Synthesis Studies and the Evaluation of C_6 Raloxifene Derivatives

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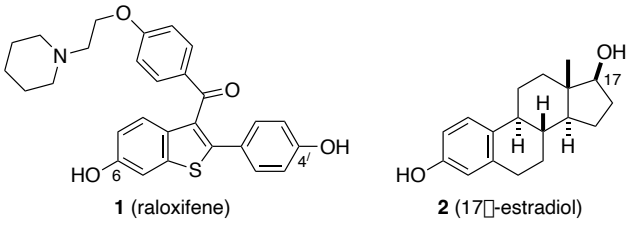
ABSTRACT: Methodology is described for synthesis of C_6 derivatives of raloxifene, a prescribed drug for the treatment and prevention of osteoporosis. Studies explore the incorporation of electron-withdrawing substituents at C_6 of the benzothiophene core. Efficient processes are also examined to introduce hydrogen bond donor and acceptor functionality. Raloxifene derivatives are evaluated with *in vitro* testing to determine estrogen receptor (ER) binding affinity and gene expression in MC3T3 cells.

KEYWORDS: *raloxifene derivatives, synthesis, methodology, estrogen binding affinity, bone properties*

Raloxifene (**1**) is a selective estrogen receptor modulator (SERM) first developed by the Lilly Research Laboratories.[1,2,3] As the FDA-approved drug, Evista®, raloxifene is used to reduce osteoporotic fractures by decreasing bone resorption and increasing bone mineral density (BMD).[4,5] However, the efficacy of **1** is far greater than what is predicted based solely on its effect on BMD.[6,7,8] Since raloxifene has high affinity for binding to the estrogen receptor (ER), it exhibits prominent side effects associated with hormone therapy.[9,10] As a result, safeguard limitations have been placed on the use of this prescribed medicine. In fact, several laboratories have presented crystallographic studies of human estrogen receptor with bound SERM derivatives[11,12,13] to identify favorable interactions for treatment of breast cancer. Recent studies have indicated that raloxifene may induce a cell-independent mechanism that leads to improved collagen quality. Collagen plays a key role in establishing the material and mechanical properties of bone that are essential to fracture resistance.[14,15] Studies have shown that the 6-hydroxy and, to a lesser extent, the 4′-hydroxy substituents of **1** are important for ER binding. These groups appear to mimic the 3- and 17β-hydroxy substituents of 17β-estradiol (17βe) (**2**). Thus, our studies have examined alterations of C_6 functionality in an effort to minimize the hormonal side effects while maintaining positive outcomes for bone strength.[16]



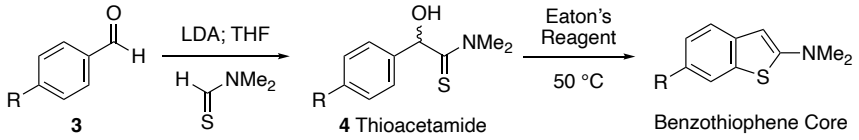
1 (raloxifene) **2** (17β-estradiol)

Figure 1. Raloxifene (**1**) and 17β-estradiol) (**2**).

Approximately one hundred derivatives of raloxifene have been prepared by studies in a number of laboratories in search of improved efficacy.[17,18,19,20] In addition, recent studies have investigated dual use properties of raloxifene analogs in a variety of diseases.[21,22] Over 90% of these variants contained C_6–OH, C_6–OCH_3, or C_6–H substitution. A large portion of this library has focused on modifications within the 2-aryl substituent attached to the core benzothiophene.[23] This letter explores the preparation and reactivity of raloxifene derivatives which incorporate C_6 substitution unavailable using the established synthetic procedures. One important goal of our studies was to replace the C_6–OH of raloxifene (**1**) with functionality which would participate in binding as hydrogen bond acceptor or donor sites, albeit with reduced affinity for the estrogen receptor. These preliminary results outline promising synthesis methods worthy of incorporation in an expanded investigation. Selected raloxifene derivatives have been examined with an in vitro ER binding assay for competitive displacement of 17βe (**2**) and with C3 gene expression in MC3T3 cells.

