Supporting Information for:

Title: Cytosolic enzymes generate cannabinoid metabolites 7-carboxy-cannabidiol and 11-nor-9-carboxy-tetrahydrocannabinol

Authors: Jessica L. Beers,[1] Aurora K. Authement,[2] Nina Isoherranen,[2] Klarissa D. Jackson[1]*

[1]Division of Pharmacotherapy and Experimental Therapeutics, University of North Carolina at Chapel Hill Eshelman School of Pharmacy, Chapel Hill, NC

[2]Department of Pharmaceutics, University of Washington School of Pharmacy, Seattle, WA

*Corresponding author: klarissa.jackson@unc.edu

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Chemicals, Reagents, and Experimental Procedures

Chemicals and reagents

7-OH-CBD, 7-COOH-CBD, 7-COOH-CBD-d_3, and 7-OH-CBD-d_3 reference standards were purchased from Cerilliant (Round Rock, TX). Stock solutions of 7-OH-CBD and 7-COOH-CBD were prepared in methanol and stored at -30ºC. Hydralazine hydrochloride (HDZ), 4-methylpyrazole (4-MP), 1-aminobenzotriazole (1-ABT), NADPH, and NAD^+ were purchased from Sigma-Aldrich (St. Lous, MO). WIN18,446 was purchased from Tocris Bioscience (Minneapolis, MN). Chemical inhibitors were prepared from stocks dissolved in dimethyl sulfoxide (DMSO) except for hydralazine hydrochloride, which was dissolved in ultrapure water.

For 7-OH-CBD incubations, mixed sex human liver S9 (HLS9, lots 1410228 and 1110370) and human liver cytosol (HLC, lots 0910313 and 1410229) pooled from 200 donors were purchased from Xenotech (Lenexa, KS). Mixed sex human liver microsomes (HLM, lots 38295 and 38296) pooled from 150 donors were purchased from Corning (Woburn, MA).

For the 11-OH-THC incubations, the following subcellular fractions were used. Mixed sex HLS9 (lot 3212595) pooled from 200 donors was purchased from BD Gentest. Mixed sex HLC (lot 0910313) pooled from 200 donors was purchased from Xenotech (Lenexa, KS). Mixed sex pooled HLM were generated by combining individual preparations of 31 donors obtained from the University of Washington Human Liver Bank. Total protein concentrations were determined by the BCA Protein Assay (Pierce, Rockford, IL, USA).

Sample preparation and LC-MS/MS analysis of CBD and THC metabolites

After each experiment, 7-COOH-CBD-containing samples were prepared by removing a 100-µL aliquot of the reaction and adding 200 µL of ice-cold acetonitrile quench solution containing the internal standard 7-COOH-CBD-d_3 (100 ng/mL). Samples were then centrifuged at 3,400 *g* at 4°C for 20 min. A 200-µL aliquot of supernatant was collected and dried under a steady flow of nitrogen gas for approximately 35 min using a Biotage SPE Dry 96 sample concentrator system (Charlotte, NC, USA). Samples were resuspended in 150 µL of starting mobile phase and re-centrifuged at 3,400 *g* at 4°C for 20 min. A 100-µL aliquot of the resulting supernatant was collected and analyzed by liquid chromatography – tandem mass spectrometry (LC-MS/MS).

CBD metabolites were measured using a Thermo TSQ Quantum Ultra triple quadrupole mass spectrometer coupled to a Waters Acquity UPLC system (method 1) and a Sciex 5500+ triple quadrupole mass spectrometer coupled to an ExionLC system (method 2). A 2.6 µm 50 x 2.1 mm Phenomenex Kinetex EVO C18 column heated to 40°C was used for analyte separation for both methods 1 and 2. The LC gradient elution program and MS quantitation settings used for analysis by method 1 have been described previously.[1] Method 2 used the following LC gradient method with a flow rate of 0.5 mL/min, in which mobile phase A was 0.1% acetic acid in LC-MS grade water, and mobile phase B was 0.1% acetic acid in LC-MS grade acetonitrile: 40% B from 0-0.5 min, increase from 40% to 90%B from 0.5-4 min, hold at 90% B from 4-4.4 min, decrease from 90%B to 40%B from 4.4-4.5 min, and hold at 40% B from 4.5-6.5 min.

