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Effect of Molecular Manipulation on the Estrogenic Activity of a Brain-Targeting Estradiol Chemical Delivery System

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The structural parameters important for biological efficacy of an estradiol chemical delivery system (CDS), a brain-targeting approach based on redox trapping, were examined by molecular manipulation of a prototype derivative, estradiol 17-(1-methyl-1,4-dihydronicotinate) (E2-CDS). Seven E2-CDS analogs in which the N-methyl substituent was altered were prepared including N-substituted short and medium straight chain alkyl, short branched chain alkyl, and aralkyl derivatives. Chemical and in vitro testing indicated that the most stable derivative was the *N*-benzyl E2-CDS. The analogs were tested in an intact male rat model to assess various central estrogenic manifestations including the rate of body weight gain, serum E2 and testosterone concentrations, and seminal vesicle, prostate and pituitary weight changes. Results indicated that all prepared CDS derivatives exerted some degree of central estrogenization with the most potent compounds being the parent E2-CDS and its ethyl homologue. Importantly, while the ethyl E2-CDS was equipotent to E2-CDS in various biological assays, it did not significantly elevate serum E2 compared to vehicle control at day 14.

Introduction

Estrogens, in general, and estradiol, in particular, exert a myriad of biological actions both in the brain and central nervous system (CNS) as well as in the periphery. Estrogen replacement therapy (ERT) has been used for some time to alleviate various vasomotor symptoms associated with menopause.[1–3] In addition, estradiol has been shown to protect neural cells in culture[4] and to improve blood flow through the mid-cerebral artery, suggesting a beneficial effect in the prevention of stroke.[5] Clinical trials have also shown that estrogen treatment can decrease certain types of memory loss associated with surgical menopause[6] and, most exciting, that estrogen replacement therapy may improve the prognosis of Alzheimer's disease by positively affecting a variety of the defining parameters.[7,8] The peripheral action of estrogen, such as its effects on circulation and bone, can also be salubrious, although systemic exposure has also been associated with increased risks of endometrial and breast cancer.[1,9] The ability to manage peripheral levels of estradiol may therefore allow optimization of therapeutic effect and minimization of potentially harmful side effects. One potential approach to this problem is brain targeting of estradiol through the use of a chemical delivery system (CDS).[10–14]

The CDS approach has been applied to a variety of pharmacologically active agents including estradiol and other sex steroids.[15–17] The method involves covalent attachment of a molecular targetor to the drug of interest to generate a molecular transport. In the case of estradiol, a 1-methyl-1,4-dihydronicotinate targetor has been connected to the 17-alcohol. The transport form (estradiol 17-(1-methyl-1,4-dihydronicotinate) (E2-CDS)) readily passes the blood–brain barrier (BBB), after which it is converted via oxidoreductases to the corresponding nicotinate salt (estradiol 17-(1-methylnicotinate), (E2-Q+)). While the E2-CDS is readily membrane permeable, the E2-Q+ has a much lower log *P* and is poorly membrane permeable. Thus, subsequent to oxidation, that portion of the CDS which is in the brain is trapped there due to its altered physicochemical and distributional profile while the polar E2-Q+ present in the systemic circulation is readily eliminated. In essence, E2-CDS provides for an intraorgan (brain) depot of the E2-Q+ which slowly hydrolyzes as a function of time to release active estradiol. The E2-Q+ is not estrogenic since manipulation of the 17-position is known to eliminate binding of the estrogen receptor.

Experimental evaluations confirm the proposed action of the E2-CDS. A single iv administration of doses as low as 0.5 mg/kg to ovariectomized rats induces prolonged pharmacological effects (3–6 weeks) as measured by luteinizing hormone (LH) suppression,[18,19] reduced rate of weight gain,[20–22] or, in castrate male rats, reestablishment of copulatory behavior.[23] Other studies have shown that a single iv administration of E2-CDS to rats was associated with rapid plasma elimination of estradiol (E2) while brain levels were high and sustained.[17,24] Yen and Sarkar reported that the action of the E2-CDS was centrally mediated at the levels of the hypothalamus since E2 release from the E2-CDS reduced portal plasma luteinizing hormone-releasing hormone (LHRH) levels and provided for increased LHRH hypothalamic concentrations.[24] Simpkins found that E2-CDS reversed the decrease in high-affinity choline uptake associated with castration in female rats, a finding which may be applicable to the action of estrogens in Alzheimer's disease.[25] Finally, E2-CDS has been successfully examined in clinical trials designed to establish the safety of this product.[26,27] Pharmacokinetic evaluations of these trials corroborate the kinetic superiority of E2-CDS as compared to E2 treatment.

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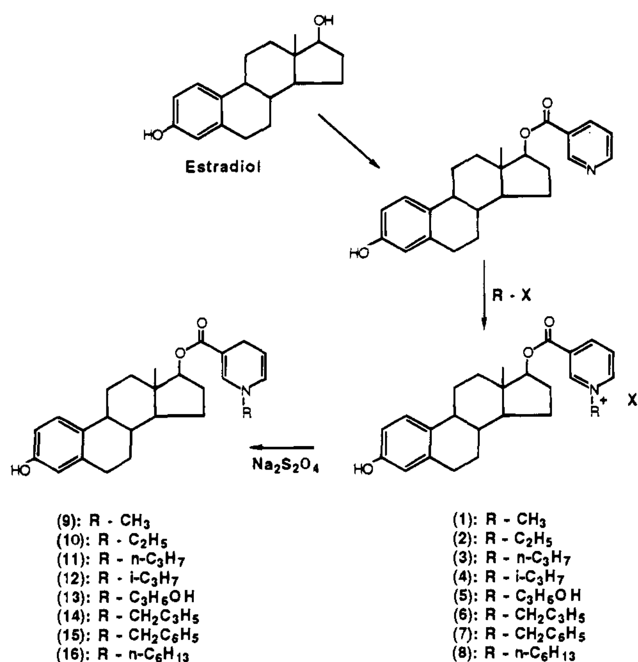
Scheme 1



While clinical evaluations are proceeding with the E2-CDS, additional manipulations have been considered to examine the structural importance of the targetor portion of the molecule. This report examines the effect of N-substitution on biological efficacy of E2-CDS.

