Chalcone-Supported Cardiac Mesoderm Induction in Human Pluripotent Stem Cells for Heart Muscle Engineering

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Figure S 1: Negligible transcription of ectodermal and endodermal marker genes in response to the ABCF+I protocol. qPCR screen for residual PSCs (OCT4), ectodermal cells (NEUROD1), and endodermal cells (SOX17; n = 3/group) in ABCF+I-derived heart muscle cells; undifferentiated HES2 and derivatives (ectodermal [Ecto] and endodermal [Endo] induction) served as reference material.
Figure S 2: Gating strategy for the assessment of cardiomyocyte derivation from hPSC by flow cytometry. Intact cells were gated on the DNA label “Hoechst 33342” fluorescence intensity (FI; left panel), then forward scatter width and height (FSC-W; FSC-H) to exclude doublets. Cardiomyocytes were identified by a positive signal after antibody labeling of sarcomeric α-actinin (ACTN2). Representative plots for IgG1 isotype control and α-actinin on the right (data from n = 6 experiments); FSC: forward scatter; FSC-A: FSC-signal area; FSC-H: FSC-signal height; FSC-W: FSC-signal width.
Figure S 3: Chalcone concentration dependent effects on cardiomyogenesis. Flow cytometry analysis of cardiomyocyte content (% α-actinin positive cells on culture day 22) after exposure of hPSCs to the AXCF+I protocol, whereas X refers to the commercially available chalcones 4’-hydroxychalcone (1) and Isoliquiritigene (2) tested at the indicated concentrations (in µmol/L; n = 3-17 biological replicates/concentration). * P < 0.05 vs. ABCF+I by ANOVA with Dunnett’s multiple comparison post-hoc test.
Figure S 4: Primary screening results. Assessment of (A) Beating Rate (BR) of the derived cardiomyocytes \((n = 3-4 /\text{group})\) and (B) Reproducibility (Repro; % of cultures with visibly beating cardiomyocytes - 4 wells analyzed/differentiation run; \(n = 4\) differentiation runs/group) at culture day 22 following the screen of 2 commercially available and 15 newly synthesized chalcones. (C) Summary of numerical values assigned for each analyzed parameter; CM: cardiomyocytes content in total cell count; DS: differentiation score, summation of the individual scores for each analyzed parameter. - : no BMP4 (ACF+I); + : ABCF+I
Figure S 5: Screen of 4-fluoro-4′-methoxychalcone (compound 5a) derivatives. Analyses were performed similarly as described for the primary screen of the first round of commercial and newly synthesized chalcones. (A) Beating Rate \((n = 12/\text{group})\) and (B) Reproducibility \((\% \text{ of cultures with visibly beating cardiomyocytes} – 4 \text{ wells analyzed/differentiation run;} \ n = 3 \text{ differentiation runs/group})\). \* \(P < 0.05\) vs. no BMP4 (-) by ANOVA with Dunnett’s multiple comparison \textit{post-hoc} test. - : no BMP4 (ACF+I); + : ABCF+I
Figure S 6: Morphological and transcript profiling of cardiomyocytes derived by the 4’-hydroxychalcone (1) as well as compound 5a and 6a supplemented AXCF+I protocol. (A) Brightfield images with arrows indicating beating cardiomyocytes clusters; refer also to Supplementary Videos. (B) Relative gene expression of pluripotent marker OCT4, early mesodermal markers T and MIXL1, cardiac mesodermal marker MESP1, early cardiac markers ISL1 and NKK2-5 and late cardiac markers MYL7 and MYL2 during differentiation using qPCR. All data were normalized to GAPDH transcript abundance (n=3-5 samples/time point).
Figure S 7: No evidence for chalcone signaling via the SMAD pathway. (A) Western blot analyses of P-SMAD1/5 and total SMAD1 in cell lysates at 0, 1 (after medium change), and 72 h post-treatment with BMP4 (5 ng/mL), 1, 5a, and 6a (all at 10 μmol/L). GAPDH was used as loading control (left). (B) Summary of the Western blot data showing relative phosphorylated protein normalized to GAPDH (right; n=3 samples/group). - : no BMP4 (ACF+I); + : ABCF+I
**Table S1.** List of Antibodies used in this study.

*(FC – Flow Cytometry, IF – Immunofluorescence, WB – Western Blot)*

| Antibody/Stain | Company          | Cat No.  | Target / Use                  | Dilution         |
|----------------|------------------|----------|-------------------------------|------------------|
| IgG1           | R&D Systems      | MAB002   | Control                       | 1:1,000 FC 1:100 IF |
| α-actinin (mouse IgG) | Sigma | A7811 | Cardiomyocyte                 | 1:4,000 FC 1:1,000 IF |
| α-smooth muscle actin (mouse IgG) | Sigma | A2547 | Stromal Cell                  | 1:4,000 FC 1:400 IF |
| Collagen I (rabbit IgG) | Abcam | Ab34710 | Stromal Cell                  | 1:2,000 FC 1:500 IF |
| Goat anti-mouse Alexafluor 488 | Invitrogen | A-11001 | Secondary Antibody            | 1:1,000 FC 1:400 IF |
| Goat anti-rabbit Alexafluor 546 | Invitrogen | A-11010 | Secondary Antibody            | 1:1,000 FC 1:400 IF |
| Phospho-SMAD1/5 (Rabbit) | Cell Signaling | 9516S | Phosphorylated SMAD1/5 (60 kDa) | 1:500 WB |
| SMAD1 (Rabbit) | Cell Signaling   | 9743S   | Total SMAD1 protein (60 kDa)  | 1:1,000 WB |
| Anti-mouse-IgG HRP conjugated | Dako | P0161   | Secondary Antibody            | 1:10,000 WB |
| Anti-rabbit-IgG HRP conjugated | Dako | 0448    | Secondary Antibody            | 1:5,000 WB |
Table S2. List of Primers used in this study