Both LC methods used a sample injection volume of 10 µL. The following MS/MS selected reaction monitoring transitions were observed using methods 1 and 2 in the negative ion mode for analyte detection: 7-OH-CBD, *m/z* 329 > 299; 7-COOH-CBD, *m/z* 343 > 299; 7-OH-CBD-d_3, *m/z* 332 > 302; and 7-COOH-CBD-d_3, *m/z* 346 > 302. Standard and quality control (QC) samples used in calibration curves to calculate metabolite formation rates were freshly prepared for each experiment in the range of 1.2-250 ng/mL and generated in concordance with current FDA recommendations for bioanalytical validation.[2] Curves developed with method 1 were generated with $1/X^2$ fitting using Thermo Xcalibur 2.2 software, and curves developed with method 2 were generated using $1/X^2$ weighting with Sciex OS 2.2. 7-COOH-CBD formation data presented in **Figs. 1-2** and **Supporting Information Figs. S1** and **S6** were collected using method 2, and data presented in **Fig. 4** and **Supporting Information Figs. S2** and **S3** were collected using method 1.

For all 11-COOH-THC experiments the reactions were terminated by adding double the reaction volume of ice-cold acetonitrile (ACN) with 11-COOH-THC-d_9 (internal standard). The samples were centrifuged at 18,000 *g* at 4°C, and the supernatants were used for LC-MS/MS analysis. This method is referred to as the ACN precipitation method in the following sections. For HLM incubations with low product formation, the incubation volume was increased to 1 mL. After 30 min, the reaction was stopped by adding three times the reaction volume of 9:1 hexane and ethyl acetate (v/v), and samples were processed using a liquid-liquid extraction (LLE) method. In this method, each sample was extracted twice with 3 mL of hexanes and ethyl acetate (in a 9:1 ratio) and centrifuged at 1,000 *g* for 10 min to facilitate phase separation. The

organic layer was transferred into a glass tube and dried under nitrogen gas flow. The residue was resuspended with 100 µL of ACN for LC-MS/MS analysis. Incubation time and concentrations of protein, substrate, inhibitor and cofactor used for incubations are specified for each experiment in the following sections. All cofactor solutions were freshly prepared before incubations. Incubations were performed in triplicate and repeated two times on separate days.

The formation of 11-COOH-THC in incubations was measured by LC-MS/MS with an AB Sciex 6500 Q/LIT mass spectrometer (AB Sciex LLC, Framingham, MA) equipped with an Agilent 1290 Infinity UHPLC (Agilent Technologies, Santa Clara, CA) and a Kinetex EVO C18 column (1.7 µm, 100 Å, 2.1 x 100 mm; Phenomenex, Torrance, CA). The mobile phase consisted of water with 20 mM ammonium formate (solvent A) and acetonitrile (ACN, solvent B). The flow rate was 0.6 mL/min and the column temperature was set at 50°C. The gradient started at 20% B, increased to 30% B within 5.0 min and then increased to 50% B within 20 min, then increased to 90% B within 1 min and held for 30 seconds, before returning to initial conditions. The injection volume was 5 µL per sample. 11-COOH-THC and the IS were monitored using positive ion electrospray ionization (ESI). 11-COOH-THC transitions were monitored with *m/z* 345.3>193.1 and 345.3>187.1 and 11-COOH-THC-d_9 was monitored with *m/z* 354.3/197.1 and 354.3/196.1. Peaks of 11-COOH-THC and IS were integrated using Analyst 1.6.3. The peak area ratio of 11-COOH-THC to IS was used to quantify 11-COOH-THC formation. For quantification, standard curves of 11-COOH-THC (six concentrations ranging from 2.72 nM to 75 nM for ACN precipitation and 1 nM to 20 nM for LLE) were constructed by spiking 11-COOH-THC into potassium phosphate buffer

with protein and standard curve samples processed in parallel with incubation samples. The ratio of 11-COOH-THC to IS peak area was plotted against 11-COOH-THC concentration (r^2 values >0.97 for all curves) and was used to calculate the concentration of 11-COOH-THC formed in incubations.

General protocol for subcellular fraction incubations with 7-OH-CBD and 11-OH-THC

Initial experiments were conducted to evaluate 7-OH-CBD substrate depletion and 7-COOH-CBD formation in HLS9. 7-OH-CBD (1 μM) was incubated with 200-donor pooled HLS9 (2 mg protein/mL) for 0-90 min with and without the cofactors NADPH (1.3 mM) (supplied by NADPH regenerating system, Corning catalog numbers 451220 and 451200) and NAD+ (2 mM)). Rate constants for the depletion of 7-OH-CBD were calculated from linear range of the semilogarithmic slope of percentage (%) remaining 7-OH-CBD for each reaction condition vs. time (5-45 min) (A) (see Table S1). Formation rates of 7-COOH-CBD were calculated by linear regression from 0-15 min (B) (see Table S2). Subsequent experiments were conducted to determine the linearity of 7-COOH-CBD formation with respect to protein concentration and time (see Fig. S2).

