Olfactory Receptor Antagonism in the OR-I7 Aldehyde Receptor and the Mammalian Olfactory System

Min Ting Liu
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OLFACTORY RECEPTOR ANTAGONISM
IN THE OR-I7 ALDEHYDE RECEPTOR
AND THE MAMMALIAN OLFACTORY SYSTEM

by

MIN TING LIU

A dissertation submitted to the Graduate Faculty in Chemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2017
Approval Page

This manuscript has been read and accepted by the Graduate Faculty in Chemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Prof. Wayne Harding

Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK
ABSTRACT

OLFACTORY RECEPTOR ANTAGONISM
IN THE OR-I7 ALDEHYDE RECEPTOR
AND THE MAMMALIAN OLFACTORY SYSTEM

by

Min Ting Liu

Advisor: Kevin Ryan

The detection of smell is initiated when an odorant binds and activates olfactory receptors (ORs) within the nose. Chapter 1 studies the structural requirements for activation of the OR-I7 aldehyde receptor. Octanal is an agonist of the OR-I7 receptor. 1, 2 4-Ethylcyclohexylacetaldehyde (1.1), with a cyclohexyl ring that locks the C4-C5 bond of octanal in the gauche conformation, was previously found to have higher potency than octanal, suggesting that the OR-I7 receptor prefers the gauche conformation of octanal during activation. 3 However, 1.1 had only been previously tested as a mixture of cis- and trans-isomers, and therefore, it was not possible to determine whether the response of 1.1 was stimulated by either or both of the isomers. In order to distinguish the response of each isomer, the cis and trans compounds were individually synthesized and tested on olfactory sensory neurons (OSNs) that co-express the OR-I7 receptor via calcium imaging. Through this study, we gained insight into the preferred conformation of the C6-C7 bond of octanal for activation of the OR-I7 receptor.

Removing the ethyl group of 4-ethylcyclohexylacetaldehyde (1.1) to form cyclohexylacetaldehyde (2.2), transforms the molecule from an activator to an inhibitor of OR-I7. 3 Chapter 2 focuses on antagonism of the rodent OR-I7 aldehyde receptor. Besides 2.2, the response of octanal can be antagonized by several short aliphatic linear and cyclic aldehydes at micromolar concentrations. 3 It was proposed that these antagonists compete with octanal for the
binding site via their aldehyde moiety, but fail to activate the receptor due to their shorter chain
lengths. The strength of antagonism for this receptor appeared to correlate with the number of
aliphatic carbons within 7 Å of the aldehyde when it was modeled in the most extended
conformation, but the data set was too small to firmly support this. To further test the possibility,
a structure-activity relationship (SAR) study was carried out in which additional carbons were
systematically substituted onto the structures of OR-I7 antagonists without exceeding 7 Å. The
synthesized analogues were tested for their ability to inhibit activation by octanal in OR-I7
receptors expressed in HEK293 cells using a cyclic AMP detection assay. This work led to more
precise information on what makes a good OR-I7 antagonist.

Currently, there is limited data on the determinants of antagonism, but several olfactory
receptors, such as OR-I7 and mOR-EG, have been reported to be antagonized by odorants which
are structurally similar to the agonist(s) of the receptor. Structural similarities may point to
similar, orthosteric binding sites within the receptor, as opposed to allosteric binding sites.
Chapter 3 looks at determinants of antagonism in the mammalian olfactory system as a whole.
For the OR-I7 receptor, the main difference between the structure of the agonist, octanal, and the
antagonists is their chain lengths, whereas the agonist of the mOR-EG receptor, eugenol, differs
from the antagonists by the functional group on the phenyl ring and the position of the double
bond. For these two receptors, these structural variants may be directing the shift from agonist to
antagonist. A study was devised to look at the extent of OR antagonism that can be stimulated by
pairs of structurally similar odorants, differing only in their carbon chain length or their oxygen
containing functional group. The response of endogenous ORs to these odorants was acquired in
live olfactory sensory neurons via calcium imaging. The response pattern of over 11,000 cells
was analyzed. Based on this study, both the chain type and functional group type can be the
determinant of OR antagonism, but the carboxyl group inhibited the highest percentage of cells out of the three functional groups tested, and cells activated by aldehydes were the most susceptible to antagonism.

In addition to the olfactory studies, which constitute the main part of this thesis, I also did some projects involving the study of pre-mRNA and DNA. Chapter 4 describes small molecule activators of the pre-mRNA 3’ cleavage reaction. The cleavage and polyadenylation of the 3’ end of pre-mRNA is an important process that occurs during the transcription of DNA to RNA. It was previously believed that besides a series of cleavage factors, ATP was also required for the cleavage step, but in an *in vitro* study, it was found that even without ATP, creatine phosphate was able to initiate cleavage between 10-60 mM concentrations.\(^5\) Aside from having a structure similar to that of an amino acid, creatine phosphate also contains a phosphate group, so it was possible that at high concentration creatine phosphate activated cleavage by inhibiting a phosphatase that normally suppresses cleavage.\(^6\) but the actual mechanism by which it acts is still unclear. Through structure-activity relationship (SAR) studies, we have found a variety of new small molecule activators of the *in vitro* pre-mRNA 3’ cleavage reaction. New activators included **cp38** (a cyclic peptide PPM1D inhibitor), dipeptide **4.12**, abscisic acid, and an arginine \(\beta\)-naphthylamide analog. The minimal concentration required for *in vitro* cleavage stimulation has been improved from 200 \(\mu\)M to the 200 nM-100 \(\mu\)M range.

Chapter 5 describes a preliminary attempt to target the molecular recognition of the DNA double helix at a DNA nick, which is a break in one of the two strands of the double helix where the phosphate group has been removed. This feature, which occurs thousands of times at any given instant in the genome of a mammalian cell due to damage, may provide an opportunity for other molecules to bind with the 3’ and 5’ hydroxyl-groups at the DNA nicks. Boronic acids are
known to reversibly bind with diols at high affinity to form boronic esters. The close proximity of the hydroxyl groups at a DNA nick resembles a diol, and could possibly bind with boronic acids in a similar, equilibrium-based manner. Boronic acids have been used for the molecular recognition of diols and polyols, and could potentially be used in the same way for DNA nicks. We tested this possibility on two model studies, one with DNA and one using a surrogate diol, polyvinyl alcohol (PVA).
To my family
ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. Kevin Ryan, for his supervision, support, and guidance, throughout the years that I’ve been working in his lab. I did not expect to major in chemistry when I first entered City College as an undergraduate. The chance to work in Dr. Ryan’s lab inspired me to continue my studies in this field and eventually pursue a doctoral degree in chemistry.

I would also like to thank my committee members, Dr. Mark Biscoe, Dr. Ryan Murelli, and Dr. Wayne Harding for their feedback, advice and suggestions. They encouraged me to look at my research at new and different angles.

Thanks to the current and past members of the Ryan lab for maintaining a safe and enjoyable working environment. Special thanks to Yadi Li for showing me laboratory techniques and training me to set up and run experiments when I first joined the lab. Thanks to Mihwa Na for training me to perform calcium imaging. Thanks to Nag Nagre for running the in vitro premRNA 3’ cleavage reactions. Thanks to Dr. Leonardo Belluscio at NIH for providing the UBI7 mouse, Jianghai Ho from the Matsunami lab for testing the odorants on OR-I7, and the Batista lab for providing homology models for our antagonists. Thanks to the Biscoe lab for sharing their reagents and instruments.

I am grateful to the faculties, staffs, and facilities offered at City College and the Graduate Center. I especially appreciate the help of Dr. Padmanava Pradhan, Cristina Veresmorte, and Dr. Glenn Kowach in showing me how to operate the NMR, GC/MS, and IR spectrometer, respectively.
Finally, I would like to thank my family and friend for their love and support. Thanks to my mom, dad, and sister for being understanding and always accommodating my schedule. Without them, I would not have made it this far.

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<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AC</td>
<td>Adenylyl cyclase</td>
</tr>
<tr>
<td>α</td>
<td>Alpha</td>
</tr>
<tr>
<td>a.k.a.</td>
<td>Also known as</td>
</tr>
<tr>
<td>et. al.</td>
<td>And others</td>
</tr>
<tr>
<td>Å</td>
<td>Angstrom</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>br. s.</td>
<td>Broad singlet</td>
</tr>
<tr>
<td>n-BuLi</td>
<td>n-Butyllithium</td>
</tr>
<tr>
<td>Ca⁺</td>
<td>Calcium 1⁺ ion</td>
</tr>
<tr>
<td>Calcd</td>
<td>Calculated</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
</tr>
<tr>
<td>δ</td>
<td>Chemical shift in parts per million (ppm)</td>
</tr>
<tr>
<td>Cpd</td>
<td>Compound</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>dec.</td>
<td>Decompose</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>CDCl₃</td>
<td>Deuterated chloroform</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>d</td>
<td>Doublet</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>EDC</td>
<td>1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FID</td>
<td>Flame Ionization Detector</td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>GC/MS</td>
<td>Gas Chromatography-Mass Spectrometry</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescence protein</td>
</tr>
<tr>
<td>EC$_{50}$</td>
<td>Half maximal effective concentration</td>
</tr>
<tr>
<td>IC$_{50}$</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>$\Delta$</td>
<td>Heat</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>HRMS</td>
<td>High-resolution mass spectrometry</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human Embryonic Kidney 293</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>H-bond</td>
<td>Hydrogen bond</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>$J$</td>
<td>J-coupling</td>
</tr>
<tr>
<td>LAH</td>
<td>Lithium Aluminum Hydride</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>Magnesium 2+ ion</td>
</tr>
<tr>
<td>Symbol</td>
<td>Term</td>
</tr>
<tr>
<td>--------</td>
<td>-------------------------------------</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>Manganese 2+ ion</td>
</tr>
<tr>
<td>m.p.</td>
<td>Melting point</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>m</td>
<td>Meter</td>
</tr>
<tr>
<td>µL</td>
<td>Microliter</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
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<tr>
<td>mL</td>
<td>Milliliter</td>
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<tr>
<td>mm</td>
<td>Millimeter</td>
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<td>Millimolar</td>
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<tr>
<td>mmol</td>
<td>Millimole</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-Morpholinopropane-1-sulfonic acid</td>
</tr>
<tr>
<td>m</td>
<td>Multiplet</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
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<tr>
<td>OR</td>
<td>Olfactory receptor</td>
</tr>
<tr>
<td>OSN</td>
<td>Olfactory sensory neuron</td>
</tr>
<tr>
<td>OMP</td>
<td>Olfactory marker protein</td>
</tr>
<tr>
<td>G-protein (Golf)</td>
<td>Olfactory-type guanine nucleotide-binding protein</td>
</tr>
<tr>
<td>Pd/C</td>
<td>Palladium on carbon</td>
</tr>
<tr>
<td>p</td>
<td>Pentet</td>
</tr>
<tr>
<td>%</td>
<td>Percent</td>
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PIP₃  Phosphatidylinositol (3,4,5) triphosphate
PI3K  Phosphoinositide 3-kinase
PVA  Polyvinyl alcohol
KBr  Potassium bromide
pre-mRNA  Precursor messenger ribonucleic acid
PP2C  Protein phosphatase 2C
PPM1  Protein phosphatase methyltransferase 1
PCC  Pyridinium chlorochromate
PVA  Polyvinyl alcohol
q  Quartet
RTP1S  a receptor-transporting protein 1 (shorter form of RTP1)
cm⁻¹  Reciprocal centimeters
RNA  Ribonucleic acid
Ser/Thr  Serine/Threonine
SV40  Simian virus 40
s  Singlet
Na⁺  Sodium 1+ ion
SAR  Structural-activity relationship
THF  Tetrahydrofuran
i.e.  That is
TLC  Thin layer chromatography
t  Triplet
ν  Wavenumber
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Chapter 1: Preference of the OR-I7 receptor for a particular C6-C7 conformation of octanal

1.1. Introduction:

1.1.1. Octanal conformation preferred by the OR-I7 receptor

Olfaction, or the sense of smell, is one of the major senses used for analyzing the physical environment. The detection of smell is initiated when odorants interact with olfactory receptors (a.k.a. odorant receptors) (ORs) expressed in the cell membranes of olfactory sensory neurons (OSNs) located within the olfactory epithelium of the nose. ORs belong to the family of G-protein coupled receptors (GPCRs). As depicted on Figure 1.1, when an odorant binds and activates an olfactory receptor, a series of intracellular events will occur resulting in the sensation of smell. The G-protein (Golf) activates adenylyl cyclase to convert ATP to cAMP, and the rise in cAMP level leads to the opening of ion channels allowing calcium and sodium ions to enter the cell. This change in the cell’s polarity results in an action potential and a signal being sent to the main olfactory bulb and then the brain. While ORs make up the largest group of GPCRs in the mammalian genome, the OR machinery remains largely unexplored due to the difficulty in stabilizing the protein for obtaining a crystal structure.

Octanal is one of the best natural product agonists of the OR-I7 receptor, a receptor specific for aliphatic aldehydes. Due to the flexibility of its aliphatic chain, octanal could theoretically adopt hundreds of different conformations with similar energies. In an effort to
learn about the conformation(s) of octanal that activates the OR-I7 receptor, the Ryan lab previously compared the response of octanal to that of 4-ethylcyclohexylacetaldehyde (1.1), which has a cyclohexyl ring that locks the C4-C5 bond of octanal in the gauche conformation (Fig. 1.2).5 (We make the assumption that only the chair conformation is accessible.)

![Diagrams showing line structures and Newman projections of octanal and its isomers](image)

**Figure 1.2**: The gauche conformation of octanal. Line structures (top) and Newman projections (bottom, front carbon: C5, back carbon: C4) of octanal, 1.1, 1.2 and 1.3 in the gauche conformation. The EC50 values were obtained from calcium imaging of the rat OR-I7 receptor.5

Compound 1.1 (EC50=1.0 µM) was found to have approximately equal potency compared to octanal (EC50=1.8 µM), suggesting that the OR-I7 receptor prefers or at least tolerates the gauche conformation of octanal during activation.5 However, 1.1 was tested as a mixture of *cis* and *trans* isomers (1:2.4), so that it was ambiguous as to whether the response of the receptor was due to activation by both or only one of the isomers. Knowing the individual response of the two isomers would not only reveal the source of the response coming from the mixture, 1.1, but also the preference of the OR-I7 receptor for the conformation of the C6-C7 bond on octanal.

The *cis-* (1.2) and *trans-*isomer (1.3) are conformationally restricted isomers of octanal. The cyclohexyl ring of 1.1 (1.2 and 1.3) locks the C4-C5 bond of octanal in the gauche
conformation, which is present in only ~10 closely related isomers of octanal. The cis- (1.2) and trans-isomer (1.3) restrict the conformation of octanal differently by locking the C5-C6 bond at two of the possible positions adopted by octanal while in the gauche conformation at C4-C5. A greater potency in either one of the isomer would indicate a conformational preference by the OR-I7 receptor for the corresponding octanal conformation simulated by the analog.

1.2. Experimental Design:

1.2.1. Synthesis of 1.2 and 1.3

To determine the response of the individual isomers, the cis-isomer (1.2) and the trans-isomer (1.3) of 1.1 were each synthesized. (The synthetic route to 1.2 and 1.3 was initially suggested by Prof. Mark Biscoe, and optimized by my colleague, Yadi Li.) The cis-isomer (1.2) was synthesized from 4-ethylcyclohexanone (Scheme 1.1A). 4-Ethylcyclohexanone was subjected to a Horner-Wadsworth-Emmons reaction to form the α,β-unsaturated ester, 1.2a. The cis-intermediate was achieved through selective reduction of 1.2a with L-selectride to obtain the cis-ester, 1.2b. The bulkiness of L-selectride directed the reaction on the less hindered side resulting in the cis-ester. The ester, 1.2b, was then LAH reduced to the alcohol, 1.2c, which was PCC oxidized to the cis-aldehyde, 1.2.

The trans-isomer, 1.3, was synthesized from the commercially available trans-4-ethylcyclohexylcarboxylic acid (Scheme 1.1B). Since the starting compound already contains the 1,4-trans structure, the trans-aldehyde, 1.3, was obtained after homologation of the functionalized chain. The carboxylic acid was LAH reduced to the alcohol, 1.3a, which was then PCC oxidized to the aldehyde 1.3b. Aldehyde 1.3b was homologated by an extra carbon via a Wittig reaction to form the alkene 1.3c. The alkene, 1.3c, was subjected to a hydroboration-oxidation reaction to form the alcohol, 1.3d, which was then PCC oxidized to form the trans-
aldehyde, 1.3. (For this study, 1.2 was synthesized from 4-ethylcyclohexanone following the procedures of Yadi Li and 1.3 was prepared from 1.3d, which was synthesized from trans-4-ethylcyclohexylcarboxylic acid by Yadi Li.8)

A

\[
\text{EtO}^- + \text{EtO}^- \xrightarrow{\text{NaH, THF}} \text{EtO}^- + \text{EtO}^- \xrightarrow{\text{L-selectride, THF}} \text{EtO}^- \xrightarrow{\text{LAH, ether}} \text{EtO}^- \xrightarrow{\text{PCC, CH}_2\text{Cl}_2} \text{EtO}^- \\
\]

1.2

1.2a

1.2b

1.2c

1.2

Scheme 1.1: Synthesis of 1.2 and 1.3.

1.2.2. Systems for testing 1.2 and 1.3

4-Ethylcyclohexylacetaldehyde (1.1) was originally tested by the Firestein lab (Zita Peterlin, Columbia University) through calcium imaging of rat olfactory sensory neurons that were infected with an adenovirus encoding the rat OR-I7 and green fluorescent protein (GFP) to mark which cells were infected and expressing OR-I7,5 but this system was no longer available for testing 1.2 and 1.3. The cis- (1.2) and trans-isomer (1.3) were thus tested first by the Matsunami lab (Jianghai Ho, Duke University) using a luciferase assay9 on modified HEK293 cells (called Hana3A cells) transfected with the OR-I7 receptor. The Matsunami lab found that the mouse I7 receptor (which differs by 15 amino acid residues compared to the homologous rat receptor, but which retains the aliphatic aldehyde pharmacology of the rat receptor10) is more highly and more reproducibly expressed in HEK293 cells, and so we switched to the mouse OR-
The luciferase technique developed in the Matsunami lab was implemented at elevated temperature (37°C) during which differential evaporation of odorants may occur (further explained in Section 2.2.1). Therefore, the cis- (1.2) and trans-isomer (1.3) of 4-ethylecyclohexylacetaldehyde (1.1) were re-tested in our lab through calcium imaging on OSNs of a transgenic UBI7 mouse11, 12 (provided by Dr. Leonardo Belluscio at NIH). The UBI7 mice have a copy of the OR-I7 transgene (two copies in homozygous mice, which we used) “knocked in” at the olfactory marker protein (OMP) genomic loci. The OMP, and hence OR-I7, is expressed in all OSNs but expression starts when the cell attains maturity, while endogenous ORs begin expression earlier in development. The concentration level of OR-I7 expressed in this recombinant way is thus lower than the normal OR expression level.

1.2.3. Limitations of the UBI7 mouse

In rodents, each mature OSN chooses only one of the ~1100 olfactory receptors for expression.13-15 However, for the transgenic UBI7 mouse (i.e. the homozygous mice that we use; there are two copies of the transgene in each OSN), mature olfactory sensory neurons very likely express an endogenous receptor chosen earlier in development by the cell in addition to the OR-I7 receptor (Figure 1.3).11, 12 (Here, expression means that the receptor’s messenger RNA, mRNA, is made by transcription of the transgene and then translated into protein that is transported to the cell membrane of the OSN in the cilia, the tip of the OSN’s dendrite.) At first, we expected octanal, an agonist of OR-I7, to activate 100% of the OSNs of the homozygous UBI7 mouse, instead of the ~6% OSNs (at 100 µM, Figure 1.4A) that are normally activated by octanal in wild-type (WT) mice. However, in UBI7 cells, the OR-I7 receptor is known to be expressed at a lower level compared to that of the endogenous receptors (possibly as low as <1% of the expression level of endogenous receptors12) due to the nature of the transgenic mouse
construction. While every cell should be expressing the OR-I7 receptor to some extent, the expression level may not be high enough to be detected by calcium imaging, which is less sensitive than the OR-I7 mRNA detection assay shown in Figure 1.3. Additionally, the expression level of the OR-I7 receptor may vary from cell to cell, due to differences in the development state of each neuron, preventing direct comparison of each cell’s absolute response. This is because the OR-I7 transgene is “knocked in” to the genomic location of both copies of the olfactory marker protein (OMP), which is turned on late during OSN development. At the time of a given experiment, some OSNs may not have very much OR-I7 on their cell surface, and may respond weakly or not at all, and the response in those cells may be dominated by the endogenous receptor, whose expression begins earlier in the development of the cell and whose identity cannot be determined in our experiments. Furthermore, the OR-I7 receptor is primarily transported to the cilia, but we lose the cilia during cell preparation (during dissociation of the olfactory epithelium tissue into individual cells), and consequently we lose a lot of the OR-I7 receptor. For these reasons, before comparing the conformationally restricted octanal analogs in UBI7 OSNs, we attempted to estimate the percentage of UBI7 OSNs that actually respond to octanal through the OR-I7 receptor versus the endogenous receptors (that, again, we have no way of identifying).
A. Expression of OR-I7 in a wild-type (WT) mouse (a slice of olfactory epithelium)

B. Expression of OR-I7 in a UBI7 mouse

Figure 1.3: Expression of OR-I7 in a wild-type mouse (top, A) and a UBI7 mouse (bottom, B) (Data is provided by Dr. Leonardo Belluscio at NIH). Expression of the OR-I7 receptor is highlighted in purple. The red arrowheads point to presumed OSNs that express the endogenous OR-I7 receptor. This experiment detects the mRNA of the receptor. Darker color indicates higher amount of OR-I7 mRNA.

1.2.4. Testing 1.2 and 1.3 on a UBI7 mouse

To our knowledge, the calcium imaging method of detecting OR-I7 OSN activation has never been tested on UBI7 mice, and therefore it was crucial to determine if the response of the expressed OR-I7 receptors could actually be detected by calcium imaging. Octanal, a natural product agonist of the OR-I7 receptor, was tested on the OSNs of the UBI7 mouse to check for the OR-I7 response. The EC$_{50}$ (concentration at which 50% of the maximal response is obtained) of octanal in OR-I7 receptor, expressed in rat neurons, is 1.8 µM, and by 10 µM octanal the receptor response is saturated.$^5$ However, we do not know if this value holds for the mouse OR-
I7 in the context of the UBI7 mouse, so we tested three concentrations, 3 µM, 10 µM, and 100 µM.

Moreover, OR-I7 is known not to be activated by the corresponding alcohol, 1-octanol. Thus, 1-octanol (at 100 µM) was also tested as a control. Activation by octanal and 1-octanol could indicate that the response is coming from an endogenous receptor that also happens to respond to octanal, and not from transgenic OR-I7. Out of the ~1100 endogenous rodent olfactory receptors, there are about 55 octanal receptors and in one experiment, 68% of receptors activated by octanal were also activated by 1-octanol, so there is a high percentage of octanal-responding cells that also respond to 1-octanol. We want to ignore these cells because they have a high chance of not responding solely through OR-I7, the receptor we intend to study.

Another consideration we must keep in mind in our calcium imaging experiments is that odorants that are applied to the cells earlier in the experiment tend to have slightly higher response than odorants applied later, and application of odorants at high concentrations could weaken or eliminate the response of odorants applied afterwards at lower concentrations, possibly due to desensitization of the receptor. (Desensitization occurs as a feedback mechanism to protect the cell from overstimulation.) To minimize the effects of desensitization, the odorants were applied at the lower concentrations first, and the sample of cells was divided into four dishes, with the odorants tested at different orders in each dish.

The solvent, DMSO, was applied to the cell as a negative control. Cells that respond to the solvent were not considered odorant-responding cells. Forskolin (10 µM), a molecule that generates a cellular response through bypassing the olfactory receptor to activate adenylyl cyclase in the signal transduction pathway, was applied at the end of the calcium imaging experiment to determine if the signal transduction pathway was still functioning at the end of the
experiment and estimate the cell’s maximum response range or full efficacy. (Efficacy describes the effect of a ligand on the functional properties of the receptor.) In the absence of a ligand, most receptors exhibit some basal (i.e. ligand-independent) activation of its G-protein. The response of a receptor could change depending on the ligand which binds to it. An inverse agonist inhibits the basal activity, whereas an antagonist does not change the basal activity but prevent other ligands from binding. A full agonist fully activates the receptor \textit{(in vitro, for example, to the strength of response similar to a saturating amount of forskolin)}, while a partial agonist only activates the receptor at partial efficacy even at saturating concentration (i.e. it reaches a plateau below the full response of forskolin.). Cells that did not respond to forskolin were excluded from the analysis (regardless of whether these cells responded to the tested compounds). The calcium imaging responses of the tested compounds were all normalized to the response of 10 µM forskolin and all cells that responded to forskolin were considered viable cells. Odorant responses that were lower than 10% of the response of forskolin were excluded.
1.3. Results and Discussion:

1.3.1. Comparison of the UBI7 mouse to wild-type (WT) mice

1.3.1.1. OR-I7 activation by octanal is detectable in UBI7 OSNs by calcium imaging

Transgenic OR-I7 expression in the UBI7 OSNs caused a ~8-fold increase in octanal-responding cells at 3 µM (WT-1.4%, UBI7-10.8%), a ~6-fold increase in octanal-responding cells at 10 µM (WT-2.2%, UBI7-13%), and a ~2.5-fold increase in octanal-responding cells at 100 µM (WT-5.8%, UBI7-14.6%) compared to wild-type OSNs (Figure 1.4A). UBI7 cells that express the OR-I7 receptor should respond to octanal, but so would some small percentage of cells via their endogenous receptors. The percentage of UBI7 cells expressing endogenous receptors that normally respond to octanal (in addition to the OR-I7 receptor) should be similar to, and could therefore be estimated by, the percentage of WT cells that respond to octanal,
which only express the endogenous receptors. The excess of UBI7 cells responding to octanal are therefore likely responding because they express the OR-I7 receptor at a sufficient expression level in addition to endogenous receptors that do not respond to octanal (at the given concentrations). Based on the difference in percentages between the WT cells and the UBI7 cells, we could estimate that 87% (10.8%-1.4%/10.8% × 100%) of the octanal-responding UBI7 cells at 3 µM, 83% (13%-2.2%/13% × 100%) at 10 µM, and 60% (14.6%-5.8%/14.6% × 100%) at 100 µM, owe their response to the expression of the OR-I7 receptor and an endogenous receptor that does not respond to octanal.

We obtained similar results when comparing the 1-octanol-positive and 1-octanol-negative responses in WT and UBI7 cells. As shown on Figure 1.4B, there was a ~6-fold increase in UBI7 cells that respond to octanal but not 1-octanol (WT: 1.6%, UBI7: 9.9%). From the difference in percentages, we could estimate that 84% (9.9%-1.6%/9.9% × 100%) of the octanal-responding cells that do not respond to 1-octanol owe their response to the expression of the OR-I7 receptor. There was a ~2.6-fold increase in UBI7 cells that respond to both 1-octanol and octanal (WT: 1.8%, UBI7: 4.7%), and a ~2.8-fold decrease in UBI7 cells that respond to 1-octanol but not octanal (WT: 1.4%, UBI7: 0.5%) compared to WT cells. The increase in cells that respond to both 1-octanol and octanal coupled with the decrease in cells that respond to 1-octanol (and not octanal) suggest that ~62% (4.7%-1.8%/4.7% × 100%) to ~64% (1.4%-0.5%/1.4% × 100%) of the UBI7 cells which express endogenous receptors that normally respond to 1-octanol but not octanal were also expressing OR-I7. (Note: For experiments on WT cells in which 1-octanol and octanal were both tested, the concentration used was 60 µM, whereas 100 µM was used for UBI7 cells. Figure 1.4B provides a crude comparison between the
WT and UBI7 cells, but there would probably be some deviation in the actual percentage differences if the same concentrations were compared.)

The percentage of UBI7 cells estimated to express the OR-I7 receptor and an endogenous receptor that do not respond to octanal (at the tested concentration) decreased as the concentration of octanal increased (87% at 3 µM, 83% at 10 µM, and 60% at 100 µM). As noted above, in rat neurons, the EC50 of octanal is about 1.8 µM and OR-I7 is saturated (reached maximum response) by 10 µM. Thus, the lowest concentration of octanal tested, 3 µM, may possibly already be sufficient to activate most of the mouse UBI7 cells that expressed an adequate level of the OR-I7 receptor. Assuming that the OR-I7 receptor was close to saturation at 3 µM, higher concentrations of octanal would only activate the same cells that were activated at 3 µM. But, as the concentration of octanal increased, various endogenous receptors (some broadly tuned perhaps) that do not respond to octanal at low concentration may start responding at the higher concentrations, and consequently the percentage of UBI7 cells estimated to respond to OR-I7 and an endogenous receptor that do not respond to octanal would drop as the concentration of octanal increased above the saturating concentration of 10 µM. Nevertheless, the increase in cells that respond to octanal for the UBI7 mouse indicates that OR-I7 was expressed at a sufficient level in at least ~60% (at 100 µM) to ~87% (at 3 µM) of the cells that respond to octanal, but the UBI7 cells’ responses at the lower concentration are probably more representative of the response of OR-I7.

1.3.1.2. Removal of 1-octanol-responding cells

UBI7 cells responding to octanal and 1-octanol are suspected to be responding to an endogenous octanal receptor that is not OR-I7 or to an endogenous receptor that responds to 1-octanol co-expressed with the OR-I7 receptor. (As OR-I7 does not respond to 1-octanol, the
response to 1-octanol unquestionably arise from the endogenous receptor.) For the UBI7 OSNs, a total of 96 cells responded to 1-octanol (@ 100 µM). Usually, over half (56% @ 60 µM (1.8%/[(1.4%+1.8%) × 100%, Figure 1.4B)) of the endogenous 1-octanol-responding cells are cells that respond to both 1-octanol and octanal. Thus, out of the 96 cells that responded to 1-octanol, we could assume that around half of them were expressing endogenous receptors that responded to both octanal and 1-octanol based on the data for WT cells. These UBI7 cells may also be expressing a detectable level of the OR-I7 receptor, but the response of octanal that was stimulated by the OR-I7 receptor would likely be overlapping with the response that was produced by the endogenous receptor (that could also respond to octanal in addition to 1-octanol). We decided to remove these cells from the analysis because the response of the endogenous receptor interferes with the OR-I7 response.

All of the 1-octanol-responding UBI7 cells were excluded from the analysis to enrich for cells responding solely through OR-I7 (96 cells excluded, 213 cells remaining). In the process, we also removed the other half of the 96 1-octanol-responding cells that express endogenous receptors responding only to 1-octanol in addition to a sufficient level of the OR-I7 receptor. (Note: There were 9 cells that express endogenous receptors responding only to 1-octanol, but the expression of OR-I7 was evidently not sufficient to stimulate a calcium imaging response. These cells were removed from the analysis.) While the octanal response of the co-expressed OR-I7 receptor would not be affected in these cells, the UBI7 cells that express these endogenous receptors were still seen as cells that respond to both 1-octanol and octanal (same as the cells that express endogenous receptors that respond to both 1-octanol and octanal). We could not distinguish the response of the endogenous receptor and the response of OR-I7 for UBI7 cells that responded to both 1-octanol and octanal. By removing all of the 1-octanol-
responding UBI7 cells, we are in effect removing half of all the octanal responses that are produced by endogenous receptors (overlapped with the octanal response produced by detectable levels of OR-I7) and increasing the signal from OR-I7. Thus, only the forskolin-responding cells that do not respond to 1-octanol were used to analyze the response of the cis- (1.2) and trans-isomer (1.3). The number of cells that responded to each odorant at 3 µM, 10 µM, and 100 µM for each dish of cells tested are given in Figure 1.5. The calcium imaging response of a representative cell that responded to forskolin but not 1-octanol is shown on Figure 1.5A. This cell responded to octanal and the trans-isomer (1.3) but not to the cis-isomer (1.2) at 3 µM, 10 µM, and 100 µM.

1.3.2. Activation of OR-I7 by octanal, 1.2, and 1.3

1.3.2.1. Trans-isomer is preferred by OR-I7 according to the number of cells activated

The trans-isomer (1.3) had a similar amount of responding UBI7 cells compared to octanal at all three concentrations for Dish 1 and 3 (Figure 1.5). The trans-isomer (1.3) gave slightly more responding cells than octanal in Dish 2 and 4, but in these two dishes the trans-isomer (1.3) was applied before octanal, and since there was a chance that the odorant applied later would give a weaker or no response due to desensitization, the difference may not be significant.

1.3.2.2. Cis-isomer has weaker response than trans-isomer and octanal

For all four dishes, the cis-isomer (1.2) activated fewer UBI7 cells compared to both the trans-isomer (1.3) and octanal at all three concentrations regardless of the order in which it was applied. We concluded that the cis-isomer (1.2) is a less potent OR-I7 agonist compared to octanal and the trans-isomer (1.3). Each cell may be expressing the OR-I7 receptor at different
levels, so it was not possible to obtain the EC$_{50}$ value for each odorant, but apparently fewer cells responded to the cis-isomer (1.2) than the trans-isomer (1.3) and octanal (Figure 1.5).

**Figure 1.5:** Response of cells to octanal, 1.2 (cis-isomer), and 1.3 (trans-isomer). (A) The calcium imaging response of a representative cell that responded to forskolin, octanal and the trans-isomer (1.3), but not the cis-isomer (1.2) nor 1-octanol. (B) Graphs representing the response of all the UBI7 cells that do not respond to 1-octanol (the total number of cells analyzed is given in the title of each graph). The odorants are shown in the order in which they were applied in the calcium imaging experiment.
1.3.3. Response strength of octanal, 1.2, and 1.3

1.3.3.1. *Trans*-isomer is preferred by OR-I7 according to response strength (agonist efficacy)

![Figure 1.6](image.png)

**Figure 1.6**: Percentage of cells responding to different odorant(s) types.

***All cells that responded to 1-octanol (96 cells) were excluded in this analysis. The number of cells responding to the odorant(s) in each category at 3, 10 and 100 µM is given in parentheses.

All of the UBI7 cells were assumed to express the OR-I7 receptor, but the expression level in some cells (at least 9%, as evident from the cells that respond to only 1-octanol (9/(9+87)) ×100%, Figure 1.4B) may not be high enough to be detected by calcium imaging. For such cells, only the response of the endogenous receptor would be detected. From comparison of the octanal responses for the WT cells and the UBI7 cells, we were also aware that ~13% of the UBI7 cells at 3 µM (1.4%/10.8% × 100%, Figure 1.4A), ~17% at 10 µM (2.2%/13% × 100%, Figure 1.4A), and ~40% at 100 µM (5.8%/14.6% × 100%, Figure 1.4A) may have responses
stimulated by the endogenous receptors. When we categorized the responses of the UBI7 cells at 3 µM, 10 µM, and 100 µM, a minority of cell types were activated as the concentration increased from 3 µM to 100 µM (Figure 1.6), which we believe to be the response of the endogenous receptor only. Since the OR-I7 receptor was expressed in a majority of the UBI7 cells (~60-87%, based on comparison of WT and UBI7 cells, Figure 1.4A), the responses from the categories of cells that made up less than 10% of the total cell population (i.e. the cells that respond to 1) the cis-isomer (1.2) and octanal only, 2) the cis-isomer (1.2) and the trans-isomer (1.3) only, 3) the cis-isomer (1.2) only, and 4) octanal only) were most likely not the response of OR-I7. However, the response of all these cells was included in the analysis on Figure 1.5.

