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Synthesis of bisboron compounds and their strong inhibitory activity on store-operated calcium entry

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ABSTRACT

Store-operated calcium entry (SOCE) is an important mechanism for replenishing intracellular calcium stores and for sustaining calcium signaling. We developed a method for synthesis of bisboron compounds that have two borinic acids or their esters in one molecule. These compounds are analogues of 2-APB, which is widely used as a membrane-permeable SOCE inhibitor. Further, we examined the effect of the newly synthesized bisboron compounds on SOCE in Jurkat T cells. All the bisboron compounds showed strong inhibitory activity on SOCE, with IC_{50} values of less than 1 μM, which were 20–45 times lower than observed with 2-APB.

Intracellular calcium signaling plays a key role in various physiological phenomena, including contraction of smooth muscle, secretion of granules, transcription, cell differentiation, and cell death.[1] Store-operated calcium entry (SOCE; also termed capacitative calcium entry, CCE), which is activated by depletion of intracellular calcium stores caused by repetitive calcium release, provides the means to replenish calcium from the extracellular milieu.[2,3] Recent studies have identified the molecular components of the calcium release-activated current (I_{CRAC}) channel, which is the major Ca^{2+} influx pathway for SOCE in T lymphocytes and mast cells. The endoplasmic reticulum-resident protein STIM and the channel pore-forming subunit Orai (also termed CRACM) constitute functional I_{CRAC} channels,[4–8] and the ablation of either markedly alters immune system function, including T cell activation and anaphylactic responses in mast cells.[6,9,10] Thus, the production of inhibitors against SOCE may provide a potential therapeutic strategy including use as immunosuppressants and allergy treatment. Two-aminoethyl diphenylborinate (2-APB) is a commonly-used SOCE inhibitor that was initially identified as a membrane-permeable inhibitor of inositol 1,4,5-trisphosphate (IP_3)-induced calcium release (IICR),[11] then later identified as an SOCE inhibitor.[12,13] Other lines of studies also identified inhibitors of SOCE.[14–17] Recently, in an attempt to improve the activity and the specificity of 2-APB, we analyzed the effects of more than 600 boron-containing 2-APB analogues on IICR and SOCE, and reported that some inhibitors exhibited 100-fold more potent SOCE inhibitory activity than 2-APB, while having less effect on IICR.[18,19] We also reported that two structural isomers of 2-APB analogues, DPB162-AE and DPB163-AE, showed different effects on SOCE in DT40, CHO, HeLa, and other cell types, and also on I_{CRAC}, which was reconstituted by STIM and CRACM subtypes in HEK293 cells, suggesting that these inhibitors may be useful tools to elucidate the mechanisms regulating CRAC channel activation.[19] In the present study, we developed a novel method for synthesis of 2-APB analogues that contain two boron atoms in the form of borinic acids and/or their esters, termed 'bisboron compounds', and examined their effects on SOCE in Jurkat T cells.

The synthetic methods for some bisboron compounds have been described previously.[20–22] The first method involves the preparation of diborinic acids in three steps[20,21]: (1) preparation of diboronic acid [$(HO)_2B–X–B(OH)_2$; X represents aromatic derivatives such as phenyl or biphenyl] by reacting di-aryl magnesium bromide with trimethoxyborane, and subsequent hydrolysis of boronate dimethyl ester; (2) esterification of diboronic acid by 2,2-dimethyl-propane-1,3-diol; and (3) treatment of the ester with aryl magnesium bromide to produce diborinic acid [RB(OH)–X–B(OH)R]. As this overall method requires purification of intermediates at each step, the total yield is low and a much longer reaction time is required. A second method uses purified diisopropoxyphenylborane (Scheme 1, **3**) to further react with diaryllithium (Scheme 1, **5**).[22] Although diisopropoxyphenylborane is purified by distillation at reduced

