4-(3′α15′β-Dihydroxy-5′β-estran-17′β-yl)furan-2-methyl Alcohol: An Anti-Digoxin Agent with a Novel Mechanism of Action

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Received June 19, 2005

The synthesis and some pharmacological properties of 4-(3′α-15′β-dihydroxy-5β-estran-17′β-yl)furan-2-methyl alcohol (**16**) have been described. The compound was synthesized by reacting a synthetic 3α-benzyloxy-5β-estr-15-en-17-one with the ethylene acetal of 4-bromo-2-furancarboxyaldehyde, followed by hydrolysis of the ethylene acetal and reduction of the aldehyde. Despite its resemblance to the structure of cardiac steroids (CS), **16** does not bind to the CS receptor on Na^+,K^+-ATPase and does not increase the force of contraction of heart muscle. However, **16** inhibited the digoxin-induced increase in the force of contraction and arrhythmias in guinea pig papillary muscle and human atrial appendages. The steroid also inhibited digoxin-induced alteration in endocytosed membrane traffic, indicating a novel mechanism of action.

Introduction

Cardenolides (i.e. digoxin, ouabain) and bufadienolides (i.e. bufalin, marinobufogenin) are steroids extracted from plants and amphibian skin, respectively, which bind to and inhibit the activity of the plasma membrane Na^+,K^+-ATPase.[1] These compounds are used in Western and Eastern medicine for the treatment of cardiac failure and certain types of arrhythmias.[2,3] In the past decade, endogenous cardenolides and bufadienolides (herein collectively termed cardiac steroids, CS) have been identified in mammals, including humans. Ouabain was identified in human plasma and adrenal gland,[4] digoxin was found in human urine,[5] an ouabain isomer was identified in the bovine hypothalamus,[6] 19-norbufalin and its peptide derivative were identified in cataractous human lenses,[7] and dihydropyrone-substituted bufadienolide was identified in human placenta.[8] In addition, the immunoreactivity of marinobufogenin-like[9] and proscillaridin A-like compounds,[10] which also belong to the bufadienolide family, has been demonstrated in human plasma. Strong evidence supports the notion that the CS are synthesized in, and released from, the adrenal gland.[11−13] The identification of endogenous CS raises new questions regarding the physiological roles and pharmacological effects of these compounds. As part of our study on digoxin-like compounds, we synthesized and screened estrane derivatives for their effects on cardiac contractility in guinea pig both in vivo and in vitro. Among the tested compounds, 4-(3′α,15′β-dihydroxy-5′β-estran-17′β-yl)-furan-2-methyl alcohol (**16**, Figure 1a) was found to be of interest and was chosen for in-depth evaluation.

Since 1969, several attempts to synthesize bufadienolides have been made.[14−17] The transformation of digoxin to a bufadienolide compound was also reported.[18] In the early 1970s, it was established that the main prerequisites for a biologically active cardenolide are the C/D cis ring junction, the 17β-butenolide moiety, and the 3β,14β-dihydroxy groups. The A/B ring junction can be either cis or trans without a dramatic change in activity.[19]

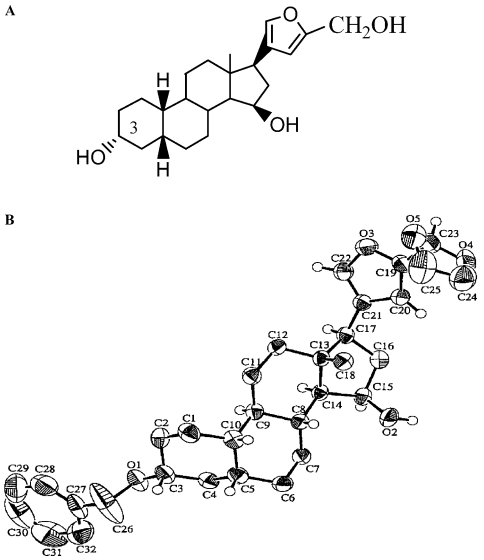

Figure 1. (a) 4-(3′α-hydroxy-15′β-hydroxy-5β-estran-17′β-yl)furan-2-methyl alcohol (compound **16**). (b) X-ray crystallography of **13**.

The most advanced synthesis of bufalin and its analogues was documented 10 years later.[20−23] According to those reports, a 3β-benzyloxy-α,β-unsaturated-17-keto steroid was reacted with the lithiated ethylene acetal of 4-bromo-2-furancarboxyaldehyde and the product was further transformed into a bufodienolide compound in a multistep process. We now describe the synthesis and in vitro pharmacological characteristics of 4-(3′α,15′β-dihydroxy-5′β-estran-17′β-yl)furan-2-methyl alcohol, which was synthesized by a multistep process.

Synthesis and Structural Characterization

The synthesis of 4-(3′α,15′β-dihydroxy-5β-estran-17′β-yl)-furan-2-methyl alcohol included three major steps: (a) synthesis of 3α-benzyloxy-5β-estr-15-en-17-one; (b) reaction of the estrane (norandrostane) moiety with the ethylene acetal of 4-bromo-2-furancarboxyaldehyde; (c) removal of the benzyl group, hydrolysis of the ethylene acetal, and reduction of the aldehyde.

(a) The synthesis of 3α-hydroxy estrane moieties was carried out using a modification of a published procedure.[20] The starting material was estrane-4-en-3,17-dione (Scheme 1) which, by hydrogenation on $RhCl_3$ hydrate, produced a 5β-estrane-3,17-

