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Naturally-Derived Molecular Ensembles in Medicine, Materials Science and Evolutionary Biology: An Interdisciplinary Study

Silvio Panettieri
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Naturally-Derived Molecular Ensembles in Medicine, Materials Science and Evolutionary Biology – An Interdisciplinary Study

by

SILVIO PANETTIERI

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
Dedicated to my family.

“The philosophy of mimicking natural processes precedes the science of understanding them.”

Sir Albert Howard
Naturally-Derived Molecular Ensembles in Medicine, Materials Science and Evolutionary Biology – An Interdisciplinary Study

by

Silvio Panettieri

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

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THE CITY UNIVERSITY OF NEW YORK
Abstract

Naturally-Derived Molecular Ensembles in Medicine, Materials Science and Evolutionary Biology – An Interdisciplinary Study

by

Silvio Panettieri

Advisor: Prof. George John

The first chapter will introduce the work carried out in collaboration with the Govind laboratory at CCNY. Our quest was set forth to investigate the intimate relationship lying between chronic inflammation and tumor development. For at least the last fifteen years much research has been conducted on this topic; yet, the level of complexity arising from exceedingly interwoven biochemical pathways in mammals has resulted in slow advancements in this field. This is why we resorted to a simple yet powerful immunogenetic model organism, the fruit fly Drosophila melanogaster, in combination with the administration of the most common anti-inflammatory drug, that is aspirin, that in recent years has shown remarkable anti-cancer properties. We induced chronic inflammation and tumor development through the constitutive expression of pro-inflammatory signalling pathways in genetically-tractable Drosophila models. Upon aspirin treatment we discovered aspirin-triggered fatty acid derivatives with known potent anti-inflammatory properties in humans. Aspirin administration concurrently restored immune and lipid homeostasis, rescued microtumor-inflammation and improved viability. Aspirin’s coordinated effects on immune signalling and bioactive lipid circuitry suggest novel but conserved chemoprotective mechanisms for therapeutic intervention at the earliest stages of tumor development.
The second chapter will present an original approach for the development and study of small fluorescent compounds that are able to respond to changes in local viscosity for *in vitro* applications. Even though these molecules were originally designed to be tested directly in live cells and image compartments with different fluidity (cell membrane, cytoplasm, etc.), we decided to take a step back in order to first have a clear idea on how these novel viscosity probes behave in highly heterogeneous environments, such as the inside of a cell, and render this question easier to address. In order to develop a robust model, we took advantage of the John lab’s expertise and employed molecular gels that in spite of their solid-like nature mostly consist of liquid-like regions in which is present a physically distinct network of orderly arranged gelator molecules. This approach allowed us to make outstanding discoveries regarding the nanoscopic architecture of the gel under study as well as the photophysics underlying the behavior of these probes. I acknowledge that some material in Chapter 2 (paragraphs 1.1 - 1.4, 3.1 and 3.2) was previously published in my Master’s Thesis with the title “Synthesis and characterization of viscosity-dependent fluorophores for bioanalytical use”.

The final chapter will show how a chemical approach can be interestingly adopted even in the case of research in evolutionary biology. This collaborative project with Dr. Lohman at CCNY had the ambitious goal to prove that two butterfly sub-populations belonging to the same species (*Elymnias hypermnestra*) develop chemically different sets of wing pigments as a result of residing in geographically distinct regions (Thailand and Indonesia). So far the evolutionary biology community has mainly been interested in a genetic approach aimed at identifying subtle changes in the organism’s DNA sequence due to adaptation to a certain environment, predator, diet, etc. The present study tries for the first time to expand the tools available to biologists
studying the evolution of butterfly as well as other organisms by looking at “chemical phenotypes”, namely variations in the chemical composition of wing pigments. Among specific aspects that will be thoroughly discussed later (Chapter 3), this project also resulted in the remarkable discovery of previously unknown pigments that broaden our knowledge in naturally-occurring compounds.
Acknowledgements

First of all, I would like to thank my PhD advisor Prof. John for supporting me during these past four years, encouraging my research and allowing me to grow as a research scientist. I sincerely appreciate that you gave me the opportunity to express my scientific creativity, attend conferences worldwide and pursue extremely exciting and enriching collaborative projects.

One of the collaborative projects started very early during my Ph.D. and was established with Dr. Govind. I cannot help thanking you for teaching me how to approach scientific discoveries with enthusiasm and rigor. You also ushered me into what used to be alien worlds for me, namely biology and genetics. I have to admit that besides uttermost curiosity, I also felt some fear when I had to start “speaking” a language so different from chemistry. I was used to working with just inanimate matter, namely pure compounds that could be named unmistakably and would not change their physical appearance or composition over time. All of the sudden I had to deal with living creatures that mate, grow, metamorphosize, fly and die. That was a much more significant cultural shock than moving to NYC almost five years ago.

Special thanks go to my thesis committee members, Prof. Stark, Prof. Bratu and Prof. Neubert for their constant support and the invaluable suggestions provided in the past years which significantly and positively changed the course of my research.

I am also grateful to Prof. Lohman for initiating a wonderful collaboration that allowed me to put myself to the test with my newly-acquired LC-MS skills and learn how fascinating butterflies are.

During these years I have created very special relationships with my labmates and colleagues. I have always felt like I had a second family ready to support me professionally,
psychologically and emotionally and now I know for sure that at least some of these people will be my true friends for the rest of my life.

In the John lab many people have come and gone and I am thankful to all of them for contributing to my growth and well-being to different extents. In particular, Julian has been a source of inspiration, amazing science and sincere friendship. Without you these years would not have been as exciting. Erisa also played an important role during these years. I know you have learned a lot from me but you will probably never know how much I learned from you. Malick is most likely the kindest and most generous person I have ever met and Satish has the unique ability to make you feel at ease and make a smile show up on your face no matter how you feel. I am so glad I could spend these years together with you guys. Mike is one of those people you have to get to know better before you start liking them. Fortunately I did and was positively surprised to see how much sincere enthusiasm towards science you have. That is something I really value.

I want to thank all the people in Govind lab I spent large part of my Ph.D. with. I feel blessed to having being able to be part of your research and life. In particular, I would like to thank Roma for teaching me most of the basics skills needed to handle fruit flies (without which the first chapter would not exist) and most importantly, for being an irreplaceable friend; and Johnny, thank you for your generosity, hard work and immense patience.

After the John lab moved to the new science building CDI followed by others, the dynamics with the surrounding labs changed for the better and all of a sudden we all started spending more time together talking about science, life, philosophy, politics, food etc. One lab in particular made these years unique, namely the Bandosz lab. Besides the scientific discussions
and collaborations I had with some of them, I will always treasure the beautiful friendships that
have been born during these years. In particular, I want to thank Lilja for making me feel at
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my very first friend in NYC and for always proving to be so ever since; Nikolina for your
incredible enthusiasm towards life and science that is certainly a source of inspiration for me.
Among my favorite people outside of the scientific community are Miriam and Mark. For me
you two will always be a beautiful example of what deep passion, love and perseverance can
create.

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you and the extremely diverse conversations I have always deeply enjoyed.
I want to thank Jiye for helping me to realize that there is an infinite potential inside every one of
us waiting to be unleashed. The secret lies in finding the right key for that to happen.
Two people that have done so much for me during these years are my spiritual siblings, Yana
and Ausar. Yana and I have gone through such profound changes during just the year and a half
we have known each other and we have done all that together. Your friendship has been beyond
what I could have ever imagined. Ausar, the most talented violinist I know, has had a very
significant impact on my life. I cannot thank you enough for the amazing music we have done
together and for proving to me that what we truly are is beyond human imagination.

Finally, my family. When you have to go through such a big adventure in your life and
your family is on the other side of the “pond”, that is tough. Fortunately, besides being far away,
they are also the best family I could ever have and they have always done their best to make me feel close as though I was sitting at the table with them.

I do not have enough words to express the deep and unconditional Love I feel for them. I just know that wherever life will take me I will always carry them inside of me. What my parents taught me and are still teaching me from afar has been of paramount importance to make the past five years unforgettable. It was so wonderful to see my siblings move on with their lives, make important decisions, make their dreams come true and at the same time be always curious about my life here, so different from what it was used to be.

No matter how far apart we will be in the future, there will always be an invisible thread keeping us as united as ever.
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Preamble

Nature is the richest source of inspiration for chemists and being a chemist myself I look at Nature as a wonderful ensemble of molecules that always continues to amaze me. Since I started to unveil that blanket of mystery around chemistry during college, I have been digging deeper and deeper into the sophisticated logic that in Nature finely influences synthesis, properties and effects of naturally-occurring compounds. I found myself trapped in an addictive curiosity that has constantly fueled my everyday life both as an individual and a scientist. With this mindset I approached my Ph.D. projects during which I had the opportunity to use my own creativity and knowledge to take advantage of what Nature so generously provides, make new discoveries starting from naturally-occurring building blocks and give a sound contribution in different fields of science.

For several years John lab has been developing new materials starting from small naturally-derived molecules\(^1\),\(^2\). Among these there is a family of compounds that has gained particular attention in the past decades, namely anacardic acids (AAs). These compounds derive from the shell of the cashew nut (\textit{Anacardium occidentale}) and all share a common structure consisting of an ortho-hydroxy benzoic acid substituted in 5 position by a 15-carbon atom chain. Prof. John accumulated a solid experience on anacardic acids and the chemistry associated with them as demonstrated by several publications featuring these compounds\(^3\),\(^4\). The presence of unsaturations on the alkyl chain and ease in functionalizing the aromatic ring combined to its amphiphilic nature make it an ideal substrate for the creation of a vast range of new derivatives with specific properties\(^4\). At the same time, AAs have been employed for centuries in Ayurvedic medicine for the treatment of several different conditions, such as fever, psoriasis, baldness, etc.\(^5\). Only recently did the scientific community start to investigate more in detail the mechanism of action of the supposed anti-inflammatory and even anti-cancer properties of AAs\(^5\). Interestingly, from a structural standpoint, AAs are highly related to another renowned molecule, that is salicylic acid (SA) originally extracted from willow tree bark. As it turned out, SA also possesses remarkable anti-inflammatory properties that the ingenious chemist Felix Hoffman was able to further enhance by synthesizing the derivative acetylsalicylic acid, also known as aspirin (ASA) in 1897\(^6\).
With the idea of expanding our knowledge of AAs by investigating their medicinal properties, during the first few months of my Ph.D. we initiated a fruitful collaboration with Govind lab that at that time was already interested in studying the anti-inflammatory and anti-cancer properties of ASA using *Drosophila melanogaster* (also known as fruit fly) as a model organism. In spite of a few months of preliminary experiments yielding promising results for both molecules, we realized that the interest on ASA as a preventive cancer medicine was gaining traction in both scientific literature and media. This led us to put AAs aside temporarily and focus on understanding ASA’s properties with the goal of contributing to the vast yet still unclear evidence regarding its mechanisms of action. This is how my journey in a very challenging and intellectually stimulating project began. In spite of its largely biological nature, this project clearly showed me that combining a chemistry-oriented mind with biology is a recipe for exciting science. This is because one has the chance to explore the science of life made up of ever-growing organisms (in this case, fruit flies) along with several biological components interacting between each other in a harmonious yet highly complex fashion; at the same time, the chemist feels the need to peer down, simplify and zero in on well-defined small biological molecules that can be isolated, fixed in space and time and named unmistakably. This collaborative project resulted in a very cohesive story that we believe will pave the way for further discoveries regarding aspirin and its use as a preventive anti-cancer drug.

The second project discussed in the present Ph.D. thesis started to take shape when at the beginning of my Master’s thesis at University of Pisa I was assigned to develop novel fluorescent molecules to use as viscosity probes *in vitro*. Fortunately, my knowledge in natural compounds at that time was good enough to make me remember that some plants such as sweet clover and meadowsweet are named for their pleasant smell, which in turn is due to high concentration of a compound called *coumarin*. Interstingly, coumarins are known not just for their pleasant smell (reason why the perfume industry used to use coumarins) but mostly for their remarkable ability to fluoresce, that is emit light upon an external light stimulus. By utilizing this simple naturally-derived basic moiety, I build upon it to design significantly more complex molecules sharing the same core, yet possessing the ability to modulate the amount of light emitted as well as its wavelength depending on the viscosity of the surroundings (very bright in...
honey-like solutions and very dim in water-like media). Such molecules belong to a vast family of compounds called *molecular rotors* as they can be depicted as a nanosized rotor/stator device in which the reciprocal rotation of the two components affect their photophysical properties. Remarkably, J. Fraser Stoddart, Jean-Pierre Sauvage, and Bernard L. Feringa won the Nobel Prize in 2016 for their outstanding work on molecular motors which are an even larger family of molecules that includes the above-mentioned rotors as well.

The rationale to develop viscosity-sensitive fluorophore lies in that several health conditions such as Alzheimer and Parkinson disease, and cardiovascular issues are associated to drastic changes in viscosity within cellular compartments of specific organs. The possibility of monitoring such alterations with a minimally-invasive approach is appealing to the medical community. Due to the complexity of biological systems, during my Ph.D. I decided to take a step back and investigate the behavior of these nanoprobes by mimicking the spatial and temporal microscopic organization present in cellular compartments with molecular gels, materials that John lab has an extended experience on. The evidence of the great potential of these molecules as viscosity probes presented itself when we observed that one of the synthesized rotors did not respond to drastic changes in viscosity upon gelation of an organic solvent brought about by a low-molecular-weight gelator. On the other hand the same molecule behaved as expected in a polymer methylcellulose gel. The main difference between the two gel systems is that in the latter the polymer chains extend in the solvent randomly, while at the microscopic level the molecular gel presents a very well-organized 3D network made up of fibers, sheets, ribbons, helices, spheres etc. just like the micro- and nano-scopic structures within a cell (cell membrane, cytoskeleton, DNA, hystones, etc.). The work showed in this thesis explores successful strategies developed in collaboration with scientists at University of Pisa to deepen our understanding of molecular rotors and further extend their use in both materials science and biology.

A minor yet exciting final project is also discussed in this thesis. This one was initiated when the evolutionary biologist Prof. Lohman (CCNY) and the John lab realized that our reciprocal expertise could be utilized to study a class of naturally-occurring compounds present in the wings of a specific species of butterfly (*Elymnias Hypermnestra*) and link their chemical
composition to a clever evolutionary strategy adopted by this species for survival. The work showed here is part of a much more vast multidisciplinary project that also involves photophysicists at Columbia University. The ultimate goal consists of taking advantage of the wondrous piece of science butterfly wings are in order to progress our understanding about evolution, natural compounds and materials science. The expertise I acquired in liquid chromatography and mass spectrometry while working on the fruit fly project allowed me to be able to confidently face the challenge of determining the butterfly wing’s chemical composition and answer intriguing questions about the way they evolved.

In conclusion, the three chapters incorporate the essence of what modern scientific research is mainly supposed to consist of, that is, collaborative efforts. Such an approach becomes essential when dealing with naturally-occurring compounds and their interaction within biological systems. This challenge is intrinsically complex and requires an array of expertises as well as physical and intellectual tools that are seldom possessed by one single individual or laboratory. In all three projects the ultimate goal was to carefully investigate relatively broad biological and biochemical problems by employing specific and tailored models that could neatly suit the purpose. Nature offered the solution.

Chapter 1: A Drosophila metainflammation-blood tumor model links aspirin-triggered eicosanoid-like mediators to JAK-STAT signalling

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1. INTRODUCTION

The acute inflammatory response is the body's first system of alarm signals that are directed toward containment of microbial and viral attacks and/or physical damage, and eventually their resolution through activation of the immune response. Whenever an inflammatory state does not result in complete resolution and re-establishment of the initial homeostasis, a chronic condition may develop. In case metabolic tissues are affected by chronic inflammation, the consequences on a biochemical and physiological level can be far more intricate as metabolic pathways become affected as well. What nowadays is referred to as metabolic inflammation or, more commonly, metainflammation has emerged as a pathophysiologic basis for many widely occurring malignancies in the general population that were not initially known to be linked to the inflammatory response, including obesity, type 2 diabetes, and cancer 1.

1.1 The intimate link between inflammation and cancer

In 1863, the pathologist Rudolf Virchow proposed that the origin of cancer was intimately linked to a state of chronic inflammation in the tissue affected by it. His hypothesis was that some classes of irritants along with tissue injury and corresponding inflammation stimulate cell proliferation 2. Normal cell proliferation does not cause cancer because the organism has sophisticated mechanisms to regulate it. Moreover, homeostatic mechanisms have been identified that help subside acute inflammation and proliferation after the healing process is completed 3. Aberrant proliferation sustained by growth/survival factors occurs in response to DNA damage and/or mutagenic assaults. Thus, tumors can be considered as wounds that are unable to heal and that have the potential to damage surrounding tissue 4.
The Nobel-prize winning virologist Peyton Rous first recognized that cancers develop from “subthreshold neoplastic states” caused by chemical or viral carcinogens that induce somatic changes. Such an initial phase is now referred to as ‘initiation’. It usually involves stable genetic alterations, that in normal tissues can remain dormant until a second type of stimulation (called ‘promotion’) occurs also via genetic mutations. Typical promoters are chemical irritants, factors released at the site of wounding, partial organ resection, hormones and chronic irritation and inflammation. Promoters play a crucial role in fuelling the overproliferation process, whether directly or indirectly, in that they recruit inflammatory cells, increase the production of reactive oxygen species (ROS) that in turn cause oxidative DNA damage as well as reduce DNA repair. In this context, cell death and/or repair programs may be partially compromised, leading to errors in DNA replication and high rates of cell proliferation. Interestingly, during a normal acute inflammation response, inflammation is self-limiting because the initial phase giving rise to the production of pro-inflammatory cytokines precedes the release of anti-inflammatory and pro-resolution cytokines. In chronic inflammation, however, the persistence of the promoters in the organism and/or the inability of the organism to initiate the necessary pro-resolution response cause the inflammatory state to become chronic.

1.2 Classical and novel eicosanoids in infection, inflammation and cancer

More than 70 years ago scientists discovered the roles of prostaglandins (PGs) in mediating acute inflammation. PGs are arachidonic acid (AA; 20:4 omega-6)-derived compounds. In the decades that followed, the biochemical evidence for the production of these pro-inflammatory bioactive lipids became clearer with the elucidation of both the eicosanoid pathway for their biosynthesis and the mechanism through which aspirin-like drugs block
prostaglandin production. Eicosanoids are C20 polyunsaturated fatty acids (PUFAs, mostly AA, eicosapentaenoic acid (EPA) and dihomo-gamma-linolenic acid (DGLA)) that can be converted both enzymatically and non-enzymatically into oxidized lipids referred to as oxylipins. (More specifically, oxylipins are compounds whose formation involves at least one step of O$_2$-dependent oxidation and PGs are examples of oxylipins.)

More recently, a new family of bioactive lipids similar to eicosanoids, referred to as ‘novel eicosanoids’, was reported. These novel eicosanoids are not derived from C20 fatty acids (as the name eicosa - twenty in Greek - would suggest) but instead either from shorter C18 or longer C22 fatty acids. In spite of this structural difference, both their biosynthesis and modes of action is similar to what is observed for classical eicosanoids and in several instances they play crucial roles in the resolution of inflammation. Thanks to the pioneering work of Charles Serhan on novel eicosanoids such as members of the resolvin and protectin families, new biomarkers of inflammation have become available. These mediators have anti-inflammatory and pro-resolution properties, thereby protecting the affected organs from further damage, promoting the clearance of inflammatory debris and triggering antimicrobial defense. Several other families of oxylipins, such as electrophilic oxo-derivatives (EFOX), have been discovered in recent years and this is contributing to delineate more and more clearly the intimate connection between bioactive lipids and immunity. The precise identification of the signalling molecules playing primary and direct roles in fuelling chronic inflammation and subsequently carcinogenesis will provide new tools to understand the strong association existing in the case of several malignancies. For example, individuals with chronic ulcerative colitis, Crohn’s disease (a chronic bowel condition), and celiac disease (genetic disease underlying an intolerance to gluten which in turn triggers a powerful inflammatory response in the gut) display exceptionally high
incidence of colon cancer. Experiments in mice have shown that knockout mutations in the prostaglandin-endoperoxide synthase (Ptgs2) encoding COX-2 gene suppress intestinal polyps induced mutations\textsuperscript{12}. In addition, individuals with Hepatitis C infection in the liver show high predisposition to liver carcinoma, schistosomiasis (also known as snail fever, a disease caused by parasitic flatworms) correlates with an increased risk of bladder and colon carcinoma, while chronic \textit{Helicobacter pylori} infection is the world’s leading cause of stomach cancer \textsuperscript{13}. In the latter case, the Gram-negative bacterium \textit{H. pylori} has been shown to be the definite carcinogen for the development of gastric cancer (the second most common type of cancer globally)\textsuperscript{13} where DNA damage in conjunction to chronic inflammation is the most likely mechanism.

