Androgen Modulators from the Roots of *Paeonia lactiflora* (Paeoniae Radix) Grown and Processed in Nara Prefecture, Japan

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The monoterpene glycoside, 3′-*O*-galloylpaeoniflorin (1), and four known compounds, 6′-*O*-galloylalbiflorin (2), pentagalloylglucose (3), 6′-*O*-benzoylpaeoniflorin (4) and 6′-*O*-galloylpaeoniflorin (5), were isolated from the roots of *Paeonia lactiflora* that had been grown and processed in Nara prefecture, Japan, as androgen modulators. Their structures were elucidated based on spectroscopic analysis. Compounds 2 and 3 showed strong androgen receptor (AR) binding activity (IC_{50} values 33.7 and 4.1 μg/ml, respectively), 1, 4 and 5 showed weak activity (20, 31 and 12% at 120 μg/ml, respectively). However, paeoniflorin (6) and albiflorin (7), the structures of which are related to 1, 2, 4 and 5, showed no activity. These results suggested that both the structure of albiflorin and the galloyl moiety are important for 2 to show strong AR binding activity. Furthermore, compounds 1—5 inhibited growth of an androgen-dependent LNCaP-FGC (prostate cancer cell line), and were indicated to be AR antagonists. Compounds 2 and 3 might be candidates as safe, natural anti-androgens.

Key words *Paeonia lactiflora*; androgen receptor; monoterpene glycoside; LNCaP-FGC (prostate cancer cell line); crude drug; Paeoniaceae

In Japan, crude drugs (*shoyaku* in Japanese) that are grown and processed in Nara prefecture are named *yamato-shoyaku*, and are of the highest class so command a high price. However, it is not known whether they are more potent than *shoyaku* from other areas. The roots of *Paeonia lactiflora* (Paeoniae radix, *shakuyaku* in Japanese) are one of the most important Japanese and Chinese crude drugs, being used in many traditional "Kampo" formulas. In particular, they are often used in "Kampo" formulas, such as Tokishakuyakusan, Shimotsuto and Keishibukuryogan, for women's hormone-related problems such as menopausal symptoms and menstrual problems.[1,2] However, the hormone modulators from Paeoniae radix have not been satisfactorily examined. Here, we have focused on hormone regulating activity and investigated the bioactive compounds from *yamato-shakuyaku*, which is a *yamato-shoyaku*. The MeOH-eluted fraction using DIAION HP-20 column chromatography of a hot-water extract of *yamato-shakuyaku*, was found to have androgen receptor (AR) binding activity.

Androgens (testosterone and its metabolite 5-α-dihydrotestosterone), steroidal hormones, play an important role in the function and development of not only male reproductive organs such as prostate and testis, but also non-reproductive organs including muscle, hair follicles and brain. Androgens are also known to act as tumor promoters, especially for prostate cancer.[3] Their biological functions are mediated by the androgen receptor, which is a member of the nuclear receptor superfamily of ligand-regulated transcription factors. Therefore, AR antagonists, which counteract the biological responses induced by androgens, are expected to be useful in the treatment of androgen-dependent tumors.[4] Flutamide, a nonsteroidal AR antagonist, is recognized worldwide as the most benefical compound for the treatment of prostate cancer when used in combination with various luteinizing hormone-releasing factor agonists. However, because this compound occasionally induces serious hepatotoxicity,[5,6] there is a need for new, safe, natural AR antagonists.

In attempts to isolate AR modulators from *yamato-shakuyaku*, this resulted in isolation of a new monoterpene glycoside, 3′-*O*-galloylpaeoniflorin (**1**), and four known compounds, 6′-*O*-galloylalbiflorin (**2**), pentagalloylglucose (**3**), 6′-*O*-benzoylpaeoniflorin (**4**) and 6′-*O*-galloylpaeoniflorin (**5**). Isolation, structure determination of **1**—**5**, and AR binding activity and prostate cancer cell growth inhibitory activity of **1**—**5**, paeoniflorin (**6**) and albiflorin (**7**) are reported herein.

Results and Discussion

Yamato-shakuyaku, Paeoniae radix (200 g), grown and processed in Nara prefecture, Japan, were ground and extracted with hot-water. The concentrated extract was subjected to DIAION HP-20, reversed-phase octadecyl silica (ODS), with final purification achieved by reversed-phase preparative HPLC (Inertsil ODS-3) and reversed-phase preparative HPLC (Cosmosil 5C_{18}-MSII) to give compounds **1** (0.6 mg), **2** (4.1 mg), **3** (12.7 mg), **4** (35.1 mg) and **5** (26.6 mg). Compounds **1**—**5** showed AR binding activity.

