(2*R*)-4-Oxo-4-[3-(Trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-*a*]pyrazin-7(8*H*)-yl]-1-(2,4,5-trifluorophenyl)butan-2-amine: A Potent, Orally Active Dipeptidyl Peptidase IV Inhibitor for the Treatment of Type 2 Diabetes

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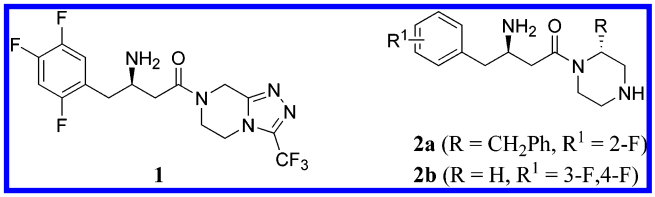
A novel series of β-amino amides incorporating fused heterocycles, i.e., triazolopiperazines, were synthesized and evaluated as inhibitors of dipeptidyl peptidase IV (DPP-IV) for the treatment of type 2 diabetes. (2*R*)-4-Oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-*a*]pyrazin-7(8*H*)-yl]-1-(2,4,5-trifluorophenyl)butan-2-amine (**1**) is a potent, orally active DPP-IV inhibitor (IC_{50} = 18 nM) with excellent selectivity over other proline-selective peptidases, oral bioavailability in preclinical species, and in vivo efficacy in animal models. MK-0431, the phosphate salt of compound **1**, was selected for development as a potential new treatment for type 2 diabetes.

Introduction

In recent years, the incretin hormone glucagon-like peptide 1 (GLP-1) has been the subject of intense research efforts related to the treatment of type 2 diabetes.[1,2] This peptide hormone is released from the gut in response to food intake. GLP-1 has a clearly established role in glucose homeostasis via stimulation of insulin biosynthesis and secretion, and inhibition of glucagon release.[2] Importantly, GLP-1 regulates insulin in a strictly glucose-dependent manner. Thus, GLP-1 therapy may pose little or no risk of hypoglycemia. Other known effects of GLP-1 therapy include slowing gastric emptying[3] and reduction of appetite,[4] although it is not known if these are physiologically important functions for this peptide. Finally, there is intriguing data in rodents suggesting a potential role in restoration of β-cell function, thus this mechanism might actually stabilize or even reverse disease progression.[5] Active GLP-1 (GLP-1[7−36]amide) is rapidly degraded in vivo through the action of dipeptidyl peptidase IV (DPP-IV), a serine protease which cleaves a dipeptide from the *N*-terminus to give the inactive GLP-1[9−36]amide.[6] Thus, a small-molecule inhibitor of DPP-IV would increase the half-life of active GLP-1 and prolong the beneficial effects of this incretin hormone. Indeed, improved glucose tolerance in diabetic patients and healthy volunteers was achieved in human clinical trials with several small molecule DPP-IV inhibitors, and inhibition of DPP-IV is emerging as a new potential therapeutic approach to the treatment of type 2 diabetes.[7]

Chart 1



With few exceptions,[8] most DPP-IV inhibitors reported to date incorporate α-amino acid moieties.[9] Recently, we reported structurally novel β-amino acid-based DPP-IV inhibitors such as **2a** (Chart 1) with high DPP-IV inhibitory potency but poor pharmacokinetic properties,[10] likely resulting from extensive metabolism of the heterocycle moiety.[10b] Our initial strategy to improve both the metabolic stability and pharmacokinetic properties was to replace piperazine moieties with metabolically robust heterocycles in the inhibitor design, in particular using fused heterocycles as piperazine replacements. A variety of fused heterocycles have been found to be effective in improving metabolic stability and pharmacokinetic properties, in addition to increasing DPP-IV inhibitory potency. Herein, we focus on the preliminary SAR of triazolopiperazine-based DPP-IV inhibitors, which led to the discovery of MK-0431, the phosphate salt of (2*R*)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-*a*]pyrazin-7(8*H*)-yl]-1-(2,4,5-trifluorophenyl)butan-2-amine (**1**), currently in clinical development.[11]

Chemistry. The β-amino acid-derived DPP-IV inhibitors in this report were synthesized by standard peptide coupling of β-amino acids with fused heterocycles.[12] Initial synthetic efforts focused on the synthesis of β-amino acids (Scheme 1). Noncommercially available β-amino acids (**9a** and **9b**) were readily available via the Arndt−Eistert homologation of the corresponding α-amino acid which were prepared using Evans's asym-

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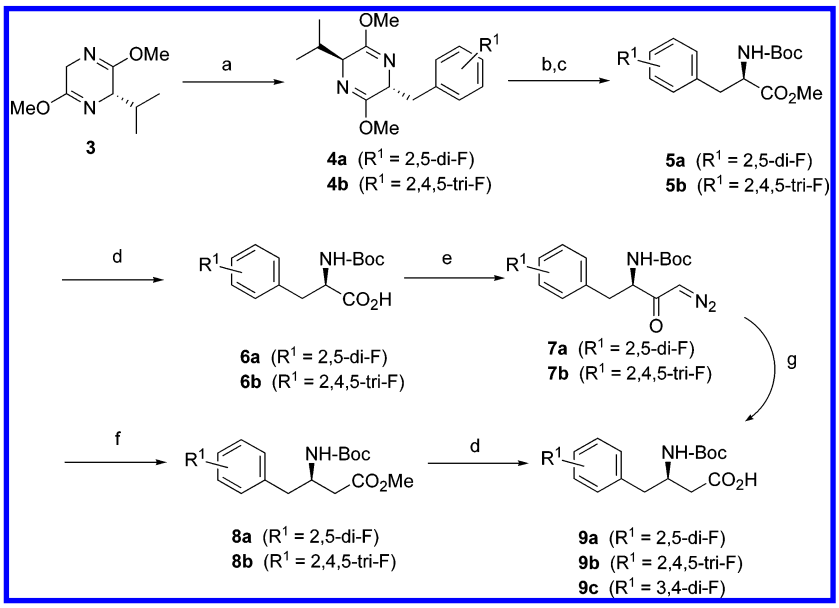
Scheme 1[a]



[a] Reagents: (a) (i) *n*-BuLi, THF, −78 °C, 20 min., (ii) 2,5-difluorobenzyl bromide (for **4a**) and 2,4,5-trifluorobenzyl bromide (for **4b**), THF; (b) (i) 1 N HCl, CH_3CN, rt, 16 h, (ii) MeOH; (c) $(Boc)_2O$, Et_3N, CH_2Cl_2; (d) LiOH, 1:1 THF/H_2O, rt, 16 h; (e) Et_2O, Et_3N, *iso*-butyl chloroformate, −30 °C, diazomethane; (f) DIPEA, MeOH, silver benzoate; (g) silver benzoate, 1,4-dioxane/H_2O (5:1), sonication, rt.

metric azidation[13] or Schöllkopf's bis-lactam methodology[14] as reported earlier.[10a,12] For the introduction of the desired (*R*)-stereochemistry in the α-amino acid **6**, the Schöllkopf reagent **3** ((2*S*)-(+)-2,5-dihydro-3,6-dimethoxyl-2-isopropylpyrazine) was employed. Alkylation of **3** with the corresponding benzyl bromide gave **4**, which was treated with hydrochloric acid followed by di-*tert*-butyl dicarbonate to give ester **5**. Hydrolysis afforded the α-amino acid **6**, which was treated with isopropyl chloroformate followed by diazomethane to give diazo ketone **7**. Subsequent rearrangement of diazo ketone **7** to ester **8** followed by hydrolysis gave the desired *β*-amino acid **9** (ee >99% by chiral HPLC analysis). The 2,4,5-trifluophenyl-substituted *β*-amino acid **9b** could also be prepared in one step by sonication of diazo ketone **7b** in the presence of silver benzoate.[15]

