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Difluoro analogue of UCS15A triggers activation of exogenously expressed c-Src in HCT 116 human colorectal carcinoma cells

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Abstract

UCS15A, an antibiotic produced by *Streptomyces sp.*, has been reported to specifically disrupt SH3 domain-mediated interactions in eukaryotic cells. Interestingly, in the case of the non-receptor tyrosine kinase Src, UCS15A was effective in suppressing the SH3 domain-mediated intermolecular rather than intramolecular interactions, and thus prevented Src interactions with certain downstream effectors without affecting Src kinase activity. Here the synthesis of a novel difluoro analogue of UCS15A is described. The effects of this compound **(8)** on Src activity were tested in HCT 116 colorectal carcinoma cells engineered for inducible expression of c-Src. The presence of compound **(8)** resulted in the increased activity of the induced c-Src implicating that **(8)** acts as a c-Src activator *in vivo*. These observations are supported by computer modelling studies which suggest that the aldehyde group of **(8)** may covalently bind to a lysine residue in the SH2-kinase linker region situated in the proximity of the SH3 domain, which could promote a conformational change resulting in increased Src activity.

Keywords: *UCS15A, c-Src, inhibitor, activator, Src homology domain, kinase activity*

Abbreviations: *SH, Src homology; GOLD, Genetic Optimisation for Ligand Docking; Dox, doxycycline*

Introduction

Correct protein-protein interactions *in vivo* are essential for the existence of every living cell. They are frequently mediated by well defined structural motifs. Multiple motifs have been described in the literature (for reviews see [1,2]). In many cases these motifs serve not only as intermolecular protein-protein interfaces, but are also involved in intramolecular interactions. This provides a mechanism to regulate the tertiary structure and consequently the functional state of the protein [1].

Src homology three (SH3) domain, found in the Src family of the non-receptor tyrosine kinases and in several other proteins, represents a good example of a multifunctional structural motif [3,4]. In the case of c-Src, the SH3 domain is normally involved in intramolecular interactions that help to maintain an inactive, closed conformation. However, following activation, the protein assumes an open, active conformation and the SH3 domain promotes interactions of c-Src with several downstream effectors [5,6].

Because abnormalities in c-Src signalling have been associated with diverse human pathologies including cancer [7], there is a significant interest in small-molecule regulators of Src activity. Several Src inhibitors have been described and some of them recently entered phase I clinical trials [8]. Based on the mode of action, Src inhibitors can be divided into three major categories: (i) molecules that interfere with Src kinase activity [9,10], (ii) agents that interfere with Src protein stability [11–13] and (iii) molecules that disrupt physical interactions of Src with its downstream effectors. The last group represents a

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relatively new category and until recently the only example was a single chemical, AP22161, that disrupts SH2 domain mediated interactions of Src with its associated proteins [14].

Recently, UCS15A, a *Streptomyces sp.* - derived compound, has been identified as a new Src inhibitor in yeast cells engineered for constitutive expression of v-Src protein [15]. More detailed studies in mammalian cells demonstrated that UCS15A interferes with molecular interactions mediated by the SH3 domain of Src [16]. Surprisingly, UCS15A did not affect the intramolecular interactions mediated by the SH3 domain and thus did not interfere with Src kinase activity per se. In addition the potency of UCS15A and its analogues in disturbing SH3 mediated protein-protein interactions *in vitro* was significantly impaired, suggesting that there are factors in the intracellular *in vivo* microenvironment that may facilitate/promote the mode of UCS15A action [15,16]. Importantly the non-sugar analogue of UCS15A (**(2)**, Figure 1) has been shown to retain all the properties of UCS15A [17].

Here the synthesis of a novel isosteric/isoelectronic difluoro non-sugar analogue of UCS15A (compound **(8)**) is described. The properties of this compound towards c-Src have been analysed *in silico* and tested in a recently described cellular system engineered for tightly regulated inducible expression of c-Src protein [18]. The data suggests that **(8)** is able to act as a Src activator *in vivo*.

Materials and methods

Chemistry

Chemicals were purchased from Sigma-Aldrich Chemical Co., Lancaster Synthesis Ltd and Fisher Scientific UK Ltd. Fresh anhydrous THF was distilled from sodium / benzophenone. Deuterated solvent ($CDCl_3$) and tetramethylsilane (TMS) were supplied by Cambridge Isotope Laboratories Inc. Andover, USA. Anhydrous diethyl ether was supplied from Aldrich SureSeal™ bottles. Column chromatography was performed using silica gel with 33–70 μm particle size, provided by VWR International, Leicestershire, UK. Thin layer chromatography (TLC), employed to monitor reactions, was conducted using VWR plates coated with silica gel $60F_{254}$ on aluminium sheets using a variety of mobile phase solvents. TLC plates were subsequently dried and visualized by short wave (254 nm) ultraviolet light. Nuclear magnetic resonance (NMR) spectra were carried out using Bruker Advance 300 spectrometer with X-Win software. ^1H, ^{13}C and ^{19}F NMR spectra were measured at 300 MHz, 75.5 MHz and 282 MHz, respectively, using $CDCl_3$ as the solvent. Chemical shifts for ^1H NMR spectra were quoted in parts per million (δ) referenced to tetramethylsilane (TMS) at zero ppm. Multiplicities were indicated by s (singlet), d (doublet), t (triplet), q (quartet), octet, m (multiplet), br (broad); or combinations thereof, for example dd (double doublet). The ^{13}C NMR spectra were recorded in parts per million (δ) referenced to tetramethylsilane (TMS) and finally ^{19}F NMR spectra were measured in parts per million (δ) referenced to trichlorofluoromethane ($CFCl_3$).

Infra-red (IR) spectra were measured by Satellite FTIR Mattson spectrometer using Mattson WinFIRST software. Mass spectra (MS) and elemental analysis were determined at the Chemistry Department in the University of Manchester. Chemical ionisation (CI), electron ionisation (EI), high resolution mass spectrometry (HRMS) were measured using an automated Fisons TRIO 2000 quadrapole spectrometer.

