Synthesis of Bioreductive Esters from Fungal Compounds

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Four new bioreductive esters (7—10) have been synthesized. Their structures composed of trimethyl lock containing quinone propionic acid with an ester linkage to the fungal cytotoxic compounds; preussomerin G (1), preussomerin I (2), phaseolinone (3) and phomenone (4). The synthesized esters are aimed to act *via* reductive activation specifically at the cancer cells, resulting from hypoxia and overexpression of reductases. Hence, the toxicity will be lessened during distribution across the normal cells. The anticancer activity was determined in cancer cell lines with reported reductase *i.e.*, BC-1 cells and NCI-H187 as well as in non-reductase containing cancer cells; KB cells. When considering each cell lines, result showed that structure modification giving to 7—10 led to less cytotoxicity than their parent compounds (1—4). Both 7 and 8 were strongly cytotoxic (IC$_{50}$≤5 μg/ml) to NCI-H187, whereas 9 and 10 were moderately cytotoxic (IC$_{50}$=6—10 μg/ml) to BC-1 cells. Additional study of stability of represented phenolic ester (8) and an alcoholic ester (9) were performed. Result illustrated that both 8 and 9 were stable in the presence of esterase. Therefore, the cytotoxicity of the synthesized compounds (8—10) might be due to partial bioreductive activation in the cancer cells.

Key words　bioreductive ester; fungal compound; cytotoxic; preussomerin I; preussomerin G; phaseolinone; phomenone

　　Thailand is known for its biodiversity of natural herbs and fungi. As a consequence, vast varieties of chemical compounds have been discovered. Previously, many cytotoxic compounds such as those found in the fungal extracts have been screened for anticancer activity.[1,2] Preussomerin G, **1** and preussomerin I, **2** were purified from *Xylaria* sp. 1067, while (+)-phaseolinone, **3** and (+)-phomenone, **4** were purified from Lichenicolous Fungus *Microsphaeropsis* sp. BCC 3050[1,2] (Fig. 1). Their cytotoxicities to cancer cell lines were reported as well as in the normal cells. **1**, **2**, **3** and **4** were found to exhibit strong cytotoxicity to the KB and BC-1 cancer cells, and also showed toxicity to the Vero cells', which represented normal cells.[1,2] This led to a limitation for further clinically study or for biopharmaceutical uses. More effort to decrease this bottle neck problem of drug discovery by adopting the bioreductive concept was thus attempted. Therefore, in this study, structure modification of **1**—**4** based on bioreductive activation has been adopted to lessen toxicity.

　　Solid tumors have been reported to be hypoxia and overexpression of reductase enzymes when compared to those in the normal cells.[3] Irregular shape of the blood vessels and condensed mass in the tumor leads to low levels of oxygen and nutrient supply, while hypoxia leads to resistance to radiation therapy.[4] The rational design of the bioreductive anticancer agents uses these properties as advantages for controlling the bioreductive activation of the bioreductive anticancer agents aiming to specifically release a potent drug only at the tumor sites. Hence this reduces toxicity during systemic distribution.

　　Quinone delivery systems containing a trimethyl lock have been designed to release toxic moieties selectively and preferentially under reductive/hypoxic conditions[5—9] (Fig. 2). A conformationally constrained bioreductive promoiety was attached to a model anticancer agent, melphalan, to create a tumor targeted drug delivery system (TDDS).[5,6] The TDDS was a substituted benzoquinone, which can also attach to any drug of interest *via* an ester or amide linker.[5—9] The conformational constraint in the quinone-containing promoiety was created by the presence of the *gem*-dimethyl group on the propionic acid spacer linking the drug and the promoiety; and the adjacent methyl group on the quinone ring ("trimethyl lock"). Upon reductive activation, the quinone was converted to its hydroquinone. The lone pair electrons form the *ortho*-OH was used in an intramolecular cyclization
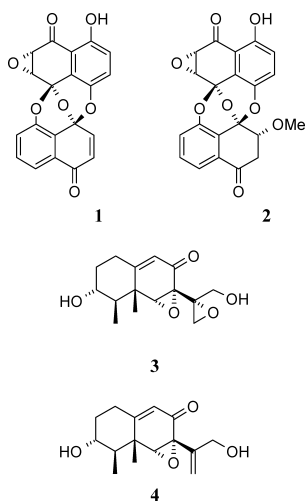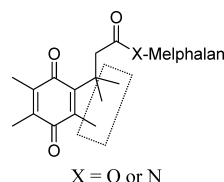

Fig. 1.　Fungal Extract Compounds

Fig. 2.　Structure of Quinone Bioreductive Agent Containing a Trimethyl Lock as Specified by Dotted Line[5,6]
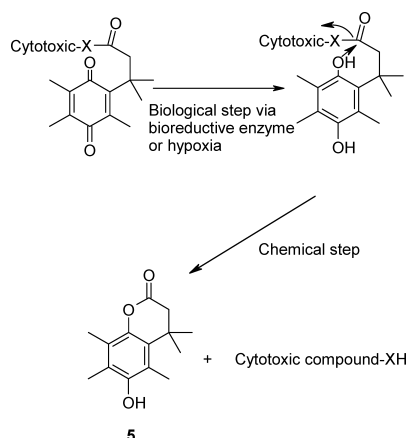

