Supplementary material

Materials and Methods

^{31}P NMR experiments were carried out in 10-mm NMR tubes and spectra were recorded on a Varian 300 MHz spectrometers using 2% H_3PO_4 in D_2O as an external standard (coaxial inner tube). Anhydrous solvents for the studies were prepared in the following way: pyridine ScanLab), acetonitrile (ScanLab) and triethylamine (Aldrich), and *t*-butylamine (Aldrich) were refluxed with CaH_2 and then distilled and stored over molecular sieves (4Å) or CaH_2 (triethylamine). Methylene chloride was freshly distilled from CaH_2. Pivaloyl chloride (Aldrich) was distilled under atmospheric pressure and stored at -20 °C in a sealed flask. 5′-*O*-(*tert*-butyldiphenylsilyl)thymidine,[1] 3′-*O*-(*tert*-butyldiphenylsilyl)thymidine,[2] 2-chloro-5,5-dimethyl-2-oxo-1,3,2-dioxaphosphinane (NEP-Cl)[3] and 4-methoxy-2-pyridine methanol 1-oxide[4] were obtained according to the published procedures. 9-Fluorenemethyl H-phosphonate **1** was prepared *via* transesterification of diphenyl H-phosphonate with 9-fluorenemethanol, analogously to other alkyl H-phosphonate monoesters.[5] Nucleosides, snake venom phosphodiesterase (SVPD, Crotalus atrox) and nuclease P1 were purchased from Sigma. All isolated compounds were of purity >98% (^1H NMR spectroscopy). Final products **6a** and **6b** obtained in theses studies were identical with genuine samples obtained on another way.

*4-Methoxy-1-oxido-2-picolyl 9-fluorenemethyl phosphorothioate, triethylammonium salt **2**.*

9-Fluorenemethyl phosphonic acid **1** and 4-methoxy-2-pyridine methanol 1-oxide were coevaporated with added pyridine - TEA (4:1 v/v, 10 mL), followed by pyridine (2 x 10 mL). The mixture was dissolved in pyridine (10 mL) and NEP-Cl (0.44 g, 2.4 mmol) was added during stirring. The reaction was allowed to stand for 10 min. Water (0.043 mL, 2.4 mmol) was added and after an additional minute sulfur (0.16 g, 4.8 mmol). After 2 h the solvent was evaporated and the resultant oil dissolved in chloroform (50 mL) washed with 1 M TEAB (50 mL) followed by sat. $Na_2S_2O_3$ (50 mL) the organic phase was dried over Na_2SO_4 and the solvent was evaporated. Silica gel column chromatography using a stepwise gradient of MeOH (0-25%) in $CHCl_3$ containing 0.1% TEA gave **2** (0.4 g, 63% yield) as a white solid. Anal. $C_{27}H_{35}N_2O_5PS$: C 61.12; H 6.65; N 5.28. Found: C 61.25; H 6.53; N 5.33. δ_H ($CDCl_3$)

0.96 (9 H, t, *J* = 7.1 Hz, CH_3), 2.69 (6 H, q, *J* = 7.1 Hz CH_2), 3.49 (3 H, s, OCH_3), 4.11 (1 H, m, J = 6.3 Hz, OCH_2), 4.27 (2 H, m, J = 7.1 Hz, CH), 5.02 (2 H, t, *J* = 8.8 Hz, OCH_2), 6.53 (1 H, dd, J = 3.3 Hz, 6′′-CH), 6.96 (1 H, d, J = 3.3 Hz, 5′′-CH), 7.24 (2 H, t, J = 2.2 and 7.1 Hz, H_{arom}), 7.59 (4 H, q, J = 7.1 Hz, H_{arom}),7.24 (2 H, t, J = 7.4 Hz, H_{arom}), 7.59 (4 H, q, J = 7.1 Hz, H_{arom}), 7.91 (1 H, d, J = 7.1 Hz, 3′′-CH) and 11.20 (1 H, s, NH). δ_P ($CDCl_3$) 56.38 ppm. δ_C($CDCl_3$) 8.61, 21.49, 32.23, 32.29, 45.69, 48.30, 48.42, 53.89, 56.26, 62.14, 62.19, 67.93, 68.01, 76.00, 76.07, 77.45, 77.88 and 78.33.