Viruses are also known to be responsible for inducing tumor growth. Infectious viral agents, such as DNA tumor viruses are thought to directly intervene on the cell’s genetic material by inserting active oncogenes, even though other mechanisms are under investigation\textsuperscript{3}. Many examples of such infectious agents are recognized in animals whereas only a few are known to target humans and they include human papilloma virus, hepatitis B virus (HBV), Epstein-Barr virus and Rous sarcoma virus\textsuperscript{5}. Generally, cancer development is effective in young children and if the individual is immune-suppressed because of HIV infection, chemotherapy or use of immunosuppressant. For instance, in Rous sarcoma virus infection, the Tumor Growth Factor-\(\beta\) and other cytokines produced by inflammatory cells are essential and actively mediate the tumor development\textsuperscript{14}. Similarly, the Epstein-Barr virus triggers uncontrolled proliferation of lymphocytes which can result in neoplasia giving rise to Burkett’s lymphoma in presence of a secondary mutation\textsuperscript{3}. Significant shifts in metabolism occur in cancer cells and highly-conserved signaling pathways activated by cytokines and lipids support the rapid expansion of the affected tissues.
These last few examples are representative of a growing body of evidence suggesting that at least 15% of malignancies worldwide arise from infections contributing to 1.2 million cases per year. As shown earlier, tumor development may arise from an infection-induced chronic inflammatory state. Researchers believe that leukocytes and other phagocytic cells recruited at the infection site generate ROS and nitrogen species to fight infection as it normally happens. Yet, repeated tissue damage and unresolved healing processes imparted by these highly oxidizing and mutagenic species induce chemical modifications of DNA resulting in irreversible genomic alterations such as point mutations, deletions, or rearrangements.

1.3 Aspirin: the wonder drug

Aspirin’s mechanism of action was discovered in the 1970s when it was observed that it irreversibly acetylates and deactivates the cyclooxygenase enzyme (prostaglandin H-synthase)\(^ {15,16}\) (Figure 1). Interestingly, COX enzymes are responsible for the biosynthesis of cyclic endoperoxides from arachidonic acid (AA) generating prostaglandins, prostacyclins and thromboxanes that have numerous functions in the body.
Salicylic acid (IUPAC name: ortho-hydroxy benzoic acid) was originally extracted from the willow tree bark. The acetylation of the hydroxyl group gives rise to the most common anti-inflammatory NSAID worldwide, i.e. acetylsalicylic acid (Aspirin).

The uniqueness of aspirin among non-steroidal anti-inflammatory drugs (NSAIDs) consists in its ability to irreversibly inactivate both COX-1 and COX-2 which is what most of its anti-inflammatory, antipyretic and analgesic effects arise from. ASA’s anticancer effects are yet to be elucidated but both COX-dependent and independent mechanisms have been proposed. Although more will be discussed in the next chapters, in this introduction we will focus on the COX-dependent mechanisms.

1.3.1 COX-dependent mechanisms

ASA’s ability to affect AA metabolism has been the most accepted explanation to the anti-cancer effect in virtue of the identification of several COX-dependent pathways/downstream targets. In fact, ASA and its primary metabolite salicylate have been shown to affect COX-2 expression at both transcriptional and post-transcriptional levels. Pro-inflammatory
prostaglandins levels are downregulated upon ASA- and salicylate-mediated reduction of the COX-2 gene transcription\(^\text{18}\). In addition, COX inhibition increases AA levels that in turn can prompt the conversion of sphingomyelin to ceramide that has well-known apoptotic properties\(^\text{19}\).

Another mechanism of aspirin concerns its anti-coagulation properties. Interestingly enough, cancer patients exhibit an increase in platelet activation that has been known to have a role in cancer progression and metastasis\(^\text{20}\). ASA is much more effective at inhibiting COX-1 in anucleate platelets owing to its short plasma half-life (15-20 min) as opposed to COX-2 in monocytes, thus causing a long-lasting defect in TXA2-dependent platelet function\(^\text{21}\). The reason for this is that nucleated cells can promptly resynthesize the inactivated enzyme making COX-2 in monocytes less sensitive and requiring higher doses of ASA and shorter dosing intervals.

Colon cancer presents overexpression of COX-2\(^\text{22,23}\). Additionally, colon cancer-overexpressed COX-2 is known to generate high levels of prostaglandin E2 (PGE2). This pro-inflammatory lipid derivative causes resistance to apoptosis, stimulation of cell migration and angiogenesis. It has also been associated to the development of a number of malignancies such as those of the lung, breast and neck\(^\text{24}\). Human studies showed that adenoma regression was enhanced upon reduction of PGE2 tissue levels through NSAID treatment\(^\text{25}\). Similar experiments with COX-2-inhibitors demonstrated that the COX-2 promoter is inducible and is regulated by NF-\(\kappa B\) signaling\(^\text{26,27}\). Because of the crucial role of COX-2 in bioactive lipid production, this strongly suggests the existence of a biochemical interaction between components of the NF-\(\kappa B\) pathway and eicosanoids.

Serhan et al. in 1995 and other researchers in more recent years\(^\text{6,28,29}\), observed that not only does the irreversible COX-2 inhibitor ASA block PG production via inhibition of COX-2
activity but it also induces a modification of its enzymatic activity leading to the generation of novel oxidized lipids. This occurs as a consequence of the ASA-mediated acetylation of a Serine residue within COX-2’s enzymatic pocket. As a result COX-2 performs an incomplete oxidation reaction that eventually produces a wide array of compounds with remarkable anti-inflammatory and anti-tumor properties. It is noteworthy to mention lipoxins, maresins, resolvins and mono-hydroxylated fatty acids. In mammalian cell cultures Groeger et al. have shown that such mono-hydroxylated lipids can undergo further oxidation mediated by cholesterol-dehydrogenase that leads to the production of a novel family of anti-inflammatory lipids, referred to as anti-inflammatory electrophilic oxo-derivatives (EFOX). As mentioned earlier, COX-2 uses arachidonic acid as a substrate to produce its natural cyclic products. Interestingly, acetylated COX-2 is able to generate mono-hydroxylated products (i.e. EFOX precursors) by accepting a broader variety of omega-6 and omega-3 polyunsaturated fatty acids (PUFAs) ranging from 18 to 22 carbon atoms. In case of linoleic acid LA (18:2, omega-6) and alfa-linolenic acid ALA (18:3, omega-3), COX-2 converts them to monohydroxylated products, i.e., 13-HODE and 13-HOTrE, respectively. Analogous to their corresponding electrophilic oxo-derivatives (13-EFOX-L_2 and 13-EFOX-LN_3), 13-HODE and 13-HOTrE were recently shown to possess anti-inflammatory properties.

1.4 Scientific problem

We sought to define the linkage between immune signaling, eicosanoid production, and the effects of ASA in chronic inflammation and tumorogenesis at the cellular and systemic levels in a simple model organism. The extreme complexity of the mammalian organ systems and the diversity of molecules in the lipidome have thwarted the delineation of clear cause-effect
mechanisms. While the immune system in Drosophila is well-characterized, the presence and function of eicosanoids is virtually unknown. We hypothesized that flies produce eicosanoids and such molecules will be less diverse than their mammalian counterparts. We therefore examined the effects of ASA on phenotypes previously defined to correspond to mammalian inflammatory conditions and then performed targeted lipidomics experiments to identify eicosanoid-like molecules. Our results set the stage of understanding how signaling networks maintain normal homeostasis.

1.5 The Drosophila tumor-inflammation model

During the course of my Ph.D. project, I took advantage of extremely powerful immuno-genetic models based on the organism Drosophila melanogaster that proved to have high molecular and phenotypic correspondence with human conditions involving inflammation and cancer described above. Drosophila lacks adaptive immunity but possesses a powerful innate immune system with cellular (blood cells or hemocytes; macrophage plasmatocytes, crystal cells and lamellocytes) and humoral (peptides secreted in circulation) arms. Humoral reactions arise in the fat body, an organ that is akin to mammalian liver.

In their natural ecosystem, fruit flies are exposed to parasitoid wasps which infect fly larvae by injecting their eggs into the host. After the wasp egg hatches, the parasitic larva starts to eat the host alive and take over the host. Soon after the parasite egg is laid, an arms race between the parasite and the host is initiated. In the host blood cells surround and choke the development of the parasite egg. This encapsulation reaction allows the host to resume its development. Interestingly, humoral immunity in the fat body is also activated in response to infection by parasitic wasps. In the presence of wasp infection, plasmatocytes differentiate into
adhesive lamellocytes to form multilayered capsules around foreign substances\textsuperscript{34}. If the encapsulation is successful the innate immune reactions are resolved within hours. Mutations in various components of the immune Toll-NF-κB (such as, Toll, cactus or dorsal) or JAK-STAT pathways can induce lamellocyte differentiation leading to formation of tumors and death of the host\textsuperscript{36}.

Qiu et al.\textsuperscript{37} showed that cactus mutants display an overabundance of hemocytes, carry melanotic capsules and die before reaching pupal stages. An additional phenotype was the enlargement of the lymph glands and a significantly high mitotic index in the hemolymph compared to wild-type hemolymph.

The mutant Ubc9 has been extensively studied in the Govind lab; Ubc9 encodes the E2 SUMO-conjugating enzyme which negatively regulates Toll-NF-κB signalling-mediated humoral and cellular immunity. Its deficit leads to systemic immune dysfunction\textsuperscript{38-40}. The mutant fat body is damaged, becomes infiltrated with large numbers of blood cells\textsuperscript{38,40}. This loss of homeostasis and infiltration is fuelled in part by massive hematopoietic overgrowth of a small, heterogeneous, transit-amplifying progenitor blood cell population, housed in the lymph gland, a multi-lobed hematopoietic organ\textsuperscript{39}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image.png}
\caption{Wild type third instar larvae (Panel A); Ubc9 third instar larvae displaying melanotic tumors as a consequence of the mutation in the Toll pathway (Panel B)\textsuperscript{40}.}
\end{figure}
Individual blood cells or small aggregates (structures < 10,000 \(\mu m^2\) in projection area), larger structures and even entire overgrown lobes (called microtumors, structures > 10,000 \(\mu m^2\) in projection area and of >50 cells)\(^{39}\) are present in circulation (Figure 2). The largest microtumors are the least abundant and remain smaller than 1 mm\(^3\) and are never vascularized, while the smaller aggregates are more abundant and make up the majority of abnormal structures\(^{38-40}\). Tumor overgrowth and ectopic immune signalling in mutants relies almost completely on functional NF-\(\kappa\)B proteins, Dorsal and Dif\(^{38,40}\).

Remarkably, low dose ASA treatment in Ubc9 larvae resulted in a significant increase of tumor-free animals. Additionally, ASA blocked the expansion of the hemapoietic organ, the lymphgland, suggesting reduced cell overproliferation, ameliorated metabolic inflammation and restored overactive Toll-NF signalling in the fat body as proven by decreased lipid droplet size and genetic expression of the antimicrobial peptide Drosomycin (Paddibhatla and Govind, unpublished results).

While the cellular and molecular mechanisms governing inflammation and immunity are well understood in Drosophila, the pathways for production and the existence of eicosanoids has not been investigated. COX-like enzymes have been reported in Drosophila\(^{41,42}\) but their roles in immunity are not known. We sought to examine if classical or novel eicosanoids are produced in flies and if so, is their level affected by aspirin? Furthermore can immune pathways modulate their levels of such eicosanoids?

1.6 \(hop^{Tum-}\): a practical genetic background for targeted lipidomics

Wasp parasitism is known to induce another important signaling pathway: the JAK-STAT pathway. This in turn leads to massive differentiation of pro-hemocytes into lamellocytes. JAKs
and STATs mediate intracellular signaling in response to cytokines. JAK tyrosine kinases are associated with the intracellular part of transmembrane proteins that form homo or heteromeric receptors (Figure 3). Ligand binding induces a conformational change that triggers pathway activation via trans-phosphorylation of JAK molecules associated with the intracellular part of the receptor. Phosphorylated (activated) JAKs then phosphorylate the receptor, creating docking sites for members of the STAT family of transcription factors, which in turn become phosphorylated. Phosphorylated (activated) STATs homo- or heterodimerize prior to nuclear translocation and transcriptional activation of target genes.

Figure 3 Simplified overview of the JAK/STAT pathway in Drosophila

Like Ubc9 mutants, hopscotch Tumorous-lethal (hopTum-1) mutants show excessive proliferation and differentiation of hemocytes in the lymph gland and in circulation. However, unlike Ubc9, a recessive loss-of-function mutation, hopTum-1 is a dominant, gain-of-function mutation. It is also temperature-sensitive due to a point mutation in the kinase domain. At all
culture temperatures (18-27°C), hop<sup>Tum-I</sup> plasmatocytes can be present in concentrations 5-20 times that of wild-type numbers<sup>47,48</sup>. At non-permissive temperatures (27°C), lamellocytes are over-represented and being adhesive<sup>49</sup> they form small aggregates or large masses, some of which eventually become melanized (Figure 4).

Figure 4 a, Arrows point to GFP-positive hematopoietic tumours in hop<sup>Tum-I</sup> whole larvae (scale bar, 1 mm). b, An aggregate of mutant cells showing GFP-positive lamellocytes and GFP-negative plasmatocytes (white arrows) (scale bar, 50 μm). c, Toll pathway components are transcriptionally active in the hop<sup>Tum-I</sup> background<sup>50</sup>. Significant increase (>1.5-fold) in mRNA levels is observed for SPE, spz, Toll, and Dif.

Additionally, the effects of this mutation result milder compared to what observed for Ubc9 in that the fat body integrity is not as compromised which renders dissection, manipulation and microscopic investigation of the tissue much more practical; also, the larva-to-adult viability is high enough (around 30%) to allow for collection of a statistically significant number of larvae (> 200) that are required for quantitative lipidomic analyses.
2. MATERIALS AND METHODS

2.1 Fly lines

The X-linked msnf9-GAL4\textsuperscript{51} insertion was recombined with the X-linked temperature sensitive hop\textsuperscript{Tum-1} mutation\textsuperscript{52}. An mCD8-GFP reporter (DBSC 5137) was introduced to distinguish plasmatocytes (not GFP-positive) from GFP-positive lamellocytes in the y w hop\textsuperscript{Tum-1} msn-GAL4/FM7; UAS-mcd8-GFP strain. STAT\textsuperscript{RNAi} line 1 is 31318 and STAT\textsuperscript{RNAi} line 2 is 31317.

2.2 ASA administration protocols

A 10 M aspirin solution was prepared in DMSO and diluted several-fold in water so that the desired final ASA concentration in fly food covered a wide range, namely 1 nM, 1 µM or 1 mM. (The final DMSO content in the fly food was negligible being at least four orders of magnitude lower than the reported cytotoxic DMSO concentration in Drosophila larvae (~ 0.3% w/w\textsuperscript{53})). The appropriate volume of the resulting aqueous solution was added directly to the fly food and thoroughly homogenized with an electric mixer for 5 min. Larvae were reared at 27ºC and collected for lipid extraction, viability assays, or dissection at the age of 5 days after egg lay, unless stated otherwise. DMSO instead of water as a vehicle for ASA administration did not impart any change in terms of biological or biochemical effects (data not shown). The highest 1 mM ASA concentration in the fly food resulted in partial lethality at pupal stages. Even higher, 1 M ASA concentration resulted in extremely high toxicity and none of the embryos hatched to larvae.

For the transgenerational experiments (Result section, Figure 9), 24-hour egglays were set up in vials containing fly food with 1 µM ASA as described above. 3-days old larvae were gently removed from the fly food and briefly washed with water. After counting them, they were
introduced into fresh fly food containing 1 μM ASA and raised at 27°C. Adults were counted 3-4 days after eclosion. These ASA-treated adults were then transferred into fresh fly food containing no ASA. A 24-hour egglay and removal of the ASA-treated adults were followed by collection and counting of 3-day old larvae which were transferred into fresh fly food. Upon eclosion the adults (G1) were counted and viability was calculated. The viability of untreated and ASA -treated y w animals resulted to be 98-100% and viability remained at the same level in the second generation (G1) (data not shown)

2.3 Linoleic acid administration protocols

The appropriate volume of pure LA (> 99.9%, Sigma Aldrich, St Louis, MO) was added directly to the fly food for a final concentration of 5 mM. The mixture was thoroughly homogenized with an electric mixer for 5 min. Wild type (y w) and hop<sup>Tum-1</sup> mutant larvae were reared at 27°C and collected for lipid extraction or viability assays at the age of 5 days after egg lay.

2.4 Analysis of aspirin-treated animals

For analysis of larvae, developmentally synchronized third instar heterozygote or mutant larvae were chosen from a timed (6-24 hour) egg lay. Larvae that were too small were presumably because of developmentally delayed and were not selected for analysis. There was no bias in terms of the sex of the selected animals.

2.4.1 Dissection and immuno-staining

Heterozygotes or mutant larvae were dissected for fat bodies, lymph glands, blood cell smears, or tumors as described previously<sup>39,54</sup>. Air-dried blood cells, aggregates, and tumors on
slides were stained following the protocols described in\textsuperscript{40,54}. Lipid droplets in the fat body were stained with Nile Red (N3013, Sigma Aldrich) as described by Greenspan et al.\textsuperscript{55}. Fluorescent dye-labeled secondary antibodies were obtained from Jackson Immunological or Invitrogen Molecular Probes. Samples were counterstained with fluorescent-labeled Phalloidin and nuclear dye Hoechst 33258 (both from Invitrogen Molecular Probes). Images were acquired in a Zeiss laser scanning confocal microscope or a Zeiss Axioscope 2 Plus fluorescence microscope, and formatted in Zeiss LSM5 or AxioVision LE 4.5 software, respectively.

2.4.2 Mitosis

To score mitotic index in smears of circulating blood in $hop^{Tum-1}$ background, the rabbit anti-phospho histone H3 (1:200) from EMD Millipore (Temecula, CA) was used followed by incubation with anti-rabbit alkaline phosphatase (1:5000) from Thermo Scientific (Waltham, MA) and staining with 125 $\mu$g/ml BCIP and 250 $\mu$g/ml NBT from Promega (Madison, WI).

\textbf{c) Synthesis of Rhodamin B-conjugated ASA and staining protocol}

\begin{center}
\textbf{Scheme 1} Synthetic protocol employed for the synthesis of RhASA.
\end{center}
In a 150 ml-two-neck round bottom flask, 2 g (4 mmol) of Rhodamine B were dissolved in 16 ml (10 mmol, 2.5 eq) of POCl₃ and the mixture were vigorously stirred at 110 °C for 2 days. Subsequently, POCl₃ was thoroughly distilled off under vacuum after which 60 ml of molecular sieve-dried acetonitrile were added to the flask along with 2.1 g (11.3 mmol, 2.8 eq) of mono-Boc-protected piperazine and 0.7 ml (5 mmol, 1.2 eq) of triethylamine. The mixture was stirred at room temperature for 24 h and for 12 h at reflux. Once thin layer chromatography (TLC) showed complete conversion of the starting material, triethylamine was distilled off and the crude product was dissolved in 20 ml of trifluoroacetic acid and stirred for 2 hours at RT. After removal of the solvent, the crude product was purified via flash chromatography (initial eluting mixture 5.5 CHCl₃ : 1 MeOH changed gradually to 2:1 ratio). After collecting the correct fractions the solvent was removed by rotavap and the final product precipitated by dissolving it in a 2 ml of methanol and adding it to 50 ml of diethyl ether dropwise.