| Gene    | F                          | R                          | Purpose | Size (bp) | Acc #                  |
|---------|-----------------------------|----------------------------|---------|-----------|------------------------|
| OCT4    | CAGTGCCCGAAACCACAC         | GGAGACCCACGCCTCAAA          | qPCR    | 161       | NM_002701, NM_203289, NM_001173531 |
| T       | TCAGCAGAGTCAGCTCCA          | CCCCACCTCCTCACTGGATT        | qPCR    | 102       | NM_001811              |
| MIXL1   | CCGAGTCCAGGATCCAGTA         | CTCTGAGCCCGAGACTGGA         | qPCR    | 58        | NM_031944              |
| NXX2-5  | ACAACTTGTAATCTGCCG         | GTGGACTCTCCGGAGTTGCTCT      | qPCR    | 82        | NM_001166175.1, NM_001166176.1, NM_004387.3 |
| MESP1   | CCACGTCGCGCGCTCTCC          | CGGTGCTCAGAGACGGCG          | qPCR    | 130       | NM_0018670             |
| ISL1    | CCCTTGAGAGTGACATAG          | GGACTGCTCACAGCTGT          | qPCR    | 147       | NM_002202.2            |
| MYL7 (MLC2a) | CAGCGCAGAGGCTGGCTGAAC | GTGCACTGGGTGTCAGGGCGA       | qPCR    | 113       | NM_021223.2            |
| MYL2 (MLC2v) | GCCGCCACCTCCACACGTTT | ACGTACACTGCCAAGGGC         | qPCR    | 149       | NM_000432.3            |
| NEUROD1 | AGCCACGATCTACCTCCTCACAG    | GCGTGCTCTCAAATGAAA         | qPCR    | 143       | NM_002500.3            |
| SOX17   | AGGAAATCTCAGAGCTCTGGTT     | CCCCAGTTCCAGAGATTGGACAGACA | qPCR    | 111       | NM_023454.3            |
| GAPDH   | CCTCAAGATCATCAGCAATGCC     | ATGTCTGGAGAGCCCGC          | qPCR    | 189       | NM_002946.3            |
Video Legends:

**Video S1:** HES2-cardiomyocytes derived in the absence of BMP4 (ACF+I protocol). Negative control: No spontaneous beating of cardiomyocytes on culture day 22.

**Video S2:** HES2-cardiomyocytes derived in the presence of BMP4 (ABCF+I protocol). Positive control: spontaneous coordinated beating of cardiomyocytes on culture day 22.

**Video S3:** HES2-cardiomyocytes derived from the AXCF+I protocol whereas X was 4'-hydroxychalcone (**compound [cpd] 1**). Beating of cardiomyocytes was recorded on culture day 22.

**Video S4:** HES2-cardiomyocytes derived from the AXCF+I protocol whereas X was 4-fluoro-4'-methoxychalcone (**compound [cpd] 5a**). Beating of cardiomyocytes was recorded on culture day 22.

**Video S5:** HES2-cardiomyocytes derived from the AXCF+I protocol whereas X was 4-fluoro-4'-hydroxychalcone (**compound [cpd] 6a**). Beating of cardiomyocytes was recorded on culture day 22.
Experimental Section

Ethics. The import and use of human embryonic stem cells (HES2-ROSA26-RFP [HES2])\(^1\) was approved by the Central Ethics Committee for Stem Cell Research according to the German Stem Cell Act (Az 1710-79-1-4-16).

PSC culture. HES2 were confirmed to be karyotypically normal, mycoplasma free and pluripotent using in vivo teratoma studies, immunofluorescence staining and flow cytometry. Feeder-cell free culture adapted HES2 were seeded at 25,000 cells/cm\(^2\) on Matrigel\(^\text{TM}\) (1:30 in phosphate-buffered saline [PBS]; BD Biosciences)-coated T75 flasks in TeSR-E8 medium (E8; Stemcell Technologies), supplemented with antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin; Gibco). After 48 h, HES2 were passaged by dispersion in TrypLE (Gibco) for 3 min at 37 °C and seeded at 50,000 cells/cm\(^2\) on Matrigel\(^\text{TM}\) coated 24-well plates (culture day - 1).

Standard cardiac differentiation protocol. We applied the ABCF+I cardiac differentiation protocol (Figure 1A),\(^2\) which we introduced previously with robust cardiomyogenesis-inducing activity in all so far tested hPSC lines, including several human embryonic and induced pluripotent stem cell lines. In brief, mesoderm induction was for 3 days with ABCF: Activin A (9 ng/ml), BMP4 (5 ng/ml), CHIR99021 (1 µmol/L) and FGF2 (5 ng/ml) in serum-free (SF) medium (RPMI 1640 supplemented with 2% B27 with insulin, 1 mmol/L sodium pyruvate [all Gibco] and 200 µmol/L L-ascorbic acid 2 phosphate sesquimagnesium salt hydrate [ASC; Sigma] and antibiotics). This was followed by cardiac specification by supplementing SF-medium with IWP4 (5 µmol/L) for 10 days beyond which cultures were maintained in SF medium until day 22.

Screening for a small molecular BMP4 replacement. We tested commercially available (4’hydroxychalcone [Cat#: CDS003892] and Isoliquiritigene [Cat#: I3766]; both from Sigma-Aldrich) and 24 newly synthesized small molecular weight compounds of the chalcone family (for details see Synthesis Schemes below) as BMP4 replacements (AXCF+I protocol). Candidate compounds were ranked according to their cardiomyogenesis inducing activity using a combined differentiation score (DS) derived from an assessment of multiple parameters: (1) reproducibility of cardiomyocyte derivation, (2) cardiomyocyte beating rate, (3) total cell number as well as (4) percentage and (5) total quantity of α-actinin positive cardiomyocytes at culture day 22. More detailed analyses were performed with the most effective compounds as indicated.
**Compound Syntheses.** A mechanically stirred solution of acetophenone (1 mmol/L) in dry MeOH (3 mL) was treated with a solution of NaOMe (5.4 M in MeOH, 5 mmol/L) at 0 °C and stirring was continued for 1 h at room temperature. Then a solution of aldehyde (1 mmol/L) in MeOH (5 mL) was added dropwise and the reaction mixture was stirred for additional 24 h. The solution was treated with H2O (20 mL), acidified to pH = 1 with HCl (5 mL of a 10% aq. solution), stirred for 10 min and extracted with EtOAc (4 × 15 mL). The combined organic layers were washed with brine (20 mL), dried over MgSO4 and concentrated under reduced pressure. Purification by flash column chromatography (EtOAc:PE = 1:9→1:2) yielded the target compounds 5a-o as indicated in **Figure 2B**; synthesis of compounds 5e[3], j[4], k[5], n[6], and o[5a, 7] were described previously. A detailed compound structure analyses of all compounds and the synthesis procedures for compounds 6 a-i (summarized also in **Figure 4A**) are listed below.

