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Synthesis of Small Combinatorial Libraries of Natural Products: Identification and Quantification of New Long-chain 3-Methyl-2-alkanones from the Root Essential Oil of *Inula helenium* L. (Asteraceae)

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ABSTRACT:

Introduction – Recently, a potent anti-staphylococcal activity of *Inula helenium* L. (Asteraceae) root essential oil was reported. Also, bioassay guided fractionation of the oil pointed to eudesmane sesquiterpene lactones and a series of unidentified constituents as the main carriers of the observed activity.

Objective – To identify nine new constituents (long-chain 3-methyl-2-alkanones) from a fraction of this root essential oil with a low minimum inhibitory concentration value (0.8 μg/mL) by employing a synthetic methodology that leads to the formation of a small combinatorial library of these compounds.

Methods – The identity of these constituents was inferred from mass spectral fragmentation patterns and GC retention data. A library of 3-methyl-2-alkanones (C_{11}–C_{19} homologous series) was synthesised in three steps starting from methyl acetoacetate and the corresponding alkyl halides. The synthetic library was also screened for *in vitro* anti-microbial activity.

Results – Gas chromatographic analyses of *I. helenium* essential oil samples with spiked compounds from the synthesised library corroborated the tentative identifications of the long-chain 3-methyl-2-alkanones. The availability of these anti-microbial compounds from this library made it possible to construct GC/FID calibration curves and determine their content in the plant material: 0.08 – 24.2 mg/100 g of dry roots.

Conclusion – The small combinatorial library approach enabled the first unequivocal identification of long-chain 3-methyl-2-alkanones as plant secondary metabolites, and, also, allowed determination of not only a single compound and biological properties, but those of a group of structurally related compounds. Copyright © 2013 John Wiley & Sons, Ltd.

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Keywords: Anti-staphylococcal activity; small combinatorial library; 3-methyl-2-alkanones; 3-methyl-2-tetradecanone; root essential oil; *Inula helenium* L

Introduction

A large number of papers dealing with essential oil analysis by GC and GC–MS usually disregarded many minor constituents, due to the lack of any positive MS library search hits for the given mass scan. These compounds could even be the contributors to organoleptic properties and/or biological activity of the essential oil. A close inspection of the mass spectral data of these minor components may give an idea of their identity to an experienced phytochemist. The knowledge of their retention data may further strengthen the tentative identification and provide the rationale for any future work on this matter. There are two possibilities: (1) to try to isolate in their pure state the constituents of interest from a usually complex matrix; or (ii) to perform a synthesis of the compound with the proposed structure. The latter approach has several advantages over the more frequent isolation and identification approach, although it is considered more time-consuming. The synthetic work gives the desired compound in the amount that allows unobstructed structural elucidation, spectroscopic characterisation and the opportunity to test the biological activity of the compound in a number of assays. In this way, two new minor volatile compounds, conmaculatin and ternanthranin, were identified from two plant species, and they were found to be strong analgaesics (Radulović *et al*., 2011, 2012a).

If only a fragment of the structure of an interesting essential oil constituent could be inferred from the available data (MS and retention index (RI)), or if the tentative identification could

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be narrowed down to a number of isomers (e.g. by biosynthetic considerations), the only comprehensive approach in this situation would be to create a small combinatorial library of all possible compounds. Such libraries provide the possibility to investigate structure–activity/property relationships within the group of synthesized compounds and provide important (spectral) data for future investigations.

Inula helenium L. (Compositae) is a perennial herb ('oman' in Serbian) used extensively in folk medicine, mostly for the treatment of respiratory conditions, digestive disorders, urinary infections and skin disorders, as well as a mosquito repellent (Tucakov, 1984).

Recently, it has been shown that *I. helenium* root essential oil possesses a potent anti-staphylococcal activity that is closely connected to its ethnopharmacological usage (Stojanović-Radić *et al.*, 2012). A bioassay-guided fractionation of the oil yielded a number of chromatographic fractions that had activity that surpassed that of the oil itself and further enabled the location of the major active principles – three bacterial cell-membrane damaging sesquiterpene lactones, alantolactone, isoalantolactone and diplophyllin (Blagojević and Radulović, 2012; Stojanović-Radić *et al.*, 2012). However, the chemical composition of another fraction with a noteworthy minimal inhibitory concentration (MIC = 0.8 μg/mL) remained unknown. This minor fraction (3.5%) contained an unidentified sesquiterpene aldehyde and a series of compounds with an analogous fragmentation pattern in their mass spectral data. The MS data hinted that the compounds of this series could be 3-methyl-2-alkanones of varying chain lengths. As the isolation of single compounds from this fraction was impossible (70 mg of a mixture of more than 10 compounds was available) a combinatorial library of long-chain 3-methyl-2-alkanones was created.

Thus, in continuation of our previous studies on the volatile anti-microbial constituents of *I. helenium* (Blagojević and Radulović, 2012; Stojanović-Radić *et al.*, 2012), in this work we report the identification, synthesis and microbiological testing of nine additional minor constituents that represent secondary metabolites found for the first time in the Plant Kingdom.

Experimental

Chemicals and reagents

All chemicals and solvents used were of analytical or pharmaceutical grade. Diethyl ether, *n*-hexane, methanol, ethanol, carbon tetrachloride and anhydrous magnesium sulphate, as well as, octanoic, decanoic, dodecanoic, tetradecanoic and hexadecanoic acids, were purchased from Sigma-Aldrich (St Louis, MS, USA). 1-Octanol, 1-decanol, 1-dodecanol and 1-tetradecanol were obtained from Carl Roth GmbH + Co.KG (Karlsruhe, Germany). Sulphuric acid, hydrochloric acid, dimethyl sulphoxide, potassium hydroxide, sodium, bromine, iodine, red phosphorus, mercuric oxide, methyl acetoacetate and methyl iodide were supplied by Merck (Darmstadt, Germany). Triphenyltetrazolium chloride and Mueller Hinton agar were also supplied by Merck, whereas Sabouraud dextrose agar was obtained from Difco Laboratories (Detroit, MI, USA). Microtitre plates were purchased from Carl Roth GmbH + Co.KG, whereas tetracycline and nystatin, used as positive controls in the anti-microbial assays, were obtained from Galenika (Belgrade, Serbia). Chromatographic separations were carried out using silica gel 60 (particle size distribution 40–63 μm) purchased from Merck. The *in vitro* anti-microbial activity was tested against a panel of laboratory control strains belonging to the American Type Culture Collection (MD, USA) including: Gram-positive *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633 and *Bacillus cereus* ATCC 9139; Gram-negative *Escherichia coli* ATCC 8739, *Proteus vulgaris* ATCC 8427 and *Pseudomonas aeruginosa* ATCC 9027; as well as a yeast organism *Candida albicans* ATCC 10231 and a mold organism *Aspergillus niger* ATCC 16404.

