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Design, synthesis and biological evaluation of nuclear receptor-degradation inducers

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ABSTRACT

Compounds that regulate the function(s) of nuclear receptors (NRs) are useful for biological studies and as candidate therapeutic agents. Most such compounds are agonists or antagonists. On the other hand, we have developed specific protein degradation inducers, which we designated as SNIPERs (Specific and Nongenetic IAPs-dependent Protein ERasers), for selective degradation of target proteins. SNIPERs are hybrid molecules consisting of an appropriate ligand for the protein of interest, coupled to a ligand for inhibitor of apoptosis proteins (IAPs), which target the bound protein for polyubiquitination and proteasomal degradation. We considered that protein knockdown with SNIPERs would be a promising alternative approach for modulating NR function. In this study, we designed and synthesized degradation inducers targeting retinoic acid receptor (RAR), estrogen receptor (ER), and androgen receptor (AR). These newly synthesized RAR, ER, and AR SNIPERs, **9**, **11**, and **13**, respectively, were confirmed to significantly reduce the levels of the corresponding NRs in live cells.

1. Introduction

Nuclear receptors (NRs), which are activated by small-molecular ligands, are transcription factors that regulate gene expression in the nucleus and play key roles in biological functions such as embryonic development and homeostasis.[1] In addition, NRs are targets for the treatment of various diseases.[2] Among NRs, retinoic acid receptors (RARα, RARβ, and RARγ) are receptors of all-*trans* retinoic acid (ATRA). Retinoids modulate the growth and differentiation of a wide variety of normal and transformed cells, and ATRA is involved in the control of embryonic development and cell differentiation. Estrogen receptors (ERα and ERβ) are activated by the endogenous hormone 17β-estradiol (E2). Estrogens play an important role in the initiation and progression of breast cancer, and overexpression of ERα appears to be associated with increased risk of breast cancer.[2c] Androgen receptor (AR) is a receptor of androgens, typically testosterone and/or its active form, 5α-dihydrotestosterone (DHT), which are endogenous ligands essential for the development and maintenance of the male reproductive system and secondary male sex characteristics. Among the pathophysiological effects elicited by androgens, a role as endogenous tumor promoters, especially for prostate tumor, is well known. This action is considered to be mediated by androgen-binding to AR.

Many agonists or antagonists for NRs have been found and synthesized, and have often been used as regulators of NRs or therapeutic agents.[3] For example, all-*trans* retinoic acid (ATRA, **1**), Am80 (**2**), and Ch55 (**3**) (Fig. 1) are agonists for retinoic acid receptors (RARs).[4] In particular, ATRA (**1**) and Am80 (**2**) have been used as therapeutic agents for patients with acute promyelocytic leukemia (APL).[5] In addition, bicalutamide[6] (**4**) (Fig. 1), which is an androgen receptor (AR) antagonist, and tamoxifen[7] (**5**) (Fig. 1), which is an estrogen receptor (ER) antagonist, have been used for the treatment of prostate cancer and breast cancer, respectively. However, although AR antagonists, which show anti-androgenic activity toward wild-type AR, are used as therapeutic agents for the treatment of prostate cancer, it has been reported that some AR antagonists shows androgenic activity toward some mutant ARs.[8] In addition, conventional AR antagonists are not effective to treat polyglutamine expansion mutation in AR such as spinal and bulbar muscular atrophy (SBMA).[9] In regard to breast cancer, acquired resistance to tamoxifen (**5**) is a substantial problem.[10] As a new approach, which might avoid some of the difficulties associated with the use of agonists or antagonists, we have focused on protein knockdown of NRs.

Several groups have reported techniques for inducing target protein-selective degradation in cells. Proteolysis-targeting chimeric molecules (PROTACs)[11] represent a pioneering approach in the field of down-regulating proteins by the use of peptides. Proteins targeted by these PROTACs include methionine aminopeptidase 2 (MetAP-2), ER, AR, aryl hydrocarbon receptor (AhR), and FK506 binding protein (FKBP). On the other hand, we have developed

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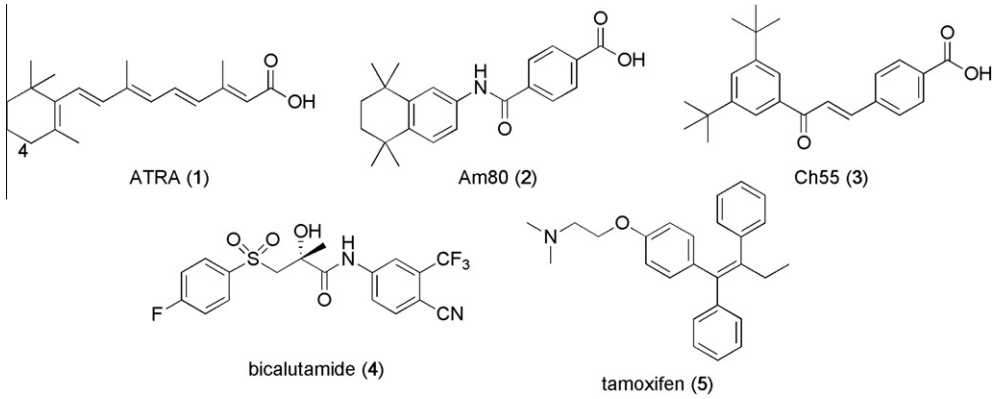


Figure 1. Structures of compounds **1**–**5**.

protein knockdown with small-molecular protein-degradation inducers (SNIPERs; Specific and Nongenetic IAPs-dependent Protein ERasers).[12] SNIPERs are bifunctional compounds which are designed by conjugating an IAPs-recognition structure with a target protein-recognition structure. This approach to targeting proteins for degradation involves three steps. First, its two recognition structures allow SNIPER to form an artificial (nonphysiological) complex linking IAPs,[13] which have ubiquitin ligase (E3) activity, with the target protein. Next, the target protein is polyubiquitinated by IAPs. Finally the polyubiquitinated protein is degraded by proteasome (Fig. 2a). Previously, we adopted methyl bestatin (MeBS, **6a**) or BE04 (**6b**) (Fig. 2b) as a ligand for cellular inhibitor of apoptosis protein 1 (cIAP1), which is one of the IAPs, and ATRA as a ligand for cellular retinoic acid binding protein II (CRABP-II), and synthesized CRABP-II degradation inducers **7** and **8**. ATRA recognizes not only CRABP-II, but also RAR. However, we utilized structure-based drug design to obtain **7** and **8**, which induce selective degradation of CRABP-II, but not RARs (introduction of a substituent at the C4 position of ATRA results in loss of ATRA's binding affinity for RARs). The protein knockdown strategy has several advantages, especially, (1) small-molecular degradation inducers possesses sufficient activity, permeability, and stability in cells to be employed in cellular assays, (2) target protein-selective protein knockdown is expected to be useful for biological studies and disease treatment, (3) suppression or degradation of cIAP1 itself, which is overexpressed in several human cancers, should not vitiate the anticancer effect, and both cancer-related protein and cIAP1 degradation would be useful for cancer treatment (actually we found that degradation of both CRABP-II and cIAP1 (**7**) is more effective than degradation of CRABP-II alone (**8**) to inhibit the proliferation of neuroblastoma cells).[12b]

This protein knockdown strategy is expected be generally adaptable to a wide range of proteins by replacing ATRA with a specific ligand for the target protein. In addition, the functions of many disease-related proteins could be inhibited by protein knockdown if suitable ligands were available. To examine the scope of the protein knockdown strategy using SNIPERs, we focused on NRs, because some NRs are known to be cancer-related, and conventional antagonists have not proved entirely satisfactory. Herein, we describe the design, synthesis and biological evaluation of SNIPERs for RAR, ER, and AR. The developed compounds were expected to exhibit antagonist-like activity via down-regulation (knockdown) of the NRs. They should be useful as tools for biological studies and candidate therapeutic agents that would overcome some of the problems encountered with conventional antagonists.

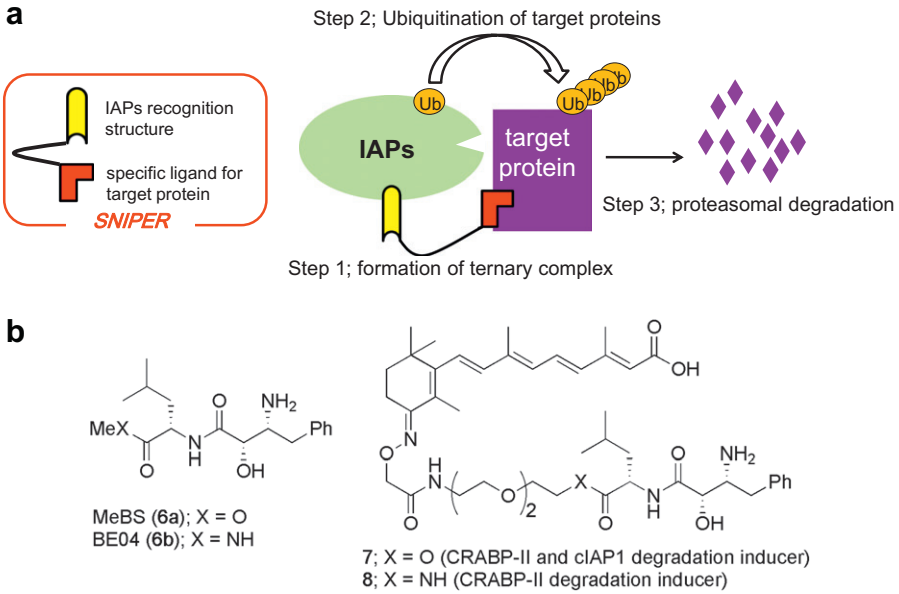


Figure 2. (a) Protein degradation (protein knockdown) by SNIPERs. (b) Chemical structures of cIAP1 ligands **6a**,**b**, and protein degradation inducers **7** and **8**.

