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ORIGINAL ARTICLE

Syntheses of some α-cyclic tripeptides as potential inhibitors for HMG-CoA Reductase

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Abstract α-Cyclic tripeptides (CtPs) are the most rigid members of the cyclic peptide family. However, due to their synthetic difficulty, biological activity has remained undisclosed. The incorporation of side-chain-protected natural amino acids into functional CtPs was performed to explore the potential biological functions. Several novel CtPs that consist of protected serine (S(Bn)) and/or glutamate (E(OBn)) were prepared from corresponding linear tripeptides by chemical synthesis. There is a strong possibility for CtPs that contain 3 phenyl groups to correlate with atorvastatin structure. The binding effects in human HMG-CoA reductase (hHMGR) activities were first evaluated by molecular docking. High docking scores were received with these CtPs for enzyme. Therefore, enzymatic assays were carried out and the compound *cyclo*(S(Bn))$_3$ was indeed able to moderately inhibit hHMGR ($IC_{50} = 110$ μM).

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Introduction

Small peptides are appealing for their cost-effectiveness (Santos et al. 2012), low toxicity, oral bioavailability (Hamman et al. 2005), and simplicity in drug designs (Kroemer 2007). Cyclization is particularly useful to improve 3-D structure (Bock et al. 2013) and specific recognition capability (Haberhauer 2008; Haberhauer et al. 2000). The 9-membered ring size of CtP and its constrained nature reduce entropic penalties for bio-macromolecules (Barbosa et al. 2010) that offer potential applications in chemical biology (Arai et al. 2013; Behera et al. 2013; Montero et al. 2011) and pharmaceutical science (Albericio and Kruger 2012; Scott et al. 2008; Marr et al. 2006). However, the formation of CtPs from linear natural amino acid sequences is formidable due to dimerization and racemization (White and Yudin 2011; Hamada and Shioiri 2005; Davies 2003). For CtP formation, cyclization to CtPs can takes place only if all 3 amide linkages are in *cis* configuration (Kartha et al. 1974; Druyan et al. 1976; Venkatachalam 1968). α-Cyclic tripeptide (CtP) generates less conformation to raise the accuracy of molecular docking due to its rigidity (Meng et al. 2011; Bombelli et al. 2004).

It is well established that proline acts as a turn inducer to facilitate cyclization through the reduction of strain barriers form *cis*-amide bonds (Rothe et al. 1965). In addition, *N*-substituted amino acids are turn induced (Kartha et al. 1974; Venkatachalam 1968). Derivatives of *cyclo*(L-Pro-L-Pro-Gly), *cyclo*(L-Pro-Gly-Gly) and *cyclo*(Gly-Gly-Gly) were synthesized with *N*-benzylglycine, *N*-methylglycine

(SAR) or *N*-allylglycine used as a glycine residue (Hioki et al. 2004; Leeuw et al. 1983; Kessler and Friedrich 1981; Kessler et al. 1978). Several functional CtPs have to be prepared using modified amino acids. For example, the *cyclo*(L-Pro-L-Pro-L-Thr) derivative and cyclic tetrapeptides that contain serine or threonine were synthesized using pseudo-proline instead of their natural forms (Fairweather et al. 2010; Skropeta et al. 2004; Riickle et al. 1999). The number of functional CtPs was limited to a handful (Debar et al. 1971; Debar et al. 1970).

HMG-CoA reductase (EC 1.1.1.88) is the rate-limiting enzyme (hHMGR) on the mevalonic acid pathway for cholesterol synthesis (Luskey and Stevens 1985). To prevent hypercholesterolemia, hHMCR has been the target for developing peptide drugs (Lin et al. 2015). Previous studies have shown that dietary proteins influence serum cholesterol level (Potter SM 1995; Sirtori et al. 1993; Zhang and Beynen 1993). Later, a novel hypocholesterolemic peptide, IIAEK (Ile-Ile-Ala-Glu-Lys), later named lactostatin, derived from β-lactoglobuline, was found to exhibit a greater hypocholesterolemic activity in comparison to medicine, β-sitosterol, in animal studies (Nagaoka et al. 2001). Soy glycinin, digested by trypsin or pepsin, also yielded peptides LPYP and IAVPGEVA with efficient hypocholesterolemic properties (Pak et al. 2005). Furthermore, synthetic cyclic peptides with 6-, 8-, or 10-membered ring were compared with their linear analogs (Pak et al. 2007, 2008). Recently, more potent hHMGR inhibitors such as SFGYVAE were shown to exhibit greater inhibitory effects than previous peptides such as LPYP (Pak et al. 2012).

Glutamate and serine are ideal candidates to verify the effects of side-chain-protected amino acid on CtPs' formation. Scheme 1, shows the structure of 3 phenyl groups on a cyclic peptide ring to be meaningful to resemble an hHMGR inhibitor atorvastatin. The actual binding ability of CtPs to the hHMGR is recorded to compare to CtPs with good correlations based on their docking scores. An attempt was made to synthesize CtPs using these two amino acids with deprotectable benzyl group.

Results and discussion

Unlike linear peptides, cyclic peptides are constrained by privileged secondary structures. They can be orally potent and resistant to protease hydrolysis in blood. For a cyclic tripeptide forming with all *cis*-amide bonds, the three Cα atoms are in the same plane. Drawing a line between each Cα atom produces a cyclic structure similar to an ordinary cyclopropane. It is rational and simpler to use Cα atoms' angle as the basis for structural description of a CtP. Cα atoms' angle is defined as the angle between the two lines of Cα atom to Cα atom. To support this concept, several real molecular models are shown in Fig. 1. These angles are very close to 60° for each CtP.

We use "Cα atoms' angle" as a term to describe the relationship of an amino acid with its neighbors. Each Cα

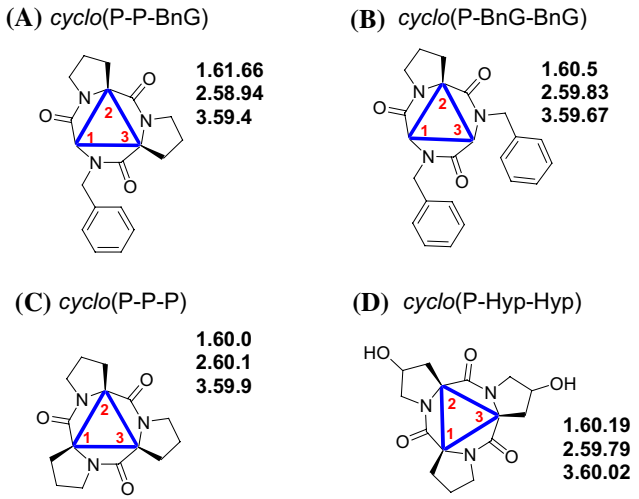
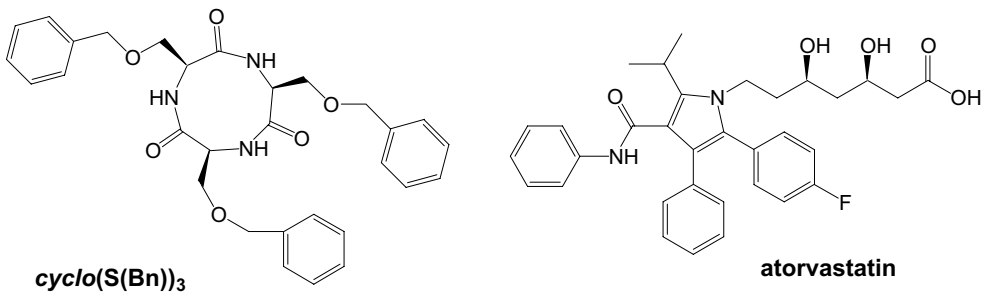


