# IMPLICATION OF SAR OF MALE MEDFLY ATTRACTANTS IN INSECT OLFACTION

V.CASAÑA-GINER[a,*], V. LEVI[b], V. NAVARRO-LLOPIS[a] AND E. B. JANG[c]

[a]*Centro de Ecología Química Agrícola, Universidad Politécnica de Valencia, Edif. Institutos ITQ, Avda. Los Naranjos s/n, Valencia 46022, Spain;* [b]*USDA-ARS, Chemicals Affecting Insect Behavior Laboratory, BARC, Bldg. 007, Rm 301, Beltsville, MD 20705, USA;* [c]*USDA-ARS, Pacific Basin Research Agricultural Center, P.O. Box 4459, Stainback Highway, Kulani RD, Hilo, HI-96720, USA*

* Corresponding author, e-mail: vcasan@itq.upv.es

Medfly (*Ceratitis capitata*) males are strongly attracted to different compounds, not described as pheromones. The best attractants reported are (+)-α-copaene, a sesquiterpene of natural source and (-)-ceralure-B1, a non-natural iodinated cyclohexane ester. Although their origin, atomic composition, chemical and physical properties are rather different, they show similar attraction to medflies. The question of why these compounds, act behaviorally in the same way, has been never addressed in research papers. We show here for the first time that these compounds have quite similar stereochemistry, water accessible surfaces, HOMO orbitals, certain local dipole moments and charges. When seven carbons, one oxygen and one iodine belonging to (-)-ceralure-B1 are selectively chosen based on topological homology with (+)-α-copaene and are overlaid with nine corresponding carbons of (+)-α-copaene, the RMS is 0.367 Å. This represents a high degree of steric resemblance. Local dipole moments and charges are similar in those regions where the molecules show topological homologies. Thus, we hypothesize that these two molecules could interact with the same odorant receptor(s). We discuss the implications of this result in future research in insect olfaction.

*Keywords*: *Ceratitis capitata*, medfly, insect olfaction, SAR, copaene, ceralure, odorant binding proteins, transmembrane proteins

## INTRODUCTION

The medfly, *Ceratitis capitata* (Wiedemann), is one of the most damaging agricultural pests worldwide [1]. The common medfly control method is aerial and terrestrial organophosphates (OPs) bait sprays. The sterile insect technique (release of gamma-ray sterilized medflies) is also used [26]. Currently, Spinosad® is being considered as an alternative to OPs [46, 47]. The timing of pesticide treatments, whichever method is employed, is mainly controlled by means of attractant-baited traps. Other control methods (mass-trapping [48], autosterilization [3]) are based on specific attractants localized in traps. Thus, medfly attractants play an important economic role in tropical and subtropical agricultural areas, since this pest has been described at least in 350 host plants [27].

The most attractive lures for the medfly are active only for males [1, 3, 5 - 9]. The biological reason for this remains unknown [28]. These lures can be divided into two main categories: natural and synthetic compounds. Natural attractants are α-ylangene and α-copaene [30, 31]. In this group, only α-copane has been extensively tested as male lure for the medfly, the most active stereoisomer being (+)-α-copaene [5, 7, 31]. Synthetic male medfly lures comprise a variety of compounds with structural similarities: siglure, medlure [29], trimedlure [10], ceralure [8] and trimedlure derivatives [14]. For 40 years, trimedlure [10], *tert*-butyl 4- and 5-chloro-*cis* and *trans*-2-methylcyclohexane-1-carboxylate (a mixture of isomers) has been used as the standard synthetic male medfly lure, despite the discovery of a more powerful male medfly attractant mixture, ceralure (ethyl 4- (and 5-) iodo-*cis* and *trans*-2-methylcyclohexane-1-carboxylate) [9, 13 - 14]. Only recently, the most synthetic male medfly lure, (-)-ceralure B1, ethyl *cis*-5-iodo-*trans*-2-methylcyclohexane-1-carboxylate,

