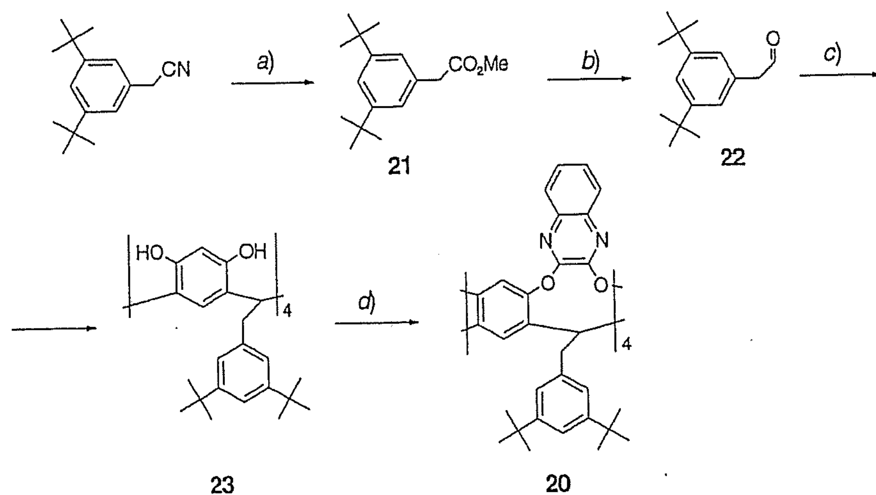
Scheme 7. *Synthesis of Cavitand* **20**



a) HCl/MeOH, reflux, 3 d; 99%. *b*) DIBAL-H, hexane, –78°, 3 h; 58%. *c*) Conc. HCl, EtOH, 60°, 5 d; 35%. *d*) 2,3-Dichloroquinoxaline, Cs_2CO_3, DMF, 60°, 3 d; 68%. DIBAL-H = diisobutylaluminum hydride.

35% yield, and subsequent bridging with 2,3-dichloroquinoxaline afforded cavitand **20** in 68% yield [4]. The molecular structure of **20** in the crystal has been previously reported [4]; *Fig. 6* depicts the *head*-to-*tail* arrangements of the cavitand *vases* in the crystal lattice, leading to infinite columns, as already described for cavitands **2b** (in the *vase*-form) and **19** (in the *kite*-form). A view into these infinite columns reveals narrow channels spanning the crystal lattice.

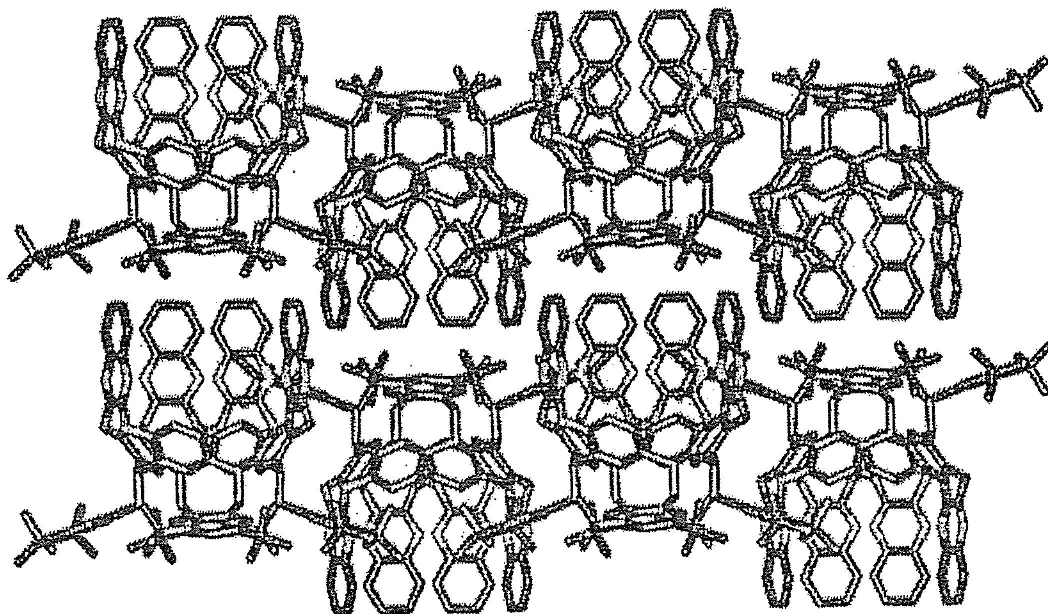
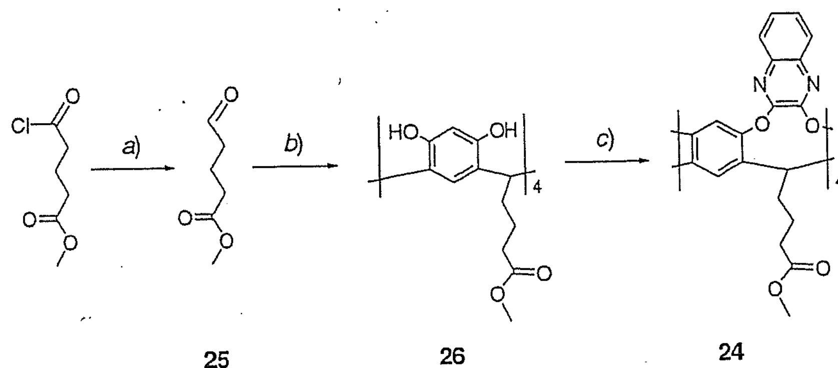


Fig. 6. *Packing diagram of cavitand* **20**

Cavitand **24**, with carboxylate legs, was prepared for *Langmuir* monolayer formation on an aqueous subphase. *Rosenmund* reduction of the acyl chloride of monomethyl glutarate gave aldehyde **25** that was condensed with resorcinol to afford octol **26** in 44% yield. Subsequent bridging with 2,3-dichloroquinoxaline provided **24** in 13% yield (*Scheme 8*). Attempts to hydrolyze the methyl ester legs proved to be fruitless; they either led to decomposition of the cavitand or left the ester intact (K_2CO_3, Li_2CO_3, or LiOH in H_2O/THF 1:3).

Scheme 8. *Synthesis of Cavitand* **24**



a) Pd/C, H_2, 2,6-dimethylpyridine, THF, 20°, 24 h. *b*) Resorcinol, conc. HCl, MeOH, 60°, 2 d; 44% (steps *a* and *b*). *c*) 2,3-Dichloroquinoxaline, Cs_2CO_3, DMF, 60°, 2 d; 13%.

Crystals of **24** were obtained by slow evaporation of a MeCN/$CHCl_3$ solution. The X-ray crystal-structure analysis at 293 K showed the cavitand in the *vase*-conformation with an asymmetric cavity (*Fig. 7*). The unit-cell contains pairs of perpendicularly aligned cavitands making *tail*-to-*tail* contacts. Disordered solvent (MeCN and, probably, H_2O) is localized within the cavity and among the ester legs.

Alkyl-thioether legs were selected due to their well-known affinity for gold surfaces under formation of stable self-assembled monolayers (SAMs) [23][24]. Cavitands **27** and **28** with alkyl-thioether legs [5] of different length were constructed employing slightly different synthetic strategies. Cavitand **27** was synthesized by acid-catalyzed condensation of resorcinol with the unsaturated aldehyde **29**, followed by bridging the resulting octol **30** to give **31** featuring legs with terminal double bonds (*Scheme 9*). The thioether moiety was introduced in the last step by 9-BBN-catalyzed radical addition of decane-1-thiol to the olefinic legs. Cavitand **28** with shorter legs was prepared by direct condensation of the thioether-containing aldehyde **32**, obtained by oxidation of alcohol **33**, with resorcinol, followed by bridging the resulting octol **34** (*Scheme 10*). SAMs formed by **31** on Au(111) were successfully prepared and imaged by UHV-STM at the molecular level showing a well-ordered monolayer [5]. On the other hand, cavitand **28**, with shorter legs, afforded only poorly ordered SAMs.

3. Conclusions. A series of *Cram*-type resorcin[4]arene-derived cavitands with modified cavity walls and legs were prepared with the aim to investigate in detail the conformational *vase–kite* switching in bulk solution as well as at the level of single molecules immobilized on surfaces. Initial STM studies of monolayers on gold surfaces

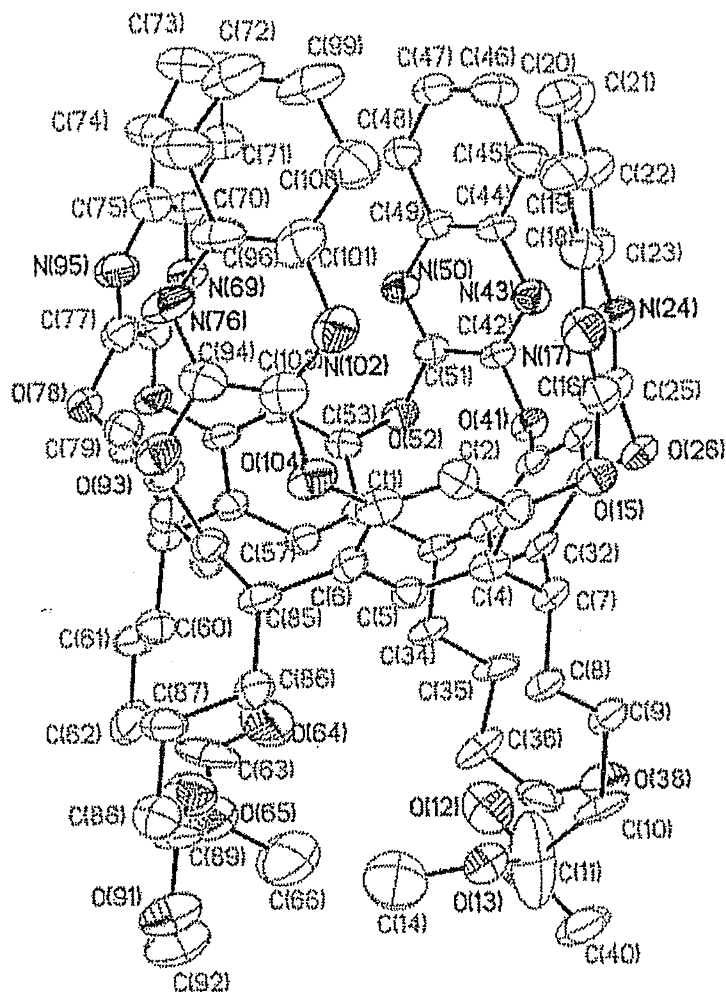
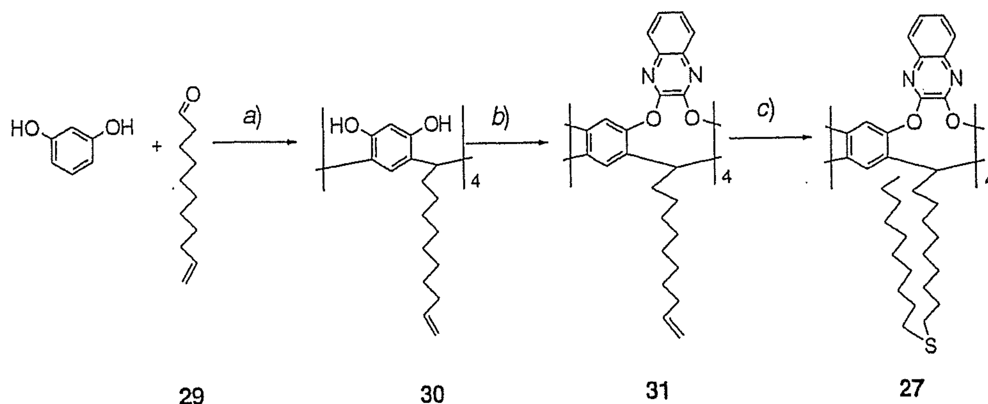


Fig. 7. *ORTEP Representation of cavitand* **24** *with atomic displacement parameters shown at the 30% probability level* (293 K). MeCN included within the cavity and solvent present among the ester tails are removed for clarity. Intramolecular N ⋯ N distances [Å]: N(17) ⋯ N(102), 4.18; N(24) ⋯ N(43), 4.30; N(50) ⋯ N(69), 4.22; N(76) ⋯ N(95), 4.47.

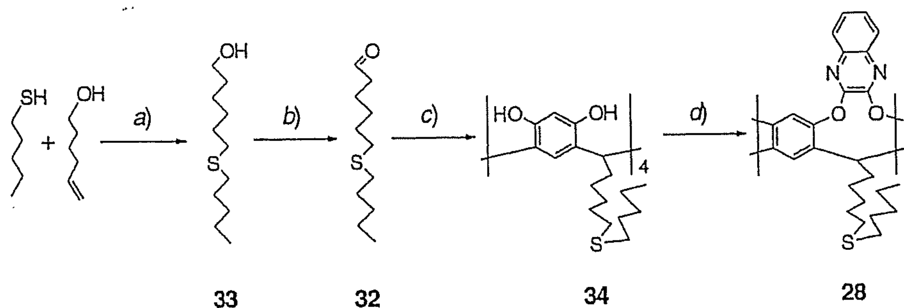
have already been reported [5], and a subsequent paper will present a detailed spectroscopic analysis of the scope and limitations of *vase–kite* switching in solution [10]. The novel substitution patterns of cavitands **15** and **16** with differential wall components open multiple opportunities for future functionalization. As an example, additional recognition sites could be attached (instead of the dye labels in **15** and **16**) for enhancing the selectivity for specific guests encapsulated in the deep cavitand cavity. Modification of the legs provided cavitands for surface immobilization and *Langmuir–Blodgett* film formation; *vase–kite* switching on surfaces and in monolayers is currently investigated in collaborative work by a variety of physical methods. This paper reports the first X-ray structural analysis of a resorcin[4]arene-based cavitand (**19**) in the *kite*-conformation. Consequently, the two different conformational

Scheme 9. *Synthesis of Cavitand* **17**



a) Conc. HCl, EtOH, 20°, 25 h; 93%. *b*) 2,3-Dichloroquinoxaline, Cs_2CO_3, Me_2SO, 20°, 2 d; 44%. *c*) Decane-1-thiol, 9-BBN, THF, 20°, 2 d; 74%. 9-BBN = 9-Borabicyclo[3.3.1]nonane.

