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Aaron D. Gross
Iowa State University

Michael J. Kimber
Iowa State University, michaelk@iastate.edu

Timothy A. Day
Iowa State University, day@iastate.edu

Paula Ribeiro
McGill University

Joel R. Coats
Iowa State University, jcoats@iastate.edu

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Abstract

Monoterpenoids are found in essential oils from numerous plant families. Octopamine is a biogenic monoamine found within various invertebrates, including insects. Octopamine exerts its physiological effects through the activation of G-protein-coupled receptors (GPCRs). GPCRs are an under-utilized receptor target in the agrochemical industry. Here we report the expression of an octopamine receptor from the brain of the American cockroach (*Periplaneta americana*) in*Saccharomyces cerevisiae,* creating a ligand-independent histidine-auxotrophic assay. The majority of monoterpenoids acted as an inverse agonist in this system. Three QSAR models show that electronic properties are most important for monoterpenoid interaction with this octopamine receptor in this yeast assay.

Disciplines

Entomology | Molecular Genetics | Parasitology | Plant Sciences

Comments

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Chapter 7

Quantitative Structure-Activity Relationships (QSARs) of Monoterpenoids at an Expressed American Cockroach Octopamine Receptor

Aaron D. Gross,[1,2] Michael J. Kimber,[2] Tim A. Day,[2] Paula Ribeiro,[3] and Joel R. Coats*,[1]

[1]Pesticide Toxicology Laboratory, Department of Entomology, Iowa State University, Ames Iowa 50011, U.S.A.
[2]Department of Biomedical Sciences, Iowa State University, Ames Iowa 50011, U.S.A.
[3]Institute of Parasitology, McGill University, Quebec, Canada H9X 3V9
***E-mail: jcoats@iastate.edu.**

Monoterpenoids are found in essential oils from numerous plant families. Octopamine is a biogenic monoamine found within various invertebrates, including insects. Octopamine exerts its physiological effects through the activation of G-protein-coupled receptors (GPCRs). GPCRs are an under-utilized receptor target in the agrochemical industry. Here we report the expression of an octopamine receptor from the brain of the American cockroach (*Periplaneta americana*) in *Saccharomyces cerevisiae,* creating a ligand-independent histidine-auxotrophic assay. The majority of monoterpenoids acted as an inverse agonist in this system. Three QSAR models show that electronic properties are most important for monoterpenoid interaction with this octopamine receptor in this yeast assay.

Introduction

Concern about the adverse health and environmental effects of conventional synthetic insecticides is evident through governmental restrictions, like the Food Quality Protection Act of 1996, limiting the availability of traditional synthetic insecticides. Therefore there is the need to identify safe but effective

compounds to control insect pests. Natural products, like essential oils and essential oil components, are becoming a valuable source for lead compounds for insecticide development. Essential oils have been known since the Middle Ages for their antibacterial properties. Essential oils can be derived from multiple tissues of plants by steam or hydro-distillation (*1*). Essential oils are complex mixtures, primarily composed of terpenoids (mono- and sesquiterpenoids) at various concentrations. The complexity of the terpenoid mixture is enhanced by the presence of various functional groups, stereochemistry, and carbon skeletal structures (*1*). The toxicity of essentials oils and their terpenoid constituents to insects has been the focus of several studies (*2–4*). While these terpenoids have been shown to be toxic to insects, their precise mechanism of how they exert this toxic action is not fully understood. Various studies have indicated that monoterpenoids may have several mechanisms of action. Mechanisms that have been reported include: inhibition of acetylcholinesterase (*5*, *6*), binding at the GABA receptor (*7–11*), binding at the nicotinic acetylcholine receptor (*12*), the octopamine receptor (*13*, *14*), and the tyramine receptor (*15*). Previous studies have also described quantitative structure-activity relationships (QSARs) to describe the toxicity of monoterpenoids and their interaction at the GABA receptor (*11*).