has been enantiomerically synthesized [15]. Each year, California, Texas and Florida states employ more than 100,000 traps baited with the male medfly attractant trimedlure [32], a chlorinated compound with noxious effects ($LC_{50}$ for trimedlure (24 hours) in rainbow trout: 1.5 ppm; in bluegill sunfish: 14.7 ppm [4]). We have tested, in field trials in Spain, (-)-ceralure B1 and (+)-α-copaene. As far as this two compounds are the most attractive male medfly lures described up to the date [6, 16], and we have our own biological activity data of both compounds, we have tried to find if there is some chemical reason for their quite similar biological activity. In this work, we have not included other lures because two main reasons: we have focussed the study on the best attractants and we do not have field data to compare attractiveness for the other lures. We have determined the shape of these molecules and solvent accessible area and other topological indexes, LUMO and HOMO orbitals, local dipole moments and charges. We have found unexpected similarities which may explain, here for the first time, why these compounds have the same biological activity in male medflies, giving also clues to understand olfaction processes in other species.

## MATHERIALS AND METHODS

### Biological Activities

#### *Chemicals*

Purity was checked by means of NMR and gas chromatography using quiral columns. Ashot Khrimian (USDA, CAIBL, Beltsville, MD) provided us (-)-Ceralure B1 (P > 99%); (+)-α-copaene (P > 97%) was obtained from *Angelica archangelica* essential oil. The α-copane from this essential oil [6], is a mixture of enantiomers, 98.6% (+) and 1.4% (-).

#### *Sexual Behavior*

Medflies (*Ceratitis capitata)* were reared in our environmental chamber, in a 16 h light : 8 h dark photoperiod with 40% relative humidity and temperature of 27ºC. Adult flies were fed a mixture of yeast autolysate and sucrose (1:4 wt:wt) before the tests. Larvae were reared on a mixture of wheat bran:sucrose:beer: yeast:nipagin:nipasol:water:hydrochloric acid (20:5:1:0.5:0.5:10:0.1). Casaña-Giner (1995, unpublished) observed that male medflies flies confined in a test tube closed with a 200 µL trimedlure-dosed cotton showed a remarkable sexual behavior (erection, copulating movements on the cotton cap and compulsive maxilary palp extension). In this work, the same test has been done with (+)-α-copaene and (-)-ceralure B1 as sexual behavior enhancers. Test tubes were observed each 15 min. during the first 5 hours, and a final observation was done 24 h after initiation of test.

### *Field Attractiveness*

To standardize the type of traps, only yellow delta traps (made in our laboratory) were used with a sticky board insert (12 × 20 cm). Each attractant was placed in the middle of the sticky board, in a glass vial (2 cm internal diameter × 3.5 cm high). A cylindrical cotton-wick (0.5 cm internal diameter × 4 cm long) was inserted in the glass vial. Sticky-board inserts and attractants were replaced every two days. Medfly catches were also recorded every two days, from day 0 until day 6 (4 counts per trap). Field trials were conducted in a mandarine (*Citrus reticulata* cultivar Marisol) orchard located in Sagunto (Valencia, Spain) during June and July, 2001. Average daily temperature ranged between 20.5 and 23.9 ºC throughout the experiment. Fruit was not yet ripped on the trees. It was designed a 4-block experiment, with 5 traps of (-)-ceralure B1, 5 of (+)-a-copaene and 5 blank (no attractant inside the cotton wick) traps. For each compund, 80 measures were recorded. For the purpose of the analyses, the four blocks were treated as trap-bait replications, thus, n = 4. Data were normalized by means of the $(x)^{1/2}$ transformation, and analyzed by one-way analysis of variance (ANOVA) using Statgraphics Plus 5.0 [49].

## Molecular descriptors

Initial molecular models were built using ChemDraw Ultra v. 6.0 (CambridgeSoft, Cambridge, MA).

The modified DelRe method can handle larger molecules and some atoms with higher atomic numbers (Br, I), but cannot handle some of the metal atom types handled by CNDO [SW].

The solvent accessible surface represents the portion of the molecule that solvent molecules can access. To determine the solvent-accessible surface, a small probe sphere simulating the solvent molecule is rolled over the surface of the molecule (van der Waals surface). The solvent-accessible surface is defined as the locus described by the center of the probe sphere

Ovality is
The ratio of the Molecular Surface Area to the Minimum Surface Area. The Minimum Surface Area is the surface area of a sphere having a volume equal to the Solvent-Excluded Volume of the molecule. Computed from the Connolly Molecular Surface Area and SolventExcluded Volume properties.

