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Antitumor agents 247. New 4-ethoxycarbonylethyl curcumin analogs as potential antiandrogenic agents☆

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Abstract—4-Ethoxycarbonylethyl curcumin (ECECur) (**3**) is a current drug candidate for the treatment of prostate cancer. Due to problems inherent in the tautomerism of ECECur, 4-fluoro-4-ethoxycarbonylethyl curcumin (**4**) and 4-ethoxycarbonylethylenyl curcumin (**5**) were designed and synthesized. These two target compounds and their synthetic intermediates (**4**–**9**) were evaluated for their inhibitory activity against androgen receptor transcription in LNCaP and PC-3 prostate cancer cell lines. While the enol–keto analogs showed varying anti-androgen potencies, the di-keto analogs showed no activity. Tetrahydropyranylation of the phenoxy groups had a positive impact on the anti-AR activity of 4-ethoxycarbonylethylenyl curcumin, but a negative impact on the activity of ECECur. With potent anti-AR activity, di-tetrapyranylated 4-ethoxycarbonylethylenyl curcumin (**9**), which exists in only one form, is a good drug lead for further structural modification. Based on the SAR information obtained from the above study, five new compounds were designed and subsequently synthesized. Among them, compound **10** was found to be the most potent anti-AR agent and is considered to be a promising drug candidate for the treatment of prostate cancer.

1. Introduction

Prostate cancer is the most prevalent cancer among males in Western countries.[2] Androgen and the androgen receptor (AR), a member of the superfamily of nuclear receptors, have been documented to play important roles in the growth of prostate cancer cells. The present treatment for prostate cancer is surgery combined with chemo-therapeutic agents.[3] The prevailing drugs are anti-androgens including steroidal agents and non-steroidal agents. However, the steroidal agents have drawbacks such as agonistic activity and overlapping effects with other hormonal systems, as well as causing some side effects.[4] These drawbacks hinder their use as ideal anti-prostate cancer drugs. The non-steroidal agents such as hydroxyflutamide and bicalutamide were thought to have no agonistic activity. However, after a long period of administration, they may result in ''anti-androgen withdrawal syndrome'' in which patients who experience an increase in prostate-specific antigen (PSA), while taking the nonsteroidal anti-androgens, have a decrease in the level of PSA after withdrawal of the drugs.[5] Due to these drawbacks with the current anti-prostate cancer drugs, we aim to develop new curcumin analogs as potential anti-androgens for treatment of prostate cancer.

Curcumin (**1**) [diferuloyl methane; 1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] (Fig. 1) is the major constituent in the rhizome of *Curcuma longa* (Zingiberaceae), commonly named turmeric. Over a long period of study, curcumin has been found to possess various biological activities, such as anti-inflammatory,[6] antioxidant,[7] anti-HIV-1 integrase,[8] chemo-preventive,[9] anti-angiogenic,[10] and anti-cancer.[11] Therefore, various groups worldwide have used curcumin as a lead compound to develop numerous analogs with different bioactivities. In our laboratory, we are interested in the design and synthesis of new curcumin analogs possessing anti-androgen receptor activity.[12,13] In a previous paper, we reported that two curcumin analogs, dimethyl curcumin (**2**) and 4-ethoxycarbonylethyl curcumin (ECECur) (**3**) (Fig. 1), showed greater potency in anti-AR assays than hydroxyflutamide, an anti-androgen, currently used clinically.[12] ECECur has also

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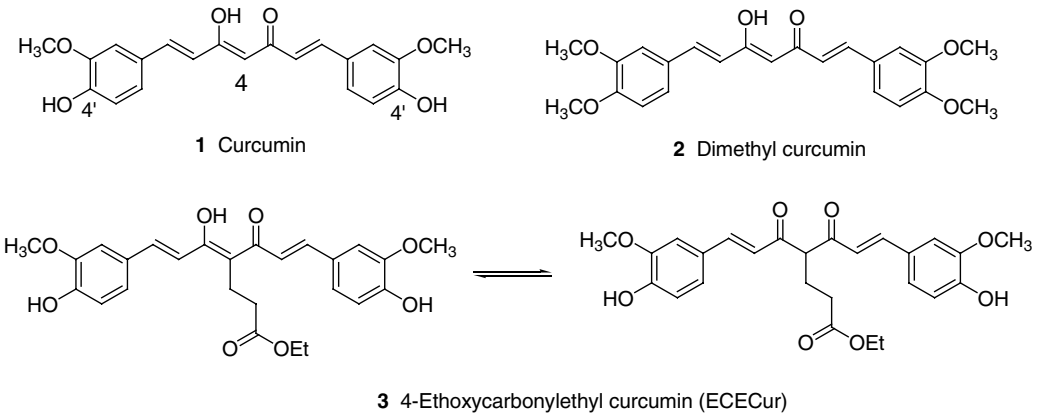


Figure 1. Structures of compounds **1**–**3**.

exhibited interesting biological activities other than potent anti-AR activity (unpublished data). It is regarded as a promising drug candidate for the treatment of prostate cancer, as well as certain other diseases. However, the tautomerism of ECECur, which causes it to exist in both enol–keto and di-keto forms, may hinder its potential as a clinically useful drug. In this paper, we present a resolution to the tautomerism of ECECur. In addition, we investigated which form of ECECur may be responsible for its anti-AR activity. This SAR study was used to guide the further design of five new analogs, among which compound **10** exhibited great potential as a drug candidate to treat prostate cancer. Extensive SAR correlations were established based on this study.

