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Short communication

Insulin-releasing activity of a series of phenylalanine derivatives

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Abstract

A series of phenylalanine derivatives were synthesized and their biological activities were evaluated. Compounds (*S*)-**3** and (*R*)-**3** exhibited more potent insulin-releasing activity than that of nateglinide, compound (*S*)-**3** also showed insulin-sensitizing activity *in vitro*. Both compounds were tested for hypoglycemia effect *in vivo*.

Keywords: Insulin-releasing activity; Insulin-sensitizing activity; Hypoglycemia effect; Phenylalanine

1. Introduction

Type 2 diabetes is a multifaceted disease characterized by insulin resistance and/or abnormal insulin secretion. This metabolic disorder accounts for more than 90% of all diabetes, its worldwide frequency is expected to grow by 6% annually, reaching a potential total of 200–300 million cases in 2010 [1]. Hyperglycemia often leads to several complications such as neuropathy, retinopathy, nephropathy and premature atherosclerosis [2]. Therefore, it is important to maintain an appropriate blood glucose level, especially during the early stage of the disease. The therapy for type 2 diabetes is often by a combination of diet, exercise, or with pharmacological agents. Current pharmacological agents available for type 2 diabetes include mainly insulin secretagogues, insulin formulations, α-glucosidase inhibitors and insulin-sensitizers [3]. Insulin secretagogues, due to their direct and fast hypoglycemic effect through stimulating the releasing of insulin from pancreatic β-cells, have been used widely in the treatment of type 2 diabetes.

Nateglinide is an insulin-releasing stimulator marketed in 1999 [4]. Nateglinide contains a carboxylic group on the right end of the structure. Another compound gliquidone, the third generation of sulfonylurea insulin secretagogue, also contains an acidic sulfonylurea group on the right of its structure, and on the left part of gliquidone's structure, there is a lipophilic aromatic alkyl group. Another type of antidiabetic agent, glitazars insulin-sensitizers, also has the structural features mentioned above [5]. So in our work searching for compounds with more potent insulin-releasing activity, we designed some nateglinide analogues by adding lipophilic substituents onto the phenyl ring of nateglinide (Fig. 1). Meanwhile, considering the configurational difference of α-carbon atom between nateglinide and insulin-sensitizing agent farglitazar, we decided to synthesize both enatiomers of all designed compounds to observe the steric effect of α-carbon atom on biological activity (Table 1).

2. Chemistry

Compound **1** is the key intermediate in the synthesis of designed target compounds. Optically active compound **1** was synthesized by condensation of L- or D-tyrosine methyl ester with *trans*-4-isopropylcyclohexylcarboxylic acid in the existence of *N*-hydroxysuccimide (HOSu) and *N*,*N*′-dicyclohexylcarbodiimide (DCC). Compound **1** and the corresponding heterocycloalkyl alcohol or 4-trifluoromethylbenzyl alcohol

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were used in a Mitsunobu condensation, followed by hydrolysis to give compounds **2**–**4** (Scheme 1).

Compound **1** condensed with *tert*-butoxycarbonyl protected 2-methylaminoethanol giving 2-[*N*-(*trans*-4-isopropylcyclohexylcarbonyl)amino]-3-[4-(*N*-methyl-*N*-(*tert*-butoxycarbonyl)aminoethoxy)phenyl]propionic acid methyl ester, which was treated with trifluoroacetic acid to remove the protective group affording 2-[*N*-(*trans*-4-isopropylcyclohexylcarbonyl)amino]-3-[4-(2-methylaminoethoxy)phenyl]propionic acid methyl ester (compound **5**). Compounds **6** and **7** were obtained by condensation of compound **5** with 2-fluoropyridine and 2-chlorobenzoxazole, respectively, and then followed by hydrolysis (Scheme 2).

Compounds **8**–**10** were afforded by saponification of the condensed products of compound **1** with corresponding alkyl halide. Compound **11** was obtained directly by hydrolysis of compound **1**. Compound **12**, the *S*-antipode of nateglinide, was synthesized by using L-phenylalanine as starting material following the method of Ref. [4].

3. Pharmacological evaluation and discussion

All synthesized compounds were screened for insulin-releasing activity in HIT-T15 cells by measuring glucose-induced insulin-releasing [6]. Nateglinide and mitiglinide were selected as positive controls. The insulin-releasing activities of all compounds were given in Table 2.

Compounds with strong insulin-releasing activity were retested, their EC_{50}s (effective concentration of causing 50% increase of insulin-releasing in HIT-T15 cells) were calculated and listed in Table 3.

For nateglinide derivatives, it was reported that *R*-configuration was necessary for insulin-releasing activity [4,7]. However, in our study we find that when certain substituents were added onto the phenyl ring of nateglinide, the compounds with *S*-configuration also showed potent insulin-releasing activity. Comparing the activities of different configurations of compounds **8** and **9** (Table 3) it could be seen that compounds with *S*-configuration even possessed a higher insulin-releasing activity than that of *R*-configuration. The maximum insulin-releasing activity was achieved by compound (*R*)-**3**, which is 63-fold more potent than the positive nateglinide *in vitro* judged from their EC_{50}s.