The Principal Moments of Inertia are the diagonal elements of the inertia tensor matrix when the Cartesian coordinate axes are the principal axes of the molecule, with the origin located at the center of mass of the molecule. In this case, the off-diagonal elements of the inertia tensor matrix are zero and the three diagonal elements, Ixx, Iyy, and Izz correspond to the Moments of Inertia about the X, Y, and Z axes of the molecule.

| Property | Description |
|---|---|
| Connolly Solvent Accessible Surface Area (Angstroms$^2$) | The locus of the center of a spherical probe (representing the solvent) as it is rolled over the molecular model. |
| Connolly Molecular Surface Area (Angstroms$^2$) | The contact surface created when a spherical probe sphere (representing the solvent) is rolled over the molecular model. |
| Connolly SolventExcluded Volume (Angstroms$^3$) | The volume contained within the contact molecular surface. |
| Ovality | The ratio of the Molecular Surface Area to the Minimum Surface Area. The Minimum Surface Area is the surface area of a sphere having a volume equal to the Solvent-Excluded Volume of the molecule. Computed from the Connolly Molecular Surface Area and SolventExcluded Volume properties. |
| Principal Moments of Inertia (X, Y, Z) (grams/mole Angstroms$^2$) | The Moments of Inertia when the Cartesian coordinate axes are the principal axes of the molecule. |

Fifth, there are the strong hydrophobic forces, due entirely to solvent entropy changes. When two nonpolar residues approach each other, the surface area exposed to solvent is reduced, increasing the entropy of all the water present and decreasing the entropy of the residues, adding to the binding energy a hydrophobic free energy of ~17 zJ/nm$^2$ of contact surface area that was formerly exposed to water [413]. In designing an artificial binding site, the above forces may be combined to achieve the desired level of affinity and specificity for a given ligand. All forces are not equally useful in this regard, however. For example, hydrophobicity is the major factor in stabilizing protein-protein associations [402]. But hydrophobicity is almost entirely nonspecific, hence contributes little to ligand discrimination. By contrast, the proper formation of hydrogen bonds and van der Waals contacts require complementarity of

the surfaces involved. Such surfaces must be able to pack closely together, creating many contact points, and charged atoms must be properly positioned to make electrostatic bonds. Thus van der Waals and polar interactions may contribute little to the dynamic stability of the ligand-receptor complex, but they do determine which molecular structures may recognize each other [402]. Other design elements of binding sites, such as directed channeling of substrates into the receptor, may also prove useful.
In analyzing molecular forces, note that at the nanoscale level, surface/surface, molecule/surface, and molecule/molecule interactions may feature very complicated behaviors. Nanodevices performing work may generate both thermodynamic and mechanical local nonequilibrium conditions, so calculations based on the general forms of interactions and on macroscopic expressions valid at equilibrium conditions should be taken only as basic estimates.

Conolly Accesible Area

(2) Computed properties were analyzed with ChemDraw Ultra V. 6.0 built-in models (ChemPropPro, ChemPropStd, MOPAC) and Gamess add-in [18].

(3) Minimized vapor-phase molecular geometries of each molecule were calculated using MOPAC (semiempirical approach), with a minimum RMS gradient of 0.001. The method chosen was AM1 with closed shell (restricted) function. Mulliken charges were also calculated.

(4) Hydrophobic surfaces were calculated as map property of the solvent accessible surface. Solvent radius used was 1.4 Å, the solvent radius for water. The rationale is that the lures investigated may be in water solution at the moment of odor recognition [43]. Ovality as defined in this paper is the ratio of the Molecular Surface Area to the Minimum Surface Area.The Minimum Surface Area is the surface area of a sphere having a volume equal to the Solvent-Excluded Volume of the molecule, computed from the Connolly Molecular Surface Area and Solvent-Excluded Volume properties. The surface area and volume calculations were performed with Michael Connolly's molecular surface areas and volumes computing algorithms [25].

(5) Similarity of molecules was calculated using the overlay option of the above mentioned program. Those atoms that showed common surface areas, after visual inspection of molecules' 3D-pictures, were selected for molecules' overlay atoms. Other

suitable overlay options were calculated, but we report here only those that showed best overlay.

