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SYNTHESIS AND BIOLOGICAL EVALUATION OF NEW CURCUMIN ANALOGS INHIBITING OSTEOCLASTOGENESIS

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Abstract – A series of curcumin analogs (**1-3**) were newly designed and synthesized for the development of therapeutic agents for osteoporosis. Among the synthesized compounds, 2,5-substituted conjugated thiophene derivative (**1a**) and the corresponding pyrazine derivative (**1c**) were shown to be potential leads for the development of anti-osteoclastogenesis agent.

INTRODUCTION

Osteoporosis, a metabolic bone disease common especially among postmenopausal women, demands urgent attention that should not be ignored.¹ Osteoporosis is affected by the balance between bone resorption with osteoclasts and ossification with osteoblasts. Therefore, compounds that can inhibit osteoclastogenesis are expected to serve as therapeutic agents for osteoporosis. In our research toward this challenging goal, we focused on curcumin, which has been reported to inhibit osteoclastogenesis induced by receptor activation of NF- κ B ligand (RANKL).² With interest in the distinctive conjugated π system of curcumin, from the perspective of the bioisosteres, we focused on curcumin analogs (**1-3**) in this study because detailed research on the chemical structure of curcumin remains to be conducted. Therefore, we decided to begin by investigating the effect of curcumin's diketone part on osteoclast differentiation and substituted aromatic rings such as thiophene or pyrazine for the diketone part based on the bioisosterism.³ We also examined the substituents of the terminal benzene rings. Furthermore, we estimated the importance of double bonds connected by two aromatic rings. The analogs (**1-3**) in this study were synthesized mainly by the Wittig reaction of dialdehydes with phosphonium salts under basic conditions. The biological evaluations of **1-3** were estimated by tartrate-resistant acid phosphatase (TRAP) assay, the most common method of detecting osteoclasts populations *in vitro*, and the XTT assay for assessing the cytotoxicity.

RESULTS AND DISCUSSION

The main compounds (**1-3**) in this study are summarized in Figure 1.

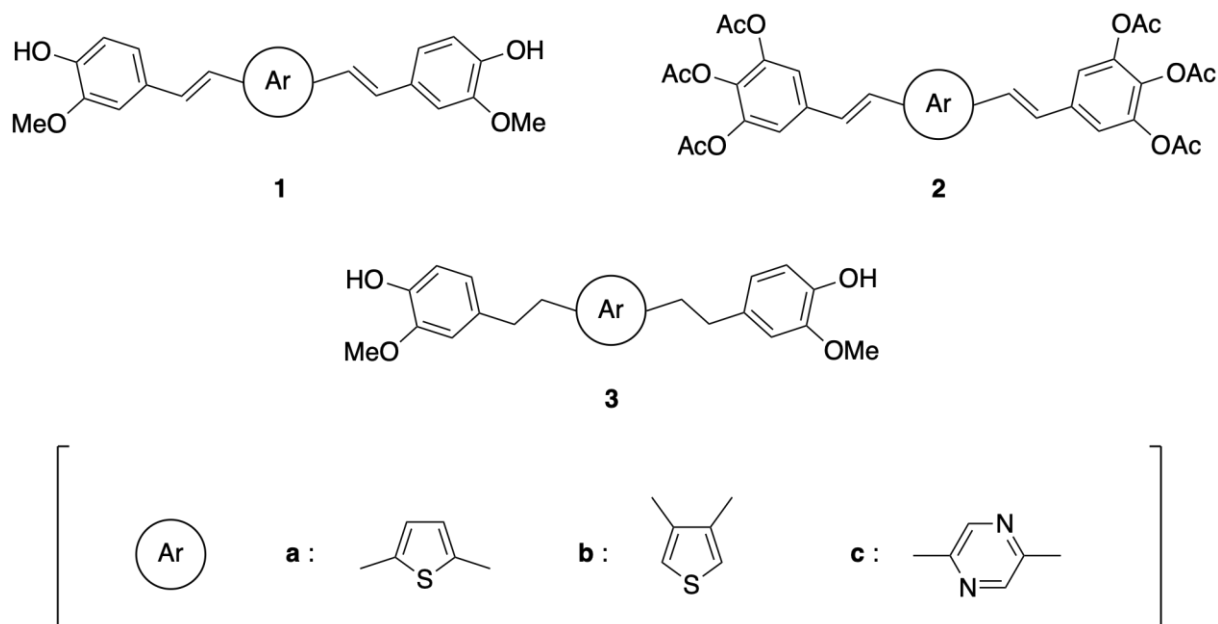


Figure 1. Target compounds (**1-3**) in this study

Dialdehydes and phosphonium salts are key intermediates for the synthesis of **1-3**. As shown in Figure 2, thiophene-2,5-dialdehyde (**4a**)⁴ and thiophene-3,4-dialdehyde (**4b**)⁴ were prepared from the corresponding dibromo derivatives according to the literature procedure, and pyrazine-2,5-dicarbaldehyde (**4c**) was prepared from the 2,5-dimethylpyridazine in four steps.⁵

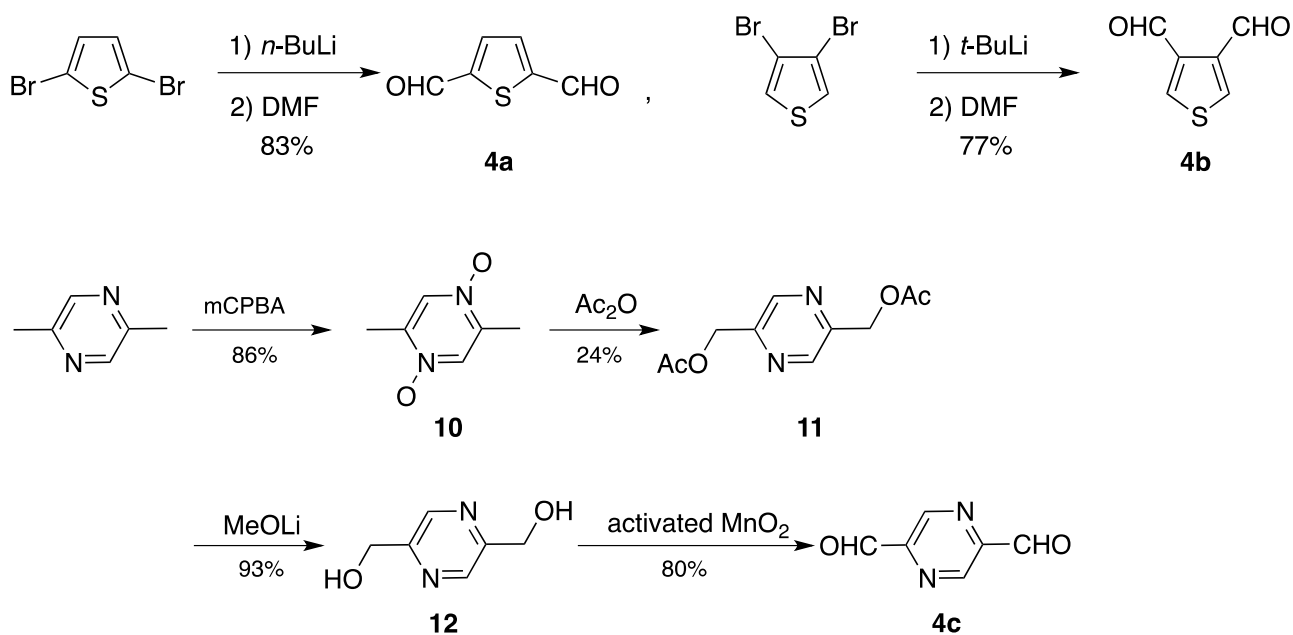


Figure 2. Preparation of dialdehydes (**4a-c**)