Fig. 1 **a** *cyclo*(P-P-BnG), (Bats and Fuess 1982). **b** *cyclo*(P-BnG-BnG), (Bats and Fuess 1980) **c** *cyclo*(P-P-P), (Druyan et al. 1976). **d** *cyclo*(P-Hyp-Hyp), (Kartha and Ambady 1975). *Blue lines* indicate the connection between the Cα atoms. The angles of the triangles are measured from their X-ray crystal structure and their values are shown on the *right* (color figure online)



Scheme 1 Structure of a benyl group-protected cyclic tripeptide and atorvastatin

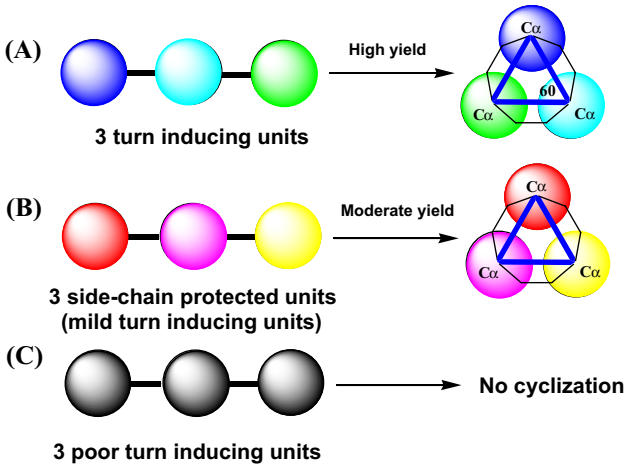


Fig. 2 Turn-inducing unit and some side-chain-protected units are able to form *all-cis* CtPs

atoms' angle of a symmetric CtP is 60°. Each Cα atoms' angle for heterotrimeric CtP has to be either slightly larger or smaller than 60°. For those CtPs that have been constructed with all *cis*-amide bonds, their Cα atoms' angle need to be preset to near 60° upon connection to another. Some units that do not meet the criterion are less likely to form *cis*-amide bonds. Hence, these units in question are difficult to incorporate into CtPs. This is probably the reason why pseudo-proline is feasible for cyclization (Fig. 2a).

The units can be classified into three categories. For those symmetric CtPs composed of the same three units, *cyclo*(P-P-P), *cyclo*(MeG-MeG-MeG), *cyclo*(BnG-BnG-BnG) and *cyclo*(AllylG-AllylG-AllylG) are symmetric and have been synthesized quantitatively (Hioki et al. 2004; Leeuw et al. 1983; Kessler and Friedrich 1981; Kessler et al. 1978). For that reason P, MeG, BnG and AllylG are turn-inducing units (Fig. 2a). Conversely, G is not a turn-inducing unit (poor turn inducer), since *cyclo*(G-G-G) was unable to form from linear G-G-G directly (Fig. 2c). Glycine has been rated as a turn inducer previously. We anticipated that some side-chain-protected amino acids could also play a role for cyclization similar to *N*-substituted amino acids with lower efficiency. We found these side-chain-protected amino acids are mild turn-inducing units (Fig. 2b).

Syntheses of cyclic tripeptides incorporated with S(Bn)

Scheme 2 shows linear tripeptides **1d** that contain the same natural amino acid S(Bn) constructed from the solution-phase method. Base hydrolysis of the methyl group on serine and coupling with pentafluorophenol with DCC was achieved. Boc protecting group was then removed with TFA. Without purification, the CtP synthesis occurred in a dilute solution of pyridine (2 mM) in 36 h. Side-chain-protected S(Bn)-S(Bn)-S(Bn) **1e** was cyclized in a low yield. Moreover, despite great effort, deprotection of the benzyl group on *cyclo*((S(Bn))$_3$ **1f** failed.

Any turn-inducing unit of a symmetric CtP must be able to incorporate with other turn-inducing units of symmetric CtPs to form heterotrimeric CtP as well as the side-chain-protected unit. The reason for this is that these side-chain-protected units are able to form *cis*-amide bonds that help to adapt an average pseudo-angle of 60°. Tentatively, S(Bn), E(OBn), or proline could be interchanged to compose a heterotrimeric CtP.

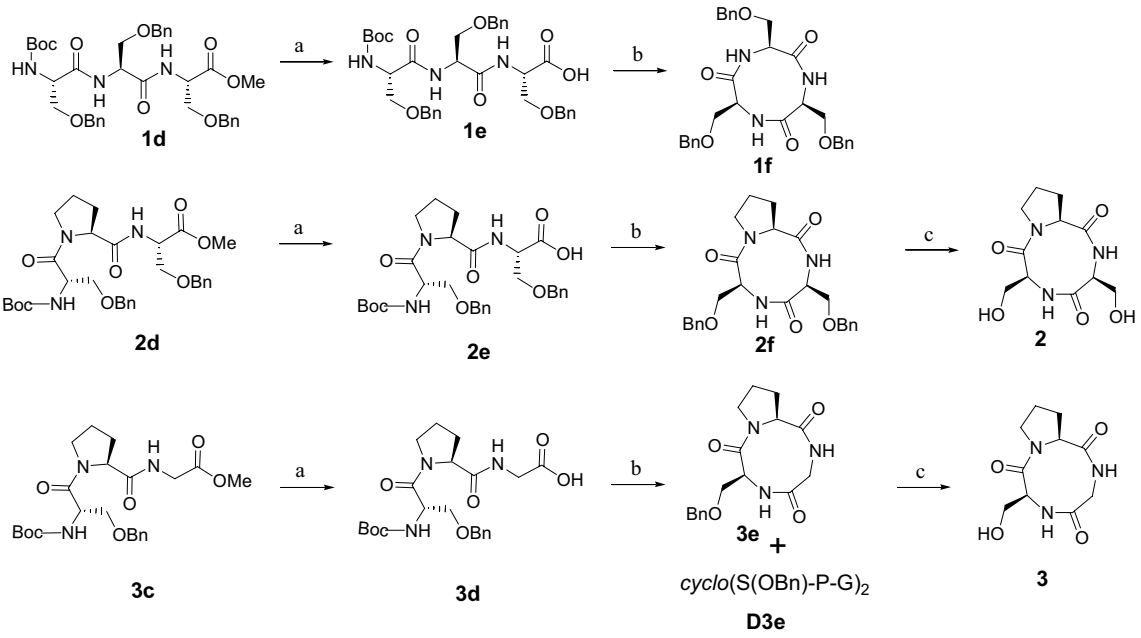
Boc-S(Bn)PS(OMe) **2e** and Boc-S(Bn)PG **3d** were synthesized to compare the ease of ring formation. Scheme 2 describes the same optimized methodology applied to the synthesis of these heterotrimeric CtPs. Two tripeptides with proline at the center were able to effectively cyclize. After deprotection of the benzyl group, *cyclo*(L-Ser-L-Pro-L-Ser) [*cyclo*(S-P-S)] **2** and *cyclo*(L-Ser-L-Pro- Gly) [*cyclo*(S-P-G)] **3** were obtained.

