- **Limonoids from *Aphanamixis polystachya* leaves and their interaction with Hsp90***

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*Dedicated to Professor Dr. Robert Verpoorte in recognition of his outstanding contribution to natural products research.

Abstract

A phytochemical study of *n*-hexane, $CHCl_3$, and $CHCl_3$-MeOH extracts of *Aphanamixis polystachya* leaves led to the isolation of ten compounds. Five of them turned out to be new natural compounds, including two mexicanolide-type (**1**, **2**) and three polyoxyphragmalin-type (**3**–**5**) limonoids, together with two known andirobin-type limonoids (**6**, **7**), and three phenolic derivatives. The structures of the new compounds were established on the basis of spectroscopic methods to be 8-hydro-14,15-en-cabralin (**1**), 3-deacetyl-8-hydro-cabralin-14,15-en-3-one (**2**), 20,22-dihydroxy-21,23-dimethoxytetrahydrofuran khayanolide A (**3**), 1-deacetyl-3-dehydroxy-3-oxokhaysenelide E (**4**), and meliaphanamixin A (**5**). All compounds were isolated for the first time from this species. The ability of the isolated limonoids to interact with the molecular chaperone Hsp90 was tested. Compounds **6** and **7** resulted the most active.

Key words

Aphanamixis polystachya, Meliaceae, limonoids, Surface Plasmon Resonance, Hsp90

Introduction

Limonoids, classified as tetranortriterpenes, whose skeleton consists in a wide range of triterpene core structure rearrangement, are the main constituents of the Meliaceae family (Sapindales order), which is among the richest and most diverse source of secondary metabolites in the Angiospermae [1]. These compounds exhibited a wide range of biological properties such as antibacterial, antifungal, antimalarial, anticancer, and antiviral activities [2].

In the last few years, our research group was involved in a project aimed to investigate natural compounds library as Heat shock protein 90 (Hsp90) interactor. Hsp90, an evolutionary conserved molecular chaperone, is implicated in a wide range of diseases, including cancer, thus representing an interesting target for drug discovery [3]. Limonoids, such as kotschyin D isolated from *Pseudocedrela kotschyi* [4] and limonol derivatives obtained from *Cedrela odorata* [5], have been found to affect the *in vitro* enzymatic activity of Hsp90. These results prompted the investigation of other Meliaceae species characterized by limonoids as main secondary metabolites, in this case *Aphanamixis polystachya* (Wall) R. Parker syn. *Aphanamixis rohituka* (Roxb.). This species is a timber tree, endemic of tropical areas of Asia, which can be also cultivated elsewhere including Northern Africa [6]. Several natural compounds have been previously reported from this plant, such as aphanamixoid-type and prieurianin-type limonoids isolated from the leaves, fruits, and seeds [7, 8], along with diterpenes, lignans, flavonoid glycosides, terpenoids, and alkaloids [9–11]. In the present study, the isolation and the structural elucidation of five new limonoids (**1**–**5**) isolated during the phytochemical investigation of *A. polystachya* leaves from Cairo Botanical Garden is reported. The ability of all isolated limonoids to interact with the molecular chaperone Hsp90 is also investigated.

Results and discussion

The dried and powdered leaves of *A. polystachya* were extracted with solvents of increasing polarity: *n*-hexane, $CHCl_3$, $CHCl_3$-MeOH (9:1), and MeOH by exhaustive maceration in order to obtain four residues, which were subjected to column chromatography over flash silica gel or Sephadex LH-20 to afford five new natural compounds, including two mexicanolide-type (**1**, **2**) and three polyoxyphragmalin-type limonoids (**3**–**5**), along with two known andirobin-type limonoids (**6**, **7**) (**Fig. 1**), and three phenolic derivatives. The structural determination of the known compounds was performed by NMR and mass spectrometry data, and their comparison with those reported in the literature. Known compounds were characterized as moluccensin N (**6**) [12], moluccensin O (**7**) [12], syringaresinol-4-*O*-β-D-glucopyranoside [13], quercetin 3-*O*-α-L-rhamnopyranoside [14], and hydrangeifolin I [15]. Copies of the original spectra are obtainable from the corresponding author.

