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Synthesis of Biotinylated Xestoquinone That Retains Inhibitory Activity Against Ca^{2+} ATPase of Skeletal Muscle Myosin

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Abstract—Xestoquinone isolated from a marine sponge binds to skeletal muscle myosin and inhibits its Ca^{2+} ATPase activity. In this study, we first examined xestoquinone and its analogues to assess the relationships between structure and myosin Ca^{2+} ATPase inhibitory activity. On the basis of the resultant data, we then designed a biotinylated xestoquinone analogue. Xestoquinone and its analogues were derived from extracts of the marine sponge *Xestospongia sapra*. Four xestoquinone analogues with a quinone structure significantly inhibited Ca^{2+} ATPase activity. In contrast, four xestoquinone analogues in which the quinone structure was converted to a quinol dimethyl ether did not inhibit Ca^{2+} ATPase activity. This suggests that the quinone moiety is essential for inhibitory activity. Then, we synthesized a biotinylated xestoquinone in which a biotin tag was introduced to a site far from the quinone moiety, and this molecule exhibited stronger inhibitory activity than that of xestoquinone. This biotinylated xestoquinone could be useful as a probe in studies of the xestoquinone-myosin binding mode.

Introduction

Marine sponges contain several compounds with various bioactivities, including antibacterial, antifungal and cytotoxic activity. Xestoquinone (**1**),[1] halenaquinone (**2**),[2] halenaquinol (**3**)[3] and halenaquinol sulfate (**4**)[3] were isolated from the marine sponge *Xestospongia sapra* or *Xestospongia exigua* (Fig. 1, Scheme 1). Each compound was found to have unique biological activities, despite their structural similarity: **1** exhibits powerful cardiotonic activity,[1] inhibits the Ca^{2+} and K^+(EDTA) ATPase activities of myosin,[4] activates the ATPase of actomyosin,[4] and releases Ca^{2+} from skeletal muscle sarcoplasmic reticulum;[5] **2** inhibits protein tyrosine kinase[6,7] and PI3 kinase;[8] **3** inhibits protein tyrosine kinase;[6,7] **4** inhibits the membrane fusion events of echinoderm gametes.[9] However, the only systematic study of the structure–activity relationships of xestoquinone and its analogues have dealt with inhibition of protein tyrosine kinase.[6,7]

Sakamoto et al. studied, in detail, the binding mode of **1** and myosin.[4] They found that reduction of Ca^{2+} ATPase activity by **1** was abolished by dithiothreitol, and that the positions C-14 and C-15 of **1** were modified by two molecules of 2-mercaptoethanol, indicating that modification of the thiol groups of myosin by **1** causes inhibition of myosin Ca^{2+} ATPase.[4] A myosin molecule contains over 40 thiol residues, with 12 or 13 residues located on each myosin head.[10] A myosin head contains two thiol groups (SH_1 and SH_2) that can be favorably modified by maleimide reagents. *N*-Ethylmaleimide can

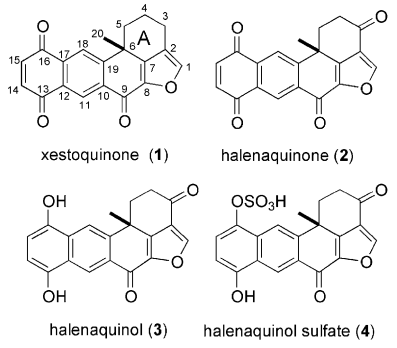

Figure 1. Xestoquinone and its analogues.

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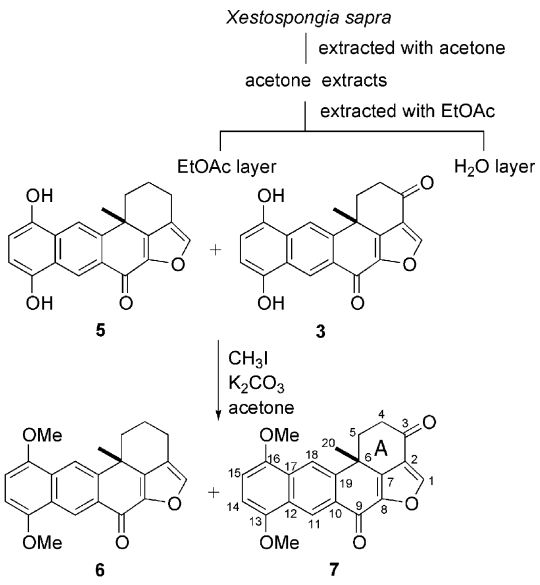


Scheme 1. Preparation of marine sponge extract, and derivatization of hydroquinone components to dimethyl ethers.