*5′-O-tert-Butyldiphenylsilylthymidin-3'-yl 9-fluorenylmethyl 1-oxido-4-methoxy-2-picolyl phosphorothioates **3a** and **3b** (fast and slow isomers).*

2 (0.36 g, 0.67 mmol) and 5′-*O*-(*tert*-butyldiphenylsilyl)thymidine (0.39 g, 0.80 mmol) were coevaporated with added pyridine (2 x 5 mL) dissolved in pyridine (5.6 mL) and NEP-Cl (0.37 g, 2.01 mmol) was added. The reaction was followed in NMR and after 40 minutes the reaction was complete. The mixture was poured into EtOAc (50 mL) and washed with sat. NaCl (50 mL). The organic phase was dried over Na_2SO_4 and the solvent was evaporated. Silica gel column chromatography using a stepwise gradient of *i*PrOH (0-10%) in $CHCl_3$ containing 0.1‰ HOAc gave fast isomer **3a** [0.29 g, 48%; R_f = 0.48 in $CHCl_3$: CH_3OH (9:1, v/v)], slow isomer **3b** [0.11 g, 18%; R_f = 0.37 in $CHCl_3$: CH_3OH (9:1, v/v)] and a mixed fraction of isomers (0.14 g, 24%). ^{1}H NMR **3a**, δ_H ($CDCl_3$) 1.07 (9 H, s, *tert*-butyl), 1.57 (3 H, s, CH_3), 2.19 (1 H, m, *J* = 6.59 Hz, 2a′-H), 2.43 (1 H, m, *J* = 5.76 Hz, 2b′-H), 3.69 (3 H, s, OCH_3), 3. 91 (2 H, m, 5′-H), 4.18 (1 H, m, 4′-H), 4.25 (1 H, t, CH), 4.51 (2 H, m, OCH_2), 5.26 (2 H, d, *J* = 9.34 Hz, OCH_2), 5.30 (1 H, m, 3′-H), 6.44 (1 H, q, 6′-H), 6.81 (1 H, dd, *J* = 3.8 Hz, 5′′-CH), 6.88 (1 H, d, *J* = 2.7 Hz, 6′′-CH), 7.25-7.73 (19 H, m, 6-H and H_{arom}), 8.22 (1 H, dd, *J* = 7.14 Hz, 3′′-CH) and 9.72 (1 H, s, NH). δ_P ($CDCl_3$) 66.93 ppm. ^{1}H NMR **3b**, δ_H ($CDCl_3$) 1.06 (9 H, s, *tert*-butyl), 1.60 (3 H, s, CH_3), 2.24 (1 H, m, *J* = 6.9 Hz, 2a′-H), 2.49 (1 H, dd, *J* = 5.5 Hz, 2b′-H), 3.73 (3 H, s, OCH_3), 3. 83 (2 H, m, 5′-H), 4.02 (1 H, m, 4′-H), 4.18 (1 H, t, CH), 4.41 (2 H, m, OCH_2), 5.28 (2 H, d, OCH_2), 5.33 (1 H, m, 3′-H), 6.38 (1 H, q, *J* = 6.0 Hz, 6′-H), 6.78 (1 H, dd, *J* = 3.8 Hz, 5′′-CH), 6.89 (1 H, d, *J* = 2.7 Hz, 6′′-CH), 7.22-7.68 (19 H, m, 6-H and H_{arom}), 8.25 (1 H, dd, *J* = 7.14 Hz, 3′′-CH) and 10.0 (1 H, s, NH). δ_P ($CDCl_3$) 66.83 ppm.