To reduce the chance of including the response of endogenous receptors in the analysis, only the cells in the most common categories, the cells that respond to 1) octanal, the cis-isomer 1.2, and the trans-isomer 1.3 (47.40%-58.22%, Figure 1.6), 2) the trans-isomer 1.3 and octanal (17.84%-34.10%, Figure 1.6), and 3) the trans-isomer 1.3 only (8.92%-16.76%, Figure 1.6) were now considered. Cells that respond only to the trans-isomer (1.3) and octanal (17.84%-34.10%) apparently indicate that the trans-isomer (1.3) and octanal are both more potent OR-I7 agonists than the cis-isomer (1.2), and cells that respond only to the trans-isomer (1.3) (8.92%-16.76%) suggest that the trans-isomer (1.3) is more potent than both the cis-isomer (1.2) and octanal. (Note: The trans-isomer (1.3) was applied before octanal in 3 out of the 4 dishes of cells tested, and that may cause the octanal response to be desensitized.) The only category in which the relative potency of the three odorants (in relation to each other) remains ambiguous is the cells that respond to the cis-isomer (1.2), the trans-isomer (1.3), and octanal (47.40%-58.22%). To compare the response strength of the cis-isomer (1.2), the trans-isomer (1.3) and octanal, we first looked at the overall average response levels (the response levels, normalized to the response of
forskolin, for these cells were averaged; forskolin is considered to activate the cell at 100%), and then at the responses of the individual cells in this category.

![Graphs showing response levels of UBI7 cells to octanal, cis- and trans- isomers.](image)

**Figure 1.7**: Response level of cells (124 total cells by 100 µM) that respond to octanal, the *cis*-isomer 1.2, and the *trans*-isomer 1.3. The graphs show the average response for all the cells in each dish (total cells on the title of the graph) that respond to all three odorants. (Average response were normalized to the response of forskolin calculated by ‘response of odorant’ / ‘response of forskolin’ × 100%. The calcium imaging response is the ratio of the fluorescence emission at 340 nm and 380 nm, F340/F380.)

Although these UBI7 cells (82 cells @ 3 µM, 104 cells @ 10 µM, and 124 cells @ 100 µM) responded to all three odorants, the average response (agonist efficacy) of the *cis*-isomer (1.2) is on average much lower (~2X lower) than the response of the *trans*-isomer (1.3) and octanal for all four dishes regardless of the order of application (Figure 1.7). This evidence further supports the idea that the OR-I7 receptor prefers the *trans*-isomer (1.3) over the *cis*-isomer (1.2) of 4-ethylcyclohexylacetaldehyde (1.1) and hence prefers the specific octanal conformation between C6-C7 that is mimicked by the *trans*-isomer (1.3) over the conformation mimicked by the *cis*-isomer (1.2).
1.3.3.2. The cis-isomer is a partial agonist of OR-I7

As an agonist of OR-I7, octanal should be able to fully activate the OR-I7 receptor, but since the expression level of OR-I7 varied from cell to cell (coupled with the possibility of desensitization), octanal may not reach the maximum response level of forskolin in every cell. However, the average response of the cis-isomer (1.2) was clearly lower than that of the trans-isomer (1.3) and octanal (regardless of the order in which it was applied) even at the highest concentration, 100 µM (Figure 1.7). Out of the cells that responded to all three odorants (the cis-isomer (1.2), the trans-isomer (1.3), and octanal), the cis-isomer (1.2) had the lowest response in 51 out of 82 cells at 3 µM (62.2%, Figure 1.8A, cell #1-51), 73 out of 104 cells at 10 µM (70.2%, Figure 1.8B, cell #1-73), and 75 out of 124 cells at 100 µM (60.5%, Figure 1.8C, cell #1-75). There exist a minority of cells in which the cis-isomer (1.2) has a 1) similar response level to the trans-isomer (1.3) and octanal (14 cells @ 3 µM (17.1%), Figure 1.8A, cell #52-65; 13 cells @ 10 µM (12.5%), Figure 1.8B, cell #74-86; 13 cells @ 100 µM (10.5%), Figure 1.8C, cell #100-112), 2) similar response level as either the trans-isomer (1.3) or octanal (17 cells @ 3 µM (20.7%), Figure 1.8A, cell #74-82; 17 cells @ 10 µM (16.3%), Figure 1.8B, cell #87-103; 32 cells @ 100 µM (26%), Figure 1.8C, cell #76-99, 113-120), or 3) higher response than both the trans-isomer (1.3) and octanal (0 cell @ 3 µM; 1 cell @ 10 µM (1%), Figure 1.8B, cell #104; 4 cells @ 100 µM (3%), Figure 1.8C, cell #121-124). However, we point out that any trend involving such small minority of cells is likely due not to OR-I7 but to an endogenous receptor, which will vary from cell to cell while OR-I7 remains constant. That is, a few endogenous receptors may be present that are selective for the cis-isomer (1.2) (and/or the trans-isomer (1.3) and/or octanal). Since the OR-I7 receptor was expressed in a majority of the UBI7 cells (at least 60% of the octanal-responding cells at 100 µM and up to ~87% at 3 µM), we attributed the
response of the most general group, the cells in which the *cis*-isomer (1.2) had the lowest response (which constitute 60.5%-70.2% of the total population of cells that respond to 1.2, 1.3, and octanal between 3 µM and 100 µM), to be the response of OR-I7. As the response of the *cis*-isomer (1.2) was systematically lower than that of octanal and the *trans*-isomer (1.3), we conclude that the *cis*-isomer (1.2) is a partial agonist of OR-I7: it activates the receptor at partial efficacy, but even at high concentration fails to achieve the maximum response. As observed for the calcium imaging response of cell C66 (Figure 1.8D), the *cis*-isomer (1.2) was unable to activate the cell at the same efficacy as the *trans*-isomer (1.3) and octanal even as its concentration increased.
Figure 1.8: Calcium imaging response of cells that respond to the cis-isomer (1.2), the trans-isomer (1.3) and octanal. (A) Relative response of the 82 cells to the cis-isomer (1.2), the trans-isomer (1.3), and octanal at 3 µM. (B) Relative response of the 104 cells to the cis-isomer (1.2), the trans-isomer (1.3), and octanal at 10 µM. (C) Relative response of the 124 cells to the cis-isomer (1.2), the trans-isomer (1.3), and octanal at 100 µM. Each row represents the relative response (normalized to the response of forskolin calculated by "response of odorant"/ "response of forskolin" × 100%) of one cell to the odorants. The cell was tested in the dish, denoted by D and the dish number, designated on the column to the right of the odorants’ responses. (The odorants’ responses were arranged in the same order for clarity, but the order of odorant application was different for each dish (Refer to Figure 1.5 or 1.7 for the order of application in each dish.), and the order in which the odorants were applied may affect the response.
level.) The response of the corresponding cell at each concentration can be identified by the cell ID number given in parentheses. Cells that do not respond to all three odorants (1.2, 1.3, and octanal) at the lower concentrations were not included in Panel A and B. (D) The calcium imaging response of a representative cell (C66) displaying partial activation by the cis-isomer (1.2).

(Note: Responses lower than 10% of the response of forskolin were usually not considered activating, but for cell C74 and C75, the cis-isomer (1.2) did elicit a response higher than 10% at the lower concentrations, but the signal probably was desensitized to be under 10% by 100 µM. For these two cells, there was a cis-isomer (1.2) response at 100 µM, but the response level was lower than 10%. Therefore, these cells were categorized as cells that respond to octanal, the cis-isomer (1.2), and the trans-isomer (1.3), instead of as cells that respond only to octanal and the trans-isomer (1.3).)

The cis- (1.2) and trans-isomers (1.3) were also tested using the luciferase assay on HEK293 cells transfected with the OR-I7 receptor gene. The relative ranking of potency (data not shown) was in agreement with our data (although the receptor did not reach saturation at the concentrations tested). The cis-isomer (1.2) was found to have a weaker response than the trans-isomer (1.3).

Lastly, we note that in a majority of the cells, the response of the trans-isomer (1.3) is the same as if not stronger than the response of octanal. As previously mentioned, one of the most common group of cells in this experiment are the cells that respond only to the trans-isomer (1.3) (Figure 1.6). Response to solely the trans-isomer (1.3) signifies that the trans-isomer (1.3) is more potent than both octanal and the cis-isomer (1.2) in these cells. From Figure 1.8A, it could already be seen that not only is the response of the trans-isomer (1.3) higher than that of the cis-isomer (1.2), but it is also higher than the response of octanal in a majority of the cells. Comparing the response level of the trans-isomer (1.3) with that of octanal in the second most common cell category (the cells that respond to the trans-isomer (1.3) and octanal, but not the cis-isomer (1.2)), the trans-isomer (1.3) gave a higher response than octanal in 71% (42 out of 59 cells) of the cells at 3 µM, 58% (28 out of 48 cells) of the cells at 10 µM, and 42% of the cells (16 out of 38 cells) at 100 µM (Figure 1.9). Only in a low percentage of the cells (3% @ 3 µM (1 cell); 10% @ 10 µM (5 cells); 18% @ 100 µM (7 cells)) were the response of octanal higher
Figure 1.9: Calcium imaging response of cells that respond to the trans-isomer (1,3) and octanal. (A) Relative response of the 59 cells to the trans-isomer (1,3) and octanal at 3 μM. (B) Relative response of the 48 cells to the trans-isomer (1,3) and octanal at 10 μM. (C) Relative response of the 38 cells to the trans-isomer (1,3) and octanal at 100 μM. Each row represents the relative response (normalized to the response of forskolin calculated by ‘response of odorant’/ ‘response of forskolin’ × 100%) of one cell to the odorants. The cell was tested in the dish, denoted by D and the dish number, designated on the column to the right of the odorants’ responses. (The odorants’ responses were arranged in the same order for clarity, but the order of odorant application was different for each dish (Refer to Figure 1.5 or 1.7 for the order of application in each dish.), and the order in which the odorants were applied may affect the response level.)

(Note: Responses lower than 10% of the response of forskolin were usually not considered activating, but for one cell (row 31) on Panel C (@ 100 μM), octanal did elicit a response higher than 10% at the lower concentrations, but the signal probably was desensitized to be under 10% by 100 μM. For this cell, there was an octanal response at 100 μM, but the response level was lower than 10%. Therefore, this cell was categorized as a cell that responds to octanal and the trans-isomer (1,3), instead of a cell that respond only to the trans-isomer (1,3).)

than that of the trans-isomer (1,3). (The rest of the cells (25-39% from 3-100 μM) gave similar responses to the trans-isomer (1,3) and octanal (Figure 1.7).) These data support the conclusion that the trans-isomer (1,3) is more potent than octanal and the geometric relationship between the aldehyde group and the last two carbons on the chain of octanal is favorably mimicked by the trans-isomer (1,3) without the loss of entropy during binding. In other words, the conformation of the trans-isomer (1,3) may be favorably pre-organized, and resemble the bound conformation of octanal in complex with rodent OR-I7.
1.3.4. A molecular explanation for our data: The OR-I7 receptor has carbon chain conformational sensing ability

Besides having a shorter chain length when in the chair conformation (cis: 6.9 Å, trans: 7.8 Å) than the trans-isomer (1.3), the ethyl group on the cis-isomer (1.2) is also at a different position from the ethyl group on the trans-isomer (1.3) in relation to the aldehyde group (Figure 1.10, Figure 1.11A). The trans-isomer (1.3) has higher potency and efficacy than the cis-isomer (1.2), so the position of the ethyl group (i.e. the orientation of the C6-C7 bond) on the trans-isomer (1.3) should be more favorable for activation of the OR-I7 receptor.

![Figure 1.10](image1.png)

**Figure 1.10:** Conformations of 1.2 and 1.3. (A) Line structures and lengths of 1.2 and 1.3 in the chair and twist-boat conformation (the distance from the carbonyl carbon to the carbon on the ethyl group, indicated by the red dashed line, was calculated on Avogadro2). (B) 3D Ball and stick model and the minimized energy for 1.2 and 1.3 in the chair and twist-boat conformation. Energy minimization/optimization was done on Avogadro2 using a MMFF94 force field with the cyclohexyl ring fixed in the chair or twist-boat conformation.

However, we noticed that if the cis-isomer (1.2) flips into the higher energy twist-boat conformation, the length of the cis-isomer (1.2) (7.5 Å) comes close to the length of the trans-isomer (1.3) (7.8 Å), and the ethyl group becomes oriented in a similar position as the ethyl group of the trans-isomer (1.3) (in the chair conformation) (Figure 1.10A and Figure 1.11B).
Our data support the idea that the cis-isomer (1.2) activates the OR-I7 receptor when in the twist-boat conformation, but the energy cost required to switch from the chair conformation (46.0 kJ/mol, calculated in Avogadro\textsuperscript{21}) to the twist-boat conformation (68.5 kJ/mol, calculated in Avogadro\textsuperscript{21}) lowers the potency of the cis-isomer (1.2) and prevents it from achieving full efficacy, hence it is a partial agonist of OR-I7.

![Overlapping 3D models. (A) 3D model of the cis-isomer 1.2 in the chair conformation (carbons: yellow; oxygen: blue) overlapping with the trans-isomer 1.3 in the chair conformation (carbons: gray; oxygen: red). (B) 3D model of the cis-isomer 1.2 in the twist-boat conformation (carbons: yellow; oxygen: blue) overlapping with the trans-isomer 1.3 in the chair conformation (carbons: gray; oxygen: red).](image)

**Figure 1.11:** Overlapping 3D models. (A) 3D model of the cis-isomer 1.2 in the chair conformation (carbons: yellow; oxygen: blue) overlapping with the trans-isomer 1.3 in the chair conformation (carbons: gray; oxygen: red). (B) 3D model of the cis-isomer 1.2 in the twist-boat conformation (carbons: yellow; oxygen: blue) overlapping with the trans-isomer 1.3 in the chair conformation (carbons: gray; oxygen: red).

***Molecular modeling and energy minimization/optimization were done on Avogadro\textsuperscript{21} using a MMFF94 force field with the cyclohexyl ring of the cis-isomer 1.2 fixed in the twist-boat conformation and the cyclohexyl ring of the trans-isomer 1.3 fixed in the chair conformation.

**1.4. Conclusions:**

4-Ethylcyclohexylacetaldehyde (1.1) (EC\textsubscript{50}=1.0 µM, as a cis-trans mixture) was previously found to have approximately equal potency compared to octanal (EC\textsubscript{50}=1.8 µM), suggesting that the OR-I7 receptor tolerates the gauche conformation of octanal during activation.\textsuperscript{5} The cis- (1.2) and trans- (1.3) isomers of 1.1 mimicked two of the possible conformations that could be adopted by octanal while in the gauche conformation. Analog 1.2 and 1.3 have been synthesized and tested on the olfactory sensory neurons of a UBI7 mouse that express the OR-I7 receptor in addition to the endogenous
receptors through calcium imaging. Based on the increase in the percentage of cells that respond to octanal, we concluded that most of the UBI7 cells (~60% at 100 µM to ~87% at 3 µM) expressed a functional level of the OR-I7 receptor. The potency of the trans-isomer (1.3) is comparable to (but consistently higher than) that of octanal, whereas the cis-isomer (1.2) was found to be a less potent OR-I7 agonist than the trans-isomer (1.3) and octanal. Thus, the conformation of the C6-C7 bond of the trans-isomer (1.3) is a better mimic of octanal’s conformation during activation of the OR-I7 receptor. The cis-isomer (1.2) could orient its ethyl group into a similar position as the trans-isomer (1.3) after converting from the chair to the twist-boat conformation, but the energy cost for adopting this conformation probably lowers the efficacy of the cis-isomer (1.2), making it a partial agonist of the OR-I7 receptor.

Since octanal may adopt hundreds of different conformations, its most stable (lowest energy) conformation, may not be its actual activating conformation, and thus there may be an entropy penalty when octanal binds OR-I7 in a preferred conformation, and this penalty may in part explain octanal’s relatively weak potency (EC50: 1.8 µM). If octanal’s structure could be constrained into its activating conformation, the efficacy would most likely rise as the entropy loss is reduced, leading to a more favorable ΔG and a more potent OR-I7 agonist. It’s not possible to test all of the conformations of octanal to find its optimal activating conformation, but the cis- (1.2) and trans-isomer (1.3) represent two of the conformations that could be adopted by octanal, and from this study, we can conclude that the conformation of octanal mimicked by the trans-isomer (1.3) is more preferable compared to that of the cis-isomer (1.2). While the trans-isomer (1.3) may not be the actual conformation of octanal when activating OR-I7, it is at least a conformation that is acceptable by the OR-I7 receptor and favorable (i.e. a closer to the actual bound, activating conformation).
The difference in potency between the cis-isomer (1.2) and the trans-isomer (1.3) indicates that the OR-I7 receptor has a preference for the conformation of its ligand during activation. As with the OR-I7 receptor, other ORs may exist with preferences for their ligands’ conformations. These results are significant because there is no structural biology information enlightening how the mammalian odorant receptors bind their ligands. Many odorants have flexible carbon chains, while protein-small molecule crystal structures, including the many GPCR X-ray structures now available, teach us that attractions like H-bonds fix small molecule conformations upon binding. But how can this apply to odorants, which in order to become volatile must have few or no H-bonds available for binding? Our results provide experimental data supporting the idea that even a flexible hydrophobic carbon chain can adopt a preferred conformation upon binding and activating an odorant receptor. It is possible that many other odorant receptors function as conformational sensors of odorant carbon chain, and this allows them to contribute to the olfactory code and through this, natural selection during evolution.

Many natural fragrances, such as the santalols, ionones, and the patchoulenes contain elaborate carbon ring systems and/or double bonds that place a degree of restriction on the conformation of the odorant. Such odorants may be conformationally restricted versions of linear compounds that activate the receptors with lower potency and efficacy.

1.5. Experimental Procedures

1.5.1. Materials and reagents

Unless otherwise stated, chemical reagents and solvents were purchased from VWR International, Fisher Scientific, or Sigma Aldrich and used without further purification. UBI7 mouse\textsuperscript{11, 12} was provided by Dr. Leonardo Belluscio (NIH/NINDS). Octanal was distilled before use. Aldehydes were stored at 4°C under vacuum prior to testing. Analytical TLC was performed
on silica gel 60 F_{254} plates. Flash chromatography\textsuperscript{22} was performed on Teledyne Isco
CombiFlash Rf-200 flash chromatography system or manually in glass columns with 230-400
mesh silica gel. \textsuperscript{1}H and \textsuperscript{13}C NMR spectra were recorded on a Varian Mercury 300 spectrometer
or a Bruker Ultrashield 500 spectrometer. HRMS was performed by Dr. Lijia Yang (CCNY
Chemistry Department staff) on a Waters LCT XE (TOF) mass spectrometer using electrospray
ionization (ESI). Infrared (IR) spectra were recorded using Thermo Nicolet 6700 FT-IR
spectrometer. GC analyses were performed on a Shimadzu GC-2010 gas chromatograph with an
FID detector using a 15 m × 0.25 mm capillary column with dimethyl polysiloxane as the
stationary phase. Compound 1.2 and 1.3 were first synthesized and characterized by Yadi Li\textsuperscript{8}
with helpful advice from Dr. Mark Biscoe on the synthetic routes to the two isomers. Compound
1.2 was synthesized for this study following his procedures, and compound 1.3 was synthesized
from 1.3d, provided by Yadi Li. (Note: The literature procedure that was applied for each
reaction is cited. The procedure may not necessarily be used for the synthesis of the same
compound as in the literature.)

**General Procedure for Lithium Aluminum Hydride (LAH) reduction\textsuperscript{5}**

The acid or ester (1 equiv.) in diethyl ether was added slowly to lithium aluminum hydride (1.1
equiv.) in diethyl ether at 0°C. The suspension was stirred at room temperature for 2-3 h and then
cooled to 0°C. Water (1 mL per g of LAH) was added, followed by 15% sodium hydroxide (1
mL per g of LAH) and water (3 mL per g of LAH). The solution was stirred for a few minutes at
room temperature, filtered through a celite pad, washed with diethyl ether, dried, and
concentrated to give the alcohol. Flash chromatography was sometimes required to purify the
product.
General Procedure for Pyridinium chlorochromate (PCC) oxidation

The alcohol (1 equiv.) was added to pyridinium chlorochromate (1.1 equiv.) and silica gel (1 g/g of PCC) in dry dichloromethane under inert atmosphere. The suspension was stirred for 2 h and then passed through a silica gel pad. The solution was concentrated to give the aldehyde. Flash chromatography or distillation was sometimes required to purify the product.

Ethyl 2-(4-ethylcyclohexylidene)acetate (1.2a). Compound 1.2a was synthesized following a literature procedure. Ethyl 2-(diethoxyphosphoryl)acetate (8.88 g, 39.6 mmol) in THF (15 mL) was slowly added to sodium hydride, 57-63% in oil, (1.6 g, 40 mmol) in THF (50 mL). The mixture was stirred at room temperature for 1 hour and 4-ethylcyclohexanone (5 g, 39.6 mmol) in THF (10 mL) was slowly added so that the temperature of the mixture was below 30°C. The solution was stirred for 15 minutes and then quenched with water. The aqueous layer was extracted with diethyl ether. The organic layer was dried, concentrated, and purified by flash chromatography eluting with hexanes/ethyl acetate (19:1) to give 1.2a (2.7 g, 13.8 mmol) as a clear liquid in 35% yield.  

\[
\begin{align*}
\text{O} & \quad \text{OEt} \\
\end{align*}
\]

\[\text{H NMR (CDCl}_3\text{) } \delta: \text{ 5.62 (s, 1H), 4.16 (q, } J = 7.2 \text{ Hz, 2H), 3.82 - 3.70 (m, 1H), 2.35 - 2.11 (m, 2H), 2.03 - 1.86 (m, 3H), 1.50 - 1.21 (m, 6H), 1.18 - 1.01 (m, 2H), 0.99 - 0.84 (m, 3H).} \]

\[\text{C NMR (CDCl}_3\text{) } \delta: \text{ 166.87, 163.56, 113.00, 59.46, 38.93, 37.31, 34.18, 33.46, 29.00, 28.95, 14.33, 11.60.} \]

IR (thin film, KBr plates), ν (cm\(^{-1}\)): 2932, 2855, 1715, 1649, 1380, 1235, 1186, 1150. Spectral data agree with those reported previously.

Ethyl 2-(cis-4-ethylcyclohexyl)acetate (1.2b). Compound 1.2a (2 g, 10.2 mmol) in THF (10 mL) was slowly added to 1 M L-selectride\(^6\)\(^7\) in THF (30.5 mL, 30.5 mmol) so that the temperature of the mixture was below -70°C. The mixture was stirred at -75°C for 1 hour and at -40°C for 4 hours. Ethanol (4.8 mL) was added
followed by ethyl acetate (14.4 mL), pH=7 phosphate buffer (0.1 M, 6.4 mL), and 30% hydrogen peroxide solution (8.8 mL). The mixture was stirred overnight. The aqueous and organic layers were partitioned and the aqueous layer was extracted with ethyl acetate. The combined organic layer was extracted with brine, dried, concentrated and purified by chromatography eluting with hexanes/ethyl acetate (49:1) to give 1.2b (0.2 g, 1 mmol) as a clear liquid in 10% yield. $^1$H NMR (CDCl$_3$) $\delta$: 4.20 - 4.09 (m, 2H), 2.29 (d, $J = 7.5$ Hz, 2H), 2.05 (m, 1H), 1.60 - 1.20 (m, 14H), 0.98 - 0.81 (m, 3H). $^{13}$C NMR (CDCl$_3$) $\delta$: 173.45, 60.08, 39.30, 37.03, 32.54, 28.76, 28.24, 26.96, 14.30, 11.84. IR (thin film, KBr plates), $\nu$ (cm$^{-1}$): 2960, 2921, 2855, 1736, 1451, 1297, 1219, 1171, 1123, 1035. HRMS (ESI) [M+Na]$^+$: Calcd for C$_{12}$H$_{22}$O$_2$ m/z = 221.1512, found m/z = 221.1511.

2-(cis-4-Ethylcyclohexyl)ethanol (1.2c). Compound 1.2b (0.2 g, 1 mmol) was LAH reduced according to the general procedure to obtain 1.2c (0.085 g, 0.54 mmol) as a clear liquid in 54% yield. $^1$H NMR (CDCl$_3$) $\delta$: 3.75 - 3.66 (m, 2H), 1.69 - 1.42 (m, 7H), 1.42 - 1.17 (m, 8H), 1.02 - 0.83 (m, 3H). $^{13}$C NMR (CDCl$_3$) $\delta$: 61.39, 37.25, 37.15, 31.76, 28.97, 28.42, 27.01, 11.88. IR (thin film, KBr plates), $\nu$ (cm$^{-1}$): 3331 (broad), 2959, 2919, 2873, 2853, 1450, 1378, 1062, 1011, 978. HRMS (ESI) [M+Na]$^+$: Calcd for C$_{10}$H$_{20}$O m/z = 179.1406, found m/z = 179.1432.

2-(cis-4-Ethylcyclohexyl)acetaldehyde (1.2). Compound 1.2c (0.04 g, 0.26 mmol) was PCC oxidized according to the general procedure to obtain 1.2 (0.03 g, 0.19 mmol) as a clear liquid in 76% yield. The product contains 95.6% of the cis-isomer 1.2 and 4.4% of the trans-isomer 1.3 according to GC analysis. $^1$H NMR (CDCl$_3$) $\delta$: 9.78 (t, $J = 2.4$ Hz, 1H), 2.41 (dd, $J = 2.4$, 7.2 Hz, 2H), 2.24 - 2.12 (m, 1H), 1.63 - 1.48 (m, 4H), 1.46 - 1.21 (m, 7H), 0.94 - 0.84 (m, 3H). $^{13}$C NMR (CDCl$_3$) $\delta$: 203.16, 48.38, 36.96, 30.08, 28.97, 28.20, 27.03,
11.81. IR (thin film, KBr plates), ν (cm⁻¹): 2960, 2921, 2855, 2711, 1726, 1451, 1378. HRMS (ESI) [M+Na]+: Calcd for C₁₀H₁₈O m/z = 177.1250, found m/z = 177.1251.

(trans-4-Ethylcyclohexyl)methanol (1.3a). (trans-4-Ethylcyclohexyl)carboxylic acid (5.7 g, 36 mmol) (purchased from OChem Incorporation) was LAH reduced according to the general procedure to obtain 1.3a (5 g, 35 mmol) as a clear liquid in 97% yield. ¹H NMR (CDCl₃) δ: ¹H NMR (CDCl₃) δ: 3.47 (d, J = 6.2 Hz, 2H), 1.90 - 1.71 (m, 4H), 1.54 - 1.36 (m, 1H), 1.29 - 0.83 (m, 11H). ¹³C NMR (CDCl₃) δ: 68.83, 40.70, 39.59, 32.26, 29.96, 29.49, 11.51. IR (thin film, KBr plates), ν (cm⁻¹): 3330 (broad), 2960, 2915, 2852, 1448, 1379, 1100, 1072, 1036, 1003, 964, 896.

(trans-4-Ethylcyclohexyl)carbaldehyde (1.3b). Compound 1.3a (3 g, 21 mmol) was PCC oxidized according to the general procedure to obtain 1.3b (2.7 g, 19 mmol) as a clear liquid in 91% yield. The aldehyde is prone to oxidation in air so it was used immediately for the next step without further purification.

trans-1-Ethyl-4-vinylcyclohexane (1.3c). Compound 1.3c was synthesized based on a literature procedure.⁵ To an ice-cooled mixture of methyl triphenylphosphonium bromide (10.33 g, 29 mmol) in dry THF (75 mL) was added 1.6 M n-BuLi solution (16.9 mL, 27 mmol). The mixture was stirred at 0°C for 30 minutes, and then cooled to -75°C. Compound 1.3b (2.7 g, 19 mmol) in THF (25 mL) was added dropwise. The mixture was stirred at -75°C for 3 hours. Ammonium chloride solution was added and the layers were separated. The organic layer was extracted with water, brine, and dried. The crude was purified by chromatography eluting with pentane to give 1.3c (2.5 g, 18 mmol) as a clear liquid in 95% yield. ¹H NMR (CDCl₃) δ: 5.78 (ddd, J = 6.6, 10.5, 17.2 Hz, 1H), 5.05 - 4.77 (m, 2H), 2.05 -
1.65 (m, 3H), 1.39 - 1.01 (m, 6H), 1.01 - 0.70 (m, 6H). $^{13}$C NMR (CDCl$_3$) $\delta$: 145.09, 111.79, 42.15, 39.31, 32.73, 31.84, 30.23, 22.91, 14.39, 11.76.

2-(trans-4-Ethylcyclohexyl)ethanol (1.3d). Compound 1.3d was synthesized based on a literature procedure.$^5$ 1 M Borane tetrahydrofuran complex solution in THF (9 mL, 9 mmol) was added dropwise to a solution of 1.3c (2.5 g, 18 mmol) in THF (50 mL). After 15 minutes, saturated sodium carbonate solution (6.2 mL) and 30% hydrogen peroxide solution (3.68 mL, 36 mmol) were added. The mixture was stirred for 2 hours. Water (50 mL) was added and the layers were separated. The aqueous layer was extracted with diethyl ether. The combined organic layers were washed with brine, dried, and concentrated. The crude product was purified by chromatography eluting with acetonitrile/dichloromethane/cyclohexane (1:9:10) to give 1.3d (1.2 g, 7.8 mmol) as a clear liquid in 43% yield.$^1$ H NMR (CDCl$_3$) $\delta$: 3.70 (t, $J = 6.8$ Hz, 2H), 1.86 - 1.65 (m, 4H), 1.57 - 1.44 (m, 3H), 1.41 - 1.29 (m, 1H), 1.27 - 0.81 (m, 10H). $^{13}$C NMR (CDCl$_3$) $\delta$: 60.99, 40.36, 39.49, 34.46, 33.30, 32.77, 30.00, 11.51. IR (thin film, KBr plates), $\nu$ (cm$^{-1}$): 3323 (broad), 2960, 2916, 2851, 1448, 1378, 1046. HRMS (ESI) [M+Na]$^+$: Calcd for C$_{10}$H$_{20}$O $m/z = 179.1406$, found $m/z = 179.1431$.

2-(trans-4-Ethylcyclohexyl)acetaldehyde (1.3). Compound 1.3d (0.3 g, 1.92 mmol) was PCC oxidized according to the general procedure to give 1.3 (0.17 g, 1.1 mmol) as a clear liquid in 57% yield.$^1$ H NMR (CDCl$_3$) $\delta$: 9.78 (t, $J = 2.4$ Hz, 1H), 2.31 (dd, $J = 2.3$, 6.7 Hz, 2H), 1.93 - 1.72 (m, 5H), 1.29 - 1.18 (m, 2H), 1.16 - 0.85 (m, 8H). $^{13}$C NMR (CDCl$_3$) $\delta$: 203.07, 51.37, 39.04, 33.17, 32.95, 32.52, 29.87, 11.46. IR (thin film, KBr plates), $\nu$ (cm$^{-1}$): 2960, 2919, 2852, 2711, 1726, 1448, 1378. HRMS (ESI) [M+Na]$^+$: Calcd for C$_{10}$H$_{18}$O $m/z = 177.1250$, found $m/z = 177.1255$. 

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1.5.2. Calcium imaging of mouse olfactory sensory neuron

Calcium imaging was performed as described previously. All animal procedures were approved by the City College of New York Institutional Animal Care and Use Committee (IACUC) and performed at the City College of New York in compliance with relevant national guidelines and regulations. Olfactory epithelium tissues were obtained from a 16 week old UBI7 mouse by my colleague, Mihwa Na. The tissues were placed into a conical tube containing an enzyme solution made with BSA (0.015 g), collagenase (0.0025 g), and 3 mL of DICAT-free Ringer solution containing sodium chloride (145 mM), potassium chloride (5.6 mM), HEPES at pH 7.4 (10 mM), glucose (10 mM), and EGTA (4 mM). The cells were incubated at 37°C for 1 h in the enzyme solution. The tissues were then allowed to settle to the bottle of the conical tube and the enzyme solution was replaced with a media containing DMEM/F12-HAM, 10% FBS (Gibco), 1% Pen/Strep (Gibco), and 1X Insulin-Transferrin-Selenium-X (Gibco). Most of the media was removed and the tissues were gently vortexed to dissociate the cells. The cells were applied onto four coverslips pre-coated with concanavalin A. The cells were allowed to recover in the media containing 0.02 M vitamin C (ascorbic acid) at 37°C for 45 minutes. The media was then replaced with 1 mL of Ringer solution (sodium chloride (138 mM), potassium chloride (5 mM), HEPES at pH 7.4 (10 mM), calcium chloride (1 mM), magnesium chloride (1.5 mM), and glucose (10 mM)) containing Fura-2 (6.25 µL) and pluronic acid (1 µL), and incubated for another 45 minutes at room temperature in the dark. The cells were placed in new Ringer solution and left at room temperature for 10 minutes. The coverslip containing the cells were viewed under a fluorescence microscope and the response was obtained using Metamorph. Odorants (400 µL) were applied for 8 seconds at 2 minutes intervals via syringe in a stream of Ringer solution flowing at a rate of 1 mL/min. (The odorants were dissolved in DMSO and then
diluted (1000X) with Ringer solution to the desired concentration.) Recordings were made at 340 nm and 380 nm. The ratio of the fluorescence reading at these two wavelengths (F340/F380) corresponds to the amount of intracellular calcium. Forskolin (10 µM) was applied at the end of the experiment to activate adenylyl cyclase. The calcium imaging response of the odorants were normalized to the response of forskolin (10 µM).
Chapter 2: Olfactory Receptor Antagonism of the OR-I7 Aldehyde Receptor

2.1. Introduction:

2.1.1. Role of antagonism in the olfactory code

According to the olfactory code, each receptor can be activated by multiple odorants, and different odorants can activate a different set of odorant receptors.\(^1\) Hence, the olfactory system identifies odors using a combinatorial receptor coding scheme, and each odorant would have a unique olfactory code for generating the smell that we experience.\(^1\) Most studies have focused on identifying the olfactory receptors that could be activated by a specific odorant or the odorants that could activate specific receptors, but odorants are also capable of antagonizing ORs that they do not activate. Antagonists bind to receptors without activating them and this competitively inhibits agonists from binding (Figure 2.1). Therefore, instead of activating all the receptors that can be activated by the individual odorants, some receptors would lose their response in a mixture, or have it lessened, perhaps to partial agonist levels, due to antagonism. Antagonism may play an important role in the olfactory code, but the extent of that role and the structural criteria of antagonism are unclear.

