ed in vacuum. The residue was diluted with water (10 mL), acidified with 2 N HCl (2 mL), and the whole was extracted with $CHCl_3$ (3× 20 mL). The combined organic layer was washed with brine (1× 20 mL), dried over $Na_2SO_4$, filtered, and the solvent was evaporated in vacuum. The residue was purified by flash chromatography (AcOEt only) to afford **13a** (65 mg, 0.156 mmol, yield 92%) as a colorless solid. Compound **13a**: colorless powder. Mp 155–159 °C (*n*-hexane). $^1$H NMR ($CDCl_3$) 1.34 (3H, s, $CH_3$), 1.39 (6H, d, $J$ = 7.1 Hz, 2C$H_3$CH), 1.43 (3H, s, $CH_3$), 1.54 (2H, m, 2CH), 1.73–1.88 (4H, m, 4CH), 2.18 (1H, d, $J$ = 8.6 Hz, CH), 2.83 (1H, d, $J$ = 16.3 Hz, CH), 3.19 (3H, s, $NCH_3$), 3.79–3.85 (1H, m, CH), 3.94 (1H, br s, $CH_3$C*H*), 7.19 (1H, br s, ArH), 10.03 (1H, br s, $CO_2H$). LRMS (FAB$^+$) *m/z* 412 ([M($^{35}$Cl$_2$)+H]$^+$), 414 ([M($^{35}$Cl$^{37}$Cl)+H]$^+$), 416 ([M($^{37}$Cl$_2$)+H]$^+$). Anal. Calcd for $C_{21}H_{27}Cl_2NO_2 \cdot 1/4H_2O$: C, 60.51; H, 6.64; N, 3.36. Found: C, 60.72; H, 6.82; N, 3.26.

**13-Isopropyl-5,8,11a-trimethyl-6-oxo-6,7,7a,8,9,10,11,11a-octahydro-5*H*-dibenzo[*b*,*d*]azepine-8-carboxylic acid methyl ester (12b).** To a suspension of NaH (60% in mineral oil, washed twice with *n*-hexane) (13 mg, 0.032 mmol, 1.1 equiv) in DMF (1 mL) was dropwise added a solution of the carboxylic acid **10b** (100 mg, 0.291 mmol) in DMF (1 mL) at 0 °C for 1 min, in a flask fitted with a $CaCl_2$ tube. After stirring at 0 °C for 15 min, MeI (0.091 mL, 1.456 mmol, 5.0 equiv) was added to the reaction mixture at 0 °C. Stirring was continued at rt for 14 h, the solvent was evaporated in vacuum, and the residue was diluted with 1NHCl and then extracted with $CHCl_3$ (3× 20 mL). The combined organic layer was washed with brine (1× 20 mL), dried over $Na_2SO_4$, filtered, and then the solvent was evaporated in vacuum. The residue was purified by flash chromatography (*n*-hexane/AcOEt = 2:1) to afford **12b** (90 mg, 0.252 mmol, yield 87%) as a pale orange oil. Compound **12b**: colorless plates. Mp 149–150 °C (*n*-hexane/AcOEt). $^1$H NMR ($CDCl_3$) 1.26 (6H, d $J$ = 7.0 Hz, 2C$H_3$CH), 1.31 (3H, s, $CH_3$), 1.48 (3H, s, $CH_3$), 1.65–1.68 (2H, m, 2CH), 1.74–1.77 (2H, m, 2CH), 1.90 (1H, d, $J$ = 13.6 Hz, CH), 1.89–1.98 (3H, m, 3CH), 2.50–2.63 (2H, m, 2CH), 2.88–2.92 (1H, m, $CH_3$C*H*), 3.27 (3H, s, $NCH_3$), 3.60 (3H, s, $CO_2CH_3$), 7.01 (1H, d, $J$ = 1.8 Hz, ArH), 7.07 (1H, dd, $J$ = 8.2 Hz, 1.9 Hz, ArH), 7.28 (1H, d, $J$ = 8.2 Hz, ArH). LRMS (FAB$^+$) *m/z* 358 ([M+H]$^+$). Anal. Calcd for $C_{22}H_{31}NO_3$: C, 73.91; H, 8.74; N, 3.92. Found: C, 73.78; H, 8.89; N, 3.97.

**13-Isopropyl-5,8,11a-trimethyl-6-oxo-6,7,7a,8,9,10,11,11a-octahydro-5*H*-dibenzo[*b*,*d*]azepine-8-carboxylic acid (13b).** A mixture of the methyl ester **12b** (50 mg, 0.141 mmol), KOH (79 mg, 1.410 mmol, 10.0 equiv) and 18-crown ether-6 (93 mg, 0.352 mmol, 2.5 equiv) in MeOH (1 mL) was stirred at 80 °C for 18 h, the mixture was cooled, and the solvent was evaporated in vacuum. The residue was diluted with water (10 mL), acidified with 2 N HCl (2 mL), and then extracted with $CHCl_3$ (3× 20 mL). The combined organic layer was washed with brine (1× 20 mL), dried over $Na_2SO_4$, filtered, and then the solvent was evaporated in vacuum. The residue was purified by flash chromatography (*n*-hexane/AcOEt = 1:2) to afford **13b** (42 mg, 0.121 mmol, yield 86%) as a colorless amorphous solid. Compound **13b**: colorless flakes. Mp 123–127 °C (*n*-hexane/AcOEt). $^1$H NMR ($CDCl_3$) 1.24 (6H, d, $J$ = 7.0 Hz, 2C$H_3$CH), 1.31 (3H, s, $CH_3$), 1.50 (3H, s, $CH_3$), 1.67–1.78 (4H, m, 2CH), 1.98–2.04 (3H, m, 3CH), 2.50–2.62 (2H, m, 2CH), 2.86–2.90 (1H, m, $CH_3$C*H*), 3.28 (3H, s, $NCH_3$), 6.97 (1H, d, $J$ = 1.8 Hz, ArH), 7.06 (1H, dd, $J$ = 8.2 Hz, 1.9 Hz, ArH), 7.26 (1H, d, $J$ = 8.2 Hz, ArH). LRMS (FAB$^+$) *m/z* 344 ([M+H]$^+$). Anal. Calcd for $C_{21}H_{29}NO_3$: C, 73.44; H, 8.51; N, 4.08. Found: C, 73.28; H, 8.31; N, 4.03.

**Toluene-4-sulfonic acid pent-4-ynyl ester (15).** To a solution of 4-pentyn-1-ol **14** (1.10 mL, 0.012 mol) and $Et_3N$ (1.81 mL, 0.013 mol, 1.1 equiv) in $CH_2Cl_2$ (8 mL) was dropwise added a solution of TsCl (2.49 g, 0.0113 mol, 1.1 equiv) in $CH_2Cl_2$ (20 mL) at 0 °C for 20 min, in a flask fitted with a $CaCl_2$ tube. Stirring was continued at rt for 20 h, and the whole was poured into icewater (50 mL). The separated aqueous layer was extracted with $CHCl_3$ (3× 30 mL). Then the combined organic layer was washed with water (1× 30 mL) and brine (1× 30 mL), dried over $Na_2SO_4$, filtered, and then the solvent was evaporated in vacuum. The residue was purified by flash chromatography (*n*-hexane/AcOEt = 4:1) to afford **15** (2.37 g, 0.010 mol, yield 84%) as a colorless liquid. Compound **15**: $^1$H NMR ($CDCl_3$) 1.85–1.89 (2H, m, $CH_2CH_2CH_2$), 1.89 (1H, t, $J$ = 2.7 Hz, HCC), 2.26 (2H, dt, $J$ = 6.9 Hz, 2.7 Hz, $CCH_2$), 2.45 (3H, s, $CH_3$), 4.15 (2H, t, $J$ = 6.1 Hz, $CH_2O$), 7.35 (2H, d, $J$ = 8.2 Hz, ArH), 7.80 (2H, d, $J$ = 8.4 Hz, ArH).

**5-Iodopent-1-yne (16).** A mixture of toluene-4-sulfonic acid pent-4-ynyl ester **15** (817 mg, 3.430 mmol) and NaI (1.234 g, 8.232 mmol, 2.4 equiv) in acetone (8 mL) was stirred at 70 °C for 1 h, the mixture was cooled, and poured into ice-water (20 mL). The whole was extracted with $Et_2O$ (3× 20 mL). The combined organic layer was washed with satd $NaHCO_3$ (1× 20 mL) and brine (1× 20 mL), dried over $Na_2SO_4$, filtered, and then the solvent was evaporated in vacuum to afford **16** (600 mg, 3.093 mmol, yield 90%) as a yellow liquid. Compound **16**: $^1$H NMR ($CDCl_3$) 2.00 (1H, t, $J$ = 2.6 Hz, HCC), 2.01 (2H, tt, $J$ = 6.7 Hz, 6.7 Hz, $CH_2CH_2I$), 2.35 (2H, dt, $J$ = 6.7 Hz, 2.6 Hz, $CCH_2$), 3.32 (2H, t, $J$ = 6.7 Hz, $CH_2I$).

**12,14-Dichloro-13-isopropyl-8,11a-dimethyl-6-oxo-5-pent-4-ynyl-6,7,7a,8,9,10,11,11a-octahydro-5*H*-dibenzo[*b*,*d*]azepine-8-carboxylic acid methyl ester (17a).** To a suspension of NaH (60% in mineral oil, washed twice with *n*-hexane) (6 mg, 0.138 mmol, 1.1 equiv) in DMF (1 mL) was dropwise added a solution of the methyl ester **10a** (52 mg, 0.125 mmol) in DMF (1.5 mL) at 0 °C for 2 min, in a flask fitted with a $CaCl_2$ tube. After stirring at 0 °C for 30 min, a solution of 5-iodopent-1-yne **16** (122 mg, 0.627 mmol, 5.0 equiv) in DMF (1.5 mL) was added to the reaction mixture at 0 °C. Stirring was continued at rt for 17 h, the solvent was evaporated in vacuum, and the residue was diluted with water (20 mL) and the whole was extracted with $CHCl_3$ (3× 20 mL). The combined organic layer was washed with brine (1× 20 mL), dried over $Na_2SO_4$, filtered, and then evaporated in vacuum. The residue was purified by flash chromatography (*n*-hexane/AcOEt = 4:1) to afford **17a** (44 mg, 0.093 mmol, yield 74%) as a colorless

oil. Compound **17a**: $^1$H NMR ($CDCl_3$) 1.42 (3H, s, $CH_3$), 1.42 (6H, d, $J$ = 7.0 Hz, 2C$H_3$CH), 1.47 (3H, s, $CH_3$), 1.72 (2H, m, 2CH), 1.83–1.91 (5H, m, 5CH), 2.01 (1H, t, $J$ = 2.3 Hz, HCC), 2.21 (1H, d, $J$ = 8.5 Hz, CH), 2.30 (2H, t, $J$ = 7.0 Hz, $CCH_2$), 2.90 (1H, d, $J$ = 16.2 Hz, CH), 3.05–3.12 (1H, m, CH), 3.66 (3H, s, $CO_2CH_3$), 3.70 (1H, dd, $J$ = 16.4 Hz, 8.9 Hz, CH), 3.96 (1H, br s, $CH_3CH$), 4.11–4.28 (1H, m, CH), 7.21 (1H, br s, ArH). LRMS (FAB$^+$) $m/z$ 478 ([M($^{35}Cl_2$)+H]$^+$), 480 ([M($^{35}Cl^{37}Cl$)+H]$^+$), 482 ([M($^{37}Cl_2$)+H]$^+$).

**12,14-Dichloro-13-isopropyl-8,11a-dimethyl-6-oxo-5-[5-(4-iodophenyl)pent-4-ynyl]-6,7,7a,8,9,10,11,11a-octahydro-5*H*-dibenzo[*b*,*d*]azepine-8-carboxylic acid methyl ester (23) and 1,4-bis[[5-(2,4-dichloro-3-isopropyl-8,11a-dimethyl-8-methoxycarbonyl-6-oxo-6,7,7a,8,9,10,11,11a-octahydro-5*H*-dibenzo[*b*,*d*]azepin-5-yl)]pent-1-ynyl]benzene (21).** A mixture of the methyl ester **17a** (44 mg, 0.093 mmol), *p*-diiodobenzene (34 mg, 0.102 mmol, 1.1 equiv), $Pd(PPh_3)_2Cl_2$ (1 mg, 0.001 mmol, 0.02 equiv), and CuI (1 mg, 0.005 mmol, 0.06 equiv) in $Et_3N$ (2 mL) was stirred at rt for 39 h under Ar atmosphere. The reaction mixture was filtered, the solvent was evaporated in vacuum, and the resultant residue was purified by flash chromatography (*n*-hexane/AcOEt = 2:1) to afford the mixture of the monosubstituted compound **25** and *p*-diiodobenzene (55 mg) as pale yellow oil and the disubstituted compound **21** (14 mg, 0.013 mmol, yield 28%) as pale yellow amorphous solid. Then the former mixture was purified by flash chromatography (*n*-hexane/AcOEt = 4:1) again to afford the monosubstituted compound **25** (32 mg, 0.046 mmol, yield 50%) as a pale orange amorphous solid. Compound **25**: $^1$H NMR ($CDCl_3$) 1.41 (6H, d, $J$ = 5.3 Hz, 2C$H_3$CH), 1.42 (3H, s, $CH_3$), 1.46 (3H, s, $CH_3$), 1.70 (2H, m, 2CH), 1.81–1.97 (5H, m, 5CH), 2.02–2.09 (1H, m, CH), 2.21 (1H, d, $J$ = 8.5 Hz, CH), 2.50 (2H, t, $J$ = 6.9 Hz, $CH_2$), 2.86 (1H, d, $J$ = 16.2 Hz, CH), 3.06–3.50 (1H, m, CH), 3.51 (3H, s, $CO_2CH_3$), 3.72 (1H, dd, $J$ = 16.1 Hz, 8.6 Hz, CH), 3.95 (1H, br s, $CH_3CH$), 4.33–4.39 (1H, m, CH), 7.14 (2H, d, $J$ = 8.1 Hz, ArH), 7.20 (1H, br s, ArH), 7.62 (2H, d, $J$ = 8.1 Hz, ArH). LRMS (FAB$^+$) $m/z$ 680 ([M($^{35}Cl_2$)+H]$^+$), 682 ([M($^{35}Cl^{37}Cl$)+H]$^+$), 684 ([M($^{37}Cl_2$)+H]$^+$). Compound **21**: $^1$H NMR ($CDCl_3$) 1.43 (12H, d, $J$ = 6.0 Hz, 4C$H_3$CH), 1.44 (6H, s, $2CH_3$), 1.48 (6H, s, $2CH_3$), 1.71 (4H, m, 4CH), 1.83–1.99 (10H, m, 10CH), 2.04–2.09 (2H, m, 2CH), 2.22 (2H, d, $J$ = 8.5 Hz, 2CH), 2.53 (4H, d, $J$ = 6.9 Hz, 4CH), 2.89 (2H, d, $J$ = 16.3 Hz, 2CH), 3.09–3.16 (2H, m, 2CH), 3.53 (6H, s, $2CO_2CH_3$), 3.74 (2H, dd, $J$ = 16.1 Hz, 8.6 Hz, 2CH), 3.97 (2H, br s, $2CH_3CH$), 4.35–4.41 (2H, m, 2CH), 7.22 (2H, br s, ArH), 7.35 (4H, s, ArH). LRMS (FAB$^+$) $m/z$ 1029 ([M($^{35}Cl_4$)+H]$^+$), 1031 ([M($^{35}Cl_3{}^{37}Cl$)+H]$^+$), 1033 ([M($^{35}Cl_2{}^{37}Cl_2$)+H]$^+$), 1035 ([M($^{35}Cl^{37}Cl_3$)+H]$^+$).

**1,4-Bis[[5-(12,14-dichloro-13-isopropyl-8,11a-dimethyl-8-methoxycarbonyl-6-oxo-6,7,7a,8,9,10,11,11a-octahydro-5*H*-dibenzo[*b*,*d*]azepin-5-yl)]pent-1-ynyl]benzene (21).** The mixture of the alkyne **17a** (17 mg, 0.036 mmol), the iodide **25** (32 mg, 0.047 mmol, 1.3 equiv), $Pd(PPh_3)_2Cl_2$ (1 mg, 0.001 mmol, 0.04 equiv), and CuI (1 mg, 0.005 mmol, 0.14 equiv) in $Et_3N$ (2 mL) was stirred at rt for 72 h under Ar atmosphere. The reaction mixture was filtered, the solvent was evaporated in vacuum, and the resultant residue was purified by flash chromatography (*n*-hexane/AcOEt = 2:1) to afford **21** (32 mg, 0.031 mmol, yield 84%) as a colorless oil.