The molecular formula of compound **1** was found to be $C_{30}H_{32}O_{15}$ [$(M-H)^-$, *m/z* 631.16612, Δ −0.7 milli mass unit (mmu), Calcd for $C_{30}H_{31}O_{15}$ 631.16684] by HR-electrospray ionization (ESI)-MS.

Detailed analysis of 1D (shown in Table 1) and 2D NMR spectroscopic data, including ^1H–^1H correlated spectroscopy (COSY), total correlation spectroscopy (TOCSY), heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond correlation (HMBC) spectra, indicated that compound **1** was a paeoniflorin derivative. The ^1H-NMR (δ_H 7.12, 2H, s) and ^{13}C-NMR (δ_C 110.4, 110.4, 121.8, 139.7, 146.4, 146.4, 168.3) data, as well as the determination of molecular formula, suggested the presence of one galloyl moiety in **1**. In addition, the low-field shifted signal at δ_H 5.11 was assigned to H-3′ of a glucose unit in **1**. Finally, the connection of the galloyl moiety and C-3′ *via* an oxygen atom was revealed by the key HMBC correlation of H-3′/C-7‴. Thus, the structure of **1** was determined as shown in Fig. 2, and named 3′-*O*-galloylpaeoniflorin (**1**). We propose that

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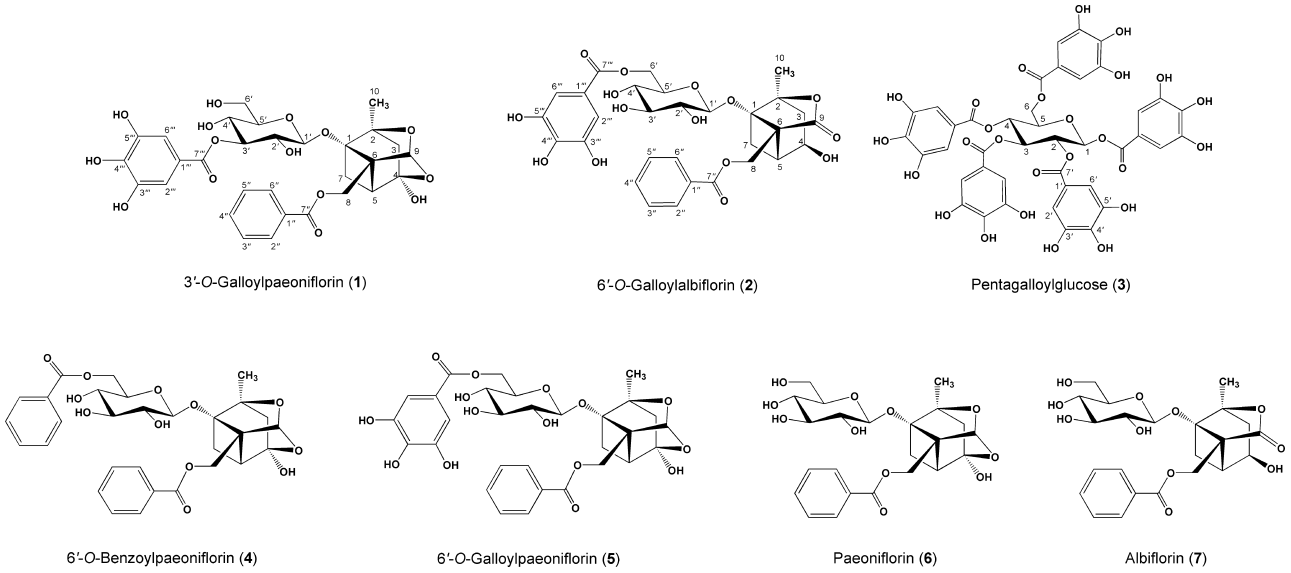

Fig. 1. Structures of Compounds **1**—**7**

Table 1. ^{1}H- (750 MHz) and ^{13}C-NMR (187.5 MHz) Spectroscopic Data for 3′-*O*-Galloylpaeoniflorin (**1**) in CD_3OD