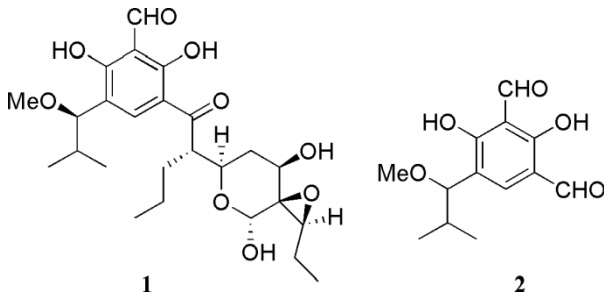


Figure 1. Structures of UCS15A **(1)** and its non-sugar analogue **(2)**.

1-(2,4-Difluorophenyl)-2-methylpropan-1-ol ***(3)***. A solution of 2,4-difluorobenzaldehyde (10.0 g, 70.4 mmol) in anhydrous diethyl ether (50 ml), chilled to −5°C (NaCl ice bath), was stirred for 15 min under nitrogen. *i*-PrMgCl (2M solution in dry diethyl ether) (45.8 mL, 91.5 mmol) was added dropwise and the mixture was stirred for 60 min at 0°C, followed by a further 60 min at room temperature. The mixture was cooled again to 0°C and a saturated solution of aqueous NH_4Cl (100 mL) was added gradually with stirring. The organic layer was washed with distilled water (2 × 100 mL), brine (2 × 100 mL), dried ($MgSO_4$) and the solvent was removed on a rotary evaporator. The residue was purified by flash column chromatography (EtOAc:Hexane, 25:75) to give 1-(2,4-difluorophenyl)-2-methylpropan-1-ol **(3)** as a yellow oil (12.3 g, 66.1 mmol, 94%); R_f 0.35 (25:75 EtOAc:Hexane). IR (neat, cm^{-1}) 2965, 1606, 1500, 1426, 1267, 1138, 964, 731 cm^{-1}. ^1H NMR: (300 MHz, $CDCl_3$) δ ppm 0.83 (3H, d, $\mathcal{J} = 6.8$ Hz, C*H*$_3$), 0.99 (3H, d, $\mathcal{J} = 6.7$ Hz, C*H*$_3$), 1.96 (1H, dqq ~ octet, $\mathcal{J} = 6.8$, C*H*(Me)$_2$), 2.02 (1H, brs, CHO*H*), 4.67 (1H, d, $\mathcal{J} = 6.7$ Hz, C*H*OH), 6.76 (1H, ddd, $^3\mathcal{J}_{HF} = 8.0$, $^3\mathcal{J}_{HF} = 6.5$, $^4\mathcal{J}_{HH} = 2.5$ Hz, Ar CH-3),

6.87 (1H, tdd, $^{3}J_{HF} = {}^{3}J_{HH} = 8.4$, $^{4}J_{HH} = 2.5$, $^{5}J_{HF} = 0.9$ Hz, Ar CH-5), 7.39 (1H, dt, $^{4}J_{HF} = 8.4$, $^{3}J_{HH} = 6.7$ Hz, Ar CH-6). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 18.3 (Me), 19.0 (Me), 35.1 (*C*H(Me)$_2$), 73.3 (*C*HOH), 103.7 (t, $^{2}J_{CF} = 25.4$ Hz, Ar-C3), 111.5 (dd, $^{2}J_{CF} = 21.0$, $^{4}J_{CF} = 3.6$ Hz, Ar-C5), 127.0 (dd, $^{2}J_{CF} = 13.6$, $^{4}J_{CF} = 3.8$ Hz, Ar-C1), 129.4 (dd, $^{3}J_{CF} = 9.6$, $^{3}J_{CF} = 6.4$ Hz, Ar-C6), 160.5 (dd, $^{1}J_{CF} = 247.7$ Hz, $^{3}J_{CF} = 11.8$ Hz, Ar-C4), 162.2 (dd, $^{1}J_{CF} = 247.7$ Hz, $^{3}J_{CF} = 11.8$ Hz, Ar-C2). ^{19}F NMR: (282 MHz, $CDCl_3$) δ_F (^{1}H-coupled) ppm −115.4 (dt, $^{3}J_{FH} = 9.3$ Hz, $^{4}J_{FF} = 8.2$ Hz, F-2), −112.5 (ddd, $^{3}J_{FH} = 9.0$ Hz, $^{4}J_{FF} = 8.4$ Hz, $^{4}J_{FH} = 7.0$ Hz, F4). δ_F (^{1}H-decoupled) ppm −115.4 (d, $^{4}J_{FF} = 7.1$ Hz), −112.5 (d, $^{4}J_{FF} = 7.1$ Hz). m/z [CI]$^+$169 (25%, [M-OH]$^+$). HRMS + EI m/z (M)$^+$ calcd for $C_{10}H_{12}O_1F_2$ 186.0851; found 186.0851.

