[Chem. Pharm. Bull.
31(10)3740—3744(1983)]

An Affinity Labeling of Estrogen Receptor. II.[1] Synthesis and Biological Activity of 3-Hydroxy-17β-(*p*-nitrophenyldithio)-1,3,5(10)-estratriene

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(Received February 28, 1983)

3-Hydroxy-17β-(*p*-nitrophenyldithio)-1,3,5(10)-estratriene (Reagent A), an affinity labeling reagent for estrogen receptor, was prepared by iodine oxidation of an alkaline mixture of 3-hydroxy-1,3,5(10)-estratriene-17β-thiol and *p*-nitrothiophenol in 50% ethanol, and was isolated in a pure state. The structure was confirmed by the physical as well as spectral properties. The biological activity of the compound was tested by the measurement of the effects of ^{3}H-thymidine incorporation into deoxyribonucleic acid (DNA) and on ^{14}C-uridine incorporation into ribonucleic acid (RNA) in a human breast cancer cell line, MCF-7. There were increases of about 2-fold in thymidine incorporation and of about 1.1- to 2-fold in uridine incorporation in cells incubated in 10^{-7} M Reagent A compared with the control.

Keywords——estrogen receptor; affinity labeling; 3-hydroxy-17β-(*p*-nitrophenyldithio)-1,3,5(10)-estratriene; MCF-7; biological activity; DNA synthesis; RNA synthesis

The mechanism by which estrogens act on their target tissues has been extensively reviewed.[2–4] The initial event in the action of a steroid hormone on a target tissue is thought to be the formation of a complex between the hormone and a specific cytoplasmic receptor protein. However, the structure of the hormone binding site on estrogen receptor is virtually unknown. Elucidation of the structure is essential to clarify the mechanism of the hormone action, especially the formation of the complex. I previously identified the presence of a thiol group at the estrogen binding site on the receptor by the use of affinity labeling with the title compound.[1] The thiol group is thought to play an important role in estrogen binding.

I now report on the preparation of the affinity labeling reagent, 3-hydroxy-17β-(*p*-nitrophenyldithio)-1,3,5(10)-estratriene (Reagent A), by iodine oxidation of an alkaline mixture of 3-hydroxy-1,3,5(10)-estratriene-17β-thiol (Reagent B) and *p*-nitrothiophenol in 50% ethanol using the method suggested by Small *et al.*[5] and Miller *et al.*,[6] and present the results of an investigation of its biological activity by the measurement of the effects of the compound on deoxyribonucleic acid (DNA) and ribonucleicacid (RNA) syntheses in a human breast cancer cell line, MCF-7.

Experimental

Mass spectra (MS) were determined with a Hitachi RMU-6MG mass spectrometer, using a direct insertion probe: electron impact energy, 20 eV; temperature 220 °C. Nuclear magnetic resonance (NMR) spectra were determined in deuteroacetone solution with a Hitachi R-22 spectrometer (90 MHz) for 3-hydroxy-17β-(*p*-nitrophenyldithio)-1,3,5(10)-estratriene and a Varian 200 spectrometer (200 MHz) for 3-hydroxy-1,3,5(10)-estratriene-17β-thiol, using tetramethylsilane (TMS) as an internal reference. The infrared (IR) spectrum was determined with a Hitachi 285 spectrophotometer. Melting points were measured with a Yanagimoto micromelting point apparatus and are uncorrected.

The following materials were obtained from the sources indicated in parentheses: estrone (Sigma Chemical Co.),

p-nitrothiophenol (Kanto Chemical Co., Tokyo), iodine (Wako Chemical Co., Osaka), potassium iodide (Wako Chemical Co.), Eagle's minimum essential medium (Nissui) and newborn calf serum (Irvine Scientific). Reagent-grade organic solvents were purchased from Wako Chemical Co. Silica gel 60 or H was obtained from E. Merck A. G., Darmstadt, Germany.