Plant material

Dried roots of *I. helenium* were purchased from a local herb shop in Niš, Serbia (manufactured by the Institute Josif Pančić, Belgrade, Serbia). The plant material was macro- and microscopically examined by one of us (Z. Stojanović-Radić) to verify the taxonomical identification of the plant species from which it originated. All tests confirmed the identity and purity of the material. A voucher specimen was deposited with the Herbarium of the Faculty of Science and Mathematics, University of Niš, under the accession number DM0112.

Essential oil – extraction and fractionation

Air-dried, to constant weight, roots of *I. helenium* (ca. 100 g) were ground and subjected to hydrodistillation with ca. 500 mL of distilled water for 3.5 h using the original Clevenger-type apparatus. The oil obtained was separated by extraction with diethyl ether and dried over anhydrous magnesium sulphate. The solvent was evaporated under a gentle stream of nitrogen at room temperature in order to exclude any loss of the essential oil and stored at −18 °C until further analysis. Once the oil yield was determined, the residue was exposed to vacuum at room temperature for a short period to eliminate the solvent completely. The pure oil was then measured on an analytical balance and multiple gravimetric measurements were taken during 24 h to ensure that all of the solvent had evaporated. The yield of the essential oil was 1.4% (w/w).

Preparative medium-pressure liquid chromatography (MPLC) was performed with a pump module C-601 and a pump controller C-610 Work-21 pump (Büchi, Switzerland) and was carried out on pre-packed column cartridges (40 × 75 mm) Silica-gel 60 (Büchi), particle size distribution 40–63 μm. Silica-gel 60 on Al plates, layer thickness 0.2 mm (Kieselgel 60 F254, Merck), was used for thin-layer chromatography (TLC). The spots on TLC were visualised by UV light (254 nm) and by spraying with 50% (v/v) aqueous sulphuric acid or phosphomolybdic acid (12 g) in ethanol (250 mL) followed by heating. A sample of the essential oil (500 mg) was subjected to MPLC (gradient diethyl ether: *n*-hexane, from pure *n*-hexane to pure diethyl ether, 100 mL). The fractions obtained (10 mL) were pooled according to TLC and/or GC−MS analyses.

Analytical and spectral analyses

GC−MS analyses. The GC−MS analyses of all samples (pure oil, MPLC fractions, reaction mixtures) were repeated three times using a Hewlett-Packard 6890 N gas chromatograph. The gas chromatograph was equipped with a fused silica capillary column DB-5 (5% phenylmethylsiloxane, 30 m × 0.25 mm, film thickness 0.25 μm; Agilent Technologies, USA) and coupled with a 5975B mass selective detector from the same company. The injector and interface were operated at 250 and 300 °C, respectively. The oven temperature was raised from 70 to 290 °C at a heating rate of 5 °C/min and then isothermally held for 10 min. Helium at 1.0 mL/min was used as a carrier gas. The samples, 1 μL of the corresponding solutions in diethyl ether (1:100), were injected in a pulsed split mode (the flow was 1.5 mL/min for the first 0.5 min and then set to 1.0 mL/min throughout the remainder of the analysis; split ratio 40:1). The mass selective detector was operated at the ionisation energy of 70 eV, in the 35–500 amu range, with a scanning speed of 0.34 s.

GC/FID analyses. The GC analyses were carried out using a Agilent 7890A GC system equipped with a single injector, one flame ionisation detector (FID) and a fused silica capillary column HP-5MS (5% phenylmethylsiloxane, 30 m × 0.32 mm, film thickness 0.25 μm; Agilent Technologies, USA). The oven temperature was programmed from 150 to 300 °C at 15 °C/min and then held isothermally at 300 °C for 5 min; the carrier gas was nitrogen at 3 mL/min; the injector temperature was held

at 250 °C. The samples, 1 μL of the corresponding solutions, were injected in a splitless mode. The parameters of the FID detector were as follows: heater temperature, 300 °C; H_2 flow, 30 mL/min; air flow, 400 mL/min; make-up flow 23.5 mL/min; signal, 20 Hz.

GC/FID quantification. The quantification of 3-methyl-2-alkanones was carried out by peak-area integration. All synthesised standards of 3-methyl-2-alkanones were injected at five different concentrations (0.009, 0.045, 0.18, 0.45 and 0.9 mg/mL) in order to build up five-point GC/FID calibration curves by plotting compound concentration versus peak area ($C = f(A)$). Each sample was analysed for three consecutive runs.

IR measurements. The IR measurements (ATR-attenuated total reflectance) were carried out using a Thermo Nicolet model 6700 FTIR instrument (Waltham, USA).

NMR measurements. The NMR spectra for all 3-methyl-2-alkanones, excluding 3-methyloctadecan-2-one, were recorded at 200 MHz (^{1}H) and 50 MHz (^{13}C) using a Varian Gemini 200 spectrometer (Palo Alto, CA, USA). The NMR spectra of 3-methyloctadecan-2-one were recorded on a Bruker Avance II + 600 (Fällanden, Switzerland; ^{1}H at 600.13 MHz; ^{13}C at 150.92 MHz). All NMR spectra were measured at 25 °C in deuterated chloroform. Chemical shifts are reported in parts per million (δ) relative to residual solvent protons as the internal standard (deuterated chloroform: 7.26 ppm for ^{1}H and 77 ppm for ^{13}C). Scalar couplings are reported in hertz.