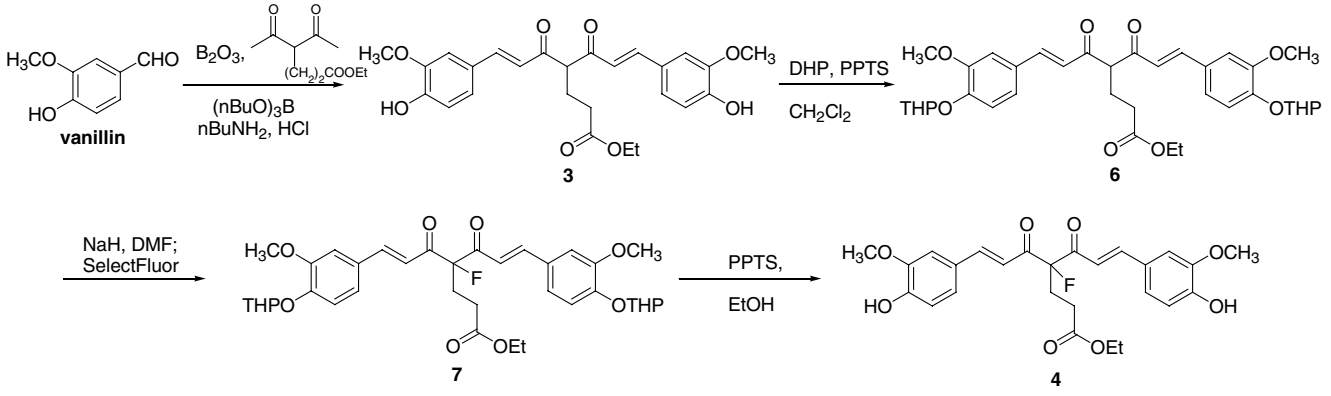
2. Chemistry

Compound **4**, the C-4 fluorinated analog of **3**, was modified from ECECur as shown in Scheme 1. Commercially available vanillin was condensed with ethyl 4-acetyl-5-oxo-hexanoate using the method of Pedersen et al.[14] to obtain compound **3**. Boric anhydride was used to form a boron complex with ethyl 4-acetyl-5-oxo-hexanoate in order to prevent Knoevenagel condensation.[14] Compound **3** was then protected as its tetrahydropyranyl (THP) ether by using DHP in the presence of PPTS in dry dichloromethane.[15] The THP ether **6** was fluorinated with 1-alkyl-4-fluoro-1, 4-diazabicyclo[2,2,2]octane salt (SelectFluor™) in the presence of sodium hydride in DMF.[16] Deprotection of compound **7** with PPTS in ethanol afforded the target compound **4**.

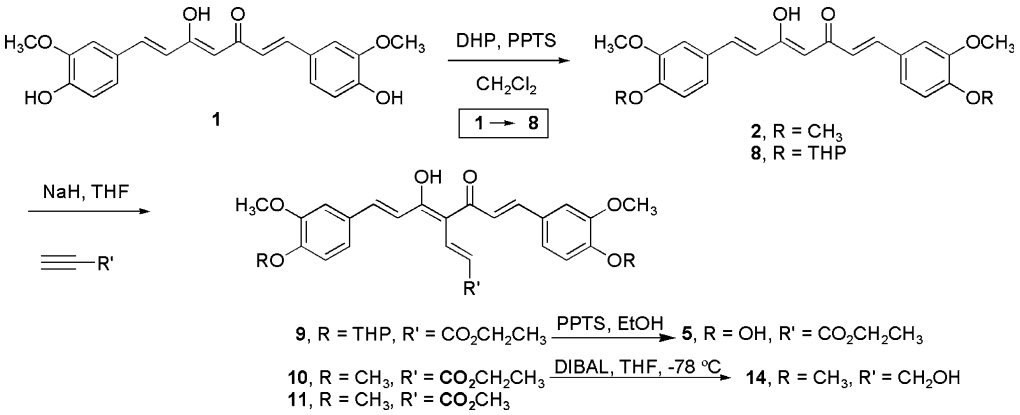
The preparation of analog **5** (Scheme 2) started from curcumin **1**, which was obtained by recrystallization of commercially available curcumin (Aldrich, Inc.). After the two phenoxy groups of **1** were protected as the THP ethers, the resulting compound **8** was alkylated at the C-4 position using sodium hydride in anhydrous tetrahydrofuran, followed by the addition of ethyl propiolate to afford monosubstituted compound **9**. The target compound **5** was obtained by cleavage of the THP ethers as described above for **4**.

Compounds **10** and **11** were derived directly from dimethyl curcumin (**2**), which was prepared from commercially available 3,4-dimethoxybenzaldehyde as described above for compound **3**. Dimethyl curcumin reacted in a Michael addition with ethyl propiolate to give compound **10** and with methyl propiolate to give compound **11** (Scheme 2).

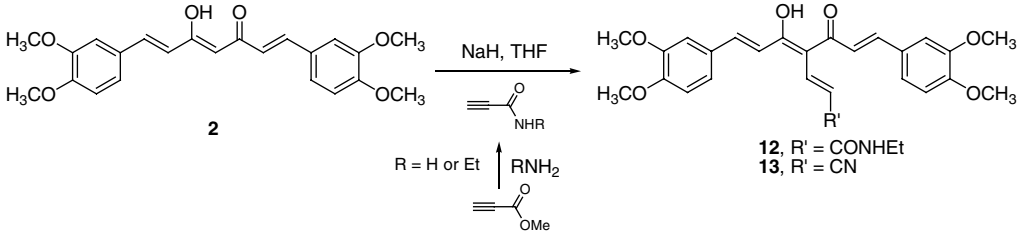
As shown in Scheme 3, compound **12** was obtained by reacting dimethyl curcumin with *N*-ethylpropiolamide, which was prepared from ethylamine by the slow addition of methyl propiolate at −30 °C.[17] Compound **13** was obtained by the reaction of dimethyl curcumin with propiolic acid amide under reflux. Propiolic acid amide



Scheme 1. Synthesis of compound **4**.