![Figure 2.1: Signal transduction pathway of the olfactory system during antagonism of the olfactory receptor. (OR=Olfactory receptor and AC=Adenylyl cyclase)](image-url)
2.1.2. Molecular receptive range and antagonism of OR-I7 receptor

<table>
<thead>
<tr>
<th>Binding Site</th>
<th>Octanal</th>
<th>Pentanal (2.1)</th>
<th>Hexanal</th>
<th>2.2</th>
<th>1.1</th>
</tr>
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<tbody>
<tr>
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<td>O</td>
<td>O</td>
<td>O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Activation Pocket</td>
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<td>H</td>
<td>H</td>
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<tr>
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<td>6.4</td>
<td>5.4</td>
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<td>-</td>
<td>1736</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</td>
<td>-</td>
<td>460</td>
<td>124</td>
<td>45</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 2.2: Agonists and antagonists of the rat OR-I7 receptor. EC<sub>50</sub> and IC<sub>50</sub> values were obtained from calcium imaging of rat OSNs.²

In a previous study, evidence of antagonism was observed in the rat olfactory receptor, OR-I7.² OR-I7 is a receptor specific for aliphatic aldehydes, with octanal, an eight carbon aliphatic aldehyde, currently as one of its most potent ligand.³ ⁴ Only aldehydes capable of adopting chain lengths longer than 6.5-6.9 Å could activate OR-I7 at micromolar concentrations.² Removal of the ethyl group on 1.1 (to form 2.2), so that the chain length becomes shorter than 6.5 Å, converts the molecule from an agonist to an antagonist of the OR-I7 receptor. Aldehydes with chain lengths shorter than 6.5 Å inhibited the response of octanal in a dose-dependent manner with micromolar potency (IC<sub>50</sub> values from 45 to 460 µM when tested by calcium imaging, Figure 2.2). Since these antagonists all have chain lengths shorter than 6.5 Å in their most extended conformation, it was proposed that these odorants bind to the receptor via their aldehyde moiety, but were not long enough to reach a hydrophobic pocket needed to trigger activation of the OR-I7 receptor. The antagonists’ potencies appear to be improving as the number of carbons in the mid-region (region in the receptor between the aldehyde binding site and the proposed hydrophobic activation pocket) increases. Hexanal (IC<sub>50</sub> = 124 µM), with one more carbon than pentanal (2.1) (IC<sub>50</sub> = 460 µM), is approximately four times more potent.
than pentanal (2.1). Cyclohexylethanal (2.2) (IC$_{50}$ = 45 µM), with two extra carbons compared to hexanal, was approximately three times as potent.

2.2. Design and synthesis of OR-I7 antagonists

![Structures of designed OR-I7 antagonists tested in Structure-Activity Relationship (SAR) study.](image)

The predicted binding free energies in a homology model for OR-I7 suggest that saturated aldehydes with five to eleven carbons may be partially stabilized by hydrophobic interactions with amino acid residues. Increasing the carbons in the mid-region of the receptor may promote additional van der Waal interactions as more hydrophobic surface area from the ligand is buried in the binding site, and consequently, the affinity and potency of the antagonists may improve. To further test this idea, a structure-activity relationship (SAR) study was conducted in which the number of carbons in the mid-region of OR-I7 antagonists, 2.1 and 2.2, was gradually increased (Figure 2.3). Analogues 2.3, 2.4, 2.5, 2.6, and 2.7 have increasing numbers of carbons, compared to 2.1, by one to five carbons, respectively. Analogues 2.7, 2.8, 2.9, 2.10, 2.11, and 2.12 have one to four more carbons compared to compound 2.2. While increasing the number of carbons, the analogues were designed to maintain chain lengths shorter than 6.5 Å to prevent the compounds from reaching the putative hydrophobic activation pocket. The described analogues were synthesized and tested in HEK293 cells transfected with the
mouse OR-17 receptor to determine if there is any correlation between the number of carbons occupying the mid-region and the antagonist’s potency.

The target compounds were synthesized from commercially available starting materials. Compounds 2.3 and 2.12 were synthesized in one step from the alcohols through PCC oxidation. Compounds 2.2 and 2.10 were synthesized in two steps from the ester and carboxylic acid, respectively, through LAH reduction followed by PCC oxidation (Scheme 2.1).

Scheme 2.1: Synthesis of 2.2, 2.3, 2.10, and 2.12.

Compound 2.4 was synthesized in four steps from 3-pentanone as previously reported (Scheme 2.2). 3-Pentanone was subjected to a Horner-Wadsworth-Emmons reaction to afford the α,β-unsaturated ester 2.4a, which was then hydrogenated to give the saturated ester 2.4b. The ester was reduced to the alcohol 2.4c and oxidized into the aldehyde 2.4.

Scheme 2.2: Synthesis of 2.4.

Compound 2.5 was also synthesized from 3-pentanone according to an established route. 3-Pentanone reacted with ethyl cyanoacetate to form the α,β-unsaturated cyanoester 2.5a, which served as the Michael acceptor in the following 1,4 addition reaction with a Grignard reagent, formed from magnesium and iodomethane, as the Michael donor, to obtain 2.5b. The ester 2.5b was hydrolyzed and decarboxylated to form the nitrile 2.5c, which was then
hydrolyzed and reduced to the alcohol 2.5d. The alcohol 2.5d was oxidized to form the aldehyde 2.5. Compound 2.6 was synthesized in a similar manner but using bromoethane instead of iodomethane to form the Grignard reagent. Compound 2.8 and 2.9 were also synthesized through the same route but starting with cyclohexanone instead of pentanone (Scheme 2.3).

Scheme 2.3: Synthetic route of 2.5, 2.6, 2.8, and 2.9.

Compound 2.7 was synthesized (Scheme 2.4) starting from crotonaldehyde. The bicyclic ring of 2.7 was obtained through two Diels-Alder reaction. First, crotonaldehyde was subjected to a nucleophilic addition reaction to form 2.7a, which was used as the diene in the following Diels-Alder reaction with ethyl acrylate as the dienophile to form 2.7b. Compound 2.7b was subjected to an elimination reaction to form the ester 2.7c, which would serve as the diene in another Diels-Alder reaction with maleic anhydride as the dienophile to form 2.7d. The alkene 2.7d was hydrogenated to the alkane 2.7e, which was then hydrolyzed to cleave the anhydride into carboxylates 2.7f. The carboxylate groups were decarboxylated to form 2.7g. The alkene 2.7g was hydrogenated to reduce the double bond on the ring to form 2.7h. The ester 2.7h was LAH reduced to the alcohol 2.7i. The alcohol 2.7i was homologated by an extra carbon via a PCC oxidation reaction, followed by a Wittig reaction and a hydroboration-oxidation reaction to form the aldehyde 2.7j, the alkene 2.7k, and the alcohol 2.7l, respectively. The alcohol 2.7l was then oxidized, as usual just before biological testing, to the aldehyde 2.7.
Scheme 2.4: Synthesis of 2.7.

Compound 2.11 (Scheme 2.5) was synthesized from (-)-b-pinene. (-)-b-Pinene was homologated by an extra carbon via a hydroboration-oxidation reaction followed by a PCC oxidation and a Wittig reaction, to form the alcohol 2.11a, aldehyde 2.11b, and alkene 2.11c, respectively. The elongated alkene 2.11c was then subjected to another hydroboration-oxidation reaction to form the alcohol 2.11d, which was oxidized to the aldehyde 2.11. (It is also possible to synthesize compound 2.11 from (-)-b-pinene in one step via a hydroformylation reaction.\textsuperscript{35,36})

Scheme 2.5: Synthesis of 2.11.
2.2.1. Evaporation problem associated with luciferase assay

The initial luciferase assay that our collaborator used (Hiroaki Matsunami lab, Duke University) is a protocol that has been employed to measure the activation of specific ORs based on the cells’ cAMP level.\textsuperscript{11} In this assay, odorants are tested in genetically modified HEK293 cells (Hana3A cells) transfected with the OR-I7 receptor gene. Transfected ORs could not easily be expressed on the cell membrane, but RTP1S, a receptor-transporting protein, appears to help promote cell surface expression. In the transfected cells, activation of the olfactory receptor results in the production of cAMP, which in turn activates a protein kinase A. Protein kinase A then activates CREB, cAMP response element binding protein, to induce the expression of the luciferase gene, causing an increase in light output so that the response of the receptor can be quantified from the luminescence reading. Since gene expression occurs slowly, several hours were usually required to see a response. As such, the odorant had to be incubated at 37°C with the transfected cell in a 75 µM buffer solution for 2-4 hours. Since the OR-I7 agonist, octanal, is an aldehyde that is prone to oxidation in air, the production of octanoic acid over time was monitored by GC/MS.
Octanal was incubated for 2 hours at 37°C in a water solution to test for oxidation. No octanoic acid formed during this time span, but there was an apparent loss of octanal from the solution (Figure 2.4A). By the end of two hours, there was less than 20% of the original amount of octanal remaining in the water solution (Figure 2.4B). Octanal is volatile and has low solubility in water, so we speculated that octanal was evaporating from the water solution during the incubation. This was a potential problem because the luciferase assay requires incubation at 37°C in an aqueous solution for several hours to promote gene expression. However, if octanal was evaporating throughout this period, we would not be testing the concentration that was initially applied before the incubation, and different odorants undergoing comparison might evaporate at different rates.
To verify if evaporation occurs during the luciferase assay carried out by our collaborators at Duke University, the amount of octanal was monitored in a mock luciferase experiment, which was identical to an actual experiment except that the transfected cells were not present. Octanal was incubated for 2 hours at 37°C in a CD293 buffer solution (the medium used in the actual luciferase experiment for promoting cell growth) with 2,2-difluoroctanal. 2,2-Difluoroctanal is an OR-I7 agonist that converts to the gem-diol form when hydrated in water, and our collaborators were testing this compound in another project. The two hydroxyl groups on the gem-diol were expected to boost the odorant’s hydrophilicity, and consequently its solubility in water. In the case of octanal, roughly 40% converts to the gem-diol form in water.12 After two hours of incubation at 37°C, the percentage of octanal went down by approximately 70%, while the percentage of 2,2-difluoroctanal in the solution remained essentially the same (Figure 2.5A and 2.5B). Thus, differential evaporation was occurring over the course of the experiment.
Figure 2.5: Disappearance rate of 2,2-difluoroctanal and octanal in CD293 buffer in a mock luciferase reaction. 2,2-Difluoroctanal and octanal (75 µL, 0.1 mM) in a CD293 buffer solution was incubated at 37 °C for 2 hours in a 96-well plate. Percentage of odorant remaining in the solution was monitored by GC/MS. The odorants were extracted from the CD293 buffer with chloroform as described in the procedure. (A) Overlapping GC/MS spectrum at 0 h (black), 1 h (blue), and 2 h (red) of incubation. (B) Percentage of odorant remaining as a function of time. Results were obtained from a single trial. Percentages were calculated from the peak area for each odorant.

To prevent octanal from escaping the 96-well plate within the two hours of incubation, several different 96-well plate covers designed to seal the plate’s opening were used, including a plastic lid with parafilm, crystallization tape, sealing mat, and a silicone mat. However, after incubation at 37°C, there was still a decrease in the percentage of octanal remaining in the buffer compared to 2,2-difluoroctanal (Figure 2.6).
Figure 2.6: Disappearance rate of 2,2-difluoroctanal and octanal in sealed 96-well plates. 2,2-Difluoroctanal and octanal (0.1 mM) were incubated at 37 °C for 4 hours in a CD293 buffer solution (300 µL, 200 µL used for sealing mat). Results were obtained from a single trial. Percentages were normalized to 2,2-difluoroctanal.

Since the covers were all ineffective in maintaining the percentage of octanal in the solution, the odorant may be interacting with the material (polystyrene with poly-D-lysine surface) of the 96-well plate in some way, possibly getting attached onto the bottom of the plate, designed to adhere to cells. Prior to the incubation with octanal and 2,2-difluoroctanal, individual 96-well plates were pre-exposed to one of the following odorants at concentrations of either 0.1 or 1 mM: 1) octanal, 2) 2,2-difluoroctanal, and 3) 1-octanol. The 96-well plates containing these odorants were incubated at 37°C for four hours and then rinsed with water and air-dried prior to the assay. Despite being pre-exposed to the odorants and covered with a sealing mat during the incubation, the percentage of octanal was still appreciably reduced by the end of two hours (Figure 2.7).
Figure 2.7: Disappearance rate of 2,2-difluoroctanal and octanal after pre-exposure. 2,2-Difluoroctanal and octanal (0.1 mM) were incubated at 37 °C for 2 hours in a CD293 buffer solution (200 µL). Results were obtained from a single trial. Percentages were calculated from the peak area.

The cover should prevent octanal from escaping into the air and the odorants do not appear to be adsorbing onto the plate. Octanal, being hydrophobic, may be vaporizing and condensing onto the walls of the 96-well plate despite being locked inside a closed chamber. To reduce the surface area inside the 96-well plate for the octanal to condense onto, the volume of the buffer solution was increased while maintaining the same concentration. With the higher volume, there was an approximately 20% increase in the amount of octanal remaining in the solution (Figure 2.8), so that was at least part of the problem.
2,2-difluoroctanal and octanal (0.1 mM) were incubated at 37 °C for 30-45 minutes in a CD293 buffer solution (75 or 225 µL). Results were obtained from a single trial. Percentages were normalized to an internal standard, bromooctane.

Although there would be less evaporation if the temperature was reduced, cooler temperature would slow gene expression too much. Instead, we decided to look at whether the evaporation rate was the same for other aldehydes besides octanal. While 2,2-difluoroctanal is an aldehyde, its ability to almost completely convert to the gem-diol form in water result in properties that are exceptionally different to other aldehydes. The evaporation rate of several other experimental aldehydes under study in our lab (A1-A11, structures shown on Figure 2.9A) with similar lengths as octanal were tested at 37 °C using 75 µL volumes. Overall, all the aldehydes have similar disappearance/evaporation rate, with around 30-50% remaining after 30 minutes (Figure 2.9B).
Next, we compared the evaporation rate of a few structural analogs of octanal and 2,2-difluoroctanal. 2,2-Dimethyloctanal is an aldehyde with opposing electronic properties (electron-donating) to that of 2,2-difluoroctanal (fluorine is electron-withdrawing). Whereas the electron-withdrawing fluoro groups on 2,2-difluoroctanal shift the equilibrium towards the gem-diol form, the electron-releasing methyl groups on 2,2-dimethyloctanal shift the equilibrium towards the aldehyde form. If there is a direct correlation between the percentage of compounds
that was converted to the gem-diol form and the disappearance of the odorants from the solution, we should see a difference in the disappearance rate of 2,2-dimethyloctanal and octanal. However, 2,2-dimethyloctanal and octanal appeared to have similar disappearance rate (Figure 2.10). Moreover, even the disappearance rate of alcohols, 2,2-difluoroctanol and 1-octanol, were similar to that of octanal (Figure 2.10). With the exception of 2,2-difluoroctanal, the other structural analogs that were tested have similar disappearance rates. Whereas the concentration of odorants in the solution may be changing over time, the ratio of the odorants to each other should be fairly similar at all time. In this sense, the disappearance of the odorants through time would not be such a significant problem for the purpose of comparing the activity of odorants within the same experiment.

![Graph showing the disappearance rate of structural analogs.](image)

**Figure 2.10:** Disappearance rate of structural analogs. Odorants (0.1 mM) were incubated at 37 °C for 4 hours in CD293 buffer solution (300 µL). (A) Results were obtained from the average of 3 trials. Bromoocetane was used as an internal standard. (B) Structures of odorants. (Odorants were provided by Yadi Li.)

While structurally analogous compounds are expected to leave the solution at similar rates, the response of the OR would probably not be stimulated by the applied concentration.
Expression of the luciferase gene required incubation of the odorant solutions at 37 °C for several hours. However, it did not appear possible to prevent volatile odorants from escaping the solution during this timeframe, and using cooler temperatures and volumes close to the capacity of the 96-well plate chambers were impractical. To limit the disappearance of the odorants, the time of the experiment need to be lowered.

2.2.2. Glosensor cAMP assay

To shorten the reaction time, our collaborators adopted the Glosensor assay to determine the cAMP level in real time without the need for any reporter gene expression. In this assay, HEK293 cells were transfected with the OR-I7 receptor and a mutated form of the firefly luciferase gene fused with a cAMP binding region. Binding with cAMP promotes an immediate increase in light output so that the response of the receptor can be quantified from the luminescence reading in less than 15 minutes and as early as 5 minutes. Additionally, the odorants do not need to be incubated at elevated temperature.

When an agonist binds to the receptor, activation of the signal transduction pathway leads to an increase in the cAMP level and subsequently a boost in the light output by the GloSensor reporter. To test for antagonism, the agonist (i.e. octanal) was co-applied with various concentrations of each of the designed OR-I7 antagonists. The luminescence reading of the binary mixture should be lower than the reading of octanal alone if the co-applied compound is actually an antagonist that is competing with octanal for the binding site.

Selected odorants (octanal, 2.1, and 2.2) have been tested using this assay with HEK293 cells transfected with the rat OR-I7 receptor and the mouse OR-I7 receptor (results not shown). The mouse OR-I7 receptor was better expressed in the cells and the Glosensor assay gave more consistent response using the mouse ortholog. For that reason, the synthesized compounds were
tested in the mouse OR-I7 receptor although prior testing using calcium imaging was done using the rat OR-I7 receptor. There are 15 amino acid differences between the rat and mouse OR-I7 receptor, but both are aliphatic aldehyde receptors that behave similarly.

2.3. Results and Discussion

2.3.1. Activation of OR-I7

![Figure 2.11: Relative cAMP response of agonist and antagonist of OR-I7. HEK293 cells transfected with the mouse OR-I7 receptor were exposed to varying concentrations of odorant. The cAMP-induced response was monitored for 24 minutes after odorant(s) addition. (A) Time course plot for increasing concentration of octanal, an agonist of the OR-I7 receptor. (B) Time course plot for increasing concentration of cyclohexylethanal (2.2), an antagonist of OR-I7. (C) Time course plot for 5 µM octanal co-applied with increasing concentration of 2.2. The average response between 3.5 min and 7 min (between dashed lines) was used to create a dose-response curve, shown on Figure 2.12 and 2.13. (Data was provided by Jianghai Ho, Matsunami lab.)](image)

The compounds were tested individually for agonist activity. If the compound is an agonist of the OR-I7 receptor, such as octanal, there would be an increase in response as the concentration of the odorant increased (Figure 2.11A). If the compound is not an agonist of OR-I7, such as 2.2, which is an antagonist of the receptor, there would be no change in the relative response as a function of concentration (Figure 2.11B). However, when an agonist is co-applied
with an antagonist, the response of the agonist is suppressed as the concentration of the antagonist increase (Figure 2.11C).

2.3.2. Inhibition of OR-I7

![Figure 2.12: Response of analogues 2.1, 2.3, 2.4, 2.5, 2.6 and 2.7. (A) Activation Dose-Response Curves. Each compound was applied to HEK293 cells expressing mouse OR-I7. The cAMP production was monitored and the average response between 3 and 7.5 min is shown versus the compound’s concentration. The responses were compared to that of octanal, a known agonist of OR-I7. (B) Inhibition Dose-Response Curves. Each set of compounds was tested for the ability to antagonize mouse OR-I7. Cells were treated with varying concentrations of the synthesized compounds co-applied with 5 µM of octanal. (C) IC\textsubscript{50} values generated from the dose-response curves. (Data was provided by Jianghai Ho, Matsunami lab.)](image)

<table>
<thead>
<tr>
<th>Structures</th>
<th>Compound</th>
<th>Relative IC\textsubscript{50} (µM)</th>
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<tr>
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Aside from octanal, and consistent with previous findings on aliphatic aldehyde shorter than 7 Å,\textsuperscript{2} none of the compounds showed any activity as an agonist up to 100 µM (Figures 2.12A, 2.13A, and 2.14A). Since the compounds are all shorter than 6.5-6.9 Å, they should not be long enough to reach the proposed binding pocket required for activation.
2.3.3. Effect of carbon numbers on antagonism

All of the tested odorants antagonized the response of octanal to some extent (Figure 2.12, 2.13, & 2.14), but there does not appear to be any apparent correlation between the number of carbons in the mid-region and the antagonist’s potency. In Set A (i.e. compounds on Figure 2.12), there was an improvement in potency after adding one carbon from 2.1 (IC50=150 µM) to 2.3 (IC50=32 µM), but the potency decreased as more carbons were added onto 2.4, 2.5, and 2.6. In Set B (i.e. compounds on Figure 2.13), the activities of 2.7 (IC50=0.37 µM) and 2.12 (IC50=1.0 µM) were comparable to that of 2.2 (IC50=0.82 µM), but the additional carbons on the rings of 2.7 and 2.12 did not significantly improve the activity compared to 2.2. Analogues 2.8 and 2.9,
with 1 and 2 extra carbons on the ring, were weaker antagonists than 2.2. Similarly, the analogues (2.10 and 2.11) in Set C (i.e. compounds on Figure 2.14), which contained 1 or 3 more carbons on the ring compared to 2.2, have weaker antagonist potency than 2.2.

Figure 2.14: Response of analogues 2.2, 2.10, and 2.11. (A) Activation Dose-Response Curves. Each compound was applied to HEK293 cells expressing mouse OR-I7. The cAMP production was monitored and the average response between 3 and 7.5 min is shown versus the compound’s concentration. The responses were compared to that of octanal, a known agonist of OR-I7. (B) Inhibition Dose-Response Curves. Each set of compounds was tested for the ability to antagonize mouse OR-I7. Cells were treated with varying concentrations of the synthesized compounds co-applied with 5 µM of octanal. (C) IC50 values generated from the dose-response curves. (Data was provided by Jianghai Ho, Matsunami lab.)

2.3.4. Effect of cyclic ring on strength of antagonism

Adding more carbons to the mid-region did not improve the antagonists’ activity, but the cyclic aldehydes appeared to have higher potency than the acyclic aldehydes. The acyclic aldehydes (2.1, 2.3, 2.4, 2.5, and 2.6) in Set A, showed weaker antagonist activity than the 2.2.2-bicyclic aldehyde, 2.7. The improvement in activity from 2.1 (IC50=460 µM) to 2.2 (IC50=45 µM) for the rat OR-I7 in the previous study might also result from this change from a linear to a cyclic system.
2.3.5. Effect of protruding groups on antagonism

The reintroduction of acyclic groups onto the cyclohexyl ring system, such as the methyl group on 2.8 and the ethyl group on 2.9, resulted in lower activity compared to 2.2, which contains no groups on its ring. The acyclic groups, which aren’t long enough to reach the activation pocket, might be clashing sterically with amino acid residues lining the binding site of the receptor, and thus interfere with binding or interactions within the mid-region. In Set B (Figure 2.13), 2.7 and 2.9 both contain the same number of carbons, but having the ethyl group of 2.9 “tied back” onto the bicyclic ring of 2.7, may possibly prevent it from sterically clashing with amino acid residues. In Set C, 2.11 (IC$_{50}$=13 µM), containing the two methyl groups on the ring system, have weaker activities compared to the other compounds tested in this set, 2.10 (IC$_{50}$=7.9 µM) and 2.2 (IC$_{50}$=0.41 µM). Ring systems that contain a globular shape, such as 2.2 (IC$_{50}$=0.82 µM), 2.7 (IC$_{50}$=0.37 µM) and 2.12 (IC$_{50}$=1.0 µM), have the highest antagonist potency in this experiment, followed by 2.10 (IC$_{50}$=7.9 µM).

2.3.6. Effect of orientation

Out of the four aldehydes containing rigid, cyclic systems (2.2, 2.7, 2.10, and 2.12), 2.10 was the least potent antagonist of OR-I7. Compound 2.10 was ~8 to 20 times less potent than the other aldehydes containing a globular structure and no protruding groups. To search for a possible explanation for this difference, the four globular aldehydes were each modeled in their most stable conformation (Figure 2.15). The orientations of the aldehydes in the receptor pocket may have some influence on the potency of the antagonists. Comparing the orientations of the four aldehydes, the ring systems for 2.2, 2.7, and 2.12 are all situated downwards from the aldehyde group, whereas the ring for 2.10 is projected out of the page, sideways to the aldehyde group. In effect, the cyclohexyl rings of 2.2, 2.7, and 2.12, may all be seen as a full hexagonal
shape on the model (Figure 2.15) with the aldehyde group placed at the same position, whereas the cyclohexyl ring of 2.10 is contracted by the 2.2.1-bridgehead to form the cyclopentyl rings. The ring system of 2.10 contained a mix of cyclohexyl and cyclopentyl rings, while 2.2, 2.7, and 2.12, all consisted only of cyclohexyl ring(s). The cyclopentyl ring on 2.10 may be less preferable for the OR-17 receptor or the particular orientations resulting from the different ring systems led to interactions with different residues on the receptor modulating the antagonists’ stability. The smaller ring system in compound 2.11 may also share this problem.

Figure 2.15: 3D Structural models of 2.2, 2.7, 2.10, and 2.12. The structures of the aldehydes were drawn in their most extended conformation. The energy was minimized using MM2 on ChemBio3D Ultra 12.0. The length was measured from the carbonyl carbon to the furthest carbon (highlighted in yellow). The structures were oriented with the aldehyde group at the same position.

2.3.7. Effect of antagonist’s size

The size of the antagonist occupying the mid-region of the receptor appears to have minimal effect on the potency of the compound. The switch from the small cyclohexyl ring of 2.2 to the larger adamantyl ring of 2.12 resulted in similar IC₅₀ values (2.2: 0.82 µM; 2.12: 1.0 µM). Large groups in the mid-region may be tolerated but not necessary for antagonizing the receptor, since 2.2 with a smaller ring system is just as potent as 2.12. Likewise, while the protruding groups of 2.8 and 2.9 may be a source of interference, they did not entirely prevent
Pentanal (2.1)  
\( \text{IC}_{50} = 150 \, \mu\text{M} \)

2.6  
\( \text{IC}_{50} = 140 \, \mu\text{M} \)

2.9  
\( \text{IC}_{50} = 13 \, \mu\text{M} \)

2.2  
\( \text{IC}_{50} = 0.82 \, \mu\text{M} \)

Figure 2.16: Homology model of the mouse OR-I7 receptor bound by selected antagonists. Homology model is based on the inactive \( \beta_2 \)-adrenergic receptor. The antagonists are colored orange (carbons) and red (oxygen). Possible interactions with amino acid residues are indicated by dashed lines. (Models were provided by the Batista lab.)
the molecules from binding to and antagonizing the response of the receptor. The mid-region seems able to accommodate a wide variety of compact organic ring systems, and as long as they do not extend beyond 7 Å, they remain antagonists and do not activate the receptor.

2.3.8. **Homology model of the mouse OR-I7 receptor bound by selected antagonists**

To better evaluate the interactions between the designed antagonists and the OR-I7 receptor, homology models were constructed with the antagonists bound to the receptor. (Models were constructed by another collaborator, Victor Batista, at Yale University.) Correlating the antagonist’s IC$_{50}$ value with its optimal position in the receptor pocket may aid in explaining the observed differences between the potency of the tested antagonists. As shown on Figure 2.16, the two weaker OR-I7 antagonists, pentanal (2.1) and 2.6 are predicted to form a H-bond with the Tyr257 residue, whereas the stronger antagonists, 2.9 (~10X as potent) and 2.2 (>100X as potent), are predicted to form a H-bond with the Lys164 residue. Thus, interaction with the Lys164 residue appears to improve the antagonists’ potency. In previous homology models, agonists of the OR-I7 receptor, including octanal, were also found to interact with the Lys164 residue. However, in another model, the gem-diol form of octanal, which was expected to form when octanal becomes hydrated in water, prefers to interact with the Tyr257 residue possibly through hydrogen bonding, and based on interaction energy calculations, the gem-diol is better accommodated (by 2-6 kcal/mol) in the OR-I7 receptor than the aldehyde. As a result, the agonist, octanal, could potentially H-bond with either the Lys164 residue or the Tyr257 residue via the gem-diol form.

In order to antagonize the response of octanal, the antagonist needs to compete with octanal for the binding site. When comparing the IC$_{50}$ values of the weaker antagonists (2.1 and 2.6) and the stronger antagonists (2.2 and 2.9) with the residues to which they are interacting
with in the homology model (Figure 2.16), binding with the Lys164 residue appears more effective in inhibiting the response of octanal than binding with the Tyr257 residue. But conversely, the most potent antagonist, 2.7, interacts with the Tyr257 residue instead of the Lys164 residue. If interaction with the Tyr257 residue is indeed less favorable compared to the Lys164 residue, then the 2.2.2-bicyclic ring of 2.7 may possibly be stabilizing the molecule through additional van der Waals interactions, or the extra interaction with the Ser260 residue, not seen for the other modeled antagonists, may be further strengthening the binding. Otherwise, binding to either residue may be just as effective in antagonizing the response of the OR-I7 receptor, since the agonist, octanal, could bind at either residue. The results may also indicate that ligands can tumble in the orthosteric binding pocket.

Based on the homology models (Figure 2.16), by increasing the carbons in the mid-region, not only the size of the molecules differs, but the orientations of the molecule in the receptor pocket also shifted to accommodate the extra carbons. For instance, 2.2, 2.7, and 2.9 all contain a cyclohexyl ring within their structure, but the ring is oriented at different positions for the three molecules. Thus, while the aldehyde group on the antagonists were only seen to bind onto one of two residues, the hydrophobic portion of the molecule are all involved in fairly diverse interactions due to the differences in their orientations, which may affect the stability of the antagonists in the receptor.
2.3.9. Attempt to synthesize 2.13

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2.2</strong></td>
<td>Front View</td>
</tr>
<tr>
<td><strong>1.1</strong></td>
<td>Side View</td>
</tr>
<tr>
<td><strong>2.7</strong></td>
<td>3D model of 2.13 overlapping with 1.1 (as the trans-isomer in the chair conformation)</td>
</tr>
<tr>
<td><strong>2.13</strong></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Binding Site</th>
<th>Mid-Region</th>
<th>Activation Pocket</th>
</tr>
</thead>
<tbody>
<tr>
<td>O</td>
<td>&lt; 6.5 Å</td>
<td>~6.5-6.9 Å</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Length (Å)</th>
<th>EC₅₀ (µM)</th>
<th>IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.4</td>
<td>-</td>
<td>0.82</td>
</tr>
<tr>
<td>7.3-7.9</td>
<td>(1.0)</td>
<td>-</td>
</tr>
<tr>
<td>5.0</td>
<td>-</td>
<td>0.37</td>
</tr>
<tr>
<td>7.5</td>
<td>-</td>
<td>?</td>
</tr>
<tr>
<td>?</td>
<td>-</td>
<td>?</td>
</tr>
</tbody>
</table>

Figure 2.17: Agonists and antagonists of the OR-I7 receptor. (A) Structures of agonists and antagonists of the OR-I7 receptor. Chain lengths are calculated on ChemBio3D Ultra 12.0. EC₅₀ values were obtained from calcium imaging of rat OR-I7. IC₅₀ values were obtained from cAMP detection assay of mouse OR-I7. (B) 3D model of 2.13 (carbons: purple, oxygen: blue) overlapping with the trans-isomer of 1.1 (carbons: gray, oxygen: red) in the chair conformation.

The 2.2.2-bicyclo aldehyde 2.7 was the only antagonist found to be slightly more potent than the original cyclohexyl aldehyde 2.2. Attaching an ethyl group onto 2.2, so that the chain length exceeds 7 Å (forming 1.1 in chapter 1), transforms 2.2 from an antagonist to an agonist of OR-I7. Similarly, attachment of an ethyl group onto the ring of 2.7 (forming 2.13, Figure 2.17A) may switch antagonist 2.7 into an agonist. Since 2.7 is a better antagonist than 2.2, 2.13 may also be a better agonist than 1.1.

As speculated in chapter 1, the cis-isomer 1.2 may be activating the OR-I7 receptor in the twist-boat conformation. In this conformation, the position of the ethyl group on the cis-isomer 1.2 is closer to the position of the ethyl group on the trans-isomer 1.3. However, there would be an energy barrier to convert the cis-isomer 1.2 from the chair conformation to the less favorable twist-boat conformation. One way to reduce the energy barrier is to pay the cost during synthesis by locking the cis-isomer (1.2) in the boat conformation using a 2.2.2-bicyclic ring, resulting in 2.13. The position of the ethyl group on 2.13 is closer to the position of the ethyl group on the
trans-isomer (1.3) (Figure 2.17B). There will be no conformational flexibility in the mid-region, and the aldehyde and ethyl groups should be locked in what we speculate would be a favorable geometric relationship for OR-I7 binding.

As an extension to this study, we attempted to synthesize compound 2.13 for analysis. Compound 2.13 would test for whether the extension of the length of 2.7 would transform the molecule from an inhibitor to an activator of the OR-I7 receptor, and the potency of 2.13 would give us feedback as to whether the cis-isomer 1.2 (in chapter 1) activates the OR-I7 receptor in the boat conformation. Our prediction is that despite the boat conformation in this ring system, the compound should be just as potent as the trans-isomer (1.3). Compound 2.13 is not commercially available and has never been synthesized before, but there are literature procedures for the synthesis of similar molecules, such as 2.13a (Scheme 2.6).14, 15 The alkene 2.13a, had been synthesized from the dialdehyde 2.13b via a Wittig reaction, and 2.13b had been synthesized from the alcohol 2.13c through oxidation (Scheme 2.6A and 2.6B).
Once 2.13a is synthesized, there are various ways to transform 2.13a to 2.13. For instance, 2.13a could be subjected to a hydroboration-oxidation to functionalize both or either one of the alkenes into hydroxyl group(s) resulting in 2.13d and/or 2.13e (Scheme 2.6C).

The remaining alkene on 2.13e could be reduced to form 2.13g, and oxidation of the hydroxyl group of 2.13g would give the product 2.13. From 2.13d, one of the hydroxyl groups could be reacted to an alkyl group through a deoxygenation reaction or by reduction of the tosylate 2.13f to arrive at 2.13g, which could then be oxidized to the aldehyde 2.13.

**Scheme 2.6**: Synthesis of 2.13. (A) Retrosynthesis of 2.13. (B) Synthesis route for 2.13a. (C) Synthesis route for 2.13 from 2.13a. Reactions that are found in the literature are indicated by solid arrows and proposed reactions are indicated by dashed arrows.
A. Route 1

![Chemical diagram for Route 1]

(Boltz, 1964) Dieckmann Condensation
\[ \text{NaH} \rightarrow \]

![Chemical diagram for 2.13h]

(Boltz, 1964) Protection
\[ \text{p-TsOH} \rightarrow \]

![Chemical diagram for 2.13i]

(Boltz, 1964) Wolff-Kishner Reduction
\[ \text{N}_2\text{H}_4, \text{KOH} \rightarrow \text{diethyl glycol} \]

B. Route 2

![Chemical diagram for Route 2]

(Della, 1985) Substitution
\[ \text{LDA, HMPA, THF} \rightarrow \]

![Chemical diagram for 2.13m]

(Della, 1985) Substitution
\[ \text{LDA, HMPA, THF} \rightarrow \]

![Chemical diagram for 2.13n]

Reduction
\[ \text{LAH} \rightarrow \]

![Chemical diagram for 2.13o]

![Chemical diagram for 2.13c]

C. Route 3

![Chemical diagram for Route 3]

(Chapman, 1970) Hell-Volhard-Zelinsky halogenation
1) \text{SOCl}_2, \text{B}_2

2) \text{MeOH} \rightarrow \]

![Chemical diagram for 2.13p]

(Chapman, 1970) dehydrohalogenation in pyridine

![Chemical diagram for 2.13q]

(Chapman, 1970) Diels-Alder reaction in reflux

![Chemical diagram for 2.13r]

(Chapman, 1970) hydrolysis & reduction
1) 20% \text{KHCO}_3

2) \text{H}_2, \text{Pd/C}

3) \text{H}^+ \rightarrow \]

![Chemical diagram for 2.13s]

(Chapman, 1970) decarboxylation
\[ \text{Pb(OAc)}_4 \rightarrow \]

![Chemical diagram for 2.13t]

(Chapman, 1970) reduction
\[ \text{H}_2, \text{Pd/C} \rightarrow \]

![Chemical diagram for 2.13o]

![Chemical diagram for 2.13c]

Scheme 2.7: Synthetic routes to arrive at 2.13c. Reactions that are found in the literature are indicated by solid arrows and proposed reactions are indicated by dashed arrows.
Before arriving at 2.13a, it is necessary to first synthesize 2.13c (available from commercial sources at ~$1000/5g). Once compound 2.13c is obtained, it could be used to synthesize 2.13a, and from 2.13a, the desired product 2.13 could be synthesized. A few possible routes have been found in the literature to synthesize 2.13c (Scheme 2.7). The first route (Scheme 2.7A) is the scheme originated by Holtz et. al. to synthesize 2.13c from diethyl succinate.\(^{16}\) The second route (Scheme 2.7B) follows the scheme used by Della et. al. to synthesize 2.13o from dimethyl cyclohexane-1,4-dicarboxylate.\(^{17}\) This route can also be used to synthesize compound 2.14 (this structure was first suggested by Dr. Ryan Murelli) from 2.14a, which would also be effective in locking the cis-isomer (1.2) in the boat conformation via the 2.2.1-bicyclo ring.

\[
\begin{align*}
&\text{2.14} \quad \Rightarrow \quad \text{2.14b} \quad \Rightarrow \quad \text{2.14a} \\
&\text{Retrosynthesis of compound 2.14 from 2.14a}
\end{align*}
\]

The third route (Scheme 2.7C) is based on the scheme used by Chapman et. al. to synthesize 2.13o from cyclohexane-1,4-dicarboxylic acid.\(^{18}\) The ester 2.13o could afterwards be reduced to the alcohol 2.13c.