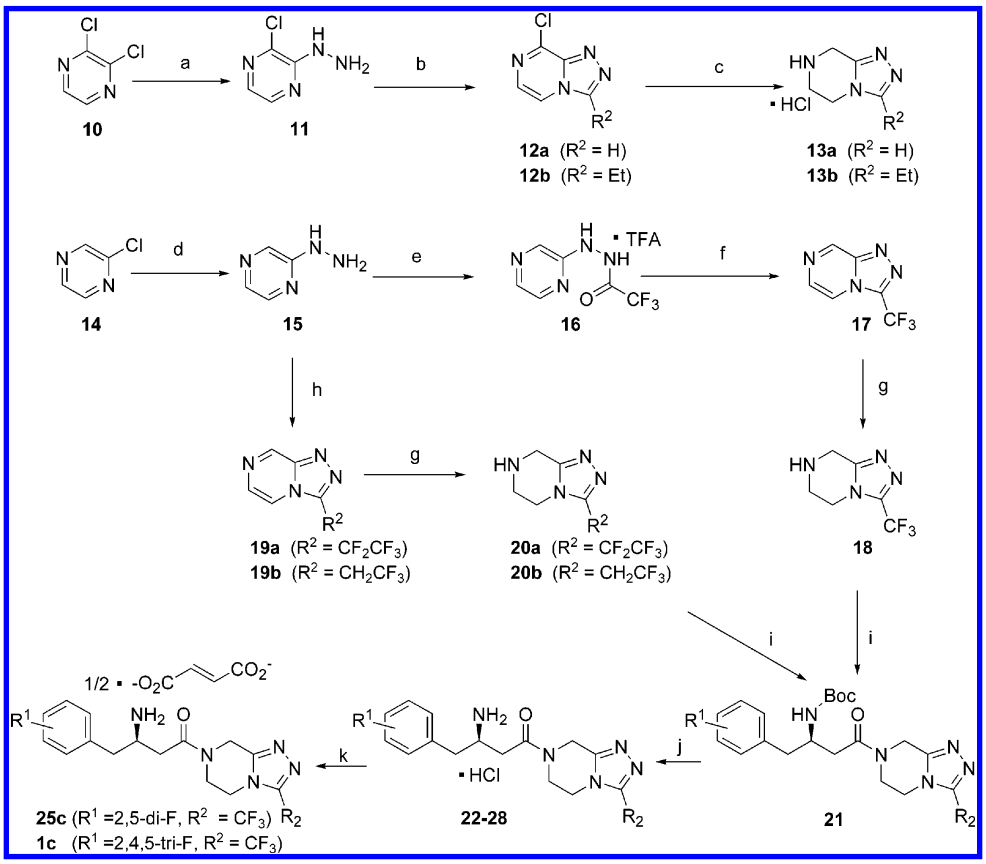
Synthetic efforts then focused on the preparation of triazolopiperazines bearing a variety of substituents at the 3-position of the ring. Three different approaches to the triazolopyrazine ring system are described in Scheme 2. First, 2,3-dichloropyrazine (**10**) was converted to 3-chloro-2-hydazinopyrazine **11** according to the literature procedure.[16] The hydrazine intermediate **11** was then cyclized to give the fused ring system **12** using an ortho ester.[16] In a second approach, hydrazinopyrazine **15**, prepared from chloropyrazine (**14**),[17] was acylated with trifluoroacetic anhydride to give **16**, which was then condensed in polyphosphoric acid (PPA) to give triazolopyrazine **17**. Alternatively, hydrazine intermediate **15** was directly condensed with carboxylic acid in PPA at elevated temperature to give the desired heterocycles **19**. Subsequent catalytic hydrogenation of fused heterocycles (**12**, **17**, and **19**) proceeded smoothly to give the target piperazine intermediates **13**, **18**, and **20**, respectively. Coupling of the *β*-amino acids (**9a**−**c**) with triazolopiperazines (**13**, **18**, and **20**) followed by deprotection of the amine provided the desired compounds **1** and **22**−**28** in Table 1. HCl salts of compounds **25** and **1** were converted to fumaric acid salts for in vivo evaluation.

Results and Discussion

Compounds **1** and **22**−**28** were evaluated in vitro for their inhibition of DPP-IV.[18] The inhibitors were also tested against DPP-IV homologues in the DPP-IV gene family, including DPP-8,[19] DPP-9,[20] fibroblast activation protein (FAP, also called seprase),[21] and other proline specific enzymes with DPP-IV-like activity, including quiescent cell proline dipeptidase (QPP, also known as DPP-II),[18,22] amino peptidase P, and prolidase. None of the compounds in this report showed any significant inhibition against these other enzymes (IC_{50}s > 10 000 nM). Since significant QPP off-target activity was often observed for the *β*-amino acid-derived DPP-IV inhibitors reported from these laboratories earlier,[10] QPP data are presented for comparison. Safety studies using a DPP8/9 selective inhibitor suggest that inhibition of DPP8 and/or DPP9 may be associated with profound toxicity in preclinical species.[23] Thus, selectivity profiles against DPP8 and DPP9 were also obtained for safety reasons.

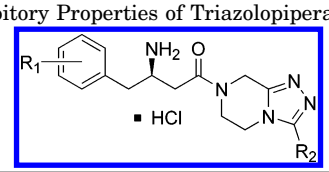
In vitro inhibitory activities for the selected triazolopiperazine-based DPP-IV inhibitors are listed in Table 1. The parent compound in this series, the unsubstituted triazole analogue **22**, was 3-fold less active than piperazine lead **2a** (DPP-IV IC_{50} = 139 nM);[10b] however, it was 7-fold more potent than the analogous 3,4-difluorophenyl analogue **2b** (IC_{50} = 3100 nM), which, like **22**, lacks the α-benzyl moiety. Ethyl analogue **23** showed a 2-fold increase in potency over the parent compound **22**. Notably, ethyl analogue **23** was stable for over 1 h in incubations with rat hepatocytes, probably due to the metabolically stable nature of the fused heterocycle. Thus, ethyl analogue **23** was further evaluated in pharmacokinetic studies in three preclinical species (Table 2). While ethyl analogue **23** suffered from poor oral bioavailability in both the rat and the rhesus monkey, it showed fair oral bioavailability in the dog.

Scheme 2[a]



[a] Reagents: (a) $NH_2NH_2 \cdot xH_2O$, EtOH, reflux; (b) $CH(OCH_3)_3$ (for **12a**) and $C_2H_5C(OC_2H_5)_3$ (for **12b**), reflux; (c) PtO_2, MeOH, H_2 (50 psi); (d) neat $NH_2NH_2 \cdot xH_2O$, 120 °C, 45 min; (e) $(CF_3CO)_2O$, 0 °C to room temperature; (f) PPA, 140 °C, 18 h; (g) H_2, 10% Pd/C, EtOH; (h) $CF_3CF_2CO_2H$ (for **19a**) and $CF_3CH_2CO_2H$ (for **19b**); (i) **9**, HOBT, EDC, DIPEA, DMF, rt, 18 h; (j) saturated HCl/MeOH; (k) (i) 1N NaOH, EtOAc, (ii) fumaric acid, EtOH, rt.

Table 1. Inhibitory Properties of Triazolopiperazine Analogues



compd	R1	R2	DPP-IV IC_{50} (nM)	QPP (nM)	DPP8 (nM)	DPP9 (nM)
22	3,4-di-F	H	455	>10000	38000	>100000
23	3,4-di-F	Et	231	64000	45000	>100000
24	3,4-di-F	CF_3	128	98000	46000	>100000
25	2,5-di-F	CF_3	27	>100000	69000	>100000
1	2,4,5-tri-F	CF_3	18	>100000	48000	>100000
26	2,4,5-tri-F	H	68	>10000	72000	>100000
27	2,4,5-tri-F	CF_2CF3	71	78000	80000	>100000
28	2,5-di-F	CH_2CF_3	103	>100000	35000	>100000

Table 2. Pharmacokinetic Profiles of Selected DPP-IV Inhibitors[a]

compd	species	Clp (mL/min/kg)	$t_{1/2}$ (h)	PO AUC_{norm} (μM h/mpk)	PO C_{max} (μM)	F (%)
23	rat	45	2.7	0.019	0.01	2
	dog	13	2.3	1.25	0.73	33
	monkey	26	1.3	0.017	0.012	1
24	rat	51	1.8	0.376	0.14	44
25	rat	43	1.6	1.0	0.19	51
1	rat	60	1.7	0.52	0.33	76
	dog	6.0	4.9	8.3	2.2	100
	monkey	28	3.7	1.0	0.33	68
26	rat	40	1.0	0.033	0.03	3
27	rat	58	2.3	0.39	0.16	61
28	rat	109	1.5	0.004	0.002	1

[a] Dose: iv: 1 mpk, po: 2 mpk.

Replacing the ethyl moiety with a trifluoromethyl group gave compound **24**, which had similar DPP-IV inhibitory potency but showed a dramatic improvement in oral bioavailability in the rat (F = 44%). A significant improvement in DPP-IV inhibitory potency was achieved using a different pattern of fluorine atoms on the phenyl in these inhibitors. 2,5-Difluorophenyl analogue **25** and 2,4,5-trifluorophenyl analogue **1** showed a 5- and 7-fold increase in the DPP-IV potency, respectively, over 3,4-difluorophenyl analogue **24**. The trend in increase in the DPP-IV inhibitory potency, from 3,4-difluoro analogue to 2,4,5-trifluoro analogue (**24**, **25**, **1**), was in line with that observed in the other two series incorporating β-amino acid moieties, reported previously from these laboratories. Both compounds **25** and **1** also exhibited improved oral bioavailability in rats (51% and 76%, respectively). In addition, compound **1** showed excellent oral bioavailability in dogs and monkeys (100% and 68%, respectively). Clearance is relatively high in rats and monkeys (60 and 28 mL/min/kg, respectively), but lower in dogs (6 mL/min/kg).

Deletion of the trifluoromethyl substituent in compound **1** resulted in a 4-fold decrease in the DPP-IV potency (**26**), and a loss of oral bioavailability in the rats (F = 3%), suggesting that the trifluoromethyl substituent was essential for the excellent pharmacokinetic profile observed with compound **1**. The pentafluoroethyl substituent is also as effective as trifluoromethyl for maintaining good pharmacokinetic profiles in the rat, as illustrated by compound **27**. While the effect of

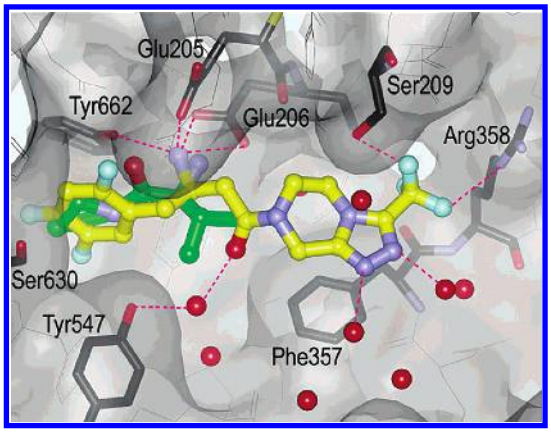


Figure 1. Compound **1** bound to DPP-IV. The overlay of compound **1** (yellow) and the substrate analog valine-pyrrolidine (green, 1N1M.pdb) shows the opposite orientation of the amino carboxylic moiety in the two compounds. The interactions made by compound **1** with DPP-IV are shown as red dotted lines. The extensive hydrogen bond network present between the ordered water molecules, the compound **1**, and protein atoms has been omitted for clarity.