Octopamine is found in numerous invertebrates and functions as a neurohormone, neurotransmitter, and neuromodulator. Octopamine has been shown to have numerous physiological actions in the insects' nervous system and several peripheral target sites. Octopamine in insects is believed to be comparable to norepinephrine in vertebrates. This is because of its similarities in its chemical structure, but also its physiological action (*16–18*). An octopamine receptor has previously been isolated and characterized from the American cockroach, *Periplaneta americana* (Pa oa1) and was used to describe monoterpenoid interactions here (*19*).

GPCRs have been studied for their possible involvement in human disease and as targets for pharmaceutical intervention. It is estimated that 30-45% of current pharmaceuticals target GPCRs (*20*). However, GPCRs have been an under-utilized target in the agrochemical industry. *Saccharomyces cerevisiae*, referred to as yeast hereafter, has emerged as an important organism for the study of heterologously expressed GPCRs (*21*, *22*). Functional expression of heterotrimeric GPCRs can be achieved by linking the expressed receptor to the endogenous pheromone response pathway, which has been performed for analysis of multiple mammalian GPCRs (*22*) and some invertebrate GPCRs (*23–25*). Previously, we have reported the expression of Pa oa1 in yeast, which resulted in a ligand-independent (constitutive) expression system (*25*). Constitutively expressed GPCRs can still yield important results about ligands interacting with the expressed receptor (*26*, *27*). For instance, constitutively active expression of GPCRs can identify compounds that act as inverse agonists or potentially as allosteric modulators (*27*). A constitutively expressed system can show the possibility of a compound interacting with a receptor resulting in various conformations of activation or inactivation. Here we expand on previously screened monoterpenoids against Pa oa1 expressed in yeast to prescribe physicochemical properties that are important for this interaction.

Materials and Methods

Insects

American cockroaches (*P. americana*) were maintained in an established colony on a 14:10 light:dark photocycle at 23 ± 2°C. These insects were provided with an unlimited supply of dry cat food and water.

Chemicals

All monoterpenoids and related aromatic compounds were purchased from Sigma (St. Louis, MO) with the exception of pulegone, which was purchased from Eastman Chemical Company (Miami, FL). For screening purposes the compounds were dissolved in certified dimethyl sulfoxide (DMSO) and serially diluted to a screening concentration of 1 x 10^{-4} M. The final concentration of DMSO to which the cells were exposed was less than 1%.

Isolation and Functional Expression of Pa oa1

The isolation and expression of the American cockroach octopamine receptor Pa oa1, was performed as previously described (*25*). Briefly, RNA was extracted from adult American cockroaches. cDNA was prepared with gene-specific primers based on the previous sequence of Pa oa1 (*19*). *NcoI* and *XbaI* restriction sites were added to the Pa oa1 open reading frame and amplified. Amplicons were ligated into the yeast expression vector, Cp4258. Yeast cells were transformed using a lithium acetate method. Specifically, yeast cell line CY 14083 (genotype: MATα PFUS1-HIS3 GPA1-Gαo(5) can1 far1Δ1442 his3 leu2 lys2 sst2Δ2 ste14::trp1::LYS2 ste18γ6-3841 Ste3Δ1156 tbt1-1 trp1 ura3) was transformed with Cp4258 carrying the Pa oa1 open reading frame (yeast cells were kindly provided by J. Broach, Princeton University). Comparisons were made to mock-transfected yeast cells that had the expression vector, Cp4258, but lacking Pa oa1. Cp4258 contains a constitutively active leucine gene, which allows for selection of yeast cells transformed with the appropriate vector.