At present, routes 1 and 2 have been attempted to synthesize 2.13c at reduced scale in our lab. For both routes, difficulties occurred during the step to produce the 2.2.2-bicyclic ring (Route 1: 2.13i, Route 2: 2.13o). Following both procedures (at reduced scale), multiple products formed from the reaction (based on TLC and 1H NMR).
The challenge in forming the 2.2.2-bicyclic ring may mainly arise from a slow intramolecular substitution reaction. To form the 2.2.2-bicyclic ring, the intermediate would need to shift from the chair conformation to the sterically hindered, less stable, boat conformation (Scheme 2.8). Meanwhile, the elimination side-products could form and intermolecular reactions could occur between the intermediates/side products.

The scale of the reaction may also be affecting the yield. Literature procedures usually perform these reactions at multi-gram scale, whereas these reactions have only been attempted in our lab at small scale. Some steps in the reactions involve keeping the temperature of the reaction stabilized and crystallization of the product. At the larger scale it would be easier to maintain the temperature of the reaction as well as promote the formation of crystals.

2.4. Conclusions

Several aldehyde analogues with chain lengths shorter than 6.5 Å were synthesized. None of these aldehydes can activate the OR-I7 receptor. This was in accordance with previous studies suggesting that the distance from the aldehyde group to the most distant carbon needs to be more than 6.9 Å in order to bridge two important contact points or pockets. The synthesized short aldehydes all inhibited the response of octanal, demonstrating that the aldehydes could compete with octanal for the binding site. The tested acyclic odorants (2.1, 2.3, 2.4, 2.5, and 2.6) have weaker antagonist potency than the compact, unbranched cyclic odorants (specifically 2.7), and cyclic odorants that contained acyclic, protruding groups (2.8, 2.9, and 2.11) were weaker antagonist than the rigid, cyclic odorants (2.2, 2.7, and/or 2.12). The OR-I7 receptor appears to
prefer rigid, cyclic groups in the mid-region, but all twelve of the synthesized odorants could antagonize the response of octanal to some extent (IC\textsubscript{50}=0.37 µM to 150 µM), suggesting that the mid-region accommodates aldehydes of different shapes and sizes. To summarize, the response of the OR-I7 receptor could be inhibited by odorants containing an aldehyde functional group, with an extended conformation of less than 6.9 Å, and preferably a rigid, cyclic structure. Although the tested odorants were all aldehydes with chain lengths shorter than 6.5 Å, they differ in many aspects, such as structural conformation, size, and flexibility. Yet, they were all able to antagonize the OR-I7 receptor. It has been established that each olfactory receptor can be activated by multiple odorants, and each odorant activates multiple receptors, but it’s noteworthy that as with OR-I7, each receptor may also be antagonized by multiple odorants. Similarly, since each odorant could activate multiple olfactory receptors, they may also be antagonizing multiple olfactory receptors, as part of their contribution to the olfactory code.

2.5. **Experimental Procedures**

Unless otherwise stated, chemical reagents and solvents were purchased from VWR International, Fisher Scientific, or Sigma Aldrich and used without further purification. CD293 medium was purchased from Life Technologies. Tetrahydrofuran (THF) was dried prior to use. Octanal and pentanal (2.1) were freshly distilled. Aldehydes were stored as a DMSO solution at 4°C in flame-sealed ampules under vacuum prior to testing. Analytical TLC was performed on silica gel 60 F\textsubscript{254} plates. Flash chromatography\textsuperscript{20} was performed on Teledyne Isco CombiFlash Rf-200 flash chromatography system. Melting points were measured on a Laboratory Devices Mel-Temp apparatus. \textsuperscript{1}H and \textsuperscript{13}C NMR spectra were recorded on a Varian Mercury 300 spectrometer or a Bruker Ultrashield 500 spectrometer. HRMS was performed by Dr. Lijia Yang (CCNY Chemistry Department staff) on a Waters LCT XE (TOF) mass spectrometer using
Electrospray ionization (ESI). Infrared (IR) spectra were recorded using Thermo Nicolet 6700 FT-IR spectrometer. GC/MS analysis was performed on Shimadzu GCMS QP-2010. (Note: The literature procedure that was applied for each reaction is cited. The procedure may not necessarily be used for the synthesis of the same compound as in the literature.)

**General Procedure for Lithium Aluminum Hydride (LAH) reduction**

The acid or ester (1 equiv.) in diethyl ether was added slowly to lithium aluminum hydride (1.1 equiv.) in diethyl ether at 0°C. The suspension was stirred at room temperature for 2-3 h and then cooled to 0°C. Water (1 mL per g of LAH) was added, followed by 15% sodium hydroxide (1 mL per g of LAH) and water (3 mL per g of LAH). The solution was stirred for a few minutes at room temperature, filtered through a celite pad, washed with diethyl ether, dried, and concentrated to give the alcohol. Flash chromatography was sometimes required to purify the product.

**General Procedure for Pyridinium chlorochromate (PCC) oxidation**

The alcohol (1 equiv.) was added to pyridinium chlorochromate (1.1 equiv.) and silica gel (1 g/g of PCC) in dry dichloromethane under inert atmosphere. The suspension was stirred for 2 h and then passed through a silica gel pad. The solution was concentrated to give the aldehyde. Flash chromatography or distillation was sometimes required to purify the product.

**General Procedure for Hydrogenation Reaction**

The alkene was hydrogenated in ethyl acetate with a catalytic amount of Pd/C for 3 h using a balloon filled with hydrogen gas. The solution was filtered through celite and concentrated to give the alkane.
**2-Cyclohexylethanol (2.2a).** Ethyl cyclohexylacetate (2 g, 11.7 mmol) was LAH reduced according to the general procedure to obtain the alcohol 2.2a (1.5 g, 11.7 mmol) as a clear liquid in 99% yield. $^1$H NMR (CDCl$_3$) $\delta$: 3.68 (t, $J = 6.7$ Hz, 2H), 1.78 - 1.59 (m, 5H), 1.51 - 1.11 (m, 7H), 1.02 - 0.84 (m, 2H). $^{13}$C NMR (CDCl$_3$) $\delta$: 60.89, 40.39, 34.22, 33.38, 26.57, 26.29. IR (thin film, KBr plates) $\nu$ (cm$^{-1}$): 3332 (broad), 2922, 2852. Spectral data match those reported previously.$^2$

**Cyclohexylacetalddehyde (2.2).** Compound 2.2a (200 mg, 1.56 mmol) was PCC oxidized according to the general procedure to obtain the aldehyde 2.2 (140 mg, 1.11 mmol) as a clear liquid in 71% yield. $^1$H NMR (CDCl$_3$) $\delta$: 9.75 (s, 1H), 2.29 (dd, $J = 1.9, 6.9$ Hz, 2H), 1.97 - 1.57 (m, 6H), 1.40 - 0.84 (m, 5H). $^{13}$C NMR (CDCl$_3$) $\delta$: 203.00, 51.38, 33.22, 32.68, 26.04, 25.97. IR (thin film, KBr plates) $\nu$ (cm$^{-1}$): 2925, 2853, 2712, 1725, 1449, 1408, 1297, 1193, 1020, 900. Spectral data match those reported previously.$^2$

**3-Methylpentanal (2.3).** 3-Methyl-1-pentanol (2 g, 19.6 mmol) was PCC oxidized according to the general procedure. The product was purified by distillation to give 2.3 (450 mg, 4.5 mmol) as a clear liquid in 23% yield as a mixture of stereoisomers. $^1$H NMR (CDCl$_3$) $\delta$: 9.80 - 9.72 (m, 1H), 2.46 - 2.31 (m, 1H), 2.21 (ddd, $J = 2.5, 7.8, 16.1$ Hz, 1H), 1.97 (qd, $J = 6.8, 13.3$ Hz, 1H), 1.45 - 1.17 (m, 2H), 1.02 - 0.78 (m, 6H). $^{13}$C NMR (CDCl$_3$) $\delta$: 203.19, 50.69, 29.74, 29.51, 19.52, 11.30. IR (thin film, KBr plates), $\nu$ (cm$^{-1}$): 2964, 2932, 2878, 2822, 2717, 1728, 1463, 1381, 1209, 1141, 1021, 946, 875, 776. Spectral data match those reported previously.$^{21-23}$

**Ethyl 3-ethylpent-2-enoate (2.4a).** Compound 2.4a was synthesized according to a literature procedure.$^6$ Triethyl phosphonoacetate (7.8 g, 34 mmol) in THF (12.5 mL) was slowly added to sodium hydride, 57-63% in oil, (1.4 g, 34
mmol) in THF (10 mL) at 0°C. The suspension was stirred for 1 h and then 3-pentanone (3 g, 34 mmol) in THF (12.5 mL) was slowly added. The solution was stirred for an hour and quenched with water. The solution was extracted with diethyl ether, and the organic layer was dried and concentrated. The crude was purified by flash chromatography eluting with hexanes/diethyl ether (99:1) to give 2.4a (1.58 g, 10.1 mmol) as a yellowish liquid in 29% yield. ¹H NMR (CDCl₃) δ: 5.59 (s, 1H), 4.14 (q, ²J = 6.7 Hz, 2H), 2.61 (q, ²J = 7.4 Hz, 2H), 2.18 (q, ²J = 7.4 Hz, 2H), 1.27 (t, ²J = 6.6 Hz, 3H), 1.06 (t, ²J = 7.4 Hz, 6H). ¹³C NMR (CDCl₃) δ: 167.30, 166.65, 113.71, 59.44, 30.74, 25.44, 14.33, 13.04, 12.02. IR (thin film, KBr plates) ν (cm⁻¹): 2972, 2936, 2877, 1717, 1645, 1463, 1380, 1307, 1273, 1205, 1148, 1105, 1040, 868. Spectral data match those previously reported.²⁴

**Ethyl 3-ethylpentanoate (2.4b).** Compound 2.4a (1.48 g, 9.47 mmol) was hydrogenated according to the general procedure to give 2.4b (1.35 g, 8.53 mmol) as a clear liquid in 90% yield. ¹H NMR (CDCl₃) δ: 4.12 (q, ²J = 7.1 Hz, 2H), 2.22 (d, ²J = 6.9 Hz, 2H), 1.74 (p, ²J = 6.4 Hz, 1H), 1.42 - 1.18 (m, 7H), 0.87 (t, ²J = 7.4 Hz, 6H). ¹³C NMR (CDCl₃) δ: 173.76, 60.08, 38.56, 37.93, 25.85, 14.28, 10.82. IR (thin film, KBr plates) ν (cm⁻¹): 2962, 2926, 2857, 1738, 1461, 1374, 1178, 1097, 1034. Spectral data match those previously reported.²⁵

**3-Ethylpentan-1-ol (2.4c).** Compound 2.4b (1.25 g, 7.9 mmol) was LAH reduced according to the general procedure and purified by flash chromatography eluting with hexanes/ethyl acetate (49:1) to give 2.4c (480 mg, 4.13) as a clear liquid in 52% yield. ¹H NMR (CDCl₃) δ: 3.64 (t, ²J = 7.2 Hz, 2H), 1.93 - 1.63 (br. s., 1H), 1.63 - 1.40 (m, 2H), 1.29 (d, ²J = 5.5 Hz, 5H), 0.84 (t, ²J = 6.9 Hz, 6H). ¹³C NMR (CDCl₃) δ: 61.37, 37.08, 36.11,
25.50, 10.78. IR (thin film, KBr plates), ν (cm$^{-1}$): 3331 (broad), 2961, 2925, 2874, 1461, 1379, 1062.

3-Ethylpentanal (2.4). Compound 2.4c (100 mg, 0.875 mmol) was PCC oxidized according to the general procedure to obtain 2.4 (32 mg, 0.280 mmol) as a clear liquid in 33% yield. $^1$H NMR (CDCl$_3$) δ: 9.77 (t, $J$ = 2.3 Hz, 1H), 2.33 (dd, $J$ = 2.5, 6.6 Hz, 2H), 1.84 (td, $J$ = 6.5, 12.8 Hz, 1H), 1.46 - 1.25 (m, 4H), 0.88 (t, $J$ = 7.4 Hz, 6H). $^{13}$C NMR (CDCl$_3$) δ: 203.43, 47.84, 35.88, 26.15, 10.92. IR (thin film, KBr plates), ν (cm$^{-1}$): 2964, 2935, 2878, 2714, 1727, 1461, 1412, 1382, 1337, 1246, 1136, 1096, 1021.

Ethyl 2-cyano-3-ethylpent-2-enoate (2.5a). Compound 2.5a was synthesized based on a literature procedure. Ethyl cyanoacetate (56.5 g, 0.5 mol), 3-pentanone (51.7 g, 0.6 mol), ammonium acetate (3.85 g, 0.05 mol), and acetic acid (6 g, 0.1 mol) were added to benzene (50 mL) in a round bottom flask connected to a Dean-Stark apparatus. The solution was refluxed for 6 h and then washed three times with water. The organic layer was dried and concentrated. The product was purified by distillation to give 2.5a (58.5 g, 0.32 mol) as a clear liquid in 64.6% yield. $^1$H NMR (CDCl$_3$) δ: 4.26 (q, $J$ = 7.2 Hz, 2H), 2.78 (q, $J$ = 7.4 Hz, 2H), 2.57 (q, $J$ = 7.4 Hz, 2H), 1.34 (t, $J$ = 7.2 Hz, 3H), 1.13 (t, $J$ = 7.4 Hz, 3H), 1.19 (t, $J$ = 7.7 Hz, 3H). $^{13}$C NMR (CDCl$_3$) δ: 184.19, 161.70, 115.62, 104.01, 61.68, 31.40, 26.51, 14.08, 12.65, 12.38. IR (thin film, KBr plates), ν (cm$^{-1}$): 2981, 2941, 2879, 2223, 1731, 1602, 1465, 1368, 1245, 1270, 1211, 1099, 1022, 912, 857, 817.

Ethyl 2-cyano-3-ethyl-3-methylpentanoate (2.5b). Compound 2.5b was synthesized based on literature procedures. Iodomethane (11.3 g, 80 mmol) in diethyl ether (10 mL) was slowly added to magnesium turnings (1.83 g, 76 mmol) in diethyl ether (10 mL) at 0°C and the solution was stirred for 30 minutes.
Copper chloride (100 mg, 1 mmol) was added, and 2.5a (9.06 g, 50 mmol) in diethyl ether (10 mL) was slowly added. The solution was stirred for 30 minutes and then added to 10% sulfuric acid (40 mL) in ice (50 g). The aqueous phase was extracted three times with diethyl ether. The combined organic layer was dried and concentrated. The resulting residue was purified by flash chromatography eluting with hexanes/ethyl acetate (9:1) to give 2.5b (4.3 g, 21.8 mmol) as a clear liquid in 43.6% yield. 

\[
\begin{align*}
\text{1H NMR (CDCl}_3) & : 4.32 - 4.20 (m, 2H), 3.45 (s, 1H), 1.64 - 1.39 (m, 4H), 1.39 - 1.22 (m, 3H), 1.08 (s, 3H), 0.98 - 0.82 (m, 6H). \\
\text{13C NMR (CDCl}_3) & : 165.57, 116.2, 62.3, 46.2, 40.47, 29.60, 28.91, 21.79, 14.06, 7.90, 7.75. \\
\text{IR (thin film, KBr plates), } & \nu (\text{cm}^{-1}): 2972, 2884, 2247, 1742, 1465, 1389, 1369, 1328, 1244, 1192.
\end{align*}
\]

3-Ethyl-3-methylpentanenitrile (2.5d). Compound 2.5d was synthesized based on a literature procedure.\(^8\) Compound 2.5b (4 g, 20 mmol) was added to a solution containing 85% potassium hydroxide (5.8 mL), water (25.4 mL), and ethanol (5 mL). The solution was refluxed for 6 h and then concentrated. The resulting residue was refluxed for 4 h in 20% sulfuric acid (31 mL). The solution was cooled to room temperature and extracted with diethyl ether. The organic layer was dried and concentrated. A catalytic amount of copper powder (70 mg) was added to the resulting residue (2.5c) and the suspension was slowly heated to 170°C. The suspension was then allowed to cool back to room temperature and was purified by flash chromatography, eluting with hexanes/ethyl acetate (19:1) to give 2.5d (1.26 g, 10.1 mmol) as a yellowish liquid in 50% yield. 

\[
\begin{align*}
\text{1H NMR (CDCl}_3) & : 2.20 (s, 2H), 1.40 (q, J = 7.2 Hz, 4H), 0.98 (s, 3H), 0.84 (t, J = 7.6 Hz, 6H). \\
\text{13C NMR (CDCl}_3) & : 118.57, 35.68, 30.88, 27.65, 23.65, 7.95. \\
\text{IR (thin film, KBr plates), } & \nu (\text{cm}^{-1}): 2969, 2941, 2883, 2247, 1742, 1465, 1389, 1369, 1328, 1244, 1192.
\end{align*}
\]

3-Ethyl-3-methylpentan-1-ol (2.5f). Compound 2.5d was hydrolyzed based on a literature procedure.\(^27\) Compound 2.5d (1.1 g, 8.8 mmol) was refluxed in a solution
with sulfuric acid (8.1 mL) and water (9.8 mL) for 6 h. The solution was cooled to room temperature, diluted with water, and extracted with diethyl ether. The organic layer was dried and concentrated. The resulting residue **2.5e** was LAH reduced according to the general procedure and purified by flash chromatography eluting with hexanes/ethyl acetate (9:1) to give **2.5f** (650 mg, 5 mmol) as a clear liquid in 56.8% yield. $^1$H NMR (CDCl$_3$) $\delta$: 3.77 - 3.57 (m, 2H), 1.90 (s, 1H), 1.75 - 1.38 (m, 2H), 1.38 - 1.02 (m, 4H), 1.02 - 0.66 (m, 9H). $^{13}$C NMR (CDCl$_3$) $\delta$: 59.50, 41.12, 34.46, 31.36, 24.23, 7.89. IR (thin film, KBr plates) $\nu$ (cm$^{-1}$): 3333 (broad), 2963, 2938, 2880, 1463, 1380.

**3-Ethyl-3-methylpentanal (2.5).** Compound **2.5f** (100 mg, 0.77 mmol) was PCC oxidized according to the general procedure to give **2.5** (58 mg, 0.45 mmol) in 59% yield. $^1$H NMR (CDCl$_3$) $\delta$: 9.84 (t, $J = 3.3$ Hz, 1H), 2.24 (d, $J = 3.3$ Hz, 2H), 1.38 (q, $J = 7.4$ Hz, 4H), 1.00 (s, 3H), 0.85 (t, $J = 7.4$ Hz, 6H). $^{13}$C NMR (CDCl$_3$) $\delta$: 204.05, 52.00, 36.33, 31.81, 24.33, 7.91. IR (thin film, KBr plates), $\nu$ (cm$^{-1}$): 2967, 2882, 2732, 1721, 1463, 1382.

HRMS (ESI) [M+H]$^+$: Calcd for C$_8$H$_{16}$O $m/z = 129.1274$, found $m/z = 129.1266$.

**Ethyl 2-cyano-3,3-diethylpentanoate (2.6b).** Compound **2.6b** was synthesized based on literature procedures.$^8$ Bromoethane (8.7 g, 80 mmol) in diethyl ether (10 mL) was slowly added to magnesium turnings (1.83 g, 76 mmol) in diethyl ether (10 mL) at 0°C and the solution was stirred for 30 minutes. Copper chloride (100 mg, 1 mmol) was added, and **2.5a** (9.06 g, 50 mmol) in diethyl ether (10 mL) was slowly added. The solution was stirred for 30 minutes and then added to 10% sulfuric acid (40 mL) in ice (50 g). The aqueous phase was extracted three times with diethyl ether. The combined organic layer was dried and concentrated. The resulting residue was purified by flash chromatography eluting with hexanes/ethyl acetate (9:1) to give **2.6b** (4.45 g, 21.1 mmol) as a
clear liquid in 42% yield. $^1$H NMR (CDCl$_3$) $\delta$: 4.31 - 4.20 (m, 2H), 3.49 - 3.43 (m, 1H), 1.65 - 1.41 (m, 6H), 1.38 - 1.25 (m, 3H), 0.99 - 0.84 (m, 9H). $^{13}$C NMR (CDCl$_3$) $\delta$: 165.74, 116.67, 62.32, 44.43, 43.12, 27.59, 13.98, 7.90. IR (thin film, KBr plates), $\nu$ (cm$^{-1}$): 2972, 2944, 2885, 2246, 1741, 1602, 1459.

3,3-Diethylpentanenitrile (2.6d). Compound 2.6d was synthesized according to a literature procedure.$^8$ Compound 2.6b (4.2 g, 20 mmol) was added to a solution containing 85% potassium hydroxide (6.1 mL), water (26.7 mL), and ethanol (5.25 mL). The solution was refluxed for 6 hours and then concentrated. The resulting residue was refluxed for 4 h in 20% sulfuric acid (32.6 mL). The solution was cooled to room temperature and extracted with diethyl ether. The organic layer was dried and concentrated. A catalytic amount of copper powder (80 mg) was added to the resulting residue (2.6c) and the suspension was slowly heated to 170°C. The suspension was then allowed to cool back to room temperature and was purified by flash chromatography, eluting with hexanes/ethyl acetate (19:1) to give 2.6d (1.32 g, 9.5 mmol) as a yellowish liquid in 47% yield. $^1$H NMR (CDCl$_3$) $\delta$: 2.19 (s, 2H), 1.44 - 1.31 (m, 6H), 0.82 (t, $J$ = 7.4 Hz, 9H). $^{13}$C NMR (CDCl$_3$) $\delta$: 118.49, 38.10, 27.87, 24.80, 7.54. IR (thin film, KBr plates), $\nu$ (cm$^{-1}$): 2968, 2943, 2882, 2245, 1735, 1461, 1425, 1383.

3,3-Diethylpentan-1-ol (2.6f). Compound 2.6d was hydrolyzed according to a literature procedure.$^{27}$ Compound 2.6d (1.1 g, 7.9 mmol) was refluxed in a solution of sulfuric acid (8.1 mL) and water (9.8 mL) for 6 h. The solution was cooled to room temperature, diluted with water, and extracted with diethyl ether. The organic layer was dried and concentrated. The resulting residue 2.6e was LAH reduced according to the general procedure and purified by flash chromatography eluting with hexanes/ethyl acetate (9:1) to give 2.6f (350 mg, 2.4 mmol) as a clear liquid in 31% yield. $^1$H NMR (CDCl$_3$) $\delta$: 3.70 - 3.57 (m, 2H),
1.56 - 1.40 (m, 3H), 1.29 - 1.13 (m, 6H), 0.83 - 0.71 (m, 9H). $^{13}$C NMR (CDCl$_3$) $\delta$: 59.28, 38.08, 36.79, 27.99, 7.46. IR (thin film, KBr plates) $\nu$ (cm$^{-1}$): 3323 (broad), 2964, 2939, 2880, 1464, 1378, 1036.

**3,3-Diethylpentanal (2.6).** Compound 2.6f (100 mg, 0.69 mmol) was PCC oxidized according to the general procedure to give 2.6 (44 mg, 0.31 mmol) in 45% yield. $^1$H NMR (CDCl$_3$) $\delta$: 9.83 (t, $J = 3.3$ Hz, 1H), 2.24 (d, $J = 3.0$ Hz, 2H), 1.45 - 1.32 (m, 6H), 0.92 - 0.76 (m, 9H). $^{13}$C NMR (CDCl$_3$) $\delta$: 204.0, 49.2, 40.98, 28.55, 7.52. IR (thin film, KBr plates), $\nu$ (cm$^{-1}$): 2967, 2882, 2733, 1721, 1462, 1381, 1300, 1155, 1093, 1028.

**(1E)-N,N-diethylbuta-1,3-dien-1-amine (2.7a).** Compound 2.7a was synthesized according to a literature procedure.$^{10}$ Crotonaldehyde (8 g, 0.114 mol) in toluene (15 mL) was added to a solution of diethylamine and potassium carbonate at 4°C. The solution was allowed to come to room temperature and was stirred for 4 h. The product was purified by distillation to give 2.7a as a yellow liquid (6.5 g, 52 mmol) in 45% yield. $^1$H NMR (CDCl$_3$) $\delta$: 6.35 - 6.17 (m, 2H), 5.03 (dd, $J = 10.7$, 13.2 Hz, 1H), 4.76 - 4.64 (m, 1H), 4.50 - 4.40 (m, 1H), 3.05 (q, $J = 7.2$ Hz, 4H), 1.19 - 0.90 (m, 6H). Spectral data match those previously reported.$^{10}$

**Ethyl 2-(diethylamino)cyclohex-3-ene-1-carboxylate (2.7b).** Compound 2.7b was synthesized according to a literature procedure.$^{10}$ To 2.7a (6.5 g, 52 mmol) in toluene (11.2 mL) was added ethyl acrylate (5.66 g, 56.5 mmol). The solution was stirred for 5 days in the dark under argon. The solution was diluted with diethyl ether and extracted three times with 2 M HCl. The combined aqueous layer was extracted twice with diethyl ether. The pH of the aqueous solution was raised to 10 by adding 6 M NaOH. The aqueous phase was extracted with diethyl ether three times. The combined ether layer was dried
and concentrated to give 2.7b (9.8 g, 43 mmol) as a yellowish liquid in 84% yield. 2.7b was used for the next step without further purification. \textsuperscript{1}H NMR (CDCl\textsubscript{3}) \( \delta \): 5.90 (br. s., 1H), 5.83 - 5.55 (m, 1H), 4.13 (dd, \( J = 6.9, 12.4 \) Hz, 2H), 3.77 - 3.51 (m, 1H), 2.75 - 2.36 (m, 5H), 2.30 - 2.10 (m, 1H), 2.05 (br. s., 1H), 1.99 - 1.83 (m, 1H), 1.80 (br. s., 1H), 1.42 - 1.11 (m, 3H), 1.10 - 0.77 (m, 6H). Spectral data match those previously reported.\textsuperscript{10}

\textbf{Ethyl cyclohexa-1,3-diene-1-carboxylate (2.7c).} Compound 2.7c was synthesized according to a literature procedure.\textsuperscript{10} Compound 2.7b (9.8 g, 43 mmol) was added to acetic acid (48 mL) and refluxed for 2 h. The reaction was quenched with ice water and extracted three times with diethyl ether. The combined organic layer was washed with water, saturated sodium bicarbonate, and brine, dried, and concentrated. The resulting residue was purified by flash chromatography, eluting with hexanes/ethyl acetate (49:1) to give 2.7c (5 g, 33 mmol) as a yellowish liquid in 75.5% yield. \textsuperscript{1}H NMR (CDCl\textsubscript{3}) \( \delta \): 6.95 (br. s., 1H), 6.16 - 5.94 (m, 2H), 4.26 - 4.09 (m, 2H), 2.50 - 2.31 (m, 2H), 2.31 - 2.13 (m, 2H), 1.35 - 1.18 (m, 3H). Spectral data match those previously reported.\textsuperscript{10}

\textbf{Ethyl 3,5-dioxo-4-oxatricyclo[5.2.2.0\textsuperscript{2,6}]undec-8-ene-1-carboxylate (2.7d).} Compound 2.7d was synthesized according to a literature procedure.\textsuperscript{9} Compound 2.7c (5 g, 33 mmol) was mixed with maleic anhydride (3.3 g, 33 mmol) and stirred for 30 minutes at 100°C and 30 minutes at 170°C. The product was purified by flash chromatography eluting with dichloromethane to give 2.7d (7.78 g, 31 mmol) as a white solid (m.p. 85-86°C) in 94.6% yield. \textsuperscript{1}H NMR (CDCl\textsubscript{3}) \( \delta \): 6.73 (d, \( J = 8.5 \) Hz, 1H), 6.38 - 6.30 (m, 1H), 4.40 - 4.28 (m, 2H), 3.68 (d, \( J = 8.5 \) Hz, 1H), 3.29 (br. s., 1H), 3.20 (d, \( J = 9.4 \) Hz, 1H), 1.98 - 1.70 (m, 2H), 1.48 (br. s., 2H), 1.35 (t, \( J = 7.2 \) Hz, 3H). \textsuperscript{13}C NMR (CDCl\textsubscript{3}) \( \delta \):
Analytical data match those previously reported.\(^9\)

**Ethyl 3,5-dioxo-4-oxatricyclo[5.2.2.0\(^2,6\)]undecane-1-carboxylate (2.7e).**

Compound 2.7d (7.78 g, 31 mmol) was hydrogenated according to the general procedure to give 2.7e (7.4 g, 29 mmol) as a white solid (m.p. 81-83°C) in 94% yield. Compound 2.7e was used for the next step without further purification. \(^1\)H NMR (CDCl\(_3\)) \(\delta\): 4.22 (q, \(J = 6.3\) Hz, 2H), 3.69 (d, \(J = 10.2\) Hz, 1H), 3.19 (d, \(J = 10.5\) Hz, 1H), 2.30 (br. s., 1H), 1.96 - 1.71 (m, 6H), 1.71 - 1.45 (m, 2H), 1.28 (t, \(J = 7.0\) Hz, 3H). \(^{13}\)C NMR (CDCl\(_3\)) \(\delta\): 173.85, 173.03, 171.66, 61.42, 45.53, 44.42, 40.36, 29.09, 26.70, 24.16, 22.65, 21.23, 14.09. Analytical data match those previously reported.\(^9\)

**Ethyl bicycle[2.2.2]oct-2-ene-1-carboxylate (2.7g).** Compound 2.7g was synthesized according to a literature procedure.\(^9\) Compound 2.7e (7.4 g, 29 mmol) and potassium carbonate (11.67 g, 65 mmol) were refluxed in water for 2 h. The solution was acidified to pH 1 with 10% HCl. The solution was extracted with ethyl acetate. The organic layer was dried and concentrated to give 2.7f (7 g, 26 mmol) as a white solid (m.p. 135-138°C), which was used for the next step without further purification. Compound 2.7f (7 g, 26 mmol) was added to acetonitrile (233 mL). Pyridine (2.8 g, 36 mmol) was added followed by lead acetate (11.66 g, 26 mmol). The solution was refluxed for 3 h and then filtered through a silica pad. The product was purified by flash chromatography eluting with hexanes/ethyl acetate (19:1) to give 2.7g (1.41 g, 7.8 mmol) as a clear liquid in 27% yield (in 2 steps from 2.7e). \(^1\)H NMR (CDCl\(_3\)) \(\delta\): 6.47 - 6.39 (m, 1H), 6.36 - 6.27 (m, 1H), 4.18 (q, \(J = 7.2\) Hz, 2H), 2.57 (br. s., 1H), 1.88 - 1.76 (m, 2H), 1.66 - 1.50 (m, 2H), 1.50 - 1.22 (m, 7H). \(^{13}\)C NMR (CDCl\(_3\)) \(\delta\): 176.72,
IR (thin film, KBr plates) ν (cm⁻¹): 2945, 2910, 2868, 1731, 1455, 1390, 1366, 1317, 1288, 1249.

**Ethyl bicyclo[2.2.2]octane-1-carboxylate (2.7h).** Compound 2.7g (1.41 g, 7.8 mmol) was hydrogenated according to the general procedure to give 2.7h (1.37 g, 7.5 mmol) as a clear liquid in 96% yield. Compound 2.7h was used for the next reaction without further purification. ¹H NMR (CDCl₃) δ: 4.07 (q, J = 7.2 Hz, 2H), 1.78 - 1.65 (m, 6H), 1.58 (d, J = 7.4 Hz, 7H), 1.22 (t, J = 7.2 Hz, 3H). ¹³C NMR (CDCl₃) δ: 178.25, 60.02, 38.19, 28.04, 25.41, 23.79, 14.21. IR (thin film, KBr plates) ν (cm⁻¹): 2945, 2921, 2866, 1726, 1457, 1365, 1336, 1243.

**Bicyclo[2.2.2]oct-1-ylmethanol (2.7i).** Compound 2.7h (1.37 g, 7.5 mmol) was LAH reduced according to the general procedure to give the alcohol 2.7i (1 g, 7.13 mmol) in 95% yield as a white solid (m.p. 60-61°C). Compound 2.7i was used for the next reaction without further purification. ¹H NMR (CDCl₃) δ: 3.21 (s, 2H), 1.68 - 1.46 (m, 7H), 1.46 - 1.08 (m, 7H). ¹³C NMR (CDCl₃) δ: 72.04, 32.44, 27.72, 25.71, 24.60.

**Bicyclo[2.2.2]octane-1-carbaldehyde (2.7j).** Compound 2.7i (1 g, 7.13 mmol) was PCC oxidized according to the general procedure to give aldehyde 2.7j, which was used for the next step without further purification. ¹H NMR (CDCl₃) δ: 9.40 (s, 1H), 1.79 - 1.51 (m, 13H). Spectral data match those reported previously.¹⁵

**1-Ethenylbicyclo[2.2.2]octane (2.7k).** Compound 2.7k was synthesized based on a literature procedure.²⁸ To methyl triphenylphosphonium bromide (5.4 g, 15 mmol) in THF (30 mL) at 0°C was added potassium butoxide (1.7 g, 15 mmol). The suspension was stirred for 1 h and then aldehyde 2.7j in THF (7 mL) was slowly added. The reaction was stirred for 2 h at room temperature and then water was added. The layers were separated and the
aqueous phase was extracted with diethyl ether. The combined organic layer was dried, concentrated and purified by flash chromatography eluting with hexanes to give the alkene \( \text{2.7k} \). 