Initial efforts have explored the incorporation of electron-withdrawing groups and nitrogen-containing functionality at C_6 of the benzothiophene core **5** (Table 1). Although intermediate thioamides **4** are generally prepared in good yields, the cyclization to the benzothiophene **5** is adversely affected by the electronic withdrawing properties of C_6 substituents. Thus, the desired cyclization fails completely using the standard conditions of catalytic methanesulfonic acid in CH_2Cl_2 at 0 °C.[24,25,26] In fact, in these experiments, no reaction is observed at reflux, indicating decreased stability in the formation of the benzylic carbocation as a prerequisite for cyclization. Several attempts to improve the leaving group capability in **4** were unsuccessful.[27] To this end, we have identified vigorous conditions using Eaton's reagent (methanesulfonic acid with P_2O_5 (10% by weight)), which has afforded modest yields of purified benzothiophenes **5** (72%), **6** (63%), **7** (24%), and **9** (53%). Reduced yields of **8** were attributed to electronic factors induced by protonation of the C_6-pyridyl product **4d**. While complete failure was observed in the attempted cyclization of **4f**, an alternative method pioneered by Yang, et al.[28] was employed to obtain the trifluoromethyl derivative **11** from the nitrile **10**, as well

Table 1. Preparation of Thioacetamides and the Benzothiophene Core



Entry	Benzaldehyde	R	Thioacetamide[a] (% yield)	Benzothiophene[b] (% yield)
1	**3a**	F_3CO—	**4a** (80)	**5** (72)
2	**3b**	Br—	**4b** (77)	**6** (63)
3	**3c**	F—	**4c** (79)	**7** (24)
4	**3d**		**4d** (72)	**8** (10)
5	**3e**		**4e** (70)	**9** (53)
6	**3f**	F_3C—	**4f** (67)	Failed

[a] Conditions: 1.1 equiv LDA, 1 equiv aldehyde (3), 1 equiv thioformamide, –78 °C to r.t., 4 h
[b] Conditions: 0.5M in Eaton's Reagent, 30 min

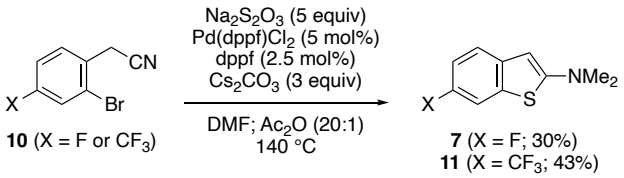


Figure 2. Palladium induced formation of the benzothiophene core.

as the problematic C_6 fluoro analog **7** (of Table 1). While the latter procedure afforded access to these electron-deficient derivatives, the expense of the starting nitriles (**10**) is prohibitive for large-scale synthesis of these particular analogs.

The C_6-bromide **6** (Table 1) is a high-value product for further elaboration as the presence of the bromide facilitates a variety of cross coupling processes. For example, Sonogashira cross couplings of **12** are generally successful and provide products as exemplified by **13** (76%). Furthermore, Buchwald—Hartwig cross couplings with cyclic secondary amines afford new C_6 derivatives such as the C_6 morpholino **14** in multigram scale reactions. Raloxifene triflates have been reported via low-yielding reactions of **1**, and these triflates also undergo Stille cross-coupling reactions in moderate yields.[29] An issue for polar amines, such as **14**, is the coelution of a persistent impurity which may hamper the isolation of highly purified quantities (>99% pure) necessary for biological evaluations. In addition to these standard techniques, the 6-bromo-benzothiophene **6** (from Table 1) readily undergoes halogen-metal exchange to provide the corresponding lithium reagent for introduction of a host of electrophiles. Table 2 illustrates four standard reactions with aldehydes, ketones, and acyl chlorides as demonstrated by the formation of **15**, **16**, **17**, and **18**.

In the cases of C_6 acylation (entries 3 and 4), the reactive lithio species from **6** leads to small amounts of ketone **19** as a byproduct (10%) which is readily separated by flash chromatography. The hydride reduction ($LiAlH_4$, THF at 0 °C) of the ethyl ester **17** leads to the corresponding primary benzylic alcohol **20** (82%) of Scheme 1. The benzylic alcohols of **15** and **20** are protected as the corresponding *tert*-butyldimethylsilyl (TBS) ether **21** and **22** in excellent yield (TBSCl, imidazole, CH_2Cl_2, r.t., 90% yield). Similarly, we have prepared the corresponding methoxymethyl (MOM) ether **23** under standard conditions.