**Chromatography.** Thin-layer chromatography was performed on silica gel plates (TLC Silica gel 60 F254; Merck). Column chromatography was performed with silica gel (Geduran® Si60, Ø = 32–64 μm; Merck).

**NMR spectroscopy.** NMR spectra were recorded on Varian “Mercury-300“, “Unity-300“, “Inova-500” and on a “AMX-300” spectrometer from Bruker. Chemical shifts are given in ppm relative to tetramethylsilane (TMS) and coupling constants $J$ in Hertz. Solvent signals serve as reference and the chemical shifts converted to the TMS scale (CHCl3: $\delta$ H = 7.24 ppm, $\delta$ C = 77.16 ppm). Multiplicities of first order signals are assigned as: s (singlet), sbr (broad singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets). Signals of higher order are declared as m (multiplet).

**IR spectroscopy.** IR spectra were recorded with a FT/IR-4100 spectrometer from JASCO (substances were applied neat on an ATR unit).

**UV spectroscopy.** UV spectra were recorded on a JASCO V-630 spectrometer.

**Mass spectrometry.** ESI-MS and ESI-HRMS spectra were recorded on a “Apex IV” spectrometer from Bruker Daltronik.

**Cell dissociation for cell counting and flow cytometry.** On culture day 22, cells were washed in PBS and dissociated by incubation in dissociation reagent (Accutase [Gibco] supplemented with 0.025% Trypsin [Gibco] and 20 μg/ml DNaseI [Merck]) for 30-45 min. The cells were
pelleted by centrifugation (300 g, 3 min) and fixed with either 4% formaldehyde (FA) for immunofluorescence staining or 70% ice cold ethanol for flow cytometry analysis.

**Flow cytometry.** Depending on the protein of interest, cells were incubated with primary antibodies (**Table S1**) raised against sarcomeric actinin (Sigma), smooth muscle actin (Sigma) and collagen type I (Abcam) in blocking buffer (containing 5% FBS, 1% bovine serum albumin [BSA], 0.5% Triton X-100 in PBS) for 45 min followed by appropriate secondary antibodies (**Table S1**) in blocking buffer and Hoechst 33342 (10 µg/mL) for 30 min at 4°C. Flow cytometry was performed with a BD LSRII SORP instrument (BD Biosciences). IgG isotype controls (R&D Systems) were used to determine non-specific staining. The gating strategy is displayed in **Figure S2**.

**Immunofluorescence staining.** Cells were plated on Matrigel™-coated glass coverslips for 24 h in SF-medium before fixation in 4% FA. After blocking for 30 minutes in blocking buffer, primary antibodies (**Table S1**) were added for 90 min followed by secondary antibodies (**Table S1**) and Hoechst 33342 (10 µg/mL) for 60 min at room temperature. Coverslips were mounted onto glass slides (SuperFrost Plus, Thermo Fisher Scientific) using Fluoromount (Dako) and imaged using a Zeiss 710 NLO confocal microscope or a Zeiss AxioImager.M2 fluorescence microscope.

**Polymerase chain reaction.** Cells were harvested and RNA extraction was performed using Trizol™ following manufacturer’s instructions (Ambion). RNA (1 µg) was treated with DNase (Roche) followed by cDNA synthesis using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR (qPCR) was performed using the Fast SYBR Green Master Mix (Applied Biosystems) in a 384-well format AB7900 HT (Applied Biosystems). Gene expression analyses were performed according to the standard curve method.**[8]** GAPDH transcript abundance was used for normalization. Primer details are given in **Table S2**.

**Immunoblotting.** Cells were lysed using CytoBuster reagent (Merck) supplemented with phosphatase- and proteinase-inhibitor cocktail (Roche). Protein concentrations were determined using a Bradford assay. Protein samples were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF, GE lifesciences) membranes. Membranes were then suspended in blocking buffer containing 5% milk in TRIS-buffered saline with Tween (TBST; 0.02 mol/L TRIS-hydrochloride, 0.17 mol/L NaCl and 0.1% Tween-20 in H₂O) for 1 hour, incubated with primary antibody (**Table S1**; in 5% BSA in TBST) overnight and with secondary antibody at room temperature for 1 hour (**Table S1**). The proteins of interest
were detected using SuperSignal HRP Substrate (Thermo Fisher Scientific) in a ChemiDoc MP Imaging system (Bio-Rad). Signals were normalized to the expression of GAPDH (Zytomed Systems).

**EHM casting.** EHM were reconstituted according to a previously described protocol.[2] Prior to casting and to enhance cardiomyocyte purity, differentiated cells were first subjected to metabolic selection[9] in RPMI (without Glucose) with 0.1 mmol/L 2-mercaptoethanol, antibiotics (all Gibco) and 2.2 mmol/L sodium lactate (Sigma) for 4 days, followed by a recovery and further maturation phase again in SF medium until culture day 22. This increased cardiomyocyte population to 66±3% for 1 and 24±4% for 5a (data collected from n=6-10 experiments). HES2-derived cardiomyocytes (1.01x10⁶ CMs/EHM) and human foreskin fibroblasts (ATCC; 0.44x10⁶ cells/EHM) were suspended in a mixture of bovine collagen (LLC Collagen Solutions, 0.4 mg/EHM) and serum-free medium (2x RPMI, 8% B27 without insulin, 200 U/ml penicillin, 200 µg/ml streptomycin). The reconstitution mixture was cast in circular molds (450 µL/EHM) and further cultured in Iscove-Medium containing 4% B27 without insulin, 1% non-essential amino acids, 2 mmol/L glutamine, 200 µmol/L ascorbic acid, 100 U/ml penicillin, and 100 µg/ml streptomycin further supplemented with 100 ng/ml IGF1 (AF-100-11), 10 ng/ml FGF-2 (AF-100-18B), 5 ng/ml VEGF₁₆₅ (AF-100-20) and 5 ng/ml TGF-β₁ (AF-100-21C; culture days 0-3 only). All growth factors were purchased from Peprotech.

