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Isoxazol-5(4*H*)one Derivatives as PTP1B Inhibitors Showing an Anti-Obesity Effect

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Dedicated to Professor Eun Lee on the occasion of his retirement and 65th birthday

Abstract: In developing inhibitors of therapeutic target enzymes, significant time and effort are committed to the preparation of large numbers of compounds. In an effort to develop a potent inhibitor of protein tyrosine phosphatase (PTP) 1B as an anti-obesity and/or anti-diabetic agent, we constructed an isoxazolone chemical library by using a simplified procedure that circumvents tedious workup and purification steps. The 10×7 isoxazolone derivatives were synthesized by coupling the two halves of the target compounds. When mixed and heated in test tubes, the precursors produced the reaction products as precipitates. After brief washing, the products were pure enough to be used for enzymatic experiments. With the precursors for the coupling reactions prepared, the 10×7 library compounds could be prepared in a day by using the present protocol. The library compounds thus obtained were examined for their inhibitory activities against PTP1B. Among them, compound **C3** was the most potent inhibitor of PTP1B with an IC_{50} of 2.3 μM. The in vivo effect of **C3** was also examined in an obesity-prone mouse strain. Diet-induced obese (DIO)/diabetic mice were divided into two groups and each group was fed a high-fat diet (HFD) or HFD+**C3** for four weeks. The group of **C3**-fed mice gained significantly less weight relative to the HFD-fed control group during the four weeks of the drug feeding period. In contrast to the anti-obesity effect of **C3**, no difference was observed in the glycemic control of the HFD and HFD+**C3** mice groups.

Keywords: diabetes · enzymes · heterocycles · inhibitors · PTP1B

Introduction

The rapid increase in the prevalence of obesity throughout the world poses a serious health threat in modern society.[1] Obesity enhances the risk of associated morbidities, including diabetes, hypertension, dyslipidemia, ischaemic heart disease, and even cancer.[2] Existing therapies for obesity are limited in number and effectiveness. Only two drugs, orlistat and sibutramine, are currently approved for the long-term treatment of obesity.[3] These drugs have limitations in their use due to adverse side effects or limited efficacy.[3] Another drug, rimonabant, recently developed as an appetite suppressant was approved in 2006 in the European Union. However, rimonabant was found to increase the risk of adverse psychiatric effects, including serious depression and suicide, and its use was suspended in 2008.[4]

To circumvent the problems of the current drugs, numerous studies have been devoted to the identification of novel therapeutic targets and significant progress has been made.[5] Among these progresses, inhibition of protein tyrosine phosphatase (PTP) 1B was recognized as a promising therapeutic strategy for the management of both obesity and diabetes.[6] Genetic ablation of PTP1B in mice suppressed weight gain and maintained insulin sensitivity upon feeding a high-fat diet (HFD). Wild-type mice, on the other hand, gained more weight and became insulin resistant.[7] Furthermore, PTP1B-depleted mice were healthy, without worrisome traits, such as enhancement of mitogenic signaling. Inhibi-

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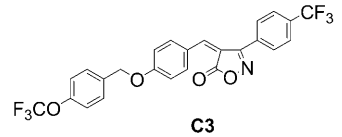
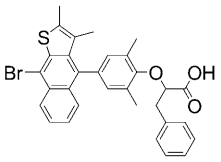
tion or reduction of the cellular abundance of PTP1B in mice also resulted in increased sensitivity to leptin and insulin, and has been shown to exhibit a protective effect against diet-induced obesity.[7–8]

Numerous active-site-directed PTP1B inhibitors have been reported, many of them with pharmacophores that mimic phosphotyrosine residues.[9] Among those, Ertiprotafib had progressed to clinical trials but it was discontinued



C3



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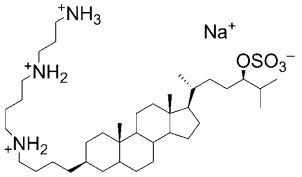
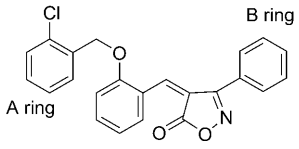


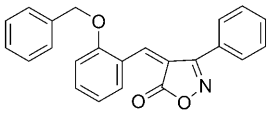
trodusquemine

in the second phase due to unwanted side effects and insufficient efficacy.[10] A noncompetitive PTP1B inhibitor, trodusquemine, has recently proceeded to the early stages of clinical trials with promising preclinical results as both an appetite suppressant and a hypoglycemic and hypocholestrolemic agent.[11] First-in-class drug targeting PTP1B being yet to be developed, intensive research is underway to develop a potent and selective PTP1B inhibitor with anti-obesity and/or hypoglycemic effects.

As an effort to find potent inhibitors of PTP1B, we previously performed virtual screening with docking simulations and identified several scaffolds that were promising for further investigation.[12] Among those, compound **L1** with a half-maximal inhibitory concentration (IC_{50}) of 22 μM was chosen as a lead compound for this study aiming to develop potent PTP1B inhibitors.



