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Diethyl-ether flower washings of *Dianthus cruentus* Griseb. (Caryophyllaceae): derivatization reactions leading to the identification of new wax constituents

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Some carnation species (*Dianthus* spp., Caryophyllaceae) exhibit a strong resistance to drought stress that is connected with the increased surface wax formation. Wax composition is unknown for the majority of *Dianthus* spp. Herein, mass spectral and gas chromatographic data, in combination with synthesis and chemical transformations (transesterification and synthesis of dimethyl disulfide adducts), enabled the identification of 151 constituents of diethyl-ether washings of fresh flowers of *Dianthus cruentus* Griseb. from Serbia. The flower wax contained, along with the dominant ubiquitous long-chain *n*-alkanes, homologous series of *n*- and branched (*iso*- and *anteiso*-) long-chain *n*-hexyl alkanoates/alkenoates and alkyl/alkenyl benzoates. The branching position in the mentioned hexyl esters was probed by synthesis of esters of three isomeric hexanols that were spectrally characterized (^{1}H- and ^{13}C-NMR, IR, MS). The washings also contained long-chain (*Z*)- and (*E*)-alkenes (C_{23}–C_{35}) with several different double bond regiochemistries. Fifty-five of these constituents (eight hexyl esters, two benzoates, and forty-five alkenes) were detected for the first time in Plantae, while 10 of these represent completely new compounds. The rare occurrence of these wax constituents makes them possible chemotaxonomic markers of this particular *Dianthus* sp.

Keywords: *Dianthus cruentus*, wax, diethyl-ether washings, branched long-chain esters, internal long-chain alkenes.

Introduction

The genus *Dianthus* L. (Caryophyllaceae) comprises over 300 species of herbaceous plants that are spread over a vast area, having a diversity hot-spot in the Mediterranean region.[1][2] Species of this genus are esteemed as ornamental plants that were either cultivated and collected from wild populations for more than 2000 years primarily due to the vibrant coloration (ranging from white to deep purple) and scent of their flowers.[3] According to the latest data, published in the second edition of the 'Flora of Serbia', the genus *Dianthus* contains 36 species;[4] however, plant species of the genus *Dianthus* originating from Serbia have never been previously investigated phytochemically. This characteristic pigmentation, among several other morphological traits, distinguishes *Dianthus* from other genera within the family Caryophyllaceae, although the evolutionary progress, diversification and the subdivision of the genus still remained controversial.[3] Species of this genus have a long history of use in folk medicine. For example, *Dianthus carthusianorum* was used by monks to relieve pain and rheumatism[2] and flowers of *D. caryophyllus* have been used in ethno medicine of northern Iraq for the treatment of gastrointestinal disorders, wounds and throat infections.[5] Secondary metabolites of *D. caryophyllus* flower extract and essential oil displayed a repellent effect against ticks (*Ixodes ricinus*) and mosquitoes (*Aedes aegypti* and *Culex pipiens*).[6][7] *Dianthus cruentus* Griseb. is an alpine carnation, native to the Balkans, having tall, upright flower stems on which clusters of pink-red flowers appear in late spring and summer (Figure S1a). This particular *Dianthus* taxon was never previously investigated.

Although ethnopharmacologically renown, *Dianthus* species were phytochemically sporadically studied, except for the classical pigment chemistry, which has been studied in detail.[3] A contemporary literature survey (SciFinder) revealed more than 4000 reports dealing with *Dianthus* species, however, less than forty reports dealt with the analysis of secondary metabolites of the taxa belonging to this genus. These report the presence of olfactory active esters of benzoic and salicylic acids, and other essential-oil components, as well as non-volatile triterpene saponins, in addition to the interesting phytoalexins derived from anthranilic acid generated as the plant's response to fungal infections.[8] Flower volatiles were investigated for their relevance for pollination biology and taxonomy.[9]

Some *Dianthus* spp. are known to exhibit a strong resistance to drought stress.[10] For example, *D. spiculifolius* Schur is believed to bare this characteristic, along with an adaptability to a wide range of environments, and this was connected with the increased wax formation on the surface of the plant species which is assumed to regulate non-stomatal water loss.[10] Cuticular waxes usually represent more or less complex mixtures of mostly saturated very-long-chain compounds (C_{20}-C_{34}) including the parent alkanes, and other poorly functionalized compounds such as primary and secondary alcohols, aldehydes, ketones, wax esters, as well as more elaborate in structure triterpenoids and flavonoids.[10] The initial, and only, analyses of wax

constituents of *Dianthus* taxa were done more than half a century ago when long-chain β-diketones (the most common constituent being tritriacontan-16,18-dione) were found to be the major components in the leaf and stem waxes from the carnation *D. caryophyllus*.[11] Only recently, the biosynthesis of these wax constituents, and gene regulation of production, in the petals of *D. caryophyllus* was elucidated.[12][13] In addition to 2-alkanones, 2-alkanols, and their esters with fatty acids,[13] other wax constituents were not reported for either *D. caryophyllus*, or *D. spiculifolius*.[10]

In this paper, as a continuation of our chemical investigation of plant species from the Serbian flora and with the aim to disclose other wax constituents of *Dianthus* species, we report the analyses of the chemical composition of *D. cruentus* Griseb. flower diethyl-ether washings. In order to identify selected esters detected by the initial GC and GC-MS analyses, a synthesis of selected behenates of isomeric hexanols was undertaken, as well as a transesterification of the 'washings' ester constituents, followed by a consideration of the structure-retention data relationships for hexyl esters of long-chain (*n*-, *iso*- and *anteiso*-) fatty acids. The identity of the detected esters of long-chain (*n*-, *iso*-, *anteiso*- and unsaturated) alcohols and benzoic acid was also corroborated in a similar manner as the mentioned hexyl esters. Homologous series of regioisomeric long-chain (*Z*)- and (*E*)-alkenes differing in the location of double bonds were identified by a combination of MS and RI data together with dimethyl disulfide (DMDS) derivatization and subsequent analysis of MS fragmentation patterns of the obtained adducts. In total, this approach allowed to detect and identify 10 new natural esters and 45 new natural alkenes.

Results and Discussion

A combination of detailed GC-MS (and GC) analyses and synthetic work (transesterification, DMDS derivatization, and synthesis of pure compounds; Figure S1c) enabled the identification of 151 volatiles from diethyl-ether washings of *Dianthus cruentus* Griseb. (Caryophyllaceae) flowers collected from a single wild-growing population at the high slopes of Šara mountain (Serbia). The identified constituents represented 84.3% of the total detected GC-peak areas (Table 1 and Figure S1b). The dominant compound classes present were the alkanes (46.8%, *n*- and branched, 38.5 and 8.3%, respectively), esters of normal/branched long-chain fatty acids or fatty alcohols (15.1%), carboxylic acids (9.8%), and alkenes (6.9%, terminal and internal alkenes, 0.1 and 6.8%, respectively), that together constituted almost 90% of all identified constituents of *D. cruentus* flower washings (Table 1). The remaining part of the identified constituents belonged to aliphatic aldehydes, shikimate metabolites, and other less abundant compound classes. The major wax constituents (relative abundance higher than 5%) were the normal-chain alkanes: heptacosane (19.5%), nonacosane (6.5%), and pentacosane (5.0%). In addition, a relatively high amount of free fatty acids (linoleic (3.5%), linolenic (3.4%), and palmitic (2.6%) acids) and branched long-chain alkanes (2-methyltriacontane (2.1%), 3-methylhentriacontane (1.5%), and 3-methylnonacosane (1.1%)) were present in the analyzed sample (Table 1).