Compound **1**, obtained as a white and amorphous powder, showed a molecular formula of $C_{29}H_{36}O_{10}$, as determined by HRESIMS at *m/z* 567.2189 $[M + Na]^+$. The negative ESIMS spectrum showed a deprotonated molecular ion peak at *m/z* 543 $[M – H]^-$, while its fragmentation pattern was characterized by peaks at *m/z* 499 $[M – H – 44]^-$, 455 $[M – H – 44 – 44]^-$, and 439 $[M – H – 44 – 60]^-$, due to the subsequent loss of two CO_2 molecules and one acetyl group, respectively. The NMR data of **1** (**Tables 1** and **2**), displayed 29 carbon resonances assignable to five methyls (one methoxy), four methylenes, nine methines (two olefinic and two oxygenated), and nine quaternary carbons (two olefinic and four carbonyls), together with signals for an acetyl group. There were 12 degrees of unsaturation evident in the molecule of **1**, of which seven were represented by four ester carbonyl, one ketone group, and two double bonds; therefore, the molecule was pentacyclic. COSY experiment showed correlations between H-3/H-2, H-2/H_2-30, H_2-30/H-8, H-8/H-9, H-9/H_2-11 and H-5/H-6. Analysis of the 2D NMR spectra, especially the HMBC data (**Fig. 2**), confirmed **1** was a rearranged mexicanolide-type limonoid with a similar structure of cabralin [16], with the only difference being the presence of a double bond between C-14 and C-15 in **1** instead of C-8 and C-14 in cabralin. The $\Delta^{14(15)}$ double bond in **1** was established by the HMBC correlations between δ 5.77/33.8 (H-15/C-8), δ 5.77/39.4 (H-15/C-13), δ 5.77/164.0 (H-15/C-16), and δ 5.09/133.9 (H-17/

C-14). The HMBC spectrum also allowed the assignment of most of the functional groups. HMBC cross-peak observed between δ 3.72/174.0 (7-OMe/C-7) enabled a methoxy group to be placed at C-7, while the HMBC correlation at δ 1.13/219.0 (Me-19/C-1) helped to establish the presence of a keto group at C-1. The signals at δ_H 7.40 (H-22) and δ_H 6.26 (H-23) observed in the ^{1}H NMR spectrum, which were correlated with the signals at δ_C 148.8 (C-22) and δ_C 97.8 (C-23) in the HSQC spectrum showed the presence of a 23-hydroxybutenolide ring moiety in **1**. The relative stereochemistry of **1** was determined by comparison of stereogenic center proton chemical shift and coupling constant with those of cabralin and similar compounds [16]. Consequently, the structure of 8-hydro-14,15-en-cabralin (**1**) was established as shown in **Fig. 1**.

Compound **2**, obtained as a white and amorphous powder, showed a molecular formula of $C_{27}H_{32}O_9$, as established by the HRESIMS spectrum (*m/z* 523.1925 $[M + Na]^+$). Its negative ESIMS spectra (*m/z* 499 $[M - H]^-$) evidenced a similar fragmentation pattern (*m/z* 455 $[M - H - 44]^-$, 411 $[M - H - 44 - 44]^-$) as that of **1**. The NMR spectroscopy data of **2** (**Tables 1** and **2**) indicated its structure to be closely related to that of **1** with the only difference being the presence of a keto group at C-3 in **2** replacing the 3-*O*-acetyl substituent in **1**. This fact was revealed by the absence of NMR spectroscopic signals corresponding to the acetyl group at C-3 along with the COSY correlation between H-2/H-3 reported for **1**, and was confirmed by the HMBC correlations between δ 3.28/213.0 (H-2/C-3) and δ 2.70/213.0 (H-5/C-3) (**Fig. 2**) and COSY correlations between H-2/H_2-30 and H_2-30/H-8, suggesting a carbonyl carbon at the C-3 position of **2**. Therefore, the structure of 3-deacetyl-8-hydro-cabralin-14,15-en-3-one was established for compound **2**.

Compound **3**, was obtained as a white and amorphous powder. Its molecular formula ($C_{29}H_{40}O_{14}$) was established from the sodiated molecular ion peak in the HRESIMS at *m/z* 635.2298 $[M + Na]^+$, indicating that **3** had ten degrees of unsaturation. The positive ESIMS spectrum showed peaks at *m/z* 635 $[M + Na]^+$, 617 $[M + Na - 18]^+$, 575 $[M + Na - 60]^+$. The ^{1}H and ^{13}C NMR data (**Tables 1** and **2**) of **3** indicated that three of the ten elements of unsaturation came from two ester and one keto groups; therefore, the molecule was heptacyclic. One and two-dimensional NMR experiments

