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Polyfluoroaromatic Stavudine (d4T) ProTides Exhibit Enhanced Anti-HIV Activity

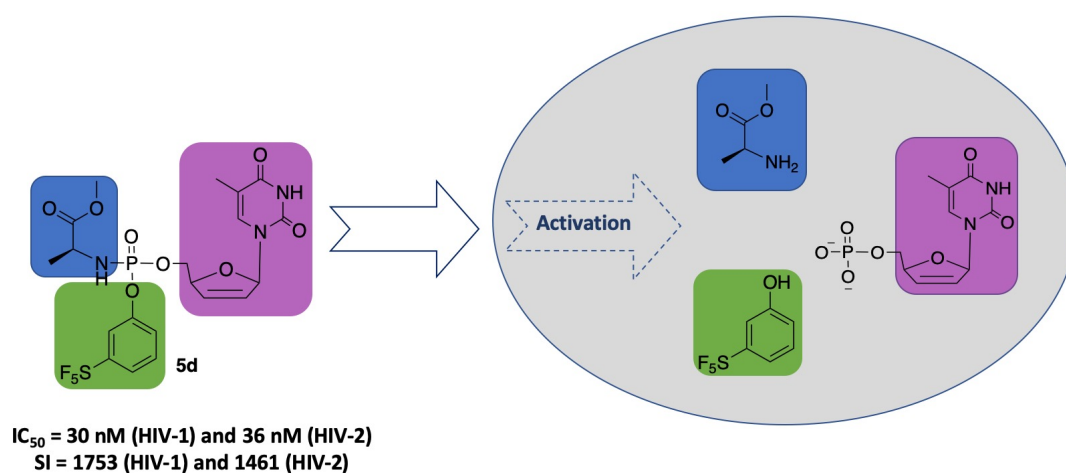
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Keywords:

Polyfluoroaromatic, pentafluorosulfanyl, SF₅, Stavudine, d4T, ProTide, Phosphoramidate, Antiviral, Nucleoside, HIV.

Graphical abstract



Highlights

- Synthesis of polyfluoroaromatic Stavudine (d4T) ProTides.
- Anti-retroviral activity against two strains; HIV-1, (III_B) and HIV-2, (ROD) in MT4 cell line.
- Evaluation of anti-HIV activity against wild type (C8166) as well as thymidine kinase deficient (C8166-TK⁻) cells.
- Nanomolar antiviral activity of novel ProTide **5d** containing 3-SF₅ aromatic ring; IC₅₀ = 30 nM, HIV-1 and IC₅₀ = 36 nM, HIV-2, more than tenfold that of d4T.
- High selectivity index of **5d**, SI = 1753 (HIV-1) and 1461 (HIV-2) twice that of d4T.
- Enzymatic activation of **5d** is monitored by ³¹P and ¹⁹F NMR.
- Polyfluoroaromatic ProTides have high *in vitro* aqueous solubility.

Human Immunodeficiency Virus (HIV) damages the immune system and leads to the life-threatening acquired immunodeficiency syndrome (AIDS). Despite the advances in the field of antiretroviral treatment, HIV remains a major public health challenge.

Nucleosides represent a prominent chemotherapeutic class for treating viral infections, however their cellular uptake, kinase-mediated activation and catabolism are limiting factors. Herein, we report the synthesis and *in vitro* evaluation of stavudine (d4T) ProTides containing polyfluorinated aryl groups against two strains; HIV-1 (strain III_B) and HIV-2 (strain ROD). ProTide **5d** containing a *meta*-substituted pentafluorosulfanyl (3-SF₅) aryl group¹⁷ showed superior antiviral activity over the parent d4T and the non-fluorinated analogue **5a**. ProTide **5d** has low nanomolar antiviral activity; (IC₅₀ = 30 nM, HIV-1) and (IC₅₀ = 36 nM, HIV-2) which is over tenfold more potent than d4T. Interestingly, ProTide **5d** showed significantly high selectivity indices (SI) = 1753 (HIV-1) and 1461 (HIV-2) which is more than twice that of the d4T. All ProTides were screened in wild type as well as thymidine kinase deficient (TK⁻) cells. Enzymatic activation of ProTide **5d** using carboxypeptidase Y enzyme and monitored using both ³¹P and ¹⁹F NMR is presented.

Human Immunodeficiency Virus (HIV) is a retrovirus, of the family *Retroviridae*, which is characterised by the presence of the reverse transcriptase (RT) enzyme¹. HIV damages the immune system and causes higher susceptibility to life-threatening opportunistic infections, known as acquired immunodeficiency syndrome (AIDS). Many advances have been made in antiretroviral therapy that have turned AIDS into a chronic condition rather than a fatal illness². However, HIV remains a major public health challenge because of the high mutation rate of HIV-RT, the adverse side effects of the antiretroviral treatment and the poor patient compliance in clinical applications³. Nucleoside / nucleotide reverse-transcriptase inhibitors (NRTIs/NtRTIs) represent the first major class of antiretroviral drugs developed⁴.

Nucleoside analogues are a well-established class of antiviral and anticancer therapeutic agents. Enzymatic phosphorylation is required to convert the nucleoside analogues to the 5'-monophosphates and subsequently to the triphosphate derivatives⁵⁻⁷. Often the three-step intracellular phosphorylation is inefficient and rate-limited by the initial monophosphorylation step. Further, reduced expression of nucleoside kinases leads to resistance to the nucleoside analogue treatment^{5,6}. Therefore, notable efforts have focused on the delivery of monophosphate prodrugs that are capable of masking the negative charge

of the phosphate group. The monophosphate phosphoramidate pro-nucleotides, known as ProTides, represent a successful strategy for bypassing the dependence on active transport and nucleoside kinase-mediated activation ^{8,9}. This is demonstrated clinically by sofosbuvir (anti-HCV, FDA approved) ⁶ and acelarin (anticancer, Phase III) ⁹, shown in **Figure 1**.

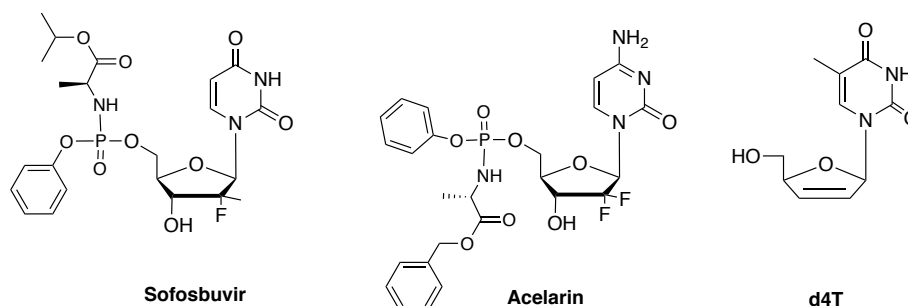


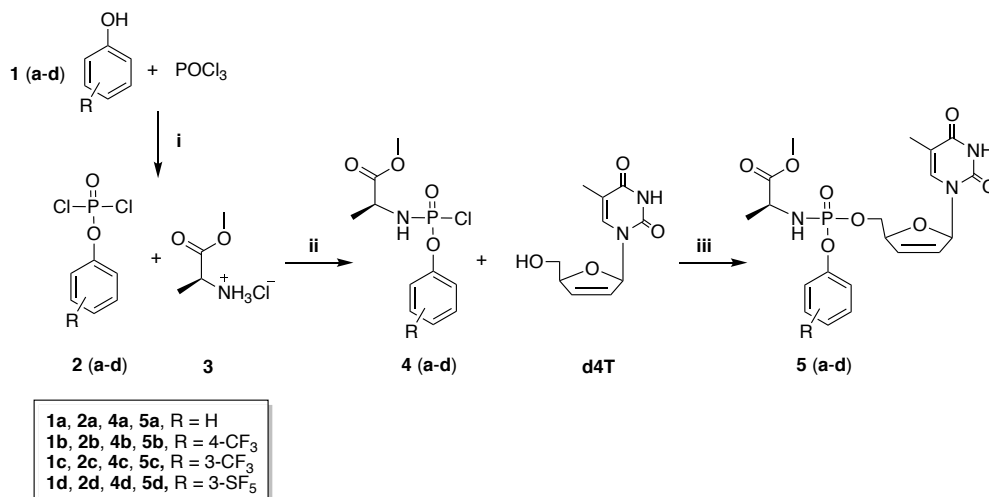
Figure 1: Chemical structures of sofosbuvir (anti-HCV, FDA approved), acelarin (anticancer, Phase III) ProTides and stavudine (d4T) nucleoside.

Stavudine (2',3'-didehydro-2',3'-dideoxythymidine, d4T) is an approved antiviral nucleoside for the prevention or treatment of HIV infection, usually as part of antiretroviral combination therapy¹⁰. In an attempt to overcome the first intracellular thymidine kinase-directed phosphorylation step of d4T, phosphoramidate derivatives were prepared and evaluated against HIV¹¹.

Generally, introduction of fluorinated substituents can provide a unique combination of electronegativity, size and lipophilicity and can also greatly affect physicochemical properties¹²⁻¹⁴. In this work we synthesised phosphoramidate derivatives of d4T (**Scheme 1**) and compared the effect of introducing different polyfluorinated variants of the aromatic ring (4-CF₃ / 3-CF₃ / 3-SF₅) and investigated whether the lipophilic and electronegative properties of the corresponding ProTides would affect their anti-HIV activity and toxicity profiles compared to the non-fluorinated ProTide.

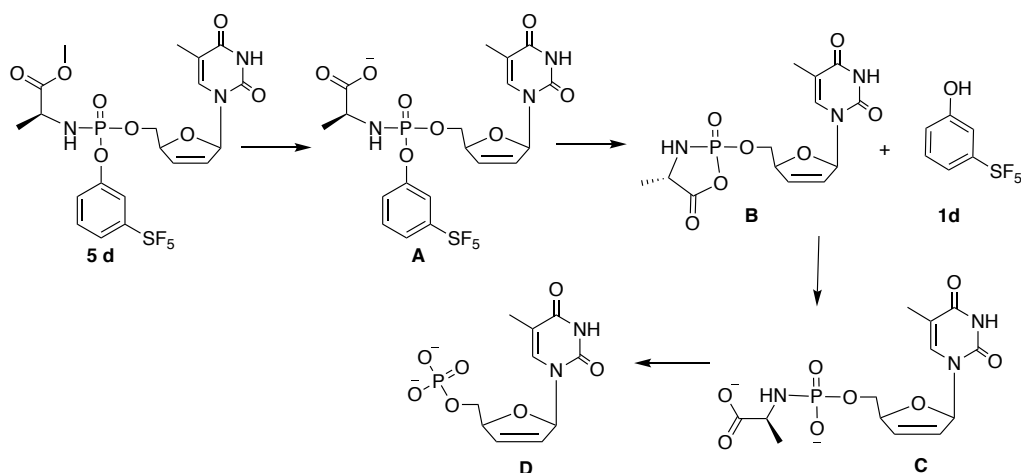
Stavudine (d4T) ProTides were prepared using previously described phosphorochloridate chemistry¹⁵. The phenyl phosphorodichloridates **2a-d** were prepared by the reaction of substituted phenols **1a-d** with POCl₃. The subsequent reaction with methyl alanine **3** to form the corresponding phosphorochloridates **4a-d** was monitored by ³¹P NMR. Next the arylaminoacyl phosphorochloridates **4a-d** were reacted with d4T in the presence of *t*-BuMgCl as a hindered base (**Scheme 1**). Stirring for 24h at ambient temperature generated the crude ProTides **5a-d** that were purified by column chromatography to provide the characteristic low to moderate yields associated with ProTide chemistry, for the final products (20–34%)¹⁶. Each

of the phosphoramidates was generated as a pair of diastereoisomers at the phosphorus centre, in a roughly 1:1 ratio, as revealed by the two closely spaced peaks in the ^{31}P NMR spectrum (see Supporting Information). It is worth mentioning that due to the instability of the intermediates it was not feasible to prepare the 4-SF₅ analogue.



Scheme 1. Synthesis of d4T phosphoramidate analogues; Reagents and conditions: i) Et₃N, anhydrous Et₂O, -78 °C to rt, 2 h, ii) Et₃N, anhydrous DCM, -78 °C, 3 h, iii) *t*-BuMgCl, anhydrous THF, -78 °C to rt, 24 h.

ProTides need to be metabolised to release the monophosphate form, which will then undergo further intracellular phosphorylation steps to generate di- and triphosphate active form. The proposed intracellular activation pathway of the most active ProTide **5d** is described in **Scheme 2**, in accordance with previous literature¹⁵. Metabolic breakdown starts with the hydrolysis of the ProTide carboxylate ester, mediated by a carboxyesterase-type enzyme to form intermediate **A**, which is followed by a spontaneous cyclisation displacing the aryl moiety via an internal nucleophilic attack of the carboxylate residue on the phosphorus centre to yield intermediate **B**. In the third step, the unstable cyclic mixed anhydride is thought to be hydrolysed to release the intermediate **C**. The final step then involves a phosphoramidase-type enzyme, which would cleave off the amino acid to generate the corresponding monophosphate **D**, trapped within the cell due to the polar nature of the monophosphate.



Scheme 2. Proposed activation pathway of **d4T** ProTide **5d** initiated by esterase or carboxypeptidase enzyme.

To probe the activation process of ProTide **5d**, **Scheme 2**, an enzymatic study using carboxypeptidase Y enzyme was carried out. ProTide **5d** was incubated with carboxypeptidase Y to verify that the enzymatic cleavage of the ester motif is sufficient to initiate the activation process and generate intermediate **C**. Compound **5d** was dissolved in acetone- d_6 in the presence of Trizma buffer (pH 7.6), treated with carboxypeptidase Y and monitored by ^{31}P NMR chemical shift changes over time, **Figure 2**. Two peaks at δ 3.61 and 3.27 ppm in the blank spectrum recorded at 25 °C correspond to the diastereoisomers of the parent compound **5d**. Within 7 mins of incubation with carboxypeptidase Y, a single peak at around δ 6 ppm started appearing, which is consistent with the single chemical shift expected for the formation of achiral intermediate **C** via the putative intermediate **B**. The peak height increased over time suggesting the accumulation of a stable product. The spectra recorded during the enzymatic reaction suggest that within 2 h of the start of the assay **5d** was almost fully converted into metabolite **C**.

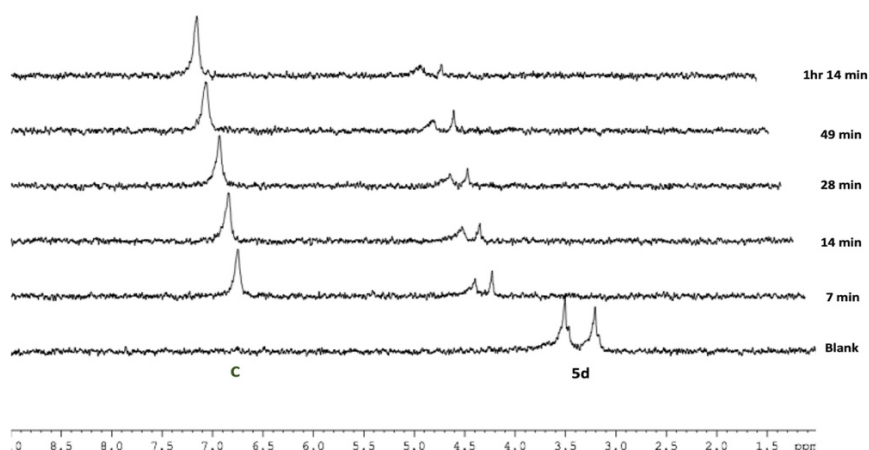


Figure 2. ^{31}P NMR spectra of **d4T** ProTide **5d** over time after carboxypeptidase Y treatment showing the signals of the metabolite **C** over time.

