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Chemistry & Drug Discovery

Supporting Information

Synthesis and Immunomodulatory Activity of Fluorine-Containing Bisphosphonates

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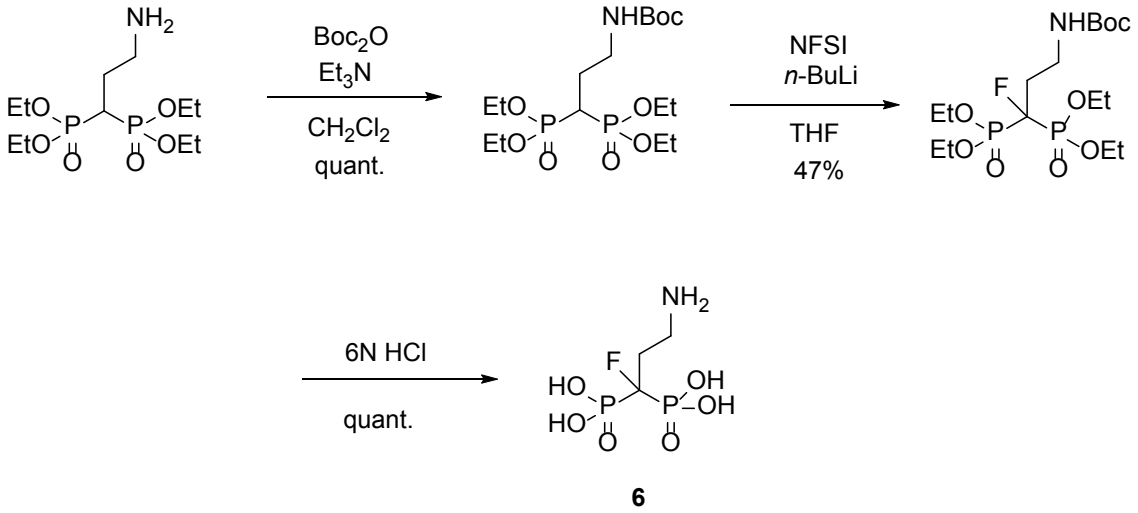
1. General Information

Unless noted otherwise, all starting materials and reagents were obtained from commercial suppliers (Merck, Darmstadt, Germany; Nacalai Tesque, Inc., Nakagyo-ku, Kyoto, Japan , Tokyo Chemical Industry Co., Ltd., Chuo-ku, Tokyo, Japan, and FUJIFILM Wako Pure Chemical Corp., Chuo-ku, Osaka, Japan) and used without further purification. All solvents were purchased from Nacalai Tesque, Inc.

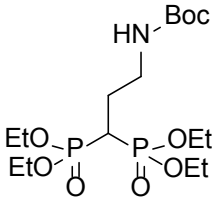
2. Experimental Procedures and Characterization Data

2.1. Synthesis of 3-amino-1-fluoro-propylidene-1,1-bisphosphonic acid (6)



Scheme S1. Synthetic procedure for the synthesis of 3-amino-1-fluoro-propylidene-1,1-bisphosphonic acid (**6**)

2.1.1. *tert*-Butyl (3,3-bis(diethoxyphosphoryl)propyl)carbamate

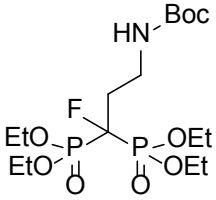


To a solution of tetraethyl-3-aminopropylidene-1,1-bisphosphonate[1] (460 mg, 1.5 mmol) in dichloromethane (20 mL) was added Boc_2O (334 μL, 1.5 mmol) and Et_3N (205 μL, 1.5 mmol) at ambient temperature. The reaction mixture was stirred for 9 h and concentrated *in vacuo* to remove volatiles to give the title compound (575 mg, 99% yield) as a colorless oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ 1.35 (t, J = 7.1 Hz, 6H), 1.53 (s, 9H), 2.06–2.15 (m, 2H),

2.40 (tt, *J* = 6.4, 23.9 Hz, 2H), 3.30–3.37 (m, 2H), 4.17–4.22 (m, 8H), 5.06 (br. s, N*H*); **^{13}C NMR** (125 MHz, $CDCl_3$) δ 16.3–16.3 (m), 27.3, 28.3, 62.6–62.7 (m), 85.1, 146.6; **HRMS** (ESI) *m/z* Calcd for $C_{16}H_{35}NNaO_8P_2$ $[M]^+$ 454.1736, found 454.1696.

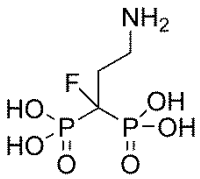
2.1.2. *tert*-Butyl (3,3-bis(diethoxyphosphoryl)-3-fluoropropyl)carbamate



To a solution of *tert*-butyl (3,3-bis(diethoxyphosphoryl)propyl)carbamate (50 mg, 0.13 mmol) in THF (2 mL) was added dropwise *n*-BuLi (90 μL, 1.6 M in hexane, 0.14 mmol) at –78°C under an argon atmosphere. After stirring for 10 min, *N*-fluorobenzenesulfonimide (45 mg, 0.14 mmol) was added to the carbanion solution and the reaction mixture was allowed to warm to room temperature over 1 h. After stirring for 7 h, the reaction was quenched by the addition of sat. aqueous ammonium chloride (10 mL). The aqueous layer was extracted with AcOEt (2 × 10 mL). The combined organic layers were dried over $MgSO_4$ and concentrated *in vacuo*, because it was difficult to purify the title compound by silica gel column chromatography (eluent: acetone/*n*-hexane = 1/1). The resulting product (28 mg) was used for following hydrolysis without further isolation steps.