Scheme 10. *Synthesis of Cavitand* **28**



a) 9-BBN, THF, 20°, 3 h; 99%. *b*) PCC, CH_2Cl_2, 20°, 5 h; 31%. *c*) Conc. HCl, EtOH, 60°, 24 h; 99%. *d*) 2,3-Dichloroquinoxaline, Cs_2CO_3, Me_2SO, 20°, 4 d; 52%. PCC = pyridinium chlorochromate.

states are now structurally well-characterized in the solid state, which also provides a sound basis for the interpretation of the results of switching studies in solution [10] and at the single-molecule level.

Support by the *Swiss National Science Foundation, via* the *NFP 'Supramolecular Functional Materials'* and the *NCCR 'Nanoscience'* is gratefully acknowledged. *Y. Y.* thanks the *Japan Science Technology Corporation Overseas Fellowship Program* for a postdoctoral fellowship.

Experimental Part

General. Reagent-grade solvents and reagents were purchased and used without further purification (except for 2,3-dichloroquinoxaline that was recrystallized from EtOH or MeOH). Octols **1a**–**1c** were prepared according to [13]; they were purified by recrystallization (2 ×) from MeOH (**1a** and **1b**) or MeOH/EtOH 4:1 (**1c**; for X-ray, see *Fig. 2*) and dried under high vacuum over P_2O_5 to afford the products as slightly pinkish powders. Diol **3a** was prepared according to [2b], and pyrazine derivatives **9** and **10** according to [1c]. Cavitands **2a** [2b], and **2b** and **2c** [1b] have been previously reported (for the X-Ray crystal structure of **2b**, see *Fig. 3*). All reactions were carried out under Ar or N_2 atmosphere. Flash chromatography (FC): SiO_2 from *Fluka* or *Merck* 230–400 mesh (particle size 40–63 μm). Anal. TLC: precoated SiO_2 glass plates with *F-254* fluorescent indicator; visualization with UVlight at 254 or 366 nm. M.p.: *Büchi Melting Point B-540*; uncorrected. The m.p.

of the highly colored BODIPY dyes could not be accurately determined. UV/VIS [nm]: *Varian Cary-500 Scan* spectrophotometer; λ_{max} in nm (ε in M^{-1} cm^{-1}). Fluorescence: *Instruments S. A. Fluorolog-3* spectrofluorometer. IR [cm^{-1}]: *Perkin-Elmer 1600-FTIR*; in CCl_4 or in KBr pellets. ^{1}H-, ^{13}C-, and ^{19}F-NMR spectra [ppm]: *Varian Mercury-300* spectrometers at r.t.; internal references [ppm]: $CDCl_3$: 7.26 (^{1}H), 77.23 (^{13}C); CD_2Cl_2: 5.32 (^{1}H), 53.80 (^{13}C); $(CD_3)_2CO$: 2.05 (^{1}H), 29.80 (^{13}C); CD_3OD: 3.31 (^{1}H), 49.15 (^{13}C); (D_8)THF (C_4D_8O): 1.73 (^{1}H), 25.20 (^{13}C); $CFCl_3$ was used as a reference for ^{19}F-NMR (0.00 ppm). FT-ICR-MALDI-MS: *Ion Spec Ultima FT-ICR-MS* (337-nm N_2-laser system); matrix: DHB (2,3-hydroxybenzoic acid) or DCTB ({(2*E*)-3-[4-(*tert*-butyl)phenyl]-2-methylprop-2-enylidene}malonitrile). EI-MS: *VG Analytical Tribrid, USA*. FAB-MS: *VG Analytical ZAB2-SEQ, USA*. Elemental analyses were performed by the *Mikrolabor* at the Laboratorium für Organische Chemie, ETH Zürich.

*r-11,c-13,c-29,c-36-Tetrahexyl-7,10:12,15:24,27:29,32-tetraethano-8,31:14,25-dimethano-11*H*,28*H*-[1,4,14,17]-tetraoxacyclohexacosino[2,3-*b*:15,16-*b'*]diquinoxaline-36,38,42,43-tetrol* (**4a**) *and r-11,c-13,c-29,c-36-Tetrahexyl-9,15-(methano[1,3]benzenomethano)-11*H*,13*H*-benzo[1'',2'':5,6;-5'',4'':5',6']bis[1,4]benzodioxonino[2,3-*b*:2',3'-*b'*]diquinoxaline-8,16,33,35-tetrol* (**5a**). To a degassed (freeze-pump) soln. of **1a** (2.48 g, 3.01 mmol) in Me_2SO (40 ml), K_2CO_3 (0.416 g, 3.01 mmol) and 2,3-dichloroquinoxaline (1.20 g, 6.02 mmol) were added, and the mixture was stirred for 1 h. More K_2CO_3 (0.832 g, 6.02 mmol) was added, and stirring under Ar was continued for 18 h at r.t. and 6 h at 50°. After cooling, the brown soln. was added to H_2O (50 ml), and the pH was adjusted to 6–7 by addition of 1M HCl. The pink precipitate formed was isolated by filtration, washed with H_2O (50 ml), and dried under high vacuum over P_2O_5. FC (SiO_2; CH_2Cl_2/AcOEt 95:5 → 85:5) afforded **2a** (107 mg, 2.7%) **3a** (603 mg, 16.6%), **4a** (103 mg, 3.2%), and **5a** (637 mg, 19.6%). *Data of* **4a**: R_f (SiO_2; CH_2Cl_2/AcOEt 90:10) 0.3. M.p. > 285° (dec.). IR (KBr): 3404 (br.), 2927, 2857, 1616, 1584, 1490, 1466, 1412, 1335, 1281, 1224, 1169, 1073, 894, 858, 760, 606. ^{1}H-NMR ($(CD_3)_2CO$, 300 MHz): 0.88–0.94 (*m*, 12 H); 1.25–1.48 (*m*, 32 H); 2.36–2.45 (*m*, 8 H); 4.56 (*t*, *J* = 7.8, 2 H); 5.51 (*t*, *J* = 7.8, 2 H); 7.15 (*s*, 4 H); 7.27–7.32 (*m*, 4 H); 7.55–7.60 (*m*, 4 H); 7.75 (*s*, 4 H); 9.04 (*s*, 4 H). ^{13}C-NMR ($(CD_3)_2CO$, 75 MHz): 14.36; 23.35; 28.88; 28.97; 32.69; 32.73; 33.99; 34.79; 34.96; 110.93; 125.33; 127.99; 129.62; 130.11; 131.69; 139.92; 152.70; 152.93; 153.08. HR-MALDI-MS (DHB): 1099.5568 ([*M* + Na]$^+$, $C_{68}H_{76}N_4O_8Na^+$; calc.: 1099.5555). *Data of* **5a**: R_f (SiO_2; CH_2Cl_2/AcOEt 85:15) 0.3. M.p. > 270° (dec.). IR (KBr): 3416 (br.), 2927, 2857, 1619, 1585, 1491, 1414, 1336, 1285, 1235, 1153, 1119, 1078, 899, 852, 759, 607. ^{1}H-NMR ($(CD_3)_2CO$, 300 MHz): 0.86–0.95 (*m*, 12 H); 1.24–1.50 (*m*, 32 H); 2.21–2.39 (*m*, 4 H); 2.41–2.51 (*m*, 4 H); 4.30 (*t*, *J* = 8.1, 2 H); 5.51 (*t*, *J* = 8.1, 2 H); 6.18 (*s*, 1 H); 7.13 (*s*, 2 H); 7.50 (*s*, 1 H); 7.62–7.81 (*m*, 8 H); 7.95 (*s*, 1 H); 8.08–8.11 (*m*, 2 H); 8.36 (*s*, 1 H); 8.72 (*s*, 4 H). ^{13}C-NMR ($(CD_3)_2CO$, 75 MHz): 14.34; 14.38; 23.33; 23.36; 28.87; 28.97; 32.60; 32.71; 34.08; 34.52; 35.15; 103.49; 110.73; 118.70; 124.39; 125.107; 125.62; 125.75; 128.37; 128.72; 130.09; 130.19; 130.47; 130.98; 137.88; 140.33; 140.38; 152.08; 152.80; 153.29; 153.37. HR-MALDI-MS (DHB): 1099.5563 ([*M* + Na]$^+$, $C_{68}H_{76}N_4O_8Na^+$; calc.: 1099.5555).

*5,6-Dichloropyrazine-2,3-dicarboxylic Acid 4-(*tert*-Butyl)phenylimide* (**6**). To **10** (0.197 g, 0.90 mmol) in THF (3 ml), 4-(*tert*-butyl)aniline (0.144 ml, 0.90 mmol) was added, and the mixture was stirred for 2 h at r.t. under Ar. Oxalyl chloride (0.085 ml, 0.99 mmol) and pyridine (0.160 ml, 1.98 mmol) were added, and the mixture was heated to 50° for 12 h. After filtration, the mixture was evaporated to dryness, and the residue was co-evaporated with heptane (3×) to remove traces of pyridine. FC (SiO_2; CH_2Cl_2/cyclohexane 3:1) gave **6** (279 mg, 89%). Pale-yellow crystals. M.p. 268–268.5°. R_f (SiO_2; CH_2Cl_2/cyclohexane 3:1) 0.25. ^{1}H-NMR ($CDCl_3$, 300 MHz): 1.36 (*s*, 9 H); 7.33–7.38 (*m*, 2 H); 7.53–7.58 (*m*, 2 H). ^{13}C-NMR ($CDCl_3$, 75 MHz): 31.54; 35.15; 125.94; 126.60; 127.53; 143.24; 152.48; 154.09; 161.34.

*7-[4-(*tert*-Butyl)phenyl]-60-*endo*,64-*endo*,68-*endo*,72-*endo*-tetrahexyl-2,12,16,27,31,42,46,57-octaoxa-4,7,10,18,25,33,40,48,55-nonaazaheptadecacyclo[$56.15.1.1^{59,73}.0^{3,11}.0^{5,9}.0^{13,71}.0^{15,69}.0^{17,26}.0^{19,24}.0^{28,67}.0^{30,65}.0^{32,41}.0^{34,39}.0^{43,63}.0^{45,61}.0^{47,56}.0^{49,54}$]pentaheptaconta-1(73),3,5(9),10,13,15(69),17,19(24),20,22,25,28,30(65),32,34(39),35,37,40,43,45(61),47,49(54),50,52,55,58(74),59(75),62,66,70-triacontaene-6,8-dione* (**7**). To **3a** (0.081 g, 0.067 mmol) and **6** (0.024 g, 0.067 mmol) in degassed Me_2SO (2.5 ml), K_2CO_3 (0.012 g, 0.084 mmol) was added, and the mixture was stirred under Ar at r.t. for 40 h. After addition of H_2O, the precipitate formed was isolated by filtration, washed with H_2O (5 ml), and dried under vacuum over P_2O_5. FC (SiO_2; CH_2Cl_2/AcOEt 100:0 → 98:2) afforded **7** (65 mg, 66%). Yellowish powder. R_f (SiO_2; CH_2Cl_2/AcOEt 98:2) 0.43. M.p. > 340° (dec.). IR (KBr): 3070, 2955, 2927, 2858, 1795, 1742, 1606, 1570, 1516, 1482, 1414, 1362, 1333, 1265, 1223, 1159, 1118, 1091, 1066, 913, 898, 761, 604. ^{1}H-NMR ($CDCl_3$, 300 MHz): 0.90–0.96 (*m*, 12 H); 1.27–1.53 (*m*, 32 H); 1.43 (*s*, 9 H); 2.20–2.32 (br. *m*, 8 H); 5.46 (*t*, *J* = 7.8, 1 H); 5.50–5.59 (*m*, 3 H); 7.11–7.15 (*m*, 2 H); 7.20 (*s*, 2 H); 7.23 (*s*, 2 H); 7.33–7.60 (*m*, 8 H); 7.81–7.93 (*m*, 6 H); 8.13 (*s*, 2 H); 8.16 (*s*, 2 H). ^{13}C-NMR ($CDCl_3$, 75 MHz): 14.42; 22.99; 28.21; 29.67; 31.65; 32.16; 32.57; 32.75; 34.66; 35.18; 118.53; 118.96; 123.50; 123.80; 125.92; 126.35; 127.58; 127.87; 128.08; 128.70; 129.23; 129.46; 135.41; 135.55; 135.81; 136.85; 139.62; 139.76; 141.14; 152.05; 152.16; 152.24;

152.37; 152.63; 152.79; 152.87; 158.44; 161.77. HR-MALDI-MS (DHB): 1480.6805 (MH^+, $C_{92}H_{90}N_9O_{10}^+$; calc.: 1480.6805).