**Isometric force measurements.** Force of contraction (FOC, i.e., systolic peak force minus diastolic tension) was measured under isometric conditions in organ baths at 37°C in gassed (5% CO₂/95% O₂) Tyrode’s solution (containing in mmol/L: 120 NaCl, 1 MgCl₂, 0.2 CaCl₂, 5.4 KCl, 22.6 NaHCO₃, 4.2 NaH₂PO₄, 5.6 glucose, and 0.56 ascorbate) as previously described.[2] EHM were analyzed at 1 Hz with 5 ms square pulses of 200 mA electrical current. EHM were mechanically stretched at intervals of 125 µm until the maximum twitch force was observed (Lmax) at 2 mmol/L calcium. Subsequently, maximal inotropic capacity was investigated under increasing extracellular calcium concentrations (0.2-4 mmol/L).

**Statistical Analyses.** Data are displayed as means with standard error of the mean (SEM) unless stated otherwise. n indicates the number of sample/biological replicates analyzed. Statistical analyses were used for each data set as indicated in the Figure legends using Graph Pad Prism. A P-value of < 0.05 was considered as an indication for significant differences between investigated groups.
Synthesis Schemes

Scheme 1: Chalcones 1, 2, and Synthesis of 5o

4’-Hydroxychalcone (4’HC)
(commercially available)

Isoquirtigenin (Iso)
(commercially available)

| 5   | a   | b   | c  | d  | e  | f  | g  | h  | i  | j  | k  | l  | m  | n  | o  |
|-----|-----|-----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| R1  | OMe | OMe | OMe | Me | OH | OH | OMe | NO2 | F  | NO2 | NO2 | NO2 | OMe | F  | F  |
| R2  | H   | H   | H   | H  | H  | H  | H  | H  | H  | H  | H  | H  | Br | H  | H  |
| R3  | H   | H   | H   | H  | H  | H  | H  | H  | H  | H  | H  | H  | H  | OMe| OH |
| R4  | F   | OMe | NaC | H  | F  | OMe | NaC | CO2 | OMe | CO2 | Me | F  | OH | Me | H  |
| R5  | H   | H   | H   | H  | H  | H  | H  | H  | H  | H  | H  | F  | H  | H  | H  |
(E)-3-(4-Fluorophenyl)-1-(4-methoxyphenyl)prop-2-en-1-one (5a):

$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 3.87 (s, 3H), 6.93 – 7.01 (m, 2H), 7.00 – 7.12 (m, 2H), 7.46 (d, $J = 15.6$ Hz, 1H), 7.56 – 7.66 (m, 2H), 7.75 (d, $J = 15.6$ Hz, 1H), 7.98 – 8.08 (m, 2H).

$^{13}$C NMR (126 MHz, CDCl$_3$): $\delta$ 55.4 (CH$_3$), 113.8 (CH), 115.8 (CH), 116.0 (CH), 121.5 (CH), 130.11 (CH), 130.18 (CH), 130.71 (CH), 130.9 (CH), 131.26 (CH), 131.28 (C), 142.5 (CH), 162.8 (C), 164.8 (C), 163.4 (C), 188.3 (C=O).

C$_{16}$H$_{13}$FO$_2$ (256.2716) [M+Na]$^+$ calc.: 279.0791
ESI-HRMS found: 279.0792

(E)-1,3-Bis(4-methoxyphenyl)prop-2-en-1-one (5b):

$^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 3.85 (s, 3H), 3.88 (s, 3H), 6.91 – 6.95 (m, 2H), 6.96 – 7.00 (m, 2H), 7.43 (d, $J = 15.6$ Hz, 1H), 7.57 – 7.63 (m, 2H), 7.78 (d, $J = 15.6$ Hz, 1H), 8.00 – 8.06 (m, 2H).

$^{13}$C NMR (126 MHz, CDCl$_3$): $\delta$ 55.4 (OCH$_3$), 113.7 (CH), 114.3 (CH), 119.5 (C), 127.7 (C), 129.9 (CH), 130.5 (CH), 131.2 (C), 143.6 (C), 161.34 (C), 163.1 (C), 188.5 (C=O).

C$_{17}$H$_{16}$O$_3$ (268.3071) [M+Na]$^+$ calc.: 291.0993
ESI-HRMS found: 291.0992
(E)-N-(4-(3-(4-Methoxyphenyl)-3-oxoprop-1-en-1-yl)phenyl)acetamide (5c):

$^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 2.20 (s, 3H), 3.89 (s, 3H), 6.95 – 7.01 (m, 2H), 7.48 (d, $J = 15.6$ Hz, 1H), 7.55 (s, 1H), 7.54 – 7.61 (m, 4H), 7.75 (d, $J = 15.6$ Hz, 1H), 8.00 – 8.06 (m, 2H).

$^{13}$C NMR (126 MHz, CDCl$_3$): $\delta$ 24.7 (CH$_3$), 55.5 (OCH$_3$), 113.7 (CH), 119.6 (CH), 120.7 (CH), 129.2 (CH), 130.6 (C), 131.0 (C), 139.7 (C), 143.2 (CH), 163.2 (C), 168.2 (CO), 188.5 (CO).

C$_{18}$H$_{17}$NO$_3$ (295.3325) [M+Na]$^+$ calc.: 318.1103

ESI-HRMS found: 318.1101

(E)-3-(4-Fluorophenyl)-1-(p-tolyl)prop-2-en-1-one (5d):

$^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 2.44 (s, 3H), 7.06 – 7.15 (m, 2H), 7.31 (dt, $J = 8.0$, 0.8 Hz, 2H), 7.46 (d, $J = 15.7$ Hz, 1H), 7.59 – 7.67 (m, 2H), 7.77 (d, $J = 15.7$ Hz, 1H), 7.89 – 7.98 (m, 2H).

