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The synthesis and biologic evaluation of anti-platelet and cytotoxic β-nitrostyrenes

Pei-Wen Hsieh [a,*], Yu-Ting Chang [a], Wen Yin Chuang [b], Hsin-Chu Shih [b], Shin-Zan Chiang [a], Chin-Chung Wu [b,*]

[a] Graduate Institute of Natural Products, Chang Gung University, Taoyuan 333, Taiwan
[b] Graduate Institute of Natural Products, Kaohsiung Medical University, Kaohsiung 807, Taiwan

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ABSTRACT

Our previous studies demonstrated that two cytotoxic β-nitrostyrene derivatives, 3,4-methylenedioxy-β-nitrostyrene (MNS) and 4-*O*-benzoyl-3-methoxy-β-nitrostyrene (BMNS) exhibit potent anti-platelet activities. In this study, a series of β-nitrostyrenes were synthesized and subjected to anti-platelet aggregation assay and cytotoxicity assay. The mono- and di-substitutions on the B ring of BMNS tended to increase the anti-platelet activity and decrease the cytotoxic activity. Of these, compounds **19** and **24** exhibited the most potent inhibitory effects on thrombin- and collagen-induced platelet aggregation ($IC_{50} \leqslant 0.7$ μM) without significant cytotoxicity on a human cancer cell line (up to 20 μM). Further studies indicated that compounds **19** and **24** inhibited platelet aggregation via prevention of glycoprotein IIb/IIIa activation. The potent and novel effects of BMNS derivatives make them attractive candidates for the development of new anti-platelet agents.

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1. Introduction

Platelets play a crucial role in the pathogenesis of atherothrombosis, which accounts for approximately 40% of all deaths in developed countries.[1] In clinical practice, aspirin, a cyclooxygenase inhibitor, in combination with clopidogrel, an ADP receptor antagonist, is the current 'gold standard' for preventing platelet aggregation.[2] However, aspirin and clopidogrel still have considerable limitations in their mode of action and efficacy.[3] In addition, some patients are refractory to these drugs. For example, patients with at least one variant CYP2C19 allele have reduced clopidogrel responsiveness and an increased risk of cardiovascular events due to decreased formation of the active metabolite.[4] Glycoprotein (GP) IIb/IIIa is a major integrin in platelets. On platelet stimulation, GPIIb/IIIa undergoes a conformational change that allows it to bind to fibrinogen with high affinity, leading to platelet aggregation. GPIIb/IIIa activation thus represents the final common pathway for platelet aggregation regardless of the stimulus used.[5] It has been proved that intravenous GPIIb/IIIa antagonists can improve the outcome of patients undergoing percutaneous interventions (PCI);[6] however, the use of oral GPIIb/IIIa antagonists has been unsuccessful. Therefore, research and development of new generation anti-platelet agents are needed and are now in progress.

Compounds with a β-nitrostyrene moiety have been recognized to have diverse biological activities, particularly antimicrobial and anticancer effects.[7–11] Furthermore, β-nitrostyrenes also exhibited inhibitory effects on protein phosphatases, human telomerase, and snake venom phospholipase.[7] Our previous studies demonstrated that two β-nitrostyrene derivatives, 3,4-methylenedioxy-β-nitrostyrene (MNS) and 4-*O*-benzoyl-3-methoxy-β-nitrostyrene (BMNS) (Fig. 1), potently prevented platelet aggregation by inhibiting the function of GPIIb/IIIa.[12,13] Unlike GPIIb/IIIa antagonists, these compounds inhibit GPIIb/IIIa activation rather than directly blocking GPIIb/IIIa. This feature may provide a new strategy for the treatment of platelet-dependent thrombosis. In this study, BMNS and MNS were chosen as model structures in an attempt to obtain more potent anti-platelet agents. In particular, the route of the test structures in the: (1) mono- or di-substitution effect on the B ring were determined; (2) substitution effect on the A ring of the C-3 and C-4 position; (3) the effect of the β-methyl fragments on bioactivity;[8] and (4) replacement of the A ring with a five or six member aromatic ring. All synthetic and commercial compounds (Tables 1 and 2) were assayed for in vitro inhibitory effects on platelet aggregation induced by collagen and thrombin, as well as cytotoxicity against a human breast cancer cell line (Tables 1 and 2). Herein, we describe the synthesis, pharmacologic data, and structure–activity relationship (SAR) of the anti-platelet and cytotoxic effects related to β-nitrostyrenes.

* Corresponding authors. Tel.: +886 3 211 8800x3105; fax: +886 3 211 8643 (P.W.H.); tel.: +886 7 3121101x2669; fax: +886 7 311 4773 (C.C.W.).
E-mail addresses: pewehs@mail.cgu.edu.tw (P.-W. Hsieh), ccwu@kmu.edu.tw (C.-C. Wu).

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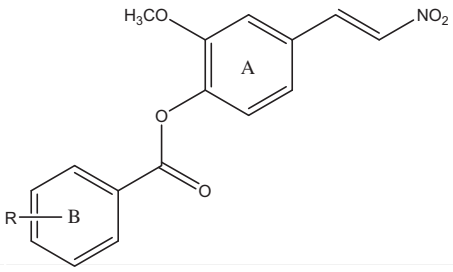
Table 1
The anti-platelet and cytotoxic activities of BMNS derivatives with substituted in B ring



Compd	R	Aggregation IC_{50} (μM)[a,b]		Cytotoxicity IC_{50} (μM) or %Inh[b,c]	
		Thrombin	Collagen	MDA-MB-231	HBL-100
BMNS	H	2.7	1.1	13.1	>20 (0%)
1	2′-F	4.7	1.6	>20 (26.5%)	>20 (4.9%)
2	2′-Cl	1.0	0.6	>20 (7.0%)	>20 (8.2%)
3	2′-Br	5.4	2.0	>20 (8.3%)	>20 (10.0%)
4	2′-CF_3	2.5	1.3	>20 (20.4%)	>20 (2.4%)
5	2′-CH_3	1.5	1.2	>20 (36.3%)	>20 (1.8%)
6	2′-OCH_3	2.1	0.8	19.4	>10 (8.8%)
7	3′-F	2.6	1.4	>20 (44.9%)	>20 (38.2%)
8	3′-Cl	1.2	0.5	18.9	>20 (23.2%)
9	3′-Br	1.3	0.9	>20 (19.1%)	>20 (18.2%)
10	3′-CF_3	3.3	1.7	>20 (34.9%)	>20 (12.5%)
11	3′-CH_3	1.7	0.9	13.4	4.1
12	3′-OCH_3	2.9	0.7	>20 (28.9%)	12.1
13	4′-F	0.9	0.8	9.8	>20 (25.1%)
14	4′-Cl	1.5	0.7	>20 (33.2%)	>20 (7.3%)
15	4′-Br	1.2	0.6	>20 (9.1%)	>20 (6.3%)
16	4′-CF_3	6.2	8.3	>20 (−4.9%)	>20 (−7.3%)
17	4′-CH_3	2.7	2.0	>20 (7.9%)	>20 (8.0%)
18	4′-OCH_3	3.5	0.8	18.1	>20 (22.7%)
19	4′-*tert*-Butyl	0.6	0.6	>20 (2.5%)	>20 (−2.1%)
20	2′,3′-F	3.3	1.6	>20 (25.5%)	>20 (4.1%)
21	2′,4′-F	3.0	2.0	>20 (18.5%)	>20 (16.0%)
22	3′,4′-F	2.9	1.1	>20 (22.1%)	>20 (0.5%)
23	3′,5′-F	3.4	1.4	>20 (24.7%)	>20 (19.0%)
24	2′,3′-Cl	0.7	0.6	>20 (−4.9%)	>20 (11.5%)
25	2′,4′-Cl	0.7	0.7	>20 (−0.1%)	>20 (9.9%)
26	3′,4′-Cl	1.0	1.8	>20 (9.4%)	>20 (15.2%)
27	3′,5′-Cl	1.9	1.4	>20 (20.0%)	>20 (15.6%)
28	2′,3′,4′-F	23.0	2.7	>20 (3.7%)	>20 (1.1%)
29	3′,4′,5′-F	1.4	0.9	12.4	>20 (16.6%)
30	3′,4′,5′-OCH_3	1.1	1.1	13.9	>20 (47.4%)