revealed that **3** had six methyl groups (three methoxy together with three tertiary methyl groups), four methylenes, nine methines (five oxygenated), and ten quaternary carbons. The presence of a quite rare 20,22-dihydroxy-21,23-dimethoxytetrahydrofurano system as E ring was recognized by HSQC correlations between δ 4.95 (s, H-21) and 111.4 (C-21), δ 4.06 (d, *J* = 3.8 Hz, H-22) and 77.3 (C-22), δ 4.99 (d, *J* = 3.8 Hz, H-23) and 113.0 (C-23), δ 3.46 (s, 21-OMe) and 55.7 (21-OMe), δ 3.43 (s, 23-OMe) and 56.0 (23-OMe) and by COSY correlations between δ 4.99 (H-23) and δ 4.06 (H-22). This assumption was confirmed by the HMBC correlations between H-21—C-20, H-21—C-22, H-21—C-23, H-21—21-OMe, and between H-23—C-21, H-23—C-22, and H-23—23-OMe (**Fig. 2**). A methoxycarbonyl moiety [δ_H 3.71 (s, 7-OMe); δ_C 52.7 (7-OMe), δ_C 176.0 (C-7)] was also identified. All protons directly bonded to carbon atoms were assigned by analysis of HSQC data and confirmed by analysis of the HMBC spectra. COSY spectrum led also to establish the correlations between H-2/H-3 (δ 2.80/3.85) and H-5/H-6 (δ 1.85/4.27). A δ-lactone ring, was characterized by the signals at [δ_H 2.98 (d, *J* = 18.0 Hz, H-15a), δ_H 2.47 (d, *J* = 18.0 Hz, H-15b), δ_H 4.93 (s, H-17); δ_C 38.0 (C-15), δ_C 83.6 (C-17), δ_C 37.3 (C-13), δ_C 65.8 (C-14), and δ_C 172.0 (C-16)], and was confirmed by HMBC correlations between H-15b—C-8, H-15b—C-13, H-17—C-8, and H-17—C-14. The cross-peak at δ 4.93/83.0 (H-17/C-20) observed in the HMBC spectrum of **3** confirmed that the position C-17 of the δ-lactone ring was linked to C-20. The NMR data suggested that **3** was a polyoxyphagmalin-type limonoid with a 4,29,1-bridge as structural features, confirmed by the HMBC correlations observed between H_2-29—C-1, H-29a—C-4, H-29b—C-10, H-29a—C-30, an oxygenated methine [δ_H 3.85 (d, *J* = 1.0 Hz, H-3); δ_C 86.3 (C-3)] at C-3 and an epoxy group between C-8 and C-14, evidenced by HMBC correlation peaks between H-2—C-8, H-9—C-8, H-11—C-14, H-17—C-14 [17, 18]. The HMBC correlations between δ 2.80/211.6 (H-2/C-30) and δ 3.85/211.6 (H-3/C-30) were in agreement with the presence of a keto group at C-30. The relative stereochemistry of **3** was established on the basis of 1D ROESY data and comparison with the literature [17]. ROESY interactions between δ 1.26/4.95 (Me-18/H-21) indicated the β-orientation

of the 21-OMe, while the correlations between δ 2.28/1.31 (H-29a/Me-19), 2.80/1.31 (H-2/Me-19), and 3.85/1.31 (H-3/Me-19) showed that these two groups were on the same side. Moreover, the coupling constant and chemical shifts of E ring showed the same configuration of entangosin [19]. On the basis of the above results, the structure of **3**, 20,22-dihydroxy-21,23-dimethoxytetrahydrofuran khayanolide A, was elucidated as shown in **Fig. 1**.