Results and Discussion

The design of compounds used in this study reflects the manipulation of two basic properties, lipophilicity and dihydronicotinate stability. Model studies on 1-substituted 1,4-dihydronicotinamides indicated that homologous extension of the 1-methyl substituent gradually increases lipophilicity without dramatic effects on compound reactivity.[28] On the other hand, the benzyl substitution strongly stabilizes the dihydropyridine to oxidation. In the preparation of the estrogen CDS's, estradiol 17-nicotinate was alkylated with a variety of alkyl halides including methyl iodide, ethyl iodide, *n*-propyl iodide, isopropyl iodide, hydroxypropyl bromide, methylcyclopropyl bromide, benzyl bromide, and *n*-hexyl iodide to give rise to the corresponding quaternary salts. The methyl (**1**), ethyl (**2**), *n*-propyl (**3**), isopropyl (**4**), hydroxypropyl (**5**), methylcyclopropyl (**6**), benzyl (**7**), and *n*-hexyl (**8**) salts were then reduced in basic aqueous sodium dithionite to yield the corresponding 1-substituted 1,4-dihydronicotinates (**9**–**16**, respectively, Scheme 1).

The lipophilicity of the estradiol chemical delivery systems largely determines the uptake of the CDS through the BBB.[29] In general, the uptake of compounds with log *P* values less than zero is BBB-limited, while the uptake of compounds with log *P*'s greater than 1.0 is blood flow-limited. For compounds with log *P* values intermediate between 0.0 and 1.0, uptake is dependent both on the permeability of the compound through the BBB and the cerebral blood flow. Lipophilicities, as measured using an R_m (reversed-phase thin-layer chromatography) technique, are collected in Table 1.[30,31] The R_m method closely reproduced the $\log_{10}$ of the octanol:water partition coefficient of E2-CDS. As expected, the lipophilicity of the E2-CDS derivatives increases with increasing N-substituent length. Branching tends to slightly reduce lipophilicity so that the *n*-propyl derivative (**11**) is 0.2 log units more lipophilic than the isopropyl-substituted CDS (**12**). The *N*-benzyl analog (**15**) is the most lipophilic compound investigated with an R_m value indicating a 7-fold increase in lipophilicity as compared to the benchmark E2-CDS (**9**).

Table 1. Lipophilicity Values As Reported Using an R_m Technique for Various Substituted E2-CDS Derivatives

compd	R_m (extrapolated)	compd	R_m (extrapolated)
9	4.52	**13**	4.89
10	4.66	**14**	3.96
11	4.79	**15**	5.39
12	4.60	**16**	5.07

[a] The corresponding log *P* was determined to be 4.50.

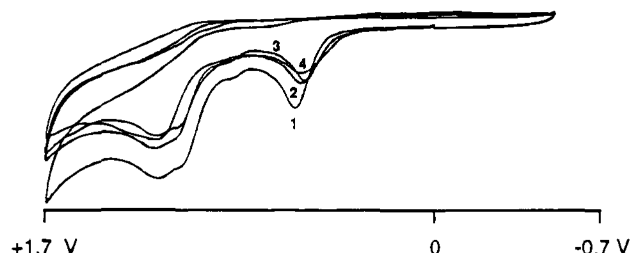


Figure 1. Cyclic voltammetry of selected E2-CDS derivatives. Solutions (1.0 mM) of various E2-CDS derivatives [1 = benzyl (**15**); 2 = *n*-propyl (**11**); 3 = hydroxypropyl (**13**); 4 = ethyl (**10**)] in acetonitrile containing 50 mM tetrabutylammonium perchlorate were examined using a Ag/AgCl reference electrode and Pt working electrode. The scan rate for all compounds was 100 mV/s at 100 μA/V. Other derivatives exhibited cyclic voltammagrams similar to 2, 3, and 4 as described above.

The stability of the dihydronicotinates is important in determining their suitability to act as delivery systems. Thus, the dihydropyridines must be sufficiently stable to be formulated and demonstrate a reasonable shelf-life but must undergo enzymatic oxidation rapidly enough to provide for E2-Q+ sequestration. Chemical stability was examined using cyclic voltammetry in which compounds were dissolved in a solution of acetonitrile containing a supporting electrolyte for electrochemical evaluation. As revealed in Figure 1, the oxidation is not reversible. The first anodic peak corresponds to an initial electron loss to give rise to the dihydropyridine radical cation.[33] In radically mediated oxidations, this is the rate-determining or partially rate-determining step.[34] The collected cyclic voltammograms suggest that most of the N-substituted E2-CDS analogs are grouped together with only small differences in their reactivity. By contrast, the *N*-benzyl derivative is conspicuously shifted to a higher potential consistent with its higher oxidative stability.

The biological stability of the CDS derivatives was evaluated spectrophotometrically in dilute rat brain and liver homogenates. Results are collected in Table 2. In all cases, the rates of degradation in the organ matrices were significantly faster than in phosphate-buffered saline ($t_{1/2}$ = days). In brain homogenates, the rates of oxidation of the short straight and branched chain *N*-alkyl-substituted compounds were similar with the *N*-propyl (**11**) derivative more reactive and the *N*-ethyl (**10**) analog less reactive than the E2-CDS (**9**) prototype. The *N*-type (**16**) and *N*-benzyl (**15**) derivatives were much less reactive due possibly to poor hydration in the case of the hexyl analog and low reactivity in the case

Table 2. Oxidation Rate Constants and First-Order Half-Lives of Various E2-CDS Derivatives in Brain and Liver Homogenate[a]

	brain homogenate		liver homogenate	
compd	$t_{1/2}$ (min) ± SEM	k (min^{-1})	$t_{1/2}$ (min) ± SEM	k (min^{-1})
9	121 ± 3	5.77×10^{-3}	32 ± 3	8.15×10^{-2}
10	198 ± 17	3.53×10^{-3}	22 ± 2	3.23×10^{-2}
11	94 ± 0.3	7.36×10^{-3}	52 ± 11	1.53×10^{-2}
12	120 ± 7	5.80×10^{-3}	26 ± 1	2.63×10^{-2}
13	123 ± 5	5.67×10^{-3}	29 ± 0.1	2.42×10^{-2}
14	114 ± 4	6.11×10^{-3}	38 ± 0.3	1.83×10^{-2}
15	288 ± 14	2.38×10^{-3}	79 ± 7	8.92×10^{-3}
16	259 ± 8	2.68×10^{-3}	56 ± 0.6	1.23×10^{-2}

[a] Data for half-lives are represented as the mean ± SEM for triplicate determinations.

of the benzyl E2-CDS. In liver homogenates, all of the compound oxidized more rapidly than in brain homogenates. The general profile of effects of substituents on reactivity was maintained, i.e., the hexyl and benzyl analogs were found to be the most stable while the other derivatives were similar to the benchmark E2-CDS in reactivity.