[a] Platelets were pre-incubated with DMSO (0.5%, control) or test compounds at 37 °C for 3 min before addition of the inducers.
[b] IC_{50} represents the 50% inhibitory concentration. Results represent the means from two independent experiments.
[c] Compounds were tested against the human breast cancer cell line, MDA-MB-231 or the non-malignant breast epithelial cell line, HBL-100 using the MTT method. The percentage of inhibition obtained at a test concentration of 20 μM is given in parentheses (%Inh).

2. Results and discussion

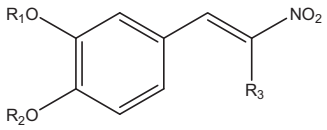
2.1. Chemistry

The BMNS derivatives (**1–30**) were prepared by acid halide esterification of *trans*-4-hydroxy-3-methoxy-β-nitrostyrene and were reacted with corresponding substituted benzoyl chlorides in a solution of dichloromethane (DCM)/ pyridine (10:1) (Scheme 1). 3-Ethoxyl-4-methoxy-β-nitrostyrene (**33**), 3-hydroxy-4-methoxy-β-nitrostyrene (**34**), and their β-methyl derivatives (**37–38**) were synthesized according to the method of Milhazes et al. (Scheme 2).[8] The benzoyl esters (**31**, **32**, **35**, and **36**) of compounds **33**, **34**, **37**, and **38** were prepared by acid halide esterification as described before (Scheme 2). Compounds **39–44**, **48**, and **49** were obtained commercially. Accordingly, compounds **1–49** (Tables 1 and 2) were fully characterized using spectroscopic data. The spectroscopic data of the new compounds are shown in the Section 4.

2.2. Cytotoxic activity

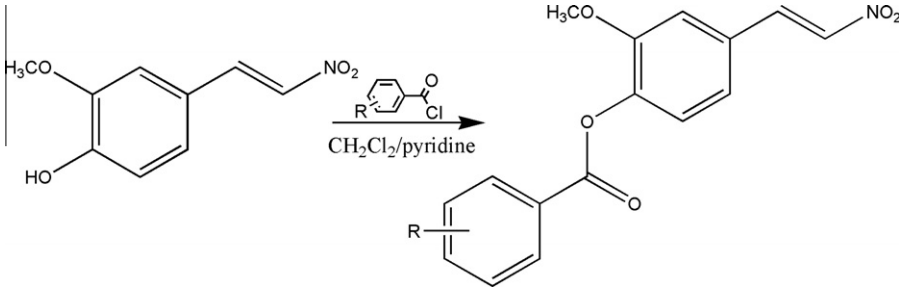
The cytotoxic properties of β-nitrostyrenes have been known for some time: these compounds induce antiproliferative and proapoptotic effects in several cancer cell lines.[7,9–11] The unsaturated nitroolefinic bond in β-nitrostyrenes has been recognized as an essential fragment in its bioactivity, as it can interact with cellular protein cysteine sulfhydryls via the Michael addition mechanism.[14,15] The reactivity of β-nitrostyrenes following the Michael addition is dependent on the position of the withdrawing group in the A ring. In contrast, methyl substitution in the β position resulted in a reduced reaction rate.[14] In the present study, the synthetic β-nitrostyrene derivatives were tested in the human breast cancer cell line MDA-MB-231 to evaluate their cytotoxicity. By comparing BMNS with MNS, it was found that the unsubstituted 4-*O*-benzoyl group did not significantly affect the cytotoxic potency. However, substitution at the benzoyl ring of BMNS resulted in a loss of cytotoxic activity (Table 1).

Table 2
The anti-platelet and cytotoxic activities of BMNS/MNS analogs modifying in the A ring of C-3 and C-4 position, and in the β-carbon of the vinyl side chain

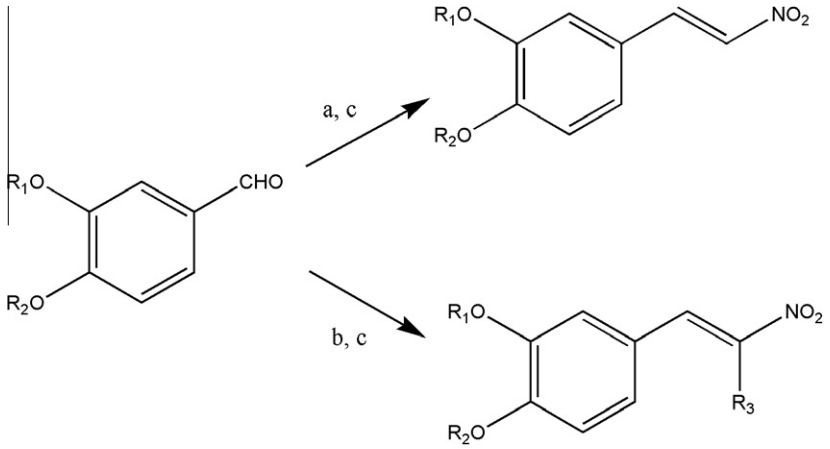


	R_1	R_2	R_3	Aggregation IC_{50}[a,b] (μM)		Cytotoxicity IC_{50} (μM) or %Inh[b,c]	
				Thrombin	Collagen	MDA-MB-231	HBL-100
BMNS	CH_3	Benzoyl	H	2.7	1.1	13.1	>20 (0%)
MNS	$-OCH_2O-$		H	14.0	7.2	14.0	>20 (39.2%)
31	CH_2CH_3	Benzoyl	H	1.2	1.0	15.3	11.6
32	Benzoyl	CH_3	H	1.9	0.8	7.7	<10 (79.8%)
33	CH_2CH_3	H	H	78.1	16.2	>20 (34.4%)	>20 (35.2%)
34	H	CH_3	H	28.5	7.2	18.9	12.8
35	CH_2CH_3	Benzoyl	CH_3	0.8	1.1	12.1	14.6
36	Benzoyl	CH_3	CH_3	2.9	1.0	6.6	7.3
37	CH_2CH_3	H	CH_3	82.7	5.8	5.3	4.5
38	H	CH_3	CH_3	61.0	11.0	7.8	6.6
39	H	OH	H	45.1	18.4	>20 (38.3%)	16.3
40	H	OCH_3	H	12.1	3.5	16.3	13.0
41	H	CH_3	H	8.9	5.9	>20 (36.6%)	15.0
42	H	Cl	H	7.5	3.5	>20 (21.2%)	15.7
43	H	H	OH	35.5	7.8	13.9	>20 (34.9%)
44	H	H	NO_2	5.7	3.0	9.9	>20 (34.2%)
45[d]	OCH_3	OH	H	66.5	16.4	NT[e]	NT[e]
46[d]	OCH_3	OCH_3	H	12.8	7.6	NT[e]	NT[e]
47[d]	H	H	H	17.8	4.9	NT[e]	NT[e]
48	—	—	—	29.8	7.7	>20 (9.9%)	>20 (28.5%)
49	—	—	—	22.9	6.8	>20 (33.3%)	17.9