After drying it under vacuum this intermediate (150 mg, 1 eq) was dissolved in 10 ml of dimethyl sulfoxide (DMSO) to which 54 mg of ASA (0.3 mmol, 1 eq), 153 µl of triethylamine and 410 mg of HBTU (1 mmol, 3.3 eq) were added in this order. The mixture was stirred for 24h at RT under nitrogen. At the end of this period 150 ml of 0.5 M NaHCO₃ were added followed by 100 ml of ethyl acetate. The biphasic mixture was transferred into a separatory funnel. Upon vigorous stirring the organic phase was recovered, whereas the aqueous phase was extracted 3 times with ethyl acetate. The combined organic fractions were dried over Na₂SO₄. After filtration on a cotton plug the solvent was removed by rotavap almost to dryness. The crude product was dissolved in 2 ml of ethyl acetate which were added to 150 ml of diethyl ether dropwise at RT. After keeping the solution at 4 °C for 1 h, the product was recovered via filtration and dried in vacuum to yield 160 mg of Rhodamine B-conjugated ASA (RhASA, 80 % yield over two steps).
All spectroscopic and MS characterization analyses confirmed the nature of the compound (data not shown).

2.4.3 Staining protocol

An 800 μM RhASA working solution in PBS was applied to dissected larval tissues. Incubation time was 30 min. Negative control used was 800 μM Rhodamine B + 800 μM ASA solution in PBS.

2.4.4 Preparation of dissected fat body for lipid droplet quantification

Fat body was dissected in PBS in 9-well glass plates and kept in PBS at all times. Before mounting, the microscope slides were modified as follows in order to avoid crushing of the tissue: a) Scotch tape was placed on the slide making sure to leave at least half a inch uncovered on both short sides; b) a thin and even layer of Vaseline was applied onto the tape with a brush; c) by using the corner of a razor blade, a half inch-wide rectangle was cut into the tape and peeled off by lifting one of the corners with the razor blade and using tweezers to remove it completely. At this point the exposed rectangular area (the well) should be clean and the surrounding tape covered with Vaseline; d) one small drop of Vectashield was placed in the middle of the exposed area and spread it on the glass evenly; e) clean tweezers were employed to take a piece of fat body from PBS extremely gently and transfer it onto the slide; f) 3-4 pieces of fat bodies were similarly placed onto each slide without crowding it; g) a glass cover slip was placed very slowly on the tissues; h) once the cover slip is in place, it was gently and uniformly pressed along the Vaseline-coated border. This step is needed for the coverslip to lie flat, the tissues to adhere to the coverslip and the Vectashield to fill as much space as possible inside the well; i) clear nail polish was then gently applied all around the edges to seal the inner well and
prevent the cover slip from sliding during handling; j) prior to imaging (which should ideally be performed as soon as possible after preparation of the slides) the slides were stored at 4°C.

Lipid droplets were manually quantified by categorizing them according to the diameter ranges we defined (small (⌀ <6.2 μm); medium (6.2< ⌀<10 μm); large (⌀>10 μm)). At least 80 cells were counted for a total of at least 500 lipid droplets per sample.

2.5 Materials for lipidomics analysis

Solid-phase extraction cartridges STRATA SPE cartridges C18-E (500 mg, 6 ml; Cat. No. 8B-S001-HCH); non-chiral RP-HPLC column: Phenomenex Luna 3 μm C18(2) 100A 150x2.0 mm; Chiral HPLC column: Chiracel OD-RH 5 μm 150x2.1 mm; MS system: 4000 QTRAP (Applied Biosystems, Foster City, CA), HPLC system: Shimadzu Prominence HPLC (Shimadzu USA, Canby, OR); NMR instrument: Varian Mercury-300.

Aspirin (>99%) and linoleic acid (≥ 99%) were purchased from Sigma Aldrich (St Louis, MO). Aspirin coated tablets (Bayer, 325 mg of active principle) were purchased from the local CVS pharmacy. MS-grade water, acetonitrile and formic acid were purchased from WorldWide Life Sciences (Bristol, PA).

The following analytical-grade standards were purchased from Cayman Chemical Co. (Ann Arbor, MI): 13(S)-HODE (>98%), 13-EFOX-L2 (>98%), 13(S)-HOTrE (>98%), 13(S)-HODE-d4 (>99% deuterated), 13-EFOX-L2-d3 (>99% deuterated).

2.6 Extraction and LC/MS analysis of oxidized lipids

To search for oxidized lipids produced in Drosophila third instar larvae we used a screening method based on SPE extraction under conditions previously reported in the literature for oxidized fatty acids56. At least three biological replicates were used per sample. No less than
200 animals at the same developmental stage (third instar) from a controlled (24 hr, 25°C) egg-lay were carefully removed from the fly food with a spatula and transferred onto a fine mesh sieve. The larvae were thoroughly washed with water, 70% ethanol briefly, and then with water again. They were gently transferred onto a Kimwipe to completely remove excess water, weighed accurately, and stored at -80°C until further use. The amounts of solvents and internal standards mentioned in the remaining section of the protocol before SPE extraction were used for 200 mg of larvae. In case larval weight exceeded 200 mg, all amounts were adjusted accordingly. For lipid extraction, larvae were transferred into a 1 ml pre-chilled glass Dounce grinder and homogenized thoroughly at 0°C with a glass pestle along with 500 μl of cold methanol. The pestle was rinsed twice with 100 μl of cold methanol each. 4 ml of ice-cold water were added to obtain a 15% methanol solution. Subsequently, 50 ng of deuterated internal standards were added to the homogenate which was left on ice in the dark. After 15 min the homogenate was centrifuged at 4°C and 4,000 rpm for 10 min. The supernatant was recovered and kept on ice. The supernatant pH was then adjusted to 3 by adding 10-20 drops of 0.1 M HCl and exactly 2 ml of the acidic extract were immediately loaded onto the SPE column which was previously conditioned with 20 ml of methanol and 20 ml of water. The cartridge was washed with 20 ml of ice-cold 15% methanol in water, 20 ml of water and 10 ml of hexane. The lipids were recovered with 12 ml of cold methyl formate which was evaporated on ice and in the dark under a gentle stream of nitrogen. The purified lipids were dissolved in 1 ml of cold, degassed ethanol, and stored at -80 °C until the LC-MS analysis.

The lipid derivatives were analyzed by reverse phase-high pressure liquid chromatography coupled to a Q-TRAP operated in triple quad mode (RP-HPLC MS/MS). The non-chiral LC-MS runs were performed after diluting the extract ten-fold in ethanol. All samples
were analyzed with a gradient solvent system consisting of A (water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid). The flow rate was 250 μl/min and the gradient used was the following: hold at 35% B for 3 min, then 35-90% B in 23 min, then 90-100% B in 0.1 min, hold for 5.9 min and 100-35% B in 0.1 min, and finally hold for 7.9 min. The oven temperature was 40°C. Chiral analyses were performed in isocratic elution with 35% A and 65% B for 25 min. The flow rate was 250 μl/min and the oven temperature was 40°C.

All the analyses were performed in Multiple Reaction Monitoring (MRM) mode whose optimal parameters and most abundant fragments were obtained experimentally for 13-HOTrE, 13-HODE, and 13-EFOX-L2 by utilizing commercially available synthetic standards. The following settings were used:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Q1 Mass (Da)</th>
<th>Q2 Mass (Da)</th>
<th>DP (Volts)</th>
<th>CE (Volts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13-HODE</td>
<td>295.170</td>
<td>280.400</td>
<td>-90</td>
<td>-26</td>
</tr>
<tr>
<td></td>
<td>295.170</td>
<td>195.200</td>
<td>-90</td>
<td>-26</td>
</tr>
<tr>
<td></td>
<td>295.170</td>
<td>180.300</td>
<td>-90</td>
<td>-28</td>
</tr>
<tr>
<td>13-EFOX-L2</td>
<td>293.130</td>
<td>249.200</td>
<td>-90</td>
<td>-26</td>
</tr>
<tr>
<td></td>
<td>293.130</td>
<td>113.100</td>
<td>-100</td>
<td>-28</td>
</tr>
<tr>
<td>13-HODE-d4</td>
<td>299.170</td>
<td>281.200</td>
<td>-95</td>
<td>-26</td>
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</tr>
<tr>
<td></td>
<td>299.170</td>
<td>198.100</td>
<td>-95</td>
<td>-26</td>
</tr>
<tr>
<td>13-EFOX-L2-d3</td>
<td>296.230</td>
<td>252.200</td>
<td>-90</td>
<td>-26</td>
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<tr>
<td></td>
<td>296.230</td>
<td>114.100</td>
<td>-95</td>
<td>-29</td>
</tr>
</tbody>
</table>

Q1: Primary mass; Q2: Target mass; DP: Difference of potential; CE: collision energy
For quantification, we made internal standard curves using 13-HODE, and 13-EFOX-L₂ as analytes and 13-HODE-d₄ and 13-EFOX-L₂-d₃ as internal standard. Quantification was carried out by employing analyte/internal standard peak area ratios. The comparison of retention times and the use of at least two MS transitions per compound rendered the detection reliable and sensitive (Figure 8).

2.7 Statistical analysis

For experiments where quantification was performed (Figure 6, Figure 8, and Figure 9), at least five biological repeats were performed unless stated otherwise. The number of samples examined is indicated for each experiment in the text, Figure legends, or Methods. For statistical analysis, either one way ANOVA with pair-wise comparison or the student \( t \)-test was applied. Data were graphed using Microsoft Excel. \( p < 0.05 \) was considered to be significant.

3. RESULTS

3.1 Anti-mitotic effects

In the Introduction we stressed ASA’s anti-proliferative properties that are most likely associated to its anti-cancer effects observed in humans. The first correspondance with human systems in the \( hop^{Tum-l} \) background was observed upon administration of 1 \( \mu \)M ASA. This resulted in considerably reduced mitosis in circulating hemocytes (Figure 5).

![Figure 5](image-url)
3.2 Metainflammation

ASA displayed surprising restorative effects on metabolically active lipid droplets (LDs) in the larval fat body (Figure 6 a). These lipid monolayer membrane-enclosed organelles produce and store triacylglycerides that serve as precursors of bioactive lipids. Aberrations in lipid droplet size, shape, or abundance are linked to metabolic diseases\textsuperscript{57}. Unlike the fat body cells of control y w larvae where small LDs predominate, the mutant hop\textsuperscript{Tum-1} fat body cells display considerable reduction in the abundance of small LDs and a significant increase in larger LDs.

![Figure 6 a and c) Representative images of Nile Red-stained larval fat body from untreated y w control, untreated and 1 μM ASA-treated hop\textsuperscript{Tum-1} animals and control hop\textsuperscript{Tum-1} and hop\textsuperscript{Tum-1} STAT RNAi (scale bar, 30 μm); b and d) Classification and quantification of lipid droplets At least 100 fat body cells were scored for each genetic background (mean ± s.d.; n > 100 cells from 6 animals; **p < 0.001, Student t-test). Arbitrary classification of lipid droplets: small (⌀ <6.2 μm); medium (6.2< ⌀<10 μm); large (⌀>10 μm).]
However, administration of 1 μM ASA reversed this anomalous phenotype, restoring the higher proportion of the small LD population (Figure 6). Similar effects of ASA were also observed on anomalous lipid droplets in Ubc9 fat body cells (not shown) and in hopTum-l larvae upon RNAi-mediated STAT knockdown in the overgrowing inflammatory blood cell population only (Figure 6 c and d). ASA’s ability to modulate lipid homeostasis in flies encouraged us to search for anti-inflammatory lipids in wild type and hopTum-l larvae.

3.3 **HODE-13 and EFOX levels**

*D. melanogaster* appears to lack fatty acids longer than C18, such as eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA)\(^{58}\), whose acetylated COX-2-mediated products are anti-inflammatory in mammals\(^6\). Nevertheless, flies clearly possess enzymes with COX-like function\(^{41,59}\) and we therefore hypothesized that ASA-treated flies might produce high levels of anti-inflammatory lipid mediators that, in mammals, derive from shorter and less unsaturated fatty acids.

LC-MS/MS-based targeted lipidomics of C18-fatty acid derivatives did not detect 13-HOTrE in any sample. Instead we found that untreated wild-type and hopTum-l third instar larvae produce 13-HODE (Figure 7 b and Figure 8 a) at levels comparable to mammalian cells (40 nM\(^60\)) and this basal level increases 3-fold upon 1 μM and 1 mM ASA treatment in both genetic backgrounds (Figure 8 a). At 1 nM however, a comparable 3-fold increase is observed in mutant samples but not in the wild type samples (Figure 8 a). To the best of our knowledge, this is the first *in vivo* demonstration of ASA-triggered 13-HODE increase. Chiral LC-MS/MS analysis resolved the R and S enantiomers and we discovered that 13(S)-HODE was twice as abundant as 13(R)-HODE (data not shown). This enantiomeric imbalance may indicate that at least some of
the oxidation product is generated by enzymatic catalysis. Interestingly, unlike the R enantiomer, 13(S)-HODE possesses anti-inflammatory properties in mammalian models.

![Diagram showing the conversion of Linoleic acid (LA) and α-linolenic acid (ALA) into 13-HODE and 13-HOTrE, respectively.]

**Figure 7 a,** In mammalian cells LA and ALA are first converted into 13-HODE and 13-HOTrE by the action of COX-2 and then further oxidized to 13-EFOX-L$_2$ and 13-EFOX-LN$_3$, respectively, via dehydrogenase enzymes. b, MRM scans monitoring for the m/z transitions 295.17/195.20 (MW of 13-HODE/loss of 99.97 m/z, see Extended Data Fig. 4) for standard 13-HODE (top panel) and a representative lipid sample (bottom panel). c, MRM scans monitoring for the m/z transitions 293.13/113.10 (MW of 13-EFOX-L$_2$/loss of 180.03 m/z) for standard 13-EFOX-L$_2$ (top panel) and a representative lipid sample (bottom panel). All signals were at least 3 times the calculated LOD.

The hydroxyl group in 13-HODE, responsible for its chiral nature, can be further oxidized by dehydrogenase enzymes to generate the electrophilic derivative 13-EFOX-L$_2$ (Figure 7 a). LC-MS/MS allowed the detection and quantification of 13-EFOX-L$_2$ in *hop*$_{Tum-1}$ larvae; the concentration of 13-EFOX-L$_2$ in these animals was 6.0 nM (Figure 8 b) which is consistent with
in vivo levels measured in mammalian cells\textsuperscript{28}. Significantly, EFOX was detected only in mutants following ASA administration at high (1 μM or 1 mM, but not at 1 nM) concentrations. 13-EFOX-L\textsubscript{2} was not detected in control y w larvae regardless of ASA-treatment (Figure 8 b). Thus, as in mammalian cells, EFOX production is triggered by ASA only in mutants, which suggests that mutants may possess higher dehydrogenase levels or activity to support this increase\textsuperscript{62}.

Figure 8 Quantification of 13-HODE (d) and 13-EFOX-L\textsubscript{2} (e) levels in y w control and hop\textsuperscript{Tum-l} animals systemically treated with 1 nM, 1 μM and 1 mM ASA, and 5 mM LA (n > 200 larvae and 3-5 biological replicates per treatment).
3.4 Effects of linoleic acid on viability and lipids

The discovery of the two LA-derived oxidized lipids in larval fly extracts prompted us to examine whether hop\textsuperscript{Tum-1} larvae would benefit from dietary supplement of the omega-6 LA precursor (13-HODE and 13-EFOX-L\textsubscript{2} are likely to be chemically unstable to be tested in similar assays). Remarkably, LA treatment increased hop\textsuperscript{Tum-1}'s larval-to-adult viability from 35\% to up to 90\% (Figure 9). However, despite a higher viability compared to ASA, LA’s beneficial effects were limited to the LA-treated parents, unlike ASA, whose effects on viability transferred to untreated progeny (Figure 9). This is the first observation of what appears to be a transgenerational effect induced by ASA treatment.

Lipidomics analysis showed that while the 13-HODE levels in \textit{y w} and hop\textsuperscript{Tum-1} larvae raised on 5 mM LA remained unchanged and comparable to those in untreated animals (Fig. 3d), the 13-EFOX-L\textsubscript{2} levels were 3- (\textit{y w}) and 7- (hop\textsuperscript{Tum-1}) fold higher than their ASA-treated counterparts (Figure 8). Thus, 13-EFOX-L\textsubscript{2} levels can be modulated \textit{in vivo} either by ASA administration or by supplementing the diet with LA. In either case, 13-EFOX-L\textsubscript{2} appears to resolve inflammation and improve the survival of tumor-ridden animals.
3.5 Identification of ASA targets

An ambitious goal that we set towards the beginning of this Ph.D. project was the identification of ASA target proteins in *Drosophila*. Unfortunately, it turned out to be more challenging and time-consuming than expected. Nevertheless, we managed to gather preliminary results showing that *Drosophila* is highly likely to possess targets that are specific to ASA. This was accomplished by synthesizing a fluorescent ASA derivative. Structurally-related fluorescent compounds were proven to retain specificity upon conjugation with Rhodamine-based fluorophores \(^6\) (see Materials and methods section). We tested this fluorescently-labeled ASA (RhASA) in four different larval organs (lymph glands, fat body, haemocytes and gut) (Figure 10). Interestingly, the negative control consisting of Rhodamine B + pristine ASA was not significantly retained by any tissue, whereas RhASA showed localization in specific subcellular regions in every single tissue which strongly suggests the presence of ASA-specific targets.
Further investigations will be carried out to identify them and determine their biochemical role in relationship to ASA.

4. DISCUSSION

4.1 Existence of HODE

ASA is commonly used for blocking inflammation and improving cardiovascular health. The widely accepted ASA mechanism of action consists of the inhibition of COX-derived pro-inflammatory prostaglandin production from the C20 fatty acid arachidonic acid\textsuperscript{64}. While such biolipids are arguably absent in flies\textsuperscript{58}, their administration rescues the loss of peroxinectin function, a COX-1-like fly gene\textsuperscript{41}. In addition, ASA has recently been shown to exert part of its anti-inflammatory activity by allowing the production of eicosanoid-like anti-inflammatory and
pro-resolution hydroxy lipids from C18-C22 fatty acids, such as i.e., 13-hydroxyicosatetraenoic acid (13-HETE)\textsuperscript{28,31}, the DHA-derived Protectin D1\textsuperscript{6}, etc. Noticeably, C20 PUFA-fed flies are able to generate the analogous 13-HETE\textsuperscript{59}. In our fly model of inflammatory tumors we found that ASA triggers the production of structurally-related and endogenous oxidized lipids (i.e., 13-HODE and 13-EFOX-L\textsubscript{2}); furthermore, their increased levels correlated with the restoration of LD morphology, among other important physiological effects such as higher viability, reduced mitosis and decreased infiltration of lamellocytes in the fat body. Interestingly, Bozza et al.\textsuperscript{65} reported on the ability of ASA to inhibit arachidonic acid-induced lipid droplet formation in mouse peritoneal macrophages and observed that the production of pro-inflammatory COX- and lipoxygenase(LOX)-pathway derived eicosanoids correlated with increased lipid droplet formation (that is, enlargement thereof). Unlike these arachidonic acid-derived lipids\textsuperscript{11}, 13-HODE and 13-EFOX-L\textsubscript{2} possess anti-inflammatory and pro-resolution activity.

\textbf{4.2 Lipid droplets}

Their increased levels correlate with the rescue of LD morphology in response to either ASA treatment or knockdown of STAT. In this respect, it is noteworthy that mammalian 5-LOX, 15-LOX and COX enzymes, localize at the interface between LDs and the cytoplasm\textsuperscript{66}. This observation supports the idea that LDs have a crucial role in preserving metabolic and immune homeostasis and they actively participate in the inflammatory response. Interestingly enough, abnormal lipid metabolism in cancer cells results in modulation of several lipogenic enzymes and accumulation of newly-formed lipids constituting LDs\textsuperscript{67} which suggests the presence of compromised cellular metabolism as a consequence of chronic and uncontrolled inflammation. We argue that enlarged LDs may be less efficient at generating and mobilizing lipid derivatives.
also in virtue of a lower overall surface area per fat body cell that limits exposure of processing enzymes and transporters towards the cytoplasmic milieu. Related to this, the endoplasmic reticulum protein Seipin plays an important role in LD morphology\textsuperscript{57} as shown, for instance, in Seipin-deficient yeast cells that displayed immature and fused LDs\textsuperscript{67}. Qiu et al.\textsuperscript{68} showed that in lipodystrophic 3T3-L1 murine adipocytes, pathogenic Seipin-A212P inhibits adipogenesis and triggers inflammatory pathways. Also, significant disruption of the delicate lipogenesis/lipolysis balance that determines, \textit{inter alia}, the size of LDs, can certainly have deleterious physiological effects. These include reduced viability that we clearly observed in tumor-ridden larvae as a consequence of a compromised metabolic state (such as, unbalanced lipid metabolism and defects in energy production) as also highlighted by Musselman et al.\textsuperscript{69}. The 3-fold increase in viability of \textit{hop}\textsuperscript{Tum-1} animals upon administration of dietary LA could, in fact, be partially explained by viewing it as the effect of a significant increase in calorie intake. Similarly, the totally saturated C18 fatty acid myristic acid only slightly increased viability (data not shown) which, in any case, suggests that the double unsaturations in LA are essential for proper biochemical transformations and the other observed beneficial effects.