3-Hydroxy-1,3,5(10)-estratriene-17β-thiol——This was prepared from 17-thiono-1,3,5(10)-estratriene by reduction with sodium borohydride.[7] Physical analysis showed: mp 193—195 °C; MS *m/e*: 288 (M^+), 255 (M−SH), 213; NMR (acetone-d_6) δ: 0.77 (3H, s, 18-C$\underline{H}_3$), 6.5—7.2 (3H, m, Ar-$\underline{H}$).

Synthesis of 3-Hydroxy-17β-(*p*-nitrophenyldithio)-1,3,5(10)-estratriene——A solution of 0.34 g (8.5 mmol) of sodium hydroxide, 0.54 g (1.9 mmol) of 3-hydroxy-1,3,5(10)-estratriene-17β-thiol and 1 g (6.5 mmol) of *p*-nitrothiophenol in 100 ml of 50% ethanol was treated with 1% I_2–KI solution with stirring at room temperature until the yellow color of *p*-nitrothiophenol vanished. The solution was left standing for several minutes, then acidified with 5% HCl and extracted with ethyl acetate. The extract was washed with water, dried over Na_2SO_4, and evaporated *in vacuo*. The resulting residue was chromatographed on a silica gel column using benzene as the eluting solvent, each eluate being checked by thin-layer chromatography (TLC) (benzene : ethyl acetate = 8 : 2, v/v). Two fractions were obtained, which showed *Rf* values of 0.60 and 0.50, respectively. These fractions were designated Compounds I and II, respectively.

Compound I: mp 182—184 °C. The melting point was not depressed on admixture with bis-(*p*-nitrophenyl) disulfide. MS *m/e* 308 (M^+).

Compound II: Repeated recrystallization from methanol–ethyl acetate–H_2O gave pale yellow plates (121 mg), mp 177.5—179.5 °C. MS *m/e*: 441 (M^+), 411 (M−NO), 255 (M−*p*-nitrophenyldithio), 213. IR ν_{max}^{Nujol} cm^{-1}: 3530, 1200 (OH), 1580, 1340 (NO_2), NMR (acetone-d_6): 0.87 (3H, s, 18-C$\underline{H}_3$), 6.5—7.2 (3H, m, Ar-$\underline{H}$), 7.8—8.4 (4H, m, Ar-$\underline{H}$). *Anal.* Calcd for $C_{24}H_{27}NO_3S_2$: C, 65.30; H, 6.17; N, 3.17. Found: C, 64.91; H, 5.80; N, 2.83.

Cell and Tissue Culture——MCF-7 cells obtained from Dr. Y. Nomura, National Kyushu Cancer Center Hospital, were maintained in Falcon plastic dishes (64 cm^2), and grown in 5% CO_2 in air at 37 °C. The growth medium consisted of autoclaved Eagle's minimum essential medium (MEM) supplemented with 1% non-essential amino acid, 2 mM L-glutamine, 8% calf serum, and 1% $NaHCO_3$. For at least one week prior to use, cells were grown in medium which was the same as the growth medium except that calf serum was replaced by charcoal-dextran-stripped calf serum prepared by incubating serum with dextran-coated charcoal twice at 40 °C for 30 min.

Effect of Reagent A on ^{14}C-Uridine and ^{3}H-Thymidine Incorporation into MCF-7 Cells——This was performed by the method of Lippman *et al.*[8] with slight modifications. Cells growing in log phase were suspended using trypsin–EDTA and plated in triplicate in plastic dishes in MEM supplemented with 1%steroid-free serum. After 24 h the medium was exchanged for fresh serum-free medium, and hormones were added in ethanol (the final concentration of ethanol was always less than 0.1%). After 24 h the medium was replaced, and hormones were again added, as mentioned above. After 36 h radioactive uridine and thymidine were added to each dish 2 h before the cells were harvested. Each dish contained 0.5 μCi of tritium and 0.25 μCi of ^{14}C-labeled precursor. Cells were harvested by washing the dishes with ice-cold phosphate-buffered saline (pH 7.4), suspending the cells in trypsin–EDTA and collecting cell pellets by centrifugation. Cell pellets were resuspended in ice water and sonically dispersed for 5 s in a sonicator at the lowest setting. Aliquots were then used for the determination of protein using the method of Lowry *et al.*[9] or for precipitation with 10% trichloroacetic acid. Acid-insoluble counts were collected and washed on 0.45-μm Millipore filters. The filters were counted in a solution containing 5.5 g of 2,3-diphenyloxazole, 667 ml of toluene and 333 ml of Triton X-100 with a Searle Mark III scintillation counter (efficiency for tritium, *ca.* 30%; efficiency for ^{14}C, *ca.* 60%).

