

1 **An *In-Silico* Investigation of Menthol Metabolism**

2 Taweetham Limpanuparb*, Wanutcha Lorpaiboon and Kritdin Chinsukserm

3 Science Division, Mahidol University International College, Mahidol University, Nakhon Pathom 73170,

4 Thailand

5 * taweetham.lim@mahidol.edu

6 **ABSTRACT**

7 Prevalence of mentholated products for consumption has brought great importance to studies on menthol's
8 metabolic pathways to ensure safety, design more potent derivatives, and identify therapeutic benefits. Proposed
9 pathways of (-)-menthol metabolism based on metabolites found experimentally in previous works by Yamaguchi,
10 Caldwell & Farmer, Madyastha & Srivatsan and Hiki et al. were not in agreement. This *in silico* approach is based
11 on the three *in vivo* studies and aims to resolve the discrepancies. Reactions in the pathways are conjugation with
12 glucuronic acid/sulfate, oxidation to alcohol, aldehyde & carboxylic acid, and formation of a four-membered/five-
13 membered ring. Gas-phase structures, standard Gibbs energies and SMD solvation energies at B3LYP/6-
14 311++G(d,p) level were obtained for 102 compounds in the pathways. This study provides a more complete
15 picture of menthol metabolism by combining information from three experimental studies and filling missing links in
16 previously published pathways.

17 **Introduction**

18 (-)-Menthol or 1*S*,3*R*,4*S*-menthol is a naturally occurring compound found in plants of the *Mentha* genus
19 commonly known as mint. It is the most abundant in nature among the 8 possible stereoisomers, and make up at
20 least 50% of peppermint (*Mentha piperita*) oil and 70-80% of corn mint (*Mentha arvensis*) oil [1]. (-)-Menthol,
21 commonly referred to as menthol, has characteristic minty smell and flavor and exerts a cooling sensation when
22 applied to the skin and mucosal membranes [2]. Other isomers differ slightly in odor and physical characteristics
23 and do not possess the cooling action [3, 4].

24 Menthol finds a wide range of applications from personal care products, medications, and confectionery to
25 pesticides and cigarettes. The popularity of the compound as a flavoring agent ranks third most important after
26 vanilla and citrus [5], and the annual production of menthol in India alone is in excess of 200 thousand metric tons
27 [6]. Mentholated products can be readily purchased as prescribed or over-the-counter medications as alleviators
28 of common cold and respiratory conditions [7], inhibitors of growth of foodborne pathogens [8], and analgesics
29 [9].

30 Considering its wide range of applications, mechanisms of action of menthol were relatively unknown until
31 recently. The cooling sensation is a result of the activation of transient receptor potential melastatin-8 (TRPM8),

32 an ion channel selective to temperature, voltage, and menthol [10]. Experimental evidence also show that (-)-
33 menthol can selectively activate κ -opioid receptors in mice and, as a result, leads to its analgesic properties [9].
34 In addition, chemical derivatives of menthol with enhanced activity have been successfully synthesized [11].
35 However, health effects of mentholated cigarettes is of great concern, not only because the improved taste may
36 facilitate initiation or inhibit quitting but also because metabolism of menthol via this route of administration has not
37 been well studied [12, 13].
38 A few studies have been conducted on toxicological effects of menthol which supports the generally accepted
39 belief that it is safe and nontoxic. No signs of toxicity were observed in rats exposed to continuous doses of up to
40 800 mg/kg/day for 28 days [5], and chronic exposure to high concentrations of menthol vapor was not reported to
41 have toxic effects in rats [14]. *In vitro* studies on various animal tissues report deterioration of biological
42 membranes at concentrations 0.32-0.76 mM [15]. The recommended daily intake for humans of 0-0.2 mg/kg
43 proposed by the WHO [16] is not supported by any toxicological data but was set to err on the side of safety
44 knowing that higher doses taken may not have produced adverse side effects.
45 To the best of our knowledge, three *in vivo* studies by Yamaguchi, Caldwell & Farmer [17], Madyastha & Srivatsan
46 [18] and Hiki et al. [19] have identified metabolites of menthol in humans and animals. Metabolites were identified
47 by GC/MS from the urine and bile of rats treated with oral doses of 500 [17] and 800 [18] mg menthol/kg body
48 weight. Over the course of 48 hours, a majority of the doses were excreted in the urine and feces. A more recent
49 randomized, double-blind, placebo-controlled study in human by Hiki et al. [19] was conducted by directly spraying
50 0.8% (-)-menthol solution at escalating doses of 10-40 mL onto the gastric mucosa. Blood and urine of the
51 participants were sampled over a 24-hour period and analyzed with GC/MS for menthol metabolites. In total, 72
52 metabolites were identified or proposed in this human study alone, compared to 9 and 18 metabolites in the
53 previous two experiments. (See S3 File for the full list of metabolites in the first worksheet in the spreadsheet file.)
54 *In vitro* investigation of metabolism in human liver microsomes revealed that the same key reactions in the
55 metabolic pathway in rats occur in the microsomes [20, 21].