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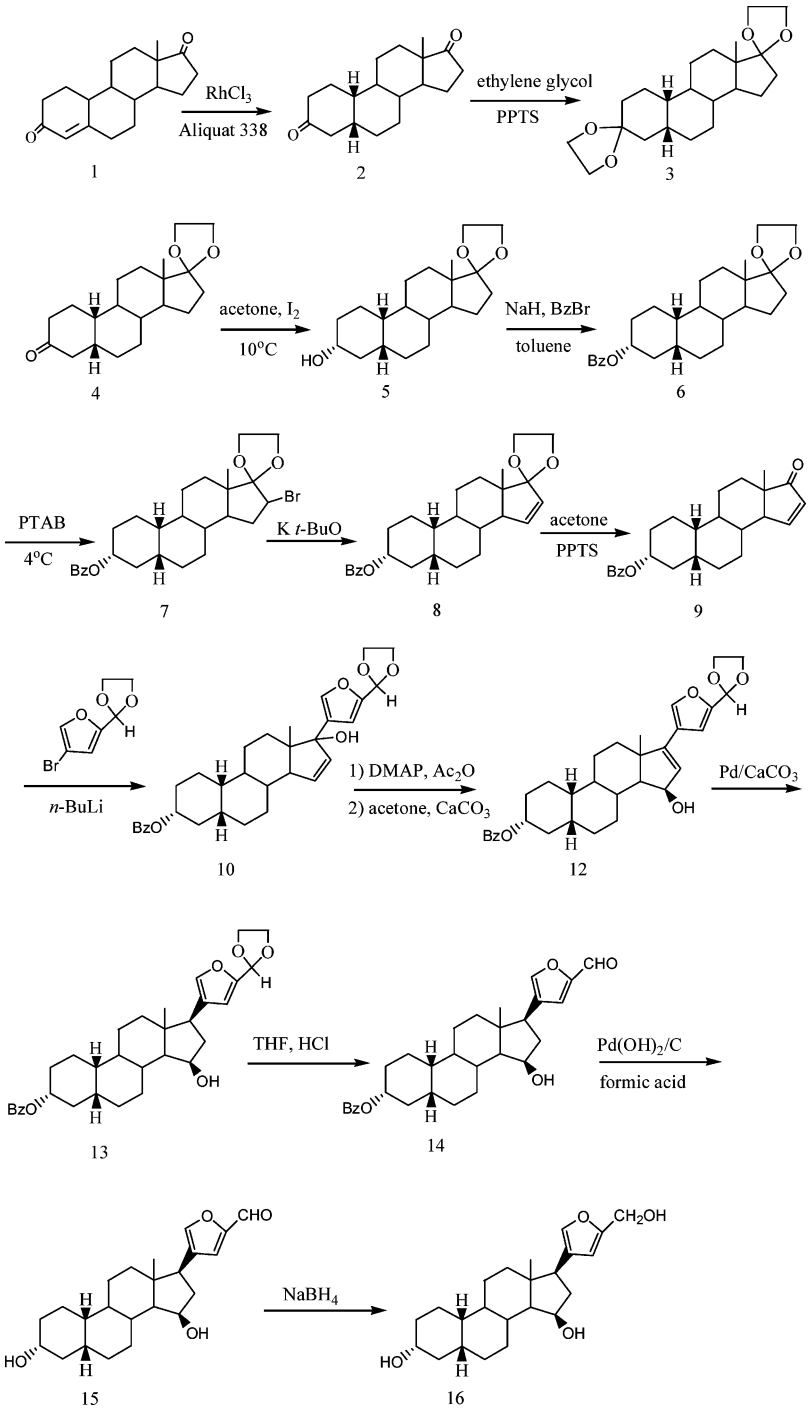
10.1021/jm0505819 CCC: $33.50
Published on Web 12/20/2005

Scheme 1



dione (compound **2**), with cis stereochemistry (80%) at the A/B ring junction.[24,25] Both ketone groups were protected as ethylene ketal, yielding compound **3**. After selective removal of the ketal at the 3 position by iodine in acetone[35] (compound **4**), the ketone was treated with $NaBH_4$ in THF/methanol, selectively producing the 3α-hydroxy-5β-estran-17-one ethylene ketal (compound **5**). The 3α-hydroxy group was protected as the benzyloxy derivative by boiling **5** with benzyl bromide in dry toluene in the presence of NaH. Selective bromination followed by dehydrobromination and removal of the ketal group produced the α,β unsaturated ketone **9** in an overall yield of 13%.

(b) Condensation of **9** with the ethylene acetal of 4-bromo-2-furancarboxyaldehyde[26,27] afforded compound **10** (Scheme 1). The purified alcohol was first acetylated with acetic anhydride in pyridine to compound **11** (not shown in Scheme 1) which, without purification, was subjected to allylic rearrangement in boiling aqueous acetone in the presence of $CaCO_3$, yielding the allylic alcohol **12**. The purified product was hydrogenated on $Pd/CaCO_3$ in methanol in the presence of traces of NaOAc, yielding the crystalline product **13**. In addition to the MS and NMR spectra, the X-ray crystallography pattern of **13** clearly indicated the ketal structure of 4-(3′α-benzyloxy-5′β-estrane-15′βhydroxy-17′β-yl)furan-2-carboxyaldehyde ethylene ketal (Figure 1b). Whereas, the orientation of the 17′β-yl furan derivative was reported previously,[20−22] the β stereochemistry of the 15′ hydroxy group was observed only by the X-ray measurement of compound **13**. Concerning the mechanism of formation, we can assume that in an earlier step (in compound **10**), the 17′ hydroxyl group can be either α or β or both conformations. Prior to the occurrence of the allylic rearrange-

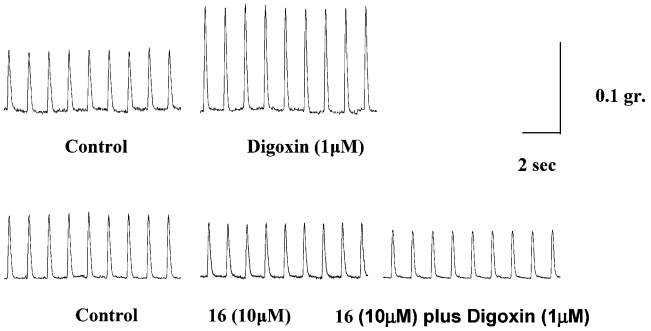


Figure 2. Inhibition of digoxin-induced increase in papillary muscle contractility by **16**. Representative records showing changes in force of contraction of guinea pig papillary muscle induced by digoxin, **16**, and the two drugs together. The experiment was repeated five times each using different muscle preparations.

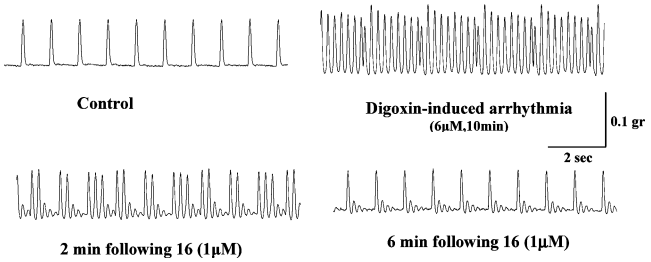


Figure 3. Inhibition of digoxin-induced arrhythmias in papillary muscle by **16**. Representative records showing changes in force of contraction and rate of guinea pig papillary muscle induced by digoxin (4 μM), **16**, and the two drugs together. The experiment was repeated five times each using different muscle preparations.

ment, a S_N2 reaction had taken place due to the alkaline environment, affording mainly the thermodynamically more stable 17′β hydroxy isomer. Further, the hydroxyl group migrates to the C-15 retaining the configuration (above the ring D) to give the 15′β hydroxy product.