The consideration of a similar structure of the side chains on the CtP backbone in the case of a heterotrimeric tripeptide that contains only two side-chain-protected groups is less certain. The synthesis was extended to explore CtPs that contain glycine. Scheme 2 shows the cyclization of S(Bn)-P-G to *cyclo*(S(Bn)-P-G) is possible. *Cyclo*(S-P-G) **3** was obtained after removal of the benzyl group. Nevertheless, cyclization generated dimer **D3e** (30 %) as the major product was verified by X-ray crystallography (Fig. S1). This phenomenon further confirmed that unprotected glycine is a poor turn-inducing unit for the cyclization process.

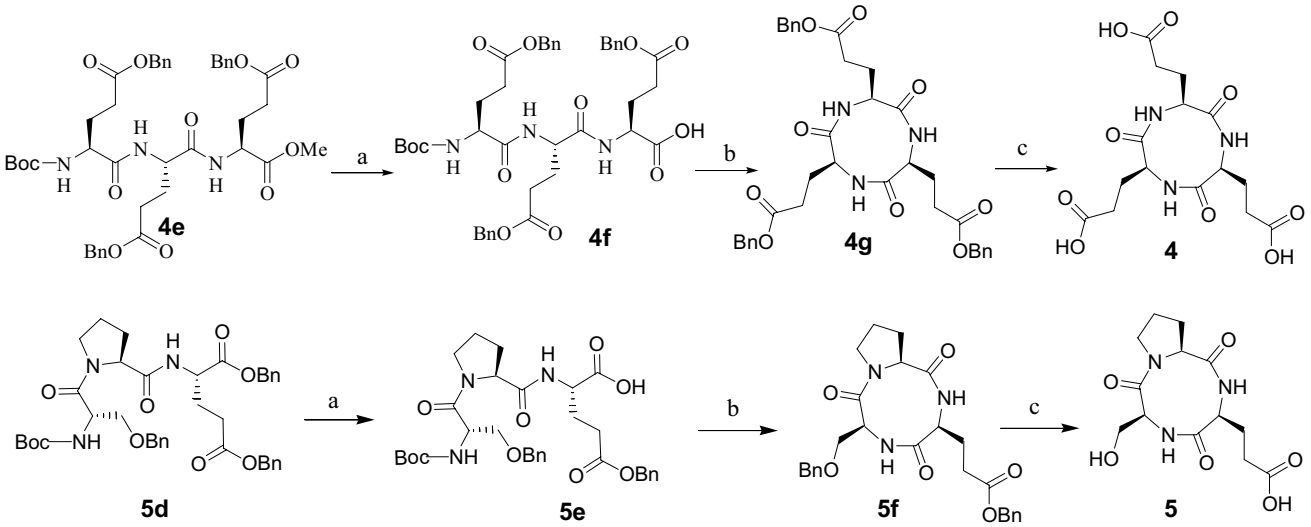
Syntheses of cyclic tripeptides incorporated with E(OBn)

The synthesis of Boc-E(OBn)-E(OBn)-E(OBn) **4e** was also carried out. Scheme 3 shows regioselective enzymatic cleavage of α-methyl group in the presence of 0.1 M phosphate buffer (Miyazawa et al. 1998) on glutamate and coupling with pentafluorophenol via DCC was achieved. Boc protecting group was then removed with TFA. CtP **4 g** was synthesized using pyridine in a dilute solution (2 mM) within 36 h without purification. Side-chain-protected E(OBn)-E(OBn)-E(OBn) was cyclized with a moderate yield. Deprotection of the benzyl group of *cyclo*((E(OBn))$_3$ went smoothly to afford *cyclo*(E-E-E) **4**. The ability of S(Bn) and E(OBn) to form homotrimeric CtPs (**1** and **2**) indicates that they act as a mild turn-inducing unit.

Boc-S(Bn)PE(OBn)$_2$ **5d** was synthesized to compare the ease of ring formation. Scheme 3 describes the same optimized methodology applied to the synthesis of this heterotrimeric CtP. Regioselective enzymatic hydrolysis of the α-benzyl ester on glutamate or base hydrolysis of methyl



Scheme 2 Synthesis of *cyclo*(S(Bn))$_3$, *cyclo*(S-P-S), *cyclo*(S-P-G). Reagents and conditions: **a** MeOH/1 N NaOH, (1:1), rt **b** (i) DCC, pentafluorophenol, DCM (ii) TFA (trifluoroacetic acid)/DCM (1:1), 0 °C (iii) pyridine, 2 mM, rt **c** H_2, $Pd(OH)_2$/C, MeOH



Scheme 3 Synthesis of *cyclo*(E-E-E) and *cyclo*(S-P-E). Reagents and conditions: **a** Amano-N/*B. licheniformis* protease (Sigma type–VIII), 0.1 M phosphate buffer pH 7, acetone, 37 °C **b** (i) DCC, pentafluorophenol, DCM (ii) TFA/DCM (1:1), 0 °C (iii) pyridine, high dilution, rt **c** H_2, $Pd(OH)_2$/C, DMF

ester on serine was achieved. The linear precursor was then activated and cyclized with pyridine to form the corresponding benzyl-protected CtP **5f**. After a simple extractive step, up to 40 and 34 % yields were obtained, respectively. Cleavage of the benzyl group of CtP finished the synthesis of *cyclo*(L-Ser-L-Pro-L-Glu) [*cyclo*(S-P-E)] **5**.

In this work, Boc-protected linear tripeptides were activated with pentafluorophenol for cyclization. To show the importance of turn-inducing unit and the related influence of side-chain-protected unit, our cyclization results and previous reported data are summarized in Table 1.

