Supplementary Information

Thiol-specific linkers for the synthesis of oligonucleotide conjugates via metal-free thiol-ene click reaction

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General Experimental Methods

All reactions were carried out under an atmosphere of argon. Reactions were monitored by TLC using aluminium backed silica gel 60 (F254) plates, visualised using UV 254 nm and vanillin dips. Flash column chromatography was carried out with silica gel (60-120 mesh). Reagents were used as received from commercial sources unless otherwise stated. All commercial amino alcohols were a mixture of cis and trans isomers unless indicated otherwise in structures or write up. Dry solvents were purchased and used as received. ^{1}H NMR spectra were recorded on a Bruker Avance 400 Spectrometer (400 MHz) at room temperature. ^{13}C NMR spectra were recorded at a frequency of 101 MHz. Chemical shifts are reported in δ units (parts per million) and coupling constants (*J*) are measured in Hertz (Hz). The residual solvent signals were used as references. ^{31}P NMR spectra (decoupled) were recorded at a frequency of 162 MHz and chemical shifts were reported in ppm. ^{19}F NMR spectra (decoupled) were recorded at a frequency of 376 MHz and chemical shifts were reported in ppm. Mass spectra were recorded on Waters LCMS system with Acquity QDa detector in positive ion detection mode (ESI^+) and analysed as m/z $[M+H]^+$. EtOAc = ethyl acetate, DIPEA = *N,N* -Diisopropylethylamine, $ClP(OCE)N(iPr)_2$ = 2-Cyanoethyl *N,N*-diisopropylchlorophosphoramidite, CH_2=$CHBF_3K$ = Potassium vinyltrifluoroborate, $Pd(dppf)Cl_2$.DCM = [1,1'-Bis(diphenylphosphino)ferrocene]dichloropalladium (II), complex with dichloromethane, DCC = *N*,*N*'-Dicyclohexylcarbodiimide, DMAP = 4-(Dimethylamino)pyridine, DMF = *N,N*-Dimethylformamide, THF = Tetrahydrofuran, DCM = Dichloromethane, rt = room temperature, eq = equivalents.

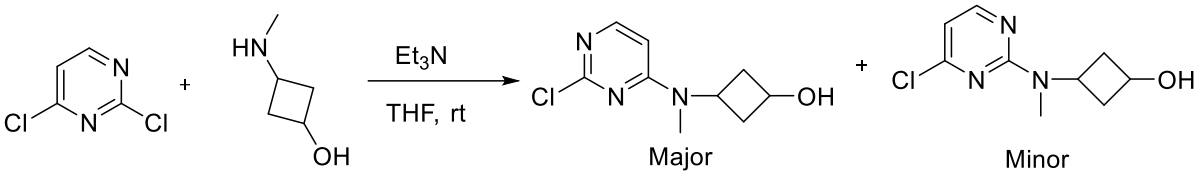
General method for synthesis of linkers

Representative Examples

Method A

Step 1

3-((2-chloropyrimidin-4-yl)(methyl)amino)cyclobutan-1-ol (2)



1 **2** **2'**

To a solution of 2,4-dichloro-pyrimidine **1** (1.00 g, 6.7 mmol) in THF (10 mL) was added Et_3N (16.75 mmol, 0.43 mL) and commercially available 3-(methylamino)cyclobutan-1-ol (cis/trans 5:1) (1.02 g, 10.05 mmol). The mixture was stirred at ambient temperature for 7 hours and concentrated under reduced pressure. The residue was dissolved in DCM (20 mL) and the solution was poured into saturated aqueous sodium bicarbonate solution (5 mL). The two layers were separated, and the aqueous layer was extracted with DCM (2 x 20 mL). The combined organic layer was dried over Na_2SO_4, filtered, concentrated under reduced pressure and purified by flash column chromatography using Hexane/EtOAc (0-100%) to afford 3-((2-chloropyrimidin-4-yl)(methyl)amino)cyclobutan-1-ol **2** (major product) (925 mg, 64.6%) as a pale yellow gum and 3-((4-chloropyrimidin-2-yl)(methyl)amino)cyclobutan-1-ol **2'** (minor product) (140 mg, 9.8%) as a yellow viscous oil.

^{1}H NMR for 3-((2-chloropyrimidin-4-yl)(methyl)amino)cyclobutan-1-ol **2** (major product)

^{1}H NMR (400 MHz, $CDCl_3$) (Cis/Trans Mixture) δ 7.86 (dd, *J* = 7.4, 5.9 Hz, 1H), 6.32 – 6.16 (m, 1H), 4.67 – 4.33 (m, 1H), 4.09 – 4.01 (m, 1H), 2.97 – 2.94 (m, 3H), 2.73 – 2.33 (m, 2H), 2.32 – 1.92 (m, 2H). (One exchangeable proton of OH was not observed).

^{13}C NMR (101 MHz, $CDCl_3$) (Cis/Trans mixture) δ 162.95, 162.90, 160.24, 160.19, 156.68, 156.60, 102.04, 63.82, 60.60, 60.54, 42.66, 38.60, 37.16, 30.68, 30.57.

LCMS (ESI-MS) m/z calculated for: $C_9H_{13}ClN_3O^+$: 214.074; found 214.079 $[M+H]^+$.

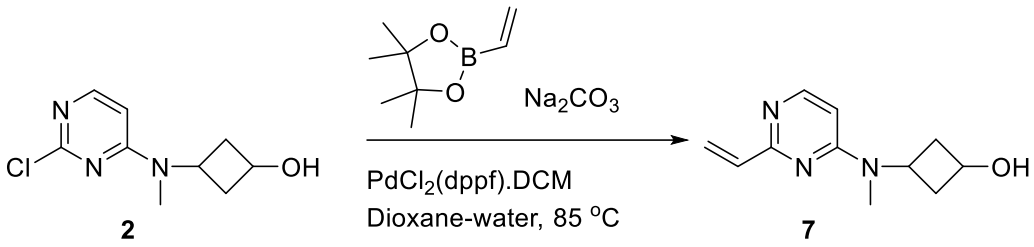
^{1}H NMR for 3-((4-chloropyrimidin-2-yl)(methyl)amino)cyclobutan-1-ol **2'** (minor product)

^{1}H NMR (400 MHz, $CDCl_3$) (Cis/Trans mixture) δ 8.08 (d, *J* = 5.1 Hz, 1H), 6.43 (dd, *J* = 5.0, 1.0 Hz, 1H), 4.55 (tt, *J* = 9.7, 7.5 Hz, 1H), 4.11 – 3.97 (m, 1H), 3.05 (d, *J* = 6.7 Hz, 3H), 2.66 - 2.58 (m, 2H), 2.21 – 1.97 (m, 3H).

LCMS (ESI-MS) m/z calculated for: $C_9H_{13}ClN_3O^+$:214.074; found 214.078 $[M+H]^+$.

Step 2

3-(methyl(2-vinylpyrimidin-4-yl)amino)cyclobutan-1-ol (7)



To a solution of 3-((2-chloropyrimidin-4-yl)(methyl)amino)cyclobutan-1-ol **2** (141 mg, 0.66 mmol) and vinylboronic acid pinacol ester (0.34 mL, 1.98 mmol) in dioxane (4 mL) and water (1 mL) under argon atmosphere was added Na_2CO_3 (245 mg, 2.31 mmol). The mixture was degassed for 5 min. Then, the catalyst Pd(dppf)Cl_2.DCM (54 mg, 0.66 mmol) was added to the mixture and further degassed for 5 min. The reaction mixture was warmed up to 85 °C and stirred at this temperature for 18 hours. The reaction mixture was cooled to room temperature, diluted with DCM and filtered through a bed of celite, washing several times with DCM (3 x 15 mL). Solvent was removed under vacuum. The residue was subjected to flash column chromatography using Heptane/Ethyl acetate (0-100%) to afford the product 3-(methyl(2-vinylpyrimidin-4-yl)amino)cyclobutan-1-ol **7** (130 mg, 96.7%) as a pale yellow gum.

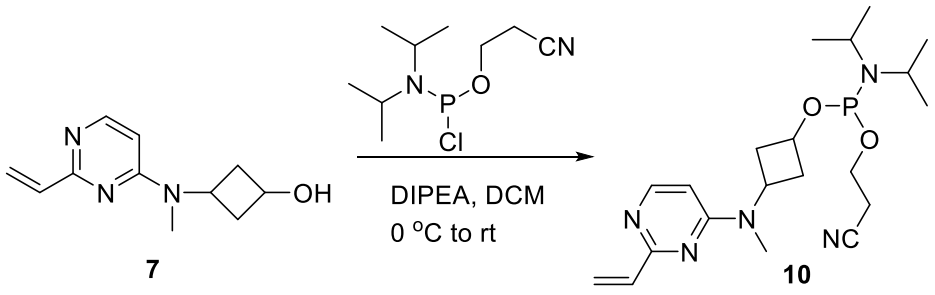
^{1}H NMR (400 MHz, $CDCl_3$) (Cis/Trans Mixture) δ 8.09 (t, *J* = 6.0 Hz, 1H), 6.67 (dd, *J* = 17.3, 10.4 Hz, 1H), 6.56 – 6.40 (m, 1H), 6.32 – 6.15 (m, 1H), 5.59 (dd, *J* = 10.4, 2.0 Hz, 1H), 4.55 – 4.30 (m, 1H), 4.10 (tt, *J* = 7.6, 6.5 Hz, 1H), 3.17 – 2.83 (m, 4H), 2.73 – 2.62 (m, 2H), 2.15 – 2.06 (m, 2H).

^{13}C NMR (101 MHz, $CDCl_3$) (Cis/Trans Mixture) δ 163.19, 163.16, 161.81, 161.77, 155.36, 155.32, 137.48, 137.45, 122.44, 122.40, 101.96, 101.90, 60.94, 60.55, 42.25, 38.89, 37.39, 30.26, 30.23.

LCMS (ESI-MS) m/z calculated for: $C_{11}H_{16}N_3O^+$: 206.129; found 206.128 $[M+H]^+$.

Step 3

2-cyanoethyl (3-(methyl(2-vinylpyrimidin-4-yl)amino)cyclobutyl) diisopropylphosphoramidite (10)



A stirred solution of 3-(methyl(2-vinylpyrimidin-4-yl)amino)cyclobutan-1-ol **7** (128 mg, 0.62 mmol) (dried overnight in high vacuum) in DCM (8 mL) was degassed by bubbling with argon for 5 min. The solution was cooled to 0 °C in an ice bath and DIPEA (0.32 mL, 1.86 mmol) was added, followed by dropwise addition of 2-Cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (0.21 mL, 0.93 mmol). The reaction mixture was allowed to gradually warm to room temperature and stirred for 6 hours. Solvent was removed under vacuum. The residue was subjected to flash column chromatography using Heptane/(1% Et_3N)/EtOAc (0-100%) to afford product 2-cyanoethyl (3-(methyl(2-vinylpyrimidin-4-yl)amino)cyclobutyl) diisopropylphosphoramidite (diastereomeric mixture) **10** (136 mg, 54.1%) as a colourless gum.

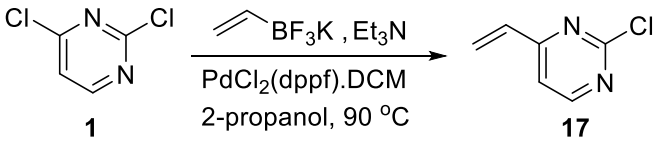
^{1}H NMR (400 MHz, CD_2Cl_2) (Mixture of diastereoisomers) δ 8.14 (d, *J* = 6.1 Hz, 0.79H), 7.98 (d, *J* = 6.1 Hz, 0.24H), 6.80 – 6.63 (m, 1H), 6.60 – 6.43 (m, 1H), 6.43 – 6.19 (m, 1H), 5.58 (dd, *J* = 10.4, 2.3 Hz, 1H), 4.67 – 4.41 (m, 1H), 4.27 – 4.09 (m, 1H), 3.92 – 3.73 (m, 2H), 3.70 – 3.60 (m, 2H), 3.05 (d, *J* = 5.6 Hz, 3H), 2.91 – 2.53 (m, 4H), 2.39 – 2.16 (m, 2H), 1.27 – 1.17 (m, 12H).

^{31}P NMR (162 MHz, CD_2Cl_2) (Mixture of diastereoisomers) δ 146.51, 145.59.

Method B

Step 1

2-chloro-4-vinylpyrimidine (17)

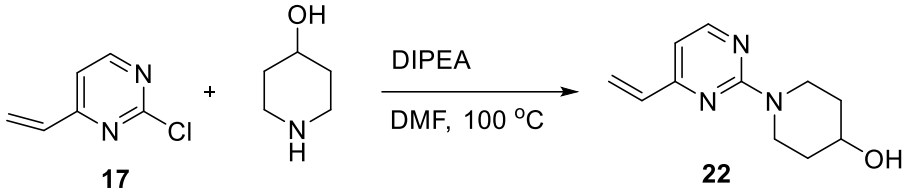


To a solution of 2,4-dichloropyrimidine **1** (519 mg, 3.48 mmol) in anhydrous 2-propanol (10 mL), potassium trifluorovinyl borate (500 mg, 3.83 mmol) and Et_3N (0.97 mL, 10.4 mmol) were added under argon atmosphere in a microwave tube. The mixture was degassed for 5 min. Then, the catalyst $Pd(dppf)Cl_2$.DCM (142 mg, 0.174 mmol) was added to the mixture and further degassed for 5 min. The tube was sealed and the reaction mixture was warmed up to 90 °C and stirred at this temperature for 5 hours. The reaction mixture was then cooled to room temperature, diluted with ethyl acetate (25 mL), filtered through a bed of celite and washed several times with ethyl acetate (3 x 10 mL). Solvent was removed under vacuum. Flash column chromatography using Heptane/EtOAc (0-100%) afforded product 2-chloro-4-vinylpyrimidine **17** (296 mg, 60.5%) as a pale-yellow oil. ^{1}H NMR data was consistent with the literature data[i].

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.57 (dd, *J* = 5.1, 1.3 Hz, 1H), 7.23 (dd, *J* = 5.1, 0.9 Hz, 1H), 6.71 (ddd, *J* = 17.4, 10.6, 1.4 Hz, 1H), 6.54 (ddd, *J* = 17.4, 1.7, 1.0 Hz, 1H), 5.80 (dt, *J* = 10.5, 1.3 Hz, 1H).

Step 2

1-(4-vinylpyrimidin-2-yl)piperidin-4-ol (22)



17 22

A solution of 2-chloro-4-vinylpyrimidine **17** (100 mg, 0.711 mmol), piperidin-4-ol (86.3 mg, 0.853 mmol) and anhydrous DIPEA (0.35 mL, 1.78 mmol) in anhydrous DMF (1.5 mL) was stirred at 100 °C in a sealed tube for 18 hours. The reaction mixture was cooled to room temperature and solvent was removed under vacuum. Flash column chromatography using Heptane/Ethyl acetate (0-100%) afforded product 1-(4-vinylpyrimidin-2-yl)piperidin-4-ol **22** (49 mg, 34%) as a pale brown gum.

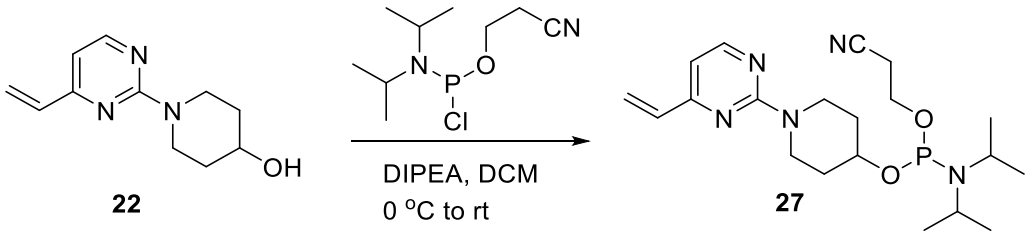
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.28 (d, *J* = 5.0 Hz, 1H), 6.59 (dd, *J* = 17.4, 10.5 Hz, 1H), 6.48 (d, *J* = 5.0 Hz, 1H), 6.37 (dd, *J* = 17.4, 1.6 Hz, 1H), 5.57 (dd, *J* = 10.5, 1.6 Hz, 1H), 4.62 – 4.40 (m, 2H), 3.94 (tt, *J* = 8.7, 4.0 Hz, 1H), 3.31 (ddd, *J* = 13.4, 10.0, 3.3 Hz, 2H), 2.00 – 1.92 (m, 3H), 1.59 – 1.50 (m, 2H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 162.89, 161.80, 158.42, 136.27, 121.48, 107.09, 68.57, 41.51, 34.37.

LCMS (ESI-MS) m/z calculated for $C_{11}H_{16}N_3O^+$: 206.129; found 206.122 $[M+H]^+$.

Step 3 – same as in Method A

2-cyanoethyl (1-(4-vinylpyrimidin-2-yl)piperidin-4-yl) diisopropylphosphoramidite (27)



22 27

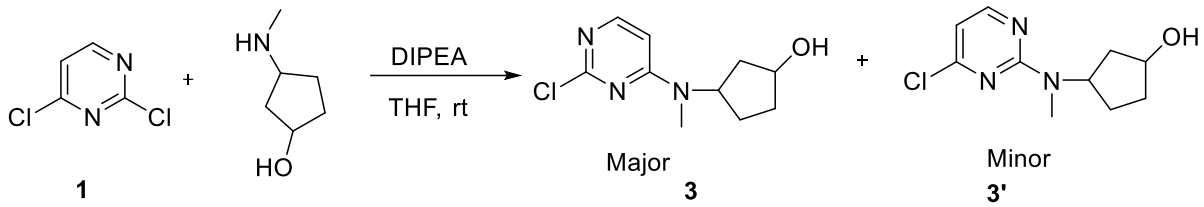
Starting from 1-(4-vinylpyrimidin-2-yl)piperidin-4-ol **22**, compound 2-cyanoethyl (1-(4-vinylpyrimidin-2-yl)piperidin-4-yl) diisopropylphosphoramidite **27** (mixture of diastereoisomers) was synthesised in 67% yield as a colourless gum, following same synthetic procedure as mentioned in **Method A Step 3**.

^{1}H NMR (400 MHz, CD_2Cl_2) (Mixture of diastereoisomers) δ 8.29 (d, *J* = 5.0 Hz, 1H), 6.61 (dd, *J* = 17.3, 10.5 Hz, 1H), 6.50 (d, *J* = 5.0 Hz, 1H), 6.40 (dd, *J* = 17.3, 1.7 Hz, 1H), 5.58 (dd, *J* = 10.5, 1.7 Hz, 1H), 4.24 – 4.10 (m, 3H), 3.91 – 3.76 (m, 2H), 3.75 – 3.60 (m, 4H), 2.67 (t, *J* = 6.3 Hz, 2H), 2.02 – 1.87 (m, 2H), 1.76 – 1.66 (m, 2H), 1.23 (dd, *J* = 6.8, 1.2 Hz, 12H).

^{31}P NMR (162 MHz, CD_2Cl_2) (Mixture of diastereoisomers) δ 146.14.

Remaining compounds

3-((2-chloropyrimidin-4-yl)(methyl)amino)cyclopentan-1-ol (3)



1 3 3'

Method A Step 1 was followed to synthesise 3-((2-chloropyrimidin-4-yl)(methyl)amino)cyclopentan-1-ol **3** (Cis/Trans mixture) starting from commercially available compounds 2,4-dichloropyrimidine **1** and 3-(methylamino)cyclopentan-1-ol (Cis/Trans mixture). Flash column chromatography using Heptane/EtOAc (0-100%) afforded compounds 3-((2-chloropyrimidin-4-yl)(methyl)amino)cyclopentan-1-ol **3** (major product) in 68% yield as a sticky white solid and 3-((4-chloropyrimidin-2-yl)(methyl)amino)cyclopentan-1-ol **3'** (minor product) in 5% yield as a yellow solid.

^{1}H NMR for 3-((2-chloropyrimidin-4-yl)(methyl)amino)cyclopentan-1-ol **3** (major product)

^{1}H NMR (400 MHz, $CDCl_3$) (Cis/Trans mixture) δ 7.96 (dd, *J* = 6.2, 2.0 Hz, 1H), 6.39 – 6.36 (m, 1H), 4.99 (brs, 1H), 4.49 – 4.34 (m, 1H), 2.99 (s, 1.65H), 2.89 (s, 1.33H) 2.78 (brs, 0.6H), 2.49 (brs, 0.4H), 2.33 – 1.42 (m, 6H).

^{13}C NMR (101 MHz, $CDCl_3$) (Cis/Trans mixture) δ 163.09, 162.84, 160.31, 160.13, 156.66, 156.64, 101.78, 101.60, 72.02, 71.81, 55.62, 54.78, 38.12, 37.82, 34.60, 34.10, 31.43, 29.89, 26.41, 26.32.

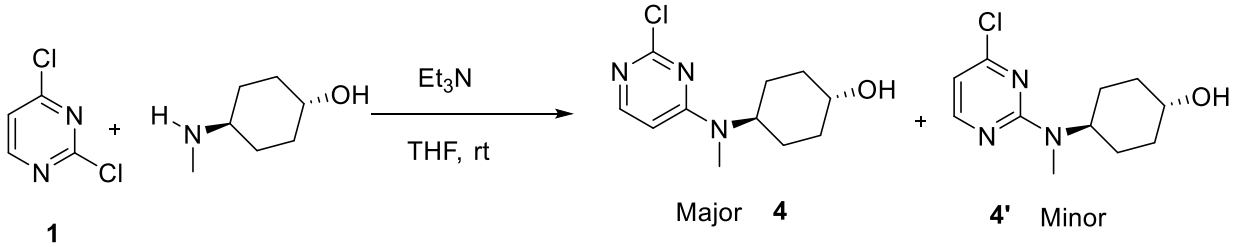
LCMS (ESI-MS) m/z calculated for: $C_{10}H_{15}ClN_3O^+$: 228.089; found 228.090 $[M+H]^+$.

^{1}H NMR for 3-((4-chloropyrimidin-2-yl)(methyl)amino)cyclopentan-1-ol **3'** (minor product)

^{1}H NMR (400 MHz, $CDCl_3$) (Cis/Trans Mixture) δ 8.12 (t, *J* = 5.2 Hz, 1H), 6.46 (dd, *J* = 7.5, 5.1 Hz, 1H), 5.49 – 5.40 (m, 0.42H), 4.78 – 4.69 (m, 0.62H), 4.47 – 4.42 (m, 0.44H), 4.34 – 4.30 (m, 0.63H), 3.15 (s, 1.84H), 2.98 (s, 1.25H), 2.24 (ddd, *J* = 14.3, 10.3, 5.9 Hz, 0.65H), 2.14 – 2.02 (m, 1.56H), 1.98 – 1.55 (m, 5H).

LCMS (ESI-MS) m/z calculated for: $C_{10}H_{15}ClN_3O^+$: 228.089; found 228.086 $[M+H]^+$.

(1*R*,4*R*)-4-((2-chloropyrimidin-4-yl)(methyl)amino)cyclohexan-1-ol (4)



1

Method A Step 1 was followed to synthesise (1*R*,4*R*)-4-((2-chloropyrimidin-4-

yl)(methyl)amino)cyclohexan-1-ol **4**, starting from commercially available compounds 2,4-dichloro-pyrimidine **1** and trans-4-(methylamino)cyclohexanol. Flash column chromatography using DCM/MeOH (0-50%) afforded compounds (1*R*,4*R*)-4-((2-chloropyrimidin-4-yl)(methyl)amino)cyclohexan-1-ol **4** (major product) in 57.1% yield as a yellow gum and (1*R*,4*R*)-4-((4-chloropyrimidin-2-yl)(methyl)amino)cyclohexan-1-ol **4'** (minor product) in 10% yield as a yellow oil.

^{1}H NMR for (1*R*,4*R*)-4-((2-chloropyrimidin-4-yl)(methyl)amino)cyclohexan-1-ol **4** (major product)

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.96 (d, *J* = 6.2 Hz, 1H), 6.29 (d, *J* = 6.2 Hz, 1H), 3.65 – 3.58 (m, 1H), 2.87 (s, 3H), 2.78 – 2.64 (m, 1H), 2.20 – 1.97 (m, 2H), 1.81 – 1.64 (m, 2H), 1.66 – 1.36 (m, 4H). (One exchangeable proton of OH was not observed).

^{13}C NMR (101 MHz, $CDCl_3$) δ 162.71, 160.33, 156.65, 101.49, 69.62, 34.34, 29.54, 27.41.

LCMS (ESI-MS) m/z calculated for $C_{11}H_{17}ClN_3O^+$: 242.105; found 242.104 $[M+H]^+$.

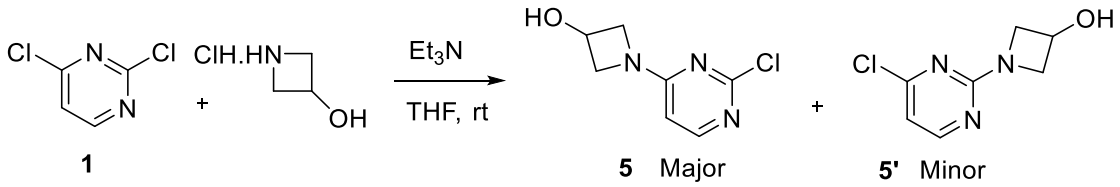
^{1}H NMR for (1*R*,4*R*)-4-((4-chloropyrimidin-2-yl)(methyl)amino)cyclohexan-1-ol **4'** (minor product)

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.12 (d, *J* = 5.1 Hz, 1H), 6.44 (d, *J* = 5.1 Hz, 1H), 4.66 – 4.45 (m, 1H), 3.63 – 3.55 (m, 1H), 2.96 (s, 3H), 2.05 – 2.02 (m, 3H), 1.80 – 1.67 (m, 2H), 1.68 – 1.41 (m, 4H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 161.67, 160.97, 158.64, 108.51, 77.48, 77.16, 76.84, 70.18, 53.33, 34.64, 29.12, 27.53.

LCMS (ESI-MS) m/z calculated for $C_{11}H_{17}ClN_3O^+$: 242.105; found 242.109 $[M+H]^+$.

1-(2-chloropyrimidin-4-yl)azetidin-3-ol (5)



Method A Step 1 was followed to synthesise 1-(2-chloropyrimidin-4-yl)azetidin-3-ol **5**, starting from commercially available compounds 2,4-dichloro-pyrimidine **1** and 3-hydroxyazetidine hydrochloride. Flash column chromatography using DCM/MeOH (0-50%) afforded compounds 1-(2-chloropyrimidin-4-yl)azetidin-3-ol **5** (major product) in 78% yield as a yellow gum and 1-(4-chloropyrimidin-2-yl)azetidin-3-ol **5'** (minor product) in 4% yield as a yellow oil.

^{1}H NMR for 1-(2-chloropyrimidin-4-yl)azetidin-3-ol **5** (major product)

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.02 (d, *J* = 5.8 Hz, 1H), 6.10 (d, *J* = 5.9 Hz, 1H), 4.84 (brs, 1H), 4.49 – 4.27 (m, 2H), 4.11 – 3.97 (m, 2H), 2.54 (brs, 1H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 163.33, 159.04, 156.46, 100.86, 62.58, 59.96, 59.69.

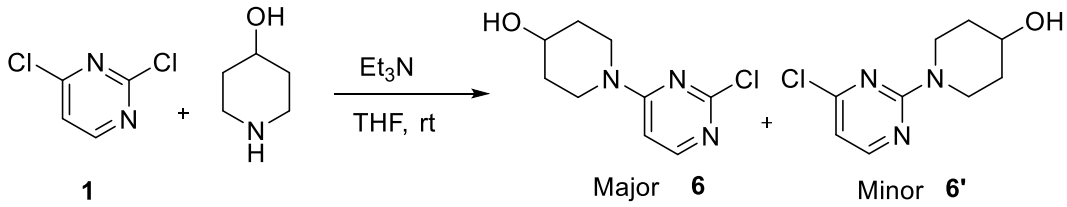
LCMS (ESI-MS) m/z calculated for $C_7H_9ClN_3O^+$: 186.043; found 186.049 $[M+H]^+$.

^{1}H NMR for 1-(4-chloropyrimidin-2-yl)azetidin-3-ol **5'** (minor product)

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.18 (d, *J* = 5.2 Hz, 1H), 6.58 (d, *J* = 5.1 Hz, 1H), 4.81 (dtd, *J* = 10.7, 6.4, 4.3 Hz, 1H), 4.44 (ddd, *J* = 10.0, 6.6, 1.4 Hz, 2H), 4.05 (ddd, *J* = 10.0, 4.3, 1.3 Hz, 2H), 2.40 (brs, 1H).

LCMS (ESI-MS) m/z calculated for $C_7H_9ClN_3O^+$: 186.043; found 186.042 $[M+H]^+$.

1-(2-chloropyrimidin-4-yl)piperidin-4-ol (6)



1 Major **6** Minor **6'**

Method A Step 1 was followed to synthesise 1-(2-chloropyrimidin-4-yl)piperidin-4-ol **6** starting from commercially available compounds 2,4-dichloropyrimidine **1** and piperidin-4-ol. Flash column chromatography using Heptane/EtOAc (0-100%) afforded compounds 1-(2-chloropyrimidin-4-yl)piperidin-4-ol **6** (major product) in 65% yield as a white solid and 1-(4-chloropyrimidin-2-yl)piperidin-4-ol **6'** (minor product) in 8% yield as a pale yellow gum.

^{1}H NMR for 1-(2-chloropyrimidin-4-yl)piperidin-4-ol **6** (major product)

^{1}H NMR (400 MHz, CD_3OD) δ 7.96 (d, *J* = 6.3 Hz, 1H), 6.71 (d, *J* = 6.4 Hz, 1H), 4.28 – 3.98 (m, 2H), 3.93 (tt, *J* = 8.3, 3.9 Hz, 1H), 3.43 – 3.33 (m, 2H), 2.00 – 1.87 (m, 2H), 1.62 – 1.44 (m, 2H). (One exchangeable proton of OH was not observed).

^{13}C NMR (101 MHz, MeOD) δ 162.94, 160.59, 156.77, 156.45, 102.33, 102.03, 101.71, 66.99, 33.91.

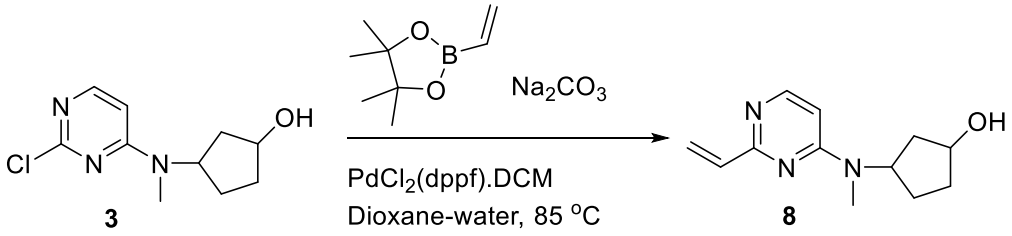
LCMS (ESI-MS) m/z calculated for: $C_9H_{13}ClN_3O^+$:214.074; found 214.077 $[M+H]^+$.

^{1}H NMR of 1-(4-chloropyrimidin-2-yl)piperidin-4-ol **6'** (minor product)

^{1}H NMR (400 MHz, CD_3OD) δ 8.18 (d, *J* = 5.1 Hz, 1H), 6.56 (d, *J* = 5.1 Hz, 1H), 4.49 – 4.25 (m, 2H), 3.88 (tt, *J* = 8.7, 4.0 Hz, 1H), 3.33 (dt, *J* = 10.2, 3.3 Hz, 2H), 2.04 – 1.82 (m, 2H), 1.47 (dtd, *J* = 13.2, 9.4, 4.0 Hz, 2H). (One exchangeable proton of OH was not observed).

LCMS (ESI-MS) m/z calculated for: $C_9H_{13}ClN_3O^+$: 214.074; found 214.071 $[M+H]^+$.

3-(methyl(2-vinylpyrimidin-4-yl)amino)cyclopentan-1-ol (8)



3 **8**

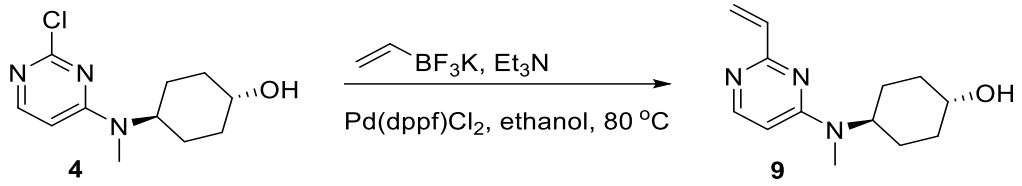
Method A Step 2 was followed to synthesise 3-(methyl(2-vinylpyrimidin-4-yl)amino)cyclopentan-1-ol **8** from 3-((2-chloropyrimidin-4-yl)(methyl)amino)cyclopentan-1-ol **3** in 30% yield as a pale yellow gum.

^{1}H NMR (400 MHz, $CDCl_3$) (Cis/Trans mixture) δ 8.07 (dd, *J* = 6.1, 2.6 Hz, 1H), 6.66 – 6.59 (m, 1H), 6.53 – 6.35 (m, 1H), 6.31 – 6.11 (m, 1H), 5.54 – 5.41 (m, 1H), 5.24 – 4.93 (m, 1H), 4.42 – 4.27 (m, 1H), 2.89 (s, 3H), 2.29 – 2.13 (m, 1H), 2.08 – 1.97 (m, 1H), 1.96 – 1.46 (m, 5H).

^{13}C NMR (101 MHz, $CDCl_3$) (Cis/Trans mixture) δ 163.20, 161.78, 155.59, 137.53, 122.37, 101.66, 72.18, 55.33, 38.10, 34.79, 31.28, 26.37.

LCMS (ESI-MS) m/z calculated for: $C_{12}H_{18}N_3O^+$: 220.144; found 220.146 $[M+H]^+$.

(1*R*,4*R*)-4-(methyl(2-vinylpyrimidin-4-yl)amino)cyclohexan-1-ol (9)



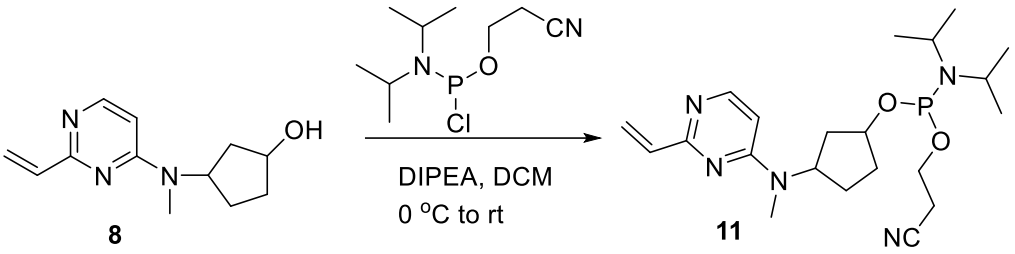
To a solution of (1*R*,4*R*)-4-((2-chloropyrimidin-4-yl)(methyl)amino)cyclohexan-1-ol **4** (140 mg, 0.58 mmol) and potassium trifluorovinyl borate (130 mg, 0.97 mmol) in ethanol (8 mL) under argon atmosphere was added Et_3N (0.17 mL, 1.22 mmol). The mixture was degassed for 5 min. Then, the catalyst $Pd(dppf)Cl_2$ (43 mg, 0.058 mmol) was added to the mixture and further degassed for 5 min. The reaction mixture was warmed up to 80 °C and stirred at this temperature for 6 hours. The reaction mixture was cooled to room temperature, diluted with DCM (10 mL), filtered through a bed of celite and washed several times with DCM (25 mL). Solvent was removed under vacuum. The residue was subjected to flash column chromatography using Heptane/Ethyl acetate (0-100%) to afford the product (1*R*,4*R*)-4-(methyl(2-vinylpyrimidin-4-yl)amino)cyclohexan-1-ol **9** (128 mg, 94.6%) as a colourless gum.

^{1}H NMR (400 MHz, CD_3OD) δ 8.23 (d, *J* = 5.0 Hz, 1H), 6.67 – 6.50 (m, 2H), 6.37 (dd, *J* = 17.4, 1.7 Hz, 1H), 5.57 (dd, *J* = 10.5, 1.7 Hz, 1H), 4.63 (tt, *J* = 10.1, 4.9 Hz, 1H), 3.56 (tt, *J* = 11.0, 4.3 Hz, 1H), 2.99 (s, 3H), 2.13 – 1.95 (m, 2H), 1.85 – 1.55 (m, 4H), 1.55 – 1.37 (m, 2H). (One exchangeable proton of OH was not observed).

^{13}C NMR (101 MHz, MeOD) δ 163.85, 162.84, 155.32, 137.74, 123.04, 102.78, 70.50, 61.51, 35.37, 29.56, 28.35.

LCMS (ESI-MS) m/z calculated for $C_{13}H_{20}N_3O^+$: 234.160; found 234.161 $[M+H]^+$.

2-cyanoethyl (3-(methyl(2-vinylpyrimidin-4-yl)amino)cyclopentyl) diisopropylphosphoramidite (11)

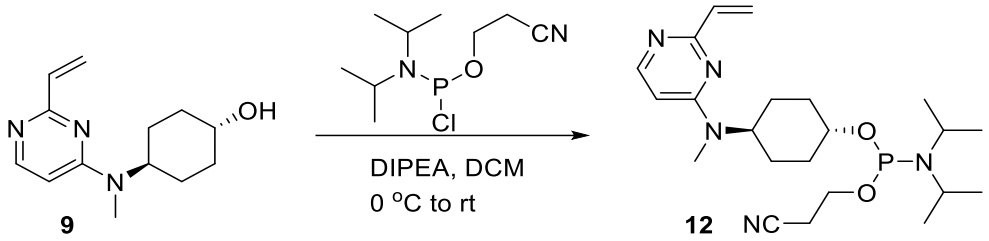


Method A Step 3 was followed to synthesise 2-cyanoethyl (3-(methyl(2-vinylpyrimidin-4-yl)amino)cyclopentyl) diisopropylphosphoramidite **11** (mixture of diastereoisomers) from 3-(methyl(2-vinylpyrimidin-4-yl)amino)cyclopentan-1-ol **8** in 50% yield as a colourless gum.

^{1}H NMR (400 MHz, CD_2Cl_2) (Mixture of diastereoisomers) δ 8.16 – 7.96 (m, 1H), 6.69 – 6.50 (m, 1H), 6.50 – 6.28 (m, 1H), 6.30 – 6.11 (m, 1H), 5.47 – 5.37 (m, 1H), 5.31 – 5.11 (m, 1H), 4.44 – 4.27 (m, 1H), 3.82 – 3.62 (m, 2H), 3.59 – 3.49 (m, 2H), 2.89 – 2.79 (m, 3H), 2.64 – 2.45 (m, 2H), 2.22 – 2.14 (m, 1H), 2.03 – 1.89 (m, 1H), 1.85 – 1.57 (m, 4H), 1.13 – 1.09 (m, 12H).

^{31}P NMR (162 MHz, CD_2Cl_2) (Mixture of diastereoisomers) δ 146.78, 146.53.

2-cyanoethyl ((1*R*,4*R*)-4-(methyl(2-vinylpyrimidin-4-yl)amino)cyclohexyl) diisopropylphosphoramidite (12)

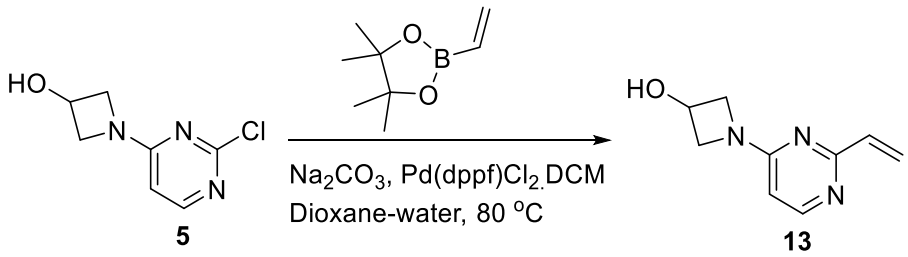


A stirred solution of (1*R*,4*R*)-4-(methyl(2-vinylpyrimidin-4-yl)amino)cyclohexan-1-ol **9** (128 mg, 0.55 mmol) (dried overnight in high vacuum) in DCM (8 mL) was degassed by bubbling with argon for 5 min. The solution was cooled to 0 °C in ice bath and DIPEA (0.29 mL, 1.65 mmol) was added followed by dropwise addition of 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (0.18 mL, 0.83 mmol). The reaction mixture was allowed to gradually warm to room temperature and stirred for 6 hours. Solvent was removed under vacuum. The residue was subjected to flash column chromatography using Heptane/(1% Et_3N)/EtOAc (0-100%) to afford product 2-cyanoethyl ((1*R*,4*R*)-4-(methyl(2-vinylpyrimidin-4-yl)amino)cyclohexyl) diisopropylphosphoramidite (diastereomeric mixture) **12** (136 mg, 57%) as a colourless gum.

^{1}H NMR (400 MHz, CD_3CN) (Mixture of diastereoisomers) δ 8.29 (d, *J* = 4.9 Hz, 1H), 6.62 (dd, *J* = 17.4, 10.5 Hz, 1H), 6.54 (d, *J* = 4.9 Hz, 1H), 6.42 (dd, *J* = 17.4, 1.8 Hz, 1H), 5.57 (dd, *J* = 10.5, 1.8 Hz, 1H), 4.76

– 4.63 (m, 1H), 3.84 – 3.72 (m, 3H), 3.68 – 3.59 (m, 2H), 3.01 (s, 3H), 2.70 – 2.65 (m, 2H), 2.16 – 2.11 (m, 1H), 2.08 – 2.04 (m, 1H), 1.75 – 1.66 (m, 4H), 1.60 – 1.48 (m, 2H), 1.20 (dd, *J* = 6.8, 2.2 Hz, 12H).
^{31}P NMR (162 MHz, CD_3CN) (Mixture of diastereoisomers) δ 145.45.

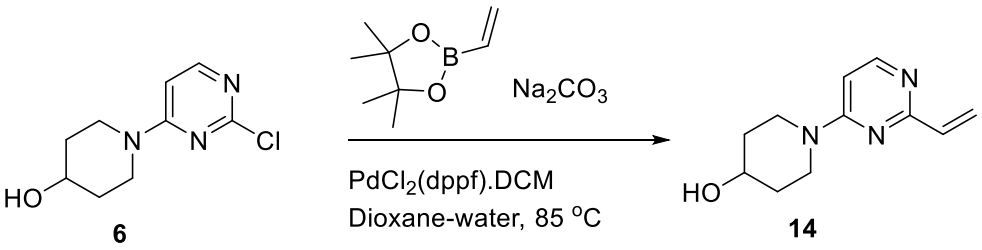
1-(4-vinylpyrimidin-2-yl)azetidin-3-ol (13)



Method A Step 2 was followed to synthesise 1-(4-vinylpyrimidin-2-yl)azetidin-3-ol **13** from 1-(2-chloropyrimidin-4-yl)azetidin-3-ol **5** in 70% yield as a pale brown gum. Flash column chromatography was performed for purification using DCM/MeOH (0-50%) solvent system.

^{1}H NMR (400 MHz, CD_3OD) δ 8.08 (d, *J* = 6.0 Hz, 1H), 6.64 (dd, *J* = 17.3, 10.5 Hz, 1H), 6.46 (dd, *J* = 17.3, 2.1 Hz, 1H), 6.25 (d, *J* = 6.0 Hz, 1H), 5.64 (dd, *J* = 10.5, 2.0 Hz, 1H), 4.76 – 4.67 (m, 1H), 4.35 (dd, *J* = 9.7, 6.6 Hz, 2H), 3.91 (dd, *J* = 9.8, 4.3 Hz, 2H). (One exchangeable proton of OH was not observed).
^{13}C NMR (101 MHz, MeOD) δ 164.37, 163.82, 154.94, 137.30, 123.62, 102.06, 62.93, 60.42.
LCMS (ESI-MS) m/z calculated for $C_9H_{12}N_3O^+$: 178.097; found 178.099 $[M+H]^+$.

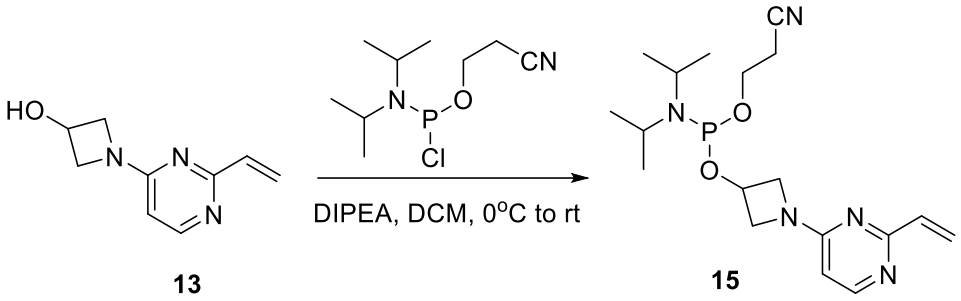
1-(2-vinylpyrimidin-4-yl)piperidin-4-ol (14)



Method A Step 2 was followed to synthesise 1-(2-vinylpyrimidin-4-yl)piperidin-4-ol **14** from 1-(2-chloropyrimidin-4-yl)piperidin-4-ol **6** in 52% yield as a pale brown gum.

^{1}H NMR (400 MHz, CD_3OD) δ 8.08 (t, *J* = 6.2 Hz, 1H), 6.60 – 6.51 (m, 2H), 6.47 (dd, *J* = 17.3, 2.1 Hz, 1H), 5.76 – 5.55 (m, 1H), 4.23 – 4.20 (m, 2H), 3.55 – 3.07 (m, 3H), 1.98 – 1.91 (m, 2H), 1.61 – 1.41 (m, 2H). (One exchangeable proton of OH was not observed).
^{13}C NMR (101 MHz, CD_3OD) δ 162.74, 161.16, 154.14, 136.11, 122.00, 101.23, 66.79, 41.10, 33.32.
LCMS (ESI-MS) m/z calculated for: $C_{11}H_{16}N_3O^+$: 206.129; found 206.125 $[M+H]^+$.

2-cyanoethyl (1-(2-vinylpyrimidin-4-yl)azetidin-3-yl) diisopropylphosphoramidite (15)

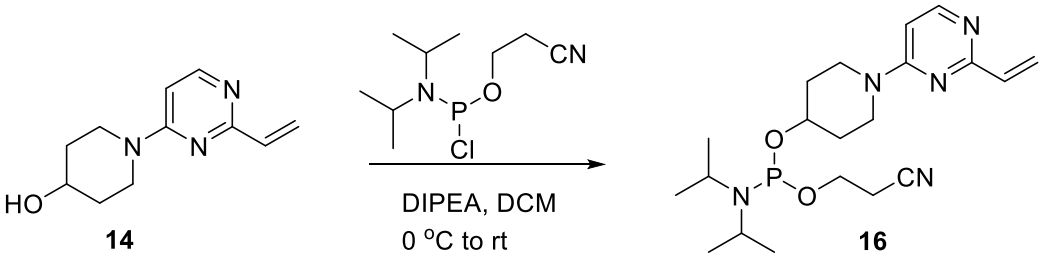


Method A Step 3 was followed to synthesise 2-cyanoethyl (1-(2-vinylpyrimidin-4-yl)azetidin-3-yl) diisopropylphosphoramidite **15** (diastereomeric mixture) from 1-(4-vinylpyrimidin-2-yl)azetidin-3-ol **13** in 51% yield as a colourless gum.

^{1}H NMR (400 MHz, CD_3CN) (Mixture of diastereoisomers) δ 8.17 (d, *J* = 5.8 Hz, 1H), 6.64 (dd, *J* = 17.3, 10.4 Hz, 1H), 6.46 (dd, *J* = 17.3, 2.4 Hz, 1H), 6.20 (d, *J* = 5.9 Hz, 1H), 5.59 (dd, *J* = 10.4, 2.4 Hz, 1H), 4.89 – 4.81 (m, 1H), 4.37 – 4.32 (m, 2H), 4.03 – 3.97 (m, 2H), 3.90 – 3.75 (m, 2H), 3.70 – 3.62 (m, 2H), 2.69 (t, *J* = 6.0 Hz, 2H), 1.21 (dd, *J* = 6.8, 2.1 Hz, 12H).

^{31}P NMR (162 MHz, CD_3CN) (Mixture of diastereoisomers) δ 147.25.

2-cyanoethyl (1-(2-vinylpyrimidin-4-yl)piperidin-4-yl) diisopropylphosphoramidite (16)

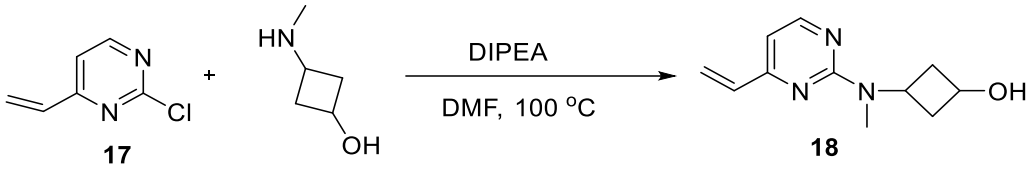


Method A Step 3 was followed to synthesise 2-cyanoethyl (1-(2-vinylpyrimidin-4-yl)piperidin-4-yl) diisopropylphosphoramidite **16** (diastereomeric mixture) from 1-(2-vinylpyrimidin-4-yl)piperidin-4-ol **14** in 78% yield as a colourless gum.

^{1}H NMR (400 MHz, CD_2Cl_2) (Mixture of diastereoisomers) δ 8.08 – 7.93 (m, 1H), 6.61 – 6.40 (m, 1H), 6.36 – 6.18 (m, 2H), 5.46 – 5.34 (m, 1H), 4.06 – 3.92 (m, 1H), 3.79 – 3.33 (m, 8H), 2.47 (t, *J* = 6.2 Hz, 2H), 1.79 – 1.68 (m, 2H), 1.66 – 1.47 (m, 2H), 1.04 (d, *J* = 6.8 Hz, 12H).

^{31}P NMR (162 MHz, CD_2Cl_2) (Mixture of diastereoisomers) δ 146.39.

3-(methyl(4-vinylpyrimidin-2-yl)amino)cyclobutan-1-ol (18)



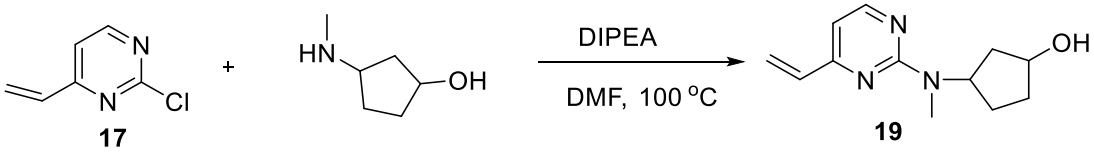
Method B Step 2 was followed to synthesise 3-(methyl(4-vinylpyrimidin-2-yl)amino)cyclobutan-1-ol **18** (mixture of Cis/Trans) from 2-chloro-4-vinylpyrimidine **17** and commercially available 3-(methylamino)cyclobutan-1-ol (mixture of Cis/Trans 5:1) in 44% yield as a yellow gum.

^{1}H NMR (400 MHz, $CDCl_3$) (Mixture of Cis/Trans) δ 8.30 (dd, *J* = 5.0, 2.0 Hz, 1H), 6.67 – 6.58 (m, 1H), 6.50 (d, *J* = 5.0 Hz, 1H), 6.39 (ddd, *J* = 17.4, 4.7, 1.6 Hz, 1H), 5.65 – 5.51 (m, 1H), 4.70 (tt, *J* = 9.7, 7.4 Hz, 1H), 4.21 – 4.06 (m, 1H), 3.17 (d, *J* = 5.6 Hz, 3H), 2.79 – 2.52 (m, 2H), 2.40 – 2.11 (m, 3H).

^{13}C NMR (101 MHz, $CDCl_3$) (Mixture of Cis/Trans) δ 162.65, 162.61, 162.14, 162.09, 158.14, 158.09, 157.11, 137.88, 136.29, 121.45, 115.15, 108.80, 107.13, 107.09, 64.58, 61.48, 48.30, 42.21, 38.78, 37.38, 32.31, 30.12.

LCMS (ESI-MS) m/z calculated for $C_{11}H_{16}N_3O^+$: 206.129; found 206.121 $[M+H]^+$.

3-(methyl(4-vinylpyrimidin-2-yl)amino)cyclopentan-1-ol (19)



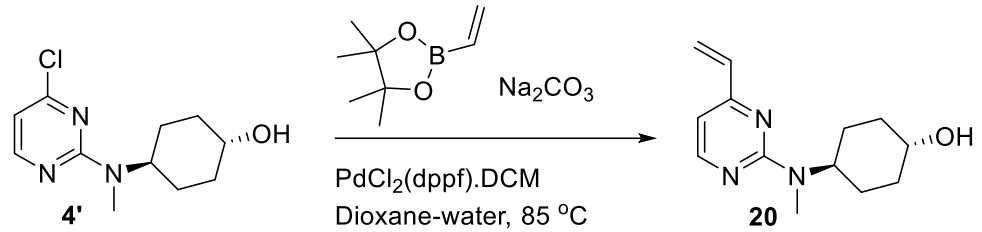
Method B Step 2 was followed to synthesise 3-(methyl(4-vinylpyrimidin-2-yl)amino)cyclopentan-1-ol **19** (mixture of Cis/Trans) from 2-chloro-4-vinylpyrimidine **17** and commercially available 3-methylamino-cyclopentan-1-ol (mixture of Cis/Trans) in 61% yield as a pale brown gum.

^{1}H NMR (400 MHz, $CDCl_3$) (Mixture of Cis/Trans) δ 8.26 (dd, *J* = 16.3, 5.0 Hz, 1H), 6.74 – 6.26 (m, 3H), 5.57 (td, *J* = 10.6, 1.6 Hz, 1H), 4.76 – 4.25 (m, 2H), 3.23 (s, 1.5H), 3.03 (s, 1.5H), 2.37 – 1.99 (m, 3H), 1.98 – 1.62 (m, 4H).

^{13}C NMR (101 MHz, $CDCl_3$) (Mixture of Cis/Trans) δ 163.05, 162.62, 162.22, 160.98, 158.13, 157.34, 136.40, 136.19, 121.70, 121.28, 106.61, 106.33, 72.60, 72.42, 58.16, 54.09, 38.14, 38.04, 34.89, 34.45, 34.38, 29.32, 26.56, 25.76.

LCMS (ESI-MS) m/z calculated for $C_{12}H_{18}N_3O^+$: 220.144; found 220.146 $[M+H]^+$.

(1*R*,4*R*)-4-(methyl(4-vinylpyrimidin-2-yl)amino)cyclohexan-1-ol (20)



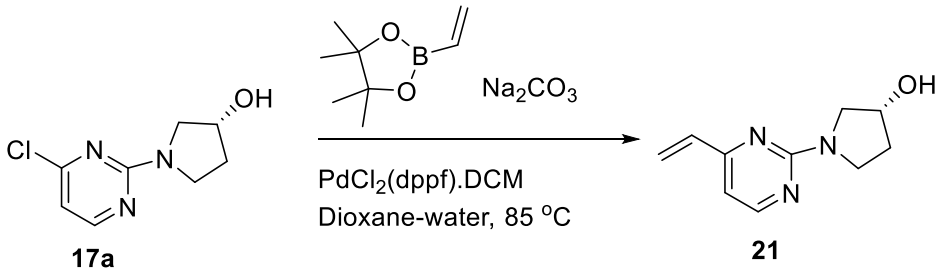
To a solution of (1*R*,4*R*)-4-((4-chloropyrimidin-2-yl)(methyl)amino)cyclohexan-1-ol **4'** (140 mg, 0.58 mmol) and vinylboronic acid pinacol ester (0.3 mL, 1.74 mmol) in dioxane (4 mL) and water (1 mL) under argon atmosphere was added Na_2CO_3 (215 mg, 2.03 mmol). The mixture was degassed for 5 min. Then the catalyst Pd(dppf)Cl_2.DCM (49 mg, 0.058 mmol) was added to the mixture and further degassed for 5 min. The reaction mixture was warmed up to 85 °C and stirred at this temperature for 18 hours. The reaction mixture was cooled to room temperature, diluted with DCM and filtered through a bed of celite and washed several times with DCM. Solvent was removed under vacuum. The residue was subjected to flash column chromatography using Heptane/Ethyl acetate (0-100%) to afford the product (1*R*,4*R*)-4-(methyl(4-vinylpyrimidin-2-yl)amino)cyclohexan-1-ol **20** (130 mg, 96%) as a pale yellow gum.

^{1}H NMR (400 MHz, CD_3OD) δ 8.08 (d, *J* = 6.3 Hz, 1H), 7.67 – 7.46 (m, 1H), 6.65 (dd, *J* = 17.4, 10.4 Hz, 1H), 6.60 – 6.37 (m, 2H), 5.63 (dd, *J* = 10.4, 2.1 Hz, 1H), 3.60 (tt, *J* = 11.1, 4.4 Hz, 1H), 2.95 (s, 3H), 2.18 – 1.97 (m, 2H), 1.75 – 1.66 (m, 4H), 1.54 – 1.42 (m, 2H). (One exchangeable proton of OH was not observed).

^{13}C NMR (101 MHz, MeOD) δ 162.45, 161.45, 153.92, 136.34, 130.86, 128.47, 121.70, 101.42, 69.11, 33.99, 28.20, 26.97, 23.66.

LCMS (ESI-MS) m/z calculated for $C_{13}H_{20}N_3O^+$: 234.160; found 234.168 $[M+H]^+$.

(*R*)-1-(4-vinylpyrimidin-2-yl)pyrrolidin-3-ol (21)



Method A Step 2 was followed to synthesise (*R*)-1-(4-vinylpyrimidin-2-yl)pyrrolidin-3-ol **21** from commercially available (*R*)-1-(4-chloropyrimidin-2-yl)pyrrolidin-3-ol **17a** in 45% yield as a yellow gum.

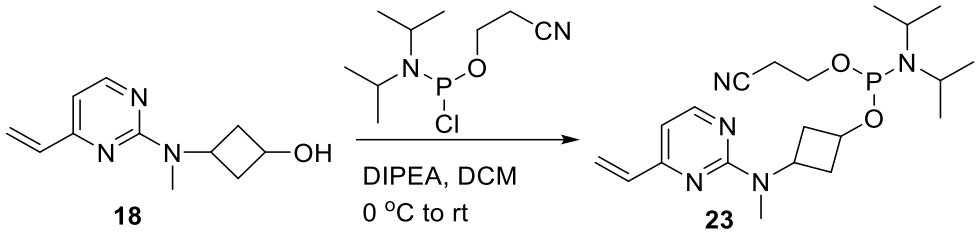
Flash column chromatography was performed for purification using Hexane/EtOAc (0-100%) solvent system.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.18 (d, *J* = 5.1 Hz, 1H), 6.50 (dd, *J* = 17.4, 10.5 Hz, 1H), 6.40 (d, *J* = 5.1 Hz, 1H), 6.28 (dd, *J* = 17.4, 1.6 Hz, 1H), 5.48 (dd, *J* = 10.5, 1.6 Hz, 1H), 4.62 – 4.37 (m, 1H), 3.77 – 3.48 (m, 4H), 2.79 (brs, 1H), 2.12 – 1.84 (m, 2H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 162.94, 160.53, 158.12, 136.18, 121.55, 106.70, 70.90, 55.09, 44.48, 34.04.

LCMS (ESI-MS) m/z calculated for: $C_{10}H_{14}N_3O^+$: 192.113; found 192.119 $[M+H]^+$.

2-cyanoethyl (3-(methyl(4-vinylpyrimidin-2-yl)amino)cyclobutyl) diisopropylphosphoramidite (23)

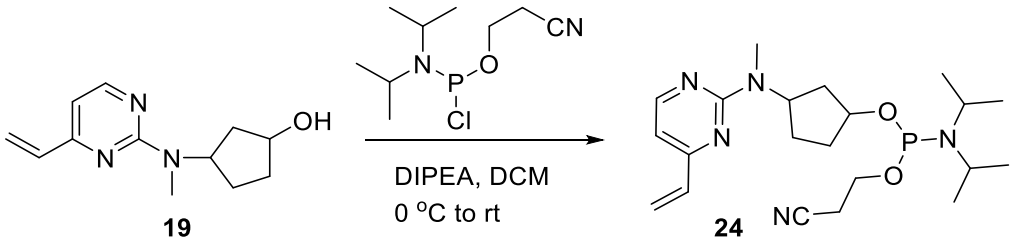


Method B Step 3 was followed to synthesise 2-cyanoethyl (3-(methyl(4-vinylpyrimidin-2-yl)amino)cyclobutyl) diisopropylphosphoramidite (diastereomeric mixture) **23** from 3-(methyl(4-vinylpyrimidin-2-yl)amino)cyclobutan-1-ol **18** in 72% yield as a colourless gum.

^{1}H NMR (400 MHz, CD_2Cl_2) (Mixture of diastereoisomers) δ 8.30 (t, *J* = 4.7 Hz, 1H), 6.62 (ddd, *J* = 17.3, 10.5, 1.3 Hz, 1H), 6.52 (d, *J* = 5.0 Hz, 1H), 6.47 – 6.36 (m, 1H), 5.58 (dd, *J* = 10.5, 1.7 Hz, 1H), 4.87 – 4.76 (m, 1H), 4.17 (dddd, *J* = 14.3, 9.2, 7.6, 6.6 Hz, 1H), 3.94 – 3.76 (m, 2H), 3.76 – 3.54 (m, 2H), 3.17 (s, 3H), 2.82 – 2.54 (m, 4H), 2.51 – 2.23 (m, 2H), 1.23 (ddd, *J* = 6.8, 3.3, 1.5 Hz, 12H).

^{31}P NMR (162 MHz, CD_2Cl_2) (Mixture of diastereoisomers) δ 146.26, 145.38.

2-cyanoethyl (3-(methyl(4-vinylpyrimidin-2-yl)amino)cyclopentyl) diisopropylphosphoramidite (24)

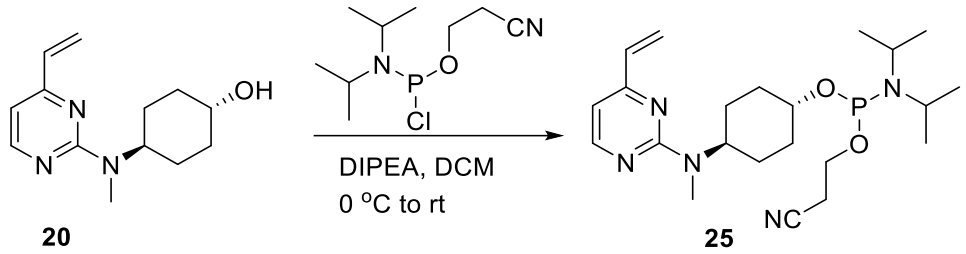


Method B Step 3 was followed to synthesise 2-cyanoethyl (3-(methyl(4-vinylpyrimidin-2-yl)amino)cyclopentyl) diisopropylphosphoramidite (diastereomeric mixture) **24** from 3-(methyl(4-vinylpyrimidin-2-yl)amino)cyclopentan-1-ol **19** in 70% yield as a colourless gum.

^{1}H NMR (400 MHz, CD_2Cl_2) (Mixture of diastereoisomers) δ 8.28 – 8.10 (m, 1H), 6.49 (ddd, *J* = 17.4, 10.5, 1.7 Hz, 1H), 6.41 – 6.22 (m, 2H), 5.51 – 5.40 (m, 1H), 4.59 – 4.20 (m, 1H), 3.80 – 3.65 (m, 2H), 3.59 – 3.49 (m, 2H), 2.99 (d, *J* = 3.2 Hz, 1.5H), 2.92 (d, *J* = 1.9 Hz, 1.5H), 2.59 – 2.50 (m, 2H), 2.23 – 1.51 (m, 7H), 1.13 – 1.08 (m, 12H).

^{31}P NMR (162 MHz, CD_2Cl_2) (Mixture of diastereoisomers) δ 146.62, 146.54, 146.43.

2-cyanoethyl ((1*R*,4*R*)-4-(methyl(4-vinylpyrimidin-2-yl)amino)cyclohexyl) diisopropylphosphoramidite (25)



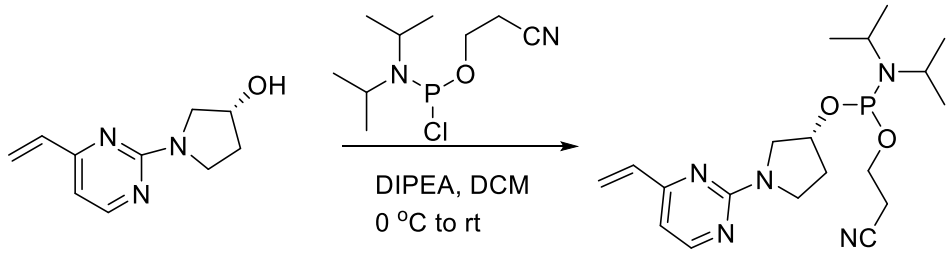
20 **25**

Method A Step 3 was followed to synthesise 2-cyanoethyl ((1*R*,4*R*)-4-(methyl(4-vinylpyrimidin-2-yl)amino)cyclohexyl) diisopropylphosphoramidite (diastereomeric mixture) **25** from (1*R*,4*R*)-4-(methyl(4-vinylpyrimidin-2-yl)amino)cyclohexan-1-ol **20** in 68% yield as a colourless gum.

^{1}H NMR (400 MHz, CD_3CN) (Mixture of diastereoisomers) δ 8.15 (d, *J* = 6.2 Hz, 1H), 6.65 (dd, *J* = 17.3, 10.4 Hz, 1H), 6.48 – 6.33 (m, 1H), 5.57 (dd, *J* = 10.4, 2.4 Hz, 1H), 3.88 – 3.72 (m, 3H), 3.69 – 3.59 (m, 2H), 2.90 (s, 3H), 2.67 (t, *J* = 6.0 Hz, 2H), 2.17 – 2.12 (m, 1H), 2.09 – 2.04 (m, 1H), 1.78 – 1.63 (m, 4H), 1.62 – 1.44 (m, 2H), 1.35 – 1.24 (m, 2H), 1.21 (dd, *J* = 6.8, 2.3 Hz, 12H).

^{31}P NMR (162 MHz, CD_3CN) (Mixture of diastereoisomers) δ 145.49.

2-cyanoethyl ((*R*)-1-(4-vinylpyrimidin-2-yl)pyrrolidin-3-yl) diisopropylphosphoramidite (26)



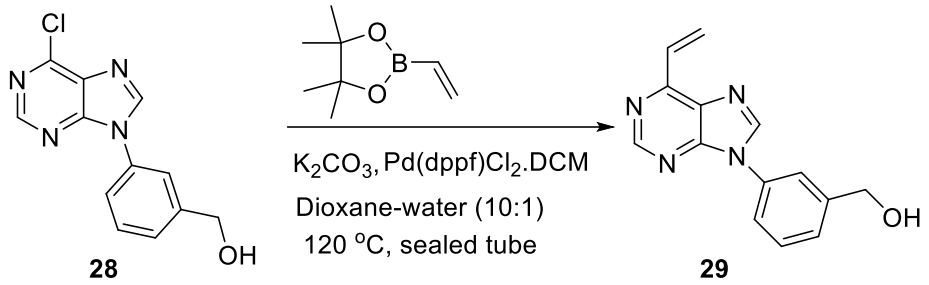
21 **26**

Method A Step 3 was followed to synthesise 2-cyanoethyl ((*R*)-1-(4-vinylpyrimidin-2-yl)pyrrolidin-3-yl) diisopropylphosphoramidite (diastereomeric mixture) **26** from (*R*)-1-(4-vinylpyrimidin-2-yl)pyrrolidin-3-ol **21** in 74% yield as a colourless gum.

^{1}H NMR (400 MHz, CD_3CN) (Mixture of diastereoisomers) δ 8.29 (dd, *J* = 5.0, 2.7 Hz, 1H), 6.76 – 6.54 (m, 2H), 6.41 (ddd, *J* = 17.4, 4.5, 1.8 Hz, 1H), 5.58 (ddd, *J* = 10.5, 2.4, 1.7 Hz, 1H), 3.98 – 3.43 (m, 9H), 2.73 – 2.59 (m, 2H), 2.18 – 2.10 (m, 2H), 1.21 – 1.14 (m, 12H).

^{31}P NMR (162 MHz, CD_3CN) (Mixture of diastereoisomers) δ 147.05, 146.98.

(3-(6-vinyl-9*H*-purin-9-yl)phenyl)methanol (29)



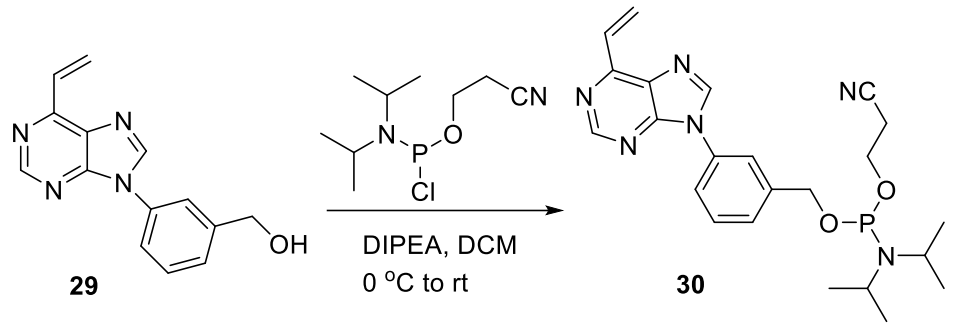
To a solution of commercially available (3-(6-chloro-9*H*-purin-9-yl)phenyl)methanol **28** (120 mg, 0.46 mmol) and vinylboronic acid pinacol ester (0.25 mL, 1.38 mmol) in dioxane (5 mL) and water (0.5 mL) in microwave tube under argon atmosphere was added K_2CO_3 (262 mg, 1.84 mmol). The mixture was degassed for 5 min. Then the catalyst Pd(dppf)Cl_2.DCM (38 mg, 0.46 mmol) was added to the mixture and further degassed for 5 min. The tube was sealed and the reaction mixture was warmed up to 120 °C and stirred at this temperature for 18 hours. The reaction mixture was cooled to room temperature, diluted with DCM (5 mL) and filtered through a bed of celite washing several times with DCM (3 x 10 mL). Solvent was removed under vacuum. The residue was subjected to flash column chromatography using Heptane/Ethyl acetate (0-100%) to afford the product (3-(6-vinyl-9*H*-purin-9-yl)phenyl)methanol **29** (60 mg, 51.7%) as a pale yellow gum.