Position	δ_C	δ_H	Position	δ_C	δ_H
1	89.6		4′	70.1	3.54 (1H, dd, *J*=9.4, 9.5 Hz)
2	87.2		5′	77.9	3.38 (1H, m)
3a	44.6	1.82 (1H, d, *J*=12.5 Hz)	6′a	62.6	3.67 (1H, dd, *J*=5.9, 11.9 Hz)
3b		2.20 (1H, d, *J*=12.5 Hz)	6′b		3.87 (1H, dd, *J*=1.5, 11.9 Hz)
4	106.4		1″	131.2	
5	43.9	2.59 (1H, d, *J*=6.8 Hz)	2″, 6″	130.7	8.05 (2H, d, *J*=7.5 Hz)
6	72.3		3″, 5″	129.7	7.49 (2H, dd, *J*=7.4, 7.5 Hz)
7a	23.4	1.98 (1H, d, *J*=10.9 Hz)	4″	134.5	7.61 (1H, t, *J*=7.4 Hz)
7b		2.51 (1H, dd, *J*=6.8, 10.9 Hz)	7″	168.1	
8	61.6	4.74 (2H, s)	1‴	121.8	
9	102.3	5.42 (1H, s)	2‴, 6‴	110.4	7.12 (2H, s)
10	19.7	1.39 (3H, s)	3‴, 5‴	146.4	
1′	100.3	4.67 (1H, d, *J*=7.7 Hz)	4‴	139.7	
2′	73.6	3.47 (1H, dd, *J*=7.7, 9.3 Hz)	7‴	168.3	
3′	79.3	5.11 (1H, dd, *J*=9.3, 9.4 Hz)			

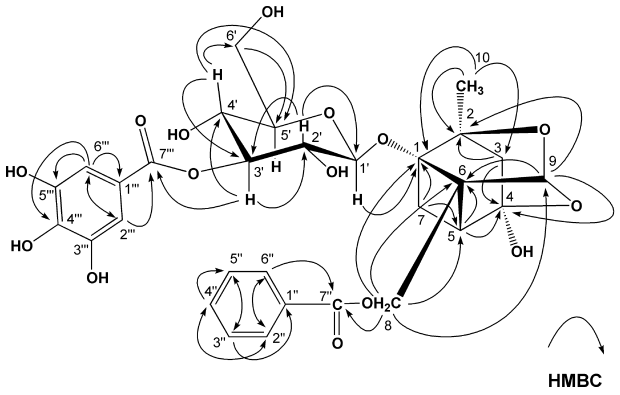

Fig. 2. The HMBC Correlations of 3′-*O*-Galloylpaeoniflorin (**1**)

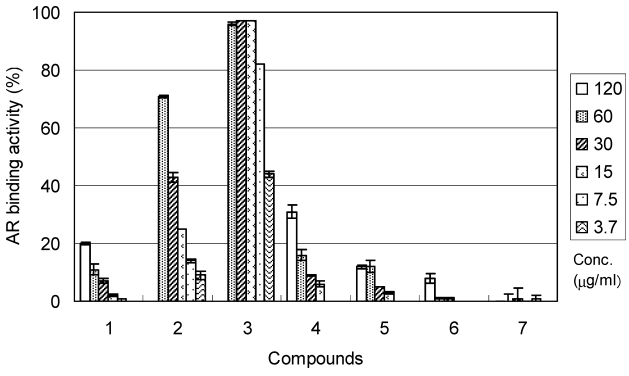

Fig. 3. AR Binding Activity of Compounds **1**—**7** (μg/ml, Mean±S.E.M., n=2)

the absolute stereochemistry of compound **1** is identical to that of paeoniflorin, since the ^{1}H–^{1}H coupling constants, as well as the ^{1}H- and ^{13}C-NMR spectroscopic data of C-1–C-1′ and C-5′–C-7″ in compound **1** were in good agreement with those of paeoniflorin.[7]

Compounds **2**—**5** were identified as 6′-*O*-galloylalbiflorin,[8] pentagalloylglucose,[9] 6′-*O*-benzoylpaeoniflorin[7] and 6′-*O*-galloylpaeoniflorin,[10] respectively, by detailed comparison of their spectroscopic data with that in the literature.

