

Supplementary Materials to the article

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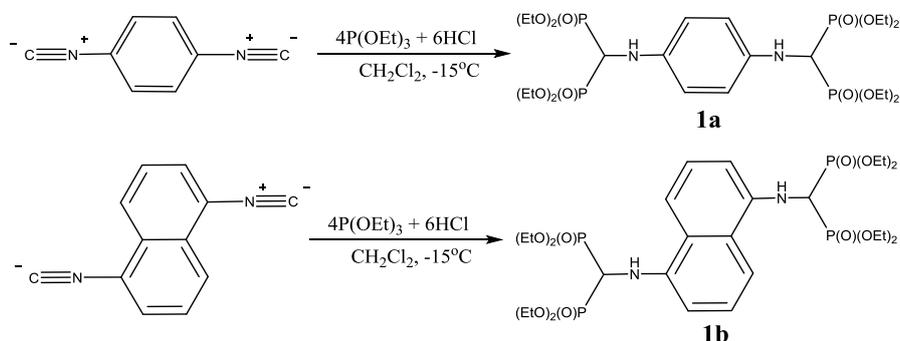
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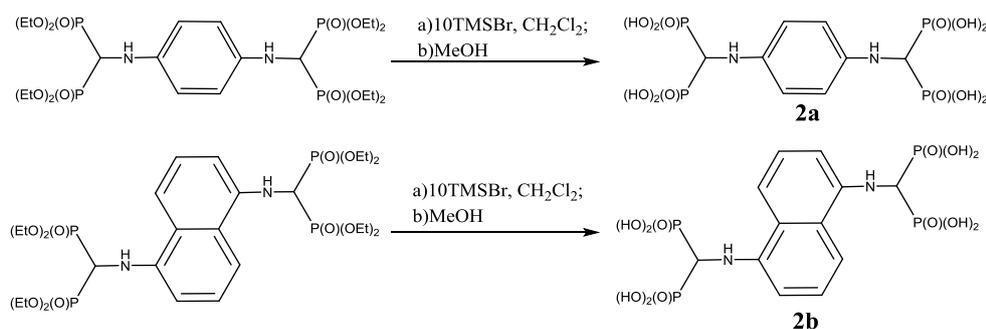
Synthesis of free benzene-1,4-bis[aminomethylidene(bisphosphonic)] acid and naphthalene-1,5-bis-[aminomethylidene(bisphosphonic)]

I step: synthesis of octaethyl benzene-1,4-bis[aminomethylidene(bisphosphonate)] **1a** and octaethyl naphthyl-1,5-bis[aminomethylidene(bisphosphonate)] **1b**.



To a cold (*ca.* -15°C , NaCl-ice bath) solution of 1,4-diisocyanobenzene (0.048 mol, 6.2 g) or 1,5-diisocyanonaphthalene (0.048 mol, 8.5 g) and triethyl phosphite (33.2 g, 0.20 mol, Sigma-Aldrich) in dry dichloromethane (300 ml) a cold solution of *ca.* 4 M HCl in 1,4-dioxane (0.3 mol, 75 ml) was added during about 30 min and the mixture was stirred for additional 1 h at the same temperature. The reaction mixture was washed with saturated NaHCO_3 solution (8 \times 50 ml), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was treated with diethyl ether/petroleum ether (150 ml, 1:2, v/v) in the case of **1a** and diethyl ether (200 ml) in the case of **1b**. The resulting solids were filtered, washed intensively with diethyl ether/petroleum ether (4 \times 30 ml, 1:2, v/v) or diethyl ether (5 \times 20 ml), respectively and dried in air giving octaethyl benzene-1,4-bis[aminomethylidene(bisphosphonate)] (15.9 g, 49%) **1a** and octaethyl naphthyl-1,5-bis[aminomethylidene(bisphosphonate)] **1b** (26.7 g, 76%). Both octaethyl esters were used in next step without further purification. The structure of **1a** and **1b** was confirmed by ^{31}P and ^1H NMR spectroscopy. **1a**: ^{31}P NMR $\{^1\text{H}\}$ (CDCl_3 , 121 MHz): δ 18.0 (s); ^1H NMR (CDCl_3 , 300 MHz): δ 1.27 (t, $J=7.1\text{Hz}$, 12H, OCH_2CH_3), 1.30 (t, $J=7.1\text{Hz}$, 12H, OCH_2CH_3), 3.80 (bs, 2H, NH), 4.07 (bt, $J=22.2\text{Hz}$, 2H, CHP) 4.14-4.22 (m, 16H, OCH_2), 6.63 (s, 4H, ArH); **2b**: ^{31}P NMR $\{^1\text{H}\}$ (CDCl_3 , 121 MHz): δ 17.5 (s); ^1H NMR (CDCl_3 , 300 MHz): δ 1.22 (t, $J=7.0\text{Hz}$, 12H, OCH_2CH_3), 1.29 (t, $J=7.0\text{Hz}$, 12H, OCH_2CH_3), 4.08-4.17 (m, 4H, OCH_2), 4.18-4.29 (m, 12H, OCH_2), 4.42 (dt, 2H, $J=10.2\text{Hz}$, $J=21.6\text{Hz}$, CHP), 4.91 (bdt, 2H, $J=4.8\text{Hz}$, $J=9.6\text{Hz}$, NH), 6.80 (t, 2H, $J=4.0\text{Hz}$, ArH), 7.36 (m, 4H, ArH). NMR data are in agreement with those reported in our previous paper [1].

