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Convergent synthesis of a steroidal antiestrogen-mitomycin C hybrid using "click" chemistry†

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Abstract

A convergent synthesis of a novel estrogen receptor-targeted drug hybrid was developed based on structures of the potent anti-proliferative mitomycin C and the steroidal anti-estrogen RU 39411. The steroidal antiestrogen was prepared with an azido-triethylene glycoloxy linker while the mitomycin C derivative (porfirimycin) incorporated a complementary 7-N-terminal alkyne. The two components were ligated using the Huisgen [3 + 2] cycloaddition ("click") reaction. Preliminary biological assays demonstrated that the final hybrid compound retained both potent anti-estrogenic and anti-proliferative activities.

Introduction

One approach for developing new chemotherapeutic agents involves conjugating two biologically active compounds to make a single hybrid agent. In the field of hormone responsive breast cancer, this strategy typically involves linking a potent estrogen receptor (ER) targeting agent to a second component, such as an anti-metabolite, intercalating agent, anti-mitotic, alkylating agent or metal chelating group.[1–19] Unfortunately, the resultant products from these efforts proved almost invariably to be less effective at each of its targets than the separate, individual components. Typically one observes loss of ER affinity, absence of cancer cell selectivity, and reduction in cytotoxicity associated with the therapeutic moiety. The objective of this project was to overcome these problems by using a different targeting strategy.

Several reviews have described the difficulties associated with designing bi-functional hybrid drugs,[20–27] but in the case of steroid receptor targeted hybrids, most of the problems are related to a reliance on chemical transformations of readily available materials or easily modified sites on those materials to prepare the target compounds. While attachment or incorporation of functional groups at the 3-,6-,17α- or 17β-positions of estradiol is relatively easy, an analysis of the crystal structures of agonist and antagonist complexes with estrogen receptor-ligand binding domain (ER-LBD) suggests that such molecular modifications seriously impair binding to the receptor[28–31] (Fig. 1). Introduction of substituents at the 7α-position of estradiol, such as those found in the anti-estrogen ICI-182,780 (faslodex),

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requires a few more steps, but leads to better retention of biological properties[32,33] (Fig. 2). However, the crystal structure of a complex using a similarly substituted ligand ICI-164,384 with ERα-LBD indicates that the steroidal scaffold is rotated around the 3–17 axis, projecting the 7α-side chain into the 11β-pocket of the receptor and causing disorder associated with helix-12.[34]

The position on the estradiol scaffold where structural modifications appear to be tolerated best by the receptor is the 11β-site. A number of studies have demonstrated that 11β-alkyl, alkenyl and aryl estradiols possess high ER binding affinity as well as a range of agonist and antagonist properties.[35–39] Because introduction of functional groups, alkyl or aryl, requires a lengthy synthetic sequence from the estradiol 3-methyl ether or 11-oxo-estradiol starting materials, relatively few research groups have exploited this route.[40,41] Because we developed expertise in preparing 11β-subsituted estradiols, selection of the 11β-(4-substituted-oxyphenyl) estradiol scaffold as the ER targeting component of our hybrid presented no significant problems.[42,43] Of equal importance for drug delivery, the 11β-(4-substituted-oxyphenyl) estradiols express high ER affinity and are potent antiestrogens.[44] As such, they would not elicit a proliferative effect in breast cancer cells.

The choice of mitomycin C as the second bioactive component, however, was based on its clinical use for the treatment of advanced breast cancer.[45–47] Although estradiol-mitomycin C conjugates had been explored previously without success, largely because of nonselectivity and toxicity, the two agents, antiestrogens plus mitomycin C, have been considered for combination chemotherapy.[48,49] Mitomycin C belongs to the class of compounds that require metabolic activation, *i.e.*, quinone reduction, prior to alkylation of the DNA.[50] It also displays a degree of sequence selectivity based upon its molecular structure.[51–54] It has also been demonstrated that structural modifications of the 7-amino group retain anticancer and DNA alkylating activity, suggesting that incorporation of a pendant group at that position would be tolerated[55–59] (Fig. 3). As a result, we selected as a preliminary target for synthesis which would incorporate all of the structural features we considered would be essential in the hybrid agent – the 11β-(4-alkoxyaryl) estradiol for anti-estrogenic effects, the 7-*N*-alkylamino mitomycin C for DNA binding, and the triethylene glycol linker to span the two functional groups (Fig. 4).

Because the optimal individual structural components for the hybrid agent were yet to be defined, our synthetic strategy needed to incorporate flexibility in the preparation of each unit as well as in the ultimate assembly process. For the preparation of the steroidal component we chose to use the Cu(I)-assisted 1,4-addition of aryl Grignard reagents to the steroidal 5,10-α-epoxide rather than 1,2-addition to the 11-oxo steroids. The latter route may be shorter but it is less effective for aryl than alkyl derivatives.[60,61] The incorporation of the protected phenolic group in the aryl moiety would subsequently permit the attachment of a variety of substituents *via* Williamson or Mitsunobu chemistry. Oligoethylene glycols provided several advantages as linkers. As bifunctional reagents, one can selectively manipulate each terminus. One end could be linked to the phenolic group using either Williamson (*via* tosylate) or Mitsunobu (*via* free alcohol) chemistry while the other could be converted to the requisite coupling group, in our case an azide. The oligoethylene glycol reagents are readily available and possess enhanced hydrophilicity which would compensate for the highly non-polar character of the steroidal component. Our strategy for the mitomycin C involved conversion to the more stable *N*-methylated aziridine derivative (porfiromycin), as well as conversion to the 7-methoxy intermediate which would undergo displacement by a variety of amines, in our case propargyl amine. Therefore, the two synthetically demanding components, the steroid and the mitomycin C and their analogs, if necessary, could be prepared separately and ultimately ligated using the Huisgen [3 + 2] cycloaddition reaction.[62,63] This procedure has the advantage that the individual

components, the alkyne and the azido group are both chemically and biologically stable, permitting the evaluation of each unit. Ultimately, the two components can be efficiently coupled to form the disubstituted triazole that is chemically and biologically stable. In this study we describe the preparation the target hybrid and initial evaluation as an ER ligand and cytotoxic agent in two breast cancer cell lines.