The synthesis of phosphonium bromides (**5** and **6**) is shown in Figure 3.⁶ Reaction of commercially available vanillin with *tert*-butyldiphenylchlorosilane (TBDPSCl) in the presence of imidazole gave TBDPS-protected aldehyde (**13**) in 89% yield. The reduction of **13** using sodium borohydride (NaBH₄) gave alcohol (**14**) in 94% yield. Bromination of **14**, followed by the reaction of the resulting bromide with triphenylphosphine (PPh₃), furnished the desired phosphonium bromide (**5**) in 88% in two steps. Phosphonium bromide (**6**) was prepared from the commercially available methyl gallate in higher yield according to a similar method used for the preparation of **5**.⁷

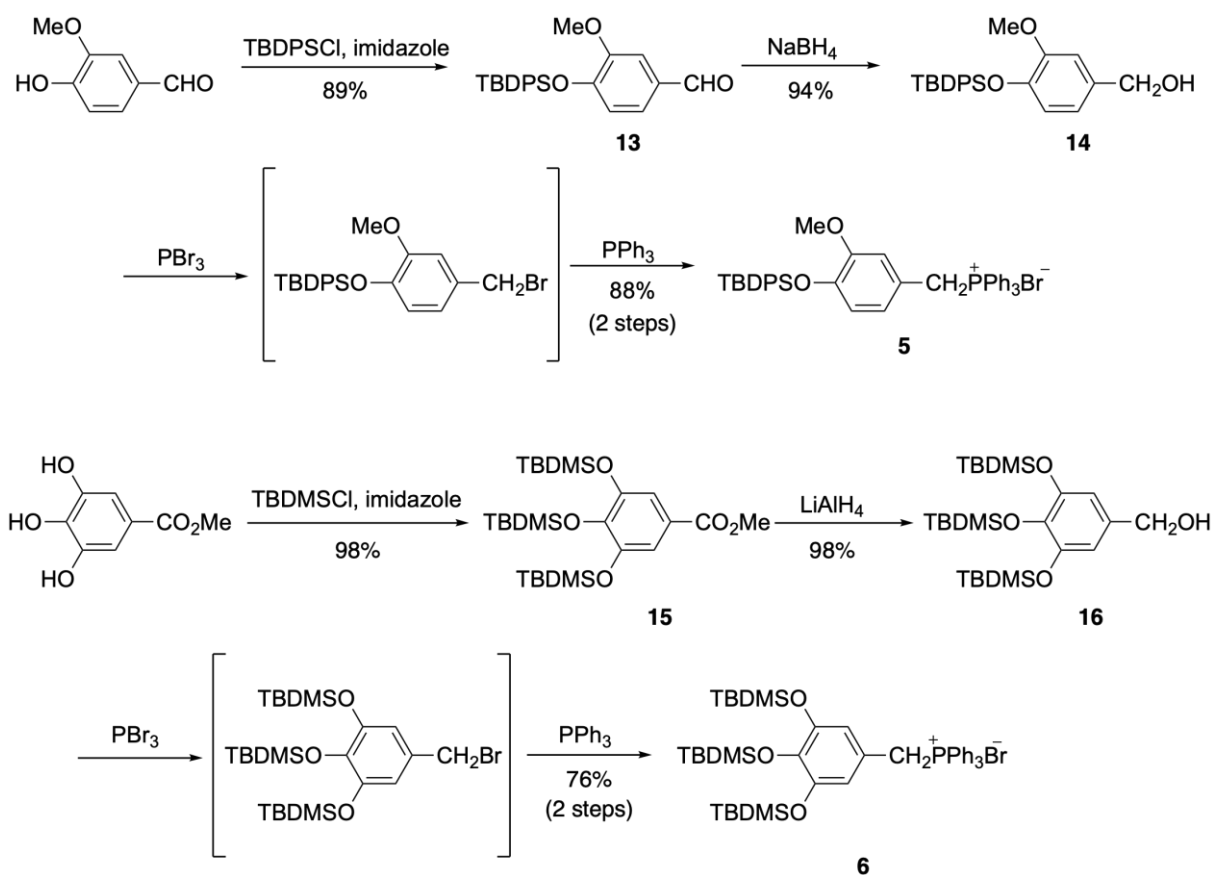


Figure 3. Preparation of phosphonium bromides (**5** and **6**)

As illustrated in Figure 4, the Wittig reaction of dialdehydes (**4a-c**) with phosphonium bromide (**5**) in the presence of potassium *tert*-butoxide (*t*-BuOK) and the following isomerization of alkene moieties, which were obtained as diastereomixture of (*Z,Z*)-, (*E,E*)-, and (*E,Z*)- isomer, gave the precursors of the target compounds (**7a-c**) in 70%, 41%, and 62% yields, respectively. The final configurations (*E, E*) of alkene parts in the isolated compounds were confirmed by ¹H-NMR (*J* = 16 Hz). Similarly, the Wittig reaction of **4a-c** with **6** under similar conditions gave the precursors of the target compounds (**8a-c**) in 86%, 76%, and 81% yields, respectively. The lower yields for the synthesis of **7b** and **8b** can be attributed to the inhibition of the Wittig reaction by the steric proximity between the 3-position of the thiophene ring and

its neighboring 4-position. Finally, the synthesis of **1a-c** was achieved in 90% (for **1a**), 94% (for **1b**), and 89% (for **1c**) yields by deprotection of the protecting group of **7a-c** by treatment with tetra-*n*-butylammonium fluoride (TBAF). Deprotection of the TBDMS group of **8a-c** in the presence of acetyl chloride (AcCl) led to the formation of compounds (**2a-c**) in 90% (for **2a**), 94% (for **2b**), and 86% (for **2c**) yields, respectively. Hydrogenation of **7a-c** over palladium catalyst (Pd/C) gave alkene-saturated derivatives (**9a-c**) in 54%, 67%, and 72% yields, respectively. By the treatment of **9a-c** with TBAF, the synthesis of **3a-c** was also achieved in 99% (for **3a**), 94% (for **3b**), and 93% (for **3c**) yields.

