Development of a Cannabinoid-Based Photoaffinity Probe to Determine the $\Delta^{8/9}$-Tetrahydrocannabinol Protein Interaction Landscape in Neuroblastoma Cells

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Abstract
Introduction: $\Delta^9$-Tetrahydrocannabinol (THC), the principle psychoactive ingredient in Cannabis, is widely used for its therapeutic effects in a large variety of diseases, but it also has numerous neurological side effects. The cannabinoid receptors (CBRs) are responsible to a large extent for these, but not all biological responses are mediated via the CBRs.

Objectives: The identification of additional target proteins of THC to enable a better understanding of the (adverse) physiological effects of THC.

Methods: In this study, a chemical proteomics approach using a two-step photoaffinity probe is applied to identify potential proteins that may interact with THC.

Results: Photoaffinity probe 1, containing a diazirine as a photocrosslinker, and a terminal alkyne as a ligation handle, was synthesized in 14 steps. It demonstrated high affinity for both CBRs. Subsequently, two-step photoaffinity labeling in neuroblastoma cells led to identification of four potential novel protein targets of THC. The identification of these putative protein hits is a first step towards a better understanding of the protein interaction profile of THC, which could ultimately lead to the development of novel therapeutics based on THC.

Keywords: photoaffinity labeling; chemical proteomics; tetrahydrocannabinol; cannabinoid receptors; protein targets

Introduction
Preparations of the plant Cannabis sativa have been used throughout history in various cultures as medicinal concoctions or therapeutics, as well as for recreational or religious purposes.1 In 1930, the isolation of cannabino1 and cannabidiol as the first active substituents was achieved,2 which was followed by the discovery of $\Delta^9$-tetrahydrocannabinol (THC) in 1964.3 THC is the psychoactive constituent of marijuana and exists in two isomers: namely $\Delta^9$-THC and $\Delta^8$-THC, of which the latter is the most thermodynamically stable isomer.4 THC treatment has been associated with therapeutic effects, such as analgesia, relaxation and fatigue, appetite stimulation,5 antiemesis,6 and reduction of nausea.5 THC is used by patients suffering from multiple sclerosis (MS),7 cancer, or AIDS.8 In addition, preclinical data of THC indicate beneficial effects in several animal models of Alzheimer’s,9 Parkinson’s,10 and Huntington’s disease.11 However, THC is also associated with many undesirable side effects, including induction of psychoactivity, anxiety, memory loss, cardiac arrhythmias, and addiction.12

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Both Δ⁹-THC and Δ⁸-THC have similar affinity to the cannabinoid receptor type 1 (CB₁R) and type 2 (CB₂R). The CB₁R is the most abundant G protein-coupled receptor (GPCR) in the mammalian brain, whereas the CB₂R is predominantly present in peripheral tissues and cells of the immune system. Most of the physiological effects of THC are mediated via the CB₁R and CB₂R as demonstrated by the use of specific CB receptor antagonists or genetically modified mice that lack the CB receptors.

It is, however, hypothesized that THC may have other non-CB receptor targets. A study, using CB₁R and CB₂R knockout mice, showed similar analgesia upon THC administration compared with the equivalent wild-type mice in the tail-flick test. This effect was not observed in the hotplate test, which requires spinal processing of nociceptive information. These observations suggest the existence of another protein target in the brain. Previously, orphan GPCRs GPR55 and GPR18 and peroxisome proliferator-activated receptor gamma were identified to bind to THC, but it is unclear whether these targets are responsible for some of the physiological effects of THC. Therefore, a more complete view of the protein interaction of THC in neuronal cells is desirable.

Photoaffinity-based protein profiling (pAβBPP) has been previously used to map the protein interaction landscape of small molecules. Photoaffinity probes use a light-responsive element to covalently crosslink the compound with its target protein upon irradiation. To circumvent the problems associated with large reporter groups, photoaffinity probes with a bioorthogonal ligation handle (e.g., alkyne), to introduce a fluorescent or affinity tag (e.g., biotin) after crosslinking to a protein, have emerged as powerful tools to visualize small molecule-protein interactions in living systems. Previously, we applied two-step pAβBPP to capture and visualize the CB₂R on human cells. Here, it was envisioned that two-step pAβBPP could be used to map the THC interaction landscape in neuroblastoma cells.

To this end, photoaffinity probe 1 (Fig. 1), a Δ⁸-THC analog carrying a diazirine as the photoreactive moiety and a terminal alkyne as the ligation handle, was developed. Probe 1 was synthesized in 14 steps and was found to have high affinity for both cannabinoid receptors (CBRs). The protein interaction landscape of THC was mapped in Neuro2A cells (a fast-growing neuroblastoma cell line with several neuronal properties), in which four putative novel targets of THC were identified.

**Materials and Methods**

**Chemistry**

**General remarks.** All reactions were performed using air- or flame-dried glassware. Solvents were purchased from Sigma-Aldrich, and dry solvents were analytically dried by storing them for 24 h on activated molecular sieves. Use of dry solvents is mentioned explicitly. Reagents were purchased from Sigma-Aldrich, Acros Organics, and Merck and used without further purification. All moisture sensitive reactions performed under an Ar atmosphere are mentioned explicitly.

¹H and ¹³C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AV 400 MHz spectrometer at 400 and 100 MHz, respectively, using CDCl₃ or CD₃OD as solvent, unless stated otherwise. Chemical shift values are reported in ppm with TMS or solvent resonance as the internal standard (CDCl₃/TMS, δ 0.00 for ¹H [TMS], δ 77.16 for ¹³C [CDCl₃]; CD₃OD, δ 3.31 for ¹H, δ 49.00 for ¹³C). Data are reported as follows: chemical shifts (δ) in ppm, multiplicity (s = singlet, d = doublet, dd = doublet of doublet, ddd = doublet of doublet of doublet, dt = doublet of triplet, t = triplet, td = triplet of doublet, q = quartet, br s = broad singlet, and m = multiplet), coupling constants J (Hz), and integration.

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**FIG. 1.** Design of photoaffinity probe 1. The photoreactive diazirine is highlighted in red, the alkyne ligation handle in blue.
indicating full reduction of residual CrO₃. The reaction was treated with additional 5 min, until all yellow color had disappeared, iPrOH was added (5 mL) and the reaction stirred at room temperature (Fig. 2). The reaction was allowed to stir for 16 h at rt. The reaction was quenched with sat. NaHCO₃ (200 mL) and transferred to a separating funnel. The pH of the aqueous layer was adjusted to pH 7 with 1 M HCl solution and subsequently extracted with DCM (2 × 200 mL) and with EtOAc (200 mL). The combined organic layers were dried over MgSO₄ and concentrated. The resulting brown syrup was dissolved in tBuOMe (20 mL), cooled in an ice bath, and ice-cold hexane (200 mL) was added. The slurry was filtered and the solids washed generously with ice-cold hexane (100 mL) to give 5 (4.56 g, 21 mmol, 99%) as off-white flaky crystals. Rₜ 0.5 (40% EtOAc/pentane).¹H NMR (400 MHz, MeOD) δ 6.47 (d, J = 2.1 Hz, 2H), 6.15 (t, J = 2.1 Hz, 1H), 5.50 (s, 1H), 3.49–3.42 (m, 2H), and 3.33–3.27 (m, 2H).