The biological activities of E2-CDS and its derivatives were examined in an intact male rat model. The eight analogs, along with a vehicle (DMSO) and estradiol control, were administered iv to rat at doses equimolar to 5.0 mg/kg of the E2-CDS. Brain-directed estradiol has significant effects on a number of biological parameters, including the rate of body weight gain in male and female rats. This effect is thought to be mediated at the level of the periventricular or ventromedial nuclei of the hypothalamus.[35] In the male rat, estradiol can interact via a negative feedback loop to reduce LHRH release at the hypothalamus and subsequently LH release at the pituitary to reduce testosterone steroidogensis at the level of the testis.[36] Direct effects of estradiol at the level of the Leydig cell are also possible. The reduced testosterone levels provoke reductions in peripheral androgen-dependent tissues such as the seminal vesicles and prostate gland. Brain-targeted E2 can also affect the pituitary even though this gland is technically outside of the BBB. This may occur because of a portal system (pituitary−hypothalamic) that connects the hypothalamus and pituitary allows for direct communication between these two loci.[37] On the basis of these interactions, the model selected examined the following endpoints: weight gain through day 14 post-drug administration, pituitary, seminal vesicle, and prostate weight at day 14, and blood estradiol and testosterone at day 14.

The effects of various drug treatment or control on body weight are shown in Figure 2. With the exception of the E2 group, all treatment groups demonstrated a significant effect on body weight at some point during the experiment. The treatment effects could be segregated into three groups. Vehicle and estradiol treatments were indistinguishable from each other and fell into a single group, while the methyl and ethyl derivatives exerted profound pharmacological effects and could be characterized as a single group. All other derivatives could be fitted into a third group with potency intermediate between the control group and the E2-CDS and ethyl homologue (**10**) groups. In terms of the dynamics of activity, body weight was already significantly different between vehicle control and E2-CDS (**9**−**12**)-

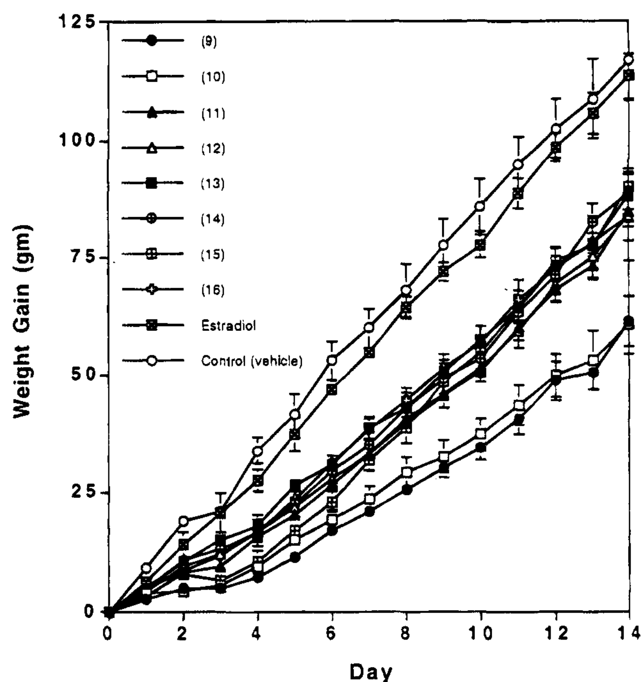


Figure 2. Effect of various E2-CDS derivatives in the rate of body weight gain in male rats. Vehicle, estradiol, or E2-CDS derivative were administered iv at a dose equimolar to 5.0 mg/kg E2-CDS (**9**). Data is represented as the mean ± SEM for six animals.

treated animals at day 1. These groups were not statistically distinguishable from each other at this time. By day 2, all of the treated animals with the exception of the E2 group had demonstrated a significantly reduced weight gain as compared to vehicle-treated animals. While there were intermittent changes in the pattern of response, most groups maintained a significantly reduced rate of weight gain through the 14 day time course of the experiment. When E2-CDS was used for comparison, differences between this compound and other analogs began to be prominent during the second week of the experiment. At days 9 and 11, E2-CDS was significantly more potent in suppressing body weight than any compound except that ethyl derivative, and at days 10, 12, and 13, E2-CDS was more effective than any compound except the ethyl (**10**) or *n*-propyl (**11**) derivative. By day 14, these differences disappeared for the most part due to increased heterogeneity in the collected data. These data indicate that E2-CDS (**9**) and the ethyl derivative of the E2-CDS (**10**) are the most potent derivatives examined while other delivery systems exerted significant but lessened action.

As indicated, E2, through its effects on the hypothalamus or by direct action on the testis, can reduce circulating testosterone levels and as a result reduce the weight of androgen-dependent tissues.[36] Serum testosterone was measured at day 14 after iv administration of vehicle or 5 mg/kg of the E2-CDS or equimolar doses of various E2-CDS derivatives. An estradiol control was also included in the study design. The results are given in Figure 3. All treatments tended to reduce serum testosterone, although significant reductions occurred only after dosing of the E2-CDS (**9**), the ethyl derivative (**10**), the methylcyclopropyl analog (**14**), and the hexyl derivative (**16**). The most potent compound in this respect was the ethyl analog which

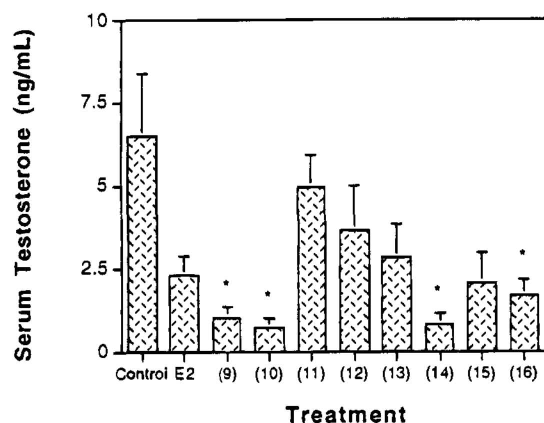


Figure 3. Effect of E2 or E2-CDS and its derivatives on serum testosterone 14 days subsequent to iv dosing. Testosterone was determined using a radioimmunoassay, and data is presented as the mean ± SEM of six animals.