Scheme 2. Synthesis of compounds **5**, **8**–**11**, and **14**.



Scheme 3. Synthesis of compounds **12** and **13**.

was prepared from concentrated aqueous ammonia and methyl propiolate at −30 °C.[18]

Compound **10** was reduced to the allylic alcohol **14** with Dibal-H at −78 °C (Scheme 2).

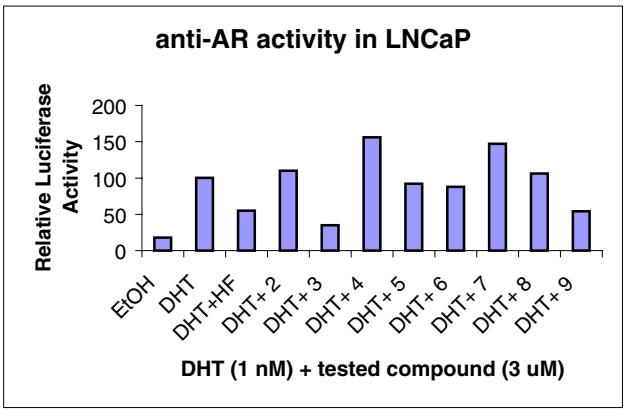
3. Results and discussion

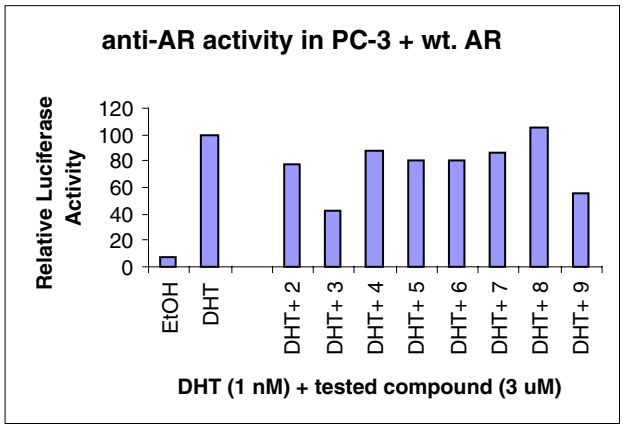
To circumvent the tautomerism of ECECur and determine the needed structural features for anti-androgen receptor activity, we designed two target compounds **4** and **5**. Ideally, target compounds should be stable in only one form as well as have minimal structural change from the parent compound ECECur in order to most likely retain similar potency.

For the di-keto ECECur analog, we designed compound **4**, in which the C-4 hydrogen has been replaced with a fluorine atom. Hydrogen and fluorine have similar atom sizes, and therefore, replacing hydrogen with fluorine does not significantly alter the overall molecular size.

We proposed compound **5** as the enol–keto ECECur analog. The only structural difference between **5** and its parent compound **3** occurs in the C-4 side chain. The ethoxycarbonylethyl side chain in ECECur contains a saturated single (C–C) bond, while the ethoxycarbonylethylenyl side chain of **5** contains an unsaturated double (C=C) bond. Compound **5** exists exclusively in an enol–keto form due to the highly conjugated polyene.

The target ECECur analogs and their synthetic intermediates were tested for inhibitory activity against AR transcription in the androgen-dependent LNCaP cell line and in the androgen-independent PC-3 cell line transfected with wild-type AR. Cytotoxicity was also examined in the LNCaP cell line. The bioassay results are shown in Figure 2 and generally were consistent over the three models. The parent compound ECECur (**3**) showed the highest activity, followed by compound **9**. Compounds **5** and **6** showed weak activity, and di-keto compounds **4**, **7**, and **8** were inactive. Compounds **5** and **9** exist exclusively in the enol–keto form; thus, the enol–keto form is likely the active form for anti-AR activity. Because fluorination of ECECur at the C-4 position (**4**) abolished activity in all bioassay models, we propose that the di-keto form may not contribute to the anti-AR activity of ECECur. In a previous paper, we also reported that a di-keto curcumin analog with di-substitution at the C4 position did not exhibit anti-AR activity in either human prostate cancer cell line.[13] The conformational difference between the di-keto and enol–keto forms of ECECur may help to explain the different anti-AR activity of these two analog types (Fig. 3). In the enol–keto conformation, the two phenyl rings and the unsaturated linker are in the same plane, because of the high degree of conjugation as well as the formation of a strong hydrogen bond between the enol proton and the carbonyl oxygen. However, in the di-keto conformation, ECECur does not stabilize in a planar structure, because the two electron-rich carbonyl oxygen atoms repel each other. Compound **6**, the THP ether of ECECur (**3**), showed decreased activity, while compound **9**, the equivalent analog of **5**, showed greater anti-AR activity. Thus, the THP-protecting group has a dissimilar influence on the anti-AR activity of different compounds. With ethoxycarbonylethyl substitution at C-4, tetrahydropyranylation of both phenols had a negative impact on the anti-AR activity. However, with





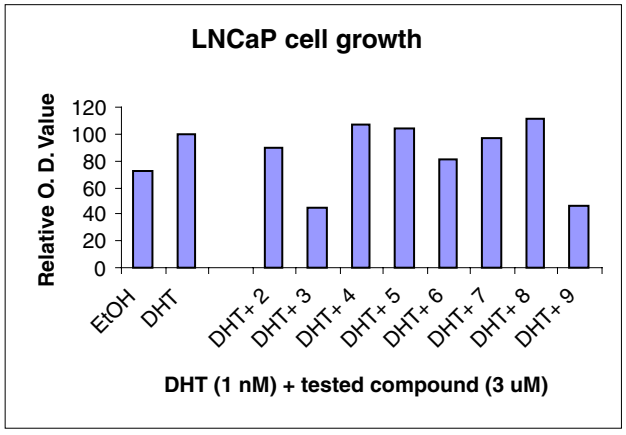


Figure 2. Anti-AR activity and cytotoxicity of compounds **2**–**9** (3 μM) in prostate cancer cells.

an ethoxycarbonylethylenyl group at C-4, it had a positive influence on the anti-AR activity. Therefore, both the C-4 position and the C-4′ moieties on the phenyl rings of curcumin analogs are important pharmacophores with respect to the anti-AR activity. In order to obtain an optimal anti-AR agent, we next focused on modification of the substituents at these positions.