modify both the SH_1 and SH_2 groups, even after modification of 2 mol of SH groups by **1**, suggesting that the binding sites of **1** are SH groups distinct from the SH_1 and SH_2 of myosin.[4] Because modification of myosin by **1** causes changes in tryptophan fluorescence intensity and the circular dichroism spectrum, it is presumed that **1** induces a conformational change via modification of myosin SH groups.[4] However, the site at which **1** binds to myosin has remained unidentified. In the present study, in an attempt to identify the site at which **1** binds to myosin, we first examined relationships between structures of xestoquinone analogues and their myosin Ca^{2+} ATPase inhibitory activity. Then, on the basis of the resultant data, we synthesized a biotinylated xestoquinone analogue.

Results and Discussion

Derivatization of xestoquinone analogues

We examined the structure–activity relationship for xestoquinone (**1**) and its analogues, to determine the most suitable position at which a biotin tag could be attached. Rather than perform total synthesis (which requires many steps), we derived the tested compounds from halenaquinol (**3**) and xestoquinol (**5**), which were obtained from extracts of the marine sponge *X. sapra*.[11–15] The acetone extract was partitioned between EtOAc and water. The EtOAc solution that contained **3** and **5** was methylated with iodomethane in the presence of potassium carbonate to give dimethyl ether (**6** and **7**). The major product **7** was selectively reduced with $NaBH_4$ in the presence of $CeCl_3$[11] to give alcohol **8** (Scheme 2). Alcohol **8** was dehydrated in the presence of an acid catalyst to give olefin-dimethyl ether **9**. The resulting dimethyl ethers **6**, **7**, **8** and **9** were oxidized by ceric ammonium nitrate (CAN), giving quinones **1**,[11] **2**,[12] **10** and **11**, respectively.

Ca^{2+} ATPase activity

Myosin Ca^{2+} ATPase activity was measured by released phosphoric acid, which was detected using malachite green.[17] A large excess (2.64 mM) of each quinone analogue (**1**, **2**, **10** and **11**) inhibited the myosin Ca^{2+} ATPase, but no dimethyl ether analogue (**6**, **7**, **8** and **9**) inhibited the myosin Ca^{2+} ATPase (Fig. 2). This suggests that the quinone structure is necessary for inhibition of the myosin Ca^{2+} ATPase, and that the A-ring structure far from the quinone moiety is not important.

When the well was washed twice with the assay buffer after incubation with **1**, inhibition was retained (Fig. 2).