Synthesis of 3-methyl-2-alkanones

Alkyl halides. Alkyl halides were obtained following two different standard procedures. A modified Hunsdiecker reaction, where free carboxylic acid is treated with a mixture of mercuric oxide and bromine in carbon tetrachloride, was applied for synthesis of odd-numbered alkyl bromides (Lampman and Aumiller, 1988), whereas even-numbered alkyl iodides were prepared by reaction of an appropriate alcohol with phosphorus triiodide generated *in situ* from red phosphorus and crystalline iodine (Vogel, 1989). All synthesised alkyl halides were purified by 'dry-flash' column chromatography and obtained in 75–85% yields.

Methyl 2-acetylalkanoates (3a–i). Metallic sodium (5 mmol) was dissolved in a solution of methyl acetoacetate (**2**, 5 mmol) in anhydrous methanol (12 mL). The reaction mixture was then cooled to 0 °C and alkyl halide (6 mmol) was slowly added dropwise with stirring during 20 min. After heating the reaction mixture under reflux for 15–25 h, the excess of methanol was evaporated *in vacuo*, 100 mL of water was added and the resulting mixture was extracted several times with diethyl ether. The organic layers were combined, washed with 10% aqueous solution of hydrochloric acid, dried over anhydrous magnesium sulphate and concentrated under reduced pressure to yield crude **3a-i**, which were used without further purification.

Compound **3d**: yield 78%. GC–MS purity: 94.2%, RI (DB-5) = 1766; MS (EI, 70 eV), *m/z* (relative intensity, %): 256 $[M]^{+\cdot}$ (0.3), 241 $[M - CH_3]^+$ (0.2), 225 $[M - OCH_3]^+$ (3.5), 214 (25.2), 196 (0.1), 185 (5.2), 171 (12.4), 157 (2.2), 143 (17.8), 129 $[C_6H_9O_3]^+$ (27.2), 116 $[C_5H_8O_3]^{+\cdot}$ (100), 101 (14.2), 87 (76.7), 74 (16.6), 55 (27.2), 43 $[CH_3CO]^+$ (70.2).

Compound **3 g**: yield 70%. GC–MS purity: 93.2%, RI (DB-5) = 2071; MS (EI, 70 eV), *m/z* (relative intensity, %): 298 $[M]^{+\cdot}$ (0.4), 283 $[M - CH_3]^+$ (0.2), 267 $[M - OCH_3]^+$ (2.3), 256 (21.9), 241 (0.1), 227 (2), 213 (10.4), 199 (4.1), 185 (2.9), 171 (2.9), 157 (4.1), 143 (13.5), 129 $[C_6H_9O_3]^+$ (26.8), 116 $[C_5H_8O_3]^{+\cdot}$ (100), 101 (11.4), 87 (64.3), 74 (14.9), 55 (21.7), 43 $[CH_3CO]^+$ (59.5).

Compound **3 h**: yield 71%. GC–MS purity: 93.8%, RI (DB-5) = 2172; MS (EI, 70 eV), *m/z* (relative intensity, %): 312 $[M]^{+\cdot}$ (0.5), 297 $[M - CH_3]^+$ (0.3), 281 $[M - OCH_3]^+$ (1.9), 270 (22.1), 241 (2.2), 227 (9.3), 213 (3), 199 (1.5), 185 (3.7), 172 (3), 154 (2), 143 (12.8), 129 $[C_6H_9O_3]^+$ (28.6), 116 $[C_5H_8O_3]^{+\cdot}$ (100), 101 (9.9), 87 (61.2), 74 (14.7), 55 (21.8), 43 43 $[CH_3CO]^+$ (60.2).

Compound **3i**: yield 70%. GC–MS purity: 93.5%, RI (DB-5) = 2272; MS (EI, 70 eV), *m/z* (relative intensity, %): 326 $[M]^{+\cdot}$ (0.6), 311 $[M - CH_3]^+$ (0.3), 295 $[M - OCH_3]^+$ (1.5), 284 (21.9), 255 (2.0), 241 (8.9), 227 (1.3), 213 (0.8), 199 (4.1), 185 (4.7), 172 (2.3), 154 (1.6), 143 (12.2), 129 $[C_6H_9O_3]^+$ (31.5), 116 $[C_5H_8O_3]^{+\cdot}$ (100), 101 (9.2), 87 (59), 74 (14.3), 55 (21.8), 43 $[CH_3CO]^+$ (61). The RI and MS data for compounds **3a**–**c**, **3e** and **3f** are given in Supporting information.

Methyl 2-acetyl-3-methylalkanoates (4a-i). Methyl 2-acetyl-3-methylalkanoates (**4a-i**) were prepared by alkylation of methyl 2-acetylalkanoates (**3a-i**) with commercially available methyl iodide following an analogous procedure described for methyl 2-acetylalkanoates.

Compound **4a**: yield 90%. GC–MS purity: 93.4%, RI (DB-5) = 1507; MS (EI, 70 eV), *m/z* (relative intensity, %): 228 $[M]^{+\cdot}$ (0.1), 197 $[M - OCH_3]^+$ (1.8), 186 (65), 169 $[M - COOCH_3]^{+\cdot}$ (1.8), 157 (3.5), 143 (30.8), 130 $[CH_3COCH(CH_3)COOCH_3]^{+\cdot}$ (60.4), 112 (5.3), 101 (100), 98 (13.6), 88 (7.7), 69 (13.6), 55 (13.3), 43 $[CH_3CO]^+$ (43.8).