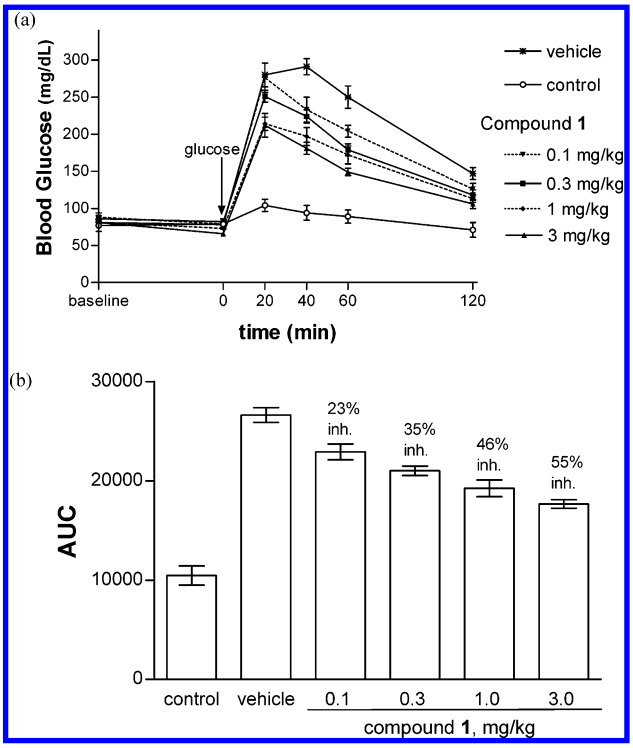


Figure 2. (a) Effects of compound **1** on glucose levels after an oral glucose tolerance test in lean C57BL/6N male mice. Compound **1** or water (vehicle) was administered 60 min prior to an oral dextrose challenge (5 g/kg). Control animals received water only. (b) The glucose AUC was determined from 0 to 120 min. Percent inhibition values for each treatment were generated from the AUC data normalized to the water-challenged controls. Data are represented as mean ± SEM (n = 7).

pentafluoroethyl substitution on DPP-IV inhibitory potency was negligible (**26** vs **27**), the excellent oral bioavailability in rats was restored (F = 61%). Incorporation of a trifluoroethyl group at the R^2 position resulted in a 4-fold decrease in the DPP-IV potency (**28**), when compared with the corresponding analogue **25**. Unlike pentafluoroethyl analogue **27**, trifluoroethyl analogue **28** had very low oral bioavailability in rats (F = 1%).

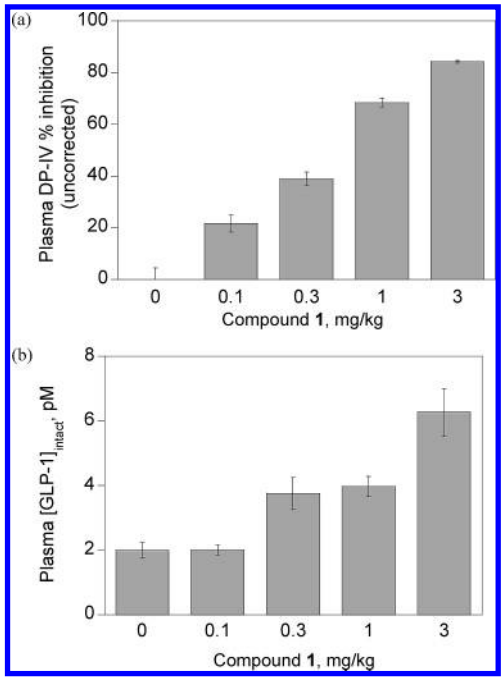


Figure 3. Effects of compound **1** on (a) DPP−IV inhibition and (b) GLP-1 levels after an oral glucose tolerance test in lean C57BL/6N male mice. Compound **1** was administered 60 min prior to an oral dextrose challenge (5 g/kg). Plasma samples were collected for analysis 20 min post-dextrose administration. Data are represented as mean ± SEM (n = 20−28/group).

As previously noted, compound **1** showed high selectivity (>1000-fold) for DPP-IV over the other proline specific peptidases. Further profiling in an extensive panel of receptor and ion channel binding and enzyme inhibition assays showed no significant activity (data not shown).

X-ray crystal structure determination shows that compound **1** binds to the active site of DPP-IV with the amide moiety in the opposite orientation of that reported for α-amino acid containing substrates and inhibitors[24] (Figure 1), in agreement with the reported SAR. The 2,4,5-trifluorophenyl moiety fully occupies the S1 hydrophobic pocket (Figure 1), and this is consistent with the observed increase in potency with this series compared to the 3,4 difluoro analogues (Table 1). The (*R*)-β-amino group forms four hydrogen bonding interactions with the side chains of a tyrosine (Tyr662) and two glutamate residues (Glu205 and Glu206). This interaction is analogous to the binding of the *N*-terminus of the substrate to DPP-IV and is consistent with data showing the (*S*)-enantiomer to be much less potent (IC_{50} = 440 nM). A water molecule bridges the carboxylic oxygen and the hydroxyl of Tyr547. Several other water-mediated interactions are also present between the nitrogen atoms of the triazolopiperazine and protein atoms. The triazolopiperazine is stacked against the side chain of Phe357. The trifluoromethyl substituent interacts with the side chains of Arg358 and Ser209, and these interactions explain the 4-fold loss in potency observed when this group is removed (Table 1, **26**). The pocket that accommodates the trifluoromethyl moiety is quite tight (Figure 1), and in fact a loss in potency is observed when the trifluoromethyl is replaced by larger

substituents (Table 1, **27**), suggesting that the trifluoromethyl may be the optimal size group in this position.

On the basis of its excellent in vitro potency, selectivity, and pharmacokinetic profile, compound **1** was chosen for in vivo evaluation. Compound **1** was assessed for its ability to improve glucose tolerance in lean mice. Administration of single oral doses reduced the blood glucose excursion in an oral glucose tolerance test (OGTT) in a dose-dependent manner from 0.1 mg/kg (23% reduction) to 3.0 mg/kg (55% reduction) when administered 60 min before an oral dextrose challenge (5 g/kg) (Figure 2). In a separate OGTT experiment, the pharmacodynamic profile of compound **1** was determined. Plasma DPP-IV inhibition, compound concentration and active GLP-1 levels were measured 20 min after dextrose challenge (Figure 3). The reduction of the blood glucose excursion at 1 mg/kg corresponded to a plasma concentration of 190 nM and 69% inhibition of plasma DPP-IV activity.[25] A 3 mg/kg dose, corresponding to a plasma concentration of 600 nM, provided 84% inhibition of DPP-IV. Maximal efficacy resulted in a 2 to 3-fold increase in active GLP-1, analogous to what is observed upon glucose challenge in DPP-IV deficient mice.[26] These results support the correlation between DPP-IV inhibition, increase in GLP-1 levels, and an improvement in glucose tolerance. Acute lowering of blood glucose was also demonstrated in diet-induced obese (DIO) mice, which are hyperglycemic and hyperinsulinemic, and show impaired glucose tolerance in response to a dextrose challenge consistent with the insulin resistance observed in type 2 diabetes mellitus. Near normalization of the glucose excursion relative to lean controls was seen following a 3 mg/kg oral dose of compound **1** (Figure 4).

Conclusions. A novel series of potent triazolopiperazine-based DPP-IV inhibitors have been discovered, with the most potent compound **1**, demonstrating excellent in vitro selectivity and in vivo efficacy. On the basis of its DPP-IV potency, selectivity, in vivo efficacy, and pharmacokinetic profile, MK-0431, the phosphate salt of (2*R*)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]-triazolo[4,3-*a*]pyrazin-7(8*H*)-yl]-1-(2,4,5-trifluorophenyl)-butan-2-amine (**1**), was chosen for development as a potential new treatment for type 2 diabetes mellitus.

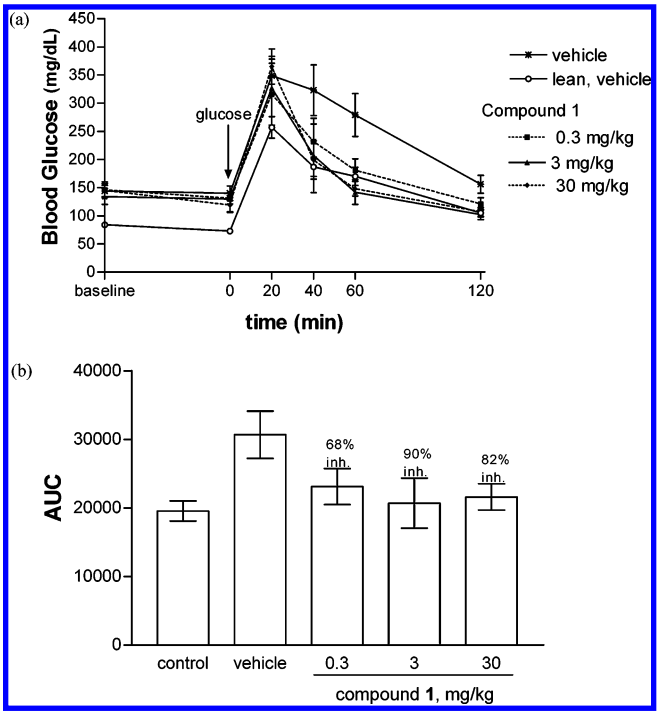


Figure 4. (a) Effects of compound **1** on glucose levels after an oral glucose tolerance test in diet induced obese (DIO) C57BL/6N male mice. Compound **1** or water (vehicle) was administered 60 min prior to an oral dextrose challenge (2 g/kg). (b) The glucose AUC was determined from 0 to 120 min. Percent inhibition values for each treatment were generated from the AUC data normalized to the dextrose-challenged lean controls. Data are represented as mean ± SEM (n = 7).

