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Synthesis and Cannabinoid Receptor Activity of Ketoalkenes from *Echinacea pallida* and Nonnatural Analogues

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Abstract: Despite its popularity and widespread use, the efficacy of *Echinacea* products remains unclear and controversial. Among the various compounds isolated from *Echinacea*, ketoalkenes and ketoalkenynes exclusively found in the pale purple coneflower (*E. pallida*) are major components of the extracts. In contrast to *E. purpurea* alkamides, these compounds have not been synthesized and studied for immunostimulatory effects. We present a practical and useful synthetic approach to the ketoalkenes using palladium-catalyzed cross-coupling reactions and the pharmaceutical results at the human cannabinoid receptors. The synthetic route developed provides overall good yields for the ketoalkenes and is applicable to other natural products with similar 1,4-diene motifs. No significant activity was observed at either receptor, indicating that the ketoalkenes from *E. pallida* are not responsible for immunomodulatory effects mediated via the cannabinergic system. However, newly synthesized non-natural analogues showed micro-molar potency at both cannabinoid receptors.

Keywords: cannabinoid receptors · cross-coupling · *Echinacea pallida* · palladium · total synthesis

Introduction

Echinacea extracts are one of the top selling herbal remedies in the USA and Europe to treat upper respiratory tract infections.[1] Some studies show that *Echinacea* is an effective immunostimulant,[2,3] while others do not,[4] and much controversy on the efficacy of these extracts exists.[5] The extracts consist of a complex mixture of caffeic acid derivatives, glycoproteins, polysaccharides and alkamides,[6] from 3 species of *Echinacea*: *E. purpura*, *E. pallida* and *E. angustifolia*.[7] The major components of *E. purpurea* and *E. angustifolia* extracts are alkamides, while *E. pallida* extracts consist of mainly ketoalkenes and ketoalkenynes.[8]

Echinacea products on the market may contain only one of the above mentioned species or mixtures of all 3 species, and are not required to guarantee content, quality, variability, or contamination of the supplement.[9] Studies have shown that *Echinacea* extracts are wide-spectrum immunomodulators, but the exact phytochemical responsible for the active nature of the extracts has not been identified.[10]

Determining the activity of *Echinacea* extracts as an effective herbal remedy is of high interest and intense research on the subject is being conducted, but further studies to elucidate the mode of action are required.[1–3,7,10]

Alkamides have been isolated and tested for biological activity and have been shown to bind with high affinity to the human cannabinoid 2 receptor (CB_2R), and may be considered a molecular mode of action explaining the immunomodulatory effects.[7,11,12] In order to investigate the biological activity of ketoalkenes and ketoalkenynes found in many *Echinacea* extracts, we report a versatile synthetic route to the ketones found in *E. pallida* (Figure 1), and present the results from functional GTPase assays of these newly synthesized compounds at the CB_1R and the CB_2R. To determine any structure activity relationship responsible for receptor activation, we describe the synthesis and testing results of structurally similar ketone containing analogues.

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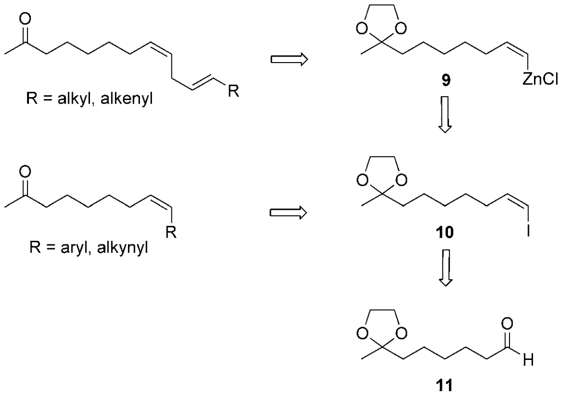
Figure 1. *Echinacea pallida*.



Scheme 1. Retrosynthetic analysis of the natural products found in *E. pallida* and their conjugated analogues.

Results and Discussion

Synthesis: In contrast to other *Echinacea* species, only *E. pallida* contains ketoalkenes and ketoalkenynes (Figure 2).

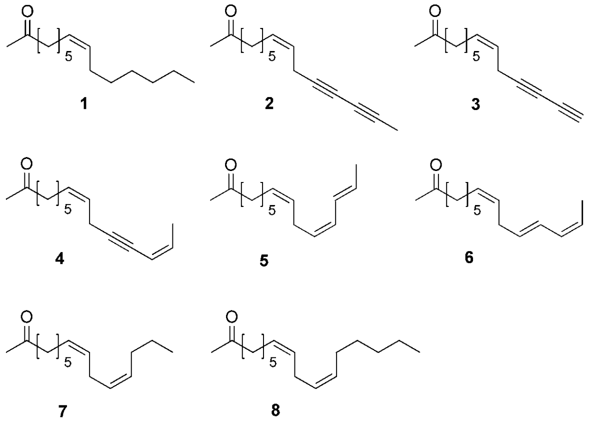
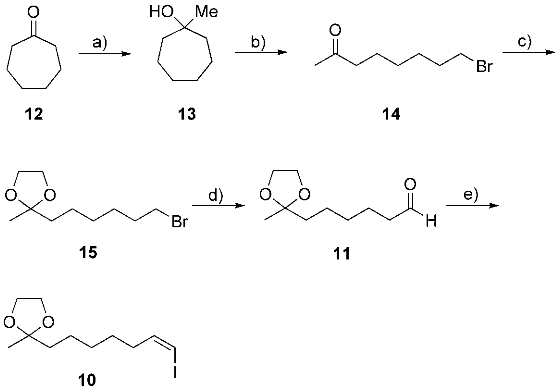


Figure 2. Ketoalkenes and ketoalkenynes isolated from *E. pallida*.

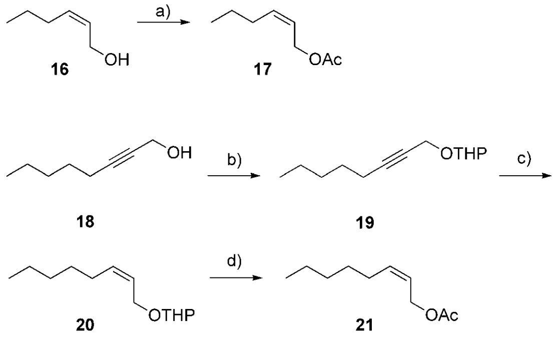
The syntheses of monoalkene **1** and bisacetylenes **2** and **3** have recently been reported by Dickschat et al[13] and Kraus et al.[14,15] Despite the large interest in obtaining synthetic samples of the remaining unsaturated compounds for biological evaluation the other ketoalkenes have not been prepared so far. We therefore planned to synthesize the polyenes **5**--**8** and test their activity at the human cannabinoid receptors in a functional GTPase assay. Our synthetic strategy was to obtain the 1,4-diene motifs via Pd-catalyzed cross-coupling reactions of appropriate allyl acetates with vinyl zinc halides that were derived from aldehyde **11**. This route not only enabled us to prepare all compounds via one aldehyde precursor but also allowed for access to non-natural conjugated analogues via Sonogashira and Suzuki cross-coupling reactions of the vinyl iodide intermediate **10** (Scheme 1).