^{1}H NMR (400 MHz, $CDCl_3$) δ 1.36 (t, *J* = 8.8 Hz, 12H), 1.42 (s, 9H), 2.33–2.43 (m, 2H), 3.45–3.48 (m, 2H), 4.22–4.31 (m, 8H), 5.18 (br. s, N*H*); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 16.3–16.4(m), 28.3, 64.0–64.2 (m), 81.8. 155.2; **^{19}C NMR** (470 MHz, $CDCl_3$) δ −193.7 (tt, *J* = 21.5, 74.5 Hz); **HRMS** (ESI) *m/z* Calcd for $C_{16}H_{34}FNNaO_8P_2$ $[M]^+$ 472.1641, found 472.1646.

2.1.3. 3-Amino-1-fluoro-propylidene-1,1-bisphosphonic acid (6)



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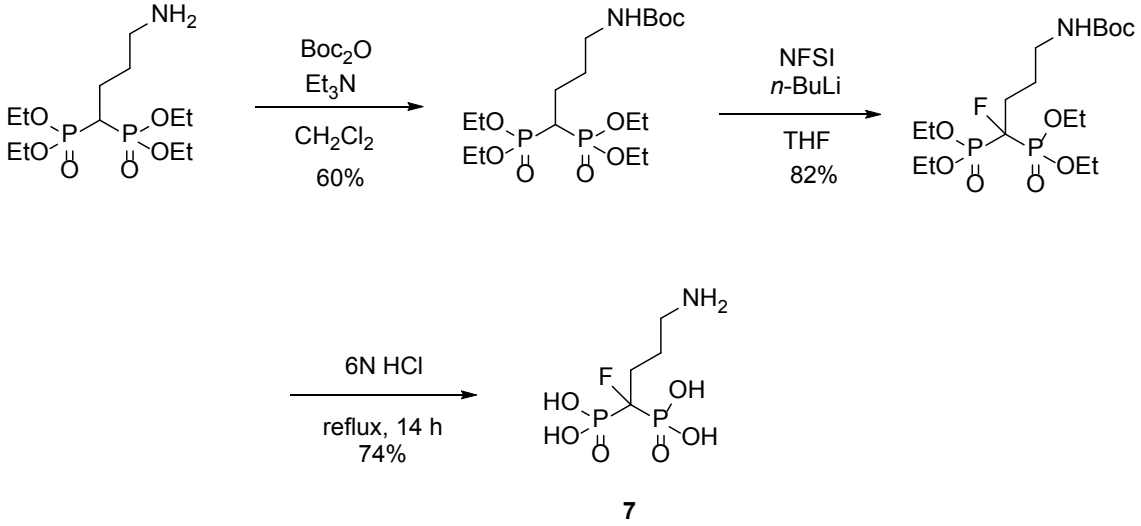
tert-Butyl (3,3-bis(diethoxyphosphoryl)-3-fluoropropyl)carbamate (28 mg, 0.06 mmol) in aqueous HCl (1 mL, 6 *N*) was refluxed for 7 h. The mixture was concentrated *in*

vacuo to remove volatiles. The residue was recrystallized from H_2O/MeOH to afford the title compound as a yellow solid. (14 mg, 45% yield).

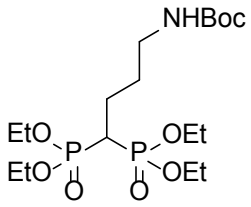
^{1}H NMR (500 MHz, D_2O) δ 2.38–2.51 (m, 2H), 3.28–3.34 (m, 2H); **^{13}C NMR** (125 MHz, D_2O) δ 29.9 (d, J = 18.0 Hz), 35.6 (d, J = 6.6 Hz); **^{19}F NMR** (470 MHz, D_2O) δ −183.4 (tt, J = 23.1, 69.2 Hz); **HRMS** (ESI) *m/z* Calcd for $C_3H_9FNO_6P_2$ $[M]^-$ 235.9889, found 235.9852.

2.2. Synthesis of 3-amino-1-fluoro-butylidene-1,1-bisphosphonic acid (7)



Scheme S2. Synthetic procedure for the synthesis of 3-amino-1-fluoro-butylidene-1,1-bisphosphonic acid (**7**)

2.2.1. *tert*-Butyl (4,4-bis(diethoxyphosphoryl)butyl)carbamate

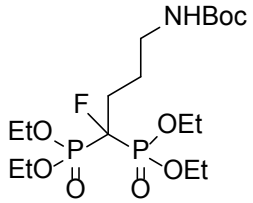


To a solution of tetraethyl-4-aminobutylidene-1,1-bisphosphonate[2] (345 mg, 1 mmol) in dichloromethane (10 mL) was added Boc_2O (218 µL, 1 mmol) and Et_3N (139 µL, 1 mmol) at room temperature. The reaction mixture was stirred for 20 h and concentrated *in vacuo* to remove volatiles. Purification of the crude mixture on silica gel column chromatography (eluent: acetone) afforded the title compound (267 mg, 60% yield) as a colorless oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ 1.29 (t, J = 7.1 Hz, 12 H), 1.38 (s, 9H), 1.71 (pent, J =

7.1 Hz, 2H), 1.85–1.96 (m, 2H), 2.24 (tt, *J* = 5.9, 23.9 Hz, 2H), 3.02–3.13 (m, 2H), 4.08–4.16 (m, 8H), 4.76 (br. s, N*H*); **^{13}C NMR** (125 MHz, $CDCl_3$) δ 16.2 (d, *J* = 2.8 Hz), 16.9 (d, *J* = 2.5 Hz), 22.6 (t, *J* = 5.1 Hz), 28.3, 28.9 (m), 30.1 (t, *J* = 132.6 Hz), 39.7, 62.4 (d, *J* = 6.5 Hz), 62.5 (d, *J* = 6.7 Hz), 78.8, 155.8; **HRMS** (ESI) *m/z* Calcd for $C_{17}H_{37}NNaO_8P_2$ $[M]^+$ 468.1892, found 468.1868.