Experimental Section

General. All commercial chemicals and solvents are reagent grade and were used without further purification unless otherwise specified. ^{1}H NMR spectra were recorded on a Varian InNova 500 MHz instrument in $CDCl_3$ or CD_3OD solutions, unless otherwise noted. Low-resolution mass spectra (MS) were determined on a Micromass Platform Liquid Chromatography−Mass Spectrometer (LC-MS). High-resolution mass spectra were acquired from a Micromass Q-TOF quadrupole-time-of-flight mass spectrometer. All MS experiments were performed using electrospray ionization in positive ion mode. Leucine enkephalin was applied as a lock-mass reference for accurate mass analysis. Elemental analyses were performed by Robertson Microlit Laboratories, Inc., Madison, NJ, and are within +0.4% of the calculated values unless otherwise noted.

(3*R*)-3-[(1,1-Dimethylethoxycarbonyl)amino]-4-(2,5-difluorophenyl)butanoic acid (9a). Step A. (2*R*,5*S*)-2,5-Dihydro-3,6-dimethoxy-2-[2′,5′-difluorophenyl)methyl]-5-isopropylpyrazine (4a). To a solution of 24.0 g (130.3 mmol) of (2*S*)-(+)-2,5-dihydro-3,6-dimethoxyl-2-isopropylpyrazine (**3**) in 500 mL of THF was added 18.4 mL (156.3 mmol) of *n*-butyllithium over 20 min at −78 °C. The reaction was stirred at −78 °C for 20 min. A cooled solution of 97.7 g (143.3 mmol) of 2,5-difluorobenzyl bromide in 30 mL of THF was then added via cannula, and the mixture was stirred at −78 °C for an additional 3.5 h. After quenching with 100 mL of water at −78 °C, the reaction mixture was concentrated and the residue was partitioned between ethyl acetate and 1 N hydrochloric acid. The aqueous layer was extracted with ethyl acetate (3×). The combined organic phase was washed with brine and dried over anhydrous magnesium sulfate. Concentration was followed by purification by chromatography using a Biotage system (silica gel, eluting sequentially with 3:100 ethyl acetate:hexanes, then 5:100 ethyl acetate:hexanes) to afford 31.6 g of the title compound as a viscous oil (75%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.21 (m, 1H), 3.00 (m, 1H), 3.24 (m, 1H), 3.60 (t, 1H, J = 3.4 Hz), 3.68(s, 3H), 3.75 (s, 3H), 4.31−4.33 (m, 1H), 5.16 (m, 1H). 6.87−7.29 (m, 3H).

Step B. (*R*)-*N*-(1,1-Dimethylethoxycarbonyl)-2,5-difluorophenylalanine Methyl Ester (5a). To a solution of 31.0 g (96.3 mmol) of (2*R*,5*S*)-2,5-dihydro-3,6-dimethoxy-2-[2′,5′-difluorophenyl)methyl]-5-isopropylpyrazine (**4a**) in 200 mL of acetonitrile was added 200 mL of 1 N hydrochloric acid, and the reaction mixture was stirred at ambient temperature for 16 h. Methanol was added, and the mixture was concentrated. This was repeated three times, and then the same process was repeated with toluene once to give 36.5 g of a solid. To a solution of the above solid in 700 mL of dichloromethane was added 134 mL (963 mmol) of triethylamine followed by 50.4 g (231.1 mmol) of di-*tert*-butyl dicarbonate. The reaction was stirred at ambient temperature for 6 h, and the solid was filtered off. The filtrate was diluted with dichloromethane, washed sequentially with 1 N hydrochloric acid and brine, and then dried over magnesium sulfate. Concentration was followed by purification by chromatography using a Biotage system (silica gel, eluting sequentially with 2:98 acetone:hexanes, 5:95 acetone:hexanes, 7:93 acetone:hexanes) to afford 18.7 g of the title compound as a solid (62%). ^{1}H NMR (500

MHz, $CDCl_3$) δ 1.43 (s, 9H), 3.19 (m, 1H), 3.23 (m, 1H), 3.77 (s, 3H), 4.61 (m, 1H), 5.10 (m, 1H). 6.87−7.05(m, 3H). MS *m/z* 216 $(M + H - t\text{-Boc})^+$.

Step C. (*R*)-*N*-(1,1-Dimethylethoxycarbonyl)-2,5-difluorophenylalanine (6a). To a solution of 18.1 g (57.4 mmol) of (*R*)-*N*-(1,1-dimethylethoxycarbonyl)-2,5-difluorophenylalanine methyl ester (**5a**) in 250 mL of THF and 250 mL of water was added 4.12 g (172.3 mmol) of lithium hydroxide. The reaction was stirred at ambient temperature for 16 h. After concentration in vacuo, the residue was diluted with ethyl acetate, washed sequentially with saturated aqueous sodium bicarbonate solution and brine, and then dried over magnesium sulfate. Concentration in vacuo gave 17.1 g of a solid, which was directly used in the next step without further purification (99%). ^{1}H NMR (500 MHz, CD_3OD) δ 1.34 (m, 9H), 2.89 (m, 1H), 3.27 (m, 1H), 4.19 (m, 1H), 6.93−7.08 (m, 3H). MS *m/z* 202 $(M + H - t\text{-Boc})^+$.

Step D. (*R*)-3-[(1,1-Dimethylethoxycarbonyl)amino]-1-diazo-4-(2,5-difluoro-phenyl)butan-2-one (7a). To a solution of 17.0 g (56.5 mmol) of (*R*)-*N*-(1,1-dimethylethoxycarbonyl)-2,5-difluorophenylalanine (**6a**) in 300 mL of diethyl ether were added sequentially 9.44 mL (67.8 mmol) of triethylamine and 8.8 mL (67.8 mmol) of isobutyl chloroformate at −30 °C. After being stirred at −30 °C for 15 min, the mixture was added to excess diazomethane in ethyl ether, prepared as follows: 1-methyl-3-nitro-1-nitrosoguanidine was added portion-wise to a mixture of diethyl ether and 40% aqueous sodium hydroxide solution at 0 °C. The reaction was stirred at 0 °C for 15 min, and the organic layer was dried over solid potassium hydroxide and used as is. The excess diazomethane was quenched by dropwise addition of acetic acid. The mixture was diluted with ethyl acetate, washed sequentially with saturated sodium bicarbonate solution and brine, and then dried over magnesium sulfate. The organic layer was concentrated in vacuo to give 16.7 g of the title compound as a solid (91%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.42 (s, 9H), 2.92 (m, 1H), 3.20 (m, 1H), 4.26 (m, 1H), 5.17 (m, 1H). 5.47(bs, 1H), 6.95 (m, 2H). 7.02 (m, 1H).

Step E. (3*R*)-3-[(1,1-Dimethylethoxycarbonyl)amino]-4-(2,5-difluorophenyl)butanoic Acid, Methyl Ester (8a). To a solution of 16.5 g (50.7 mmol) of (*R*)-3-[(1,1-dimethylethoxycarbonyl)amino]-1-diazo-4-(2,5-difluoro-phenyl)butan-2-one (**7a**) in 300 mL of methanol at −30 °C were added sequentially 26.5 mL (152.1 mmol) of diisopropylethylamine and 2.3 g (10.1 mmol) of silver benzoate. The reaction was warmed to ambient temperature and stirred for 2.5 h. After methanol was evaporated in vacuo, the residue was diluted with 200 mL of dichloromethane, and the black residual material was filtered off through Celite. The filtrate was concentrated and purified by chromatography using a Biotage system (silica gel, eluting sequentially with 5:100 ethyl acetate: hexanes, 1:9 ethyl acetate:hexanes, and 1:4 ethyl acetate: hexanes) to afford 10.5 g of the title compound as a solid (63%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.41 (s, 9H), 2.59 (m, 2H), 2.92 (m, 2H), 3.72 (s, 3H), 4.20 (m, 1H), 5.16 (m, 1H). 6.87−7.05-(m, 3H). MS *m/z* 316 $(M + H)^+$.

Step F. (3*R*)-3-[(1,1-Dimethylethoxycarbonyl)amino]-4-(2,5-difluorophenyl)butanoic acid (9a). To a solution of 19.45 g (61.7 mmol) of (3*R*)-3-[(1,1-dimethylethoxycarbonyl)-amino]-4-(2,5-difluorophenyl)butanoic acid, methyl ester (**8a**) in 250 mL of THF and 250 mL of water was added 4.43 g (185.5 mmol) of lithium hydroxide. The reaction was stirred at ambient temperature for 16 h. After concentration, the reaction mixture was diluted with ethyl acetate, washed sequentially with saturated sodium bicarbonate and brine, and then dried over anhydrous magnesium sulfate. Concentration in vacuo gave 18.3 g of the title compound as a white solid, which was used without further purification (94%). ^{1}H NMR (500 MHz, CD_3OD) δ 1.34 (m, 9H), 2.52 (m, 2H), 2.73 (m, 1H), 2.93 (m, 1H), 4.18(m, 1H), 6.55−7.13 (m, 3H). MS *m/z* 216 $(M + H - t\text{-Boc})^+$.

(3*R*)-*N*-(*tert*-Butoxycarbonyl)-3-amino-4-(2,4,5-trifluorophenyl)butanoic Acid (9b). Step A. (2*S*,5*R*)-2,5-Dihydro-3,6-dimethoxy-2-isopropyl-5-(2′,4′,5′-trifluorobenzyl)-pyrazine (4b). The title compound (3.81 g) was prepared from 3.42 g (18.5 mol) of (2*S*)-2,5-dihydro-3,6-dimethoxy-2-isopropylpyrazine (**3**) using the procedure analogous to that described in Step A for the synthesis of **9a**, except that 2,4,5-trifluorobenzyl bromide was employed in place of 2,5-difluorobenzyl bromide. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.01 (m, 1H), 6.85 (m, 1H), 4.22 (m, 1H), 3.78 (m, 3H), 3.64 (m, 3H), 3.61 (m, 1H), 3.20 (m, 1H), 2.98 (m, 1H), 2.20 (m, 1H), 0.99 (d, 3H, J = 8 Hz), 0.62 (d, 3H, J = 8 Hz).

