PROGRESS TOWARDS THE DEVELOPMENT OF G-PROTEIN COUPLED RECEPTOR BASED LOGIC GATES

A Thesis
Presented to
The Academic Faculty

By

Vincent P. Peterson

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science in Chemistry & Biochemistry

Georgia Institute of Technology
May 2016
Copyright © 2016 by Vincent P. Peterson
PROGRESS TOWARDS THE DEVELOPMENT OF G-PROTEIN COUPLED RECEPTOR BASED LOGIC GATES

Approved by:

Dr. Pamela Peralta-Yahya, Advisor
School of Chemistry and Biochemistry
Georgia Institute of Technology

Dr. Inga Schmidt-Krey
School of Chemistry and Biochemistry
Georgia Institute of Technology

Dr. Donald Doyle
School of Chemistry and Biochemistry
Georgia Institute of Technology

Dr. Julie Champion
School of Chemical and Biomolecular Engineering
Georgia Institute of Technology

Date Approved: April 29, 2016
ACKNOWLEDGMENTS

I thank everyone that made this project possible. First thank my lab partners, Amy, Emily, and Katherine for their support in lab. In particular I thank Stephen Sarria for constructing several of the plasmids I would later use during this project and use to make some of my plasmids, and Dr. Kuntal Mukherjee, who provided much of the training and background required to complete this work.

Secondly, I thank Georgia Tech for the opportunity to pursue a PhD program, and for allowing me to complete my Master’s Degree. In a similar line, I thank the people numerous that provided me with the support and education required to complete this work.

Third, I thank my committee members, Drs. Donald Doyle, Inga Schmidt-Krey, and Amit Reddi for their feedback on my work and for reviewing my thesis. Most importantly, I thank Dr. Pamela Peralta-Yahya for her extensive work training me, editing my writing, and making me into a proficient synthetic biologist. I hope to make all of these people proud.
# TABLE OF CONTENTS

ACKNOWLEDGMENTS iii  
LIST OF TABLES vi  
LIST OF FIGURES vii  
SUMMARY viii  

## CHAPTERS

1. Introduction  
   1.1 Biological Logic Gates 1  
   1.2 Biosensors 1  
   1.3 Cell Surface Receptors 2  
   1.4 Hypothesis 3  
   1.5 G Protein Coupled Receptor based Yeast Sensors 4  
   1.6 Ligands of Interest 6  
   1.7 The Receptors GPR40 & MOR-EG 7  

2. Materials and Methods  
   2.1 Strain Construction 8  
   2.2 Plasmid Construction 8  
   2.3 Yeast Transformation 9  
   2.4 Flow Cytometry Procedure 9  
   2.5 Data Analysis 10  

3. Results and Discussion  
   3.1 Vectors Required 11  
   3.2 Overview of signaling cascades 11  
   3.3 Testing MAP Kinase Pathway and the GPR40 GPCR with Decanoic Acid 12  
   3.4 Testing cAMP Cascade and the MOR-EG GPCR with Eugenol 12
LIST OF TABLES

1.4 OR and NOR Truth Table 3

A1 Table of Yeast Strains 21

A2 Table of Plasmids 21

A3 Table of Primers 22
LIST OF FIGURES

1.4 OR Gate Simple Schematic 4

1.5 Native Mitogen-activated Protein Kinase Pathway and cyclic Adenosine Monophosphate Cascade 6

1.7 Schematic of the pathways developed in this study 7

3.4 Dose dependent response of the MAPK pathway-dependent GPR40 sensor with decanoic acid and cAMP cascade-dependent MOR-EG sensor with eugenol 12

3.5 MAPK pathway with the GPR40 GPCR and using integrated $P_{GAL4(5x)}$ 14

3.5 cAMP cascade with the MOR-EG GPCR and using integrated $P_{GAL4(5x)}$ 15

3.5 MAPK pathways with the GPR40 GPCR and decanoic acid or eugenol 16

3.5 cAMP cascades with the MOR-EG GPCR and decanoic acid or eugenol 17

4.1 Proposed OR gate schematic using the GPR40 GPCR and integrated $P_{GAL4(5x)}$, and the MOR-EG GPCR and integrated $P_{CRE}$ 18
SUMMARY

The yeast *Saccharomyces cerevisiae* along with the bacterium *Escherichia coli* have been popular model organisms for the creation or modification of chemical producing or chemical sensing strains. Yeast has been used to express G-protein coupled receptors (GPCRs), seven-transmembrane cell-surface receptors found in eukaryotes responsible for the detection toxins, pheromones, drugs, nutrients, and light. GPCRs have been used in the development of biosensors in yeast through the use of the signaling cascades, such as the endogenous yeast mating pathway and the heterologous cyclic adenosine monophosphate (cAMP) cascades. Use of two cascades simultaneously would allow for the creation of complex logic gates in yeast. Development of logic gates in yeast would allow for the detection of multiple chemicals by a single yeast cell. Here I present work toward the development of logic gates in yeast through the use of an engineered yeast mating pathway and a heterologous mammalian cAMP cascade.

Chapter 1 provides an overview of previous work that sets the stage to the development of GPCR-based logic gates. Chapter 2 outlines the methods used to generate the strains and plasmids used in this study, and the methods used to collect and analyze the data. Chapter 3 presents the initial results, demonstrating the use of the yeast mating pathway and the cAMP signaling cascade independently. Chapter 4 summarizes the conclusions that can be drawn, and provides a path to the establishment of GPCR-based logic gates.
CHAPTER 1: INTRODUCTION

1.1 Biological Logic Gates

The interconnectivity of regulatory elements in cells, be they ribonucleic acids (RNA)-, small molecule-, or protein-based, resembles that of electronic circuits\(^1,2\). Living cells require highly dynamic signaling systems to monitor and execute appropriate physiological responses to varying internal and external states\(^3\). The dissection of these regulatory systems has in several cases been simplified to ignore the biochemical interactions between signaling molecules in favor of simplified logic-based models in order to examine the system in its entirety\(^4,5\). The field of systems biology has tended to use these logical models in order to provide a more complete understanding of the system in question\(^5-7\) and provide a foundation to analyze individual components\(^8\). There is an increasing interest to use logical models to design synthetic signaling circuits\(^5,9,10\). Synthetic circuits have been of interest as a means to investigate and modify existing pathways\(^11,12\), as well as to create new ones\(^13,14\). Notable examples include RNA-based translational regulators such as aptamers\(^15,16\) and antisense RNA\(^17\), the protein-based tetracycline repressor protein (TetR)\(^18\), and CRISPR/Cas9 based regulators\(^19\).

1.2 Biosensors

In addition to investigating and building regulatory pathways, synthetic signaling circuits have been adapted to make biosensors. Simple, single-input sensors have been shown to be an excellent means to detect chemicals\(^20\). Biosensors can detect chemicals through the production of a fluorescent signal. Previously generated biosensors detect chemicals such as the drug theophylline and the antimicrobial eugenol using green fluorescent protein (GFP) as a reporter\(^15,16,21\), and the retinoid-like compound LG335, which was detected by adenine production\(^22\). More advanced biosensors use multiple inputs to produce more advanced responses. Multi-input sensors are required for cells to recognize and respond to the complex extracellular conditions they are subjected to, including pH, temperature, and osmotic pressure\(^23,24\). Several multi-input systems have been generated that function in a manner resembling Boolean digital logic gates.
seen in computers, including transcription factor based chemical sensors in *E. coli*\(^{23, 25, 26}\), light-based edge detectors\(^{24}\), and mammalian transcriptional systems\(^{25}\). The development of cellular logic gates and customized signaling cascades have the potential to act as important tools for cell-based therapeutics\(^{27}\). This method has been proposed for induced pluripotent stem cells, where logic gates would allow for the reprogramming of chromatin and generation of specific cell types. In addition, biosensors have the potential to guide evolution for the development of drug and biofuel producing microbes, allowing for the rapid screening and selection of highly producing strains\(^{28}\). While there has been extensive work in the area of intracellular sensors\(^{20}\), there has been less work using cell-surface receptors as a sensing unit\(^{29}\). The use of cell-surface receptors has the potential to expand the number of multi-input systems by detecting chemicals regardless of cell permeability.