Moreover, due to the presence of the 3-SF₅ group in **5d**, we considered that the activation process of ProTide **5d** using carboxypeptidase Y enzyme can be also monitored using ¹⁹F NMR chemical shift changes over time, **Figure 3**. The appearance of two doublet peaks at δ 62.15 and 62.48 ppm in the blank spectrum corresponds to the diastereoisomers of the parent compound **5d**. The new doublet signal appearing at δ 61.86 and 62.21 (corresponding to the four fluorine atoms of the SF₅ group) indicating the release of 3-SF₅ phenol (**1d**).

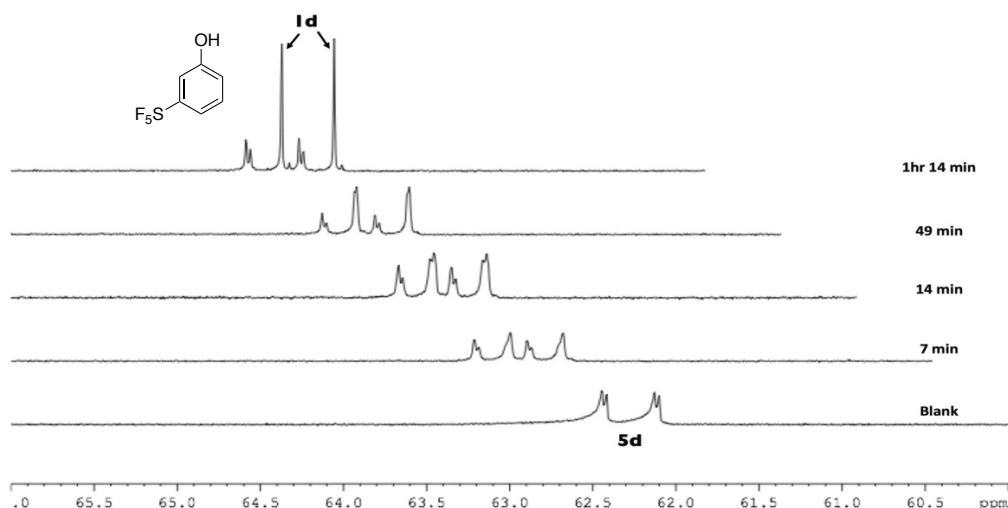


Figure 3. ¹⁹F NMR spectra of ProTide **5d** over time after carboxypeptidase Y treatment showing the signals of the metabolites over time.

Both ³¹P NMR and ¹⁹F NMR monitoring experiments, **Figure 2** and **3**, indicate that ProTide **5d** activation is initiated by the carboxypeptidase enzyme Y and that after 1.25h of incubation most of the ProTide is metabolised. Quantification of intracellular drug levels would be the focus of future investigation.

Nucleoside reverse transcriptase inhibitors (NRTIs) and nucleotide analogue reverse-transcriptase inhibitors (NtRTIs) act as chain terminators for both viral and host DNA synthesis⁴. The former describes their antiviral activity, while the latter describes their drug toxicity/side effects. Hence, d4T ProTides **5a-d** were screened for both antiviral activity and cytotoxicity.

The antiviral activity profile of compounds **5a-d** was evaluated in MT4 cells against two HIV strains; HIV-1 (III_B) and HIV-2 (ROD) in parallel with stavudine (d4T) as a positive control. The biological results are expressed as IC₅₀, CC₅₀ and SI (selectivity index; CC₅₀/IC₅₀ ratio).

All ProTides showed markedly enhanced antiviral activity compared to the parent d4T except the non-fluorinated ProTide **5a** which is slightly more potent than d4T. The best activity was

observed across both HIV types in ProTide **5d** with the *meta*-pentafluorosulfanyl (3-SF₅) substituent attached to its aromatic ring. ProTide **5d** has low nanomolar antiviral activity; (IC₅₀ = 30 nM, HIV-1) and (IC₅₀ = 36 nM, HIV-2) which is over tenfold more potent than d4T, **Table 1**. Additionally, ProTide **5d** showed significantly high selectivity indices (SI) = 1753 (HIV-1) and 1461 (HIV-2) which is more than twice that of the d4T. This could be attributed to its enhanced lipophilic and/or electronegative properties imparted by the presence of five fluorine atoms. Also, the position of the CF₃ substituent in the phenyl ring of **5b** and **5c** has little influence on the anti-HIV activity but more effect on the toxicity profile which was in favour of the *meta*-CF₃ (**5c**) over the *para*-CF₃ (**5b**) as observed in the selectivity index values; SI (**5c**) = 1612 (HIV-1) and 1349 (HIV-2) compared to SI (**5b**) = 1159 (HIV-1) and 921 (HIV-2), **Table 1**.

comp	HIV-1 (III _B)			HIV-2 (ROD)		
	IC ₅₀ (μM)	CC ₅₀ (μM)	SI	IC ₅₀ (μM)	CC ₅₀ (μM)	SI
d4T	0.40±0.09	313±7	782	0.45±0.09	313±7	695
5a	0.21±0.09	251±7	1195	0.27±0.11	251±7	930
5b	0.037±0.002	42.9±12.6	1159	0.047±0.022	42.9±12.6	912
5c	0.041±0.006	66.1±24.8	1612	0.049±0.017	66.1±24.8	1349
5d	0.030±0.012	52.6±4.9	1753	0.036±0.020	52.6±4.9	1461

Table 1. Anti-HIV-1 and 2 activity and cytotoxicity of stavudine (d4T) and ProTide analogues **5a-d**.

Further, we tested our compounds in the C8166 cell line and a variant thymidine kinase deficient variant (C8166-TK⁻) to probe the effect of TK deficiency on the activity of the test compounds and the degree to which the ProTides could bypass this dependence.

As shown in **Table 2**, the fluorinated ProTides **5b-d** displayed greatly enhanced antiviral potency than the non-fluorinated analogue **5a** or the parent d4T across. ProTide **5b** (4-CF₃) has around 180-fold higher activity than d4T, while ProTide **5d** (3-SF₅) is around 75-fold more active than d4T. It is interesting to observe that compounds **5a**, **5b** and **5d** can retain their activity in TK deficient cells while d4T has completely lost its activity. While ProTide **5c** (IC₅₀ = 0.039 μM) shows better activity than **5a** (IC₅₀ = 0.30 μM) and d4T (IC₅₀ = 4.06 μM), it is not active in TK⁻ cells. This is most probably because **5c** uptake is enhanced but it falls back to its free nucleoside form and cannot be re-phosphorylated in these cells, **Table 2**.

	HIV-1 (III _B)
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ID	IC ₅₀ WT (μM)	IC ₅₀ TK ⁻ (μM)
d4T	4.06±0.18	NA
5a	0.30±0.04	0.32±0.15
5b	0.022±0.006	0.075±0.000
5c	0.039±0.007	NA
5d	0.054±0.039	0.335±0.004

Table 2. Antiviral activity of stavudine (d4T) and ProTide analogues **5a-d** against HIV-1 strain III_B in the C8166 cell line and the thymidine kinase deficient (C8166-TK⁻) cell line. NA: not active

Introduction of fluorine atoms into molecular structures increases their hydrophobicity. Thus, we investigated the *in vitro* turbidimetric aqueous solubility of the polyfluorinated ProTides **5b-d** using five concentrations (1, 3, 10, 30 and 100 μM) in phosphate buffered saline (PBS; pH 7.4) solution (1% DMSO), which were incubated at 37°C for 2 hr. The estimated precipitation range (lower and upper bound) and a mid-range value were calculated for compounds **5b-d**. Nicardipine and pyrene were used as control compounds. The results show that ProTides with polyfluoroaromatic substituents **5b-d** retain high aqueous solubility with estimated precipitation range 100 μM (lower bound) and >100 μM (upper bound), **Table 3**, confirming their potential advantage for intestinal absorption.

Compound	MW	ClogP ^a	Estimated Precipitation range (μM)			Estimated solubility (μM)
			Lower bound	Upper bound	Calculated mid-range	
d4T	224.22	-0.4875	100	100	100	> 100
5b	533.40	1.9788	100	100	100	> 100
5c	533.40	1.9788	100	100	100	> 100
5d	591.44	2.3288	100	100	100	> 100
Nicardipine	-	-	10	30	20	10-30
pyrene	-	-	3	10	6.5	3-10

Table 3. Aqueous solubility of **5b-d** versus control compounds (nicardipine and pyrene) in PBS (pH=7.4) buffered aqueous solution. ^aCalculated by PerkinElmer ChemDraw 16.0 software.

In this study, a series of polyfluoroaromatic ProTides of d4T were synthesised. The antiviral activity was evaluated *in vitro* against the two HIV types; HIV-1 (strain III_B) and HIV-2 (strain ROD). Our findings identified compound **5d** featuring a *meta*-pentafluorosulfanyl (SF₅) in its aromatic ring as promising lead. ProTide **5d** has low nanomolar antiviral activity; (IC₅₀ = 30 nM, HIV-1) and (IC₅₀ = 36 nM, HIV-2) which is over tenfold more potent than d4T. Moreover,

5d has the best activity / toxicity profile with a selectivity index (SI) = 1753 (HIV-1) and 1461 (HIV-2), which is more than twice that of the parent d4T (SI) = 782 (HIV-1) and 695 (HIV-2). Additionally, ProTides **5a**, **5c** and **5d** retained some activity in the thymidine kinase deficient (C8166-TK⁻) cell line. Furthermore, the polyfluorinated ProTides **5b-d** retained aqueous solubility comparable to that of the parent d4T. Considering the above findings, it is concluded that **5d** represents a promising basis for further development of NtRTI anti-HIV therapies.

Acknowledgment

The authors would like to thank the Welsh Government (A4B-Academic Expertise for Business). The technical assistance of Mrs. Kristien Erven, Mr. Kris Uyttersprot and Mrs. Cindy Heens for the HIV experiments is gratefully acknowledged. The authors also would like to dedicate this article to the memory of Prof. Christopher McGuigan.

Supplementary data

Supplementary data including chemistry and carboxypeptidase Y enzymatic assay procedures. ¹H, ³¹P, ¹⁹F and ¹³C NMR spectra of Protides **5a-d** as well as HPLC analysis reports associated with this article can be found, in the online version.

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16. General experimental procedure for the preparation of ProTides (5a-d)

d4T phosphoramidates (**5a-d**) were prepared applying the previously described phosphochloridate chemistry. To a stirring solution of d4T (1.0 eq.) in THF, *tert*-butylmagnesium chloride (1.0 M in THF) (1.1 eq) was added dropwise over 1 min, and the appropriate phosphochloridate (3.0 eq.) in anhydrous THF was added at room temperature and stirred for 16 h. The solvent was removed under reduced pressure and the yellow oil obtained was dissolved in DCM and washed with 0.5 M HCl and water. The organic layer was dried over MgSO₄, filtered, reduced to dryness and purified by flash chromatography.

17. Spectral data of ProTide 2',3'-Didehydro-2',3'-dideoxythymidine-5'-(3-(pentafluorosulfanyl) phenyl methoxy alaninyl phosphate) (5d); ¹H NMR (CDCl₃): δ 9.11 (bs, 1H, NH), 7.54-7.49 (m, 2H, ArH), 7.41-7.31 (m, 2H, ArH), 7.21-7.11 (2m, 1H, H-6), 6.97-6.93 (2m, 1H, H-1'), 6.28-6.20 (2m, 1H, H-2'), 5.88-5.82 (2m, 1H, H-3'), 5.00-4.92 (2m, 1H, H-4'), 4.35-4.17 (2m, 2H, H-5'), 3.98-3.88 (m, 1H, Ala CH), 3.64, 3.64 (2s, 3H, OCH₃), 1.82 (bs, 1H, NH), 1.78, 1.74 (2d, J = 1 Hz, 3H, CH₃-5), 1.32, 1.27 (2d, J = 6.5 Hz, 3H, Ala CH₃); ³¹P NMR (CDCl₃): δ 3.10, 2.60; ¹⁹F NMR (CDCl₃): δ 83.21 (p, J = 149.9 Hz, 1F), 62.82, 62.80 (2d, J = 149.9, 150.4 Hz, 4F); ¹³C NMR (CDCl₃): 173.88, 173.66 (2d, ³J_{C-P} = 6.5, 7.4 Hz, C=O ester), 163.68, 163.63 (ArC, C-4), 154.41 (m, ArC, CSF₅), 150.76 (C=O, C-2), 150.18, 150.04 (2d, ²J_{C-P} = 5.8 Hz, ArC *ipso* phenyl), 135.64, 135.44 (ArCH, C-6), 133.05, 132.77 (C-3'), 129.91 (ArCH)

,127.73, 127.63 (C-2'), 123.80, 123.60 (2d, $^2J_{C-P} = 3.8$, 5 Hz, ArCH), 122.66 (m, ArCH), 118.55 (m, ArCH), 111.45, 111.27 (ArC, C-5), 89.96, 89.68 (C-1'), 84.48, 84.42 (2d, $^3J_{C-P} = 2.5$ Hz, C-4'), 67.58, 66.86 (2d, $^2J_{C-P} = 4$, 3.8 Hz, C-5'), 52.69 (OCH₃), 50.25, 50.16 (Ala CH), 20.93, 20.82 (2d, 5, 6.3 Hz, Ala CH₃), 12.35, 12.25 (CH₃, C-5). MS (ES+) m/z: 614.1 (M+Na⁺), C₂₀H₂₃F₅N₃O₈PS, Reverse-phase HPLC, eluting with H₂O/CH₃CN from 90/10 to 0/100 in 30 min; flow = 1 mL/min, t_R = 16.01 min. yield 34%.

Supporting Information

Polyfluoroaromatic Stavudine (d4T) ProTides Exhibit Enhanced Anti-HIV Activity

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Contents

Chemistry and carboxypeptidase Y enzymatic assay procedures. ¹H, ³¹P, ¹⁹F and ¹³C NMR spectra of Protides **5a-d** as well as HPLC analysis reports.

S1. Chemistry.

S2. Carboxypeptidase Y enzymatic assay procedure.