Compound **4** was a white and amorphous powder and had a molecular formula of $C_{28}H_{34}O_{12}$ as deduced from the molecular ion $[M + Na]^+$ at *m/z* 585.1967 observed in the HRESIMS, indicating that **4** had twelve degrees of unsaturation. Analysis of ^{13}C NMR chemical shift (**Table 2**) of **4** showed that this molecule was heptacyclic. The ^1H NMR spectroscopy data of **4** displayed the existence of three tertiary methyls [δ_H 1.17 (s, Me-18), 1.35 (s, Me-19), 1.13 (s, Me-28)], one geminal coupled methylene at C-29 [δ_H 2.17 (d, J = 12.0 Hz, H-29a) and 1.86 (d, J =12.0 Hz, H-29b)], and other geminal coupled methylene at C-15 [δ_H 3.17 (d, J = 18.0 Hz, H-15a) and 2.78 (d, J = 18.0 Hz, H-15b], which proton signals had correlation in the HSQC spectrum with δ_C 13.0 (C-18), 19.0 (C-19), 15.0 (C-28), 44.8 (C-29), and 33.3 (C-15), respectively. COSY spectrum revealed the presence of H-2/H-30, H-5/H-6, H-9/H_2-11, H_2-11/H_2-12, and H-22/H-23 correlations. Thus, compound **4** was established to be a rearranged phragmalin-type limonoid [20, 21]. Additionally, the signals at δ_H 7.42 (br s, H-22), 5.98 (br s, H-23), and 3.55 (s, 23-OMe) observed in the ^1H NMR, which correlated with the signals at δ_C 149.0 (C-22), 103.1 (C-23), and 57.1 (23-OMe) in the HSQC spectrum, were in agreement with the presence of a 23-methoxybutenolide ring moiety in **4**. This conclusion was confirmed by the HMBC correlations between H-17—C-20, H-17—C-22, H-22—C-21, H-22—C-23, which also indicated that the 23-methoxybutenolide ring was connected to C-17 (**Fig. 2**). Extensive analyses of 1D and 2D NMR data revealed that **4** shared a common structure with khaysenelide E [22], but with two different substitution patterns. The 2D NMR data indicated that a hydroxyl group at C-1 and a keto group at C-3 in **4** replaced the *O*-acetyl substituent at C-1 and the hydroxyl group at C-3 in khaysenelide E. The presence of the keto group at C-3 in **4** was confirmed by the HMBC correlations between H-2—C-3, H-5—C-3, Me-28—C-3, H_2-29—C-3,

and H-30—C-3. The configuration of all the chiral centres of **4** was determined to be the same as in khaysenelide E by analysis of ^{1}H NMR data and 1D ROESY spectra. Thus, the structure of 1-deacetyl-3-dehydroxy-3-oxokhaysenelide E was established for **4**.

Compound **5**, isolated in a trace amount, showed a sodiated molecular ion peak at *m/z* 601.1889 $[M + Na]^+$ in the HR-ESIMS, corresponding to the molecular formula $C_{28}H_{34}O_{13}$. Other fragmentation peaks were at *m/z* 573.1925 $[M + Na - 28]^+$, 557.1990 $[M + Na - 44]^+$, 541.1677 $[M + Na - 60]^+$, 513.1727 $[M + Na - 44 - 44]^+$. The NMR spectroscopic data (**Tables 1** and **2**) of **5** showed similarities to **4**. COSY spectrum revealed the presence of H-5/H-6 and H-9/H_2-11 correlations. The 2D NMR data indicated that the positions 23 and 2 of **5** were substituted by a hydroxyl group and a methoxy group, replacing, respectively, the methoxy group at C-23 and the methyne proton at C-2 in **4**. In fact, the ^{1}H NMR spectrum of **5** showed a signal at δ_H 3.51 (s, 2-OMe), which correlated in the HMBC with δ 101.1 (C-2). Other correlations observed in the HMBC spectrum of **5** at δ 2.88/101.0 (H-30/C-2) (**Fig. 2**), confirmed the presence of a quaternary oxygenated carbon at C-2. Thus, the structure of **5**, called meliaphanamixin A, was established as shown in **Fig.1**.

Since our previous results suggested that limonoids could modulate the Hsp90 activity, compounds **1**–**7** were studied by Surface Plasmon Resonance (SPR) experiments to investigate their ability to interact with this chaperone; radicicol and 17-*N*-allylamino-17-demethoxygeldanamycin (17-AAG), two well known Hsp90 inhibitors, were used as positive control [4, 5]. Compounds **6** and **7** bound the immobilized protein (**Table 3**) showing the highest affinity towards the chaperone as indicated by the obtained K_D values of 0.133 ± 0.018 μM K_D for the Hsp90/**6** and 0.183 ± 0.029 μM K_D for the Hsp90/**7** complex. Interestingly, both compounds **6** and **7** showed an affinity toward the chaperone similar to that of 17-AAG. These data confirmed that limonoids, having different structural features are able to link Hsp90 protein.

The cytotoxic activity of the most active compounds **6** and **7** was evaluated in Jurkat (T-cell leukemia) cell line. None of the compounds were cytotoxic, as they all had EC_{50} values in excess of 100 μM.

The limonoids of *A. polystachya* identified in this work, having mexicanolide, polyoxyphragmalin, and andirobin-type skeleton, are in accordance with the ones found in other Meliaceae plants [23]. The differences between the chemical content of the plant leaves from Egypt and those of other studied species from Asian regions confirmed that diverse environments could partially modify their metabolism.