The AR binding activity of compounds **1**—**5**, paeoniflorin (**6**), albiflorin (**7**) and testosterone (positive control) was as-

Table 2. IC_{50} Values for AR Binding Activity by Compounds **1**—**7**

Sample name	IC_{50} μg/ml (μM)
3′-*O*-Galloylpaeoniflorin (**1**)	>120.0 (>190.0)
6′-*O*-Galloylalbiflorin (**2**)	33.7 (53.3)
Pentagalloylglucose (**3**)	4.1 (4.3)
6′-*O*-Benzoylpaeoniflorin (**4**)	>120.0 (>205.0)
6′-*O*-Galloylpaeoniflorin (**5**)	>120.0 (>190.0)
Paeoniflorin (**6**)	>120.0 (>250.0)
Albiflorin (**7**)	>120.0 (>250.0)
Testosterone	0.0012 (0.0042)

sessed as described in Experimental (shown in Fig. 3 and Table 2). Compounds **2** and **3** displayed strong AR binding activity (IC_{50} values 33.7 and 4.1 μg/ml, respectively), whereas AR binding activities of **1**, **4** and **5** were weak (20, 31, 12%, respectively) at 120 μg/ml. The AR binding activity observed was in a dose-dependent manner. Interestingly, compounds **6** and **7**, the structures of which are related to **1**, **2**, **4** and **5**, showed no AR binding activity at 120 μg/ml.

To evaluate whether compounds **1**—**7** were either AR agonists or antagonists, their ability to inhibit growth of LNCaP-FGC (prostate cancer cell line) was assessed (using mitomycin as positive control) as described in Experimental (shown in Fig. 4 and Table 3), because the growth of LNCaP-FGC was an androgen-dependent.[11] Compounds **1**—**5** inhibited cell growth (**1**: 43% at 120 μg/ml, IC_{50} values **2**: 58.0 μg/ml, **3**: 22.0 μg/ml, **4**: 84.0 μg/ml, **5**: 141.0 μg/ml, respectively). Compounds **6** and **7**, by contrast, showed weak inhibitory activity (37, 40%, respectively) at 200 μg/ml. The inhibitory activity of compounds **1**—**7** was in a dose-dependent manner. On the basis of these results, compounds **1**—**5** were indicated to be AR antagonists.

We have thus isolated the first hormone modulators from the roots of *P. lactiflora*. This is the first report of paeoniflorin and albiflorin derivatives such as **1**, **2**, **4** and **5** showing AR binding activity. Interestingly, compounds **6** and **7** showed no AR binding activity. The AR binding activity of 6′-*O*-galloylalbiflorin (**2**) was much stronger than that of the galloylpaeoniflorin derivatives (**1**, **5**). These results suggested that both the structure of albiflorin and the galloyl moiety were important for **2** to show strong AR binding activity. Additionally, 6′-*O*-galloylpaeoniflorin (**5**) displayed the weakest AR binding activity of the paeoniflorin derivatives (**1**, **4**, **5**). The only structural differences between **2** and **5** are at C-4 and C-9, so the galloyl moiety of **2** might interact with the carbonyl group of C-9 and/or the hydroxyl group of C-4.

It has been reported that **3** can inhibit growth of prostate cancer LNCaP cells by two aspects including inhibition of 5-α-reductase activity and expression of AR protein levels; however, the AR binding activity of **3** has not been reported.[12] We propose that **3** inhibits prostate cancer cell growth partly by acting as an AR antagonist.

The AR binding activity of compound **3** was equivalent to flutamide (IC_{50} 5.0 μM),[13] which is in clinical use, and the activity of compound **2** was also relatively strong. Additonally, because Paeoniae radix was taken in long time as a crude drug, compounds **2** and **3** might be candidates as safe, natural AR antagonists.

HPLC analysis of Paeoniae radix grown and processed in other areas established that these compounds were not specific to *yamato-shakuyaku*, although plants grown in different areas contained different quantitative ratios of these compounds (data not shown). Consequently, these compounds might be useful as new quality markers of Paeoniae radix based on AR regulating activity.