*5′-O-tert-Butyldiphenylsilylthymidin-3'-yl 1-oxido-4-methoxy-2-picolyl phosphorothioate, triethylammonium salt **4a**.*

3a (1. 13 g, 1.26 mmol) was dissolved in pyridine–*tert*-butylamine (9:1, v/v, 50 mL) and the reaction mixture was stirred at room temperature for 15 min. The solvent was

evaporated in vacuum and the residue was partitioned between $CHCl_3$ (2 x 100 mL) and 1M TEAB (100 mL). The organic phase was dried Na_2SO_4 and the solvent was evaporated. Residual pyridine was removed by evaporation of added MeCN (2 x 50 mL).

Silica gel chromatography using a stepwise gradient of MeOH (0-25%) in $CHCl_3$ containing TEA (0.1%) afforded the diester **4a** (0.87 g, 69 %) as a white foam. Anal. $C_{29}H_{51}N_4O_9PSSi$: C 50.42; H 7.44; N 8.11. Found: C 50.31; H 7.53; N 8.03. δ_H ($CDCl_3$) 1.04 (9 H, s, *tert*-butyl), 1.32 (9 H, t, 3 x CH_3), 1.51 (3 H, s, CH_3), 2.18 (1 H, m, 2a′-H), 2.64 (1 H, m, 2b′-H), 3.10 (6 H, q, 3 x CH_2), 3.73 (3 H, s, OCH_3), 3. 93 (2 H, m, 5′-H), 4.28 (1 H, m, 4′-H), 5.22 (2 H, d, OCH_2), 5.35 (1 H, m, 3′-H), 6.38 (1 H, q, 6′-H), 6.73 (1 H, dd, 5′′-CH), 7.12 (1 H, d, 6′′-CH), 7.27-7.66 (11 H, m, 6-H and H_{arom}), 8.21 (1 H, dd 3′′-CH), 9.69 (1 H, s, NH), 11.72 (1 H, s, NH). δ_P ($CDCl_3$) 57.78 ppm.

*5′-O-tert-Butyldiphenylsilylthymidin-3'-yl 1-oxido-4-methoxy-2-picolyl phosphorothioate, triethylammonium salt **4b**.*

The triester **3b** (0.49 g, 0.43 mmol) was dissolved in pyridine-tert-butylamine (9:1, v/v, 20 mL) and the reaction mixture was stirred at room temperature for 15 min. The solvent evaporated in vacuum and the residue was partitioned between chloroform (2 x 50 mL) and 1 M TEAB (50 mL). The organic phase was dried Na_2SO_4 and the solvent was evaporated. Residual pyridine was removed by evaporation of added MeCN (2 x 50 mL).

Silica gel chromatography using a stepwise gradient of MeOH (0-25%) in $CHCl_3$ containing TEA (0.1 %) afforded **4b** (0.33 g, 83 %) as a white foam. Anal. $C_{29}H_{51}N_4O_9PSSi$: C 50.42; H 7.44; N 8.11. Found: C 50.28; H 7.49; N 8.15. δ_H ($CDCl_3$) 1.03 (9 H, s, *tert*-butyl), 1.28 (9 H, t, 3 x CH_3), 1.45 (3 H, s, CH_3), 2.10 (1 H, m, 2a′-H), 2.53 (1 H, m, 2b′-H), 3.08 (6 H, q, 3 x CH_2), 3.77 (3 H, s, OCH_3), 3. 95 (2 H, m, 5′-H), 4.29 (1 H, m, 4′-H), 5.28 (2 H, d, OCH_2), 5.35 (1 H, m, 3′-H), 6.34 (1 H, q, 6′-H), 6.75 (1 H, dd 5′′-CH), 7.17 (1 H, d, 6′′-CH), 7.29-7.69 (11 H, m, 6-H and H_{arom}), 8.37 (1 H, dd, 3′′-CH), 9.70 (1 H, s, NH), 11.73 (1 H, s, NH). δ_P ($CDCl_3$) 57.94 ppm.