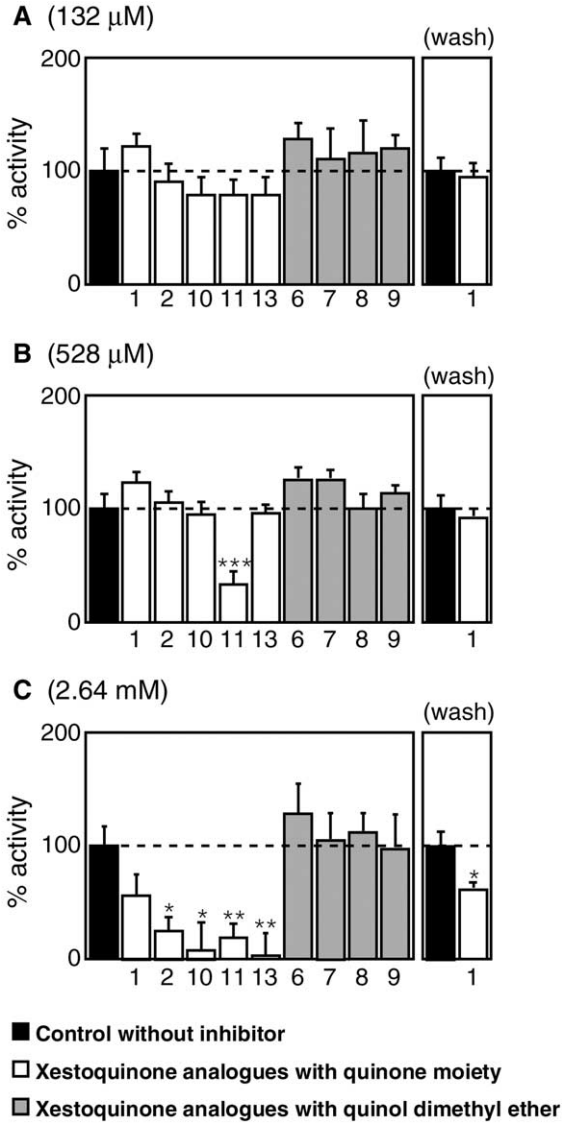
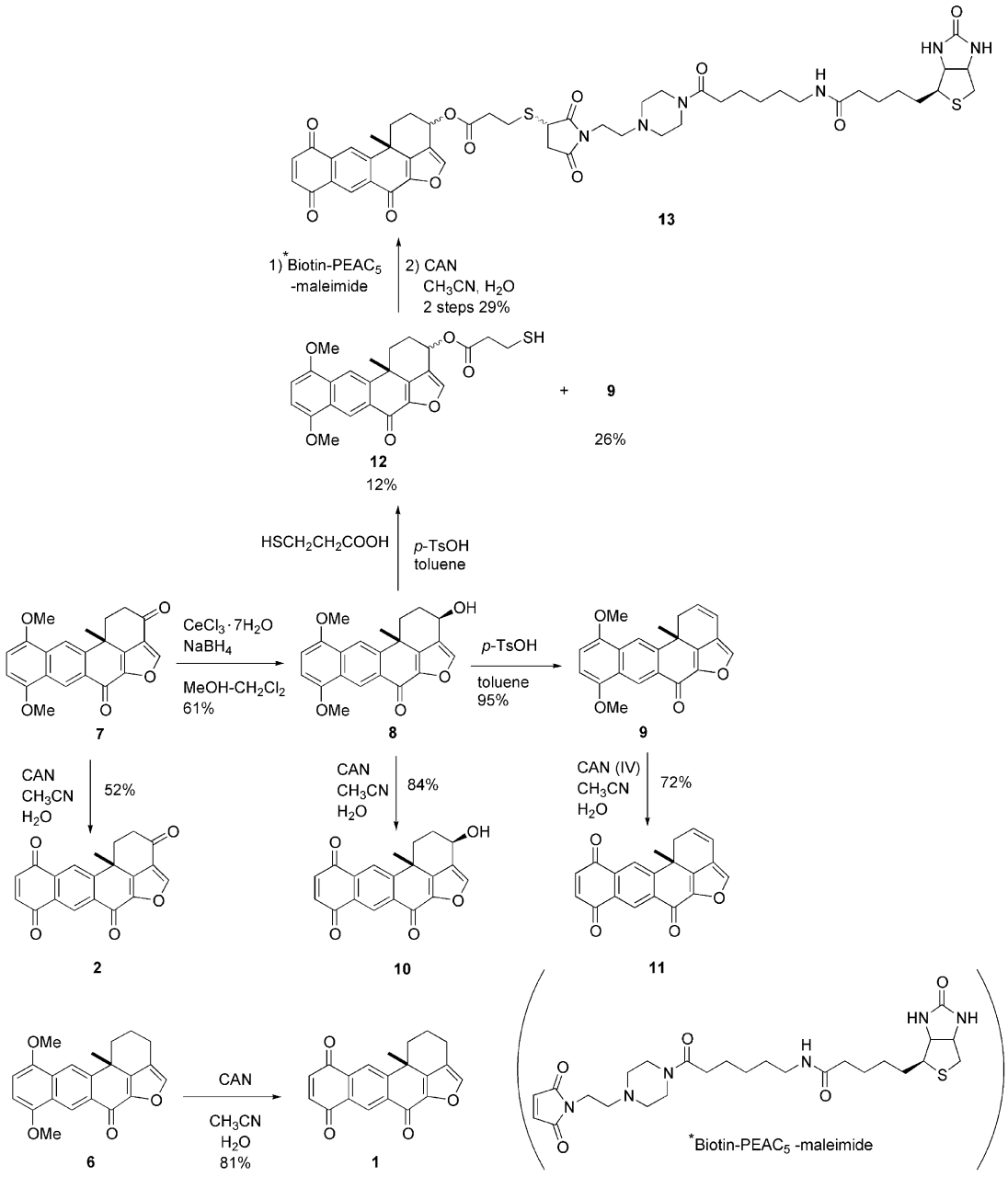


Figure 2. Effects of xestoquinone (**1**) and its analogues on myosin Ca^{2+} ATPase activity: (A) test at substance concentration of 132 μM; (B) test at substance concentration of 528 μM; (C) test at substance concentration of 2.64 mM. 'Wash' indicates that the well was washed with the assay buffer after incubation. The ordinate gives the relative phosphoric acid value; the phosphoric acid value corresponding to lack of inhibitor was designated as 100% (dashed line). Values are given as means ± SE ($n = 4$). Values significantly different from the control value (without inhibitor, filled bar) are indicated by asterisks (Student's t-test, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$).



Scheme 2. Derivatization of various xestoquinone analogues.