### 1.3 Cell Surface Receptors

Cell surface receptors have the unique advantage over intracellular receptors of being able to detect chemicals that cannot penetrate the cell surface. Among them are receptor histidine kinases (RHK), receptor tyrosine kinases (RTKs), and GPCRs. Receptor histidine kinases are two component signaling systems, and are among the most widely used in nature\(^{30}\), and have been used previously to make light-based biosensors\(^{24}\). RHKs are abundant amongst prokaryotes to detect osmotic conditions, nutrients, and light\(^{30, 31}\), and are used for hormone detection in several eukaryotes\(^{32}\), such as with the fruiting hormone ethylene in plants\(^{33}\). No RHKs have been found in the animal kingdom\(^{30}\). RTKs are also two-component signaling systems that are primarily hormone detectors found in eukaryotes, detecting hormones, such as epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and insulin\(^1, 34, 35\). GPCRs are seven-transmembrane domain receptors found in eukaryotes, where they are used to detect toxins, pheromones, drugs, nutrients, and even photons\(^{36, 37}\). GPCRs are highly relevant in the medical field as drug targets\(^{38}\). GPCRs are particularly appealing as a sensing unit due to the wide variety of ligands they can detect\(^{39}\).
1.4 Hypothesis

Here I hypothesize that orthogonal GPCR-based sensors can be used to make logic gates by leveraging two different parallel signal processing units, i.e. the yeast mating pathway and the cAMP cascade, and connecting each of these cascades to a different heterologous GPCR for the sensing of two inputs independently (Fig. 1). The independence of each signal processing unit would allow for the generation of more complex signaling outputs. The advantage of using GPCRs as the sensor unit of logic gates is that they naturally detect a large variety of chemicals, thus logic gates could be generated with a number of different chemical inputs. Here I demonstrate progress towards the production of an OR gate (Table 1). In the future, GPCR-based sensors could be used to construct the universal NOR gate (Table 1), which can be used to generate any computational operation by layering just a single type of logic gate.

Table 1: OR and NOR truth table. Given the presence of an input (1) or its absence (0), specific patterns of outputs emerge based upon the type of gate present. For an OR gate, the presence of either input A or input B provides an output. A NOR gate only provides an output when neither A or B are present.
Figure 1: OR Gate Simple Schematic. Two different G-protein coupled receptors bind their ligands and induce the expression of a fluorescent protein independently of one another.

1.5 G-Protein Coupled Receptor-based yeast sensors

The yeast *Saccharomyces cerevisiae* is an attractive target for the expression of heterologous GPCRs, and has been previously used to de-orphanize GPCRs. The expression of heterologous GPCRs in yeast has had some success, but is still a difficult task. Recently, the Peralta-Yahya laboratory has engineered the yeast mating pathway to transmit a chemical binding event from a heterologous GPCR on the yeast cell surface to a transcription factor resulting in the expression of green fluorescent protein. The yeast mating pathway uses a mitogen-activated protein kinase (MAPK) pathway in order to relay signal through the recruitment of various protein kinases to a structural scaffold. The yeast MAPK signaling pathway has been extensively studied and has been the subject of several engineering projects in an attempt to improve and better understand the signal. The MAPK pathway in *S. cerevisiae* detects the yeast mating pheromone using the STE2 or STE3 GPCR, which transmits signal to STE20 via the Gβ/Gγ complex, STE4 and STE18, respectively. STE20 activates proteins anchored to the STE5 scaffold: STE11, STE7, and finally FUS3. FUS3 goes on to activate the transcription factor STE12, which targets several pheromone response promoters, particularly P_FUS1 and P FIG1 (Fig. 2A). The Peralta-Yahya laboratory has deleted genes in the MAPK pathway to enable the
use of GPCRs as sensors\textsuperscript{46}, including \textit{far1}, which leads to cell cycle arrest following MAPK activation, \textit{sst2}, which spontaneously inactivates the GPCR signal by aiding in GTP hydrolysis in the G\textsubscript{a} subunit, and \textit{ste2}, the native GPCR that would compete with the heterologous GPCR for cell surface expression\textsuperscript{50, 51}.

MAPK pathways are relatively uncommon compared to cyclic AMP (cAMP) and inositol trisphosphate/diacylglycerol (IP3/DAG) cascades\textsuperscript{52}. Components of the mammalian cAMP cascade have been heterologously expressed in yeast\textsuperscript{21, 53, 54}. The cAMP cascade is found in olfactory neurons, using cyclic adenosine monophosphate as a secondary messenger in the cascade\textsuperscript{39}. The cascade presented here uses an olfactory GPCR and the G\textsubscript{olf} type G\textsubscript{a} subunit to activate the membrane-imbedded protein adenylate cyclase type III, which converts ATP into cyclic AMP. cAMP in turn binds to ion channels to stimulate Ca\textsuperscript{2+} influx required for signal propagation along neurons, and protein kinase A to regulate cellular activity, often through cAMP response elements (CRE) and cAMP response element binding protein (CREBP) (Fig. 2B)\textsuperscript{55, 56}. The core components of this cascade have been expressed in yeast previously to stimulate the transcription of green fluorescent protein (GFP)\textsuperscript{21}. 


Figure 2: Native Mitogen-Activated Protein Kinase Pathway and Cyclic Adenosine Monophosphate Cascade. (A) The yeast mitogen-activated protein kinase (MAPK) cascade found in MATα type yeast. The yeast mating hormone secreted by MATα type yeast, α-factor, binds STE2 and leads to the activation of the MAPK pathway resulting cell cycle arrest and the activation of mating pathway genes. (B) The mammalian cyclic AMP (cAMP) cascade. Detection of a ligand by the olfactory receptor leads to an accumulation of cAMP inside the cell, which stimulates an influx of Ca^{2+} and activates Protein Kinase A (PKA).

1.6 Ligands of Interest

Decanoic acid and eugenol were selected as the ligands of interest. Decanoic acid is a biofuel precursor\(^{57}\) which can be converted to fatty acid methyl esters (FAME), an advanced biodiesel\(^{58}\). Decanoic acid and other medium-chain fatty acids produce FAMEs with better cold properties than diesel fuel\(^{59,60}\), and are challenging to produce in microorganisms\(^{61-63}\). Use of a decanoic acid biosensor could be extremely useful to advance the engineering of medium chain fatty acid production strains, and has already been generated\(^{46}\). Eugenol is a phenylpolypropene that is abundant in clove oil and has been used in dentistry due to its anesthetic, antibacterial, and anti-inflammatory properties\(^{64}\). Eugenol and other phenylpropenes have also been explored as possible therapeutic agents due to their potent antifungal properties\(^{65,66}\), and has thus been investigated as a possible biosynthetic target\(^{67}\).
1.7 The Receptors GPR40 & MOR-EG

The human pancreatic GPCR, GPR40, detects medium-chain fatty acids in the bloodstream and is of interest in the study of diabetes. GPR40 was used to signal through the yeast mating pathway upon addition of decanoic acid following the deletion of ste2, sst2, far1 (Fig. 3A). The mouse GPCR mOR-EG has a high sensitivity for vanillin and eugenol, and is found in the olfactory bulb of mice. The ligand-binding domain of MOR-EG was inserted between the first and seventh helix of the rat I7 GPCR, which has coupled to the heterologously expressed cAMP signaling cascade and expressed in yeast to induce signal via eugenol (Fig. 3B).