Histidine-Auxotrophic Assay

The histidine-auxotrophic assay is the expression of Pa oa1, or an exogenous GPCR, which couples to the yeast's endogenous pheromone-response pathway (the yeast's reproductive system). This is a modified auxotorophic yeast strain, which carries a *His3* reporter gene under transcriptional control of the pheromone-responsive *Fus1* promoter. *His3* expression results in the synthesis of histidine. Therefore, when the receptor is in the active state, histidine will be produced, and yeast cells will grow when they are present in histidine-deficient medium. The histidine-auxotrophic assay was performed similarly to previous reports from our laboratories (*23*, *25*).Briefly, 2 mL of selective medium (-Leu) was inoculated with transformed yeast cells and allowed to grow overnight on an orbital shaker (30°C and 250 RPM (OD_{600} 1.0-2.0)). Cells were pelleted at 5,000 x *g* at room temperature and washed three times with medium which was deficient in leucine

and histidine. The pellet was resuspended in 1 mL of leucine/histidine-deficient media supplemented with 10 mM 3-amino-1,2,4-triazole (3-AT;Sigma) to help control growth by interfering with histidine synthesis. Cells were dispensed into a 96-well clear Costar® plate with a total volume of 200 µL at 15-20 cells/µL (OD_{600} of 0.01), which included 10 µL of vehicle or test compound solution. Cells were allowed to grow at 30°C and 98% humidity for 24 hr at which time optical density readings (absorbance of 600 nm) were taken using a Spectramax 190 (Molecular Devices, Inc. Sunnyvale, CA). Experiments were performed in quadruplicate on 96-well plates for one experiment, with a total of five experiments performed for statistical analysis.

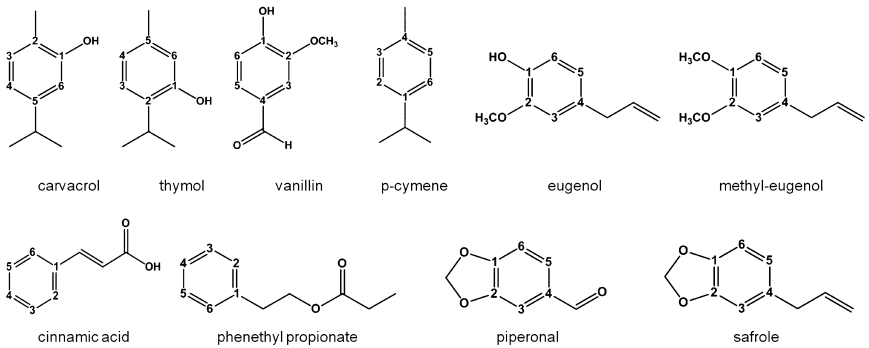

Figure 1. This figure shows numbering of the aromatic monoterpenoids used in the yeast assay and used for Model 1.

QSAR Calculations and Analysis

Descriptors were chosen to measure classical and semi-empirical quantum parameters. Descriptors included the water-octanol partition coefficient (*Log P*), lowest unoccupied molecular orbital (LUMO), highest occupied molecular orbital (HOMO), dipole moment, Mulliken charge, Lwdin charge, and polarizability. Calculations were performed using GAMESS interfaced with ChemBio3D Ultra 12. (Cambridge Software Corporation, Cambridge MA). The energy and geometry of candidate monoterpenoids were optimized and analyzed using a split valence basis set and a polarization function (6-31*d). Electrotopological state of candidate monoterpenoids was calculated with E-Calc (Scivision INC., Burlington, MA). Prior to calculation of selected descriptors, six of the monoterpenoid carbons were numbered, and this was focused on a six-member ring (Figure 1 and Figure 2). In aromatic monoterpenoids, the six carbons of the aromatic ring were numbered (Figure 1). In acyclic monoterpenoids, carbon skeleton structures were drawn in a conformation that gave a structure similar to cyclic monoterpenoids, and carbons were numbered 1-6 (Figure 2). Carbon numbering was based on substituents. For monoterpenoids that did not contain a

heteroatom (oxygen), the lowest number was assigned to the largest substituent (i.e. propyl versus methyl). The next lowest number was given to the carbon bonded to the next closest substituent; priority was always given to the next largest substituent, if applicable. A monoterpenoid that contained a heteroatom that was directly attached to one of the six numbered carbon atoms was given a lower number than a heteroatom attached to a carbon that was not directly numbered 1-6 (carbonyl). In compounds that contained two heteroatoms, a lower number was given to a hydroxyl versus an ether.