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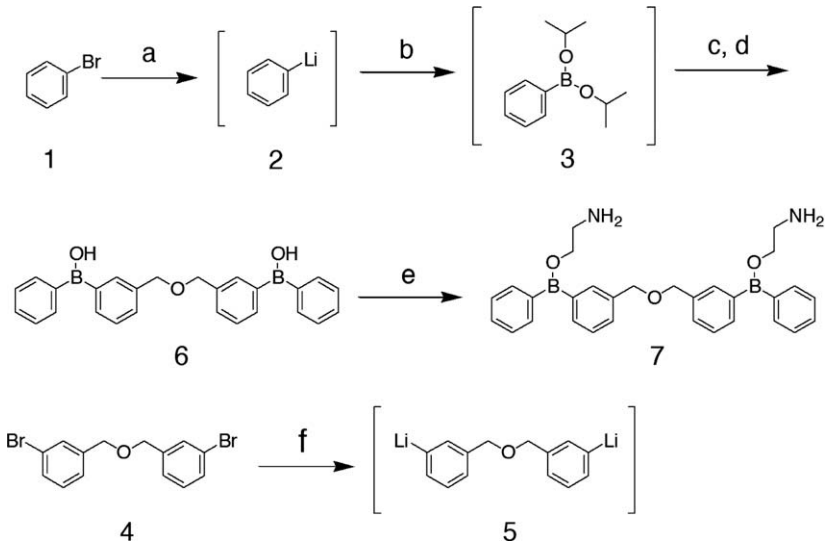

Scheme 1. Reagents and conditions: (a) *sec*-BuLi, ether, −98 °C; (b) $B(OiPr)_3$, ether, −78 °C; (c) compound **5**, ether, gradual warming from −78 °C to rt; (d) 1 M HCl; (e) 2-aminoethanol, ethanol, rt; (f) *sec*-BuLi, ether, −78 °C.

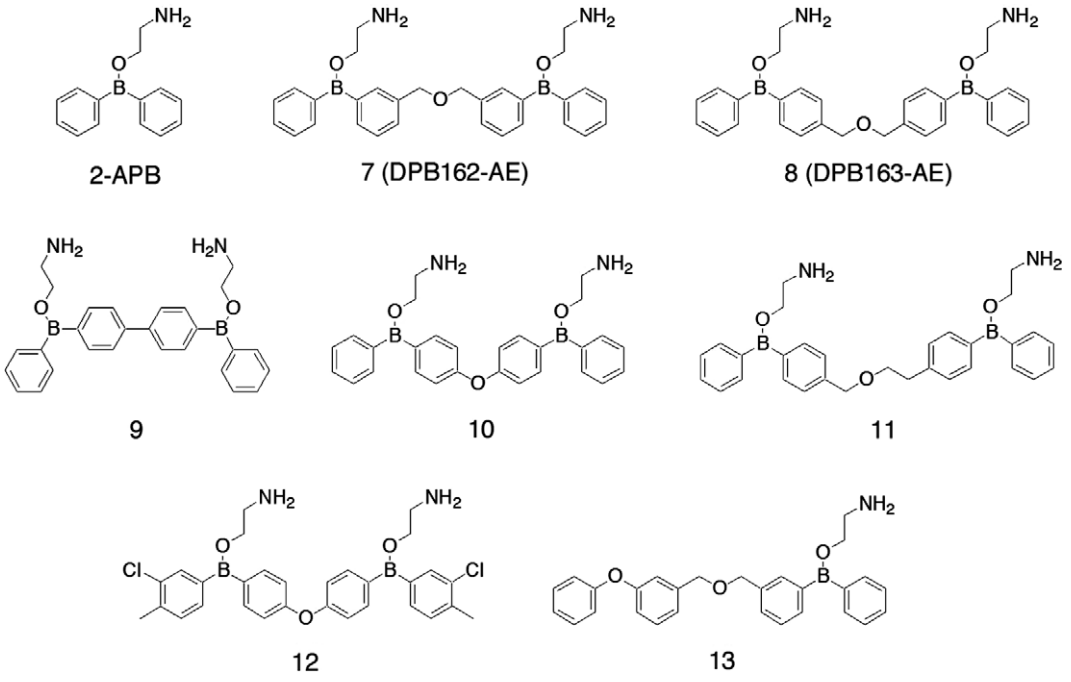

Figure 1. Chemical structures of 2-APB and synthesized borinate esters.