**1,4-Bis[[5-(12,14-dichloro-13-isopropyl-8,11a-dimethyl-8-hydroxycarbonyl-6-oxo-6,7,7a,8,9,10,11,11a-octahydro-5*H*-dibenzo[*b*,*d*]azepin-5-yl)]pent-1-ynyl]benzene (29).** A mixture of the methyl ester **21** (46 mg, 0.045 mmol), KOH (25 mg, 0.445 mmol, 10.0 equiv) and 18-crown ether-6 (59 mg, 0.223 mmol, 5.0 equiv) in MeOH (2 mL) was stirred at 80 °C for 19 h, the mixture was cooled, and the solvent was evaporated in vacuum. The residue was diluted with water (20 mL), acidified with 2 N HCl (2 mL) and then extracted with $CHCl_3$ (3× 20 mL). The combined organic layer was washed with brine (1× 20 mL), dried over $Na_2SO_4$, filtered, and then the solvent was evaporated in vacuum. The residue was purified by flash chromatography (AcOEt/MeOH = 10:1) to afford **29** (29 mg, 0.029 mmol, yield 65%) as a colorless solid. Compound **29**: colorless powder. Mp 211–214 °C (*n*-hexane/AcOEt). $^1$H NMR ($CD_3OD$) 1.31 (6H, s, $2CH_3$), 1.43–1.49 (12H, m, 2C$H_3$CH and $2CH_3$), 1.76–2.18 (14H, m, 14CH), 2.27 (2H, d, $J$ = 8.6 Hz, 2CH), 2.54–2.55 (4H, m, 4CH), 3.15 (2H, d, $J$ = 16.5 Hz, 2CH), 3.65–3.72 (2H, m, 2CH), 4.02 (2H, br s, $2CH_3CH$), 4.30 (2H, m, 2CH), 7.32 (4H, s, ArH), 7.35 (2H, br s, ArH). LRMS (FAB$^+$) $m/z$ 1001 ([M($^{35}Cl_4$)+H]$^+$), 1003 ([M($^{35}Cl_3{}^{37}Cl$)+H]$^+$), 1005 ([M($^{35}Cl_2{}^{37}Cl_2$)+H]$^+$), 1007 ([M($^{35}Cl^{37}Cl_3$)+H]$^+$), 1009 ([M($^{37}Cl_4$)+H]$^+$). HRMS (FAB$^+$) found: 1001.3432. Calcd for $C_{56}H_{64}{}^{35}Cl_4N_2O_6$: 1001.3449. Anal. Calcd for $C_{56}H_{64}Cl_4N_2O_6 \cdot 3/2H_2O$: C, 65.30; H, 6.56; N, 2.72. Found: C, 65.34; H, 6.59; N, 2.60.

**13-Isopropyl-8,11a-dimethyl-6-oxo-5-pent-4-ynyl-6,7,7a,8,9,10,11,11a-octahydro-5*H*-dibenzo[*b*,*d*]-azepine-8-carboxylic acid methyl ester (17d).** To a suspension of NaH (60% in mineral oil, washed twice with *n*-hexane) (12 mg, 0.292 mmol, 1.1 equiv) in DMF (1 mL) was dropwise added a solution of the methyl ester **10b** (91 mg, 0.265 mmol) in DMF (1.5 mL) at 0 °C over 1 min, in a flask fitted with a $CaCl_2$ tube. After stirring at 0 °C for 30 min, a solution of 5-iodopent-1-yne **16** (257 mg, 1.326 mmol, 5.0 equiv) in DMF (1.5 mL) was added to the reaction mixture at 0 °C. Stirring was continued at rt for 18 h, and the solvent was evaporated in vacuum, and the residue was diluted with water (20 mL) and then extracted with $CHCl_3$ (3× 20 mL). The combined organic layer was washed with brine (1× 20 mL), dried over $Na_2SO_4$, filtered and the solvent was evaporated in vacuum. The residue was purified by flash chromatography (*n*-hexane/AcOEt = 4:1) to afford **17b** (76 mg, 0.85 mmol, yield 70%) as a colorless oil. Compound **17b**: $^1$H NMR ($CDCl_3$) 1.27 (6H, d, $J$ = 6.9 Hz, 2C$H_3$CH), 1.32 (3H, s, $CH_3$), 1.48 (3H, s, $CH_3$), 1.66–1.68 (2H, m, 2CH), 1.75–1.78 (2H, m, 2CH), 1.90 (1H, d, $J$ = 13.3 Hz, CH), 1.95–2.04 (2H, m, 2CH), 1.96 (1H, t, $J$ = 2.6 Hz, HCC), 2.10–2.17 (1H, m, CH), 2.19–2.37 (2H, m, 2CH), 2.50–2.59 (2H, m, 2CH), 2.88–2.96 (1H, m, $CH_3CH$), 3.48–3.56 (1H, m, CH), 3.60 (3H, s, $CO_2CH_3$), 3.90–3.97 (1H, m, CH), 7.08 (1H, dd, $J$ = 8.2 Hz, 1.9 Hz, ArH), 7.11 (1H, d,

$J$ = 1.9 Hz, ArH), 7.29 (1H, d, $J$ = 8.2 Hz, ArH). LRMS ($FAB^+$) $m/z$ 410 ($[M+H]^+$).

**12,14-Dichloro-13-isopropyl-8,11a-dimethyl-6-oxo-5-[5-(3-iodophenyl)pent-4-ylnyl]-6,7,7a,8,9,10,11,11a-octahydro-5*H*-dibenzo[*b*,*d*]azepine-8-carboxylic acid methyl ester (24) and 1,3-bis[[5-(12,14-dichloro-13-isopropyl-8,11a-dimethyl-8-methoxycarbonyl-6-oxo-6,7,7a,8,9,10,11,11a-octahydro-5*H*-dibenzo[*b*,*d*]azepin-5-yl)]pent-1-ynyl]benzene (20).** A mixture of the alkyne **17b** (62 mg, 0.129 mmol), *m*-diiodobenzene (21 mg, 0064 mmol, 0.5 equiv), $Pd(PPh_3)_2Cl_2$ (1 mg, 0.001 mmol, 0.01 equiv), and CuI (1 mg, 0.005 mmol, 0.04 equiv) in $Et_3N$ (3 mL) was stirred at rt for 49 h under Ar atmosphere. The reaction mixture was filtered, the solvent was evaporated in vacuum. The resultant residue was purified by flash chromatography (*n*-hexane/AcOEt = 4:1) to afford the monosubstituted compound **24** (32 mg, 0.046 mmol, yield 36%) as an orange oil and the disubstituted compound **20** (31 mg, 0.030 mmol, yield 47%) as an orange oil. Compound **24**: $^1H$ NMR ($CDCl_3$) 1.41 (6H, d, $J$ = 5.9 Hz, $2CH_3CH$), 1.42 (3H, s, $CH_3$), 1.47 (3H, s, $CH_3$), 1.70–1.71 (2H, m, 2CH), 1.82–1.93 (5H, m, 5CH), 2.03–2.10 (1H, m, CH), 2.20 (1H, d, $J$ = 8.5 Hz, CH), 2.51 (2H, t, $J$ = 7.0 Hz, $CH_2$), 2.87 (1H, d, $J$ = 16.2 Hz, CH), 3.07–3.14 (1H, m, CH), 3.52 (3H, s, $CO_2CH_3$), 3.72 (1H, dd, $J$ = 16.1 Hz, 8.6 Hz, CH), 3.96 (1H, br s, $CH_3CH$), 4.32–4.39 (1H, m, CH), 7.02 (1H, t, $J$ = 7.9 Hz, ArH), 7.20 (1H, br s, ArH), 7.37 (1H, td, $J$ = 7.8 Hz, 1.2 Hz, ArH), 7.61 (1H, td, $J$ = 8.5 Hz, 1.4 Hz, ArH), 7.77 (1H, t, $J$ = 1.6 Hz, ArH). LRMS ($FAB^+$) $m/z$ 680 ($[M(^{35}Cl_2)+H]^+$), 682 ($[M(^{35}Cl^{37}Cl)+H]^+$), 684 ($[M(^{37}Cl_2)+H]^+$). Compound **20**: $^1H$ NMR ($CDCl_3$) 1.41 (6H, s, $2CH_3$), 1.41 (12H, d, $J$ = 6.3 Hz, $4CH_3CH$), 1.47 (6H, s, $2CH_3$), 1.69–1.70 (4H, m, 4CH), 1.81–1.93 (10H, m, 10CH), 2.03–2.08 (2H, m, 2CH), 2.20 (2H, d, $J$ = 8.5 Hz, 2CH), 2.50 (4H, d, $J$ = 7.0 Hz, 4CH), 2.88 (2H, d, $J$ = 16.2 Hz, 2CH), 3.07–3.14 (2H, m, 2CH), 3.51 (6H, s, $2CO_2CH_3$), 3.73 (2H, dd, $J$ = 16.2 Hz, 8.7 Hz, 2CH), 3.96 (2H, br s, $2CH_3CH$), 4.32–4.39 (2H, m, 2CH), 7.20 (2H, br s, ArH), 7.21 (2H, t, $J$ = 7.7 Hz, ArH), 7.32 (2H, d, $J$ = 7.8 Hz, ArH), 7.44 (1H, s, ArH). LRMS ($FAB^+$) $m/z$ 1029 ($[M(^{35}Cl_4)+H]^+$), 1031 ($[M(^{35}Cl_3{}^{37}Cl)+H]^+$), 1033 ($[M(^{35}Cl_2{}^{37}Cl_2)+H]^+$), 1035 ($M(^{35}Cl^{37}Cl_3)+H)^+$).

**1,3-Bis[[5-(12,14-dichloro-13-isopropyl-8,11a-dimethyl-8-methoxycarbonyl-6-oxo-6,7,7a,8,9,10,11,11a-octahydro-5*H*-dibenzo[*b*,*d*]azepin-5-yl)]pent-1-ynyl]benzene (20).** A mixture of the alkyne **17a** (22 mg, 0.046 mmol), the iodide **24** (32 mg, 0.046 mmol), $Pd(PPh_3)_2Cl_2$ (1 mg, 0.001 mmol, 0.03 equiv), and CuI (1 mg, 0.005 mmol, 0.1 equiv) in $Et_3N$ (2 mL) was stirred at rt for 48 h under Ar atmosphere. The reaction mixture was filtered, and the solvent was evaporated in vacuum, and the residue was purified by flash chromatography (*n*-hexane/AcOEt = 2:1) to afford **20** (33 mg, 0.032 mmol, yield 70%) as an orange oil.

**1,3-Bis[[5-(12,14-dichloro-13-isopropyl-8,11a-dimethyl-8-hydroxycarbonyl-6-oxo-6,7,7a,8,9,10,11,11a-octahydro-5*H*-dibenzo[*b*,*d*]azepin-5-yl)]pent-1-ynyl]benzene (28).** A mixture of the methyl ester **20** (69 mg, 0.067 mmol), KOH (38 mg, 0.672 mmol, 10.0 equiv), and 18-crown ether-6 (89 mg, 0.336 mmol, 5.0 equiv) in MeOH (2 mL) was stirred at 80 °C for 18 h, the whole was cooled, and the solvent was evaporated in vacuum. The resultant residue was diluted with water (20 mL), acidified with 2 N HCl (2 mL), and then extracted with $CHCl_3$ (3× 20 mL). The combined organic layer was washed with brine (1× 20 mL), dried over $Na_2SO_4$, filtered, and then the solvent was evaporated in vacuum. The residue was purified by flash chromatography (AcOEt/MeOH = 10:1) to afford the desired compound **28** (50 mg, 0.050 mmol, yield 75%) as a colorless solid. Compound **28**: colorless powder. Mp 207–213 °C (*n*-hexane/$CHCl_3$). $^1H$ NMR ($CDCl_3$) 1.29 (6H, s, $2CH_3$), 1.32 (12H, d, $J$ = 7.1 Hz, $4CH_3CH$), 1.33 (6H, s, $2CH_3$), 1.61 (4H, m, 4CH), 1.75–1.89 (10H, m, 10CH), 2.13 (2H, d, $J$ = 8.6 Hz, 2CH), 2.21 (4H, m, 4CH), 2.92–3.00 (2H,, m, 2CH), 2.98 (2H, d, $J$ = 16.7 Hz, 2CH), 3.50–3.57 (2H, m, 2CH), 3.91 (2H, br s, $2CH_3CH$), 4.13–4.20 (2H, m, 2CH), 7.09 (3H, m, ArH), 7.24 (2H, br s, ArH), 7.30 (1H, s, ArH). LRMS ($FAB^+$) $m/z$ 1001 ($[M(^{35}Cl_4)+H]^+$), 1003 ($[M(^{35}Cl_3{}^{37}Cl)+H]^+$), 1005 ($[M(^{35}Cl_2{}^{37}Cl_2)+H]^+$), 1007 ($[M(^{35}Cl^{37}Cl_3)+H]^+$), 1009 ($[M(^{37}Cl_4)+H]^+$). HRMS ($FAB^+$) found: 1001.4380. Calcd for $C_{56}H_{65}{}^{35}Cl_4N_2O_6$: 1001.4302. Anal. Calcd for $C_{56}H_{64}Cl_4N_2O_6{\cdot}3/2H_2O$: C, 65.30; H, 6.56; N, 2.72. Found: C, 65.03; H, 6.78; N, 2.62.

**13-Isopropyl-8,11a-dimethyl-6-oxo-5-[5-(3-iodophenyl)pent-4-ylnyl]-6,7,7a,8,9,10,11,11a-octahydro-5*H*-dibenzo[*b*,*d*]azepine-8-carboxylic acid methyl ester (26) and 1,3-bis[[5-(13-isopropyl-8,11a-dimethyl-8-methoxycarbonyl-6-oxo-6,7,7a,8,9,10,11,11a-octahydro-5*H*-dibenzo[*b*,*d*]azepin-5-yl)]pent-1-ynyl]-benzene (22).** A mixture of the alkyne **17b** (66 mg, 0.161 mmol), *m*-diiodobenzene (53 mg, 0.161 mmol), $Pd(PPh_3)_2Cl_2$ (1 mg, 0.001 mmol, 0.01 equiv), and CuI (1 mg, 0.005 mmol, 0.03 equiv) in $Et_3N$ (2 mL) was stirred at rt for 19 h under Ar atmosphere. The reaction mixture was filtered, the solvent was evaporated in vacuum, and the resultant residue was purified by flash chromatography (*n*-hexane/AcOEt = 4:1–2:1) to afford the monosubstituted compound **26** (63 mg, 0.103 mmol, yield 64%) as yellow oil and the disubstituted compound **22** (26 mg, 0.029 mmol, yield 36%) as a yellow oil. Compound **26**: $^1H$ NMR ($CDCl_3$) 1.23 (6H, d, $J$ = 6.9 Hz, $2CH_3CH$), 1.34 (3H, s, $CH_3$), 1.50 (3H, s, $CH_3$), 1.68–1.69 (2H, m, 2CH), 1.77–1.79 (2H, m, 2CH), 1.92 (1H, d, $J$ = 8.3 Hz, CH), 1.97–2.00 (2H, m, 2CH), 2.09 (1H, m, CH), 2.22–2.23 (1H, m, CH), 2.46–2.64 (4H, m, 4CH), 2.83–2.90 (1H, m, $CH_3CH$), 3.55–3.61 (1H, m, CH), 3.62 (3H, s, $CO_2CH_3$), 3.95–4.02 (1H, m, CH), 7.00 (1H, t, $J$ = 7.9 Hz, ArH), 7.08 (1H, dd, $J$ = 8.2 Hz, 1.8 Hz, ArH), 7.13 (1H, d, $J$ = 1.8 Hz, ArH), 7.30 (1H, t, $J$ = 8.3 Hz, ArH), 7.32 (1H, dt, $J$ = 8.0 Hz, 1.4 Hz, ArH), 7.60 (1H, td, $J$ = 7.9 Hz, 1.2 Hz, ArH), 7.73 (1H, t, $J$ = 1.6 Hz, ArH). LRMS ($FAB^+$) $m/z$ 612 ($[M+H]^+$). Compound **22**: $^1H$ NMR ($CDCl_3$) 1.19 (12H, d, $J$ = 6.9 Hz, $4CH_3CH$), 1.33 (6H, s, $2CH_3$), 1.48 (6H, s, $2CH_3$), 1.67–1.68 (4H, m, 4CH), 1.75–1.77 (4H, m, 4CH), 1.90 (2H, d, $J$ = 7.3 Hz, 2CH), 2.01–2.11 (2H, m, 2CH), 2.17–2.24 (2H, m, 2CH), 2.40–2.62 (8H, m, 8CH), 2.80–2.90 (2H, m, $2CH_3CH$), 3.42–3.60 (2H, m, 2CH), 3.61 (6H, s, $2CO_2CH_3$), 3.92–4.00 (2H, m, 2CH), 7.05 (2H, dd, $J$ = 8.2 Hz, 1.9 Hz, ArH), 7.11 (2H, d, $J$ = 1.9 Hz, ArH), 7.16 (1H, d,

$J$ = 8.4 Hz, ArH), 7.24 (2H, dd, $J$ = 8.6 Hz, 1.5 Hz, ArH), 7.28 (2H, d, $J$ = 8.3 Hz, ArH), 7.36 (1H, m, ArH). LRMS ($FAB^+$) $m/z$ 893 ($[M+H]^+$).