L1



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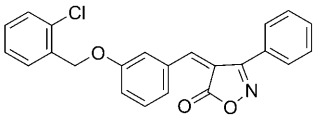

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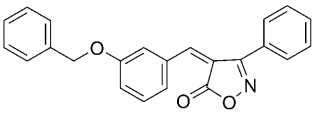
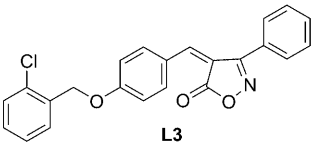
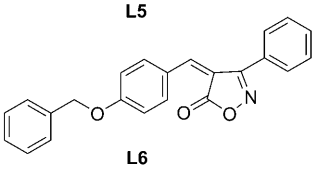


L5



L3



L6

All mouse experiments were performed following the protocols approved by the Institutional Animal Care and Use Committee of Inha University, Incheon, Korea.

Results and Discussion

Library Construction

With compound **L1** as a lead, a chemical library was designed with structural variations on the two aromatic rings (A and B rings) linked to the isoxazol-5(4*H*)one moiety. To find a proper orientation of the substituent on the A ring, three regioisomers, **L1**–**L3**, with a chlorobenzyloxy substituent at the *ortho*, *meta*, and *para*-positions, respectively, on the A ring of the core structure were synthesized and examined for their inhibitory potency against the enzyme activity of PTP1B. Compounds **L1**–**L3** exhibited IC_{50} values of 22, 25, and 15 μM, respectively, which suggest the *para*-position as the choice for derivatization (Table 1). To confirm this result, we also synthesized analogous compounds, **L4**–**L6**, bearing a benzyloxy substituent at the *ortho*, *meta*, and *para*-positions, respectively. Among those, *para*-isomer (**L6**) was the most potent PTP1B inhibitor (Table 1). Based on these observations, a 3,4-diarylisoxazol-5-one chemical library was prepared with the substituent on the A ring being fixed at the *para*-position (Scheme 1).

The strategy for the library synthesis is shown in Scheme 1. The condensation reaction of **I** and **II** was accomplished without catalyst by heating the reaction mixture at 65 °C.[13] In this study, all the condensation reactions proceeded in the absence of piperidine or any other catalysts. Furthermore, the product precipitated on cooling of the reaction mixture, which eliminated workup process and saved

Abstract in Korean:

의약개발시 표적효소에 대한 억제제 개발과정에서 다수의 화합물들 합성에 상당한 시간과 노력이 소모된다. 비만 및 당뇨 치료제로 사용될 수 있는 PTP1B 억제제 개발을 위하여 옥사졸론 화합물 라이브러리를 제작하였는데 반응 후 처리와 정제과정을 생략할 수 있는 방법을 사용하였다. 표적화합물들의 반쪽에 해당하는 두 개의 전구체들을 축합하여 10 × 7 옥사졸론 유도체들을 합성하였는데, 전구체들을 시험관 내에서 혼합 가열하면 생성물이 침전형태로 얻어졌다. 약간의 용매로 세척하는 것만으로 효소 실험에 사용하기에 충분한 순도의 화합물을 얻을 수 있었다. 축합반응을 위한 전구체들이 준비된 상태에서 10 × 7 라이브러리를 하루 동안에 제작할 수 있었다. 라이브러리 화합물들에 대하여 PTP1B 억제활성을 측정한 결과 가장 강력한 억제제는 화합물 **C3**로 IC_{50} 값은 2.3 μM 이었다. 비만이 되기 쉬운 생쥐 모델을 이용하여 화합물 **C3**의 생체 내 효과를 실험하였다. 생쥐들에게 고지방식을 먹여 비만/당뇨를 유발한 뒤 생쥐들을 두 그룹으로 나누고 각각 고지방식 또는 고지방식 + **C3**를 4주간 먹였다. 화합물 **C3** 투여 그룹은 고지방식만 먹은 그룹에 비해 체중 증가가 현저하게 감소하였다. 화합물 **C3**에 의한 혈당조절효과는 관찰되지 않았다.

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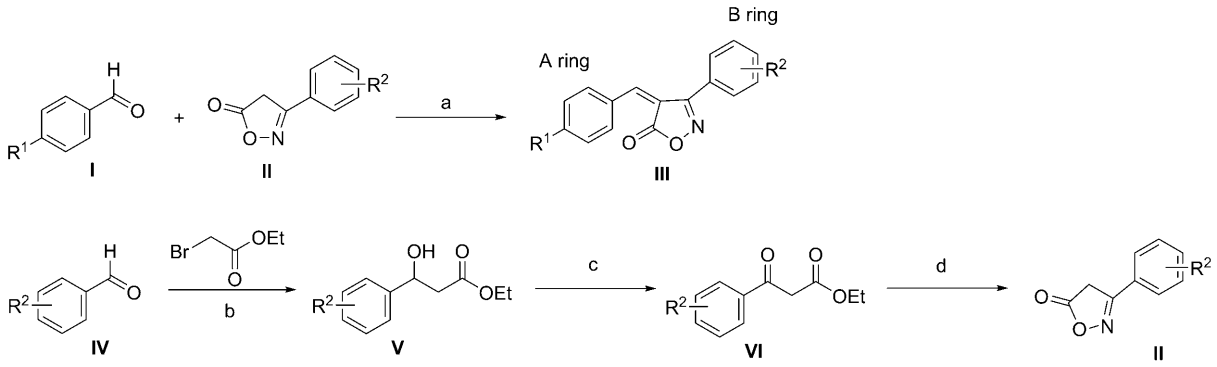

Scheme 1. Synthesis of isoxazolone derivatives: a) *i*PrOH, 65 °C; b) Zn, NH_4Cl; c) MnO_2, CH_2Cl_2; d) $H_2NOH{\cdot}HCl$, EtOH, pyridine, reflux.