This is consistent with the findings of Sakamoto et al.,[4] who suggested that the quinone moiety of **1** was modified by the thiol groups of the Cys residues of myosin. Interestingly, only the olefinic analogue **11** exhibited strong inhibitory activity at a low concentration (528 μM); this novel molecule may be useful in studies of the ATPase activity of myosin.

Derivatization of a biotinylated analogue and its Ca^{2+} ATPase inhibitory activity

Because modifications at the A-ring structure far from the quinone moiety of **1** were not expected to affect myosin Ca^{2+} ATPase activity (Fig. 2) (as discussed above), a biotin tag was introduced at the position C-3 of **1**. Condensation of alcohol **8** with mercaptopropionic acid in the presence of an acid catalyst gave **12** and **9** with epimerization at the position C-3 (Scheme 2). The ester **12** was treated with biotin-$PEAC_5$ maleimide, and then oxidized by CAN, to give the biotinylated xestoquinone analogue **13**. Reversed-phase high-performance liquid chromatography (RP-HPLC) analysis of **13** gave two major and one minor peaks (asterisks in Fig. 3), and each fraction gave identical pseudomolecular ions at m/z 971.4 in MALDI-TOF-MS. Furthermore, the major peaks (29.6 and 30.4 min in Fig. 3) and the minor one (24.8 min in Fig. 3) were equilibrated each other within 18 h (data not shown), indicating that they were all isomers of **13**. We examined inhibition of myosin ATPase by the biotinylated analogue **13**, and found that its inhibitory activity was higher than that of **1** at a concentration of 2.64 mM (Fig. 2). This is further evidence that biotinylation at the position C-3 had little effect on inhibition of myosin Ca^{2+} ATPase, and again

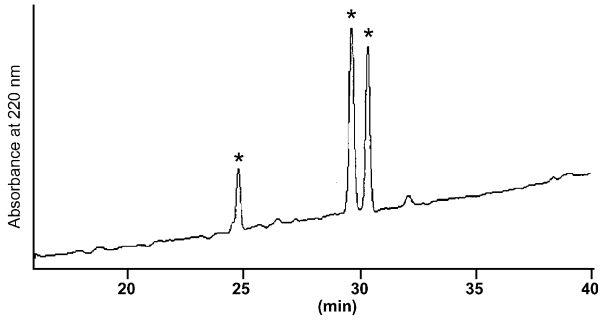


Figure 3. HPLC analysis of biotinylated analogue (**13**), which was eluted using a linear gradient of 20–80% acetonitrile containing 0.1% TFA in 60 min. Peaks were detected at 220 nm. Three peaks corresponding to each isomers of **13** are marked with an asterisk.

suggests that the quinone moiety of **1** is the structure that is essential for this inhibitory activity. Biotinylated compounds that retain original bioactivities are sometimes very useful in biochemical and physiological analysis. The novel biotinylated xestoquinone **13** may be useful as a probe in studies of myosin–xestoquinone interaction, particularly for determining the site where **1** binds to myosin.

Conclusion

We firstly identified the importance of the quinone moiety of xestoquinone (**1**) in inhibition of myosin ATPase, by analyzing structure–activity relationships of **1** and its derived analogues. Based on the resultant data, we then designed a biotin-tagged xestoquinone (**13**), in which the biotin tag was introduced at the position C-3 of **1**, far from the essential quinone moiety. The synthesized **13** exhibited myosin ATPase inhibitory activity that was equal to or stronger than that of **1**. We also found that the olefin **11** exhibited much stronger myosin ATPase inhibitory activity than **1**. The two novel compounds **11** and **13** may be useful for further studies of xestoquinone–myosin interaction.

Experimental

General procedures

Melting points were measured with a Yanaco MP-J3 micro melting point apparatus and uncorrected. IR spectra were obtained using a JASCO FT/IR-8300 spectrometer. ^{1}H NMR spectra were obtained using a Varian Gemini-2000 (300 MHz), Bruker ARX-400 (400 MHz) or Bruker AMX-600 (600 MHz) spectrometer. Chemical shifts (δ) were given in parts per million, relative to tetramethylsilane (δ 0.00) as an internal standard, and coupling constants (*J*) in Hz. MALDI-TOF mass spectrometry (MS) was performed by an Applied Biosystems Voyager DE Pro. High-resolution (HR) ESI-TOF-MS was performed using an Applied Biosystems Mariner Biospectrometry Workstation, using an angiotensin I/neurotensin mixture as a standard for internal calibration. Reactions were monitored using TLC (60F-254, Merck, Art 5715). Preparative TLC was performed using 60F-254 plates (Merck, Art 5774). Silica gel for column chromatography was obtained from Wakogel C-300 (Wako chemicals). RP-HPLC was performed using a JASCO 1500 system with a Develosil ODS-HG-5 column (4.6×250 mm, Nomura Chemicals). Preparative RP-HPLC was performed using a Develosil ODS-5 column (20×250 mm, Nomura Chemicals).