pressure,[23] aryldiisopropoxyborane cannot be distilled if the molecular weight of the aryl group is high. As such, this method is unsuitable for the synthesis of a wide variety of bisboron compounds. To overcome these problems, we developed a convenient method for the production of diborinic acids from commercially available or easily prepared materials, without purification of the intermediates. This method is summarized in Scheme 1. Lithiation of bromobenzene **1** with *sec*-butyllithium (*sec*-BuLi) at −98 °C in diethylether and subsequent reaction with triisopropoxyborane at −78 °C afforded diisopropoxyphenylborane **3**,[24] which was used in a further reaction without purification to avoid loss of yield. Treatment of diisopropoxyphenylborane **3** with the bis-phenyllithium **5**, which was prepared by lithiation of 3,3′-dibromodibenzyl ether **4** under the same conditions, produced diborinic acid **6** by gradual warming of the reaction mixture to room temperature and following addition of 1 M HCl. Esterification of the borinic acid **6** with 2-aminoethanol produced the bisboron compound **7**.[25] The different reactivities of triisopropoxyborane, aryldiisopropoxyborane, and

Table 1
Total yield of synthesized borinate esters and their inhibitory activities on SOCE in Jurkat T cells

Compound	Yield (%)	IC_{50} (μM)
2-APB	—	15
7	31.9	0.32
8	24.1	0.43
9	52.3	0.45
10	41.1	0.41
11	47.1	0.31
12	41.1	0.64
13	60.2	1.9

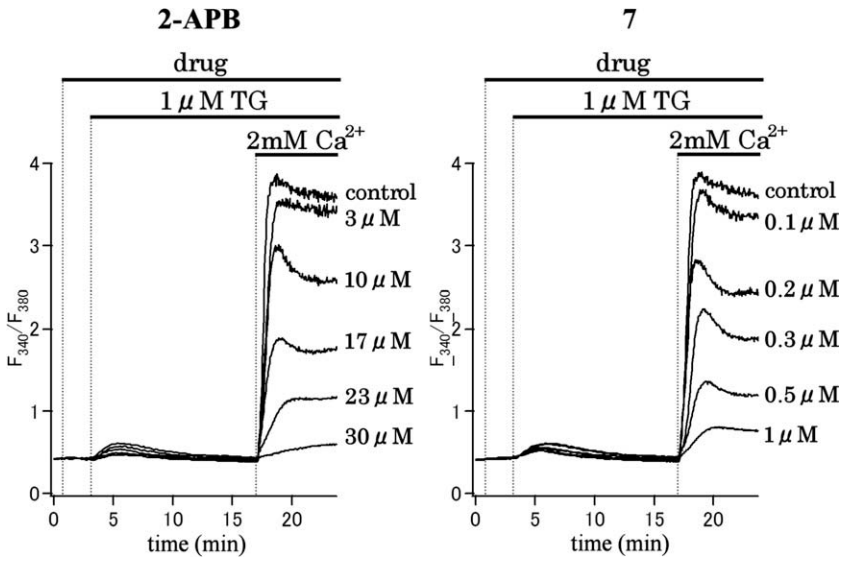

Figure 2. Effects of 2-APB and bisboron compound **7** on TG-induced SOCE in Jurkat T cells.

