Supporting Information

Synthesis and Study of Alendronate Derivatives as Potential Prodrugs of Alendronate Sodium; for the Treatment of Low Bone Density and Osteoporosis

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Experimental, General: Unless specified otherwise, all materials were purchased form Sigma-Aldrich (Milwaukee, WI) and used as received. Sodium alendronate trihydrate was obtained from Merck Research Laboratories Sample Collection (Rahway, NJ), naphthalene-2,3-dicarboxaldhyde was purchased from Molecular Probes (Eugene, OR) and Male Sprague Dawley rats with body weight of 0.3 kg were from Charles River Laboratories (Wilmington, MA). ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded using a Brooker 400MHz instruments; observed HRMS values represent an average of two runs per sample. Preparative reverse phase liquid chromatography (RPHPLC) was performed using Waters MS Directed Purification System consisting of 2525 Binary Gradient Pump, 2767 Injector/Collector and 2996 PDA UV detector, mobile phase: gradient of water and acetonitrile (each cont. 0.1% TFA), column: Waters Xterra (50x3mm, 3.5 micron packing material). The quantitation of alendronate in urine, plasma, and in vitro buffers was accomplished by HPLC with fluorescent detection according to the previously published method; the assay's limit of reliable quantitation is 1ng/mL in urine and 5ng/mL in plasma and buffers.[1]

1: A 100-mL round bottomed flask was charged with alendronate sodium trihydrate (3.25g, 10mmol), benzyl chloroformate (2.5g, 15mmol) and 2M aqueous solution of sodium hydroxide (30mL, 60mmol) and the reaction was aged at room temperature for 36h. The crude reaction mixture was acidified with 1M aqueous hydrochloric acid to pH = 1 and concentrated, the solid residue combined with ethyl ether (100mL) and concentrated. The crude product was combined with water (20mL) and resulting suspension was stirred vigorously for 5h, filtered, solids were rinsed with water (2 x 10mL) and dried in desicator: ^{1}H NMR (400 MHz, CD_3OD) δ 1.88 (m, 2H), 2.03 (m, 2H), 3.15 (m, 2H), 5.04 (s, 2H), 2.27 (m, 1H), 2.32 (m, 4H); ^{13}C NMR (400 MHz, CD_3OD) δ 25.3, 32.4, 42.4, 49.6, 67.3, 113.5, 128.7, 128.9, 129.4, 159.0; ^{31}P NMR (400 MHz, CD_3OD) δ 21.4; HRMS theor. *m/z* 383.0535, obs. *m/z* 383.0537.

[1] Kline, W. F.; Matuszewski, B. K *J. Chromatogr.* **1992**, *583*, 183.

4: A 1000mL-oven-dried round bottomed flask under inert atmosphere of nitrogen was charged with 4-chlorobutyryl chloride (14g, 0.10mol). Triethyl phosphite (16.6g, 0.10mol) was added drop wise at 0°C via syringe over 5 minutes with stirring. The resulting mixture was allowed to reach room temperature and stirred additional 15 minutes after which, anhydrous methylene chloride (300mL) was added via canella. Diethyl phosphite (15.2g, 0.11mol) and imidazole (6.8g, 0.1mol) were added sequentially and the mixture was aged at room temperature for 1h, washed with 1M aqueous hydrochloric acid (2 x 300mL), dried over sodium sulfate and concentrated. Analytically pure **4** (15.6g, 41%) was obtained by column chromatography on silica gel using Biotage 75L (eluent: 15% ethanol in ethyl acetate): ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.28 (t, *J* = 7Hz, 12H), 2.06 (m, 4H), 3.48 (t, *J* = 4.3Hz, 2H), 4.15 (m, 8H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 16.1 (t, *J* = 3.1Hz), 25.4 (t, *J* = 6.1Hz) 31.5, 45.2, 63.5 (dt, *J* = 5.8, 13Hz), 73.8; ^{31}P NMR (400 MHz, $CDCl_3$) δ 20.7 HRMS theor. *m/z* 380.0921, obs. *m/z* 380.0917. Following are analytical data for rearranged byproduct **5**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.34 (m, 12H), 2.02 (m, 2H), 2.08 (m, 2H), 3.56 (m, 2H), 4.17 (m, 8H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 15.9 (m), 16.4 (m), 28.12, 28.2, 28.4, 44.2, 62.9 (m), 64.1 (m); ^{31}P NMR (400 MHz, $CDCl_3$) δ 20.5 (d, *J* = 22Hz), 0.11 (d, *J* = 22Hz); HRMS theor. *m/z* 380.0921, obs. *m/z* 380.0914.

6a: A 50mL-oven-dried round bottomed flask under inert atmosphere of nitrogen was charged with **4** (3.8g, 10mmol) and myristoyl chloride (7.4g, 30mmol) and the resulting mixture was heated to 60°C for 36h. Analytically pure **6a** (0.89g, 15%) was obtained by column chromatography on silica gel using Biotage 40L (eluent: ethyl acetate): ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, *J* = 7Hz, 3H), 1.25 (m, 22H), 1.36 (td, *J* = 7.5, 0.8Hz, 12H), 1.62 (m, 2H), 2.38 (m, 2H), 2.44 (m, 2H), 3.54 (t, *J* = 8Hz, 2H), 4.24 (m, 8H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 13.9, 16.6, 22.9, 25.0, 27.4 (m), 29.3, 29.5, 29.6, 29.6, 29.7, 29.7, 29.8, 29.9, 29.9, 30.1, 32.1, 34.5, 45.2, 64.0 (dt, J = 22, 4), 172; ^{31}P NMR (400 MHz, $CDCl_3$) δ 17.6; HRMS theor. *m/z* 590.2904, obs. *m/z* 590.2901.

7a: A 100mL-oven-dried round bottomed flask under inert atmosphere of nitrogen was charged with **6a** (0.59g, 1mmol), anhydrous dimethylformamide (25mL) and sodium azide (72mg, 1.1mmol) and the reaction was aged at to 60°C for 10h. The resulting mixture was combined with water (100mL) and methylene chloride (100mL) and organic layer seprated, washed with water (2 x 100mL), dried with sodium sulfate and concentrated. Analytically pure **7a** (100mg, 17%) was obtained by column chromatography on silica gel using Biotage 40L (eluent: ethyl acetate): ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.89 (t, *J* = 8Hz, 3H), 1.25 (m, 22H), 1.36 (td, *J* = 7.5, 0.7Hz, 12H), 1.62 (m, 2H), 2.39 (m, 2H), 2.47 (m, 2H), 3.27 (t, *J* = 8Hz, 2H), 4.29 (m, 8H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 14.0, 16.7, 22.7, 25.2, 27.4 (m), 29.4, 29.5, 29.6, 29.7, 29.7, 29.7, 29.8, 29.9, 29.9, 30.2, 32.1, 34.5, 41.2, 64.0 (dt, J = 21, 5), 172; ^{31}P NMR (400 MHz, $CDCl_3$) δ 17.7; HRMS theor. *m/z* 597.3308, obs. *m/z* 597.3306.

9a: A 100mL-3-neck-oven-dried round bottomed flask under inert atmosphere of nitrogen was charged with **7a** (100mg, 0.17mmol), anhydrous methylene chloride (30mL), acetic acid (0.1mL) and palladium on carbon (10% w/w, 0.017mmol) and the resulting mixture was hydrogenated under 1atm of hydrogen for 2h. The reaction mixture was filtered and concentrated to afford quantitatively **9a**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.86 (t, *J* = 6Hz, 3H), 1.27 (m, 20H), 1.36 (t, *J* = 7.5Hz, 12H), 1.62 (m, 2H), 1.84 (m, 2H), 2.03 (m, 2H), 2.02 (t, *J* = 7.1 Hz, 2H), 3.16 (m, 2H), 4.24 (m, 8H), 6.04 (s, 1H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 13.9, 22.6, 24.8, 29.0, 29.2, 29.2, 29.2,

29.3, 29.3, 29.4, 29.4, 29.5, 29.5, 29.6, 29.6, 29.9, 30.0, 34.1, 51.8, 159.9; ^{31}P NMR (400 MHz, $CDCl_3$) δ 20.1; HRMS theor. *m/z* 571.3403, obs. *m/z* 571.3399.