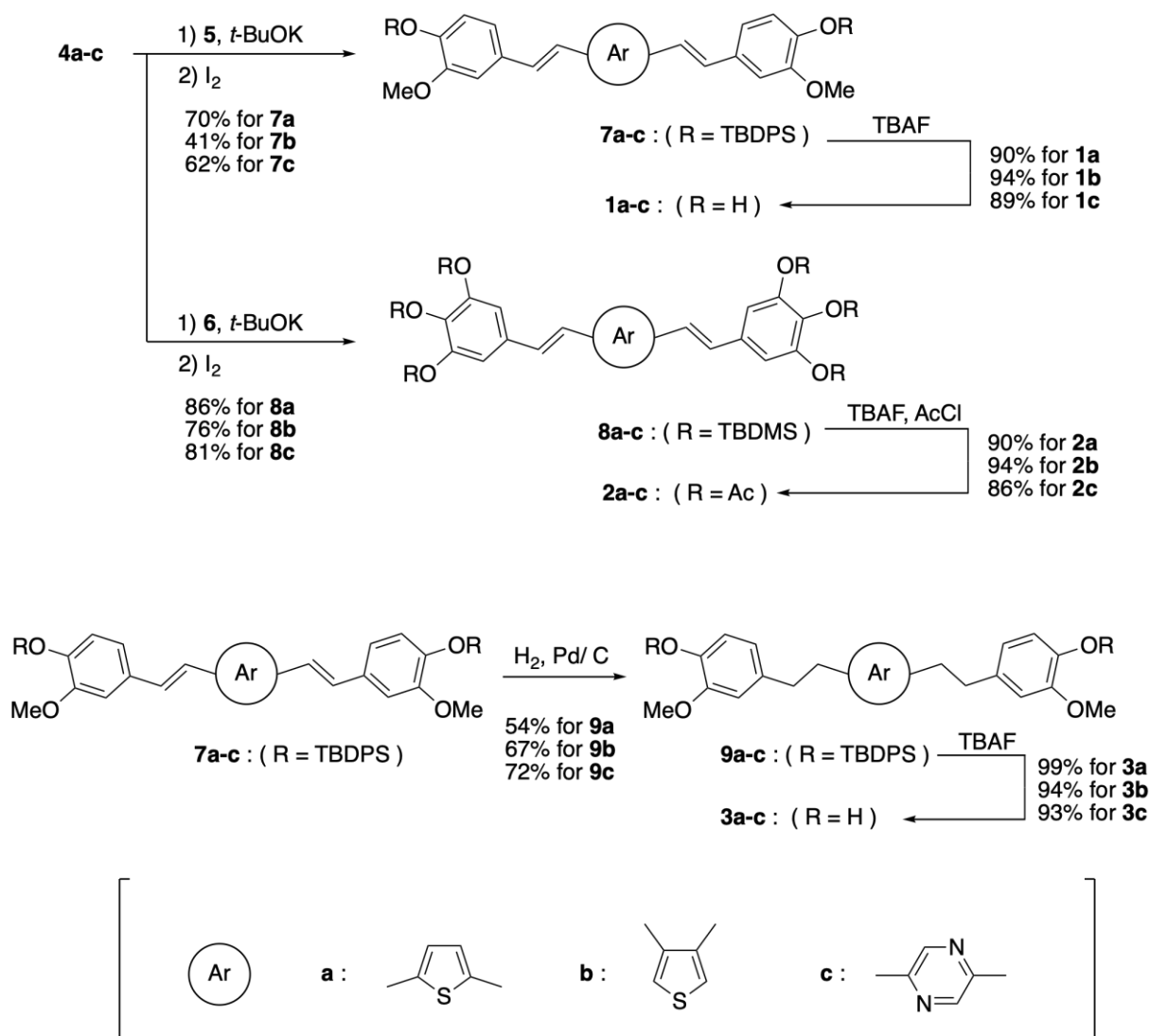


Figure 4. Synthesis of target compounds (**1-3**) via Wittig reaction

The inhibitory effects of our compounds (**1-3**) on osteoclast differentiation of RAW264.7 cells were estimated by the tartrate-resistant acid phosphatase (TRAP) assay *in vitro*.⁸

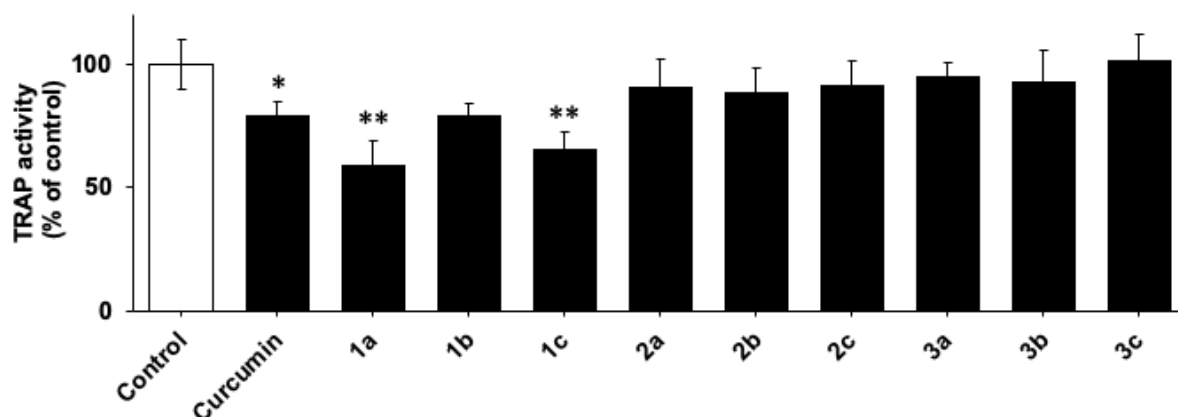


Figure 5. Inhibitory effect of compounds (**1-3**) on osteoclast differentiation

RAW264.7 cells were cultured with the test compound (3 μ M) in the presence of RANKL for two days. TRAP activity was measured using TRACP and ALP Kit (Takara Bio Inc.). The data are expressed as mean \pm SD (n = 8). *P < 0.001 versus the control; **P < 0.001 versus curcumin.

As shown in Figure 5, 2,5-substituted thiophene derivative (**1a**) and 2,5-substituted pyrazine derivative (**1c**) had higher inhibitory effects than curcumin. The difference in the inhibitory effects between **1a** and **1b** might result from the shape of each compound: compound **1a** has a linear conjugated structure, while compound **1b** has a V-shaped one. We would also need to consider several conformers such as *s-cis/s-trans* structures of vinyl-thiophene bonds. The reason why the inhibitory effects of tris(acetoxy)compounds (**2**) had less effect than those of compounds (**1**) might be because compounds (**2**) had no hydroxy group. Deacetylated compounds of **2** were expected to be better compounds for comparison with those of **1**. However, we had no choice but to give up on the preparation of deacetylated compounds of **2** due to their structural instability. The lower inhibitory effects of compounds (**3**) compared to compounds (**1**) suggest that the conjugated system among three aromatic rings could play a critical role in the inhibition of osteoclastogenesis.

To confirm that the inhibitory effect mentioned above was not caused by the cytotoxicity of our compounds, their cell viability was assessed by XTT assay.⁹ Figure 6 illustrates that none of the compounds (**1-3**) in this study were cytotoxic at a concentration of 3 μ M. This evidence supports that the inhibitory effects of **1a** and **1b** are not due to their cytotoxicity.

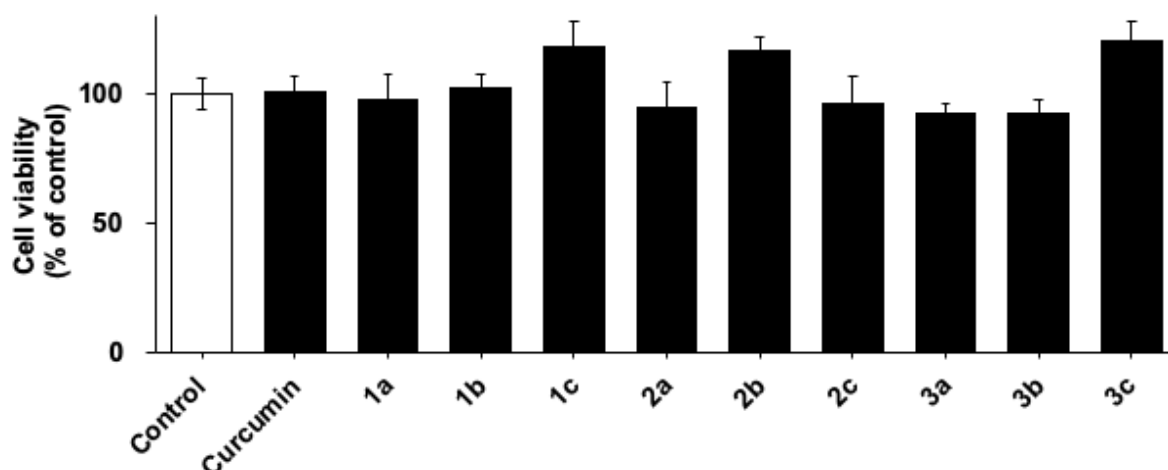


Figure 6. Effects of compounds on cell viability

RAW264.7 cells were cultured with the test compound (3 μ M) for two days. Cell viability was measured using Cell Proliferation Kit (Biological Industries). The data are expressed as mean \pm SD (n = 4).