Fig. 3. Schematic Representing Anticipated Bioreductive Activation of Bioreductive Agent (Where X is O or N) in the Cancer Cells Containing Reductase Enzyme
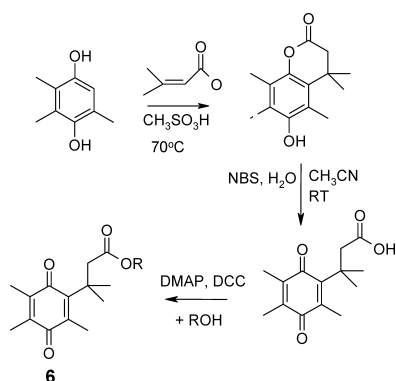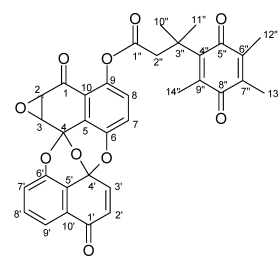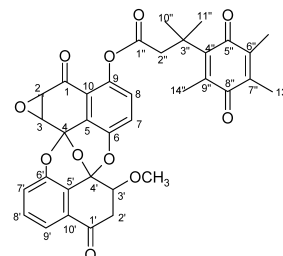
Adapted from refs. 5, 6.

Fig. 4. Synthesis Steps for Bioreductive Esters when R is the Fungal Cytotoxic Compounds 1—4

Fig. 5. Structures of Synthesized Bioreductive Esters from Fungal Compounds

to release the potent drug resulting in the formation of non-toxic lactone species (Fig. 3).[5,6] The drug will only be released efficiently in the presence of the tri-methyl lock system. The trimethyl lock-containing structure has been reported to undergo bioreductive activation with rate of reaction giving potential specific delivery of many types of model compounds such as amines, amino acids, peptides, alcohol, fluorophores and other anticancer agents.[7,9,10—19]

Various substituents on the benzoquinone ring with different electronic properties such as a methyl-substituted benzoquinone were reported to enhance reductase enzyme selectivity and stability of the bioreductive promoiety, while redox properties govern the rate of release of the potent drug.[5,6] Therefore, a methyl-substituted benzoquinone containing a trimethyl lock was selected as a template for the design of a new bioreductive agent from fungal cytotoxic compounds. Based on the hydroxyl-containing structure of selected fungal compounds (1—4), structure modification as bioreductive agent has been designed by using an ester linkage. In this study, four bioreductive agents were synthesized and structures were elucidated by using spectrometry. Then, anticancer activities in cancer cells as well as Vero cells were evaluated in comparison to their parent compounds.

Discussion and Conclusion

The structures of the bioreductive ester of fungal cytotoxic compounds are shown in Fig. 5. It should be noted that 2.5 and 4 eq of quinone propionic acid (6) was required for the reaction with 3 and 4 to get the ester products 9 and 10, respectively. When an attempt to use a lower equivalent of 6 (1 eq) was conducted as in the synthesis of bioreductive esters 7 and 8, no bioreductive ester product was formed. After structure elucidation, it was found that 9 and 10 were coupled with two molecules of quinone propionic acid. That might be due to the structures of 3 and 4 possessing free –OH on both sides of their structures.

The standard anticancer agents, ellipticin and doxorubicin were used as positive controls. It was found that ellipticin and doxorubicin were strongly cytotoxic in all cancer cell types as well as in the Vero cell line. Upon bioreductive activation of the synthesized compounds, it is supposed that the ester compound is cleaved at ester bond and releases the cytotoxic compound as well as its lactone by-product (Fig. 3). Results showed that lactone, 5 exhibited no cytotoxicity as the IC_{50} was higher than 50 μg/ml. The cytotoxicity of 6 was also determined in case it might be presented in the cell because of an unpredicted reaction. It was found that 6 did not show any cytotoxicity as it was inactive (IC_{50}>50 μg/ml) in all cell lines studied.

Compound 8
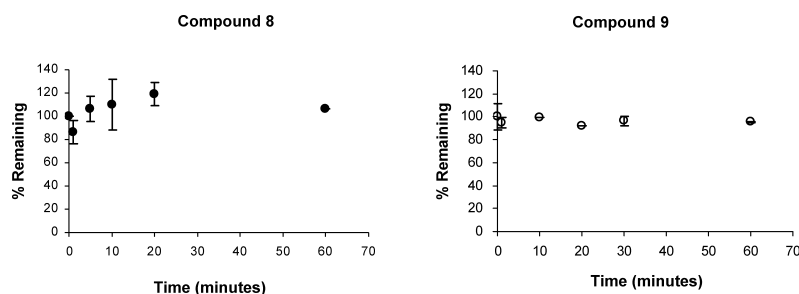
Compound 9

Fig. 6. Stability Profile of Compounds **8** and **9** in the Presence of Esterase under 0.05 M Phosphate Buffer Solution, pH 7.4 at 37 °C Which Was Determined by Gas Chromatography Spectrometer

All data are average of three determinations±S.E.M. The % remaining on Y axis was calculated from the percent of peak area ratio between bioreductive ester and the internal standard caffeine compared to the peak area of the bioreductive ester in an absence of esterase enzyme.