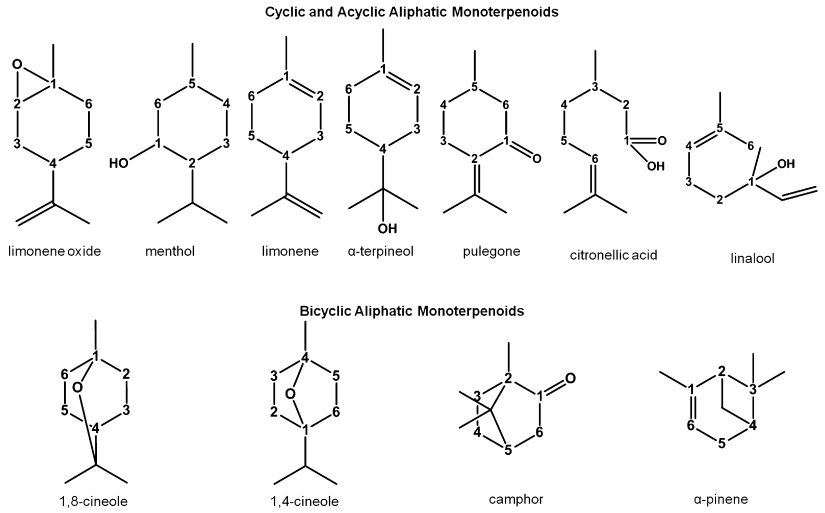

Figure 2. This figure shows the numbering of the aliphatic acyclic, cyclic, and bicyclic monoterpenoids used in this assay to produce Model 2 and Model 3.

Selected descriptors and the growth results, log transformed, from the ligand-independent yeast histidine-auxotrophic assay were analyzed with simple linear and multiple linear regressions. The square of the correlation coefficient (R^2) of ≥ 0.8 was required to describe activity. Regression models were validated using the leave-one-out method (Q^2), shown in the equation below. Simple and multiple linear regression models that had an R^2 of ≥ 0.8 and a Q^2 of ≥ 0.6 were suggested to have a non-random relationship (*28*).

$$Q^2 = 1 - \frac{PRESS}{SSTO}$$

where,

$$PRESS = \sum_Y (Y_{predicted} - Y_{actual})^2$$

Statistical Analysis

Statistical analysis of data obtained from the yeast growth assay was obtained using Analysis of Variance (ANOVA) with α=0.05, using SAS 9.2 (SAS Institute Inc., Cary, NC). Yeast growth was normalized with respect to the vehicle control, and log transformations were performed to achieve an accurate fit model. Linear and multiple regression models were obtained with SAS 9.2.

Results

Yeast Histidine-Auxotrophic Assay

Monoterpenoid activity at Pa oa1 was determined using a histidine-auxotrophic yeast functional expression assay. However, expression of Pa oa1 in a modified auxotrophic yeast strain CY 14083 resulted in a 35-fold increase in growth over yeast cells not expressing Pa oa1 (*25*). When cells expressing Pa oa1 were exposed to octopamine and its immediate synthetic precursor, tyramine, there was not a significant result from the vehicle (Table 1). However, three octopaminergic compounds, phentolamine, synephrine, and chlordimeform, resulted in decreases in yeast growth (Table 1).

Table 1. The effect of octopaminergic compounds on yeast growth by using a yeast expression of a heterologous Pa oa1. Growth that was significantly affected is shown in bold with an asterisk (ANOVA, α = 0.05).