The Purity of Reagents A and B——This was discussed previously.[1]

Results and Discussion

Preparation of 3-Hydroxy-17β-(*p*-nitrophenyldithio)-1,3,5(10)-estratriene

Compounds I and II were obtained by iodine oxidation of an alkaline solution of a mixture of *p*-nitrothiophenol and 3-hydroxy-1,3,5(10)-estratriene-17β-thiol.

Compound I was identified as bis-(*p*-nitrophenyl) disulfide because the melting point was not depressed on admixture with authentic bis-(*p*-nitrophenyl) disulfide and the MS displayed a peak at *m/e* 308 (M^+).

The molecular formula $C_{24}H_{27}NO_3S_2$ was assigned to Compound II from the elemental analysis and MS (M^+ at *m/e* 441). The IR spectrum of Compound II showed hydroxyl absorptions at 3530 and 1200 cm^{-1} and nitro group absorptions at 1580 and 1340 cm^{-1}.

The NMR spectrum of Compound II (Fig. 1) showed a four-proton multiplet due to the *p*-nitrophenyl group at δ 7.8—8.4[10,11] and three aromatic protons which were assigned to the

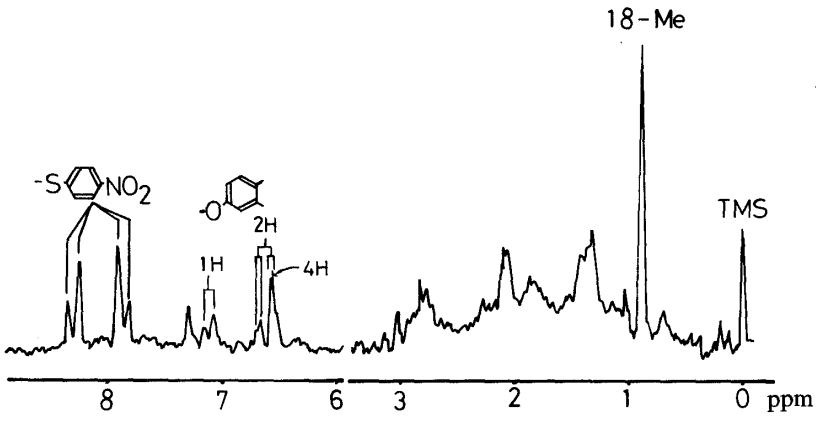

Fig. 1. NMR Spectrum of Compound II

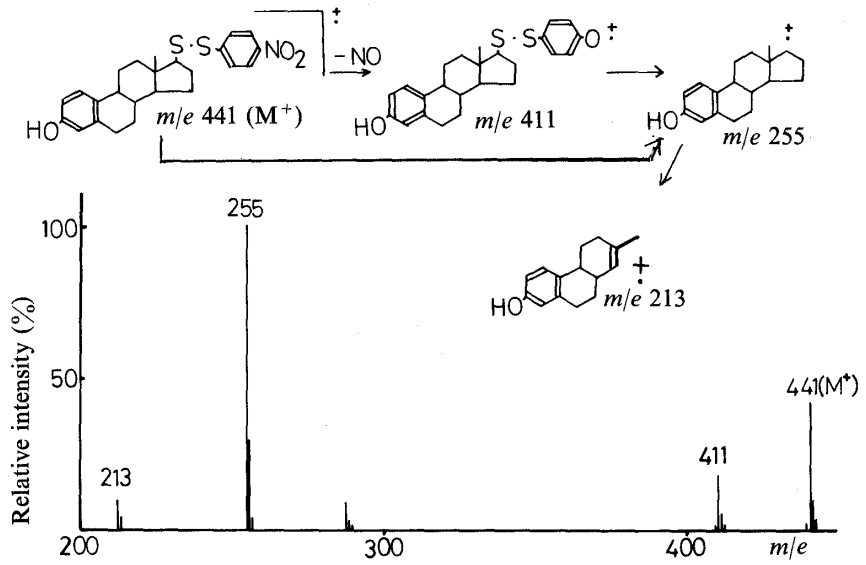

Fig. 2. Mass Spectrum and Fragmentation Pattern of Compound II

C-1 proton at δ 7.16, the C-2 proton at δ 6.67 and the C-4 proton at δ 6.63.[12] The spectrum showed a three-proton singlet due to the C-18 methyl group at δ 0.87. The spectrum of 3-hydroxy-1,3,5(10)-estratriene-17β-thiol showed a signal due to the C-18 methyl group at δ 0.77. The downfield shift (0.10 Hz) indicates a steric and/or diamagnetic anisotropy effect(s) of the *p*-nitrophenyl group on the angular methyl group.[13,14]