Compound (*S*)-**3** also exhibited a high insulin-releasing activity *in vitro*. Considering the structural similarity between (*S*)-**3** and farglitazar, the insulin-sensitizing activity of (*S*)-**3** was tested on 3T3-L1 preadipocyte cells following the literature's method by measuring the triglyceride accumulation generating from insulin-regulated differentiation of preadipocyte versus adipocyte [8]. The triglyceride accumulation increase caused by (*S*)-**3** at concentrations of 1 μM and 10 μM were 38.14% and 46.01%, respectively; for rosiglitazone at concentrations of 1 μM and 10 μM, the triglyceride accumulation increased by 38.81% and 35.87%, respectively. The results

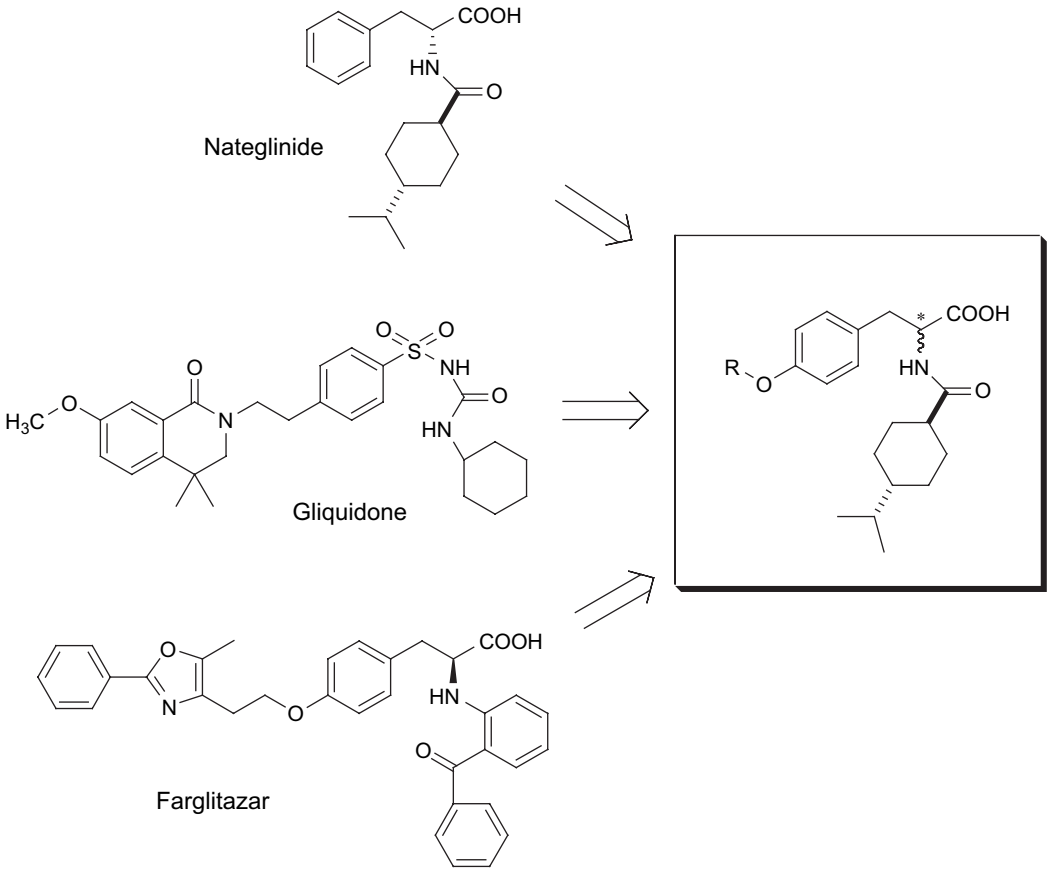
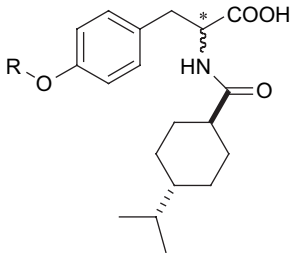
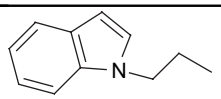
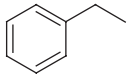
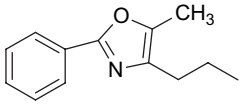
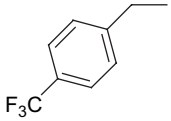
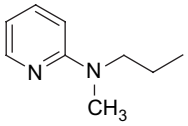
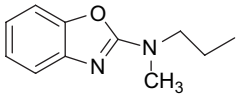


Fig. 1. Design of phenylalanine derivatives.

Table 1
Structure of phenylalanine derivatives



Compounds[a]	R	Compounds[a]	R
2		**8**	
3		**9**	*n*-Bu
4		**10**	Et
6		**11**	H
7			

[a] Both (*S*) and (*R*) configurations of α-carbon atom of all compounds were synthesized for activity-screening.

indicated that (*S*)-**3** showed good insulin-sensitizing activity almost equivalent to the positive control rosiglitazone.

To test *in vivo* activities of (*S*)-**3** and (*R*)-**3**, both compounds were administered by oral gavage to glucose-loading normal SD rats at a dose of 3 mg/kg body weight. The serum-glucose levels were measured at several time points after dosing, and the results were given as decreasing percentage of plasma-glucose level compared to control (Table 4). In this test, compound (*R*)-**3** showed impressive glucose reduction at the time point of 30 min and 60 min after dosing. The reduction percentage amounts to 36% at the point of 30 min, which was nearly equal to the control drug mitiglinide. However, the plasma-glucose level after that gradually got back to the level of negative control group, and was maintained till the end of test. Meanwhile, mitiglinide exhibited a continuing lowering action on plasma-glucose throughout the test. Compound (*S*)-**3** showed no lowering action at all time points. The biological activity *in vivo* exhibits a poor correlation with the results obtained *in vitro*, the underlying reasons may include inferior bioavailability and unstable metabolic properties, which need further exploration.

In the study of antidiabetic agents, designing of compounds with both insulin-sensitizing activity and insulin-releasing activity is also an important research field. Although the synthesized compounds in our work didn't show satisfactory activity *in vivo*, it is no doubt that the type of compounds could offer us a hint to design and synthesize more compounds with higher insulin-releasing activity or even with higher insulin-sensitizing activity.

