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Synthesis and Characterization of Photoaffinity Labelling reagents Towards the Hsp90 C-terminal Domain

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Glucosyl-novobiocin-based diazirine photoaffinity labelling reagents (PALs) were designed and synthesized to probe the Hsp90 C-terminal domain unknown binding pocket and structure-activity relationship. Five PALs were successfully synthesized from novobiocin in six consecutive steps employing a phase transfer catalytic glycosylation. Reactions were monitored and guided by analytical LC/MS which led to different strategies of adding either PAL precursor or sugar moiety first. The structures and bonding linkages of these compounds were characterised by various 2D-NMR spectroscopy and MS techniques. The synthetic technique provides powerful probes for unknown protein binding pocket.

Introduction

Heat Shock Protein 90 kDa (Hsp90) is a molecular chaperone involved in folding up to 200 proteins with the assistance of 20 co-chaperones.[1] It has been the target of research in genetics and epigenetics,[2] neurodegeneration[3] and cell mobility.[4] Hsp90 is particularly important for cancer cell survival.[5, 6] So far seventeen Hsp90 inhibitors have been developed to various stages of clinical trials, however, candidates were suspended at different clinical stages.[7,8] While these results validate Hsp90 inhibition as a relevant anti-cancer strategy, there is no FDA-approved drug.[1, 9, 10], due to the high cytotoxicity from N-terminal domain targeting inhibitors.

Hsp90 consists of three domains: an N-domain that contains an ATP, drug-binding site and co-chaperone-interacting motifs; an M-domain for client proteins and co-chaperones; and a C-terminal domain (CTD) contains a dimerization MEEVD motif.[11] Inhibition of the Hsp90CTD decreases chaperone dimerization, diminishes ATPase activity and impairs the formation of the Hsp90 protein complex. Novobiocin based CTD inhibitors have shown some superior activity against various cancer cell lines in apoptosis[12] or proliferation[13] compared to N-terminal inhibitors. Indeed, CTD inhibitors have attracted significant attention recently.[10, 14]

Due to the lack of a high resolution crystal structure, however, the binding site and structure-activity relationship (SAR) of Hsp90CTD is still unclear. Most high resolution Hsp90 crystal structure does not contain CTD. Only 4 Hsp90 crystals reported low resolution CTD: 2IOQ (*E. coli.*),[15] 2CG9 (Yeast),[11] 2O1U (Dog)[16] and 3Q6N (Human, which has 18 amino acid residues missing in the CTD).[17] Previous research indicated a few possible locations for the binding pockets[18, 19] in CTD, however, no confirmation has been obtained. Experimental-based SAR can provide limited guidance in drug design (Fig. 1A), in-depth understanding of the exact binding of ligand molecules to individual amino acid residues in the polypeptide is urgently required.

Previously we demonstrated that the single glycosylation the 4'-hydroxyl group of novobiocin (Fig. 1A) could increase its anticancer activity[20, 21] via binding to the Hsp90CTD[22]. Although protein modelling data indicate the formation of strong hydrogen bonds with Hsp90CTD peptides, the exact binding details is not clear.[20] We hereby designed and synthesized five glucosyl-novobiocin (Glc-Nov, Fig. 1B) based trifluoromethyl diazirine-type photoaffinity labelling reagents (PALs, **1-5**) to probe the binding site of Hsp90CTD.

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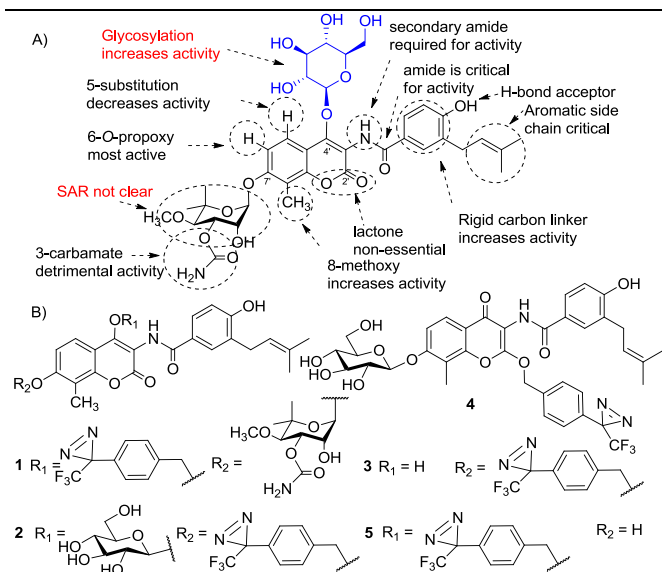
Figure. 1 Experimental based Hsp90CTD SAR (A) and five PALs (B)

These five compounds were designed to probe the binding of sugars (4'-glucose or 7'-noviose) with Hsp90CTD. Compound **1** mimics Glc-Nov with the PAL in the 4'-OH position to probe the 4'-*O*-glucose moiety binding sites. Compound **2** mimics Glc-Nov with the PAL in the 7'-OH and glucose in the 4'-OH positions respectively. Compound **3** mimics novobiocin with the PAL in the 7'- OH position to probe the 7'-*O*-noviose moiety binding sites in the absence of the 4'-*O*-glucose moiety. Compound **4** tests the potential binding in the 2'-*O* position and compound **5** tests the binding site with only the PAL in the 4'-O position, but no substitution on the 7'-OH group.

Trifluoromethyl diazirine has an excellent chemical stability and is highly resistant toward a number of factors such as temperature, nucleophiles, acidic and basic conditions and oxidizing/reducing reagents. The activation by UV (350 nm) yields an extremely reactive flexible carbene, which can insert into C-H, N-H and O-H bonds[23] with low non-specific binding [24].

Results & Discussion

Synthesis of compound **1** was achieved by reaction of novobiocin with diazirine bromide (**6**) in a polar aprotic solvent (DMF). Complete NMR assignment of all individual proton and carbon resonances has been achieved using ^{1}H, ^{13}C-NMR, DEPT-135, COSY, HSQC and HMBC (Fig. 2, S1a-c & Table S1). The long range correlation between the H1''' and coumarin ring C4' in the HMBC spectrum indicates that the C1''' was linked with the 4'-OH of novobiocin (Fig. 2).