1-(2,4-Difluorophenyl)-2-methylpropyl methyl ether ***(4)***. A mixture of KOH (1.01 g, 18.0 mmol) and DMSO (5 ml) was stirred for 5 min. A solution of 1-(2,4-difluorophenyl)-2-methylpropan-1-ol (1.09 g, 3.00 mmol) in DMSO (15 mL) was added, followed by MeI (1.12 ml, 18.0 mmol). The mixture was stirred for 5 h. The solution was poured into distilled water (100 mL) and the product was extracted with DCM (3 × 50 mL), washed with water (5 × 100 mL), dried ($MgSO_4$) and evaporation of the solvent yielded 1-(2,4-difluorophenyl)-2-methylpropyl methyl ether **(4)** as a yellow oil (6.73 g, 33.6 mmol, 90%). IR (neat, cm^{-1}) 2964, 1605, 1499, 1425, 1248, 1095, 964, 730 cm^{-1}. ^{1}H NMR: (300 MHz, $CDCl_3$) δ ppm 0.79 (3H, d, $J = 6.9$ Hz, C*H*$_3$), 0.98 (3H, d, $J = 6.7$ Hz, C*H*$_3$), 2.0 (1H, dqq ~ octet, $J = 0.7$, $J = 6.8$ Hz, C*H*(Me)$_2$), 3.19 (3H, s, OC*H*$_3$), 4.17 (1H, d, $J = 7.1$ Hz, C*H*OCH$_3$), 6.76 (1H, ddt, $^{3}J_{HF} = 9.0$, $^{3}J_{HF} = 7.1$, $^{4}J_{HH} = 2.5$ Hz, Ar CH-3), 6.90 (1H, tdd, $^{3}J_{HF} = {}^{3}J_{HH} = 8.0$, $^{4}J_{HH} = 2.4$, $^{5}J_{HF} = 0.8$ Hz, Ar CH-5), 7.32 (1H, dt, $^{4}J_{HF} = 8.5$, $^{3}J_{HH} = 6.7$ Hz, Ar CH-6). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 18.8 (Me), 18.9 (Me), 34.6 (*C*H(Me)$_2$), 57.5 (OC H$_3$), 82.1 (*C*HOCH$_3$), 103.7 (dd, $^{2}J_{CF} = 25.2$, $^{2}J_{CF} = 26.6$ Hz, Ar-C3), 111.7 (dd, $^{2}J_{CF} = 21.0$, $^{4}J_{CF} = 3.7$ Hz, Ar-C5), 124.5 (dd, $^{2}J_{CF} = 14.2$, $^{4}J_{CF} = 3.7$ Hz, Ar-C1), 129.5 (dd, $^{3}J_{CF} = 9.5$ Hz, $^{3}J_{CF} = 6.0$ Hz, Ar-C6), 161.1 (dd, $^{1}J_{CF}$ 247.6, Hz, $^{3}J_{CF} = 11.8$ Hz, Ar-C2 or Ar-C4), 162.8 (dd, $^{1}J_{CF} = 247.6$, $^{3}J_{CF} = 11.8$ Hz, Ar-C2 or Ar-C4). ^{19}F NMR: (282 MHz, $CDCl_3$) δ_F (^{1}H-coupled) ppm −115.5 (dt, $^{4}J_{FH} = 8.2$ Hz, $^{3}J_{FH} = {}^{4}J_{FF} = 8.5$, F-2), −112.6 (ddd, $^{4}J_{FF} = 8.5$ Hz, $^{3}J_{FH} = 8.2$ Hz, $^{4}J_{FH} = 6.9$ Hz, F-4). δ_F (^{1}H-decoupled) ppm −115.5 (d, $^{4}J_{FF} = 7.1$ Hz), −112.6 (d, $^{4}J_{FF} = 6.9$ Hz). m/z [EI]$^+$218 (4%, [M + NH$_4$]$^+$), 186 (38%, [M-CH$_2$]$^+$), 169 (38%, [M-OCH$_3$]$^+$), 157 (100%, [M-C$_3$H$_7$]$^+$), 127 (22%, [M-C$_4$H$_9$O]$^+$).

2,6-Difluoro-3-(1-methoxy-2-methylpropyl)benzaldehyde ***(5)***. 1-(2,4-Difluorophenyl)-2-methylpropyl methyl ether (500 mg, 2.50 mmol) was dissolved in anhydrous THF (10 ml) and stirred for 15 min under argon. The reaction was cooled −78°C, followed by dropwise addition of sec-BuLi to (3.75 ml, 5.20 mmol) and stirred for 2 h. Methyl formate (0.22 mL, 3.75 mmol) was added and the mixture was stirred for 1.5 h at −78°C. H_2SO_4 (1M, 20 mL) was added gradually and the mixture was stirred for another 5 min. The product was extracted with diethyl ether (3 × 30 mL), washed with water (100 mL), HCl (1 M, 100 mL), brine (100 mL), dried ($MgSO_4$) and evaporation of the solvent yielded 2,6-difluoro-3-(1-methoxy-2-methylpropyl)benzaldehyde **(5)** as a colourless oil (545.6 mg, 2.40 mmol, 96%); R_f 0.40 (EtOAc:Hexane, 15:85). IR (neat, cm^{-1}) 2963, 1701 (C═O), 1616, 1478, 1364, 1251, 1090, 986 cm^{-1}. ^{1}H NMR: (300 MHz, $CDCl_3$) δ ppm 0.75 (3H, d, $J = 6.8$ Hz, C*H*$_3$), 0.90 (3H, d, $J = 6.7$ Hz, C*H*$_3$), 1.85 (1H, dqq ~ octet, $J = 6.7$ Hz, C*H*(Me)$_2$), 3.14 (3H, s, OC*H*$_3$), 4.17 (1H, d, $J = 6.8$ Hz, C*H*OCH$_3$), 6.95 (1H, td, $^{3}J_{HF} = {}^{3}J_{HH} = 8.7$, $^{5}J_{HF} = 1.1$ Hz, Ar CH-5), 7.54 (1H, dt, $^{3}J_{HH} = 8.2$, $^{4}J_{HF} = 6.6$ Hz, Ar CH-4), 10.29 (1H, s, C*H*O). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 17.2 (Me), 17.3 (Me), 33.2 (*C*H(Me)$_2$), 56.4 (OC H$_3$), 80.1 (*C*HOCH$_3$), 111.4 (dd, $^{2}J_{CF} = 20.9$, $^{4}J_{CF} = 4.1$ Hz Ar-C5), 112.5 (t, $^{2}J_{CF} = 22.5$ Hz, Ar-C1), 124.5 (dd, $^{2}J_{CF} = 14.1$, $^{4}J_{CF} = 4.1$ Hz, Ar-C3), 133.9 (t, $^{3}J_{CF} = 8.0$ Hz Ar-C4), 159.6 (dd, $^{1}J_{CF} = 262.6$ Hz, $^{3}J_{CF} = 5.5$ Hz, Ar-C6), 161.4 (dd, $^{1}J_{CF} = 262.6$ Hz, $^{3}J_{CF} = 5.6$ Hz, Ar-C2), 183.7 (t, $^{3}J_{CF} = 4.7$ Hz, *C*HO). ^{19}F NMR: (282 MHz, $CDCl_3$) δ_F (^{1}H-coupled) ppm −121.0 (d, $^{4}J_{FF} = {}^{4}J_{FH} = 7.4$ Hz, F-2), −117.0 (dt, $^{3}J_{FH} = 9.8$ Hz, $^{4}J_{FF} = {}^{4}J_{FH} = 6.6$ Hz, F-6). δ_F (^{1}H-decoupled) ppm −121.0 (d, $^{4}J_{FF} = 7.2$ Hz), −117.0 (d, $^{4}J_{FF} = 7.1$ Hz). m/z [CI]$^+$245 (99%, [M + NH$_3$]$^+$). HRMS + ES m/z (M + NH$_4$)$^+$ calcd for $C_{12}H_{18}O_2N_1F_2$ 246.1300; found 246.1302.

