FACTA UNIVERSITATIS
Series: **Physics, Chemistry and Technology** Vol. 13, N° 2, Special Issue, 2015, pp. 83- 89
DOI: 10.2298/FUPCT1502083G

A NOTE ON THE BIOSYNTHESIS OF LONG-CHAIN 3-METHYL-2-ALKANONES FROM THE ROOT ESSENTIAL OIL OF *INULA HELENIUM* L. (ASTERACEAE)[†]

UDC 581.192 : 547.284

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Abstract. *A predominantly odd-numbered, Gaussian-like distribution of the relative amounts of 3-methyl-2-alkanones, from I. helenium root essential oil, was observed. This distribution pattern indicates that their biosynthesis is related to that of fatty acids and related compounds. Simple (non-branched) 2-alkanones also show an odd carbon number prevalence in plants and other organisms, and it was shown that their biosynthesis indeed proceeds via the acetate pathway. In this paper, we propose three possible biosynthetic pathways by which 3-methyl-2-alkanones could be formed in the plant tissues. The essential distinction between them lies in the way the branching methyl group is introduced. The Gaussian parameter σ for the observed distribution of these ketones could be interpreted as the error introduced by the first elongase enzyme system involved in the biosynthesis of fatty acid-derived compounds.*

Key words: *3-methyl-2-alkanones*, Inula helenium *L*, *essential oil*, *Gaussian-like distribution*, *biosynthesis*, *acetate pathway*

1. INTRODUCTION

Inula helenium L. (Compositae) is a perennial herb native to Southeastern Europe. In Serbia, it is a widespread plant species ("oman" in Serbian), used in folk medicine mostly for the treatment of respiratory conditions, disorders of digestion, urinary infections, and for skin disorders (Tucakov, 1984).

Recently, we reported that *I. helenium* root essential oil possesses a very potent antistaphylococcal activity, with obvious membrane-damaging effects. A bioassay guided fractionation of the oil yielded a number of chromatographic fractions that had the

Received October 01st, 2015; accepted November 26th, 2015.
[†] Acknowledgement: The authors acknowledge the Ministry of Education, Science and Technological Development of Serbia for the financial support (Project No. 172061). This work is a part of the PhD thesis of Marija S. Genčić supervised by Niko S. Radulović.
Dedicated to Professor Radosav Palić on the happy occasion of his 70th birthday.
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activity that surpassed that of the oil itself and further enabled the location of active principles (Stojanović-Radić et al., 2012). One of these fractions (eluting with 5% diethyl ether in hexane), highly active against *S. aureus*, contained an unidentified sesquiterpene aldehyde and a series of nine compounds showing regularities in their GC retention behavior and possessing analogous mass spectra (Fig. 1). The MS data (*e.g.* base peak at *m/z* 72) hinted that the compounds of this series could be 3-methyl-2-alkanones of varying chain lengths (C_{11}–C_{19}) that would represent secondary metabolites found for the first time in the Plant Kingdom. Since the isolation of single compounds from this fraction was impossible, we opted for the creation of a small synthetic combinatorial library of these long chain 3-methyl-2-alkanones that enabled their unequivocal identification, as well as, the determination of their potential biological role (Radulović et al., 2014).

Thus, in continuation of our previous studies on the volatile constituents of *I. helenium*, in this paper, based on the available evidence related to the biosynthesis of the mentioned long-chain 3-methyl-2-alkanones, we propose the most likely biosynthetic pathways by which these rare secondary metabolites could form in the plant tissues.