Incubations were performed according to the following protocol unless otherwise stated: HLC, HLM, and HLS9 were first pre-warmed at 37°C for 5 min in 100 mM potassium phosphate buffer (pH 7.4) in a final volume of 200-250 μL. Substrate concentrations were 1 μM 11-OH-THC or 1 μM 7-OH-CBD, and reactions were initiated with the addition of cofactor(s). The incubation times were as described for each experiment in the following sections. All the incubation times were sufficiently short to

avoid time-dependent loss of or decrease in enzyme activity and were performed under time and protein linearity (see Fig. S2 and S5).

Cofactor dependence of 7-COOH-CBD and 11-COOH-THC formation in HLM, HLC, and HLS9

To examine the roles of NAD^+ vs. NADPH-dependent enzymes in 7-COOH-CBD formation, 7-OH-CBD (1 µM) was incubated with HLS9, HLM, and HLC (0.25 mg/mL protein) for 20 min with the cofactors NADPH (1 mM), NAD^+ (2 mM), and/or vehicle control (100 mM potassium phosphate buffer, pH 7.4). Reactions (250 µL total) were initiated with the addition of substrate and incubated at 37°C in a shaking water bath for the duration of the experiment (20 min). Results are the average ± range of two independent experiments each performed in triplicate.

To test cofactor dependence of 11-COOH-THC formation, all subcellular fractions were incubated with 1 µM 11-OH-THC in the absence or presence of various cofactors. Tested cofactors and their final concentrations in incubations were 1 mM NADPH, 2 mM NAD^+, and a mixture of 1 mM NADPH/2 mM NAD^+ for all subcellular fractions. To measure 11-COOH-THC formation from 11-OH-THC with HLM, 0.05 mg/mL HLMs were incubated for 30 min with 1 µM 11-OH-THC. Samples were processed with the previously mentioned LLE method. To measure 11-COOH-THC formation from 11-OH-THC with HLC and HLS9, 0.075 mg protein/mL (HLC) and 0.05 mg protein/mL (HLS9) were incubated for 25 min and 10 min, respectively. Samples were processed with the ACN precipitation method.

Inhibition of 7-COOH-CBD and 11-COOH-THC formation in HLC and HLS9 with inhibitors hydralazine, 4-methylpyrazole, 1-aminobenzotriazole, and WIN18,446

To determine the relative contributions of cytosolic and microsomal drug-metabolizing enzymes to 7-COOH-CBD formation, 7-OH-CBD (1 μM) was incubated with HLS9 and HLC (0.25 and 0.5 mg/mL protein, respectively) for 20 min using the methods described above with the addition of 1-ABT (nonselective cytochrome P450 inhibitor; 500 μM), hydralazine (aldehyde oxidase, AOX inhibitor; 25 μM), 4-methylpyrazole (alcohol dehydrogenase, ADH inhibitor; 100 μM), WIN18,446 (aldehyde dehydrogenase, ALDH inhibitor; 250 μM), or DMSO vehicle control (0.1 % v/v). Reactions were initiated with the addition of cofactors NADPH (1 mM), NAD^+ (2 mM), or vehicle control. Results are the mean and range of two independent experiments performed in triplicate.

Inhibition assays with 11-OH-THC were completed as previously described for incubations in HLC and HLS9 with 2 mM NAD^+ and 1 mM NADPH cofactor. Due to a lack of cytochrome P450 enzymes in HLC fractions, 1-ABT and NADPH were not included in inhibition assays with 11-OH-THC.

Determination of IC_{50} of WIN18,446 for 7-COOH-CBD formation and hydralazine for 11-COOH-THC formation

The concentration of WIN18,446 resulting in 50% inhibition (IC_{50}) of 7-COOH-CBD formation was determined with HLC (0.5 mg/mL protein) using a concentration range of 0.01 – 100 μM (0.01, 0.025, 0.05, 0.1, 0.5, 1, 5, 10, and 100 μM WIN) in the presence of 2 mM NAD^+. Reactions (200 μL total) were initiated with the addition of

cofactor and conducted over 30 min. Results are the average ± SD of three independent experiments performed in triplicate. The IC_{50} of WIN was calculated by fitting data normalized to vehicle control to a nonlinear dose-response inhibition model in GraphPad Prism.