Results and discussion

The synthesis of the estradiol component began with the estra-5-(10), 9(11)-diene 3,17 diethylene ketal **2**, an intermediate that we had previously synthesized[64,65] (Scheme 1). Epoxidation using hydrogen peroxide and hexafluoroacetone under basic conditions gave the 5,10-α-epoxide **3** and the 5,10-β-isomer **4** in a 76% isolated yield (3: 1 ratio). Cu(I)-catalyzed 1,4- addition of 4-(trimethylsilyloxy) phenylmagnesium bromide followed by dehydration and deketalization provided the 11β-(4-hydroxyphenyl)-estra-4,9-diene-3,17-dione **5** in a 90% yield for the three steps.[66–68] It should be noted that under these conditions, the α-4-hydroxyphenyl steroid generated from the β-isomer **4** undergoes isomerization to the more stable 11β-product **5**. Stereochemistry is clearly established by the upfield shift of the C-18 methyl group. Tosylation of triethylene glycol proceeded in high yield to give the ditosylate derivative **6**[69] which underwent Williamson ether synthesis with the 4-hydroxyphenyl steroid **5**. Subsequent displacement of the terminal tosylate group with sodium azide in ethanol gave the azido-triethylene glycoloxyphenyl derivative **7** in 10% isolated yield (two steps).[70,71] Aromatization of the estradienedione with acetic anhydride-acetyl bromide, acetate saponification and stereoselective borohydride reduction of the 17-ketone gave the 11β-(4-azido-triethylene glycoloxyphenyl) estradiol intermediate **8** in 56% isolated yield for the three steps.

Preparation of the mitomycin C component began with *N*-methylation of mitomycin C **9** with methyl iodide to give porfiromycin **10** in 61% yield[72,73] (Scheme 2). Subsequent hydrolysis of the quinone amine to the hydroxy derivative, followed by methylation with diazomethane gave the intermediate methyl ether. The intermediate underwent the displacement reaction with propargylamine to give the desired 7-(*N*-propargyl)-porfiromycin **11** in 55% yield for the three steps. Modifications of the amination step and combining the last three steps into a single pot method significantly improved the overall yield.

Ligation was accomplished using the Huisgen [3 + 2]-cycloaddition reaction between the terminal alkynyl and azido groups[73–76] (Scheme 3). A slight modification of the conventional method was used, resulting in an isolated yield of 81% for the antiestrogen-mitomycin C hybrid **1**, which was characterized by ^{1}H-,^{13}C-NMR and HRMS. Analysis indicated a single cycloaddition product in which the two coupling moieties were 1,4- to one another. None of the 1,5-isomer was detected by NMR.

Initial biological evaluation of hybrid **1** as an ERα-LBD targeting group used competitive binding assays with estradiol.[77–79] The hybrid compound **1** competitively displaced estradiol from ERα-LBD with relative binding affinity (RBA) value of 7 ± 1%, compared to estradiol 100%. The intermediate azido-estradiol derivative **8**, in the same assay system had an RBA = 26 ± 9%, indicating that the presence of the additional mitomycin-C group at the terminus of the linker did not have an adverse effect on ER-LBD binding. Antagonist activity for hybrid **1** was determined using the induction of alkaline phosphatase in Ishikawa cells.[80] The hybrid **1** did not stimulate the production of alkaline phosphatase at any dose level, however, the compound potently blocked the stimulation caused by 1 nM estradiol. This is a typical antiestrogenic (antagonist) response. The antiestrogenic effect was similar to that shown by the azido-estradiol derivative **8** which was a potent antagonist (K_i = 2.4 ± 0.6

nM). Again, the results demonstrated that the presence of the mitomycin C moiety did not interfere with the receptor binding or the inhibition of the transcriptional response. The *N*-propargyl-porfiromycin **11**, as expected, exhibited very low binding affinity for ERα-LBD (RBA = 0.3 ± 0.2%). Compound **11** did exhibit a low level of inhibition in the alkaline phosphatase assay (K_i = 40 ± 4 nM) suggesting that at higher doses of the antibiotic analog, that there may be some cytotoxic effects rather than anti-estrogenic action. Therefore, one of the key criteria for a hybrid drug was achieved, namely the presence of the mitomycin component did not compromise the affinity or efficacy of the ER-binding group.

The second series of biological assays evaluated the effect of the mitomycin moiety on cellular proliferation of MCF-7 (ER-positive) and MDA-MB-231 (ER-negative) breast cancer cell lines.[32,33] Control studies using mitomycin C indicated that it is marginally more potent in the MCF-7 *vs.* the MDA-231 cell lines. Incorporation of the anti-estrogen moiety linked through the triethylene glycol to the mitomycin C in hybrid **1** had little additional effect compared to mitomycin C on the anti-proliferative response in the MCF-7 or MDA-MB-231 cells. The hybrid was neither more potent than mitomycin C (IC50 in the low μM range) nor more selective for the ER(+)-MCF-7 cells. The second criterion was also achieved as the presence of the anti-estrogenic moiety in hybrid **1**, did not interfere with the biological activity of the cytotoxin. However, the last objective was not entirely successful as the hybrid did not appear to achieve potentiation of the biological response or enhancement of the cell selectivity *via* a ER-mediated effect. [Graphs of the cytotoxicity assays are shown in the ESI†]

An examination of the crystal structures of anti-estrogens bound to the ERα-LBD suggests that the substituent at the 11β-position of estradiol is functionally equivalent to the dialkyl-aminoalkylphenyl group of the triarylethylene anti-estrogens (hydroxytamoxifen and raloxifene). Therefore, the second oxygen of the linker occupies the same site as the amino group in exerting its effect on helix-12 and specifically on aspartic acid-351.[28,29,81] Essentially all atoms beyond that point are external to the receptor surface and should be accessible for other interactions with either solvent or other proteins. Further interactions with the surface of the estrogen receptor would be significant only if those interactions provide complementary binding to the protein, and in this case, they do not appear to be significant. As shown in this study, the addition of substituents beyond the second oxygen of the triethylene glycoxy group does not dramatically reduce binding (indicating low steric/electronic demands) nor does it enhance binding (evidence of absence of complementary interactions). Therefore, the triethylene glycoxy group successfully provided a means for tethering a second molecular component to the steroidal scaffold without compromising ER binding. Regarding the mitomycin-C component, binding studies suggest that interactions of the electrophilic methoxylated carbon and carbamoylated carbon with guanyl residues of DNA (mono or bis alkylation) occur on one face of the MMC molecule. The amino component associated with the benzoquinone moiety remains solvent accessible and therefore should not provide additional interactions with the DNA.[51–54,82] The data indicate that there are relatively few differences in biological activity between mitomycin C and the conjugated derivative **1** in their ability to induce an anti-proliferative response in MCF-7 and MDA-MB-231 cell lines. Therefore, as with the ER binding, extended triethylene glycoxy moiety therefore did not participate in the alkylation events responsible for the anti-proliferative effect. The apparent absence of synergy between the two groups suggests that the individual components do not recruit the complementary protein/DNA targets and therefore are not providing simultaneous binding.