Molecular docking of CtPs with hHMGR

The 3-D structure (PDB 1HWK) was chosen as the receptor for the docking process to evaluate the potential binding

Table 1 Cyclization of the tripeptides with activate ester

Entry	CtP	Yield (%)	No. of turn-inducing unit	No. of side-chain-protected unit	Precursor
1	*cyclo*(S(Bn))$_3$ **1f**	11	0	3	Boc-S(Bn)-S(Bn)-S(Bn)-Pfp
2	*cyclo*(S(Bn)-P-S(Bn)) **2f**	36	1	2	Boc-S(Bn)-P-S(Bn)-Pfp
3	*cyclo*(S(Bn)-P-G) **3e**	25	1	1	Boc-S(Bn)-P-G-Pfp
4	*cyclo*(E(OBn))$_3$ **4 g**	19	0	3	Boc-[E(OBn)]$_3$-Pfp
5	*cyclo*(S(Bn)-P-E(OBn)) **5f**	42	1	2	Boc-S(Bn)-P-E(OBn)-Pfp
6	*cyclo*(S-P-E(OBn))	0	1	1	Boc-S-P-E(OBn)-Pfp
7	*cyclo*(P-P-P)	88	3	0	Boc-P-P-P-Pnp (Rothe et al. 1965)
8	*cyclo*(P-P-Hyp)	12	2	0	Boc-P-P-Hyp-Pnp (Debar et al. 1971)
9	*cyclo*(AllylG)$_3$	11	0	3	Boc-(AllylG)$_3$-Tcp (Hioki et al. 2004)

Pfp pentafluorophenol, *Pnp p*-nitrophenol, *Tcp* 2,4,5-trichlorophenol, *Hyp* 3-hydroxyproline

Table 2 Docking results of the selected tripeptides with hHMGR

Entry	Compound	DOCK	-PMF	Predicted H-bond position
1	*cyclo*(E(OBn))$_3$	90.22	167.25	Asn755
2	*cyclo*(E-E-E)	54.49	95.27	Ser852, Lys691, Ser661
3	*cyclo*(S(Bn))$_3$	80.93	117.41	Arg590
4	*cyclo*(S(Bn)-P-E(OBn))	76.24	135.32	Arg590
6	*cyclo*(S(Bn)-P-S(Bn))	71.53	121.98	No
8	S(Bn)-S(Bn)-S(Bn)	61.78	78.54	Arg836, Arg650
9	E(OBn)-E(OBn)-E(OBn)	68.89	62.28	Arg836, Arg650

capabilities of the CtPs with hHMGR. The potential binding sites on one of the monomers of PDB IHWK were predicted. The largest site was approximately 1000 A^3 in volume and includes important amino acid residues: Arg590, Val683, Ser684, Asp690, Lys691, and Lys692 (as previously determined, Istvan and Deisenhofer 2001) were used as targets of docking from the use the Ligand Fit algorithm of the DS 2.0 modeling tool (Huang et al. 2013). Previously, we found that DOCK scores of a few known statin molecules were in the range of 70 by similar maneuvers (data not shown). The binding potentials of each CtP molecule with the receptor were estimated by the docking scores -PMF and DOCK.

Table 2 shows 5 out of 10 CtP molecules received the docking scores successfully. Among them, 4 molecules received the DOCK scores even greater than 70. The potential hydrogen bonds of these CtPs with hHMGR were also predicted and listed. Two linear tripeptides were also estimated and received the DOCK scores less than 70. In general, benzyl group-protected CtPs exhibited stronger activities against hHMGR than those without. The docking possesses the compound *cyclo*(E(OBn))$_3$. Figure 3 displays

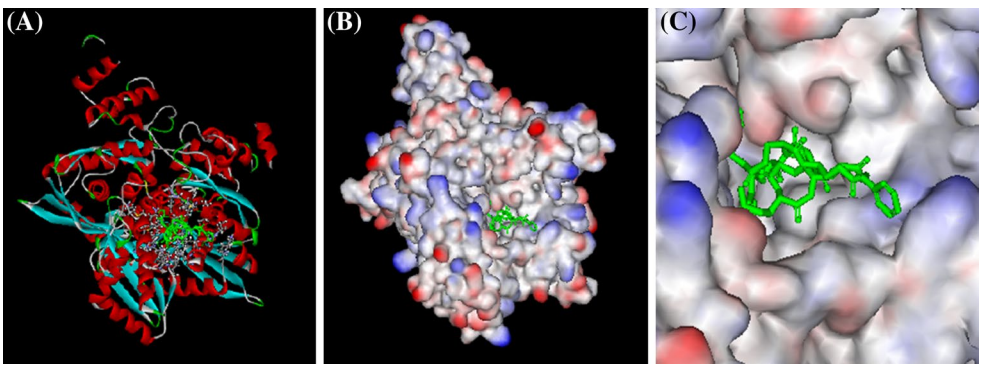


Fig. 3 Molecular docking. **a** Compound *cyclo*(EOBn)$_3$ (*green*) with hHMGR ribbon structure. **b** Compound *cyclo*(E(OBn))$_3$ with surface of hHMGR **c** A closer look of compound *cyclo*(EOBn)$_3$ in the binding site of hHMGR surface (color figure online)

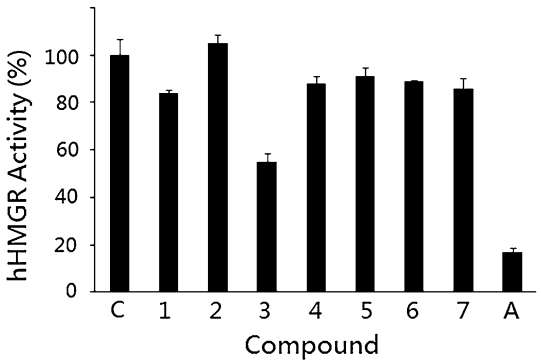


Fig. 4 Inhibitory effects of the cyclic tripeptides on hHMGR activities. The concentrations of the tripeptide are 100 μM (200 μL final volume containing 1 % DMSO). The compounds examined are *1 cyclo*(E(OBn))$_3$, *2 cyclo*(E–E-E), *3 cyclo*(S(Bn))$_3$, *4 cyclo*(S(Bn)-P-E(OBn)), *5 cyclo*(S-P-E), *6 cyclo*(S(Bn)-P-S(Bn)), *7 cyclo*(S(Bn)-P-G). The control (C) reaction contains the same composition except no cyclotripeptide. The positive control (A) uses the same mixture with atorvastatin (3 nM). The experiments have been repeated for three times and the standard deviations calculated

that CtP received highest docking scores. The residue Asn755 was predicted to form a hydrogen bond with the tripeptide compound (Fig. 3c).

Enzymatic activity assays using recombinant hHMGR

The hHMGR was cloned, over-expressed and purified as described in "Materials and methods". The hHMGR enzymatic activities can be assessed by measuring the dependent oxidation of NAPDH (Pak et al. 2006). Using the data from the Lineweaver–Burk plot, it was found that under the experimental condition, the Km value was 121.4 ± 13.5 μM and the V_{max} was 2.63 ± 0.52 μmole △NADPH/min/mg. These values are reasonably close to previously published data from HMG-CoA-dependent oxidation of NADPH or a radioisotope method (Pak et al. 2006). The potential inhibitory effects of these CtPs on hHMGR enzyme activity were experimentally examined.