*5′-O-tert-Butyldiphenylsilylthymidin-3′-yl 3′-O-tert-Butyldiphenylsilylthymidin-5′-yl(1-oxido-4-methoxy-2-picolyl) phosphorothioate **5a** (fast isomer).*

4a (0.71 g, 0.87 mmol) and 3-*O-tert*-butyldiphenylsilylthymidine (0,63 g, 1,3 mmol) were coevaporated with CH_2Cl_2-MeCN (1:1, v/v, 2 x 10 mL) and dissolved in CH_2Cl_2 (7.2 mL). Pyridine (0.21 mL, 2.6 mmol) was added followed by NEP-Cl (0.48 g, 2.6 mmol). After 15 minutes the reaction was poured into 1 M TEAB (100 mL) and extracted with CH_2Cl_2 (2 x

20 mL), dried Na_2SO_4, filtered and the solvent was evaporated. Column chromatography using $CHCl_3$ as an eluent with a gradient of isopropanol (0- 10 %) gave **5a** (0.7 g, 70%) as a white foam. δ_H ($CDCl_3$) 1.04 (18 H, s, *tert*-butyl), 1.58 (3 H, s, CH_3), 1.80 (3 H, s, CH_3), 2.18 (2 H, m, 2 x 2a´-H), 2.36 (2 H, m, 2 x 2b´-H), 3.77 (3 H, s, OCH_3), 3. 93 (4 H, m, 2 x 5´-H), 4.37 (2 H, m, 2 x 4´-H), 5.24 (2 H, d, OCH_2), 5.30 (2 H, m, 2 x 3´-H), 6.33 (1 H, q, 6´-H), 6.40 (1 H, q, 6´-H), 6.78 (1 H, dd, 5´´-CH), 6.87 (1 H, d, 6´´-CH), 7.27-7.66 (22 H, m, 6-H and H_{arom}), 8.21 (1 H, d 3´´-CH), 9.95 (2 H, d, 2 x NH). δ_P ($CDCl_3$) 67.90 ppm.

*5´-O-tert-Butyldiphenylsilylthymidin-3´-yl 3´-O-tert-butyldiphenylsilylthymidin-5´-yl(1-oxido-4-methoxy-2-picolyl) phosphorothioate **5b** (slow isomer).*

4b (0.33 g, 0.40 mmol) and 3-*O*-*tert*-butyldiphenylsilylthymidine (0.29 g, 0.6 mmol) were coevaporated with CH_2Cl_2-MeCN (1:1, v/v, 2 x 4 mL) and dissolved in dichloromethane (3.3 mL). Pyridine (0.1 mL, 1.2 mmol) was added followed by NEP-Cl (0.22 g, 1.2 mmol). After 15 minutes the reaction was poured into 1M TEAB (50 mL) and extracted with CH_2Cl_2 (2 x 10 mL), dried Na_2SO_4, filtered and the solvent was evaporated. Column chromatography using $CHCl_3$ as an eluent with a gradient of isopropanol (0- 10 %) gave **5b** (0.35 g, 75%) as a white foam. δ_H ($CDCl_3$) 1.08 (18 H, s, *tert*-butyl), 1.60 (3 H, s, CH_3), 1.81 (3 H, s, CH_3), 2.09 (2 H, m, 2 x 2a´-H), 2.29 (2 H, m, 2 x 2b´-H), 3.82 (3 H, s, OCH_3), 4.01 (4 H, m, 2 x 5´-H), 4.35 (2 H, m, 2 x 4´-H), 5.33 (2 H, d, OCH_2), 5.30 (2 H, m, 2 x 3´-H), 6.34 (1 H, q, 6´-H), 6.42 (1 H, q, 6´-H), 6.78 (1 H, dd, 5´´-CH), 6.95 (1 H, d, 6´´-CH), 7.36-7.70 (22 H, m, 6-H and H_{arom}), 7.92 (1 H, d 3´´-CH), 9.86 (2 H, d, 2 x NH). δ_P ($CDCl_3$) 67.24 ppm.

