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Methylenedisalicylic acid derivatives: New PTP1B inhibitors that confer resistance to diet-induced obesity

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Abstract—Methylenedisalicylic acid derivatives were synthesized and their inhibitory activities against protein tyrosine phosphatases (PTPases) examined. Two of the compounds, **8** and **9**, showed K_i values of 9.4 and 6.3 μM against PTP1B, 4- and 7-fold lower values compared to those against TC-PTP. They were reversible and slow-binding inhibitors against PTP1B. When compound **8** was fed to a mouse model, the weight gain and adipocyte fat storage induced by a high-fat-diet were significantly suppressed.

Reversible tyrosine phosphorylation of cellular proteins is a pivotal mechanism for the control of a diverse range of cellular processes including the maintenance of homeostasis.[1] Hydrolytic cleavage of the phosphoryl group from the phosphotyrosine residue of a protein is catalyzed by the protein tyrosine phosphatase (PTPase) family of enzymes, which is composed of 107 highly homologous members.[2] Physiological substrates of the PTPases have been the focus of research, with PTP1B being one of the most intensively studied enzymes.[3] PTP1B is known to dephosphorylate the insulin receptor (IR) in skeletal muscle and liver, and Jak2 in the hypothalamus.[4] These substrate proteins are involved in the control of IR and leptin signaling, respectively, and these signaling events eventually result in the homeostatic regulation of the blood glucose level and body weight.[5] Based on these research results, the inhibition of PTP1B has emerged as a novel therapeutic strategy for the treatments of type 2 diabetes and obesity.

Numerous active site-directed PTPase inhibitors have been reported, and their chemical structures include a diverse range of the imaginable pharmacophores that mimic phosphotyrosine residues.[6] Of these, Ertiprotafib (**1**) progressed to clinical trials but these were discontinued in the second phase.[7] Because of the high homology of the PTPase family of enzymes, the design of inhibitors that discriminate the active site of each of the PTPases is a challenging hurdle. To achieve selectivity toward PTP1B, a second phosphate-binding site near the PTP1B active site has been utilized.[8] The noncompetitive inhibitors that bind to the surface region of PTP1B, not conserved between PTPases, were also reported.[9] Another issue is the bioavailability of the drug candidate, but many of the reported phosphotyrosine mimics are highly charged, which makes them less attractive in this respect. Recently, (*E*,*E*)-1-iodo-2,5-bis(3-hydroxycarbonyl-4-hydroxy) styrylbenzene (ISB), which contains two salicylate moieties, has been used as a brain imaging reagent, and is known to penetrate the plasma membrane by diffusion and, to a lesser extent, the blood-brain-barrier.[10] This result drew our attention to the use of disalicylic acid derivatives as therapeutically promising PTP1B inhibitors. In this study, various derivatives of methylenedisalicylic acids (MDSAs, **3**–**10**) were synthesized and their in vitro inhibitory activity against PTP1B and in vivo efficacy to prevent diet-induced weight gain in animal model tested (Fig. 1).

The MDSA derivatives were synthesized as depicted in Scheme 1.[11] Treatment of methyl 3-phenylsalicylate with

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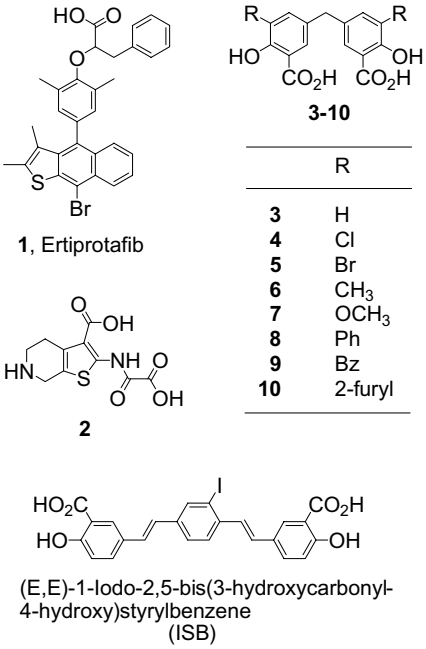


Figure 1. Compounds used or referred in this study.