2-(Dimethoxymethyl)-1,3-difluoro-4-(1-methoxy-2-methylpropyl)benzene ***(6)***. Dry MeOH (20 mL) was added to 2,6-difluoro-3-(1-methoxy-2-methylpropyl)benzaldehyde (244.2 mg, 1.07 mmol), trimethyl orthoformate (179.1 mg, 1.61 mmol), TsOH (305.3 mg, 1.61 mmol) and the mixture was heated at reflux for 3 h under argon. The mixture was diluted with diethyl ether (100 mL), washed with a solution of NaOH (15%): brine (1:1 ratio) (100 mL), distilled water (100 mL), brine (100 mL), dried ($MgSO_4$) and evaporation of the solvent yielded 2-(dimethoxymethyl)-1,3-difluoro-4-(1-methoxy-2-methylpropyl)benzene **(6)** as a colourless oil (243.1 mg, 0.89 mmol, 83%); R_f 0.26 (EtOAc:Hexane, 10:90). IR (neat, cm^{-1}) 2962, 1623, 1483, 1384, 1249, 1205, 1092, 1029,

965 cm^{-1}. ^1H NMR: (300 MHz, $CDCl_3$) δ ppm 0.80 (3H, d, $\mathcal{J} = 6.8$ Hz, CH_3), 0.96 (3H, d, $\mathcal{J} = 6.7$ Hz, CH_3), 1.91 (1H, dqq ~ octet, $\mathcal{J} = 6.7$ Hz, CH(Me)$_2$), 3.19 (3H, s, OCH_3), 3.47 (3H, s, OCH_3), 3.49 (3H, s, OCH_3), 4.21 (1H, d, $\mathcal{J} = 6.8$ Hz, CHOCH$_3$), 5.61 (1H, s, CH(OCH$_3$)$_2$), 6.93 (1H, dd, $^3\mathcal{J}_{HF} = 9.6$, $^3\mathcal{J}_{HH} = 8.7$ Hz, Ar CH-6), 7.33 (1H, dd, $^4\mathcal{J}_{HF} = 8.0$, $^3\mathcal{J}_{HH} = 6.6$ Hz, Ar CH-5). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 18.3 (Me), 18.5 (Me), 34.3 (CH(Me)$_2$), 55.0 (OCH$_3$), 55.4 (OCH$_3$), 57.3 (OCH$_3$), 81.5 (CHOCH$_3$), 99.5 (t, $^3\mathcal{J}_{CF} = 3.2$ Hz, (CH(OCH$_3$)$_2$), 111.8 (dd, $^2\mathcal{J}_{CF} = 22.5$ Hz, $^4\mathcal{J}_{CF} = 3.8$ Hz, Ar-C6), 113.5 (t, $^2\mathcal{J}_{CF} = 33.4$ Hz, Ar-C2), 124.6 (dd, $^2\mathcal{J}_{CF} = 15.3$, $^4\mathcal{J}_{CF} = 3.8$ Hz, Ar-C4), 129.0 (t, $^3\mathcal{J}_{CF} = 7.0$ Hz, Ar-C5), 157.7 (dd, $^1\mathcal{J}_{CF} = 251.2$ Hz, $^3\mathcal{J}_{CF} = 7.8$ Hz, Ar-C1), 161.0 (dd, $^1\mathcal{J}_{CF} = 251.2$ Hz, $^3\mathcal{J}_{CF} = 7.7$ Hz, Ar-C3). ^{19}F NMR: (282 MHz, $CDCl_3$) δ_F (^{1}H-coupled) ppm −119.8 – −119.7 (m, F-3), −115.9 – −115.8 (m, F-1). δ_F (^{1}H-decoupled) ppm −119.7 (d, $^4\mathcal{J}_{FF} = 4.8$ Hz), −115.8 (d, $^4\mathcal{J}_{FF} = 4.8$ Hz). m/z [EI]$^+$273 (17%, [M-H]$^+$), 259 (23%, [M-CH$_3$]$^+$), 243 (14%, [M-OCH$_3$]$^+$), 231 (100%, [M-C$_3$H$_7$]$^+$), 185 (47%, [M-C$_5$H$_{13}$O]$^+$), 157 (48%, [M-C$_6$H$_{13}$O$_2$]$^+$).