Coumarin ring **7** (Table S2) and 4-hydroxy-3(3-methylbut--enyl) benzoic acid **8** (Table S3) were obtained by acid hydrolysis of novobiocin following reported procedures, but with slight modification as shown in the experimental section.[25] Coupling was performed using Steglich esterification reaction employing 1-Ethyl-3-(3dimethylaminopropyl) to obtain aminocoumarin **9** with 62% of yield. The NMR spectrum of **9** showed 4'-OH (br), 7'-OH and NH at 11.73, 10.52 and 9.44 ppm respectively (Fig. S2, Table S4). The long-range heteronuclear (H-C) correlations between 7'-OH and C7', and C8' and C9' and NH coupled to C4' and C1 supported the assignment and also indicated the formation of the amide bond (Fig. S2c).
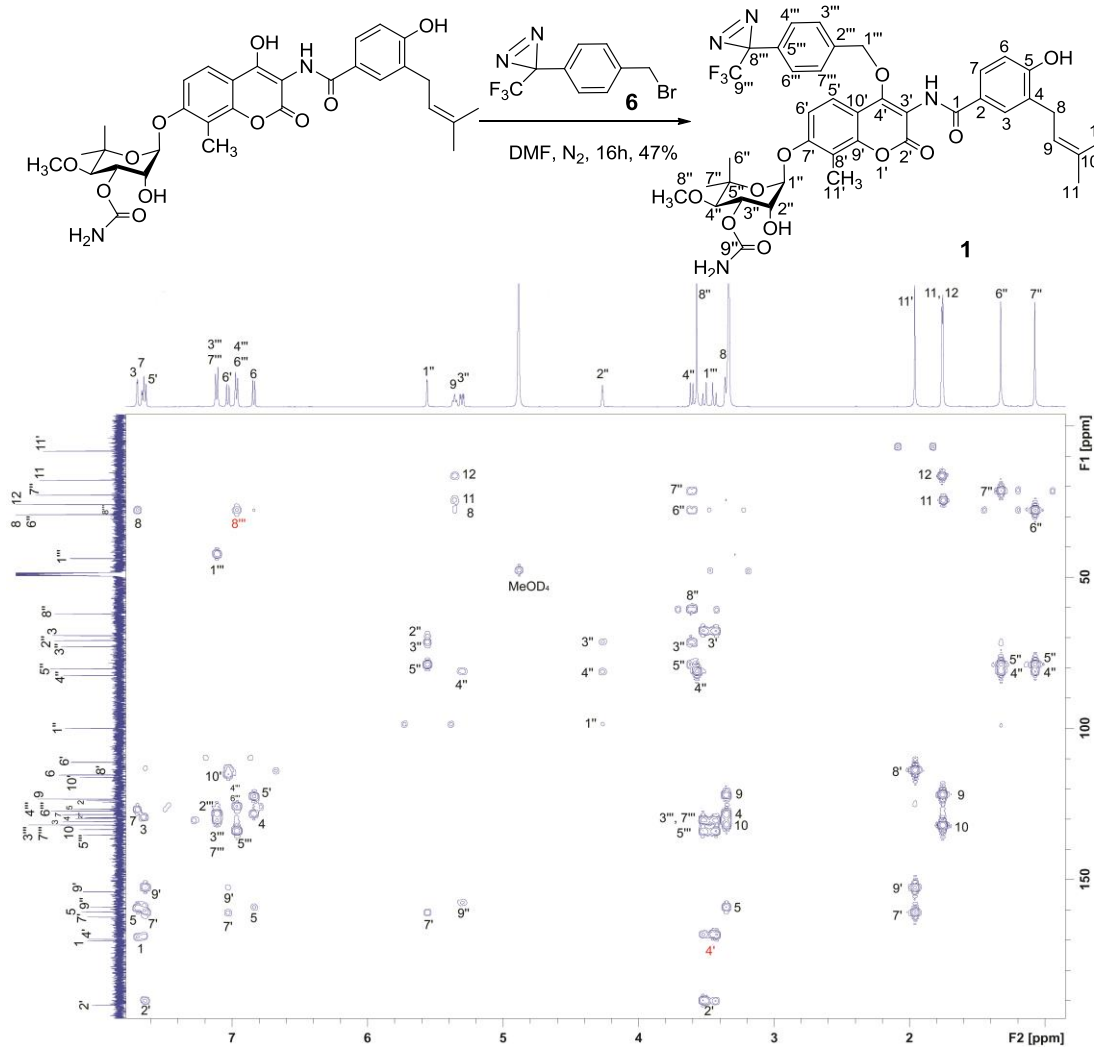
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Figure 2. Synthesis of compound **1**. Top: Synthesis scheme. Bottom: HMBC NMR assignment of compound **1**. Long range coupling between H1''' and C4' (Red 4'). It also shows the coupling between C4'''/C6''' and C8''' (red 8''').
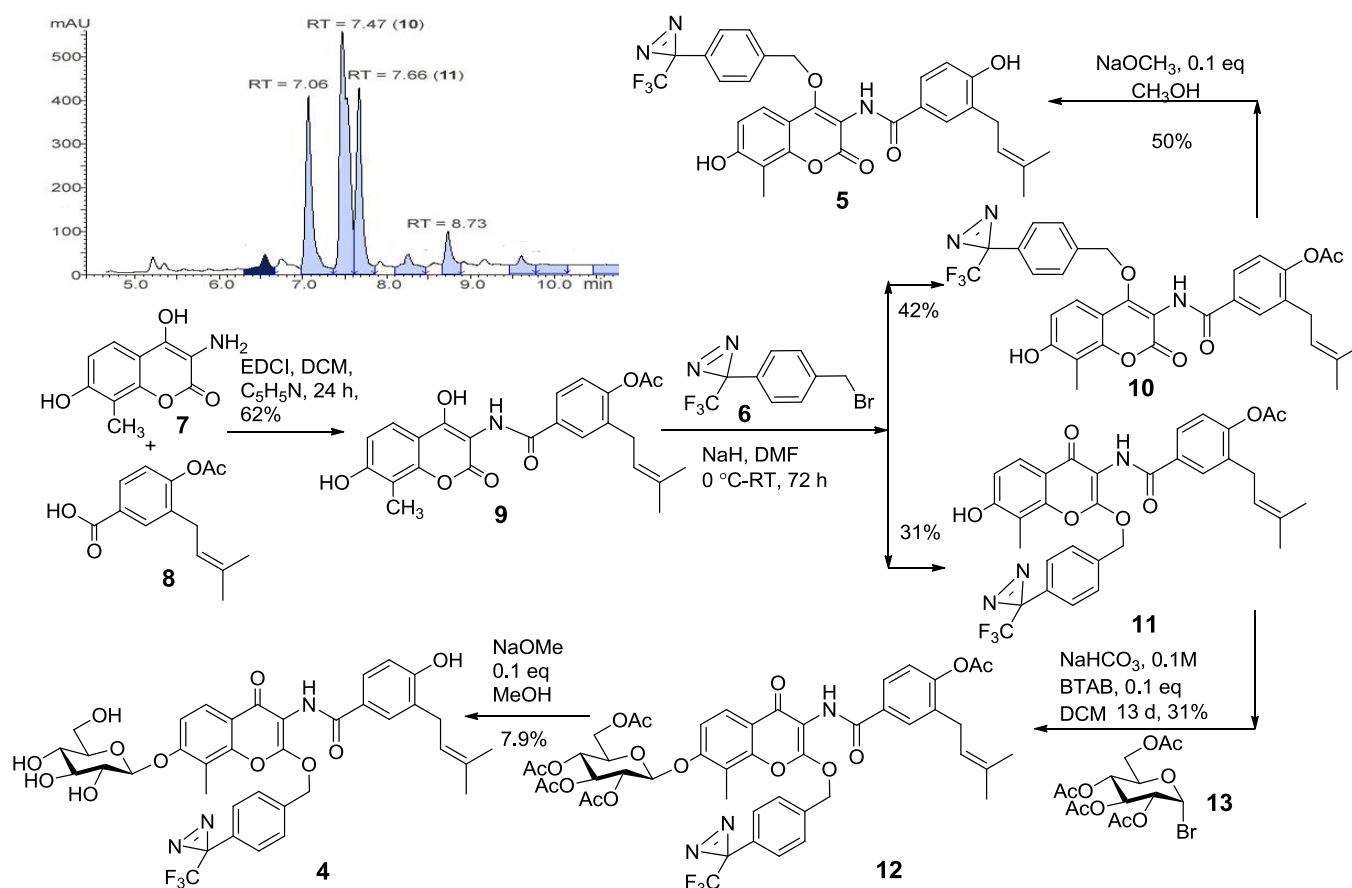
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Figure 3. Scheme to synthesis of compound **4** & **5** (inset, top left, analytical LC-MS spectrum of compound **10** &**11**).

