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Pyridinium Lipids with the Dodecaborate Cluster as Polar Headgroup: Synthesis, Characterization of the Physical−Chemical Behavior, and Toxicity in Cell Culture

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We have prepared nine new dodecaborate cluster lipids with potential use in boron neutron capture therapy of tumors. This new generation of boron lipids is only singly negatively charged and consists of a pyridinium core with C_{12}, C_{14}, and C_{16} chains as lipid backbone, connected through the nitrogen atom via a butylene, pentylene, or ethyleneoxyethylene linker to the oxygen atom on the dodecaborate cluster as headgroup. The lipids were obtained by nucleophilic attack of 4-(bisalkylmethyl)pyridine on the tetrahydrofurane, the dioxane, and a newly prepared tetrahydropyrane derivative, respectively, of *closo*-dodecaborate. All of these boron lipids are able to form closed vesicles in addition to some bilayers in the pure state and in the presence of helper lipids. The thermotropic behavior was found to be increasingly complex and polymorphic with increasing alkyl chain length. Except for two lipids, all lipids have low *in vitro* toxicity, and longer alkyl chains lead to a significant decrease in toxicity. The choice of the linker plays no major role with respect to their ability to form liposomes and their thermotropic properties, but the toxicity is influenced by the linkers in the case of short alkyl chains.

INTRODUCTION

Therapy for patients with malignant neoplasms, especially high-grade gliomas, melanomas, and their metastatic manifestations, has been only marginally successful with conventional treatments such as surgery or chemotherapy (*1*−*3*). Boron neutron capture therapy (BNCT) is focused on the treatment of these types of cancer. The therapy is based on the nuclear reaction that occurs when boron-10 is irradiated with thermal neutrons followed by nuclear fission to high-energy α-particles and lithium-7 nuclei. These products only act in a short range (cell diameter), which offers a selective damaging of the cancer cells if the boron-10 is selectively accumulated in the tumor and not in the surrounding healthy tissue. For successful treatment, a high amount of boron is necessary (10^9 B atoms per cell, or 20−30 μg per gram of tumor).

Only two substances are in clinical trials, boronophenylalanine (BPA) and mercaptododecaborate (BSH), but they do not reach "ideal" boron concentration in the tumor (*2*) and are hence not optimal boron delivery agents.

In the past, many prospective boron agents have been synthesized, but most of them show disqualifying features (*4*). Low molecular weight boron-containing nucleosides and nucleotides have been prepared for targeting hyperproliferating malignant cells (*5, 6*). Sugar derivatives are synthesized for cell uptake via specific transporters into cells with increased metabolism (*7*). Another strategy is to obtain boron-containing agents for which target receptors are present in the plasma membrane of the tumor cell, possibly followed by translocation into the cell (*4*). Porphyrin and phthalocyanine derivatives (*8*−*12*) are a part of this category; they often exhibit high toxicity, however. High molecular weight boronated agents, such as antibodies and their fragments, have been prepared that can recognize tumor-associated epitopes (*13*−*15*). Their rapid clearance by the reticuloendothelial system and the necessity to couple enough boron to an antibody molecule for successful therapy are, however, disadvantageous.

Liposomes are promising transport systems for boron agents, as they can carry a large amount of boron. In addition, targeting with tumor-seeking entities, e.g., folate (*16*), EGF (*17*), or transferrin (*18*), makes selective transfer possible. The encapsulation of low molecular weight boron agents into the inner liposomal core has been studied in a variety of experimental systems (*19, 20*). For effective delivery of boron, low encapsulation efficiency, leakage upon storage or in contact with serum and the effect of the liposome structure in the presence of charged boron clusters (*21*) are disadvantages. These problems can be avoided by incorporating boron-containing lipids directly into liposomal membranes.

The first dodecaborate ether lipid has been described by Lee et al. (*22*). The subsequently published dodecaborate lipids have BSH as the headgroup (*23, 24*). These lipids show similarly low toxicity to BSH. All these boron lipids are doubly negatively charged.

Recently, we published the first dodecaborate cluster lipids with only one negative charge (*25*). The chemical structure of these boron lipids are shown in Scheme 1.

The new boron lipids in this study also have a single negative charge. We used ring-opening reactions of different oxonium derivatives of the dodecaborate cluster with *p*-bisalkylmethylpyridine as nucleophile. This allows facile preparation and the possibility to vary the structure of the hydrophobic part of the lipid. Commonly, *N*-methyl-*p*-bisalkylmethylpyridinium is

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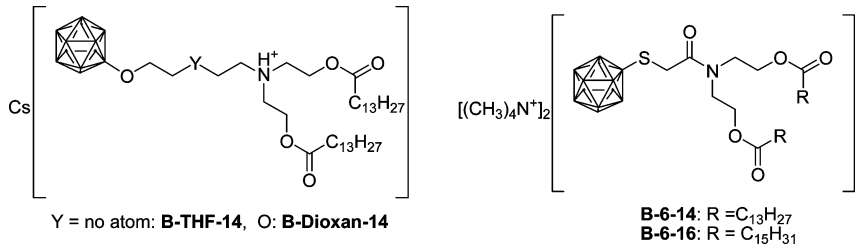
Scheme 1. Structures of the Boron Lipids: B-THF-14 and B-Dioxan-14 (left) (*25*) as well as B-6-14 and B-6-16 (right) (*24*)[a]



Y = no atom: **B-THF-14**, O: **B-Dioxan-14**

B-6-14: R = $C_{13}H_{27}$
B-6-16: R = $C_{15}H_{31}$

[a] In the icosahedral structures, each unsubstituted corner represents a B−H unit, and the substituted corner, a B atom. The cluster carries two negative charges which are omitted for clarity.

applied in lipoplexes (so-called SAINTs (synthetic amphiphiles for interdisciplinary nucleic acid therapy)) for gene therapy (*26*−*28*). We adapted this basic structure for the first time for boron lipids with potential use in BNCT.

EXPERIMENTAL PROCEDURES

General. A Bruker Esquire spectrometer was used for electrospray mass spectrometry. For boron-containing compounds, the peak with the highest intensity is given. To identify the charge of the compound isotope satellite peaks were used. NMR spectra were recorded on a Bruker DPX 200 spectrometer. IR spectra were collected on a BioRad FTS 155 using KBr pellet. Melting points were measured on a Büchi 512 melting point apparatus. Purity of the compounds was assessed by ESI mass spectrometry and ^{1}H-, ^{13}C-, and ^{11}B NMR spectroscopy, as it is known that the elemental analysis of dodecaborate containing compounds is not reliable (*29, 30*).

Chemistry. *1-Tetramethylene-(3-oxa)-oxonium-closo-undecahydrododecaborate (−1), Tetrabutylammonium Salt (***1a***).* The convenient method recently described was used, in which closo-dodecaborate was reacted with hydrogen chloride in 1,4-dioxane in the presence of $NaBF_4$ (*31*).

*1-Tetramethyleneoxonium-closo-undecahydrododecaborate (−1), Tetrabutylammonium Salt (***1b***).* The same method as for **1a** was used, but replacing hydrogen chloride with *p*-toluenesulfonic acid (2 equiv) and tetrahydrofurane as solvent. The compound was identical to that described by Sivaev et al. (*32*).