**1,3-Bis[[5-(13-isopropyl-8,11a-dimethyl-8-hydroxycarbonyl-6-oxo-6,7,7a,8,9,10,11,11a-octahydro-5*H*-dibenzo[*b*,*d*]azepin-5-yl)]pent-1-ynyl]benzene (30).** A mixture of the methyl ester **22** (26 mg, 0.029 mmol), KOH (16 mg, 0.289 mmol, 10.0 equiv), and 18-crown ether-6 (38 mg, 0.145 mmol, 5.0 equiv) in MeOH (2 mL) was stirred at 80 °C for 17 h, the whole was cooled, and the solvent was evaporated in vacuum. The residue was diluted with water (20 mL), acidified with 2 N HCl (2 mL), and then extracted with $CHCl_3$ (3× 20 mL). The combined organic layer was washed with brine (1× 20 mL), dried over $Na_2SO_4$, filtered, and then the solvent was evaporated in vacuum. The residue was purified by flash chromatography (AcOEt/MeOH = 10:1) to afford **30** (9 mg, 0.010 mmol, yield 36%) as a colorless amorphous solid. Compound **30**: colorless powder. Mp 159–163 °C (*n*-hexane/$CHCl_3$). $^1H$ NMR ($CD_3OD$) 1.04 (12H, d, $J$ = 6.9 Hz, $4CH_3CH$), 1.22 (6H, s, $2CH_3$), 1.33 (6H, s, $2CH_3$), 1.57–1.68 (8H, m, 8CH), 1.84–1.88 (8H, m, 8CH), 2.21–2.27 (2H, m, 2CH), 2.38–2.48 (8H, m, 8CH), 2.73–2.78 (2H, m, $2CH_3CH$), 3.30–3.38 (2H, m, 2CH), 3.80 (2H, m, 2CH), 7.01–7.08 (6H, m, ArH), 7.23–7.25 (4H, m, ArH). LRMS ($FAB^+$) $m/z$ 866 ($[M+H]^+$). Anal. Calcd for $C_{56}H_{68}N_2O_6{\cdot}2H_2O$: C, 74.64; H, 8.05; N, 3.11. Found: C, 74.45; H, 8.31; N, 2.83.

**12,14-Dichloro-13-isopropyl-8,11a-dimethyl-6-oxo-5-[5-(2-iodophenyl)pent-4-ynyl]-6,7,7a,8,9,10,11,11a-octahydro-5*H*-dibenzo[*b*,*d*]azepine-8-carboxylic acid methyl ester (23) and 1,10-bis[5-(12,14-dichloro-13-isopropyl-8,11a-dimethyl-8-methoxycarbonyl-6-oxo-6,7,7a,8,9,10,11,11a-octahydro-5*H*-dibenzo[*b*,*d*]azepin-5-yl)]decane-4,6-diyne (18).** A mixture of the alkyne **17a** (77 mg, 0.161 mmol), *o*-diiodobenzene (0.010 mL, 0.080 mmol, 0.5 equiv), $Pd(PPh_3)_2Cl_2$ (1 mg, 0.001 mmol, 0.01 equiv), and CuI (1 mg, 0.005 mmol, 0.03 equiv) in $Et_3N$ (2 mL) was stirred at rt for 48 h under Ar atmosphere. The reaction mixture was filtered, the solvent was evaporated in vacuum and the resultant residue was purified by flash chromatography (*n*-hexane/AcOEt = 2:1) to afford the monosubstituted compound **23** (17 mg, 0.025 mmol, yield 15%) as orange oil and the homodimeric compound **18** (52 mg, 0.054 mmol, yield 67%) as an orange oil. Compound **23**: $^1H$ NMR ($CDCl_3$) 1.43 (6H, d, $J$ = 7.5 Hz, $2CH_3CH$), 1.44 (3H, s, $CH_3$), 1.49 (3H, s, $CH_3$), 1.70 (2H, m, 2CH), 1.85–1.92 (4H, m, 2CH), 2.02–2.03 (1H, m, CH), 2.12–2.17 (1H, m, CH), 2.22 (1H, d, $J$ = 8.5 Hz, CH), 2.61 (2H, t, $J$ = 6.9 Hz, $CH_2$), 2.91 (1H, d, $J$ = 16.3 Hz, CH), 3.20–3.27 (1H, m, CH), 3.52 (3H, s, $CO_2CH_3$), 3.77 (1H, dd, $J$ = 16.1 Hz, 8.6 Hz, CH), 3.98 (1H, br s, $CH_3CH$), 4.34–4.38 (1H, m, CH), 6.98 (1H, dt, $J$ = 7.7 Hz, 1.7 Hz, ArH), 7.22 (1H, br s, ArH), 7.29 (1H, dt, $J$ = 7.6 Hz, 1.2 Hz, ArH), 7.44 (1H, dd, $J$ = 7.7 Hz, 1.6 Hz, ArH), 7.84 (1H, dd, $J$ = 8.0 Hz, 1.6 Hz, ArH). LRMS ($FAB^+$) $m/z$ 680 ($[M(^{35}Cl_2)+H]^+$), 682 ($[M(^{35}Cl^{37}Cl)+H]^+$), 684 ($[M(^{37}Cl_2)+H]^+$). Compound **18**: $^1H$ NMR ($CDCl_3$) 1.40 (6H, s, $2CH_3$), 1.41 (12H, d, $J$ = 5.8 Hz, $4CH_3CH$), 1.46 (6H, s, $2CH_3$), 1.71 (4H, m, 4CH), 1.79–1.92 (10H, m, 10CH), 1.95–2.04 (2H, m, 2CH), 2.20 (2H, d, $J$ = 8.5 Hz, 2CH), 2.36 (4H, d, $J$ = 7.1 Hz, 4CH), 2.83 (2H, d, $J$ = 16.2 Hz, 2CH), 2.98–3.05 (2H, m, 2CH), 3.67 (6H, s, $2CO_2CH_3$), 3.69 (2H, dd, $J$ = 16.0 Hz, 8.6 Hz, 2CH), 3.94 (2H, br s, $2CH_3CH$), 4.26–4.32 (2H, m, 2CH), 7.19–7.23 (2H, br s, ArH). LRMS ($FAB^+$) $m/z$ 1029 ($[M(^{35}Cl_4)+H]^+$), 1031 ($[M(^{35}Cl_3{}^{37}Cl)+H]^+$), 1033 ($[M(^{35}Cl_2{}^{37}Cl_2)+H]^+$), 1035 ($[M(^{35}Cl^{37}Cl_3)+H]^+$), 1037 ($[M(^{37}Cl_4)+H]^+$).

**1,2-Bis[[5-(12,14-dichloro-13-isopropyl-8,11a-dimethyl-8-methoxycarbonyl-6-oxo-6,7,7a,8,9,10,11,11a-octahydro-5*H*-dibenzo[*b*,*d*]azepin-5-yl)]pent-1-ynyl]benzene (19).** A mixture of the alkyne **17a** (15 mg, 0.032 mmol), the iodide **23** (22 mg, 0.032 mmol), $Pd(PPh_3)_4$ (2 mg, 0.002 mmol, 0.05 equiv), and CuI (1 mg, 0.010 mmol, 0.3 equiv) in $Et_3N$ (1 mL) was stirred at rt for 19 h under Ar atmosphere. The reaction mixture was filtered, the solvent was evaporated in vacuum, and the resultant residue was purified by flash chromatography (*n*-hexane/AcOEt = 2:1) to afford **19** (11 mg, 0.011 mmol, yield 33%) as an orange oil. Compound **19**: $^1H$ NMR ($CDCl_3$) 1.41 (6H, s, $2CH_3$), 1.42 (12H, d, $J$ = 6.6 Hz, $4CH_3CH$), 1.46 (6H, s, $2CH_3$), 1.68 (4H, m, 4CH), 1.81–1.92 (8H, m, 8CH), 1.95–2.00 (2H, m, 2CH), 2.07–2.14 (2H, m, 2CH), 2.20 (2H, d, $J$ = 8.0 Hz, 2CH), 2.57 (4H, t, $J$ = 6.9 Hz, $2CH_2$), 2.89 (2H, d, $J$ = 16.2 Hz, 2CH), 3.13–3.20 (2H, m, 2CH), 3.47 (6H, s, $2CO_2CH_3$), 3.74 (2H, dd, $J$ = 16.2 Hz, 8.6 Hz, 2CH), 3.96 (2H, br s, $2CH_3CH$), 4.33–4.38 (2H, m, 2CH), 7.20 (2H, dd, $J$ = 5.7 Hz, 3.3 Hz, ArH), 7.19–7.20 (2H, m, ArH), 7.41 (2H, dd, $J$ = 5.7 Hz, 3.4 Hz, ArH). LRMS ($FAB^+$) $m/z$ 1029 ($[M(^{35}Cl_4)+H]^+$), 1031 ($[M(^{35}Cl_3{}^{37}Cl_3)+H]^+$), 1033 ($[M(^{35}Cl_2{}^{37}Cl_2)+H]^+$), 1035 ($[M(^{35}Cl^{37}Cl_3)+H]^+$).

**1,2-Bis[[5-(12,14-dichloro-13-isopropyl-8,11a-dimethyl-8-hydroxycarbonyl-6-oxo-6,7,7a,8,9,10,11,11a-octahydro-5*H*-dibenzo[*b*,*d*]azepin-5-yl)]pent-1-ynyl]benzene (27).** A mixture of the methyl ester **19** (11 mg, 0.011 mmol), KOH (60 mg, 0.108 mmol, 10.0 equiv), and 18-crown ether-6 (14 mg, 0.054 mmol, 5.0 equiv) in MeOH (2 mL) was stirred at 80 °C for 16 h, the whole was cooled, and the solvent was evaporated in vacuum, and the resultant residue was diluted with water (20 mL), acidified with 2 N HCl (2 mL) and then extracted with $CHCl_3$ (3× 20 mL). The combined organic layer was washed with brine (1× 20 mL), dried over $Na_2SO_4$, filtered, and then the solvent was evaporated in vacuum. The residue was purified by flash chromatography (AcOEt/MeOH = 20:1) to afford **27** (4 mg, 0.004 mmol, yield 41%) as a colorless solid. Compound **27**: pale yellow powder. Mp 181–184 °C (*n*-hexane/AcOEt). $^1H$ NMR ($CD_3OD$) 1.28 (6H, s, $2CH_3$), 1.34 (12H, m, $2CH_3CH$ and $2CH_3$), 1.73 (14H, m, 14CH), 2.21 (6H, m, 6CH), 2.98 (4H, m, 4CH), 3.54 (2H, m, 2CH), 3.91 (2H, br s, $2CH_3CH$), 4.15 (2H, m, 2CH), 7.06 (2H, s, ArH), 7.27 (4H, m, ArH). LRMS ($FAB^+$) $m/z$ 1001 ($[M(^{35}Cl_4)+H]^+$), 1003 ($[M(^{35}Cl_3{}^{37}Cl)+H]^+$), 1005 ($[M(^{35}Cl_2{}^{37}Cl_2)+H]^+$), 1007 ($[M(^{35}Cl^{37}Cl_3)+H]^+$), 1009 ($[M(^{37}Cl_4)+H]^+$). Anal. Calcd for $C_{56}H_{64}Cl_4N_2O_6{\cdot}3/2H_2O$: C, 65.30; H, 6.56; N, 2.72. Found: C, 65.17; H, 6.79; N, 2.69.

**12,14-Dichloro-13-isopropyl-8,11a-dimethyl-6-oxo-5-pent-4-ynyl-6,7,7a,8,9,10,11,11a-octahydro-5*H*-dibenzo[*b*,*d*]azepine-8-carboxylic acid (31).** A mixture of the methyl ester **17a** (41 mg, 0.086 mmol), KOH (48 mg, 0.863 mmol, 10.0 equiv) and 18-crown ether-6 (57 mg, 0.216 mmol, 2.5 equiv) in MeOH (1 mL) was stirred at 80 °C for 17 h, the whole was cooled, and the solvent was evaporated in vacuum. The residue was diluted with water (10 mL), acidified with 2 N HCl (2 mL), and then extracted with $CHCl_3$ (3× 20 mL). The combined organic layer was washed with brine (1× 20 mL), dried over $Na_2SO_4$, filtered, and then the solvent was evaporated in vacuum. The residue was purified by flash chromatography (AcOEt/MeOH = 10:1) to afford **31** (29 mg, 0.0624 mmol, yield 72%) as a colorless solid. Compound **31**: colorless flakes. Mp 122–126 °C (*n*-hexane/AcOEt). $^1$H NMR ($CDCl_3$) 1.39 (3H, s, $CH_3$), 1.40 (6H, d, $J$ = 5.5 Hz, 2C$H_3$CH), 1.44 (3H, s, $CH_3$), 1.69–1.74 (2H, m, 2CH), 1.73 (1H, t, $J$ = 2.4 Hz, HCC), 1.81–1.89 (5H, m, 5CH), 2.16 (1H, d, $J$ = 8.5 Hz, CH), 2.50–2.53 (2H, m, 2CH), 2.94–3.01 (1H, s, CH), 3.04 (1H, d, $J$ = 16.4 Hz, CH), 3.74 (1H, dd, $J$ = 15.9 Hz, 9.0 Hz, CH), 3.95 (1H, br s, $CH_3$C*H*), 4.36–4.43 (1H, m, CH), 7.19 (1H, br s, ArH). LRMS (FAB$^+$) *m*/*z* 464 ([M($^{35}$Cl$_2$)+H]$^+$), 466 ([M($^{35}$Cl$^{37}$Cl)+H]$^+$), 468 ([M($^{37}$Cl$_2$)+H]$^+$). Anal. Calcd for $C_{25}H_{31}Cl_2NO_3$: C, 64.65; H, 6.73; N, 3.02. Found: C, 64.63; H, 6.87; N, 2.94.

**12,14-Dichloro-13-isopropyl-8,11a-dimethyl-6-oxo-5-(5-phenylpent-4-ynyl)-6,7,7a,8,9,10,11,11a-octahydro-5*H*-dibenzo[*b*,*d*]azepine-8-carboxylic acid methyl ester (32c).** A mixture of the alkyne **17a** (20 mg, 0.063 mmol), iodobenzene (0.008 mL, 0.075 mmol, 1.2 equiv), $Pd(PPh_3)_2Cl_2$ (1 mg, 0.001 mmol, 0.02 equiv), and CuI (1 mg, 0.005 mmol, 0.08 equiv) in $Et_3N$ (1 mL) was stirred at rt for 14 h under Ar atmosphere. The reaction mixture was filtered, the solvent was evaporated in vacuum, and the resultant residue was purified by flash chromatography (*n*-hexane/AcOEt = 5:1) to afford **32a** (37 mg, quantitative yield) as a pale yellow amorphous solid. Compound **32a**: $^1$H NMR ($CDCl_3$) 1.42 (6H, d, $J$ = 6.7 Hz, 2C$H_3$CH), 1.42 (3H, s, $CH_3$), 1.47 (3H, s, $CH_3$), 1.69 (2H, m, 2CH), 1.81–1.98 (5H, m, 5CH), 2.06–2.11 (1H, m, CH), 2.20 (1H, d, $J$ = 8.5 Hz, CH), 2.52 (2H, t, $J$ = 7.0 Hz, $CH_2$), 2.89 (1H, d, $J$ = 16.3 Hz, CH), 3.10–3.17 (1H, m, CH), 3.48 (3H, s, $CO_2CH_3$), 3.73 (1H, dd, $J$ = 16.2 Hz, 8.5 Hz, CH), 3.96 (1H, br s, $CH_3$C*H*), 4.32–4.39 (1H, m, CH), 7.20 (1H, br s, ArH), 7.27–7.29 (3H, m, ArH), 7.41 (2H, dd, $J$ = 7.9 Hz, 1.9 Hz, ArH). LRMS (FAB$^+$), *m*/*z* 554 ([M($^{35}$Cl$_2$) +H]$^+$), 556 ([M($^{35}$Cl$^{37}$Cl)+H]$^+$), 558 ([M($^{37}$Cl$_2$)+H]$^+$).