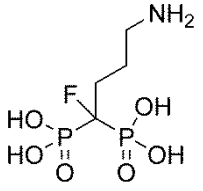
2.2.2. *tert*-Butyl (3,3-bis(diethoxyphosphoryl)-3-fluorobutyl)carbamate



To a solution of *tert*-butyl (4,4-bis(diethoxyphosphoryl)butyl)carbamate (220 mg, 0.49 mmol) in THF (12 mL) was added dropwise *n*-BuLi (338 μL, 1.6 M in hexane, 0.54 mmol) at –78°C under an argon atmosphere. After stirring for 10 min, *N*-fluorobenzenesulfonimide (170 mg, 0.54 mmol) was added to the carbanion solution and the reaction mixture was allowed to warm to room temperature over 1 h. After stirring for 12 h, the reaction was quenched by the addition of sat. aqueous ammonium chloride (10 mL). The aqueous layer was extracted with AcOEt (2 × 10 mL). The combined organic layers were dried over $MgSO_4$ and concentrated *in vacuo*, because it was difficult to purify the title compound on silica gel column chromatography (eluent: acetone/AcOEt = 1/1). The resulting product (184 mg, <82% yield) was used for following procedure without further isolation steps.

^{1}H NMR (500 MHz, $CDCl_3$) δ 1.36 (t, *J* = 7.1 Hz, 12H), 1.43 (s, 9H), 1.82–1.88 (m, 2H), 2.12–2.26 (m, 2H), 3.09–3.20 (m, 2H), 4.20–4.33 (m, 8H), 4.62 (br. s, N*H*); **^{13}C NMR** (125 MHz, $CDCl_3$) δ 16.4–16.5 (m), 23.6–23.8 (m), 28.4, 30.4 (d, *J* = 20.1 Hz), 40.5, 64.0 (dt, *J* =3.5, 26.1 Hz), 79.0, 95.4 (dt, *J* = 155.5, 187.1 Hz), 155.9; **^{19}F NMR** (470 MHz, D_2O) δ −192.6 (tt, *J* = 23.8, 75.4 Hz); **HRMS** (ESI) *m/z* Calcd for $C_{17}H_{36}FNNaO_8P_2$ $[M]^+$ 486.1798, found 486.1811.

2.2.3. 3-Amino-1-fluoro-butylidene-1,1-bisphosphonic acid (7)



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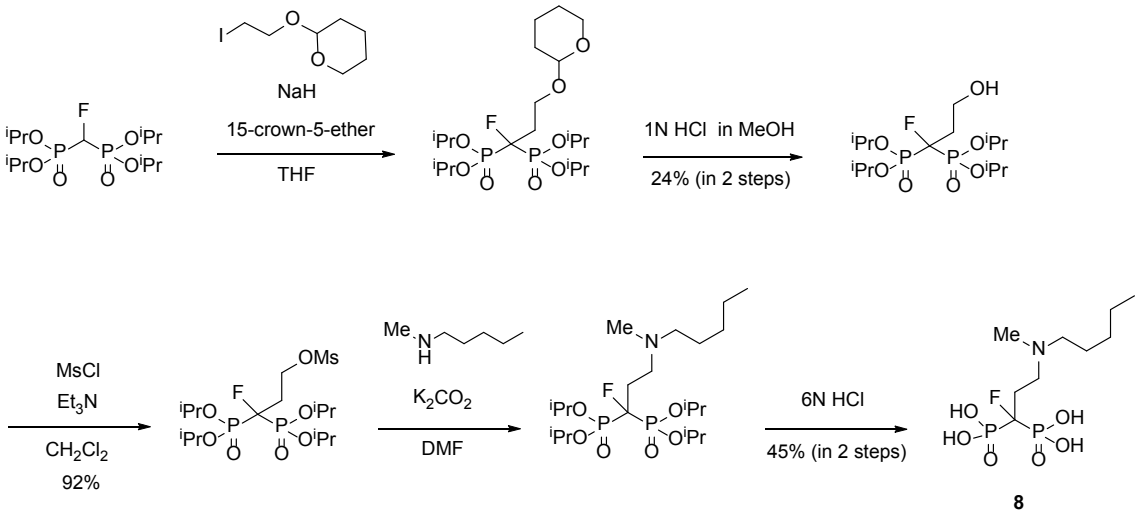
tert-Butyl (3,3-bis(diethoxyphosphoryl)-3-fluoropropyl)carbamate (100 mg, 0.22 mmol) in aqueous HCl (2 mL, 6 *N*) was refluxed for 15 h. The mixture was concentrated *in vacuo* to remove volatiles. The residue was recrystallized form H_2O/MeOH to afford the title compound as a white solid. (15 mg, 74% yield).

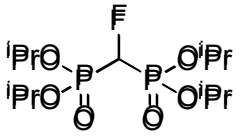
^{1}H NMR (500 MHz, D_2O) δ 1.93–2.04 (m, 2H), 2.08–2.23 (m, 2H), 2.95–3.05 (m, 2H); **^{13}C NMR** (125 MHz, D_2O) δ 21.7 (q, J = 6.0 Hz), 29.3 (d, J = 19.6 Hz), 39.5; **^{19}F NMR** (470 MHz, D_2O) δ −189.5 (tt, J =23.8, 72.5 Hz); **HRMS** (ESI) *m/z* Calcd for $C_9H_{19}FNO_8P_2$ $[M]^-$ 250.0046, found 250.0069.

