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**Design, synthesis and evaluation of a diazirine photoaffinity probe for ligand-based
receptor capture targeting G protein-coupled receptors**

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A ligand-based receptor capture probe targeting GPCRs

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Abbreviations used: 6xHis, hexa-histidine tag; Ac, acetyl; COSY, correlation spectroscopy; CuAAC, copper(I)-catalyzed azide alkyne cycloaddition; DCM, dichloromethane; DIPEA, N,N-diisopropylethylamine; DMEM, Dulbecco's modification of Eagle's medium; DMF, dimethylformamide; DMSO, dimethylsulfoxide; Dox, doxycycline; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; eGFP, enhanced green fluorescent protein; FBS, fetal bovine serum; GPCR, G protein-coupled receptor; HA, haemagglutinin; HBSS, Hanks' balanced salt solution; HBTU, hexafluorophosphate benzotriazole tetramethyl uronium; HCTU, hexafluorophosphate chlorobenzotriazole tetramethyl uronium; HPLC, high-performance liquid chromatography; IP1, inositol monophosphate; HRMS, high resolution mass spectrometry; HSQC, heteronuclear single quantum coherence spectroscopy; HTRF, homogenous time-resolved fluorescence; LC-MS, liquid chromatography-mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LRC, ligand-based receptor capture; MS, mass-spectrometry; MTBE, methyl tert-butyl ether; NaAsc, sodium ascorbate; NHS, N-hydroxysuccinimide; NK₁, neurokinin 1; Nle, norleucine; NMR, nuclear magnetic resonance; NP40, nonyl phenoxypolyethoxylethanol 40; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PEG, polyethylene glycol; RT, room temperature; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SP, substance P; TFA, trifluoroacetic acid; TIS, triisopropylsilane; UV, ultra-violet;

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Abstract

Chemoproteomic approaches to identify ligand-receptor interactions have gained popularity. However, identifying trans-membrane receptors remains challenging. A new tri-functional probe to aid the non-biased identification of such receptors was developed and synthesized using a convenient 7-step synthesis. This probe contained three functional groups: (1) an NHS-ester for ligand-coupling through free amines; (2) a diazirine moiety to capture the receptor of interest upon irradiation with UV-light; and (3) a biotin group which allowed affinity purification of the final adduct using streptavidin. The interaction between the G protein-coupled tachykinin NK₁ receptor, expressed in an inducible manner using the Flp-InTM T-RExTM system, and the peptidic ligand substance P was employed as a test system. Liquid chromatography–mass spectrometry analysis confirmed successful coupling of the probe to substance P, while inositol monophosphate accumulation assays demonstrated that coupling of the probe did not interfere substantially with the substance P-NK₁ receptor interaction. Confocal microscopy and western blotting provided evidence of the formation of a covalent bond between the probe and the NK₁ receptor upon UV activation. As proof of concept, the probe was used in full ligand-based receptor capture experiments to identify the substance P-binding receptor via LC-MS/MS, resulting in the successful identification only of the NK₁ receptor. This provides proof-of-concept towards general utilization of this probe to define interactions between ligands and previously unidentified plasma-membrane receptors.

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Introduction

Membrane proteins are vital components of drug discovery, being targeted by some 60% of the currently marketed therapeutic medicines, with roughly half of these interacting with one or more members of the G protein-coupled receptor (GPCR) superfamily (Overington *et al.*, 2006; Hauser *et al.*, 2017; Santos *et al.*, 2017). Identification of transmembrane receptor targets of poorly characterized ligands can provide new starting points for drug innovation, provide valuable information about off-target effects, and enhance mechanistic understanding of molecular pathways (Hopkins and Groom, 2002; Schirle and Jenkins, 2016).

Combining affinity chromatography with mass-spectrometry (MS) is a popular chemoproteomic method that has been used to identify intracellular ligand-receptor interactions (Lenz *et al.*, 2011), however, transmembrane receptors possess some unfavorable characteristics which hamper this approach. These include that their hydrophobicity and high number of post-translational modifications can result in aggregation and loss of ligand-binding activity upon solubilization. Moreover, these receptors are regularly of low abundance and their interactions with ligands are often transient in nature, making it challenging to maintain ligand-receptor interactions throughout experiments (Wright, 2009; Helbig *et al.*, 2010; Savas *et al.*, 2011; Vuckovic *et al.*, 2013). Various groups have endeavored to overcome these issues by developing tri-functional probes either structurally based on a ligand of interest or capable of coupling to such a ligand (Frei *et al.*, 2012; Blex *et al.*, 2017; Thomas *et al.*, 2017; Tremblay and Hill, 2017; Sobotzki *et al.*, 2018). Apart from a ligand or ligand-binding moiety, such probes must contain a receptor-capture moiety, which enables formation of a covalent bond between the probe and target receptor, and an affinity tag to assist with receptor-purification. The covalent bond formed between the probe and target receptor prevents dissociation during such studies. Moreover, as such a bond will stay intact during cell lysis, it allows for ligand-receptor binding and receptor-capture to be

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performed on living cells thus circumventing binding-issues faced following receptor solubilization.

One of the first tri-functional probes specifically designed to identify membrane receptors on living cells is TRICEPS, a potentially universal probe that is coupled to ligands of interest through an amine-reactive N-hydroxysuccinimide (NHS) ester (Frei *et al.*, 2012). The ligand-probe adduct is then used to capture and purify glycosylated target receptors, as trans-plasma membrane proteins routinely become N-glycosylated during transport from the endoplasmic reticulum. Conceptually the target can then be identified through MS analysis. Although this elegant ligand-based receptor capture (LRC) strategy has resulted in successful identification of certain membrane receptors (Li *et al.*, 2016, 2018; Ponda and Breslow, 2016), identification can only occur for receptors carrying an N-linked glycan in close proximity to the ligand-binding site. To overcome this glycan-dependency others have recently developed ligand-derived probes harboring photoaffinity labels to capture target receptors (Blex *et al.*, 2017; Thomas *et al.*, 2017). Such labels, which are activated upon irradiation with UV-light, are nowadays commonly used to enhance capture of cytosolic receptors (Hatanaka, 2015).

Described herein is the design, synthesis and evaluation of a new tri-functional probe for LRC, that combines the universality of a ligand-binding NHS-ester with receptor-capture through a photoaffinity label. The interaction between the tachykinin NK₁ receptor and substance P (SP) was employed as a test system. The NK₁ receptor is a GPCR belonging to the tachykinin family and the undecapeptide SP is its major and most potent endogenous ligand. Interaction between the peptide and the NK₁ receptor is associated with various physiological processes, including mitogenesis, wound healing, and neuronal transmission related to pain, depression, stress, anxiety and emesis (Garcia-Recio and Gascón, 2015). Initially, this system was used to investigate individual steps in the LRC protocol, including

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ligand-coupling, potential interference of the probe on the ligand-receptor interaction, and ability of the probe to covalently couple to the receptor. Finally, it was also used in a full LRC experiment to provide a proof of concept, demonstrating that this novel probe can be used as a general tool to help identify target receptors for a variety of ligands.

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Materials and Methods

Unless otherwise stated, all (bio)chemicals and reagents were from Sigma-Aldrich (Dorset, UK). Tissue culture reagents and buffers were from Thermo Fisher Scientific (Paisley, UK). Molecular biology enzymes and reagents were from New England BioLabs (Hitchin, UK). Primers were ordered at Eurofins Genomics (Ebersberg, Germany). For western blotting the following primary antibodies were used: mouse anti-tubulin (ab7291; 1:4,000; Abcam, Cambridge, UK); rabbit anti-6xHis (ab9108; 1:4,000; Abcam, Cambridge UK); and sheep anti-GFP (produced in house). Secondary antibodies (800CW goat anti-mouse; 680RD goat anti-mouse; 800CW donkey anti-rabbit; and 800CW donkey anti-goat) and 680LT streptavidin were purchased from LI-COR (Cambridge, UK) and were all used at 1:10,000. Ac-Nle-SP and Probe **1** were synthesized as described below.

Chemical synthesis

All chemicals were commercially obtained and used without further purification, unless stated otherwise. Reactions involving air-sensitive reagents and dry solvents were performed in flame-dried glassware under nitrogen atmosphere. Reactions were monitored by TLC, using plates with a UV fluorescent indicator (60 F₂₅₄, 0.25 mm; Merck, Feltham, UK). Compounds were visualized either by UV absorption or by dipping in molybdenum or a ninhydrin-based stain, followed by heating. Column chromatography was performed using silica gel 60 (35-70 μ m; Merck, Feltham, UK) or with an Isolera One purification system (Biotage, Ystrad Mynach, UK) and use of prepacked silica ULTRA SNAP cartridges (Biotage, Ystrad Mynach, UK). An Alpha-2-4 lyophilizer (Christ, Osterode am Harz, Germany) equipped with a high vacuum pump was used for lyophilization. All NMR spectra were recorded using 400/500 NMR spectrometers (Bruker, Glasgow, UK). δ_{H} values are given in ppm, relative to an internal signal of Me₄Si (0.00 ppm), the signal of CDCl₃ (7.26

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ppm). δ_c values are given in ppm relative to the signal of Me₄Si (0.00 ppm), the signal of CDCl₃ (77.16), or the signal of (CD₃)₂SO (39.52). For ¹H-NMR spectra integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, sept = septet, m = multiplet, br = broad, or a combination of these), coupling constants J (Hz) and assignments are also reported. For ¹³C-NMR spectra multiplicity and coupling constants are reported where appropriate. High resolution mass spectrometry (HRMS) was performed by the analytical service of the University of Glasgow, either on a MStation JMS-700 instrument (Jeol, Welwyn Garden City, UK) using positive chemical ionization (CI) or a positive ion impact (EI), or on a micro TOFq High Resolution instrument (Bruker) using positive ion electrospray (ESI). Analytical high-performance liquid chromatography (HPLC) was performed on the Prominence HPLC system (Shimadzu, Wolverton, UK) with a UV-detector operating at 214 nm and 254 nm using either a Phenomenex Gemini C18 column (110 Å, 5 µm, 250 x 4.60 mm) or a Dr Maisch Reprosil Gold C18 column (200 Å, 5 µm, 250 x 4.6 mm) at a flow rate of 1 mL/min using a 40 min protocol (Buffer A for 2 min, a linear gradient of 0-100% Buffer B in buffer A for 28 min, Buffer B for 2 min, , a linear gradient of 100-0% Buffer B in buffer A for 3 min and Buffer A for 5 min). Buffer A [H₂O/MeCN 95:5 with 0.1% TFA] Buffer B [MeCN/H₂O 95:5 with 0.1% TFA]. Liquid chromatography mass spectrometry (LC-MS) was carried out with a LCQ Fleet quadrupole mass spectrometer (Thermo Fisher Scientific, Paisley, UK) and an Ultimate 3000 LC (Dionex, Camberley, UK) with use of a Dr. Maisch Reprosil Gold 120 C18 column (150 Å, 3 µm, 150 x 4 mm). Buffers and protocols used were identical to those of analytical HPLC.

Synthesis Probe 1

For long-term storage diazirine containing products were protected from light. During, synthesis, however, no extra care was taken to avoid light and no activation of the diazirine moiety was observed.

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Compound 2 - (9H-fluoren-9-yl)methyl *tert*-butyl (6-oxo-6-(prop-2-yn-1-ylamino)hexane-1,5-diyl)(*S*)-dicarbamate

Fmoc-Lys(Boc)-OH (23.61 g, 50 mmol), Oxyma (10.48 g, 74 mmol) and propargylamine (3.4 mL, 53 mmol) were dissolved in DCM (1 L) and cooled on ice. EDC (18.87 g, 98 mmol) was added after which the, now turbid, mixture was stirred at RT for 40 min. The reaction mixture was washed with subsequently H₂O (500 mL, 3x), citric acid (1 M, 500 mL, 2x), H₂O (1 L), NaHCO₃ (sat, 500 mL, 2x) and H₂O (1 L). The organic layer was dried over MgSO₄ and concentrated under vacuum to yield a yellow solid (26.52 g, 95%). Analytical data corresponded to previously reported data (Hartwig and Hecht, 2010).

Compound 3 - *tert*-butyl (*S*)-(6-oxo-6-(prop-2-yn-1-ylamino)-5-(3-(4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)phenyl)propanamido)hexyl)carbamate

Compound 2 (748.4 mg, 1.5 mmol) was dissolved in Piperidine/DMF (1:15 (v/v) 25 mL) and the mixture was stirred at RT. After 10 min the mixture was concentrated under vacuum and the piperidine was removed by co-evaporations with toluene. This crude mixture was dissolved in 10 mL DCM and 3-(4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)phenyl)propanoic acid (304.0 mg, 1.2 mmol, synthesized as described before by Geurink *et al.*, (2010)), HBTU (448.1 mg, 1.2 mmol) and DIPEA (790 μ L, 4.6 mmol) were added. The total mixture was stirred for 2 h at RT before it was washed with H₂O (50 mL), KHSO₄ (1 M, 50 mL) and H₂O (50 mL). The organic layer was dried over MgSO₄, concentrated under vacuum and purified by flash chromatography (DCM/MeOH 20:0 \rightarrow 19:1) yielding a white solid (556 mg, 91%).