^{1}H NMR (400 MHz, CD_3OD) δ 8.86 – 8.84 (m, 1H), 8.77 – 8.76 (m, 1H), 7.84 (brs, 1H), 7.73 (d, *J* = 7.9 Hz, 1H), 7.58 (t, *J* = 7.8 Hz, 1H), 7.50 (d, *J* = 7.7 Hz, 1H), 7.33 (dd, *J* = 17.5, 11.0 Hz, 1H), 7.03 (dd, *J* = 17.5, 1.7 Hz, 1H), 6.00 (dd, *J* = 11.0, 1.7 Hz, 1H), 4.74 (s, 2H). (One exchangeable proton of OH was not observed).

^{13}C NMR (101 MHz, MeOD) δ 154.71, 153.66, 153.16, 146.50, 145.24, 135.74, 132.39, 130.81, 127.81, 127.32, 123.56, 123.08, 116.01, 64.49.

LCMS (ESI-MS) m/z calculated for: $C_{14}H_{13}N_4O^+$: 253.108; found 253.109 $[M+H]^+$.

2-cyanoethyl (3-(6-vinyl-9*H*-purin-9-yl)benzyl) diisopropylphosphoramidite (30)

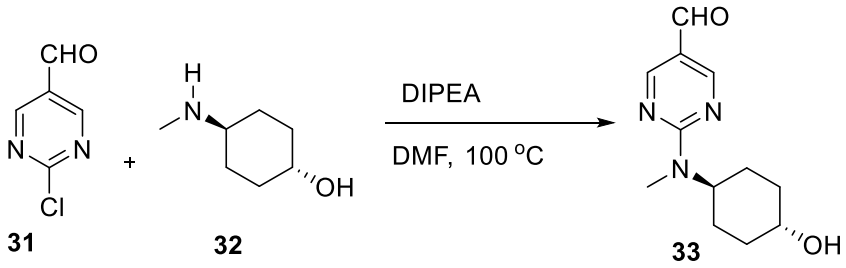


Method A Step 3 was followed to synthesise 2-cyanoethyl (3-(6-vinyl-9*H*-purin-9-yl)benzyl) diisopropylphosphoramidite (diastereomeric mixture) **30** from (3-(6-vinyl-9*H*-purin-9-yl)phenyl)methanol **29** in 76% yield as a colourless gum.

^{1}H NMR (400 MHz, CD_2Cl_2) (Mixture of diastereoisomers) δ 8.92 (s, 1H), 8.39 (s, 1H), 7.81 (s, 1H), 7.73 – 7.62 (m, 1H), 7.62 – 7.52 (m, 1H), 7.47 (dt, *J* = 7.9, 1.2 Hz, 1H), 7.35 (dd, *J* = 17.5, 10.9 Hz, 1H), 7.07 (dd, *J* = 17.5, 1.8 Hz, 1H), 5.96 (dd, *J* = 10.9, 1.9 Hz, 1H), 4.96 – 4.71 (m, 2H), 3.95 – 3.80 (m, 2H), 3.73 – 3.62 (m, 2H), 2.65 (t, *J* = 6.2 Hz, 2H), 1.22 (dd, *J* = 6.8, 1.3 Hz, 12H).

^{31}P NMR (162 MHz, CD_2Cl_2) (Mixture of diastereoisomers) δ 148.79.

2-((1*R*,4*R*)-4-hydroxycyclohexyl)(methyl)amino)pyrimidine-5-carbaldehyde (33)



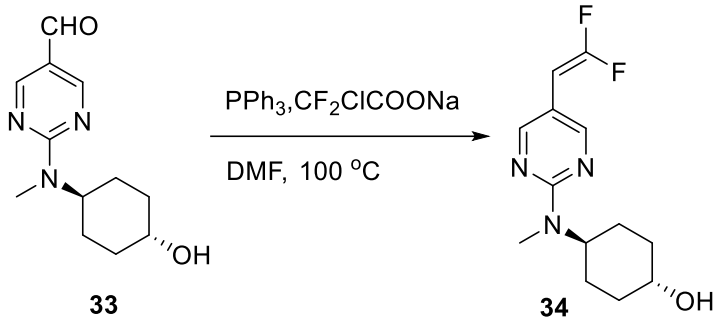
Method B Step 2 was followed to synthesise 2-((1*R*,4*R*)-4-hydroxycyclohexyl)(methyl)amino)pyrimidine-5-carbaldehyde **33** from commercially available 2-chloropyrimidine-5-carbaldehyde **31** and commercially available trans-4-(methylamino)cyclohexan-1-ol **32** in 65% yield as a yellow gum. Flash column chromatography was performed for purification using Hexane/EtOAc (0-100%) solvent system.

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.70 (s, 1H), 8.67 (s, 2H), 4.75 (tt, *J* = 11.7, 4.0 Hz, 1H), 3.60 (tt, *J* = 10.8, 4.3 Hz, 1H), 3.07 (s, 3H), 2.65 (s, 1H), 2.12 – 2.01 (m, 2H), 1.81 – 1.40 (m, 6H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 187.86, 162.53, 160.46, 160.16, 119.37, 69.76, 53.96, 34.39, 29.49, 27.48.

LCMS (ESI-MS) m/z calculated for: $C_{12}H_{18}N_3O_2^+$: 236.139; found 236.142 $[M+H]^+$.

(1*R*,4*R*)-4-((5-(2,2-difluorovinyl)pyrimidin-2-yl)(methyl)amino)cyclohexan-1-ol (34)

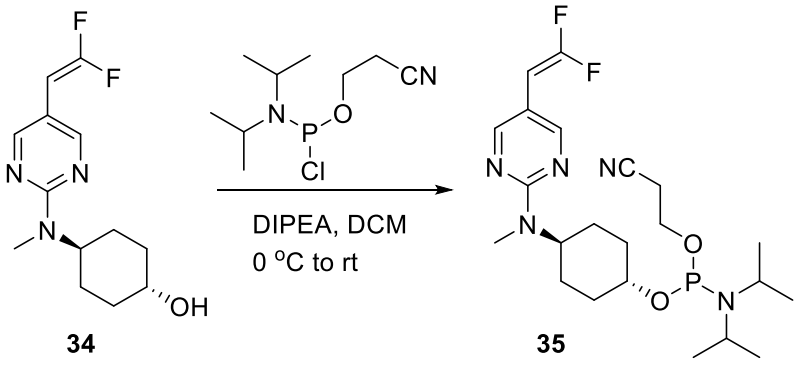


33 **34**

Aldehyde **33** (165 mg, 0.70 mmol) and triphenylphosphine (368 mg, 1.4 mmol) were dissolved in DMF (2.5 mL) under argon atmosphere and heated to 100 °C. Sodium chlorodifluoroacetate (314 mg, 1.47 mmol) was added portion-wise at 100 °C and the reaction mixture was stirred at this temperature for 24 hours. The reaction mixture was cooled to room temperature and solvent was removed under vacuum. The residue was dissolved in DCM (10 mL) and washed with water (2.5 mL), 3% H_2O_2 in water (2.5 mL) and brine (2.5 mL). The organic layer was dried over sodium sulphate and solvent was removed under vacuum. Flash column chromatography using Heptane/Ethyl acetate (0-100%) afforded (1*R*,4*R*)-4-((5-(2,2-difluorovinyl)pyrimidin-2-yl)(methyl)amino)cyclohexan-1-ol **34** (76 mg, 41%) as a pale brown oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.21 (s, 2H), 4.99 (dd, *J* = 27.3, 2.8 Hz, 1H), 4.56 (tt, *J* = 11.7, 4.0 Hz, 1H), 3.55 (tt, *J* = 10.6, 4.3 Hz, 1H), 2.93 (s, 3H), 2.07 – 1.80 (m, 3H), 1.78 – 1.60 (m, 2H), 1.59 – 1.33 (m, 4H).
^{19}F NMR (376 MHz, $CDCl_3$) δ -83.19 (d, *J* = 36.1 Hz), -85.40 (d, *J* = 36.1 Hz).
^{13}C NMR (101 MHz, $CDCl_3$) δ 160.39, 156.19 (dd, *J* = 6.3, 3.2 Hz), 155.88 (dd, *J* = 296.4, 288.5 Hz), 152.98, 112.15 (t, *J* = 6.2 Hz), 76.58 (dd, *J* = 31.2, 16.6 Hz), 70.17, 53.12, 34.66, 28.88, 27.52.
LCMS (ESI-MS) m/z calculated for: $C_{13}H_{18}F_2N_3O^+$: 270.141; found 270.148 $[M+H]^+$.

2-cyanoethyl ((1*R*,4*R*)-4-((5-(2,2-difluorovinyl)pyrimidin-2-yl)(methyl)amino)cyclohexyl) diisopropylphosphoramidite (35)



34 **35**

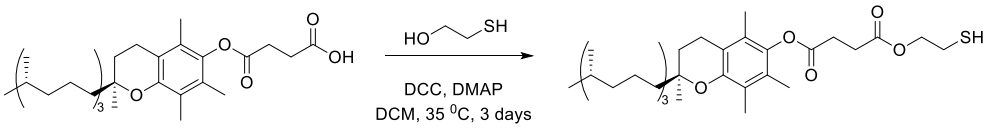
Method B Step 3 was followed to synthesise 2-cyanoethyl ((1*R*,4*R*)-4-((5-(2,2-difluorovinyl)pyrimidin-2-yl)(methyl)amino)cyclohexyl) diisopropylphosphoramidite **35** from (1*R*,4*R*)-4-((5-(2,2-difluorovinyl)pyrimidin-2-yl)(methyl)amino)cyclohexan-1-ol (diastereomeric mixture) **34** in 75% yield as a colourless gum.

^{1}H NMR (400 MHz, CD_2Cl_2) (Mixture of diastereoisomers) δ 8.18 (s, 2H), 5.01 (dd, *J* = 27.6, 3.0 Hz, 1H), 4.54 (tt, *J* = 11.3, 4.1 Hz, 1H), 3.80 – 3.61 (m, 3H), 3.57 – 3.48 (m, 2H), 2.90 (s, 3H), 2.55 (t, *J* = 6.3 Hz, 2H), 2.15 – 1.87 (m, 2H), 1.67 – 1.40 (m, 6H), 1.10 (dd, *J* = 6.9, 1.2 Hz, 12H).

^{31}P NMR (162 MHz, CD_2Cl_2) (Mixture of diastereoisomers) δ 145.90.

^{19}F NMR (376 MHz, CD_2Cl_2) (Mixture of diastereoisomers) δ -84.17 (d, *J* = 38.1 Hz), -86.61 (d, *J* = 38.1 Hz).

Synthesis of mercaptoethyl-D-α-tocopheryl succinate (37)



36 **37**

Under argon atmosphere, DCC (933 mg, 4.52 mmol), DMAP (92.1 mg, 0.753 mmol) and D-α-Tocopheryl succinate **36** (2.00 g, 3.77 mmol) were added and dissolved in anhydrous DCM (15 mL). After stirring at room temperature for 10 min, β-mercaptoethanol (0.4 mL, 4.60 mmol) was added to the mixture and the reaction mixture was stirred at 35 °C for 3 days away from light. The solution was then filtered and washed thrice with 0.05 M aqueous HCl, thrice with saturated aqueous sodium bicarbonate solution and thrice with water. The resultant solution was washed with saturated brine solution (3 x 10 mL). After drying over Na_2SO_4, the organic phase was filtered and the solvent was removed under reduced pressure to afford the crude mercaptoethyl-D-α-tocopheryl succinate (1.87 g) which was dried under high vacuum and used in the next reaction without further purification. The crude ^{1}H NMR of mercaptoethyl-D-α-tocopheryl succinate (TocoSH) **37** reported below corresponds to that of the ^{1}H NMR reported in the literature.[ii]

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.24 (t, *J* = 6.7 Hz, 2H), 2.94 (t, *J* = 6.9 Hz, 2H), 2.82 – 2.72 (m, 3H), 2.58 (t, *J* = 6.8 Hz, 2H), 2.08 (s, 3H), 2.01 (s, 3H), 1.97 (s, 3H), 1.87 – 1.69 (m, 4H), 1.64 – 1.47 (m, 5H), 1.45 – 1.24 (m, 12H), 1.18 – 1.01 (m, 7H), 0.85 (dd, *J* = 9.3, 6.8 Hz, 12H).

Oligonucleotide synthesis

General method for the synthesis of oligonucleotides containing novel linkers

Solid phase oligonucleotide synthesis

Oligonucleotides were synthesised on a 1 µmol scale with the MM12 synthesiser (LGC Biosearch Technologies) using 1000 Å Universal Controlled Pore Glass (CPG) (loading 47.7 µmol/g, LGC Biosearch Technologies). Cleavage of the 4,4'-dimethoxytrityl (DMTr) group was performed with 3% trichloroacetic acid in dichloromethane (DCM) (LGC Biosearch Technologies). DNA and LNA phosphoramidites were prepared as 0.1 M solutions in dry acetonitrile (ACN) (LGC Biosearch Technologies). Linker phosphoramidites were dissolved in a 50:50 THF:DCM mixture (0.08 M). Solvents were moisture controlled with less than 30 ppm water content. 0.25 M solution of 5-ethylthio-1*H*-tetrazole in dry ACN (LGC Biosearch Technologies) was used as an activator. Failed sequences were capped with a 1:1 mixture of Capping Mix A (Tetrahydrofuran/Lutidine/Acetic anhydride, 8:1:1, v/v/v, LGC Biosearch Technologies) and Capping Mix B (16% *N*-methylimidazole in tetrahydrofuran, LGC Biosearch Technologies). The oxidising step was performed with 0.02 M iodine in tetrahydrofuran/pyridine/water (7:2:1, v/v/v, LGC Biosearch Technologies).

Oligonucleotide synthesis parameters:

Deblock: 2×60 s, coupling times: 3×180 s (standard DNA phosphoramidites), 3×480 s (LNA phosphoramidites) capping: 2x60 s, oxidation: 1×60 s. For modified phosphoramidites (linker at the 5'-end of oligonucleotide) the coupling time was 4 x 360 s and the capping step was removed.

Oligonucleotides (**ON**) with linkers **a** – **l** at the 5'-end were synthesised with the following sequences:

ON1: 5'-dCdGdAdCdGdCdTdTdGdCdAdGdCdT-3'

ON2: 5'-dCdTdAdCdAdCdTdTdCdCdAdTdCdT-3'

ON3: 5'-dGd^{m}CdAdTdTd^{m}CdTdAdAdTdAdGd^{m}CdAdGd^{m}C-3'

ON4: 5'-lGl^{m}ClAdTdTd^{m}CdTdAdAdTdAdGd^{m}ClAlGl^{m}C-3'

Capital letter is base code: G = guanine, A = adenine, C = cytosine, ^{m}C = 5-methylcytosine, T = thymine. Small letters are sugar codes: l = locked nucleic acid (LNA) sugar, d = deoxyribose sugar.

General method for cleavage and deprotection of linker-containing oligonucleotides

Method I:

5 mg of the CPG containing linker-modified oligonucleotide was placed in a vial, treated with 100 µL of 0.4 M sodium hydroxide (NaOH) in methanol/water (4:1) solution and shaken (Eppendorf ThermoMixerC) for 20 min at 80 °C. The sample was left to cool down to room temperature, spun down and filtered. The filtrate was frozen and freeze-dried. The sample was desalted on Glen Gel-Pak™ 0.2 Desalting Column (catalogue no 61-5002-50, Glen Research) following the manufacturer's protocol. The collected sample was frozen and freeze-dried.

Method II:

5 mg of the CPG containing linker-modified oligonucleotide was placed in vial, treated with 100 µL of 32% aqueous ammonia solution and shaken (Eppendorf ThermoMixerC) overnight at 23 °C. Sample was spun down and filtered. The filtrate was frozen and freeze-dried.

General method for oligonucleotide purification

Sample was purified on the Waters preparative high-performance liquid chromatography (HPLC) system (XBridge BEH C18 OBD Prep Column; 130 Å; 10 mm x 250 mm, 5 µm; Waters) with a flow rate of 4 mL/min. Eluent A: 0.1 M triethylammonium acetate (TEAA) in water; eluent B: 0.1 M TEAA in 50% ACN; gradient 15 - 45% B in 20 min. Collected fractions were directly desalted on Glen Gel-Pak™ 1.0 Desalting Column (catalogue no 61-5010-50, Glen Research) following the manufacturer's protocol. The collected sample was frozen and freeze-dried. The sample was dissolved in water and analysed by liquid chromatography mass spectrometry (LCMS) (Waters LCMS system with Acquity QDa detector, ACQUITY PREMIER Oligonucleotide BEH C18 column (130 Å; 2.1 x 50 mm, 1.7 µm; Waters)) at 65 °C with a flow rate of 0.3 mL/min. Eluent A: 7 mM triethylamine (TEA), 80 mM hexafluoroisopropanol (HFIP) in water; eluent B: 3.5 mM TEA, 40 mM HFIP in 50% ACN; gradient 5 - 30% B in 8 min. Samples were run in the negative mode (ESI^-) and analysed as m/z $[M]^-$ or $[M-H]^-$. The purity of the oligonucleotides was auto measured using the integration function on MassLynx software V4.2 (Waters).

General method for analysis of the oligonucleotide conjugates

Unless stated otherwise, oligonucleotide conjugates were analysed on liquid chromatography mass spectrometry (Waters LCMS system with Acquity QDa detector, ACQUITY PREMIER Oligonucleotide BEH C18 column (130 Å; 2.1 x 50 mm, 1.7 µm; Waters)) at 65 °C with a flow rate of 0.3 mL/min. Eluent A: 7 mM TEA, 80 mM HFIP in water; eluent B: 3.5 mM TEA, 40 mM HFIP in 50% ACN; gradient 5 - 30% B in 8 min. Samples were run in the negative mode (ESI^-) and analysed as m/z $[M]^-$ or $[M-H]^-$.

Thiolation experiments

General method for Thiol-Ene Click Reactions

Conjugation with β-mercaptoethanol

- General method

A solution of 100 mM β-mercaptoethanol (β-ME) in the phosphate-buffered saline (PBS) (pH = 7.45) was freshly prepared: 1.4 µL of β-mercaptoethanol was added to 200 µL of 5x PBS. Then, the oligonucleotide sample (1 nmol dissolved in 50 µL of water) was mixed with 25 µL of conjugation buffer (containing β-mercaptoethanol) and incubated for 2 h at 37 °C. LCMS analysis (conditions as in general method) was performed immediately after mixing (0 h), and then after 2 h (by mixing 5-10 µL of sample with 10 µL of water) to verify formation of the conjugated product.

For **ON3-b**, analogous experiment was also performed using 20 eq of β-ME (time points: 0 h, 2 h, 4 h and 22 h).

In case of reactivity assay with **ON1-b** and **ON1-c**, LCMS analysis was performed after 0 h, 1 h and 2 h.

- Method for the conjugation of linker **i**

A solution of 100 mM β-mercaptoethanol in water was freshly prepared: 1.4 µL of β-mercaptoethanol was added to 200 µL of water. Then, the oligonucleotide sample (2 nmol dissolved in 50 µL of water) was mixed with 25 µL of β-mercaptoethanol solution and incubated for 6 h at 65 °C. LCMS analysis (conditions as in general method) was performed immediately after mixing (0 h), and then after 2 h, 4 h and 6 h (by mixing 5 µL of sample with 10 µL of water) to verify formation of the conjugated product.

- Method for the conjugation of linker **j**

2 nmol of the oligonucleotide sample was dissolved in 10 µL of water. Then, 65 µL of β-mercaptoethanol was added and reaction mixture was incubated at 65 °C for 6 h. LCMS analysis (conditions as in general method) was performed immediately after mixing (0 h), and then after 2 h, 4 h and 6 h (by mixing 5 µL of sample with 10 µL of water) to verify formation of the conjugated product.

Conjugation with glutathione

A stock solution of 200 mM reduced glutathione (GSH) in water was freshly prepared: 12.3 mg (40 µmol) of GSH was added to 200 µL of water. Then, the oligonucleotide sample (2 nmol dissolved in 50 µL of water) was mixed with 25 µL of the stock solution (containing GSH) and incubated for 6 h

at 37 °C. LCMS analysis (conditions as in general method) was performed immediately after mixing (0 h), and then every 2 h (for 6 h in total) to verify formation of the conjugated product (by mixing 5 µL of sample with 5 µL of water).

Conjugation with peptide

Peptide P1 sequence (**P1**): NH_2- SYQGWC -COOH

Peptide P2 sequence (**P2**): NH_2- SYQGWA -COOH

100 nmol of **P1**/**P2** was dissolved in 50 µL of 5x PBS buffer. Then, the oligonucleotide sample (1 nmol dissolved in 50 µL of water) was mixed with 25 µL of the buffer solution (containing **P1** or **P2**) and incubated at 37 °C. LCMS analysis (conditions as in general method) was performed immediately after mixing (0 h), and then after 1 h, 2 h, 3 h, 4 h, 6 h and 22 h (for conjugation with **P1**) or 1 h, 2 h and 4 h (for control experiment with **P2**) to verify formation of the conjugated product (by mixing 5-10 µL of sample with 10 µL of water).

For **ON4-b**, analogous experiment was also performed using 20 eq of P1 (time points: 0 h, 2 h, 18 h and 25 h) or 3 eq of P1 (time points: 0 h, 3 h, 21 h and 27 h).

In a control experiment, 3 nmol of **P1** was dissolved in 25 µL of 5x PBS buffer. Then, 50 µL of water was added and the solution was incubated at 37 °C. LCMS analysis (conditions as in general method) was performed immediately after mixing (0 h), and then after 3 h, 5 h and 23 h.

Conjugation with lipid

A 4 mM stock solution of mercaptoethyl-D-α-tocopheryl succinate (**37**) in acetonitrile was freshly prepared: 23.6 mg (40 µmol) of **37** was dissolved in 10 mL of acetonitrile. Oligonucleotide sample (1 nmol dissolved in 50 µL of water) was mixed with 25 µL (100 nmol) of the stock solution containing lipid-thiol and incubated at 37 °C. LCMS analysis (conditions as described below) was performed immediately after mixing (0 h), and then after 2 h and 4 h to verify formation of the conjugated product (by mixing 5 µL of sample with 5 µL of water).

In a control experiment, 100 nmol of **37** was dissolved in 25 µL of acetonitrile. Then, 50 µL of water was added and the solution was incubated at 37 °C. LCMS analysis (conditions as described below) was performed immediately after mixing (0 h), and then after 2 h, and 4 h.

LCMS analysis conditions:

Eluent A: 7 mM TEA, 80 mM HFIP in water; eluent B: 3.5 mM TEA, 40 mM HFIP in 90% ACN; gradient 0 - 20% B 1 to 6 min, then 20 - 85% 6 to 13 min. Samples were run in the negative mode (ESI^-) and analysed as m/z $[M]^-$ or $[M-H]^-$.

Conjugation with GalNAc

A 4 mM stock solution of commercially available α-GalNAc-PEG3-Thiol in water was freshly prepared: 1.48 mg (4 µmol) of α-GalNAc-PEG3-Thiol was dissolved in 1 mL of water. Oligonucleotide sample (1 nmol dissolved in 50 µL of water) was mixed with 25 µL (100 nmol) of the GalNAc-thiol-containing stock solution and incubated at 37 °C for 4 h. LCMS analysis (conditions as in general method) was performed immediately after mixing (0 h), and then after 2 h and 4 h to verify formation of the conjugated product (by mixing 5 µL of sample with 5 µL of water).

For **ON3-b,** analogous experiment was also performed using 20 eq (time points: 0 h, 2 h, 4 h and 23 h) or 3 eq of α-GalNAc-PEG3-Thiol (time points: 0 h, 3 h, 5 h, 24 h, 28 h, 33 h and 50 h).

LCMS chromatograms

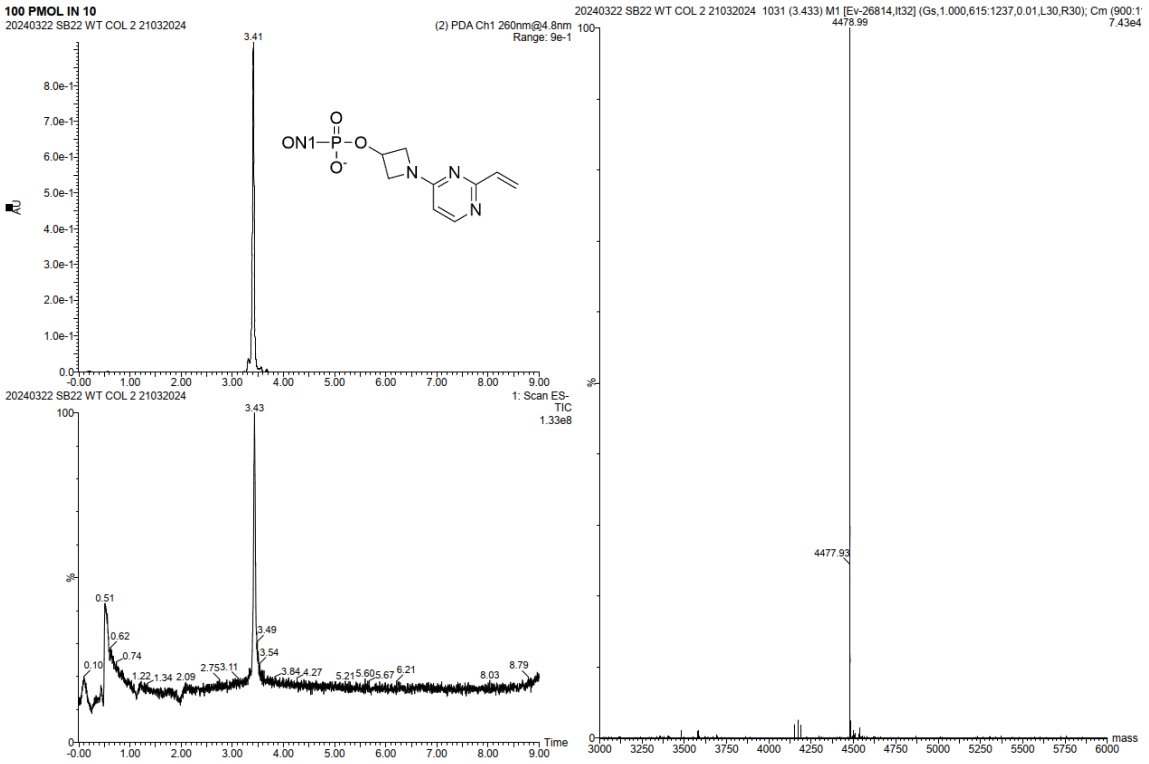
Oligonucleotide synthesis

Table 1: Summary of the synthesised linker-modified oligonucleotides.

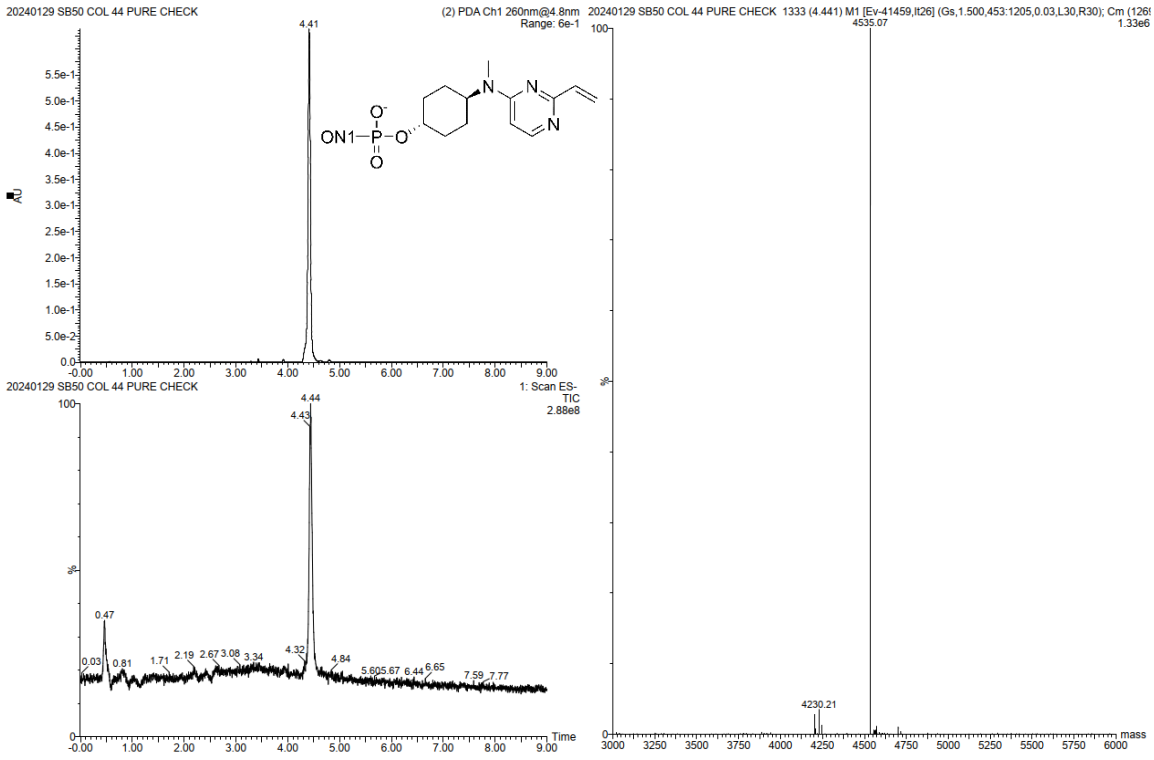
Oligonucleotide	Linker	Deprotection method	Mass calc.	Mass found	Purity [%]
ON1-a	a	I	4478.96 $[M]^-$	4478.99	91.8
ON1-b	b	I	4535.07 $[M]^-$	4535.07	91.5
ON1-c	c	I	4535.07 $[M]^-$	4535.50	91.0
ON1-d	d	I	4507.02 $[M]^-$	4507.12	89.6
ON1-e	e	I	4521.05 $[M]^-$	4521.32	88.5
ON1-f	f	I	4507.02 $[M]^-$	4507.50	83.5
ON1-g	g	I	4507.02 $[M]^-$	4507.00	89.0
ON1-h	h	I	4492.99 $[M]^-$	4493.19	91.2
ON1-i	i	II	4553.03 $[M-H]^-$	4571.00*	89.0
ON1-j	j	II	4571.05 $[M]^-$	4571.23	96.7
ON1-k	k	I	4507.02 $[M]^-$	4507.00	88.0
ON1-l	l	I	4521.05 $[M]^-$	4521.73	93.7
ON2-a	a	I	4372.92 $[M]^-$	4373.42	90.8
ON2-b	b	I	4429.03 $[M]^-$	4429.00	90.4
ON2-c	c	I	4429.03 $[M]^-$	4429.52	94.0
ON2-d	d	I	4400.98 $[M]^-$	4401.69	96.5
ON2-e	e	I	4415.01 $[M]^-$	4415.53	95.8
ON2-j	j	II	4465.01 $[M]^-$	4466.05	96.0
ON3-b	b	I	5215.61 $[M-H]^-$	5215.52	95.0
ON4-b	b	I	5384.67 $[M]^-$	5384.85	96.3

*mass identified as $[M-H+H_2O]^-$

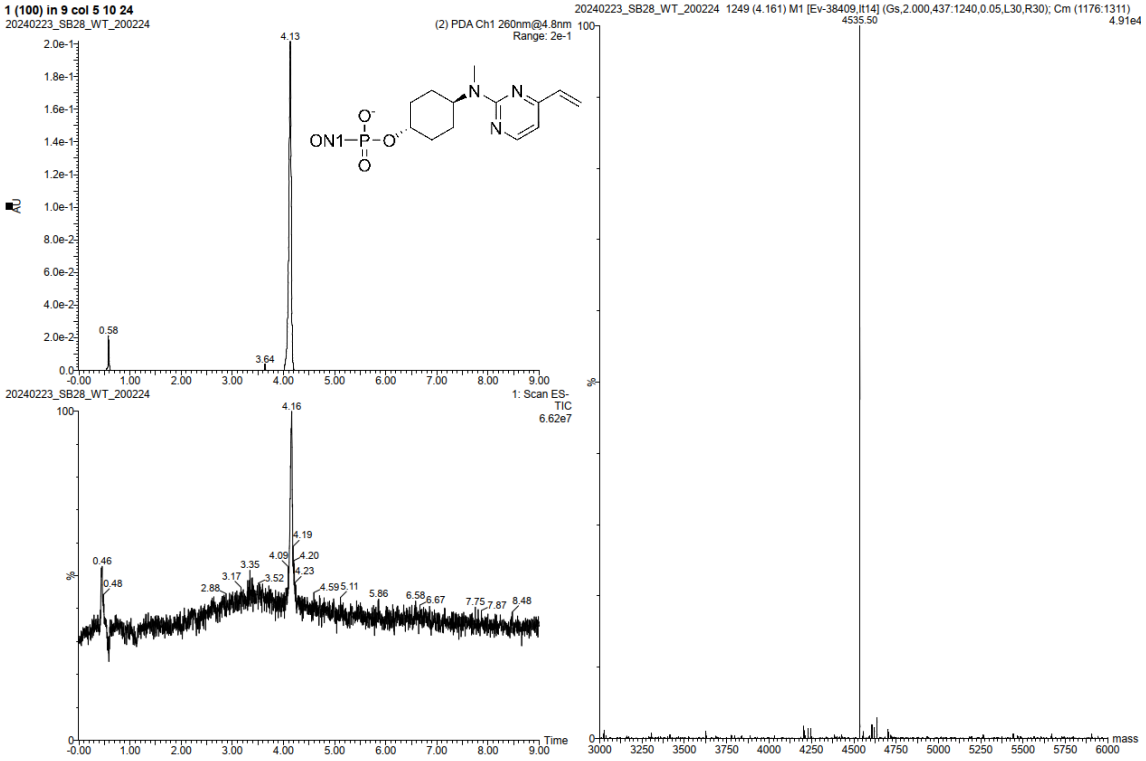
ON1-a


100 PMOL IN 10
20240322 SB22 WT COL 2 21032024
(2) PDA Ch1 260nm@4.8nm
Range: 9e-1
3.41
AU
ON1
20240322 SB22 WT COL 2 21032024
1: Scan ES-
TIC
1.33e8
3.43
0.51
0.62
0.74
0.10
1.22
1.34
2.09
2.75
3.11
3.49
3.54
3.84
4.27
5.21
5.60
5.67
6.21
8.03
8.79
Time
20240322 SB22 WT COL 2 21032024 1031 (3.433) M1 [Ev-26814,It32] (Gs,1.000,615:1237,0.01,L30,R30); Cm (900:1
4478.99
7.43e4
4477.93
mass

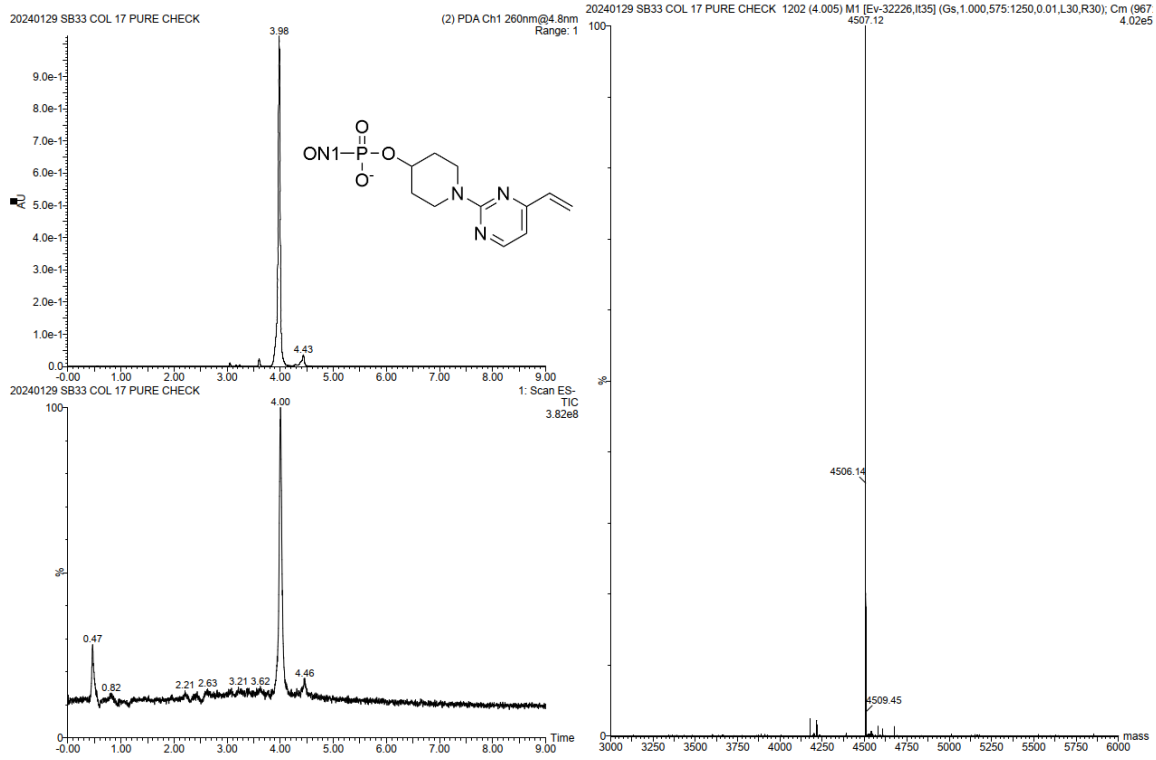
ON1-b


20240129 SB50 COL 44 PURE CHECK
(2) PDA Ch1 260nm@4.8nm
Range: 6e-1
4.41
AU
ON1
20240129 SB50 COL 44 PURE CHECK
1: Scan ES-
TIC
2.88e8
4.44
4.43
0.47
0.03
0.81
1.71
2.19
2.67
3.08
3.34
4.32
4.84
5.60
5.67
6.44
6.65
7.59
7.77
Time
20240129 SB50 COL 44 PURE CHECK 1333 (4.441) M1 [Ev-41459,It26] (Gs,1.500,453:1205,0.03,L30,R30); Cm (126
4535.07
1.33e6
4230.21
mass

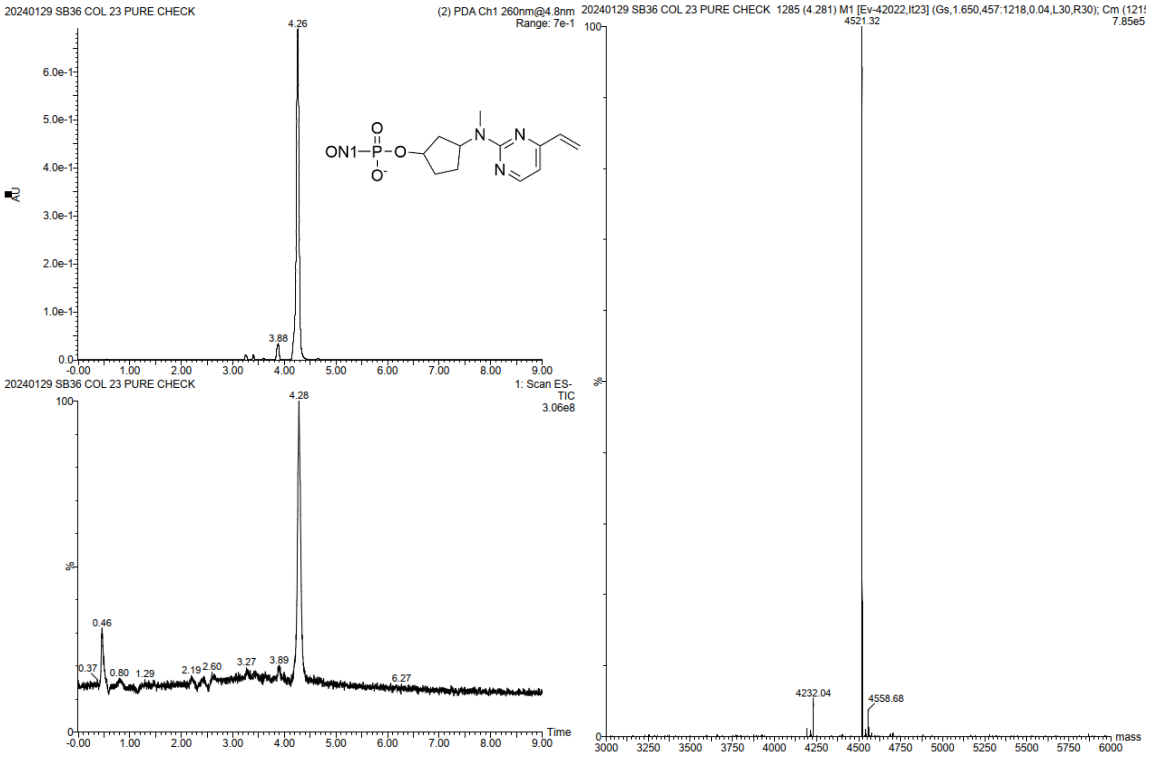
ON1-c


1 (100) in 9 col 5 10 24
20240223_SB28_WT_200224
(2) PDA Ch1 260nm@4.8nm
Range: 2e-1
4.13
0.58
3.64
AU
ON1
20240223_SB28_WT_200224
1: Scan ES-
TIC
6.62e7
4.16
4.19
4.09
4.20
4.23
0.46
0.48
3.35
3.17
3.52
2.88
4.59
5.11
5.86
6.58
6.67
7.75
7.87
8.48
Time
20240223_SB28_WT_200224 1249 (4.161) M1 [Ev-38409,It14] (Gs,2.000,437:1240,0.05,L30,R30); Cm (1176:1311)
4535.50
4.91e4
mass

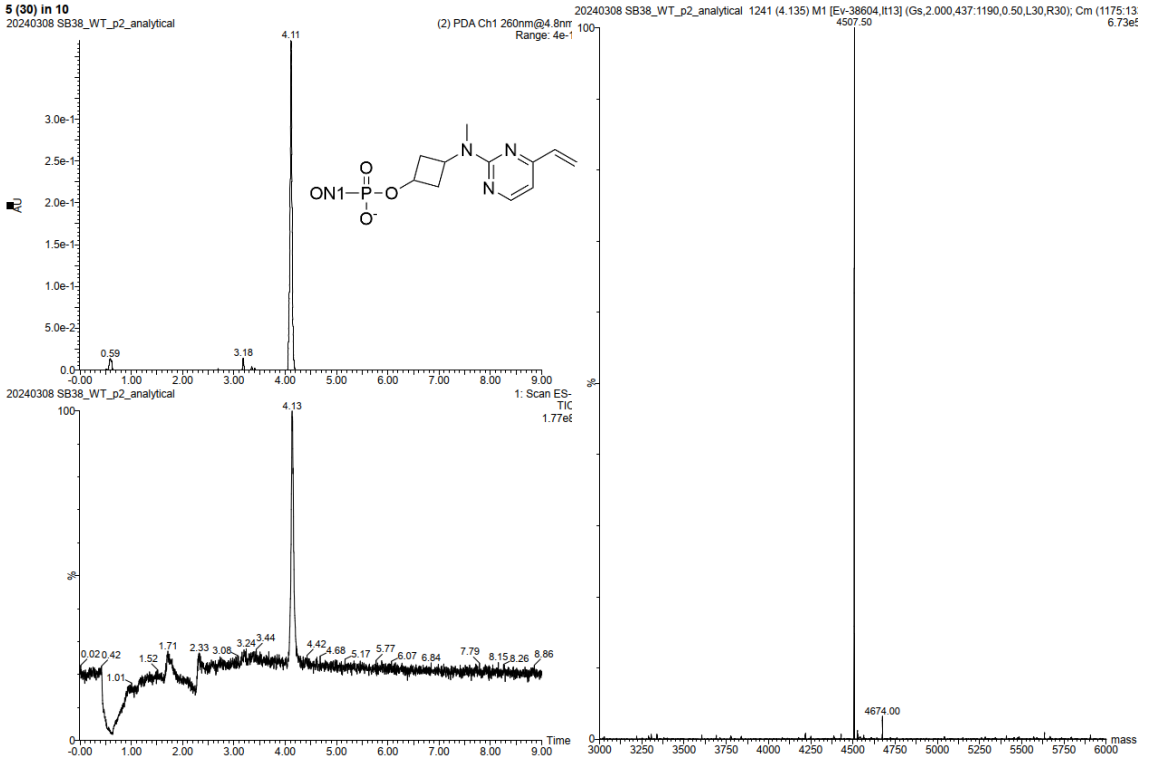
ON1-d


20240129 SB33 COL 17 PURE CHECK
(2) PDA Ch1 260nm@4.8nm
Range: 1
3.98
4.43
AU
ON1
20240129 SB33 COL 17 PURE CHECK
1: Scan ES-
TIC
3.82e8
4.00
0.47
0.82
2.21
2.63
3.21
3.62
4.46
Time
20240129 SB33 COL 17 PURE CHECK 1202 (4.005) M1 [Ev-32226,It35] (Gs,1.000,575:1250,0.01,L30,R30); Cm (967
4507.12
4.02e5
4506.14
4509.45
mass

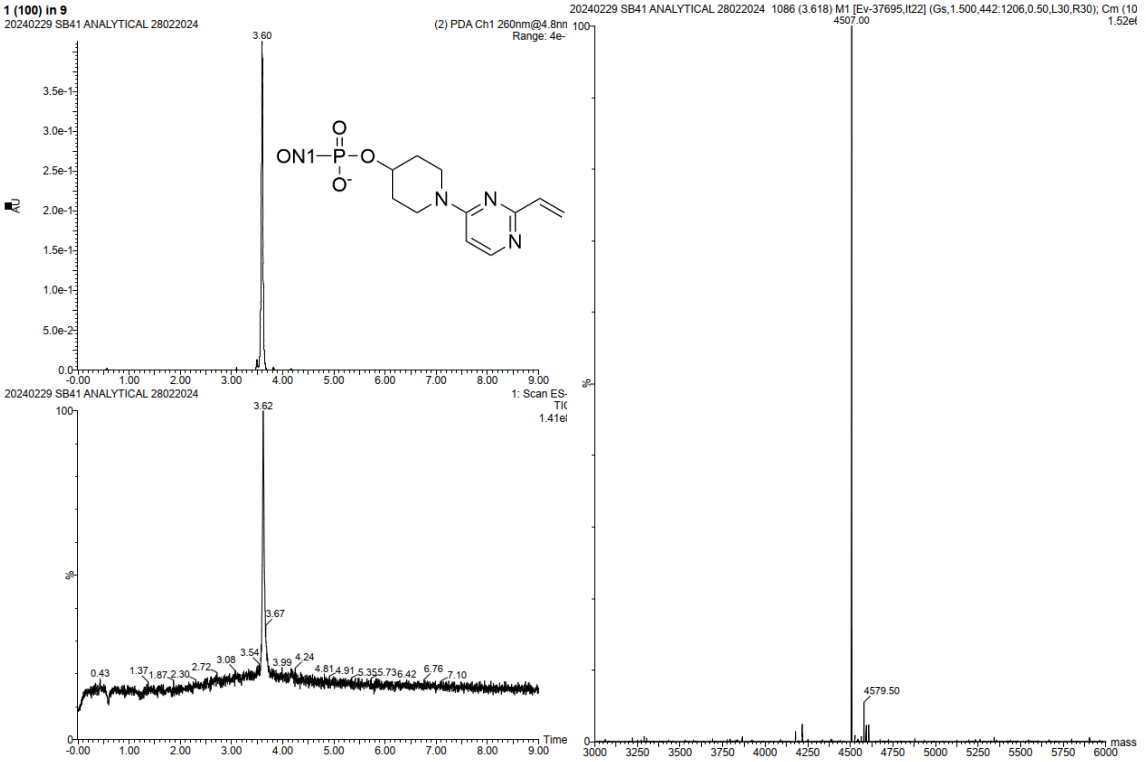
ON1-e


20240129 SB36 COL 23 PURE CHECK
(2) PDA Ch1 260nm@4.8nm
Range: 7e-1
4.26
3.88
AU
20240129 SB36 COL 23 PURE CHECK
1: Scan ES-
TIC
3.06e8
4.28
0.46
0.37
0.80
1.29
2.19
2.60
3.27
3.89
6.27
%
Time
20240129 SB36 COL 23 PURE CHECK 1285 (4.281) M1 [Ev-42022,It23] (Gs,1.650,457:1218,0.04,L30,R30); Cm (121
4521.32
7.85e5
4232.04
4558.68
mass

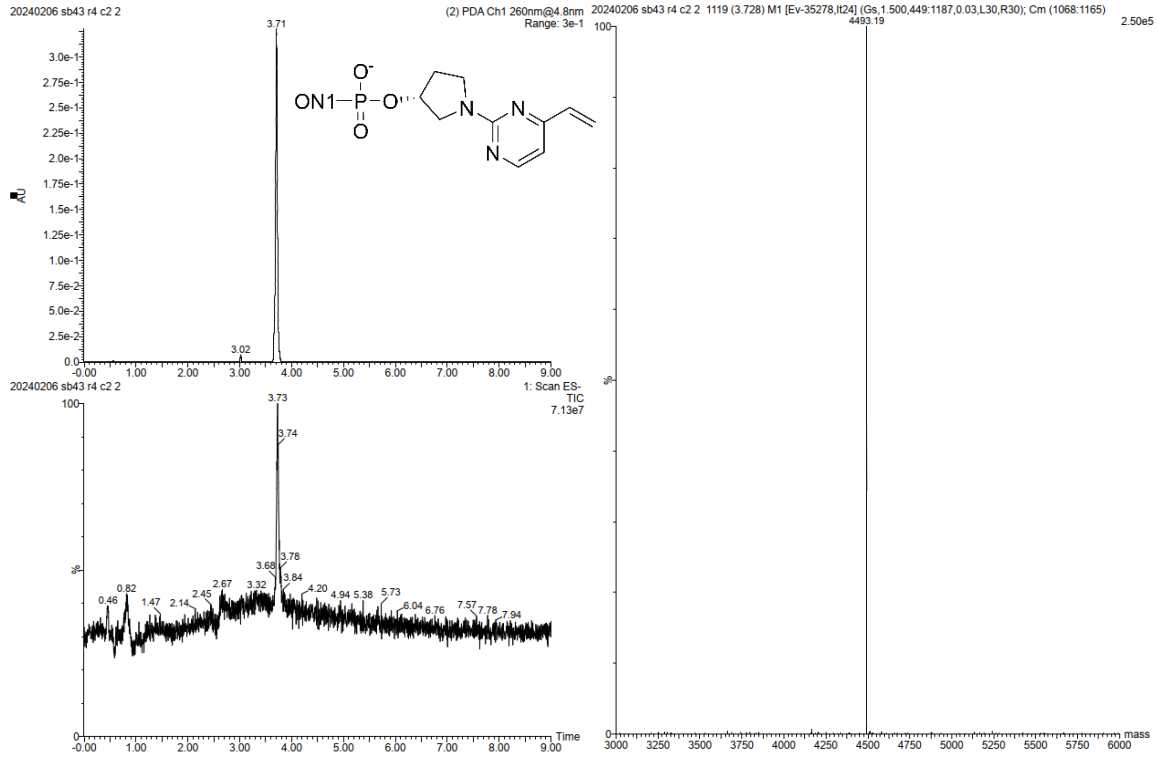
ON1-f


5 (30) in 10
20240308 SB38_WT_p2_analytical
(2) PDA Ch1 260nm@4.8nm
Range: 4e-1
4.11
0.59
3.18
AU
20240308 SB38_WT_p2_analytical
1: Scan ES-
TIC
1.77e8
4.13
0.02
0.42
1.01
1.52
1.71
2.33
3.08
3.24
3.44
4.42
4.68
5.17
5.77
6.07
6.84
7.79
8.15
8.26
8.86
%
Time
20240308 SB38_WT_p2_analytical 1241 (4.135) M1 [Ev-38604,It13] (Gs,2.000,437:1190,0.50,L30,R30); Cm (1175:13
4507.50
6.73e5
4674.00
mass

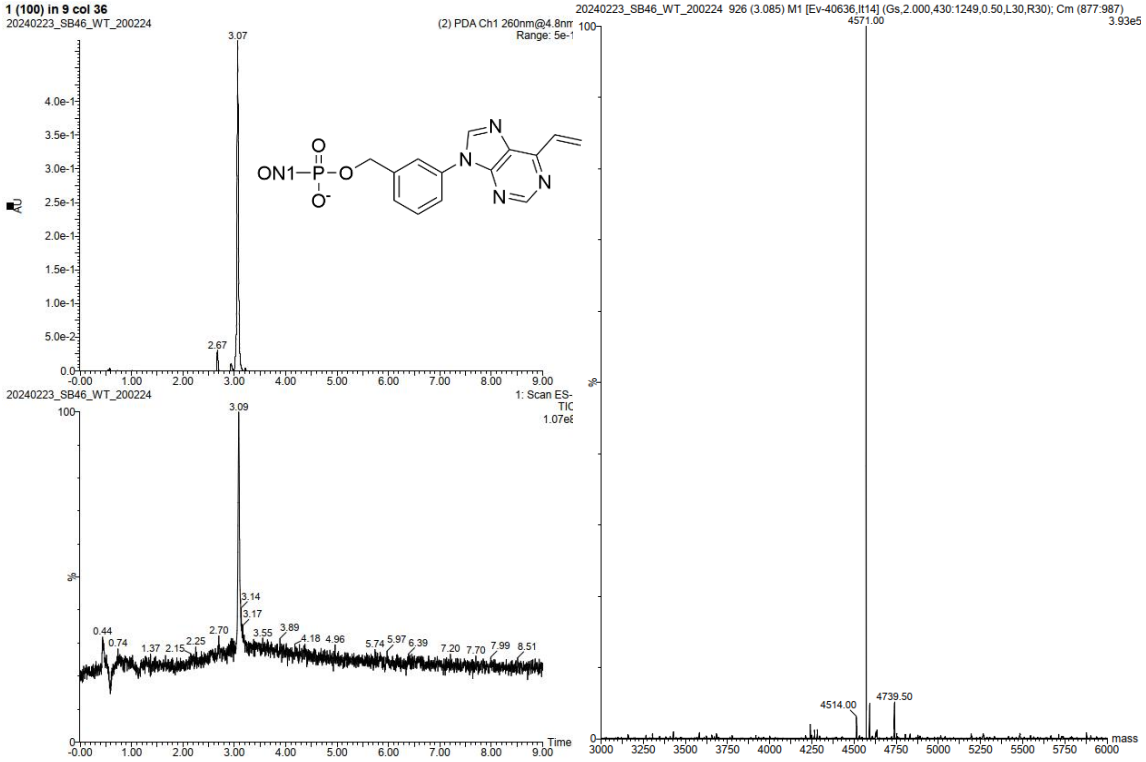
ON1-g


1 (100) in 9
20240229 SB41 ANALYTICAL 28022024
(2) PDA Ch1 260nm@4.8nm
Range: 4e-
3.60
ON1—P—O
AU
20240229 SB41 ANALYTICAL 28022024
1: Scan ES-
TIC
1.41e
3.62
3.67
3.54
3.08
3.99
4.24
0.43
1.37
1.87
2.30
2.72
4.81
4.91
5.35
5.73
6.42
6.76
7.10
Time
20240229 SB41 ANALYTICAL 28022024 1086 (3.618) M1 [Ev-37695,It22] (Gs,1.500,442:1206,0.50,L30,R30); Cm (10
4507.00
4579.50
mass

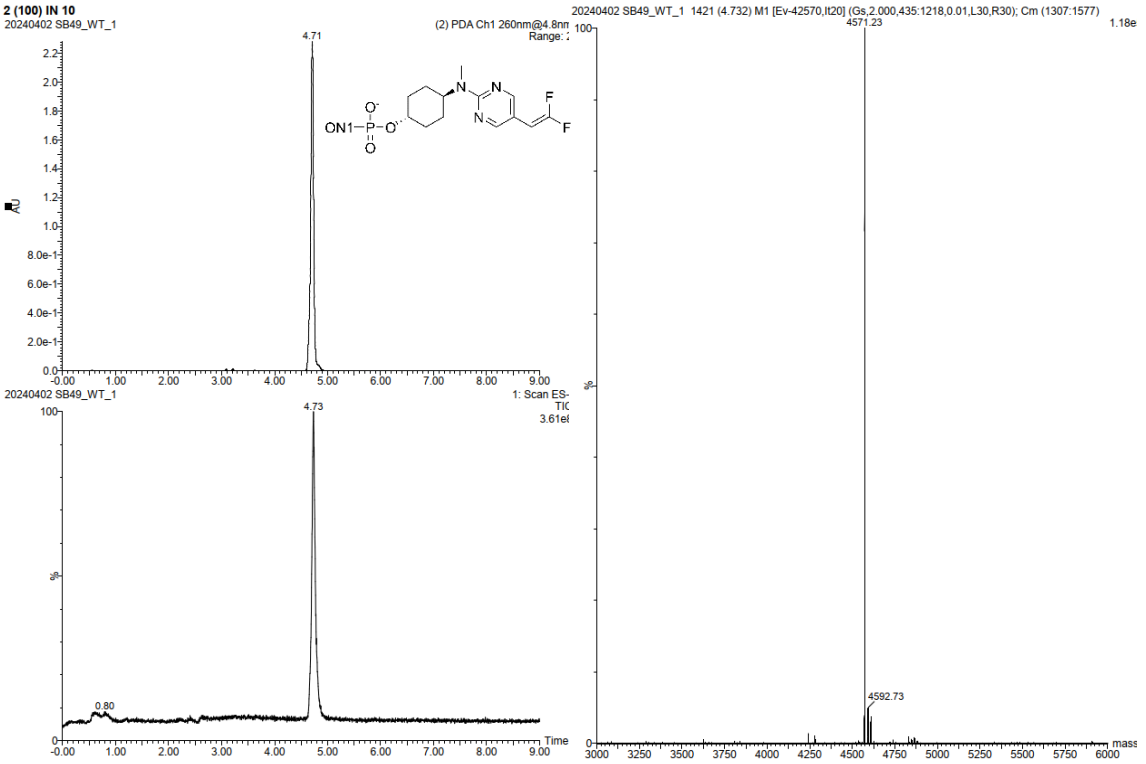
ON1-h


20240206 sb43 r4 c2 2
(2) PDA Ch1 260nm@4.8nm
Range: 3e-1
3.71
3.02
ON1—P—O
AU
20240206 sb43 r4 c2 2
1: Scan ES-
TIC
7.13e7
3.73
3.74
3.78
3.68
3.84
3.32
2.67
2.45
0.82
0.46
1.47
2.14
4.20
4.94
5.38
5.73
6.04
6.76
7.57
7.78
7.94
Time
20240206 sb43 r4 c2 2 1119 (3.728) M1 [Ev-35278,It24] (Gs,1.500,449:1187,0.03,L30,R30); Cm (1068:1165)
4493.19
mass

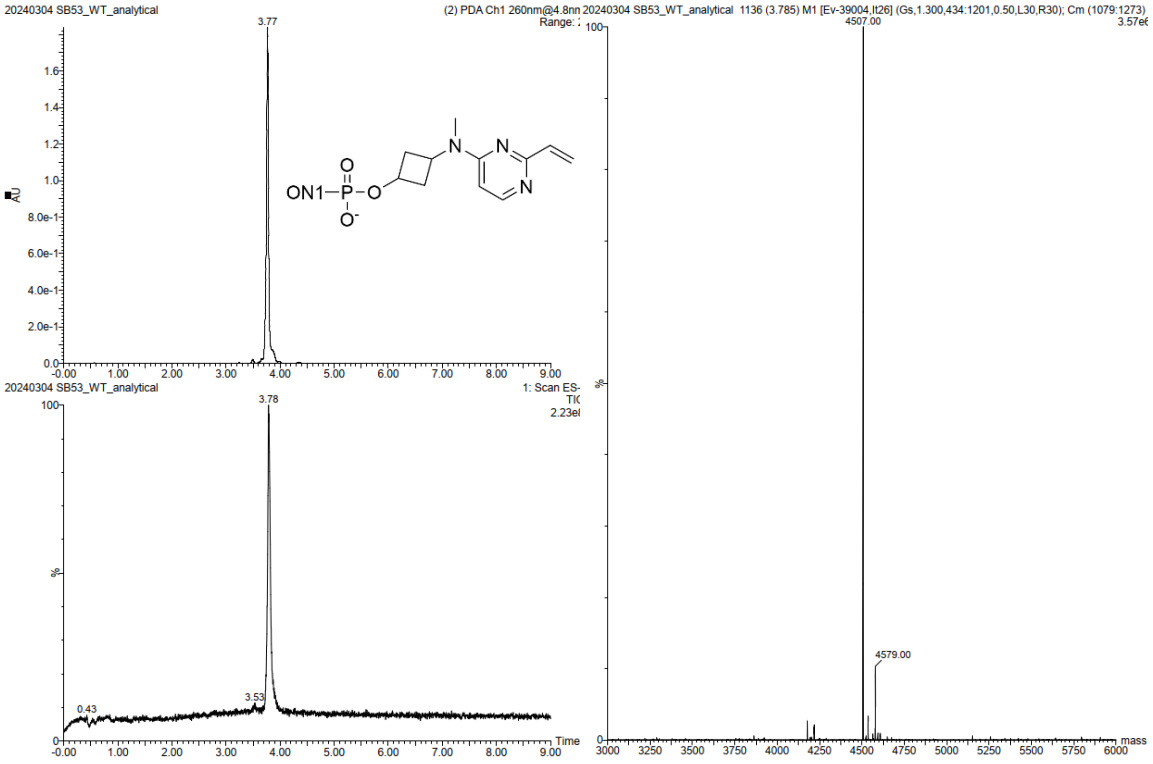
ON1-i


1 (100) in 9 col 36
20240223_SB46_WT_200224
(2) PDA Ch1 260nm@4.8nm
Range: 5e-1
3.07
2.67
AU
20240223_SB46_WT_200224
1: Scan ES-
TIC
1.07e8
3.09
3.14
3.17
0.44
0.74
1.37
2.15
2.25
2.70
3.55
3.89
4.18
4.96
5.74
5.97
6.39
7.20
7.70
7.99
8.51
%
Time
ON1
20240223_SB46_WT_200224 926 (3.085) M1 [Ev-40636,It14] (Gs,2.000,430:1249,0.50,L30,R30); Cm (877:987)
4571.00
3.93e5
4514.00
4739.50
mass

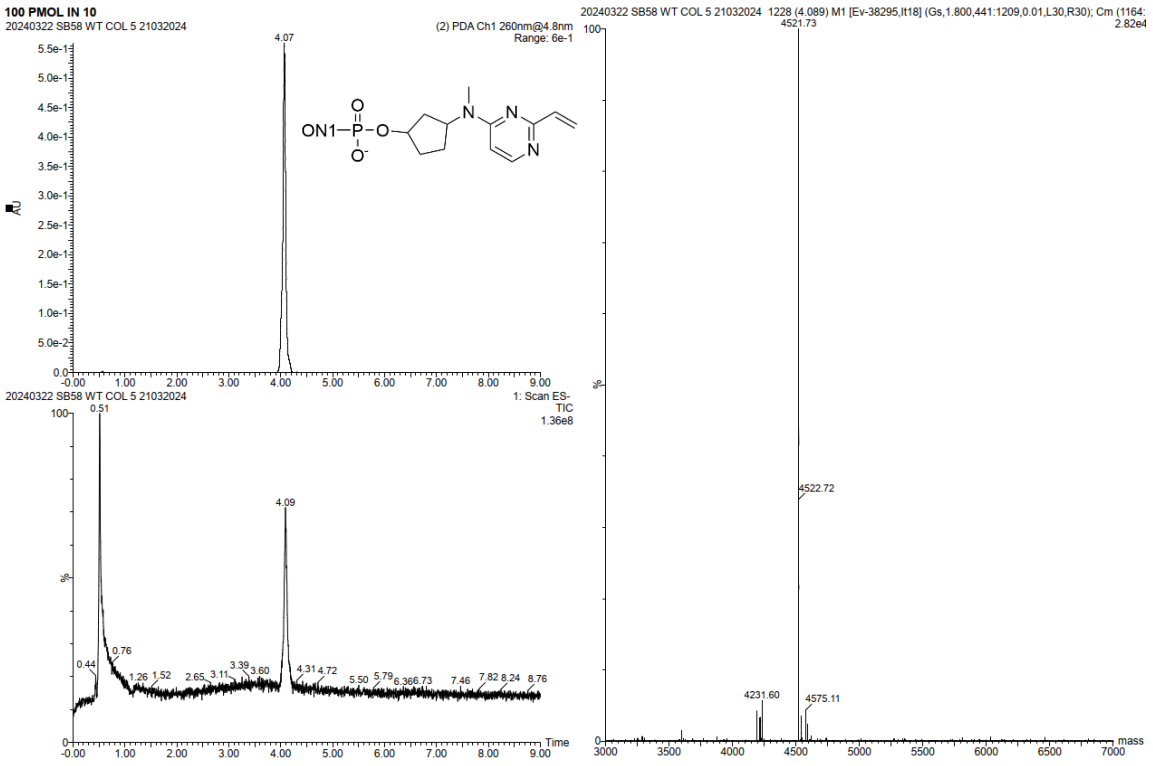
ON1-j


2 (100) IN 10
20240402 SB49_WT_1
(2) PDA Ch1 260nm@4.8nm
4.71
AU
20240402 SB49_WT_1
1: Scan ES-
TIC
3.61e8
4.73
0.80
%
Time
ON1
F
20240402 SB49_WT_1 1421 (4.732) M1 [Ev-42570,It20] (Gs,2.000,435:1218,0.01,L30,R30); Cm (1307:1577)
4571.23
1.18e
4592.73
mass

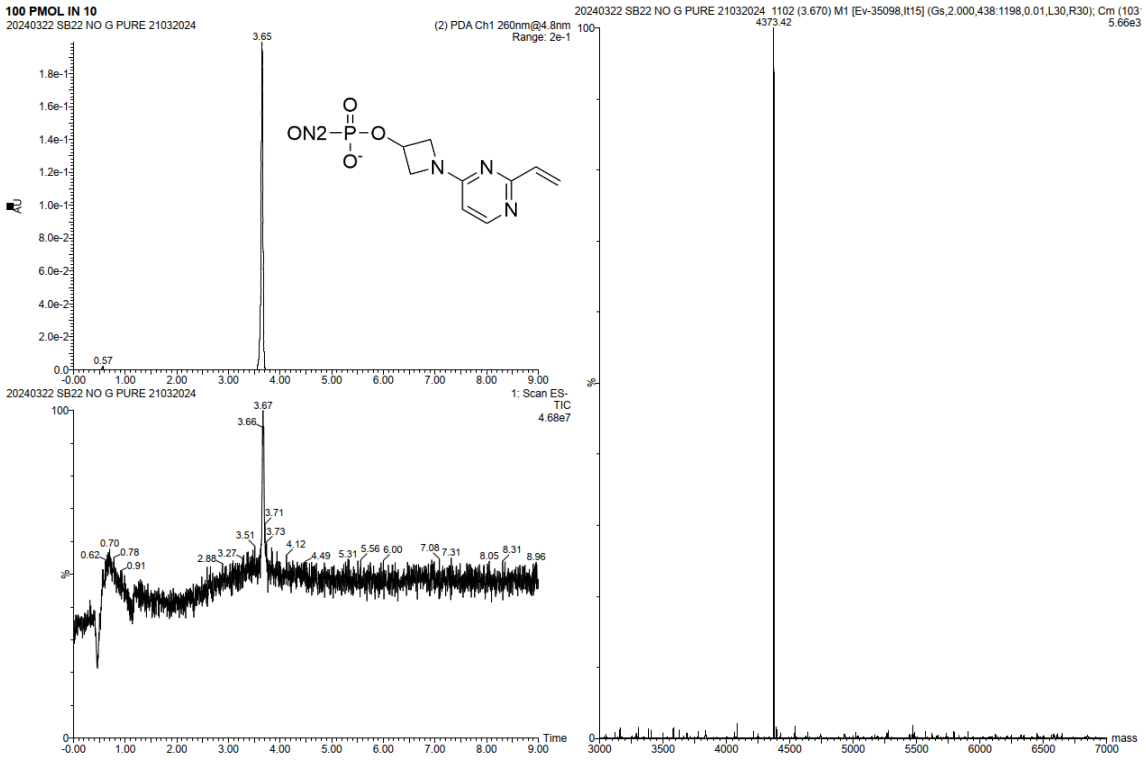
ON1-k


20240304 SB53_WT_analytical
(2) PDA Ch1 260nm@4.8nm
3.77
ON1
20240304 SB53_WT_analytical
1: Scan ES-
3.78
0.43
3.53
Time
20240304 SB53_WT_analytical 1136 (3.785) M1 [Ev-39004,It26] (Gs,1.300,434:1201,0.50,L30,R30); Cm (1079:1273)
4507.00
4579.00
mass