The search for a mild acylation method has led to an alternative process that offers opportunities for broad applications via low temperature lithiation at C_3. A general and high-yielding process for C_3 bromination of the benzothiophene core is exemplified by the examples of **21**, **22**, and **23** (Scheme 1). The purified bromides **24abc** are subsequently used for halogen-metal exchange at –78 °C to give a reactive lithio species which provides the ketones **25abc** upon reaction with the Weinreb amide **26**. Furthermore, the alcohol **25d** was also readily available via the treatment of **25a** with TBAF for deprotection of the silyl ether (95% yield). The preparation and introduction of **26** offers an important advantage since it is readily purified by flash chromatography and avoids use of the acid chloride salt which has been used in previously published acylation procedures. In these cases, the acid chloride salt consumes one equivalent of lithium reagent. Based on 2:1 stoichiometry of the aryl lithium and the acylation reagent, we observed complete consumption of the starting bromides **24abc** and often recovered 15%–20% of **26**. Yields of the

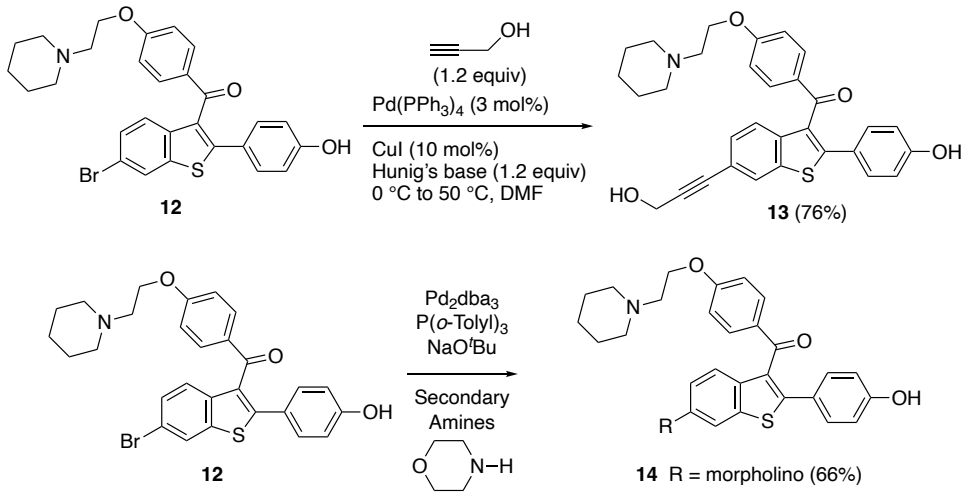
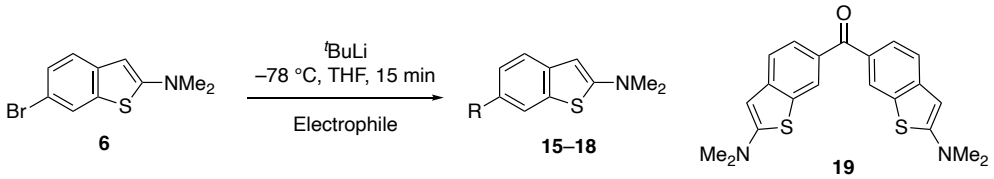
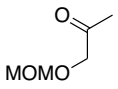
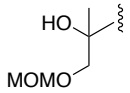


Figure 3. Examples of cross-coupling reactions of the C_6-bromide **12**.

Table 2. The synthesis of 6-substituted benzothiophenes *via* halogen-metal exchange.



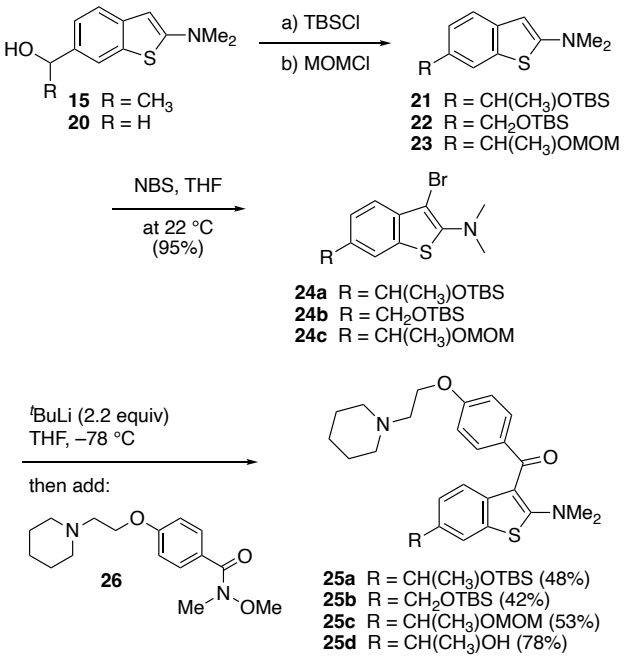
Entry	Electrophile	R	Product (% yield)
1			**15** (71)
2			**16** (43)
3			**17** (63)
4			**18** (68)

