Stereochemistry of NaBH₄ Reduction of a 19-Carbonyl Group of 3-Deoxy Androgens. Synthesis of [19S-³H]- and [19R-³H]-Labeled Aromatase Inhibitors Having a 19-Hydroxy Group

Mitsuteru NUMAZAWA,* Norishige SOHTOME, and Masao NAGAOKA

Tohoku Pharmaceutical University; 4–4–1 Komatsusima, Aoba-ku, Sendai 981–8558, Japan.

Received February 4, 2004; accepted March 9, 2004

To study the stereochemical aspects of the aromatase reaction of androst-4-en-17-one (1) and its 5-ene isomer 4, competitive inhibitors of aromatase, the [19S-³H]- and [19R-³H]-labeled 19-hydroxy derivatives 2 and 5, were synthesized through NaB³H₄ reduction of the corresponding 19-aldehydes 3 and 6 as a key reaction. The hitherto unknown stereochemistry of the NaB³H₄ reduction was established based on the deuteration-labeling experiments with NaB³H₄. A comparison of 1H-NMR spectra of the NaB³H₄ reduction products of 19-als 3 and 6 with those of the respective authentic steroids revealed that there will be a difference in the stereochemistry of the corresponding 19-ols 2 and 6. Jones oxidation of the [19S-³H]19-ols 2 and 5, followed by the non-labeled NaBH₄ reduction, gave the corresponding [19R-³H]19-ols 4 and 6 (R-³H:S-³H=90:10 for steroid 2 and 70:30 for steroid 5). The stereoselectively ³H-labeled compounds 2 and 5 were similarly obtained in these sequences.

Key words aromatase inhibitor; isotope labeling; stereochemistry; [19-³H]19-hydroxy androgen; [19-²H]19-hydroxy androgen; NaB³H₄ reduction

Human placental aromatase is a cytochrome P-450 enzyme complex that catalyzes the conversion of androgens into estrogens.¹⁻³ This process is illustrated by the conversion of androst-4-ene-3,17-dione (androstenedione, AD) into estrone through three steps, each of which required 1 mol of O₂ and 1 mol of NADPH (Fig. 1).⁴ The first two steps occurred at the C-19 position to produce 19-hydroxy and 19-oxo intermediates, respectively.⁵⁻⁹) The stereospecific 19-pro R hydrogen loss of the 19-oxo intermediate in the second step, C-19 and 19-oxygenation has been established using the [19R-²H] hydrogen loss of the 19-hydroxy intermediate in the second step, C-19 and 19-oxygenation has been established using the [19R-²H] isotope labeling method. The [19S-³H]19-hydroxy sterol is stereospecifically obtained by the reduction of 19-oxoAD with NaB³H₄, and the oxidation of the 19 S steroid followed by the reduction with NaBH₄ stereospecifically yields the [19R-³H] stereoisomer. The last step, C-19 and 19-O₂ losses were eliminated as formic acid and water, respectively.¹¹⁻¹³)

Our laboratory has previously reported that 3-deoxy androgens, androst-4-en-17-one (1)¹⁴) and its 5-ene isomer 4,¹⁵) are powerful and good competitive inhibitors of aromatase (Fig. 2). The structure–activity relationships of 3-deoxy androgen analogs have indicated that the binding geometries of the two 3-deoxy steroids 1 and 4 in the active site of aromatase are different principally in the region of an A–B ring system of the steroid molecule.¹⁴⁻¹⁶) Gas chromatography-mass spectrometric analysis of the aromatase reaction of the inhibitors 1 and 4 has revealed that they are converted into the 19-oxo derivatives 3 and 6, through their 19-hydroxy intermediates 2 and 5, by aromatase, respectively, where the more potent inhibitor 1 is the less effective substrate. In contrast, the less potent inhibitor 4 is the more effective substrate.¹⁷) On the basis of these previous findings, it is predicted that there will be a difference in the stereochemistry of the 19-hydrogen loss between 19-ols 2 and 5 in the conversion into the corresponding 19-als 3 and 6. To determine this, we needed the stereoselectively 19-²H-labeled steroids 2 and 5. Thus, in this study, we initially explored the stereochemistry of the NaBH₄ reduction of 3-deoxy 19-als 3 and 6, then synthesized [19S-³H]19-ols 2 and 5 and their [19R-³H] isomers.