II step: synthesis of benzene-1,4-bis[aminomethylidene(bisphosphonic)] acid **2a** and naphthalene-1,5-bis-[aminomethylidene(bisphosphonic)] acid **2b**.



To a cold (*ca.* 0°C, ice bath) solution of octaethyl benzene-1,4-bis[aminomethylidene(bisphosphonate)] **1a** (0.020 mol, 13.6 g) or octaethyl naphthyl-1,5-bis[aminomethylidene(bisphosphonate)] **1b** (0.020 mol, 14.6 g) in dry dichloromethane (200 ml) a bromotrimethylsilane (26.4 ml, 0.20 mol) was added dropwise over 30 min. The mixture was stirred for about 1 h at 0°C and overnight at room temperature. Then was concentrated *in vacuo*, the resulting residue was treated with methanol (200 ml) and stirred for about 30 minutes at room temperature. The reaction mixture was evaporated again and a methanol-acetone mixture (300 ml, 2:1, v/v) was added. The resulting solid products were filtered off, washed with a methanol-acetone mixture (3×30 ml, 2:1, v/v), water-acetone mixture (2×50 ml, 1:1, v/v), acetone (2×50 ml) and dried in air giving benzene-1,4-bis[aminomethylidene(bisphosphonic)] acid **2a** (8.2 g, 90%) and naphthalene-1,5-bis[aminomethylidene(bisphosphonic)] acid **2b** (6.8 g, 67%). Obtained free acids were directly used for the preparation of their tetrasodium salts (WG 12399C and WG12592A).

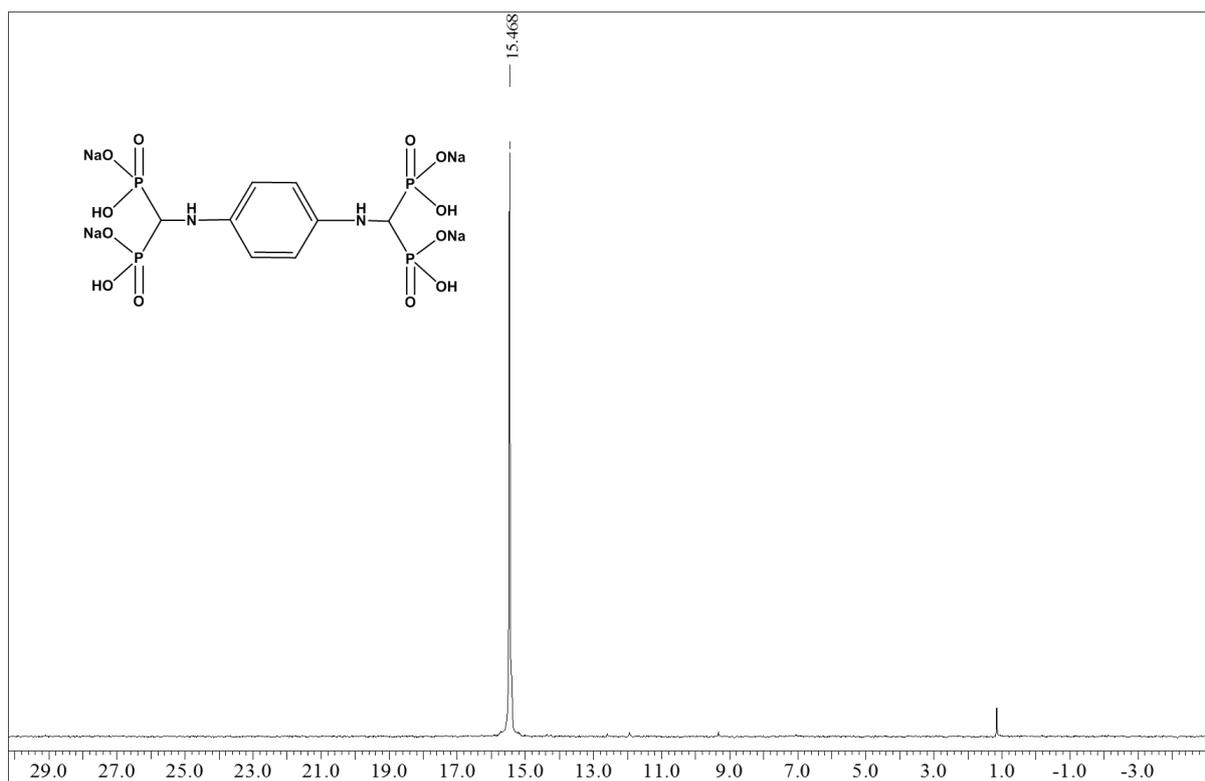


Figure S1. $^{31}\text{P}\{^1\text{H}\}$ NMR spectrum of WG12399C in D_2O .