Step B. (*R*)-*N*-(*tert*-Butoxycarbonyl)-2,4,5-trifluorophenylalanine Methyl Ester (5b). To a solution of 3.81 g (11.6 mmol) of (2*S*,5*R*)-2,5-dihydro-3,6-dimethoxy-2-isopropyl-5-(2′,4′,5'trifluorobenzyl)pyrazine (**4b**) in 20 mL of acetonitrile was added 20 mL of 2 N hydrochloric acid. The reaction was stirred for 72 h and concentrated in vacuo. The residue was dissolved in 30 mL of dichloromethane, and 10 mL (72 mmol) of triethylamine and 9.68 g (44.8 mmol) of di-*tert*-butyl dicarbonate were added. The reaction was stirred for 16 h, diluted with ethyl acetate, and washed sequentially with 1 N hydrochloric acid and brine. The organic phase was dried over sodium sulfate, concentrated in vacuo, and purified by flash chromatography (silica gel, 9:1 hexanes:ethyl acetate) to afford 2.41 g of the title compound. ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.99 (m, 1H), 6.94 (m, 1H), 5.08 (m, 1H), 4.58 (m, 1H), 3.78 (m, 3H), 3.19 (m, 1H), 3.01 (m, 1H), 1.41 (s, 9H).

Step C. (*R*)-*N*-(*tert*-Butoxycarbonyl)-2,4,5-trifluorophenylalanine (6b). The title compound (2.01 g) was prepared from 2.41 g (7.5 mol) of (*R*)-*N*-(*tert*-butoxycarbonyl)-2,4,5-trifluorophenylalanine methyl ester (**5b**) using the procedure analogous to that described in Step C for the synthesis of **6a**. MS *m/z* 220.9 $(M + 1H - t\text{-BOC})^+$.

Step D. (3*R*)-*N*-(*tert*-Butoxycarbonyl)-3-amino-4-(2,4,5-trifluorophenyl)butanoic Acid (9b). To a solution of 0.37 g (1.16 mmol) of (*R*)-*N*-(*tert*-butoxycarbonyl)-2,4,5-trifluorophenylalanine (**6b**) in 10 mL of diethyl ether at −20 °C were added sequentially 0.193 mL (1.3 mmol) of triethylamine and 0.18 mL (1.3 mmol) of isobutyl chloroformate, and the reaction was stirred at this temperature for 15 min. A cooled ethereal solution of diazomethane was then added until the yellow color persisted and stirring was continued for 1 h. The excess diazomethane was quenched by dropwise addition of acetic acid. The reaction was diluted with ethyl acetate, washed sequentially with saturated aqueous sodium bicarbonate solution and brine, dried over magnesium sulfate, and concentrated in vacuo. Purification by flash chromatography (silica gel, 3:1 hexane:ethyl acetate) afforded 0.36 g of diazoketone. To a solution of 0.35 g (1.15 mmol) of the diazoketone dissolved in 12 mL of 1,4-dioxane: water (5:1) was added 26 mg (0.113 mmol) of silver benzoate. The resultant solution was sonicated for 2 h before diluting with ethyl acetate and washing sequentially with 1 N hydrochloric acid and brine, drying over magnesium sulfate, and concentrating in vacuo. Purification by flash chromatography (silica gel, 97:2:1 dichloromethane: methanol:acetic acid) afforded 401 mg of the title compound. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.06 (m, 1H), 6.95 (m, 1H), 5.06 (bs, 1H), 4.18 (m, 1H), 2.98 (m, 2H), 2.61 (m, 2H), 1.39 (s, 9H).

8-Chlorotriazolo[4,3-*a*]pyrazine (12a). To 3-chloro-2-hydrazinopyrazine **11** (1.0 g, 6.92 mmol), prepared from 2,3-dichloropyrazine and hydrazine using a procedure analogous to that described in the literature,[16] was added 15 mL of trimethyl orthoformate. After refluxing for 10 h, the reaction was cooled to room temperature and the precipitate was filtered. The solid was collected to give 0.635 g of the title compound as a solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.77 (d, 2H, J = 5.0 Hz), 8.50 (d, 2H, J = 5.0 Hz). 9.40 (s, 1H).

5,6,7,8-Tetrahydro[1,2,4]triazolo[4,3-*a*]pyrazine, Hydrochloride (13a). 13a was prepared from 8-chlorotriazolo-[4,3-*a*]pyrazine **12a** (0.50 g, 4.8 mmol), platinum oxide (0.25 g), and palladium on carbon (0.1 g) in 100 mL of methanol in a Paar shaker under H_2 (50 psi) for 14 h. Filtration through Celite followed by concentration gave the title compound as a solid. ^{1}H NMR (500 MHz, CD_3OD) δ 3.34 (s, 2H), 3.78 (t, 2H, J = 6.0 Hz), 4.78 (t, 2H, J = 6.0 Hz), 8.65 (s, 1H).

3-Ethyl-8-chloro-triazolo[4,3-*a*]pyrazine (12b). To 3-chloro-2-hydrazinopyrazine (**11**) (3.0 g, 20.75 mmol), prepared from 2,3-dichloropyrazine and hydrazine using a procedure analogous to that described in the literature,[16] was added 8 mL of triethyl orthopropionate. After refluxing for 10 h, the reaction was cooled to room temperature and the precipitate was filtered. The solid was purified by flash chromatography (100% ethyl acetate, then 10% methanol in ethyl acetate) to give 2.73 g of the title compound as a solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.54 (t, 3H, J = 7.6 Hz), 3.16 (q, 2H, J = 7.8 Hz), 7.70 (d, 1H, J = 4.5 Hz). 7.83 (d, 1H, J = 4.8 Hz).

Ethyl-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyrazine, Hydrochloride (13b). The title compound was prepared from 3-ethyl-7-chlorotriazolo[4,3-*a*]pyrazine (**12b**) (2.70 g, 14.8 mmol) and platinum oxide (0.4 g) in 200 mL of methanol in a Paar shaker under H_2 (50 psi) for 14 h. Filtration through Celite followed by concentration gave the title compound as a solid. ^{1}H NMR (500 MHz, CD_3OD) δ 1.36 (t, 3H, J = 6.0 Hz), 2.84 (q, 2H, J = 6.0 Hz), 3.70 (t, 2H, J = 8.0 Hz), 4.28 (t, 2H, J = 8.0 Hz), 4.06(s, 2H). MS *m/z* 153 $(M + H)^+$.

2-Hydrazinopyrazine (15). This compound was prepared essentially following the literature procedure.[17a] To 75 mL of hydrazine hydrate in a round-bottom flask at room temperature was added dropwise 25.1 g (219 mmol) of chloropyrazine. The resultant mixture was stirred at 120 °C for 45 min. The solution was then maintained at 2 °C for 24 h. A white precipitate was collected by filtration and recrystallized from benzene to yield 11.75 g (49%) of the title compound. ^{1}H NMR (500 MHz, CD_3OD): δ 7.72 (d, 1H, H=2.7 Hz), 7.99−8.00 (m, 1H), 8.10 (d, 1H, J = 1.1 Hz).

3-(Trifluoromethyl)-1,2,4-triazolo[4,3-*a*]pyrazine (17). To 2-hydrazinopyrazine **15** (17.51 g, 159 mmol, from step A) was added 150 mL of trifluoroacetic anhydride dropwise at 0 °C (highly exothermic!). After being stirred at room temperature for 2 h, the reaction mixture was concentrated to give a brown colored solid (51.13 g). To the above solid (50.01 g) was added 200 mL of polyphosphoric acid, and the reaction was stirred at 140 °C for 18 h. The hot PPA solution was added to ice and neutralized by the addition of ammonium hydroxide (highly exothermic!). The aqueous solution was extracted with ethyl acetate (3×), washed with brine, and dried over anhydrous magnesium sulfate. Concentration followed by flash chromatography (silica gel, 1:1 hexanes:ethyl acetate, then 100% ethyl acetate) afforded the title compound as a solid (16.21 g, 54%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.17∼8.20 (m, 2H), 9.54 (s, 1H). MS *m/z* 189 $(M + H)^+$.

3-(Trifluoromethyl)-5,6,7,8-tetrahydro-1,2,4-triazolo[4,3-*a*]pyrazine (18). 3-(Trifluoromethyl)-1,2,4-triazolo[4,3-*a*]pyrazine **17** (2.01 g, 10.67 mmol) was hydrogenated under atmospheric hydrogen with 10% Pd/C (400 mg) as a catalyst in ethanol (20 mL) at ambient temperature for 18 h. The reaction mixture was filtered through Celite and concentrated. Purification by flash chromatography (silica gel, 10% methanol/dichloromethane) gave 1.77 g (86%) of the title compound as a viscous oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.21 (br, 1H), 3.29 (t, 2H, J = 5.5 Hz), 4.09 (t, 2H, J = 5.5 Hz), 4.24 (s, 2H). MS *m/z* 193 $(M + H)^+$.