1,3,5-trioxane, in presence of glacial acetic acid at 95 °C, afforded compound **8**.[12] Applying similar procedures, compounds **3**–**7** and **9** were synthesized from the methyl esters of their corresponding salicylate derivatives.[13] For the preparation of **10**, Stille coupling[14] procedure was adopted. The protected form of **14** was treated with 2-(tributylstannyl)furan to introduce a furanyl functional group.

Compounds **3**–**10** were then tested for their ability to inhibit PTP1B, using *p*-nitrophenyl phosphate (*p*NPP) as a substrate. The enzyme and compounds were preincubated for 10 min prior to initiation of the enzyme reaction by addition of the substrate. The IC_{50} and K_i values for each compound determined under these conditions are presented in Table 1. Salicylic acid is known to be a weak competitive inhibitor of PTP1B, with an inhibition constant of 19.4 mM.[15] As previously reported in our laboratory, MDSA was not a good inhibitor of PTP1B (IC_{50} = 3600 μM).[16] The dihalogenated compounds, **4** and **5**, were found to be more potent than MDSA, but substitution with electron-donating substituents, such as methyl or methoxy group, resulted in a significant loss of potency, as shown with **6** and **7** (IC_{50} > 1 mM). Compounds **8** and **9** were found to be the most potent of the series of MDSA derivatives, with K_i values of 9.4 and 6.3 μM, respectively. The K_i values were compared with those of reference compounds, **1** and **2**. Under our assay conditions, Ertiprotafib showed a K_i value of 1.5 μM. However, compound **2** exhibited a K_i value of 102 μM, proving that compounds **8** and **9** were 11- and 16-fold more potent than **2**. Compound **2** has previously been reported as a potent PTP1B inhibitor, with a K_i = 5.5 μM at pH = 7.0.[19] The inhibitory activities of compounds **8** and **9** against other PTPases, including TC-PTP, were also tested in order to verify their specificities. As shown in Table 2, both compounds **8** and **9** demonstrated ⩾10-fold selectivities against the most homologous TC-PTP, with excellent to modest

Table 1. Inhibitory effect of the compounds on PTP1B[a]

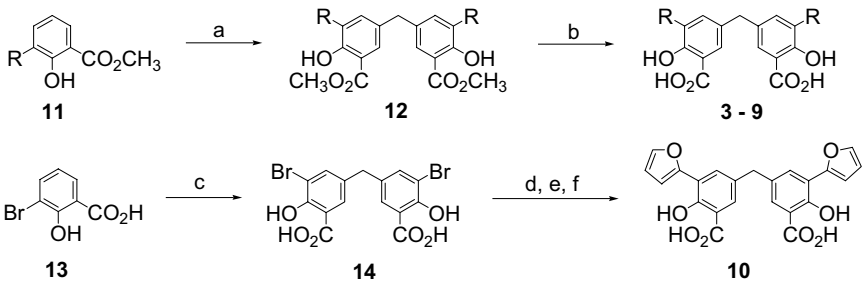
Compound	IC_{50}[b] (μM)	K_i[c] (μM)
3	3600 ± 800	
4	242 ± 56	
5	78 ± 9	
6	>1000	
7	>1000	
8	20 ± 1	9.4
9	15 ± 4	6.3
10	39 ± 7	
1, Ertiprotafib	1.4 ± 0.1	1.5
2	500 ± 100	102[d]

[a] The enzyme reaction was initiated by the addition of *p*NPP to the PTP1B in assay buffer, preincubated for 10 min with inhibitors dissolved in DMSO. The final assay mixture contained: 2 mM *p*NPP, 40 nM PTP1B, 100 mM Hepes, and 5 mM EDTA, at pH 7, plus 10% enzyme dilution buffer (25 mM Hepes, 5 mM EDTA, 1 mM DTT, and 1 mg/mL bovine serum albumin, at pH 7.3). After incubation at 37 °C for 3 min, 0.5 M NaOH (0.95 mL) was added, and the A_{405} measured to determine the amount of *p*-nitrophenol released. The IC_{50} values of the inhibitors were determined by measuring the PTPase activity in a range of different inhibitor concentrations.

[b] Values are means ± standard deviations of three experiments. The kinetic data were analyzed using the GraFit 5.0 program (Erithacus Software).