*3-(Dimethoxymethyl)-2,4-difluoro-5-(1-methoxy-2-methylpropyl)benzaldehyde **(7)***. 2-(Dimethoxymethyl)-1,3-difluoro-4-(1-methoxy-2-methylpropyl)benzene (220.0 mg, 0.80 mmol) was dissolved in anhydrous THF (10 mL) and stirred for 15 min under argon. The reaction was cooled to −78°C followed by dropwise addition of sec-BuLi (0.86 mL, 1.20 mmol) and stirred for 2 h. Methyl formate (0.07 mL, 1.20 mmol) was added and the mixture was stirred for 1.5 h at −78°C. H_2SO_4 (1M, 20 mL) was added gradually and the mixture was stirred for another 5 min. The product was extracted with diethyl ether (3 × 30 mL), washed with water (100 mL), HCl (1 M, 100 mL), brine (100 mL), dried ($MgSO_4$) and evaporation of the solvent yielded 3-(dimethoxymethyl)-2,4-difluoro-5-(1-methoxy-2-methylpropyl)benzaldehyde **(7)** as a colourless oil (181.4 mg, 0.60 mmol, 75%); R_f 0.41 (EtOAc:Hexane, 20:80). IR (neat, cm^{-1}) 2963, 1696 (C=O), 1607, 1466, 1236, 1191, 1073, 994 cm^{-1}. ^1H NMR: (300 MHz, $CDCl_3$) δ ppm 0.82 (3H, d, $\mathcal{J} = 6.6$ Hz, CH_3), 0.95 (3H, d, $\mathcal{J} = 6.7$ Hz, CH_3), 1.93 (1H, dqq ~ octet, $\mathcal{J} = 6.7$ Hz, CH(Me)$_2$), 3.21 (3H, s, OCH_3), 3.50 (3H, s, OCH_3), 3.52 (3H, s, OCH_3), 4.21 (1H, d, $\mathcal{J} = 6.6$ Hz, CHOCH$_3$), 5.64 (1H, s, CH(OCH$_3$)$_2$), 7.93 (1H, t, $^4\mathcal{J}_{HF} = 7.8$ Hz, Ar CH-6), 10.32 (s, CHO). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 18.4 (Me), 18.8 (Me), 34.5 (CH(Me)$_2$), 55.7 (OCH$_3$), 55.9 (OCH$_3$), 57.9 (OCH$_3$), 81.9 (CHOCH$_3$), 99.6 (t, $^3\mathcal{J}_{CF} = 3.2$ Hz, CH(OCH$_3$)$_2$), 115.2 (dd, $^2\mathcal{J}_{CF} = 33.4$ Hz, $^4\mathcal{J}_{CF} = 2.2$ Hz, Ar-C1), 121.5 (dd, $^2\mathcal{J}_{CF} = 12.7$, $^4\mathcal{J}_{CF} = 5.5$ Hz, Ar-C5), 126.7 (t, $^2\mathcal{J}_{CF} = 20.3$ Hz, Ar-C3), 129.1 (dd, $^3\mathcal{J}_{CF} = 8.6$, $^3\mathcal{J}_{CF} = 8.2$ Hz, Ar-C6), 161.9 (dd, $^1\mathcal{J}_{CF} = 261.2$ Hz, $^3\mathcal{J}_{CF} = 8.8$ Hz, Ar-C2), 163.7 (dd, $^1\mathcal{J}_{CF} = 261.2$ Hz, $^3\mathcal{J}_{CF} = 8.9$ Hz, Ar-C4), 186.3 (d, $^3\mathcal{J}_{CF} = 7.2$ Hz, CHO). ^{19}F NMR: (282.4 MHz, $CDCl_3$) δ_F (^{1}H-coupled) ppm −124.4 (t, $^4\mathcal{J}_{FF} = {}^4\mathcal{J}_{FH} = 8.0$ Hz), −107.6 (t, $^4\mathcal{J}_{FF} = {}^4\mathcal{J}_{FH} = 9.1$ Hz). δ_F (^{1}H-decoupled) ppm −124.4 (d, $^4\mathcal{J}_{FF} = 8.5$ Hz), −107.6 (d, $^4\mathcal{J}_{FF} = 8.4$ Hz). m/z [CI]$^+$287 (12%, [M-CH$_3$]$^+$), 271 (51%, [M-OCH$_3$]$^+$), 256 (100%, [M-C$_2$H$_6$O]$^+$); HRMS + ES m/z (M + NH$_4$)$^+$ calcd for $C_{15}H_{24}O_4N_1F_2$ 320.1668; found 320.1667.

*2,4-Difluoro-5-(1-methoxy-2-methylpropyl)isophthalaldehyde **(8)***. A solution of 3-(dimethoxymethyl)-2,4-difluoro-5-(1-methoxy-2-methylpropyl) benzaldehyde (110.2 mg, 0.36 mmol), trifluoroacetic acid (50% aqueous solution) (5 mL) and $CHCl_3$ (10 mL) were stirred at room temperature for 2 h. DCM (50 mL) was added, which was washed with distilled water (50 mL), NaOH (1M) (3 × 50 mL), $NaHCO_3$ (50 mL), brine (50 mL), dried over $MgSO_4$ and evaporation of the solvent furnished 2,4-difluoro-5-(1-methoxy-2-methylpropyl)isophthalaldehyde **(8)** as a colourless oil (20.4 mg, 0.08 mmol, 22%); R_f 0.43 (EtOAc:Hexane, 20:80). IR (neat, cm^{-1}) 2965, 1697 (C=O), 1602, 1461, 1244, 1087, 984, 731 cm^{-1}. ^1H NMR: (300 MHz, $CDCl_3$) δ ppm 0.85 (3H, d, $\mathcal{J} = 6.8$ Hz, CH_3), 0.97 (3H, d, $\mathcal{J} = 6.7$ Hz, CH_3), 1.96 (1H, dqq ~ octet, $\mathcal{J} = 6.7$ Hz, CH(Me)$_2$), 3.32 (3H, s, OCH_3), 4.26 (1H, d, $\mathcal{J} = 6.6$ Hz, CHOCH$_3$), 8.19 (1H, t, $^4\mathcal{J}_{HF} = 7.7$ Hz, Ar CH-6), 10.36 (1H, s, CHO), 10.40 (1H, s, CHO). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 18.0 (Me), 18.4 (Me), 34.2 (CH(Me)$_2$), 57.7 (OCH$_3$), 81.1 (CHOCH$_3$), 113.9 (dd, $^2\mathcal{J}_{CF} = 22.5$ Hz, $^4\mathcal{J}_{CF} = 2.0$ Hz, Ar-C1), 121.3 (dd, $^2\mathcal{J}_{CF} = 11.9$, $^4\mathcal{J}_{CF} = 4.2$ Hz, Ar-C5), 127.7 (dd, $^2\mathcal{J}_{CF} = 15.0$ Hz, $^2\mathcal{J}_{CF} = 14.9$ Hz, Ar-C3), 133.9 (t, $^3\mathcal{J}_{CF} = 9.5$ Hz, Ar-C6), 163.3 (dd, $^1\mathcal{J}_{CF} = 270.6$ Hz, $^3\mathcal{J}_{CF} = 6.6$ Hz, Ar-C2), 165.1 (dd, $^1\mathcal{J}_{CF} = 270.8$ Hz, $^3\mathcal{J}_{CF} = 6.7$ Hz, Ar-C4), 183.6 (t, $^3\mathcal{J}_{CF} = 4.8$ Hz, ArC3-CHO), 184.8 (d, $^3\mathcal{J}_{CF} = 7.1$ Hz, ArC1-CHO). ^{19}F NMR: (282.4 MHz, $CDCl_3$) δ_F (^{1}H-coupled) ppm −126.4 (t, $^4\mathcal{J}_{FF} = {}^4\mathcal{J}_{FH} = 8.0$ Hz), −109.5 (t, $^4\mathcal{J}_{FF} = {}^4\mathcal{J}_{FH} = 7.8$ Hz). δ_F (^{1}H-decoupled) ppm −126.4 (d, $^4\mathcal{J}_{FF} = 8.2$ Hz), −109.5 (d, $^4\mathcal{J}_{FF} = 8.1$ Hz). m/z [CI]$^+$290 (40%, [M + 2NH$_2$ (imine)]$^+$), 274 (27%, [M· + NH$_4$]$^+$), 213 (17%, [M-C$_3$H$_7$]$^+$), 198 (14%, [M-C$_4$H$_{10}$]$^+$); HRMS + ES m/z (M + NH$_4$)$^+$ calcd for $C_{13}H_{18}O_3N_1F_2$ 274.1249; found 274.1252.