2.3. Synthesis of 1-fluoro-3-(methyl(pentyl)amino)propylidene-1,1-bisphosphonic acid (8)



Scheme S3. Synthetic procedure for the synthesis of 1-fluoro-3-(methyl(pentyl)amino) propylidene-1,1-bisphosphonic acid (**8**)

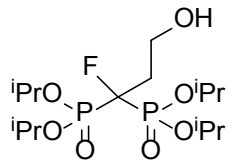
2.3.1. Tetraisopropyl monofluoromethylenediphosphonate



Tetraisopropyl monofluoromethylenediphosphonate was prepared by literature procedure. [3] Tetraisopropyl methylendiphosphonate (2.5 g, 7.3 mmol) in DMF (20 mL) was cooled to 0ºC in an ice-bath. NaH (386 mg, 16.6 mmol, 60%) was suspended in

DMF (20 mL) in another flask, which was cooled to 0°C for 5 min. The suspension was added dropwise to the cooled solution of tetraisopropyl methylendiphosphonate. The mixture was stirred at 0°C for 10 min and allowed to warm to room temperature. After stirring for 1 h, a solution of Selectfluor (1-chloromethyl-4-fluoro-1,4-diazobicylco [2.2.2]octane bis (tetrafluoroborate) (5.7 g, 16.6 mmol) in DMF was added to the mixture. After the reaction mixture was stirred for 6 h at ambient temperature and diluted with dichloromethane (50 mL), the reaction was quenched by the addition of sat. aqueous ammonium chloride (50 mL). The aqueous layer was extracted with dichloromethane (2 × 50 mL). The combined organic layers were dried over $MgSO_4$ and concentrated *in vacuo*. Purification on Silica gel column chromatography (gradient: AcOEt/*n*-hexane = 1/2 to AcOEt) afforded mono fluoro bisphosphonate (746 mg, 22%) and difluoro bisphosphonate (615 mg, 28% yield).

2.3.2. Tetraisopropyl-1-fluoro-3-hydroxypropane-1,1-bisphosphonate

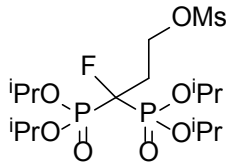


To a suspension of NaH (96 mg, 2.4 mmol, 60%) in THF (15 mL) was added a solution of tetraisopropyl monofluoromethylenediphosphonate (724mg, 2 mmol) in THF (5 mL) at 0°C under an argon atmosphere. After being stirred for 30 min, a solution of 2-(2-iodoethoxy)tetrahydro-2*H*-pyran (615 mg, 2.4 mmol) and 15-crown-5-ether (88 mg, 0.4 mmol) in THF (2.0 mL) was added to the mixture. The reaction mixture was stirred for 24 h at room temperature before being quenched with sat. aqueous NH_4Cl. The aqueous solution was extracted with AcOEt (2 × 50 mL) and combined organic layers were washed with water and brine, dried over $MgSO_4$, filtered, and concentrated *in vacuo*. Then, the crude tetraisopropyl-1-fluoro-3-((tetrahydro-2*H*-pyran-2-yl)oxy) propylidene-1,1-bisphosphonate was dissolved in 1 *N* HCl in MeOH (2 mL). After being stirred for 10 min, the reaction mixture was concentrated *in vacuo*. Purification on silica gel column chromatography (eluent: acetone/*n*-hexane = 1/1) afforded tetraisopropyl-1-fluoro-3-hydroxypropane-1,1-bisphosphonate (198 mg, 24%) as a colorless oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ 1.37–1.38 (m, 24 H), 2.42 (dtt, *J* = 5.4, 15.2, 27.5 Hz, 2H), 3.88 (t, *J* = 5.1 Hz, 2H), 4.11 (br. s, O*H*), 4.89–4.90 (m, 4H); **^{13}C NMR** (125 MHz, $CDCl_3$) δ 23.7–23.8 (m), 24.2–24.3 (m), 36.8 (d, *J* = 19.2 Hz), 73.1 (t, *J* = 3.7 Hz), 73.2 (t, *J* = 3.5 Hz); **^{19}F NMR** (470 MHz, $CDCl_3$) δ −193.8 (tt, *J* = 22.7, 78.0 Hz); **HRMS**

(ESI) *m/z* Calcd for $C_{15}H_{33}FN_2NaO_7P_2$ $[M]^+$ 429.1583, found 429.1543

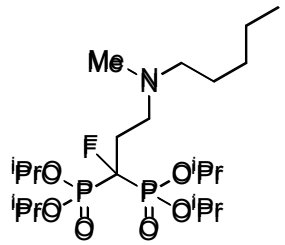
2.3.3. 2,2-Bis(diisopropoxyphosphoryl)2-fluoroethyl methanesulfonate



To a solution of tetraisopropyl-1-fluoro-3-hydroxypropane-1,1-bisphosphonate (190 mg, 0.47 mmol) in dichloromethane (5 mL) was added sequentially triethylamine (78 μL, 0.56 mmol) and methanesulfonyl chloride (43 μL, 0.56 mmol) at room temperature. The reaction mixture was stirred for 7 h before quenching with sat. aqueous $NaHCO_3$. The aqueous solution was extracted with AcOEt (2 × 50 mL) and combined organic layers were washed with water and brine, dried over $MgSO_4$, filtered, and concentrated *in vacuo*. Purification of the crude preparation on silica gel column chromatography (eluent: acetone/*n*-hexane = 1/1) afforded the title compound (209 mg, 92%) as a yellow oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ 1.36–1.38 (m, 24 H), 2.53–2.66 (m, 2H), 3.01 (s, 3H), 4.54 (t, *J* = 7.8 Hz, 2H), 4.82–4.91 (m, 4H); **^{13}C NMR** (125 MHz, $CDCl_3$) δ 23.7 (dt, *J* = 2.8, 12.9 Hz), 24.2 (d, *J* = 28.6 Hz), 32.9 (d, *J* = 20.1 Hz), 37.3, 65.3 (q, *J* = 6.9 Hz), 73.2 (t, *J* = 3.7 Hz), 73.5 (t, *J* = 3.7 Hz); **^{19}F NMR** (470 MHz, $CDCl_3$) δ −195.0 (tt, *J* = 23.1, 75.1 Hz); **HRMS** (ESI) *m/z* Calcd for $C_{16}H_{35}FNaO_9P_2S$ $[M]^+$ 507.1359, found 507.1353