Nucleophilic substitution reaction between compound **9** and the photoaffinity labelling regent precursor **6** resulted in two compounds (Fig. 3, inset, S3 & S4) with desired product masses (Fig. S3d, S3e, S4e, S4f) of 4'- and 7'- linked products. Preparative HPLC indeed separated those two compounds, the one with lower retention time (95 min, data not shown) was characterised to be the 4'-*O* compound **10** (Fig. 3, S3a-c and Table S5,) with the evidence of H1'' coupled to C4' in HMBC (Fig. S3b). However, the fraction at 100 min was surprisingly not the expected 7'-O-compound as the NMR did not show evidence of linkage to the 7'-C position (Fig. S4b). The 7'-OH and NH protons were still present in the ¹H-NMR (Fig. S4, Table S6). The spectrum showed a shift for H1'' from 3.3 ppm to 5.5 ppm and coupling to a carbon atom in a very similar position to C4' (Fig. S4d), which was shown to be C2'. These results indicated that there is an equilibrium between the 4' and 2' positions in the coumarin ring and that compound **11** is linked to the 2'-OH in the enol form.

It is worth noting that conventional catalysts for glycosylation, such as Ag₂O, Ag₂CO₃, AgOTf, TMSOTf and Hg(CN)₂, did not work

efficiently with glycosyl acceptors **10** and **11** (data not shown). Here we adopted a different route employing phase transfer glycosylation reaction. An optimised reaction condition was obtained by conducting a number of experiments using varying reactant ratios, catalyst equivalents, base concentrations, organic solvents and even solid phase transfer conditions (these investigations will be reported in future paper). Phase transfer glucosylation of compound **11** using Benzyl-tri-butyl ammonium bromide (BTAB) gave **12** (Fig. S5, Table S7) with the glucose moiety linked with the 7'-OH of the coumarin ring. The disappearance of the 7'-OH signal and the coupling between H1''' and C7' demonstrated the correct assignment (Fig. S5a-c). But, under the same conditions, glucosylation of compound **10** gave two different compounds (Fig. S6) which are probably due to early activation of diazirine. Analytical LC/MS data showed that glucosylation happened but under such conditions, the compound lost N₂ to form a radical, which led to either an insertion reaction (938, [M+H]⁺, Fig. S6b); or the removal of one additional acetyl group

(896, [M+H]+, Fig. S6c). Possible structures are proposed in Fig. S6b and S6c based on the MS data and mechanism.

Since directly coupling compound **6** to the 7'-OH position of the coumarin was not possible, an alternative approach was ~~adopted by changing the sequence of reactions. Reaction of~~

compound **9** with sugar donor **13** gave the expected product **14** (Fig. 4, S7, Table S8) and a di-glucosyl product (data not shown). Compound **14** reacted with PAL precursor **6** to give compound **15** at moderate yield (Fig. S8, Table S9).
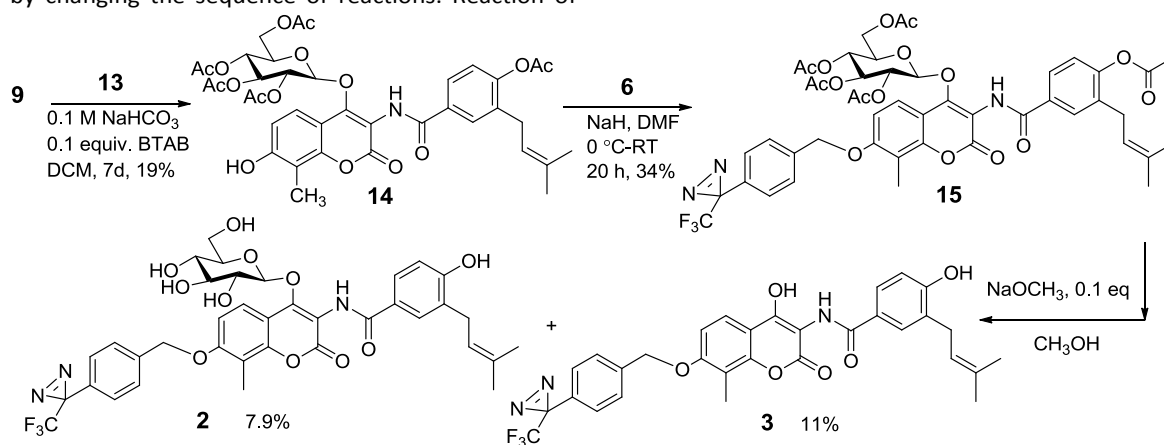

Figure 4. Synthesis of compound **2** & **3**

Deprotection of the acetyl groups using NaOMe was slow and step by step deacetylation was observed and confirmed by LC/MS, e.g. with one acetyl group (Fig. S9c&f) four acetyl group (Fig. S9c&g) being observed. In addition, cleavage of the glycoside bond was also observed (Fig. S9c&h) with the mass of 592 in negative mode probably due to unavoidable basic conditions caused by trace amount of water. The deprotection of compound **15** gave the desired compound **2** (Fig. S9a-c, Table S10) as well as compound **3** (Fig. S10, Table S11). Compound **4** (Fig. S11, Table S12) and **5** (Fig. S12, Table S13) were obtained by deprotecting compounds **12** and **10**, respectively.

Conclusions

In conclusion, the synthesis of five glucosyl-novobiocin based PALs was achieved in six steps. This accentuates that the phase transfer glycosylation is an effective way of for the incorporation of sugar moieties to molecules of interest with difficulties in conventional glycosylation methods. The structures have been confirmed by different NMR technology. This strategy of glucosyl-novobiocin modification provides a valuable approach for further development of enhanced glucosyl novobiocin mimetic. Preliminary data indicate the binding between Hsp90 CTD with compound **1**. and tandem MS analysis indicated the exact peptide the PAL binds to. This method provide an effective synthetic route for multifunctional compounds, and a concise chemical biology tool to probe protein unknown binding pocket SAR.

Experimental Section

General experiment details

Unless otherwise stated, all reactions were carried out in anhydrous condition. Reactions were monitored by thin layer chromatography

(TLC) and/or a Shimadzu single quadrupole LC/MS. Flash column chromatography was performed on Merck silica Gel 60 (particle size 40-63μm). ^{1}HNMR and ^{13}CNMR spectra were obtained using Bruker 400 MHz or a Bruker 500 MHz spectrometers. Multiplicity is abbreviated as follows (br = broad, s = singlet, d = doublet, dd = double doublet, t = triplet, m = multiplet, *etc.*) and coupling constants were obtained in Hertz. Assignments were aided by DEPT 90, DEPT 135, COSY, NOESY, HSQC and HMBC. High resolution mass spectra were obtained using Water Q-Tof MS. Chemicals were purchased from Sigma-Aldrich.

General procedure for phase transfer glycosylation

Brominated glycosyl donor (1.1-3.0 equiv), glycosyl acceptor (1 equiv.) and benzyl tributyl ammonium bromide (0.2 equiv.) in dichloromethane (DCM, 10 mL) was stirred for 5 min at room temperature. The 0.1 M NaHCO$_3$ (6 mL) was then introduced to the reaction mixture. The reaction mixture was stirred for specified time at room temperature. Upon completion (as indicated by TLC and LC/MS), the aqueous phase was washed with DCM (3 × 30 ml). The dichloromethane extracts were combined and washed with saturated NaHCO$_3$ (2 × 30 ml), water (2 × 30 ml) and dried (MgSO$_4$), filtered and solvents removed *in vacuo* to give the corresponding glycosides. The crude material was purified by column chromatography (ethyl acetate: hexane 1:2 to 1:3 v:v depending on the product) then by preparative HPLC using methods 1-3.

HPLC purification methods

Waters 2555 pump and 2489 UV detector with Waters C-18 (25 × 200 mm) column. Solvent A 0.1% TFA in Water, Solvent B 0.1% TFA in MeOH. Flow rate: 10 mL/min. Three methods, HPLC-1 (100 min), HPLC-2 (175 min) and HPLC-3 (255 min).

