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Aza analogues of equol: Novel ligands for estrogen receptor β

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Abstract—3-Aryl-tetrahydroquinolines, aza analogues of equol, are synthesized and evaluated for their binding properties to the estrogen receptors ERα and ERβ. Several of these compounds exhibited binding selectivity for ER similar to that of genistein. Compounds 8c and 8d were found to have dual actions: antagonists for ERα and agonists for ERβ in a yeast two-hybrid assay. These compounds have no estrogenic effects on the uterus and bone in vivo.

1. Introduction

The discovery of a second estrogen receptor, termed ERβ,[1] has prompted intensive studies on respective functions of the two estrogen receptor subtypes, ERα and ERβ. It is known that the distribution of these two subtypes is different in human tissues. ERβ is widely expressed in the lung, brain,[2] and prostate,[3] but is not the dominant subtype in the uterus and breast. These observations suggest that estrogen receptors might be tissue specific and ER subtype-selective ligands may produce different biological effects.[4] ERα mediates many of the well-documented activities elicited by estrogens in mammary gland, bone, and uterine tissues. Elucidation of the roles that ERβ plays would reveal its therapeutic value. For instance, an ERβ selective agonist was shown to exert potent action on experimental inflammation, pointing to its potential to become a drug target.[5]

Although the two subtypes share about 56% sequence homology, the ligand-binding pocket for ERβ differs from that of ERα by only two amino acids (ERα Leu 384 → ERβ Met336; ERα Met421 → ERβ ILe373).[6] Considering this small change in the ligand-binding pocket, it is not surprising that 17β-estradiol displays a similar affinity for both subtypes. A handful of compounds that display some selectivity for ERβ, including genistein,[7] DPN,[8] ERB-041,[9] and tetrahydrofluorenone[10] (Fig. 1), have been reported, but the desire of developing more potent and selective ERβ modulators remains strong. In this paper, we describe the discovery and characterization of a new class of ligands that are selective for ERβ.

Equol, a key metabolite of daidzein, is a unique compound which selectively mimics estrogen actions[11] and in the meanwhile antagonizes the effects of dihydrotestosterone.[12] Equol and dehydroequol were reported to

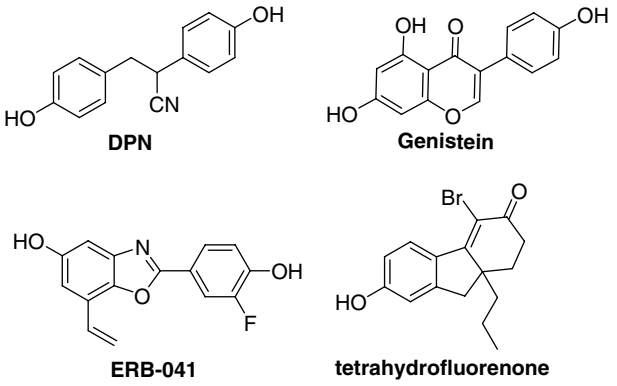

Figure 1. Selective estrogen receptor β ligands.

Keywords: 3-Phenyl-tetrahydroquinoline; ERβ; Equol.
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possess modest selectivity for ERβ (8- to 90-fold),[13] and the former could also inhibit bone loss in ovariectomized mice.[14] In order to study the structure-activity relationship (SAR) of this class of compounds, we chose tetrahydroquinoline as the scaffold because of its easy modification. The different groups on nitrogen should change the dominative conformations of the 3-aryl-tetrahydroquinolines and might promote selectivity for ERβ. Thus, a series of 7-hydroxy-3-phenyl-1,2,3,4-tetrahydroquinoline derivatives were prepared according to a previously disclosed synthetic procedure.[15]

2. Results and discussion

2.1. Chemistry

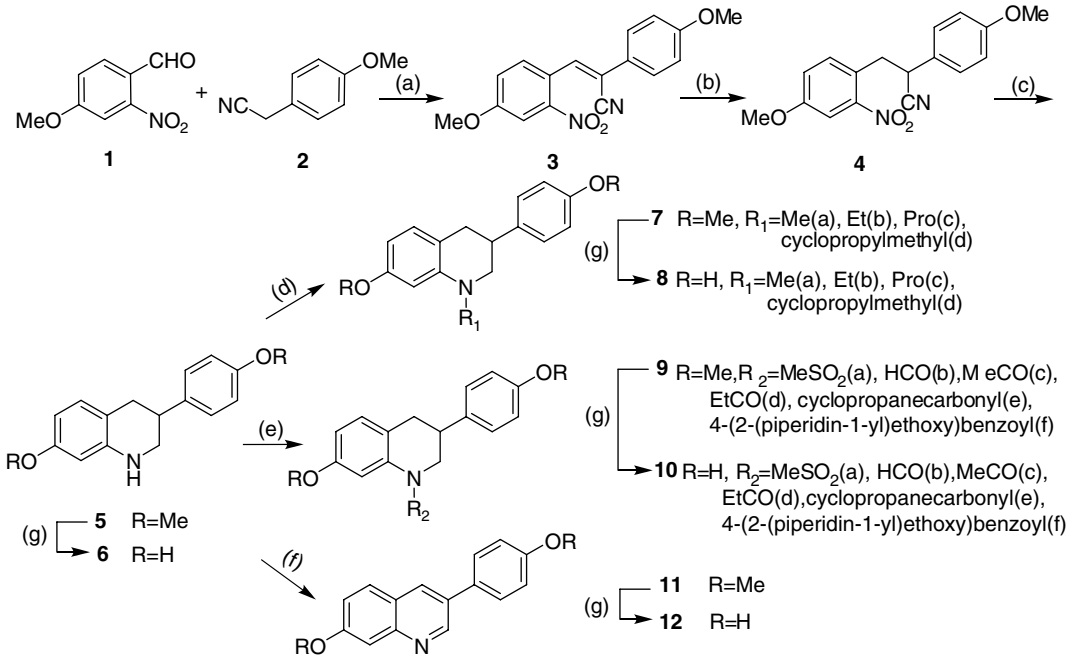
The starting material 4-methoxy-2-nitrobenzaldehyde was made from inexpensive and commercially available 4-methoxy-2-nitro-aniline according to the procedure described in the literature.[16] As shown in Scheme 1, the 2-phenyl-3-(2-nitro-phenyl)-acrylonitrile derivative **3** was easily obtained by condensation of 4-methoxy-2-nitrobenzaldehyde **1** with 4-methoxyphenylacetonitrile **2**. Reduction of the acrylonitrile **3** was carried out with $NaBH_4$ in methanol and THF, thereby yielding propionitrile **4**. 3-Aryl-1,2,3,4-tetrahydro-quinoline **5** was directly obtained by Pd/C-catalyzed hydrogenation of **4**. Deprotection of the methoxy group of **5** was readily achieved with BBr_3 in CH_2Cl_2 to afford **6**. Alkylation of the nitrogen of **5** was effected with sodium borohydride and neat carboxylic acids in THF to yield **7** and removal of the methoxy protecting group was once again achieved with BBr_3 to afford *N*-alkyl tetrahydroquinolines **8**. N-Sulfonylation/N-acylation of **5** was readily accomplished by treating **5** with methanesulfonyl chloride or R-carbonyl chloride and Et_3N in CH_2Cl_2 to yield sulfonamide/amides **9** and subsequent deprotection (BBr_3) afforded **10**. Finally, dehydrogenation of **5** with palladium on activated charcoal at 270 °C gave the aromatic ring **11** and deprotection with BBr_3 in the same way afforded **12**.