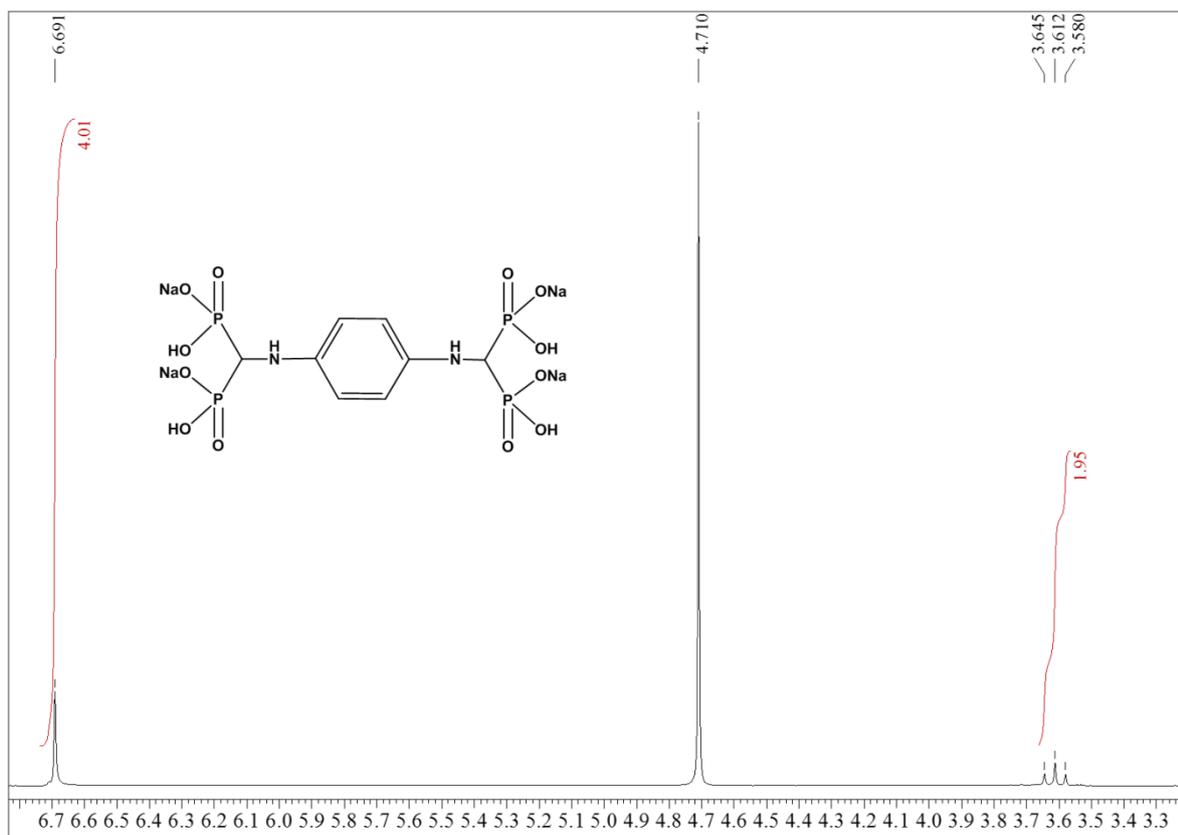


Figure S2. ^1H NMR spectrum of WG12399C in D_2O .

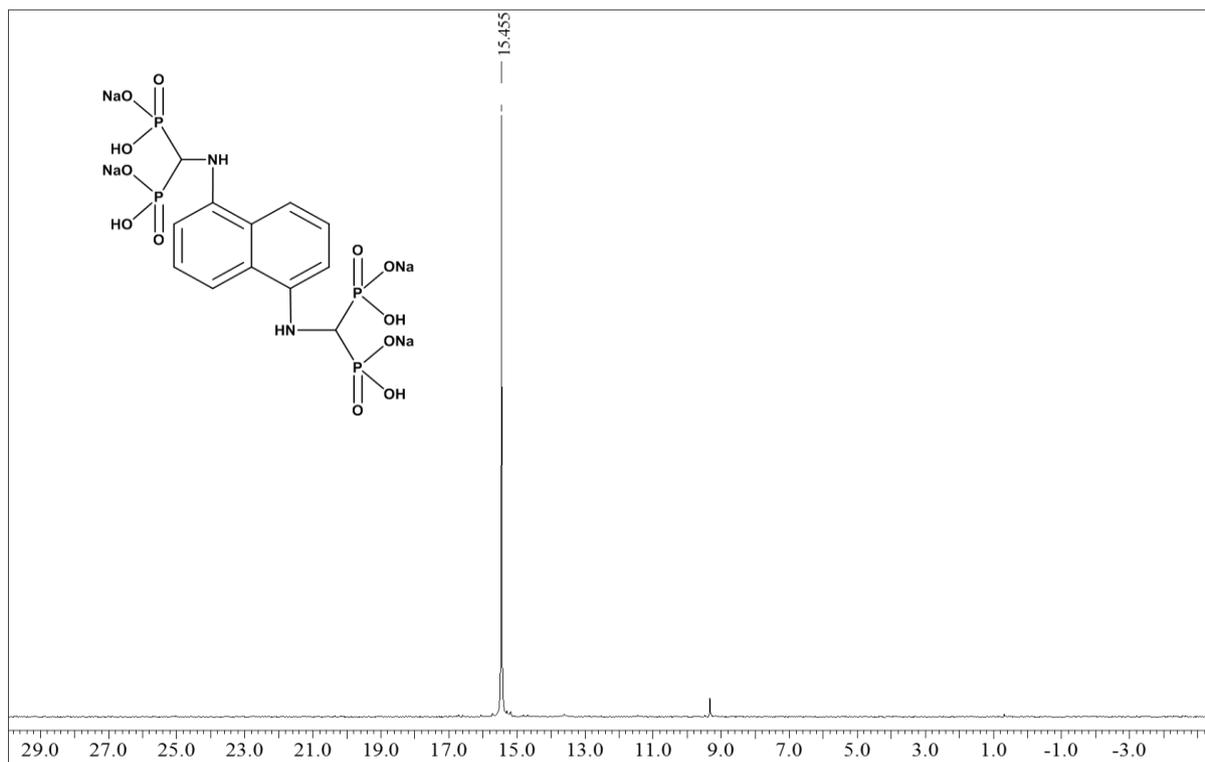


Figure S3. $^{31}\text{P}\{^1\text{H}\}$ NMR spectrum of WG12592A in D_2O .