Based on the preceding SAR information, we designed and synthesized compounds **10**–**14**, which have C-4′ methoxy groups on both phenyl rings and various ethylenyl side chains at C-4. As shown in Figure 4, among the five new analogs, compound **10** exhibited the highest potency in all three bioassay models. Compound **13** showed moderate anti-AR activity in LNCaP cells, weak anti-AR activity in PC-3 cells transfected with wild-type androgen receptor, and weak inhibitory activity against the growth of LNCaP cells. Compounds **11**, **12**, and **14** showed either weak or no activity in the prostate cancer cells. Compared with ECECur (**3**), compound **10** showed similar anti-AR potency in the LNCaP cell line, was slightly less potent in PC-3 cells transfected with wild-type androgen receptor, and was more cytotoxic in the LNCaP cell growth assay (Fig. 3). Since compound **10** showed significant activity and does not undergo or be limited by tautomerism, it is regarded as a very promising drug candidate for in vivo investigation. Our SAR conclusions were extended on the basis of the structural features and bioactivity of these five new analogs. Although the structures of these analogs vary only in the side chain at C-4, the replacement of ethoxy (compound **10**) with methoxy (compound **11**) or *N*-ethylamino (compound **12**) resulted in loss of activity, which implies that a long chain ester may be more favorable. The reduction of ester to alcohol also led to loss of activity, emphasizing the necessity for an ester in the side chain. From comparing compounds **10** and **13**, the nitrile functional group does not enhance the anti-AR activity or the cytotoxicity in LNCaP cells. Therefore, in our future study, we will focus on optimization of the ester length.

4. Conclusion

In summary, we eliminated the tautomerism problem of 4-ethoxycarbonylethyl curcumin **3** by synthesis of 4-fluoro-4-ethoxycarbonylethyl curcumin **4** and 4-ethoxycarbonylethylenyl curcumin **5**. Evaluation of the anti-androgen activity of these ECECur analogs (**3**–**14**) in LNCaP cells and PC-3 cells indicated that di-keto

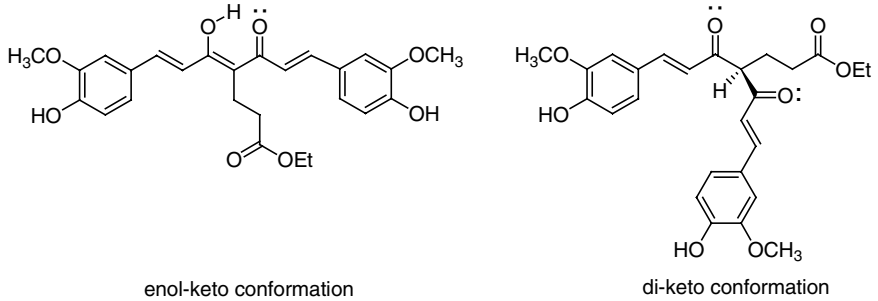


Figure 3. The conformations of ECECur.

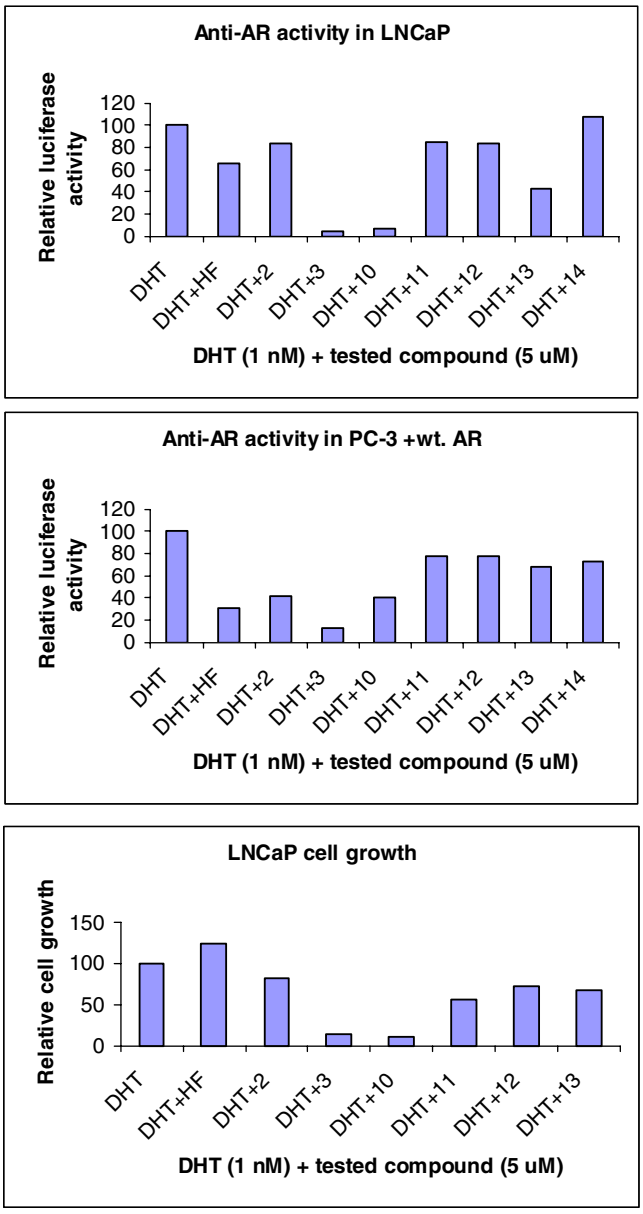


Figure 4. Anti-AR activity and cytotoxicity of compounds **2**, **3**, and **10**–**14** (5 μM) in prostate cancer cells.