†Electronic supplementary information (ESI) available: Biological assays and graphs/figures, and spectral data for intermediates and final compound. See DOI: 10.1039/c2ob25902h

Conclusions

In summary, we have described the synthesis of a steroidal anti-estrogen conjugated to the therapeutic agent, mitomycin C, hybrid **1**, in which both components retained their full biological properties. The individual targeting and cytotoxic components were synthesized separately and ligated by means of a heterobifunctional triethylene glycol derivative. Binding and functional assays indicated that the antiestrogenic component, as well as the intact hybrid **1**, retained high affinity for ERα-LBD and possessed potent antiestrogenic activity in ER-responsive cells. Cell proliferation assays with two breast cancer cell lines indicated that the mitomycin-C component, as well as the intact hybrid **1**, retained potent cytotoxic effects. The results indicated that although hybrid **1** was not selective for ER(+)-MCF-7 cells as compared to ER(−)-MDA-MB-231 cells, the individual components within the intact hybrid retained their biological properties. Although the objectives of maintaining ER affinity and cytotoxic activity were achieved, synergy between the two components was not established. The absence of cellular selectivity and lack of enhancement of toxicity suggest that additional factors in hybrid design, such as modification of the linker or choice of therapeutic group may be required. The synthetic steps needed to adapt the mitomycin C component for conjugation make it less than optimal for such studies, particularly for evaluation the effects of linker length and conformational flexibility. Nevertheless, this seminal work provides the basis for defining the roles of the steroid component, the linker properties and the therapeutic component. Studies related to those factors are in progress and will be described in subsequent publications.

Experimental section

General information

All reagents and solvents were purchased from Aldrich or Fisher Scientific. THF and toluene were distilled from sodium/benzophenone. Reactions were monitored by TLC, performed on 0.2 mm silica gel plastic backed sheets containing F-254 indicator. Visualization on TLC was achieved using UV light, iodine vapor and/or phosphomolybdic acid reagent. Column chromatography was performed with 32–63 μm silica gel packing. Melting points were determined using an Electrotherm capillary melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded with a Varian Mercury 300 MHz, a Varian 500 MHz or a Bruker 700 MHz spectrometer. DEPT and ^{13}C experiments were performed on a Varian Mercury instrument at 75 MHz. NMR spectra chemical shifts are reported in parts per million downfield from TMS and referenced either to TMS, or internal standard for chloroform-d, acetone-d_6, methanol-d_4, and THF-d_8 solvent peak. Coupling constants are reported in hertz. High-resolution mass spectra were obtained by electron impact (EI) or fast atom bombardment (FAB) on MStation JMS700 (JEOL) by University of Massachusetts Amherst, Mass Spectrometry Center using sodium iodide as an internal standard.

3,3,17,17-Diethylenedioxy-5,10-α-epoxy-estr-9(11)-ene 3 and 3,3,17,17-diethylenedioxy-5,10-β-epoxy-estr-9(11)-ene 4—Estra-5-(10),9(11)-diene 3,17 diethylene ketal **2** (1 g, 2.79 mmol), hexafluoroacetone trihydrate (0.04 mL, 0.279 mmol), pyridine (0.005 mL), 50% hydrogen peroxide (0.3 mL, 4.74 mmol, *ca.* 18 M) and dichloromethane (10 mL) were charged into a round bottom flask at room temperature under argon atmosphere. The mixture was stirred for 20 h at room temperature (TLC monitoring: ethyl acetate: hexanes, 3: 7). After reductive workup (aqueous sodium thiosulfate solution, 2 g in 50 mL of water), the organic layer was washed with water (25 mL × 2), extracted with dichloromethane (30 mL × 2). The organic layer was dried over magnesium sulfate and concentrated under reduced pressure to give a mixture of **3** and **4** (ratio of $\alpha:\beta \approx 3:1$, ^{1}H NMR). The mixture was purified from other components by chromatographic separation on

a silica gel column (25 g, ethyl acetate: hexanes, 1: 4). The combined fractions containing the products were concentrated under reduced pressure.

Yield = 0.81 g, 76%. R_f = 0.4 (ethyl acetate–hexanes 5: 1). ^{1}H NMR (300 MHz, $CDCl_3$, δ 6.05 (m, 1H, **3**), 5.86 (m, 1H, **4**), 0.88 (s, 3H, **3**), 0.89 (s, 3H, **4**). ^{13}C NMR (75 MHz, $CDCl_3$) δ 191.1, 164.9, 132.2, 114.5, 55.8.

11β-(4-Hydroxyphenyl)estra-4,9-diene-3,17-dione 5 (one pot reaction)— Copper(I) chloride (35 mg, 0.35 mmol) was added at room temperature to a *ca.* 1 M solution of 4-(trimethylsilyloxy)-phenyl magnesium bromide in THF (10 mL) under argon atmosphere. solution of the mixture of **3** and **4** (ratio ≈ 3: 1) (760 mg, 2.03 mmol) in THF (10 mL) was added during ~30 min at room temperature (exothermic). The mixture was then stirred for 1 h at room temperature (TLC monitoring: ethyl acetate: hexanes = 3: 7). When the reaction was complete, the solution was poured into a biphasic mixture of aqueous ammonium chloride (15 equiv, 6 mL) and methylene chloride (8 mL) at 10–15 °C. The organic layer was separated, washed with water (20 mL × 2), concentrated the total volume to ~5 mL, and diluted with methylene chloride (5 mL). Aqueous hydrochloric acid (6 equiv, 0.47 g in 2.6 mL of water) was added at 0–5 °C. This biphasic mixture was stirred for 2 h at 0–5 °C (pH < 1, pH paper) and then diluted with water (20 mL). The organic phase was separated, washed with water (20 mL × 2) and carefully neutralized to pH ≈ 8 (10% sodium bicarbonate, ~1.5 mL, pH ~ 7–8). The neutralized solution was washed with water (30 mL × 2). The combined organic layer was dried over magnesium sulfate. Compound **5** (0.66 g, 90%) was isolated from a silica gel flash column chromatography (ethyl acetate–hexanes, 3: 7).