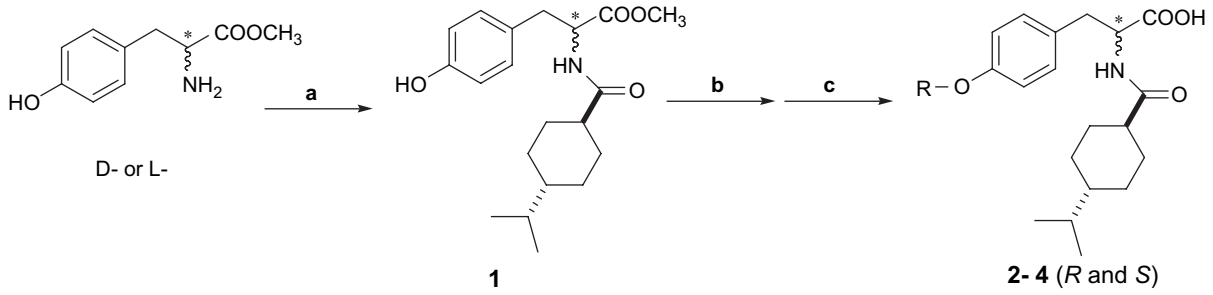
4. Conclusions

In summary, we found that the *S*-configuration derivatives of nateglinide possess potent insulin-releasing activity *in vitro*, and compound (*S*)-**3** even exhibited insulin-sensitizing activity *in vitro*. Among all synthesized compounds, both compounds (*S*)-**3** and (*R*)-**3** possessed more potent insulin-releasing activity than that of nateglinide and mitiglinide in HIT-T15 cells, but both compounds showed a weak *in vivo* activity in rat model. Further structure–activity relationship exploration of this type of compounds is still undergoing.

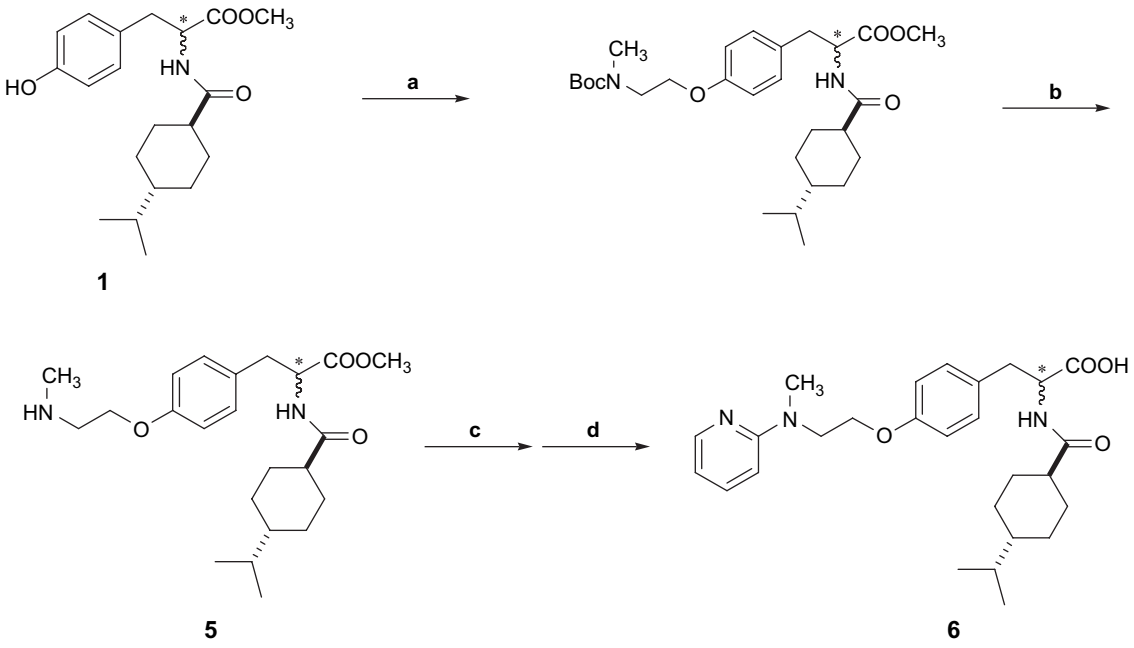
5. Experimental protocols

5.1. General chemistry

All melting points were determined on MEL-TEMP melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Varian Mercury 400 NMR spectrometer with TMS as internal standard, chemical shifts are expressed as δ (ppm). Mass spectra were determined using Finnigan MAT 95, EI: 70 eV. High resolution mass spectra (HRMS) were performed on a Kratos MS80 instrument. Elemental analyses (C, H, N) were performed on a Leco CHN-2000



Scheme 1. (a) *trans*-4-Isopropylcyclohexylcarboxylic acid, HOSu, DCC; (b) Ph_3P, diethyl azodicarboxylate, ROH, THF and (c) 1 N LiOH, THF/MeOH (3:1).



Scheme 2. (a) 2-(*N*-Boc-*N*-methlyamino) ethanol, Ph_3P, diethyl azodicarboxylate, THF; (b) trifluoroacetic acid, CH_2Cl_2; (c) 2-fluoropyridine, reflux and (d) 1 N LiOH, THF/MeOH (3:1).

elemental analyzer and all compounds are within ±0.4% of theory. Flash chromatography was carried out on silica gel (200–300 mesh), and chromatographic solvent proportions are expressed on a volume:volume basis. Optical rotations were measured with a Perkin–Elmer 241 polarimeter at room temperature (20 °C). IR spectrums were recorded on a Bruker Tensor 27 FTIR spectrometer. Synthetic procedures of *S*-configuration compounds are listed below; the synthetic procedures of *R*-configuration compounds are the same with their corresponding antipodes. All analytic data of *R*-configuration compounds are nearly identical with that of *S*-configuration except for the optical rotations, which exhibited opposite rotation but with a nearly identical value compared to the corresponding *S*-configuration compounds.