The bioreductive ester of fungal compounds, **7**, **8**, **9** and **10** demonstrated lesser cytotoxicity in normal cells (*e.g.*, Vero cell) compared to their parents; **1**, **2**, **3**, and **4**, respectively. When considering of cytotoxicity in non reductase-containing cancer cells (*e.g.*, KB cells) between the synthesized bioreductive esters and their parents (**1** *vs.* **7**; **2** *vs.* **8**; **3** *vs.* **9** and **4** *vs.* **10**), the synthesized bioreductive esters were less toxic than their parents.

It was found that, **1** was strongly toxic to all cell lines with IC_{50} ranged from 0.1—0.4 µg/ml. The KB cell (non reductase-containing cell) and NCI-H187 cancer cells (a reductase-containing cells) were more sensitive to **1** than other cancer cell lines. After structural change into bioreductive ester (**7**), the NCI-H187 was the highest sensitive cancer cells to **7** giving IC_{50} of 0.82 µg/ml, while the KB cells was less sensitive giving IC_{50} of 1.15 µg/ml. **2** was strongly toxic to all cell lines with IC_{50} ranged from 0.1—0.6 µg/ml. The NCI-H187 cancer cell was shown to be the most sensitive to **2** than the KB cell and other cancer cell lines. After structural change into **8**, the NCI-H187 was the highest sensitive cancer cells to **8** yielding IC_{50} of 1.22 µg/ml, whereas the KB cells yielding IC_{50} of 4.8 µg/ml.

3 was toxic to all cell lines with IC_{50} ranged from 0.7—1.5 µg/ml. The BC-1 cancer cells (a reductase-containing cells) was highly sensitive to **3**. After structural change into **9**, the BC-1 cell was the highest sensitive cells to **9** giving IC_{50} of 5.3 µg/ml. **4** was toxic to KB and BC-1 cell lines with IC_{50} ranged from 0.51—1.2 µg/ml. The BC-1 cancer cells (a reductase-containing cells) was highly sensitive to **4** compared to the KB cells (non-reductase-containing cancer cells). After structural change into **10**, the BC-1 cell was the highest sensitive cells to **10** giving IC_{50} of 7.3 µg/ml.

When compare the chemical structures, the parent phenolic compounds (**1**, **2**) were generally more potent than the parent alcoholic compounds (**3**, **4**). And the same pattern of cytotoxicity was also observed for the synthesized bioreductive esters from the same chemical structures. In other word, the chemosensitivity to the cancer cells of the same set of chemical structures (*e.g.*, a set of **1**, **2**, **7**, **8**; and a set of **3**, **4**, **9**, **10**) were similar in each sets. This may be due to the compounds in each sets are structural related and thus demonstrating intuitive chemosensitivity to the same cancer cell line.

In general, phenolic esters are more easily hydrolyzed than alcohol ester. Our result demonstrated that the synthesized bioreductive esters **7** and **8** illustrated more cytotoxicity to

Table 1. Cytotoxicity Results for Compounds **1**—**10**

Compounds	#	IC_{50} (µg/ml)			
		Vero cells	KB cells	BC-1 cells	NCI-H187
Standard ellipticine		0.6	0.27	0.21	0.15—0.49
Standard doxorubicin		1.9	0.17	0.14	0.02—0.04
Lactone	(**5**)	>50	>50	>50	>50
Quinone propionic acid	(**6**)	>50	>50	>50	>50
Preussomerin G	(**1**)	0.1	0.4	0.7	0.4
Preussomerin G ester	(**7**)	1.3	1.15	2.9	0.82
Preussomerin I	(**2**)	0.1	0.5	0.6	0.2
Preussomerin I ester	(**8**)	0.6	4.8	2.14	1.22
Phaseolinone	(**3**)	0.7	1.0	0.9	1.5
Phaseolinone ester	(**9**)	4.4	7.6	5.3	7.04
Phomenone	(**4**)	nd	1.2[a]	0.51[a]	nd
Phomenone ester	(**10**)	9.5	12.3	7.3	>20

Values were means of three experiments (nd=not determined). Data interpretation: IC_{50} (µg/ml)>20=inactive, 10—20=weekly active, 6—10=moderately active and ≤5=strongly active. Cytotoxicity test in Vero cell, KB cells and BC-1 cells were performed by using sulforhodamine B (SRB) assay, while cytotoxicity test in NCI-H187 cells were using MTT assay. *a*) Data was obtained from ref. 4.