[c] K_i values of compounds **1**, **8**, and **9** were determined using the relationships: $K_i^{app} = ([S] + K_m)/(K_m/K_i + [S]/\alpha K_i)$ and $\upsilon_s/\upsilon_o = 1/([I]/K_i^{app} + 1)$. For **2**, $K_i^{app} = K_i(1 + [S]/K_m)$.[17,18]

[d] The K_i value of 5.5 μM at pH = 7.0 was reported previously by Anderson et al.[19] We observed K_i = 11.5 μM under their assay conditions.



Scheme 1. Synthetic strategy for the synthesis of methylenedisalicylic acid derivatives (**3**–**10**). Reagents and conditions: (a) $(CH_2O)_3$, H_2SO_4, CH_3CO_2H, 95 °C; (b) 10% aq. NaOH, dioxane, 80 °C; (c) 37% aq. CH_2O, H_2SO_4, CH_3OH, H_2O; (d) Ac_2O, H_3PO_4, 80 °C; CH_3I, K_2CO_3, acetone, reflux, 5 h, 57%; (e) 2-(tributylstannyl)furan, $Pd(PPh_3)_2Cl_2$, dioxane, 100 °C, 7 h, 76%; (f) 1 M NaOH, 1,4-dioxane, H_2O, reflux, 1 h, 99%.

Table 2. Inhibition of PTPases by compounds **8** and **9**

Compound	IC_{50}[a] (μM) (K_i[b],μM)				
	PTP1B	LAR-D1	TC-PTP	SHP-1cat	YPTP1
8	20 ± 1 (9.4)	>2000	291 ± 9 (64)	51 ± 2 (17)	234 ± 11
9	15 ± 4 (6.3)	>2000	146 ± 5 (25)	32 ± 6 (14)	103 ± 10

[a] Values are means ± standard deviations of three experiments.
[b] Values are from a single measurement.

selectivities against LAR-D1 and YPTP1. Less selectivity was observed against SHP-1cat. Of the compounds **8** and **9**, the former was used for further study due to the convenience of its chemical synthesis.

The nature of PTP1B inhibition was also investigated using kinetic experiments. The inhibition by compound **8** exhibited mixed-type patterns in Lineweaver-Burk plot analysis (data not shown). Continuous assay of the enzyme reaction in the presence of **8** showed a time-dependent decrease in the reaction rate, followed by a steady-state reaction rate, characteristic of slow-binding or irreversible inhibition (Fig. 2). To distinguish the mode of inhibition between these potential possibilities, PTP1B was incubated in the presence of compound **8**; therefore, essentially all of the enzymes would form complexes with compound **8**. This mixture was then diluted 50-fold into the assay buffer, containing *p*NPP, to reduce the inhibitor concentration to 14 μM, lower than the IC_{50} value of compound **8**.

After incubation for specified time periods, aliquots of the mixture were removed at different times to measure the progress of the enzyme reaction (Fig. 3). Rapid restoration of the enzyme activity to about 30% of the control reaction suggested the inhibition was reversible. Taken together, these results indicated that compound **8** was a reversible slow-binding inhibitor of PTP1B.

K_i^{app} values were obtained for various *p*NPP concentrations, using the relationship: $v_s/v_o = 1/([I]/K_i^{app} + 1)$,

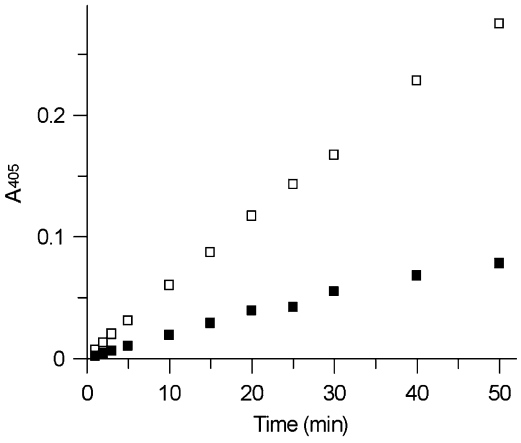


Figure 3. Reversibility of the PTP1B inhibition due to compound **8**. PTP1B was preincubated for 20 min at 25 °C in the absence (□) or presence of 700 μM **8** (■) in enzyme dilution buffer (PTP1B 15 μg/mL). A 12 μL aliquot of the mixture was then added into a solution containing 120 μL of 5× buffer A (500 mM Hepes, 25 mM EDTA, pH 7), 60 μL of 100 mM *p*NPP, and 408 μL H_2O. After incubation at 25 °C for specified times, a 50 μL aliquot of the mixture was taken, quenched with 0.95 mL of 0.5 M NaOH, and the A_{405} measured.