Table 1. The chemical composition of the diethyl-ether washings of fresh flowers of *Dianthus cruentus* from Serbia.

No[a]	RI[b]	Compound	Class[c]	[%][d]	ID[e]
1	802	Hexanal	ALD	tr	MS, RI, CoI
2	834	2-Methylbutanoic acid	AC	tr	MS, RI, CoI
3	852	(*Z*)-Hex-3-en-1-ol	O	tr	MS, RI, CoI
4	867	1-Hexanol	O	tr	MS, RI, CoI
5	903	Heptanal	ALD	0.2	MS, RI, CoI
6	959	Benzaldehyde	SK	tr	MS, RI, CoI
7	1036	Benzyl alcohol	SK	0.4	MS, RI, CoI
8	1047	Phenylacetaldehyde	SK	0.1	MS, RI, CoI
9	1065	Heptanoic acid	AC	0.3	MS, RI, CoI
10	1106	Nonanal	ALD	0.1	MS, RI, CoI
11	1120	Maltol	O	0.4	MS, RI
12	1122	Phenethyl alcohol	SK	0.3	MS, RI, CoI
13	1160	Benzoic acid	SK	tr	MS, RI, CoI
14	1170	1-Nonanol	O	tr	MS, RI, CoI
15	1206	Decanal	ALD	tr	MS, RI, CoI
16	1217	4-Vinylphenol	SK	tr	MS, RI
17	1245	Phenylacetic acid	SK	tr	MS, RI, CoI
18	1266	Nonanoic acid	AC	tr	MS, RI, CoI

19	1308	Undecanal	ALD	tr	MS, RI, CoI
20	1316	2-Methoxy-4-vinylphenol	SK	tr	MS, RI
21	1425	(*E*)-Caryophyllene	O	tr	MS, RI, CoI
22	1453	Vanillyl alcohol	SK	tr	MS, RI
23	1462	Undecanoic acid	AC	tr	MS, RI, CoI
24	1570	Vanillic acid	SK	0.5	MS, RI
25	1683	Methyl 3-(4-hydroxy-3-methoxyphenyl)propanoate (*syn*. methyl dihydroferulate)	SK	tr	MS, RI
26	1688	3,4-Dimethoxyphenylacetic acid	SK	tr	MS, RI
27	1736	3-(4-Hydroxy-3-methoxyphenyl)propionic acid (*syn*. dihydroferulic acid)	SK	tr	MS, RI
28	1793	*p*-Coumaric acid	SK	1.4	MS, RI, CoI
29	1818	Hexadecanal	ALD	0.1	MS, RI
30	1861	Pentadecanoic acid	AC	tr	MS, RI, CoI
31	1870	(*E*)-Ferulic acid	SK	0.2	MS, RI, CoI
32	1900	Nonadecane	A	tr	MS, RI, CoI
33	1913	*O*,*O*-Dimethylcaffeic acid	SK	tr	MS, RI
34	1927	Methyl hexadecanoate (*syn*. methyl palmitate)	AE	tr	MS, RI, CoI
35	1961	Palmitic acid	AC	2.6	MS, RI, CoI
36	2022	Octadecanal	ALD	0.1	MS, RI, CoI
37	2063	2-Methyleicosane	BA	tr	MS, RI
38	2100	Heneicosane	A	0.2	MS, RI, CoI
39	2123	Nonadecanal	ALD	tr	MS, RI
40	2134	(*Z*,*Z*)-9,12-Octadecadienoic acid (*syn*. linoleic acid)	AC	3.5	MS, RI, CoI
41	2151	(*Z*,*Z*,*Z*)-9,12,15-Octadecatrienoic acid (*syn*. linolenic acid)	AC	3.4	MS, RI, CoI
42	2200	Docosane	A	0.1	MS, RI, CoI
43	2226	Eicosanal	ALD	0.1	MS, RI
44	2264	2-Methyldocosane	BA	0.2	MS, RI
45	2273	(*E*)-Tricos-10-ene	AL	tr[f]	MS, DMDS
46	2273	(*E*)-Tricos-9-ene	AL		MS, RI, DMDS
47	2282	(*E*)-Tricos-7-ene	AL	0.1	MS, RI, DMDS
48	2300	Tricosane	A	2.0	MS, RI, CoI
49	2327	Heneicosanal	ALD	0.1	MS, RI
50	2373	3-Methyltricosane	BA	0.1	MS, RI
51	2400	Tetracosane	A	0.4	MS, RI, CoI
52	2464	2-Methyltetracosane	BA	0.4	MS, RI
53	2475	(*E*)-Pentacos-11-ene	AL		MS, DMDS
54	2475	(*E*)-Pentacos-10-ene	AL	tr[f]	MS, DMDS
55	2475	(*E*)-Pentacos-9-ene	AL		MS, RI, DMDS
56	2483	(*E*)-Pentacos-7-ene	AL	0.1	MS, RI, DMDS
57	2496	Pentacos-1-ene	1-AL	0.1	MS, RI
58	2500	Pentacosane	A	5.0	MS, RI, CoI
59	2563	2-Methylpentacosane	BA	tr	MS, RI
60	2574	3-Methylpentacosane	BA	0.2	MS, RI
61	2600	Hexacosane	A	1.2	MS, RI, CoI
62	2612	1-Docosyl acetate	AE	0.5	MS, RI
63	2662	2-Methylhexacosane	BA	0.9	MS, RI

64	2675	(*E*)-Heptacos-13-ene	AL		MS, DMDS
65	2675	(*E*)-Heptacos-12-ene	AL		MS, RI, DMDS
66	2675	(*E*)-Heptacos-11-ene	AL	0.4[f]	MS, RI, DMDS
67	2675	(*E*)-Heptacos-10-ene	AL		MS, RI, DMDS
68	2675	(*E*)-Heptacos-9-ene	AL		MS, RI, DMDS
69	2684	(*E*)-Heptacos-7-ene	AL	0.3	MS, RI, DMDS
70	2694	Hydroxy-1-(hydroxymethyl)ethyl (*Z*,*Z*)-9,12-octadecadienoate (*syn*. 2-monolinolein)	AE	0.3	MS, RI
71	2700	Heptacosane	A	19.5	MS, RI, CoI
72	2764	2-Methylheptacosane	BA	tr	MS, RI
73	2785	Hexyl eicosanoate	AE	0.1	MS, RI, Tr
74	2800	Octacosane	A	0.7	MS, RI, CoI
75	2815	1-Tetracosyl acetate	AE	0.2	MS, RI
76	2831	*all*-(*E*)-Squalene	O	0.2	MS, RI, CoI
77	2839	(*Z*)-Nonacos-13-ene	AL		MS, DMDS
78	2839	(*Z*)-Nonacos-12-ene	AL		MS, DMDS
79	2839	(*Z*)-Nonacos-11-ene	AL	0.3[f]	MS, DMDS
80	2839	(*Z*)-Nonacos-10-ene	AL		MS, DMDS
81	2839	(*Z*)-Nonacos-9-ene	AL		MS, DMDS
82	2863	2-Methyloctacosane	BA	0.8	MS, RI
83	2874	(*E*)-Nonacos-13-ene	AL		MS, RI, DMDS
84	2874	(*E*)-Nonacos-12-ene	AL		MS, DMDS
85	2874	(*E*)-Nonacos-11-ene	AL	0.6[f]	MS, RI, DMDS
86	2874	(*E*)-Nonacos-10-ene	AL		MS, DMDS
87	2874	(*E*)-Nonacos-9-ene	AL		MS, RI, DMDS
88	2886	(*E*)-Nonacos-7-ene	AL	0.3	MS, RI, DMDS
89	2900	Nonacosane	A	6.5	MS, RI, CoI
90	2948	Hexyl 20-methylheneicosanoate	AE	0.1	MS, TR
91	2963	2-Methylnonacosane	BA	0.1	MS, RI
92	2974	3-Methylnonacosane	BA	1.1	MS, RI
93	2984	Hexyl docosanoate	AE	0.1	MS, RI, CoI
94	3000	Triacontane	A	0.6	MS, RI, CoI
95	3014	1-Hexacosyl acetate	AE	0.1	MS, RI
96	3038	1-Eicosyl benzoate	BE	0.3	MS, RI
97	3042	(*Z*)-Hentriacont-15-ene	AL		MS, DMDS
98	3042	(*Z*)-Hentriacont-14-ene	AL		MS, DMDS
99	3042	(*Z*)-Hentriacont-13-ene	AL		MS, DMDS
100	3042	(*Z*)-Hentriacont-12-ene	AL	0.4[f]	MS, DMDS
101	3042	(*Z*)-Hentriacont-11-ene	AL		MS, DMDS
102	3042	(*Z*)-Hentriacont-10-ene	AL		MS, DMDS
103	3042	(*Z*)-Hentriacont-9-ene	AL		MS, DMDS
104	3063	2-Methyltriacontane	BA	2.1	MS, RI
105	3078	(*E*)-Hentriacont-15-ene	AL		MS, RI, DMDS
106	3078	(*E*)-Hentriacont-14-ene	AL	2.6[f]	MS, RI, DMDS
107	3078	(*E*)-Hentriacont-13-ene	AL		MS, RI, DMDS
108	3078	(*E*)-Hentriacont-12-ene	AL		MS, DMDS