To build an IC_{50} curve of hydralazine-mediated inhibition of 11-COOH-THC formation, 3 μM 11-OH-THC and 9 concentrations of HDZ (0.01-10,000 nM) were incubated for 20 min in 0.1 mg/mL of HLS9. All samples were processed with the ACN precipitation method. Control groups were incubations with no inhibitor. Percentage of control activity was calculated and plotted against inhibitor concentrations. The IC_{50} values were determined by non-linear regression using GraphPad Prism 5.0.

To build an IC_{50} curve of WIN-mediated inhibition of 11-COOH-THC formation, 3 μM 11-OH-THC and 6 concentrations of WIN (0.03-300 μM) were incubated for 20 min in 0.1 mg/mL of HLS9. All samples were processed with the ACN precipitation method. Control groups were incubations with no inhibitor. Percentage of control activity was calculated and plotted against inhibitor concentrations. The IC_{50} values were determined by non-linear regression using GraphPad Prism 5.0.

Safety: No unexpected or unusually high safety hazards were encountered.

Table S1. 7-OH-CBD depletion in HLS9 in the presence and absence of cofactors.[a]

Cofactors(s)	7-OH-CBD depletion rate constant (min^{-1})
NADPH/NAD^+	-0.023
NADPH	-0.027
NAD^+	-0.002
No cofactor	-0.003

[a]7-OH-CBD (1 μM) was incubated with 200-donor pooled HLS9 (2 mg protein/mL) for 0-90 min with and without the cofactors NADPH (1.3 mM) (supplied by NADPH regenerating system, Corning catalog numbers 451220 and 451200) and NAD^+ (2 mM). Rate constants for the depletion of 7-OH-CBD were calculated from the linear range of the semilogarithmic slope of percentage (%) remaining 7-OH-CBD for each reaction condition vs. time (5-45 min) (see Fig. S3A) using GraphPad Prism.

Table S2. 7-COOH-CBD formation in HLS9 in the presence and absence of cofactors.[a]

Cofactor(s)	7-COOH-CBD formation (pmol/min/mg protein)
NADPH/NAD^+	4.26
NADPH	4.24
NAD^+	1.67
No cofactor	0.50

[a]7-OH-CBD (1 μM) was incubated with 200-donor pooled HLS9 (2 mg protein/mL) for 0-90 min with and without the cofactors NADPH (1.3 mM) (supplied by NADPH regenerating system, Corning catalog numbers 451220 and 451200) and NAD^+ (2 mM). Formation rates of 7-COOH-CBD were calculated by linear regression from 0-15 min (see Fig. S3B).

Figure S1.

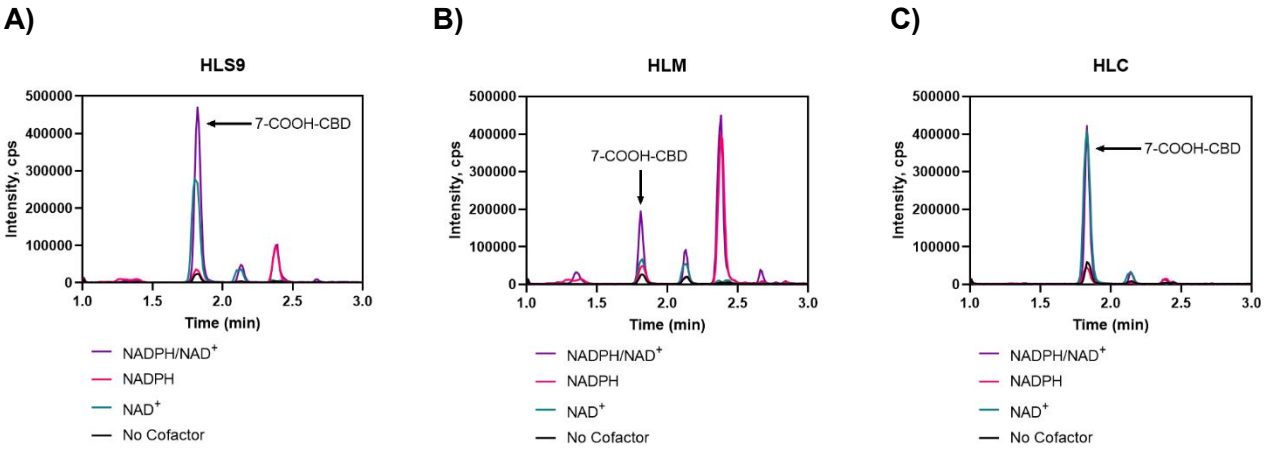

Figure S1. Representative LC-MS/MS chromatograms of cofactor dependence of 7-COOH-CBD formation. Formation of 7-COOH-CBD in A) HLS9, B) HLM, and C) HLC was measured via LC-MS/MS using method 2 in the negative ion mode (*m/z* 343>299). Quantitation was performed by measuring the peak area ratio of 7-COOH-CBD to internal standard 7-COOH-CBD-d_3 (*m/z* 346>302). The chromatographic peak representing 7-COOH-CBD is indicated.