(c) Product **13** was hydrolyzed in diluted hydrochloric acid, yielding the aldehyde **14** and deprotected with $Pd(OH)_2$/C in the presence of formic acid, producing **15**, which was further reduced with $NaBH_4$ to 4-(3′α,15′β-dihydroxy-5′β-estrane-17β-yl)furan-2-methyl alcohol (**16**). **16** was dissolved in ethyl alcohol and diluted with saline, and the biological activity was measured.

Biological Activity

The effect of **16** on guinea pig papillary muscle contractility was determined using conventional techniques.[28] Preliminary experiments demonstrated that in this preparation at short time (minutes) periods, 0.5−4 μM digoxin elicited significant increase in contractility and arrhythmias at the high concentration. Compound **16**, on the other hand, up to 20 μM, did not significantly affect muscle contractility and rhythm (data not shown). Thus, these concentration ranges were chosen in the following experiments. The addition of digoxin (1 μM) to the muscle bathing solution elicited a 60% increase in the force of contraction within 10 min. Compound **16** (10 μM) slightly reduced the force of contraction. However, when digoxin and **16** (at the above concentrations) were added simultaneously, a complete blockade of digoxin-induced increase in contraction was apparent (Figure 2). An anti-digoxin effect of **16** was also observed by measuring digoxin-induced arrhythmias (Figure 3). Papillary muscle exposed to 4 μM digoxin developed severe arrhythmias within several min. Digoxin-induced arrhythmias had an average time for onset of 7 ± 1 min (mean ± SD) and were sustained for more than 20 min. The addition of **16** (1 μM) 1 min after the onset of arrhythmias lowered arrhythmias

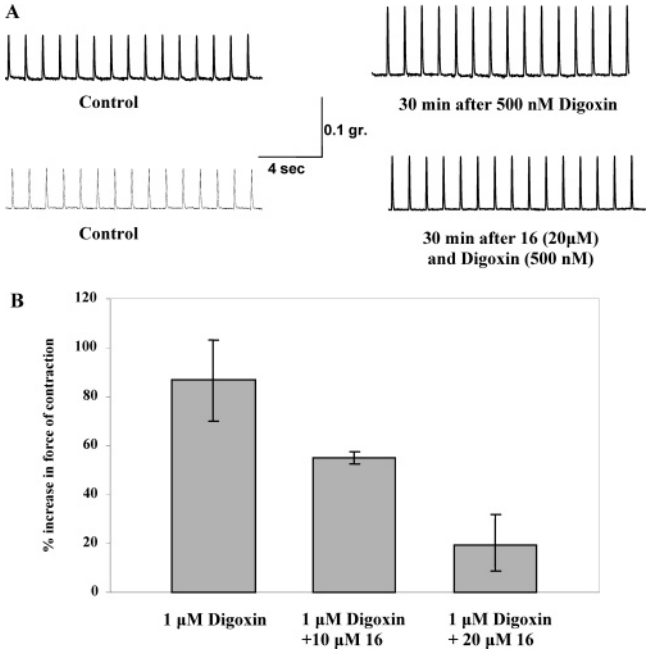


Figure 4. Inhibition of digoxin-induced increase in human heart strips contractility by **16**. (a) Representative records showing changes in force of contraction of human atrial strips, induced by digoxin, **16**, and the two drugs together. The experiment was repeated 10 times each using different muscle preparations. (b) Effect of increasing **16** concentrations on digoxin-induced increased force of contraction of human atrial strips. The columns represent the average ± SE of six experiments (87 ± 16, 57 ± 2.5, and 19 ± 13 for 1 μM digoxin, 1 μM digoxin, and 10 μM **16**, and 1 μM digoxin and 20 μM **16**, respectively).

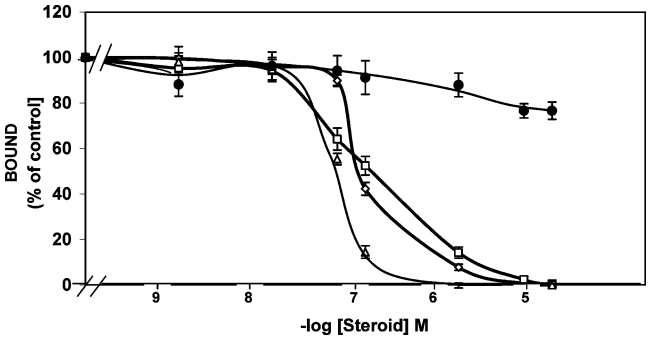


Figure 5. Displacement of ^{3}H-digoxin by digoxin, ouabain, bufalin, and **16** from guinea pig crude synaptosomal fraction. Crude synaptosomal fraction preparation and binding assays are described in the Biological Methods. A 200 μL volume (60 μg of protein) was incubated with ^{3}H-digoxin in the presence of increasing concentrations of digoxin (open triangles), ouabain (open circles), bufalin (open squares), or **16** (closed circles). Following 1 h incubation, the membranes were passed through Whatman GF/B filters, which were then washed twice. The radioactivity retained on the filters was measured by β-counter liquid scintillation. The experiments resulted in IC_{50} of 10, 20, and 30 nM for digoxin, ouabain, and bufalin, respectively, and undefined value for **16**.

irregularity, and the rhythm returned to the normal stimulatory rate after within 10 ± 3 min (Figure 3).