Aldehyde **11** was previously synthesized from methylacetoacetate in 8 steps with an overall yield of 38%.[16] Alternatively, we started from commercially available cycloheptanone which was converted into methylcycloheptanol in quantitative yield. Retro-Barbier fragmentation[17] and protection of the resulting keto group resulted in bromide **15**, which was then oxidized to aldehyde **11** (Scheme 2).[18] The synthesis of aldehyde **11** could thus be shortened by 4 steps and the overall yield increased to 59%. Conversion to the vinyl iodide **10** was finally achieved by the method of Stork and Zhao in good yield and high stereoselectivity.[19]



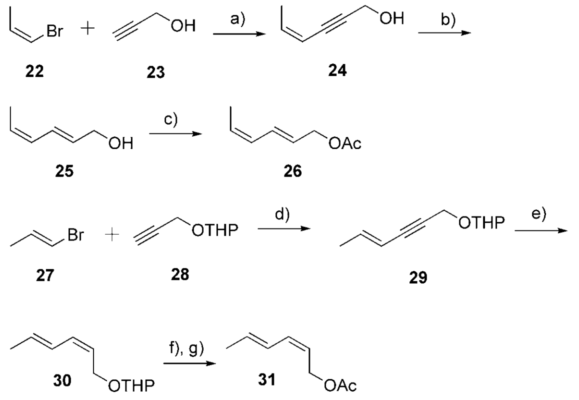
Scheme 2. Improved synthesis of aldehyde **11** and its conversion into the vinyl iodide intermediate **10**. a) MeLi (1.1 equiv), Et_2O, −78°C, 100%; b) Br_2, K_2CO_3, $CHCl_3$, 0°C, 93%; c) ethylene glycol, *p*TsOH, toluene, reflux, 92%; d) trimethylammonium-*N*-oxide, DMSO, RT, 69%; e) ICH_2PPh_3I, NaHMDS, RT; **11**, −78°C, 81%.

The synthesis of the 1,4-dienes required the selective preparation of *E*- and *Z*-allyl acetates. (*Z*)-Hex-2-enyl acetate **17** was directly obtained from the commercially available corresponding alcohol, whereas (*Z*)-oct-2-enyl acetate **21** was prepared by hydroboration of the THP protected propargyl alcohol **19**, followed by deprotection and acetylation. Reduction of the propargyl acetate was also attempted but turned out to be less selective and gave higher amounts of the undesired *E*-isomer. Direct conversion of the THP protected alcohol **20** into the acetate[20] **21** was inferior to the sequence of deprotection followed by acetylation because the yield was lower and separation was more difficult (Scheme 3).



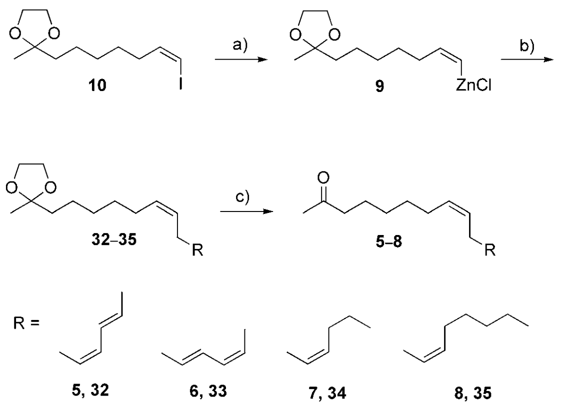
Scheme 3. Synthesis of allyl acetates **17** and **21**. a) Ac_2O, DMAP, NEt_3, CH_2Cl_2, RT, 99%; b) DHP, *p*TsOH, CH_2Cl_2, RT, 96%; c) $BH_3{\cdot}Me_2S$, cyclohexene, Et_2O, 0°C to RT; **19**, 0°C to RT; HOAc, 0°C to RT, 89%; d) 1) *p*TsOH, MeOH, RT, 87%; 2) Ac_2O, DMAP, NEt_3, CH_2Cl_2, RT, 99%.

Diene coupling partners **26** and **31** were prepared from 1,3-enynes **24** and **29**, which were synthesized by Sonogashira cross-coupling of *cis*- or *trans*-1-bromo-1-propene and (THP-protected) propargyl alcohol. The *E*-allyl acetate **26** was then prepared via trans-hydroalumination while the *Z*-isomer **31** was again synthesized as described above (Scheme 4).



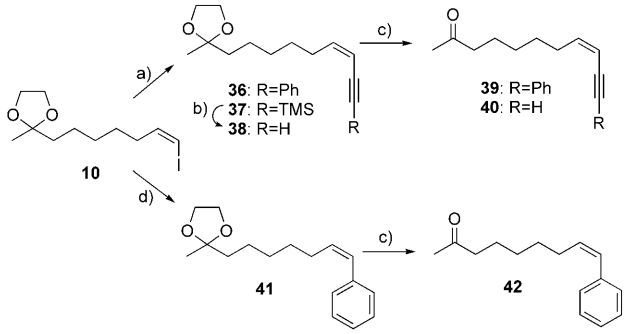
Scheme 4. Synthesis of the diene coupling partners. a) $[PdCl_2(PPh_3)_2]$ (2 mol%), CuI (4 mol%), piperidine, RT, 91%; b) $LiAlH_4$, THF, −10°C to RT, 97%; c) Ac_2O, DMAP, NEt_3, CH_2Cl_2, RT, 99%; d) $[PdCl_2(PPh_3)_2]$ (2 mol%), CuI (4 mol%), piperidine, RT, 92%; e) $BH_3{\cdot}Me_2S$, cyclohexene, Et_2O, 0°C to RT; **29**, 0°C to RT; HOAc, 0°C to RT, 91%; f) *p*TsOH, MeOH, RT, 84%; g) Ac_2O, DMAP, NEt_3, CH_2Cl_2, RT, 99%.

Pd-catalyzed cross-coupling between the vinyl zinc chloride **9** and the corresponding allyl acetates gave the desired 1,4-dienes which were finally deprotected to yield the ketoalkenes **5–8** (Scheme 5).

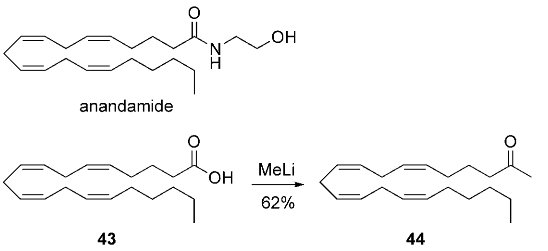


Scheme 5. Synthesis of the 1,4-dienes **5–8** via Pd-catalyzed cross-coupling. a) *t*BuLi, THF, −78°C, then $ZnCl_2$, −78°C to RT; b) $[PdCl_2(PPh_3)_2]$ (3 mol%), ROAc, RT, 20–27%; c) 1 M HCl, THF, RT, quant.