56

57 **FIGURE 1** Metabolic pathway of menthol in rats and in human, an adaptation from Yamaguchi, Caldwell, & Farmer [17], Madyastha & Sirvastan
58 [18] and Hiki et al. [19]. Red, Green, and Blue texts indicate that menthol metabolites were found in both rats and human, only in rats, and only
59 in human respectively. Gray and Black texts indicate menthol metabolites proposed by previous experiments and by this paper respectively.
60 Arrows to the right and arrows upward indicate oxidation reactions $+\frac{1}{2} O_2$ and $-H_2$ respectively. Downward arrows indicate conjugation with
61 sulfate. Dashed arrows indicate reactions of four-membered ring metabolites. Diagonal arrows toward top left indicate dehydration reaction.
62 Main pathways are shown on the left and pathways containing glucuronide metabolites with similar possible connections are shown on the right.
63 Lists of compounds and reactions are provided in Table 1 and Table 2, respectively.

64

65 This *in silico* investigation is based on the metabolites identified experimentally by the three *in vivo* studies [17-19].
66 We aim to resolve discrepancies and missing links found in these three studies by proposing more complete
67 pathways in Figure 1 where all 73 experimentally identified metabolites, 5 previously proposed intermediates and

68 24 newly proposed intermediates are included. Possible reactions involved in the pathways are conjugation with
69 glucuronic acid/sulfate, oxidation to alcohol, aldehyde & carboxylic acid, and formation of a four/five-membered
70 ring at position 3, 7, 8, 9 and 10 of the parent compound. In this paper, we calculated Gibbs energies of reactions
71 and associated them with the type, the position and the step of reaction in the pathways.

72 Materials and methods

73 Gas-phase structures were calculated based on the B3LYP/6-311++G(d,p) level and were confirmed to be at
74 minimum energy on the electronic potential energy surfaces by frequency calculations. The solvation energies in
75 water of the gas-phase structures were calculated with the SMD model [22]. The calculation of Gibbs energies in
76 solution phase is the same as in our previous work [23, 24] where there is a special treatment for water [25-28].
77 All quantum chemical calculations were performed using the Q-Chem 5.1 program package [29]. (Shell script,
78 spreadsheet templates, and Mathematica [30] notebook used were modified from our previous work [23, 24]. All
79 output files and other associated codes to obtain the standard Gibbs energies of the reaction are provided in S1
80 File and S2 File respectively.) The abbreviated names for each of the metabolites in this study are as in Table 1.
81 For simplicity, we based the naming system of menthol metabolites on their five substitutable positions, namely
82 position 3, 7, 8, 9 and 10. A menthol metabolite is referred to as a five-character sequence named according to its
83 substituted functional groups at these positions with the abbreviation explained below in Table 1.

84 All DFT calculations were completed with no imaginary frequencies, showing that each of the structures obtained
85 from gas-phase calculations were minima on the potential energy surfaces. The lowest energy structure of (-)-
86 menthol is a chair conformer of hexane where all three substituent groups are in equatorial positions as shown in
87 Table 1. This is consistent with previous computational result at B3LYP/6-31G(d,p) level [31]. Benchmark
88 calculations were also performed at MP2/6-311++G(d,p) level for metabolites along the most likely pathways in
89 Figure 2. Reaction energies obtained from MP2 and B3LYP are in good agreement. (Coefficient of determination
90 $r^2=0.9999$ and mean absolute error, MAE=2.25 kcal/mol. See the third worksheet of the Excel file in S3 File for
91 details.) These results are in line with earlier studies and confirm that B3LYP/6-311++G** yields acceptable
92 results at a reasonable computational cost. [23, 32]

93

94 Results

95 The present study has combined the different published metabolic pathways of menthol and offers the relative
96 stabilities of each metabolite based on thermodynamic calculations for each step involved as reported in Figures 1
97 to 4. Reaction energies were computed with the relevant additional reagents (oxygen, sulfate group, hydronium
98 ion and glucuronic acid) and product (water and hydrogen) added to the scheme. They may not be the actual
99 compound in the reactions but they serve as simple reference points for the thermodynamic calculations for

100 reactions of interest. The full list of compounds and reaction energies is in the first worksheet of the Excel file in

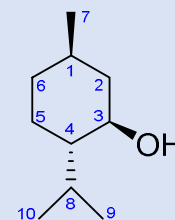
101 S3 File.