Figure 3: Schematic of the pathways developed in this study. (A) The mitogen activated protein kinase signaling pathway. The native STE2 G-protein coupled receptor was deleted and replaced with GPR40. In addition, sst2 and far1 were also deleted to enhance flux through the pathway. PFIG1 controls the expression of green fluorescent protein. (B) The cAMP signaling cascade. Gα, Gβ, Gγ, Adenylate cyclase III, cAMP response element binding protein, and chimeric I7/MOR-EG G-protein coupled receptor were transformed in yeast, using the yeast’s protein kinase A to activate cAMP response element binding protein and produce green fluorescent protein under control of PCRE.
CHAPTER 2: MATERIALS & METHODS

2.1 Yeast Strain Construction

The yeast haploid strain W303 (MATa, leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15) Δsst2, Δfar1, Δste2::GSHU:: (PPY110) was used in this study. Various other strains were generated from this strain using Delitto perfetto integration technique. The GSHU core cassette contains the I-SceI gene under control of the inducible P_GAL1 promoter, as well as the hygromycin resistance marker and a counter selectable K. lactis URA3 marker gene. P_FIG1-eGFP-T_CYC1 was amplified from pRS415-Leu2-P_FIG1-eGFP (PPY586) using primers VP139/VP140 and integrated at ste2 after induction of the I-SceI gene and transformation via standard lithium acetate protocol to generate strain VP1. P_CRE-eGFP-T_CYC1 was amplified from pESC-Leu2-P_CRE-eGFP-P_TEF1-MOREGChimera (PPY429) using primers VP161/VP140 and integrated into PPY110 at ste2 using a standard lithium acetate protocol to make strain VP8. P_GAL4(5x)-eGFP-T_CYC1 was amplified from pRS415-Leu2-P_GAL4(5x)-eGFP (PPY528) using primers VP157/VP140 and integrated into strain PPY110 at ste2 using a standard lithium acetate protocol to make strain VP7. Sequences were confirmed through amplification via primers VP167/VP168 from the genome, which were 100 base pairs upstream and downstream of the gene. The sample was gel purified and the size confirmed, and then sequenced with the amplification primers.

2.2 Plasmid Construction

To construct plasmid pESC-His3-P_TEF1-ACIII-P_ADH1-GY-P_HXT7-MOREG (VP4), pESC-His3-P_TEF1-ACIII-P_ADH1-GY (SS38) was amplified using primers VP90/VP91, P_HXT7 was amplified from pESC-Ura3-P_HXT7-G_y-P_TEF1-Gβ (SS41) using primers VP134/VP135, MOREG was amplified from pESC-Leu2-P_CRE-eGFP-P_TEF1-MOREG (SS39) using VP67/VP68, and T_HXT7 was amplified from pESC-Leu2-P_TEF1-P_HXT7 (SS75) using primers VP86/VP87. The fragments were combined and re-amplified with primers VP68/VP86, then cloned into the amplified SS38 fragment using Gibson assembly, maintaining the ACIII and G_y genes present in SS38.
pESC-Ura3-P_{HXT7-G_{oil}}-P_{TEF1-G_{β}-P_{ADH1}}-GPR40 (VP3) was constructed in three parts; \( P_{ADH1} \) was cloned from pESC-Trp1-P_{ADH1-CREBP} (SS39) using primers VP136/VP137, GPR40 was amplified from pESC-His3-P_{TEF1-GPR40} (PPY469) using primers VP78/126, and \( T_{HXT7} \) was amplified from SS75 using VP80/VP81. The fragments were combined and re-amplified with primers VP126/VP80 and cloned into SS41 using Gibson assembly at \( \text{PacI/Sacl} \), maintaining the \( G_{oil} \) and \( G_{β} \) genes present in the original SS41.

Synthetic Transcription Factor 3 (STF3) was amplified from pCRG-141 \( \Delta 11/86^{77} \), synthesized by Operon, using primers SS195/AME229 and cloned at \( \text{PacI/Ncol} \) in SS39 to generate pESC-Trp1-P_{ADH1-STF3} (PPY716).

### 2.3 Yeast Transformation

For the decanoic acid sensor strain, pKM469 or pKM685 was transformed into strain VP1 and VP7, respectively, via standard lithium acetate protocol.

The eugenol sensor strain was constructed by transforming VP4, SS41, and SS39 or pESC-Trp1-P_{ADH1-STF3} into strains VP8 and VP7 via standard lithium acetate protocol.

### 2.4 Flow Cytometry Procedure

**Decanoic Acid Detection:** The cells were grown overnight in synthetic complete media with 2% glucose and lacking histidine (SD glu (H-)). The next day, the cells were used to inoculate 20 mL of SD glu (H-) in a 250 mL flask to an \( \text{OD}_{660} = 0.06 \) and incubated for 18 hrs at 15 \( °C \) (150 r.p.m.). The cells were centrifuged at 1800g for 2 minutes, re-suspended in 1 mL of water, and 50 \( \mu \)L of cells were used to inoculate 5 mL of SD glu (H-) in a 5 mL tube containing 50 \( \mu \)L of decanoic acid or eugenol dissolved in DMSO (0-800 \( \mu \)M) and the final concentration of DMSO was no greater than 1% vol/vol, well below the IC\(_{50}\) of 10% v/v\(^{72}\). The 800 \( \mu \)M maximum was still below the reported solubility of decanoic acid in water\(^{73}\), so precipitation was not a concern. The cells were incubated at 30 \( °C \) for 4 hours (250 r.p.m) before reading cell fluorescence using a flow cytometer.
**Eugenol Detection:** The cells were grown overnight in synthetic complete media with 2% glucose and lacking histidine, uracil, and tryptophan (SD glu (H’U’W’)). The next day, the cells were used to inoculate 20 mL of SD glu (H’U’W’) in a 250 mL flask to an OD$_{600}$= 0.06 and incubated for 18 hrs at 15 °C (150 r.p.m.). The cells were centrifuged at 1800 g for 2 minutes, re-suspended in 1 mL of water, and 50 μL of cells were used to inoculate 5 mL of SD glu (H’U’W’) in a 5 mL tube containing 50 μL of decanoic acid or eugenol dissolved in DMSO (0-800 μM) and the final concentration of DMSO was no greater than 1% vol/vol. The cells were incubated at 30 °C for 4 hours (250 r.p.m) before reading cell fluorescence using a flow cytometer.

GFP fluorescence was measured using a BD LSRII flow cytometer with the following settings: 488 nm laser line, 515–545 nm filter, FSC: 178 V, SSC: 122 V, FITC: 600 V. Fluorescence data was collected from 10,000 viable cells for each experiment.