Extraction of crude material containing halenaquinol (3) and xestoquinol (5), and derivatization of xestoquinol dimethyl ether (6) and halenaquinol dimethyl ether (7). *X. sapra* was collected on 9 November 2000, at Akajima, Okinawa, Japan. The sponge was frozen immediately, and then stored at −30 °C until used. The sponge (739 g) was homogenized in a blender with acetone (1000 mL), and then filtered. The residue was washed twice with acetone (500 mL×2). The filtrates were combined and the solvent was then evaporated. The aqueous residue was extracted with EtOAc (500 mL×2, 200 mL×3), and the combined organic layers were dried over anhydrous $MgSO_4$. Evaporation of the solvent in vacuo gave a crude pitch-black oil, which contained **3** and **5** (3.9 g).

The pitch-black oil (3.9 g) was dissolved in acetone (60 mL), and anhydrous potassium carbonate (9.7 g) and iodomethane (36 mL) were then added under a nitrogen atmosphere. After being refluxed in dim light for 14 h, the reaction mixture was diluted with ethyl acetate (250 mL), washed with aqueous $NaHCO_3$ and brine, and then dried over anhydrous $MgSO_4$. Evaporation in vacuo and purification by chromatography on silica gel (CH_2Cl_2/Et_2O 9:1) afforded xestoquinol dimethyl ether (**6**, 30 mg, brown-orange powder) and halenaquinol dimethyl ether (**7**, 332 mg, lemon-yellow powder). **6**:[11] ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.54 (3H, s), 1.82 (1H, td, *J* = 13, 4 Hz), 2.10–2.23 (2H, m), 2.57–2.67 (2H, m), 2.88 (1H, dd, *J* = 17, 7 Hz), 3.98 (6H, s), 6.70 (1H, d, *J* = 8 Hz), 6.82 (1H, d, *J* = 8 Hz), 7.48 (1H, t, *J* = 1.4 Hz), 8.28 (1H, s) and 9.28 (1H, s). **7**:[18] ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.69 (3H, s), 2.34 (1H, td, *J* = 5, 13 Hz), 2.79–3.10 (3H, m), 4.00 (6H, s), 6.74 (1H, d, *J* = 8 Hz), 6.86 (1H, d, *J* = 8 Hz), 8.23 (1H, s), 8.32 (1H, s) and 9.31 (1H, s).

3-Hydroxyxestoquinol dimethyl ether (8). Halenaquinol dimethyl ether (**7**, 607 mg, 1.68 mmol) was dissolved in dichloromethane (50 mL) and methanol (50 mL), followed by addition of $CeCl_3 \cdot 7H_2O$ (6.4 g, 17.2 mmol). This mixture was stirred at room temperature for 10 min. Sodium borohydride (16.4 mg, 0.43 mmol) was then added, followed by stirring for 20 min, which was repeated three times. This reaction mixture was poured into water and extracted with ethyl acetate. The organic layer was washed with brine and evaporated in vacuo. The crude product was purified by column chromatography on silica gel (hexane/EtOAc 1:2) to give dimethyl ether **8** (373 mg, 61%, bright yellow powder):[18] ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD = 9:1) δ 1.63 (3H, s), 1.94 (1H, td, *J* = 13, 4 Hz), 2.20–2.30 (1H, m), 2,55 (1H, m), 2.63 (1H, dt, *J* = 4, 13 Hz), 3.98 (6H, s), 5.00 (1H, brt, *J* = 8 Hz), 6.71 (1H, d, *J* = 8 Hz), 6.83 (1H, d, *J* = 8 Hz), 7.78 (1H, d, *J* = 0.6 Hz), 8.24 (1H, s) and 9.27 (1H, s).

3,4-Dehydroxestoquinol dimethyl ether (9). Hydroxydimethyl ether (**8**, 14.3 mg, 0.039 mmol) and *p*-TsOH (1

mg, 5 μmol) in toluene (2 mL) were refluxed for 15 min under an argon atmosphere. The crude products were purified by preparative TLC (hexane/EtOAc 1:1) to give olefin dimethyl ether (**9**, 13 mg, 95%, yellowish powder). **9**:[12] ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.53 (3H, s), 2.67 (1H, brd, J = 17 Hz), 3.17 (1H, dd, J = 17, 6 Hz), 3.99 (6H, s), 6.12 (1H, m), 6.62 (1H, dd, J = 10, 2 Hz), 6.72 (1H, d, J = 8 Hz), 6.83 (1H, d, J = 8 Hz), 7.58 (1H, s), 8.27 (1H, s) and 9.31 (1H, s).