Analytical data: ¹H NMR (400 MHz, Chloroform-*d*) δ 7.26 (br s, 1H, NH), 7.20 (d, *J* = 8.4 Hz, 2H, 2x C^{AR}-H), 7.09 (d, *J* = 8.0 Hz, 2H, 2x C^{AR}-H), 6.73 (d, *J* = 8.0 Hz, 1H, NH), 4.79 (t, *J* = 6.0 Hz, 1H, NH), 4.52 (td, *J* = 7.8, 5.8 Hz, 1H, Lys- ^{α} CH), 4.06 – 3.85 (m, 2H, CH₂C-CH), 3.08 (dd, *J* = 11.9, 6.2 Hz, 2H, Lys- ^{ϵ} CH₂), 2.94 (td, *J* = 7.6, 4.4 Hz, 2H, CH₂CH₂C^{AR}),

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2.52 (t, $J = 7.7$ Hz, 2H, $\text{CH}_2\text{CH}_2\text{C}^{\text{AR}}$), 2.20 (t, $J = 2.5$ Hz, 1H, C-CH), 1.92 – 1.19 (m, 15H, $\text{Lys-}^{\beta,\gamma,\delta}\text{CH}_2$ and Boc- CH_3). ^{13}C NMR (101 MHz, CDCl_3) δ 172.0, 171.6, 156.2, 142.5, 128.8, 127.1, 126.7, 122.16 (q, $J = 274.7$ Hz, CF_3), 79.3, 77.3, 71.6, 52.7, 40.0, 37.4, 32.1, 31.1, 29.6, 29.1, 28.5 28.33 (q, $J = 40.4$ Hz, $\text{CN}_2\text{-CF}_3$), 22.5. HSQC and COSY spectra were in accordance with assignments given above. HRMS: calcd $\text{C}_{25}\text{H}_{32}\text{F}_3\text{N}_5\text{O}_4$ $[\text{M}+\text{Na}]^+$ 546.2299, found 546.2284

Compound 4 - (S)-4-oxo-4-((6-oxo-6-(prop-2-yn-1-ylamino)-5-(2-(4-(3-(trifluoromethyl)-3H-diazirin-3-yl)phenyl)acetamido)hexyl)amino)butanoic acid

Compound 3 (554.2 mg, 1.1 mmol) was dissolved in DCM/TFA (1:1 (v/v) 60 mL) and stirred for 10 min before it was concentrated under vacuum. The last traces of TFA were removed by 3 co-evaporations with toluene. The yellow solid that yielded was dissolved in DMF (50 mL) and succinic anhydride (128.4 mg, 1.3 mmol) and DIPEA (880 μL , 5.1 mmol) were added. The mixture was stirred for 45 min at RT after which it was concentrated under vacuum. The remaining solid was dissolved in DCM (100 mL) and extracted with NaHCO_3 (sat, 150 mL, 2x). The aqueous layers were acidified with HCl (37%) and extracted with EtOAc (100 mL, 3x). The organic layers were combined, dried over MgSO_4 , concentrated under vacuum and purified by flash chromatography (DCM/MeOH/AcOH, 19:1:0.2) to yield the acid as a white solid (418 mg, 75% over 2 steps).

Analytical data: ^1H NMR (400 MHz, Methanol- d_4) δ 7.32 (d, $J = 8.4$ Hz, 2H, 2x $\text{C}^{\text{AR}}\text{-H}$), 7.16 (d, $J = 8.0$ Hz, 2H, 2x $\text{C}^{\text{AR}}\text{-H}$), 4.25 (dd, $J = 8.7, 5.4$ Hz, 1H, $\text{Lys-}^{\alpha}\text{CH}$), 4.05 – 3.79 (m, 2H, $\text{CH}_2\text{C-CH}$), 3.14 (t, $J = 7.0$ Hz, 2H, $\text{Lys-}^{\epsilon}\text{CH}_2$), 2.94 (t, $J = 7.7$ Hz, 2H, $\text{CH}_2\text{CH}_2\text{C}^{\text{AR}}$), 2.66 – 2.50 (m, 5H, $\text{CH}_2\text{CH}_2\text{C}^{\text{AR}}$ and $\text{CH}_2\text{CH}_2\text{COOH}$), 2.45 (t, $J = 6.9$ Hz, 2H, $\text{CH}_2\text{CH}_2\text{COOH}$), 1.83 – 1.20 (m, 6H, $\text{Lys-}^{\beta,\gamma,\delta}\text{CH}_2$). ^{13}C NMR (101 MHz, MeOD) δ 176.4, 174.8, 174.5, 174.0, 144.7, 130.3, 127.8, 127.7, 126.4 (q, $J = 274.0$ Hz, CF_3), 80.5, 72.3,

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54.5, 40.0, 38.0, 32.7, 32.3, 31.7, 30.5, 29.9, 29.6 (q, $J = 39.7$ Hz, $\text{CN}_2\text{-CF}_3$), 29.5, 24.0.

HSQC and **COSY** spectra are in accord with assignments given above. **HRMS**: calcd

$\text{C}_{24}\text{H}_{28}\text{F}_3\text{N}_5\text{O}_5$ $[\text{M}+\text{Na}]^+$ 546.1935, found 546.1916.

Compound 5 – 4-oxo-4-(((S)-6-oxo-6-(((1-(15-oxo-19-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-4,7,10-trioxa-14-azanonadecyl)-1H-1,2,3-triazol-4-yl)methyl)amino)-5-(3-(4-(3-(trifluoromethyl)-3H-diazirin-3-yl)phenyl)propanamido)hexyl)amino)butanoic acid

Compound 4 (58.1 mg, 111 μmol) and biotin-PEG-azide (65.1 mg, 138 μmol) (synthesized as described before by Chambers *et al.*, (2013)) were dissolved in MeOH (3 mL). Next, aqueous CuSO_4 (10 mM, 2.8 mL) and aqueous sodium ascorbate (30 mg/mL, 335 μL) were added. The mixture was stirred under N_2 atmosphere for 2 h before it was concentrated under vacuum. The crude product was purified by flash chromatography (DCM/MeOH/AcOH, 19:1:0.2 \rightarrow 16:4:0.2) to yield the biotinylated product as a white solid (71 mg, 65%).

Analytical data: ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 8.44 (br s, 1H, NH), 8.16 (br s, 1H, NH), 7.90 – 7.83 (m, 2H, NH and triazole-CH), 7.76 (t, $J = 5.7$ Hz, 1H, NH), 7.35 (d, $J = 8.0$ Hz, 2H, 2x $\text{C}^{\text{AR}}\text{-H}$), 7.17 (d, $J = 7.9$ Hz, 2H, 2x $\text{C}^{\text{AR}}\text{-H}$), 6.44 (s, 1H, NH), 6.37 (s, 1H, NH), 4.37 (t, $J = 7.0$ Hz, 2H, $\text{PEG-CH}_2\text{N}^{\text{triazole}}$), 4.33 – 4.24 (m, 3H, $\text{NHCH}_2\text{C}^{\text{triazole}}$ and biotin-SCH₂CH), 4.24 – 4.14 (m, 1H, Lys- $^{\alpha}\text{CH}$), 4.13 (ddd, $J = 7.3, 4.5, 1.8$ Hz, 1H, biotin-SCH₂CH), 3.54 – 3.34 (m, 12H, 2x $\text{PEG-OCH}_2\text{CH}_2\text{O}$ and 2x $\text{PEG-CH}_2\text{CH}_2\text{CH}_2\text{O}$, overlapping with water signal), 3.13 – 3.02 (m, 3H, biotin-SCH and $\text{PEG-CH}_2\text{NH}$), 2.96 (q, $J = 6.5$ Hz, 2H, Lys- $^{\epsilon}\text{CH}_2$), 2.88 – 2.78 (m, 3H, , biotin-SCH₂ (1H) and $\text{CH}_2\text{CH}_2\text{C}^{\text{AR}}$), 2.58 (d, $J = 12.4$ Hz, 1H, biotin-SCH₂ (1H)), 2.50 – 2.40 (m, 2H, $\text{CH}_2\text{CH}_2\text{C}^{\text{AR}}$), 2.26 (s, 4H, $\text{CH}_2\text{CH}_2\text{COOH}$), 2.09 – 1.96 (m, 4H, $\text{PEG-CH}_2\text{CH}_2\text{N}^{\text{triazole}}$ and biotin- $^{\alpha}\text{CH}$), 1.81 – 1.09 (m, 14H, Lys- $^{\beta,\gamma,\delta}\text{CH}_2$, biotin- $^{\beta,\gamma,\delta}\text{CH}_2$ and $\text{PEG-CH}_2\text{CH}_2\text{NH}$). ^{13}C NMR (126 MHz, DMSO) δ

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171.9, 171.2, 162.7, 144.8, 144.0, 129.3, 126.3, 125.1, 122.8, 122.0 (d, $J = 275.5$ Hz, CF_3), 69.8, 69.7, 69.6, 69.5, 68.1, 66.9, 61.1, 59.2, 55.4, 46.5, 40.0, 38.2, 36.2, 35.7, 35.2, 34.3, 31.6, 30.6, 30.0, 29.4, 28.7, 28.2, 28.0, 28.0 (d, $J = 39.8$ Hz, $\text{CN}_2\text{-CF}_3$), 25.3, 22.7. **HSQC**, **HMBC**, **COSY** and **TOCSY** spectra are in accord with assignments given above. **HRMS**: calcd $\text{C}_{44}\text{H}_{64}\text{F}_3\text{N}_{11}\text{O}_{10}\text{S}$ $[\text{M}+\text{Na}]^+$ 1018.4403, found 1018.4394.

Probe 1 - 2,5-dioxopyrrolidin-1-yl 4-oxo-4-(((S)-6-oxo-6-(((1-(15-oxo-19-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-4,7,10-trioxa-14-azanonadecyl)-1H-1,2,3-triazol-4-yl)methyl)amino)-5-(3-(4-(3-(trifluoromethyl)-3H-diazirin-3-yl)phenyl)propanamido)hexyl)amino)butanoate

Compound **5** (27.5 mg, 27.6 μmol) was dissolved in 3 mL dry DMF and NHS (33.5 mg, 291 μmol) and EDC (55.3 mg 288 μmol) were added. The mixture was stirred under N_2 atmosphere for 2.5h, after which it was concentrated under vacuum. The crude yellow oil was triturated with ice-cold H_2O (3x 1mL) to yield a white solid (13.5 mg, 45%).

Analytical data: LC-MS data shown in **Supplementary Figure S1**. **HRMS**: calcd $\text{C}_{48}\text{H}_{67}\text{F}_3\text{N}_{12}\text{O}_{12}\text{S}$ $[\text{M}+\text{Na}]^+$ 1115.4566, found 1115.4607.

Synthesis Ac-Nle-SP

Ac-Nle-SP was assembled by solid phase peptide synthesis on an automatic Tribute-UV peptide synthesizer (Protein Technologies, Manchester, UK) on a 0.25 mmol scale using Rink Amide resin (0.24 mmol/g; Rapp Polymere, Tübingen, Germany). First, the resin was allowed to swell in DMF (3x 10 min). The Fmoc-group was then removed with a mix of piperidine/DMF (8 mL, 1:4, v/v), using of the Tribute-UV peptide synthesizer RV_top_UV_Xtend protocol, followed by washes with DMF (5x 30 s). Next, the correct Fmoc-protected amino acid was pre-activated for 2 min with HCTU (4 eq) and DIPEA (8 eq) in

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DMF, before it was added to the resin and shaken (20 min). The resin was washed with DMF (6x 30 s), after which Fmoc-deprotection and coupling of the next amino acid took place as before. After the coupling of the last amino acid one final Fmoc deprotection step took place. The resin was then manually treated with Ac₂O (250 µL, 2.3 mmol) and DIPEA (185 µL, 1 mmol) in DMF (10 mL) for 30 min. The resin was washed with DMF (5x 30 s) and DCM (5x 30 s) and dried under high vacuum and peptides were then cleaved and deprotected in TFA/H₂O/TIS (10 mL, 95/2.5/2.5, v/v/v, 1 h). To precipitate the peptide, the TFA mixture was added dropwise to an ice-cold solution of MTBE/hexanes (90 mL, 1:1, v/v). After centrifugation (2000 x g, 5 min) the supernatant was discarded and the peptide was washed twice more with MTBE/hexanes. The peptide was then dissolved in tBuOH/H₂O (15 mL, 1:1, v/v) and lyophilized to yield a crude peptide. This crude was further purified using the Agilent technologies 1260 Infinity LC System equipped with a Gemini C18 column (110 Å, 10 µm, 250 x 20 mm) using a 60 min linear gradient of 0-100% Buffer B in Buffer A at 12.5 mL/min (for buffers see general information). Auto-collection of fractions based on the UV measurements at 214 nm was used. Fractions containing pure peptide, as assessed by analytical HPLC, were combined and lyophilized to give Ac-Nle-SP with a 13% yield

Analytical data: for HPLC trace see **Supplementary Figure S2**. LC-MS: m/z calcd for C₆₆H₁₀₂N₁₈O₁₄ [M+2H]²⁺ 686.40 found 686.50.