109	3078	(*E*)-Hentriacont-11-ene	AL		MS, RI, DMDS
110	3078	(*E*)-Hentriacont-10-ene	AL		MS, DMDS
111	3078	(*E*)-Hentriacont-9-ene	AL		MS, RI, DMDS
112	3088	(*E*)-Hentriacont-7-ene	AL	0.9	MS, RI, DMDS
113	3100	Hentriacontane	A	tr	MS, RI, CoI
114	3114	1-Heptacosyl acetate	AE	2.8	MS, RI, TR
115	3124	2-Nonacosanone	O	0.7	MS, RI
116	3143	Vitamin E (*syn*. α-tocopherol)	O	0.8	MS, RI, CoI
117	3150	Hexyl 22-methyltricosanoate	AE	1.4	MS, TR
118	3151	3,4',5,7-Tetrahydroxyflavone (*syn*. kaempferol)	O	tr	MS, RI
119	3176	3-Methylhentriacontane	BA	1.5	MS, RI
120	3187	Hexyl tetracosanoate	AE	0.5	MS, RI, TR
121	3200	Dotriacontane	A	0.3	MS, RI, CoI
122	3208	20-Methylheneicosyl benzoate	BE	0.3	MS, TR
123	3216	1-Octacosyl acetate	AE	0.3	MS, RI
124	3234	Docos-15-en-1-yl benzoate[g]	BE	0.4	MS, TR, DMDS
125	3247	1-Docosyl benzoate	BE	1.0	MS, TR
126	3263	2-Methyldotriacontane	BA	0.5	MS, RI
127	3278	(*E*)-Tritriacont-15-ene	AL		MS, DMDS
128	3278	(*E*)-Tritriacont-14-ene	AL		MS, DMDS
129	3278	(*E*)-Tritriacont-13-ene	AL		MS, RI, DMDS
130	3278	(*E*)-Tritriacont-12-ene	AL	0.6[f]	MS, RI, DMDS
131	3278	(*E*)-Tritriacont-11-ene	AL		MS, DMDS
132	3278	(*E*)-Tritriacont-10-ene	AL		MS, DMDS
133	3278	(*E*)-Tritriacont-9-ene	AL		MS, DMDS
134	3288	(*E*)-Tritriacont-7-ene	AL	0.2	MS, DMDS
135	3300	Tritriacontane	A	1.3	MS, RI, CoI
136	3314	1-Nonacosyl acetate	AE	0.8	MS, RI, TR
137	3351	Hexyl 24-methylpentacosanoate	AE	0.6	MS, TR
138	3373	3-Methyltritriacontane	BA	0.4	MS, RI
139	3390	Hexyl hexacosanoate	AE	2.2	MS, RI, TR
140	3453	1-Tetracosyl benzoate	BE	0.7	MS, RI, TR
141	3463	Hexyl 24-methylhexacosanoate	AE	0.3	MS, TR
142	3500	Pentatriacontane	A	0.7	MS, RI, CoI
143	3515	1-Hentriacontyl acetate	AE	0.7	MS, RI, TR
144	3550	Hexyl 26-methylheptacosanoate	AE	0.3	MS, TR
145	3588	Hexyl octacosanoate	AE	0.5	MS, TR
146	3658	1-Hexacosyl benzoate	BE	tr	MS, TR
147	3662	Hexyl 26-methyloctacosanoate	AE	0.2	MS, TR
148	3750	Hexyl 28-methylnonacosanoate	AE	0.1	MS, TR
149	3788	Hexyl triacontanoate	AE	0.2	MS, TR
150	3862	Hexyl 28-methyltriacontanoate	AE	0.1	MS, TR
151	3862	1-Octacosyl benzoate	BE	tr	MS, TR
		Total identified [%]		**84.3**	
		n-Alkanes	A	38.5	

Branched alkanes (*iso*- and *anteiso*-)	BA	8.3
1-Alkenes	1-AL	0.1
Internal alkenes	AL	6.8
Aldehydes	ALD	0.7
Carboxylic acid	AC	9.8
Aliphatic esters	AE	12.4
Benzoates	BE	2.7
Shikimate pathway metabolites	SK	2.9
Others	O	1.7

[a] Compounds listed in order of elution from a GC column. [b] Retention indices determined experimentally on the DB-5MS column relative to a series C_8-C_{40}*n*-alkanes. [c] Values are means of three individual analyses; tr, trace amounts (< 0.05%). [d] The abbreviations of the compound classes are given at the end of the table. [e] ID = Compound identification: MS, mass spectra matching; RI, retention indices matching with literature data; CoI, co-injection with a pure reference compound; TR, identification by transesterification; DMDS, identification by derivatization with dimethyl disulfide. [f] Alkenes with different double bond locations (C_9 – C_{15}) represented one wide peak in GC chromatogram and for that reason it was not possible to determine their distinct relative amount in the washings. [g] Exact stereochemistry of the double bond was not determined.

In addition to the ubiquitous constituents (e.g. the above mentioned fatty acids, alkanes etc.), some of the detected wax components have a very restricted natural occurrence. Two classes of constituents caught our attention: several series of long-chain fatty acid esters and long-chain fatty alcohols, as well as alkenes (monoenes) with different regiochemistries of their double bonds (we will address the identification of these alkenes later on in the paper).

Analogous fragmentation patterns visible in the mass spectra of seven constituents with high RI values (2785, 2984, 3187, 3390, 3588 and 3788) on a DB-5MS column suggested that these constituents might represent homologous hexyl esters of long-chain saturated fatty acids. This tentative identification was based on the observed characteristic fragmentation pattern: the base peak at *m/z* 84 (present in the MS of hexyl esters[14]) and relatively intense peaks of RCO_2H^+, $RCO_2H_2^+$ ions (for example, *m/z* 340 and 341 in the case of docosanoates). The identity of several of the detected *n*-hexyl esters (*n*-hexyl eicosanoate, docosanoate, tetracosanoate, hexacosanoate, octacosanoate and triacontanoate) were easily corroborated by a comparison of their retention indices with literature values[14] and for *n*-hexyl docosanoate (syn. *n*-hexyl behenate) also by GC co-chromatography of the washings with a synthetically obtained pure standard (see Experimental part). A literature search showed that the above mentioned *n*-hexyl esters were only sporadically reported as plant/animal species metabolites, but neither of these reports included a *Dianthus* taxon or the plant family Caryophyllaceae (Table S1).