[a] Conditions: 1 equiv thiophene, 2.2 equiv ^{t}BuLi, 0.1M in THF, –78 °C then add electrophile (excess) at –78 °C.

ketones **25abc** generally ranged from 44% to 60% with isolation of as much as 30% of the reduced benzothiophene. Prolonged reaction times and high temperatures failed to provide improved yields of product. When these reactions were quenched with D_2O, no evidence of deuterium incorporation was found. In fact, we have measured approximately 70% deuterium incorporation after directly quenching the halogen-metal exchange with D_2O. The choice of solvent is significant as THF led to increased amounts of reduced benzothiophene, whereas pentane in ether (60:40 by volume) led to the best results for deuterium incorporation. While the aryl lithium may have limited stability in anhydrous THF, the amide **26** was insoluble in pentane/ether, and further attempts to improve the stability of the C_3 lithio species by addition of TMEDA, DMPU, or HMPA in THF solutions also led to reduced yields. These experiments have demonstrated great potential for the use of two sequential lithiations at C_6 and C_3 of the core benzohiophene to construct a wide variety of raloxifene derivatives, and therefore, we continue to examine alternative solvents to gain better overall yields.

Our studies have also examined the Friedel–Crafts acylation of novel C_6-substituted benzothiophenes from Tables 1 and 2 enroute to raloxifene analogs.[30,31] We have prepared the acid chloride salt **27** of Table 3 by treatment of the known carboxylic acid[32] with oxalyl chloride in CH_2Cl_2 solution containing small amounts of DMF. The resulting hydrochloride salt **27** is filtered and triturated with small amounts of solvent. As a white powder, it is easily stored at room temperature under argon to maintain an anhydrous condition. Unfortunately, electron-withdrawing groups at C_6 of the benzothiophene dramatically reduce the reactivity of the enamine moiety. While the solid **27** is readily measured and introduced into these

Scheme 1. An alternative acylation procedure via C3 bromination.

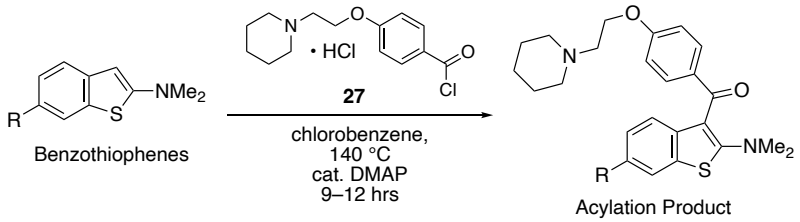


reactions, it shows low solubility in most organic solvents. As shown by the examples of Table 3, acylations using the chloride **27** require prolonged reaction times and higher temperatures (140 °C) as compared to the usual published procedures. In the presence of catalytic 4-dimethylaminopyridine (DMAP), the desired ketones **28** through **34** (Table 3) are obtained in 53% to 72% yields. As expected, derivatives **21**, **22**, and **23** from Scheme 1 were not amenable to these robust acylation conditions.

Our preliminary studies have demonstrated the synthesis of novel raloxifene derivatives via the installation of the 2-aryl component upon 1,4-conjugate addition of 4-(*tert*-butyldimethylsiloxy)phenyl magnesium bromide reagent with our enamide acylation products. Five representative examples illustrate the formation of novel raloxifenes **35** through **39**. These derivatives have not been readily accessible via standard protocols. Flash silica gel chromatography of the crude reaction mixture following the Grignard addition has directly led to TBS silyl ether cleavage using *tert*-*n*-butylammonium fluoride (TBAF). The final products are obtained by flash chromatography to derive C_6-subtituted raloxifenes in >96% purity for biological evaluations.