*7,21-Bis[4-(*tert*-butyl)phenyl]-59*-endo,*63*-endo,*67*-endo,*71*-endo-*tetrahexyl-2,12,16,26,30,41,45,56-octaoxa-4,7,10,18,21,24,32,39,47,54-decaazaheptadecacyclo[55.15.1.1^{58,72}.0^{3,11}.0^{5,9}.0^{13,70}.0^{15,68}.0^{17,25}.0^{19,23}.0^{27,66}.0^{29,64}.0^{31,40}.0^{33,38}.0^{42,62}.0^{44,60}.0^{46,55}.0^{48,53}]tetraheptaconta-1(72),3,5(9),10,13,15(68),17,19(23),24,27,29(64),31,33(38),34,36,39,42,44(60),46,48(53),49,51,54,57(73),58(74),61,65,69-octacosaene-6,8,20,22-tetrone* (**8**). To **5a** (0.060 g, 0.056 mmol) and **6** (0.039 g, 0.11 mmol) in degassed Me_2SO (2 ml), K_2CO_3 (0.019 g, 0.139 mmol) was added, and the mixture was stirred under Ar at r.t. for 24 h. H_2O (5 ml) was added, and the formed precipitate was isolated by filtration. Washing with H_2O (5 ml), drying under vacuum over P_2O_5, and FC (SiO_2; CH_2Cl_2/AcOEt 100:0 → 97:3) afforded **8** (46 mg, 50%). Yellowish powder. R_f = (SiO_2; CH_2Cl_2/AcOEt 98:2) 0.45. M.p. > 350° (dec.). IR (KBr): 3073, 2956, 2927, 2859, 1797, 1741, 1696, 1579, 1517, 1483, 1413, 1369, 1333, 1265, 1223, 1202, 1160, 1090, 1065, 914, 900, 817, 764; 603. ^{1}H-NMR ((D_8)THF, 300 MHz): 0.93–0.97 (*m*, 12 H); 1.36–1.54 (*m*, 32 H); 1.43 (*s*, 18 H); 2.36–2.47 (*m*, 8 H); 5.67 (*t*, *J* = 8.1, 2 H); 5.78 (*t*, *J* = 8.1, 2 H); 7.04–7.09 (*m*, 4 H); 7.32–7.60 (*m*, 12 H); 7.96–7.99 (*m*, 5 H); 8.24–8.25 (*m*, 3 H). ^{13}C-NMR ((D_8)THF, 75 MHz): 14.34; 23.47; 28.81; 28.85; 30.06; 30.11; 31.72; 32.77; 32.92; 34.93; 35.04; 35.29; 119.61; 119.99; 124.18; 124.63; 125.28; 125.95; 127.11; 128.38; 129.38; 129.64; 129.86; 130.17; 136.57; 136.72; 137.21; 137.76; 140.15; 140.27; 142.62; 142.96; 151.39; 152.66; 152.92; 153.07; 153.47; 153.57; 157.60; 157.85; 161.57; 162.27. HR-MALDI-MS (DHB): 1631.7306 (MH^+, $C_{100}H_{99}N_{10}O_{12}^+$; calc.: 1631.7444).

*2,8-Diethyl-5,5-difluoro-1,3,7,9-tetramethyl-10-(4-nitrophenyl)-dipyrrolo[1,2-*c*:2,1-*f*][1,3,2]diazaborinin-4-ium-5-uide* (**11**). A soln. of 2,4-dimethyl-3-ethyl-1*H*-pyrrole (3.21 g, 26.1 mmol) and 4-nitrobenzylaldehyde (1.97 g, 13.0 mmol) in CH_2Cl_2 (500 ml) was degassed (bubbling N_2 for 1 h), and TFA (0.10 ml, 1.3 mmol) was added. After stirring for 2 h at r.t., the mixture was washed with sat. aq. $NaHCO_3$ soln., H_2O, and sat. aq. NaCl soln., dried (Na_2SO_4), and evaporated to yield the intermediate dipyrromethane (4.98 g). This compound (4.01 g, 10.6 mmol) was dissolved in PhMe (50 ml), and DDQ (2,3-dichloro-5,6-dicyano-*p*-benzoquinone, 2.41 g, 10.6 mmol) was added as a suspension in PhMe (100 ml). After stirring for 1 h at r.t., Et_3N (4.4 ml, 32 mmol) was added to the black mixture, and, 10 min later, $BF_3 \cdot OEt_2$ (6.7 ml, 53 mmol) was added. The mixture was stirred at r.t. for 30 min, then heated to 50° for 1 h. After cooling, the mixture was filtered through a short plug (SiO_2; toluene), and the soln. was evaporated to dryness. FC (SiO_2; PhMe CH_2Cl_2/cyclohexane 3:2) gave **11** (1.71 g, 38%). Dark-red powder; purple soln. in $CHCl_3$. M.p. 193.5–196°. R_f (SiO_2; CH_2Cl_2/cyclohexane 3:2) 0.4. UV/VIS ($CHCl_3$): 533 (70000). Fluorescence ($CHCl_3$): 540. IR (CCl_4): 2966, 2930, 2873, 2853, 1600, 1543, 1530, 1475, 1411, 1388, 1346, 1320, 1192, 1161, 1114, 1083, 1054, 980, 856. ^{1}H-NMR ($CDCl_3$, 300 MHz): 0.98 (*t*, *J* = 7.5, 6 H); 1.26 (*s*, 6 H); 2.32 (*q*, *J* = 7.5, 4 H); 2.54 (*s*, 6 H); 7.51–7.55 (*m*, 2 H); 8.35–8.40 (*m*, 2 H). ^{13}C-NMR ($CDCl_3$, 75 MHz): 12.32; 12.91; 14.88; 17.36; 77.43; 124.36; 129.99; 133.57; 136.88; 137.76; 142.92; 148.25; 154.99. ^{19}F-NMR ($CDCl_3$, 282.5 MHz): −145.96 (*q*, *J* = 35). HR-MALDI-MS (DHB): 425.2083 (M^+, $C_{23}H_{26}BF_2N_3O_2^+$; calc.: 425.20807). X-Ray: see *Fig. 4*.

*10-[4-Aminophenyl]-2,8-diethyl-5,5-difluoro-1,3,7,9-tetramethyldipyrrolo[1,2-*c*:2,1-*f*][1,3,2]diazaborinin-4-ium-5-uide* (**12**). To **11** (485 mg, 1.14 mmol) in CH_2Cl_2/EtOH 1:1 (30 ml), Pd/C 10% (62 mg) was added, and the mixture was stirred under H_2 for 12 h. Filtration through *Celite*, evaporation to dryness, and FC (SiO_2; CH_2Cl_2) afforded **12** (301 mg, 67%). Orange powder; bright-orange soln. in $CHCl_3$. M.p. 280–285° (dec.). R_f (SiO_2; CH_2Cl_2) 0.47. UV/VIS ($CHCl_3$): 526 (71000). Fluorescence ($CHCl_3$): 536. IR (KBr): 3507, 3415, 2962, 2925, 2869, 1620, 1529, 1474, 1402, 1318, 1276, 1193, 1161, 1114, 1083, 1055, 1016, 976, 867, 831, 798, 761, 707, 659, 612, 534. ^{1}H-NMR ($CDCl_3$, 300 MHz): 0.98 (*t*, *J* = 7.5, 6 H); 1.40 (*s*, 6 H); 2.31 (*q*, *J* = 7.5, 4 H); 2.52 (*s*, 6 H); 3.82 (br. *s*, 2 H); 6.75–6.80 (*m*, 2 H); 6.99–7.03 (*m*, 2 H). ^{13}C-NMR ($CDCl_3$, 75 MHz): 12.26; 12.80; 14.99; 17.41; 115.55; 125.71; 129.33; 131.47; 132.58; 138.62; 141.13; 146.90; 153.26. ^{19}F-NMR ($CDCl_3$, 282.5 MHz): −145.29 (*q*, *J* = 35). HR-MALDI-MS (DHB): 395.2341 (M^+, $C_{23}H_{28}BF_2N_3^+$; calc.: 395.23389). Anal. calc. for $C_{23}H_{28}BF_2N_3$ (395.296): C 69.88, H 7.14, B 2.73, F 9.61, N 10.63; found: C 69.71, H 6.91, N 10.47.

*10-[4-(2,3-Dichloro-5,7-dioxo-6,7-dihydro-5*H*-pyrrolo[3,4-*b*]pyrazin-6-yl)phenyl]-2,8-diethyl-5,5-difluoro-1,3,7,9-tetramethyldipyrrolo[1,2-*c*:2,1-*f*][1,3,2]diazaborinin-4-ium-5-uide* (**13**). To **7** (0.109 g, 0.497 mmol) in THF (4 ml), **12** (0.187 g, 0.473 mmol)) was added, and the soln. was stirred for 1 h at r.t. under Ar. Oxalyl chloride (0.048 ml, 0.57 mmol) and pyridine (0.115 ml, 1.42 mmol) were added, and the mixture was heated to 50° for 12 h. The soln. obtained after filtration was evaporated to dryness, and the residue co-evaporated with heptane (3 ×) to remove traces of pyridine. FC (SiO_2; CH_2Cl_2) afforded **13** (212 mg, 72%). Red powder; bright-purple soln. in $CHCl_3$. M.p. 285–295° (dec.). R_f = (SiO_2; CH_2Cl_2) 0.55. UV/VIS ($CHCl_3$): 530 (73000). Fluorescence ($CHCl_3$): 537. IR (KBr): 2965, 2930, 2871, 1803, 1740, 1541, 1518, 1477, 1388, 1373, 1320, 1272, 1234, 1194, 1161, 1116, 1067, 979, 803, 760, 702, 536. ^{1}H-NMR ($CDCl_3$, 300 MHz): 0.99 (*t*, *J* = 7.5, 6 H); 1.35 (*s*, 6 H); 2.31 (*q*, *J* = 7.5, 4 H); 2.52 (*s*, 6 H); 7.48–7.52 (*m*, 2 H); 7.62–7.66 (*m*, 2 H). ^{13}C-NMR ($CDCl_3$, 75 MHz): 12.34;

12.87; 14.94; 17.42; 77.43; 126.70; 129.56; 130.67; 131.03; 133.26; 136.62; 138.35; 143.01; 154.34; 160.92. ^{19}F-NMR ($CDCl_3$, 282.5 MHz): −145.14 (*q*, *J* = 35). HR-MALDI-MS (DHB): 595.1523 (M^+, $C_{29}H_{26}BCl_2F_2N_5O_2^+$; calc. 595.15192).

Compound **14** (for nomenclature and full characterization, see [9]). Preparation as described for **7** from **3a** (0.060 g, 0.050 mmol), **13** (0.030 g, 0.050 mmol), and K_2CO_3 (0.0086 g, 0.062 mmol) in Me_2SO (2.5 ml). CC (SiO_2; CH_2Cl_2/AcOEt 100:0 → 98:2) afforded **14** (63 mg, 73%). Bright-red solid.

Compound **15** (for nomenclature and full characterization, see [9]). To a degassed soln. of **4a** (0.018 g, 0.0167 mmol) and **13** (0.022 g, 0.0367 mmol) in Me_2SO (1 ml), K_2CO_3 (0.0051 g, 0.0367 mmol) was added. After stirring under Ar at r.t. for 4 h, additional K_2CO_3 (0.0051 g, 0.0367 mmol) was added. A dark-red precipitate formed within 30 min, H_2O (5 ml) was added, and the precipitate was isolated by filtration, washed with H_2O (5 ml), and dried under vacuum over P_2O_5. FC (SiO_2; CH_2Cl_2/AcOEt 100:0 → 97:3) provided **15** (19 mg, 54%). Bright-red solid.

Compound **16** (for nomenclature and full characterization, see [9]). Preparation as described for **8** from **5a** (0.035 g, 0.032 mmol), **13** (0.038 g, 0.064 mmol), and K_2CO_3 (0.009 g, 0.064 mmol) in Me_2SO (1.5 ml). FC (SiO_2; CH_2Cl_2/AcOEt 100:0 → 97:3) gave **16** (45 mg, 66%). Bright-red solid.

52-endo,*56*-endo,*60*-endo,*64*-endo-*Tetrahexyl-2,4,8,19,23,34,38,49-octaoxa-10,17,25,32,40,47-hexaazapentadecacyclo[48.15.1.1^{51,65}.0^{5,63}.0^{7,61}.0^{9,18}.0^{11,16}.0^{20,59}.0^{22,57}.0^{24,33}.0^{26,31}.0^{35,55}.0^{37,53}.0^{39,48}.0^{41,46}]heptahexaconta-1(65),5,7(61),9,11(16),12,14,17,20,22(57),24,26(31),27,29,32,35,37(53),39,41(46),42,44,47,50(66),51(67),54,58,62-heptacosaene* (**17**). To a degassed soln. of **3a** (0.088 g, 0.073 mmol) in Me_2SO (4 ml), K_2CO_3 (0.015 g, 0.109 mmol) and CH_2BrCl (0.019 ml, 0.29 mmol) were added, and the mixture was stirred for 12 h at 45° under Ar. Additional K_2CO_3 (0.10 g, 0.073 mmol) and CH_2BrCl (0.019 ml, 0.29 mmol) were added, and the mixture was stirred at 55° for 30 h. After addition of cold H_2O (10 ml), the precipitate formed was isolated by filtration and dried (P_2O_5). FC (SiO_2; CH_2Cl_2/AcOEt 100:0 → 97:3) provided **17** (49 mg, 55%). Colorless amorphous solid. R_f (SiO_2; CH_2Cl_2/cyclohexane 3:1) 0.37. M.p. 335° (dec.). IR (KBr): 3067, 2954, 2927, 2857, 1607, 1579, 1570, 1485, 1415, 1334, 1276, 1222, 1162, 1118, 1068, 972, 913, 896, 759, 606. ^{1}H-NMR ($CDCl_3$, 300 MHz): 0.91–0.98 (*m*, 12 H); 1.32–1.55 (*m*, 32 H); 2.22–2.37 (*m*, 8 H); 4.10 (*d*, *J* = 7.2, 1 H); 4.71 (*t*, *J* = 8.1, 1 H); 5.66 (*d*, *J* = 7.2, 1 H); 5.71 (*m*, 3 H); 7.22 (*s*, 2 H); 7.23 (*s*, 2 H); 7.24 (*s*, 2 H); 7.44–7.59 (*m*, 6 H); 7.68–7.72 (*m*, 2 H); 7.86–7.93 (*m*, 4 H); 8.29 (*s*, 2 H). ^{13}C-NMR ($CDCl_3$, 75 MHz): 14.30; 22.89; 28.07; 28.18; 29.62; 29.70; 30.62; 32.09; 32.34; 32.55; 34.30; 34.46; 36.55; 99.63; 117.38; 118.92; 121.88; 123.94; 128.02; 129.28; 129.36; 129.49; 135.42; 136.12; 136.53; 138.66; 139.90; 139.94; 152.18; 152.67; 152.88; 152.92; 153.09; 155.44. HR-MALDI-MS (DHB): 1215.5966 (MH^+, $C_{77}H_{79}N_4O_8^+$; calc.: 1215.59602).