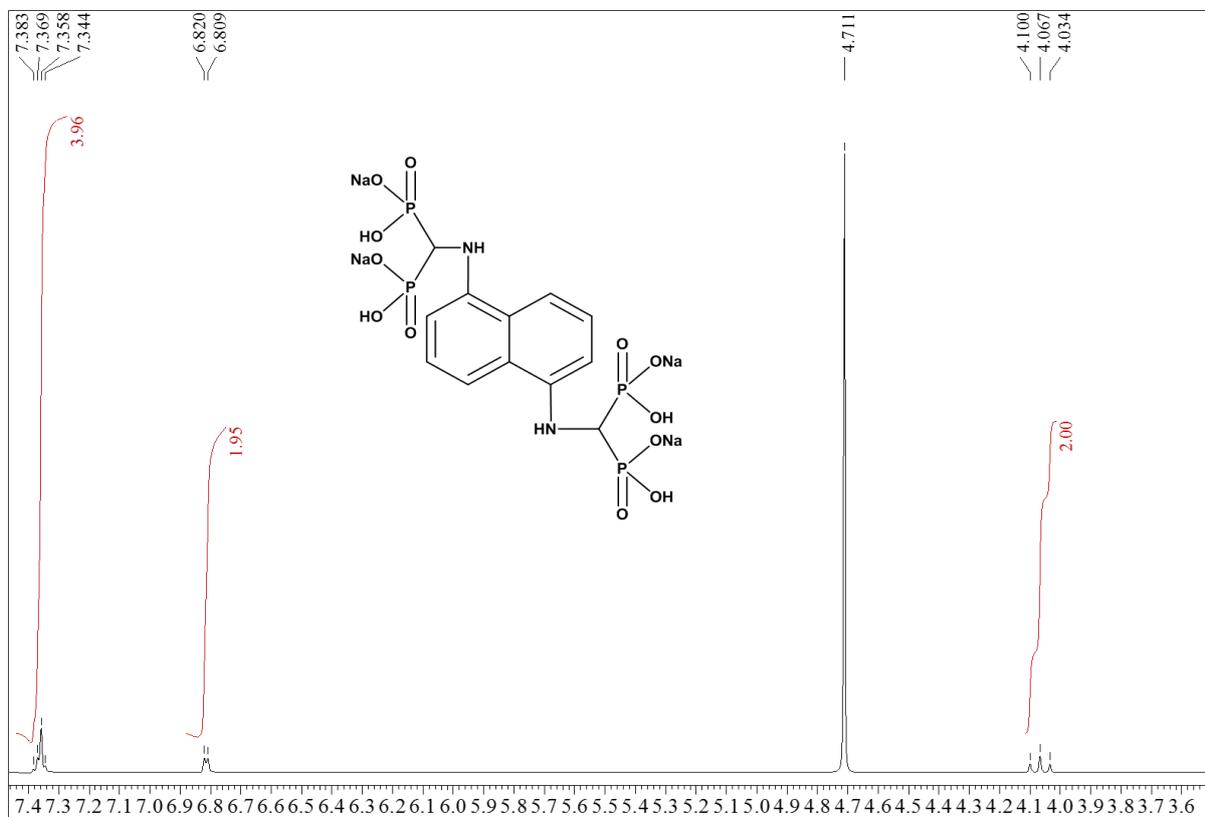
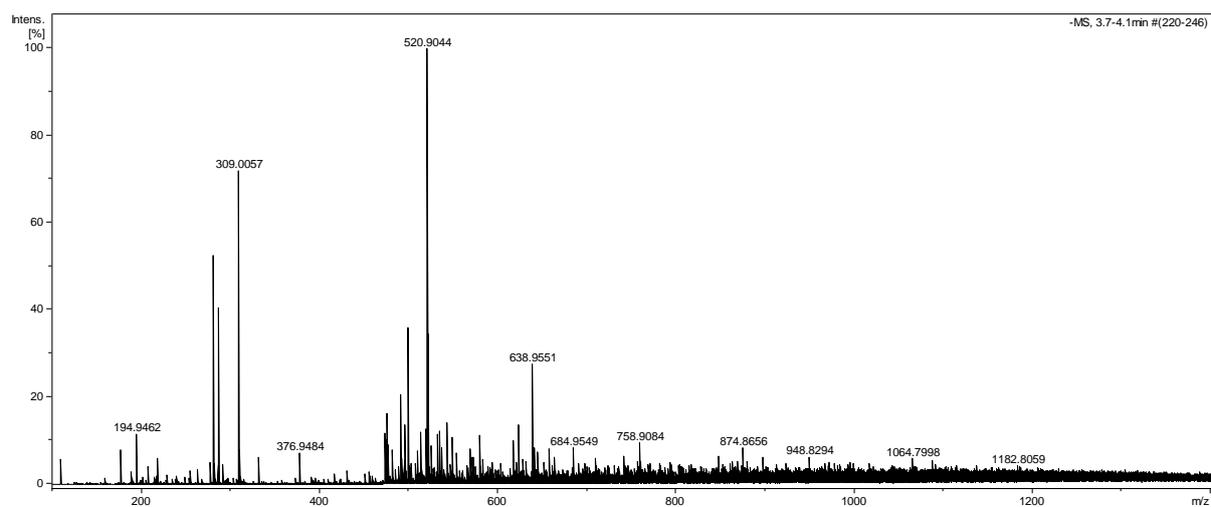


Figure S4. ^1H NMR spectrum of WG12592A in D_2O .