**12,14-Dichloro-13-isopropyl-8,11a-dimethyl-6-oxo-5-(5-phenylpent-4-ynyl)-6,7,7a,8,9,10,11,11a-octahydro-5*H*-dibenzo[*b*,*d*]azepine-8-carboxylic acid (33a).** A mixture of the methyl ester **32a** (37 mg, 0.067 mmol), KOH (38 mg, 0.669 mmol, 10.0 equiv), and 18-crown ether-6 (44 mg, 0.167 mmol, 2.5 equiv) in MeOH (1 mL) was stirred at 80 °C for 15 h, the whole was cooled, and the solvent was evaporated in vacuum. The residue was diluted with water (10 mL), acidified with 2 N HCl (2 mL), and then extracted with $CHCl_3$ (3× 20 mL). The combined organic layer was washed with brine (1× 20 mL), dried over $Na_2SO_4$, filtered, and then evaporated in vacuum. The residue was purified by flash chromatography (*n*-hexane/AcOEt = 10:1) to afford **33a** (31 mg, 0.058 mmol, yield 87%) as a colorless oil. Compound **33a**: colorless powder. Mp 127–130 °C (*n*-hexane/AcOEt). $^1$H NMR ($CDCl_3$) 1.37–1.44 (12H, m, 4$CH_3$), 1.70 (2H, m, 2CH), 1.79–1.88 (5H, m, 5CH), 1.90–2.04 (1H, m, CH), 2.11 (1H, d, $J$ = 8.5 Hz, CH), 2.47 (2H, t, $J$ = 6.9 Hz, $CH_2$), 2.89 (1H, d, $J$ = 16.3 Hz, CH), 3.07–3.14 (1H, m, CH), 3.48 (1H, dd, $J$ = 16.1 Hz, 8.6 Hz, CH), 3.94 (1H, br s, $CH_3$C*H*), 4.27–4.36 (1H, m, CH), 7.13–7.36 (6H, m, ArH). LRMS (FAB$^+$) *m*/*z* 540 ([M($^{35}$Cl$_2$)+H]$^+$), 542 ([M($^{35}$Cl$^{37}$Cl)+H]$^+$), 544 ([M($^{37}$Cl$_2$)+H]$^+$). Anal. Calcd for $C_{22}H_{29}Cl_2NO_3$: C, 68.88; H, 6.53; N, 2.59. Found: C, 68.62; H, 6.65; N, 2.58.

**13-Isopropyl-8,11a-dimethyl-6-oxo-5-(5-phenylpent-4-ynyl)-6,7,7a,8,9,10,11,11a-octahydro-5*H*-dibenzo[*b*,*d*]azepine-8-carboxylic acid methyl ester (32b).** A mixture of the alkyne **17b** (88 mg, 0.215 mmol), iodobenzene (0.029 mL, 0.258 mmol, 1.2 equiv), $Pd(PPh_3)_2Cl_2$ (2 mg, 0.002 mmol, 0.01 equiv), and CuI (1 mg, 0.005 mmol, 0.02 equiv) in $Et_3N$ (1 mL) was stirred at rt for 21 h under Ar atmosphere. The reaction mixture was filtered, the solvent was evaporated in vacuum, and the resultant residue was purified by flash chromatography (*n*-hexane/AcOEt = 2:1) to afford **32b** (81 mg, 0.167 mmol. yield 78%) as a pale orange amorphous solid. Compound **32b**: $^1$H NMR ($CDCl_3$) 1.20 (6H, d, $J$ = 6.9 Hz, 2C$H_3$CH), 1.33 (3H, s, $CH_3$), 1.49 (3H, s, $CH_3$), 1.66–1.68 (2H, m, 2CH), 1.74–1.77 (2H, m, 2CH), 1.91 (1H, d, $J$ = 13.3 Hz, CH), 1.92–1.98 (2H, m, 2CH), 2.05–2.12 (1H, m, CH), 2.16–2.27 (1H, m, CH), 2.41–2.62 (4H, m, 4CH), 2.79–2.90 (1H, m, $CH_3$C*H*), 3.56–3.63 (1H, m, CH), 3.60 (3H, s, $CO_2CH_3$), 3.95–4.02 (1H, m, CH), 7.06 (1H, dd, $J$ = 8.2 Hz, 1.7 Hz, ArH), 7.13 (1H, d, $J$ = 1.9 Hz, ArH), 7.24–7.26 (3H, m, ArH), 7.28 (1H, d, $J$ = 8.3 Hz, ArH), 7.34–7.37 (2H, m, ArH). LRMS (FAB$^+$) *m*/*z* 486 ([M+H]$^+$).

**13-Isopropyl-8,11a-dimethyl-6-oxo-5-(5-phenylpent-4-ynyl)-6,7,7a,8,9,10,11,11a-octahydro-5*H*-dibenzo[*b*,*d*]azepine-8-carboxylic acid (33b).** A mixture of the methyl ester **32b** (67 mg, 0.139 mmol), KOH (93 mg, 0.167 mmol, 1.2 equiv), and 18-crown ether-6 (92 mg, 0.347 mmol, 2.5 equiv) in MeOH (1 mL) was stirred at 80 °C for 15 h, the whole was cooled, and the solvent was evaporated in vacuum. The residue was diluted with water (20 mL), acidified with 2 N HCl (2 mL), and then extracted with $CHCl_3$ (3× 20 mL). The combined organic layer was washed with brine (1× 20 mL), dried over $Na_2SO_4$, filtered, and then evaporated in vacuum. The residue was purified by flash chromatography (*n*-hexane/AcOEt = 1:1) to afford the unreacted compound **32b** (24 mg, 0.049 mmol, yield 35% after recrystallization) as a colorless amorphous solid and the desired compound **33b** (32 mg, 0.068 mmol, yield 49%) as a colorless amorphous solid. Compound **33b**: pale yellow powder. Mp 76–79 °C (*n*-hexane). $^1$H NMR ($CDCl_3$) 1.24 (6H, d, $J$ = 7.0 Hz, 2C$H_3$CH), 1.26 (3H, s, $CH_3$), 1.46 (3H, s, $CH_3$), 1.65–1.77 (4H, m, 4CH), 1.88–2.03 (2H, m, 2CH), 1.97 (1H, d, $J$ = 13.3 Hz, CH), 2.05–2.12 (1H, m, CH), 2.46–2.57 (4H, m, 4CH), 2.84–2.91 (1H, m, $CH_3$C*H*), 3.23–3.31 (1H, m, CH), 3.74–3.80

(1H, m, CH), 7.05 (1H, dd, $J = 8.2$ Hz, 1.8 Hz, ArH), 7.08 (1H, d, $J = 1.8$ Hz, ArH), 7.18 (2H, d, $J = 8.2$ Hz, ArH), 7.21–7.25 (2H, m, ArH), 7.29 (1H, d, $J = 7.4$ Hz, ArH). LRMS ($FAB^+$) *m/z* 472 ($[M+H]^+$). Anal. Calcd for $C_{31}H_{37}Cl_2NO_3$: C, 78.95; H, 7.91; N, 2.97. Found: C, 78.65; H, 8.07; N, 2.80.

### 5.2. Biological assays

**5.2.1. Electrophysiological measurements.** Human BK channel subunits (hslo α and hslo β1) were subcloned into pcDNA3 (Invitrogen, CA, USA) and pTracer-CMV2 (Invitrogen), respectively, and transiently co-expressed in TSA201 cells with FuGENE6 transfection reagent (Roche Diagnostics Corporation, IN, USA). BK channel currents were measured with the inside-out patch clamp technique (Patch/Whole Cell Clamp Amplifier CEZ-2400, Nihon Koden). Experiments was performed within 24–48 h after the transfection. All the experiments were carried out at room temperature (20–25 °C). Test compounds were diluted in the intracellular solution (in mM; 140 K-Mes (Alfa Aesar, MA, USA), 10 HEPES (Sigma–Aldrich, Inc., MO, USA), 5 HEDTA (Nacalai Tesque, Inc., Kyoto, Japan), and 100 nM free-$Ca^{2+}$, pH 7.4) at the final concentration of 10 μM and applied to the intracellular side of the patch membrane. The resistance of the patch pipette was 2–5 MΩ when filled with the pipette solution. Test voltages from −100 mV to 190 mV were applied at 10 mV steps. The BK channel conductance was determined by measuring the tail current at each test voltage, and plotted as conductance–voltage curves. $V_{1/2}$ (half-maximal activation voltage) values were calculated based on Boltzmann's fit of the conductance–voltage curves. The BK channel opening activities of tested compounds were evaluated as the hyperpolarizing shift of $V_{1/2}$.

**5.2.2. Measurement of isolated detrusor smooth muscle relaxation.** The experimental protocol complied with the Guidelines for Animal Experiments approved by Toho University. Urinary bladders were isolated from male Japanese white rabbits (3–4 kg, 3–4 months old) that had been euthanized with an overdose of pentobarbital. The isolated tissue was immediately immersed in Krebs-bicarbonate solution (in mM; 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 2.6 $CaCl_2$, 1.2 $KH_2PO_4$, 25 $NaHCO_3$, and 11 glucose). The longitudinal strips of detrusor smooth muscle (5–7 mm length, 2–3 mm width), after removal of mucosa by dissection, were suspended in an organ bath chamber containing 10 mL of Krebs solution, maintained at 37 °C and bubbled with 95% $O_2$/5% $CO_2$ gas. Muscle tension was increased isotonically using a force–displacement transducer (TB-612T, Nihon Koden, Tokyo, Japan) connected to a carrier amplifier (AP-601G, Nihon Koden) with a basal preload of ca. 2 gW as initial tension. The tissues were allowed to equilibrate for 1 h. The strips were precontracted with high-$K^+$ (30 mM or 120 mM) Krebs solution by replacement of the whole bath solution and allowed to equilibrate for 5–30 min (typically) before the addition of test compounds. When high-$K^+$ solutions were used to contract preparations, equimolar concentrations of $Na^+$ in Krebs solution were replaced with $K^+$ to maintain isotonicity. The relaxant activities of the test compounds were compared with that of vehicle (0.1% DMSO) measured in the same muscle strip. All values are expressed as means ± SEM. Statistical significance of differences was determined by paired Student's *t*-test, and *P* values less than 0.05 were considered to be significant.

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# Molecular Mechanisms for Large Conductance $Ca^{2+}$-Activated $K^+$ Channel Activation by a Novel Opener, 12,14-Dichlorodehydroabietic Acid

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## ABSTRACT

Our recent study has revealed that 12,14-dichlorodehydroabietic acid (diCl-DHAA), which is synthetically derived from a natural product, abietic acid, is a potent opener of large conductance $Ca^{2+}$-activated $K^+$ (BK) channel. Here, we examined, by using a channel expression system in human embryonic kidney 293 cells, the mechanisms underlying the BK channel opening action of diCl-DHAA and which subunit of the BK channel ($\alpha$ or $\beta$1) is the site of action for diCl-DHAA. BK channel activity was significantly enhanced by diCl-DHAA at concentrations of 0.1 $\mu$M and higher in a concentration-dependent manner. diCl-DHAA enhanced the activity of BK$\alpha$ by increasing sensitivity to both $Ca^{2+}$ and membrane potential without changing the single channel conductance. It is notable that the increase in BK channel open probability by diCl-DHAA showed significant inverse voltage dependence, i.e., larger potentiation at lower potentials. Since coexpression of $\beta$1 subunit with BK$\alpha$ did not affect the potency of diCl-DHAA, the site of action for diCl-DHAA is suggested to be BK$\alpha$ subunit. Moreover, kinetic analysis of single channel currents indicates that diCl-DHAA opens BK$\alpha$ mainly by decreasing the time staying in a long closed state. Although reconstituted voltage-dependent $Ca^{2+}$ channel current was significantly reduced by 1 $\mu$M diCl-DHAA, BK channels were selectively activated at lower concentrations. These results indicate that diCl-DHAA is one of the most potent BK channel openers acting on BK$\alpha$ and a useful prototype compound to develop a novel BK channel opener.

Large conductance $Ca^{2+}$-activated $K^+$ (BK) channels are expressed in many different types of excitable cells and have significant physiological roles in the regulation of frequency of firing, action potential repolarization, and/or afterhyperpolarization (for reviews, see Vergara et al., 1998; Kaczorowski and Garcia, 1999). BK channel activation by the $Ca^{2+}$-induced $Ca^{2+}$ release during excitation-contraction coupling significantly contributes to action potential repolarization/afterhyperpolarization in some smooth muscle cells (Imaizumi et al., 1998; Ohi et al., 2001).

In addition, the negative feedback control of intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) by BK channels works to protect cells from $Ca^{2+}$ overload during pathophysiological conditions (Lawson, 2000). Hyperpolarization of neuronal cells by BK channel activation down-regulates the activity of voltage-dependent $Na^+$ and $Ca^{2+}$ channels and may prevent cell death, which is mainly caused by excess intracellular $Ca^{2+}$ in the setting of brain ischemia following stroke. In smooth muscle cells, BK channels are also activated by spontaneous $Ca^{2+}$ release from sarcoplasmic reticulum (Nelson and Quayle, 1995; Bolton and Imaizumi, 1996; Imaizumi et al., 1999) and are thought to be one of the essential regulators of resting membrane potential. Accumulated evidence indicates that the control of BK channel activity in arterial smooth muscle is one of the major determinants of vascular tone and that its abnormality can be a cause of hypertension (Brenner

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**ABBREVIATIONS:** BK, large conductance $Ca^{2+}$-activated $K^+$; $[Ca^{2+}]_i$, intracellular $Ca^{2+}$ concentration; BMS-204352, (3*S*)-(+)-(5-chloro-2-methoxyphenyl)-1,3-dihydro-3-fluoro-6-(trifluoromethyl)-2*H*-indole-2-one; L-735,334, 14-hedroxy 8-daucene-3,4-diol oleate; diCl-DHAA, 12,14-dichlorodehydroabietic acid; CaV, voltage-dependent $Ca^{2+}$; HEK, human embryonic kidney; I-V, current-voltage; SK, small conductance $Ca^{2+}$-activated $K^+$; IK, intermediate conductance $Ca^{2+}$-activated $K^+$; CGS-7181, ethyl 2-hydroxy-1-[[(4-methylphenyl)amino]oxo]-6-trifluoromethyl-1*H*-indole-3-carboxylate; CGS-7184, ethyl 1-[[(4-chlorophenyl)amino]oxo]-2-hydroxy-6-trifluoromethyl-1*H*-indole-3-carboxylate; NS-1608, *N*-(3-(trifluoromethyl)phenyl)-*N*′-(2-hydroxy-5-chlorophenyl)urea; NS-1619, 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2*H*-benzimidazol-2-one; NS-8, 2-amino-3-cyano-5-(2-fluorophenyl)-4-methylpyrrole.

et al., 2000b; Wellman and Nelson, 2003; Fernández-Fernández et al., 2004).

Agents that enhance BK channel activity (BK channel openers) may therefore be effective in protecting neurons from damage following an ischemic stroke and/or in suppressing excess activity of smooth muscle tissues (Lawson, 2000). Many compounds that were found from natural products or were synthesized have been reported to be BK channel openers (Coghlan et al., 2001). Most of these BK channel openers, including BMS-204352 (Gribkoff et al., 2001), are not highly potent activators ($EC_{50}$ values >300 nM) under the resting cellular conditions where intracellular $Ca^{2+}$ concentration is 50 to 150 nM (Schrøder et al., 2003). Terpenoids derived from natural products—dehydrosoyasaponin-I, maxikdiol, and L-735,334—have been reported as BK channel openers (Kaczorowski and Garcia, 1999). In addition, 17β-estradiol (Valverde et al., 1999) and epoxyeicosatrienoic acids (Fukao et al., 2001) may be endogenous BK channel openers, and some transmitters and hormones can enhance BK channel activity via kinase activation (Vergara et al., 1998).

In our previous study (Imaizumi et al., 2002), novel compounds, including pimaric acid, were discovered from terpenoids, which have chemical structures similar to that of maxikdiol, a moderate BK channel opener (Singh et al., 1994). Moreover, our recent study (Ohwada et al., 2003) has revealed that chemical modification of abietic acid, an inactive compound of resin acid derivatives, to dehydroabietic acid resulted in BK channel opening, and further chemical modification to 12,14-dichlorodehydroabietic acid (diCl-DHAA) led to finding of a potent BK channel opener. However, the underlying mechanisms of diCl-DHAA-induced activation of BK channel and the selectivity against voltage-dependent $Ca^{2+}$ (CaV) channel have not been defined. The present study was therefore undertaken to identify molecular mechanisms of diCl-DHAA-induced activation of BK channels and to examine the selectivity against inhibition of CaV channels by using human embryonic kidney (HEK) 293 cells as an expression system.

## Materials and Methods

**Vector Constructs, Cell Culture, and Transfection.** Restriction enzyme-digested DNA fragments of BKα (KpnI/XbaI-double digested) and BKβ1 (EcoRI/XbaI-double digested) were ligated into mammalian expression vectors pcDNA3.1(+) and pcDNA3.1/Zeo(+) (Invitrogen, Carlsbad, CA), respectively, using the TaKaRa ligation kit version 1 (TaKaRa, Osaka, Japan) (Yamada et al., 2001). HEK293 cell lines were obtained from Health Science Research Resources Bank (Tokyo, Japan) and maintained in minimal essential medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (JRH Biosciences, Lenexa, KS), 100 units/ml penicillin (Wako Pure Chemicals, Osaka, Japan), and 100 μg/ml streptomycin (Meiji Seika, Tokyo, Japan). Stable expression of BKα and BKβ was achieved by using calcium phosphate coprecipitation transfection techniques as reported previously (Imaizumi et al., 2002). G418- and G418/zeocin-resistant cells were selected as those which were BKα-expressing and BKβ1-coexpressing, respectively.