Table 2
Insulin-releasing activities of the compounds (mean ± sd, $n = 3$)

Compounds	Insulin-releasing activity[a]		
	at 1 μM	at 10 μM	at 100 μM
(*S*)-**2**	35.7 ± 3.3	74.9 ± 0.8	146.6 ± 6.8
(*R*)-**2**	37.8 ± 3.8	58.8 ± 4.1	111.7 ± 17.2
(*S*)-**3**	68.5 ± 6.0	131.2 ± 6.2	329.2 ± 12.6
(*R*)-**3**	105.2 ± 7.1	200.9 ± 21.7	487.4 ± 44.6
(*S*)-**4**	47.9 ± 5.1	76.7 ± 16.2	156.9 ± 6.5
(*R*)-**4**	29.8 ± 2.7	85.6 ± 12.4	152.1 ± 33.8
(*S*)-**6**	26.0 ± 2.0	41.5 ± 12.5	83.7 ± 20.3
(*R*)-**6**	24.4 ± 6.4	36.2 ± 6.7	59.4 ± 7.6
(*S*)-7	35.5 ± 4.1	72.8 ± 11.3	126.9 ± 5.5
(*R*)-7	24.8 ± 3.3	81.7 ± 12.6	145.1 ± 23.6
(*S*)-**8**	53.1 ± 2.0	147.5 ± 12.5	225.9 ± 49.9
(*R*)-**8**	38.9 ± 3.8	94.4 ± 10.5	171.1 ± 25.7
(*S*)-**9**	73.4 ± 5.6	136.7 ± 12.9	326.2 ± 13.0
(*R*)-**9**	58.9 ± 9.3	107.1 ± 4.4	261.6 ± 19.5
(*S*)-**10**	55.8 ± 3.6	99.9 ± 12.8	151.3 ± 3.8
(*R*)-**10**	76.2 ± 4.6	107.3 ± 11.7	183.8 ± 8.7
(*S*)-**11**	17.5 ± 1.6	35.4 ± 2.4	64.8 ± 8.6
(*R*)-**11**	34.2 ± 8.8	69.6 ± 9.3	122.2 ± 40.2
(*S*)-Nateglinide (**12**)	26.4 ± 7.2	35.3 ± 11.7	61.1 ± 14.2
Nateglinide	36.3 ± 5.8	66.8 ± 3.7	124.3 ± 6.9
Mitiglinide	52.5 ± 1.62	66.0 ± 1.55	120 ± 8.16

[a] Insulin-releasing activities were given as the increasing percentage of insulin-releasing from HIT-T15 cells than that of the negative group (DMSO).

5.2. (2*S*)-2-[*N*-(trans-4-isopropylcyclohexylcarbonyl) amino]-3-(4-hydroxyphenyl)propionic acid methyl ester ((*S*)-*1*)

To a solution of 0.7 g (4.1 mmol) of *trans*-4-isopropylcyclohexylcarboxylic acid and 0.53 g (4.6 mmol) of HOSu in 14 mL of chloroform was added 0.95 g (4.6 mmol) of DCC portionwise. The resulting solution was stirred at room temperature for 3 h. The precipitate produced was filtered off. Acetic acid (0.4 mL) was added into the filtrate and stirred at room temperature for 2 h. Saturated $NaHCO_3$ (10 mL) was added into the mixture and stirred together, then the water layer was removed. The chloroform layer was washed with 5 mL of H_2O and brine, then dried over Mg_2SO_4 and filtered. L-Tyrosine methyl ester (0.80 g, 4.1 mmol) was added, and stirred at room temperature for 24 h. The solution was washed with 1 N HCl, water, then dried on Mg_2SO_4 and filtered. After removal of the solvent in vacuo, the residue was dissolved in methanol, kept at −20 °C, a white solid was separated out. The mother liquor was concentrated and then another portion of solid was collected. Title compound (0.53 g) was afforded together. Yield: 37.8%. M.p. 123–126 °C. $[\alpha]_D^{25}$ 54.3 (*c* 0.50, $CHCl_3$). ^1H NMR ($CDCl_3$): $\delta = 0.82$ (d, $J = 6.9$ Hz,

Table 3
EC_{50}s of the compounds ($n = 3$)

Compounds	(*S*)-**3**	(*R*)-**3**	(*S*)-**8**	(*R*)-**8**	(*S*)-**9**	(*R*)-**9**	(*S*)-**10**	(*R*)-**10**	Nateglinide	Mitiglinide
EC_{50} (μM)	0.54	0.12	1.05	2.82	0.55	0.65	0.96	0.26	7.59	0.708

6H), 0.89–1.12 (m, 3H), 1.30–1.43 (m, 3H), 1.70–1.90 (m, 4H), 2.05 (m, 1H), 2.96 (dd, $J = 6.5$ Hz, 14.2 Hz, 1H), 3.09 (dd, $J = 5.5$ Hz, 14.7 Hz, 1H), 3.72 (s, 3H), 4.85 (m, 1H), 6.00 (d, $J = 8.1$ Hz, 1H), 6.70 (d, $J = 8.4$ Hz, 2H), 6.90 (d, $J = 8.2$ Hz, 2H). IR (KBr, cm^{-1}): 3408.8 (O–H), 3325.7 (N–H), 1622.1 (C=O); MS (EI) *m/e* (%): 347 (19, M^+), 170 (100). Anal. Calcd for $C_{20}H_{29}NO_4$ (%): C, 69.14; H, 8.41; N, 4.03. Found: C, 69.25; H, 8.75; N, 3.88.