The computations were carried out on a HP Pavillion workstation featuring an Intel Pentium III processor and 533 MHz clock.

## RESULTS AND DISCUSION

### Behavior test

### Field trials

Results of the field trial did show a moderately higher, attraction of (+)-α-copaene over (-)-ceralure B1. Male medfly catches (mean ± s.d.) of (+)-α-copaene were 12.85 ± 3.21 per trap and day; that of (-)-ceralure B1 were 9.2 ± 1.89. Both showed significant differences with the blank (0.1 ± 0.0) ($n=4$, $P < 0.000$). However, no significant differences were found in between these two compounds. More extensive field trials would increase the statistical power (chance of finding significant differences when they actually exist) of the analysis, and establish if (+)-α-copaene is actually a better lure than (-)-ceralure B1.

### Molecular Modeling

Biological data showed that male medflies have the same biological response (attraction in the field and sexual behavior for both (+)-α-copaene and (-)-ceralure B1). We have tried to find similarities in both molecules that could justify their biological activity, rather than obtain quantitive structure active relationships depending on changes in a model structure.

The rationale of computing the stereochemistry, as we have done, has been explained previously [11]. In brief, the molecules with the lowest energies represent the

conformation of the molecule that would predominate in the vapor phase at room temperature *in vacuo*. This is particularly important since the same energy-minimized conformations in the vapor phase at approximately the same temperature would be impinging upon the male medfly tests [12]; whether or not these minimized conformations would be modified [18] by the receptor to a higher energy state is unknown, but it seems unlikely due to the energy barrier to the higher state conformation. Moreover, attractiveness to the receptor should depend upon an existing minimal energy conformation in the vapor phase. Thus, there is no rationale for predicting which one of the many higher energy states should interact with the receptor [11].

Warthen et al. [11, 33] have described QSAR of trimedlure as attractants for the medfly. In [11], 270 random linear correlations between catches of eight trimedlure isomers and molecular descriptors (molecular volume, molecular surface area, torsion angle and an average interatomic distance) were made. Of these correlationships, some with significant $r^2$ may have occurred purely by chance, even when $r^2$ is well above of that of random correlation. Statistically, a high $r^2$ of a linear regression, does not necessary means a good fit, because it depends on the number of data points, only eight in that case [11] (the more data, the more representative godness of fit is $r^2$). In fact, in the same paper, the best regresson line fails to predict the ranking of attraction of two trimedlure isomers (out of the eight isomers studied) [11]. We have tried to apply the equation described in [11] to predict the different attractiveness of ceralure isomers. Obviously, we had to modify it according to their different structures (chlorine and *tert*-butil ester moieties in trimedlure, iodine and ethyl ester in ceralure) the value of chlorine torsion angle replaced with that of iodine, and the interatomic distances of the *tert*-butil ester replaced with those of the ethyl ester. According to field

attractiveness data of four ceralure isomers, published by the same group [9], results of equation described in [11] are meaningless when applied to ceralure (no relationship of attraction predicted values with actual ones).

This novel and initial effort of Warthen et al. have been put aside. We want to present real possibilities of such studies in improving insect pheromones and attractants and understanding insect olfaction.

Water accessible surface of energy minimized (+)-α-copaene and (-)-ceralure B1 are shown in Figure 1. With an adequate sterical positioning, it is said, overlaying atoms that after visual inspection show similar water accessible surface patterns in each molecule, the resemblance is surprisingly high, having in mind their quite different chemical formulas (Figure 2), elementary analysis and physical and chemical properties derived from their chemical nature, one as an halogenated cyclohexane ethyl ester and the other a sesquiterpene.

In Table II we present some molecular descriptors that are somewhat similar in between the two molecules (hereinafter, percentages are always expressed with reference to the lowest value, in each descriptor). The boiling point differ about 45 °K, molar refractivity differs about 8%, electronic energy (MOPAC) in 20%, and total energy (Gamess) in 10%. Although difference in LUMO energy is considerable, we realized that in the graphical study of LUMO orbitals (alpha and beta regions) (results not shown) that comparable regions in the matching rings are very similar. The most important results came from the steric resemblance. The Connolly Accesible Area differs surprisingly only in 0.2%, being also the other Connolly descriptors very similar. Ovality, as a general descriptor for the geometry of the molecule has only a 3.5% deviation from one molecule to another. Concerning principal moments of inertia, they are rather different, except in the X axis (difference of 14%). The lipophilicity of the

molecule, expressed as the coefficient octanol / water, is repeatedly higher in (+)-α-copaene by 25% in each of the three fragmentation methods studied (Table II).