ECECur analogs did not exhibit anti-AR activity, but enol–keto ECECur analogs showed varying anti-AR potencies. Our SAR study revealed that: (1) the enol–keto moiety is responsible for the anti-AR activity, and the di-keto form probably is not an active form; (2) tetrahydropyranylation or methylation of the phenoxy groups impacts the anti-AR potency; (3) the –CH=CHCOOEt side chain is superior to –CH=CHCOOMe, –CH=CHCONHEt, –CH=CHCN, and –CH=CHCH$_2$OH. Proper substitutions at the C-4 and C-4′ positions are vital for anti-AR activity as well as cytotoxicity in LNCaP cells. Compound **10**, which bears the optimal features identified to date for potent anti-AR activity and exists exclusively in the enol–keto form, has been identified as a promising curcumin analog and is undergoing continuing evaluation as a potential clinical trial candidate for the treatment of prostate cancer.

5. Experimental

Melting points were determined with a Fisher–Johns melting apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Varian Gemini-300 spectrometer. The chemical shifts are presented in terms of parts per million with TMS as the internal reference. MS spectra were recorded on an API-3000 LC/MS/MS spectrometer. Column chromatography was carried out on CombiFlash® Companion™ (Isco, Inc.), and thin-layer chromatography was performed on pre-coated silica gel or aluminum plates (Aldrich, Inc.). Elemental analyses were performed by Atlantic Microlab Inc., Norcross, GA, and agreed with theoretical values to within ±0.4%.

5.1. Ethoxycarbonylethyl curcumin (3)

The synthetic procedure was modified from the method first introduced by Pedersen et al.[14] Ethyl 4-acetyl-5-oxo-hexanoate (1.87 mL, 10 mmol) and boric anhydride (0.5 g, 7 mmol) were dissolved in 10 mL EtOAc. The solution was stirred for 30 min at 40 °C. Vanillin (3.1 g, 20 mmol) and tributyl borate (5.4 mL, 20 mmol) were added, and the mixture was stirred for 30 min. Butylamine (1.49 mL, 15 mmol) dissolved in 10 mL EtOAc was added dropwise during 15 min. The stirring was continued for 24 h at 40 °C. The mixture was then hydrolyzed by adding 30 mL of 4 N hydrochloric acid and heating for 30 min at 60 °C. The organic layer was separated, and the aqueous layer was extracted three times with EtOAc. The combined organic layers were washed until neutral and dried over anhydrous sodium sulfate. The solvent was removed in vacuo. The crude product was purified by CombiFlash® chromatography eluting with hexane–EtOAc.

Dimethyl curcumin (**2**) was prepared from 2,4-pentanedione and 3,4-dimethoxybenzaldehyde using the same procedure.

The structures of **1**–**3** were confirmed by comparison of their physical data with those reported in the literature.[12,14]

5.2. 7-[3-Methoxy-4-(tetrahydropyran-2-yloxy)-phenyl]-4-{3-[3-methoxy-4-(tetrahydropyran-2-yloxy)-phenyl]-acryloyl}-5-oxo-hept-6-enoic acid ethyl ester (6)

Compound **3** (153.1 mg, 0.33 mmol) and dihydropyran (0.73 mL, 7.32 mmol) were dissolved in 3 mL of dry dichloromethane containing PPTS (8.3 mg, 0.033 mmol). The solution was stirred at rt for 48 h. The solution was then washed with water, and the solvent was removed in vacuo. The crude product was purified by CombiFlash® chromatography eluting with hexane–EtOAc to give **6** (134 mg), 59% yield, yellow powder, mp 60–61 °C (acetone); ESI MS *m/z* 635.2 $(M-H)^+$; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.25 (3H, t), 1.57–2.17 (12H, m), 2.96 (0.57H, t), 3.62 (4H, t), 3.91 (6H, s), 4.13 (2H, q), 5.47 (2H, t), 6.72 (2H, d, J = 15.6 Hz), 6.90–7.18 (6H, m), 7.44 (2H, d, J = 15.6 Hz); Anal. ($C_{36}H_{44}O_{10}$) C, H.