The synthesis of 3-aryl-tetrahydroquinoline analogues was accomplished using the synthetic transformations shown in Scheme 2. The substituted *trans-o*-nitro-α-phenyl-cinnamic acid was prepared by condensation of 4-methoxy-2-nitrobenzaldehyde with 4-methoxyphenylacetic acid in the presence of acetic anhydride and triethylamine under reflux, and catalytic hydrogenation of the corresponding (*E*)-*o*-nitro-α-phenylcinnamic acid over Pd/C afforded 3,4-dihydro-3-phenylcarbostyril **13** in good yield.

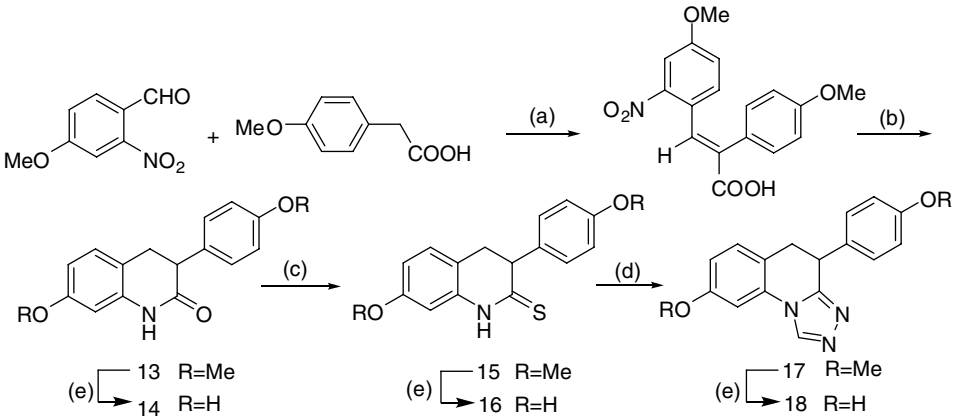
The conversion of **13** to the corresponding thiolactam **15** (96%) was achieved by the Lawesson's reagent[17] in boiling toluene. The thiolactam **15** reacted further with formic anhydrazine in cyclohexanol to afford compound **17**.[18] In the preparation of compound **17**, the reaction should be carried out under nitrogen atmosphere and low boiling point solvents should not be used. The methoxy protecting group of **13**, **15**, **17** was removed using BBr_3 to unmask the phenol giving the desired analogues **14**, **16**, **18**, respectively.

2.2. Biological activity

The compounds described above were primarily tested for intrinsic activity in an ER binding assay with [^{3}H]17β-estradiol and full-length recombinant human ERα and ERβ proteins. First, these compounds were tested at 10 and 1 μM, and their binding activities for



Scheme 1. Reagents and condition: (a) Na, CH_3CH_2OH; (b) $NaBH_4$, THF-CH_3OH; (c) H_2, Pd/C, THF-CH_3OH; (d) R_2COOH, $NaBH_4$, THF; (e) R_2COX, TEA, CH_2Cl_2; (f) Pd/C, heating under N_2; (g) BBr_3, CH_2Cl_2.



Scheme 2. Reagents: (a) TEA, Ac_2O; (b) H_2, Pd/C, THF-CH_3OH; (c) Lawesson's reagent, THF; (d) $HCONHNH_2$, Cyclohexanol; (e) BBr_3, CH_2Cl_2.

ERα and ERβ are shown in Figure 1. We found that all the carbonyl amides of tetrahydroquinoline displayed lower affinities to both ERs, and the hydrophobic property may improve this feature. For example, *N*-alkyl compounds of tetrahydroquinoline showed higher affinities than amides, thioamide **16** also displayed higher affinity than compound **14/18** because **16** is more hydrophobic. Then we examined the IC_{50} values of several active compounds chosen from Figure 2 and the results are summarized in Table 1. They generally exhibited poor binding properties compared to the control compound, and the IC_{50} of the most active compound **8b** was at least 12-fold less potent than genistein for ERβ. Interestingly, compound **10a** with a methanesulfonyl group showed an ERβ binding selectivity close to that of genistein.

To assess whether this class of compounds are of agonist or antagonist nature, some of them were additionally evaluated in a yeast two-hybrid assay. This system is based on the ligand-dependent interaction between two proteins, a hormone receptor and a co-activator, and estrogenic activity is detected by α-galactosidase activity.[19]

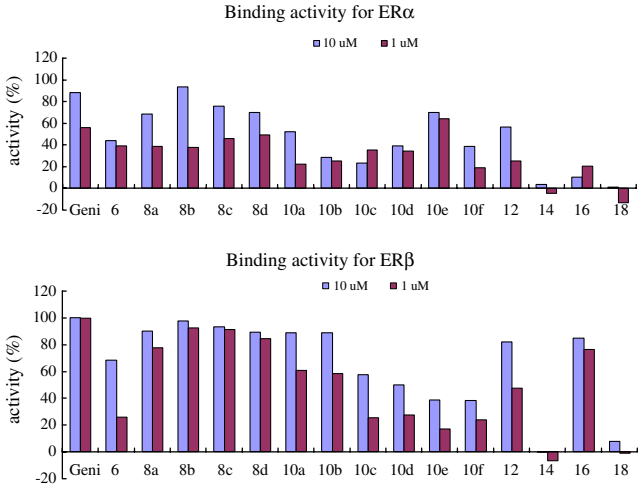


Figure 2. Estrogen receptor binding characteristics of various 3-aryl-tetrahydroquinoline analogues.

Table 1. Binding property and selectivity for human ERα and ERβ

Compound	ERα IC_{50}(μM)	ERβ IC_{50}(μM)	fold Selectivity for ERβ
Genistein	0.58	0.0068	85.1
8a	2.95	0.22	13.3
8b	1.41	0.083	17.0
8c	2.47	0.14	17.9
8d	1.63	0.23	7.1
10a	32.10	0.52	61.5
10e	NA	2.95	—
16	NA	4.90	—

NA, not active.

We determined the EC_{50} values using the ERβ agonist genistein as a control.[20] As shown in Tables 2 and 3, all these compounds behaved as agonists on ERβ; most of them (except 8c and 8d) also displayed ERα agonism; compounds **8c** and **8d** exhibited antagonist features on ERα and significantly inhibited the effect induced by

Table 2. In Yeast Binding Affinity for Selected ERβ Ligands (1)[a]

Compound	ERα EC_{50} (μM)	ERβ EC_{50} (μM)	ERβ selectivity[b]
Genistein	0.81 ± 0.06	0.028 ± 0.001	29
6	>100	9.7 ± 0.66	N/A[c]
8a	15.2 ± 2.02	0.3 ± 0.01	50
8b	24.33 ± 3.9	0.58 ± 0.02	41
12	>100	37.5 ± 1.52	N/A

[a] Dose-depedent effect of genistein and test compounds on ERα/β-SRC-1 interactions as determined by the yeast two-hybrid assay.
[b] Selectivity is reported as EC_{50} (ERα)/ EC_{50} (ERβ).
[c] N/A, selectivity could not be accurately determined under the assay conditions used. Values are means ± SD from three independent experiments.