Table 1. Inhibitory potency of compounds **L1**–**L6** against PTP1B.

Compounds	IC_{50} [μM][a]
L1	22 ± 2
L2	25 ± 1
L3	15 ± 1
L4	102 ± 10
L5	71 ± 4
L6	43 ± 3

[a] Data expressed as means ± standard deviations of two experiments. The kinetic data were analyzed by using the GraFit 5.0 program (Erithacus Software).

the time and effort for the isolation of the desired product. This feature is one of the conspicuous virtues in the library synthesis for the present study. 3-Arylisoxazol-5-one derivatives (**II**) for the B ring side of the 3,4-diarylisoxazol-5-one derivatives (**III**) were synthesized as in the lower part of Scheme 1.[14]

To minimize the time and effort required for the preparation of a chemical library of 70 compounds, we developed a protocol that uses test tubes as reaction vessels and eliminates the aqueous workup step. Briefly, 70 test tubes (10 mL capacity) were set in a 10 × 7 array. Solutions of benzaldehyde derivatives (**A**–**J**; Scheme 2) and isoxazolone derivatives (**1**–**7**) (0.05 mmol each) dissolved in isopropanol were placed in glass test tubes in 10 × 7 combinations. The volumes of isopropanol used to dissolve each of the compounds are shown in the Experimental Section. By following heating for four hours at 65 °C in a dry heating block and cooling to room temperature, the reaction products had separated out as precipitates in most of the reaction tubes. In some cases, in which no precipitate had formed, the addition of hexane (0.5 mL) and cooling to −50 °C effectively induced precipitation. Removal of the solvent and washing the precipitate with isopropanol (0.5 mL) afforded a solid product that showed essentially a single spot in TLC analysis. The purity of the crude products was also proved by the ^1H NMR spectra of randomly selected samples (see the Supporting Information). Even though the products can be further purified by recrystallization or column chromatography, the precipitates were used as such for enzyme experiments.

Inhibition of PTP1B and other PTPs by Compound C3

The 10 × 7 isoxazolone chemical library was tested in the PTP1B assay for inhibitory activity at a 10 μM concentration by using *para*-nitrophenol phosphate (*p*NPP) as a substrate. As summarized in Figure 1, the compounds in column **C** and row **3** exhibited higher potency relative to other columns and rows. Six of the compounds, **A3**, **B3**, **C2**, **C3**, **C5**, and **C7**, inhibited more than 90% of the PTP1B enzyme activity and these compounds were further purified by recrystallization in isopropanol for IC_{50} determination and structural characterization. The six compounds exhibited IC_{50} values of 2.3–7.2 μM, with compound **C3** exhibiting the lowest value at 2.3 μM (Table 2). There was a good correlation between the IC_{50} values obtained by using the crude and the purified **C3**, which suggests that the data obtained with the crude compounds provide a reliable indication of their true activity.

The inhibitory activity of **C3** against other PTPs was tested to examine the PTP1B specificity of **C3**, the results of which are summarized in Table 3. Compound **C3** demonstrated a 10-fold greater selectivity over T-cell PTP (TC-PTP), the most homologous with PTP1B among the human PTPs, and a 2.9-fold greater selectivity over the catalytic domain of Src homology phosphatase-1 (SHP-1cat). Compound **C3** showed a 12-fold greater selectivity for PTP1B versus vaccinia H1-related phosphatase (VHR), a dual specificity phosphatase. YPTP1, a yeast PTP, was also tested to compare PTPs of different origin, and it exhibited 4.6-fold selectivity.

Enzyme Kinetic Experiments

To investigate the mode of inhibition of **C3**, the enzyme activity of PTP1B was determined by varying the concentration of the substrate, *p*NPP. Lineweaver–Burk plot analysis of the results by plotting the reciprocal of the enzyme reaction rates versus 1/[*p*NPP] revealed the competitive pattern of inhibition, which indicates that **C3** binds to the active site of the enzyme (data not shown).

