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CHANGES IN MONOTERPENE COMPOSITION OF *MENTHA AQUATICA* PRODUCED BY GENE SUBSTITUTION FROM A HIGH LIMONENE STRAIN OF *M. CITRATA*

MERRITT J. MURRAY
A M Todd Company, Kalamazoo, MI 49005, U S A.

and

FRIEDRICH W HEFENDEHL
Institut fur Pharmazeutische Biologie der Albert-Ludwigs-Universitat,
78 Freiburg, Schanzlestrasse 9, Germany

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Abstract—The dominant gene *Lm* that causes 60–90% limonene/cineole was substituted into *M aquatica* by four convergent backcrosses The natural strain of *M aquatica* has 7 7% cineole, 4 9% limonene, traces of terpinolene and pulegone, 0 1% menthone, 0 2% menthol, and 66 4% menthofuran The two modified hybrid strains with dominant gene *Lm* have 53 8 and 78 7% limonene/cineole and a total of only 1 0–3 8% 3-oxygenated compounds in contrast to a total of 66 7% found in the natural strain The postulate is made that the *Lm* gene largely prevents either the conversion of α-terpineol → terpinolene or of limonene → isopiperitenone and that in these strains the recessive *cc* genotype largely but not completely prevents the conversion of limonene → carvone resulting in limonene accumulation *Mentha* species almost invariably have either 2-oxygenated or 3-oxygenated compounds, not both Close coupling phase linkage of the genes *Lm* and *C* explains why the self-pollinated progeny of *M spicata* or *M crispa* C–*Lm*/c-*lm* have a ratio of 3 carvone/dihydrocarvone 1 pulegone/menthone rather than a ratio of 9 carvone 3 limonene 3 carvone and menthone 1 menthone which would be expected if the genes *Lm* and *C* were independently inherited

INTRODUCTION

THE FACT that all *Mentha* species have 1–25% *l*-limonene has led to general agreement that limonene must have an important role in oil biogenesis, but there is no agreement on the exact role of limonene in the synthesis of 2- and 3-oxygenated compounds despite a number of chemical studies The extreme view is that limonene is not a precursor of any oxygenated compound Fujita[1] has postulated that limonene and cineole are both derived from α-terpineol and that limonene is the precursor of both the 2-oxygenated compound carvone and the 3-oxygenated compounds isopiperitenone and piperitenone, whereas Loomis[2] has postulated that α-terpineol produces either limonene/carvone or terpinolene/piperitenone The 1954 report[3] that a single dominant gene *C* caused carvone in *Mentha crispa* while the homozygous recessive *cc* genotype caused menthone may have seemed foolish to many chemists since methone and carvone obviously could not be synthesized from a common

[1] FUJITA, Y (1960) *Koryo* **59**, 41
[2] LOOMIS, W D (1967) in *Terpenoids in Plants* (PRIDHAM, J B , ed), p 59, Academic Press, New York
[3] MURRAY, M J and REITSEMA, R H (1954) *J Am Pharm Assoc Sci Ed* **43**, 612

precursor Nonetheless, the statement is true that *C* allows carvone formation whereas the recessive *c* does not Other true breeding (homozygous) dominant or recessive genes in *M crispa* determine that menthone is the major end-product in a *cc* genotype There is universal agreement among geneticists that genes exert their effect by allowing or not allowing the production of a specific enzyme To determine which conversion the gene *C* controls, one must study its breeding behavior in crosses to known genotypes, or substitute dominant *C* into different known genotypes by convergent backcrossing [4]

The dominant gene *Lm* found in specific and exceptional *M citrata* × *M aquatica* hybrids definitely prevents the development of menthone in hybrids with several tester strains having a known ketone composition [5]

The gene *Lm* also seemed to prevent the development of carvone, but this conclusion was not based on the most critical evidence and needs verification There was difficulty in accurately separating individuals having limonene/carvone from individuals having limonene from their herbage odor The proper tester strain should have been a homozygous *CC* strain with oil from F_1 individuals analyzed for oil composition

The present gene substitution study was one of several experimental approaches designed to determine which biogenetic conversion the dominant gene *Lm* controlled and its interrelationship with other genes