2.3.4. Tetraisopropyl-1-fluoro-3-(methyl(pentyl)amino)propylidene-1,1-bisphosphonate

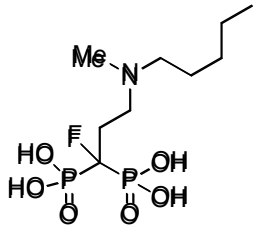


To a solution of 2,2-bis(diisopropoxyphosphoryl)2-fluoroethyl methanesulfonate (150 mg, 0.31 mmol) in DMF (2.5 mL) was added K_2CO_3 (129 mg, 0.93 mmol) and a solution of *N*-methylpentylamine (63 mg, 0.62 mmol). The reaction mixture was stirred at 80°C for 19 h. After being quenched by the addition of water, the aqueous layer was extracted with AcOEt (2 × 50 mL). Combined organic layers were washed with brine, dried over $MgSO_4$ and concentrated *in vacuo*. Purification of the crude preparation on

silica gel column chromatography (eluent: acetone/*n*-hexane = 1/1) afforded the title compound (31 mg, 21% yield) as a colorless oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ 0.90 (t, *J* = 7.4 Hz, 3H), 1.27–1.39 (m, 28H), 1.44–1.52 (m, 2H), 2.23–2.40 (m, 7 H), 2.72–2.78 (m, 2H), 4.83–4.94 (m, 4H); **^{13}C NMR** (125 MHz, $CDCl_3$) δ 14.0, 22.6, 23.7 (dt, *J* = 3.0, 18.0 Hz), 24.3, (dt, *J* = 1.4, 32.6 Hz), 27.0, 29.7, 30.3 (d, *J* = 19.1 Hz), 42.0, 50.9 (q, *J* = 6.2 Hz), 57.3, 72.6 (t, *J* = 3.5 Hz), 72.9 (t, *J* = 3.7 Hz); **^{19}F NMR** (470 MHz, $CDCl_3$) δ −193.6 (tt, *J* = 23.4, 76.2 Hz); **HRMS** (ESI) *m/z* Calcd for $C_{21}H_{46}FNNaO_6P_2$ $[M]^+$ 512.2682, found 512.2686.

2.3.5. 1-Fluoro-3-(methyl(pentyl)amino)propylidene-1,1-bisphosphonic acid (8)



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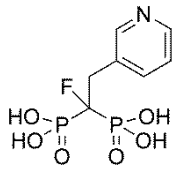
Tetraisopropyl-1-fluoro-3-(methyl(pentyl)amino)propylidene-1,1-bisphosphonate (30 mg, 0.06 mmol) in aqueous HCl (1 mL, 6 *N*) was refluxed for 7 h. The mixture was concentrated *in vacuo* to remove volatiles to give the title compound (20 mg, 99% yield) as a sticky oil.

^{1}H NMR (500 MHz, D_2O) δ 0.79 (t, *J* = 7.1 Hz, 3H), 1.21–1.29 (m, 4H), 1.57–1.72 (m, 2H), 2.42–2.53 (m, 2H), 2.79 (s, 3H), 2.99–3.04 (m, 1H), 3.11–3.17 (m, 1H), 3.26–3.33 (m, 1H), 3.46 (m, 1H); **^{13}C NMR** (125 MHz, D_2O) δ 12.9, 21.4, 23.1, 27.3 (d, *J* = 19.9 Hz), 27.7, 39.4, 51.5–51.7 (m), 56.3; **^{19}F NMR** (470 MHz, D_2O) δ −189.5 (tt, *J* = 21.6, 69.9 Hz); **HRMS** (ESI) *m/z* Calcd for $C_9H_{21}FNNaO_6P_2$ $[M]^-$ 320.0828, found 320.0843.

2.4. 1-Fluoro-2-(pyridin-3-yl)ethylidene-1,1-bisphosphonic acid (9)



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1-Fluoro-2-(pyridinyl)-ethylidene-1,1-bisphosphonic acid (**9**) was prepared as described.[4]