diarylisopropoxyborane allowed step-by-step addition of aryllithium to triisopropoxyborane,[23] resulting in selective production of bisboron compound **6**. The advantage of this method is that diborinic acid is prepared in a one-pot reaction from readily available starting materials, and therefore in high yield. For example, we improved the yield of borinate esters **9** to 52% (Fig. 1) from the previously described 13%.[20,21] Further, we produced DPB162-AE and DPB163-AE (**7**, **8**), as well as newly synthesized bisboron compounds 9–**12**, in high yield using the same method (Table 1).[26] Borinate ester **13**, in which one boron was substituted with oxygen, was synthesized using 3-bromobenzyl-3′-phenoxybenzyl ether instead of bisarylbromide.[26]

Next, we examined the effect of the borinate esters on SOCE in Jurkat T cells. Ca^{2+} entry in fura-2-loaded cells was measured after depletion of internal Ca^{2+} stores induced by thapsigargin (TG), a sarcoplasmic and endoplasmic reticulum calcium ATPase inhibitor, with or without borinate esters.[27] Consistent with previous Letters,[18,19] store depletion and subsequent addition of external Ca^{2+} evoked a rapid and large elevation in the concentration of cytoplasmic Ca^{2+}. Application of 2-APB or borinate esters before the restoration of external Ca^{2+} inhibited Ca^{2+} entry in a drug dose-dependent manner (Fig. 2). The IC_{50} values of the different drugs can be seen in Table 1. All bisboron compounds showed a marked inhibitory effect on SOCE, with IC_{50} values of less than 1 μM, regardless of the linker length between borinate esters (**8–11**), substituents in the phenyl ring (**10**, **12**), and orientation in benzyl ether (**7**, **8**); these IC_{50} values were 20–45 times lower than for 2-APB. The monoboron compound **13**, in which one boron atom was substituted with oxygen, was more potent than 2-APB. However, compound **13** was less effective than a half amount of bisboron compounds, suggesting that the two boron atoms might exert a synergistic effect on the target molecule, although the bulkiness of diarylborinate group or phenoxyphenyl group would be also important for the interaction between boron compound and the target molecule. Our previous Letter indicated that compounds **7** and **8** inhibit SOCE via STIM protein, an activator of SOCE.[19] Therefore, further study of the effect of bisboron compounds for STIM will provide insights into where and how bisboron compounds interact with STIM and inhibit SOCE.

In conclusion, we established a facile method for preparing bisboron compounds, which have been demonstrated to be potent inhibitors of SOCE. These compounds may help to further elucidate the molecular mechanism of CRAC channel activation.[19] Our method of bisboron compound synthesis will allow us to prepare a wide variety of bisboron compounds for further screening to identify more potent and selective SOCE inhibitors, which may be more suitable as therapeutics for autoimmune diseases and allergic disorders.