The ketoalkenes of *E. pallida* share two common structural motifs: a skipped conjugation in the unsaturated part and the combination of a methyl ketone and a polyene moiety connected by a flexible alkyl chain. In order to explore whether the skipped conjugation plays an important role in a potential mechanism of action we prepared a series of conjugated analogues via Suzuki and Sonogashira cross-coupling reactions of the vinyl iodide intermediate **10** (Scheme 6) and compared their activity at the CBRs to the natural products. We also investigated the influence of an amide versus a methylketone head group, which is the main difference between *E. purpurea* alkamides and *E. pallida* ketoalkenes. For that reason we replaced the amide group of the endogenous ligand anandamide by a simple methylketone, which was prepared by derivatization of arachidonic acid (Scheme 7).



Scheme 6. Preparation of non-natural conjugated analogues. a) RCCH (1.2 equiv), $[PdCl_2(PPh_3)_2]$ (5 mol%), CuI (5 mol%), NEt_3, RT, 67–71%; b) TBAF, THF, RT, 76%; c) 1 M HCl, THF, RT, 80–96% d) $PhB(OH)_2$ (1.1 equiv), $Pd(OAc)_2$ (5 mol%), NaOH (2.5 equiv), THF, reflux, 61%.



Scheme 7. Structure of anandamide and synthesis of its keto analogue **44**.

Pharmacology: Cannabinoid receptors couple to G_i/G_o proteins and the nucleotide exchange can be monitored as an increase in steady-state GTPase activity.[21] All synthesized compounds were tested for agonistic activity at the CBRs in a steady-state GTPase assay (see Table 1). Compounds were considered to be CBR agonists when GTPase activation was more than 20% compared to basal GTPase activity [3% (v/v) DMSO]. The maximal stimulatory effect of the most abundant endogenous CBR agonist 2-arachidonoyl glycerol (2-AG) was set at 100% to determine E_{max} values of active compounds. The second endogenous CBR agonist, anandamide (ANA), was a full agonist at the CB_1R and a partial agonist at the CB_2R. Compounds **36** and **39** are analogues of the natural ketoalkenynes from *E. pallida* with a keto group at C2, a *cis*-double bond at C8 and a triple bond at C10. Introduction of a phenyl ring in compound **39** led to partial agonism at both CBRs with a potency in the micromolar range. Protection of the keto group at C2 (**36**) resulted in a complete loss of efficacy at the CB_1R and partial agonism at the CB_2R. The potency of compound **36** at the CB_2R was in the micromolar range. Compound **44**, an analogue of the endogenous agonist anandamide, was a full agonist at both CBRs. Replacement of the isobutylamide head group by a methyl ketone even led to a higher efficacy at the CB_2R compared to anandamide. However, the $\log EC_{50}$ values of compound **44** were only in the micromolar range compared to anandamide or 2-AG. This finding suggests that substitution of the carboxyl group at C1 with a larger or an amine-containing group, for example, ethanolamine (anandamide) or glycerol (2-AG) is necessary for a high potency at CBRs.

Conclusion

We have developed a versatile synthetic route for the preparation of *E. pallida* ketoalkenes and new non-natural conjugated analogues. A particular advantage of our synthetic strategy is the possibility of preparing different conjugated and non conjugated polyene motifs found in many natural products starting from only one common precursor and following the same reaction procedures. The simplicity of the reactions involved and the inexpensive commercially available starting materials are also advantageous.

Both classes of compounds were tested for their ability to activate the human cannabinoid receptors 1 and 2 (CB_1R and CB_2R). In contrast to the alkamides of *E. purpurea*, which have been shown to be CB_2R agonists,[7] no agonistic activity at either receptor was found in the functional GTPase assay for the ketoalkenes from *E. pallida*. If the observed immunomodulatory activity of *Echinacea* products is mediated via the CBR system, *E. pallida* ketoalkenes will not contribute to this effect. However, a different mode of action for these compounds or synergic effects cannot be excluded and may be responsible for the observed physiological activity of *Echinacea* extracts. Interestingly, the non-natural phenylacetylene containing analogue **39** as well as its keto-protected precursor **36** showed micromolar activity at both receptors. The same was true for the keto analogue **44** of anandamide, indicating that an amide or ester moiety is not necessarily important for activation but for high affinity at the CBRs.

Table 1. Stimulation of GTPase activity, E_{max} values and $\log EC_{50}$ values of 2-AG, anandamide (ANA) and synthesized compounds.[a]

Compound (CBR)	GTPase activation [%]	E_{max} [%]	$\log EC_{50}$
2-AG (CB_1R)	69 ± 6	100	-6.60 ± 0.12
2-AG (CB_2R)	74 ± 25	100	-6.53 ± 0.51
ANA (CB_1R)	80 ± 8	116 ± 19	-6.80 ± 0.10
ANA (CB_2R)	49 ± 6	66 ± 15	-6.36 ± 0.44
36 (CB_1R)	<20	n.a.	n.a.
36 (CB_2R)	33 ± 3	45 ± 6	-5.23 ± 0.62
39 (CB_1R)	41 ± 20	59 ± 35	-5.28 ± 0.27
39 (CB_2R)	44 ± 11	59 ± 18	-5.84 ± 0.19
44 (CB_1R)	68 ± 6	99 ± 9	-5.71 ± 0.44
44 (CB_2R)	85 ± 7	114 ± 9	-5.46 ± 0.29

[a] GTPase activity was determined in Sf9 insect cell membranes expressing CBRs, $G\alpha_{i2}$, $G\beta_1\gamma_2$ and RGS4. Results are expressed as percentages of mean values ±S.D. compared to basal GTPase activation assessed by 3% (v/v) DMSO. E_{max} values represent the stimulation relative to the endogenous agonist 2-AG (defined as 100% stimulation). Data represents three independent experiments performed with different membrane preparations.