The mass fragmentation pattern of Compound II showed major peaks at *m/e* 441, 411, 255 and 213, as shown in Fig. 2.[15,16] Prominent peaks in the MS of 3-hydroxy-1,3,5(10)-estratriene-17β-thiol appeared at *m/e* 288 (M^+), 255 (M−SH) and 213. The coincidence of the ions at *m/e* 255 and 213 suggests that both compounds have the same structure except for the *p*-nitrophenylthio group.

These results showed that Compound II is 3-hydroxy-17β-(*p*-nitrophenyldithio)-1,3,5(10)-estratriene.

Biological Activity of 3-Hydroxy-17β-(*p*-nitrophenyldithio)-1,3,5(10)-estratriene

The biological activity of the compound was tested by measurement of the effects on ^{3}H-thymidine incorporation into DNA and ^{14}C-uridine incorporation into RNA in a human breast cancer cell line, MCF-7, which shows marked stimulation of incorporation of

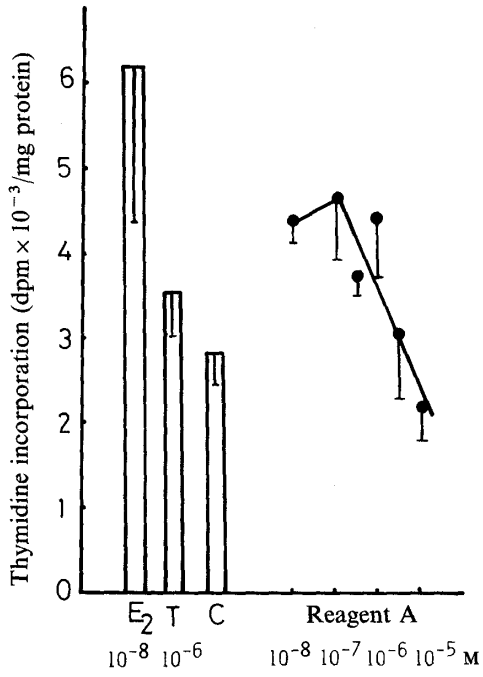

Fig. 3. Effects of Reagent A on Thymidine Incorporation on MCF-7 Cells *in Vitro*

Cells were incubated for 60 h in media containing Reagent A, estradiol (E_2), tamoxifen (T) or vehicle (C) at the concentrations shown, and each dish was pulsed with 0.5 μCi of [^{3}H]thymidine for 2 h prior to harvesting of the cells. All values are means of triplicate determinations ± S.D.