In conclusion, we have designed and synthesized curcumin analogs (**1-3**) and evaluated their inhibitory effects on RANKL-induced osteoclast formation. Among them, compounds (**1a**) and (**1c**) exhibited potent inhibitory effects by the TRAP assay. Their cell viability showed no cytotoxicity at 3 μ M. The structure-activity relationships of compounds (**1a**) and (**1c**) and their higher screening assays, such as TRAP-staining assay, pit formation assay, and Western blot analysis, could lead us to the development of a new type of chemical tool for the treatment of osteoporosis.

EXPERIMENTAL

All conventional reactions were carried out under an argon atmosphere with dry solvents under anhydrous conditions unless otherwise noted. Solvents and reagents were purified by literature methods where necessary.¹⁰ Flash column chromatography was performed according to the literature.¹¹ RANKL was purchased from PeproTech, Inc. All melting points were determined on a Büchi melting point apparatus (B-540) and are uncorrected. Infrared (IR) spectra were recorded on a JASCO FT/IR-4100 FT-IR spectrometer. ¹H NMR spectra were recorded on a JEOL JNM-ECA500 (500 MHz), JEOL JNM-ECZ500R (500 MHz), or Bruker AC400-P (400 MHz) spectrometer; chemical shifts (δ) are reported in parts per million relative to tetramethylsilane (TMS). Splitting patterns are designated as s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad; dsp, double of septets. Mass spectra (MS) were recorded on a Shimadzu LCMS-IT-TOF or a JEOL MS-700T mass spectrometer. Column chromatography was performed on Kanto Chemical silica gel 60N (spherical, neutral).

Thiophene-2,5-dialdehyde (4a)⁴

This compound was synthesized according to the literature procedure.⁴ Colorless needles; 83%; mp 110.5-111.0 °C (lit. mp 113-114 °C); ¹H NMR (400 MHz, CDCl₃) δ 10.0 (s, 2H), 7.85 (s, 2H); ¹³C NMR (101 MHz, CDCl₃) δ 183.5, 149.3, 135.2.

Thiophene-3,4-dialdehyde (4b)⁴

This compound was synthesized according to the literature procedure.⁴ Colorless crystals; 77%; mp 72.0-73.5 °C (lit. mp 76-77 °C); ¹H NMR (400 MHz, CDCl₃) δ 10.3 (s, 2H), 8.22 (s, 2H); ¹³C NMR (101 MHz, CDCl₃) δ 187.5, 140.4, 137.8.

2,5-Dimethylpyrazine 1,4-dioxide (10)⁵

m-Chloroperbenzoic acid (mCPBA) (60.0 g, 243 mmol) was dissolved in dry ethyl acetate (EtOAc) (200 mL). The solution was washed with brine, and then dried over anhydrous MgSO₄. After removal of the desiccant, EtOAc solution of mCPBA was prepared. Separately, to a solution of 2,5-dimethylpyrazine (8.77 g, 81.1 mmol) in EtOAc (50 mL) was added EtOAc solution of mCPBA (200 mL) at 0 °C. After being stirred at room temperature for 16 h, the precipitate was collected. The precipitate was washed with EtOAc to give **10** (9.83 g, 86%) as white powder. Mp 290.0-290.5 °C (dec) (lit. mp 286 °C (dec)); ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.61 (s, 2H), 2.35 (s, 6H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 143.9, 134.8, 13.9.

2,5-Di(acetoxymethyl)pyrazine (11)⁵

After a suspension of **10** (8.50 g, 60.7 mmol) in acetic anhydride (100 mL) was refluxed for 7 h, the reaction mixture was cooled to room temperature. After being stirred at room temperature for 12 h, the reaction solvent was removed. The residue was washed with Et₂O (300 mL) and then filtered through a pad of Celite. After removal of the solvent, the residue was purified by flash column chromatography (silica gel, *n*-hexane/EtOAc = 1/1, v/v) to give **11** (3.27 g, 24%) as colorless crystals. Mp 75.0-75.5 °C (dec) (lit. mp 78-79 °C (dec)); ¹H NMR (500 MHz, CDCl₃) δ 8.63 (s, 2H), 5.27 (s, 4H), 2.17 (s, 6H); ¹³C NMR (126 MHz, CDCl₃) δ 170.3, 150.4, 142.8, 64.4, 20.6.

2,5-Di(hydroxymethyl)pyrazine (12)⁵

To a solution of **11** (1.60 g, 7.14 mmol) in dry MeOH (60 mL) was added dropwise lithium methoxide (7.14 mL in 1 mol/L MeOH). After being stirred at room temperature for 30 min, to the reaction mixture was added solid ammonium chloride (0.5 g). After removal of the solvent, the residue was purified by flash column chromatography (silica gel, MeOH/CHCl₃ = 1/4, v/v) to give **12** (0.930 g, 93%) as a colorless solid; ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.60 (s, 2H), 5.60 (t, *J* = 6.0 Hz, 2H), 4.63 (d, *J* = 6.0 Hz, 4H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 154.9, 141.1, 62.5.

Pyrazine-2,5-dialdehyde (4c)⁵

To a suspension of **12** (0.930 g, 6.64 mmol) in 1,4-dioxane (300 mL) was added activated manganese dioxide (90% active, 3.21 g, 33.2 mmol). After being refluxed for 24 h, the reaction mixture was cooled to room temperature and filtered through a pad of Celite. The residue was washed with heated EtOAc and CHCl₃, and then the filtrate was removed to give **4c** (0.723 g, 80%) as a pale yellow solid. Mp 90.0-90.5 °C (lit. mp 96-98 °C); ¹H NMR (400 MHz, CDCl₃) δ 10.2 (s, 2H), 9.31 (s, 2H); ¹³C NMR (101 MHz, CDCl₃) δ 191.8, 148.7, 143.4.

4-((tert-Butyldiphenylsilyl)oxy)-3-methoxybenzaldehyde (13)⁶

To a solution of imidazole (7.95 g, 117 mmol) and vanillin (5.92 g, 38.9 mmol) in DMF (100 mL) was added *tert*-butyldiphenylchlorosilane (TBDPSCl) (10.0 mL, 38.9 mmol). After being stirred for 16 h, the reaction was quenched with water and extracted with EtOAc. The organic layer was washed with brine and then dried over anhydrous MgSO₄. After removal of the solvent, the residue was purified by flash chromatography (silica gel, *n*-hexane-EtOAc = 9:1, v/v) to give **13** (13.5 g, 89%) as colorless oil. ¹H NMR (400 MHz, CDCl₃) δ 9.76 (s, 1H), 7.69 (d, *J* = 6.8 Hz, 4H), 7.42-7.34 (m, 6H), 7.30 (d, *J* = 1.6 Hz, 1H), 7.18 (dd, *J* = 1.6, 8.0 Hz, 1H), 6.79 (d, *J* = 8.0 Hz, 1H), 3.63 (s, 3H), 1.12 (s, 9H); ¹³C NMR (101 MHz, CDCl₃) δ 191.2, 151.5, 151.3, 135.4, 132.9, 130.9, 130.1, 127.9, 127.8, 120.2, 110.3, 55.4, 26.7, 20.0.