Experimental Section

General: Commercial reagents and starting materials were purchased from Aldrich, Fluka, Lancaster or Acros and used without further purification. THF was distilled from sodium/benzophenone. Flash chromatography was performed on Acros silica gel 60 A (35–70 μm). TLC was performed on alumina plates coated with silica gel (Merck silica gel 60 F_{254}, thickness 0.2 mm) and products were detected after staining with 15% phosphomolybdic acid in ethanol. NMR spectra were recorded with Bruker Avance 300 (^{1}H: 300.1 MHz, ^{13}C: 75.5 MHz, $T = 300$ K), Bruker Avance 400 (^{1}H: 400.1 MHz, ^{13}C: 100.6 MHz, $T = 300$ K) and Bruker Avance 600 (^{1}H: 600.1 MHz, ^{13}C: 150.1 MHz, $T = 300$ K) instruments. Chemical shifts are reported in δ ppm relative to external standards and coupling constants J are given in Hz. Abbreviations for the characterization of the signals are: s = singlet, d = doublet, t = triplet, m = multiplet, brs = broad singlet, dt = double triplet. The relative number of protons is determined by integration. Error of reported values: chemical shift 0.01 ppm (^{1}H NMR), 0.1 ppm (^{13}C NMR), coupling constant 0.1 Hz. The used solvent for each spectrum is reported. Mass spectra were recorded with Finnigan MAT SSQ 710 A (CI) and Finnigan MAT 95 (HRMS), IR spectra with a Bio-Rad FT-IR Excalibur FTS 3000 MX spectrometer and UV/Vis spectra with a Cary BIO 50 UV/Vis/NIR spectrometer (Varian). The following compounds were prepared as described above and have been reported before: 1-methylcycloheptanol (**13**),[22] 8-bromooctan-2-one (**14**),[17] 2-(6-bromohexyl)-2-methyl-1,3-dioxolane (**15**),[23] (iodomethyl)triphenylphosphonium iodide,[24] 6-(2-methyl-1,3-dioxolan-2-yl)-hexanal (**11**),[16] (*Z*)-hex-2-enyl acetate (**17**),[25] 2-(oct-2-ynyloxy)-tetrahydro-2*H*-pyran (**19**),[26] (*Z*)-oct-2-en-1-ol,[27] (*Z*)-hex-4-en-2-yn-1-ol (**24**),[28] (2*E*,4*Z*)-hexa-2,4-dien-1-ol (**25**),[28] (*E*)-2-(hex-4-en-2-ynyloxy)-tetrahydro-2*H*-pyran (**29**),[29] (2*Z*,4*E*)-hexa-2,4-dien-1-ol.[30]

General procedure A for the semi-reduction of alkynes to the corresponding (Z)-alkenes: Borane/dimethylsulfide complex (1.1 equiv) was added to dry Et_2O (≈1 M) and cooled to 0 °C. Cyclohexene (2.2 equiv) was slowly added by syringe and the mixture was stirred at 0 °C for 15 min before it was warmed to RT and stirred for 1 h. The white suspension was again cooled to 0 °C and the alkyne (1 equiv) was slowly added via syringe. The mixture was stirred for 1 h at 0 °C and 1.5 h at RT until the white precipitate completely dissolved, then the reaction mixture was again cooled to 0°C and acetic acid ($\approx$9 equiv) was slowly added. The ice bath was removed and the solution was stirred at RT for 2 h. Et_2O was added and the organic phase was washed with water (4×), dried over $MgSO_4$, concentrated and purified by flash chromatography on silica gel (petroleum ether/EtOAc).

General procedure B for the acetylation of allyl alcohols: To a 0.3 M solution of the alcohol in dry CH_2Cl_2, triethylamine (1.3 equiv), freshly distilled acetic anhydride (1.1 equiv) and catalytic amounts of DMAP were added. The mixture was stirred at RT until complete consumption of the alcohol ($\approx$4 h), diluted with CH_2Cl_2 and washed with water. The aqueous phase was extracted with CH_2Cl_2 and the organic phases were dried over $MgSO_4$. The solvent was removed and the product purified by flash chromatography on silica gel (pentane/Et_2O).

General procedure C for the Sonogashira cross-coupling of 1-bromo-1-propene and different acetylenes: To a 0.3 M solution of bromopropene (1.1 equiv) in freshly distilled piperidine, $[PdCl_2(PPh_3)_2]$ (2 mol%), the terminal acetylene (1 equiv) and CuI (4 mol%) were added. The mixture was stirred at RT until complete consumption of the acetylene, quenched with saturated solution of NH_4Cl and extracted with Et_2O. The combined organic phases were washed with brine, dried with $MgSO_4$, concentrated and purified by flash chromatography on silica gel (pentane/Et_2O).

General procedure D for the Pd-catalyzed cross-coupling of vinyl zinc chlorides and allyl acetates: Under an argon atmosphere a 0.2 M solution of vinyl iodide **11** in freshly distilled dry THF (Na/benzophenone) was cooled to −78°C. *t*BuLi (1.6 M in pentane, 2.2 equiv) was added via syringe and the mixture was stirred for 1 h at −78°C. Dry $ZnCl_2$ (1.1 equiv) was added and the mixture was slowly warmed to RT. $[PdCl_2(PPh_3)_2]$ (5 mol%) and the allyl acetate were then added and the mixture was stirred at RT overnight, concentrated under a stream of nitrogen, quenched with saturated NH_4Cl and extracted with CH_2Cl_2. The combined organic phases were washed with brine, dried with $MgSO_4$, concentrated and purified by flash chromatography on silica gel (petroleum ether/EtOAc).

General procedure E for the deprotection of 1,3-dioxolane-protected ketones: The dioxolane was dissolved in THF (5 mL) and 1 M HCl (1 mL) was added. The mixture was stirred at RT overnight, diluted with Et_2O, washed with aqueous saturated solution of $NaHCO_3$ and dried with $MgSO_4$. The solvents were removed under reduced pressure and the products were purified by flash chromatography on silica gel (petroleum ether/EtOAc).

(*Z*)-2-(7-Iodohept-6-enyl)-2-methyl-1,3-dioxolane (10): To a suspension of (iodomethyl)triphenylphosphonium iodide (5.1 g, 9.7 mmol) in dry THF (75 mL), 1 M sodium bis-(trimethylsilyl)amide in THF (11 mmol) was added. The orange suspension was cooled to −78°C and *N*,*N'*-dimethylpropyleneurea (DMPU) (5.9 mL) was added. After 15 min aldehyde **11** (1.2 g, 6.4 mmol) was added via syringe and the mixture was slowly warmed to RT. The solvent was removed under reduced pressure and the resulting brown oil was dissolved in CH_2Cl_2, washed with water and concentrated. Byproducts were removed by precipitation with petroleum ether, the mother liquor was concentrated and the product was purified by flash chromatography on silica gel to yield vinyl iodide **10** as colourless oil (1.6 g, 81%). $R_f = 0.31$ (petroleum ether/EtOAc 9:1); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 6.19$–6.12 (m, 2H; CH), 3.98–3.88 (m, 4H; OCH_2), 2.17–2.10 (m, 2H; CH_2), 1.66–1.61 (m, 2H; CH_2), 1.46–1.33 (m, 6H; CH_2), 1.31 ppm (s, 3H; CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 141.3$ (+), 110.1 (C_{quat}), 82.3 (+), 64.6 (−), 39.1 (−), 34.6 (−), 29.3 (−), 27.9 (−), 23.9 (−), 23.8 ppm (+); IR (neat): $\tilde{\nu} = 2981$, 2937, 1375, 1069 cm^{-1}; MS (CI, NH_3): *m*/*z* (%): 311 (100) $[M+H^+]$, 328 (12) $[M+NH_4^+]$.