Table 3. Effect of E2-CDS Derivatives on Seminal Vesicle, Prostate, and Pituitary Weights in Male Rats[a]

compd	seminal vesicle mg % ± SEM	prostate mg % ± SEM	pituitary mg % ± SEM
control (vehicle)	126.08 ± 4.56	87.69 ± 4.00	3.71 ± 0.16
estradiol	107.36 ± 3.66	62.26 ± 6.31[b]	3.88 ± 0.08
9	68.15 ± 7.85[b]	35.75 ± 3.85[b]	6.46 ± 0.46[b]
10	70.75 ± 6.81[b]	32.06 ± 3.27[b]	6.17 ± 0.33[b]
11	85.97 ± 5.68[b]	46.40 ± 3.14[b]	5.32 ± 0.17[b]
12	104.62 ± 4.98	68.64 ± 4.99	4.86 ± 0.27
13	106.49 ± 10.27	75.22 ± 2.31	4.60 ± 0.08
14	97.29 ± 8.39	54.31 ± 2.76[b]	4.40 ± 0.13
15	97.43 ± 4.38	67.38 ± 6.35	4.86 ± 0.17
16	95.66 ± 6.80	66.26 ± 4.31	5.28 ± 0.36[b]

[a] Data is represented as organ weight per 100 body weight ± SEM for six animals. [b] Significantly different from vehicle control.

reduced serum testosterone by almost 90% compared to vehicle-treated animals. The reduced serum testosterone was associated with reduced seminal vesicle and prostate gland weights. When organ weights were examined, all treatments except estradiol itself resulted in a significant decline in seminal vesicle weights and all treatments including the estradiol control induced significant involution in the case of the prostate. Since body weights were differentially reduced across the treatment groups, raw organ weights were converted to mg % values (Table 3) wherein organ weights were represented per 100 g of body weight. When these values were compared to DMSO controls, only E2-CDS (**9**) and the ethyl (**10**) and propyl (**11**) derivatives provided for a significant reduction in seminal vesicle weight. The prostate tended to be more sensitive to the effects of estrogen than did the seminal vesicles in that significant reduction in organ weights (mg %) were observed after estradiol as well as compounds **9**–**11** and **14**.

Pituitary hypertrophy can be related not only to estradiol in blood but also with estradiol delivery and release in brain due to a portal system that shunts blood directly from the hypothalamus to the pituitary.[37] The data indicated that E2-CDS (**9**) and the ethyl E2-CDS (**10**) derivative significantly increased the weight of the pituitary while other derivative tended to induce hypertrophy. When organ weights are normalized for body weight, several other treatments were found to induce significant hypertrophy including the *n*-propyl (**11**) and *n*-hexyl (**16**) E2-CDS treatment groups (Table 3).

Table 4. Effect of E2 or E2-CDS and Its Derivatives on Serum Estradiol 14 Days Subsequent to a Single iv Dose (Equimolar to 5 mg/kg of the E2-CDS (**9**)[a]

compd	serum E2 (pg/mL)	compd	serum E2 (pg/mL)
control (vehicle)	16.29 ± 1.09	**12**	43.64 ± 6.20
estradiol	22.20 ± 2.51	**13**	44.30 ± 5.96[b]
9	52.96 ± 5.64[b]	**14**	40.42 ± 4.14
10	37.68 ± 4.99	**15**	41.98 ± 3.48
11	42.93 ± 5.66	**16**	37.39 ± 6.10

[a] Estradiol was determined by means of a radioimmunoassay. [b] Significantly different from vehicle control.

Peripheral estradiol levels were examined at day 14 using radioimmunoassay. Results are collected in Table 4. All treatments of the estradiol derivatives tended to increase circulating estradiol levels, although only the prototype E2-CDS (**9**) and the hydroxypropyl E2-CDS (**13**) did so in a significant manner. Estradiol levels in rats at day 14 were a little more than 3-fold higher in E2-CDS-treated rats and 2.7 times higher in hydroxypropyl E2-CDS-dosed rats compared to vehicle-treated animals. Previous studies in rodents have indicated that E2 and E2-Q+ derived from E2-CDS are readily cleared from blood.[19,24] However, since E2-CDS is converted to the E2-Q+ in brain which serves as a long-term depot, estradiol is continuously being released from the CNS to blood as long as E2-Q+ is present in brain. Various studies have shown that doses of E2-CDS between 0.5 and 3.0 mg/kg provide for detectable levels of the E2-Q+ in brain for up to 1 month.[17] Pharmacokinetic studies in the rat suggest that after an initial distribution phase of 1–2 days, much of the estradiol detected in blood can be attributed to material initially delivered to the brain.[24] These data suggest that the ethyl derivative of E2-CDS (**10**) is as active as E2-CDS in the various biological parameters examined but does not significantly elevate estradiol levels compared to vehicle controls.

Pharmaceutically, while DMSO was used as a vehicle in these experiments, the further development of these drugs will require an acceptable dosage form. Chemically modified cyclodextrins have been useful in developing formulations of E2-CDS, and clinical trials have proceeded based on these systems. The degree of complexation of E2-CDS and (2-hydroxypropyl)-β-cyclodextrin (HPβCD) is approximately 40 mg/g. The analogs prepared in this study were screened for their solubility in HPβCD which may serve as a formulation adjunct. Comparative results are given in Figure 4. In general, the analogs were solubilized to a lesser extent than the parent compound (**9**) with the exception of the hydroxypropyl E2-CDS. The hydroxy group in the side chain may increase the equilibrium solubility of this derivative. The ethyl (**10**), propyl (**11**), and methylcyclopropyl (**14**) derivatives of the E2-CDS demonstrated solubilization of between 25 and 40% for the methyl benchmark (**9**), and the longer chain hexyl E2-CDS (**16**) and bulky benzyl E2-CDS (**15**) were solubilized only to an extent of 10% compared to the E2-CDS. Given the potency of these compounds, the extent of solubilization of the short chain alkyl derivatives is well within the useful range. Optimization of complexation conditions is expected to improve the degree of interaction.

In conclusion, several E2-CDS derivatives have been made and tested in various in vitro and in vivo models.

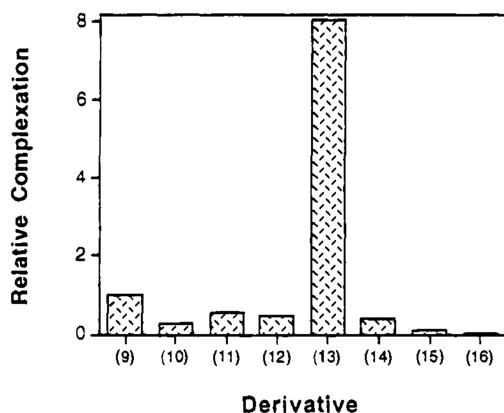


Figure 4. Relative degree of complexation of various E2-CDS derivatives with (2-hydroxypropyl)-β-cyclodextrin. In this figure, E2-CDS (**9**) is given a value of 1.0.