*1-Pentamethyleneoxonium-closo-undecahydrododecaborate (−1), Tetrabutylammonium Salt (***1c***).* The same method as for **1b** was used, but the solvent was tetrahydropyrane instead of tetrahydrofurane. Yield 70%, mp 159 °C (lit. mp 159 °C (*33*). MS (ESI, acetonitrile, *m*/*z*) negative 227.1 [A$^-$], 695.6 [2 A$^-$ + N(n-$C_4H_9)_4^+$], 141.1 [$B_{12}H_{11}^-$]; positive 242.4 [N(n-$C_4H_9)_4^+$]. ^{1}H NMR (200 MHz, [D3] CD_3CN, 25 °C, TMS): δ = 1.32 (t, 12 H, −C*H*$_3$), 1.54 (m, 10 H, N$^+$−CH$_2$−CH$_2$−C*H*$_2$-, -O$^+$−CH$_2$−CH$_2$−C*H*$_2$-), 1.59 (m, 8 H, -N$^+$−CH$_2$−C*H*$_2$-), 1.64 (m, 8 H, -O$^+$−CH$_2$−C*H*$_2$-), 3.07 (m, 8 H, -N$^+$−C*H*$_2$-), 4.50 (t, 4 H, -O$^+$−C*H*$_2$-). ^{13}C NMR (200 MHz, [D3] CD_3CN, 25 °C, TMS): δ = 13.25, 19.76, 20.70, 23.74, 25.07, 58.76, 82.54. ^{11}B NMR (200 MHz, [D3] CD_3CN, 25 °C): −19.79 (1 B), −17.00 (10 B), 8.86 (1 B). IR (KBr): ν = 2962 (−C−H), 2873 (−C−H), 2492 (B−H), 1469 (−C−H), 1045 (−C−O−C−).

*4-(Bisdodecylmethyl)pyridine (***2a***), 4-(Bistetradecylmethyl)pyridine (***2b***), and 4-(Bishexadecylmethyl)pyridine (***2c***)* were synthesized as described by Meekel et al. *(34).*

General method for preparation of dodecaborate derivatives of SAINT lipids. **2** (0.42 g, 1 mmol) was suspended in 30 mL of dry acetonitrile and **1** (0.66 mmol) was added. The reaction mixture was refluxed for 4 days. The solvent was evaporated and the residue dissolved in methanol. After addition of cesium fluoride (0.15 g, 1 mmol) dissolved in methanol, a white precipitate as product was formed and filtered off. It was dried in oil pump vacuum.

4-(Bisdodecylmethyl)pyridinio-N-butoxy-undecahydro-closo-dodecaborate (−1), Cesium Salt (THF-SAINT-12). **2a** and **1b** were used. Yield 0.33 g (65%). MS (ESI, acetonitrile, *m*/*z*) negative 642.5 [A$^-$]; positive 132.8 [Cs$^+$]. ^{1}H NMR (200 MHz, [D6] DMSO, 25 °C, TMS): δ = 0.82 (t, 6H, −C*H*$_3$), 1.19 (m, 42 H, -(C*H*$_2$)$_{10}$CH$_3$, −O−CH$_2$−C*H*$_2$-), 1.62 (m, 6H, −C*H*$_2$−(C*H*$_2$)$_{10}$CH$_3$, −O−C*H*$_2$-), 1.91 (m, 2H, −C*H*$_2$−CH$_2$−N$^+$-), 2.86 (m, 1H, −C*H*-), 4.67 (m, 2H, −C*H*$_2$−N$^+$-), 7.93 (d, 2H, -C═C*H*-), 9.01 (d, 2H, -N$^+$═CH−). ^{13}C NMR (200 MHz, [D6] DMSO, 25 °C, TMS): δ = 14.84, 22.99, 27.56, 29.86, 30.79, 32.17, 35.65, 45.78, 60.25, 68.83, 127.39, 145.59, 166.68. ^{11}B NMR (200 MHz, [D6] DMSO, 25 °C): −23.28 (1 B), −17.24 (10 B), 6.62 (1 B). IR (KBr): ν = 2925 (−C−H), 2854 (−C−H), 2477 (−B−H), 1641 (-C═C−), 1468 (−C−H), 1159 (−C−N-), 1056 (−B−O−C-), 721 (═C−H).

4-(Bisdodecylmethyl)pyridinio-N-ethoxy-ethoxy-undecahydro-closo-dodecaborate (−1), Cesium Salt (Dioxan-SAINT-12). **2a** and **1a** were used. Yield 70%. MS (ESI, acetonitrile, *m*/*z*) negative 658.6 [A$^-$]; positive 132.9 [Cs$^+$]. ^{1}H NMR (200 MHz, [D6] DMSO, 25 °C, TMS): δ = 0.82 (t, 6H, −C*H*$_3$), 1.19 (m, 40H, -(C*H*$_2$)$_{10}$−CH$_3$), 1.61 (m, 4H, −C*H*$_2$−(CH$_2$)$_{10}$−CH$_3$), 2.86 (1 H, −C*H*-), 3.41 (m, 2H, B−O−C*H*$_2$-), 3.53 (m, 2H, −CH$_2$−C*H*$_2$−O−), 3.84 (m, 2H,−O−C*H*$_2$−CH$_2$−N$^+$-), 4.73 (m, 2H,-C*H*$_2$−N$^+$-), 7.92 (d, 2H, -C═C*H*-), 9.09 (d, 2H, -N$^+$═CH−). ^{13}C NMR (200 MHz, [D6] DMSO, 25 °C, TMS): δ = 13.96, 22.10, 26.70, 29.00, 31.28, 34.76, 44.90, 59.52, 67.27, 68.73, 72.63, 126.26, 143.35, 165.76. ^{11}B NMR (200 MHz, [D6] DMSO, 25 °C): −22.96 (1 B), −17.42 (10 B), 6.63 (1 B). IR (KBr): ν = 2919 (−C−H), 2852 (−C−H), 2478 (−B−H), 1643 (-C═C−), 1471 (−C−H), 1059 (−B−O−C-, −C−O−C-), 719 (═C−H).

4-(Bisdodecylmethyl)pyridinio-N-pentoxy-undecahydro-closo-dodecaborate (−1), Cesium Salt (Pyran-SAINT-12). **2a** and **1c** were used. Yield: 50%. MS (ESI, acetonitrile, *m*/*z*) negative 656.6 [A$^-$]; positive 132.8 [Cs$^+$]. ^{1}H NMR (200 MHz, [D6] DMSO, 25 °C, TMS): δ = 0.82 (t, 6H, −C*H*$_3$), 1.19−1.38 (m, 44H, -(C*H*$_2$)$_{10}$−CH$_3$, −O−CH$_2$−(C*H*$_2$)$_2$-), 1.61 (m, 6H, −C*H*$_2$−(CH$_2$)$_{10}$−CH$_3$, −C*H*$_2$−CH$_2$−N$^+$-), 1.89 (m, 2H, −O−C*H*$_2$-), 2.85 (m, 1H, −C*H*-), 4.53 (m, 2H, −CH$_2$−C*H*$_2$−N$^+$-), 7.97 (d, 2H, -C═C*H*-), 9.00 (d, 2H, -N$^+$═CH−). ^{13}C NMR (200 MHz, [D6] DMSO, 25 °C, TMS): δ = 14.10, 22.24, 26.76, 29.12, 30.61, 31.42, 34.87, 45.04, 60.03, 67.55, 126.88, 144.45, 166.04. ^{11}B NMR (200 MHz, [D6] DMSO, 25 °C): −22.99 (1 B), −17.42 (10 B), 6.60 (1 B). IR (KBr): ν = 3056 (═C−H), 2942 (−C−H), 2854 (−C−H), 2473 (−B−H), 1642 (-C═C−), 1468 (−C−H), 1160 (−C−N-), 1055 (−B−O−C-), 722 (═C−H).