A.



B.

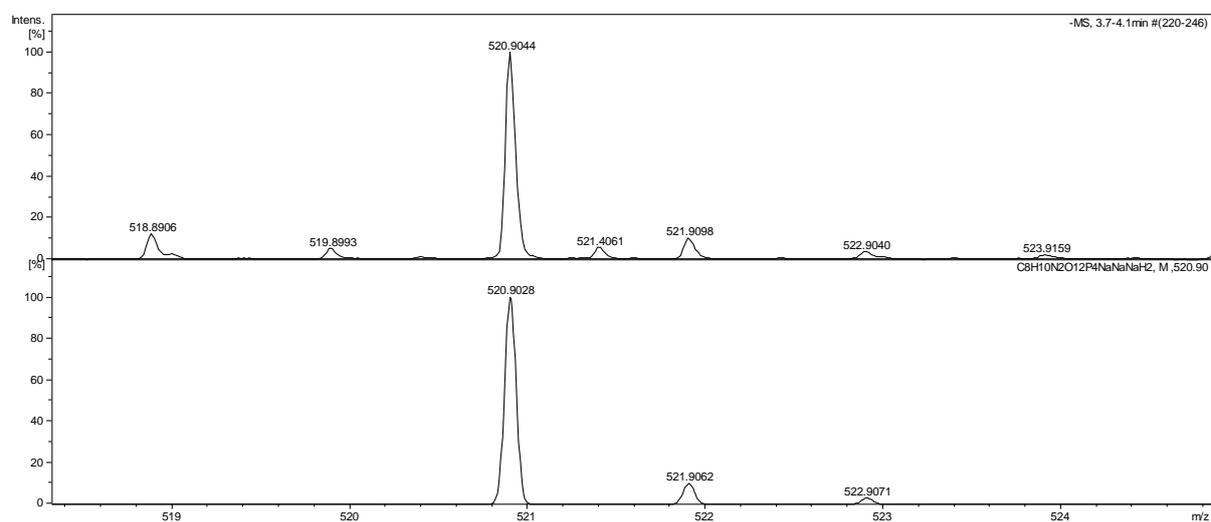
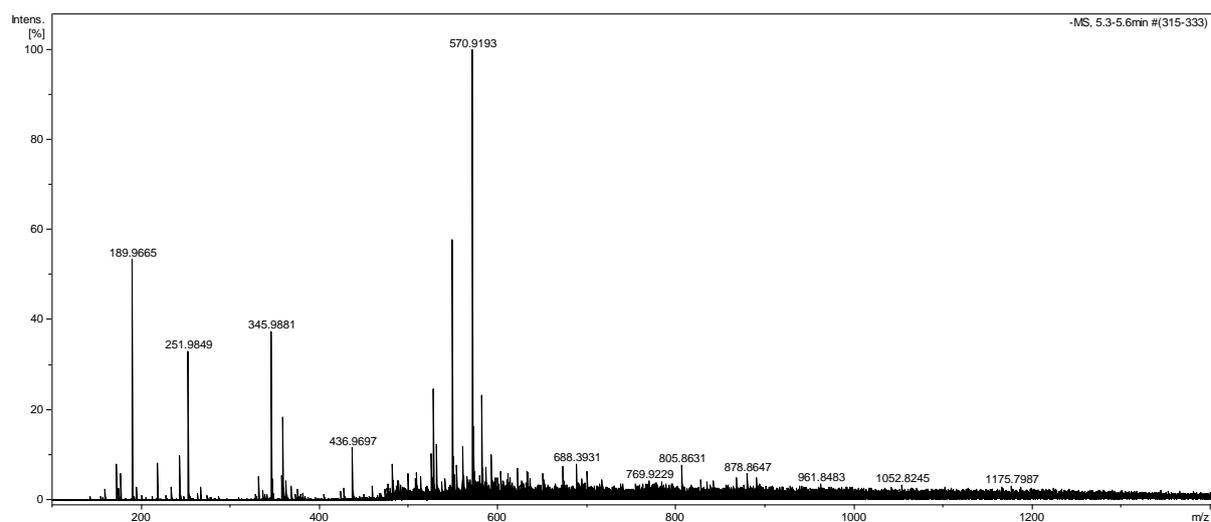


Figure S5. HRMS-ESI spectrum of WG12399C. A. Full HRMS-ESI spectrum. B. Expanded HRMS-ESI spectrum with measured (top) and simulated (bottom) $[M-4H+3Na]^-$ peak.

A.



B.