1H NMR (CDCl\(_3\)) \( \delta \): 5.72 - 5.64 (m, 1H), 4.85 - 4.77 (m, 2H), 1.63 - 1.51 (m, 7H), 1.51 - 1.40 (m, 6H). Spectral data agree with those reported previously.\(^{15}\)

\[
\text{2-(Bicyclo[2.2.2]oct-1-yl)ethanol (2.7l). Compound 2.7l was subjected to a hydroboration-oxidation reaction.}\(^{29}\)
\]

Compound \( \text{2.7k} \) was dissolved in THF and cooled to 0°C. 1 M Borane tetrahydrofuran complex solution in THF (2.4 mL, 2.4 mmol) was slowly added and the solution was stirred for 1 h at room temperature. The solution was cooled to 0°C and water (500 µL) was added followed by 3 M NaOH (750 µL) and 30% hydrogen peroxide (750 µL). The solution was stirred at room temperature for 2 h. The reaction was quenched with water and the layers were separated. The aqueous layer was extracted with diethyl ether and the combined organic layer was dried and concentrated. The product was purified by flash chromatography eluting with hexanes/ethyl acetate (9:1) to give the alcohol \( \text{2.7l} \) (330 mg, 2.17 mmol) in 30% yield (over 3 steps from \( \text{2.7i} \)). 1H NMR (CDCl\(_3\)) \( \delta \): 3.83 - 3.63 (m, 2H), 1.64 - 1.32 (m, 15H), 1.28 (br. s., 1H). 13C NMR (CDCl\(_3\)) \( \delta \): 59.49, 44.86, 31.15, 29.24, 26.17, 24.17. IR (thin film, KBr plates) \( \nu \) (cm\(^{-1}\)): 3331 (broad), 2930, 2859, 1455, 1409, 1268.

HRMS (ESI) [M+Na]\(^+\): Calcd for C\(_{10}\)H\(_{16}\)O \( m/\zeta = 175.1099 \), found \( m/\zeta = 175.1098 \).

\[
\text{Bicyclo[2.2.2]oct-1-ylacetaldehyde (2.7). Compound 2.7l (100 mg, 0.65 mmol) was PCC oxidized according to the general procedure to the aldehyde 2.7 (70 mg, 0.46 mmol) as a clear liquid in 71% yield. } \]

1H NMR (CDCl\(_3\)) \( \delta \): 9.84 - 9.79 (m, 1H), 2.12 (d, \( J = 3.0 \) Hz, 2H), 1.65 - 1.47 (m, 13H). 13C NMR (CDCl\(_3\)) \( \delta \): 203.78, 55.19, 31.31, 30.70, 25.98, 24.11. IR (thin film, KBr plates), \( \nu \) (cm\(^{-1}\)): 2937, 2862, 2728, 1721, 1456, 1409, 1268. HRMS (ESI) [M+Na]\(^+\): Calcd for C\(_{10}\)H\(_{16}\)O \( m/\zeta = 175.1099 \), found \( m/\zeta = 175.1098 \).
**Ethyl cyano(cyclohexylidene)acetate (2.8a).** Compound 2.8a was synthesized based on a literature procedure. Ethyl cyanoacetate (56.5 g, 0.5 mol), cyclohexanone (58.9 g, 0.6 mol), ammonium acetate (3.85 g, 0.05 mol), and acetic acid (6 g, 0.1 mol) were added to benzene (50 mL) in a round bottom flask connected to a Dean and Stark apparatus. The solution was refluxed for 6 h and then washed three times with water. The organic layer was dried and concentrated. The product was purified by distillation to give 2.8a (60.6 g, 0.31 mol) as a clear liquid in 63% yield. \(^1\)H NMR (CDCl\(_3\)) \(\delta\): 4.26 (q, \(J = 7.0\) Hz, 2H), 2.97 (t, \(J = 5.8\) Hz, 2H), 2.70 - 2.60 (m, 2H), 1.85 - 1.57 (m, 6H), 1.34 (t, \(J = 7.2\) Hz, 3H). \(^{13}\)C NMR (CDCl\(_3\)) \(\delta\): 180.03, 162.03, 115.63, 102.06, 61.72, 36.91, 31.59, 28.59, 28.26, 25.65, 14.09. IR (thin film, KBr plates) \(\nu\) (cm\(^{-1}\)): 2939, 2861, 2224, 1729, 1601, 1447, 1367, 1201. Spectral data agree with those previously reported.\(^{30}\)

**Ethyl cyano(1-methylcyclohexyl)acetate (2.8b).** Compound 2.8b was synthesized based on the literature procedures.\(^{8, 26}\) Iodomethane (11 g, 77 mmol) in diethyl ether (10 mL) was slowly added to magnesium turnings (1.83 g, 76 mmol) in diethyl ether (10 mL) at 0°C and the solution was stirred for 30 minutes. Copper chloride (100 mg, 1 mmol) was added, and 2.8a (9.66 g, 50 mmol) in diethyl ether (10 mL) was slowly added. The solution was stirred for 30 minutes and then added to 10% sulfuric acid (40 mL) in ice (50 g). The aqueous phase was extracted three times with diethyl ether. The combined organic layer was dried and concentrated. The resulting residue was purified by flash chromatography eluting with hexanes/ethyl acetate (9:1) to give 2.8b (10.4 g, 49.7 mmol) as a yellowish liquid in 99% yield. \(^1\)H NMR (CDCl\(_3\)) \(\delta\): 4.32 - 4.19 (m, 2H), 3.45 (s, 1H), 1.62 - 1.37 (m, 10H), 1.31 (t, \(J = 7.0\) Hz, 3H), 1.14 (s, 3H). \(^{13}\)C NMR (CDCl\(_3\)) \(\delta\): 165.26, 115.88, 62.20, 48.21, 37.62, 36.25, 35.36, 25.45, 21.93, 21.60, 21.55, 14.04. IR (thin film, KBr plates), \(\nu\) (cm\(^{-1}\)): 2939, 2861, 2224, 1729, 1601, 1447, 1367, 1201.
2933, 2861, 2247, 1742, 1601, 1448, 1388, 1370, 1325. Spectral data match those previously reported.²⁶

\[\begin{align*}
\text{(1-Methylcyclohexyl)acetonitrile (2.8d). Compound 2.8d} \text{ was synthesized based on a literature procedure.}^8 \text{ Compound 2.8b (5.2 g, 25 mmol) was added to a solution containing 85% potassium hydroxide (7.5 mL), water (33 mL), and ethanol (6.5 mL). The solution was refluxed for 6 hours and then concentrated. The resulting residue was refluxed for 4 h in 20% sulfuric acid (40.3 mL). The solution was cooled to room temperature and extracted with diethyl ether. The organic layer was dried and concentrated. A catalytic amount of copper powder (70 mg) was added to the resulting residue (2.8c) and the suspension was slowly heated to 170°C. The suspension was then allowed to cool back to room temperature and was purified by flash chromatography, eluting with hexanes/ethyl acetate (19:1) to give 2.8d (2.2 g, 16 mmol) as a yellowish liquid in 64% yield. }^{1} \text{H NMR (CDCl}_3) \delta: 2.25 (s, 2H), 1.63 - 1.31 (m, 10H), 1.07 (s, 3H). \text{ }^{13} \text{C NMR (CDCl}_3) \delta: 118.44, 36.95, 33.01, 30.33, 25.75, 25.24, 21.88. \text{ IR (thin film, KBr plates) } \nu (\text{cm}^{-1}): 2929, 2855, 2242, 1742, 1453, 1422, 1384.}
\end{align*}\]

\[\begin{align*}
\text{2-(1-Methylcyclohexyl)ethanol (2.8f). Compound 2.8d} \text{ was hydrolyzed based on a literature procedure.}^{27} \text{ Compound 2.8d (1.89 g, 13.8 mmol) was refluxed in a solution with sulfuric acid (14 mL) and water (16.8 mL) for 6 h. The solution was cooled to room temperature, diluted with water, and extracted with diethyl ether. The organic layer was dried and concentrated. The resulting residue 2.8e was LAH reduced according to the general procedure and purified by flash chromatography eluting with hexanes/ethyl acetate (9:1) to give 2.8f (710 mg, 5 mmol) as a yellowish liquid in 36% yield. }^{1} \text{H NMR (CDCl}_3) \delta: 3.75 - 3.65 (m, 2H), 1.61 - 1.48 (m, 2H), 1.48 - 1.17 (m, 11H), 0.90 (s, 3H). \text{ }^{13} \text{C NMR (CDCl}_3) \delta: 59.45, 44.49,
\end{align*}\]
(1-Methylcyclohexyl)acetaldehyde (2.8). Compound **2.8d** (100 mg, 0.70 mmol) was PCC oxidized according to the general procedure to give **2.8** (62 mg, 0.44 mmol) in 63% yield. $^1$H NMR (CDCl$_3$) $\delta$: 9.86 (t, $J = 3.3$ Hz, 1H), 2.29 (d, $J = 3.0$ Hz, 2H), 1.55 - 1.31 (m, 10H), 1.08 (s, 3H). $^{13}$C NMR (CDCl$_3$) $\delta$: 203.96, 54.53, 38.18, 33.69, 26.01, 25.77, 21.78. IR (thin film, KBr plates), $\nu$ (cm$^{-1}$): 3330 (broad), 2925, 2851, 1453, 1043, 1019.

**Ethyl cyano(1-ethylcyclohexyl)acetate (2.9b).** Compound **2.9b** was synthesized based on literature procedures.\(^8\) \(^26\) Bromoethane (8.7 g, 80 mmol) in diethyl ether (10 mL) was slowly added to magnesium turnings (1.83 g, 76 mmol) in diethyl ether (10 mL) at 0°C and the solution was stirred for 30 minutes. Copper chloride (50 mg, 0.5 mmol) was added, and **2.8a** (9.06 g, 50 mmol) in diethyl ether (10 mL) was slowly added. The solution was stirred for 30 minutes and then added to 10% sulfuric acid (40 mL) in ice (50 g). The aqueous phase was extracted three times with diethyl ether. The combined organic layer was dried and concentrated. The resulting residue was purified by flash chromatography eluting with hexanes/ethyl acetate (9:1) to give **2.9b** (3.6 g, 16.2 mmol) as a clear liquid in 32% yield. $^1$H NMR (CDCl$_3$) $\delta$: 4.25 (q, $J = 7.2$ Hz, 2H), 3.61 (s, 1H), 1.76 - 1.40 (m, 12H), 1.32 (t, $J = 7.0$ Hz, 3H), 0.89 (t, $J = 7.4$ Hz, 3H). $^{13}$C NMR (CDCl$_3$) $\delta$: 165.56, 116.26, 62.29, 45.57, 40.50, 32.54, 32.37, 26.66, 25.39, 21.31, 21.28, 14.08, 7.42. IR (thin film, KBr plates) $\nu$ (cm$^{-1}$): 2935, 2865, 2246, 1741, 1456, 1387, 1369, 1323. HRMS (ESI) [M+Na]$^+$: Calcd for C$_{13}$H$_{21}$NO$_2$ m/z = 246.1464, found m/z = 246.1471.

(1-Ethylcyclohexyl)acetonitrile (2.9d). Compound **2.9d** was synthesized based on a literature procedure.\(^8\) Compound **2.9b** (3.5 g, 15.7 mmol) was added to a solution
containing 85% potassium hydroxide (5.1 mL), water (22.2 mL), and ethanol (4.38 mL). The solution was refluxed for 6 h and then concentrated. The resulting residue was refluxed for 4 h in 20% sulfuric acid (27.1 mL). The solution was cooled to room temperature and extracted with diethyl ether. The organic layer was dried and concentrated. A catalytic amount of copper powder (60 mg) was added to the resulting residue (2.9c) and the suspension was slowly heated to 170°C. The suspension was then allowed to cool back to room temperature and was purified by flash chromatography, eluting with hexanes/ethyl acetate (19:1) to give 2.9d (1.4 g, 9.3 mmol) as a clear liquid in 59% yield. \(^1\)H NMR (CDCl\(_3\)) \(\delta\): 2.28 (s, 2H), 1.58 - 1.36 (m, 12H), 0.92 - 0.81 (m, 3H). \(^{13}\)C NMR (CDCl\(_3\)) \(\delta\): 118.49, 35.38, 34.65, 30.00, 26.07, 25.80, 21.52, 7.36. IR (thin film, KBr plates) \(\nu\) (cm\(^{-1}\)): 2966, 2929, 2857, 2243. HRMS (ESI) [M+Na]+: Calcd for C\(_{10}\)H\(_{17}\)N m/z = 174.1253, found m/z = 174.1266.

\(-\text{(1-Ethylcyclohexyl)ethanol (2.9f).}\) Compound 2.9d was hydrolyzed based on a literature procedure.\(^{27}\) Compound 2.9d (1.26 g, 8.3 mmol) was refluxed in a solution of sulfuric acid (9.3 mL) and water (11.2 mL) for 6 hours. The solution was cooled to room temperature, diluted with water, and extracted with diethyl ether. The organic layer was dried and concentrated. The resulting residue 2.9e was LAH reduced according to the general procedure and purified by flash chromatography eluting with hexanes/ethyl acetate (9:1) to give 2.9f (690 mg, 4.4 mmol) as a yellowish liquid in 53% yield. \(^1\)H NMR (CDCl\(_3\)) \(\delta\): 3.71 - 3.60 (m, 2H), 1.61 - 1.47 (m, 2H), 1.47 - 1.20 (m, 13H), 0.79 (t, \(J = 7.4\) Hz, 3H). \(^{13}\)C NMR (CDCl\(_3\)) \(\delta\): 59.15, 39.20, 35.79, 34.37, 29.67, 26.47, 21.60, 7.42. IR (thin film, KBr plates) \(\nu\) (cm\(^{-1}\)): 3329 (broad), 2925, 2859, 1460, 1379.
(1-Ethylcyclohexyl)acetaldehyde (2.9). Compound 2.9d (100 mg, 0.64 mmol) was PCC oxidized according to the general procedure to give 2.9 (70 mg, 0.45 mmol) in 71% yield. $^1$H NMR (CDCl$_3$) $\delta$: 9.84 (t, $J = 3.3$ Hz, 1H), 2.31 (d, $J = 3.3$ Hz, 2H), 1.53 - 1.35 (m, 12H), 0.90 - 0.80 (m, 3H). $^{13}$C NMR (CDCl$_3$) $\delta$: 204.15, 49.99, 36.47, 35.80, 30.58, 26.10, 21.48, 7.48. IR (thin film, KBr plates), $\nu$ (cm$^{-1}$): 2928, 2856, 2726, 1720, 1456, 1381. HRMS (ESI) [M+Na]$^+$: Calcd for C$_{10}$H$_{18}$O $m/z = 177.1255$, found $m/z = 177.1275$.

2-(Bicyclo[2.2.1]hept-2-yl)ethanol (2.10a). 2-Norbornaneacetic acid (1 g, 6.5 mmol) was LAH reduced according to the general procedure to the alcohol 2.10a (780 mg, 5.6 mmol) in 86% yield as the exo (cis) product. $^1$H NMR (CDCl$_3$) $\delta$: 3.61 (t, $J = 6.7$ Hz, 2H), 2.18 (br. s., 1H), 1.94 (br. s., 1H), 1.66 - 1.26 (m, 8H), 1.23 - 0.96 (m, 4H). $^{13}$C NMR (CDCl$_3$) $\delta$: 61.66, 41.14, 39.94, 38.34, 38.10, 36.55, 35.31, 30.07, 28.76. IR (thin film, KBr plates), $\nu$ (cm$^{-1}$): 3330 (broad), 2948, 2869, 1455, 1313, 1053, 1030, 986. Spectral data match those previously reported.$^{31-33}$

Bicyclo[2.2.1]hept-2-ylacetaldheyde (2.10). Compound 2.10a (100 mg, 0.71 mmol) was PCC oxidized according to the general procedure to the aldehyde 2.10 (72 mg, 0.52 mmol) in 73% yield as the exo (cis) product. $^1$H NMR (CDCl$_3$) $\delta$: 9.71 (t, $J = 1.9$ Hz, 1H), 2.45 - 2.19 (m, 3H), 2.00 - 1.89 (m, 2H), 1.61 - 1.42 (m, 3H), 1.34 - 1.01 (m, 5H). $^{13}$C NMR (CDCl$_3$) $\delta$: 202.58, 51.07, 41.11, 37.93, 36.69, 35.86, 35.21, 29.77, 28.50. IR (thin film, KBr plates), $\nu$ (cm$^{-1}$): 2950, 2870, 2818, 2716, 1724.

(6,6-dimethylbicyclo[3.1.1]hept-2-yl)methanol (2.11a). Compound 2.11a was synthesized from a hydroboration-oxidation reaction.$^{29}$ (-)-b-Pinene (10 g, 73.4 mmol) was dissolved in THF (160 mL) and 1M borane tetrahydrofuran complex solution in THF (36 mL, 36 mmol) was slowly added. The solution was stirred for 1 h at room
temperature. The solution was cooled to 0°C and water (7.4 mL) was slowly added to quench the reaction followed by 3M NaOH (10 mL) and 30% hydrogen peroxide (10 mL). The solution was stirred at room temperature for 2 h and then water (100 mL) was added. The layers were separated and the aqueous layer was extracted with diethyl ether. The combined organic layer was dried, concentrated, and the product was purified by flash chromatography eluting with hexanes/ethyl acetate (49:1) to give the alcohol 2.11a (6.8 g, 44 mmol) as a clear liquid in 60% yield. \(^1\)H NMR (CDCl\(_3\)) \(\delta\): 3.67 - 3.51 (m, 2H), 2.42 - 2.31 (m, 1H), 2.25 (t, \(J = 7.4 \) Hz, 1H), 2.06 - 1.82 (m, 5H), 1.56 - 1.29 (m, 2H), 1.29 - 1.14 (m, 3H), 1.01 - 0.88 (m, 4H). \(^13\)C NMR (CDCl\(_3\)) \(\delta\): 67.85, 44.49, 42.88, 41.49, 38.66, 33.15, 27.98, 26.00, 23.34, 18.77. IR (thin film, KBr plates) \(\nu\) (cm\(^{-1}\)): 3330 (broad), 2948, 2869.

6,6-Dimethylbicyclo[3.1.1]heptanes-2-carbaldehyde (2.11b). Compound 2.11a (6.8 g, 44 mmol) was PCC oxidized according to the general procedure to the aldehyde 2.11b which was purified by flash chromatography eluting with hexanes/ethyl acetate (19:1) to give 2.11b (2.1 g, 13.8 mmol) as a clear oil in 31% yield. \(^1\)H NMR (CDCl\(_3\)) \(\delta\): 9.76 (s, 1H), 2.79 - 2.69 (m, 1H), 2.58 - 2.50 (m, 1H), 2.44 - 2.19 (m, 2H), 1.98 - 1.79 (m, 4H), 1.30 - 1.16 (m, 4H), 0.71 (s, 3H). Spectral data match those previously reported.\(^{34}\)

2-Ethenyl-6,6-dimethylbicyclo[3.1.1]heptanes (2.11c). Compound 2.11c was synthesized based on a literature procedure.\(^2\) 1.6 M Butyllithium in hexane (16.8 mL, 26.9 mmol) was added to a solution of methyltriphenyl phosphonium bromide (12.3 g, 34.4 mmol) in THF at 0°C. After 30 minutes, the solution was cooled to -75°C and 2.11b (3.5 g, 23.0 mmol) was slowly added. The solution was stirred for 3 h at -75°C. The reaction was quenched with ammonium chloride solution and the organic layer was dried and concentrated. The product was purified by flash chromatography, eluting with hexanes to give 2.11c (820 mg, 5.46 mmol)
in 24% yield. $^1$H NMR (CDCl$_3$) $\delta$: 6.05 - 5.92 (m, 1H), 4.96 - 4.82 (m, 2H), 2.71 (br. s., 1H), 2.32 (br. s., 1H), 1.97-1.88 (m, 5H), 1.65 (d, $J = 9.4$ Hz, 1H), 1.19 (s, 3H), 1.03 - 0.92 (m, 3H).

2-(6,6-Dimethylbicyclo[3.1.1]hept-2-yl)ethanol (2.11d). Compound 2.11c was subjected to a hydroboration-oxidation reaction.$^{29}$ Compound 2.11c (800 mg, 5.32 mmol) was dissolved in THF (12.8 mL) and 1 M borane tetrahydrofuran complex solution in THF (5.0 mL, 5 mmol) was slowly added. The solution was stirred for 1 h at room temperature. The solution was cooled to 0°C and water was slowly added to quench the reaction followed by 3 M NaOH (0.8 mL) and 30% hydrogen peroxide (1 mL, 8.82 mmol). The solution was stirred at room temperature for 2 h and then quenched with water. The layers were separated and the aqueous layer was extracted with diethyl ether. The combined organic layer was dried, concentrated, and the product was purified by flash chromatography eluting with hexanes/ethyl acetate (9:1) to give the alcohol 2.11d in 67% yield. $^1$H NMR (CDCl$_3$) $\delta$: 3.65 (dt, $J = 3.4$, 6.8 Hz, 2H), 2.33 (d, $J = 7.2$ Hz, 1H), 2.11 (d, $J = 7.4$ Hz, 1H), 2.02 - 1.78 (m, 4H), 1.68 (q, $J = 7.1$ Hz, 2H), 1.59 - 1.31 (m, 2H), 1.18 (s, 3H), 1.04 - 0.80 (m, 5H). $^{13}$C NMR (CDCl$_3$) $\delta$: 61.71, 46.40, 41.43, 40.74, 38.71, 37.47, 33.63, 28.18, 26.44, 23.25, 22.35. IR (thin film, KBr plates) $\nu$ (cm$^{-1}$): 3325 (broad), 2935, 2907, 1468, 1383, 1366.

(6,6-dimethylbicyclo[3.1.1]hept-2-yl)acetaldehyde (2.11). Compound 2.11d (100 mg, 0.6 mmol) was PCC oxidized according to the general procedure to the aldehyde 2.11 (24 mg, 0.14 mmol) in 24% yield (95.5% cis product by GC/MS). $^1$H NMR (CDCl$_3$) $\delta$: 9.71 (t, $J = 2.1$ Hz, 1H), 2.70 - 2.30 (m, 4H), 2.14 - 1.79 (m, 6H), 1.52 - 1.39 (m, 1H), 1.19 (s, 3H), 1.06 - 0.85 (m, 5H). $^{13}$C NMR (CDCl$_3$) $\delta$: 203.39, 52.51, 46.78, 41.55, 39.16, 35.53, 33.89, 28.42, 26.62, 23.66, 22.51. IR (thin film, KBr plates), $\nu$ (cm$^{-1}$): 2984, 2941, 2909, 2869, 2814, 2713, 1725, 1468, 1408, 1384, 1367. Spectral data match those previously reported.$^{35,36}$
**2-(1-Adamantyl)ethanal (2.12).** 2-(1-Adamantyl)ethanol (200 mg, 1.1 mmol was PCC oxidized according to the general procedure to the aldehyde 2.12 (140 mg, 0.79 mmol) in 71% yield. This compound was noticed to have a camphoraceous odor. \(^1\)H NMR (CDCl\(_3\)) \(\delta\): 9.87 (t, \(J = 3.2\) Hz, 1H), 2.13 (d, \(J = 3.0\) Hz, 2H), 1.99 (br. s., 3H), 1.78 - 1.59 (m, 12H). \(^{13}\)C NMR (CDCl\(_3\)) \(\delta\): 203.59, 57.25, 42.73, 36.65, 33.34, 28.49. IR (thin film, KBr plates), \(\nu\) (cm\(^{-1}\)): 2903, 2848, 2728, 1721, 1450, 1406, 1362, 1346. Spectral data match those previously reported.\(^{37,38}\)

**Mouse OR-I7 HEK293 Glosensor Assay** (from collaborator Jianghai Ho experiments in Matsunami Lab, Duke University Medical Center)

The GloSensor cAMP Assay System (Promega) was used according to manufacturer’s instructions with slight modifications as previously described.\(^2\) A plasmid encoding Rho-tagged mouse OR-I7 (80 ng/well) was transfected into the Hana3A cell line in 96-well plate format along with plasmids encoding the human receptor trafficking protein, RTP1S (10 ng/well), type 3 muscarinic acetylcholine receptor (M3-R) (10 ng/well), and pGloSensor TM-22F (10 ng/well). Then, 18 to 24 h following transfection, cells were loaded with 2% GloSensor reagent for 2 h and treated with compounds in a total volume of 74 \(\mu\)L. Luminescence was measured using a Polarstar Optima plate reader (BMG) with a time interval of 90 seconds per well. Data was analyzed and IC\(_{50}\)s were estimated using Prism 5.0 and Microsoft Excel. Responses over \(t=3-7.5\) minutes were summed, base-lined, normalized, and plotted versus odorant concentration (Figures 2.12, 2.13, and 2.14).

**Homology Model Construction and Ligand Docking** (Experiments done in the lab of Victor Batista, Yale University, Department of Chemistry)
**General procedure for evaporation assay**

These experiments were mock experiments, done without the transfected cells, but otherwise identical to our collaborator’s lab at Duke University. The solute evaporation was monitored to learn whether there were different odorant evaporation rates in the biological testing. The odorant was diluted with DMSO to 1.0 M, and the 1.0 M DMSO solution was diluted with CD293 buffer (or water) to 0.1 mM. Designated volumes of the 0.1 mM solution were transferred into 96-well plates and incubated at 37°C for the designated timespan. The 96-well plate was then placed on top of ice and the odorant solutions were pooled. An aliquot of the pooled solution was extracted with an equal volume of chloroform or a 0.1 mM 1-bromoctane solution in chloroform as an internal standard. The layers were separated and the organic layer was diluted with 2.0 mL of chloroform, dried with magnesium sulfate and analyzed by GC/MS. GC/MS analyses were obtained on a Shimadzu GC/MS-QP2010 instrument. Temperature program for GC analysis was as follows: Injection temperature = 250°C; held at 65°C for 1 minute, heated from 65°C to 80°C at 5°C/minute, held at 80°C for 1 minute, heated from 80°C to 120°C at 15°C/minute, held at 120°C for 1 minute, heated from 120°C to 200°C at 40°C/minute, and held at 200°C for 3 minutes. The amount of odorant in the solution was determined relative to the peak area or relative to 2,2-difluoroctanal or 1-bromoctane (as an internal standard). The percentage of odorant remaining over time was compared to a sample that was extracted prior to the incubation, designated as time = 0 minute.

**Pre-exposure assay**

For the experiment requiring pre-exposure to the odorants, 400 μL of the following solution in water was separately added into the 96-well plates prior to the experiment: water, 0.1 mM octanal, 0.1 mM 1-octanol, 0.1 mM 2,2-difluoroctanal, 1 mM octanal, 1 mM 1-octanol. The
solutions were incubated at 37°C for 4 hours uncovered. Afterwards, the solutions were discarded, and the well plates were rinsed three times with water and left to dry overnight. During the experiment, 200 µL of 0.1 mM octanal and 2,2-difluoroctanal was added to the well plates. The 96-well plates were covered with sealing mats (ThermoScientific, catalog #4411-11) and treated as described in the general procedure.
Chapter 3: Olfactory Receptor Antagonism in the Mammalian Olfactory System

3.1. Introduction

3.1.1. Combinatorial receptor coding

Olfaction is one of the major senses used to analyze the physical environment. Rodents have about 1100 olfactory receptor genes and humans have about 390 olfactory receptor genes, plus many pseudogenes.\(^1\) Each odorant can activate multiple olfactory receptors and each olfactory receptor can be activated by multiple odorants, suggesting that each odorant has a specific combinatorial olfactory code for generating its unique smell.\(^1\) An odor was generally believed to be linked to the activation of a set of receptors by specific odorant(s), but mixtures have long been noted to elicit lower response than the individual components,\(^2\) suggesting the possibility of odorants to act as inhibitors as well as activators. Recently, odorants were identified that antagonized olfactory receptors through binding to the receptors without activating them.\(^3\)\(^-\)\(^5\) These antagonists inhibit the action of agonists. Thus, in a mixture, some receptors may have their response modulated due to antagonism, and the original olfactory code of the individual odorants might become much more complex, but currently, it is uncertain whether antagonism is an important contributor to the olfactory code in natural product mixtures and whole animals.

3.1.2. Antagonism at the receptor level

<table>
<thead>
<tr>
<th>OR-I7</th>
<th>mOR-EG</th>
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<tbody>
<tr>
<td>Octanal</td>
<td>Eugenol</td>
</tr>
<tr>
<td>Agonist</td>
<td>Agonist</td>
</tr>
<tr>
<td>Cyclohexylethanal</td>
<td>Methyl isoeugenol</td>
</tr>
<tr>
<td>Antagonist</td>
<td>Antagonist</td>
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</table>

Agonist and antagonist of OR-I7 and mOR-EG.
Octanal, an eight-carbon, aliphatic aldehyde, activates the aldehyde receptor OR-I7 when applied alone, but its response has been shown to decrease when co-applied with citral\textsuperscript{3} and cyclohexylethanal,\textsuperscript{5} both now considered antagonists of the OR-I7 receptor. Octanal’s olfactory code, and consequently its smell, would presumably be modified in a mixture with these compounds. Similarly, the response of eugenol (100 µM) in the mOR-EG receptor was eliminated in a mixture containing high concentration (1 mM) of methyl isoeugenol.\textsuperscript{4} For each of these rodent receptors, the antagonists were structurally similar to the agonist, which may mean they act at the orthosteric site. Octanal differs from cyclohexylethanal by only its extended chain length, and eugenol differs from methyl isoeugenol by the functional groups on the phenyl ring and the position of a double bond. Antagonism had also been seen in human olfactory receptors. The human receptor, OR1G1, could be activated by several odorants, with 9-carbons being the optimal chain length, and the agonist-induced response was inhibited by several shorter 6-carbons odorants.\textsuperscript{6} These findings demonstrated that the response of odorants can be antagonized at the receptor, and structural variations in chain length and functional group, can change an agonist into an antagonist. In these cases, where the structure of the antagonist is related to the structure of the agonist (activating odorant ligand), it is assumed that the antagonist targets the same or overlapping binding site as the activating ligand, though this does not need to be the case as allosteric antagonists are known for other GPCRs.\textsuperscript{7}

While the aforementioned receptors are one of the many receptors that are activated by octanal and eugenol, they cannot be representative of the whole receptor set that generate the smell of each of these odorant. Octanal at 30 µM was found to activate 144 cells (6%) out of 2301 cells from rats,\textsuperscript{3} and eugenol at 100 µM could activate 95 cells (3%) out of 3000 cells\textsuperscript{4} in previous experiments. Since each mature olfactory sensory neuron (OSN) express only one
olfactory receptor, it’s possible that octanal’s and eugenol’s olfactory code involve several olfactory receptors. However, antagonizing the response of a single receptor may be sufficient to change the odorant’s smell. Currently, little is known about the degree at which antagonism affects the olfactory code and there is limited information on the determinants of antagonism in olfactory receptors. One way to study antagonism is to look at all (or a sampling of) the ORs in the mammalian olfactory system. Activation of an OR leads to an increase in the intramolecular calcium level, allowing a large repertoire of olfactory receptors to be screened and profiled at once via calcium imaging. Since each olfactory sensory neuron expresses only one olfactory receptor, the response of an OR can be determined, with and without an antagonist, by studying the calcium level of olfactory sensory neurons.

<table>
<thead>
<tr>
<th>Designated Agonist</th>
<th>Tested as Antagonist</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Functional Group vs Length/Conformation</td>
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<tr>
<td><strong>Experiment A:</strong></td>
<td></td>
</tr>
<tr>
<td>1-Octanol (3.1)</td>
<td>Octanal (3.2) Octanoic acid (3.3) Cyclohexylethanol (3.4)</td>
</tr>
<tr>
<td><strong>Experiment B:</strong></td>
<td></td>
</tr>
<tr>
<td>Octanal (3.2)</td>
<td>1-Octanol (3.1) Octanoic acid (3.3) Cyclohexylethanal (3.5)</td>
</tr>
<tr>
<td><strong>Experiment C:</strong></td>
<td></td>
</tr>
<tr>
<td>Octanoic acid (3.3)</td>
<td>1-Octanol (3.1) Octanal (3.2) Cyclohexylethanoic acid (3.6)</td>
</tr>
</tbody>
</table>

Table 3.1: Agonists and antagonists tested in OSNs. In Exp. A, 1-octanol (3.1) was treated as the agonist. In Exp. B, octanal (3.2) was treated as the agonist. In Exp. C, octanoic acid (3.3) was treated as the agonist. The individual odorants were first applied alone and then co-applied with the agonist.

### 3.1.3. Factors causing mixture suppression

In a mixture, reduced activation, compared to the individual components, may be attributed to other factors besides competition with the agonist for the active site. Other than
competing with the agonist for the orthosteric or active site, an odorant may also inhibit the receptor through binding to an allosteric site, another site on the receptor besides the orthosteric site. Additionally, an odorant may cause reduction in activity by activating alternative pathways. For instance, ORs have been found to display phosphoinositide 3-kinase (PI3K)-dependent antagonism with odorant pairs.\textsuperscript{10} Activation of PI3K would stimulate the production of phosphatidylinositol (3,4,5) triphosphate (PIP\textsubscript{3}) which negatively regulates the sensitivity of the ion channel and thereby reduce the signal of the receptor. However, the activation of two functionally distinct targets is expected to require very different ligands. The orthosteric and allosteric sites usually bind to structurally distinct molecules, and PI3K-dependent antagonism was achieved by odorant pairs with few structural similarities. For instance, the only structural similarity between citral and 1-octanol, which could be inhibited by citral in a PI3K-dependent manner,\textsuperscript{10} was their extended carbon chain, whereas their degree of saturation, functional groups, and the number of carbons are all different. In summary, very little is known about the structural attributes that distinguish an agonist from an antagonist in the orthosteric site of a receptor. It is expected that they will share some structural features, but it’s not clear what these features are.

\textbf{3.1.4. Experimental design: Length vs. functional group}

In this chapter, we aim to uncover the relative importance of the functional group (alcohol, aldehyde, or carboxylic acid) and of the carbon chain (linear vs. cyclic 8-carbon chain) in a presumed orthosteric antagonist. To reduce the possibility of an odorant modifying the response of a receptor through an allosteric site or a separate pathway, there should be minimal structural differences between the two odorants tested in the binary mixtures. The odorants in our study were designed to only differ from each other by either their chain length or their functional group. When the chain length is changed, the number of carbons is kept constant, but they are
conformationally restricted in a cyclohexyl ring. Thus, any decline in activity would most likely be linked to these structurally-related odorants competing for the same binding site on the receptor rather than stimulating distant sites or pathways. Accordingly, these two variations, chain length and functional group, were simultaneously evaluated as possible determinants of antagonism. In this study, the degree of antagonism elicited by binary mixtures in olfactory sensory neurons was analyzed (Table 3.1).

The first experiment (please refer to Table 3.1) focused on all of the receptors that can be activated by 1-octanol (3.1). In the activated cells, 1-octanol (3.1) was applied as a binary mixture with octanal (3.2), octanoic acid (3.3), or cyclohexylethanol (3.4). Octanal (3.2) and octanoic acid (3.3) have the same number of carbons and unconstrained chain length as 1-octanol (3.1), but a different functional group. These mixtures tested the functional group as a decisive factor for antagonism. Cyclohexylethanol (3.4) has the same number of carbons and functional group as 1-octanol (3.1) but a shorter chain length. The mixture of the two tested for chain length as the source of antagonism. Since the structure of 1-octanol (3.1) is similar to that of octanal (3.2), octanoic acid (3.3), and cyclohexylethanol (3.4) (with the exception of the functional group or chain length), any antagonism seen, would most likely arise from competition for the orthosteric site, rather than from binding to a distinct allosteric site. The second experiment was conducted in a similar manner, except the designated agonist was switched from 1-octanol (3.1) to octanal (3.2). Octanal (3.2) was tested in a mixture with 1-octanol (3.1) or octanoic acid (3.3) to look at functional group and with cyclohexylethanal (3.5) to look at carbon chain length or shape as the determinant of antagonism. The same was done with octanoic acid (3.3) as the agonist. Octanoic acid (3.3) was tested in binary mixtures with 1-octanol (3.1) or octanal (3.2) to study the effect of functional group and cyclohexylethanoic acid (3.6) to study the effect of chain
length on antagonism. The response of endogenous ORs to the individual compounds and their binary mixtures in live mouse olfactory sensory neurons was obtained through calcium imaging.