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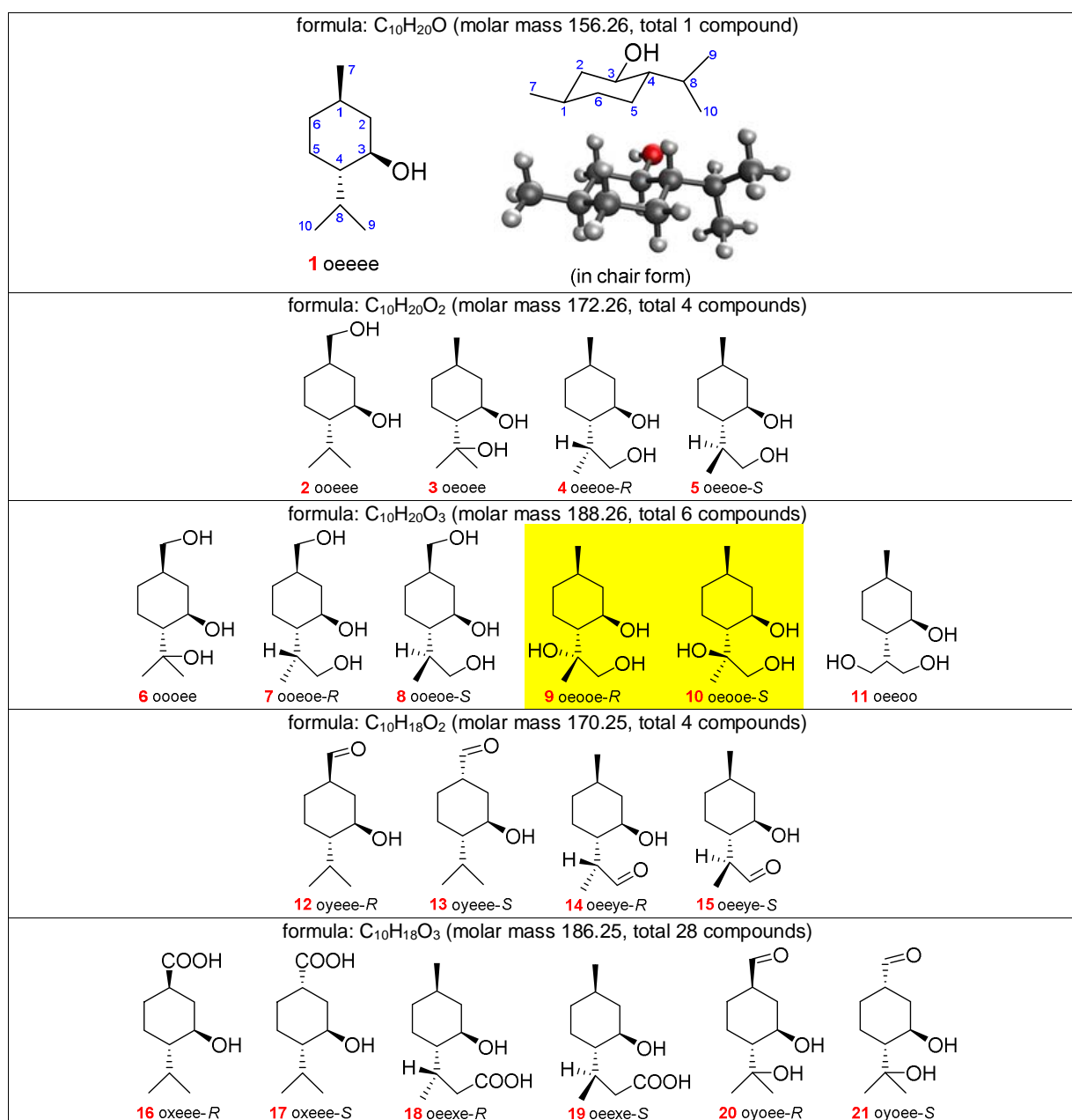
104 **TABLE 1** Abbreviations for the nomenclature of menthol metabolites referred to by the present study and a
 105 list of 102 compounds in this study grouped by molecular formula.
 106