5-(1,3-Dithiolan-2-yl)-2-((1R,2S,5S)-4,6,6-trimethyl bicyclo[3.1.1]hept-3-en-2-yl)benzene-1,3-diol (6): A 500 mL round bottom flask was charged with a magnetic stirring bar, dithiolane 5 (2.2 g, 10.3 mmol, 1 eq), and purged with Ar. Dry CHCl₃ (90 mL) was added, along with anhydrous campshorsulfonic acid (0.26 g, 1.03 mmol, 0.1 eq), and the flask purged with Ar again. (S)-cis-Verbenol (1.73 g, 11.35 mmol, 1.1 eq, 50% ee) in dry CHCl₃ (10 mL) was added dropwise, and the reaction allowed to stir at rt for 3 h. Upon completion, the reaction was quenched with an aqueous solution of 1:4 (v/v) sat. NaHCO₃/brine (100 mL), and transferred to a separating funnel. The pH of the aqueous layer was adjusted to pH 7 with 1 M HCl solution, and subsequently extracted with CHCl₃ (2 × 120 mL) and with EtOAc (120 mL). The combined organic layer was dried over MgSO₄ and concentrated. After concentration, the crude residue (~4 g) was purified by flash column chromatography (150 g silica, eluting with 10% EtOAc/pentane (8 CV) to give 6 (2.17 g, 6.24 mmol, 60%) as a viscous yellow oil, which forms a foamy amorphous white solid under reduced pressure at rt. Rₜ 0.65 (20% EtOAc/pentane).¹H NMR (400 MHz, CDCl₃) δ...
6.53 (s, 2H), 5.68 (d, J = 1.3 Hz, 1H), 5.48 (s, 1H), 3.92 (dd, J = 5.0, 2.5 Hz, 1H), 3.46–3.43 (m, 2H), 3.33–3.30 (m, 2H), 2.33–2.23 (m, 2H), 2.19–2.16 (m, 1H), 1.85 (s, 3H), 1.50–1.46 (m, 1H), 1.32 (s, 3H), and 0.95 (s, 3H).13CN M R (100 MHz, CDCl3) d 153.2, 140.5, 116.3, 115.0, 55.8, 48.0, 47.1, 40.9, 40.2, 38.1, 28.0, 26.1, 23.9, and 20.6. LC-MS (ESI+) m/z: calculated for C19H25O2S2 [M + H]+: 349.13, found 349.07.

**FIG. 2.** Synthesis of probe 1. Reagents and conditions: (a) BH3Me2S, B(OMe)3, THF, rt, 16 h, 99%; (b) CrO3, H2SO4, Acetone, 0°C, 10 min, 86%; (c) 1,2-ethanediithiol, BF3 Et2O, THF, rt, 16 h, 99%; (d) (S)-cis-verbenol, CSA, CHCl3, rt, 2 h, 60%; (e) BF3 Et2O, DCM, 0°C–rt, 1.5 h, 62%; (f) AgNO3, EtOH/H2O (10:1), rt, 18 h; (g) TBSi, imidazole, DCM, rt, 3 h, 87% (yield over two steps); (h) LiBH4, THF, rt, 30 min, 99%; (i) CBr4, PPh3, DCM, rt, 1 h, 98%; (j) Thiourea, EtOH, 40°C, 1 h; (k) 1 M NaOH (aq.), EtOH, rt, 1 h, 80% (two steps); (l) LDA, THF, –40°C, 30 min; then propargyl bromide, 0°C, 1 h, 76%; (m) ethylene glycol, TsOH, PhMe, reflux in Dean-Stark apparatus, 3 h; (n) LiAlH4, THF, 0°C, 1 h, 92% (two steps); (o) TsOH, 19:1 acetone/H2O, 50°C, 2 h, 98%; (p) NH3 (l), reflux, 5 h; then NH3SO3H in MeOH, rt, 1 h; (q) I2, Et2N, DCM, 0°C, 83% (two steps); (r) I2, PPh3, imidazole DCM, rt, 1 h, 90%; (s) K2CO3, 2:1 THF/DMF, 30°C, 22 h; (t) TBAF, THF, 0°C, 15 min, 84% (two steps). DMF, dimethylformamide; rt, room temperature; THF, tetrahydrofuran.
over 5 min, upon which the reaction was allowed to warm to rt, and was stirred for 1.5 h. Upon completion, the reaction was quenched with an aqueous solution of 1:4 (v/v) sat. NaHCO₃/brine (450 mL) and was transferred to a separating funnel. The pH of the aqueous layer was adjusted to pH 7 with 1 M HCl aq. solution, and subsequently extracted with CHCl₃ (2 × 450 mL). The combined organic layer was dried over MgSO₄, and concentrated. After concentration, the crude residue (~ 6 g) was purified by flash column chromatography (225 g silica), eluting first with 6% EtOAc/pentane (6 CV), then 8% EtOAc/pentane (8 CV) to give 7 (3.39 g, 10.3 mmol, 62%) as a viscous dark yellow oil, which forms a foamy amorphous yellow solid under reduced pressure at rt. Rᵋ: 0.55 (1% TFA/DCM).³¹H NMR (400 MHz, CDCl₃) δ 6.56 (s, 1H), 6.48 (d, J = 1.1 Hz, 1H), 5.48 (s, 1H), 5.41 (d, J = 3.8 Hz, 1H), 5.26 (s, 1H), 3.49–3.37 (m, 2H), 3.35–3.25 (m, 2H), 3.19 (dd, J = 10.8, 4.6 Hz, 1H), 4.53 (d, J = 10.9 Hz, 3H), 1.69 (s, 3H), 1.37 (s, 3H), and 1.09 (s, 3H). ³¹C NMR (100 MHz, CDCl₃) δ 155.3, 155.0, 140.2, 134.8, 119.4, 113.2, 109.9, 106.7, 77.13, 55.8, 44.8, 40.2, 40.1, 35.8, 31.80, 28.0, 27.6, 23.6, and 18.7. LC-MS (ESI+) m/z: calculated for C₁₉H₂₃O₂S₂ [M + H]+ 349.13, found 349.07.