(*Z*)-2-(Oct-2-enyloxy)-tetrahydro-2*H*-pyran (20): The compound was prepared following general procedure A and isolated as colourless oil. Yield: 1.39 g, 83%. $R_f = 0.29$ (petroleum ether/EtOAc 19:1); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 5.63$–5.50 (m, 2H), 4.64 (t, $^3J = 2.2$ Hz, 1H), 4.29–4.23 (m, 1H), 4.10–4.04 (m, 1H), 3.92–3.85 (m, 1H), 3.55–3.47 (m, 1H), 2.10–2.04 (m, 2H; CH_2), 1.88–1.50 (m, 6H; CH_2), 1.41–1.21 (m, 6H; CH_2), 0.88 ppm (t, $^3J = 6.7$ Hz, 3H; CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 133.9$, 125.7, 97.9, 62.8, 62.2, 31.4, 30.7, 29.3, 27.5, 25.5, 22.5, 19.6, 14.1 ppm; IR (neat): $\tilde{\nu} = 2925$, 2856, 1455, 1025 cm^{-1}; CI-MS (NH_3): *m*/*z* (%): 102 (100) $[DHP+NH_4^+]$, 230 (18) $[M+NH_4^+]$.

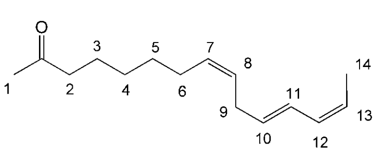
(*Z*)-Oct-2-enyl acetate (21): The compound was prepared following general procedure B and isolated as colourless oil. Yield: 0.56 g, 99%. $R_f = 0.31$ (petroleum ether/EtOAc 19:1); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 5.69$–5.48 (m, 2H; CH), 4.61 (d, $^3J = 6.6$ Hz, 2H; OCH_2), 2.12–2.03 (m, 5H; CH_2/CH_3), 1.42–1.23 (m, 6H; CH_2), 0.88 ppm (t, $^3J = 7.0$ Hz, 3H; CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 171.0$ (C_{quat}), 135.6 (+), 123.2 (+), 60.4 (−), 31.4 (−), 29.1 (−), 27.5 (−), 22.5 (−), 21.0 (+), 14.0 ppm (+); IR (neat): $\tilde{\nu} = 3024$, 2928, 1739, 1372, 1225 cm^{-1}; CI-MS (NH_3): *m*/*z* (%): 188 (100) $[M+NH_4^+]$.

(2*E*,4*Z*)-Hexa-2,4-dienyl acetate (26): The compound was prepared following general procedure B and isolated as colourless oil. Yield: 0.48 g, 99%. $R_f = 0.33$ (petroleum ether/EtOAc 9:1); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 6.65$–6.55 (m, 1H), 6.01 (dt, $^4J = 1.4$, $^3J = 11.0$ Hz, 1H), 5.73 (dt, $^3J = 7.0$, $^3J = 15.4$ Hz, 1H), 5.57 (dq, $^3J = 7.3$ Hz, $^3J = 11.0$ Hz, 1H), 4.62 (d, $^3J = 6.6$ Hz, 2H; CH_2), 2.07 (s, 3H; CH_3), 1.77 ppm (dd, $^3J = 7.1$, $^4J = 1.6$ Hz, 3H; CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 170.8$ (C_{quat}), 129.6 (+), 128.2 (+), 128.2 (+), 125.9 (+), 65.0 (−), 21.0 (+), 13.4 ppm (+); IR (neat): $\tilde{\nu} = 3023$, 1738, 1373, 1222 cm^{-1}; CI-MS (NH_3): *m*/*z* (%): 158 (10) $[M+NH_4^+]$, 98 (100) $[M+NH_4^+-HOAc]$.

2-((2*Z*,4*E*)-Hexa-2,4-dienyloxy)-tetrahydro-2*H*-pyran (30): The compound was prepared following general procedure A and isolated as colourless oil. Yield: 1.32 g, 91%. $R_f = 0.36$ (CH_2Cl_2); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 6.40$–6.29 (m, 1H), 6.11 (t, $^3J = 11.0$ Hz, 1H), 5.75 (dq, $^3J = 6.7$, $^3J = 14.8$ Hz, 1H), 5.44 (dt, $^3J = 7.1$, $^3J = 11.0$ Hz, 1H), 4.66–4.64 (m, 1H), 4.38–4.16 (m, 2H), 3.93–3.86 (m, 1H), 3.56–3.49 (m, 1H), 1.78 (d, $^3J = 6.3$ Hz, 3H; CH_3), 1.88–1.50 ppm (m, 6H; CH_2); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 131.8$ (+), 131.6 (+), 126.6 (+), 124.4 (+), 97.8 (+), 62.8 (−), 62.3 (−), 30.7 (−), 25.5 (−), 19.5 (−), 18.3 ppm (+); IR (neat): $\tilde{\nu} = 3024$, 2940, 1117, 1021 cm^{-1}; CI-MS (NH_3): *m*/*z* (%): 102 (100) $[DHP+NH_4^+]$, 200 (22) $[M+NH_4^+]$.

(2*Z*,4*E*)-Hexa-2,4-dienyl acetate (31): The compound was prepared following general procedure B and isolated as colourless oil. Yield: 0.73 g, 99%. $R_f = 0.39$ (CH_2Cl_2); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 6.39$–6.30 (m, 1H), 6.15 (t, $^3J = 11.1$ Hz, 1H), 5.81 (dq, $^3J = 7.0$, $^3J = 14.8$ Hz, 1H), 5.40 (dt, $^3J = 7.5$, $^3J = 11.0$ Hz, 1H), 4.72 (d, $^3J = 7.4$ Hz, 2H; CH_2), 2.07 (s, 3H; CH_3), 1.80 ppm (d, $^3J = 6.0$ Hz, 3H; CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 171.0$ (C_{quat}), 133.2 (+), 132.9 (+), 126.0 (+), 121.5 (+), 60.5 (−), 21.0 (+), 18.4 ppm (+); IR (neat): $\tilde{\nu} = 3031$, 1735, 1372, 1223 cm^{-1}; CI-MS (NH_3): *m*/*z* (%): 158 (71) $[M+NH_4^+]$, 98 (100) $[M+NH_4^+-HOAc]$.

2-Methyl-2-((6*Z*,9*E*,11*Z*)-trideca-6,9,11-trienyl)-1,3-dioxolane (33): The compound was prepared following general procedure D and isolated as colourless oil. Yield: 70 mg, 27%. $R_f = 0.36$ (CH_2Cl_2); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 6.39$–6.30 (m, 1H), 6.03–5.93 (m, 1H), 5.63 (dt, $^3J = 7.0$ Hz, $^3J = 15.1$ Hz, 1H), 5.49–5.34 (m, 3H), 3.98–3.88 (m, 4H; OCH_2), 2.84 (t, $^3J = 6.4$ Hz, 2H; CH_2), 2.07–2.01 (m, 2H, CH_2), 1.73 (dd, $^3J = 6.9$ Hz, $^4J = 1.6$ Hz, 3H; CH_3), 1.65–1.58 (m, 2H; CH_2), 1.43–1.31 (m, 6H; CH_2), 1.30 ppm (s, 3H; CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 132.3$ (+), 131.0 (+), 129.4 (+), 126.9 (+), 125.6 (+), 124.4 (+), 110.2 (C_{quat}), 64.6 (−), 39.2 (−), 30.6 (−), 29.6 (−), 29.5 (−), 27.1 (−), 24.0 (−), 23.7 (+), 13.3 ppm (+); IR (neat): $\tilde{\nu} = 2981$, 2935, 1375, 1064 cm^{-1}; CI-MS (NH_3): *m*/*z* (%): 265 (100) $[M+H^+]$, 282 (26) $[M+NH_4^+]$.