Figure S2.

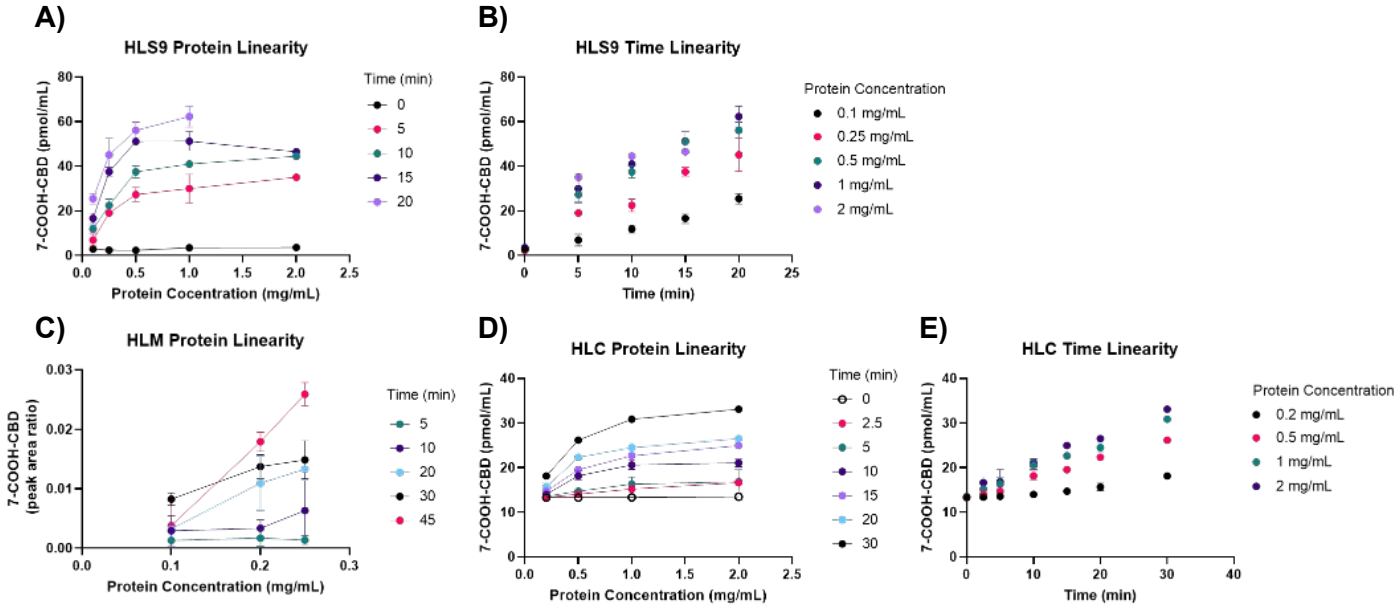

Figure S2. Protein-time linearity of 7-COOH-CBD formation in HLC, HLM, and HLS9. To determine optimal conditions for reaction phenotyping experiments, increasing concentrations of pooled HLS9 (A and B), HLM (C) and HLC (D and E) (ranging from 0.1 – 2 mg/mL protein) were incubated with 1 µM 7-OH-CBD. Experiments performed with HLM were supplemented with NADPH (1 mM) as a cofactor, and experiments performed with HLC and HLS9 were supplemented with NAD^+ (2 mM) as a cofactor.

Figure S3.