UV activation of probe 1

The free acid derivative of the probe (**5**) was dissolved in MeOH (1 mg/mL) and either kept in the dark or exposed to light of 365 nm for 15 min with a high intensity UV-lamp (SB-100p/FB; Spectroline, Westbury, NY). Samples were analyzed using LC-MS.

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Probe 1 coupling to Ac-Nle-SP and Glycine.

Probe **1** in DMSO (50 mM, 25 μ L) was added to a solution of Ac-Nle-SP in Phosphate Buffered Saline (PBS) (pH 8.2, 1.05 mM, 475 μ L) to form Ac-Nle-SP-probe **8**. The mixture was incubated for 1 h at RT during which time a precipitate had formed. This was re-dissolved by addition of 500 μ L DMSO yielding a 0.5 mM solution of **8** (**Supplementary Figure S3A**). Glycine (Gly)-probe **9**, was prepared in a similar fashion using a solution of glycine in PBS (pH 8.2, 1 mg/mL, 475 μ L) (**Supplementary Figure S3B**). Although no precipitate had formed, this mixture was also diluted with 500 μ L DMSO. Moreover, to keep the amount of probe, probe by-products and DMSO between Ac-Nle-SP-probe and Gly-probe equal, in all experiments the volume of Gly-probe used was always identical to the volume of Ac-Nle-SP-probe used. Both mixtures were stored at -20°C and went through numerous freeze-thaw cycles over the span of a year without any obvious loss of activity.

Generation of Flp-InTM T-RExTM 293 cell lines

HA-NK₁-eGFP: A human NK₁ construct in pcDNA3.1+ (TACR100000; cDNA Resource Center, Bloomsberg, CA) was used as PCR template. Primers were designed to introduce an HA-epitope (YPYDVPDYA) on the N-terminus of the NK₁ construct. Moreover, XhoI and BamHI restriction sites were introduced at the 3' and 5' termini respectively. The primers used were 5'

ACGTACGTACGTCTCGAGGCCACCATGTACCCCTATGACGTGCCCCGATTACGCGG
ATAAC GTCCTCCCGGTGGACTCAGACCTCTCC 3' and

3' ACGTACGTACGTTGGATCCCGGGAGAGCACATTGGAGGAGAAGCTGAAGC 5' .

The resulting DNA fragment was subcloned between the XhoI and BamHI sites of the pEGFP-N1-plasmid harboring monomeric Ala206Lys enhanced GFP (eGFP), a variant of eGFP that is less prone to dimerization (von Stetten *et al.*, 2012), resulting in a HA-NK₁-

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eGFP construct. HA-NK₁-eGFP was excised from pEGFP-N1 with NheI and NotI. The NheI end was blunted and the fragment subcloned into pcDNA5/FRT/TO (Thermo Fisher Scientific, Paisley, UK) between EcoRV and NotI. This plasmid was used in the Flp-InTM T-RExTM system to create cells inducibly able to express HA-NK₁-eGFP as described previously (Ward *et al.*, 2011).

HA-NK₁-6xHis: Using NotI and AgeI, eGFP was excised from the HA-NK₁-eGFP construct in pcDNA5/FRT/TO. DNA encoding a hexa-His-tag, created by annealing the two primers 5' CCGGTCCACCATCACCATCACCATTAGGGCGCGCCGC 3' and 3' AGGTGGTAGTGGTAGTGGTAATCCCGCGCGGCGCCGG 5', was subcloned into the same site, thereby creating a HA-NK₁-6xHis construct. This was used in the Flp-InTM T-RExTM system as above to create cells able to inducibly express HA-NK₁-6xHis.

Cell maintenance

Flp-InTM T-RExTM 293 cells able to express either HA-NK₁-eGFP or HA-NK₁-6xHis upon addition of doxycycline (Dox) were maintained in complete Dulbecco's Modification of Eagle's Medium (DMEM) with glucose (4500 mg/L) and sodium bicarbonate, without sodium pyruvate, supplemented with 10% (v/v) foetal bovine serum, penicillin (100 units/mL), streptomycin (100 µg/mL), hygromycin (200 µg/mL), and blasticidin (5 µg/mL) in a humidified 5% CO₂ atmosphere at 37 °C and sub-cultured every three days.

Dox titration – western blotting

Flp-InTM T-RExTM 293 cells harboring HA-NK₁-eGFP or HA-NK₁-6xHis were grown in poly-D-lysine coated 6-well plates and incubated with various concentrations of Dox overnight. Cells were harvested and in radioimmunoprecipitation assay (RIPA) buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 10 mM NaF, 5 mM EDTA, 10 mM Na₂HPO₄, 5% (v/v)

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ethylene glycol, pH 7.3), passed through a 25 gauge needle (6x), rotated for 45 min at 4 °C and centrifuged for 10 min at $21,000 \times g$ at 4 °C to get cleared lysates. Lysates were analyzed by western blotting.

Dox-titration – epifluorescence quantification

Flp-InTM T-RExTM cells harboring HA-NK₁-eGFP were cultured on black clear bottom 96-well plates coated with poly-D-lysine. After induction with various concentration of Dox the plates were incubated overnight. Cells were washed twice with HBSS before they were incubated for 20 min at 37°C with 10 µg/mL Hoechst nuclear stain to enable cell number determination. After two more washes with HBSS fluorescence was measured with a CLARIOstar plate reader (BMG Labtech, Ortenberg, Germany). To quantify the Hoechst stained nuclei an excitation wavelength of 355/20 nm and an emission wavelength of 455/30 nm were used for eGFP 470/15 nm and 515/20 nm were used.

Inositol phosphate accumulation assays

Flp-InTM T-RExTM 293 cells harboring HA-NK₁-eGFP or HA-NK₁-6xHis were grown in T75 tissue culture flasks and induced by addition of 100 ng/mL Dox overnight. Uninduced cells, grown in a similar fashion, were used as negative controls. Inositol monophosphate (IP1) experiments were performed using a homogenous time-resolved fluorescence (HTRF)-based detection kit (CisBio Bioassays, Codolet, France) according to the manufacturer's protocol. Cells were plated at 7500 cells/well into white, solid-bottom, 384-well plates with appropriate ligand concentrations present in the wells and incubated at 37 °C for 2 h. The reactions were stopped by addition of the supplied lysis buffer, supplemented with the HTRF reagents. Plates were incubated for 1-24 h at RT before the output was measured with a Pherastar FS plate reader (BMGLabtech, Ortenberg, Germany).

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Live cell imaging assays

Flp-InTM T-RExTM 293 cells harboring HA-NK₁-eGFP were grown on poly-D-lysine coated coverslips and induced with 100 ng/mL Dox in serum free medium overnight. Uninduced cells, grown in a similar fashion, were used as negative control. All subsequent steps, apart from the actual imaging, were performed on ice or at 4°C. Hank's Balanced Salt Solution (HBSS) was used for all washes and dilutions. Cells were washed three times, incubated with Ac-Nle-SP-probe **8** (1 µM) or Gly-probe **9** for 1 h in the dark and washed a further three times. For competition experiments, Ac-Nle-SP (**7**) (10 µM) was added and cells were incubated for 3 h at 4°C in the dark, followed by three more washes. The cells were exposed to light of 365 nm for 15 min with a high intensity UV-lamp (SB-100p/FB; Spectroline, Westbury, NY). Cells were washed three times and incubated with DyLightTM 594-conjugated streptavidin (15 µg/mL; Vectorlabs, Peterborough, UK) for 30 min at RT in the dark. After three final washes, cells were imaged using an 880 laser scanning confocal microscope (Zeiss, Cambridge, UK).

Simultaneous dual color eGFP/DyLightTM 594 live cell microscopy imaging

A Zeiss 880 laser scanning confocal microscope, (invert configuration), equipped with a 63x oil immersion Plan Apochromat objective lens, (numerical aperture = 1.4), was used to acquire high resolution, 12 bit depth channel images, (image size = 512 x 512). Using the single track multiple channel capability of the Zeiss Zen Black software, (version 2.3), HA-NK₁-eGFP and DyLightTM 594 labelled ligand were excited simultaneously at 488 and 561 nm respectively. Well separated spectral emission detection windows, (495 – 555 nm for eGFP and 600-700 nm for DyLightTM 594), were set up to ensure that the emission signals elicited from each fluorophore were recorded without any bleed through or time delay issues. The pinhole diameter was set to 1 Airy unit and frame averaging was set to 4 to minimize

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noise and optimize signal collection. Bright field transmission images were simultaneously detected along with the fluorescence images using the dedicated transmitted light detector.

eGFP /DyLight™ 594 co-localization analysis

Each recorded channel image was exported into Metamorph, (version 7.8.13, Molecular Devices, San Jose, CA), and a green or red look up table was assigned to the eGFP and DyLight™ 594 channel images respectively. The total background level of autofluorescence, was subtracted (using a black colored region adjacent to fluorescing cell) from every matrix pixel used to form each 512 x 512 channel image. To quantify the co-localization between HA-NK₁-eGFP and DyLight™ 594, green-red pixel intensity scatterplots were generated for each image. Pearson correlation coefficient values were generated from the generated scatter plots that described the degree by which eGFP and DyLight™ 594 fluorescence at each homologous pixel varied from a perfect positive correlation slope value of 1.0. This was done for 4 representative images for each experimental group. Significant statistical difference, between the experimental groups was determined using ANOVA with Tukey's multiple comparison test.

Receptor capture by Ac-Nle-SP-probe and Gly-probe

Flp-In™ T-REx™ 293 cells harboring HA-NK₁-6xHis were grown in a 10 cm dish and induced with 100 ng/mL Dox overnight. If needed for a negative control, uninduced cells were grown in a similar fashion. Cells were harvested using Versene. All subsequent steps were performed on ice or at 4°C. Cells were resuspended in HBSS (600 µL) supplemented with Ac-Nle-SP-probe **8** (1 µM) or Gly-probe **9**. Cells were incubated for 1 h in the dark and under constant agitation. Cells were spun down at 300 x g for 3 min and washed twice with HBSS, before they were then resuspended in HBSS (600 µL), transferred to a 12-well plate and exposed to light of 365 nm for 15 min (SB-100p/FB; Spectroline, Westbury, NY). Cells

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were transferred back to a 15 mL Falcon tube, centrifuged at 3200 x g for 5 min and washed twice with PBS before they were frozen at -20°C for at least an hour. From here on all buffers were supplemented with cOmplete™ EDTA-free Protease Inhibitor Cocktail (Roche, Burgess Hill, UK). Frozen cell pellets were resuspended in PBS buffer (500 µL), passed through a 25 gauge needle (10x), homogenized using a glass on Teflon homogenizer (50x), and again passed through a needle (10x). To remove cell debris, the mixture was centrifuged at 200 x g for 5 min, after which the supernatant was centrifuged at 50,000 x g for 30 min. The pellets were resuspended in PBS supplemented with 1% (v/v) NP40 (150 µL) after which the samples were rotated for 1 h. The lysates were then centrifuged at 21,000 x g for 10 min to get rid of any non-solubilized material. Lysates were analyzed by western blotting or used for the full LRC experiment.

Western blotting

Lysates of the Dox titration and receptor capture experiments were analyzed through western blotting. A bicinchoninic assay (BCA) (Expedeon, Cambridge, UK) was used according to the manufacturer's protocol, to determine and equalize the protein concentrations of the samples. SDS-PAGE sample buffer was added to the samples and they were heated to 65 °C for 5 min. 10-20 µg protein per sample was loaded into wells of 4 to 12% BisTris precast NuPAGE or BOLT gels (Thermo Fisher Scientific, Paisley, UK) and subjected to SDS-PAGE in NuPAGE or BOLT MOPS SDS running buffer (Thermo Fisher Scientific, Paisley, UK). The proteins were then electrophoretically transferred to a nitrocellulose membrane, which was blocked in PBS blocking buffer (LI-COR, Cambridge, UK) and subsequently probed for HA-NK₁-eGFP, HA-NK₁-6xHis, tubulin and/or biotin using the appropriate primary and secondary antibodies, or streptavidin diluted in PBS blocking buffer (LI-COR,

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Cambridge, UK) supplemented with 0.2% Tween 20. An Odyssey Scanner (LI-COR, Cambridge, UK) was used to image the membranes.