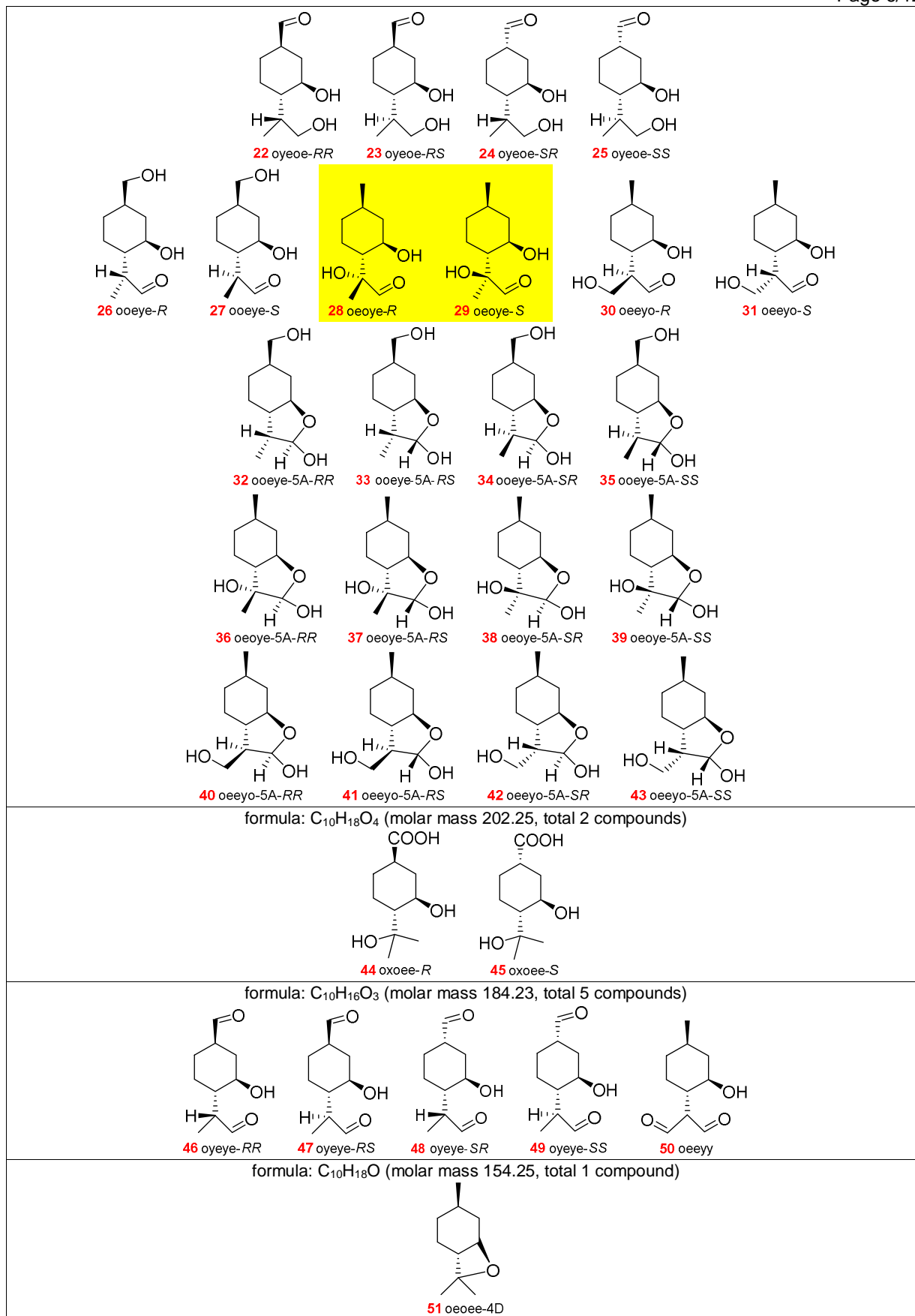
Group \ Position	3	7	8	9	10
Original form	o	e	e	e	e
Alkane	-	e	e	e	e
Alcohol	o	o	o	<u>o</u>	o
Aldehyde	y	<u>y</u>	-	<u>y</u>	y
Carboxylic acid	-	<u>x</u>	-	<u>x</u>	-
Dehydration	-4D for four-membered ring formation at positions 3 and 8				
Aldol reaction	-5A for four-membered ring formation at positions 3 and 9				
Glucuronic acid	O	<u>O</u> , <u>X</u>	-	<u>O</u> , <u>X</u> , <u>Y</u>	-
Sulfate group	s	-	-	-	-