To verify that our analogs had reduced ER binding affinity, fluorescence polarization (FP) tests were performed, where selected derivatives **13**, **35**, **36**, **37**, and **39**, were compared to 17βe (**2**) using an ER-alpha-competitor assay kit (PolarScreen™ ER Alpha Competitor Assay Green, Thermo Fisher). FP of fluorochrome tracers bound to ER was measured (EnVision 2102 Multilabel Plate Reader, Perkin Elmer) in 8 triplicate serial dilutions of compound concentrations

Table 3. Friedel–Crafts acylation of benzothiophenes at 140 °C.



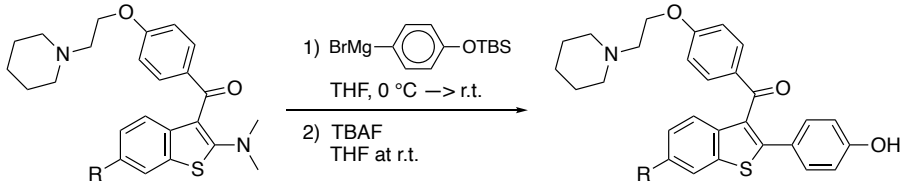
Entry	Benzothiophene	R	Acylation Product (% yield)
1	**5**	F_3CO—	**28** (71)
2	**6**	Br—	**29** (70)
3	**7**	F—	**30** (65)
4	**8**		**31** (53)
5	**9**		**32** (72)
6	**17**		**33** (67)
7	**18**		**34** (63)

[a] Conditions: 1 equiv thiophene 5, 1.1 equiv HCl salt 40, cat. DMAP, 1M in chlorobenzene, 140 °C for 9–12 hours.

ranging from 10^{-10} to 10^{-6} M. The output degree of light polarization for each well was plotted versus compound concentration and fit to a nonlinear curve in GraphPad PRISM (9.5.1) to produce an IC50 value for each compound (half of the maximal concentration required to reduce tracer displacement due to binding). Results indicated that analogs **35** and **39** had significantly lower ER binding affinity compared to 17βe (**2**), as shown by the high compound concentration needed to detect a change in tracer binding (Figure 4). We also sought confirmation of these results by assessing *in vivo* effects, C3 gene expression was analyzed in MC3T3-E1 Subclone 4 (ATCC® CRL-2593; Manassas, VA) murine pre-osteoblasts fed media dosed with analog treatments or DMSO at concentrations of 1, 10, and 100 nM for each treatment, with 2 replicates. RNA extractions were performed after 2 days of growth using Bioline kit without Trizol (High Capacity RNA to cDNA synthesis kit 4387406). Gene expression was performed, with all samples assessed in triplicates (Life Technologies Taqman Fast Advanced Buffer and Assay Mm00437838, assessed in a QuantSTudio 3 Real-Time PCR), and qPCR data was analyzed using the Livak method. C3 expression was not significantly upregulated in most analogs compared to controls (Figure 4), further indicating that ER binding affinity was successfully reduced.

The goal of our studies is to identify raloxifene analogs with little or no estrogen receptor (ER) signaling while modulating bone quality and mechanical properties. Preliminary efforts selected compound **39** for these investigations. The G610C mouse model of osteogenesis imperfecta (OI) was used in these *in vivo* studies, and mice were bred in-house with wildtype (WT) females to produce G610C and WT offspring. A description of the data and methodology from the *in vivo* studies is too extensive to include in this letter, but it appears in a separate publication.[33] The proof of concept shows that **39** has low ER affinity and positive impacts on the ability of OI bone to resist fracture at the expense of reduced pre-yield mechanical behavior. In fact, treatment with **39** did not improve pre-yield mechanical properties, but post-yield and total displacement were significantly increased. Analog **39**, together with loading, increased 4-pt bending displacement, strain, and toughness of G610C bone. The strongest effects were apparent in loaded bone where treatment with **39** is combined with a bone anabolic stimulus. Our findings suggest that toughness of *de novo* bone tissue may be positively impacted by treatment with **39**. This communication details procedures that offer a robust protocol for the evaluation of a wide variety of derivatives made available by our investigation.

Table 4. Grignard reactions for the formation of raloxifene derivatives.