4-((tert-Butyldiphenylsilyl)oxy)-3-methoxyphenyl)methanol (14)⁶

To a solution of **13** (13.3 g, 34.1 mmol) in dry EtOH (70 mL) was added slowly sodium borohydride (1.93 g, 51.0 mmol) at 0 °C. After being stirred at room temperature for 3 h, the solvent was evaporated in vacuo. To the residue was added EtOAc and 2 mol/L HCl aq. The organic layer was washed with brine, and then dried over anhydrous MgSO₄. After removal of the solvent, the residue was purified by flash chromatography (silica gel, *n*-hexane-EtOAc = 2:1, v/v) to give **14** (12.6 g, 94%) as colorless oil. ¹H NMR (400 MHz, CDCl₃) δ 7.71 (dd, *J* = 1.6, 7.6 Hz, 4H), 7.39 (t, *J* = 7.6 Hz, 2H), 7.34 (t, *J* = 7.6 Hz, 4H), 6.81 (d, *J* = 8.0 Hz, 1H), 6.67 (d, *J* = 8.0 Hz, 1H), 6.61 (dd, *J* = 2.0, 8.0 Hz, 1H), 4.53 (s, 2H), 3.58 (s, 3H), 1.11 (s, 9H); ¹³C NMR (101 MHz, CDCl₃) δ 150.8, 144.8, 135.5, 134.3, 133.7, 129.7, 127.6, 120.1, 119.4, 111.6, 65.5, 55.5, 26.8, 19.9.

4-((tert-Butyldiphenylsilyl)oxy)-3-methoxyphenyl)methyltriphenylphosphonium bromide (5)

To a solution of **14** (12.5 g, 31.8 mmol) in dry Et₂O (100 mL) was added phosphorous tribromide (1.51 mL, 15.9 mmol) at 0 °C. After being stirred at room temperature for 12 h, the reaction mixture was quenched with water and then extracted with Et₂O. The organic layer was washed with brine, and then dried over anhydrous MgSO₄. To the residue was added triphenylphosphine (8.35 g, 31.8 mmol) in dry toluene (100 mL) after removal of the solvent. The reaction mixture was refluxed for 12 h, and cooled to room temperature. The precipitate was collected, and then washed with Et₂O to give **5** (20.1 g, 88% by

two steps) as white powder. Mp 202.5-204.0 °C; IR (KBr) ν 1432, 1282, 1159, 1109, 897, 698 cm^{-1} ; ^1H NMR (400 MHz, CDCl_3) δ 7.74 (t, $J = 8.0$ Hz, 4H), 7.69-7.64 (m, 9H), 7.61-7.56 (m, 6H), 7.40 (s, 2H), 7.32 (t, $J = 8.0$ Hz, 4H), 6.77 (s, 1H), 6.45 (d, $J = 8.0$ Hz, 1H), 6.17 (d, $J = 8.0$ Hz, 1H), 5.21 (d, $J = 13.6$ Hz, 2H), 3.31 (s, 3H), 1.08 (s, 9H); ^{13}C NMR (101 MHz, CDCl_3) δ 150.7, 145.2(d), 135.4, 134.9(d), 134.5(d), 133.2, 130.1(d), 129.8, 127.5, 123.4(d), 120.3(d), 119.6(d), 117.8(d), 115.6(d), 55.4, 30.7(d), 26.7, 19.8; MS (FAB) m/z 637 (M-Br) $^+$; Anal. Calcd for $\text{C}_{42}\text{H}_{42}\text{BrO}_2\text{PSi}$: C, 70.28; H, 5.90. Found: C, 70.28; H, 5.81.

Methyl 3,4,5-(tris(*tert*-butyldimethylsilyl)oxy)benzoate (15)⁷

This compound was synthesized according to the literature procedure.⁷ Colorless crystals; 98%; ^1H NMR (500 MHz, CDCl_3) δ 7.20 (s, 2H), 3.85 (s, 3H), 0.99 (s, 9H), 0.95 (s, 18H), 0.23 (s, 12H), 0.13 (s, 6H).

(3,4,5-(Tris(*tert*-butyldimethylsilyl)oxy)phenyl)methanol (16)⁷

This compound was synthesized according to the literature procedure.⁷ Pale yellow oil; 98%; ^1H NMR (500 MHz, CDCl_3) δ 6.49 (s, 2H), 4.48 (d, $J = 5.9$ Hz, 2H), 1.44 (t, $J = 5.9$ Hz, 1H), 0.99 (s, 9H), 0.93 (s, 18H), 0.20 (s, 12H), 0.11 (s, 6H).

(3,4,5-(Tris(*tert*-butyldimethylsilyl)oxy)phenyl)methyltriphenylphosphonium bromide (6)

This compound was synthesized from alcohol **16** according to the method used for the preparation of **5** from alcohol **14**. Colorless plates; 76%; mp 272.0-273.0 °C; IR (KBr) ν 2931, 1857, 1493, 1438, 1250 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 7.79-7.76 (m, 3H), 7.74-7.70 (m, 6H), 7.66-7.63 (m, 6H), 6.17 (d, $J = 2.5$ Hz, 2H), 5.14 (d, $J = 13.5$ Hz, 2H), 0.96 (s, 9H), 0.82 (s, 18H), 0.091 (s, 6H), 0.015 (s, 12H); ^{13}C -NMR (126 MHz, CDCl_3) δ 149.2(d), 139.0(d), 135.0(d), 134.4(d), 130.3(d), 118.7(d), 118.3(d), 116.7(d), 30.9(d), 26.25, 26.17, 18.7, 18.5, -3.64, -3.76; MS (FAB) m/z 743 (M-Br) $^+$; Anal. Calcd for $\text{C}_{43}\text{H}_{64}\text{BrO}_3\text{PSi}_3$: C, 62.67; H, 7.83. Found: C, 62.42; H, 7.48.

(*E, E*)-2,5-Bis(4'-((*tert*-butyldiphenylsilyl)oxy)-3'-methoxystyryl)thiophene (7a)

To a solution of phosphonium bromide **5** (1.22 g, 1.70 mmol) in dry THF (20 mL) was added potassium *tert*-butoxide (*t*-BuOK) (0.368 g, 3.28 mmol). After being stirred at room temperature for 10 min, dialdehyde **4a** (0.200 g, 1.43 mmol) in dry THF (5 mL) was added at the same temperature. After being stirred at room temperature for 2 h, the reaction mixture was quenched with saturated aqueous NH_4Cl and extracted with Et_2O . The organic layer was washed with brine, and then dried over anhydrous MgSO_4 . After removal of the solvent, the residue was purified by flash column chromatography (silica gel, *n*-hexane/ $\text{EtOAc} = 9/1$, v/v) to give the isomer mixture. To the mixture was added catalytic amount of iodine in dry toluene. After being stirred at 100 °C for 3 h, the reaction mixture was quenched with saturated aqueous sodium hydrogen sulfite (NaHSO_3), and then extracted with Et_2O . The organic layer was washed with brine, and then dried over anhydrous MgSO_4 . Removal of the solvent gave **7a** (0.857 g, 70%) as yellow powder. Mp 54.0-55.5 °C; IR (KBr) ν 1509, 1282, 910 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3)

δ 7.71 (dd, $J = 1.5, 7.0$ Hz, 8H), 7.40 (tt, $J = 1.5, 7.0$ Hz, 4H), 7.34 (td, $J = 1.5, 7.0$ Hz, 8H), 6.95 (d, $J = 16.0$ Hz, 2H), 6.87 (d, $J = 2.0$ Hz, 2H), 6.83 (s, 2H), 6.74 (dd, $J = 2.0, 8.0$ Hz, 2H), 6.73 (d, $J = 16.0$ Hz, 2H), 6.66 (d, $J = 8.0$ Hz, 2H), 3.61 (s, 6H), 1.11 (s, 18H); ^{13}C NMR (126 MHz, CDCl_3) δ 150.7, 145.1, 141.7, 135.3, 133.5, 130.7, 129.6, 128.3, 127.5, 126.4, 120.3, 120.0, 119.4, 109.8, 55.4, 26.6, 19.8; HR-MS (FAB) Calcd for $\text{C}_{54}\text{H}_{56}\text{O}_4\text{SSi}_2$ (M) $^+$ 856.3438. Found: 856.3398.