where v_s/v_o was approximated by ratio of the enzyme activities in the presence/absence of the inhibitor when the inhibitor-binding reached equilibrium.[17] The K_i^{app} values were virtually unchanged with changes in the *p*NPP concentration, which was indicative of the non-competitive nature of the inhibition (Fig. 4).[18] Structural

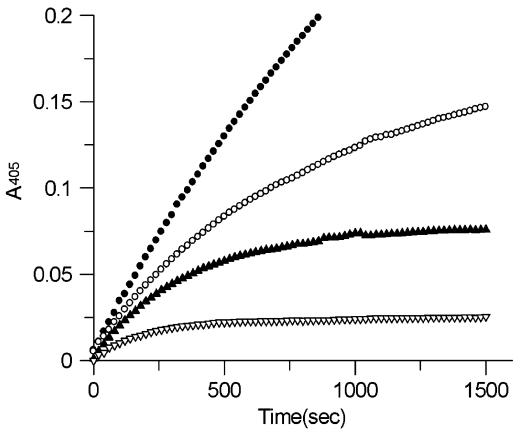


Figure 2. Progress curves for the inhibition of PTP1B due to compound **8**. Hydrolysis of *p*NPP by PTP1B was continuously monitored in the absence (●) and presence of 100 μM (○), 200 μM (▲), and 300 μM (▽) of **8**. The PTP1B and *p*NPP concentrations were 0.7 μg/mL and 0.1 mM, respectively.

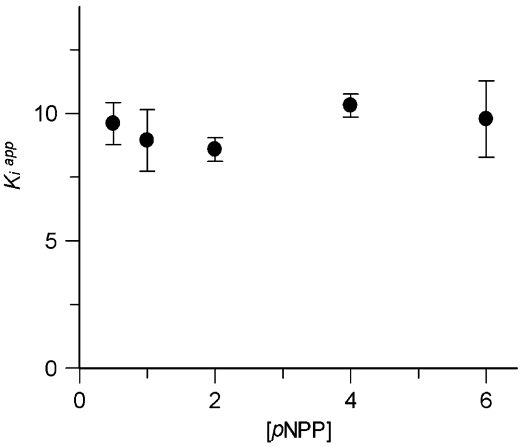


Figure 4. Noncompetitive nature of PTP1B inhibition due to compound **8**. K_i^{app} values were obtained using the relationship: $v_s/v_o = 1/([I]/K_i^{app} + 1)$ where v_s/v_o was approximated by the ratio of the enzyme activity after prolonged incubation with an inhibitor/uninhibited PTP1B activity.[17]

Table 3. In vivo effect of compound **8** in diet-induced obese mice[a]

	Body weight[b] (g)		Liver weight[c] (g)	Adipose tissue weight[c] (g)	
	Initial	Final		Epididymal	Retroperitoneal
LFD[d]	25.2 (±1.4)	26.2 (±1.4)*	0.99 (±0.10)	0.52 (±0.09)*	0.15 (±0.04)*
HFD[e]	27.9 (±2.4)	33.4 (±2.8)	1.14 (±0.12)	1.80 (±0.49)	0.51 (±0.11)
HFD + **8**[f]	27.9 (±2.9)	29.2 (±2.5)*	1.03 (±0.13)	0.86 (±0.24)*	0.26 (±0.08)*

[a] Three groups (8 mice each) of 4-week-old mice (C57BL/6J Jms Slc male, Japan SLC, Inc. Haruno Breeding branch) were acclimatized for 1 week. One and two groups were fed LFD and HFD, respectively, for 8 weeks. The three groups were then fed LFD, HFD or HFD + **8** for 4 weeks. The LFD was provided in pellet form throughout the experiment. On the other hand, HFD and HFD + **8** groups were fed HFD in pellet form for 8 weeks of obesity-developing period and HFD or HFD + **8** in powdered form during inhibitor-feeding period. Mice were individually housed and maintained in a 12 h light/dark cycle at 22 ± 2 °C and fed ad libitum. All values are mean values ± SEM.