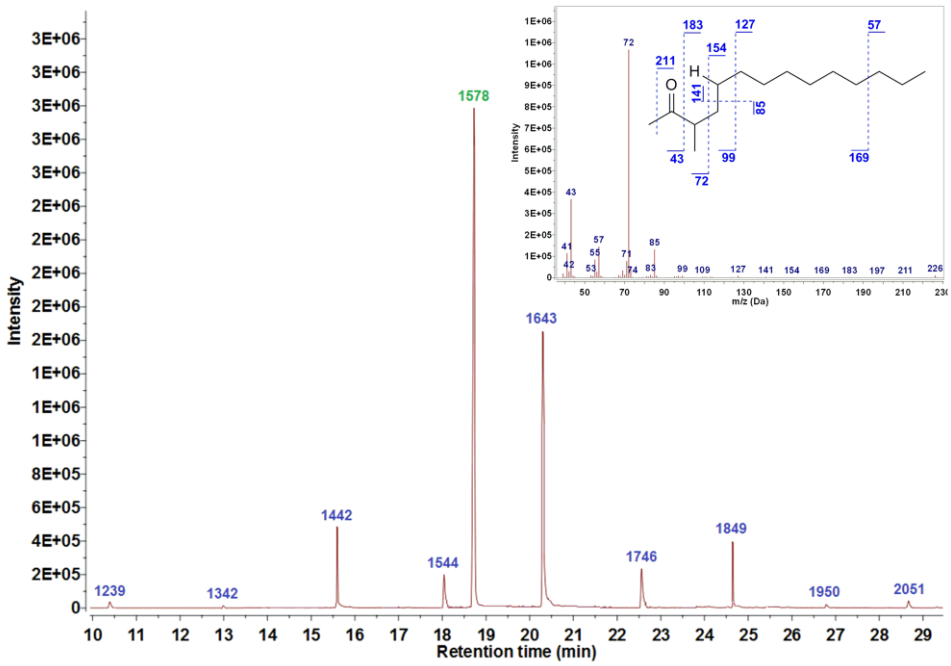

Fig. 1 TIC chromatogram of the fraction of *I. helenium* root essential oil (5% diethyl ether in hexane) displaying peaks (blue colored values of retention indices) corresponding to 3-methyl-2-alkanones and the mass spectrum of 3-methyltetradecan-2-one (RI = 1643, green colored value).

2. MATERIALS AND METHODS

2.1. Plant material

Dried roots of *I. helenium* were purchased from a local herb shop in Niš, Serbia (produced by Josif Pančić Institute, Belgrade, Serbia). The plant material was macro- and microscopically examined by a trained botanist 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 Sciences and Mathematics, University of Niš, under the accession numbers DM0112.

2.2. Essential-oil isolation

Air-dried to constant weight, roots of *I. helenium* (*ca.* 100 g) were grounded and subjected to hydrodistillation with *ca*. 500 mL of distilled water for 3.5 h using the original Clevenger-type apparatus. The obtained oil was separated by extraction with diethyl ether (Et_2O) and dried over anhydrous $MgSO_4$. The solvent was evaporated under a gentle stream of N_2 at room temperature in order to exclude any loss of the essential oil and stored at −18 °C until further analysis. The yield of the essential oil was 1.4% (w/w).

2.3. Essential-oil fractionation

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, particle size distribution 40-63 μm, Büchi. 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 visualized by UV light (254 nm) and by spraying with 50% (v/v) aqueous sulfuric 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 Et_2O : hexane, from pure hexane to pure Et_2O, 100 mL). The obtained fractions (10 mL) were pooled according to TLC and/or GC/MS analyses.

2.4. GC/MS analyses

The GC/MS analyses of the pure oil and MPLC fractions were repeated three times using a Hewlett-Packard 6890N gas chromatograph. The gas chromatograph was equipped with a fused silica capillary column DB-5MS (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 ºC, respectively. The oven temperature was raised from 70 to 290 ºC at a heating rate of 5 ºC min^{-1} and then isothermally held for 10 min. As a carrier gas helium at 1.0 mL min^{-1} was used. 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^{-1} for the first 0.5 min and then set to 1.0 mL min^{-1} throughout the remainder of the analysis; split ratio 40 : 1). Mass selective detector was operated at the ionization energy of 70 eV, in the 35–700 amu range with a scanning speed of 0.34 s. The percentage composition was computed from the GC peak areas without the use of correction factors.