An anti-digoxin effect of **16** was also demonstrated on human atrial appendages. Human atrial appendages are routinely discarded during open heart surgery and were used in the following experiments. The addition of digoxin (500 nM) increased the force of contraction of the human atrial strip by ∼60% (Figure 4). However, in the presence of **16** (1 μM), the same concentration of digoxin elicited an increase of only ∼20% in the force of contraction (Figure 4). The effect of different concentrations of **16** on the digoxin-induced increase in the force of contraction of the human atrial appendages is shown in Figure

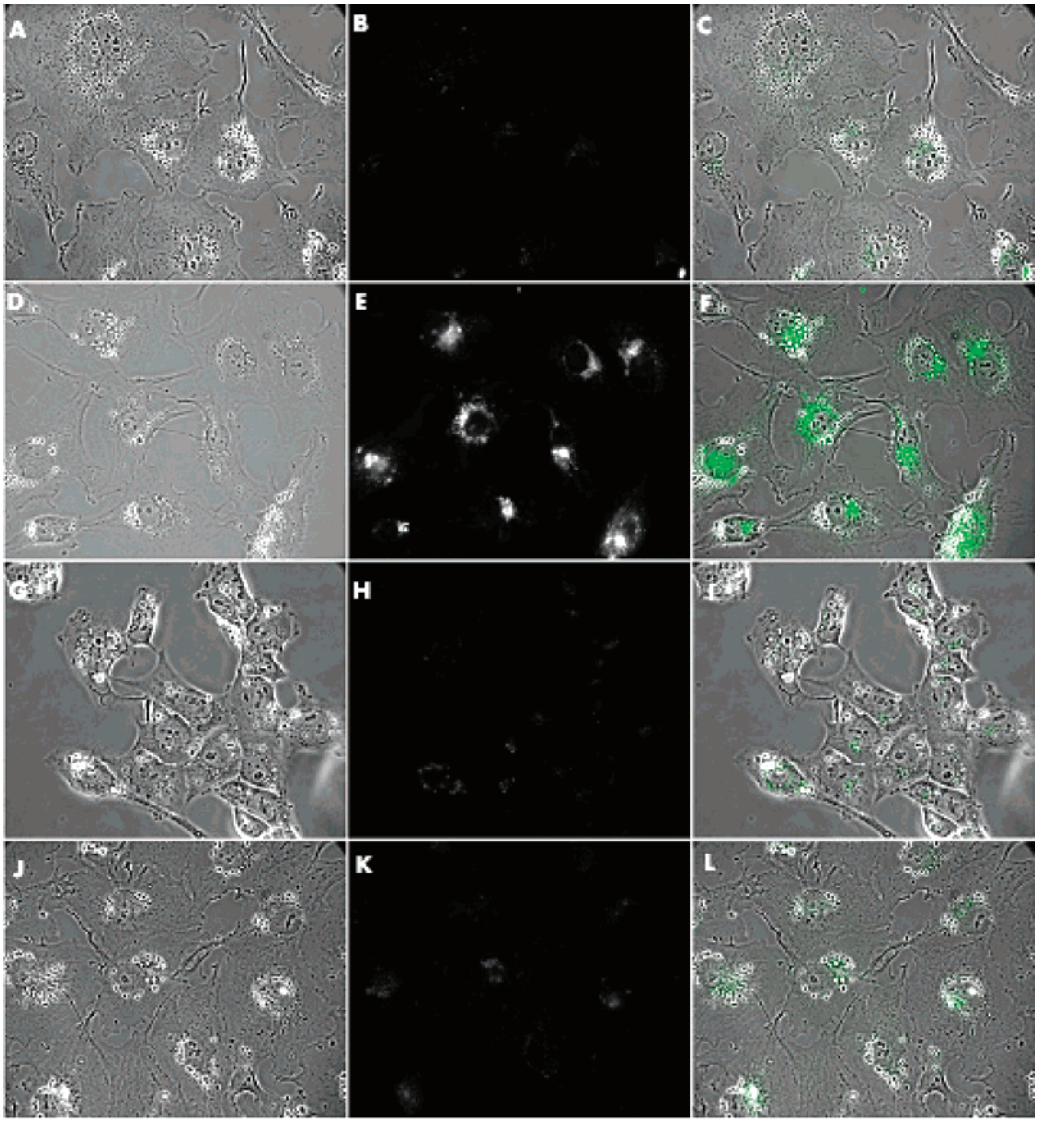


Figure 6. Inhibition of digoxin-induced transferrin accumulation in NT2 cells by **16**. NT2 cells were grown in DMEM-F12 on glass coverslips for 24 h. The DMEM-F12 was then replaced with drug-free medium (A−C) or medium containing 20 nM digoxin (D−F), 20 nM **16** (G−I), or 20 nM digoxin, and 20 nM **16** (J−L). Following 20 min incubation, the medium was removed and the cells were incubated in medium containing transferrin Alexa Fluor, as described in Materials and Methods. Following 4 h incubation, the cells were observed under a fluorescence microscope. Phase contrast images (A, D, G, and J), fluorescent images (B, E, H, and K), and the merged images of the two (C, F, I, and L) are shown. The green color corresponds to transferrin signals. Scale bar, 20 μm.

5. Clearly, the effect of **16** is dose-dependent, and an almost complete block of the digoxin-induced effect was obtained at a ratio of 20:1 (**16**:digoxin).

It is generally accepted that the mechanism of action of CS-induced increase in the force of contraction of heat muscle is mediated by the inhibition of the plasma membrane Na^+,K^+-ATPase.[1,2] Although alternative mechanisms were recently suggested,[29] it is believed that this inhibition results in a local increase in intracellular Na^+ and a consequent reduction in Na^+/Ca^{2+} exchange and increased intracellular Ca^{2+} and contractility.[30] Thus, a plausible mechanism for the inhibition of the digoxin-induced effect by **16** is the inhibition by this steroid of digoxin binding to its receptor on Na^+,K^+-ATPase. Binding experiments, however, do not support this notion. The displacement of ^{3}H-digoxin by digoxin, ouabain, bufalin, and **16** from rat brain synaptosomal fraction is shown in Figure 6. Whereas digoxin, ouabain, and bufalin displaced ^{3}H-digoxin in a dose-dependent manner (IC_{50} 10 and 20 nM, and 30 nM, respectively), **16** had only a marginal effect. Thus, it can be concluded that **16** does not interact with the CS binding site on Na^+,K^+-ATPase, and its digoxin antagonism is mediated by a different pathway.