Material and Methods

General experimental procedures

Optical rotations were measured on a Perkin–Elmer 241 polarimeter equipped with a sodium lamp (589 nm) and a 1 dm microcell. 1D and 2D NMR experiments (^{1}H, ^{13}C, 1D-TOCSY, 1D-ROESY, COSY, HSQC, and HMBC) were performed at 300 K in CD_3OD or $CDCl_3$ on a Bruker DRX-600 spectrometer (Bruker BioSpin, GmBH) equipped with a Bruker 5 mm TCI CryoProbe. HRESIMS were acquired in the positive ion mode on a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific). ESIMS were obtained from an LCQ Advantage ThermoFinnigan spectrometer (ThermoFinnigan), equipped with Xcalibur software. Column chromatographies (CC) were performed over Sephadex LH-20 (40–70 µm, Pharmacia) and Isolera Biotage purification system (flash Silica gel 60 SNAP cartridge). Reverse phase - high performance liquid chromatography (RP-HPLC) separations were conducted on a Shimadzu LC-8A series pumping system equipped with a Shimadzu RID-10A refractive index detector and Shimadzu injector on a C_{18} µ-Bondapak column (30 cm x 7.8 mm, 10 µm Waters, flow rate 2.0 mL/min). Thin Layer Chromatography (TLC) analyses were carried out using precoated Kieselgel 60 F254 (0.20 mm thickness) plates (Merck); compounds were detected by cerium disulfate/sulfuric acid (Sigma-Aldrich).

Plant material

The leaves of *A. polystachya* were collected in June 2013 in Al-Zoharia Botanical Garden, Cairo, Egypt and identified by Dr. Momdouh Shokry. A voucher specimen has been deposited at the Herbarium Horti Botanici Pisani, Pisa, Italy (n. 4185 *Aphanamixis polystachya* (Wall.) R. Parker/1, Flora Aegyptiaca).

Extraction and isolation

The dried leaves of *A. polystachya* (900 g) were extracted for 72 h with solvents of increasing polarity: *n*-hexane, $CHCl_3$, $CHCl_3$-MeOH (9:1), and MeOH by exhaustive maceration (2 L) to give 12.7, 28.4, 30.8, and 46.5 g of the respective residues. The *n*-hexane extract was partitioned between *n*-hexane and a MeOH-H_2O (3:2) mixture to afford a MeOH-H_2O residue (1.1 g), which was subjected to flash silica gel column chromatography by Biotage (SNAP 100 g, flow rate 50 mL/min, collection volume 20 mL) eluting with *n*-hexane followed by increasing concentrations of $CHCl_3$ in *n*-hexane (between 1% and 100%) continuing with $CHCl_3$ followed by increasing concentrations of MeOH in $CHCl_3$ (between 1% and 100%) and collecting six major fractions (A–F). Fractions B (47.2 mg) and E (59.1 mg) were subjected to RP-HPLC on a C_{18} μ-Bondapak column with MeOH-H_2O (1:1) as eluent to give compounds **2** (1.0 mg, t_R 12 min) and **1** (0.9 mg, t_R 15 min) from fraction B; compounds **2** (1.2 mg, t_R 12 min), **1** (1.6 mg, t_R 15 min), and **6** (1.7 mg, t_R 17 min) from fraction E. Fraction C (63.9 mg) was purified by RP-HPLC on a C_{18} μ-Bondapak column with MeOH-H_2O (3:2) as eluent, to give compound **7** (1.7 mg, t_R 12 min). Part of the $CHCl_3$ extract (4.6 g) was submitted to flash silica gel column chromatography by Biotage (SNAP 340 g column, flow rate 90 mL/min, collection volume 30 mL), eluting with $CHCl_3$ followed by increasing concentrations of MeOH in $CHCl_3$ (between 1% and 30%) and collecting six major fractions (A-F). Fractions B (116.0 mg) and D (163.3 mg) were subjected to RP-HPLC on a C_{18} μ-Bondapak column with MeOH-H_2O (55:45) as eluent, to give compounds **7** (3.4 mg, t_R 14 min) from fraction B and **6** (3.6 mg, t_R 12 min) from fraction D. Fraction C (43.8 mg) was subjected to RP-HPLC on a C_{18} μ-Bondapak column with MeOH-H_2O (1:1) as eluent, to give compound **6** (2.3