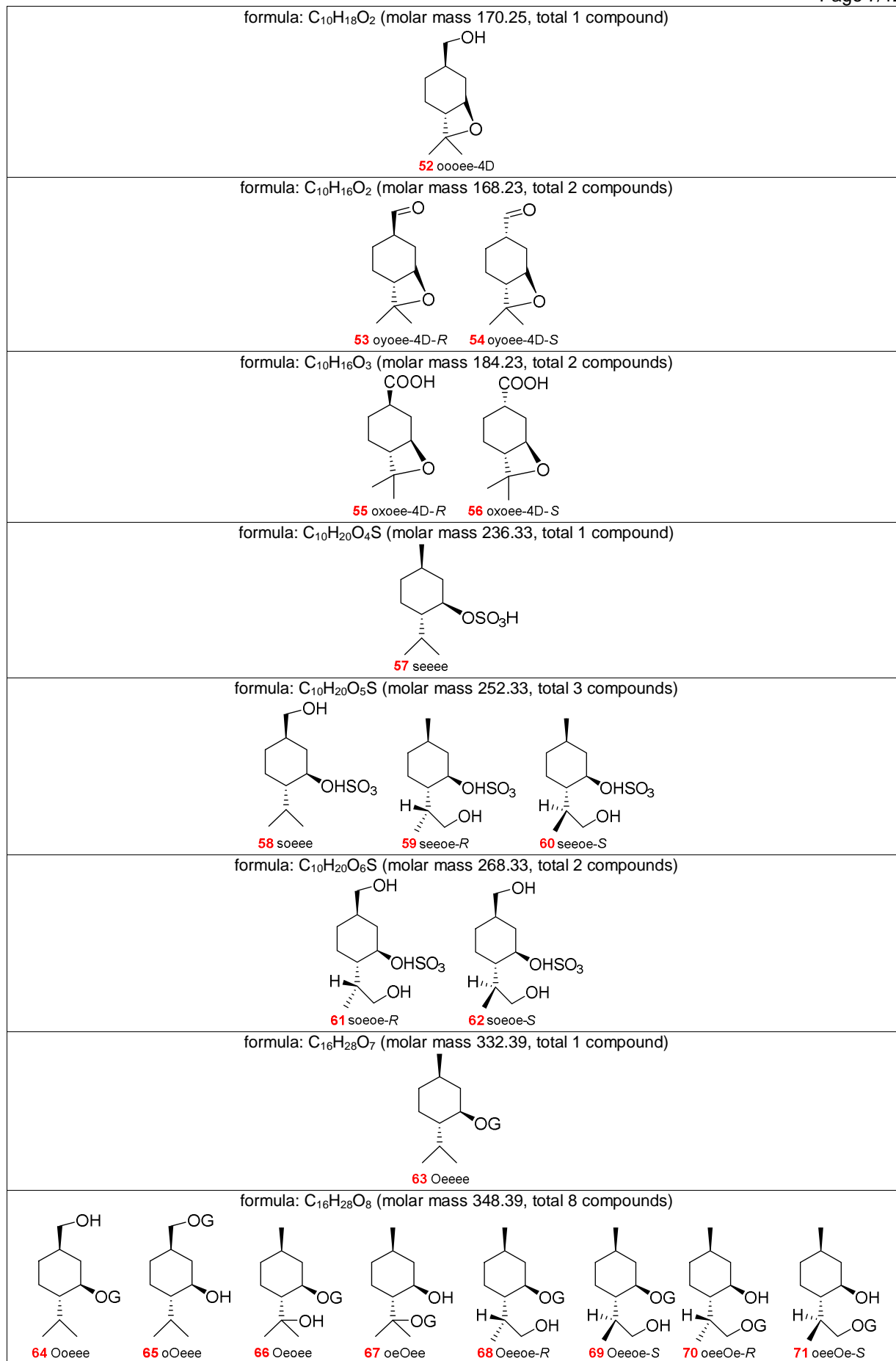


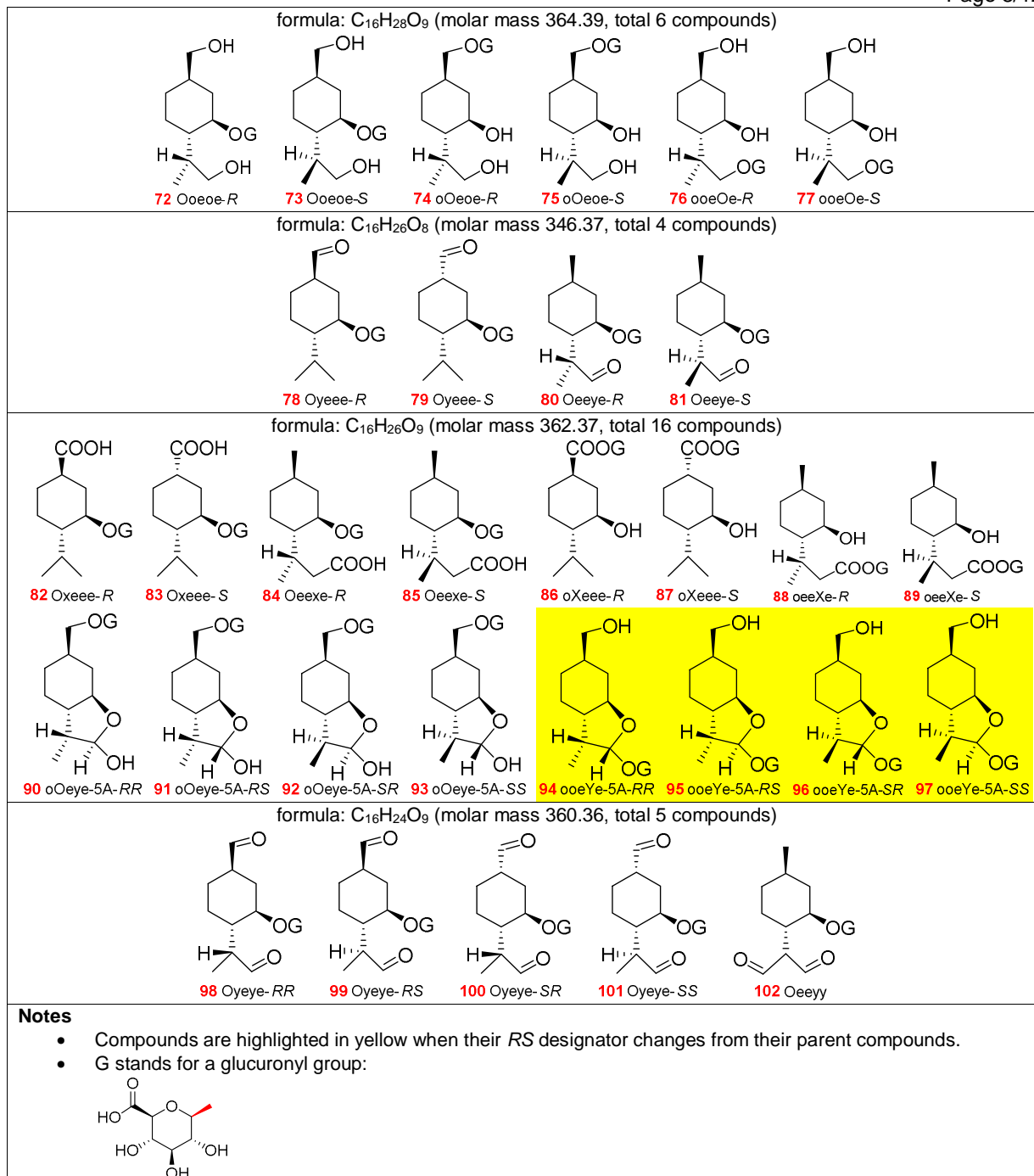
- An underlined indicates that there are *R* and *S* stereoisomers due to the substitution.
- Substitution at position 9 leads to a new chiral center if it is not the same as 10.
- Substitution at position 10 is forced to have lower or the same oxidation state for the carbon atom when compared to position 9.
- Dashes are where substitution with the functional group at that respective position cannot occur