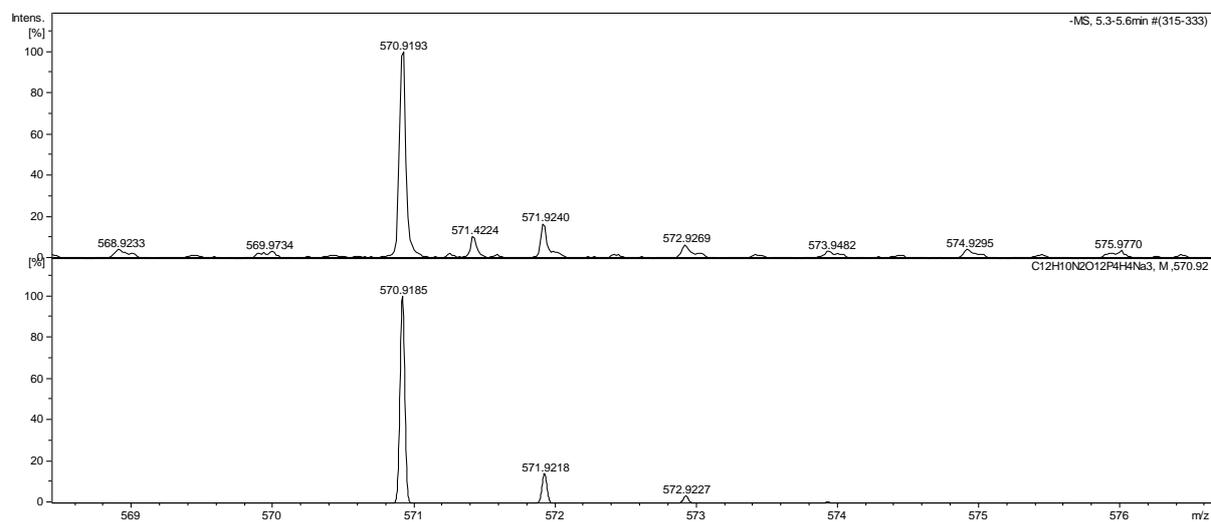


Figure S6. HRMS-ESI spectrum of WG12592A. A. Full HRMS-ESI spectrum. B. Expanded HRMS-ESI spectrum with measured (top) and simulated (bottom) $[M-4H+3Na]^+$ peak.

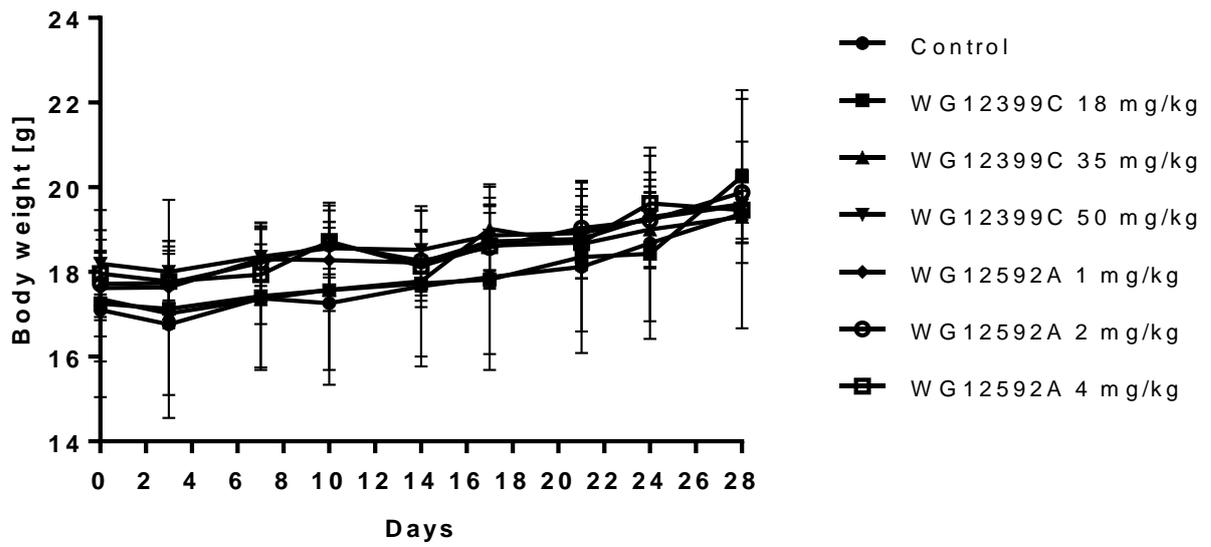
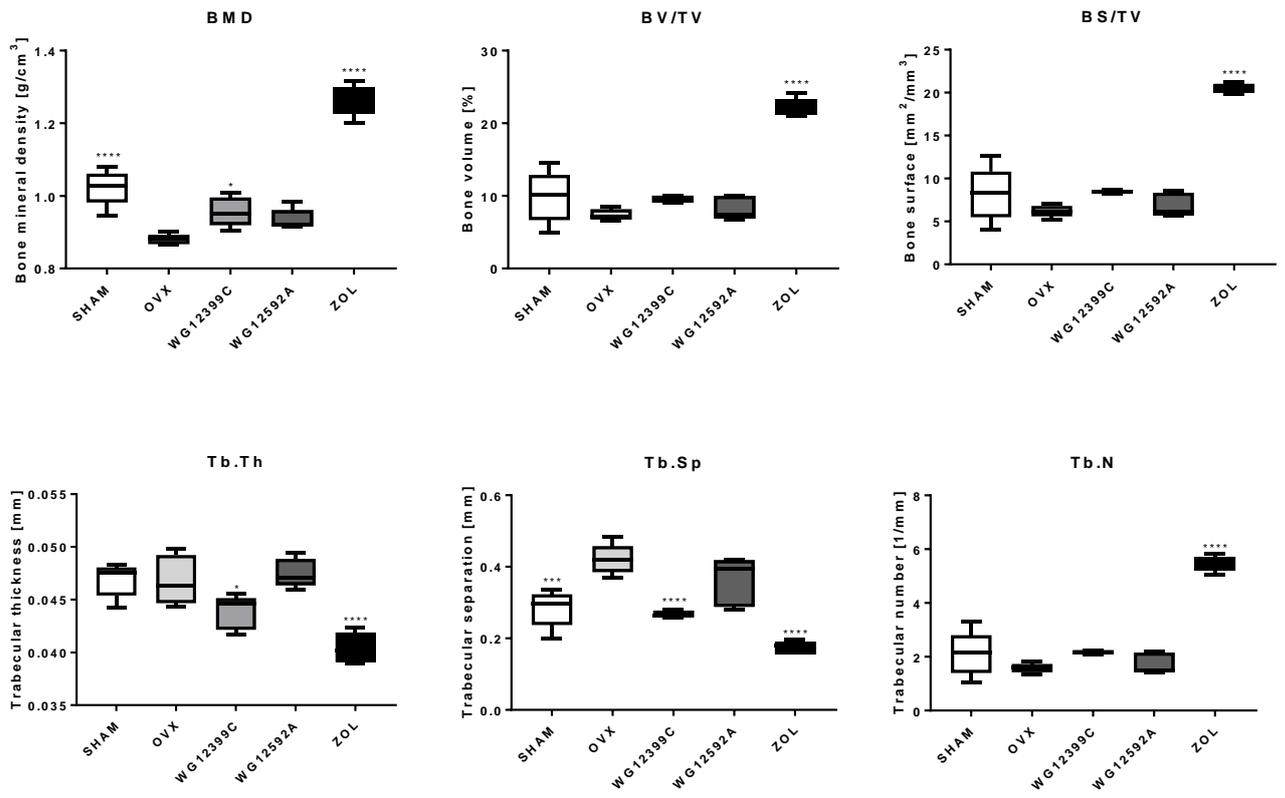