	Octopaminergics
Compound	***% Yeast growth ± SEM***
Vehicle (control)	100%
Octopamine	99 ± 2%
Tyramine	96 ± 4%
Phentolamine	**63 ± 2% ***
Synephrine	**65 ± 3% ***
Chlordimeform	**76 ± 2% ***

The expressed Pa oa1 was screened against 21 monoterpenoids with various effects on its activity (Table 2 and Table 3). In the ligand-independent system, all of the aliphatic and most of the aromatic monoterpenoids acted as inverse agonists. Only one of the tested monoterpenoids, carvacrol, resulted in an increase in yeast growth (Table 2).

Table 2. The effect of aromatic monoterpenoids on yeast growth by using a yeast expression of a heterologous Pa oa1. Growth that was significantly affected is shown in bold with an asterisk (ANOVA, α = 0.05).

Aromatic Monoterpenoids	
Compound	***% Yeast growth ± SEM***
vehicle (control)	100%
carvacrol	**130 ± 7%***
Thymol	103 ± 2%
Safrole	**93 ± 3%***
Cymene	**80 ± 3%***
phenethyl propionate	**80 ± 4%***
cinnamic acid	**65 ± 4%***
Eugenol	**30 ± 2%***
Methyl eugenol	**17 ± 10%***

MODEL 1

$$Y = 1.55(\pm 0.23) + 7.56\ (\pm 1.00)[\mathrm{MCC3}] + 0.72(\pm 0.24)[\mathrm{MCC1}] - 0.12(\pm 0.02)[\mathrm{DM}]$$

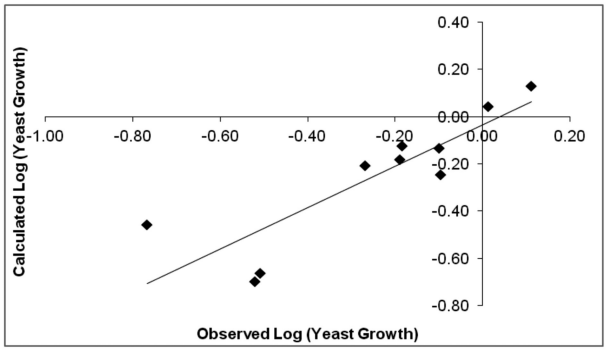

Figure 3. This figure shows the observed log of yeast growth versus the calculated log of yeast growth for the aromatic monoterpenoids. It resulted in Model 1: Y = 1.55(±0.23) + 7.56 (±1.00) [MCC3] + 0.72 (±0.24) [MCC1] – 0.12 (±0.02)[DM] with an R^2 = 0.9109, F=20.45, and a Q^2 = 0.8582.

QSAR Analysis and Models

The biological data along with the output calculations of physicochemical properties allowed for simple and multiple linear regressions to be performed. Initially, all screened terpenoids (aromatic, cyclic, acyclic, and bicyclic) were placed in the same training set; however, it did not result in an $R^2 > 0.8$ or $Q^2 > 0.6$, which were the preset guidelines. Therefore, an aromatic monoterpenoid (Figure 1) training set was used to identify a model that predicts growth activity as a result of aromatic monoterpenoids interacting with the expressed Pa oa1. A multiple linear regression model (Model 1), within the identified limits ($R^2 \geq 0.8$ and $Q^2 \geq 0.6$), for 10 aromatic monoterpenoids (Figure 1) was determined (Model 1, Figure 3, Table 4). Model 1 resulted in an R^2 of 0.9109, F=20.45, and a Q^2 of 0.8582. Model 1 shows that an increase in the Mulliken charge at carbon-3 (MCC3) and the Mulliken charge at carbon-1 (MCC1) (indicating an increase in electron density at these two carbons) cause a greater interaction with expressed Pa oa1. Furthermore, a decrease in the dipole moment (DM) of the aromatic monoterpenoids is also important to the interaction of aromatic monoterpenoids with expressed Pa oa1.