Results and Discussion

A strategy of the stereoselective ³H-labeling at C-19 of 19-hydroxy-4-en-17-oxo steroid 2 and its 5-ene isomer 5 employed a reduction of the corresponding 19-aldehydes 3 and 6 with NaB³H₄ as a key reaction. The hitherto unknown stereochemistry of the reduction was initially determined using the deuterium-labeling experiments and ³H-NMR spectroscopies; thus, the authentic [19S-³H]-labeled 19-hydroxy

Fig. 1. Mechanism of Androstenedione (AD) Aromatization

Fig. 2. Structures of 3-Deoxy Androgens

* To whom correspondence should be addressed. e-mail: numazawa@tohoku-pharm.ac.jp © 2004 Pharmaceutical Society of Japan
compounds 2 and 18 were synthesized, respectively, starting from [19S-2H]19-hydroxyAD and [19S-2H]3β-acetoxyandrost-5-ene-17β,19-diol (12) of which the stereochemistry at C-19 has previously been determined.10)

The former starting material [a ratio of S to R/H11005 90 : 10; 1H-NMR δ: 3.92 ppm (0.90H, H R) and 4.06 ppm (0.10H, H S)] was obtained by the reaction of 19-oxoAD with NaB 2H 4.10) Treatment of this with tert-butyldimethylsilyl (TBDMS) chloride in the presence of imidazole in DMF followed by a reductive deoxygenation with LiAlH 4 and AlCl 3 in THF 18) yielded [19S-2H]3-deoxy-17β-ol 8 (Fig. 3). This compound was oxidized with pyridinium dichromate in CH 2Cl2 to give the [19-2H]17-keto derivative 9 that was converted into the authentic [19S-2H]19-hydroxy-4-en-17-one 2 by treatment with diluted HCl.

Another starting material [17α,19S-2H2]17β,19-diol 12 [a 70 : 30 ratio of S to R; 1H-NMR δ: 4.47 ppm (0.70H, H R) and 3.94 ppm (0.30H, H S) as the triacetate derivative 13], that was obtained by treatment of 17,19-dione 11 with NaB 2H 4, was converted into [17α,19]-bis-TBDMS ether 14 by treatment with TBDMS chloride, as described above (Fig. 4). Hydrolysis of [3H2]compound 14 with NaOH, followed by treatment with p-toluene-sulfonyl (Ts) chloride in pyridine, gave [3H2]3β-tosylate 16. Reductive deoxygenation of compound 16 with zinc powder and NaI,19) followed by hydrolysis of the two TBDMS groups with diluted HCl, produced the authentic [17α,19S-2H2]17β,19-diol 18.

We then explored the stereochemistry of the NaB2H4 reduction of 3-deoxy-19-oxo steroids 3 and 6 (Fig. 5). The two 19-oxo compounds were separately treated with NaB2H4 to give [17α,19-2H2] diols 19 and 18 as well as the [19-2H]19-hydroxy-17-oxo derivatives 2 and 5, respectively. In the experiment with the 5-ene steroid 6, [17α-2H]17β-hydroxy-19-one 20 was also isolated, in addition to the two products, suggesting that a 17-carbonyl function of compound 5 would be more reactive toward NaBH4 than the 19-carbonyl function under the conditions used. A comparison of 1H-NMR spectra of the 2H-labeled compounds 2 and 18 with those of the respective authentic samples obtained above revealed that the major product was [19S-2H]-labeled product in each case, where the stereoselectivity of the reactions was S:R = 90 : 10 for 4-ene 3 and 70 : 30 for 5-ene 6 (Fig. 6). This indicates that the stereoselective si-face attack of the borohydride reagent gives rise principally to the (19S) introduction of the labeled group. The stereoselectivities obtained were almost the same as those previously reported in the borohydride reductions of the corresponding 3-deoxygenated compounds, 19-oxoAD and 3β-acetoxy-5-ene steroid 10.10) A 19-carbonyl function of 19-oxoAD and steroid 10 is thought to be oriented in the out-of-ring position, then the borohydride reagent approaches the carbonyl carbon from the less hindered over-A-ring side rather than the crowded over-C-ring side, thereby stereoselectively giving the [19S-2H]-labeled 19-hydroxyAD and compound 12.10,20) We have previously reported the conformational analysis of 4-en-19-oxo steroid.
ably from the C(4)–C(5) edge, resulting in the reagent from the less hindered over-A-ring side, presum-
ably similar to that involved in the reduction of the 3 18-isomer and its 5-ene.