  HPLC-1: *t*=0 min, 30% B, *t*=90 min, 95% B, *t*=100 min, 95% B, *t*=101 min, 30% B, *t*=105 min, 30% B.

HPLC-2: *t*=0 min, 30% B, *t*=170 min, 95% B, *t*=175 min, 95% B, *t*=176 min, 30% B, *t*=177 min, 30% B.

HPLC-3: *t*=0 min, 30% B, *t*=250 min, 95% B, *t*=255 min, 95% B, *t*=256 min, 30% B, *t*=257 min, 30% B.

Deprotection of acetate

To a solution of the acetylated compound (1 mmol) in dry methanol (10 ml vol. based on acetate groups) was added sodium methoxide (0.1 mmol). The mixture was stirred at room temperature and monitored by LC/MS. The reaction mixture was neutralised with a drop of acetic acid, stirred for 0.5 hr. The reaction mixture was filtered and concentrated under reduced pressure and then purified by preparative HPLC.

4-(4-(3-(trifluoromethyl)-3H-diazirin-3-yl)phenyl)methanyl-novobiocin (1)

Novobiocin sodium salt and 3-(4-(bromomethyl)phenyl)-3-(trifluoromethyl)-3H-diazirine (88 mg, 0.316 mmol) were dissolved in 5 ml of anhydrous DMF under the protection of nitrogen. The mixture was protected from light and stirred vigorously at room temperature for 16h. The solvent were removed under vacuum and syrup was purified by flash chromatography (ethyl acetate, Rf = 0.5) to yield compound **1** as pale yellow solid (120mg, 47%). $[\alpha]_D^{20}$: -130.5°, (ACN). ^{1}HNMR (400 MHz, MeOD$_4$): δ = 7.55 (1 H, s, H3), 6.69 (1 H, d, J = 8.9 Hz, H6), 7.51 (1 H, d, J = 8.4 Hz, H7), 3.21 (2 H, m, H8), 5.20 (1 H, t, J = 7.4 Hz, H9), 1.60 (6H, s, H11, H12), 7.50 (1 H, d, J = 7.4 Hz, H5'), 6.88 (1 H, d, J = 8.4 Hz, H6'), 1.81 (3 H, s, H11'), 5.41 (1 H, s, H1''), 4.12 (1 H, s, H2''), 5.15 (1 H, d, J = 10.8 Hz, H3''), 3.46 (1 H, d, J = 10.8 Hz, H4''), 1.18 (3 H, s, H6''), 0.93 (3 H, s, H7''); 3.42 (3 H, s, H8''); 3.33 (2 H, d, J = 11.8, 36.9 Hz, H1'''), 6.96 (2 H, d, J = 7.9 Hz, H3''', H7'''), 6.81 (2 H, d, J = 7.9 Hz, H4''', H6'''). ^{13}CNMR (125 MHz, MeOD$_4$): δ = 169.70 (C1), 123.89 (C2), 130.91 (C3), 129.71 (C4), 160.6 (C5), 115.43 (C6), 128.51(C7), 29.33 (C8), 123.40 (C9), 133.50 (C10), 26.10(C11), 17.93 (C12), 191.50 (C2'), 69.34 (C3'), 170.30 (C4'), 126.60 (C5'), 111.25 (C6'), 162.50 (C7'), 115.33 (C8'), 154.10 (C9'), 116.21 (C10'), 8.30 (C11'), 100.07 (C1''), 71.01 (C2''), 72.96 (C3''), 82.55 (C4''), 80.34 (C5''), 29.34 (C6''), 22.92 (C7''), 62.08 (C8''), 159.17 (C9''), 43.90 (C1'''), 127.34 (C2'''), 131.94 (C3'''), 129.72 (C4''')135.31 (C5'''), 127.34 (C6'''), 131.94 (C7'''), 29.10 (C8'''), 123.20 (C9'''). ESI$^+$811 [M+H]$^+$, HRMS: 811.2767, calculated for $C_{40}H_{42}F_3N_4O_{11}$: 811.2802.

3-amino-4,7-dihydroxy-8-methyl-2H-chromen-2-one (7)

Novobiocin sodium salt (20 g, 0.0315 mol) was dissolved into a mixture of pyridine and acetic anhydride (5:1, 240 mL), and heated under reflux for 4 hours. After being cooled to room temperature, the mixture was then acidified with 5N-HCl drop by drop, the pH was monitored by the pH paper (pH=1) and the temperature of the reaction mixture was kept below 25°C by ice bath. Then the brown syrup precipitated, the aqueous phase was decanted. The brown syrup was washed with a small amount of diethyl ether (liquid kept for compound **8**), and later the precipitation of the crude product was collected. The crude product was further washed by diethyl ether again (liquid kept

for compound **8**) until getting the light grey powder (9.5 g, 69%): R$_f$ =0.13, petroleum ether/ethyl acetic 1:2 v/v. The above solid (7.0 g, 0.03 mol) was dissolved in anhydrous methanol (105 mL) and 10 % HCl/methanol (190 mL) and the mixture was refluxed for 2 hrs. The clear black solution obtained was evaporated *in vacuo* until precipitation started, then kept the reaction mixture at 4 °C overnight. The precipitation was filtered and washed with ice cold methanol to give the light grey powder. Then the filtrate was evaporated again and the procedure was repeated twice, and afforded the yellow solid (5.9 g, 95 %). R$_f$= 0.15, chloroform: methanol 4:1 v/v. ^{1}HNMR (400 MHz, MeOD$_4$): δ = 7.77 (1 H, d, J = 8.03 Hz, H5), 6.95 (1 H, d, J = 8.56 Hz, H6), 2.28 (3 H, s, H11). ^{13}CNMR (125 MHz, MeOD$_4$): δ = 164.2 (C2), 96.2 (C3), 161.7 (C4), 122.6 (C5), 113.7 (C6), 162.3 (C7), 113.5 (C8), 153.6 (C9), 108.1 (C10), 9.2 (C11). ESI$^+$208 [M+H]$^+$.

Synthesis of 4-acetoxy-3-(3-methylbut-2-en-1-yl)benzoic acid (8)

The filtrate from above procedure was concentrated to get light brown syrup and was extracted using ethyl acetate (3 × 25 ml) and with dilute hydrochloric acid (3 × 20 ml of a 3% aqueous solution). The organic extracts were combined and washed sequentially with saturated aqueous solution of NaHCO$_3$ (2 × 20 ml) and water (2 × 20 ml). The combined organic extracts were dried over magnesium sulphate, filtered and concentrated *in vacuo* and purified via column chromatography(petroleum ether:ethyl acetate 80:20) to afford white crystalline powder (1.2 g, 16 %). ^{1}HNMR (500 MHz, CDCl$_3$): δ = 8.03 (1H, s, H3), 7.17 (1H, d, J = 8.3 Hz, H6), 8.01 (1H, d, J = 8.3 Hz, H7), 3.31 (2H, d, J = 7.4 Hz, H8), 5.28 (1H, t, J = 7.1 Hz, H9), 1.79 (3H, s, H11), 1.75 (3H, s, H12), 2.34 (3H, s, H14). ^{13}CNMR (125 MHz, CDCl$_3$): δ = 171.8 (C1), 127.1(C2), 132.3 (C-3), 129.3 (C4), 163.4 (C5), 123.6 (C6), 129.5 (C7), 29.3 (C8), 121.0 (C9), 134.1 (C10), 26.3 (C11), 17.7 (C12) 168.9 (C13), 21.0 (C14). ESI$^+$ 249 [M+H]$^+$.