Xestoquinone (1). Xestoquinol dimethyl ether (**6**, 8.0 mg, 0.023 mmol) was dissolved in acetonitrile (2 mL), followed by addition of a solution of ceric ammonium nitrate (CAN) (IV) (37.2 mg, 0.0679 mmol) in H_2O (1 mL) at 0 °C, and this mixture was stirred for 10 min. This reaction mixture was washed with aqueous $NaHCO_3$ and brine, and then dried over anhydrous $MgSO_4$. Evaporation in vacuo gave a crude product, which was purified by preparative TLC (hexane/EtOAc 1:1) to give xestoquinone (**1**, 5.9 mg, 81%, bright brown powder). **1**:[11] ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.54 (3H, s), 1.76 (1H, td, J = 13, 5 Hz), 2.17–2.30 (2H, m), 2.55–2.71 (2H, m), 2.99 (1H, dd, J = 17, 8 Hz), 7.04 (1H, d, J = 11 Hz), 7.07 (1H, d, J = 11 Hz), 7.55 (1H, t, J = 1.5 Hz), 8.25 (1H, s) and 9.06 (1H, s).

Halenaquinone (2). Halenaquinol dimethyl ether (**7**, 13 mg) was oxidized with CAN (IV), as described above, to give halenaquinone (**2**, 6.2 mg, 52%, bright brown powder). **2**:[12] ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.70 (3H, s), 2.29 (1H, dt, J = 6, 12 Hz), 2.86 (2H, m), 3.03 (1H, m), 7.07 (1H, d, J = 12 Hz), 7.11 (1H, d, J = 12 Hz), 8.29 (2H, s) and 9.31 (1H, s).

(3*R*)-3-Hydroxyxestoquinone (10). 3-Hydroxyxestoquinol dimethyl ether (**8**, 10.0 mg) was oxidized with CAN (IV), as described above, to give quinone **10** (7.7 mg, 84%) as a bright brown powder: mp 225–228 °C; $[\alpha]_D^{25}$ +27 (*c* 0.15, $CHCl_3$–MeOH 1:9); UV (MeOH) λ_{max} 217 (ε 16,600), 256 (17,500), 292 (12,600) and 341 nm (6700); IR (KBr) ν_{max} 3536, 2360, 1668, 1671, 1601 and 1559 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$–CD_3OD 9:1) δ 1.64 (3H, s, H-20), 1.93 (1H, dt, J = 3.6, 13.3 Hz, H-5α), 2.25 (1H, ddt, J = 9.0, 3.6, 13.3 Hz, H-4β), 2.51 (1H, ddt, J = 13.3, 7.4, 3.6 Hz, H-4α), 2.62 (1H, dt, J = 13.3, 3.6 Hz, H-5β), 4.93 (1H, dd, J = 9.0, 7.4 Hz, H-3), 7.06/7.09 (1H each, d, J = 10.8 Hz, H-14 and 15), 7.87 (1H, d, J = 1.0 Hz, H-1), 8.23 (1H, s, H-18) and 9.00 (1H, s, H-11); ^{13}C NMR (100 MHz, $CDCl_3$–CD_3OD 9:1) δ 30.2 (t), 32.5 (t), 33.5 (q), 37.6 (s), 61.9 (d), 123.3 (d), 125.7 (s), 127.2 (d), 130.6 (s), 133.6 (s), 137.9 (s), 138.9 (d), 139.5 (d), 144.1 (s), 148.0 (d), 148.0 (s), 156.4 (s), 170.8 (s), 184.1 (s) and 184.9 (s); selected NOESY correlations H-3/H-4α, H-3/H-5α, H-4β/H-20, H-5β/H-20, H-4α/H-5α, H-4β/H-5β, H-5β/H-18, and H-18/H-20; HRMS (ESI-TOF) calcd for $C_{20}H_{15}O_5$ (M + H) 335.0914, found 335.0923. Anal. calcd for $C_{20}H_{14}O_5$ 2/3H_2O: C, 69.36; H, 4.45%; found: C, 69.30; H, 4.17%.