[b] Body weights were recorded after feeding LFD or HFD for 8 weeks (initial) and after 4 weeks of drug treatment (final).

[c] Liver and adipose tissue weights were determined at the end of the study.

[d] Low-fat-diet control group; maintained on LFD (16 kcal % fat, 3.9 kcal/g, D10012G, Research Diets, New Brunswick, NJ, USA) throughout the study.

[e] High-fat-diet control group; maintained on HFD (45 kcal % fat, 4.7 kcal/g, D12451, Research Diets, New Brunswick, NJ, USA) throughout the study.

[f] Drug test group: maintained on HFD for the first 8 weeks and then HFD + **8** for 4 weeks. The concentration of **8** in the diets was 5 g/kg (0.5% w/w).

* Significantly different from the HFD group ($p < 0.005$, one-way ANOVA).

analysis of the complex of PTP1B and **8** might provide further information for the design of selective PTP1B inhibitors.

To examine the effect of compound **8** on an animal model, 5 week-old obesity-prone mice (C57BL/6J Jms Slc) were fed a high-fat-diet (HFD) for 8 weeks, followed by a HFD + **8** for 4 weeks. For lean and obese controls, two groups of mice were fed a low-fat-diet (LFD) or HFD for the entire study period. As shown in Table 3, feeding of a HFD + **8** for 4 weeks resulted in an increase of 1.3 g in the body weight compared to a 5.5 g increase in the obese control, which was comparable with the 1.0 g increase observed in the lean control group. Consistent with these observations, a reduction in the adiposity was observed in both the epididymal and retroperitoneal fat weights of the drug-fed mice. The adipose tissue weight was significantly lower than in the obese control group and, moreover, was only marginally higher than in the lean control group, where a LFD had been provided for the 12 week feeding period. The liver weights were not significantly different between the obese and lean control groups and the outward appearance of liver showed no overt toxicity upon treatment with compound **8**. The in vivo results demonstrated the potential of compound **8** as a lead for the development of an anti-obesity drug.

In summary, methylenedisalicylic acid derivatives exhibited inhibitory activities against PTP1B, of which, compound **8** was the second most potent, showing a 7-fold PTP1B selectivity against TC-PTP, and was a reversible and slow-binding inhibitor of PTP1B. Compound **8** was found to significantly suppress the weight gain and adipocyte fat storage induced by a HFD.

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12. Compound **8** was prepared according to the following procedure. 1,3,5-trioxane (32 mg) was added to a solution of methyl 3-phenylsalicylate (513 mg) in glacial acetic acid

(0.46 mL) and the mixture was heated to 90 °C. A 1:5 mixture of sulfuric acid and glacial acetic acid (27 μL) was then added and stirred at 90–96 °C for 3 h. The reaction mixture was cooled to rt, poured into water, and extracted with EtOAc. Organic extracts were combined, washed with water, and the solvent was removed. Column chromatography of the resulting oil afforded dimethyl ester of **8** as a white solid (306 mg): mp 150–153 °C; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 11.16 (s, 2H), 7.68 (d, J = 2.4 Hz, 2H), 7.57–7.51 (m, 4H), 7.46–7.29 (m, 8H), 3.95 (s, 6H, CH_3), 3.93 (s, 2H, CH_2). Hydrolysis of the ester (200 mg) in hot 1,4-dioxane (1.0 mL) and 10% NaOH (2.0 mL) gave **8** as a white solid (185 mg): mp 261 °C (dec); ^{1}H NMR (Acetone-d_6, 200 MHz) δ 11.54 (s, 2H, exchangeable with D_2O), 7.86 (d, J = 2.6 Hz, 2H), 7.60–7.54 (m, 6H), 7.44–7.27 (m, 6H), 4.07 (s, 2H, CH_2); ^{13}C NMR (Acetone-d_6, 100 MHz) δ 173.24 (CO_2H), 158.89, 138.31, 138.27, 133.10, 131.09, 130.39, 130.26, 128.93, 128.12, 113.32 (C_{arom}), 40.25 (CH_2); LRMS m/z 440 (M^+); HRMS calcd for $C_{27}H_{20}O_6$ 440.1260 (M^+), found 440.1262.