Figure 4 shows various amounts of CtPs were reacted with the recombinant hHMGR. Though the compound *cyclo*(E(OBn))$_3$ received the best docking scores, its inhibitory effect on hHMGR is less than *cyclo*(S(Bn))$_3$, which have poorer docking scores. Among the cyclic tripeptides, only *cyclo*(S(Bn))$_3$ effectively inhibits hHMGR. Its IC_{50} was determined to be approximately 110 μM. Though this value is weaker than that of statins (IC_{50} in the nM ranges), the present study demonstrated that the novels *cyclo*(S(Bn))$_3$ can act as inhibitors of hHMGR. Today, treatment of hypercholesterolemia with hHMGR inhibitor drugs is a mature protocol. CtPs still have a great potential to become safer drug candidates to treat aging-associated diseases such as hypercholesterolemia.

Conclusion

A reliable methodology has been developed to widen the scope of CtPs synthesis. Two new side-chain-protected units, (E(OBn) and S(Bn)) were revealed and new functional CtPs were generated. Through virtual screening, compounds *cyclo*(E(OBn))$_3$ and *cyclo*(S(Bn))$_3$ were found to act as inhibitors of hHMGR. Further analysis revealed that these compounds are placed in the same binding site as of the substrate HMG-CoA in the catalytic domain and may form two hydrogen bonds with Arg590 of hHMGR. Though the IC_{50} value (110 μM) of compound a *cyclo*(S(Bn))$_3$ against hHMGR is milder than statins, we have demonstrated for the first time that CtPs have an inhibitory effect on hHMGR. The overall clinical outcomes of CtPs still need to be thoroughly investigated and optimized by structure modifications. CtPs derivatives with different protection groups may be critical for fitting in the catalytic domain. A shorter distance for phenyl group may increase the chance to generate desired CtPs with a better inhibiting activity. CtPs can also serve as a new lead for new drug discovery through docking. The formation of CtPs with suitable functional group may also facilitate the interaction with other proteins for other applications.

Materials and methods

All reactions involving air or moisture sensitive reagents were carried out under a dry argon or nitrogen atmosphere using freshly distilled solvents. Pyridine and dioxane were distilled from CaH_2. The NMR spectra were recorded on a Bruker DRX 400, (^{1}H at 400.13 MHz, and ^{13}C at 100.03 MHz). Chemical shifts (δ) are reported in ppm relative to the residual solvent peak ($CHCl_3$, δ = 7.26, $^{13}CDCl_3$, δ = 77.0; CD_2HOD, δ = 3.34, $^{13}CD_3OD$, δ = 49.0; D_2O, and δ = 4.47). MALDI-TOF was performed on a Bruker Autoflex MALDI-TOF mass spectrometer (Bruker Daltonics, Breman, Germany). High-resolution electrospray ionization mass spectrometry (ESI–MS) was performed on a Shimadzu-LCMS-IT-TOF mass spectrometer. Infrared spectra were recorded on a Perkin Elmer Spectrum one FT-IR spectrometer using KBr pellets (4000–400 cm^{-1}).

Bacterial strains and materials

The bacterial strains used in the present study include: *E. coli* strain DH5α (*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*) for molecular cloning and *E. coli* strain BL21Star [F$^-$ *ompT hsdS B* (*r B*$^-$ *m B*$^-$) *gal dcm rne131*] for over-expression of the cloned genes. Vector pET28a and *E. coli* strains

were obtained from Invitrogen (USA). Atorvastatin was used a positive control in the hHMGR enzymatic assays and was purchased from Sigma-Aldrich Co. (USA).

General procedure for synthesis of compound 4f and 5e

To a solution of Amano-N/*B. licheniformis* protease (Sigma type–VIII) (60 mg) in buffer (0.1 M phosphate, pH 7; 100 mL) in a 250-mL round-bottom flask at 37 °C was added dropwise a solution of Boc-protected tripeptide dibenzyl ester (1 mmol) in acetone (25 mL). The reaction mixture was stirred for 10 h at 37 °C and monitored by TLC until the complete disappearance of starting material. The solution was then basified to pH 8 and unchanged ester was removed by ethyl acetate. The aqueous layer was acidified to pH 2 and centrifuged to remove enzyme. The filtrate was extracted with ethyl acetate and dried over sodium sulfate. The extract was evaporated and purified by column chromatography using DCM/MeOH (96:4) as eluent to obtain Boc-protected tripeptide α-acid γ-benzyl ester.

General procedure for synthesis of compound 1e, 2e, and 3d

To a solution of Boc-protected tripeptide methyl ester in methanol (20 mL) in a 50-mL round-bottom flask was slowly added 1 N NaOH (20 mL). After the addition was completed, the reaction mixture was stirred for 1 h at RT and monitored by TLC. After evaporation of methanol, the resultant mixture was acidified with 5 % citric acid and extracted with ethyl acetate. The combined organic extracts were dried over sodium sulfate and concentrated. The resultant mixture was purified by column chromatography using DCM/MeOH (96:4) to obtain acid unprotected tripeptides.

General procedure for synthesis of cyclic peptide 1f, 2f, 3e, 4g, and 5f

To a solution of acid-free tripeptide (1 mmol) in dichloromethane (10 mL) in a 25-mL round-bottom flask was added pentafluorophenol (1.1 mmol) and DCC (1 mmol) at 0 °C. The reaction mixture was allowed to stir for 1 h at 0 °C and then 22 h at RT. The solvent was evaporated and the residue was dissolved in ethyl acetate and filtered to remove DCU. The filtrate was evaporated to obtain pentafluorophenol-protected tripeptide and was placed in a (25-mL) round-bottom flask and cooled to 0 °C. To this, TFA/DCM (4 mL, 1:1) was added and stirred 1 h at 0 °C. The solvents were then concentrated. After triturating with ether, followed by decanting to remove pentafluorophenol, the residue was then dried in a vacuum and used directly in the ensuing cyclization.

To a solution of pyridine (500 mL) in a 1-L round-bottom flask was added drop wise a solution of crude Boc-deprotected tripeptide in dioxane (20 mL) with efficient stirring. Addition was completed after 6 h and the reaction mixture was stirred for 36 h at RT. Then the solvent was distilled off. The resultant mass was dissolved in DCM and washed successively with 5 % citric acid solution, water, 5 % sodium bicarbonate solution, and water. The organic layer was dried over sodium sulfate and concentrated. The resultant mixture was purified by column chromatography using DCM/MeOH (97:3) as eluent to obtain protected cyclic tripeptide.

General procedure for synthesis of cyclic peptide 3, 4, and 5

To a solution of benzyl-protected cyclic tripeptide in DMF/MeOH in a 10-mL round-bottom flask under nitrogen was added 20 % palladium hydroxide on carbon. The vessel was purged three times with nitrogen and then three times with hydrogen, and the mixture was allowed to stir for 2 h under hydrogen at atmospheric pressure. The catalyst was removed by filtration and the filtrate was evaporated to obtain unprotected cyclic tripeptide.