43-endo,*47*-endo,*51*-endo,*55*-endo-*Tetrahexyl-2,4,8,10,14,25,29,40-octaoxa-16,23,31,38-tetraazatridecacyclo[39.15.1.1^{42,56}.0^{5,54}.0^{7,52}.0^{11,50}.0^{13,46}.0^{15,24}.0^{17,22}.0^{26,46}.0^{28,44}.0^{30,39}.0^{32,37}]octapentaconta-1(56),5,7(52),11,13(48),15,17(22),18,20,23,26,28(44),30,32(37),33,35,38,41(57),42(58),45,49,53-docosaene* (**18**). To a degassed soln. of **5a** (0.070 g, 0.066 mmol) in Me_2SO (3 ml), K_2CO_3 (0.036 g, 0.26 mmol) and CH_2BrCl (0.042 ml, 0.65 mmol) were added, and the mixture was stirred under Ar for 12 h at 55°. Additional K_2CO_3 (0.018 g, 0.13 mmol) and CH_2BrCl (0.021 ml, 0.325 mmol) were added, and the mixture was stirred for 24 h. After addition of cold H_2O (10 ml), the precipitate formed was isolated by filtration and dried (P_2O_5). FC (SiO_2; CH_2Cl_2/AcOEt 100:0 → 99.5:0.5) afforded **18** (34 mg, 47%). Colorless powder. R_f (SiO_2; CH_2Cl_2/cyclohexane 3:1) 0.42. M.p. 295° (dec.). IR (KBr): 3068, 2954, 2927, 2857, 1607, 1579, 1486, 1414, 1333, 1284, 1261, 1222, 1118, 1073, 973, 914, 896, 760, 719, 606. ^{1}H-NMR ($CDCl_3$, 300 MHz): 0.89–0.97 (*m*, 12 H); 1.30–1.57 (*m*, 32 H); 2.19–2.35 (*m*, 8 H); 4.16 (*d*, *J* = 7.2, 2 H); 4.71 (*t*, *J* = 8.1, 2 H); 5.65 (*d*, *J* = 7.2, 2 H); 5.73 (*t*, *J* = 8.1, 2 H); 6.33 (*s*, 1 H); 7.14 (*s*, 1 H); 7.19 (*s*, 1 H); 7.21 (*s*, 2 H); 7.32 (*s*, 2 H); 7.55–7.67 (*m*, 4 H); 7.83 (br. *d*, *J* = 7.8, 2 H); 8.01 (br. *d*, *J* = 7.8, 2 H); 8.34 (*s*, 1 H). ^{13}C-NMR ($CDCl_3$, 75 MHz): 14.41; 22.98; 23.01; 28.14; 28.30; 29.73; 29.79; 30.51; 32.18; 32.21; 32.28; 34.54; 36.60; 99.54; 116.61; 117.15; 118.72; 120.28; 121.97; 124.33; 128.03; 129.38; 129.65; 135.34; 136.25; 137.86; 139.01; 139.88; 151.95; 152.46; 152.76; 153.08; 154.80; 155.39. HR-MALDI-MS (DHB): 1101.5748 (MH^+, $C_{70}H_{77}N_4O_8^+$; calc.: 1101.57423).

2,3:2′,3′:2″,3″:2‴,3‴-[2-endo,*8*-endo,*14*-endo,*20*-endo-*Tetrahexylpentacyclo[19.3.1.1^{3,7}.1^{9,13}.1^{15,19}]octacosa-1(25),3,5,7(28),9,11,13(27),15,17,19(26),21,23-dodecaene-4,24:6,10:12,16:18,22-octayloctaoxy]tetrakis(5,6-dinitropyrazine)* (**19**). To **1a** (0.890 g, 1.08 mmol) and 1,2-difluoro-3,4-dinitrobenzene (0.969 g, 4.75 mmol) in DMF (30 ml), Et_3N (2.41 ml, 17.3 mmol) was added dropwise, and the mixture was heated to 70° for 12 h. After cooling, the mixture was poured into 1M HCl (200 ml), and the precipitate formed was isolated by filtration, washed with 1M HCl (200 ml), H_2O (200 ml), and dried (P_2O_5). FC (SiO_2; CH_2Cl_2) afforded **19** (814 mg, 51%). Slightly yellowish powder. M.p. 295–315° (dec.). R_f (SiO_2; CH_2Cl_2) 0.65; R_f = (SiO_2; CH_2Cl_2/cyclohexane 3:1) 0.13. IR (KBr): 3056, 2930, 2858, 1594, 1542, 1486, 1362, 1326, 1287, 1192, 1142, 1078, 899, 851, 823, 752, 653. ^{1}H-NMR ($CDCl_3$, 300 MHz): 0.84–0.89 (*m*, 12 H); 1.09-1.19 (*m*, 32 H); 1.96–2.08 (*m*, 8 H); 3.90–3.96 (*m*,

4 H); 6.21 (br. *s*, 2 H); 7.01 (*s*, 2 H); 7.02 (*s*, 2 H); 7.22 (br. *s*, 2 H); 7.63 (*s*, 4 H); 7.66 (*s*, 4 H). ^{13}C-NMR ($(CD_3)_2CO$, 75 MHz): 14.24; 23.28; 27.81; 31.85; 32.52; 37.14; 153.72 (other peaks not resolved due to conformational equilibration). HR-MALDI-MS (DHB): 1503.4538 ($[M+Na]^+$, $C_{76}H_{72}N_8O_{24}Na^+$; calc.: 1503.45569). X-Ray: see *Fig. 5*.

*Methyl 2-[3,5-Di(*tert*-butyl)phenyl]acetate* (**21**) [25]. HCl Gas was bubbled for 20 min through a soln. of 2-[3,5-di-(*tert*-butyl)phenyl]acetonitrile [22] (38.8 g, 169 mmol) in anh. MeOH (350 ml), and the mixture was heated to reflux for 3 d under Ar. Evaporation under reduced pressure gave a residue that was dissolved in CH_2Cl_2 (1000 ml). The soln. was washed with 1M HCl (400 ml), dried ($MgSO_4$), and evaporated. Filtration of the resulting oil through a short column (5 cm SiO_2; CH_2Cl_2) gave **21** (43.8 g, 99%). Pale-yellow oil. R_f (SiO_2; hexane/CH_2Cl_2 1:1) 0.42. ^{1}H-NMR (300 MHz, $CDCl_3$): 1.33 (*s*, 18 H); 3.66 (*s*, 2 H); 3.70 (*s*, 3 H); 7.13 (*d*, $J = 1.9$, 2 H); 7.34 (*t*, $J = 1.9$, 1 H). ^{13}C-NMR (75 MHz, CD_3OD): 30.5; 34.2; 40.8; 51.0; 120.7; 123.2; 133.4; 150.8; 173.2. EI-MS: 262 (M^+), 247 ($[M - Me]^+$).

*2-[3,5-Di(*tert*-butyl)phenyl]acetaldehyde* (**22**) [26]. To a soln. of **21** (43.8 g, 167 mmol) in hexane (350 ml), cooled to $-78°$, DIBAL-H (1M in hexane, 200 ml) was added *via* cannula under N_2, and the mixture was stirred at $-78°$ for 3 h. After cautious addition of MeOH (30 ml), the mixture was poured into sat. aq. Na/K tartrate soln. (400 ml). AcOEt (400 ml) was added, and the mixture was stirred for 12 h. The org. layer was separated, and the aq. layer was washed with AcOEt (2 × 300 ml). The combined org. layers were dried (K_2CO_3) and evaporated under reduced pressure. Distillation (114–116° 0.8 Torr) gave **22** (22.4 g, 58%). Yellow oil. R_f (SiO_2; CH_2Cl_2) 0.58. UV/VIS (MeOH): 256 (1120). IR (CH_2Cl_2): 2964, 1718, 1595, 1472, 1395, 1364, 1262, 1190, 1159, 1097, 1000, 892, 867, 815, 697. ^{1}H-NMR (300 MHz, $CDCl_3$): 1.35 (*s*, 18 H); 3.68 (*d*, $J = 2.4$, 2 H); 7.08 (*d*, $J = 1.8$, 2 H); 7.40 (*t*, $J = 1.8$, 1 H); 9.77 (*t*, $J = 2.4$, 1 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 31.5; 34.9; 51.3; 121.7; 124.0; 124.4; 131.2; 151.9; 200.3. EI-MS: 232.2 (M^+), 217.2 ($[M - Me]^+$), 203.2 ($[M - CHO]^+$).

Compound **23** (for nomenclature and full characterization, see [4]). To resorcinol (2.47 g, 22.5 mmol) and **22** (5.20 g, 22.4 mmol) in EtOH (50 ml) at 0° under N_2, conc. HCl (40 ml) was added dropwise over 30 min. The soln. was stirred at 60° for 5 d, then poured into H_2O (1500 ml). After stirring for 2 h at r.t, the precipitate formed was collected by filtration and recrystallized (MeCN) to give **23** (2.57 g, 35%). Pale-brown solid.

Compound **20** (for nomenclature and full characterization including X-ray structure analysis (*Fig. 6*), see [4]). A soln. of **22** (0.56 g, 0.43 mmol), 2,3-dichloroquinoxaline (0.39 g, 1.96 mmol), and Cs_2CO_3 (1.18 g, 3.62 mmol) in anh. DMF (60 ml) was stirred under N_2 for 2 d at 60°. After cooling, CH_2Cl_2 (400 ml) was added. Washing with H_2O, drying (Na_2SO_4), and evaporation provided a solid that was purified by FC (SiO_2; CH_2Cl_2/MeOH 99.5:0.5) to give **20** (0.53 g, 68%). Off-white solid.

Tetramethyl 4,4',4'',4'''-(4,6,10,12,16,18,22,24-Octahydroxypentacyclo[19.3.1.1^{3,7}.1^{9,13}.1^{15,19}]octacosa-1(25),3,5,7(28),9,11,13(27),15,17,19(26),21,23-dodecaene-2,8,14,20-tetrayl)tetrabutanoate (**26**). Pd/C (10%) (2.9 g) was flushed with H_2, freshly distilled THF (900 ml) was added, and the soln. was reflushed with H_2. 2,6-Dimethylpyridine (21.2 g, 198 mmol) and glutaric acid monomethyl ester chloride (29.8 g, 181 mmol) were added, and the soln. was stirred at r.t. under H_2 for 24 h. Filtration through *Celite* and evaporation under reduced pressure left a residue that was taken up in CH_2Cl_2 (500 ml). Washing with H_2O (300 ml), 1M HCl (300 ml), and H_2O (300 ml) and evaporation under reduced pressure provided **25** (25.3 g). Colorless oil. ^{1}H-NMR (300 MHz, $CDCl_3$): 1.80–2.30 (*m*, 2 H); 2.38 (*t*, $J = 7.5$, 2 H); 2.49–2.60 (*m*, 2 H); 3.68 (*s*, 3 H); 9.79 (*t*, $J = 1.2$, 1 H). To **25** (25.3 g) and resorcinol (19.9 g, 181 mmol) in MeOH (300 ml) at 0°, conc. HCl (5 ml) was added over 30 min, and the soln. was stirred under Ar at r.t. for 2 h, then at 60° for 2 d. The soln. was poured into H_2O (1500 ml), and the resulting cream-colored precipitate was collected by filtration and recrystallized (MeCN) to give **26** (17.59 g, 44%). White solid. M.p. 205–210°. UV/VIS (MeOH): 286 (17300). ^{1}H-NMR (300 MHz, CD_3OD): 1.58 (*quint.*, $J = 7.5$, 8 H); 2.25 (*q*, $J = 7.8$, 8 H); 2.42 (*t*, $J = 7.4$, 8 H); 3.66 (*s*, 12 H); 4.31 (*t*, $J = 7.9$, 4 H); 6.23 (*s*, 4 H); 7.24 (*s*, 4 H). ^{13}C-NMR (75 MHz, CD_3OD): 24.7; 34.1; 34.6; 34.8; 52.1; 104.1; 125.3; 153.2; 176.0. HR-MALDI-MS (DHB): 911.3460 ($[M+Na]^+$, $C_{48}H_{56}NaO_{16}^+$; calc.: 911.3461). Anal. calc. for $(C_{48}H_{56}O_{16})_2 \cdot H_2O \cdot MeCN$ (1836.965): C 64.08, H 6.42 N 0.76; found: C 63.7, H 6.35, N 0.74.

*2,3:2',3':2'',3'':2''',3'''-[2-*endo*,8-*endo*,14-*endo*,20-*endo*-Tetrakis[3-(methoxycarbonyl)propyl]pentacyclo[19.3.1.1^{3,7}.1^{9,13}.1^{15,19}]octacosa-1(25),3,5,7(28),9,11,13(27),15,17,19(26),21,23-dodecaene-4,24:6,10:12,16:18,22-octayloctaoxy]tetraquinoxaline* (**24**). A soln. of 2,3-dichloroquinoxaline (1.51 g, 7.57 mmol), **26** (1.50 g, 1.69 mmol), and Cs_2CO_3 (4.50 g, 13.8 mmol) in anh. DMF (250 ml) was stirred under Ar at 60° for 2 d. The mixture was poured into dilute aq. AcOH soln., and the resulting precipitate was collected by filtration. Recrystallization (MeCN) gave **24** (0.305 g, 13%). Colorless solid. M.p. > 250°. UV/VIS ($CHCl_3$): 315 (36500), 328 (32000). ^{1}H-NMR (300 MHz, $CDCl_3$): 1.68–1.82 (*m*, 8 H); 2.34–2.46 (*m*, 8 H); 2.54 (*t*, $J = 7.3$, 8 H); 3.74 (*s*, 12 H); 5.65 (*t*, $J = 8.0$, 4 H); 7.34 (*s*, 4 H); 7.44–7.51 (*m*, 8 H); 7.77–7.83 (*m*, 8 H); 8.19 (*s*, 4 H). ^{13}C-NMR

(75 MHz, $CDCl_3$): 23.5; 31.7; 33.7; 34.4; 51.7; 119.0; 123.8; 127.9; 129.1; 135.7; 139.8; 152.6; 152.7; 174.1. HR-MALDI-MS (DHB): 1415.4494 ($[M+Na]^+$, $C_{80}H_{64}N_8NaO_{16}^+$; calc.: 1415.4338). X-Ray: see *Fig. 7.*

2,8,14,20-Tetrakis(dec-9-enyl)pentacyclo[19.3.1.1^{3,7}.1^{9,13}.1^{15,19}]octacosa-1(25),3,5,7(28),9,11,13(27),15,17,19(26),21,23-dodecaene-4,6,10,12,16,18,22,24-octol (**30**). To resorcinol (11.2 g, 0.1 mol) and **29** (17.7 g, 0.1 mol) in EtOH (100 ml), conc. HCl (16 ml) was added dropwise over 30 min at 0° under Ar. After stirring for 25 h at r.t., the mixture was poured into H_2O (300 ml). The precipitate formed was isolated by filtration, triturated with H_2O, and dried under vacuum at 70° for 20 h to give **30** (24.3 g, 93%). Orange-colored solid. R_f = (SiO_2; $CHCl_3$/MeOH 5:1) 0.60; R_f (SiO_2; CH_2Cl_2/MeOH 10:1) 0.42. M.p. > 250° (EtOH). IR (KBr): 3316, 2924, 2866, 1639, 1617, 1504, 1461, 1436, 1367, 1344, 1300, 1267, 1194, 1166, 1150, 1083, 989, 907, 848, 828, 722, 628, 611, 556, 512. ^1H-NMR (($(CD_3)_2CO$, 200 MHz): 1.20–1.70 (*m*, 56 H); 2.28 (*m*, 8 H); 4.30 (*t*, *J* = 7.9, 4 H), 4.93 (*m*, 8 H); 5.82 (*m*, 4 H); 6.25 (*s*, 4 H); 7.55 (*s*, 4 H); 8.50 (br. *s*, 8 H). FAB-MS: 1040.6 (M^+). HR-MALDI-MS: 1063.6995 ($[M+Na]^+$, $C_{68}H_{96}NaO_8^+$; calc.: 1063.7003).