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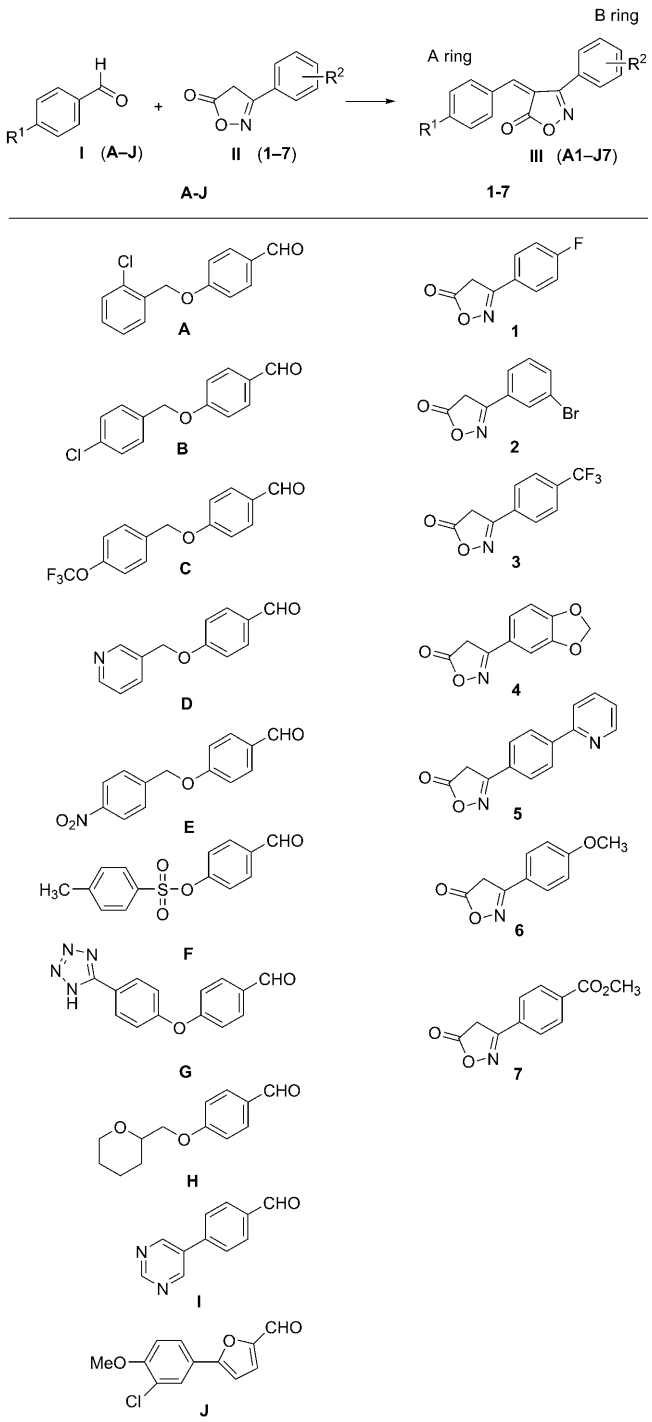

Scheme 2. Construction of the isoxazolone chemical library.

In Vivo Effect of C3 in DIO Mice

The most potent PTP1B inhibitor **C3** was selected, and its in vivo efficacy as an anti-obesity and/or hypoglycemic agent was evaluated. Five-week-old mice susceptible to obesity/diabetes (C57BL/6J Jms Slc male) were fed a HFD ad libitum for eight weeks, followed by a HFD+**C3** for four weeks. For lean and obese controls, two other groups of mice were fed a low-fat diet (LFD) or HFD throughout the

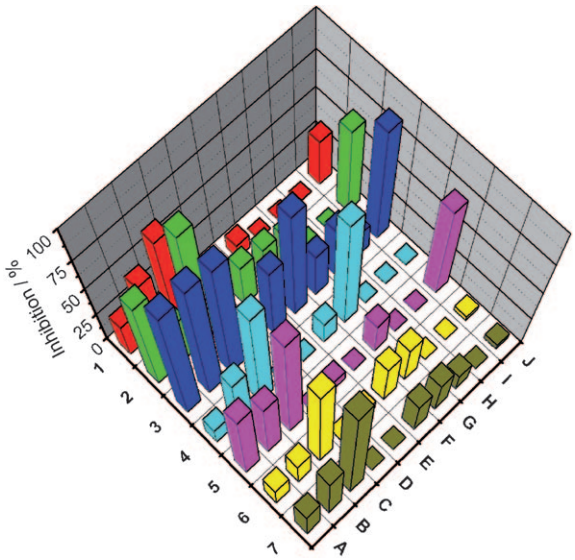

Figure 1. Percent inhibition of PTP1B by the library compounds (**A1**–**J7**) at 10 μM concentrations when *p*NPP was used as a substrate for the assay.

Table 2. Inhibitory potency of the six most potent compounds against PTP1B.

Compounds	IC_{50} [MM][a]
A3	2.5±1.3
B3	3.9±1.1
C2	2.4±0.1
C3	2.3±0.0
C5	4.2±0.0
C7	7.2±0.5

[a] Data expressed as means ±standard deviations of two experiments. The kinetic data are analyzed by using the GraFit 5.0 program (Erithacus Software).

Table 3. Inhibitory effect of compound **C3** on PTPs.

Compound	IC_{50} [μM][a]				
	PTP1B	TC-PTP	SHP-1cat	YPTP1	VHR
C3	2.3±0	24±3	6.7±0.9	11±0.4	27±1

[a] Data expressed as means ±standard deviations of two experiments. The kinetic data were analyzed by using the GraFit 5.0 program (Erithacus Software).

12 week study period. For the drug-fed group, **C3** was provided mixed with the food (1.0 g of **C3** per kg of diet). The daily uptake of **C3** was approximated as 2.7 mg day^{-1}/mouse, equivalent to 88 mg day^{-1} kg^{-1} of mouse weight. As shown in Figure 2, feeding **C3** to the mice significantly suppressed body weight gain in DIO mice ($p<0.05$). The drug-fed group of mice showed an average increase of 5.9 g in their body weight relative to the average 7.5 g increase in the HFD-fed obese control group. In the case of the lean control group, a 2.6 g increase of body weight was observed. Reduction of adiposity was also clearly observed in both the epididymal and retroperitoneal fat weights of the **C3**-fed mice, which were significantly lower than in the obese con-