Of the synthesized analogs, the *N*-benzyl E2-CDS, appeared to be the most stable in organ homogenates and to electrochemical oxidation. N-Substitution by short straight or branched chain alkyl groups had only small effect on these parameters. When administered iv into male rats, the estradiol CDS's exerted potent and long-lasting suppression on the rate of weight gain, reduced serum testosterone, and concomitantly reduced the weight of androgen-dependent tissues and induced pituitary hypertrophy. The most potent compounds were consistently the E2-CDS (**9**) and its ethyl homologue (**10**). Interestingly, while the E2-CDS significantly elevated E2 levels in the male rat at day 14 after dosing compared to a vehicle control, the ethyl analog did not. This finding may suggest that the ethyl E2-CDS may be a potent brain-targeting CDS with an even lower potential to increase peripheral E2 levels than E2-CDS.

Experimental Section

Chemistry. Microcombustion analysis of compounds synthesized was performed by Atlantic Microlabs, Atlanta, GA. Uncorrected melting points (mp) were determined with either an Electrothermal or Thomas-Hoover melting point apparatus. Ultraviolet spectra (UV) were obtained on either a Hewlett-Packard 8451A diode array or Shimadzu UV-160 rapid scan spectrophotometer. Infrared spectra (IR) were recorded using a Perkin-Elmer 1600 Series FTIR. Samples were prepared in compressed KBr pellets. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were obtained on a Varian XL 300 (300 MHz, FT mode). Skeletal steroid absorbances were consistent in all structures and were, therefore, not reported. Samples were dissolved in an appropriate solvent and chemical shifts (δ) reported relative to tetramethylsilane. Thin-layer chromatography for in-process synthetic examination was performed on EM reagents DC-aluminum foil plates coated to a thickness of 0.2 mm with indicated silica gel (60 mesh). All chemicals were reagent grade and were obtained from either Aldrich Chemical Co. for Sigma Chemical Corp. Estradiol was obtained from Schering AG (Berlichem, Batch No. 27045909). Estradiol 17-nicotinate, E2-Q$^+$ (**1**), and E2-CDS (**9**) were prepared based on previously published methods.[15,16]

General Procedure for the Preparation of the Nicotinate Salts (E2-Q$^+$). To a solution of 13.8 g (36 mmol) of estradiol nicotinate in 100 mL of nitromethane was added 40.2 mmol of the appropriate alkyl halide. The solution was allowed to stir at 50 °C for 24 h. The solvent was removed in vacuo and the residue recrystallized from methanol or acetone.

1-Ethyl-3-[[[3-hydroxyestra-1,3,5(10)-trien-17β-yl]oxy]-carbonyl]pyridinium iodide (ethyl E2-Q+, 2): yield 65%; mp 263.1–263.7 °C; IR (KBr) ν (cm^{-1}) 3179 (phenolic OH stretch), 1718 (C=O stretch), 1617 (C=C stretch), 1299 (C–O–C asymmetric stretch), 1184 (C–O–C symmetric stretch), 745 (=CH out of plane bending); ^{1}H NMR (DMSO-d_6) δ ppm 9.60 (1H, s, pyridinium C-2), 9.41 (1H, m, pyridinium C-6), 9.02 (1H, m, pyridinium C-4), 8.35 (1H, m, pyridinium C-5), 7.05 (1H, d, phenol C-1), 6.55 (1H, d, phenol C-2), 6.45 (1H, s, phenol C-4), 4.99 (1H, t, steroid C-17), 4.84 (2H, q, N$^+$C*H*$_2$CH$_3$), 1.60 (3H, t, N$^+$CH$_2$C*H*$_3$), 0.98 (3H, s, steroid C18). Anal. ($C_{26}H_{31}O_3NI$) C, H, N, I.

1-Propyl-3-[[[3-hydroxyestra-1,3,5(10)-trien-17β-yl]oxy]-carbonyl]pyridinium iodide (propyl E2-Q+, 3): yield 75%; mp 271.6–272.0 °C; IR (KBr) ν (cm^{-1}) 3221 (OH stretch), 1731 (C=O stretch), 1614, 1579 (C=C stretch), 1495, 1457 (aromatic ring), 1305 (C–O–C asymmetric stretch), 1172 (C–O–C symmetric stretch), 1121 (C–O stretch), 873, 749 (=CH out of plane bending); ^{1}H NMR (DMSO-d_6) δ ppm 9.62 (1H, s, pyridinium C-2), 9.41 (1H, d, pyridinium C-6), 9.02 (1H, d, pyridinium C-4), 8.35 (1H, t, pyridinium C-5), 7.05 (1H, d, phenol C-1), 6.55 (1H, d, phenol C-2), 6.45 (1H, d, phenol C-4), 4.97 (1H, t, steroid C-17), 4.79 (2H, t, N$^+$CH$_2$), 0.98 (3H, s, steroid C-18), 0.95 (3H, t, CH$_2$CH$_3$ C-17). Anal. ($C_{27}H_{34}O_3NI$) C, H, N, I.

1-(2-Methylethyl)-3-[[[3-hydroxyestra-1,3,5(10)-trien-17β-yl]oxy]carbonyl]pyridinium iodide (isopropyl E2-Q+, 4): yield 45.8%; mp 269.8–270.4 °C; IR (KBr) δ (cm^{-1}) 3222 (OH stretch), 1723 (C=O stretch), 1615, 1579 (C–C stretch), 1747, 1459 (aromatic ring), 1303 (C–O–C asymmetric stretch), 1167 (C–O–C symmetric stretch), 1129 (C–O stretch); ^{1}H NMR (DMSO-d_6) δ ppm 9.60 (1H, s, pyridinium C-2), 9.50 (1H, d, pyridinium C-6), 9.02 (1H, d, pyridinium C-4), 8.35 (1H, t, pyridinium C-5), 7.05 (1H, d, phenol C-1), 6.52 (1H, d, phenol C-2), 6.45 (1H, d, phenol C-4), 5.29 (1H, m, N$^+$CH), 4.95 (1H, t, steroid C-17), 1.60 (6H, d, CH(C*H*$_3$)$_2$), 0.97 (3H, s, steroid C-18). Anal. ($C_{27}H_{34}O_3NI$) C, H, N, I.

1-(3-Hydroxypropyl)-3-[[[3-hydroxyestra-1,3,5(10)-trien-17β-yl]oxy]carbonyl]pyridinium bromide (hydroxypropyl E2-Q+, 5): yield 79.5%; mp 270.6–271.0 °C; IR (KBr), ν (cm^{-1}) 3344, 3211 (OH stetch), 1726 (C=O stretch), 1617, 1581 (C=C stretch), 1497, 1457 (aromatic ring), 1306 (C–O–C asymmetric stretch), 1178 (C–O–C symmetric stretch), 1120 (C–O stretch); ^{1}H NMR (DMSO-d_6) δ ppm 9.58 (1H, s, pyridinium C-2), 9.38 (1H, d, pyridinium C-6), 9.0 (1H, d, pyridinium C-4), 8.31 (1H, t, pyridinium C-5), 7.05 (1H, d, phenol C-1), 6.52 (1H, dd, phenol C-2), 6.46 (1H, d, phenol C-4), 4.94 (1H, t, steroid C-17), 4.86 (2H, t, N$^+$CH$_2$), 3.50 (2H, t, CH$_2$-OH), 0.98 (3H, s, steroid C-18). Anal. ($C_{27}H_{34}O_4NBr$) C, H, N, Br.