mg, t_R 17 min). Fraction F (325.4 mg) was subjected to RP-HPLC on a C_{18} μ-Bondapak column with MeOH-H_2O (25:75) as eluent, to give compound **5** (0.6 mg, t_R 16 min). The $CHCl_3$-MeOH extract (10.2 g) was submitted to Sephadex LH-20 column (5 x 75 cm), flow rate 1.0 mL/min, collection volume 15 mL, using MeOH as eluent and collecting five major fractions (A–E). Fraction E yielded quercetin 3-*O*-α-L-rhamnopyranoside (47.6 mg). Fractions B (1.8 g) and C (1.2 g) were subjected to flash silica gel column chromatography by Biotage (SNAP 100 g column, flow rate 40 mL/min, collection volume 20 mL), eluting with $CHCl_3$ followed by increasing concentrations of MeOH in $CHCl_3$ (between 1% and 70%) and collecting seven subfractions (B1–B7) from fraction B and three subfractions (C1–C3) from fraction C, respectively. Subfractions B3 (80.9 mg) and B5 (117.6 mg) were subjected to RP-HPLC on a C_{18} μ-Bondapak column with MeOH-H_2O (35:65) as eluent, to give compound **3** (1.8 mg, t_R 9 min) from subfraction B3; syringaresinol-4-*O*-β-D-glucopyranoside (1.4 mg, t_R 20 min) from subfraction B5. Subfraction B7 (78.4 mg) was subjected to RP-HPLC on a C_{18} μ-Bondapak column with MeOH-H_2O (25:75) as eluent, to give hydrangeifolin I (2.3 mg, t_R 14 min). Subfraction C2 (29.6 mg) was subjected to RP-HPLC on a C_{18} μ-Bondapak column with MeOH-H_2O (35:65) as eluent, to give compound **4** (1.6 mg, t_R 14 min).

Compound **1**: white amorphous powder; $[\alpha]_D$ + 56.0 (*c* 0.1, MeOH); ^1H and ^{13}C NMR data, see **Tables 1** and **2**; ESIMS *m/z* 543 $[M - H]^-$, 499 $[M - H - 44]^-$, 471 $[M - H - 44 - 18]^-$, 455 $[M - H - 44 - 44]^-$, 439 $[M - H - 44 - 60]^-$; HRESIMS *m/z* 567.2189 $[M + Na]^+$ (calcd. for $C_{29}H_{36}O_{10}Na$, 567.2206), 507.1966 $[M + Na - 60]^+$, 463.2070 $[M + Na - 60 - 44]^+$, 419.2173 $[M + Na - 60 - 44 - 44]^+$.

Compound **2**: white amorphous powder; $[\alpha]_D$ + 83.0 (*c* 0.1, MeOH); ^1H and ^{13}C NMR data see **Tables 1** and **2**; ESIMS *m/z* 499 $[M - H]^-$, 455 $[M - H - 44]^-$, 411 $[M - H - 44 - 44]^-$, 381 $[M - H - 44 - 74]^-$; HRESIMS *m/z* 523.1925 $[M + Na]^+$ (calcd. for $C_{27}H_{32}O_9Na$, 523.1944).

Compound **3**: white amorphous powder; $[\alpha]_D$ + 33.7 (*c* 0.1, MeOH); ^1H and ^{13}C NMR data, see **Tables 1** and **2**; ESIMS *m/z* 635 $[M + Na]^+$, 617 $[M + Na - 18]^+$, 575 $[M + Na - 60]^+$; HRESIMS *m/z* 635.2298 $[M + Na]^+$ (calcd. for $C_{29}H_{40}O_{14}Na$ 635.2316).

Compound **4**: white amorphous powder; $[\alpha]_D$: + 24.3 (*c* 0.1, MeOH); ^1H and ^{13}C NMR data, see **Tables 1** and **2**; ESIMS *m/z* 585 $[M + Na]^+$; HRESIMS *m/z* 585.1967 $[M + Na]^+$, 541.2034 $[M + Na - 44]^+$, 497 $[M + Na - 44 - 44]^+$, (calcd. for $C_{28}H_{34}O_{12}Na$ 585.1948).