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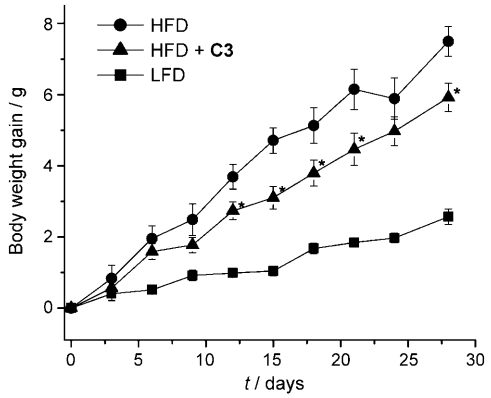

Figure 2. The effect of LFD, HFD, and HFD+**C3** on the body weight of DIO mice. Data points represent means ±SEM; $n=8$/group. Statistical comparisons between HFD and the HFD+**C3** group were performed by using one-way ANOVA, in which * represents $p<0.05$. ●: HFD; ▲: HFD+**C3**; ■: LFD.

trol group ($p=0.051$ for epididymal fat weight, $p<0.01$ for retroperitoneal fat weight; Table 4). Food consumption was not significantly different between the **C3**-treated and obese

Table 4. Effect of compound **C3** on fat pad weight and feed efficiency.[a]

Mice group	Epididymal fat [g]	Retroperitoneal fat [g]	Feed efficiency [wt. gain/ kcal×100]
HFD	2.08±0.17	0.71±0.06	1.96±0.08
HFD+**C3**	1.63±0.14[b]	0.47±0.04**	1.63±0.08**
LFD	0.71±0.04	0.10±0.01	0.87±0.07

[a] Values are means ±SEM; $n=8$/group. Significance of the difference between the HFD group and **C3**-fed group was calculated by one-way ANOVA, in which ** represents $p<0.01$. [b] $p=0.051$.

control mice groups ($p>0.05$, data not shown). Feed efficiency (weight gain per calorie of food intake) of the **C3**-fed mice group was significantly lower relative to the HFD control group ($p<0.01$), which suggests that the in vivo effect of **C3** was due to an increase in metabolic rate, not a decrease in food intake.

The effect of **C3** on glucose metabolism in the mice was also examined after a four-week drug-feeding period. Fasting glucose levels were checked after fasting for eight hours. The fasting glucose level of the **C3**-fed group was not significantly lower than that seen in the DIO control group (data not shown). Glucose tolerance was checked right after the measurement of the fasting glucose level. After loading extra glucose (1.0 g kg^{-1} of body weight) by intraperitoneal injection, the blood glucose concentration was measured over a 120 min period at 20 or 30 min intervals. The **C3**-fed groups did not normalize their blood glucose concentration significantly faster than the HFD control group (data not shown).

Conclusions

The time and effort required for the preparation of a large number of molecules is a hurdle in the early stages of drug development. In the present study, an isoxazolone chemical library was constructed by using a simple procedure that circumvents tedious workup and purification steps. The 10×7 isoxazolone derivatives were synthesized by coupling various aldehyde derivatives and small-sized isoxazolone derivatives. The reaction progressed by mixing and heating the precursors in isopropanol in test tubes without catalysts. After the reaction, the products precipitated on cooling and all of them exhibited essentially a single spot on TLC. The precursors for the coupling reaction being synthesized, the 10×7 library was prepared in a day by using the present protocol.

Among the 70 library compounds, compound **C3** was the most potent PTP1B inhibitor, exhibiting the IC_{50} value of 2.3 μM. Compound **C3** also exhibited good PTP1B selectivity over other PTPs, including TC-PTP, the most homologous with PTP1B among the human PTPs. The in vivo effect of **C3** was examined in a mouse model system. After feeding a HFD for eight weeks to induce obesity and diabetes in mice, **C3** was fed to the obese/diabetic mice for four weeks to examine the effect of **C3**. The **C3**-fed mice group gained significantly less weight relative to the HFD-fed control group during the four weeks of the drug feeding period. In contrast to the anti-obesity effect of **C3**, an anti-hyperglycemic effect was not observed in the HFD+**C3** mice group.

The present study demonstrates that the coupling protocol described in this report is an effective method for synthesizing a large number of isoxazolone derivatives. Compound **C3** synthesized by this method proved to be a potent PTP1B inhibitor and a promising lead compound for the control of obesity.

Experimental Section

Material and Methods

^{1}H and ^{13}C NMR spectra were recorded on a Varian Inova 400 (400 MHz) or Varian Vnmrs 400 (400 MHz) spectrometer by using $CDCl_3$ or [D_6]DMSO as a solvent. Chemical shifts are reported in units of parts per million (ppm) from tetramethylsilane with the solvent resonance as the internal standard ($CHCl_3$: $\delta=7.26$ ppm for ^{1}H, $CDCl_3$: $\delta=77.0$ ppm for ^{13}C NMR spectra). Data are reported as follows: chemical shift, multiplicity (s; singlet, d; doublet, t; triplet, q; quartet, m; multiplet, br; broad), coupling constants (Hz), and integration. IR spectra were recorded on a Bruker Vertex 80 V spectrophotometer. All reactions were monitored by TLC by using E. Merck silica gel precoated plates (60F-254, 0.25 mm). TLC visualization was done with UV light and/or 5% ethanolic *p*-anisaldehyde. All of the reagents were purchased from Aldrich (St. Louis, USA), Sigma (St. Louis, USA), or TCI (Tokyo, Japan) and used as received. The native form of PTP1B, YPTP1, VHR, and the catalytic domain of SHP-1 (SHP-1cat) were overexpressed in *E. coli*, and purified as described previously.[15] TC-PTP was purchased from New England Biolabs, (Beverly, USA). A substrate, *p*NPP, for the PTP assay was purchased from Sigma in the di(Tris) salt form. Absorbances were measured by using a Novaspec-II spectrophotometer (Amersham pharmacia Biotech, Cambridge, UK). Mice (C57BL/6J Jms Slc) were purchased from Japan SLC, Haruno Breeding branch (Syuchi-gun, Shizuoka Prefecture, Japan). Mouse diets were either HFD (D12451) and LFD (D10012G) containing 45 and 16% of the calories from fat, respectively (Research Diets, New Brunswick, NJ, USA).