1-(Cyclopropylmethyl)-3-[[[3-hydroxyestra-1,3,5(10)-trien-17β-yl]oxy]carbonyl]pyridinium bromide (cyclopropyl E2-Q+, 6): yield 28.6%; mp 247.0–253.6 °C: IR (KBr), ν (cm^{-1}) 3158 (OH stetch), 1726 (C=O stretch), 1616, 1578 (C=C stretch), 1496, 1458 (aromatic ring), 1307 (C–O–C asymmetric stretch), 1167 (C–O–C symmetric stretch), 1124 (C–o stretch), 750 (=CH out of plane bending); ^{1}H NMR (DMSO-d_6) δ ppm 9.65 (1H, s, pyridinium C-2), 9.45 (1H, d, pyridinium C-6), 9.05 (1H, d, pyridinium C-4), 8.35 (1H, t, pyridinium C-5), 7.05 (1H, d, phenol C-1), 6.55 (1H, d, phenol C-2), 6.45 (1H, s, phenol C-4), 4.95 (1H, t, steroid C-17), 4.70 (2H, d, N$^+$C*H*$_2$), 0.98 (3H, s, steroid C-18), 0.66 (4H, d, 2 × (CH$_2$) cyclopropyl). Anal. ($C_{28}H_{34}O_3NBr{\cdot}H_2O$) C, H, N, Br.

1-(Phenylmethyl)-3-[[[3-hydroxyestra-1,3,5(10)-trien-17β-yl]oxy]carbonyl]pyridinium bromide (benzyl E2-Q+, 7): yield 57%; mp 253.0–253.6 °C; IR (KBr) ν (cm^{-1}) 3444 (OH stretch), 1738 (C=O stretch), 1634, 1580 (C=C stretch), 1496, 1457 (aromatic ring), 1293 (C–O© asymmetric stretch), 1162 (C–O–C symmetric stretch), 709 (=CH out of plane bending); ^{1}H NMR (DMSO-d_6) δ ppm 9.78 (1H, s, pyridinium C-2), 9.57 (1H, d, pyridinium C-6), 9.02 (1H, d, pyridinium C-4), 8.38 (1H, t, pyridinium C-5), 7.7 (2H, m, =CH *m*-, *m'*-phenyl), 7.50 (3H, m, =CH o-, o'-, *p*-phenyl), 7.05 (1H, d, phenol C-1), 6.55 (1H, d, phenol C-2), 6.45 (1H, s, phenol C-4), 6.20 (2H, s, N$^+$C*H*$_2$), 4.95 (1H, t, steroid C-17), 0.94 (3H, s, steroid C-18). Anal. ($C_{31}H_{34}O_3NBr$) C, H, Br.

1-Hexyl-3-[[[3-hydroxyestra-1,3,5(10)-trien-17β-yl]oxy]-carbonyl]pyridinium iodide (hexyl E2-Q+, 8): yield 50.6%; mp 209.6–210.1 °C; IR (KBr) ν (cm^{-1}) 3139 (OH stretch), 1734 (C=O stretch), 1619, 1581 (C=C stretch), 1498, 1459 (aromatic

ring), 1299 (C—O—C asymmetric stretch), 1168 (C—O—C symmetric stretch), 1127 (C—o stretch), 876, 753 (=CH out of plane bending); ^{1}H NMR (DMSO-d_6) δ ppm 9.60 (1H, s, pyridinium C-2), 9.40 (1H, d, pyridinium C-6), 9.02 (1H, d, pyridinium C-4), 8.35 (1H, t, pyridinium C-5), 7.05 (1H, d, phenol C-1), 6.51 (1H, d, phenol C-2), 6.45 (1H, s, phenol C-4), 4.95 (1H, t, steroid C-17), 4.77 (2H, t, N^+CH_2), 0.98 (3H, s, steroid C-18), 0.87 (3H, m, $(CH_2)_5CH_3$). Anal. ($C_{30}H_{40}O_3NI$) C, H, N, I.

General Procedure for the Preparation of the Dihydronicotinates (E2-CDS). To a ice-cold solution of 18.2 mmol of the appropriate estradiol 17-(1-substituted 1,4-dihydronicotinate) in 1.6 L of 50:50 acetonitrile:water was added 238 mmol (20 g) of $NaHCO_3$ and 173 mmol (30 g) of $Na_2S_2O_4$. After the mixture was stirred for 1.5 h under argon, 150 mL of methanol was added which induced separation of an oil. The residue was collected, recrystallized from methanol, and dried in vacuo (35 °C) to constant weight.

3-Hydroxy-17β-[[(1-ethyl-1,4-dihydropyridin-3-yl)carbonyl]oxy]estra-1,3,5(10)-triene (ethyl E2-CDS, 10): yield 83%; mp 191.7–193.2 °C; UV (MeOH) 358, 281 nm; IR (KBr) ν (cm^{-1}) 3402 (OH stretch), 1681 (C=O stretch), 1658, 1576 (C=C stretch), 1502, 1443 (aromatic ring), 1285 (C=O—C asymmetric stretch), 1177 (C—O—C symmetric stretch), 1069 (C—O stretch), 868 (=CH out of plane bending); ^{1}H NMR (DMSO-d_6) δ ppm 9.0 (1H, s, OH), 7.0–7.1 (2H, m, C-1 phenol + C-2 pyridine), 6.5 5(1H, d, C-2 phenol), 6.45 (1H, s, C-4 phenol), 5.9 (1H, d, C-6 pyridine), 4.7 (1H, m, C-5 pyridine), 4.5 (1H, m, C-17 steroid), 3.2 (2H, q, CH_2CH_3), 3.0 (2H, s, C-4 pyridine), 1.1 (3H, t, CH_2CH_3), 0.80 (3H, s, C-18 steroid). Anal. ($C_{26}H_{33}O_3N$) C, H, N.