**2.5 Data Analysis**

Flow cytometry histogram analysis was done using FlowJo software. Samples were exported from the BD LSRII in fcs3.0 format, and imported into FlowJo. The population was gated on a forward vs side scatter plot to remove significant size outliers, and the median green fluorescence of the sub-population was measured. This was repeated in triplicate for each sample. The measured fluorescence had the fluorescence of a control strain subtracted from them, using strains VP1 or VP7 transformed via standard lithium acetate protocol with plasmid pESC-His3 for the decanoic acid strains, and VP8 or VP7 containing plasmids pESC-His3, pESC-Ura3, and pESC-Trp1 for the eugenol and combined strains. Resulting values were averaged and graphed versus concentration.
CHAPTER 3: RESULTS & DISCUSSION

3.1 Strains Required

For this project, several yeast strains needed to be constructed\textsuperscript{21, 46}. First, the reporter gene $P_{FIG1}$-eGFP needed to be integrated into the yeast strain lacking $ste2$, $far1$, and $sst2$ (PPY110)\textsuperscript{46}, given that the GPR40-based sensor carrying the reporter gene in a centromeric plasmid had a significant amount of noise\textsuperscript{46}. The reporter gene of the cAMP cascade, $P_{CRE}$-eGFP, was also integrated. The olfactory cAMP cascade reconstituted in yeast\textsuperscript{21} was modified to use the constitutive promoters $P_{TEF1}$ rather than galactose-inducible promoters. Integration of the reporters will not only reduce noise, but also plasmid burden. The reporter gene $P_{GAL4(5x)}$-eGFP was also integrated into strain PPY110 and used with synthetic transcription factors in an attempt to obtain a greater fold signal from receptors GPR40 and MOR-EG. The native transcription factor STE12 was not deleted and is still active in strains VP1, VP7, and VP8.

3.2 Overview of Signaling Cascades

To determine that the MAPK and cAMP signaling cascades could be used to make orthogonal GPCR-based biosensors, each pathway had to be constructed and tested individually. Prior precedent existed for the MAPK pathway using GPR40\textsuperscript{46}, which was altered to use an integrated $P_{FIG}$-eGFP.

Previously, the rat $G_{\alpha}$, $G_{\beta2}$, $G_{\gamma5}$, and Adenylate Cyclase III (ACIII) along with a human cyclic AMP response element binding protein (CREBP) and chimeric rat I7 GPCR had been transformed in yeast under galactose inducible promoters\textsuperscript{21}. These proteins were instead cloned here under the TEF1, HXT7, and ADH1 constitutive promoters, and the GPCR was modified to include the same 1\textsuperscript{st} and 7\textsuperscript{th} helices of the rat I7 GPCR, but the 2\textsuperscript{nd} through 6\textsuperscript{th} helix of the receptor MOR-EG, which has a high affinity for eugenol and vanillin\textsuperscript{39}. In this manner, addition of eugenol should lead to production of cAMP via ACIII and use the yeast protein kinase A (PKA) to activate CREBP and lead to transcription of eGPF under control of the cyclic AMP response element promoter ($P_{CRE}$).
3.3 Testing MAP Kinase Pathway and the GPR40 GPCR with Decanoic Acid

Initial testing of the MAPK pathway using GPR40 as the GPCR and decanoic acid generated a dose-dependent response. As can be seen in Figure 4A, addition of even 50 μM of decanoic acid leads cells containing GPR40 leads an increase in fluorescence, with a linear range of ~50-400 μM decanoic acid ($R^2 = 0.937$) and a ~2-fold dynamic range across the linear range.

3.4 Testing cAMP Cascade and the MOR-EG GPCR with Eugenol

Incubation of the cAMP cascade with eugenol and the MOR-EG GPCR also produced a dose-dependent response (Fig. 4B). To maintain consistency, eugenol was also dissolved in DMSO despite being already in a liquid phase and having a higher solubility in water than decanoic acid. For the concentration range tested, green fluorescence did not appear to reach a maximum, providing a linear range of ~50-800 μM ($R^2 = 0.995$) and a ~2.5-fold dynamic range across the linear range. Vanillin was also tested, but generated no significant signal (data not shown).

Figure 4: Dose dependent response of the MAPK pathway-dependent GPR40 sensor with decanoic acid and cAMP cascade-dependent MOR-EG sensor with eugenol. (A) Use of integrated $P_{FIG}$-GFP and GPR40 with the MAPK pathway. (B) Use of integrated $P_{CRE}$-GFP and MORE-EG with the cAMP cascade. Autofluorescence was measured with strain VP1 bearing blank plasmid pESC-His3 (for A) and strain VP8 bearing blank plasmids pESC-His3, pESC-Ura3, and pESC-Trp1 (for B) and subtracted from the sensor response. Each point was measured in triplicate and the error bars represent the standard deviation from the mean.
3.5 Use of Synthetic Transcription Factors

To improve performance of the sensors, five Gal4 binding sites were placed in a minimal promoter to make a synthetic Gal4(5x) promoter46. The yeast Gal4 DNA binding protein is a potent regulator that has been well studied as a model regulatory mechanism75 and has a high binding affinity for its target sequence76. However, the STE12 and CREBP transcription factors used previously are incapable of binding to P_{Gal4(5x)}, so a different transcription factor must be used. For the MAPK pathway, the synthetic transcription factor STF1, bearing the Gal4 DNA binding domain, a B42 activation domain, and the STE12 phosphorylation domain was used in a manner identical to previous work46, creating the network shown in Fig. 5A. The method presented here is different, however, in that the transcription factor STE12 was not deleted. This creates a system where the native transcription factor and synthetic transcription factor are phosphorylated. Despite this fact, increasing amounts of decanoic acid led to a dose-dependent response similar to what was seen using STE12 and P_{Fig1} (Fig. 5B), with a linear range of ~50-400 μM decanoic acid (R^2 = 0.993) and a ~4.5-fold dynamic range across the linear range. This result is unsurprising given the high affinity of the Gal4 DNA binding domain for its sequence, and demonstrates that the modified pathway is still effective despite the presence of STE12.
Figure 5: MAPK pathway with the GPR40 GPCR and using integrated P_{GAL4(5x)} (A) Use of P_{GAL4(5x)} requires a synthetic transcription factor, synthetic transcription factor 1 (STF1), to interface between the MAPK pathway and GFP. (B) Decanoic Acid stimulates green fluorescence in a dose-dependent manner when using STF1 and integrated P_{GAL4(5x)}. Autofluorescence was measured with strain VP7 bearing blank plasmid pESC-His3 and was subtracted from the sensor response. Each point was measured in triplicate and the error bars represent the standard deviation from the mean.

The cAMP cascade was also modified to use P_{GAL4(5x)} and a synthetic transcription factor. To have signal upon eugenol activation, the yeast Gal4 DNA binding domain was fused to a minimal CRE phosphorylation and activation domain, creating the signal transduction pathway in Fig. 6A. P_{CRE} and CREBP have been reported to have a K_D of 2 ± 1 nM, while the GAL4 DNA binding domain and the GAL4 binding sequence have a reported K_D of 13 ± 4 nM. Adding eugenol led to a dose-dependent response, with a linear range of ~50-800 μM (R^2 = 0.835) and a ~2-fold dynamic range across the linear range (Fig. 6B), also not reaching a maxima for the concentrations tested.
3.6 Examination of Cross-Talk

Before the OR gate was examined, the pathways were tested using alternate ligands to confirm that the cAMP cascade was incapable of being activated by decanoic acid, and that the MAPK pathway was incapable of being activated by eugenol. The results demonstrate that eugenol has an insignificant influence upon the MAPK pathway with either the integrated P<sub>FIG1</sub> promoter (Fig 7A) or the integrated P<sub>GAL4(5x)</sub> promoter (Fig 7B). It should be noted that STE12 is still active in the P<sub>GAL4(5x)</sub> strain.

When decanoic acid was incubated with the cAMP cascade, however, a significant change in fluorescence from background could be seen, but decanoic acid appeared to cause a decrease, rather than an increase in fluorescence for both the integrated P<sub>CRE</sub> promoter (Fig 8A) and integrated P<sub>Gal4(5x)</sub> promoter (Fig 8B). This leads to the conclusion that decanoic acid is incapable of stimulating the cAMP cascade.