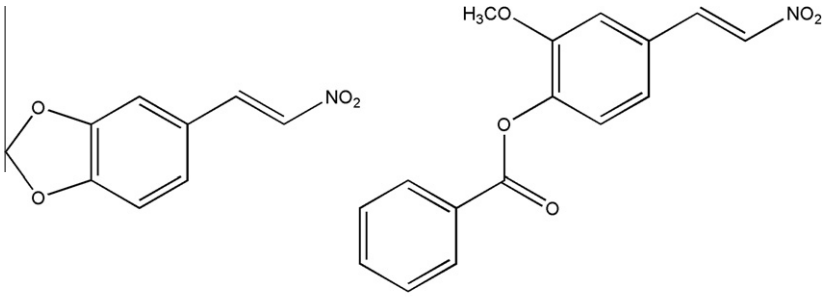
[a] Platelets were pre-incubated with DMSO (0.5%, control) or test compounds at 37 °C for 3 min before addition of the inducers.
[b] IC_{50} represents the 50% inhibitory concentration. Results represent the means from two independent experiments.
[c] Compounds were tested against the human breast cancer cell line, MDA-MB-231 or the non-malignant breast epithelial cell line, HBL-100 using the MTT method. The percentage of inhibition obtained at a test concentration of 20 μM is given in parentheses (%Inh).
[d] The biological data of compounds **45–47** were came from literature directly.[12]
[e] None test.



Scheme 1. The straightforward synthesis of BMNS analogues (**1–30**).



Scheme 2. Reagents and conditions: (a) nitromethane, ammonium acetate, reflux, 24 h; (b) nitroethane, ammonium acetate, reflux, 24 h; (c) benzoyl chloride, DCM/pyridine (10:1), 24 h.



MNS BMNS

Figure 1. The chemical structures of MNS and BMNS.

Table 2 shows that β-methyl substituted nitrostyrenes (**35**, **36**, **37**, and **38**) were more potent than β-nitrostyrenes (**31**, **32**, **33**, and **34**) in inhibiting the viability of MDA-MB-231 cells. These findings conflict with those report where β-methyl substitution reduced the reactivity of the nitroolefinic group with sulfhydryl and, suggests another mechanism for the action of β-methyl substituted nitrostyrenes. In addition, we also tested the cytotoxicity of the β-nitrostyrenes in a non-malignant breast epithelial cell line HBL-100. As shown in Tables 1 and 2, the cytotoxic activities of most of these compounds in HBL-100 epithelial cells were less than or equal to that observed in MDA-MB-231 cancer cells, except compounds **11**, **12**, **39**, **41**, **42**, and **49**.

2.3. Anti-platelet activity

All β-nitrostyrenes (**1**–**49**) were subjected to an anti-platelet aggregation assay with collagen (Col) and thrombin (Thr) as inducers. The results showed that in most cases, the mono- and di-substitutions on the B ring of BMNS led to increased anti-platelet activity and decreased cytotoxic activity (Table 1). Of these, compounds **2**, **13**, **19**, **24**, and **25** exhibited dual inhibitory effects on Thr- and Col-induced platelet aggregation with IC_{50} values of 1.0 and 0.6, 0.9 and 0.8, 0.6 and 0.6, 0.7 and 0.6, as well as 0.7 and 0.7 μM, respectively.

The anti-platelet aggregation assay data and SAR analysis confirmed that activity depended on the presence of the B ring. Furthermore, most chloro-substitution(s) at the B ring were more favorable than others. However, difluoro derivatives were unfavorable compared with mono-fluoro substituted derivatives (Table 1). Conversely, exchanging the substituents at C-3 and C-4, or replacing methoxyl with an ethoxyl group at C-3 slightly enhanced the anti-platelet aggregation activity. In addition, substitution at the β position with a methyl group did not affect anti-platelet activity (Table 2). On the other hand, analysis of the anti-platelet data of MNS analogs (Table 2) showed that activity depended on the hydrophobicity of the substitution at C-4. Compared with the data of compounds **33**, **34**, and **39**, with a hydroxyl at C-4, more hydrophobic groups at C-3 showed decreased activities, however, this phenomenon was reversed by replacing a hydroxyl with a methoxyl group at C-4 (**34** and **46**). Moreover, when the A ring was modified with a five member ring, for example, furan or thiophene, activity was also diminished (Table 2).

Because compounds **19** and **24** exhibited potent dual inhibitory effects on platelet aggregation induced by Thr and Col, which both are critical for thrombosis, and no cytotoxicity was found, these compounds were chosen for further studies on the mechanisms involved in the anti-platelet effect. Protein kinase C activation and $[Ca^{2+}]_i$ rise are the major signaling pathways involved in the induction of platelet aggregation. In the present study, the calcium ionophore A23187 (500 nM) and the protein kinase C (PKC) activator tetradecanoyl phorbol acetate (TPA, 200 nM), were used to directly induce platelet aggregation without receptor activation. Both **19** and **24** completely prevented A23187-induced platelet aggregation with IC_{50} values of 1.1 ± 0.2 and 3.3 ± 0.1 μM, respectively (n = 3). In a similar manner, **19** and **24** also abolished TPA-induced platelet aggregation with IC_{50} values of 3.5 ± 0.6 and 5.3 ± 0.4 μM, respectively (n = 3). These results suggested that **19** and **24** inhibited platelet aggregation by acting at sites downstream of the Ca^{2+}/PKC pathway. We thus examined the effects of **19** and **24** on the activation of GPIIb/IIIa, which represents the final common pathway for platelet aggregation regardless of the stimulus used. In the present study, we used flow cytometry and FITC-conjugated PAC-1 monoclonal antibody, which only binds to the activated form of GPIIb/IIIa, to determine whether the β-nitrostyrene derivatives were able to prevent GPIIb/IIIa activation. As shown in Figure 2, BMNS, **19**, and **24** inhibited thrombin-induced PAC-1 binding in a concentration-dependent manner. Furthermore, the rank order of potency of these compounds in preventing GPIIb/IIIa activation (**19** > **24** > BMNS) was correlated with their ability to inhibit platelet aggregation, suggesting that the β-nitrostyrene derivatives inhibited platelet aggregation by preventing GPIIb/IIIa activation.