(*E, E*)-3,4-Bis(4'-((*tert*-butyldiphenylsilyl)oxy)-3'-methoxystyryl)thiophene (7b)

This compound was synthesized from aldehyde **4b** and phosphonium bromide **5** according to the method used for the preparation of **7a**. Colorless powder; 41%; mp 53.0-55.5 °C; IR (KBr) ν 1512, 1282, 822 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 7.72 (dd, $J = 1.5, 7.5$ Hz, 8H), 7.40 (tt, $J = 1.5, 7.5$ Hz, 4H), 7.35 (t, $J = 7.5$ Hz, 8H), 7.28 (s, 2H), 6.93 (d, $J = 16.0$ Hz, 2H), 6.89 (d, $J = 2.0$ Hz, 2H), 6.81 (d, $J = 16.0$ Hz, 2H), 6.78 (dd, $J = 2.0, 8.5$ Hz, 2H), 6.67 (dd, $J = 8.5$ Hz, 2H), 3.60 (s, 6H), 1.12 (s, 18H); ^{13}C NMR (126 MHz, CDCl_3) δ 150.6, 145.1, 138.7, 135.3, 133.5, 131.1, 130.6, 129.6, 127.5, 120.5, 120.3, 119.9, 119.4, 110.3, 55.5, 26.7, 19.8; HR-MS (FAB) Calcd for $\text{C}_{54}\text{H}_{57}\text{O}_4\text{SSi}_2$ [$\text{M}+\text{H}$] $^+$ 857.3516; found: 857.3550.

(*E, E*)-2,5-Bis(3'-((*tert*-butyldiphenylsilyl)oxy)-4'-methoxystyryl)pyrazine (7c)

This compound was synthesized from aldehyde **4c** and phosphonium bromide **5** according to the method used for the preparation of **7a**. Yellow sticky oil; IR (KBr) ν 3441, 1510, 1285, 910 cm^{-1} ; ^1H NMR (400 MHz, CDCl_3) δ 8.49 (s, 2H), 7.73-7.70 (m, 8H), 7.55 (d, $J = 16.0$ Hz, 2H), 7.43-7.33 (m, 12H), 7.01 (d, $J = 2.0$ Hz, 2H), 6.94 (d, $J = 16.0$ Hz, 2H), 6.89 (dd, $J = 1.6, 8.0$ Hz, 2H), 6.70 (d, $J = 8.0$ Hz, 2H), 3.62 (s, 6H), 1.12 (s, 18H); ^{13}C NMR (101 MHz, CDCl_3) δ 150.9, 149.1, 146.3, 143.0, 135.5, 134.2, 133.5, 130.2, 129.8, 127.7, 122.3, 120.9, 120.5, 110.6, 55.5, 26.8, 20.0; HR-MS (FAB) Calcd for $\text{C}_{54}\text{H}_{57}\text{N}_2\text{O}_4\text{SSi}_2$ [$\text{M}+\text{H}$] $^+$ 853.3857; found: 853.3860.

(*E, E*)-2,5-Bis((3',4',5'-tris(*tert*-butyldimethylsilyl)oxy)styryl)thiophene (8a)

This compound was synthesized from aldehyde **4a** and phosphonium bromide **6** according to the method used for the preparation of **7a**. Yellow crystals; mp 168.0-169.0 °C; IR (KBr) ν 2933, 2858, 1490, 1255, 1085 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 6.90 (d, $J = 16.0$ Hz, 2H), 6.89 (s, 2H), 6.69 (d, $J = 16.0$ Hz, 2H), 6.61 (s, 4H), 1.00 (s, 18H), 0.96 (s, 36H), 0.24 (s, 24H), 0.13 (s, 12H); ^{13}C NMR (126 MHz, CDCl_3) δ 148.9, 141.8, 138.8, 129.4, 128.6, 126.7, 120.2, 112.6, 26.38, 26.36, 19.0, 18.6, -3.43, -3.78; Anal. Calcd for $\text{C}_{56}\text{H}_{100}\text{O}_6\text{SSi}_6$: C, 62.86; H, 9.42. Found: C, 62.51; H, 9.08.

(*E, E*)-3,4-Bis((3',4',5'-tris(*tert*-butyldimethylsilyl)oxy)styryl)thiophene (8b)

This compound was synthesized from aldehyde **4b** and phosphonium bromide **6** according to the method used for the preparation of **7a**. A colorless solid; 76%; mp 88.5-89.5 °C; IR (KBr) ν 2937, 2859, 1491, 1254, 1085 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 7.33 (s, 2H), 6.90 (d, $J = 16.0$ Hz, 2H), 6.74 (d, $J = 16.0$ Hz, 2H), 6.64 (s, 4H), 1.00 (s, 18H), 0.94 (s, 36H), 0.21 (s, 24H), 0.13 (s, 12H); ^{13}C NMR (126 MHz,

CDCl₃) δ 148.8, 138.8, 138.6, 130.9, 129.9, 120.8, 120.1, 112.7, 26.4(d), 18.9, 18.6, -3.46, -3.80; HR-MS (FAB) Calcd for C₅₆H₁₀₁O₆SSi₆ [(M+H)⁺] 1069.5934; found: 1069.5970.

(*E,E*)-2,5-Bis((3',4',5'-tris(*tert*-butyldimethylsilyl)oxy)styryl)pyrazine (8c)

This compound was synthesized from aldehyde **4c** and phosphonium bromide **6** according to the method used for the preparation of **7a**. Yellow powder; mp 156.1-157.0 °C; IR (KBr) ν 2944, 2863, 1481, 1249, 1084 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 8.53 (s, 2H), 7.50 (d, *J* = 16.0 Hz, 2H), 6.89 (d, *J* = 16.0 Hz, 2H), 6.76 (s, 4H), 1.00 (s, 18H), 0.96 (s, 36H), 0.25 (s, 24H), 0.14 (s, 12H); ¹³C NMR (126 MHz, CDCl₃) δ 143.1, 139.9, 134.4, 128.8, 122.3, 113.6, 26.4(d), 19.0, 18.6, -3.43, -3.77; Anal. Calcd for C₅₆H₁₀₀N₂O₆Si₆: C, 63.10; H, 9.46; N, 2.36. Found: C, 63.04; H, 9.25; N, 2.34.

(*E,E*)-2,5-Bis(4'-hydroxy-3'-methoxystyryl)thiophene (1a)

To a solution of **7a** (0.241 g, 0.281 mmol) in dry THF (20 mL) was added slowly tetra-*n*-butylammonium fluoride (TBAF) (1.05 mL in 1 mol/L THF, 1.05 mmol) at 0 °C. After being stirred at room temperature for 2 h, the reaction mixture was quenched with saturated aqueous NH₄Cl and extracted with EtOAc. The organic layer was washed with brine, and then dried over anhydrous MgSO₄. After removal of the solvent, the residue was purified by flash column chromatography (silica gel, *n*-hexane/EtOAc = 1/1, v/v) to give **1a** (0.104 g, 90%) as yellow green crystals. Mp 184.0-184.5 °C (dec); IR (KBr) ν 3470, 3420, 1510, 1236, 1026, 948 cm⁻¹; ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.19 (s, 2H), 7.24 (d, *J* = 16.0 Hz, 2H), 7.19 (d, *J* = 2.0 Hz, 2H), 7.03 (s, 2H), 6.96 (dd, *J* = 2.0, 8.0 Hz, 2H), 6.80 (d, *J* = 16.0 Hz, 2H), 6.76 (d, *J* = 8.0 Hz, 2H), 3.84 (s, 6H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 147.9, 146.8, 141.2, 128.25, 128.23, 126.7, 120.1, 119.2, 115.6, 109.6, 55.6; MS (FAB) *m/z* 380 (M)⁺; Anal. Calcd for C₂₂H₂₀O₄S: C, 69.45; H, 5.30. Found: C, 69.27; H, 5.47.