3-Hydroxy-17β-[[(1-propyl-1,4-dihydropyridin-3-yl)carbonyl]oxy]estra-1,3,5(10)-triene (propyl E2-CDS, 11): yield 88%; mp 165.0–166.4 °C; UV (MeOH) 358, 281 nm; IR (KBr) ν (cm^{-1}) 3408, 3277 (OH stretch), 1681 (C=O stretch), 1657, 1575 (C=C stretch), 1502, 1440 (aromatic ring), 1285 (C—O—C asymmetric stretch), 1177 (C—O—C symmetric stretch), 1087 (C—OH stretch), 991 (=CH out of plane bending); ^{1}H NMR (DMSO-d_6) δ ppm 9.0 (1H, s, OH), 7.0–7.1 (2H, m, C-1 phenol + C-2 pyridine), 6.5 (1H, d, C-2 phenol), 6.45 (1H, s, C-4 phenol), 5.9 (1H, d, C-6 pyridine), 4.7 (1H, m, C-5 pyridine), 4.6 (1H, t, C-17 steroid), 3.1 (2H, m, NCH_2), 3.0 (2H, s, C-4 pyridine), 0.85 (3H, t, $(CH_2)_2$-CH_3), 0.80 (3H, s, C-18 steroid). Anal. ($C_{27}H_{35}O_3N{\cdot}0.5H_2O$) C, N, H.

3-Hydroxy-17β-[[[1-(2-methylethyl)-1,4-dihydropyridin-3-yl]carbonyl]oxy]estra-1,3,5(10)-triene (isopropyl E2-CDS, 12): yield 61%; mp 188.0–189.3 °C; UV (MeOH) 358, 281 nm; IR (KBr) ν (cm^{-1}) 3407 (OH stretch), 1679 (C=O stretch), 1656, 1577 (C=C stretch), 1500, 1439 (aromatic ring), 1261 (C—O—C asymmetric stretch), 1180 (C—O—C symmetric stretch), 713 (=CH out of plane bending); ^{1}H NMR (DMSO-d_6) δ ppm 9.0 (1H, s, OH), 7.0–7.1 (2H, m, C-1 phenol + C-2 pyridine), 6.5 (1H, d, C-2 phenol), 6.48 (1H, s, C-4 phenol), 6.0 (1H, d, C-6 pyridine), 4.7 (1H, m, C-5 pyridine), 4.5 (1H, t, C-17 steroid), 3.51 (1H, m, N-CH), 3.0 (2H, s, C-4 pyridine), 1.12 (6H, d, $CH(CH_3)_2$), 0.79 (3H, s, C-18 steroid). Anal. ($C_{27}H_{35}O_3N$) C, H, N.

3-Hydroxy-17β-[[[1-(3-hydroxypropyl)-1,4-dihydropyridin-3-yl]carbonyl]oxy]estra-1,3,5(10)-triene (hydroxypropyl E2-CDS, 13): yield 78.5%; mp 175.5–176.4 °C; UV (MeOH) 358, 281 nm; IR (KBr) ν (cm^{-1}) 3395 (OH stretch); 1678 (C=O stretch), 1656, 1594 (C=C stretch), 1500, 1448 (aromatic ring), 1285 (C—O—C asymmetric stretch), 1177 (C—O—C symmetric stretch), 1087 (C—O stretch), 870, 711 (=CH out of plane bending); ^{1}H NMR (DMSO-d_6) δ ppm 9.0 (1H, s, OH), 7.0–7.1 (2H, m, C-1 phenol + C-2 pyridine), 6.5 (1H, d, C-2 phenol), 6.44 (1H, s, C-4 phenol), 5.9 (1H, d, C-6 pyridine), 4.7 (1H, m, C-5 pyridine), 4.5 (3H, m, C-17 steroid + NCH_2), 3.2 (2H, t, CH_2OH), 3.0 (2H, s, C-4 pyridine), 0.79 (3H, s, C-18 steroid). Anal. ($C_{27}H_{35}O_4N$) C, H, N.

3-Hydroxy-17β-[[[1-(cyclopropylmethyl)-1,4-dihydropyridin-3-yl]carbonyl]oxy]estra-1,3,5(10)-triene (cyclopropylmethyl E2-CDS, 14): yield 61.6%; mp 180.1–180.8 °C; UV (MeOH) 358, 281 nm; IR (KBr) ν (cm^{-1}) 3369 (OH stretch), 1681 (C=O stretch), 1648, 1580 (C=C stretch), 1500, 1392 (aromatic ring), 1283 (C—O—C asymmetric stretch), 1177 (C—O—C symmetric stretch), 1091 (C—O stretch), 698 (=CH out of plane bending); ^{1}H NMR (DMSO-d_6) δ ppm 9.0 (1H, s, OH), 7.1 (1H, s, C-2 pyridine), 7.0 (1H, d, C-1 phenol), 6.5 (1H, d, C-2 phenol), 6.45 (1H, s, C-4 phenol), 5.9 (1H, d, C-6 pyridine), 4.7 (1H, m, C-5 pyridine), 4.5 (1H, t, C-17 steroid), 2.9–3.0 (2H, m, NCH + C-4 pyridine), 0.79 (3H, s, C-18 steroid), 0.46 (2H, d, cyclopropyl CH_2 trans), 0.2 (2H, d, cyclopropyl CH_2 cis). Anal. ($C_{28}H_{35}O_3N$) C, H, N.

3-Hydroxy-17β-[[[1-(phenylmethyl)-1,4-dihydropyridin-3-yl]carbonyl]oxy]estra-1,3,5(10)-triene (benzyl E2-CDS, 15): yield 82%; mp 167.8–169.0 °C; UV (MeOH) 358, 281 nm; IR (KBr) ν (cm^{-1}) 3374 (OH stretch), 1685 (C=O stretch), 1654, 1592 (C=C stretch), 1499, 1446 (aromatic ring), 1283 (C—O—C asymmetric stretch), 1184 (C—O—C symmetric stretch), 1077 (C—O stretch), 706 (=CH out of plane bending); ^{1}H NMR (DMSO-d_6) δ ppm 9.0 (1H, s, OH), 7.4 (2H, m-, m′-phenyl), 7.3 (3H, m-, p-, o-, o′-phenyl), 7.2 (1H, s, C-2 pyridine), 7.0 (1H, d, C-1 phenol), 6.55 (1H, d, C-2 phenol), 6.45 (1H, s, C-4 phenol), 5.9 (1H, d, C-6 pyridine), 4.7 (1H, m, C-5 pyridine), 4.6 (1H, t, C-17 steroid), 4.35 (2H, s, N-CH_2), 3.0 (2H, s C-4 pyridine), 0.75 (3H, s, C-18 steroid). Anal. ($C_{31}H_{35}O_3N$) C, H, N.