*5.3. (2S)-2-[N-(trans-4-isopropylcyclohexylcarbonyl) amino]-3-[4-[2-(1-indolyl)ethoxy]phenyl]propionic acid ((S)-**2**)*

Triphenylphosphine (0.61 g, 2.31 mmol) was added into a stirred solution of 0.24 g (1.54 mmol) of 2-(1-indolyl)ethanol and 0.54 g (1.54 mmol) of (*S*)-**1** in 30 mL of anhydrous tetrahydrofuran, then 0.37 mL (2.31 mmol) of diethyl azodicarboxylate was added dropwise at 0 °C. The resulting solution was stirred at room temperature for 24 h. After solvent was removed under reduced pressure, the residue was diluted with ether and a solid separated out, the solid was filtered out and recrystallized from methanol to give a white solid. The solid was dissolved in 1 mL of mixed solvent of tetrahydrofuran–methanol (3:1), then 1 mL of 1 N solution of LiOH in H_2O was added, the resulting solution was stirred at room temperature for 24 h. The pH of the solution was adjusted to 5 with 1 N HCl and 5 mL of H_2O was added and then this mixture was stirred, filtered, and the filter cake was recrystallized from methanol to give 0.29 g of the title compound. Yield: 39.6%. M.p. 165–167 °C (dec). $[\alpha]_D^{25}$ 26.7 (*c* 0.545, $CHCl_3$). ^1H NMR ($CDCl_3$): $\delta = 0.81$ (d, $J = 6.9$ Hz, 6H), 0.80–1.10 (m, 3H), 1.30–1.45 (m, 3H), 1.70–1.90 (m, 4H), 1.99 (m, 1H), 3.07 (dd, $J = 14.3$ Hz, 6.2 Hz, 1H), 3.15 (dd, $J = 14.3$ Hz, 5.2 Hz, 1H), 4.23 (t, $J = 5.5$ Hz, 2H), 4.50 (t, $J = 5.5$ Hz, 2H), 4.75 (m, 1H), 5.89 (d, $J = 6.6$ Hz, 1H), 6.50 (d, $J = 3.9$ Hz, 1H), 6.76 (d, $J = 8.4$ Hz, 2H), 7.00 (d, $J = 8.4$ Hz, 2H), 7.11 (t, $J = 7.3$ Hz, 1H), 7.24 (m, 2H), 7.40 (d, $J = 8.1$ Hz, 1H), 7.62 (d, $J = 7.7$ Hz, 1H); IR (KBr, cm^{-1}): 3305.4 (N–H), 1712.5 (C=O), 1650.8 (C=O); MS (EI) *m/e* (%): 476 (11, M^+), 144 (100); HRMS: 476.2670 ($C_{29}H_{36}N_2O_4$).

Table 4
Decreasing percentage of plasma-glucose level after administration of compound (*S*)-**3** and (*R*)-**3**[a]

Group	Decreasing percentage of serum-glucose level compared to control group (%)					
	0 min	15 min	30 min	60 min	120 min	180 min
(*S*)-**3**	−8.37	−4.73	−8.51	−4.47	−11.3	−2.07
(*R*)-**3**	−0.853	−21.2	−36.4	−33.7	−17.3	−11.4
Mitiglinide	8.72	−16.5	−36.2	−61.7	−87.3	−70.3

[a] Dosage: 3 mg/kg body weight, $n = 8$–10.

*5.4. (2S)-2-[N-(trans-4-isopropylcyclohexylcarbonyl) amino]-3-[4-[2-(5-methyl-2-phenyl-4-oxazole) ethoxy]phenyl]propionic acid ((S)-**3**)*

Using (*S*)-**1** and 2-(5-methyl-2-phenyl-4-oxazole)ethanol as starting material, the title compound was prepared following the procedure in 5.3. Yield: 77.2%. M.p. 151–153 °C (dec). $[\alpha]_D^{25}$ 81.1 (*c* 0.545, $CHCl_3$). ^1H NMR (DMSO-d_6): $\delta = 0.80$ (d, $J = 7.6$ Hz, 6H), 0.85–1.0 (m, 3H), 1.10–1.40 (m, 3H), 1.50–1.72 (m, 4H), 2.00 (m, 1H), 2.35 (s, 3H), 2.75 (dd, $J = 14$ Hz, 9.9 Hz, 1H), 2.90 (t, $J = 6.6$ Hz, 2H), 2.96 (dd, $J = 14$ Hz, 4.7 Hz, 1H), 4.17 (t, $J = 6.6$ Hz, 2H), 4.21 (m, 1H), 6.81 (d, $J = 8.8$ Hz, 2H), 7.13 (d, $J = 8.8$ Hz, 2H), 7.50 (m, 3H), 7.92 (m, 3H), 12.6 (s, 1H); IR (KBr, cm^{-1}): 3282.3 (N–H), 1710.6 (C=O), 1631.5(C=O); MS (EI) *m/e* (%): 518 (1, M^+), 186 (100); Anal. Calcd for $C_{31}H_{38}N_2O_5$ (%): C, 71.81; H, 7.34; N, 5.41. Found: C, 71.49; H, 7.24; N, 5.36.