3. Conclusion

In this study, we synthesized approximately forty β-nitrostyrene derivatives and compared their anti-platelet and cytotoxic activities. The analysis of structure–activity relationship revealed that substitutions on ring B of BMNS improved the anti-platelet activity and decreased the cytotoxic activity. Alteration of the

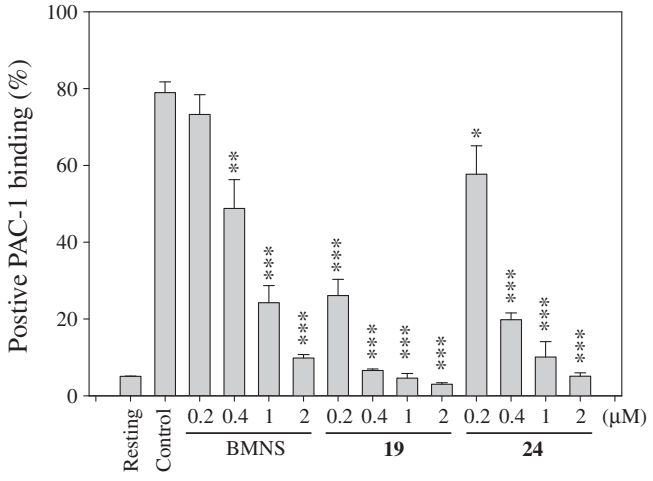


Figure 2. Effects of BMNS analogs on platelet GPIIb/IIIa activation analyzed by flow cytometry as described in Materials and Methods. Results are presented as mean ± S.E.M. (n = 3). *P <0.05, **P <0.01, ***P <0.001 as compared with the control.

bioactivity of BMNS derivatives implied that a more specific mechanism might be involved in the anti-platelet effect. Our study has demonstrated that the BMNS derivatives **19** and **24** inhibited platelet aggregation via prevention of GPIIb/IIIa activation. The potent and novel effects of BMNS derivatives make them attractive candidates for the development of new anti-platelet agents.

4. Experimental

4.1. General

The NMR spectra using $CDCl_3$ or DMSO-d_6 as solvents were obtained on a Bruker AVANCE-400 MHz FT-NMR spectrometer. Chemical shifts were internally referenced to the solvent signals in TMS. Low-resolution EI-MS were recorded on a Quattro GC/MS spectrometer having a direct inlet system, low-resolution and high-resolution ESI-MS spectra on a Bruker Daltonics APEX II 30e spectrometer.

4.2. General procedure for synthesis of β-nitrostyrene benzoyl esters (1–32, and 35–36)

To a mixture solution of β-nitrostyrenes (1.0 mmol) in DCM/pyridine (10:1) was added the suitable benzoyl chlorides (2.0 mmol). The reaction mixture was stirred at room temperature for 24 h. The solvent was evaporated at reduced pressure. The residue was purified by flash column chromatography (Si-Gel) to afford the products.

4.3. General procedure for synthesis of compounds 33–34 and 37–38

The synthetic method as described by Milhazes et al.[8] Briefly, compound 3-hydroxy-4-methoxybenzaldehyde or 3-ethoxy-4-hydroxybenzaldehyde (1.0 mmol) dissolved in nitromethane or nitroethane (3.0 ml), respectively, were added ammonium acetate (1.0 mmol). The reaction mixture was reflux for 24 h. The solvent was evaporated at reduced pressure. The residue was purified by column chromatography (Si-Gel) using solution of $CHCl_3$/hexane (3:4) or acetone/hexane (6:1) mixture to afford the products.

4.4. (*E*)-2-Methoxy-4-(2-nitrovinyl)phenyl 2-fluorobenzoate (1)

Yield (17%) from *trans*-4-hydroxy-3-methoxy-β-nitrostyrene (Aldrich) and 2-fluorobenzoyl chloride (Aldrich). Mp 120–122 °C; ^{1}H NMR ($CDCl_3$) δ 7.98 (1H, d, J = 13.6 Hz, H-α), 7.58 (1H, d, J = 13.6 Hz, H-β), 7.14 (1H, br s, H-2), 7.25 (1H, J = 8.4 Hz, H-5), 7.21 (1H, dd, J = 1.6, 8.4 Hz, H-6), 7.21 (1H, m, H-3′), 7.62 (1H, m, H-4′), 7.29 (1H, t, J = 8.0 Hz, H-5′), 8.12 (1H, td, J = 1.2, 8.0 Hz, H-6′), 3.87 (3H, s, OCH_3). ^{13}C NMR ($CDCl_3$) δ 138.9 (d, C-α), 137.7 (d, C-β), 129.5 (s, C-1), 112.6 (d, C-2), 143.3 (s, C-3), 152.3 (s, C-4), 124.4 (d, C-5), 123.0 (d, C-6), 117.8 (s, J_{C-F} = 13 Hz, C-1′), 162.8 (s, J_{C-F} = 160 Hz, C-2′), 117.7 (d, J_{C-F} = 22 Hz, C-3′), 136.0 (d, J_{C-F} = 9 Hz, C-4′), 133.1 (d, C-5′), 124.7 (d, J_{C-F} = 4 Hz, C-6′), 162.1 (s, C=O), 56.5 (q, OCH_3). ESI-MS (m/z, %): 340 $[M+Na]^+$ (100). HRES-I-MS m/z 340.0596 $[M+Na]^+$ (calcd for $C_{16}H_{12}FNO_5Na$ 340.0597).

4.5. (*E*)-2-Methoxy-4-(2-nitrovinyl)phenyl 2-(trifluoromethyl)-benzoate (4)

Yield (77%) from *trans*-4-hydroxy-3-methoxy-β-nitrostyrene (Aldrich) and 2-(trifluoromethyl)benzoyl chloride (Aldrich). Mp 158–160 °C; ^{1}H NMR ($CDCl_3$) δ 7.99 (1H, d, J = 13.6 Hz, H-α), 7.57 (1H, d, J = 13.6 Hz, H-β), 7.14 (1H, br s, H-2), 7.27 (1H, J = 8.0 Hz, H-5), 7.22 (1H, br.d, J = 8.0 Hz, H-6), 7.84 (1H, m, H-3′), 7.70 (2H, m, H-4′, H′-5), 8.12 (1H, m, H-6′), 3.91 (3H, s, OCH_3). ^{13}C NMR ($CDCl_3$) δ 138.7 (d, C-α), 137.8 (d, C-β), 129.7 (s, C-1), 112.7 (d, C-2), 143.0 (s, C-3), 152.4 (s, C-4), 124.1 (d, C-5), 122.9 (d, C-6), 127.3 (s, J_{C-F} = 5 Hz, C-1′), 129.4 (s, J_{C-F} = 33 Hz, C-2′), 127.4 (d, J_{C-F} = 5 Hz, C-3′), 132.4 (d, C-4′), 132.3 (d, C-5′), 131.3 (d, C-6′), 164.2 (s, C=O), 56.5 (q, OCH_3), 123.7 (s, J_{C-F} = 272 Hz, CF_3). EI-MS (m/z, %): 367 $[M]^+$ (8), 173 (100), 145 (96). HRESI-MS m/z 368.0748 $[M+H]^+$ (calcd for $C_{17}H_{13}F_3NO_5$ 368.0746).