10: A 1000mL-oven-dried round bottomed flask under inert atmosphere of nitrogen was charged with 4-chlorobutyryl chloride (14g, 0.10mol). Triethyl phosphite (16.6g, 0.10mol) was added drop wise at 0°C via syringe over 5 minutes with stirring. The resulting mixture was allowed to reach room temperature and stirred additional 15 minutes after which, anhydrous methylene chloride (300mL) was added via canella. Diethyl phosphite (15.2g, 0.11mol) and 4-(dimethylamino)pyridine (12.2g, 0.10mol) were added sequentially, the reaction mixture was aged at room temperature for 1h and *tert*-butyldimethylsilyl chloride (16.5g, 0.11mol) was added and the reaction aged at room temperature for another 15h. The reaction mixture was washed with 0.1M aqueous hydrochloric acid (2 x 300mL), dried over sodium sulfate and concentrated. Analytically pure **10** (22.3g, 45%) was obtained by column chromatography on silica gel using Biotage 75L (eluent: 50% ethyl acetate in n-heptane): ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.13 (s, 6H), 0.83 (s, 9H), 1.30 (t, *J* = 7.0 Hz, 12H), 2.07 (m, 2H), 2.16 (m, 2H), 3.49 (t, *J* = 6.4 Hz, 2H), 4.07 (m, 8H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 16.4, 19.0, 25.1, 27.1, 33.4, 45.4, 53.7, 64.2 (m); ^{31}P NMR (400 MHz, $CDCl_3$) δ 19.21; HRMS theor. *m/z* 494.1785, obs. *m/z* 494.1789.

11: A 100mL-oven-dried round bottomed flask under inert atmosphere of nitrogen was charged with **10** (4.95g, 10mmol), anhydrous dimethylformamide (50mL) and sodium azide (1.3g, 20mmol) and the reaction was aged at 75°C for 2h. The resulting mixture was combined with water (100mL) and dichloromethane (200mL) and organic layer seprated, washed with water (2 x 200mL), dried with sodium sulfate and concentrated. Anaytically pure **11** (4.06g, 82%) was obtained by column chromatography on silica gel using Biotage 40L (eluent: ethyl acetate): ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.205 (s, 6H), 0.913 (s, 9H), 1.352 (t, *J* = 7.0 Hz, 12H), 1.950–2.240 (m, 4H), 3.277 (t, *J* = 6.4 Hz, 2H), 4.160–4.320 (m, 8H); ^{13}C NMR (400 MHz, $CDCl_3$) δ -2.618, 16.469, 19.010, 23.703, 25.779, 33.175, 51.841, 62.970 (m); ^{31}P NMR (400 MHz, $CDCl_3$) δ 20.492; HRMS theor. *m/z* 501.2267, obs. *m/z* 501.2277.

12: A 100mL-3-neck-oven-dried round bottomed flask under inert atmosphere of nitrogen was charged with **10** (4.0g, 8.1mmol), anhydrous ethyl acetate (100mL), and palladium on carbon (10% w/w, 0.8mmol) and the resulting mixture was hydrogenated under 50psi of hydrogen for 15h. The reaction mixture was filtered and concentrated yielding quantitatively analytically pure **12**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.151 (s, 6H), 0.872 (s, 9H), 1.354 (q, *J* = 6.8 Hz, 12H), 1.950–2.230 (m, 4H), 2.981 (br, 2H), 4.197 (m, 8H), 8.048 (br, 2H); ^{13}C NMR (400 MHz, $CDCl_3$) δ -2.242, 16.478, 18.991, 22.801, 25.772, 33.176, 40.359, 63.896, 64.117; ^{31}P NMR (400 MHz, $CDCl_3$) δ 19.500; HRMS theor. *m/z* 475.2362, obs. *m/z* 475.2365.

13a: A 250mL-oven-dried round bottomed flask under inert atmosphere of nitrogen was charged with **12** (4.75g, 10mmol), anhydrous methyle chloride (100mL) and triethyl amine (20.2g, 20mmol). To this mixture, myristoyl chloride (5.0g, 20mmol) was added dropwise 0°C via syringe over 5 minutes and the resulting mixture was aged at room temperature for 2h. The resulting mixture was combined with saturated solution of sodium bicarbonate (200mL) and organic layer seprated, washed with 0.1M aqueous solution of HCl (150mL) and water (200mL), dried with sodium sulfate and concentrated. Anaytically pure **13a** (5.2g, 76%) was obtained by column chromatography on silica gel using Biotage 40L (eluent: gradient of 0 → 100% of ethyl

acetate in hexanes): ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.187 (s, 6H), 0.896 (s, 9H), 1.349 (t, *J* = 7.2 Hz, 12H), 2.000–2.200 (m, 4H), 2.300–2.700 (m, 27H), 3.262 (t, *J* = 6.4 Hz, 2H), 4.100–4.300 (m, 8H), 6.578 (s, 1H); ^{13}C NMR (400 MHz, $CDCl_3$) δ -2.444, 14.099, 16.380, 18.913, 22.673, 23.622, 25.701, 25.895, 29.276, 29.341, 29.450, 29.597, 29.632, 29.663, 31.909, 33.001, 36.447, 40.010, 63.574, 63.609, 159.170; ^{31}P NMR (400 MHz, $CDCl_3$) δ 19.823; HRMS theor. *m/z* 685.4346, obs. *m/z* 685.4363.

13b (5.3g, 87%) was prepared from **12** and acetyl chloride by the procedure described for **13a**. **13b**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.187 (s, 6H), 0.898 (s, 9H), 1.347 (t, *J* = 6.8 Hz, 12H), 1.992 (s, 3H), 2.000–2.150 (m, 4H), 3.245 (dt, *J* = 6.4, 6.4 Hz, 2H), 4.150–4.270 (m, 8H) 6.034 (s, 1H); ^{13}C NMR (400 MHz, $CDCl_3$) δ -2.211, 16.694, 19.200, 23.007, 23.738, 26.008, 33.350, 40.421, 63.304, 63.458, 171.365; ^{31}P NMR (400 MHz, $CDCl_3$) δ 20.500; HRMS theor. *m/z* 517.2468, obs. *m/z* 517.2462.

13c: A 100mL-oven-dried round bottomed flask under inert atmosphere of nitrogen was charged with **12** (475mg, 1mmol), anhydrous methyle chloride (25mL), EDC chloride (192mg, 1mmol), oleic acid (282mg, 1mmol) and diisopropylethyl amine (400mg, 3mmol) and the reaction was aged at room temperature for 2h. Purification was accomplished using MS directed reverse-phase liquid chromatography. Anaytically pure **13c** (4.06g, 82%) was obtain after lyophilization: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.184 (s, 6H), 0.895 (s, 9H), 1.220–1.320 (m, 13H), 1.347 (t, *J* = 7.2 Hz, 12H), 1.623 (m, 2H), 1.878 (m, 2H), 1.970–2.280 (m, 18H), 3.256 (m, 2H), 4.209 (m, 8H), 5.338 (m, 2H), 6.063 (s, 1H); ^{13}C NMR (400 MHz, $CDCl_3$) δ -2.448, 14.095, 16.434, 18.944, 22.665, 25.755, 25.868, 27.181, 27.204, 29.140, 29.260, 29.299, 29.512, 29.725, 29.756, 31.889, 33.102, 36.602, 39.909, 63.148, 63.276, 129.743, 129.976; ^{31}P NMR (400 MHz, $CDCl_3$) δ 20.307; HRMS theor. *m/z* = 739.4816, obs. *m/z* = 739.4813.