*[R_P]-Thymidin-3'-yl thymidin-5´-yl phosphorothioate, sodium salt **6a**.*

5a (0.7 g, 0.6 mmol) was dissolved in pyridine-TEA-PhSH (1:1:1, v/v/) and was stirred at RT for 2 h. Silica gel chromatography using a stepwise gradient of MeOH (0-10%) in chloroform containing 0.1‰ TEA gave (0.32 g 46%) of the diester. δ_H ($CDCl_3$) 1.06 (18 H, d, *tert*-butyl), 1.20 (9 H, t, 3 x CH_3), 1.50 (3 H, s, CH_3), 1.97 (3 H, s, CH_3), 2.09 (2 H, m, 2 x 2a´-H), 2.26 (2 H, m, 2 x 2b´-H), 2.93 (6 H, q, 3 x CH_2), 3.69 (2 H, m, 5´-H), 3. 95 (2 H, m, 5´-H), 4.11 (1 H, m, 2 x 4´-H), 4.26 (1 H, m, 2 x 4´-H), 4.50 (1 H, m, 3´-H), 5.21 (1 H, m, 3´-H), 6.36 (1 H, q, 6´-H), 6.54 (1 H, q, 6´-H), 7.31-7.58 (10 H, m, H_{arom}), 7.45 (1 H, d 6-H), 7.58-7.70 (10 H, m, H_{arom}), 7.81 (1 H, d 6-H), 9.10 (1 H, s, NH) 9.32 (1 H, s, NH), 11.63 (1 H, s, NH).δ_P ($CDCl_3$) 56.90 ppm. Then, the diester (0.32 g, 0.28 mmol) and tetrabutylammonium fluoride trihydrate (0.27 g, 0.84 mmol) were dissolved in THF (0,26 mL) and stirred at RT over night. H_2O (5 mL) was added and the solution was washed with ether (3 x 2 mL). The water phase was evaporated to near dryness and the product was passed through an ion-

exchange column Dowex 50-x2, 100 - 200 mesh (dry), strongly acidic cation exchange, ion form Na^+. Then the water was evaporated. The product was passed through a gel filtration column Sephadex G 10 and lyophilized giving **6a** (142 mg, 87%).δ_H (D_2O) 1.74 (3 H, s, CH_3), 1.79 (3 H, s, CH_3), 2.21 (3 H, m, 2 x 2a′-H and 2b′-H), 2.41 (1 H, m, 2b′-H), 3. 69 (2 H, m, 5′-H), 4.01 (2 H, m, 5′-H), 4.07 (1 H, m, 4′-H), 4.44 (1 H, m, 4′-H), 4.65 (1 H, m, 3′-H), 4.82 (1 H, m, 3′-H), 6.07 (1 H, t, 6′-H), 6.18 (1 H, t, 6′-H), 7.52 (1 H, d 6-H), 7.58 (1 H, d 6-H), δ_P (D_2O) 55.79 ppm. δ_C (D_2O) 166.27, 166.21, 151.65, 151.47, 137.42, 137.38, 111.63, 111.46, 85.85, 85.76, 85.38, 85.29, 75.77, 75.71, 71.03, 61.14, 39.08, 37.80, 11.98, 11.85.