(6A,R,10aR)-1-(((tert-Butyldimethylsilyl)oxy)-6,6,9-trimethyl-6a,7,10,10a-tetrahydro-6H-benzo[c]chromene-3-yl)methanol (9): A flame-fried 10 mL round bottom flask was charged with a magnetic stirring bar, aldehyde 8 (193 mg, 0.5 mmol, 1 eq), and the flask was purged with Ar. Dry THF was added (2 mL) and the flask cooled to 0°C in an ice water bath. 2 M LiBH₄ in THF (0.375 mL, 0.75 mmol, 1.5 eq) was added drop-wise, upon which the reaction was allowed to warm to rt, and was stirred for 30 min. Upon completion, the reaction was quenched with H₂O (50 mL) and transferred to a separating funnel. The aqueous layer was extracted with Et₂O (3 × 40 mL), and the combined organic layer was dried over MgSO₄ and concentrated. After concentration, the residue (~ 200 mg) was filtered through a short pad of silica (5 g), eluting with CHCl₃, to give primary alcohol 9 (192 mg, 495 μmol, 99%) as a turbid, colorless syrup. Rᵋ: 0.4 (CHCl₃). ¹H NMR (400 MHz, CDCl₃) δ 6.46 (d, J = 1.2 Hz, 1H), 6.39 (d, J = 1.2 Hz, 1H), 5.41 (d, J = 3.6 Hz, 1H), 4.53 (s, 2H), 3.30–3.14 (m, 1H), 2.59 (td, J = 10.8, 4.2 Hz, 1H), 2.26–2.02 (m, 1H), 1.91–1.57 (m, 4H), 1.68 (s, 3H), 1.37 (s, 3H), 1.07 (s, 3H), 1.00 (s, 9H), 0.27 (s, 3H), and 0.15 (s, 3H). ¹³C NMR (100 MHz, CDCl₃) δ 155.5, 155.1, 140.4, 135.0, 119.4, 116.7, 109.9, 109.4, 76.9, 65.3, 45.4, 36.1, 32.5, 28.2, 27.5, 26.1, 23.5, 18.4, –3.4, and –4.2.

(6A,R,10aR)-3-(Bromomethyl)-6,6,9-trimethyl-6a,7,10,10a-tetrahydro-6H-benzo[c]chromene-1-yl)oxy (tert-butyl)dimethylsilyl (10): A 10 mL round bottom flask was charged with a magnetic stirring bar, 9 (192 mg, 495 μmol, 1 eq), DCM (2.5 mL), and CBr₄
(172 mg, 519 μmol, 1.05 eq). The flask was cooled to 0°C in an ice-water bath and, PPh₃ (136 mg, 0.519 mmol, 1.05 eq) was added. The reaction was purged with Ar and allowed to come to rt and stirred for 1 h. Upon completion, the reaction was concentrated under reduced pressure, and hexane (1 mL) was added. The resulting slurry was purified by flash column chromatography (15 g silica), eluting first with pentane (6 CV), then with 25% Et₂O/pentane (6 CV) to give 10 (218 mg, 483 μmol, 98%) as a clear, viscous oil. Rf 0.35 (pentane).

H NMR (400 MHz, CDCl₃) δ 6.48 (d, J = 1.6 Hz, 1H), 6.40 (d, J = 1.5 Hz, 1H), 5.41 (d, J = 2.5 Hz, 1H), 4.41–4.27 (m, 2H), 3.22 (dd, J = 16.6, 3.6 Hz, 1H), 2.58 (td, J = 10.8, 4.2 Hz, 1H), 2.20–2.05 (m, 1H), 1.84–1.72 (m, 3H), 1.63 (s, 3H), 1.37 (s, 3H), 1.04 (s, 3H), 1.00 (s, 9H), 0.28 (s, 3H), and 0.15 (s, 3H). 13C NMR (100 MHz, CDCl₃) δ 155.2, 155.0, 136.7, 135.0, 119.4, 117.8, 112.1, 111.6, 76.9, 45.3, 36.0, 32.5, 28.2, 27.6, 26.1, 23.5, 18.5, –3.4, and –4.2.

((6aR,10aR)-1-((tert-Butyldimethylsilyl)oxy)-6,6,9-trimethyl-6a,7,10,10a-tetrahydro-6H-benzo[c]chromen-3-yl)methanethiol (11): A 10 mL round bottom flask was charged with a magnetic stirring bar, bromide 10 (31 mg, 68 μmol, 1 eq), and EtOH (1.3 mL). Thiourea (10 mg, 134 μmol, 2 eq) was added, the reaction heated to 40°C in a warm water bath and stirred for 1 h. Upon completion, the reaction was cooled, dry N₂ gas was bubbled through the reaction for 5 min, and subsequently 1 M NaOH (0.2 mL) was added, and the reaction was stirred another 1 h. Upon completion, the reaction was quenched with 0.1 M HCl (10 mL) and transferred to a separating funnel. The aqueous layer was extracted with Et₂O (20 mL), and the organic layer was washed with sat. NaHCO₃ (10 mL), H₂O (10 mL), brine (10 mL), dried over MgSO₄, and concentrated. After concentration, the crude residue (~25 mg) was purified by flash column chromatography (2 g silica), eluting first with pentane (4 CV), then 5% CHCl₃/ pentane (8 CV) to give 11 (22 mg, 54 μmol, 80% over two steps) as a turbid, viscous oil. Rf 0.5 (5% CHCl₃/ pentane). H NMR (400 MHz, CDCl₃) δ 6.41 (d, J = 1.7 Hz, 1H), 6.35 (d, J = 1.7 Hz, 1H), 5.41 (d, J = 3.8 Hz, 1H), 3.66–3.53 (m, 2H), 3.22 (dd, J = 16.6, 4.3 Hz, 1H), 2.57 (td, J = 10.9, 4.3 Hz, 1H), 2.23–2.07 (m, 1H), 1.92–1.68 (m, 3H), 1.68 (s, 3H), 1.36 (s, 3H), 1.07 (s, 3H), 1.00 (s, 9H), 0.27 (s, 3H), and 0.15 (s, 3H). 13C NMR (100 MHz, CDCl₃) δ 155.02, 140.3, 135.0, 119.4, 116.2, 111.2, 110.5, 76.9, 45.4, 36.1, 32.4, 28.9, 28.2, 27.6, 26.1, 23.5, 18.5, –3.4, and –4.2.