Biology

HCT116 cells (obtained from the ATCC/LCG Promochem, Teddington, U.K) and HCT116 cells engineered for inducible expression of c-Src in the presence of Dox (clone wt23, [18]) were grown in

McCoy's 5A media (Gibco/Invitrogen, Paisley, U.K) supplemented with 10% fetal calf serum, 10U/mL penicillin and 10 μg/mL streptomycin. Cells were maintained at 37°C, 5% CO_2 in a humidified incubator. Cells were seeded at 1×10^6/3mL media/well in six well tissue culture plates (Corning, Corning, NY, cat. No:3516). After 24 h, the media was replaced with a fresh media containing the appropriate treatment. The treatments included a combination of Dox (Clontech, Palo Alto, CA, cat. no: 8634-1, prepared and stored as a 2 mg/mL stock in H_2O at −20°C), PP2 (Calbiochem, Nottingham, U.K, cat.no:529576), compound **(8)** (prepared and stored as both a 10 mM and a 40 mM stock in DMSO at −20 °C) and DMSO (Sigma, Poole, U.K cat. no:154938). The used concentrations of the drugs were: 2 μg/mL Dox, 10μM PP2 and 0–40μM compound **(8)** as indicated in the results section. As an additional control (+ Dox + DMSO), 2 μg/mL Dox + 1 μl DMSO/mL media was used to ensure that the results obtained were not due to the presence of DMSO. After a further 24 h, the cells were harvested directly into 2x Laemmli buffer (Biorad, Hercules, CA cat. no:161-0737) and the lysates were analyzed by SDS polyacrylamide gel electrophoresis as described previously [19]. Proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Perkin-Elmer, Boston, MA) and incubated with the desired primary antibody followed by the appropriate horseradish peroxidase-coupled anti-mouse or anti-rabbit secondary antibody (Dako, Glostrop, Denmark). Primary antibodies used were: mouse monoclonal anti-phosphotyrosine (Upstate, Chandlers Ford, U.K, clone 4G10, cat. no: 16-105), mouse monoclonal anti-Src (Upstate, Chandlers Ford, U.K, clone GD11, cat. no: 05-184), polyclonal rabbit anti-Src [Y418] (Biosource, Camarillo, CA, cat. no: 44-660), rabbit monoclonal anti-GAPDH (Cell Signaling, Hitchin, U.K, cat. no: 2118) and mouse monoclonal anti-actin (Sigma, Poole, U.K, Clone AC40, cat. no: A 4700).

Molecular modelling

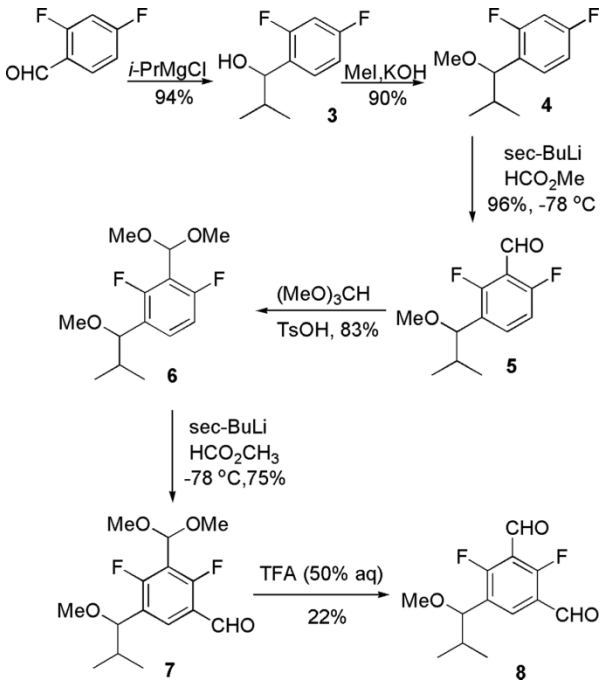
The crystal structure of an active form of Src was obtained from the Brookhaven protein database, entry code 1Y57 (1.91 Å) [20]. Water molecules were deleted from the protein crystal structure, hydrogen atoms were added, tautomeric and ionization states for selected residues such as Asp, Glu and His were checked. The 3D structure of the difluoro analogue **(8)** was modelled using the program SYBYL (Tripos, Inc., St. Louis, 2005). Final structures were energy minimized using the Tripos force field.

The genetic algorithm based docking program GOLD v3.0.1 [21] was used to covalently dock **(8)** to exposed lysine residues at the interface between the SH3 domain and the SH2-kinase linker region, modelling imine formation. Subsequently docking solutions produced by the program were saved, visualized and compared for analysis. Quantum chemical calculations including Mulliken population analysis were performed for **(8)** using the quantum chemistry package, Gaussian 03 [22].

Results

Chemical synthesis

The synthesis of 2,4-difluoro-5-(1-methoxy-2-methylpropyl)isophthalaldehyde **(8)** is shown in Scheme 1. 2,4-Difluorobenzaldehyde was reacted with isopropyl magnesium chloride [23] (isopropyl magnesium bromide gave a poor yield) to give 1-(2,4-difluorophenyl)-2-methylpropan-1-ol **(3)** in an excellent yield of 94%. Alcohol **(3)** was methylated with methyl iodide [24] to give **(4)** in 90% yield. Formylation of **(4)** with sec-BuLi [17] and methyl formate at −78°C added the first aldehyde **(5)** in a yield of 96%. Aldehyde **(5)** was subsequently protected with trimethyl orthoformate [25] to give acetal **(6)** in 83% yield. Formylation with methyl formate and sec-BuLi introduced the second aldehyde group to give **(7)** in 75% yield. Deprotection of the acetal in a poor 22% yield accomplished the formation of the target compound **(8)** (Scheme 1). All compounds were fully characterized using a range of analytical techniques including IR spectroscopy, ^1H, ^{13}C and ^{19}F NMR spectroscopy. The analogues showed appropriate coupling patterns to fluorine.



Scheme 1. Synthesis of the difluoro analogue of UCS15A **(8)**.