the cancer cells studied compared to **9** and **10**. To rule out that possible hydrolysis reaction, the stability of the bioreductive esters was thus performed. Due to the low quantity of the ester product from the synthesis were obtained, the ester compound **8** and **9** were selected for further stability test. The structure of **8** represented a phenolic bioreductive ester, while **9** represented an alcoholic bioreductive ester. The stability of **8** and **9** was conducted in the presence of the esterase enzyme in a phosphate buffer solution (0.05 M) pH 7.4. The degradation was determined by using gas chromatography (GC) spectrometer to detect a decrease peak area of **8** or **9** and an increase peak area of lactone that might be occurred *via* the same route of bioreductive activation. Result showed that **8** and **9** were stable under the condition studied (Fig. 6). There was no decreasing of the peak area of **8** or **9** and no lactone peak or any new peak observed based on the GC chromatogram.

Structural modification at the hydroxyl group of fungal compounds *via* the ester linkage led to less cytotoxicity in all cancer cell lines studied either possessing or not possessing reductase activity. It is speculated that the ester linkage at the hydroxyl group of the cytotoxic compound somehow influenced the toxic activity of fungal compounds. The difference in reductase enzymatic activity between cell lines has been suggested to be responsible for their variable sensitivity to

the cytotoxicity of antitumor quinones.[20] Different cell lines might possess different reductase levels and each compound might have different susceptibility to the bioreductive activation. When consideration of the reductase-containing cancer cells (*e.g.*, BC-1 and NCI-H187 cells), almost all of the synthesized bioreductive esters showed less toxicity than their parents. The synthesized esters may undergo partial bioreductive activation in reductase-containing cancer cells (*e.g.*, BC-1 and NCI-H187 cells) and hence less toxic moieties were released in the cancer cells compared to what happened to their parent compounds. However, another possibility that the new esters themselves expressed the cytotoxicity regardless the bioreductive activation could not be ruled out. Toxicity of the synthesized bioreductive esters is inconclusive and more experimental studies need to be performed. Therefore, further experiments regarding reductase activated bioreduction of the esters has been now performed in our laboratory.

Experimental

General Experimental Procedure The melting points were measured with an Electrothermal digital 900 melting point apparatus (U.K.). The IR spectra were determined with a FT/IR spectrophotometer Spectrum One (PerkinElmer, U.K.) by a KBr disk method. ^{1}H- and ^{13}C-NMR were recorded using a NMR Varian AS 400 NMR spectrometer operating at 400 mHz with residual chloroform (CDCl$_3$) as an internal reference, and chemical shifts are expressed in δ (ppm). TOF mass spectra were measured with a Micromass LCT mass spectrometer (U.S.A.). Optical rotations were measured using a JASCO DIP-1000 polarimeter. Preparative HPLC was performed on a WATER 600 instrument (WATERS 996 photodiode array detector). TLC was conducted with Kieselgel 60 F$_{254}$ plates (Merck, Germany). Silica gel GF 254 (Merck, Germany) and Sephadex LH 20 (Amersham Bioscience, Sweden) were used for column chromatography. Gas chromatography spectrometer was performed on a GC (Hewlett® Packard, HP 6890 series), HP-5 Column (Hewlett® Packard, 5% diphenyl and 95% methylpolysiloxane, film thickness 0.25 μm, length 30 m, column ID 0.32). Nitrogen, helium, hydrogen and oxygen gases were from Harris Calorific.

Extraction, Separation and Purification of Preussomerin G (1) and Preussomerin I (2) Separation and purification of **1** and **2** followed the method of Seephonkai *et al.*[1] Briefly, *Microsphaeropsis* sp. BCC 3050 was macerated with 1 l methanol at room temperature for 2 d. The methanolic extract was mixed with water and washed with hexane. The methanol layer was evaporated to concentrate and was dissolved in ethyl acetate. The crude extract from the ethyl acetate layer was further separated by using column chromatography (CC) (3.5×26 cm) using Sephadex LH 20 as the stationary phase and 100% methanol as the mobile phase. Fraction numbers 150—325 were collected for further CC separation by using a silica gel column with stepwise gradient elution: dichloromethene/hexane in order of 20 : 80, 30 : 70, 40 : 60, 50 : 50, 80 : 20, respectively. Four fractions were collected and fraction number 2 contained **1**, while fraction number 3 contained **1**, **2** and other compounds. Fraction number 2 was purified by using a silica gel column with gradient mobile phase: dichloromethene/hexane to get pure compound, **1**. Fraction number 3 was purified by using silica gel column with gradient mobile phase: CH$_2$Cl$_2$/hexane to get pure compound, **2**. The products **1** and **2** were yellow crystals. The structure elucidation of **1** and **2** were identified and were found to be identical to those previously reported.[1]