$^{13}$C NMR (126 MHz, CDCl$_3$): $\delta$ 21.7 (CH$_3$), 115.9 (CH), 116.0 (CH), 121.7 (CH), 128.5 (CH), 129.2 (CH), 130.1 (CH), 131.1 (C), 135.4 (CH), 142.9 (CH), 143.5 (C), 162.8 (C), 164.8 (C), 189.5 (CO).

C$_{16}$H$_{13}$FO (240.2722) [M+Na]$^+$ calc.: 263.0838

ESI-HRMS found: 263.0843
(E)-3-(4-Methoxyphenyl)-1-(4-hydroxyphenyl)prop-2-en-1-one (5e)\[^3\]:
\[\text{C}_{16}\text{H}_{14}\text{O}_3\] (254.2806)

(E)-N-(4-(3-(4-Hydroxyphenyl)-3-oxoprop-1-en-1-yl)phenyl)acetamide (5f):
\[\text{C}_{17}\text{H}_{15}\text{NO}_3\] \([\text{M}+\text{H}]^+\) calc.: 282.1118
ESI-HRMS found: 282.1125

Methyl (E)-4-(3-(4-methoxyphenyl)-3-oxoprop-1-en-1-yl)benzoate (5g):
\[\text{C}_{18}\text{H}_{16}\text{O}_4\] \([\text{M}+\text{Na}]^+\) calc.: 319.0953
ESI-HRMS found: 319.091
(E)-3-(4-Methoxyphenyl)-1-(4-nitrophenyl)prop-2-en-1-one (5h):

$^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 3.88 (s, 3H), 6.93 – 6.99 (m, 2H), 7.36 (d, $J = 15.6$ Hz, 1H), 7.63 (d, $J = 15.6$ Hz, 1H), 7.79 – 7.86 (m, 1H), 8.10 – 8.16 (m, 2H), 8.32 – 8.38 (m, 2H).

$^{13}$C NMR (126 MHz, CDCl$_3$): $\delta$ 55.5 (OCH$_3$), 114.5 (CH), 118.9 (CH), 123.7 (CH), 123.9 (CH), 126.9 (CH), 129.2 (CH), 129.3 (C), 130.5 (CH), 143.3 (C), 146.6 (CH), 149.8 (C), 162.1 (C), 188.8 (CO).

C$_{16}$H$_{13}$NO$_4$ (283.2787)  
[M+Na]$^+$ calc.: 306.0728 
ESI-HRMS found: 306.0737

Methyl (E)-4-(3-(4-fluorophenyl)-3-oxoprop-1-en-1-yl)benzoate (5i):

$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 3.91 (s, 3H), 7.08 – 7.22 (m, 2H), 7.55 (d, $J = 15.7$ Hz, 1H), 7.66 (d, $J = 8.3$ Hz, 2H), 7.78 (d, $J = 15.7$ Hz, 1H), 8.05 (ddd, $J = 8.8$, 3.9, 2.1 Hz, 4H).

$^{13}$C NMR (126 MHz, CDCl$_3$): $\delta$ 52.2 (OCH$_3$), 115.6 (CH), 115.8 (CH), 123.5 (s), 126.5 (C), 128.1 (CH), 129.8 (CH), 130.0 (CH), 131.0 (CH), 131.5 (s), 134.1 (C), 138.8 (CH), 143.2 (CH), 164.6 (C), 166.2 (C), 166.6 (OC=O), 188.2 (C=O).

C$_{17}$H$_{13}$FO$_3$ (284.2817)  
[M+Na]$^+$ calc.: 307.0739 
ESI-HRMS found: 307.0741
\((E)-3-(4\text{-Fluorphenyl})-1-(4\text{-nitrophenyl})\text{prop-2-en-1-one (5j)}^{[4]}:\)
\(\text{C}_{15}\text{H}_{10}\text{FNO}_{3}\) (271.2432)

\((E)-3-(4\text{-Hydroxyphenyl})-1-(4\text{-nitrophenyl})\text{prop-2-en-1-one (5k)}^{[5]}:\)
\(\text{C}_{15}\text{H}_{11}\text{NO}_{4}\) (269.2521)

\((E)-1-(4\text{-Nitrophenyl})-3-(p\text{-tolyl})\text{prop-2-en-1-one (5l)}:\)
\(\text{H NMR} \ (500 \text{ MHz, CDCl}_3): \delta \ 2.41 \text{ (s, 3H)}, 7.25 \text{ (dt, } J = 8.4, 0.8 \text{ Hz, 2H}), 7.44 \text{ (d, } J = 15.7 \text{ Hz, 1H}), 7.53 - 7.59 \text{ (m, 2H)}, 7.83 \text{ (d, } J = 15.7 \text{ Hz, 1H}), 8.11 - 8.17 \text{ (m, 2H)}, 8.32 - 8.39 \text{ (m, 2H)}.
\(\text{C}_{16}\text{H}_{13}\text{NO}_{3}\) (267.2793) \ [M+H]^+ \text{ calc.: 268.0965}
\text{ESI-HRMS found: 268.0968}
(E)-3-(2-Bromo-5-fluorophenyl)-1-(4-methoxyphenyl)prop-2-en-1-one (5m):

$^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 3.89 (s, 3H), 6.94 – 7.02 (m, 3H), 7.38 – 7.45 (m, 2H), 7.58 (dd, $J = 8.8$, 5.3 Hz, 1H), 7.98 – 8.07 (m, 3H).

$^{13}$C NMR (126 MHz, CDCl$_3$): $\delta$ 55.5 (OCH$_3$), 113.8 (CH), 114.2 (CH), 114.4 (CH), 118.3 (CH), 119.8 (C), 125.7 (CH), 130.4 (CH), 130.8 (CH), 134.6 (CH), 136.8 (C), 140.9 (CH), 160.7 (C), 162.7 (C), 163.5 (C), 187.8 (CO).