4.3 \textit{Electrophilic-oxo derivatives (EFOX)}

Groeger et al.\textsuperscript{28} shed light on the potent anti-inflammatory effects of the PUFA-derived electrophilic lipid derivatives EFOX. We showed that the production of LA-derived 13-EFOX-L\textsubscript{2} is upregulated \textit{in vivo} upon administration of medium and high dose ASA (1 μM and 1mM). According to Figure 7 a, 13-EFOX-L\textsubscript{2}’s immediate precursor is 13-HODE. From a mechanistic standpoint, this oxidation reaction can take place either enzymatically (enantiospecific conversion) or via radical intervention (racemic conversion). Of relevance to the latter case,
*hop*<sup>Tum-1</sup> mutants are characterized by high levels of radical oxygen species (ROS) and through a mechanism of peroxyl radical transfer can lead to the complete oxidation of the sensitive allyl alcohol group in 13-HODE to an α,β-unsaturated ketone.

Regarding the enzymatic HODE/EFOX conversion, Groegor et al. have shown that hydroxylated PUFAs can undergo dehydrogenase-mediated oxidation. Among the potential candidates performing this step, 15-hydroxyprostaglandin dehydrogenase (15-HPGD) has been proposed. Interestingly, in our study 13-EFOX-L<sub>2</sub> production appears to be ASA-dose dependent as EFOX production occurs only at 1 μM and above. To this regard, a recent report by Fink et al. showed that while 15-HPGD is down-regulated in colorectal cancer cells, prolonged ASA treatment resulted in significantly higher 15-HPGD expression in cancerous cells only. This also supports our finding whereby ASA treatment leads to high levels of the precursor 13-HODE but no EFOX production in *yw* animals that arguably experience modest dehydrogenase levels as well as low oxidative stress.

### 4.4 Genetics helps delineate cause-and-effect

Genetic intervention through the haemocyte-specific knockdown of STAT in *hop*<sup>Tum-1</sup> animals provided remarkable information regarding the complex and still poorly understood cross-talk seeming to be present between lipid metabolism and immune pathways. Among the effects observed is the increased levels of 13-HODE and 13-EFOX-L<sub>2</sub>. According to the model developed by Groeger et al., a putative fly COX-ortholog would be unable to generate linear hydroxylated fatty acids in absence of ASA-mediated acetylation of the active site.
On the other hand, the oxidizing enzyme 15-LOX is known to hydroxylate a large variety of PUFAs\(^6\) in mammals. Interestingly, it was shown that 15-LOX production is completely lost in pancreatic cancer cells\(^71\); also, its overexpression in colorectal\(^72\) and pancreatic cancer cells as well as administration of its arachidonic acid-derived metabolites\(^71\) was shown to greatly suppress tumor growth. Even though a 15-LOX ortholog has not yet been identified in flies, the presence of high levels of 13-HODE in ASA-untreated STAT knockdown \(hop^{Tum-1}\) animals suggests that 15-LOX-like enzymes might be responsible for its significant increase. This finding can be interpreted in virtue of a putative upregulation of 15-LOX-like enzymes in the tumor-forming hemocytes (where the knockdown occurs). In addition to 13-HODE increase, this cell population-specific STAT knockdown was also associated to the rescue of the LD phenotype in the fat body. This phenomenon seems likely to result from a tissue-nonautonomous effect of the mutation (Figure 11). In this regard, although further investigations are still on-going, there is data pointing at the role of 13-HODE in regulating cell adhesion and tumor cell growth\(^73\). Buchanan et al.\(^73\) have shown in human endothelial cells that 13-HODE localization within cellular “vesicles” (most likely, lipid droplets) and its release into the extracellular matrix vary

![Figure 11](#) Schematics summarizing the major findings of this research consisting of role of linoleic acid-derivatives in rescuing the tumor and the LD phenotype as well as the nonautonomous effect observed upon STAT knockdown in \(hop^{Tum-1}\) larvae.
depending on the cellular state (either unchallenged or challenged by cytokines) which, in turn, affects cell adhesivity and spreading. Interestingly, one of the most striking phenotypes associated to the hop<sup>Tum-l</sup> mutation is the loss of integrity of the fat body tissue and compromised integrity of fat body cells among each other. In support of the role of the two LA-metabolites proposed earlier, this phenotype is largely reversed upon ASA treatment or STAT (Figure 11) which both increase their levels. In the latter case, we hypothesize that 13-HODE (and most likely, 13-EFOX-L<sub>2</sub> as well) acts a secondary messenger released by the overproliferating hemocytes to the fat body. Here, a feedback mechanism, yet to be elucidated, causes the fat body cells to readjust their metabolic state and restore lipid homeostasis (rescue of the LD morphology) (Figure 11).

### 4.5 Epigenetic effects

A particularly striking discovery was the transgenerational rescue of viability in fly mutants that had never been reported before. Even though at the moment we do not have any biological explanation for this phenomenon, this finding suggests that in addition to genetic effects, ASA may also act via epigenetic mechanisms. In support of this hypothesis, ASA is known to possess several protein targets, other than COXs, that can undergo acetylation and the experiment with the fluorescently-labeled ASA (Figure 10) clearly shows the presence of tissue regions that are subject to ASA uptake, most likely in virtue of the expression of putative ASA targets. Furthermore, radio-labelling experiments with <sup>3</sup>H or <sup>14</sup>C demonstrated that ASA acetylates numerous proteins both <em>in vitro</em> and <em>in vivo</em>, such as albumin, fibrinogen as well as haemoglobin, DNA, RNA, histones, transglutaminase and other plasma constituents including hormones and enzymes. Bateman et al. reported the ability of aspirin to acetylate
120 proteins in human adenocarcinoma cells using an alkyne–aspirin chemical reporter and leveraging the alkyne functional group to bind fluorophores via click chemistry. The identified enzymes included several metabolic pathway enzymes, structural proteins, proteins involved in translation, proteasomal subunits, mitochondrial proteins and histones. Among these, histones appear to be most intriguing owing to their prominent role in transcriptional regulation. Histones are highly alkaline proteins found in eukaryotic cell nuclei that package and order the DNA into structural units called nucleosomes \(^{84}\). Histones undergo acetylation mediated by the enzymes Histone acetyl transferases (HAT). The acetylation of histones by a number of HATs is known to play an important role in coordinating gene expression, cell-cycle progression as well as cancer development\(^{85}\). Histone acetylation can cause charge neutralization of basic lysine residues leading to changes in the chromatin structure and transcription of genes. It is possible that chemical acetylation by aspirin may have a role in shifting the equilibrium of the enzymatic acetylation/deacetylation process that occurs naturally in cells bringing about major changes in gene expression.

In sum, by integrating genetics with chemistry we discovered a surprisingly diverse spectrum of beneficial effects of ASA in a single and powerful model system. ASA restores 1) homeostasis in NF-κB signalling, 2) reduces mitosis, 3) attenuates lipid droplet morphology, and 4) improves overall viability. Our results suggest the existence of multiple conserved *bona fide* targets of ASA in *Drosophila*, some of which must link cell and tissue signalling with cell cycle control and inflammation, while others may regulate lipid metabolism and energy production, and influence gene expression via genetic modifications.
Our system presents an unprecedented opportunity to identify novel ASA targets, examine mechanisms of tumor growth in their earliest stages, and work out the complex and interlinked genetics of immune signalling and bioactive lipid action.

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The manuscript associated to this work is under preparation.
6. REFERENCES


CHAPTER 2: Direct probing of molecular self-assembly with multifunctional nano-viscosity sensors

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1. INTRODUCTION

Viscosity is one of the most relevant environmental properties that affect cell function. Viscosity has a significant role in determining the diffusion rate of species in liquid media. In biosystems, changes in viscosity have been connected to diseases and malfunction at the cellular level\textsuperscript{1-3}. These perturbations are brought about by changes in mobility of chemicals within and among cells, influencing fundamental processes such as signaling and transport and the efficiency of bimolecular processes governed by diffusion of short-lived intermediates, such as the diffusion of reactive oxygen species during an oxidative stress attack.

\textbf{Figure 1} Microscopic dynamic model of a biological membrane (figure from Luby-Phelps, 2000\textsuperscript{1})
Though cytoplasm is mainly composed of water, fluid-phase cytoplasm viscosity is on average about 28% higher than water, possibly on account of the high concentration of colloids such as proteins\textsuperscript{1,4}. Some estimates show that translational diffusion of both small and large biomolecules can be 5-50 times lower than in pure water, although macromolecular crowding also contributes to this effect. Other cell compartments show an even more marked high viscosity value: measurements in cell vesicles demonstrated that the local microviscosity can be as high as 140 cP\textsuperscript{1} (cP = centipoises, water viscosity is about 1 cP). Because of its large-scale molecular order, cell plasma membrane has intrinsically high viscosity. Yet, cell membrane is by no means a homogeneous environment since it comprises phospholipids with different hydrophobic chains and hydrophilic heads (polar portion linked to choline, galactose, serine, etc…) as well as transmembrane and membrane proteins which deeply affect the local physicochemical properties (Figure 1). Alterations in cell membrane microviscosity have been linked to atherosclerosis, cell malignancy, hypercholesterolemia and diabetes\textsuperscript{2,3,5-7}. For instance, colorectal cancer was shown to lead to alteration in membrane fluidity\textsuperscript{8}. Several authors related the adaptation and resistance of microorganisms to environmental stress to relevant changes in the fluidity properties of phospholipid and biological membranes that are locally produced by the same external agents. These changes in membrane fluidity can be viewed as the “input” signal that initiates the regulation, activating effectors that in turn influence the chemical composition of the membrane in a feedback mechanism. This adaptation strategy allows for the maintenance of the physical characteristic of membranes and, thereby, of their functionality.

Despite the remarkable role played local viscosity has on cell functions, the need of imaging such parameter at high spatial resolution non-destructively in living specimens greatly restrict the number of available bioanalytical viscosity indicators. In recent years, much attention has been
devoted to the development of fluorescent molecules whose emission properties in the visible and near infrared range are strongly modulated by viscosity\textsuperscript{5,9-11}. Such probes are meant to be applied in cell microscopy fluorescence imaging by confocal microscopy, a technique that revolutionized the study of cell biology through the direct real-time visualization of molecular processes at sub-micrometer scale\textsuperscript{12}. Ideally, one of these probes should report on the viscosity of its nanoenvironment in real-time, affording a “viscosity map” of the cell in physiological as well as non-physiological conditions. Some probes of this kind have been already reported in the literature\textsuperscript{8,10}, but there is still much room for improving their properties, especially their brightness and sensing selectivity.

From the previous paragraphs it was clear that the cellular environment is extremely heterogeneous. This signifies that unless we take a step back and deconstruct the system in much simpler terms, the application of these viscosity probes within the cell would result in a superficial understanding of the cellular architecture itself as well as its photophysical behavior. With the goal in mind of eventually testing such probes \textit{in vitro}, we decided to first apply these novel fluorophores in systems that elegantly mimic the heterogeneity of cells, namely molecular gels. Since these systems offer spatially distinct, reproducible and molecularly dynamic solid-like and liquid-like regions, mimicking cells where numerous organelles are inserted within a liquid-like medium (the cytoplasm) that also has its own microscopic fibrillar backbone.

With the support of powerful synthetic, microscopic, spectroscopic and computational tools, it will be shown how it was possible to gain remarkable insights into both the architectural composition of the gel as well as the photophysical features of these novel viscosity sensors. In
the near future more investigations will be set forth to test the probes presented in this thesis, as well as analogs, in biological systems.

1.1 The molecular rotors

Excitation of a fluorophore induces the motion of an electron from one orbital to another. If the initial and final orbitals are separated in space, the electronic transition is accompanied by an almost instantaneous change in the dipole moment of the fluorophore. When the fluorophore structure is characterized by an electron-donating group (e.g. -NH$_2$, -OCH$_3$) conjugated to an electron-withdrawing group (e.g. -C=O, -CN), the increase in dipole moment can be quite large$^{13}$. Consequently, the excited state reached upon excitation (usually referred to as the locally excited state, LE, Figure 2) is not in equilibrium with the surrounding solvent molecules when the latter are polar. In a sufficiently fluid medium, the solvent molecules rotate during the lifetime of the excited state until the solvation shell is again in thermodynamic equilibrium with the fluorophore. The fluorophore eventually reaches a relaxed excited state whose energy is lower than that of the LE state; the relaxed state, characterized by maximum charge separation, is usually referred to as intramolecular charge transfer (ICT) state$^{14}$. Note that in poorly viscous solvents the solvent rearrangement takes place in a few picoseconds, being therefore almost instantaneous with respect to excited state lifetime.

As anticipated, the presence of electron donating and electron withdrawing groups in conjugated structures strongly favors the formation of ICT. In addition to the $\sigma$ and $\pi$ orbitals (a typical transition usually occurs between $\pi$ and $\pi^*$), electrons can also be photoexcited from nonbonding orbitals $n$ such as those belonging to oxygen or nitrogen contained in the donor group. The $\pi$-conjugation allows interaction between the donor and acceptor groups so that the
nonbonding electrons of the donor can be delocalized upon photoexcitation to the unoccupied orbitals of the acceptor, usually $\sigma^*$ or $\pi^*$\textsuperscript{13}.

Relaxation towards an ICT state may be accompanied by internal rotation within the fluorophore. The formation of a twisted state TICT can be interpreted as a dominant vibrational state that leads to major energy loss\textsuperscript{13,15}. The most studied fluorophore displaying this phenomenology is 4-N,N-dimethylaminobenzonitrile (DMABN, Figure 2)\textsuperscript{12,16,17}. In spite of its structural simplicity, DMABN exhibits a complex photophysics\textsuperscript{9}.

![Figure 2](image)

**Figure 2** a) Intramolecular twisting in DMABN; b) ground-state ($S_0$) and excited-state ($S_1$) energies in the planar (LE) and twisted configurations (TICT) of a molecular rotor as a function of the intramolecular rotation angle ($\phi$).

In the ground state, the molecule is almost planar, allowing the maximum conjugation between the dimethylamino group and the phenyl ring. According to the Franck–Condon principle, the LE state is still planar, but solvent relaxation induces a concomitant rotation of the dimethylamino group until it is twisted at right angles and the conjugation is lost (Figure 2)\textsuperscript{13}. Such twisted state maintains large charge separation and is therefore named twisted intermolecular charge transfer (TICT) state. The LE $\rightarrow$ TICT conversion competes with
vibrational relaxation in LE planar configuration\textsuperscript{18}. Fluorophores displaying LE\textarrow{TICT} conversion are generally referred to as molecular rotors (Figure 2).

A qualitative sketch of the energy levels of a molecular rotor can be seen in Figure 2. The ground-state energy level is higher in the twisted configuration, and when electrons are not excited the molecule is mostly in planar state. The excited-level TICT state has a lower energy than the LE state, but the states are separated by a transition energy that needs to be overcome when the molecule enters the TICT state from the LE state. The energy barrier between the LE and TICT states is solvent-dependent. For DMABN, a polar solvent lowers the barrier, and viscous solvents generally elevate the barrier, thereby yielding solvent-dependent conversion rates. The fluorescent lifetimes $\tau_f$ and $\tau'_f$ have been determined to be near 2.5–3 ns\textsuperscript{9}. The TICT formation rate constant $k_a$ is in the order of $10^{10}$ s\textsuperscript{-1}, and the return rate $k_d$ is strongly solvent-dependent and has been found to vary in magnitude from $10^8$ to $10^{10}$ s\textsuperscript{-1}. The actual rotational motion takes place in less than 20 ps.

A significant number of fluorophores can be classified as molecular rotors. In DMABN the energy gap between the TICT and ground state is about 30\% smaller than the gap between LE and ground state. When the energy gap between TICT and $S_0$ becomes much smaller, non-radiative internal conversion pathways increase their efficiency and TICT becomes non-emissive. Molecular rotors whose TICT state is non-emissive exhibit only a single emission band but their quantum yield and lifetime are dependent on the viscosity of the environment. Figure 3 depicts the Jablonski diagram for molecular rotors.
1.2 Viscosity-dependent behavior of molecular rotors

In viscosity-sensing applications, molecular rotors with a single emission band are most widely used. LE-state quantum yield $\Phi_F$ and bulk viscosity $\eta$ follow a power-law relationship that is widely referred to as the Förster-Hoffmann equation, Eq.1:

$$\log \Phi_F = C + x \cdot \log \eta$$

where $C$ and $x$ solvent and dye-dependent constants. This relationship has been experimentally shown to be valid in a wide range of viscosities and in both polar and nonpolar fluids. Eq. 1 has become so popular that in some instances the existence of this power-law relationship has been used to support TICT behavior of specific molecules. Figure 4 shows the log-log dependence expressed in Eq. 1 for the molecular rotors CCVJ and DCVJ, as experimentally obtained from the rotors’ emissions measured at different solvent viscosities.
Figure 4 Emission spectra of the molecular rotors CCVJ (a) and DCVJ (b) in a viscosity gradient with different mixture ratios of ethylene glycol and glycerol. An higher emission intensity, caused by a higher fluorescent quantum yield, is seen in higher-viscosity solvents. Plotting the peak intensities as a function of viscosity in a double-logarithmic scale reveals the power–law relationship described in Eq. 1, and the constants x and C can be determined from the diagram (spectra and graphs from Heidekker, 2010).

1.3 Chemical classes of Molecular Rotors

The ICT mechanism is associated with a large number of fluorophores. It is therefore not surprising that the formation of TICT states has been detected in several structurally different compounds. Although this structural diversity, there is a common motif for all molecular rotors that consists in π-conjugation between an electron withdrawing (EW) and an electron donor (ED) group, as shown in Figure 1.
Figure 5 Mesomeric structures of a TICT probe. Upon photoexcitation, the groundstate structure (on the left) is converted to a dipolar quinoid structure (on the right).

The most popular electron-donating (ED) group is a disubstituted nitrogen atom that can be dialkylated or inserted in a cycle (julolidinic or morpholinic framework), whereas different electron-withdrawing (EWG) groups were published, including functionalities such as nitriles, carboxylic esters, and aromatic rings. The EDG and EWG groups are linked via a $\pi$-conjugation system that makes possible electron transfer from donor to acceptor upon photoexcitation.

DMABN (1) is a representative and well-investigated example of this structural family. In the class of DMABN-related structures may be included every chemical and structural modification that does not alter the extent of the described $\pi$-conjugation systems. It is also important to notice that the increase of the ED-EW group distance can deeply change the fluorescent profile of such probes, as it happens stepping from DMABN to (p-(dialkylamino)-benzylidene)malononitrile, DBMN (2).
In reality, the increase of the distance between the two groups in 2 lowers the excitation energy and leads to non-radiative decay from the TICT state because of the very small S1-S0 energy gap in the twisted state. Other structures related to DBMN are (pseudo-)stilbenes (such as DAMPSI, 3) and 2-cyanomethylene-3cyano-2,5-dihydrofuran (DCDHF, 4). Structurally-different examples of molecular rotors include triphenylmethane-based fluorophores (crystal violet, 5), porphyrins (such as 6) and BODIPYs (such as 7) shown in Figure 6.