3,4-Dehydroxestoquinone (11). 3,4-Dehydroxestoquinol dimethyl ether (**9**, 31.2 mg) was oxidized with CAN (IV), as described above, to give quinone **11** (20.6 mg, 72%) as a dark yellow powder: mp 213–215 °C, $[\alpha]_D^{25}$ −55 (*c* 0.39, $CHCl_3$); UV (MeOH) λ_{max} 242 (ε 23,500), 295 (10,200) and 339 nm (sh, 6400); IR (KBr) ν_{max} 3071, 2361, 1772, 1670, 1653, 1559 and 1541 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.53 (3H, s, C-20), 2.60 (1H, brd, J = 16.6 Hz, H-5), 3.10 (1H, dd, J = 16.6, 6.2 H_2, H-5), 6.12 (1H, ddd, J = 9.7, 6.2, 2.1 Hz, H-4), 6.64 (1H, dd, J = 9.7, 3.0 Hz, H-3), 7.04/7.07 (1H each, d, J = 10.6 Hz, H-14 and 15), 7.62 (1H, s, H-1), 8.22 (1H, s, H-18) and 9.04 (1H, s, H-11); ^{13}C NMR (100 MHz, $CDCl_3$) δ 31.0 (q), 34.3 (t), 36.3 (s), 117.6 (d), 121.0 (s), 123.6 (d), 127.1 (d), 128.1 (d), 130.5 (s), 133.5 (s), 138.0 (s), 138.6 (d), 139.4 (d), 142.5 (d), 143.6 (s), 144.7 (s), 155.0 (s), 170.1 (s), 183.7 (s) and 184.6 (s); HRMS (ESI-TOF) calcd for $C_{20}H_{13}O_4$ (M + H) 317.0808, found 317.0794. Anal. calcd for $C_{20}H_{12}O_4$ H_2O: C, 71.85; H, 4.22%; found C, 71.57; H, 3.71%.

3-(3-Mercaptopropionyloxy)xestoquinol dimethyl ether (12). A solution of mercaptopropionic acid (92 μL, 1.06 mmol) and hydroxy dimethyl ether (**8**, 389.7 mg, 1.096 mmol) in toluene (80 mL) was refluxed for 1 h under an argon atmosphere. The crude products were purified by preparative TLC (hexane/EtOAc, 2:1), to give the SH compound (**12**, 29.5 mg, 12%, lemon-yellow powder) and olefin-dimethyl ether (**9**, 93.8 mg, 26%). The SH compound **12** was used immediately in the relevant reactions, because of its instability. **12**: mp 125–127 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.52 (1.5H, s), 1.64 (1.5H, s), 1.99 (0.5H, td, J = 13, 3 Hz), 2.17 (0.5H, td, J = 14, 3 Hz), 2.25–2.88 (7H, m), 3.98 (6H, s), 5.92 (0.5H, brt, J = 8 Hz), 5.99 (0.5H, d, J = 4 Hz), 6.71 (1H, d, J = 8.4 Hz), 6.83 (1H, d, J = 8.4 Hz), 7.79 (1H, s), 8.26 (0.5H, s), 8.27 (0.5H, s) and 9.27 (1H, s).

Biotinylated xestoquinone analogue (13). The SH compound (**12**, 29.5 mg, 0.065 mmol) was dissolved in CH_3CN/H_2O (1:1, 3 mL) with biotin-$PEAC_5$-maleimide (38.8 mg, 0.066 mmol, Dojindo Laboratories), and stirred at room temperature for 4.5 h. After the mixture was allowed to stand at 0 °C, a solution of CAN (IV) (107.7 mg, 0.196 mmol) in CH_3CN/H_2O (2:1, 0.3 mL) was added, and stirred for 20 min at 0 °C. This reaction mixture was filtered and lyophilized. The crude product was purified by preparative HPLC (eluted with a linear gradient of 20–80% acetonitrile in 0.1% TFA in 60 min, 5 mL/min) to collect the major peaks to give biotin compound **13** (18.1 mg, 29%, lemon-yellow powder). The equilibrium experiments of **13** were performed as follows. Each HPLC peak (Fig. 3) was lyophilized, dissolved in CH_3CN/H_2O (1:1), incubated at rt for 18 h, and analyzed by RP-HPLC. **13**: analytical RP-HPLC; t_R = 24.8, 29.6 and 30.4 min (linear gradient, 20–80% acetonitrile in 0.1% TFA in 60 min, 0.8 mL/min); mp 140–144 °C, $[\alpha]_D^{25}$ −21 (*c* 0.91, $CHCl_3$–MeOH 1:1); UV (MeOH) λ_{max} 216 (ε 21,200), 255 (18,400), 289 (13,500) and 336 nm (7000); IR (KBr) ν_{max} 1702, 1675, 1635, 1459, 1440, 1321, 1241 and 1137 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$–CD_3OD 4:1) δ 1.36 (2H, m), 1.43 (2H, m), 1.52 (2H, m), 1.56/1.68 (1.5H each, s, H-20), 1.62 (2H, m), 1.61–1.74 (4H, m), 1.94 (0.5H, dt, J = 4.1 and 13.6 Hz, H-5), 2.12 (0.5H, brt, J = 12.6 Hz, H-5), 2.20 (2H, m), 2.34 (2H, m), 2.38-2.55 (6H, m), 2.55-2.73 (5.5H, m), 2.74 (1H, d, J = 12.9 Hz), 2.85 (0.5H, m), 2.93