RESULTS AND DISCUSSION

Genetic Data on Gene Substitution

The dominant gene *Lm* found in two *M citrata* × *M aquatica* F_1 hybrids has been substituted by 4–5 backcrosses into both the German and Dutch strains of *M aquatica* known from previous work[4] to be true breeding for a high menthofuran oil composition and morphological appearance In addition to the extensive first and second backcross progenies previously summarized,[5] later second backcross progenies had 296 limonene odored to 288 menthofuran odored like the *M aquatica* parental strain The 3rd–5th backcross progenies also had 1 1 ratios and the total progeny consisted of 310 limonene odored to 308 menthofuran odored No exceptional individuals having distinctive odors were found in these progenies All individuals that might possibly be different were field grown and studied One may conclude that either the gene *Lm* is actually a single gene or that no crossing over occurred between two closely linked genes in 1202 carefully studied individuals As will be shown, these genetic results are interpretable without assuming that 1 gene affects 2 enzymes or that limonene produces an unknown intermediate compound that can be converted to either 2- or 3-oxygenated compounds

Chemical Composition of M aquatica *Hybrids and Control*

The oil composition of control *M aquatica* and two hybrids are given in Table 1 A number of hydrocarbon constituents were not identified since they are of little interest in this study and should be qualitatively the same as those previously identified in *M aquatica* [4 6]

Table 2 shows that oil from normal *M aquatica* strains has 66 7% 3-oxygenated compounds and no 2-oxygenated compounds In hybrid 2 of this study, the substitution of one dominant *Lm* gene into the *M aquatica* genotype almost entirely prevents the development of

[4] HEFENDEHL, F W and MURRAY, M J (1972) *Phytochemistry* **11**, 189
[5] LINCOLN, D E , MARBLE, P M , CRAMER, F J and MURRAY, M J (1971) *Theoret Appl Genet* **41**, 365
[6] MURRAY, M J and HEFENDEHL, F W (1972) *Phytochemistry* **11**, 2469

TABLE 1 OIL COMPOSITION OF TWO LIMONENE-ODORED HYBRIDS HAVING THE GENOTYPE *Lm lm* SUBSTITUTED BY 4 BACKCROSSES FOR THE *lm lm* GENOTYPE OF NORMAL MENTHOFURAN-ODORED *Mentha aquatica*

Oil constituent*	Hybrid 1 (%)	Hybrid 2 (%)	*Mentha aquatica* (%)
α-Pinene	1 5	1 5	1 8
Camphene	(Trace)	(Trace)	(Trace)
β-Pinene	2 6	2 5	1 9
Sabinene	1 4	1 4	0 8
Myrcene	1 2	0 7	1 3
Limonene	39 7	65 2	4 9
β-Phellandrene	0 3	0 2	0 1
Cineole	14 1	13 5	7 7
cis- *β*-Ocimene	3 3	Trace	1 8
γ-Terpinene	0 2	0 7	
trans- *β*-Ocimene	1 3	Trace	0 5
p-Cymene	0 9	0 1	0 1
Unknown Ester		0 1	
Terpinolene	(Trace)	(Trace)	Trace
Octyl acetate		Trace	Trace
Octen-3-yl acetate		Trace	
Octanol-3		Trace	Trace
1-Octen-3-ol		0 1	0 3
Menthone	0 5	0 2	0 1
Menthofuran	Trace	0 1	66 4
iso-Menthone	0 3	Trace	Trace
Unknown HC	0 1		
β-Bourbonene	1 7	0 1	
Linalool	0 6	0 1	0 3
Unknown HC		0 1	
Linalylacetate	Trace	Trace	
Menthyl acetate	0 7		Trace
Neoiso-Menthylacetate	0 8		
Unknown HC	0 5		
Unknown oxygenated	0 3	0 2	
Unknown HC	0 6		
Caryophyllene	6 7	6 6	7 0
neoiso-Menthol	Trace		
Menthol	1 4	0 6	0 2
Unknown HC		0 3	
Pulegone	Trace	0 1	Trace
Unknown HC	0·2		
Unknown HC	0 1		
β-Farnesene	0 8	0 7	
Dihydrocarvyl acetate	5 0		
Unknown HC	0 2	0 8	
α-Terpineol	(Trace)	0 7	
Germacrene D	2 0	0 9	
Piperitone	(0 1)	(Trace)	
Carvone	0 8	0 4	
Dihydrocarveol	(Trace)		
Dihydrolimonene-10-yl acetate		0 5	
cis-Carvylacetate	5 8		
trans-Carveol	0 2		
cis-Carveol	Trace		
Limonene-10-ylacetate	0 2	0 2	
Dihydrolimonene-10-ol	2 5		
Unknown oxygenated		1 0	
Humulene			0 4
ε-Muurolene			3 2
Trace peaks, all under 0 1%	1 4	0 4	1 2
Total	100 0	100 0	100 0