4.6. (*E*)-2-Methoxy-4-(2-nitrovinyl)phenyl 3-bromobenzoate (9)

Yield (89%) from *trans*-4-hydroxy-3-methoxy-β-nitrostyrene (Aldrich) and 3-bromobenzoyl chloride (Aldrich). Mp 170–172 °C; ^{1}H NMR ($CDCl_3$) δ 8.00 (1H, d, J = 14.0 Hz, H-α), 7.58 (1H, d, J = 14.0 Hz, H-β), 7.14 (1H, br s, H-2), 7.25 (1H, J = 8.8 Hz, H-5), 7.21 (1H, dd, J = 1.6, 8.8 Hz, H-6), 8.34 (1H, t, J = 1.6 Hz, H-2′), 7.78 (1H, br.d, J = 8.0 Hz, H-4′), 7.40 (1H, t, J = 8.0 Hz, H′-5), 8.13 (1H, br.d, J = 8.0 Hz, H-6′), 3.87 (3H, s, OCH_3). ^{13}C NMR ($CDCl_3$) δ 138.8 (d, C-α), 137.7 (d, C-β), 129.4 (s, C-1), 112.6 (d, C-2), 143.3 (s, C-3), 152.3 (s, C-4), 124.3 (d, C-5), 122.9 (d, C-6), 131.2 (s, C-1′), 133.7 (d, C-2′), 123.1 (s, C-3′), 137.2 (d, C-4′), 130.6 (d, C-5′), 129.6 (d, C-6′), 163.4 (s, C=O), 56.5 (q, OCH_3). ESI-MS (m/z, %): 400 $[M+Na]^+$ (25), 381 (100). HRESI-MS m/z 399.9798 $[M+Na]^+$, (calcd for $C_{16}H_{12}BrNO_5Na$ 399.9797).

4.7. (*E*)-2-Methoxy-4-(2-nitrovinyl)phenyl 3-(trifluoromethyl)-benzoate (10)

Yield (67%) from *trans*-4-hydroxy-3-methoxy-β-nitrostyrene (Aldrich) and 3-(trifluoromethyl)benzoyl chloride (Alfa Aesar). Mp 155–157 °C; ^{1}H NMR ($CDCl_3$) δ 8.00 (1H, d, J = 13.6 Hz, H-α), 7.59 (1H, d, J = 13.6 Hz, H-β), 7.15 (1H, d, J = 1.6 Hz, H-2), 7.26 (1H, J = 8.0 Hz, H-5), 7.23 (1H, dd, J = 1.6, 8.0 Hz, H-6), 8.47 (1H, br s, H-2′), 7.92 (1H, br.d, J = 8.0 Hz, H-4′), 7.68 (1H, t, J = 8.0 Hz, H-5′), 8.39 (1H, br.d, J = 8.0 Hz, H-6′), 3.88 (3H, s, OCH_3). ^{13}C NMR ($CDCl_3$) δ 138.7 (d, C-α), 137.8 (d, C-β), 129.8 (s, C-1), 112.7 (d, C-2), 143.2 (s, C-3), 152.3 (s, C-4), 124.3 (d, C-5), 122.9 (d, C-6), 130.2 (s, C-1′), 127.7 (d, J_{C-F} = 4 Hz, C-2′), 131.8 (s, J_{C-F} = 33 Hz, C-3′), 130.7 (d, J_{C-F} = 3 Hz, C-4′), 129.7 (d, C-5′), 134.0 (d, C-6′), 163.4 (s, C=O), 56.5 (q, OCH_3), 124.0 (s, J_{C-F} = 270 Hz, CF_3). EI-MS (m/z, %): 367 $[M]^+$ (19), 173 (100), 145 (95). HRESI-MS m/z 368.0750 $[M+H]^+$ (calcd for $C_{17}H_{13}F_3NO_5$ 368.0746).

4.8. (*E*)-2-Methoxy-4-(2-nitrovinyl)phenyl 4-fluorobenzoate (13)

Yield (37%) from *trans*-4-hydroxy-3-methoxy-β-nitrostyrene (Aldrich) and 4-fluorobenzoyl chloride (Aldrich) Mp 186–188 °C; ^{1}H NMR ($CDCl_3$) δ 8.03 (1H, d, J = 13.6 Hz, H-α), 7.60 (1H, d, J = 13.6 Hz, H-β), 7.16 (1H, br s, H-2), 7.25 (1H, d, J = 8.8 Hz, H-5), 7.21 (1H, br.d, J = 8.8 Hz, H-6), 8.25 (2H, dd, J = 5.6, 8.8 Hz, H-2′,6′), 7.23 (2H, m, H-3′,5′), 3.90 (3H, s, OCH_3). ^{13}C NMR ($CDCl_3$) δ 138.9 (d, C-α), 137.7 (d, C-β), 129.4 (s, C-1), 112.6 (d, C-2), 143.5 (s, C-3), 152.4 (s, C-4), 124.4 (d, C-5), 123.0 (d, C-6), 118.5 (s, C-1′), 133.5 (d, J_{C-F} = 11 Hz, C-2′, 6′), 116.3 (d, J_{C-F} = 11 Hz, C-3′, 5′), 166.3 (s, J_{C-F} = 210 Hz, C-4′), 163.8 (s, C=O), 56.5 (q, OCH_3). ESI-MS (m/z, %): 340 $[M+Na]^+$ (100). HRESI-MS m/z 340.0599 $[M+Na]^+$ (calcd for $C_{16}H_{12}FNO_5Na$ 340.0597).

4.9. (*E*)-2-Methoxy-4-(2-nitrovinyl)phenyl 4-(trifluoromethyl)-benzoate (16)

Yield (79%) from *trans*-4-hydroxy-3-methoxy-β-nitrostyrene (Aldrich) and 4-(trifluoromethyl)benzoyl chloride (Alfa Aesar). Mp 197–199 °C; ^{1}H NMR ($CDCl_3$) δ 8.01 (1H, d, J = 14.0 Hz, H-α), 7.59 (1H, d, J = 14.0 Hz, H-β), 7.15 (1H, d, J = 1.2 Hz, H-2), 7.27 (1H, d, J = 8.0 Hz, H-5), 7.23 (1H, dd, J = 1.2, 8.0 Hz, H-6), 8.33

(2H, d, J = 8.0 Hz, H-2′,6′), 7.80 (2H, d, J = 8.0 Hz, H-3′,5′), 3.88 (3H, s, OCH_3). ^{13}C NMR ($CDCl_3$) δ 138.7 (d, C-α), 137.8 (d, C-β), 129.7 (s, C-1), 112.6 (d, C-2), 143.2 (s, C-3), 152.3 (s, C-4), 124.3 (d, C-5), 122.9 (d, C-6), 118.5 (s, C-1′), 131.2 (d, C-2′, 6′), 126.1 (d, J_{C-F} = 3 Hz, C-3′, 5′), 135.7 (s, J_{C-F} = 32 Hz, C-4′), 163.5 (s, C=O), 56.5 (q, OCH_3), 123.9 (s, J_{C-F} = 271 Hz, CF_3). EI-MS (m/z, %): 367 $[M]^+$ (4), 337 (5), 173 (100), 145 (61). HREI-MS m/z 368.0748 $[M+H]^+$ (calcd for $C_{17}H_{13}F_3NO_5$ 368.0746).