Alongside these seven 1-hexyl esters of *n*-chain acids, additional two series (grouped based on their regular change of RI values) of related constituents with very similar MS-fragmentation patterns were found to elute slightly faster from the GC column than the *n*-chain hexyl homologs. This implied the existence of branched-chain (in the alcohol or acid moieties) isomers (methylpentyl esters of *n*-chain fatty acids or *vice versa*, 1-hexyl esters of branched fatty acids). The type of branching, i.e. the most likely existence of esters of *iso*-and *anteiso*-chained fatty acids or 3- and 4-methylpentanols, and the exclusion of other isomers (such as secondary alcohols, or the presence of multiple branching), was inferred from the small differences in the RI values compared with the straight-chain isomers.[15] The first group of branched esters had 26 units lower RI values compared to the identified *n*-chain esters and this was indicative of *anteiso*-branched compounds.[15] The second group was characterized by eluting 11 additional RI units faster (their RI values were 37 units lower than the *n*-isomers) which is typical for *iso*-branched counterparts.[15] This gas chromatographic behavior of *n*-, *iso*-and *anteiso*-compounds was also observable in the identified alkanes in the washings (e.g. 2-methylnonacosane, 3-methylnonacosane and triacontane with RI = 2963, 2974 and 3000, respectively; Table 1), additionally confirming the ΔRI values between the *n*-, *iso*- and *anteiso*-isomers.

The specific *iso*- and *anteiso*-long-chain fatty acids, needed to prepare the synthetic samples of 1-hexyl esters for a direct comparison, are commercially unavailable. For that reason, we followed a non-standard approach that included two parts: the synthesis of 3- and 4-methylpentyl, and 1-hexyl behenates (all synthetized behenates were completely spectrally characterized (^{1}H- and ^{13}C-NMR, MS, and IR; Experimental part and Figures S2-S22), and a transesterification (methanolysis) of a part of the washings. It is worth noting that 4-methylpentyldocosanoate (*syn.* isohexyl behenate) and 3-methylpentyldocosanoate represent completely new compounds. Our assumption was that the rejection of isomers baring a branch in the alcohol moiety, and the transesterification of a hexyl ester to a methyl one, would provide sufficient information to permit us to positively identify the structures of these esters. For example, if the washings contained branched hexyl esters of *n*-behenic acid (the three synthesized esters corresponded to non-overlapping peaks in the chromatogram with RI values 2949, 2957 and 2984, for 4-methylpentyl-, 3-methylpentyl- and 1-hexyl behenates, respectively) after transesterification with MeONa in MeOH, one would expect the onset of only one peak of the corresponding methyl behenate. On the other hand, if the sample consisted of *n*-hexyl esters of *n*-, *iso*- and/or *anteiso*-long-chain fatty acids, transesterification would yield three methyl esters with similar MS fragmentation patterns (e.g. a chromatogram would contain peaks of methyl *n*-, *iso*- and/or *anteiso*-behenates). Since, besides the hexyl esters, we did not detect any other ester of long-chained fatty acids in the washings, a detailed GC-MS analysis of the transesterified washings proved the presence of

several branched-chain fatty acid moieties in the detected constituents (methyl esters of *iso*- (20-methylheneicosanoic, 22-methyltricosanoic, 24-methylpentacosanoic, 26-methylheptacosanoic, and 28-methylnonacosanoic acids) and *anteiso*-fatty acids (24-methylhexacosanoic, 26-methyloctacosanoic,and28-methyltriacontanoic acids)). The identification of these methyl esters was based on an analysis of their mass spectra and the comparison of the obtained RI data with the previously published ones for lower homologs from the series of *n*-, *iso*- and/or *anteiso*-long-chain fatty acids methyl esters.[16] Thus, in this way we proved the presence of *n*-hexyl esters of the above-mentioned branched *iso*- or *anteiso*-fatty acids (C_{22} – C_{31}; Table 1) in the washings. Based on a detailed literature survey (Table S1), all identified hexyl esters of *iso*- or *anteiso*-fatty acids (8 constituents in total) represent new compounds and newly discovered natural products (Figure 1).

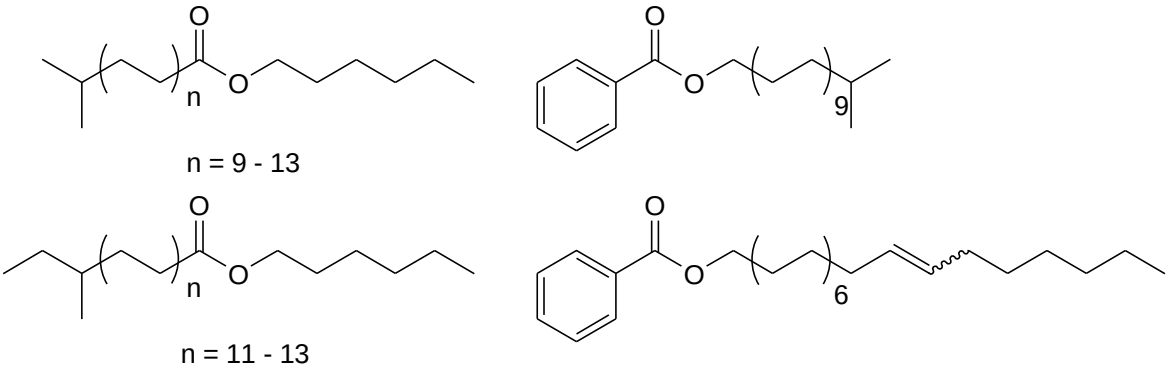

Figure 1. Structures of new natural compounds identified in the diethyl-ether washings of *Dianthus cruentus* Griseb. (Caryophyllaceae) flowers

Almost identical mass spectra of the esters of *n*-hexanol and *iso*-acids, and the esters of the corresponding 4-methyl-1-pentanol and *n*-acids (e.g. hexyl 20-methylheneicosanoate and 4-methylpentyldocosanoate), as well as the practically identical retention indices of the two sets of compounds (e.g. 2948 and 2949 for hexyl 20-methylheneicosanoate and 4-methylpentyldocosanoate, respectively), disclose that identification based solely on these two parameters is not possible. The situation is the same for the *anteiso*-branched acid and the corresponding alcohol esters. It seems that, in the case of such very long-chain esters, transesterification is a necessary method for the differentiation of the branching position.

Alongside the *n*-hexyl esters, a homologous series of five constituents, in trace amounts or up to 1% abundance, was tentatively identified as long-chain alkyl benzoates (esters of benzoic acid with eicosanol, docosanol, tetracosanol, hexacosanol, and octacosanol). Besides the analysis of the MS fragmentation patterns, in several cases, the identification was confirmed by matching of the values of the corresponding retention indices with literature data (Table 1).[17] All MSes displayed a base ion at *m/z*123 ($C_6H_5CO_2H_2^+$) and other intense ions at *m/z* 105 and 77 ($C_7H_5O^+$ and C_6H_5, respectively) which are indicative of benzoates; the presence of the molecular ion gave the opportunity to allocate the number of carbon atoms to the alcoholic moiety. Additional related constituents with the MS fragmentations analogous to that of the mentioned benzoates were located by extracting partial-ion current chromatograms for *m/z* 123 and105. Among the now more clearly visible peaks containing the two fragment ions, a constituent eluting 40 units faster (RI = 3208) than 1-docosyl benzoate (RI = 3248) was identified as 20-methylheneicosyl benzoate (a new natural product; Tables 1 and S1; Figure 1) in the manner already discussed for the hexyl esters; in addition to MS and ΔRI data, the transesterification experiment verified the presence of branched fatty alcohols.