(1H, dd, $J=12.9$, 5.0 Hz), 2.98 (0.5H, m), 3.08 (0.5H, m), 3.13–3.30 (5H, m), 3.45 (2H, m), 3.56 (2H, m), 3.62–3.72 (2H, m), 3.80 (0.5H, m), 3.88 (0.5H, m), 4.32 (1H, dd, $J=7.8$ and 4.5 Hz), 4.52 (1H, dd, $J=7.8$ and 4.9 Hz), 5.92 (0.5H, t, $J=8.1$ Hz, H-3α), 5.99 (0.5H, d, $J=4.9$ Hz, H-3β), 7.11 (2H, s, H-14 and 15), 7.90 (0.5H, d, $J=2.9$ Hz, H-1), 7.93 (0.5H, d, $J=2.9$ Hz, H-1), 8.28/8.29 (0.5H each, s, H-18) and 9.02/9.03 (0.5H each, s, H-11); ^{13}C NMR (150 MHz, $CDCl_3$–CD_3OD 4:1) δ 24.9 (t), 25.8 (t), 26.1 (t, C-4), 26.6/26.9 (0.5C each, t), 26.8 (t), 28.0/32.6 (0.5C each, t, C-5), 28.3 (t), 28.6 (t), 29.1 (t), 32.2/33.2 (0.5C each, q, C-20) 33.2 (t), 34.6 (t), 36.0 (3C, t), 37.4/37.5 (0.5C each, s, C-6), 39.3 (t), 39.5 (d), 40.5 (t), 41.8 (t), 45.8 (t), 52.9 (t), 53.1 (t), 54.7 (t), 55.8 (d), 60.4 (d), 62.2 (d), 62.8 (0.5C, d, C-3 of α-OCOR isomer), 64.7 (0.5C, d, C-3 of β-OCOR isomer), 121.6/122.0 (0.5C each, s, C-2), 123.3/123.7 (0.5C each, d, C-18), 127.2/127.3 (0.5C each, d, C-11), 130.8 (s, C-12), 133.8 (s, C-17), 137.2 (s, C-8), 137.7/137.8 (s, C-10), 139.1/139.6 (1C each, d, C-14 and 15), 140.8/141.6 (0.5C each, s, C-19), 149.1/149.9 (0.5C each, d, C-1), 155.7/156.0 (0.5C each, s, C-7), 164.3 (s), 171.0 (s, C-9), 171.8/172.2 (0.5C each, s, COO), 172.4 (s, CON<), 174.3 (s, CONH), 175.3/177.1 (1C each, s, CONCO), 184.2 (s, C-13) and 184.9 (s, C-16); MS (ESI-TOF) (rel. int.) m/z 993.42 $(M+Na)^+$ (8), 971.43 $(M+H)^+$ (51), 497.22 $(M+H+Na)^{2+}$ (18), 486.22 $(M+2H)^{2+}$ (100); HRMS (ESI-TOF) calcd for $C_{49}H_{60}N_6O_{11}S_2$ (M + 2H) 486.1875, found 486.1884 $(M+2H)^{2+}$. Anal. calcd for $C_{49}H_{58}N_6O_{11}S_2$ $3CF_3COOH$: C, 50.30; H, 4.68%; found C, 50.70; H, 4.62%.

Ca^{2+} ATPase assays. Malachite green reagent was prepared as reported.[16,17] Multi-well plates (96-well Maxisorp plate, Nunc) were coated (25 μL/well) with 6.6 nmol rabbit skeletal muscle myosin (SIGMA) in Tris–HCl buffer (20 mM Tris–HCl, 150 mM NaCl, pH 7.4) for 12 h at 4 °C. Wells that were not coated with myosin were used as blanks. The wells were washed with the assay buffer (20 mM $CaCl_2$, 100 mM Tris–HCl and 660 mM KCl, pH 7.6), and were then incubated at 4 °C for 1 h with 25 μL of various concentrations of xestoquinone analogues in the assay buffer. The well treated with xestoquinone (**1**) was also washed twice with the ice-cold assay buffer after incubation for 'wash' (see Fig. 2). Twenty-five microliters of 1 mM ATP were added, and incubated for 20 min at 37 °C, and then added 200 μL of malachite green reagent. Absorption at a wavelength of 630 nm was measured within 5 min using a microplate reader (Wako Spectra Max 250, Wako Chemicals). Four independent measurements were performed for each set of conditions.

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