2. Chemistry

Compounds **9**–**13** prepared for this study are shown in Figure 3. Scheme 1 shows the preparation of compound **24**, which is an intermediate for synthesis of compounds **9** and **10**. Acid **14** was treated with 2.2 equiv of MeLi to give ketone **15**. Compound **16** was prepared by bromination of **15**, followed by Arbuzov reaction. Compound **18** was obtained from compound **17** via protection of the hydroxyl group, bromination and nucleophilic substitution reaction. Solvolysis of acetate **18** with a catalytic amount of sodium methoxide and oxidation with MnO_2 yielded aldehyde **19**. Horner–Wadsworth–Emmons reaction of **16** and **19** afforded olefin **20** (E/Z mixture). Treatment of BBr_3 with **20** gave compound **21** by deprotection of the methoxy group and

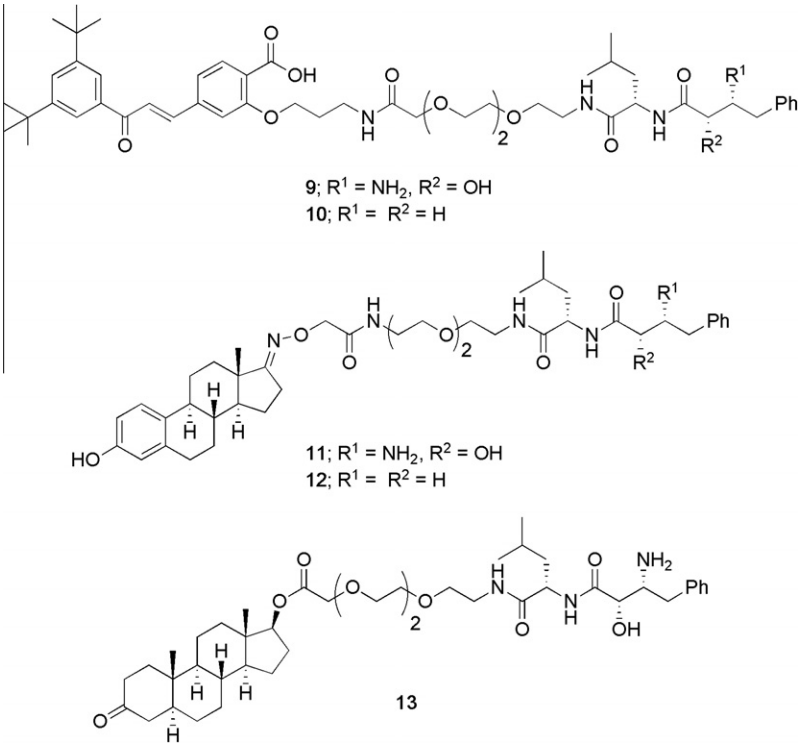
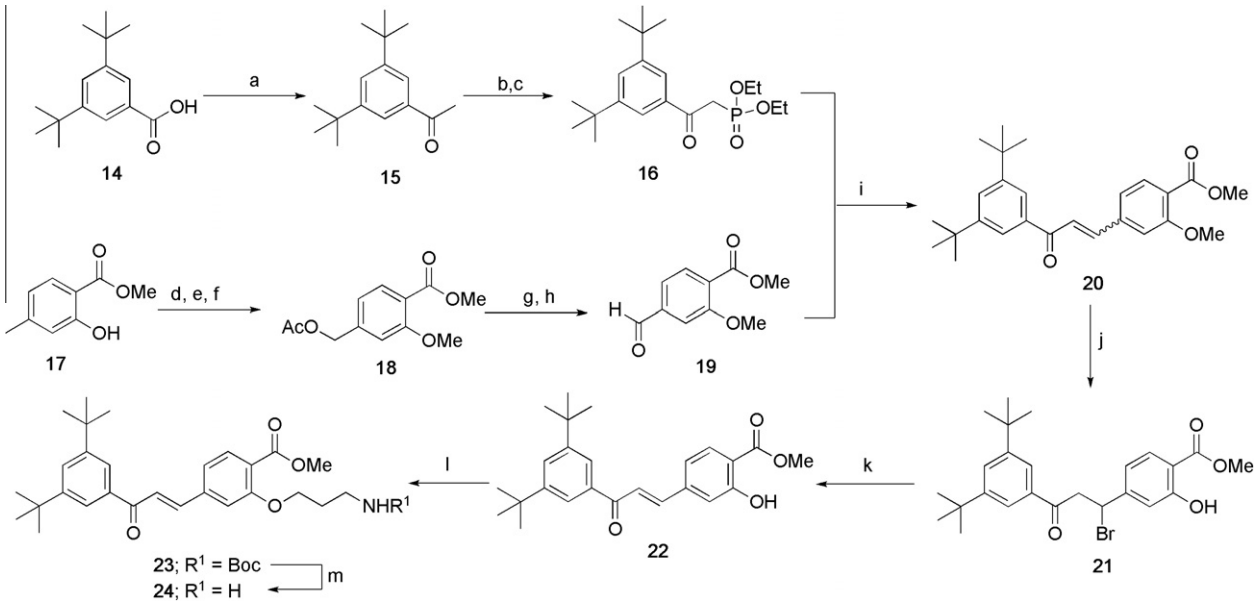


Figure 3. Structures of NR SNIPERs and their negative controls.

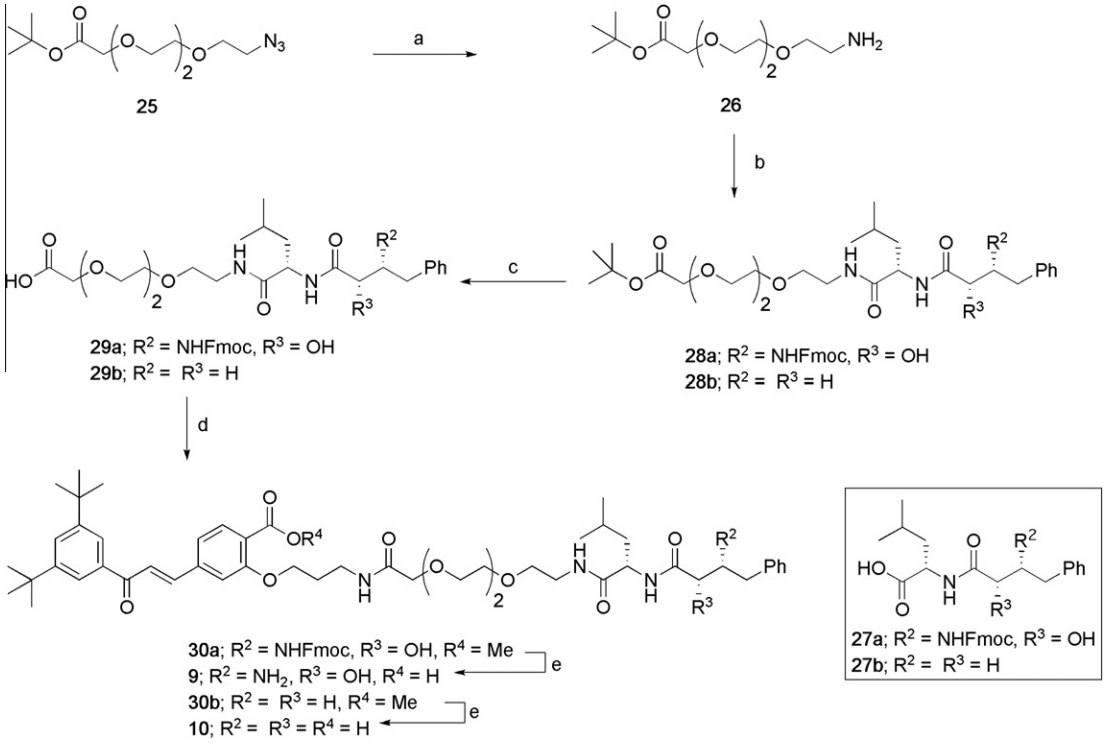


Scheme 1. Reagents and conditions: (a) MeLi, THF, Et_2O, −78 °C to room temperature, 85%; (b) Br_2, AcOH, 0 °C; (c) $P(OEt)_3$, THF, 60 °C, 59% (2 steps); (d) NaH, MeI, DMF, 0 °C to room temperature, 100%; (e) *N*-bromosuccinimide (NBS), azobisisobutyronitrile (AIBN), CCl_4, 70 °C; (f) AcOK, DMF, room temperature, 57% (2 steps); (g) NaOMe, MeOH, room temperature, 86%; (h) MnO_2, CH_2Cl_2, room temperature, 94%; (i) NaOMe, MeOH, room temperature, 76%; (j) BBr_3, CH_2Cl_2, 0 °C; (k) DBU, toluene, reflux, 61% (2 steps); (l) *tert*-butyl 3-bromopropylcarbamate, K_2CO_3, DMF, 90 °C, 98%; (m) HCl, 1,4-dioxane, CH_2Cl_2, room temperature, quant.