Synthesis of 4-((4,7-dihydroxy-8-methyl-2-oxo-2H-chromen-3-yl)carbamoyl)-2-(3-methylbut-2-en-1-yl)phenyl acetate (9)

Compound **7** (1.0 g, 4.8 mmol) was dissolved in a mixture of anhydrous dichloromethane and pyridine (7:3). Then the N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDCI, 1.0 g, 5.2 mmol) and the compound **8** was added dropwise dissolved in a mixture of DCM:pyridine (84:36) and stirred at room temperature under nitrogen. After 12 hrs of reaction was washed with aq. NaHCO$_3$ (30 ml) and water (30 mL × 2), the organic phase was obtained and the excess solvent was removed *in vacuo* and the residue was purified *via* solid chromatography (petroleum ether: ethyl acetate, 3:1) to afford a yellow solid (1.3 g, 62%). ^{1}HNMR(500 MHz, DMSO-d$_6$): δ = 7.90 (1H, s, H3), 7.22 (1H, d, J = 8.1 Hz, H6), 7.89 (1H, d, J = 8.1 Hz, H7), 3.28 (2H, d, J = 7.1 Hz, H8), 5.22 (1H, t, J = 6.8 Hz, H9), 1.71 (6H, s, H11, H12), 2.33 (3H, s, H14), 7.61 (1H, d, J = 8.1 Hz, H5'), 6.90 (1H, d, J = 8.1 Hz, H6'), 2.19 (3H, s, H11'), 11.73 (1H, br, 4'-OH), 10.52 (1H, s, 7'-OH), 9.44 (1H, s, NH). ^{13}CNMR(125 MHz, DMSO-d$_6$): δ =165.94 (C1), 133.13 (C2), 129.96 (C3), 132.50 (C4), 150.99 (C5), 122.44 (C6), 126.94 (C7), 28.99 (C8), 121.55 (C9), 131.75 (C10), 26.36 (C11), 18.51 (C12), 168.89 (C13), 21.26 (C14), 160.57 (C2'), 99.72 (C3'), 160.85 (C4'), 121.52 (C5'), 111.83 (C6'), 159.10

(C7'), 110.43 (C8'), 151.47 (C9'), 108.07 (C10'), 8.88 (C11'). HMBC: NH linked with C4' and C1. ESI⁺ 438 ([M+H]⁺). HRMS 438.1552, calculated 438.1553 ($C_{24}H_{24}NO_7$).

4-((7-hydroxy-8-methyl-2-oxo-4-((4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzyl)oxy)-2H-chromen-3-yl)carbamoyl)-2-(3-methylbut-2-en-1-yl)phenyl acetate (10)

To an oven dried flask equipped with magnetic stirrer bar, rubber septa and a nitrogen inlet, compound **9** (0.2 g, 0.45 mmol) and 10 mL of anhydrous DMF were added. The solution was cooled to 0°C and sodium hydride (4.2 mg of 60% mineral oil dispersion) was added, allowed the reaction mixture to stir for 30 min at 0°C. Compound **6**(0.14 g, 0.49 mmol) dissolved in DMF was added drop wise to the reaction flask. The reaction was allowed to slowly warm up to room temperature and was stirred for 72 h). The reaction mixture was then extracted with ethyl acetate (2 × 20 ml) and water. The combined organic extracts was dried over magnesium sulfate. The drying agent was filtered off and the organic extract was concentrated *in vacuo* to give the compound as yellow foam. The desired products was isolated *via* preparative HPLC-1 with retention time of 95 min and freeze dried to obtain a light yellow solid (0.12 g, 42 %). ¹HNMR (500 MHz, DMSO-d_6): δ = 7.86 (1H, s, H3), 7.34 (1H, d, J = 8.2 Hz, H6), 7.92 (1H, d, J = 8.8 Hz, H7), 3.34 (2H, d, J = 7.6 Hz, H8), 5.28 (1H, t, J = 7.1 Hz, H9), 1.79 (3H, s, H11), 1.77(3H, s, H12), 2.42 (3H, s, H14), 7.51 (1H, d, J = 9.5 Hz, H5'), 6.77 (1H, d, J = 8.9 Hz, H6'), 1.90 (3H, s, H11'), 3.4 (2H, m, H1''), 7.21 (2H, d, J = 7.6 Hz, H3'', H7''), 7.09 (2H, d, J = 7.6 Hz, H4'', H6''), 10.96 (1H, s, 7'-OH), 9.93 (1H, s, NH). ¹³CNMR(125 MHz, DMSO-d_6): δ = 166.05 (C1), 129.46 (C2), 129.82 (C3), 133.61 (C4), 151.56 (C5), 122.84 (C6), 126.72 (C7), 28.92 (C8), 121.24 (C9), 132.80 (C10), 26.06 (C11), 18.45 (C12), 169.90 (C13), 8.19 (C14), 188.40 (C2'), 67.69 (C3'), 167.57 (C4'), 125.24 (C5'), 112.05 (C6'), 163.17 (C7'), 111.78 (C8'), 152.69 (C9'), 110.68 (C10'), 7.49 (C11'). 42.4 (C1''), 126.84 (C2''), 130.78 (C3''), 125.87 (C4''), 134.30 (C5''), 125.87 (C6''), 130.78 (C7''), 28.43(C8''), 123.76 (C9''). HMBC: NH linked with C4' and C1. ESI⁺ 636 [M+H]⁺. HRMS: 636.1959, calculated for $C_{33}H_{29}F_3N_3O_7$: 636.1958.

4-((7-hydroxy-8-methyl-4-oxo-2-((4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzyl)oxy)-4H-chromen-3-yl)carbamoyl)-2-(3-methylbut-2-en-1-yl)phenyl acetate (11)

To an oven dried flask equipped with magnetic stirrer bar, rubber septa and a nitrogen inlet, compound **9** (0.2 g, 0.45 mmol) and 10 mL of anhydrous DMF were added. The solution was cooled to 0°C and sodium hydride (4.2 mg of 60% mineral oil dispersion) was added, allowed the reaction mixture to stir for 30 min at 0°C. Compound **6**(0.14 g, 0.49 mmol) dissolved in DMF was added drop wise to the reaction flask. The reaction was allowed to slowly warm up to room temperature and was stirred for 72 h). The reaction mixture was then extracted with ethyl acetate (2 × 20 ml) and water. The combined organic extracts was dried over magnesium sulfate. The drying agent was filtered off and the organic extract was concentrated *in*

vacuo to give the compound as yellow foam. The desired products was isolated *via* preparative HPLC-1 with a retention time of 100 min and freeze dried to obtain a light yellow solid (0.09 g, 31 %). ¹HNMR (500 MHz, DMSO-d_6): δ = 7.83 (1H, s, H3), 7.22 (1H, d, J = 8.8 Hz, H6), 7.82 (1H, d, J = 5.8 Hz, H7), 3.27 (2H, d, J = 7.4 Hz, H8), 5.20 (1H, t, J = 5.7 Hz, H9), 1.69 (6H, s, H11, H12), 2.33 (3H, s, H14), 7.50 (1H, d, J = 8.9 Hz, H5'), 6.90 (1H, d, J = 8.8 Hz, H6'), 2.19 (3H, s, H11'), 5.46 (2H, s, H1''), 7.55 (2H, d, J = 8.1 Hz, H3'', H7''), 7.27 (2H, d, J = 7.5 Hz, H4'', H6''). ¹³CNMR(125 MHz, DMSO-d_6): δ = 166.23 (C1), 131.17 (C2), 129.70 (C3), 133.49 (C4), 151.38 (C5), 122.76 (C6), 126.62 (C7), 28.39 (C8), 121.34 (C9), 132.63 (C10), 25.36 (C11), 17.59 (C12), 168.90 (C13), 20.81 (C14), 160.97 (C2'), 48.71 (C3'), 161.16 (C4'), 121.75 (C5'), 112.27 (C6'), 159.37 (C7'), 108.52 (C8'), 150.83 (C9'), 110.66 (C10'), 8.12 (C11'). 73.10 (C1''), 127.50 (C2''), 128.52 (C3''), 126.62 (C4''), 138.46 (C5''), 126.62 (C6''), 128.52 (C7''), 28.56 (C8''), 124.0 (C9''). HMBC: H1'' liked with C2'. ESI⁺ 636 [M+H]⁺. HRMS: 636.1963, calculated for $C_{33}H_{29}F_3N_3O_7$: 636.1958.