(8*Z*,11*E*,13*Z*)-Pentadeca-8,11,13-trien-2-one (6): The compound was prepared following general procedure E and isolated as colourless oil in quantitative yield (30 mg). $R_f = 0.33$ (CH_2Cl_2); ^{1}H NMR (600 MHz, C_6D_6, COSY): $\delta = 6.48$–6.44 (m, 1H, H(11)), 6.10–6.06 (m, 1H, H(12)), 5.62 (dt, $^3J = 6.9$, $^3J = 15.1$ Hz, 1H, H(10)), 5.52–5.42 (m, 2H, H(7)/H(8)), 5.34 (dq, $^3J = 7.1$, $^3J = 10.7$ Hz, 1H, H(13)), 2.81 (t, $^3J = 6.7$ Hz, 2H, CH_2; H(9)), 1.99–1.95 (m, 2H, CH_2; H(6)), 1.90 (t, $^3J = 7.3$ Hz, 2H, CH_2; H(2)), 1.64 (s, 3H, CH_3; H(1)), 1.62 (dd, $^3J = 7.2$, $^4J = 1.8$ Hz, 3H, CH_3; H(14)), 1.43

(q, $^3J=7.6$ Hz, 2H, CH_2; H(3)), 1.23 (q, $^3J=7.5$ Hz, 2H, CH_2; H(5)), 1.14–1.09 ppm (m, 2H, CH_2; H(4)); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=$ 209.3 (C_{quat}), 132.2 (+), 130.7 (+), 129.3 (+), 127.1 (+), 125.6 (+), 124.4 (+), 43.7 (−), 30.6 (−), 29.9 (+), 29.4 (−), 28.8 (−), 27.0 (−), 23.7 (−), 13.3 ppm (+); IR (neat): $\tilde{\nu}=3014$, 2932, 2856, 1716, 1358 cm^{-1}; CI-MS (NH_3): m/z (%): 238 (100) $[M+NH_4^+]$.

2-Methyl-2-((6*Z*,9*Z*,11*E*)-trideca-6,9,11-trienyl)-1,3-dioxolane (32): The compound was prepared following general procedure D and isolated as colourless oil. Yield: 71 mg, 21%. $R_f=0.27$ (petroleum ether/EtOAc 24:1); ^1H NMR (300 MHz, $CDCl_3$): $\delta=6.03$–5.28– (m, 6H), 3.94–3.84 (m, 4H; OCH_2), 2.88–2.73 (m, 2H; CH_2), 2.00 (quint, $^3J=6.0$ Hz, 2H; CH_2), 1.69 (d, $^3J=6.6$ Hz, 3H; CH_3), 1.62–1.57 (m, 2H; CH_2), 1.40–1.29 (m, 6H; CH_2), 1.27 ppm (s, 3H; CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=131.5$ (+), 130.8 (+), 130.5 (+), 129.9 (+), 127.2 (+), 127.1 (+), 110.2 (C_{quat}), 64.6 (−), 39.2 (−), 30.3 (−), 29.6 (−), 29.5 (−), 27.1 (−), 24.0 (−), 23.7 (+),18.0 ppm (+); CI-MS (NH_3): m/z (%): 187 (100) $[(C_{11}H_{22}O_2)H^+]$, 204 (61) $[(C_{11}H_{22}O_2)NH_4^+]$.

(8Z,11Z,13*E*)-Pentadeca-8,11,13-trien-2-one (5): The compound was prepared following general procedure E and isolated as colourless oil in quantitative yield (39 mg). $R_f=0.47$ (petroleum ether/EtOAc 4:1); ^1H NMR (300 MHz, $CDCl_3$): $\delta=6.07$–5.33 (m, 6H), 2.92–2.77 (m, 2H; CH_2), 2.41 (t, $^3J=7.7$ Hz, 2H; CH_2), 2.13 (s, 3H; CH_3), 2.04 (quint, $^3J=6.3$ Hz, 2H; CH_2), 1.73 (d, $^3J=6.9$ Hz, 3H; CH_3), 1.62–1.52 (m, 2H; CH_2), 1.41–1.25 ppm (m, 4H; CH_2); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=$ 209.3 (C_{quat}), 131.5 (+), 130.6 (+), 130.5 (+), 129.8 (+), 127.3 (+), 127.2 (+), 43.7 (−), 30.3 (−), 29.9 (+), 29.4 (−), 28.8 (−), 26.9 (−), 23.7 (−), 18.1 ppm (+); IR (neat): $\tilde{\nu}=3013$, 2927, 2855, 1716, 1359 cm^{-1}; CI-MS (NH_3): m/z (%): 238 (100) $[M+NH_4^+]$, 221 (4) $[M+H^+]$.

2-Methyl-2-((6*Z*,9*Z*)-pentadeca-6,9-dienyl)-1,3-dioxolane (35): The compound was prepared following general procedure D and isolated as colourless oil. Yield: 56 mg, 20%. $R_f=0.42$ (petroleum ether/EtOAc 9:1); ^1H NMR (300 MHz, $CDCl_3$): $\delta=5.50$–5.34 (m, 4H; CH), 4.00–3.90 (m, 4H; OCH_2), 2.74 (t, $^3J=5.5$ Hz, 2H; CH_2), 2.08–1.96 (m, 4H; CH_2), 1.67–1.60 (m, 2H; CH_2), 1.46–1.28 (m, 15H; CH_2/CH_3), 0.90 ppm (t, $^3J=7.0$ Hz, 3H; CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=130.9$ (+), 130.3 (+), 128.3 (+), 127.9 (+), 110.2 (C_{quat}), 64.6 (−), 39.2 (−), 32.6 (−), 31.4 (−), 30.5 (−), 29.6 (−), 29.5 (−), 29.2 (−), 27.1 (−), 24.0 (−), 23.7 (+), 22.6 (−), 14.1 ppm (+); IR (neat): $\tilde{\nu}=2925$, 2855, 1376, 1067 cm^{-1}; CI-MS (NH_3): m/z (%): 295 (100) $[M+H^+]$, 312 (16) $[M+NH_4^+]$.