4.10. (*E*)-2-Methoxy-4-(2-nitrovinyl)phenyl 2,3-difluorobenzoate (20)

Yield (50%) from *trans*-4-hydroxy-3-methoxy-β-nitrostyrene (Aldrich) and 2,3-difluorobenzoyl chloride (Aldrich). Mp 167–169 °C; ^1H NMR ($CDCl_3$) δ 7.99 (1H, d, J = 13.6 Hz, H-α), 7.55 (1H, d, J = 13.6 Hz, H-β), 7.15 (1H, br s, H-2), 7.25 (1H, d, J = 8.0 Hz, H-5), 7.22 (1H, br.d, J = 8.0 Hz, H-6), 7.25 (1H, m, H-4′), 7.46 (1H, br.q, J = 8.0 Hz, H-5′), 7.88 (1H, br.t, J = 8.0 Hz, H-6′), 3.88 (3H, s, OCH_3). ^{13}C NMR ($CDCl_3$) δ 138.3 (d, C-α), 137.3 (d, C-β), 129.3 (s, C-1), 112.3 (d, C-2), 142.6 (s, C-3), 151.8 (s, C-4), 123.8 (d, C-5), 122.5 (d, C-6), 119.7 (s, J_{C-F} = 40 Hz, C-1′), 151.1 (s, J_{C-F} = 228 Hz, C-2′, 6′), 151.3 (s, J_{C-F} = 249 Hz, C-3′), 122.4 (d, J_{C-F} = 21 Hz, C-4′), 124.0 (d, J_{C-F} = 5 Hz, C-5′), 127.2 (d, J_{C-F} = 3 Hz, C-6′), 160.9 (s, C=O), 56.1 (q, OCH_3). EI-MS (m/z, %): 335 $[M]^+$ (44), 141 (100), 113 (94). HRESI-MS m/z 336.0683 $[M+H]^+$ (calcd for $C_{16}H_{12}F_2NO_5$ 336.0684).

4.11. (*E*)-2-Methoxy-4-(2-nitrovinyl)phenyl 2,4-difluorobenzoate (21)

Yield (28%) from *trans*-4-hydroxy-3-methoxy-β-nitrostyrene (Aldrich) and 2,4-difluorobenzoyl chloride (Aldrich). Mp 170–172 °C; ^1H NMR ($CDCl_3$) δ 7.99 (1H, d, J = 13.6 Hz, H-α), 7.57 (1H, d, J = 13.6 Hz, H-β), 7.14 (1H, d, J = 1.2 Hz, H-2), 7.25 (1H, d, J = 8.0 Hz, H-5), 7.21 (1H, dd, J = 1.2, 8.0 Hz, H-6), 7.00 (1H, m, H-3′), 6.96 (1H, m, H-5′), 8.16 (1H, br.t, J = 8.4 Hz, H-6′), 3.88 (3H, s, OCH_3). ^{13}C NMR ($CDCl_3$) δ 138.3 (d, C-α), 137.3 (d, C-β), 129.2 (s, C-1), 112.3 (d, C-2), 142.8 (s, C-3), 151.9 (s, C-4), 123.9 (d, C-5), 122.5 (d, C-6), 112.2 (s, J_{C-F} = 24 Hz, C-1′), 162.0 (s, J_{C-F} = 210 Hz, C-2′, 6′), 105.6 (d, J_{C-F} = 26 Hz, C-3′), 165.2 (d, J_{C-F} = 280 Hz, C-4′), 111.9 (d, J_{C-F} = 25 Hz, C-5′), 134.6 (d, J_{C-F} = 10 Hz, C-6′), 160.8 (s, C=O), 56.1 (q, OCH_3). EI-MS (m/z, %): 335 $[M]^+$ (14), 141 (100), 113 (80). HRESI-MS m/z 336.0682 $[M+H]^+$ (calcd for $C_{16}H_{12}F_2NO_5$ 336.0684).

4.12. (*E*)-2-Methoxy-4-(2-nitrovinyl)phenyl 3,4-difluorobenzoate (22)

Yield (48%) from *trans*-4-hydroxy-3-methoxy-β-nitrostyrene (Aldrich) and 3,4-difluorobenzoyl chloride (Aldrich). Mp 207–209 °C; ^1H NMR ($CDCl_3$) δ 8.00 (1H, d, J = 13.6 Hz, H-α), 7.58 (1H, d, J = 13.6 Hz, H-β), 7.14 (1H, br s, H-2), 7.25 (1H, d, J = 8.4 Hz, H-5), 7.22 (1H, dd, J = 1.6, 8.0 Hz, H-6), 7.99 (1H, m, H-2′), 7.31 (1H, br.q, J = 8.4 Hz, H-5′), 8.02 (1H, m, H-6′), 3.88 (3H, s, OCH_3). EI-MS (m/z, %): 335 $[M]^+$ (37), 141 (100), 113 (97). HRESI-MS m/z 336.0683 $[M+H]^+$ (calcd for $C_{16}H_{12}F_2NO_5$ 336.0684).

4.13. (*E*)-2-Methoxy-4-(2-nitrovinyl)phenyl 3,5-difluorobenzoate (23)

Yield (47%) from *trans*-4-hydroxy-3-methoxy-β-nitrostyrene (Aldrich) and 3,5-difluorobenzoyl chloride (Alfa Aesar). Mp 206–208 °C; ^1H NMR ($CDCl_3$) δ 8.00 (1H, d, J = 13.6 Hz, H-α), 7.58 (1H, d, J = 13.6 Hz, H-β), 7.14 (1H, br s, H-2), 7.25 (1H, d, J = 8.0 Hz, H-5), 7.22 (1H, dd, J = 1.6, 8.0 Hz, H-6), 7.73 (2H, m, H-2′, 6′), 7.09 (1H, m, H-4′), 3.88 (3H, s, OCH_3). ^{13}C NMR (DMSO-d_6) δ 138.5 (d, C-α), 138.4 (d, C-β), 129.8 (s, C-1), 113.2 (d, C-2), 141.8 (s, C-3), 151.1 (s, C-4), 123.6 (d, C-5), 123.5 (d, C-6), 131.7 (s, J_{C-F} = 2 Hz, C-1′), 113.1 (d, J_{C-F} = 24 Hz, C-2′, 6′), 162.4 (s, J_{C-F} = 247 Hz, C-3′), 109.9 (d, J_{C-F} = 26 Hz, C-4′), 162.3 (s, J_{C-F} = 248 Hz, C-5′), 161.6 (s, C=O), 56.3 (q, OCH_3). EI-MS (m/z, %): 335 $[M]^+$ (44), 141 (100), 113 (84). HRESI-MS m/z 336.0682 $[M+H]^+$ (calcd for $C_{16}H_{12}F_2NO_5$ 336.0684).

4.14. (*E*)-2-Methoxy-4-(2-nitrovinyl)phenyl 2,3-dichlorobenzoate (24)

Yield (85%) from *trans*-4-hydroxy-3-methoxy-β-nitrostyrene (Aldrich) and 2,3-dichlorobenzoyl chloride (Alfa Aesar). Mp 132–134 °C; ^1H NMR ($CDCl_3$) δ 7.99 (1H, d, J = 13.6 Hz, H-α), 7.55 (1H, d, J = 13.6 Hz, H-β), 7.15 (1H, br s, H-2), 7.27 (1H, d, J = 8.0 Hz, H-5), 7.21 (1H, br.d, J = 8.0 Hz, H-6), 7.69 (1H, d, J = 8.0 Hz, H-4′), 7.35 (1H, t, J = 8.0 Hz, H-5′), 7.88 (1H, d, J = 8.0 Hz, H-6′), 3.90 (3H, s, OCH_3). ^{13}C NMR ($CDCl_3$) δ 138.2 (d, C-α), 137.3 (d, C-β), 129.2 (s, C-1), 112.2 (d, C-2), 142.6 (s, C-3), 151.7 (s, C-4), 123.7 (d, C-5), 122.5 (d, C-6), 132.4 (s, C-1′), 131.1 (s, C-2′), 134.9 (s, C-3′), 134.0 (d, C-4′), 127.2 (d, C-5′), 129.8 (d, C-6′), 162.5 (s, C=O), 56.1 (q, OCH_3). ESI-MS (m/z, %): 390 $[M+Na]^+$ (93), 368 $[M+H]^+$ (64). HRESI-MS m/z 389.9914 $[M+Na]^+$ (calcd for $C_{16}H_{11}Cl_2NO_5Na$ 389.9912).