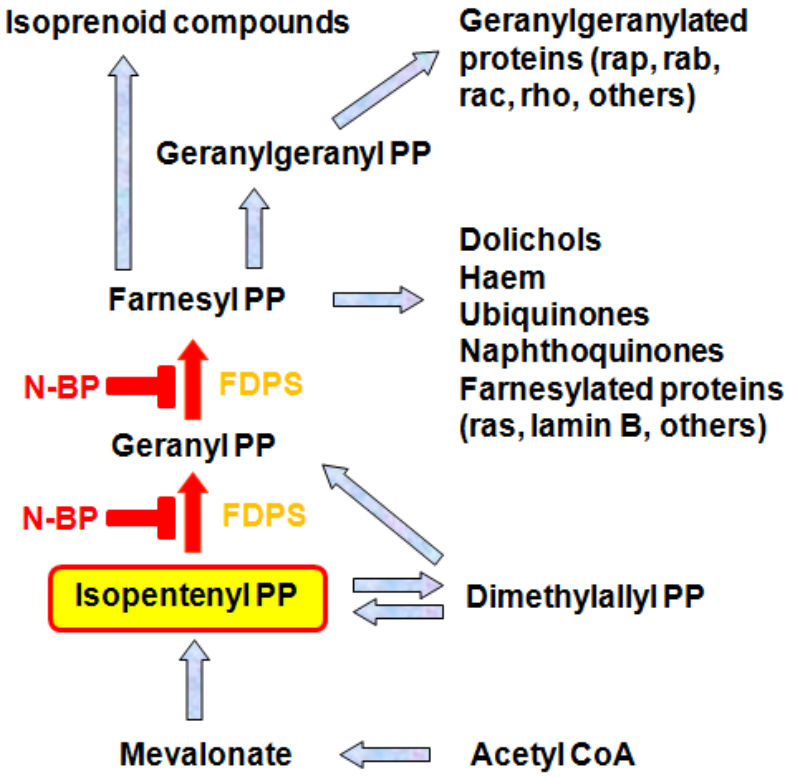


Figure S1. Mode of action of nitrogen–containing bisphosphonates (N-BPs). Isoprenoid metabolites such as steroid hormones, bile acids, lipoproteins and cholesterol are biosynthesized via mevalonate pathway. Acetyl CoA is sequentially converted to acetoacetyl CoA, 3-hydroxy-3-methyl-glutaryl CoA, mevalonate, mevalonate-5-phosphate, mevalonate-5-diphosphate, isopentenyl diphosphate (IPP) and then dimethyallyl diphosphate (DMAPP). IPP and DMAPP condense to form geranyl diphosphate (GPP) by the action of farnesyl diphosphate synthase (FDPS). This is followed by a second condensation with IPP to form farnesyl diphosphate (FPP) through the catalysis by the same enzyme, from which a series of isoprenoid metabolites including geranylgeranyl diphosphate (GGPP) are biosynthesized. Aliphatic portions of FPP and GGPP are transferred to small G proteins, which is essential for their membrane anchoring and physiological functions. When the cells are treated with N-BPs, FDPS is specifically inhibited, resulting in the intracellular accumulation of IPP and DMAPP. The inhibition of FDPS also leads to a decrease in the intracellular concentration of FPP and GGPP, resulting in the dysfunction of small G proteins, such as Ras, Rap, Rho and Rac, which are essential for the survival and growth of tumor cells.

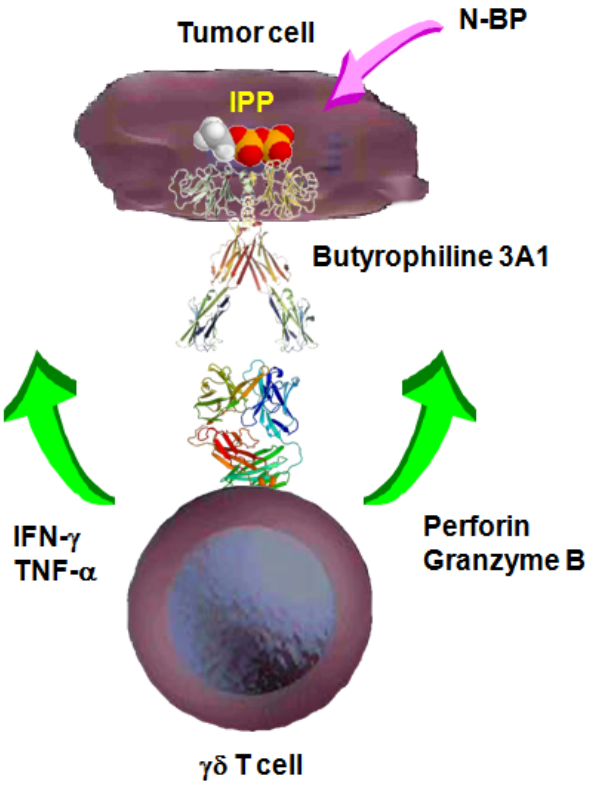
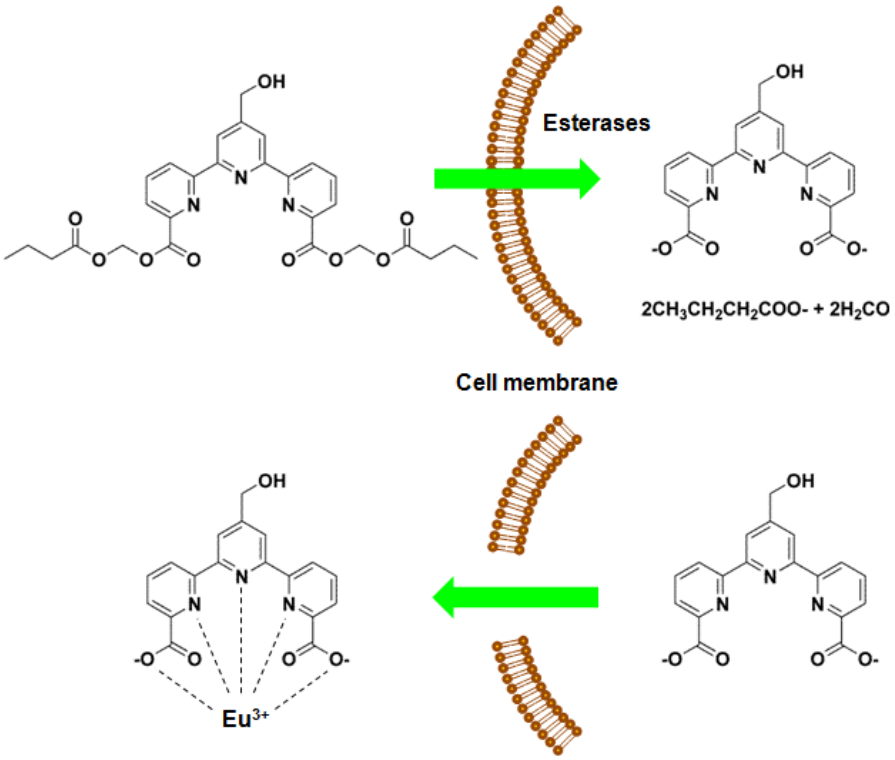
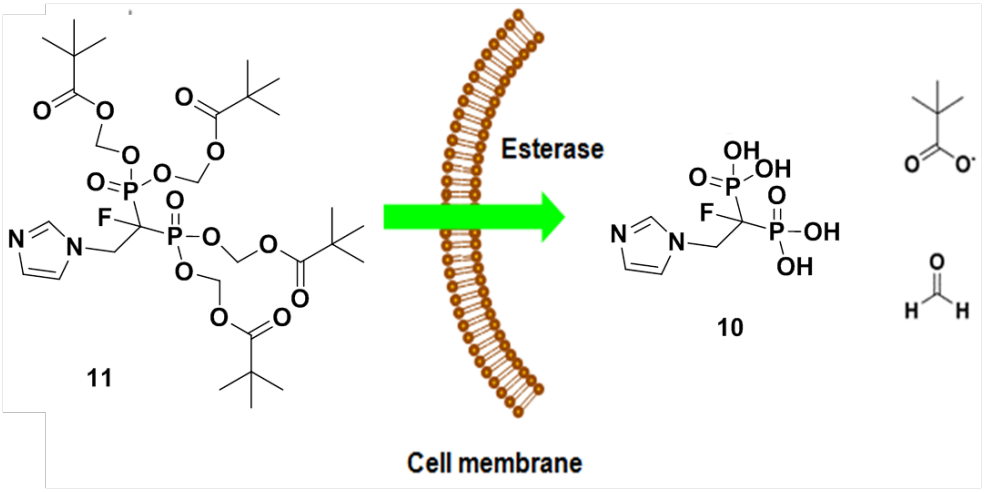