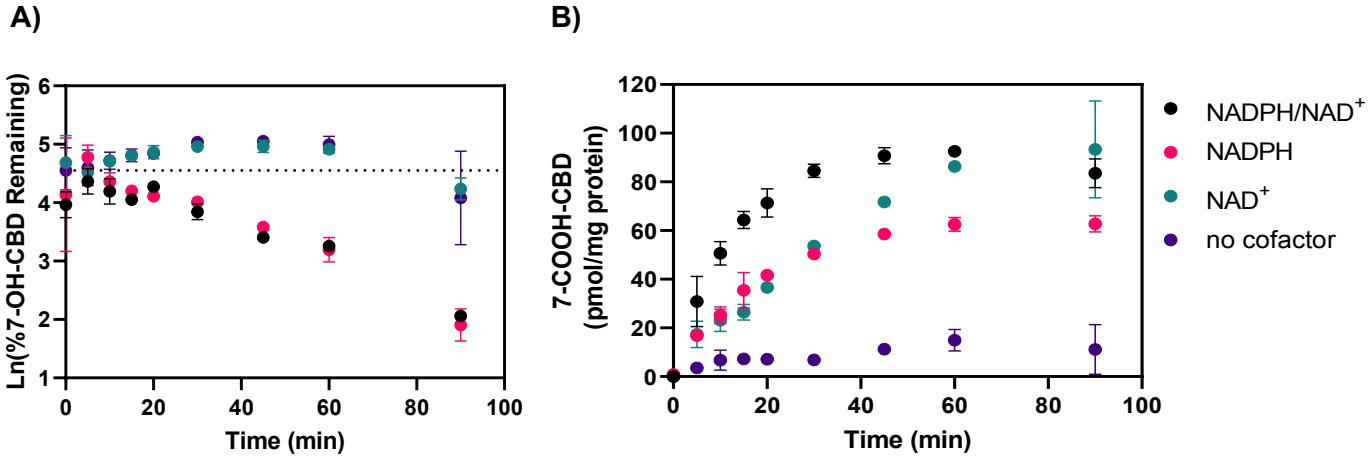

Figure S3. 7-OH-CBD substrate depletion and 7-COOH-CBD formation in HLS9. 7-OH-CBD (1 μM) was incubated with 200-donor pooled HLS9 (2 mg protein/mL) for 0-90 min with and without the cofactors NADPH (1.3 mM) (supplied by NADPH regenerating system, Corning catalog numbers 451220 and 451200) and NAD^+ (2 mM). Rate constants for the depletion of 7-OH-CBD were calculated from linear range of the semilogarithmic slope of percentage (%) remaining 7-OH-CBD for each reaction condition vs. time (5-45 min) (A) (see Table S1). Formation rates of 7-COOH-CBD were calculated by linear regression from 0-15 min (B) (see Table S2).

Figure S4.

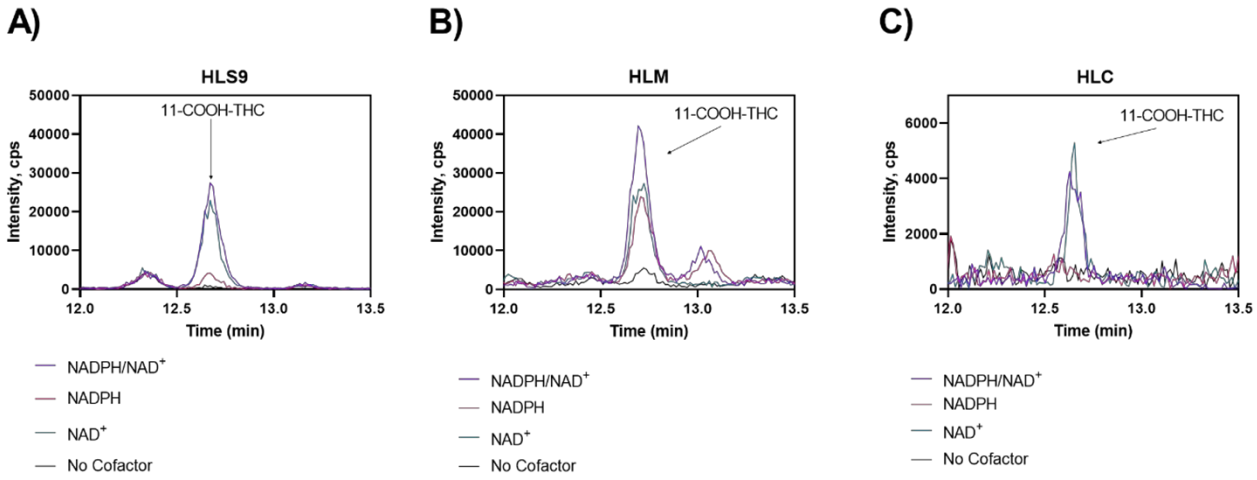

Figure S4. Representative LC-MS/MS chromatograms of cofactor dependence of 11-COOH-THC formation. Formation of 11-COOH-THC in A) HLS9, B) HLM, and C) HLC was measured via LC-MS/MS in the positive ion mode using the mass transitions *m/z* 345.3>193.1 and 345.3>187.1. Quantification was performed by measuring the peak area ratio of 11-COOH-THC to internal standard 11-COOH-THC-d_9 (*m/z* 354.3>197.1 and 354.3>196.1). The chromatographic peak representing 11-COOH-THC is indicated.