*2,3:2',3':2'',3'':2''',3'''-[2-*endo*,8-*endo*,14-*endo*,20-*endo*-Tetrakis(dec-9-enyl)pentacyclo[19.3.1.1^{3,7}.1^{9,13}.1^{15,19}]-octacosa-1(25),3,5,7(28),9,11,13(27),15,17,19(26),21,23-dodecaene-4,24:6,10:12,16:18,22-octayloctaoxy]tetraquinoxaline* (**31**). A soln of **30** (2.08 g, 2.0 mmol), 2,3-dichloroquinoxaline (1.66 g, 8.0 mmol), and Cs_2CO_3 (2.90 g, 8.8 mmol) in dry Me_2SO (70 ml) was stirred under Ar at r.t. for 2 d. The mixture was poured into ice-water, and the brownish precipitate formed was isolated by filtration. The filtrate was extracted with CH_2Cl_2, and the org. layer was washed with H_2O and evaporated to provide additional solid crude product that was combined with the first precipitate. FC (SiO_2 (150 g); CH_2Cl_2/AcOEt 98:2 → 90:10) afforded **31** (1.285 g, 44%). Colorless flakes. R_f (SiO_2; CH_2Cl_2) 0.23; R_f = (SiO_2; CH_2Cl_2/AcOEt 20:1) 0.66. M.p. > 250° (CH_2Cl_2/Me_2CO). IR (KBr): 3066, 2925, 2852, 1711, 1639, 1606, 1570, 1482, 1416, 1400, 1361, 1265, 1222, 1160, 1117, 1061, 1017, 994, 950, 911, 896, 759, 722, 606, 583, 522, 461. ^1H-NMR ($CDCl_3$, 200 MHz): 1.20–1.50 (*m*, 48 H); 1.99–2.09 (*m*, 8 H); 2.24 (*m*, 8 H); 4.95 (*m*, 8 H); 5.53 (*t*, *J* = 8.2, 4 H); 5.81 (*m*, 4 H); 7.18 (*s*, 4 H); 7.40–7.49 (*m*, 8 H); 7.72–7.81 (*m*, 8 H); 8.12 (*s*, 4 H). ^{13}C-NMR ($CDCl_3$, 75 MHz): 27.8; 28.9; 29.0; 29.4; 29.5; 32.3; 33.7; 34.1; 114.2; 118.7; 123.4; 127.8; 129.8; 135.8; 139.3; 139.7; 152.5; 152.6. FAB-MS: 1546 (MH^+). HR-MALDI-MS: 1567.7950 ($[M+Na]^+$, $C_{100}H_{104}N_8NaO_8^+$; calc.: 1567.7875), 1545.8168 (MH^+, $C_{100}H_{105}N_8O_8^+$; calc.: 1545.8055). Anal. calc. for $C_{100}H_{104}N_8O_8 \cdot$ AcOEt (1634.05): C 76.44, H 6.91, N 6.86; found: C 76.15, H 6.82, N 6.78.

*2,3:2',3':2'',3'':2''',3'''-[2-*endo*,8-*endo*,14-*endo*,20-*endo*-Tetrakis[10-(decylsulfanyl)delcyl]pentacyclo-[19.3.1.1^{3,7}.1^{9,13}.1^{15,19}]octacosa-1(25),3,5,7(28),9,11,13(27),15,17,19(26),21,23-dodecaene-4,24:6,10:12,16:18,22-octayloctaoxy]tetraquinoxaline* (**27**). To **27** (474 mg, 0.33 mmol) in freshly distilled THF (20 ml), decane-1-thiol (0.7 ml, 3.3 mmol) was added, and the mixture was stirred under Ar at 0° for 1 h. After addition of 9-BBN (0.5M in THF, 33 ml, 16.5 mmol), the mixture was stirred under Ar for 2 d at r.t., then poured into H_2O (200 ml), and extracted with CH_2Cl_2 (200 ml). Washing with H_2O (2 × 200 ml), drying ($MgSO_4$), evaporation, and FC (SiO_2 (50 g); CH_2Cl_2/AcOEt 99:1 → 50:50) gave **27** (543 mg, 0.242 mmol, 74%). Colorless powder. R_f (SiO_2; CH_2Cl_2) 0.25. M.p. 53–55° (CH_2Cl_2/Me_2CO). IR (KBr): 2924, 2851, 1482, 1417, 1336, 1266, 1160, 897, 758, 607. ^1H-NMR ($CDCl_3$, 200 MHz): 0.86 (*t*, *J* = 6.6, 12 H); 1.20–1.70 (*m*, 128 H); 2.22 (*m*, 8 H); 2.48 (*m*, 16 H); 5.54 (*t*, *J* = 8.2, 4 H); 7.18 (*s*, 4 H); 7.40–7.49 (*m*, 8 H); 7.73–7.81 (*m*, 8 H); 8.13 (*s*, 4 H). ^{13}C-NMR ($CDCl_3$, 75 MHz): 14.0; 22.6; 27.8; 28.9–29.7 (15 ×); 31.8; 32.1; 34.1; 118.8; 123.4; 127.8; 129.0; 135.8; 139.7; 152.5; 152.6. MALDI-MS: 2242.5 (MH^+, $C_{140}H_{193}N_8O_8S_4^+$; calc.: 2242.3823), 2264.5 ($[M+Na]^+$, $C_{140}H_{192}N_8NaO_8S_4^+$; calc.: 2264.3643); 2281.4 ($[M+K]^+$, $C_{140}H_{192}KN_8O_8S_4^+$; calc.: 2280.3382). Anal. calc. for $C_{140}H_{192}N_8O_8S_4$ (2243.34): C 74.96, H 8.63, N 4.99; found: C 74.85, H 8.41, N 5.01.

6-(Pentylsulfanyl)hexan-1-ol (**33**). To hex-6-en-1-ol (1.22 ml, 1.0 g, 10 mmol) in freshly distilled THF (20 ml), 9-BBN (0.5M in THF, 100 ml, 50 mmol) was added at 0° under N_2. Pentane-1-thiol (1.28 ml, 1.24 g, 10 mmol) was added at 0°, and the mixture was stirred for 3 h at r.t. The mixture was poured into sat. aq. NaCl soln. (100 ml) and extracted with CH_2Cl_2 (3 × 100 ml). Drying ($MgSO_4$), evaporation, and FC (SiO_2; CH_2Cl_2/AcOEt 5:2) provided **33** (2.2 g, 99%). Colorless oil. R_f = (SiO_2; hexane/AcOEt 2:1) 0.35–0.38. IR (neat): 3356, 2922, 2856, 1461, 1378, 1300, 1267, 1217, 1056, 895, 728. ^1H-NMR ($CDCl_3$, 300 MHz): 0.87 (*t*, *J* = 7.2, 3 H); 1.20–1.64 (*m*, 14 H); 2.47 (*t*, *J* = 7.5, 2 H); 2.49 (*t*, *J* = 7.5, 2 H); 3.62 (br. *m*, 2 H). ^{13}C-NMR ($CDCl_3$, 75 MHz): 13.8; 22.2; 25.3; 28.6; 29.3; 29.5; 31.0; 32.0; 32.1; 32.5; 62.9. EI-MS: 204 (M^+), 157 ($[M-MeS]^+$), 143 ($[M-EtS]^+$), 131 ($[M-BuO]^+$), 115 ($[M-BuS]^+$). Anal. calc. for $C_{11}H_{24}OS$ (204.3737): C 64.65, H 11.84; found: C 64.59, H 11.83.

6-(Pentylsulfanyl)hexanal (**32**). To a suspension of PCC (1.6 g, 7.5 mmol) and *Celite* (1.6 g) in freshly distilled CH_2Cl_2 (20 ml), a soln. of **33** (1.02 g, 5 mmol) in freshly distilled CH_2Cl_2 (5 ml) was added dropwise under Ar at r.t. After stirring for 5 h under Ar at r.t., the mixture was filtered through a pad of SiO_2, which was subsequently washed with CH_2Cl_2. The combined filtrates were concentrated to yield **32** (313 mg, 31%). Colorless oil. R_f (SiO_2; CH_2Cl_2)0.59. IR (neat): 2929, 2856, 2711, 2357, 2333, 1722, 1458, 1300, 1272, 1217, 1156,

1078. ^{1}H-NMR ($CDCl_3$, 300 MHz): 0.87 (*t*, *J* = 7.2, 3 H); 1.24 – 1.47 (*m*, 6 H); 1.50 – 1.69 (*m*, 6 H); 2.37 – 2.45 (*m*, 2 H); 2.47 (*t*, *J* = 7.2, 2 H); 2.49 (*t*, *J* = 7.2, 2 H); 9.75 (*t*, *J* = 1.7, 1 H). ^{13}C-NMR ($CDCl_3$, 75 MHz): 13.8; 21.6; 22.2; 28.3; 29.3 (2 ×); 31.0; 31.8; 32.1; 43.7; 202.7. EI-MS: 202 (M^+), 174 ($[M - CO]^+$). Anal. calc. for $C_{11}H_{22}OS$ (202.3578): C 65.29, H 10.96; found: C 65.28, H 10.77.

2,8,14,20-Tetrakis[5-(pentylsulfanyl)pentyl]pentacyclo[19.3.1.1^{3,7}.1^{9,13}.1^{15,19}]octacosa-1(25),3,5,7(28),9,11,13(27), 15,17,19(26),21,23-dodecaene-4,6,10,12,16,18,22,24-octol (**34**). To **32** (282 mg, 1.39 mmol) and resorcinol (156 mg, 1.39 mmol) in EtOH (1.4 ml), conc. HCl (0.22 ml) was added dropwise over 30 min at 0° under N_2. The mixture was stirred at r.t. for 1 h and at 60° for 24 h, then it was poured into ice-water. The formed precipitate was isolated by filtration and dried under vacuum for 2 d to give **34** (412 mg, 99%). Colorless powder. M.p. > 250° (EtOH). IR (KBr): 3254, 2927, 2856, 1618, 1500, 1450, 1367, 1328, 1294, 1256, 1211, 1167, 1089, 900, 833. ^{1}H-NMR ($(CD_3)_2CO$, 300 MHz): 0.89 (*t*, *J* = 7.1, 12 H); 1.22 – 1.42 (*m*, 24 H); 1.42 – 1.64 (*m*, 24 H); 2.27 – 2.35 (*m*, 8 H); 2.50 (*t*, *J* = 7.3, 16 H); 4.30 (*t*, *J* = 7.8, 4 H); 6.24 (*s*, 4 H); 7.55 (*s*, 4 H); 8.48 (*s*, 8 H). ^{13}C-NMR ($(CD_3)_2CO$, 50 MHz): 14.3; 22.9; 29.6; 30.1; 30.4; 30.8; 31.8; 32.5; 32.6; 34.2; 34.3; 103.7; 125.3; 125.5; 152.8. HR-MALDI-MS: 1199.6497 ($[M + Na]^+$, $C_{68}H_{104}NaO_8S_4^+$; calc.: 1199.6512).

*2,3:2′,3′:2″,3″:2‴,3‴-[*2-endo,8-endo,14-endo,20-endo-*tetrakis[5-(pentylthio)pentyl]pentacyclo[19.3.1.1^{3,7}.1^{9,13}.1^{15,19}]octacosa-1(25),3,5,7(28),9,11,13(27),15,17,19(26),21,23-dodecaene-4,24:6,10:12,16:18,22-octayloctaoxy]tetraquinoxaline* (**28**). A soln. of **34** (133 mg, 0.113 mmol), 2,3-dichloroquinoxaline (94 mg, 0.45 mmol), and Cs_2CO_3 (163 mg, 0.50 mmol) in dry Me_2SO (5 ml) was stirred at r.t. under Ar for 4 d. The mixture was poured into ice-water, and the precipitate formed was isolated by filtration, triturated with H_2O, and then dissolved in Me_2CO/CH_2Cl_2. The soln. was dried ($MgSO_4$) and concentrated to give a dark-brown solid that was purified by FC (SiO_2; CH_2Cl_2/AcOEt 98:2 → 80:20) to give **28** (99 mg, 52%). Colorless flakes. M.p. 227 – 230° (EtOH). IR (KBr): 3067, 2926, 2856, 1606, 1572, 1483, 1467, 1417, 1400, 1361, 1336, 1265, 1233, 1222, 1160, 1117, 1072, 1044, 1011, 950, 906, 896, 867, 757, 722, 606, 583. ^{1}H-NMR ($CDCl_3$, 200 MHz): 0.89 (*t*, *J* = 7.1, 12 H); 1.28 – 1.50 (*m*, 24 H); 1.50 – 1.75 (*m*, 24 H); 2.27 (*m*, 8 H); 2.52 (*t*, *J* = 7.3, 8 H); 2.55 (*m*, 8 H); 5.59 (*t*, *J* = 8.1, 4 H); 7.18 (*s*, 4 H); 7.41 – 7.50 (*m*, 8 H); 7.71 – 7.80 (*m*, 8 H); 8.16 (*s*, 4 H). ^{13}C-NMR ($CDCl_3$, 50 MHz): 13.9; 22.2; 27.5; 28.7; 29.4; 29.6; 31.1; 32.1; 32.2 (2 ×); 34.0; 118.9; 123.3; 127.8; 129.0; 135.7; 139.7; 152.5; 152.6. HR-MALDI-MS: 1703.7388 ($[M + Na]^+$, $C_{100}H_{112}N_8NaO_8S_4^+$; calc.: 1703.7384), 1681.7578 (MH^+, $C_{100}H_{113}N_8O_8S_4^+$; calc.: 1681.7656). Anal. calc. for $C_{100}H_{112}N_8O_8S_4$ (1682.2724): C 71.40, H 6.71, N 6.66; found: C 71.50, H 7.02, N 6.86.