3 with semiempirical molecular orbital PM3 calculations, indica-
ting that the 19-oxo function favors the over-A-ring con-
formation with semiempirical molecular orbital PM3 calculations, in-
31°. 21) This conformation would also allow the attack of the 
borohydride conformation of a C-19-carbonyl group of another 19-al, respectively) through Jones oxidation, the non-labeled 
NaBH₄ reduction, and the HPLC purification. Their chemical and radiochemical purities were determined to be more than 98%, based on the HPLC analysis.

To understand the steric course of the aromatase reaction of 3-deoxy-19-ols 2 and 5, incubation studies of the stereose-
lectively [3H]-labeled substrates 2 and 5 with human pla-
cental aromatase are now underway.

Experimental
Melting points were measured on a Yanagimoto melting point apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer FT-IR 1725 spectrometer in KBr pellets, and 1H-NMR spectra were obtained in CDCl₃ solution with a JEOL EX270 (270 MHz) spectrometer using tetra-
ethylsilane as an internal standard. Mass spectra (MS) was obtained with a JEOL JMS-DX303 spectrometer. Column chromatography was conducted with silica gel (E. Merck, Darmstadt, Germany, 70–230 mesh) (solvent: hexane–EtOAc). TLC was performed on E. Merek precoated TLC silica gel plates (silica gel 60F-254, layer thickness 0.25 and 0.5 mm for analytical and radiochemical purities were determined to be more than 98%, based on the HPLC analysis.

Synthesis of the Authentic [19S-2H]19-Hydroxyandrost-4-en-17-one [2] [19S-2H]19-Hydroxy-AD (150 mg, 0.50 mmol), which was synthesized by treatment of 19-oxo AD with NaBH₄ according to the method of Osawa’s group,10) was initially treated with TBDMS chloride (112 mg, 0.74 mmol) and imidazole (50 mg, 0.74 mmol) in DMF (3 ml), according to the method previously reported,22) to give [19S-2H]-3-Hydroxyandrost-4-en-17-one (150 mg, 87%) (Fig. 3): mp 156—

157 °C (from acetone) (lit. 22) mp 156—157 °C for non-labeled 7). 1H-NMR δ: 0.03 and 0.04 (3H, s, 19-H), 3.89 (0.10H, s, 19-H), 5.88 (1H, dd, J=8.1, 8.7 Hz, 17α), 3.77 (0.90H, s, 19-H), 3.81 (0.10H, s, 19-H), 5.40 (1H, m, 4-H). A solution of the [3H]19-silyloxy steroid 7 (180 mg, 0.43 mmol) in THF (4 ml) was treated with a mixture of LiAlH₄ (44 mg, 1.15 mmol) and AlCl₃ (515 mg, 3.86 mmol) in ether (10 ml) for 1 h under reflux, according to the method previously reported,19) and then the product was purified by column 
HPLC (C18 column, CH3CN–H2O) in the former experiment. The products were purified by reverse-phase HPLC (C₁₈ column, CH₃CN–H₂O) in the latter experiment. The products were purified by reverse-phase HPLC (silica, hexane–THF) in the later, giving the corresponding [19S-2H]19-ols 2 and 5 (specific activity: 0.61 and 0.39 mCi/mmol, respectively). The [19S-2H] compounds were converted into the corresponding [19R-2H] isomers (specific activity: 0.55 and 0.21 mCi/mmol for 2 and 5, respectively) by Jones oxidation, the non-labeled 
NaBH₄ reduction, and the HPLC purification. Their chemical and radiochemical purities were determined to be more than 98%, based on the HPLC analysis.

To understand the steric course of the aromatase reaction of 3-deoxy-19-ols 2 and 5, incubation studies of the stereose-
lectively [3H]-labeled substrates 2 and 5 with human pla-
cental aromatase are now underway.