Yield = 0.66 g, 90%. ^{1}H NMR (300 MHz, $CDCl_3$, δ 6.75 and 7.07 (AA′BB′, 4H), 5.81 (s, 1H), 4.35 (d, J = 7.2 Hz, 1H), 4.01 (t, J = 6 Hz, 2H), 2.85 (t, J = 6 Hz, 2H), 2.63 (q, J = 7 Hz, 4H), 0.56 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$,): δ 219.3, 200.0, 156.6, 154.3, 145.6, 135.8, 130.2, 128.2, 123.5, 115.9, 50.8, 47.9, 39.8, 38.2, 38.0, 36.9, 35.6, 31.1, 26.9, 26.0, 22.1, 14.6.

Triethylene glycol ditosylate 6—To a solution of triethylene glycol (3 g, 0.02 mol) in diethyl ether (40 mL), triethylamine (7.8 g, 0.04 mol) was added at room temperature, followed by addition of *p*-toluenesulfonyl chloride (7.8 g, 0.04 mol) under argon atmosphere. The mixture was stirred at room temperature for 18 h (TLC monitoring, ethyl acetate: hexanes, 1: 4). The organic solvents were removed under reduced pressure. The residue was dissolved in methylene chloride (20 mL), washed with sodium bicarbonate (20 mL, saturated), water (20 mL × 2), brine (30 mL), dried over magnesium sulfate and concentrated under vacuum. Colorless crystals of triethylene glycol ditosylate were obtained from ethyl acetate.

Yield = 5.8 g, 64%. mp: 75–77 °C. R_f = 0.5 (ethyl acetate: hexanes, 1: 4). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.78 and 7.34 (AA′BB′, 8H), 4.12 (d, 4H), 3.65 (t, 4H), 3.53 (s, 4H), 2.45 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$, δ 145.1, 133.3, 130.1, 128.2, 70.9, 69.4, 69.0, 21.8.

11β-(4-((2-(2-(2-Azidoethoxy)ethoxy)ethoxy)phenyl)-estra-4,9-diene-3,17-dione 7—To a solution of **5** (150 mg, 0.41 mmol) in acetonitrile (20 mL), potassium carbonate (230 mg, 1.64 mmol) was added, and the mixture was heated at ~90 °C for 30 min. The bis α,ώ-toluenesulfonyl triethylene glycol **6** (380 mg, 0.82 mmol) was charged and stirred at ~90 °C for 18 h (TLC monitoring: ethyl acetate: hexanes, 1: 1). The mixture was allowed to cool to room temperature, and diluted with a mixture of methylene chloride (20 mL) and cold water (~0 °C, 20 mL). After stirring for 30 min, the aqueous layer was extracted with methylene chloride (30 mL × 2). The combined organic layers were washed with water (20

mL), brine (20 mL), dried over magnesium sulfate and concentrated to dryness under vacuum. The intermediate (50 mg, 19% yield) was isolated through a silica gel column (50 g) chromatography (ethyl acetate: hexanes, 2: 3).

Yield = 50 mg, 19%. R_f = 0.4 (ethyl acetate: hexanes = 1: 1). ^{1}H NMR (300 MHz, $CDCl_3$,): δ 7.79 and 7.33 (AA′BB′, J = 7.8 Hz, J = 8.1 Hz, 4H), 7.33 and 6.83 (AA′BB′, J = 7.1, J = 8.7 Hz, 4H), 5.79 (s, 1H), 4.38 (d, J = 6.6 Hz, 1H), 4.13 (t, J = 10.2 Hz, 2H), 4.08 (t, J = 4.8 Hz, 2H), 3.82 (t, J = 3.3 Hz, 2H), 3.69 (t, J = 3.3 Hz, 2H), 3.65 (m, 2H), 3.62 (m, 2H), 0.55 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$,): δ 219.0, 199.4, 157.2, 156.1, 145.1, 145.0, 136.3, 133.3, 130.3, 130.0, 128.2, 128.1, 123.6, 115.0, 71.0, 70.9, 70.0, 69.4, 69.0, 67.6, 57.4, 57.1, 50.9, 47.9, 39.8, 38.2, 37.0, 35.6, 27.0, 26.1, 22.1, 21.8, 14.6.

To a solution of the intermediate (59 mg, 0.1 mmol) in 95% ethanol (5 mL), sodium azide (13 mg, 0.2 mmol) was added. The mixture was stirred at ~90 °C for 18 h, and then evaporated to dryness under reduced vacuum. Compound **7** was isolated using silica gel column chromatography (ethyl acetate: hexanes, 2: 3).

Yield = 35 mg, 67%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.08 and 6.84 (AA′BB′, J = 8.7 Hz, J = 9.0 Hz, 4H), 5.80 (s, 1H, C_4–H), 4.38 (d, J = 6.6 Hz, 2H, $C_{11\alpha}$–H), 4.10 (t, J = 4.8 Hz, 2H), 3.86 (t, J = 5.4 Hz, 2H), 3.74 (m, 2H), 3.68 (m, 2H), 3.39 (t, J = 5.1 Hz, 2H), 0.55 (s, 3H, C_{18}–CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 219.0, 199.4, 157.2, 156.1, 145.1, 136.3, 130.3, 128.5, 123.6, 114.9, 71.1, 70.9, 70.3, 67.6, 50.9, 50.9, 47.9, 39.8, 38.2, 38.0, 37.0, 35.6, 31.1, 27.0, 26.1, 22.1, 14.6.

11β-(4-((2-(2-(2-Azidoethoxy)ethoxy)ethoxy)phenyl)estradiol 8—To a solution of **7** (284 mg, 0.55 mmol) in methylene chloride (20 mL), acetic anhydride (0.05 mL, d = 1.080 g mL^{-1}, 0.55 mmol) was added slowly under argon atmosphere at room temperature, followed by acetyl bromide (169 mg, 1.375 mmol). The mixture was stirred at room temperature for 5 h (TLC monitoring: ethyl acetate: hexanes, 1: 1) and then carefully poured into an aqueous solution of sodium bicarbonate (50 mg in 10 mL ice-water). After stirring for 15 h at room temperature, the mixture was diluted with methylene chloride (50 mL). The organic layer was separated, washed with sodium hydroxide (1N, 25 mL × 2), water (25 mL × 3, to pH ≈ 7), dried over magnesium sulfate, and concentrated to dryness under reduced vacuum. The crude product (310 mg, 0.55 mmol) was dissolved in methanol (20 mL), and cooled to ~0 °C in an ice-water bath. Potassium hydroxide (62 mg, 1.10 mmol) was added under argon atmosphere. The mixture was stirred at 0 °C for 1.5 h (TLC monitoring: ethyl acetate: hexanes, 1: 1). Without further work up and purification, sodium borohydride (50 mg, 1.32 mmol) was added, and stirred for additional 2.5 h at 0 °C (TLC monitoring: ethyl acetate: hexanes, 1: 1). After removal of the solvent under reduced pressure, the residue was diluted with methylene chloride (50 mL) and ice-water (50 mL). The aqueous layer was extracted with methylene chloride (25 mL × 2). Combined organic layers were washed with water (25 mL), dried over magnesium sulfate, and concentrated to dryness under reduced vacuum. The purification step was performed using column chromatography (silica gel-ethyl acetate: hexanes, 1: 1). Compound **8** was collected from combined fractions.