Figure 6: cAMP cascade with the MOR-EG GPCR and using integrated P<sub>GAL4(5x)</sub> (A) Use of P<sub>GAL4(5x)</sub> requires a synthetic transcription factor, synthetic transcription factor 3 (STF3), to interface between the cAMP cascade and GFP. (B) Eugenol stimulates green fluorescence in a dose-dependent manner when using STF3 and integrated P<sub>GAL4(5x)</sub>. Autofluorescence was measured with strain VP7 bearing blank plasmids pESC-His3, pESC-Ura3, and pESC-Trp1 and subtracted from the sensor response. Each point was measured in triplicate and the error bars represent the standard deviation from the mean.
Figure 7: MAPK pathways with the GPR40 GPCR and decanoic acid or eugenol. (A) The MAPK pathway using integrated P$_{NIG1}$ demonstrates a minor increase in fluorescence when exposed to eugenol, compared to a massive increase from decanoic acid. The decanoic acid curve is the same as in Figure 4A. (B) MAPK using integrated P$_{GAL4(5x)}$ demonstrates an insignificant increase in fluorescence when exposed to eugenol, compared to a massive increase from decanoic acid. The decanoic acid curve is the same as in Figure 5B. Autofluorescence was measured with strain VP1 (for A) or VP7 (for B) bearing blank plasmid pESC-His3, and subtracted from the sensor response. Each point was measured in triplicate and the error bars represent the standard deviation from the mean.

Figure 8: cAMP cascades with the MOR-EG GPCR and decanoic acid or eugenol. (A) cAMP using integrated P$_{CRE}$ demonstrates a significant increase in fluorescence when exposed to eugenol, compared to a significant decrease from decanoic acid. The eugenol curve is the same as seen in Figure 4B. (B) cAMP using integrated P$_{GAL4(5x)}$ demonstrates a significant increase in fluorescence when exposed to eugenol, compared to a significant decrease from decanoic acid. The decanoic acid curve is the same as seen in Figure 6B. Autofluorescence was measured from strain VP8 (for A) or VP7 (for B) bearing blank plasmids pESC-His3, pESC-Ura3, and pESC-Trp1 and subtracted from the sensor response. Each point was measured in triplicate and the error bars represent the standard deviation from the mean.
CHAPTER 4: CONCLUSION & FUTURE SCOPE

4.1 Completion of OR Gate

Preliminary data has been presented here to make a GPCR-based OR gate in yeast. A previous method by which the MAPK signaling pathway in *S. cerevisiae* was used as a biosensor was modified by integrating the reporter gene. A cAMP-dependent GPCR-based sensor was also modified that does not rely upon galactose for induction. Both signaling cascades were modified to use a synthetic transcription factor in an attempt to achieve a greater dynamic range, and demonstrated a greater increase in fluorescence from the MAPK pathway, but a smaller dynamic range from the cAMP cascade. In the future, the MAPK pathway and the cAMP cascade should be able to be combined to create an OR gate. Thus, using a modified MAPK pathway with a minimal Gal4 promoter and a deleted *ste12*, combined with the cAMP pathway using the CRE promoter, a robust OR gate using parallel, orthogonal pathways should be able to be created. This would allow for more customization of signal than simply co-expressing two GPCRs using the same pathway.

In order to construct a GPCR-based logic gate, the two signaling cascades would need to be combined into a single cell in a manner that minimized cross-talk between the two pathways. While an OR gate must simply demonstrate that decanoic acid and eugenol can both produce a fluorescent signal from a single cell, meaning that cross-talk becomes irrelevant, other logic gates are more particular. To accomplish this goal, the GAL4(5x) strain, VP7, should have P<sub>CRE</sub>-mKATE2 integrated (Fig. 9). In addition, STE12 needs to be deleted to direct the MAPK pathway towards P<sub>GAL4(5x)</sub>. In this manner, a two color system may be used to monitor the signal coming through both cascades. As has been demonstrated, the GAL4(5x) promoter is more effective at activating GFP expression via the MAPK pathway than the FIG promoter, even with STE12 competing for transcription factor activation. The CRE promoter is more effective at activating GFP expression for the cAMP cascade than the GAL4(5x) promoter. I hypothesize that deleting *ste12* would enhance activity of the MAPK pathway to produce GFP. Therefore, GPR40 and STF1
need only to be combined with the cAMP cascade controlling GFP expression under the CRE promoter. mKATE2 has been shown to be a highly effective red fluorescent protein in *S. cerevisiae* and would thus make an ideal reporter for the cAMP cascade.

**Figure 9: Proposed OR gate schematic using the GPR40 GPCR and integrated P<sub>GAL4(5X)</sub>, and the MOR-EG GPCR and integrated P<sub>CRE</sub>.** Introduction of either eugenol or decanoic acid would lead to the generation of a fluorescent signal, green for decanoic acid, and red for eugenol. The absence of a red signal in the presence of decanoic acid would demonstrate that the MAPK pathway is minimally interacting with the cAMP cascade, as would the absence of a green signal in the presence of eugenol.

To generate the proposed OR gate, I propose that the following steps need to be taken: (1) create and test the OR gate, and (2) improve the signal transduction through the use of stronger promoters to drive the expression of proteins in the cAMP cascade. The cAMP cascade uses a combination of TEF1, ADH1, and HXT7 promoters. While P<sub>TEF1</sub> and P<sub>ADH1</sub> are known to be strong, P<sub>HXT7</sub> has been shown to be much weaker. Given that both MOR-EG chimera and Gα subunit expression are controlled by P<sub>HXT7</sub>, flux through the cascade may be limited at this point by low quantities of MOR-EG and Gα protein necessary to propagate signal, thus leading to a lower overall fluorescence. Use of stronger promoters would provide a means to test this hypothesis. MOR-EG and Gα are the entry points of signal into the cascade, binding eugenol and transmitting signal to ACIII, respectively, and reduced protein levels may be limiting the maximum
amount of signal that can be transmitted through the cascade. The PGK1 and TDH3 promoters have both been shown to have similar strengths to ADH1 and TEF179. Use of a more powerful constitutive promoter such as \( P_{\text{PGK1}} \) or \( P_{\text{TDH3}} \) instead of \( P_{\text{HXT7}} \) could therefore improve the effectiveness of cAMP signaling.

4.2 Generation of Other Logic Gates

Moving beyond this project, generation of a universal NOR or NAND logic gate would be of particular importance. NOR and NAND gates are important in computation because the can be layered to make any other logic gate that exists, as compared to OR gates, which can only make more OR gates. A NOR gate would complement the OR gate that would be developed here. Although several synthetic NOR gates have been generated using transcription factors80-82, and a few with RNA based sensors19, 83, only a single NOR gate has been generated using a GPCR84. The previous GPCR-based NOR gate was established across different cells to communicate with one another. Several strategies have been considered through the development of this project, primarily involving making repressors instead of activators. The first idea tried was moving the TATA box, required for RNA polymerase binding, upstream of the transcription factor’s binding domain85, but this was found to be ineffective. As an alternative, the yeast Cyc8-Tup1 complex was examined as a possible means to create a repressor. Cyc8, also referred to as Ssn6, binds to 4 units of Tup1 to both block the activation domain of recruiting proteins and by histone deacytylation86. Use of a truncated Cyc8 protein (residues 1-351) fused to the LexA DNA binding domain has demonstrated increased fold repression over a simple LexA-Cyc8 fusion87, and could be adapted to make a repressor. A functional repressor would allow for the construction of the universal NOR and NAND gates that are important in computation.