* Quantitative results in brackets indicate, that this compound was identified only by addition analysis All other compounds were identified by spectroscopic methods (see Experimental)

the 3-oxygenated compounds, namely the ketones piperitenone, piperitone, pulegone, menthone, their alcohols and esters, or menthofuran, the oxidation product of pulegone Slightly larger amounts are found in hybrid 1 Less variability between individuals might be expected in fifth or sixth backcross progenies than in the fourth backcross ones studied

TABLE 2 SUMMARY GIVING TOTALS OF 2- AND 3- OXYGENATED COMPOUNDS FOUND IN EACH HYBRID AND CONTROL

Total	Hybrid 1	Hybrid 2	*M aquatica*
Limonene/cineole	53 8	78 7	12 6
2-Oxygenated compounds	11 8	0 4	0 0
3-Oxygenated compounds	3 8	1 0	66 7

Genotype *M aquatica* $c_1c_1\ c_2c_2\ lm_1lm_1\ lm_2lm_2$ from Refs 4 and 5
Genotype hybrids $c_1c_1\ c_2c_2\ Lm_1lm_1\ lm_2lm_2$ or $c_1c_1\ c_2c_2\ lm_1lm_1\ Lm_2lm_2$

The oil of hybrid 2 could not have 65 2% limonene if limonene were readily converted to carvone or to 3-oxygenated compounds or if this strain had genes that caused the rapid conversion of limonene → alcohol → ester The *R* gene has been shown to cause the conversion of menthone, carvone, and dihydrocarvone to their alcohols,[6] but this gene apparently has no effect on the hydrocarbon terpene, limonene The alcohols and esters of limonene and dihydrolimonene were more readily identified in the hybrid strains than in other assayed material even though reference to Table 1 will show that the amounts did not exceed 2 5%

One might expect that the amount of α-terpineol would be increased in strains having large amounts of limonene if α-terpineol is the precursor of limonene and cineole as postulated [1,2] Hybrid 2 had 0 7% α-terpineol whereas the amounts in *M aquatica* are so small that none has been identified [7,8] These data do not provide evidence on the role of α-terpineol in oil biogenesis

CONCLUSIONS

The dominant gene *Lm* largely prevents the conversion of limonene → isopiperitenone as postulated by Fujita[1] or of α-terpineol → terpinolene as postulated by Loomis [2] This conclusion is based upon the logical assumption that if the *Lm* gene prevented the conversion of terpinolene → piperitenone, or isopiperitenone → piperitenone that terpinolene or isopiperitenone would be accumulated The same statement applies to the conversion of piperitenone → pulegone No isopiperitenone or piperitenone were found and only trace amounts of terpinolene All usually identified 3-oxygenated oil constituents of *M aquatica* were greatly reduced Conversely, there was definitely no increase in any 3-oxygenated constituent

The recessive *cc* genotype of *M aquatica* and of the hybrids largely prevents the conversion of limonene → carvone as postulated on the basis of chemical structure,[1,2] and chemical synthesis

[7] HEFENDEHL, F W (1967) *Arch Pharmaz* **300**, 438
[8] HANDA, K L , SMITH, D M , NIGAM, I C and LEVI, L (1964) *J Pharm Sci* **53**, 1407

This interpretation assumes that a genotype having the dominant *Lm* and recessive *cc* genes will result in the accumulation of limonene, whereas a genotype having both recessive genes *lm lm cc* would allow the 3-oxygenated compounds and prevent carvone formation Conversely, an individual having both dominant genes in the genotype would produce carvone and could not produce 3-oxygenated compounds An individual having the recessive *lm* and dominant *C* genes should be able to produce both 2- and 3-oxygenated compounds New recombinations could not be expected from backcrossing the limonene strain having the genotype *cc Lm lm lm lm* to *M aquatica* having the genotype *cc lm lm lm lm* since both are true breeding for the recessive *cc* gene