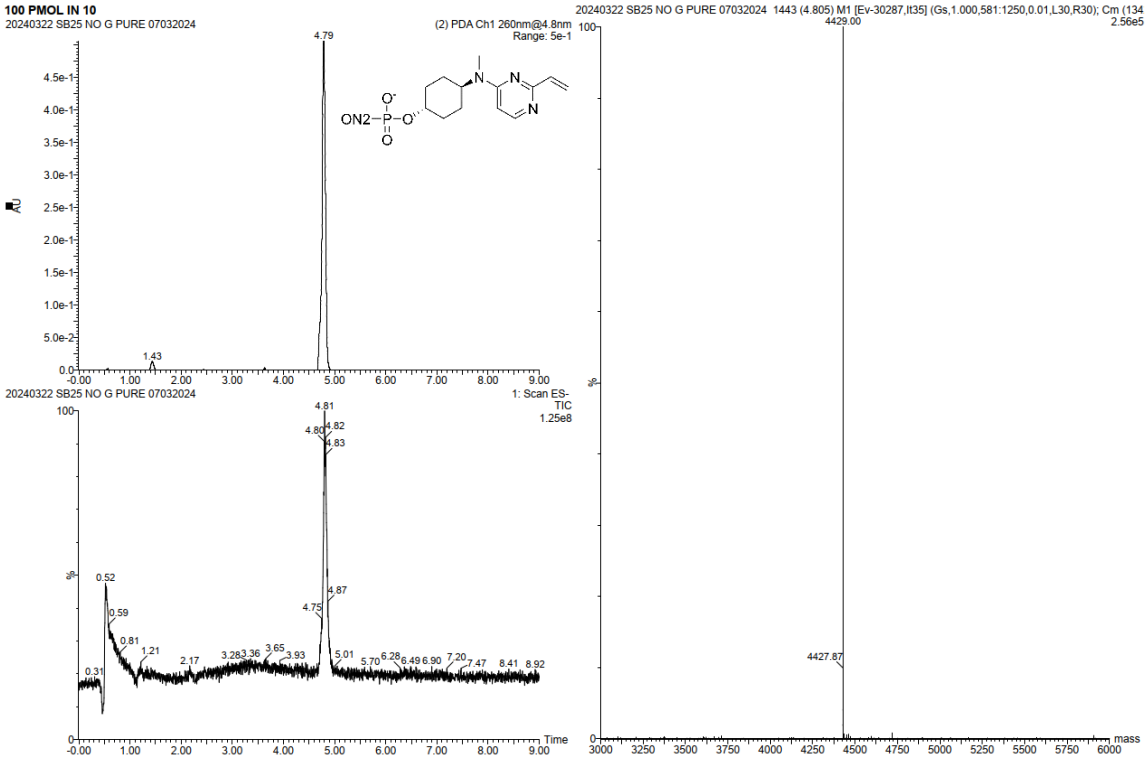
ON1-l


100 PMOL IN 10
20240322 SB58 WT COL 5 21032024
(2) PDA Ch1 260nm@4.8nm
4.07
ON1
20240322 SB58 WT COL 5 21032024
1: Scan ES-
0.51
4.09
0.76
0.44
Time
20240322 SB58 WT COL 5 21032024 1228 (4.089) M1 [Ev-38295,It18] (Gs,1.800,441:1209,0.01,L30,R30); Cm (1164:
4521.73
4522.72
4231.60
4575.11
mass

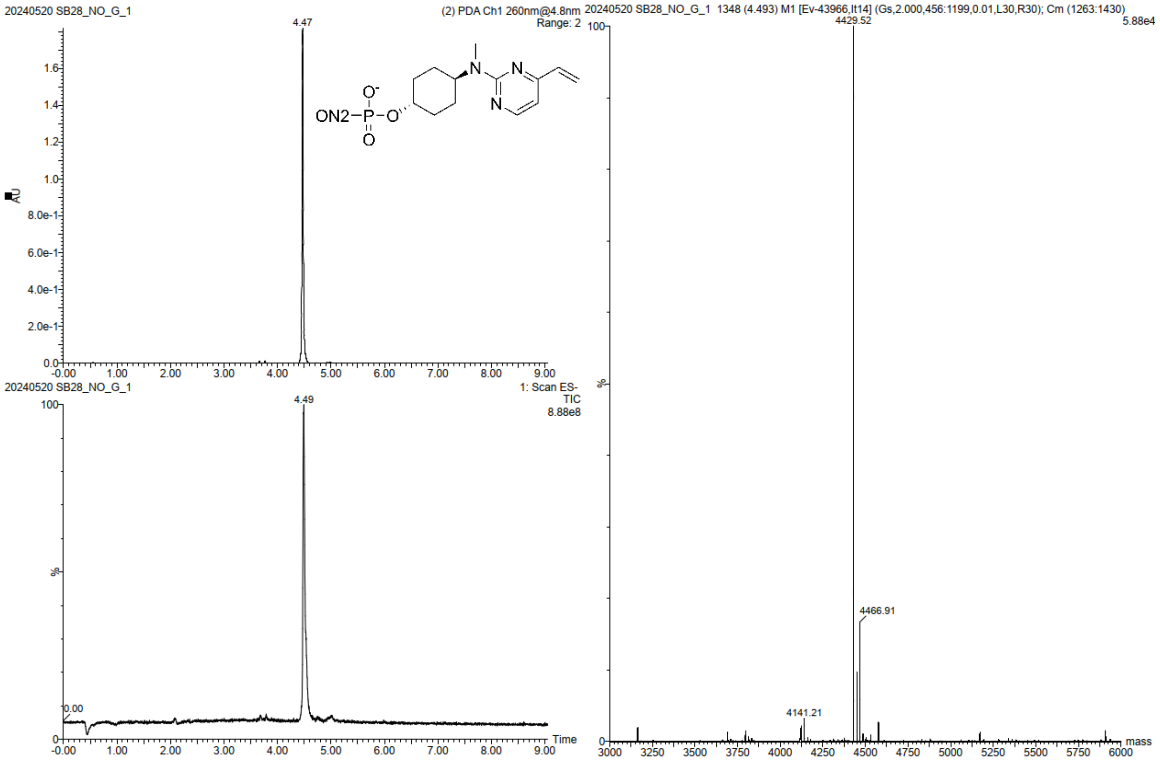
ON2-a


100 PMOL IN 10
20240322 SB22 NO G PURE 21032024
(2) PDA Ch1 260nm@4.8nm
Range: 2e-1
3.65
0.57
ON2
20240322 SB22 NO G PURE 21032024
1: Scan ES-
TIC
4.68e7
3.67
3.66
3.71
3.51
3.73
4.12
0.70
0.62
0.78
0.91
2.88
3.27
4.49
5.31
5.56
6.00
7.08
7.31
8.05
8.31
8.96
Time
20240322 SB22 NO G PURE 21032024 1102 (3.670) M1 [Ev-35098,It15] (Gs,2.000,438:1198,0.01,L30,R30); Cm (103
4373.42
5.66e3
mass

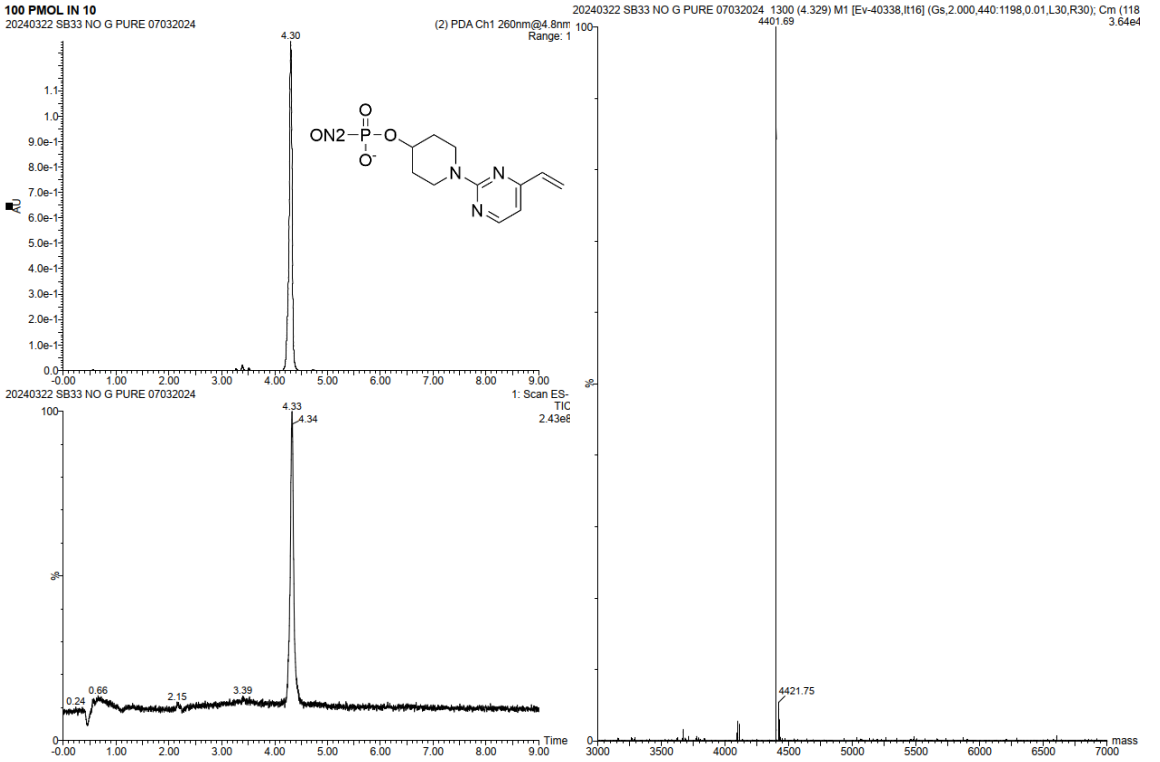
ON2-b


100 PMOL IN 10
20240322 SB25 NO G PURE 07032024
(2) PDA Ch1 260nm@4.8nm
Range: 5e-1
4.79
1.43
ON2
20240322 SB25 NO G PURE 07032024
1: Scan ES-
TIC
1.25e8
4.81
4.80
4.82
4.83
0.52
0.59
0.81
1.21
2.17
3.28
3.36
3.65
3.93
4.75
4.87
5.01
5.70
6.28
6.49
6.90
7.20
7.47
8.41
8.92
0.31
Time
20240322 SB25 NO G PURE 07032024 1443 (4.805) M1 [Ev-30287,It35] (Gs,1.000,581:1250,0.01,L30,R30); Cm (134
4429.00
2.56e5
4427.87
mass

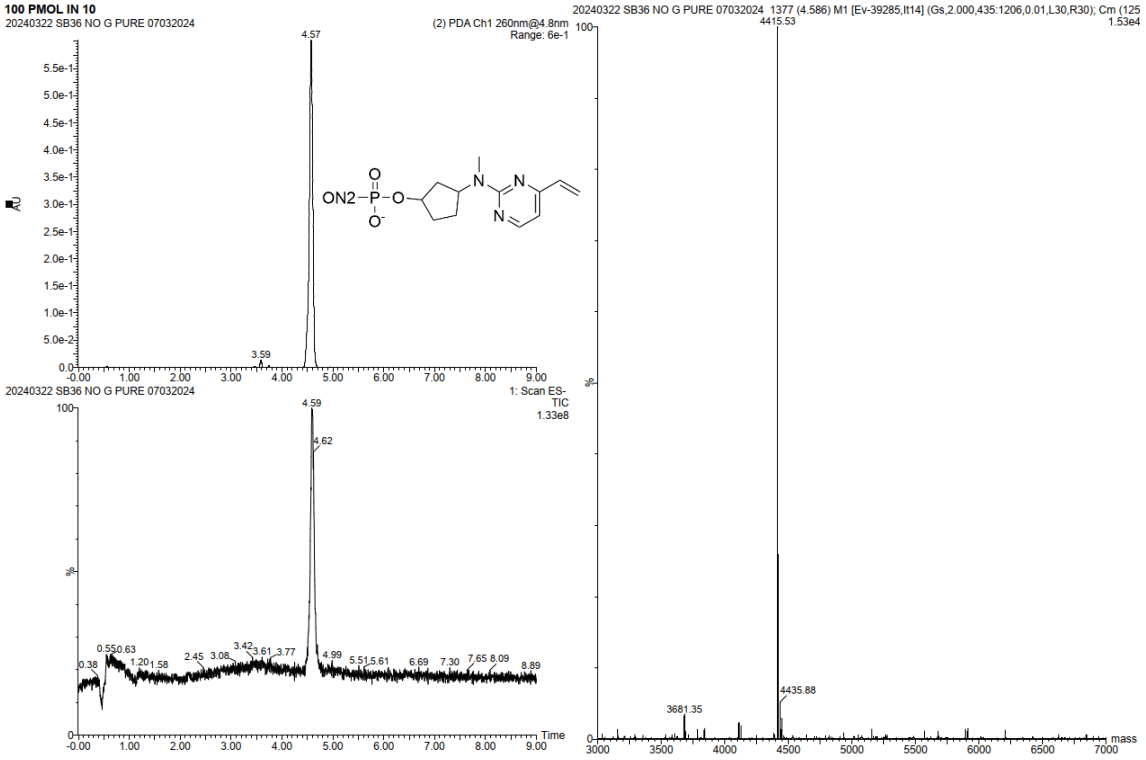
ON2-c


20240520 SB28_NO_G_1
(2) PDA Ch1 260nm@4.8nm
Range: 2
4.47
AU
20240520 SB28_NO_G_1
1: Scan ES-
TIC
8.88e8
4.49
%
0.00
Time
20240520 SB28_NO_G_1 1348 (4.493) M1 [Ev-43966,It14] (Gs,2.000,456:1199,0.01,L30,R30); Cm (1263:1430)
4429.52
5.88e4
4466.91
4141.21
mass

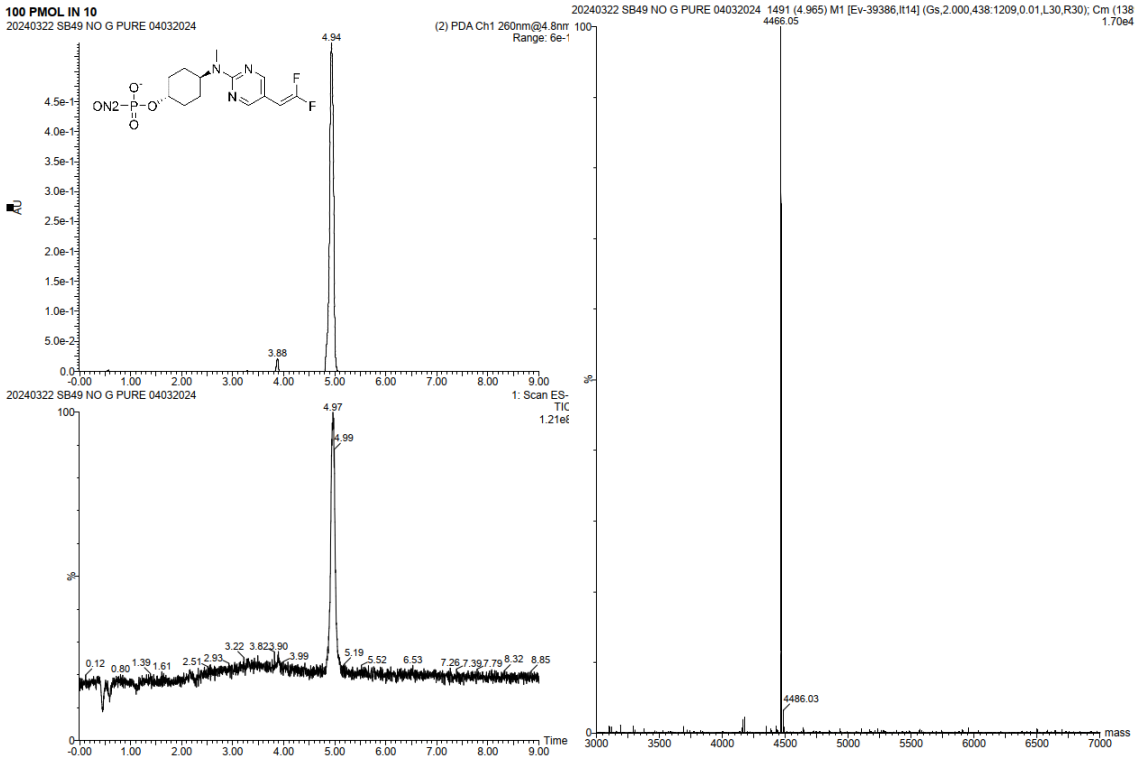
ON2-d


100 PMOL IN 10
20240322 SB33 NO G PURE 07032024
(2) PDA Ch1 260nm@4.8nm
Range: 1
4.30
AU
20240322 SB33 NO G PURE 07032024
1: Scan ES-
TIC
4.33
4.34
%
0.24
0.66
2.15
3.39
Time
20240322 SB33 NO G PURE 07032024 1300 (4.329) M1 [Ev-40338,It16] (Gs,2.000,440:1198,0.01,L30,R30); Cm (118
4401.69
3.64e4
4421.75
mass

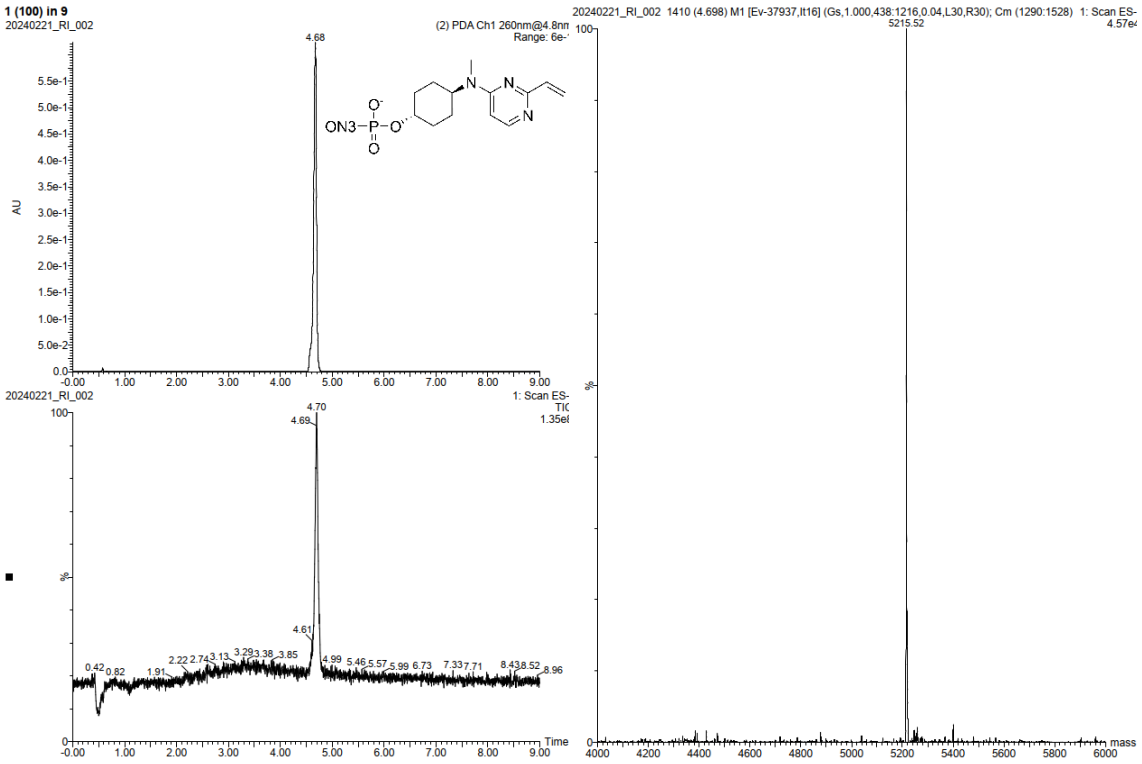
ON2-e


100 PMOL IN 10
20240322 SB36 NO G PURE 07032024
(2) PDA Ch1 260nm@4.8nm
Range: 6e-1
4.57
3.59
AU
ON2
20240322 SB36 NO G PURE 07032024
1: Scan ES-
TIC
1.33e8
4.59
4.62
Time
20240322 SB36 NO G PURE 07032024 1377 (4.586) M1 [Ev-39285,It14] (Gs,2.000,435:1206,0.01,L30,R30); Cm (125
4415.53
1.53e4
4435.88
3681.35
mass

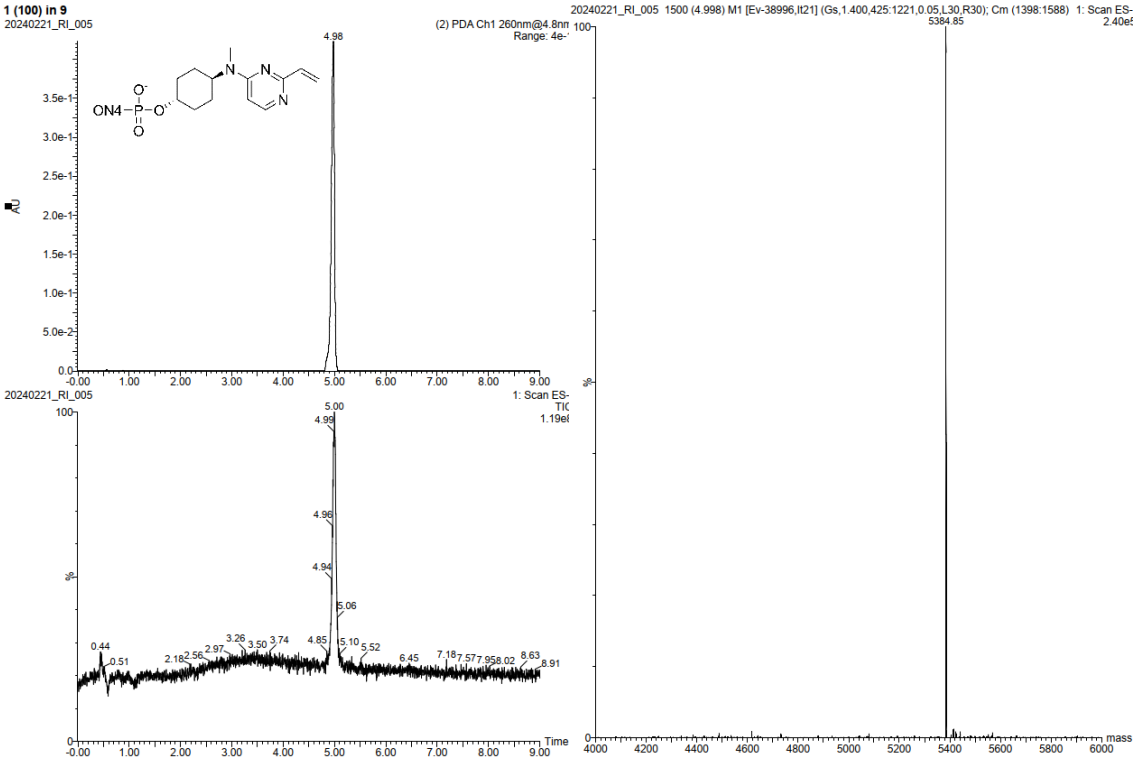
ON2-j


100 PMOL IN 10
20240322 SB49 NO G PURE 04032024
(2) PDA Ch1 260nm@4.8nm
Range: 6e-1
4.94
3.88
AU
ON2
F
F
20240322 SB49 NO G PURE 04032024
1: Scan ES-
TIC
1.21e8
4.97
4.99
Time
20240322 SB49 NO G PURE 04032024 1491 (4.965) M1 [Ev-39386,It14] (Gs,2.000,438:1209,0.01,L30,R30); Cm (138
4466.05
1.70e4
4486.03
mass

ON3-b


1 (100) in 9
20240221_RI_002
(2) PDA Ch1 260nm@4.8nm
Range: 6e-
ON3
AU
4.68
20240221_RI_002
1: Scan ES-
TIC
1.35e
4.70
4.69
4.61
Time
20240221_RI_002 1410 (4.698) M1 [Ev-37937,It16] (Gs,1.000,438:1216,0.04,L30,R30); Cm (1290:1528) 1: Scan ES-
5215.52
4.57e
mass

ON4-b


1 (100) in 9
20240221_RI_005
(2) PDA Ch1 260nm@4.8nm
Range: 4e-
ON4
AU
4.98
20240221_RI_005
1: Scan ES-
TIC
1.19e
5.00
4.99
4.96
4.94
5.06
Time
20240221_RI_005 1500 (4.998) M1 [Ev-38996,It21] (Gs,1.400,425:1221,0.05,L30,R30); Cm (1398:1588) 1: Scan ES-
5384.85
2.40e
mass

Thiolation experiments

Table 2: Summary of the conjugation experiments.

Oligonucleotide	Linker	Conjugation with	Product	Mass calc.	Mass found	Yield[1]
ON1-a	a	β-ME	ON1-a'	4557.09 $[M]^-$	4557.66	full conversion[2, 3]
ON1-b	b	β-ME	ON1-b'	4613.20 $[M]^-$	4613.40	full conversion[2, 3]
ON1-c	c	β-ME	ON1-c'	4613.20 $[M]^-$	4613.00	93%[2]
ON1-d	d	β-ME	ON1-d'	4585.15 $[M]^-$	4585.20	86%
ON1-e	e	β-ME	ON1-e'	4599.17 $[M]^-$	4599.34	98%[2]
ON1-f	f	β-ME	ON1-f'	4585.15 $[M]^-$	4585.70	94%
ON1-g	g	β-ME	ON1-g'	4584.15 $[M-H]^-$	4584.50	83%[2]
ON1-h	h	β-ME	ON1-h'	4571.12 $[M]^-$	4571.60	89%
ON1-i	i	β-ME	ON1-i'	4631.16 $[M-H]^-$	4631.90	4%
ON1-j	j	β-ME	ON1-j'	4648.18 $[M-H]^-$	4648.70	33%
ON1-k	k	β-ME	ON1-k'	4584.15 $[M-H]^-$	4584.00	94%[2]
ON1-l	l	β-ME	ON1-l'	4599.17 $[M]^-$	4599.49	full conversion[2, 3]
ON2-a	a	β-ME	ON2-a'	4451.04 $[M]^-$	4451.44	98%[2]
ON2-b	b	β-ME	ON2-b'	4507.15 $[M]^-$	4507.45	full conversion[2, 3]
ON2-c	c	β-ME	ON2-c'	4507.15 $[M]^-$	4507.40	full conversion[2, 3]
ON2-d	d	β-ME	ON2-d'	4479.10 $[M]^-$	4479.55	93%
ON2-e	e	β-ME	ON2-e'	4493.12 $[M]^-$	4493.45	80%[2]
ON3-b	b	β-ME	ON3-b'	5294.74 $[M]^-$	5295.00	90%
ON4-b	b	β-ME	ON4-b'	5462.80 $[M]^-$	5463.40	full conversion[2, 3]
ON1-a	a	GSH	ON1-a''	4786.29 $[M]^-$	4786.87	full conversion[2, 3]
ON1-b	b	GSH	ON1-b''	4842.39 $[M]^-$	4842.79	full conversion[2, 3]
ON1-c	c	GSH	ON1-c''	4842.39 $[M]^-$	4843.15	80%[2]
ON2-d	d	GSH	ON2-d''	4708.29 $[M]^-$	4708.89	full conversion[2, 3]
ON2-e	e	GSH	ON2-e''	4722.31 $[M]^-$	4723.07	full conversion[2, 3]
ON3-b	b	P1	ON3-b-P1	5959.42 $[M]^-$	5959.00	79%
ON4-b	b	P1	ON4-b-P1	6127.48 $[M]^-$	6127.30	92%
ON3-b	b	P2	ON3-b	5216.61 $[M]^-$	5217.50	n/a
ON4-b	b	P2	ON4-b	5384.67 $[M]^-$	5385.60	n/a
ON3-b	b	GalNAc-PEG3-SH	ON3-b-GalNAc	5586.04 $[M]^-$	5586.45	full conversion[3]
ON3-b	b	Toco-SH, 37	ON3-b-TF	5807.52 $[M]^-$	5807.45	13%

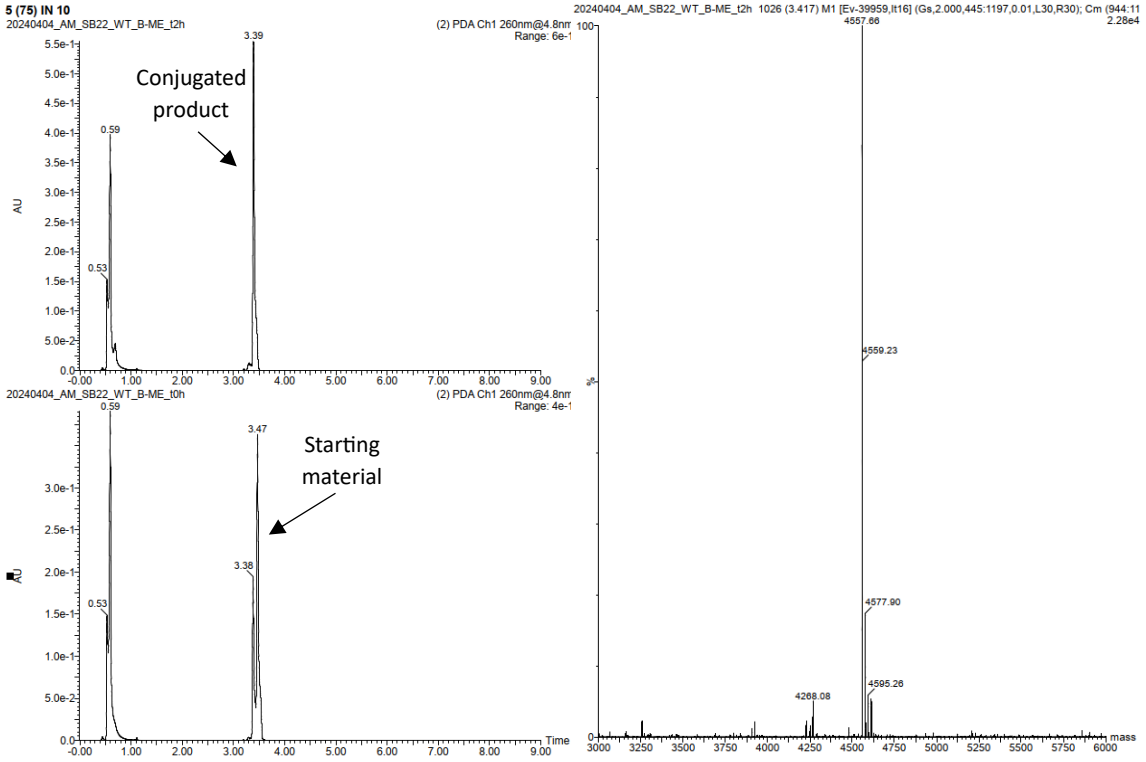
In all thiolation experiments, crude samples were injected on the LCMS.

[1]The conjugation yield was calculated based on the measured AUC (260 nm) for peaks corresponding to the starting material (0 h) and conjugated product (final time point). Due to the potential variability between injections (samples prepared manually), these results should be treated as rough estimation.

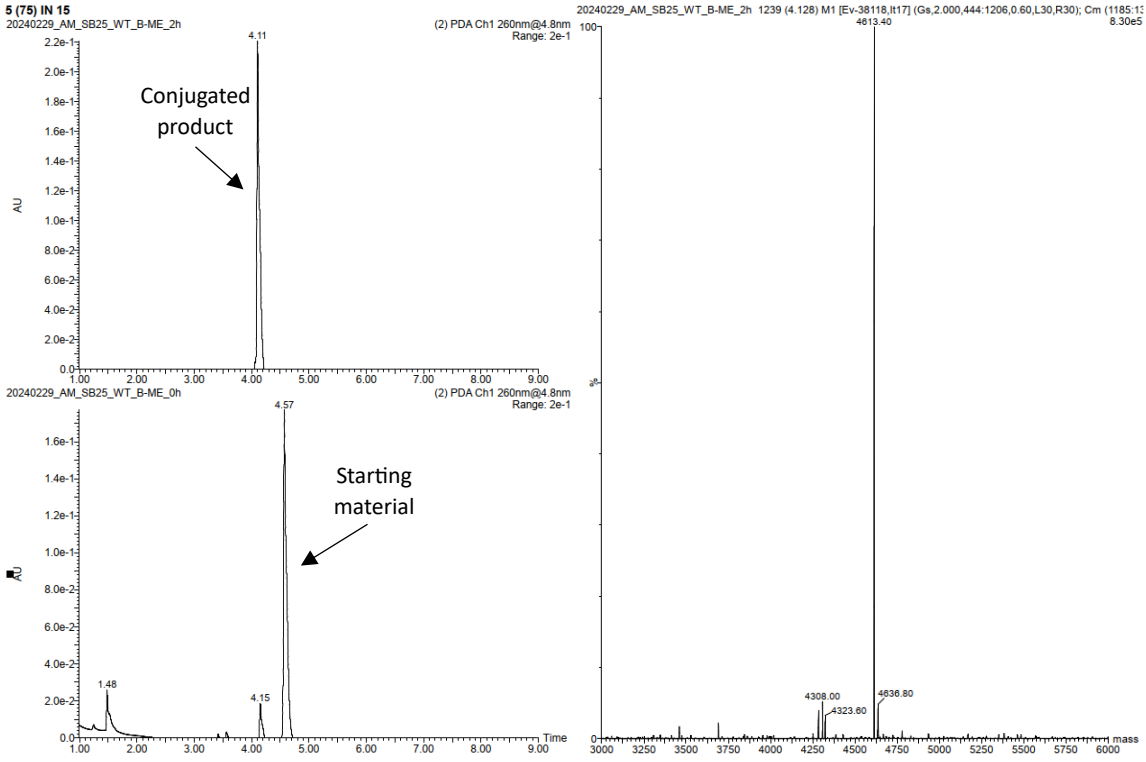
[2]For some of the compounds, a significant amount of the conjugated product was detected already at 0 h. In these cases, product AUC value at 0 h was subtracted from the product AUC value at final time point and the yield was calculated for the remaining product.

[3]Calculated yield was quantitative.

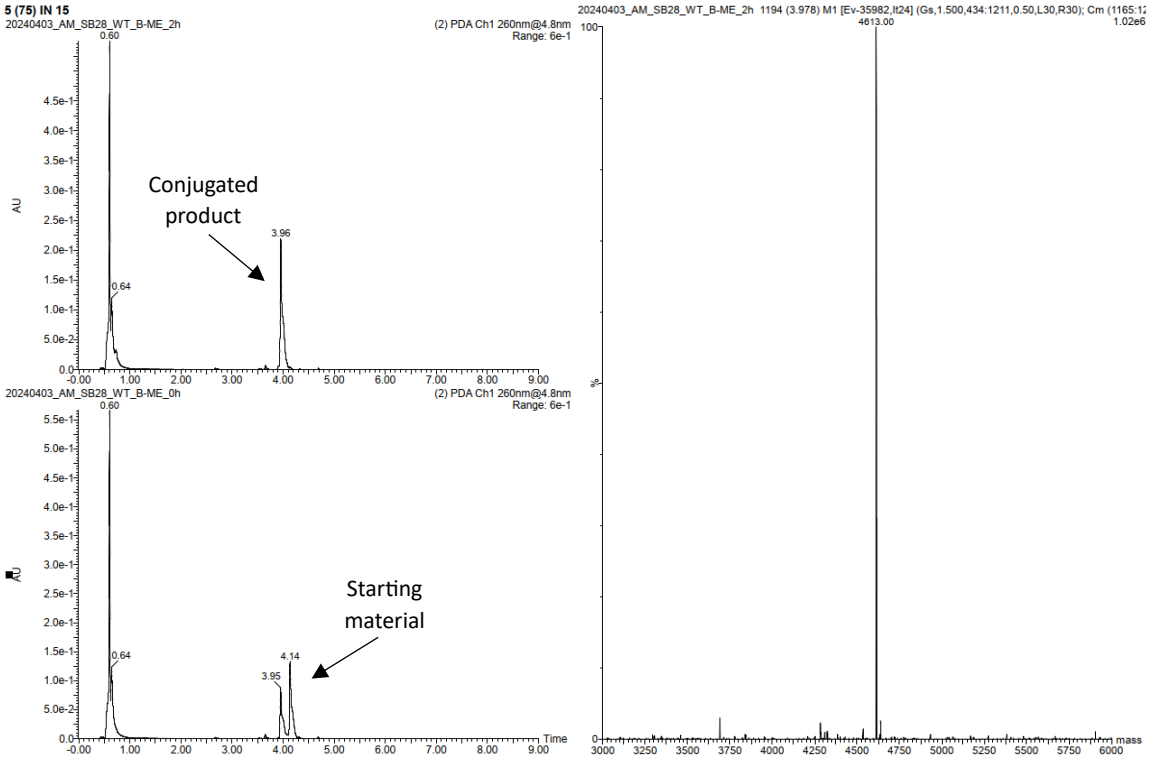
ON1-a’


5 (75) IN 10
20240404_AM_SB22_WT_B-ME_t2h
(2) PDA Ch1 260nm@4.8nm
Range: 6e-1
Conjugated product
3.39
0.59
0.53
AU
20240404_AM_SB22_WT_B-ME_t0h
(2) PDA Ch1 260nm@4.8nm
Range: 4e-1
Starting material
3.47
3.38
0.59
0.53
AU
Time
20240404_AM_SB22_WT_B-ME_t2h 1026 (3.417) M1 [Ev-39959,It16] (Gs,2.000,445:1197,0.01,L30,R30); Cm (944:11
2.28e4
4557.66
4559.23
4577.90
4595.26
4268.08
%
mass

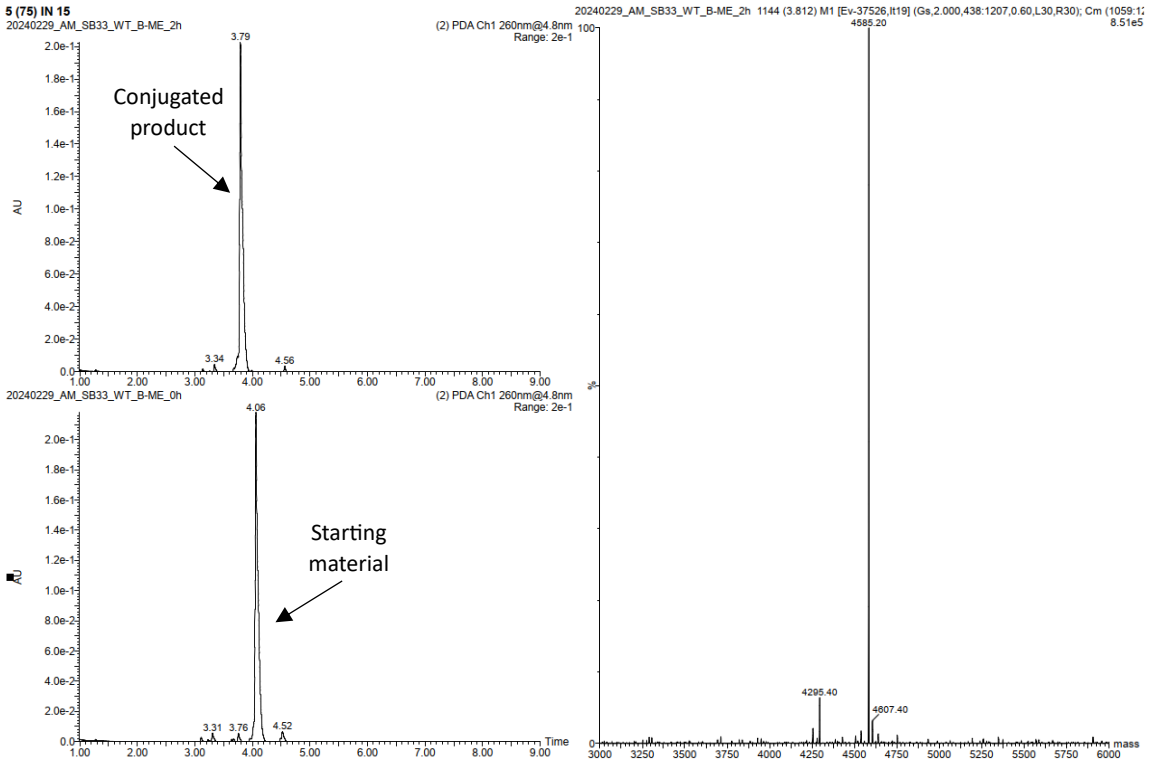
ON1-b’


5 (75) IN 15
20240229_AM_SB25_WT_B-ME_2h
(2) PDA Ch1 260nm@4.8nm
Range: 2e-1
Conjugated product
4.11
AU
20240229_AM_SB25_WT_B-ME_0h
(2) PDA Ch1 260nm@4.8nm
Range: 2e-1
Starting material
4.57
1.48
4.15
AU
Time
20240229_AM_SB25_WT_B-ME_2h 1239 (4.128) M1 [Ev-38118,It17] (Gs,2.000,444:1206,0.60,L30,R30); Cm (1185:1
8.30e5
4613.40
4636.80
4308.00
4323.60
%
mass

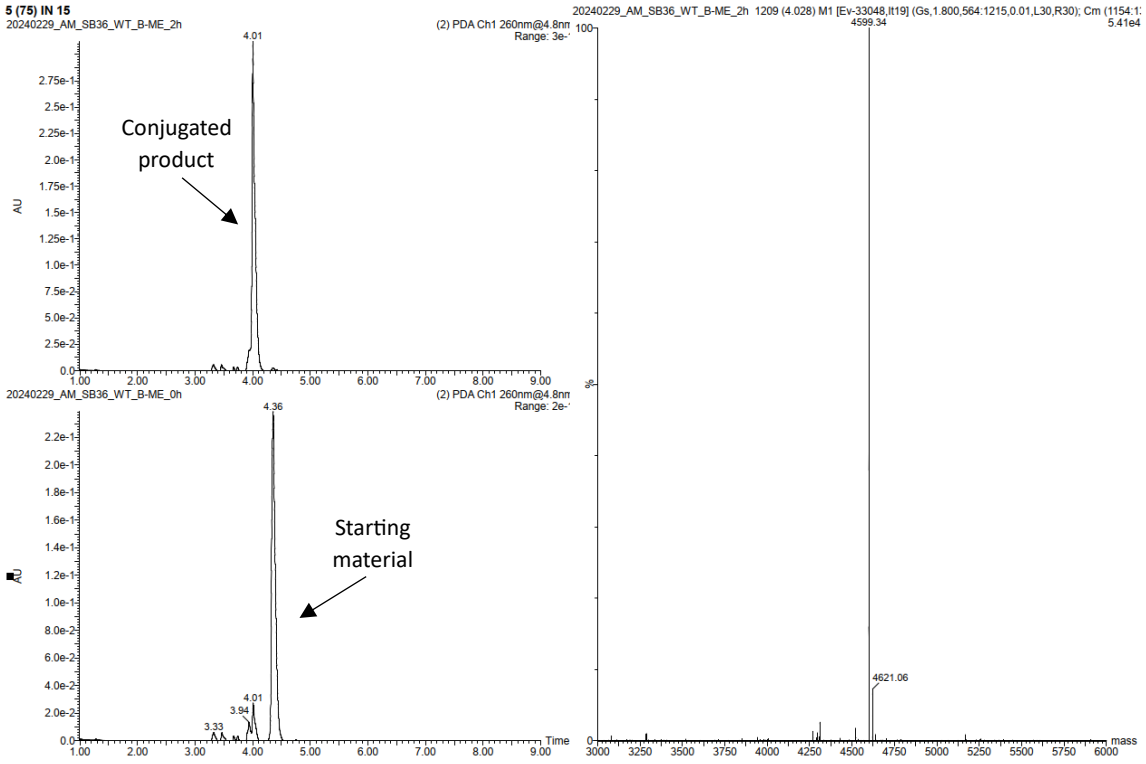
ON1-c’


5 (75) IN 15
20240403_AM_SB28_WT_B-ME_2h
(2) PDA Ch1 260nm@4.8nm
Range: 6e-1
Conjugated product
Starting material
AU
Time
20240403_AM_SB28_WT_B-ME_2h 1194 (3.978) M1 [Ev-35982,It24] (Gs,1.500,434:1211,0.50,L30,R30); Cm (1165:1
4613.00
1.02e6
mass

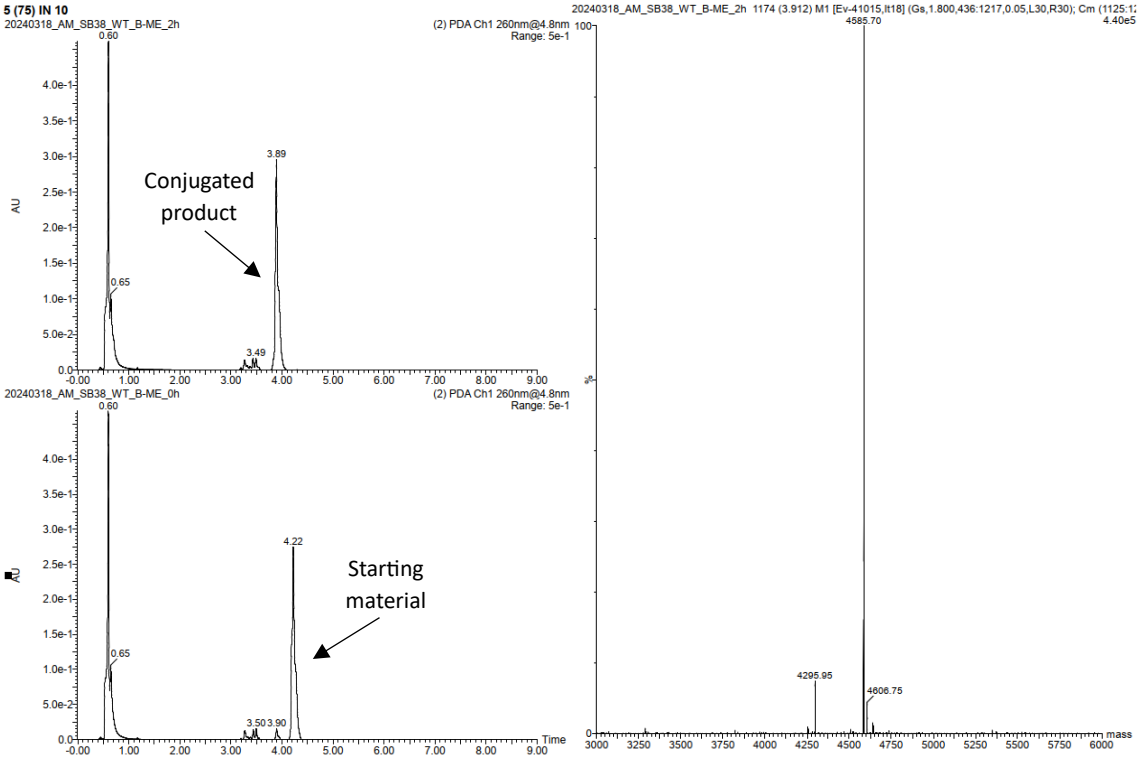
ON1-d’


5 (75) IN 15
20240229_AM_SB33_WT_B-ME_2h
(2) PDA Ch1 260nm@4.8nm
Range: 2e-1
Conjugated product
Starting material
AU
Time
20240229_AM_SB33_WT_B-ME_2h 1144 (3.812) M1 [Ev-37526,It19] (Gs,2.000,438:1207,0.60,L30,R30); Cm (1059:1
4585.20
8.51e5
4295.40
4607.40
mass

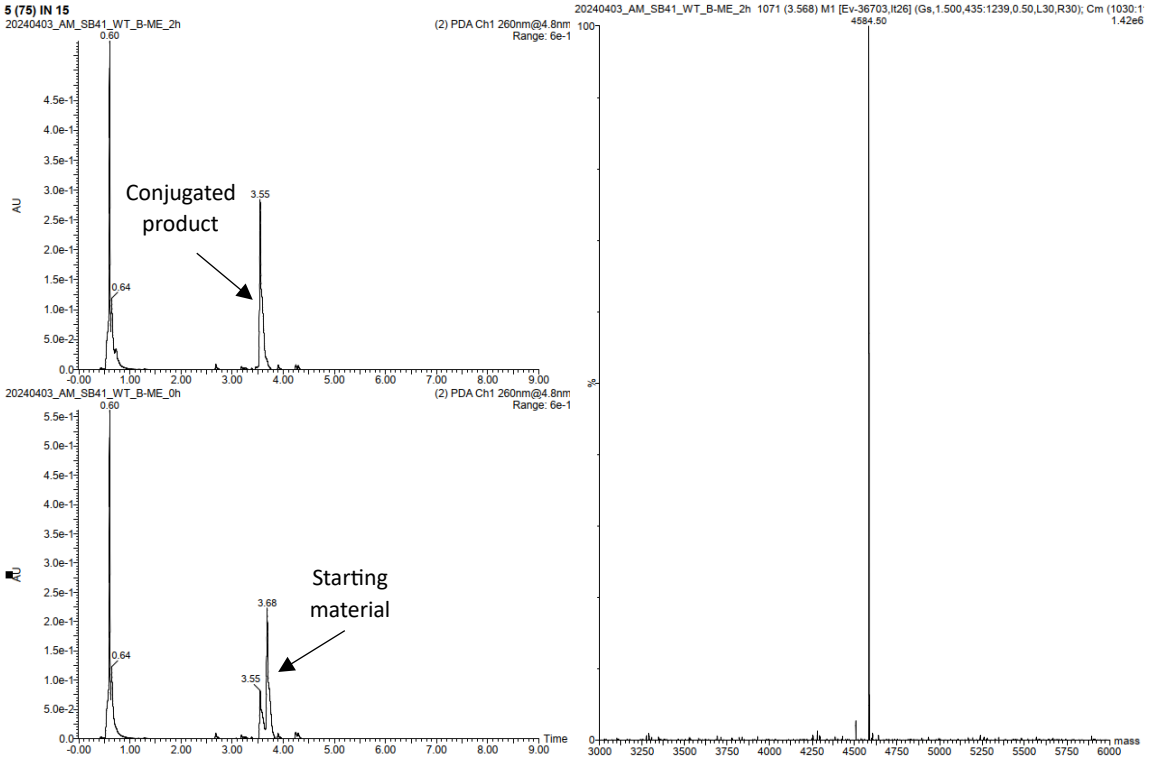
ON1-e’


5 (75) IN 15
20240229_AM_SB36_WT_B-ME_2h
(2) PDA Ch1 260nm@4.8nm
Range: 3e-
4.01
Conjugated product
AU
20240229_AM_SB36_WT_B-ME_0h
(2) PDA Ch1 260nm@4.8nm
Range: 2e-
4.36
Starting material
4.01
3.94
3.33
Time
20240229_AM_SB36_WT_B-ME_2h 1209 (4.028) M1 [Ev-33048,It19] (Gs,1.800,564:1215,0.01,L30,R30); Cm (1154:1
4599.34
5.41e4
100
%
4621.06
mass

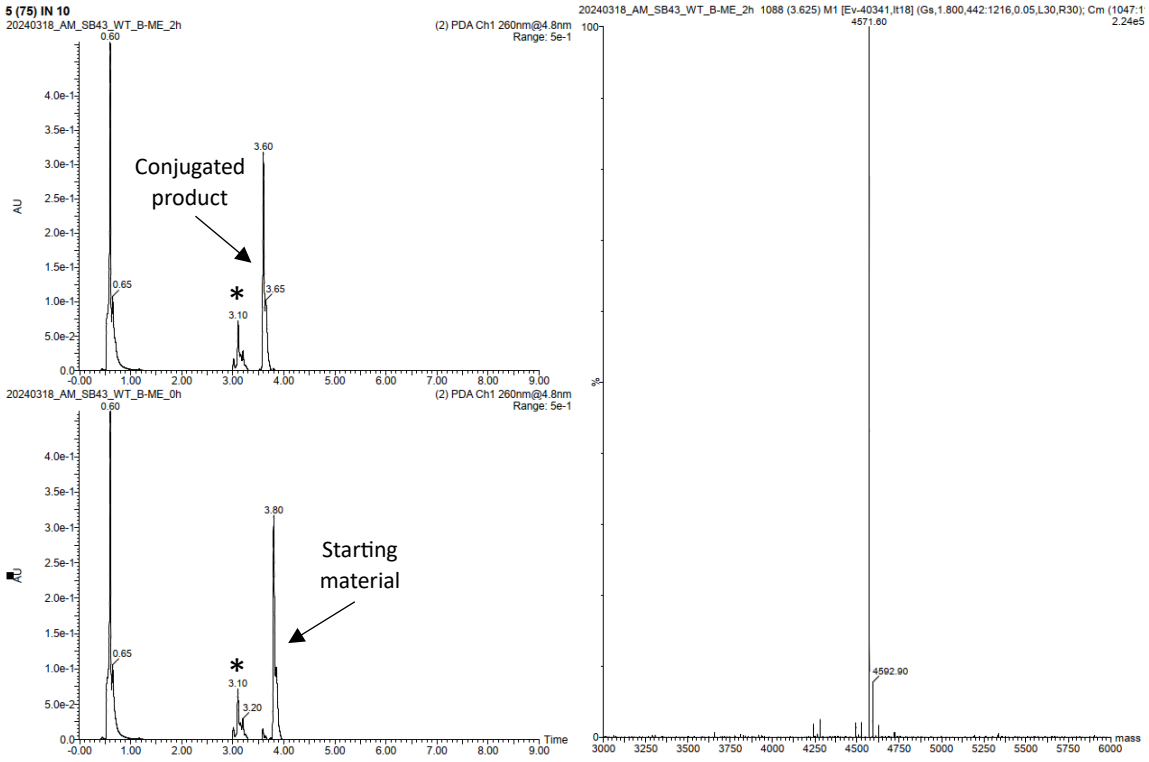
ON1-f’


5 (75) IN 10
20240318_AM_SB38_WT_B-ME_2h
(2) PDA Ch1 260nm@4.8nm
Range: 5e-1
0.60
3.89
Conjugated product
0.65
3.49
AU
20240318_AM_SB38_WT_B-ME_0h
(2) PDA Ch1 260nm@4.8nm
Range: 5e-1
0.60
4.22
Starting material
0.65
3.50
3.90
Time
20240318_AM_SB38_WT_B-ME_2h 1174 (3.912) M1 [Ev-41015,It18] (Gs,1.800,436:1217,0.05,L30,R30); Cm (1125:1
4585.70
4.40e5
100
%
4295.95
4606.75
mass

ON1-g'

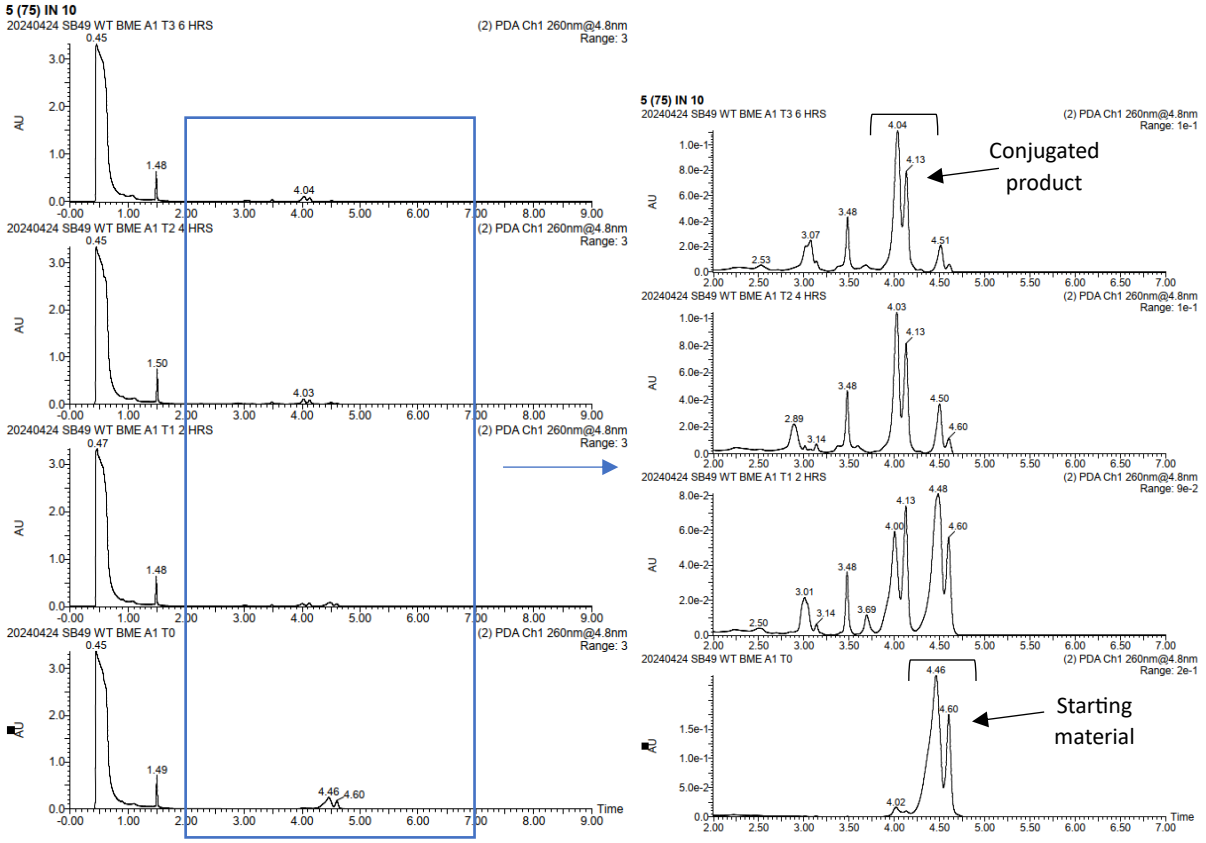


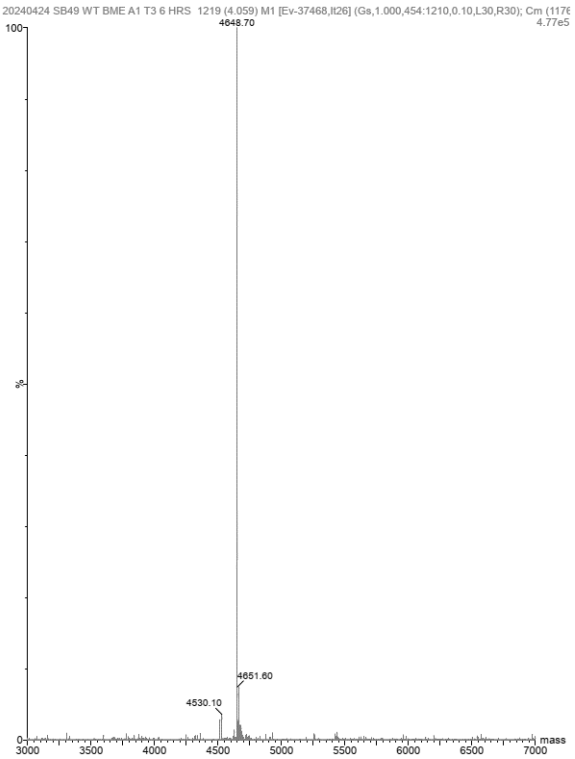
ON1-h'



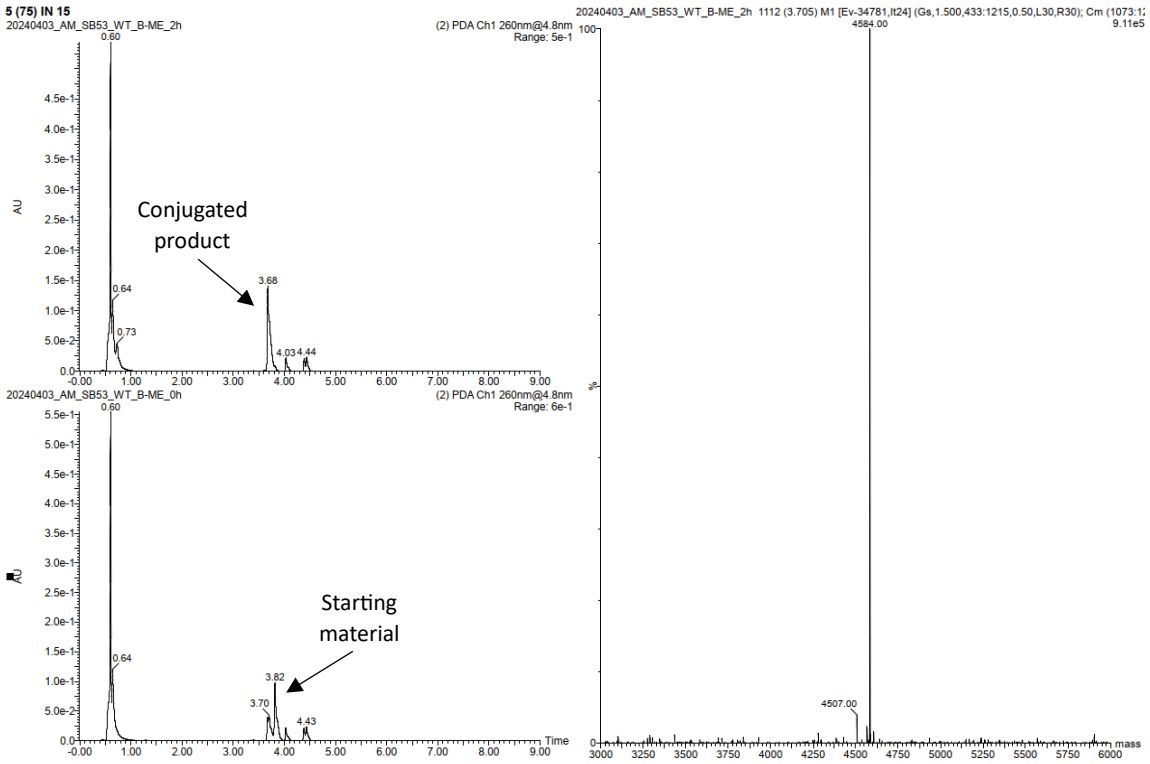
*Peak eluting at 3.10 min has a mass corresponding to the starting material.