13. Compounds **3**–**8** and **9**–**10** were synthesized starting from appropriate 3-substituted methyl salicylate derivatives in the similar conditions as for the synthesis of **8**. For example, **9** was synthesized starting from methyl 3-benzylsalicylate.
Compound **4**: mp 284 °C (dec); ^{1}H NMR (200 MHz, Acetone-d_6) δ 11.58 (s, 2H, OH), 7.78 (d, J = 2.2 Hz, 2H), 7.60 (d, J = 2.2 Hz, 2H), 3.99 (s, 2H, CH_2).
Compound **5**: mp 283–285 °C (dec); ^{1}H NMR (Acetone-d_6, 200 MHz) δ 11.39 (br s, 2H), 7.82 (d, J = 2.2 Hz, 2H), 7.76 (d, J = 2.2 Hz, 2H), 3.99 (s, 2H); ^{13}C NMR (Acetone-d_6, 100 MHz) δ 172.12 (COOH), 157.99, 140.37, 134.07, 130.71, 114.36, 111.50, 39.32 (CH_2).
Compound **6**: mp 268 °C (dec), rep 296–298 °C; ^{1}H NMR (DMSO-d_6, 200 MHz) δ 11.47 (br s, 2H, exchangeable with D_2O), 7.46 (s, 2H), 7.25 (s, 2H), 3.77 (s, 2H, CH_2), 2.14 (s, 6H, CH_3); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 173.09 (CO_2H), 158.73, 137.52, 131.99, 127.81, 126.36, 112.33, (C_{arom}), 40.75 (CH_2), 16.01 (CH_3).
Compound **7**: mp 239 °C (dec); ^{1}H NMR (Acetone-d_6, 200 MHz) δ 11.20 (br s, 2H, exchangeable with D_2O), 7.35 (d, J = 1.4 Hz, 2H), 7.14 (d, J = 1.8 Hz, 2H), 3.91 (s, 2H, CH_2), 3.80 (s, 6H, OCH_3); ^{13}C NMR (Acetone-d_6, 100 MHz) δ 173.00 (CO_2H), 152.28, 149.67, 132.54, 121.84, 119.30, 112.95, (C_{arom}), 56.55 (CH_3), 41.16 (CH_2); LRMS m/z 348 (M^+); HRMS calcd for $C_{17}H_{16}O_8$ 348.0845 (M^+), found 348.0842.
Compound **9**: mp 248 °C (dec); ^{1}H NMR (Acetone-d_6, 200 MHz) δ 11.36 (br s, 2H, exchangeable with D_2O), 7.61 (d, J = 2.2 Hz, 2H), 7.29 (d, J = 2.2 Hz, 2H), 7.24–7.13 (m, 10H), 3.95 (s, 4H, CH_2), 3.85 (s, 2H, CH_2); ^{13}C NMR (Acetone-d_6, 100 MHz) δ 173.12 (CO_2H), 159.51, 141.56, 138.16, 132.59, 130.67, 129.72, 129.19, 129.10, 126.82, 112.68 (C_{arom}), 44.35, 35.89 (CH_2); LRMS m/z 468 (M^+); HRMS calcd for $C_{29}H_{24}O_6$ 468.1573(M^+), found 468.1574.
Compound **10**: mp >300 °C (dec); ^{1}H NMR (Acetone-d_6, 200 MHz) δ 12.03 (s, 2H), 7.97 (d, J = 2.2 Hz, 2H), 7.79 (d, J = 2.2 Hz, 2H), 7.60 (d, J = 1.6 Hz, 2H), 7.11 (d, J = 3.4 Hz, 2H), 6.57 (m, 2H), 4.10 (s, 2H); ^{13}C NMR (Acetone-d_6, 100 MHz) δ 173.69 (CO_2H), 158.07, 150.49, 143.20, 133.49, 133.05, 130.31, 121.17, 114.04, 113.29, 112.18, 40.93 (CH_2); LRMS m/z 420 (M^+); HRMS calcd for $C_{23}H_{16}O_8$ 420.0845 (M^+), found 420.0840.

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