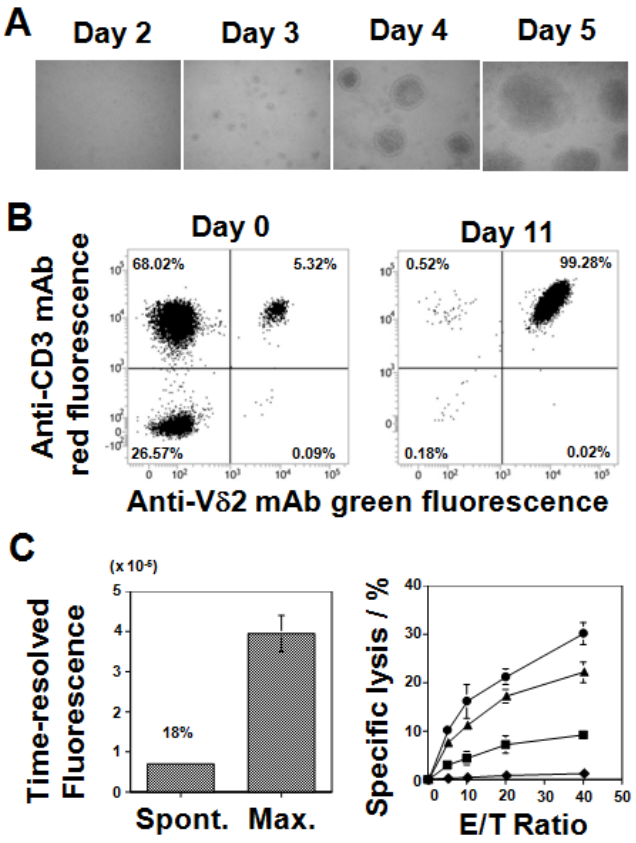
Figure S2. Butyrophilin 3A1-dependent recognition by Vγ2Vδ2 T cells of tumor cells pretreated with nitrogen–containing bisphosphonates. Butyrophilin 3A1 (BTN3A1) is one of the immunoglobulin superfamily members with IgV and IgC domains, a transmembrane stretch, an intracellular spacer and an intracellular B30.2 domain. When tumor cells are treated with a high concentration of nitrogen–containing bisphosphonates (N-BPs), the negatively–charged N-BPs are internalized into tumor cells via fluid–phase endocytosis. The intracellular N-BPs inhibit farnesyl diphosphate synthase (FDPS) in the mevalonate pathway, resulting in the accumulation of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Then, IPP and DMAPP interact with B30.2, an intracellular domain of BTN3A1. The interaction between IPP/DMAPP and B30.2 is recognized by Vγ2Vδ2 T cells in a T cell receptor (TCR)–dependent manner. Although the precise mechanism for the BTN3A1–mediated recognition remains unknown, Vγ2Vδ2 T cells secrete cytokines such as IFN-γ and TNF-α and kill tumor cells through a perforin and granzyme B–dependent cytotoxic pathway. It is worthy of note that the fluid–phase endocytosis by tumor cells is generally inefficient and that a high concentration of N-BP acids is required to sensitize tumor cells.



Supplementary Figure S3. Schematic representation of a non-radioactive cellular cytotoxicity assay system. When tumor cells are treated with bis(butyryloxymethyl) 4'-hydroxymethyl-2,2':6',2"- terpyridine-6,6"-dicarboxylate (BM-HT), the hydrophobic BM-HT permeates into cell membrane and is subject to hydrolysis by intracellular esterases to 4'-hydroxymethyl-2,2':6',2"- terpyridine-6,6"-dicarboxylate (HT), butanoic acid and formaldehyde. The nascent HT is negatively–charged and retains in the cytoplasm. When the HT–labeled tumor cells are challenged by immune effector cells like Vγ2Vδ2^{+} γδ T cells, the integrity of the tumor cell membrane is disturbed and HT is released from the cytoplasm into culture media. On addition of Eu^{3+} solution to the culture media, HT forms a chelate complex with Eu^{3+}. The HT/Eu^{3+} chelate emits long–life fluorescence upon laser excitation, thereby the specific lysis of the tumor cells can be determined by measuring time–resolved fluorescence. Because the spontaneous release rate of HT is smaller than that of other Eu^{3+}–chelate forming reagent, the BM-HT–based cellular cytotoxicity assay system yields more reliable results than other non-radioactive cell–mediated cytotoxicity assay methods. In addition, the release rate of HT is greater than that of [^{51}Cr]-sodium chromate, allowing the implementation of a rapid cytotoxicity assay system, compared to the conventional [^{51}Cr]-chromium release assay.