Yield = 148 mg, 56% (one pot reaction, three-steps combined). ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.63 (d, J = 4.8 Hz, 2H), 6.48 (d, J = 5.4 Hz, 1H), 6.29 (d, J = 5.1 Hz, 2H), 6.25 (d, J = 1.5 Hz, 1H), 6.08 (dd, J = 5 Hz, J = 1. 5 Hz, 1H), 4.00 (t, J = 2.7 Hz, 2H), 3.92 (t, 1H, H-11α), 3.79 (t, J = 3.3 Hz, 1H), 3.70 (m, 3H), 3.65 (m, 4H), 1.77 (t, J = 3 Hz, 1H), 2.96 (t, J = 9 Hz, 1H), 2.84 (s, 1H), 2.80 (d, J = 3 Hz, 1H), 2.49 (dd, J = 1.2 Hz, J = 7.7 Hz, 1H), 1.00–2.30 (m, 10H), 0.32 (s, 3H, C_{18}–CH_3) ^{13}C NMR (75 MHz, $CDCl_3$, δ155.9, 153.3, 137.9, 136.2, 130.8, 127.9, 115.5, 113.8, 113.5, 82.8, 71.0, 70.9, 70.3, 70.1, 67.3, 52.0, 50.9,

47.6, 45.7, 43.8, 38.5, 35.6, 30.7, 30.4, 28.2, 23.4, 13.1. HRMS calcd for $C_{30}H_{39}N_3O_5$ m/z 521.2890, found m/z 521.2840.

***N*-Methyl mitomycin C 10 (porfiromycin)—**To a reaction flask, mitomycin C **9** (200 mg, 0.6 mmol), K_2CO_3 (0.83 g, 6 mmol), and anhydrous acetone (30 mL) were charged at room temperature under argon; then methyl iodide (0.75 mL, 1.2 mmol) was added to the mixture. The mixture was heated at reflux for 16 h (TLC monitoring: methanol: dichloromethane = 1: 9). When the reaction was complete, the mixture was cooled to room temperature, filtered, and washed with a small volume of anhydrous acetone. The filtrate was concentrated to dryness (470 mg). The crude product was purified using column chromatography (silica gel, 10 g, methanol: dichloromethane = 10: 90). The combined fractions containing the product were concentrated under reduced pressure to give **10**.

Yield = 128 mg, 61%. R_f = 0.4 (methanol: dichloromethane = 10: 90). ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.43 (s, 1H), 5.01 (s, 2H), 4.63 (dd, J = 6 Hz, 1H), 4.26 (q, 2H), 3.51 (dd, J = 6 Hz, 1H), 3.44 (dd, J = 5 Hz, 1H), 3.21 (s, 3H), 4.63 (dd, J = 6 Hz, 1H), 2.20 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$,) δ 179.0, 176.0, 157.0, 155.7, 148.5, 109.6, 106.1, 103.7, 62.5, 49.8, 46.2, 43.3, 43.0, 42.7, 32.7, 9.8.

Preparation of 7-*N*-propargyl-*N*-methylmitomycin C 11 without purification of intermediates

Methylation: To a reaction flask, mitomycin C **9** (200 mg, 0.6 mmol), potassium carbonate (380 mg, 6 mmol, anhydrous, ~120 °C for 2 h), and acetone (30 mL, anhydrous) were charged under argon, then methyl iodide (1.7 g, 0.75 mL d = 2.275 g mL^{-1}, 12 mmol) was added at room temperature under argon. The mixture was heated to reflux for 18 h (TLC monitoring: methanol: dichloromethane = 1: 9, R_f = 0.4, reaction mixture; R_f = 0.1, mitomycin C). When the reaction went to completion, the mixture was allowed to cool to room temperature, then filtered, washed with small amount of anhydrous acetone. The filtrate was evaporated to dryness. Weight of the intermediate compound was 341 mg. Without further purification, the material was used for the next step.

Hydrolysis: A solution of the crude product (341 mg) in 0.1 N solution of sodium hydroxide (30 mL) was stirred for 4 h at room temperature (TLC monitoring: methanol: dichloromethane = 1: 9). One new spot (R_f = 0.3) was formed (R_f = 0.4, starting material) by TLC analysis.

Acidification: The resultant mixture was allowed to cool to ~0 °C in an ice bath; then 1 N sulfuric acid (~2.6 mL) was added dropwise to pH ~ 4. The acidified mixture was extracted with ethyl acetate (20 mL × 3). The organic layer was separated, dried over magnesium sulfate, and concentrated to dryness. Weight of the crude intermediate was 274 mg.

Methylation: The acidified crude intermediate in anhydrous diethyl ether (15 mL) was allowed to cool to −10 °C to −15 °C (ice-sodium chloride). Diazomethane (gas) was passed into the pre-cooled solution for 15 min, then the mixture was stirred for 4 h at ~0 °C (TLC monitoring: methanol: dichloromethane = 1: 9). When the reaction was complete, the mixture was allowed to warm to room temperature and concentrated to dryness under reduced vacuum in a fume hood. During the drying process, the product was treated with anhydrous methanol (10 mL × 3). Weight of the crude product was 218 mg.

7-Propargylaminomitosane formation: To a solution of the intermediate (218 mg) in anhydrous methanol (12 mL), propargylamine (0.19 mL, 1 eq) was added at room temperature under argon. The mixture was stirred at room temperature for 2 h (TLC

monitoring: methanol: dichloromethane = 1: 9). The mixture was evaporated to dryness under a reduced vacuum, treated with anhydrous methanol (10 mL × 3) during the evaporation process. The crude product was isolated (298 mg) and subsequently purified using column chromatography (silica gel, 10 g; methanol: dichloromethane = 15: 85). The fractions containing the product were combined and concentrated under reduced pressure to give the product **11**.

Yield = 127 mg, 55%. (five-steps).