4.3 Potential Application: Flocculation

An application for the OR gate involves making a biosensor capable of rapid removal from media when desired, and provides flexibility by allowing two different chemicals to act as activators. Yeast have been proposed for use as a sensor strain for bioproduction, and have seen
extensive use as a screening tool. A cheap and simple reporter would be yeast flocculation. Flocculation is the process by which yeast sediment together, and is critical in the brewing industry, where flocculated yeast are removed from fermentation batches in order for use in subsequent fermentations. Flocculation is caused by cell-surface flocculation proteins binding to mannose chains found on the cell walls of other yeast, which causes aggregation of cells into multicellular flocs. The W303 strain of S. cerevisiae is incapable of flocculation due to an inactive flo8 gene, which is essential for activation of several proteins essential for flocculation, including FLO1 and FLO11. However, overexpression of gts1, a gene responsible for budding and cell size, is capable of inducing flocculation in W303 yeast. As such, controlling the expression of gts1 under P_CRE or P_FIG1 would allow the yeast to flocculate in the presence of a ligand. An OR gate capable of flocculation would allow for simultaneous sensing of two ligands at the same time with a rapid, easily detectable response capable of viewing with the naked eye. Thus, two equally useful ligands could be detected simultaneously without the use of any special instrumentation.
# APPENDIX

## Table A.1 Table of Yeast Strains

<table>
<thead>
<tr>
<th>Strain #</th>
<th>Name</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPY110</td>
<td>PPY110</td>
<td>W303 (MATa, leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15), Δsst2, Δfar1, Δste2::GSHU::</td>
<td>Mukherjee et. al. ACS Synthetic Biology, (2015).</td>
</tr>
<tr>
<td>PPY870</td>
<td>VP1</td>
<td>W303, Δsst2, Δfar1, Δste2::P_FIG1-eGFP::</td>
<td>This study</td>
</tr>
<tr>
<td>PPY960</td>
<td>VP7</td>
<td>W303, Δsst2, Δfar1, Δste2::P_GAL4(5x)-eGFP::</td>
<td>This study</td>
</tr>
<tr>
<td>PPY962</td>
<td>VP8</td>
<td>W303, Δsst2, Δfar1, Δste2::P_CRE-eGFP::</td>
<td>This study</td>
</tr>
</tbody>
</table>

## Table A.2 Table of Plasmids

<table>
<thead>
<tr>
<th>Strain #</th>
<th>Name</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPY34</td>
<td>pESC-His3</td>
<td>pESC-His3</td>
<td>Agilent</td>
</tr>
<tr>
<td>PPY35</td>
<td>pESC-Ura3</td>
<td>pESC-Ura3</td>
<td>Agilent</td>
</tr>
<tr>
<td>PPY36</td>
<td>pESC-Trp1</td>
<td>pESC-Trp1</td>
<td>Agilent</td>
</tr>
<tr>
<td>PPY428</td>
<td>SS38</td>
<td>pESC-His3-P_TEF1-ACIII-P_ADH1-Gγ</td>
<td>Stephen Sarria</td>
</tr>
<tr>
<td>PPY429</td>
<td>SS39</td>
<td>pESC-Leu2-P_CRE-eGFP-P_TEF1-MOREGChimera</td>
<td>Stephen Sarria</td>
</tr>
<tr>
<td>PPY441</td>
<td>SS41</td>
<td>pESC-Ura3-P_HXT7-Golf-P_TEF1-Gβ</td>
<td>Stephen Sarria</td>
</tr>
<tr>
<td>PPY466</td>
<td>SS49</td>
<td>pESC-Trp1-P_TEF1-P_ADH1-CREBP</td>
<td>Stephen Sarria</td>
</tr>
<tr>
<td>PPY469</td>
<td>pKM469</td>
<td>pESC-His3-P_TEF1-GPR40</td>
<td>Mukherjee et. al. ACS Synthetic Biology, (2015).</td>
</tr>
<tr>
<td>PPY528</td>
<td>PKM528</td>
<td>pESC-Leu2-P_GAL4(5x)-eGFP</td>
<td>Mukherjee et. al. ACS Synthetic Biology, (2015).</td>
</tr>
<tr>
<td>PPY586</td>
<td>pKM586</td>
<td>pRS415-Leu2-P_Fig1-GFP</td>
<td>Mukherjee et. al. ACS Synthetic Biology, (2015).</td>
</tr>
<tr>
<td>PPY685</td>
<td>pKM685</td>
<td>pESC-His3-P_TEF1-GPR40-P_ADH1-STF1</td>
<td>Mukherjee et. al. ACS Synthetic Biology, (2015).</td>
</tr>
<tr>
<td>PPY697</td>
<td>SS75</td>
<td>pESC-Leu2-P_TEF1-P_HXT7</td>
<td>Stephen Sarria</td>
</tr>
<tr>
<td>PPY716</td>
<td>pESC-Trp1-ADH-STF3</td>
<td>pESC-Trp1-P_ADH1-STF3</td>
<td>Stephen Sarria</td>
</tr>
<tr>
<td>PPY829</td>
<td>pESC-Trp1-TEF-SF1-ADH-STF3</td>
<td>pESC-Trp1-P_TEF1-STF1-P_ADH1-STF3</td>
<td>This study</td>
</tr>
<tr>
<td>PPY893</td>
<td>VP3</td>
<td>pESC-Ura3-P_HXT7-Golf-P_TEF1-Gβ-P_ADH1-GPR40</td>
<td>This study</td>
</tr>
<tr>
<td>PPY894</td>
<td>VP4</td>
<td>pESC-His3-P_TEF1-ACIII-P_ADH1-Gγ-P_HXT7-MOREG</td>
<td>This study</td>
</tr>
</tbody>
</table>
### A.3 Table of Primers

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP67</td>
<td>ACAAAAAAGTTTTTTAATTTTAATCAAAAAATGGGAGAGGAGGATTCAC</td>
</tr>
<tr>
<td>VP68</td>
<td>TAGACAGCGCCACAACCTTGGAGGTGCTTAAACCAATCTTGAGACCTTT</td>
</tr>
<tr>
<td>VP78</td>
<td>CCAAGCATACAATCAACTATCTATATCAATGGATTTGCCACCACAATT</td>
</tr>
<tr>
<td>VP80</td>
<td>ACAAGGATGACGACGATAGTCTGCTTGGACCAACACTTTTATTATTAC</td>
</tr>
<tr>
<td>VP81</td>
<td>ATATTGTACACCGAAACAACAAAAAGATTCTTTAAAGTTTTCTTCTCC</td>
</tr>
<tr>
<td>VP86</td>
<td>TGGTAATAACAAATGTAATTAATTAATTGTGGAACACTTTTATTATTC</td>
</tr>
<tr>
<td>VP87</td>
<td>CAAATTCCTGATCACCCCCTCATTTCCACGGTCTTTAAAGTTTTCTTCTCC</td>
</tr>
<tr>
<td>VP90</td>
<td>TTAATTAATACAAATTGTTTATTAC</td>
</tr>
<tr>
<td>VP91</td>
<td>GAGCTCCATCAAGGTTGTCGG</td>
</tr>
<tr>
<td>VP107</td>
<td>TACGACTCACTATAGGGCCGGCCGGCTGACATGAAAGCTACTGTCTTCTA</td>
</tr>
<tr>
<td>VP109</td>
<td>TCGGTAGAGCGCGATCTTAGCTAGCCGCGTTAGAACCATTATTGTGGG</td>
</tr>
<tr>
<td>VP126</td>
<td>CCAACCTCTGGCGAAGGATTGTTTAAATTAATACCTTTTGAGATTCACC</td>
</tr>
<tr>
<td>VP134</td>
<td>CCGTGAAATGAGGGGTAT</td>
</tr>
<tr>
<td>VP135</td>
<td>TTTTTGATTTAAAAATAAAAACTTTTTTTGTTTTGTTTAT</td>
</tr>
<tr>
<td>VP136</td>
<td>TGTATATGAGATAGTTGTGATGTGCTTGG</td>
</tr>
<tr>
<td>VP137</td>
<td>ATCCTTTTGTGTTTCCGCGT</td>
</tr>
<tr>
<td>VP139</td>
<td>AATTGTTGACTTTAAATGCTACGGTTAGAACCATATCCAAAGAAATCAAAATGTCACCTGACATGACCTT</td>
</tr>
<tr>
<td>VP140</td>
<td>ACCTTATAACGTAAGCAGTTAAGAATTACTTTTTTCAAGGCCGTAATTGCCCTT</td>
</tr>
<tr>
<td>VP157</td>
<td>AATTGTTGACTTTAAATGCTACGGTTAAGAACCATATCCAAAGAATCAAACCAGTCTTTACCCGG</td>
</tr>
<tr>
<td>VP167</td>
<td>TGCTATTAATGACCTTTTTTGAAC</td>
</tr>
<tr>
<td>VP168</td>
<td>ATCAAAATAAGGAAAGATACCATT</td>
</tr>
</tbody>
</table>
I7/MOR-EG GPCR Sequence