Besides incorporating *n*- and *iso*-alcohols, an additional benzoate with a slightly lower RI value (3234) compared to that of 1-docosyl benzoate (3247) was also observed. Ions at *m/z* 306 ($C_{22}H_{42}^+$) and 428 ($M^{+\cdot}$) implied a benzoate of a docosenol (Figure S23). The position of the double bond was inferred from another derivatization - the reaction of a sample of the washings with dimethyl disulfide (DMDS; Table 2). The obtained DMDS-derivatized sample was subjected to GC-MS analysis and a careful inspection of the chromatogram allowed the location of a peak belonging to the adduct of the unsaturated benzoate, and furthermore, a consideration of its MS fragmentation led to the regiochemistry of the double bond. The MS of the DMDS adduct showed two intense fragment ions at *m/z* 377 and 145 $[M - 377]^+$. A third fragment ion was also observed at *m/z* 255 corresponding to the loss of benzoic acid from the ion at *m/z* 377 (Figure S24). Such ions could arise from an isomer in which the double bond was between C-15-C-16. This meant that docos-15-en-1-yl benzoate was also present in the washings of *D. cruenthus* flowers, i.e. we identified a new natural product (Figure 1).

The second group of compounds that seemed to deserve our attention was a homologous series of long-chain alkenes that also nicely reacted with DMDS and yielded easily observable adduct-peaks in the chromatogram of the derivatized sample. These alkenes seemed to display several different double bond locations. For example, besides 1-pentacosene, which was identified based on a comparison of the experimental RI value and its mass spectrum with those from the available commercial libraries, the chromatogram contained other groups of peaks with a similar mass spectral fragmentation (Table 1). Since RI and MS data are not sufficient (very similar for regioisomeric internal alkenes) to permit a definite identification of an internal alkene, we turned to the DMDS derivatized chromatogram. For example, in the case of the isomeric hentriacontenes which were of the highest relative abundance in the washings (2.6%), the peaks corresponding to the original alkenes (RI = 3042, 3078 and 3088) disappeared from the

chromatogram of the DMDS derivatized sample and this was accompanied by the appearing of three peaks of DMDS adducts having the appropriate molecular weight (at *m/z* = 528, which comes from 434 + 2x47). Since there are 15 regioisomeric hentriacontenes, that would give rise to a specific combination of fragment ions at *m/z* [M – $CH_3(CH_2)_nCHSCH_3$] and [M –$CH_3(CH_2)_nCHSCH_3$], we inspected the chromatogram of the derivatized sample for the presences of all combinations. The presence of some adducts was only revealed through the examination of partial ion chromatograms (PIC) and the "co-elution" of the two ion currents of the corresponding pairs of fragment ions (Supplementary Figures S25-S59). These DMDS adducts eluted very closely one to each other or practically co-eluted, and this was when the use of mass spectral deconvolution was of utmost importance. Thus, this approach allowed finding and positively identifying a number of alkenes that would have been missed out in standard analyses. The possibility that positional (or other) isomerization took place was ruled out by DMDS derivation and further analysis of a pure commercial sample of methyl oleate which did not show any signs of double bond migration or *E*/*Z* isomerization during the derivation and analysis procedures (a single peak of the adduct was observed and only a single expected combination of fragment ions).

Table 2 provides the combinations of values of fragment ions at *m/z* [M – $CH_3(CH_2)_nCHSCH_3$] and [M –$CH_3(CH_2)_nCHSCH_3$], for n = 0 – 16,that were observed in the TIC or PICs (Figures S25-S59). In this way we discovered adducts of 48 different (*E*) and (*Z*) long-chain alkenes with double-bond positions at C-7, C-9 – C-15 (Table 1). The formation of DMDS adducts is believed to be entirely stereospecific,[17] and that means, if both (*Z*) and (*E*)-diastereoisomers of, for example, hentriacont-10-ene were present in the washings, we could expect two GC-peaks representing the *threo*- and *erythro*-DMDS derivatives (formed from the *Z*- and *E*-isomers, respectively). Also, previously it was demonstrated that the *threo*- and *erythro*-DMDS derivatives eluted separately from both polar and apolar GC columns and that the DMDS adduct derived from the *Z*-isomer is the faster eluting one.[18] Thus, for a pair of DMDS adduct peaks, possessing the same mass fragmentation, the one having a shorter Rt originated from the *Z*-isomer, for example: Rt = 51.08 min was assigned to the DMDS adduct of (*Z*)-hentriacont-10-ene and the one at Rt = 51.68 min to the adduct of the *E*-isomer ((*E*)-hentriacont-10-ene). Thus, this implied the presence of (*Z*)-hentriacont-10-ene and (*E*)-hentriacont-10-enein the washings with the values of the retention indices of 3043 and 3078, respectively (Table 1). The difference between the RI values of *E* and *Z* geometrical isomers for the same position of the double bond in a long-chain alkene becomes progressively larger with the length of the hydrocarbon chain,[19] and follows the following trend for the $CH_3(CH_2)_nCH{=}CH(CH_2)_nCH_3$ isomers: RI = 97.23 x (2n+4) + 11.43.

Table 2. Identified DMDS adducts in GC chromatogram of, with dimethyl disulfide, derivatized crude diethyl ether extract sample.

RI[a]	Compound	MW[b]	*m/z*[c]	
			1	**2**
2924	*erythro*-(Tricosane-10,11-diyl)*bis*(methylsulfane)	416	229	187
2924	*erythro*-(Tricosane-9,10-diyl)*bis*(methylsulfane)	416	243	173
2931	*erythro*-(Tricosane-7,8-diyl)*bis*(methylsulfane)	416	271	145
3124	*erythro*-(Pentacosane-11,12-diyl)*bis*(methylsulfane)	444	243	201
3124	*erythro*-(Pentacosane-10,11-diyl)*bis*(methylsulfane)	444	257	187
3127	*erythro*-(Pentacosane-9,10-diyl)*bis*(methylsulfane)	444	271	173
3136	*erythro*-(Pentacosane-7,8-diyl)*bis*(methylsulfane)	444	299	145
3329	*erythro*-(Heptacosane-13,14-diyl)*bis*(methylsulfane)	472	243	229
3329	*erythro*-(Heptacosane-12,13-diyl)*bis*(methylsulfane)	472	257	215
3330	*erythro*-(Heptacosane-11,12-diyl)*bis*(methylsulfane)	472	271	201
3330	*erythro*-(Heptacosane-10,11-diyl)*bis*(methylsulfane)	472	285	187
3330	*erythro*-(Heptacosane-9,10-diyl)*bis*(methylsulfane)	472	299	173
3341	*erythro*-(Heptacosane-7,8-diyl)*bis*(methylsulfane)	472	327	145
3495	*threo*-(Nonacosane-13,14-diyl)*bis*(methylsulfane)	500	271	229
3495	*threo*-(Nonacosane-12,13-diyl)*bis*(methylsulfane)	500	285	215
3495	*threo*-(Nonacosane-11,12-diyl)*bis*(methylsulfane)	500	299	201
3495	*threo*-(Nonacosane-10,11-diyl)*bis*(methylsulfane)	500	313	187
3495	*threo*-(Nonacosane-9,10-diyl)*bis*(methylsulfane)	500	327	173
3530	*erythro*-(Nonacosane-13,14-diyl)*bis*(methylsulfane)	500	271	229
3531	*erythro*-(Nonacosane-12,13-diyl)*bis*(methylsulfane)	500	285	215
3531	*erythro*-(Nonacosane-11,12-diyl)*bis*(methylsulfane)	500	299	201
3531	*erythro*-(Nonacosane-10,11-diyl)*bis*(methylsulfane)	500	313	187
3335	*erythro*-(Nonacosane-9,10-diyl)*bis*(methylsulfane)	500	327	173
3344	*erythro*-(Nonacosane-7,8-diyl)*bis*(methylsulfane)	500	355	145
3697	*threo*-(Hentriacontane-15,16-diyl)*bis*(methylsulfane)	528	271	257