**C$_{16}$H$_{12}$BrFO$_2$** (335.1677)  
[M+H]$^+$  
calc.: 335.0081  
ESI-HRMS  
found: 335.0077

(E)-3-(3-Methoxyphenyl)-1-(4-fluorphenyl)prop-2-en-1-one (5n)\(^6\):

**C$_{16}$H$_{13}$FO$_2$** (256.2716)

(E)-3-(3-Hydroxyphenyl)-1-(4-fluorphenyl)prop-2-en-1-one (5o)\(^{5a, 7}\):

**C$_{15}$H$_{11}$FO$_2$** (242.2450)
Scheme 2: Synthesis of 6a-c

(2E)-3-(4-Fluorophenyl)-1-(4-hydroxyphenyl)prop-2-en-1-one (6a)[10]: BBr₃ (2.34 mL, 2.34 mmol) in CH₂Cl₂ (3 mL) was added dropwise to a solution of 5a[11] (200 mg, 0.78 mmol) in CH₂Cl₂ (3 mL) at 0°C. The reaction mixture was stirred at room temperature for 1.5 h and the reaction quenched with MeOH at 0°C, then the mixture was diluted with water and extracted with CH₂Cl₂ (3 x). The organic phase was dried (Na₂SO₄) and concentrated. The residue was purified by column chromatography on silica gel using 5 – 20% ethyl acetate in petroleum ether to obtain compound 6a (91 mg, Yield: 48%) as a light orange solid.

¹H NMR (300 MHz, DMSO-d₆) δ 10.36 (s, 1H), 8.06 (d, J = 8.7 Hz, 2H), 7.97 – 7.89 (m, 2H), 7.85 (d, J = 15.6 Hz, 1H), 7.67 (d, J = 15.6 Hz, 1H), 7.27 (t, J = 8.8 Hz, 2H), 6.90 (d, J = 8.7 Hz, 2H).

¹³C NMR (126 MHz, DMSO-d₆) δ 186.78, 163.92, 161.95, 141.15, 131.35, 130.90, 130.70, 128.91, 121.92, 115.61, 115.16.

C₁₅H₁₁FO₂ (242.2450) [M+Na]⁺ calc.: 265.0635

ESI-HRMS found: 265.0635
A mixture of compound 5a (300 mg, 1.17 mmol), NH₂OH-HCl (244 mg, 3.51 mmol) and pyridine (1 mL) in EtOH (10 mL) was heated at 60°C for 16 h. Then the mixture was concentrated in vacuo, the residue acidified with 1N aq HCl and extracted with ethyl acetate (3 x). The organic phase was dried (Na₂SO₄) and evaporated in vacuo. The obtained crude material was purified by column chromatography on silica gel using 5 – 15% ethyl acetate in petroleum ether to get compound 6b (314 mg, Yield: 99%) as a white solid.

\[ \text{C}_{16}\text{H}_{14}\text{FNO}_2 \] (271.2863) \[ [\text{M+H}]^+ \] calc.: 272.1081

ESI-HRMS found: 272.1081

A mixture of compound 5a (300 mg, 1.17 mmol), MeONH₂-HCl (293 mg, 3.51 mmol) and pyridine (1 mL) in EtOH (10 mL) was heated at 60°C for 16 h. The mixture was concentrated in vacuo, the obtained residue acidified with 1N aq HCl and extracted with ethyl acetate (3 x). The organic phase was dried (Na₂SO₄) and evaporated in vacuo. The obtained crude material was purified by column chromatography on silica gel using 5 – 15% ethyl acetate in pet ether to get compound 6c (321 mg, Yield: 96%) as a white solid.

\[ \text{C}_{17}\text{H}_{16}\text{FNO}_2 \] (285.3128) \[ [\text{M+H}]^+ \] calc.: 286.1239

ESI-HRMS found: 286.1238
Scheme 3: Synthesis of 6d and 6e

Ethyl (2Z,4E)-5-(4-fluorophenyl)-3-(4-methoxyphenyl)penta-2,4-dienoate (6d): A solution of triethylphosphonoacetate (874 mg, 3.9 mmol) in dimethoxyethane (1 mL) and a solution of compound 5a (1 g, 3.9 mmol) in dimethoxyethane (7 mL) were successively added to a suspension of 60% NaH (156 mg, 3.9 mmol) in dimethoxyethane (7 mL) at room temperature. The reaction mixture was heated at 80°C for 4 h, then cooled to room temperature, diluted with water (100 mL), and extracted with CH2Cl2 (3 x). The organic phase was dried (Na2SO4) and evaporated in vacuo. The obtained crude material was purified by column chromatography on silica gel using 5 – 10% ethyl acetate in petroleum ether to afford compound 6d (206 mg, Yield: 16%) as a colourless thick liquid.

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 8.40 (dt, $J$ = 16.2, 0.8 Hz, 1H), 7.50 – 7.38 (m, 2H), 7.30 (d, $J$ = 8.7 Hz, 2H), 6.99 (t, $J$ = 8.7 Hz, 2H), 6.91 (d, $J$ = 8.7 Hz, 2H), 6.59 (d, $J$ = 16.2 Hz, 1H), 5.78 (d, $J$ = 0.9 Hz, 1H), 4.22 (q, $J$ = 7.1 Hz, 2H), 3.84 (s, 3H), 1.32 (t, $J$ = 7.1 Hz, 3H).

$^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 166.35, 163.84, 161.86, 159.92, 155.27, 153.70, 137.90, 132.87, 132.12, 130.19, 128.96, 126.00, 117.03, 115.59, 113.63, 59.96, 55.33, 14.45.

C$_{20}$H$_{19}$FO$_3$ (326.3615) [M+Na]$^+$ calc.: 349.1205 ESI-HRMS found: 349.1210
(2Z,4E)-5-(4-Fluorophenyl)-3-(4-methoxyphenyl)penta-2,4-dienoic acid (6e): A mixture of compound 6d (120 mg, 0.37 mmol) and NaOH (147 mg, 3.67 mmol) in EtOH (6 mL) and water (1.5 mL) was heated at 80°C for 4 h. The reaction mixture was cooled to room temperature, acidified with 2N HCl, extracted with ethyl acetate (3 x). The organic phase was dried (Na₂SO₄) and concentrated under vacuum. The resulting solid was washed with pet ether to get the product 6e (97 mg, Yield: 90%) as a white solid.