The cDNAs encoding voltage-dependent $Ca^{2+}$ channel α1C subunit of the rabbit (rCaVα1C) and β3 subunit of the mouse (mCaVβ3) were kind gifts from Dr. Veit Flockerzi (Institut für Pharmakologie und Toxikologie, Universität des Saarlandes, Hamburg, Germany) and were ligated into mammalian expression vectors pcDNA3.1(+) and pTracer(+), respectively (Murakami et al., 2003). These plasmid vectors were transfected into HEK293 cells for transient expression. The functional coexpression of rCaVα1C and mCaVβ3 was successfully determined by the appearance of the inward currents and green fluorescent protein fluorescence.

**Solutions.** The standard HEPES-buffered solution for electrophysiological recording had an ionic composition of 137 mM NaCl, 5.9 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 14 mM glucose, and 10 mM HEPES. The pH of the solution was adjusted to 7.4 with NaOH. The pipette solution for whole-cell recordings of $K^+$ currents contained 140 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, 2 mM $Na_2ATP$, and 5 mM EGTA. The pCa and pH of the pipette solution were adjusted to 6.5 and 7.2 by adding $CaCl_2$ and KOH, respectively. For recordings of single BK channel currents in the excised inside-out patch configuration, the pipette solution contained the standard HEPES-buffered solution or $K^+$-rich HEPES-buffered solution that was prepared by replacement of 134.1 mM NaCl in the standard HEPES-buffered solution with equimolar KCl. The bathing solution contained 140 mM KCl, 1.2 mM $MgCl_2$, 14 mM glucose, 10 mM HEPES, and 5 mM EGTA. Selected pCa of the bathing solution was obtained by adding adequate amount of $CaCl_2$, and the pH was adjusted to 7.2 with NaOH. The pipette solution for whole-cell recording of $Ca^{2+}$ inward currents had an ionic composition of 140 mM CsCl, 1 mM $MgCl_2$, 10 mM HEPES, 2 mM $Na_2ATP$, and 5 mM EGTA. The pH of the pipette solution was adjusted to 7.2 by adding CsOH.

**Electrophysiological Experiments.** The whole-cell and inside-out patch clamps were applied to single cells using CEZ-2400 amplifier (Nihon Kohden, Tokyo, Japan) and EPC-7 amplifier (List Electronics, Darmstadt, Germany), respectively. The procedures of electrophysiological recordings and data acquisition/analysis for whole-cell recording have been described previously (Imaizumi et al., 1989). The resistance of the pipette was 1.5 to 3 MΩ for whole-cell and 15 to 25 MΩ for inside-out patch configurations when filled with the pipette solutions. The series resistance was partly compensated electrically under whole-cell voltage clamp. Whole-cell and single channel recordings were carried out at room temperature (24 ± 1°C). Single channel current analyses were done using software PAT V7.0C (developed by Dr. J. Dempster, University of Strathclyde, Glasgow, Scotland). The open probability ($P_o$) was measured from the event histogram plotted against current amplitude. The number of channels in a patch was determined from recordings at pCa = 3.5, and the analyses were performed only when the number of channels in a patch was less than six.

**Chemicals.** Most of pharmacological agents were obtained from Sigma-Aldrich (St. Louis, MO), unless mentioned otherwise. Iberiotoxin was obtained from Peptide Institute Inc. (Osaka, Japan). Pimaric acid, abietic acid, dehydroabietic acid, and diCl-DHAA were obtained from Helix Biotech (New Westminster, BC, Canada). The test compounds were dissolved with dimethyl sulfoxide. The final concentration of dimethyl sulfoxide was 0.03% or lower.

**Statistics.** Data are expressed as means ± S.E.M. Statistical significance between two groups and among multiple groups was evaluated using Student's $t$ test and Scheffé's test after F-test or one-way analysis of variance, respectively.

## Results

**Effects of diCl-DHAA on Macroscopic BK Channel Currents.** Effects of diCl-DHAA on BK channel currents were examined in single HEKBKαβ1 under whole-cell voltage-clamp mode. The $Ca^{2+}$ concentration in the pipette solution was fixed at pCa = 6.5 using a $Ca^{2+}$-EGTA buffer (see *Materials and Methods*). Depolarization from −60 to +10 mV induced outward currents in both native HEK and HEKBKαβ1, whereas the current density was approximately 4 times larger in the latter cells as reported previously (Imaizumi et al., 2002). Application of diCl-DHAA in a concentration range of 0.1 to 1.0 μM increased the outward

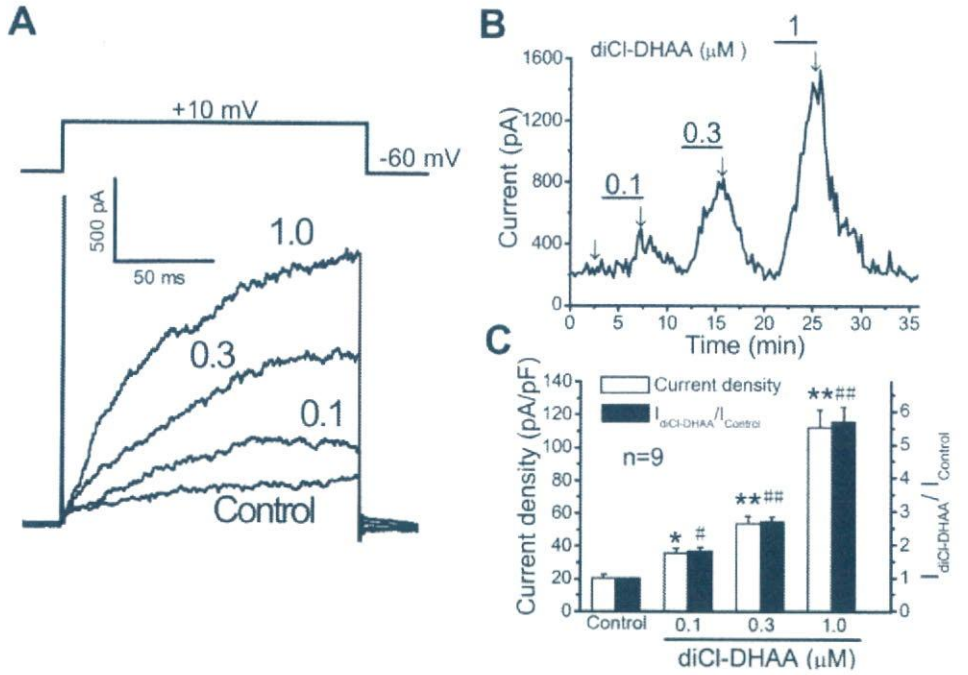


**Fig. 1.** Effects of diCl-DHAA on macroscopic membrane currents in HEKBK$\alpha\beta$1. A and B, a single HEKBK$\alpha\beta$1 was depolarized from −60 to +10 mV for 150 ms under whole-cell voltage-clamp mode. diCl-DHAA was applied in a concentration range of 0.1 and 1 $\mu$M. Original current recordings at different concentrations were superimposed and are shown in A. The peak outward current amplitude at +10 mV was measured and plotted against time in B. The original traces were obtained at the time indicated by vertical arrows in the time course. It is notable that effects of 0.1 to 1 $\mu$M diCl-DHAA were completely removed by washout. C, concentration-response relationships for diCl-DHAA. Experiments were carried out in a manner typically shown in A. Data about current density at +10 mV, which was obtained by dividing peak current amplitude with cell capacitance in each cell (pA/pF), are summarized as open columns. The relative amplitude of peak outward current at +10 mV in the presence of diCl-DHAA ($I_{diCl\text{-}DHAA}/I_{control}$) was also determined by taking the amplitude in the absence of diCl-DHAA as unity (closed columns). Means ± S.E.M. are shown by columns and vertical bars, respectively. */#, $p < 0.05$ and **/##, $p < 0.01$ versus control.

currents in HEKBK$\alpha\beta$1 in a dose-dependent manner (Fig. 1, A and B) but not in native HEK cells (data not shown). The enhancement of the outward currents in HEKBK$\alpha\beta$1 was completely removed by washout of diCl-DHAA (Fig. 1B). The relationship between concentrations of diCl-DHAA and corresponding responses is summarized in Fig. 1C. The increase in outward current density by diCl-DHAA was significant at a concentration of 0.1 $\mu$M and higher. Taking the current density at +10 mV in the control as unity, the relative amplitude of peak outward currents in the presence of 0.1, 0.3, and 1 $\mu$M diCl-DHAA was also plotted against concentration (Fig. 1C).

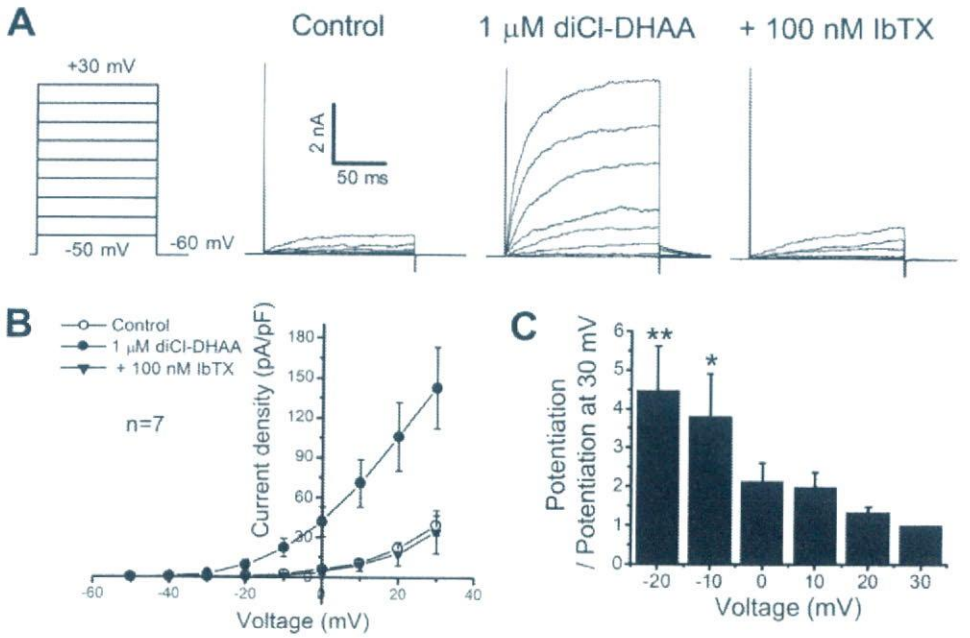


**Fig. 2.** Effects of diCl-DHAA on I-V relationship in BK$\alpha\beta$1. A, each single HEKBK$\alpha\beta$1 was depolarized from −60 mV by 10-mV steps for 150 ms under whole-cell voltage-clamp mode. The outward currents elicited by depolarization was markedly enhanced by application of 1 $\mu$M diCl-DHAA and then reduced by the addition of 100 nM iberiotoxin. B, I-V relationships were obtained in the control (open circles), in the presence of diCl-DHAA (closed circles), and after the addition of 100 nM iberiotoxin (closed triangles) in experiments such as typically shown in A. Number of examples is seven. C, voltage dependence of the potentiation of BK channel current by diCl-DHAA was reevaluated from the data shown in B. The relative potentiation of the outward currents by 1 $\mu$M diCl-DHAA was plotted against test potentials by taking that at +30 mV as unity. *, $p < 0.05$ and **, $p < 0.01$ versus unity at +30 mV.

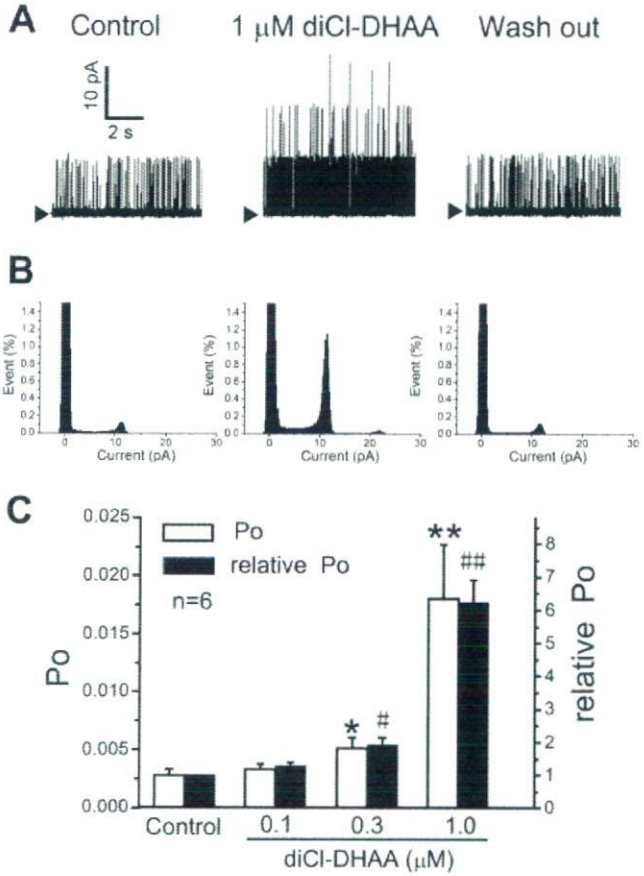


**Fig. 3.** Effects of diCl-DHAA on single BK$\alpha$ channel currents recorded using an inside-out patch-clamp techniques. A, single channel currents were recorded at +40 mV in a patch from HEKBK$\alpha$ under symmetrical 140 mM $K^+$ conditions. The free $Ca^{2+}$ in the bathing solution was adjusted to pCa = 7.0. The original current traces were recorded before and after application of 1 $\mu$M diCl-DHAA and after the washout of diCl-DHAA. A closed triangle on the left side of each trace indicates the zero current level. B, amplitude histograms in the control, in the presence of 1 $\mu$M diCl-DHAA, and after the washout were obtained from the recordings shown in A. The ordinate expresses the relative area (percentage) at the corresponding amplitude in each bin (0.2 pA). C, summarized data demonstrate the relationship between concentrations of diCl-DHAA and $P_o$ of BK$\alpha$. $P_o$ was calculated from the histogram shown in B as the relative time spent at open state based on the total number of BK$\alpha$ channels in the patch, which was determined by elevating $Ca^{2+}$ concentration to pCa = 3.5 (open columns). The relative $P_o$ was obtained taking the $P_o$ in the absence of diCl-DHAA as unity (closed columns). Number of examples is six. */#, $p < 0.05$ and **/##, $p < 0.01$ versus control.

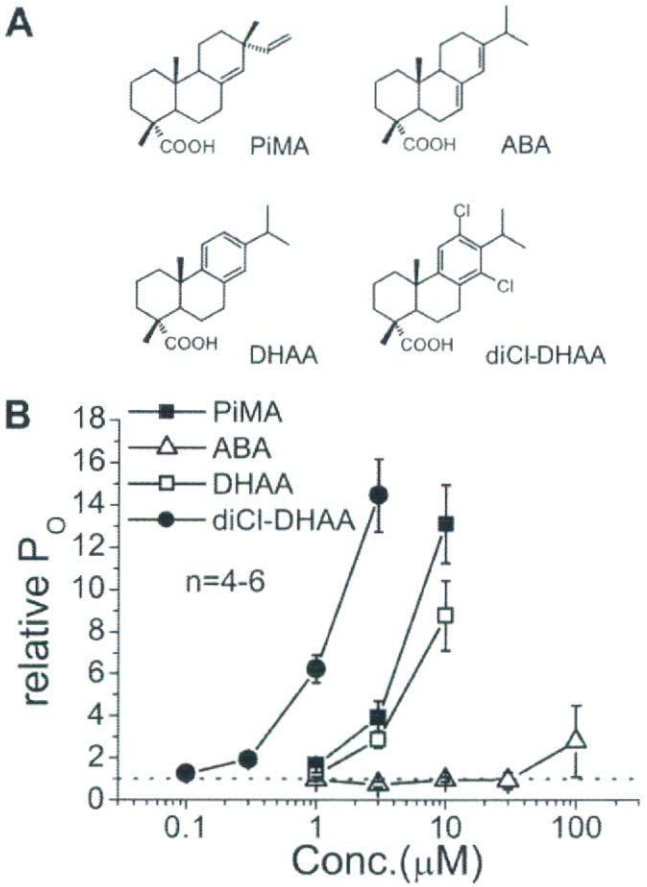


**Fig. 4.** Structure-activity relationships of abietic acid derivatives on $P_o$ of BK$\alpha$. A, chemical structures of test compounds pimaric acid, abietic acid, dehydroabietic acid, and diCl-DHAA. B, concentration-response relationships for compounds listed in A. Effects of abietic acid (open triangles), DHAA (open squares), and diCl-DHAA (closed circles) were examined in experiments identical to that shown in Fig. 3 for diCl-DHAA. The data for pimaric acid (closed squares) are taken from a previous study (Imaizumi et al., 2002). The relative $P_o$ was determined taking the $P_o$ in the absence of compounds as unity (a dotted line). Means ± S.E.M. are shown by symbols and vertical bars, respectively. Number of experiments is four to six for each compound.