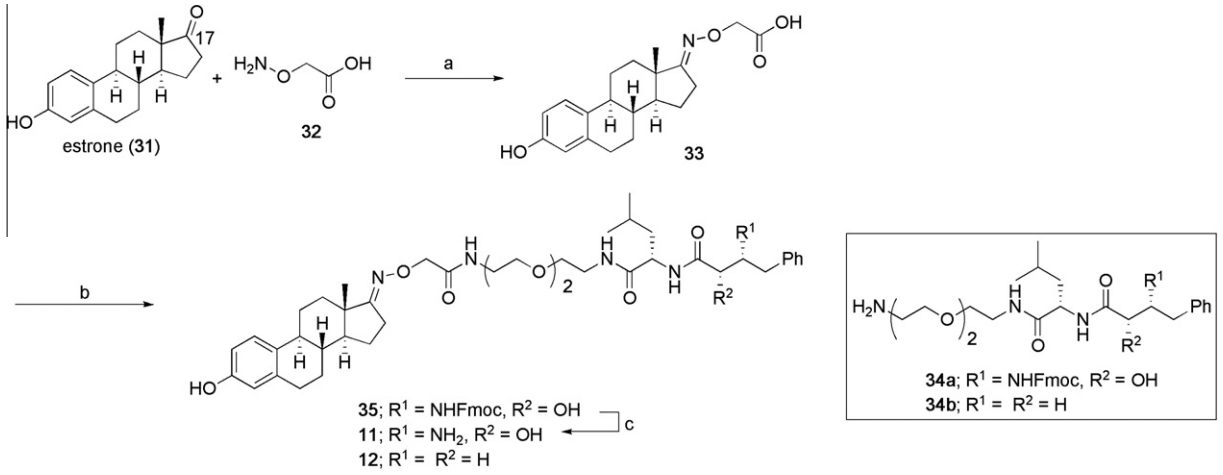
1,4-addition of bromine. HBr elimination from **21** using DBU afforded *E*-olefin **22** as a single geometrical isomer. Treatment of **22** with *tert*-butyl 3-bromopropylcarbamate,[14] followed by deprotection of the *tert*-butoxycarbonyl (Boc) group, gave amine **24**. Compounds **9** and **10** were prepared by means of the procedure outlined in Scheme 2. Reduction of azide **25**[15] in the presence of PPh_3 and H_2O yielded amine **26**. Condensation of amine **26** with acids **27**[12a,b] afforded amides **28**. Hydrolysis of esters **28** under an acidic condition gave acids **29**. Condensation of acids **29** with amine **24** afforded amides **30**. Removal of the Fmoc group and methyl group of **30** gave compounds **9** and **10**.

Compound **11** and negative control **12** were synthesized by means of the procedure shown in Scheme 3. Compound **33** was prepared by oximation of estrone (**31**) and *O*-(carboxylmethyl)hydroxylamine (**32**). Amidation of **33** and **34a,b**[12b] gave amides **35** and **12**. Removal of the Fmoc group of **35** with DBU[12a,b,16] gave compound **11**.

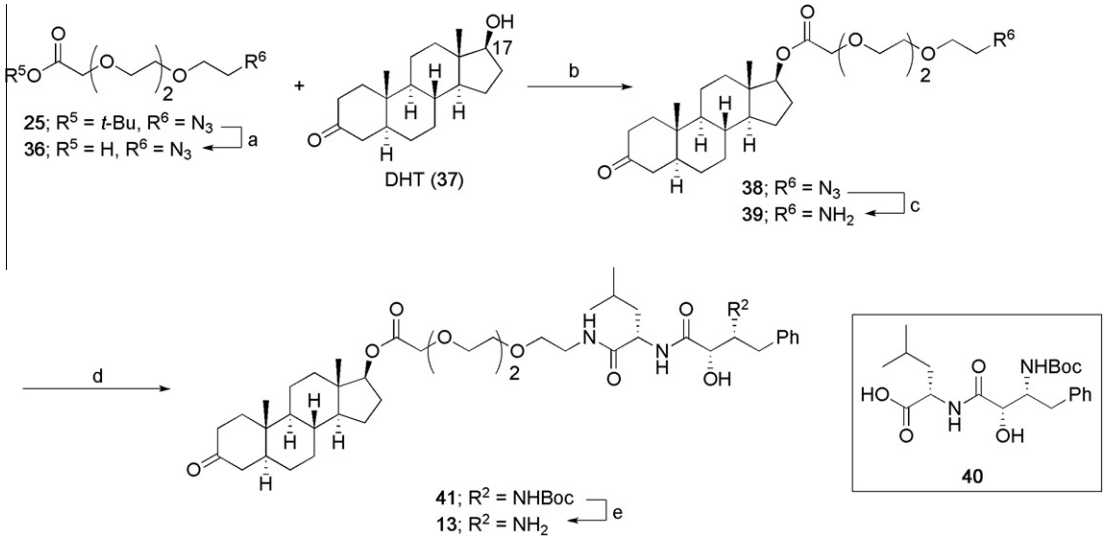
Compound **13** was synthesized from compound **25**, dihydrotestosterone (DHT; **37**) and compound **40**[12b] via the route shown in Scheme 4. Removal of the *tert*-butyl group of ester **25** gave acid **36**. Condensation of DHT (**37**) and **36** afforded ester **38**, and reduction of the azide group of **38** resulted in the formation of amine **39**. Amine **39** was treated with acid **40** to provide amide **41**. Finally,



Scheme 2. Reagents and conditions: (a) PPh_3, H_2O, THF, room temperature; (b) **27a** or **27b**, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI), 1-hydroxybenzotriazole hydrate ($HOBt{\cdot}H_2O$), THF, room temperature, 89–100% (from **27**); (c) HCl, 1,4-dioxane, CH_2Cl_2, room temperature, quant; (d) **24**, EDCI, $HOBt{\cdot}H_2O$, THF, room temperature, 51–71% (from **29**); (e) LiOH, MeOH, H_2O, room temperature, 61–83%.



Scheme 3. Reagents and conditions: (a) pyridine, room temperature, 100%; (b) **34a** or **34b**, EDCI, $HOBt{\cdot}H_2O$, CH_2Cl_2, room temperature, 60–64%; (c) DBU, n-$C_{12}H_{25}SH$, CH_2Cl_2, room temperature, 61%.



Scheme 4. Reagents and conditions: (a) HCl, 1,4-dioxane, CH_2Cl_2, room temperature; (b) EDCI, *N*,*N*-dimethylaminopyridine (DMAP), CH_2Cl_2, room temperature, 45% (2 steps; from **25**); (c) PPh_3, H_2O, THF, room temperature; (d) **40**, EDCI, HOBt·H_2O, CH_2Cl_2, room temperature, 97% (2 steps; from **38**); (e) HCl, 1,4-dioxane, CH_2Cl_2, 0 °C, 90%.

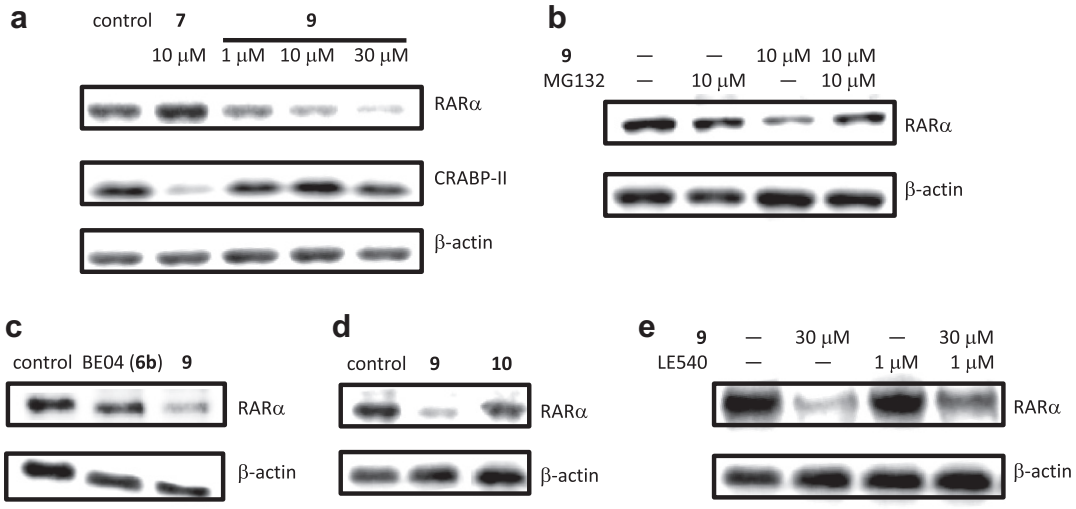


Figure 4. (a) Western blot-detection of RARα and CRABP-II levels in HT1080 cells expressing FLAG-tagged cIAP1 after 24-h treatment with each reagent. (b) Western blot-detection of RARα in HT1080 after 6-h treatment with MG132, which was added to the culture 30 min prior to the addition of **9**. (c) The influence of BE04 (**6b**) on RARα level. Western blot detection of RARα in HT1080 after 24-h treatment with each reagent (30 μM). (d) Western blot detection of RARα in HT1080 cells expressing FLAG-tagged cIAP1 after 24-h treatment with each reagent (30 μM). (e) Western blot detection of RARα in HT1080 cells after 6-h treatment with LE540, which was added to the culture 30 min prior to the addition of **9**.

