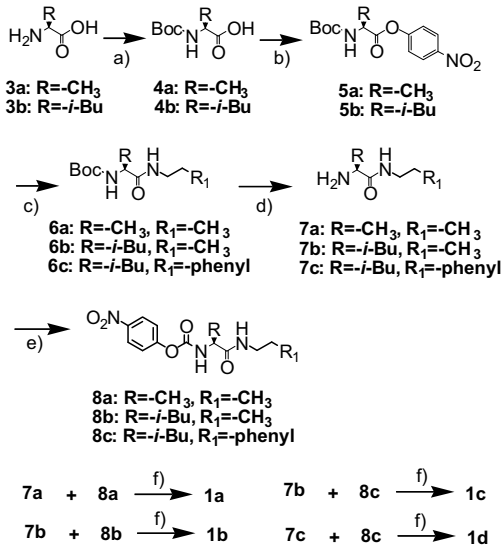
Scheme 1. Chemical structures of the urea-dipeptides (**1**) and a selected natural sequence (**2**).

interactions are unknown yet. Accordingly, we synthesized a set of urea-dipeptides (**1**)—the shortest urea-linked peptidomimetics and a natural sequence **2**. A comparison study of the solution behavior between **1** and **2** indicates that the urea-dipeptides show much stronger H-bonding propensity to nucleate the β-sheetlike assembly.

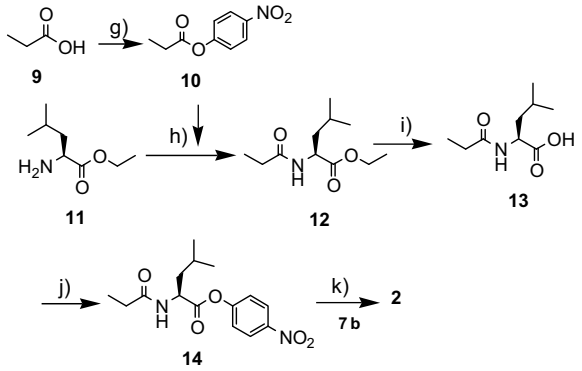
2. Results and discussion

2.1. Synthesis of the dipeptides

The urea-dipeptides **1** and the selected natural sequence **2** were successfully synthesized from the commercial amino acids by following 5–6 steps, as shown in Schemes 2 and 3. We selected 4-nitrophenyl as an activating group[31a,32] to convert either carboxylic acid to amide or amine to urea. The commercial L-alanine or L-leucine (**3**) was first converted into *N*-Boc-protected amino acid (**4**) in 10:1 water/THF mixture, in which about 10% THF was used to dissolve $(Boc)_2O$ to afford a homologous reaction solution. Next, 1-propyl amine or 2-phenylethyl amine in acetonitrile efficiently replaced the activating 4-nitrophenoyl group in **5** at rt to give **6**. Reacting the Boc-deprotected **7** and 4-nitrophenyl chloroformate yielded compound **8**. Finally, coupling between compounds **7** and **8** in acetonitrile at rt successfully yielded the urea-dipeptides **1** as white precipitate.



Scheme 2. Synthesis of the urea-dipeptides (**1**). Reaction conditions: (a) $(Boc)_2O$, KOH, H_2O/THF (10:1), 50 °C, 85–95%; (b) 4-nitrophenol/DCC, DCM, 80–90%; (c) 1-propyl amine or 2-phenylethyl amine, acetonitrile, 90–95%; (d) TFA, DCM, ~100%; (e) 4-nitrophenyl chloroformate, THF, 0 °C, 60–70%; (f) acetonitrile, rt, 50–80%. Note: THF, tetrahydrofuran; DCC, *N,N′*-dicyclohexyl carbodiimide; DCM, dichloromethane; TFA, trifluoroacetic acid.



Scheme 3. Synthesis of the natural sequence **2**. Reaction conditions: (g) 4-nitrophenol/DCC, DCM, 90%; (h) acetonitrile/rt, 90–95%; (i) NaOH, EtOH/H_2O, ~95%; (j) 4-nitrophenol/DCC, DCM, 80–90%; (k) acetonitrile (containing 5% methanol), rt, 76%.

The dipeptide **2**[31b] was synthesized by following a procedure as shown in Scheme 3. 1-Propyl acid was first activated by using 4-nitrophenoyl and then reacted with *N*-unprotected L-leucine ethyl ester (**11**) at rt to give *N*-ethyl-carbomoyl L-leucine ethyl ester (**12**), which was further hydrolyzed to *N*-ethyl-carbomoyl L-leucine (**13**) by using sodium hydroxide in 1:1 ethanol/water. Then, carboxylic acid group of **13** was again functionalized with 4-nitrophenoyl to yield **14**. Reaction of **14** with **7b** at rt in acetonitrile afforded the expected dipeptide **2**.

2.2. Solubility

Compound **1a** is insoluble in chloroform, while **1b** shows improved solubility in chloroform with a value of ~3 mM at rt. The concentration of **1b** can reach up to 20 mM upon heating, while the compound may precipitate within several minutes, depending on the concentrations, for example, **1b** precipitates within about 3 min when cooling the 20 mM chloroform solution. This 'temporal' solubility makes it be possible to measure the ^{1}H NMR spectra approximately. The solubility of the urea-dipeptides can be further improved largely by replacing one or two *n*-propyl (*n*-Pr) groups with phenylethyl (ϕe) units, for example, the solubility of both **1c** and **1d** can reach up than 70 mM at rt.

2.3. Interpeptide H-bonding interactions

In general, the amide-protons engaged in intermolecular H-bonds give a characteristic of nonlinear upfield shift with an increase of the concentration, whereas, the chemical shifts of intramolecularly H-bonded amide-protons show an nearly independence on concentration.

Figure 1a reveals that compounds **1b**–**1d** exhibit a typically nonlinear concentration dependence of the δ_{-NH}s, characteristically indicating intermolecularly H-bonded-NHs, and therefore suggesting the β-sheetlike aggregates of the urea-dipeptides. As expected, the amide-protons of **1b**–**1d** all show a distinct concentration-dependent behavior to that of compound **2** (Fig. 1b). At a low concentration level, typically, 1–7 mM, the amide-protons in the natural peptide **2** do not show any downfield shift with the increase of concentration, suggesting that the amide-protons remain most likely free of H-bonds. Conversely, the δ_{-NH}s of **1b**–**1d** all show a dramatical increase as the increase of concentration, revealing that the urea-dipeptides hold much stronger H-bonding propensity than the natural sequence. Such a strong H-bonding propensity is supported by the fact that no plateau was observed even when the concentration is down to 0.1 mM, a concentration being about two orders of magnitude lower than that concentration (about 7 mM) for the natural sequence starting to show H-bonding