HRMS calcd for $C_{19}H_{22}N_4O_5$ m/z 386.1590, found $m + 1/z$ 387.1662.

Steroidal antiestrogen-mitomycin C hybrid 1—To a reaction flask, compound **8** (7.5 mg, 0.0195 mmole) and compound **11** (10.1 mg, 0.0194 mmole) were suspended in a 1: 1 mixture of water and *t*-butyl alcohol (0.6 mL) (0.3 mL of water, 0.3 mL of *t*-butyl alcohol) at room temperature. Copper(II) sulfate pentahydrate (0.01 eq, 1.9×10^{-4} mmol, 3 uL of freshly prepared 0.065 M solution in water) was added, followed by sodium ascorbate (0.05 eq, 9.7×10^{-4} mmol, 4 uL of freshly prepared 0.25 M solution in water). The heterogeneous mixture was stirred at room temperature for 18 h (TLC monitoring: methanol: dichloromethane = 1: 9). To the mixture, ice (~1 g) was added and stirred for 5 min, then extracted with dichloromethane (5 mL × 3). The organic layer was separated, dried over magnesium sulfate, and concentrated under reduced vacuum. The crude product was purified through a silica gel column (10 g) chromatography (methanol: dichloromethane = 1: 9). The combined fractions containing the product were concentrated under reduced pressure to give a white solid **1**.

Yield = 14.4 mg, 82%. ^{1}H NMR ($CDCl_3$, δ 7.67 (s, 1H), 6.94 (d, J = 8.4 Hz, 2H) 6.74 (d, J = 8.7 Hz, 1H), 6.61 (d, J = 9 Hz, 2H), 6.55 (d, J = 2.4 Hz, 1H), 6.51 (d, J = 6.6 Hz, 1H), 6.33 (dd, J = 2.4 Hz, J = 8.3 Hz, 1H), 6.29 (s, br, 1H), 4.80 (br, 2H), 4.69 (q, 3H), 4.63 (d, J = 4.5 Hz, 1H), 4.47 (t, J = 4.5 Hz, 2H), 4.36 (t, J = 10.5 Hz, 1H), 4.25 (d, J = 13.2 Hz, 1H), 4.01 (t, J = 4.2 Hz, 1H), 3.90 (t, J = 5.4 Hz 1H), 3.81 (t, J = 5.1 Hz, 1H), 3.75 (t, J = 4.8 Hz, 1H), 3.67 (d, J = 8.1 Hz, 1H), 3.63 (d, J = 3.6 Hz, 1H), 3.58 (d, J = 3.6 Hz, 1H), 3.53 (d, J = 4.5 Hz, 1H), 3.45 (dd, J = 1.8 Hz, J = 13.2 Hz, 1H), 3.16 (s, 3H, OCH_3), 2.94 (d, J = 12.9 Hz, 1H), 2.81 (d, J = 14.7 Hz, 2H), 2.48 (d, J = 13.5 Hz, 1H), 2.0–1.0 (see COSY, appendix), 2.25 (s, 3H, CH_3), 1.92 (s, 3H, CH_3), 0.31 (s, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$, Appendix: Fig. 90, 91, 92) δ 179.5, 176.3, 156.8, 155.8, 155.2, 153.6, 147.0, 145.0, 137.9, 136.5, 130.9, 130.4, 127.8, 123.3, 115.6, 113.7, 113.5, 110.2, 106.2, 105.0, 82.8, 70.8, 70.0, 69.5, 67.5, 62.8, 52.0, 50.6, 49.9, 49.8, 47.5, 46.4, 45.7, 43.9, 43.3, 43.0, 42.8, 41.0, 38.6, 35.7, 30.6, 30.4, 28.2, 13.1, 9.9. HRMS calcd. for $C_{49}H_{61}N_7O_{10}$ m/z 907.4480, found m/z 907.4445; 1D NOESY; COSY spectra are shown in ESI[†]

Biological assays

Competitive binding to human LBD-ER*α* and human LBD-ER*α* ER*β*—Binding affinities of the steroidal derivatives relative to E_2 were performed in incubations with the LBD of ERα in lysates of *Escherichia coli* in which the LBD of human ERα (M_{250}–V_{595}). The assay was performed overnight in phosphate buffered saline + 1 mM EDTA at room temperature. The competition for binding of [^{3}H]E_2 to the LBD of the E_2-derivatives in comparison to E_2, relative binding affinity (RBA) was determined over a range of concentrations from 10^{-12} to 10^{-6} M. After incubation, the media is aspirated, the plates are washed 3 times and the receptor bound radioactivity absorbed to the plates are extracted with methanol and counted. The results, as RBAs compared to E_2, of all receptor studies are from at least 3 separate experiments performed in duplicate. RBAs represent the ratio of the EC_{50} of E_2 to that of the steroid analog × 100 using the curve fitting program Prism to determine the EC_{50}.

Estrogenic potency in Ishikawa cells—The potency of the anti-estrogen-mitomycin C hybrid **1** was determined in an estrogen bioassay, the induction of alkaline phosphatase in human endometrial adenocarcinoma cells (Ishikawa) grown in 96-well microtiter plates. The cells are grown in phenol red free medium with estrogen depleted (charcoal stripped) bovine serum in the presence or absence of varying amounts of the steroids, across a dose range of at least 6 orders of magnitude. After 3 days, the cells are washed, frozen and thawed, and then incubated with 5 mM *p*-nitrophenyl phosphate, a chromogenic substrate for the alkaline phosphatase enzyme, at pH 9.8. To ensure linear enzymatic analysis, the plates are monitored kinetically for the production of *p*-nitrophenol at 405 nm. For antagonists, the effect (K_i) of each compound tested at a range of 10^{-6} M to 10^{-12} M was measured for the inhibition of the action of 10^{-9} M E_2 (EC_{50}–0.2 nM). Each compound was analyzed in at least 3 separate experiments performed in duplicate. The K_i and RSA (RSA = ratio of 1/EC_{50} of the steroid analog to that of E_2 × 100) were determined using the curve fitting program Prism.