3. RESULTS AND DISCUSSION

The 3-methyl-2-alkanones, found in the second most active fraction of *I. helenium* root essential oil, had a predominantly odd-numbered distribution centered at C_{15}, with an average chain-length (ACL) of 14.94, accompanied by a very high carbon preference index (CPI) value of 9.96. CPI and ACL were calculated according to a modified formula proposed by Reddy et al. (2000). The prevalence in plants, as well as in other organisms, of simple 2-alkanones with an odd-over-even carbon number predominance suggests that their biosynthesis is related to that of fatty acids and related compounds (Fridman et al., 2005). During the biosynthesis of 2-alkanones, 3-ketoacyl-ACP (ACP, acyl carrier protein) intermediates are diverted from the normal pathway leading to fatty acids prior to reduction of the 3-carbonyl moiety and are instead converted to the final 2-alkanones *via* two sequential enzyme-catalyzed reactions: first, hydrolysis of the ACP-linked 3-ketoacyl group and, second, decarboxylation of the resultant 3-ketoacid (Fig. 2) (Auldridge et al., 2012). Due to their structural similarity and the analogy in their distribution patterns, it could be assumed that the biosynthesis of 3-methyl-2-alkanones proceeds in an analogous manner as for 2-alkanones. However, there are three possible modes of introduction of the branching methyl group to the primarily formed polyketide chain: 1) methylation of a β-keto ester with a suitable biological electrophilic agent such as *S*-adenosylmethionine (Dewick, 2002), 2) through a starter possessing the branching methyl group in the appropriate position (Youping et al., 2010) or 3) by replacement of malonyl–CoA with methylmalonyl–CoA at specific points of the fatty acid chain elongation (Chase et al., 1992). Having this in mind, we can envisage at least three different biosynthetic pathways (A-C) leading to 3-methyl-2-alkanones (Fig. 2). Both pathways A and C begin with the same starter - propionyl-CoA. In pathway C the methyl group is introduced from methylmalonyl-CoA in the penultimate elongation step, whereas pathway A requires an additional *C*-methylation step followed by a final Claisen condensation with the subsequent hydrolysis of the thioester and decarboxylation (Fig. 2). Pathway B diverges most strikingly from the mentioned two as the methyl group in the position C-3 is to be introduced through an appropriate starter-C_6-3-methylpentanoyl-CoA (derived, for example, from homoisoleucine) and the corresponding ketone is set up by a regiospecific hydroxylation of an odd-numbered *anteiso*-alkane followed by an oxidation of the obtained alcohol (Chase et al., 1992).

Another fact that sustains the biosynthesis of 3-methyl-2-alkanones by the acetate pathway is their Gaussian-like distribution which can be readily perceived from Fig. 3. Very recently, we observed during a study dealing with *Lycopus europaeus* L. fruit waxes that the feature of epicuticular-wax (*n*-, *iso*- and *anteiso*-) alkane profiles is their Gaussian-like appearance and we proposed that these normal distributions could be interpreted as the end result of work of elongase enzyme systems where the Gaussian parameter μ should match the length of an "ideal" fatty acid biosynthesized and σ would represent the error introduced by this enzyme system. These curve parameters were shown to correspond excellently to ACL and CPI values usually utilized to describe the natural distribution of wax alkanes (Radulović et al., 2012). The distribution of odd-numbered 3-methyl-2-alkanones has the following Gaussian parameters: $\mu = 14.95$, $\sigma = 2.17$, $R^2 = 0.9999$. There is a remarkable matching between the values of parameters μ and ACL. Interestingly, the standard deviation σ for 3-methyl-2-alkanones has a value of