13d (4.9g, 81%) was prepared from **12** and benzyl chloroformate by the procedure described for **13a**. **13d**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.175 (s, 6H), 0.888 (s, 9H), 1.330 (dt, *J* = 7.2, 1.2 Hz, 12H), 1.800–1.910 (m, 2H), 2.000–2.200 (m, 2H), 3.150–3.260 (m, 2H), 4.100–4.300 (m, 8H), 4.900 (s, 1H), 5.091 (s, 2H), 7.270–7.400 (m, 5H); ^{13}C NMR (400 MHz, $CDCl_3$) δ -2.529, 16.384, 18.936, 25.724, 32.927, 41.276, 63.260, 63.365, 66.595, 128.063, 128.485; ^{31}P NMR (400 MHz, $CDCl_3$) δ 20.108; HRMS theor. *m/z* = 609.2730, obs. *m/z* = 609.2738.

14a: A 100mL-oven-dried round bottomed flask under inert atmosphere of nitrogen was charged with **13a** (685mg, 1mmol), anhydrous acetonitrile (25mL) and trimethylsilyl iodide (1.6g, 8mmol) and the reaction was aged at room temperature for 2h. Methanol (20mL) was added to the mixture via syringe over 10 minutes and the reaction aged at room temperature additional 30 minutes and concentrated. Purification was accomplished using MS directed reverse-phase liquid chromatography. Anaytically pure **14a** (520mg, 90%) was obtain after lyophilization: ^{1}H NMR (400 MHz, CD_3OD) δ 0.21 (s, 6H), 0.91 (s, 9H), 1.22 (m, 21H), 1.58 (m, 2H), 1.89 (m, 2H), 2.04 (m, 2H), 2.15 (t, *J* = 7.4 Hz, 2H), 3.15 (t, *J* = 5.4 Hz, 2H), ^{13}C NMR (400 MHz, CD_3OD) δ -2.444, 14.099, 16.39, 18.7, 23.6, 25.4, 25.8, 29.1, 29.4, 29.5, 29.7, 29.7, 29.8, 31.7, 36.3, 40.0, 63.52, 63.61, 152.13; ^{31}P NMR (400 MHz, $CDCl_3$) δ 20.5; HRMS theor. *m/z* = 573.3016, obs. *m/z* = 573.3018.

15a: A 5mL-oven-dried round bottomed flask under inert atmosphere of nitrogen was charged with **14a** (573mg, 1mmol) and 1M solution of TBAF in tetrahydrofuran (6mL, 6mmol), the reaction was aged at room temperature for 2h and concentrated. Purification was accomplished using MS directed reverse-phase liquid chromatography. Anaytically pure **15a** (450mg, 98%) was obtain after lyophilization: ^{1}H NMR (400 MHz, CD_3OD) δ 0.83 (t, J = 7.0, 3H), 1.27 (m, 21H), 1.58 (m, 2H), 1.88 (m, 2H), 2.04 (m, 2H), 2.15 (t, *J* = 7.4 Hz, 2H), 3.18 (t, *J* = 5.8 Hz, 2H), ^{13}C NMR (400 MHz, CD_3OD) δ 13.9, 22.0, 24.0, 26.5, 28.3, 29.7, 30.3, 30.4, 30.5, 30.5, 30.6, 30.6, 30.7, 31.7, 32.7, 35.1, 37.1, 176.5; ^{31}P NMR (400 MHz, $CDCl_3$) δ 21.4; HRMS theor. *m/z* = 459.2151, obs. *m/z* = 459.2147.

15b (240mg, 82%) was prepared from **13b** by procedure described for **15a**. **15b**: ^{1}H NMR (400 MHz, CD_3OD) δ 1.800–1.960 (m, 2H), 1.919 (s, 3H), 1.960–2.120 (m, 2H), 3.168 (t, *J* = 6.8 Hz, 2H); ^{13}C NMR (400 MHz, CD_3OD) 21.322, 23.557, 31.181, 39.867, 57.050, 172.174; ^{31}P NMR (400 MHz, CD_3OD) δ 21.390; HRMS theor. *m/z* = 291.0273, obs. *m/z* = 291.0269.

In vivo testing of alendronate pro-drugs: The dosing solutions were prepared by dissolving alendronic acid and **1** in water and **15a** in 2% polysorbate-80, respectively. Four groups of rats (n = 3 rats/group) were dosed by i.v. with alendronic acid, **1**, **15a**, and **15b**, respectively, at a dose equivalent 0.1mpk of free alendronic acid. For oral bioavailability studies, the rats fasted for approximately 12h prior to the oral dosing, then the food was returned to the rats 12h post-dosing. Each group of rats (n = 3/group) was orally dosed with alendronic acid, **1**, **15a** and **15b**, at equivalent to free alendronic acid of 5mpk, 2mpk, and 1mpk. Urine was collected for 24h pre-dosing, and for 24h and post-dosing for all the animals (no significant additional amounts of alendronic acid were excreted in 24-48h period post-dosing).

In vitro testing of alendronate pro-drugs: Uptake and conversion of alendronate prodrug **15a** across rat intestinal tissue was studied using chamber setup following existing protocols.[2] Briefly, small sections of excised rat intestine (upper jejunum or lower ileum), 2.5 to 3 cm in length, were opened along the mesenteric border and secured in side-by-side diffusion chambers. Assembled chambers were placed in a 37°C heating block, connected to a 95% O_2/5% CO_2 airlift, and filled with 5ml of 37°C Krebs-Ringer Bicarbonate buffer, pH 7.4. The diffusion chambers and the airlift/heating block assembly were purchased from Navicyte/Harvard Apparatus. After a 15-min equilibration period, the original buffer was replaced with warm fresh buffer or drug solution in the same buffer. Drug concentration used was 0.25-25μg/mL. All experiments were conducted in the mucosal (donor)-to-serosal (receiver) direction. Transport was allowed to proceed for 1 or 2h periods. At the end of the incubation period samples were taken from donor and receiver chambers. Samples were analyzed by an HPLC-UV method that allows for alendronate detection.

The stability of the prodrugs in urine at acidic condition: 50 uL of alendronic acid (15 ug/mL), 50 uL of **1** (15 ug/mL), and 50 uL of **15a** (approx 15 ug/mL), respectively, were spiked into three sets of test tubes each containing 5mL of urine. The pH was then adjusted to 2.0 using 6M aqueous HCl. One test tube of each set was placed in a 5°C refrigerator as control. One test tube of each set was incubated at 60°C for 1.5h, and another was incubated at 60°C for 3h. All samples were extracted and analyzed using the above method. Data indicated no detectable conversion of **1** and **15a** to alendronic acid in the acidified urine samples.

[2] Li, L.Y.; Amidon, G.L.; Kim, J.S.; Heimbach, T.; Kesisoglou, F.; Topliss, J.T.; Fleisher, D. *J. Pharm. Exp. Ther.* **2002**, *301(2)*, 586.

The stability of the prodrugs in fresh human plasma: Buffered saline with 50mM HEPES at pH 7.8 was used as a control medium; alendronic acid, **1**, and **15a** stock solution were spiked into the plasma and the control medium to make final samples of 10 ug/mL which were incubated at 37°C for 0h, 1h, 2h, and 16h. No detectable alendronic acid was found in the samples containing **1** and **15a** in either plasma or buffered saline.

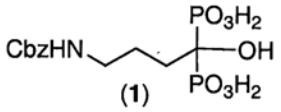
Conditions for the HPLC analyses; with the exception of compounds **1**, **15a** and **15b**, all HPLC analyses reflect mass ionization (Electrospray Positive Ionization, Full Scan mode) as the absence of any chromophore in compounds **4-14** rules out the UV detection. HPLC traces for the key compounds immediately follow the HPLC conditions description (pages S-7 to S-18).