The odorant vehicle, or solvent, DMSO, was first applied to the cell as a negative control. Cells that respond to the solvent were not considered odorant-responding cells. Individual odorants were applied to the cells at the beginning of the experiment and again at the end of the experiment. Cells that failed to respond at the beginning or end (possibly due to sensitization or desensitization of the receptor) were not considered as an odorant-responding cell. Cells that gave irregular responses (i.e. noisy responses or a response when no odorant was applied) were not considered odorant-responding cells. Forskolin was applied at the end of the calcium imaging experiment to determine if the signal transduction pathway was still functioning. Forskolin is a molecule that bypasses the olfactory receptor to activate adenylyl cyclase. All cells that responded to forskolin were considered viable cells regardless of whether they responded to the odorants (viable cells may respond to functional groups and carbon chain other than the ones that were tested in this experiment). During calcium imaging screens with individual organic odorants, it is common to activate between 0 - 8% of the set of forskolin-responding cells (about 2000 cells per mouse).

Different ORs will probably be activated and antagonized at different ligand concentrations. Since we cannot test all concentrations, 60 µM was the concentration chosen for this study. The odorant responses were in all cases normalized to the response of forskolin. Responses (of individual odorants) that were consistently lower than 10% of the response of forskolin were omitted. An inhibitory, and possibly antagonistic, response was defined as a 70-100% reduction in the agonist’s response (compared to when the agonist was applied alone).
3.2. Results and Discussion:

The rodent olfactory system consists of around 1100 ORs, and the response profiles of over 10,000 viable cells (forskolin responding cells) were obtained and analyzed in total. Although the identity of the OR expressed in each cell was unknown, we aim to profile a majority of the olfactory receptor types through analyzing 10-fold the number of receptors. Olfactory sensory neurons were exposed to 60 µM of each odorant and then binary mixtures that contain the designated agonist and one other odorant at 60 µM. If the odorant is not an antagonist for the receptor expressed in an observed cell, then there should be no reduction in the response of the binary mixtures compared to the response of the agonist, but if the odorant is antagonizing the response of the agonist, the binary mixture will display a reduction in signal (Figure 3.1).

\[
\% \text{ Antagonism} = \frac{y}{x+y} \times 100\%
\]

Figure 3.1: Calcium imaging response of cells. A baseline was drawn from the DMSO response to the forskolin response and a line was drawn between the two peak signals of the designated agonist, applied before and after the binary mixtures. The height of the binary mixture response, \(x\), and the height between the binary mixture response and the interpolated agonist response, \(y\), were measured. To calculate the percent antagonism (signal reduction), \(y\) was divided by \(x+y\) and multiplied by 100%. Only cells that show at least 70% reduction from the agonist response to the binary mixture response were considered to be inhibited. (The odorant designated as the antagonist was applied 4 seconds before and during application of the agonist.)
Figure 3.2: Percent activation and percent antagonism. Each row represents the relative response of one cell to the four odorants. The response of the cell to the designated agonist in each experiment is shown in the first column. The response of the cell to the other three odorants tested in the experiment is shown in the following three columns. The response of the agonist in each cell was antagonized by the odorant(s) whose compound number is bolded in the last column. The percentage of antagonism (% reduction of the agonist’s response) is given in parentheses.

In order to be considered an antagonist, the molecule must not respond to the cell on its own (partial agonists could also lower the response of full agonists).

3.2.1. Summary of antagonism

The response of the agonist was reduced by at least 70% in 38 total cells (out of 11707 viable cells tested) (Figures 3.2, 3.3). In the first experiment with 1-octanol (3.1) as the agonist, 125 cells responded to 1-octanol (3.1) (out of 3830 viable cells). Out of these 125 cells, antagonism was seen in 7 cells (5.6%). Octanoic acid (3.3) was the inhibitor in 2 cells (1.6%), cyclohexylethanol (3.4) was the inhibitor in 4 cells (3.2%), and 1 cell (0.8%) was inhibited by both octanoic acid (3.3) and cyclohexylethanol (3.4). In the
second experiment with octanal (3.2) as the agonist, 82 cells were activated by octanal (out of 3478 viable cells). Out of these 82 cells, inhibition was seen in 31 cells (37.8%). Two cells (2.4%) were inhibited by 1-octanol (3.1), 12 cells (14.6%) were inhibited by octanoic acid (3.3), 12 cells (14.6%) were inhibited by 3.5, 1 cell (1.2%) was inhibited by both 3.1 and 3.5, 3 cells (3.7%) were inhibited by both 3.3 and 3.5, and 1 cell (1.2%) was inhibited by both 3.1 and 3.3. In the experiment with octanoic acid (3.3) as the agonist, 39 cells (out of 4399 cells) were activated by octanoic acid (3.3), but inhibition was never seen in any of these cells. Out of the three tested agonists, 3.1, 3.2, and 3.3, the cells activated by octanal (3.2) were the most susceptible to inhibition by one or more of the other compounds tested, being inhibited in 37.8% of the cells, followed by 1-octanol (3.1), which was inhibited in 5.6% of the cells, and cells activated by octanoic acid (3.3) was the least susceptible to inhibition (These data are summarized in Figure 3.3).
3.2.2. Competitive Inhibition

We limited our study to only a few structurally similar molecules, so that antagonism would most likely arise from competitive inhibition, but there would always be the chance that reduction in activity was due to other types of inhibition, in addition to the initiation of inhibitory G-protein pathways. To ask if some of the cells showing a reduction in response were due to an antagonist competitively inhibiting the active site, additional experiments were conducted to test
for competitive inhibition.\textsuperscript{4, 11, 12} We note that in all of our experiments, the identity of the OR expressed by the cells is unknown. Due to the nature of the experimental apparatus, we cannot inspect the data during the experiment and change to a different type of experiment on the same day. Consequently, after finding evidence for inhibition (Figure 3.2 and 3.3) we planned a new experiment specifically searching for evidence of pharmacologic antagonism. To determine if the inhibition was competitive, first the concentration of the antagonist was gradually increased, and then the concentration of the agonist was gradually increased. We should see a reduction in the agonist’s response as more antagonists compete with the agonist for the binding site, and we should see the agonist’s response recovering as more agonist is applied to compete with the antagonist for the binding site.

Thus, in a new experiment, where one odorant was kept constant and the other varied by dose, out of 1775 forskolin responding cells, 8 cells (0.45%; compared to 0.46% in Experiment B) were found to show dose-dependent inhibition by a compound designated as an antagonist. As seen for one of the 8 cells in Figure 3.4A, the concentration of the agonist, octanal, was kept constant at 60 µM, while the concentration of the antagonist, 3.5, increased from 30 to 300 µM. The response of the agonist, octanal (3.2), was gradually reduced as higher concentrations of the antagonist, cyclohexylethanal (3.5), were applied. Conversely, in a separate experiment, an example of which is shown in Figure 3.4B, the concentration of the antagonist, 3.5, was kept constant at 60 µM, while the concentration of the agonist, octanal (3.2), increased from 30 to 300 µM. Out of 1473 cells, the response of octanal was rescued in 2 cells (0.14%; compared to 0.46% in Experiment B). In these cells, the response of the agonist, octanal (3.2), was lower when co-applied with 3.5. As the concentration of octanal (3.2) increased, the response of octanal (3.2) was recovered.
In comparison, the percentage of cells that were antagonized by 3.5 in these two experiments (0.45% and 0.14%) was lower than that in Experiment B (0.46%). Since a higher concentration (up to 300 µM compared to only 60 µM in Experiment B) of the antagonist, 3.5, was applied, we expected a higher percentage of cells to be antagonized. Due to experimental limitations, we have no control over the types of receptors that were sampled in each experiment, and the cells that were collected per experiment probably account for less than 1% of the total population of cells in the olfactory epithelium, so each of our experiment entail a stochastic sampling, that is, we cannot be sure the same receptors show up in every sampling. Furthermore, only a low number of cells were antagonized in each separate experiment so that the inclusion or omission of 1 or 2 cells as an antagonized cell would lead to a significant change in the percentages. Differences between experiments are inevitable, but we hope to converge to some general statistics as the total number of cells profiled increase.
Figure 3.4: Cells showing competitive inhibition. (A) Dose-dependent inhibition of octanal (3.2) by cyclohexylethanal (3.5). (B) Response of octanal (3.2) in the absence and presence of cyclohexylethanal (3.5).

3.2.3. Trends between the ability to activate, to antagonize, and to be antagonized (Based on analysis of data from Experiments A, B, and C)

Octanal (3.2), an aldehyde, activated the highest percentage of forskolin-positive cells (3.323%) (in agreement with previous work\textsuperscript{3, 13}), followed by 1-octanol (3.1) (3.152%), an alcohol, and lastly octanoic acid (3.3), a carboxylic acid (0.880%) (Figure 3.5). This ordering holds true for their shorter cyclic counterparts as well, with 3.5, an aldehyde, activating the highest percentage of cells (1.67%), followed by 3.4 (0.52%), an alcohol, and 3.6 (0.21%), a carboxylic acid (Table 3.2). Although octanal (3.2) activated the most cells, it was also antagonized in the highest number of cells (31 cells, 37.8%, compared to 5.6% for octanol and 0% for octanoic acid). Octanoic acid activated the fewest cells, but was antagonized in the least
number of cells (0 cells). Thus, the cells that were activated by the highest-activating odorant (octanal) were also the most susceptible to antagonism and vice versa (i.e. the cells that were activated by the least-activating odorant (octanoic acid) were the least susceptible to antagonism). However, since antagonism could only be seen for cells that were activated, it’s not entirely unexpected for the agonist that activated the highest percentage of cells to also be inhibited the most. One might also state that odorant GPCRs appear to like to bind aldehydes more than alcohols and acids, and binding may lead to activation or antagonism.

In the tested binary mixtures (3.1&3.2, 3.1&3.3, and 3.2&3.3), antagonism was always observed to some extent. In the case of the binary mixtures containing 3.1 and 3.2, 3.2 never inhibited the response of 3.1, but 3.1 could inhibit the response of 3.2 in 4 cells (4.9%). For binary mixtures containing 3.1 and 3.3, 3.1 never inhibited the response of 3.3, whereas 3.3 inhibited the response of 3.1 in 3 cells (2.4%). For binary mixtures of 3.2 and 3.3, 3.2 never inhibited the response of 3.3, but 3.3 inhibited the response of 3.2 in 16 cells (19.5%).
Figure 3.5: Status of each odorant in the 11,707 cells as 1) designated agonist, 2) designated antagonist, and 3) an agonist whose response is inhibited (susceptibility to antagonism). The percentages are all relative to the total 11,707 cells tested.

The response to octanoic acid (3.3), when it was tested as the designated agonist, was never inhibited by any of the other odorants, but octanoic acid (3.3) was able to inhibit the response of the other odorants in several cells. (The odorants were diluted in solutions buffered to pH = 7.3. The odorants do not change the pH of the solution, eliminating the possibility that inhibition occurred due to change in the pH.) With the same chain length, but a different functional group, octanoic acid (3.3) inhibited the response of a total of 3 (out of 125) 1-octanol-responding cells and 16 (out of 82) octanal-responding cells. In comparison, octanal (3.2) did not inhibit the response of any odorant, and antagonism by 1-octanol (3.1) was substantially lower, comprising of only 4 (out of 82) octanal-responding cells and 0 (out of 39) octanoic acid-responding cells. In these experiments, the ability of one odorant to act as the antagonist appeared to be the reverse of their susceptibility to be antagonized (Figure 3.5). Octanal (3.2),
which did not inhibit the response of any cell, was antagonized in a total of 31 cells (out of 82), whereas octanoic acid (3.3), which antagonized a total of 19 cells, was never antagonized in any cell (out of 39). 1-Octanol (3.1) antagonized 4 cells and was antagonized in 7 cells. Hence, the best antagonist is the worst at being antagonized and vice versa.

Although octanal (3.2) activated the most cells, it was never observed to inhibit the response of any cell activated by the other odorants tested. As the least activating odorant of the three functional groups tested, octanoic acid (3.3) was actually found to be the best inhibitor. Concurrently, octanal (3.2) is known to have a strong, citrus, fruity odor, whereas octanoic acid’s rancid smell is much fainter in comparison (though perhaps in part due to its higher boiling point and lower vapor pressure). In regards to the olfactory code, this characteristic of octanoic acid (3.3) suggest that weakly activating odorants might be triggering signal transduction in very few ORs, but they could still be binding to many receptors. Odorants with a faint smell may activate few receptors, but they may nevertheless be effective in masking the odor of highly activating, strong odorants. This speculative statement is suggested by our data.

3.2.4. Effect of chain length and functional group on activation and antagonism

Multiple ORs were seen to be inhibited by the linear carbon chain odorants and/or the shorter cyclic analog (Figure 3.2 and 3.3). The molecular features of chain length and functional group probably play a role in determining antagonism in ORs other than OR-I7 and mOR-EG (mentioned above), but overall, in our limited study, neither emerged as the clearly more important antagonist structural feature since the number of cells antagonized by each category, antagonists with a different chain type, linear or cyclic, (16 out of 38 antagonized cells) and antagonists with different functional groups (17 out of antagonized 38 cells), were similar (the
remaining 5 cells were antagonized by both antagonists with different chain length and antagonists with different functional group).

However, cells that responded to only one odorant, and therefore only one of the three functional groups tested, were more commonly antagonized by the cyclic odorant bearing the same functional group (13 out of 20 antagonized cells, Figure 3.2, top: C1-C3, C4-C5, C7-C11, C14, C19-C20) than were antagonized by the linear odorants bearing a different functional group (5 out of 20 antagonized cells, Figure 3.2, top: C6, C12, C15, C17-C18 (the two remaining antagonized cells, C13 and C16, were inhibited by both a cyclic odorant bearing the same functional group and a linear odorant bearing a different functional group)). These types of cells appear to be selective for the odorant’s functional group, with antagonism or activation being determined by structural features of the carbon chain (in our study, the number of carbons in the chain did not vary).

A majority of the antagonized cells that responded to more than one odorant (11 out of 18 antagonized cells, Figure 3.2, bottom: C21-C22, C25-C26, C28-C33, C35) were inhibited by only octanoic acid (3.3). Since these cells could bind with multiple odorants, binding to these cells conceivably relies on the odorants’ affinity to the active site. These cells may be antagonized by octanoic acid (3.3) due to the higher affinity of octanoic acid for the binding site, possibly through stronger hydrogen bonding with the amino acid residues on the receptor.14
3.2.4.1. Linear vs. cyclic

For all cells that are specific for functional groups, the response of the linear odorants was never higher than that of their cyclic counterpart. Cells which were specific for functional groups usually responded more strongly for the shorter, cyclic odorant. Within the group of cells that responded to both the linear and cyclic odorant bearing the same functional group, cells which responded more strongly for the linear odorant were never encountered though equipotent examples were observed (Figure 3.6A). The responses of the linear odorant were either the same as or lower than the response of the cyclic odorant. Since the linear and cyclic odorants contain the same number of carbons and the same functional group, but differ in their size and chain length, the cells that showed similar responses for the linear and cyclic odorants probably have little preference for the size or chain length. Conversely, the cells showing higher responses for the cyclic odorant probably prefer its more compact, rotationally restricted shape.

The more flexible, linear odorants can adopt a conformation with a similar size and length as the cyclic odorants (Figure 3.6B). While the response of the linear odorant would be weaker due to unfavorable conformational entropy loss and/or steric clashes with amino acids in the receptor, it might still elicit a response in cells that prefer the cyclic odorant. No cells were
observed in which the response of the linear odorant was higher (at the same concentration) than
the response of the cyclic odorant, but there should be cells that prefer the size and length of the
linear odorant. In contrast to the flexible linear odorant, the constrained cyclic odorant could not
adopt the chain length of the longer linear odorants (Figure 3.6B). As with the OR-I7 receptor,
the cyclic odorant might act as an antagonist in this type of cell, binding to the receptor but not
activating it due to failure to reach both the aldehyde and small hydrophobic activation pockets
at the same time.5 Notably, there were as many cells that responded to only the linear odorant but
were inhibited by only the cyclic odorant (3 for 1-octanol, Figure 3.2: C1-C3; 10 for octanal,
Figure 3.2: C4-C5, C7-C11, C14, C19-C20; not applicable to octanoic acid) as the number of
cells that responded to only the linear and cyclic odorants with the same functional group (2 for
1-octanol, Figure 3.6A:C39-C40; 9 for octanal, Figure 3.6A: C41-C49). These inhibited cells
may be cells that were more selective for the chain length of the linear odorant. As with the OR-
I7 receptor, the cyclic odorant was able to compete with the linear odorant for the binding site,
but was unable to activate the receptor, probably because of its shorter chain length.
Cells that responded to octanal (3.2) and octanoic acid (3.3) displayed a similar response pattern as the cells that responded to the linear and cyclic odorants bearing the same functional group. For all cells that responded to both octanal (3.2) and octanoic acid (3.3), the response of octanal (3.2) was never higher than the response of octanoic acid (3.3) (for C55-C56, the response of octanal and octanoic acid were both over 90% when normalized to forskolin, but the response of octanoic acid was noticeably higher (data not shown); the response of octanal was less than 70% of the response of octanoic acid in both C55 and C56) (Figure 3.7A).

At equilibrium in the buffer solution (pH ~ 7.3), octanal (3.2) is partially hydrated to the gem-diol form and octanoic acid (3.3) would be fully deprotonated to a carboxylate. The carboxylate may be able to form stronger hydrogen bonding or carboxylate ionic interactions\textsuperscript{15, 16} with the OR compared to the gem-diol (Figure 3.7B), causing all the cells to produce a weaker response towards octanal (3.2).

The higher affinity that the carboxylate may have for the receptors might explain why octanoic acid (3.3) was the best antagonist out of the three functional groups tested. Octanoic acid (3.3) was able to inhibit the response of octanal (3.2) in 16 cells, whereas octanal (3.2) was
never seen to inhibit the response of octanoic acid (3.3). It is worth mentioning that in several cells that initially responded to both octanal (3.2) and octanoic acid (3.3) individually (octanal giving the lower response), the cells stopped responding to octanal (3.2) by the end of the experiment, but they still responded to octanoic acid (3.3) (these cells were excluded from the final data due to their inconsistent response to octanal, which consequently lowered the actual percentage of octanal and octanoic acid responding cells). Exposure of the receptor to a strong ligand, octanoic acid (3.3), may possibly be desensitizing the response of the weaker ligand, octanal (3.2).

### 3.2.5. Correlation between activation and inhibition

![Chemical structures](image)

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*Tested in Experiments A, B, and C; *aTested in Experiment A; *bTested in Experiment B; *cTested in Experiment C

**Table 3.2:** Percentage of cells that were activated by 60 µM of odorant(s). The responses of 11707 viable cells were analyzed. Four odorants were tested in each experiment. The response to 1-octanol (3.1), octanal (3.2), and octanoic acid (3.3) was tested in every cell. All responses were normalized to the response of forskolin at 10 µM. To calculate the percentages, the number of cells activated by the indicated odorant(s) was divided by the total number of forskolin responding cells and multiplied by 100%.
3.2.5.1 Narrowly vs. broadly tuned cells

Cells that were specific for one odorant among the small set of related compounds tested here (these cells will be referred to as ‘narrowly tuned’ cells) were more common than cells that responded to more than one odorant (these cells will be referred to as ‘broadly tuned’ cells, though in the literature broadly tune usually means capable of being activated by a wide variety of functional groups and carbon chain shapes) (Table 3.2). Of the total 11707 viable cells tested at 60 μM, 5.6% responded to only one of the four odorants tested, 2.3% responded to two out of four odorants, 0.75% responded to three odorants and 0.13% responded to all four odorants. The percentage of responding cells were similar in the individual experiments (Figure 3.8 - Experiment A, with 1-octanol (3.1) tested as the agonist, Experiment B, with octanal (3.2) tested as the agonist, and Experiment C, with octanoic acid (3.3) tested as the agonist). Over half of the odorant-responding cells, responded to only one out of the four odorants tested, suggesting that most cells distinguish between these three closely related functional groups.

![Figure 3.8: Percentage of OSNs responding to 1, 2, 3, or 4 odorants in each experiment. In Experiment A, 1-octanol (3.1) was tested as the agonist. There were 3830 forskolin-responding cells. In Experiment B, octanal (3.2) was tested as the agonist. There were 3478 forskolin-responding cells. In Experiment C, octanoic acid (3.3) was tested as the agonist. There were 4399 forskolin-responding cells.](image-url)
The highest number of antagonized cells (20 cells) came from the ‘narrowly tuned’ cells that responded to only one of the four odorants. The number of antagonized cells gradually went down for the less common cell types - cells responding to two odorants (11 antagonized cells) and cells responding to three odorants (7 antagonized cells). Since the “narrowly tuned” cells were most common, these cells should statistically have a higher chance of being antagonized compared to the less common cell types.

In Figure 3.2, the cells showing antagonism were divided into two groups, those that are ‘narrowly tuned,’ responding to only one odorant, in this case the agonist (Fig. 3.2, top), and those that respond to more than one odorant, including the agonist (Fig. 3.2, bottom). Among the cells that responded to only one out of the four odorants tested, a majority of the cells were antagonized by the cyclic odorant of the same functional group (13 out of 20 antagonized cells), whereas among cells that responded to more than one odorant, a majority were antagonized by octanoic acid (3.3) (11 out of 18 cells showing antagonism). The total number of cells inhibited by antagonists with a shorter chain length than the agonist (16 cells) was similar to the number of cells inhibited by antagonists with a different functional group from the agonist (17 cells), but chain length appeared to be a more common determinant of antagonism among ‘narrowly tuned’ cells, whereas functional group, specifically the carboxyl group of 3.3, appeared to be a more common determinant among ‘broadly tuned’ cells.

Of the ‘broadly tuned’ cells that showed antagonism, the odorant that was antagonized in each cell was never the one that stimulated the highest response. Consequently, the response of the designated agonist was never inhibited in a ‘broadly tuned’ cell wherein the agonist gave the strongest signal. In these antagonized cells, the agonist had the same or a weaker signal than the other odorants that could activate those cells (Figure 3.2, bottom). The agonist may possibly
have a similar or lower efficacy and/or affinity for these receptors, and consequently its response would be easier to antagonize in these cells, just as it was more difficult to antagonize the response of octanoic acid (3.3), which was expected to have a higher affinity for the receptors due to ionic interactions with charged amino acids in addition to hydrogen bonding.

3.2.5.2. 1-Octanol and octanoic acid responding cells

![Diagram of chemical structures]

**Figure 3.9**: Cells that respond to 1-octanol (3.1) and octanoic acid (3.3), but not octanal (3.2).

Among the antagonized cells, there were no cells that could bind to 1-octanol (3.1) and octanoic acid (3.3) (as an antagonist), without also binding to octanal (3.2). In other words, cells responding to the alcohol and acid appeared obligated to also bind the aldehyde. This may relate to how a receptor recognizes and distinguishes these functional groups. The cells that respond only to 1-octanol (3.1) (Fig. 3.2, top) were only antagonized by the cyclic alcohol (3.4), and the cells that were antagonized by octanoic acid (3.3) (Fig. 3.2, bottom) responded to octanal (3.2) in addition to 1-octanol (3.1). Hence, none of the antagonized cells were able to bind with only 1-octanol (3.1) and octanoic acid (3.3).
In related work published by Araneda et al. in 2004, no cells were found to respond only to 1-octanol (3.1) and octanoic acid (3.3), without also responding to octanal (3.2) at 30 µM and 300 µM. However, this observation was based on the response of less than 150 cells that responded to 1-octanol (3.1), octanal (3.2), and/or octanoic acid (3.3). Under our experimental conditions, we analyzed over 500 cells that responded to 1-octanol (3.1), octanal (3.2), and/or octanoic acid (3.3) at 60 µM out of the 11,707 cells that responded to forskolin. Of these cells, there were two cells that responded to 1-octanol (3.1) and octanoic acid (3.3), but not octanal (3.2), (Figure 3.9). Aside from the difference in relative intensity compared to forskolin, the response pattern of the two cells was nearly identical. These cells

Figure 3.10: Categories of hypothetical receptors. (A) Receptor that is selective for alcohols. (B) Receptor that is selective for the carbonyl group. (C) Receptor that is selective for the hydroxyl group. (D) Receptor that is selective for molecules containing a single hydroxyl group.
likely expressed the same receptor, suggesting that there is at least one olfactory receptor in the mouse genome that can bind 1-octanol (3.1) and octanoic acid (3.3) but not octanal (3.2). In this case, it occurs in 0.017% of all viable cells (roughly 1 out of 5000 cells). Due to experimental limitations, it was not possible to look at every single cell, and thus every receptor, in the olfactory epithelium, but the more cells that are analyzed the closer we would be to analyzing all the olfactory receptors.

Theoretically, given a constant carbon chain shape, distinguishing the alcohol and carboxyl group from the aldehyde group should be difficult. A receptor that is specific for alcohols would bind with 1-octanol (3.1) and the gem-diol form of octanal (3.2) (Figure 3.10A). A receptor that is specific for the carbonyl group would bind with the aldehyde form of octanal (3.2) and octanoic acid (3.3) (Figure 3.10B). A receptor that is specific for the hydroxyl group (or hydrogen donors) would be expected to bind only 1-octanol (3.1) and octanoic acid (3.3), but because octanal (3.2) forms a significant amount of the gem-diol form,17-19 such a receptor could bind to all three functional groups (Figure 3.10C). For a receptor to interact with 1-octanol (3.1) and octanoic acid (3.3) but not octanal (3.2), either the concentration of the gem-diol form (present at equilibrium in roughly half the amount of octanal (3.2) applied) was not high enough to activate the receptor selective for hydrogen donors (1-octanol (3.1), octanal (3.2), and octanoic acid (3.3) were all tested at the same concentration (60 µM), so the fraction of octanal (3.2) that became hydrated to the gem-diol would be considerably lower compared to the concentration of 1-octanol and octanoic acid present) (i.e. the receptor depicted on Figure 3.10C), or the receptor could only accommodate one hydroxyl group, whereas there is two present on the gem-diol form of octanal (3.2) (i.e. the receptor on Figure 3.10D).
3.2.5.3. 1-Octanol and octanal responding cells

The percentage of cells that responded selectively to one odorant was always observed in our experiment to be higher than the percentage of cells responding to more than one odorant, with one exception: cells that respond to both 1-octanol (3.1) and octanal (3.2). The percentage of cells responding to this pair of odorants (1.44%) was comparable to or even higher than the percentage of cells that responded to single odorants, with the percentage of cells responding to a single odorant being highest for 1-octanol (1.32%) and octanal (1.31%). Cells responding to the other pairs of odorants were noticeably lower (Table 3.2, column 2), with the percentage of the next most common cell type (aldehyde-specific cells responding only to 3.2 and 3.5, 0.29%) being around 5-fold lower. Much of the cells appeared to be unable to distinguish between the alcohol and aldehyde group.³,13 Of all the cells that responded to octanal (390 cells), 53% (205 cells) were found to also respond to 1-octanol at 60 µM (49%³ and 68%¹⁹ of octanal-responding cells were found to also respond to 1-octanol in rat OSNs at 30 µM). The high percentage of cells responding to alcohol 3.1 and aldehyde 3.2 might be attributed to the gem-diol form of aldehyde 3.2. It was previously found that 42% of cells that appear to require (i.e. specific for) the aldehyde group for activation responded to the gem-diol form.¹⁹ The gem-diol form differs from the aldehyde form in terms of geometry, hydrogen bonding, and dipole strength and direction to such an extent that it could be considered a different functional group. The hydration equilibrium constant (K_H) for aliphatic aldehydes is around 0.83 at 25°C,¹⁷-¹⁹ so accordingly, approximately 45% of the aldehyde is converted to the gem-diol in water at equilibrium. Since aldehydes convert to the gem-diol form in approximately a one to one ratio in the presence of water, some of the cells activated by octanal (3.2) may possibly be activated by its gem-diol form. Cells that responded to both 1-octanol (3.1) and octanal (3.2) may express receptors that
are specific for the hydroxyl group of alcohols (Figure 3.10A). A portion of the 1-octanol/octanal-responding cells may be responding to 1-octanol (3.1) and the gem-diol form of octanal (3.2). At the same time, for cells where octanal (3.2) was antagonized, it might actually be the gem-diol form that was antagonized by the antagonists with the different chain length and/or functional group.

3.3. Conclusions

In this study, limited to the main oxygen functional groups with different oxidation states (alcohol, aldehyde, and carboxylic acid) with either an eight carbon linear chain or an eight carbon shorter chain with a cyclohexyl ring, a few notable trends were observed regarding antagonism. There did not appear to be a clear preference for either chain type or functional group type as the determinant of inhibition, but octanoic acid (3.3) inhibited the highest percentage of cells out of the three functional groups tested. Carboxylic acids may have higher affinity for ORs compared to aldehyde and alcohol possibly due to strong hydrogen bonding and charge-charge interactions. While octanoic acid (3.3) inhibited the response of the most cells compared to 1-octanol (3.1) and octanal (3.2), it was also the weakest activator, activating the lowest percentage of cells. In comparison, although octanal (3.2) did not inhibit the response of any cells, it was the best activator, suggesting a possible inverse-correlation between the ability of an odorant to activate versus inhibit ORs. None of the functional group-specific receptors responded more strongly to the linear odorant compared to the cyclic odorant, suggesting that chain length/shape may be a dominant feature of functional group-selective ORs. When an odorant contains a chain below a certain length (number of carbons), it may bind but fail to satisfy some distal steric requirement for activation, and becomes an antagonist. A relatively small set of odorants were tested in this study to ensure the viability of the cells by the end of the
experiment. In order to confirm the observed trends, a larger set of odorants would need to be tested.

In this study, agonism appears to be more common than antagonism, but in comparison to agonism, it’s more difficult to identify all the cells that were antagonized. Activation of a cell is determined from the rise in the calcium level, but since there would be no change in the calcium level when a ligand binds but not activate a receptor, it is not possible to check for antagonism (i.e. binding) if an agonist of the receptor was not present. In this study, we only see antagonism in the cells that were activated by one of the odorants designated as an agonist. Each odorant might be binding a higher percentage of cells than what was observed, but evidence of antagonism was only limited to the cells in which an agonist was applied. In addition, only one concentration (60 μM) was used for the agonist and antagonist. Even though the response of the agonist might not be inhibited at 60 μM by an antagonist, it may be inhibited if a higher concentration was applied, and in designing such experiments, it is not clear what concentrations are the most physiologically relevant. To ensure that the reduction in signal was due to antagonism and not signal fluctuation, only cells showing over 70% reduction in activity were considered to be antagonized (we did not want to mistake response-to-response variation for antagonism), but there may also be cells that were antagonized, and suppressed by less than 70% at the tested concentration. Thus, we used a stringent criterion for calling antagonism.

The percentage of antagonism in cells may be underestimated in this study, but still, antagonism was seen in several cells and as long as antagonism occurs, the original olfactory code of the odorant would be modified. All of the tested binary mixtures (3.1&3.2, 3.1&3.3, and 3.2&3.3; not applicable to the mixtures containing the cyclic odorants because antagonism of the cyclic odorant by the linear odorant was not tested in all possible combinations, but signs of
antagonism were already seen for 3.1&3.4 and 3.2&3.5) displayed some degree of antagonism. In each pair of odorants, one of the two odorants would serve as the antagonist, while the other acted as the agonist. Unlike rodents, humans are predicted by their genomic DNA to have only a few hundred olfactory receptors, but we may be exposed to hundreds of different odorants at once. As more receptors become activated, everything would be expected to eventually converge to the same smell if antagonism, or some other kind of mixture suppression, were not at work in the mixture. Mixtures clearly do not all converge to the same smell. Antagonism of the receptors may possibly act as a fine tuning mechanism to produce the smell that we experience, with our larger brains sorting out the greater combinatorial complexity of signals generated in the nose.

3.4. Experimental Procedures

Materials and reagents
Unless otherwise stated, chemical reagents and solvents were purchased from VWR International, Fisher Scientific, or Sigma Aldrich and used without further purification. C57BL/6 mice were purchased from Charles River Laboratories. Octanal (3.2) was distilled before use. 1-Octanol (3.1) and octanoic acid (3.3) were purified by flash chromatography prior to testing. Aldehydes were stored at 4°C under vacuum prior to testing. Analytical TLC was performed on silica gel 60 F254 plates. Flash chromatography was performed on Teledyne Isco CombiFlash Rf-200 flash chromatography system. 1H and 13C NMR spectra were recorded on a Varian Mercury 300 spectrometer or a Bruker Ultrashield 500 spectrometer. Infrared (IR) spectra were recorded using Thermo Nicolet 6700 FT-IR spectrometer. (Note: The literature procedure that was applied for each reaction is cited. The procedure may not necessarily be used for the synthesis of the same compound as in the literature.)
**2-Cyclohexylethanol (3.4)** (Compound 2.2a in chapter 2). Compound 3.4 was synthesized according to a literature procedure. Ethyl cyclohexylacetate (2 g, 11.7 mmol) in diethyl ether (10 mL) was added slowly to lithium aluminum hydride (0.49 g, 13 mmol) in diethyl ether (40 mL) at 0°C. The suspension was stirred at room temperature for 2 h and then cooled to 0°C. Water (0.5 mL) was added, followed by 15% sodium hydroxide (0.5 mL) and water (1.5 mL). The solution was stirred for a few minutes at room temperature, filtered through a celite pad, washed with diethyl ether, dried, and concentrated. The crude was purified by flash chromatography eluting with ethyl acetate/hexanes (1:9) to obtain the alcohol 3.4 (1.5 g, 11.7 mmol) as a clear liquid in 99% yield. \(^1\)H NMR (CDCl\(_3\)) \(\delta\): 3.68 (t, \(J = 6.7 \text{ Hz}, 2\)H), 1.78 - 1.59 (m, 5H), 1.51 - 1.11 (m, 7H), 1.02 - 0.84 (m, 2H). \(^13\)C NMR (CDCl\(_3\)) \(\delta\): 60.89, 40.39, 34.22, 33.38, 26.57, 26.29. IR (thin film, KBr plates) \(\nu \text{ (cm}^{-1})\): 3332 (broad), 2922, 2852, 1448, 1051, 1011, 978. Spectral data match those reported previously.