Figure S5.

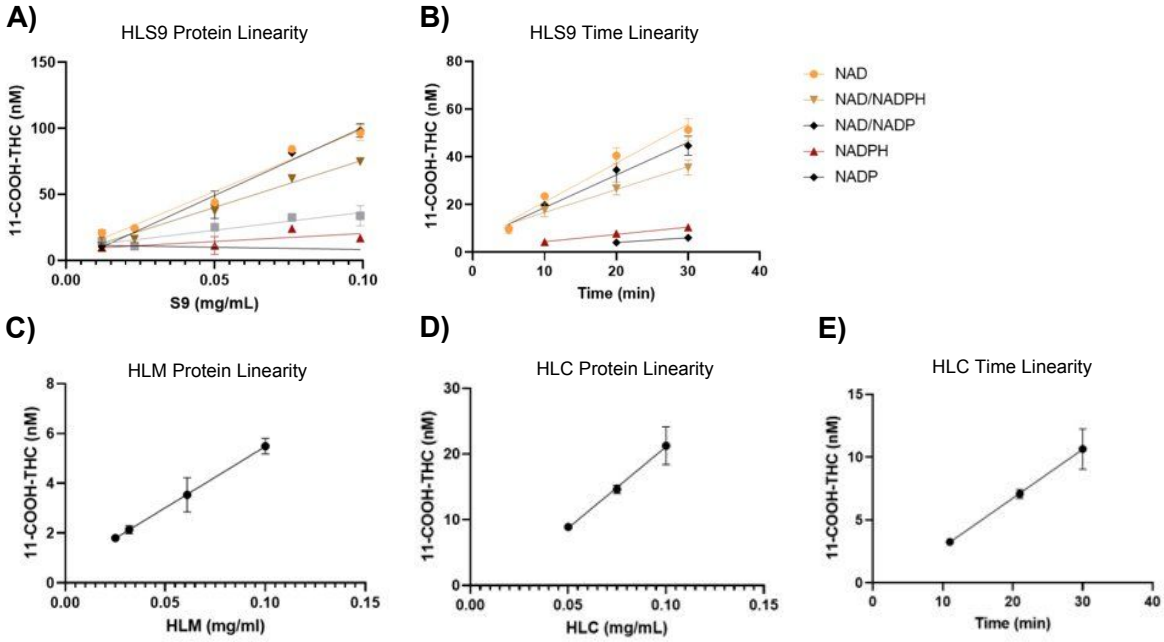

Figure S5. Protein-time linearity of 11-COOH-THC formation in HLC, HLM, and HLS9. To determine optimal reaction time and protein concentration for experiments, increasing concentrations of pooled HLS9 (A and B) and HLC (D and E) (0.05 – 0.1 mg/mL protein) were incubated with 1 µM 11-OH-THC and NADPH (1 mM), NADP (2 mM), and NAD^+ (2 mM) for 5 to 30 min and 10 to 30 min for HLS9 and HLC, respectively. To determine optimal protein concentrations, pooled HLM (C) (0.025 – 0.1 mg/mL) were incubated with 3 µM 11-OH-THC and NADPH (1 mM) for 30 min.

Figure S6.