Ethyl 3-oxohept-6-ynoate (13): A flame-dried Schlenk tube was charged with a stirring bar and purged multiple times with Ar. Dry THF (30 mL) and then freshly distilled diisopropylamine (9.71 mL, 69.28 mmol) were added, and the solution cooled to −78°C. 1.6 M nBuLi in hexanes (39.38 mL, 63 mmol) was added dropwise, and stirred for 15 min. The generated LDA solution (0.8 M by titration, 73 mL, 2.12 eq) was transferred via cannula to a flame-dried 250 mL round bottom flask. The flask was cooled to −40°C, upon which ethyl acetoacetate 12 (3.47 mL, 27.5 mmol, 1 eq) in dry THF (25 mL) was added dropwise. The reaction was stirred for 30 min, upon which propargyl bromide (80% in toluene, 3 mL, 28 mmol, 1.01 eq) was added dropwise, and the reaction was allowed to warm to 0°C and stirred for 1 h. Upon completion, the reaction was quenched with 0.5 M HCl (200 mL) and transferred to a separating funnel. The aqueous layer was extracted with Et₂O (2 × 200 mL). The combined organic layers were washed with brine (100 mL), dried over MgSO₄, and concentrated. The resulting amber syrup (4.80 g) was purified by fractional distillation (118°C, 15 mbar) to give 13 (3.49 g, 20.8 mmol, 76%) as a clear oil. Rf 0.4 (50% CHCl₃/hexane). H NMR (400 MHz, CDCl₃) δ 4.20 (q, J = 7.1 Hz, 2H), 3.47 (s, 2H), 2.82 (t, J = 7.2 Hz, 2H), 2.53–2.43 (m, 2H), 1.97 (t, J = 2.7 Hz, 1H), and 1.29 (t, J = 7.1 Hz, 3H). 13C NMR (100 MHz, CDCl₃) δ 200.7, 167.0, 90.2, 82.6, 69.1, 61.6, 49.3, 41.7, 14.2, and 12.9.

2-(2-(But-3-yn-1-yl)-1,3-dioxolan-2-yl)ethan-1-ol (14): A 50 mL round bottom flask was charged with a magnetic stirring bar, equipped with a Dean-Stark apparatus, and purged with Ar. Thirteen (383 mg, 2.27 mmol, 1 eq) in toluene (35 mL) was added, along with ethylene glycol (211 mg, 1.5 eq), followed by para-toluene sulfonic acid (39 mg, 0.23 mmol, 0.1 eq), and the reaction heated to reflux for 3 h. Upon completion, the reaction was quenched with sat. NaHCO₃ (25 mL), diluted with EtOAc (25 mL), and transferred to a separating funnel. The organic layer was washed with H₂O (50 mL), brine (50 mL), dried over MgSO₄, and concentrated. The crude ester was dissolved in dry THF (10 mL) and added dropwise to a flame-dried 25 mL round bottom flask previously cooled to 0°C, purged with Ar, and containing LiAlH₄ (150 mg, 3.97 mmol, 1.75 eq) suspended in dry THF (10 mL). Upon addition, the reaction was allowed to warm to rt and stirred for 1 h. Upon completion, the reaction was quenched with 10 mL EtOAc, stirred for 5 min,
and aq. 10 wt.% Rochelle’s salt (50 mL) was added and stirred for an additional 10 min. The reaction was transferred to a separating funnel, upon which the organic layer was washed with brine (30 mL), dried over MgSO4, and concentrated. After concentration, the crude residue was filtered through a short pad of silica (~10 g), eluting with CHCl3, to give 14 (357 mg, 2.09 mmol, 92% over two steps), as a pale yellow oil. Rf: 0.25 (pentane). 1H NMR (400 MHz, CDCl3) δ 4.00 (m, 4H), 3.75 (t, J = 5.7 Hz, 2H), 2.73 (s, 1H), 2.42–2.21 (m, 2H), and 2.05–1.79 (m, 5H). 13C NMR (100 MHz, CDCl3) δ 111.0, 84.0, 68.3, 65.0, 58.6, 38.4, 36.0, and 13.1.

1-Hydroxyhept-6-yn-3-one (15): A 25 mL round bottom flask was charged with a magnetic stirring bar, and 14 (357 mg, 2.09 mmol, 1 eq) in acetone (9.5 mL) was added, followed by para-toluenesulfonic acid (99 mg, 0.52 mmol, 0.25 eq), and millipore H2O (0.5 mL), and the reaction was heated to 50°C for 2 h. Upon completion, the reaction was quenched with sat. NaHCO3 (10 mL), diluted with EtOAc (30 mL), and transferred to a separating funnel. The organic layer was washed with brine (20 mL), dried over MgSO4, and concentrated. After concentration, the crude residue was filtered through a short pad of silica (~15 g), eluting with CHCl3, to give 15 (258 mg, 2.05 mmol, 98%) as a pale yellow oil. Rf: 0.25 (CHCl3). 1H NMR (400 MHz, CDCl3) δ 3.87 (t, J = 5.5 Hz, 2H), 2.71 (dd, J = 9.1, 5.5 Hz, 4H), 2.55–2.40 (m, 3H), and 1.97 (t, J = 2.5 Hz, 1H). 13C NMR (100 MHz, CDCl3) δ 209.1, 82.9, 69.0, 57.8, 44.7, 41.9, and 12.9.

3-(But-3-yn-1-yl)-3(2-iodoethyl)-3H-diazirine (17): A 25 mL amber flask was charged with a magnetic stirring bar, and 16 (234 mg, 1.69 mmol, 1 eq). DCM (7.5 mL) was added. The flask was cooled to 0°C in an ice bath, and imidazole (345 mg, 5.07 mmol, 3 eq), was added, followed by I2 (515 mg, 2.03 mmol, 1.2 eq) and PPh3 (488 mg, 1.86 mmol, 1.1 eq). The reaction was purged with Ar, allowed to come to rt, and stirred for 1 h. Upon completion, the reaction was quenched with aq. 10 wt.% Na2S2O3 (10 mL) and transferred to a separating funnel. The aqueous layer was extracted with CHCl3 (3 × 20 mL). The combined organic layer was dried over MgSO4 and concentrated. After concentration, the crude residue (~500 mg) was purified by flash column chromatography (25 g silica), eluting with pentane (6 CV), then 5% EtOAc/pentane (6 CV) to give 17 (378 mg, 1.52 mmol, 90%) as a clear oil. Rf: 0.25 (pentane). 1H NMR (400 MHz, CDCl3) δ 2.90 (t, J = 7.6 Hz, 2H), 2.13 (t, J = 7.6 Hz, 2H), 2.07–1.96 (m, 3H), and 1.69 (t, J = 7.1 Hz, 2H). 13C NMR (100 MHz, CDCl3) δ 82.6, 77.4, 69.6, 37.7, 32.0, 28.8, 13.4, and –3.9.