1.4 Coumarin-related fluorescent probes

One of the most widespread fluorescent dye motif which encountered countless applications in chemistry, medicine, biochemistry and biophysics is the coumarin (2H-cromen-2-one) core$^{21-24}$, shown in
Soon after their discovery, coumarin derivatives found applications in pharmaceutical field, since they can have interesting anticoagulant properties depending on their structure\textsuperscript{24}. Indeed, the coumarin coumarin is the precursor of a number of synthetic anticoagulant pharmaceuticals, such as Warfarin and some even more potent rodenticides that act \textit{in vivo} as vitamin-K antagonist\textsuperscript{25}. More recently, new coumarin derivatives were synthesized and produced for their unique photophysical features\textsuperscript{26,27}: coumarins are usually characterized by high quantum yields (up to 0.90), high molar extinction coefficients (10000 – 40000 M\textsuperscript{-1} cm\textsuperscript{-1}), large Stokes shifts (up to 160 nm), and their optical features can be efficiently tuned by adjusting the coumarin structure. For instance, coumarins are used as dyes, sensors for metal cations, and dopants for OLEDs\textsuperscript{28}. The well-known commercial fluorophores Alexafluor 350 and Alexafluor 430 are based on a coumarin core. Recently, coumarins were thoroughly investigated in view of synthesizing new probes for biological application, in particular for imaging of living cells\textsuperscript{11}.

\section{MATERIALS AND METHODS}

\subsection{Reagents}

Raspberry ketone glucoside was provided by Beijing Brilliance Bio, and vinyl esters were purchased from TCI America. Lipase acrylic resin (Novozymes 435) from Candida antarctica
(≥5,000 U/g), recombinant, expressed in Aspergillus niger was provided by Novozymes. 9-Formyl-8-hydroxyjulolidin, diethyl malonate, piperidine and 4-ethynylphenylacetonitrile, 1-Azido-1-deoxy-β-D-glucopyranoside and 8-bromooctanoic acid were purchased from Sigmaaldrich (St Louis, MO). Sodium azide, Silica Gel (200-300 Mesh), toluene, hexanes, ethyl acetate, ethanol and acetone were purchased from Thermofisher (NY, NY). The following solvents were dried with the indicated essicant and distillated before use under dry N₂: triethylamine with CaH₂, THF with Na, dichloromethane with P₂O₅. Bruker ScanAssyst-Air AFM tips (70 kHz 70, 0.4 N/m, silicon nitride), Ted Pella, Inc. AFM specimen discs (12 mm diameter, stainless steel).

2.2 Instruments

NMR Spectrometer Bruker 300 (300 MHz); Mass spectrometer 4000 QTRAP (Applied Biosystems, Foster City, CA); HPLC system: Shimadzu Prominance HPLC (Shimadzu USA, Canby, OR); UV/Vis Spectrophotometer Thermoscientific Evolution 300; Fluorimeter HORIBA Jobin Ivon FluoroMax-3; J-810 Circular Dichroism Spectropolarimeter Jasco; Atomic Force Microscope Bruker MultiMode 8 AFM; Stimulated Emission Depletion microscope Leica TCS SP8 STED 3X; Ubbelohde Viscosimeter (k = 0.332 and k = 1.055)

2.3 Synthesis

2.3.1 Raspberry Ketone Glucoside Fatty Acid Ester Synthesis.

In a 500 mL screw-cap Erlenmeyer flask, solid Novozymes 435 lipase catalyst (0.3 g/mmol glucoside) was added to mixture of raspberry ketone glucoside (2.0 mmol, 0.652 g), and
vinyl octanoate (3:1 mmol acyl donor/glucoside ratio) containing 50 mL of dried acetone. The reaction proceeded in an orbital shaker at 250 rpm and 50 °C. The reaction was monitored by thin-layer chromatography (TLC) with an ethyl acetate eluent and visualized using 5% sulfuric acid solution in water and gentle heating. After 24 h, the bottom glucoside spot ($R_f = 0.1$) faded and a product spot appeared ($R_f = 0.4$). Before the solution was allowed to cool to room temperature, the enzymes were filtered out and rinsed with acetone until the washings showed no further product on TLC before they were air-dried and stored for reuse. Acetone was evaporated under vacuum from the filtrate, leaving behind a crude solid mixture of glucoside–ester product. The solid mixture was triturated thrice with 50 mL of hexanes at 50 °C to remove the excess fatty acid and derivatives from the opaque light yellow bulk solid. To remove trace elements of unreacted sugar, ester, or acids, the product (0.85 g) was dissolved in 25 mL of methanol and coated by evaporation onto 5.0 g of silica gel before being spread onto a short silica plug (40.0 g). The column was twice eluted to dryness with 200 mL ethyl acetate, and the solvent was evaporated from the second fraction via rotary evaporation to afford the pure fatty acid glucoside ester product (95% yield).

2.3.2 2,3,6,7-tetrahydro-1H,5H,11H-pyrano[2,3-f]pyrido[3,2,1-ij]quinolin-11-one (10)

In a 25 ml two necked round bottom flask equipped with condenser, stirrer and CaCl$_2$ tower, containing julolidine derivative (13, 1.00 g, 4.6 mmol) and diethylmalonate 12 (1.47 g, 1.4 ml, 2 eq) dissolved in ethanol (15 ml), piperidine (0.4 g, 0.46 ml, 1 eq) was added. The mixture was stirred under reflux for 6 h. After confirmation by TLC that the starting material (1) had been fully consumed, the solvent was carefully removed under reduced pressure.
The intermediate **11** (deep orange solid) was suspended in diluted HCl (20 ml) and glacial acetic acid (2 ml) and the mixture was kept under reflux for 12 h. After this time the mixture was left to cool down to RT and the pH was adjusted to 4.5 by adding small amount of NaHCO$_3$(sat).

Afterwards, the crude product was extracted with dichloromethane (3 x 20 ml) and the combined organic layers were dried with Na$_2$SO$_4$, filtered and concentrated under reduced pressure. The resultant green solid was purified by flash chromatography (silica gel; petroleum ether / ethyl acetate, 3:2) to give **10** (1.29 g) in 74 % yield as a dark yellow solid.

$^1$H-NMR (CDCl$_3$, 298 K) δ (ppm): 7.74 (d, 1H, J = 9.20 Hz), 6.83 (s, 1H), 5.99 (d, 1H, J = 9.20 Hz), 3.24 (q, 4H, J = 5.60 Hz), 2.87 (t, 2H, J = 6.40 Hz), 2.74 (t, 2H, J = 6.40 Hz), 1.95 (m, 4H)

$^{13}$C-NMR (CDCl$_3$, 298 K) δ (ppm): 162.70, 151.70, 145.90, 144.00, 125.00, 118.31, 108.43, 106.77, 50.02, 49.63, 27.53, 21.59, 20.66, 20.35

ESI-MS (m/z): 242.3 (M+H$^+$)

2.3.3 11-oxo-2,3,6,7-tetrahydro-1H,5H,11H-pyranol[2,3-f]pyrido[3,2,1-ij]quinolin-10-carboxaldehyde (**9**)

In a 50 ml three-necked bottom flask, equipped with condenser, stirrer, CaCl$_2$ tower and N$_2$ inlet tube, containing POCl$_3$ (0.3 ml), anhydrous DMF (0.3 ml) was added dropwise to yield a scarlet homogenous solution which was stirred for 30 min at 45 °C. Afterwards, **10** (220 mg, 0.91 mmol) was dissolved in DMF (2 ml) and introduced dropwise after which the mixture was stirred at 60°C for 6 h. After cooling to RT, the solution was slowly poured in cracked ice (10 g) and vigorously stirred for further 15 min. The pH was adjusted to about 5.5 by adding a small amount of NaOH$_{conc.}$. The large amount of yielded solid was filtrated in vacuum, washed with
cold water, dried and recrystallized with ethanol to give 9 (216 mg) in 88 % yield as iridescent dark green crystals.

\[ {^1}H-\text{NMR (DMSO, 298 K)} \delta \text{ (ppm): 9.85 (s, 1H), 8.23 (s, 1H), 7.25 (s, 1H), 3.36 (m, 4H), 2.70 (m, 4H), 1.86 (m, 4H, J = 5.90 Hz)} \]

\[ {^{13}}C-\text{NMR (DMSO, 298 K)} \delta \text{ (ppm): 187.35, 153.93, 150.12, 146.10, 129.40, 120.40, 108.43, 105.80, 50.50, 50.11, 27.45, 21.11, 20.12} \]

ESI-MS (m/z): 270.3 (M+H+) 

2.3.4 10-((2′Z)-2′-(4′-ethynyl)phen-1-yl-prop-2′-en-3′-yl)-2,3,6,7-tetrahydro-1H,5H,11H-pyranono[2,3-f]pyrido[3,2,1-ij]quinolin-11-one (C1)

In a 25 ml two necked round bottom flask equipped with condenser and stirrer, containing 9 (180 mg, 0.67 mmol) and 4-ethynyl phenylacetonitrile 8 (0.104 g, 1.1 eq) dissolved in MeOH (13 ml), a solution of piperidine (12 mg, 14 μl, 0.2 eq) in MeOH (1 ml) was added. The mixture was stirred under reflux for 6 h. After evaporation of the solvent the crude product was recrystallized with acetone and diethyl ether, filtered in vacuum with an Hirsch funnel, carefully washed with cold ether and dry in vacuum to yield C1 (126 mg) in 48 % yield as a dark red crystalline solid.

\[ {^1}H-\text{NMR (DMSO, 298 K)} \delta \text{ (ppm): 8.50 (s, 1H), 7.80 (s, 1H), 7.67 (d, 2H, J = 9.10 Hz), 7.57 (d, 2H, J = 9.10 Hz) 7.16 (s, 1H), 4.34 (s, 1H), 3.25 (m, 4H), 2.72 (m, 4H), 1.88 (m, 4H)} \]

\[ {^{13}}C-\text{NMR (DMSO, 298 K)} \delta \text{ (ppm): 158.90, 153.68, 150.12, 138.28, 137.75, 130.10, 129.63, 128.43, 122.82, 120.74, 119.21, 119.10, 117.96, 112.26, 110.33, 84.28, 77.67, 51.55, 26.56, 22.93, 22.87, 22.21} \]

ESI-MS (m/z): 393.2 (M+H+)
2.3.5 Synthetic protocol for Oct-C1

4.5 mmol of 8-bromoocatanoic acid (1.00 g) and 1.6 eq of sodium azide were added to 6 ml of DMSO. The solution was stirred at RT for 24 hours after which 70 ml of H2O were added and the product was extracted 2x40 ml of diethyl ether. The combined organic phases were washed 3x50 ml H2O and 2x50 ml NaCl sat. The solvent was evaporated via rotavapor to yield 650 mg of the crude product as a clear liquid. 10 mg of crude 8-azido caprylic acid (5, 0.05 mmol) were added to 1 eq of C1 in 0.4 ml of DCM and 0.3 ml of H2O. After complete dissolution, 1.5 mg of CuSO4 pentahydrate (1.5 eq) and 3 mg of sodium ascorbate (2 eq) dissolved in 0.1 ml of H2O were added. The biphasic reaction was stirred vigorously for 4 hours. 20 ml of H2O and 40 ml of DCM were added. The organic phase was recovered via separatory funnel, dried on anhydrous MgSO4 and the solvent removed via rotavap to yield 11 mg of Oct-C1 as a dark orange solid.

1H-NMR (DMSO, 298 K) δ (ppm): 8.69 (s, 1H), 8.54 (s, 1H), 7.96 (d, 2H), 7.82 (s, 1H), 7.76 (d, 2Hz), 7.18 (s, 1H), 4.41 (t, 2H), 2.75 (dd, 4H), 2.30-2.40 (2H), 2.00-1.80 (6H), 1.40-1.55 (2H), 1.40-1.15 (8H).

13C-NMR (DMSO, 298 K) δ (ppm): 177.0, 162.0, 148.8, 143.7, 140.1, 138.6, 135.7, 134.9, 134.0, 131.0, 130.5, 125.9, 125.7, 124.6, 120.2, 117.5, 117.2, 116.3, 111.7, 58.5, 53.2, 35.8, 34.3, 29.9, 29.7, 27.8, 27.2, 25.1, 19.9.

ESI-MS (m/z): 576.6 [M-H]−

2.3.6 Synthetic protocol for Glu-C1

2 mg of C1 (0.02 mmol) was dissolved in 0.4 ml of DCM and 0.3 ml of H2O in a 15 ml-brown glass vial. 4 mg of 1-Azido-1-deoxy-β-D-glucopyranoside (0.02 mmol, 1 eq) dissolved in
50 μl of H₂O were added to the biphasic C1 solution. 1.5 mg of CuSO₄ pentahydrate (1.5 eq) and 3 mg of sodium ascorbate (2 eq) were dissolved in 0.1 ml of H₂O and finally added to the reaction vial. The reaction was stirred at RT for 48 hours. The product was purified via flash chromatography on silica gel by using pure ethyl acetate followed by 1:1 ethyl acetate:methanol to recover the product in 30% yield as a dark orange solid.

¹H-NMR (DMSO, 298 K) δ (ppm): 8.95 (s, 1H), 8.56 (s, 1H), 8.03 (s, 1H), 7.84 (d, 2H), 7.81 (s, 2H), 7.20 (d, 2H), 6.65 (s, 1H), 5.61 (bb, 1H), 5.34 (bb, 3H), 3.25 (m, 4H), 2.72 (m, 4H), 1.88 (m, 4H).

¹³C-NMR (DMSO, 298 K) δ (ppm): 162.0, 148.8, 143.0, 140.1, 138.6, 134.8, 126.6, 126.4, 135.5, 132.4, 129.0, 130.5, 124.6, 120.2, 117.5, 116.9, 116.3, 111.8, 82.7, 72.7, 69.9, 66.6, 68.3, 61.8, 58.4, 58.2, 34.3, 34.1, 29.6, 19.7.

ESI-MS (m/z): 619.8 (M+Na⁺)

### 2.4 Gel characterization

#### 2.4.1 Preparation of R8 Molecular Gels

Gels were prepared by adding the solid glassy glycosides (10.0 - 50.0 mg gelator) to toluene (1 mL). The mixture was then heated to disperse the gelator at 5°C below the boiling point of the solvents, to produce a homogeneous sol. The sol was kept at this temperature for 5 min under constant agitation to fully disperse the gelator. The sol was then allowed to cool to room temperature to allow for self-assembly. No free flow of the 1% gels upon inversion was observed after 6 min. The samples were inverted to confirm gel formation after 1h.
2.4.2 Rheological Characterization of Gels

Oscillatory rheological measurements were performed on a stress-controlled rheometer (AR 2000 ex, TA Instruments) with a cone and plate geometry (1° 58’ 47” angle and 40 mm diameter with a truncation gap of 45 μm). 1 mL of gel was loaded onto the plate, and the cone was lowered to minimize the truncation gap. Precautions were taken to minimize shear-induced disruption of the gel network: before experiments samples were equilibrated within the geometry for 10 minutes. Excess gel was trimmed away from the cone to ensure optimal filling. Thixotropic/repeating yield strain ($\sigma_y$) was examined for toluene gels by performing oscillatory strain sweep measurements from 0.01 to 100% deformation at a fixed frequency of 1 Hz and then ramping through this cycle several times.

2.5 Spectroscopic measurements

1500 μL samples were used in quartz cuvette (ThorLabs, Newton, NJ) with optical path ($l$) of 1 cm. The temperature of the cell compartment was set at 25 °C, by a built-in Peltier cooler (Varian). Absorption data were recorded at 25 °C using 1 nm band-pass, 1 nm resolution and 200 nm/ min scanning speed. Fluorescence intensity measurements were carried out employing an excitation and emission band-pass of 2.5 nm, 120 nm/ min scan rate and 600 V PMT detector voltage. Absorption data were recorded at 25°C using 1 nm band-pass, 1 nm resolution and 200 nm/ min scanning speed.

Quantum yields were determined according to a reported procedure$^{29}$, using rhodamine G6 as reference dye. Extinction coefficients were obtained by accurately determining the fluorophore sample concentration via NMR analysis (using acetonitrile as internal standard) and
performing the measurements after diluting the sample in the appropriate solvent. Molar extinction coefficients were determined by Lambert – Beer law.

The time-transient fluorescence emission measurements were obtained by adding the appropriate volume of a stock solution of the fluorophores (C1, Glu-C1 and Oct-C1) in hot R8 toluene solution (1% w/w) for a final concentration of 100 nM. The cuvette was kept sealed with a Teflon cap throughout the duration of the experiment to ensure no variations in concentration.

2.5.1 AFM measurements

An R8 1% solution (w/v) was prepared by adding the solid gelator to 3 ml of toluene (1 mL). The mixture was then heated and stirred at 100°C to produce a homogeneous sol. 5 μL of sample solutions were drop-casted onto microscope cover glasses (Fisher Scientific, 12-542A, NY, NY) that were kept at 50°C on a hot plate. The time points considered for AFM imaging were immediately after R8 completely dissolved (time 0 min) and after 0.5, 1, 1.5, 2, 4 and 6 min. After complete evaporation of the solvent (about 5 sec) the samples were further air-dried in a dust-free environment for 12 h and then glued onto magnetic discs prior AFM imaging. The samples were scanned in ScanAssyst mode.

2.5.2 STED measurements

10 μl of 1% R8 toluene gel doped with either Glu-C1 or Oct-C1 (100 nM) were deposited onto a microscope cover glass kept at 50°C on a hot plate to ensure quick evaporation of the solvent. After further drying overnight at RT in a dust-free environment, the samples were imaged.
2.6 Quantum Mechanics Calculations

Table 1 reports the ground-state energies for the various C1-triazol conformers Z-s-cis and Z-s-trans (See Figure 4 of main text), evaluated at the DFT-PBE0 level, with the inclusion of solvent effects by the Polarizable Continuum Method (PCM). Excitation energies were calculated by TD-DFT CAM-B3LYP, both in the gas phase and using PCM.

<table>
<thead>
<tr>
<th></th>
<th>Dipole (Debye) (PBE0/6-31G*)</th>
<th>Delta Ground State Energy (kJ/mol) (PBE0/6-31G*)</th>
<th>Excitation wavelength (nm) (CAM-B3LYP/6-31+G*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-s-cis/Z-s-trans</td>
<td>∆E=E(Z-s-trans) – E(Z-s-cis)</td>
<td>Z-s-cis</td>
<td>Z-s-trans</td>
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<td>gas phase</td>
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<td>12.9</td>
<td>29.6</td>
</tr>
<tr>
<td>CCl4</td>
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<tr>
<td>water</td>
<td>12.5</td>
<td>20.2</td>
<td>12.9</td>
</tr>
</tbody>
</table>

Table 1 Ground state energies, permanent dipole moments and excitation wavelengths of s-cis and s-trans conformers of C1-triazol in the gas phase and in various solvents.

In all examined cases the Z-s-cis conformer is the most stable, though the ∆E decreases (i.e. Z-s-trans gets less unstable) in more polar solvents, because of the sizably larger dipole moment of the Z-s-trans conformer. The excitation energy of Z-s-trans is ~ 30-35 nm blue shifted. Both Z-s-trans and Z-s-cis display a slight blue shift in going from apolar to polar solvents. In the Z-s-cis structures the CN groups is coplanar with the coumarin ring system. By contrast in Z-s-trans the C15-C14-C16-C17 dihedral angle is ~30 deg, because of steric clash with the coumarin carbonyl.
2.6.1 Coarse-grained MD simulations

a) Preparation of the system and formation of the R8 “double helix”

Aggregation of the R8 gelator in toluene was first studied by coarse-grained simulations with the Martini force field (for the parameterization procedure see Methods). To speed up aggregation we used model 1 of Toluene (with C4/SC4 beads, at 350K to prevent toluene freezing). Indeed this model better segregates the R8 molecules, resulting in faster formation of a single cluster. We began with 216 R8 molecules, evenly spaced in a 16.3-nm cube with 23544 toluene molecules. After 200ns of simulation time, three large clusters formed. We then reduced the number of toluene molecules in the system to 8000, and a single cluster formed. In this aggregate the R8 molecules present the lipid tails both to the external solvent and to the interior of the cluster, the glucose rings being halfway between the hydrophobic regions. Already at this early stage, the cluster features glucose-ring stacking.