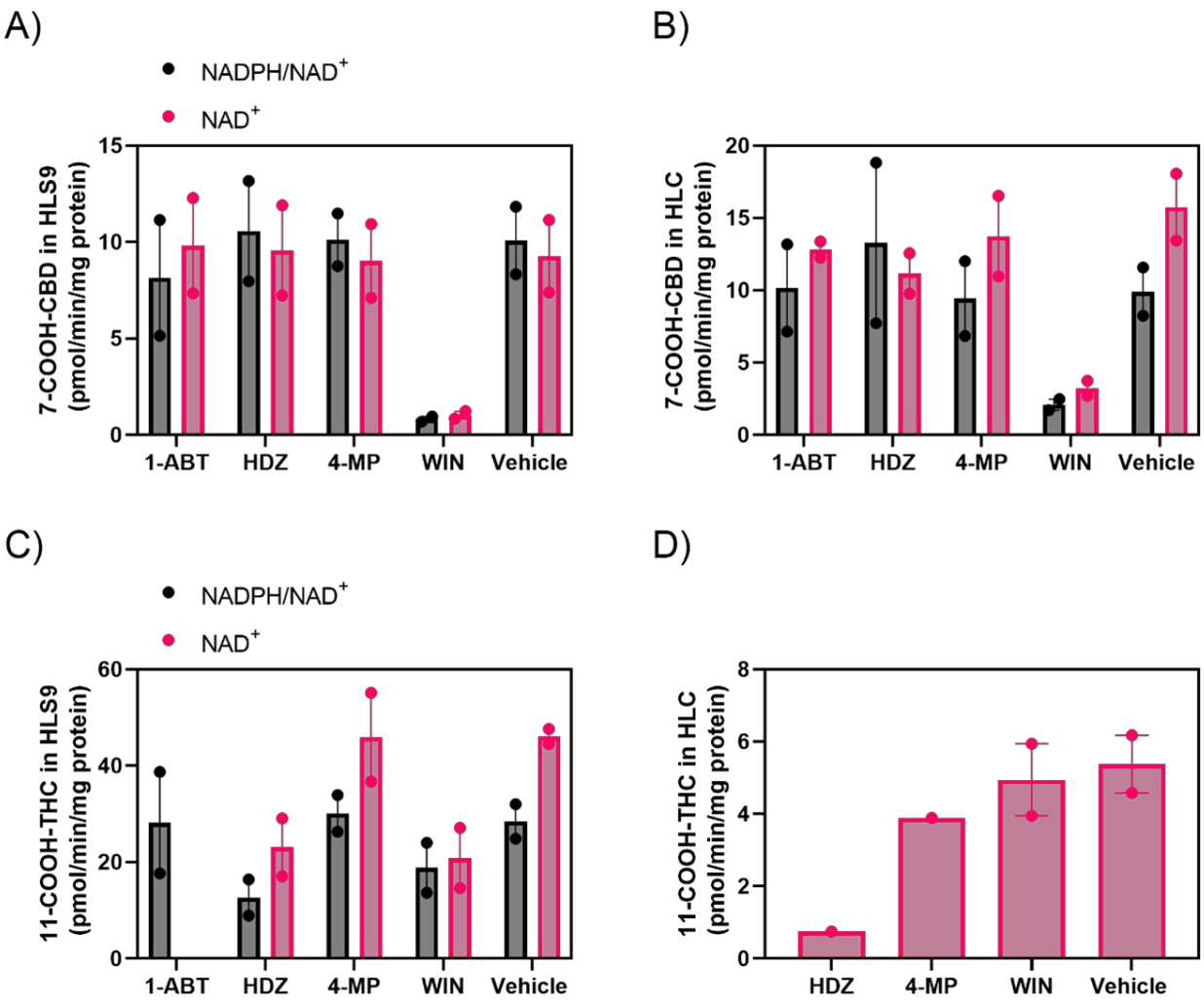

Figure S6. Formation rates of cannabinoid metabolites in the presence of selected chemical inhibitors. These data show the calculated formation rates of 7-COOH-CBD and 11-COOH-THC with inhibitors which are shown as normalized percentages in Fig. 3 of the main text. Pooled HLS9 and HLC were incubated with DMSO vehicle (0.5% v/v), 1-aminobenzotriazole (1-ABT, P450 inhibitor, 500 μM), hydralazine (HDZ, AOX inhibitor, 25 μM), 4-methylpyrazole (4-MP, ADH inhibitor, 250 μM), or WIN18,466 (WIN, ALDH inhibitor, 250 μM) with NADPH (1 mM) and

NAD^+ (2 mM). A-B show 7-COOH-CBD formation, and C-D show 11-COOH-THC formation. For C, experiments with 1-ABT were performed only with NADPH and NAD^+ together, and for D, experiments were performed with HLC in the presence and absence of NAD^+ only. Bars and points represent the mean ± range of two independent experiments performed in triplicate. Black bars = reactions performed with NADPH and NAD^+, pink bars = reactions performed with 7-OH-CBD or 11-OH-THC and NAD^+. N.A. = not applicable (experiment not performed).

Supporting Information References

(1) Beers, J. L.; Fu, D.; Jackson, K. D. Cytochrome P450-Catalyzed Metabolism of Cannabidiol to the Active Metabolite 7-Hydroxy-Cannabidiol. *Drug Metab. Dispos.* **2021**, *49*, 882–891.

(2) U.S. Food and Drug Administration. Bioanalytical Method Validation - Guidance for Industry. *U.S. Department of Health and Human Services, Food and Drug Administration* **2018**, 1–41.