Since 1962, LogP has been used to simulate both transport and distribution in biological systems as well as interactions at receptor sites [19]. As far as odor recognition is involved in such a process, and the insect olfactory cells are bathed in an aqueous medium, the sensillum lymph, comparable to the mucus of the olfactory epithelium in vertebrates [20], LogP might be of crucial importance in the olfactory recognition of pheromones and/or attractants together with the molecular shape. Even when the two molecules are computationally overlaid, the hydrophobic surface, along with molecular shape, is quite similar in between all three models (Figure 3). Figure 2 and Table I, show the atoms selected, after a screening seeking for a good fit for overlay. In Table I we can see how close atoms are after computation. Similarities in the hydrophobic surfaces presented in Figures 1 and 3, comparable ovalities of both attractants, are additional clues pointing to this hypothesis.

Buck and Axel [21] identied a novel multigene family in rat that provided a molecular basis for odor recognition. Vosshall et al. (1999), in a semiempirical way, identified a family of seven transmebrane domain proteins (G7-TM), in *Drosophila melanogaster* Meigen, encoded by 100 to 200 genes [22]. Odorant Receptors (ORs) belong to the G7-TM protein family. They are "serpentine" G proteins that traverse the olfactory sensory neuron's membrane seven times. In several vertebrate species, and in the invertebrate *Caenorhabditis elegans*, as many as 1000 genes encode ORs, suggesting that 1%-5% of the coding potential in these organisms is devoted to the recognition of olfactory sensory stimuli [22].

The original approach to the discovery of *D. melanogaster* ORs was partly due to previous biocomputation of public *D. melanogaster* gene databases, searching for likely

odorant receptors DNA expressing sequences. For this purpose, 10% of the *D. melanogaster* was subjected to GENSCAN analysis [23] to predict the intron-exon of all sequences within the database.

The molecular components of olfactory signal transduction in insects are, to some extent, known. Signal transduction is initiated when odorants (either alone or in complexes with odorant binding proteins) bind ORs. This binding causes a conformational change to the OR that allows it to interact with heterotrimeric G-proteins ($\alpha\beta\gamma$) and thereby releasing the G$\alpha$ subunit that in turn, will activate downstream effector enzymes [24].

One question that arises from our present work is if it is possible that only one receptor, is able to recognize both (+)-$\alpha$-copaene and (-)-ceralure B1. If that is true, then it might lead to a new focusing of identical ORs that may accommodate to different attractants, giving a new debate about specificity of the ORs to single molecules, and optimization via molecular modeling of new attractants for insect control.

## CONCLUDING REMARKS

Hydrophobic surfaces and molecular shape seem to be crucial to the biological activity in male medflies. However, parameters such electronic descriptors, may also be important. In fact, some of these descriptors (total energy, electronic energy and HOMO and LUMO orbitals are in some extent similar in both (+)-α-copaene and (-)-ceralure B1.

Research in molecular biology of olfaction has been usually based on relatively low or medium response-triggereing odors [34-41], when compared to the biological activity effects of (+)-α-copaene and (-)-ceralure B1 in male medflies, as described in this paper, high attractiveness in the field, erection and other sexual behaviors. However, noncognitive aspects of olfactory behavior (those related to mating, alarm reactions, kin recognition, homing, etc.) may occur in different regions of the brain than that for the hedonistic analysis of odors [42].

We admire the work began by Buck and Axel in finding ORs. However, one question arise in the process of understanding insect olfaction: we have an insect, the medfly, that shows an extremely high biological response to an odorant (sexual behavior – e.g. copulating movements when exposed to ceralure and copaene- and high attractiveness); we also have *two different molecules* that seem to act the same way, and we have genetic information in medfly genes... is it not the time to join molecular biology, computational chemistry and entomology to better understand insect olfaction? These three separate fields, molecular biology, computational chemistry and entomology, joined similarly in Pharma research, should be companions in insect

olfaction research, in special, in the identification of new pheromones or searching for improved attractants.