3-(Pentafluoroethyl)-1,2,4-triazolo[4,3-*a*]pyrazine (19a). To 2-hydrazinopyrazine **15** (2.04 g, 18.55 mmol) was added pentafluoropropionic acid (6.08 g, 37.09 mmol) followed by 50 mL of polyphosphoric acid, and the reaction was stirred at 155 °C for 8 h. The hot PPA solution was added to ice and neutralized by the addition of ammonium hydroxide (highly exothermic!). The aqueous solution was extracted with ethyl acetate (3×), washed with brine, and dried over anhydrous magnesium sulfate. Concentration followed by flash chromatography (silica gel, 1:1 hexanes: ethyl acetate) afforded the title compound as a solid (399 mg, 9%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.17 (d, 1H, J = 4.8 Hz), 8.23 (d, 1H, J = 4.6 Hz), 9.57 (s, 1H). MS *m/z* 239 $(M + H)^+$.

3-(Pentafluoroethyl)-5,6,7,8-tetrahydro-1,2,4-triazolo[4,3-*a*]pyrazine (20a). 19a (394 mg, 1.66 mmol) was hydrogenated under atmospheric hydrogen with 10% Pd/C (200 mg) as a catalyst in ethanol (5 mL) at ambient temperature for 18 h. The reaction mixture was filtered through Celite and concentrated. Purification by flash chromatography (silica gel, 10% methanol/dichloromethane) gave 295 mg (73%) of the title compound as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.89 (br, 1H), 3.30 (t, 2H, J = 5.5 Hz), 4.15 (t, 2H, J = 5.5 Hz), 4.29 (s, 2H). MS *m/z* 243 $(M + H)^+$.

3-(2,2,2-Trifluoroethyl)-1,2,4-triazolo[4,3-*a*]pyrazine (19b). To 2-hydrazinopyrazine **15** (1.60 g, 14.55 mmol) was added 3,3,3-trifluoropropionic acid (5.59 g, 43.66 mmol) followed by 20 mL of polyphosphoric acid, and the reaction was stirred at 110 °C for 18 h. The hot PPA solution was added to ice and neutralized by the addition of ammonium hydroxide (highly exothermic!). The aqueous solution was extracted with ethyl acetate (3×), washed with brine, and dried over anhydrous magnesium sulfate. Concentration followed by flash chromatography (silica gel, 1:1 hexanes: ethyl acetate) afforded the title compound as a viscous oil (148 mg, 5%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.14 (q, 2H, J = 9.4 Hz), 8.00 (d, 1H, J = 4.1 Hz), 8.04 (d, 1H, J = 4.8 Hz), 9.44 (d, 1H, J = 1.4 Hz). MS *m/z* 202 $(M + H)^+$.

3-(2,2,2-Trifluoroethyl)-5,6,7,8-tetrahydro-1,2,4-triazolo-[4,3-*a*]pyrazine (20b). 19b (141 mg, 0.70 mmol) was hydrogenated under atmospheric hydrogen with PtO_2 (70 mg) as a catalyst in methanol (5 mL) at ambient temperature for 2 h. The reaction mixture was filtered through Celite and concentrated to give 110 mg (77%) of the title compound as a viscous oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.27 (t, 2H, J = 5.7 Hz), 3.66 (q, 2H, J = 9.8 Hz), 3.94 (t, 2H, J = 5.2 Hz), 4.22 (s, 2H). MS *m/z* 207 $(M + H)^+$.

General Procedure for the Synthesis of 21. To a solution of triazolopiperazine (**13a**, **13b**, **18**, **20a**, or **20b**) and a β-amino acid (**9a**, **9b**, or **9c**) in dichloromethane or DMF was added HOBT followed by EDC and DIPEA. After being stirred at ambient temperature for 14−18 h, DMF was evaporated to give a viscous residue, which was partitioned between ethyl acetate and saturated aqueous sodium bicarbonate solution. The aqueous layer was extracted three times with ethyl acetate. The combined organic layers were dried over anhydrous magnesium sulfate, filtered, concentrated, and chromatographed on silica gel. The crude product was also purified by HPLC (Gilson; column: YMC−Pack Pro C18 100 × 20 mm I.D.; solvent gradient system starting from 10% acetonitrile, 90% water, and 0.1% trifluoroaceticic acid, increased to 90% acetonitrile, 10% water, and 0.1% trifluoroaceticic acid).

General Procedure for *tert*-Butyloxycarbonyl (Boc) Deprotection. To a solution of **21** in methanol was added saturated methanolic hydrogen chloride solution at 0 °C. After being stirred at room temperature for 1 h, the solution was concentrated to give a white foamy solid.

7-[(3*R*)-3-Amino-4-(3,4-difluorophenyl)butanoyl]-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyrazine, dihydrochloride (22). ^{1}H NMR (500 MHz, CD_3OD): δ 3.10(m, 4H), 3.80−5.50 (m, 7H), 7.26 (m, 3H), 9.56 (s, 1H). MS *m/z* 322 $(M + H)^+$; HPLC A 0.9 min. HRMS (ES^+) calcd for $C_{15}H_{18}F_2N_5O$ $(M + H^+)$ *m/e* 322.1479, found *m/e* 322.1482. Anal. ($C_{15}H_{19}Cl_2F_2N_5O$) C, H; N: calcd, 17.76; found, 17.17.

7-[(3*R*)-3-Amino-4-(3,4-difluorophenyl)butanoyl]-3-ethyl-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyrazine, dihydrochloride (23). ^{1}H NMR (500 MHz, CD_3OD): δ 1.45(t, 3H), 2.93−3.07 (m, 6H), 3.90−4.31(m, 5H), 5.08 (m, 2H), 7.16 (s, 1H), 7.31 (m, 2H). MS *m/z* 350 $(M + H)^+$; HRMS (ES^+) calcd for $C_{17}H_{22}F_2N_5O$ $(M + H^+)$ *m/e* 350.1792, found *m/e* 350.1788. Anal. ($C_{17}H_{23}Cl_2F_2N_5O$) C, H, N.

7-[(3*R*)-3-Amino-4-(3,4-difluorophenyl)butanoyl]-3-(trifluoromethyl)-5,6,7,8-tetrahydro-1,2,4-triazolo[4,3-*a*]pyrazine, hydrochloride (24). ^{1}H NMR (500 MHz, CD_3OD) δ 2.75∼3.10 (m, 4H), 3.80∼4.40 (m, 5H), 4.95∼5.10 (m, 2H), 7.03∼7.20 (m, 3H). MS *m/z* 390 $(M + H)^+$; HRMS (ES^+) calcd for $C_{16}H_{17}F_5N_5O$ $(M + H^+)$ *m/e* 390.1353, found *m/e* 390.1346. Anal. ($C_{16}H_{17}ClF_5N_5O$) N; C: calcd, 45.13; found, 43.96; H: calcd, 4.02; found 3.61.

7-[(3*R*)-3-Amino-4-(2,5-difluorophenyl)butanoyl]-3-(trifluoromethyl)-5,6,7,8-tetrahydro-1,2,4-triazolo[4,3-*a*]pyrazine, (2*E*)-2-butenedioate (2:1) (25c). Step A. 7-[(3*R*)-

3-[(1,1-Dimethylethoxycarbonyl)amino]-4-(2,5-difluorophenyl)butanoyl]-3-(trifluoromethyl)-5,6,7,8-tetrahydro-1,2,4-triazolo[4,3-*a*]pyrazine (21a). (3*R*)-3-[(1,1-Dimethylethoxycarbonyl)amino]-4-(2,5-difluorophenyl)butanoic acid (**9a**, 18.23 g, 57.88 mmol) and 3-(trifluoromethyl)-5,6,7,8-tetrahydro-1,2,4-triazolo[4,3-*a*]pyrazine (**18**, 10.58 g, 55.12 mmol) were dissolved in anhydrous DMF (150 mL). To the above solution was added HOBT (8.94 g, 66.14 mmol) followed by EDC (12.68 g, 66.14 mmol) at 0 °C. After being stirred at room temperature for 16 h, DMF was evaporated to give a viscous residue, which was partitioned between ethyl acetate and saturated aqueous sodium bicarbonate solution. The aqueous layer was extracted with ethyl acetate (3×). The combined organic phase was washed with brine and dried over anhydrous magnesium sulfate. Concentration was followed by purification by flash chromatography (Biotage system, with ethyl acetate:hexanes = 70:30, ethyl acetate:hexanes = 80:20) to afford 24.88 g of the title compound as a foamy solid (92%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.38 (s, 9H), 2.57∼3.05 (m, 4H), 3.85∼4.30 (m, 5H), 4.90 (s, 1H), 4.95∼5.15 (m, 1H), 5.22∼5.40 (br, 1H), 6.86∼7.24 (m, 3H). MS *m/z* 390 $(M + H - t\text{-Boc})^+$.

Step B. 7-[(3*R*)-3-Amino-4-(2,5-difluorophenyl)butanoyl]-3-(trifluoromethyl)-5,6,7,8-tetrahydro-1,2,4-triazolo[4,3-*a*]pyrazine, hydrochloride (25a). 7-[(3*R*)-3-[(1,1-Dimethylethoxycarbonyl)amino]-4-(2,5-difluorophenyl)butanoyl]-3-(trifluoromethyl)-5,6,7,8-tetrahydro-1,2,4-triazolo[4,3-*a*]pyrazine (**21a**, 19.1 mg, 0.039 mmol, from step A) was dissolved in 4 M HCl solution of 1,4-dioxane (1.5 mL) at room temperature. After being stirred at room temperature for 30 min, the solution was concentrated to give a white foamy solid. ^{1}H NMR (500 MHz, CD_3OD) δ 2.75∼3.16 (m, 4H), 3.86∼4.35 (m, 5H), 4.95∼5.05 (m, 2H), 7.13∼7.27 (m, 3H). MS *m/z* 390 $(M + H)^+$.; HRMS (ES^+) calcd for $C_{16}H_{17}F_5N_5O$ $(M + H^+)$ *m/e* 390.1353, found *m/e* 390.1351. Anal. ($C_{16}H_{17}ClF_5N_5O$) C, H, N.