We recently demonstrated that the bufadienolide bufalin induces the accumulation of vesicles in the perinuclear region of human cells.[31] On the basis of the effect of CS on the accumulation of FM1-43 in colocalization with specific Rab proteins, these vesicles were identified as late endosomes, and we suggested that endogenous CS-like compounds are involved in the regulation of endocytosed membrane traffic.[31] Thus, it can be postulated that CS exert their action on heart contractility and rhythm, partially by their effect on intracellular membrane traffic. This possibility is supported by recent findings that CS effects are inhibited by endocytosis inhibitors.[32,33] The effect of digoxin on the accumulation of transferrin in human neuronal NT2 cells is shown in Figure 6. In the absence of digoxin, no accumulation of transferrin was apparent (Figur 6a−c). Like bufalin,[31] digoxin induced a 20-fold increase in the accumulation of transferrin (Figure 6d−f), indicating reduced recycling of the plasma membrane. Interestingly, the new steroid **16** completely blocked the digoxin-induced transferrin accumulation. These findings support the hypothesis that **16** antagonism of the digoxin-induced effect on cardiac muscle is mediated by

the inhibition of digoxin-induced changes in intracellular membrane traffic.

CS are used for the treatment of cardiac failure and certain types of arrhythmias,[2,3] despite the frequent toxic side effects of these drugs.[2] In severe cases of CS toxicity, treatment with specific digoxin antibodies is recommended.[34] The discovery that **16** is an antagonist of CS action raises the possibility of developing such compounds and their derivatives for the treatment of CS toxicity.

Materials and Methods

General Procedures. Solvents were purchased from Biolab (Jerusalem, Israel), Aldrich Chemical Co. (Milwaukee, WI), and Frutarom (Haifa, Israel). Chemical reagents $RhCl_3 \cdot H_2O$, Aliquat-336, Pd $(OH)_2$/10%C, *n*-butyllithium, pyridinium tribromide, ethylene glycol, and potassium *tert*-butoxide were purchased from Aldrich Chemical Co. Estrane-4-ene-3,17-dione was purchased from Taizhou Xingye Chemical Co., Ltd, Shanghai 201507, P.R. China. 4-Bromo-2-furaldehyde was synthesized as described previously.[12,13]

Electron impact mass spectra were obtained using a ThermoQuest Trace MS (San Jose, CA) mass spectrometer, operating at 70 eV and with the ion source heated to 200 °C. The samples were inserted by direct inlet with external heating of up to 220 °C or by a GC inlet provided with a capillary column Phenomenex (Torrance, CA) ZB 5, 5% phenyl−95% dimethyl polysiloxane (25m × 0.25 mm and 0.25 μm film thickness). Separation was performed in a splitless mode with a temperature program. The starting temperature was 80 °C which, after 6 min, was increased to 260 °C, 6 °C /min, maintained for 10 min, and then decreased to the starting temperature. The ^{1}H NMR (300 MHz) measurements were performed using a Varian VXR-300S (Palo Alto, CA) spectrometer in $CDCl_3$. IR spectra were recorded with a Perkin-Elmer FT-2000 or an Analect FT-IR spectrometer (Analect Instruments FX-6160, Irvine, CA). Liquid samples were recorded as a film between two NaCl plates. Solid samples were pressed into KBr pellets. The melting point was determined using an electrothermal apparatus (Electrothermal, England) and was uncorrected. Elemental analyses were performed by the Microanalytical Laboratory at The Hebrew University, Jerusalem, Israel. Crystallographic structure analysis was done on an ENRAF-NONIUS CAD-4 computer-controlled diffractometer, and all crystallographic computing was done on a VAX9000 computer at the Hebrew University of Jerusalem. The HPLC measurement was performed with a Hewlett-Packard (HP) 1050 Series chromatograph provided with a HP 104OM detection system and an Agilent ChemStation (Waldbron, Germany). The detector was set to 225 nm and the chromatography of samples was performed on a Luna C-18, 5 μm column (250 × 4.6 mm) (Phenomenex, Torrance, CA) with an isocratic 60% acetonitrile/water system with a flow of 0.8 mL/min. One milligram of sample was dissolved in 1.0 mL ethyl alcohol in an autosample vial, and 10 μL was injected into chromatograph for HPLC analysis.

5β-Estrane-3,17-dione (2). A solution of estran-4-ene-3,17-dione (5 g, 18.38 mmol) in dichloromethane (90.0 mL) was mixed with the catalyst previously prepared from $RhCl_3 \cdot H_2O$ (40 mg) and Aliquat-336 (200 mg) in water (2 mL) and dichloromethane (10.0 mL). The catalyst solution was added to the steroid solution and hydrogenated overnight at 50 psi. The product was filtered, washed twice with water (40 mL), and dried over Na_2SO_4, yielding 80% of the cis isomer. Recrystallization from ethyl acetate produced compound **2** (3.0 g, 60% yield) as a white solid. Crystallization from ethyl acetate produced a white solid, mp 182−184 °C. MS: *m*/*z* ($M^{+\cdot}$) 274. IR 1737, 1705 cm^{-1}. ^{1}H NMR: δ 2.58 (t, 1H, J = 16 Hz, 4-H_{ax}), 0.88 (s, 3H, CH_3[18]). Elemental Analysis: $C_{18}H_{26}O_2$ (MW 274), Calcd C (78.8%), H (9.48%), Found C (78.64%), H (9.21%).

5β-Estrane-3,17 Ethylene Diketal (compound 3). Compound **2** (5 g, 18.2 mmol) was dissolved in dry benzene (100 mL), mixed with ethylene glycol (25 mL, 280 mmol) and pyridinium *p*-toluenesulfonate (1 g, 3.9 mmol), and refluxed, using a Dean−Stark separator overnight or until no starting material was detected by TLC. The benzene was removed at low pressure, the residue was dissolved in EtOAc (100 mL), washed twice with brine, and dried over Na_2SO_4, and the solvents were removed under reduced pressure, affording 6.5 g (98.5% yield) of the title compound as an oil. MS: *m*/*z* ($M^{+\cdot}$) 362. ^{1}H NMR: δ 3.81 (m, 4H,OCH_2CH_2O), 0.87 (s, 3H, CH_3[18]).

3-Oxo-5β-estrane 17-Ethylene Ketal (compound 4). Diketal **3** (300 mg, 1, 5 mmol) was dissolved in acetone (15 mL) and cooled in an ice bath to 10 °C, and iodine (90 mg, 0.16 mmol) was then added. The solution was stirred at 10 °C for 20 min. The reaction was terminated by the addition of 10% aqueous $Na_2S_2O_3$ until the color of the iodine disappeared. Most of the acetone was then removed under vacuum, and the residue was extracted with dichloromethane (50 mL) and washed with brine. The organic layer was separated, dried over Na_2SO_4, and filtered. The solvent was removed, affording an oily product (300 mg, 99% yield) that was reduced with $NaBH_4$ without additional purification. MS: *m*/*z* ($M^{+\cdot}$) 318. IR 3737 cm^{-1}. ^{1}H NMR: δ 3.62 (m, 1H, CHOH), 3.81 (m, 4H, OCH_2CH_2O), 0.87 (s, 3H, CH_3[18]).