Compound **5**: white amorphous powder; $C_{28}H_{34}O_{13;}$ $[\alpha]_D$ + 27.3 (*c* 0.1, MeOH); ^1H and ^{13}C NMR data, see **Tables 1** and **2**; ESIMS *m/z* 577 $[M - H]^-$, 533 $[M - H - 44]^-$, 517 $[M - H - 60]^-$; HRESIMS *m/z* 601.1889 $[M + Na]^+$, 573.1925 $[M + Na - 28]^+$, 557.1990 $[M + Na - 44]^+$, 541.1677 $[M + Na - 60]^+$, 513.1727 $[M + Na - 44 - 44]^+$ (calcd. for $C_{28}H_{34}O_{13}Na$ 601.5463).

Surface Plasmon Resonance analyses

SPR experiments were performed as described elsewhere [5]. Briefly, analyses were carried out using a Biacore 3000 optical biosensor equipped with research-grade CM5 sensor chips (GE Healthcare). Two separate recombinant Hsp90 surfaces, a Bovine Serum Albumin (BSA) surface and an unmodified reference surface, were prepared for simultaneous analyses. Proteins (100 µg/mL in 10 mM sodium acetate, pH 5.0) were immobilized on individual sensor chip surfaces at a flow rate of 5 µL/min using standard amine-coupling protocols to obtain densities of 8−12 kRU. Compounds **1**–**7**, as well as 17-AAG and radicicol used as a positive controls, were dissolved in 100% DMSO to obtain 4 mM solutions and diluted 1:1000 (v/v) in Phosphate-Buffered Saline (PBS) (10 mM NaH_2PO_4, 150 mM NaCl, pH 7.4) to a final DMSO concentration of 0.1%. A series of concentrations were prepared as 2-fold dilutions into running buffer: for each sample, the complete binding study was performed using a six-point concentration series, typically spanning 0.025-4 µM, and triplicate aliquots of each test compound were dispensed into single-use vials. Included in each analysis were multiple blank samples of running buffer alone. Binding experiments were performed at 25 °C, using a flow rate of 50 µL/min, with 60 s monitoring of association and 250 s monitoring of dissociation. Simple interactions were adequately fit to a single-site bimolecular interaction model (A+B = AB), yielding a single K_D. Sensorgram elaborations were performed using the Biaevaluation software provided by GE Healthcare.

Cell culture and cytotoxic assay

Jurkat cell lines were obtained from the American Type Cell Culture (ATCC). Cells were maintained in RPMI 1640, supplemented with 10% fetal bovine serum, 100 mg/L streptomycin and penicillin 100 IU/mL at 37 °C in a humidified atmosphere of 5% CO_2. To ensure logarithmic growth, cells were subcultured every two days. Stock solutions (50 mM) of purified compounds in DMSO were stored in the dark at 4 °C. Appropriate dilutions were prepared in culture medium immediately prior to use. In all experiments, the final concentration of DMSO did not exceed 0.15% (v/v). Cells were seeded in 96-well plates and incubated for the established times in the absence (vehicle only) and in the presence of different concentrations of compounds **6** and **7**. The day before treatments, cells were seeded at a cell density of 1×10^4 cells/well. The number of viable cells was quantified by MTT assay by using etoposide, E1383, synthetic, ≥98%, Sigma-Aldrich, as positive control [24]. Absorption at 550 nm for each well was assessed using a microplate reader (LabSystems). In some experiments cell viability was also checked by Trypan Blue exclusion assay using a Bürker counting chamber.

Supporting information

NMR spectra of compounds **1–5** and SPR sensorgrams of tested compounds are available as Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

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Legends for figures

Fig. 1 Structures of compounds **1**–**7**.

Fig. 2 Key HMBC correlations of compounds **1**–**5**.

Table 1 ^{1}H NMR data of compounds **1–5** (CD_3OD, 600 MHz, *J* in Hz)