Biological evaluation

Considering the fact that in the previously published studies UCS15A has been reported to have a profound effect on the function of the non-receptor tyrosine kinase Src, we decided to analyze the effects of **(8)** on c-Src activity in HCT 116 human colorectal carcinoma cells. Treatment of wild type HCT 116 cells for 24 h with concentrations of **(8)** ranging from 5μM to 40μM had no visible effect on the overall intracellular tyrosine phosphorylation pattern (Figure 2A, lane: - Dox and data not shown). Subsequently, in order to be able to study the effects of **(8)** on c-Src activity in HCT 116 cells more precisely, we decided to employ the recently developed HCT 116 cells in which expression of high levels of wild type (wt) c-Src can be inducibly triggered by treatment with low concentrations of doxycycline (Dox). These cells (HCT116 wtSrc23) preserve the characteristics of parental HCT 116 cells and thus represent a good experimental model system for analysis of c-Src function *in vivo* [18]. Importantly Dox did not effect the tyrosine phosphorylation pattern in HCT 116 cells either alone nor in the presence of **(8)** (Figure 2A, + Dox lanes). Induction of HCT 116 wtSrc23 cells with Dox for 24 h resulted in a significant increase in the total level of c-Src protein. Also the fraction of active, autophosphorylated [P-Tyr 418] Src was significantly higher (Figure 2B, compare lanes −Dox and + Dox). Surprisingly, induction of wt c-Src in the presence of **(8)** at concentration 40μM resulted in an even higher fraction of activated c-Src. This was accompanied by a significant elevation in the total intracellular tyrosine phosphorylation signal (Figure 2B, lane + Dox +40μM **(8)**). Importantly, the increases observed in c-Src phosphorylation on Tyr 418 and in the global intracellular tyrosine phosphorylation could be attributed to Src kinase activity because they could be prevented by treatment with PP2, a specific inhibitor of Src kinase activity (Figure 2B, compare lines: + Dox +40μM **(8)** and + Dox +40μM **(8)** + PP2). Taken together these data indicate that **(8)** may act as a weak c-Src activator *in vivo*.

This unexpected effect of **(8)** suggested the establishment of a stabilisation mechanism of Src protein in the active conformation in the colon cancer cells, which will enhance the activity of Src protein. Therefore, we observe an increase in cellular phosphorylation as a final outcome of the process.

Modelling of the potential covalent interaction of the difluoro analogue of UCS15A with Src

Although the mechanism of action of **(8)** is unknown, given the reactive nature of **(8)**, it may well involve Schiff base formation of the reactive aldehyde groups of **(8)** with the amino groups of lysines in the protein to give an imine. Here computational prediction of imine formation between the aldehyde groups of **(8)** and lysines in the SH3/SH2-kinase linker region of Src

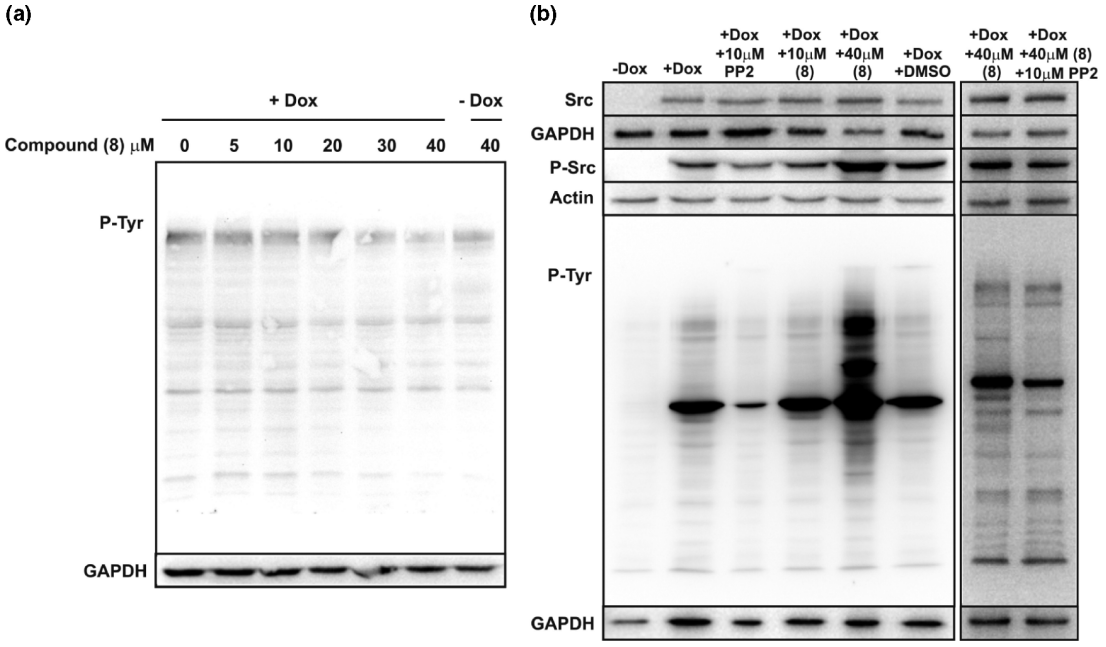


Figure 2. The effects of compound **(8)** on tyrosine phosphorylation in wild type HCT 116 cells **(A)** and in HCT 116 wtSrc23 cells engineered for inducible expression of c-Src **(B)**. (A) Total phosphotyrosine signal (P-Tyr) detected in HCT 116 cells after 24 h of treatment with the indicated concentrations of compound **(8)** in the presence (+ Dox) or absence (− Dox) of doxycycline. GAPDH blot provides the loading control. (B) Total phosphotyrosine signal (P-Tyr) detected in HCT 116 wtSrc23 cells 24 h after indicated treatments (see Materials and Methods section for details). Src and P-Src blots illustrate accompanying levels of total c-Src and phospho-c-Src (Tyr 418 phosphorylation) respectively. GAPDH and Actin blots provide the loading controls.