4-(Bistetradecylmethyl)pyridinio-N-butoxy-undecahydro-closo-dodecaborate (−1), Cesium Salt (THF-SAINT-14). **2b** and **1b** were used. Yield 60%. MS (ESI, acetonitrile, *m*/*z*) negative 698.8 [A$^-$]; positive 132.9 [Cs$^+$]. ^{1}H NMR (200 MHz, [D6] DMSO, 25 °C, TMS): δ = 0.82 (t, 6H, −C*H*$_3$), 1.20 (m, 50 H, -(C*H*$_2$)$_{12}$CH$_3$, −O−CH$_2$−C*H*$_2$-), 1.61 (m, 6H, −C*H*$_2$−(CH$_2$)$_{12}$CH$_3$, −O−C*H*$_2$-), 1.91 (m, 2H, −C*H*$_2$−CH$_2$−N$^+$-), 2.86

(m, 1H, −C*H*-), 4.67 (m, 2H, −CH_2−N^+-), 7.93 (d, 2H, -C═C*H*-), 9.01 (d, 2H, -N^+═CH−). ^{13}C NMR (200 MHz, [D6] DMSO, 25 °C, TMS): δ = 13.93, 22.10, 26.65, 29.03, 29.93, 31.31, 34.73, 44.87, 59.30, 67.92, 126.46, 144.73, 165.76. ^{11}B NMR (200 MHz, [D6] DMSO, 25 °C): −22.52 (1 B), −17.86 − (−16.83) (10 B), 6.66 (1 B). IR (KBr): ν = 2924 (−C−H), 2854 (−C−H), 2477 (−B−H), 1642 (-C═C−), 1468 (−C−H), 1157 (−C−N-), 1056 (−B−O−C-), 721 (═C−H).

4-(Bistetradecylmethyl)pyridinio-N-ethoxy-ethoxy-undecahydro-closo-dodecaborate (−1), Cesium Salt (Dioxan-SAINT-14). **1a** and **2b** were used. Yield 60%. MS (ESI, acetonitrile, *m/z*) negative 714.8 [A^-]; positive 132.9 [Cs^+]. ^{1}H NMR (200 MHz, [D6] DMSO, 25 °C, TMS): δ = 0.82 (t, 6H, −CH_3), 1.2 (m, 48H, -(CH_2)$_{12}$−CH_3), 1.61 (m, 4H, −CH_2−(CH_2)$_{12}$−CH_3), 2.86 (1 H, −C*H*-), 3.40 (m, 2H, B−O−CH_2-), 3.54 (m, 2H, −CH_2−CH_2−O−), 3.84 (m, 2H,−O−CH_2−CH_2−N^+-), 4.73 (m, 2H,-CH_2−N^+-), 7.91 (d, 2H, -C═C*H*-), 9.10 (d, 2H, -N^+═CH−). ^{13}C NMR (200 MHz, [D6] DMSO, 25 °C, TMS): δ = 14.82, 22.99, 27.56, 29.9, 32.19, 35.59, 45.73, 60.41, 68.16, 69.65, 73.52, 127.15, 146.24, 166.62. ^{11}B NMR (200 MHz, [D6] DMSO, 25 °C): −22.67 (1 B), −17.65 − (−16.68) (10 B), 6.93 (1 B). IR (KBr): ν = 3059 (═C−H), 2924 (−C−H), 2853 (−C−H), 2473 (−B−H), 1643 (-C═C−), 1468 (−C−H), 1167 and 1109 (−C−N-), 1057 and 1027 (−B−O−C-, −C−O−C-), 722 (═C−H).

4-(Bistetradecylmethyl)pyridinio-N-pentoxy-undecahydro-closo-dodecaborate (−1), Cesium Salt (Pyran-SAINT-14). **1c** and **2b** were used. Yield 45%. MS (ESI, acetonitrile, *m/z*) negative 712.9 [A^-]; positive 132.8 [Cs^+]. ^{1}H NMR (200 MHz, [D6] DMSO, 25 °C, TMS): δ = 0.84 (t, 6H, −CH_3), 1.20−1.40 (m, 52H, -(CH_2)$_{12}$−CH_3, −O−CH_2−(CH_2)$_2$-), 1.63 (m, 6H, −CH_2−(CH_2)$_{12}$−CH_3, −CH_2−CH_2−N^+-), 1.91 (m, 2H, −O−CH_2-), 2.86 (m, 1H, −C*H*-), 4.55 (m, 2H, −CH_2−N^+-), 7.96 (d, 2H, -C═C*H*-), 9.02 (d, 2H, -N^+═CH−). ^{13}C NMR (200 MHz, [D6] DMSO, 25 °C, TMS): δ = 14.10, 22.24, 26.70, 29.15, 30.63, 31.45, 34.79, 44.98, 60.03, 67.55, 126.88, 144.45, 166.04. ^{11}B NMR (200 MHz, [D6] DMSO, 25 °C): −22.67 (1 B), −17.65 − (−16.68) (10 B), 6.93 (1 B). IR (KBr): ν = 3056 (═C−H), 2942 (−C−H), 2854 (−C−H), 2473 (−B−H), 1642 (-C═C−), 1468 (−C−H), 1160 (−C−N-), 1055 (−B−O−C-), 722 (═C−H).

4-(Bishexadecylmethyl)pyridinio-N-butoxy-undecahydro-closo-dodecaborate (−1), Cesium Salt (THF-SAINT-16). **2c** and **1b** were used. Yield 50%. MS (ESI, acetonitrile, *m/z*) negative 754.9 [A^-]; positive 132.8 [Cs^+]. ^{1}H NMR (200 MHz, [D6] DMSO, 25 °C, TMS): δ = 0.82 (t, 6H, −CH_3), 1.20 (m, 58H, -(CH_2)$_{14}$−CH_3, −O−CH_2−CH_2-), 1.62 (m, 6H, −CH_2−(CH_2)$_{14}$$CH_3$, −O−C$H_2$-), 1.92 (m, 2H, −C$H_2$−$CH_2$−$N^+$-), 2.86 (m, 1H, −C*H*-), 4.68 (m, 2H, −CH_2−N^+-), 7.92 (d, 2H, -C═C*H*-), 9.01 (d, 2H, -N^+═CH−). ^{13}C NMR (200 MHz, [D1] $CDCl_3$, 25 °C, TMS): δ = 13.98, 22.13, 26.65, 29.03, 30.72, 31.31, 34.73, 44.84, 59.35, 67.95, 126.46, 144.46, 149.34. ^{11}B NMR (200 MHz, [D6] DMSO, 25 °C): −19.82 (1 B), −17.00 (10 B), 7.13 (1 B). IR (KBr): ν = 2920 (−C−H), 2853 (−C−H), 2475 (−B−H), 1641 (-C═C−), 1468 (−C−H), 1160 (−C−N-), 1059 (−B−O−C-), 720 (═C−H).