Compound **4b**: yield 86%. GC–MS purity: 94.9%, RI (DB-5) = 1608; MS (EI, 70 eV), *m/z* (relative intensity, %): 242 $[M]^{+\cdot}$ (0.1), 211 $[M - OCH_3]^+$ (1.5), 200 (64.8), 183 $[M - COOCH_3]^+$ (1.8), 171 (3.3), 157 (26.7), 143 (22.8), 130 $[CH_3COCH(CH_3)COOCH_3]^{+\cdot}$ (49), 112 (5.2), 101 (100), 98 (13), 88 (7.9), 69 (13.1), 55 (13), 43 $[CH_3CO]^+$ (43.1).

Compound **4c**: yield 86%. GC–MS purity: 93.9%, RI (DB-5) = 1709; MS (EI, 70 eV), *m/z* (relative intensity, %): 256 $[M]^{+\cdot}$ (0.1), 225 $[M - OCH_3]^+$ (1.3), 213 (64.4), 197 $[M - COOCH_3]^+$ (1.6), 185 (3.1), 171 (7.6), 157 (25.3), 143 (11.3), 130 $[CH_3COCH(CH_3)COOCH_3]^{+\cdot}$ (48.6), 112 (5), 101 (100), 98 (12.9), 88 (8), 69 (12.8), 55 (12.7), 43 $[CH_3CO]^+$ (42.5).

Compound **4d**: yield 85%. GC–MS purity: 95.9%, RI (DB-5) = 1810; MS (EI, 70 eV), *m/z* (relative intensity, %): 270 $[M]^{+\cdot}$ (0.1), 239 $[M - OCH_3]^+$ (1.4), 228 (64.3), 211 $[M - COOCH_3]^{+\cdot}$ (1.5), 199 (3), 185 (7.9), 171 (15.0), 157 (15.8), 143 (10.8), 130 $[CH_3COCH(CH_3)COOCH_3]^{+\cdot}$ (48.5), 112 (5), 101 (100), 98 (13.1), 88 (7.9), 69 (12.7), 55 (12.6), 43 43 $[CH_3CO]^+$ (42.2).

Compound **4e**: yield 82%. GC–MS purity: 95.9%, RI (DB-5) = 1910; MS (EI, 70 eV), *m/z* (relative intensity, %): 284 $[M]^{+\cdot}$ (0.1), 253 $[M - OCH_3]^+$ (1.3), 242 (64.7), 225 $[M - COOCH_3]^+$ (1.4), 213 (2.9), 199 (7.6), 185 (14.9), 171 (2.4), 157 (15.9), 143 (10.7), 130 $[CH_3COCH(CH_3)COOCH_3]^{+\cdot}$ (48.6), 112 (5), 101 (100), 98 (12.9), 88 (7.9), 69 (12.8), 55 (12.7), 43 $[CH_3CO]^+$ (42.5).

Compound **4f**: yield 84%. GC–MS purity: 95.8%, RI (DB-5) = 2012; MS (EI, 70 eV), *m/z* (relative intensity, %): 298 $[M]^{+\cdot}$ (0.1), 267 $[M - OCH_3]^+$ (1.2), 256 (67.3), 239 $[M - COOCH_3]^+$ (1.2), 213 (6.6), 199 (14.6), 185 (4.2), 171 (2), 157 (16.9), 143 (9.7), 130 $[CH_3COCH(CH_3)COOCH_3]^{+\cdot}$ (52.6), 112 (5.1), 101 (100), 98 (12.6), 88 (8.5), 69 (13.7), 55 (13.7), 43 $[CH_3CO]^+$ (45.6).

Compound **4 g**: yield 80%. GC–MS purity: 95.4%, RI (DB-5) = 2114; MS (EI, 70 eV), *m/z* (relative intensity, %): 312 $[M]^{+\cdot}$ (0.2), 281 $[M - OCH_3]^+$ (1.1), 270 (73.8), 253 $[M - COOCH_3]^+$ (1.1), 227 (5.7), 213 (13.9), 199 (4.3), 185 (1.9), 171 (4.0), 157 (19.4), 143 (8.6), 130 $[CH_3COCH(CH_3)COOCH_3]^{+\cdot}$ (57.7), 112 (5.5), 101 (100), 98 (12.4), 88 (9), 69 (14.3), 55 (13.4), 43 $[CH_3CO]^+$ (43.6).

Compound **4 h**: yield 81%. GC–MS purity: 95.2%, RI (DB-5) = 2213; MS (EI, 70 eV), *m/z* (relative intensity, %): 326 $[M]^{+\cdot}$ (0.1), 295 $[M - OCH_3]^+$ (1), 284 (75.6), 267 $[M - COOCH_3]^+$ (1.5), 241 (3.4), 227 (13.7), 213 (3.3), 199 (2.8), 185 (4.0), 171 (2.3), 157 (17.4), 143 (8.3), 130 $[CH_3COCH(CH_3)COOCH_3]^{+\cdot}$ (60.8), 112 (5.5), 101 (100), 98 (12), 88 (9.3), 69 (14.4), 55 (14.5), 43 $[CH_3CO]^+$ (47.6).

Compound **4i**: yield 84%. GC–MS purity: 95.6%, RI (DB-5) = 2313; MS (EI, 70 eV), *m/z* (relative intensity, %): 340 $[M]^{+\cdot}$ (0.1), 309 $[M - OCH_3]^+$ (0.8), 298 (79.7), 281 $[M - COOCH_3]^+$ (1.8), 265 (1.2), 255 (5.4), 241 (13.9), 227 (1.5), 213 (3.9), 199 (5.3), 185 (4.1), 171 (1.3), 157 (16.4), 143 (8.7), 130 $[CH_3COCH(CH_3)COOCH_3]^{+\cdot}$ (64.5), 112 (6.4), 101 (100), 98 (11.7), 88 (9.7), 69 (14.3), 55 (15.1), 43 $[CH_3CO]^+$ (50.7).