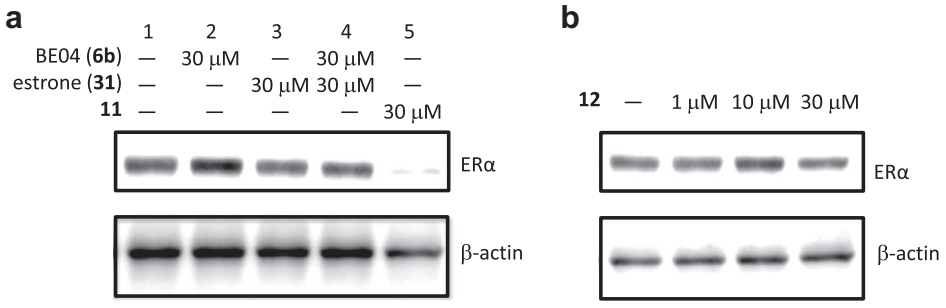


Figure 5. (a) Western blot detection of ERα in MCF-7 cells after 24-h treatment with (a) BE04 (**6b**), estrone (**31**), compound **11** and (b) negative control **12**.

compound **13** was obtained by removal of the Boc group under acidic conditions.

3. Results and discussion

3.1. RAR SNIPER

First, we attempted to identify an RAR SNIPER in order to confirm that the protein knockdown strategy is applicable to NRs. ATRA (**1**), Am80 (**2**), and Ch55 (**3**) are RAR agonists, but ATRA (**1**) and Am80 (**2**) also bind to CRABP-II.[17] On the other hand, the binding affinity of Ch55 (**3**) for CRABP-II is quite low (almost no binding).[4b,c] Therefore we expected to obtain high selectivity for RAR degradation by the use of Ch55 (**3**) for an RAR SNIPER. Additionally, previous studies suggested that introduction of a substituent at the *ortho*-position to the carboxyl group of Ch55 (**3**) would not affect the binding affinity of the compound for RAR.[14] Using BE04 (**6b**) (Fig. 2b) as the binding moiety for cIAP1 has been established to be effective.[12b,18] Based on these results, we designed and synthesized compound **9** as an RAR SNIPER (Fig. 3). We also prepared a negative control **10** (Fig. 3), which would not bind to cIAP1.[12b]

We evaluated the RARα degradation induced by compound **9** by means of Western blot analysis. Treatment of HT1080 cells expressing FLAG-tagged cIAP1 with compound **9** decreased the RARα level in a concentration-dependent manner (Fig. 4a). Compound **9** had no influence on the amount of CRABP-II, while compound **7** (Fig. 2b), which induces degradation of CRABP-II, did not induce RARα degradation (Fig. 4a). This indicated that compound **9** shows selectivity for RAR, as we had expected.

We investigated the influence of proteasome inhibitors on the RARα level in **9**-treated cells (Fig. 4b). The reduction of RARα by **9** was blocked by proteasome inhibitor MG132. Thus, the decrease of RARα induced by **9** can be attributed to proteasomal degradation.

We confirmed that the negative control **10** and BE04 (**6b**) had no effect on RARα level (Fig. 4c and d). In addition, RAR antagonist, LE540, inhibited degradation of RARα induced by compound **9** (Fig. 4e). Thus, conjugation of Ch55 (**3**) and BE04 (**6b**) within a single molecule, thah is, affording a compound which binds simultaneously to both RARα and cIAP1, is essential for RARα degradation-inducing activity. Collectively, these data confirm that compound **9** degraded RARα by protein knockdown. It is noteworthy that CRABP-II and RARα could be independently and selectively degraded by the use of **7** and **9**, respectively, despite the fact that both proteins recognize ATRA.

3.2. ER and AR SNIPERs

Next, we attempted to create SNIPERs targeting other NRs, that is, ER and AR. We used the structures of an ER agonist, estrone (**31**; Scheme 3) and an AR agonist, DHT (**37**; Scheme 4) for these SNIPERs, respectively. It has been reported that introduction of substituents to C-17 position of steroid structure kept binding affinity to the corresponding NRs.[11c,19] Therefore, we designed compounds **11** and **13** using a similar strategy to that which we had employed for the development of the RAR SNIPER (Fig. 3).

The biological activities of synthetic compounds **11** and **13** were evaluated by Western blotting using human mammary tumor MCF-7 cells, which express ERα and AR. Compound **11** significantly down-regulated ERα levels (Fig. 5a). We confirmed that the decrease in ERα was not caused by a partial structure of **11** or by a mere mixture of BE04 (**6b**) and estrone (**31**). BE04 (**6b**, 30 μM; lane 2), estrone (**31**, 30 μM; lane 3), and the mixture of BE04 (**6b**) and estrone (**31**) (30 μM each; lane 4) did not decrease ERα as much as did **11** (30 μM; lane 5). Thus, conjugation of BE04 (**6b**) and estrone (**31**) within a single molecule is important for ERα degradation-inducing activity. In addition, the negative control **12** (Fig. 3), which would not bind to cIAP1, did not decrease ERα (Fig. 5b). Collectively, these data indicate that compound **11** induced protein knockdown of ERα.

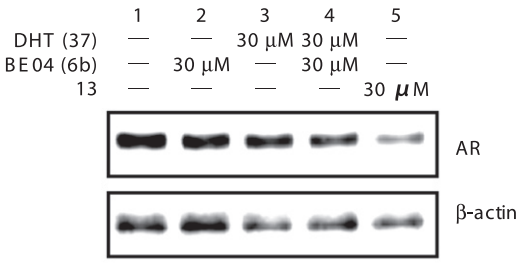


Figure 6. Western blot detection of AR in MCF-7 cells after 24-h treatment with BE04 (**6b**), DHT (**37**) and compound **13**.

The result of protein knockdown for AR is shown in Figure 6. Compound **13** (30 μM; lane 5) decreased the AR level, compared with the control (lane 1), and did so more potently than BE04 (**6b**, 30 μM; lane 2), DHT (**37**, 30 μM; lane 3), or the mixture of BE04 (**6b**) and DHT (**37**) (30 μM each; lane 4). Thus, conjugation of BE04 (**6b**) and DHT (**37**) within a single molecule is also important for AR degradation-inducing activity. These results suggested that compounds **11** and **13** effectively decrease ERα and AR levels, respectively, and may be useful both as tools for biological studies and candidate anticancer agents, serving to inhibit NR functions through knockdown of the receptors.

4. Conclusion

Based on the structures of the RAR agonist Ch55 (**3**), AR agonist DHT (**37**), and ER agonist estrone (**31**), we designed and synthesized RAR, AR, and ER degradation inducers, consisting of the appropriate NR ligand coupled to a ligand of cellular inhibitor of apoptosis protein 1 (cIAP1), which targets the bound NR protein for polyubiquitination and proteasomal degradation. We confirmed that these newly synthesized RAR, AR, and ER SNIPERs, **9**, **11**, and **13**, respectively, were effective for protein knockdown of the corresponding NR, significantly reducing the levels of RAR, AR, and ER, respectively, in live cells. These compounds are expected to be useful as new NR regulators for biological studies and treatment of NR-related diseases.

5. Experimental section

5.1. Chemistry

Proton nuclear magnetic resonance spectra (^{1}H NMR) and carbon nuclear magnetic resonance spectra (^{13}C NMR) were recorded on a JEOL JNMGX500 (500 MHz) spectrometer in the indicated solvent. Chemical shifts (δ) are reported in parts per million relative to the internal standard tetramethylsilane. High-resolution mass spectra (HRMS) and fast atom bombardment (FAB) mass spectra were recorded on a JEOL JMA-HX110 mass spectrometer. Other chemical reagents and solvents were purchased from Aldrich, Merck, Tokyo Kasei Kogyo, Wako Pure Chemical Industries, and Kanto Kagaku and used without purification. Flash column chromatography was performed using silica gel 60 (particle size 0.060–0.210 mm) supplied by Kanto Kagaku.

5.1.1. 1-(3,5-Di-*tert*-butylphenyl)ethanone (15)

1.6 N MeLi in Et_2O (300 μL, 0.480 mmol) was added to a solution of **14** (50.7 mg, 0.0216 mmol) in THF (1.0 mL) at −78 °C. The mixture was stirred at room temperature for 5 h, then poured into water and extracted with AcOEt. The organic layer was washed

with brine and dried over $MgSO_4$. Filtration, evaporation of the solvent in vacuo and purification by flash column chromatography (AcOEt/*n*-hexane = 1/25) gave 42.5 mg (85%) of **15** as a yellow oil; ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm) 7.81 (2H, d, J = 1.8 Hz), 7.65 (1H, t, J = 1.8 Hz), 2.63 (3H, s), 1.36 (18H, s); MS (FAB) *m/z*: 233 (MH^+).

5.1.2. Diethyl 3,5-di-*tert*-butylbenzoylmethylphosphonate (16)

Br_2 (620 mg, 3.38 mmol) was added to solution of **15** (694 mg, 2.99 mmol) in AcOH/THF (5.0 mL/5.0 mL) at 0 °C. The reaction mixture was stirred for 4 h, then concentrated and purified by flash column chromatography (AcOEt/*n*-hexane = 1/10) to afford a crude bromine compound that was used in the next reaction without further purification. A mixture of the crude bromine compound and $P(OEt)_3$ (623 μL, 3.60 mmol) in MeCN (5.0 mL) was heated at reflux temperature for 8 h. The reaction mixture was concentrated and purified by flash column chromatography (AcOEt/*n*-hexane = 1/11) to give 647 mg (59%; 2 steps) of **16** as a colorless solid; ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm) 7.86 (2H, d, J = 1.8 Hz), 7.66 (1H, t, J = 1.8 Hz), 4.15 (4H, m), 3.67 (1H, s), 3.63 (1H, s), 1.36 (18H, s), 1.29 (6H, t, J = 6.7 Hz); MS (FAB) *m/z*: 369 (MH^+).