Supplementary Figure S4. Mode of action of a nitrogen–containing bisphosphonate prodrug in stimulating Vγ2Vδ2 T cells. When tumor cells are treated with tetrakispivaloxylmethyl-1-fluoro-2-(1*H*-imidazol-1-yl)ethylidene-1,1-bisphosphonate (**11**), the hydrophobic nitrogen–containing bisphosphonate (N-BP) prodrug permeates into cell membrane, where the compound is hydrolyzed by intracellular esterases to give1-fluoro-2-(imidazol-1-yl)ethylidene-1,1-bisphosphonic acid (**10**), pivalic acid and formaldehyde. The nascent N-BP acid inhibits farnesyl diphosphate synthase (FDPS) in the cytoplasm, resulting in the intracellular accumulation of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). The resulting IPP and DMAPP, in turn, bind to B30.2, an intracellular domain of butyrophilin 3A1 (BTN3A1), whose interaction is sensed by Vγ2Vδ2 T cells in a Vγ2Vδ2 TCR-dependent manner. It is noteworthy that the compound (**11**) is highly hydrophobic and efficiently permeates into cell membrane. By contrast, conventional N-BPs, such as the second generation N-BP pamidronate and the third generation N-BP zoledronic acid, are negatively–charged and fail to permeate freely into tumor cell membrane, because the N-BP acids are designed to interact with bones for the treatment of patients with bone–related diseases like Paget's disease, osteoporosis, and hypercalcemia of malignancy. It is also worthy of note that the compound (**11**) is efficiently internalized into antigen–presenting cells, such as macrophages and dendritic cells, leading to the intracellular accumulation of IPP/DMAPP and the expansion of peripheral blood Vγ2Vδ2 T cells. It is thus probable that the N-BP prodrug is superior to conventional N-BP acids in both sensitizing tumor cells and stimulating Vγ2Vδ2 T cells *in vitro*.



Supplementary Fig. S5. Immunomodulatory effects of **11** on Vγ2Vδ2 T cells from a healthy adult donor. (**A**) Expansion by **11** of Vγ2Vδ2 T cells from PBMCs from a healthy adult donor. PBMCs were stimulated with **11** and cell clustering was monitored on days 2, 3, 4, and 5. (**B**) Flow cytometric analysis of Vγ2Vδ2 T cells before (day 0) and after (day 11) expansion with **11**. Cells on days 0 and 11 were analyzed for the expression of Vδ2 and CD3 through a FACS Verse flow cytometer. (**C**) Spontaneous release of chelate-forming HT and specific lysis of H520 lung caicinoma cells by **11**–expanded Vγ2Vδ2 T cells. (Left panel) H520 cells were treated with 25 μM of BM-HT at 37ºC for 15 min. After being washed with RPMI1640 medium, the cells were incubated in the presence or absence of 0.125% detergent in DMSO for 40 min. The spontaneous release rate was determined through a PHERAStar multiplate spectrophotometer. (Right panel) H520 cells were pretreated with 0 μM (♦), 1.25 μM (■), 2.5 μM (▲) or 5 μM (●) of **11**. The cells were further treated with 25 μM of BM-HT at 37ºC for 15 min. After being washed with RPMI1640 medium, the cells were challenged by Vγ2Vδ2 T cells for 40 min. The specific lysis was determined using a PHERAStar multiplate spectrophotometer. Data show mean ± SD and are representative of three independent experiments.

Supplementary Table S1. Comparison of Vγ2Vδ2 T cell–mediated cytotoxicity against 786-0 renal cell carcinoma. The specific lysis (%) at the effector–to–target ratio of 20 and at a fluorine–containing bisphosphonate concentration of 1,000 μM shown in Fig. 2 is listed. Data show mean ± SD and are representative of three independent experiments.

Compound No.	Specific lysis (%)	Compound No.	Specific lysis (%)
1	14.7 ± 0.6	**6**	1.7 ± 0.3
2	30.1 ± 3.1	**7**	7.7 ± 0.9
3	58.8 ± 0.6	**8**	14.1 ± 0.7
4	50.6 ± 2.5	**9**	41.9 ± 3.3
5	46.0 ± 2.9	**10**	35.2 ± 2.4

Supplementary Table S2. Comparison of Vγ2Vδ2 T cell–mediated cytotoxicity against various tumor cells. The specific lysis (%) at the effector–to–target ratio of 20 and at a compound **11** concentration of 5 μM shown in Fig. 3 is listed. Data show mean ± SD and are representative of three independent experiments.

Tumor cell line	Specific lysis (%)
786-0	37.8 ± 2.9
UOK121	48.2 ± 5.9
EJ-1	20.7 ± 1.5
U937	45.7 ± 1.1

Supplementary Table S3. Species specificity of Vγ2Vδ2 T cell–mediated cytotoxicity against various tumor cells. The specific lysis (%) against human and mouse tumor cell lines at the effector–to–target ratio of 20:1 and at a compound **11** concentration of 5 μM is summarized.

Tumor cell line	Specific lysis (%)	Species	Origin	Source
SW982	18.3 ± 2.7	Human	Synovial sarcoma	1
MKN74	34.6 ± 3.0	Human	Gastric carcinoma	2
A431	62.0 ± 3.6	Human	Epidermoid carcinoma	2
RAMOS-RAI	45.3 ± 1.5	Human	Burkitt lymphoma	2
OST	11.0 ± 2.3	Human	Osteosarcoma	3
HCT-4	23.5 ± 2.1	Human	HTLV-1–induced T cell line	4
YAC-1	0.1 ± 0.4	Mouse	Mo-MuLV–induced lymphoma	1
B16F10	0.2 ± 0.5	Mouse	Melanoma	2

Tumor cell lines were pretreated with 5 μM of **11** at 37ºC for 2 h and treated with 25 μM of BM-HT at 37ºC for 15 min. After being washed with RPMI1640 medium, the cells were challenged by Vγ2Vδ2 T cells at an effector–to–target ratio of 20:1 for 40 min. After the supernatants were mixed with Eu^{3+} solution, the specific lysis was determined by measuring time–resolved fluorescence using a PHERAStar multiplate spectrophotometer. Data show mean ± SD and are representative of three independent experiments. Tumor cell lines indicated as 1 were purchased from American Type Culture Collection, Manassas, VA, 2 from Health Science Research Resources Bank, Sennan, Osaka, Japan, 3 kindly provided by Prof. Junya Toguchida, Kyoto University, Sakyo-ku, Kyoto, Japan and 4 kindly provided by Professor Tatsufumi Nakamura, Nagasaki University, Sakamoto, Nagasaki, Japan.

7. References

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