S1. Chemistry

The anhydrous solvents and any other commercially available reagents were purchased from Sigma-Aldrich (U.K.) and used without further purification. Amino acid esters were purchased from Carbosynth (U.K.). Carboxypeptidase Y and buffers were purchased from Sigma-Aldrich (U.K.). All reactions were carried out under nitrogen atmosphere. Reactions were monitored with analytical TLC on silica gel 60-F254 precoated aluminium plates and visualised under UV (254 nm) and/or using ³¹P NMR spectra. Column chromatography was performed on silica gel (35-70 µm). Preparative TLC plates (20 cm × 20 cm, 500-2000 µm) were purchased from Merck. Proton (¹H), carbon (¹³C), Fluorine (¹⁹F) and phosphorus (³¹P) NMR spectra were recorded on a Bruker Avance 500 MHz spectrometer at 25 °C. Spectra were autocalibrated to the deuterated solvent peak, and all ¹³C NMR and ³¹P NMR were proton decoupled. The purity of the final compounds was verified to be >95% by HPLC analysis using either i) ThermoSCIENTIFIC, SPECTRA SYSTEM P4000, detector SPECTRA SYSTEM UV2000, Varian Pursuit XRs 5 C18, 150 x 4.6 mm (as an analytic column) or ii) Varian Prostar (LC Workstation-Varian Prostar 335 LC detector), Thermo SCIENTIFIC Hypersil Gold C18, 5 µm, 150 x 4.6 mm (as an analytical column) with a gradient elution of H₂O/ CH₃CN from 100/0 to 0/100 in 35

min, flow = 1 mL/min, λ = 275 nm. Mass spectra were performed on Bruker Daltonics microTOF-LC, (atmospheric pressure ionisation, electron spray mass spectroscopy) in either positive or negative mode.

2',3'-Dideoxy-2',3'-didehydrothymidine 5'-(phenyl methoxyalaninyl phosphate) (5a)

^1H NMR (CDCl_3): δ 8.80, 8.76 (2bs, 1H, NH), 7.36-7.13 (m, 6H, ArH, H-6), 7.05-6.99 (2m, 1H, H-1'), 6.37-6.26 (2dt, J = 6, 2 Hz, 1H, H-2'), 5.93 -5.85 (2m, 1H, H-3'), 5.06-4.98 (2m, 1H, H-4'), 4.42-4.26 (2m, 2H, H-5'), 4.04-3.93 (m, 1H, Ala CH), 3.71, 3.70 (2s, 3H, OCH_3), 2.00 (bs, 1H, NH), 1.86, 1.82 (2d, J = 0.3 Hz, 3H, CH_3 - 5), 1.36, 1.32 (2d, J = 7 Hz, 3H, Ala CH_3); ^{31}P NMR (CDCl_3): δ 3.10, 2.49; ^{13}C NMR (CDCl_3): 173.96, 173.82 (2d, $^3J_{\text{C-P}}$ = 7.1 Hz, C=O ester), 163.69, 163.63 (ArC-4), 150.79, 150.75 (C=O, C-2), 150.42, 150.28 (2d, $^2J_{\text{C-P}}$ = 6.3 Hz, ArC ipso phenyl), 135.88, 135.61 (ArCH, C-6), 133.37, 132.07 (C-3'), 129.80, 129.74 (ArCH), 127.49, 127.33 (C-2'), 125.25, 125.19 (ArCH), 120.19, 120.05 (2d, $^2J_{\text{C-P}}$ = 3.8, 5 Hz, ArCH), 111.41, 111.29 (C-5), 89.85, 89.62 (C-1'), 84.71, 84.64 (C-4'), 67.16, 66.55 (2d, $^2J_{\text{C-P}}$ = 5, 3.8 Hz, C-5'), 52.60, 52.58 (OCH_3), 50.21, 50.09 (AlaCH), 20.96, 20.90 (2d, 5 Hz, Ala CH_3), 12.34, 12.30 (CH_3 , C-5). MS (ES^+) m/z : 488.1 ($\text{M}+\text{Na}^+$), $\text{C}_{20}\text{H}_{24}\text{N}_3\text{O}_8\text{P}$, Reverse-phase HPLC, eluting with $\text{H}_2\text{O}/\text{CH}_3\text{CN}$ from 90/10 to 0/100 in 30 min; flow = 1 mL/min, t_R = 15.00 min. yield 28%.

2',3'-Didehydro-2',3'-dideoxythymidine 5'-(3-trifluoromethylphenylmethoxyalaninyl phosphate) (5b)

^1H NMR (CDCl_3): δ 9.24, 9.21 (2bs, 1H, NH), 7.49-7.38 (m, 4H, ArH), 7.29-7.20 (2m, 1H, H-6), 7.05-7.00 (2m, 1H, H-1'), 6.36-6.27 (2dt, J = 6, 2 Hz, 1H, H-2'), 5.94 -5.87 (2m, 1H, H-3'), 5.07-4.99 (2m, 1H, H-4'), 4.43-4.24 (2m, 2H, H-5'), 4.11-3.94 (m, 2H, Ala CH, NH), 3.71, 3.70 (2s, 3H, OCH_3), 1.86, 1.81 (2d, J = 1.5 Hz, 3H, CH_3 - 5), 1.38, 1.35 (2d, J = 7 Hz, 3H, Ala CH_3); ^{31}P NMR (CDCl_3): δ 3.25, 2.63; ^{19}F NMR (CDCl_3): δ -62.74, -62.75; ^{13}C NMR (CDCl_3): 173.90, 173.74 (2d, $^3J_{\text{C-P}}$ = 7.5 Hz, C=O ester), 163.83, 163.79 (ArC-4), 150.86 (C=O, C-2), 150.63, 150.50 (2d, $^2J_{\text{C-P}}$ = 6.3, 5 Hz, ArC ipso phenyl), 135.68, 135.48 (ArCH, C-6), 133.09, 132.83 (C-3'), 130.47 (ArCH), 130.06 (m, ArC), 127.68, 127.58 (C-2'), 123.85, 123.68 (2d, $^2J_{\text{C-P}}$ = 3.8, 5 Hz, ArCH), 123.39 (q, $^1J_{\text{C-P}}$ = 270.8 Hz, CF_3), 121.96 (m, ArCH), 117.39 (m, ArCH), 111.44, 111.27 (C-5), 89.92, 89.65 (C-1'), 84.54, 84.51 (2d, $^2J_{\text{C-P}}$ = 3.8, 5 Hz, C-4'), 67.47, 66.80 (2d, $^2J_{\text{C-P}}$ = 3.8, 5 Hz, C-5'), 52.64 (OCH_3), 50.24, 50.13 (AlaCH), 20.89, 20.80 (2d, 5.6.3 Hz, Ala CH_3), 12.34, 12.26 (CH_3 , C-5). MS (ES^+) m/z : 556.1 ($\text{M}+\text{Na}^+$), $\text{C}_{21}\text{H}_{23}\text{F}_3\text{N}_3\text{O}_8\text{P}$, Reverse-phase HPLC, eluting with $\text{H}_2\text{O}/\text{CH}_3\text{CN}$ from 90/10 to 0/100 in 30 min; flow = 1 mL/min, t_R = 14.97 min. yield 20%.

2',3'-Didehydro-2',3'-dideoxythymidine-5'-(4-(trifluoromethyl)phenyl methoxy alaninyl phosphate) (5c).

^1H NMR (CDCl_3): δ 8.49, 8.45 (2bs, 1H, NH), 7.54 (d, J = 8 Hz, 2H, ArH), 7.26, 7.23 (d, J = 8.5, 9 Hz, 2H, ArH), 7.21-7.13 (2m, 1H, H-6), 6.98-6.93 (2m, 1H, H-1'), 6.31-6.21 (2dt, J = 6, 2 Hz, 1H, H-2'), 5.88 -5.82 (2m, 1H, H-3'), 5.01-4.93 (2m, 1H, H-4'), 4.35-4.16 (2m, 2H, H-5'), 3.98-3.86 (m, 1H, Ala CH), 3.76-3.66 (m, 1H, NH), 3.64, 3.63 (2s, 3H, OCH_3), 1.81, 1.76 (2d, J = 1 Hz, 3H, CH_3 - 5), 1.31, 1.27 (2d, J = 7 Hz, 3H, Ala CH_3); ^{31}P NMR (CDCl_3): δ 2.95, 2.41; ^{19}F NMR (CDCl_3): δ -62.21, -62.22; ^{13}C NMR (CDCl_3): 173.74, 173.60 (C=O ester), 163.44, 163.41 (ArC-4), 153.04, 152.84 (ArC ipso phenyl), 150.62 (C=O, C-2), 135.68, 135.46 (ArCH, C-6), 133.18, 132.87 (C-3'), 127.70, 127.55 (C-2'), 127.21 (m, ArCH), 124.83 (ArC), 122.93 (ArC), 120.49 (2d, J = 5 Hz, ArCH), 111.42, 111.27 (C-5), 89.96, 89.71 (C-1'), 84.54, 84.47 (C-4'), 67.49, 66.80 (2d, $^2J_{\text{C-P}}$ = 3.8, 5 Hz, C-5'), 52.69 (OCH_3), 50.21, 50.14 (AlaCH), 20.98, 20.90 (2d, 5 Hz, Ala CH_3), 12.39, 12.36 (CH_3 , C-5). MS (ES^+) m/z : 556.1 ($\text{M}+\text{Na}^+$), $\text{C}_{21}\text{H}_{23}\text{F}_3\text{N}_3\text{O}_8\text{P}$, Reverse-phase HPLC, eluting with $\text{H}_2\text{O}/\text{CH}_3\text{CN}$ from 90/10 to 0/100 in 30 min; flow = 1 mL/min, t_R = 17.08 min. yield 24%.

2',3'-Didehydro-2',3'-dideoxythymidine-5'-(3-(pentafluorosulfanyl)phenyl methoxy alaninyl phosphate) (5d)

^1H NMR (CDCl_3): δ 9.11 (bs, 1H, NH), 7.54-7.49 (m, 2H, ArH), 7.41-7.31 (m, 2H, ArH), 7.21-7.11 (2m, 1H, H-6), 6.97-6.93 (2m, 1H, H-1'), 6.28-6.20 (2m, 1H, H-2'), 5.88 -5.82 (2m, 1H, H-3'), 5.00-4.92 (2m, 1H, H-4'), 4.35-4.17 (2m, 2H, H-5'), 3.98-3.88 (m, 1H, Ala CH), 3.64, 3.64 (2s, 3H, OCH_3), 1.82 (bs, 1H, NH), 1.78, 1.74 (2d, J = 1 Hz, 3H, CH_3 - 5), 1.32, 1.27 (2d, J = 6.5 Hz, 3H, Ala CH_3); ^{31}P NMR (CDCl_3): δ 3.10, 2.60; ^{19}F NMR (CDCl_3): δ 83.21 (p, J = 149.9 Hz, 1F), 62.82, 62.80 (2d, J = 149.9, 150.4 Hz, 4F); ^{13}C NMR (CDCl_3): 173.88, 173.66 (2d, $^3J_{\text{C-P}}$ = 6.5, 7.4 Hz, C=O ester), 163.68, 163.63 (ArC, C-4), 154.41 (m, ArC, CSF_5), 150.76 (C=O, C-2), 150.18, 150.04 (2d, $^2J_{\text{C-P}}$ = 5.8 Hz, ArC ipso phenyl), 135.64, 135.44 (ArCH, C-6), 133.05, 132.77 (C-3'), 129.91 (ArCH), 127.73, 127.63 (C-2'), 123.80, 123.60 (2d, $^2J_{\text{C-P}}$ = 3.8, 5 Hz, ArCH), 122.66 (m, ArCH), 118.55 (m, ArCH), 111.45, 111.27 (ArC, C-5), 89.96, 89.68 (C-1'), 84.48, 84.42 (2d, $^3J_{\text{C-P}}$ = 2.5 Hz, C-4'), 67.58, 66.86 (2d, $^2J_{\text{C-P}}$ = 4, 3.8 Hz, C-5'), 52.69 (OCH_3), 50.25, 50.16 (Ala CH), 20.93, 20.82 (2d, 5, 6.3 Hz, Ala CH_3), 12.35, 12.25 (CH_3 , C-5). MS (ES^+) m/z : 614.1 ($\text{M}+\text{Na}^+$), $\text{C}_{20}\text{H}_{23}\text{F}_5\text{N}_3\text{O}_8\text{PS}$, Reverse-phase HPLC, eluting with $\text{H}_2\text{O}/\text{CH}_3\text{CN}$ from 90/10 to 0/100 in 30 min; flow = 1 mL/min, t_R = 16.01 min. yield 34%.

S2. Carboxypeptidase Y enzymatic assay procedure

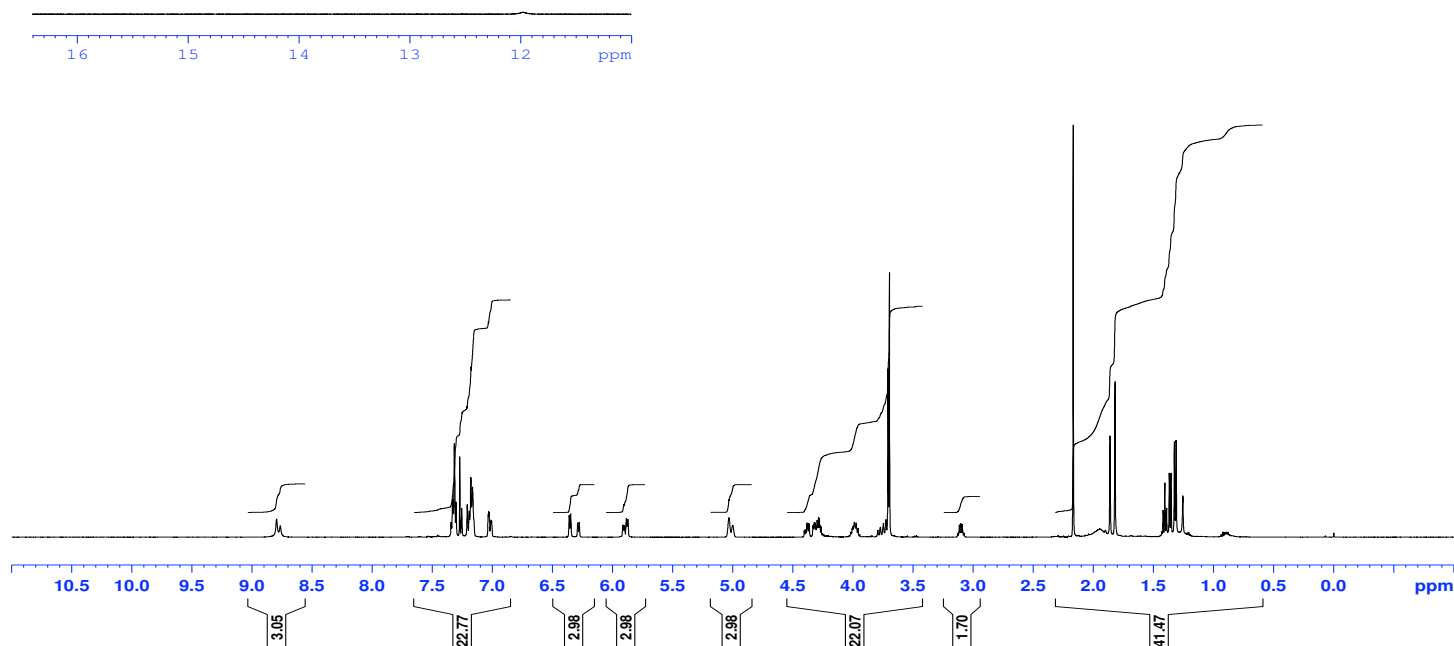
The enzymatic activation of the protides towards carboxypeptidase Y was studied using *in situ* ^{31}P NMR. The experiment was carried out by dissolving **5d** (5.0 mg) in d_6 -acetone (0.15 mL) and adding Trizma buffer pH 7.6 (0.30 mL). The resulting solution was placed in an NMR tube and a ^{31}P NMR experiment at 25 °C was recorded as the blank experiment. The enzyme carboxypeptidase Y (0.1 mg) was dissolved in Trizma (0.15 mL) and added to the solution of the phosphoramidate derivative in the NMR tube. The ^{31}P -NMR experiment was performed recording the experiment every 7 min at 25 °C.