**Cyclohexylacetaldehyde (3.5)** (Compound 2.2 in chapter 2). Compound 3.5 was synthesized according to a literature procedure. The alcohol 3.4 (200 mg, 1.56 mmol) was added to pyridinium chlorochromate (370 mg, 1.7 mmol) and silica gel (370 mg) in dry dichloromethane (10 mL) under inert atmosphere. The suspension was stirred for 2 h and then passed through a silica gel pad. The solution was concentrated to give the aldehyde 3.5 (140 mg, 1.11 mmol) in 71% yield. \(^1\)H NMR (CDCl\(_3\)) \(\delta\): 9.75 (s, 1H), 2.29 (dd, \(J = 1.9, 6.9 \text{ Hz}, 2\)H), 1.97 - 1.57 (m, 6H), 1.40 - 0.84 (m, 5H). \(^13\)C NMR (CDCl\(_3\)) \(\delta\): 203.00, 51.38, 33.22, 32.68, 26.04, 25.97. IR (thin film, KBr plates) \(\nu \text{ (cm}^{-1})\): 2925, 2853, 2712, 1725, 1449, 1408, 1297, 1193, 1020, 900. Spectral data match those reported previously.
**Cyclohexylethanoic acid (3.6).** Compound 3.6 was synthesized based on a literature procedure.\textsuperscript{21} Ethyl cyclohexylacetate (936 mg, 5.5 mmol) was placed in 1 M NaOH (90% methanol in water, 30 mL) and stirred for 3 h. The mixture was concentrated and water (25 mL) was added. The solution was acidified with 5 M HCl (6 mL) and extracted with ethyl acetate (3 × 20 mL). The organic layer was washed with brine, dried, and concentrated. The crude was purified by flash chromatography eluting with ethyl acetate/hexanes (1:9) to give the carboxylic acid 3.6 (560 mg, 3.9 mmol) in 72% yield. \textsuperscript{1}H NMR (CDCl\textsubscript{3}) \(\delta\): 11.57 (s, 1H), 2.23 (d, \(J = 6.3\) Hz, 2H), 1.91 - 1.52 (m, 6H), 1.44 - 1.08 (m, 3H), 1.08 - 0.69 (m, 2H). \textsuperscript{13}C NMR (CDCl\textsubscript{3}) \(\delta\): 179.55, 41.90, 34.66, 32.97, 26.09, 26.00. IR (thin film, KBr plates) \(\nu\) (cm\textsuperscript{-1}): 3029 (broad), 2925, 2853, 1707, 1449, 1411, 1297. Spectral data match those reported previously.\textsuperscript{22, 23}

**Calcium imaging of mouse olfactory sensory neuron**

Calcium imaging was performed as described previously.\textsuperscript{5} All animal procedures were approved by the City College of New York Institutional Animal Care and Use Committee (IACUC) and performed at the City College of New York in compliance with relevant national guidelines and regulations. Dissociated olfactory epithelium tissue was obtained from 6-8 weeks old C57B/6 mice by my colleague, Mihwa Na. The tissues were placed into a 15 mL Corning conical polypropylene centrifuge tube containing an enzyme solution made with BSA (0.015 g), collagenase (0.0025 g), and 3 mL of DICAT-free Ringer solution containing sodium chloride (145 mM), potassium chloride (5.6 mM), HEPES at pH 7.4 (10 mM), glucose (10 mM), and EGTA (4 mM). The cells were incubated at 37°C for 1 h in the enzyme solution. The tissues were then allowed to settle to the bottle of the conical tube and the enzyme solution was replaced with a media containing DMEM/F12-HAM, 10% FBS (Gibco), 1% Pen/Strep (Gibco), and 1X...
Insulin-Transferrin-Selenium-X (Gibco). Most of the media was removed and the tissues were gently vortexed to dissociate the cells. The cells were allowed to adhere to coverslips pre-coated with the protein concanavalin A. The cells were allowed to recover in the media containing 0.02 M vitamin C (ascorbic acid) at 37°C for 45 minutes. The media was then replaced with 1 mL of Ringer solution (sodium chloride (138 mM), potassium chloride (5 mM), HEPES at pH 7.4 (10 mM), calcium chloride (1 mM), magnesium chloride (1.5 mM), and glucose (10 mM)) containing Fura-2 (6.25 µL) and pluronic acid (1 µL), and incubated for another 45 minutes at room temperature in the dark. The cells were placed in new Ringer solution and left at room temperature for 10 minutes. The coverslip containing the cells were viewed under a fluorescence microscope and the response was obtained using Metamorph. Odorants were applied via in-line syringes hooked up to a manifold for 8 seconds at 2 minutes intervals and the antagonist was applied 4 seconds before and during application of the agonist.
Chapter 4: Small molecular activators of the pre-mRNA 3’ cleavage reaction

Parts of this chapter have been published in *Bioorganic and Medicinal Chemistry*, 22 (2), Liu, M.; Nagre, N. N.; Ryan, K., Structurally diverse low molecular weight activators of the mammalian pre-mRNA 3’ cleavage reaction, 834-841.

4.1. Introduction

Eukaryotic pre-messenger RNA (pre-mRNA) undergoes multiple processing steps prior to translation, and each of these steps provides the cell with an opportunity for gene expression regulation.\(^1\) The pre-mRNA 3’ cleavage step, a site-specific RNA hydrolysis reaction that specifies where the polyadenylate (poly(A)) tail will be added to the mRNA, can take place at different locations near the 3’ end of a nascent mRNA, downstream of the translation stop codon. This variability leads in many genes to alternative mRNA isoforms with different 3’ untranslated regions (3’UTRs) between the stop codon and the poly(A) tail.\(^2\)\(^,\)\(^3\) The pre-mRNA 3’ cleavage step can influence the stability of the mature mRNA because many 3’UTRs contain destabilizing sequences and miRNA binding sites, the loss of which, when 3’ cleavage occurs relatively close to the stop codon, may slow turnover and allow escape from repression.\(^2\)\(^,\)\(^4\)-\(^6\) Shorter 3’UTRs resulting from alternative polyadenylation in some oncogenes have been correlated with cellular proliferation and may contribute to oncogenesis.\(^7\)\(^,\)\(^8\) The 3’ cleavage and polyadenylation reactions are coupled *in vivo* but can be studied separately *in vitro*. Natural product inhibitors of the pre-mRNA polyadenylation step have recently been found.\(^9\)\(^,\)\(^10\) However, low molecular weight compounds that influence the 3’ cleavage step in cells are not yet available, but would enable new experimental inquiries into the multi-protein complex that carries out 3’ cleavage, and might provide a chemical tool to influence alternative polyadenylation.
We previously found *in vitro* evidence that a kinase-phosphatase pair may exert influence over the 3’ cleavage reaction.\(^{11}\) Considering this possibility led us to propose that creatine phosphate, long known to stimulate the *in vitro* reaction at high concentration,\(^{12,13}\) might do so by acting as a serendipitous inhibitor of an unknown 3’ cleavage-suppressing protein phosphatase. This model led to experiments identifying two protein phosphatase 2C (PP2C, a.k.a. PPM1) family inhibitors that stimulated 3’ cleavage in place of creatine phosphate.\(^{14}\) In humans, the PPM1 superfamily consists of at least eighteen different Mg\(^{2+}\)- or Mn\(^{2+}\)-dependent Ser/Thr phosphatases defined by shared sequence homology.\(^{15-18}\) In plants, the PPM1 family is greatly expanded, with at least 80 family members in Arabidopsis.\(^{19}\) The *in vitro* 3’ cleavage reaction is carried out in the presence of excess EDTA, where free Mg\(^{2+}\) and Mn\(^{2+}\) concentrations are very low. Thus, if a PPM1 family member is involved *in vitro*, it would likely act in a manner that does not require its metal-dependent enzymatic activity, perhaps acting allosterically or by influencing the multi-protein 3’ cleavage factor complexes.

While progress in other major protein phosphatase families has benefited from the identification of natural product inhibitors,\(^{20}\) inhibitor discovery for the PPM1 family has been slower. The inhibitors we previously identified as 3’ cleavage activators had been discovered using the PPM1A family member.\(^ {21}\) They had micromolar potency against PPM1A and only modest selectivity for the PPM1 family over the other major protein phosphatase families.\(^ {21}\) By analogy with the toxin inhibitors of the other protein phosphatase families, we expect PPM1 inhibitors to show a measure of family-specific activity, enabling 3’ cleavage activation even if PPM1A is not the actual family member involved in pre-mRNA 3’ cleavage. Here, we have tested more potent PPM1 inhibitors for pre-mRNA 3’ cleavage activity, and carried out structure-activity relationships for two previously identified 3’ cleavage activators. Though we
are not yet able to identify the protein targeted by these small molecules, our results strengthen a
model in which a PPM1 family member or related protein acts to suppress 3’ cleavage in vitro.

4.2. Results and Discussion

4.2.1. Pre-mRNA 3’ cleavage activation by a potent PPM1D cyclopeptide inhibitor

Cyclic phosphopeptides based on substrates of the PPM1D family member, also known
as Wip1, are among the most potent inhibitors of any PPM1 family phosphatase.\textsuperscript{22, 23} We used
one of these, cyclic peptide \textsuperscript{38} (cp38, Fig. 4.1A),\textsuperscript{22} in place of 50 mM creatine phosphate in the
in vitro pre-mRNA 3’ cleavage reaction. This peptide inhibits PPM1D with a \( K_i \) of 150 nM.\textsuperscript{22} In
the 3’ cleavage assay, a standard in vitro pre-mRNA 3’ cleavage substrate adapted from the
simian virus 40 (SV40) late poly(A) signal\textsuperscript{24} is exposed to partially purified 3’ cleavage factors
from HeLa cell nuclear extract.\textsuperscript{11, 13, 25, 26} Cleavage of the radiolabeled substrate into its 5’ and 3’
fragments can be observed and quantitated following resolution on a denaturing polyacrylamide
gel. In the absence of creatine phosphate, or other activator, this reaction is inefficient. In all
experiments presented here, the amount of cleavage brought about by an activator (5’
fragment/(5’ fragment+uncleaved SV40L RNA)) is normalized to that brought about by 50 mM
creatine phosphate (R.C., or relative cleavage in figures). As shown in Fig. 4.1B, at 200 nM,
cp38 activated a level of RNA cleavage comparable to that produced by 50 mM creatine
phosphate. Removal of cp38’s serine and tyrosine phosphate groups with calf intestinal alkaline
phosphatase (CIP) prior to cleavage, led to complete loss of activation, while removal of only the
tyrosine phosphate, using a tyrosine-specific phosphatase, did not reduce activity. (Residual CIP
activity was inhibited\textsuperscript{11} before cp38 was added to the in vitro reaction to prevent possible
dephosphorylation of the cleavage factor proteins.) A repeat of this experiment is shown on
Figure 4.1D. Cyclopeptide cp38 is therefore ~1000-fold more potent than the most potent 3’
cleavage activator previously identified,\textsuperscript{14} and phosphorylation of its serine, which is required for PPM1 inhibition,\textsuperscript{23} is required to activate pre-mRNA 3’ cleavage. PPM1D is present at very low levels in HeLa cells,\textsuperscript{27} an observation that lead us to suppose that \textbf{cp38} is likely not working through this PPM1 family member, but may instead be a family-specific inhibitor that activates 3’ cleavage through another family member, or homologous protein.
Figure 4.1: Pre-mRNA in vitro 3’ cleavage activation by PP2C inhibitors. (A) Structure of cyclic peptide \textbf{cp38} and \textbf{(S)-(+)}-abscisic acid (ABA). (B) Denaturing gel analysis of SV40L pre-mRNA 3’ in vitro cleavage reactions activated by \textbf{cp38}. Creatine phosphate (CP) or \textbf{cp38} was added, with or without pre-treatment with CIP (calf intestinal alkaline phosphatase), mock CIP (CIP buffer only) or tyrosine phosphatase (YOP). Residual CIP activity was completely removed before mixing \textbf{cp38} with cleavage factor proteins. Relative cleavage (R.C. = [5’ fragment/(5’ fragment + uncleaved RNA)]X100) was normalized to \textbf{CP}, which is set to R.C. = 1.00. (C) Denaturing gel analysis of in vitro cleavage using ABA in place of CP. DMSO lane corresponds to reaction without ABA. (D) Repeat of pre-mRNA 3’ cleavage reaction using \textbf{cp38} as activator. (E and F) Repeat of pre-mRNA 3’ cleavage reaction using ABA as activator.
4.2.2. Pre-mRNA 3’ cleavage activation by abscisic acid

To further test the hypothesis that a PPM1 family member acts to suppress 3’ cleavage, we used the isoprenoid plant hormone (S)-(+)‐abscisic acid (ABA). This natural product is a potent inhibitor of the group A plant PPM1 enzymes. It works by forming a ternary complex with the PPM1 enzyme and a co-receptor from the PYR/PYL/RCAR family of ABA receptors.\textsuperscript{28} For example, ABA inhibits the plant PPM1s ABI1 and ABI2 with IC\textsubscript{50} values of 60 nM and 70 nM, respectively, when RCAR1 is present.\textsuperscript{29} ABA is bound mainly by the PYR/PYL/RCAR receptor, but also makes contact with the PPM1.\textsuperscript{30, 31} In the absence of a PYR/PYL/RCAR co-receptor, ABA can directly inhibit some plant PP2C/PPM1 enzymes \textit{in vitro}, though with much lower potency. For example, ABA reduces ABI2 phosphatase activity \textit{in vitro} by about 15% at ABA concentrations above 3 μM.\textsuperscript{29} We found no pre-mRNA 3’ cleavage activation when nanomolar concentrations of ABA were used in place of creatine phosphate (not shown). However, at and above 100 μM ABA, approximately 10%-15% of the normal 3’ cleavage activity was observed (cf. 50 mM creatine phosphate) (Fig. 4.1C). Two repeats of this experiment are shown on Figures 4.1E and 4.1F. We consider two possible interpretations of this result. First, the absence of activation at nanomolar ABA concentration may indicate that there are no conserved mammalian proteins that can act in the manner of the plant PYR/PYL/RCAR co-receptors, though ABA is found in animal cells.\textsuperscript{32} A BLAST search did not reveal any human RCAR homologs. Second, homology between the plant and human PPM1 proteins may be sufficient to conserve the direct but much less potent inhibition of PPM1 family members. In either case, though its cleavage activation efficiency was low, compared to our first generation compounds, ABA is a relatively potent 3’ cleavage activator. This result adds to the
accumulating though circumstantial evidence that a PPM1 family member can influence pre-mRNA processing.

4.2.3. Structure-activity study of arginine β-naphthylamide: Aryl group and side chain

Prior to the present work, compound 4.1 (Fig. 4.2A) was identified as a 3’ cleavage activator through a limited structure-activity relationship study undertaken using commercially available compounds related to our initial lead, leucine β-naphthylamide, a weak PPM1A inhibitor. To search for a more potent activator than 4.1, we synthesized a series of analogs in which the naphthalene ring was altered (Fig. 4.2A, 4.2-4.4) or the guanidino group was alkylated or replaced by another positively charged group (Fig. 4.2A, 4.5-4.6).
Figure 4.2: Structure-activity study of compound 4.1, previously found to activate pre-mRNA 3' cleavage in vitro. (A) Analogs included changes to the naphthyl group and the arginine side-chain of 4.1. (B) In vitro 3' cleavage results for duplicate cleavage reactions using the indicated analogs in place of 4.1, all at 1 mM. R. C., relative cleavage, defined in Fig. 4.1, normalizes all compounds to activation by 50 mM CP.
The synthesis of compounds 4.2-4.6 (Scheme 4.1, A and B) was accomplished by coupling the relevant aryl amine to the suitably protected arginine, ornithine or lysine, followed by guanidinylation where necessary, and finally deprotection.

**Scheme 4.1**: (A and B) Synthesis of the arginine-β-naphthylamide analogs used in Fig. 4.2. (C) Synthesis of morpholine analog 4.7 used in Fig. 4.3.
We evaluated each of the compounds shown in Fig. 4.2A for their ability to activate \textit{in vitro} 3’ cleavage at 1 mM in place of, and compared to, 50 mM creatine phosphate. The results of duplicate reactions are shown in four independent 3’ cleavage reactions in Fig. 4.2, B-E. Changes to the naphthalene ring and guanidino group of \textit{4.1} led to loss of activity (compounds \textit{4.2, 4.3, 4.4, 4.5}), and the guanidino group could not be replaced by trimethylammonium (compound \textit{4.1} vs. \textit{4.6}), even though both groups have a positive charge.

\textbf{4.2.4. Arginine \(\beta\)-naphthylamide modified at the \(\alpha\)-position retains potency}

In previous work we found that acetylation of the \(\alpha\)-amino group of leucine \(\beta\)-naphthylamide led to some loss of that compound’s 3’ cleavage activity, as well as a significant decrease in solubility, a point of practical importance due to the high activator concentrations needed for activity. In view of the poor results changing the naphthyl and guanidino groups of lead compound \textit{4.1}, we decided to modify the \(\alpha\)-amino group. To minimize a reduction in solubility we chose the morpholine group for its high water solubility (compound \textit{4.7}, Fig. 4.3, and Scheme 4.1C). Unlike the other changes, this modification retained activity. A representative \textit{in vitro} 3’ cleavage comparison of \textit{4.7} with \textit{4.1} over 25 to 200 \(\mu\)M (as usual, in place of, and compared to, creatine phosphate) is shown in Fig. 4.3B. Corroborating experiments are shown on Figure 4.3C and 4.3D. Compound \textit{4.7} proved to be slightly more potent than lead compound \textit{4.1}, and to have a similar maximum relative cleavage (Fig. 4.3B). While only slightly more potent than \textit{4.1}, the activity of compound \textit{4.7} indicates that modifications of the alpha amine can be tolerated, and that this part of the molecule merits a larger SAR study beyond the scope of the present work.
Figure 4.3: Structure and 3' cleavage activity of a morpholine-modified arginine-β-naphthylamide analog. (A) Structure of 4.7. (B) Side by side comparison of 4.1 and 4.7 in an SV40L pre-mRNA 3' in vitro cleavage reaction. R. C., relative cleavage, as defined in Fig. 4.1, with activation by 50 mM creatine phosphate (CP) set to 1.00. (C and D) Repeats of pre-mRNA 3' cleavage reaction.

4.2.5. Structure-activity study on phosphocholine

We previously identified phosphocholine as a pre-mRNA 3' cleavage activator. Like creatine phosphate, it activates 3' cleavage over the 10-50 mM range. Its ability to inhibit PPM1 family proteins is unknown. Phosphocholine is an intermediate in the biosynthesis of phosphatidylcholines, and it is unlikely to be a physiological cofactor in 3' cleavage. However, understanding the determinants of 3' cleavage activity of this very simple structure might help us to design more potent activators. To learn whether all three methyl groups are necessary for activity, we synthesized the mono- and dimethyl phosphocholine analogs, 4.9 and 4.10 (Scheme 4.2A). Their synthesis is shown in Scheme 4.2B. We also made an analog of phosphocholine with an extra carbon between the charged groups (compound 4.11, Scheme 4.2A, C). None of these compounds showed detectable in vitro 3' pre-mRNA cleavage activity (not shown).
lack of activity in these analogs showed that all three methyls of the trimethylammonium group are required, and the two-carbon spacing between this group and the phosphate is critical.

Scheme 4.2: Phosphocholine (PhC) analogs. (A) Structures of the analogs. (B) Synthesis of analogs 4.9 and 4.10. (C) Synthesis of analog 4.11.

4.2.6. Phosphocholine as a possible modified histone protein mimic

Trimethyllysine and phosphoserine residues are commonly found among the histone proteins of chromatin, where in vivo transcription takes place. Since several of the 3’ cleavage factor proteins associate with transcribing RNA polymerase II and the 3’ cleavage reaction is coupled to transcription and begins co-transcriptionally, it is conceivable that a histone protein could, within the chromatin, locally interact with RNA polymerase II-associated cleavage factor proteins to play a role in 3’ cleavage. In trying to understand how phosphocholine activates 3’ cleavage in vitro, we have considered the possibility that it may mimic a modified histone protein having closely situated trimethyllysine and phosphoserine (or threonine) residues (Fig. 4.4A). In an initial test of this idea, we replaced 50 mM creatine phosphate in a 3’ cleavage
reaction with dipeptide 4.12, Ac-Lys(Me)-Ser(P04)-CO2H (Fig. 4.4B). Peptide 4.12 may also be viewed as a derivative of phosphoserine which, like creatine phosphate, activates 3’ cleavage in the 10 to 50 mM range.\(^{38}\) Peptide 4.12 activated \emph{in vitro} 3’ cleavage at ~1 mM (Fig. 4.4C), while phosphoserine and phosphocholine\(^{14}\) have no effect at this concentration. A repeat of this experiment is shown on Figure 4.4D. This result shows that placing the two functional groups of phosphocholine in the structural context of a dipeptide, where they can approach each other as closely as they do in phosphocholine, reduced the concentration necessary for them to activate 3’ cleavage. Viewed differently, placing phosphoserine in a dipeptide coupled to N-(acetyl)trimethyllysine (to create 4.12) led to increased potency. While far from directly implicating a histone protein in the 3’ cleavage reaction, this result indirectly supports the possibility that phosphocholine works by mimicking such a cleavage-promoting modified histone variant. We note that the partially purified protein 3’ cleavage factors used in our assay are fractionated from soluble nuclear proteins, and not from the chromatin-rich insoluble nuclear pellet, so a cleavage-enhancing chromatin protein would be depleted or lost during cleavage factor preparation. Interestingly, the PPM1G family member plays a role in histone H2A-H2B exchange into chromatin.\(^{39}\) PPM1G is also involved in splicesomal splicing,\(^{40}\) a pre-mRNA processing step that can be coupled to 3’ cleavage.\(^{41}\) In view of this context, this result justifies additional study of suitably modified lysine, serine and threonine-containing peptide sequences taken from the heavily modified histone protein tails.
**Figure 4.4**: Phosphocholine (PhC) as a mimic of a modified histone protein tail. (A) Phosphocholine holds a trimethyl ammonium group close to a phosphate, as a histone protein can do with trimethylated lysine and phosphoserine (or phosphothreonine). (B) Structure of dipeptide 4.12. (C) SV40L pre-mRNA 3’ cleavage activation by peptide 4.12 compared to phosphocholine (PhC), creatine phosphate (CP) and phosphoserine (PS). R.C., relative cleavage, as defined in Fig. 4.1. (D) Repeat of pre-mRNA 3’ cleavage reaction.

### 4.2.7. Inhibitory activity of pre-mRNA 3’ cleavage activators

The small molecules that we have found to activate pre-mRNA cleavage were either inhibitors of a protein phosphate or analogs of inhibitors. Therefore, it is of interest to determine whether the small molecules were activating cleavage through inhibition of a protein phosphatase. While we speculate that a protein phosphatase may be involved in the cleavage reaction, we are uncertain as to which protein is actually involved. As a preliminary test, PPM1A was used as a representative enzyme. PPM1A may not be the exact protein phosphatase involved in the cleavage reaction, but it should be structurally homologous to the other protein phosphatase in the family and possibly share some family-specific activity.
Figure 4.5: Inhibitory activity of small molecule activators. The ability of the small molecule cleavage activators (creatine phosphate (CP), phosphocholine (PhC), arginine β-naphthylamide (4.1), 4.2 (an analog that is not a cleavage activator at the tested concentration), 4.7, dipeptide 4.12, abscisic acid (ABA), and cyclic peptide 38 (cp38)) to inhibit PPM1A was tested using para-nitrophenylphosphate (pNPP) as the substrate.

The inhibitory activities for a selected group of small molecules were tested at the concentration at which they activate cleavage using p-nitrophenylphosphate as the substrate. Unfortunately, besides creatine phosphate and phosphocholine, which stimulated over 40% reduction in activity, the other cleavage activators were not able to significantly inhibit PPM1A at the concentration in which they activate pre-mRNA 3’ cleavage. This result signifies that either the compounds were not activating cleavage through inhibiting a protein phosphatase or that the compounds were suppressing a protein phosphatase which was not PPM1A.

4.3. Conclusions

Guided by a model proposing that a PPM1 phosphatase acts to suppress or negatively regulate the pre-mRNA 3’ cleavage reaction, an obligatory step in the maturation of nearly all eukaryotic mRNAs, we have assayed a group of structurally diverse PPM1 inhibitors and new synthetic small molecules for their ability to activate this reaction in vitro. Our results lend further, though circumstantial, support to this model. The minimal concentration required for in
vitro cleavage has been improved from 200 μM to the 200 nM-100 μM range. In addition, our results point to future experiments aiming to further the goal of developing membrane-permeable small molecule activators of 3’ cleavage sufficiently potent for use in tissue culture experiments.

4.4. Experimental procedures

4.4.1. Materials and methods

Unless otherwise stated, commercial reagents and solvents were used without additional purification. Solvents were purchased from VWR. Cyclic peptide 38 (cp38) was provided by E. Appella and R. Hayashi and made as previously described.22 (S)-(+-)Abscisic acid (ABA), phosphocholine (PhC), and phosphoserine (PS) were purchased from TCI America. Creatine phosphate (CP) was purchased from Calbiochem. Compound 4.1 was purchased from Bachem. Compound 4.8 was purchased from Fluka. Compound 4.12 was purchased from Shanghai Apeptide Co., Ltd. Unless otherwise stated, starting reagents were purchased from VWR. Analytical TLC was performed on silica gel 60 F254 plates. Flash chromatography was performed manually in glass columns on 230-400 mesh silica gel (Alfa Aesar). Analytical samples were dried overnight in vacuo over phosphorus pentoxide prior to testing. Melting points were measured on a Laboratory Devices Mel-Temp apparatus. 1H and 13C NMR spectra were recorded on a Varian Mercury 300 spectrometer or Varian Inova 500 spectrometer. High-resolution mass spectra (HRMS) were measured using electrospray ionization (ESI) on a Waters LCT XE (TOF) mass spectrometer by Dr. L. Yang (CCNY). Infrared (IR) spectra were recorded using a Thermo Nicolet 380 FT-IR spectrometer or Thermo Nicolet 6700 FT-IR spectrometer.

4.4.2. General Procedure for the synthesis of compounds 4.2-4.4

Z3-Arg-OH (Z, benzyloxycarbonyl) was dissolved in dichloromethane. 1.2 equiv. of the amine was added followed by 2.5 equiv. of 1-hydroxy-7-azabenzotriazole (HOAt). 1.5 equiv. of 1-
ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was dissolved in dichloromethane and slowly added to the reaction at room temperature. Reaction progress was monitored by TLC (ethyl acetate:dichloromethane). The solution was washed sequentially with 0.5 M HCl solution, water, brine, dried, and concentrated. The crude was purified by flash chromatography. The resulting product was hydrogenated in methanol using a latex balloon with catalytic amount (approximately a spatula tip) of palladium hydroxide (Pd 20% on carbon, nominally 58% water) for 3 hours. The solution was then filtered through celite and concentrated to yield the final product.

2-Amino-5-guanidino-pentanoic acid phenylamide (4.2).

White solid (yield 72%, m.p. 110°C dec.). $^1$H NMR (D$_2$O) $\delta$: 7.17 - 7.32 (m, 4H), 7.10 (m, 1H), 3.89 (t, $J = 6.33$ Hz, 1H), 2.95 - 3.11 (m, 2H), 1.70 - 1.87 (m, 2H), 1.43 - 1.61 (m, 2H). $^{13}$C NMR (D$_2$O) $\delta$: 169.49, 156.78, 135.98, 129.51, 129.35, 126.24, 122.05, 121.83, 53.80, 53.51, 40.43, 28.65, 23.63. IR (neat) $\nu$ (cm$^{-1}$): 3175, 1655, 1596, 1546, 1442, 1360, 1309, 1253, 752, 691. HRMS (ESI) [M+H]$^+$: Calcd for C$_{12}$H$_{19}$N$_5$O $m/z$ = 250.1668, found $m/z$ = 250.1655.

2-Amino-5-guanidino-pentanoic acid naphthalen-1-ylamide (4.3).

White solid (yield 62%, m.p. 166°C dec.). $^1$H NMR (D$_2$O) $\delta$: 7.57 - 7.75 (m, 3H), 7.21 - 7.41 (m, 4H), 4.18 (t, $J = 6.33$ Hz, 1H), 2.99 (t, $J = 6.60$ Hz, 2H), 1.79 - 1.99 (m, 2H), 1.47 - 1.73 (m, 2H). $^{13}$C NMR (D$_2$O) $\delta$: 169.52, 156.62, 133.93, 130.49, 128.71, 128.46, 128.05, 127.07, 125.63, 124.10, 121.95, 121.66, 53.35, 53.11, 40.34, 28.33, 23.86. IR (neat) $\nu$ (cm$^{-1}$): 3149, 1658, 1536, 1497, 1348, 793, 770. HRMS (ESI) [M+H]$^+$: Calcd for C$_{16}$H$_{21}$N$_5$O $m/z$ = 300.1824, found $m/z$ = 300.1816.
2-Amino-5-guanidino-pentanoic acid quinolin-3-ylamide (4.4).

Yellow solid (yield 30%, m.p. 113°C dec.). $^1$H NMR (D$_2$O) $\delta$: 9.34 (s, 1H), 8.97 (s, 1H), 8.02 (d, $J = 8.80$ Hz, 1H), 8.07 (d, $J = 8.53$ Hz, 1H), 7.91 (t, $J = 7.70$ Hz, 1H), 7.72 - 7.84 (m, 1H), 4.19 (t, $J = 6.46$ Hz, 1H), 3.11 (t, $J = 6.74$ Hz, 2H), 1.97 (m, 2H), 1.51 - 1.73 (m, 2H). $^{13}$C NMR (D$_2$O) $\delta$: 168.80, 156.79, 138.45, 138.08, 135.98, 135.76, 135.28, 134.63, 131.59, 130.88, 129.07, 120.31, 119.94, 53.65, 53.39, 40.37, 28.01, 23.66. IR (neat) $\nu$ (cm$^{-1}$): 3159, 1663, 1558, 1491, 1466, 1365, 782, 747. HRMS (ESI) [M+H]$^+$: Calcd for C$_{15}$H$_{20}$N$_6$O $m/z$ = 301.1777, found $m/z$ = 301.1786.

2-Amino-5-(N'-ethylguanidino)-pentanoic acid naphthalen-1-ylamide (4.5). Boc-Ornithine(Z)-OH (500 mg, 1.36 mmol) was dissolved in dichloromethane (25 mL). 1-Naphthylamine (234 mg, 1.64 mmol) was added followed by HOAt (464 mg, 3.41 mmol) and EDC (392 mg, 2.05 mmol). Reaction progress was monitored by TLC. The resulting material was purified by flash chromatography, eluting with dichloromethane/ethyl acetate (9:1) to give 4.5a (531 mg, 1.08 mmol, 79% yield). Compound 4.5a (531 mg, 1.08 mmol) was hydrogenated for 2 h in methanol (50 mL) with a catalytic amount of palladium hydroxide as described in section 4.4.2. The solution was filtered through celite and concentrated to give 4.5b. Compound 4.5b was dissolved in acetonitrile (7 mL) and ethylated N, N'-bis-tert-butoxycarbonylpyrazole-1-carboxamidine (364 mg, 1.08 mmol) was added followed by diisopropylethylamine (181 mg, 2.34 mmol). The solution was stirred overnight, concentrated and purified by flash chromatography, eluting with dichloromethane/ethyl acetate (4:1) to give 520 mg (0.83 mmol) of a white, sticky solid. The resulting compound (170 mg, 0.27 mmol) was dissolved in dichloromethane (2 mL) and deprotected with trifluoroacetic acid (2 mL) overnight. The solution was concentrated,
suspended in fresh dichloromethane, and extracted with water. The aqueous layer was concentrated and dried overnight in a drying pistol with phosphorus pentoxide to give 4.5 as a sticky, white solid in 68% yield. $^1$H NMR (D$_2$O) $\delta$: 7.59 - 7.81 (m, 3H), 7.24 - 7.45 (m, 4H), 4.11 - 4.23 (m, 1H), 3.05 (m, 2H), 2.85 (q, $J = 6.88$ Hz, 2H), 1.85 - 2.02 (m, 2H), 1.60 (m, 2H), 0.81 (t, $J = 7.01$ Hz, 3H). $^{13}$C NMR (D$_2$O) $\delta$: 169.49, 163.23, 162.76, 155.38, 133.99, 130.53, 128.39, 127.09, 126.75, 125.64, 123.90, 122.11, 118.25, 114.38, 53.35, 53.13, 40.15, 36.20, 28.26, 23.84, 13.02. IR (neat) $\nu$ (cm$^{-1}$): 3208, 3051, 1659, 1542, 1181, 1131, 798, 721. HRMS (ESI) [M+H]$^+$: Calc'd for C$_{18}$H$_{25}$N$_5$O m/z = 328.2137, found m/z = 328.2121.

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\text{[5-Amino-5-(naphthalen-2-ylcarbamoyl)-pentyl-trimethylammonium (4.6).]}
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Boc-Lys(Z)-OH (1 g, 2.6 mmol) was dissolved in dichloromethane (50 mL). 2-Naphthylamine$^{44}$ (0.37 g, 2.6 mmol) was added followed by HOAt (0.89 g, 6.54 mmol) and EDC (0.76 g, 3.96 mmol). After 1.5 h, the solution was concentrated and purified by flash chromatography, eluting with chloroform/ethyl acetate (4:1) to give 4.6a (800 mg, 1.58 mmol), in 61% yield. Compound 4.6a (288 mg, 0.57 mmol) was suspended in methanol (25 mL) and hydrogenated with a catalytic amount of palladium hydroxide. After 1.5 h, the solution was filtered through celite and concentrated to give 4.6b (212 mg, 0.57 mmol) in quantitative yield. Compound 4.6b (205 mg, 0.55 mmol) was then dissolved in acetonitrile (1 mL) and potassium carbonate (191 mg, 1.38 mmol) was added followed by iodomethane (392 mg, 2.76 mmol). The solution was stirred overnight, concentrated, suspended in dichloromethane, and filtered. The filtrate was concentrated and the crude compound was dissolved in dichloromethane (2 mL) and trifluoroacetic acid (2 mL) was added. The solution was stirred for 1.5 h and then concentrated, suspended in fresh dichloromethane, and extracted with water. The aqueous layer was
concentrated and dried overnight over phosphorus pentoxide to give 4.6 as an orange, sticky solid in 85% yield. $^1$H NMR (D$_2$O) δ: 7.88 (s, 1H), 7.66 - 7.78 (m, 3H), 7.28 - 7.40 (m, 3H), 3.99 (t, J = 6.60 Hz, 1H), 3.06 - 3.15 (m, 2H), 2.84 (s, 9H), 1.83 - 1.94 (m, 2H), 1.66 (m, 2H), 1.34 (m, 2H). $^{13}$C NMR (D$_2$O) δ: 168.48, 162.78, 133.88, 133.41, 131.29, 129.43, 127.94, 127.84, 127.33, 126.38, 121.10, 119.01, 66.12, 53.81, 53.05, 30.65, 22.31, 21.32. IR (neat) ν (cm$^{-1}$): 3049, 1671, 1198, 1128, 832, 799, 720. HRMS (ESI) [M]$^+$: Calcd for [C$_{19}$H$_{28}$N$_3$O]$^+$ m/z = 314.2232, found m/z = 314.2223.