5.1.3. Methyl 4-(acetoxymethyl)-2-methoxybenzoate (18)

A solution of **17** (3000 mg, 18.1 mmol) in DMF (5.0 mL) was added to a suspension of 60% NaH in oil (1019 mg, 25.5 mmol) in DMF (10 mL) with cooling in an ice-bath and the mixture was stirred for 30 min, then MeI (3.5 mL, 56.2 mmol) was added to it. Stirring was continued at room temperature for 18 h, then the mixture was acidified with 2 N HCl and extracted with AcOEt. The organic layer was washed with brine, and dried over $MgSO_4$. Filtration, evaporation of the solvent in vacuo and purification of the residue by flash column chromatography (AcOEt/*n*-hexane = 1/4) gave 3252 mg (100%) of methyl 2-methoxy-4-methylbenzoate as a colorless oil; ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm): 7.72 (1H, d, J = 8.5 Hz), 6.79 (1H, d, J = 6.7 Hz), 6.78 (1H, s), 3.90 (3H, s), 3.87 (3H, s), 2.37 (3H, s); MS (FAB) *m/z*: 181 (MH^+).

AIBN (578 mg, 3.52 mmol) was added to a solution of methyl 2-methoxy-4-methylbenzoate (3172 mg, 17.6 mmol) and NBS (3446 mg, 19.4 mmol) in CCl_4 (40 mL) and the resulting mixture was heated at 70 °C for 8 h. After cooling, the reaction mixture was concentrated and purified by flash column chromatography (AcOEt/*n*-hexane = 1/6 to 1/3) to give a crude bromine compound that was used in the next reaction without further purification. AcOK (9062 mg, 92.3 mmol) was added to a solution of the crude bromine compound in DMF (10 mL) and the resulting mixture was stirred at room temperature for 15 h, then poured into water and extracted with AcOEt. The organic layer was washed with brine, and dried over $MgSO_4$. Filtration, evaporation of the solvent in vacuo and purification of the residue by flash column chromatography (AcOEt/*n*-hexane = 1/4 to 1/1) gave 2395 mg (57%; 2 steps) of **18** as a colorless solid; ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm): 7.80 (1H, d, J = 7.9 Hz), 6. 69 (1H, d, J = 7.9 Hz), 6.95 (1H, s), 5.12 (2H, s), 3.92 (3H, s), 3.89 (3H, s), 2.13 (3H, s); MS (FAB) *m/z*: 239 (MH^+).

5.1.4. Methyl 4-formyl-2-methoxybenzoate (19)

A solution of **18** (227 mg, 0.953 mmol) in MeOH (2.0 mL) was added to a solution of NaOMe (10.0 mg, 0.185 mmol) in MeOH (1.0 mL) with cooling in an ice-bath and the mixture was stirred for 22 h. The reaction mixture was acidified with 2 N HCl and extracted with AcOEt. The organic layer was washed with brine, and dried over $MgSO_4$. Filtration and evaporation of the solvent in vacuo gave 160 mg (86%) of methyl 4-(hydroxymethyl)-2-methoxybenzoate as a colorless oil; ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm): 7.80 (1H, d, J = 7.9 Hz), 7.03 (1H, s), 6.94 (1H, d, J = 8.5 Hz), 4.75 (2H, d, J = 6.1 Hz), 3.93 (3H, s), 3.88 (3H, s), 1.79 (1H, t, J = 6.1 Hz); MS (FAB) *m/z*: 197 (MH^+).

A mixture of methyl 4-(hydroxymethyl)-2-methoxybenzoate (738 mg 3.76 mmol) and MnO_2 (3270 mg, 37.6 mmol) in CH_2Cl_2 (20 mL) was stirred at room temperature for 28 h. The MnO_2 was removed by filtration and the filtrate was concentrated in vacuo. The residue was purified by flash column chromatography (AcOEt/*n*-hexane = 1/2 to AcOEt only) to give 687 mg (94%) of **19** as a colorless oil; ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm): 10.03 (1H, s), 7.90 (1H, d, J = 7.9 Hz), 7.48 (2H, m), 3.98 (3H, s), 3.93 (3H, s), 1.79 (1H, t, J = 6.1 Hz); MS (FAB) *m/z*: 195 (MH^+).

5.1.5. (*E*)-Methyl 4-[3-(3,5-di-*tert*-butylphenyl)-3-oxoprop-1-enyl]-2-hydroxybenzoate (22)

A solution of **16** (132 mg, 0.680 mmol) in MeOH (3.0 mL) was added to a solution of NaOMe (109 mg, 2.02 mmol) in MeOH (3.0 mL) with cooling in an ice-bath and the mixture was stirred for 30 min. A solution of **19** (240 mg, 0.651 mmol) in MeOH (4.0 mL) was added, and the resulting mixture was stirred at room temperature for 36 h, then acidified with 2 N HCl and extracted with AcOEt. The organic layer was washed with brine, and dried over $MgSO_4$. Filtration and evaporation of the solvent in vacuo gave 202 mg of **20** (E/Z mixture) as a yellow oil. 1 N BBr_3 in CH_2Cl_2 (2.0 mL, 2.0 mmol) was added to a solution of **20** (201 mg, 0.492 mmol) in 3.0 mL of CH_2Cl_2 at 0 °C. The mixture was stirred for 1 h, poured into water and extracted with AcOEt. The organic layer washed with brine, and dried over $MgSO_4$. Filtration, evaporation of the solvent in vacuo and purification of the residue by flash column chromatography (AcOEt/*n*-hexane = 1/10) gave 155 mg of a yellow solid. A mixture of the yellow solid (134 mg) and DBU (43 μL, 0.288 mmol) in toluene (1.0 mL) was heated at reflux temperature for 30 min, then cooled, concentrated and purified by flash column chromatography (AcOEt/*n*-hexane = 1/6 to 1/3) to give 103 mg (46%; 3 steps) of **22** as a yellow solid; ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm): 10.83 (1H, s), 7.88 (1H, d, J = 7.9 Hz), 7.83 (1H, d, J = 1.8 Hz), 7.70 (1H, d, J = 18.0 Hz), 7.65 (1H, t, J = 1.2 Hz), 7.55 (1H, d, J = 16.9 Hz), 7.25 (1H, s), 7.14 (1H, dd, J = 7.9, 1.8 Hz), 3.98 (3H, s), 1.39 (18H, s); MS (FAB) *m/z*: 395 (MH^+).

5.1.6. (*E*)-Methyl 2-[3-(*tert*-butoxycarbonyl amino)propoxy]-4-[3-(3,5-di-*tert*-butylphenyl amino)-3-oxoprop-1-enyl]benzoate (23)

A mixture of **22** (33.1 mg 0.0839 mmol), K_2CO_3 (47.2 mg, 0.342 mmol), KI (20.8 mg, 0.125 mmol) and *tert*-butyl 3-bromopropylcarbamate (79.6 mg, 0.334 mmol) in DMF (2.0 mL) was heated at 90 °C for 5 h. After cooling, the reaction mixture was poured into water and extracted with AcOEt. The organic layer washed with brine, and dried over $MgSO_4$. Filtration, evaporation of the solvent in vacuo and purification of the residue by flash column chromatography (AcOEt/*n*-hexane = 1/3) gave 45.2 mg (98%) of **23** as a yellow oil; ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm): 7.94 (1H, d, J = 8.5 Hz), 7.82 (1H, d, J = 1.8 Hz), 7.72 (1H, d, J = 18.0 Hz), 7.69 (1H, t, J = 1.2 Hz), 7.51 (1H, d, J = 16.9 Hz), 7.30 (1H, d, J = 7.9, Hz), 7.14 (1H, s), 5.92 (1H, br), 4.19 (2H, t, J = 5.4 Hz), 3.92 (3H, s), 3.43 (2H, m), 2.08 (2H, m), 1.46 (9H, s), 1.39 (18H, s); MS (FAB) *m/z*: 552 (MH^+).

5.1.7. (*E*)-Methyl 2-(3-aminopropoxy)-4-[3-(3,5-di-*tert*-butylphenyl)-3-oxoprop-1-enyl]benzoate hydrochloride (24·HCl)

4 N HCl in 1,4-dioxane (500 μL, 2.00 mmol) was added to a solution of **23** (28.1 mg, 0.0509 mmol) in CH_2Cl_2 (0.50 mL) with cooling in an ice-bath and the resulting mixture was stirred at room temperature for 4 h, then concentrated in vacuo to give crude **24**·HCl, which was used in the next step without further purification; MS (FAB) *m/z*: 452 (MH^+).

5.1.8. *tert*-Butyl 2-{2-[2-(2-aminoethoxy)ethoxy]ethoxy}acetate (26)

Triphenyl phosphine (151 mg, 0.576 mmol) was added to a solution of **25** (151 mg, 0.522 mmol) and H_2O (150 μL, 8.31 mmol) in THF (3.0 mL) and the resulting mixture was stirred at room temperature for 24 h. The reaction mixture was concentrated in vacuo to give crude **26**, which was used in the next step without further purification.