Acknowledgments

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25. To a solution of bromobenzene (211 μL, 2.0 mmol) in diethyl ether (8 mL), we added 0.99 M *sec*-BuLi (2.15 mL, 2.13 mmol) at −98 °C, gradually warmed to −78 °C to complete lithiation, and then added triisopropoxyborane (459 μL, 2.0 mmol). The reaction mixture was stirred for 80 min at −78 °C. At the same time, 3,3′-dibromodibenzyl ether (355 mg, 1.0 mmol), which was prepared by conventional methods, was dissolved in diethyl ether (10 mL), then reacted with 0.99 M *sec*-BuLi (2.15 mL, 2.13 mmol) and stirred for 1 h at −78 °C. The reaction mixture added to the mixture of diisopropoxyphenylborane, gradually warmed to room temperature, and then stirred overnight. The reaction was quenched with 1 N HCl, the diethyl ether layer was collected, and the water layer then extracted twice with diethyl ether. The combined diethyl ether layers were dried over $MgSO_4$ and concentrated. The crude residue was purified by flash column chromatography on a silica gel (*n*-hexane/EtOAc = 3:1) to give 3,3′-(hydroxyphenylboryl)dibenzyl ether (153 mg, 0.377 mmol, 37.7%) as an oil. The borinic acid was dissolved in ethanol (4 mL), and 2-aminoethanol (49.8 μL, 0.831 mmol) then added. The reaction mixture was stirred for 15 min at room temperature, the ethanol removed in vacuo, and the mixture then purified by reprecipitation in diisopropylether/$CHCl_3$ to give 3,3′-[(2-aminoethoxy)phenylboryl]dibenzyl ether **7** (157 mg, 0.319 mmol, 84.6%, total 31.9%) as a light yellow powder.
26. *Spectroscopic data for* **7**: light yellow powder; ^{1}H NMR (270 MHz, DMSO-d_6) δ 7.40–7.23 (m, 8H), 7.14–7.00 (m, 10H), 6.06 (br, 4H, NH_2), 4.40 (s, 4H), 3.75 (t, 4H, J = 6.3 Hz), 2.81 (tt, 4H, J = 6.3 Hz, 5.9 Hz) ^{13}C NMR (67.5 MHz, DMSO-d_6) δ 136.0, 131.3, 130.9, 130.6, 126.4, 126.3, 124.7, 124.4, 72.2, 62.4, 41.4 HRMS(FAB) (m/z): calcd for $C_{30}H_{35}O_3N_2B_2^+$ [M+H$^+$] 493.2834, found 493.2849. Compound **8**: light yellow powder; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.39–7.35 (m, 8H), 7.14–7.00 (m, 10H), 6.04 (br, 4H, NH_2), 4.37 (s, 4H), 3.75 (t, 4H, J = 6.0 Hz), 2.81 (t, 4H, J = 6.0 Hz) ^{13}C NMR (100 MHz, DMSO-d_6) δ 134.9, 131.5, 131.4, 126.6, 126.2, 124.9, 71.6, 62.4, 41.3 HRMS(FAB) (m/z): calcd for $C_{30}H_{35}O_3N_2B_2^+$ [M+H$^+$] 493.2834, found 493.2838. Compound **9**: white powder; ^{1}H NMR (400 MHz, CD_3OD) δ 7.10–7.07 (m, 12H), 6.86–6.75 (m, 6H), 3.61 (t, 4H, J = 6.0 Hz), 2.69 (t, 4H, J = 6.0 Hz) ^{13}C NMR (100 MHz, CD_3OD) δ 140.5, 133.4, 133.0, 128.1, 126.7, 126.5, 64.0, 42.6 HRMS(FAB) (m/z): calcd for $C_{28}H_{31}O_2N_2B_2^+$ [M+H$^+$] 449.2571, found 449.2570. Compound **10**: light yellow powder: ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.40–7.23 (m, 8H), 7.15–7.02 (m, 6H), 6.73 (d, 4H, J = 6.8 Hz), 6.03 (br, 4H, NH_2), 3.75 (t, 4H, J = 6.0 Hz), 2.82 (t, 4H, J = 6.0 Hz) ^{13}C NMR (100 MHz, DMSO-d_6) δ 155.1, 132.8, 131.4, 126.6, 124.8, 116.8, 62.4, 41.3 HRMS(FAB) (m/z): calcd for $C_{28}H_{31}O_3N_2B_2^+$ [M+H$^+$] 465.2520, found 465.2502. Compound **11**: light yellow powder; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.38–7.27 (m, 8H), 7.14–6.97 (m, 10H), 6.02 (br, 4H, NH_2), 4.35 (s, 2H), 3.75 (t, 2H, J = 6.4 Hz), 3.74 (t, 2H, J = 6.4 Hz), 3.52 (t, 2H, J = 7.2 Hz), 2.82 (t, 2H, J = 6.4 Hz) 2.78 (t, 2H, J = 6.4 Hz) 2.71 (t, 2H, J = 7.2 Hz) ^{13}C NMR (100 MHz, DMSO-d_6) δ 135.1, 134.8, 131.5, 131.5, 131.4, 127.2, 127.2, 126.6, 126.5, 126.3, 124.9, 124.8, 72.3, 70.8, 62.4, 41.3 HRMS(FAB) (m/z): calcd for $C_{31}H_{37}O_3N_2B_2^+$ [M+H$^+$] 507.2990, found 507.3000. Compound **12**: white powder; ^{1}H NMR (270 MHz, $CDCl_3$) δ 7.40 (s, 2H), 7.33 (d, 4H, J = 7.0 Hz), 7.12 (d, 2H, J = 7.5 Hz), 7.12 (d, 2H, J = 7.5 Hz), 6.97 (d, 4H, J = 7.0 Hz), 3.97 (t, 4H, J = 5.9 Hz), 3.02 (t, 4H, J = 5.9 Hz), 2.32 (s, 6H) ^{13}C NMR (100 MHz, DMSO-d_6) δ 155.1, 132.8, 132.4, 131.6, 131.2, 130.2, 129.8, 116.9, 62.4, 41.3, 19.3 HRMS(FAB) (m/z): calcd for $C_{30}H_{31}O_3N_2Cl_2B_2^1$ [M−H$^+$] 559.1898, found 559.1906. Compound **13**: light yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34–7.15 (m, 12H), 7.10–6.89 (m, 6H), 4.49 (s, 2H), 4.47 (s, 2H), 4.14 (br, 2H, NH_2), 3.72 (t, 2H, J = 6.0 Hz), 2.73 (br, 2H) ^{13}C NMR (100 MHz, DMSO-d_6) δ 140.3, 136.5, 131.6, 131.5, 131.4, 129.8, 129.8, 127.7, 127.6, 126.4, 126.3, 123.2, 122.7, 118.9, 118.2, 118.0, 73.4, 72.1, 63.0, 42.1 HRMS(FAB) (m/z): calcd for $C_{28}H_{29}O_3N_1B_1^+$ [M+H$^+$] 438.2241, found 438.2244.
27. Jurkat T cells (1.25×10^7 cells) grown in RPMI-1640 medium (Gibco) containing 10% fetal calf serum were collected by centrifugation (420*g*), washed with Ca^{2+}(+) assay buffer (137 mM NaCl, 5.4 mM KCl, 4.2 mM $NaHCO_3$, 1.0 mM $CaCl_2$, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 5.6 mM glucose, 20 mM HEPES, pH 7.4), and then loaded with 5 μM Fura-2/acetoxymethyl ester (Fura-2-AM) at room temperature in the dark for 60 min. The fura-2-loaded cells were washed twice with Ca^{2+}(−) assay buffer (137 mM NaCl, 5.4 mM KCl, 4.2 mM $NaHCO_3$, 0.1 mM EGTA, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 5.6 mM glucose, 20 mM HEPES, pH 7.4), resuspended at 1.25×10^6 cells/mL with Ca^{2+}(−) assay buffer, and then transferred into 96-well plates (80 μL per well; $\sim 10^5$ cells). Ca^{2+} measurements were performed using the Functional Imaging Cell-Sorting System (FDSS/IMACS; Hamamatsu Photonics).Fura-2 fluorescence intensities were measured at 510 nm with excitation at 340 nm (F_{340}) and 380 nm (F_{380}), and emission ratios (F_{340}/F_{380}) were monitored in each well. Experiments were performed according to the following time schedule; 1 min: addition of 20 μL of 5X concentration of borinate ester in Ca^{2+}(−) assay buffer, which was prepared as a 1000× stock solution in DMSO; 3 min: addition of 20 μL of 6 μM TG in Ca^{2+}(−) assay buffer containing borinate ester of interest concentration (total 1 μM of TG); 16 min: addition of 20 μL of 14 mM $CaCl_2$ in Ca^{2+}(−) assay buffer containing borinate ester of interest concentration (total 2 mM of Ca^{2+}); and 22 min: end of measurement. Control experiments were performed in each plate, in which 0.1% DMSO was added instead of borinate ester solutions. The maximal emission ratio in control wells were regarded as the maximal Ca^{2+} entry of Jurkat T cells in same plate, and the inhibitory activities of borinate esters were calculated from dividing the maximal emission ratio in the presence of borinate esters by the maximal emission ratio in control.