(8Z,11Z)-Heptadeca-8,11-dien-2-one (8): The compound was prepared following general procedure E and isolated as colourless oil in quantitative yield (36 mg). $R_f=0.20$ (petroleum ether/EtOAc 19:1); ^1H NMR (300 MHz, $CDCl_3$): $\delta=5.50$–5.33 (m, 4H; CH), 2.74 (t, $^3J=5.6$ Hz, 2H; CH_2), 2.44 (t, $^3J=7.5$ Hz, 2H; CH_2), 2.15 (s, 3H, CH_3), 2.09–1.96 (m, 4H, CH_2), 1.65–1.55 (m, 2H, CH_2), 1.44–1.26 (m, 10H, CH_2), 0.90 ppm (t, $^3J=6.7$ Hz, 3H; CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=209.2$ (C_{quat}), 130.9 (+), 130.1 (+), 128.2 (+), 128.0 (+), 43.8 (−), 32.5 (−), 31.4 (−), 30.5 (−), 29.9 (+), 29.4 (−), 29.2 (−), 28.8 (−), 26.9 (−), 23.7 (−), 22.6 (−), 14.1 ppm (+); IR (neat): $\tilde{\nu}=3008$, 2925, 2855, 1717 cm^{-1}; HRMS (PI-EI): m/z: calcd for $C_{17}H_{30}O$: 250.2297, found: 250.2295 $[M]^+$.

2-Methyl-2-((6*Z*,9*Z*)-trideca-6,9-dienyl)-1,3-dioxolane (34): The compound was prepared following general procedure D and isolated as colourless oil (116 mg, 26%). $R_f=0.31$ (pentane/Et_2O 9:1); ^1H NMR (300 MHz, $CDCl_3$): $\delta=5.42$–5.32 (m, 4H), 3.98–3.88 (m, 4H; OCH_2), 2.72 (t, $^3J=5.2$ Hz, 2H; CH_2), 2.09–1.98 (m, 4H; CH_2), 1.65–1.60 (m, 2H; CH_2), 1.42–1.25 (m, 11H), 0.88 ppm (t, $^3J=8.0$ Hz, 3H; CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=130.6$ (+), 130.3 (+), 128.5 (+), 127.9 (+), 110.2 (C_{quat}), 64.6 (−), 39.2 (−), 34.7 (−), 30.5 (−), 29.6 (−), 29.5 (−), 27.1 (−), 24.0 (−), 23.7 (+), 22.7 (−), 13.7 ppm (+); IR (neat): $\tilde{\nu}=2933$, 2873, 1069, 627 cm^{-1}; CI-MS (NH_3): m/z (%): 267 (100) $[M+H^+]$, 284 (13) $[M+NH_4^+]$.

(8Z,11Z)-Pentadeca-8,11-dien-2-one (7): The compound was deprotected following general procedure E and obtained in quantitative yield (74 mg). $R_f=0.29$ (pentane/Et_2O 9:1); ^1H NMR (300 MHz, $CDCl_3$): $\delta=$ 5.39–5.29 (m, 4H), 2.68 (t, $^3J=5.5$ Hz, 2H; CH_2), 2.38 (t, $^3J=8.0$ Hz, 2H; CH_2), 2.09 (s, 3H; CH_3), 2.03–1.89 (m, 4H; CH_2), 1.59–1.49 (m, 2H; CH_2), 1.40–1.22(m, 6H; CH_2), 0.84 ppm (t, $^3J=8.0$ Hz, 3H; CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=209.3$ (C_{quat}), 130.6 (+), 130.1 (+), 128.4 (+), 128.0 (+), 43.8 (−), 34.7 (−), 30.5 (−), 29.9 (+), 29.4 (−), 28.8 (−), 26.9 (−), 23.7 (−), 22.7 (−), 13.7 ppm (+); IR (neat): $\tilde{\nu}=2936$, 2867, 1711, 631 cm^{-1}; CI-MS (NH_3): m/z (%): 240 (100) $[M+NH_4^+]$.

Trimethyl((*Z*)-9-(2-methyl-1,3-dioxolan-2-yl)non-3-en-1-ynyl)silane (37): To a dry flask **10** (0.23 g, 0.74 mmol), $[PdCl_2(PPh_3)_2]$ (10 mg, 0.032 mmol), CuI (5 mg, 0.026 mmol), and NEt_3 (3 mL) was added under an inert atmosphere. To the cloudy yellow solution TMS-acetylene (0.10 g, 1.03 mmol) was added. The reaction was stirred at RT for 4 h, quenched with water (15 mL), extracted with ether (4×15 mL), washed with brine and purified by flash chromatography to afford 0.15 g (71%) of product. $R_f=0.40$ (petroleum ether/EtOAc 9:1); ^1H NMR (300 MHz, $CDCl_3$): $\delta=5.99$–5.90 (m, 1H), 5.48 (dt, $^4J=1.4$, $^3J=10.8$ Hz, 1H), 3.97–3.91 (m, 4H; OCH_2), 2.37–2.27 (m, 2H; CH_2), 1.68–1.61 (m, 2H; CH_2), 1.47–1.35 (m, 6H; CH_2), 1.32 (s, 3H; CH_3), 0.20 ppm (s, 9H; $SiMe_3$); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=145.5$, 110.1, 109.1, 102.8, 102.1, 64.6, 39.1, 30.2, 29.4, 28.7, 23.9, 23.7, 0 ppm; IR (neat): $\tilde{\nu}=2941$, 2878, 2146 cm^{-1}; HRMS (PI-EI): m/z: calcd for $C_{16}H_{28}O_2Si$: 280.1859, found: 280.1855 $[M]^+$.

(*Z*)-Undec-8-en-10-yn-2-one (40): The compound was prepared following general procedure E and isolated as colourless oil in 83% yield (23 mg). ^1H NMR (300 MHz, $CDCl_3$): $\delta=6.03$–5.93 (m, 1H), 5.48–5.41 (m, 1H), 3.07 (d, $^4J=1.9$ Hz, 1H), 2.43 (t, $^3J=7.3$ Hz, 2H; CH_2), 2.36–2.30 (m, 2H; CH_2), 2.13 (s, 3H; CH_3), 1.65–1.54 (m, 2H; CH_2), 1.46–1.28 ppm (m, 4H; CH_2); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=209.2$, 145.8, 108.3, 81.3, 80.5, 43.7, 30.0, 29.9, 28.6, 28.5, 23.6 ppm; IR (neat): $\tilde{\nu}=3290$, 3263, 2934, 2858, 2151, 2022, 1712 cm^{-1}; HRMS (PI-EI): m/z: calcd for $C_{11}H_{15}O$: 163.1123, found: 163.1123 $[M+H^+]$.