Full ligand-based receptor capture experiment

For the full LRC experiment, receptor capture took place as described above with the exception that 5 confluent T150 flasks were used, buffer quantities were multiplied by 10, and UV-activation was performed in a 10 cm dish. All subsequent steps were performed on ice or at 4°C and all buffers were supplemented with cOmplete™ EDTA-free Protease Inhibitor Cocktail (Roche, Burgess Hill, UK). Lysates were added to Pierce™ Streptavidin Agarose beads (250 µL; Thermo Fisher Scientific, Paisley, UK) and the total volume was made up to 3 mL using PBS supplemented with 1% (v/v) NP40. The beads were incubated overnight, before they were washed 4 times with RIPA buffer, 4 times with PBS supplemented with 880 mM NaCl, and 4 times with PBS. Beads were frozen at -80°C until they were analyzed by LC-MS/MS. For each condition 3 biological repeats were performed.

LC-MS/MS analysis

Proteomics was carried out by the University of Leicester Proteomics Facility (PNACL, University of Leicester). The streptavidin-conjugated beads were re-suspended by gentle mixing and transferred to a 30,000 molecular weight cut-off filtration unit (Vivacon 500; Satorius, Göttingen, Germany). Excess liquid was removed by spinning at 14,000 x g for 15 min. The beads were then re-suspended a total of 2 times in 200 µL of freshly prepared 8 M Urea in 0.1 M Tris/HCL pH 8.5 and spun at 14,000 x g for 15 min, discarding the flow through solution. Reduction was carried out by addition of 100 µL 5 mM dithiothreitol solution for 30 min at 60 °C. After cooling, samples were incubated with addition of 100 µL of 50 mM iodoacetamide solution for 30 min at RT in the dark. After incubation, solutions were removed by spinning at 14,000 x g for 15 min. Beads were then re-suspended a total of

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two times in 100 μ L of 8 M urea in 0.1 M Tris/HCL pH 8.5 and spun at 14,000 x g for 15 min, discarding the flow through solution. Beads were then re-suspended twice in 100 μ L of 50 mM triethylammonium bicarbonate and spun at 14,000 x g for 15 min, discarding the flow through solution. Just prior to use, lyophilized trypsin was re-suspended in 50 mM triethylammonium bicarbonate and 5 μ g was added to each sample. Digestion took place overnight at 37 °C in a humidified chamber. Filter units were transferred to a clean collection tube and samples were spun down for 10 min at 14,000 x g. A further 40 μ L of 50 mM triethylammonium bicarbonate was added to beads and samples spun again for 10 min at 14,000 x g. The samples were concentrated to approximately 20 μ L volume in a SpeedVac centrifuge and digests were then acidified with formic acid (final concentration 0.1%). LC-MS/MS was carried out using an RSLCnano HPLC system (Dionex, Camberley, UK) and an LTQ-Orbitrap-Velos mass spectrometer (Thermo Fisher Scientific, Paisley, UK). Samples were loaded at high flow rate onto a reverse-phase trap column (0.3 mm i.d. x 1 mm), containing 5 μ m C18 300 Å Acclaim PepMap media (Dionex, Camberley, UK) maintained at a temperature of 37 °C. The loading buffer was 0.1% formic acid / 0.05% trifluoroacetic acid / 2% acetonitrile. Peptides were eluted from the trap column at a flow rate of 0.3 μ L/min and through a reverse-phase capillary column (75 μ m i.d. x 250 mm) containing Symmetry C18 100 Å media (Waters, Elstree, UK) that was manufactured in-house using a high pressure packing device (Proxeon Biosystems, Odense, Denmark). The output from the column was sprayed directly into the nanospray ion source of the LTQ-Orbitrap-Velos mass spectrometer. The LTQ-Orbitrap-Velos mass spectrometer was set to acquire a 1 microscan FTMS scan event at 60000 resolution over the m/z range 300-2000 Da in positive ion mode. The maximum injection time for MS was 500 ms and the AGC target setting was $1e^6$. Accurate calibration of the FTMS scan was achieved using a background ion lock mass for $C_6H_{10}O_{14}S_3$ (401.922718 Da). Subsequently up to 10 data dependent HCD MS/MS were triggered from

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the FTMS scan. The isolation width was 2.0 Da, normalized collision energy 42.5. Dynamic exclusion was enabled. The maximum injection time for MS/MS was 250 ms and the AGC target setting was $5e^4$. The .raw data file obtained from each LC-MS/MS acquisition was processed using Proteome Discoverer (version 1.4; Thermo Fisher Scientific, Paisley, UK), searching each file in turn using Mascot (version 2.2.04, Matrix Science Ltd., London, UK; Perkins *et al.*, 1999) against the human reference proteome downloaded from UniProtKB (Proteome ID: UP000005640; UniProt Consortium, 2010). The peptide tolerance was set to 5 ppm and the MS/MS tolerance was set to 0.05 Da. Fixed modifications were set as carbamidomethyl (C) with variable modification of oxidation (M) and phosphorylation (S), (T) and (Y). Trypsin was selected as the enzyme and up to 3 missed cleavages were allowed. A decoy database search was performed. The output from Proteome Discoverer was further processed using Scaffold Q+S (version 4.0.5, Proteome Software; Searle, 2010). Upon import, the data was searched using X!Tandem (The Global Proteome Machine Organization; Craig and Beavis, 2004). PeptideProphet (Keller *et al.*, 2002) and ProteinProphet (Institute for Systems Biology; Nesvizhskii *et al.*, 2003) probability thresholds of 95% were calculated from the decoy searches and Scaffold was used to calculate an improved 95% peptide and protein probability threshold on the data from the two different search algorithms. Protein identifications were accepted if they contained at least 3 identified peptides.

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Results

Design and synthesis of the probe: A probe for ligand-based receptor capture should permit the attachment of a ligand, enable receptor capture and allow enrichment for identification via, for example, MS-analysis (**Figure 1A**). Similar to other probes, an NHS-ester was chosen to attach the ligand to the probe. Under physiological conditions the NHS-ester reacts with primary amines, present in peptide and protein ligands, to yield amide bonds (Anderson *et al.*, 1963; Mädler *et al.*, 2009). To enable receptor capture, a photoaffinity label was introduced. Irradiation of such labels results in covalent coupling to nearby molecules (Sumranjit and Chung, 2013). A diazirine moiety was chosen over other photoaffinity labels due to its reported short activation time, high stability and low background photo-crosslinking (Kleiner *et al.*, 2017; Hill and Robertson, 2018). For enrichment of the captured receptor(s) after cell lysis a biotin moiety was incorporated into the probe. The required functional groups were introduced into a tri-functional molecular scaffold based on lysine, creating probe **1** which can be used for ligand-based receptor capture (**Figure 1**). Probe **1** was conveniently synthesized in 7 steps from Fmoc-N- ϵ -Boc-lysine with an overall yield of 19% (**Figure 1B**). The synthesis started with the coupling of propargylamine to the free carboxylic acid creating an alkyne handle in amide **2**. This was followed by Fmoc-deprotection and coupling of the photoaffinity label 3-{4-[3-(trifluoromethyl)-3H-diazirin-3-yl]phenyl}propanoic acid, leading to **3** (Geurink *et al.*, 2010). After Boc-deprotection, succinic anhydride was used to incorporate a carboxylic acid moiety, thereby affording compound **4**. Addition of biotin-PEG-azide via copper(I)-catalyzed azide alkyne cycloaddition (CuAAC) resulted in the formation of compound **5**, which was activated with an NHS-ester to yield the final product, probe **1**. Before proceeding to the biological evaluation, it was confirmed that UV-irradiation of the probe did indeed activate the diazirine

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moiety followed by insertion of the resulting carbene into nearby molecules (**Supplementary Figure S4**).

Synthesis of substance P and coupling to the probe: The NK₁ receptor and its ligand SP were chosen as a test system. A slightly modified version of SP (Ac-Nle-SP), in which the C-terminal methionine residue was replaced with a norleucine (Nle) residue and the N-terminus was capped with an acetyl (Ac) group to leave only one free amine available, was synthesized using solid phase peptide synthesis (**Figure 2A and Supplementary Figure S2**). Addition of probe **1** (2.5 eq) to Ac-Nle-SP in PBS (pH 8.2) resulted in coupling of Ac-Nle-SP to probe **1** yielding Ac-Nle-SP-probe **8**, while no free Ac-Nle-SP remained (**Figure 2B and Supplementary Figure S3A**). The remainder of the probe either hydrolyzed back to the free acid **5** or formed cyclisation product **6** (**Supplementary Figure S3A**). To function as a negative control, probe **1** was also coupled to glycine, resulting in a similar mixture of Gly-probe **9** (**Figure 2C**), and hydrolyzed probe **5** and cyclisation product **6** (**Supplementary Figure S3B**).

Generation of NK₁-expressing cell lines: The Flp-InTM T-RExTM 293 system was used to create cell lines designed to allow inducible expression of a receptor of interest upon addition of the antibiotic doxycycline (Dox) (**Figure 3**). In the first of these an NK₁ receptor construct was generated in which the haemagglutinin (HA) epitope tag (amino acid sequence YPYDVPDYA) was engineered into the extracellular N-terminal domain and enhanced green fluorescent protein (eGFP) was added in-frame with the intracellular C-terminal tail of the receptor (HA-NK₁-eGFP). In a second iteration eGFP was replaced with a hexa-histidine tag (HA-NK₁-6xHis) to facilitate detection using an anti-6xHis antiserum.

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Binding of Ac-Nle-SP-probe to the NK₁ receptor: The NK₁ receptor interacts with G proteins of the G_q class and this results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate. To confirm that Ac-Nle-SP-probe retained affinity for the receptor, accumulation of the inositol monophosphate (IP1) end-products of this reaction was assessed in Flp-InTM T-RExTM 293 cells that had been treated with Dox to induce expression of either HA-NK₁-eGFP or HA-NK₁-6xHis. These studies showed that although the potency of Ac-Nle-SP-probe was reduced by a factor of between 4-8 fold compared to free Ac-Nle-SP, it retained binding and agonism at the receptor (**Figure 4**). Control experiments confirmed that no response was obtained in cells not induced to express the NK₁ receptor constructs or in cells treated with the Gly-probe.

To study the binding of Ac-Nle-SP-probe to the NK₁ receptor directly, confocal microscopy was performed on HA-NK₁-eGFP expressing cells. The eGFP component of this receptor construct can be visualized directly when the receptor is induced, and indeed, cell surface localization of the expressed receptor was clearly observed in Dox-induced cells but not in uninduced cells (**Figure 5, upper panels**). Following treatment of cells with 1 μ M Ac-Nle-SP-probe for 1 h at 4°C, after which cells were washed and exposed to UV-light for 15 minutes, DyLightTM 594-conjugated streptavidin, which interacts with the biotin component of the probe, was utilized to visualize binding of Ac-Nle-SP-probe to HA-NK₁-eGFP (**Figure 5, middle panels**). This was obvious only when the receptor was expressed and was not replicated when Gly-probe was used (**Figure 5, middle panels**). Merging of the images showed co-localization at the level of light microscopy of the eGFP-containing receptor and the DyLightTM 594 labelled probe (**Figure 5, lower panels**).

NK₁ receptor capture by Ac-Nle-SP-probe: To confirm that the observed interaction between probe **1** and HA-NK₁-eGFP was indeed covalent and not simply a reflection of a

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slow off-rate of Ac-Nle-SP-probe from the receptor following initial binding, competition experiments were performed. As before, cells expressing HA-NK₁-eGFP were treated with Ac-Nle-SP-probe followed by irradiation with UV-light. Now, however, before adding the DyLight™ 594-conjugated streptavidin to visualize probe binding to the receptor, cells were incubated with excess free Ac-Nle-SP. Treatment with free ligand did not result in reduction of co-localization between the probe and the receptor for cells exposed to UV-light (**Figure 6A,B, samples 1 vs 3**). By contrast, when cells were maintained in the dark there was a significant ($p \leq 0.001$) reduction in co-localization (**Figure 6A, B, samples 2 vs 4**), indicating partial dissociation of Ac-Nle-SP-probe from the receptor over this time period. This suggests that UV-activation did indeed induce covalent linkage between the probe and the NK₁ receptor and although dissociation of Ac-Nle-SP-probe from the receptor in the absence of cross-linking was slow, it did occur.

To further confirm UV-induced formation of a covalent bond between the photoaffinity probe and HA-NK₁-eGFP, western blotting was performed. Since Flp-In™ T-REx™ 293 cells constitutively express a number of endogenously biotinylated proteins, which unfortunately migrated at the same molecular mass on SDS-PAGE gels as the HA-NK₁-eGFP receptor, these experiments were performed on cells expressing the HA-NK₁-6xHis-receptor construct, which is predicted to have a substantially lower molecular mass (by some 27 kDa). Following Dox-induced expression of HA-NK₁-6xHis cells were treated with Ac-Nle-SP-probe, washed and exposed to UV-light. Cells were then lysed and membrane preparations collected. Streptavidin detection confirmed the presence of a biotinylated protein of the same mobility as the HA-NK₁-6xHis receptor in UV-irradiated samples treated with Ac-Nle-SP-probe (**Figure 7, sample 2**), but not in samples treated with Gly-probe (**Figure 7, sample 3**), nor in samples that were not exposed to UV-light (**Figure 7, sample 4**), or in samples not

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expressing the NK₁-receptor (**Figure 7, sample 5**). This confirmed that a covalent bond was indeed formed between Ac-Nle-SP-probe and the NK₁ receptor but not between Gly-probe and the receptor.