107
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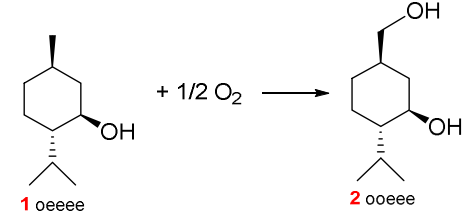
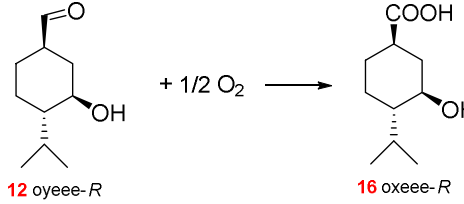
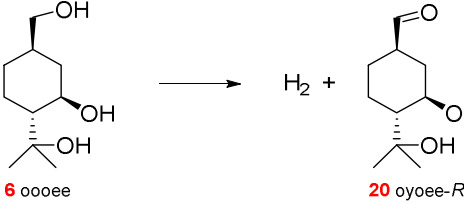
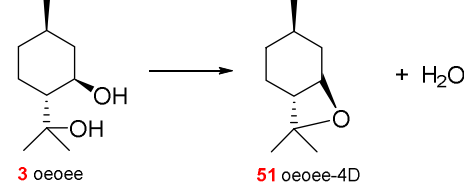
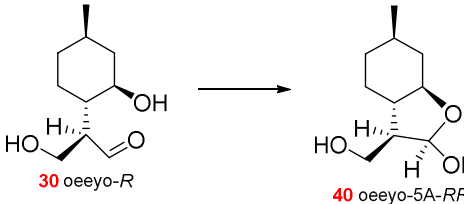
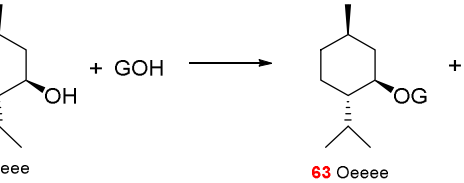
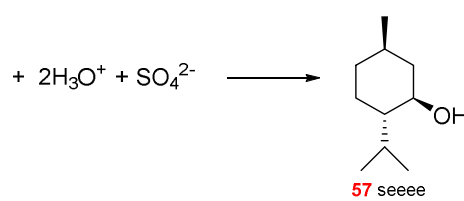








110 **TABLE 2** Representative of oxidation reactions to alcohol, aldehyde and carboxylic acid, ring formation
 111 (dehydration reaction and aldol reaction) and conjugation with glucuronic acid/sulfate group.

Abbreviation/explanation	Example
o1 for oxidation from alkane to alcohol	 <p style="text-align: center;">1 oeeee 2 oeeee</p>
o2 for oxidation from aldehyde to carboxylic acid	 <p style="text-align: center;">12 oyeee-R 16 oxeee-R</p>
o3 for oxidation from alcohol to aldehyde	 <p style="text-align: center;">6 ooooo 20 oyeee-R</p>
4D for dehydration (four-membered ring formation)	 <p style="text-align: center;">3 oeeee 51 oeeee-4D</p>
5A for aldol reaction (five-membered ring formation)	 <p style="text-align: center;">30 oeeeyo-R 40 oeeeyo-5A-RR</p>
g for conjugation with glucuronic acid	 <p style="text-align: center;">1 oeeee 63 Oeeee</p>
s for conjugation with sulfate	 <p style="text-align: center;">1 oeeee 57 seeee</p>

112 **FIGURE 2** Average reaction energies in solution phase and gas phase for oxidation from alkane to alcohol (o1), oxidation from
 113 aldehyde to carboxylic acid (o2), oxidation from alcohol to aldehyde (o3), dehydration or four-membered ring formation (4D),
 114 aldol reaction or five-membered ring formation (5A), conjugation with glucuronic acid (g) and conjugation with sulfate (s) at five
 115 different positions of (-)-menthol. Representative reactions of each type are shown in Table 2.

116 **FIGURE 3** Relative stability of 102 (-)-menthol metabolite compounds.

117 **FIGURE 4** Lowest energy diagram for each step of menthol metabolism

Discussion

119
120

121 The standard Gibbs energies for each reaction in both gas and solution phase listed in the first worksheet of
122 the spreadsheet of S3 File are summarized in Figure 2. With the exception of sulfation (s-3), reaction
123 energies in gas phase are lower in magnitude but have the same sign as their equivalents in solution phase.
124 Oxidation reactions (o1 and o2, addition of $\frac{1}{2}\text{O}_2$) and conjugation with sulfate are the most exergonic and
125 should occur easily. This may be explained by the fact that oxidation tends to introduce polar functional
126 groups whose interactions with water serve to stabilize the compound. The reaction energies of sulfation are
127 very exothermic in gas phase but not solution phase. This large difference in energy is explained by the
128 presence of charged species in the reactant side which is greatly stabilized by the solvent – water – when
129 compared to the product side that receives little stabilization upon solvation. The most endergonic reactions
130 are four-membered ring formation (4D) and oxidation from alcohol to aldehyde (o3, removal of H_2). The four-
131 membered ring formation was proposed based on experimental evidence [18] published in 1988 and should
132 be verified in further experiment. Difference in reaction energies due to position effect can be mostly
133 explained by steric hindrance (i.e. g-8 has the highest reaction energy.) and inductive effect (i.e. o-8
134 producing secondary alcohol is the most exergonic.).