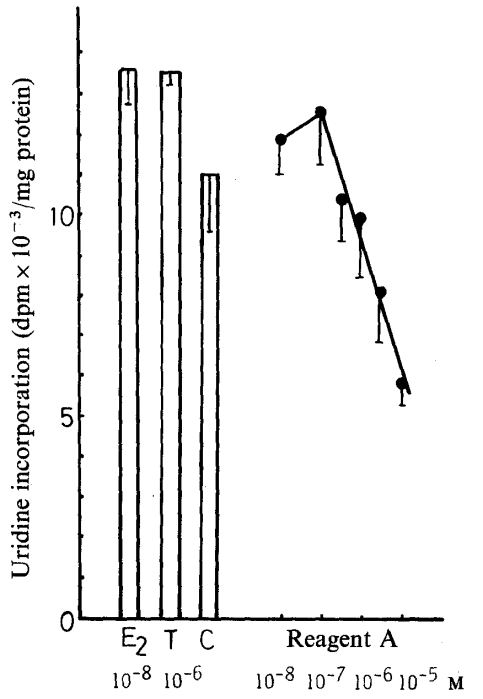

Fig. 4. Effects of Reagent A on Uridine Incorporation on MCF-7 Cells *in Vitro*

Cells were incubated for 60 h in media containing Reagent A, estradiol (E_2), tamoxifen (T) or vehicle (C) at the concentrations shown, and each dish was pulsed with 0.25 μCi of [^{14}C]uridine for 2 h prior to harvesting of the cells. All values are means of triplicate determinations ± S.D.

precursors into macromolecules by estradiol.[8] This experiment was performed under entirely serum-free conditions because Reagent A might react with serum albumin, as shown in the previous paper.[1] In Fig. 3, the effects of Reagent A addition on ^{3}H-thymidine incorporation into DNA in MCF-7 cells are shown. Thymidine incorporations in 10^{-8}—10^{-6} M Reagent A were approximately the same; there was an about 1.7-fold increase in thymidine incorporation in cells incubated in 10^{-7} M Reagent A compared with control values. This value is about 76% of the estradiol-stimulated value. The inhibition by 5×10^{-6} or 10^{-5} M Reagent A may be due to nonspecific toxicity.[8] The effects of Reagent A addition on ^{14}C-uridine incorporation into RNA in MCF-7 cells are shown in Fig. 4. The inhibition of ^{14}C-uridine incorporation into RNA in 5×10^{-6} or 10^{-5} M Reagent A may be due to nonspecific toxicity, as in the case of ^{3}H-thymidine incorporation into DNA. When the cells were incubated in 10^{-7} M Reagent A, the values of uridine incorporation show about 1.1-fold increase over the control although this increase cannot be regarded as statistically significant.

The effects of Reagent A addition on ^{3}H-thymidine incorporation into DNA and ^{14}C-uridine incorporation into RNA in MCF-7 cells were also measured in comparison with those of Reagent B. The results for DNA synthesis were as follows: 1740 for Reagent B (10^{-7} M), 1162 for Reagent A (10^{-7} M) and 548 dpm/mg protein for control. Those on RNA synthesis were as follows: 6565 for Reagent B (10^{-7} M), 7053 for Reagent A (10^{-7} M) and 3437 dpm/mg protein for control. The values are means of triplicate determinations.

Thus, Reagent A at 10^{-8}—10^{-6} M stimulated DNA synthesis about 1.7- to 2-fold compared to the control (about 70% of the Reagent B-stimulated value at 10^{-7} M), and 10^{-7} M Reagent A stimulated RNA synthesis about 1.1- to 2-fold compared to the control (approximately equal to the Reagent B-stimulated value at 10^{-7} M).

These results suggest that Reagent A associates with the estrogen receptor in the cell, forms a covalent bond with a thiol group at or near its estrogen binding site[1)] and then translocates into the cell nucleus, where it interacts with the chromatin.

Acknowledgement I thank Professor K. Hosokawa for his encouragement and Professor M. Yamato and Dr. T. Ishikawa, Faculty of Pharmaceutical Sciences, Okayama University, for the NMR spectral measurements (Hitachi R-22 spectrometer) and elemental analysis. This work was supported in part by Research Project Grants (No. 53-403, 54-404, 55-302, 55-306) from Kawasaki Medical School and by a Grant-in-Aid for Scientific Research, No. 244027, from the Ministry of Education, Science and Culture of Japan.

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