(Figure 3A and Table I) was performed using the docking program GOLD and a crystal structure of Src (1Y57, 1.91 Å). The most favourable Schiff base was formed between the 3-aldehyde group of **(8)** with the sidechain of Lys249 of the linker region (Figure 3B), with a predicted binding energy of −6.5 kcal/mol (Table 1). To further rationalise covalent bond formation at the 3-aldehyde, compound **(8)** was geometry optimised at the B3LYP/6-31+G* level of theory. Mulliken charges were then derived from the MP2/aug-cc-pVDZ density matrix. The Mulliken charge obtained for the aldehyde carbon at the 3-position was +1.16 e, and for the carbon of the 1-aldehyde group was +1.00 e. This suggests that nucleophiles would have a similar propensity to attack at either aldehyde, with perhaps a small preference for

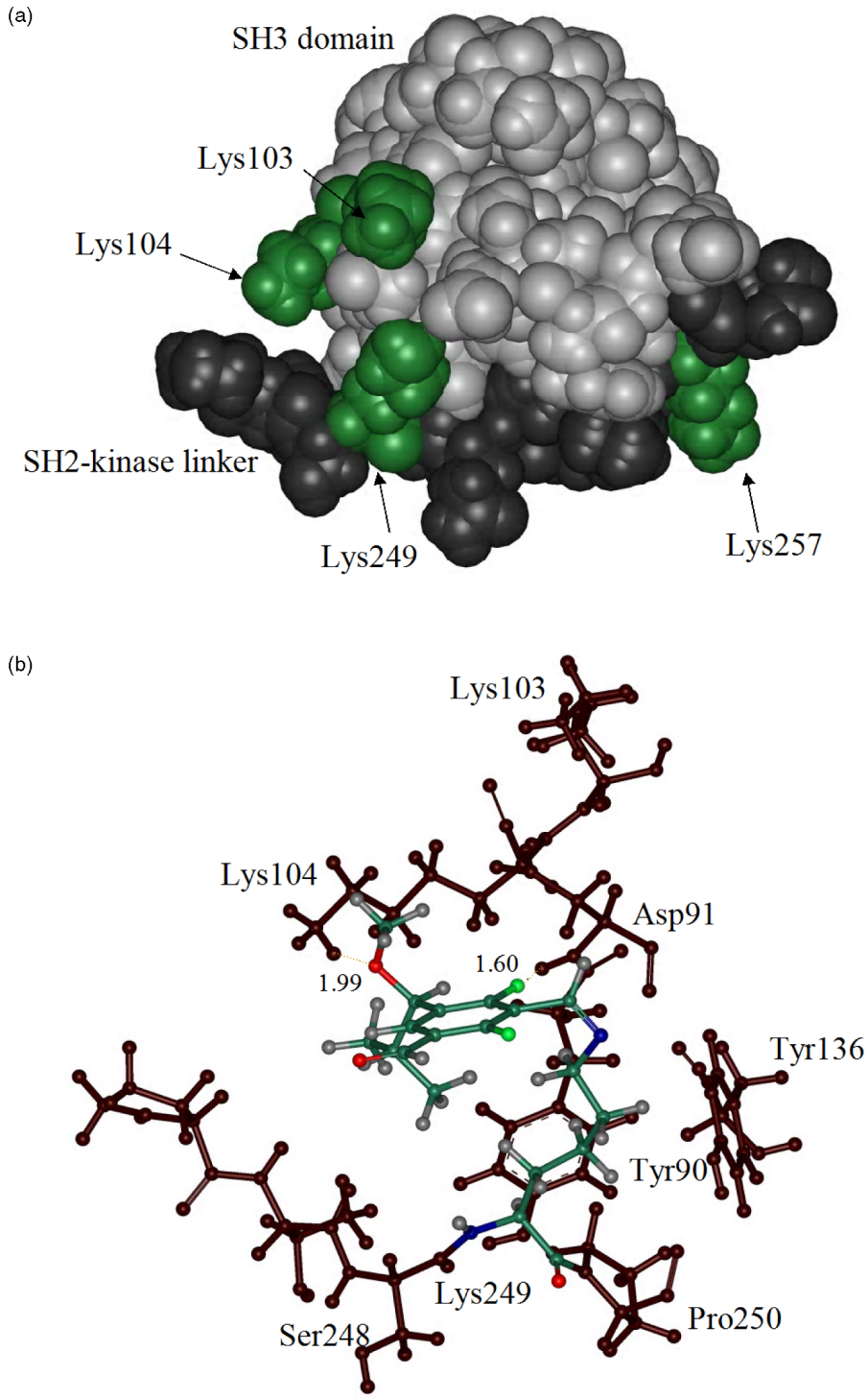


Figure 3. (A) Lysine residues targeted for covalent docking of **(8)** at SH3/SH2-kinase linker interface. (B) Difluoro analogue **(8)** covalently bound to Lys249 in Src. Hydrogen bond distances in Å.

Table I. Scored interactions (ΔG) from the covalent docking of the aldehyde group of **(8)** with the lysine side-chains in the Src SH3/linker region using ChemScore (kcal/mol). Hydrogen bonding residues and distances (Å) are shown.

covalent interaction	ΔG	protein-ligand hydrogen bond distances
Lys103/3-CHO	−2.6	1.90 (Lys249 $NH_2H\cdots$OMe)
Lys103/1-CHO	−1.9	2.04 (Ser101 OH···OCH)
Lys104/3-CHO	−5.6	–
Lys104/1-CHO	−5.6	2.06 (Asp91 N-H···OMe)
Lys249/3-CHO	−6.5	1.99 (Lys104 $NH_2H\cdots$OMe)
		1.60 (Asp91 N-H···F4)
Lys249/1-CHO	−5.2	1.98 (Lys104 $NH_2H\cdots$OMe)
Lys257/3-CHO	0.2	–
Lys257/1-CHO	2.4	–

the 3-position. In addition to this, the higher fitness of **(8)** docked at the 3-position suggests stabilisation from non-covalent forces such as hydrogen bonding, established in concert with covalent bond formation.

The bound docked complex shows that **(8)** forms good interactions with exposed residue Lys249, being stabilised by two hydrogen bonds (Table 1, Figure 3). It should also be noted that covalent interactions with nearby residues in the SH3 domain, Lys104 and to a lesser extent Lys103, are also predicted as potential sites of reaction for **(8)** (Table I).

In summary, the synthesis of the isosteric/isoelectronic difluoro non-sugar analogue of UCS15A **(8)** has been achieved in good yield. Biological evaluation of this compound has revealed an interesting property, namely activation of Src-dependent phosphorylation on long-term exposure of **(8)**. It is postulated that formation of a Schiff base in the SH3/SH2-kinase linker region could potentially disrupt initial steps in activation of the scaffold function of Src, through introduction of new non-covalent interactions between domains, and inhibit subsequent deactivation through covalent modification of the linker and/or SH3 domain. However, further biophysical and biochemical elucidation of the mode of action of **(8)** and the basis of Src activity is required.

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