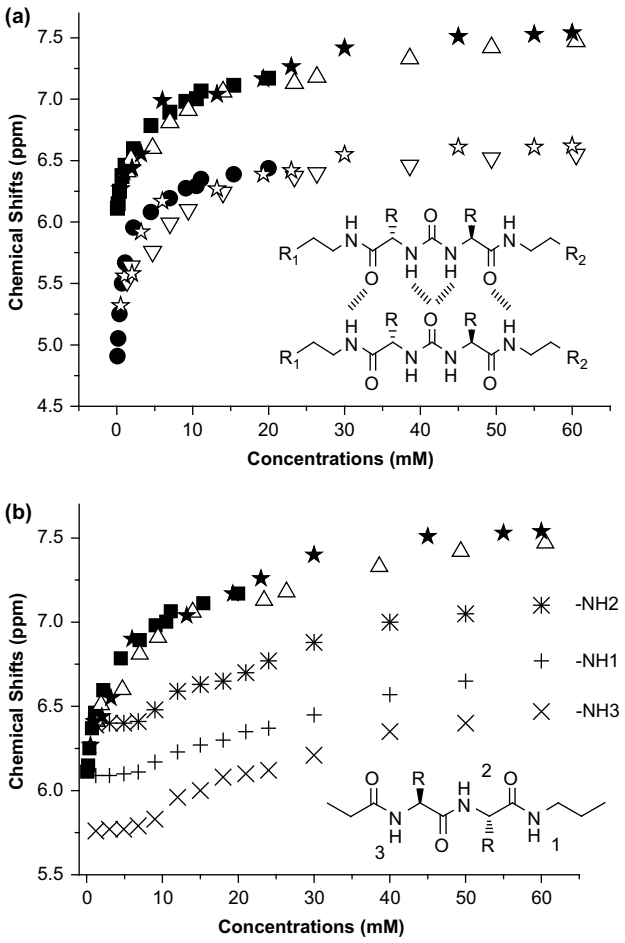


Figure 1. (a) Concentration-dependent δ_{-NH}s of the amide-protons and urea-protons of **1b** (■ and ●), **1c** (★ and ☆) and **1d** (△ and ▽). Inset shows self-pairing and β-sheetlike assembly of **1** in $CDCl_3$. (b) Concentration-dependent δ_{-NH}s of the amide-protons of **2** in $CDCl_3$ (+, ⁎, and ×). Inset shows NH numbers in dipeptide **2**, assigned by both COSY and NOE spectra, as compared, it also shows the δ_{-NH}s of the amide-NHs of **1b**–**1d** at different concentrations.

propensity. When concentration exceeds 10 mM, the amide-protons of **1b**–**1d** nearly reach their plateau, indicating maturation of interstrand H-bonding interactions and β-sheetlike aggregates. The amide-protons of **2**, however, continuously increase higher and higher, suggesting the propensity to form H-bonds at this high concentration level.

2.4. Characterizations of the β-sheetlike assembly

2.4.1. $^3J_{HN\alpha}$ coupling constants. The magnitude of the $^3J_{HN\alpha}$ coupling constant for a peptide residue is dependent on the ϕ-angle (N–C^{α}), therefore on the local conformation of the peptide chain.[33] A β-folding conformation generally gives a $^3J_{HN\alpha}$ coupling constant in the range of 8–10 Hz, while an unstructured random coil conformation yields a $^3J_{HN\alpha}$ coupling constant ranging from 5.8 to 7.3 Hz. Additionally, Wishart et al.[34] have produced a simple method for secondary structure determination by analyzing the difference between the α-proton chemical shift for each residue and for the same residue type in a 'random coil' conformation. Accordingly, β-strands consistently show greater chemical shifts than that of the random coil. In our cases, the α-protons of **1b**–**1d** all show a characteristically nonlinear shift downfield from 4.17 ppm (a chemical shift relative to the leucine residue type in random coil conformations) to 4.30 ppm when the concentration increases. And the α-protons of **1b**–**1d** give a $^3J_{HN\alpha}$ coupling constant of 8.0 Hz at a high concentration level (typically >10 mM). These results indicate that the dipeptide chains adopt β-folding conformations at high concentration levels, at which the peptide molecules form matured β-sheetlike assembly (typically, >10 mM).

2.4.2. NOE spectra. The NOE experiment is an ideal tool to characterize formation of β-sheetlike structures. In β-sheetlike assembly, interstrand NOE signals are generally observed between amide-protons and α-protons of opposite residues, and that between side-chains and between backbone protons and side-chain protons. Other evidences include intramolecular NOE contacts in a pleated peptide chain. These NOE signals characterize that the peptide chains are intermolecularly H-bonded together to form β-sheetlike assembly. As shown by NOE experiment of **1d** (Fig. 2), for example, the strong interstrand NOE coupling between the urea- and amide-NHs and NOEs from side-chains to backbones demonstrate the formation of the β-sheetlike assembly. The intramolecular NOE signals between the amide-NHs and α-protons and that between the ϕe-αC-protons and the phenyl-protons all indicate the β-folding conformations of the peptide chains, further confirming the β-sheetlike assembly. In addition, the β-sheetlike assembly is consistent with the fact that the presence of phenyl π–π stacks in **1c** or **1d** intensifies as concentration increases (Fig. 3).

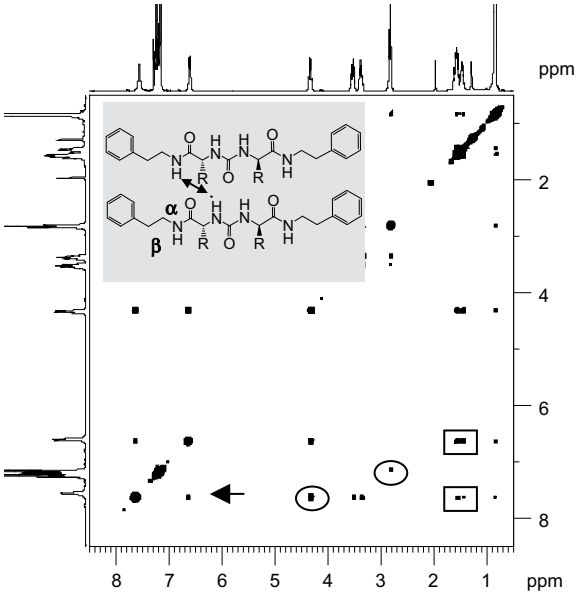


Figure 2. NOESY spectrum of **1d** in $CDCl_3$ solution carried out at 74 mM and 25 °C (mixing time 0.8 s). The interstrand NOE is indicated by arrow, the intrastrand NOE is labeled by unfilled circles, and NOEs of side-chain to backbone are pointed by unfilled squares.

2.4.3. CD spectra. CD is a main and direct evidence to characterize formation of the β-sheetlike assembly. In general, peptides with natural sequences forming β-sheet assembly give a negative CD band around 216 nm.[35] As shown in Figure 4, CD spectra of **1b**–**1d** conducted in chloroform solutions all show a negative CD band around 217 nm, suggesting the formation of the β-sheetlike assembly. In addition, all three compounds also yield a negative CD band around 225 nm. This CD signal is corresponding to the absorption band peaking at 220 nm originated from the n→π transitions of the urea and/or amide units[35] (most likely, it is originated from the urea unit since the natural sequence and reported natural peptides do not show this CD band. However, the originity needs to be investigated in more details). This fact suggests that the n→π transitions of the urea-dipeptides adopting β-folding conformations yields two negative CD bands around 217 and 225 nm. This is surprised. Another interesting thing is that the intensity of the

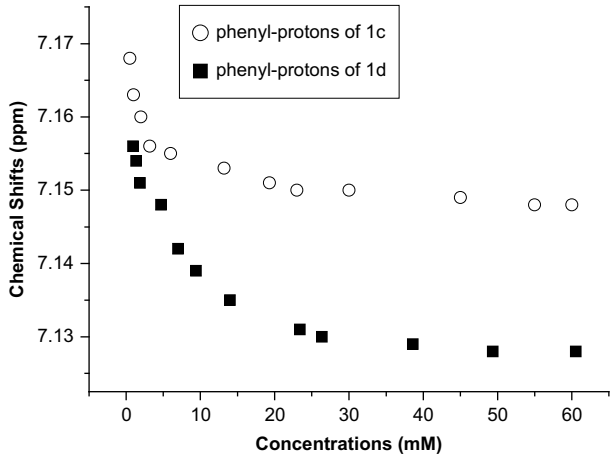


Figure 3. Concentration-dependent δ_{-NH}s of the phenyl-protons of **1c** and **1d**.