(6aR,10aR)-3-(((2-(3-(But-3-yn-1-yl)-3H-diazirin-3-yl)ethyl)thio)methyl)-6,6,9-trimethyl-6a,7,10,10a-tetrahydro-6H-benzoc[6]chromen-1-ol (1): A 10 mL round bottom flask was charged with a magnetic stirring bar, tricyclic probe precursor 11 (20 mg, 49 μmol, 1 eq), and THF (0.5 mL), and the reaction cooled to 0°C in an ice water bath. Minimalist linker 17 (19 mg, 75 μmol, 1.53 eq) in THF (0.5 mL) was added, followed by anhydrous K2CO3 (13.5 mg,
98 μmol, 2 eq), and DMF (0.5 mL). The reaction was purged with Ar again, allowed to come to rt, then warmed to 30°C, and stirred for 22 h. Upon completion, H2O (10 mL) was added, and the reaction was transferred to a separating funnel. The aqueous layer was extracted with CHCl3 (2 × 10 mL), and the combined organic layer was washed with brine (10 mL), dried over MgSO4, and concentrated. The crude silyl ether was subsequently dissolved in THF (0.5 mL) and transferred to a 10 mL round bottom flask, purged with Ar, and cooled to 0°C in an ice water bath. 1 M TBAF in THF (98 μL, 98 μmol, 2 eq) was added, and the reaction stirred for 15 min at 0°C. Upon completion, the reaction was quenched with H2O (10 mL), and transferred to a separating funnel. The aqueous layer was extracted with Et2O (10 mL) and the organic layer was washed with brine (10 mL), dried over MgSO4, and concentrated. After concentration, the crude residue (~ 25 mg) was purified by flash column chromatography (2g silica), eluting first with 20% CHCl3/pentane (4 CV), 40% CHCl3/pentane (4 CV), and then 50% CHCl3/pentane (4 CV) to give probe 1 (17 mg, 41 μmol, 84% over two steps) as a clear, viscous oil. Rf 0.3 (50% CHCl3/pentane). 1H NMR (400 MHz, CDCl3) δ 6.34 (d, J = 1.2 Hz, 1H), 6.26 (s, J = 1.6 Hz, 1H), 5.43 (d, J = 3.8 Hz, 1H), 4.87 (s, 1H), 3.52 (s, 2H), 3.19 (dd, J = 16.0, 4.0 Hz, 1H), 2.70 (td, J = 10.7, 4.5 Hz, 1H), 2.25 (m, 3H), 1.99 (m, 3H), 1.81 (t, J = 8.9 Hz, 3H), 1.70 (s, 3H), 1.62 (dd, J = 14.7, 7.5 Hz, 5H), 1.38 (s, 3H), and 1.10 (s, 3H). 13C NMR (100 MHz, CDCl3) δ 155.4, 155.2, 137.7, 134.8, 119.5, 111.0, 107.7, 77.1, 69.4, 44.9, 36.1, 36.0, 33.0, 32.30, 31.8, 29.9, 28.0, 27.7, 25.7, 23.6, 18.6, and 13.4. LC-MS purity found >95%. High resolution mass spectrometry (HRMS) (ESI+) m/z calculated for C24H31N2O2S [M + H]+: 411.2101, found 411.2100.

Biology

General remarks. All common reagents were purchased from commercial sources and used as received. Probe 1 was synthesized as described above, Δ9-THC, Δ8-THC and CY5-N3 were synthesized according to previously published procedures29,30 and biotin-N3 was purchased from Bio-Connect Life Sciences. [3H]CP55940 (specific activity 141.2 Ci/mmol) and GF-B/GF-C filters were purchased from Perkin Elmer (Waltham, MA). The CHO-K1 CNR1 and CNR2 cell lines (catalog numbers 93-0959C2 and 93-0706C2, respectively) were obtained from DiscoveRx. Cell culture plates were purchased from Sarstedt.

Cannabinoid receptor ligands CP55940 and AM630 were obtained from Sigma Aldrich (St. Louis, MO), and rimonabant was obtained from F. Hoffmann-La Roche Ltd. (Basel, Switzerland). Reagents used for the pulldown procedure are: avidin-agarose from egg white (50% glycerol suspension from Sigma Aldrich), 10× phosphate buffered saline (PBS) (proteomics grade, Sigma Aldrich) and Trypsin, sequencing grade (Promega). The CaproBox™ was kindly provided by Caprotec Bioanalytics GmbH, Berlin. All buffers and solutions were prepared using Millipore water (deionized using a MilliQ A10 Biocel™, with a 0.22 μm filter) and analytical grade reagents and solvents. Buffers are prepared at rt and stored at 4°C, unless stated otherwise.

Cell culture and membrane preparation. CHOK1hCB1_bgal and CHOK1hCB2_bgal (source; DiscoveRx, Fremont, CA) were cultured in Ham’s F12 Nutrient Mixture supplemented with 10% fetal calf serum, 1 mM glutamine, 50 μg/mL penicillin, 50 μg/mL streptomycin, 300 mg/mL hygromycin, and 800 μg/mL genetin in a humidified atmosphere at 37°C and 5% CO2. Cells were subcultured twice a week at a ratio of 1:20 on 10-cm ø plates by trypsinization. For membrane preparation, the cells were subcultured 1:10 and transferred to large 15 cm diameter plates. Next, the cells were detached by scraping them into 5 mL PBS and collected and centrifuged at 1000 g for 5 min. Pellets derived from 30 plates were added together and resuspended in 20 mL ice-cold buffer (50 mM Tris-HCl, 5 mM MgCl2, pH 7.4). An UltraThurrax homogenizer was used to homogenize the cell suspension. Membranes and the cytosolic fraction were separated by ultracentrifugation (100,000 g, with a Ti-70 rotor in a Beckham Coulter Ultracentrifuge) at 4°C for 20 min. The supernatant was discarded and the pellet was resuspended in 10 mL of the same buffer and the homogenization and centrifugation steps were repeated. Supernatant was discarded and the pellet was resuspended in 5 mL buffer. Aliquots of 200 μL were frozen at −80°C until further use. Protein concentration was determined using the BCA method.³¹

³¹[3H]CP55940 displacement assay. The affinity of probe 1 on CBRs was determined on membrane fractions of CB1R- or CB2R overexpressing CHO cells, as described previously.¹³ Membrane aliquots containing 5 μg (CHOK1hCB1_bgal) or 1 μg (CHOK1hCB2_bgal) of membrane protein in 100 μL assay buffer (50 mM Tris-
HCl, 5 mM MgCl₂, 0.1% BSA, pH 7.4) were incubated at 30°C for 1 h, in presence of 3.5 nM (CHO1hCB₂_bgal) or 1.5 nM [³H]CP55940 (CHO1hCB₂_bgal). Non-specific binding was determined in the presence of 10 μM SR141716A (CHO1hCB₂_bgal) or 10 μM AM630 (CHO1hCB₂_bgal). Incubation was terminated by rapid filtration performed on GF/C filters (Whatman International, Maidstone, United Kingdom), presoaked for 30 min with 0.25% polyethylene-imine (PEI), using a Brandel harvester (Brandel, Gaithersburg, MD). Filter-bound radioactivity was determined by scintillation spectrometry using a Tri-Carb 2900 TR liquid scintillation counter (Perkin Elmer, Boston, MA).