ON1-j’

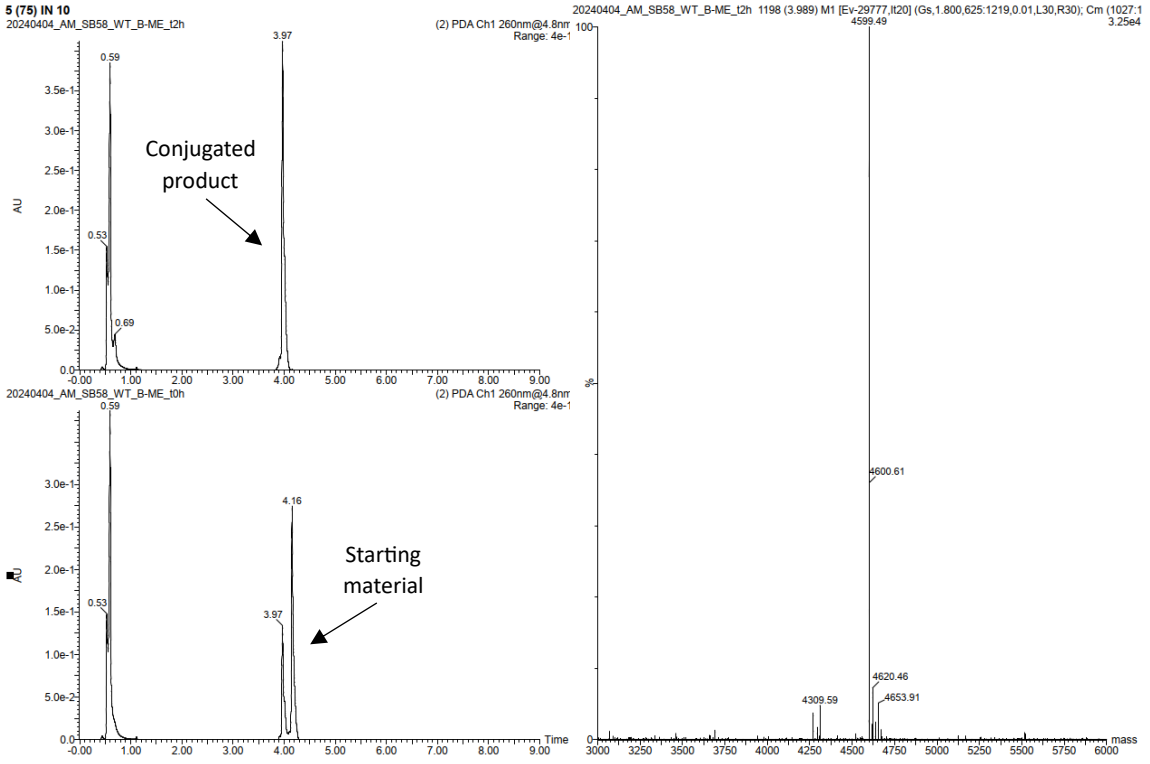

5 (75) IN 10
20240424 SB49 WT BME A1 T3 6 HRS
(2) PDA Ch1 260nm@4.8nm
Range: 3
20240424 SB49 WT BME A1 T2 4 HRS
20240424 SB49 WT BME A1 T1 2 HRS
20240424 SB49 WT BME A1 T0
AU
Time
Range: 1e-1
Range: 9e-2
Range: 2e-1
Conjugated product
Starting material


20240424 SB49 WT BME A1 T3 6 HRS
4648.70
4651.60
4530.10
mass

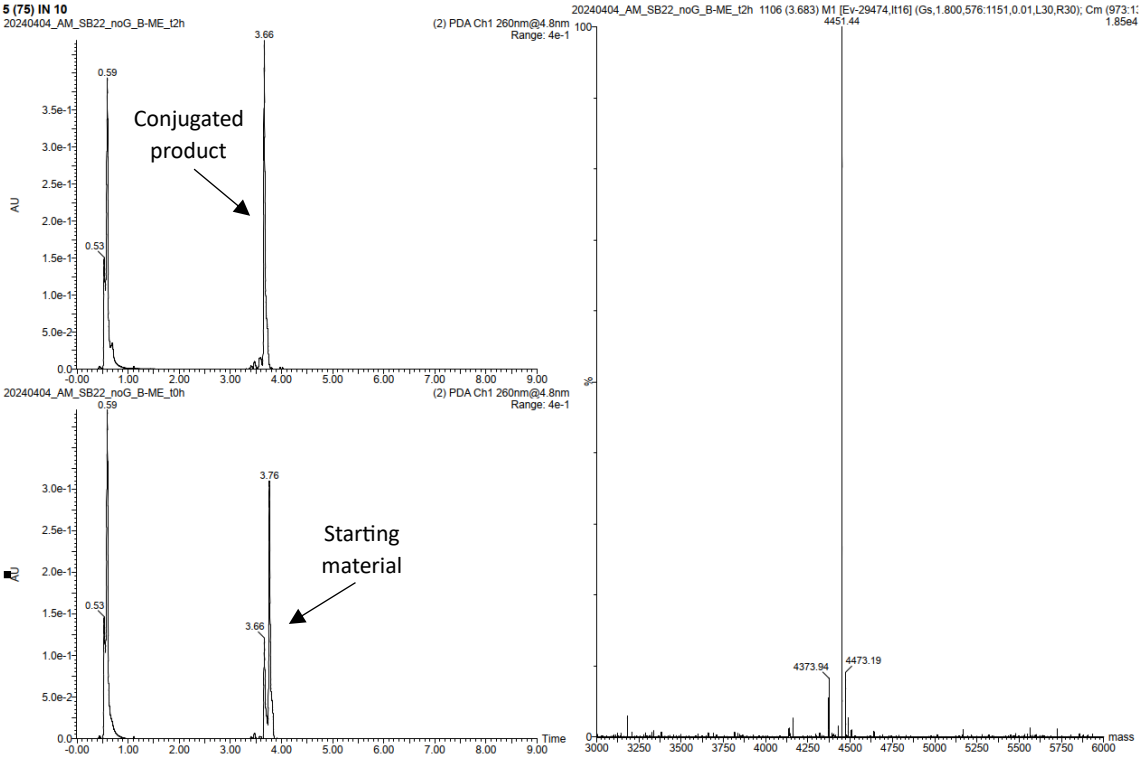
ON1-k’


5 (75) IN 15
20240403_AM_SB53_WT_B-ME_2h
(2) PDA Ch1 260nm@4.8nm
Range: 5e-1
Conjugated product
20240403_AM_SB53_WT_B-ME_0h
(2) PDA Ch1 260nm@4.8nm
Range: 6e-1
Starting material
AU
Time
20240403_AM_SB53_WT_B-ME_2h 1112 (3.705) M1 [Ev-34781,It24] (Gs,1.500,433:1215,0.50,L30,R30); Cm (1073:1
4584.00
9.11e5
4507.00
%
mass

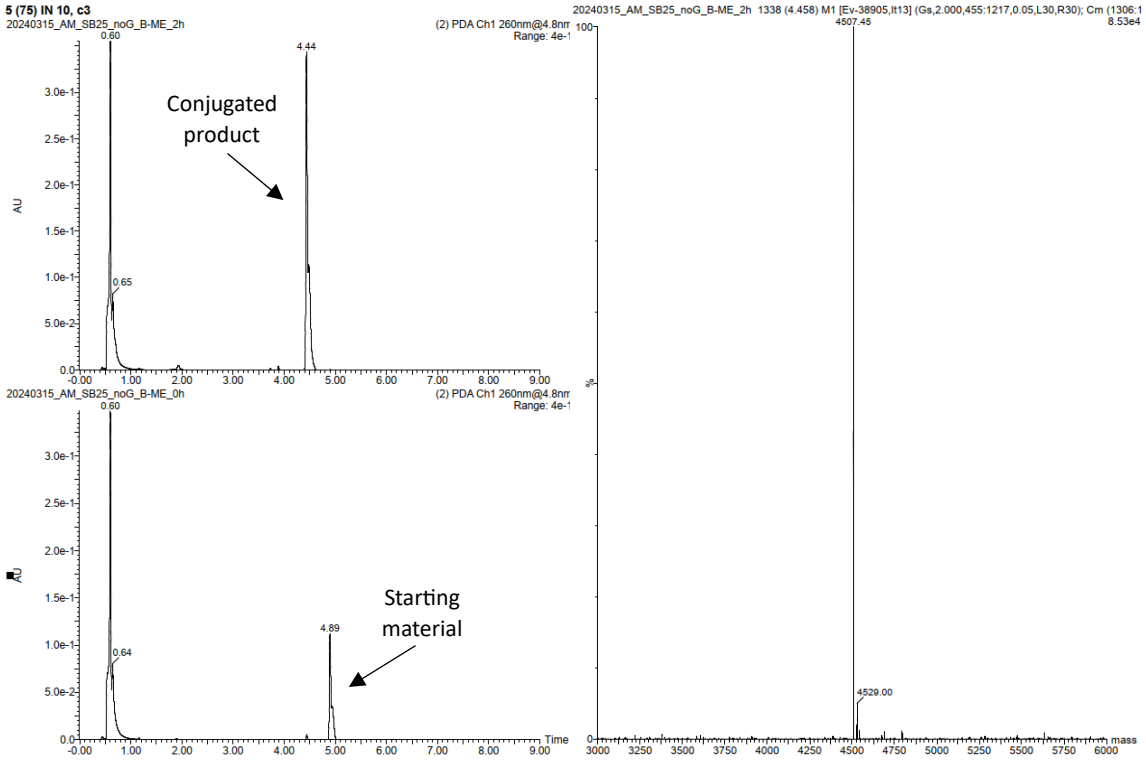
ON1-l’


5 (75) IN 10
20240404_AM_SB58_WT_B-ME_t2h
(2) PDA Ch1 260nm@4.8nm
Range: 4e-1
Conjugated product
20240404_AM_SB58_WT_B-ME_t0h
(2) PDA Ch1 260nm@4.8nm
Range: 4e-1
Starting material
AU
Time
20240404_AM_SB58_WT_B-ME_t2h 1198 (3.989) M1 [Ev-29777,It20] (Gs,1.800,625:1219,0.01,L30,R30); Cm (1027:1
4599.49
3.25e4
4600.61
4620.46
4653.91
4309.59
%
mass

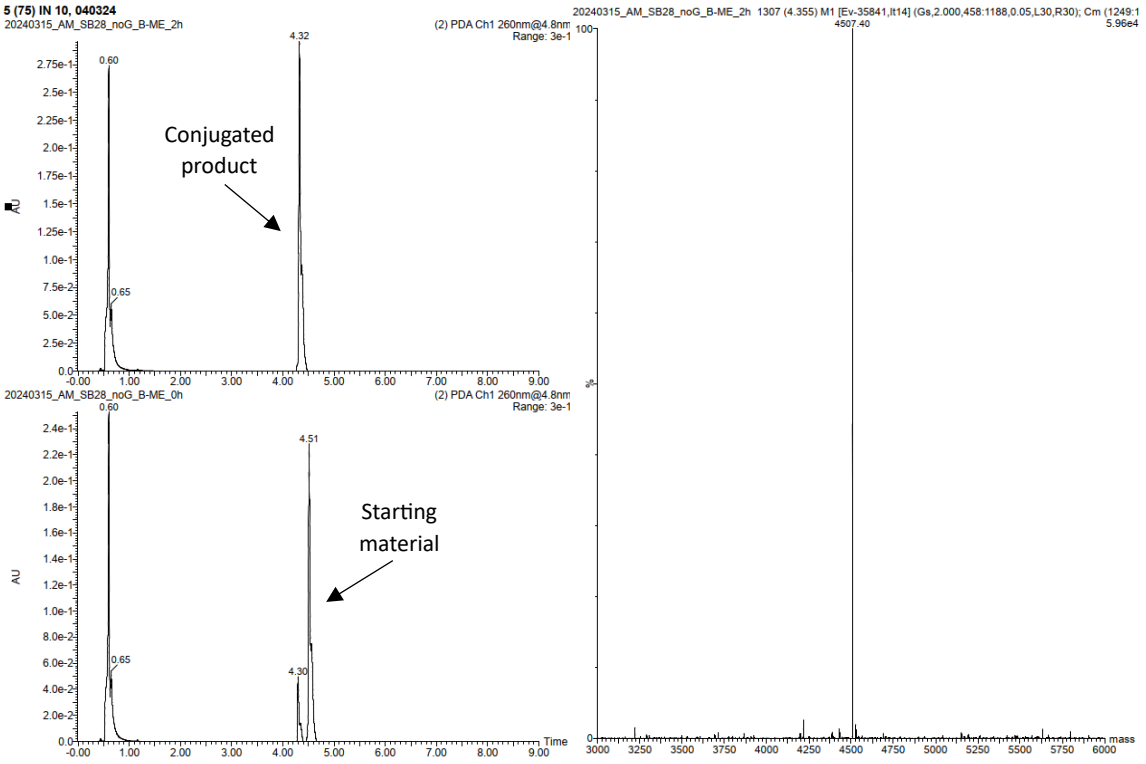
ON2-a’


5 (75) IN 10
20240404_AM_SB22_noG_B-ME_t2h
(2) PDA Ch1 260nm@4.8nm
Range: 4e-1
Conjugated product
20240404_AM_SB22_noG_B-ME_t0h
Starting material
AU
Time
20240404_AM_SB22_noG_B-ME_t2h 1106 (3.683) M1 [Ev-29474,It16] (Gs,1.800,576:1151,0.01,L30,R30); Cm
4451.44
4373.94
4473.19
mass

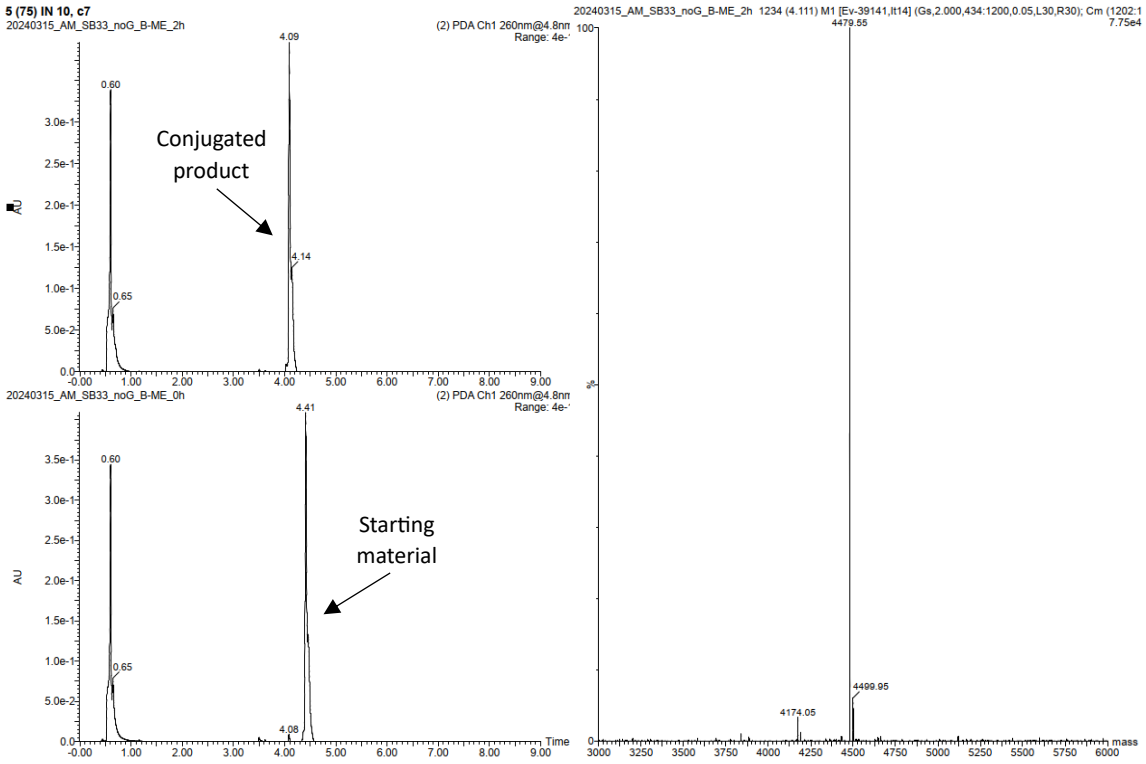
ON2-b’


5 (75) IN 10, c3
20240315_AM_SB25_noG_B-ME_2h
(2) PDA Ch1 260nm@4.8nm
Range: 4e-1
Conjugated product
20240315_AM_SB25_noG_B-ME_0h
Starting material
AU
Time
20240315_AM_SB25_noG_B-ME_2h 1338 (4.458) M1 [Ev-38905,It13] (Gs,2.000,455:1217,0.05,L30,R30); Cm
4507.45
4529.00
mass

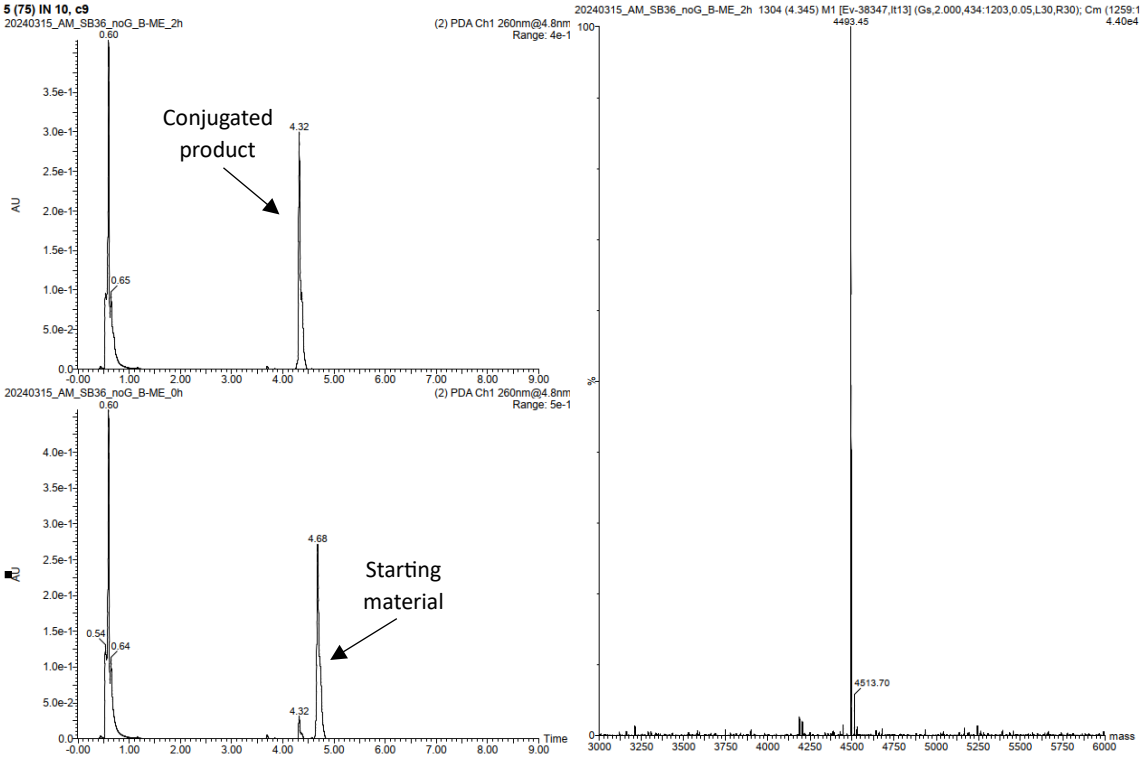
ON2-c’


5 (75) IN 10, 040324
20240315_AM_SB28_noG_B-ME_2h
(2) PDA Ch1 260nm@4.8nm
Range: 3e-1
Conjugated product
4.32
0.60
0.65
AU
20240315_AM_SB28_noG_B-ME_0h
(2) PDA Ch1 260nm@4.8nm
Range: 3e-1
Starting material
4.51
4.30
Time
20240315_AM_SB28_noG_B-ME_2h 1307 (4.355) M1 [Ev-35841,It14] (Gs,2.000,458:1188,0.05,L30,R30); Cm (1249:1
4507.40
5.96e4
%
mass

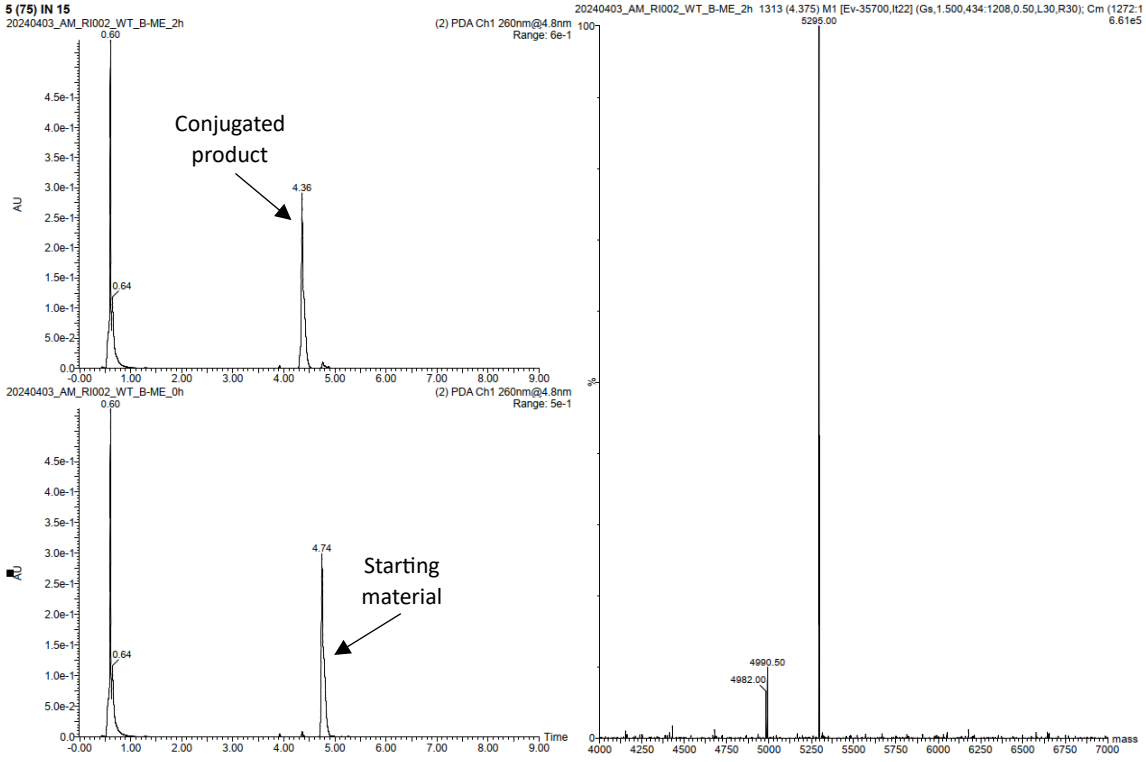
ON2-d’


5 (75) IN 10, c7
20240315_AM_SB33_noG_B-ME_2h
(2) PDA Ch1 260nm@4.8nm
Range: 4e-
Conjugated product
4.09
0.60
4.14
0.65
AU
20240315_AM_SB33_noG_B-ME_0h
(2) PDA Ch1 260nm@4.8nm
Range: 4e-
Starting material
4.41
4.08
Time
20240315_AM_SB33_noG_B-ME_2h 1234 (4.111) M1 [Ev-39141,It14] (Gs,2.000,434:1200,0.05,L30,R30); Cm (1202:1
4479.55
7.75e4
4499.95
4174.05
%
mass

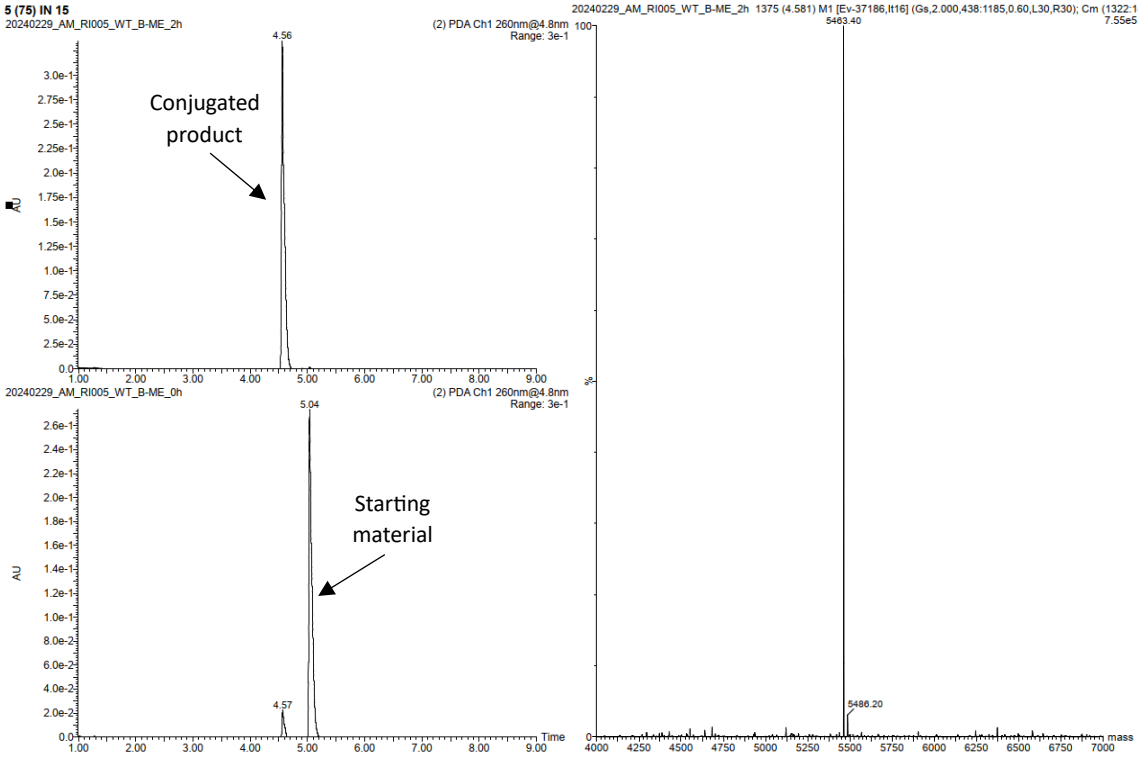
ON2-e’


5 (75) IN 10, c9
20240315_AM_SB36_noG_B-ME_2h
(2) PDA Ch1 260nm@4.8nm
Range: 4e-1
0.60
4.32
0.65
Conjugated product
AU
20240315_AM_SB36_noG_B-ME_0h
(2) PDA Ch1 260nm@4.8nm
Range: 5e-1
0.60
4.68
0.54
0.64
4.32
Starting material
Time
20240315_AM_SB36_noG_B-ME_2h 1304 (4.345) M1 [Ev-38347,It13] (Gs,2.000,434:1203,0.05,L30,R30); Cm (1259:1
4493.45
4.40e4
100
%
4513.70
mass

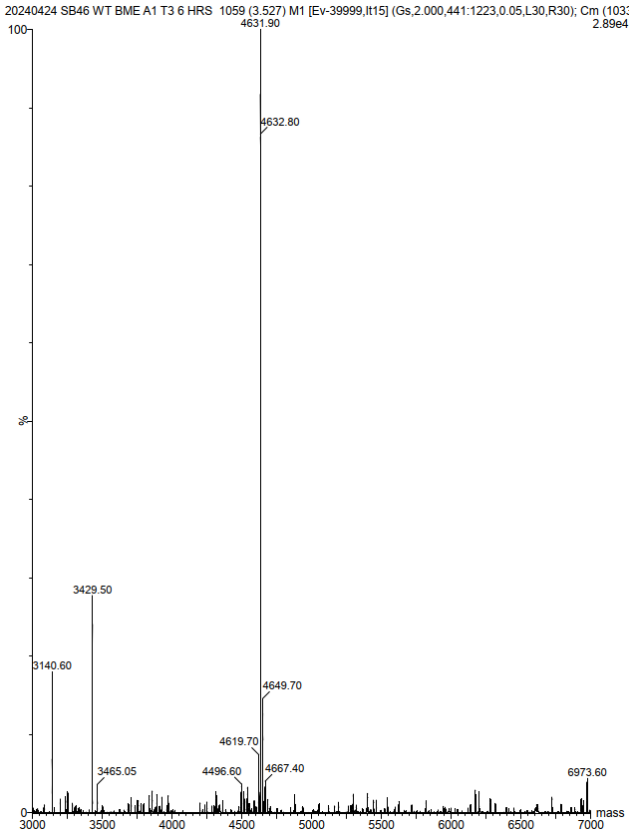
ON3-b’


5 (75) IN 15
20240403_AM_RI002_WT_B-ME_2h
(2) PDA Ch1 260nm@4.8nm
Range: 6e-1
0.60
4.36
0.64
Conjugated product
AU
20240403_AM_RI002_WT_B-ME_0h
(2) PDA Ch1 260nm@4.8nm
Range: 5e-1
0.60
4.74
0.64
Starting material
Time
20240403_AM_RI002_WT_B-ME_2h 1313 (4.375) M1 [Ev-35700,It22] (Gs,1.500,434:1208,0.50,L30,R30); Cm (1272:1
5295.00
6.61e5
100
%
4990.50
4982.00
mass

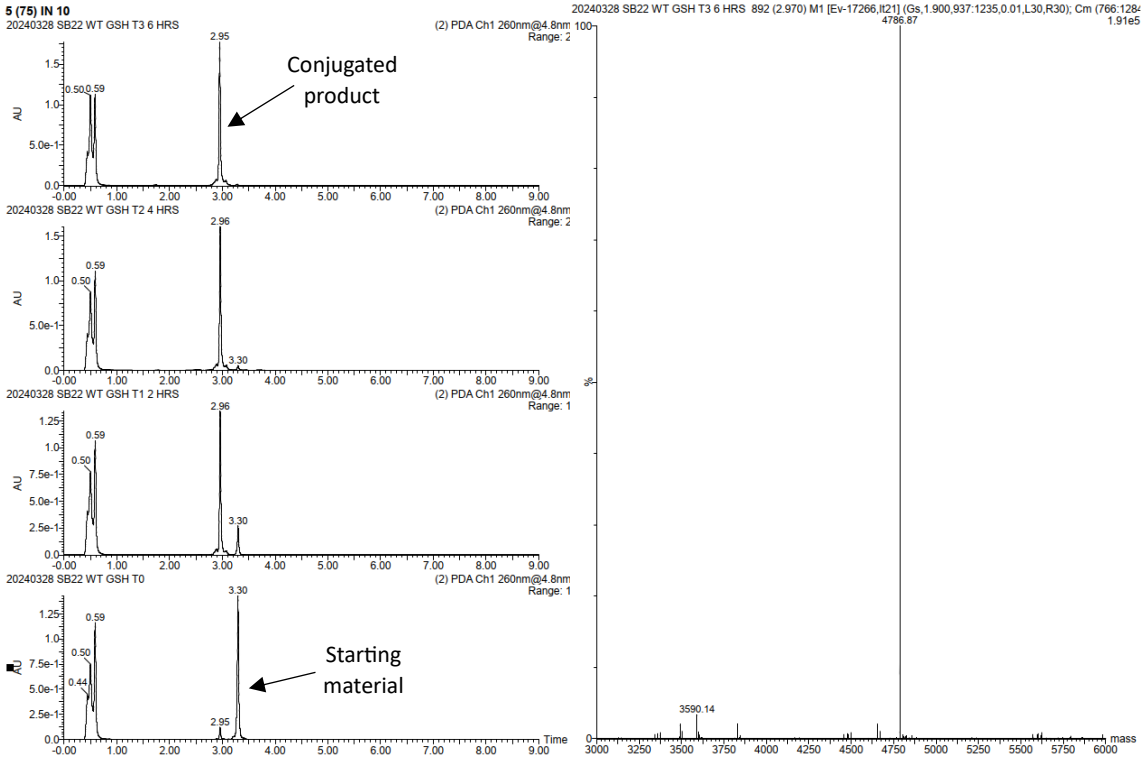
ON4-b’


5 (75) IN 15
20240229_AM_RI005_WT_B-ME_2h
(2) PDA Ch1 260nm@4.8nm
Range: 3e-1
4.56
Conjugated product
AU
20240229_AM_RI005_WT_B-ME_0h
(2) PDA Ch1 260nm@4.8nm
Range: 3e-1
5.04
Starting material
4.57
Time
20240229_AM_RI005_WT_B-ME_2h 1375 (4.581) M1 [Ev-37186,It16] (Gs,2.000,438:1185,0.60,L30,R30); Cm (1322:1
5463.40
7.55e5
%
5486.20
mass

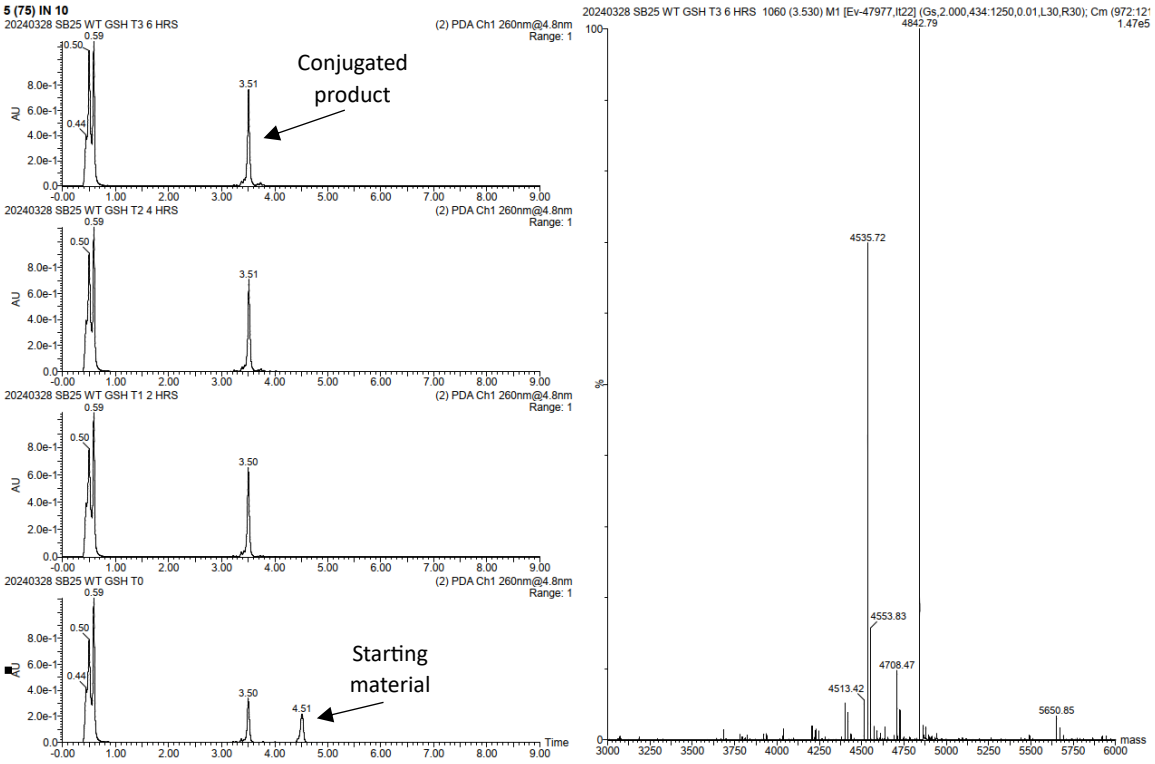
ON1-i’


20240424 SB46 WT BME A1 T3 6 HRS 1059 (3.527) M1 [Ev-39999,It15] (Gs,2.000,441:1223,0.05,L30,R30); Cm (103
4631.90
2.89e4
4632.80
3429.50
3140.60
4649.70
4619.70
3465.05
4496.60
4667.40
6973.60
%
mass

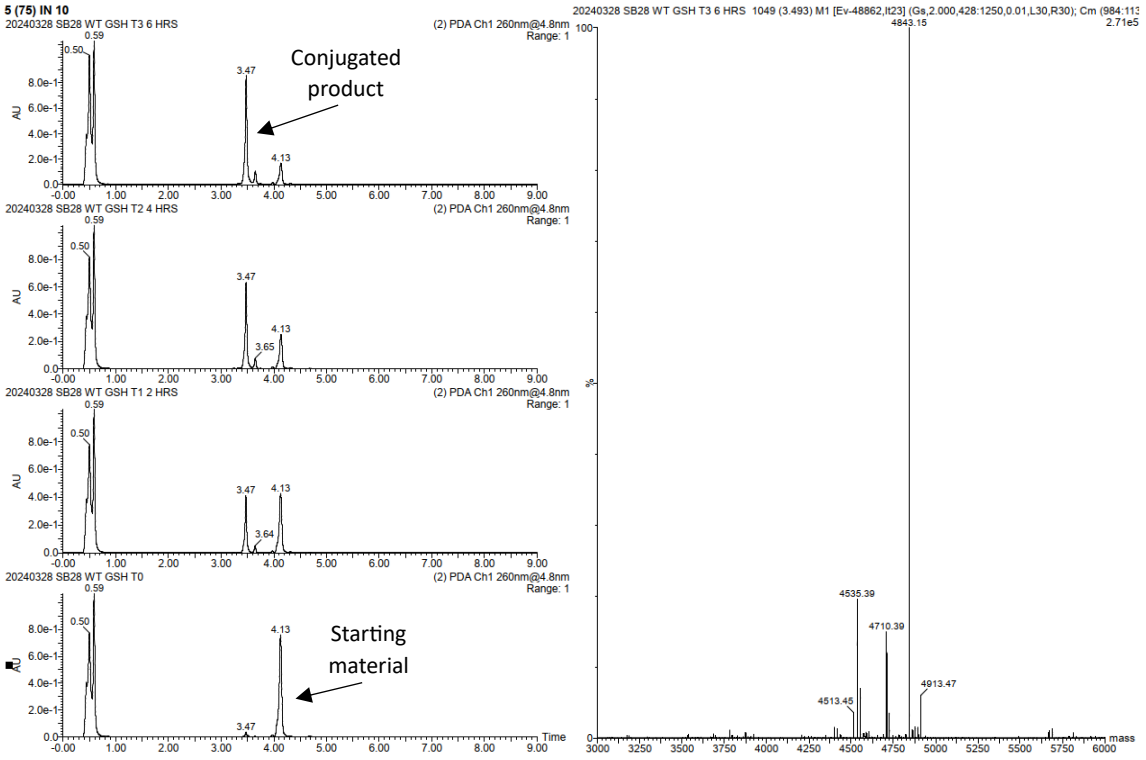
ON1-a''


5 (75) IN 10
20240328 SB22 WT GSH T3 6 HRS
(2) PDA Ch1 260nm@4.8nm
Conjugated product
20240328 SB22 WT GSH T2 4 HRS
20240328 SB22 WT GSH T1 2 HRS
20240328 SB22 WT GSH T0
Starting material
AU
Time
20240328 SB22 WT GSH T3 6 HRS 892 (2.970) M1 [Ev-17266,It21] (Gs,1.900,937:1235,0.01,L30,R30); Cm (766:128
4786.87
3590.14
%
mass

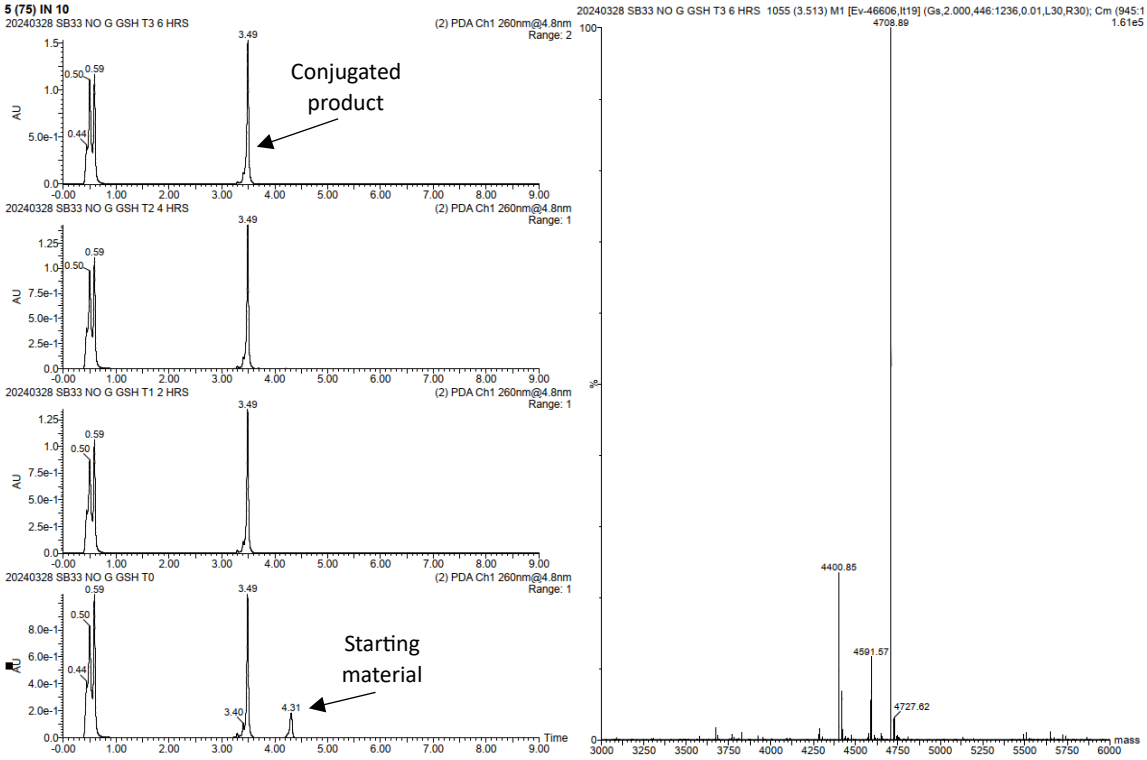
ON1-b''


5 (75) IN 10
20240328 SB25 WT GSH T3 6 HRS
(2) PDA Ch1 260nm@4.8nm
Conjugated product
20240328 SB25 WT GSH T2 4 HRS
20240328 SB25 WT GSH T1 2 HRS
20240328 SB25 WT GSH T0
Starting material
AU
Time
20240328 SB25 WT GSH T3 6 HRS 1060 (3.530) M1 [Ev-47977,It22] (Gs,2.000,434:1250,0.01,L30,R30); Cm (972:12
4842.79
4535.72
4553.83
4708.47
4513.42
5650.85
%
mass

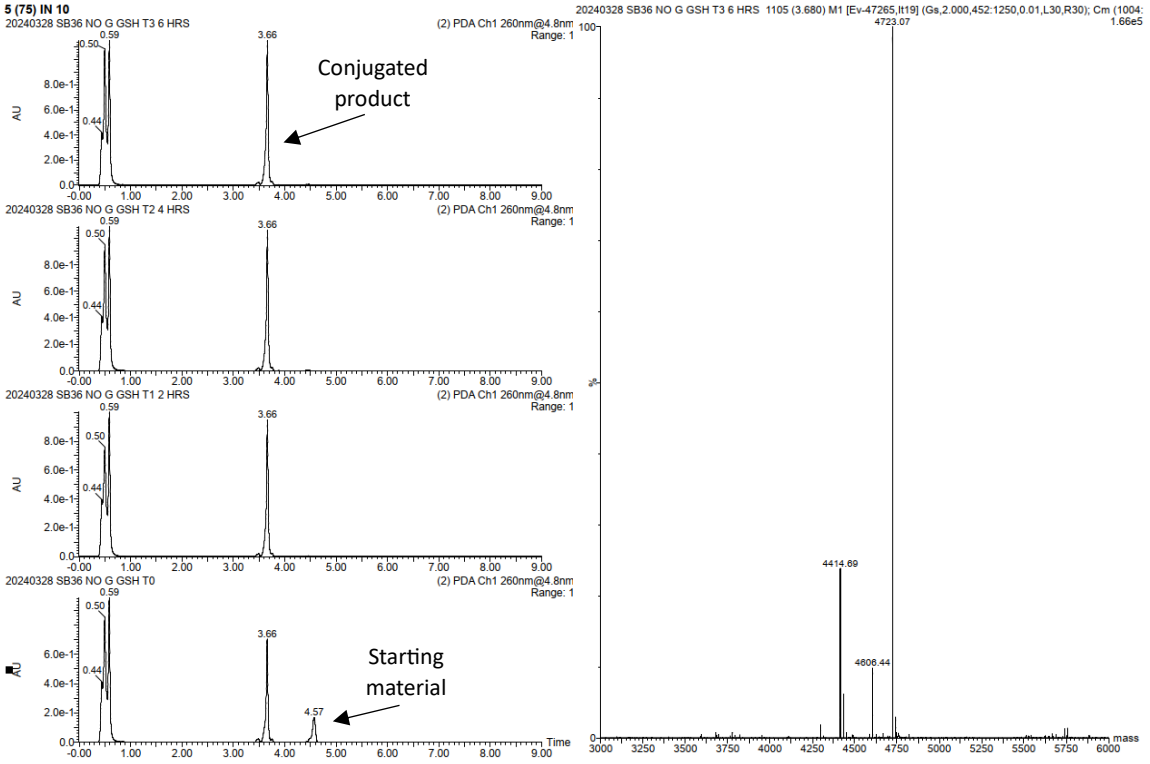
ON1-c''


5 (75) IN 10
20240328 SB28 WT GSH T3 6 HRS
(2) PDA Ch1 260nm@4.8nm
Range: 1
Conjugated product
20240328 SB28 WT GSH T2 4 HRS
20240328 SB28 WT GSH T1 2 HRS
20240328 SB28 WT GSH T0
Starting material
AU
Time
20240328 SB28 WT GSH T3 6 HRS 1049 (3.493) M1 [Ev-48862,It23] (Gs,2.000,428:1250,0.01,L30,R30); Cm (984:113
2.71e5
4843.15
4535.39
4710.39
4913.47
4513.45
%
mass

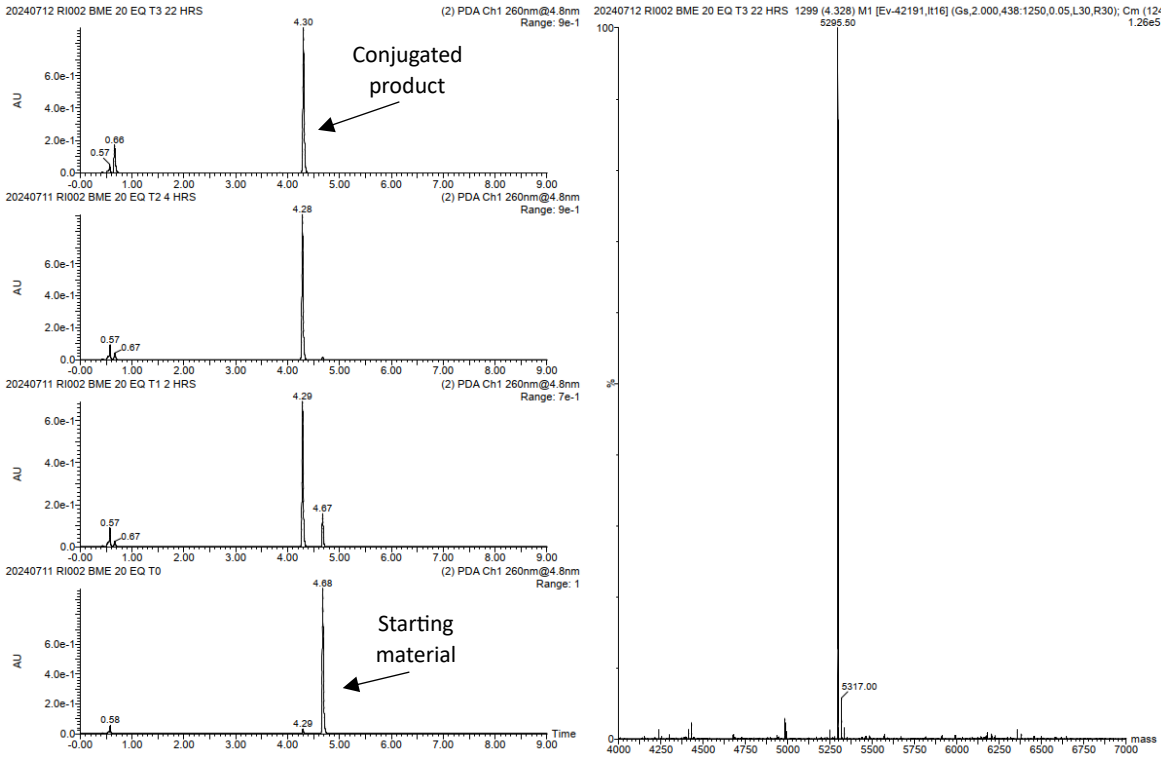
ON2-d''


5 (75) IN 10
20240328 SB33 NO G GSH T3 6 HRS
(2) PDA Ch1 260nm@4.8nm
Range: 2
Conjugated product
20240328 SB33 NO G GSH T2 4 HRS
Range: 1
20240328 SB33 NO G GSH T1 2 HRS
20240328 SB33 NO G GSH T0
Starting material
AU
Time
20240328 SB33 NO G GSH T3 6 HRS 1055 (3.513) M1 [Ev-46606,It19] (Gs,2.000,446:1236,0.01,L30,R30); Cm (945:1
1.61e5
4708.89
4400.85
4591.57
4727.62
%
mass

ON2-e''



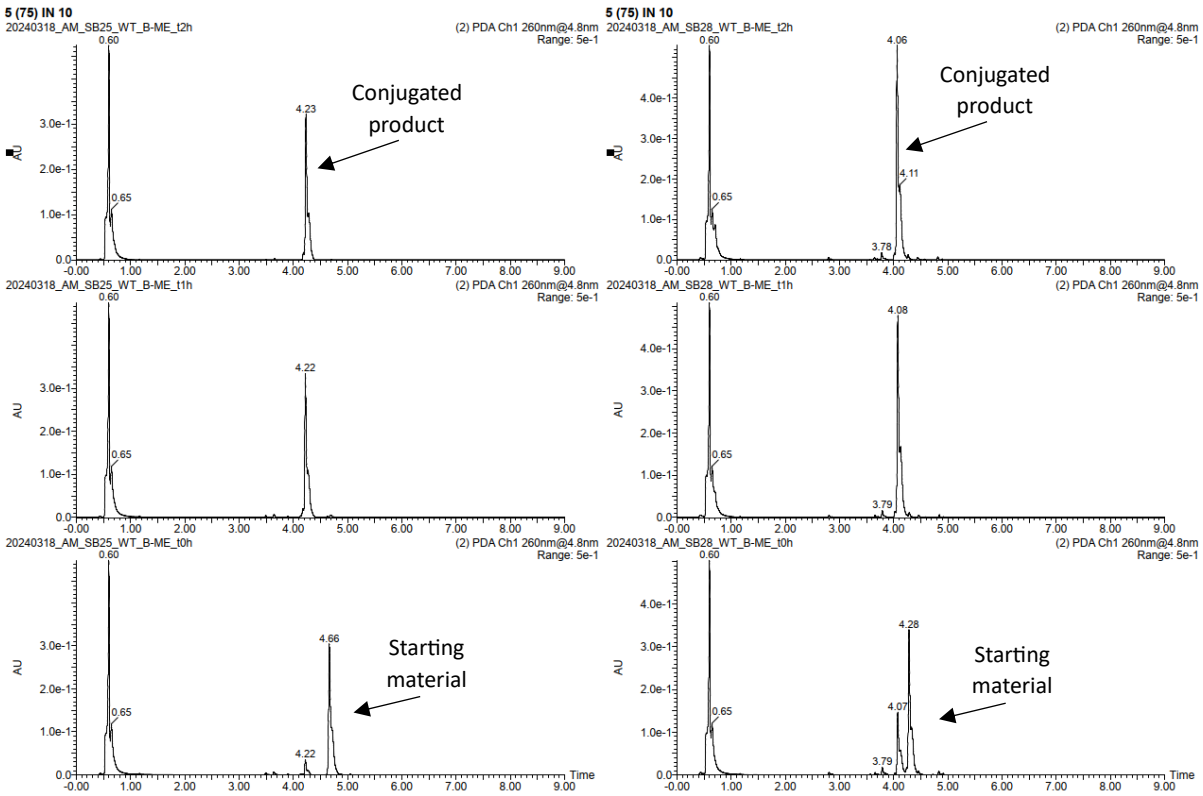
ON3-b' – 20 eq experiment



Estimated conversion[1]: 84%.

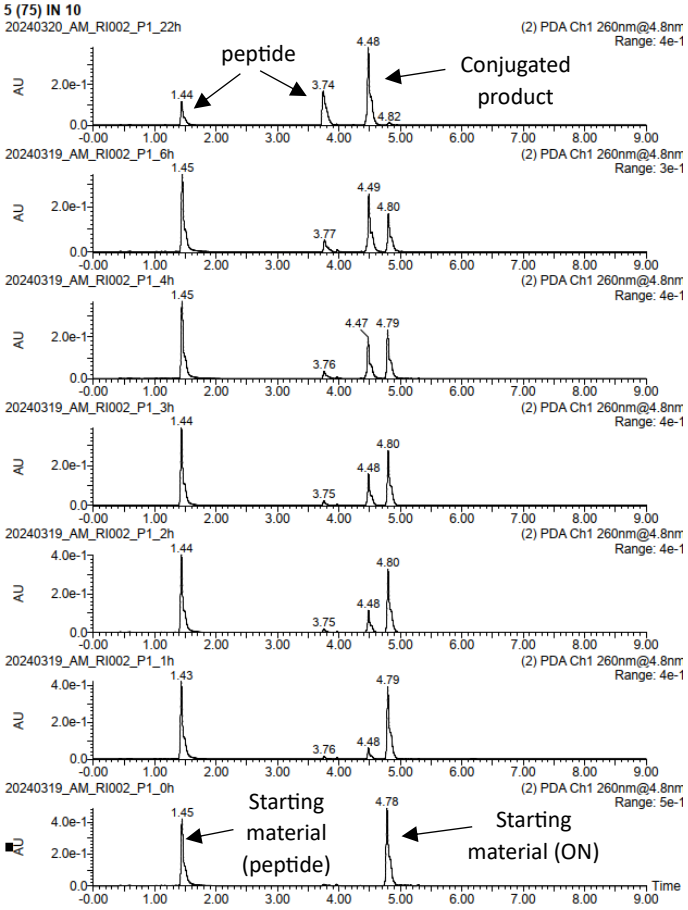
Reactivity comparison

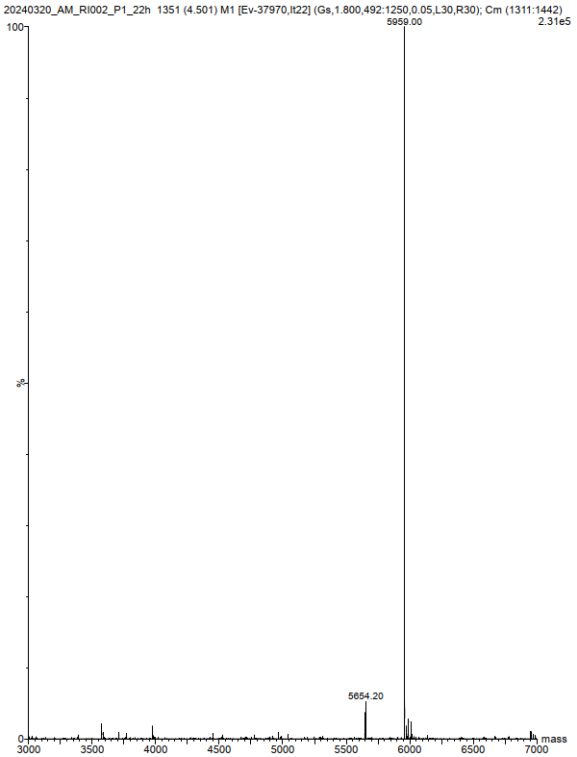
ON1-b' vs ON1-c'



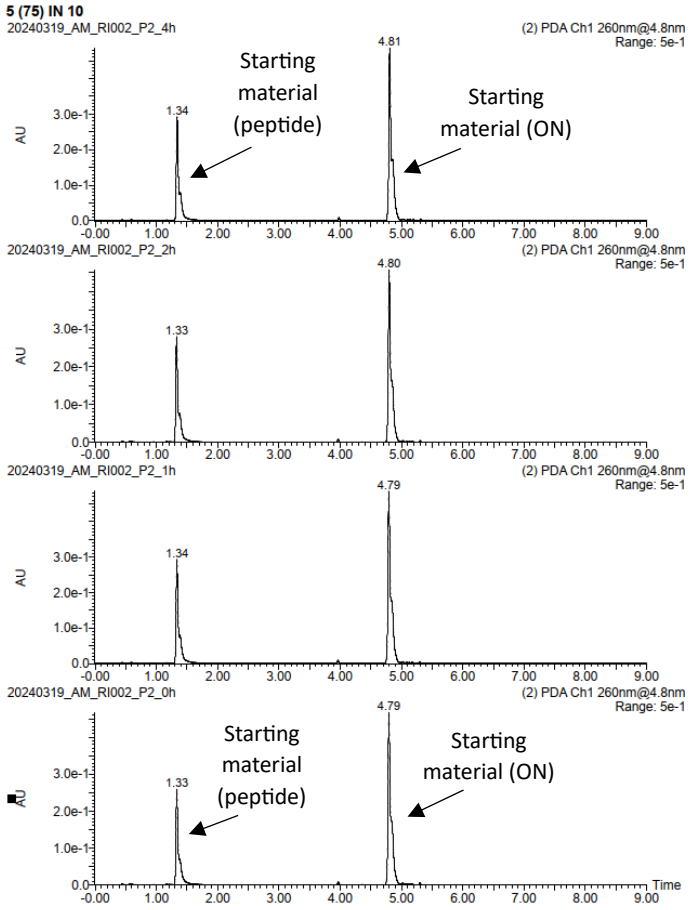
Peptide conjugation

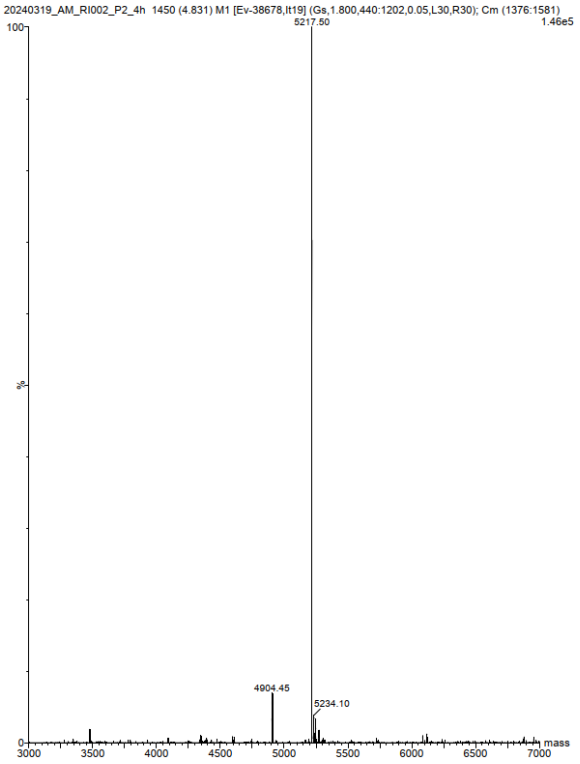
ON3-b-P1



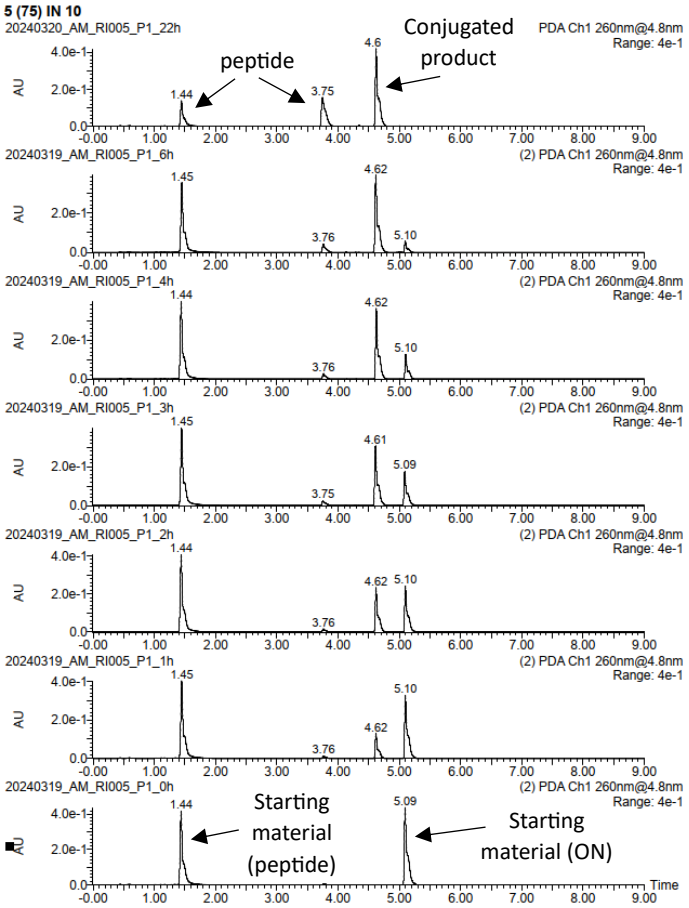


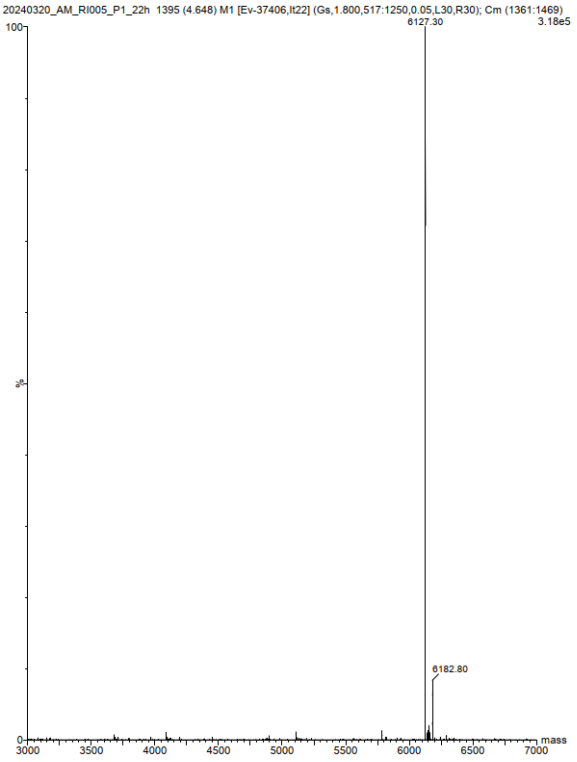
ON3-b (conjugation with P2)



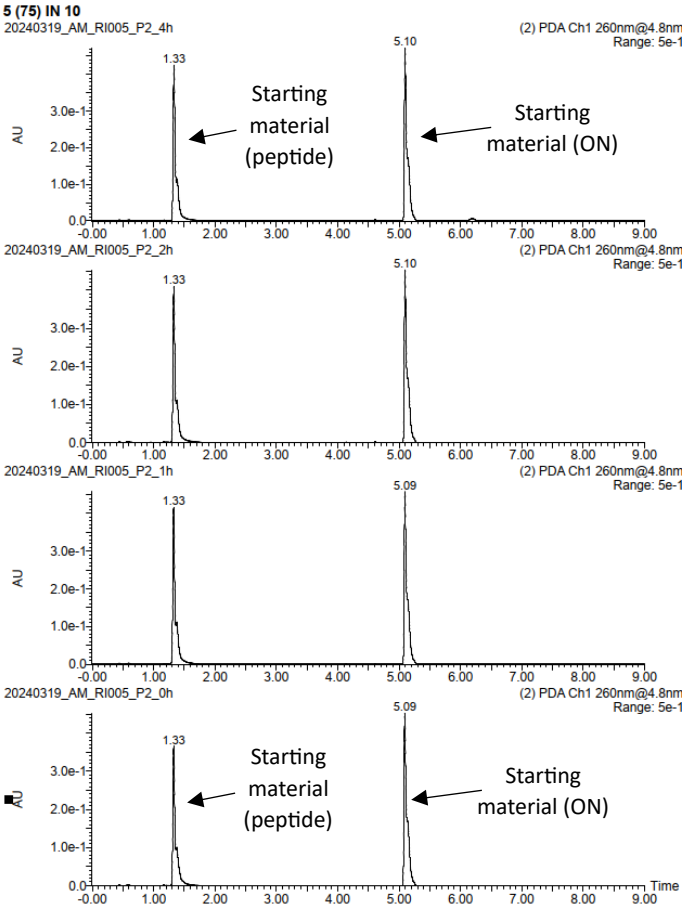


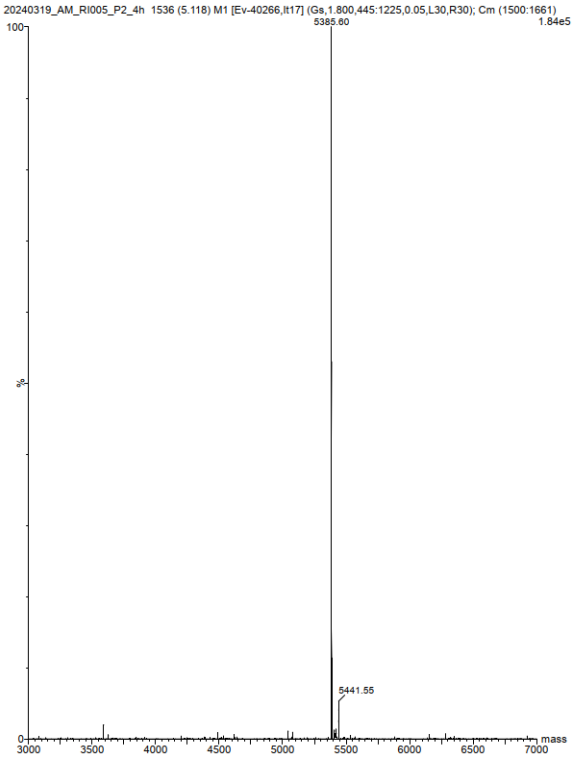
ON4-b-P1


5 (75) IN 10
20240320_AM_RI005_P1_22h
PDA Ch1 260nm@4.8nm
Range: 4e-1
peptide
Conjugated product
4.6
1.44
3.75
20240319_AM_RI005_P1_6h
(2) PDA Ch1 260nm@4.8nm
Range: 4e-1
1.45
4.62
3.76
5.10
20240319_AM_RI005_P1_4h
1.44
4.62
3.76
5.10
20240319_AM_RI005_P1_3h
1.45
4.61
5.09
3.75
20240319_AM_RI005_P1_2h
1.44
4.62
5.10
3.76
20240319_AM_RI005_P1_1h
1.45
5.10
4.62
3.76
20240319_AM_RI005_P1_0h
1.44
5.09
Starting material (peptide)
Starting material (ON)
AU
Time

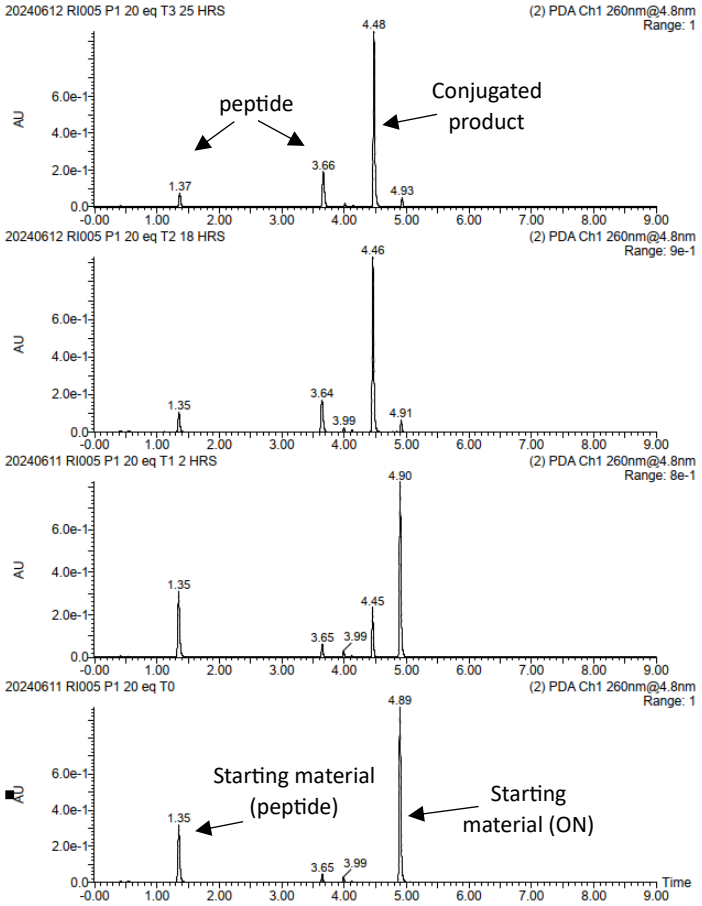

20240320_AM_RI005_P1_22h 1395 (4.648) M1 [Ev-37406,It22] (Gs,1.800,517:1250,0.05,L30,R30); Cm (1361:1469)
6127.30
3.18e5
6182.80
%
mass

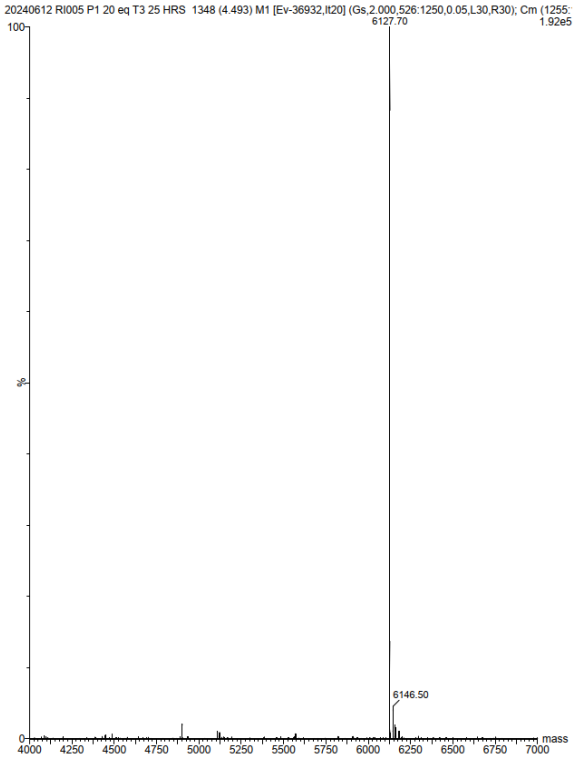
ON4-b (conjugation with P2)





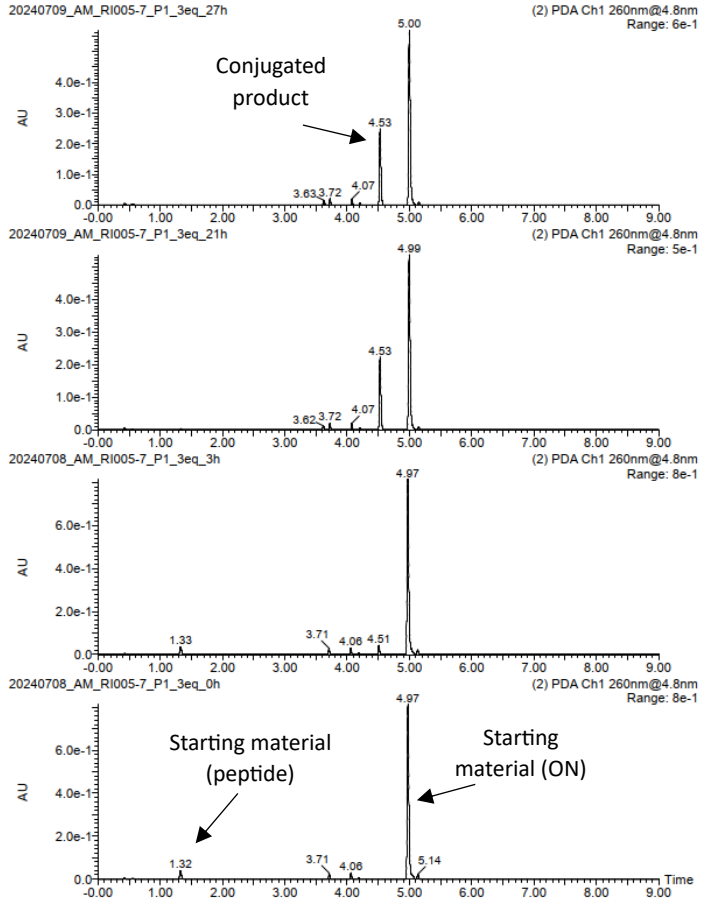
ON4-b-P1 – 20 eq experiment

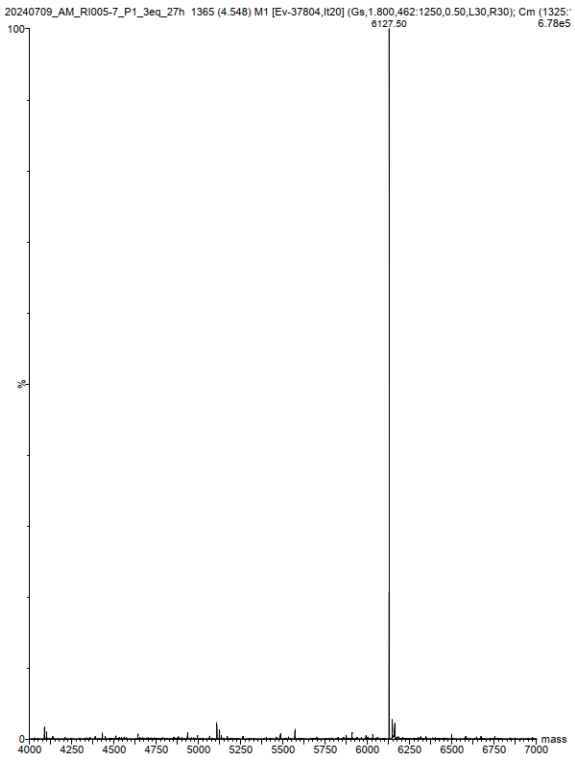




Estimated conversion[1]: 22%.

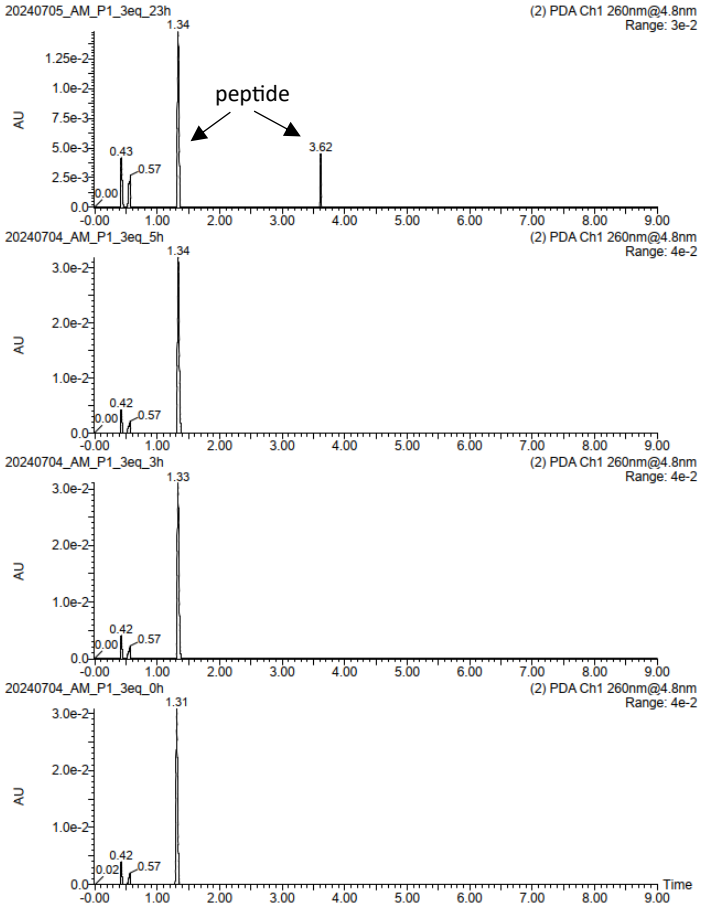
ON4-b-P1 – 3 eq experiment

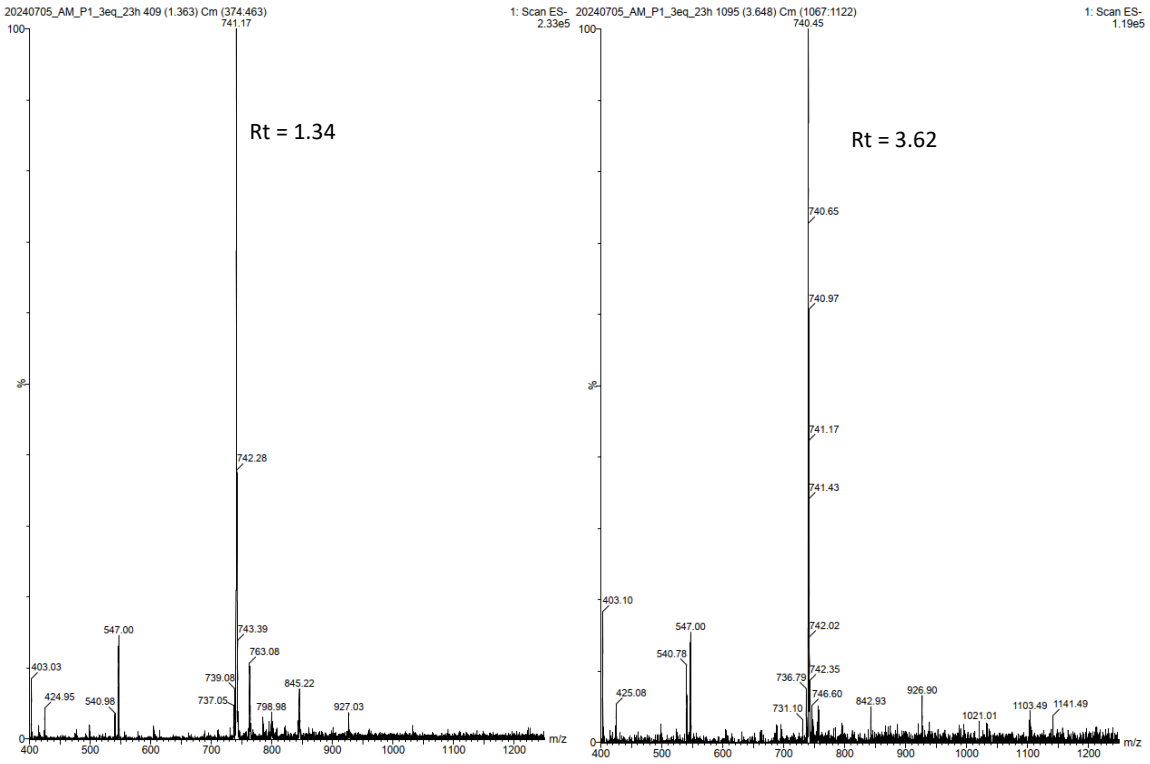




Estimated conversion[1]: 27%.