5.3. 4-Fluoro-7-[3-methoxy-4-(tetrahydropyran-2-yloxy)-phenyl]-4-{3-[3-methoxy-4-(tetrahydropyran-2-yloxy)-phenyl]-acryloyl}-5-oxo-hept-6-enoic acid ethyl ester (7)

A DMF solution (3 mL) of **6** (30 mg, 0.047 mmol) was added to an oil-free suspension of NaH (10 mg of 60%, 6 mg, 0.25 mmol) in DMF (2 mL) under nitrogen at 0 °C. The solution was stirred at 0 °C for 30 min and then at rt for 2 h. To the sodium salt solution, SelectFluor™ (70 mg, 0.2 mmol) in DMF (2 mL) was added. After stirring for 1 h, the solution was extracted with EtOAc and washed with 5% H_2SO_4 (10 mL) and subsequently with saturated $NaHCO_3$ solution (10 mL). The solvent was evaporated in vacuo, and the crude product was purified by CombiFlash® chromatography eluting with hexane–EtOAc to afford **7** (4 mg), 13% yield, yellow powder, mp 59–60 °C; ESI MS *m/z* 677.3 $(M+Na)^+$, 655.3 $(M+H)^+$; ^1H NMR (300 MHz, $CDCl_3$): δ 1.25 (3H, t), 1.5–2.1 (12H, m), 2.45 (2H, m), 2.65 (2H, m), 3.62 (4H, t), 3.90 (6H, s), 4.13 (2H, q), 5.49 (2H, t), 7.06 (2H, dd, $J = 3$ Hz, $J = 15.6$ Hz), 7.12–7.15 (6H, m), 7.75 (2H, d, $J = 15.6$ Hz); Anal. ($C_{36}H_{44}FO_{10}$) C, H.

5.4. 4-Fluoro-7-(4-hydroxy-3-methoxyphenyl)-4-[3-(3-methoxy-4-methylphenyl)-acryloyl]-5-oxo-hept-6-enoic acid ethyl ester (4)

The EtOH (3 mL) solution of compound **7** (12.5 mg, 0.019 mmol) and PPTS (5 mg, 0.02 mmol) was stirred at rt for 3 h. The solution was evaporated in vacuo, and compound **4** (9 mg) was obtained by CombiFlash® chromatography eluting with hexane–EtOAc. 97% yield, yellow powder, mp 63–63.5 °C; EIMS *m/z* 509.3 $(M+Na)^+$, 487.3 $(M+H)^+$; ^1H NMR (300 MHz, $CDCl_3$): δ 1.25 (3H, t), 2.45 (2H, m), 2.65 (2H, m), 3.95 (6H, s), 6.93 (2H, d, $J = 7.8$ Hz), 7.06 (2H, dd, $J = 3$ Hz, $J = 15.6$ Hz), 7.09 (2H, d, $J = 1.8$ Hz), 7.16 (2H, dd, $J = 1.8$ Hz, $J = 7.8$ Hz), 7.75 (2H, d, $J = 15.6$ Hz); Anal. ($C_{26}H_{27}FO_8 \cdot 3/4H_2O$) C, H.

5.5. 5-Hydroxy-1,7-bis-[3-methoxy-4-(tetrahydropyran-2-yloxy)-phenyl]-hepta-1,4,6-trien-3-one (8)

Recrystallized curcumin (**1**, 1.08 g, 2.94 mmol) was protected as its tetrahydropyranyl ethers by reaction with dihydropyran (2 mL, 20 mmol) as described above for conversion of **3** to **6**. CombiFlash® chromatography eluting with hexane–EtOAc gave pure **8** (1.12 g), 66.8% yield, yellow powder, mp 67–69 °C; ESI MS *m/z* 535.0 $(M-H)^+$; ^1H NMR (300 MHz, $CDCl_3$): δ 1.57–2.17 (12H, m), 3.62 (4H, t), 3.91 (6H, s), 5.47 (2H, t), 5.83 (1H, s), 6.50 (2H, d, $J = 15.9$ Hz), 7.09–7.16 (6H, m), 7.60 (2H, d, $J = 15.9$ Hz); Anal. ($C_{31}H_{36}O_8 \cdot 1/4H_2O$) C, H.

5.6. 5-Hydroxy-7-[3-methoxy-4-(tetrahydropyran-2-yloxy)-phenyl]-4-{3-[3-methoxy-4-(tetrahydropyran-2-yloxy)-phenyl]-acryloyl}-hepta-2,4,6-trienoic acid ethyl ester (9)

A THF solution (3 mL) of **8** (55 mg, 0.10 mmol) was added to an oil-free suspension of NaH (7 mg of 60%, 4.2 mg, 0.17 mmol) in THF (2 mL) under nitrogen at 0 °C. The solution was stirred at 0 °C for 30 min and then at rt for 2 h. To the sodium salt solution, ethyl propiolate (0.02 mL, 0.20 mmol) was added. After stirring for 2 h, the solution was extracted with EtOAc and washed with 5% H_2SO_4 (10 mL) and saturated $NaHCO_3$ solution (10 mL). Then the solvent was evaporated in vacuo. The crude product was purified by CombiFlash® chromatography eluting with hexane–EtOAc to afford **9** (39.4 mg), 62% yield, orange powder; mp 72–73 °C; ESI MS *m/z* 634.7 $(M+H)^+$; ^1H NMR (300 MHz, $CDCl_3$): δ 1.34 (3H, t), 1.5–2.2 (12H, m), 3.62 (4H, t), 3.92 (6H, s), 4.28 (2H, q), 5.49 (2H, t) 5.96 (H, d, $J = 15.6$ Hz), 7.00 (2H, d, $J = 15.6$ Hz), 7.08–7.16 (6H, m), 7.76 (2H, d, $J = 15.3$ Hz), 7.83 (H, d, $J = 15.9$ Hz); Anal. ($C_{36}H_{42}O_{10}$) C, H.

5.7. 5-Hydroxy-7-(4-hydroxy-3-methoxyphenyl)-4-[3-(4-hydroxy-3-methoxyphenyl)-acryloyl]-hepta-2,4,6-trienoic acid ethyl ester (5)

Compound **9** was converted to **5** using the same procedure described above for **4** from **7**. 93% yield, orange powder, mp 106–106.5 °C; ESI MS *m/z* 465.2 $(M-H)^+$; ^1H NMR (300 MHz, $CDCl_3$): δ 1.34 (3H, t), 3.95 (6H, s), 4.29 (2H, quart), 5.96 (2H, d, $J = 15.6$ Hz), 6.95 (2H, d, $J = 8.2$ Hz), 6.96 (1H, d, $J = 15.6$ Hz), 7.05 (2H, d, $J = 2.1$ Hz), 7.17 (2H, dd, $J = 8.2$ Hz, $J = 2.1$ Hz), 7.75 (2H, d, $J = 15.3$ Hz), 7.90 (1H, d, $J = 15.9$ Hz); Anal. ($C_{26}H_{26}O_8 \cdot 11/8H_2O$) C, H.