(*Z*)-11-Phenylundec-8-en-10-yn-2-one (39): The compound was prepared following general procedure E and isolated as colourless oil in 80% yield (30 mg). ^1H NMR (300 MHz, $CDCl_3$): $\delta=7.45$–7.41 (m, 2H), 7.34–7.28 (m, 3H), 5.71–5.65 (m, 1H), 6.10–5.91 (m, 1H), 2.46–2.38 (m, 4H; CH_2), 2.11 (s, 3H; CH_3), 1.67–1.30 ppm (m, 6H; CH_2); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=209.2$, 143.9, 131.4, 128.3, 128.1, 109.3, 86.4, 43.7, 30.2, 29.9, 28.7, 28.6, 23.7 ppm; IR (neat): $\tilde{\nu}=3019$, 2929, 2855, 2197 cm^{-1}; HRMS (PI-EI): m/z: calcd for $C_{17}H_{20}O$: 240.1514, found: 240.1517 $[M]^+$.

(*Z*)-9-Phenylnon-8-en-2-one (42): The compound was prepared following general procedure E and isolated as colourless oil in 96% yield (23 mg). ^1H NMR (300 MHz, $CDCl_3$): $\delta=7.32$–7.13 (m, 5H), 6.36 (m, 1H), 5.59 (m, 1H), 2.36 (t, $^3J=7.4$ Hz, 2H; CH_2), 2.34–2.22 (m, 2H; CH_2), 2.07 (s, 3H; CH_3), 1.46–1.16 ppm (m, 6H; CH_2); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=$ 209.2, 137.7, 132.8, 128.9, 128.7, 128.1, 126.5, 43.7, 29.9, 29.7, 28.8, 28.4, 23.7 ppm; IR (neat): $\tilde{\nu}=3006$, 2929, 2855, 2206, 2006 cm^{-1}; HRMS (PI-EI): m/z: calcd for $C_{15}H_{20}O$: 216.1514, found: 216.1513 $[M]^+$.

(6Z,9Z,12Z,15Z)-Henicosa-6,9,12,15-tetraen-2-one (44): Arachidonic acid **43** (0.25 g, 0.8 mmol) was dissolved in dry THF (2 mL). MeLi (1.6 M in Et_2O, 2.1 mL, 3.3 mmol) was added at 0°C resulting in a dark red solution. After 2 h, freshly distilled TMSCl (0.6 mL, 4.8 mmol) was added and the mixture was allowed to warm to RT. The resulting colourless solution was treated with 2 M HCl (2 mL), diluted with Et_2O, washed with water and brine, dried ($MgSO_4$) and concentrated. Purification by flash chromatography on silica gel gave **44** as colourless oil (150 mg, 62%). $R_f=0.40$ (hexane/Et_2O 4:1); ^1H NMR (300 MHz, $CDCl_3$): $\delta=5.44$–5.28 (m, 8H), 2.85–2.78 (m, 6H; CH_2), 2.43 (t, $^3J=7.9$ Hz, 2H; CH_2), 2.13 (s, 3H; CH_3), 2.11–2.02 (m, 4H; CH_2), 1.64 (quint, $^3J=7.8$ Hz, 2H; CH_2), 1.39–1.25 (m, 6H; CH_2), 0.88 ppm (t, $^3J=6.7$ Hz, 3H; CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=208.9$, 130.5, 129.2, 128.8, 128.6, 128.2, 128.2, 127.9, 127.5, 43.0, 31.5, 29.9, 29.3, 27.2, 26.5, 25.7, 23.6, 22.6, 14.1 ppm; IR (neat): $\tilde{\nu}=3011$, 2925, 2857, 1715, 631 cm^{-1}; CI-MS (NH_3): m/z (%): 320 (100) $[M+NH_4^+]$.

Pharmacological materials: GTP, ATP, adenylyl imidodiphosphate, creatine kinase, creatine phosphate, bovine serum albumin and salts (highest purity available) were purchased either from Roche (Mannheim, Germany) or Sigma (St. Louis, MO, USA). Dimethyl sulfoxide was from Merck (Darmstadt, Germany). Tris base was purchased from USB (Cleveland, OH, USA). The cannabinoid receptor ligands 2-arachidonyl glycerol and anandamide were purchased from Tocris Cookson (Ballwin, MO, USA). [γ-^{32}P]GTP was synthesized through enzymatic phosphorylation of GDP and [^{32}P]orthophosphoric acid (8000 Ci $mmol^{-1}$, Perkin–Elmer Life Sciences, Boston, MA, USA) as described previously.[31] Mono(cyclohexyl)-ammoniumphosphoenolpyruvate, pyruvate kinase and myokinase were from Sigma (St. Louis, MO, USA). Stock solutions of 2-arachidonyl glycerol, anandamide and synthesized compounds (10 mM each) were prepared in dimethyl sulfoxide. Dilutions of ligands were prepared in such a way that the dimethyl sulfoxide concentration was 30% (v/v) and that the final dimethyl sulfoxide concentration in the GTPase assay tubes was 3% (v/v).

Pharmacological methods: The steady-state GTPase assay is an established approach to study G-protein coupling of GPCRs at a very proximal point of the signal transduction cascade. The GTPase assay was performed as described previously.[32] Recombinant baculo viruses encoding FLAG- and hexahistidine-tagged hCB_1R and hCB_2R, $G\alpha_{i2}$, $G\beta_1\gamma_2$ and RGS4 (Regulator of G-protein Signaling 4) were generated in Sf9 insect cells using the BaculoGOLD transfection kit (BD PharMingen, San Diego, CA, USA).[21,32,33] For transfection, cells were seeded (cell density 3.0×10^6 cells mL^{-1}) and infected with a 1:100 dilution of high-titer baculovirus stocks. Cells were cultured for 48 h, Sf9 membranes were prepared as described previously[33] and used in the steady-state GTPase assay. Assay tubes contained Sf9 membranes (15 μg of protein per tube), 1.0 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM ATP, 100 nM GTP, 0.1 mM adenylyl imidodiphosphate, 5 mM creatine phosphate, 40 μg of creatine kinase, 0.2% (w/v) bovine serum albumin in 50 mM Tris/HCl, pH 7.4, and synthesized or reference compounds at various concentrations. Reaction mixtures (80 μL) were incubated for 2 min at 25 °C before the addition of 20 μL of [γ-^{32}P]GTP (0.1 μCi/tube). All stock and work dilutions of [γ-^{32}P]GTP were prepared in 20 mM Tris/HCl, pH 7.4. Reactions were conducted for 20 min at 25 °C. Reactions were terminated by the addition of 900 μL of slurry consisting of 5% (w/v) activated charcoal and 50 mM NaH_2PO_4, pH 2.0. Charcoal absorbs nucleotides but not $^{32}P_i$. Charcoal-quenched reaction mixtures were centrifuged for 7 min at room temperature at 15000 × g. 600 μL supernatant fluid of reaction mixtures was removed, and $^{32}P_i$ was determined by Čerenkov radiation in 3 mL water. Enzyme activities were corrected for spontaneous degradation of [γ-^{32}P]GTP. Spontaneous [γ-^{32}P]GTP degradation amounted to <1% of the total amount of radioactivity added. The experimental conditions chosen ensured that not more than 10% of the total amount of [γ-^{32}P]GTP added was converted to $^{32}P_i$. The GTPase activity was calculated and expressed as pmoles of P_i released per mg of membrane protein per min.

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