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Synthesis

The syntheses of aldehydes, **A** to **J**, and isoxazolones, **1** to **7**, are described in the Supporting Information.

Library Synthesis Procedure

The solubility of the aldehydes and isoxazolones in isopropanol at 65 °C was determined by dissolving 0.05 mmol of aldehydes **A**–**J** and isoxazolones **1**–**7** in isopropanol (Table 5). Seventy test tubes (10 mL capacity)

Table 5. Volume of isopropanol used to dissolve 0.05 mmol of aldehydes and isoxazolnes at 65 °C.

Aldehydes	*i*PrOH [mL]	Isoxazolones	*i*PrOH [mL]
A	0.2	**1**	0.4
B	0.2	**2**	0.4
C	0.2	**3**	0.4
D	0.2	**4**	3.0
E	1.6	**5**	4.0
F	0.2	**6**	1.4
G	0.4	**7**	3.0
H	0.2[a]		
I	1.0		
J	1.2		

[a] **H** was a liquid and dissolved in 0.2 mL of *i*PrOH for convenience.

were set in a 10×7 array. A solution of a benzaldehyde derivative **A** (0.05 mmol) in isopropanol was placed in the first column of test tubes (10 tubes). A solution of **B** was then placed in the second column. Other aldehyde derivatives were placed in a similar way in each column of test tubes. In the second step, a solution of isoxazolone derivative **1** (0.05 mmol) in isopropanol was added into the first row of test tubes (7 tubes). Solutions of **2**–**7** were then added into the next rows of test tubes. The total volume of isopropanol in each tube was 0.60 to 6.6 mL. The resulting solutions were kept at 65 °C in a dry heating block for 4 h. In most of the test tubes, precipitates formed during the reaction or on cooling to room temperature. In some cases (**F2**, **F3**, **F4**, **F6**, **F7**, **G2**, **G3**, **G4**) no precipitate formed, but TLC showed complete consumption of starting materials. Addition of hexane (0.5 mL) and cooling to −50 °C resulted in precipitation of the reaction product. The supernatant liquid was removed and the precipitate was washed with isopropanol (0.5 mL). The solvent was evaporated by heating in a dry heating block and the product analyzed by TLC, in which all the products exhibited essentially a single spot. ^{1}H NMR spectra of randomly selected crude samples of **A7**, **E4**, and **J7** also revealed the absence of noticeable impurities as shown in the Supporting Information. The 70 compounds, thus obtained, were dissolved in DMSO to a 100 μM concentration and used for enzyme assay to determine the inhibitory activity against PTP1B and other PTPs. Six of the compounds that inhibited more than 90% of the PTP1B enzyme activity were further purified by recrystallization in isopropanol for IC_{50} determination and structural characterization including elemental and spectroscopic analysis.

*(Z)-4-[4-(2-Chlorobenzyloxy)benzylidene]-3-[4-(trifluoromethyl)phenyl]isoxazol-5(4H)one (**A3**)*

R_f=0.5 (EtOAc/*n*-hexane 1:4); m.p.: 171 °C; IR (KBr): $\tilde{\nu}$=1746, 1588, 1563, 1327, 1277, 1174, 1114, 1092 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ= 5.25 (s, 2H), 7.10 (d, J=8.4 Hz, 2H), 7.29–7.30 (m, 2H), 7.42–7.44 (m, 1H), 7.47 (brs, 1H), 7.50–7.52 (m, 1H), 7.74 (d, J=8.8 Hz, 2H), 7.84 (d, J=8.4 Hz, 2H), 8.43 ppm (d, J=8.8 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ=67.4, 115.1, 115.3, 115.9 (d, J=3.8 Hz), 119.0, 120.5 (d, J= 3.8, Hz), 121.1, 122.2, 124.1, 126.1, 127.0, 128.8, 129.5 (3C), 130.6, 130.9, 132.4 (q, J=32.6 Hz), 132.7, 133.3, 137.3, 151.9, 156.8, 157.10 (1C), 163.4, 163.6 (1C), 168.6 ppm; elemental analysis: calcd (%) for $C_{24}H_{15}ClF_3NO_3$ (457.8): C 62.96, H 3.30, N 3.06; found: C 62.93, H 2.84, N 3.27.