5.8. 7-(3,4-Dimethoxyphenyl)-4-[3-(3,4-dimethoxy phenyl)-acryloyl]-5-hydroxy-hepta-2,4,6-trienoic acid ethyl ester (10)

Dimethylated curcumin (**2**) (360 mg, 1 mmol) was reacted with NaH (24 mg, 1 mmol) and ethyl propiolate (0.2 mL, 1.97 mmol), as described above for preparation of **9**, to afford **10** (268 mg), 54% yield, red powder, mp 170–171 °C; ESI MS *m/z* 494.6 $(M+H)^+$; ^1H NMR (300 MHz, $CDCl_3$): δ 1.34 (3H, t), 3.95 (12H, s), 4.29 (2H, quart), 5.98 (2H, d, $J = 15.6$ Hz), 6.95 (2H, d, $J = 8.4$ Hz), 7.00 (1H, d, $J = 15.6$ Hz), 7.08 (2H, d, $J = 1.8$ Hz), 7.22 (2H, dd, $J = 8.4$ Hz, $J = 1.8$ Hz), 7.77 (2H, d, $J = 15.3$ Hz), 7.91 (1H, d, $J = 15.6$ Hz). Anal. ($C_{28}H_{30}O_8 \cdot 1/4H_2O$) C, H.

5.9. 7-(3,4-Dimethoxyphenyl)-4-[3-(3,4-dimethoxy phenyl)-acryloyl]-5-hydroxy-hepta-2,4,6-trienoic acid methyl ester (11)

Dimethylated curcumin (**2**) (200 mg, 0.5 mmol) was reacted with NaH (20 mg of 60%, (4.2 mg, 0.5 mmol) in THF (3 mL) then with methyl propiolate (0.08 mL, 1 mmol), as described above for preparation of **9**, to afford **11** (121 mg), 50% yield, orange powder; mp 167–168 °C; ESI MS *m/z* 481.6 $(M+H)^+$; ^1H NMR (300 MHz, $CDCl_3$): δ 3.82 (3H, s), 3.93 (12H, s), 5.98 (H, d, $J = 15.6$ Hz), 6.90 (2H, d, $J = 8.4$ Hz), 6.98 (2H, d, $J = 15.6$ Hz), 7.07 (2H, d, $J = 1.8$ Hz), 7.20 (2H, dd, $J = 8.4$ Hz, $J = 1.8$ Hz), 7.76 (2H, d, $J = 15.6$ Hz), 7.90 (H, d, $J = 15.9$ Hz); Anal. ($C_{27}H_{28}O_8 \cdot 1/4H_2O$) C, H.

5.10. 7-(3,4-Dimethoxyphenyl)-4-[3-(3,4-dimethoxy phenyl)-acryloyl]-5-hydroxy-hepta-2,4,6-trienoic acid ethylamide (12)

Ethylamine (6 mL, 2.0 M in MeOH) and water (6 mL) were cooled in dry ice-isopropyl alcohol. Methyl propiolate (840 mg, 10 mmol, 0.84 mL) was added gradually to the stirring solution. After 10 min, the solvent was evaporated in vacuo. Crude *N*-ethylpropionamide was obtained. A THF solution (3 mL) of **2** (100 mg, 0.25 mmol) was added to an oil-free suspension of NaH (10 mg of 60%, 2.4 mg, 0.25 mmol) in THF (3 mL) under nitrogen at 0 °C. The solution was stirred at 0 °C for 30 min and then at rt for 2 h. To the sodium salt solution, crude *N*-ethylpropionamide (0.40 mL) was added. After stirring for 22 h, the solution was extracted with EtOAc and washed with 5% H_2SO_4 (10 mL) and subsequently saturated $NaHCO_3$ solution (10 mL). Then the solvent was evaporated in vacuo. The crude product was purified by CombiFlash® chromatography eluting with hexane–EtOAc to afford **12** (20 mg), yellow powder, mp 219–221 °C; ESI MS *m*/*z* 516.2 $(M+Na)^+$; ^1H NMR (300 MHz, $CDCl_3$): δ 1.32 (3H, t), 3.92 (6H, s), 3.92 (6H, s), 4.00 (2H, quart), 5.85(H, d, J = 15.3 Hz), 6.88 (2H, d, J = 8.4 Hz), 6.95 (2H, d, J = 15.6 Hz), 7.06 (2H, d, J = 1.5 Hz), 7.18 (2H, dd, J = 8.4 Hz, J = 1.5 Hz), 7.73 (2H, d, J = 15.6 Hz), 7.82 (H, d, J = 15.3 Hz); Anal. ($C_{28}H_{31}NO_7 \cdot 3/2H_2O$) C, H.