Boc-L-seryl(Bn)-L-seryl(Bn)-L-serine(Bn), 1e

Compound **1e** was obtained as a white solid (2.16 g, 90 %) by following general procedure. $[\alpha]_D^{28} = +14(c0.1, CHCl_3)$. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.42 (s, 9H), 3.57–3.87 (m, 6H), 4.42–4.51 (m, 7H), 4.67–4.78 (m, 2H), 5.56–5.62 (m, 1H), 7.22–7.26 (m, 15H), 7.50–7.52 (d, 1H, $J = 7.2$ Hz), 7.57 (s, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 28.3, 53.1, 54.2, 54.5, 69.4, 69.7, 73.2, 73.3, 73.3, 73.4, 80.4, 127.7, 127.7, 127.8, 127.8, 128.5, 137.4, 137.5, 137.6, 155.7, 170.1, 170.9, 172.8. IR (KBr) 3314, 2929, 1718, 1654, 1516, 1454, 1367, 1252, 1165, 1106, 1028, 747, 698 cm^{-1}. HRMS (ESI): m/z calcd. for $C_{35}H_{44}N_3O_9$ $[M+H]^+$ 650.3072; found 650.3062.

***Cyclo*(L-seryl(Bn)-L-seryl(Bn)-L-seryl(Bn)), 1f**

Compound **1f** was obtained as a white solid (58 mg, 11 %) by following general procedure. $[\alpha]_D^{21} = +9(c0.1, MeOH)$. ^{1}H NMR (DMSO d_6, 400 MHz) δ 3.58 (s, 6H), 4.41–4.47 (m, 6H), 4.66 (s, 3H), 7.24 (s, 15H), 8.19 (s, 3H). ^{13}C NMR (DMSO d_6, 100 MHz) δ 53.1, 70.3, 72.6, 127.9, 128.1, 128.6, 138.5, 169.6. IR (KBr) 3284, 3060, 2862, 1953, 1875, 1632, 1521, 1454, 1362, 1308, 1256, 1209, 1104, 1026, 908, 736, 695, 607, 459 cm^{-1}. MALDI-TOF: m/z 532.27 $[M+H]^+$. HRMS (ESI): m/z calcd. for $C_{30}H_{34}N_3O_6$ $[M+H]^+$ 532.2442; found 532.2495.

Boc-L-seryl(Bn)-L-prolyl-L-serine(Bn), 2e

Compound **2e** was obtained as a white solid (3.5 g, 95 %). $[\alpha]_D^{27} = -21(c0.1, CHCl_3)$ by following general procedure. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.40 (s, 9H), 1.91–2.04 (m, 3H), 2.15–2.16 (m, 1H), 3.59–3.79 (m, 6H), 4.30–4.33 (d, 2H, $J = 12.28$ Hz), 4.39 (s, 3H), 4.44–4.53 (m, 2H), 4.61–4.75 (m, 3H), 7.19–7.27 (m, 10H), 7.38–7.39 (d, 1H, $J = 7.48$ Hz), 8.41 (s, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 24.7, 28.4, 28.5, 47.9, 51.7, 52.7, 60.6, 69.6, 70.3, 73.1, 73.2, 80.0, 127.6, 127.7, 128.4, 128.4, 137.6, 137.7, 155.5, 171.4, 172.4. IR (KBr) 3318, 2978, 1708, 1643, 1524, 1454, 1367, 1251, 1166, 1107, 1027, 747, 699 cm^{-1}. HRMS (ESI): m/z calcd. for $C_{30}H_{40}N_3O_8$ $[M+H]^+$ 570.2810; found 570.2801.

***Cyclo*(L-seryl(Bn)-L-prolyl-L-seryl(Bn)), 2f**

Compound **2f** was obtained as a white solid (163 mg, 36 %) by following general procedure. $[\alpha]_D^{27} = -204(c0.1, MeOH)$. ^{1}H NMR ($CD_3OD$, 400 MHz) δ 1.73–1.85 (m, 1H), 1.91–2.03 (m, 1H), 2.08–2.17 (m, 1H), 2.55–2.61 (m, 1H), 3.52–3.54 (d, 2H, $J = 9.2$ Hz), 3.64–3.72 (m, 2H), 3.85–3.90 (m, 1H), 4.00–4.02 (d, 2H, $J = 9.08$ Hz), 4.12 (s, 1H), 4.39 (s, 1H), 4.46 (s, 2H), 4.50 (s, 2H), 4.57–4.63 (m, 1H), 7.23–7.33 (m, 10H). ^{13}C NMR (CD_3OD, 100 MHz) δ 21.6, 28.9, 44.6, 57.4, 58.6, 69.7, 70.6, 72.9, 73.1, 127.6, 127.7, 127.7, 128.1, 128.3, 137.5, 137.8, 161.6, 164.4. IR (KBr) 3432, 1671, 1455, 1359, 1206, 1137, 802, 723, 700 cm^{-1}. MALDI-TOF: m/z 452.21 $[M+H]^+$; HRMS (ESI): m/z calcd. for $C_{25}H_{30}N_3O_5$ $[M+H]^+$ 452.2180; found 452.2179.

***Cyclo*(L-seryl-L-prolyl-L-seryl), 2**

To a solution of *cyclo* (L-seryl(Bn)-L-prolyl-L-seryl(Bn)) (100 mg, 0.22 mol) in MeOH (2 mL) in a 250 mL bottle was added 20 % palladium hydroxide on carbon (10 mg) under nitrogen. The reaction vessel was purged three times with nitrogen and then the bottle was shaken at 50 lbs per sq inch hydrogen pressure in a parr shaker for 6 h. The catalyst was removed by filtration and the filtrate was evaporated to obtain compound **2** as a white solid (57 mg, 95 %). $[\alpha]_D^{21} = +11^0(c1, MeOH)$. ^{1}H NMR ($CD_3OD$, 400 MHz) δ 2.02–2.16 (m, 3H), 2.68 (s, 1H), 3.60 (s, 2H), 3.86 (s, 2H), 3.98–4.05 (m, 3H), 4.14 (s, 1H), 4.36 (s, 1H), 4.77 (s, 1H). ^{13}C NMR (CD_3OD, 100 MHz) δ 21.6, 28.9, 44.7, 53.4, 57.5, 59.8, 61.5, 63.3, 162.5, 164.5. IR (KBr): 3384, 1675, 1456, 1343, 1314, 1206, 1141, 1062, 846, 801, 725, 603, 520 cm^{-1}. MALDI-TOF: m/z 272.39 $[M+H]^+$. HRMS (ESI): m/z calcd. for $C_{11}H_{18}N_3O_5$ $[M+H]^+$ 272.1241; found 272.1236.

Boc-L-seryl(Bn)-L-prolyl-glycine, 3d

Compound **3d** was obtained as a white solid (1.87 g, 92 %) by following general procedure. $[\alpha]_D^{27} = -41(c0.1, CHCl_3)$. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.34 (s, 9H), 1.86–2.08 (m, 4H), 3.41–3.76 (m, 6H), 4.32–4.44 (m, 2H), 4.56–4.57 (m, 1H), 4.70–4.76 (m, 1H), 4.84–4.86 (d, 2H, $J = 8$ Hz), 7.15 (s, 1H), 7.05–7.35 (m, 5H), 10.99 (s, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 24.3, 28.3, 28.8, 40.7, 47.7, 51.4, 60.2, 70.8, 73.5, 80.1, 127.4, 128.1, 128.6, 137.2, 155.5, 170.9, 171.6, 171.8. IR (KBr) 3322, 2978, 1712, 1647, 1532, 1454, 1367, 1252, 1166, 1102, 1048, 1025, 870, 747, 700 cm^{-1}. HRMS (ESI): m/z calcd. for $C_{22}H_{32}N_3O_7$ $[M+H]^+$ 450.2235; found 450.2226.