Ligand-based receptor capture using Ac-Nle-SP-probe: Confidence in the ability to selectively label the NK₁ receptor now allowed attempts to identify the covalently captured receptor in a non-biased manner via ligand-based receptor capture and MS according to the procedure depicted in **Figure 1**. After treatment of cells expressing HA-NK₁-6xHis with either the Ac-Nle-SP-probe or Gly-probe, followed by exposure to UV, membrane preparations were obtained, detergent solubilized and added to streptavidin-coated beads. An ‘on-bead’ trypsin digest was performed and resulting peptides were analyzed using LC-MS/MS. This resulted in a total list of 49 identified human proteins, 48 of which were identified in both Gly-probe and Ac-Nle-SP-probe treated samples (**Figure 8A and Supplementary data**). This group mostly consisted of endogenously biotinylated proteins including carboxylases and common contaminants such as keratin. The NK₁ receptor (uniprot ID P25103), which was recovered as five distinct peptide fragments (**Table 1, Figure 8B**), was the only protein identified in Ac-Nle-SP-probe but not in Gly-probe treated samples. Moreover, apart from NK₁, none of the other protein hits showed significant differences ($p \leq 0.05$) in either total spectrum count, or total ion current between the samples, illustrating the specificity of the system and probe developed (**Supplementary data**).

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Discussion

Herein the development, synthesis and evaluation of a new tri-functional probe for ligand-based receptor capture have been documented. This probe uses a diazirine photoaffinity label to capture nearby target receptors. This has several advantages over other capture-techniques: (1) All receptors with an extracellular domain can be captured in intact cells by such a photo-reactive group. This is in contrast to, for example, the protected hydrazine group utilized in TRICEPS, which can only capture receptors that are N-glycosylated in proximity to the ligand binding site (Frei *et al.*, 2012). (2) A washing step after ligand-receptor binding but before receptor capture can be introduced thus reducing non-specific binding and background labelling by the probe. (3) As these groups are very stable in the absence of light the ligand can be coupled to the probe and the complex can be stored, limiting the need to synthesize new batches of the probe for each experimental run. For affinity purification a biotin moiety was incorporated into the probe. Biotin is the most widely used affinity tag, due to its high affinity binding to streptavidin (Hermanson, 2013), however, a new trend in the development of cytosolic probes has emerged recently. This involves the incorporation of an alkyne or azide handle into the probe instead of the bulky biotin group, which would hinder cell entrance of the probe. CuAAC can then be used to couple biotin, or any other reporter tag, to the probe after cell lysis, or even couple the probe directly to azide-conjugated beads (Smith and Collins, 2015). Originally such a strategy was explored and a variant of probe **1**, bearing an alkyne handle, instead of a biotin moiety, was created by directly activating compound **4** with an NHS-ester. This probe was coupled to Ac-Nle-SP and added to cells expressing the NK₁ receptor. It was then attempted to perform CuAAC on lysates of these cells in order to couple this probe to either biotin or azide-conjugated agarose beads. In our hands, however, this strategy was not successful. For the purpose of identifying

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transmembrane receptors on living cells, no cell entrance is required and hence it was decided to attach biotin to the probe from the outset, resulting in probe **1**.

It should be noted that probe **1** was not fully purified. LC-MS analysis revealed the presence of some hydrolyzed probe **5**, as well as a probe product which had undergone cyclic imide formation (**6**) (**Supplementary Figure S1**). Whether these by-products were formed during the analysis or whether they were actually present in the product was not investigated, since they were not expected to interfere with coupling of a ligand. For ligand-coupling, probe **1** was dissolved in PBS (pH 8.2) and partial hydrolysis as well as intramolecular cyclization of the active NHS-ester were inevitable under these conditions (**Supplementary Figure S3**) (Lim *et al.*, 2014; Klykov and Weller, 2015). These probe by-products were easily eliminated when the cells were washed before irradiation. Indeed, when cells lacking expression of NK₁-receptor were treated with the Ac-Nle-SP-probe, no substantial background labelling was observed (**Figure 5, right panels**).

For ligand-based receptor capture experiments to be successful it is important that the ligand-probe adduct binds to receptor(s) of interest. In this case this was tested by using accumulation of IP1, which is a consequence of binding of SP and related peptides to the NK₁ receptor. After binding of the ligand-probe adduct to the receptor it is vital that the ligand remains associated with the receptor until the probe is activated by UV-light.

Incubation of cells with Ac-Nle-SP-probe was first performed at 37°C, however, it was soon appreciated that the NK₁ receptor rapidly (3 min) internalizes upon activation by its agonist SP (Garland *et al.*, 1994). Internalization of the NK₁-SP complex is known to be linked with dissociation and degradation of SP, therefore it was important to perform receptor-capture experiments at lower temperatures, preventing internalization of the receptor (Garland *et al.*, 1994). That the Ac-Nle-SP-probe still bound to the NK₁ receptor at 4°C, but did not result in

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substantial internalization of the receptor was apparent from the microscopy studies. These low temperatures also slowed down the dissociation of the ligand from the receptor.

Potentially, this may be beneficial for receptor capture, as it should keep the probe in close proximity to the receptor. It did, however, limit dissociation and competition of the non-cross-linked Ac-Nle-SP-probe from the NK₁ receptor in the presence of an excess of free Ac-Nle-SP.

After initial characterization of the probe, a proof of concept for probe **1** was provided in which Ac-Nle-SP was coupled to this probe and used to identify the GPCR NK₁ through LC-MS/MS. This is one of the few successful examples in which a GPCR has been identified using a universal probe and proteomics. TRICEPS was used previously to identify the apelin receptor, however, this probe clearly has shortcomings with respect to the requirement for glycosylation of the receptor (Frei *et al.*, 2012). Whilst we were working on the development of these photoreactive universal probes others also realised the benefit of incorporating photoreactive groups into probes targeting transmembrane receptors. Two reports describing such probes specifically targeting GPCRs have been published recently. In both cases the probes were designed on known pharmacological ligands rather than being potentially universal. Blex *et al.*, (2017) generated the probe CPT-00031 based on the structure of sertindole, a well-known dopamine D₂ receptor antagonist, whilst Thomas *et al.*, (2017) created a probe based on the structure of an active parent compound known to bind G protein-coupled receptor 39 (GPR39). Akin to the probe described herein both these probes also contained a 3-aryl-3-(trifluoromethyl)diazirine moiety and a biotin group and were tested on systems overexpressing the receptor of interest.

A major challenge in progressing studies of this nature to true unbiased detection of genuinely novel ligand-receptor interactions is the number of ‘off-target’ interactions that are

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identified and including appropriate negative controls is an important aspect of LRC experiments. In the studies using CPT-00031 on dopamine D₂ receptor expressing HEK293 cells a competition experiment with an excess of sertindole was performed as a potential negative control. However, there were over 150 proteins enriched in the non-competition over the competition samples (Blex et al., 2017). Although this study did attempt to assess sensitivity by diluting transfected cells with non-transfected control cells there were always other proteins identified as well as the dopamine D₂ receptor. Moreover, when diluting the transfected cells to a level in which the target receptor was not enriched, 18 other membrane proteins were. Whether any of these 18 proteins truly bound to sertindole was not further investigated. Unfortunately, Thomas et al., (2018), who used a similar competition experiment as negative control, did not include the full data set obtained with their LC-MS/MS experiments and it is, therefore, hard to ascertain how many proteins were identified in the LRC studies. It is clear, however, that there were at least seven transmembrane receptors, including GPR39, enriched in the non-competition samples (Thomas *et al.*, 2017). In the studies reported herein, instead of competition experiments, an LRC experiment using Gly-probe was used as a negative control. In contrast to the two probes discussed above, comparison between the Gly-probe and the Ac-Nle-SP-probe treated samples resulted in the identification of only one protein: the NK₁ receptor.

Establishing that probe **1** can indeed be used to identify the NK₁ receptor as the Ac-Nle-SP-binding receptor in a test system is an important proof of concept towards general utilization of this probe to help identify unknown plasma-membrane receptors for ligands of interest. Nevertheless, the goal of developing a truly high specificity and universal probe for ligand-based receptor capture that can be used with confidence in natively expressing cells and tissue remains a substantial challenge.

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Authorship Contribution

Participated in research design: Müskens, Ward, Langemheen, Tobin, Liskamp, Milligan

Conducted experiments: Müskens

Contributed new reagents or analytic tools: Müskens, Herkt

Performed data analysis: Müskens

Wrote or contributed to the writing of the manuscript: Müskens, Liskamp, Milligan

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Footnotes

FMM thanks University of Glasgow for a Lord Kelvin-Adam Smith studentship

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Legends for Figures

Figure 1: A flow chart of ligand-based receptor capture and the synthesis of probe 1

A) To initiate ligand-based receptor capture the probe is first coupled to the ligand of interest (top of scheme) or a control ligand (glycine – bottom of scheme), after which the adduct is added to cells potentially expressing a target receptor. After an appropriate incubation time during which the ligand binds the receptor, cells are washed to rinse away unbound probe. Cells are then exposed to UV-light to activate the diazirine moiety which captures the receptor. Cells are lysed, and lysates are added to streptavidin beads. Captured proteins are digested on the beads with trypsin and released peptides are analyzed using mass spectrometry. Comparison between the proteins identified for ligand-treated and glycine-treated cells should reveal the ligand-binding receptor. **B)** Synthesis of probe 1.

Figure 2: Structures of Ac-Nle-SP, Ac-Nle-SP-probe and Gly-probe

Figure 3: Doxycycline-dependent expression of HA-NK₁-eGFP and HA-NK₁-6xHis

Flp-InTM T-RExTM 293 cells expressed HA-NK₁-eGFP (**A-C**) or HA-NK₁-6xHis (**D**) following induction by treatment with doxycycline (Dox). **A)** Expression of HA-NK₁-eGFP following overnight treatment with various concentrations of Dox was visualized by immunoblotting with anti-GFP after cell lysates were resolved with SDS-PAGE and transferred to blotting membrane. An anti-tubulin antiserum provided a loading control. **B)** Expression of HA-NK₁-eGFP following overnight treatment of cells with various concentrations of Dox and treatment with Hoechst nuclear stain (10 µg/mL, 30 min) was

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quantified with a microplate reader by measuring fluorescence of eGFP and the Hoechst stained nuclei. **C)** Cells as above were treated in the absence (**left panel**) or presence of Dox (100 ng/mL; **right panel**) overnight and imaged using a confocal microscope while HA-NK1-eGFP was excited. **D)** Expression of HA-NK₁-6xHis following overnight treatment with various concentration of Dox was visualized by immunoblotting with anti-6xHis after cell lysates were resolved with SDS-PAGE and transferred to blotting membrane. As in **A**, an anti-tubulin antiserum provided a loading control.

Figure 4: Ac-Nle-SP-probe activates the NK₁ receptor constructs

IP1 accumulation was assessed in both Dox-induced and uninduced Flp-InTM T-RExTM 293 cells harboring either the HA-NK₁-eGFP (**A**) or the HA-NK₁-6xHis (**B**) construct. The Ac-Nle-SP-probe was clearly active although less potent than native Ac-Nle-SP. Data are means \pm SD pooled from n = 3 independent experiments each performed in triplicate.

Figure 5: Ac-Nle-SP-probe binds to the NK₁ receptor constructs at the cell surface

Dox-induced or uninduced Flp-InTM T-RExTM 293 cells harboring HA-NK₁-eGFP were treated with Ac-Nle-SP-probe or Gly-probe (1 μ M at 4°C for 1 h) before exposure to UV-light (15 min) and were then treated with DyLightTM 594-conjugated streptavidin before they were imaged using a confocal microscope. HA-NK₁-eGFP and the DyLightTM 594 labelled probe were excited simultaneously at 488 nm (**upper panels**) and 561 nm (**middle panels**) respectively. Co-localization between the receptor and the probe was visualized (yellow color; **lower panels**). The bright field image demonstrates that cells were present in the uninduced -Dox panels.

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Figure 6: Ac-Nle-SP-probe covalently captures NK₁ receptor constructs upon UV-activation

A) Dox-induced Flp-InTM T-RExTM 293 cells expressing HA-NK₁-eGFP were treated with Ac-Nle-SP-probe (1 μ M at 4°C for 1 h) before they were activated with UV-light for 15 min (samples 1 and 3) or kept in the dark (samples 2 and 4). Cells were then either treated immediately with DyLightTM 594-conjugated streptavidin (samples 1-2) or were first exposed to Ac-Nle-SP (10 μ M at 4°C for 3h) (samples 3-4). They were then imaged using a confocal microscope. HA-NK₁-eGFP and the DyLightTM 594 labelled probe were excited simultaneously at 488 nm (**upper panels**) and 561 nm (**middle panels**) respectively. Co-localization between the receptor and the probe was visualized (yellow color; **lower panels**).