*(Z)-4-[4-(4-Chlorobenzyloxy)benzylidene]-3-[4-(trifluoromethyl)phenyl]isoxazol-5(4H)one (**B3**)*

R_f=0.5 (EtOAc/*n*-hexane 1:4); m.p.: 195 °C; IR (KBr): $\tilde{\nu}$=1737, 1585, 1558, 1330, 1183, 1092 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ=5.15 (s, 2H), 7.06 (d, J=9.2 Hz, 2H), 7.36 (brs, 4H), 7.46 (brs, 1H) 7.73 (d, J= 8 Hz, 2H), 7.83 (d, J=7.6 Hz, 2H), 8.42 ppm (d, J=9.2 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ=69.5, 115.02, 115.3, 115.9 (d, J=3.8 Hz), 119.0, 120.5 (d, J=3.8 Hz), 121.1, 122.2, 124.1, 126.1, 128.7, 128.8, 129.5, 130.6, 130.9, 132.4 (q, J=30.2 Hz), 134.1, 134.2 (1C) 137.3, 151.9, 156.8, 157.1 (1C), 163.4, 163.7 (1C), 168.6 ppm; elemental analysis: calcd (%) for $C_{24}H_{15}ClF_3NO_3$ (457.8): C 62.96, H 3.30, N 3.06; found: C 63.41, H 2.78, N 3.37.

*(Z)-3-(3-Bromophenyl)-4-[4-[4-(trifluoromethoxy)benzyloxy]benzylidene}isoxazol-5(4H)one (**C2**)*

R_f=0.44 (EtOAc/*n*-hexane 1:4); m.p.: 123 °C; IR (KBr): $\tilde{\nu}$=1753, 1585, 1515, 1277, 1178, 1098 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ=5.17 (s, 2H), 7.07 (d, J=9.2 Hz, 2H), 7.24–7.70 (m, 3H), 7.41–7.51 (m, 4H), 7.52 (d, J=6.8 Hz, 1H), 7.72 (d, J=8 Hz, 1H), 7.75 (brs, 1H), 8.42 ppm (d, J=9.2 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ=69.4, 114.9, 115.3, 120.3 (q, J=256.2 Hz; OCF_3), 121.1, 123.2, 126.1, 127.3, 128.9, 129.5, 130.7, 131.5, 133.8, 134.3, 137.3, 149.1, 151.9, 162.9, 163.7, 168.5 ppm; elemental analysis: calcd (%) for $C_{24}H_{15}BrF_3NO_4$ (518.3): C 55.62, H 2.92, N 2.70; found: C 55.43, H 2.68, N 2.91.

*(Z)-4-[4-[4-(Trifluoromethoxy)benzyloxy]benzylidene}-3-[4-(trifluoromethyl)phenyl]isoxazol-5(4H)one (**C3**)*

R_f=0.4 (EtOAc/*n*-hexane 1:4); m.p.: 169 °C; IR (KBr): $\tilde{\nu}$=1737, 1588, 1558, 1332, 1282, 1179, 1092 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ=5.17 (s, 2H), 7.07 (d, J=8.8 Hz, 2H), 7.25 (d, J=8.8 Hz, 2H), 7.45–7.47 (m, 3H), 7.43 (d, J=8 Hz, 2H), 7.83 (d, J=8.4 Hz, 2H), 7.62 ppm (d, J= 9.2 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ=69.5, 115.2, 115.4, 115.9, 119.1, 120.6, 121.2, 122.3, 124.2, 126.2, 129.0, 129.6, 130.7, 130.4, 132.4 (q, J=33.3 Hz), 134.4, 137.4, 149.1, 152.0, 156.9, 157.2 (1C), 163.5, 164.0 (1C), 168.7 ppm; elemental analysis: calcd (%) for $C_{25}H_{15}F_6NO_4$ (507.4): C 59.18, H 2.98, N 2.76; found: C 59.01, H 2.89, N 2.91.

*(Z)-3-[4-(Pyridin-2-yl)phenyl]-4-[4-[4-(trifluoromethoxy)benzyloxy]benzylidene}isoxazol-5(4H)one (**C5**)*

R_f=0.06 (EtOAc/*n*-hexane 1:4); m.p.: 189 °C; IR (KBr): $\tilde{\nu}$=1737, 1585, 1558, 1288, 1266, 1178, 1092, 870, 776 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ=5.20 (s, 2H), 7.07 (d, J=8.8 Hz, 2H), 7.24–7.26 (m, 3H), 7.30–7.31 (m, 1H), 7.46 (d, J=8.8 Hz, 2H), 7.56 (brs, 1H), 7.70 (d, J=8.4 Hz, 2H), 7.80–7.82 (m, 2H), 8.18 (d, J=8.0 Hz, 2H), 8.42 (d, J=8.8 Hz, 2H), 8.74 ppm (d, J=5.2 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ=69.4, 115.4, 115.7, 120.4 (q, J=253.3 Hz OCF_3), 120.8, 121.2, 122.9, 126.3, 127.7, 128.0, 129.0, 129.2, 134.4, 137.0, 137.3, 141.8, 149.9, 152.0, 156.2, 163.6, 164.0, 168.9 ppm; elemental analysis: calcd (%) for $C_{29}H_{19}F_3N_2O_4$ (516.1): C 67.44, H 3.71, N 5.42; found: C 67.29, H 3.50, N 5.58.