***Cyclo*(L-seryl(Bn)-L-prolyl-glycyl), 3e**

Compound **3e** was obtained as a white solid (83 mg, 25 %). $[\alpha]_D^{27} = -99(c0.1, MeOH)$ by following general procedure. ^{1}H NMR (CD_3OD, 400 MHz) δ 1.91–2.14 (m, 3H), 2.55–2.58 (m, 1H), 3.49–3.60 (m, 2H), 3.75 (dd, 1H, $J = 4.5$, 8.2 Hz), 3.93 (s, 2H), 3.96 (s, 1H), 4.25 (s, 1H), 4.51 (s, 2H), 4.65–4.69 (m, 1H), 7.27–7.35 (m, 5H). ^{13}C NMR (CD_3OD, 100 MHz) δ 21.7, 28.5, 44.7, 45.4, 57.2, 58.1, 70.7, 73.1, 127.7, 127.8, 128.3, 137.6, 162.3, 164.1. IR (KBr) 3419, 3065, 2957, 1675, 1456, 1385, 1310, 1204, 1131, 1074, 1027, 835, 801, 745, 721, 700 cm^{-1}. MALDI-TOF: m/z 332.24 $[M+H]^+$; HRMS (ESI): m/z calcd. for $C_{17}H_{22}N_3O_4$ $[M+H]^+$ 332.1605; found 332.1600.

***Cyclo*(L-seryl-L-prolyl-glycyl), 3**

Compound **3** was obtained as a white solid (73 mg, 95 %) by following general procedure. $[\alpha]_D^{21} = +19(c1, MeOH)$. ^{1}H NMR ($CD_3OD$, 400 MHz) δ 2.00 (s, 2H), 2.15 (s, 1H), 2.57 (s, 1H), 3.58–3.59 (d, 2H, $J = 7.4$ Hz), 3.82–3.88 (m, 1H), 3.92–3.95 (m, 2H), 3.99 (s, 1H), 4.09 (s, 1H), 4.70 (s, 1H). ^{13}C NMR (CD_3OD, 100 MHz) δ 21.7, 28.7, 44.6, 45.6, 57.2, 60.4, 63.6, 161.9, 165.2. IR (KBr) 3430, 2347, 1675, 1459, 1386, 1312, 1206, 1137, 1064, 840, 803, 724 cm^{-1}. MALDI-TOF: m/z 242.10, $[M+H]^+$. HRMS (ESI): m/z calcd. for $C_{10}H_{16}N_3O_4$ $[M+H]^+$ 242.1135; found 242.1126.

Boc-L-glutamyl(OBn)-L-glutamyl(OBn)-L-glutamyl-α-acid γ-benzyl ester, 4f

Compound **4f** was obtained as a white solid (7.1 g, 82 %) by following general procedure. $[\alpha]_D^{28} = -48(c0.1, CHCl_3)$. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.37 (s, 9H), 1.90–2.04 (m, 3H), 2.10–2.22 (m, 3H), 2.44 (t, 6H, $J = 6.84$), 4.21 (s, 1H), 4.48 (s, 1H), 4.55 (s, 1H), 5.06-5.08 (d, 6H,

$J = 5.12$ Hz), 5.60 (s, 1H), 7.29–7.31 (d, 15H, $J = 5.2$ Hz), 7.52 (s, 2H), 8.76 (s, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 26.6, 27.4, 28.3, 29.7, 30.2, 30.4, 52.5, 52.8, 54.0, 66.5, 66.6, 66.6, 80.3, 127.3, 128.2, 128.5, 135.8, 135.8, 135.9, 156.0, 171.5, 172.4, 172.9, 173.2, 173.5, 174.8. IR (KBr) 3322, 2977, 1731, 1656, 1517, 1455, 1392, 1367, 1259, 1167, 750, 698 cm^{-1}. HRMS (ESI): m/z calcd. for $C_{41}H_{50}N_3O_{12}$ $[M+H]^+$ 776.3389; found 776.3398.

Cyclo(L-glutamyl(OBn)-L-glutamyl(OBn)-L-glutamyl(OBn)), 4g

Compound **4g** was obtained as a white solid (125 mg, 19 %) by following general procedure. $[\alpha]_D^{21} = -11^0(c0.1,$ DCM) . ^{1}H NMR (DMSO d_6, 400 MHz) δ 1.78-1.80 (d, 3H, $J = 5.24$ Hz), 1.91 (s, 3H), 2.35 (s, 6H), 4.25 (s, 3H), 5.02 (s, 6H), 7.30 (s, 15H), 8.06 (s, 3H). ^{13}C NMR (DMSO d_6, 100 MHz) δ 27.7, 30.5, 52.3, 65.9, 128.3, 128.4, 128.9, 136.6, 171.3, 172.7. IR (KBr) 3289, 3065, 3034, 2951, 1735, 1693, 1624, 1524, 1455, 1387, 1259, 1166, 1082, 1003, 739, 698 cm^{-1}. MALDI-TOF: m/z 668.44 $[M+H]^+$; HRMS (ESI): m/z calcd. for $C_{37}H_{40}N_3O_9$ $[M+H]^+$ 658.2759; found 658.2756.

Cyclo(L-glutamyl-L-glutamyl-L-glutamyl), 4

Compound **4** was obtained as a white solid (56 mg, 95 %) by following general procedure. ^{1}H NMR (D_2O/pyridine d_5, 400 MHz) δ 1.80 (s, 3H), 1.90 (s, 3H), 2.16 (s, 6H), 4.17 (s, 3H). ^{13}C NMR (D_2O/pyridine d_5, 100 MHz) δ 27.4, 32.9, 53.7, 173.6, 180.2, 180.5, 180.7. IR (KBr) 3349, 2948, 2371, 1736, 1607, 1542, 1411, 1278, 1168, 1114, 1065, 785, 633 cm^{-1}. HRMS (ESI): m/z calcd. for $C_{15}H_{22}N_3O_9$ $[M+H]^+$ 388.1351; found 388.1377.