¹H NMR spectrum of ProTide (5a)

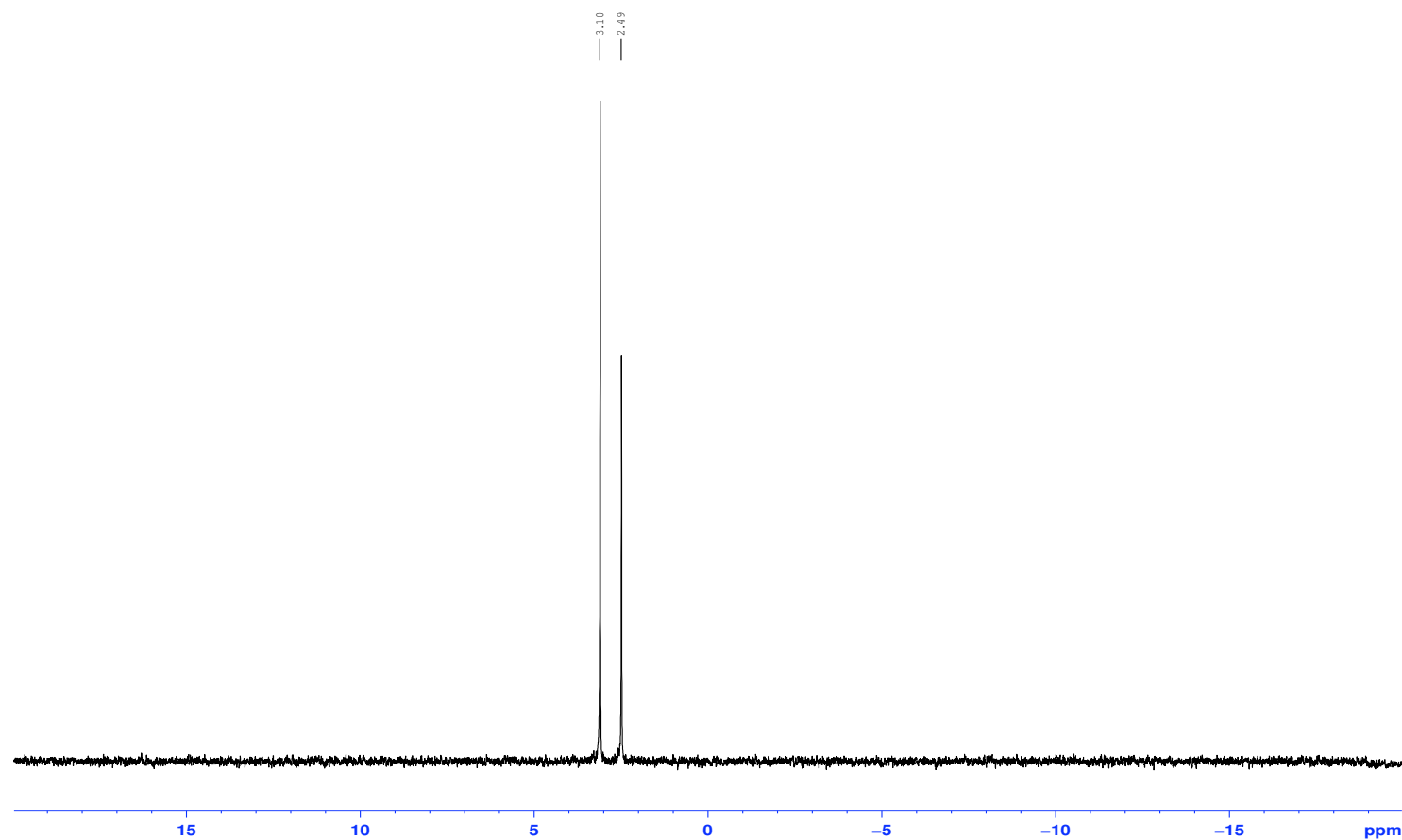


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PROCNO 1
Date_ 20140809
Time_ 12.47
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PULPROG zg30
TD 65536
SOLVENT CDCl3
NS 16
DS 2
SWH 10330.578 Hz
FIDRES 0.157632 Hz
AQ 3.1719923 sec
RG 287
DW 48.400 use
DE 6.50 use
TE 298.1 K
D1 1.00000000 sec
TD0 1

===== CHANNEL f1 =====
NUC1 1H
P1 11.50 use
PL1 -1.00 dB
PL1W 11.38419914 W
SFO1 500.1330885 MHz
SI 32768
SF 500.1300003 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00



³¹P NMR spectrum of ProTide (5a)

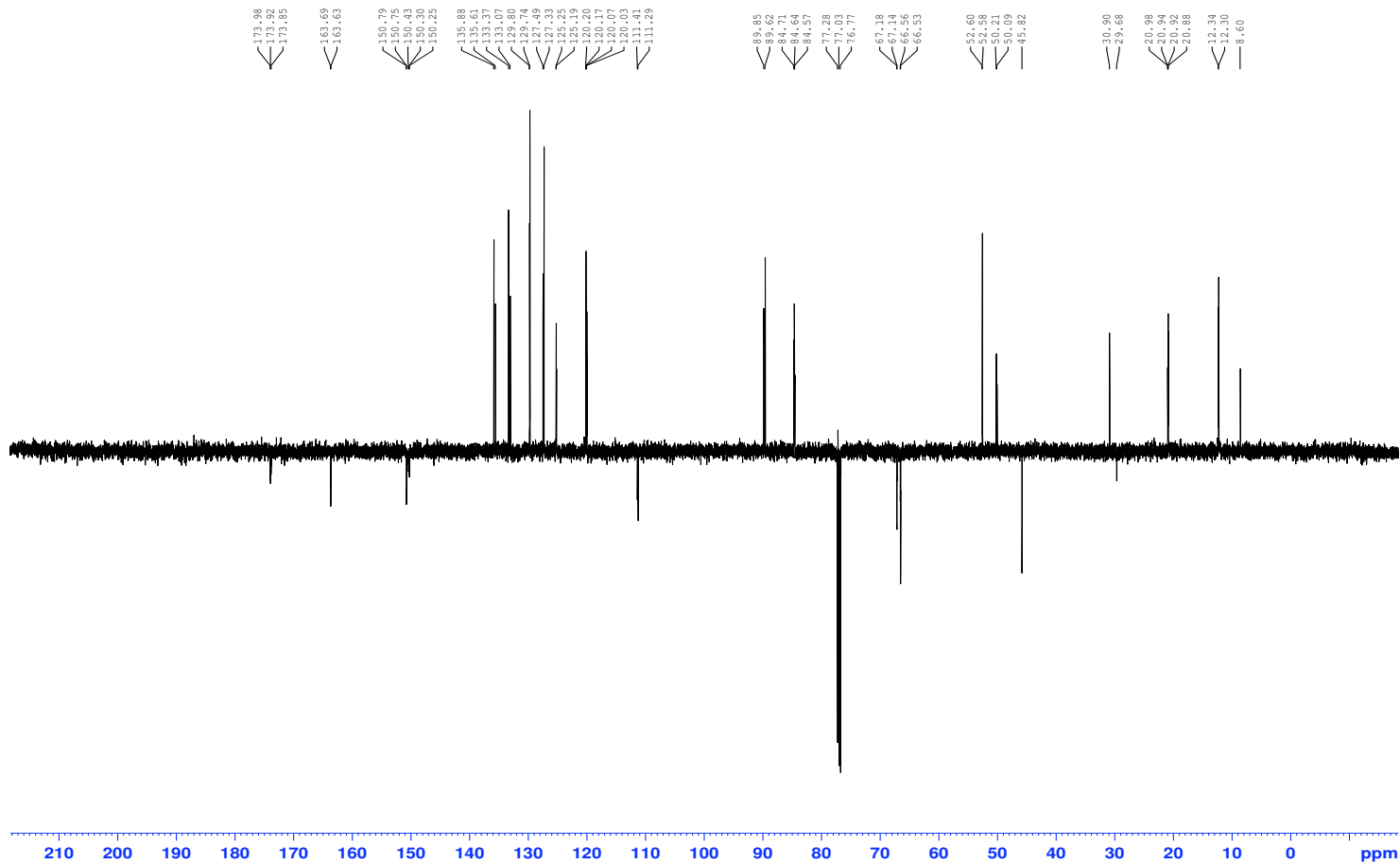


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PROCNO 1
Date_ 20140730
Time 13.54
INSTRUM Avance500
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PULPROG zgpg30
TD 16384
SOLVENT CDCl3
NS 16
DS 4
SWH 8081.896 Hz
FIDRES 0.493280 Hz
AQ 1.0136734 sec
RG 3250
DW 61.867 use
DE 12.00 use
TE 298.1 K
D1 1.65999997 sec
D11 0.03000000 sec
TDO 1

===== CHANNEL f1 =====
NUC1 31P
P1 10.40 use
PL1 4.00 dB
PL1W 24.3400015 W
SFO1 202.4563350 MHz

===== CHANNEL f2 =====
CPDPRG2 waltz16
NUC2 1H
PCPD2 80.00 use
PL2 -2.00 dB
PL12 14.85 dB
PL13 120.00 dB
PL2W 14.33185768 W
PL12W 0.29600734 W
PL13W 0.00000000 W
SFO2 500.1320005 MHz
SI 32768
SF 202.4563350 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
FC 1.40

¹³C NMR spectrum of ProTide (5a)



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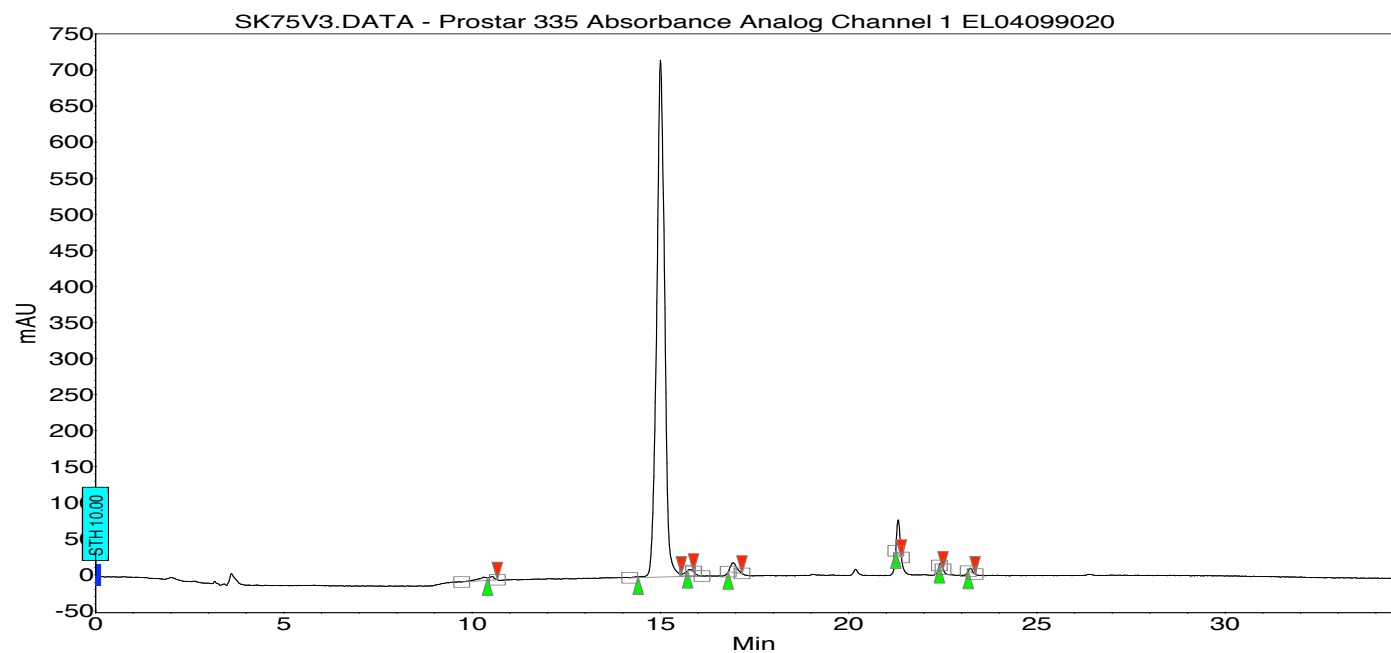
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PROCNO 1
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Time 12.24
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PULPROG pendant
TD 65536
SOLVENT CDC13
NS 512
DS 4
SWH 29761.904 Hz
FIDRES 0.454131 Hz
AQ 1.1010548 sec
RG 3250
DE 16.800 usec
TE 12.00 usec
TD 298.1 K
CNST2 145.000000
D1 2.00000000 sec
D2 0.00172414 sec
D12 0.00020000 sec
D15 0.00431034 sec
D20 0.00345000 sec
TD0 4
===== CHANNEL f1 =====
NUC1 13C
P1 7.20 usec
F2 14.40 usec
PL1 -2.00 dB
PLW 101.27846527 W
SF01 125.7703643 MHz
===== CHANNEL f2 =====
CPDPRG2 waltz16
NUC2 1H
P1 11.50 usec
F2 23.00 usec
PCPD2 80.00 usec
PL2 -2.00 dB
PL12 14.85 dB
PLW2 14.33185768 W
P12W 0.0290754 W
SF02 500.1320005 MHz
SI 32768
SF 125.7577890 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40
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HPLC analysis spectrum of ProTide (5a)

Chromatogram : SK75V3_channel1

System : HPLC_Analytical
Method : 5FU-Blanka ACN 30+5min ok
User : User1
Acquisition time: 8/22/2014 4:43:10 PM
Date: 8/22/2014
Time: 5:26:41 PM