5.1.9. *tert*-Butyl 2-[2-(2-{2-[(*S*)-2-((2*S*,3*R*)-3-{[(9*H*-fluoren-9-yl)methoxy]carbonyl amino}-2-hydroxy-4-phenylbutanamido)-4-methylpentanamido]ethoxy}ethoxy)ethoxy]acetate (28a)

EDCI (46.2 mg, 0.241 mmol) was added to a solution of crude **26**, **27a** (45.2 mg, 0.0852 mmol) and HOBt·H_2O (52.1 mg, 0.340 mmol) in CH_2Cl_2 (3.0 mL) with cooling in an ice-bath. The resulting mixture was stirred at room temperature for 17 h. The reaction was quenched with water, and the mixture was extracted with AcOEt. The organic layer was washed with brine, and dried over $MgSO_4$. Filtration, evaporation of the solvent in vacuo and purification of the residue by flash column chromatography (MeOH/$CHCl_3$ = 1/30) gave 59.1 mg (89%; from **27a**) of **28a** as a colorless oil; ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm): 7.75 (2H, d, J = 7.3 Hz), 7.51 (1H, d, J = 7.3 Hz), 7.39 (2H, t, J = 7.3 Hz), 7.35–7.16 (6H, m), 6.81 (1H, br), 5.49 (1H, d, J = 8.5 Hz), 5.14 (1H, m), 4.49 (1H, m), 4.28 (4H, m), 4.13 (1H, m), 4.03 (3H, m), 3.80–1.38 (12H, m), 3.04 (1H, m), 2.96 (1H, m), 1.73 (1H, m), 1.58 (2H, m), 1.48 (9H, s), 0.86 (3H, d, J = 6.1 Hz), 0.86 (3H, d, J = 6.1 Hz); MS (FAB) *m/z*: (MH^+); 798 (MNa^+), 776 (MH^+).

5.1.10. 2-[2-(2-{2-[(*S*)-2-((2*S*,3*R*)-3-{[(9*H*-Fluoren-9-yl)methoxy]carbonyl amino})-2-hydroxy-4-phenylbutanamido)-4-methylpentanamido]ethoxy}ethoxy)ethoxy]acetic acid (29a)

4 N HCl in 1,4-dioxane (1000 μL, 4.00 mmol) was added to a solution of **28a** (31.8 mg, 0.0513 mmol) in CH_2Cl_2 (0.50 mL) with cooling in an ice-bath and the resulting mixture was stirred at room temperature for 5 h. The reaction mixture was concentrated in vacuo to give crude **29a**, which was used in next step without further purification; MS (FAB) *m/z*: 720 (MH^+).

5.1.11. Methyl 2-(3-{2-[2-(2-{2-[(*S*)-2-((2*S*,3*R*)-3-{[(9*H*-fluoren-9-yl)methoxy]carbonyl amino}-2-hydroxy-4-phenylbutanamido)-4- methylpentanamido]ethoxy}ethoxy)ethoxy]acetamido} propoxy)-4-[(*E*)-3-(3,5-di-*tert*-butylphenyl)-3-oxoprop-1-enyl] benzoate (30a)

Compound **30a** (yield; 30.0 mg, 51%; 2 steps from **28a**) was prepared from crude **24** and **29a** using the same procedure as described for **28a**; Colorless oil; ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm): 7.89 (1H, d, J = 7.9 Hz), 7.82 (2H, s), 7.75–7.68 (4H, m), 7.53–7.46 (4H, m), 7.38 (5H, m), 7.30–7.13 (7H, m), 5.60 (1H, d, J = 8.5 Hz), 5.47 (1H, m), 4.51 (1H, m), 4.32–4.09 (7H, m), 3.89 (3H, s), 3.72–3.40 (14H, m), 3.04 (1H, m), 2.93 (1H, m), 2.07 (2H, m), 1.73 (1H, m), 1.58 (2H, m), 1.38 (18H, s), 0.85 (6H, d, J = 5.5 Hz); MS (FAB) *m/z*: 1175 (MNa^+), 1153 (MH^+).

5.1.12. 2-[3-(2-{2-[2-(2-{(*S*)-2-[(2*S*,3*R*)-3-Amino-2-hydroxy-4-phenylbutanamido]-4-methylpentanamido}ethoxy)ethoxy] ethoxy}acetamido)propoxy]-4-[(*E*)-3-(3,5-di-*tert*-butylphenyl)-3-oxoprop-1-enyl]benzoic acid (9)

LiOH·H_2O (36.0 mg, 0.858 mmol) was added to a solution of **30a** (16.0 mg, 0.0139 mmol) in MeOH/H_2O (1.0 mL/0.25 mL) and the resulting mixture was stirred at room temperature for 2 h. The reaction mixture was neutralized and concentrated in vacuo. The residue was extracted with AcOEt. The organic solution was washed with brine, concentrated in vacuo and purified by PTLC ($CHCl_3$/MeOH/25% NH_3 aqueous solution = 6/1/1) to give 683 mg (64%) of **9** as a colorless solid; ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm): 8.19 (1H, br), 7.90 (2H, m), 7.82 (2H, d, J = 1.8 Hz), 7.76 (1H, m), 7.69 (1H, d, J = 15.8 Hz), 7.67 (1H, t, J = 1.8 Hz), 7.48 (1H, d, J = 15.8 Hz), 7.32–7.18 (5H, m), 7.14 (1H, s), 4.30 (2H, m), 4.14 (3H, m), 4.00–3.40 (21H, m), 3.26 (1H, m), 3.13 (1H, m), 2.04 (2H, m), 1.80 (1H, m), 1.61 (2H, m), 1.38 (18H, s), 0.90 (3H, d, J = 6.7 Hz) 0.89 (3H, d, J = 6.7 Hz); ^{13}C NMR ($CDCl_3$, 125 MHz, δ; ppm): 191.49, 172.61, 171.91, 169.91, 169.74, 151.33, 143.48, 137.76, 129.36, 128.99, 127.74, 127.29, 127.23, 122.75, 120.51, 119.27, 113.92, 70.96, 70.10, 70.02, 69.69, 69.03, 69.39, 68.04, 67.02, 55.01, 52.79, 40.08, 39.12, 36.39, 36.22, 35.03, 31.41, 29.69, 24.81, 23.04, 21.57; MS (FAB) *m/z*: 917 (MH^+); HRMS (FAB) calcd for $C_{51}H_{73}N_4O_{11}^+$, 917.5276; found 917.5297.

5.1.13. (*S*)-*tert*-Butyl 2-[2-(2-{2-[4-methyl-2-(4-phenylbutanamido)pentanamido]ethoxy}ethoxy) ethoxy]acetate (28b)

Compound **28b** (yield; 60.0 mg, 100% from **27b**) was prepared from **27b** (36.1 mg, 0.114 mmol) and crude **26** using the same procedure as described for **28a**; Colorless oil; ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm): 7.27 (2H, t, J = 7.3 Hz), 7.19–7.14 (3H, m), 6.82 (1H, m), 6.22 (1H, m), 4.49 (1H, dt, J = 3.6, 4.8 Hz), 4.01 (2H, s), 3.70 (4H, m), 3.62 (4H, m), 3.55 (2H, t, J = 4.9 Hz), 3.44 (2H, t, J = 5.4 Hz), 2.64 (2H, t, J = 7.6 Hz), 2.21 (2H, t, J = 7.6 Hz), 1.95 (2H, quin, J = 7.3 Hz), 1.64 (2H, m), 1.53 (1H, m), 1.52 (9H, s), 0.93 (3H, d, J = 6.1 Hz), 0.92 (3H, d, J = 6.1 Hz); MS (FAB) *m/z*: 523 (MH^+).

5.1.14. (*S*,*E*)-Methyl 4-[3-(3,5-di-*tert*-butylphenyl)-3-oxoprop-1-enyl]-2-(3-{2-[2-(2-{2-[4-methyl-2-(4-phenylbutanamido) pentanamido]ethoxy}ethoxy)ethoxy]acetamido}propoxy) benzoate (30b)

Compound **30b** (yield; 30.8 mg, 72%; 2 steps from **28b**) was prepared from crude **24** and **28b** (24.5 mg, 0.0469 mmol) using the same procedure as described for **28a** and **30a**; Colorless oil; ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm): 7.87 (1H, d, J = 7.9 Hz), 7.82 (2H, d, J = 1.8 Hz), 7.72 (1H, d, J = 15.9 Hz), 7.68 (1H, t, J = 1.8 Hz), 7.53 (1H, t, J = 15.8 Hz), 7.42 (1H, br), 7.16 (4H, m), 6.92 (1H, m), 5.52 (1H, m), 4.49 (1H, m), 4.17 (2H, m), 4.09 (2H, s), 3.91 (3H, s), 3.80–3.37 (14H, m), 2.62 (2H, t, J = 7.6 Hz), 2.21 (2H, t, J = 7.6 Hz), 2.19 (2H, t, J = 7.6 Hz), 1.96 (2H, m), 1.68 (2H, m), 1.51 (1H, m), 1.39 (18H, s), 0.92 (6H, d, J = 4.8 Hz); MS (FAB) *m/z*: 922 (MNa^+), 900 (MH^+).