4-(Bishexadecylmethyl)pyridinio-N-ethoxy-ethoxy-undecahydro-closo-dodecaborate (−1), Cesium Salt (Dioxan-SAINT-16). **1a** and **2c** were used. Yield. 60% MS (ESI, acetonitrile, *m/z*) negative 770.9 [A^-]; positive 132.9 [Cs^+]. ^{1}H NMR (200 MHz, [D6] DMSO, 25 °C, TMS): δ = 0.82 (t, 6H, −CH_3), 1.20 (m, 56H, -(CH_2)$_{14}$−CH_3), 1.61 (m, 4H, −CH_2−(CH_2)$_{14}$−CH_3), 2.86 (1 H, −C*H*-), 3.40 (m, 2H, B−O−CH_2-), 3.54 (m, 2H, −CH_2−CH_2−O−), 3.84 (m, 2H,−O−CH_2−CH_2−N^+-), 4.73 (m, 2H,-CH_2−N^+-), 7.91 (d, 2H, -C═C*H*-), 9.10 (d, 2H, -N^+═CH−). ^{13}C NMR (200 MHz, [D6] DMSO, 25 °C, TMS): δ = 13.93, 22.10, 26.65, 29.03, 30.66, 34.68, 44.81, 59.52, 67.30, 68.76, 72.66, 126.23, 145.38, 165.73. ^{11}B NMR (200 MHz, [D6] DMSO, 25 °C): −23.46 (1 B), −17.24 (10 B), 6.66 (1 B). IR (KBr): ν = 2922 (−C−H), 2853 (−C−H), 2476 (−B−H), 1642 (-C═C−), 1468 (−C−H), 1171 (−C−N-), 721 (═C−H).

4-(Bishexadecylmethyl)pyridinio-N-pentoxy-undecahydro-closo-dodecaborate (−1), Cesium Salt (Pyran-SAINT-16). **1c** and **2c** were used. Yield 40%. MS (ESI, acetonitrile, *m/z*) negative 769.8 [A^-]; positive 132.9 [Cs^+]. ^{1}H NMR (200 MHz, [D6] DMSO, 25 °C, TMS): δ = 0.85 (t, 6H, −CH_3), 1.22−1.41 (m, 60H, -(CH_2)$_{14}$−CH_3, −O−CH_2−(CH_2)$_2$-), 1.63 (m, 6H, −CH_2−(CH_2)$_{14}$−CH_3, −CH_2−CH_2−N^+-), 1.92 (m, 2H, −O−CH_2-), 2.85 (m, 1H, −C*H*-), 4.55 (m, 2H, −CH_2−CH_2−N^+-), 7.96 (d, 2H, -C═C*H*-), 8.99 (d, 2H, -N^+═CH−). ^{13}C NMR (200 MHz, [D6] DMSO, 25 °C, TMS): δ = 14.10, 22.24, 26.76, 29.12, 30.61, 31.42, 34.87, 45.04, 60.03, 67.55, 126.88, 144.45, 166.04. ^{11}B NMR (200 MHz, [D6] DMSO, 25 °C): −23.69 (1 B), −17.30 (10 B), 7.13 (1 B). IR (KBr): ν = 2923 (−C−H), 2853 (−C−H), 2475 (−B−H), 1641 (-C═C−), 1466 (−C−H), 1159 (−C−N-), 1056 (−B−O−C-), 722 (═C−H).

N-Methyl-4-(dihexydecylmethyl)pyridinium Iodide (Me-SAINT-16). The compound was synthesized according to Sudhölter et al. (1982) (*35*) and Meekel et al. (*34*).

Preparation of Liposomes. The boron-containing lipids were either used in pure form or mixed with equal molar amounts of DSPC and cholesterol plus 2 mol % DSPE-PEG_{2000}. The lipid, or lipid mixture, was dissolved in chloroform and dried to a thin lipid film in a round-bottom flask. The lipid film was hydrated and dispersed by vortexing in 10 mM HEPES[1]buffer saline, pH 7.4 (150 mM NaCl, 10 mM HEPES). The resulting suspension was frozen and thawed in 10 cycles followed by extrusion (21 times) through a polycarbonate membrane with a pore diameter of 100 nm (Avestin, Mannheim, Germany) at a temperature of 50 °C. Final lipid concentration was 10 mM. Lipid content was measured by the Stewart assay (*36*), using appropriate standard curves for the individual lipids and ICP-MS measurements.

Liposomes could not be prepared from Dioxan-SAINT-12; the suspension obtained after hydration of the lipid film yielded larger particles, which could not be extruded even after ultrasound treatment.

For DSC measurements, the pure lipids were dispersed from a lipid film (obtained as described above) by hydration with 10 mM Hepes 100 mM NaCl, pH 7.4, through ten freeze−thaw cycles, and stored at 4 °C prior to measurement.

Inductively Coupled Plasma Mass Spectrometry (ICP-MS). ICP-MS was used to quantify boron in liposome preparations. The analysis was carried out in a ThermoElectron Element 2 ICP-MS apparatus, by measuring the signal for the boron-11 nuclide. The samples were treated with concentrated nitric acid (HNO_3) in the first dilution step and further diluted with water to concentration ranges appropriate for the sensitivity of the apparatus. The water produced by a Milli-Q system was used. The boron concentrations were determined by a calibration curve obtained from samples with known boron content. Indium was added as internal standard.

Cryotransmission Electron Microscopy (cryo-TEM). A small amount (∼1 μL) of the liposome suspension (final lipid

[1]Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BNCT, boron neutron capture therapy; DSPC, distearoylphosphatidylcholine; BSH, $Na_2B_{12}H_{11}SH$; DSC, differential scanning calorimetry; DPPC, dipalmitoylphosphatidylcholine; TEM, transmission electron microscopy; ATP, adenosine triphosphate; BPA, boronphenylalanine; EGF, epidermal growth factor; SAINT, *s*ynthetic *a*mphiphiles for *i*nterdisciplinary *n*ucleic acid *t*herapy; ESI, electrospray ionization; ICP-MS, inductively coupled plasma mass spectrometry; DSPE, distearoylphosphatidylethanolamine.

concentration 5 mM) was transferred to a polymer-coated copper grid and shock-frozen from 25 °C by injection into liquid ethane. The vitrified samples were examined in a Zeiss EM LEO 912Ω electron microscope, operating at an accelerating voltage of 80 keV in filtered bright field image mode at $\Delta E = 0$ eV. The stage temperature was kept below 108 K, and images were recorded at defocus settings between 1 and 3 μm. Images were recorded by a slow scan charge-coupled device (SSCCD) camera using the minimal dose focusing device. To assess the reproducibility of the results, several images were recorded in different areas of the specimen.

Differential Scanning Calorimetry Measurements. Differential scanning calorimetry (DSC) measurements were carried out on a VP-DSC microcalorimeter from Microcal (Northhampton, MA), using the pure boron lipid (final lipid concentration 10 mM). The samples were degassed under vacuum prior to the measurement. For the up- and down-scans, a scan rate of 60 °C/h and a filtering period of 2 s were used. From each scan, a buffer background scan was subtracted. For data analysis, the software package ORIGIN (Microcal) was used. The buffer system for this measurement was 10 mM HEPES with 100 mM sodium chloride, pH = 7.4.

Viability Assay. The cell viability was determined with the CellTiter-Glo assay (Promega GmbH, Mannheim, Germany), which is based on the quantification of ATP, which is in turn proportional to the number of metabolically active cells.

The cell line V79 (lung fibroblasts of Chinese hamster) was cultivated with Ham's F10 medium and 10% fetal calf serum at 37 °C and 5% CO_2. Cells (20 000 per well) were seeded into 48-well plates and grown for 24 h. Then, the cells were incubated with different concentrations of boron-containing liposomes (DSPC/cholesterol/boron lipid (1:1:1 molar ratio) plus 2 mol % DSPE-PEG_{2000}) for 24 h. Then, 300 μL CellTiter-Glo reagent was added, and the luminescence was measured after few minutes (Microlumat Plus LB 96, EG & Bertold, Bad Wildbad, Germany). The average of two wells was compared with the average of eight untreated samples ($n = 2$).