Figure S7. Body weight changes in mice receiving WG12399C and WG12592A. Starting from the day 0 mice were administered intravenously with the following total doses divided into four weekly injections: WG12399C - 18, 35 and 50 mg/kg; WG12592A - 1, 2 and 4 mg/kg. Data are expressed as mean \pm standard deviation, n=5.

A.



B.

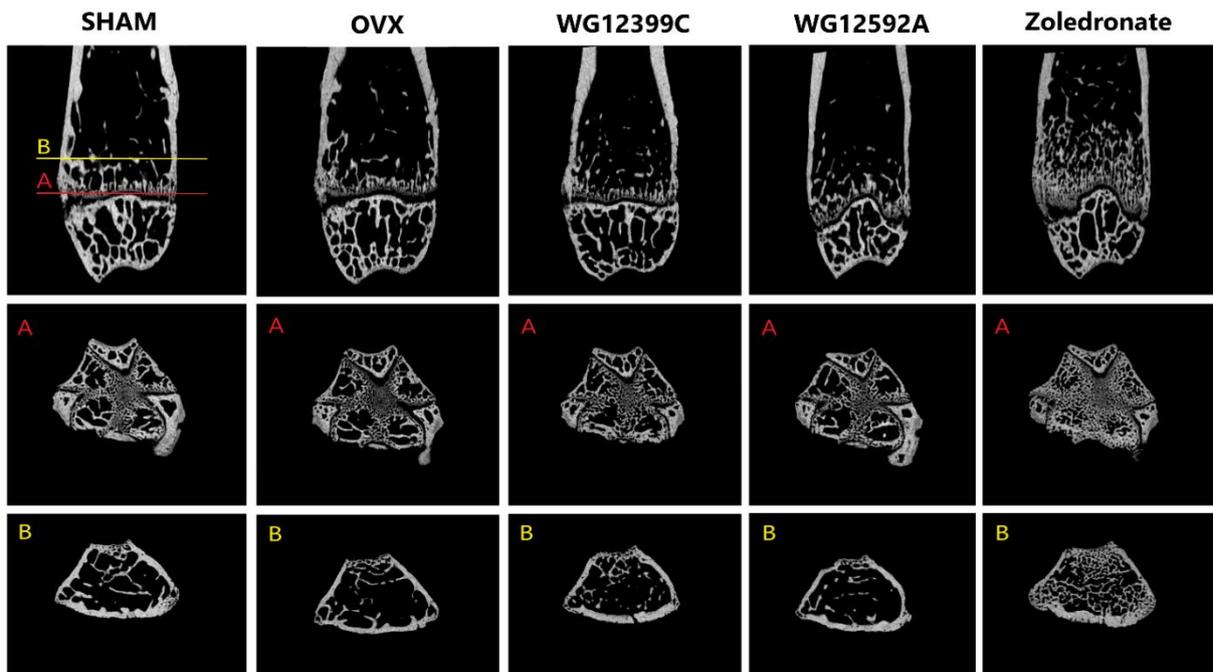


Figure S8. The effect of bisphosphonates on trabecular bone architecture of the distal femoral metaphysis. Mice were bilaterally ovariectomized and then administered intravenously with WG12399C 60 mg/kg, WG12592A 6 mg/kg or zoledronate 120 µg/kg in weekly doses. Vehicle-treated (OVX) and sham-operated (SHAM) mice served as controls. A. Histograms representing the structural parameters of the trabecular bone measured with µ-CT: BMD – bone mineral density; BV/TV - bone volume, BS/TV - bone surface, Tb. Th – trabecular thickness, Tb.Sp – trabecular separation, Tb.N – trabecular number. Data are expressed as mean ± standard deviation, n=5, *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001 vs OVX group were assessed with one-way analysis of variance. B. µ-CT images of the distal femurs (two upper lines, axial view of the metaphyseal region; bottom, longitudinal view).