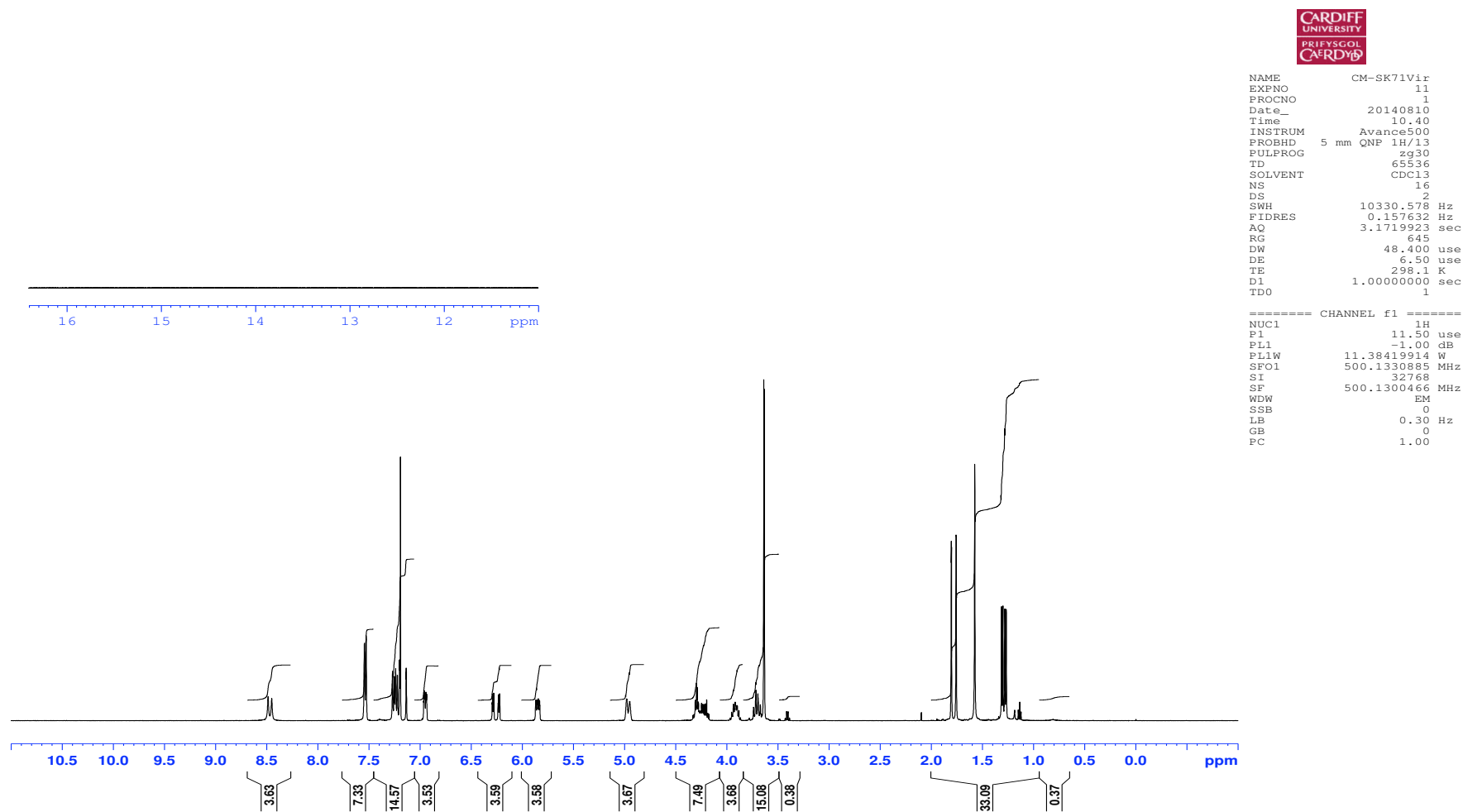
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Printed : 8/22/2014 5:26:41 PM
Method: 5FU-Blanka ACN 30+5min ok



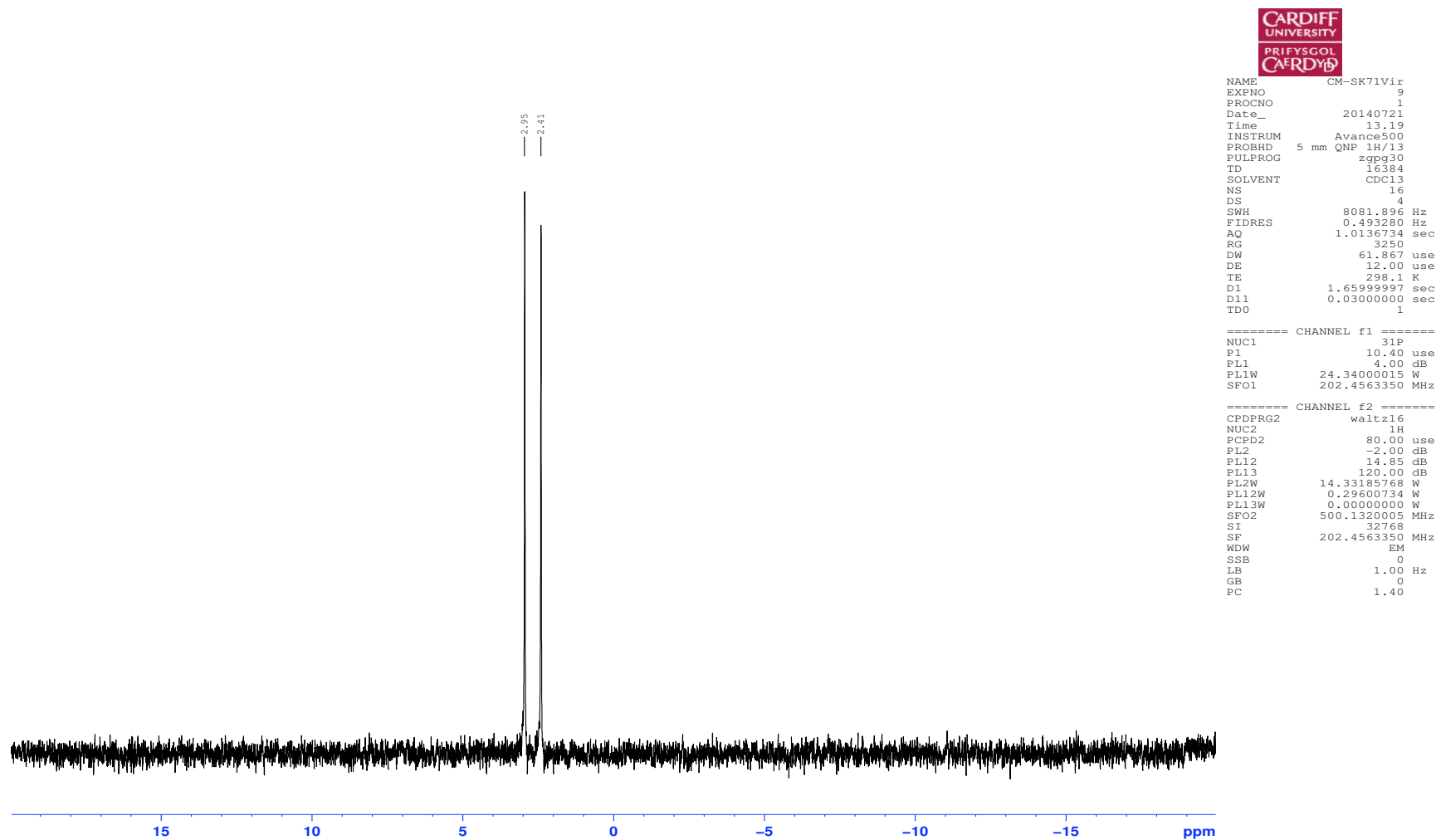
Peak results :

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2	UNKNOWN	15.00	95.44	716.6	174.4	95.442
3	UNKNOWN	15.84	0.12	1.8	0.2	0.117
4	UNKNOWN	16.93	1.31	13.0	2.4	1.315
5	UNKNOWN	21.32	2.25	46.8	4.1	2.254
6	UNKNOWN	22.45	0.17	4.9	0.3	0.173
7	UNKNOWN	23.24	0.26	5.4	0.5	0.261
Total			100.00	793.7	182.8	100.000

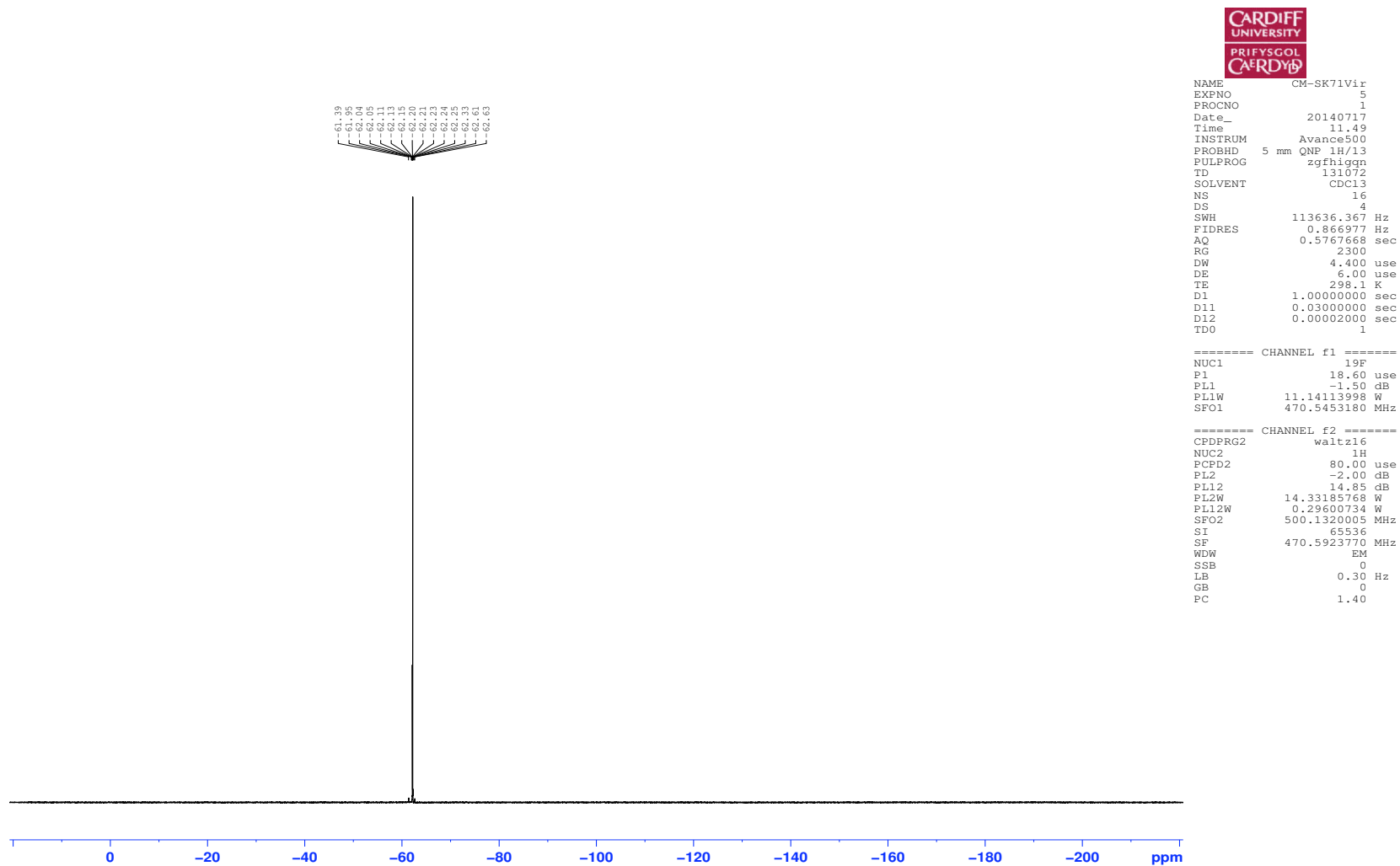
^1H NMR spectrum of ProTide (5b)



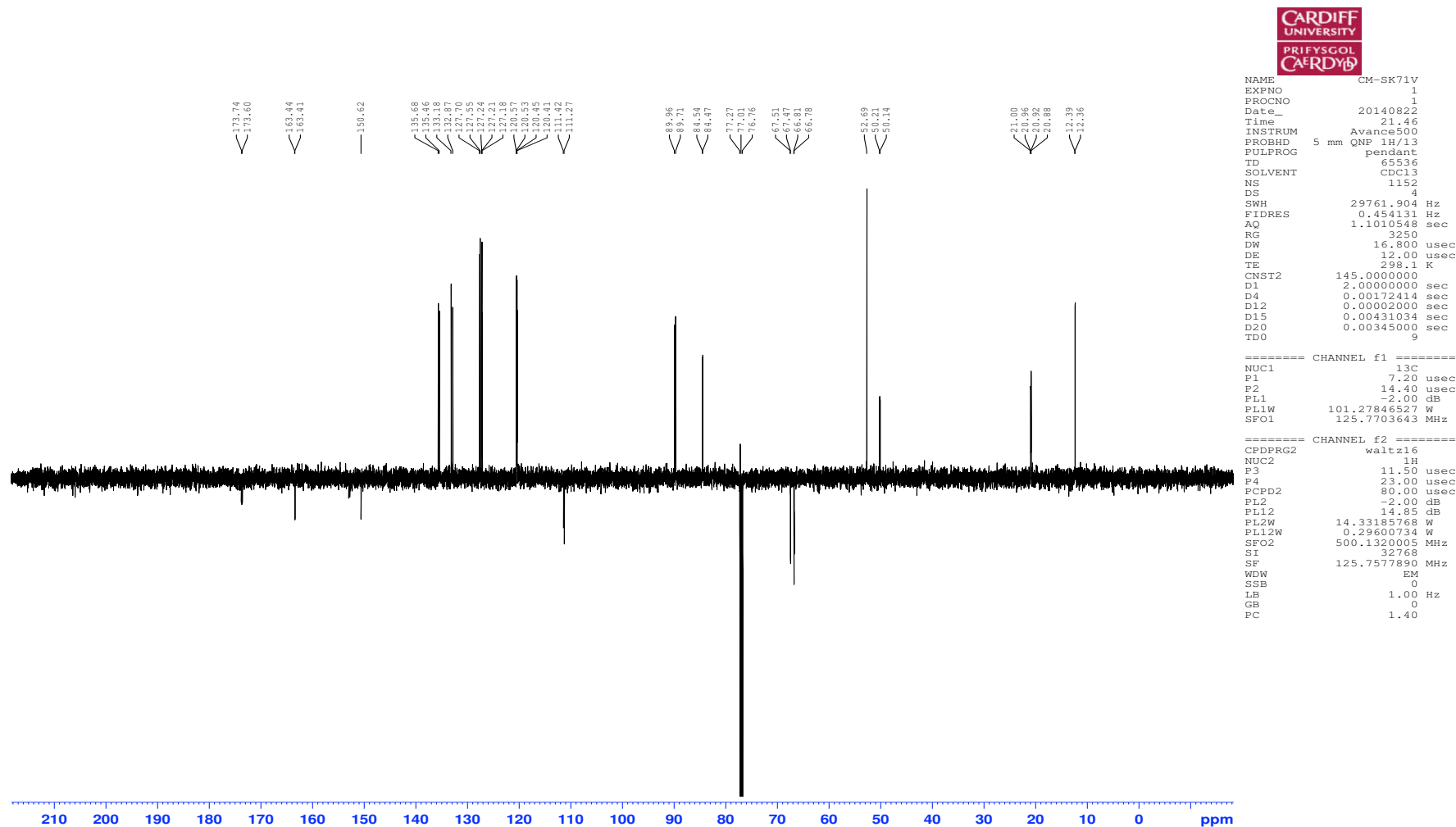
³¹P NMR spectrum of ProTide (5b)