HPLC Conditions 1: Mass Spectrometer: Micromass ZQ single quadrupole, Electrospray Positive Ionization, Full Scan mode (150-750amu in 0.5s); HPLC: Agilent 1100, Binary Pump; DAD UV detector: Hardware/software Waters/Micromass MassLynx 4.0; Column: Waters Xterra, 3.0 mm Width, 50 mm Length, 3.5 micron packing material; Runtime: 5.5 min; Flow Rate: 1.0 ml /min.; Mobile Phase A = Water + 0.05% TFA, B = Acetonitrile + 0.05% TFA; Gradient: Time/%A/%B: 0.00/90/10, 3.75/2/98, 4.75/2/98, 4.76/90/10, 5.5/90/10.

HPLC Conditions 2: The HPLC/DAD/MS instrument consisted of a Waters Micromass ZQ ESI probe and Agilent 1100 HPLC. Purity determination was performed by HPLC/DAD/MS analysis under the following condition: gradient [A = H2O + 10 mM Ammonium formate, B = MeOH + 10 mM Ammonium formate, 5% B to 100% B in 11.3 minutes, 100% B for 1.7 minutes, 100% B to 5% B in 0.2 minutes, 5% B for 1.8 minutes] at 1.4 ml/min on the column Sunfire C18 (5 μ, 4.6 x 100 mm) at 40°C with diode array detection and injection volume of 2 or 5 μL.

HPLC Conditions 3: Mass Spectrometer: Micromass ZQ single quadrupole, Electrospray Positive Ionization, Full Scan mode (150-750amu in 0.5s); HPLC: Agilent 1100, Binary Pump; DAD UV detector: Hardware/software Waters/Micromass MassLynx 4.0; Column: Waters Xterra, 3.0 mm Width, 50 mm Length, 3.5 micron packing material; Runtime: 5.5 min; Flow Rate: 1.0 ml /min.; Mobile Phase A = Water + 0.05% TFA, B = Acetonitrile + 0.05% TFA; Gradient: Time/%A/%B: 0.00/90/10, 1.80/0/100, 4.75/0/100, 4.76/90/10, 5.5/90/10.

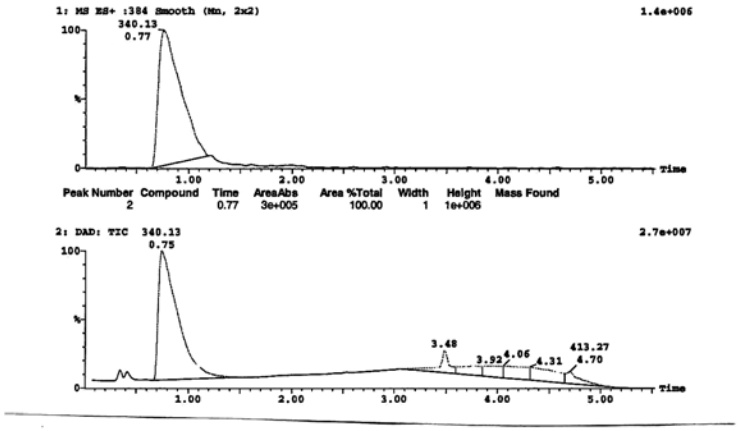
HPLC Conditions 4: Waters MS Directed System: 2525 Binary Gradient Pump, 2767 Injector, 2996 PDA UV detector, Controlled by Waters/Micromass MassLynx 4.0 software, Mass Spectrometer: Micromass ZQ single quadrupole, Electrospray Positive Ionization, Full Scan mode (150-1000amu), Column: Waters Sunfire 30 mm Width, 100 mm Length, 5micon packing material 19 x 10 mm Pre-Column, Runtime: 15 min, Flow Rate: 50mL/min, Mobile Phase: A = Water + .1 % TFA, B= Acetonitrile + .1 % TFA, Gradient: Time/%A/%B: 0.00/60/40, 2.00/60/40, 10.5/0/100, 13.5/0/100, 13.6/60/40, 15/60/40.



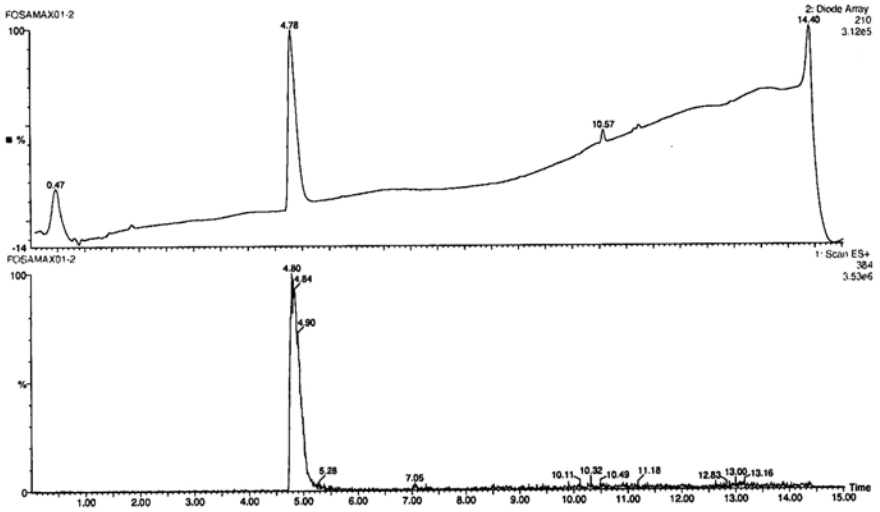
(**1**)

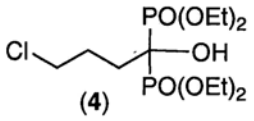
HPLC analyses in two diverse systems

HPLC Conditions 1



HPLC Conditions 2

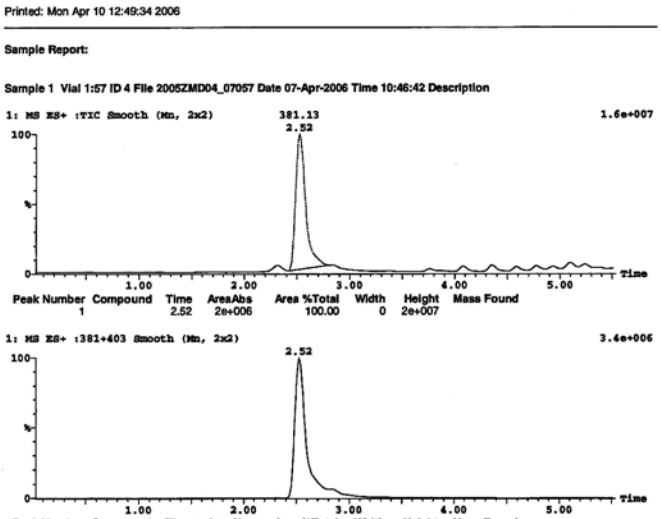




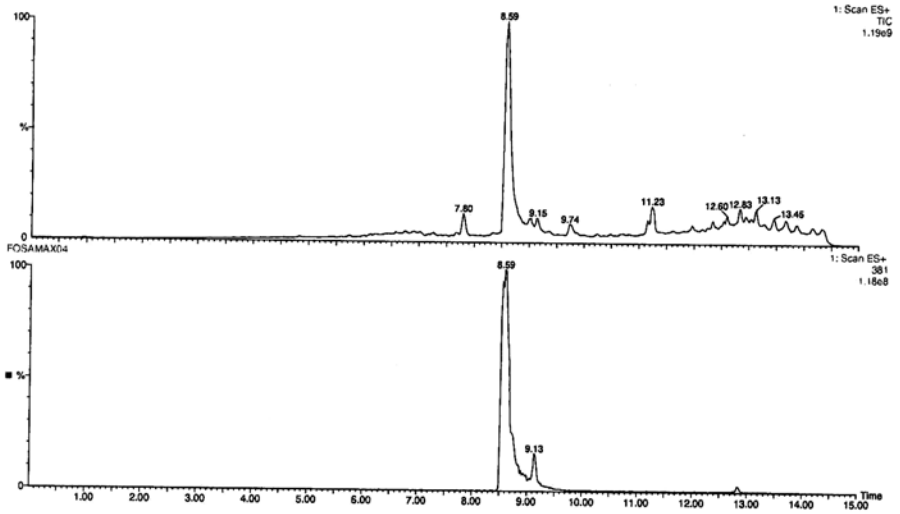
(**4**)