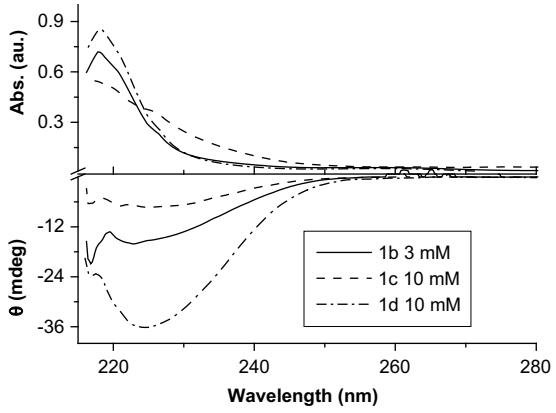


Figure 4. UV–vis and CD spectra of **1b**–**1d** in the chloroform solutions.

225 nm signal is dependent on both the structure and concentration. For examples, **1c** has a much stronger 225 nm CD signal than that at 217 nm, while **1d** with a same concentration (10 mM) shows a reversal intensity of the 225 band over the 217 nm band (Fig. 4). Concentration-dependent CD experiments shown in Figure 5 reveal that the intensity of the 225 nm CD signal of **1c**, for example, decreases more quickly than that of the 217 nm signal with decreasing the concentration. In particular, the existence of the 217 nm signal at a concentration of as low as 0.5 mM indicates again that the strong propensity of the urea-dipeptides to nucleate the formation of the β-sheetlike assembly.

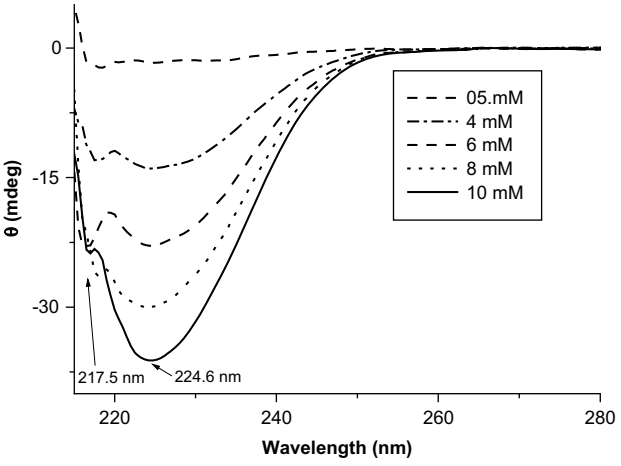


Figure 5. CD spectra of **1c** at different concentrations.

2.4.4. XRD characteristics. X-ray diffraction (XRD) experiments may give better insights into the structural information of the β-sheet assembly. The XRD scattering shown in Figure 6 carried out by depositing the **1b**/$CHCl_3$ solution, for example, onto the Si substrate shows a strong and a weak reflections, respectively, at 2θ=7.0 and 18.4°, which are corresponding to two *d*-spacings of 12.7 and 4.8 Å. Other reflections in the range of 2θ=5–35° can be assigned to the higher or lower orders of these two reflections. The *d*-values of 12.7 and 4.8 Å are, respectively, relative to the stacking periodicity of the β-sheets and the spacing between peptide backbones running orthogonal to the β-sheet axis, typically characteristic of the β-sheet structures of **1b**.[36]

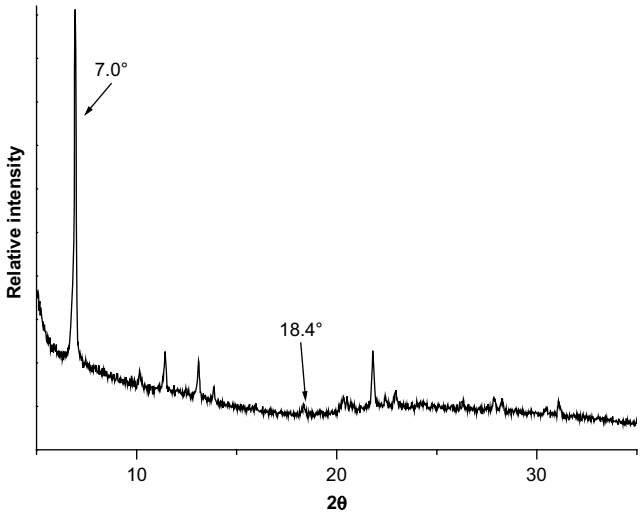


Figure 6. XRD scattering of **1b** deposited onto the Si substrate.

2.5. Asymmetrical β-sheetlike assembly

Particularly, **1c** bears asymmetrical terminal groups at two ends, while, the NMR evidences indicate that it tends to form 'asymmetrical' β-sheetlike assembly, in which the urea-dipeptide molecules self-organize mainly in a parallel fashion, not in the antiparallel way or both (Fig. 7). The first evidence comes from the

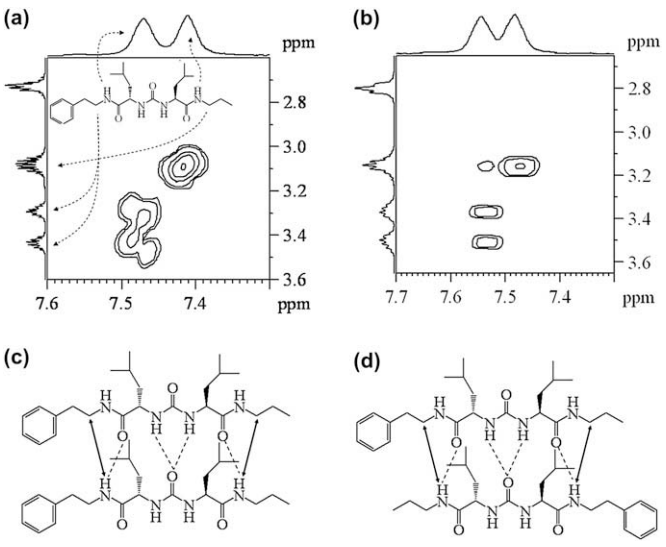


Figure 7. Part of COSY (a) and NOE spectra (b) of **1c** conducted at 60 mM and rt; parallel (c) and anti-parallel (d) self-organizations and the NOE contacts.

concentration-dependent behavior of the amide-NHs. The two amide-NHs attached at the ϕe and *n*-Pr ends, respectively, give two distinct triplets in the NMR spectra at high concentration levels, which are undoubtedly assignable to the amide-NH, respectively, linking with the *n*-Pr (at downfield) or ϕe group (at upfield) by the COSY spectrum (Fig. 7a). As further indicated in Figure 8, the $\Delta\delta_{\text{a-mide-NH}}$ representing the difference between the chemical shifts of these two amide-NHs shows an increase with the concentration. This fact suggests that the aggregate process into the β-sheetlike assembly may produce different chemical environments around the end amide-protons.

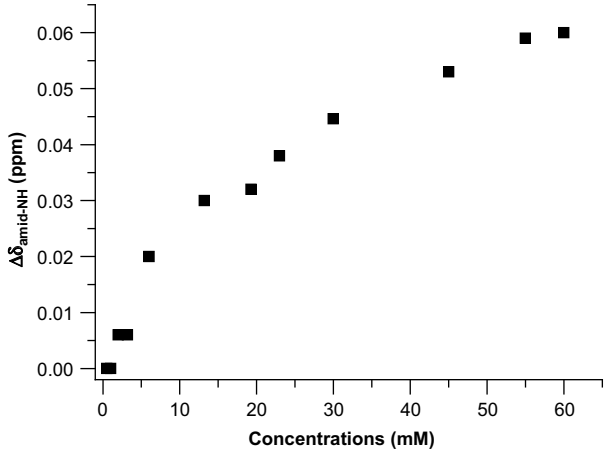


Figure 8. A plot of the $\Delta\delta_{\text{amide-NH}}$ (difference between the chemical shifts of the amide-NH at the ϕe end and that at the *n*-Pr end) of **1c** versus concentrations.