Table 3. In Yeast Binding Affinity for Selected ERβ Ligands (2)

Compound	ERα IC_{50}[a] (μM)	ERβ EC_{50} (μM)
8c	2.65 ± 0.49	4.75 ± 0.27
8d	5.57 ± 0.30	6.89 ± 0.74

[a] IC_{50}, the concentration of a test compound required to inhibit 50% of the maximal α-galactosidase activity by 1 nM of 17β-estradiol ± SEM.

17β-estradiol (1 nM) (IC_{50} = 2.65 and 5.57 μM, respectively).

The above compounds were next examined in vivo for their abilities to affect uterine wet weight and bone mineral density. In contrast to raloxifene (control), the 10 compounds tested did not show any estrogenic effects in ovariectomized mice including the ERα agonists (Table 4). We presumed that the ERα agonists were easily transformed into hydrophilic metabolites that are less active and eliminated quickly in vivo. The biotransformation of these compounds reduced estrogenic property to a minimum.

3. Conclusion

In summary, we have identified 3-aryl-tetrahydroquinolines as a new class of ERβ-selective ligands. Substitution at the nitrogen position, particularly with methanesulfonyl group, is preferable for ERβ selectivity. Compounds **8c** and **8d** were found to be antagonists for ERα and agonists for ERβ in the yeast two-hybrid assay. And this series of compounds did not display any observable estrogen-like activities in vivo.

4. Experimental

4.1. General

The ^{1}H NMR spectra were recorded on Bruker AMX 400 MHz NMR spectrometer. The NMR data were reported in parts per million relative to TMS or referenced to the solvent in which they were run. Melting points (uncorrected) were determined on a Buchi-510 capillary apparatus. The MS(ESI) and HRMS (ESI) spectra were obtained on an Finnigan MAT 95 mass spectrometer, EI: 70 eV. The solvent was removed by rotary evaporation under reduced pressure, and flash column chromatography was performed on silica gel (200–300 mesh). Anhydrous solvents were obtained by distilling from sodium wire.

Table 4. Effects on uterine wet weight and bone mineral density

Compound	Bone mineral density (mg/cm^3)	Uterine wet weight (mg)
Raloxifene	608 ± 34***	69.5 ± 12.7***
6	467 ± 68*	31.4 ± 5.8*
8a	455 ± 53*	31.8 ± 4.0*
8b	454 ± 36*	31.7 ± 3.9*
8c	439 ± 28*	28.3 ± 3.3*
8d	461 ± 39*	30.8 ± 12.2*
OVX+DW	461 ± 19	31.4 ± 3.5
Sham + DW	587 ± 67***	120.0 ± 29.0***
Raloxifene	555 ± 54***	62.8 ± 12.2**
10a	475 ± 48*	32.5 ± 6.9*
10b	463 ± 36*	36.4 ± 10.4*
10c	451 ± 28*	29.1 ± 3.2*
12	430 ± 22**	33.1 ± 4.8*
16	483 ± 22*	38.7 ± 16.8*
OVX+DW	461 ± 9	36.8 ± 11.4
Sham + DW	556 ± 36***	129.1 ± 16.7***

Two months old ovariectomized Kunming mice were treated (sc) with the test compounds at a dose of 4 μmol/kg qd for 4 weeks. Uterine wet weight was measured on the day when mice were sacrificed (n = 5) per group; mean ± SD.

* $p > 0.05$.

** $p < 0.05$.

*** $p < 0.01$ versus distilled water (DW) treated animals.

4.2. Synthesis

4.2.1. Synthesis of (*Z*)-3-(4-methoxy-2-nitro-phenyl)-2-(4-methoxy-phenyl)-acrylonitrile (3). A mixture of 4-methoxy-2-nitrobenzaldehyde (1.38 g, 7.6 mmol) and 4-methoxyphenyl acetonitrile (1.12 g, 7.6 mmol) was added to a solution of sodium (0.17 g, 7.6mmol) in absolute ethanol (10 mL) while stirring. A few minutes later, the mixture became warm, turned cloudy, and precipitated. Stirring was continued for another 5 h. The mixture was cooled and the product was separated by filtration. The crude product was washed with distilled water (20 mL), then with 95% ethanol (50 mL) to afford a pure, bright yellow powder (1.82 g, 76.9%). Mp 143–144 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.86 (s, 3H), 3.94 (s, 3H), 6.97 (d, J = 8.8 Hz, 2H), 7.28 (m, 1H), 7.64 (d, J = 8.8 Hz, 2H), 7.70 (d, J = 2.8 Hz, 1H),7.85 (s, 1H), 7.91 (d, J = 8.5 Hz, 1H). MS (EI): *m/e* (%) 310 (80M$^+$), 293 (100), 149 (65), 122 (70).

4.2.2. Synthesis of 3-(4-methoxy-2-nitro-phenyl)-2-(4-methoxy-phenyl)-propionitrile (4). (*Z*)-3-(4-Methoxy-2-nitro-phenyl)-2-(4-methoxy-phenyl)-acrylonitrile (1.55 g, 5 mmol) and sodium borohydride (0.28 g, 7.5 mmol) were added to a mixture of THF (25 mL) and MeOH (5 mL), stirred at ambient temperature for 4 h, and then poured the mixture into cold water and neutralized with 1 mol L^{-1} HCl. The aqueous solution was extracted with EtOAc. The combined organic phase was washed successively with water, saturated $NaHCO_3$, and brine and then dried with anhydrous sodium sulfate. Removal of the solvent afforded 3-(4-methoxy-2-nitro-phenyl)-2-(4-methoxy-phenyl)-propionitrile (1.53 g, 98.2%), which was used for the next step without further purification. Mp 107-109 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.15 (dd, J = 10.3 Hz, 13.5 Hz, 1H), 3.43 (dd, J = 5.2 Hz, 13.2 Hz, 1H), 3.82 (s, 3H), 3.88 (s, 3H), 4.28 (dd, J = 5.3 Hz, 10.1 Hz, 1H), 6.92 (d, J = 8.7 Hz, 2H), 7.14 (dd, J = 2.9 Hz, 8.6 Hz, 1H), 7.33 (d, J = 8.3 Hz, 1H), 7.34 (d, J = 8.8 Hz, 2H), 7.57 (d, J = 2.7 Hz, 1H). MS (EI): *m/e* (%) 312 (7M$^+$), 166 (100), 146 (85).