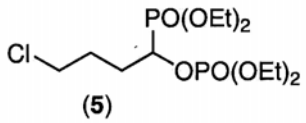
HPLC analyses in two diverse systems

HPLC Conditions 1



HPLC Conditions 2

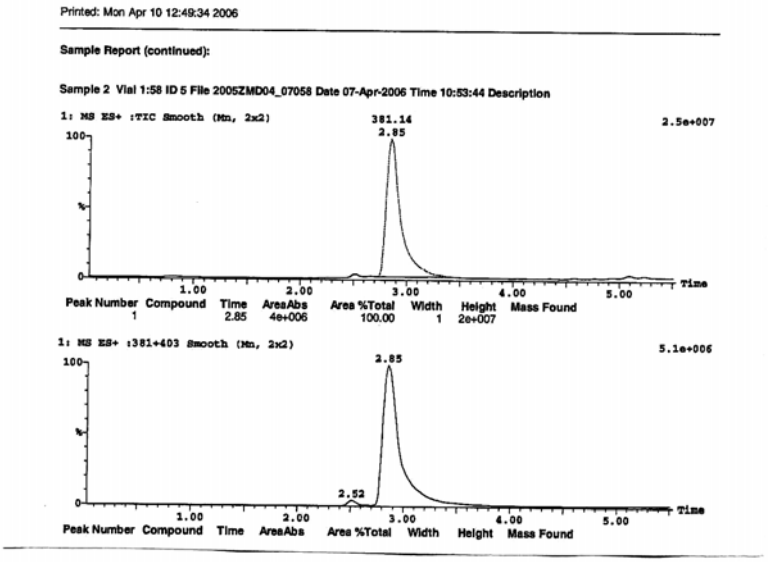




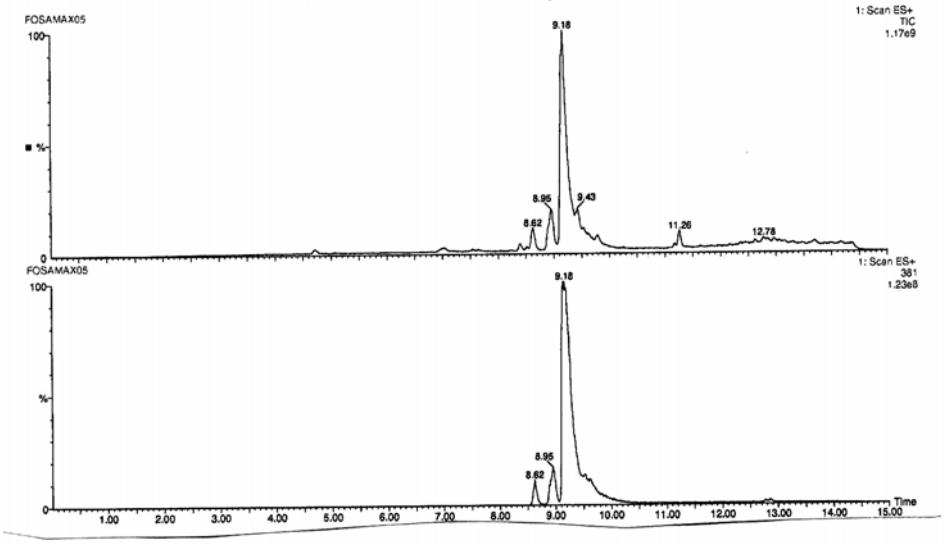
(**5**)

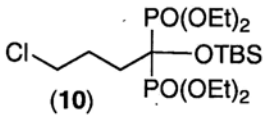
HPLC analyses in two diverse systems

HPLC Conditions 1



HPLC Conditions 2

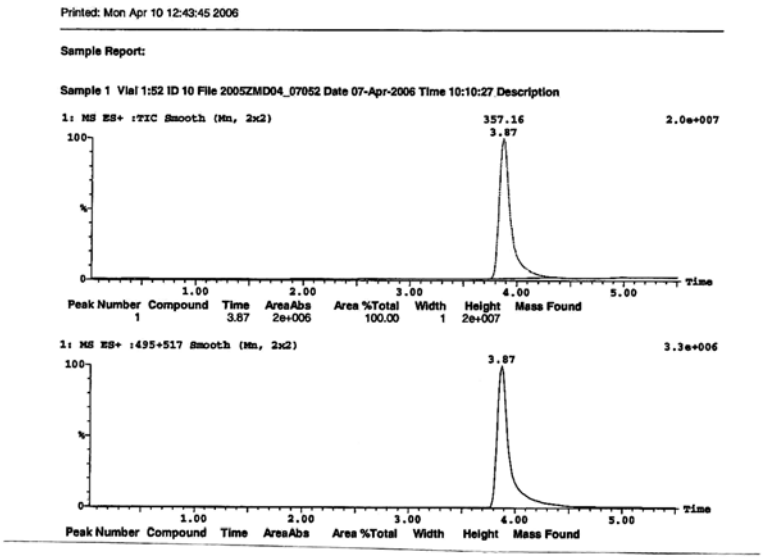




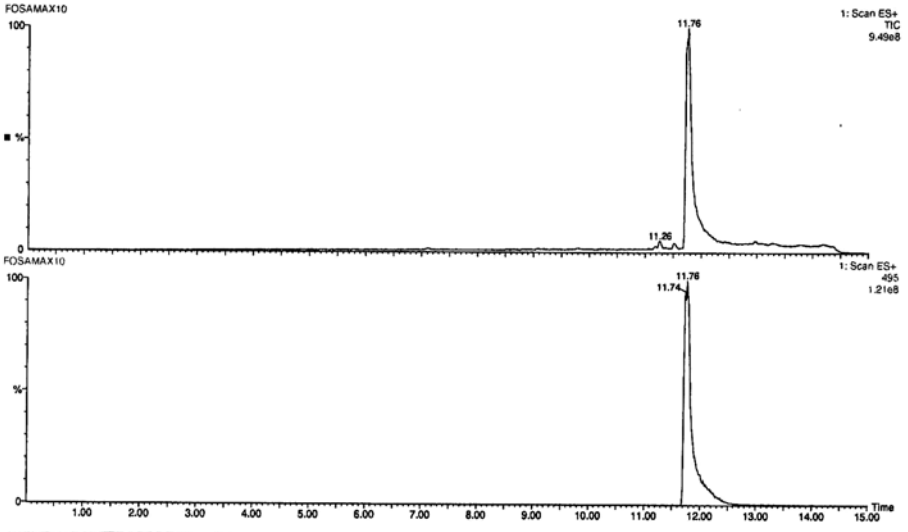
(**10**)