Step C. 7-[(3*R*)-3-Amino-4-(2,5-difluorophenyl)butanoyl]-3-(trifluoromethyl)-5,6,7,8-tetrahydro-1,2,4-triazolo[4,3-*a*]pyrazine (25b). To 7-[(3*R*)-3-[(1,1-Dimethylethoxycarbonyl)amino]-4-(2,5-difluorophenyl)butanoyl]-3-(trifluoromethyl)-5,6,7,8-tetrahydro-1,2,4-triazolo[4,3-*a*]pyrazine (**21a**, 24.75 g, 50.62 mmol, from step A) in 1000 mL round-bottom flask was added 30 mL of methanol followed by 70 mL of HCl saturated methanol solution at 0 °C. After being stirred at room temperature for 1 h, the solution was concentrated to give a white foamy solid. The above solid was partitioned between ethyl acetate (500 mL) and 1 N aqueous sodium hydroxide solution (100 mL). The aqueous layer was extracted with ethyl acetate (3×). The combined organic phase was washed with brine and dried over anhydrous magnesium sulfate. Concentration was followed by purification by flash chromatography on a Biotage system (eluting with CH_2Cl_2:MeOH:NH_4OH = 900:50:2.5, then CH_2Cl_2:MeOH:NH_4OH = 900:100:5) to afford 16.79 g of the title compound as a viscous oil (85%). ^{1}H NMR (500 MHz, CD_3OD) δ 2.50−2.70 (m, 2H), 2.75∼2.90 (m, 2H), 3.48∼3.60 (m, 1H), 3.95∼4.12 (m, 2H), 4.15∼4.35 (m, 2H), 4.85∼5.10 (m, 2H), 6.95∼7.12 (m, 2H); MS *m/z* 390 $(M + H)^+$.

Step D. 7-[(3*R*)-3-Amino-4-(2,5-difluorophenyl)butanoyl]-3-(trifluoromethyl)-5,6,7,8-tetrahydro-1,2,4-triazolo[4,3-*a*]pyrazine, (2*E*)-2-butenedioate (2:1) (25c). To a solution of fumaric acid (75.5 mg, 0.650 mmol) in ethanol (2 mL) was added 506 mg (1.301 mmol) of 7-[(3*R*)-3-amino-4-(2,5-difluorophenyl)butanoyl]-3-(trifluoromethyl)-5,6,7,8-tetrahydro-1,2,4-triazolo[4,3-*a*]pyrazine **25b** in ethanol (4 mL) via pipet. To the above solution was added anhydrous diethyl ether dropwise until the solution became cloudy. The white cloudy heterogeneous mixture was vigorously stirred at room temperature for 20 min. White solid was filtered and washed with diethyl ether. Filtrate was concentrated and dissolved in ethanol (2 mL). To the above ethanol solution was added diethyl ether dropwise until the solution became cloudy. The white cloudy heterogeneous mixture was vigorously stirred at room temperature for 20 min. White solid was filtered and washed with diethyl ether. Combined white solid was dried in vacuo to give 261 mg of the title compound as a white solid (45%). ^{1}H NMR (500 MHz, CD_3OD) δ 2.75−2.95 (m, 2H), 3.00∼3.15 (m, 2H), 3.85∼3.90 (m, 1H), 3.90∼4.35 (m, 4H), 4.90∼5.05 (m, 2H), 6.65 (s, 1H), 7.00∼7.20 (m, 2H); MS *m/z* 390 $(M + H)^+$; HRMS (ES^+) calcd for $C_{16}H_{17}F_5N_5O$ $(M + H^+)$ *m/e* 390.1353, found *m/e* 390.1346. Anal. ($C_{18}H_{18}F_5N_5O_3$) H, N; C: calcd, 48.33; found, 47.37.

(2*R*)-4-Oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-*a*]pyrazin-7(8*H*)-yl]-1-(2,4,5-trifluorophenyl)butan-2-amine, (2*E*)-2-butenedioate (2:1) (1c). Step A. *tert*-Butyl{(1*R*)-3-oxo-1-(2,4,5-trifluorobenzyl)-3-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-*a*]pyrazin-7(8*H*)-yl]propyl}carbamate (21b). (3*R*)-3-[(1,1-Dimethylethoxycarbonyl)-amino]-4-(2,4,5-trifluorophenyl)butanoic acid (**9b**, 8.044 g, 24.16 mmol) and 3-(trifluoromethyl)-5,6,7,8-tetrahydro-1,2,4-triazolo[4,3-*a*]pyrazine (**18**, 4.638 g, 24.16 mmol) were dissolved in anhydrous DMF (75 mL). To the above solution was added HOBT (3.997 g, 28.99 mmol) followed by EDC (5.557 g, 28.99 mmol) at 0 °C. After being stirred at room temperature for 16 h, DMF was evaporated to give a viscous residue, which was partitioned between ethyl acetate and saturated aqueous sodium bicarbonate solution. The aqueous layer was extracted with ethyl acetate (3×). The combined organic phase was washed with brine and dried over anhydrous magnesium sulfate. Concentration was followed by purification by flash chromatography (Biotage system, with ethyl acetate:hexanes = 70:30, then ethyl acetate:hexanes = 80:20) to afford 8.67 g of the title compound as a foamy solid (71%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.37 (s, 9H), 2.61∼3.00 (m, 4H), 3.92∼4.30 (m, 5H), 4.93 (s, 1H), 4.95∼5.12 (m, 1H), 5.22∼5.35 (br, 1H), 6.83∼6.95 (m, 1H), 7.02∼7.12 (m, 1H). MS *m/z* 452 $(M + H - t\text{-Boc})^+$.

Step B. (2*R*)-4-Oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-*a*]pyrazin-7(8*H*)-yl]-1-(2,4,5-trifluorophenyl)butan-2-amine, hydrochloride (1a). *tert*-Butyl{(1*R*)-3-oxo-1-(2,4,5-trifluorobenzyl)-3-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-*a*]pyrazin-7(8*H*)-yl]propyl}carbamate (**21b**, 22 mg, 0.043 mmol, from step A) was dissolved in 2 mL of saturated methanolic hydrogen chloride solution at room temperature. After being stirred at room temperature for 1 h, the solution was concentrated to give a white foamy solid. ^{1}H NMR (500 MHz, CD_3OD) δ 2.75∼3.15 (m, 4H), 3.82∼4.35 (m, 5H), 4.90∼5.05 (m, 2H), 7.16∼7.25 (m, 1H), 7.30∼7.42 (m, 1H). MS *m/z* 408 $(M + H)^+$. HRMS (ES^+) calcd for $C_{16}H_{16}F_6N_5O$ $(M + H^+)$ *m/e* 408.1259, found *m/e* 408.1258. Anal. ($C_{16}H_{16}ClF_6N_5O$) C, H, N.

Step C. (2*R*)-4-Oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-*a*]pyrazin-7(8*H*)-yl]-1-(2,4,5-trifluorophenyl)butan-2-amine (1). To a solution of *tert*-butyl{((1*R*)-3-oxo-1-(2,4,5-trifluorobenzyl)-3-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-*a*]-pyrazin-7(8*H*)-yl]propyl}carbamate (**21b**, 8.66 g, 17.08 mmol) in methanol (20 mL) was added 20 mL of saturated methanolic hydrogen chloride solution at 0 °C. After being stirred at room temperature for 1 h, the solution was concentrated to give a white foamy solid. The above solid partitioned between ethyl acetate (300 mL) and 1 N aqueous sodium hydroxide solution (300 mL). The aqueous layer was extracted with ethyl acetate (3×). The combined organic phase was washed with brine and dried over anhydrous magnesium sulfate. Concentration was followed by purification by flash chromatography using a Biotage system (eluting with CH_2Cl_2:MeOH:NH_4OH = 900:50:2.5, then CH_2Cl_2:MeOH:NH_4OH = 900:100:5) to afford 5.787 g of the title compound as a viscous oil (83%). ^{1}H NMR (500 MHz, CD_3OD) δ 2.60−2.83 (m, 2H), 2.83∼3.00 (m, 2H), 3.63∼3.73 (m, 1H), 3.95∼4.15 (m, 2H), 4.15∼4.35 (m, 2H), 4.90∼5.06 (m, 2H), 7.12∼7.22 (m, 1H), 7.30∼7.38 (m, 1H); MS *m/z* 408 $(M + H)^+$. Anal. ($C_{16}H_{15}F_6N_5O$) C, H, N.

Step D. (2*R*)-4-Oxo-4-(3-trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-*a*]pyrazin-7(8*H*)-yl]-1-(2,4,5-trifluorophenyl)butan-2-amine, (2*E*)-2-butenedioate (2:1) (1c). Fumaric acid (8.5 mg, 0.073 mmol) and (2*R*)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-*a*]pyrazin-7(8*H*)-yl]-1-(2,4,5-trifluorophenyl)butan-2-amine (**1**, 49.6 mg, 0.122 mmol) were dissolved in ethanol (150 μL). The reaction mixture

was stirred for 2 min and gently heated with a heat gun until the solution became cloudy. To the above cloudy solution was added anhydrous diethyl ether dropwise. The white cloudy heterogeneous mixture was vigorously stirred at room temperature for 5 min. White solid was filtered and washed with diethyl ether (3×). The filtered solid was dried in vacuo at 100 °C for 30 min to give 37.5 mg of the title compound as a white solid (45%). ^{1}H NMR (500 MHz, CD_3OD) δ 2.75−2.95 (m, 2H), 3.00∼3.15 (m, 2H), 3.85∼3.90 (m, 1H), 3.90∼4.35 (m, 4H), 4.90∼5.05 (m, 2H), 6.65 (s, 1H), 7.00∼7.20 (m, 2H); MS *m/z* 390 $(M + H)^+$; HRMS (ES^+) calcd for $C_{16}H_{16}F_6N_5O$ $(M + H^+)$ *m/e* 408.1259, found *m/e* 408.1263. Anal. ($C_{18}H_{17}F_6N_5O_3$) C, H, N, F.