The IC_{50} values were obtained by fitting a sigmoidal curve with the following equation:

$$f = \frac{a}{[1 + \exp(-(x - IC_{50})/b)]}$$

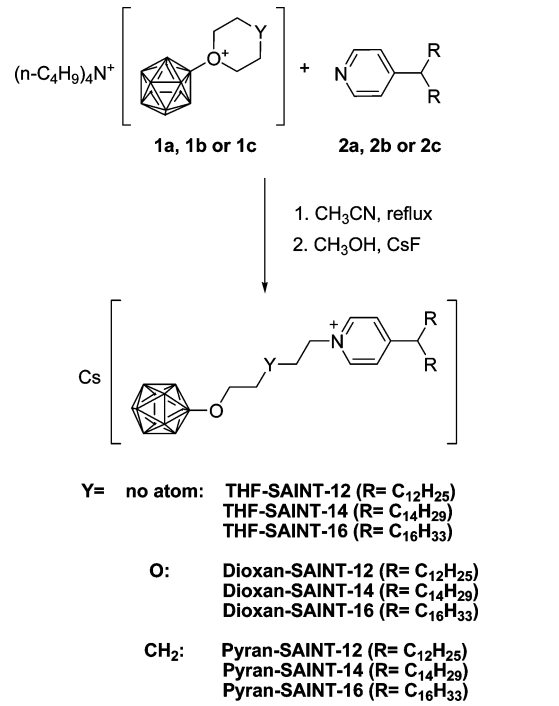
in which f corresponds to the percentage survival of cells, a corresponds to the response without inhibition, x to the concentration of the tested substance, b to the slope of the response curve at $c = IC_{50}$, and IC_{50} to the concentration of the tested substance that provokes 50% reduction of cell viability.

RESULTS

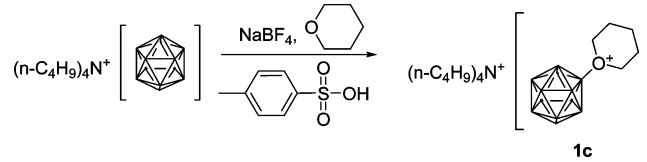
Chemistry. In this study, nine different dodecaborate cluster lipids were synthesized. All of them carry only one negative net charge. Recently, we published (*25*) the first boron lipids with a net charge of −1, but with a completely different basic structure. The lipids prepared here carry a bisalkylmethyl group on the *p*-position of a pyridine, which is *N*-alkylated with a linker carrying the dodecaborate cluster as polar headgroup. The lipids differ both in the alkyl chain length of the tails and in the linkers. For the connection of the dodecaborate cluster with the lipid backbone nucleophilic ring-opening reactions of tetrahydrofurane (THF), dioxane and tetrahydropyrane (THP) derivatives of the cluster with the alkylpyridinium compound (**2a**,**b**,**c**) were used (Scheme 2).

Semioshkin et al. (*37*) recently reported reactions of oxonium derivatives (THF and dioxane) with amines (also pyridine), but under different conditions and not applied to boron lipids. For the first time, we accomplished a ring-opening reaction with

Scheme 2. Synthesis of the Boron Lipids via Ring-Opening Reactions between the Alkylpyridnium Compound (2a,b,c) and the THF, Dioxane, and THP Derivative of the Dodecaborate Cluster, Respectively



Scheme 3. Synthesis of the Tetrahydropyrane (THP) Derivative of the Dodecaborate Cluster



the THP derivative of the cluster. The THP derivative had first been published by Peymann et al. (*33*) and had been prepared by alkylation of hydroxyundecahydro-*closo*-dodecaborate with dibromopentane, which requires two synthesis steps from the dodecaborate cluster to the THP derivative. We developed a reaction procedure that leads to the THP derivative from the unsubstituted cluster in one step and in good yields (Scheme 3).

The dodecaborate cluster is suspended in tetrahydropyrane, and 2 equiv *p*-toluenesulfonic acid and 5 equiv $NaBF_4$ are added. The product is obtained with a yield of 70%.

The synthesis of the alkylpyridinium compounds followed the work of Meekel et al. (*34*). The ring-opening reactions are carried out with the THF, dioxane, or THP derivatives of the cluster and 1.5 equiv alkylpyridinium compound (**2a**,**b**,**c**) in acetonitrile. The reaction yields are 50−70% for the THF and dioxane derivatives and 40−50% for the THP derivative, slightly depending on the length of the alkyl chain of the pyridine unit.

Physical Characterization and Liposome Preparation. The lipid film hydration and extrusion method at 50 °C was used to prepare liposomes from pure boron lipid. The structures formed were investigated by cryo-TEM (Figure 1).

The shortest chains with 12 carbons produce a mixture of liposomes and some open structures. THF-SAINT-12 (Figure 1a) forms predominantly closed vesicles but also some thick bilayers. The closed structures are heterogeneous in size; some of them are smaller than 100 nm, which is the diameter of the extrusion membrane pores. Pyran-SAINT-12 (Figure 1f) is able

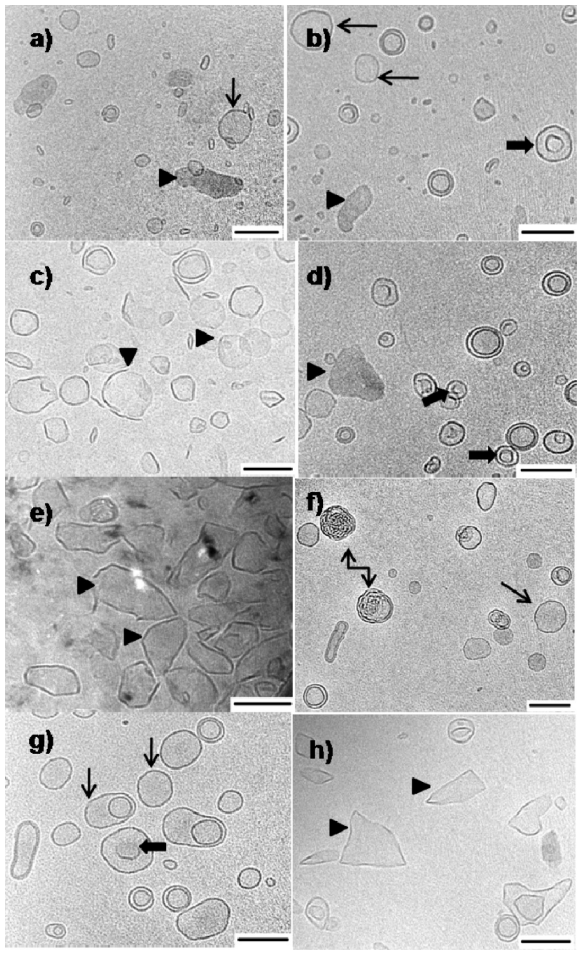


Figure 1. Cryo-TEM pictures from pure boron lipids: (a) THF-SAINT-12, (b) THF-SAINT-14, (c) THF-SAINT-16, (d) Dioxan-SAINT-14, (e) Dioxan-SAINT-16, (f) Pyran-SAINT-12, (g) Pyran-SAINT-14, (h) Pyran-SAINT-16. Scale bar 200 nm. Regular liposomes are indicated by ←, open structures by ▼, invaginated structures by thick arrows, and inverted structures by double-headed arrows.

to form liposomes, and inverted structures are observed. Closed structures are found in greatest abundance for all the three derivatives with 14-carbon chains. Only for THF-SAINT-14 and Dioxan-SAINT-14 are some bilayers observed. Interestingly, some of the liposomes show invagination. For the longer 16-carbon chains, the structures formed appear to be almost exclusively open. Several bilayers have edges bent upward, giving at first glance an impression of closed structures.