Boc-L-seryl(Bn)-L-prolyl-L-glutamic acid γ-benzyl ester, 5e

Compound **5e** was obtained as a white solid (690 mg, 85 %) by following general procedure. $[\alpha]_D^{27} = -36(c0.1,$ $CHCl_3)$. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.40 (s, 9H), 1.69–1.72 (m, 1H), 1.93–2.09 (m, 4H), 2.16–2.33 (m, 3H), 3.65–3.67 (m, 3H), 3.82–3.85 (m, 1H), 4.41–4.59 (m, 4H), 4.75–4.77 (d, 1H, $J = 8$ Hz), 5.05 (s, 9H), 5.77–5.79 (d, 1H, $J = 8$ Hz), 7.20–7.32 (m, 10H), 9.00 (s, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 24.8, 27.0, 28.3, 30.3, 47.9, 51.6, 60.7, 66.4, 70.5, 73.3, 80.1, 127.4, 127.9, 128.3, 128.5, 128.6, 135.8, 137.4, 155.5, 171.3, 171.4, 172.5, 173.5. IR (KBr) 3321, 2977, 1736, 1648, 1524, 1454, 1392, 1367, 1253, 1166, 1116, 1026, 869, 746, 699 cm^{-1}. HRMS (ESI): m/z calcd. for $C_{32}H_{42}N_3O_9$ $[M+H]^+$ 612.2916; found 612.2915.

Cyclo(L-seryl(Bn)-L-prolyl-L-glutamyl(OBn)), 5f

Compound **5f** was obtained as a white solid (207 mg, 42 %) by following general procedure. $[\alpha]_D^{27} = -177(c0.1,$ MeOH). ^{1}H NMR (CD_3OD, 400 MHz) δ 1.83–1.99 (m, 2H), 2.09–2.35 (m, 4H), 2.55–2.57 (d, 1H, $J = 8$ Hz), 3.53–3.55 (d, 1H, $J = 8$ Hz), 3.70–3.72 (d, 1H, $J = 8$ Hz), 3.94–3.96 (d, 1H, $J = 8$ Hz), 4.22 (s, 1H), 4.50 (q, 2H), 4.58–4.64 (m, 1H), 5.10 (s, 2H), 7.29–7.32 (m, 10H). ^{13}C NMR (CD_3OD, 100 MHz) δ 21.7, 25.9, 28.9, 30.0, 44.6, 57.4, 58.5, 66.2, 70.8, 73.1, 127.6, 127.7, 127.9, 128.0, 128.3, 128.3, 136.1, 137.6, 161.7, 164.3, 173.3. IR (KBr) 3286, 3035, 2955, 1671, 1498, 1455, 1204, 1131, 1027, 909, 800, 741, 720, 698 cm^{-1}. MALDI-TOF: m/z 494.48 $[M+H]^+$; HRMS (ESI): m/z calcd. for $C_{27}H_{32}N_3O_6$ $[M+H]^+$ 494.2286; found 494.2277.

Cyclo(L-seryl-L-prolyl-L-glutamyl), 5

Compound **5** was obtained as a white solid (60 mg, 96 %) by following general procedure. $[\alpha]_D^{21} = +24(c1,$ MeOH). ^{1}H NMR (CD_3OD, 400 MHz) δ 1.97–2.10 (m, 2H), 2.14–2.21 (m, 2H), 2.36–2.66 (m, 4H), 3.58–3.60 (m, 2H), 3.79 (dd, 1H, $J = 2.6$, 2.48 Hz), 4.02 (dd, 1H, $J = 2.32$, 2.28 Hz), 4.12 (s, 1H), 4.42 (s, 1H), 4.76–4.80 (m, 1H). ^{13}C NMR (CD_3OD, 100 MHz) δ 21.7, 26.1, 28.9, 30.7, 44.7, 48.5, 57.4, 59.8, 63.3, 162.2, 164.4, 177.3. IR (KBr) 3331, 2957, 1665, 1448, 1338, 1312, 1202, 1135, 1063, 837, 800, 722, 599 cm^{-1}. MALDI-TOF: m/z 314.27 $[M+H]^+$. HRMS (ESI): m/z calcd. for $C_{13}H_{20}N_3O_6$ $[M+H]^+$ 314.1347; found 314.1355.

Protein structure and molecular docking

The 3-D structure PDB 1HWK of human HMG-CoA reductase was downloaded from the Protein Data Bank (http://www.rcsb.org/pdb) as the molecular target (Istvan and Deisenhofer 2001). A tetramer was chosen and fixed with the forcefield CHARMM (Chemistry at Harvard Macromolecular mechanics) equipped in DS 2.0 (http://accelrys.com/ products/discovery-studio) to add up the hydrogen atoms, partial charges, and missing residues to be properly used for molecular docking processes (Brooks et al. 1983; Huang et al. 2013). After PDB IHWK was downloaded, three of the tetrameric structures were removed, and the potential binding sites were predicted by the DS 2.0 modeling tool. The largest binding site was found to correlate with the binding sites of the 6 statins (Istvan and Deisenhofer 2001). The newly synthesized tripeptides were docked and analyzed using potential of mean force (PMF) scores and DOCK scores (Brooks et al. 1983; Muegge and Martin 1999; Venkatachalam et al. 2003).

Cloning, over-expression and purification of recombinant hHMGR

The 1380 bp DNA encoding the catalytic domain (residue 460 to residue 888) and part of the linker region (residue 429 to residue 460) of human HMGR gene was amplified by PCR. The mRNA of hHMGR gene was collected from HepG2 cells, reverse transcribed to cDNA, and used as PCR template using the forward primer: 5′-GGT CTC GAG TAT TCA GGC TGT CTT CTT-3 and reverse primer: 5′-ACA GGA TCC AAC TCC TCC TTA CTC GAT-3′. Then the DNA fragment was subcloned into the *Xho*I–*Bam*HI site of vector pET28a to obtain plasmid pET28a/hHMGR. The pET28a vector carries an N-terminal His-tag/thrombin/T7-tag sequence and a *lac*I operator between T7 promoter and the ribosomal binding site RBS. Then, the plasmid pET28a/hHMGR was used to transform *E. coli* BL21 Star and the expression of the cloned gene was induced by the addition of 1 mM IPTG at OD_{600} ~ 0.6. After growing for another 3 h, the cells were collected and sonicated with Ultrasonic Processor (Microson/M8726). The recombinant His-HMG protein was then purified using the HiTrap Chealth column (Amersham, USA) connected to an AKTA prime fractional collector (GE Healthcare, USA). The collected protein was further concentrated by passing through Centricon (Millipore, USA) and finally dissolved in 0.1 M KH_2PO_4 buffer (Lin et al. 2012; Luskey and Stevens 1985).

Characterization and measurement of hHMGR activity

The HMG-CoA reductase (HMGR)-dependent oxidation of NAPDH was measured for assessing the hHMGR activity. Testing compounds were added to the assay mixtures (200 μL final volume) containing 100 μM HMG-CoA, 200 μM NADPH, 10 mM DTT, 10 mM EDTA, 200 mM KCl and KH_2PO_4 buffer, pH 7.4. Then, 20 μg HMGR was added to each reaction mixture and the reactions were measured by following OD_{340} (at a 30-min interval) using an EnSpire Multimode ELISA plate reader (Pak et al. 2005). One unit (U) of hHMGR was defined as the amount of enzyme that catalyzes the oxidation of 1 mol of NADPH per minute. Protein concentrations were determined by the Bradford method (Bradford 1976).

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Conflict of interest The authors declare that they have no conflict of interest.

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