*[S_P]-Thymidin-3'-yl thymidin-5′-yl phosphorothioate, sodium salt **6b**.*

5b (0.20 g, 0.18 mmol) was dissolved in pyridine-TEA-PhSH (1:1:1, v/v/v) and was stirred at RT for 2 h. Silica gel chromatography using a stepwise gradient of MeOH (0-10%) in chloroform containing 0.1% TEA gave (0.086 g 47%) of the diester. NMR, δ_H ($CDCl_3$) 1.06 (18 H, d, *tert*-butyl), 1.24 (9 H, t, 3 x CH_3), 1.56 (3 H, s, CH_3), 1.86 (1 H, m, 2a′-H), 1.98 (3 H, s, CH_3), 2.09 (1 H, m, 2a′-H), 2.14 (1 H, m, 2b′-H), 2.55 (1 H, m, 2b′-H), 3.01 (6 H, q, 3 x CH_2), 3.75 (2 H, m, 5′-H), 3. 98 (2 H, m, 5′-H), 4.01 (1 H, m, 2 x 4′-H), 4.20 (1 H, m, 2 x 4′-H), 4.30 (1 H, m, 3′-H), 5.29 (1 H, m, 3′-H), 6.34 (1 H, t, J = 6.9 Hz, 6′-H), 6.52 (1 H, t, J = 6.9 Hz, 6′-H), 7.23-7.75 (21 H, m, 6-H and H_{arom}), 7.84 (1 H, d, 6-H), 9.54 (1 H, s, NH) 9.76 (1 H, s, NH), 11.81 (1 H, s, NH).δ_P ($CDCl_3$) 57.18 ppm. Then, the diester (0.10 g, 0.086 mmol) and tetrabutylammonium fluoride trihydrate (81 mg, 0.26 mmol) were dissolved in THF (0,26 mL) and stirred at RT over night. H_2O (5 mL) was added and the solution was washed with ether (3 x 2 mL). The water phase was evaporated to near dryness and the product was passed through an ion-exchange column Dowex 50-x2, 100 - 200 mesh (dry), strongly acidic cation exchange, ion form Na^+. Then the water was evaporated and the product passed through a gel filtration column Sephadex G 10 and lyophilized giving **6b** (46 mg, 92%). NMR, δ_H (D_2O) 1.74 (3 H, s, CH_3), 1.78 (3 H, s, CH_3), 2.22 (3 H, m, 2 x 2a′-H and 2b′-H), 2.41 (1 H, m, 2b′-H), 3.69 (2 H, m, 5′-H), 4.03 (2 H, m, 5′-H), 4.07 (1 H, m, 4′-H), 4.46(1 H, m, 4′-H), 4.65 (1 H, m, 3′-H), 4.82 (1 H, m, 3′-H), 6.08(1 H, t, J = 6.9 Hz, 6′-H), 6.18 (1 H, t, J = 6.9 Hz, 6′-H), 7.53 (1 H, d 6-H), 7.60 (1 H, d 6-H), δ_P (D_2O) 55.79 ppm. δ_C (D_2O) 166.45, 166.36, 151.74, 151.59, 137.44, 111.65 111.53, 85.72, 85.63, 85.29, 85.16, 75.35, 75.27, 70.83, 65.50, 61.07, 38.97, 37.86, 11.88, 11.76.

*Enzymatic digestion of **6a** and **6b**.*

The following stock solutions were prepared: dinucleoside phosphorothioate **6a** or **6b** (5 mg, 0.009 mmol) was dissolved in buffer A [0.250 mL; 30 mM $(NH_4)_2SO_4$ and 0.44 mM

$ZnSO_4$] and in buffer B [0.250 mL;50 mM Tris-HCl and 0.2 mM $MgCl_2$]. Nuclease P1 (1 mg) was dissolved in the buffer A (0.5 mL) and snake venom phosphodiesterase (SVPD, 1.6 mg), in buffer B (0.5 mL).

The enzymatic digestion was carried out by mixing a sample of **6a** or **6b** in buffer A (0.05 mL) with nuclease P1 in buffer A (0.05 mL) or with SVPD in buffer B (0.05 mL), and the reaction mixtures were incubated at 37°C over night. TLC isopropanol-ammonia-water (7:2:1, v/v/v) revealed that nuclease P1 hydrolyzed the isomer **6b** and snake venom phosphodiesterase (SVPD) hydrolyzed the isomer **6a**. This identified configuration at the phosphorus centre in **6a** as [R_P], and the configuration in **6b** as [S_P].