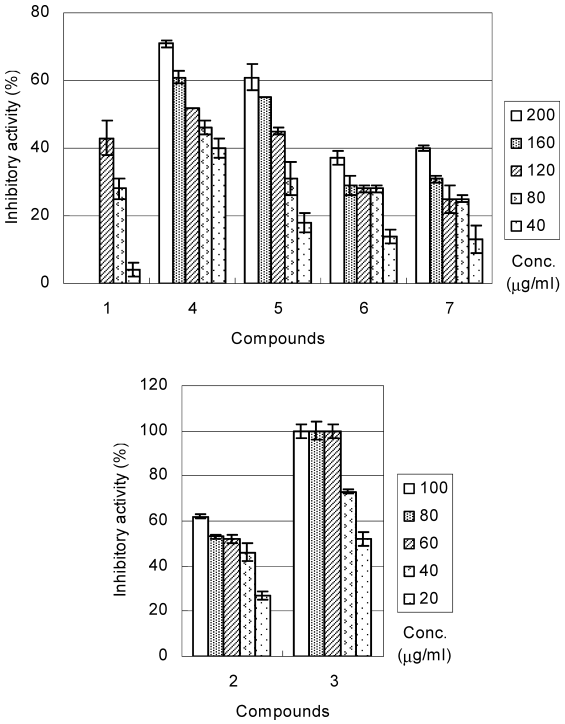

Fig. 4. Inhibition of Growth of Prostate Cancer LNCaP-FGC Cell by Compounds **1**—**7** (μg/ml, Mean±S.E.M., n=2)

Table 3. IC_{50} Values for Inhibitory Activity on Prostate Cancer LNCaP-FGC Cell Growth by Compounds **1**—**7**

Sample name	IC_{50} μg/ml (μM)
3′-*O*-Galloylpaoniflorin (**1**)	>120.0 (>190.0)
6′-*O*-Galloylalbiflorin (**2**)	58.0 (91.8)
Pentagalloylglucose (**3**)	22.0 (23.4)
6′-*O*-Benzoylpaeoniflorin (**4**)	84.0 (144.0)
6′-*O*-Galloylpaeoniflorin (**5**)	141.0 (223.0)
Paeoniflorin (**6**)	>200.0 (>417.0)
Albiflorin (**7**)	>200.0 (>417.0)
Mitomycin	0.033 (0.1)

Conclusion

In summary, a new monoterpene glycoside, 3′-*O*-galloylpaeoniflorin (**1**), and four known compounds (**2**—**5**), were isolated from *yamato-shakuyaku* as AR modulators. Interestingly, while these compounds showed AR binding activity, paeoniflorin and albiflorin, whose structures are related to these compounds, did not. These results suggested that both the structure of albiflorin and the galloyl moiety were important for 6′-*O*-galloylalbiflorin (**2**) to show strong AR binding activity. Further studies on their action mechanism and structure–activity relationships of these AR modulators are in progress.

Experimental

General Optical rotations were measured using a DIP-1000 digital polarimeter (Jasco), whereas UV spectra were acquired with a V-630 spec-

trophotometer (Jasco). IR spectra were obtained using an IR Prestige-21 FTIR-8400S (Shimadzu), and NMR (^{1}H-NMR: 750, 400 MHz, ^{13}C-NMR: 187.5 MHz) spectra were measured on AVANCE-750 (Bruker Biospin) and JNM-GSX-400 (JEOL). MS were obtained using an Apex-Q94e (Bruker Daltonics). Radioactivity was measured in a wallac 1450 microbeta TRILUX (PerkinElmer). Fluorescence intensities were measured using a GENios Plus microplate reader (Tecan).

Plant Material *Yamato-shakuyaku*, Paeoniae radix, grown and processed in Nara, Japan, were purchased from Fukuda shoten (Sakurai, Nara, Japan) and Ruta Corporation (Osaka, Osaka, Japan). Voucher specimens were deposited in the core laboratory of Nara Prefectural Small and Medium-sized Enterprises Support Corporation (Kashihara, Nara, Japan).

Chemicals [H^3]-Mibolerone and unlabeled mibolerone were purchased from PerkinElmer, U.S.A. Alamarblue was purchased from AbD Serotec, U.K. Paeoniflorin, albiflorin and the other reagents were analytical-grade products from Wako Pure Chemical Industries, Japan.