3-Methyl-2-alkanones (1a-i). A slightly modified procedure of Ohnuma *et al.* (1989) was applied. A mixture of methyl 2-acetyl-3-methylalkanoates (**4a–i**, 3 mmol), 2 mol/L aqueous solution of potassium hydroxide (20 mL) and ethanol (3.3 mL) was stirred at room temperature for 24 h. The reaction mixture was then acidified with 10% aqueous solution of hydrochloric acid and extracted three times with diethyl ether. Combined ether extracts, dried over anhydrous magnesium sulphate, were concentrated under reduced pressure. The crude products obtained were fractioned by 'dry-flash' column chromatography using a gradient of diethyl ether and *n*-hexane (from pure *n*-hexane to 2% diethyl ether in *n*-hexane with an increment step of 1%) to yield pure 3-methyl-2-alkanones.

Compound **1 g**: yield 93%. GC–MS purity: 99.1%, RI (DB-5) = 1849; MS (EI, 70 eV), *m/z* (relative intensity, %): 254 $[M]^{+\cdot}$ (0.6), 239 $[M-CH_3]^+$ (0.1), 211 $[M-CH_3CO]^+$ (0.1), 197 $[M-C_4H_9]^+$ (0.1), 182 $[M-C_4H_8O]^{+\cdot}$ (0.1), 169 (0.1), 155 (0.1), 141 (0.3), 127 (0.6), 109 (0.4), 97 (0.9), 85 (11.4), 72 $[C_4H_8O]^{+\cdot}$ (100), 71 $[CH_3COCHCH_3]^+$ (6.5), 57 $[C_4H_9]^+$ (12.6), 43 $[CH_3CO]^+$ (32.3); FTIR-ATR (neat) cm^{-1}: 2921, 2852, 1714 (C = O), 1461, 1347, 1119, 715; ^{1}H-NMR (200 MHz, $CDCl_3$) δ 2.50 (h, *J* = 6.9 Hz, 1H, CH), 2.13 (s, 3H, CH_3CO), 1.32–1.22 (m, 24H, 12 × CH_2), 1.08 (d, *J* = 6.9 Hz, 3H, $CHCH_3$), 0.88 (t, *J* = 7.0 Hz, 3H, CH_2CH_3); ^{13}C-NMR (50 MHz, $CDCl_3$) δ 212.9 (C = O), 47.2 (CH), 32.9 (CH_2), 31.9 (CH_2), 29.7 (5 × CH_2), 29.6 (CH_2), 29.5 (CH_2), 29.3 (CH_2), 27.9 ($CH_3C=O$), 27.2 (CH_2), 22.7 (CH_2), 16.1 (CH_3), 14.1 (CH_3).

Compound **1 h**: yield 86%. GC–MS purity: 99.4%, RI (DB-5) = 1950; MS (EI, 70 eV), *m/z* (relative intensity, %): 268 $[M]^{+\cdot}$ (0.8), 255 $[M-CH_3]^+$ (0.1), 225 $[M-CH_3CO]^+$ (0.1), 211 $[M-C_4H_9]^+$ (0.1), 196 $[M-C_4H_8O]^{+\cdot}$ (0.1), 169 (0.1), 155 (0.1), 141 (0.1), 127 (0.3), 113 (0.6), 109 (0.5), 97 (1.1), 85 (11.5), 72 $[C_4H_8O]^{+\cdot}$ (100), 71 $[CH_3COCHCH_3]^+$ (6.2), 57 $[C_4H_9]^+$ (11.1), 43 $[CH_3CO]^+$ (28.7); FTIR-ATR (neat) cm^{-1}: 2920, 2851, 1713 (C = O), 1460, 1352, 1123, 720; ^{1}H-NMR (200 MHz, $CDCl_3$) δ 2.52 (h, *J* = 6.9 Hz, 1H, CH), 2.15 (s, 3H, CH_3CO), 1.39–1.21 (m, 26H, 13 × CH_2), 1.10 (d, *J* = 6.9 Hz, 3H, $CHCH_3$), 0.90 (t, *J* = 7.0 Hz, 3H, CH_2CH_3); ^{13}C-NMR (50 MHz, $CDCl_3$) δ 213.1 (C = O), 47.2 (CH), 33.0 (CH_2), 31.9 (CH_2), 29.7 (6 × CH_2), 29.6 (CH_2), 29.5 (CH_2), 29.4 (CH_2), 28.0 ($CH_3C=O$), 27.3 (CH_2), 22.7 (CH_2), 16.2 (CH_3), 14.2 (CH_3).

Compound **1i**: yield 85%. GC–MS purity: 99.5%, RI (DB-5) = 2051; MS (EI, 70 eV), *m/z* (relative intensity, %): 282 $[M]^{+\cdot}$ (0.7), 267 $[M-CH_3]^+$ (0.1), 239 $[M-CH_3CO]^+$ (0.1), 225 $[M-C_4H_9]^+$ (0.1), 210 $[M-C_4H_8O]^+$ (0.1), 183 (0.1), 169 (0.1), 155 (0.1), 141 (0.3), 127 (0.6), 109 (0.5), 97 (1.1), 85 (11.5), 72 $[C_4H_8O]^{+\cdot}$ (100), 71 $[CH_3COCHCH_3]^+$ (6.2), 57 $[C_4H_9]^+$ (11.1), 43 $[CH_3CO]^+$ (28.7); FTIR-ATR (neat) cm^{-1}: 2919, 2852, 1713 (C = O), 1461, 1352, 1124, 720; ^{1}H-NMR (600 MHz, $CDCl_3$) δ 2.52 (h, *J* = 6.9 Hz, 1H, CH), 2.15 (s, 3H, CH_3CO), 1.39–1.21 (m, 28H, 14 × CH_2), 1.10 (d, *J* = 6.9 Hz, 3H, $CHCH_3$), 0.90 (t, *J* = 7.0 Hz, 3H, CH_2CH_3); ^{13}C-NMR (151 MHz, $CDCl_3$) δ 213.1 (C = O), 47.2 (CH), 33.0 (CH_2), 31.9 (CH_2), 29.7 (7 × CH_2), 29.6 (CH_2), 29.5 (CH_2), 29.4 (CH_2), 28.0 ($CH_3C=O$), 27.3 (CH_2), 22.7 (CH_2), 16.2 (CH_3), 14.2 (CH_3). Spectroscopic data (RI, MS, IR, ^{1}H NMR and ^{13}C NMR) of compounds **1a**–**f** are given in Supporting information.