4.2.3. Synthesis of 7-methoxy-3-(4-methoxy-phenyl)-1,2,3,4-tetrahydro-quinoline (5). After several vacuum/H_2 cycles to remove air from the reaction flask, the stirred mixture of the 3-(4-methoxy-2-nitro-phenyl)-2-(4-methoxy-phenyl)-propionitrile (1.62 g, 5.2 mmol), 10% Pd/C (0.49 g, 30 wt% of the propanenitrile) in THF (20 mL) and MeOH (5 mL) was hydrogenated at ordinary pressure and at temperature (ca. 40 °C) for 48 h. The reaction mixture was filtered using a membrane filter and filtrate was concentrated under reduced pressure. The crude mixture was purified by flash silica gel column chromatography, using petroleum ether/ethyl acetate (V/V: 10/1) as eluent, thus obtaining the white crystal

(0.85 g, 60.6%). Mp 146–147 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.92–2.97 (m, 2H), 3.26–3.31 (m, 2H), 3.59–3.61 (m, 1H), 3.75 (s, 3H), 3.81 (s, 3H), 6.53 (dd, J = 2.4 Hz, 8.3 Hz, 1H), 6.6 (d, J = 2.2 Hz, 1H), 6.89 (d, J = 8.8 Hz, 2H), 7.00 (d, J = 8.5 Hz, 1H), 7.19 (d, J = 8.7 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 33.9, 37.9, 48.4, 55.1, 55.2, 99.2, 102.9, 113.9, 114.0, 128.0, 130.0, 135.9, 144.7, 158.2, 158.9. MS (EI): *m/e* (%) 269 (100 M^+), 148 (55).

4.2.4. General procedure for alkylation of the tetrahydroquinoline nitrogen.

4.2.4.1. 7-Methoxy-3-(4-methoxy-phenyl)-1-methyl-1,2,3,4-tetrahydro- quinoline (7a). Compound **5** (0.27 g, 1 mmol) was dissolved in 10 mL of THF under N_2, chilled to 0 °C, and 0.28 g (7.5 mmol) of $NaBH_4$ added, followed by slow addition of 3 mL of HCOOH. The reaction mixture was allowed to warm to room temperature and stirred overnight. The solvent was removed, the residue was slurried in water and made basic with 1 mol L^{-1} NaOH, and the product was extracted into EtOAc and chromatographed on a silica gel column. Elution with petroleum ether/EtOAc (10/1) gave a white solid (0.11 g, 38.1%). Mp 99–100 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.90–2.93 (m, 2H), 2.95 (s, 3H), 3.31 (d, J = 6.1 Hz, 2H), 3.14–3.20 (m, 1H), 3.80 (s, 3H), 3.81 (s, 3H), 6.30 (s, 2H), 6.88 (d, J = 8.6 Hz, 2H), 6.93 (d, J = 8.1 Hz, 1H), 7.16 (d, J = 8.9 Hz, 2H). MS (EI): *m/e* (%) 283 (100 M^+), 162 (35).

4.2.4.2. 1-Ethyl-7-methoxy-3-(4-methoxy-phenyl)-1,2,3,4-tetrahydro-quinoline (7b). This compound was prepared from compound **5** with acetic acid according to the similar procedure to **7a** as a solid in 88.2%. Mp 77–78 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.15 (t, J = 7.2 Hz, 3H), 2.88–2.91 (m, 2H), 3.03–3.15 (m, 1H), 3.20–3.33 (m, 3H), 3.40–3.51 (m, 1H), 3.80 (s, 3H), 3.81 (s, 3H), 6.18 (dd, J = 2.6 Hz, 8.1 Hz, 1H), 6.23 (d, J = 2.2 Hz, 1H), 6.89 (d, J = 8.5 Hz, 2H), 6.91 (d, J = 8.1 Hz, 1H), 7.17 (d, J = 8.4 Hz, 2H). MS (EI): *m/e* (%) 297 (100 M^+), 282 (85), 121 (60).

4.2.4.3. 7-Methoxy-3-(4-methoxy-phenyl)-1-propyl-1,2,3,4-tetrahydro-quinoline (7c). This compound was prepared from compound **5** with propionic acid according to the similar procedure to **7a** as a solid in 87.4%. Mp 84–85 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.93 (t, J = 7.3 Hz, 3H), 1.52–1.71 (m, 2H), 2.86–2.93 (m, 2H), 3.03–3.36 (m, 5H), 3.79 (s, 3H), 3.81 (s, 3H), 6.15–6.26 (m, 2H), 6.88 (d, J = 8.5 Hz, 2H), 6.89 (m, 1H), 7.16 (d, J = 8.7 Hz, 2H). MS (EI): *m/e* (%) 311 (50M^+), 282 (100).

4.2.4.4. 1-Cyclopropylmethyl-7-methoxy-3-(4-methoxy-phenyl)-1,2,3,4-tetrahydro-quinoline (7d). This compound was prepared from compound **5** with cyclopropanecarboxylic acid according to the similar procedure to **7a** with acetic acid as a solid in 36.2%. Mp 97–98 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.20–0.25 (m, 2H), 0.52–0.55 (m, 2H), 0.92–0.94 (m, 1H), 2.91–3.07 (m, 3H), 3.08–3.20 (br, 1H), 3.41–3.45 (br, 3H), 3.80 (s, 3H), 3.81 (s, 3H), 6.20–6.46 (m, 2H), 6.89 (d, J = 8.8 Hz, 2H), 6.93 (m, 1H), 7.17 (d, J = 8.8 Hz, 2H). MS (EI): *m/e* (%) 323 (100M^+), 149 (50), 121 (50).

4.2.5. General procedure for sulfonylation/acylation of the tetrahydroquinoline nitrogen.

4.2.5.1. 1-Methanesulfonyl-7-methoxy-3-(4-methoxy-phenyl)-1,2,3,4- tetrahydro-quinoline (9a). The THF solution of the methanesulfonyl chloride (0.09 g, 0.825mmol) was added dropwise to a solution of **5** (0.21 g, 0.75 mmol), triethylamine(0.11 mL, 0.825 mmol) in dry THF (10 mL) blanketed under nitrogen and cooled to 0 °C. When the addition was complete, the reaction mixture was stirred at room temperature for 2 h. After removal of THF, the residue was loaded on silica gel column and eluted with petroleum ether-ethyl acetate to afford the corresponding product (0.13 g, 51.9%). Mp 135.5–136 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.83 (s, 3H), 2.87–3.20 (m, 3H), 3.50–3.60 (m, 1H), 4.22–4.30 (m, 1H), 3.80 (s, 3H), 3.81 (s, 3H), 6.68 (dd, J = 2.3 Hz, 8.2 Hz, 1H), 6.88 (d, J = 8.8 Hz, 2H), 7.09 (d, J = 8.4 Hz, 1H), 7.17 (d, J = 8.7 Hz, 2H), 7.40 (d, J = 2.4 Hz, 1H). MS (EI): *m/e* (%) 347 (60M^+), 214 (80), 121 (100).

4.2.5.2. 7-Methoxy-3-(4-methoxy-phenyl)-3,4-dihydro-2*H*-quinoline-1-carbaldehyde (9b). This compound was prepared from compound **5** with formic acid and acetic anhydride according to the similar procedure to **9a** as a solid in 72.3%. Mp 112–113 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.84–3.17 (m, 3H), 3.33–3.41 (m, 1H), 3.81 (s, 3H), 3.83 (s, 3H), 4.38–4.42 (m, 1H), 6.71 (dd, J = 2.4 Hz, 8.5 Hz, 1H), 6.74 (d, J = 2.4 Hz, 1H), 6.88 (d, J = 8.7 Hz, 2H), 7.12 (d, J = 8.3 Hz, 1H), 7.17 (d, J = 8.7 Hz, 2H), 8.82 (s, 1H). MS (EI): *m/e* (%) 297 (100M^+), 134 (70).