position	**1**[a]	**2**[a]	**3**	**4**	**5**
2	3.14 m	3.28 m	2.80 d (1.0)	4.37 d (10.5)	
3	5.05 d (9.5)		3.85 d (1.0)		
5	3.24 br d (10.4)	2.70 dd (10.0, 5.0)	1.82 br d (5.0)	3.23 d (6.0)	2.90 d (7.0)
6a	2.50 br t (13.0)	2.60 dd (17.0, 5.0)	4.27 d (5.0)	4.29 d (6.4)	4.28 d (7.0)
6b	2.26[b]	2.40 dd (17.0, 5.0)			
8	3.22 m	2.55 m			
9	1.71 m	1.85 m	1.95 dd (13.6, 5.7)	2.30 d (9.0)	2.29 dd (9.0, 4.0)
11a	1.84[b]	1.88 m	1.18[b]	1.92 m	1.90[b]
11b			1.04 m	1.80 m	
12a	1.84[b]	1.84[b]	2.52 br dd (14.0, 4.0)	2.02 ddd (18.0, 14.0, 4.7)	1.82 m
12b		1.36 m	1.15[b]	2.06 m	1.63 m
15a	5.77 s	5.86 s	2.98 d (18.0)	3.17 d (18.0)	3.19 s
15b			2.47 d (18.0)	2.78 d (18.0)	
17	5.09 br s	5.06 s	4.93 s	5.40 s	-[c]
18	1.12 s	1.10 s	1.26 s	1.17 s	1.14 s
19	1.13 s	1.18 s	1.31 s	1.35 s	1.33 s
21			4.95 s		
22	7.40 br s	7.43 br s	4.06 d (3.8)	7.42 br s	-[c]
23	6.26 s	6.24 s	4.99 d (3.8)	5.98 br s	-[c]
28	0.86 s	1.20 s	1.32 s	1.13 s	1.15 s
29a	0.83 s	0.92 s	2.28 d (13.0)	2.17 d (12.0)	2.13 d (12.0)
29b			2.04 d (13.0)	1.86 d (12.0)	1.88 d (12.0)
30a	2.26[b]	2.93 ddd (12.8, 8.7, 4.5)		2.82 d (10.5)	2.88 s
30b	1.63 m	1.83[b]			
2-OMe					3.51 s
7-OMe	3.72 s	3.75 s	3.71 s	3.74 s	3.74 s
21-OMe			3.46 s		
23-OMe			3.43 s	3.55 s	
3-OAc	2.20 s				

J values are in parentheses and reported in Hz; chemical shifts are given in ppm; assignments were confirmed by COSY, 1D-TOCSY, HSQC, and HMBC experiments. [a] Data measured in $CDCl_3$. [b] Overlapped signal. [c] Signal can't be observed clearly from 1D and 2D NMR. Weak signals are due presumably to an instable hemiacetal function and tautomerism of the butenolide ring in solution.

Table 2 ^{13}C NMR data of compounds **1–5** (CD_3OD, 150 MHz)

position	**1**[a]	**2**[a]	**3**	**4**	**5**
1	219.0	211.9	86.5	85.3	85.0
2	46.0	58.3	62.7	75.4	101.0
3	78.8	213.0	86.3	207.2	204.0
4	38.7	40.5	43.7	50.3	50.6
5	41.6	41.3	46.2	43.4	43.5
6	34.0	35.0	73.0	72.0	71.2
7	174.0	173.0	176.0	175.0	174.7
8	33.8	33.3	77.7	87.6	87.0
9	49.0	49.6	56.6	56.0	58.6
10	53.2	50.7	58.4	60.9	61.0
11	19.0	18.5	20.3	16.7	18.0
12	27.2	26.9	30.7	27.2	26.9
13	39.4	38.8	37.3	38.7	40.0
14	133.9	134.1	65.8	85.4	85.0
15	113.9	114.7	38.0	33.3	36.5
16	164.0	163.8	172.0	171.6	172.3
17	79.0	80.1	83.6	79.5	-[b]
18	20.0	20.0	18.3	13.0	15.0
19	18.6	20.3	20.0	19.0	18.2
20	133.2	133.0	83.0	133.6	-[b]
21	170.0	170.0	111.4	171.2	-[b]
22	148.8[b]	149.7[b]	77.3	149.0	-[b]
23	97.8[b]	98.2[b]	113.0	103.1	-[b]
28	22.5	22.0	18.4	15.0	15.0
29	20.9	19.9	45.5	44.8	44.7
30	35.2	38.5	211.6	64.0	72.8
2-OMe					52.6
7-OMe	53.2	53.5	52.7	51.6	52.1
21-OMe			55.7		
23-OMe			56.0	57.1	
3-$\underline{CO}CH_3$	171.0				
3-$CO\underline{CH_3}$	21.3				

[a] Data measured in $CDCl_3$. [b] Signal can't be observed clearly from 1D and 2D NMR. Weak signals are due presumably to an instable hemiacetal function and tautomerism of the butenolide ring in solution.

Table 3 Thermodynamic constants measured by Surface Plasmon Resonance for the interaction between compounds **1–7** and immobilized Hsp90

compound	K_D (nM)[a]
1	NB
2	NB
3	NB
4	6087 ± 820
5	NB
6	133 ± 18
7	183 ± 29
radicicol	1.8 ± 0.3
17 AAG	376 ± 81

[a] Results were given mean ± standard deviation

NB: no binding