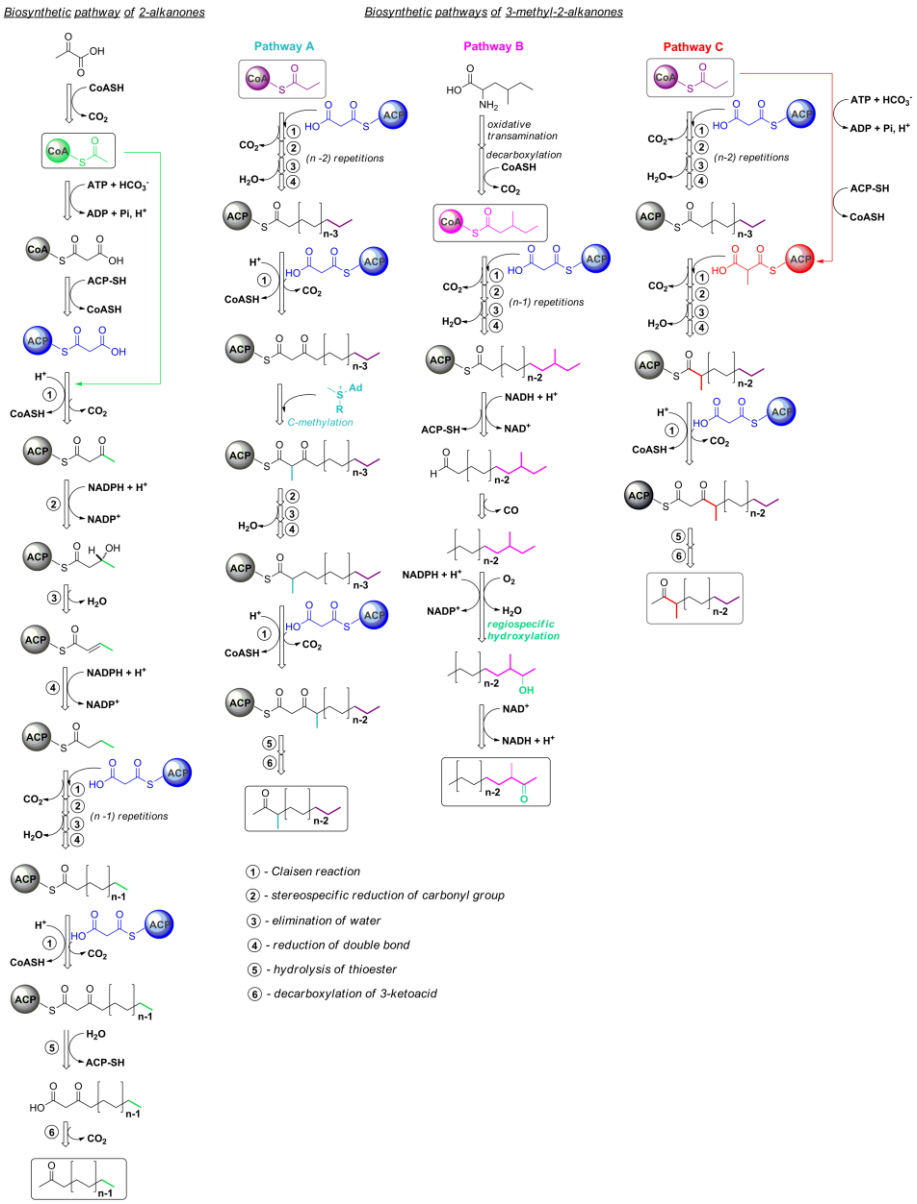

Fig. 2 Differences in the (proposed) biosynthetic pathways of odd-numbered 2-alkanones (Auldridge et al., 2012; Fridman et al., 2005) and 3-methyl-2-alkanones

ca. 2 C atoms (one acetate unit), whereas this error is for the four alkane distributions of *L. europaeus* (C_{21}–C_{34}) *ca.* two acetate (1.7-2.5) units (Radulović et al., 2012). This difference could be connected to the type of the elongation system(s) involved in the biosynthesis of these two classes of compounds. In general, the biosynthesis of major