We switched to semi-isotropic pressure coupling in order to guide the formation of a protofiber along the z axis. This was done by increasing the pressure along the z-axis to 30 bar while leaving that on the x-y plane to 1 bar, in a 10-ns simulation. As a result the box z side decreased and the cluster interacted with its image to form a continuous aggregate along z. The squeezing along z was stopped when the lateral width of the aggregate was compatible with the diameter of the fiber from the experimental measurement (i.e. ~ 6nm). The simulation was then switched to isotropic pressure coupling. Due to the over-aggregating tendency of model 1 of toluene, keeping semi-isotropic coupling with equal 1-bar pressure on z and x-y resulted in further reduction of the box z length, and a thicker aggregate. Model 2 of toluene preserves the formed aggregate, but considerably slows the aggregation. Indeed only around 85% of R8 molecules are aggregated, while the remaining 15% exchange with the solvent.
The emerging structure consists of segments of stacked \textbf{R8} molecules, arranged in a way similar to a double helix. The stacking is made of 5 staggered \textbf{R8} molecules, with the glucose rings at the center and the lipid and benzenoid tails on the outside. Such structure is stable over the $\mu$s simulated timescale at 310K with model 2 of toluene.

\textit{b) Interaction of \textit{Glu-C1} and \textit{Oct-C1} with the double helix fiber}

In order to study the interaction of the fluorophores (\textit{Glu-C1} and \textit{Oct-C1}) with the \textbf{R8} supramolecular structure we started with the molecules in the solvent part of the simulation box. We then partially melted the double helix by increasing the temperature to 350K for 100ns. We then decreased the temperature to 310K so as to let the double helix renature. As a result of the process both \textit{Oct-C1} and \textit{Glu-C1} get inserted in the double helix and remain stacked between the various \textbf{R8} molecules in the $3\mu$s time of the simulations. By visual inspection it is apparent that the glucose ring of \textit{Glu-C1} is constantly stacked among the \textbf{R8} glucose rings, while \textit{Oct-C1} octanoic acid stays in contact with \textbf{R8} lipid tails. This arrangement causes a different positioning of the coumarin in the two cases, though the twisted geometry of the double helix prevents a simple assessment of these features.

\textit{c) Insertion of \textit{Glu-C1} and \textit{Oct-C1} in the single fibril}

To better characterize the interaction of \textit{Glu-C1} and \textit{Oct-C1} with the \textbf{R8} supramolecular structure, we isolated a single fibril from the double helix structure. This was done by taking two stacked quintuplets and replicating them 4 times in the z direction. The resulting structure is stable over the $\mu$s-simulated timescale.

\textbf{Glu-C1} and \textbf{Oct-C1} were initially put in the solvent and inserted in the fibril by pulling their center of mass toward the axis of the fibril by a constant 500 kJ mol$^{-1}$nm$^{-1}$ force for 10 ns. Then the two systems were run for 4 $\mu$s and the analysis performed on the last 2 $\mu$s. The resulting
positioning of the two molecules is analogous to the spontaneous insertion in the double helix. With respect to the axis of the fibril, the different arrangement of the two derivatives emerges very clearly, and is quantified by the radial distribution function with respect to the fibril axis. The glucose of **Glu-C1** is aligned with the **R8** glucose rings within the central region of the fibril. The coumarin part prevalently points outwards, in contact with **R8** lipid tails. By contrast, **Oct-C1** coumarin is more internal to the fibril and **Oct-C1 C1** octanoid acid tails project outwards.

2.6.2 All atom MD simulations

a) Structure of the **R8** fibril

The local ordering from the CG simulations was used as a template for the all-atom model of the **R8** aggregate structure. This was achieved by using the coordinates of the stacked glucose-ring beads (the most stable groups within the aggregate) as restraints for the corresponding atoms in the all-atom representation. More in detail, 10 **R8** molecules in toluene were simulated with increasingly strong restraints on their glucose ring atoms, forcing them to a configuration of two stacked **R8** quintuplets taken from the CG simulation. The system was then replicated four-fold along the z axis in order to obtain a (periodic) 8 **R8** quintuplet stack.

The system was then simulated with no constraints. During a 100ns simulation a regular structure emerges with 4 instead of 5 **R8** molecules per stack (Figure 1e-g in the main text). This difference between the CG and all-atom prediction stems from the less directional and “softer” description of inter bead interactions in the CG representation. It comes as no surprise that the CG method is able to capture the general architecture of the assembly but may fail in the fine details, for which the all-atom description is clearly more reliable. As a consequence, we
extracted one quadruplet and replicated it 6 times along z (24 total R8 molecules). The system was then simulated for 50ns at 300K and other 50ns at 310K (with semi isotropic pressure coupling, in order to allow for adjustment along z). The resulting aggregate structure is remarkably stable during the simulation. Such stability is the result of the tightly packed sugar rings, interacting with each other by establishing several H-bonds. In addition, the phenyl rings are rather regularly π-stacked. The alkyl chains are less ordered though some degree of mutual contact is present along the z-axis.

The discovered quadruplet arrangement is not symmetric, insofar as it does not maximize the in-plane inter phenyl tail and inter alkyl chains contact. We tried a more symmetrical quadruplet structure, in which the phenyl tails and the alkyl chains are paired. However such an arrangement is much less stable during the simulated 50ns at 310K, and in particular the regular stacking is lost at the end of the simulation.

b) Insertion of Glu-C1 and Oct-C1

Again, the results of the CG simulations in terms of the interaction of Glu-C1 and Oct-C1 with the fibril were used to guide the initial structures for the all-atom simulations. The CG simulations predicted two possible arrangements in the Glu-C1 case, both having Glu-C1 glucose blocked within the sugar core of the fibril, sandwiched with the other glucose rings of the stack. In the first, the C1 is positioned out of the sugar core, in contact with phenyl and/or alkyl chains. In the second it is midway between the sugar core and the external part of the fiber. The second case is not feasible in the all-atom case, because the C1 part is sizably tilted w.r.t. Glu-C1 glucose ring, so it cannot be accommodated between the stacks once the glucose ring intercalates with the other glucoses. (The structure was feasible in the CG case because of the softer and less directional glucose-glucose interactions).
We started from the regular R8-quadruplet stack from the previous simulations, and substituted one R8 molecule with Glu-C1, in such a way as to fill the left void with Glu-C1 glucose ring. Due to the presence of the C1 substituent the glucose ring of Glu-C1 cannot be positioned in the same orientation of the missing R8 glucose. Nonetheless, several H-bonds are established with the rest of the R8 matrix, and the structure is stable over the 50ns simulation at 310K.

In the CG simulation Oct-C1 is positioned with the C1 part within the sugar core of the fibril, and its octanoyl acid tail points outward and is in contact with the other alkyl chains and phenyl ring tails. This arrangement was translated into the all-atom simulation by removing two R8 molecules and filling the empty space with Oct-C1 and then letting the system relax for 50ns.

2.6.3 Free-energy calculations

We studied how the different arrangement of C1 in the two cases (Glu-C1 and Oct-C1) impacts the relative population of the two C1 isomers (Z-s-cis and Z-s-trans). Indeed the polarity experienced by C1 in the two cases (low for Glu-C1 and high for Oct-C1) would point to spectral shifts that are opposite w.r.t the measured spectra. However, we show that the interaction of Glu-C1/Oct-C1 with the R8 stack modifies the population of the two isomers leading to agreement with the experiments.

2.6.4 QM calculations

Optimization of the structures was performed at the DFT PBE0 6-31G(d) level. The excitation energies were calculated on the optimized structure using TD-DFT CAM-B3LYP with the 6-31+G(d) basis set. Solvent effects were introduced within an implicit solvent treatment by the Polarizable Continuum Model (PCM). Gaussian in the g09 version was used.
3. RESULTS

The compound investigated in my PhD project has several similarities with known molecular rotors. Indeed, as demonstrated by several examples in introduction, rational modifications of the basic rotor configuration may lead to significant changes in the viscosity-dependent behavior. Beside its spectroscopic features, this compound was designed to allow for facile conjugation to specific chemical moieties.

The terminal alkyne group is known to take part in an important click chemistry reaction, i.e. copper(I)-catalyzed Huisgen 1,3-dipolar cycloaddition with organic azides to yield 1,2,3-triazoles with extremely high 1,4 regioselectivity (Scheme 1).

![Scheme 1](image)

**Scheme 1** Copper(I)-catalyzed Huisgen 1,3-dipolar cycloaddition for biomolecule functionalization.

The general structure was designed to integrate a push-pull system and the aforementioned terminal alkyne. In particular, the amino ED group and two EW groups (cyano and a para-alkynyl phenyl) were separated by the extended pi-conjugation of the coumarin core (necessary for efficient electron delocalization).

This general structure is sketched in Figure 8 along with the 3-styryl coumarin derivative C1.
Figure 8 Sketched representation of the chemical structure of C1 along with the identification of the four different subunits (julolidine framework, coumarin core, stilbene-like portion and terminal alkyne group).

The molecular rotor C1 has been designed to combine different structural features belonging to molecular rotors classes previously discussed. In fact, the chemical structure of fluorophore C1 is based on four different motifs (Figure 8): a) tricyclic julolidine framework which completely prohibits rotation across the C-N bond, removing this nonradiative deactivation pathway; b) the coumarin core which, as discussed previously, gives to the fluorophore unique photophysical properties; c) a (Z)-stilbene-like portion (heterocyclic ring - double bond - cyano group/phenyl ring), which contains the bond involved in the viscosity-dependent rotation; d) a terminal alkyne group perfectly suitable for further functionalization via click-chemistry reactions.
3.1 Synthetic approach for 3-styryl-coumarin molecular rotor (C1)

As illustrated in the retrosynthetic Scheme 2, the synthesis of coumarin C1 required four synthetic steps starting from the valuable julolidine derivative 8-hydroxy-2,3,6,7-tetrahydro-1H,5H-pyrido[3,2,1-ij]quinoline-9-carbaldehyde (13).

![Scheme 2 Retrosynthetic scheme for the synthesis of 3-styryl-coumarin C1.](image)

Intermediate coumarin derivative 10 was synthesized following a published procedure30. Julolidine 13 has been converted into the intermediate coumarin derivative 11 via condensation reaction with diethyl malonate (12) followed by lactonization (Scheme 3).
In the first step, 13 and the active methylene compound 12 were condensed in ethanol at reflux for 6 h in presence of catalytic piperidine. Prior to the subsequent step, it was essential to remove all the reaction solvent under reduced pressure even though this was not explicitly reported in the published procedure. In the second step, the deep orange residue containing ethyl 10-carboxylate derivative 11 was suspended in diluted HCl and glacial acetic acid. This reaction (acid hydrolysis followed by decarboxylation) was initially performed at RT as reported in the reference article but the product was obtained in very poor yield. On account of this unexpected result, the reaction was performed under reflux conditions. After few minutes from the beginning of the reflux, CO₂ release indicated the advancement of the reaction. After 12 h, the mixture was cooled down to RT and the pH was adjusted to 4.5 by adding small amount of a saturated solution of NaHCO₃. Note that coumarin derivatives are very unstable in alkaline solutions and the final pH must be carefully controlled. Flash chromatography of the worked out compound gave 10 in 74 % yield as a dark yellow solid. The determination of the melting point and NMR analysis were in perfect agreement with literature data and confirmed the high purity of 10.
The synthesis of 10-carboxaldehyde derivative 9 was accomplished via Vielsmeier reaction (Scheme 4). This reaction was proved to be totally regioselective in position 3 of the electron-rich coumarin core since 9 resulted the sole regioisomer.

![Scheme 4 Synthesis of compound 30.](image)

In more detail, coumarin 10 in DMF was slowly added to a solution of phosphoryl chloride in DMF previously stirred for 30 min at 45 °C; then, reflux was maintained for 6 h. As for the previously reported Vilsmeier, the reaction was quenched on ice. Work-up included pH adjustment was to about 5.5 by concentrated NaOH and filtration. The impure solid was recrystallized from ethanol to give 9 in 88 % yield as iridescent dark green crystals. NMR analysis confirmed the structural features and purity of aldehyde 9 (> 98 %).
This synthesis of C1 was carried out as shown in Scheme 5:

![Scheme 5 Synthesis of 3-styryl coumarin C1.](image)

Aldehyde 9 and arylacetonitrile 8 were mixed in MeOH in presence of catalyst piperidine, and reflux was maintained for 6 h. Flash-chromatography proved unsuitable for purification of C1, because it was clearly proven that silica-gel catalyzed the decomposition of C1 into the precursors 9 and 8. Thus, the product was recrystallized from acetone and diethyl ether to afford C1 in 48 % yield as a dark red crystalline solid. NMR analysis confirmed the structural features of molecular rotor C1.

### 3.2 NMR-based stereochemical characterization of C1

The piperidine-catalyzed condensation reaction between an aromatic aldehyde (such as 9) and an active methylene compounds (such as 8) is diastereoselective, since the formation of the (Z)-diastereoisomer is usually preferred over the (E)-diastereoisomer. In order to obtain consistent and reproducible spectroscopic data, the determination of the diastereoisomeric excess (d.e.) of each molecular rotors was needed. Surprisingly, Heidekker et al.\(^5\) did not report any stereochemical characterization of their molecular rotors (such as d.e. values, spectroscopic evidences etc.). In this thesis, C1 has been characterized from a stereochemical standpoint via
NMR by performing either cycleNOE. CycleNOE is a peculiar steady-state measurement of NOE based on the subtraction between two monodimensional $^1$H-NMR spectra. The first of these spectra ($sp1$) is collected after a saturation pulse in resonance with the nucleus whose surroundings have to be analyzed; the second one ($sp2$) is a $^1$H spectrum associated with off-resonance saturation pulse. In $sp1$ the irradiated nucleus resonance vanishes, and NOE is transferred to spatially close nuclei thus modifying their signal intensity (an increase is usually observed in small molecules). Accordingly, $sp1-sp2$ results in a spectrum where the irradiated proton resonance is inverted and only those signals belonging to proximal nuclei survive and are positive$^{46}$. It is possible also to see negative peaks in particular cases when there is a linear disposition through the space of a three-spin system$^{31}$. This fact can provide even more in-depth information about the reciprocal spatial disposition of molecular moieties.

The $^1$H-NMR spectrum of C1 in benzene-d6 displays the presence of a single stereochemical form (Figure 9). In the aromatic region (ranging from 6.42 ppm to 8.70 ppm) the protons H-7, H-8 and H-9 appear as three sharp singlets ($\delta_{H-7} = 6.42$ ppm, $\delta_{H-6} = 7.96$ ppm and $\delta_{H-8} = 8.70$ ppm), while the protons H-10 and H-11 as two roofing doublets ($\delta_{H-10} = 7.30$ ppm and $\delta_{H-11} = 7.23$ ppm, $J_{H10-11} = 7.5$ Hz) partially covered by the solvent signal at 7.10 ppm.
Yet, the $^1$H-NMR spectrum in DMSO-d$_6$ showed the presence of two distinguishable stereoisomers, in 70/30 molar ratio. Figure 10 reports the comparison between the aromatic resonances observed in benzene and DMSO-d$_6$ at RT. The major component in the DMSO mixture (red spectrum) retains the same pattern of the compound observed in benzene (black spectrum). Yet, the minor component visible in the spectrum recorded in DMSO-d$_6$ displays striking differences: its putative protons H-8 and H-9 (enclosed by a red rectangular frame in Figure 10) are strongly shifted upfield with respect to the more abundant peaks.
Figure 10 1H-NMR aromatic region enlargement spectra (300 MHz, RT; 6.00 – 9.00 ppm) of C1 recorded in DMSO-d6 (red spectrum) and in benzene-d6 (black spectrum): the resonances of proton H-8 and H-9 of the two isomeric forms are enclosed by rectangular frames.

The *cycle*NOE experiments were performed by sequentially saturating the resonances of proton H-7, H-8 and H-9 as reported in Figure 11 b, d and c. It is important to notice that the selective saturation of proton H-10 was not possible because of the partial superimposition of its resonance with that of H-11. In Figure 11 b (saturation of H-7) a well-definite positive singlet (proton H-8) appears at 8.70 ppm. This means that the proton H-8 is affected by the NOE effect due to its spatial closeness to proton H-7, as expected from the chemical structure of the coumarin core of C1.
Then, proton H-9 was saturated: a positive NOE effect becomes visible as a positive doublet centered at 7.30 ppm along with a less intense negative doublet at higher field (δ = 7.24 ppm) (Figure 11 c): the former doublet was easily assigned to protons H-10 while the latter doublet should belong to H-11. This finding strongly supports the attribution of Z-configuration to the styril double bond, as predicted by steric energy considerations. Yet, no NOE effect was transferred to H-8. Consistently, when the coumarinic proton H-8 was saturated, detectable NOE effect was found for the proton H-7, but not for H-9 (Figure 11 d). The last two findings can be reconciled with the Z-configuration of the styril double bond if the presence of two...
atropoisomers (i.e. energetically-distinguishable stereoisomers resulting from hindered rotation about single bonds) is taken into account. In such a case, the two atropoisomers are identifiable with \textit{s-trans-}(Z)-C1 and \textit{s-cis-}(Z)-C1 (Scheme 6); the observed lack of NOE transfer between H-8 and H-9 in cycleNOE spectra indicates that \textit{s-cis-}(Z)-C1 atropoisomer is predominant in benzene.

**Scheme 6** Atropoisomeric equilibrium of (Z)-C1.

The equilibrium between two atropoisomers of the Z-configuration nicely explains the presence of a stereochemical mixture. The notable downfield shift of the resonances attributable to H-8 and H-9 in \textit{s-cis-}(Z)-C1 (major component) compared to \textit{s-trans-}(Z)-C1 (minor component) can be rationalized by considering the deshielding cone of the nearby cyano and carbonyl group, as showed in Figure 12.
Note that 1,3-diene derivatives (in which coumarin C1 can be included) are known to undergo fast equilibrium between s-cis and s-trans rotamers, i.e. these are conformational isomers and are equiprobable at RT owing to the low interconversion energetic barrier. Since no example of atropisomeric equilibrium is reported in literature for 1,3-diene derivatives, the obtained results appear highly relevant and novel: in fact, the stereochemical investigation led to the discovery of the first atropisomeric 1,3-diene derivative. Moreover, the atropisomeric equilibrium of C1 proved to be fully shifted towards the s-cis atropisomer in solvents such as benzene or water thereby allowing the easy isolation of this atropisomer.

Finally, theoretical calculations were carried out in order to obtain more consistent data on the stereochemical features of C1. The geometry optimization and the relative thermodynamic stability of s-cis and s-trans of both diastereoisomers (E and Z) were performed by DFT/6-31G* adopting the PCM (Polarizable Continuum Model) method and toluene as implicit solvent. Remarkably, the results were in perfect agreement with the discussed experimental data. In fact, atropoisomer s-cis-(Z)-C1 was associated with the lowest energy, whereas the atropoisomer s-
trans-(Z)-C1 resulted 2.91 kcal/mol less; s-cis and the s-trans atropoisomers of diastereoisomer E were respectively 3.28 kcal/mol and 5.08 kcal/mol higher than s-cis-(Z)-C1.

3.3 The gelation process

Viscosity refers to a bulk property of the matter that is strictly linked to the nature of the constituent molecules. The viscosity-dependent photophysical behavior of molecular rotors has a molecular-scale dependence. Note that nanoviscosity need not be the same as bulk viscosity, the latter referring to a property measured over a spatial scale several orders of magnitude larger. Many researchers have tried to give rational interpretation to friction effects at nanoscale, especially for non-homogeneous mixtures; “equivalent viscosity” is the viscosity of a homogeneous medium in which the response of the fluorescent probe is the same. Arguably, the values for equivalent viscosity and nano-viscosity happen to match only if the environment in which the molecular probe finds itself is completely isotropic. This is generally true for completely miscible solvents (such as, glycerol and methanol) as well as for miscible polymers that due to their random arrangement within the solvent (associated to Brownian motion of polymer subunits) tend to form an overall isotropic matrix.

There is a peculiar and rather extreme situation, however, where equivalent viscosity and nanoviscosity fail to overlap, that is molecular gels. On the microscopic level these systems are highly anisotropic by virtue of the bidimensionally ordered network that spans the solvent. In most cases gel-forming molecules - also referred to as gelators - are needed at very low concentrations (usually below 2% w/w). Whenever gelation is effective, the viscosity of the system increases from that of the solvent to virtually infinite after gelation takes place, as complete absence of the solvent’s free flow demonstrates. Gelators are amphiphilic in nature.
which means that they are characterized by distinct hydrophobic and hydrophilic portions. As a consequence of this, gelator molecules will tend to self-assemble in an ordered way by interacting with each other in a like-assembles-with-like fashion. Therefore, relatively strong H-bonds will be established among polar heads, whereas more hydrophobic tails will interact via pi-pi stacking, Van der Vaals forces, etc. (Figure 13).