(2S,3R,5R,6R)-2-((3-(4-acetoxy-3-(3-methylbut-2-en-1-yl)benzamido)-8-methyl-4-oxo-2-((4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzyl)oxy)-4H-chromen-7-yl)oxy)-6-(acetoxymethyl)tetrahydro-2H-pyran-3,4,5-triyl triacetate (12)

Compound **11** (0.05 g, 0.07 mmol) was reacted with glucosyl bromide (compound **13**, 0.12 g, 0.30 mmol, 3 equiv.) using the general phase transfer glycosylation procedure (in DCM) for 13 days. The product was purified by preparative HPLC-2 with a retention time of 96 min afford compound **12** as a colourless white powder (LCMS yield : 52 %, isolated yield, 0.02 g, 31 %). $[\alpha]_D^{20}$: -22.41°, (CHCl₃). ¹HNMR (500 MHz, CDCl₃): δ = 7.88 (1H, d, J = 2.33 Hz, H3), 7.20 (1H, d, J = 8.33 Hz, H6), 7.82 (1H, dd, J = 2.46, 9.49 Hz, H7), 3.34 (2H, d, J = 7.02 Hz, H8), 5.25 (1H, m, H9), 1.79 (3H, s, H11), 1.75 (3H, s, H12), 2.38 (3H, s, H14), 7.70 (1H, d, J = 9.02 Hz, H5'), 7.01 (1H, d, J = 9.07 Hz, H6'), 2.29 (3H, s, H11'), 5.38 (2H, s, H1''), 7.50 (2H, d, J = 8.60 Hz, H3'', H7''), 7.21 (2H, d, J = 8.21 Hz, H4'', H6''), 5.15 (1H, d, J = 7.68 Hz, H1'''), 5.39 (1H, m, H2'''), 5.36 (1H, m, H3'''), 5.24 (1H, m, H4'''), 3.93 (1H, m, H5'''), 4.34 (1H, m, H6a'''), 4.22 (1H, m, H6b'''), 2.09-2.11 (12 H, s, 4 * C$\underline{H_3}$CO), 7.75 (1H, s, NH). ¹³CNMR(125 MHz, CDCl₃): δ = 166.62 (C1), 130.82 (C2), 129.97 (C3), 134.78 (C4), 152.50 (C5), 123.11 (C6), 126.38 (C7), 28.92 (C8), 120.78 (C9), 134.27 (C10), 25.71 (C11), 17.90 (C12), 168.98 (C13), 20.82 (C14), 157.78 (C2'), 104.95 (C3'), 162.43 (C4'), 122.03 (C5'), 111.62 (C6'), 157.33 (C7'), 116.11 (C8'), 150.00 (C9'), 113.19 (C10'), 8.4 (C11'). 72.49 (C1''), 129.64 (C2''), 128.26 (C3''), 126.82 (C4''), 137.73 (C5''), 126.82 (C6''), 128.26 (C7''), 28.19 (C8''), 122.6 (C9''), 99.22 (C1'''), 70.89 (C2'''), 72.18 (C3'''), 68.22 (C4'''), 72.23 (C5''') 62.01 (C6a'''), 62.01 (C6b'''), 20.69 (4 * C$\underline{H_3}$CO), 170.51 (CH₃$\underline{C}$O), 170.22 (CH₃$\underline{C}$O), 169.41 (CH₃$\underline{C}$O), 169.21 (CH₃$\underline{C}$O).HMBC: H1''' linked with C7' and H1'' lined with C2'. ESI⁺ 966 [M+H]⁺. HRMS: 7966.2873, calculated for $C_{47}H_{47}F_3N_3O_{16}$: 966.2873.

Glycosylation of compound 10

The same procedure was followed in the synthesis of compound **12**, i.e. Compound **10** (0.05 g, 0.07 mmol) was reacted with glucosyl bromide (compound **13**, 0.12 g, 0.30 mmol, 3 equiv.) using the general phase transfer glycosylation procedure (in DCM) for 13 days. However, no desired glycosylation product formed but new reactions with N_2 removal and possible insert reactions were discovered by LC/MS.

(2S,3R,5R,6R)-2-((3-(4-acetoxy-3-(3-methylbut-2-en-1-yl)benzamido)-7-hydroxy-8-methyl-2-oxo-2H-chromen-4-yl)oxy)-6-(acetoxymethyl)tetrahydro-2H-pyran-3,4,5-triyl triacetate (14)

Compound **9** (0.3 g, 0.68 mmol) was reacted with glucosyl bromide (**13**, 0.84 g, 2.0 mmol) using the general phase transfer glycosylation procedure (in DCM) for 7 days. The product was purified by preparative HPLC-2 with a retention time of 112 min to afford compound **14** as a white powder (0.10 g, 19%). $[\alpha]_D^{20}$: -66.03°, (CHCl₃). ¹HNMR (500 MHz, CDCl₃): δ = 7.95 (1H, s, H3), 7.23 (1H, d, J = 9.15 Hz, H6), 7.87 (1H, d, J = 8.56 Hz, H7), 3.37 (2H, d, J = 7.85 Hz, H8), 5.27 (1H, m, H9), 1.79 (3H, s, H11), 1.76 (3H, s, H12), 2.38 (3H, s, H14), 7.35 (1H, d, J = 9.18 Hz, H5'), 6.75 (1H, d, J = 9.21 Hz, H6'), 2.21 (3H, s, H11'), 5.36 (1H, m, H1''), 5.36 (1H, m, H2''), 5.24 (1H, m, H3''), 5.12 (1H, t, J = 9.39 Hz, H4''), 3.64 (1H, m, H5''), 4.17 (1H, m, H6a''), 4.24 (1H, m, H6b''), 2.00-2.07 (12 H, s, 4 * C$\underline{H}_3$CO), 7.81 (1H, s, NH). ¹³CNMR(125 MHz, CDCl₃): δ = 167.51 (C1), 130.57 (C2), 130.19 (C3), 134.87 (C4), 152.49 (C5), 123.00 (C6), 126.49 (C7), 29.05 (C8), 120.79 (C9), 134.06 (C10), 25.76 (C11), 17.96 (C12), 168.85 (C13), 20.55 (C14), 157.55 (C2'), 107.02 (C3'), 160.98 (C4'), 121.41 (C5'), 112.64 (C6'), 158.71 (C7'), 109.01 (C8'), 151.16 (C9'), 112.10 (C10'), 8.08 (C11'). 98.95 (C1''), 71.20 (C2''), 72.35 (C3''), 67.98 (C4''), 72.57 (C5'') 61.49 (C6''), 20.54-20.63 (4 * C$\underline{H}_3$CO), 170.56 (CH₃$\underline{C}$O), 170.05 (CH₃$\underline{C}$O), 169.39 (CH₃$\underline{C}$O), 169.33 (CH₃$\underline{C}$O). ESI⁻ 766 [M-H]⁻. HRMS: 768.2504, calculated for $C_{38}H_{42}NO_{16}$: 768.2504.