X-Ray Crystal Structure of **1c**. Crystal data at 233 K for $C_{44}H_{56}O_8 \cdot 2(MeOH) \cdot MeCH_2OH$ (M_r 823.04): monoclinic, space group *C2/c* (No. 15), D_c = 1.207 g/cm^3, *Z* = 8, *a* = 36.049(7), *b* = 11.564(2), *c* = 21.773(5) Å, β = 93.71(2)°, *V* = 9058(3) Å^3, *Nonius CAD4* diffractometer, CuK_α radiation, λ = 1.5418 Å. A crystal, obtained by slow cooling of a MeOH/EtOH soln. (linear dimensions *ca.* 0.2 × 0.18 × 0.16 mm) was mounted at low temp. to prevent evaporation of enclosed solvent. The structure was solved by direct methods (*SIR97*) [27] and refined by full-matrix least-squares analysis (SHELXL-97) [28], by using an isotropic extinction correction, and $w = 1/[\sigma 2(F_o^2) + (0.0933P)^2 + 49.1608P]$, where $P = (F_o^2 + 2F_c^2)/3$. The subunit C(10)–C(13) and one MeOH are disordered over two orientations. For C(11), C(12), C(13), and O(200), two sets of atomic parameters were refined with population parameters of 0.7 and 0.3, resp. In *Fig. 2*, only one orientation is shown for clarity. All heavy atoms were refined anisotropically (H-atoms of the ordered structure isotropically, whereby H-positions are based on stereochemical considerations). Final $R(F)$ = 0.084, $wR(F^2)$ = 0.241 for 626 parameters and 5047 reflections with $I > 2\sigma(I)$ and $2.46 < \theta < 56.96°$ (corresponding *R* values based on all 6098 reflections are 0.098 and 0.252 resp.). CCDC-214738.

X-Ray Crystal Structure of **2b**. Crystal data at 293 K for $C_{80}H_{72}N_8O_8 \cdot CH_2Cl_2 \cdot 2\,MeCN$ (M_r 1440.51): monoclinic, space group *C2/c* (No. 15), D_c = 1.224 g/cm^3, *Z* = 4, *a* = 19.007(14), *b* = 17.623(13), *c* = 24.57(2) Å, β = 109.63(6)°, *V* = 7752(11) Å^3; *Picker-Stoe* diffractometer, CuK_α radiation, λ = 1.5418 Å. Prismatic crystals (linear dimensions *ca.* 0.15 × 0.1 × 0.1 mm) were obtained by slow evaporation from $MeCN/CH_2Cl_2$. The structure was solved by direct methods (SHELXS-86) [29] and refined by full-matrix least-squares analysis (SHELXL-93) [30]. The subunit C(35)–C(36) and both MeCN molecules are disordered over two orientations. In *Fig. 3*, only one orientation is shown for clarity. All heavy atoms were refined anisotropically, H-atoms were fixed isotropically with atomic positions based on stereochemical considerations. Final $R(F)$ = 0.0602, $wR(F^2)$ = 0.1681 for 511 parameters and 3228 reflections with $I > 2\sigma(I)$ and $3.52 < \theta < 49.98°$. CCDC-214733.

X-Ray Crystal Structure of **11**. Crystal data at 295 *K* for $C_{23}H_{26}BF_2N_3O_2$ (M_r 425.28): triclinic, space group $P\bar{1}$ (No. 2), D_c = 1.042 g/cm^3, *Z* = 6, *a* = 13.9886(4), *b* = 17.0634(5), *c* = 18.9111(6) Å, α = 71.360(2)°, β = 72.540(2)°, γ = 88.770(2)°, *V* = 4066.3(2) Å^3; *Bruker-Nonius Kappa-CCD* diffractometer, MoK_α radiation, λ = 0.7107 Å. A dark-red crystal (linear dimensions *ca.* 0.3 × 0.3 × 0.02 mm) was obtained by slow evaporation of a $CHCl_3$ soln. The structure was solved by direct methods (SIR97) [27] and refined by full-matrix least-

squares analysis (SHELXL-97) [28], by using an isotropic extinction correction and $w = 1/[\sigma2(F_o^2) + (0.1974P)^2 + 1.6862P]$, where $P = (F_o^2 + 2F_c^2)/3$. All heavy atoms were refined anisotropically (H-atoms isotropically, whereby H-positions are based on stereochemical considerations). Final $R(F) = 0.087$, $wR(F^2) = 0.258$ for 839 parameters and 4959 reflections with $I > 2\sigma(I)$ and $3.13 < \theta < 20.03°$ (corresponding R values based on all 7142 reflections are 0.118 and 0.298 resp.). CCDC-214739.

X-Ray Crystal Structure of **19**. Crystal data at 253 K for $C_{76}H_{72}N_8O_{24} \cdot 2\ Me_2CO$ (M_r 1597.57): monoclinic, space group $P2_1/m$ (No. 11), $D_c = 1.234$ g/cm³, $Z = 2$, $a = 12.3125(3)$, $b = 27.0211(7)$, $c = 13.1175(4)$ Å, $\beta = 99.780(1)°$, $V = 4300.7(2)$ Å³; *Bruker-Nonius Kappa-CCD* diffractometer, MoK_α radiation, $\lambda = 0.7107$ Å. An orange crystal, obtained by evaporation of a Me_2CO soln. (linear dimensions *ca.* 0.3 × 0.3 × 0.28 mm) was mounted at low temp. in epoxy resin to prevent evaporation of enclosed solvent. The crystals shatter below *ca.* 230 K. At 253 K, they desintegrate within a few hours. The structure was solved by direct methods (SIR97) [27] and refined by full-matrix least-squares analysis (SHELXL-97) [28], by using an isotropic extinction correction and $w = 1/[\sigma2(F_o^2) + (0.1188P)^2 + 2.8782P]$, where $P = (F_o^2 + 2F_c^2)/3$. All heavy atoms were refined anisotropically (H-atoms isotropically, whereby H-positions are based on stereochemical considerations). Final $R(F) = 0.081$, $wR(F^2) = 0.209$ for 568 parameters and 4932 reflections with $I > 2\sigma(I)$ and $1.75 < \theta < 24.11°$ (corresponding R values based on all 6523 reflections are 0.104 and 0.231 resp.). CCDC-214740.

X-Ray Crystal Structure of **24**. Crystal data at 293 K for $C_{80}H_{64}N_8O_{16} \cdot MeCN$ (M_r 1434.46): monoclinic, space group $P2(1)/c$ (No. 14), $D_c = 1.311$ g/cm³, $Z = 4$, $a = 21.08(2)$, $b = 17.07(2)$, $c = 20.99(3)$ Å, $\beta = 104.03(9)°$, $V = 7332(15)$ Å³; *Picker-Stoe* diffractometer, CuK_α radiation, $\lambda = 1.5418$ Å. Prismatic crystals (linear dimensions *ca.* 0.1 × 0.1 × 0.07 mm) were obtained by slow evaporation from $MeCN/CHCl_3$. The structure was solved by direct methods (SHELXS-86) [29] and refined by full-matrix least-squares analysis (SHELXL-93) [30]. All heavy atoms were refined anisotropically, H-atoms fixed isotropically with atomic positions based on stereochemical considerations. Final $R(F) = 0.1088$, $wR(F2) = 0.2957$ for 977 parameters and 3500 reflections with $I > 2\sigma(I)$ and $2.16 < \theta < 42.50°$. CCDC-214734.

Crystallographic data (excluding structure factors) for the structures reported in this paper have been deposited with the *Cambridge Crystallographic Data Centre* (*CCDC*). Copies of the data can be obtained, free of charge, on application to the *CCDC*, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44(1223)336033; e-mail: deposit@ccdc.cam.ac.uk).

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Enhancing effect of poly(L-lactide) on the differentiation of mouse osteoblast-like MC3T3-E1 cells

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Abstract

Poly(L-lactide) (PLLA) has bioabsorbability and biocompatibility, and it is used as biodegradable screws, pins and plates for internal bone fixation. The purpose of this study was to clarify the effects of low molecular weight (Mw) PLLA on the proliferation and differentiation of mouse osteoblast-like MC3T3-E1 cells. MC3T3-E1 cells were cultured with the concentration of 5–50 μg/ml of PLLA with weight average Mw of 5000 (PLLA-5k) and 10,000 (PLLA-10k) for 2 weeks using the micromass culture. Both PLLAs did not affect the proliferation of MC3T3-E1 cells. However, the calcifications of MC3T3-E1 cells were stimulated with increasing the concentration of the PLLAs. Then PLLA-5k increased the calcification of MC3T3-E1 cells more than PLLA-10k. Additionally, both PLLAs increased the alkaline phosphatase (ALP) activity and calcium content of MC3T3-E1 cells up to the similar level to the calcification. These results indicated that low Mw PLLA enhanced the differentiation of MC3T3-E1 cells with no effect on the proliferation. Moreover, it was suggested that the increase of the ALP activity was a key step to stimulate the calcification of MC3T3-E1 cells. The osteoconductivity of implanted PLLA would be based on the enhancing effect of low Mw PLLA on the differentiation of the osteoblasts.
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Keywords: Poly(L-lactide); Osteoblast; MC3T3-E1 cell; Calcification; Differentiation; Micromass culture

1. Introduction

Poly(L-lactide) (PLLA) with a high molecular weight (Mw) is used as biodegradable screws, pins and plates for internal bone fixation in the orthopedics. Bos et al. reported that the mass loss of PLLA was observed after 26 weeks, and no acute or chronic inflammatory reaction to PLLA was observed until 143 weeks with exception of the early part implant period, by subcutaneous implantation into rats [1]. Otto et al. observed lamellar bone formation around the PLLA wire at 2 and 6 months after intramedullary implantation into rat tibiae [2]. Mainil-Varlet et al. also observed decreasing Mw of PLLA after 4 weeks and bone formation around the PLLA pin at 1 month after implantation into the cortex of sheep tibiae [3]. Thus, there have been many reports on the bioabsorbability and biocompatibility of PLLA.

Recently, there were reported that the lower change in the Mw of PLLA by heat treatment [4] and γ-ray irradiation [5] was responsible for enhancing the differentiation of mouse osteoblast-like MC3T3-E1 cells cultured on the PLLA. It was expected that the low Mw PLLA produced by degradation should enhance the differentiation of osteoblasts. Ikarashi et al. examined the response of MC3T3-E1 cells cultured on several PLLAs with different Mws. The alkaline phosphatase (ALP) activity increased when the cells were cultured on the PLLA with weight average Mw of 20,000 for 2 weeks, but not on the PLLA with Mw of 270,000 and 1,370,000. They also reported that the ALP activity increased when MC3T3-E1 cells were cultured with low Mw poly(DL-lactide) (PDLLA) for 2 weeks [6]. However, the proliferation and differentiation of MC3T3-E1 cells cultured with low Mw PLLA have not been clarified, and it is insufficient to discuss osteoblast differentiation only based on the ALP activity.

In the present study, MC3T3-E1 cells were cultured with low Mw PLLA using the micromass culture, and the differentiation of the cells was synthetically

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evaluated from the ALP activity and calcification. Thus, it was possible to clarify the effects of low Mw PLLA on the proliferation and differentiation of osteoblast-like MC3T3-E1 cells.

2. Materials and methods

2.1. Materials

PLLA with weight average Mw of 5000 (PLLA-5k) and 10,000 (PLLA-10k) were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). The polydispersity indexes, which were calculated as the ratio of the weight average Mw to the number average Mw, of the PLLA-5k and PLLA-10k were, respectively 2.5 and 2.8, by gel permeation chromatography. The PLLAs were used without any refining.

PLLA-5k and PLLA-10k were, respectively, dissolved in dimethyl sulfoxide (DMSO) to a concentration of 50 mg/ml, and sterilized by filtration through a 0.22 μm filter. The sterilized PLLA solutions were serially diluted with DMSO to give concentrations of 5, 10 and 25 mg/ml.

2.2. Cells

Mouse osteoblast-like MC3T3-E1 cells were obtained from RIKEN Cell Bank (Saitama, Japan). MC3T3-E1 cells were grown in alpha minimum essential medium (α-MEM) (Gibco Laboratories, Grand Island, New York, USA) supplemented with 20% fetal bovine serum (Intergen, Purchase, New York, USA), 100 μg/ml penicillin and 100 mU/ml streptomycin in a 37°C humidified atmosphere of 5% CO_2. The cells were passaged with 0.05% trypsin and 0.1% ethylenediaminetetraacetic acid tetrasodium salts solution (Gibco Laboratories).

2.3. Micromass culture

Cell suspensions were prepared in the culture medium and adjusted to give 2×10^6 cells/ml. A 20 μl aliquot of the cell suspensions was delivered into each well of type I collagen coated 24-well plate (Iwaki Glass, Tokyo, Japan). After the spot-like cells were attached on the well, 1 ml of the culture medium containing 10 mM disodium β-glycerophosphate (β-GP) (Sigma Chemical Co., St. Louis, MO, USA) and 1 μl of the serially diluted PLLA solution was added. As a control, 1 μl of DMSO was added to the culture medium instead of the PLLA solution. The culture medium containing each chemical at the same concentration was changed three times a week, and the cells were cultured for 2 weeks.