Table 3. The effect of aliphatic monoterpenoids on yeast growth by using a yeast expression of a heterologous Pa oa1. Growth that was significantly affected is shown in bold with an asterisk (ANOVA, α = 0.05).

Aliphatic Monoterpenoids	
Compound	***% Yeast growth ± SEM***
vehicle (control)	100%
limonene oxide	99 ± 3%
α-terpineol	92 ± 5%
linalool	91 ± 2%
1,8-cineole	89 ± 5%
1,4-cineole	**78 ± 6%***
citronellic acid	**74 ± 4%***
pulegone	**73 ± 2%***
limonene	**68 ± 5%***
camphor	**56 ± 4%***

Data from the 11 aliphatic monoterpenoids (Figure 2) resulted in a multiple linear regression model (Model 2 Figure 4, Table 5). This multiple linear regression model was within the identified model limits ($R^2 \geq 0.8$ and $Q^2 \geq 0.6$)

for all of the aliphatic compounds. Model 2 resulted in an $R^2 = 0.8175$, $F = 5.60$, and $Q^2 = 0.6358$. This model shows that a decrease in the Mulliken charge around carbon-1 (MCC1) with an increase in the Lwdin charge at carbon-1 (LCC1), an increase in the HOMO, and the electrotopological state at carbon-6 (ES6) are important factors in aliphatic monoterpenoids interacting with Pa oa1 and thereby affecting the growth of the yeast cells. While Model 2 fits within the parameters initially identified, removal of the four bicyclic aliphatic monoterpenoids resulted in a model with a higher R^2 and Q^2 (Model 3 Figure 5, Table 6).

Table 4. This table shows the values for the observed and predicted yeast growth to form Model 1. It also shows the residual for the observed and predicted yeast growth.

Aromatic monoterpenoid	*Observed yeast growth*	*Predicted yeast growth*	*Residual*
carvacrol	0.11	0.13	0.02
thymol	0.01	0.04	0.03
cinamic acid	-0.19	-0.18	0.01
cymene	-0.10	-0.13	0.03
eugenol	-0.52	-0.70	0.18
methyl-eugenol	-0.77	-0.46	0.31
phenethyl propionate	-0.18	-0.12	0.06
piperonal	-0.27	-0.21	0.06
safrole	-0.10	-0.25	0.15
vanillin	-0.51	-0.66	0.15

Model 3 is produced by reducing the training set from 11 to seven aliphatic monoterpenoids, which increased the R^2, Q^2, and F-value to 0.9211, 0.8716, and 29.94, respectively. Model 3 shows that a decrease in the Lwdin charge at carbon-3 (LCC3), along with an increase of the Mulliken charge at carbon-4 (MCC4) and an increase in the electrotopological state at carbon-3 (ES3) causes an increase the growth of yeast, presumably by the monoterpenoid interaction with Pa oa1. Again, this is showing the electron density around certain carbons to be important in describing the interaction of the aliphatic monoterpenoids with Pa oa1.

MODEL 2

$$Y = 1.10(\pm 0.56) - 1.52\,(\pm 0.39)[MCC1] + 1.57(\pm 0.54)[LCC1] + 0.08(\pm 0.04)[HOMO] + 0.08\,(\pm 0.03)[ES6]$$

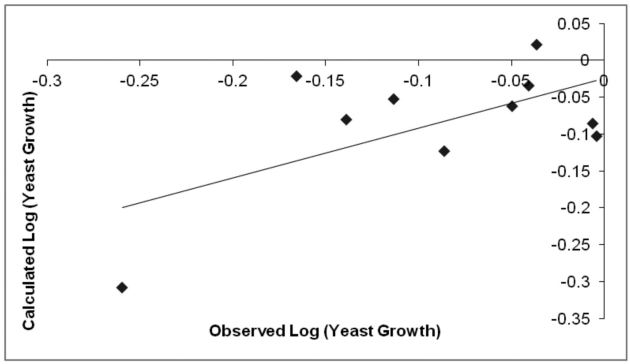

Figure 4. This figure shows observed log of yeast growth versus calculated log of yeast growth for all aliphatic monoterpenoids. This was used to form Model 2: Y = 1.10 (±0.56) – 1.52 (±0.39)[MCC1] + 1.57 (0.54)[LCC1] + 0.08(±0.04)[HOMO] + 0.08 (±0.03)[ES6] with an R^2=0.8175, F = 5.60, and Q^2 =0.6358.