(3R,5R,6R)-2-((3-(4-acetoxy-3-(3-methylbut-2-en-1-yl)benzamido)-8-methyl-2-oxo-7-((4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzyl)oxy)-2H-chromen-4-yl)oxy)-6-(acetoxymethyl)tetrahydro-2H-pyran-3,4,5-triyltriacetate (15)

To an oven dried flask equipped with magnetic stirrer bar, rubber septa and a nitrogen inlet, compound **14** (0.05 g, 0.07 mmol) and 10 mL of anhydrous DMF were added. The solution was cooled to 0°C and sodium hydride (2.1 mg of 60% mineral oil dispersion) was added, allowed the reaction mixture to stir for 30 min at 0°C. Compound **6** (0.02g, 0.08mmol) dissolved in DMF was added drop wise to the reaction flask. The reaction was allowed to slowly warm up to room temperature and was stirred for 20 h. The reaction mixture was then extracted with ethyl acetate (2 × 20 ml) and water. The combined organic extracts was dried over magnesium sulfate. The drying agent was filtered off and the organic extract was concentrated *in vacuo* to give the compound as yellow foam. The desired products was isolated *via* preparative HPLC-2 with a retention of 160 min and freeze dried to obtain a light yellow solid (LCMS

yield : 35 %, isolated yield, 0.02 g, 34 %). $[\alpha]_D^{20}$: -43.73°, (CHCl₃). ¹HNMR (500 MHz, CDCl₃): δ = 7.89 (1H, s, H3), 7.20 (1H, d, J = 8.42 Hz, H6), 7.83 (1H, d, J = 8.91 Hz, H7), 3.34 (2H, d, J = 7.02 Hz, H8), 5.26 (1H, m, H9), 1.78 (3H, s, H11), 1.75 (3H, s, H12), 2.34 (3H, s, H14), 7.63 (1H, d, J = 9.22 Hz, H5'), 6.90 (1H, d, J = 8.76 Hz, H6'), 2.29 (3H, s, H11'), 5.23 (2H, s, H1''), 7.50 (2H, d, J = 8.64 Hz, H3'', H7''), 7.26 (2H, d, J = 8.21 Hz, H4'', H6''), 5.40 (1H, d, J = 8.63Hz, H1'''), 5.36 (1H, t, J = 7.90 Hz, H2'''), 5.22 (1H, m, H3'''), 5.13 (1H, t, J = 8.77 Hz, H4'''), 3.64 (1H, m, H5'''), 4.13 (1H, dd, J = 5.69, 12.8 Hz, H6a'''), 3.90 (1H, dd, J = 2.13, 12.09 Hz, H6b'''), 1.96-2.03 (12 H, s, 4 * C$\underline{H}_3$CO). ¹³CNMR(125 MHz, CDCl₃): δ = 166.50 (C1), 130.84 (C2), 129.95 (C3), 134.77 (C4), 152.29 (C5), 122.98 (C6), 126.32 (C7), 28.93 (C8), 120.77 (C9), 134.12 (C10), 25.75 (C11), 18.00 (C12), 168.84 (C13), 20.89 (C14), 161.29 (C2'), 106.98 (C3'), 156.61 (C4'), 122.08 (C5'), 108.63 (C6'), 159.49 (C7'), 114.54 (C8'), 150.58 (C9'), 110.65 (C10'), 8.48 (C11'). 69.67 (C1''), 129.03 (C2''), 127.42 (C3''), 126.89 (C4''), 138.08 (C5''), 126.89 (C6''), 127.42 (C7''), 28.56 (C8''), 117.37 (C9''), 98.57 (C1'''), 71.15 (C2'''), 72.34 (C3'''), 67.86 (C4'''), 72.46 (C5'''), 61.34 (C6'''), 20.53-20.62 (4 * C$\underline{H}_3$CO), 170.34 (CH₃$\underline{C}$O), 169.98 (CH₃$\underline{C}$O), 169.52 (CH₃$\underline{C}$O), 169.33 (CH₃$\underline{C}$O). HMBC: H1''' linked with C4' and H1'' lined with C7'. ESI⁺ 1953 [2M+Na⁺]⁺. HRMS: 966.2930, calculated for $C_{47}H_{47}F_3N_3O_{16}$: 966.2908.

4-hydroxy-N-(8-methyl-2-oxo-7-((4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzyl)oxy)-4-(((3R,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)-2H-chromen-3-yl)-3-(3-methylbut-2-en-1-yl)benzamide (2) and 4-hydroxy-N-(4-hydroxy-8-methyl-2-oxo-7-((4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzyl)oxy)-2H-chromen-3-yl)-3-(3-methylbut-2-en-1-yl)benzamide (3)

Compound **15** (0.02 g, 0.02 mmol) was reacted with sodium methoxide (catalytic percentage) using the general acetate deprotection procedure (in CH₃OH) for 26 h. The product was purified by preparative HPLC-3 with a retention of 201 min to afford compound **2** as a white powder (1.2mg, 7.9 %) and 256 min for compound **3** as white powder (3 mg, 11%).

compound **2**: $[\alpha]_D^{20}$: -66.7°, (CHCl₃). ¹HNMR (500 MHz, MeOD₄): δ = 7.82 (1H, s, H3), 6.87 (1H, d, J = 8.63 Hz, H6), 7.77 (1H, d, J = 8.61 Hz, H7), 3.42 (2H, m, H8), 5.38 (1H, m, H9), 1.77 (6H, s, H11, H12), 7.97 (1H, d, J = 8.61 Hz, H5'), 7.12 (1H, d, J = 7.75 Hz, H6'), 2.38 (3H, s, H11'), 5.33 (2H, s, H1''), 7.63 (2H, d, J = 7.75 Hz, H3'', H7''), 7.33 (2H, d, J = 8.62 Hz, H4'', H6''), 5.22 (1H, d, J = 8.82 Hz, H1'''), 3.49 (1H, m, H2'''), 3.30 (1H, m, H3'''), 3.36 (1H, m, H4'''), 3.12 (1H, m, H5'''), 3.68 (1H, d, J = 11.8 Hz, H6a'''), 3.52 (1H, m, H6b'''). ¹³CNMR(125 MHz, MeOD₄): δ = 170.34 (C1), 125.26 (C2), 130.98 (C3), 129.96 (C4), 160.66 (C5), 115.53 (C6), 128.52 (C7), 29.33 (C8), 123.41 (C9), 133.57 (C10), 26.1 (C11), 18.0 (C12), 168.84 (C13), 163.09 (C2'), 108.87 (C3'), 161.14 (C4'), 124.12 (C5'), 110.06 (C6'), 160.88 (C7'), 114.92 (C8'), 152.38 (C9'), 111.92 (C10'), 8.4 (C11'). 70.82 (C1''), 129.71 (C2''), 129.04 (C3''), 127.92 (C4''), 140.59 (C5''), 127.92 (C6''), 129.04 (C7''), 29.41 (C8''), 123.75 (C9''), 103.76 (C1'''), 75.35 (C2'''), 77.74 (C3'''), 70.82 (C4'''), 78.76 (C5'''), 61.96 (C6'''). HMBC: H1''' linked with C4' and H1'' lined with C7'. ESI⁻ 754 [M-H]⁻. HRMS:756.2365, calculated for $C_{37}H_{37}F_3N_3O_{11}$: 756.2380.