wax components occurs *via* a sequential elongation of a primer with C_2 units derived from malonyl-CoA (Shepherd and Griffiths, 2006). In this condensation-elongation process, acyl chains of up to C_{16} and C_{18} formed by *de novo* synthesis are further extended to C_{30} or higher by a second elongation system. Finally, a modification of the acyl chain gives products including alkanes, aldehydes, primary alcohols, alkyl esters, secondary alcohols, ketones and various polyoxygenated compounds (Youping et al., 2010). It is proposed that the wax-alkane distribution is genetically predetermined by the depth of the hydrophobic pocket in the enzyme membrane complex of the second elongation system (as well as, by the number of these elongation enzymes) and that the distribution around this ideal (mean) would always have to correspond to the most probable errors two acetate units apart (Denic and Weissman, 2007; Radulović et al., 2012). Regarding the chain length of homologous 3-methyl-2-alkanones (C_{11}–C_{19}), it seems that their distribution reflects the work of the first elongation system, firstly, by the overall number of acetate units built into the longest detected homologue, and secondly, this system appears to be genetically encoded in such a way that it is less prone to error (*ca*. 1 acetate unit) than the second elongation system (*ca*. 2 acetate units).

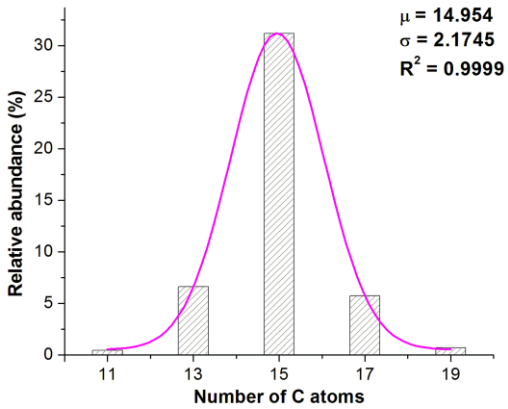

Fig. 3 Gaussian-like distributions of odd-numbered 3-methyl-2-alkanones relative contents

4. CONCLUSIONS

Homologous 3-methyl-2-alkanones, found in the *I. helenium* root essential oil, had a predominantly odd-numbered, Gaussian-like distribution which indicated that their biosynthesis probably proceeds *via* the acetate pathway. A similar distribution pattern has been previously observed for simple 2-alkanones whose biosynthesis is well studied. Thus, we assumed that biosynthesis of these two groups of constitutional isomers proceed in an analogous manner. However, the introduction of the branching methyl group of 3-methyl-2-alkanones requires additional steps. Up to now, for the biosynthesis of natural products derived primarily from the combinations of acetate units, three different modes of the branching methyl introduction group were recognized, so there are at least three plausible biosynthetic pathways leading to 3-methyl-2-alkanones. These were discussed in detail in this paper. Further studies are required to reveal which of

them is operational in the case of 3-methyl-2-alkanones. Finally, the Gaussian parameter σ could be interpreted as error in the biosynthesis introduced by the first elongase enzyme system, involved in the biosynthesis of fatty acid-derived compounds.

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BIOSINTEZA 3-METIL-2-ALKANONA DUGOG UGLJOVODONIČNOG LANCA IZ ETARSKOG ULJA KORENA BILJNE VRSTE *INULA HELENIUM* L. (ASTERACEAE)

3-Metil-2-alkanoni dugog ugljovodoničnog lanca su nađeni u etarskom ulju korena biljne vrste I. helenium *L. Uočena je Gausova raspodela njihovih relativnih količina, pri čemu su homolozi sa neparnim brojem C-atoma bili zastupljeniji. Ovakva raspodela ukazuje na to da njihova biosinteza protiče veoma slično kao i biosinteza masnih kiselina i srodnih jedinjenja. 2-Alkanoni sa neparnim brojem C-atoma su, takođe, zastupljeniji kod biljaka i ostalih organizama, a za njih je dokazano da zaista nastaju po gore pomenutom acetatnom biosintetskom putu. Imajući sve ovo u vidu, predložili smo tri moguća biosintetska puta kojim bi 3-metil-2-alkanoni mogli nastati u biljnim tkivima. Osnovna razlika između predloženih puteva je u načinu na koji se uvodi metil račva. Gausov parametar σ uočenih raspodela količina alkana bi se mogao posmatrati kao greška prvog enzimskog sistema elongaze koji učestvuje u biosintezi masnih kiselina i jedinjenja koja se iz njih izvode.*

Ključne reči: *3-metil-2-alkanoni, Inula helenium L, etarsko ulje, Gausova raspodela, biosinteza, acetatni biosintetski put*