2.4. Proliferation assay

The proliferation of MC3T3-E1 cells was determined by using a cell proliferation assay reagent, TetraColor ONE (Seikagaku Co., Tokyo, Japan). The cell cultures were exchanged with the culture medium containing 0.1 mM 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt, 4 μM 1-methoxy-5-methylphenazinium methylsulfate and 3 mM NaCl, and were incubated for 2 h. The absorbance of the medium was read at 450 nm (reference at 600 nm) with a plate reader (μQUANT, Bio-Tek Instruments, Inc., Winooski, VT, USA). It has been proven that the absorbance and cell population show the linear relationship.

2.5. Calcification assay

The calcium depositions of MC3T3-E1 cell cultures were stained by alizarin red S solution. The alizarin red S solution was freshly made; 0.1 ml of 28% ammonia solution in 100 ml distilled water was added to 1 g of alizarin red S in 100 ml distilled water to make it pH 6.36–6.40. After the proliferation was determined, the cell cultures were washed three times with Dulbecco's phosphate-buffered saline without calcium and magnesium salts (PBS(−)) and fixed by the addition of 10% formalin dissolved in PBS(−) solution. After fixing, the cell cultures were washed five times with distilled water and stained by alizarin red S solution for 5 min. The transmission digital images of alizarin red S stained cultures were obtained with a color image scanner (GT-9500WIN, SEIKO EPSON Co., Nagano, Japan) with a transparency unit (GT95FLU, SEIKO EPSON Co.), and then, the alizarin red S stained areas were measured using an image processing and analysis software, Scion Image (Scion Co., Frederick, MD, USA).

2.6. Preparation of cell lysates

The ALP activity and calcium content of MC3T3-E1 cells cultured with low Mw PLLA for 2 weeks were measured using the cell lysates [7]. The cell cultures were washed twice with PBS(−). The cells were recovered by trypsinization and washed twice with PBS(−) by centrifugation at 1000 rpm for 2 min. The residues were resuspended in 1 ml of 0.2% Nonidet P-40 and sonicated in an ice bath for 2 min using an ultrasonic processor (VC-50T, Sonic & Materials Inc., Danbury, CT, USA). The cell lysates were stored frozen at −20°C until measurement of the ALP activity and calcium content.

2.7. ALP activity

The ALP activity of cell lysates was measured according to the method of Ikarashi et al. [4]. The same

quantity of 2 mM $MgCl_2$ in 0.1 M carbonate buffer (pH 10.2) and 20 mM *p*-nitrophenylphosphate were mixed as the substrate solution, and then, this substrate solution was pre-incubated at 37°C. Twenty microliters of the cell lysates was incubated with 1 ml of the substrate solution at 37°C for 30 min. The enzymatic reaction was stopped by adding 2 ml of 0.25 N NaOH, and the absorbance of *p*-nitrophenol liberated was read at 410 nm. The calibration curve of ALP activity was made by the standard solutions that diluted calf intestine ALP (Boehringer Mannheim GmbH, Germany) at the various concentrations. Total protein content of cell lysates was measured by the method of Lowry et al. [8] with minor modification using bovine serum albumin (Wako Pure Chemical Industries, Ltd.) as a reference standard [5]. The ALP activity of cell lysate was normalized for total protein content of the cell lysate.

2.8. Calcium content

The calcium content of cell lysates was determined by using a diagnostic kit, Calcium C (Wako Pure Chemical Industries, Ltd.). The same quantity of the cell lysates and 1 N HCl were mixed, and decalcified for 15 h at room temperature [9]. Ten microliters of the decalcifying solution and 1.0 ml of 0.88 M monoethanolamine buffer (pH 11.0) were mixed, and 100 μl of 0.63 mM *o*-cresolphthalein complexon and 69 mM 8-hydroxyquinoline was added. After 15 min at room temperature, the absorbance of the reaction solution was read at 570 nm. The standard calcium solutions of various concentrations were also operated by the same manner in order to make the calibration curve.

2.9. Statistical analysis

All measured values were collected in four sets and expressed in means ± standard deviation (SD). Differences among the groups were evaluated with one-way or two-way analysis of variance (ANOVA). When significant differences among the groups were found, Tukey–Kramer test was applied for multiple comparisons. A *P*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Proliferation

Mouse osteoblast-like MC3T3-E1 cells were cultured with the concentration of 5–50 μg/ml of PLLA-5k or PLLA-10k for 2 weeks using the micromass culture. In microscopic observation, the proliferations of MC3T3-E1 cells cultured with PLLA-5k and PLLA-10k were almost the same as that of the control group during the culture period. Fig. 1 shows the effects of PLLA-5k and PLLA-10k on the proliferation of MC3T3-E1 cells. There was no significant difference between the proliferation of the cells cultured with and without PLLA-5k ($P = 0.7537$) and PLLA-10k ($P = 0.7521$) by one-way ANOVA. PLLA-5k and PLLA-10k up to 50 μg/ml did not affect the proliferation of MC3T3-E1 cells.

3.2. Calcification

The calcium depositions of MC3T3-E1 cell cultures were stained by alizarin red S solution (Fig. 2). Because alizarin red S combines with the calcium and forms the

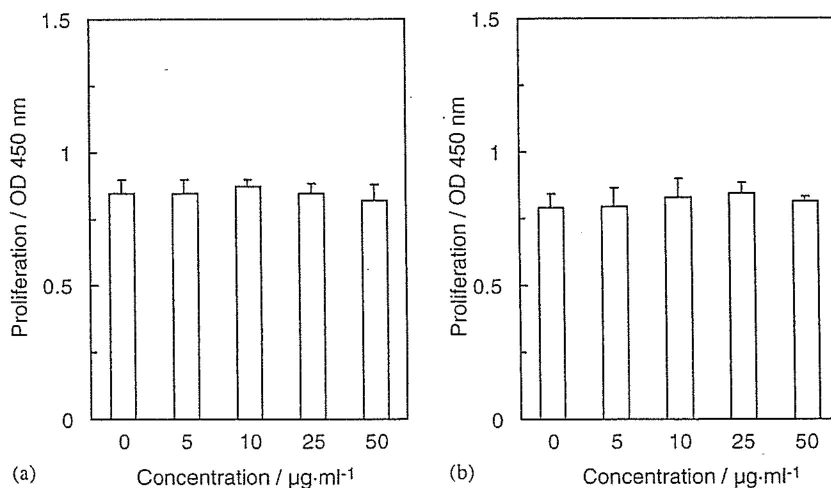

Fig. 1. The effect of PLLA on the proliferation of MC3T3-E1 cells. MC3T3-E1 cells were cultured with the concentration of 5–50 μg/ml of PLLA-5k (a) or PLLA-10k (b) for 2 weeks using the micromass culture. The proliferation of MC3T3-E1 cells cultured with the PLLA was determined using a cell proliferation assay reagent, TetraColor ONE (Seikagaku Co.). Values are means ± SD for four dishes. Significant difference at $P < 0.05$ was not found among the groups by one-way ANOVA.

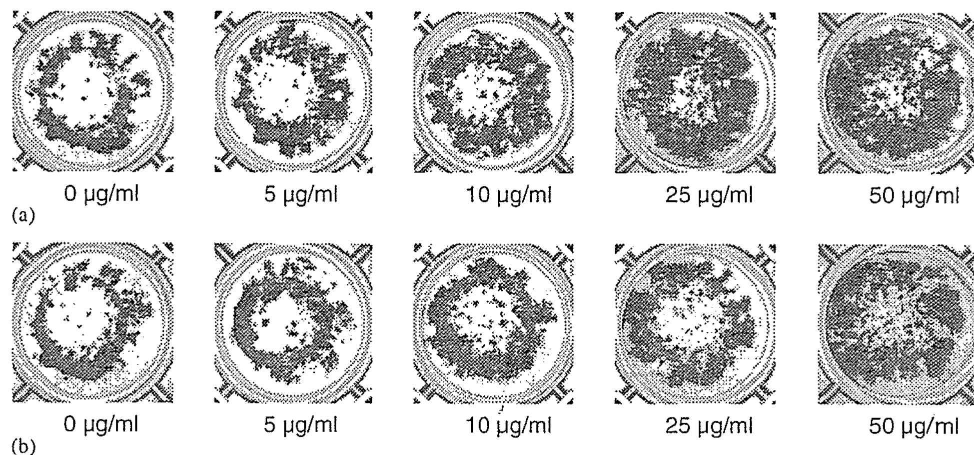

Fig. 2. The appearance of alizarin red S staining of MC3T3-E1 cells cultured with PLLA. MC3T3-E1 cells were cultured with the concentration of 5–50 μg/ml of PLLA-5k (a) or PLLA-10k (b) for 2 weeks using the micromass culture. The culture of MC3T3-E1 cells was stained by alizarin red S solution.

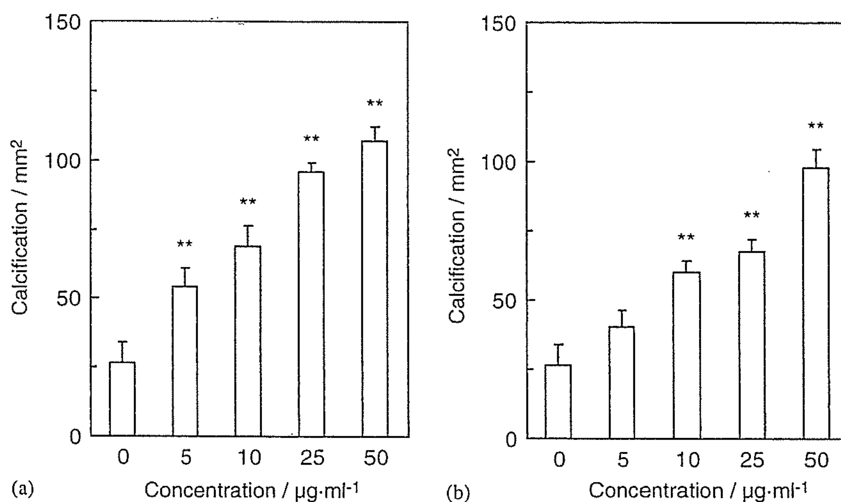

Fig. 3. The effect of PLLA on the calcification of MC3T3-E1 cells. MC3T3-E1 cells were cultured with the concentration of 5–50 μg/ml of PLLA-5k (a) or PLLA-10k (b) for 2 weeks using the micromass culture. The calcification of MC3T3-E1 cells cultured with the PLLA was determined by measuring the alizarin red S stained areas using an image processing and analysis software, Scion Image (Scion Co.). Values are means±SD for four dishes. Significant difference compared with control (without PLLA) at $^{**}P<0.01$ by Tukey–Kramer test with one-way ANOVA.

lac of poor solubility, the calcification parts in cell cultures are stained dark-red. The calcification parts clearly increased with increasing the concentrations of PLLA-5k and PLLA-10k. The total area of the calcification parts in the well was measured to determine the extent of the calcification of cell cultures. Fig. 3 shows the effect of PLLA-5k and PLLA-10k on the calcification of MC3T3-E1 cells. The calcification of MC3T3-E1 cells cultured with PLLA-5k increased 2.1-fold at 5 μg/ml and increased 4-fold at 50 μg/ml (Fig. 3(a)), and then the calcifications of the cells were significantly increased with increasing the concentrations of the PLLA-5k by one-way ANOVA ($P<0.0001$). On the other hand, the calcification of MC3T3-E1 cells cultured with PLLA-10k increased 1.5- and 3.7-fold at 5 and 50 μg/ml, respectively (Fig. 3(b)), and then the calcifications of the cells were also significantly increased with increasing the concentrations of the PLLA-10k by one-way ANOVA ($P<0.0001$). PLLA-5k and PLLA-10k stimulated the differentiation of MC3T3-E1 cells cultured with the PLLAs, dose-dependently ($A = 11.86\ \mathrm{sqrt}(C_{5k}) + 28.99$, $r = 0.9880$, $P = 0.0016$, A: total area of calcification parts (mm^2), C_{5k}: concentration of PLLA-5k (μg/ml); $A = 9.93\ \mathrm{sqrt}(C_{10k}) + 23.73$, $r = 0.9807$, $P = 0.0032$, C_{10k}: concentration of PLLA-10k (μg/ml)). Moreover, when it was compared at the weight concentration of PLLA, PLLA-5k increased the calcification of MC3T3-E1 cells more than PLLA-10k significantly ($P = 0.0075$ by two-way ANOVA).

3.3. *ALP activity and calcium content*

ALP is a representative enzyme of osteoblastic differentiation, and then ALP activity was determined

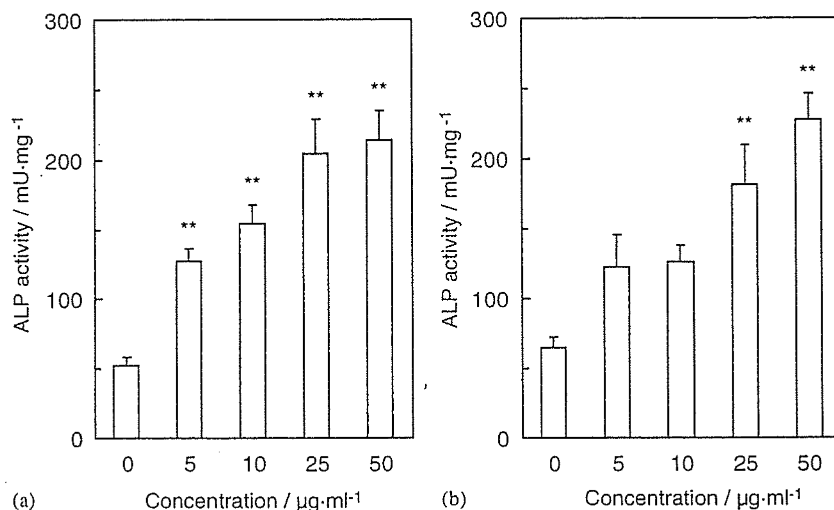

Fig. 4. The ALP activity of MC3T3-E1 cells cultured with PLLA. MC3T3-E1 cells were cultured with the concentration of 5–50 µg/ml of PLLA-5k (a) or PLLA-10k (b) for 2 weeks using the micromass culture. The ALP activity of cell lysate of MC3T3-E1 cells cultured with the PLLA was determined using p-nitrophenylphosphate as a substrate. Values are means ± SD for four dishes. Significant difference compared with control (without PLLA) at $^{**}P<0.01$ by Tukey–Kramer test with one-way ANOVA.