¹⁹F NMR spectrum of ProTide (5b)



¹³C NMR spectrum of ProTide (5b)

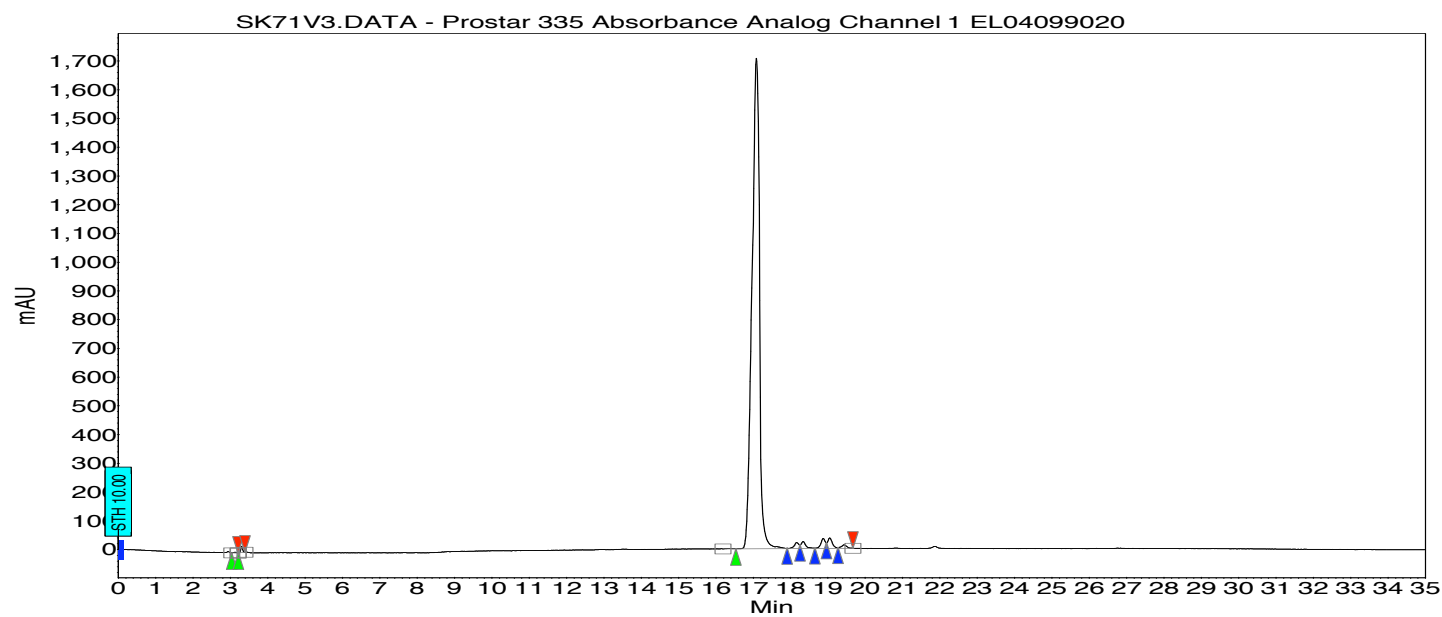


HPLC analysis spectrum of ProTide 5b

Chromatogram : SK71V3_channel1

System : HPLC_Analytical
Method : 5FU-Blanka ACN 30+5min ok
User : User1
Acquisition time: 8/22/2014 11:30:01 AM
Date: 8/22/2014
Time: 12:16:05 PM

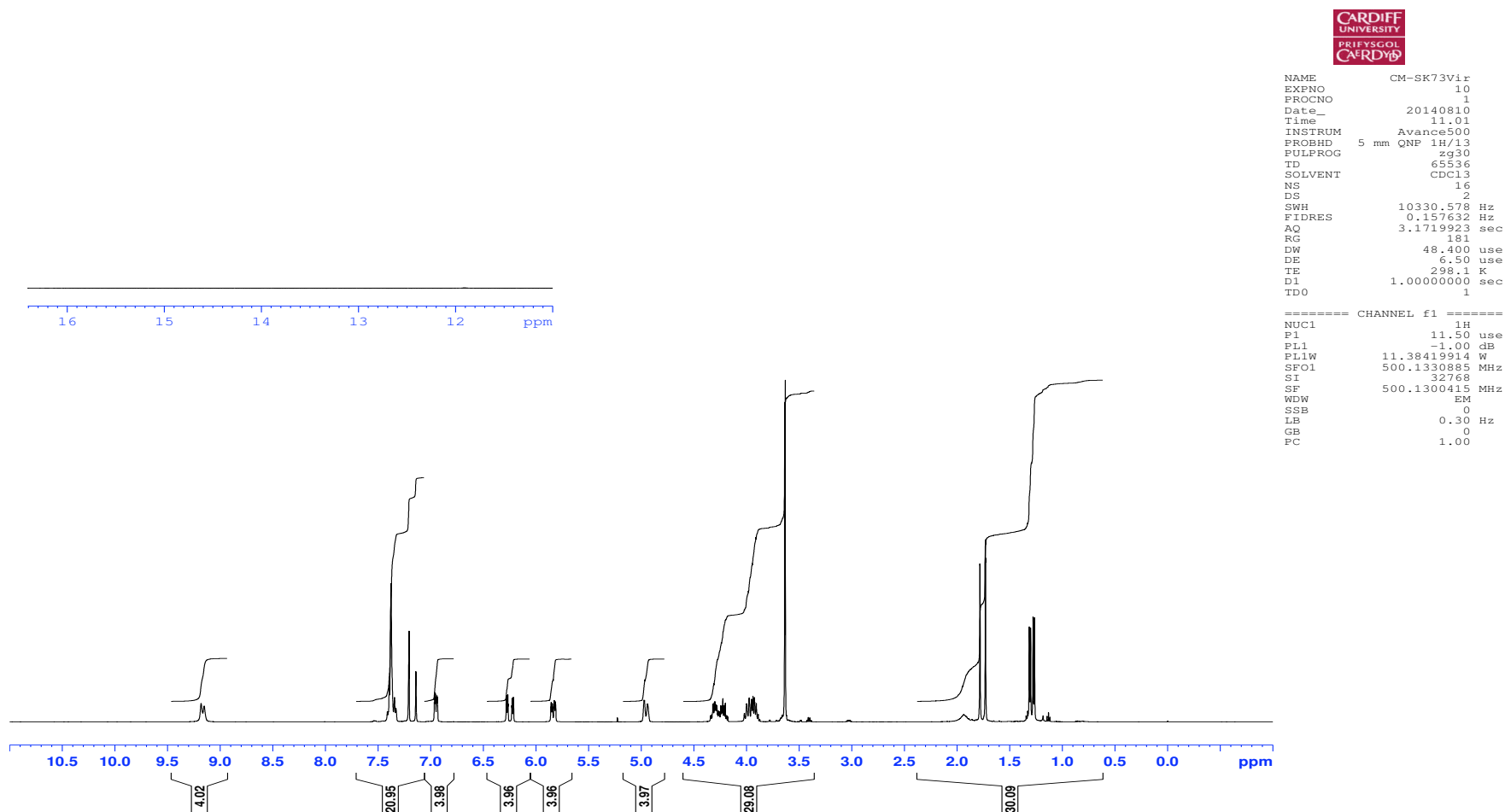
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Method: 5FU-Blanka ACN 30+5min ok



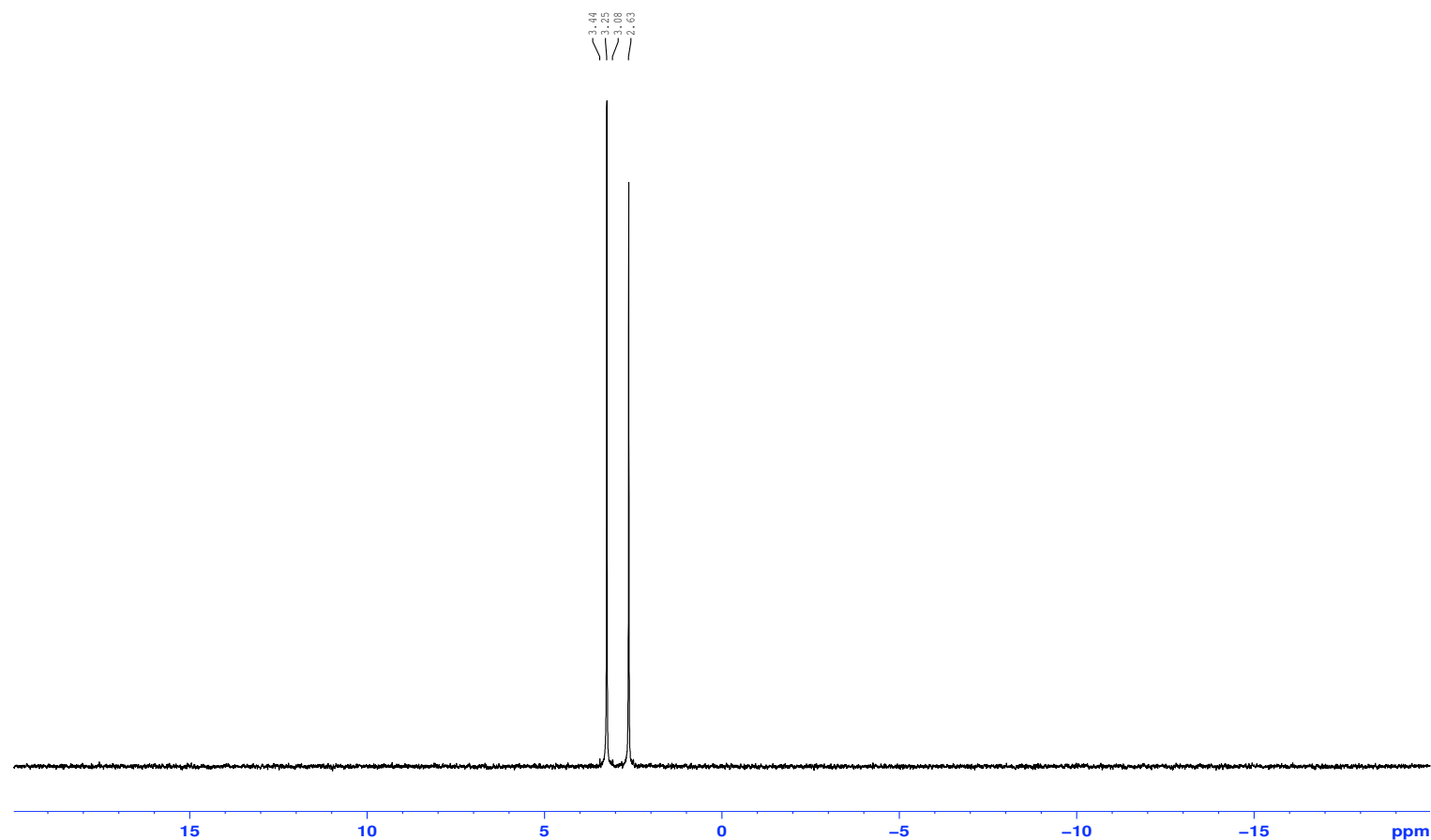
Peak results :

Index	Name	Time [Min]	Quantity [% Area]	Height [mAU]	Area [mAU.Min]	Area % [%]
1	UNKNOWN	3.15	0.05	2.2	0.2	0.050
8	UNKNOWN	3.29	0.30	21.9	1.2	0.297
2	UNKNOWN	17.08	95.15	1706.9	394.5	95.146
3	UNKNOWN	18.16	0.74	20.9	3.1	0.741
4	UNKNOWN	18.33	0.85	24.1	3.5	0.852
5	UNKNOWN	18.88	1.14	34.3	4.7	1.145
6	UNKNOWN	19.05	1.29	36.3	5.4	1.292
7	UNKNOWN	19.43	0.48	12.1	2.0	0.478
Total			100.00	1858.8	414.7	100.000

¹H NMR spectrum of ProTide (5c)



³¹P NMR spectrum of ProTide (5c)



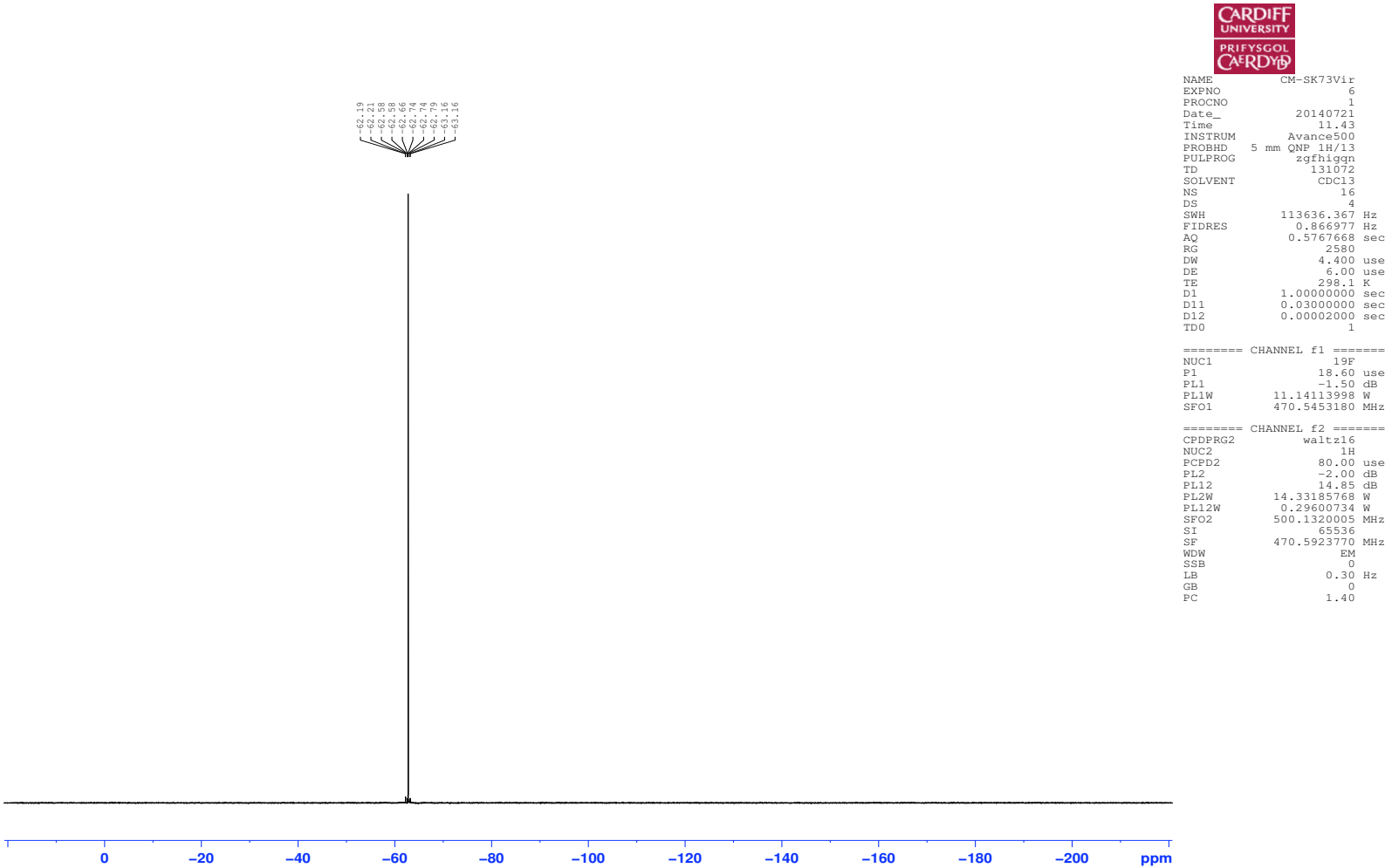
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PROCNO        1
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Time          11.27
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PULPROG       zgpg30
TD            16384
SOLVENT       CDCl3
NS            16
DS            4
SWH           8081.896 Hz
FIDRES        0.493280 Hz
AQ            1.0136734 sec
RG            3250
DW            61.867 use
DE            12.00 use
TE            298.1 K
D1            1.65999997 sec
D11           0.03000000 sec
TD0           1