Cell proliferation assays—Measurement of the toxicity of **1** toward MCF-7 (ER+) and MDA-MB231 (ER−) cell lines. Toxicity experiments were performed on breast cancer cell lines MCF-7 and MDA-MB231 obtained from the American Type Culture Collection. Both cell lines were grown in Minimal Essential Media (MEM) containing phenol red (GIBCO BRL, Rockville, MD) supplemented with 10% fetal bovine serum (Biowhittaker, Walkersville, MD), 2 mM glutamine and 1 mM sodium pyruvate in a 5% CO2/95% air atmosphere. Cells were seeded at 2 × 105 in 6-well plates and, 48 h later, exposed to test compounds in growth medium for 2 h. After treatment, cells were washed once and incubated in fresh growth medium for 24 h, after which they were trypsinized and replated at 103 cells per 6 cm dish. After 11 days, colonies were fixed with acetic acid/methanol and stained with GIEMSA stain and counted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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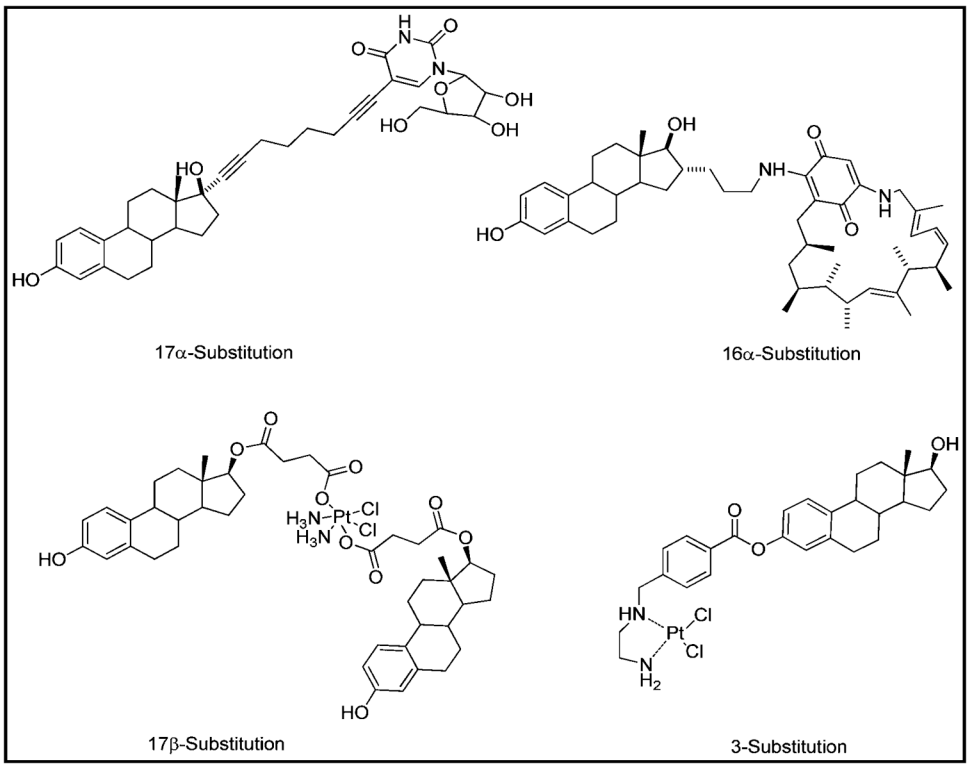


Fig. 1.
Representative estradiol-based ER-targeted hybrids substituted at 3, 16α, 17α or 17β position.[10,13,19]

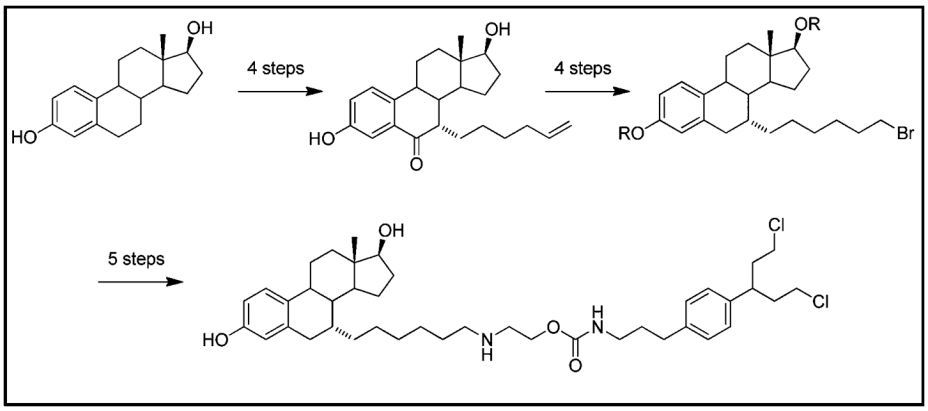


Fig. 2.
Preparation of a 7α-substituted estradiol-chlorambucil hybrid.[32]

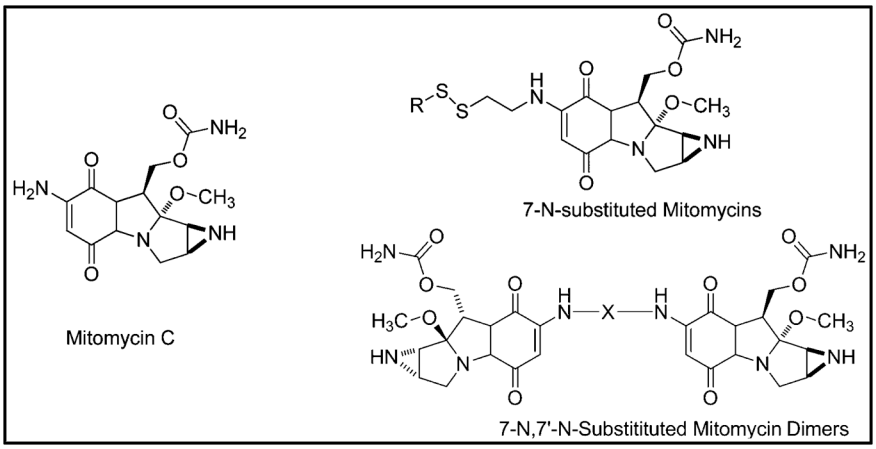


Fig. 3.
Mitomycin C and structural modifications that retain anti-tumor activity.[50,51]