Compound **3**:

^{1}HNMR (500 MHz, MeOD$_4$): δ = 7.61 (1H, s, H3), 6.78 (1H, d, J = 8.99 Hz, H6), 7.61 (1H, d, m, H7), 3.30 (2H, d, 7.64, H8), 5.17 (1H, s, H9), 1.68 (6H, s, H11, H12), 7.70 (1H, d, J = 9.13 Hz, H5'), 6.79 (1H, d, J = 8.67 Hz, H6'), 2.25 (3H, s, H11'), 5.07 (2H, s, H1''), 7.36 (2H, d, J = 9.18 Hz, H3'', H7''), 7.11 (2H, d, J = 9.18 Hz, H4'', H6''), 13.89 (1H, s, 4'-OH), 8.60 (1H, s, NH). ^{13}CNMR(125 MHz, MeOD$_4$): δ = 166.95 (C1), 123.81 (C2), 129.92 (C3), 127.58 (C4), 158.97 (C5), 116.22 (C6), 127.64 (C7), 29.77 (C8), 120.59 (C9), 136.27 (C10), 25.82 (C11), 18.11 (C12), 161.76 (C2'), 103.06 (C3'), 153.40 (C4'), 122.57 (C5'), 108.67 (C6'), 159.12 (C7'), 114.15 (C8'), 149.81 (C9'), 111.07 (C10'), 8.50 (C11'). 69.91 (C1''), 129.02 (C2''), 127.41 (C3''), 126.85 (C4''), 138.29 (C5''), 126.85 (C6''), 127.41 (C7''), 28.70 (C8''), 122.10 (C9''). HMBC: H1'' lined with C7'. ESI$^-$ 592 [M-H]$^-$. HRMS: 594.1842, calculated for C$_{31}$H$_{27}$F$_3$N$_3$O$_6$: 594.1852.

4-hydroxy-N-(8-methyl-4-oxo-2-((4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzyl)oxy)-7-(((2S,3R,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)-4H-chromen-3-yl)-3-(3-methylbut-2-en-1-yl)benzamide (4)

Compound **12** (0.01 g, 0.01 mmol) was reacted with sodium methoxide (catalytic percentage) using the general acetate deprotection procedure (in CH$_3$OH) for 22 h. The product was purified by preparative HPLC-3 with a retention time of 136 min to afford compound **4** as a white powder (1.2mg,7.9 %).[α]$_D^{20}$: -41.9° (ACN).^{1}HNMR (500 MHz, MeOD$_4$): δ = 7.79 (1H, s, H3), 6.87 (1H, d, J = 6.4 Hz, H6), 7.70 (1H, m, H7), 3.4 (2H, m, H8), 5.38 (1H, m, H9), 1.76 (6H, s, H11, H12), 7.97 (1H, d, J = 8.61 Hz, H5'), 6.85 (1H, d, J = 6.3 Hz, H6'), 2.39 (3H, s, H11'), 5.51 (2H, d, J = 3.3 Hz, H1''), 7.48 (2H, m, H3'', H7''), 7.23 (2H, d, J = 7.23 Hz, H4'', H6''), 5.06 (1H, d, J = 7.9 Hz, H1'''), 3.56 (1H, m, H2'''), 3.49 (1H, m, H3'''), 3.46 (1H, m, H4'''), 3.41 (1H, m, H5'''), 3.92 (1H, d, J = 12.15 Hz, H6a'''), 3.72 (1H, dd, J = 5.5, 12.15 Hz, H6b'''), 7.71 (1H, s, NH). ^{13}CNMR(125 MHz, MeOD$_4$): δ = 164.0 (C1), 131.2 (C2), 131.0 (C3), 133.7 (C4), 148.6 (C5), 123.40 (C6), 128.47 (C7), 29.32 (C8), 123.39 (C9), 135.5 (C10), 26.00 (C11), 17.96 (C12), 163.81 (C2'), 105.9 (C3'), 164.2 (C4'), 127.99 (C5'), 115.53 (C6'), 160.53 (C7'), 116.3 (C8'), 154.4 (C9'), 112.9 (C10'), 8.63 (C11'). 75.20 (C1''), 130.4 (C2''), 129.33 (C3''), 112.97 (C4''), 139.8 (C5''), 112.97 (C6''), 129.33 (C7''), 28.19 (C8''), 126 (C9''), 102.21 (C1'''), 74.86 (C2'''), 78.17 (C3'''), 71.26 (C4'''), 78.39 (C5'''), 62.52 (C6'''). HMBC: H1''' linked with C7' and H1'' lined with C2'. ESI$^-$ 755 [M-H]$^-$. HRMS: 756.2365, calculated for C$_{37}$H$_{37}$F$_3$N$_3$O$_{11}$: 756.2380.

4-hydroxy-N-(7-hydroxy-8-methyl-2-oxo-4-((4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzyl)oxy)-2H-chromen-3-yl)-3-(3-methylbut-2-en-1-yl)benzamide (5)

Compound **10**(0.01 g, 0.02 mmol) was reacted with sodium methoxide (catalytic percentage) using the general acetate deprotection procedure (in CH$_3$OH) for 15 h. The product was purified by preparative HPLC using 30-95 % methanol water in 180 min gradient elution to afford compound **5** as a white powder (6.6 mg, 50 %). ^{1}HNMR (500 MHz, DMSO-d$_6$): δ = 7.63 (1H, d, J = 2.01Hz, H3), 6.83 (1H, d, J = 8.01 Hz, H6), 7.57 (1H, m, H7), 3.38 (2H, d, J = 7.51 Hz, H8), 5.31 (1H, m, H9), 1.80 (3H, s, H11), 1.70 (3H, s, H12), 2.42 (3H, s, H14), 7.57 (1H, m, H5'),

6.53 (1H, d, J = 8.61 Hz, H6'), 2.01 (3H, s, H11'), 3.44 (2H, m, H1''), 7.13 (2H, d, J = 8.03 Hz, H3'', H7''), 7.00 (2H, d, J = 8.03 Hz, H4'', H6''). ^{13}CNMR(125 MHz, DMSO-d$_6$): δ = 162.3 (C1), 127.5 (C2), 130.0 (C3), 127.5 (C4), 158.4 (C5), 115.7 (C6), 127.4 (C7), 25.8 (C8), 120.9 (C9), 135.7 (C10), 29.5 (C11), 18.1 (C12), 169.90 (C13), 8.19 (C14), 188.9 (C2'), 67.4 (C3'), 168.0 (C4'), 127.5 (C5'), 112.1 (C6'), 162.1 (C7'), 112.3 (C8'), 153.4 (C9'), 126.0 (C10'), 8.0 (C11'). 43.3 (C1''), 129.2 (C2''), 130.5 (C3''), 126.5 (C4''), 132.8 (C5''), 126.5 (C6''), 130.5 (C7''), 43.0 (C8''), 120.6 (C9''). HMBC: 1'' lined with C4'. ESI$^+$ 594 [M+H]$^+$; HRMS: 594.1863, calculated for C$_{31}$H$_{27}$F$_3$N$_3$O$_6$: 594.1852.

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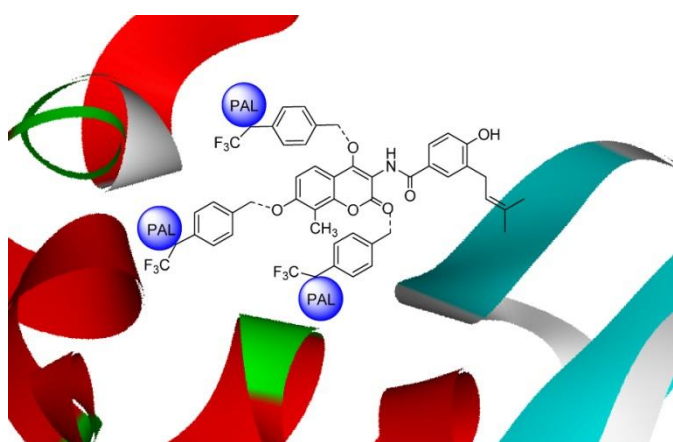
Synthesis of diazirine type of photoaffinity labelling reagents to probe Hsp90 C-terminal Domain binding pocket and structure-activity relationship. Structure to illustrate probe positions only.