**Voltage Dependence of diCl-DHAA-Induced Enhancement of BK Channel Currents.** In Fig. 2, the voltage-dependent enhancement of BK channel currents was examined by analyzing effects of diCl-DHAA on the current-voltage (I-V) relationship. HEKBK$\alpha\beta$1 was depolarized from a holding potential of −60 mV to test potentials in the range between −50 and +30 mV with 10-mV steps (Fig. 2A). Application of 1 $\mu$M diCl-DHAA increased the currents at any test potential. Addition of 100 nM iberiotoxin, a specific BK channel blocker, removed the enhancement of outward currents, supporting that the action of diCl-DHAA was selective to BK channel currents. Figure 2B summarizes the relationships between current density of peak outward currents and test potentials in the absence and presence of 1 $\mu$M diCl-DHAA and after addition of 100 nM iberiotoxin. The current density at +30 mV was increased from 39.13 ± 7.34 to 142.55 ± 30.81 pA/pF ($n = 7$; $p < 0.01$). In Fig. 2C, the voltage dependence of diCl-DHAA-induced enhancement of BK channel currents was determined as the relationship between the relative potentiation of outward currents and test potentials by taking the potentiation at +30 mV in the presence of 1 $\mu$M diCl-DHAA as unity. The potentiation at −20 and −10 mV was significantly greater than that at +30 mV (4.48 ± 1.15 and 3.80 ± 1.09 times at −20 and −10 mV, respectively, $p < 0.01$, versus unity at +30 mV).

**Activation of Single BK$\alpha$ Channel Current by diCl-DHAA and Related Compounds.** Effects of diCl-DHAA on single BK$\alpha$ channel currents were examined in excised inside-out patch configuration. The bathing and pipette solution contained symmetrical 140 mM $K^+$. The free $Ca^{2+}$ concentration in the bathing solution was pCa7. Under these conditions, the unitary current amplitude and open probability ($P_o$) at +40 mV was 10.1 ± 0.2 pA and 0.0028 ± 0.0005 ($n = 6$), respectively. The application of 1 $\mu$M diCl-DHAA increased channel activity without change in the unitary

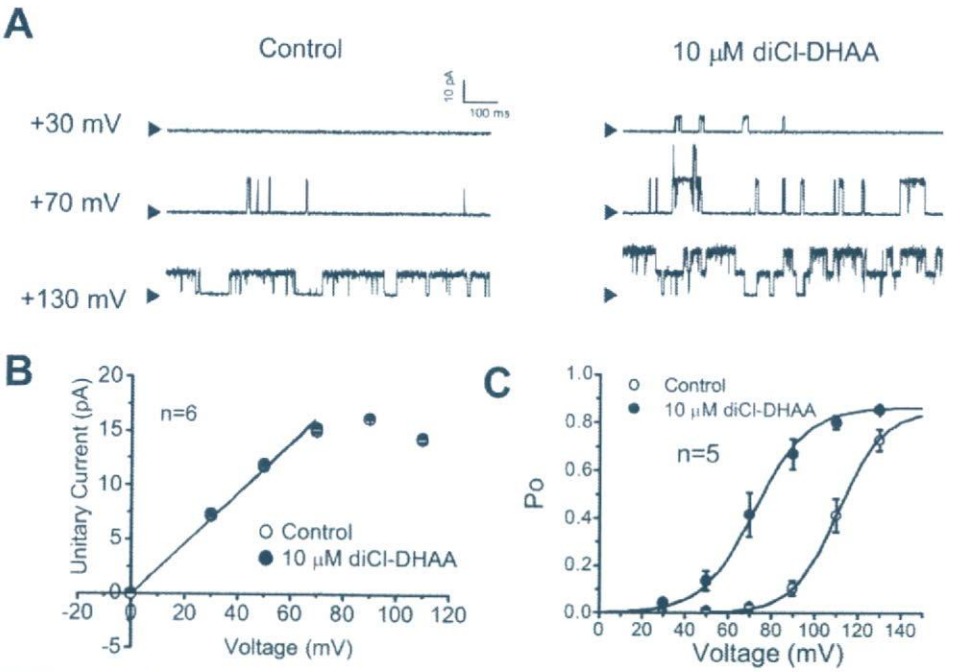


**Fig. 5.** Effects of diCl-DHAA on single channel conductance and voltage dependence of BKα. Single channel currents of BKα were recorded in inside-out patch configuration at pCa = 7.0 in symmetrical 140 mM $K^+$ conditions. Recordings were obtained at several test potentials in the range of 0 to +130 mV in the absence and presence of 10 μM diCl-DHAA. Experimental conditions, except applied potentials, are the same as those shown in Fig. 3. A, original current traces at +30, +70, and +130 mV in the absence (left) and presence of 10 μM diCl-DHAA (right). B, relationship between unitary current amplitude and test potentials was plotted in a range of 0 and +70 mV in the absence (open circles) and presence of 10 μM diCl-DHAA (closed circles) and was fitted by a linear line. The single channel conductance was determined from the slope ($n$ = 6). C, effects of diCl-DHAA on voltage dependence of BKα. The relationships between $P_o$ and test potentials were obtained in the absence (open circles) and presence of 10 μM diCl-DHAA (closed circles). Number of examples is five for each. The data were fitted using the Boltzmann equation (see "Results"). The fitted lines are based on the following values of $V_{1/2}$, $S$, and $C$: 110.7 mV, 10.5 mV, and 0.15 in the absence and 72.3 mV, 12.2 mV, and 0.13 in the presence of 10 μM diCl-DHAA, respectively.

current amplitude (10.43 ± 0.28 pA, 0.0180 ± 0.0047, $n$ = 6; Fig. 3, A and B). It is notable that diCl-DHAA was effective on BKα even when applied to the cytosolic phase. This effect of diCl-DHAA was completely removed by the washout. The $P_o$ was significantly increased by diCl-DHAA at 0.3 μM and higher concentrations (Fig. 3C), and the relative $P_o$ determined by taking $P_o$ in the control as unity was 1.26 ± 0.13, 1.92 ± 0.19, 6.24 ± 0.67, and 14.45 ± 1.72 in the presence of 0.1, 0.3, 1.0, and 3.0 μM diCl-DHAA, respectively ($n$ = 4–6). These results are mostly comparable with those obtained under whole-cell clamp conditions (Fig. 1C). In Fig. 4, the potency of diCl-DHAA to activate BKα was compared with that of abietic acid and dehydroabietic acid, an aromatic derivative of abietic acid. The data for pimaric acid, a potent BK channel opener, were also taken from a previous study, where the potency of pimaric acid was determined under the same experimental conditions (Imaizumi et al., 2002). Even a high concentration of abietic acid at 30 and 100 μM failed to increase the activity of BKα, whereas dehydroabietic acid at 3 and 10 μM increased activity significantly. Nevertheless, diCl-DHAA was much more potent as an activator of BKα than dehydroabietic acid and pimaric acid.

**Effects of diCl-DHAA on Characteristics of Single BKα Channel Currents.** In Fig. 5, effects of diCl-DHAA on characteristics of single BKα channel currents were systematically examined in the excised inside-out patch configuration. The bathing and pipette solution contained symmetrical 140 mM $K^+$. The pCa in the bathing solution was 7. The conductance of BKα, which was determined by slope of the regression line between 0 and +70 mV, was 224.8 ± 4.1 and 224.8 ± 4.3 pS in the absence and presence of 10 μM diCl-DHAA, respectively ($n$ = 6; $p > 0.05$; Fig. 5B), indicating that diCl-DHAA did not affect BKα channel conductance. The inward rectification shown at high potentials (more than +80 mV) was consistent with that reported as voltage-dependent block of BK channel by $Na^+$ (Yellen, 1984) and was not affected by diCl-DHAA. Moreover, the $P_o$ in the absence and presence of 10 μM diCl-DHAA was calculated and plotted against test potentials in Fig. 5C. Under these conditions, the increase in $P_o$ was voltage-dependent in the range of +30 to +130 mV, and a set of data were well described by Boltzmann relationship:

$$P_o = (1 - C)/[1 + \exp\{(V_{1/2} - V_m)/S\}] \quad (1)$$

where $V_{1/2}$, $V_m$, $S$, and $C$ is the voltage required for half-maximum activation, membrane potential, slope factor, and constant, respectively. Application of 10 μM diCl-DHAA neither changed $S$ nor $C$ ($S$, 10.5 ± 1.5 and 12.2 ± 1.3 mV; $C$, 0.15 ± 0.03 and 0.13 ± 0.02 in the absence and presence of diCl-DHAA, respectively; $n$ = 5), whereas it significantly shifted $V_{1/2}$ to a more negative potential (110.7 ± 3.3 and 72.4 ± 5.7mV, respectively; $n$ = 5; $p < 0.01$).

In Fig. 6, the effects of diCl-DHAA on $Ca^{2+}$ sensitivity of BKα were examined at 0 mV in asymmetrical 5.9/140 mM $K^+$ conditions. When $Ca^{2+}$ concentration in the bathing solution was elevated in a pCa range between 7.0 and 5.0, the $P_o$ was increased in a concentration-dependent manner (Fig. 6, A and B). The relationship between $Ca^{2+}$ concentration and the $P_o$ of BKα was fitted by the following equation:

$$P_o = (1 - C)/1 + (K_d/[Ca^{2+}]_i)^m \quad (2)$$

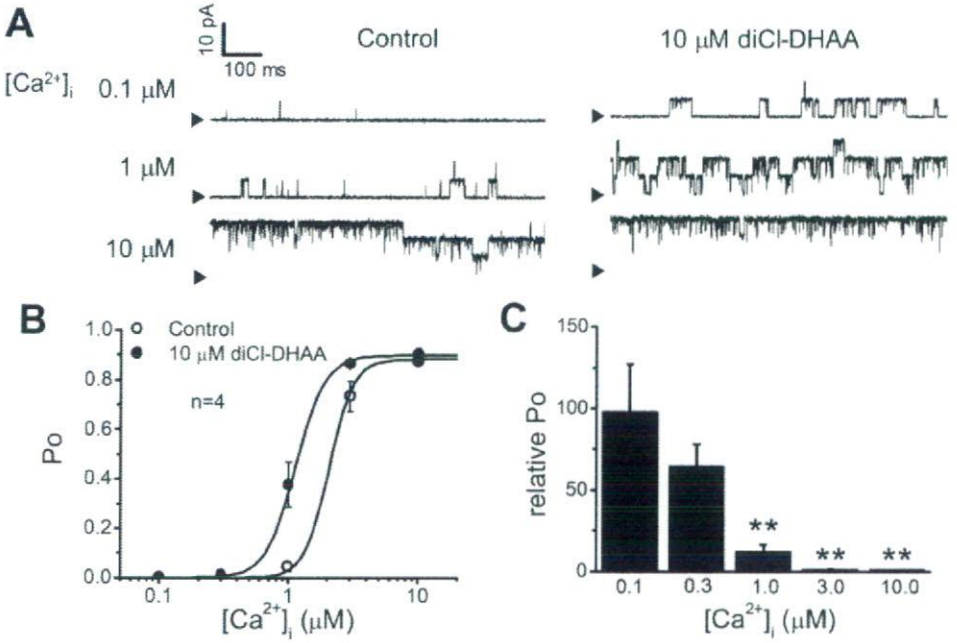


**Fig. 6.** Effects of diCl-DHAA on $Ca^{2+}$ dependence of BK$\alpha$. A, single channel currents of BK$\alpha$ were recorded in inside-out patch configuration at 0 mV in asymmetrical $K^+$ conditions (5.9/140 mM $K^+$) in the absence and presence of 10 $\mu$M diCl-DHAA. Recordings were obtained when $[Ca^{2+}]_i$ was 0.1, 1, or 10 $\mu$M. B, relationships between $P_o$ and $[Ca^{2+}]_i$ were obtained in the absence (open circles) and presence of 10 $\mu$M diCl-DHAA (closed circles). Number of examples is four for each experiment. The data were fitted with the Hill equation (see *Results*). The fitted lines were plotted based on the following values of $K_d$, $m$, and $C$: 2.06, 4.65, and 0.15$\mu$M in the absence and 1.14, 3.83, and 0.10 $\mu$M in the presence of 10 $\mu$M diCl-DHAA, respectively. C, relative $P_o$ in the presence of 10 $\mu$M diCl-DHAA versus that in the absence of 10 $\mu$M diCl-DHAA was re-evaluated at various $[Ca^{2+}]_i$ from the data shown in B. **, $p < 0.01$ versus relative $P_o$ at 0.1 $\mu$M $Ca^{2+}$.

where $K_d$ is an apparent dissociation constant of $Ca^{2+}$, $[Ca^{2+}]_i$ is the pCa in the bathing solution, $m$ is a Hill coefficient, and $C$ is a constant. Under the control conditions, $K_d$, $m$, and $C$, which were obtained from the best fitting, were pCa = 5.68 ± 0.03, 4.65 ± 0.92, and 0.152 ± 0.029, respectively ($n$ = 4). In the presence of 10 $\mu$M diCl-DHAA, $K_d$ was changed to pCa = 5.94 ± 0.03 ($n$ = 4; $p < 0.05$), whereas the Hill coefficient, which indicates binding of one $Ca^{2+}$ to each $\alpha$ subunit in the tetrameric complex of a functional BK$\alpha$ channel, was not significantly affected (3.83 ± 0.45; $p > 0.05$ versus control). $C$ was 0.102 ± 0.008 and not affected by diCl-DHAA ($p > 0.05$ versus control). The relative $P_o$ in the presence of 10 $\mu$M diCl-DHAA to that of the control was plotted against $[Ca^{2+}]_i$ in Fig. 6C. The lower the $P_o$ in the control conditions, the larger the enhancement by diCl-DHAA.

Effects of diCl-DHAA on the kinetic properties of BK$\alpha$ were examined in excised inside-out patches, which included only one channel (Fig. 7). These patches had a single channel event even when $[Ca^{2+}]_i$ was elevated to pCa = 3.5. Figure 7A shows original current traces of BK$\alpha$ at pCa = 6.5 in the absence and presence of 10 $\mu$M diCl-DHAA. The data for open and closed dwell time in Fig. 7A were reconstituted as distribution histograms in Fig. 7, B and C, respectively. These histograms were well fitted by a double and triple exponential function, respectively (Fig. 7, B and C). As shown in Table 1, application of 10 $\mu$M diCl-DHAA caused a marked decrease in the mean closed time ($\tau_{Cs}$) and its relative magnitude ($A_{Cs}$) of the slow component (4–5 times change), whereas other parameters were moderately changed ($\tau_{Os}$, $\tau_{Cf}$, $\tau_{Ci}$, and $A_{Of}$) or were not affected ($\tau_{Of}$, $A_{Os}$, $A_{Cf}$, and $A_{Ci}$) ($n$ = 5).