(*E,E*)-3,4-Bis(4'-hydroxy-3'-methoxystyryl)thiophene (1b)

This compound was synthesized from **7b** according to the method used for the synthesis of **1a**. Colorless crystals; 94%; mp 150.0-150.5 °C (dec); IR (KBr) ν 3425, 1513, 1264, 1028, 953, 810 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 7.33 (s, 2H), 7.02-7.00 (m, 4H), 7.01 (d, *J* = 16.0 Hz, 2H), 6.91 (d, *J* = 8.0 Hz, 2H), 6.89 (d, *J* = 16.0 Hz, 2H), 5.67 (s, 2H), 3.93 (s, 6H); ¹³C NMR (101 MHz, CDCl₃) δ 146.9, 145.8, 138.9, 130.8, 130.3, 120.8, 120.4, 119.9, 114.8, 108.6, 56.1; MS (EI) *m/z* 380 (M)⁺; Anal. Calcd for C₂₂H₂₀O₄S: C, 69.45; H, 5.30. Found: C, 69.24; H, 5.30.

(*E,E*)-2,5-Bis(4'-hydroxy-3'-methoxystyryl)pyrazine (1c)

This compound was synthesized from **7c** according to the method used for the synthesis of **1a**. Orange powder; mp 250.3-251.0 °C (dec); IR (KBr) ν 3450, 1633, 1280 cm⁻¹; ¹H NMR (400 MHz, DMSO-*d*₆) δ 9.38 (s, 2H), 8.65 (s, 2H), 7.65 (d, *J* = 16.0 Hz, 2H), 7.31 (s, 2H), 7.22 (d, *J* = 16.0 Hz, 2H), 7.09 (d, *J* = 8.0 Hz, 2H), 6.80 (d, *J* = 8.0 Hz, 2H), 3.85 (s, 6H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 148.7, 148.0, 147.8,

143.0, 133.6, 127.9, 121.5, 121.4, 115.6, 110.2, 55.6; MS (EI) m/z 376 (M)⁺; Anal. Calcd for C₂₂H₂₀N₂O₄: C, 70.20; H, 5.36; N, 7.44. Found: C, 69.92; H, 5.45; N, 7.19.

(*E,E*)-2,5-Bis((3',4',5'-tris(acetoxy)styryl)thiophene (2a)

To a solution of **8a** (0.200 g, 0.187 mmol) in dry THF (30 mL) was added dropwise acetyl chloride (0.132 mL, 1.87 mmol) and TBAF (7.29 mL, 7.29 mmol) at 0 °C, and then added acetyl chloride (1.00 mL) again. The reaction mixture was quenched with saturated aqueous sodium hydrogen carbonate (NaHCO₃) and extracted with EtOAc. The organic layer was washed with brine, and then dried over anhydrous MgSO₄. After removal of the solvent, the residue was purified by flash column chromatography (silica gel, *n*-hexane/EtOAc = 1/3, v/v) to give **2a** (0.116 g, 97%) as yellow crystals. Mp 237.8-238.5 °C; IR (KBr) ν 1773, 1197 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 7.20 (s, 4H), 7.09 (d, *J* = 16.0 Hz, 2H), 6.95 (s, 2H), 6.79 (d, *J* = 16.0 Hz, 2H), 2.30 (s, 12H), 2.29 (s, 6H); ¹³C NMR (126 MHz, CDCl₃) δ 168.0, 167.2, 143.7, 141.8, 135.7, 133.8, 128.2, 126.5, 123.8, 118.4, 20.8, 20.3; MS (FAB) m/z 636 (M)⁺; Anal. Calcd for C₃₂H₂₈O₁₂S: C, 60.37; H, 4.43. Found: C, 60.22; H, 4.44.

(*E,E*)-3,4-Bis((3',4',5'-tris(acetoxy)styryl)thiophene (2b)

This compound was synthesized from **8b** according to the method used for the synthesis of **2a**. Colorless crystals; 99%; mp 173.8-174.1 °C; IR (KBr) ν 1770, 1196 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 6.37 (s, 2H), 7.22 (s, 4H), 7.06 (d, *J* = 16.0 Hz, 2H), 6.83 (d, *J* = 16.0 Hz, 2H), 2.30 (s, 12H), 2.29 (s, 6H); ¹³C NMR (126 MHz, CDCl₃) δ 168.0, 167.2, 143.7, 138.0, 136.1, 134.0, 128.8, 123.7, 122.5, 118.7, 20.8, 20.3; MS (FAB) m/z 637 [(M+H)⁺]; Anal. Calcd for C₃₂H₂₈O₁₂S: C, 60.37; H, 4.43. Found: C, 60.39; H, 4.45.

(*E,E*)-2,5-Bis((3',4',5'-tris(acetoxy)styryl)pyrazine (2c)

This compound was synthesized from **8c** according to the method used for the synthesis of **2a**. Yellow powder; mp 262.0-263.5 °C; IR (KBr) ν 1775, 1194 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 8.55 (s, 2H), 7.66 (d, *J* = 16.0 Hz, 2H), 7.34 (s, 4H), 7.10 (d, *J* = 16.0 Hz, 2H), 2.314 (s, 12H), 2.307 (s, 6H); ¹³C NMR (126 MHz, CDCl₃) δ 167.9, 167.1, 148.9, 143.8, 143.7, 135.0, 134.8, 132.4, 126.0, 119.4, 20.8, 20.3; MS (FAB) m/z 633 [(M+H)⁺]; Anal. Calcd for C₃₂H₂₈N₂O₁₂: C, 60.76; H, 4.46; N, 4.43. Found: C, 60.48; H, 4.48; N, 4.37.

2,5-Bis(2-(4'-((*tert*-butyldiphenylsilyloxy)-3'-methoxyphenyl)ethyl)thiophene (9a)

The reaction mixture of **7a** (0.241 g, 0.281 mmol), palladium 10% on carbon (1.32 g) in EtOH-EtOAc (50 mL, 1:1, v/v) was degassed with N₂ gas, and then flashed with H₂ gas. The reaction mixture was stirred under a balloon of H₂ atmosphere for 10 h. The reaction mixture was purged with N₂ gas, and filtered through a pad of Celite. The residue was washed with Et₂O. After removal of the solvent, the residue was purified by flash column chromatography (silica gel, *n*-hexane/EtOAc = 10/1, v/v) to give **9a** (0.287 g, 54%) as colorless oil; IR (neat) ν 2934, 2856, 1512, 1286, 910 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 7.70

(d, $J = 7.2$ Hz, 8H), 7.40-7.31 (m, 12H), 6.62 (d, $J = 8.0$ Hz, 2H), 6.55 (d, $J = 2.0$ Hz, 2H), 6.45 (dd, $J = 2.0, 8.0$ Hz, 2H), 6.45 (s, 2H), 3.52 (s, 6H), 2.95 (t, $J = 8.0$ Hz, 4H), 2.78 (t, $J = 8.0$ Hz, 4H), 1.10 (s, 18H); ^{13}C NMR (101 MHz, CDCl_3) δ 150.3, 143.4, 142.4, 135.5, 134.7, 133.9, 129.7, 127.6, 123.9, 120.4, 120.1, 112.9, 55.5, 37.9, 32.4, 26.8, 19.9; MS (FAB) m/z 859 [(M-H) $^+$].