It is possible to design new lead drugs ignoring the explicit mechanism of action, but only by topological similarity with other active compounds (analgesics, bronchodilators, antivirals, pesticides, organic conductors, etc.) [44].

The odors used for understanding *Drosophila* or rat olfaction have not the extremely high biological activity of that of (+)-α-copaene and (-)-ceralure B1 do have in the medfly; responses in *Drosophila*. Thus, there is an open field to investigate these processes in the medfly, maybe giving more clues to ORs than what has been done already with other "moderate-response triggering" odors . We do believe that the era of "error and trial" or "observation and isolation" for finding pheromones and insect attractants is becoming to an end, and molecular biology together with SAR and QSAR studies will drive the next wave of pheromone's and attractant's discoveries.

**Table I** Atomic distances in between overlaid atoms and overlay RMS.

| *Atom Labels* | | *Distance* (Å) |
|---|---|---|
| (-)-ceralure B1 | (+)-α-copaene | |
| 4 | 10 | 0.302 |
| 5 | 8 | 0.201 |
| 6 | 6 | 0.183 |
| 7 | 5 | 0.376 |
| 8 | 4 | 0.148 |
| 9 | 9 | 0.269 |
| 10 | 11 | 0.593 |
| O (12) | 12 | 0.271 |
| I (13) | 3 | 0.621 |
| | overlay RMS: | 0.367 Å |

**Table II** Computerized molecular descriptors for (-)-ceralure B1 and (+)-α-copaene.

| *Properties Server* | *Descriptor* | *Computed Properties* | |
|---|---|---|---|
| | | (-)-ceralure B1 | (+)-(α)-copaene |
| CAS number | | 119164-08-8 | 14912-44-8 |
| ChemPropPro | Molecular formula | $C_{10}H_{17}IO_2$ | $C_{15}H_{24}$ |
| ChemPropPro | Molecular weight | 296.149 amu | 204.356 amu |
| ChemPropPro | Normal boiling point | 563 ± 20 ºK | 515 ± 20 ºK |
| Experimental | Boiling point | 354 - 351 ºK (0.3 Torr) † | 519 - 524 ºK (760 Torr) * |
| ChemPropPro | Critical volume | 640.5 cm $^3$/mol | 714.5 cm $^3$/mol |
| Crippen's fragmentation | LogP (octanol / water) | 3.11 ± 0.47 | 4.23 ± 0.47 |
| Viswanadhan's fragmentation | LogP (octanol / water) | 2.98 ± 0.49 | 4.08 ± 0.49 |
| Broto's framentation | LogP (octanol / water) | 2.95 ± 0.68 | 3.79 ± 0.43 |
| Viswanadhan's fragmentation | Molar refractivity | 60.96 ± 0.13 | 65.77 ± 0.77 |
| Experimental | Molar refractivity | - | 65.61* |
| ChemPropStd | Connolly Accesible Area | 430.993 Å $^2$ | 430.035 Å $^2$ |
| ChemPropStd | Connolly Molecular Area | 222.195 Å $^2$ | 229.682 Å $^2$ |
| ChemPropStd | Connoly Solvent-Excluded Volume | 204.311 Å $^3$ | 226.658 Å $^3$ |
| ChemPropStd | Ovality | 1.32451 | 1.2776 |
| ChemPropStd | Principal Moment of Inertia - X | 581.525 gr / (mole * Å $^2$) | 502.216 gr / (mole * Å $^2$) |
| ChemPropStd | Principal Moment of Inertia - Y | 2397.81 gr / (mole * Å $^2$) | 1119.96 gr / (mole * Å $^2$) |
| ChemPropStd | Principal Moment of Inertia - Z | 2800.55 gr / (mole * Å $^2$) | 1298.5 gr / (mole * Å $^2$) |
| Mopac | Electronic Energy | -13169 eV | -16697.5 eV |
| Mopac | HOMO ENERGY | -10.5416 eV | -9.0724 eV |
| Mopac | LUMO ENERGY | 0.206253 eV | 1.18687 eV |
| Gamess | Total Energy | -2251.19 eV | -2504.59 eV |