In order to get an insight into this, we conducted the NOE experiment. As shown in Figure 7b, the NOE spectrum shows very strong NOE signals both between the ϕe-αC-protons and amide-protons at the ϕe end and between the *n*-Pr-αC-protons and amide-protons at the *n*-Pr end. While the NOE signal between ϕe-αC-protons and amide-protons at the *n*-Pr end and that between the *n*-Pr-αC-protons and amide-protons at the ϕe end are very weak. This fact illustrates the parallel to parallel self-organization of the urea-dipeptide **1c** as the dominant type of aggregates. Obviously, the π–π stacks between the phenyl-units residing at one side of the assembled ribbon-like structure would produce different environments for the amide-NHs linked with the ϕe unit to that ones attached to the *n*-Pr group. The above results suggest that the phenyl π–π stacking may play a role in directing molecules of **1c** into the 'asymmetrical' β-sheetlike assembly.

2.6. Disruption of β-sheetlike assembly

H-bonding solvents such as methanol effectively disrupt the β-sheetlike assembly, even only several percent of methanol. For example, addition of 5% methanol into a 20 mM **1d**/$CDCl_3$ solution resulted in complete disruption of the intermolecular H-bonds and formation of new competitive H-bonds between methanol and –NHs, which displayed an obvious upfield shift for the urea-protons and a little downfield shift for the amide-protons (Fig. 9). This upfield shift of the urea-protons or slightly downfield shift of the amide-protons almost remains well if the solution is further diluted using 5% methanol/$CDCl_3$ (v/v), indicating the initial addition of methanol have completely consumed the interstrand H-bonds, and the competitive H-bonding opportunities between the dipeptide and methanol molecules are also kept well by the further addition of methanol in 5% methanol/$CDCl_3$ since the concentration of

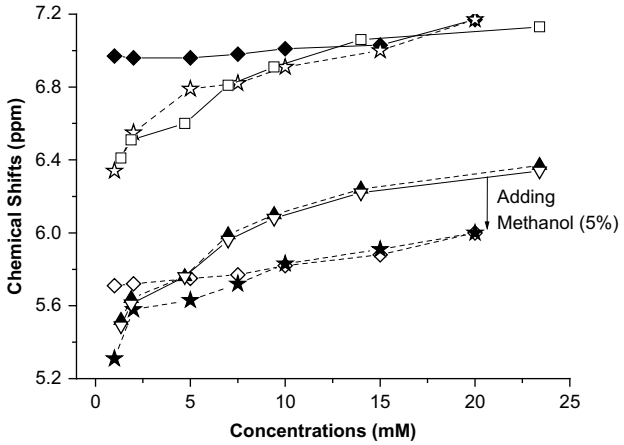


Figure 9. Concentration-dependent $\delta_{\text{-NH}}$s of the amide- and urea-NHs of **1d** from a 20 mM solution of 5% of $CH_3OH/CDCl_3$ (v/v) diluted by using either 5% $CH_3OH/CDCl_3$ (◆ and ◇) or pure $CDCl_3$ (☆ and ★). As compared, it also shows concentration-dependent $\delta_{\text{-NH}}$s of the amide- (□) and urea-NHs (▲ and ▽) of **1d** from a 23 mM $CDCl_3$ solution diluted with pure $CDCl_3$.

methanol in the solution remains constantly. When the solution is further diluted using pure $CDCl_3$, however, all the –NHs show a large upfield shift as the chloroform molecules have replaced some methanol molecules, effectively reducing H-bonding opportunities.

3. Conclusions

The solution NMR and CD studies demonstrate that the urea-dipeptides adopt β-folding conformations in non-H-bonding solvents, and self-organize into the β-sheetlike assembly. Most surprisingly, the urea-dipeptides show much stronger H-bonding propensity to nucleate the formation of the β-sheetlike assembly than the natural sequence. Additionally, the urea-dipeptides show very different solution behavior to the imide-dipeptides[31] although they have similar structural features. These distinct features of the H-bonding nature suggest that the urea-dipeptide segment may be an alternative interesting linear β-mimetic motif to be incorporated with the imide-dipeptide segment together to generate new peptidomimetics, which contain both the urea- and imide-dipeptide segments, which is undergoing in our lab.

4. Experimental part

4.1. Apparatus and materials

NMR measurements. NMR spectra were recorded on Brucker instruments (300, 400, or 600 kHz), NOESY and COSY spectra were made at rt on the 600 kHz instrument.

Mass spectrometry. ESI-mass spectra (ESI=electro spray ionization) were obtained with a Shimadzu LC–MS 2010 mass spectrometer. A proper solvent such as dichloromethane, acetonitrile, methanol, water, was used for dissolving the sample.

CD spectrometry. CD spectra were obtained on a JASCO model J-815 spectropolarimeter. Each CD spectrum was obtained by integrating three repeated scans with a scan rate of 500 nm/min and was background-corrected with a 0.1 mm quartz cell.

XRD measurements. XRD measurements were performed by using a X-ray powder diffraction (XRD, Bruker D8 Focus) with Cu Kα as the radiation source (λ=1.5418 Å) and operated at 40 kV and 40 mA.

Materials. Starting materials are all commercially available reagents and solvents used as received except for statements.

Tetrahydrofuran (THF) was distilled on sodium. Dichloromethane and acetonitrile were dried on anhydrate Na_2CO_3. Reactions were monitored by thin-layer chromatography on pre-coated silica gel plates (Yantai Shi Huagxue Gongye Yanjiusuo) and visualized using UV irradiation (254 nm) or colored by iodine. Flash chromatography was performed on silica gel H60 (Giandao Haiyang Huagongchang).

4.2. Synthesis of the dipeptides

*4.2.1. General procedure for synthesis of N-Boc-amino acid (**4**).* 10 mmol of commercial amino acid and 11 mmol of KOH were dissolved in a mixture of water (40 mL) and THF (4 mL). To it 11 mmol of di-*tert*-butyl-dicarbonate was added, and the resultant solution was allowed to react at 50 °C. 2–3 h later, the reaction mixture was cooled down to rt and stirred overnight. Then, 11 mL of 1 M HCl was added dropwise to adjust pH value of the solution to about 5. The solution was extracted with ethyl acetate (50 mL, ×3) and dichloromethane (50 mL, ×3), respectively. The organic phases were mixed and dried with anhydrate Na_2SO_4. Removal of organic solvents afforded expected product.

*4.2.1.1. N-Boc-L-alanine acid (**4a**).* 4.5 g (50 mmol) of L-alanine and 3.8 g (55 mmol) of KOH, 13.0 g (55 mmol) of di-*tert*-butyl-dicarbonate were mixed in a mixture of water (200 mL) and THF (20 mL). The reaction afforded **4a** as an oil-like solid (9 g, 47 mmol, 95%). ^{1}H NMR δ (ppm, $CDCl_3$, 400 MHz): 10.66 (s, broad, 1H, acid-H), 5.08 (s, 1H, carbamate-H), 4.35 (s, 1H, α-proton), 1.53 (d, 3H, $-CH_3$, ^3J=7.6 Hz), 1.45 (s, 9H, Boc-Hs). ESI-MS: 188 ($-H^+$), 189, 211 ($-H^+$ and $+Na^+$), 227 ($-H^+$ and $+K^+$).