Data analysis
Graphs and statistics were performed with GraphPad Prism 7, using the results of three independent experiments performed in duplicate. The nonlinear regression analysis for one site—Fit Kᵢ (constrains: top = 100 and bottom = 0) was used to obtain logKᵢ values, which are provided by Prism by direct application of the Cheng–Prusoff equation

\[
Kᵢ = IC₅₀/(1 + ([L]/K_D)),
\]

where [L] is the exact concentration of [³H]CP55940 determined per experiment (i.e., ~3.5 or ~1.5 nM) and K_D = 0.10 (CB₁R) or 0.33 (CB₂R) nM of [³H]CP55940.

Two-step photoaffinity labeling, gel-based analysis
Wild type (WT)CHO, CB₁R, and CB₂R membrane aliquots were diluted to 2 μg/μL and homogenized for 20 sec with a Heidolph Silent crusher at 25,000 rpm, and benzonase was added (1:10,000 dilution from working stock of 2,500,000 U/mL, assay concentration: 250 U/mL). Eighteen microliters of protein was added per well of a 96-well flat bottom plate and 20 μM CP55940 or MilliQ water with the same% of dimethylsulfoxide (DMSO) was added, but the sample without UV was kept in an Eppendorf tube protected with aluminia foil. After incubation of 30 min at rt, 2 μM LEI121 or probe 1, or MilliQ water with the same% of DMSO was added, and the protein was again incubated for 30 min at rt. The samples were then diluted with 30 μL 50 mM Hepes buffer and irradiated for 5 min with CaproBox, preset at 350 nm and cooled during irradiation. The ligation reaction was then performed with 5 μL click master mix per sample (0.455 mM CuSO₄, 2.73 mM NaAsc, 0.09 mM THPTA, 3.6 μM Cy₅-N₃). The click mix is prepared as follows: 2.5 μL 10 mM CuSO₄ and 1.5 μL 100 mM NaAsc were mixed together until the copper is fully reduced (visible by the change from the rusty brown color to bright yellow), then 0.5 μL 10 mM THPTA and 0.5 μL 0.4 mM Cy₅-N₃ were added. After incubation in the dark for 1 h, the protein was denatured with 18 μL 4 × Laemmli sample buffer, and the samples were resolved on a 12.5% acrylamide gel (12 μL per sample per well). Bio-Rad ImageLab was used for gel analysis and quantification.

Chemoproteomic profiling of THC protein targets
Neuro2A cells were cultured at 37°C with 7% CO₂ in DMEM supplemented with 10% New Born Calf serum, 10% fetal calf serum, 1 mM glutamine, 50 μg/mL penicillin, and 50 μg/mL streptomycin and passaged twice a week. Cells were washed with PBS, then pretreated in PBS, containing 1 mM MgCl₂ and 1 mM CaCl₂, with or without 10 μM THC, for 30 min at 37°C. Then, 1 or 10 μM probe 1 (or the same amount of DMSO for the untreated control) was added (final concentration in a total volume of 3 mL) and incubated for 30 min at 37°C. The solution was removed from the cells and replaced by 1.5 mL PBS containing 1 mM MgCl₂ and 1 mM CaCl₂, then the plates were immediately irradiated (except the No UV control) with CaproBox (350 nm) for 5 min, and the cells were harvested by scraping.

The cells were pelleted (10 min, 1200 g, 4°C), supernatant removed, and resuspended in 250 μL 50 mM Hepes buffer. The cells were destroyed with the Heidolph Silent Crusher (20 sec, 25,000 rpm). Samples were sonicated for 10 × 2.5 sec with 0.5 sec interval (using a probe sonicator from Branson, Digital Sonifier) and 2 μL of 10% sodium dodecyl sulfate (SDS) was added. If samples were frozen at ~80°C before continuation of the experiment, the samples were sonicated again for 10 × 0.5 sec with 0.5 sec interval using a probe sonicator. The protein content was quantified using Bradford and the experiment was continued using the same amount of protein for each sample. Sample volumes were adjusted to 400 μL with 50 mM Hepes buffer, then the ligation reaction with biotin-N₃ was performed with 43.7 μL click mix per sample (1.25 mM CuSO₄, 7.5 mM NaAsc, 0.25 mM THPTA, 22.5 μM biotin-N₃) for 1 h at rt in the dark. For step-by-step preparation of the click reagents, mix 21.85 μL 25 mM CuSO₄, 13.15 μL 250 mM NaAsc, 4.37 μL 25 mM THPTA, and 4.37 μL 2.25 mM biotin-N₃ in this order.

To remove all click components, the protein was precipitated by the addition of 666 μL MeOH, 166 μL CHCl₃, and 300 μL MilliQ and centrifuged at 20,238 g.
for 10 min. The supernatant was removed and the pellet was resuspended in 600 μL MeOH using sonication (6 × 0.5 sec, interval 0.5 sec). The protein was pelleted at 20,238 g for 10 min and the supernatant removed. The protein was then denatured in 15 min at rt with 500 μL 1% SDS containing 25 mM NH4HCO3, followed by reduction (65°C, 15 min, 700 rpm shaking) using 5 μL 1 M DTT per sample. Samples were cooled to rt before alkylation with 40 μL 0.5 M IAA per sample for 30 min at rt in the dark. One hundred forty microliters of 10% SDS was added per sample, and each sample was added to 6 mL PBS containing 50 μL avidin beads (prewashed with PBS 3×, pelleting at 2000 g for 2 min), and incubated for 2 h at rt while rotating. Beads were pelleted (2000 g, 2 min) and washed with PBS with 0.5% SDS (1×) and with PBS (3×).

On-bead digest of peptides was performed overnight at 37°C, at 1000 rpm with digestion buffer (250 μL per sample, recipe: 300 μL 1 M Tris, 300 μL 1 M NaCl, 3 μL of 1 M CaCl2, 60 μL ACN, 3 μL 0.5 μg/μL Trypsin and 2334 μL MilliQ). Samples were quenched with 12.5 μL formic acid (FA) and beads were removed using a Biospin column (600 g, 2 min). Samples were added on C18 StageTips (conditioned with 50 μL MeOH, then 50 μL of 0.5% (v/v) FA in 80% (v/v) ACN/MilliQ (solution B), then 50 μL 0.5% (v/v) FA in MilliQ (solution A), each conditioning step was performed using centrifugation for 2 min at 600 g) by spinning for 15 min at 800 g, then washed with solution A for 10 min at 800 g, and eluted with solution B for 5 min at 800 g into low-binding Eppendorf tubes. Samples were evaporated using an Eppendorf SpeedVac (Eppendorf Concentrator Plus 5301) and 50 μL of LC/MS solution was added (recipe for 2 mL: 1900 μL MilliQ, 60 μL ACN, 2 μL FA, 40 μL of 1 nmol/μL yeast enolase stock). Samples were measured using a NanoACQUITY UPLC System coupled to a SYNAPT G2-Si high-definition mass spectrometer (Waters). The peptides were separated using an analytical column (HSS-T3 C18 1.8 μM, 75 μM × 250 mm, Waters) with a concave gradient (5–40% ACN in H2O with 0.1% FA). [Glu1]-fibrinopeptide B was used as lock mass. Mass spectra were acquired using the UDMA method. The mass range was set from 50 to 2000 Da with a scan time of 0.6 sec in positive, resolution mode. The collision energy was set to 4 V in the trap cell for low-energy MS mode. For the elevated energy scan, the transfer cell collision energy was ramped using drift time-specific collision energies.