Experimental
Melting points were measured on a Yanagimoto melting point apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer FT-IR 1725 spectrometer in KBr pellets, and 1H-NMR spectra were obtained in CDCl₃ solution with a JEOL EX270 (270 MHz) spectrometer using tetra-
ethylsilane as an internal standard. Mass spectra (MS) was obtained with a JEOL JMS-DX303 spectrometer. Column chromatography was conducted with silica gel (E. Merck, Darmstadt, Germany, 70–230 mesh) (solvent: hexane–EtOAc). TLC was performed on E. Merek precoated TLC silica gel plates (silica gel 60F-254, layer thickness 0.25 and 0.5 mm for analytical and preparation use, respectively; solvent: hexane–EtOAc). The HPLC system consisted of a Waters model 510 pump (Milford, MA, U.S.A.) and a Soma UV detector (Tokyo, Japan).

Synthesis of the Authentic [19S-2H]19-Hydroxyandrost-4-en-17-one [2] [19S-2H]19-Hydroxy-AD (150 mg, 0.50 mmol), which was synthesized by treatment of 19-oxo AD with NaBH₄ according to the method of Osawa’s group,10) was initially treated with TBDMS chloride (112 mg, 0.74 mmol) and imidazole (50 mg, 0.74 mmol) in DMF (3 ml), according to the method previously reported,22) to give [19S-2H]-3-Hydroxyandrost-4-en-17-one (150 mg, 87%) (Fig. 3): mp 156—

157 °C (from acetone) (lit. 22) mp 156—157 °C for non-labeled 7). 1H-NMR δ: 0.03 and 0.04 (3H, s, 19-H), 3.89 (0.10H, s, 19-H), 5.88 (1H, dd, J=8.1, 8.7 Hz, 17α), 3.77 (0.90H, s, 19-H), 3.81 (0.10H, s, 19-H), 5.40 (1H, m, 4-H).

A solution of the [3H]19-silyloxy steroid 7 (180 mg, 0.43 mmol) in THF (4 ml) was treated with a mixture of LiAlH₄ (44 mg, 1.15 mmol) and AlCl₃ (515 mg, 3.86 mmol) in ether (10 ml) for 1 h under reflux, according to the method previously reported,19) and then the product was purified by column chromatography, yielding [19S-2H]-3-Hydroxyandrost-4-en-17-one (8) (54 mg, 32%): mp 106–106 °C (from acetone) (lit.14) mp 105.5—

106 °C for non-labeled 8). 1H-NMR δ: 0.03 and 0.04 (3H, each, s, OSiMe₂), 0.87 (9H, s, SiCMe₃), 0.88 (3H, s, 18-Me), 3.61 (1H, dd, J=8.1, 8.7 Hz, 17α), 3.77 (0.90H, s, 19-H), 3.81 (0.10H, s, 19-H), 5.40 (1H, m, 4-H). The [3H]17β-ol (8) (49 mg, 0.12 mmol) was treated with pyridinium dichro-
matate (53 mg, 0.14 mmol) in CH₃Cl, 2 ml) at room temperature for 10 h, di-
luted with EtOAc (50 ml), washed with 5% HCl, 5% NaHCO₃ solution, and H₂O, sequentially, and dried with NaSO₄. Evaporation of the solvent that was a solid was recrystallized from MeOH, yielding [19S-2H]-3-Hydroxyandrost-4-en-17-one (9) (28 mg, 57%): mp 66–66.5 °C (lit.14) mp 65.5—66 °C for non-labeled 9). 1H-NMR δ: 0.03 and 0.04 (3H, each, s, OSiMe₂), 0.88 (9H, s, SiCMe₃), 0.89 (3H, s, 18-Me), 3.79 (0.90H, s, 19-H), 3.83 (0.10H, s, 19-H), 5.42 (1H, m, 4-H).

The [3H]19-silyloxy compound 9 (28 mg, 0.07 mmol) was hydrolyzed with 3 m HCl (0.3 ml) in THF (0.5 ml) and propan-2-ol (0.8 ml) at room tempera-
ture, according to the previous method,19) affording [19S-2H]-19-hydroxy-