*4.2.1.2. N-Boc-L-leucine acid (**4b**).* 1.31 g (10 mmol) of L-leucine, 0.75 g (11 mmol) of KOH, and 2.4 g (11 mmol) of di-*tert*-butyl-dicarbonate were dissolved in a mixture of water (40 mL) and THF (4 mL). The reaction afforded **4b** as an oil-like solid (2.2 g, 9.0 mmol, 90%). ^{1}H NMR δ (ppm, $CDCl_3$, 400 MHz): 6.20 (s, broad, 1H, acid-H), 4.91 (s, 1H, carbamate-H), 4.31 (s, 1H, α-proton), 1.76–1.60 (m, 2H, $-CH_2$, ^3J=6.4 Hz), 1.59–1.49 (m, 1H, –CH, ^3J=6.6 Hz), 1.44 (s, 9H, Boc-Hs), 0.96 (d, 6H, $-CH_3$, ^3J=6.4 Hz). ESI-MS: 230 ($-H^+$), 231, 253 ($-H^+$ and $+Na^+$), 269 ($-H^+$ and $+K^+$).

*4.2.1.3. 4-Nitrophenoyl N-Boc-L-alanine ester (**5a**).* 1.9 g (10 mmol) of **4a**, 1.7 g (12 mmol) of 4-nitrophenol, and 2.8 g (12 mmol) of DCC were mixed with 100 mL of CH_2Cl_2 and stirred overnight. Then, the precipitate was filtered out and the solvents of the filtration were removed. The resultant residue was applied to flash chromatography with petrol ether/dichloromethane/ethyl acetate=10:2:1 as eluents to afford **5a** as a light-yellow solid (2.6 g, 8.4 mmol, 85%, R_f=0.2). ^{1}H NMR δ (ppm, $CDCl_3$, 400 MHz): 8.30 (d, 2H, phenyl-Hs, ^3J=9.0 Hz), 7.32 (d, 2H, phenyl-Hs, ^3J=9.0 Hz), 5.11 (s, 1H, carbamate-H), 4.54 (t, 1H, α-proton, ^3J=7.1 and 6.5 Hz), 1.58 (d, 3H, $-CH_3$, ^3J=7.4 Hz), 1.47 (s, 9H, Boc-Hs). ^{13}C NMR δ (ppm, $CDCl_3$, 100 MHz): 171.4, 155.3, 155.2, 145.6, 125.4, 122.4, 80.5, 49.7, 28.4, 18.1. ESI-MS: 333 ($+Na^+$).

*4.2.1.4. 4-Nitrophenoyl N-Boc-L-leucine ester (**5b**).* 2.3 g (10 mmol) of **4b**, 1.7 g (12 mmol) of 4-nitrophenol, and 2.8 g (12 mmol) of DCC were mixed with 100 mL of CH_2Cl_2 and stirred overnight. After the precipitate was filtered out and the solvents of the filtration were removed, the residue was applied to flash chromatography with petrol ether/dichloromethane/ethyl acetate=10:4:1 as eluents to afford **5b** as a light-yellow solid (2.8 g, 8 mmol, 80%, R_f=0.2). ^{1}H NMR δ (ppm, $CDCl_3$, 400 MHz): 8.28 (d, 2H, phenyl-Hs, ^3J=8.9 Hz), 7.31 (d, 2H, phenyl-Hs, ^3J=8.9 Hz), 4.93 (s, 1H, carbamate-H), 4.51 (s, 1H, α-proton), 1.69 (m, 2H, $-CH_2$, ^3J=6.4 Hz), 1.47 (s, 10H, Boc-Hs, –CH of leucine), 1.02 (d, 6H, $-CH_3$, ^3J=4.4 Hz). ^{13}C NMR δ (ppm, $CDCl_3$, 100 MHz): 171.8, 155.6, 155.4, 145.6, 125.4, 122.5, 80.5, 55.9, 41.3, 28.4, 23.0, 21.9. ESI-MS: 375 ($+Na^+$).

*4.2.2. General procedure for synthesis of **6**.* 2 mmol of **5** was dissolved in 10 mL of dried CH_3CN, excess amine was injected inside, and stirred overnight. Then, acetonitrile was removed under vacuum and the residue was re-dissolved in 10 mL of dichloromethane and washed, respectively, using satd Na_2CO_3 aqueous and water (10 mL, ×3) and then dried with anhydrate Na_2SO_4. After removal of organic solvent, the residue was applied to flash chromatography to afford **6** as a white solid.

*4.2.2.1. tert-Butyl N-[(S)-1-propylcarbamoyl-ethyl]carbamate (**6a**).* 620 mg (2 mmol) of **5a** and 330 μL (7.8 mmol) of 1-propyl amine were mixed in 10 mL of CH_3CN. The reaction residue was applied to flash chromatography with petrol ether/ethyl acetate=4:1 as eluents to afford **6a** as a white solid (420 mg, 1.8 mmol, 91%, R_f=0.2). ^{1}H NMR δ (ppm, $CDCl_3$, 400 MHz): 6.61 (s, 1H, amide-H), 5.33 (s, 1H, carbamate-H, ^3J=7.2 Hz), 4.21 (t, 1H, α-proton, ^3J=5.0 Hz), 3.25 (q, 2H, propyl-CH_2, ^3J=6.4 Hz), 1.53 (m, 2H, propyl-CH_2, ^3J=7.2 Hz), 1.46 (s, 9H, Boc-Hs), 1.38 (d, 3H, $-CH_3$, ^3J=7.0 Hz), 0.94 (t, 3H, propyl-CH_3, ^3J=7.4 Hz). ^{13}C NMR δ (ppm, $CDCl_3$, 100 MHz): 172.7, 155.6, 79.9, 50.0, 41.1, 29.7, 22.8, 18.6, 11.3. ESI-MS: 253 ($+Na^+$).

*4.2.2.2. tert-Butyl N-[(S)-3-methyl-1-propylcarbamoyl-butyl]carbamate (**6b**).* 705 mg (2 mmol) of **5b** and 330 μL (7.8 mmol) of 1-propyl amine were mixed in 10 mL of CH_3CN. The reaction residue was applied to flash chromatography with petrol ether/ethyl acetate=5:1 as eluents to afford **6b** as a white solid (510 mg, 1.9 mmol, 94%, R_f=0.2). ^{1}H NMR δ (ppm, $CDCl_3$, 400 MHz): 6.15 (s, 1H, amide-H), 4.84 (d, 1H, carbamate-H, ^3J=8.8 Hz), 4.06 (t, 1H, α-proton, ^3J=7.4 Hz), 3.20 (q, 2H, propyl-CH_2, ^3J=6.4 Hz), 1.67 (s, 2H, $-CH_2$), 1.60–1.43 (m, 3H, propyl-CH_2 and –CH, ^3J=7.2 Hz), 1.40 (s, 9H, Boc-Hs), 0.89–0.83 (q, 9H, propyl-CH_3 and $-CH_3$, ^3J=7.2 Hz). ^{13}C NMR δ (ppm, $CDCl_3$, 100 MHz): 172.5, 155.8, 79.9, 53.1, 41.3, 41.1, 28.3, 25.0, 22.9, 22.1, 11.3. ESI-MS: 295 ($+Na^+$).