B) Co-localization was quantified by generating green-red pixel intensity scatterplots for each pixel and determining the Pearson correlation coefficient for 4 representative images for each condition. Data are means \pm SD. Significant statistical difference was determined using ANOVA with Tukey's multiple comparison test (*** $p \leq 0.001$).

Figure 7: Detection of Ac-Nle-SP-probe NK₁ receptor interactions

Dox-induced (**lanes 1-4**) or uninduced (**lane 5**) Flp-InTM T-RExTM 293 cells harboring HA-NK₁-6xHis were treated with either Ac-Nle-SP-probe (**lanes 2,4,5**) or Gly-probe (**lane 3**) and then exposed to UV light (**lanes 1-3, 5**) or maintained in the dark (**lane 4**). Following cell lysis, membrane preparations were generated and resolved by SDS-PAGE. These were immunoblotted with an anti-6xHis antiserum (**left panel**), or streptavidin (**centre panel**). Merging of these two images and pseudo-color labelling identifies the receptor-probe complex as yellow (**right panel**).

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Figure 8: Identification of proteins by mass spectrometry after ligand-based receptor capture.

A) Schematic view of human proteins identified by LC-MS/MS after ligand-based receptor capture (LRC) experiments with either Gly-probe or Ac-Nle-SP-probe. **B)** The amino acid sequence of the human NK₁ receptor is displayed. Peptides that were identified using LC-MS/MS after LRC with Ac-Nle-SP-probe are colored. See **Table 1** for further details (1 = blue, 2 = green, 3 = yellow, 4 = purple. 5 = green).

Tables

Table 1: Peptide sequences from NK₁ receptor identified by mass spectrometry

	Peptide sequence	Total spectra (#)	Found in # samples	Modifications
1	(R)LETTISTVVGAHEEPEP GPK(A)	2	2	
2	(R)YLQTQGSVYKVS(L)	1	1	
3	(K)ATPSSDLTSNcSSR(S)	2	2	Carbamidomethyl
4	(R)YMAIIHPLQPR(L)	1	1	
5	(R)YLQTQGSVYK(V)	1	1	