Extraction, Separation and Purification of Phaseolinone (3) and Phomenone (4) Separation and purification of **3** and **4** followed the method of Isaka *et al.*[2] Briefly, *Xylaria* sp. BCC 1067 was macerated with ethyl acetate. The ethyl acetate crude extract was separated by using two column chromatography systems; Sephadex LH 20 as the stationary phase and 100% methanol as the mobile phase; and silica gel 60H with a gradient mobile phase (100% dichloromethene-dichloromethene/methanol=95 : 5). Preparative HPLC with a reverse phase column (Prep Nova-Pak® HR C18 column, 6 μm, 40×100 mm), 80% water/20% methanol as mobile phase, flow rate of 10 ml/min, and UV-detector at 220 nm was conducted. Pure (+)-phaseolinone, **3** and (+)-phomenone, **4** were eluted at a retention time of 14 min and 23 min, respectively. The products **3** and **4** were colorless crystals. The structure elucidation of **3** and **4** were identified and were identical to those previously reported.[2]

Synthesis of Bioreductive Agents A schematic diagram of the synthe-

sis steps of the bioreductive agent is shown in Fig. 4.

Lactone (5)[5,7—9] Trimethylhydroquinone 0.5 g (3.3 mmol) was added into methanesulfonic acid 5 ml followed by the addition of 3,3-dimethylacrylic acid 0.4 g (4 mmol). The mixture was allowed to react at 70 °C for 30 min, and then cooled to room temperature. The reaction mixture was diluted with water (65 ml) and extracted three times with 25 ml dichloromethane followed by water, saturated sodium chloride and dried over anhydrous sodium sulphate. Solvent removal of dichloromethane by rotary evaporator and recrystallization from hexane afforded the pure lactone **5** as white crystals in 72% yield (1.1 g). Structure elucidation was confirmed by using IR, HR-MS and NMR. ^{1}H-NMR (CDCl$_3$) δ: 1.48 (6H, s, H-10″-11″), 2.22 (3H, s, H-12″), 2.25 (3H, s, H-13″), 2.39 (3H, s, H-14″), 2.58 (2H, s, H-2″), 4.8 (1H, s, 8″-OH). ^{13}C-NMR (CDCl$_3$) δ: 12.3, 12.5, 14.4, 27.7, 35.5, 46.1, 119.0, 121.9, 123.4, 128.2, 143.5, 148.8, 168.9. IR (KBr) cm^{-1}: 3441, 2962, 1743, 1614, 1418, 1295, 1261, 1192. HR-MS (ESI-TOF, negative) *m/z*: 257.1164 [M+Na]$^+$, Calcd for C$_{14}$H$_{18}$O$_3$Na 257.1104. MS *m/z*: 234 (M$^+$).

Quinone Propionic Acid (6)[5,7—9] *N*-Bromosuccinimide (NBS) 0.3 g (1.7 mmol) was dissolved in 30 ml of water. Lactone solution was prepared by dissolving 0.3 g (1.3 mmol) of lactone in 45 ml of acetonitrile. NBS solution was added into the lactone solution. The reaction mixture was allowed to react at room temperature for 45 min, then 75 ml of diethyl ether was added. The ether layer was extracted with 5% sodium bicarbonate 75 ml, 2 times. The pH of sodium bicarbonate was adjusted to acidic pH, then extracted with 30 ml of diethyl ether, three times. The ether layer was dried over anhydrous sodium bicarbonate. Solvent was removed by rotary evaporation. Recrystallization in acetonitrile afforded yellow crystalline quinone propionic acid **6** in 63% yield (0.2 g). Structure elucidation was confirmed by using IR, MS and NMR. ^{1}H-NMR (CDCl$_3$) δ: 1.43 (6H, s, H-10″-11″), 1.93 (3H, s, H-12″), 1.95 (3H, s, H-13″), 2.14 (3H, s, H-14″), 3.02 (2H, s, H-2″). ^{13}C-NMR (CDCl$_3$) δ: 12.0, 12.4, 14.2, 28.8, 37.9, 47.0, 138.4, 139.0, 142.9, 151.9, 177.1, 187.4, 190.8. IR (KBr) cm^{-1}: 3434, 2971, 1706, 1645, 1436, 1379, 1226. HR-MS (ESI-TOF, negative) *m/z*: 273.1095 [M+Na]$^+$, (Calcd for C$_{14}$H$_{18}$O$_4$Na 273.1103). MS *m/z*: 250 (M$^+$).