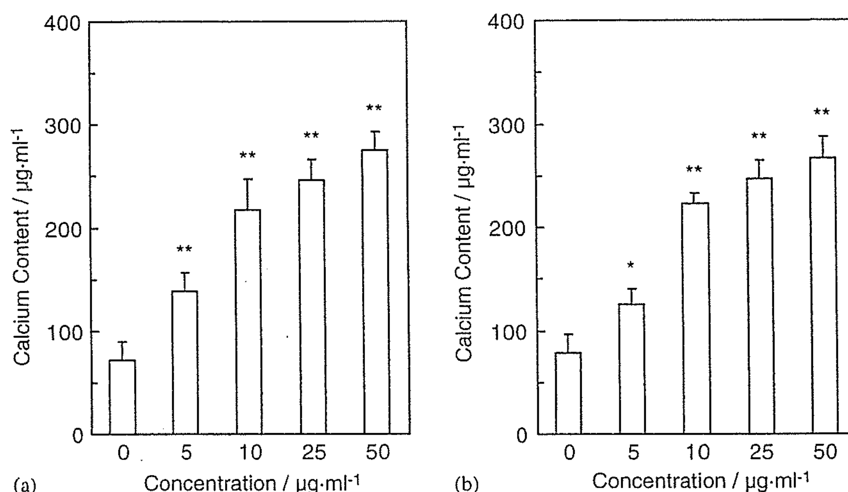

Fig. 5. The calcium content of MC3T3-E1 cells cultured with PLLA. MC3T3-E1 cells were cultured with the concentration of 5–50 µg/ml of PLLA-5k (a) or PLLA-10k (b) for 2 weeks using the micromass culture. The calcium content of cell lysate of MC3T3-E1 cells cultured with the PLLA was determined using a diagnostic kit, Calcium C (Wako Pure Chemical Industries, Ltd.). Values are means ± SD for four dishes. Significant difference compared with control (without PLLA) at $^{*}P<0.05$ or $^{**}P<0.01$ by Tukey–Kramer test with one-way ANOVA.

as an indicator of osteoblastic differentiation of MC3T3-E1 cells cultured with PLLA-5k or PLLA-10k. Fig. 4 shows the effects of PLLA-5k and PLLA-10k on the ALP activity of MC3T3-E1 cells. ALP activities of the cells cultured with the PLLA-5k ($P<0.0001$) and PLLA-10k ($P<0.0001$) significantly increased with increasing the concentrations of the PLLAs by one-way ANOVA.

Calcification is also an important indicator of osteoblastic differentiation, and the calcium content was determined in order to verify the result of the above calcification assay. Fig. 5 shows the effects of PLLA-5k and PLLA-10k on the calcium content of MC3T3-E1 cells. Calcium contents of the cells cultured with the PLLA-5k ($P<0.0001$) and PLLA-10k ($P<0.0001$) significantly increased with increasing the concentrations of the PLLAs by one-way ANOVA. The results of calcium content completely agreed with the results of calcification assay of the cells cultured with PLLA-5k and PLLA-10k.

4. Discussion

The implanted PLLA nonenzymatically hydrolyzes in vivo, and the Mw of the PLLA decreases. Bergsma et al. determined the PLLA particles with number average molecular weight (Mn) of 5600 and 5400 at 3.3 and 5.7

Table 1
The comparison among the cell responses of MC3T3-E1 cells cultured with PLLA

Sample	Concentration (μg/ml)	Proliferation (%)	Calcification (%)	ALP activity (%)	Calcium content (%)
PLLA-5k	0	100	100	100	100
	5	100	205	241	193
	10	103	260	293	302
	25	100	361	388	343
	50	97	404	407	384
PLLA-10k	0	100	100	100	100
	5	100	152	188	161
	10	105	227	193	285
	25	106	254	277	316
	50	103	369	349	341

The ratios of the proliferation, calcification, ALP activity and calcium content of MC3T3-E1 cells cultured with PLLA-5k or PLLA-10 to the control (without PLLA) were calculated.

years, respectively, after the implantation of PLLA plates or screws (Mn of 760,000) into patients [10]. Bos et al. reported that the Mw of PLLA rapidly decreased in the first 12 weeks, but the mass loss of the PLLA continued after 78 weeks, after the subcutaneous implantation of PLLA (viscosity average molecular weight (Mv) of 900,000) into rats [1]. Mainil-Varlet et al. also observed that the weight average Mw of PLLA implanted into the cortex of sheep tibiae decreased from 61,900 to 42,600 at 4 weeks, to 20,800 at 12 weeks and to 9206 at 52 weeks [3]. Kinoshita et al. reported that the degradation and absorption of PLLA mesh (Mw of 200,000) implanted subcutaneously in the back and subperiosteally at the calvaria of rats continued after 30 months, and then many macrophages appeared in the circumferential tissue of hydrolyzed PLLA particles [11]. The purpose of the present study was to clarify the effects of low Mw PLLA, which would be produced by degradation, on the proliferation and differentiation of mouse osteoblasts in vitro.

In the present experiment, we added 10 mM β-GP to the α-MEM medium, and MC3T3-E1 cells were micromass cultured with low Mw PLLA. β-GP displays synergistic action with ascorbic acid to further stimulate collagen accumulation and ALP activity in osteoblasts, and mature osteoblasts require β-GP for mineralization [12]. Moreover, Quarles et al. also reported that MC3T3-E1 cells actively proliferated before attaining confluence, but failed to express ALP activity and did not accumulate mineralized extracellular collagenous matrix at this stage. After the cultures underwent growth arrest owing to the attainment of confluence, ALP activity and mineralized extracellular collagenous matrix were expressed [12]. The cell density of the micromass culture is extremely high, and the situation of the micromass culture is similar to state of confluence from initial stage of culture. Ikarashi et al. cultured MC3T3-E1 cells in the α-MEM without β-GP on the monolayer, and observed the calcification of MC3T3-E1 cells by alizarin red S staining after the culture as long as 4 weeks [6]. However, we cultured MC3T3-E1 cells in the α-MEM with β-GP using the micromass culture, and succeeded in the detection of the calcification of MC3T3-E1 cells in the short period of 2 weeks (Fig. 2).

The osteogenesis is the most important phenotype of the osteoblasts and it has been confirmed that MC3T3-E1 cells form the calcified bone tissue in vitro [13,14]. Therefore, the calcification was an index of the final differentiation of MC3T3-E1 cells for the present experiment. PLLA-5k and PLLA-10k did not affect the proliferation of MC3T3-E1 cells cultured with the PLLAs (Fig. 1). On the other hand, the calcification of MC3T3-E1 cells cultured with the PLLAs increased with increasing the concentration of the PLLAs dose-dependently (Fig. 3). These results indicate that the low Mw PLLA stimulated the differentiation of MC3T3-E1 cells cultured with the PLLA with no effect on the proliferation. Ikarashi et al. also observed that the poly(DL-lactide) (PDLLA) with weight average Mw of 5000 and 10,000 did not affect the proliferation, but remarkably increased the calcification of MC3T3-E1 cells [6].

The cell responses of MC3T3-E1 cells cultured with the PLLAs were compared (Table 1). As described above, the PLLAs did not effect on the proliferation of MC3T3-E1 cells, but the PLLAs stimulated the calcification of MC3T3-E1 cells. The calcification of MC3T3-E1 cells cultured with 50 μg/ml of PLLA-5k and PLLA-10k increased approximately 4- and 3.7-fold, respectively. ALP activity and calcium content of MC3T3-E1 cells cultured with the PLLAs also increased to the similar level to the calcification. Thompson and Puleo observed that the ALP activity, osteocalcin content and calcium amount of bone marrow stromal cells greatly rose in the later stage of culture. They indicated that the osteoprogenitor cells first differentiated into immatute osteoblasts characterized by the expression of ALP and then into mature osteoblasts characterized by the

expression of osteocalcin and calcification [15]. The increase rates of ALP activity and calcification of MC3T3-E1 cells cultured with the PLLAs were almost equal in the present results. As the ALP activity increasing during the process of culture with PLLAs, the calcification of MC3T3-E1 cells would be stimulated. Otto et al. reported when mouse primary osteoblasts were cultured with the PLLA (Mv of 21,500) wire for 48 h, DNA content did not change, but ALP activity increased by 28% [16]. Ikarashi et al. also observed that the ALP activity of MC3T3-E1 cells remarkably increased with no effect on the proliferation, when the cells were cultured on the PLLA (weight average Mw of 20,000) and with the PDLLA for 2 weeks [6]. Our results corresponded to their results.

Ikarashi et al. reported that heat treatment decreased the Mw of PLLA, and then the heat treated PLLA did not effect the proliferation, but increased the differentiation of MC3T3-E1 cells cultured on the PLLA [4]. Isama et al. also reported that the Mw of PLLA was decreased by γ-irradiation, and then the irradiated PLLA increased the differentiation of MC3T3-E1 cells cultured on the PLLA with no effect the proliferation [5]. They describe that lower change in the Mw of PLLA would be responsible for enhancing the differentiation of MC3T3-E1 cells cultured on the heat irradiated or γ-irradiated PLLA. The present result that the low Mw PLLA enhanced the differentiation of MC3T3-E1 cells strongly supports their proposal.

We examined the effects of the low Mw PLLA only in the mouse osteoblat-like MC3T3-E1 cells. The MC3T3-E1 cells are the most widely used for the research of the bone formation. However, culture osteoblasts show various phenotypes according to cell lines [13]. It would be required that further studies to analyze the effect of PLLA on other osteoblasts, such as human osteoblasts if considering clinical use of PLLA.

In conclusion, the effects of low Mw PLLA on the proliferation and differentiation of mouse osteoblast-like MC3T3-E1 cells were investigated. The PLLA did not affect the proliferation of MC3T3-E1 cells. However, the ALP activity and calcification of MC3T3-E1 cells increased with increasing the concentration of the PLLA dose-dependently. These results indicated that the low Mw PLLA enhances the differentiation of MC3T3-E1 cells. Moreover, the increase rates of ALP activity and calcification of MC3T3-E1 cells cultured with the PLLA were almost equal. The increase of the ALP activity would be a critical step to stimulate the calcification of MC3T3-E1 cells. The present findings show that the osteoconductivity of implanted PLLA is based on the enhancing effect of low Mw PLLA on the differentiation of osteoblasts.

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Neural differentiation of midbrain cells on various protein-immobilized polyethylene films

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Abstract: The effect of surface modification of polyethylene (PE) film on differentiation of midbrain (MB) cells obtained from rat embryos was determined by their micromass culture system. When cultured on untreated PE film, cell differentiation was suppressed to approximately two-thirds of that observed in a control culture dish. On the contrary, type I collagen-immobilized PE film increased differentiated foci of the MB cells more than did the untreated PE film. RGDS (Arg-Gly-Asp-Ser) peptide immobilization onto PE film resulted in almost the same differentiation activity as the collagen immobilized PE film. Bovine serum albumin (BSA) immobilization onto PE film enhance the differentiation activity more than did the untreated PE film, but not up to the levels of collagen- and RGDS-immobilized PE. The number of differentiated foci of the MB cells on untreated PE film were increased by the addition of the condition medium prepared from the collagen-immobilized PE film. However, the number of foci was not increased by the addition of other condition media obtained from control dish, untreated, BSA-, and RGDS-immobilized PE. On the other hand, none of these condition media enhanced a differentiation of the neuronal cell line of PC12 cells, suggesting that some factors effectively differentiate midbrain cells, composed of neuronal epithelial and mesenchymal cells, but not the PC12 cells secreted in the condition media prepared from collagen-immobilized PE. In addition, it is probable that neural growth factor was not secreted in these condition media, which could not induce the differentiation of PC12 cells. © 2003 Wiley Periodicals, Inc. J Biomed Mater Res 64A: 439–446, 2003

Key words: midbrain cells; cell differentiation; micromass culture system; surface modification; collagen

INTRODUCTION

Implantation of biomaterials still induces many side effects, although recent advances in technology have made it possible to improve biomaterials more safely and effectively. Undesirable inflammatory reactions were often observed around the biomaterials after their implantation, resulting in not only the loss of their expected function but also severe side effects for the patients. For studies on interactions between the biomaterials and the body, in other words, many types of cells should be carefully investigated *in vitro*. The effect of the materials on cell proliferation, differentiation, and function must be estimated in order to improve biomaterials that can be applied to humans. Previously, we reported that when cells were cultured on a polyethylene film, gap-junctional intercellular communication (GJIC) of cells, which is an important function to maintain cell and tissue homeostasis, was inhibited.

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Furthermore, the inhibitory level decreased when the surface of the film was modified with collagen.[1] Thus, we suggested that surface modification of biomaterial is one way to diminish undesirable effects on cell and tissue homeostasis. However, it is not clear whether cell differentiation function can be improved with recovery of the GJIC by a surface characteristic of the biomaterial. It is important to design a surface characteristic suitable for normal cell differentiation in order to develop biomaterials, such as a biocompatible scaffold, for tissue engineering. To clarify the effect of biomaterial surface characteristics on cell differentiation and the relationship of biomaterial effects on differentiation and GJIC, we applied a midbrain (MB) micromass culture to estimate the effect. The MB micromass culture system is a convenient *in vitro* assay originally developed for *in vitro* teratogenicity tests.[2] In addition, it has already been suggested that the differentiation level of the MB cells might be related to GJIC inhibitory activity of the biomaterial.[3] In this study, we examined the effect of surfaces modified with various kinds of proteins on cell differentiation under *in vitro* experimental conditions to clarify suitable surface characteristics for producing excellent biomaterials.