Figure 13 Schematic of the gelation process: the amphiphilic nature of gelator molecules favors the formation of specific intermolecular interactions. This drives the isotropic growth of nanoscopic fibrils (in this case, helices) and the subsequent formation of microscopic fibers.

Interestingly, molecular gelators display poor solubility in the solvent of choice and increased solubility at higher temperature. The importance of such chemophysical property lies in that the self-assembly occurs as a consequence of increasingly lower solubility as the temperature of the system decreases. During this intermediate and highly dynamic phase driven by a complex and kinetically-controlled series of events, the molecules can either aggregate anisotropically and precipitate out of solution as crystals or assemble isotropically giving rise to
ordered nanoscopic structures such as helices, sheets, ribbons etc. (Figure 13) and, in some cases, to optically clear materials\textsuperscript{33}. Curiously enough, since the gelator molecules constitute just a minimal fraction overall (<2\%), most of the space in the system will still be occupied by solvent molecules. Here, the local viscosity will not be significantly different from that of the pure solvent\textsuperscript{34}. On the other hand, we can hypothesize that the complex architecture of the gel network will present regions with drastically higher nanoviscosity owing to the aforementioned intra- and inter-molecular interactions. These specific interactions define the reciprocal geometrical arrangement of gelator ‘monomers’ on a nanoscopic scale as well as the rheological properties of the resulting material.

3.4 Characterization of toluene gels of the gelator R8

Inspired by previous reports of materials characterization via molecular rotors\textsuperscript{34-36}, we decided to explore the photophysical behavior of C1 in a gel system which, to the best of our knowledge, has previously never been successfully reported. This represented the perfect experimental platform to expand our knowledge regarding both molecular viscosity probes and gel formation.

The self-assembled system in which C1 was tested consisted of low-concentration toluene gels of the raspberry ketone glucoside caprylate gelator (R8, Figure 14 a and b\textsuperscript{33}) A thorough characterization was carried out to elucidate the physicochemical properties of its assembly, and the mechanical properties of the gelled materials. With a minimum gelation concentration of 0.46 wt. \% in toluene and a temperature of gelation at 1.0 wt. \% of 45-47\degree C, optically clear gels can be formed through thermoreversible gelation\textsuperscript{33}. Oscillatory dynamic
rheology demonstrates the thixotropic (mechano-reversible) behavior of the toluene gels (data not shown) common to a variety of polymeric and molecular gels$^{37}$.

![Chemical structure of the gelator R8](image1.png)

**Figure 14** a) chemical structure of the gelator R8; b) 1% toluene solution of R8 before gelation (left vial) and after (right vial); c) CD spectra of R8 toluene solutions in different conditions (R8 sol in ethanol at RT (red line), 1% R8 sol in toluene (green line) and 1% R8 gel in toluene (blue line); d) AFM image of a drop-cast 1% R8 toluene gel showing right-handed helices (scale bar: 40 nm).

In apolar solvents X-ray diffraction highlights the $\beta'$ conformation of the fatty acid tails on the exterior of the 3D-network indicating that the gelator packs in a fashion similar to natural triglycerides in a 3D-epitaxial arrangement$^{33}$. UV-Vis circular dichroism (CD) of the unassembled gelator in ethanol and the 1.0% toluene sol above the Tg (Figure 14 c, red and green line, respectively) demonstrate primarily a small positive band corresponding to the ketone
absorption at 270 nm. Interestingly, no change in peak intensity was observed before the sol-gel transition occurred (data not shown). However, once the solvent started to gel after 4 min, the system exhibited a substantial negative ‘excitonic’ CD signal\textsuperscript{38} arising from the alkyl phenol moiety at 290 nm while the ketone absorption barely changed (blue line, Figure 14 c). These results suggest that the supramolecular gelator architecture is itself chiral in nature. This observation matches well with atomic force microscopy images (AFM) of a drop-cast 1.0% toluene gel clearly showing right-handed nanoscopic helices about 7 nm-thick and more than 200 nm-long (Figure 14 d).

Figure 15 a) Views of the R8 quadruplet stacks: top view of the R8 quadruplet from a minimized MD snapshot. The 4 molecules are not on the same plane; b) lateral view of the glucose rings only, showing the inter-layer network of H-bonds; c) lateral view of 11 stacks.

Molecular and structural investigation of the fibrils were performed by Dr. Riccardo Nifosí at Scuola Normale Superiore di Pisa and extensively used to support the spectroscopic and microscopic observations regarding the self-assembly process. Via coarse-grained molecular dynamics simulations we showed that the non-polar alkyl tail and phenolic head reside on the outside of the fibril whereas the hydrophilic glucose moiety faces inwards, as expected in toluene (Figure 15). Remarkably, large-scale MD simulations confirmed the handedness of the fibrils
(Figure 16) and suggested a helical nature of two intertwined tubular structures each of which consists of four-molecule discs stacked longitudinally (Figure 15). Here, we propose that the gelator’s hydrophobic tails extending out from adjacent stacks interact with each other therefore causing unit fibrils to fuse together and drive the sol-gel transition. In support of this finding the width and the pitch measured from the AFM images are both comparable with the computational model, being these dimensions about 8 nm and 4 nm, respectively (Figure 16).

**Figure 16** Comparison between a gel double-helix obtained via AFM and the MD-simulated double-helix (scale bar: 1 nm).
3.5 Application of C1 and its derivatives in the gel system

The results described in the previous paragraph can then be integrated to the information gleaned by introducing the probes into the system in order to investigate nano-viscosity of unconventional liquid-like systems and help build a model of the gelator’s dynamic assembly. The viscosity-dependence of C1 was assessed in glycerol-methanol mixtures (methanol: \( \varepsilon = 33.6, \eta = 0.59 \) cSt; glycerol: \( \varepsilon = 40.1, \eta = 1400 \) cSt) (Figure 1a). Remarkably, C1’s fluorescence intensity displayed an excellent linear double-logarithmic dependence on viscosity, (Figure 17). This strongly supports the classical photophysical behavior of molecular rotors accounting for emission from the local excited state and modulation of the overall quantum yield via Twisted Intramolecular Charge Transfer (TICT) transition state as the viscosity of the medium increases.

![Figure 17](image)

**Figure 17** C1’s fluorescence emission was measured in glycerol/methanol solutions characterized by increasing viscosity. The double logarithmic plot of fluorescence intensity vs viscosity shows an excellent linear behavior.

Monitoring the fluorescence of R8 toluene gels doped with the C1 probe exhibited a lack of response (data not shown) which was also observed by Geiger et al. by employing a merocyanine viscosity-sensitive dye in a squaraine-cholesterol gel\(^{34}\). We can infer, therefore, that C1 does not effectively intercalate within the gelator fibrils and remains mostly in regions of the

\[ y = 0.187x + 13.962 \quad R^2 = 0.988 \]
gel network with a liquid-like viscosity ($\eta_{\text{calc}} = 2.2$ cSt; $\eta_{\text{lit. toluene}} = 0.55$ cSt). Thus to develop derivatives which can investigate not merely the viscosity of the liquid phase but also dynamically probe the gelator architecture, tailored C1 analogues were designed and synthesized to have a specific affinity to portions of the gelator assemblies (Figure 18).

Figure 18 Chemical structure and calibration curves at increasing viscosity of the molecular rotor Glu-C1 (a) and Oct-C1 (b).

Glucose and octanoic acid moieties, both of which comprise components of the gelator structure, were introduced onto C1 via azide-alkyne Huisgen cycloaddition without significantly altering its photophysical features to give rise to the two molecular rotors Glu-C1 and Oct-C1, respectively (Figure 18). Following the idea that the gelator components aggregate in a ‘like
assembles with like’ fashion, the glucose moiety and the octanoic acid chain were chosen in order to interact with the hydrophilic head and the hydrophobic tails of R8, respectively. Coarse grained and all-atom MD simulations strongly supported this molecular design confirming that such chemical modifications cause the viscosity-sensitive portions of the two probes to reside in two spatially distinct regions of the gelator fibril: either exposed towards the solvent (Glu-C1, Figure 19 a) or within the core (Oct-C1, Figure 19 b). As highlighted in Figure 19 b, the positioning of the bulky coumarin portion of Oct-C1 in correspondence of the sugar core causes partial perturbation of the H-bond network stabilizing successive sugar layers (Figure 15). At the same time, the probe forms labile H-bonds with adjacent hydroxyl groups through its lactone carbonyl and cyano group. Combined with Glu-C1, such a situation presents the unique opportunity to investigate not only changes in viscosity at the fibril-fibril interface but also architectural modifications of the fibril’s architecture.
Figure 19 a) Top view of Glu-C1 inserted within a gel fibril from a minimized all-atom MD snapshot; b) Top view of Z-s-cis Oct-C1 inserted within a gel fibril from a minimized all-atom MD snapshot; c) Fluorescence emission spectra of Glu-C1 acquired throughout the gelation process of 1% R8 in toluene; d) Fluorescence emission spectra of Oct-C1 acquired throughout the gelation process of 1% R8 in toluene.
Figure 20 The images (a-f) show the epitaxial growth of the gelator fibers over the course of gelation (6 min). Tubular structures are visible already after 30 sec (b). Between 4 and 6 min (e and f, respectively), the elongated fibers exhibit a torsion around their longitudinal axis that results in the formation of right-handed double-helices (f) (scale bar: 25 nm).

Solutions of R8 in toluene (1% w/w) were gelled in presence of the two probes, separately. Super-resolution fluorescence microscopy (STED) performed on doped gels clearly shows the uptake of the fluorescent probes into grown microscopic fibers (Figure 13). Also, fluorescence spectra were monitored over the course of gelation starting from the hot sol (~100 °C) to the gel at RT. Figure 19 c and d show the marked difference between the fluorescence emission spectra of Glu-C1 and Oct-C1, respectively. Representative of the successive stages of
gelation, the time-point curves correspond to the evolution of the helical fibrils starting from unassembled gelator molecules.

According to the computational model in Figure 19 a, the interaction of the glucose ring in Glu-C1 with the gelator’s sugar moieties causes its coumarin portion to project from the fibrils and be exposed to the toluene phase. Such a peculiar spatial arrangement along the R8 fibrils allows Glu-C1 to report on the change in local nano-viscosity occurring at the hydrophobic interface of the fibrils. Figure 20 shows time-transient AFM images of drop-cast 1% R8 gels obtained over the course of gelation (0-6 min). Starting from an apparently unassembled system (Figure 20 a), protofibrils begin to grow in tight contact with each other forming highly oriented bundles (Figure 20 b-e). This shows that the “velcro”-like longitudinal association among fibrils, captured by Glu-C1, and their epitaxial growth take place simultaneously, instead of constituting two distinct steps in the gelation process. Interestingly, after about 4 min the increase in fluorescence emission plateaus suggesting little response of the probe towards the helix formation observed after that time (Figure 20 e-f). Surprisingly, all normalized fluorescence intensities fall markedly outside of the linear range (data not shown) suggesting that the viscosity at the fibril-fibril interface reaches solid-like values arguably owing to the high level of interdigitation of R8 hydrophobic portions.
After minute 4, the red-shifted band (556 nm) gradually decreases in intensity along with the blue-shifted band peaking at 528 nm that instead becomes preponderant (Figure 19 d). We attributed this phenomenon to two co-existing conformers of **Oct-C1** and supported this hypothesis by recording fluorescence emission spectra at different temperatures in pure toluene in absence of **R8** gelator. Figure 22 shows that at RT the red-shifted band is the main one. However, at higher temperature the blue-shifted band surpasses the red-shifted one in intensity, clearly suggesting that there exists an energy barrier between the two putative species that can be overcome thermally. Interestingly, this conversion is completely reversible upon cooling.
Based on these observations we hypothesized that similarly to its parent compound C1, in these particular conditions also Oct-C1 exhibits two atropisomeric forms arising from the partially restricted rotation around the coumarin-styblene single bond (Figure 21 a). TD-DFT calculations (using 6-31+G*, cam-b3lyp, PCM toluene) confirmed that in toluene the ground state energy of the s-cis isomer is 6.2 kcal/mol lower than the s-trans one. Moreover, similar to what observed in both the gelation experiment (Figure 19 d) and the temperature-transient measurements (Figure 22), TD-DFT calculations also confirmed a $\Delta \lambda_{exc} \approx 30$ nm between the two isomers with the s-cis conformer’s emission being red-shifted. Moreover, RP-HPLC-MS analysis supports this evidence showing that Oct-C1 exists as two chemically distinguishable isomers having the same m/z value but clearly distinct retention times (Figure 22).

![Figure 22](image)

**Figure 22** Fluorescence spectra showing the variation of the s-cis/s-trans Oct-C1 ratio at different temperatures (left) (red: RT, green: 50 °C, blue: 100 °C). RP-HPLC-MS of Oct-C1 dissolved in ethanol performed on a C18 achiral HPLC column showing that the two atropisomers are chemically distinguishable (retention times are 13.57 min and 14.86 min) and have the same mass (576.3 m/z for the mono-deprotonated adduct) (right).

Interestingly, the fibrillar architecture greatly affects this equilibrium in a highly dynamic fashion: according to Figure 19 d, at the very initial phase (curve 1) the two atropisomers co-exist in a ~1:1 ratio; arguably, this ratio slightly differs from what is observed in pure toluene at high temperature (~1:1.3, s-cis:s-trans) in virtue of the presence of nascent R8 protofibrils in
solution. As the system cools down, the s-cis isomer becomes more and more abundant which is in line with the temperature-transient experiment (Figure 22). Concurrently, as Oct-C1 molecules get incorporated into elongating fibrils and the H-bond network of the latter becomes more compact, the probe experiences steric hindrance (i.e. higher local viscosity) which, in turn, induces an increased fluorescence emission (Figure 19 d, curves 1-2). In the second phase (Figure 19 d, curve 2-3), although the system is approaching RT, surprisingly, the emission from the more thermodynamically unstable s-trans isomer rises, indicating that the atropisomeric equilibrium is significantly shifted towards this form (final s-cis:s-trans ratio $\approx 1:1.3$). Remarkably, this situation is indefinitely stable at RT and diametrically opposite to what was found in pure toluene where the s-cis isomer is instead approximately 1.5 times more abundant (Figure 22, left panel). Furthermore, the transition observed spectroscopically takes place simultaneously with the formation of chiral structures evident via AFM (Figure 20 f and g) and CD (Figure 14 c).

![Figure 23](image.png)

**Figure 23** Potential of mean force for torsion around the dihedral describing Zs cis and Zs trans isomers.
To prove that it is an intrinsic H-bond rearrangement of the fibril’s sugar core that causes the fibers’ architectural change, we show via all-atom simulations that the H-bond interactions between the glucose hydroxyl groups and the coumarin portion are affected. In fact, the atropisomeric equilibrium results highly perturbed due to a significant stabilization of the s-trans isomer (Figure 23) through a three-H-bond-network established between the lactone carbonyl and the cyano group with two adjacent hydroxyl groups of two spatially close glucose rings (Figure 21 b). Based on these findings we can infer that the interactions stabilizing the s-trans isomer can be established only in the fully formed fiber owing to fine rearrangements in the local structure at the end of gelation.

4. DISCUSSION

We have demonstrated the application of two novel coumarin derivatives belonging to the class of molecular rotors for the investigation of the complex self-assembly mechanism of a low-molecular weight gelator. Our strategy consisted of tailoring the viscosity-sensitive core of the probe C1 with moieties that imparted both high chemical affinity with the stacked gelator molecules and preferential spatial localization within individual fibers. A particularly striking discovery was related to the observation of solid-like viscosity values at the interface of reversed fibrils where the hydrophobic tails reside. This finding yields important insights into the nature of the interfibrillar adhesive forces taking place within larger fibers. Also, owing to instantaneous fluorescent response upon environmental changes these probes allowed the monitoring the growth of the nanofibrils in real-time. We showed that the fibril twisting underlies a rearrangement of the H-bond network within the hydrophilic core. This was clearly
sensed by Oct-C1 that revealed how 3-styryl-coumarin’s intrinsic s-cis/s-trans atropisomeric equilibrium can serve as a tool to monitor subtle intermolecular interactions. Furthermore, computational simulations helped us to understand and interpret adequately both microscopic and spectroscopic results. This gave us the opportunity to clearly show for the first time that coumarin-based molecular rotors can be successfully employed in heterogeneous systems, such as molecular gels, by taking advantage of simple yet essential chemical modifications that render the probes prone to interact with the gel architecture. This approach could be easily extended to other classes of molecular rotors with the goal of studying self-assembly as well as biological systems more in detail.

5. ACKNOWLEDGEMENTS

Dr. Julian Silverman designed and synthesized the molecular gelator R8 and was directly involved in the testing and characterizing the molecular rotors in toluene R8 gels. We thank Dr. Ranieri Bizzarri and Dr. Giovanni Signore at Scuola Normale Superiore di Pisa for their guidance in designing and synthesizing the original molecular rotor C1 and performing the preliminary spectroscopic characterization. The computational studies have been performed by Dr. Riccardo Nifosí at University of Pisa and we deeply thank him for his remarkable contribution. We are also grateful to Dr. Tai-De Li and Dr. Tong Wang at the Advanced Science Research Center (ASRC) for their help with the imaging experiments and Dr. Lorenzo Di Bari for the useful discussions.

The manuscript associated to this work is under preparation.
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Chapter 3: Different ommochrome pigment mixtures enable sexually dimorphic Batesian mimicry in geographically isolated populations of the common palmfly, *Elymnias hypermnestra*

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1. INTRODUCTION

1.1 Pigmentation in butterfly wings

Butterflies are universally considered attractive because of their bright coloration patterns. The color patterns are due to a variety of numerous small scales, each with a distinct color, which together create the species-characteristic appearance\(^1\). The scale colors can have a structural and/or a pigmentary origin, depending on the scale anatomy and its pigmentation\(^2\) (Figure 1). Striking structural colors are widespread among butterflies, with Morpho butterflies being the most famous examples, but also many lycaenids and papilionids feature iridescent colors due to intricate photonic systems\(^3\,4\). The scale elements creating structural colors can be almost perfect thin films\(^5\), (perforated) multilayers in the scale lumen, multilayers in the scale ridges\(^6,7\).

The pigments contributing to coloration vary among the butterfly families. The black scales of all butterfly families contain melanin. White scales may be presumed to be unpigmented, but the white scales of pierids contain leucopterin, a purely UV-absorbing pigment, whereas the yellow, orange and red scales of pierids contain the violet- and blue-absorbing xanthopterin and/or erythropterin\(^8\). The pigments of the colored scales of papilionids contain another pigment class, the papiliochromes, derived from the precursor kynurenine\(^9\), which is also used by nymphalids to produce 3-hydroxy-kynurenine (3-OH-kynurenine) and various ommochromes, pigments that predominantly determine their wing coloration\(^10,11\). The wing scales of most nymphalids are essentially flattened sacs; in each, the lower lamina is connected by a series of pillars (the trabeculae) to the upper lamina, which consists of the ridges
and crossribs\textsuperscript{6} (Figure 1). Light reflected by these components together with the wavelength-selective absorption by the scale’s pigment determines the scale color.

**Figure 1** a) Photograph of the dorsal forewing of a *Graphium sarpedon* (scale bar: 2 mm); b) Magnification of the previous area showing aligned microscopic green scales (scale bar: 150 μm); c) SEM image of individual scales (scale bar: 40 μm); d) Further magnification of the previous area showing a ladder-like structure (scale bar: 5 μm)\textsuperscript{7}.

On the other hand, ommochromes are synthesized in a complex metabolic pathway that transforms the amino acid tryptophan into red, orange, or yellow pigments with the aid of several enzymes and transporter molecules\textsuperscript{12,13}. Ommochromes are ubiquitous as filtering pigments in insect eyes, but their deployment as wing pigments is known only from the Nymphalidae\textsuperscript{1,12,14}. 
This dual function of ommochromes in some Nymphalidae might explain some of the group’s phenotypic diversity. Runaway sexual selection is posited to lead to elaborate sexually selected traits, and results when there is tight linkage or pleiotropy between genes responsible for the trait and behavioral preference for it in the opposite sex\textsuperscript{15}. Polymorphic \textit{Heliconius} butterflies mate assortatively, and genetic mapping has demonstrated tight linkage between genes for wing patterns and mate preference\textsuperscript{16}. Similarly, the fact that ommochromes function as wing and eye pigments may indicate a functional link\textsuperscript{14}, and provide the necessary basis for runaway selection to have contributed to elaborate pattern variation in butterflies.