Extraction and Isolation *Yamato-shakuyaku* (200 g), Paeoniae radix grown and processed in Nara, Japan, were ground and extracted with hot-water (4 l) at 75 °C for 1.5 h. The concentrated extract (77.1 g) was applied to a DIAION HP-20 (ϕ65×45 mm) column with H_2O–MeOH (10 : 0, 8 : 2, 7 : 3, 6 : 4, 4 : 6, 2 : 8, 0 : 10) to give seven fractions; F1, F2, F3, F4, F5, F6 and F7. Fractions F2—5 were combined and subjected to chromatography on a reversed-phase ODS (Cosmosil 140-C_{18} OPN, ϕ30×65 mm) using H_2O–MeOH (9 : 1, 8 : 2, 7 : 3, 0 : 10) as eluent and on a reversed-phase preparative HPLC (Inertsil ODS-3, ϕ20×250 mm) using H_2O–MeOH–formic acid (64.9 : 35 : 0.1) to give seven fractions; F25A, F25B, F25C, F25D, F25E, F25F and F25G. Fraction F25E was applied to a reversed-phase preparative HPLC (Cosmosil 5C_{18}-MSII, ϕ20×250 mm) with H_2O–acetonitrile (80 : 20) to give compound **1** (0.6 mg). Fraction F25C was subjected to reversed-phase preparative HPLC (Cosmosil 5C_{18}-MSII, ϕ20×250 mm) using H_2O–MeOH (70 : 30) to give compounds **2** (4.1 mg) and **3** (12.7 mg). Fraction F25F, following reversed-phase preparative HPLC (Cosmosil 5C_{18}-MSII, ϕ20×250 mm) with H_2O–acetonitrile (85 : 15), gave compound **5** (26.6 mg). Fractions F6 and 7 were combined and subjected to DIAION HP-20 chromatography (ϕ10×130 mm) with H_2O–MeOH (4 : 6, 2 : 8, 0 : 10) to give three fractions; F67A, F67B and F67C. Fraction F67C was subjected to reversed-phase preparative HPLC (Inertsil ODS-3, ϕ20×250 mm) with H_2O–MeOH–formic acid (49.9 : 50 : 0.1) to give compound **4** (35.1 mg).

3′-*O*-Galloylpaeoniflorin (**1**): A yellow-brown amorphous powder; $[\alpha]_D^{30}$ +16.0° (c=0.27, MeOH); UV_{max} (MeOH, ε) 219.8 nm (119000), 274.4 nm (87600); IR (film) 3383, 1705 and 1607 cm^{-1}. HR-ESI-MS *m/z*: 631.16612 [(M−H)$^-$, 631.16684 for $C_{30}H_{31}O_{15}$]; For ^{1}H- (750 MHz) and ^{13}C-NMR (187.5 MHz) spectroscopic data, see in Table 1.

Measurement of Androgen Receptor (AR) Binding Activity Compounds **1**—**7** and testosterone (positive control) were evaluated for their AR binding activity by measuring the binding [H^3]-mibolerone to AR. Membrane (rat) preparation obtained from Panvera (Cat # P2719) was prepared in modified triphosphate pH 7.4 buffer using standard techniques. An aliquot (78 ng) of membrane preparation was incubated with 1.5 nM [H^3]-mibolerone in either the presence or absence of a test sample for 4 h at 4 °C. Non-specific binding was estimated in the presence of 10 μM mibolerone. The reaction mixture was incubated with a hydroxyapatite slurry over 15 min and filtered. The filters were washed 3 times and counted to determine [H^3]-mibolerone specifically bound. Test compounds were screened at 120—60—30—15—7.5 (compounds **1**, **4**—**7**) or 60—30—15—7.5—3.8 (compounds **2**, **3**) μg/ml. AR binding activity was calculated by the equation as described below:

AR binding activity (%)=100×[1−([H^3]-miborelone specifically bound in the presence of test sample−non specific binding in the presence of test sample)/([H^3]-miborelone specifically bound in the control−non specific binding in the control)]

Measurement of Inhibitory Activity on Cell Growth of an Androgen Responsive Prostate Cancer LNCaP-FGC Cell Line Compounds **1**—**7** and mitomycin (positive control) were evaluated for their inhibitory activity on cell growth of an androgen responsive LNCaP-FGC (prostate cancer cell line). LNCaP-FGC cells (2.5×10^3/well in 90% RPMI-1640 medium plus 10% fetal bovine serum [FBS]), obtained from a human prostate cancer cell line (ATCC CRL-10995), were pre-incubated in 96-well plates in an atmosphere of 5% CO_2 at 37 °C for 24 h. Test compounds and/or vehicle was then added to each well with final concentration of 0.4% DMSO/DW in RPMI-1640 medium plus FBS for an additional 72 h. After a further 6-h incubation in the presence of alamarblue, fluorescence intensity was measured using a spectroflour plus plate reader with excitation at 530 nm and emission at 590 nm. Test compounds were screened at 200—160—120—80—40 (compounds **4**—**7**) or 120—80—40 (compound **1**) or 100—80—60—40—20 (compounds **2**, **3**) μg/ml.

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