*4.2.2.3. tert-Butyl N-[(S)-3-methyl-1-phenethyl-carbamoyl-butyl]carbamate (**6c**).* 705 mg (2 mmol) of **5c** and 1 mL (8.5 mmol) of 2-phenylethyl amine were mixed in 10 mL of CH_3CN. The reaction residue was applied to flash chromatography with petrol ether/ethyl acetate=5:1 as eluents to afford **6c** as a white solid (600 mg, 1.8 mmol, 90%, R_f=0.2). ^{1}H NMR δ (ppm, $CDCl_3$, 300 MHz): 7.30–7.14 (m, 5H, phenyl-Hs, ^3J=6.8 Hz), 6.62 (s, 1H, amide-H), 5.18 (d, 1H, carbamate-H, ^3J=7.3 Hz), 4.09 (s, 1H, α-proton), 3.54–3.50 (m, 1H, phenylethyl-CH_2, ^3J=6.8 Hz), 3.42–3.37 (m, 1H, phenylethyl-CH_2, ^3J=6.1 Hz), 2.80–2.75 (t, 2H, phenylethyl-CH_2, ^3J=7.1 Hz), 1.58 (m, 2H, $-CH_2$, ^3J=7.0 Hz), 1.46 (m, 1H, –CH, ^3J=7.9 Hz), 1.40 (s, 9H, Boc-Hs), 0.89 (d, 6H, $-CH_3$, ^3J=5.4 Hz). ^{13}C NMR δ (ppm, $CDCl_3$, 100 MHz): 172.8, 155.8, 138.8, 128.8, 128.6, 126.5, 79.8, 53.2, 41.6, 40.7, 35.7, 28.3, 24.8, 22.9, 22.1. ESI-MS: 357 ($+Na^+$).

*4.2.3. General procedure for synthesis of **7**.* 0.5 mmol of **6** was dissolved in dichloromethane (10 mL) and 1 mL of TFA was added. 2 h later, 10 mL×3 of satd Na_2CO_3 aqueous and water were, respectively, used to wash the solution. Organic phase was collected and dried with anhydrate Na_2SO_4. The solvents were removed to afford **7** as an oil-like solid. The residue was used for further synthesis without any other purification.

*4.2.4. General procedure for synthesis of **8**.* The above obtained **7** was mixed with 10 mL of dried THF and 200 μL of DIEA, then, a solution of 120 mg of 4-nitrophenyl chloroformate in 10 mL of THF was added dropwise at 0 °C and then stirred overnight. After

removal of organic solvent, the residue was applied to flash chromatography to afford **8**.

*4.2.4.1. 4-Nitrophenyl N-[(S)-1-propylcarbamoyl-ethyl]carbamate (**8a**).* The reaction residue was applied to flash chromatography with petrol ether/dichloromethane/ethyl acetate=1:1:1 as eluents to afford **8a** as a light-yellow solid (130 mg, 0.9 mmol, 90%, R_f=0.2). ^{1}H NMR δ (ppm, $CDCl_3$, 400 MHz): 8.27 (d, 2H, 4-nitro-phenyl-Hs, ^3J=8.7 Hz), 7.31 (d, 2H, 4-nitro-phenyl-Hs, ^3J=8.8 Hz), 6.10 (s, 1H, amide-H), 5.86 (s, 1H, carbamate-H), 4.31 (q, 1H, α-proton, ^3J=7.0 Hz), 3.35 (q, 2H, propyl-CH_2, ^3J=6.6 Hz), 1.6–1.5 (m, 5H, propyl-CH_2 and –CH_3), 1.04 (t, 3H, propyl-CH_3, ^3J=7.3 Hz). ^{13}C NMR δ (ppm, $CDCl_3$, 100 MHz): 171.4, 155.7, 152.7, 144.9, 125.2, 122.1, 50.8, 41.5, 22.8, 19.2, 11.3. ESI-MS: 296 ($+H^+$), 318 ($+Na^+$).

*4.2.4.2. 4-Nitrophenyl N-[(S)-1-propylcarbamoyl-ethyl]carbamate (**8b**).* The reaction residue was applied to flash chromatography with petrol ether/dichloromethane/ethyl acetate=2:1:1 as eluents to afford **8b** as a light-yellow solid (140 mg, 0.4 mmol, 84%, R_f=0.2). ^{1}H NMR δ (ppm, $CDCl_3$, 400 MHz): 8.26 (d, 2H, 4-nitro-phenyl-Hs, ^3J=9.1 Hz), 7.32 (d, 2H, 4-nitro-phenyl-Hs, ^3J=9.1 Hz), 5.80 (s, 1H, amide-H), 5.84 (s, 1H, 4-nitrophenyl-carbamate-H, ^3J=8.3 Hz), 4.2–4.13 (q, 1H, α-proton, ^3J=6.3 Hz), 3.35–3.20 (m, 2H, propyl-CH_2, ^3J=6.0 Hz), 1.7–1.6 (m, 2H, –CH_2 of leucine, ^3J=4.5 Hz), 1.6–1.5 (m, 3H, propyl-CH_2 and –CH), 1.00 (d, 3H, propyl-CH_3, ^3J=5.7 Hz), 0.91 (t, 6H, –CH_3, ^3J=5.2 Hz). ^{13}C NMR δ (ppm, $CDCl_3$, 100 MHz): 172.0, 155.8, 153.3, 144.7, 125.1, 122.0, 54.0, 41.9, 41.4, 24.8, 22.8, 22.6, 22.2, 11.3. ESI-MS: 338 ($+H^+$), 360 ($+Na^+$).

*4.2.4.3. 4-Nitrophenyl N-[(S)-3-methyl-1-phenethyl-carbamoyl-butyl]carbamate (**8c**).* The reaction residue was applied to flash chromatography with petrol ether/dichloromethane/ethyl acetate=2:1:1 as eluents to afford **8c** as a light-yellow solid (180 mg, 0.5 mmol, 91%, R_f=0.2). ^{1}H NMR δ (ppm, $CDCl_3$, 400 MHz): 8.18 (d, 2H, 4-nitro-phenyl-Hs, ^3J=9.2 Hz), 7.35–7.24 (m, 5H, phenyl-Hs, ^3J=5.9 Hz), 6.90 (d, 2H, 4-nitro-phenyl-Hs, ^3J=9.2 Hz), 6.05 (d, 1H, 4-nitrophenyl-carbamate-H, ^3J=6.9 Hz), 5.86 (s, 1H, amide-H), 4.12 (q, 1H, α-proton, ^3J=7.1 Hz), 3.70–3.59 (m, 2H, phenylethyl-CH_2, ^3J=6.8 Hz), 2.98–2.89 (t, 2H, phenylethyl-CH_2, ^3J=6.8 Hz), 1.72 (m, 2H, –CH_2, ^3J=4.5 Hz), 1.40–1.30 (m, 1H, –CH, ^3J=6.7 Hz), 0.97–0.86 (m, 6H, –CH_3, ^3J=5.7 Hz). ^{13}C NMR δ (ppm, $CDCl_3$, 100 MHz): 174.1, 161.3, 157.0, 142.2, 138.5, 129.0, 128.5, 126.7, 126.3, 115.7, 55.6, 40.9, 39.7, 33.9, 25.3, 23.0. 21.6. ESI-MS: 400 ($+H^+$), 422 ($+Na^+$).