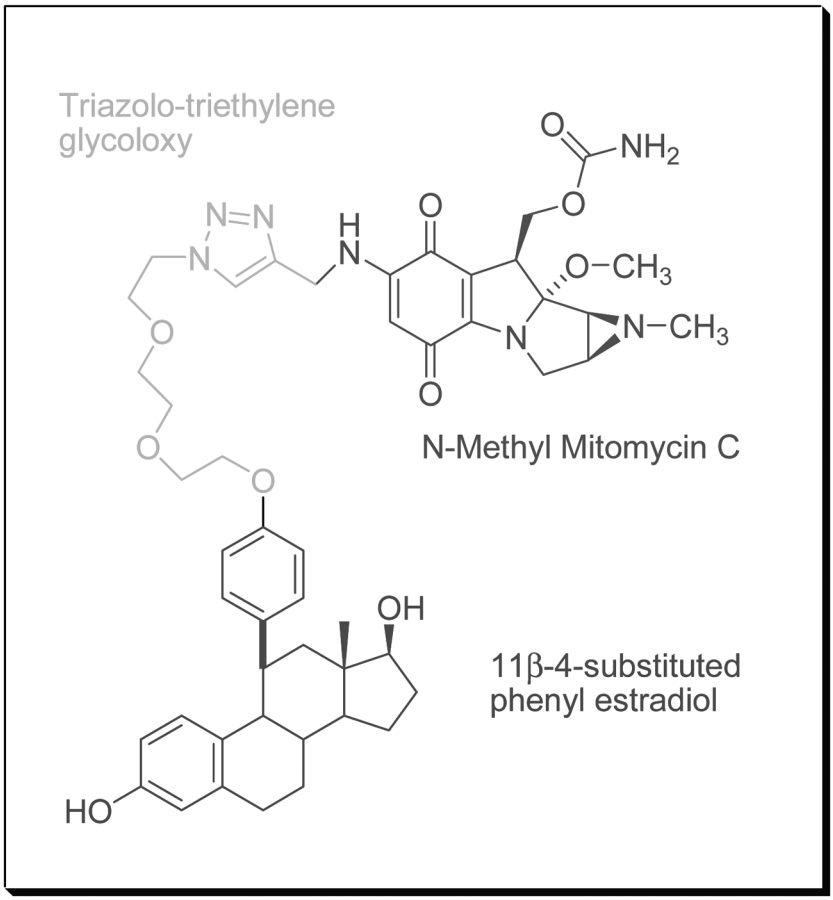
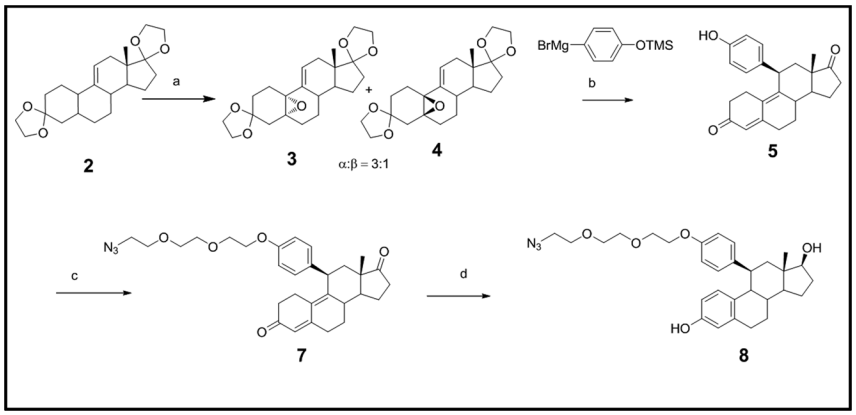


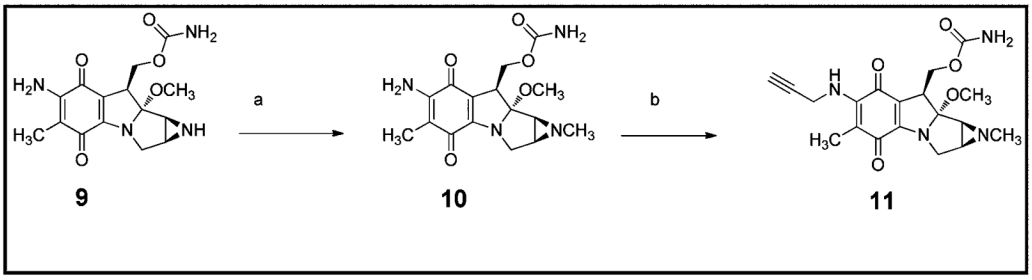
Fig. 4.

7-*N*-Propargyl-*N*′-methyl mitomycin C (*N*-propargyl porfirimycin) linked to 11β-(4-alkoxyphenyl) estradiol antiestrogen through an triethylene glycol linker to give target hybrid **1**. Linkage avoids detrimental interactions with target proteins (estrogen receptor) or DNA intercalation sites.



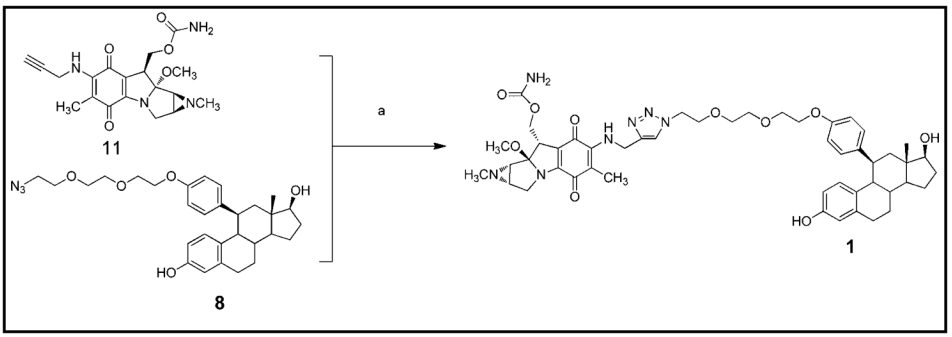
Scheme 1.

Preparation of the 11β-[4-(ώ-azido-triethyleneglycoloxy)-phenyl]estradiol. Reagents and conditions (a) CF_3COCF_3, H_2O_2, pyridine, rt; (b) [1] Cu(I), [2] HCl; (c) [1] **6**, K_2CO_3, CH_3CN, reflux, [2] NaN_3, ethanol, reflux; (d) [1] AcBr, Ac_2O, CH_2Cl_2, rt, [2] KOH, methanol, 0–5 °C, [3] $NaBH_4$, KOH, methanol.



Scheme 2.

Preparation of the mitomycin C component. Reagents and conditions (a) CH_3I, K_2CO_3, acetone, reflux; (b) [1] 0.1 N NaOH, rt, [2] 1 N H_2SO_4, 0 °C, [3] CH_2N_2, ether, [4] propargylamine, methanol, rt.



Scheme 3.

Ligation of mitomycin C and estradiol components to form target hybrid **1**. Reagents and conditions (a) 0.02 eq $CuSO_4 \cdot 5H_2O$, 0.10 eq sodium ascorbate, H_2O-*tert*-BuOH (1: 1), rt, 20 h.