7-[(3*R*)-3-Amino-4-(2,4,5-trifluorophenyl)butanoyl]-3-5,6,7,8-tetrahydro-1,2,4-triazolo[4,3-*a*]pyrazine, dihydrochloride (26). ^{1}H NMR (500 MHz, CD_3OD): δ 9.49 (s, 1H), 9.53 (s, 1H), 7.45 (m,1H), 5.15 (m, 2H), 4.47 (bs,1H), 4.32−4.45(m,1H), 4.05−4.25 (m, 2H), 3.90−3.99 (m,1H), 2.89−3.20 (m, 4H); MS *m/z* 340 $(M + H)^+$.; HRMS (ES^+) calcd for $C_{15}H_{17}F_3N_5O$ $(M + H^+)$ *m/e* 340.1385, found *m/e* 340.1383. Anal. ($C_{15}H_{18}Cl_2F_3N_5O$) C, H, N.

7-[(3*R*)-3-Amino-4-(2,4,5-trifluorophenyl)butanoyl]-3-(pentafluoroethyl)-5,6,7,8-tetrahydro-1,2,4-triazolo[4,3-*a*]pyrazine, hydrochloride (27). ^{1}H NMR (500 MHz, CD_3OD) δ 2.75∼3.15 (m, 4H), 3.85∼4.40 (m, 5H), 4.90∼5.05 (m, 2H), 7.16∼7.30 (m, 1H), 7.30∼7.42 (m, 1H). MS *m/z* 458.2 $(M + H)^+$; HRMS (ES^+) calcd for $C_{17}H_{16}F_8N_5O$ $(M + H^+)$ *m/e* 458.1227, found *m/e* 458.1223. Anal. ($C_{17}H_{16}ClF_8N_5O$) C, H, N.

7-[(3*R*)-3-Amino-4-(2,5-difluorophenyl)butanoyl]-3-(2,2,2-trifluoroethyl)-5,6,7,8-tetrahydro-1,2,4-triazolo[4,3-*a*]pyrazine, dihydrochloride (28). ^{1}H NMR (500 MHz, CD_3OD) δ 2.85∼3.20 (m, 4H), 3.90∼4.40 (m, 7H), 5.00∼5.20 (m, 2H), 7.05∼7.22 (m, 3H). MS *m/z* 404 $(M + H)^+$; HRMS (ES^+) calcd for $C_{17}H_{19}F_5N_5O$ $(M + H^+)$ *m/e* 404.1510, found *m/e* 404.1507. Anal. ($C_{17}H_{20}Cl_2F_5N_5O$) C, H, N.

X-ray Crystallographic Analysis. DPP-IV (residues 39−766) was crystallized in the presence of compound **1** following the reported conditions.[24a] A 99.9% complete, 7-fold redundant X-ray diffraction data set to 2.1 Å was collected from a single-crystal cooled to 100 K, using the beamline 32-ID at the Advanced Photon Source (Argonne, IL). The structure was solved using Molecular Replacement procedures (MOLREP[27]) and the 1N1M.pdb coordinate file, without water, ligand and sugar molecules. The structure was refined against all available data to 2.1 Å. using CNX (Accelrys[28]) to a crystallographic *R*-factor of 19.3% and an R_{free} of 22.8%. The root-mean-square deviation between the model and ideal bond distances and bond angles are 0.009 Å and 1.44°, respectively. Coordinates have been deposited with the Protein Data Bank, accession code 1X70. Data collection and refinement statistics and further refinement details are available as Supporting Information.

Oral Glucose Tolerance Test in Lean Mice. Male C57BL/6N mice (7−12 weeks of age) from Taconic Farms, Germantown, NY, were housed 10 per cage and given access to normal diet rodent chow (Teklad 7012) and water ad libitum. Mice (n = 7/group) were randomly assigned to treatment groups and fasted overnight (∼18−21 h). Baseline (t = −60 min) blood glucose concentration was determined by glucometer from tail nick blood. Animals were then treated orally with vehicle (0.25% methylcellulose, 5 mL/kg) or compound **1** (3, 1, 0.3, and 0.1 mg/kg; 5 mL/kg). Blood glucose concentration was measured 1 h after treatment (t = 0 min) and mice were then orally challenged with dextrose (5 g/kg, 10 mL/kg). One group of vehicle-treated mice was challenged with water as a negative control. Blood glucose levels were determined from tail bleeds taken 20, 40, 60, and 120 min after dextrose challenge. The blood glucose excursion profile from t = 0 to t = 120 min was used to integrate an area under the curve (AUC) for each treatment. Percent inhibition values for each treatment were generated from the AUC data normalized to the water-challenged controls.

Lean Mouse Pharmacodynamic Assay. Male C57BL/6N mice (7−12 weeks of age, 19−26 g) from Taconic Farms, Germantown, NY were housed 10 per cage and given access to normal diet rodent chow (Teklad 7012) and water ad libitum. Mice (n = 20−28/group) were randomly assigned to treatment groups and fasted overnight (∼18−21 h). Baseline (t = −60 min) blood glucose concentration was determined by glucometer from tail nick blood. Animals were then treated orally with vehicle (0.25% methylcellulose, 5 mL/kg) or compound **1** (3, 1, 0.3, and 0.1 mg/kg; 5 mL/kg) and the blood glucose concentration determined 1 h after treatment (t = 0 min). After the blood glucose determination at t = 0, mice were orally challenged with dextrose (5 g/kg, 10 mL/kg). One group of vehicle-treated mice was challenged with water as a negative control. Blood glucose levels were determined from tail bleeds taken 20 min after dextrose challenge. Mice were then immediately euthanized and terminal blood samples collected by cardiac puncture. Blood samples were collected into EDTA and the plasma harvested by centrifugation. Aliquots of plasma samples were stored at −70 °C until analysis.

Measurement of Plasma DPP-IV Activity.[25] Plasma DPP-IV activity was measured using a continuous fluorometric assay with the substrate Gly-Pro-AMC, which is cleaved by DPP-IV to release the fluorescent AMC leaving group. A typical reaction contains 50% plasma, 50 μM Gly-Pro-AMC, and buffer (100 mM HEPES, pH 7.5, 0.1 mg/mL BSA) in a total reaction volume of 50 to 70 μL (depending on the availability of sample). Liberation of AMC was monitored continuously in a 96-well plate fluorometer (SpectramMAX Gemini, Molecular Devices), using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Under these conditions, approximately 5 μM AMC is produced in 5 min at 37 °C. The platereader used was a SpectramMAX Gemini (Molecular Devices). The assay exhibits linear rates only for about 5 min due to the rapid substrate depletion. Therefore, it was important to preincubate all assay components to the assay temperature prior to the assay. The data are reported as % inhibition calculated as follows: %Inhibition = $100\ (1 - (V_t/V_c))$, where V_t is the rate of reaction of treated sample and V_c is the rate of reaction of control sample.

Measurement of Plasma Active (intact) GLP-1. Plasma intact GLP-1 was measured using a 96-well ELISA kit for active hormone, purchased from Linco Research Inc (St. Charles, MO, cat # EGLP-35K). The assay has a detection limit of 2 pM and is selective for active GLP-1 (GLP-1[7−36] amide and GLP-1[7−37]). The DPP-IV inhibitor valine thiazolidide (100 μM) was added to plasma aliquots for active GLP-1 measurements to prevent degradation of the hormone.

Determination of Plasma Concentration of Compound 1. Plasma concentrations of compound **1** were determined 20 min after dextrose challenge and 80 min postcompound administration by liquid chromatography/tandem mass spectrometry. Plasma was prepared for MS analysis by solid-phase extraction using OASIS-HLB 96-well extraction plates. The typical limit of quantitation was 10 nM.

Oral Glucose Tolerance Test in Diet Induced Obese (DIO) Mice. Male C57BL/6N mice were purchased from Taconic Farms, Germantown, NY. At 5 weeks of age they were placed on a high fat diet F-3282 (35% fat by weight) supplied by BioServ, New Jersey for 27 weeks. An age-matched cohort of mice was fed a normal diet rodent chow (Teklad 7012) to provide a lean control group. All mice were given access to food and water ad libitum. At approximately 7 months of age, mice were used for the following OGTT. DIO animals were randomly assigned to treatment groups. The DIO (45−55 g) and lean mice (28−32 g) (n = 7−8/group) were fasted overnight (18−21 h) and baseline (t = −60 min) blood glucose concentration was determined by glucometer from tail nick blood. DIO animals were then treated orally with vehicle (0.25% methylcellulose, 5 mL/kg) or compound **1** (30, 3, 0.3 mg/kg; 5 mL/kg). Lean control mice received only vehicle. Blood glucose concentration was measured 1 h after treatment (t = 0 min), and mice were then orally challenged with dextrose (2 g/kg,

10 mL/kg). Blood glucose levels were determined from tail bleeds taken 20, 40, 60, and 120 min after dextrose challenge. The blood glucose excursion profile from $t = 0$ to $t = 120$ min was used to integrate an area under the curve (AUC) for each treatment. Percent inhibition values for each treatment were generated from the AUC data normalized to the dextrose-challenged lean controls.

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Supporting Information Available: The X-ray crystallographic data of compound **1** bound to DPP-IV and purity data for compounds **1** and **22**−**28**. This material is available free of charge via the Internet at http://pubs.acs.org..

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