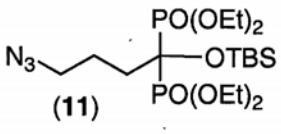
HPLC analyses in two diverse systems

HPLC Conditions 1



HPLC Conditions 2

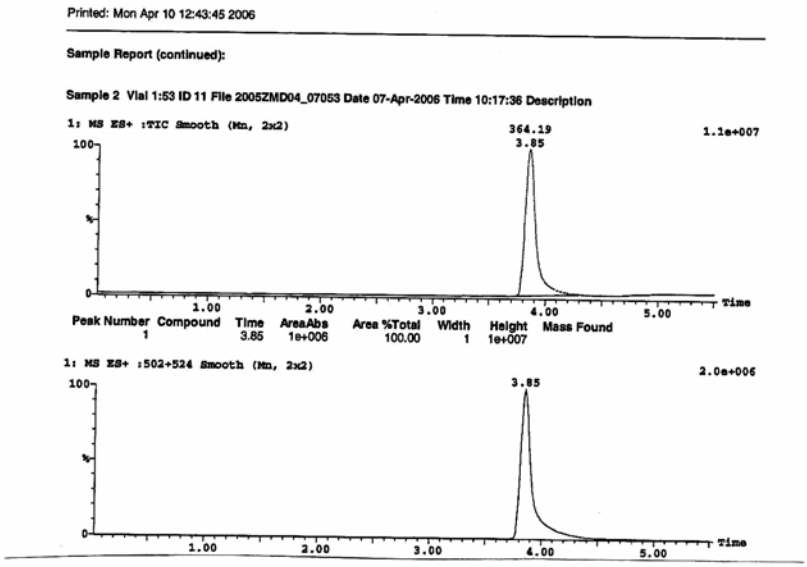




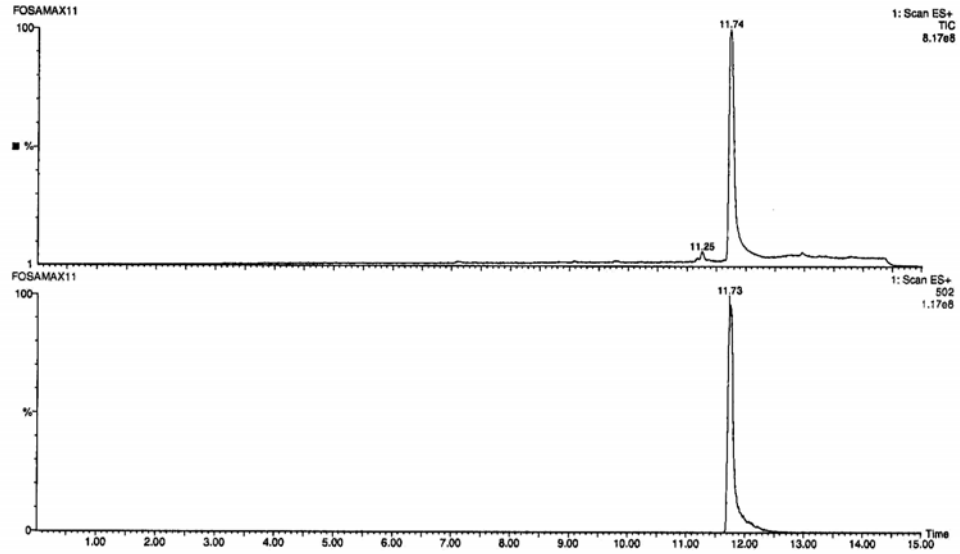
(**11**)

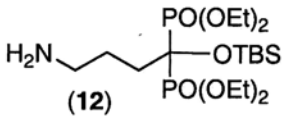
HPLC analyses in two diverse systems

HPLC Conditions 1



HPLC Conditions 2

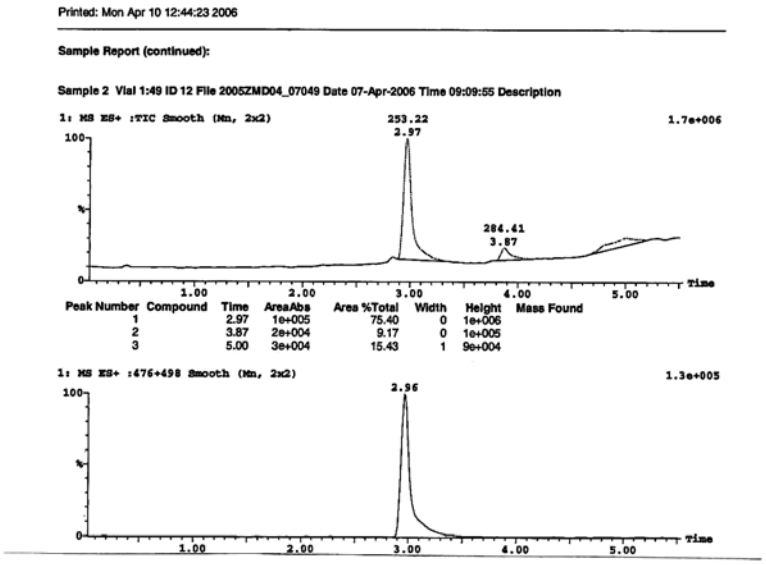




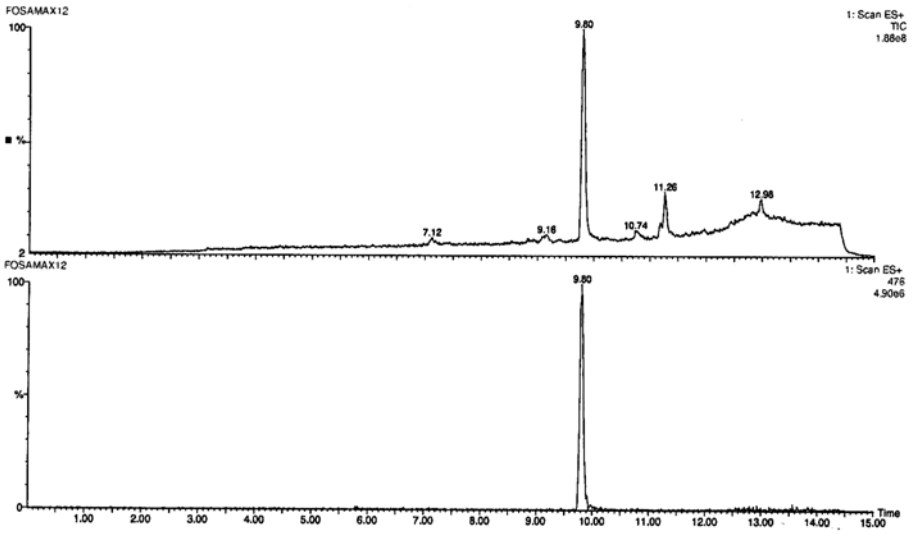
(**12**)