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† US Patent No. 4,764,366

**Figure 1** Hydrophobic surfaces of (-)-ceralure B1 (left) and (+)-α-copaene (right).

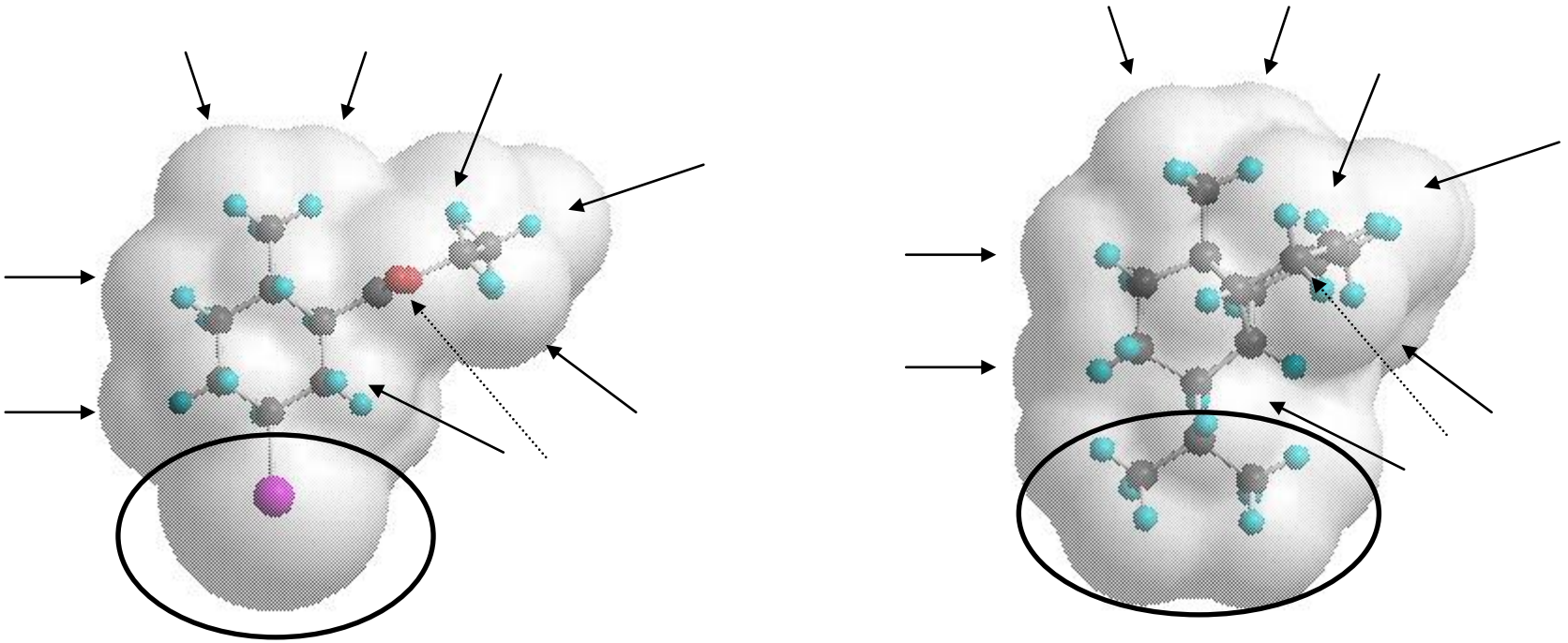
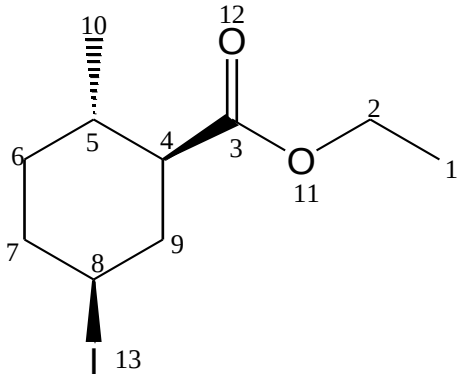