*4.2.4.4. Urea-dipeptide (**1a**).* 70 mg (0.54 mmol) of **7a** and 170 mg (0.58 mmol) of **8a** were dissolved in 5 mL of dried CH_3CN and stirred at rt overnight. A day later, the white precipitate was collected by filtration and washed with water to afford urea-dipeptide **1a** (130 mg, 0.45 mmol) as a white solid with a yield of 84%. ^{1}H NMR δ (ppm, DMSO-d_6, 400 MHz): 7.81 (t, 2H, amide-NHs, ^3J=5.2 Hz), 6.27 (d, 2H, urea-NHs, ^3J=7.2 Hz), 4.08 (quintet, 2H, α-proton, ^3J=7.2 Hz), 2.99, (m, 4H, *n-Pr*-αC–Hs, ^3J=6.0 Hz), 1.38 (m, 4H, *n-Pr*-βC–Hs, ^3J=7.2 Hz), 1.13 (6H, –CH_3, ^3J=6.8 Hz), 0.82 (t, 6H, –CH_3, ^3J=7.7 Hz) (see below). ^{13}C NMR δ (ppm, DMSO-d_6, 100 MHz): 173.4, 157.1, 49.1, ~40 (overlapping with DMSO), 22.8, 20.2, 11.8. ESI-MS: 287($+H^+$), 309 ($+Na^+$).

*4.2.4.5. Urea-dipeptide (**1b**).* 90 mg (0.52 mmol) of **7b** and 190 mg (0.56 mmol) of **8b** were dissolved in 5 mL of dried CH_3CN and stirred at rt overnight. A day later, the white precipitate was collected by filtration and washed with water to afford urea-dipeptide **1b** (80 mg, 0.22 mmol) as a white solid with a yield of 42%. ^{1}H NMR δ (ppm, $CDCl_3$, 400 MHz, 20 mM): 7.12 (s, broad, 2H, amide-NHs), 6.38 (d, 2H, urea-NHs, ^3J=8.0 Hz), 4.29–4.23 (q, 2H, α-proton, $^3J_{HN\alpha}$=8.0 Hz), 3.22–3.12 (m, 4H, *n-Pr*-αC–Hs, ^3J=6.4 Hz), 1.66–1.56 (m, 4H, β-Hs of Leu, ^3J=5–7 Hz), 1.53–1.46 (m, 6H, γ-Hs of Leu and *n-Pr*-βC–Hs), 0.91–0.88 (t, 18H, δ-Hs of Leu and *n-Pr*-γC–Hs, ^3J=6.8 Hz). Elementary Analysis for $C_{19}H_{38}N_4O_3$: Calcd C, 61.59; H, 10.34; N, 15.12; Exp. C, 61.89; H, 10.30; N, 14.86; ESI-MS: 371 ($+H^+$), 393 ($+Na^+$).

*4.2.4.6. Urea-dipeptide (**1c**).* 90 mg (0.52 mmol) of **7b** and 220 mg (0.55 mmol) of **8c** were dissolved in 5 mL of dried CH_3CN and stirred at rt overnight. A day later, the white precipitate was collected by filtration and washed with water to afford urea-dipeptide **1c** (60 mg, 0.14) as a white solid with a yield of 27%. ^{1}H NMR δ (ppm, $CDCl_3$, 400 MHz, 13 mM): 7.28–7.13 (m, 5H, ph-Hs, ^3J=7.6 Hz), 7.04 (s, 1H, amide-NH), 7.01(s, 1H, amide-NH), 6.27–6.21 (quartet, 2H, urea-NHs, ^3J=8.8 and 8.4 Hz), 4.28–4.23 (sextet, 2H, α-proton, $^3J_{HN\alpha}$=7.8 Hz), 3.57–3.48 (m, 1H, phenylethyl-αC–Hs, ^3J=7.8 Hz), 3.42–3.34 (m, 1H, phenylethyl-αC–Hs, ^3J=8.0 Hz), 3.23–3.09 (m, 2H, *n*-propyl-Hs, ^3J=8.0 Hz), 2.81–2.78 (m, 2H, phenylethyl-βC–Hs, ^3J=7.4 Hz), 1.63–1.46 (m, 8H, β-Hs of Leu, γ-Hs of Leu, and *n-Pr*-βC–Hs, ^3J=3–8 Hz), 0.91–0.85 (q, 15H, δ-Hs of Leu and *n-Pr*-γC–Hs, ^3J=6.0, ^3J=6.8 Hz). ^{13}C NMR δ (ppm, $CDCl_3$, 100 MHz): 172.9, 157.2, 138.1, 127.7, 127.4, 125.3, 57.4 (weak), 51.7, 40.9, 40.8, 40.2, 39.8, 34.7, 30.9 (weak), 28.7 (weak), 23.9, 23.8, 21.8, 21.7, 21.6, 17.4 (weak), 13.1 (weak), 10.4. ESI-MS: 433 ($+H^+$), 455 ($+Na^+$).

*4.2.4.7. Urea-dipeptide (**1d**).* 120 mg (0.51 mmol) of **7c** and 220 mg (0.55 mmol) of **8c** were dissolved in 5 mL of dried CH_3CN and stirred at rt overnight. A day later, the white precipitate was collected by filtration and washed with water to afford urea-dipeptide **1d** (90 mg, 0.18 mmol) as a white solid with a yield of 36%. ^{1}H NMR δ (ppm, $CDCl_3$, 400 MHz, 20 mM): 7.26–7.13 (m, 12H, ph-Hs and amide-NHs, ^3J=7.2 Hz), 6.38 (d, 2H, urea-NHs, ^3J=8.4 Hz), 4.31–4.24 (q, 2H, α-proton, $^3J_{HN\alpha}$=8.0 Hz), 3.54–3.49 (quartet, 2H, phenylethyl-αC–Hs, ^3J=7.0 Hz), 3.38–3.23 (m, 2H, phenylethyl-αC–Hs, ^3J=6.8 Hz), 2.81–2.78 (m, 4H, phenylethyl-βC–Hs, ^3J=7.4 Hz), 1.58–1.50 (m, 4H, β-Hs of Leu, ^3J=6–7 Hz), 1.44–1.41 (m, 2H, γ-Hs of Leu, ^3J=6.8 Hz), 0.84–0.82 (q, 12H, δ-Hs of Leu, ^3J=6.5, ^3J=2.8 Hz). ^{13}C NMR δ (ppm, $CDCl_3$, 100 MHz): 172.8, 157.1, 138.0, 127.7, 127.4, 125.3, 57.4 (weak), 51.7, 40.9, 39.8, 34.7, 28.7 (weak), 23.8, 21.7, 21.6, 17.4 (weak). TOF-MS: 494. ESI-MS: 495 ($+H^+$), 517 ($+Na^+$).

*4.2.5. 4-Nitro-phenyl n-propyl ester (**10**).* 1.4 mL (20 mmol) of *n*-propyl acid (9), 1.2 g (10 mmol) of 4-nitrophenol, and 2.1 g (10 mmol) of DCC were mixed with 100 mL of CH_2Cl_2 and stirred overnight. Then the precipitate was filtered out. After removal of organic solvents, the residue was applied to flash chromatography with petrol ether/dichloromethane=4:1 as eluents to afford **10** as a white solid (1.7 g, 8.7 mmol, R_f=0.2) with a yield of 87%. ^{1}H NMR δ (ppm, $CDCl_3$, 400 MHz): 8.18 (d, 2H, phenyl-Hs, ^3J=8.8 Hz), 7.16 (d, 2H, phenyl-Hs, ^3J=8.8 Hz), 2.51 (quartet, 2H, –CH_2, ^3J=7.6 Hz), 1.14 (d, 3H, –CH_3, ^3J=7.8 Hz). ^{13}C NMR δ (ppm, $CDCl_3$, 100 MHz): 171.8, 155.4, 145.0, 124.9, 122.3, 27.4, 8.6. ESI-MS: 195, 218 ($+Na^+$).