3-Hydroxy-5β-estrane 17-Ethylene Ketal (compound 5). Compound **4** (3.18 g, 10.0 mmol) was dissolved in a 20% solution of methanol in THF (50 mL), $NaBH_4$ (1.0 g, 26.0 mmol) was slowly added, and the solution was stirred overnight at room temperature. The reaction was terminated by the addition of water (20 mL) and stirred for 10 min. Removal of the organic solvent at reduced pressure produced a white residue that was dissolved in diethyl ether, washed with water, and dried over Na_2SO_4, affording an oily product (3.0 g, 93.7% yield). MS: *m*/*z* ($M^{+\cdot}$) 320. ^{1}H NMR: δ 3.62 (m, 1H, CHOH), 3.81 (m, 4H, OCH_2CH_2O), 0.87 (s, 3H, CH_3[18]).

3α-Benzyloxy-5β-estran-17-one Ethylene Ketal (6). A solution of compound **5** (6.0 g, 18.7 mmol) in dry toluene (80 mL), NaH powder (1.5 g, 60 mmol), and benzyl bromide (3.2 g, 18.7 mmol) was boiled under nitrogen for 6 h. After cooling, the toluene solution was poured slowly over ice. The organic phase was separated, washed with water, and dried over Na_2SO_4. Removal of the solvent resulted in an oily product. Flash chromatography on silica gel with diethyl ether:hexane (1:9) produced compound **6** (6.2 g, 78% yield) as a white solid. MS: *m*/*z* ($M^{+\cdot}$) 410. ^{1}H NMR: δ 7.35 (m, 5H, Ph), 4.50 (s, 2H, CH_2Ph), 3.89 (m, 4H, OCH_2CH_2O), 3.38 (m,1H, H−C(3)), 0.83 (s, 3H, CH_3[18]).

3α-Benzyloxy-16-bromo-5β-estran-17-one Ethylene Ketal (7). Pyridinium tribromide (9 g, 25.2 mmol) was added slowly to a stirred solution of compound **6** (9 g, 21.9 mmol) in dry THF (45 mL). After 20 h at 4 °C, the mixture was poured into a cold solution of 5% $NaHCO_3$ (30 mL) and 10% $Na_2S_2O_3$ (30 mL). The organic phase was extracted with hexane (2 × 100 mL), washed with brine, and dried over Na_2SO_4, and the solvent was removed under reduced pressure, yielding 9 g (75.3% yield) of the title compound as an oil. The crude oil was subjected to dehydrobromination without additional purification. MS: *m*/*z* ($M^{+\cdot}$) 488, 490.

3α-Benzyloxy-5β-estr-15-en-17-one Ethylene Ketal (8). The dry compound **7** (12 g, 24.4 mmol) was dissolved in dry benzene (100 mL) and dry DMSO (50 mL), and potassium *tert*-butoxide (8.0 g) was slowly added. The mixture was stirred and heated to 60 °C under nitrogen. After 20 h, the product was cooled and brine (50 mL) was added. The mixture was extracted with benzene and washed with brine. Solvent removal afforded compound **8** (5.6 g, 57% yield) as an oily compound, from which the protecting group was removed without additional purification. MS: *m*/*z* ($M^{+\cdot}$) 408. ^{1}H NMR: δ 7.34 (m, 5H, Ph), 6.19 (d, 1H, H−C[16]) 5.68 (d × d, 1H, H−C[15]), 4.59(m, 2H CH_2Ph), 3.93 (m, 4H, OCH_2CH_2O), 3.39 (m, 1H, H−C[3]) 0.93 (s, 3H, CH_3[18′]).

3α-Benzyloxy-5β-estr-15-en-17-one (9). Pyridinium *p*-toluenesulfonate (1 g, 4 mmol) was added to a solution of compound **8** (5.6 g, 13.7 mmol) in acetone:water 10:2 (120 mL). The mixture was stirred overnight, and deprotection was monitored by TLC. The acetone was removed under reduced pressure, and the product was extracted with dichloromethane. The dichloromethane solution was washed with brine and dried over Na_2SO_4. Solvent removal produced compound **9** (4.0 g, 80% yield) as an oil. Flash chromatography on silica gel with diethyl ether:hexane (3:7) and crystallization afforded a white solid (3.4 g, 68%), mp 92−94 °C (from diethyl ether). MS: *m*/*z* ($M^{+\cdot}$) 364. IR 1707 cm^{-1}. ^{1}H

NMR: δ 7.52 (d, 1H, H−C[16]), 7.34 (m, 5H, Ph), 6.02 (d × d, 1H, H−C[15]), 4.56 (m, 2H CH_2Ph), 3.4 (m, 1H, H−C[3]) 1.06 (s, 3H, CH_3[18′]). Anal. $C_{25}H_{32}O_2$ (MW 364), Calcd C 82.42%, H 8.79%; Found C 82.10%, H 8.80%.

Ethylene Acetal of 4-(3′a-Benzyloxy-17′-hydroxy-5′β-estr-15′-en-17′-yl)furan-2-carboxyaldehyde (10). A 4.0 mL volume (10 mmol) of 2.5 M *n*-butyllithium was slowly added to an ethylene acetal of 4-bromo-2-furancarboxyaldehyde (2.5 g, 11.4 mmol) solution in freshly distilled dry THF (10 mL), and the solution was cooled to −70 °C under N_2. After 30 min at −70 °C, compound **9** (2.0 g, 5.5 mmol) dissolved in dry THF (20 mL) was added through a syringe. After 1 h at −70 °C, the mixture was allowed to warm to −10 °C and citric acid (100 mM, 10 mL) was slowly added. The product was extracted with diethyl ether, washed with 100 mM citric acid (30 mL) and then with water, and dried over Na_2SO_4. Solvent removal and purification by flash chromatography on silica gel with diethyl ether:hexane (7:3) afforded compound **10** (2.3 g, 40% yield) as a white solid. MS: *m*/*z* ($M^{+\cdot}$) 504, IR: 3550 cm^{-1}. ^1H NMR: δ 7.33 (m, 5H, Ph), 7.18 (s, 1H, H−C[5]), 6.42 (s, 1H, H−C[3]), 6.06 (d, 1H, H−C[16′]), 5.89 (s, 1H, H−C−C[2]), 5.68 (d × d, 1H, H−C[15′]), 4.54 (s, 2H CH_2Ph), 4.08 (m,4H, OCH_2-CH_2O), 3.36 (m, 1H, H−C[3′]) 1.02 (s, 3H, CH_3 [18′]).