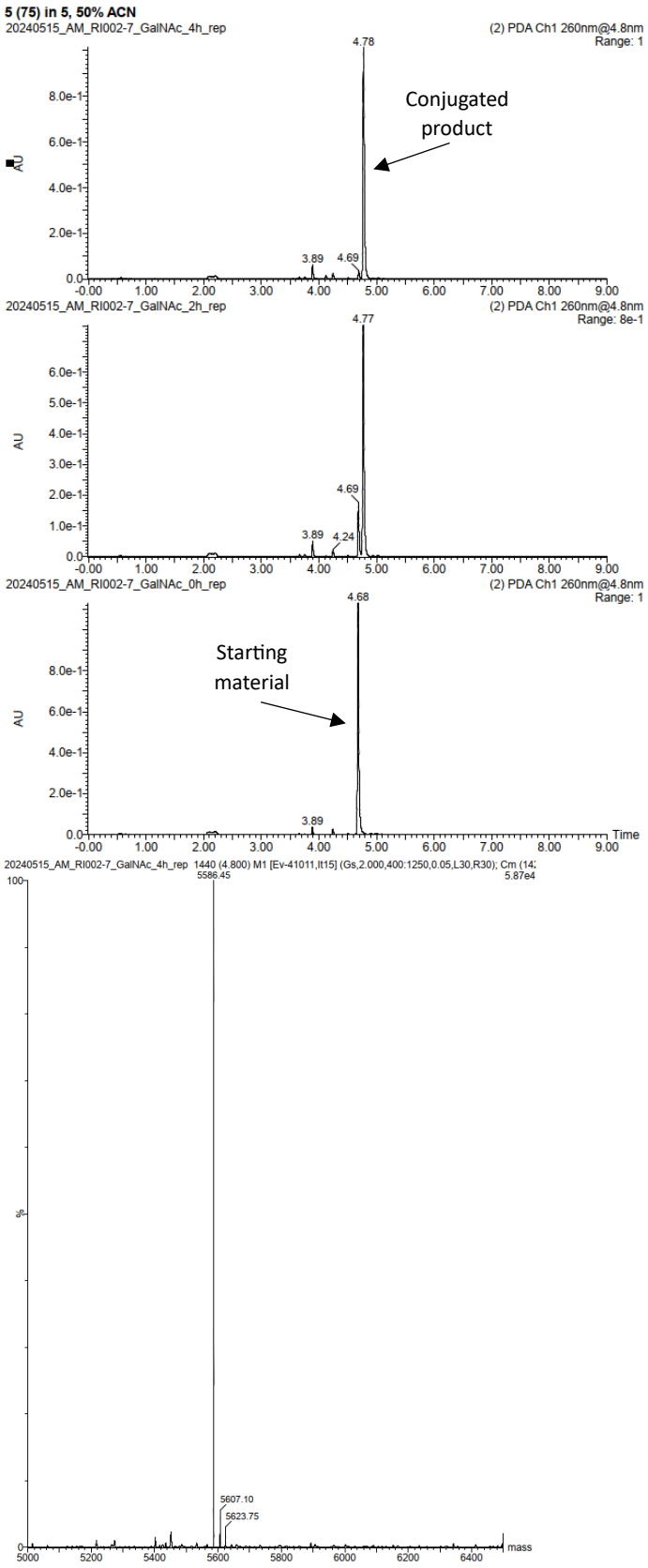
P1 – control experiment



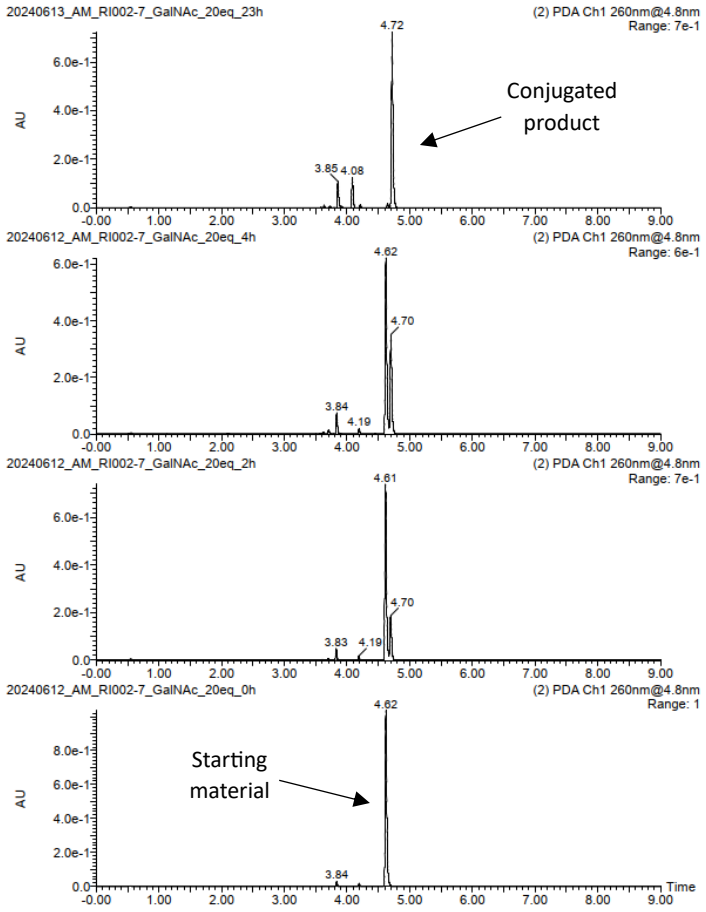


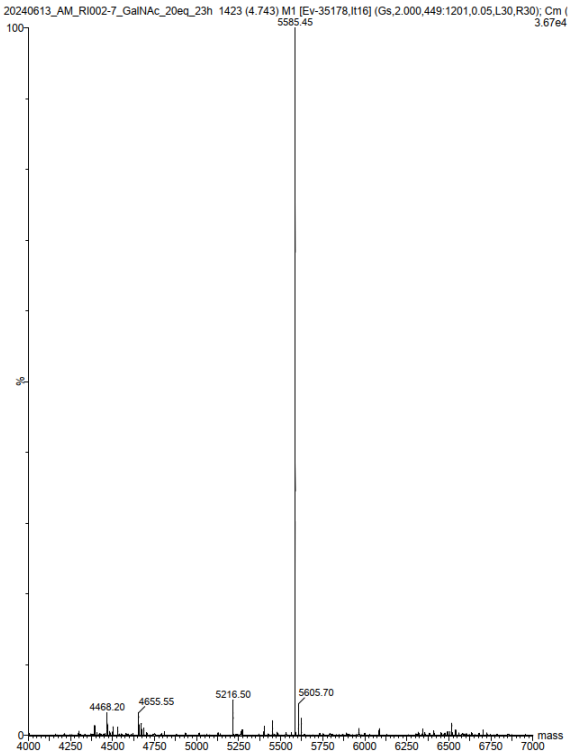
GalNAc conjugation

ON3-b-GalNAc



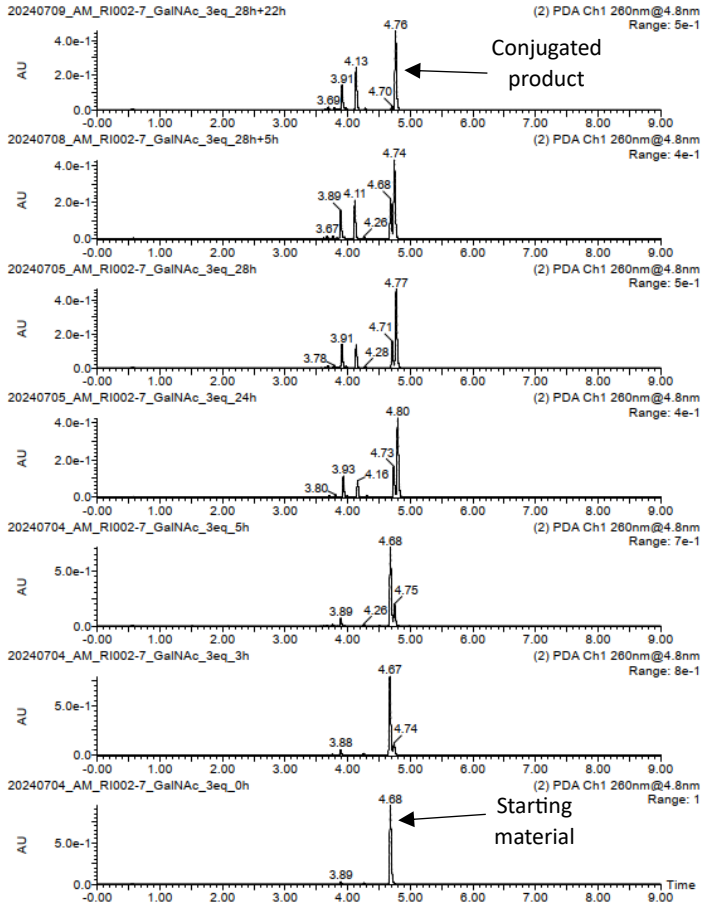
ON3-b-GalNAc – 20 eq experiment

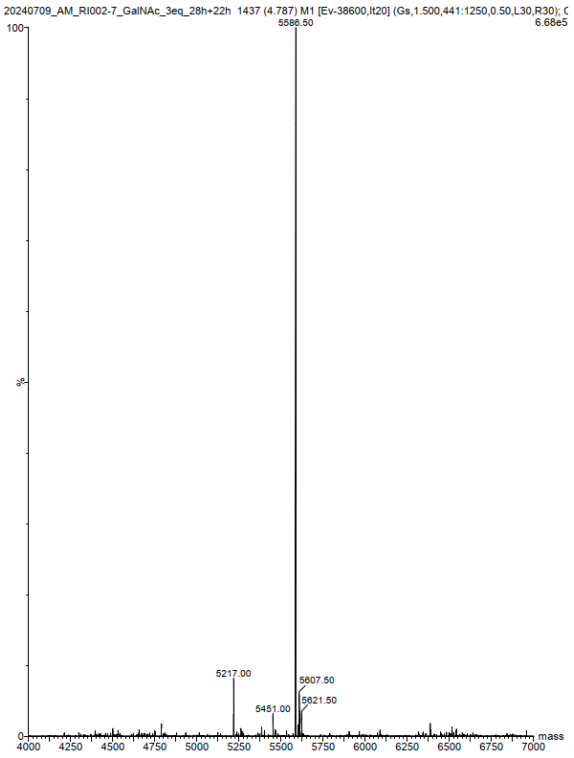




Estimated conversion[1]: 70%.

ON3-b-GalNAc – 3 eq experiment

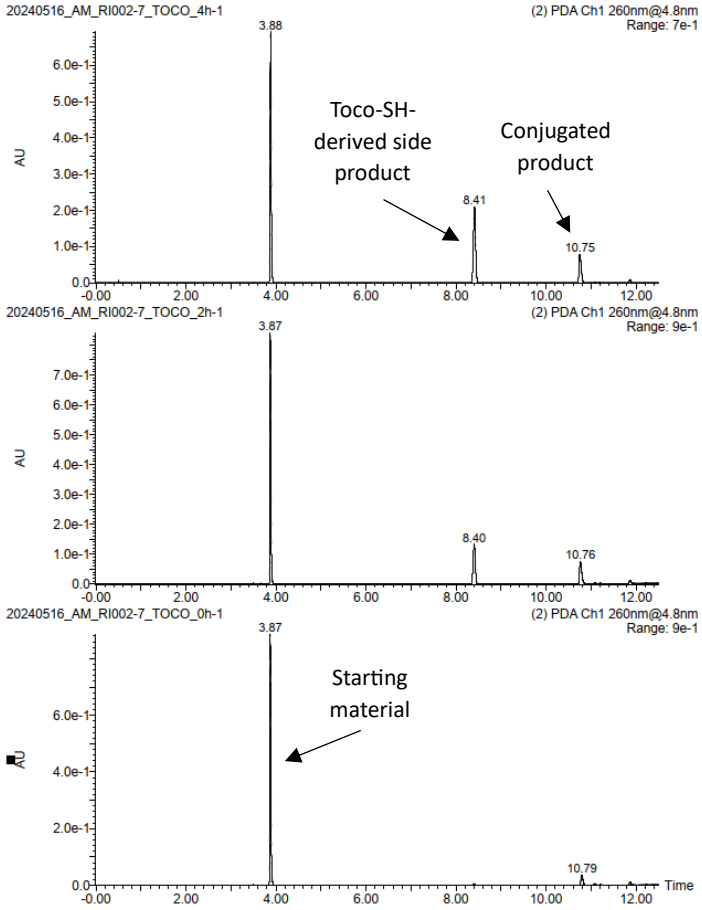


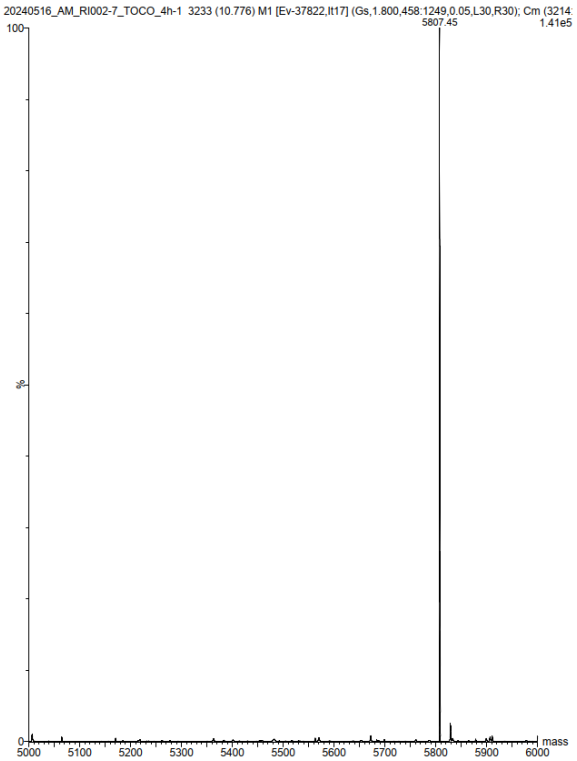


Estimated conversion[1]: 49%.

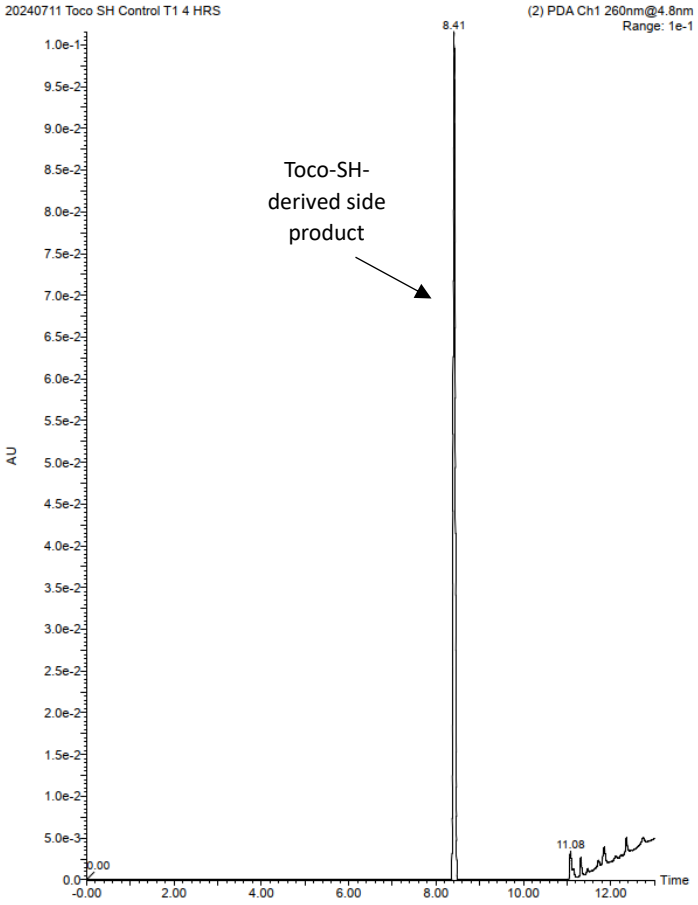
Lipid conjugation

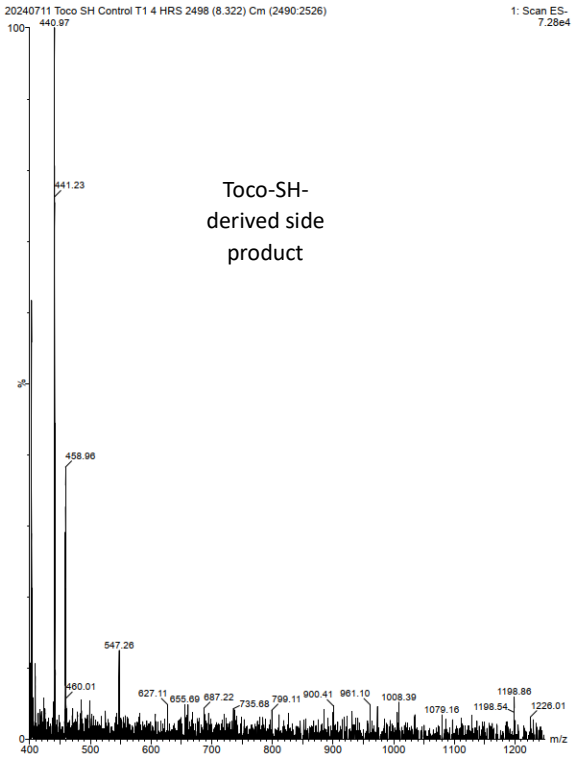
ON3-b-TF

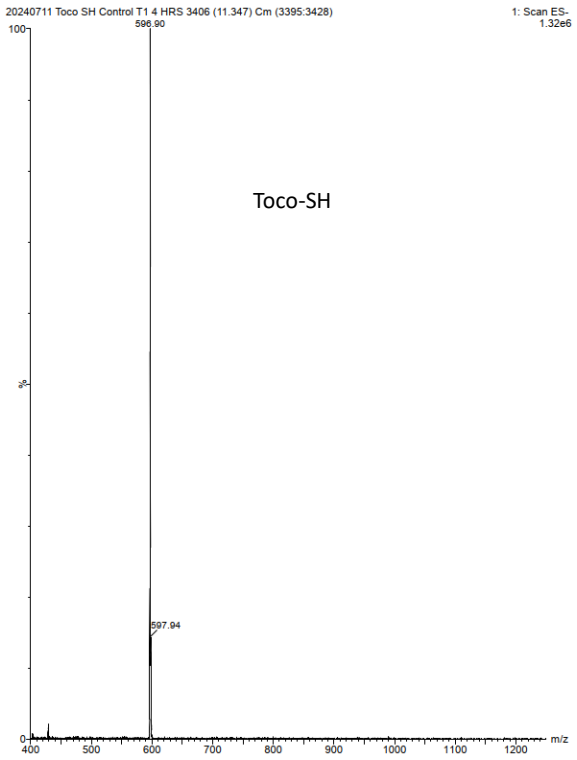




TF – control experiment



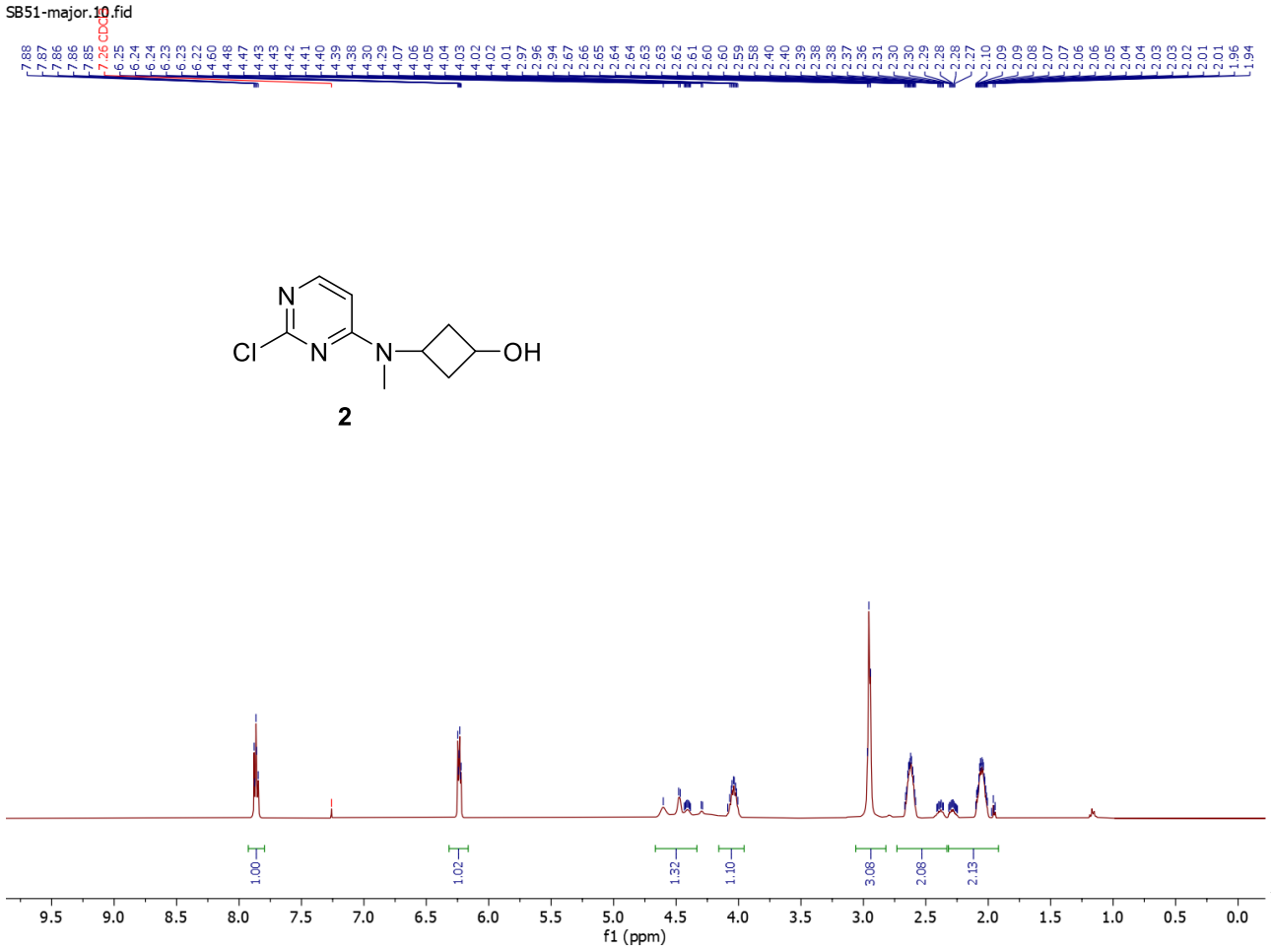




NMR spectra

3-((2-chloropyrimidin-4-yl)(methyl)amino)cyclobutan-1-ol (Cis/Trans Mixture) (2)

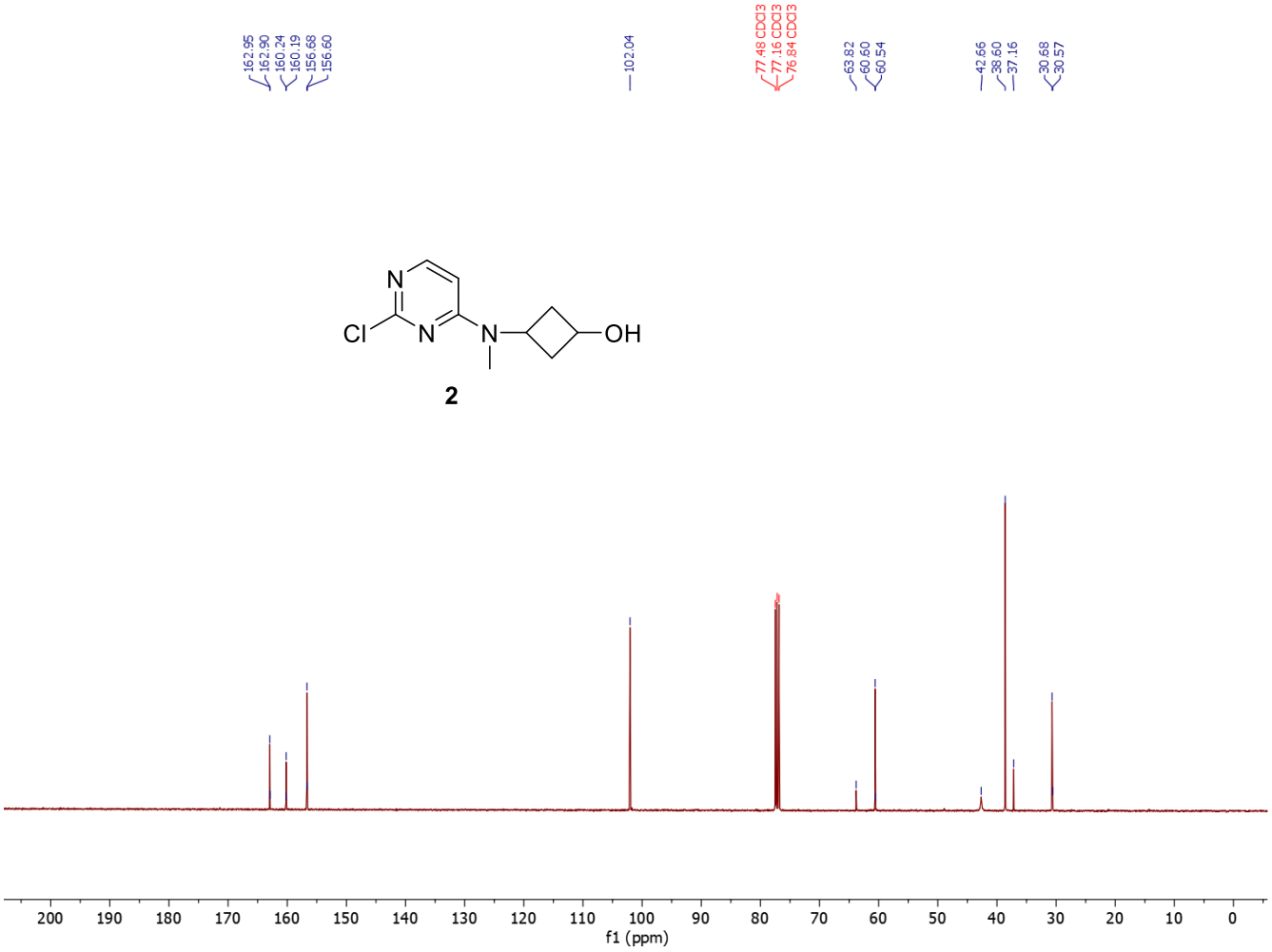
^{1}H NMR (400 MHz, $CDCl_3$)



2

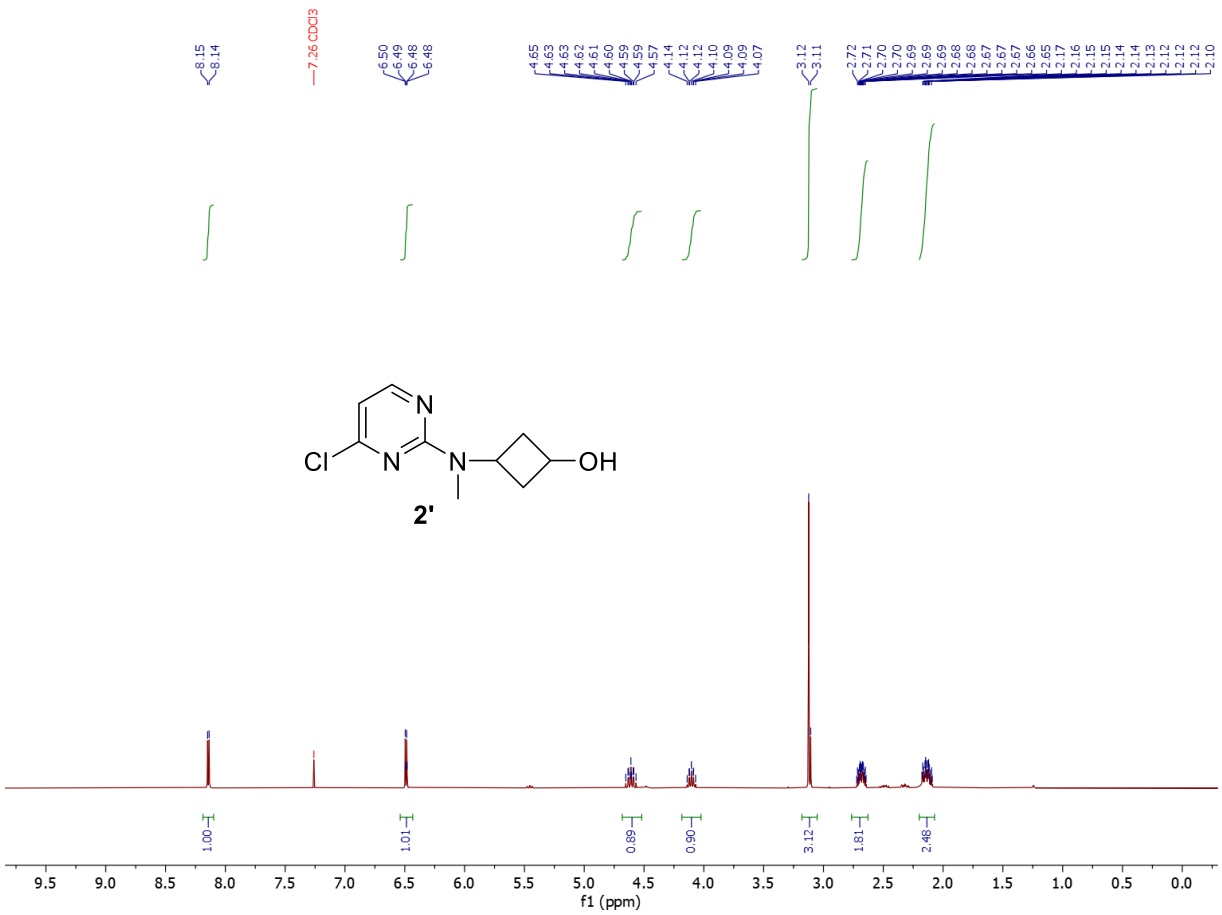
3-((2-chloropyrimidin-4-yl)(methyl)amino)cyclobutan-1-ol (Cis/Trans Mixture) (2)

^{13}C NMR (101 MHz, $CDCl_3$)



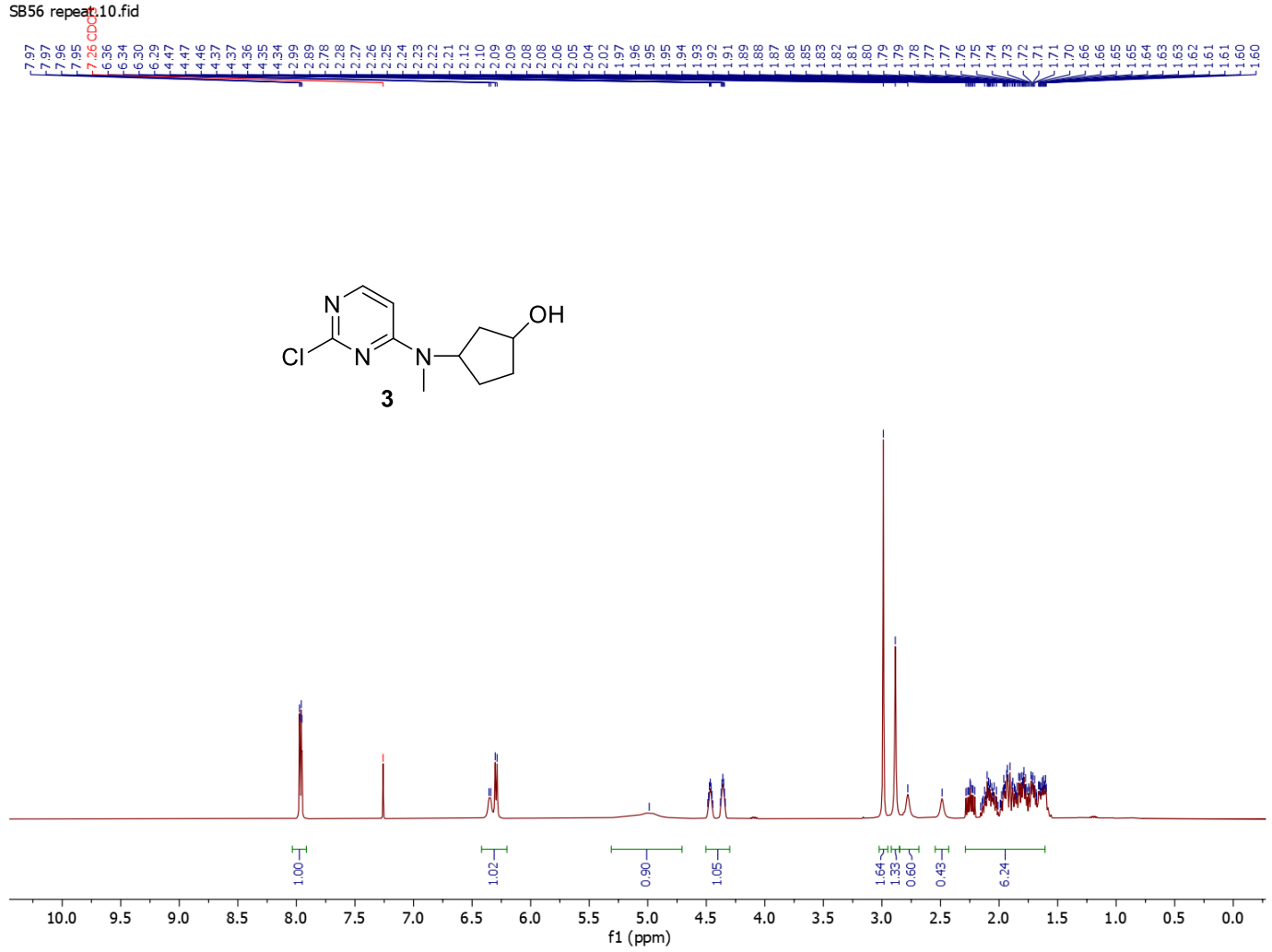
3-((4-chloropyrimidin-2-yl)(methyl)amino)cyclobutan-1-ol (Cis/Trans Mixture) (2')

^{1}H NMR (400 MHz, $CDCl_3$)



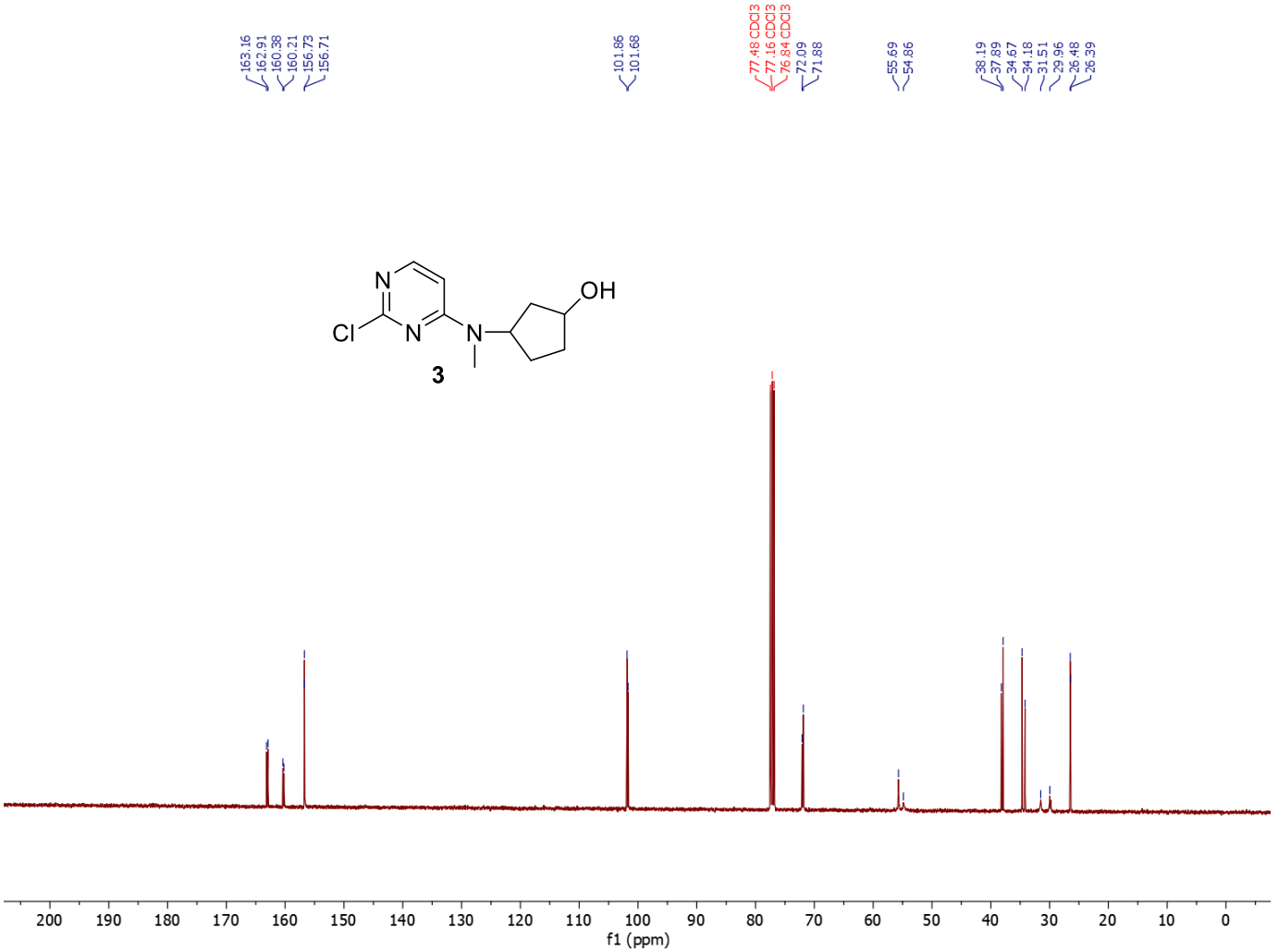
3-((2-chloropyrimidin-4-yl)(methyl)amino)cyclopentan-1-ol (Cis/Trans Mixture) (3)

^{1}H NMR (400 MHz, $CDCl_3$)



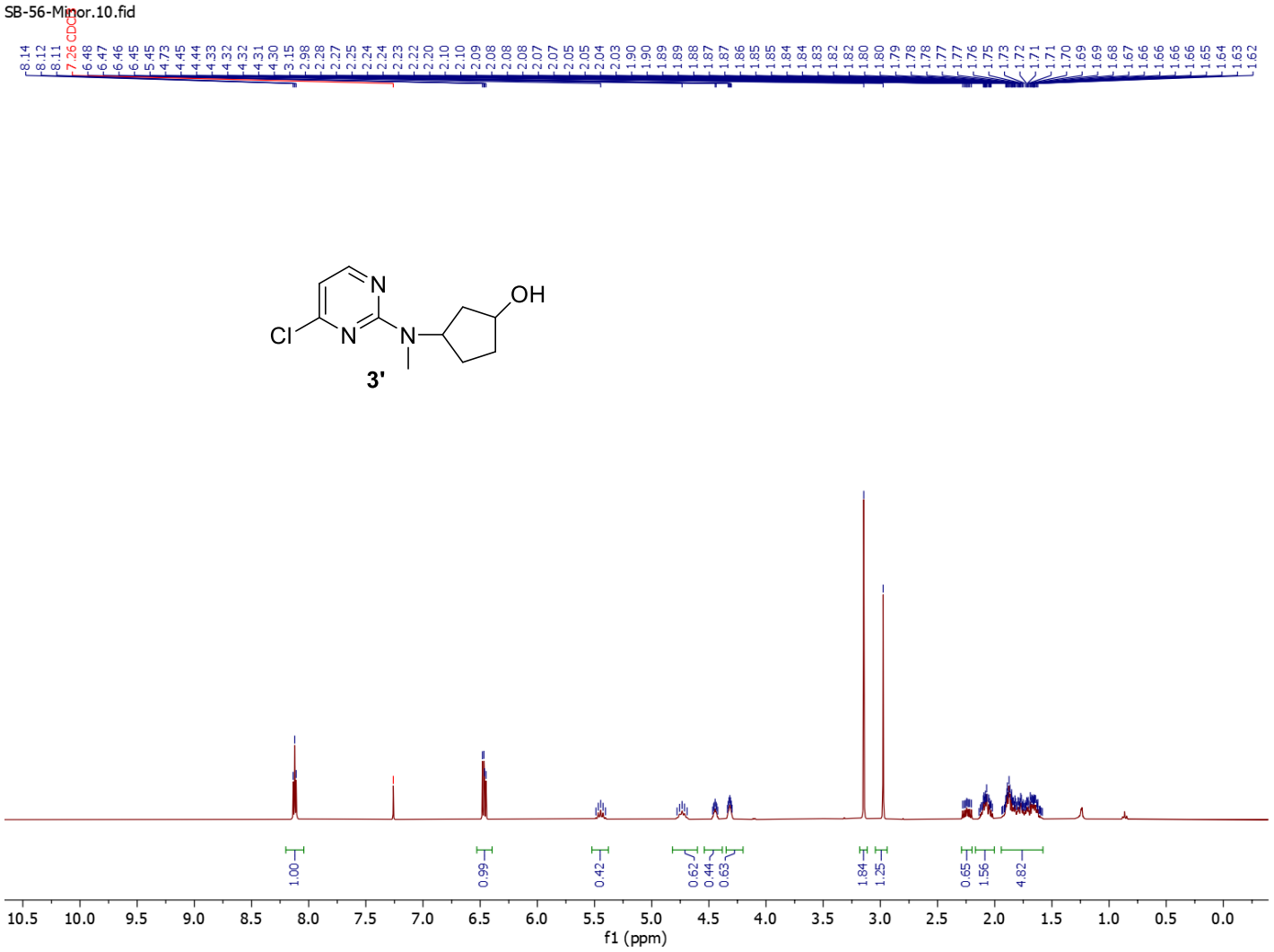
3-((2-chloropyrimidin-4-yl)(methyl)amino)cyclopentan-1-ol (Cis/Trans Mixture) (3)

^{13}C NMR (101 MHz, $CDCl_3$)



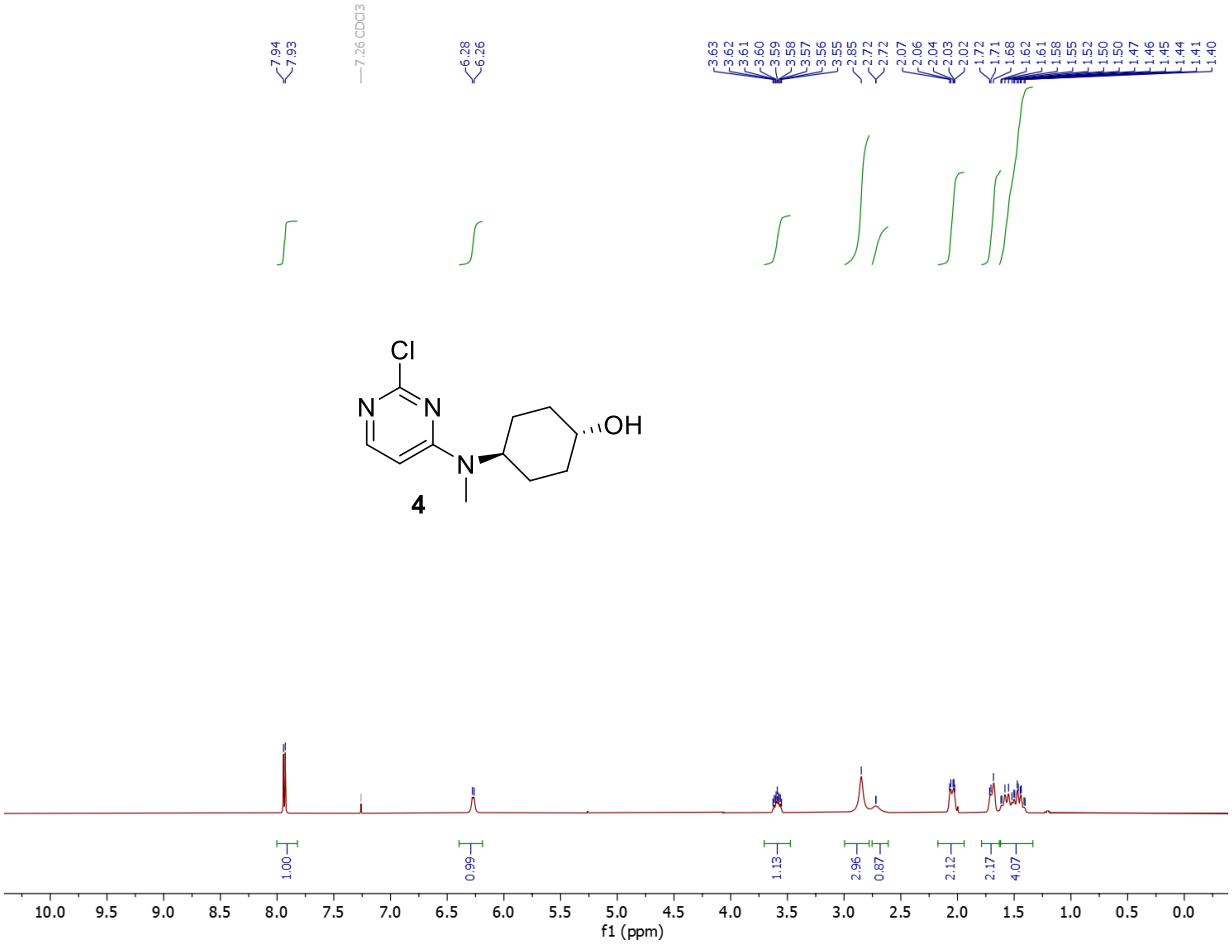
3-((4-chloropyrimidin-2-yl)(methyl)amino)cyclopentan-1-ol (Cis/Trans Mixture) (3')

^{1}H NMR (400 MHz, $CDCl_3$)



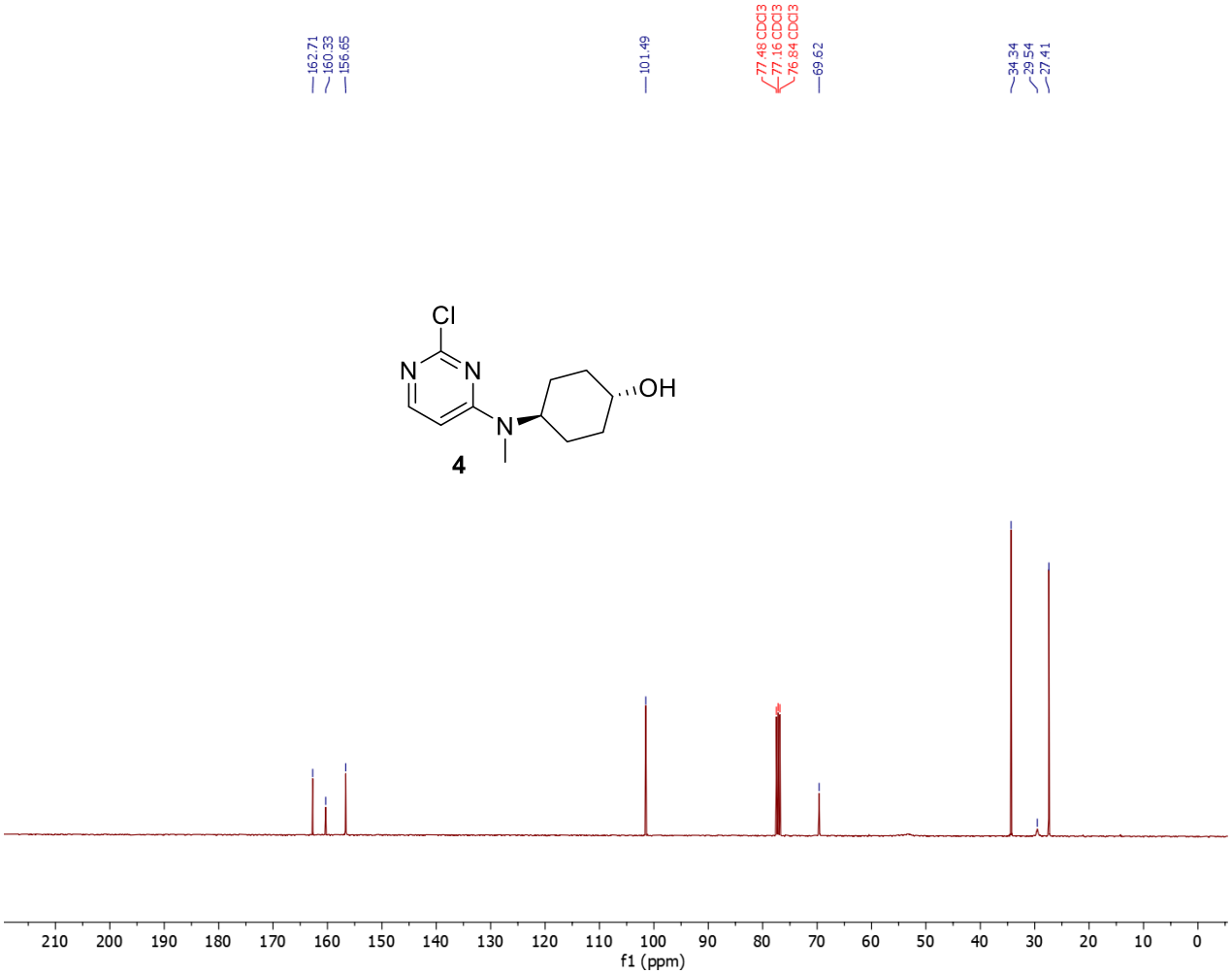
(1*R*,4*R*)-4-((2-chloropyrimidin-4-yl)(methyl)amino)cyclohexan-1-ol (4)

^{1}H NMR (400 MHz, $CDCl_3$)



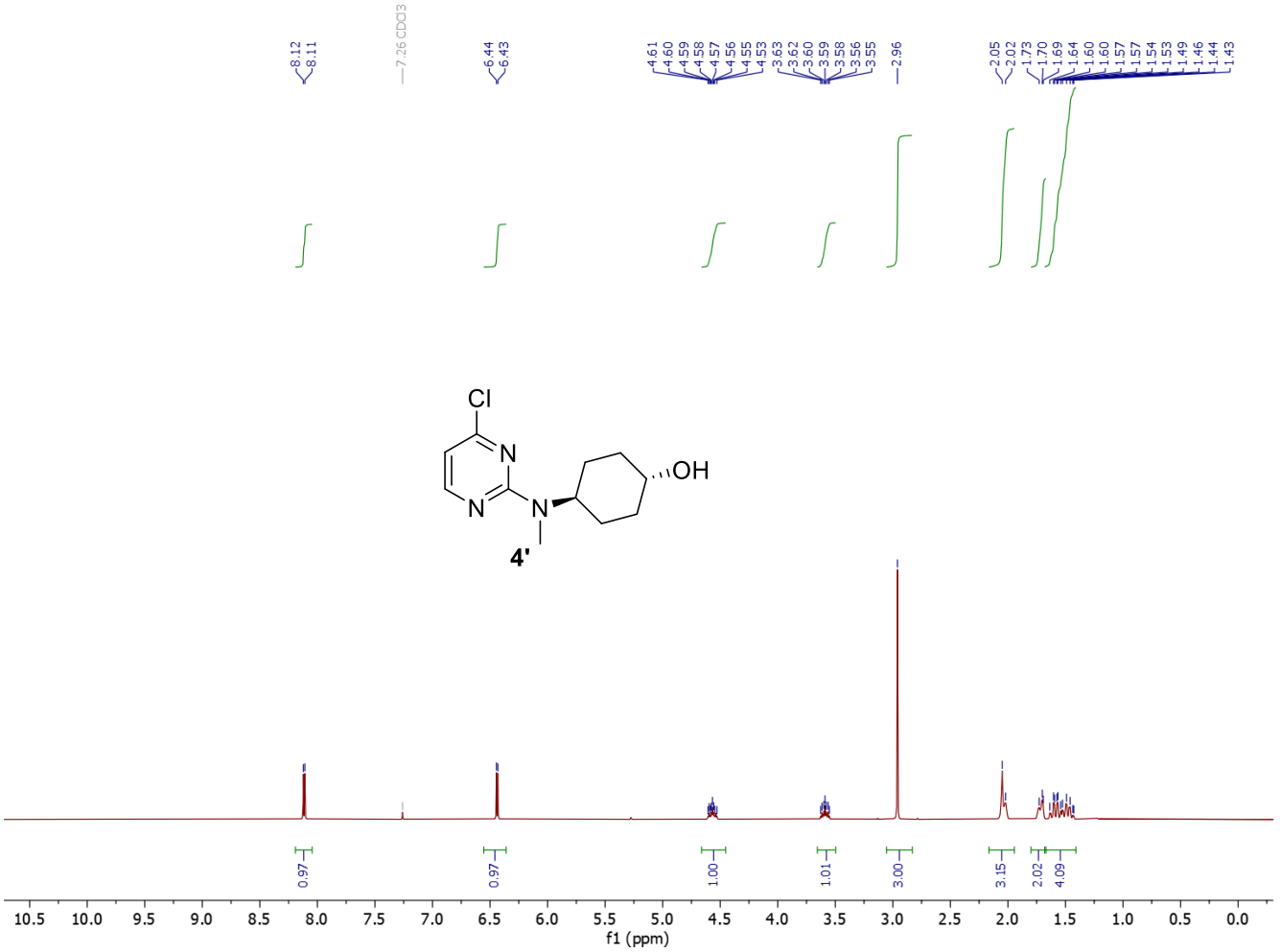
(1*R*,4*R*)-4-((2-chloropyrimidin-4-yl)(methyl)amino)cyclohexan-1-ol (4)

^{13}C NMR (101 MHz, $CDCl_3$)



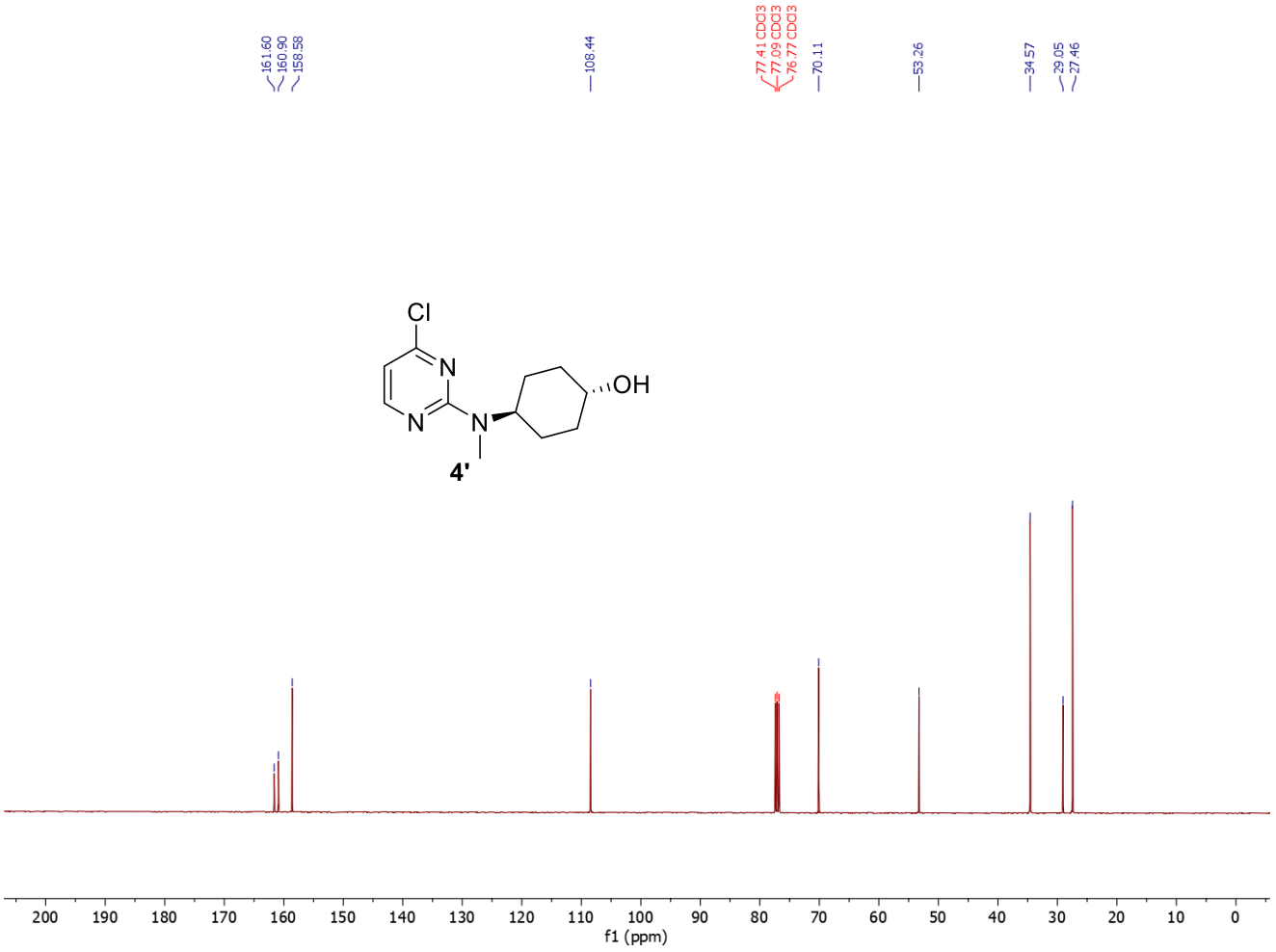
(1*R*,4*R*)-4-((4-chloropyrimidin-2-yl)(methyl)amino)cyclohexan-1-ol (4')

^{1}H NMR (400 MHz, $CDCl_3$)



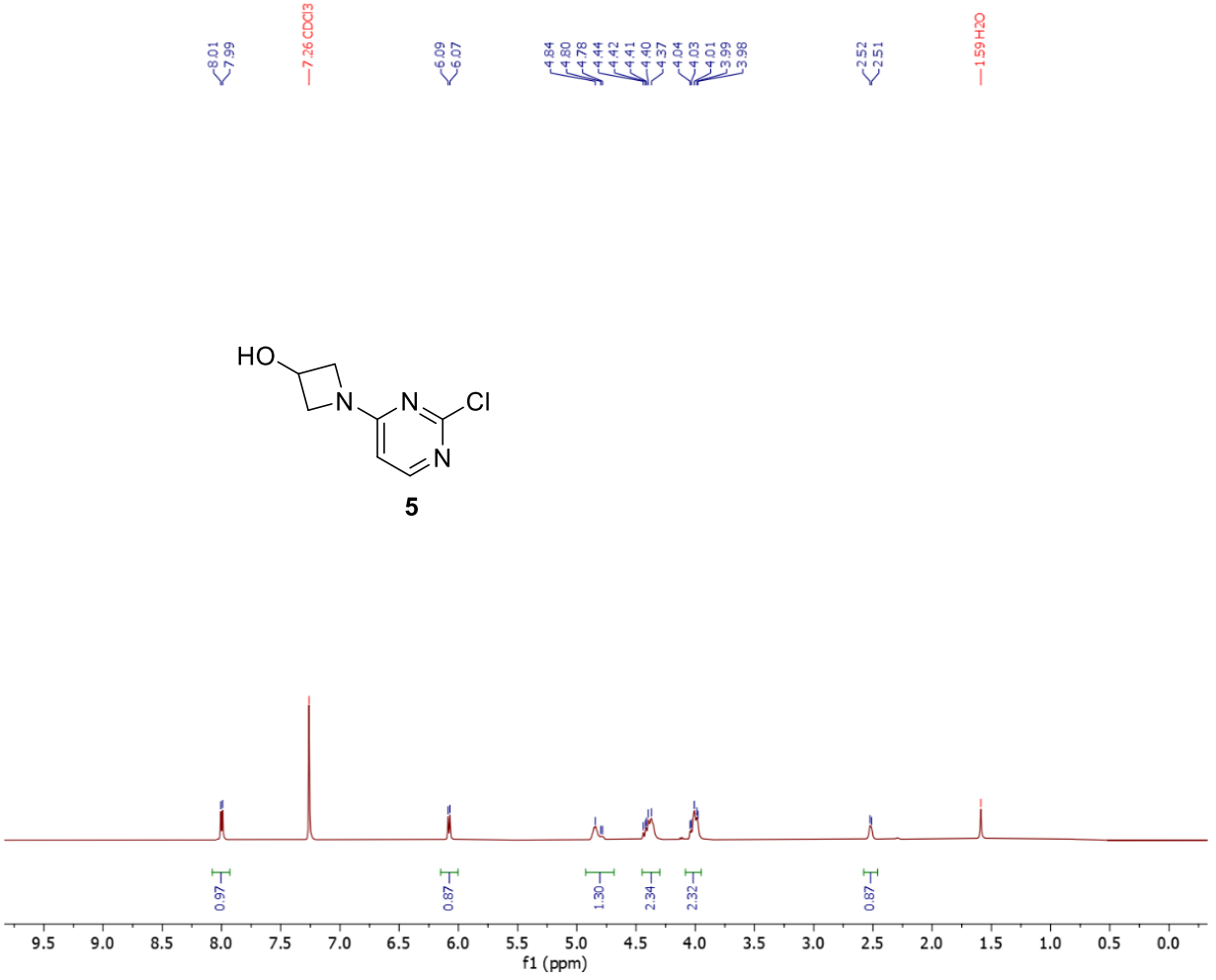
(1*R*,4*R*)-4-((4-chloropyrimidin-2-yl)(methyl)amino)cyclohexan-1-ol (4')

^{13}C NMR (101 MHz, $CDCl_3$)



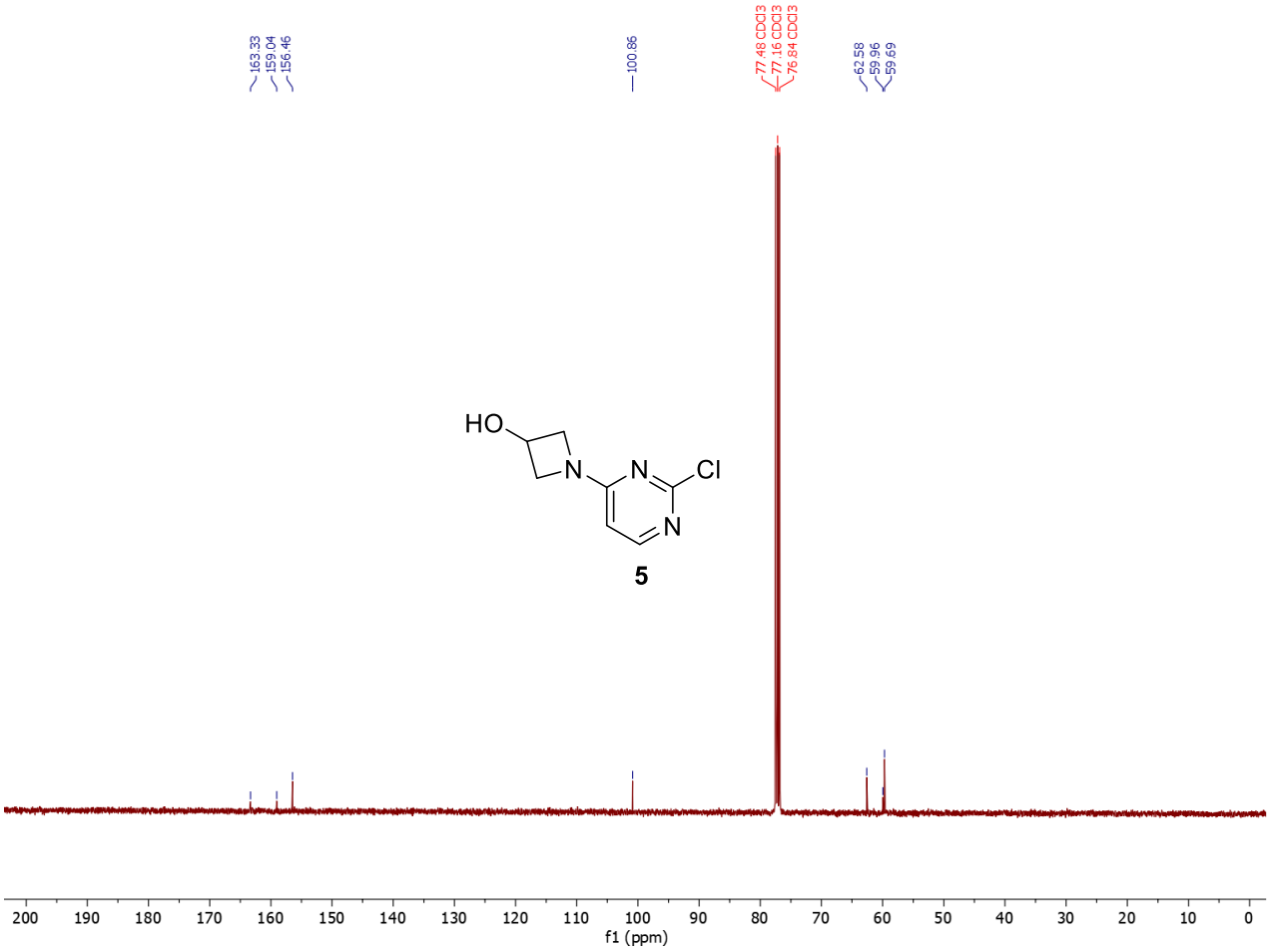
1-(2-chloropyrimidin-4-yl)azetidin-3-ol (5)

^{1}H NMR (400 MHz, $CDCl_3$)



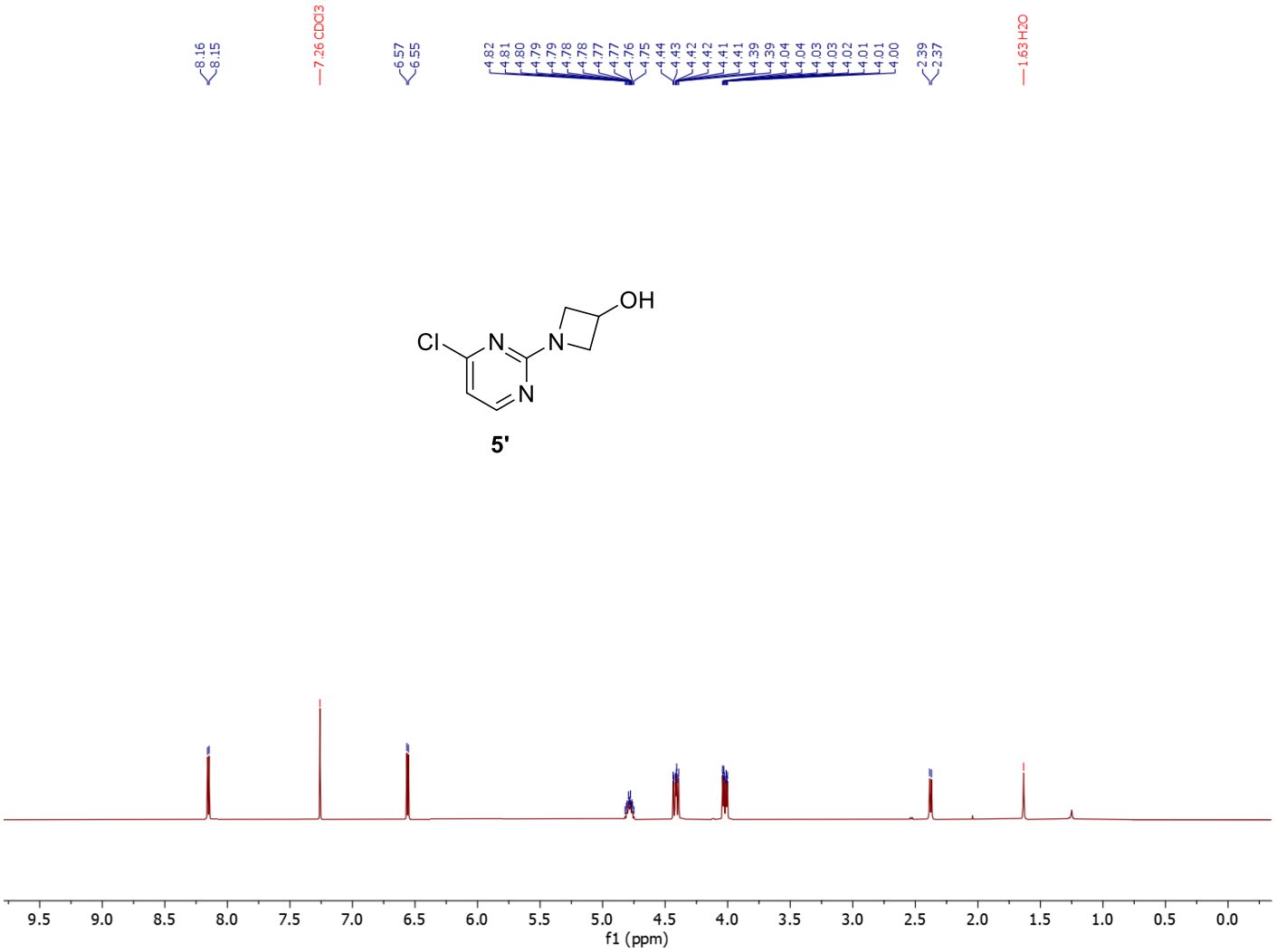
1-(2-chloropyrimidin-4-yl)azetidin-3-ol (5)