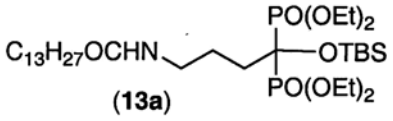
HPLC analyses in two diverse systems

HPLC Conditions 1



HPLC Conditions 2

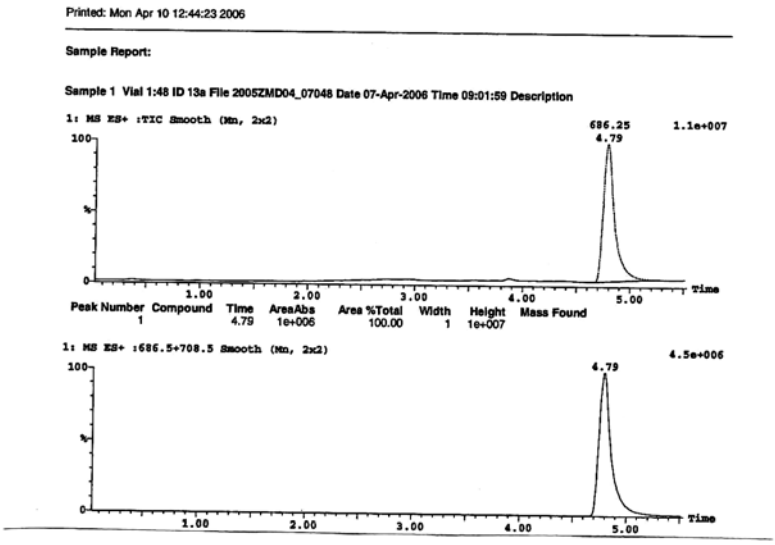




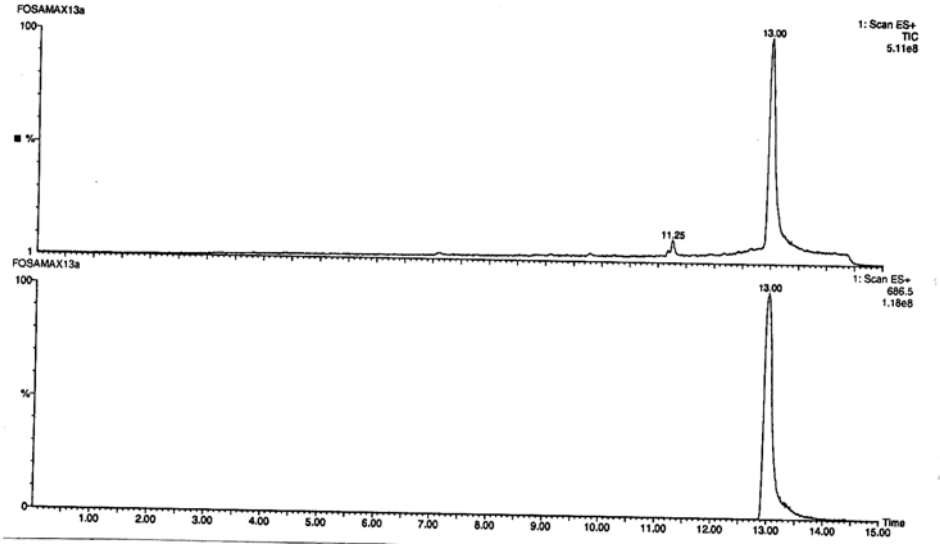
(13a)

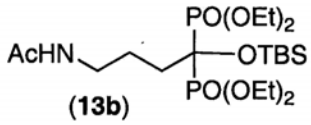
HPLC analyses in two diverse systems

HPLC Conditions 1



HPLC Conditions 2

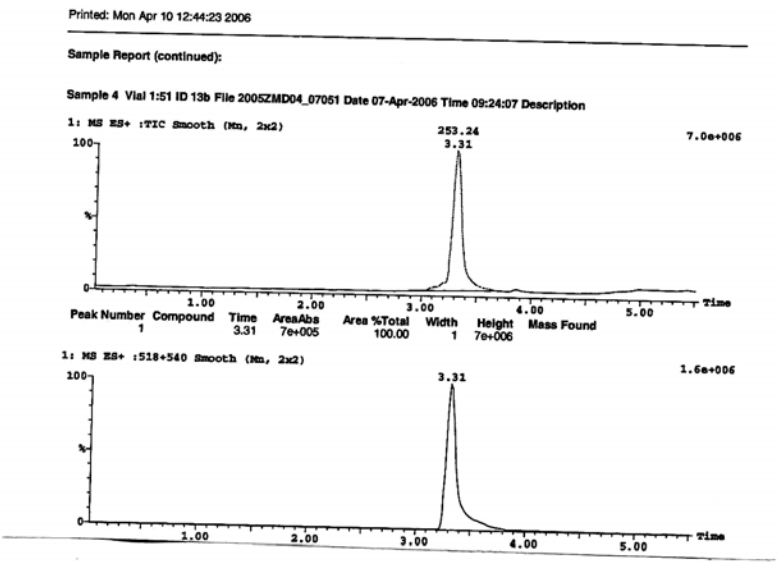




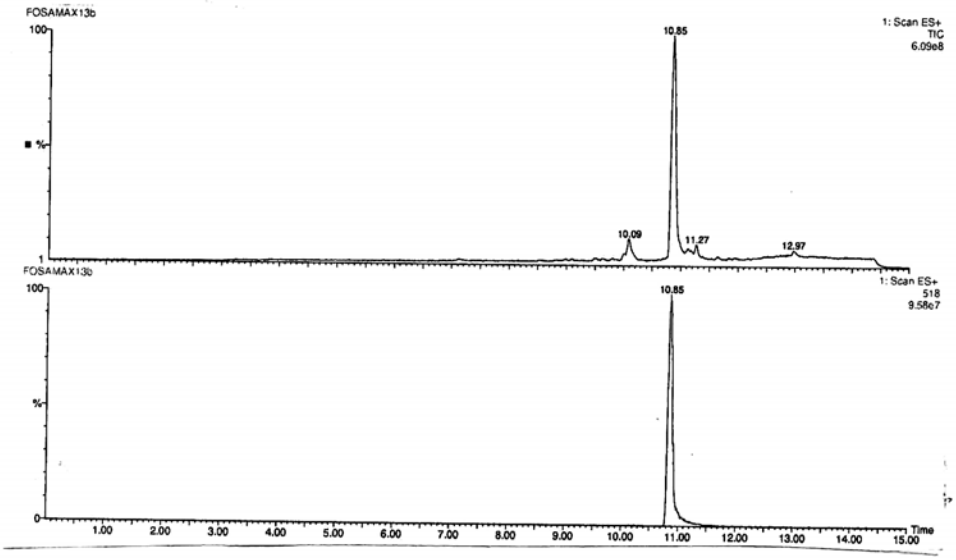
(13b)

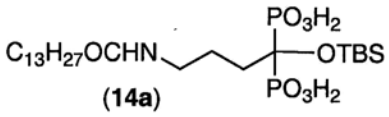
HPLC analyses in two diverse systems

HPLC Conditions 1



HPLC Conditions 2

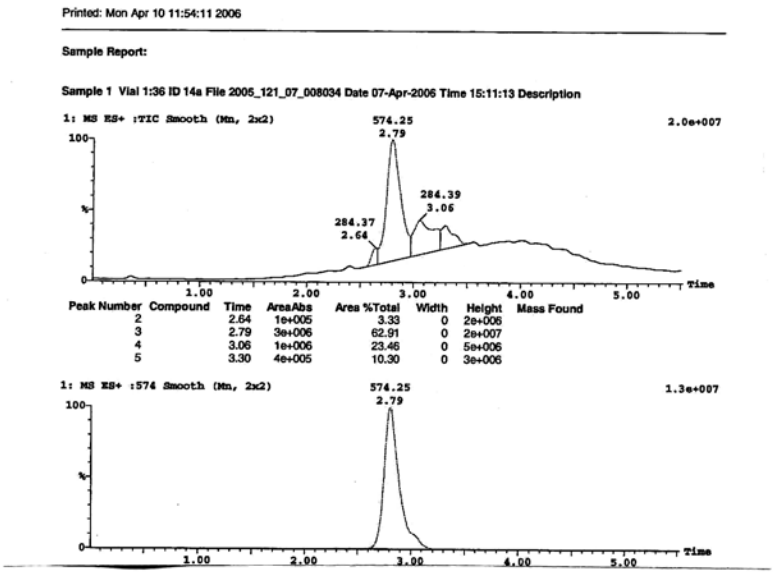




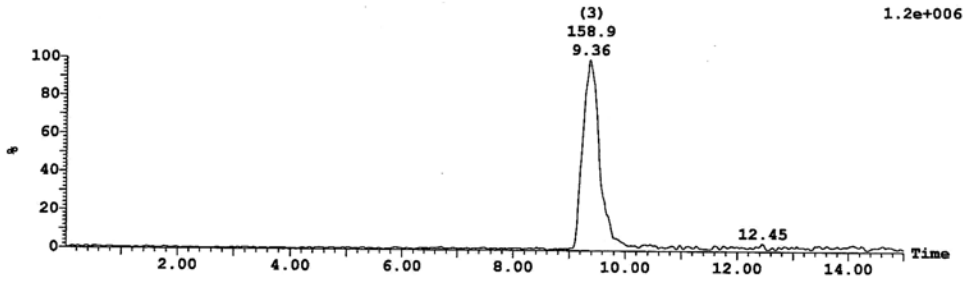
(**14a**)