\[ {^3}H-NMR \delta: 19-H; 3.52 \text{ppm (0.10H, } H_r) \text{ and } 3.95 \text{ppm (0.90H, } H_s) \text{ for 2 and 3.89 ppm (0.30H, } H_r) \text{ and 3.59 ppm (0.70H, } H_s) \text{ for 5.} \]
The crude [1H]compound 16 (210 mg) was, without isolation, treated with Zn powder (110 mg, 0.88 mmol) and NaI (543 mg, 1.21 mmol) in ethylendioxy dimethyl ether (6 ml) and H2O (0.5 ml) under reflux for 2 h. After this, the mixture was diluted with EtOAc (200 ml), washed with H2O, and dried with Na2SO4. Evaporation of the solvent gave an oil that was purified by column chromatography and recrystallized from acetone, yielding [17a,19S,H]-17β-bis(tert-butyldimethylsiloxy)androsten-5-ene (7 mg, 32%), 74—75 °C. 1H-NMR δ: 0.002, 0.009, 0.020 and 0.031 (1H each, s, OMeS), 0.74 (3H, s, 18-Me), 0.87 and 0.89 [9H each, s, SiCMe3], 3.56 (0.30H, s, 19-H), 3.94 (0.70H, s, 19-H), 3.60 (1H, m, 17α-H), 5.46 (1H, m, 6-H). MS m/z: (relat. int.): 520 (M-1, 1), 463 (300), 36 (36), and 331 (20). Non-labeled compound 17 was also obtained as described above. Compound 17: mp 74—75 °C. 1H-NMR δ: 0.002, 0.009, 0.020 and 0.031 (1H each, s, OMeSi), 0.74 (3H, s, 18-Me), 0.87 and 0.89 [9H each, s, SiMe3], 3.56 and 3.94 (1H each, d, J=10.5 Hz, 19-CH), 3.60 (1H, m, 17α-H), 5.46 (1H, m, 6-H). Anal. Caled for C21H32O4: C, 72.44; H, 9.36. Found: C, 72.44; H, 9.36.

Reaction of 19-Oxo-4-ene Steroid 3 with NaBtH4. NaBtH4 (1.9 mg, 50 μmol) was added to a solution of 19-oxo-4-ene 3 (67 mg, 0.23 mmol) in dry MeOH (1 ml) under ice-cooling, and the mixture was stirred for 1 h. After this time, NaBH4 (1.2 mg, 31 μmol) was added to the mixture, and the mixture was further stirred at 0 °C for 2.7 h, diluted with EtOAc (50 ml), washed with H2O, and dried with Na2SO4. After evaporation of the solvent, the residue obtained (71 mg) was purified by preparative TLC (hexane–EtOAc: 3:1, v/v, multiple developments), yielding two products (Fig. 5). The less polar product was recrystallized from acetone to give [19S-H]-19-hydroxyandrosten-4-ene-17,19-dione (7 mg, 70%), mp 126—127 °C (mp for non-labeled 18S, 137—138 °C). 1H-NMR δ: 0.82 (3H, s, 18-Me), 3.57 (0.30H, s, 19-H), 3.84 (0.70H, s, 19-H), 5.68 (1H, m, 6-H). MS m/z: (relat. int.): 292 (M+, 22), 260 (100), 242 (40) (d1; 11; d2; 89).