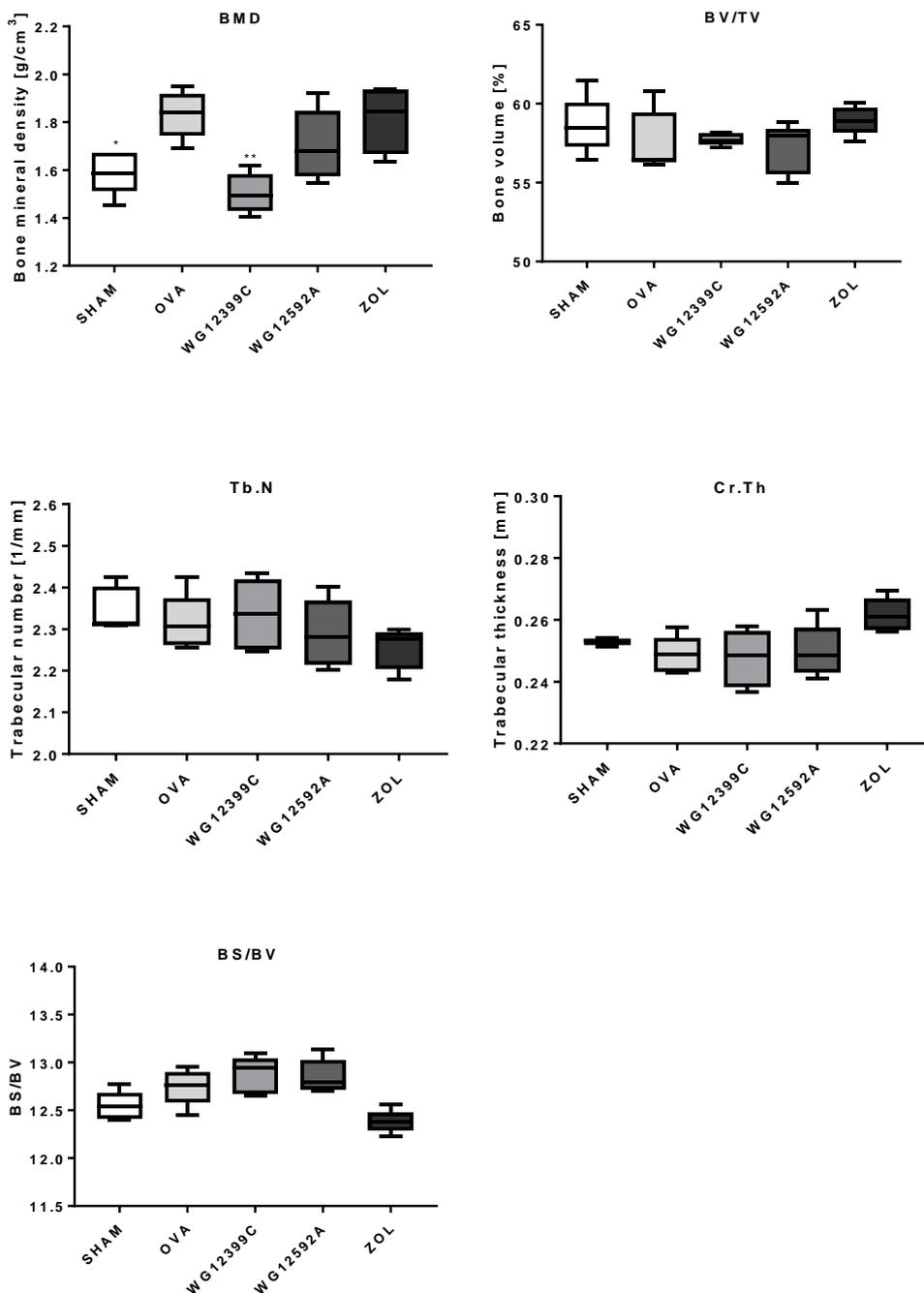


Figure S9. The effect of bisphosphonates on cortical bone architecture of the femoral shaft. Mice were bilaterally ovariectomized and then administered intravenously with WG12399C 60 mg/kg, WG12592A 6 mg/kg or zoledronate 120 μ g/kg in weekly doses. Vehicle-treated (OVX) and sham-operated (SHAM) mice served as controls. A. Histograms representing the structural parameters of the cortical bone measured with μ -CT: BMD – bone mineral density; BV/TV - bone volume; Tb.N – trabecular number; Cr.Th – trabecular thickness; BS/BV - bone surface/bone volume ratio. Data are expressed as mean \pm standard deviation, n=5, **p<0.01 vs OVX group were assessed with one-way analysis of variance.

Supplementary references:

1. Goldeman, W.; Nasulewicz-Goldeman, A. Synthesis and antiproliferative activity of aromatic and aliphatic bis[aminomethylidene(bisphosphonic)] acids. *Bioorganic Med. Chem. Lett.* **2014**, *24*, 3475–3479, doi:10.1016/j.bmcl.2014.05.071.