Figures

Figure 1

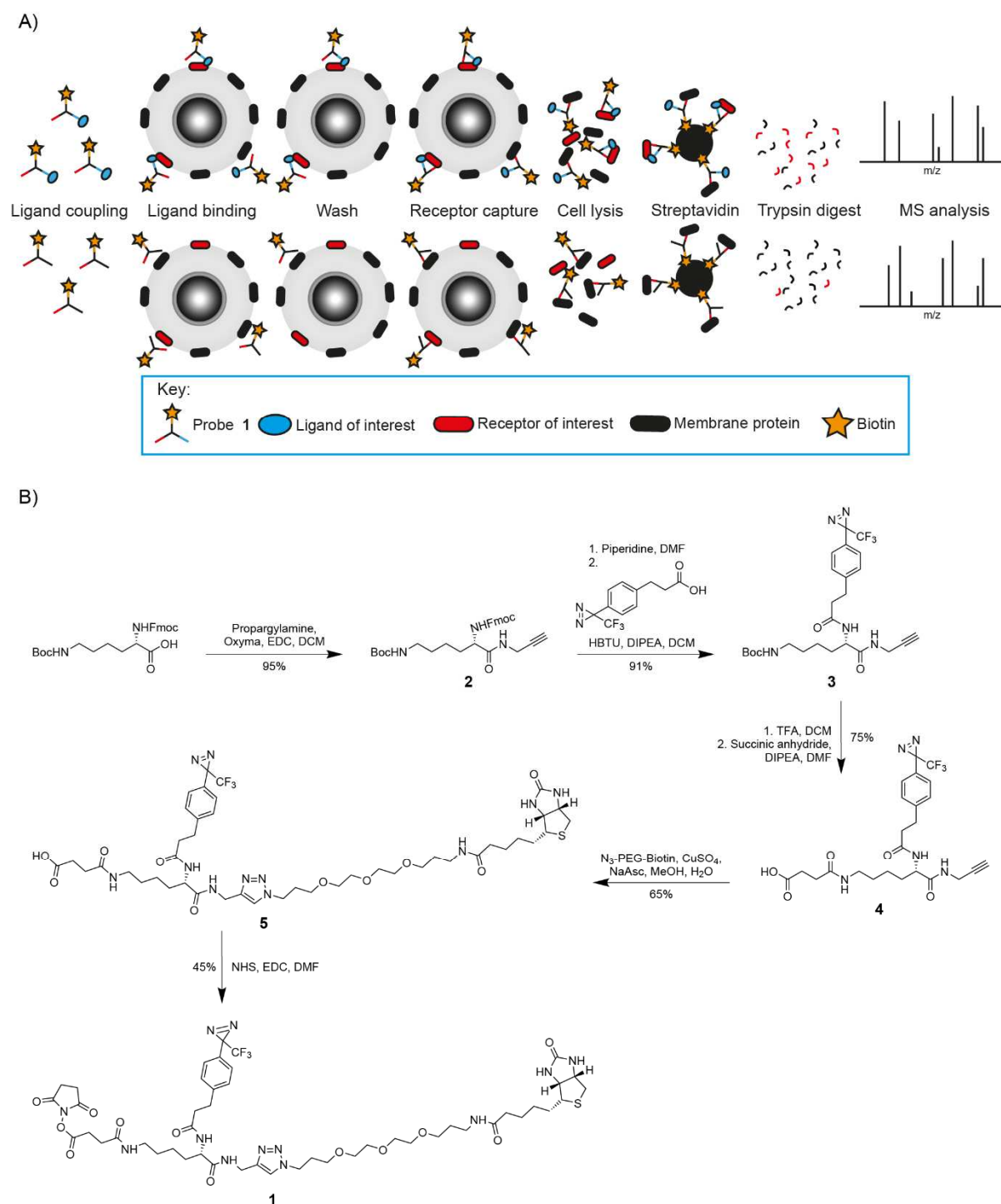


Figure 2

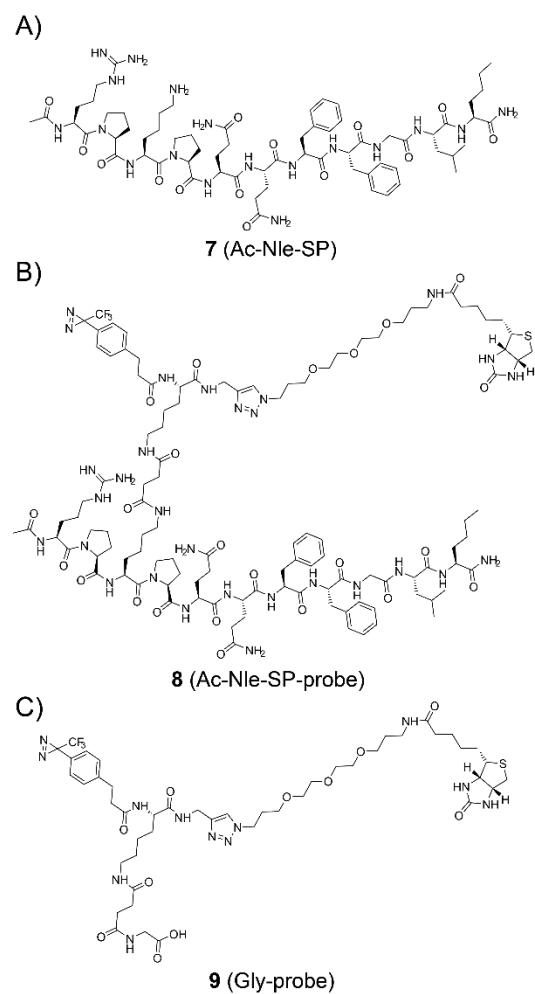


Figure 3

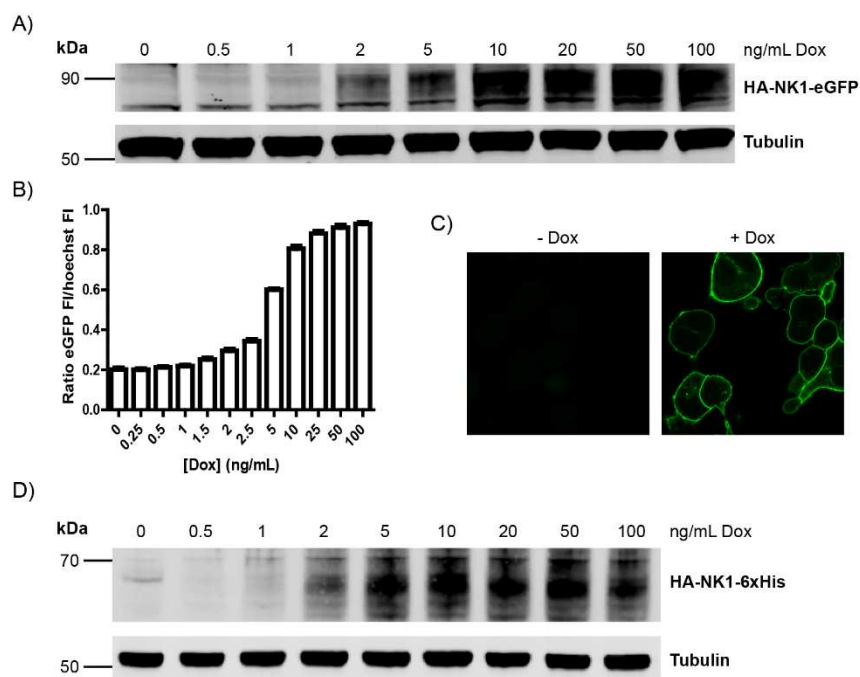


Figure 4

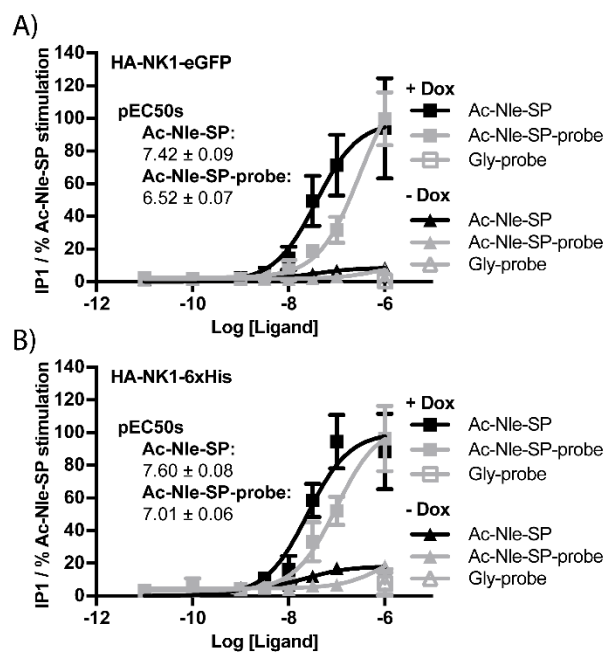


Figure 5

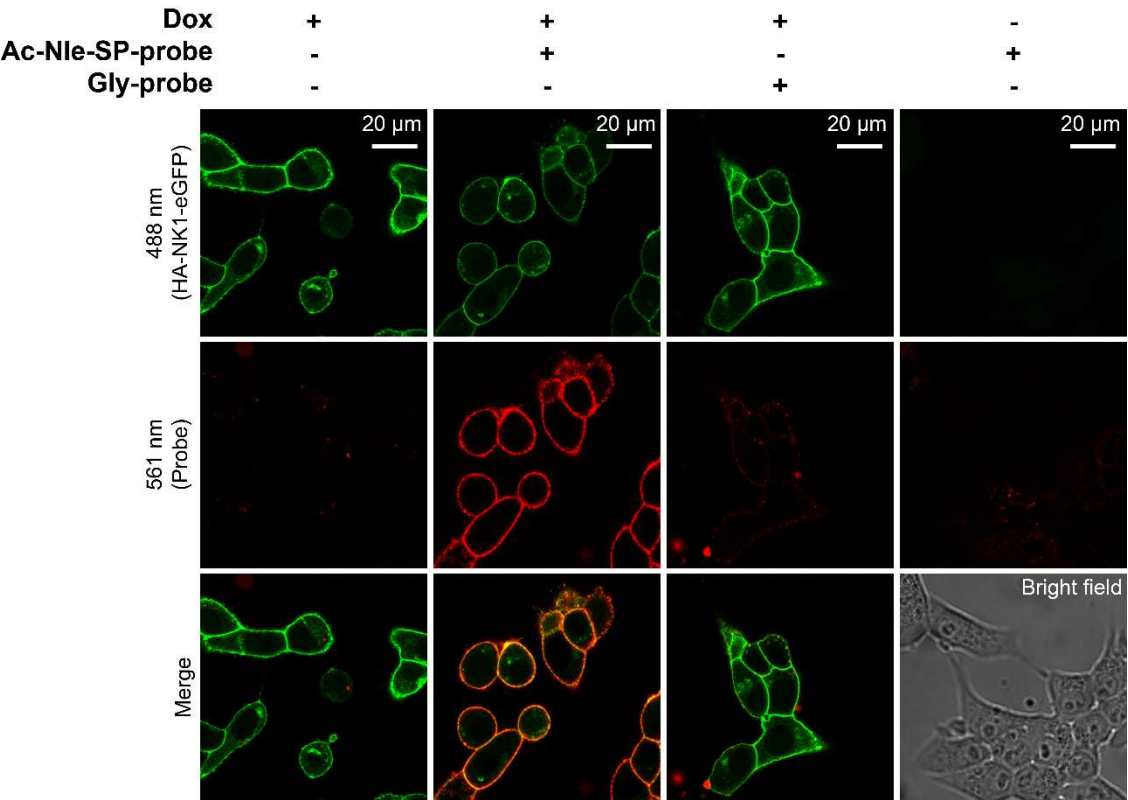


Figure 6

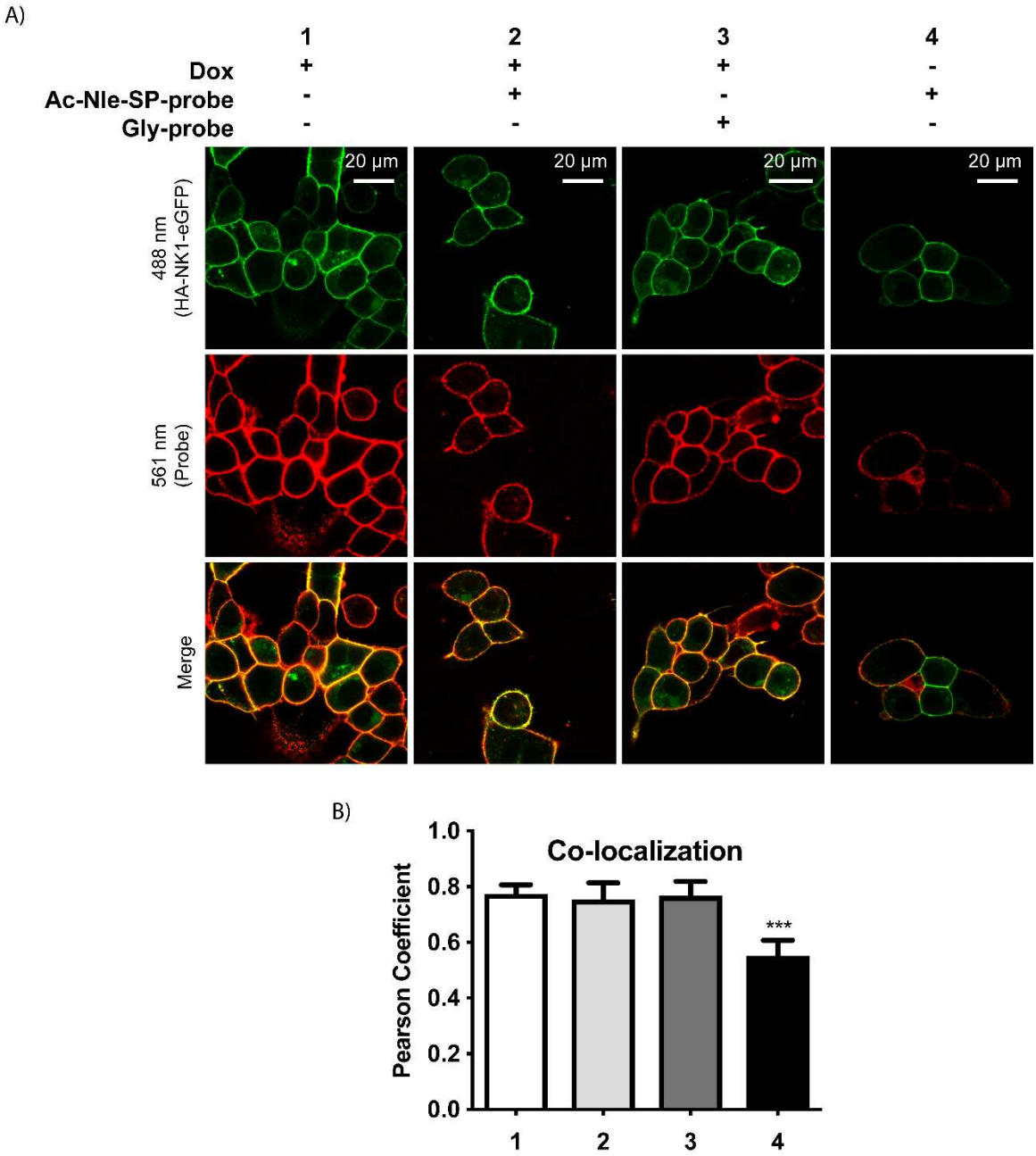


Figure 7

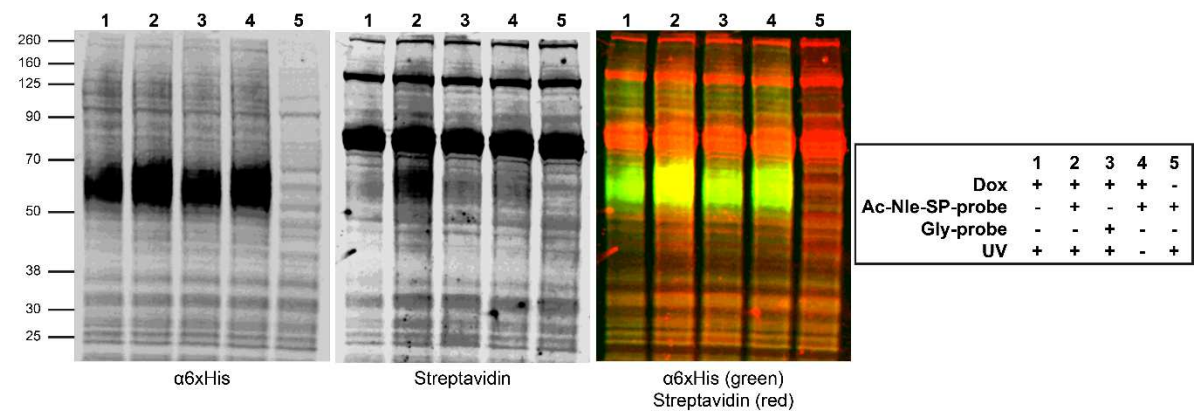
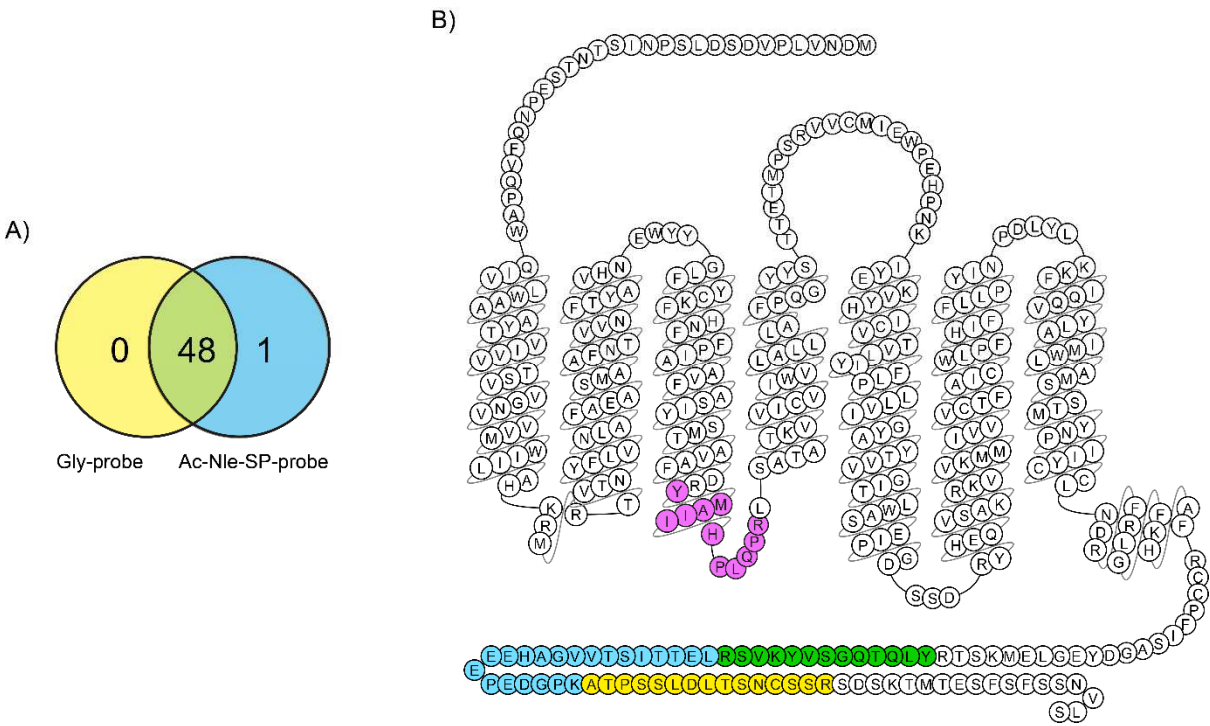


Figure 8



Design, synthesis and evaluation of a diazirine photoaffinity probe for ligand-based receptor capture targeting G protein-coupled receptors

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Molecular Pharmacology

Supplementary Data

Figure S1. Analysis of the purity of probe 1.

Figure S2. Analysis of the purity of Ac-Nle-SP.

Figure S3. Coupling of probe 1 to Ac-Nle-SP and Gly. Figure S4. UV-activation of probe 1.

Figure S4. UV-activation of probe 1.

LCMS Data available in separate excel file

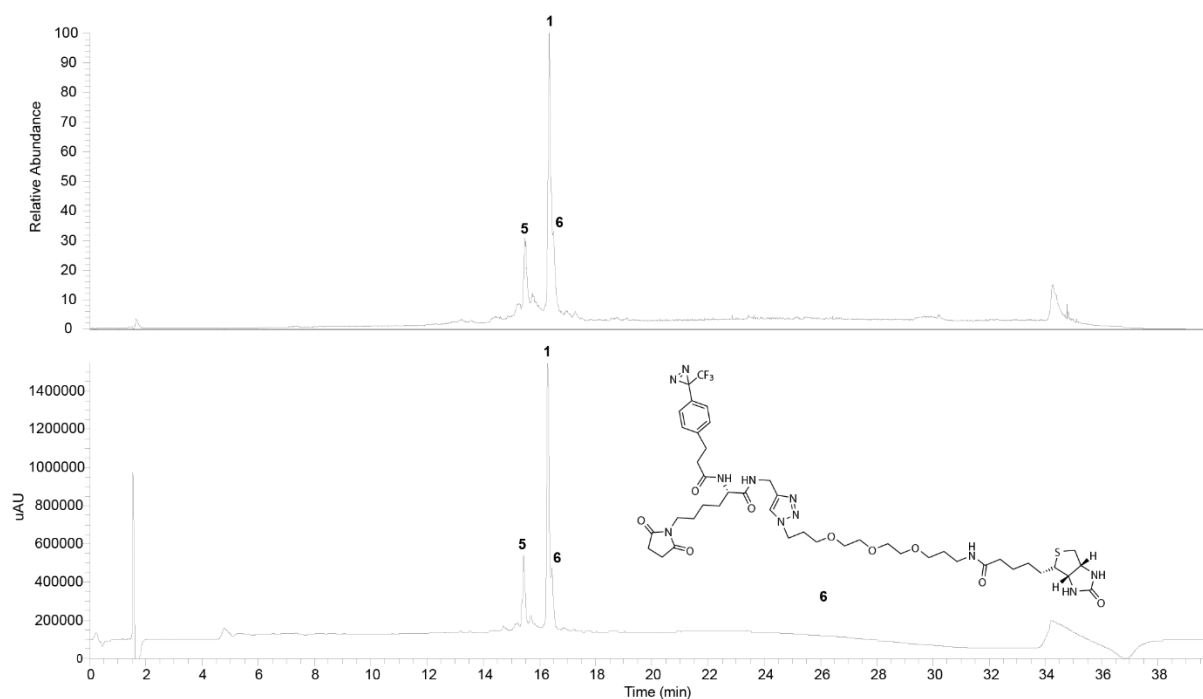


Figure S1. Analysis of the purity of probe 1. Probe 1 was analysed by LC-MS using a C18 column with a 5-95% gradient of MeCN in H₂O over 28 minutes (2-30 min). The UV-trace (**bottom panel**) and mass-trace (**top panel**) are depicted and reveal the presence of three compounds: the desired probe **1**, hydrolysed probe **5** and intermolecularly cyclised probe **6**. For the structures of **1** and **5** see main article.