*4.2.6. Ethyl N-ethyl-carbonyl-L-leucine acid ester (**12**).* 0.7 g (3.6 mmol) of **10**, 0.6 g (3.1 mmol) of ethyl L-leucine acid ester hydrochloride (**11**), and 2.0 mL of triethyl amine were dissolved in 5 mL of dried CH_3CN and stirred overnight. After CH_3CN was removed under vacuum, the residue was re-dissolved into 10 mL of dichloromethane and washed using satd Na_2CO_3 aqueous (10 mL×3) and water (10 mL×3), Then the collected organic phase was dried with anhydrate Na_2SO_4. The solvents were removed and the resulted residue was applied to flash chromatography with petrol ether/ethyl acetate=5:1 as eluents to afford **12** as a light-yellow oil-like solid (0.6 g, 2.8 mmol, R_f=0.2) with a yield of 90%. ^{1}H NMR δ (ppm, $CDCl_3$, 400 MHz): 5.84 (d, 1H, amide-H, ^3J=7.3 Hz), 4.62 (m, 1H, α-proton, ^3J=4.0 Hz), 4.18 (quartet, 2H, –CH_2, ^3J=7.2 Hz), 2.25 (quartet, 2H,

–CH_2, ^3J=7.8 Hz), 1.66 (m, 2H, –CH_2, ^3J=6.4 Hz), 1.53 (m, 1H, –CH, ^3J=8.0 Hz), 1.29 (t, 3H, –CH_3, ^3J=7.2 Hz), 1.17 (t, 3H, –CH_3, ^3J=7.8 Hz), 0.95 (quartet, 6H, –CH_3, ^3J=5.6 Hz). ^{13}C NMR δ (ppm, $CDCl_3$, 100 MHz): 173.7, 173.3, 61.0, 50.4, 41.3, 29.1, 24.6, 22.6, 21.7, 13.9, 9.6. ESI-MS: 215, 238 ($+Na^+$).

*4.2.7. N-Ethyl-carbonyl-L-leucine acid (**13**).* 600 mg (2.8 mmol) of **12** and 240 mg (6 mmol) of NaOH were dissolved in 5 mL of C_2H_5OH and 5 mL of water and stirred for a week. Then the solution was neutralized using 1 M HCl to pH=3.0. 10 mL×3 of dichloromethane was then used for extraction. The organic phases were collected and dried with anhydrate Na_2SO_4. The solvents were removed to afford **13** as a white solid (500 mg, 2.7 mmol) with a yield of 96%. ^{1}H NMR δ (ppm, $CDCl_3$, 300 MHz): 8.92 (s, broad, 1H, acid-H), 5.97 (d, 1H, amide-H, ^3J=7.2 Hz), 4.62 (m, 1H, α-proton, ^3J=6.0 Hz), 2.29 (quartet, 2H, –CH_2, ^3J=7.2 Hz), 1.75 (m, 2H, –CH_2, ^3J=6.0 Hz), 1.60 (m, 1H, –CH, ^3J=8.8 Hz), 1.17 (t, 3H, –CH_3, ^3J=7.5 Hz), 0.95 (quartet, 6H, –CH_3, ^3J=5.6 Hz). ESI-MS: 186 ($-H^+$), 187, 209 ($-H^+$ and $+Na^+$), 225 ($-H^+$ and $+K^+$).

*4.2.8. 4-Nitro-phenyl N-ethyl-carbonyl-L-leucine acid ester (**14**).* 500 mg (2.7 mmol) of **13**, 560 mg (4 mmol) of 4-nitrophenol, and 800 mg (4 mmol) of DCC were mixed with 20 mL of CH_2Cl_2 and stirred overnight. Then the precipitate was filtered out. After removal of organic solvents, the residue was applied to flash chromatography with petrol ether/dichloromethane/ethyl acetate=10:10:1 as eluents to afford **14** as a light-yellow solid (700 mg, 2.3 mmol, R_f=0.2) with a yield of 85%. ^{1}H NMR δ (ppm, $CDCl_3$, 400 MHz): 8.30 (d, 2H, phenyl-Hs, ^3J=9.0 Hz), 7.31 (d, 2H, phenyl-Hs, ^3J=9.0 Hz), 5.78 (d, 2H, amide-H, ^3J=6.9 Hz), 4.83 (m, 1H, α-proton, ^3J=9.6, 5.4, 8.4 Hz), 2.33 (quartet, 2H, –CH_2, ^3J=7.6 Hz), 1.81 (m, 2H, –CH_2, ^3J=6.4 Hz), 1.22 (t, 3H, –CH_3, ^3J=7.8 Hz), 1.17 (t, 1H, –CH, ^3J=7.8 Hz), 1.05 (t, 6H, –CH_3, ^3J=5.6 Hz). ^{13}C NMR δ (ppm, $CDCl_3$, 100 MHz): 173.9, 171.2, 155.2, 145.6, 125.3, 122.4, 51.1, 41.1, 31.0, 25.1, 22.9, 21.9, 9.6. ESI-MS: 309 ($+H^+$), 331 ($+Na^+$), 347 ($+K^+$).

*4.2.9. Dipeptide **2**.* 156 mg (0.51 mmol) of **14**, 90 mg (0.52 mmol) of **7b**, and 2.0 mL of triethyl amine were dissolved in 2 mL of CH_3CN (containing 5% of methanol) and stirred overnight. After CH_3CN was removed under vacuum, the residue was re-dissolved into 10 mL of dichloromethane and washed using satd Na_2CO_3 aqueous water (respectively, 10 mL×3). The organic phases were collected and then dried with anhydrate Na_2SO_4. The solvents were removed and the resulted residue was applied to flash chromatography with petrol ether/dichloromethane/ethyl acetate=5:5:1 as eluents to afford **2** as a white solid (135 mg, 0.39 mmol, R_f=0.2) with a yield of 76%. ^{1}H NMR δ (ppm, $CDCl_3$, 400 MHz, 24 mM): 6.77–6.75 (d, 1H, amide-NH, No. 2, ^3J=8.1 Hz), 6.36 (t, 1H, amide-NH, No. 1, ^3J=8.0 Hz), 6.11–6.09 (d, 1H, amide-NH, No. 3, ^3J=7.9 Hz), 4.54–4.46 (m, 1H, α-proton, $^3J_{HN\alpha}$=8.4 Hz), 4.43–4.36 (m, 1H, α-proton, $^3J_{HN\alpha}$=8.4 Hz), 3.22–3.16 (q, 2H, *n*-Pr-αC–Hs, ^3J=6.6 Hz), 2.26–2.20 (q, 2H, –CH_2, ^3J=6.0 Hz), 1.80–1.45 (m, 8H, β-Hs of Leu, γ-Hs of Leu, and *n-Pr*-βC–Hs, ^3J=3–8 Hz), 1.17–1.12 (t, 3H, –CH_3, ^3J=7.5 Hz), 0.93–0.88 (q, 15H, δ-Hs of Leu and *n-Pr*-γC–Hs, ^3J=6.0, ^3J=6.8 Hz). ^{13}C NMR δ (ppm, $CDCl_3$, 100 MHz): 174.0, 172.3, 171.6, 51.9, 51.6, 41.3, 41.2, 40.8, 29.7, 29.5, 24.9, 24.8, 22.8, 22.7, 22.3, 22.2, 14.1, 11.3. ESI-MS: 341, 364 ($+Na^+$).

Acknowledgements

We thank NSFC (Nos. 20872145 and 20733006), the Chinese Academy of Sciences, the National Research Fund for Fundamental Key Project 973 (2006CB806200, 2007CB936401), and the CAS/SAFEA International Partnership Program for Creative Research Teams.

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