4.15. (*E*)-2-Methoxy-4-(2-nitrovinyl)phenyl 3,5-dichlorobenzoate (27)

Yield (68%) from *trans*-4-hydroxy-3-methoxy-β-nitrostyrene (Aldrich) and 3,5-dichlorobenzoyl chloride (Aldrich). Mp 244–245 °C; ^1H NMR ($CDCl_3$) δ 8.00 (1H, d, J = 13.6 Hz, H-α), 7.57 (1H, d, J = 13.6 Hz, H-β), 7.14 (1H, br s, H-2), 7.25 (2H, br.d, J = 10.8 Hz, H-5, 6), 8.07 (2H, br s, H-2′, 6′), 7.64 (1H, s, H-4′), 3.88 (3H, s, OCH_3). ESI-MS (m/z, %): 390 $[M+Na]^+$ (25). HRESI-MS m/z 389.9909 $[M+Na]^+$ (calcd for $C_{16}H_{11}Cl_2NO_5Na$ 389.9912).

4.16. (*E*)-2-Methoxy-4-(2-nitrovinyl)phenyl 2,4,5-trifluorobenzoate (28)

Yield (42%) from *trans*-4-hydroxy-3-methoxy-β-nitrostyrene (Aldrich) and 2,4,5-trifluorobenzoyl chloride (Aldrich). Mp 229–231 °C; ^1H NMR ($CDCl_3$) δ 8.00 (1H, d, J = 13.6 Hz, H-α), 7.58 (1H, d, J = 13.6 Hz, H-β), 7.14 (1H, br s, H-2), 7.25 (1H, d, J = 8.8 Hz, H-5), 7.22 (1H, dd, J = 1.2, 8.8 Hz, H-6), 7.85 (2H, br.t, J = 6.8 Hz, H-3′, 6′), 3.88 (3H, s, OCH_3). ^{13}C NMR (DMSO-d_6) δ 138.5 (d, C-α), 138.5 (d, C-β), 129.8 (s, C-1), 113.2 (d, C-2), 141.8 (s, C-3), 151.1 (s, C-4), 123.6 (d, C-5), 123.5 (d, C-6), 124.7 (s, C-1′), 150.4 (s, J_{C-F} = 236 Hz, C-2′), 103.8 (d, C-3′), 150.4 (s, J_{C-F} = 236 Hz, C-4′), 139.8 (s, J_{C-F} = 278 Hz, C-5′), 115.1 (d, J_{C-F} = 23 Hz, C-6′), 161.2 (s, C=O), 56.3 (q, OCH_3). EI-MS (m/z, %): 353 $[M]^+$ (32), 159 (100), 131 (86). HRESI-MS m/z 354.0587 $[M+H]^+$ (calcd for $C_{16}H_{11}F_3NO_5Na$ 354.0589).

4.17. (*E*)-2-Methoxy-4-(2-nitrovinyl)phenyl 3,4,5-trifluorobenzoate (29)

Yield (73%) from *trans*-4-hydroxy-3-methoxy-β-nitrostyrene (Aldrich) and 3,4,5-trifluorobenzoyl chloride (Aldrich). Mp 183–185 °C; ^1H NMR ($CDCl_3$) δ 8.00 (1H, d, J = 13.6 Hz, H-α), 7.58 (1H, d, J = 13.6 Hz, H-β), 7.14 (1H, d, J = 1.2 Hz, H-2), 7.25 (1H, d, J = 8.8 Hz, H-5), 7.22 (1H, dd, J = 1.2, 8.8 Hz, H-6), 7.96 (1H, m, H-2′), 7.08 (1H, m, H-6′), 3.89 (3H, s, OCH_3). ^{13}C NMR ($CDCl_3$) δ 138.2 (d, C-α), 137.4 (d, C-β), 129.4 (s, C-1), 112.2 (d, C-2), 142.4 (s, C-3), 152.6 (s, C-4), 123.7 (d, C-5), 122.5 (d, C-6), 120.5 (s, J_{C-F} = 28 Hz, C-1′), 107.4 (d, J_{C-F} = 38 Hz, C-2′), 151.1 (s, J_{C-F} = 278 Hz, C-3′, 5′), 143.9 (s, J_{C-F} = 298 Hz, C-4′), 107.4 (d, J_{C-F} = 38 Hz, C-6′), 159.9 (s, C=O), 56.1 (q, OCH_3). EI-MS (m/z, %): 353 $[M]^+$ (22), 159 (100), 131 (73). HRESI-MS m/z 354.0586 $[M+H]^+$ (calcd for $C_{16}H_{11}F_3NO_5$ 354.0589).

4.18. (*E*)-2-Ethoxy-4-(2-nitrovinyl)phenyl benzoate (31)

Yield (55%) from **33** and benzoyl chloride (Aldrich). Mp 134–135 °C; ^{1}H NMR ($CDCl_3$) δ 7.96 (1H, d, J = 13.6 Hz, H-α), 7.56 (1H, d, J = 13.6 Hz, H-β), 7.12 (1H, d, J = 1.2 Hz, H-2), 7.23 (1H, d, J = 8.0 Hz, H-5), 7.17 (1H, dd, J = 1.2, 8.0 Hz, H-6), 8.12 (2H, d, J = 7.6 Hz, H-2′, 6′), 7.51 (2H, t, J = 7.6 Hz, H-3′, 5′), 7.65 (1H, t, J = 7.6 Hz, H-4′), 4.07 (2H, q, J = 6.8 Hz, OCH_2CH$_3$), 1.31 (3H, t, J = 6.8 Hz, OCH$_2$$CH_3$). ^{13}C NMR ($CDCl_3$) δ 138.5 (d, C-α), 137.0 (d, C-β), 128.9 (s, C-1), 113.3 (d, C-2), 143.5 (s, C-3), 151.3 (s, C-4), 123.8 (d, C-5), 122.4 (d, C-6), 128.7 (s, C-1′), 130.2 (d, C-2′, 6′), 128.5 (d, C-3′, 5′), 133.7 (d, C-4′), 164.3 (s, C=O), 64.7 (t, OCH_2CH$_3$), 14.5 (q, OCH$_2$$CH_3$). ESI-MS ($m/z$, %): 336 [M+Na]$^+$ (100), 331 (85), 314 [M+H]$^+$ (19). HRESI-MS m/z 336.0847 [M+Na]$^+$ (calcd for $C_{17}H_{15}NO_5Na$ 336.08448).