ATGGAGAGAAGGAATCACTCTGGTAGGGTTTCTGAATTCGTTCTATTGGGATTCCCAGCTC
CAGCTCCTCTAAGAGTCTTGCTATTCTTTCTATCTCTATTGTACGTTCTAGTCTTTAT
GCTTTGGGATTCACTTGCTACTACTTCACTTACACTGCTACTGCTACTTCGCTTCTTGCTT
GTGAACGAAATTGACCTTCTTCTCTGCGAGCTATCATCCTATTGGGATTCCCAGCTC
CAGCTCCTCAGAGTCTTGCTATTCTTTCTATCTCTATTGTACGTTCTAGTCTTTAT
GCTTTGGGATTCACTTGCTACTACTTCACTTACACTGCTACTGCTACTTCGCTTCTTGCTT
GTGAACGAAATTGACCTTCTTCTCTGCGAGCTATCATCCTATTGGGATTCCCAGCTC
I7/MOR-EG GPCR Sequence

G\textsubscript{\alpha} olfactory sequence (UniProt: P38406)

G\textsubscript{\beta} sequence (UniProt: P54313)
TGTGCTTTGGGATATTGAAACTGGTCAACAACTGTTGGTTTTGCTGGTCATTCCGGTGATGTTATGTCCTTGTCCTTGGCTCCAGATGGTAGAACTTTTGTTTCTGGTGCTTGTGATGCTTCCATTAAGTTGTGGGATGTTAGAGATTCCATGTGTAGACAAACTTTTATTGGTCACGAATCGATATTAATGCTGTTGCTTTTCTTCCCAAATGGTTATGCTTTTACTACTGGTTCCGATGATGCTACTTGTAGATTGTTTGATTTGAGAGCTGATCAAGAATTGTTGATGTATTCCCACGATAATATTATTTGTGGTATTACTTCCGTTGCTTTCTCCAGATCCGGTAGATTGTTGTTGGCTGGTTATGATGATTTTAATTGTAATATTTGGGATGCTATGAAGGGTGATAGAGCTGGTGTTTTGGCTGGTCATGATAATAGAGTTTCCTGTTTGGGTGTTACTGATGATGGTATGGCTGTTGCTACTGGTTCTGGGATTCCTTCTTGAAGATTTGGAATTAA

\(G\gamma 5\) sequence (UniProt: P63219)

ATGGGTATTATTTTGAATATTCCAAATATTGGTAGAGAAATGGCTAGATGGTTGAGAGCTTTGGCTTTGCAATTGGTTATGTTGTCCTCCGATCCAGCTACTCATGTTAAATCCTGGGCTTGGTTGTATATGCCAGTTGCTACTGCTTTAAGAGGAGATGGTTTGTTGGCTGCTTCTGAAGGTAAAGTTGTTATTTCTTCCGTTAGAACTAGACAAAAGGGTATTCAAGTTCCAGATACTGCTATGGGGCCTCATACTGGTAATAAACAATTGTAA

Adenylate cyclase III sequence (UniProt: P21932)

ATGACTGAAAGTCAAGGTTTCTCCGATCCAGATGAGGATTTGTTGATGAATCAGACAAATTATCCGTGAATTTCCGCTGAATCTCATCCTGTTCTTCTTCTTCTTCTTATTACCCACTTGGTGGTAGAACTCACGAAATTTCCGTCAGAATTCCGGTTCCTGTTTGTGTTTGCCAAGATTTATGAGATTGACTTTTGTCCCAGAATCTTGGAAAATTTGTACCAAACTTATTTTAAGAGACAAAGACACGAAACTTTGTTGGTCTTGGTCGTCCTTCGCTGCTTTGTTTGATTGTTATGTCGTCGTCATGTGTGCTGTCGTCTTCTCCTCGATAGGTTGGCTCCATTGATGGTCGCTGGTGTCGGTTTGGTCTTGGATATTATTTTGTTTGTCTTGATGTAAGAAGGGTTTGTTGCCAGATAGAGTCTCCAGAAAGGTCGTCCCATACTTGTTGTGGTTGTTGATTACTGCTCAAATTTTCTCCTACTTGGGTTTGAATTTCTCCAGAGCACATGCTGCTTCCGATACTGTCGGTTGGCAAGCATTTTTCGTCTTCTCCTTCTTCATTACTTTGCGATTTCGCTTTCCGATCCAGCCATTTGGGCAAGCTTTTCTCCGCTTGTTCCGCTCAAGAATTGGTCAAGTTGTTGAATGAATTGTTTGCTAGATTTGATAAGTTGGCTGCTAAGTATCATCAATTGAGAATTAAGATTTTGGGTGATTGTTATTATTGTATTTGTGGTTTGCCAGATTATAGAAGAGATCCGGTAGAGATAAGCTGCAATATGATGTCTGGTCCACTGATGTCACTGTCGCTAATAAAATGGAAGCTGGTGGTATTCCAGGTAGAGTCATATTTCCCAATCCACTATGGATTGTTTGAAGGGTGAATTTGATGTCGAACCAGGTGATGGTGTTCTAGATGTGATTATTTGGATGAAAAGGGTATTGAAACTTATTTGATTATTGCTTCTAAGCCAGAAGCTTTTCTCTATGCAAGAAGACTGCTCAAAATGGTTTGAATGGTTCCGCTTTGCCAAATGCGTTCCAGCTTCCAAGCCATCCTCCCCAGCTTTGATTGAAACTAAGGAACCAAATGGTTCTGCTCATGCATCTGGTTCTACTTCTGAAGAAGCTGAAGAACAAGAAGCTCAAGCTGATAATCCATCCTTTCCAAATCCAAGAAGAAGATTGAGATTGCAAGATTTGGCTGATAGAGTCGTCGATGCTCCGAAGATGAACACGAATTGAATCAATTGTTGAATGAAGCATTGTTGGAAAGAGAATCC

G\gamma 5\) sequence (UniProt: P63219)

ATGGGTATTATTTTGAATATTCCAAATATTGGTAGAGAAATGGCTAGATGGTTGAGAGCTTTGGCTTTGCAATTGGTTATGTTGTCCTCCGATCCAGCTACTCATGTTAAATCCTGGGCTTGGTTATGATGATTTTAATTGTAATATTTGGGATGCTATGAAGGGTGATAGAGCTGGTGTTTTGGCTGGTCATGATAATAGAGTTTCCTGTTTGGGTGTTACTGATGATGGTATGGCTGTTGCTACTGGTTCTGGGATTCCTTCTTGAAGATTTGGAATTAA