¹H NMR (300 MHz, DMSO-d₆) δ 12.27 (s, 1H), 8.29 (d, J = 16.3 Hz, 1H), 7.53 (d, J = 8.7, 2H), 7.33 (d, J = 8.6 Hz, 2H), 7.21 (t, J = 8.9 Hz, 2H), 7.01 (d, J = 8.7 Hz, 2H), 6.59 (d, J = 16.3 Hz, 1H), 5.72 (s, 1H), 3.81 (s, 3H).

¹³C NMR (126 MHz, DMSO-d₆) δ 166.68, 163.01, 161.05, 159.48, 153.50, 136.40, 132.69, 131.28, 129.83, 128.85, 125.83, 117.88, 115.59, 113.74, 55.07.

C₁₈H₁₅FO₃ (298.3083) [M+Na]+ calc.: 321.0893
ESI-HRMS found: 321.0897
Scheme 4: Synthesis of 6f and 6g

3-(4-Fluorophenyl)-1-(4-methoxyphenyl)propan-1-one (7)[13]: To a solution of 5a (200 mg, 0.78 mmol) in EtOH (50 mL) was added a solution of ammonium acetate (6 g, 78 mmol) in water (8 mL). Then zinc powder (382 mg, 5.85 mmol, 7.5 eq) was put in at room temperature in 5 portions at intervals of 15 min. After completion of the reaction within 70 min (monitored by TLC), the mixture was filtered, the filter cake washed with EtOH (2 x 15 mL), and the combined filtrates concentrated in vacuo. The residue was diluted with water, extracted with ethyl acetate (3 x) and the organic phase was dried (Na₂SO₄) and concentrated in vacuo. Crude product was purified by column chromatography on silica gel using 5% ethyl acetate in pet ether to obtain compound 7 (133 mg, Yield: 73%) as a colourless thick liquid.

\[ \text{C}_16\text{H}_{15}\text{FO}_2 (258.2875) \]

**1H NMR** (300 MHz, CDCl₃) δ 7.99 – 7.79 (m, 2H), 7.27 – 7.06 (m, 2H), 7.03 – 6.79 (m, 4H), 3.84 (s, 3H), 3.20 (ddd, \( J = 8.0, 6.9, 0.9 \) Hz, 2H), 3.01 (t, \( J = 7.5 \) Hz, 3H).

**13C NMR** (126 MHz, CDCl₃) δ 197.33, 163.33, 162.17, 160.23, 136.93, 130.15, 129.81, 129.69, 115.10, 113.65, 55.45, 40.07, 29.49.

\[ [\text{M+Na}]^+ \]

clac.: 281.0949

ESI-HRMS found: 281.0948
Ethyl (2Z)-5-(4-fluorophenyl)-3-(4-methoxyphenyl)pent-2-enoate (6f): Triethylphosphonoacetate (1.15 mL, 5.8 mmol) was added to a suspension of 60% NaH (232 mg, 5.8 mmol) in dimethoxyethane (20 mL) at room temperature and the mixture stirred for 1 h. A solution of compound 7 (500 mg, 1.93 mmol) in dimethoxyethane (5 mL) was slowly added at room temperature and the reaction mixture heated at 80°C for 4 h. After cooling to room temperature, the mixture was diluted with water (100 mL) and extracted with CH₂Cl₂ (3 x). The organic phase was dried (Na₂SO₄) and evaporated in vacuo. The obtained crude material was purified by column chromatography on silica gel using 5 – 10% ethyl acetate in petroleum ether to afford compound 6f (241 mg, Yield: 38%) as a colourless thick liquid.

**1H NMR** (300 MHz, CDCl₃) δ 7.40 (d, J = 9.0 Hz, 2H), 7.20 – 7.11 (m, 2H), 6.97 – 6.85 (m, 4H), 6.02 (s, 1H), 4.17 (q, J = 7.1 Hz, 2H), 3.83 (s, 3H), 3.40 – 3.26 (m, 2H), 2.75 – 2.62 (m, 2H), 1.29 (t, J = 7.1 Hz, 3H).

**13C NMR** (126 MHz, CDCl₃) δ 166.33, 162.17, 160.35, 158.43, 137.14, 132.91, 129.76, 127.94, 116.09, 114.86, 113.97, 59.79, 55.37, 34.55, 32.95, 14.44.

**C₂₀H₂₁FO₃** (328.3773) [M+Na]⁺ calc.: 351.1369

ESI-HRMS found: 351.1367

(2Z)-5-(4-Fluorophenyl)-3-(4-methoxyphenyl)pent-2-enoic acid (6g): A mixture of 6f (61 mg, 0.185 mmol) and NaOH (74 mg, 1.85 mmol) in EtOH (4 mL) and water (1 mL) was heated at 80°C for 4 h. The reaction mixture was cooled to room temperature, acidified with 2N HCl, and extracted with ethyl acetate (3 x). The organic phase was dried (Na₂SO₄) and concentrated.
in vacuo. The resulting solid was washed with petroleum ether to get the desired compound 6g (51 mg, Yield: 93%) as a white solid.

$^1$H NMR (300 MHz, DMSO-$d_6$) δ 12.06 (s, 1H), 7.52 (d, $J = 8.8$ Hz, 2H), 7.22 (dd, $J = 8.6$, 5.7 Hz, 2H), 7.07 (t, $J = 8.9$ Hz, 2H), 6.98 (d, $J = 8.8$ Hz, 2H), 6.00 (s, 1H), 3.80 (s, 3H), 3.37 – 3.26 (m, 2H), 2.69 – 2.57 (m, 2H).

$^{13}$C NMR (126 MHz, DMSO-$d_6$) δ 166.91, 161.32, 159.80, 159.41, 156.88, 137.18, 131.93, 129.64, 127.75, 116.16, 114.60, 113.92, 55.10, 33.90, 31.60.