*5.5. (2S)-2-[N-(trans-4-isopropylcyclohexylcarbonyl) amino]-3-[4-(4-trifluoromethylphenylmethoxy)phenyl] propionic acid ((S)-**4**)*

Using (*S*)-**1** and 4-trifluoromethylbenzyl alcohol as starting materials, ethyl ether was used as solvent, the title compound was prepared following the procedure in 5.3. Yield: 60.6%. M.p. 171–172 °C. $[\alpha]_D^{25}$ 53.9 (*c* 0.285 in $CHCl_3$). ^1H NMR ($CDCl_3$): $\delta = 0.81$ (d, $J = 6.7$ Hz, 6H), 1.0 (m, 3H), 1.40 (m, 3H), 1.70–1.90 (m, 4H), 2.05 (m, 1H), 3.08 (dd, $J = 5.5$ Hz, 14.1 Hz, 1H), 3.19 (dd, $J = 5.5$ Hz, 14.3 Hz, 1H), 4.80 (m, 1H), 5.08 (s, 2H), 5.98 (d, $J = 6.4$ Hz, 1H), 6.88 (d, $J = 8.2$ Hz, 2H), 7.08 (d, $J = 8.2$ Hz, 2H), 7.52 (d, $J = 8.0$ Hz, 2H), 7.62 (d, $J = 8.2$ Hz, 2H). IR (KBr, cm^{-1}): 3328.6 (N–H), 1731.8 (C=O), 1612.2 (C=O); MS (EI) *m/e* (%): 491 (3, M^+), 159 (100). Anal. Calcd for $C_{27}H_{32}F_3NO_4 \cdot 1/2H_2O$: C, 64.80; H, 6.60; N, 2.80. Found: C, 65.20; H, 6.43; N, 3.01.

*5.6. (2S)-2-[N-(trans-4-isopropylcyclohexylcarbonyl) amino]-3-[4-[2-[N-methyl-N-(2-pyridyl)amino] ethoxy]phenyl]propionic acid ((S)-**6**)*

A stirred solution of 0.19 g (1.1 mmol) of 2-[(*N-tert*-butoxycarbonyl)-methylamino]ethanol and 0.35 g (1 mmol) of (*S*)-**1** in 15 mL of anhydrous tetrahydrofuran was treated with 0.38 g (1.5 mmol) of triphenylphosphine, then 240 μL (1.5 mmol) of diethyl azodicarboxylate was added dropwise at 0 °C. The

resulting solution was stirred at room temperature for 24 h. The solvent was removed in vacuo, and the residue was chromatographed over silica gel using petroleum/ethyl acetate (2:1) as eluent to afford 0.21 g of compound (*S*)-**5** as a colorless syrupy. The colorless syrupy was dissolved in 4.8 mL of dichloromethane, then 4.8 mL of trifluoroacetic acid was added. The resulting solution was stirred at room temperature for 1 h. Then parts of the solvent were removed in vacuo at room temperature. The residual solution was neutralized with saturated $NaHCO_3$ and extracted with dichloromethane (10 mL × 2), The combined organics were washed with H_2O, dried on Mg_2SO_4, filtered and concentrated in vacuo. The residue was refluxed with 2 mL of 2-fluoropyridine for 24 h. Excessive 2-fluoropyridine was removed under reduced pressure, the residue was dissolved in small amount of acetone, chromatographed over silica gel using acetone/petroleum (1:2) as eluent afforded 0.081 g of white solid. Hydrolysis of the white solid following the same procedure in Section 5.3 provided 0.06 g of the title compound. Yield: 76.8%. M.p. 158–160 °C. $[\alpha]_D^{25}$ 30.0 (*c* 0.26, $CHCl_3$). ^1H NMR ($CDCl_3$): $\delta = 0.80$ (d, $J = 7.6$ Hz, 6H), 0.80–1.00 (m, 3H), 1.13–1.40 (m, 3H), 1.62–1.82 (m, 4H), 1.96 (m, 1H), 3.03 (d, $J = 4.4$ Hz, 2H), 3.10 (s, 3H), 3.88 (t, $J = 6.5$ Hz, 2H), 3.98 (t, $J = 6.6$ Hz, 2H), 4.62 (m, 1H), 5.40 (s, 1H), 6.21 (d, $J = 6.9$ Hz, 1H), 6.55 (m, 1H), 6.75 (d, $J = 8.5$ Hz, 2H), 6.98 (d, $J = 8.5$ Hz, 2H), 7.48 (m, 1H), 8.03 (m, 1H). IR (KBr, cm^{-1}): 3299.7 (N–H), 1720.2 (C=O), 1637.3 (C=O); MS (EI) *m/e* (%): 467 (2, M^+), 135 (100). Anal. Calcd for $C_{27}H_{37}N_3O_4 \cdot 1/2H_2O$: C, 68.07; H, 7.98; N, 8.82. Found: C, 67.86; H, 7.75; N, 8.79.

5.7. (2S)-2-[N-(trans-4-isopropylcyclohexylcarbonyl) amino]-3-[4-[2-[N-methyl-N-(2-benzooxazolyl)amino] ethoxy]phenyl]propionic acid ((S)-7)