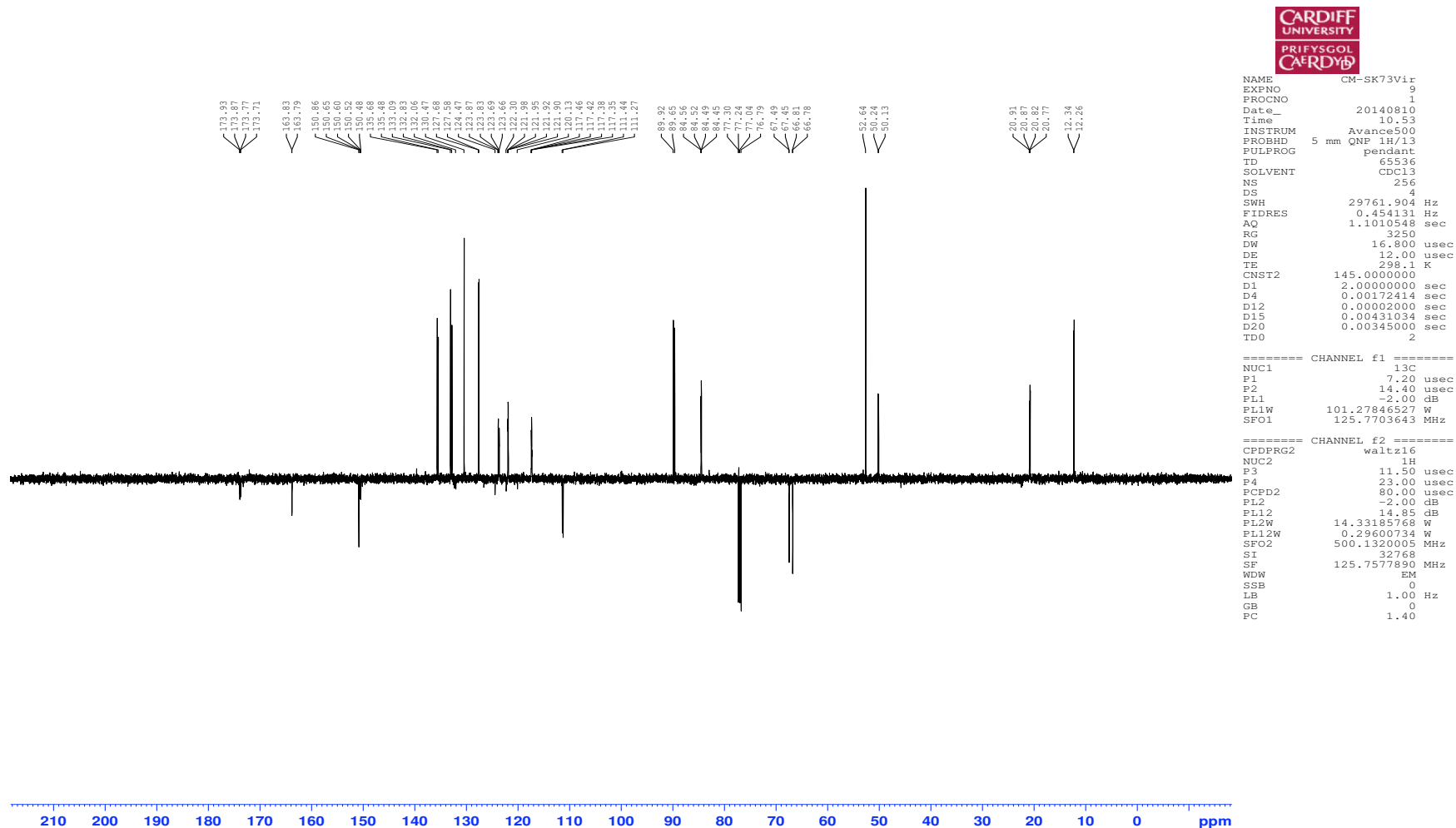
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PL1           4.00 dB
PL1W          24.34000015 W
SFO1          202.4563350 MHz

===== CHANNEL f2 =====
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NUC2          1H
PCPD2         80.00 use
PL2           -2.00 dB
PL12          14.85 dB
PL13          120.00 dB
PL2W          14.33185768 W
PL12W         0.29600734 W
PL13W         0.00000000 W
SFO2          500.1320005 MHz
SI            32768
SF            202.4563350 MHz
WDW           EM
SSB           0
LB            1.00 Hz
GB            0
PC            1.40
    
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^{19}F NMR spectrum of ProTide (5c)



¹³C NMR spectrum of ProTide (5c)

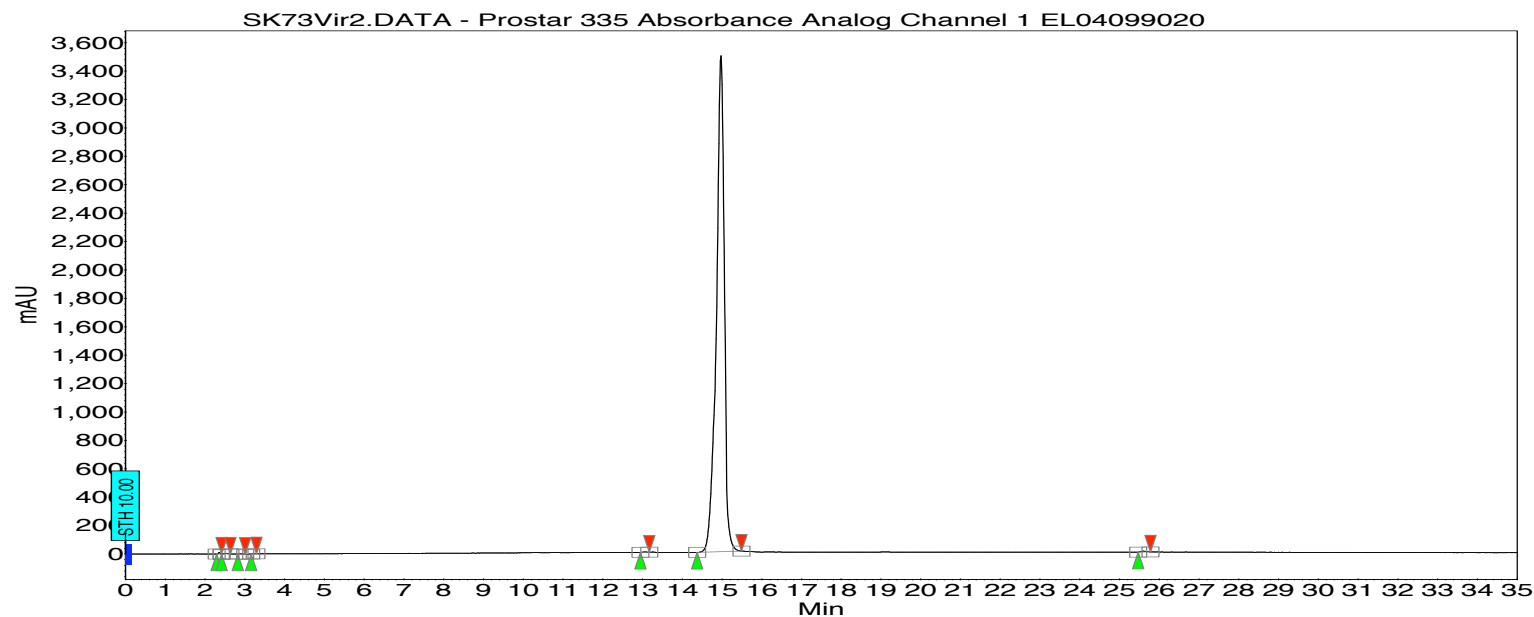


HPLC analysis spectrum of ProTide 5c

Chromatogram : SK73Vir2_channel1

System : HPLC_Analytical
Method : 5FU-Blanka ACN 30+5min ok
User : User1
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Date: 7/21/2014
Time: 11:56:32 AM

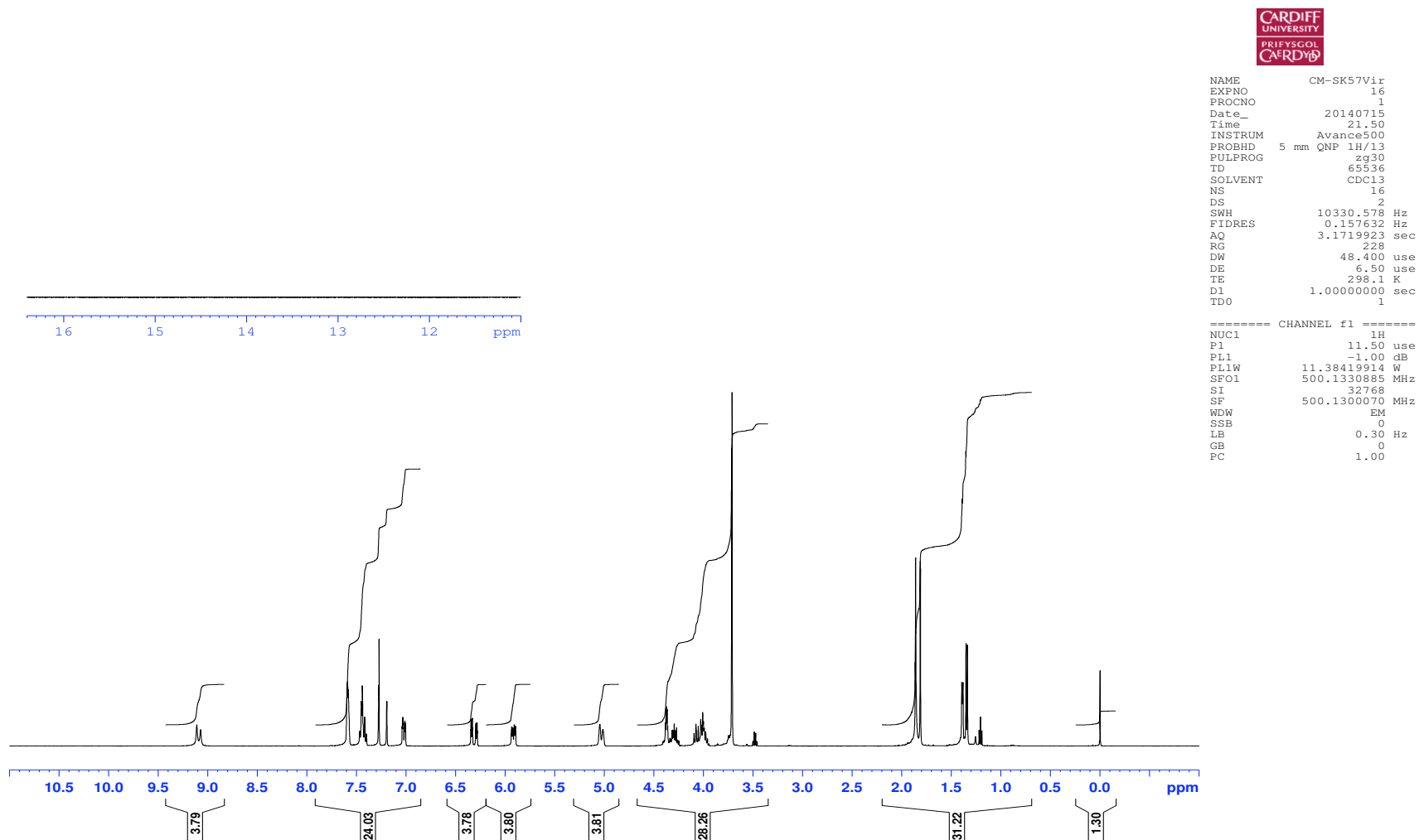
Acquired : 7/21/2014 11:11:44 AM
Processed : 7/21/2014 11:48:53 AM
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Method: 5FU-Blanka ACN 30+5min ok