^{13}C NMR (101 MHz, $CDCl_3$)



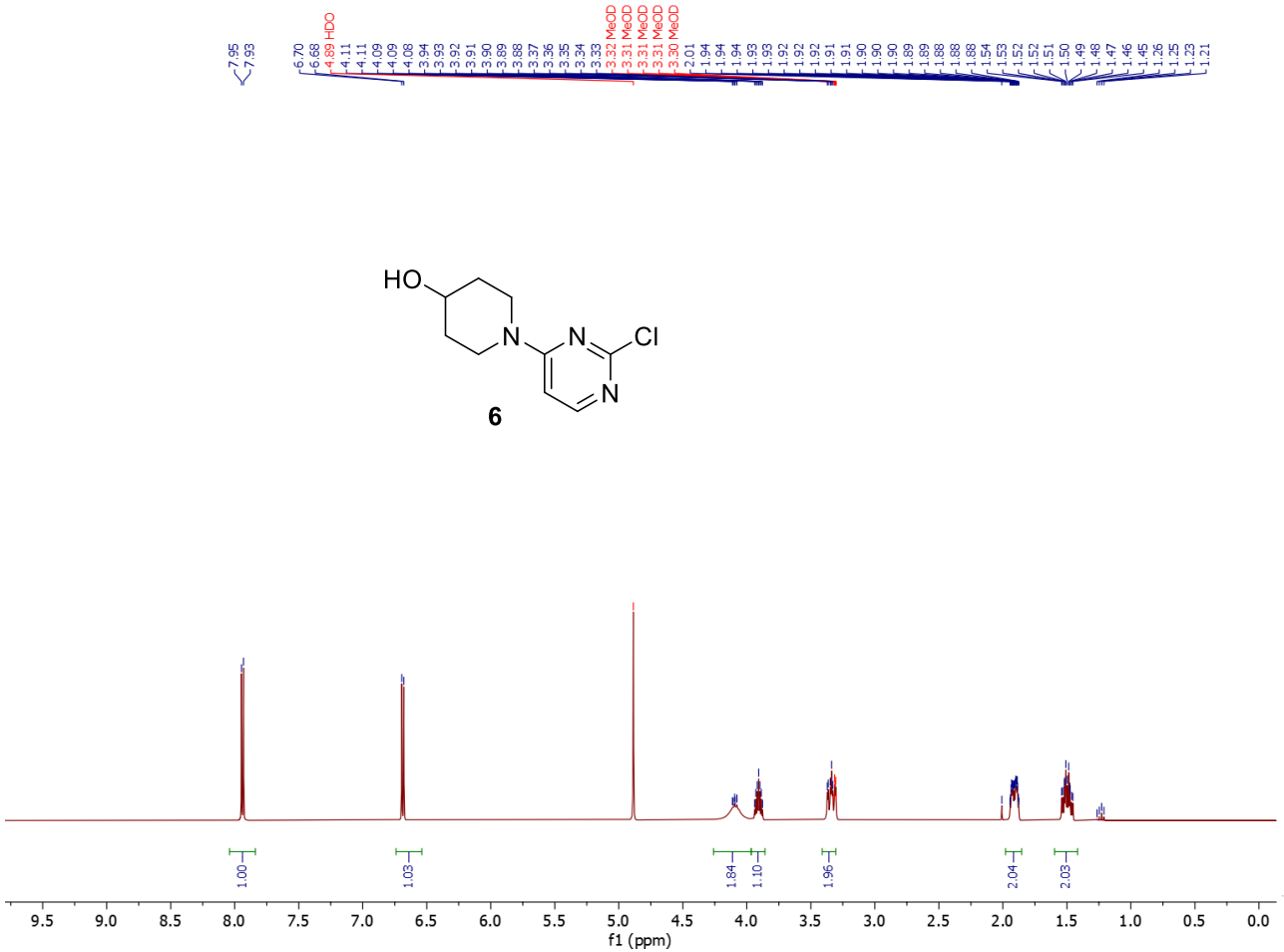
1-(4-chloropyrimidin-2-yl)azetidin-3-ol (5')

^{1}H NMR (400 MHz, $CDCl_3$)



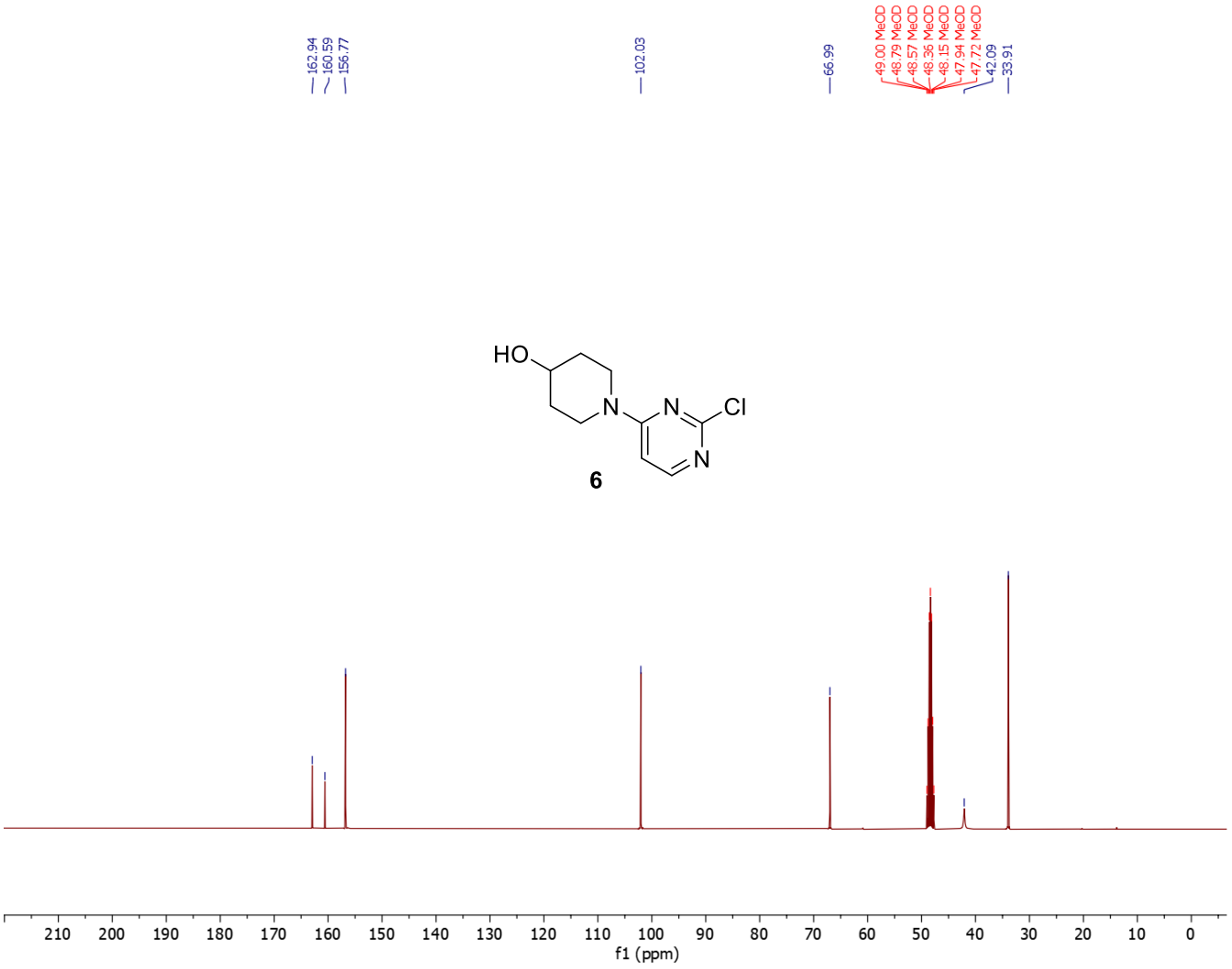
1-(2-vinylpyrimidin-4-yl)piperidin-4-ol (6)

^{1}H NMR (400 MHz, Methanol-d4)



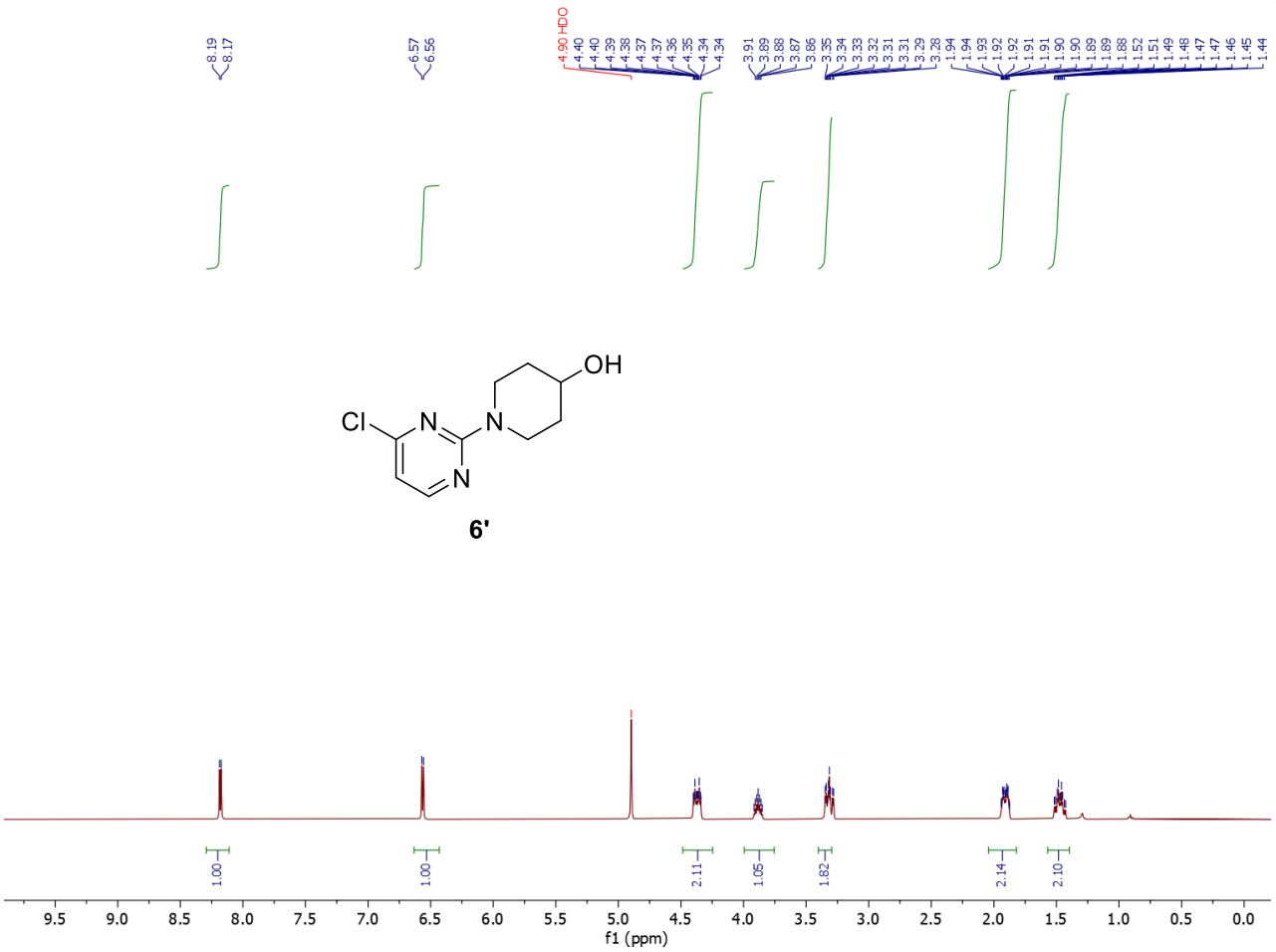
1-(2-vinylpyrimidin-4-yl)piperidin-4-ol (6)

^{13}C NMR (101 MHz, Methanol-d4)



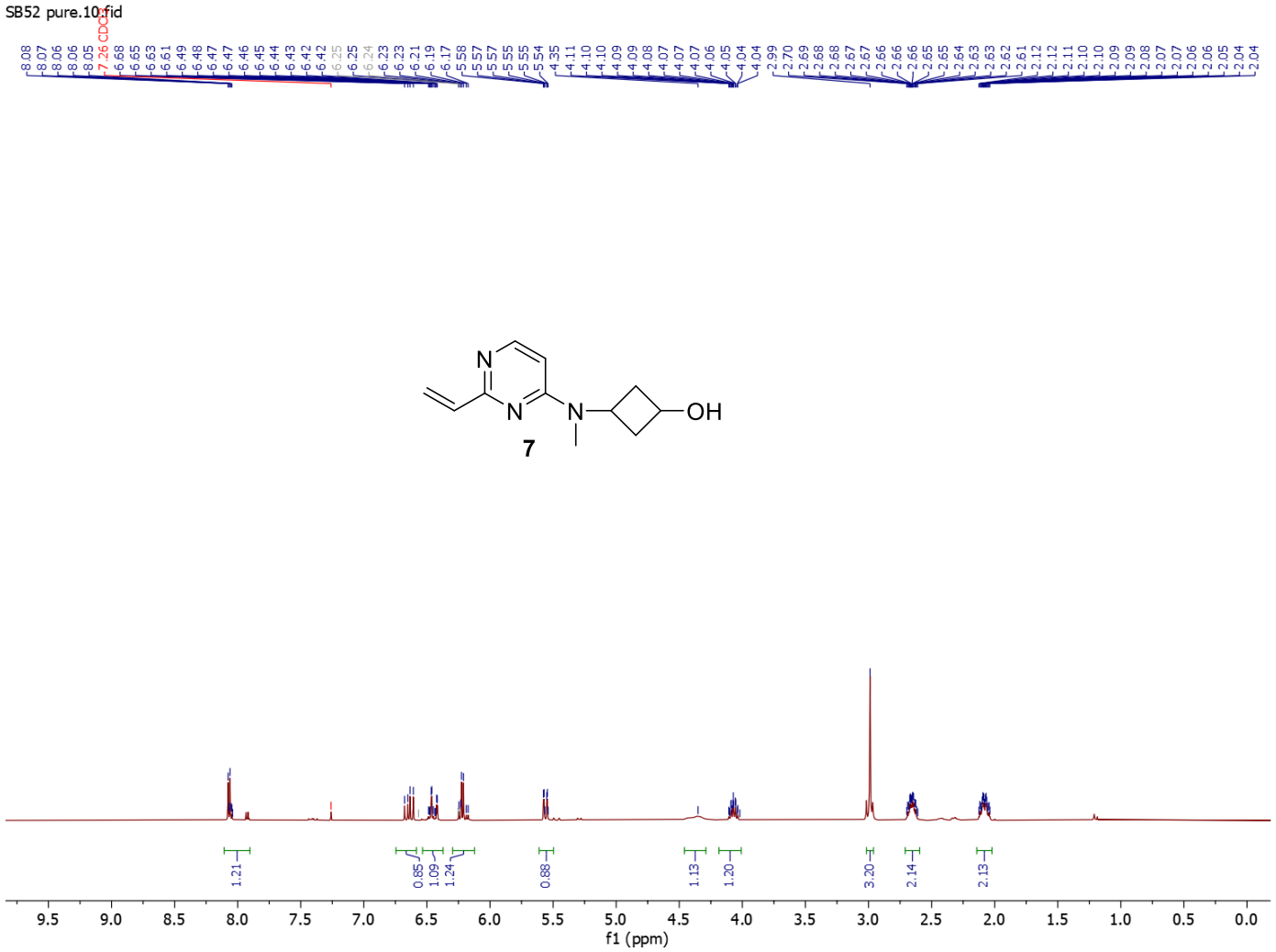
1-(4-chloropyrimidin-2-yl)piperidin-4-ol (6')

^{1}H NMR (400 MHz, Methanol-d4)



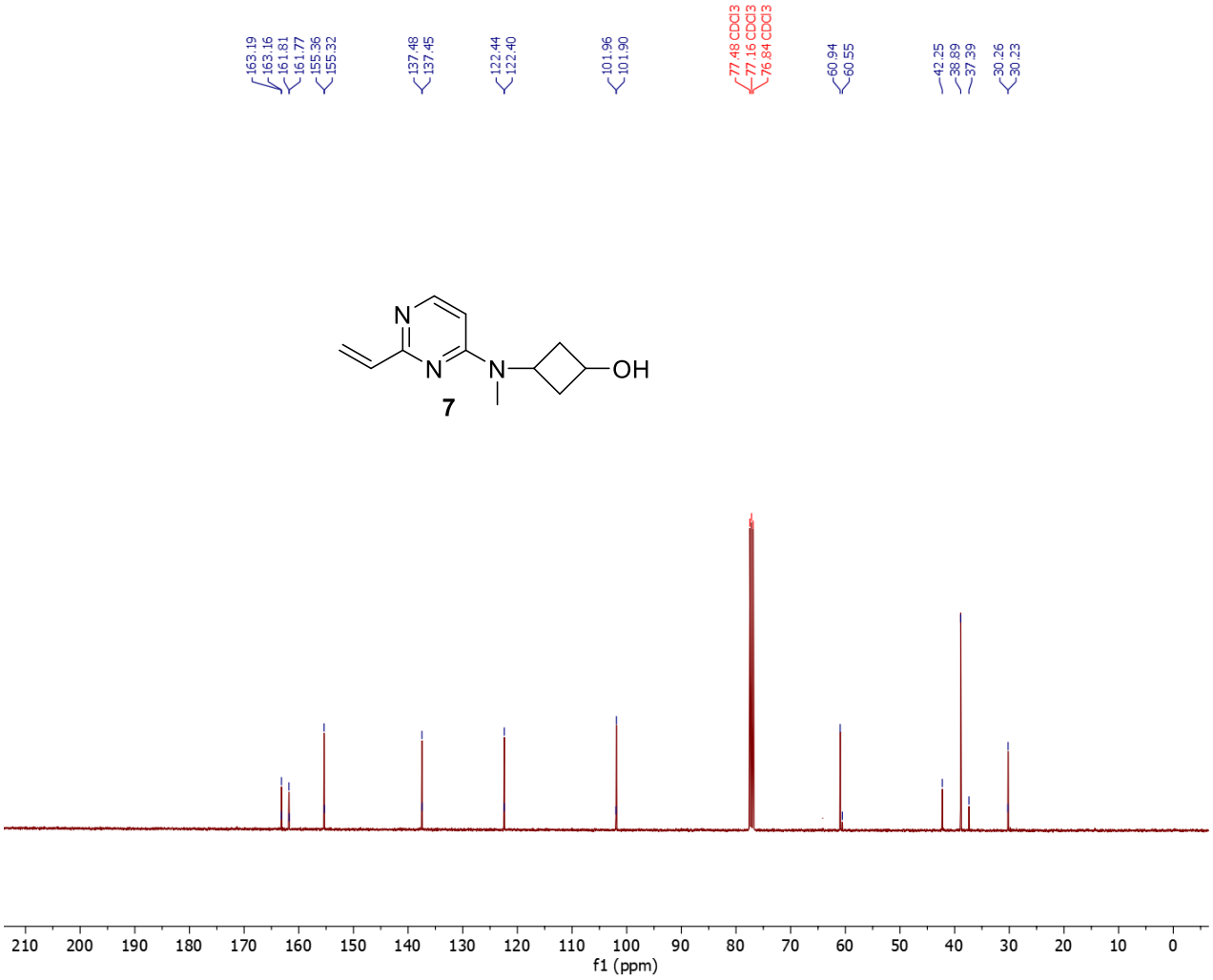
3-(methyl(2-vinylpyrimidin-4-yl)amino)cyclobutan-1-ol (Cis/Trans Mixture) (7)

^{1}H NMR (400 MHz, Methanol-d4)



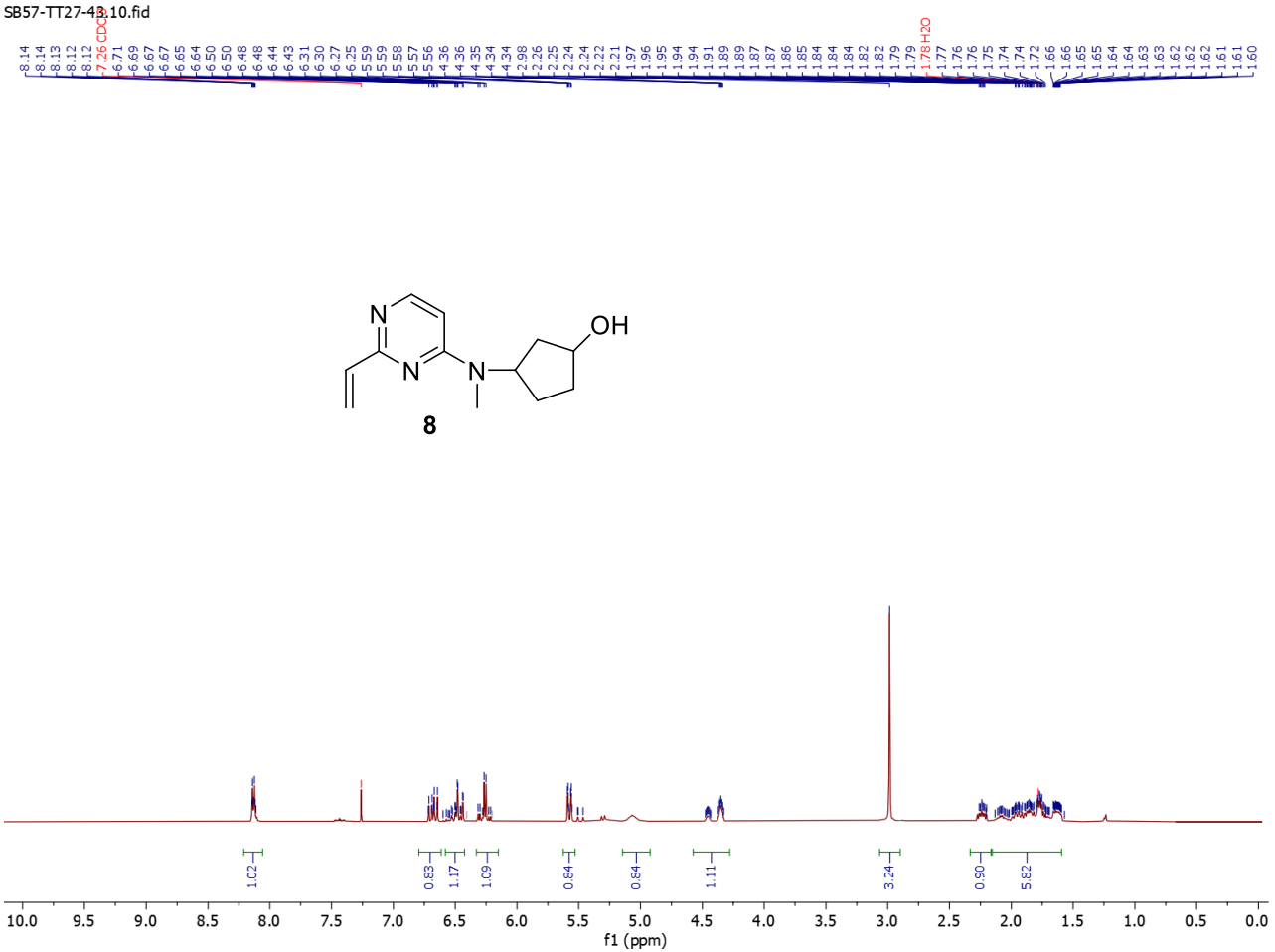
3-(methyl(2-vinylpyrimidin-4-yl)amino)cyclobutan-1-ol (Cis/Trans Mixture) (7)

^{13}C NMR (101 MHz, Methanol-d4)



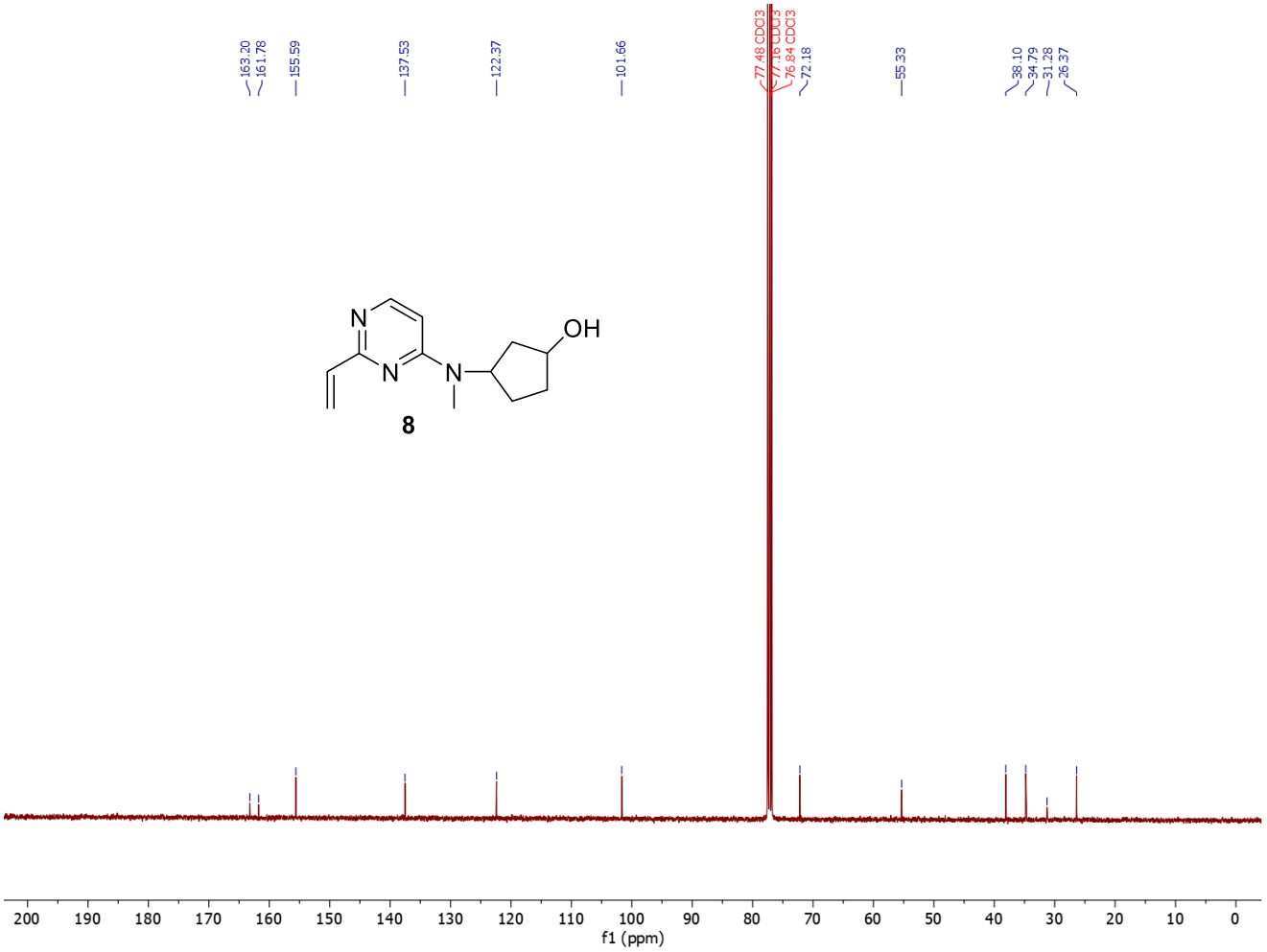
3-(methyl(2-vinylpyrimidin-4-yl)amino)cyclopentan-1-ol (Cis/Trans Mixture) (8)

^{1}H NMR (400 MHz, $CDCl_3$)



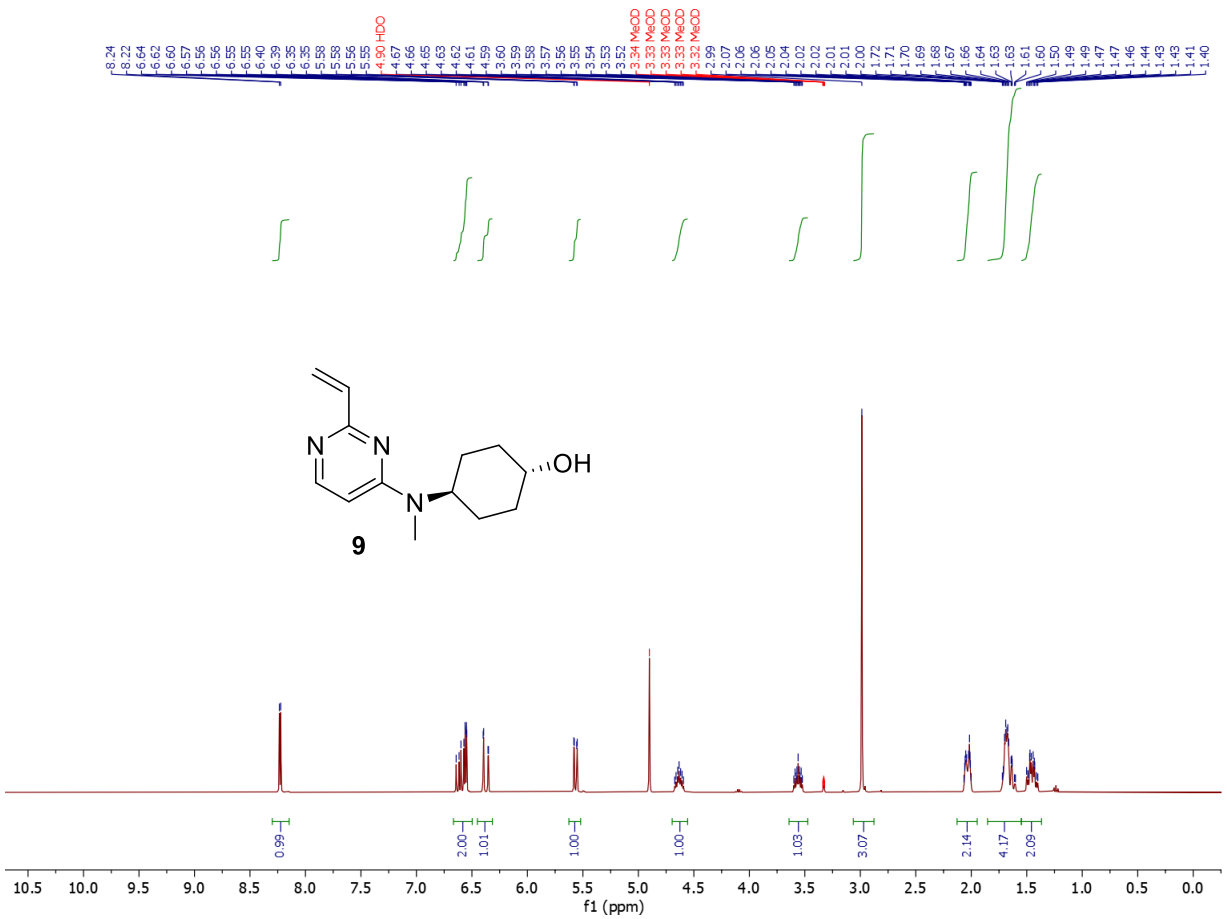
3-(methyl(2-vinylpyrimidin-4-yl)amino)cyclopentan-1-ol (Cis/Trans Mixture) (8)

^{13}C NMR (400 MHz, $CDCl_3$)



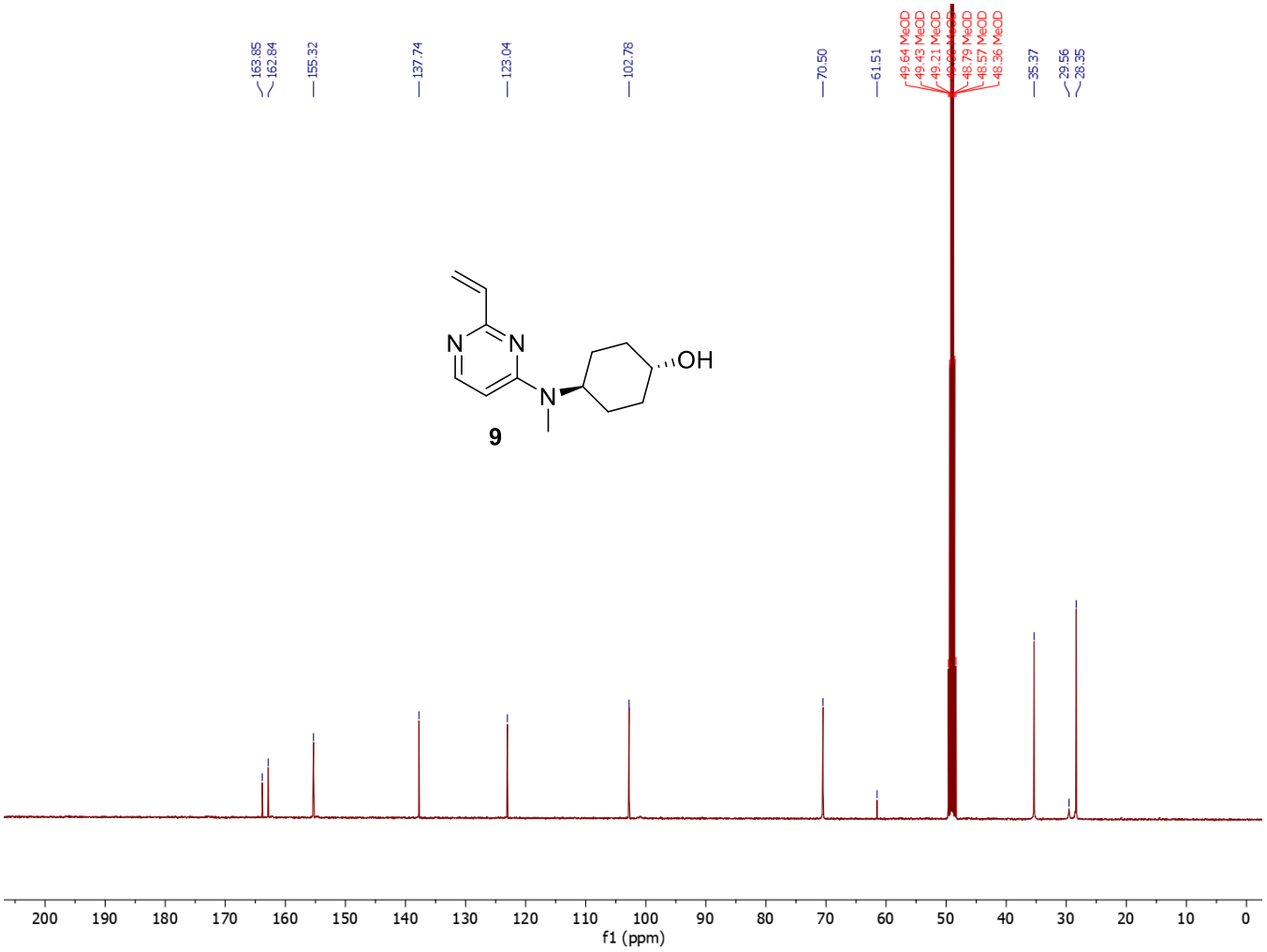
(1*R*,4*R*)-4-(methyl(2-vinylpyrimidin-4-yl)amino)cyclohexan-1-ol (9)

^{1}H NMR (400 MHz, Methanol-d4)



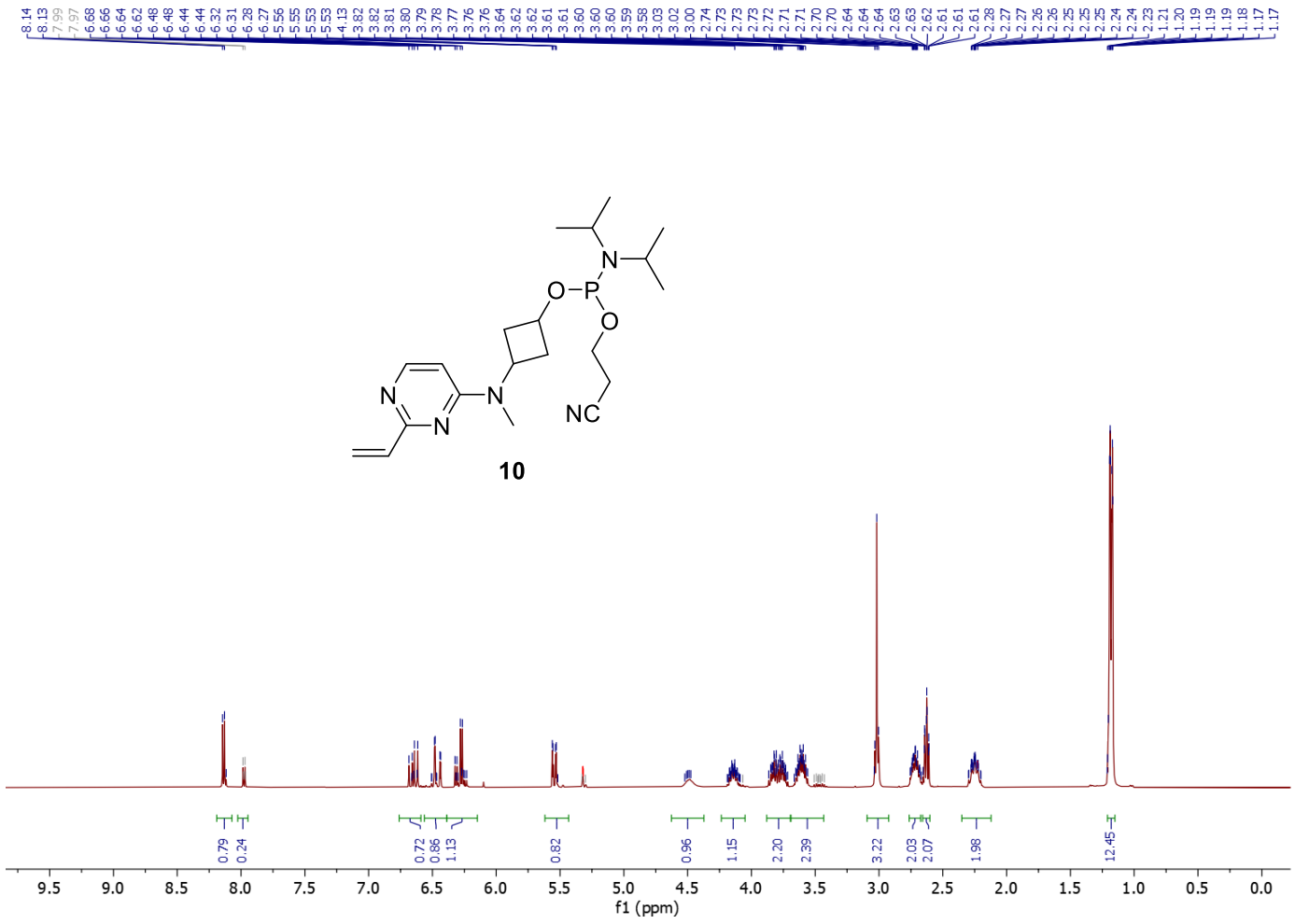
(1*R*,4*R*)-4-(methyl(2-vinylpyrimidin-4-yl)amino)cyclohexan-1-ol (9)

^{13}C NMR (101 MHz, Methanol-d4)



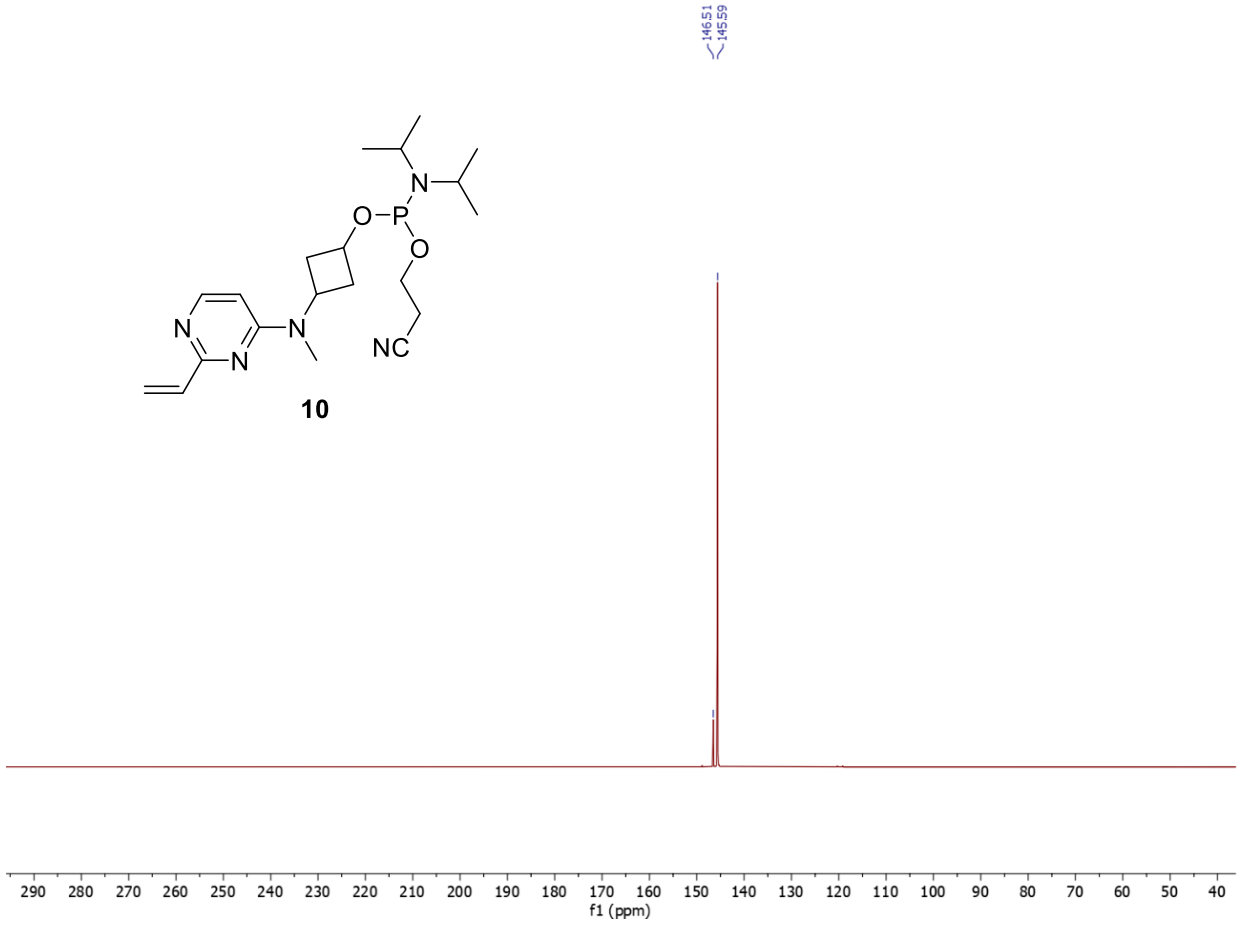
2-cyanoethyl (3-(methyl(2-vinylpyrimidin-4-yl)amino)cyclobutyl) diisopropylphosphoramidite (Mixture of Diastereoisomers) (10)

^{1}H NMR (400 MHz, CD_2Cl_2)



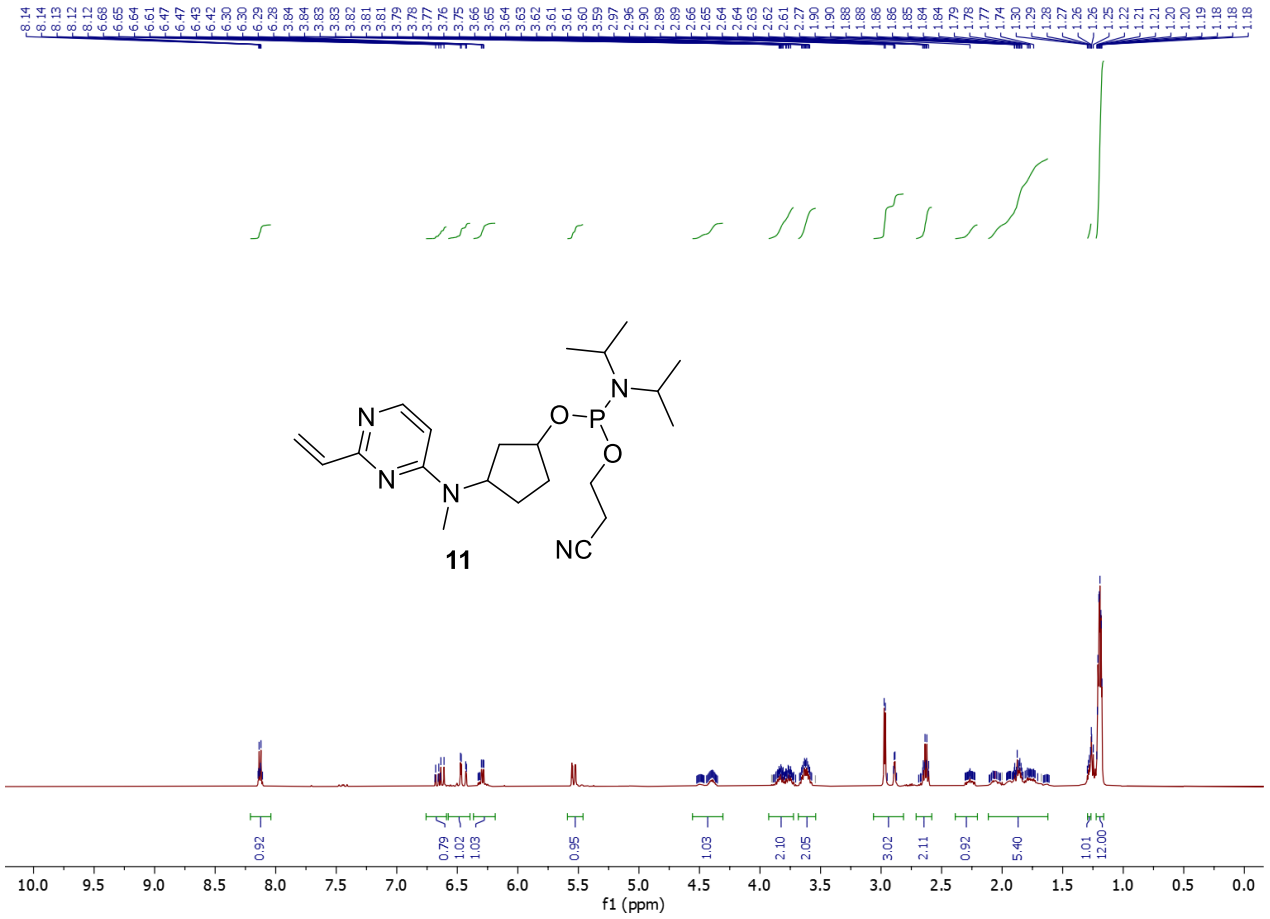
2-cyanoethyl (3-(methyl(2-vinylpyrimidin-4-yl)amino)cyclobutyl) diisopropylphosphoramidite (Mixture of Diastereoisomers) (10)

^{31}P NMR (162 MHz, CD_2Cl_2)



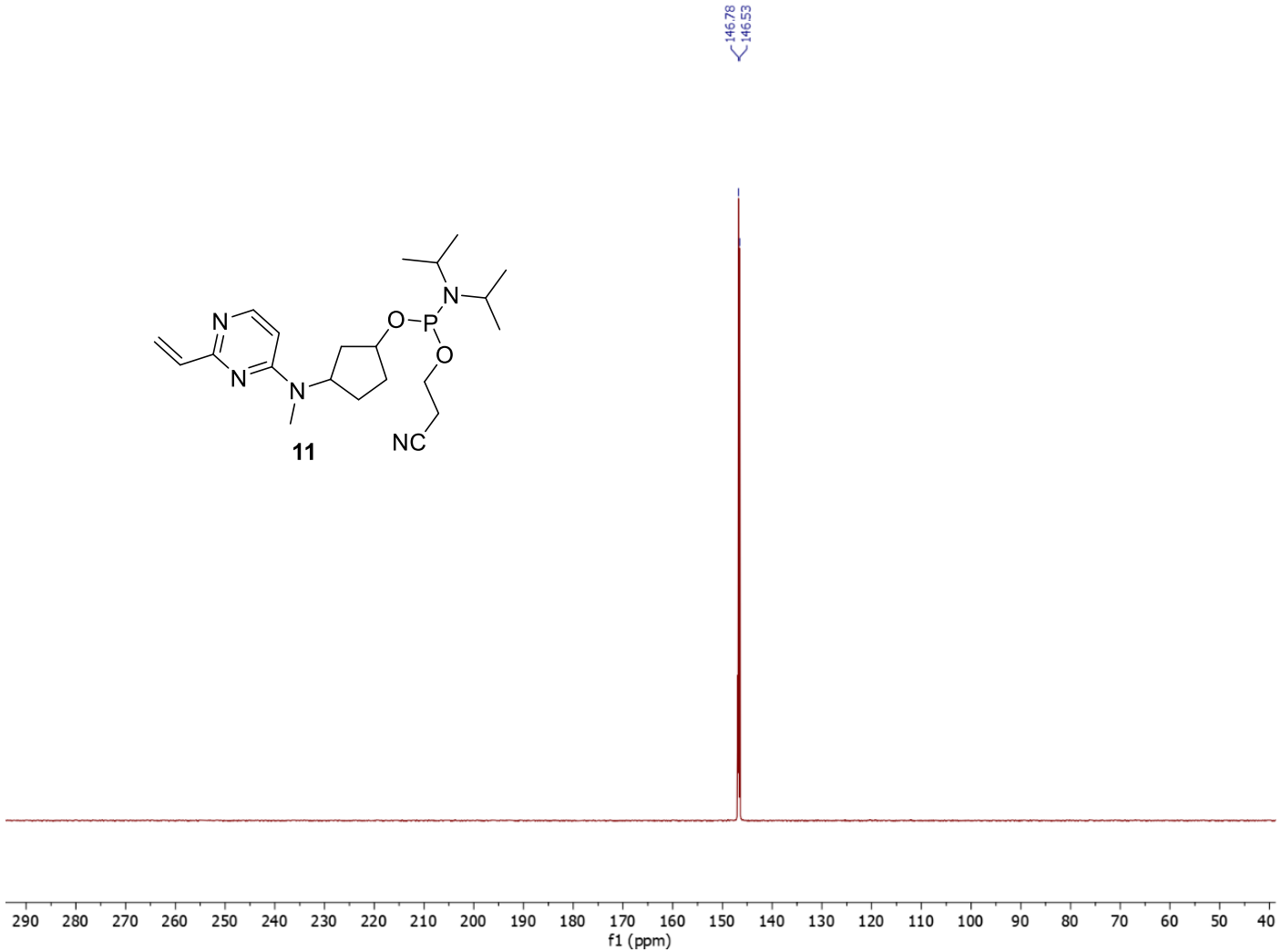
2-cyanoethyl (3-(methyl(2-vinylpyrimidin-4-yl)amino)cyclopentyl) diisopropylphosphoramidite (Mixture of Diastereoisomers) (11)

^{1}H NMR (400 MHz, CD_2Cl_2)



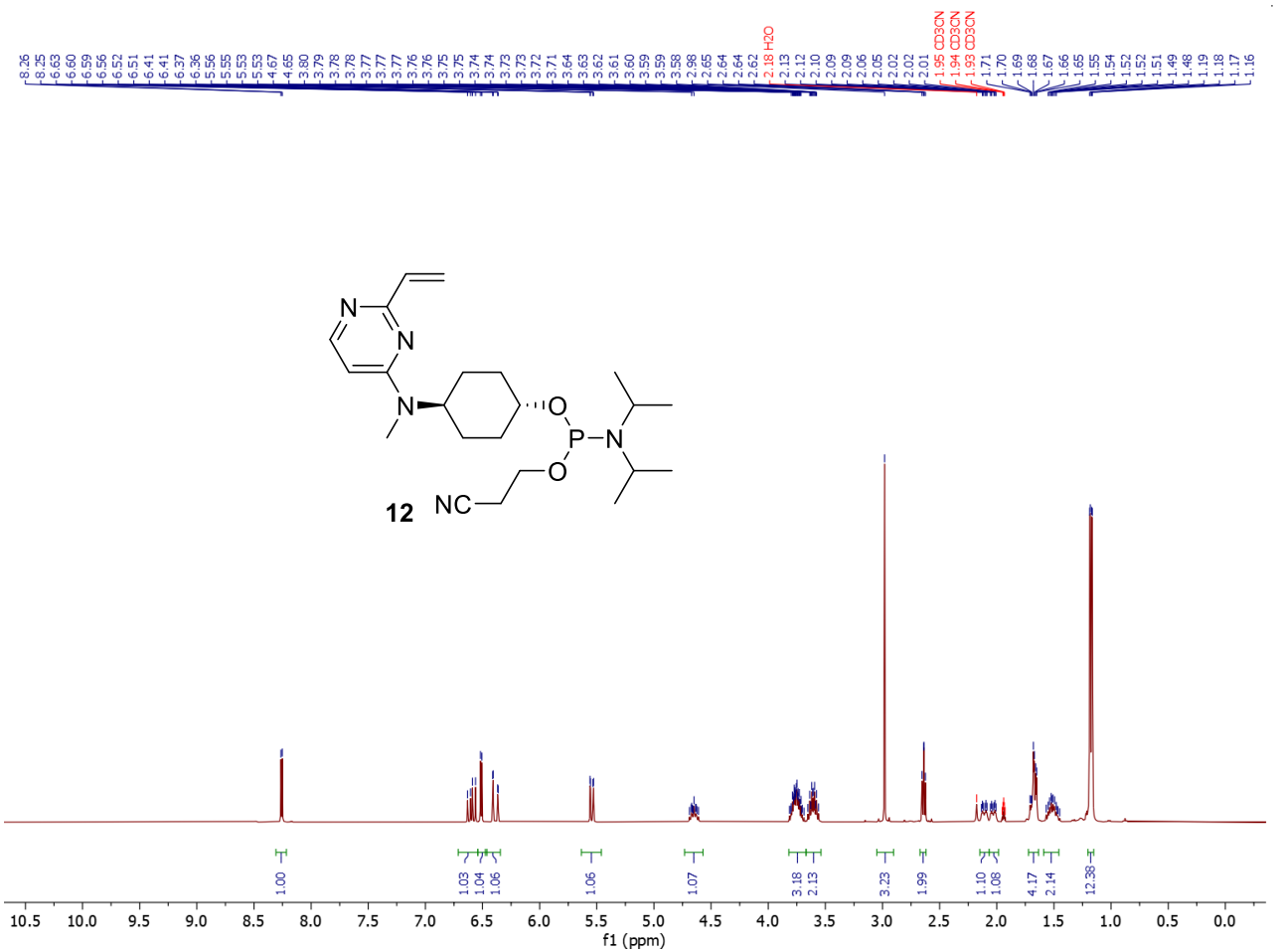
2-cyanoethyl (3-(methyl(2-vinylpyrimidin-4-yl)amino)cyclopentyl) diisopropylphosphoramidite (Mixture of Diastereoisomers) (11)

^{31}P NMR (162 MHz, CD_2Cl_2)



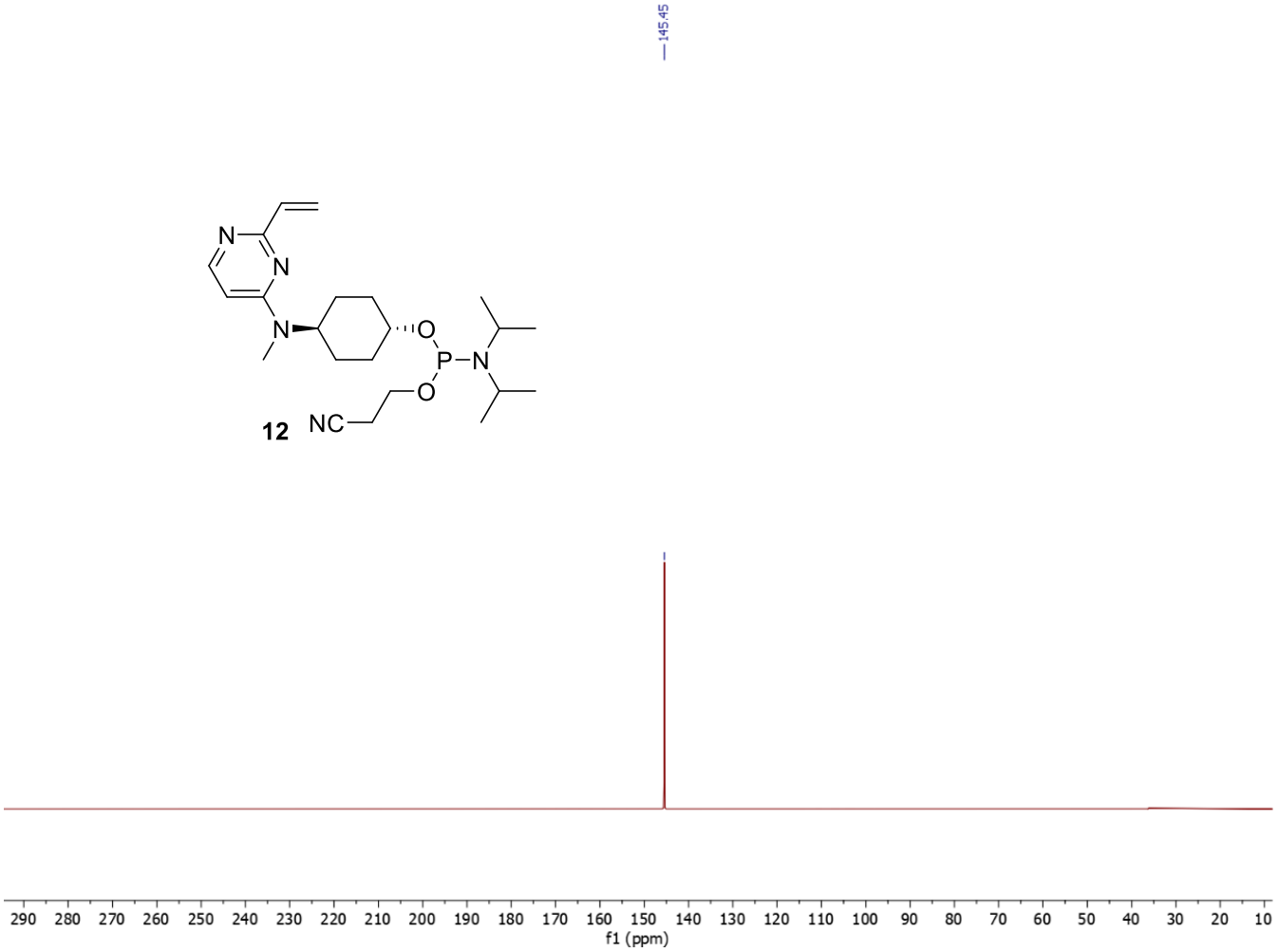
2-cyanoethyl ((1*R*,4*R*)-4-(methyl(2-vinylpyrimidin-4-yl)amino)cyclohexyl) diisopropylphosphoramidite (Mixture of Diastereoisomers) (12)

^{1}H NMR (400 MHz, CD_3CN)



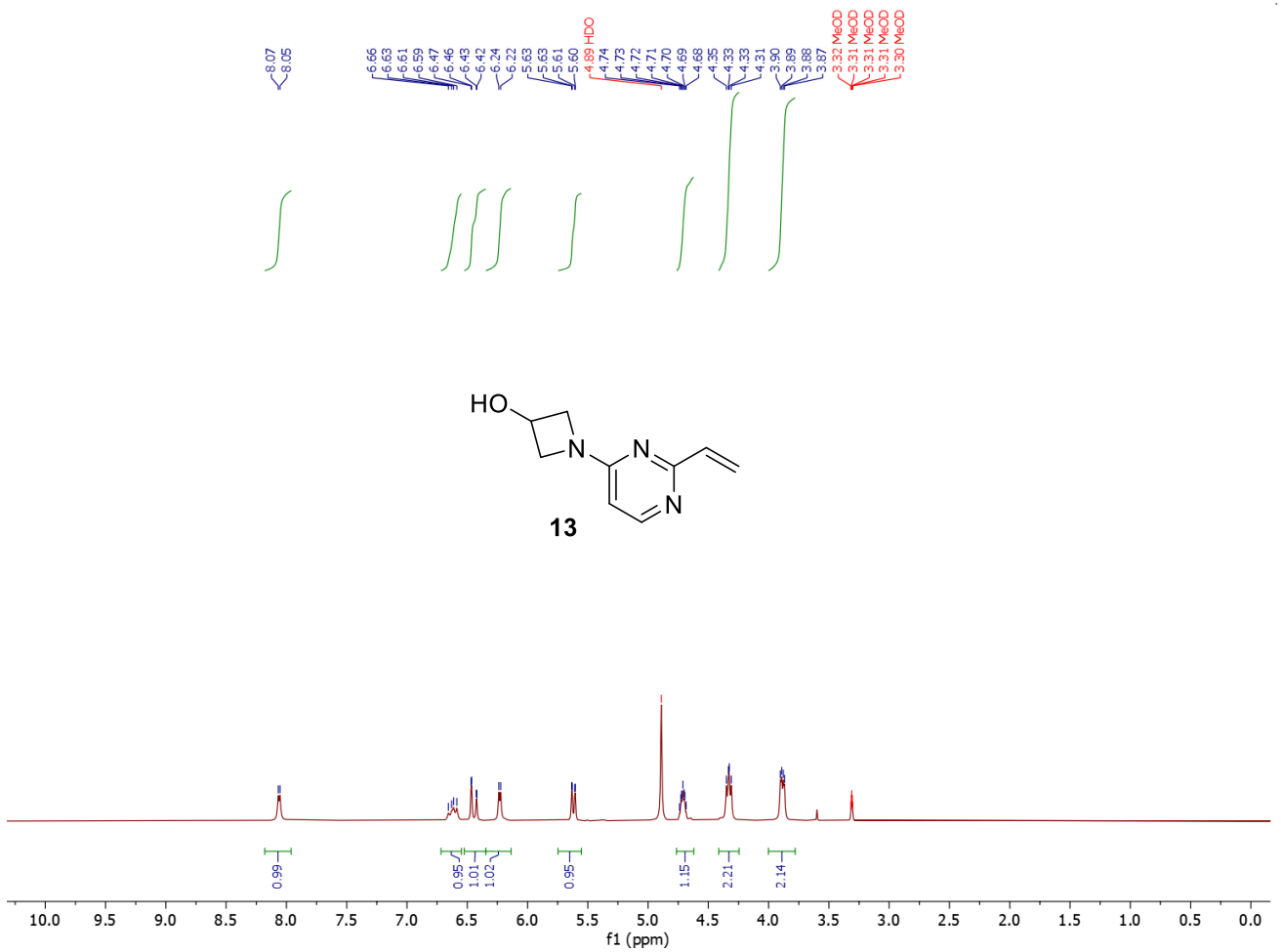
2-cyanoethyl ((1*R*,4*R*)-4-(methyl(2-vinylpyrimidin-4-yl)amino)cyclohexyl) diisopropylphosphoramidite (Mixture of Diastereoisomers) (12)

^{31}P NMR (162 MHz, CD_3CN)



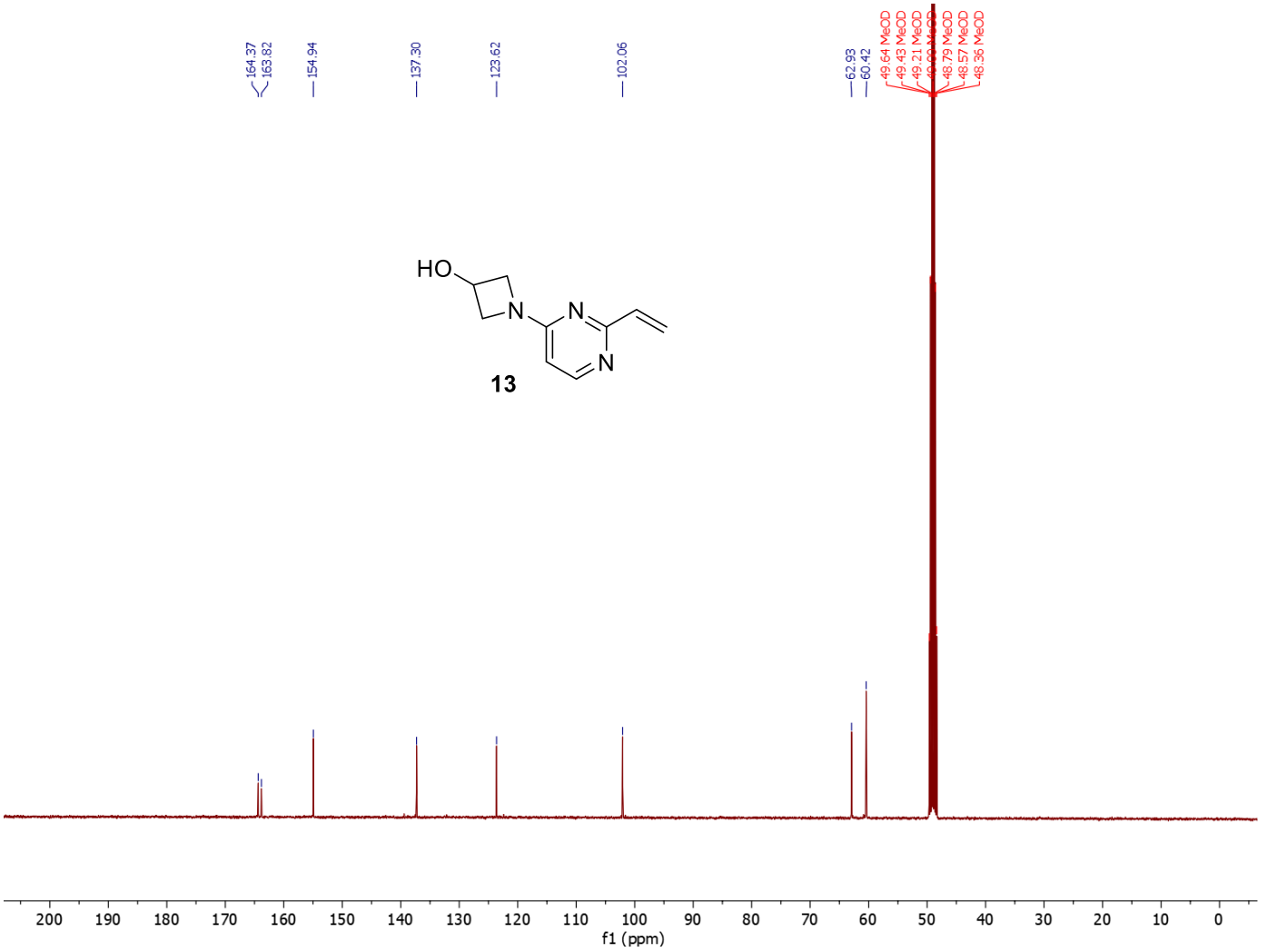
1-(4-vinylpyrimidin-2-yl)azetidin-3-ol (13)

^{1}H NMR (400 MHz, Methanol-d4)