At the temperature of extrusion (50 °C) and preparation for cryo-TEM (25 °C), SAINT-16 derivatives are in the gel phase (see DSC data below), which is less favorable for the formation of liposomes. Extrusion at 65 °C and subsequent storage at 4 °C produces bilayer disks as well as closed liposomes (Figure 2a,b) in the case of THF-SAINT-16 and Dioxan-SAINT-16. This behavior is different from that of the lipid B-6−14, which forms very large bilayer sheets from liposomes below the phase transition temperature (*24*).

Interestingly, the choice of the spacer has no drastic influence on the morphology of the liposomes. Recently, we found drastic changes in the vesicle shape between the boron lipids B-THF-14 and B-Dioxan-14 (*25*) with change of the linker.

Cryo-TEM was employed also to visualize the structures formed when the boron-containing lipids had been mixed with helper lipids. For liposomal preparations, DSPC, cholesterol, and boron-containing lipid in the molar ratio of 1:1:1 plus 2 mol % DSPE-PEG_{2000} were used. THF-SAINT-12 (Figure 3a), THF-SAINT-14 (Figure 3b), and THF-SAINT-16 (Figure 3c) form liposomes in heterogeneous size. The liposome diameters differ from 100 nm, which is the pore size of the extrusion membrane, to 300 nm.

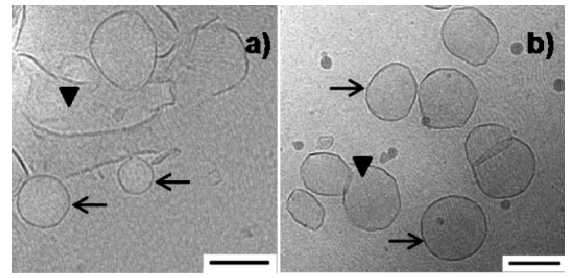


Figure 2. Samples from THF-SAINT-16 (a) and Dioxan-SAINT-16 (b) extruded at 65 °C and stored at 4 °C. Scale bar 200 nm. Regular liposomes are indicated by ←, open structures by ▼.

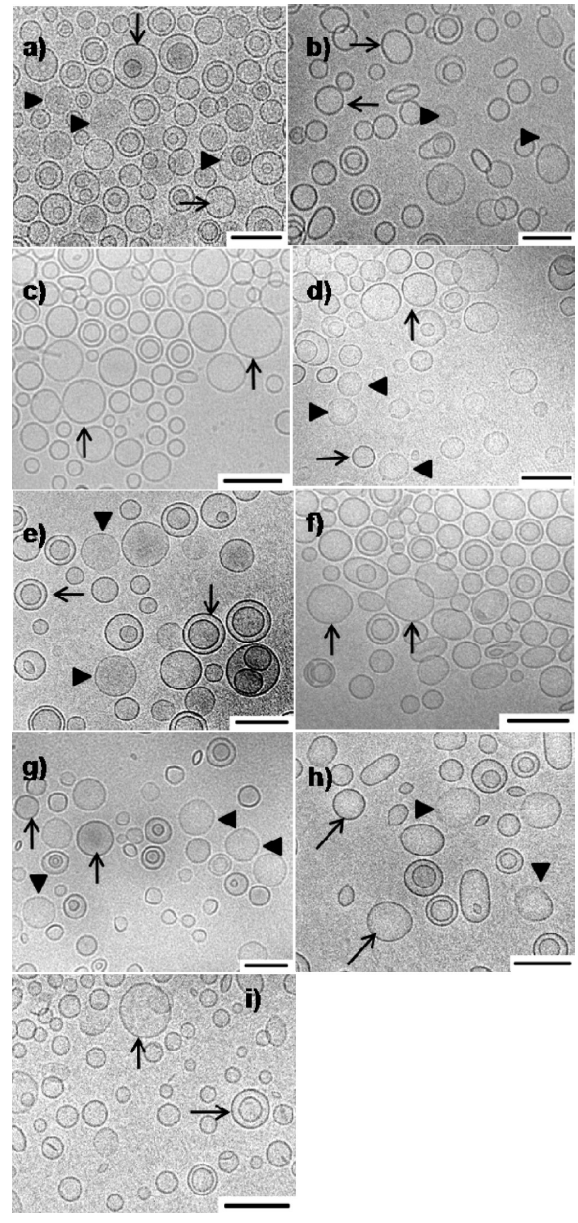


Figure 3. Cryo-TEM pictures of the liposomal preparations with helper lipids: DSPC/cholesterol/boron lipid (1:1:1) plus 2 mol % DSPE-PEG_{2000}: (a) THF-SAINT-12, (b) THF-SAINT-14, (c) THF-SAINT-16, (d) Dioxan-SAINT-12, (e) Dioxan-SAINT-14, (f) Dioxan-SAINT-16, (g) Pyran-SAINT-12, (h) Pyran-SAINT-14, (i) Pyran-SAINT-16. Scale bar 200 nm. Regular liposomes are indicated by ←, open structures by ▼.

For Dioxan-SAINT-12 (Figure 3d), Dioxan-SAINT-14 (Figure 3e), and Dioxan-SAINT-16 (Figure 3f), also liposomes can be observed in different sizes. By adding helper lipids, liposomal vesicles are also found for Pyran-SAINT-12 (Figure 3g), Pyran-SAINT-14 (Figure 3h), and Pyran-SAINT-16 (Figure 3i) with a size distribution. In general, the formation of liposomes is influenced by the chain length of the tails in the SAINTs. With mixtures containing the short-chain derivatives, an increased tendency to form open structures can be seen.

In the DSC measurements, no phase transition could be detected for any of the three SAINT-12 derivatives. This fact is, however, not surprising because Me-SAINT-12 (1-methyl-4-(bisdodecylmethyl)pyridinium chloride) has a phase transition at 0 °C (*34*), which is outside the range accessible by the DSC used.

The DSC profile of pure THF-SAINT-14 is shown in Figure 4a. From 4 to 20 °C, a broad peak with complex shape can be seen, which consists of two transitions at 13.8 and 16.8 °C. Both transitions change only insignificantly between the first and the second upscan. The two transitions might arise from the heterogeneity of the vesicle shapes and sizes (see Figure 1b). In the literature, it is known that small unilamellar vesicles (SUVs) give rise to a main transition different from that of multilamellar (MLVs) or large unilamellar vesicles (LUVs) (*38*). Interestingly, in the first upscan two further transitions at 33.0 and 66.5 °C can be observed, which disappear in the following upscans. Metastable subtransitions are described, e.g., for DPPC (dipalmitoylphosphatidylcholine) bilayers in gel phase (*39, 40*), which are reversible after storage at low temperatures for a few days. The transitions for THF-SAINT-14 are surprising in the liquid phase, and we have no evidence about the reversibility or explanation for structural changes in these phases. In cryo-TEM, no change in the vesicle shape was found after heating (pictures not shown here).