Bioreductive Esters The syntheses of bioreductive ester compounds were accomplished within one step by esterification of purified fungal compounds with quinone propionic acid, **6** based on the previously reported methods with modification (Fig. 4).[5,7—9,21]

Preussomerin G Ester (7) and Preussomerin I Ester (8) Quinone propionic acid (53 mg, 0.2 mmol) was dissolved in dry dichloromethane and reacted with preussomerin G or preussomerin I (0.05 mmol) under nitrogen gas. 4-(*N*,*N*-dimethylamino)pyridine (DMAP) (0.04 mmol) was added into reaction mixture followed by *N*,*N*′-dicyclohexylcarbodiimide (DCC) (0.26 mmol) in dry dichloromethane solution. The resulting reaction mixture was stirred at 40 °C for 2 h with observed formation of a white precipitate. The filtrate was collected after filtering, cold 1 N HCl was added and it was then extracted with dichloromethane and adjusted to neutral pH by 5% sodium bicarbonate. The aqueous layers was washed with dichloromethane and dried over anhydrous sodium sulphate. Solvent was removed by rotary evaporation. The residue was purified by column chromatography (Sephadex LH 20: 100% methanol) giving yellow crystals of **7** and **8**. Percent yields of **7** and **8** were 38% (12 mg) and 80% (24 mg), respectively. Structure elucidation was confirmed by using IR, MS and NMR.

Preussomerin G Ester (**7**) (Chart 1): ^{1}H-NMR (CDCl$_3$) δ: 1.51 (3H, s, H-10″), 1.59 (3H, s, H-11″), 1.89 (6H, s, H-12″, 13″), 2.18 (3H, s, H-14″), 3.22 (H, d, *J*=17 Hz, H-2″), 3.40 (1H, d, *J*=17 Hz, H-2″), 3.81 (1H, d, *J*=4.3 Hz, H-2), 4.19 (1H, d, *J*=4.3 Hz, H-3), 6.60 (1H, d, *J*=9.75 Hz, H-2′), 6.98 (1H,

7

Chart 1

Chart 2

d, $J=8.58\,\text{Hz}$, H-8), 7.01 (1H, d, $J=8.17\,\text{Hz}$, H-7′), 7.04 (1H, d, $J=8.58\,\text{Hz}$, H-7), 7.21 (1H, d, $J=9.75\,\text{Hz}$, H-3′), 7.40 (1H, dd, $J=8.17$, 6.6 Hz, H-8′), 7.62 (1H, d, $J=6.63\,\text{Hz}$, H-9′). ^{13}C-NMR (CDCl$_3$) δ: 12.0, 12.5, 14.3, 28.9, 29.1, 38.1, 47.3, 52.1, 52.6, 53.4, 89.9, 93.9, 119.0, 120.0, 120.9, 121.0, 122.8, 127.1, 130.2, 131.2, 133.6, 138.6, 139.2, 140.4, 142.7, 142.8, 148.1, 148.7, 151.8, 171.4, 183.6, 187.5, 189.5, 190.7. IR (KBr) cm^{-1}: 2963, 1725, 1645, 1439, 1187, 1136. HR-MS (ESI-TOF, negative) m/z: 617.1424 [M+Na]$^+$, Calcd for C$_{34}$H$_{26}$O$_{10}$Na 617.1424. MS m/z: 594 (M$^+$). [α]$_D^{28}$: +65° (c=0.0045, acetonitrile).

Preussomerin I Ester (**8**) (Chart 2): ^{1}H-NMR (CDCl$_3$) δ: 1.51 (3H, s, H-10″), 1.58 (3H, s, H-11″), 1.89 (6H, s, H-12″, 13″), 3.10 (1H, dd, $J=17.9$, 2.7 Hz, H-2′a), 3.22 (1H, d, $J=16.7\,\text{Hz}$, H-2″a), 3.37 (1H, dd, $J=17.9$, 2.7 Hz, H-2′b), 3.38 (1H, d, $J=17.1\,\text{Hz}$, H-2″b), 3.53 (3H, s, 3′-OCH$_3$), 3.80 (1H, d, $J=4.3\,\text{Hz}$, H-2), 4.25 (1H, d, $J=4.3\,\text{Hz}$, H-3), 4.33 (1H, t, $J=2.7\,\text{Hz}$, H-3′), 7.00 (1H, d, $J=8.9\,\text{Hz}$, H-8), 7.04 (1H, d, $J=8.9\,\text{Hz}$, H-7), 7.05 (1H, d, $J=8.5\,\text{Hz}$, H-7′), 7.39 (1H, t, $J=8\,\text{Hz}$, H-8′), 7.65 (1H, d, $J=7.8\,\text{Hz}$, H-9′). ^{13}C-NMR (CDCl$_3$) δ: 12.1, 12.5, 14.3, 28.9, 29.1, 38.1, 40.3, 47.3, 52.6, 53.4, 59.3, 79.1, 93.5, 94.7, 119.3, 120.5, 120.8, 121.9, 122.5, 127.1, 130.7, 131.3, 138.6, 139.2, 142.4, 142.7, 142.9, 147.6, 149.7, 151.8, 171.4, 187.4, 189.7, 190.7, 193.2. IR (KBr) cm^{-1}: 2927, 1761, 1696, 1642, 1476, 1284, 1107. HR-MS (ESI-TOF, negative) m/z: 649.1699 [M+Na]$^+$, Calcd for C$_{35}$H$_{30}$O$_{11}$Na 649.1686. MS m/z: 626 (M$^+$). [α]$_D^{28}$: −258° (c=0.0044, acetonitrile).