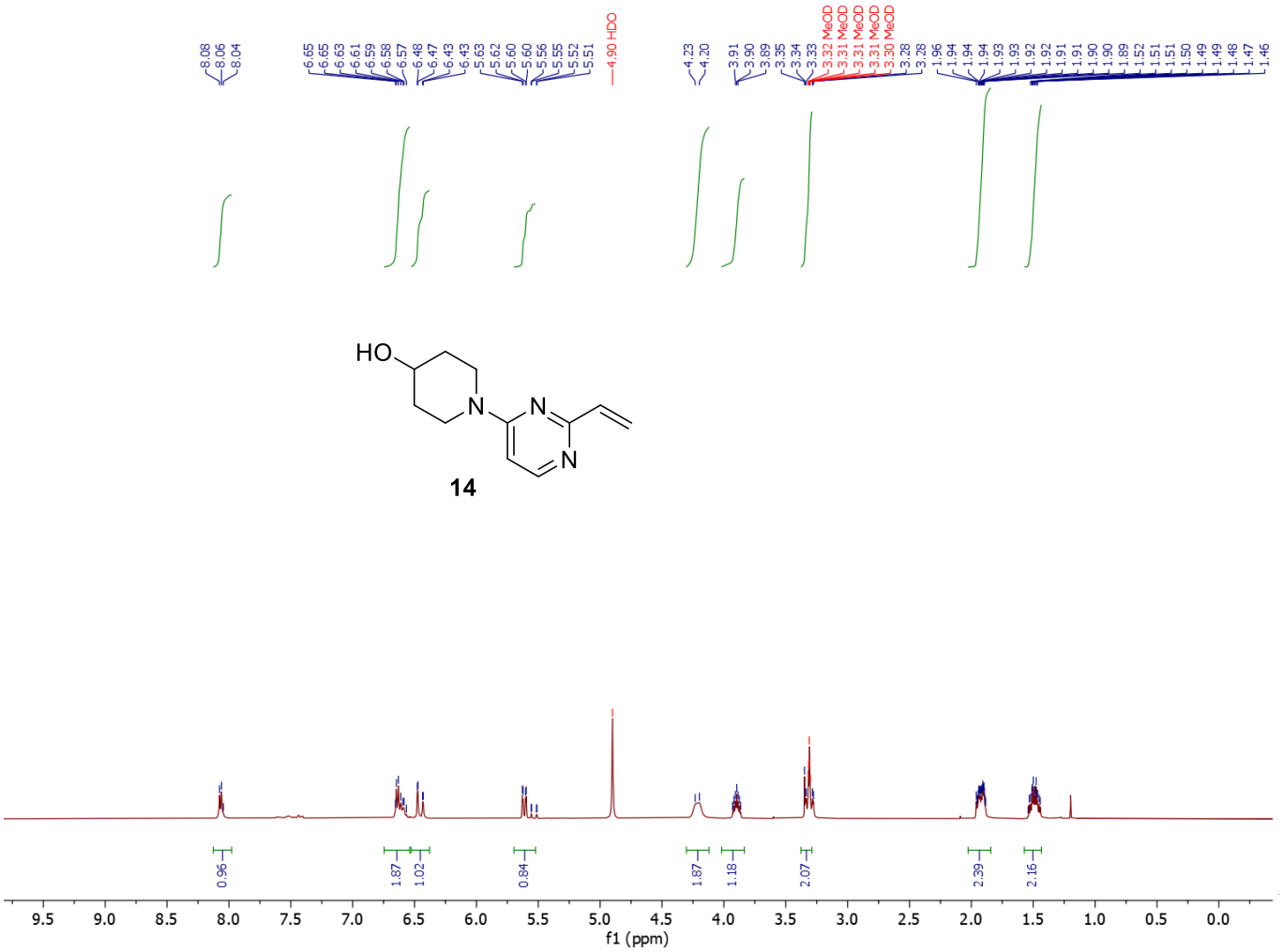
1-(4-vinylpyrimidin-2-yl)azetidin-3-ol (13)

^{13}C NMR (101 MHz, Methanol-d4)



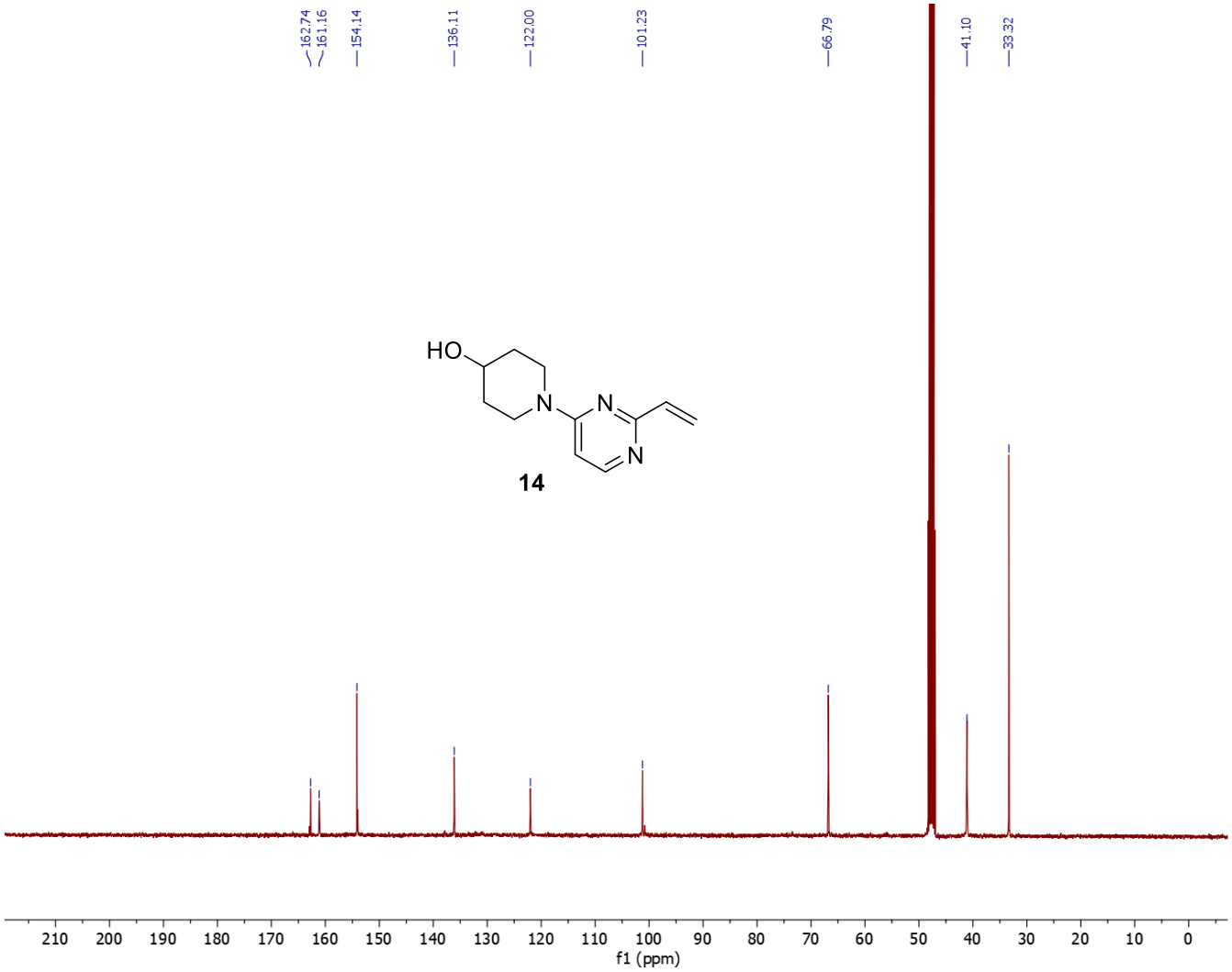
1-(2-vinylpyrimidin-4-yl)piperidin-4-ol (14)

^{1}H NMR (400 MHz, Methanol-d4)



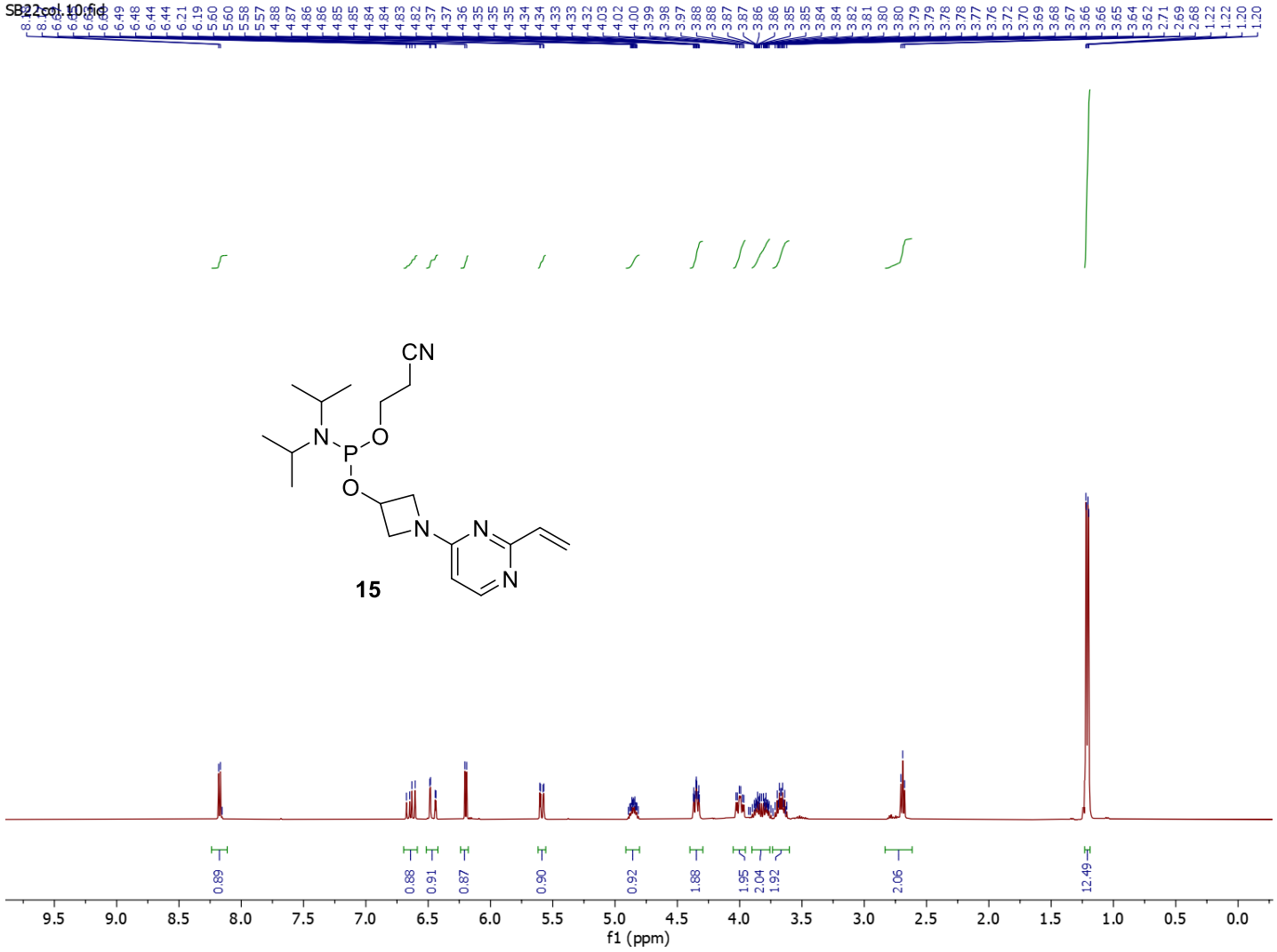
1-(2-vinylpyrimidin-4-yl)piperidin-4-ol (14)

^{13}C NMR (101 MHz, Methanol-d4)



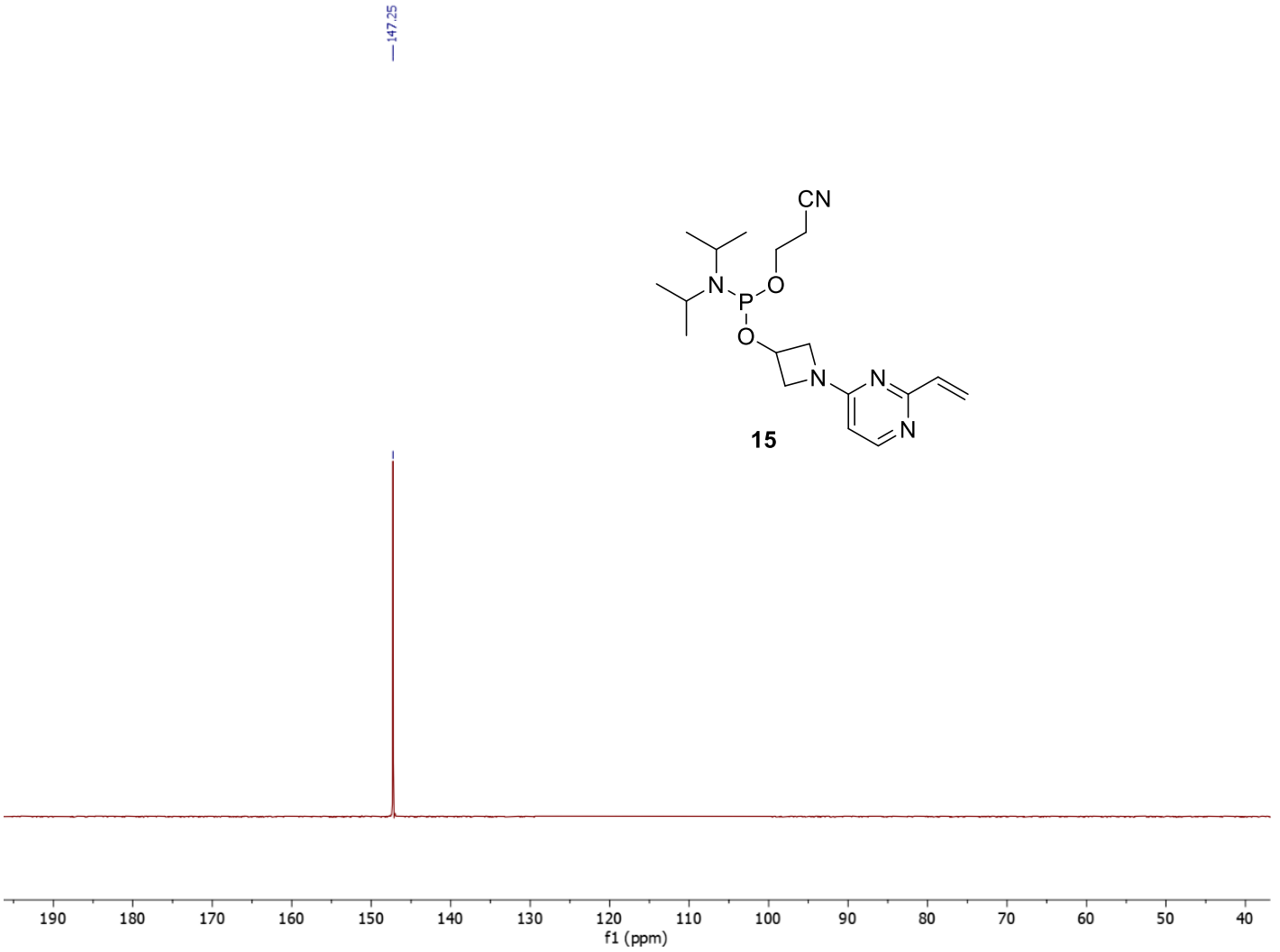
2-cyanoethyl (1-(2-vinylpyrimidin-4-yl)azetidin-3-yl) diisopropylphosphoramidite (Mixture of Diastereoisomers) (15)

^{1}H NMR (400 MHz, CD_3CN)



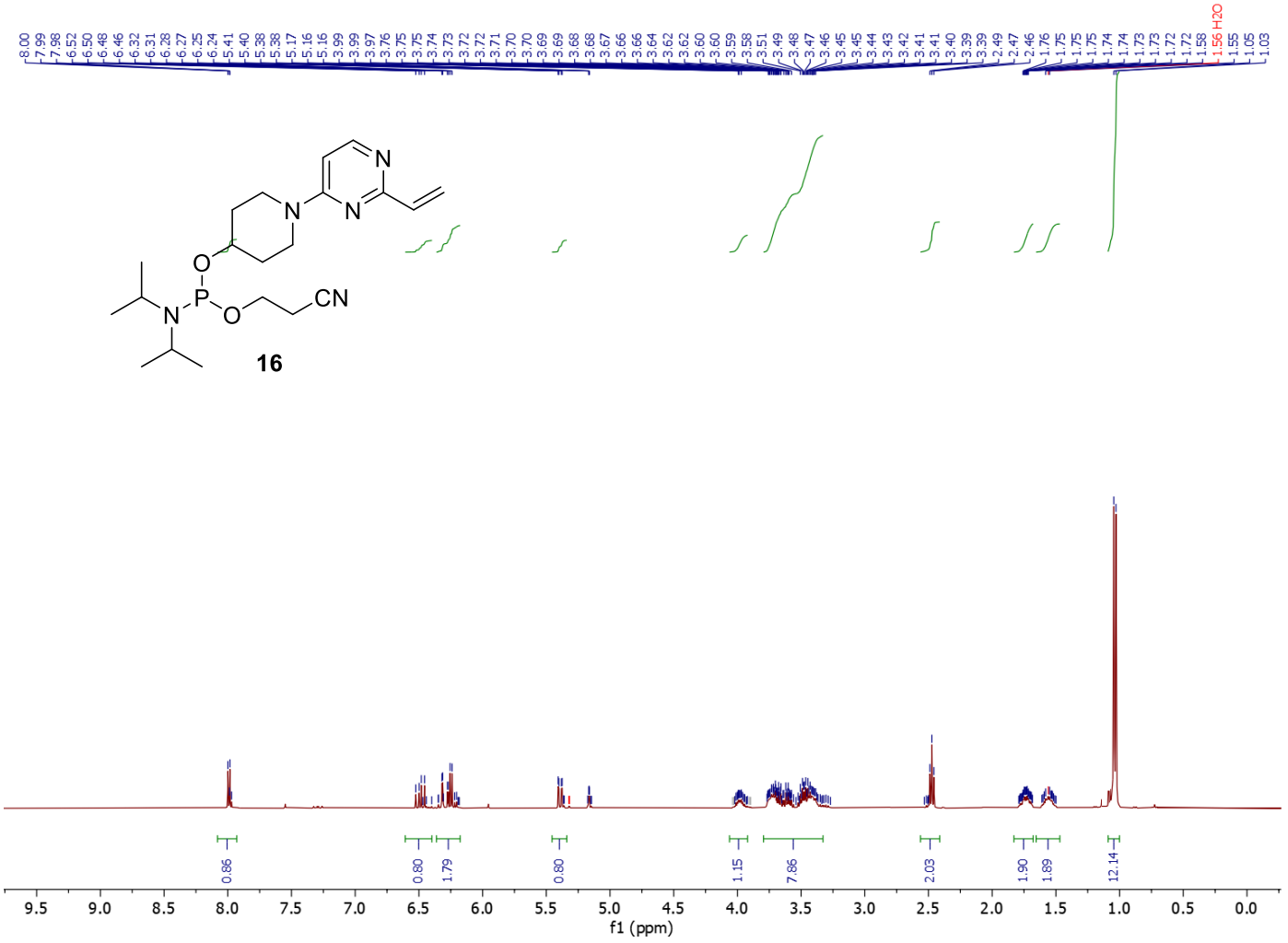
2-cyanoethyl (1-(2-vinylpyrimidin-4-yl)azetidin-3-yl) diisopropylphosphoramidite (Mixture of Diastereoisomers) (15)

^{31}P NMR (162 MHz, CD_3CN)



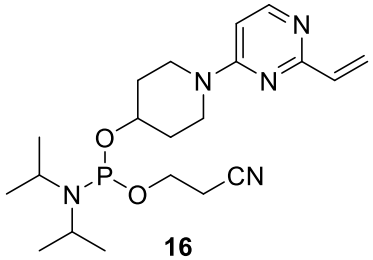
2-cyanoethyl (1-(2-vinylpyrimidin-4-yl)piperidin-4-yl) diisopropylphosphoramidite (Mixture of Diastereoisomers) (16)

^{1}H NMR (400 MHz, CD_2Cl_2)



2-cyanoethyl (1-(2-vinylpyrimidin-4-yl)piperidin-4-yl) diisopropylphosphoramidite (Mixture of Diastereoisomers) (16)

^{31}P NMR (162 MHz, CD_2Cl_2)



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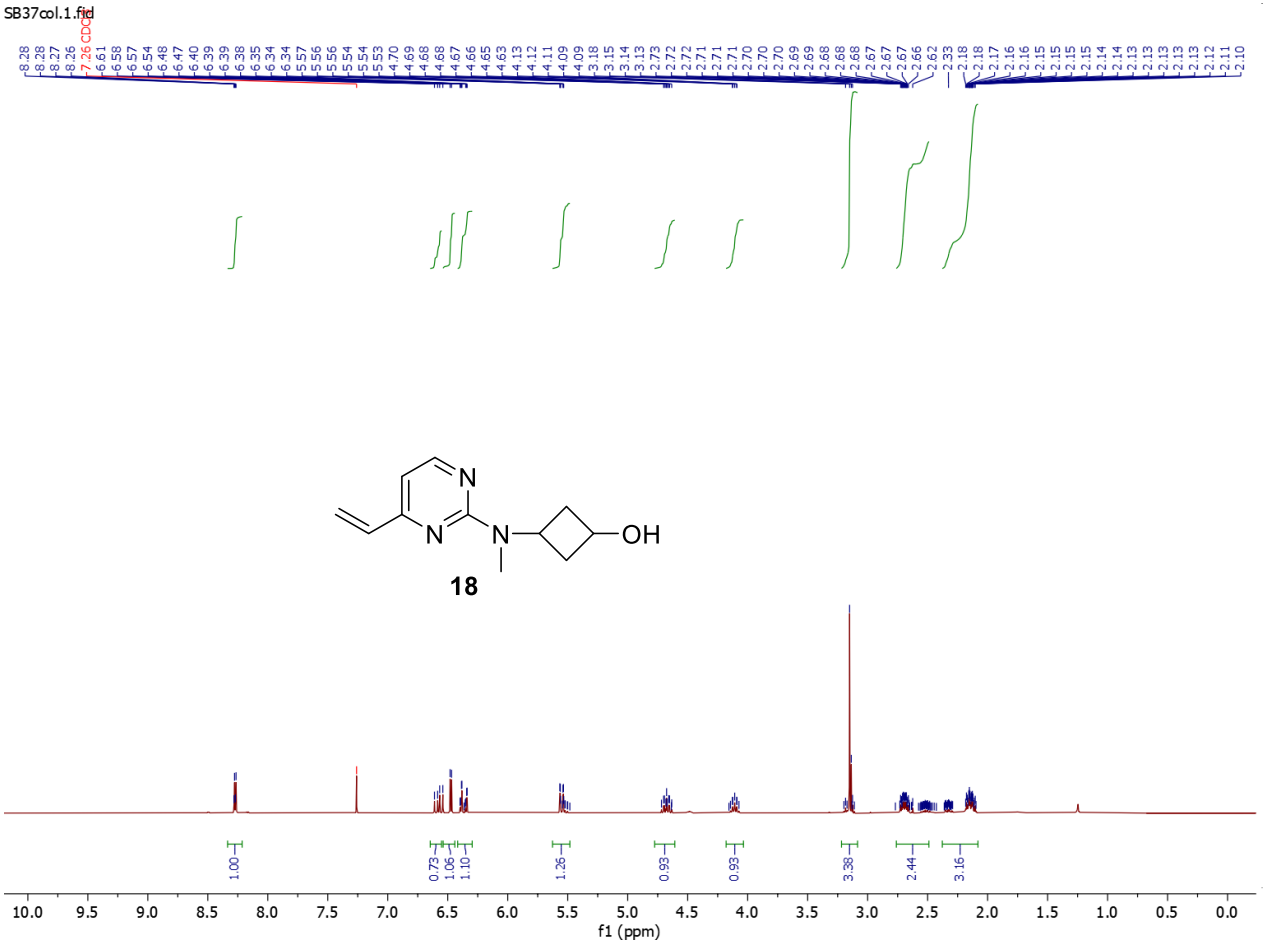
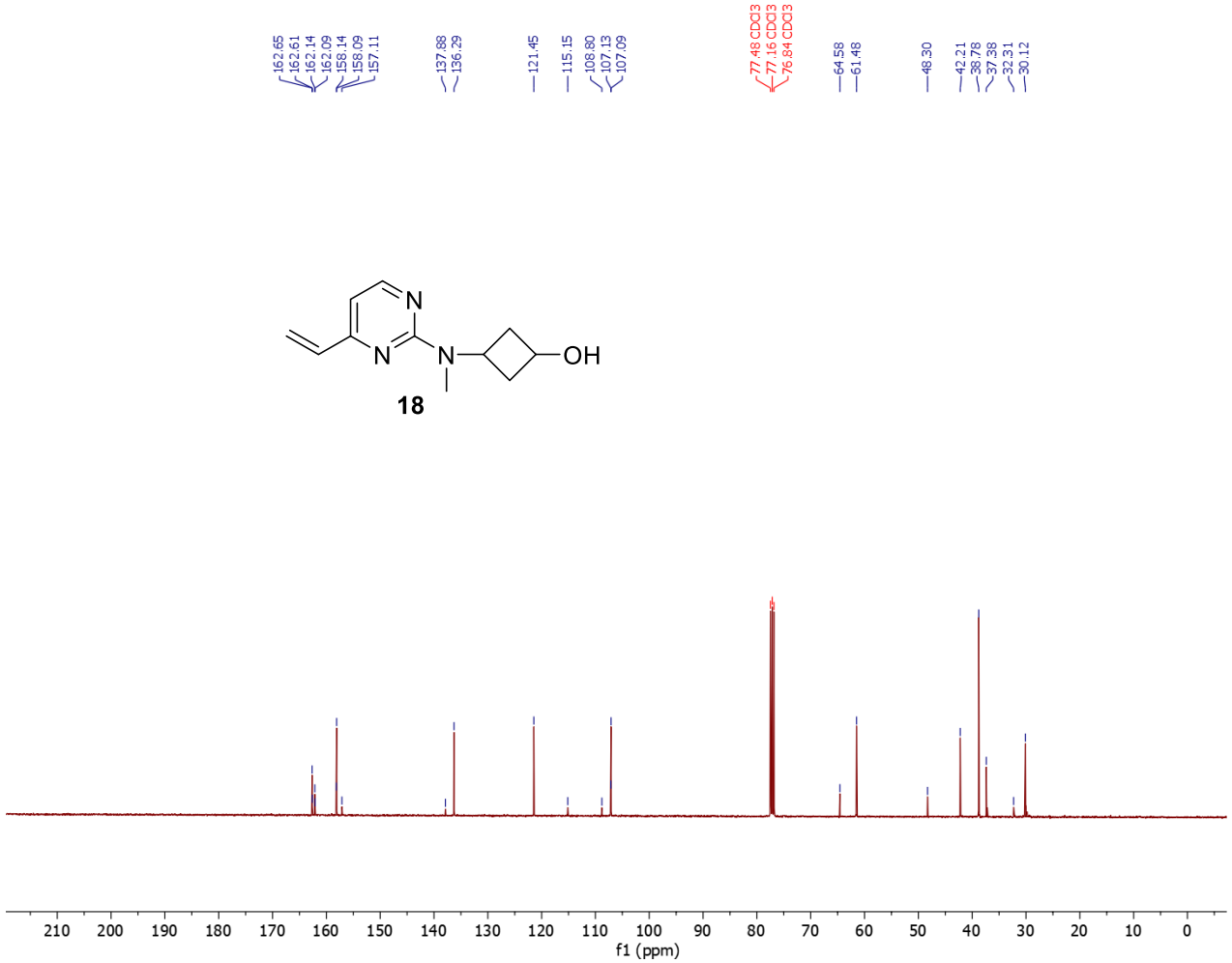
3-(methyl(4-vinylpyrimidin-2-yl)amino)cyclobutan-1-ol (18) (Mixture of Cis/Trans)

^{1}H NMR (400 MHz, $CDCl_3$)



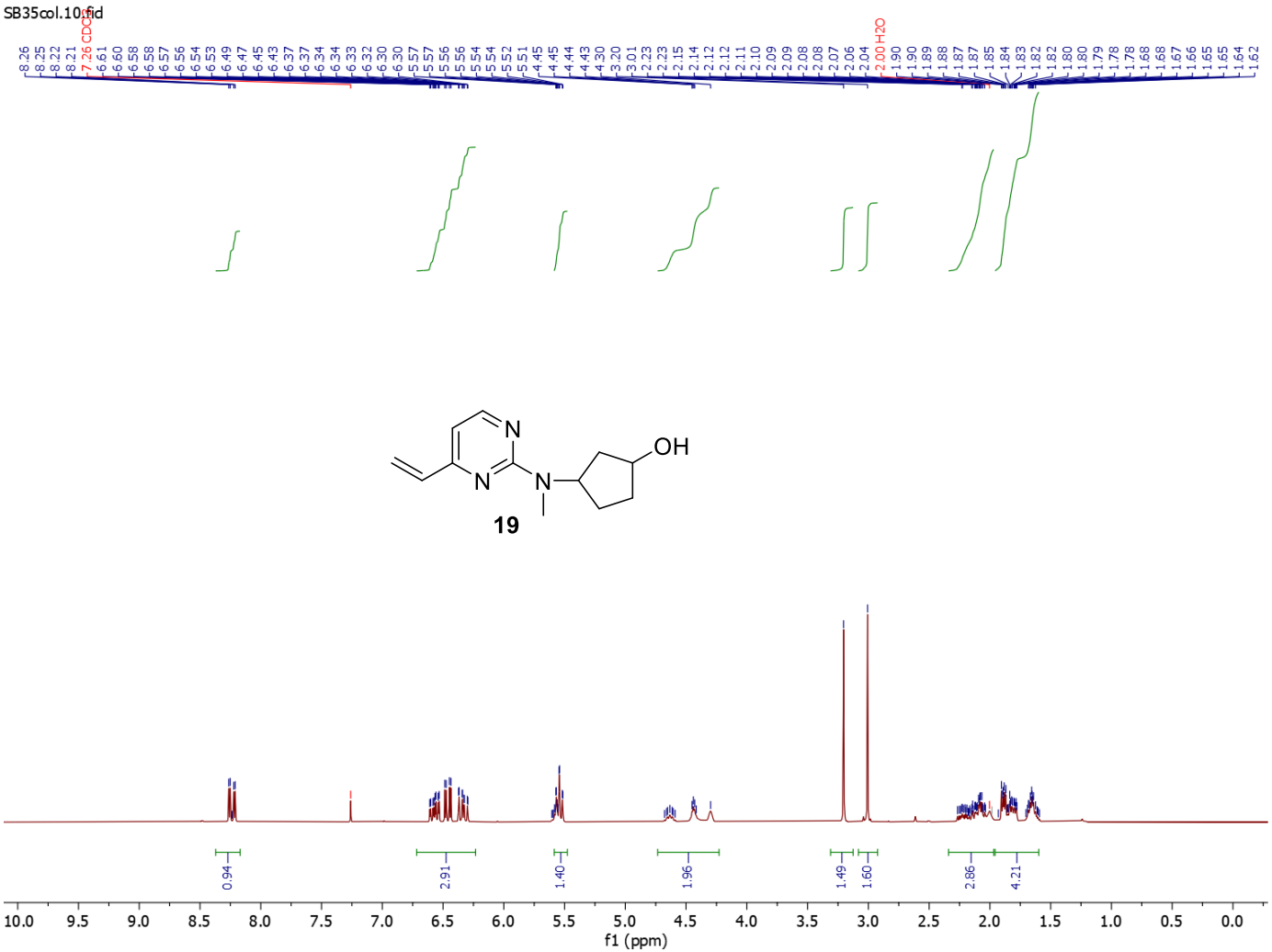
3-(methyl(4-vinylpyrimidin-2-yl)amino)cyclobutan-1-ol (18) (Mixture of Cis/Trans)

^{13}C NMR (400 MHz, $CDCl_3$)



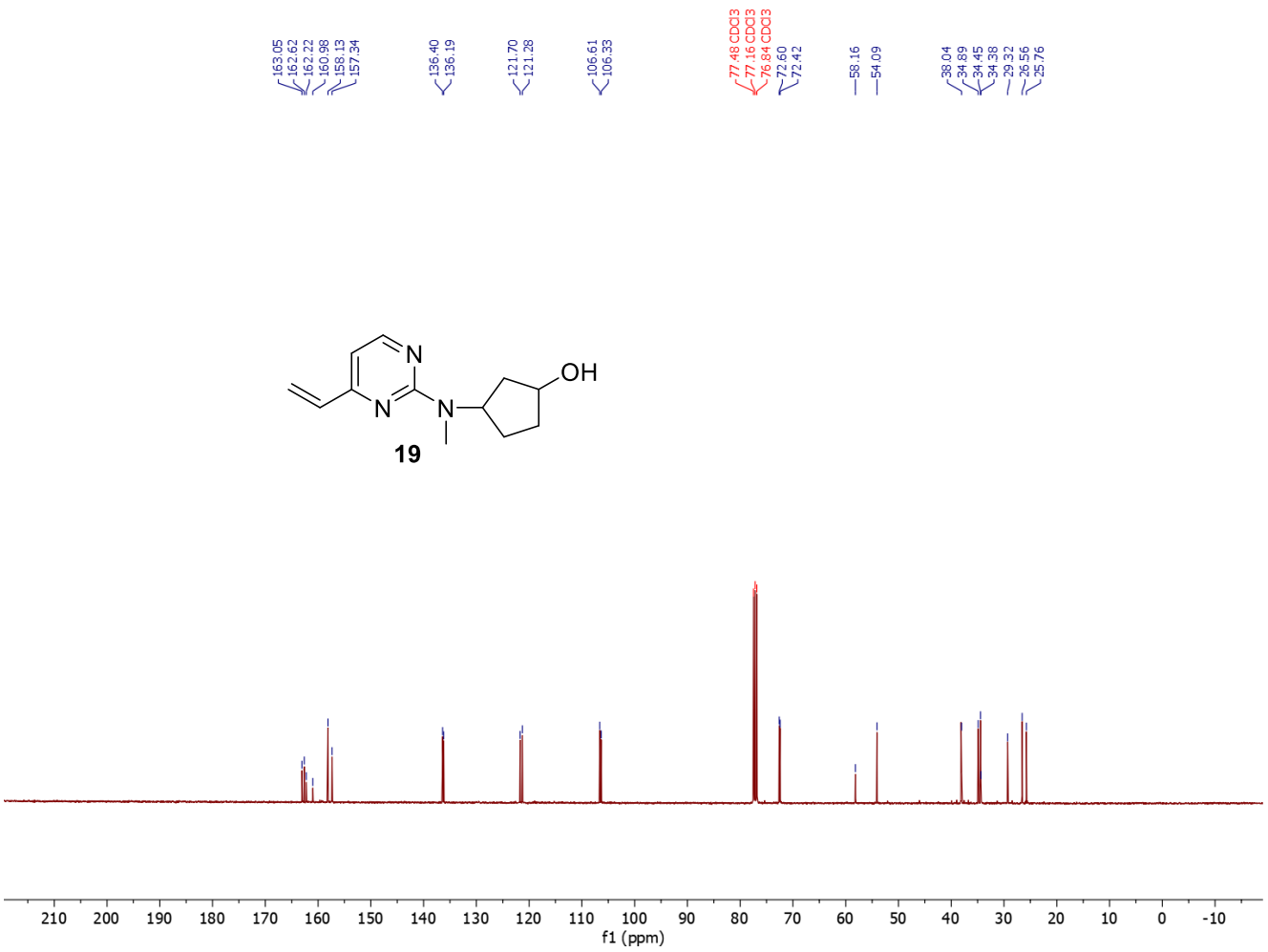
3-(methyl(4-vinylpyrimidin-2-yl)amino)cyclopentan-1-ol (19) (Mixture of Cis/Trans)

^{1}H NMR (400 MHz, $CDCl_3$)



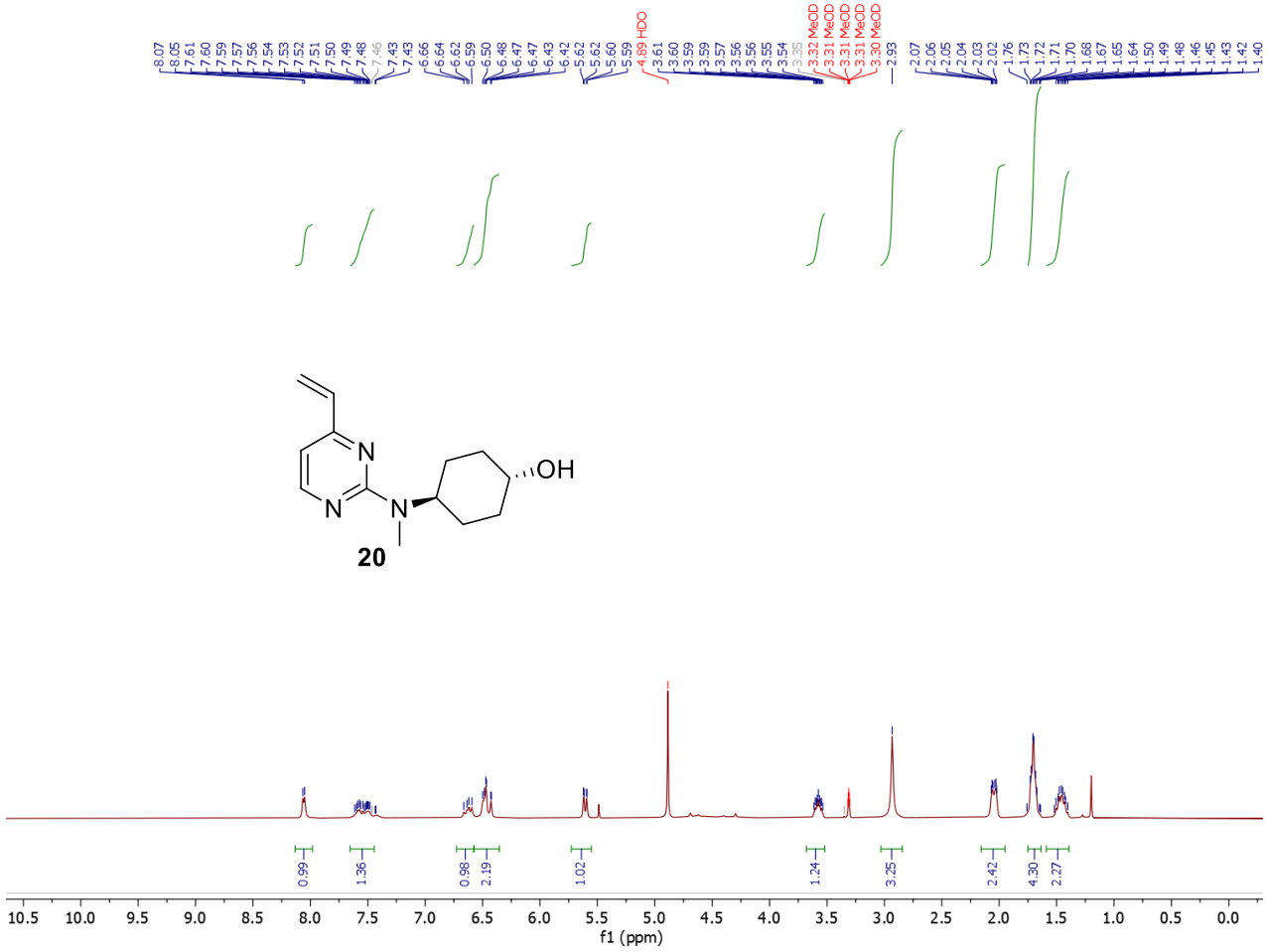
3-(methyl(4-vinylpyrimidin-2-yl)amino)cyclopentan-1-ol (19) (Mixture of Cis/Trans)

^{13}C NMR (400 MHz, $CDCl_3$)



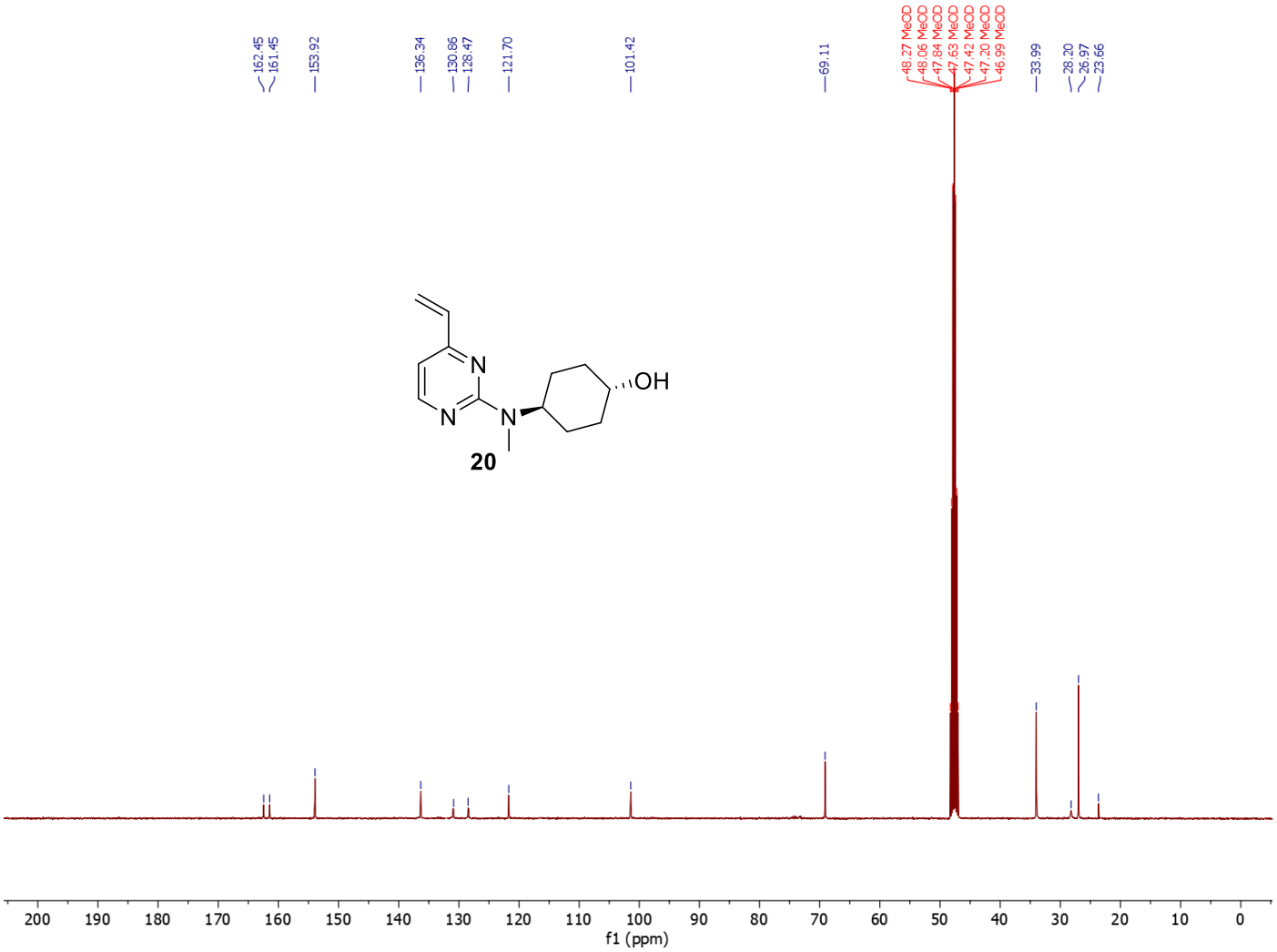
(1*R*,4*R*)-4-(methyl(4-vinylpyrimidin-2-yl)amino)cyclohexan-1-ol (20)

^{1}H NMR (400 MHz, Methanol-d4)



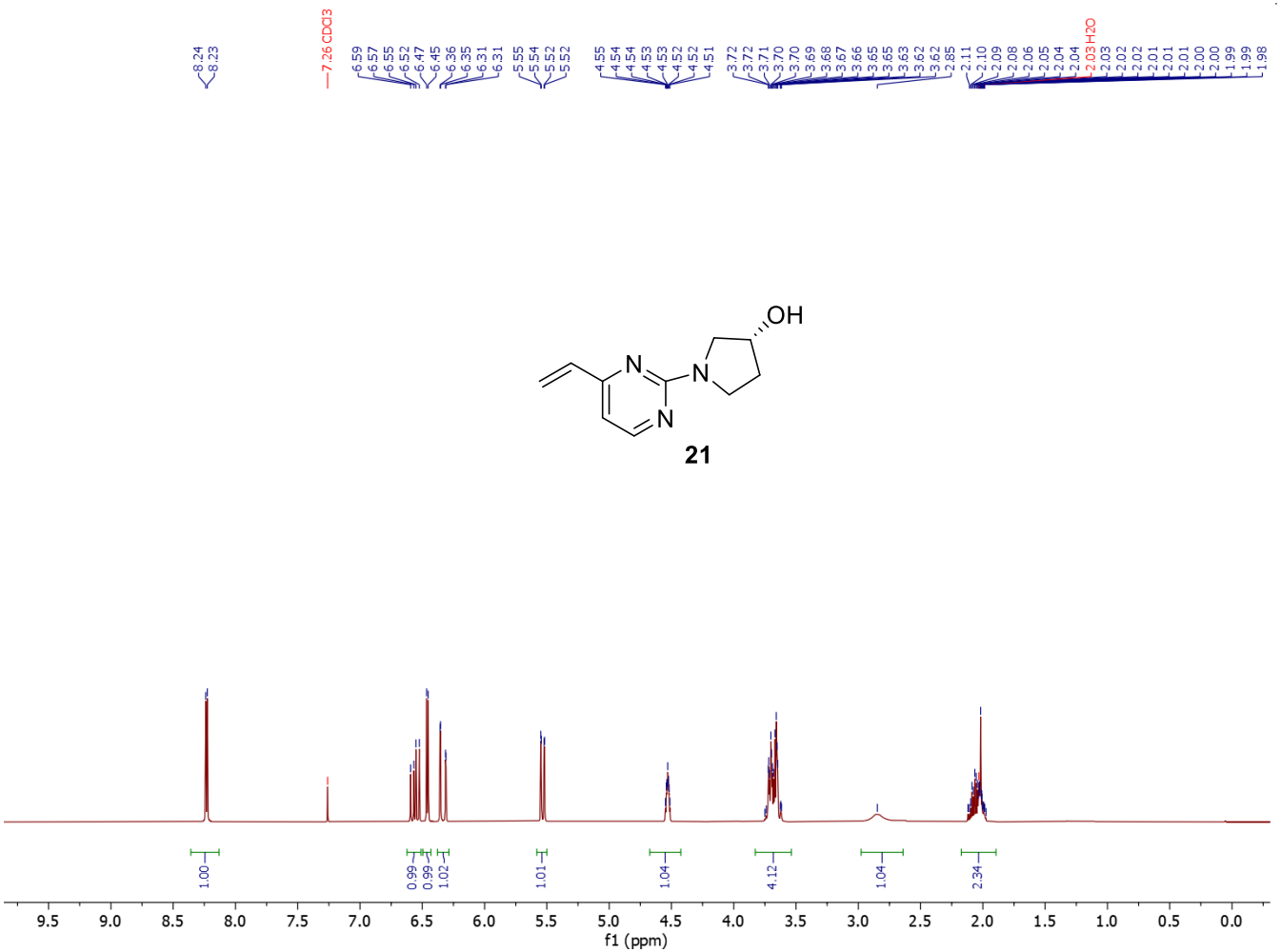
(1*R*,4*R*)-4-(methyl(4-vinylpyrimidin-2-yl)amino)cyclohexan-1-ol (20)

^{13}C NMR (101 MHz, Methanol-d4)



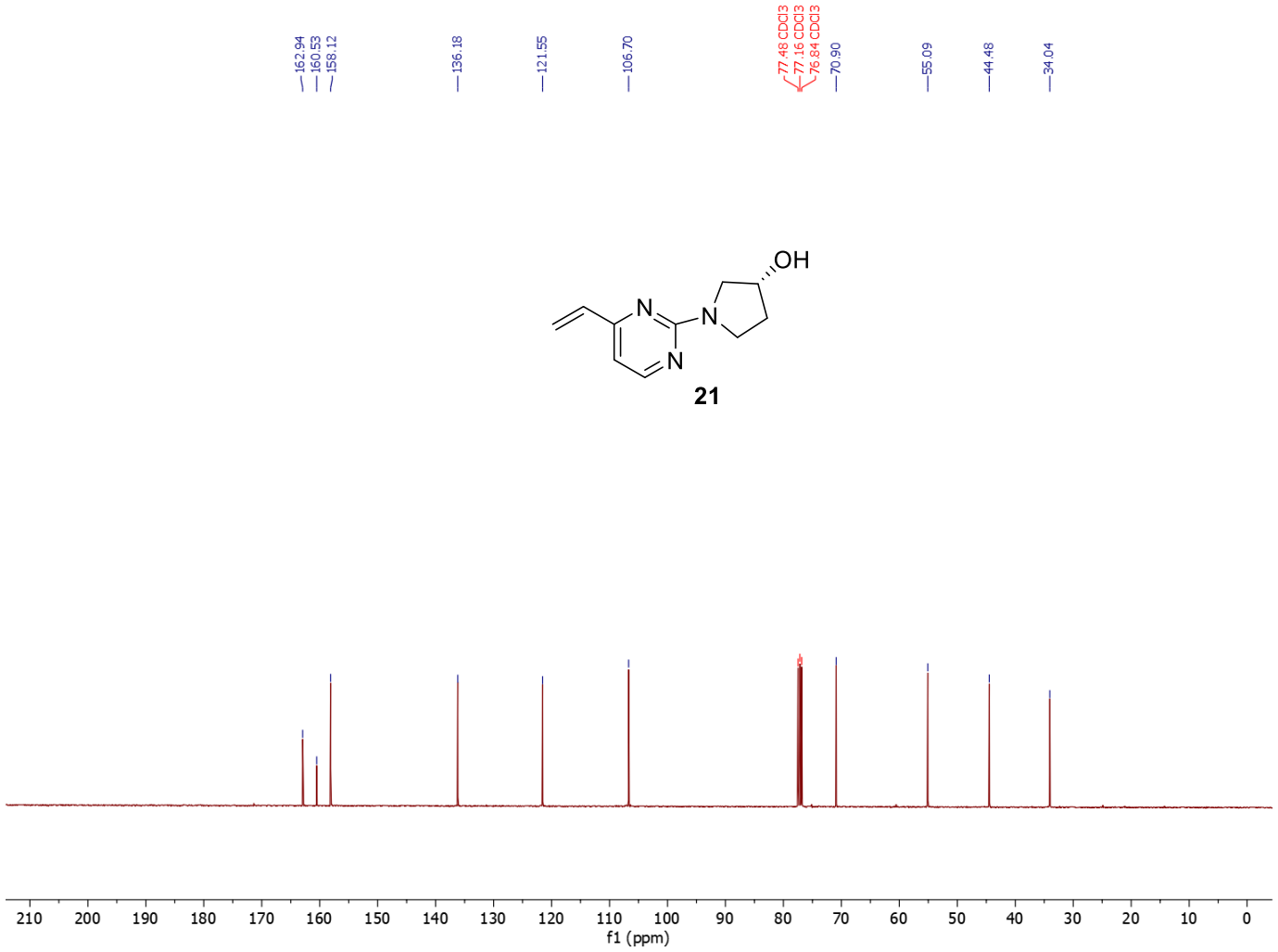
(*R*)-1-(4-vinylpyrimidin-2-yl)pyrrolidin-3-ol (21)

^{1}H NMR (400 MHz, $CDCl_3$)



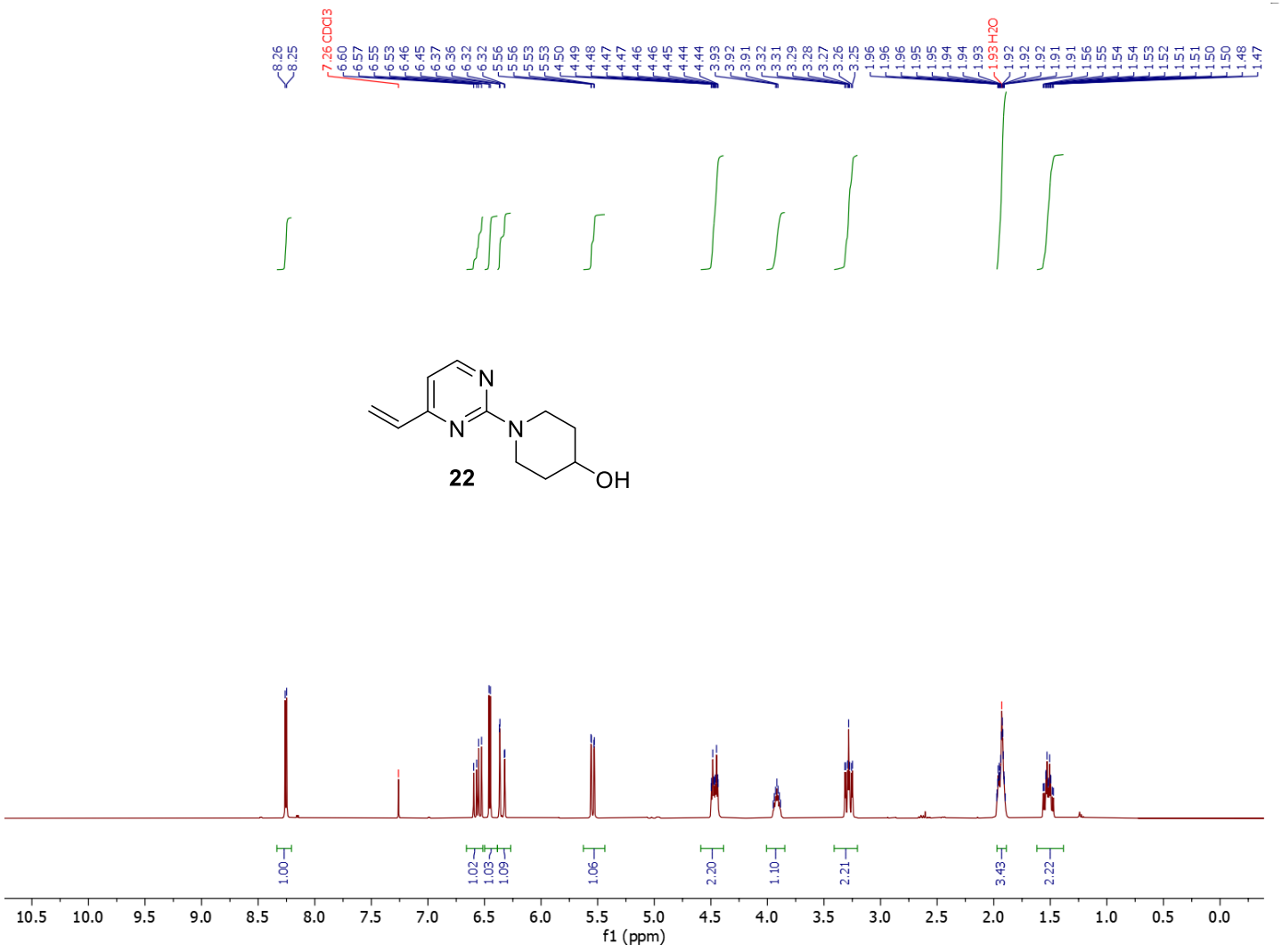
(*R*)-1-(4-vinylpyrimidin-2-yl)pyrrolidin-3-ol (21)

^{13}C NMR (400 MHz, $CDCl_3$)



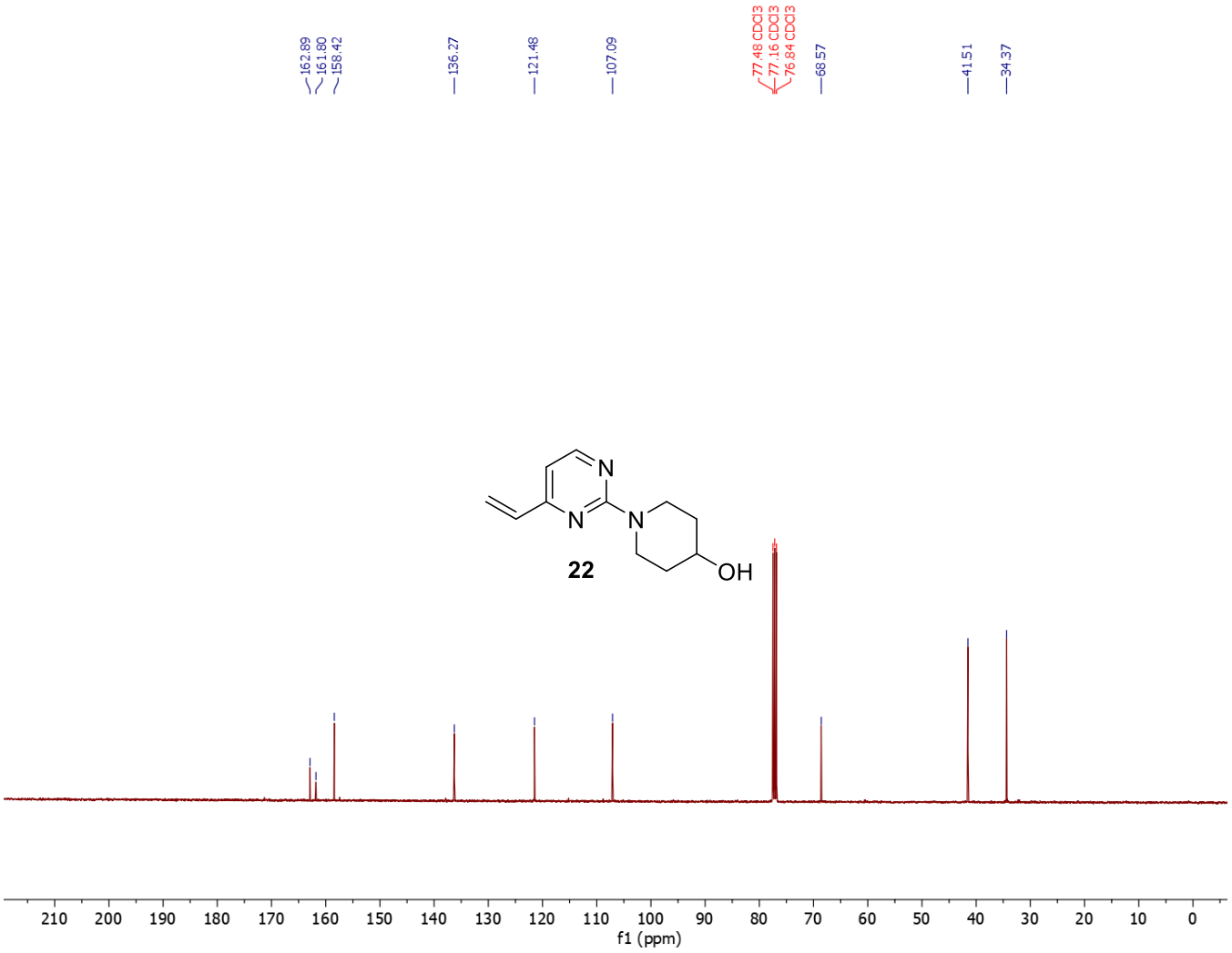
1-(4-vinylpyrimidin-2-yl)piperidin-4-ol (22)

^{1}H NMR (400 MHz, $CDCl_3$)



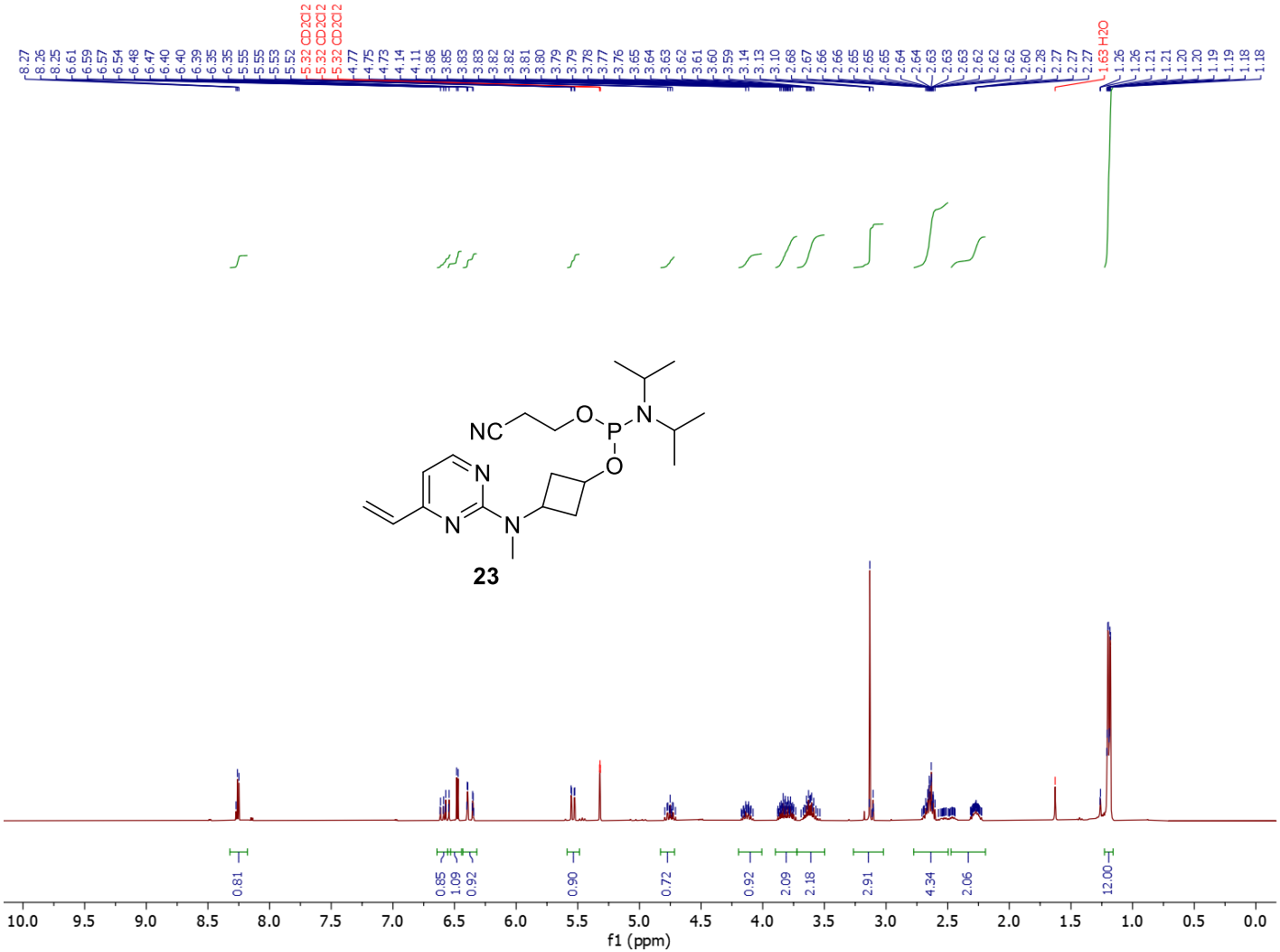
1-(4-vinylpyrimidin-2-yl)piperidin-4-ol (22)

^{13}C NMR (101 MHz, $CDCl_3$)



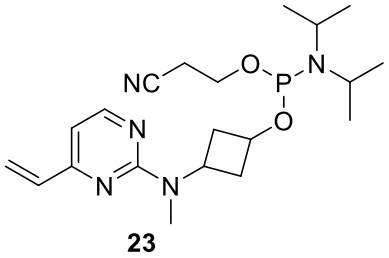
2-cyanoethyl (3-(methyl(4-vinylpyrimidin-2-yl)amino)cyclobutyl) diisopropylphosphoramidite (Mixture of Diastereoisomers) (23)

^{1}H NMR (400 MHz, CD_2Cl_2)



2-cyanoethyl (3-(methyl(4-vinylpyrimidin-2-yl)amino)cyclobutyl) diisopropylphosphoramidite (Mixture of Diastereoisomers) (23)

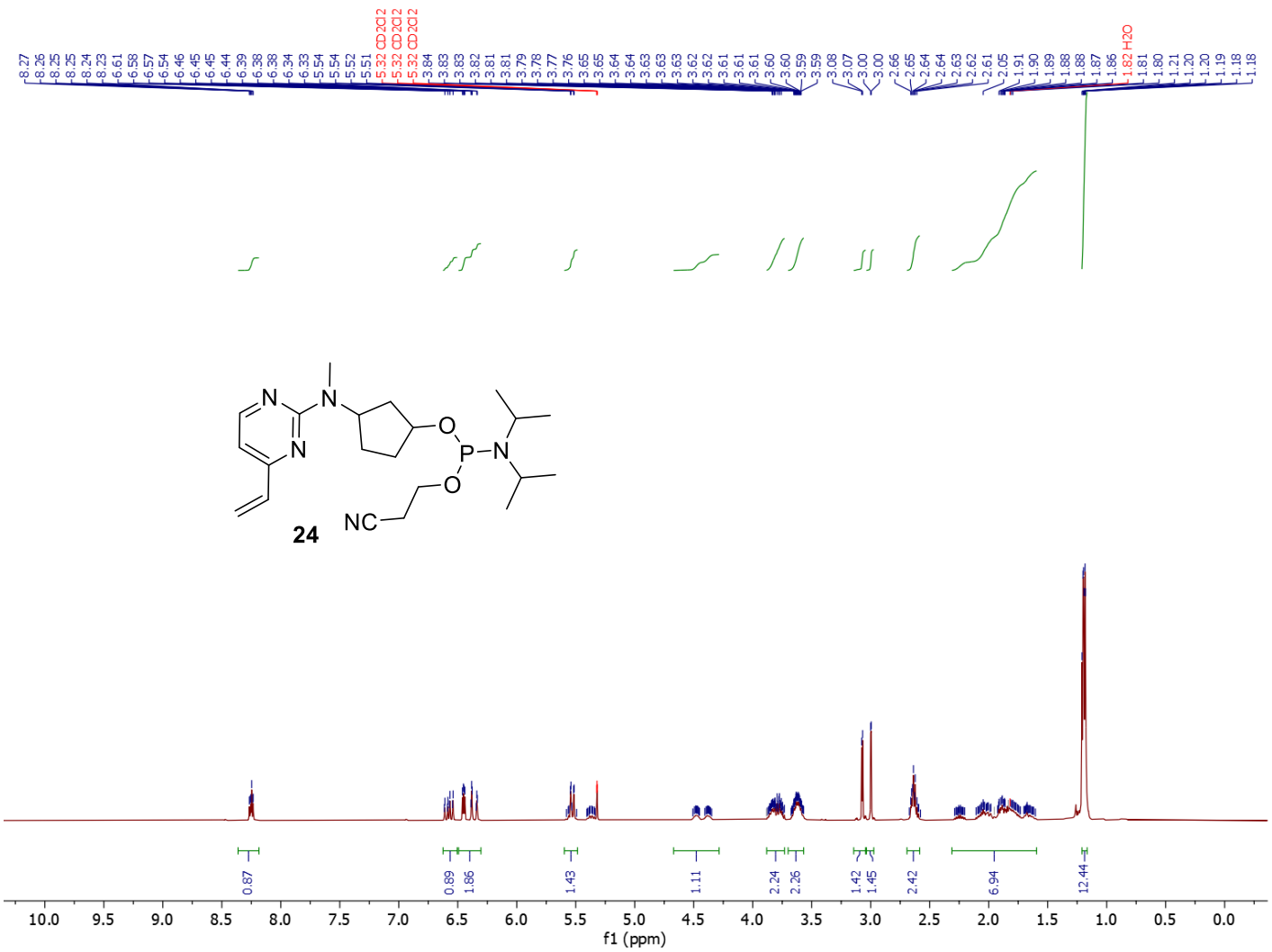
^{31}P NMR (400 MHz, CD_2Cl_2)



23

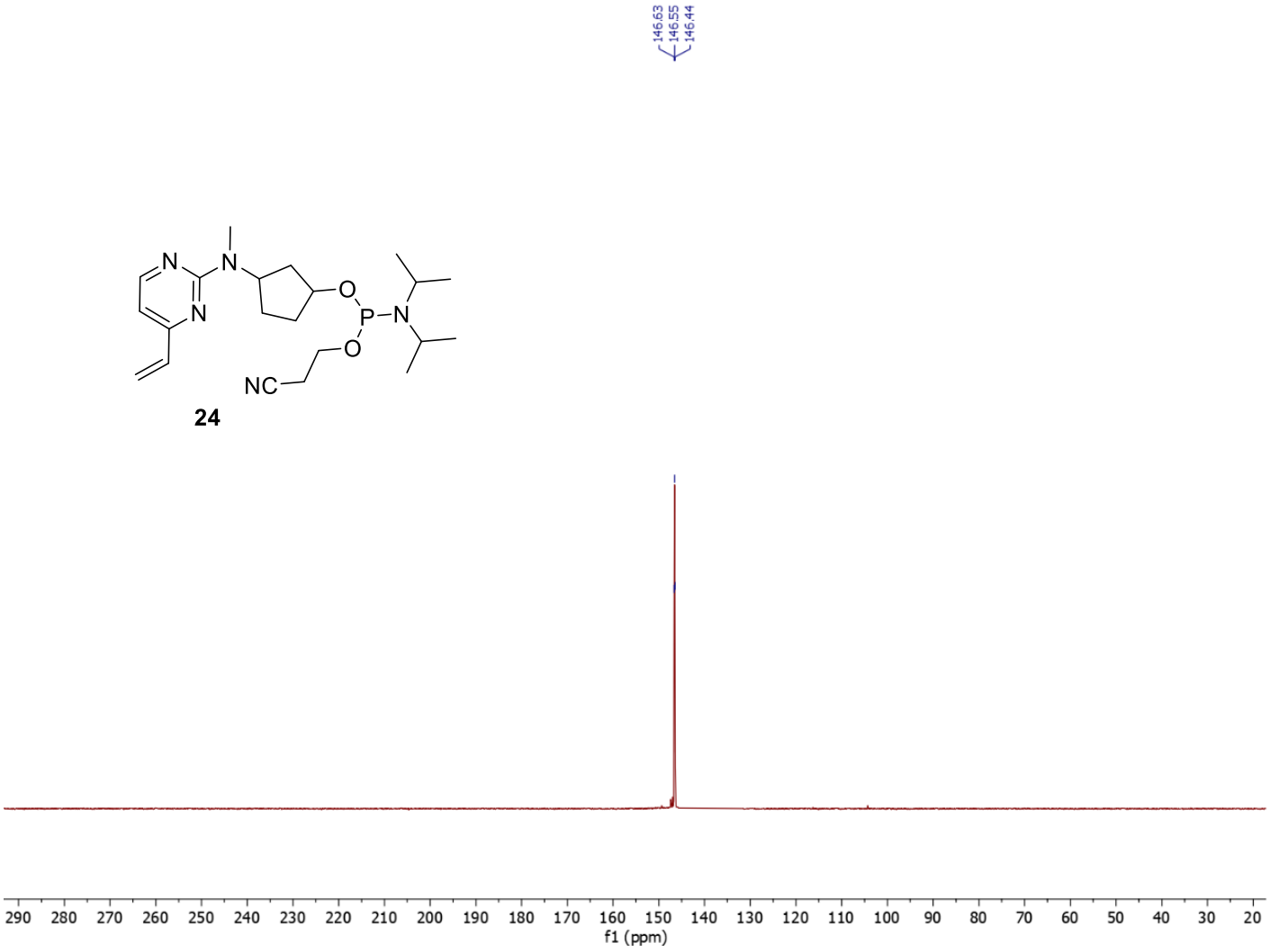
2-cyanoethyl (3-(methyl(4-vinylpyrimidin-2-yl)amino)cyclopentyl) diisopropylphosphoramidite (Mixture of Diastereoisomers) (24)