135 Gas phase energies were used to calculate solution phase energies, but as discussed previously they are
136 not representative of the reactions that occur in biological systems. Since these reactions happen in solution,
137 only the energies in solution phase were considered for each metabolite. Standard Gibbs energies for each
138 metabolite are shown relative to the parent compound (**1** oeeee) in Figure 3. The step numbers correspond
139 to the number of reactions required to generate each compound from **1** oeeee according to Figure 1. The
140 first step from the parent compound tends to be the most exergonic with an average at -38.6 kcal/mol and
141 the average reaction energy decreases monotonically to around -3.1 kcal/mol at the fifth step.

142 Compounds with the lowest energy from each step were identified and are shown in Figure 4 with additional
143 intermediates for completion. In general, the metabolite with the lowest relative energy in a step was the
144 starting material for the lowest energy metabolite in the next.

145 • This energy diagram (Figure 4) is in agreement with major aspects of published metabolic
146 pathways, in particular the conversion of menthol to *p*-menthane-3,8-diol (**3** oeoe). Partly due to
147 increased solubility, the compounds **3** oeoe and its glucuronic acid conjugates, **66** Oeoe/67
148 oeOee, were found to be major metabolites excreted in the urine of both rats and humans [17-19].
149 In contrast, *p*-menthane-3,7-diol (**2** oeeee) and *p*-menthane-3,9-diol (**4** oeoe-R, **5** oeoe-S)
150 excreted from both rats and humans in small quantities. Figure 2 reports that oxidation from alkane
151 to aldehyde at either position 7, 8, 9, or 10 is equally exothermic with a slight preference for position

152 8. Published evidence that **3** oeoee is formed as a product of enzymatic activity [18] and this
153 observed thermodynamic preference explain the disproportionately large amount of **3** oeoee isolated
154 experimentally compared to its isomers.
155 • Oxidation from alcohol to aldehyde is an endothermic reaction, hence metabolites containing
156 aldehyde groups are either not detected or detected in small quantities and serve as intermediates
157 to products of intramolecular aldol condensation to form cyclic ethers or further exothermic oxidation
158 to carboxylic acid. In rats, no metabolites containing aldehyde groups were detected in the plasma,
159 urine, bile, or feces. [17, 18] The published metabolic pathways show a direct conversion from
160 alcohol to carboxylic acid. Only the most recent study conducted by Hiki et al. [19] reported
161 detection of aldehyde menthol glucuronides in human urine at very low levels; the pathway proposed
162 by Hiki et al shows further conversion to cyclic ethers and carboxylic acid. Since oxidation is a
163 stepwise process, Figure 4 shows this stepwise conversion from **6** ooeee to **44** oxoee-*R*.

164 Concluding remarks

165 In this study, gas-phase structures of menthol and its metabolites (a total of 102 compounds and 151
166 reactions) were obtained by quantum calculations at B3LYP/6-311++G(d,p) level. The standard Gibbs
167 energies of their respective reactions in solution were calculated with the SMD solvation model and
168 corrected for standard state conditions. The lowest energy diagram (Figure 4) reported was largely in
169 agreement with previously published experimental results. Information obtained in this study opens
170 possibilities for further investigation of the pharmacological effects of menthol and its metabolites. Given that
171 oxidation metabolites of menthol are energetically favorable, potency and toxicity of these oxidized
172 derivatives should be further investigated. Different stereoisomer of menthol as well as MD-based
173 approaches could also be explored in future research.

174 Supporting information

175 S1 File All Q-CHEM output files (zip)

176 S2 File Wolfram Mathematica notebook, shell script and template (zip)

177 S3 File Microsoft Excel spreadsheet and associated files (zip)

178 Acknowledgement

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180 Author Contributions

181 [This is to be copied from Editorial Manager after acceptance of the manuscript.]

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