**Comparison of diCl-DHAA-Induced Effects on BK$\alpha$ with Those on BK$\alpha\beta$1.** The enhancement of single BK$\alpha$ channel activity by diCl-DHAA indicated the direct action of this compound on BK$\alpha$. It has been, however, established that coexpression of $\beta$1 subunit with BK$\alpha$ increases the sensitivity of BK$\alpha$ to $Ca^{2+}$ and voltage (Wallner et al., 1996; Cox

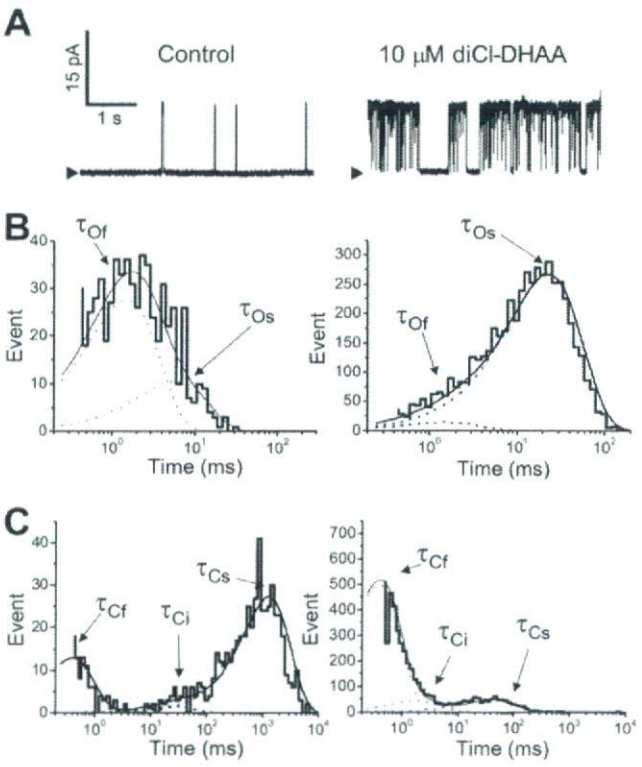


**Fig. 7.** Kinetics of diCl-DHAA-induced activation of BK$\alpha$. A, single channel currents of BK$\alpha$ were recorded at +50 mV in inside-out patch configuration at pCa = 6.5 in symmetrical 140 mM $K^+$ conditions. B, dwell-time histograms of open times before (left) and after (right) application of 10 $\mu$M diCl-DHAA. The open-time histogram in the absence and presence of diCl-DHAA was fitted by a double exponential function. $\tau_{Of}$ and $\tau_{Os}$ represent time constants of the fast and slow components of the open times in BK$\alpha$ kinetics, respectively. Continuous lines show the sum of individual components (dotted lines). C, dwell-time histograms of closed times before (left) and after (right) application of 10 $\mu$M diCl-DHAA. The closed time histogram was fitted by a triple exponential function. $\tau_{Cf}$, $\tau_{Ci}$, and $\tau_{Cs}$ represent time constants of the fast, intermediate, and slow components of closed times, respectively.

and Aldrich, 2000). To determine whether diCl-DHAA also acts on the functional coupling between BK$\alpha$ and $\beta$1 subunits, the increase in $P_o$ by diCl-DHAA in BK$\alpha\beta$1 was compared with that in BK$\alpha$ in inside-out patches. The $P_o$ of BK$\alpha$ at pCa = 7.0 was increased at any test potential by coexpression with $\beta$1 subunit (Fig. 8A). Since the increase in $P_o$ by diCl-DHAA depended on a basal $P_o$ before application (Fig. 6C), effects of diCl-DHAA on BK$\alpha$ at +40 mV were compared with those on BK$\alpha\beta$1 at +20 mV. The basal $P_o$ values were comparable with each other (0.0028 ± 0.0006 versus 0.0023 ± 0.0006; $n$ = 5; Fig. 8B). The application of 1 $\mu$M diCl-DHAA increased the $P_o$ to 0.0180 ± 0.0058 in BK$\alpha$ ($n$ = 5; $p < 0.05$) and to 0.0208 ± 0.0037 in BK$\alpha\beta$1 ($n$ = 5; $p < 0.01$). The ratio of $P_o$ in BK$\alpha$ in the presence and absence of diCl-DHAA was 7.2 ± 0.8 and therefore not significantly different from that in BK$\alpha\beta$1 (11.1 ± 3.1; $p > 0.05$). This finding strongly suggests that coexpression of $\beta$1 subunit did not affect the diCl-DHAA-induced enhancement of BK$\alpha$.

**Selectivity of diCl-DHAA on BK Channel Versus Voltage-Dependent $Ca^{2+}$ Channel.** To examine whether the action of diCl-DHAA is selective to BK channels over CaV channels, effects of 0.3 and 1 $\mu$M diCl-DHAA on BK$\alpha$ were compared with those on CaV channel currents in HEK293 cells, which coexpressed $\alpha$1 subunit of rabbit CaV channel and $\beta$3 subunit of mouse CaV$\alpha$1C$\beta$3. Here, effects of pimaric acid on CaV$\alpha$1C$\beta$3 were also examined. The inward currents through CaV$\alpha$1C$\beta$3 were elicited upon depolarization from a holding potential of −60 mV to test potentials in a range of −50 and +40 mV by 10-mV step every 10 s. The maximum amplitude was obtained at +10 mV (peak amplitude, 193 ± 54.4 pA; $n$ = 6; Fig. 9A). CaV$\alpha$1C$\beta$3 channel currents were not inhibited by 0.3 $\mu$M diCl-DHAA or pimaric acid, whereas they were significantly inhibited by both compounds at 1 $\mu$M (only diCl-DHAA; Fig. 9A). Data about effects of diCl-DHAA and pimaric acid on BK$\alpha$ are those shown in Fig. 3 and provided in a previous study (Imaizumi et al., 2002) and were obtained in inside-out patches at +40 mV and pCa = 7.0 under symmetrical 140 mM $K^+$ conditions. Enhancement of BK channel activity by diCl-DHAA was significant at 0.3 and 1 $\mu$M, indicating that 0.3 $\mu$M diCl-DHAA is selective for the BK channel over the CaV channel.

TABLE 1
Time constants and relative weights

| | Control | diCl-DHAA | diCl-DHAA/ Control |
|---|---|---|---|
| Time constants (ms) | | | |
| $\tau_{Of}$ | 1.49 ± 0.17 | 1.53 ± 0.22 | 1.02 ± 0.10 |
| $\tau_{Os}$ | 10.34 ± 3.75 | 23.73 ± 5.38 | 2.68 ± 0.52* |
| $\tau_{Cf}$ | 0.65 ± 0.05 | 0.74 ± 0.04 | 1.14 ± 0.04* |
| $\tau_{Cs}$ | 43.32 ± 11.05 | 11.33 ± 3.96 | 0.41 ± 0.16* |
| $\tau_{Cs}$ | 1955.19 ± 560.17 | 264.08 ± 77.97 | 0.21 ± 0.07** |
| Relative weights | | | |
| $A_{Of}$ | 0.53 ± 0.11 | 0.16 ± 0.04 | 0.40 ± 0.12** |
| $A_{Os}$ | 0.47 ± 0.11 | 0.84 ± 0.04 | 2.15 ± 0.44 |
| $A_{Cf}$ | 0.40 ± 0.08 | 0.82 ± 0.02 | 2.43 ± 0.54 |
| $A_{Cs}$ | 0.12 ± 0.04 | 0.11 ± 0.01 | 1.74 ± 0.73 |
| $A_{Cs}$ | 0.44 ± 0.11 | 0.07 ± 0.02 | 0.23 ± 0.07** |

* $p < 0.05$ and ** $p < 0.01$ vs. unity ($n$ = 5).

## Discussion

In the present study, we have demonstrated that diCl-DHAA is one of the most potent synthesized activators affecting the BK$\alpha$ subunit via changing the voltage and $Ca^{2+}$ sensitivity of the channel. Chemical modification of abietic acid, an inactive compound, to dehydroabietic acid and diCl-DHAA provides a potent and selective BK channel opener. The latter has an inverse voltage dependence for BK$\alpha$ channel activation and is one of the most potent openers available by application from outside of the cell membrane.

BK channels consist of channel-forming $\alpha$ subunits and accessory $\beta$ subunits ($\beta$1–$\beta$4) arranged in tetramers (Vergara et al., 1998). Each $\beta$ subunit interacts with N-terminal region of an $\alpha$ subunit (Wallner et al., 1996) and regulates the activity of the $\alpha$ subunit by changing $Ca^{2+}$ and voltage sensitivity and/or channel kinetics. Although only one major type of $\alpha$ subunit with splice variants has been defined, the combination of the BK channel $\alpha$ subunit encoded by KCNMA1 and $\beta$ subunits encoded by KCNMB1-4 provides the

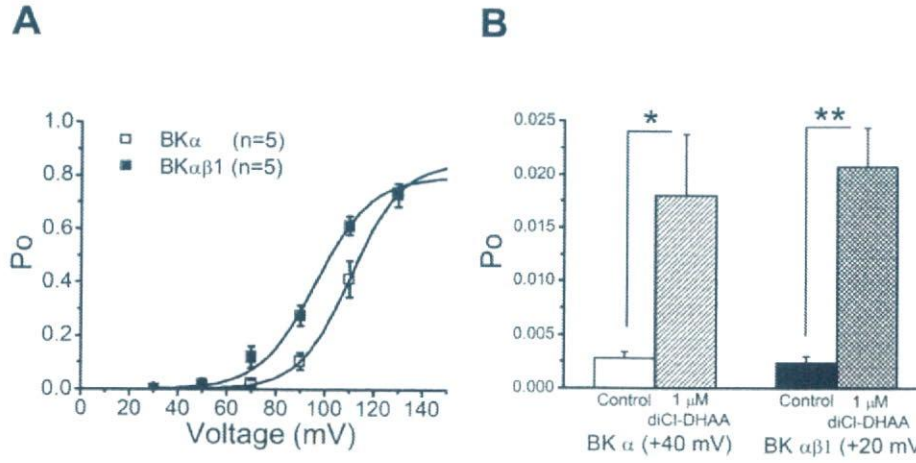


**Fig. 8.** Comparison of effects of diCl-DHAA on single channel currents due to BK$\alpha$ or BK$\alpha\beta$1. A, relationships between $P_o$ and test potentials were obtained in HEKBK$\alpha$ (open squares) and HEKBK$\alpha\beta$1 (closed squares). Recordings were obtained at pCa = 7.0 in symmetrical 140 mM $K^+$ conditions. Number of examples is five for each experiment. The data were fitted using the Boltzmann equation (see *Results*). The fitted lines are based on the following values of $V_{1/2}$, $S$, and $C$: 110.7 mV, 10.5 mV, and 0.15 in HEKBK$\alpha$ and 96.3 mV, 11.4 mV, and 0.20 in HEKBK$\alpha\beta$1, respectively. B, effect of 1 $\mu$M diCl-DHAA on $P_o$ of BK$\alpha\beta$1 was compared with that of BK$\alpha$. The $P_o$ of BK$\alpha$ in the absence of diCl-DHAA was 0.00382 ± 0.00104 at +40 mV and close to $P_o$ of BK$\alpha\beta$1 at +20 mV (0.00346 ± 0.00079; $p > 0.05$). Number of examples is five for each. *, $p < 0.05$ and **, $p < 0.01$ versus the corresponding control.

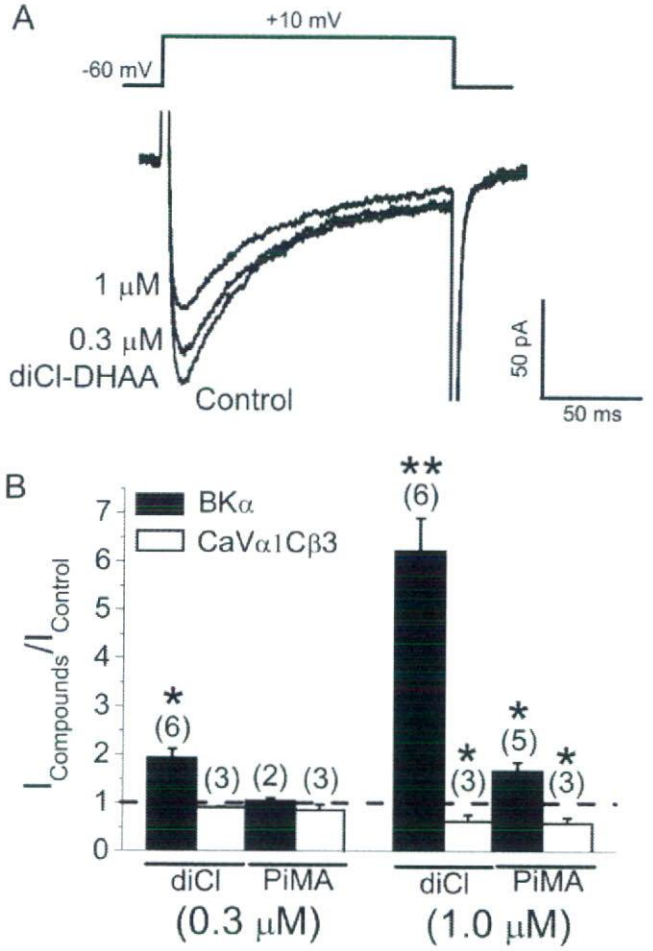


**Fig. 9.** Inhibitory effects of diCl-DHAA and pimaric acid on CaV channel currents. A, representative current traces obtained from HEKs coexpressing rabbit $\alpha$1C and mouse $\beta$3 with GFP (see *Materials and Methods*). The inward currents were elicited by 150-ms depolarizing pulses to +10 mV from a holding potential of −60 mV in the absence and presence of 0.3 or 1 $\mu$M diCl-DHAA. B, summary of effects of diCl-DHAA and pimaric acid on CaV channel currents (closed columns). For comparison, their effects on BK$\alpha$ are also illustrated. Inhibitory effects of diCl-DHAA and pimaric acid on CaV channel currents were examined in experiments identical to that shown in A, and the relative amplitude of inward currents at +10 mV in the presence of each compound was determined taking the amplitude in the absence as unity. For revaluation of effects of diCl-DHAA and pimaric acid on BK$\alpha$, the same set of data shown in Fig. 3 (present study) and Fig. 5 (Imaizumi et al., 2002) were used. Means ± S.E.M. are shown by columns and vertical bars, respectively. The number in parenthesis denotes number of cells used. *, $p < 0.05$ and **, $p < 0.01$ versus unity.

diversity of BK channels (McManus et al., 1995; Brenner et al., 2000a; Uebele et al., 2000), which offers opportunities of development of new therapeutic agents. Benzimidazolone derivatives such as biarylureas (NS-1608), NS-1619, BMS-204352 (Gribkoff et al., 2001), arylpyrrole (NS-8), and indole-3-carboxylic acid esters (CGS-7181 and CGS-7184) have been characterized as effective BK channel openers (Coghlan et al., 2001).

Natural products have also been evaluated as BK channel openers, and terpenoids such as dehydrosoyasaponin I (McManus et al., 1993), maxikdiol (Singh et al., 1994), and L-735,334 (Lee et al., 1995) have been identified as active BK channel openers. Our pioneer work of pimarane compounds, which have a close structural similarity to maxikdiol, has revealed that pimaric acid is a potent BK channel opener that interacts with BK$\alpha$ subunit but not with the BK$\beta$1 subunit (Imaizumi et al., 2002). Pimaric acid activates BK channels in HEKBK$\alpha\beta$1 when applied externally as well as when applied to the "internal phase" in inside-out patches. Its potency seems to be slightly higher than that of maxikdiol. An important comparative result in the previous study is that abietic acid did not show BK channel opening action despite of the fact that abietic acid is a structural isomer ($C_{20}H_{30}O_2$) of pimaric acid.

Of importance is our recent finding that chemical modification of abietic acid to dehydroabietic acid as well as diCl-DHAA produced compounds active to the open BK channel (Ohwada et al., 2003). In the present study, we provided new information about mechanisms of diCl-DHAA-induced activation of BK channels. diCl-DHAA activated BK channels in HEKBK$\alpha$ when applied externally as well as when applied to the internal phase in inside-out patches. Its potency was obviously higher than that of pimaric acid in whole-cell recording under the same experimental conditions, since significant activation was observed at 0.1 $\mu$M diCl-DHAA (Imaizumi et al., 2002). Dehydrosoyasaponin-I (Giangiacomo et al., 1998), 17$\beta$-estradiol (Valverde et al., 1999), and tamoxifen (Dick et al., 2001) interact with $\beta$ subunits of BK channels to increase the channel activity. In contrast, NS-1619 (Ahring et al., 1997), epoxyeicosatrienoic acid (Fukao et al., 2001), Evans blue (Yamada et al., 2001), and pimaric acid (Imaizumi et al., 2002) act on the BK$\alpha$ subunit. Our results clearly showed that diCl-DHAA interacts with the BK$\alpha$ subunit but may not interact with the BK$\beta$1 subunit. We also found that 100 nM iberiotoxin completely removed the diCl-DHAA-induced potentiation of the macroscopic BK$\alpha\beta$1 channel currents. This finding suggests that diCl-DHAA does not affect the iberiotoxin binding to BK$\alpha\beta$1, although effects of diCl-DHAA on $^{125}$I-iberiotoxin binding were not examined in this study. Consistently, the concentration-response relationship of charybdtoxin for the inhibition of macroscopic BK$\alpha\beta$1 channel currents was not affected by the presence of 10 $\mu$M pimaric acid, which has a close structural analogy to diCl-DHAA (Imaizumi et al., 2002).

It is obvious from the present results that diCl-DHAA activates BK channels in a voltage- and $Ca^{2+}$-dependent manner. It is very notable that the potentiation of BK channel activity by diCl-DHAA was significantly larger at negative potentials as well as at lower $Ca^{2+}$ concentrations. In contrast, BMS-204352, a potent BK channel opener, caused activation of BK channel currents only at positive potentials (more than +30 mV; Gribkoff et al., 2001; Schrøder et al., 2003). To our knowledge, diCl-DHAA, and presumably pimaric acid as well, are the only compounds that show marked inversed voltage dependence for potentiation among various types of BK channel openers. This characteristic feature of diCl-DHAA can be considered particularly effective to prevent membrane depolarization, hyperexcitability, and/or excess $Ca^{2+}$ influx to the cell and may be advantageous for clinical use. Moreover, diCl-DHAA decreased the time for the channels to stay in the prolonged closed states. It can be, therefore, suggested that this kinetic change in the presence of diCl-DHAA causes activation of BK channel. Niflumic acid opens BK channels mainly by decreasing the time in the long-closed states (Ottolia and Toro, 1994).