^{1}H NMR (400 MHz, CD_2Cl_2)



2-cyanoethyl (3-(methyl(4-vinylpyrimidin-2-yl)amino)cyclopentyl) diisopropylphosphoramidite (Mixture of Diastereoisomers) (24)

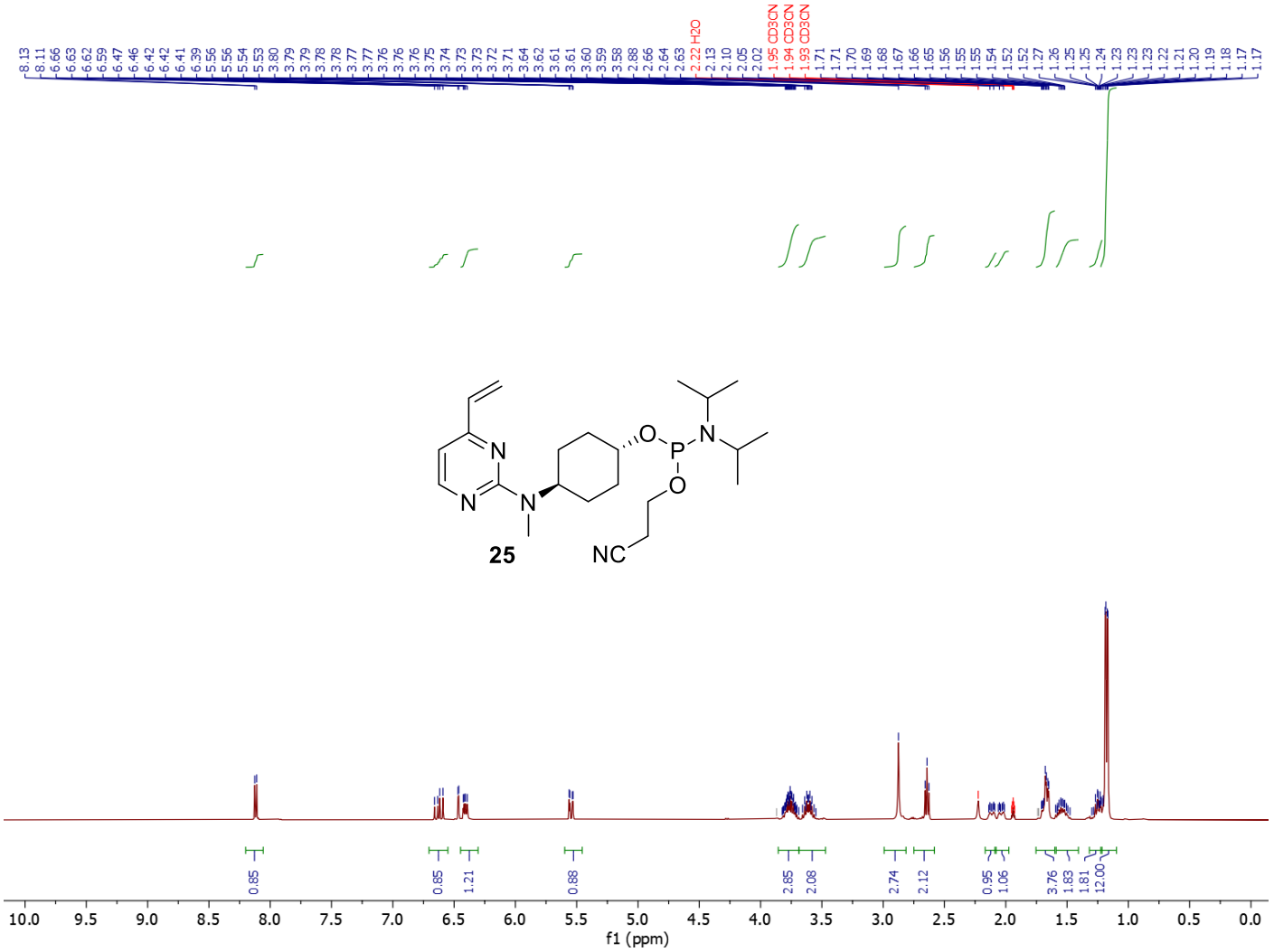
^{31}P NMR (162 MHz, CD_2Cl_2)



24

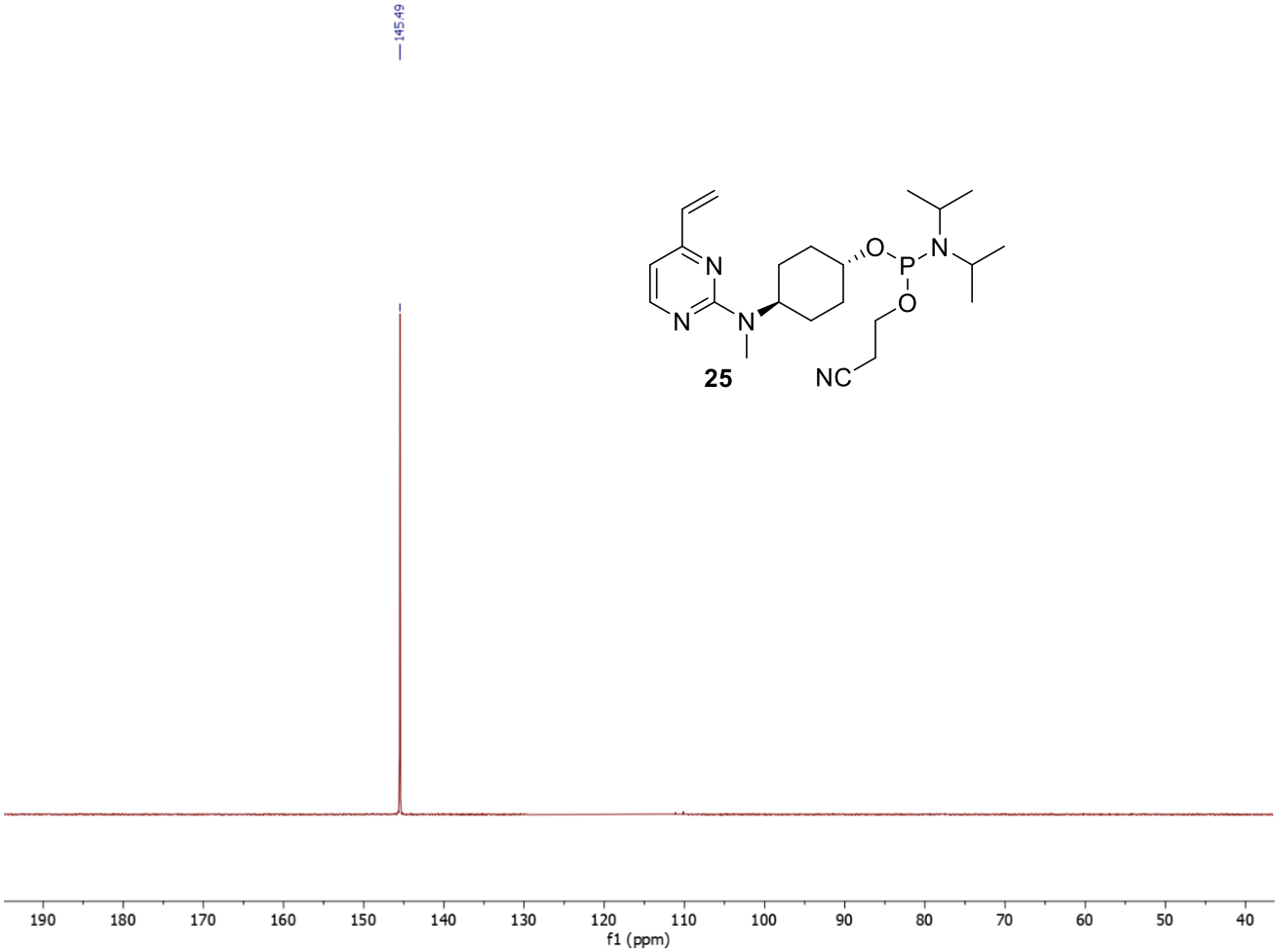
2-cyanoethyl ((1*R*,4*R*)-4-(methyl(4-vinylpyrimidin-2-yl)amino)cyclohexyl) diisopropylphosphoramidite (Mixture of Diastereoisomers) (25)

^{1}H NMR (400 MHz, CD_3CN)



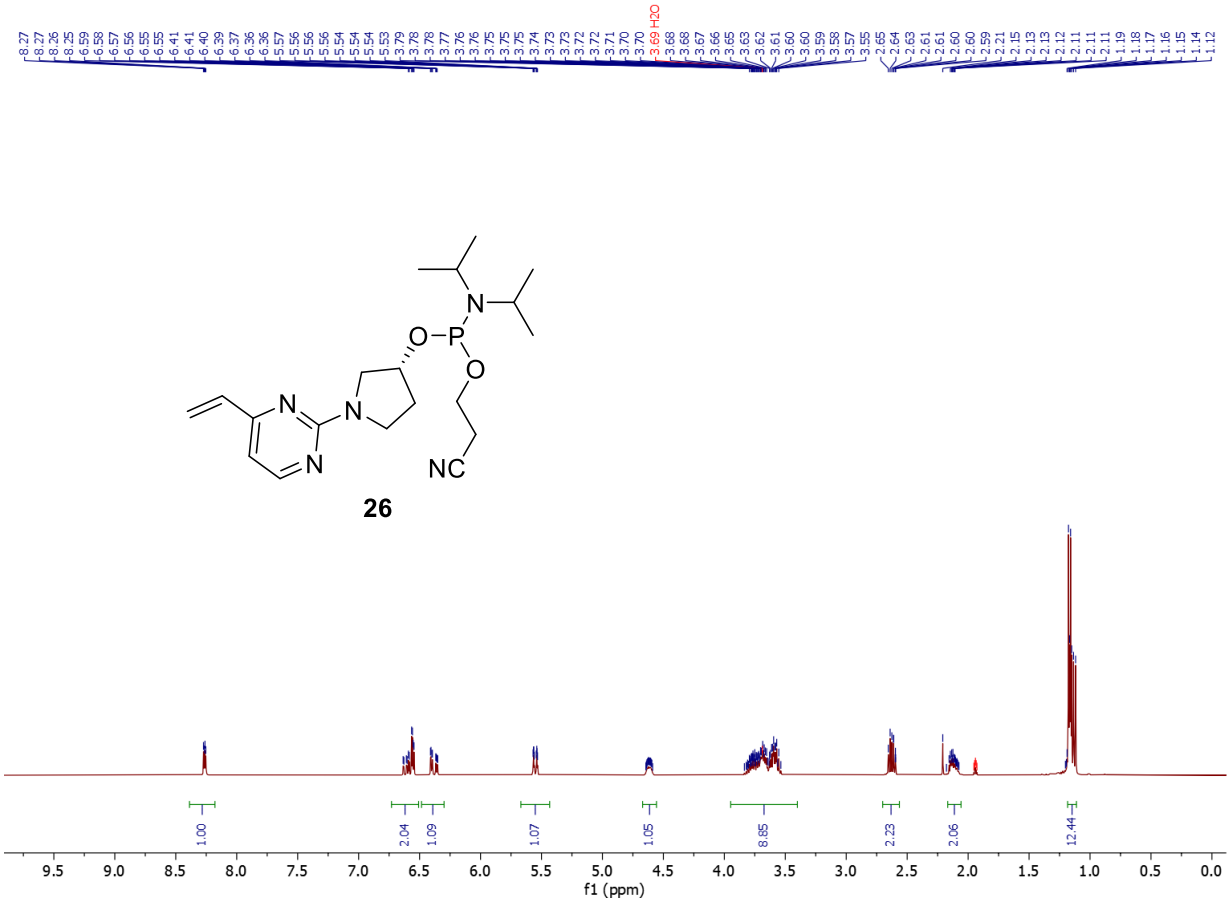
2-cyanoethyl ((1*R*,4*R*)-4-(methyl(4-vinylpyrimidin-2-yl)amino)cyclohexyl) diisopropylphosphoramidite (Mixture of Diastereoisomers) (25)

^{31}P NMR (400 MHz, CD_3CN)



2-cyanoethyl ((*R*)-1-(4-vinylpyrimidin-2-yl)pyrrolidin-3-yl) diisopropylphosphoramidite (Mixture of Diastereoisomers) (26)

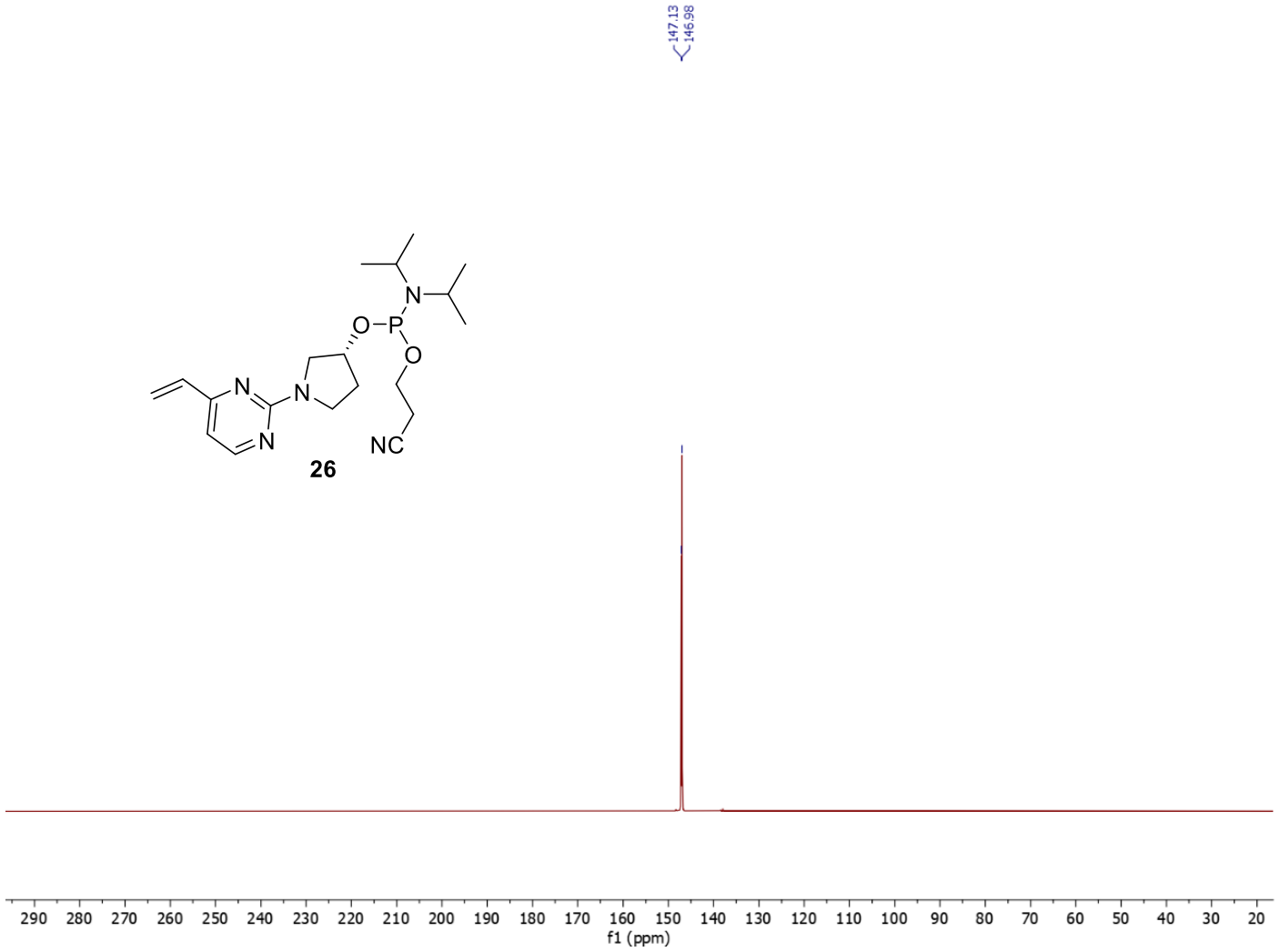
^{1}H NMR (400 MHz, CD_3CN)



26

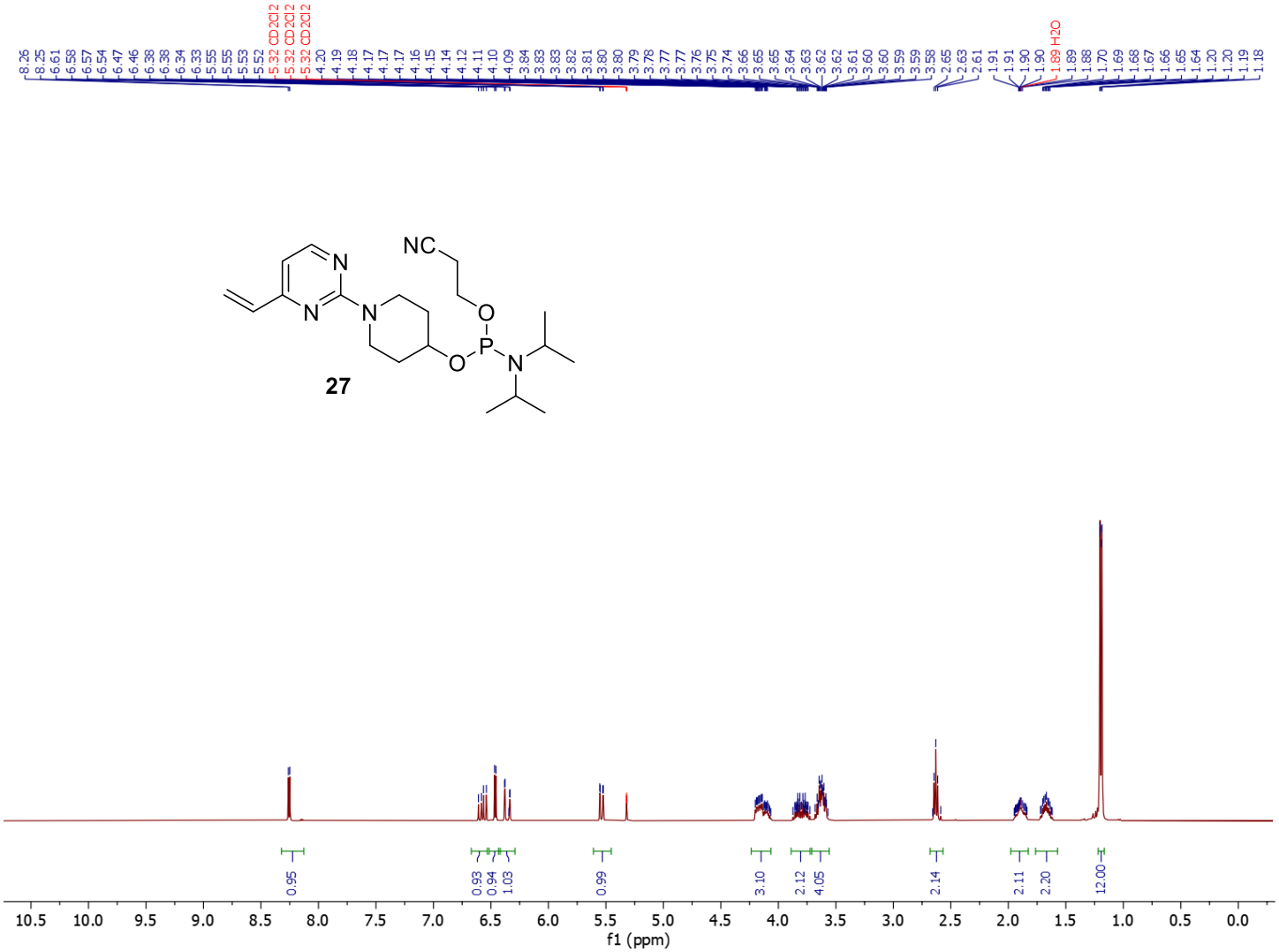
2-cyanoethyl ((*R*)-1-(4-vinylpyrimidin-2-yl)pyrrolidin-3-yl) diisopropylphosphoramidite (Mixture of Diastereoisomers) (26)

^{31}P NMR (400 MHz, CD_3CN)



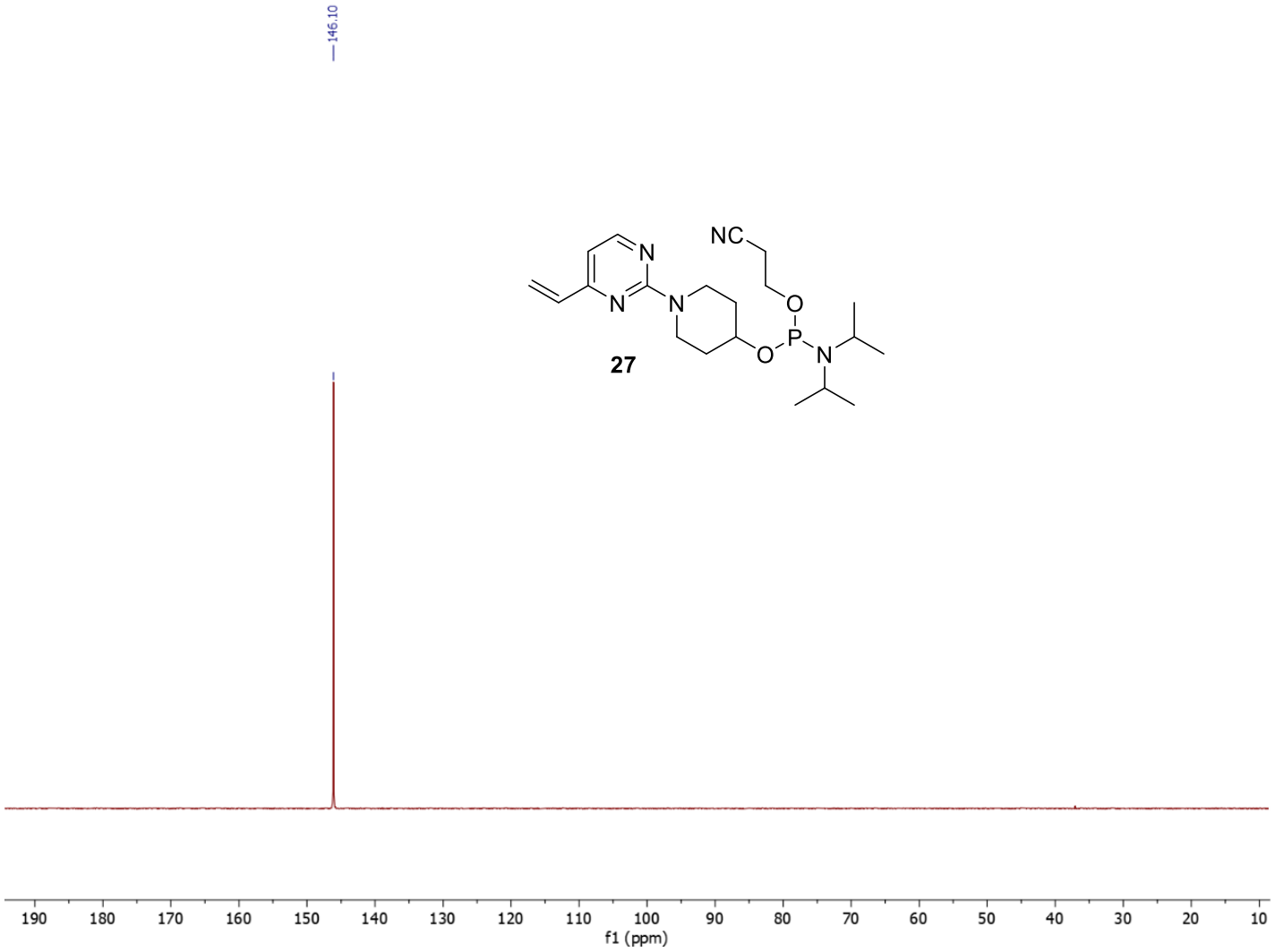
2-cyanoethyl (1-(4-vinylpyrimidin-2-yl)piperidin-4-yl) diisopropylphosphoramidite (Mixture of Diastereoisomers) (27)

^{1}H NMR (400 MHz, CD_2Cl_2)



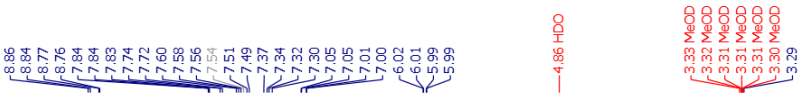
2-cyanoethyl (1-(4-vinylpyrimidin-2-yl)piperidin-4-yl) diisopropylphosphoramidite (Mixture of Diastereoisomers) (27)

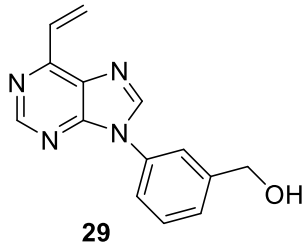
^{31}P NMR (400 MHz, CD_2Cl_2)



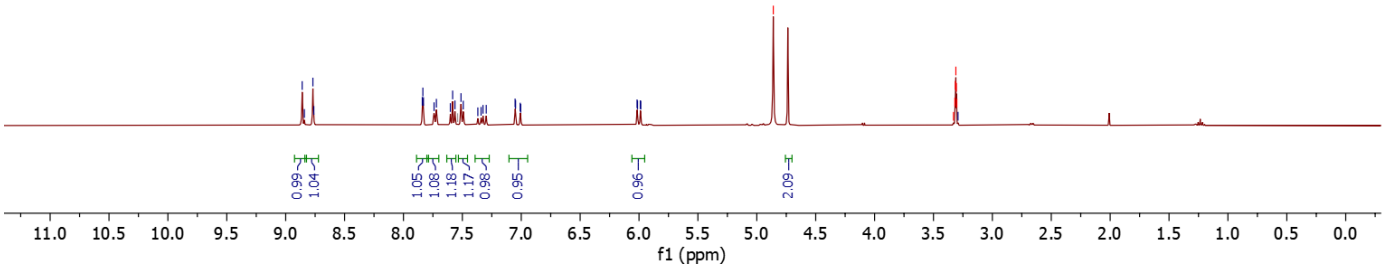
(3-(6-vinyl-*9H*-purin-9-yl)phenyl)methanol (29)

^{1}H NMR (400 MHz, Methanol-d4)



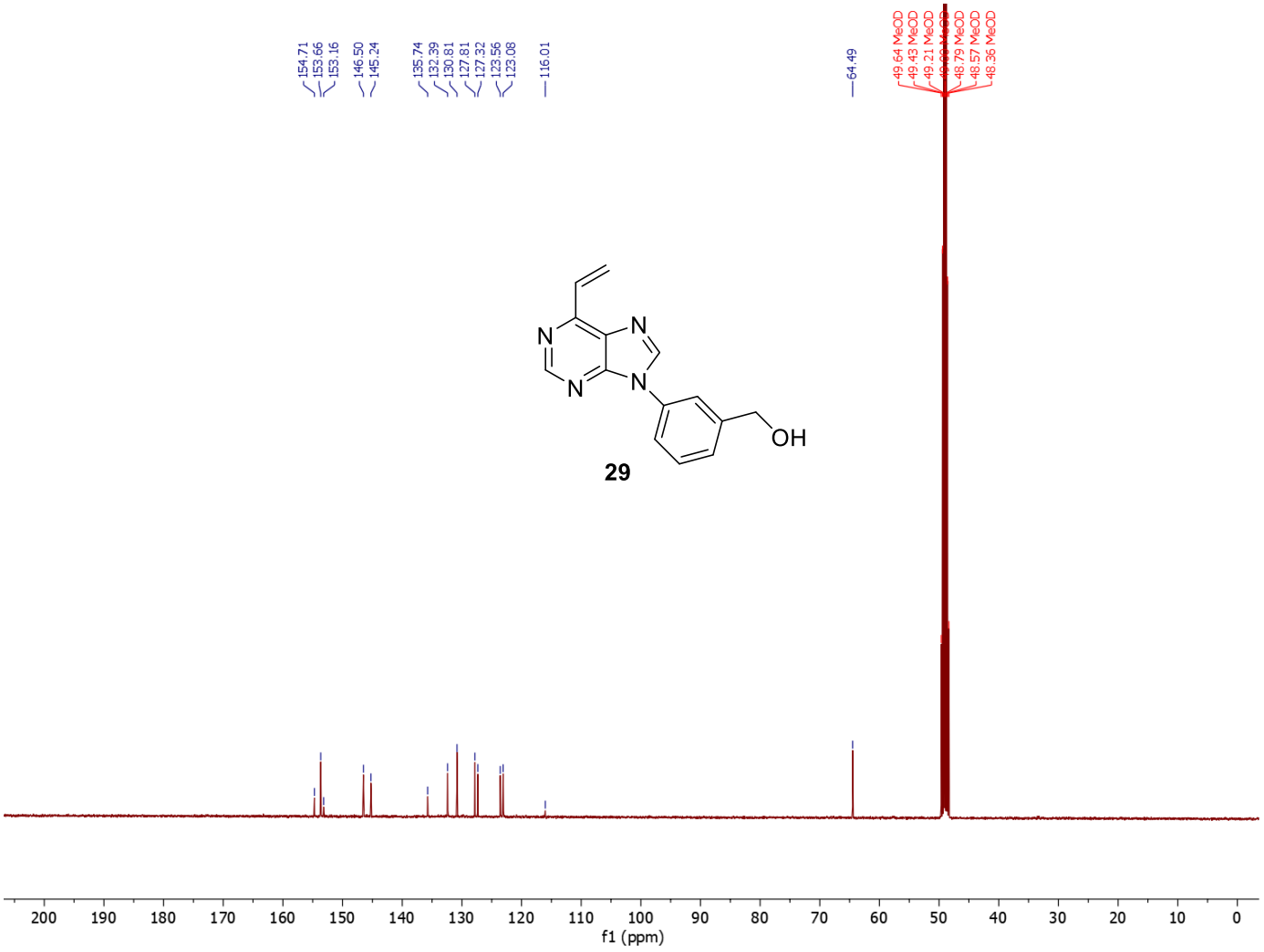


29



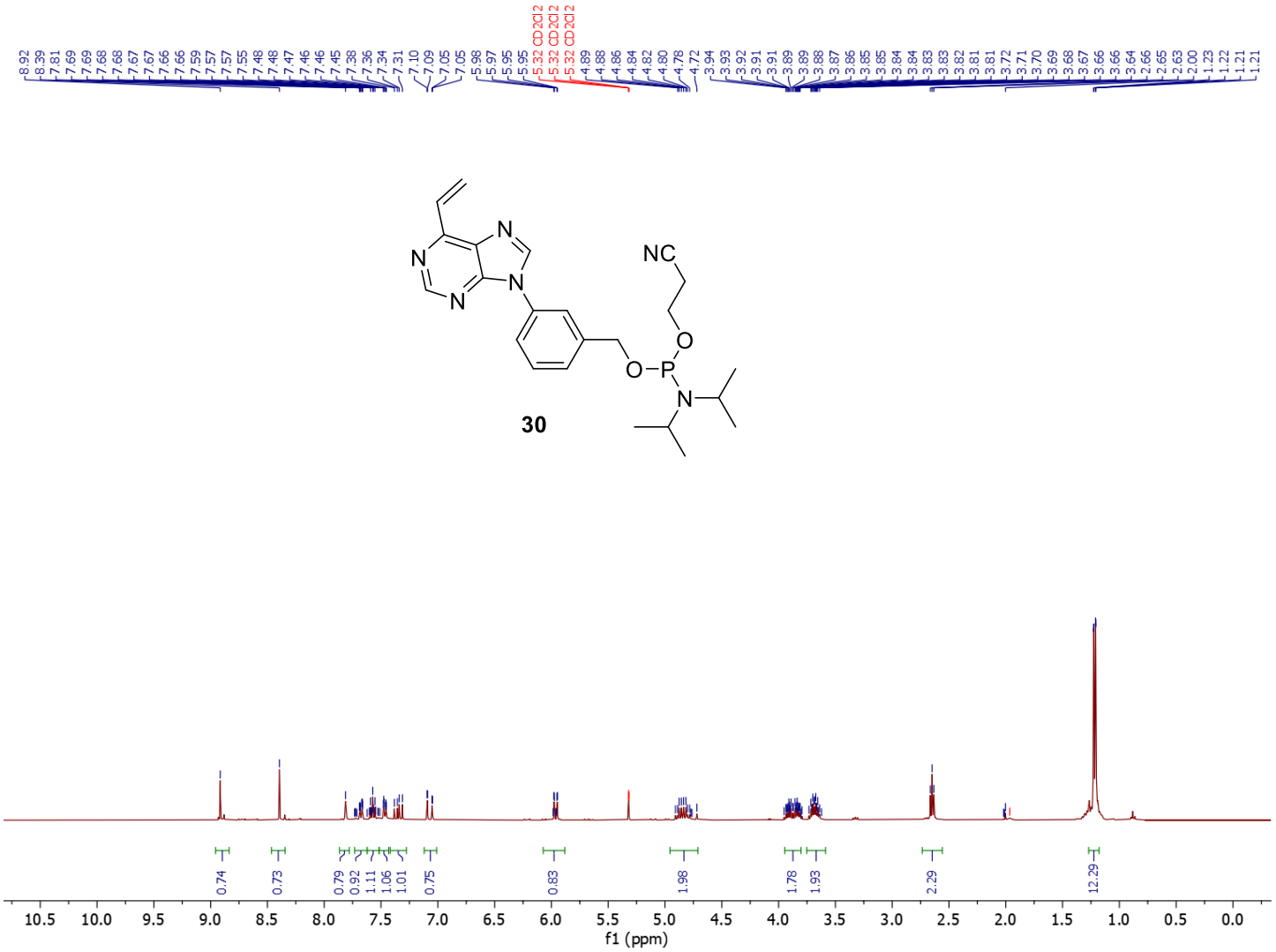
(3-(6-vinyl-9*H*-purin-9-yl)phenyl)methanol (29)

^{13}C NMR (101 MHz, Methanol-d4)



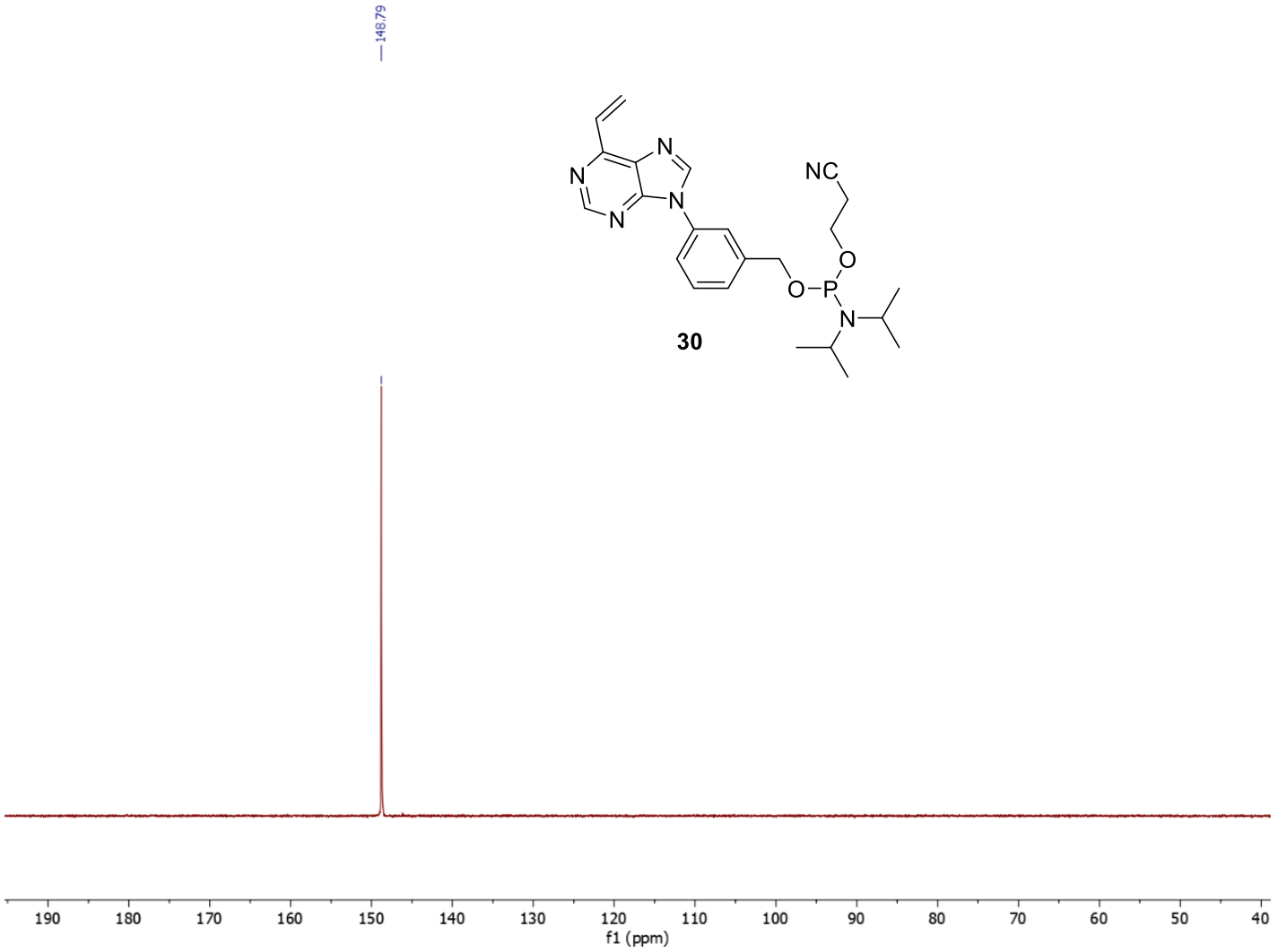
2-cyanoethyl (3-(6-vinyl-9*H*-purin-9-yl)benzyl) diisopropylphosphoramidite (Mixture of Diastereoisomers) (30)

^{1}H NMR (400 MHz, CD_2Cl_2)



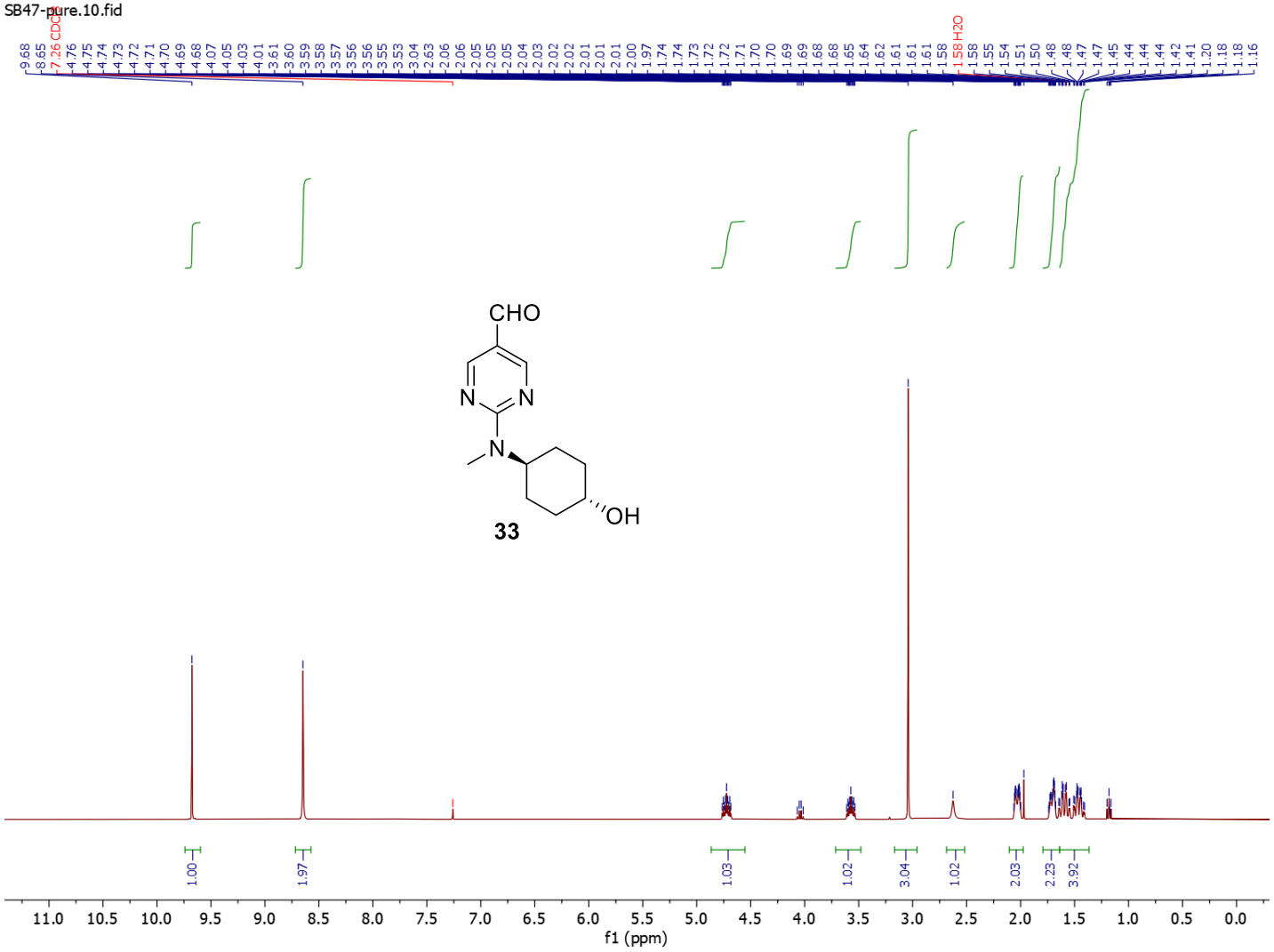
2-cyanoethyl (3-(6-vinyl-9*H*-purin-9-yl)benzyl) diisopropylphosphoramidite (Mixture of Diastereoisomers) (30)

^{31}P NMR (400 MHz, CD_2Cl_2)



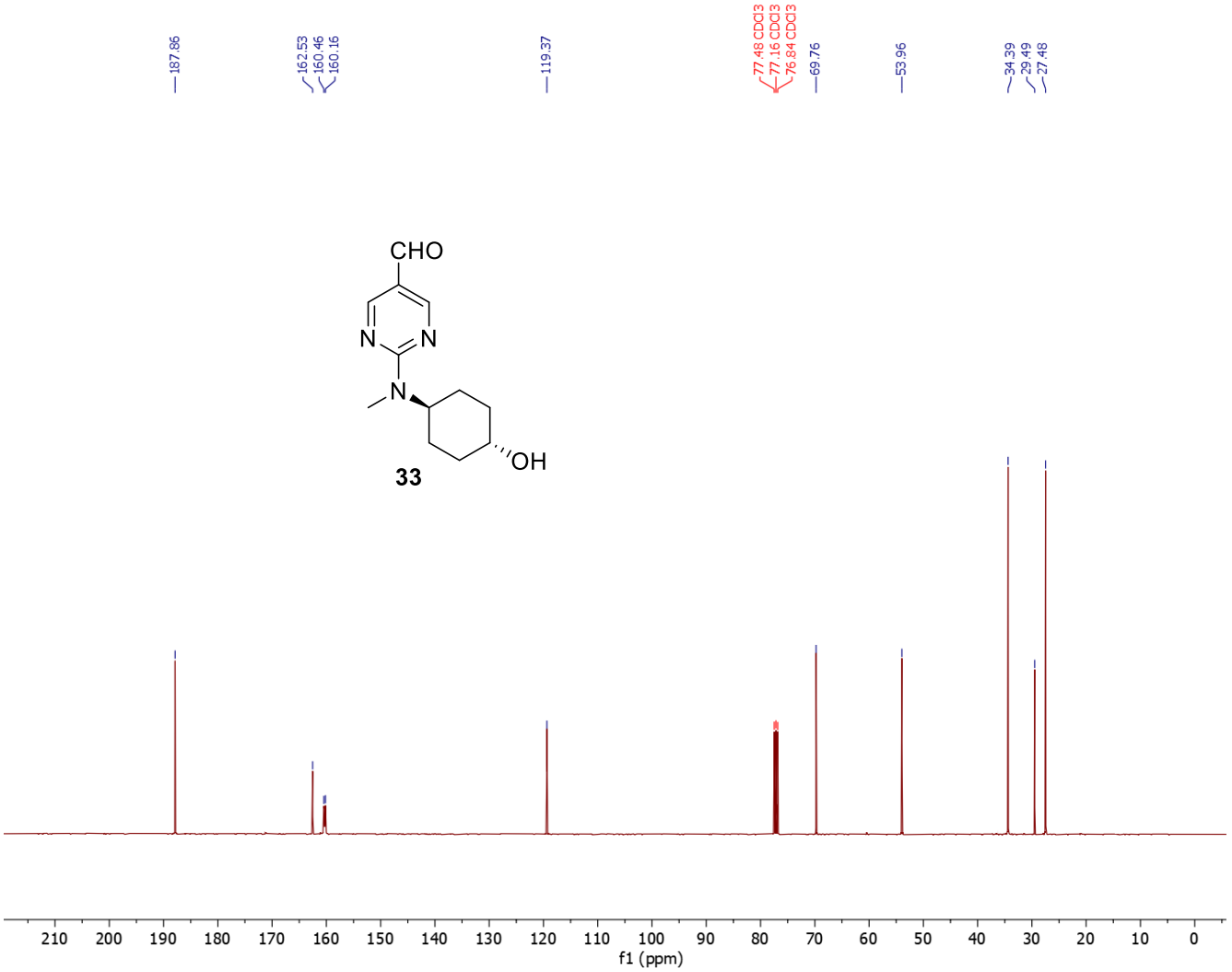
2-((1*R*,4*R*)-4-hydroxycyclohexyl)(methyl)amino)pyrimidine-5-carbaldehyde (33)

^{1}H NMR (400 MHz, $CDCl_3$)



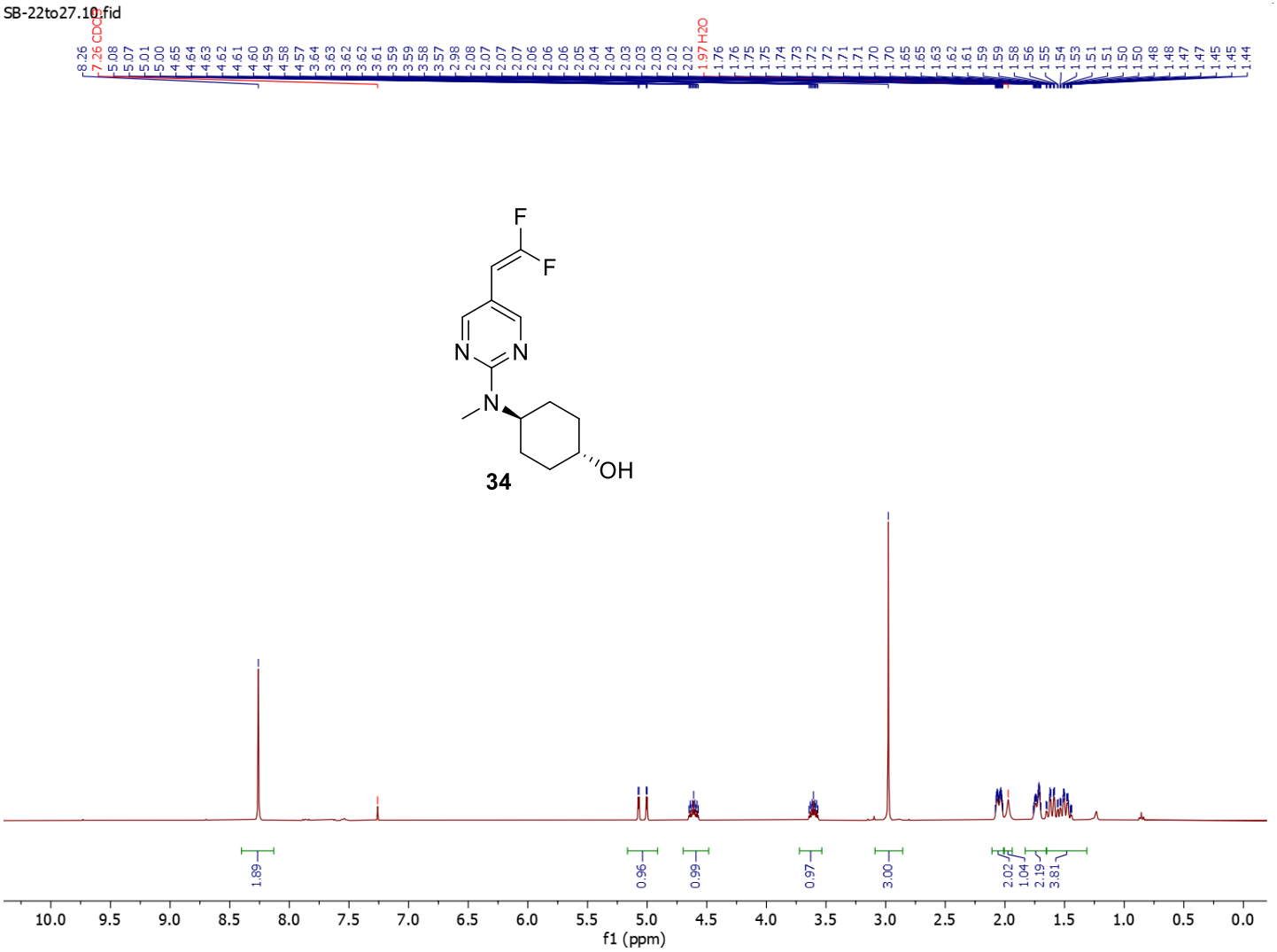
2-((1*R*,4*R*)-4-hydroxycyclohexyl)(methyl)amino)pyrimidine-5-carbaldehyde (33)

^{13}C NMR (101 MHz, $CDCl_3$)



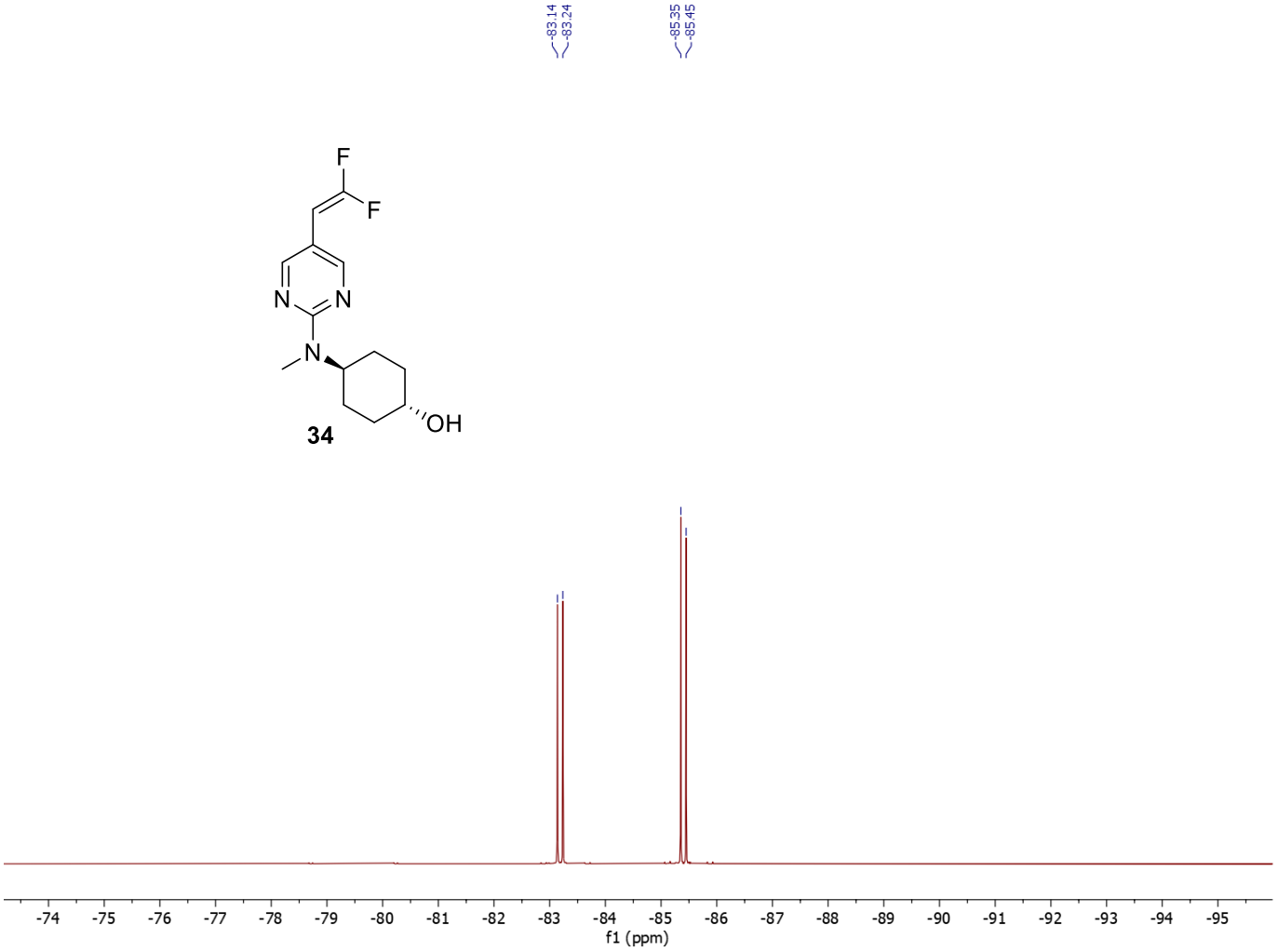
(1*R*,4*R*)-4-((5-(2,2-difluorovinyl)pyrimidin-2-yl)(methyl)amino)cyclohexan-1-ol (34)

^{1}H NMR (400 MHz, $CDCl_3$)



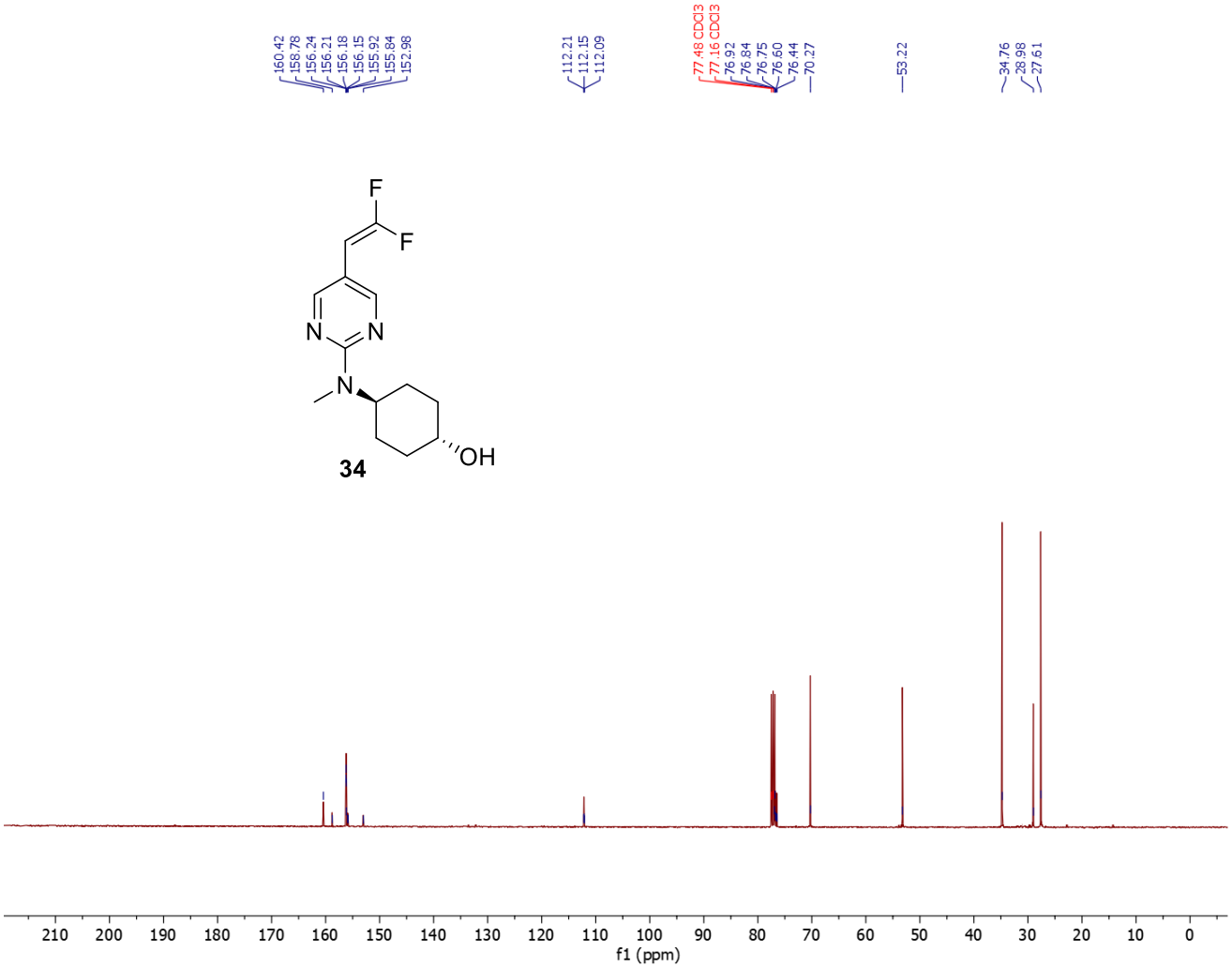
(1*R*,4*R*)-4-((5-(2,2-difluorovinyl)pyrimidin-2-yl)(methyl)amino)cyclohexan-1-ol (34)

^{19}F NMR (376 MHz, $CDCl_3$)



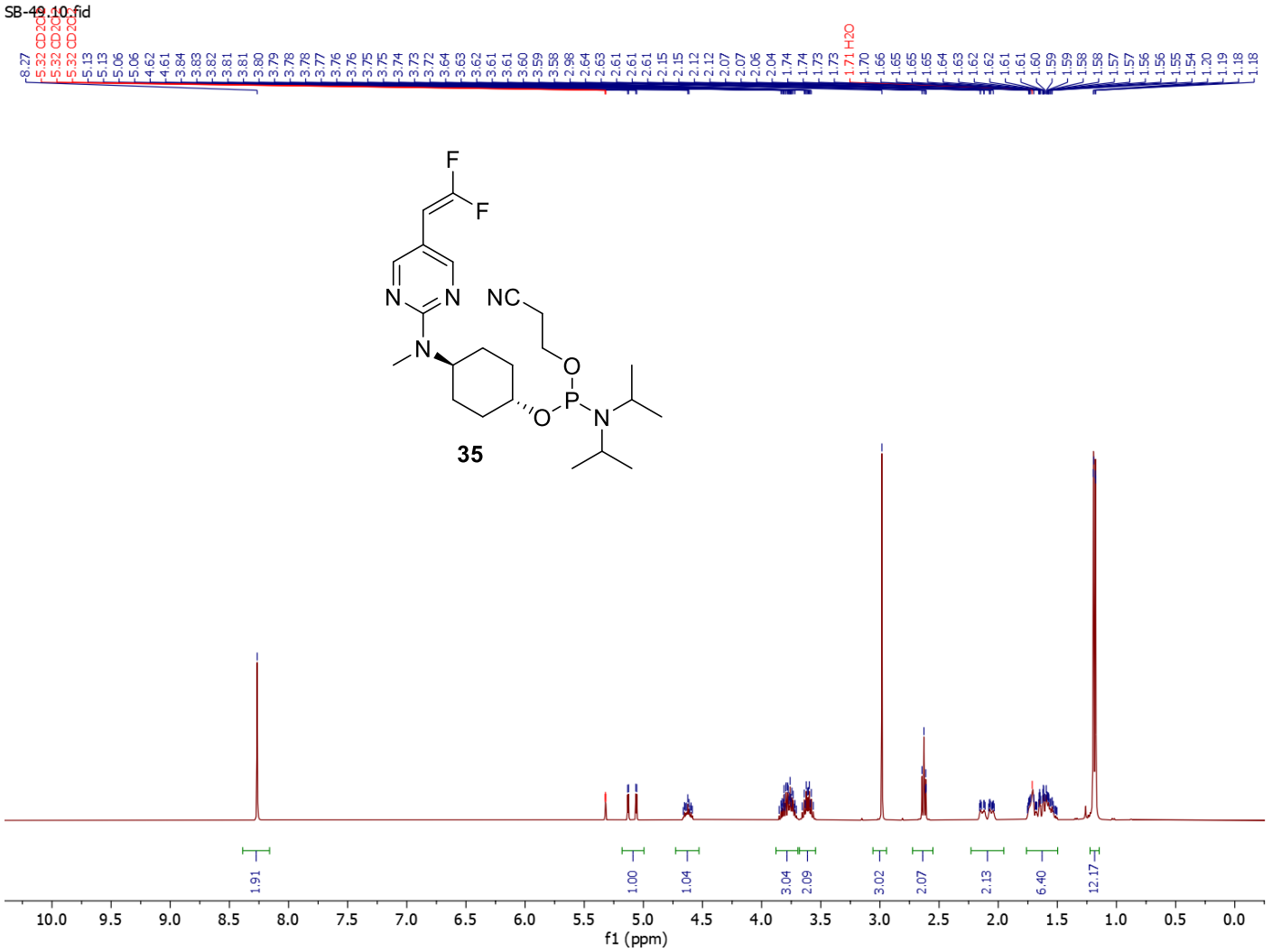
(1*R*,4*R*)-4-((5-(2,2-difluorovinyl)pyrimidin-2-yl)(methyl)amino)cyclohexan-1-ol (34)

^{13}C NMR (101 MHz, $CDCl_3$)



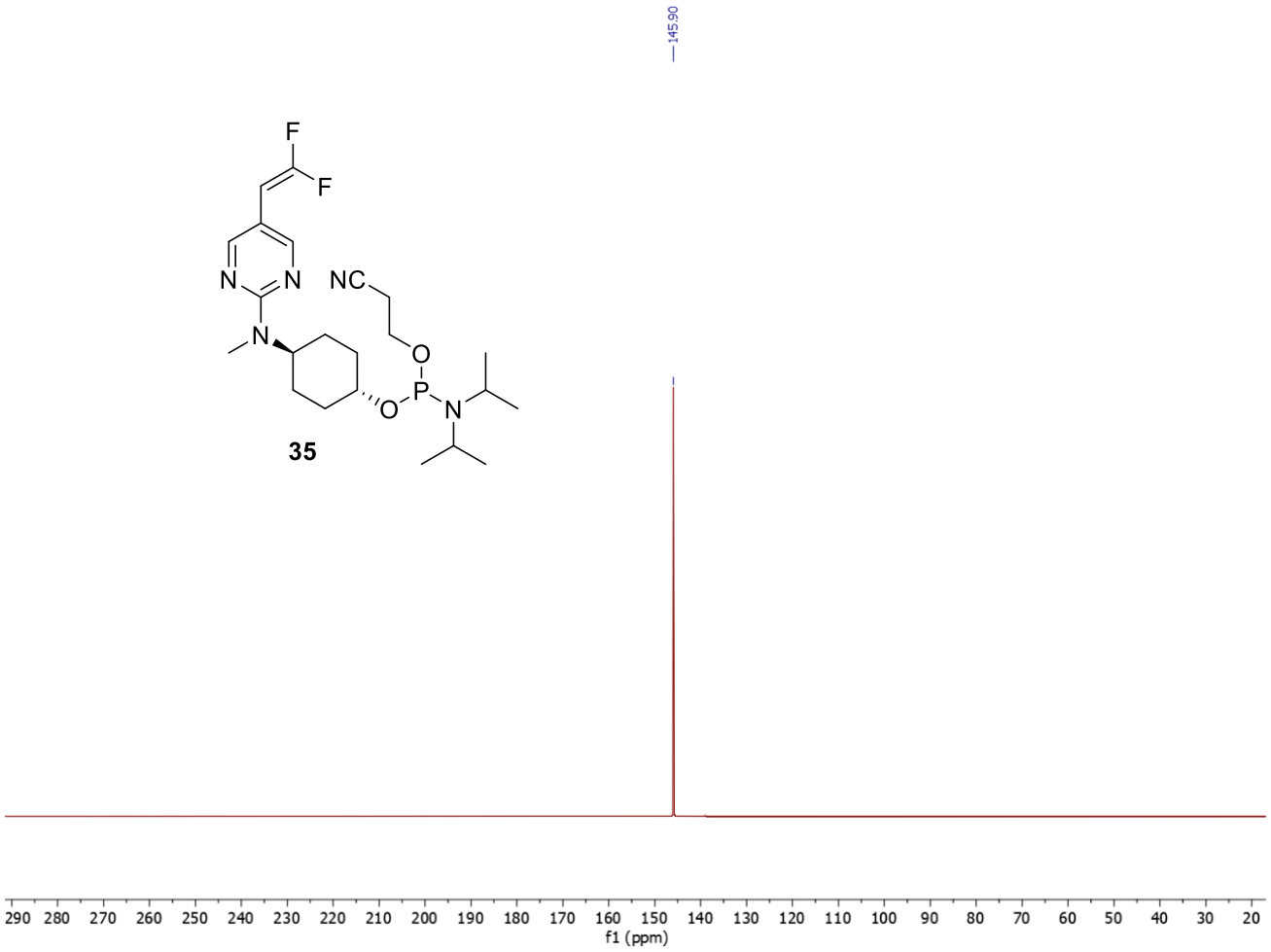
2-cyanoethyl ((1*R*,4*R*)-4-((5-(2,2-difluorovinyl)pyrimidin-2-yl)(methyl)amino)cyclohexyl) diisopropylphosphoramidite (Mixture of Diastereoisomers) (35)

^{1}H NMR (400 MHz, CD_2Cl_2)



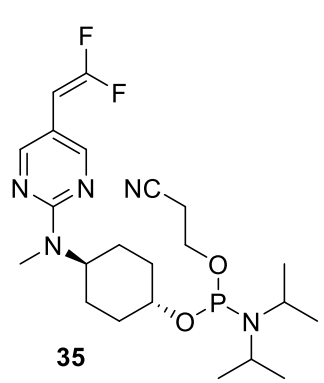
2-cyanoethyl ((1*R*,4*R*)-4-((5-(2,2-difluorovinyl)pyrimidin-2-yl)(methyl)amino)cyclohexyl) diisopropylphosphoramidite (35)

^{31}P NMR (400 MHz, CD_2Cl_2)

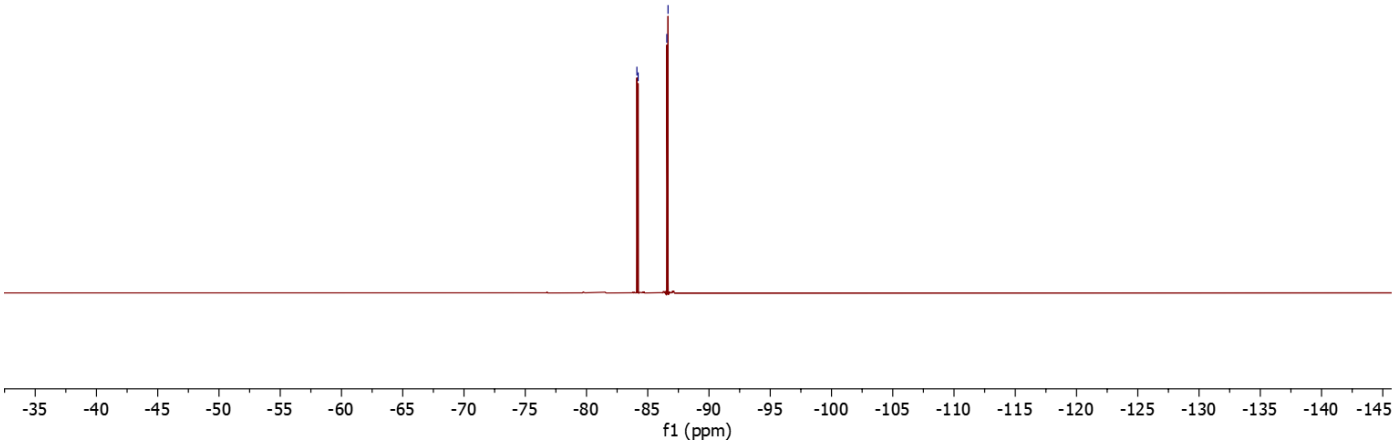


2-cyanoethyl ((1*R*,4*R*)-4-((5-(2,2-difluorovinyl)pyrimidin-2-yl)(methyl)amino)cyclohexyl) diisopropylphosphoramidite (35)

^{19}F NMR (376 MHz, CD_2Cl_2)



35



References

[i] Raux, E.; Klenc, J.; Blake, A.; Sączewski, J.; Strekowski, L. *Molecules* **2010**, *15*(3), 1973-1984.
[ii] Vanslambrouck, S.; Riva, R.; Ucakar, B.; Préat, V.; Gagliardi, M.; Molin, D.G.M.; Lecomte, P.; Jérôme, C. *Molecules* **2021**, *26*, 1750.