Antimicrobial assay

The anti-microbial activity was evaluated using a microdilution broth method (Radulović *et al.*, 2012b). Stock solutions of 3-methyl-2-alkanones were prepared in 0.05% (v/v) aqueous Tween 80 and serial dilutions tested in the range 37.00–0.14 mg/mL. Tetracycline and nystatin served as positive controls, whereas the corresponding solvent (0.05% (v/v) Tween 80) was used as the negative control.

Results and discussion

In a previous study of *I. helenium* root oil 45 compounds were identified, but additional constituents that did not appear in the original total ion chromatogram (TIC) of the unfractionated oil were detected after a chromatographic separation on SiO_2 of the oil. One of these fractions (eluting with 5% diethyl ether in *n*-hexane), highly active against *S. aureus*, contained a sesquiterpene aldehyde and a series of nine compounds (**1a–i**) showing regularities in their GC retention behaviour and possessing analogous mass spectral data. In the mass spectra of compounds **1a–i** (Fig. 1) the base peaks at *m/z* 72 arising from a probable McLafferty fragmentation and the abundant acetyl ion fragment at *m/z* 43 hinted that these compounds might constitute a series of 3-methyl-2-alkanones (Francke, 2010). Additionally, the difference of 14 amu (one CH_2 group) between the molecular ions of any two consecutive compounds in the TIC, separated by ca. 100 RI units, pointed to the existence of a C_{11}–C_{19} homologous series (Fig. 1). Unfortunately, we found no retention data in the literature on these compounds and relied on the following rule of thumb for the structure–retention data relationship: the difference between literature RI values for isomeric compounds 2-tridecanone and 6,10-dimethyl-2-undecanone was more than 90 units in favour of the first, which is consistent with the presence of two methyl branches (Setzer *et al.*, 2005; Wang *et al.*, 2006). Hence, the existence of additional branches in the chain of compounds **1a–i** was ruled out because compound **1c** from the *I. helenium* oil

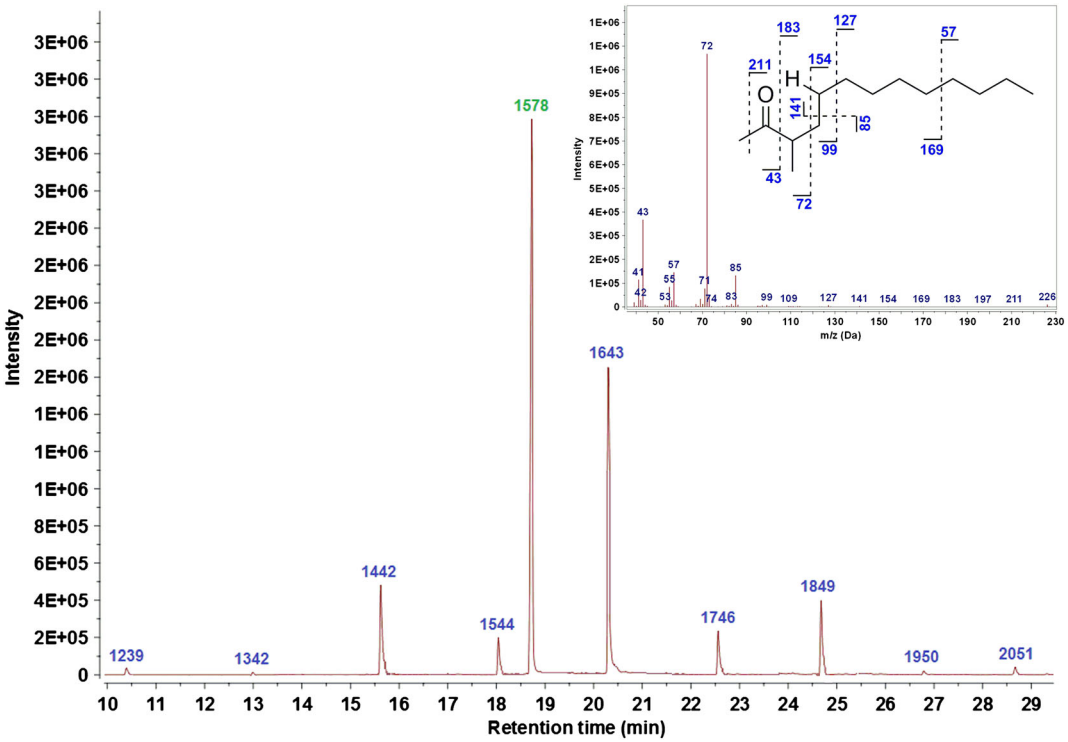

Figure 1. TIC chromatogram of the fraction of *I.* helenium root essential oil (5% diethyl ether in *n*-hexane) showing peaks corresponding to an unidentified sesquiterpene aldehyde (RI 1578) and 3-methyl-2-alkanones (**1a–i**) and the mass spectrum of 3-methyltetradecan-2-one (**1e**).

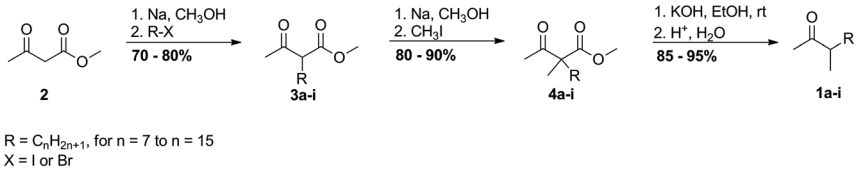

Figure 2. Synthetic route to 3-methyl-2-alkanones (**1a–i**)

fraction that corresponds to this molecular mass has an RI value of ca. 50 units less that the straight-chain isomer.