3,4-Bis(2-(4'-((*tert*-butyldiphenylsilyl)oxy)-3'-methoxyphenyl)ethyl)thiophene (9b)

This compound was synthesized from **7b** according to the method used for the preparation of **9a**. Colorless oil; IR (neat) ν 2932, 2857, 1513, 1286, 914 cm^{-1} ; ^1H NMR (400 MHz, CDCl_3) δ 7.70 (dd, $J = 7.5, 1.5$ Hz, 2H), 7.38 (tt, $J = 7.5, 1.5$ Hz, 4H), 7.33 (t, $J = 7.5$ Hz, 8H), 6.76 (s, 2H), 6.60 (d, $J = 8.0$ Hz, 2H), 6.48 (d, $J = 2.0$ Hz, 2H), 6.42 (dd, $J = 8.0, 2.0$ Hz, 2H), 3.49 (s, 6H), 2.75-2.72 (m, 4H), 2.69- 2.66 (m, 4H), 1.10 (s, 18H); ^{13}C NMR (101 MHz, CDCl_3) δ 150.3, 143.4, 141.1, 135.6, 135.1, 133.9, 129.7, 127.6, 120.7, 120.4, 120.1, 113.0, 55.5, 35.9, 30.9, 26.8, 19.9; MS (FAB) m/z 859 [(M-H) $^+$].

2,5-Bis(2-(4'-((*tert*-butyldiphenylsilyl)oxy)-3'-methoxyphenyl)ethyl)pyrazine (9c)

This compound was synthesized from **7c** according to the method used for the preparation of **9a**. Colorless oil; IR (KBr) ν 2931, 2856, 1514, 1286, 921 cm^{-1} ; ^1H NMR (400 MHz, CDCl_3) δ 8.21 (s, 2H), 7.70 (dd, $J = 1.2, 8.0$ Hz, 8H), 7.41-7.31 (m, 12H), 6.60 (d, $J = 8.0$ Hz, 2H), 6.55 (d, $J = 2.0$ Hz, 2H), 6.43 (dd, $J = 2.0, 8.0$ Hz, 2H), 3.51 (s, 6H), 3.00-2.96 (m, 4H), 2.90-2.86 (m, 4H), 1.10 (s, 18H); ^{13}C NMR (101 MHz, CDCl_3) δ 153.9, 150.4, 143.7, 143.5, 135.5, 134.3, 133.8, 129.7, 127.6, 120.4, 120.1, 112.8, 55.5, 37.2, 35.4, 26.8, 19.9; MS (FAB) m/z 857 [(M+H) $^+$].

2,5-Bis(2-(4'-hydroxy-3'-methoxyphenyl)ethyl)thiophene (3a)

This compound was synthesized from **9a** according to the synthesis of **1a** from **7a**. colorless plates; IR (KBr) ν 3427, 2927, 2849, 1514, 1227 cm^{-1} ; ^1H NMR (400 MHz, CDCl_3) δ 6.78 (d, $J = 2.0$ Hz, 2H), 6.77 (d, $J = 8.0$ Hz, 2H), 6.67 (dd, $J = 2.0, 8.0$ Hz, 2H), 6.54 (s, 2H), 5.58 (s, 2H), 3.87 (s, 6H), 3.01 (t, $J = 7.2$ Hz, 4H), 2.85 (t, $J = 7.2$ Hz, 4H); ^{13}C NMR (101 MHz, CDCl_3) δ 145.6, 145.1, 142.4, 134.8, 123.9, 119.9, 114.8, 110.7, 56.1, 37.5, 32.3; MS (EI) m/z 384 (M^+); Anal. Calcd for $\text{C}_{22}\text{H}_{24}\text{O}_4\text{S}$: C, 68.72; H, 6.29. Found: C, 68.66; H, 6.28.

3,4-Bis(2-(4'-hydroxy-3'-methoxyphenyl)ethyl)thiophene (3b)

This compound was synthesized from **9b** according to the synthesis of **1a** from **7a**. Colorless powder; IR (KBr) ν 3421, 2926, 2845, 1514, 1260 cm^{-1} ; ^1H NMR (400 MHz, CDCl_3) δ 6.92 (s, 2H), 6.82 (d, $J = 8.0$ Hz, 2H), 6.67 (dd, $J = 2.0, 8.0$ Hz, 2H), 6.58 (d, $J = 2.0$ Hz, 2H), 5.49 (s, 2H), 3.82 (s, 6H), 2.85-2.81 (m, 4H), 2.75-2.72 (m, 4H); ^{13}C NMR (101 MHz, CDCl_3) δ 146.4, 143.9, 141.2, 133.8, 121.0, 120.7, 114.3, 111.2, 56.0, 36.0, 31.1; MS (EI) m/z 384 (M^+); Anal. Calcd for $\text{C}_{22}\text{H}_{24}\text{O}_4\text{S}$: C, 68.72; H, 6.29. Found: C, 68.49; H, 6.27.

2,5-Bis(2-(4'-hydroxy-3'-methoxyphenyl)ethyl)pyrazine (3c)

This compound was synthesized from **9c** according to the synthesis of **1a** from **7a**. Colorless powder; mp 176.2-176.5 °C; IR (KBr) ν 3466, 2947, 1523, 1215 cm^{-1} ; ^1H NMR (400 MHz, DMSO- d_6) δ 8.71 (s, 2H), 8.38 (s, 2H), 6.73 (d, $J = 1.6$ Hz, 2H), 6.64 (d, $J = 8.0$ Hz, 2H), 6.54 (dd, $J = 1.6, 8.0$ Hz, 2H), 3.70 (s, 6H), 2.98 (t, $J = 7.6$ Hz, 4H), 2.86 (t, $J = 7.6$ Hz, 4H); ^{13}C NMR (101 MHz, DMSO- d_6) δ 153.6, 147.4, 144.6, 143.3, 131.8, 120.4, 115.3, 112.5, 55.5, 36.2, 34.4; MS (EI) m/z 380 (M) $^+$; Anal. Calcd for $\text{C}_{22}\text{H}_{24}\text{N}_2\text{O}_4$: C, 69.46; H, 6.36; N, 7.36. Found: C, 69.29; H, 6.45; N, 7.11.

Cell culture

RAW264.7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 0.1 mM Non-Essential Amino Acids solution (NEAA), and Penicillin-Streptomycin-L-Glutamine solution (100 units/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin, and 2 mM glutamine). These cells were incubated at 37 °C in 5% CO_2 in humidified air.

TRAP assay⁸

To estimate the inhibitory effect of our compounds on osteoclastogenesis, RAW 264.7 cells (1×10^4 cells/well) were seeded on a 24-well plate. The medium was changed to alpha-Modified Eagle Minimum Essential Medium ($\text{MEM}\alpha$) containing 3 μM our compound in DMSO and 50 ng/mL RANKL, and incubated for an additional 48 h. Each well was treated with the solution including TRACP & ALP Kit (Takara Bio) according to the manufacturer's instructions, and then absorbances at 405 nm was measured as TRAP activity by a microplate reader.

XTT assay⁹

To estimate the cytotoxicity of our compounds, RAW 264.7 cells (2×10^3 cells/well) were seeded on a 96-well plate. The medium was changed to $\text{MEM}\alpha$ containing 3 μM our compound in DMSO, and incubated for an additional 48 h. Each well was treated with the solution including Cell Proliferation Kit (Biological Industries) according to the manufacturer's instructions, and then absorbances at 450 nm was measured as cytotoxicity by a microplate reader.

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