3697	*threo*-(Hentriacontane-14,15-diyl)*bis*(methylsulfane)	528	285	243
3697	*threo*-(Hentriacontane-13,14-diyl)*bis*(methylsulfane)	528	299	229
3697	*threo*-(Hentriacontane-12,13-diyl)*bis*(methylsulfane)	528	313	215
3698	*threo*-(Hentriacontane-11,12-diyl)*bis*(methylsulfane)	528	327	201
3700	*threo*-(Hentriacontane-10,11-diyl)*bis*(methylsulfane)	528	341	187
3735	*erythro*-(Hentriacontane-15,16-diyl)*bis*(methylsulfane)	528	271	257
3735	*erythro*-(Hentriacontane-14,15-diyl)*bis*(methylsulfane)	528	285	243
3735	*erythro*-(Hentriacontane-13,14-diyl)*bis*(methylsulfane)	528	299	229
3735	*erythro*-(Hentriacontane-12,13-diyl)*bis*(methylsulfane)	528	313	215
3737	*erythro*-(Hentriacontane-11,12-diyl)*bis*(methylsulfane)	528	327	201
3739	*erythro*-(Hentriacontane-10,11-diyl)*bis*(methylsulfane)	528	341	187
3741	*erythro*-(Hentriacontane-9,10-diyl)*bis*(methylsulfane)	528	355	173
3751	*erythro*-(Hentriacontane-7,8-diyl)*bis*(methylsulfane)	528	383	145
3930	15,16-*bis*(Methylthio)docosyl benzoate	522	377	145
3940	*erythro*-(Tritriacontane-15,16-diyl)*bis*(methylsulfane)	556	299	257
3940	*erythro*-(Tritriacontane-14,15-diyl)*bis*(methylsulfane)	556	313	243
3940	*erythro*-(Tritriacontane-13,14-diyl)*bis*(methylsulfane)	556	327	229
3940	*erythro*-(Tritriacontane-12,13-diyl)*bis*(methylsulfane)	556	341	215
3942	*erythro*-(Tritriacontane-11,12-diyl)*bis*(methylsulfane)	556	355	201
3945	*erythro*-(Tritriacontane-10,11-diyl)*bis*(methylsulfane)	556	369	187
3946	*erythro*-(Tritriacontane-9,10-diyl)*bis*(methylsulfane)	556	383	173
3956	*erythro*-(Tritriacontane-7,8-diyl)*bis*(methylsulfane)	556	411	145

[a] Retention indices determined experimentally on the DB-5MS column relative to a series C_8-C_{40}*n*-alkanes. [b] MW = molecular weight. [c] *m/z* = characteristic fragmentation pattern in DMDS adducts: 1 = $[M - CH_3(CH_2)_nCHSCH_3]^+$ and 2 = $[CH_3(CH_2)_nCHSCH_3]^+$.

Forty-five of these long-chain alkenes with the double-bond position at C-7, C-9 – C-15 represent new natural products. The limited occurrence of the detected compounds can be illustrated by the data given in Table S1. For that reason, these natural products could be used as chemotaxonomic markers of *Dianthus cruentus*, and possibly of the genus *Dianthus* and/or Caryophyllaceae plant family. However, the chemotaxonomic significance is yet to be verified and this will be one of our aims in the future.

All of the identified compounds, due to their hydrophobic nature, surely contribute to the resistance of this particular carnation to drought stress via regulating non-stomatal water loss.[10] The heterogeneity of the structures of the detected wax constituents could also impart to the not-rigidity (lower degree of crystallinity) of the surface wax making it more adaptable to flower tissues growth. Additionally, the benzoates might contribute to the UV-protection of this high-mountain species by absorbing this part of the Sun's spectrum. A high number of detected compounds baring an unsaturation could also have an antioxidant role by trapping reactive oxygen species and not allowing them to make their way into the plant tissues.

Conclusions

Detailed GC and GC-MS analysis of a diethyl-ether washings sample obtained from fresh flowers of *Dianthus cruentus* Griseb. (Caryophyllaceae) enabled the identification of 151 constituents, including ten completely new compounds (Figure 1): hexyl 20-methylheneicosanoate, hexyl 22-methyltricosanoate, hexyl 24-methylpentacosanoate, hexyl 24-methylhexacosanoate, hexyl 26-methylheptacosanoate, hexyl 26-methyloctacosanoate, hexyl 28-methylnonacosanoate, hexyl 28-methyltriacontanoate, 20-methylheneicosyl benzoate, and docos-15-en-1-yl benzoate. The identity of the wax constituents was unambiguously confirmed by GC co-injection of the washings sample with appropriate synthesized standards and/or by a non-standard approach that included transesterification or DMDS derivatization of crude extract sample. The current approach implies that the identification of long-chain esters and/or alkenes necessitates transesterification and DMDS derivatization as complementary identification methods as RI data and fragmentation pattern in mass spectra are practically indistinguishable for certain isomeric species within these series. Based on a detailed literature search, fifty-eight of these constituents (eight hexyl esters, two benzoates, and forty-eight alkenes) represent new natural products in Plantae. Two additional synthetic hexyl isomers, which were fully characterized (^{1}H- and ^{13}C-NMR, IR, MS), represent completely new compounds.

Experimental Section

General

All solvents (HPLC grade) were purchased from Sigma-Aldrich (St Louis, MO, USA). Chemicals for synthetic use, including 4-(dimethylamino)pyridine (DMAP), *N*,*N*′-dicyclohexylcarbodiimide (DCC), anhydrous magnesium sulfate, 3-methylpentanol, 4-methylpentanol, 1-hexanol, and docosanoic (*syn*.

behenic) acid were purchased from Sigma-Aldrich or Carl Roth (Karlsruhe, Germany). Preparative MPLC was performed with a pump module C-601 and a pump controller C-610 Work-21 pump (Büchi Labortechnic, Flawil, Switzerland) and was carried out on a pre-packed column cartridge (40 x 75 mm, silica-gel 60, particle size distribution 40-63 μm, Büchi), whereas precoated Al silica gel plates (Merck (Darmstadt, Germany), Kieselgel 60 F_{254}, 0.2 mm) were used for analytical TLC analyses. The spots on TLC were visualized by UV light (254 nm) and by spraying with 50% (*v/v*) aq. H_2SO_4 or 10% (w/v) ethanolic solution of phosphomolybdic acid, followed by 10 min of heating at 110 °C. IR measurements (ATR-attenuated total reflectance) were carried out using a Thermo Nicolet model 6700 FTIR instrument (Waltham, USA). Elemental analyses (microanalysis of carbon, hydrogen, and oxygen) were carried out with a Carlo Erba Elemental Analyzer model 1106 (Carlo Erba Strumentazione, Milan, Italy) as previously described.[20]

Plant material

Flowers of *Dianthus cruentus* Griseb. (Caryophyllaceae) originated from the high slopes of Šara mountain (in the village Sevce, meadows at the site of Miruš at 1200 m above sea level). Voucher specimens were deposited in the Herbarium of the Faculty of Sciences and Mathematics, University of Niš, Serbia, under the acquisition number HMN 13637. The identity of the plant material was determined by one of the authors (V.R.), a professor of botany.