4.2.5.3. 1-[7-Methoxy-3-(4-methoxy-phenyl)-3,4-dihydro-2*H*-quinolin-1-yl]-ethanone (9c). This compound was prepared from compound **5** with acetyl chloride according to the similar procedure to **9a** as a solid in 96.5%. Mp 102–103 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.21 (s, 3H), 2.80–2.89 (m, 1H), 2.99–3.14 (m, 2H), 3.56–3.64 (m, 1H), 3.79 (s, 3H), 3.81 (s, 3H), 4.11–4.22 (m, 1H), 6,71 (dd, J = 2.4 Hz, 8.5 Hz, 1H), 6.74 (d, J = 2.4 Hz, 1H), 6.86 (d, J = 8.6 Hz, 2H), 7.08 (d, J = 8.5 Hz, 1H), 7.14 (d, J = 8.5 Hz, 2H). MS (EI): *m/e* (%) 311 (5M^+), 149 (100).

4.2.5.4. 1-[7-Methoxy-3-(4-methoxy-phenyl)-3,4-dihydro-2*H*-quinolin-1-yl]-propan-1-one (9d). This compound was prepared from compound **5** with propionyl chloride according to the similar procedure to **9a** as a solid in 99%. Mp 89–90 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.14 (t, J = 7.4 Hz, 3H), 2.50 (q, J = 7.2 Hz, 2H), 2.80–2.89 (m, 1H), 2.99–3.18 (m, 2H), 3.57–3.62 (m, 1H), 3.80 (s, 3H), 3.81 (s, 3H), 4.11–4.20 (m, 1H), 6.71 (dd, J = 2.5 Hz, 8.5 Hz, 1H), 6.74 (d, J = 2.4 Hz, 1H), 6.86 (d, J = 8.7 Hz, 2H), 7.08 (d, J = 8.4 Hz, 1H), 7.15 (d, J = 8.6 Hz, 2H). MS (EI): *m/e* (%) 325 (100M^+), 269 (100).

4.2.5.5. Cyclopropyl-[7-methoxy-3-(4-methoxy-phenyl)-3,4-dihydro-2*H*-quinolin-1-yl]-methanone (9e). This compound was prepared from compound **5** with cyclo-

propanecarbonyl chloride according to the similar procedure to **9a** as a solid in 93.5%. Mp 106–107 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.80 (m, 2H), 1.12 (m, 2H), 2.00–2.09 (m, 1H), 2.80–2.91 (m, 1H), 2.99–3.18 (m, 2H), 3.57 (dd, J = 10.0 Hz, 12.7 Hz, 1H), 3.79 (s, 3H), 3.81 (s, 3H), 4.25 (dd, J = 0.9 Hz, 5.4 Hz, 1H), 6.70 (dd, J = 2.6 Hz, 8.3 Hz, 1H), 6.85 (d, J = 8.9 Hz, 2H), 7.05 (d, J = 2.5 Hz, 1H), 7.10 (d, J = 8.3 Hz, 1H), 7.15 (d, J = 8.8 Hz, 2H). MS (EI): *m/e* (%) 337 (95M$^+$), 269 (100).

4.2.5.6. [7-Methoxy-3-(4-methoxy-phenyl)-3,4-dihydro-2*H*-quinolin-1-yl]-[4-(2-piperidin-1-yl-ethoxy)-phenyl]-methanone (9f). This compound was prepared from compound **5** with 4-(2-(piperidin-1-yl)ethoxy)benzoyl chloride according to the similar procedure to **9a** as a solid in 96.8%. Mp 166–168 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.63–1.71 (m, 4H), 1.82–1.97 (m, 4H), 2.20–2.37 (m, 2H), 2.71–2.84 (m, 2H), 2.91–3.02 (m, 1H), 3.07–3.25 (m, 2H), 3.54 (s, 3H), 3.71 (dd, J = 9.1 Hz, 12.6 Hz, 1H), 3.79 (s, 3H), 4.24 (dd, J = 4.7 Hz, 12.6 Hz, 1H), 4.54–4.60 (m, 2H), 6.45-6.46 (m, 1H), 6.63 (dd, J = 2.2 Hz, 8.4 Hz, 1H), 6.79 (d, J = 8.8 Hz, 2H), 6.86 (d, J = 8.4 Hz, 2H), 7.08 (d, J = 8.3 Hz, 1H), 7.15 (d, J = 8.6 Hz, 2H), 7.33 (d, J = 8.9 Hz, 2H). MS (EI): *m/e* (%) 500 (10 M$^+$), 98 (100).

4.2.6. Methoxy-3-(4-methoxy-phenyl)-quinoline (11). A mixture of **5** (0.27 g, 1mmol) and palladium on activated charcoal (10%) (54 mg) was heated at 270–280 °C, under nitrogen with stirring for 30 min. On cooling, methanol was added and the reaction mixture was filtered through Celite. The solvent was evaporated and the residue was purified by flash silica gel column chromatography using petroleum ether-ethyl acetate as eluent to afford white solid (0.25 g, 96.2%). Mp 142–143 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.88 (s, 3H), 3.98 (s, 3H), 7.05 (d, J = 8.8 Hz, 2H), 7.22 (dd, J = 2.4 Hz, 8.9 Hz, 1H), 7.47 (d, J = 2.4 Hz, 1H), 7.63 (d, J = 8.9 Hz, 2H), 7.75 (d, J = 9.0 Hz, 1H), 8.20 (d, J = 2.3 Hz, 1H), 9.07 (d, J = 2.3 Hz, 1H). MS (EI): *m/e* (%) 265 (100M$^+$), 250 (60).

4.2.7. 7-Methoxy-3-(4-methoxy-phenyl)-3,4-dihydro-1*H*-quinolin-2-one (13). A mixture of 1.81 g (10 mmol) of 4-methoxy-2-nitrobenzaldehyde, 2.41 g (14.5 mmol) of 4-methoxyphenylacetic acid, 5 mL (54 mmol) of acetic anhydride, and 1 g (10 mmol) of triethylamine was refluxed for 15 minutes in a 25-mL flask. The solution was cooled to 90 °C, and 5 mL of cold water was added over a 5-min period at a rate that maintained the temperature above 90 °C. The solution was cooled to 20 °C and the substituted *trans-o*-nitro-α-phenylcinnamic acid precipitated in the form of light orange crystals. It was separated by filtration and washed with 10 mL of 50% acetic acid and with water. After recrystallization from 50 mL of ethanol, the dried acid was in the form of yellow prisms weighing 2.39 g (71%) and melting at 205–206 °C. The obtained (*E*)-3-(4-methoxy-2-nitrophenyl)-2-(4-methoxy-phenyl)-acrylic acid (1.65 g, 5mmol) was hydrogenated with 10% Pd/C (0.17 g) in THF and MeOH at room temperature under atmospheric pressure for 12 h. The catalyst and the solvent were removed and the residue was recrystallized with ethanol to give 13 (1.32 g, 93%), Mp 182–183 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.00–3.06 (m, 2H), 3.68 (s, 3H), 3.69 (s, 3H), 3.72–3.79 (m, 1H), 6.45–6.47 (m, 1H), 6.49–6.51 (m, 1H), 6.82 (d, J = 9.0 Hz, 2H), 7.05 (d, J = 8.1 Hz, 1H), 7.10 (d, J = 8.5 Hz, 2H). MS (EI): *m/e* (%) 283 (100 M$^+$), 149 (55), 57 (50).