Enamide Acylation Product — C_6-Raloxifene Derivative

Entry	Enamide (R)	C_6-Raloxifene Derivative (% yield)
1	**28** R = OCF_3	**35** R = OCF_3 (75)
2	**29** R = Br	**36** R = Br (73)
3	**30** R = F	**37** R = F (73)
4	**25d** R = $CH(CH_3)OH$	**38** R = $CH(CH_3)OH$ (44)
5	**34** R = $C(O)NMe_2$	**39** R = $C(O)NMe_2$ (70)

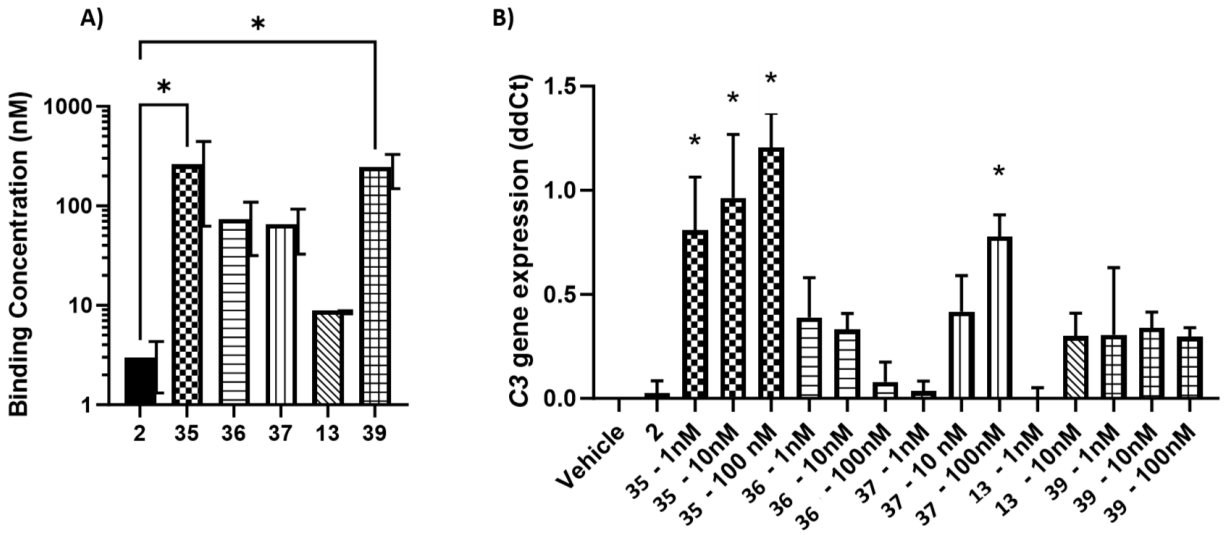


Figure 4. *Analog characterization and in vitro testing.* Solutions (nM) of derivatives were prepared in the buffered medium supplied in the commercial test kits. **A**) IC50 values from repeated fluorescence polarization tests indicating estrogen-binding affinity. P-values from one-way ANOVA-post hocs shown with * for $p<0.05$. **B**) C3 gene expression in MC3T3 cells treated with various analog concentrations, normalized by GAPDH. P-values from one-way ANOVA post hocs shown with * indicating $p<0.0001$.

In conclusion, this study has examined new opportunities for the preparation of raloxifene derivatives. Specifically, the scope of C_6 substitution has been limited in the prior art. In this preliminary study, synthesis methods and techniques have been devised to expand the scope of available compounds. Substitution at C_6 has addressed the preparation of benzothiophenes which provide reduced binding affinity for the ER receptor. Results also outline pathways for the introduction of various hydrogen bond donor and acceptor functionality at C_6 of the raloxifene core. Further studies to assess the biology of C_6 raloxifene derivatives is currently in progress.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.X.xXXXX

Experimental procedures; full characterization data for all final products; characterizations of intermediates in the synthesis sequence listed as compounds **4a**, **5**, **4b**, **6**, **4c**, **7**, **4d**, **8**, **4e**, **9**, **4f**, **11**, **13**, **14**, **15**–**18**, **20**–**26**, and **28**–**39**; HPLC proof of purity for products **13**, **35**, **36**, **37**, **38**, and **39** (PDF).

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Notes

The authors declare that they have no known competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.

ABBREVIATIONS

ER – estrogen binding; SERM – selective estrogen receptor modulator; BMD – bone mineral density; 17βe – 17β-estradiol

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