Raw data were processed using Progenesis QI for Proteomics (3.0, Waters), with lock mass correction (7,858,426 Da) and a database search was performed against the proteomic database of *Mus musculus*, with trypsin as digestion reagent, max two missed cleavages, carbamidomethyl C as a fixed modification, oxidation M as a variable modification, and FDR set to 1%. Relative quantitation using Hi-3 was performed after filtering the peptides on score (cutoff 5).

Data analysis
The average normalized abundance of proteins in sample replicates of two independent experiments was used to calculate the ratio of proteins in the probe-treated sample and the “No UV” sample, to determine the level of enrichment by UV-irradiation (Fig. 5A). Protein targets that were enriched >2× by probe 1 are shown in Supplementary Table S1. Proteins that were <2-fold enriched and highly abundant (>20%) in the “CRAPome” database www.crapome.org/, version 1.1) were excluded from further analysis. Gene ontology data of the ~150 resulting putative probe targets (Fig. 5B, C) were derived using the DAVID Bioinformatics Database (https://david.ncifcrf.gov/home.jsp, version 6.8).

In THC competition experiments, the normalized abundance of proteins in sample replicates of three independent experiments was used to calculate the ratio of proteins in THC-pretreated samples over probe-treated samples. The average of the mean ratios of the triplicate samples of each independent experiment was used to calculate the effect of THC (as fold change), and a Student’s t-test was used to determine whether the fold change was significantly lower than 1, indicating a significant reduction of the abundance of that particular protein in the THC-treated samples (Fig. 5D). A p-value less than 0.05 was considered statistically significant. Proteins that showed <50% inhibition (Supplementary Table S2) were excluded from gene ontology analysis. This analysis yielded one putative protein target of Δ9-THC and three putative targets of Δ8-THC (Fig. 5A, B, [red dots] and E). Gene ontology and KEGG pathway analysis of the resulting putative protein targets was derived using the DAVID Bioinformatics software (https://david.ncifcrf.gov/home.jsp, version 6.8). In addition, it was investigated whether these proteins are associated with pathophysologies or diseases using the Online Mendelian Inheritance in Man (OMIM) database (www.omim.org/, September 2017).
Results and Discussion

Synthesis of photoaffinity probe 1

To identify the best position in THC to introduce the photoreactive group and the ligation tag, an analysis of previously reported structure-activity relationship data of THC analogs was conducted. This led to the design of probe 1, which contains a diazirine and ligation handle on the alkyl side chain of THC. An advantage of this design is the direct coupling of the bifunctional side chain as “minimalist linker.”

The synthesis of probe 1 commenced with reduction of commercially available 3,5-dihydroxybenzoic acid 2 to corresponding benzyl alcohol 3 in near-quantitative yield, using dimethyl sulfide complex of borane, along with co-reagent trimethoxyborate (Fig. 2). Benzyl alcohol 3 was oxidized to aldehyde 4 using a stoichiometric amount of Jones reagent, which prevented...
overoxidation to the benzoid acid. Protection of the aldehyde was performed under Lewis acidic conditions, which resulted in 1,3-dithiolane 5 in excellent yield. Electrophilic aromatic substitution of resorcinol derivative 5 under acidic conditions with the commercially available chiral monoterpene (S)-cis-verbenol yielded bicyclic intermediate 6 in moderate yield.

The tricyclic intermediate 7 was obtained in moderate yield by ring-closing rearrangement of bicyclic dithiolane 6, due to the generation of side products.
$\Delta^8$-THC was synthesized in two steps from olivetol and (S)-cis-verbenol using the same procedures, in a similar yield and comparable to literature.\textsuperscript{30} Intermediate 7 was deprotected by Ag(I) salts, using a AgNO\textsubscript{3}/wet EtOH system.\textsuperscript{40} Overoxidation of the resulting aldehyde to the equivalent benzoic acid was prevented using a modified workup, comprised additional washing steps with 10 wt.% Na\textsubscript{2}SO\textsubscript{3} (aq.), on top of the sole filtration step described in the literature.\textsuperscript{40} The resulting aldehyde was not isolated but subjected directly to phenol protection with TBS ether, to yield aldehyde 8 in excellent yield over 2 steps. Reduction of 8 to benzyl alcohol 9 with LiBH\textsubscript{4} proceeded with near-quantitative yield, and a subsequent Appel reaction afforded benzyl bromide 10 in excellent yield. Benzyl mercaptan 11 was obtained by substitution of the bromide by thiourea, followed by cleavage of the amidine moiety from the sulfur atom with NaOH (aq).

The synthesis of minimalist linker 17 started with the functionalization of commercially available ethyl acetoacetate 12 to propargyl ketoester 13 via generation of the dienolate under strongly basic conditions, followed by regiospecific electrophilic attack by propargyl bromide.\textsuperscript{37} Ketoester 13 was then protected with ethylene glycol to the corresponding ketal, with azeotropic removal of water under acidic conditions, followed by direct reduction of the ester group with LiAlH\textsubscript{4}, afforded corresponding alcohol 14 with excellent yield over 2 steps.\textsuperscript{41} Deprotection of ketal 14 afforded ketone 15 in a near-quantitative yield, which was next functionalized by refluxing in liquid NH\textsubscript{3}, followed by addition of hydroxylamine-O-sulfonic acid. The resulting crude diaziridine was subsequently oxidized to diazirine 16 using molecular iodine in mild basic conditions and was obtained in high yield over 2 steps. Sixteen then underwent a modified Appel reaction to generate minimalist linker 17 as alkyl iodide, in excellent yield.

Finally, minimalist linker 17 was coupled overnight at 30°C to resorcinol mercaptan 11 using K\textsubscript{2}CO\textsubscript{3} in a 2:1 THF/DMF solvent system and the crude sulfide underwent rapid TBS ether deprotection in the presence of TBAF, affording target probe 1 in high yield over two steps. Overall, probe 1 was synthesized from commercially available 3,5-dihydroxybenzoic acid 2 in 14 steps, with a total yield of 18%.