To a solution of 36 mg (0.21 mmol) of compound (*S*)-**5** in 2 mL of tetrahydrofuran were added 240 μL (0.51 mmol) of triethylamine and 40 mg (0.26 mmol) of 2-chlorobenzoxazole. The resulting solution was stirred at room temperature for 24 h. Tetrahydrofuran was removed under reduced pressure, the residue was mixed with 4 mL of ethyl acetate, then added 4 mL of saturated solution of $NaHCO_3$ in H_2O and stirred, the organic layer was separated, dried over anhydrous Na_2SO_4 and concentrated, the residue was mixed with a mixed solvent of petrolether/ethyl acetate (1:1), a white solid separated. The solid was hydrolyzed with LiOH to give 42 mg of the title compound. Yield: 39.6%. M.p. 179–180 °C (dec). $[\alpha]_D^{25}$ −81.1 (*c* 0.535, $CHCl_3$). ^1H NMR (DMSO-d_6): $\delta = 0.81$ (d, $J = 6.9$ Hz, 6H), 0.80–1.0 (m, 3H), 1.15–1.40 (m, 3H), 1.60 (m, 4H), 1.95 (m, 1H), 2.78 (dd, $J = 13.5$ Hz, 7.3 Hz, 1H), 2.96 (dd, $J = 13.5$ Hz, 4.8 Hz, 1H), 3.20 (s, 3H), 3.82 (t, $J = 5.5$ Hz, 2H), 4.10 (m, 1H), 4.20 (t, $J = 5.5$ Hz, 2H), 6.78 (d, $J = 8.4$ Hz, 2H), 6.95 (t, $J = 7.7$ Hz, 1H), 7.00 (d, $J = 8.4$ Hz, 2H), 7.10 (t, $J = 7.0$ Hz, 1H), 7.28 (d, $J = 8.3$ Hz, 1H), 7.35 (d, $J = 8.0$ Hz, 1H), 7.40 (d, $J = 7.3$ Hz, 1H). IR (KBr, cm^{-1}): 3322.8 (N–H), 1731.8 (C=O), 1648.9 (C=O); MS (EI) *m/e* (%): 507 (12, M^+), 148 (100). Anal. Calcd for $C_{29}H_{37}N_3O_5$: C, 68.64; H, 7.30; N, 8.28. Found: C, 68.45; H, 7.49; N, 8.21.

5.8. (2S)-2-[N-(trans-4-isopropylcyclohexylcarbonyl) amino]-3-(4-phenylmethoxyphenyl)propionic acid ((S)-8)

A solution of 137 mL (1.14 mmol) of benzyl bromide and 0.13 g (0.38 mmol) of (*S*)-**1** in 1 mL of DMF was treated with 0.16 g (1.14 mmol) of powered K_2CO_3. The resulting mixture was stirred at 70 °C for 12 h. After cooling to 0 °C, 5 mL of H_2O was added, the resulting solution was extracted with EtOAc (5 mL × 2). The combined extract was washed with H_2O, dried on anhydrous Na_2SO_4. Solvent was removed under reduced pressure, the residue mixed with ether, and a white solid separated. The solid was hydrolyzed with LiOH to give 0.10 g of the title compound. Yield: 62.5%. M.p. 140–142 °C (dec). $[\alpha]_D^{25}$ 70.9 (*c* 0.83, $CHCl_3$). ^1H NMR ($CDCl_3$): $\delta = 0.81$ (d, $J = 6.9$ Hz, 6H), 1.0 (m, 3H), 1.40 (m, 3H), 1.70–1.90 (m, 4H), 2.05 (m, 1H), 3.04 (dd, $J = 5.5$ Hz, 13.9 Hz, 1H), 3.19 (dd, $J = 5.5$ Hz, 13.9 Hz, 1H), 4.81 (m, 1H), 5.01 (s, 2H), 6.12 (d, $J = 7.3$ Hz, 1H), 6.91 (d, $J = 8.4$ Hz, 2H), 7.08 (d, $J = 8.4$ Hz, 2H), 7.39 (m, 5H); IR (KBr, cm^{-1}): 3328.6 (N–H), 1731.8 (C=O), 1648.9 (C=O); MS (EI) *m/e* (%): 423 (4, M^+), 91 (100). Anal. Calcd for $C_{26}H_{33}NO_4 \cdot 1/2H_2O$: C, 72.22; H, 7.87; N, 3.24. Found: C, 72.34; H, 7.71; N, 3.47.

5.9. (2S)-2-[N-(trans-4-isopropylcyclohexylcarbonyl) amino]-3-(4-butoxyphenyl)propionic acid ((S)-9)

Taking *n*-butyl bromide and (*S*)-**1** as starting material, the title compound was prepared following the procedure as in Section 5.8. Yield: 70.3%. M.p. 120–121 °C. $[\alpha]_D^{25}$ 87.9 (*c* 1.145, $CHCl_3$). ^1H NMR ($CDCl_3$): $\delta = 0.82$ (d, $J = 6.9$ Hz, 6H), 0.96 (m, 6H), 1.38 (m, 3H), 1.50 (m, 2H), 1.75–1.90 (m, 6H), 2.05 (m, 1H), 3.04 (dd, $J = 5.8$ Hz, 14.3 Hz, 1H), 3.19 (dd, $J = 5.5$ Hz, 14.2 Hz, 1H), 3.91 (t, $J = 6.6$ Hz, 2H), 4.80 (m, 1H), 5.99 (d, $J = 7.3$ Hz, 1H), 6.81 (d, $J = 8.4$ Hz, 2H), 7.07 (d, $J = 8.4$ Hz, 2H). IR (KBr, cm^{-1}): 3305.4 (N–H), 1716.4 (C=O), 1646.9 (C=O); MS (EI) *m/e* (%): 389 (7, M^+), 163 (100). Anal. Calcd for $C_{23}H_{35}NO_4 \cdot 1/3H_2O$: C, 69.87; H, 9.03; N, 3.54. Found: C, 69.60; H, 8.88; N, 3.64.