Preparation of flower washings

Intact fresh flowers (30.46 g) of *D. cruentus*, handled one by one, in such a way as to minimize any contact with human skin, were shortly (5-7 s) separately immersed in a vessel with 500 mL of diethyl ether, while being exposed to ultrasonic waves (the glass beaker was inside an ultrasonic bath, Elmasonic S30 (Elma, Germany) operating at a frequency of 37 kHz, with an effective ultrasonic power of 30 W and a peak of 240 W), at room temperature. To remove all the insoluble material, the washings were gravity filtered through a small column packed with several grams of Celite® (Merck, Germany), and dried over anhydrous $MgSO_4$, then concentrated to 10 mL at room temperature using a stream of N_2 before GC and GC-MS analyses. The yield of the washings (%, w/w), obtained by complete evaporation of the solvent in vacuo, was 1.73%.

Gas chromatography-mass spectrometry (GC-MS) analyses

The GC-MS analyses (three repetitions) of the washings and pure synthesized esters were carried out using a Hewlett-Packard 6890N gas chromatograph equipped with a fused silica capillary column DB-5MS (5% phenylmethylsiloxane, 30 m × 0.25 mm, film thickness 0.25 μm, Agilent Technologies, Palo Alto, CA, USA) and coupled with a 5975B mass selective detector from the same company. The injector and interface were operated at 250 °C and 320 °C, respectively. Oven temperature was raised from 70 °C to 315 °C at a heating rate of 5 °C/min and the program ended with an isothermal period of 30 min. As a carrier gas helium at 1.0 mL/min was used. The samples, 1.0 μl of the diethyl ether solutions of the washings (1.0 mg per 1.0 mL), 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). MS conditions were as follows: ionization voltage of 70 eV, acquisition mass range 35-650, scan time 0.32 s.

NMR measurements

The ^{1}H- (including ^{1}H-NMR spectra with homonuclear decoupling), ^{13}C- (with and without heteronuclear decoupling) nuclear magnetic resonance (NMR) spectra, distortion less enhancement by polarization transfer (DEPT90 and DEPT135) and 2D (^{1}H–^{1}H COSY, NOESY, gHSQC and gHMBC) NMR spectra of 3-methylpentyl, 4-methylpentyl and hexyl behenates were recorded on a Bruker Avance III 400 MHz NMR spectrometer (Fällanden, Switzerland; ^{1}H at 400 MHz, ^{13}C at 101 MHz) equipped with a 5-11 mm dual ^{13}C/^{1}H probe head. All NMR spectra were measured at 25 °C in $CDCl_3$ with tetramethylsilane (TMS) as an internal standard. Chemical shifts are reported in ppm (δ) and referenced to TMS (δ_H = 0 ppm) in ^{1}H-NMR spectra and/or to solvent (deuterated chloroform: δ_H = 7.26 ppm and δ_C = 77.16 ppm) in ^{13}C- and heteronuclear 2D spectra. Scalar couplings are reported in hertz (Hz).

Component identification

Diethyl-ether washings constituents were identified by comparison of their linear retention indices (relative to C_8-C_{40} alkanes on a DB-5MS column[21]) with literature values and their mass spectra with those of authentic standards, as well as those from Wiley 7, NIST14, MassFinder 2.3, and a homemade MS library with the spectra corresponding to pure substances, and, wherever possible, by co-injection with an authentic sample (see Table 1, column Identification). Additionally, samples of the washings were subjected to derivatization reactions that included transesterification, and formation of dimethyl disulfide (DMDS) adducts, described in details below, and afterwards to additional GC-MS analyses.

Synthesis of esters

A solution of docosanoic acid (syn. behenic acid; 200 mg, 0.59 mmol), 3-methylpentyl, 4-methylpentyl or hexyl alcohol (55 mg, 0.54 mmol), DMAP (20 mg, 0.16 mmol) and DCC (111 mg, 0.54 mmol) in 20 mL of dry CH_2Cl_2 was stirred under N_2 overnight at room temperature.[22] The urea precipitate was filtered off and the filtrate was concentrated *in vacuo*. The addition of cold *n*-pentane provoked another crop of the urea to separate out. The crude ester was

purified by MPLC on silica gel using 3% (*v/v*) diethyl ether in hexane. The purity of the esters was checked by TLC, GC-MS, and NMR. Mass spectra, IR, 1D and 2D NMR spectra of 3-methylpentyl, 4-methylpentyl and hexyl behenates are given in the Supplementary data file (Figures S2 – S22). Spectral data are given below:

3-Methylpentyl behenate: yield 78%; white waxy solid. RI (DB-5MS): 2957. IR (ATR): 2958, 2915, 2848, 1737, 1465, 1417, 1377, 1321, 1301, 1285, 1269, 1239, 1205, 1188, 1168, 1110, 1061, 964, 909, 720. MS (EI), (*m/z*, (relative abundance, %)): 425(2), 424(M^+, 7), 341(13), 340(7), 323(6), 145(4), 129(6), 97(6), 85(56), 84(100), 83(9), 73(5), 71(10), 70(4), 69(31), 67(4), 57(32), 56(14), 55(25), 43(48), 42(5), 41(20). ^{1}H-NMR (400 MHz, $CDCl_3$) 4.16 – 4.04 (*m*, two diastereotopic strongly coupled H, H-(C1′), 2 H); 2.28 (*t*, *J* = 7.5, H-(C2), 2 H); 1.71 – 1.57 (overlapping signals, H-(C3) and one of the diastereotopic H-(C2′), 3 H); 1.53-1.31 (overlapped signals, center at ~1.45 H-(C3′), ~1.43 one diastereotopic from H-(C2′), ~1.36 one of the diastereotopic from H-(C4′), 1.32 – 1.23 (overlapping signals, H-(C4) – H-(C21), 36 H); 1.24 – 1.13 (*m*, one of the diastereotopic H from H-(C4′), 1 H); 0.90 (*d*, *J* = 6.4, H-(C6′), 3 H); 0.88 (*t*, *J* = 7.3, H-(C5′) and H-(C22), 6 H). ^{13}C-NMR (101 MHz, $CDCl_3$): 174.0 (C(1)); 62.9 (C(1′)); 35.2 (C(2′)); 34.5 (C(2)); 32.0 (C(20)); 31.5 (C(3′)); 29.7, 29.6, 29.5, 29.4, 29.3 (C(4′), C(5) – C(19)); 29.2 (C(4)); 25.1 (C(3)); 22.7 (C(21)); 19.0 (C(6′)); 14.1 (C(22)); 11.2 (C(5′)). Anal. calc. for $C_{28}H_{56}O_2$ (424.74): C 79.18, H 13.29, O 7.53; found: C 79.21, H 13.28, O 7.51.