Reaction of 19-Oxo-4-ene Steroid 2 with NaBtH4. NaBtH4 (1.9 mg, 50 μmol) was added to a solution of 19-oxo-4-ene 3 (67 mg, 0.23 mmol) in dry MeOH (1 ml) under ice-cooling, and the mixture was stirred for 1 h. After this time, NaBH4 (1.2 mg, 31 μmol) was added to the mixture, and the mixture was further stirred at 0 °C for 2.7 h, diluted with EtOAc (50 ml), washed with H2O, and dried with Na2SO4. After evaporation of the solvent, the residue obtained (71 mg) was purified by preparative TLC (hexane–EtOAc: 3:1, v/v, multiple developments), yielding two products (Fig. 5). The less polar product was recrystallized from acetone to give [19S-H]-19-hydroxyandrosten-4-ene-17,19-dione (7 mg, 70%), mp 126—127 °C (mp for non-labeled 18S, 137—138 °C). 1H-NMR δ: 0.82 (3H, s, 18-Me), 3.57 (0.30H, s, 19-H), 3.84 (0.70H, s, 19-H), 5.68 (1H, m, 6-H). MS m/z: (relat. int.): 292 (M+, 22), 260 (100), 242 (40) (d1; 11; d2; 89).
After evaporation of the solvent, the oily product obtained was purified by preparative TLC (hexane- EtOAc, 2:1, v/v), affording two stereoidal fractions. The less polar fraction (R_f=0.65) was further purified by normal phase HPLC using a silica gel column (R-SIL-5-06, 4.6 x 250 mm) (YMC, Kyoto, Japan) and hexane-THF (75: 25, v/v, 1 ml/min) as a mobile phase, giving two compounds. [19-3H]-19-hydroxyandrosten-5-ene-17β,19-diol (5) (6 mg, 8%) (δ=8.1 min) and [17α-3H]-17α-hydroxyandrosten-5-ene-19-one (20) (23 mg, 28%) (δ=9.6 min). [19-3H]-Compounds 5: mp 86–90 °C (lit. mp 120–124 °C for non-labeled 6). [1H]-NMR δ: 0.94 (3H, s, 18-Me), 3.59 (0.30H, d, J=13.5 Hz, 19-H), 3.89 (0.70H, d, J=13.5 Hz, 19-H), 5.72 (1H, s, 6-H). MS (relat. int.): 287 (M⁺, 29), 250 (100), 239 (40) (δ 14, d; 90). [1α-3H]-Compounds 20: mp 125–131 °C (lit. mp 145–151 °C for non-labeled 19). 1H-NMR δ: 0.70 (3H, s, 18-Me), 5.75 (1H, m, 6-H), 6.69 (1H, d, J=1.5 Hz, 19-CHO). MS (relat. int.): 287 (M⁺, 11), 257, 240, 224 (50) (δ 10, d; 90). The starting material (6 mg, 41%) was recovered from the more polar fraction on TLC (R_f=0.40). Treatment of the recovered steroid 6 (41 mg, 0.143 mmol) with an excess of NaBH₄ (0.11 mmol) and a longer reaction time (2.5 h), followed by the preparative TLC and the subsequent HPLC, as described above, yielded [17α,19S]-19-hydroxy-3α,5α-androstan-17β-ol (2), 19,21-diol (18) (9 mg, 22%) (R_f=0.40) as well as [17α-3H]-19-ol (1.7 mg, 4%) and [17α-3H]-17β-ol (20) (23 mg, 56%). [17α-3H]-Diol 18: mp 79–85 °C (lit. mp 137–138 °C for non-labeled 18). 1H-NMR δ: 0.82 (3H, s, 18-Me), 3.57 (0.30H, br s, 19-H), 3.83 (0.70H, br s, 19-H), 5.68 (1H, m, 6-H). MS (relat. int.): 292 (M⁺, 18), 260 (100), 242 (54) (δ 2, d; 12, d; 86).

Synthesis of [19-3H]-19-hydroxy-5-ene Steroid 5 Jones reagent was added dropwise to a solution of [17α,19S]-19-ol,19-diol (20) (60 mg, 0.21 mmol) in acetone (2 ml) until the orange color of the reagent remained, and the mixture was stirred at 0°C for 5 min. After this time, the reaction mixture was poured into ice-water (10 ml), then extracted with EtOAc (100 ml). The organic layer was washed with saturated NaHCO₃ solution and H₂O, and dried with Na₂SO₄. Evaporation of the solvent gave a solid that was purified by preparative TLC (hexane-EtOAc=4:1, v/v) to give [19-3H]-19-hydroxy-5-ene-isomer 5 (36.5 mg, 56%). mp 106–112 °C (lit. mp 148–151 °C for non-labeled 17). [19-2H]-Compound 5 (1.1 mg, 3.8 µmol) was similarly converted into the [19-3H]-19-oxo derivative 6 by treatment of Jones reagent (Jones reagent, 6 µl; acetone, 0.2 ml; reaction period 3 min), then this was treated with non-labeled NaBH₄ (NaBH₄ (28.8 mg, 0.76 mmol) in 1 ml of 0.1 M NaOH solution, 110 µl) and MeOH (1 ml) at 0°C for 30 min, and the product was purified by the normal phase HPLC, as described above, producing [19-3H]-Compounds 5 (radiochemical yield: 3.3%; specific activity 0.21 mCi/mmol).

The chemical and radiochemical purities of the [19-3H]-Compounds were determined by HPLC analysis under the conditions described above, respectively, to be more than 98%.

Acknowledgements This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References