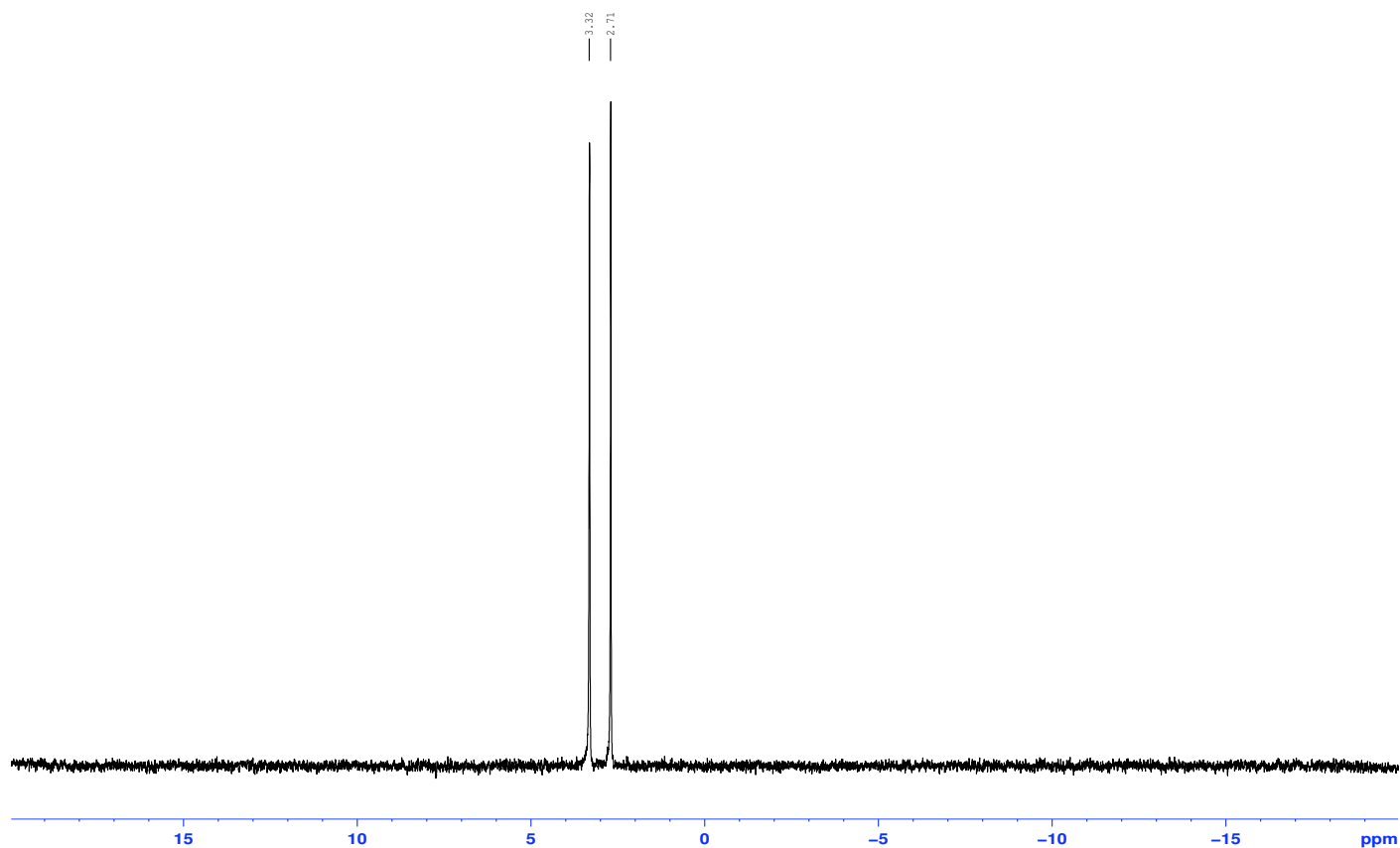
Peak results :

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2	UNKNOWN	2.55	0.05	3.0	0.4	0.050
3	UNKNOWN	2.91	0.02	1.9	0.1	0.018
4	UNKNOWN	3.20	0.01	0.9	0.1	0.009
5	UNKNOWN	13.07	0.03	2.0	0.2	0.028
6	UNKNOWN	14.97	99.72	3491.3	810.1	99.721
7	UNKNOWN	25.60	0.11	6.3	0.9	0.107
Total			100.00	3517.0	812.3	100.000

¹H NMR spectrum of ProTide (5d)



³¹P NMR spectrum of ProTide (5d)



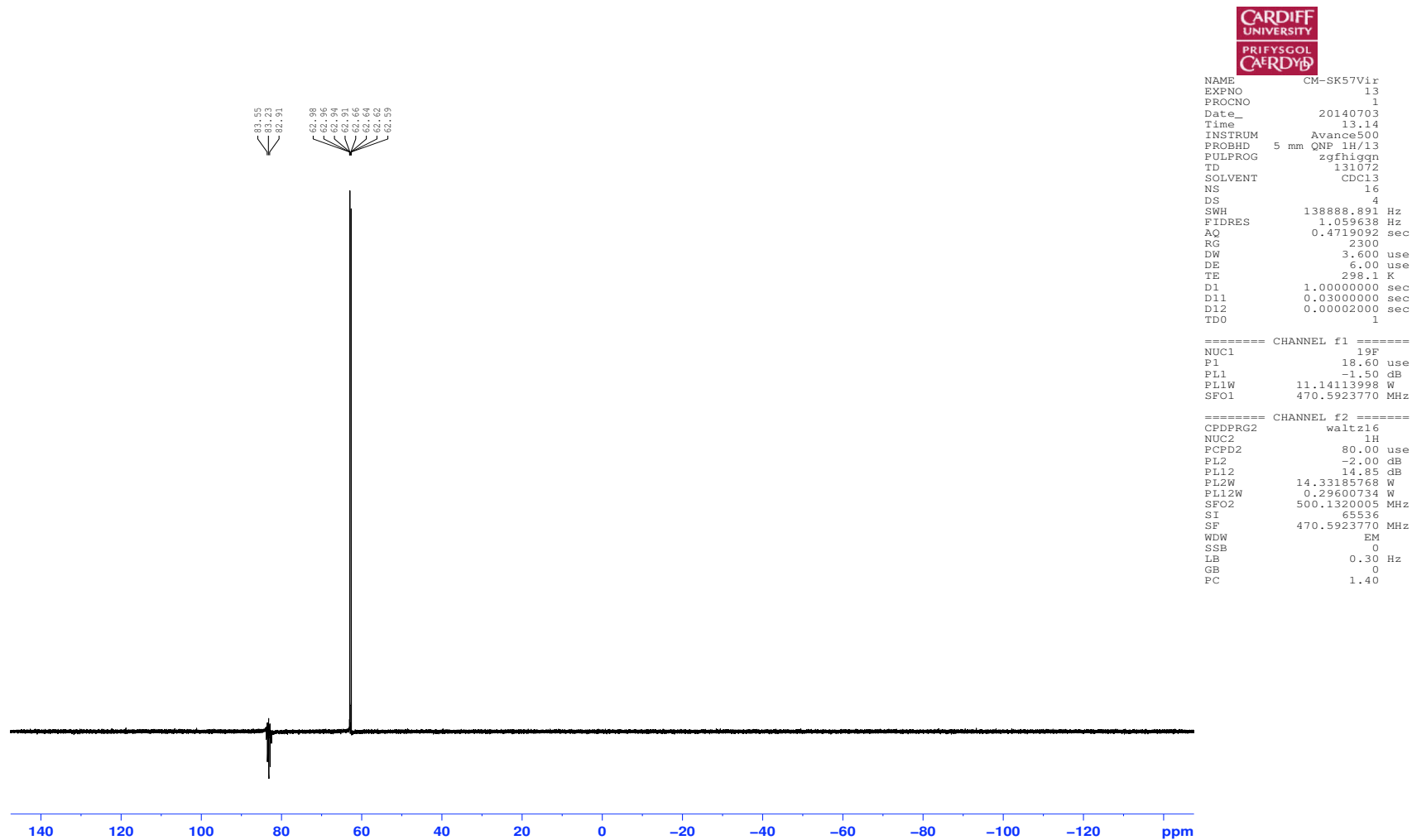
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PROCNO        1
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TD            16384
SOLVENT       CDCl3
NS            16
DS            4
SWH            8081.896 Hz
FIDRES        0.493280 Hz
AQ            1.0136734 sec
RG            3250
DW            61.867 use
DE            12.00 use
TE            298.1 K
D1            1.6599997 sec
D11           0.03000000 sec
TD0           1

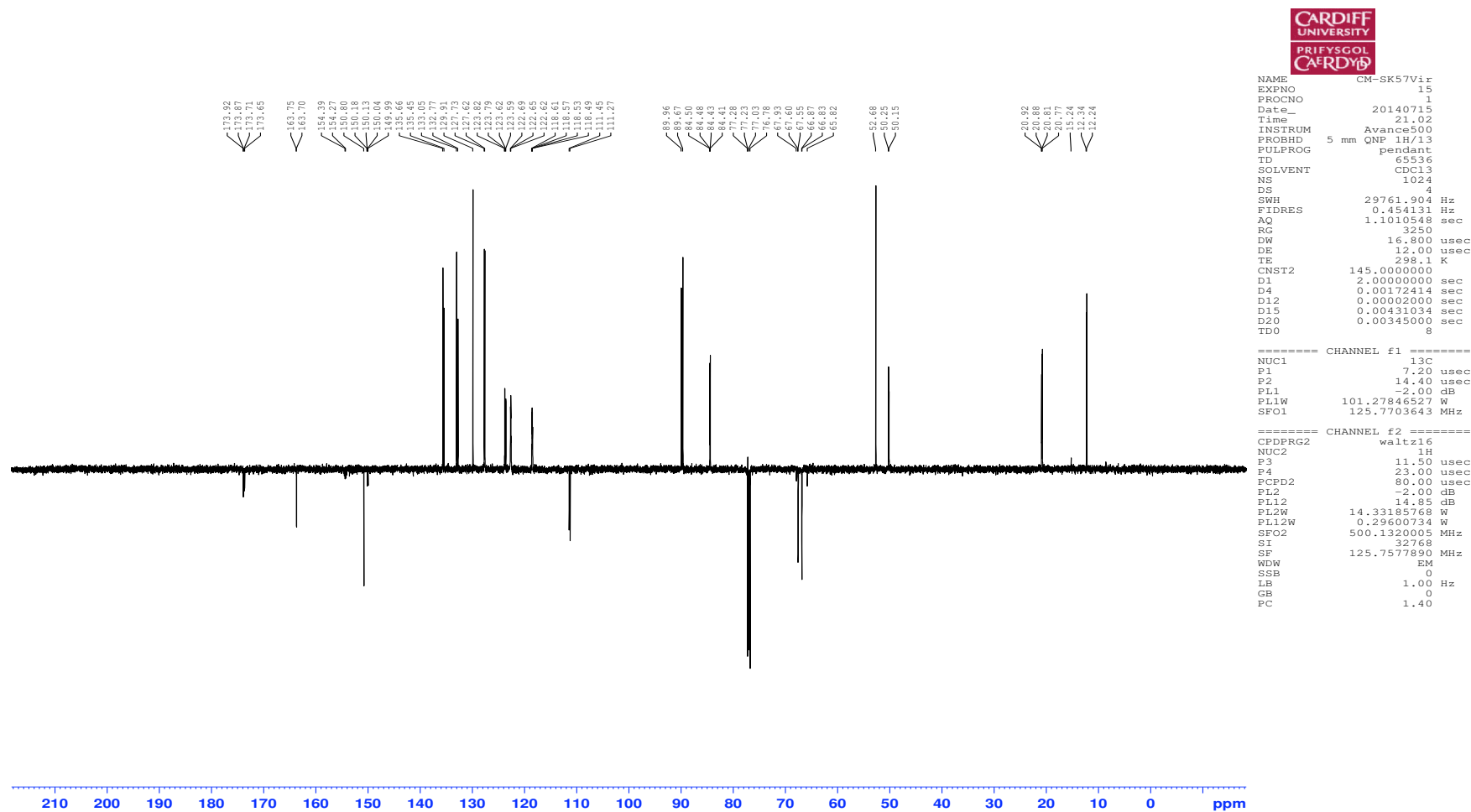
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PL1W          24.34000015 W
SFO1          202.4563350 MHz

===== CHANNEL f2 =====
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NUC2          1H
PCPD2         80.00 use
PL2           -2.00 dB
PL12          14.85 dB
PL13          120.00 dB
PL2W          14.33185768 W
PL12W         0.29600734 W
PL13W         0.00000000 W
SFO2          500.1320005 MHz
SI            32768
SF            202.4563350 MHz
WDW           EM
SSB           0
LB            1.00 Hz
GB            0
PC            1.40
    
```


¹⁹F NMR spectrum of ProTide (5d)



¹³C NMR spectrum of ProTide (5d)

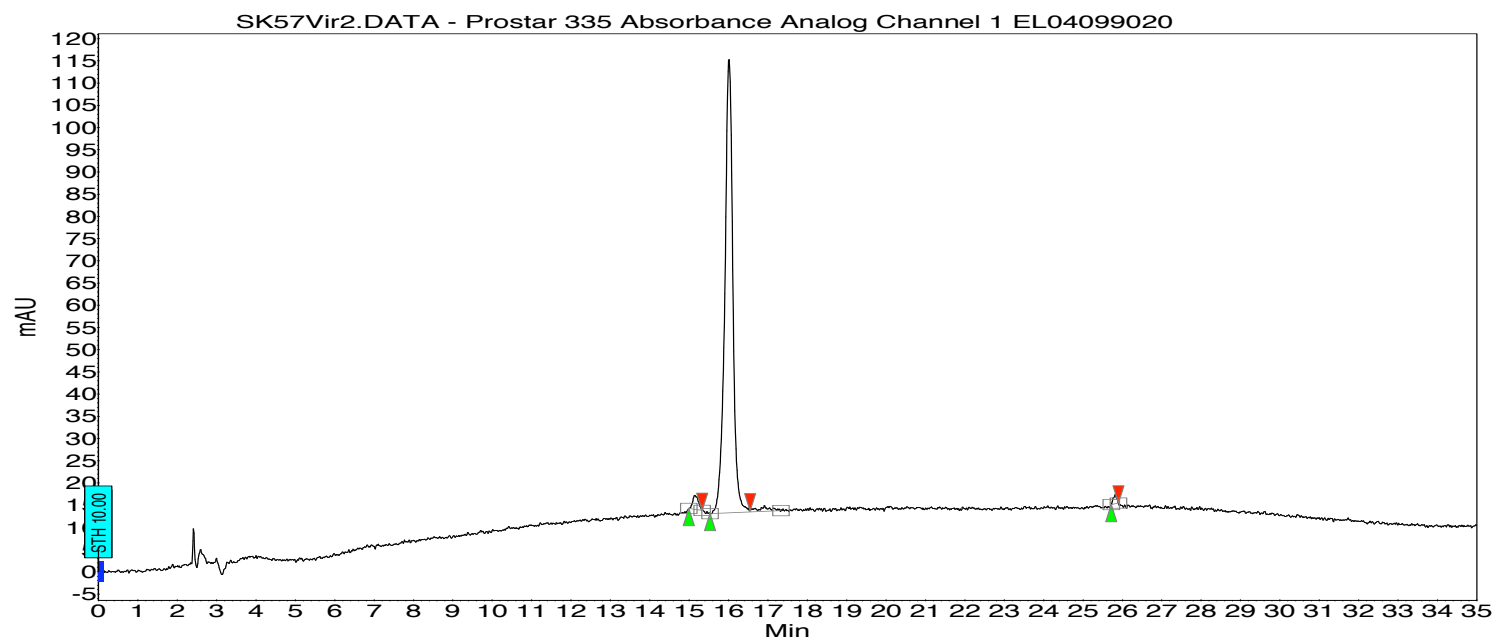


HPLC analysis spectrum of ProTide 5d

Chromatogram : SK57Vir2_channel1

System : HPLC_Analytical
Method : 5FU-Blanka ACN 30+5min ok
User : User1
Acquisition time: 7/21/2014 11:59:40 AM
Date: 7/21/2014
Time: 12:57:36 PM

Acquired : 7/21/2014 11:59:40 AM
Processed : 7/21/2014 12:57:23 PM
Printed : 7/21/2014 12:57:36 PM
Method: 5FU-Blanka ACN 30+5min ok



Peak results :

Index	Name	Time [Min]	Quantity [% Area]	Height [mAU]	Area [mAU.Min]	Area % [%]
3	UNKNOWN	15.16	2.06	2.9	0.5	2.055
1	UNKNOWN	16.01	97.15	102.0	24.2	97.145
2	UNKNOWN	25.81	0.80	2.1	0.2	0.800
Total			100.00	107.1	24.9	100.000