4.2.8. 7-Methoxy-3-(4-methoxy-phenyl)-3,4-dihydro-1*H*-quinoline-2-thione (15). A mixture of **13** (1.92 g, 6.8 mmol) and Lawesson's reagent (2.06 g, 5.1 mmol) was suspended in anhydrous toluene (25 mL) and then refluxed under nitrogen atmosphere for 5 h. The solution was then cooled to room temperature and evaporated. The residue was purified by flash chromatography on silica gel (eluant CH_2Cl_2–MeOH, 50:1) providing **15** (1.95 g) in 96% yield as a pale yellow solid: Mp 179–180 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.00 (dd, J = 6.1 Hz, 16.1 Hz, 1H), 3.24 (dd, J = 6.3 Hz, 15.9 Hz, 1H), 3.74 (s, 3H), 3.78 (s, 3H), 4.27 (t, J = 6.0 Hz, 1H), 6.44–6.47 (m, 1H), 6.60–6.62 (m, 1H), 6.78 (d, J = 8.5 Hz, 2H), 7.04 (d, J = 8.2 Hz, 1H), 7.13 (d, J = 8.6 Hz, 2H). MS (EI): *m/e* (%) 299 (100M$^+$).

4.2.9. 8-Methoxy-4-(4-methoxy-phenyl)-4,5-dihydro-[1,2,4]triazolo[4,3-*a*]quinoline (17). A mixture of compound **15** (0.3 g, 1 mmol) and formic hydrazine 0.08 g (1.2 mmol) in 5 mL cyclohexanol was refluxed for 6 h under a nitrogen atmosphere. After the solvent was removed under reduced pressure, the residue was dissolved in EtOAc and loaded on silica gel column and eluted with CH_2Cl_2-MeOH (50:1) to afford a brown solid 0.28 g (90.2%), Mp 90–92.5 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.16–3.31 (m, 2H), 3.75 (s, 3H), 3.86 (s, 3H), 4.51 (t, J = 7.1 Hz, 1H), 6.78 (d, J = 2.5 Hz, 1H), 6.82 (d, J = 8.8, 2H), 6.96 (d, J = 2.5 Hz, 1H), 7.16 (d, J = 8.5 Hz, 2H), 7.25 (d, J = 8.6 Hz, 1H), 8.71 (s, 1H). MS (EI): *m/e* (%) 307 (5M$^+$), 84 (100).

4.2.10. General procedure for protecting-group cleavage.

4.2.10.1. 3-(4-Hydroxy-phenyl)-1,2,3,4-tetrahydro-quinolin-7-ol (6). The substrate **5** (0.20 g, 0.75 mmol) was dissolved in CH_2Cl_2 (10mL) and cooled to −10 °C and BBr_3 (0.75 g, 0.29mL, 3.0mmol) in CH_2Cl_2 was added dropwise with stirring. As the solution of boron tribromide was added, a white precipitate was formed. The reaction mixture was allowed to attain room temperature overnight with stirring, when a clear, brownish yellow solution was obtained. The reaction mixture was then hydrolyzed by careful shaking with 5 mL of water, thus precipitating a white solid which was dissolved by the addition of 1 mol L^{-1} sodium hydroxide, then neutralized with dilute hydrochloric acid, extracted with 50 mL of ethyl acetate, and the organic layer was dried over anhydrous magnesium sulfate. On removal of the ethyl acetate under reduced pressure, a brownish yellow oil remained which was purified by flash chromatography on silica gel (eluant petroleum ether/ethyl acetate, 2:1) providing **6** (0.20 g) in 99% yield as a white solid. Mp 228–229 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.61–2.83 (m, 3H), 3.0–3.07 (m, 1H), 3.15–3.21 (m, 1H), 5.87 (dd, J = 2.2 Hz, 7.8 Hz, 1H), 5.92 (d,

J = 2.4 Hz, 1H), 6.63 (d, J = 8.2 Hz, 1H), 6.69 (d, J = 8.4 Hz, 2H), 7.04 (d, J = 8.3 Hz, 2H). MS (EI): *m/e* (%) 241 (100M^+), 149 (60), 134 (65). HRMS (EI) calcd for $C_{15}H^{15}NO_2$, 241.1103; found, 241.1106.

4.2.10.2. 3-(4-Hydroxy-phenyl)-1-methyl-1,2,3,4-tetrahydro-quinolin-7-ol (8a). This compound was prepared according to the similar procedure to **6** as a solid in 84.5%. Mp 203–204 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.70–2.73 (m, 2H), 2.78 (s, 3H), 2.93 (s, 1H), 3.11–3.15 (m, 2H), 5.98–6.00 (m, 2H), 6.68–6.70 (m, 3H), 7.05 (d, J = 8.0 Hz, 2H), 8.96 (s, 1H), 9.36 (s, 1H). MS (EI): *m/e* (%) 255 (100 M^+), 148 (75). HRMS (EI) calcd for $C_{16}H_{17}NO_2$, 255.1259; found, 255.1262.

4.2.10.3. 1-Ethyl-3-(4-hydroxy-phenyl)-1,2,3,4-tetrahydro-quinolin-7-ol (8b). This compound was prepared according to the similar procedure to **6** as a solid in 93.6%. Mp 174–175 °C. 1H NMR (300 MHz, DMSO-*d*6) δ 1.01 (t, J = 6.6 Hz, 3H), 2.64–2.78 (m, 2H), 3.09–3.19 (m, 2H), 3.52–3.60 (m, 3H), 5.87–5.94 (m, 1H), 6.02 (s, 1H), 6.67–6.70 (m, 3H), 7.05 (d, J = 8.2 Hz, 2H), 8.91 (s, 1H), 9.36 (s, 1H). MS (EI): *m/e* (%) 269 (100 M+), 254 (95). Anal. calcd for $C_{17}H_{19}NO_2$·0.25-H2O: C, 74.56; H, 7.18; N. 5.11; found: C, 74.89; H, 7.10; N. 4.85.

4.2.10.4. 3-(4-Hydroxy-phenyl)-1-propyl-1,2,3,4-tetrahydro-quinolin-7-ol (8c). This compound was prepared according to the similar procedure to **6** as a solid in 78.8%. Mp 144-145 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.84 (t, J = 7.4 Hz, 3H), 1.43–1.55 (m, 2H), 2.60–2.78 (m, 2H), 2.80–2.90 (m, 1H), 2.99–3.09 (m, 1H), 3.14–3.25 (m, 3H), 5.88 (dd, J = 1.8 Hz, 7.6 Hz, 1H), 5.99 (d, J = 2.0 Hz, 1H), 6.64 (m, 1H), 6.67 (d, J = 8.6 Hz, 2H), 7.03 (d, J = 8.8 Hz, 2H). MS (EI): *m/e* (%) 283 (55 M^+), 254 (100). HRMS (EI) calcd for $C_{18}H_{21}NO_2$, 283.1572; found, 283.1562.