**Figure 2** Atom labeling of (-)-ceralure B1 and (+)-α-copaene used for overlay.




(-)-ceralure B1

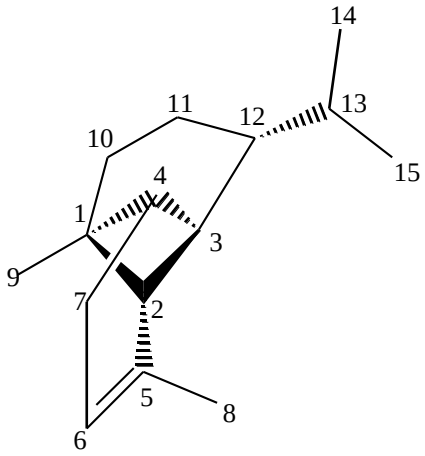
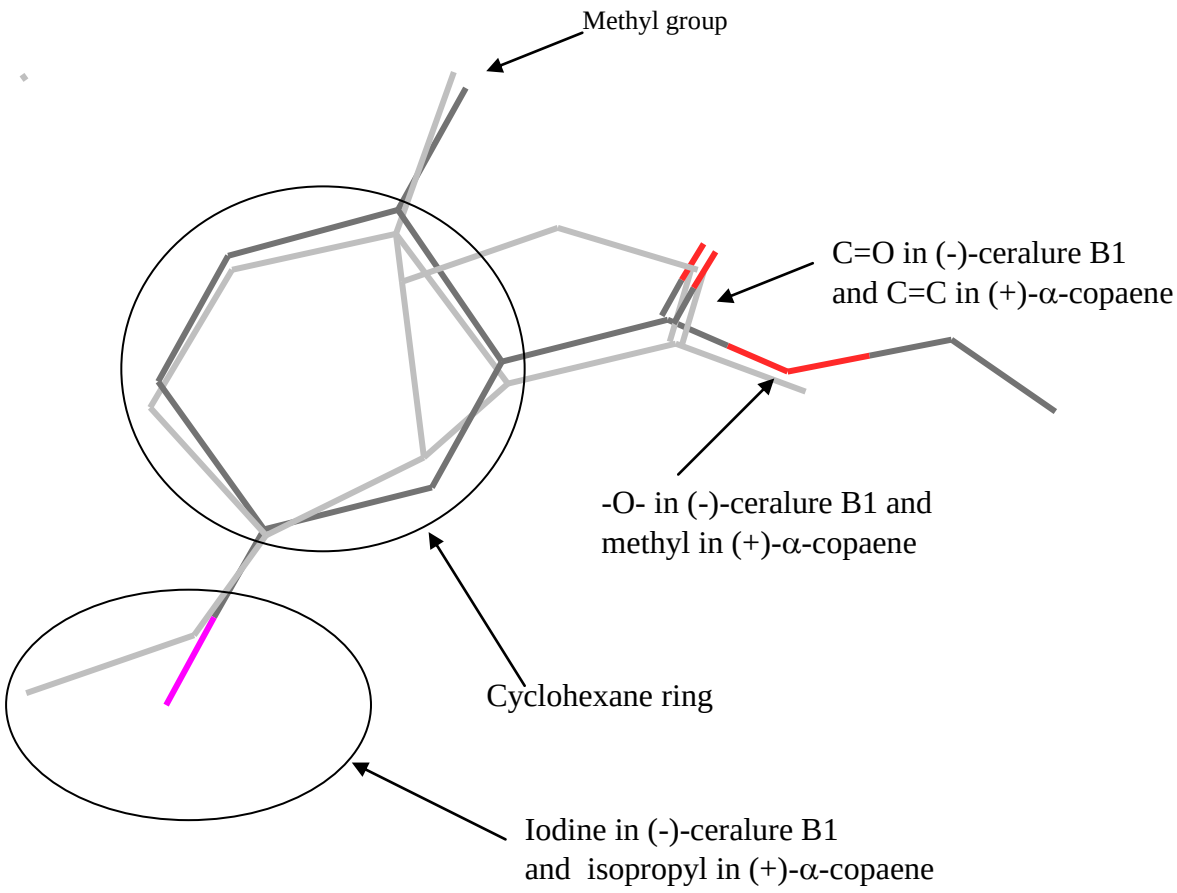


**Figure 3** Overlay of (-)-ceralure B1 and (+)-α-copaene.



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