Table 5. This table shows the values for the observed and predicted yeast growth to form Model 2. It also shows the residual for the observed and predicted yeast growth.

Aromatic monoterpenoid	*Observed yeast growth*	*Predicted yeast growth*	*Residual*
1,8-cineol	-0.05	-0.06	0.01
1,4-cineol	-0.11	-0.05	0.06
α-terpineol	-0.04	0.02	0.06
α-pinene	0.00	-0.10	0.10
camphor	-0.26	-0.31	0.05
limonene	-0.17	-0.02	0.14

Continued on next page.

Table 5. (Continued). This table shows the values for the observed and predicted yeast growth to form Model 2. It also shows the residual for the observed and predicted yeast growth.

Aromatic monoterpenoid	*Observed yeast growth*	*Predicted yeast growth*	*Residual*
limonene oxide	-0.01	-0.09	0.08
menthol	-0.09	-0.12	0.04
pulegone	-0.14	-0.08	0.06
linalool	-0.04	-0.04	0.01

Model 3

$$Y = -5.41(\pm 0.73) - 20.30(\pm 2.71)[LCC3] + 2.01(\pm 0.31)[MCC4] + 0.16(\pm 0.04)[ES3]$$

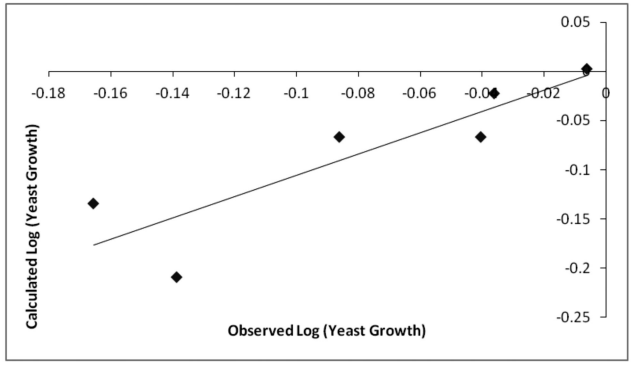

Figure 5. This figure shows observed log of yeast growth versus calculated log of yeast growth for acyclic and cyclic aliphatic monoterpenoids. This was used to form Model 3: Y = -5.41(±0.73) – 20.30(±2.71)[LCC3] + 2.01(±0.31)[MCC4] + 0.16(±0.04)[ES3] with an R^2 =0.9211, F-value 29.94 , and Q^2=0.8716.

Table 6. This table shows the values for the observed and predicted yeast growth to form Model 3. It also shows the residual for the observed and predicted yeast growth.

Aliphatic monoterpenoid	*Observed yeast growth*	*Predicted growth yeast*	*Residual*
α-terpineol	-0.04	-0.02	0.01
limonene	-0.17	-0.13	0.03
limonene oxide	-0.01	0.00	0.01
menthol	-0.09	-0.07	0.02
pulegone	-0.14	-0.21	0.07
linalool	-0.04	-0.07	0.03

Discussion

The expression of Pa oa1 in yeast resulted in a ligand-independent expression system; the lack of response to the previously identified ligand, octopamine, suggests that the receptor is fully activated (Table 1). However, other octopaminergic compounds interacted with the Pa oa1, which is constitutively active, and decrease the response of this receptor in this system. This probably is not related to the *in vivo* function of these ligands but shows an interaction with Pa oa1 (Table 1). Several octopaminergic compounds and monoterpenoids were shown to interact with the octopamine receptor, significantly altering the growth rate of yeast cells. It has been previously suggested that a ligand-independent screening system is beneficial in identifying molecules that can block this activity. Further, this type of assay is advantageous in the detection of compounds that can regulate the function of Pa oa1 independent of its ligand, octopamine (*27*). Previously, several constitutively active human GPCRs, formed by over-expression in *Xenopus laevis* melanophores, were used to search for potential new drugs (*26*).