The temperature difference (approximately 4 °C) of the transition peaks between the up- and down-scans is remarkable; such strong hysteresis has only been described for the boron lipid B-THF-14 (*25*). Furthermore, it seems that the transitions in the downscan do not end at 4 °C and perhaps more transitions might follow. In contrast, Dioxan-SAINT-14 (Figure 4b) has a broad peak with a maximum at 12.3 °C. The broadness indicates that the liposome composition is heterogeneous, as can also be observed in cryo-TEM (see Figure 3d). The temperature difference (approximately 4 °C) of the transition peaks between the up- and down-scans is again remarkable. The transition does not end at 4 °C in the downscan. The DSC profile of Pyran-SAINT-14 (Figure 4c) shows a broad peak from 4 to 18 °C, similar to THF-SAINT-14. The maxima are located at 8.5 and 12.4 °C and are probably the main transitions. Meekel et al. (*34*) recorded a main transition of Me-SAINT-14 (1-Methyl-4-(ditetradecylmethyl)pyridinium chloride) at approximately 16 °C. We again attribute the two transitions to the heterogeneity of vesicle formation and size. No transitions in the downscan can be observed, which indicates that they are located outside of the temperature range in which the measurements were performed.

The DSC profile is more complex for all SAINT-16 derivatives (Figure 5). All of them show multiple transitions, which occur at similar temperatures; the transitions recur during all up- and down-scans. THF-SAINT-16 (Figure 5a) has a main transition at 56.0 °C. Sudhölter et al. (*35*) reported a main transition for Me-SAINT-16 (1-Methyl-4-(dihexadecylmethyl)pyridinium chloride) at 64 °C and we also detected it, although at 58 °C (data not shown here). THF-SAINT-16 passes through three further transitions at 24.9, 39.0, and 62.8 °C. A rich polymorphism in the DSC is known for N-methylated pyridinium cores depending on the counterion, with solid−solid

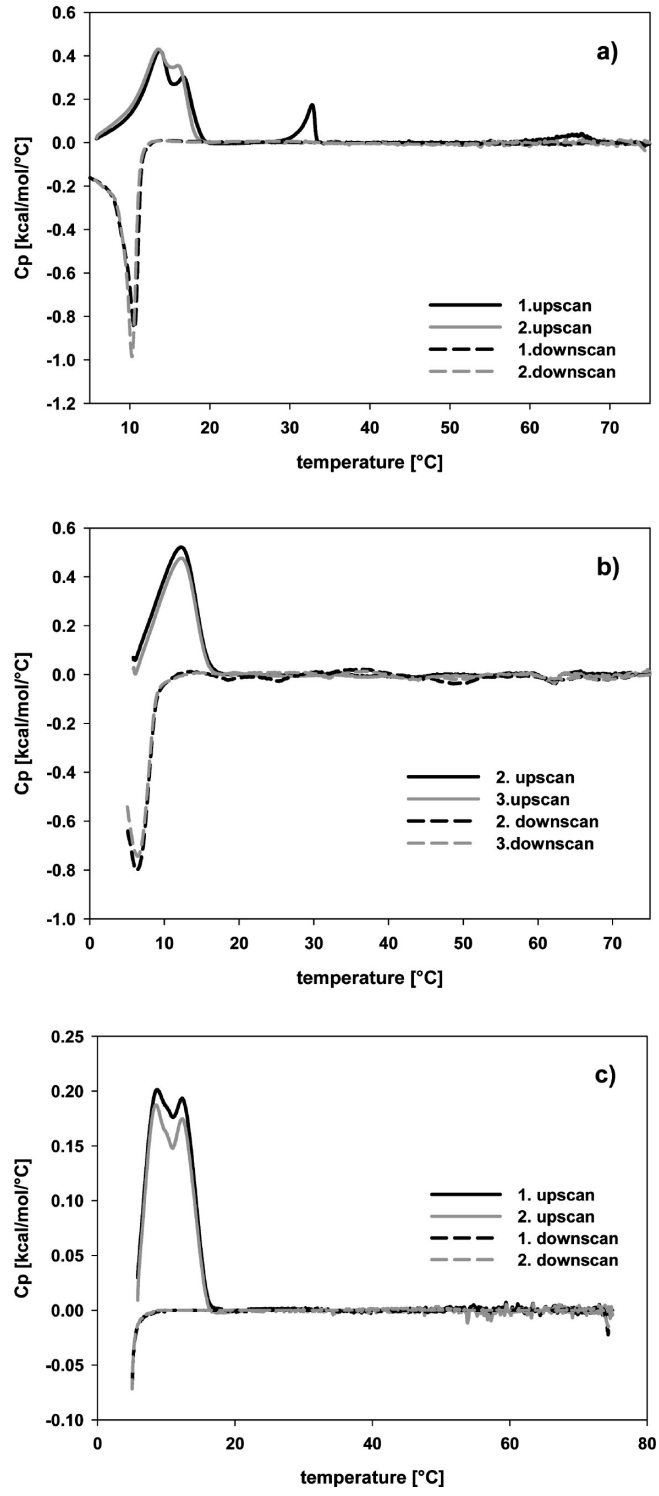


Figure 4. DSC of pure films of THF-SAINT-14 (a), Dioxan-SAINT-14 (b), and Pyran-SAINT-14 (c). Lipid concentration 5 mM.

transitions and transitions between smectic phases (*35*). Therefore, solid−solid and liquid−liquid transitions are not very special for this boronated pyridinium lipid.

A tilted smectic phase is described by Sudhölter et al. (*35*) for Me-SAINT-16 iodide. Molecular dynamics simulation would certainly be required for THF-SAINT-16 to answer precisely the question of lipid packing. In the downscan, only two transitions are visible. As can be seen in Figure 5b, Dioxan-SAINT-16 also has four transitions, which are located at 26.8, 39.9, 55.2, and 62.8 °C. Two solid−solid transitions can be noted, as well as a main transition at 55.2 °C and a liquid−liquid