4.2.10.5. 1-Cyclopropylmethyl-3-(4-hydroxy-phenyl)-1,2,3,4-tetrahydro-quinolin-7-ol (8d). This compound was prepared according to the similar procedure to **6** as a solid in 92.5%. Mp 124–125 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.17 (m, 2H), 0.41–0.43 (m, 2H), 0.75–0.80 (m, 1H), 2.60–2.75 (m, 2H), 2.75–2.89 (m, 2H), 3.24–3.29 (m, 3H), 5.93 (d, J = 8.3 Hz, 1H), 6.11 (s, 1H), 6.67–6.70 (m, 3H), 7.05 (d, J = 8.3 Hz, 2H), 8.92 (s, 1H), 9.36 (s, 1H). MS (EI): *m/e* (%) 295 (60M^+), 149 (50), 115 (100). HRMS (EI) calcd for $C_{19}H_{21}NO_2$, 295.1572; found, 295.1568.

4.2.10.6. 3-(4-Hydroxy-phenyl)-1-methanesulfonyl-1,2,3,4-tetrahydro-quinolin-7-ol (10a). This compound was prepared according to the similar procedure to **6** as a solid in 77.6%. Mp 231–232 °C. 1H NMR (300 MHz, DMSO-d_6) δ 2.69–2.89 (m, 2H), 2.98 (s, 3H), 3.36–3.50 (m, 2H), 3.92–4.05 (m, 1H), 6.51 (dd, J = 2.5 Hz, 8.3 Hz, 1H), 6.72 (d, J = 8.5 Hz, 2H), 7.00 (d, J = 8.1 Hz, 1H), 7.12 (d, J = 2.6 Hz, 1H), 7.13 (d, J = 8.6 Hz, 2H). MS (EI): *m/e* (%) 319 (5 M+), 200 (100), 121 (75). Anal. calcd for $C_{16}H_{17}NO_4S$·0.5H_2O: C, 5, 5.52; N, 4.27; found: C, 58.38; H, 5.65; N, 4.178.52; H.

4.2.10.7. 7-Hydroxy-3-(4-hydroxy-phenyl)-3,4-dihydro-2*H*-quinoline-1-carbaldehyde (10b). This compound was prepared according to the similar procedure to **6** as a solid in 100%. Mp 238–239 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.78–2.95 (m, 2H), 3.28 (dd, J = 10.2 Hz, 12.4 Hz, 1H), 4.01 (dd, J = 7.2 Hz, 14.2 Hz, 1H), 4.11 (dd, J = 3.7 Hz, 13.1 Hz, 1H), 6.55 (dd, J = 2.4 Hz, 8.1 Hz, 1H), 6.71 (d, J = 8.4 Hz, 2H), 6.78 (d, J = 2.3 Hz, 1H), 7.03 (d, J = 8.0 Hz, 1H), 7.10 (d, J = 8.7 Hz, 2H), 8.78 (s, 1H). MS (EI): *m/e* (%) 269 (100M^+). HRMS (EI) calcd for $C_{16}H_{15}NO_3$, 269.1052; found, 269.1044.

4.2.10.8. 1-[7-Hydroxy-3-(4-hydroxy-phenyl)-3,4-dihydro-2*H*-quinolin-1-yl]-ethanone (10c). This compound was prepared according to the similar procedure to **6** as a solid in 77.0%. Mp 201–202 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.09 (s, 3H), 2.68–2.78 (m, 1H), 2.82-3.04 (m, 2H), 3.40–3.52 (m, 1H), 3.98–4.06 (m, 1H), 6.53 (dd, J = 2.4 Hz, 8.3 Hz, 1H), 6.70 (d, J = 8.4 Hz, 2H), 6.78 (d, J = 2.3 Hz, 1H), 7.03 (d, J = 8.5 Hz, 1H), 7.07 (d, J = 8.4 Hz, 2H). MS (EI): *m/e* (%) 283 (100M^+), 241 (65), 134 (55). HRMS (EI) calcd for $C_{17}H_{17}NO_3$, 283.1208; found, 283.1200.

4.2.10.9. 1-[7-Hydroxy-3-(4-hydroxy-phenyl)-3,4-dihydro-2*H*-quinolin-1-yl]-propan-1-one (10d). This compound was prepared according to the similar procedure to **6** as a solid in 73%. Mp 204-205 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.94 (t, J = 7.4 Hz, 3H), 2.36–2.41 (m, 2H), 2.62–2.73 (m, 1H), 2.83–2.93 (m, 2H), 3.41–3.46 (m, 1H), 3.87–3.91 (m, 1H), 6.51 (dd, J = 2.4 Hz, 8.3 Hz, 1H), 6.67 (d, J = 8.5 Hz, 2H), 6.78 (d, J = 2.3 Hz, 1H), 6.96 (d, J = 8.4 Hz, 1H), 7.03 (d, J = 8.5 Hz, 2H). MS (EI): *m/e* (%) 297 (70 M^+), 241 (100), 134 (45). HRMS (EI) calcd for $C_{18}H_{19}NO_3$, 297.1365; found, 297.1369.

4.2.10.10. Cyclopropyl-[7-hydroxy-3-(4-hydroxy-phenyl)-3,4-dihydro-2*H*-quinolin-1-yl]-methanone (10e). This compound was prepared according to the similar procedure to **6** as a solid in 69%. Mp 267–268 °C. 1H NMR (300 MHz, DMSO-d_6) δ 0.76–0.85 (m, 4H), 1.97–2.01 (m, 1H), 2.62–2.78 (m, 1H), 2.86–2.93 (m, 2H), 3.41–4.46 (m, 1H), 4.00–4.08 (m,1H), 6.55 (dd, J = 2.4 Hz, 8.2 Hz, 1H), 6.68 (d, J = 8.6 Hz, 2H), 6.91 (d, J = 2.4 Hz, 1H), 7.02 (d, J = 8.2 Hz, 1H), 7.06 (d, J = 8.5 Hz, 2H). MS (EI): *m/e* (%) 309 (40 M+), 241 (100), 84 (75). HRMS (EI) calcd for $C_{19}H_{19}NO_3$, 309.1365; found, 309.1356.

4.2.10.11. [7-Hydroxy-3-(4-hydroxy-phenyl)-3,4-dihydro-2*H*-quinolin-1-yl]-[4-(2-piperidin-1-yl-ethoxy)-phenyl]-methanone (10f). This compound was prepared according to the similar procedure to **6** as a solid in 80%. Mp 135-136 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.49–1.55 (m, 2H), 1.63–1.69 (m, 4H), 2.66–2.74 (m, 4H), 2.90–2.98 (m, 3H), 3.03–3.12 (m, 2H), 3.73 (dd, J = 8.0 Hz, 12.4 Hz, 1H), 4.06 (dd, J = 4.4 Hz, 12.7 Hz, 1H), 4.17 (t, J = 5.6 Hz, 2H), 6.33–6.36 (m, 1H), 6.52 (dd, J = 2.3 Hz, 8.1 Hz, 1H), 6.70 (d, J = 9.0 Hz, 2H), 6.88 (d, J = 8.7 Hz, 2H), 7.02–7.06 (m, 3H), 7.25 (d, J = 8.7 Hz, 2H). MS (EI): *m/e* (%) 472 (15M^+), 98

(100). HRMS (EI) calcd for $C_{29}H_{32}N_2O_4$, 472.2362; found, 472.2357.