4.19. (*E*)-2-Ethoxy-4-(2-nitroprop-1-enyl)phenyl benzoate (35)

Yield (12%) from **37** and benzoyl chloride (Aldrich). Mp 85–87 °C; ^{1}H NMR ($CDCl_3$) δ 8.07 (1H, s, H-α), 7.04 (1H, br s, H-2), 7.25 (1H, d, J = 8.4 Hz, H-5), 7.07 (1H, d, J = 8.4 Hz, H-6), 8.21 (2H, d, J = 7.6 Hz, H-2′, 6′), 7.52 (2H, t, J = 7.6 Hz, H-3′, 5′), 7.65 (1H, t, J = 7.6 Hz, H-4′), 4.07 (2H, q, J = 6.8 Hz, OCH_2CH$_3$), 1.32 (3H, t, J = 6.8 Hz, OCH$_2$$CH_3$), 2.48 (3H, β-CH$_3$). ^{13}C NMR ($CDCl_3$) δ 133.6 (d, C-α), 147.7 (d, C-β), 129.1 (s, C-1), 115.2 (d, C-2), 141.6 (s, C-3), 150.9 (s, C-4), 123.4 (d, C-5), 122.5 (d, C-6), 131.1 (s, C-1′), 130.2 (d, C-2′, 6′), 128.5 (d, C-3′, 5′), 133.0 (d, C-4′), 164.5 (s, C=O), 64.7 (t, OCH_2CH$_3$), 14.5 (q, OCH$_2$$CH_3$), 14.0 (q, β-CH$_3$). ESI-MS ($m/z$, %): 350 [M+Na]$^+$ (66), 328 [M+H]$^+$ (9). HRESI-MS m/z 350.1002 [M+Na]$^+$ (calcd for $C_{18}H_{17}NO_5Na$ 350.1004).

4.20. Preparation of washed human platelets

Human blood anticoagulated with acid citrate dextrose (ACD) was obtained from healthy human volunteers who had not taken any drugs within the last two weeks. The platelet suspension was then prepared according to the washing procedure previously described.[15] Platelets were finally suspended in Tyrode's solution containing Ca^{2+} (2 mM), glucose (11.1 mM) and bovine serum albumin (3.5 mg/ml) at a concentration of 3×10^8 platelets/ml.

4.21. Measurement of platelet aggregation[16]

Platelet aggregation was measured turbidimetrically with a light-transmission aggregometer (Chrono-Log Co., USA). The platelet suspension was incubated with dimethyl sulfoxide (DMSO, vehicle) or test compounds at 37 °C for 3 min under a stirring condition (1200 rpm) prior to the addition of the platelet aggregation inducers. The extent of platelet aggregation was measured as the maximal increase of light transmission within 5 min after the addition of inducers.

4.22. Measurement of PAC-1 binding by flow cytometry[12]

Washed human platelets (3×10^7 platelets/ml) were pre-incubated with DMSO or test compounds for 3 min, and then treated with or without thrombin in the presence of FITC-conjugated PAC-1 monoclonal antibody for 15 min at room temperature. The samples were then fixed with 1% paraformaldehyde. Flow cytometric analysis was performed on a Beckman Coulter EPICS XL flow cytometer with EXPO32 ADC software. Platelets were identified by logarithmic signal amplification for forward and side scatter. The levels of PAC-1 binding were expressed as the percentages of cells positive for PAC-1.

4.23. Cell viability assays[17]

Compounds were tested against MDA-MB-231 cells (a human breast cancer cell line) and HBL-100 cells (a non-malignant breast epithelial cell line) using the MTT method as described before.[17] Freshly trypsinized cell suspensions were seeded in 96 well microtiter plates at densities of 8000 cells per well with tested compounds added from DMSO-diluted stock. After 72 h in culture, attached cells were incubated with MTT (0.5 mg/mL, 1 h) and subsequently solubilized in DMSO for determining of IC_{50}. The cytotoxic activity of compounds was expressed as the concentration inhibiting cell growth by 50% (IC_{50}) calculated from the survival curves.

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Supplementary data

Supplementary data (additional information on compound purity, including HPLC analysis of the target compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.08.039.

References and notes

1. Franchini, M.; Mannucci, P. M. *Eur. J. Int. Med.* **2009**, *20*, 733.
2. Angiolillo, D. J.; Bhatt, D. L.; Gurbel, P. A.; Jennings, L. K. *Am. J. Cardiol.* **2009**, *103*, 40A.
3. De Meyer, S. F.; Vanhoorelbeke, K.; Broos, K.; Salles, I. I.; Deckmyn, H. *Br. J. Haematol.* **2008**, *142*, 515.
4. Mega, J. L.; Close, S. L.; Wiviott, S. D.; Shen, L.; Hockett, R. D.; Brandt, J. T.; Walker, J. R.; Antman, E. M.; Macias, W.; Braunwald, E.; Sabatine, M. S. *N. Engl. J. Med.* **2009**, *360*, 354.
5. Blue, R.; Murica, M.; Karan, C.; Jirouskova, M.; Coller, B. S. *Blood* **2008**, *111*, 1248.
6. Varon, D.; Spectre, G. *Hematology* **2009**, 267.
7. Pettit, R. K.; Pettit, G. R.; Hamel, E.; Hogan, F.; Moser, B. R.; Wolf, S.; Pon, S.; Chapuis, J. C.; Schmidt, J. M. *Bioorg. Med. Chem.* **2009**, *17*, 6606.
8. Milhazes, N.; Calheiros, R.; Marques, M. P. M.; Garrido, J.; Cordeiro, M. N. D. S.; Rodrigues, C.; Quinteira, S.; Novais, C.; Peixe, L.; Borges, F. *Bioorg. Med. Chem.* **2006**, *14*, 4078.
9. Kaap, S.; Quentin, I.; Tamiru, D.; Shaheen, M.; Eger, K.; Steinfelder, H. J. *Biochem. Pharmacol.* **2003**, *65*, 603.
10. Werner, J. M.; Eger, K.; Jürgen Steinfelder, H. *Apoptosis* **2007**, *12*, 235.
11. Carter, K. C.; Finnon, Y. S.; Daeid, N. N.; Robson, D. C.; Waddell, R. *Immunopharmacol. Immunotoxicol.* **2002**, *24*, 187.
12. Wang, W. Y.; Wu, Y. C.; Wu, C. C. *Mol. Pharmacol.* **2006**, *70*, 1380.
13. Wang, W. Y.; Hsieh, P. W.; Wu, Y. C.; Wu, C. C. *Biochem. Pharmacol.* **2007**, *74*, 601.
14. Gullner, G.; Cserhati, T.; Mikite, G. *Pest. Biochem. Physiol.* **1991**, *39*, 1.
15. Louis-Ferdinand, R. T.; Fuller, G. C. *J. Pharm. Sci.* **1969**, *58*, 1155.
16. Wu, C. C.; Wang, T. W.; Wang, W. Y.; Hsieh, P. W.; Wu, Y. C. *Eur. J. Pharmacol.* **2005**, *527*, 37.
17. Yen, C. T.; Wu, C. C.; Lee, J. C.; Chen, S. L.; Morris-Natschke, S. L.; Hsieh, P. W.; Wu, Y. C. *Eur. J. Med. Chem.* **2010**, *45*, 2494.