5.11. 7-(3,4-Dimethoxyphenyl)-4-[3-(3,4-dimethoxy phenyl)-acryloyl]-5-hydroxy-hepta-2,4,6-trienenitrile (13)

Compound **2** was reacted with propionamide prepared from concentrated aqueous ammonia (14 mL) and methyl propiolate (4.2 g, 50 mmol) giving 2.1 g of off-white prisms with cooling below 0 °C as described above in the preparation of **12**. CombiFlash® chromatography eluting with hexane–EtOAc afforded **13** (40 mg). mp 204–205 °C; ESI MS *m*/*z* 448.3 $(M+H)^+$; ^1H NMR (300 MHz, DMSO): δ 3.70 (3H, s), 3.75 (3H, s), 3.79 (3H, s), 3.80 (3H, s), 6.32 (H, d, J = 9.6 Hz), 6.99 (2H, dd, J = 8.4 Hz, J = 1.5 Hz), (2H, d, J = 15.6 Hz), (2H, d, J = 1.8 Hz), (2H, dd, J = 8.4 Hz, J = 1.8 Hz), (2H, d, J = 15.6 Hz), (H, d, J = 15.9 Hz); Anal. ($C_{26}H_{25}NO_6 \cdot 9/4H_2O$) C, H.

5.12. 1,7-Bis-(3,4-dimethoxyphenyl)-5-hydroxy-4-(3-hydroxypropenyl)-hepta-1,4,6-trien-3-one (14)

Compound **10** (49.4 mg, 0.1 mmol) was dissolved in anhydrous THF (10 mL). At −78 °C, Dibal-H (0.4 mL 1.5 M in toluene, 0.6 mmol) was added to the THF solution. Stirring was continued for 75 min at −78 °C. Water was then added to quench the excess Dibal-H. The mixture was filtered and extracted with EtOAc. The filtrate was washed with water and saturated NaCl solution, and then dried over anhydrous Na_2SO_4. The organic solvent was removed in vacuo. PTLC offered **14** (8.4 mg), red powder, mp 178–179 °C; ESI MS *m*/*z* 453.2 $(M+H)^+$; ^1H NMR (300 MHz, $CDCl_3$): δ 3.92 (6H, s), 3.93 (6H, s), 4.40 (2H, d, J = 4.5 Hz), 5.30 (0.4H, s), 5.88 (H, triplet of doublet, J = 15.6 Hz, J = 4.5 Hz), 6.59 (H, d, J = 15.6 Hz), 6.88 (2H, d, J = 8.4 Hz), 6.97 (2H, d, J = 15.6 Hz), 7.06 (2H, d, J = 1.8 Hz), 7.17 (2H, dd, J = 8.4 Hz, J = 1.8 Hz), 7.68 (2H, d, J = 15.6 Hz); Anal. ($C_{26}H_{28}O_7 \cdot 3/4H_2O$) C, H.

5.13. Cell culture and gene transfection

Human prostate cancer LNCaP and PC-3 cells were maintained in RPMI medium and Dulbecco's minimum essential medium (DMEM), respectively. Both media were strengthened with penicillin (25 units/mL), streptomycin (25 μg/mL), and 10% fetal calf serum. Androgen receptor transactivation assay, an androgen-dependent reporter gene transcription test, was employed as the primary screening for potential anti-androgen identification. This assay was first performed in LNCaP cells, which express a clinically relevant mutant AR. Once anti-androgenic activity was detected in the LNCaP AR transactivation assay, compounds were re-examined for their potential activity against wild-type AR. Wild-type AR transactivation assay was performed in PC-3 host cells, which lack an endogenous, functional AR. The method and conditions of cell and gene transfection have been described previously.[12,13] In brief, cells were plated in 24-well tissue culture dishes 24 (PC-3 cells) or 48 (LNCaP cells) hours prior to transfection. Subsequently, LNCaP cells were transfected with a reporter gene, MMTV-luciferase, which contains MMTV-LTR promoter and androgen receptor binding element, and PRL-SV40, which served as an internal control for transfection efficiency. PC-3 cells were transfected with a wild-type AR expression plasmid, pSG5AR, in addition to the above-mentioned MMTV-luciferase reporter gene and PRL-SV40 internal control. SuperFect (Qiagen, Chatsworth, CA) was employed as the transfection reagent following manufacturer's recommendations. At the end of a 5 h transfection, the medium was changed to DMEM or RPMI supplemented with 10% charcoal dextran-stripped, that is, androgen-depleted, serum. Twenty-four hours later, the cells were treated with 1 nM DHT and/or test compounds at the designated concentration for another 24 h. The cells were harvested for luciferase activity assay using Dual Luciferase Assay System (Promega, Madison, WI). The derived data were expressed as relative luciferase activity normalized to the internal luciferase control. Cells cultured in medium containing DHT (androgen), as a positive control, induced a marked reporter gene expression. Test compounds capable of significantly suppressing this DHT-induced reporter gene expression were identified as potential anti-androgens.

5.14. LNCaP cell growth assay

As mentioned above, LNCaP cells contain a substantial amount of mutant AR, and thus the growth of these cells can be significantly activated upon androgen incubation. LNCaP cell growth assay was used to further confirm the anti-androgenic activity detected by the above-mentioned AR transactivation assay. An MTT assay, which relies upon the conversion of a colorless substrate to reduced color tetrazolium by the mitochondrial dehydrogenase (possessed by all viable cells), was used to measure cell growth. The experimental conditions have been detailed elsewhere.[13] Briefly, cells were plated in

96-well tissue culture plates and then incubated for five consecutive days in the presence of 1 nM DHT and/or test compounds in 10% charcoal dextran-stripped serum containing RPMI. At five days of incubation, the cells were given MTT (5 mg/mL in PBS) for 3 h at 37 °C. The resultant precipitate was dissolved in a lysis buffer and then quantitated at a wavelength of 595 nm using a microplate reader. To ensure the accuracy of data derived from the MTT analysis, cell count was performed using duplicate samples. Test compounds that displayed an adverse effect on the androgen-induced prostate tumor cell growth were identified as potential anti-androgens and anti-prostate cancer agents.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2005.11.034.

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