$\text{C}_{18}\text{H}_{17}\text{FO}_3$ (300.3242)  
$[^{\text{M+Na}}]^+$  
calc.: 323.1050

ESI-HRMS  
found: 323.1054
Scheme 5: Synthesis of 6h

2-Fluoro-1-(4-methoxyphenyl)ethan-1-one (9): A mixture of 2-bromo-4-methoxyacetophenone (500 mg, 2.18 mmol; labelled as compound 8), KF (634 mg, 10.9 mmol) and 18-crown-6 ether (115 mg, 0.44 mmol) in CH$_3$CN (10 mL) was heated at 80°C for 20 h under Argon atmosphere. The reaction mixture was concentrated and the residue was diluted with water, extracted with ethyl acetate (3 x). Combined organic layer was washed with brine, dried (Na$_2$SO$_4$), and concentrated under vacuum. Crude product was purified on silica gel column chromatography using 0 – 5% ethyl acetate in pet ether to get the desired compound 9 (310 mg, Yield: 85%) as a white solid.

$^{1}$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.93 – 7.78 (m, 2H), 7.03 – 6.82 (m, 2H), 5.56 – 5.37 (m, 2H), 3.86 (s, 3H).

C$_9$H$_9$FO$_2$ (168.1650)

(2Z)-2-Fluoro-3-(4-fluorophenyl)-1-(4-methoxyphenyl)prop-2-en-1-one (6h): 20% aq KOH (0.1 mL) was added to a solution of compound 9 (100 mg, 0.59 mmol) and 4-fluorobenzaldehyde (73.5 mg, 0.59 mmol) in MeOH (2 mL) at room temperature and the mixture was stirred at room temperature for 16 h. Ice cold water was added and the reaction mixture acidified with 2N aq HCl. The resulting precipitate was filtered off, washed with water...
(4 x 7 mL) and allowed to dry at the air. The product was purified by column chromatography on silica gel using 5% ethyl acetate in petroleum ether to afford compound 6h (56 mg, Yield: 35%) as a white solid.

$^1$H NMR (300 MHz, CDCl₃) $\delta$ 7.96 – 7.90 (m, 2H), 7.72 – 7.63 (m, 2H), 7.09 (t, $J = 8.7$ Hz, 2H), 6.96 (d, $J = 8.9$ Hz, 2H), 6.82 (d, $J = 36.4$ Hz, 1H), 3.87 (s, 3H).

$^{13}$C NMR (126 MHz, CDCl₃) $\delta$ 185.61, 164.08, 163.53, 162.08, 155.69, 153.51, 132.36, 131.84, 128.52, 127.73, 117.37, 115.93, 113.73, 55.52.

C₁₆H₁₂F₂O₂ (274.2621) [M+Na]$^+$ calc.: 297.0700

ESI-HRMS found: 297.0698
(2Z)-2-Bromo-3-(4-fluorophenyl)-1-(4-methoxyphenyl)prop-2-en-1-one (10): Et₄NBr (984 mg, 4.68 mmol) was added to a stirred suspension of Dess–Martin periodinane (DMP) (1.98 g, 4.68 mmol) in CH₂Cl₂ (8 mL) at room temperature and stirring was continued for 10 min with formation of a pale yellow solution. Compound 5a (1 g, 3.9 mmol) was added to the mixture in portions at room temperature and stirring was continued at room temperature for 24 h. Then the mixture was diluted with CH₂Cl₂ (50 mL), washed with a sat solution of NaHSO₃ and a sat solution of NaHCO₃ and then with water. The organic phase was dried (Na₂SO₄) and concentrated in vacuo. Purification by column chromatography on silica gel using 5% ethyl acetate in petroleum ether yielded compound 10 (970 mg, Yield: 74%) as a pale-yellow thick liquid.

**1H NMR** (300 MHz, CDCl₃) δ 7.96 – 7.89 (m, 2H), 7.25 (s, 1H), 7.18 – 7.09 (m, 2H), 6.91 – 6.82 (m, 4H), 3.83 (s, 3H).

**13C NMR** (126 MHz, CDCl₃) δ 190.34, 164.49, 163.39, 161.40, 133.91, 132.37, 129.88, 126.09, 115.64, 114.21, 113.81, 55.56.

**C₁₆H₁₂BrFO₂** (335.1677)  
[M+Na]⁺  
calc.: 356.9886  
ESI-HRMS found: 356.9897
(2Z)-3-(4-Fluorophenyl)-1-(4-methoxyphenyl)-2-(trifluoromethyl)prop-2-en-1-one (6i): A mixture of compound 10 (200 mg, 0.7 mmol), CuI (136 mg, 0.72 mmol) and methyl 2,2-difluoro-2-(fluorosulfonyl) acetate (0.55 mL, 4.35 mmol) in dry DMF (20 mL) was heated at 75°C for 3 days under an argon atmosphere. Then, the reaction mixture was cooled to room temperature, diluted with ether (50 mL), filtered, the filter cake washed with ether and the combined filtrates mixed with water (150 mL) and extracted with ether. The organic phase was washed with brine, dried (Na₂SO₄) and concentrated in vacuo. Purification by column chromatography on silica gel using 10% ethyl acetate in petroleum ether yielded compound 6i (89 mg, Yield: 46%) as colourless thick liquid.

**1H NMR** (300 MHz, CDCl₃) δ 7.85 (d, J = 9.0 Hz, 2H), 7.36 (d, J = 1.8 Hz, 1H), 7.29 – 7.19 (m, 2H), 6.92 – 6.79 (m, 4H), 3.81 (s, 3H).

**13C NMR** (126 MHz, Chloroform-d) δ 190.61, 164.54, 164.29, 162.29, 134.63 (q, J = 5.9 Hz), 132.03, 131.46 (d, J = 8.6 Hz), 128.33, 128.24 (d, J = 3.4 Hz), 123.29, 121.12, 115.85 (d, J = 21.9 Hz), 114.12, 55.53.

C₁₇H₁₂F₄O₂ (324.2696) [M+Na]⁺ calc.: 347.0667
ESI-HRMS found: 347.0666
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