5.1.15. (*S*,*E*)-4-[3-(3,5-Di-*tert*-butylphenyl)-3-oxoprop-1-enyl]-2-(3-{2-[2-(2-{2-[4-methyl-2-(4-phenylbutanamido) pentanamido]ethoxy}ethoxy)ethoxy]acetamido}propoxy) benzoic acid (10)

Compound **10** (yield; 18.6 mg, 61%) was prepared from **30b** (30.8 mg, 0.0342 mmol) using the same procedure as described for **9**; Colorless oil; ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm): 7.98 (1H, d, J = 7.9 Hz), 7.83 (2H, d, J = 1.8 Hz), 7.72 (1H, d, J = 15.2 Hz), 7.69 (1H, t, J = 1.8 Hz), 7.52 (1H, d, J = 15.8 Hz), 7.33 (1H, d, J = 7.3 Hz), 7.26 (1H, d, J = 7.3 Hz), 7.16 (3H, m), 6.39 (1H, m), 4.55 (1H, q, 6.1 Hz), 4.21 (2H, t, J = 5.4 Hz), 4.04 (2H, m), 3.72–3.32 (14H, m), 2.62 (2H, t, J = 7.3 Hz), 2.23 (2H, t, J = 7.6 Hz), 2.10 (2H, t, J = 6.1 Hz), 1.96 (2H, quin, J = 7.3 Hz), 1.62 (2H, m), 1.53 (1H, m), 1.38 (18H, s), 0.93 (3H, d, J = 6.7 Hz), 0.92 (3H, d, J = 6.7 Hz); ^{13}C NMR ($CDCl_3$, 125 MHz, δ; ppm): 191.32, 173.26, 172.63, 170.30, 158.23, 151.37, 142.99, 141.32, 137.61, 132.87, 128.43, 128.39, 128.37, 127.35, 125.35, 124.94, 122.75, 122.35, 120.16, 113.28, 70.68, 70.54, 70.47, 70.13, 69.89, 69.58, 67.70, 57.24, 51.80, 41.37, 39.01, 36.57, 35.68, 35.41, 35.03, 31.39, 28.98, 27.08, 24.80, 22.79, 22.21; MS (FAB) *m/z*: 908 (MNa^+), 886 (MH^+); HRMS (FAB) calcd for $C_{51}H_{72}N_3O_{10}^+$, 886.5218; found 886.5235.

5.1.16. 2-({(*E*)-[(8*R*,9*S*,13*S*,14*S*)-3-Hydroxy-13-methyl-7,8,9,11,12,13,15,16-octahydro-6*H*-cyclopenta[*a*]phenanthren-17(14*H*)-ylidene]amino}oxy)acetic acid (33)

A mixture of estrone (**31**) (50.0 mg, 0.185 mmol) and *O*-(carboxylmethyl)hydroxylamine (**32**) (61.0 mg, 0.555 mmol) in pyridine (2.0 mL) was stirred at room temperature for 17 h. The reaction mixture was poured into 10% aqueous citric acid and extracted with AcOEt. The organic layer was washed with brine and dried over $MgSO_4$. Filtration, evaporation of the solvent in vacuo and purification of the residue by flash column chromatography ($CHCl_3$/MeOH = 6/1) gave 64.0 mg (100%) of **33** as a colorless solid; ^{1}H NMR (CD_3CD, 500 MHz, δ; ppm): 7.09 (1H, d, J = 8.6 Hz), 6.56 (1H, dd, J = 8.6, 2.5 Hz), 6.50 (1H, d, J = 2.4 Hz), 4.53 (2H, s), 4.84–4.77 (2H, m), 2.66–2.55 (2H, m), 2.40–2.35 (1H, m), 2.26–2.21 (1H, m), 2.03 (1H, td, J = 12.8, 3.1 Hz), 1.98–1.91 (2H, m), 1.62 (1H, dt, J = 13.4, 4.3 Hz), 1.54–1.40 (5H, m), 0.97 (3H, s); MS (FAB) m/z: 344 (MH^+).

5.1.17. (9*H*-Fluoren-9-yl)methyl [(14*S*,17*S*,18*R*)-17-hydroxy-1-({(*E*)-[(8*R*,9*S*,13*S*,14*S*)-3-hydroxy-13-methyl-7,8,9,11,12,13,15,16-octahydro-6*H*-cyclopenta[*a*]phenanthren-17(14*H*)-ylidene]amino}oxy)-14-isobutyl-2,13,16-trioxo-19-phenyl-6,9-dioxa-3,12,15-triazanonadecan-18-yl]carbamate (35)

Compound **35** (yield; 43.0 mg, 64%) was prepared from **34a** (35.0 mg, 0.102 mmol) and **33** (51.0 mg, 0.0772 mmol) using the same procedure as described for **28a**; Colorless solid; ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm): 7.75 (2H, d, J = 7.3 Hz), 7.50 (2H, d, J = 7.3 Hz), 7.39 (2H, t, J = 7.3 Hz), 7.31–7.18 (5H, m), 7.11 (1H, d, J = 8.6 Hz), 6.76 (1H, br s), 6.70 (1H, t, J = 4.3 Hz), 6.62 (1H, dd, J = 10.0, 2.5 Hz), 6.57 (1H, d, J = 2.5 Hz), 4.48 (2H, s), 4.46 (1H, m), 4.30–4.18 (4H, m), 4.12 (1H, br), 3.50 (12H, m), 3.30 (1H, m), 3.04–2.94 (2H, m), 2.84–2.81 (2H, m), 2.60–2.46 (2H, m), 2.34–2.30 (1H, m), 2.22 (1H, m), 2.20 (1H, m), 2.03–2.00 (1H, m), 1.92–1.87 (2H, m), 1.73–1.34 (8H, m), 0.92 (3H, s), 0.86 (3H, d, J = 6.1 Hz), 0.84 (3H, d, J = 6.1 Hz); MS (FAB) m/z: 987 (MH^+).

5.1.18. (*S*)-2-[(2*S*,3*R*)-3-Amino-2-hydroxy-4-phenylbutanamido]-*N*-[2-(2-{2-[2-({(*E*)-[(8*R*,9*S*,13*S*,14*S*)-3-hydroxy-13-methyl-7,8,9,11,12,13,15,16-octahydro-6*H*-cyclopenta[*a*]phenanthren-17(14*H*)-ylidene] amino}oxy) acetamido]ethoxy}ethoxy)ethyl]-4-methylpentanamide (11)

DBU (12.0 μL, 0.0759 mmol) was added to a solution of **35** (25.0 mg, 25.3 mmol) and dodecyl mercaptan (8.00 μL, 0.0336 mmol) in CH_2Cl_2 (1.0 mL). The reaction mixture was stirred at room temperature for 2 h, then purified by PTLC ($CHCl_3$/MeOH/25% NH_3 aqueous solution = 15/1/0.1) to give 11.9 mg (61%) of **11** as a colorless oil; ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm): 7.55 (1H, d, J = 8.5 Hz), 7.32–7.29 (2H, m), 7.24–7.21 (1H, m), 7.12 (1H, d, J = 8.6 Hz), 6.90 (1H, t, J = 5.5 Hz), 6.70 (1H, t, J = 5.5 Hz), 6.64 (1H, dd, J = 8.6, 2.5 Hz), 6.58 (1H, d, J = 4.5 Hz), 4.50 (2H, s), 4.45–4.42 (1H, m), 4.00 (1H, d, J = 2.4 Hz), 3.58–3.34 (14H, m), 2.97 (1H, dd, J = 13.4, 4.9 Hz), 2.83 (2H, m), 2.61–2.49 (3H, m), 2.35 (1H, dd, J = 13.4, 3.4 Hz), 2.22 (1H, dt, J = 13.4, 3.4 Hz), 2.05–2.01 (2H, m), 1.91 (2H, m), 1.73–1.37 (8H, m), 0.93 (6H, s), 0.90 (3H, s); ^{13}C NMR ($CDCl_3$, 125 MHz, δ; ppm): 173.13, 172.50, 171.63, 170.49, 153.46, 137.95, 137.69, 131.68, 129.06, 128.47, 126.44, 126.16, 115.12, 112.64, 72.31, 70.29, 70.06, 69.93, 69.64, 69.50, 54.28, 52.71, 51.50, 44.42, 43.73, 40.44, 39.03, 38.94, 38.50, 37.85, 33.83, 29.43, 29.22, 26.97, 25.89, 25.85, 24.64, 22.75, 22.72, 21.48, 17.04; MS (FAB) m/z: 764 (MH^+); HRMS (FAB) calcd for $C_{42}H_{62}N_5O_8^+$, 764.4598; found 764.4590.

5.1.19. *N*-[2-(2-{2-[2-({(*E*)-[(8*R*,9*S*,13*S*,14*S*)-3-Hydroxy-13-methyl-7,8,9,11,12,13,15,16-octahydro-6*H*-cyclopenta[*a*]phenanthren-17(14*H*)-ylidene]amino}oxy)acetamido]ethoxy}ethoxy)ethyl]-4-methyl-(*S*)-2-(4-phenylbutanamido)pentanamide (12).