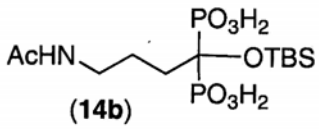
HPLC analyses in two diverse systems

HPLC Conditions 3



HPLC Conditions 4





(14b)

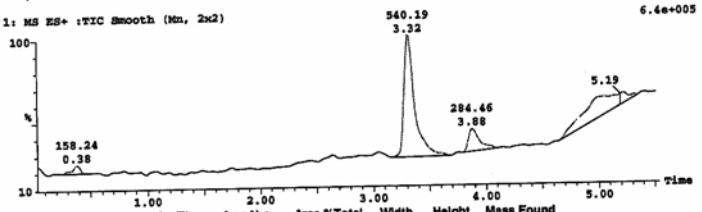
HPLC analyses in two diverse systems

HPLC Conditions 1

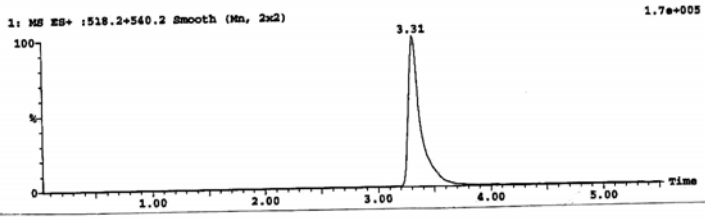
Printed: Mon Apr 10 11:54:55 2006

Sample Report:

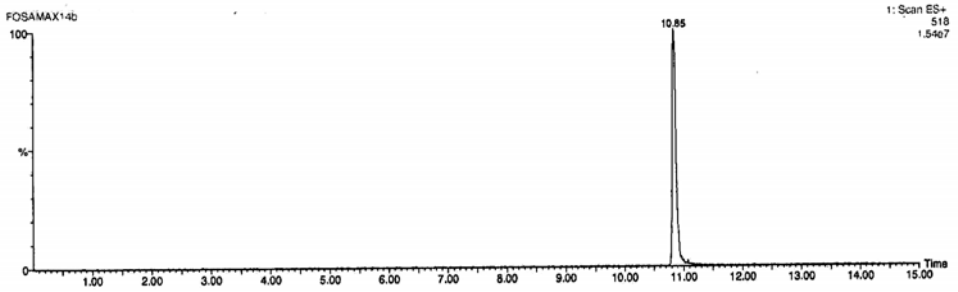
Sample 1 Vial 1:75 ID 14b File 2005ZMD04_07075 Date 07-Apr-2006 Time 14:19:03 Description

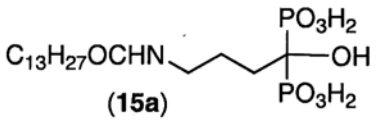


Peak Number	Compound	Time	AreaAbs	Area %Total	Width	Height	Mass Found
1		0.38	3e+003	2.79	0	3e+004	
2		3.32	5e+004	55.13	1	5e+005	
3		3.88	1e+004	10.50	0	9e+004	
4		5.19	3e+004	28.02	1	4e+004	
5		5.22	3e+003	3.56	0	4e+004	



HPLC Conditions 2

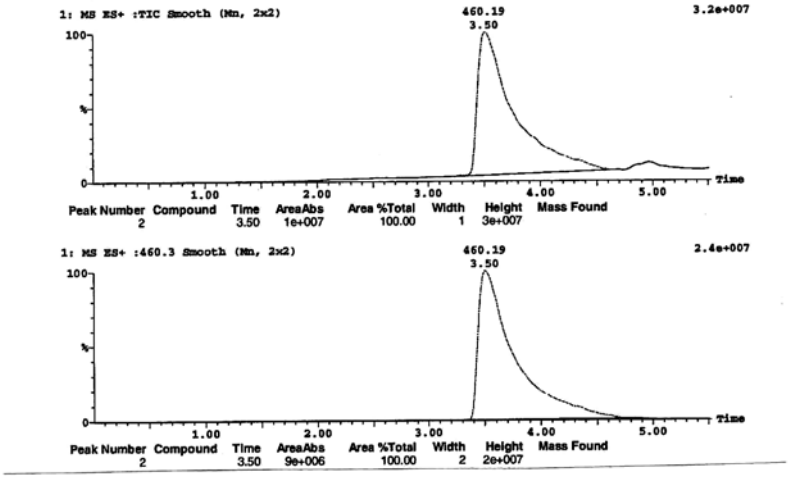




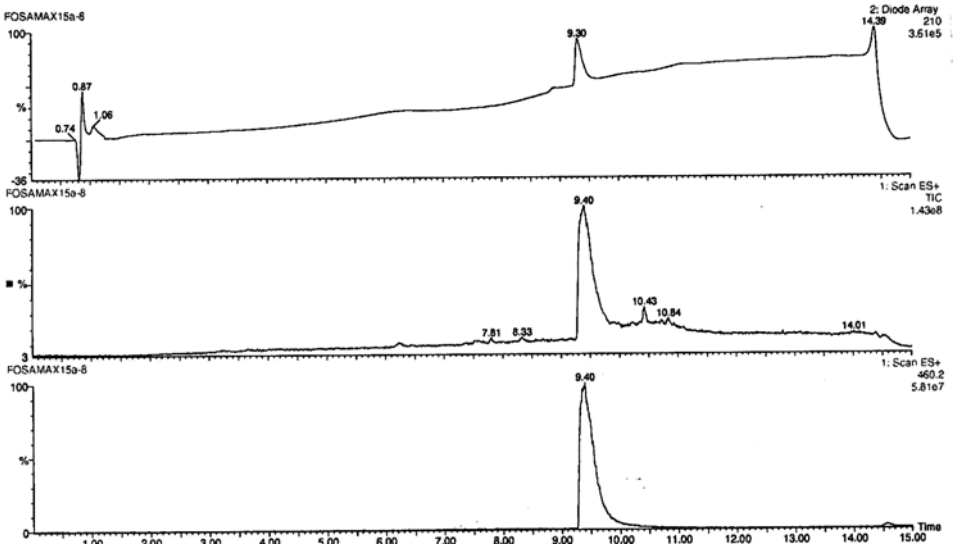
(15a)

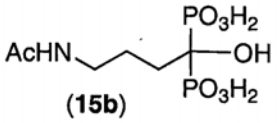
HPLC analyses in two diverse systems

HPLC Conditions 1



HPLC Conditions 2

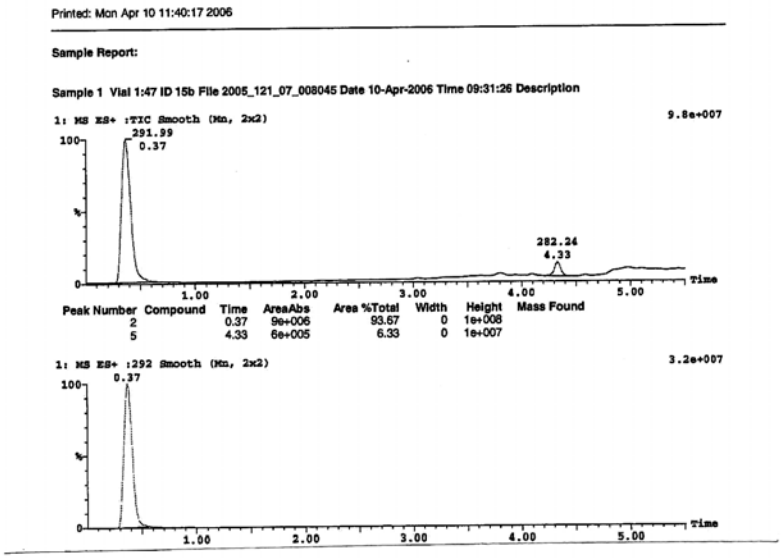




(15b)

HPLC analyses in two diverse systems

HPLC Conditions 1



HPLC Conditions 2