\textbf{1.2 Batesian mimicry in butterflies}

Insects comprise more than half of all described species\textsuperscript{17}, and—with over 150,000 described species—butterflies and moths (Order Lepidoptera) are a major component of insect diversity\textsuperscript{18}. The group is distinguished by scale-covered wings, and it is speculated that these detachable scales evolved to enable the insects to escape from spider webs\textsuperscript{19}. However, the colors and patterns of scales on the wings of most day-flying species have evolved other functions for inter- and intraspecific communication including crypsis (cryptic coloration similar to camouflage), aposematism (warning coloration), sexual signaling, and mimicry\textsuperscript{20,21}.

Mimicry in butterflies has received much attention, as it provides a clear and easily understood example of natural selection, and has been recognized as such for over a century\textsuperscript{20,22}. Mimicry can be manifested in many ways\textsuperscript{23}. Perhaps the simplest categorization discriminates Batesian mimicry, in which a palatable mimic resembles an unpalatable model\textsuperscript{24}, and Müllerian mimicry, in which two or more unpalatable or otherwise noxious species converge on a similar phenotype\textsuperscript{25}. Within a given area, predators learn to associate unpleasant taste with particular
visual phenotypes. Batesian mimics enjoy reduced predation from being mistaken for a noxious species that predators learn to avoid, while Müllerian mimics benefit from sharing the cost among different species of “educating” predators on the association between the shared phenotype and its unpalatability \(^{20}\). Mimetic species can often be distinguished from their models by human observers (Figure 2). Yet, lower visual acuity of the predators may favor the mimetic species in succeeding \(^{20}\). Also, this strategy is aided by blur caused by beating wings, and a more subtle mimicry such as behavioral mimicry in which the mimetic species imitates the height, speed, and other characteristics of the model’s flight \(^{26,27}\).

As mentioned earlier, mimetic and other types of visual signals in butterflies are made possible by structural color and pigments of wing scales. Glittering blue butterflies in the genus *Morpho* (Nymphalidae: Morphinae) are a frequently cited example of structural color \(^4\). Most butterfly wing colors are due to pigments, and the vast majority of butterfly wing pigments are either melanins, flavonoids, pterins, and ommochromes \(^1\), though other compounds may be associated to coloration in specific populations \(^{28,29}\).

### 1.3 The Batesian mimetic species *Elymnias hypermnestra*

The genetic, genomic, and developmental basis of butterfly wing patterns are the subject of much contemporary research \(^{11,30-34}\). Much of this work focuses on 1) identifying genomic regions that produce wing pigments; or 2) spatial patterns of gene expression in developing wings that result in the mosaic of colors that cause an individual’s wing pattern. However, the chemical identity of wing pigments has received less attention. Although there are ca. 18,500 species of butterflies (Gerardo Lamas, unpublished data), which constitute the majority of day-flying Lepidoptera, wing pigments from a limited number of butterfly species
have been characterized chemically\textsuperscript{1,35}. Most studies that investigate ommochrome pigments in butterflies focus on a small number of species with pigments that were characterized years ago\textsuperscript{10,36,37}. Few studies try to explore more in detail the complexity of butterfly diversity to look for novel pigments or investigate whether putatively ancestral developmental pathways are dormant and re-activated over the course of evolutionary history.

We investigated the chemical identity of orange ommochrome wing pigments in female 
*Elymnias hypermnestra* butterflies (Lepidoptera: Nymphalidae: Satyrinae), a Batesian mimetic species that is facultatively sexually dimorphic (that is, a condition where the two genders of the
same species display different characteristics beyond the differences in their sexual organs). This is the most widely distributed species in the genus *Elymnias*, and its ca. 23 subspecies range throughout most of South and Southeast Asia\(^3\). The larvae feed on a variety of palm species Arecaceae; \(^3\), and adults of *E. hypermnestra hainana* are readily targeted by birds (S.-H. Yen, pers. comm.), indicating that the species does not seem to acquire or produce any harmful defensive chemicals that might render them unpalatable to predators. However, males and females of the species are protected by different unpalatable model species that predators learn to avoid. The wings of every male *E. hypermnestra* subspecies have a dark brown/black ground color with a bluish apical band on the forewing; some subspecies have light brown marginal bands on the hindwings. This phenotype resembles several species of *Euploea* butterfly (Nymphalidae: Danainae) including *Eu. tulliolus* and *Eu. eunice*\(^3,4\).
Wing patterns of female *E. hypermnestra* are variable. In eastern Indochina, peninsular Malaysia, Borneo, Sumatra, and the Lesser Sundas east of Wallace’s Line, females resemble males, and populations are more or less monomorphic (namely no difference between the two sexes). In South Asia, western Indochina, Java, Bali, Seram, and Buru, females have orange wings rimmed with black and a white subapical band on the forewings, which closely resemble wing patterns of the unpalatable species *Danaus chrysippus* and *D. genutia* (Nymphalidae: Danainae)

(Figure 3). Interestingly, the distribution of *E. hypermnestra* on the territory is partially shared with putative model species of *Euploea* and *Danaus* (Figure 3). Noxious defensive chemicals are obtained by larvae of these *Euploea* and *Danaus* species when they feed on their...
host plants in the families Apocynaceae and Moraceae\cite{42,43}. These compounds provide protection from predators throughout the larval, pupal, and adult stages, and the distinctive color patterns that advertise their unpalatability are copied by a variety of Müllerian and Batesian mimics in several butterfly and moth families, including other *Elymnias*\cite{22,39,44}.

It is unclear whether orange *E. hypermnestra* females from geographically unrelated populations (Thailand and Indonesia) are more closely related to each other than to the monomorphic populations that are present in intermediate regions. It is also uncertain whether their orange coloration is imparted by the same compound or suite of compounds. Since the shade of orange on wings of specimens from Thai and Balinese populations differs slightly, we hypothesized that different pigments are responsible for wing coloration in the two populations. Any differences detected would suggest local adaptation to different models and—in line with other data—might implicate Batesian mimicry as a powerful force for generating biochemical novelty.

2. MATERIALS AND METHODS

2.1 Specimen Acquisition and Pigment Extraction

Adult female *Elymnias hypermnestra baliensis* specimens were obtained from Bali, Indonesia, and adult female *Elymnias hypermnestra tinctoria* specimens were obtained from Chiang Mai, Thailand. Two extracts were made: one from pooled wings of 10 female *E. h. baliensis* specimens (4 wings per butterfly, 40 wings per extraction), and another from 4 female *E. h. tinctoria* specimens (we were unable to obtain 10 females of this subspecies). The orange regions of all wings (both forewings and both hindwings; Fig. 1) were excised with scissors and macerated in a single 1.5 ml tube with 500μL of 70% methanol in water before being ground...
with a plastic pestle. Each sample mixture was then sonicated for 15 minutes using an ultrasonicator (Brenson 5510), vortexed at maximum speed for 2 min, and centrifuged for 5 min at 8000 rpm. The supernatant was discarded and the tissue was dried under a gentle flow of nitrogen. The wing tissue was then placed in a solution of 0.1% HCl_{Conc} in methanol (v/v), and ground using a pestle. The sample was sonicated, vortexed, and centrifuged as before. The supernatant was then recovered and filtered on a PES microfilter (EMD Millipore, pore size 0.22 μm). The extract, which was dark brown, was stored at 4 °C until analysis. An initial, unsuccessful attempt to extract omochromes followed the protocol of Nijhout \(^{10}\) and did not include ultrasonication and vortexing; we found these steps were necessary for successful pigment extraction.

2.2 HPLC-MS Analyses

Samples were analyzed on an Agilent 1290 Infinity high performance liquid chromatography system coupled to an Agilent 6550 Q-ToF (Time-of-Flight) mass spectrometer using an Agilent Poroshell 120 SB-C18 column (2.7μm, 2.1x50mm) at 45 degrees °C and a linear gradient of 5-95% methanol in water (0.1% formic acid) with a flow rate of 0.4 mL/min. MS analysis were run with the following parameters: VCap = 3500 V, Gas temp = 250 °C. MS/MS analyses were run with CE = 30 V.
3. RESULTS

3.1 LC-MS/MS analysis on the butterfly wing extracts

We identified six ommochrome pigments in *E. h. tinctoria* and five in *E. h. baliensis*; only two compounds were found in the wings of both subspecies. With the exception of xanthommatin, which was found only in *E. h. tinctoria*, none of the compounds have apparently been identified before. The differences in composition were apparent in the LC-MS chemical profiles of wing pigments from the two subspecies (Figure 4). Detection and resolution of the orange pigments was made possible by employing a UV-Vis detector set to 450 nm along with a high-resolution mass spectrometer (HRMS).

![Chromatograms of pigment mixtures extracted from orange wings of female E. h. tinctoria (upper), and E. h. baliensis (lower). Letters (b-j) refer to ommochrome compounds identified from each extract (Figure 5). Peak f and g are shared by both butterfly populations.](image)

**Figure 4** Chromatograms of pigment mixtures extracted from orange wings of female *E. h. tinctoria* (upper), and *E. h. baliensis* (lower). Letters (b-j) refer to ommochrome compounds identified from each extract (Figure 5). Peak f and g are shared by both butterfly populations.
We expected to find pigment molecules that shared the tetracyclic heteroaromatic ommochrome core structure 4H-7-oxa-4,12-diaza-benzo[a]anthracene-1,5-dione (Fig. 4a), which is biosynthesized using tryptophan as a precursor. Our interpretation of each compound’s mass spectrum reasonably assumes that its structure includes this core. To elucidate the structures in detail, we took advantage of HRMS to determine empirical formulae and predict putative structures and MS/MS to gain more detailed structural information.

We isolated and characterized six different pigments in *E. h. tinctoria* (Figure 4 and Figure 5), including xanthommatin (Figure 5c). The mass spectrum of peak b (Figure 4) has a molecular ion ([M+H]$^+$) of 380.0875 Da that corresponds to the molecular formula C$_{19}$H$_{13}$N$_3$O$_6$. The MS/MS spectrum yields evidence for the presence of an alanine residue (fragment 307.0708 m/z; see SI) that—combined with the inferred molecular formula—suggests a structure lacking the carboxylic acid moiety in position 11 (Figure 5b). This newly discovered pigment was named tinctoriommatin, after the subspecific name of the butterfly population from which the compound was isolated.

The mass and the corresponding empirical formula for peak c (Figure 4) perfectly match with that of xanthommatin. With a retention time of 3.6 min, the corresponding mass spectrum has the fragments 317.1 m/z (loss of the side chain in position 11 plus neutral H$_2$O loss) and 307.1 m/z (loss of the side chain in position 11 only; see SI). These data are consistent with the structure of xanthommatin (Figure 5c). Interestingly, the mass relative to peak f (retention time 4.7 min; Figure 4) is the same as xanthommatin. MS/MS analysis revealed that it corresponds with high confidence to an isomeric form having of an alanine residue attached to the carboxylic acid in position 3 via an amide bond (Figure 5f). Also, the keto-amino acid portion in position 11
seems to have undergone oxidative cleavage, giving rise to a carboxylic acid substituent. The fragments 305.0437 and 317.0549 in the MS/MS spectra (see SI) are clearly the result of the combined loss of the alanine portion via the labile amide cleavage in two different positions and either CO₂ or H₂O. Since this compound was found in both populations of *Elymnias hypermnestra* that we examined, we named it elyynniommatin.

![Chemical Structures](image)

**Figure 5** Hypothesized chemical structures and trivial names of ommochrome pigment compounds isolated from the wings of orange female *Elymnias hypermnestra*; all compounds share a common 4-ring core (a); modifications to this core are indicated in pink. Six compounds were isolated from *E. h. tinctoria* in Thailand (b-g) and five were isolated from *E. h. baliensis* from Bali, Indonesia (f-j). Two compounds (f-g) were found in both populations. With the exception of xanthommatin (c), all compounds were characterized for the first time in this study.
The mass of peak g (C_{21}H_{15}N_{3}O_{8}; Figure 4) differs from xanthommatin by only 14 Da (+CH_{2}). The only structural modification that would be biosynthetically reasonable and would not affect the spectroscopic properties significantly involves methylation of the aliphatic carboxylic acid (Figure 5g,) giving rise to a methyl ester moiety. The fragment [M-MeOH]^{+} with m/z = 407.0501 supports this proposed structure (see SI). We named this compound methylxanthommatin; it was isolated from both populations.

Peak d (Figure 4) has a mass of 453.09 Da with the corresponding empirical formula C_{22}H_{16}N_{2}O_{9}. A thorough analysis of the fragments obtained via MS/MS supports the hypothesis that the molecule is the doubly methylated version of xanthommatin (Figure 5d) with the substitution of the amino group in alpha position with a hydroxyl radical, leading us to name this compound dimethyl hydroxyxanthommatin. We observed the neutral H_{2}O loss from the hydroxyl group in alpha position (435.1 m/z) in its MS/MS spectrum, the loss of the fragment -COOCH_{3} (375.1 m/z), and the mass 304.0 m/z arising from the loss of the latter fragment along with part of the side chain (see Supplementary Information (SI)).

The MS/MS spectrum of peak e (Figure 4) shows a neutral H_{2}O loss fragment (349.04 m/z) along with another intense fragment with mass 289.02 m/z that can be associated to the loss of CO_{2}+MeOH (M-76.1 m/z, see SI). By combining these findings with its empirical formula C_{18}H_{10}N_{2}O_{7}, we propose a pigment containing a methyl carboxylate in position 11 and a carboxylic acid in position 3 (Figure 5e). We named this compound somommatin after the Thai word for orange: sôm.
The orange-brown pigment fraction of *E. h. baliensis* contained five distinct compounds (Figure 4) with masses and empirical formulae that were consistent with xanthommatin derivatives. Interestingly, we did not detect xanthommatin itself, but variously substituted xanthommatin-derived molecules, two of which were also found in *E. h. tinctoria* wing extracts: elymniommatin and methylxanthommatin (Figure 5f,g).

With a formula of \( \text{C}_{19}\text{H}_{13}\text{N}_{3}\text{O}_{6} \) and \([\text{M+H}]^{+} = 380.0865\), peak h (Figure 4) appears to be an analog having the keto-amino acid portion in position 11 and lacking the carboxylic group in position 3 (Figure 5h). Accordingly, the fragment with mass 291.0 m/z corresponds to the loss of the aminoacidic portion, whereas the fragment 363.1 and 334.1 m/z arise from \( \text{H}_{2}\text{O} \) and \( \text{CO}_{2} \) loss, respectively (see SI). We named this pigment baliommatin after the island on which this subspecies is found, Bali, which is also the etymological root of the subspecific name of *E. hypermnestra baliensis*.

Peak i (Figure 4) is the lightest of the detected pigments with \([\text{M+H}]^{+} = 353.0402\) m/z and having the formula \( \text{C}_{17}\text{H}_{8}\text{N}_{2}\text{O}_{7} \). Based on this information, we proposed that the compound’s substituents are merely two carboxylic acid groups in position 3 and 11 (Figure 5i). The MS/MS spectrum provides little structural information as expected due to the high stability of the chromophore core but it presents two significant fragments, namely 307.0331 m/z and 289.0231 m/z, which correspond to \([\text{M-CO}_{2}]^{+}\) and \([\text{M-CO}_{2}\text{-H}_{2}\text{O}]^{+}\) (see SI). These support the presence of two carboxylic acid groups and exclude the possibility of more complex substituents on the central core. We named this pigment oranyeommatin after the Balinese and Bahasa Indonesian word for orange: oranye.
Related to methylxanthommatin is the structure of the compound in peak j (Figure 4), which has the formula C$_{21}$H$_{14}$N$_2$O$_9$. Compared to compound methylxanthommatin, the empirical formula for this molecule differs in having one additional oxygen atom and one less nitrogen atom (Figure 5j). Assuming that the chromophore core is not affected, we hypothesize that the amino acidic portion underwent a deamination reaction that introduced a hydroxyl group in the alpha position. Accordingly, a fragment with m/z = 307.0712 arises from the cleavage of the aromatic carbon-carbonyl carbon bond and the loss of the whole side chain (see SI). We called this compound methyl hydroxyxanthommatin.

4. DISCUSSION

The presence of different ommochrome mixtures pigmenting the orange wings of females from distinct Elymnias hypermnestra-populated sites suggests differential gene expression or independent evolution in these two geographically separated populations. If the two orange populations are sister to each other (i.e., if they share a most recent common ancestor with orange females), then the presence of different arrays of pigment molecules might result from different selective conditions in the tropical (Bali) and subtropical (Thailand) habitats where these organisms are found. For example, different populations of model species may differ in hue or in UV reflectance, and these Elymnias mimics may be adapting to local variation in their models. Alternatively, different host plant species or other factors during larval development may trigger different development pathways resulting in biosynthesis of different pigment molecules.
However, if each of these populations with orange females is more closely related to monomorphic populations with dark females—as suggested by preliminary molecular phylogenetic work on this species—then orange pigmentation may have evolved twice. It is highly unlikely that the entire biosynthetic pathway evolved de novo more than once, but there are several alternatives that would explain this finding. A mutation might silence the ommochrome pathway in populations with dark females, and a previously silent mutation might have restored it in populations with orange females. Another possibility is that orange pigmentation is still produced in dark females but is masked by melanin-derived pigments. However, the presence of unique compounds in each population suggests that evolutionary elaboration of a core developmental pathway may have occurred in one or both of these populations.

These results suggest that visually similar mimetic phenotypes have a different biochemical basis, likely resulting from disconnected evolutionary processes in geographically separated populations. The large number of novel compounds that we describe suggests that there may be much pigment diversity waiting to be discovered in other mimetic butterflies. We speculate that Batesian mimics unrelated to their model species are more likely to yield other novel pigments. This is because their phenotypic similarity to model species almost certainly results from evolutionary convergence rather than recent common ancestry as has been documented in *Heliconius* Müllerian mimics  45.

This novel comparative study opens up new questions related to pigment distribution and phenotypic adaptation. It would be interesting to determine whether orange pigments are present in the wings of males and/or females in “monomorphic” populations, but covered by melanin
pigments. Similarly, we could investigate if the putative models *Danaus genutia* and *D. chrysippus* produce the same orange pigments as *E. hypermnestra* females.

The discovery of xanthurenic acid in *Junonia coenia* butterfly wings prompted Daniels and Reed\(^{46}\) to conclude that the ommochrome synthesis pathway in butterflies must diverge from the model developed for *Drosophila* eyes by Linzen\(^{12}\), which could not accommodate synthesis of that molecule. Similarly, our findings prompt novel hypotheses for the biosynthetic pathways capable for generating these molecules.

We propose two main biosynthetic routes for the novel pigments found in this study. In the first (Figure 6a) the putative molecular scaffold is represented by oranyeommatin that bears two carboxylic acid moieties as side chains. This pigment is likely to derive from an oxidative condensation between anthranilic acid and 7-amino xanthurenic acid. While somommatin is the fruit of a monomethylation reaction, both elymniommatin and tinctoriommatin contain an alanine residue linked to the chromophoric core via an amide bond. Interestingly, along with anthranilic acid, alanine results from the oxidative cleavage of kynurenine (Figure 6). We speculate that during the progression of wing pigmentation, this amino acid is first produced via kynurenine’s degradation and is then reincorporated into more complex molecules, therefore contributing to the wide array of pigments that characterize *Elymnias hypermnestra*.

In the second biosynthetic route (Figure 6b), both the keto-aminoacidic side chain of xanthommatin and its carboxylic acid undergo a series of modifications. The loss of the aromatic carboxylic acid gives rise to baliommatin. Methyl esterification of the aliphatic carboxylic acid leads to methylxanthommatin. Upon its oxidative deamination that generates a putative alfa-keto derivative and its successive regioselective reduction to hydroxyl group, methyl
hydroxyxanthommatin is formed. Finally, a second methylation of the remaining carboxylic acid group yields dimethyl hydroxyxanthommatin.

Figure 6 Hypothesized biochemical pathways for synthesis of the ommochrome compounds identified in this study
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The manuscript associated to this work is under preparation.
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7. SUPPLEMENTARY INFORMATION

Peak b - Xanthommatin

Peak c - Tinctoriommatin

Peak d - Somommatin