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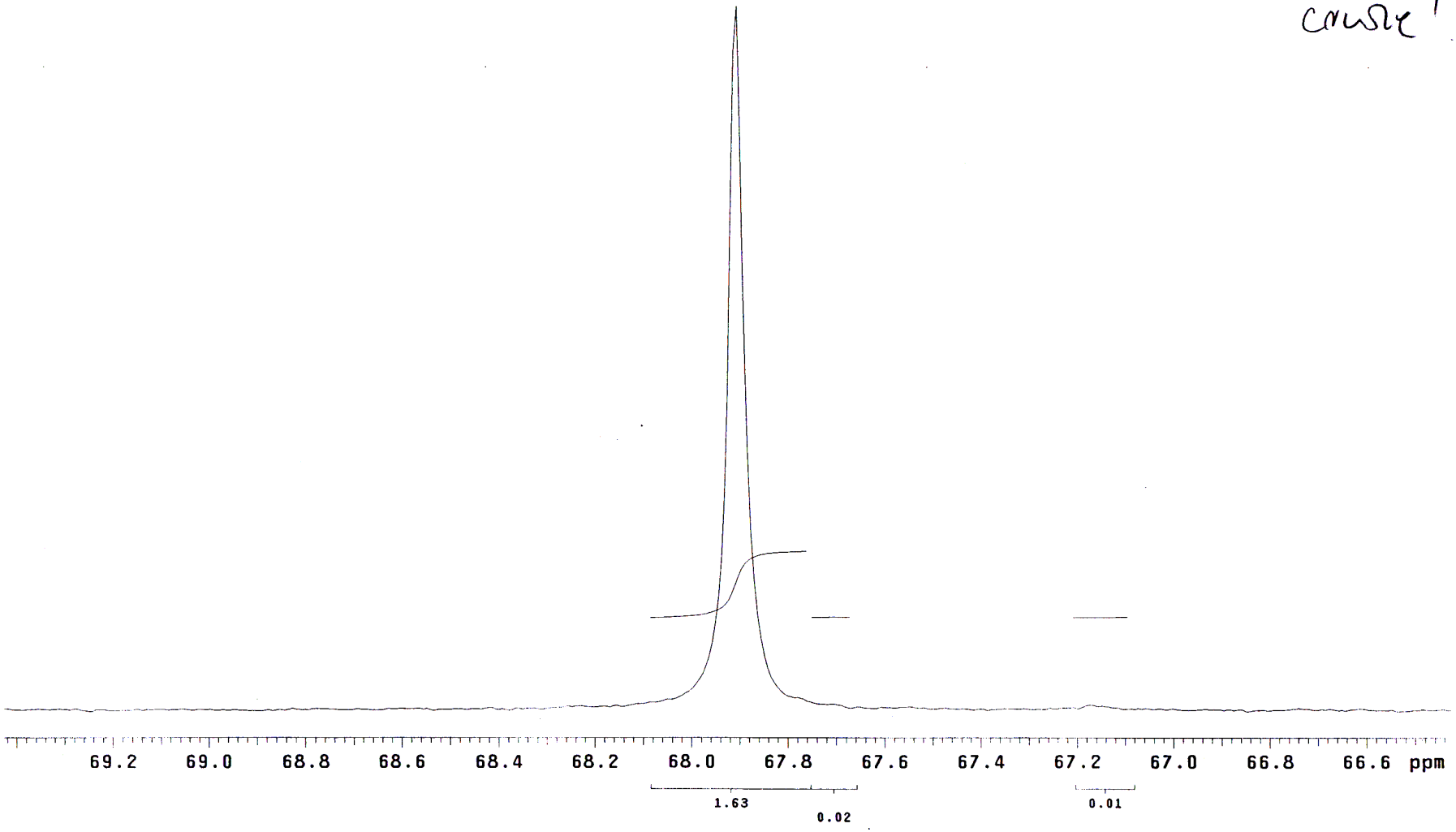
99 coupling test TPSONa
Pulse Sequence: s2pul

INDEX	FREQUENCY	PPM	HEIGHT
1	8407.507	69.243	-0.5
2	8244.792	67.903	118.3
3	8182.018	67.386	-0.3
4	8155.587	67.168	0.7
5	8116.767	66.848	-0.5
6	8103.551	66.739	0.1

31 P NMR

Tb (from 4b)
crude



slow TPSONox coupling 5-OHT

Pulse Sequence: s2pul

INDEX	FREQUENCY	PPM	HEIGHT
1	8164.673	67.243	121.0

31P NMR

Ta (from 4a) crude!