Ethylene Acetal of 4-(3′a-Benzyloxy-17′-acetyl-5′β-estr-15′-en-17′-yl)furan-2-carboxyaldehyde (11). Compound **10** (2.3 g, 4.5 mmol) was dissolved in a mixture of pyridine (20 mL), acetic anhydride (10 mL), and dimethylaminopyridine (100 mg) and stirred overnight at room temperature. The reaction was terminated by the addition of 0.5 M HCl (30 mL). The product was extracted with diethyl ether, washed with 5% $NaHCO_3$, and dried over Na_2SO_4. Solvent removal afforded compound 11 (2.4 g, 96% yield) as an oily product. MS: *m*/*z* $[M - 60]^{\cdot+}$ 486. ^1H NMR: δ 7.32 (m, 5H, Ph), 7.19 (s, 1H, H−C[5]), 6.39 (d, 1H, H−C[15′]), 6.28 (s, 1H, H−C[3]) 6.10 (d, 1H, H−C[16′]) 5.87 (s, 1H, H−C−C[2]), 5.68 (d × d, 1H, H−C[15′]), 4.52 (s, 2H, CH_2Ph), 4.08 (m,4H, OCH_2-CH_2O), 3.36 (m, 1H, H−C[3′]), 2.02 (s, 3H, $OCOCH_3$), 1.02 (s, 3H, CH_3 [18′]).

Ethylene Acetal of 4-(3′a-Benzyloxy-15′β-hydroxy-5′β-estr-16′-en-17′-yl)furan-2-carboxyaldehyde (12). Compound **11** (1.8 g, 3.3 mmol) was dissolved in acetone (75 mL) and water (15 mL), and $CaCO_3$ (1 g) was added. The mixture was stirred and boiled for 3 days and the reaction monitored by TLC. After filtration, the acetone was removed under reduced pressure and the product dissolved in diethyl ether, washed with water, and dried over Na_2-SO_4. Solvent removal and flash chromatography on silica gel with diethyl ether:hexane (3:7) afforded compound **12** (0.9 g, 54% yield) as a yellow solid. MS: *m*/*z* ($M^{+\cdot}$) 504. IR 3550 cm^{-1}. ^1H NMR-($CDCl_3$) δ 7.52 (s, 1H, H−C[5]), 7.32 (m, 5H, Ph), 6.57 (s, 1H, H−C[3]), 5.94 (d, 1H, H−C[16′]), 5.90 (s, 1H, H−C−C[2]), 4.57 (m, 1H, H−C[15′] and s, 2H, CH_2Ph), 4.08 (m,4H, OCH_2CH_2O), 3.40 (m, 1H, H−C[3′]) 1.28 (s, 3H, CH_3[18′]).

Ethylene Acetal of 4-(3′a-Benzyloxy-15′β-hydroxy-5′β-estran-17′β-yl)furan-2-carboxyaldehyde (13). Compound **12** (0.5 g, 1.0 mmol) was dissolved in THF (9 mL), methanol (20 mL), and 5% NaOAc (0.6 mL), mixed with 10% Pd/$CaCO_3$ (150 mg), and hydrogenated at 50 psi for 6 h.. The solution was filtered and the solvent evaporated under reduced pressure. The product was dissolved in dichloromethane, washed with water, and dried over Na_2SO_4. Removal of the solvent afforded compound **13** (0.5 g, 99% yield) as a white solid. Crystallization afforded a white solid, mp 147−149 °C (from hexane:diethyl ether). X-ray diffraction measurements of compound **13** (Figure 1b) showed the 3′α-benzyloxy-15′β-hydroxy--5′β-estran-17′β-yl configuration. MS: *m*/*z* ($M^{+\cdot}$) 506. IR 3550 cm^{-1}. ^1H NMR: δ 7.34 (m, 5H, Ph), 7.20 (s, 1H, H−C[5]), 6.38 (s, 1H, H−C[3]), 5.85 (s, 1H, H−C−C[2]), 4.56 (s, 2H, CH_2Ph), 4.38 (m, 1H, H−C[15′]), 4.08 (m,4H, OCH_2CH_2O), 3.40 (m, 1H, H−C[3′]) 0.78 (s, 3H, CH_3 [18′]).

4(3′a-Benzyloxy-15′β-hydroxy-5′β-estran-17′β-yl)furan-2-carboxyaldehyde (14). A solution of **13** (200 mg, 0.39 mmol) in THF (14 mL) and 1 N HCl (7 mL) was stirred for 3 h. The solution was diluted with water and extracted twice with diethyl ether. The organic solution was washed with 5% $NaHCO_3$ and water and dried over anhydrous Na_2SO_4. Removal of the solvent afforded compound **14** (120 mg, 66% yield) as an oily product. MS: *m*/*z* ($M^{+\cdot}$) 464. ^1H NMR: δ 7.34 (m, 5H, Ph), 7.20 (s, 1H, H−C[5]), 6.38 (s, 1H, H−C[3]), 5.85 (s, 1H, H−C−C[2]), 4.56 (s, 2H, CH_2Ph), 4.38 (m, 1H, H−C[15′]), 3.40 (m, 1H, H−C[3′]) 0.78 (s, 3H, CH_3 [18′]).

4-(3′a,15′β-Dihydroxy-5′β-estran-17′β-yl)furan-2-carboxyaldehyde (15). A solution of compound **14** (300 mg, 0.65 mmol) in 10% formic acid in ethanol (10 mL) and 10% $Pd(OH)_2$/C (300 mg) was boiled for 60 min. The solution was filtered, and water (5 mL) was added. The product was extracted with dichloromethane, washed with water, and dried over Na_2SO_4. Removal of the solvent afforded compound **15** (220 mg, 91% yield) as a yellow solid. MS: *m*/*z* ($M^{+\cdot}$) 372.