A detailed literature survey showed that long-chain 3-methyl-2-alkanones had rarely been reported in samples of natural origin, with no reference to their existence in the Plant Kingdom. 3-Methyl-2-decanone was found in territorial marking fluids of the male Bengal tiger, *Panthera tigris* (Burger *et al*., 2008), whereas this compound and its homologue (3-methyl-2-undecanone) were detected in the normal urine of male wolves, *Canis lupus* (Raymer *et al*., 1986). Three longer chain homologues, 3-methyl-2-tridecanone, 3-methyl-2-tetradecanone and 3-methyl-2-pentadecanone, were found in Dufour glands' secretions of a species of desert-dwelling ants of the *Cataglyphis bicolor* group (Gökçen *et al*., 2002). Motivated by the interesting properties of these ketones and their limited natural occurrence, the tentative structure assignment was confirmed by comparing the chromatographic and spectral properties of these, up to now unknown, *I. helenium* constituents to that of synthetic 3-methyl-2-alkanones. It was virtually impossible to isolate these compounds from the complex oil fraction matrix and the synthesis gave the opportunity to ascertain to what extent these compounds had contributed to the previously observed strong anti-staphylococcal activity of the fraction in question.

Commercially available methyl acetoacetate (**2**) was the starting material in this synthetic effort. The route began with a base-catalysed alkylation of β-ketoester **2** with an appropriate halogen alkane to obtain methyl 2-acetylalkanoates (**3a–i**) that were further methylated, in an analogous manner. Subsequent tandem mild basic hydrolysis and decarboxylation of the methylation products **4a–i** yielded 3-methyl-2-alkanones (**1a–i**) (as depicted in Fig. 2). These three step transformations were achieved in 50–65% overall yields. The structural assignments of the target molecules were undertaken by spectral means (^{1}H- and ^{13}C-NMR, IR, MS). After the co-injection of the nine synthesised compounds with the root essential oil of *I. helenium*, the originally proposed hypothesis was unambiguously corroborated by these methods. All nine 3-methyl-2-alkanones were found herein for the first time in the Plant Kingdom. Additionally, four of them (C_{13}, $C_{17}-C_{19}$) are reported here to occur in a living organism for the first time.

The GC/FID calibration curves for the synthesised 3-methyl-2-alkanones were built up in order to quantify these compounds in the plant material. For example, the calibration curve (amount = $1.3285\times10^{-4}\times$ area + 5.0526×10^{-3}) for 3-methyl-2-octadecanone, in the concentration range 0.009-0.9 mg/mL, had an excellent correlation coefficient $R^2 = 0.99989$. All other 3-methyl-2-alkanones from this series had comparable curves and similar response factors. The content of 3-methyl-2-alkanones determined per 100 g of dry *I. helenium* roots was within the range 0.08–24.2 mg (Table 1).

The contribution of the identified 3-methyl-2-alkanones to the overall anti-microbial action of the original fraction of *I. helenium* essential oil was evaluated in microdilution assays against six pathogenic bacterial and two fungal strains. Surprisingly, 3-methyl-2-alkanones did not show

Table 1. GC/FID calibration range, calibration curve equation, correlation coefficient (R^2) and quantitative results for each 3-methyl-2-alkanone

Designation	Compound name	Calibration range (mg/mL)	Calibration curve equation	Correlation coefficient (R^2)	Content (mg/100 g of dry roots)
1a	3-methyldecan-2-one	0.009–0.9	$C = 1.3807\times10^{-4}A + 5.0699\times10^{-3}$	0.9997	0.32
1b	3-methylundecan-2-one		$C = 1.3690\times10^{-4}A + 5.0678\times10^{-3}$	0.9997	0.21
1c	3-methyldodecan-2-one		$C = 1.3579\times10^{-4}A + 5.0626\times10^{-3}$	0.9998	5.1
1d	3-methyltridecan-2-one		$C = 1.3542\times10^{-4}A + 5.0606\times10^{-3}$	0.9999	2.1
1e	3-methyltetradecan-2-one		$C = 1.3436\times10^{-4}A + 5.0595\times10^{-3}$	0.9999	24.2
1f	3-methylpentadecan-2-one		$C = 1.3379\times10^{-4}A + 5.0566\times10^{-3}$	0.9997	2.5
1 g	3-methylhexadecan-2-one		$C = 1.3336\times10^{-4}A + 5.0544\times10^{-3}$	0.9998	4.5
1 h	3-methylheptadecan-2-one		$C = 1.3379\times10^{-4}A + 5.0531\times10^{-3}$	0.9999	0.08
1i	3-methyloctadecan-2-one		$C = 1.3285\times10^{-4}A + 5.0526\times10^{-3}$	0.9999	0.54

Table 2. Anti-microbial activity of 3-methyl-2-alkanones

Fungal strain	Compound[a] MIC (mg/mL)									Nystatin MIC (μg/mL)
	1a	**1b**	**1c**	**1d**	**1e**	**1f**	**1 g**	**1 h**	**1i**	
Candida albicans	3.70	3.70	3.70	3.70	3.70	3.70	3.70	3.70	3.70	0.78

MIC, minimum inhibitory concentrations.

[a] In the tested concentrations, none of the compounds (**1a–i)** were active against *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Escherichia coli*, *Proteus vulgaris, Pseudomonas aeruginosa* and *Aspergillus niger*.

any anti-staphylococcal activity in the concentrations tested. Furthermore, 3-methyl-2-alkanones manifested selectivity toward just one fungal strain – *Candida albicans* (MIC = 3.7 mg/mL, Table 2). This implies that the very high activity against *S. aureus* of this oil fraction comes from the still unidentified sesquiterpene aldehyde with a mass spectrum resembling that of bicyclogermacrenal (Stojanović-Radić *et al*., 2012). The observed anti-candidal activity of 3-methyl-2-alkanones (*C. albicans* is a yeast species that can be found in the soil; Marples, 1966; Hube, 2004) suggests that these compounds could be plant-root defence metabolites against attack of such pathogens and a number of previous studies on related compounds confirm this (McDowell *et al*., 1988; Fridman *et al*., 2005; Ntalli *et al*., 2011).