### CBR binding affinity of probe 1

To test the affinity of probe 1 on both the CB\textsubscript{1}R and CB\textsubscript{2}R, a $[^3H]$CP55940 displacement assay was used.
Probe 1 bound to the CB1R and CB2R with a pKi value of 8.5–0.1 and 8.0–0.4, respectively, which is similar as previously reported for Δ⁹-THC (pKi = 8.5–0.1 and 8.2–0.2, respectively) and Δ⁸-THC (pKi = 7.4–0.1 and 7.4–0.2, respectively). Two-step photoaffinity labeling of CB1R and CB2R
The ability of probe 1 to label CBRs in membranes of CB2R- or CB1R-overexpressing CHO cells was tested using a two-step photoaffinity labeling assay for gel-based imaging as previously reported. Probe 1 at a concentration of 2 μM, which is more than sufficient to fully occupy the binding site of the receptors, did not label either one of the CBRs (Fig. 4). Of note, positive control LEI121, a CB2R-selective photoaffinity probe previously reported, did show profound labeling of CB2R. This may indicate that the diazirine of probe 1, positioned on the “flexible” alkylic side chain, is not in close proximity to the amino acid residues in the binding site of CB1R or CB2R to form a covalent bond with the protein. This observation is in line with previous results showing that the position of the photoactive diazirine on the scaffold of CBR ligands is essential to capture the CBR.

Chemoproteomic profiling of THC protein targets using probe 1
The ability of probe 1 as a chemical tool to identify additional, non-CBR, protein targets of THC was evaluated next. Live Neuro2A cells (a fast-growing neuroblastome cell line with neuronal properties and therefore a suitable test system) were incubated with probe 1 (10 μM). Vehicle-treated and nonirradiated cells were used as control. Ligation with biotin-N₃ for affinity enrichment on avidin agarose beads enabled identification of nearly 800 proteins by mass spectrometry-based proteomics (Fig. 5A).

Nearly 200 proteins were more than twofold enriched by probe 1 compared to the untreated control, of which ~50 proteins were also found in the “CRAPome” database (Contaminant Repository for Affinity Purification). The CRAPome database constitutes a list of frequently identified proteins (e.g., ribosomal proteins or histones) in photoaffinity labeling experiments regardless of the type of probe. These CRAPome proteins can, therefore, be considered as false positives, suggesting that nearly 150 unique probe targets were identified. Gene ontology analysis revealed that protein targets of probe 1 are mostly located in the endoplasmic reticulum, mitochondria and membranes or in the cytoplasm (Fig. 5B). The proteins are mostly associated with energy metabolism and protein transport (Fig. 5C). Probe targets that were more than twofold enriched are shown in Supplementary Table S1.

To assess which of the probe targets also interact with THC, competition experiments with probe 1 (1 μM) and Δ⁹-THC (10 μM) or Δ⁸-THC (10 μM) were performed. This resulted in one putative protein target of Δ⁹-THC (Cox4i1) and three for Δ⁸-THC (Reep5, Mtxh2, Gnb1) (Fig. 5A, D [red dots]) for which the labeling of the protein by probe 1 was reduced by 40–70% (Fig. 5E, Supplementary Table S2). It should be noted that putative protein target Reep5 was enriched only 1.5-fold by probe 1, but is listed because it had the largest reduction after THC-pretreatment (69%–6%). The absence of complete inhibition by THC may be due to a low affinity to these proteins, because an inhibition between 40% and 70% indicates an IC₅₀ in the micromolar range. However, as this was measured in

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<th>Gene name</th>
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<th>OMIM database</th>
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<tbody>
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<td>Δ⁹-THC Reep5</td>
<td>Ras signaling pathway, Chemokine signaling pathway, PI3K-Akt signaling pathway, Circadian entrainment, Retrograde endocannabinoid signaling, Glutamatergic synapse, Cholinergic synapse, Serotonergic synapse, GABAergic synapse, Dopaminergic synapse, Phototransduction, Morphine addiction, Alcoholism, Pathways in cancer</td>
<td>Neuropathy, spastic paraplegia</td>
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<tr>
<td>Gnb1</td>
<td>Δ⁸-THC Cox4i1</td>
<td>Acute somatic leukemia</td>
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Inhibition data are the mean ± SEM (N = 3, n = 3). Putative protein targets were analyzed using the KEGG and OMIM database and were enriched ~ 2× or more after UV-irradiation.

OMIM, Online Mendelian Inheritance in Man.

Table 2. Putative THC Protein Targets with Hits in the KEGG and/or OMIM Database

<table>
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Soethoudt, et al.; Cannabis and Cannabinoid Research 2018, 3.1
http://online.liebertpub.com/doi/10.1089/can.2018.0003
presence of 1 μM probe, the actual pK₈ of THC for these proteins may be a bit higher.

Cox4i1 is involved in energy metabolism, whereas Reep5, Metch2, and Gnb1 are associated with protein modification and transport, energy metabolism, apoptosis and DNA maintenance, or signal transduction, respectively (Table 1). Interestingly, these four putative protein targets are associated with various neurological diseases as reported in the KEGG and OMIM database (Table 2).²²,²³

Conclusions
The aim of this study was to identify unknown protein targets of THC using photoaffinity labeling and chemical proteomics. To this end, Δ⁸-THC-derived probe 1 was synthesized in 14 steps with a total yield of 18%. Probe 1 had nanomolar affinity for both CBRs, but was not able to undergo a covalent addition with the CBRs and therefore unable to visualize the CBRs in an established gel-based photoaffinity labeling assay. Different positioning of the photoactive group in the probe, for example, on the more rigid tricyclic core of the scaffold to enable a stronger interaction between diazirine and amino acid residues, might allow the covalent capturing of CBRs.

Photoaffinity labeling of the proteome of live Neuro2A cells resulted in the identification of ~150 target proteins. Competition studies with THC significantly reduced enrichment of four proteins by probe 1, which suggests that THC has a limited interaction profile in Neuro2A cells. Reep5, Metch2, and Gnb1 were identified as putative protein targets of Δ⁷-THC, whereas Cox4i1 was targeted by Δ⁹-THC. These targets are mostly involved in protein handling, energy metabolism, apoptosis or DNA maintenance, which may suggest that long-term exposure of THC may affect a variety of (epigenetic) functions of brain cells. Of note, the affinity and functional activity of THC on these four proteins need to be further validated in orthogonal experiments using recombinant expression systems, followed by experiments to identify a mechanistic link between these proteins and physiological effects of THC.

Taken together, the identification of the putative protein hits described is a first step toward a better understanding of the protein interaction profile of THC, which could ultimately lead to the development of novel therapeutics based on THC.

Acknowledgments
The authors thank Caprotec for kindly providing the CaproBox. Hans van den Elst and Fons Lefeber are acknowledged for technical support. We thank the University of Granada for a predoctoral FPU fellowship to MDMG. Funding: MS, LHH and MvdS were supported by an ECHO-STIP grant from the Netherlands Organization for Scientific Research (NWO, grant no.: 711.014.009).

Authors’ Contributions

Author Disclosure Statement
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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