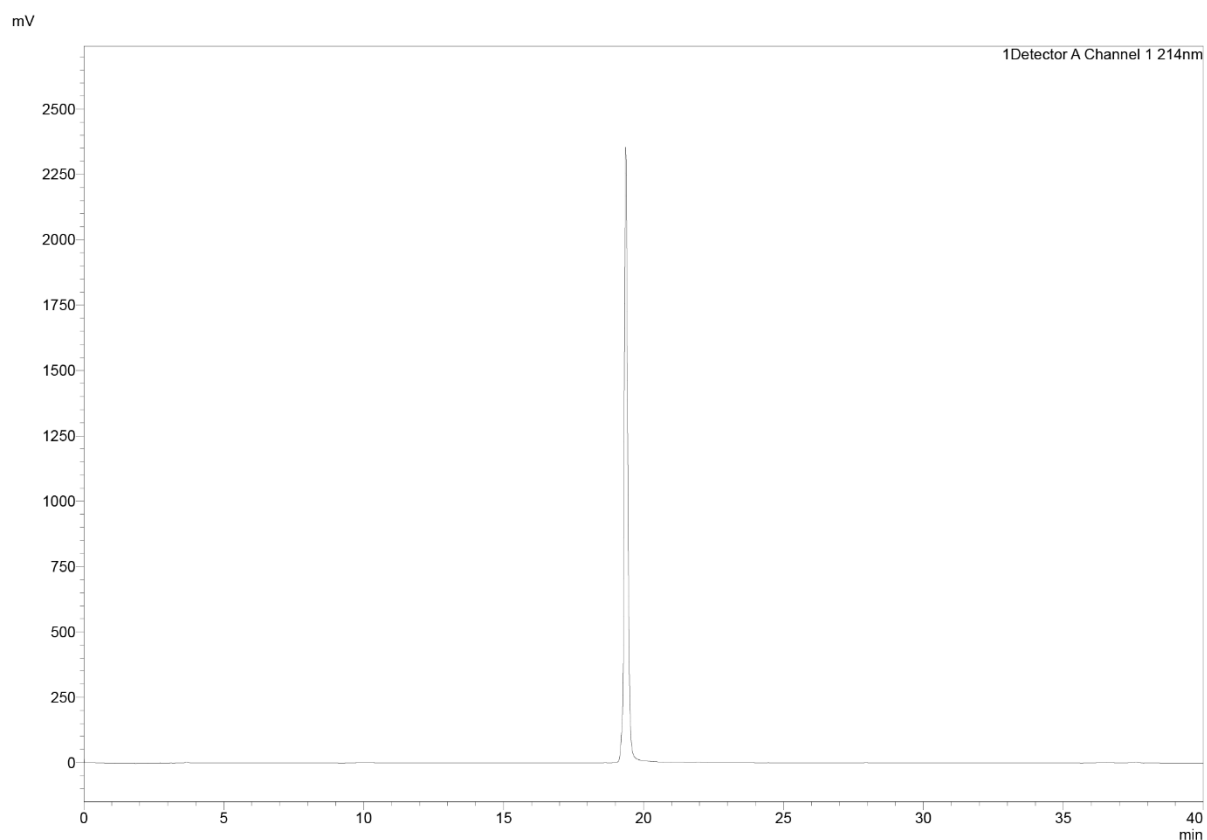


Figure S2. Analysis of the purity of Ac-Nle-SP. UV-trace of HPLC analysis of Ac-Nle-SP using a C18 column with a 5-95% gradient of MeCN in H₂O over 28 minutes (2-30 min).

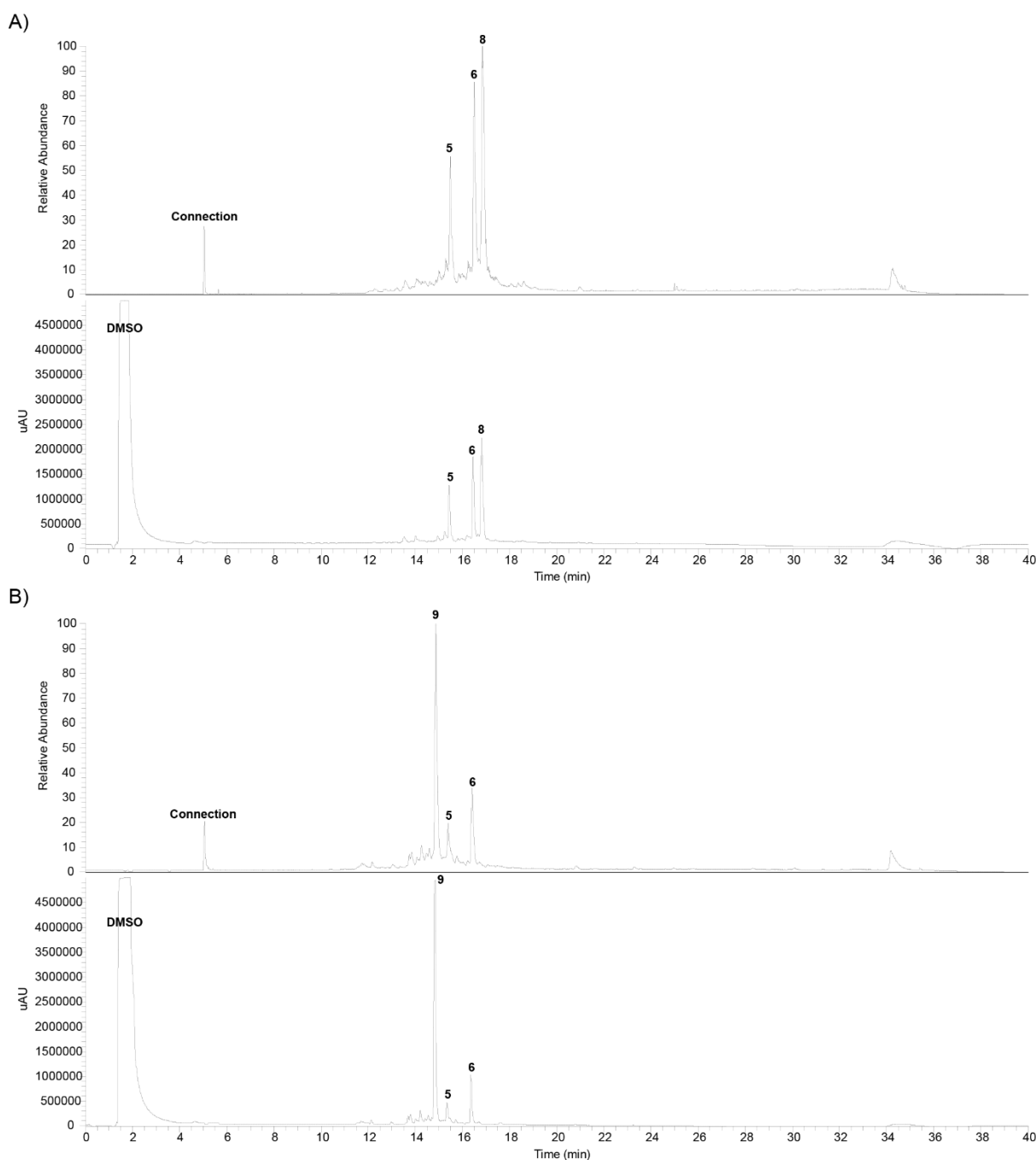


Figure S3. Coupling of probe 1 to Ac-Nle-SP and Gly. 1 μ M Ac-Nle-SP (**A**) or 1 mg/mL Gly (**B**) was incubated with 2.5 μ M probe 1 for 1 h at RT in PBS (pH 8.2), the mixture was then diluted with DMSO (1:1, v/v) and analysed with LC-MS using a C18 column with a 5-95% gradient of MeCN in H₂O over 28 minutes (2-30 min). The UV-traces (**bottom panels**) and mass-traces (**top panels**) are depicted and reveal the presence of three compounds for each mixture: the desired adducts **8** and **9**, hydrolysed probe **5** and intermolecularly cyclised probe **6**. No free Ac-Nle-SP or Gly was observed. To prevent DMSO from getting into the MS, the MS was only connected after 5 min, this connection resulted in the appearance of a peak in mass trace. For the structures of **5**, **8** and **9** see main article, for **6** see **Figure S1**.

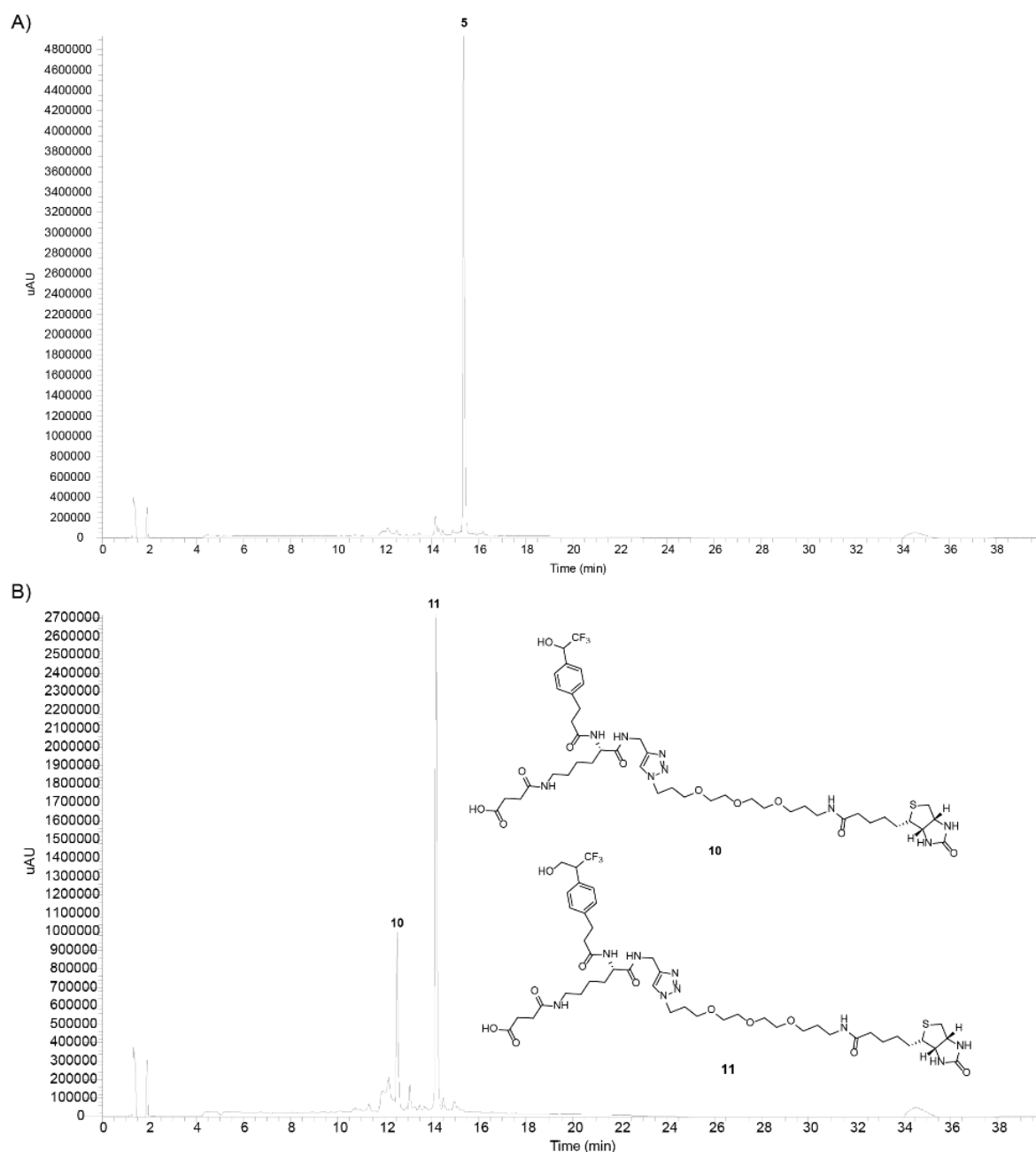


Figure S4. UV-activation of probe 1. Since probe **1** was obtained as a mixture (Figure S1), compound **5** was used to test the UV-activation of the diazirine moiety on the probe. Upon activation with UV, compound **5** formed adducts with H₂O (**10**) and MeOH (**11**). The free acid derivative of the probe (**5**) was dissolved in MeOH (1 mg/mL) and either kept in the dark (**A**) or exposed to UV-light of 365 nm for 15 min (**B**). Samples were then analysed by LC-MS using a C18 column with a 5-95% gradient of MeCN in H₂O over 28 minutes (2-30 min). The UV-traces are depicted. For the structure **5** see main article.