Conclusion

In conclusion, the identification and synthesis of nine additional constituents from a fraction of *I. helenium* root essential oil with a low MIC value (0.8 μg/mL) against *S. aureus* were reported here. The existence of a C_{11}–C_{19} homologous series of 3-methyl-2-alkanones was corroborated by synthesis in three steps with the overall yields of 50–65% starting from methyl acetoacetate. The construction of GC/FID calibration curves ($R^2 = 0.9997$–0.9999) enabled the determination of the content of 3-methyl-2-alkanones in the plant material: 0.08–24.2 mg/100 g of dry roots. The *in vitro* anti-microbial activity assay revealed that 3-methyl-2-alkanones were not active against *S. aureus* at the tested concentrations, but manifested a selectivity toward one fungal strain – *C. albicans* (MIC = 3.7 mg/mL). Finally, these long-chain 3-methyl-2-alkanones have a rather restricted occurrence in samples of natural origin and this is the very first report of their occurrence in the Plant Kingdom.

SUPPORTING INFORMATION

Supporting information can be found in the online version of this article.

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References

Blagojević PD, Radulović NS. 2012. Conformational analysis of antistaphylococcal sesquiterpene lactones from *Inula helenium* essential oil. *Nat Prod Commun* **7**: 1407–1410.

Burger BV, Viviers MZ, Bekker JPI, le Roux M, Fish N, Fourie WB, Weibchen G. 2008. Chemical characterization of territorial marking fluid of male Bengal tiger, *Panthera tigris*. *J Chem Ecol* **34**: 659–671.

Francke W. 2010. Structure elucidation of some naturally occurring carbonyl compounds upon coupled gas chromatography/mass spectrometry and micro-reactions. *Chemoecology* **20**: 163–169.

Fridman E, Wang J, Iijima Y, Froehlich JE, Gang DR, Ohlrogge J, Pichersky E. 2005. Metabolic, genomic, and biochemical analyses of glandular trichomes from the wild tomato species *Lycopersicon hirsutum* identify a key enzyme in the biosynthesis of methylketones. *Plant Cell* **17**: 1252–1267.

Gökçen OA, Morgan ED, Dani FR, Agosti D, Wehner R. 2002. Dufour gland contents of ants of the *Cataglyphis bicolor* group. *J Chem Ecol* **28**: 71–87.

Hube B. 2004. From commensal to pathogen: stage- and tissue-specific gene expression of *Candida* albicans. *Curr Opin Microbiol* **7**: 336–341.

Lampman GM, Aumiller JC. 1988. Mercury(II) oxide-modified Hunsdiecker reaction: 1-bromo-3-chlorocyclobutane. *Org Synth Coll Vol* **6**: 179.

Marples MJ. 1966. Some observations on the ecology of *Candida albicans*, a potential mammalian pathogen. *New Zeal J Ecol* **13**: 29–34.

McDowell PG, Lwande W, Deans SG, Waterman P. 1988. Volatile resin exudate from stem bark of *Commiphora rostrata*: Potential role in plant defence. *Phytochemistry* **27**: 2519–2521.

Ntalli NG, Manconi F, Leonti M, Maxia A, Caboni P. 2011. Aliphatic ketones from *Ruta chalepensis* (Rutaceae) induce paralysis on root knot nematodes. *J Agric Food Chem* **59**: 7098–7103.

Ohnuma S-I, Ito M, Koyama T, Ogura K. 1989. Undecaprenyl diphosphate synthase reaction with artificial substrate homologues – novel behavior in the termination of prenyl chain elongation. *Tetrahedron* **45**: 6145–6160.

Radulović NS, Miltojević AB, McDermott M, Waldren S, Parnell JA, Pinheiro MM, Fernandes PD, de Sousa Menezes F. 2011. Identification of a new antinociceptive alkaloid isopropyl N-methylanthranilate from the essential oil of *Choisya ternata* Kunth. *J Ethnopharmacol* 2011 **135**: 610–619.

Radulović N, Đorđević N, Denić M, Pinheiro MM, Fernandes PD, Boylan F. 2012a. A novel toxic alkaloid from poison hemlock (*Conium maculatum* L., Apiaceae): identification, synthesis and antinociceptive activity. *Food Chem Toxicol* **50**: 274–279.

Radulović N, Denić M, Stojanović-Radić Z, Skropeta D. 2012b. Fatty and volatile oils of the gypsywort *Lycopus europaeus* L. and the Gaussian-like distribution of its wax alkanes. *J Am Oil Chem Soc* **89**: 2165–2185.

Raymer J, Wiesler D, Novotny M, Asa C, Seal US, Mech LD. 1986. Chemical scent constituents in urine of wolf (*Canis lupus*) and their dependence on reproductive hormones. *J Chem Ecol* **12**: 297–314.

Setzer WN, Noletto JA, Lawton RO, Haber WA. 2005. Leaf essential oil composition of five *Zanthoxylum* species from Monteverde, Costa Rica. *Mol Divers* **9**: 3–13.

Stojanović-Radić ZZ, Čomić LR, Radulović NS, Blagojević PD, Denić M, Miltojević AB, Rajković J, Mihajilov-Krstev TM. 2012. Antistaphylococcal activity of *Inula helenium* L. root essential oil: eudesmane sesquiterpene lactones induce cell membrane damage. *Eur J Clin Microbiol* **31**: 1015–1025.

Tucakov J. 1984. *Lečenje biljem*. Izdavačka radna organizacija Rad: Belgrade; 521–522.

Vogel AI. 1989. *Vogel's Textbook of Practical Organic Chemistry*, 5th edn. Longman Scientific and Technical: Harlow; 568–569.

Wang Q, Yang Y, Zhao X, Zhu B, Nan P, Zhao J, Wang L, Chen F, Liu Z, Zhong Y. 2006. Chemical variation in the essential oil of *Ephedra sinica* from northeastern China. *Food Chem* **98**: 52–58.