Compound **12** (yield; 43.0 mg, 60%) was prepared from **34b** (35.0 mg, 0.102 mmol) and **33** (51.0 mg, 0.0772 mmol) using the same procedure as described for **28a**; colorless solid; ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm); 7.27 (1H, t, J = 7.3 Hz), 7.20–7.12 (3H, m), 6.69 (1H, t, J = 5.5 Hz), 6.64 (1H, dd, J = 8.3, 2.8 Hz), 6.59 (2H, d, J = 2.8 Hz), 6.14 (1H, d, J = 8.3 Hz), 4.50 (s, 2H), 4.44 (1H, dt, J = 5.5, 8.6 Hz), 3.64–3.56 (6H, m), 3.52–3.50 (4H, m), 3.47–3.38 (2H, m), 2.86–2.82 (2H, m), 2.63 (2H, t, J = 7.3 Hz), 2.59–2.49 (2H, m), 2.35–2.32 (1H, m), 2.21 (2H, t, J = 7.3 Hz), 2.19–1.89 (5H, m), 1.63–1.35 (10H, m), 0.93 (6H, d, J = 1.8 Hz), 0.92 (3H, s); ^{13}C NMR ($CDCl_3$, 125 MHz, δ; ppm): 172.83, 172.65, 172.26, 170.61, 153.88, 141.34, 137.89, 131.79, 128.46, 128.41, 126.37, 125.99, 115.40, 112.93, 72.58, 70.58, 70.38, 70.16, 69.93, 69.68, 52.96, 51.57, 44.64, 43.98, 41.55, 39.26, 38.71, 38.17, 35.76, 35.16, 34.08, 29.48, 27.23, 27.09, 26.13, 26.07, 24.80, 22.97, 22.89, 22.14, 17.28; MS (FAB) m/z: 733 (MH^+); HRMS (FAB) calcd for $C_{42}H_{61}N_4O_7^+$, 733.4540; found 733.4554.

5.1.20. (5*S*,8*R*,9*S*,10*S*,13*S*,17*S*)-10,13-Dimethyl-3-oxohexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl 2-{2-[2-(2-aminoethoxy)ethoxy]ethoxy}acetate (38)

4 N HCl in 1,4-dioxane (2.00 mL, 8.00 mmol) was added to a solution of **25** (220 mg, 0.760 mmol) in CH_2Cl_2 (2.0 mL) with cooling in an ice-bath and the resulting mixture was stirred at room temperature for 24 h. The reaction mixture was concentrated in vacuo to give crude **36**. EDCI (250 mg, 1.30 mmol) was added to a solution of crude **36**, DHT (**37**) (221 mg, 0.760 mmol) and DMAP (140 mg, 1.14 mmol) in CH_2Cl_2 (7.6 mL) with cooling in an ice-bath. The resulting mixture was stirred at room temperature for 18 h. The reaction was quenched with water, and the mixture was extracted with AcOEt. The organic layer was washed with brine, and dried over $MgSO_4$. Filtration, evaporation of the solvent in vacuo and purification of the residue by flash column chromatography (AcOEt/*n*-hexane = 1/1) gave 174 mg (45%; 2 steps) of **38** as a colorless solid; ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm): 4.68 (1H, t, J = 7.9 Hz), 4.14 (2H, s), 3.75–3.67 (10H, m), 3.39 (2H, t, J = 4.9 Hz), 2.43–1.99 (6H, m), 1.77–0.73 (22H, m); MS (FAB) m/z: (MH^+); 506 (MH^+).

5.1.21. (14*S*,17*S*,18*R*)-[(5*S*,8*R*,9*S*,10*S*,13*S*,14*S*,17*S*)-10,13-Dimethyl-3-oxohexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl] 18-(*tert*-butylcarbonylamino)-17-hydroxy-14-isobutyl-13,16-dioxo-19-phenyl-3,6,9-trioxa-12,15-diazanonadecan-1-oate (39)

Crude **39** was prepared from **38** (36.0 mg, 0.0712 mmol) using the same procedure as described for **26**. Compound **39** was used in the next step without further purification.

5.1.22. (14*S*,17*S*,18*R*)-[(5*S*,8*R*,9*S*,10*S*,13*S*,14*S*,17*S*)-10,13-Dimethyl-3-oxohexadecahydro-1*H*-cyclopenta[*a*]phenanthren-1-7-yl] 18-(*tert*-butylcarbonylamino)-17-hydroxy-14-isobutyl-13,16-dioxo-19-phenyl-3,6,9-trioxa-12,15-diazanonadecan-1-oate (41)

Compound **41** (yield; 20.0 mg, 96%; 2 steps from **38**) was prepared from crude **39** (36.0 mg, 0.0712 mmol) and **40** (9.70 mg, 0.0237 mmol) using the same procedure as described for **28a**; yellow oil; ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm): 7.30–7.20 (5H, m), 6.87 (1H, s), 5.43 (1H, s), 5.04 (1H, s), 4.67 (1H, t, J = 9.2 Hz), 4.50–4.44 (1H, m), 4.16–4.08 (3H, m), 3.74–3.36 (7H, m), 3.06–2.98 (2H, m), 2.40–2.07 (5H, m), 2.02–1.98 (1H, m), 1.75–1.25 (24H, m), 1.17 (1H, dt, J = 10.4, 4.3 Hz), 1.07–1.03 (1H, m) 1.01 (3H, s), 0.93 (3H, d, J = 6.1 Hz), 0.90 (3H, d, J = 6.2 Hz), 0.79 (3H, s), 0.75 (1H, dt, J = 10.4, 4.3 Hz); MS (FAB) m/z: 770 (MH^+).

5.1.23. (14*S*,17*S*,18*R*)-[(5*S*,8*R*,9*S*,10*S*,13*S*,14*S*,17*S*)-10,13-Dimethyl-3-oxohexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl] 18-amino-17-hydroxy-14-isobutyl-13,16-dioxo-19-phenyl-3,6,9-trioxa-12,15-diazanonadecan-1-oate (13)

Compound **13** (yield; 2.40 mg, 90%) was prepared from **41** (3.00 mg, 0.0034 mmol) using the same procedure as described for **24**; yellow amorphous solid; ^{1}H NMR (CD_3OD, 500 MHz, δ; ppm): 7.40–7.31 (5H, m), 4.69 (1H, t, J = 8.6 Hz), 4.38 (1H, dt, J = 8.6, 6.7 Hz), 4.17 (2H, s), 4.14 (2H, d, J = 3.1 Hz), 3.72–3.59 (9H, m), 3.53 (2H, t, J = 6.1 Hz), 3.43–3.40 (2H, m), 3.12 (1H, dd, J = 14.0, 7.9 Hz), 2.94 (1H, dd, J = 7.3 Hz), 2.50 (1H, dt, J = 6.7, 15.3 Hz), 2.38 (1H, t, J = 14.0 Hz), 2.26–2.14 (2H, m) 2.07–2.01 (2H, m), 1.80–1.10 (18H, m), 1.08 (3H, s), 0.98 (3H, d, J = 6.1 Hz), 0.97 (3H, d, J = 6.1 Hz), 0.85 (3H, s), 0.84–0.77 (1H, m); ^{13}C NMR ($CDCl_3$, 125 MHz, δ; ppm): 211.73, 172.14, 171.08, 135.20, 129.67, 128.95, 127.49, 84.22, 53.71, 53.57, 50.35, 46.50, 44.60, 42.79, 38.43, 38.09, 36.81, 35.68, 35.13, 31.13, 28.70, 27.48, 24.87, 23.49, 23.20, 21.36, 20.87, 12.19, 11.44; MS (FAB) m/z: 770 (MH^+); HRMS (FAB) calcd for $C_{43}H_{68}N_3O_9^+$, 770.4956; found 770.4956.

5.2. Biology

5.2.1. Cell culture conditions

Human fibrosarcoma HT-1080 cells were cultured in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO_2 in air. Human mammary tumor MCF-7 cells were cultured in D-MEM medium containing 10% FBS, penicillin and streptomycin mixture, and physiological amounts of endogenous agonists, E2 (2 nM; for ER assay)[20] and DHT (10 nM; for AR assay),[21] respectively, at 37 °C in a humidified atmosphere of 5% CO_2 in air.

5.2.2. FLAG-IAPs transfection

The transfection experiments were carried out according to the method reported in Ref. 18.

5.2.3. Western blotting

HT1080, FLAG-IAPs HT1080 and MCF-7 (1×10^6) cells were treated for the indicated period with BE04 (**6b**),[12b] compound **7**,[12a] MG132 (Peptide Institute Inc.), and/or synthetic compounds at the indicated concentrations, then the cells were collected and extracted with SDS buffer. Protein concentrations of the lysates were determined using BCA protein assay. Equivalent amounts of protein from each lysate were resolved in 10–20% SDS–polyacrylamide gels and transferred onto PVDF membranes. The transblotted membranes were blocked with TBS-T containing 5% skim milk, and probed with rabbit polyclonal CRABP-II antibody (Novus Biologicals, Inc.) (1:1000 dilution), mouse monoclonal RARα antibody (Perseus Proteomics Inc.) (1:1000 dilution), mouse monoclonal AR antibody (Santa Cruz Biotechnology, Inc.) (1:200 dilution), rabbit polyclonal ERα antibody (Santa Cruz Biotechnology, Inc.) (1:200 dilution), anti-goat IgG-horseradish peroxidase conjugates (Kirkegaard & Perry Laboratories, Inc.) (1:5000 dilution), anti-mouse IgG-horseradish peroxidase conjugates (Chemicon) (1:2000 dilution), goat anti-rabbit IgG-horseradish peroxidase conjugates (Amersham) (1:2000 dilution), β-actin antibody (Santa Cruz Biotechnology, Inc.) (1:2000 dilution) as appropriate in can-get-signal solution (Toyobo). After probing, the membrane was washed twice more with TBS-T. The immunoblots were visualized by enhanced chemiluminescence with Immobilon™ Western Chemiluminescent HRP Substrate (Millipore).

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