It is important to characterize the selectivity of diCl-DHAA to BK channels over that of other ion channels. We found that diCl-DHAA at concentrations of 0.3 $\mu$M or less increased BK channel activity without inhibiting CaV channels. Moreover, even though diCl-DHAA-induced inhibition of CaV channels at 1 $\mu$M was comparable with that by 1 $\mu$M pimaric acid (~30% of the control), the activation of BK channel currents by diCl-DHAA was significantly greater than that by pimaric acid, suggesting that the potent activation of BK channels by diCl-DHAA provides more selectivity against CaV channels than that by pimaric acid. The selectivity of BK channel openers against CaV channels has not been well defined, but nordihydroguaiaretic acid or NS-1619-induced inhibition of CaV channel currents was comparable with or slightly more potent than the activation of BK channels, respectively (Holland et al., 1996; Yamamura et al., 1999). For development of potent and selective BK channel openers, scaffoldings of dehydroabietic acid may be useful (Ohwada et al., 2003).

Effects of diCl-DHAA on small (SK) and intermediate (IK) conductance $Ca^{2+}$-activated $K^+$ channels were not examined systematically in this study. BK channel openers reported so far, including pimaric acid, are however selective over SK and IK channels (Kaczorowski and Garcia, 1999; Coghlan et al., 2001; Imaizumi et al., 2002), and our preliminary data suggest that 1 $\mu$M diCl-DHAA did not affect the activities of SK2 and SK4 channels (K. Sakamoto, unpublished data). Genetically, and even functionally in some aspects, KCNMA (BK) is closer to voltage-dependent $K^+$ channels than KCNN (SK and IK), because of the presence of its voltage-sensitive domain (Vergara et al., 1998). It is therefore worth examining the effects of diCl-DHAA on cloned voltage-dependent $K^+$ channels, which remain to be determined.

In conclusion, our results provide new information of mechanisms underlying diCl-DHAA-induced activation of BK channels and the selectivity against CaV channels. diCl-DHAA is effective from either side of cell membrane and acts on BK$\alpha$ subunit to increase $Ca^{2+}$ and voltage sensitivity. In contrast to many other BK channel openers, the effect of diCl-DHAA on BK$\alpha$ significantly showed inverse voltage dependence, i.e., larger potentiation at lower membrane potentials. In this respect, diCl-DHAA may be one of the most potent BK channel openers ever known to sensitize the negative feedback control of $[Ca^{2+}]_i$ regulation via activation of BK channels, which suppress depolarization from resting membrane potential, and subsequently, membrane excitability. diCl-DHAA at low concentrations (<1 $\mu$M) shows selectivity to the BK channel over CaV channels and possesses higher selectivity to BK channels than pimaric acid. Dehydroabietic acid, including diCl-DHAA, is a new prototype scaffolding as a potent BK$\alpha$ channel opener.

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# Multiple transcripts of $Ca^{2+}$ channel $\alpha_1$-subunits and a novel spliced variant of the $\alpha_{1C}$-subunit in rat ductus arteriosus

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**Yokoyama, Utako, Susumu Minamisawa, Satomi Adachi-Akahane, Toru Akaike, Isao Naguro, Kengo Funakoshi, Mari Iwamoto, Masamichi Nakagome, Nobuyuki Uemura, Hideaki Hori, Shumpei Yokota, and Yoshihiro Ishikawa.** Multiple transcripts of $Ca^{2+}$ channel $\alpha_1$-subunits and a novel spliced variant of the $\alpha_{1C}$-subunit in rat ductus arteriosus. *Am J Physiol Heart Circ Physiol* 290: H1660–H1670, 2006. First published November 4, 2005; doi:10.1152/ajpheart.00100.2004.—Voltage-dependent $Ca^{2+}$ channels (VDCCs), which consist of multiple subtypes, regulate vascular tone in developing arterial smooth muscle, including the ductus arteriosus (DA). First, we examined the expression of VDCC subunits in the Wistar rat DA during development. Among $\alpha_1$-subunits, $\alpha_{1C}$ and $\alpha_{1G}$ were the most predominant isoforms. Maternal administration of vitamin A significantly increased $\alpha_{1C}$- and $\alpha_{1G}$-transcripts. Second, we examined the effect of VDCC subunits on proliferation of DA smooth muscle cells. We found that 1 μM nitrendipine (an L-type $Ca^{2+}$ channel blocker) and kurtoxin (a T-type $Ca^{2+}$ channel blocker) significantly decreased [$^3$H]thymidine incorporation and that 3 μM efonidipine (an L- and T-type $Ca^{2+}$ channel blocker) further decreased [$^3$H]thymidine incorporation, suggesting that L- and T-type $Ca^{2+}$ channels are involved in smooth muscle cell proliferation in the DA. Third, we found that a novel alternatively spliced variant of the $\alpha_{1C}$-isoform was highly expressed in the neointimal cushion of the DA, where proliferating and migrating smooth muscle cells are abundant. The basic channel properties of the spliced variant did not differ from those of the conventional $\alpha_{1C}$-subunit. We conclude that multiple VDCC subunits were identified in the DA, and, in particular, $\alpha_{1C}$- and $\alpha_{1G}$-subunits were predominant in the DA. A novel spliced variant of the $\alpha_{1C}$-subunit gene may play a distinct role in neointimal cushion formation in the DA.

alternative spliced; development; gene expression; fetal circulation

THE DUCTUS ARTERIOSUS (DA) is a fetal arterial connection between the pulmonary artery and the descending aorta. After birth, the DA closes immediately, in accordance with its smooth muscle contraction. An increase in oxygen tension and a dramatic decline in circulating prostaglandins are the most important triggers of DA contraction (5). Generally, vascular smooth muscle contraction is induced by $Ca^{2+}$/calmodulin-dependent phosphorylation of the regulatory myosin light chain, which is mediated by an increase in intracellular $Ca^{2+}$. $Ca^{2+}$ influx through voltage-dependent $Ca^{2+}$ channels (VDCCs) and $Ca^{2+}$ release from intracellular stores are major sources of this increase (8, 26). Thus VDCCs must play an important role in vascular myogenic reactivity and tone of the DA.

VDCCs are classified, according to their distinct electrophysiological and pharmacological properties, into low (T-type) and high (L-, N-, P-, Q-, and R-type) VDCCs (20, 39). VDCCs consist of different combinations of $\alpha_1$-subunits and auxiliary subunits. The $\alpha_1$-subunit forms the ion-conducting pore, the voltage sensor, and the interaction sites for $Ca^{2+}$ channel blockers and activators (15). Therefore, $\alpha_1$-subunits principally determine the channel character of VDCCs. Ten $\alpha_1$-subunit isoforms have been identified. Four $\alpha_{2\delta}$-subunit complexes and four β-subunits, which modulate the trafficking and the biophysical channel properties of $\alpha_1$-subunits (1), have been identified (3). Although some studies have investigated the role of VDCCs in the DA (28, 37), characterization of VDCCs, including the composition of each subunit, the developmental change in their expression, and their physiological roles, remains poorly understood.

In addition to their role in determining contractile state, a growing body of evidence has demonstrated that VDCCs play an important role in regulating differentiation and remodeling of vascular smooth muscle cells (SMCs) (14, 17, 41). The DA dramatically changes its morphology during development. Intimal cushion formation during development is a characteristic feature of vascular remodeling of the DA (10, 30). Intimal cushion formation involves many cellular processes, including an increase in SMC migration and proliferation, production of hyaluronic acid under the endothelial layer, and impairment of elastin fiber assembly. The role of VDCCs in vascular remodeling of the DA has not been investigated.

In the present study, we identified multiple VDCC subunits in the DA by semiquantitative and quantitative RT-PCR and immunodetection. In particular, $\alpha_{1C}$- and $\alpha_{1G}$-subunits were predominant in the DA. Furthermore, we will demonstrate the identification of a novel spliced variant of the $\alpha_{1C}$-subunit gene that may play a role in neointimal cushion formation of the DA.

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Table 1. *Oligonucleotides used for RT-PCR*

| Gene | Accession No. | Forward (5′-3′) | Position | Reverse (5′-3′) | Position | Size, bp | Annealing Temperature, °C |
|---|---|---|---|---|---|---|---|
| $Ca_v1.1$ ($\alpha_{1S}$) | U31816 | cgcgaggtcatggacgtggag | 572–592 | gatcaccagccagtagaagac | 695–715 | 144 | 60 |
| $Ca_v1.2$ ($\alpha_{1C}$)-1 | AF394939 | ggagagttttccaaagagagg | 1342–1362 | gatcaccagccagtagaagac | 1699–1719 | 378 | 60 |
| $Ca_v1.2$ ($\alpha_{1C}$)-2 | AF394939 | tggaaactcagctctaagag | 5551–5570 | tcctggtaggagagtatatc | 5695–5714 | 164 | 54 |
| $Ca_v1.3$ ($\alpha_{1D}$) | NM_017298 | attctgaacatggtcttcacg | 4246–4265 | gattctattgctctcttcaga | 4405–4425 | 180 | 55 |
| $Ca_v1.4$ ($\alpha_{1F}$) | NM_053701 | gcagatggcccttcaatctc | 001–020 | ccatgtcggatcccaggaag | 833–852 | 852 | 57 |
| $Ca_v3.1$ ($\alpha_{1G}$) | NM_031601 | aatggcaagtcggcttca | 3648–3665 | caggagacgaaaccttga | 3837–3854 | 207 | 50 |
| $Ca_v3.2$ ($\alpha_{1H}$) | AF290213 | gacgaggataagacgtct | 3111–3128 | ggagacgcgtagcctgtt | 3906–3923 | 813 | 57 |
| $Ca_v3.3$ ($\alpha_{1I}$) | NM_020084 | gatgaggaccagagctca | 2768–2785 | tctggctgcagtgagaggc | 2943–2961 | 194 | 60 |
| $\alpha_{2\delta1}$ | NM_012919 | tggtgtgatgggcgttgatgtgtc | 1741–1764 | gtcattgc-agtattcccttggtgc | 2234–2258 | 518 | 56 |
| $\alpha_{2\delta2}$ | NM_175592 | aggttcttcagtgaggtggat | 2749–2769 | ttataggacgcgttcaccgag | 3081–3101 | 353 | 62 |
| $\alpha_{2\delta3}$ | NM_175595 | ggcacagatgtcccagtaaaaga | 1483–1505 | tgtgtagtagtagtcattggtcat | 1780–1803 | 321 | 58 |
| $\beta_{1\text{-}1}$ | NM_017346 | tgacaactccagttccag | 624–641 | tcaagaagtcaaacaacg | 835–852 | 229 | 62 |
| $\beta_{1\text{-}2}$ | NM_017346 | tcaatgtccaaatagcag | 1163–1180 | tgtcagcatcaaaggtgtc | 1555–1573 | 411 | 56 |
| $\beta_2$ | NM_053851 | acagagagcaaagcaagggaat | 771–792 | tctccttgagaaccctgtgaatt | 1693–1715 | 945 | 56 |
| $\beta_3$ | NM_012828 | gtggtgttggatgctgac | 892–909 | attgtggtcatgctccga | 1483–1500 | 609 | 58 |
| $\beta_4$ | XM_215742 | cgattcggcaagagagagaacagcaag | 396–422 | gtttgggcagcctcaaagcctatgtcg | 1730–1756 | 1361 | 58 |
| GAPDH | AF106860 | cccatcaccatcttccaggagcg | 1060–1082 | gcagggatgatgttctgggctgcc | 1446–1469 | 410 | 55 |

## MATERIALS AND METHODS

*Animals.* All animals were cared for in compliance with the guiding principles of the American Physiological Society. The experiments were approved by the Ethical Committee of Animal Experiments of Yokohama City University School of Medicine.

*Maternal vitamin A administration.* Maturation of the fetal DA was accelerated by maternal administration of vitamin A, as described previously (25, 42). Briefly, vitamin A [retinyl palmitate, 33 mg, which is equivalent to 18 mg of retinol (Eisai, Tokyo, Japan)] was diluted in polyoxyethylene castor oil and injected intramuscularly into pregnant Wistar rats daily from *day 17* of gestation in a dose of 1 mg (3,000 IU)/kg body wt.

*Tissue collection and preparation.* For developmental studies, we used pooled tissues obtained from Wistar rat embryos on embryonic *days 19* (e19, $n = 60$) and *21* (e21, $n = 90$) and from neonates on the day of birth (*day 0*, $n = 60$). After excision, tissues were immediately frozen in liquid nitrogen and stored at −80°C.

*Semiquantitative and quantitative RT-PCR.* Total RNA was isolated from the tissues using TRIzol, as recommended by the manufacturer (Invitrogen, La Jolla, CA). Genomic DNA was digested by DNase I before RT reaction. After annealing to random hexamer, 2 μM total RNA was reverse transcribed to cDNA by SuperScript II RT (Invitrogen). For semiquantitative RT-PCR analyses of VDCC subunits, the primers for PCR amplification were designed on the basis of the rat nucleotide sequences of VDCCs (Table 1) to amplify cDNA between two exons, except $\alpha_{1S}$-subunit primers. The PCR cycle consisted of denaturation at 94°C for 30 s, annealing at each temperature for 30 s, and elongation at 72°C for 30 s; 35–40 forty cycles were performed. Amplification products were analyzed by 2% agarose gel electrophoresis and ethidium bromide staining. A fragment of GAPDH was amplified as internal control. For quantitative RT-PCR analysis, sequences for PCR primers are listed in Table 2. The spliced variant of the $\alpha_{1C}$-subunit and nonspliced isoform were detected simultaneously by $Ca_v1.2$ ($\alpha_{1C}$)-4 primer. Amplification and detection were performed as described previously (40). Each template was tested at least three times to confirm the reproducibility of the assays. The abundance of each gene was determined relative to GAPDH using TaqMan rodent GAPDH control reagents kits (Applied Biosystems, Foster City, CA). For each RT-PCR experiment, we included RT negative control and confirmed no amplification in each reaction.

*Restriction enzyme analysis.* The relative abundance of high-voltage-activated (HVA) channels of the $\alpha_1$-subunits was determined in the DA as described previously (29). Briefly, all HVA channels of the $\alpha_1$-subunits were amplified by RT-PCR using the following set of primers: at(c/t) (a/g)tc acc ttc cag gag ca (forward) and gcg tag atg aag aa(a/g/c) agc at (reverse). Five restriction enzymes selectively cut the $\alpha_{1A}$-, $\alpha_{1B}$-, $\alpha_{1C}$-, $\alpha_{1D}$-, and $\alpha_{1E}$-isoforms of the PCR products. The intensity of the digested fragments in correspondence to each isoform was measured by a FujiFilm image analysis system (Image Gauge version 3.41).

*Generation of polyclonal antibody against spliced variant of $\alpha_{1C}$-subunit.* We generated a polyclonal antibody against spliced variant of rat $\alpha_{1C}$-subunit using a keyhole limpet hemocyanin-conjugated synthetic peptide for immunization. Antigens for spliced variant of anti-rat $\alpha_{1C}$-subunit antibody were derived from the I-II cytoplasmic loop region spanning amino acids 59–71 of the spliced variant of rat

Table 2. *Oligonucleotides used for quantitative RT-PCR*

| Gene | Accession No. | Forward (5′-3′) | Position | Reverse (5′-3′) | Position | Probe (5′-3′) | Position | Size, bp |
|---|---|---|---|---|---|---|---|---|
| $Ca_v1.2$ ($\alpha_{1C}$)-3 | AF394939 | tgattgttgtgg-gtagcattgtt | 3992–4014 | tcatagagggaga-gcattgggtat | 4049–4072 | tagcaatcaccgaggtacacc-cagctg (FAM) | 4019–4072 | 81 |
| $Ca_v1.2$ ($\alpha_{1C}$)-4 | AY323810 | aatgaggacgag-ggcatgg | 136–154 | gcccacaagtgag-actgagtctg | 258–280 | agggaaatttgcttggtttag-tcactccaca (FAM) | 210–241 | 145 |
| | AF394939 | | | | | tgaagacaaaccccgaaacat-gagca (VIC) | 1497–1522 | 70 |
| $Ca_v1.3$ ($\alpha_{1D}$) | NM_017298 | gaagaggacgag-cctgaggtt | 3028–3048 | ttttctccttcat-gttcaactctga | 3076–3100 | (SYBR) | | 73 |
| $Ca_v3.1$ ($\alpha_{1G}$) | NM_031601 | cctgatttcttt-tcgcccag | 3054–3073 | tggcaaaaggc-tctttcgtag | 3138–3158 | (SYBR) | | 105 |