5.10. (2S)-2-[N-(trans-4-isopropylcyclohexylcarbonyl) amino]-3-(4-ethoxyphenyl)propionic acid ((S)-10)

Taking ethyl bromide and compound (*S*)-**1** as starting material, the title compound was prepared following the procedure as in Section 5.8. Yield: 75.0%. M.p. 168–170 °C. $[\alpha]_D^{25}$ 93.2 (*c* 1.13, $CHCl_3$). ^1H NMR ($CDCl_3$): $\delta = 0.81$ (d, $J = 6.9$ Hz, 4H), 1.0 (m, 3H), 1.40 (m, 5H), 1.75–1.90 (m, 4H), 2.05 (m, 1H), 3.04 (dd, $J = 5.5$ Hz, 14.0 Hz, 1H), 3.17 (dd, $J = 5.1$ Hz, 14.3 Hz, 1H), 4.00 (m, 2H), 4.80 (m, 1H), 5.97 (d, $J = 7.6$ Hz, 1H), 6.81 (d, $J = 8.4$ Hz, 2H), 7.03 (d, $J = 8.3$ Hz, 2H). IR (KBr, cm^{-1}): 3303.5 (N–H), 1724.1 (C=O), 1643.1 (C=O); MS (EI) *m/e* (%): 361 (16, M^+),

192 (100). Anal. Calcd for $C_{21}H_{31}NO_4 \cdot 1/3H_2O$: C, 68.66; H, 8.63; N, 3.81. Found: C, 68.93; H, 8.43; N, 3.86.

5.11. (2S)-2-[N-(trans-4-isopropylcyclohexylcarbonyl) amino]-3-(4-hydroxyphenyl)propionic acid ((S)-**11**)

The title compound was prepared by hydrolysis of compound (*S*)-**1** with LiOH. Yield: 81.1%. M.p. 156–157 °C. $[\alpha]_D^{25}$ 76.4 (*c* 0.845, $CHCl_3$). ^1H NMR (DMCO-d_6): $\delta = 0.81$ (d, $J = 7.0$ Hz, 6H), 1.0 (m, 3H), 1.40 (m, 3H), 1.75–1.90 (m, 4H), 2.15 (m, 1H), 2.90 (dd, $J = 8.1$ Hz, 13.9 Hz, 1H), 3.10 (dd, $J = 5.1$ Hz, 13.9 Hz, 1H), 4.82 (m, 1H), 6.72 (d, $J = 8.4$ Hz, 2H), 7.05 (d, $J = 8.4$ Hz, 2H). IR (KBr, cm^{-1}): 3303.5 (N–H), 1619.9 (C=O); MS (EI) *m/e* (%): 333 (10, M^+), 170 (100). Anal. Calcd for $C_{19}H_{27}NO_4 \cdot 2H_2O$: C, 61.79; H, 8.40; N, 3.79. Found: C, 61.94; H, 8.17; N, 3.84.

5.12. Insulin-releasing screening in HIT-T15 cells

HIT-T15 cells from American Type Culture Collection (ATCC, USA) were seeded into a 96-well plate at a density of 5×10^4 cells/well, and cultured with Ham's F12 medium (supplemented with 10% fetal bovine serum, 100 unit/mL penicillin, and 100 μg/mL streptomycin) for 2 days at 37 °C in 5% CO_2–95% air. After being washed twice with Krebs–Ringer bicarbonate–HEPES buffer (KRB–HEPES buffer, containing 129 mM NaCl, 4.7 mM KCl, 5 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.8 mM glucose, 10 mM HEPES, pH 7.4) supplemented with 0.1% bovine serum albumin, the subconfluent cells were pre-incubated with KRB–HEPES buffer for 1 h, then incubated for another 1 h with KRB–HEPES buffer containing one of the agents being tested, nateglinide or mitiglinide at a range of concentrations (both incubations being at 37 °C in 5% CO_2–95% air, all agents being dissolved in DMSO and the same final concentration of the solvent being added to the control medium). Aliquots of the culture media were collected for measurement of the insulin concentration using an insulin radioimmunoassay kit (Atomic Energy Research Institute, Beijing, China).

5.13. Insulin-sensitizing screening in 3T3-L1 cells

3T3-L1 cells were obtained from the ATCC and maintained at 37 °C in an atmosphere of 5% CO_2 in Dulbecco's modified Eagle medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS). The 3T3-L1 pre-adipocytes were grown in 96-well plates until 2 days postconfluence. The differentiation was induced by addition of 5 μg/mL insulin (Lilly), 0.5 mM isobutylmethylxanthine and 1 μM dexamethasone (Sigma). The induction medium was removed 2 days after incubation. After an additional 2 days of incubation in DMEM supplemented with 10% FBS and 5 μg/mL insulin, the medium was changed every other day with DMEM supplemented with 10% FBS. Cells were challenged during the first 4 days of differentiation with different compounds at 1 and 10 μmol/L, respectively, with rosiglitazone as positive control and 0.1% DMSO as negative control. The addition of compounds to the medium was accomplished by dissolving the drug in DMSO and diluting the drug 1000-fold with medium. Seven days after the induction of 3T3-L1 cells, oil red O staining was used to detect triglyceride accumulation in 3T3-L1 cells. The precipitation of oil red O in 3T3-L1 adipocytes was dissolved with isopropyl alcohol, and OD value at 510 nm was determined by ELISA spectrometry. The results were based on three or four independent experiments.

5.14. In vivo screening in glucose-loading normal rats

Normal SD rats weighting 180–220 g were fasted overnight and divided into six groups with 8–10 rats each. Following oral administration of 3 mg/kg of one of the agents being tested, mitiglinide (positive control) or 0.5% of carboxylmethyl-cellulose sodium (CMC-Na, vehicle control), the rats were immediately administered oral glucose 2.5 g/kg. Blood samples were drawn before glucose and drug administration and at 15, 30, 60, 120 and 180 min after administration from the caudal vein, and then serum-glucose levels were measured by the glucose oxidase method.

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