In this study, octopaminergic compounds were shown to affect the growth of yeast by interacting with Pa oa1. In a ligand-independent system octopaminergic compounds acted as inverse agonists. Therefore, octopaminergic compounds are interacting in some manner with Pa oa1 that changes the conformation of the receptor, decreasing the affinity for the endogenous G-protein, thereby decreasing the signaling through the endogenous pheromone response pathway and decreasing the production of histidine and therefore decreasing yeast cell growth. However, one of the monoterpenoids tested, carvacrol, was shown to increase growth of the yeast cells (Figure 2). This suggests that this aromatic monoterpenoid interacts with Pa oa1 altering the conformation of the receptor and increasing the affinity for the endogenous G-protein. This increases the

production of histidine and therefore an increase in yeast cell growth. The current system provides a response that can be characterized as interacting with Pa oa1 at various degrees of efficacy (at one concentration). Therefore, we suggest that this assay is good for providing an initial indication of the interaction of a monoterpenoid with the receptor, but further analysis should be performed to determine the exact efficacy.

Twenty-one monoterpenoids were tested in this study and used to create models to predict activity at a constitutively active Pa oa1 receptor. It has previously been suggested that octopamine receptor activity is enhanced with the presence of an oxygen atom (*13*). However, p-cymene, which lacks an oxygen substituent, showed a significant result in our assay, but was not as effective as other monoterpenoids that contained an oxygen substituent. Methyl-eugenol showed the best response in this assay. Methyl-eugenol contains a hydroxyl and ether directly attached to the aromatic ring. In contrast, the bicyclic aliphatic monoterpenoid, camphor, which contains a ketone, was the most active aliphatic monoterpenoid tested. Limonene was the next best aliphatic monoterpenoid and does not contain an oxygen substituent; this again shows that an oxygen atom was not necessary for activity in this system.

Various structural features of the tested monoterpenoids were quantified using physicochemical properties. This resulted in the formation of three models to describe the activity of monoterpenoids at the ligand-independent octopamine receptor (Pa oa1). It was evident that electronic parameters are important in prescribing this interaction. Electronic parameters were also important in QSAR models using similar compounds at the insect GABA receptor (*11*). In the training set composed of all aromatic monoterpenoids: as the electronic density at carbon-3 and carbon-1 increased, a compound's interafction with Pa oa1 also increased (a decrease or increase in growth). A decrease in the molecule's dipole moment also increased aromatic monoterpenoids' interaction with Pa oa1. Electronic parameters were demonstrated to be important in acyclic, cyclic, and bicyclic aliphatic monoterpenoids with increases in the HOMO and increases in the electronic accessibility (E-state) at carbon-6 causing greater interaction with Pa oa1. However, removing bicyclic aliphatic monoterpenoids from Model 2 resulted in a better model (Model 3). In Model 3, electronic parameters at carbons 3 and 4 were important in the interaction of acyclic and cyclic aliphatic monoterpenoids.

Conclusion

Electronic parameters are important in determining the effect of various monoterpenoids' activities at the octopamine receptor. Decreasing the aliphatic training set to exclude bicyclic monoterpenoids increased the quality of the model but limited the size of the training set. Future studies should focus on increasing the number of molecules within a training set to get more comprehensive models. This may include the introduction of synthetic derivatives of naturally occurring monoterpenoids. Expression of Pa oa1 in a mammalian-based cell system also may result in a more effective testing plateform.

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