Mentha species and hybrids almost invariably have either 2-oxygenated or 3-oxygenated compounds, not both The genetic postulate is advanced that there is close coupling phase linkage between the two genes *Lm* and *C* As a result, the self-pollinated progeny of the *Lm-C/lm-c* genotype of *M spicata* and *M crispa* produces 3 carvone/dihydrocarvone 1 pulegone/menthone individuals If the two genes were independently inherited, a ratio of 9 carvone 3 high limonene 3 having carvone and menthone 1 menthone would be expected and is not found

The genes *C* and *Lm* occur on a chromosome pair that is duplicated in *M spicata* and in *M aquatica* Many strains of *M spicata* have the genotype *Cc cc* and give a self-pollinated ratio of 3 carvone/dihydrocarvone 1 pulegone/menthone A few strains have the genotype *Cc Cc* and give a self-pollinated duplicate gene ratio of 15 carvone 1 menthone Certain high limonene strains have the genotype *Lm lm Lm lm* whereas others have the genotype *Lm lm lm lm* The simplest comparison is that of studying the oil composition of *M aquatica* having the known genotype $c_1c_1\ c_2c_2\ lm_1lm_1\ lm_2lm_2$ and the hybrids of Table 2 which have the genotype $c_1c_1\ c_2c_2\ Lm_1lm_1\ lm_2lm_2$ or $c_1c_1\ c_2c_2\ lm_1lm_1\ Lm_2lm_2$

EXPERIMENTAL

Plant material and oil isolation The hybridizations and genetic ratios based on herbage odor were done in the A M Todd Co laboratories The plots of Hybrids 1 and 2 were grown at Mentha, Michigan, U S A at latitude 42° 22′ with oil distillation in a 19 9-l experimental still [9]

Fractionation of the oil (a) *Prefractionation* 2 ml of oil has been separated by 'Dry Column-Chromatography'[10,11] on silicic acid, Woelm 60–150 mesh, activity III into four fractions Column dimensions 56 × 2 cm, nylon tube *Fraction* 1 development with 100 ml hexane, 23 cm of the lower part of the column is cut off → hydrocarbons *Fraction* 2 Rest of hexane, being in the remaining part of the column (33 cm) → some hydrocarbons, menthofuran *Fractions* 3 and 4 development with 60 ml propylchloride dividing of the column into 2 sections, upper section (17 cm) → alcohols, unsaturated ketones, lower section (16 cm) → ketones, esters Extraction of compounds from silicic acid by Et_2O (cineole is present in fractions 3 and 4) (b) *Fractionation* into single compounds by preparative GLC[4] on columns 1, 2, 3, 4 (see 'Gas chromatography')

Gas chromatography GC-Varian Aerograph 1860-4 with TC (preparative analyses) and FID (analytic analyses)

Columns SS-Steel, deactivated [4] *Preparative* 3 m × 6 mm (15% liquid phase), for refractionations 3 m × 3 mm (10% liquid phase) Stationary phases were all on Chromosorb W, DMCS-treated 60–80 or 80–100 mesh (1) PEG 20 M, (2) Apiezon L, (3) QF-1, (4) β,β′-Oxidipropionitril *Analytic* Thin-film capillary columns 100 m × 0 25 mm or 50 m × 0 25 mm, (5) PEG 20 M or 1540, (6) QF-1, (7) Apiezon L, (8) OV-17 Addition analyses on analytical columns 5–8 with isolated peaks (preparative GC) to minimize incidental coincidence

[9] Reitsema, R H and Baarman, V J (1953) *J Am Pharm Assoc Sci Educ* **42**, 734
[10] Loev, B and Snader, K M (1965) *Chem Ind (London)* 15
[11] Loev, B and Goodman, M M (1967) *Chem Ind (London)* 2026

Quantitative analysis Digital-integrator Mod 477, Varian MAT Internal normalization,[12] values corrected, corresponding to their C-number and structure [13]

Identification of isolated compounds IR as film (NaCl or AgCl plates or reflection plates Wilks MIR-15 GC-IR analyser) *UV* Zeiss-Spectrophotometer PMQ II in EtOH or iso-octane Identifications of esters and difference analyses (Reaction chromatography) see Ref 4

Acknowledgement—F W H thanks the Deutsche Forschungsgemeinschaft for financial support

[12] BAYER, E (1959) *Gaschromatgraphie*, Springer, Berlin
[13] ACKMAN, R G (1964) *J Gaschromatog* **2**, 173