4.2.10.12. 3-(4-Hydroxy-phenyl)-quinolin-7-ol (12). This compound was prepared according to the similar procedure to **6** as a solid in 70%. Mp 185-186 °C. ^{1}H NMR (300 MHz, DMSO-d_6) 6.94 (d, J = 8.8 Hz, 2H), 7.35 (d J = 8.3 Hz, 2H), 7.71 (d, J = 8.9 Hz, 2H), 8.03 (dd, J = 8.8 Hz, 21.7 Hz, 2H), 8.87 (s, 1H), 9.20 (dd, J = 2.1 Hz, 16.0 Hz, 1H). MS (EI): *m/e* (%) 236 (25M$^+$), 208 (65). HRMS (EI) calcd for $C_{15}H_{11}NO_2$, 237.0790; found, 237.0784.

4.2.10.13. 7-Hydroxy-3-(4-hydroxy-phenyl)-3,4-dihydro-1*H*-quinolin-2-one (14). This compound was prepared according to the similar procedure to **6** as a solid in 100%. Mp > 300 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.91–3.10 (m, 2H), 3.57–3.62 (m, 1H), 6.30 (dd, J = 2.4 Hz, 8.0 Hz, 1H), 6.34 (d, J = 2.1 Hz, 1H), 6.65 (d, J = 8.6 Hz, 2H), 6.92 (d, J = 8.1 Hz, 1H), 6.97 (d, J = 8.6 Hz, 2H). MS (EI): *m/e* (%) 255 (100 M$^+$), 122 (50). HRMS (EI) calcd for $C_{15}H_{13}NO_3$, 255.0895; found, 255.0890.

4.2.10.14. 7-Hydroxy-3-(4-hydroxy-phenyl)-3,4-dihydro-1*H*-quinoline-2-thione (16). This compound was prepared according to the similar procedure to **6** as a solid in 100%. Mp 262-264 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.81 (dd, J = 4.1 Hz, 16.2 Hz, 1H), 3.08 (dd, J = 6.3 Hz, 16.1 Hz, 1H), 4.11 (dd, J = 4.0 Hz, 5.9 Hz, 1H), 6.41 (dd, J = 2.4 Hz, 7.9 Hz, 1H), 6.57–6.59 (m, 2H), 6.60 (s, 1H), 6.90–6.94 (m, 3H). MS (EI): *m/e* (%) 271 (25 M$^+$), 149 (30), 61 (100). HRMS (EI) calcd for $C_{15}H_{13}NO_2S$, 271.0667; found, 271.0658.

4.2.10.15. 4-(4-Hydroxy-phenyl)-4,5-dihydro-[1,2,4]-triazolo[4,3-*a*]quinolin-8-ol (18). This compound was prepared according to the similar procedure to **6** as a solid in 100%. Mp 205 °C dec. ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.05–3.18 (m, 2H), 4.44 (t, J = 6.8 Hz, 1H), 6.65 (d, J = 8.9 Hz, 2H), 6.68 (d, J = 2.2 Hz, 1H) 6.95 (d, J = 8.6 Hz, 2H), 7.16–7.19 (m, 2H), 9.24 (s, 1H). MS (EI): *m/e* (%) 278 (25M-1^+), 210 (100). HRMS (EI) calcd for $C_{16}H_{13}N_3O_2$, 279.1008; found, 279.0999.

4.3. Ligand binding competition experiments

The compounds were tested for intrinsic activity in an ER binding assay with [^{3}H]17β-estradiol and full-length recombinant human ERα and ERβ proteins according to the procedure described in the literature.[20]

4.4. Yeast two-hybrid assay

4.4.1. Yeast transformation and culture. The yeast strain AH109 was obtained from Clontech (Palo Alto,CA), and transformation was performed according to the lithium acetate method.[21] Briefly, 500 ng of plasmid DNA was added to 50 μL of the competent cells and mixed with 36 μL of 1 M lithium acetate, 240 μL of 50% PEG3350, and 50 ng single-strain DNA at 30 °C for 30 min followed by heat-shock (250 rpm) at 42 °C for 30 min. The mixture was subsequently spread on a drop-out-agar plate without leucine and tryptophan(SD–T$^-$L$^-$). The plates were incubated at 30 °C for 48 h for yeast growth. PCR was used to confirm transformation with the target gene.

4.4.2. α-Galactosidase activity assay. The quantitative α-galactosidase activity assays were carried out by using *p*-nitrophenyl α-D-galactopyranoside (PNP-α-Gal) as the substrate according to the Clontech manual. The corresponding yeast cells were cultured in SD minimal medium (3 mL) without leucine and tryptophan (T$^-$L$^-$) (yeast nitrogen base without amino acids 6.7 g L^{-1}, D-(+)-glucose 20 g L^{-1}, yeast synthetic drop-out medium supplement without leucine and tryptophan 1.54 g L^{-1}) at 30 °C with shaking (250 rpm). When the OD_{600} of the cells had reached about 1.0, the cultures were diluted 50 times with fresh T$^-$L$^-$ medium, and different concentrations of ligands (1 μL) or DMSO (1 μL; as a blank) were then added to diluted cultures (999 μL). After further incubation at 30 °C for 16 h, each sample (200 μL) was transferred to a 96-well plates (Corning Costar 96-well flat-bottomed plate), and the OD_{600} value was measured by a Benchmark PlusTM microplate spectrophotometer (Bio-Rad). the cells were centrifuged at 3000 rpm for 30 min and 16 μL of supernatants was transferred into a 96-well plate with fresh assay buffer (48 μL; 1 volume of 100 mM PNP-α-Gal + 2 volumes of 0.5 M sodium acetate, pH 4.5 per well). After a further 60-min incubation at 30 °C, the reaction was terminated by the addition of stop solution (136 μL, 1 M Na_2CO_3) in each well, and the OD_{410} was measured. One unit of a-galactosidase is defined as the amount of enzyme that hydrolyzes 1 μmol PNP-α-Gal to *p*-nitrophenol and D-galactose in 1 min at 30 °C in acetate buffer (pH 4.5).[22] The α-galactosidase activity was calculated according to the following formula:

$$\text{α-Galactosidase activity [milliunits/(mL} \times \text{cell)]} = OD_{410} \times V_f \times 1000/[(\xi \times b) \times t \times V_i \times OD_{600}]$$

t Elapsed time (min) of incubation.
V_f Final volume of assay (200 μL).
V_i Volume of culture medium supernatant added (16 μL).
OD_{600} Optical density of overnight culture.
$\xi \times b$ *p*-Nitrophenol molar absorbtivity b at 410 nm× the light path (cm) = 10.5 (mL mol^{-1}; Yeast Protocols Handbook PT3024–1, Clontech).

4.5. Bioassay on the OVX rat model

The bone mineral density (BMD) of the tibial diaphysis was measured by peripheral quantitative computed tomography (pQCT) using an XCT Reaearch SA + pQCT machine (Stratec Medizintechnik, Pforzheim, Germany). The measurements were made with a collimator opening of 0.2 mm on specimens embedded in methylmethacrylate. One slice in the middiaphysis of the tibias located 2.0 mm proximal from the tibiofibular junction was measured. A voxel size of 0.09 mm and a threshold of 710 mg/cm^3 were used

for calculation of cortical BMD. Statistics were computed using *t*-test.

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