*(Z)-Methyl 4-(5-oxo-4-[4-[4-(trifluoromethoxy)benzyloxy]benzylidene}-4,5-dihydroisoxazol-3-yl)benzoate (**C7**)*

R_f=0.2 (EtOAc/*n*-hexane 1:4); m.p.: 172 °C; IR (KBr): $\tilde{\nu}$=1768,1711, 1583, 1517, 1277, 1179, 1087, 771 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ= 4.00 (s, 3H), 5.17 (s, 2H), 7.07 (d, J=9.2 Hz, 2H), 7.24–7.26 (m, 3H), 7.45–7.48 (m, 4H), 7.68 (d, J=8 Hz, 2H), 8.22 (d, J=8.4 Hz, 2H), 8.41 ppm (d, J=9.2 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ=52.5, 69.5, 115.2, 115.4, 120.3 (q, J=256.2 Hz; OCF_3), 121.2, 126.2, 128.8, 128.9, 129.0, 130.3, 131.9, 132.3, 134.3, 137.4, 149.1, 151.9, 163.5, 163.8, 166.2, 168.6, 174.0 ppm; elemental analysis: calcd (%) for $C_{26}H_{18}ClF_3NO_6$ (457): C 62.78, H 3.65, N 2.82; found: C 62.68, H 3.57, N 2.81.

Enzyme Assay: IC_{50} Determination

The enzyme activity was measured at 37 °C by monitoring the hydrolysis of *p*NPP in an enzyme reaction buffer. The absorbance at 405 nm was measured to determine the amount of *p*-nitrophenol released. Enzymes were diluted before the experiment to appropriate concentrations in

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enzyme dilution buffer (20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 1.0 mM ethylenediaminetetraacetic acid (EDTA), 1.0 mM dithiothreitol (DTT), pH 6.0 for VHR; 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5.0 mM EDTA, 1.0 mM DTT, 1.0 mg mL^{-1} bovine serum albumin, pH 7.3 for other PTPs). Inhibitors were dissolved in dimethyl sulfoxide (DMSO). The enzyme reaction buffer was buffer A (50 mM HEPES, 5.0 mM EDTA, pH 7.0) except for VHR, for which buffer B (50 mM acetate, 25 mM Tris-HCl, 25 mM Bis-Tris propane, 1 mM DTT, pH 6.0) was used. For a typical 50 μL reaction, inhibitor (5.0 μL) was added to a reaction mixture containing enzyme (5.0 μL), 5 × enzyme reaction buffer (10 μL), and H_2O (25 μL) and the resulting mixture was incubated at 37 °C for 10 min. The enzyme reaction was initiated by addition of *p*NPP (20 mM, 5.0 μL). After 3–5 min at 37 °C, the reaction was quenched by addition of 0.5 M NaOH (950 μL) and the absorbance at 405 nm was measured. IC_{50} values of the inhibitors were determined by measuring the *p*NPP hydrolase activity at a range of different inhibitor concentrations. The concentrations of enzymes in the assay mixture were 40 nM for PTP1B, 100 nM for SHP-1cat, 15 nM for YPTP1, 500 nM for VHR, and 33 units (manufacturer's definition)/mL for TC-PTP.

LB Plot Analysis

For LB plot analysis, the enzyme activity of PTP1B was measured at a series of different *p*NPP concentrations in the presence and absence of inhibitor **C3**. The kinetic data were analyzed by using the GraFit 5.0 program (Erithacus Software).

Mouse Experiment: Biological Effect of C3 in Diet-Induced Obese Mice

Twenty-four mice (C57BL/6J Jms Slc, 4 week-old male, 17–19 g) were individually housed and maintained in a 12 h light/dark cycle at 25 ± 2 °C with food and water available ad libitum. Mouse diets were either HFD or LFD containing 45 and 16 % calories from fat, respectively, and were either in pellet or powder form. All mice were acclimatized for 1 week (LFD), with 16 mice fed a HFD for the first 8 weeks of the study for the development of obesity and diabetes; the remaining 8 were fed a LFD. The mice assigned to the LFD group were maintained on a LFD throughout the study, as a lean control group. At week 8, all the HFD-fed mice were assigned to 1 of 2 groups, each containing 8 mice. The first group remained on a HFD throughout the study, as an obese/diadetic control group. The remaining group was fed a HFD containing **C3** for 4 weeks. The concentration of **C3** in the diets was 1.0 g kg^{-1} of diet (0.1 % w/w). The LFD was provided in pellet form throughout the experiment. Conversely, the **C3**-treated mice group was fed with powdered HFD mixed with a solution of **C3**. Briefly, 0.20 g of **C3** was dissolved in 0.8 mL of DMSO, homogenized (Superfine Homogenizer, Fluko Instrument Shanghai, Shanghai, China) with 20 mL of 1 % aqueous solution of polyglycerol fatty acid ester, mixed into powdered food (200 g) and it was kneaded to form a dough. The diet for the HFD control group of mice was prepared as above, but without **C3**.

The body weight and food intake were recorded every 3 days throughout the study. The effect of **C3** on glucose metabolism was examined at the end of the 4 weeks of the drug-feeding period. The fasting glucose levels of the mice were checked after fasting for 8 h. Immediately after that, a glucose tolerance test was performed by intraperitoneal injection of glucose (1.0 g kg^{-1} of body weight) and measurement of blood glucose levels from tail bleeds with a glucometer (Accu check active, Roche diagnostics, Ireland) at 0 (prior to glucose administration, used as a fasting glucose level), 20, 40, 60, 90, and 120 min after glucose injection. The data for the mice were analyzed by using the one-way ANOVA with the SPSS version 11.5 statistical package for windows (SPSS, Chicago, Illinois). Differences were considered significant at 2 levels, $p < 0.05$ or $p < 0.01$.

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