Phaseolinone Ester (9) or Phomenone Ester (10) Synthesis of phaseolinone ester (**9**) or phomenone ester (**10**) was similar to the method used for synthesis of **7** and **8**. For synthesis of phaseolinone ester (**9**), quinone propionic acid (0.08 mmol) in dry dichloromethane, phaseolinone (11.4 mg, 0.03 mmol), DMAP (0.011 mmol) and DCC (0.11 mmol) were used for the reaction. Preparative thin layer chromatography was used to purify the residue of **9** by using silica gel as the stationary phase and ethyl acetate : dichloromethane (5 : 95) as the mobile phase giving yellow crystals. The Rf value of **9** was 0.59. For the synthesis of phomenone (**10**), quinone propionic acid (0.14 mol) in dry dichloromethane, phomenone (9 mg, 0.34 mmol), DMAP (0.017 mmol) and DCC (0.17 mmol) were used for the reaction. The residue of **10** from the reaction was purified by preparative TLC with silica gel as the stationary phase and ethyl acetate : dichloromethane (5 : 95) as the mobile phase giving yellow crystals and Rf value of 0.55. Percent yields of **9** and **10** were 73% (18 mg) and 71% (20 mg), respectively. The organic solvent was removed and structures were elucidated by using IR, MS and NMR.

Phaseolinone Ester (**9**): ^{1}H-NMR (CDCl$_3$) δ: 1.04 (3H, d, $J=6.6\,\text{Hz}$, H-13), 1.12 (3H, s, H-14), 1.34 (1H, m, H-5a), 1.41 (6H, s, H-10″), 1.43 (6H, s, H-11″), 1.93—1.96 (12H, s, H-12″, 12″, 13″, 13″), 2.01—2.07 (2H, m, H-7, 5b), 2.12 (3H, s, H-14″), 2.14 (3H, s, H-14″), 2.31 (1H, m, H-4a), 2.42 (1H, m, H-4b), 2.57 (1H, d, $J=5.0\,\text{Hz}$, H-15a), 2.94 (H, d, $J=5\,\text{Hz}$, H-15b), 2.99 (4H, m, H-2″), 3.02 (4H, s, H-2″, 2″), 3.50 (1H, s, H-9), 4.34 (1H, d, $J=12\,\text{Hz}$, H-12a), 4.54 (1H, d, $J=12\,\text{Hz}$, H-12b), 4.74 (1H, m, H-6), 5.70 (1H, m, H-2). ^{13}C-NMR (CDCl$_3$) δ: 11.2, 12.1, 12.5, 12.6, 14.2, 14.3, 18.5, 28.7, 28.8, 28.9, 29.6, 30.4, 31.1, 37.9, 38.3, 41.1, 41.3, 47.4, 47.8, 48.6, 54.8, 61.7, 62.5, 63.4, 72.6, 120.6, 120.8, 138.4, 138.7, 138.8, 138.9, 142.8, 142.9, 152.2, 152.3, 162.5, 172.0, 172.4, 187.4, 187.5, 190.6, 190.8, 192.3. IR (KBr) cm^{-1}: 2934, 1638, 1526, 1450, 1374, 1226. HR-MS (ESI-TOF, negative) m/z : 745.3591 [M+H]$^+$, Calcd for C$_{43}$H$_{52}$O$_{11}$ 745.3588. MS m/z: 744 (M$^+$). [α]$_D^{28}$: +82° (c=0.0039, acetonitrile).

Phomenone Ester (**10**): ^{1}H-NMR (CDCl$_3$) δ: 1.07 (3H, d, $J=6.63\,\text{Hz}$, H-13), 1.27 (3H, s, H-14), 1.32 (1H, m, H-5a), 1.38 (3H, s, H-10″), 1.39 (3H, s, H-10″), 1.44 (6H, s, H-11″), 1.89 (3H, s, H-12″), 1.94 (3H, s, H-12″), 1.97

(6H, s, H-13″), 2.06 (1H, m, H-5b), 2.12 (3H, s, H-14″), 2.14 (3H, s, H-14″), 2.30 (1H, m, H-4a), 2.51 (1H, m, H-4b), 2.82 (1H, d, $J=16.7\,\text{Hz}$, H-2a″), 3.03 (1H, d, $J=16.7\,\text{Hz}$, H-2b″), 3.30 (1H, s, H-9), 4.63 (1H, d, $J=13.2\,\text{Hz}$, H-12a), 4.70 (1H, d, $J=13.2\,\text{Hz}$, H-12b), 4.78 (1H, dt, $J=11$, 4.3 Hz, H-6), 5.32 (1H, s, H-15a), 5.36 (1H, s, H-15b), 5.73 (1H, s, H-2). ^{13}C-NMR (CDCl$_3$) δ: 11.2, 12.1, 12.6, 12.7, 14.3, 18.4, 28.6, 28.7, 28.8, 28.9, 29.7, 30.5, 31.4, 37.8, 38.3, 41.2, 41.4, 47.5, 47.9, 61.3, 64.7, 68.4, 72.7, 117.3, 121.1, 138.4, 138.5, 138.7, 138.8, 142.8, 142.9, 152.2, 152.4, 162.2, 172.1, 172.4, 187.4, 190.6, 190.9, 191.9. IR (KBr) cm^{-1}: 2927, 1642, 1533, 1450, 1374, 1223. HR-MS (ESI-TOF, negative) m/z: 751.3465 [M+Na]$^+$, Calcd for C$_{43}$H$_{52}$O$_{10}$Na 751.3458. MS m/z: 728 (M$^+$). [α]$_D^{28}$: +72.5°(c=0.0038, acetonitrile).