4-(3′a15′β-dihydroxy-5′β-estran-17′β-yl)furan-2-methanol (16). $NaBH_4$ (200 mg) was slowly added to a solution of compound **15** (200 mg, 0.53 mmol) in THF (15 mL) and methanol (3 mL). After the exothermic reaction was completed, the solution was stirred for 1 h. Water (10 mL) was added, and the solution was stirred for 15 min. The organic solvents were removed under low pressure, and the remaining solution was extracted with diethyl ether, washed with water, and dried Na_2SO_4. Removal of the solvent afforded compound **16** (170 mg, 85% yield) as a white solid. HPLC measurement showed a large peak with a λ_{max} at 220 nm with more than 90% compound **16**. MS: *m*/*z* ($M^{+\cdot}$) 374. ^1H NMR: δ 7.20 (s, 1H, H−C[5]), 6.20 (s, 1H, H−C[3]), 4.58 (s, 2H, CH_2−O), 4.38 (m, 1Ht, H−C[15′]), 3.63 (m, 1H, H−C[3′]) 0.78 (s, 3H, CH_3 [18′]).^{13}C NMR: δ 153.96 (C2), 139.55 (C5), 125.69 (C4), 109.51 (C3), 71.91 (C3′), 71.04 (C6), 59.54 (C15′), 57.93 (C14′), 15.85 (C18′)

Biological Methods. Measurements of Heart Muscle Contractility. All experiments were carried out in accordance with the guidelines of The Hebrew University Ethics Committee. Guinea pigs were sacrificed by cervical dislocation. The hearts were immediately excised in Krebs−Henseleit bicarbonate buffer (composition in mM: 118.4 NaCl, 4.7 KCl, 25 $NaHCO_3$, 1.2 KH_2PO_4, 2 $CaCl_2$, 1.2 $MgSO_4$, and 5.5 glucose, pH 7.4). Papillary muscles were excised from the left and right ventricles, secured with silk thread to a polypropylene tissue holder, and mounted vertically in a 12 mL bath. The nutrient solution was aerated with 95% O_2/5% CO_2 and maintained at 36 °C. The papillary muscles were driven by a pair of platinum electrodes (filed stimulation) with a rectangular current pulse (1 Hz, 0.5 ms, about 1.2× threshold voltage) generated by an electronic stimulator (Master-8, A.M. P.I and a custom-made isolated current amplifier). The developed tension was measured isometrically with a force-displacement transducer (FSG-01, Experimetria) connected to a bridge amplifier (Lablinc, Coulbourn Instruments). The data were displayed and recorded on a PC based PowerLab/16sp system.

Human atrial appendages are routinely discarded in the course of open heart surgery. The tissue was transferred within minutes to the laboratory, dissected into isolated trabecula (5−10 mm long and 2−6 mm in diameter), and mounted on the same system as described above. The trabecula was driven at 0.5 Hz field stimulation, and the force of contraction was followed as described for the papillary muscle preparation.

Membrane Preparation. A crude synaptosomal membrane preparation was prepared from guinea pig brain by homogenizing whole brain (minus cerebellum) in 10 volumes (w/v) of ice-cold 0.32 M sucrose, using a Teflon−glass homogenizer (Zivan, Haifa, Israel). The homogenate was centrifuged at 1000*g* for 10 min at 4 °C. The supernatant was decanted and centrifuged at 28500*g* for 10 min at 4 °C. The pellet was re-suspended in the sucrose solution and recentrifuged under the same conditions. The resulting pellet was resuspended in 50 mM Tris buffer, pH 7.4, and dispersed, using the glass−glass homogenizer. The preparation was diluted to a final protein concentration of 5−10 mg/mL and was stored at −70 °C in aliquots of 1 mL until used.

^{3}H-Digoxin Binding to Synaptosomal Fraction. Two hundred microliters of synaptosomes (60 μg of protein) was incubated for 1 h at 37 °C with 300 μL of a solution containing final concentrations of 30 mM Tris-HCl buffer, pH 7.4, 0.4 mM EDTA, 80 mM NaCl, 4 mM $MgSO_4$, 2 mM ATP (Tris salt, vanadium-free), 40 nM ^{3}H-digoxin, and varying concentrations of nonradioactive digoxin, ouabain, bufalin, or **16**. The reactions were terminated

by the addition of 3 mL of ice-cold 50 mM Tris-HCl, pH 7.4, followed by passage over Whatman GF/B filters (Whatman International Ltd., Maidstone, U.K.). The filters were washed twice with 3 mL of Tris buffer, dried, and counted in a liquid scintillation counter. Specific binding was calculated by subtracting the binding observed in the presence of 100 μM unlabeled digoxin from that observed in the absence of unlabeled digoxin and representing 93% of the total binding under control conditions.

Cell Culture. Human neuronal precursor cells (NT2)) were obtained from Stratagene Cloning Systems (La Jolla, CA). The cells were grown in T25 tissue culture flasks in DMEM-F12 supplemented with 2 mM glutamine, 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, in an atmosphere of 5% CO_2 at 37 °C, as described previously.[31] The cells were grown to confluence and then split 1:4−1:5 by trypsinization and replated. In the fluorescence microscopy studies, cells were grown on glass coverslips, which were mounted with neutral contact glue on a 1.4 × 2.4-cm aperture in the center of 6-cm Petri dishes, 24−48 h before the experiments.

Fluorescence Microscopy. Conventional fluorescence microscopy was performed as previously described.[31] Images were acquired with a cooled SensiCAM charge-coupled device camera and analyzed using the IP Plus 4.1 version (Signal Analytics, San Diego, CA) software. Unlabeled cells were used to determine autofluorescence. The image background was corrected as follows: Two to three regions were selected from cell-free areas in each field and the average intensity of these regions was considered the background value for that field. This value was then subtracted from each pixel in the field. An integrated fluorescence power reading for each image was recorded after background correction. Images were saved in TIFF format and transferred to Adobe Photoshop version 5.5 software for printing.

Acknowledgment. This study was supported in part by The Nophar program of the Chief Scientist Office, Ministry of Industry, Trade & Labor, State of Israel.

Note Added after ASAP Publication. In the version of this paper posted December 20, 2005, an incorrect compound number (**16**) was present in part b of the Figure 1 caption. The compound number was changed to **13**, and the paper was reposted December 23, 2005.

Supporting Information Available: Detailed X-ray data is available free of charge via the Internet at http://pubs.acs.org.

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JM0505819