5-Guanidino-2-morpholin-4-yl-pentanoic acid naphthalen-2-ylamide (4.7). Boc-Orn(Z)-OH (1 g, 2.73 mmol) was dissolved in dichloromethane (50 mL). 2-Naphthylamine$^{44}$ (470 mg, 3.28 mmol) was added followed by HOAt (929 mg, 6.83 mmol) and EDC (785 mg, 4.09 mmol). Reaction progress was monitored by TLC. The resulting material was purified by flash chromatography, eluting with dichloromethane/ethyl acetate (9:1) to give 4.7a (570 mg, 1.56 mmol, 49% yield). Compound 4.7a (320 mg, 0.65 mmol) was dissolved in dichloromethane (2 mL) with trifluoroacetic acid (2 mL) and stirred at room temperature overnight. The solution was concentrated and used for the next step without further purification. The crude compound was dissolved in acetonitrile (6 mL) and refluxed with potassium carbonate (360 mg, 2.6 mmol) and bis(2-bromoethyl)ether (181 mg, 0.78 mmol) overnight. The next day, the solution was filtered and purified by flash chromatography (first 1:1 ethyl acetate/dichloromethane then 100% ethyl acetate) to give 4.7b (100 mg, 0.22 mmol) as a yellowish solid in 33% yield. Compound 4.7b was hydrogenated in methanol (12 mL) with catalytic amount of palladium hydroxide. After 2 h, the solution was filtered through celite and concentrated. The resulting compound was dissolved in acetonitrile (1 mL) and N,N’-bis-tert-
butoxycarbonylpyrazole-1-carboxamidine\(^{45}\) (27 mg, 0.87 mmol) was added followed by DIPEA (15 mg, 0.113 mmol). The reaction was stirred overnight, concentrated and purified by flash chromatography, eluting with ethyl acetate/hexanes (4:1) to give 4.7c (20 mg, 0.035 mmol) as a sticky, white solid in 41% yield. Compound 4.7c was dissolved in dichloromethane (1 mL) and trifluoroacetic acid (1 mL) was added. The solution was stirred overnight and then concentrated, suspended in dichloromethane, and extracted with water. The aqueous layer was concentrated and dried overnight over phosphorus pentoxide to give 4.7 in 85% yield. \(^1\)H NMR (D\(_2\)O) \(\delta\): 7.96 (s, 1H), 7.70 - 7.85 (m, 3H), 7.34 - 7.48 (m, 3H), 3.18 - 4.05 (m, 9H), 3.11 (t, \(J = 6.46\) Hz, 2H), 1.86 - 2.15 (m, 2H), 1.45 - 1.72 (m, 2H). \(^{13}\)C NMR (D\(_2\)O) \(\delta\): 166.00, 162.95, 156.95, 133.31, 131.47, 129.47, 127.91, 127.36, 126.56, 120.97, 119.33, 117.67, 115.35, 69.27, 63.74, 40.48, 24.62, 23.86. IR (neat) \(\nu\) (cm\(^{-1}\)): 3360; 2877, 2718, 1668, 1563, 1508, 1435, 1364, 1200, 800, 723, 707. HRMS (ESI) [M+H]\(^+\): Calcd for C\(_{20}\)H\(_{27}\)N\(_5\)O\(_2\) m/z = 370.2243, found m/z = 370.2224.

Phosphoric acid mono-(2-methylamino-ethyl) ester (4.9). 2-

Methylaminoethanol (500 mg, 6.66 mmol) was stirred with sodium bicarbonate (1.12 g, 13.31 mmol) in diethyl ether (6.6 mL). Benzyl chloroformate, 30-35% in toluene, (3.6 g, 6.66 mmol) was slowly added at 0°C. The solution was warmed to room temperature after 15 minutes. After 1 h, the solution was concentrated and purified by flash chromatography, eluting with dichloromethane/ethyl acetate (1:1) to give 4.9a in 63% yield. Compound 4.9a (300 mg, 1.43 mmol) was dissolved in diethyl ether (5 mL) and diphenyl chlorophosphate (578 mg, 2.15 mmol) was added followed by triethylamine (290 mg, 2.87 mmol). After 2 h, the solution was filtered and purified by flash chromatography, eluting with dichloromethane/ethyl acetate (9:1) to give 4.9b (633 mg) in 81% yield. Compound 4.9b (500 mg, 1.13 mmol) was added to a solution containing sodium hydride, 57-63% in oil, (86 mg, 2.15
mmol) and benzyl alcohol (368 mg, 3.42 mmol) in diethyl ether (5 mL). After 1 h, the solution was purified by flash chromatography, eluting with dichloromethane/ethyl acetate (9:1) to give 4.9c (324 mg) in 60% yield. Compound 4.9c (324 mg, 0.69 mmol) was hydrogenated in methanol (25 mL) with catalytic amount of Pd/C (10% Pd, 50% wet with water) for 1 h. The solution was filtered through celite, and the celite pad was washed with water. The solution was concentrated and dried overnight over phosphorus pentoxide to give a 4.9 (90 mg, 0.58 mmol) as a white solid (m.p. 204 - 206°C) in 84% yield. $^1$H NMR (D$_2$O) $\delta$: 3.76 - 3.94 (m, 2H), 2.97 - 3.13 (m, 2H), 2.49 (s, 3H). $^{13}$C NMR (D$_2$O) $\delta$: 60.25, 49.11, 48.99, 32.77, 32.41. IR (neat) $\nu$ (cm$^{-1}$): 3017, 2729, 2520, 1146, 1075, 1026, 923, 815. HRMS (ESI) [M+H]$^+$: Calcd for C$_3$H$_{10}$NO$_4$P $m/z$ = 156.0426, found $m/z$ = 156.0424.

Phosphoric acid mono-(2-dimethylamino-ethyl) ester (4.10). 2-Dimethylaminoethanol, 4.10a, (500 mg, 5.61 mmol) was dissolved in ether (5.6 mL) and diphenyl chlorophosphate (1.8 g, 6.73 mmol) was added followed by triethylamine (1.135 g, 11.22 mmol). After 1 h, the solution was concentrated and purified by flash chromatography, eluting with dichloromethane/triethylamine (9:1) to give 4.10b (1.37 g) in 76% yield. Compound 4.10b (540 mg, 1.68 mmol) was added to a solution containing sodium hydride, 57-63% in oil, (121 mg, 3.03 mmol) and benzyl alcohol (545 mg, 5.04 mmol) in ether (5.4 mL). After 1 h, the solution was concentrated and purified by flash chromatography, eluting with ethyl acetate/methanol (19:1) to give 4.10c (340 mg) in 58% yield. Compound 4.10c (166 mg, 0.48 mmol) was hydrogenated in methanol (25 mL) with catalytic amount of palladium hydroxide for 1 h. The solution was filtered through celite, concentrated, and dried overnight over phosphorus pentoxide to give 4.10 (70 mg) as a white solid (m.p. 155 - 157°C) in 87% yield. $^1$H NMR (D$_2$O) $\delta$: 3.92 (m, 2H), 3.19 (m, 2H), 2.71 (s, 6H). $^{13}$C NMR (D$_2$O) $\delta$: 57.87,
IR (neat) ν (cm⁻¹): 3415, 2629, 2404, 1163, 1093, 1026, 993, 951, 931, 768.

HRMS (ESI) [M+H]^+: Calcd for CaH₁₂NO₄P m/z = 170.0582, found m/z = 170.0609.

Trimethyl-(3-phosphonooxypropyl)-ammonium iodide (4.11). 3,3-

Dimethylamino-1-propanol (500 mg, 4.846 mmol) was dissolved in ether (4.8 mL) and diphenyl chlorophosphate (1.56 g, 5.816 mmol) was added, followed immediately by triethylamine (1 g, 9.692 mmol). After 1 h, the solution was concentrated and purified by flash chromatography, eluting with ethyl acetate/triethylamine (19:1) to give 4.11a (1.45 g) in 92% yield. Compound 4.11a (1.45 g, 4.32 mmol) was added to a solution of sodium hydride, 57-63% in oil, (519 mg, 12.97 mmol) and excess benzyl alcohol in diethyl ether (14.5 mL). After 1 h, the reaction was concentrated and the crude was purified by flash chromatography, eluting with ethyl acetate/triethylamine (19:1) to give 4.11b (246 mg) in 15% yield. Compound 4.11b (246 mg, 0.68 mmol) was dissolved in acetonitrile (1 mL) and iodomethane (192 mg, 1.35 mmol) was added. After 1 h, the solution was concentrated to give 4.11c (337 mg), which was used in the next step without further purification. Compound 4.11c (337 mg, 0.67 mmol) was first hydrogenated in 50 ml methanol:water (1:1) with a catalytic amount of palladium hydroxide. These conditions removed only one of the two benzyl groups. The second benzyl group was removed by latex balloon hydrogenation in 150 ml 30% (v/v) aqueous formic acid with a catalytic amount of Pd/C. The reaction contents were filtered through a celite pad, concentrated under reduced pressure, and washed with dichloromethane. The aqueous layer was further evaporated under reduced pressure, and the residue was exhaustively dried overnight over phosphorus pentoxide to give 4.11 (80 mg) as a white solid (m.p. 230°C dec.) in 35% yield. ¹H NMR (D₂O) δ: 3.75 (m, 2H), 3.14 - 3.34 (m, 2H), 2.93 (s, 9H), 1.92 (m, 2H). ¹³C NMR (D₂O) δ: 64.41, 60.60, 52.96, 24.29, 24.20. IR (neat) ν (cm⁻¹): 3379, 3020, 2957,
2939, 2887, 2432, 1190, 1054, 932. HRMS (ESI) [M]+: Calcd for C₆H₁₇NO₄P⁺ m/z = 198.0890, found m/z = 198.0879.

4.4.3. Low molecular weight activator stock solutions

Cyclic peptide 38 (cp38), kindly provided by E. Appella, was dissolved in water (1 M) and then serially diluted with same. Dephosphorylation of cp38 with calf intestinal alkaline phosphatase (CIP; Promega) and YOP Protein Tyrosine Phosphatase (NEB) were carried out as described for other proteins. The possibility of exposure of the cleavage factors to residual CIP activity was eliminated by using an established and validated procedure. (S)-(+) Abscisic acid (ABA) was dissolved in DMSO (500 mM) and then serially diluted with water. Compounds 4.1-4.7 were placed in water and treated with 1-3 equiv. of 1.00 M HCl to dissolve the compounds. Compound 4.1 was purchased as the HCl salt and dissolved in water directly. Compounds 4.8-4.11 were dissolved in water and treated with NaOH until the pH reached 6 - 7.9. (1.1 equiv. of NaOH was added to compound 4.8 and 4.9; 1.0 equiv. of NaOH was added to compound 4.10; 1.5 equiv. of NaOH was added to compound 4.11; 0.25 equiv. of NaOH was added to compound 4.12.)

4.4.4. Cleavage factor fractionation from HeLa cell nuclear extract (Kindly carried out by my colleague, N. Nagre)

HeLa cell pellets were purchased from the National Cell Culture Center (Biovest International). Nuclear extract and 3’ cleavage factor DEAE-sepharose fractions (CPSF, CstF and CFm) were prepared as described in detail elsewhere. Active fractions from each cleavage factor were pooled, concentrated by 70% ammonium sulfate precipitation and dialyzed 2 X 3 h at 4°C against Buffer D50AS (20% glycerol, 20 mM Na-HEPES, pH 7.9, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT and 50 mM ammonium sulfate). Total protein concentration of cleavage
factor fractions was estimated by Bio-Rad Bradford protein assay with bovine serum albumin as the standard and determined to be: CPSF (3 mg/mL); CstF (3.5 mg/mL); CFm (3 mg/mL). The amount of 3’ cleavage factor fractions used in a 12.5 µL cleavage reaction were: CPSF (0.8 µL); CstF (0.6 µL); CFm (3 µL).

4.4.5. RNA substrate (Kindly made by my colleague, N. Nagre)

SV40 late pre-mRNA (233 nt) was transcribed in vitro by SP6 RNA polymerase (Promega) from the pG3SVL-A plasmid linearized at the DraI site. Substrate RNA was uniformly labeled by including [α-32P]-UTP (Perkin-Elmer Life Sciences) and 5’ capped during transcription using the 5’Me7G(5’)(ppp(5’)G-Cap analog (NEB). The transcript was purified on a 0.4 mm denaturing polyacrylamide gel, located by shadowing on film, and extracted as previously described.

4.4.6. In vitro 3’ cleavage reactions (Kindly carried out by my colleague, N. Nagre)

Cleavage reactions were carried out as described in detail elsewhere in 12.5 µL and contained, in addition to the cleavage activators indicated in the figures, tRNA (0.1 mg/mL), pH 8 EDTA (2 mM), 2’-dATP (2 mM), DTT (0.41 mM), BSA (Roche; 40 ng/mL), placental RNase inhibitor (Promega, 0.32 u/mL), polyvinyl alcohol (2.5%), 10% glycerol, 10 mM HEPES-NaOH (pH 7.9), 25 mM ammonium sulfate, the cleavage factor fractions described in section 4.4, and the RNA substrate (1–5 nM). The reactions were incubated at 30°C for 2 h and then digested with Proteinase K, phenol-CHCl3 extracted, ethanol precipitated, and resolved on a 6% denaturing polyacrylamide gel. After drying, the gel was exposed to a Molecular Dynamics Storm PhosphorImager screen and the bands were quantitated using ImageQuant software. Relative cleavage (R.C.) was calculated as [5’ fragment/ (5’ fragment + uncleaved RNA)] x 100. The total recovered RNA varied from lane to lane due to losses during phenol-CHCl3 extraction and
ethanol precipitation. Use of the R.C. ratio standardizes the amount cleaved and allows comparison of gel lanes with different amounts of total recovered RNA.

4.4.7. PPM1A inhibition assay

A 16.25 µL solution containing 154 mM Tris-HCl (pH 7.9), 15% glycerol, 31 mM MgCl₂, and 154 mM ammonium sulfate was placed in a microfuge tube on ice. To this was added 26.25 µL of a mixture containing 0.95 mg/mL BSA, 0.95 mM DTT, and 38 mM pNPP (all in water). Five µL of a 10X stock solution of the tested compound was then added. To start the reaction, 2.5 µL of a 0.05 mg/mL (approximately 1 µM) solution of PPM1A in 152 mM Tris-HCl, 5 mM imidazole, 3.75 mM NaCl, 30 mM MgCl₂, 140 mM ammonium sulfate, and 15% glycerol was added. Final concentration of all components were as follows: 58 mM Tris-HCl, 6% glycerol, 12 mM MgCl₂, 53 mM ammonium sulfate, 0.5 mg/mL BSA, 0.5 mM DTT, 20 mM pNPP, 250 µM imidazole, 187 µM NaCl, ~50 nM PPM1A, plus the inhibitor concentration as follows: CP, PhC: 10 and 50 mM; 4.1, 4.2: 0.2 and 1 mM; 4.7: 25, 100, 200 µM; 4.12: 0.1 and 1 mM; ABA: 50, 100, 200 µM; cp38: 150, 200 nM. The solution was gently mixed, centrifuged, and incubated at 37°C for 2.5 h. 10 µL of 500 mM EDTA (pH 10) was added to stop the reaction and the absorbance at 405 nm was taken on a NanoDrop spectrophotometer.

Acknowledgments. This work was supported by Grant 5SC1GM083754 from the National Institutes of Health (NIH). Additional infrastructural support at the City College of New York (CCNY) was provided by the NIH National Center for Research Resources (2G12RR03060-26A1) and the National Institute on Minority Health and Health Disparities (8G12MD007603-27).
Chapter 5: Use of boronic acids towards molecular recognition of DNA nicks

5.1. Introduction

Deoxyribonucleic acid (DNA) is a macromolecule that carries genetic information essential for life. The nucleotides making up DNA are linked with phosphodiester bonds, but some of these bonds may be broken due to damage or enzymatic activities resulting in nicks on the DNA. Theoretically, these DNA nicks could provide a point of recognition for designed small molecules to bind with the 3’ and 5’ hydroxyl-groups at the breaks. In this study, we aimed to substitute the phosphate group at DNA nicks with a boronate group (Scheme 5.1).

![Scheme 5.1](image-url)

Scheme 5.1: Representation of a DNA fragment with the phosphate group replaced with a boronate group.

Boronic acids are known to reversibly bind with diols with modest affinity to form boronic esters, and as a result, they have been used for various applications, including the development of saccharides sensors, nucleotide and carbohydrate transporters, affinity ligands, and antibody mimics.¹ The close proximity of the hydroxyl groups at the DNA nick resembles a diol, and could possibly bind with boronic acids in a similar way. Indeed, a DNA assembly had been synthesized with the phosphate linkage replaced with a boronate linkage,² demonstrating...
that nucleotides on DNA could be bonded within a boronate linkage in a stable manner. Although the phosphate group could potentially be replaced with a boronate group, whether boronic acids easily bind to DNA nicks is still uncertain, due to the only modest affinity between the boronic acid and the diol. We expect that the equilibrium shown in Scheme 5.1 (right) favors the middle structure, i.e. nick not filled with boronate. Our long term idea is to add the boronic acid to other DNA-recognizing structures to increase affinity and impart selectivity for the DNA break.

5.2. Experimental design

To determine whether boronic acids readily bind onto DNA nicks, we could simply mix the two together and observe if binding occurs. In order to replicate a DNA that contains nicks, we used two short DNA strands, DNA 1 – 5’-GTCGCC-3’ and DNA 2 – 5’-GACGCG-3’, which could form partial complementary pairings, leaving the OH at the ends to participate in binding (Figure 5.1).

Figure 5.1: Expected interaction between boronic acid, 5.4, and the nicked DNA form from DNA 1 and DNA 2.
Complementary base-pairing occurs at physiological pH (~pH 7.4). At high pH, the guanine and thymine bases become deprotonated, leading to less hydrogen bonding and therefore less pairing. The stability of the boronate ester is also pH-dependent. Boronate ester formation only occurs near or above the pKa of the boronic acid, so it was necessary to use a boronic acid with a lower pKa than the physiological pH of 7.4. The pKa’s of boronic acids are typically above pH 8, but the benoxaborole was found to have a pKa around physiological pH, ~7.2-7.3. Unfortunately, benoxaborole only contains the boronic group on one end of the phenyl ring, and thus it would not be effective in producing cross-links on DNA. To produce a boronic acid with a pKa around physiological pH and that is able to cross-link with DNA nicks, benoxaborole 5.4 was synthesized. Benoxaborole 5.4, which contains two boronic acid groups, was expected to bind onto the DNA nicks at around pH 7.4 (Figure 5.1).

One way to assess that binding has occurred was from the formation of a gel. Gels form when polymers are linked together. An especially well-known gel formation reaction is formulated using polyvinyl alcohol (PVA) and sodium tetraborate (borax). The borate acts as cross-linker for PVA, and the degree of cross-linking could be modulated by changing the concentration of the borate or the pH of the solution. If the benoxaborole 5.4 is indeed linking together chains of DNA, then it’s likely that a gel would form. However, it’s of importance to ensure that the ratio of the linker and polymer to the water content is adequate for gel formation. To determine the appropriate ratio of linker to polymer that would lead to gel formation, preliminary testing was done using compounds 5.1, 5.2, and 5.3. These boronic acids were tested for gel formation with polyvinyl alcohol (PVA), a polymer containing a multitude of hydroxyl (-OH) groups (as a simple though imperfect mimic of a diol). Boric acid 5.1, 5.3, and 5.4, were expected to form a gel with PVA through cross-linking, whereas the negative control,
phenylboronic acid 5.2, which contains only one boronic acid group, should not be able to form a gel (Scheme 5.2). Since the pKa of 5.1 (9.24), 5.2 (8.8), and 5.3 (~8.8) are all above 8, they need to be tested at high pH in order for gel formation to occur. (For the purpose of this experiment, pH 10 was used). Once a ratio was found which afford gel formation between boric acid 5.1 and PVA, the same ratio was used to test 5.2, which should not form a gel, and 5.3 and 5.4, which should form a gel with PVA. If gel formation was seen using 5.4 with PVA, then 5.4 would be tested with DNA at physiological pH.

Scheme 5.2: Expected interaction of PVA with 5.1, 5.2, 5.3, and 5.4.
5.3. Results and Discussion

Compounds 5.1, 5.2, and 5.3 were commercially available. The diboronic acid 5.4 was synthesized starting from toldidine. Tolidine was subjected to a Sandmeyer reaction to convert the amino groups to bromo-groups forming 5.4a. A free-radical halogenation reaction using N-bromosuccinimide (NBS) and AiBN as the initiator was employed to brominate the methyl groups on 5.4a to obtain 5.4b. The primary bromo-groups on 5.4b were reacted into acetyl-groups using potassium acetate to form 5.4c. Compound 5.4c was then subjected to a Miyaura borylation reaction to form 5.4d. The pinacol protecting groups on 5.4d was removed using sodium periodate giving the boronic acid 5.4e. Deprotection of the acetyl-groups gave the final product 5.4. The compounds (Table 5.1) were first tested on PVA for gel formation.
Compounds

5.1 Boric acid

5.2 Phenylboronic acid

5.3 4,4'-Biphenylbisboronic acid

5.4 Synthesized boronic acid

Table 5.1: Tested compounds.

<table>
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<th>Test #</th>
<th>Buffer conc. (mM)</th>
<th>PVA conc. (mM)</th>
<th>Boric acid, 5.1. conc. (mM)</th>
<th>Mole Ratio</th>
<th>OH Ratio</th>
<th>Gel?</th>
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Table 5.2: Optimized conditions for gel formation. Carbonate buffer at pH = 10 was used. Highlighted rows are conditions that led to the formation of a strong gel. OH ratio for boric acid was determined from the assumption that each molecule of boric acid could bind with four OH groups. The PVA used for the experiment consisted of polymers with molecular weights ranging from 30000 to 70000 g/mol. The average molecular weight, 50000 g/mol, was employed for the purpose of calculating the number of monomers, and therefore the number of OH units, on each molecule of PVA. (Testing was done by MS Biochemistry student, Jane Tania.)
Before testing the synthesized benzoxaborole, 5.4, on nicked DNA, we need to determine the ratio of benzoxaborole to DNA that would result in the formation of a gel. Preliminary optimization was done with commercially available polyvinyl alcohol (PVA) and boric acid, 5.1. Various concentrations of PVA and boric acid (5.1) were tested to achieve gel formation. From the data, gel formation was most apparent at a PVA: boric acid (5.1) mole ratio of 1:10, but gel formation may not occur when the PVA and/or boric acid (5.1) concentration are too low, as exemplified by test # 5 and 11 (Table 5.2), in which the mole ratio was almost the same as the mole ratio for test # 4 and 12 (Table 5.2), but gel formation did not occur. Additionally, gel did not form at pH = 7.4, which is below the pKa of boric acid (5.1) (results not shown). Since the other boronic acids (5.2, 5.3, and 5.4) are less soluble in water compared to boric acid, 5.1, a few additional tests were done to determine the lowest concentration of boric acid that could be used to afford gel formation with PVA (Table 5.3).

<table>
<thead>
<tr>
<th>Test #</th>
<th>Buffer conc. (mM)</th>
<th>PVA conc. (mM)</th>
<th>Boric acid, 5.1, conc. (mM)</th>
<th>Mole Ratio</th>
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Table 5.3: Lowering boric acid concentration for gel formation. Carbonate buffer at pH = 10 was used.

Gel formation could not occur at a concentration lower than 10 mM of boric acid (Table 5.3), so this concentration was used to test the other compounds (Table 5.4).

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Table 5.4: Gel formation of 5.2, 5.3, and 5.4 with PVA. Carbonate buffer at pH = 10 was used.
As expected, phenylboronic acid, 5.2, did not form a gel with PVA at any of the concentrations tested (Table 5.4). Since phenylboronic acid (5.2) could only bond with two OH groups at a time, it is less likely to crosslink molecules of PVA together. Boronic acid 5.3 was able to form a partial gel with PVA. Due to the low water solubility of 5.3, it forms a gel locally before it could homogenize with the solution. Since benzoxaborole 5.4 has a higher solubility in water, it was able to form a strong gel with PVA (Table 5.4, test # 33 and 34), but here the pH was 10. We wanted to eventually test the benzoxaborole 5.4 with DNA at pH 7.4, so 5.4 was tested with PVA at pH 7.4 (Table 5.5).

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Table 5.6: Gel formation of 5.4 with DNA1 (5’-GTCGCC-3’) and DNA 2 (5’-GACGGC-3’).

Benzoxaborole 5.4 could not be tested at 10 mM as planned since it was not soluble at even 2 mM in the DNA solution at either pH 7.4 or pH 10 (Table 5.6, test # 43 and 44).
Table 5.7: Gel formation of 5.4 with higher concentration of DNA 1 (5'-GTCGCC-3') and DNA 2 (5'-GACGGC-3').

During the testing, the concentration of both 5.4 and the DNA were lowered to ensure that 5.4 remained dissolved and concurrently achieve the OH ratio to achieve gel formation from the tests with PVA (Table 5.4, test #33 and 34). With PVA, 5.4 formed a strong gel at a ratio of ~ 30-40(PVA):1(5.4) (Table 5.4, test #33 and 34). The DNA was adjusted to be ~10 – 50 times more concentrated in OH sites than the boronic acid. Unfortunately, gel formation did not occur. The DNA solution may be too dilute to observe gel formation.

Table 5.8: Solubility at pH 7.4 (~10% DMSO).

Although gel formation with DNA did not occur at the tested conditions (Table 5.7), it was still of interest to determine if 5.4 could affect gel formation at pH 7.4. Thus, 5.4 was further tested on PVA. While 5.4 is slightly more soluble than 5.3 at pH 7.4, they both were not able to form a gel at the concentrations in which they were soluble (Table 5.8).
<table>
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<tr>
<th>Test #</th>
<th>Cpd</th>
<th>Buffer conc. (mM)</th>
<th>PVA conc. (mM)</th>
<th>Cpd. conc. (mM)</th>
<th>Mole Ratio</th>
<th>OH Ratio</th>
<th>Gel?</th>
<th>Notes</th>
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Table 5.9: Solubility at pH 7.4 using MOPS buffer.

To ensure that the phosphate buffer is not interfering with the solubility of 5.4, the compound was retested at pH 7.4 using another buffer, MOPS. As shown on Table 5.9, 5.4 behaved similarly in the MOPS buffer. As with the phosphate buffer (Table 5.8, test # 64 and 65), 5.4 was soluble at 0.75 mM but not 1 mM (Table 5.9, test # 66 and 67). Since 5.4 is soluble and could form a gel at 10 mM in a pH 10 buffer (Table 5.4, test # 33 and 34), the lower pH of the phosphate and MOPS buffer is likely affecting the solubility of the benzoxaborole 5.4.

<table>
<thead>
<tr>
<th>Test #</th>
<th>Cpd</th>
<th>Buffer conc. (mM)</th>
<th>PVA conc. (mM)</th>
<th>Cpd. conc. (mM)</th>
<th>Mole Ratio</th>
<th>OH Ratio</th>
<th>Gel?</th>
<th>Notes</th>
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</thead>
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</table>

Table 5.10: Solubility at pH = 7.8 using phosphate buffer.

Since the phosphate buffer does not appear to be affecting the solubility of the compound compared to other buffer (MOPS), 5.4 was again tested in a phosphate buffer with the pH increased to 7.8. At pH 7.4, 5.4 could only dissolve at concentrations lower than 0.75 mM, but at pH 7.8, 5.4 could dissolve at 1.3 mM (Table 5.10, test #71). There was an improvement in the solubility at pH 7.8, but unfortunately there were still not enough linkers in the solution to affect gel formation at physiological pH.

5.4. Conclusions

The synthesized benzoxaborole 5.4 was able to form a gel with PVA at pH 10, but it was not able to form a gel with DNA at any of the tested concentrations. This does not necessarily indicate that 5.4 was not binding with the DNA, seeing as the solution may be too diluted for a gel to form. Although the ratio (of the OH groups) of 5.4 to DNA was similar to the ratio of 5.4...
to PVA that led to gel formation, there was a large difference in the amount of OH groups on the DNA compared to PVA that was in the solution, and thus the hydroxyl group concentration was much lower. The DNA was tested at a concentration of 5 mM whereas PVA was only tested at 1.0-1.5 mM, but each molecule of PVA could have at least 680 OH groups, while the two DNA strands produce only four OH groups. Even at 1 mM, PVA has at least 34 times more OHs that are available for binding compared to DNA at 5 mM. In order to mimic the conditions for gel formation using PVA, the concentration of DNA may have to be over 100 mM, which is not practical for a biopolymer. Instead, other methods such as NMR, viscometry or calorimetry might be better to use to test for binding.

5.5. Experimental Procedures

Unless otherwise stated, chemical reagents and solvents were purchased from VWR International, Fisher Scientific, or Sigma Aldrich and used without further purification. Analytical TLC was performed on silica gel 60 F254 plates. Flash chromatography was performed on Teledyne Isco CombiFlash Rf-200 flash chromatography system. Melting points were measured on a Laboratory Devices Mel-Temp apparatus. 1H and 13C NMR spectra were recorded on a Varian Mercury 300 spectrometer or a Bruker Ultrashield 500 spectrometer. (Note: The literature procedure that was applied for each reaction is cited. The procedure may not necessarily be used for the synthesis of the same compound as in the literature.)

4,4′-Dibromo-3,3′-dimethyl-biphenyl (5.4a). Compound 5.4a was synthesized based on literature procedure. Tolidine (5 g, 23.5 mmol) was dissolved in a solution of 50% sulfuric acid (25 mL), water (100 mL), and acetonitrile (100 mL). The solution was cooled to 0°C and sodium nitrite (4.1 g, 60 mmol) in water (10 mL) was added. The mixture was stirred briefly and copper bromide (7.5 g, 52 mmol)
in 48% hydrobromic acid (100 mL) was added. The mixture was warmed to room temperature and refluxed overnight. The crude mixture was passed through a silica pad. The filtrate was concentrated and purified by column chromatography eluting with 100% hexanes to obtain the product **5.4a** (3.6 g, 10.6 mmol) as a white solid (m.p. 63-65°C) in 45% yield. $^1$H NMR (CDCl$_3$) δ: 7.57 (d, $J = 8.3$ Hz, 2H), 7.46 - 7.31 (m, 2H), 7.29 - 7.16 (m, 2H), 2.55 - 2.39 (s, 6H). $^{13}$C NMR (CDCl$_3$) δ: 139.39, 138.30, 132.75, 129.31, 125.84, 124.29, 23.08.

![4,4'-Dibromo-3,3'-bis-bromomethyl-biphenyl](image)

**4,4'-Dibromo-3,3'-bis-bromomethyl-biphenyl (5.4b).** Compound **5.4b** was synthesized based on literature procedure.$^8$ Compound **5.4a** (2.45 g, 7.2 mmol), N-bromosuccinimide (2.56 g, 14.4 mmol), and a catalytic amount of azobisisobutyronitrile (AiBN) were suspended in chloroform (30 mL). The suspension was refluxed overnight. The next day, the suspension was cooled and filtered to obtain the crude product **5.4b** (3.1 g) as a white solid (m.p. 211-214°C), which was used for the next step without further purification. $^1$H NMR (CDCl$_3$) δ: 7.68 - 7.61 (m, 4H), 7.35 (dd, $J = 2.2$, 8.3 Hz, 2H), 4.66 (s, 4H). $^{13}$C NMR (CDCl$_3$) δ: 139.40, 137.73, 133.96, 129.62, 128.49, 124.11, 33.10.

![Acetic acid 3'-acetoxyethyl-4,4'-dibromo-biphenyl-3-ylmethy](image)

**Acetic acid 3'-acetoxyethyl-4,4'-dibromo-biphenyl-3-ylmethy ester (5.4c).** Compound **5.4c** was synthesized based on a literature procedure.$^9$ The crude product **5.4b** (3 g) was refluxed with potassium acetate (3 g, 30.6 mmol) in dimethylformamide (50 mL). After 1 h, the solution was cooled, diluted with water, and extracted three times with diethyl ether. The diethyl ether solution was extracted with brine, dried, and concentrated. The resulting material was purified by flash chromatography, eluting with hexanes/ethyl acetate (9:1) to obtain the product **5.4c** (950 mg, 2.08 mmol) as a white solid (m.p. 112-113°C) in 29% yield in two steps. $^1$H NMR (CDCl$_3$)
\( \delta \): 7.67 - 7.62 (m, \( J = 8.3 \) Hz, 2H), 7.57 (s, 2H), 7.40 - 7.35 (m, \( J = 8.0 \) Hz, 2H), 5.25 (s, 4H), 2.16 (s, 6H). \(^{13}\)C NMR (CDCl\(_3\)) \( \delta \): 170.61, 139.35, 135.87, 133.42, 128.51, 128.21, 123.09, 65.79, 20.93.

**Acetic acid 3'-acetoxyethyl-4,4'-bis-(4,4,5,5-tetramethyl-[1,3,2]diazaborol-2-yl)-biphenyl-3-ylmethyl ester (5.4d).** Compound 5.4c was synthesized based on a literature procedure.\(^1\) Compound 5.4c (950 mg, 2.08 mmol) was dissolved in dioxane (10.4 mL) and the solution was bubbled for 15 minutes with argon. Potassium acetate (0.98 g, 9.98 mmol), bis(pinacolato)diboron (BPin)\(_2\) (1.16 g, 4.58 mmol), and Pd(PPh\(_3\))\(_2\)Cl\(_2\) (234 mg, 0.334 mmol) were added and the suspension was stirred at 80°C for 3.5 h under argon. The suspension was filtered and concentrated. The resulting residue was purified by flash chromatography eluting with hexanes/ethyl acetate (19:1) to obtain the product 5.4d (970 mg, 1.76 mmol) as a yellowish oil in 85% yield. \(^1\)H NMR (CDCl\(_3\)) \( \delta \): 7.93 (d, \( J = 7.7 \) Hz, 2H), 7.62 - 7.53 (m, 4H), 5.43 (s, 4H), 2.10 (s, 6H), 1.35 (s, 24H). \(^{13}\)C NMR (CDCl\(_3\)) \( \delta \): 170.78, 143.25, 142.50, 138.86, 129.01, 128.75, 126.20, 126.13, 116.86, 102.35, 56.85, 28.01, 21.12.

**3H,3'H-[5,5']Bi[benzo[c][1,2]oxaborolyl]-1,1'-diol (5.4).** Compound 5.4d was oxidized based on a literature procedure.\(^1\) Compound 5.4d (420 mg, 0.76 mmol) was placed in THF (8.4 mL) and water (2.1 mL), and sodium periodate (980 mg, 4.6 mmol) was added. The suspension was stirred overnight. 2 M Hydrochloric acid (0.168 mL) was added and the mixture was stirred for another 24 hours. Methanol (35 mL) was added and the mixture was filtered. The filtrate was diluted with ethyl acetate, dried, concentrated to give the crude product 5.4e, which was used for the next reaction without further purification. The crude product 5.4e was stirred with potassium carbonate (1.79
g, 13 mmol) in methanol for 2 hours. The suspension was acidified with 5% HCl until the solution became clear. The solution was diluted with water and extracted with ethyl acetate, dried, and concentrated. The resulting solid was triturated in methanol and then hexanes to obtain the product 5.4 (75 mg, 0.28 mmol) as a white solid (m.p. 339°C dec.) in 37% yield in two steps. 

\[ ^1\text{H NMR (DMSO)} \delta: 9.21 (s, 2H), 7.81 (d, J = 7.4 \text{ Hz}, 2H), 7.72 - 7.63 (m, 4H), 5.04 (s, 4H). \]

\[ ^{13}\text{C NMR (DMSO)} \delta: 155.33, 143.03, 131.51, 126.50, 120.38, 70.44. \]

**General gel formation assay**

PVA (0.1-1.7 mM) or DNA (0.5-5 mM), buffer solution (5-50 mM), and water were added into an eppendorf tube and vortexed briefly. The boronic acid (0.1-30 mM) was added and the solution was immediately vortexed and left at room temperature overnight. If gel had not formed by the next day, solutions were sonicated, heated, placed at 0°C, or mixed to promote gel formation. After each manipulation, the reaction was examined for gel formation or thickening.
References

Abstract


Chapter 1


**Chapter 2**


6. Rawat, V.; Chouthaiwale, P. V.; Chavan, V. B.; Suryavanshi, G.; Sudalai, A. A facile enantioselective synthesis of (S)-N-(5-chlorothiophene-2-sulfonyl)-beta,beta-diethylalaninol


Chapter 3


**Chapter 4**


10. Overy, D.; Calati, K.; Kahn, J. N.; Hsu, M. J.; Martin, J.; Collado, J.; Roemer, T.; Harris, G.; Parish, C. A. Isolation and structure elucidation of parafungins C and D, isoxazolidinone-


**Chapter 5**