Cytotoxicity Study Cytotoxicity was evaluated in cancer cell lines that has been reported to contain reductase enzymes (human breast cancer BC-1 and human lung cancer NCI-H187), or without reductase (human epidermoid carcinoma, KB) as well as in normal cells (African green monkey kidney fibroblast, Vero cells).[1] The cytotoxicities of the bioreductive esters were compared to their unchanged form (parent compounds) by using colorimetric methods; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay[22] and Sulforhodamine B (SRB).[23] MTT was used for study in NCI-H187 cells, while SRB was used for study in Vero, KB and BC-1 cells.

In this assay, NCI-H187 cells were seeded on 96-well plates (10^5 cells/well) containing 200 μl RPMI 1640 media supplemented with 10% FBS, 1% NEAA, 0.5% penicillin–streptomycin solution (×100) at 37 °C with 5% carbon dioxide. Vero, KB and BC-1 cells were seeded on 96-well plates (10^5 cells/well) containing 200-μl DMEM media supplemented with 10% FBS, 1% non-essential amino acid, 0.5% penicillin–streptomycin at 37 °C with 5% carbon dioxide. The test compounds were added into cultured cells, 24 h after seeding.

MTT Method: After treatment for 21 h, the MTT solution (5 mg/ml of MTT) was added to each well. After 3 more hours (treatment of total 24 h), 100 μl of solution mixture (10% SDS and 0.01 N HCl) was added to each well. Cell viability of the treated cells was determined by measuring absorbance of the blue formazan at 570 nm using an ELISA plate reader with a reference wavelength of 620 nm for background subtraction. The experiments were performed as triplicates. The MTT transformation of MTT by the media-treated cells was set as zero percent-cytotoxicity.

Sulforhodamine B (SRB) Method: After exposure of the cells with test compound for 24 h, the medium was removed by suction. The cells were fixed with 40% trichloroacetic acid (TCA) (Sigma, St. Louis, MO, U.S.A.) and the plates were kept at 4 °C for 1 h. The TCA was removed by suction, and the plates were rinsed with water and stained with SRB (Sigma) 0.4% w/v in 1% acetic acid for 30 min. Excess dye was washed out by 1% acetic acid (Bio Lab Ltd., Jerusalem, Israel), repeatedly 4 times. The stained cells were then extracted and solubilized with 10 nM Tris base (tris(hydroxymethyl)aminomethane) (Sigma) (0.5—2.0 ml) and then was measured by using a microtiter plate reader at 564 nm. The numbers of viable cells were expressed as a percentage of the untreated control cell cultures.

Stability Study The bioreductive esters **8** and **9** were selected as representative of phenolic and alcoholic structures. Stability study of **8** (200 μM) or **9** (1600 μM) was conducted separately under the condition containing the esterase enzyme (4 and 8 units/ml, respectively) in the phosphate buffer 0.05 M, pH 7.4 at 37 °C. At each time points, the reaction mixture was pipetted out to another tube containing acetonitrile to stop the reaction. The reaction mixture was frozen, thawed, and centrifuged at 12000 rpm for 15 min to precipitate the enzyme. Only 100 μl of supernatant was pipetted and determined by using the GC and caffeine was used as an internal standard with a final concentration of 100 μg/ml.

GC Chromatographic Assay: In order to follow the degradation of bioreductive esters, the detection of a decrease peak area of the bioreductive ester as well as an increase peak area of the lactone must be detected. Briefly, the injection port set to splitless mode was adopted for the quantitative analysis of those two peaks. HP-5 (5% diphenyl and 95% methylpolysiloxane) column and flame ionization detector (FID) were used. Nitrogen gas was used as a carrier gas with flow rate of 1.0 ml/min. The injected volume of **8** and **9** was 2 μl. The syringe temperature was kept at 290 °C. Volatile samples were separated on the HP-5 column. For compound **8**, the column was kept at 200 °C for 5 min. The temperature was then increased at 280 °C with the rate of 30 °C/min and kept for 9 min at this temperature. For compound **9**, the column was kept at 150 °C for 5 min. The temperature was then increased at 280 °C with the rate of 30 °C/min and kept for 7 min at this temperature. The column outlet was connected to an FID detector, which was set at 290 °C.

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