4-Methylpentyl behenate: yield 81%; white waxy solid. RI (DB-5MS): 2949. IR (ATR): 2955, 2915, 2870, 2848, 1737, 1473, 1463, 1414, 1367, 1330, 1316, 1301, 1285, 1270, 1254, 1238, 1205, 1187, 1167, 1111, 1085, 1068, 993, 729, 719. MS (EI), (*m/z*, (relative abundance, %)): 425(4), 424(M^+, 14), 381(6), 342(21), 341(90), 340(15), 320(4), 297(5), 185(4), 157(4), 145(8), 129(8), 116(9), 111(6), 98(6), 97(12), 95(5), 87(4), 86(5), 85(83), 84(98), 83(18), 81(5), 73(10), 71(20), 70(6), 69(33), 68(4), 67(7), 60(5), 57(52), 56(59), 55(34), 44(4), 43(100), 42(11), 41(37). ^{1}H-NMR (400 MHz, $CDCl_3$): 4.05 (*t*, *J* = 6.8, H-(C1′), 2 H); 2.29 (*t*, *J* = 7.5, H-(C2), 2 H); 1.66 – 1.58 (overlapping signals, H-(C3) and H-(C2′)); 1.56 (*n*, *J* = 6.6, H-(C4′), 1 H); 1.34 – 1.24 (overlapping signals, H-(C4) – H-(C21), 36 H); 1.25 – 1.19 (*m*, H-(C3′), 2 H); 0.89 (*d*, *J* = 6.6, H-(C5′) and H-(C6′), 6 H); 0.88 (*t*, *J* = 7.1, H-(C22), 3 H). ^{13}C-NMR (101 MHz, $CDCl_3$): 174.0 (C(1)); 64.7 (C(1′)); 35.1 (C(3′)); 34.4 (C(2)); 32.0 (C(20)); 29.7, 29.6, 29.5, 29.4, 29.3 (C(5) – C(19)); 29.2 (C(4)); 27.8 (C(4′)); 26.6 (C(2′)); 25.1 (C(3)); 22.7 (C(21)); 22.5 (C(6′) and C(5′)); 14.1 (C(22)). Anal. calc. for $C_{28}H_{56}O_2$ (424.74): C 79.18, H 13.29, O 7.53; found: C 79.17, H 13.29, O 7.54.

Hexyl behenate: yield 83%; white waxy solid. RI (DB-5MS): 2984. IR (ATR): 2955, 2915, 2848, 1737, 1473, 1463, 1399, 1378, 1331, 1317, 1302, 1285, 1270, 1254, 1238, 1221, 1204, 1188, 1170, 1111, 1070, 1011, 908, 728, 720. MS (EI), (*m/z*, (relative abundance, %)): 425(8), 424(M^+, 25), 342(21), 341(90), 340(20), 324(4), 323(17), 297(10), 241(10), 213(5), 185(7), 171(4), 157(8), 145(10), 129(21), 125(5), 115(6), 111(9), 109(4), 101(5), 99(5), 98(8), 97(18), 95(7), 87(7), 85(33), 84(65), 83(22), 82(4), 81(7), 73(22), 71(26), 70(7), 69(37), 68(4), 67(8), 61(28), 60(11), 58(4), 57(58), 56(42), 55(47), 54(4), 44(4), 43(100), 42(16), 41(40), 39(5). ^{1}H-NMR (400 MHz, $CDCl_3$): 4.06 (*t*, *J* = 6.7, H-(C1′), 2 H); 2.29 (*t*, *J* = 7.5, H-(C2), 2 H); 1.66 – 1.57 (overlapping peaks, H-(C3) and H-(C2′); 1.40 – 1.18 (*m*, H-(C4) – H-(C21) and H-(C3′) – H-(C5′), 42 H); 0.89 (*t*, *J* = 6.7, H-(C6′), 3 H); 0.88 (*t*, *J* = 6.8, H-(C22), 3 H). ^{13}C-NMR (101 MHz, $CDCl_3$): 174.0 (C(1)); 64.4 (C(1′)); 34.4 (C(2)); 32.0 (C(20)); 31.5 (C(4′)); 29.7, 29.6, 29.5, 29.4, 29.3 (C(5) – C(19)); 29.2 (C(4)); 28.7 (C(2′)); 25.6 (C(3′)); 25.1 (C(3)); 22.7 (C(21) and C(5′)); 14.1 (C(22)); 14.0 (C(6′)). Anal. calc. for $C_{28}H_{56}O_2$ (424.74): C 79.18, H 13.29, O, 7.53; found: C 79.20, H 13.29, O 7.51.

Transesterification of diethyl ether washings

The washings (0.1 g) were added in small portions with stirring in refluxing MeONa (a solution of sodium methoxide was prepared by dissolving 0.1 g of metallic sodium in anhydrous methanol (20 mL)). The reaction mixture was brought to reflux ($CaCl_2$ tube), quenched with excess ice-water and extracted with diethyl ether (3 × 50 mL). The organic layers were combined, dried over anhydrous $MgSO_4$ and the solvent was removed in vacuo. Successfulness of the transesterification was confirmed by GC-MS analysis.

Dimethyl disulfide (DMDS) derivatization

The sample of the washings was dissolved in DMDS (0.25 mL per mg of the sample) and a solution (0.05 mL per mg of the sample) of I_2 in diethyl ether (60 mg/mL) was added. The mixture was stirred at room temperature overnight. Then diethyl ether was added, and the obtained mixture was washed with 10% aq. $Na_2S_2O_3$, dried over anhydrous $MgSO_4$ and evaporated to dryness. The residue was taken up in fresh diethyl ether and directly analyzed by GC-MS.

Supplementary Material

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/MS-number.

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Author Contribution Statement

Plant material identification: V.R.; Plant material collection and preparation of the washings: M. R., V. D., N. R. R. and N. S. R.; GC-MS analyses: M. M., N. R. R. and N. S. R.; Synthesis of esters: M. M., B. D. and M. R.; Preparation of the first draft: M. M., B. D. and V. D. Recording of NMR spectra N. S. R. Analyses of the obtained data: M. M., M. R. and N. S. R.; Contribution of reagents and analytical tools: N. S. R.; Preparation of the final version of the manuscript: N. S. R., V. D., V. R. and M. R.

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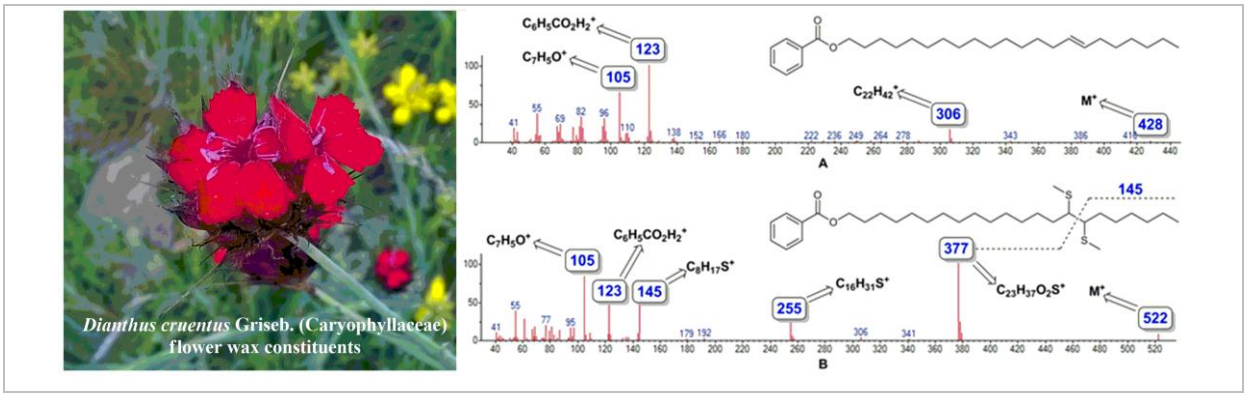

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GC-MS and synthetic workled to the identification of 151 constituents (55 new natural products) from diethyl-ether flower washings of *Dianthus cruentus* Griseb. (Caryophyllaceae).