3-Hydroxy-17β-[[(1-hexyl-1,4-dihydropyridin-3-yl)carbonyl]oxy]estra-1,3,5(10)-triene (hexyl E2-CDS, 16): yield 85.7%; mp 156.7–158.4 °C; UV (MeOH) 358, 281 nm; IR (KBr) ν (cm^{-1}) 3326 (OH stretch), 1685 (C=O stretch), 1648, 1587 (C=C stetch), 1497, 1459 (aromatic ring), 1272 (C—O—C asymmetric stretch), 1174 (C—O—C symmetric stretch), 1093 (C—O stretch), 870, 718 (=CH out of plane bending); ^{1}H NMR (DMSO-d_6) δ ppm 9.0 (1H, s, OH), 7.0–7.1 (2H, m, C-1 phenol + C-2 pyridine), 6.5 (1H, d, C-2 phenol), 6.45 (1H, s, C-4 phenol), 5.86 (1H, d, C-6 pyridine), 4.75 (1H, m, C-5 pyridine), 4.55 (1H, t, C-17 steroid), 3.1 (2H, t, NCH_2), 3.0 (2H, s, C-4 pyridine), 0.83 (3H, t, $(CH_2)_5CH_3$), 0.78 (3H, s, C-18 steroid). Anal. ($C_{30}H_{41}O_3N{\cdot}0.25H_2O$) C, H, N.

Lipophilicity (R_m) Determinations. The lipophilicity of various E2-CDS derivatives was determined using an R_m method. Five microliters of a solution of **9–16** in methylene chloride were spotted 1 cm above the bottom of a C8 reversed-phase TLC plate (20 cm × 20 cm, Whatman, C8, 200 μm, with fluorescent indicator, no. 4808–820). The plates were then developed with various mixtures of acetone:water. The solvent was allowed to elute 11 cm up the plate (10 cm past the compound origin). The R_m was determined from the expression:

$$R_m = \log\left(\frac{1}{|R_f - 1|}\right)$$

where R_f is the distance traveled by the compound divided by the distance traveled by the solvent from (10 cm). Extrapolation of R_m values to 100% water gave the reported values. In all cases, the correlation coefficient of the extrapolated line was >0.99.

Cyclic Voltammetry of E2-CDS Derivatives. Cyclic voltammagrams were recorded on a BAS Model CV-1B cyclic voltammograph connected to a Houston Instruments Omnigraphic X—Y 100 recorder. Acetonitrile containing 50 mM of tetrabutylammonium perchlorate as a supporting electrolyte was used in all evaluations. Solutions were degassed with argon prior to all determinations. One mM solutions of the appropriate dihydronicotinate were scanned at 100 mV/s using a glassy carbon working electrode and a Ag/AgCl reference electrode at 100 μA/V sensitivity. Electrodes were polished with alumina powder (0.3 μm) and cleaned with deionized water and acetonitrile.

Determination of Relative Degree of Complexation of E2-CDS derivatives with 2-(Hydroxypropyl)-β-cyclodextrin. A solution of 43.5% w/v HPβCD was prepared by dissolving 100 g of the cyclodextrin in 200 mL of degassed, deionized (18.3 Ω) water. An excess of E2-CDS and each of the derivatives was then added to 10 mL of cyclodextrin solution and stirred for 6 h (protected from light). The suspensions were then centrifuged (3000g) and filtered (through 0.45 μm membranes), and the concentration of either E2-CDS or the E2-CDS derivatives was determined spectrophotometri-

cally at 360 nm. In this study, all of the E2-CDS derivatives have negligible solubility in water. Standard curves were prepared using a methanolic stock and were linear over the examined concentration range (>0.999). The solubilities of the derivative in the HPβCD solutions were **9** = 16.13 ± 0.42 mg/mL, **10** = 3.89 ± 0.11 mg/mL, 11 = 9.11 ± 0.65 mg/mL, **12** = 7.29 ± 0.85 mg/mL, **13** = 130.0 ± 8.92 mg/mL, **14** = 6.25 ± 1.08 mg/mL, **15** = 1.51 ± 0.44 mg/mL, and **16** = 0.34 ± 0.14 mg/mL.

In Vitro Stability in Brain and Liver Homogenate. Adult, male Sprague–Dawley rats were anesthetized with pentobarbital (65 mg/kg), the thoracic cavity was opened, the vena cava was cut, and 20 mL of phosphate-buffered saline was perfused through the left ventricle. The brain and 1.0 g of liver were removed and homogenized in a sufficient volume of phosphate-buffered saline to give a 2% w/v homogenate of brain and a 1.0% w/v homogenate of liver. Brain or liver homogenate (2.75 mL) was then placed in a cuvette fitted with a Teflon septum, and the cuvette was placed in the thermostated cell holder of a Hewlett-Packard 8451A diode array spectrophotometer and equilibrated at 37 °C. At time zero, 27.5 μL of a 5 × 10^{-3} DMSO solution of the appropriate dihydronicotinate were introduced into the cuvette via a Hamilton syringe. The disappearance of the band III absorbance (360 nm) was monitored. The pseudo-first-order rate constants were calculated by plotting the change in the log ($absorbance_{360}$) with time using an HP 85 dedicated microprocessor, and all determinations were made in triplicate.

In Vivo Animal Evaluation. Adult, male Sprague–Dawley rats (BW = 175–200 g) were obtained from Harlan Sprague–Dawley, Inc. (Indianapolis, IN) and housed in our vivarium. Animals were maintained at 23 ± 2 °C at a relative humidity of 55–70%. The light:dark cycle was 12:12 h with lights on at 0700 h. After acclimatization, conscious restrained (Broome-type) animals were injected via the lateral tail vein with vehicle (DMSO, 0.5 mL/kg) or E2-CDS or its analogs at a dose of 0.025 mmol/kg. This corresponds to a dose of 5 mg/kg of the E2-CDS. Animals were weighed before drug treatment and daily for 14 days subsequent to drug administration. At day 14, animals were euthanized, and blood was taken for serum E2 and testosterone determinations. Seminal vesicles, prostates, and pituitary glands were removed and weighed. Serum estradiol and testosterone were determined (in duplicate) by radioimmunoassay technique using a Coat-a-Count kit (Diagnostic Products Co., Los Angeles, CA). Cross-reactivity of the antibody used was <0.1% with E2-Q+, and the limits of detection for serum estradiol and testosterone were 10 pg/mL and 0.07 ng/mL, respectively.

Statistics. The significance of differences in mean body weight gain, organ weights (in mg or mg % of body weight), and serum estradiol and testosterone were analyzed using one-way ANOVA with post hoc Turkey's comparison. For all tests, the level of probability was $p < 0.05$.

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