Adenylate cyclase III sequence (UniProt: P21932)

ATGACTGAAAGTCAAGGTTTCTCCGATCCAGATGAGGATTTGTTGATGAATCAGACAAATTATCCGTGAATTTCCGCTGAATCTCATCCTGTTCTTCTTCTTCTTCTTATTACCCACTTGGTGGTAGAACTCACGAAATTTCCGTCAGAATTCCGGTTCCTGTTTGTGTTTGCCAAGATTTATGAGATTGACTTTTGTCCCAGAATCTTGGAAAATTTGTACCAAACTTATTTTAAGAGACAAAGACACGAAACTTTGTTGGTCTTGGTCGTCCTTCGCTGCTTTGTTTGATTGTTATGTCGTCGTCATGTGTGCTGTCGTCTTCTCCTCGATAGGTTGGCTCCATTGATGGTCGCTGGTGTCGGTTTGGTCTTGGATATTATTTTGTTTGTCTTGATGTAAGAAGGGTTTGTTGCCAGATAGAGTCTCCAGAAAGGTCGTCCCATACTTGTTGTGGTTGTTGATTACTGCTCAAATTTTCTCCTACTTGGGTTTGAATTTCTCCAGAGCACATGCTGCTTCCGATACTGTCGGTTGGCAAGCATTTTTCGTCTTCTCCTTCTTCATTACTTTGCGATTTCGCTTTCCGATCCAGCCATTTGGGCAAGCTTTTCTCCGCTTGTTCCGCTCAAGAATTGGTCAAGTTGTTGAATGAATTGTTTGCTAGATTTGATAAGTTGGCTGCTAAGTATCATCAATTGAGAATTAAGATTTTGGGTGATTGTTATTATTGTATTTGTGGTTTGCCAGATTATAGAAGAGATCCGGTAGAGATAAGCTGCAATATGATGTCTGGTCCACTGATGTCACTGTCGCTAATAAAATGGAAGCTGGTGGTATTCCAGGTAGAGTCATATTTCCCAATCCACTATGGATTGTTTGAAGGGTGAATTTGATGTCGAACCAGGTGATGGTGTTCTAGATGTGATTATTTGGATGAAAAGGGTATTGAAACTTATTTGATTATTGCTTCTAAGCCAGAAGCTTTTCTCTATGCAAGAAGACTGCTCAAAATGGTTTGAATGGTTCCGCTTTGCCAAATGCGTTCCAGCTTCCAAGCCATCCTCCCCAGCTTTGATTGAAACTAAGGAACCAAATGGTTCTGCTCATGCATCTGGTTCTACTTCTGAAGAAGCTGAAGAACAAGAAGCTCAAGCTGATAATCCATCCTTTCCAAATCCAAGAAGAAGATTGAGATTGCAAGATTTGGCTGATAGAGTCGTCGATGCTCCGAAGATGAACACGAATTGAATCAATTGTTGAATGAAGCATTGTTGGAAAGAGAATCC
GCTCAAGTCGTAAGAAGAGAAATACTTTTCTTGTGTACTATGAGATTATAGTGACCAGAAAT
GAAACTAGATATTCGGCGAAGAGAAAAGCAATTCGGCGTCTTCTCTCTCTTCTTCTCTCTTGT
GTCGACTCTTTGTACTGTAGATGCAAAATTTTGTATTGACCTTTGTTGAGTGAATTATATTAT
GTCAGCTTTGTCGTCGCGTGAAAGTCTTTGTCTTCTTTGTCTTGTGGTAGACTTACATTAT
TTTCAAGAAGACTTTTCTTCCAAAGAAGATTGGTCGTTTCTCTCTCTCTCTCTCTCTCTCTCT
GGAAGTGAAGTGTCTTTCTTTGCTTTGTTTTGTTGAGATTTGTCTTTCTCTCTCTCTCTCTCT
ATGTGTGTCTTTGCTTTGTCTTTGTCTTTGTCTTTGTCTTTGTCTTTGTCTTTGTCTTTGTCTT
ATTTTGACTATTGAGTTCTGGTGCTGATAATCAACAATCTTCTTGTAAAGATTTGAAAAGATT
GTTCTGGTACTCAAATTTCTACTATTGCTGAATCTGAAGATTCTCAAGAATCTGTTGATTCTG
ACTGATTCTCAAAAAATTTTGTCTAGAAGACCATCTTATAGAAAAATTTTGAATGATTTGTCTT
CTATTGAACAATTTGGAATAAATATTGGGTACTATGATAGATTGGCTTCTGTTGAAACTGATAT
GCCATTGACTTTGAGACAACATAGAATTCTGCTACTTCTTCTTCTGAAGAATCTTCTAATAAAG
GTCAAAAGACAATTGACTGTTTCTATTGATTCTGCTGCTCATCATGATAATTCTACTATTCCATT
GGATTTTATGCCAAGAGATGCTTTGCAAGAAGATTGGTAGACTACTGATTGCTGACTTCAGAT
GTCATAACTAATGTTGTTTTACTTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT
ATGTTTCTTGTGGAAGATGGTGCCACTAAGATGTTTATGGAATAATGGAGAGAT
GGAATGAAGCATTTGCTGACTATGATATGCTGAGGAAATGTTTCTCTCTCTCTCTCTCTCTCTCT
CTTCTGCAAATTTTTGCTATGAAATGTTAATAAAGATGCTGTTACTGATAGATTGGCTTCTGTTG
AAACTGATATTGGAATATTAAAGCTTTGTTGACTGGTTTGTTTGTTCAAGATAATGTTAATAAAG
ATGCTGTTACTGATAGATTGGCTTCTGTTGAAACTGATATGCCATTGACTTTGAGACAACATAGA
ATTCTGCTACTTCTCTTCTGAAGAATCTTCTTCTTCTAAAGTGGAAGACATTTGCTGACTTCTCTT
GATTTGCTGACTTCTTTCTTTGCTGACTTCTTTCTTTGCTGACTTCTTTCTTTGCTGACTTCTCTT
GCTGAATAGGCTATTGCTTACTATGGAATATTGCTGACTTTGCTGACTTTGCTGACTTTGCTGAC
TTACTGATAGAATTGGCTATTGCTGACTTTGCTGACTTTGCTGACTTTGCTGACTTTGCTGACTT
ATGCTAATTTTCTTTGCTGACTTTGCTGACTTTGCTGACTTTGCTGACTTTGCTGACTTTGCTGAC
TTCTGCTACTTCTCTTCTGAAGAATCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCT
GATTTGCTGACTTCTTTGCTGACTTTGCTGACTTTGCTGACTTTGCTGACTTTGCTGACTTTGCTGAC
TTCTGCTACTTCTCTTCTGAAGAATCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCT
GATTTGCTGACTTCTTTGCTGACTTTGCTGACTTTGCTGACTTTGCTGACTTTGCTGACTTTGCTGAC
TTCTGCTACTTCTCTTCTGAAGAATCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCT
GATTTGCTGACTTCTTTGCTGACTTTGCTGACTTTGCTGACTTTGCTGACTTTGCTGACTTTGCTGAC
TTCTGCTACTTCTCTTCTGAAGAATCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCT
REFERENCES


[73] Decanoic Acid, National Center for Biotechnology Information, PubChem Compound Database.

[74] Eugenol, National Center for Biotechnology Information, PubChem Compound Database.