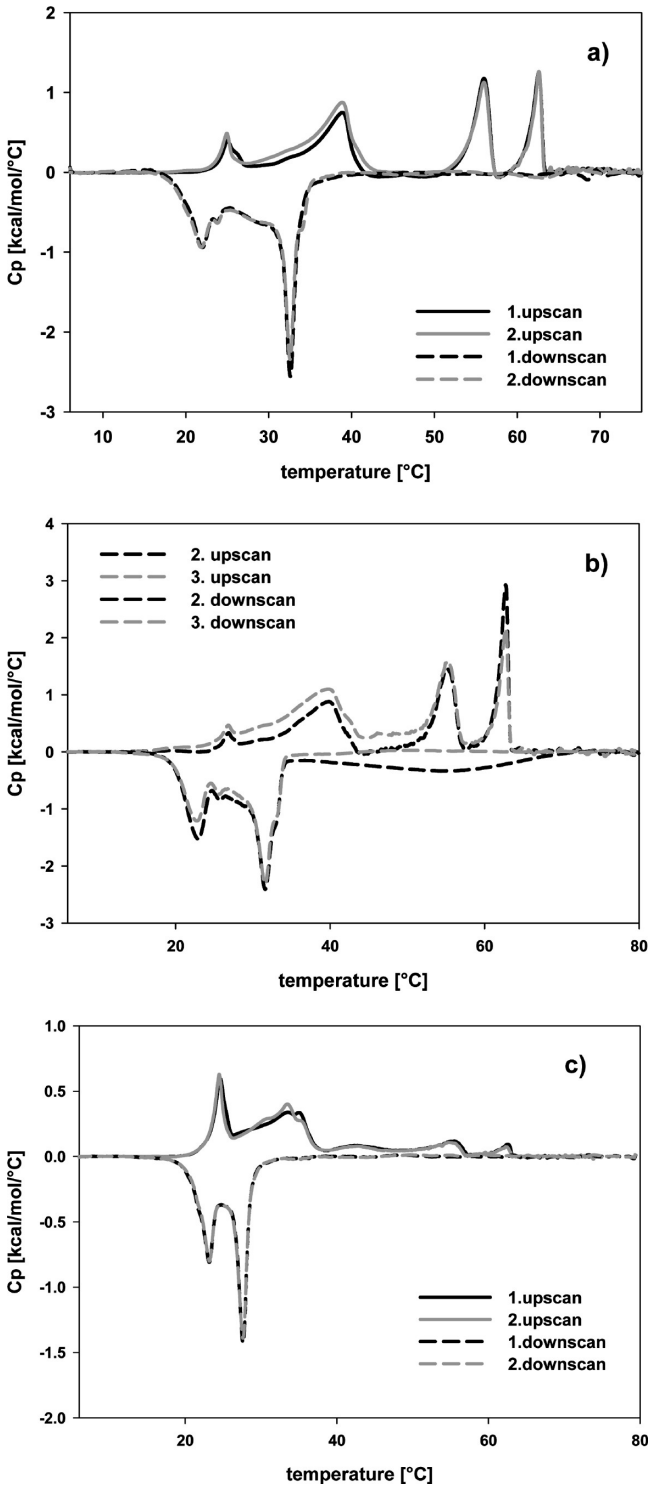


Figure 5. DSC of pure films of THF-SAINT-16 (a), Dioxan-SAINT-16 (b), and Pyran-SAINT-16 (c). Lipid concentration 10 mM.

transition at 62.8 °C, which is comparable to THF-SAINT-16. Again, only two peaks in the downscan can be observed. Pyran-SAINT-16 shows transitions in the same temperature region (24.4, 33.6, 55.1, and 62.4 °C) as THF-SAINT-16 and Dioxan-SAINT-16, but for Pyran-SAINT-16, the transitions at higher temperatures have very small enthalpies. We have no explanation for this, but it might be assumed that the transitions concern the same event of transitions as in THF-SAINT-16 and Dioxan-SAINT-16.

As summarized in Table 1, all investigated lipids have a low impact on cell viability except for Dioxan-SAINT-12 and Dioxan-SAINT-14 (Table 1). It seems that the choice of the linker influences the viability in the case of short alkyl chains. The dioxane linker leads to a higher toxic effect as compared to the THF or THP linker. This is contrary to the boron lipids B-THF-14 and B-Dioxan-14 where the introduction of an ether function in the hydrocarbon spacer leads to a decrease of toxicity (*25*).

Table 1. IC_{50} Values and Standard Deviations for All of the Tested Lipids[a]

name	log IC_{50} ± SD	IC_{50} [mM]
THF-SAINT-12	0.18 ± 0.02	1.5
THF-SAINT-14	0.51 ± 0.07	3.3
THF-SAINT-16	0.68 ± 0.01	4.8
Dioxan-SAINT-12	−0.56 ± 0.03	0.3
Dioxan-SAINT-14	−0.16 ± 0.14	0.7
Dioxan-SAINT-16	0.49 ± 0.02	3.0
Pyran-SAINT-12	0.27 ± 0.04	1.9
Pyran-SAINT-14	0.14 ± 0.02	1.4
Pyran-SAINT-16	0.54 ± 0.03	3.4

[a] For the determination of the cell viability, hamster V79 fibroblasts and the CellTiter-Glo assay were used.

The increasing alkyl chains lead to a significant decrease of toxicity in the case of THF-SAINT and Dioxan-SAINT lipids. This tendency is not well pronounced for the Pyran-SAINT lipids; however, the toxicity decreases by half from Pyran-SAINT-12 to Pyran-SAINT-16. Previously, we found a similar trend for the lipids B-6-14 and B-6-16 (*24*).

It seems that the linker influences the viability for longer alkyl chains less powerfully because the IC_{50} values are all in the same concentration range (4 mM ± 1.5 mM). Thus, for the SAINT-16 derivatives, the influence on cell viability depends only slightly on the nature of the linker, whereas this is not the case for shorter alkyl chains.

DISCUSSION

Synthesis of nine *closo*-dodecaborate cluster containing lipids was achieved. These SAINT derivatives are the first boron lipids with an alkylpyridinium core as lipid backbone. The lipids differ in their alkyl chain length and in the linker connecting the dodecaborate cluster with the lipid backbone. All of them are only singly negatively charged; thus, they are the second generation of boron lipids with a net charge of −1 (*25*). The net charge of liposomes influences the biological properties. The incorporation of negatively charged lipids into the liposomal membrane accelerates the opsonization and consequently the clearance of liposomes from the bloodstream (*41*). The decrease of lipid charge may, by reduced clearance, lead to longer retention times in the body in comparison to doubly negatively charged dodecaborate cluster lipids. Thus, dodecaborate cluster lipids with reduced net charge (up to a neutral molecule) might be desirable in regard to therapeutic efficacy.

The change in the negative net charge does not influence the toxicity of the dodecaborate cluster lipids against cells. Thus, the IC_{50} values of the SAINTs lie in the same concentrations range as B-6-14 (*24*). Studies of cell uptake are in progress for the SAINTs, B-Dioxan-14, B-6-14, and B-6-16, in order to investigate the influence of the different net charges in more detail.

Depending on the preparation temperature, the SAINT lipids are able to form closed liposomes in the absence of helper lipids and are stable after storing at 4 °C. The liposomes from pure boron lipid are able to transfer high amounts of boron to the tumor. Targeting with tumor-seeking entities probably allows selective accumulation.

Interestingly, the choice of the linker plays no major role with respect to liposome formation or thermotropic behavior. Recently, we reported about the lipids B-THF-14 and B-Dioxan-14 in which only the linker differs. For those lipids, the linker influences the vesicle formation as well as the DSC profile (*25*). The absence of the effect of the linker on toxicity observed here is very unexpected and makes it difficult to propose an optimal linker for further syntheses.

The length of the alkyl chains influences the properties of the lipids with regard to their thermotropic behavior, liposome formation in the absence and in the presence of helper lipids, and cell toxicity. Higher temperatures are necessary during the preparation of liposomes when the length of the chains increases. It must be pointed out that the helper and boron lipids should not differ significantly in their tail lengths. Therefore, DSPC, which is commonly used as helper lipid for *in vivo* experiments, is suitable for the SAINT-16 derivatives. Longer lipid tails lead to a decrease of toxicity and are consequently recommended for dodecaborate cluster lipids.

All SAINT lipids have been prepared as cesium salts. The purification of the lipids by precipitation from methanol could be achieved by addition of a solution of cesium fluoride in methanol; this is an elegant procedure and consequently recommended for synthesis strategies of boron lipids. In contrast to, e.g., the tetramethylammonium ion (which is toxic *in vivo*), the cesium ion does not carry a substantial toxicity on its own.

The new lipids, with the exception of Dioxan-SAINT-12 and Dioxan-SAINT-14, have low *in vitro* toxicity and hence might represent suitable boron carriers for BNCT. *In vivo* experiments on mice are in progress to proceed to the next stage toward successful treatment with BNCT.

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Supporting Information Available: ^{1}H NMR, ^{13}C NMR, and ^{11}B NMR spectra; ESI mass spectra, and IR spectra of compounds **1c** and all SAINT lipids. This material is available free of charge via the Internet at http://pubs.acs.org.

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