Article

# In Search of Biological Activity

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There are many reasons why a chemist might become interested in a new molecule - it may present an opportunity to develop or apply new synthetic methodology, give new insights into fundamental aspects of structure, or it may have a valuable function. Of these functions, one of the most quoted is *biological activity*. Yet for all the interest in biological activity, it is a poorly understood and highly complex concept. Discussed below are some of the ways of viewing biological activity at the cellular level, and an exploration of some of the methodologies now available to determine the underlying biological mechanisms involved.

## An introduction to biological activity

A living cell contains an intricate set of interacting pathways. In a highly simplified view, at its centre is a nucleus which contains the nucleic acid DNA that carries information coded in the form of a sequence of the nitrogen-rich bases adenine, cytosine, guanine and thymine. This stored information encodes for the production of proteins. All cells in the human body contain the same DNA but those in the brain are distinguished from those in the toes by the parts of the DNA information utilized for the production of proteins. Proteins are produced through a process that starts with copying the relevant coding section of DNA into a related nucleic acid, RNA. This RNA copy initially carries sections present in the original DNA sequence that are interruptions of the protein coding sequence; these non-coding regions are excised and the RNA is spliced together to create a molecule which carries only protein coding sequence. The RNA is then checked for obvious errors and then used as a template for protein production. By controlling the sections of DNA that are copied into RNA, and the rate at which the RNA molecules are used for the production of proteins, a cell can take on the form required by its environment. It can also respond to changes in that environment, which can include treatment with a biologically active molecule.

The biological activity of a compound reported in the chemical literature is commonly measured by one of three methods – ability to inhibit an isolated protein target, a gross impact on the growth of cells in culture, or the impact on a disease, *e.g.* anticancer, antibiotic, immunosuppressant. Each of these provides a valid and useful way to understand how a small molecule interacts with a biological system, but the bigger picture can be so much more interesting and open up whole new avenues for research.

Determining the activity of a compound against a single, isolated protein target is an important way of comparing a series of compounds. Generally, it can be undertaken in high throughput on large compound sets and, in the hands of a medicinal chemist, can be used to guide the production of new compounds with improved properties through structure-activity relationship analysis. Measuring activity in this way has been the backbone of many research and development programmes. However, it presupposes that the functional target of the molecule is known, and that it is the only significant target. What is becoming clearer as more and more detailed studies into the mode of action are undertaken is that many compounds, potentially the majority of compounds with biological activity, have a set of biological targets through which they function, and not the ideal single target.

For example, it is well known that caffeine (**1**; Chart 1) is a psychostimulant that it exerts its effect through adenosine receptors. However, the way this effect is mediated by different adenosine receptor subtypes, or dimers of receptor subtypes, is still a matter of investigation.[1] The biological activity of caffeine does not stop there, however, but impacts on cellular processes including cell growth and DNA synthesis. In one of the more eloquent descriptions of a compound having multiple activities through disparate targets, Kaufmann *et al*. note *Caffeine occupies an important niche in the cell cycle checkpoint field. Not only does it help bleary-eyed scientists concentrate on their experiments, it directly inhibits the checkpoint kinases, ATM and ATR*.[2] Whilst this discovery may sound of only esoteric interest, such action of caffeine allows a cell to progress through its cycle while carrying damaged DNA; it avoids the checkpoint in which the damage would be repaired or the cell committed to death. This checkpoint is designed not only to ensure that harmful DNA mutations are not propagated into future generations of cells, but also it aids in providing resistance to tumours from radiotherapy and DNA damaging therapeutic agents. Thus, caffeine could be seen as sensitising tumours towards a number of therapeutic interventions. However, the concentration of caffeine required to inhibit the activity of its checkpoint targets is *ca*. 500-fold higher than that which gives its psychostimulatory effects on the adenosine receptors. Kaufmann's team can therefore use caffeine to help them stay awake to the wee small hours (studying checkpoint kinases) without putting themselves at risk of acquisition of DNA mutation.

Whilst discussing caffeine, it is clear that only one particular target set, the adenosine receptors, are the realistic targets in the body. For other molecules this is not so clear. Many clinical agents, even though ascribed as having the same mode of action through a common primary target, have discernible *flavours* of activity through secondary targets. This is exemplified by the anti-leukemic compound imatinib (**2**; Chart 1) [Gleevec® (Glivec® Europe/Australia) as its mesylate salt] and its analogues. A number of these drugs were specifically developed as imatinib analogues and yet each compound has its unique range of targets. This includes one analogue that binds to

over 30 different kinases and another with a functional target from a quite unrelated set of proteins.[3]

Caffeine and imatinib are but two examples from many that illustrate the growing literature on the promiscuity of drug-like compounds. The human genome is believed to contain approximately 25,000 genes that hold the information which becomes translated into proteins. However, the RNA copy of the coding DNA is commonly manipulated by the cell on the way to the production of proteins, allowing a gene to code for multiple protein variants from the same gene. Following that, the proteins can undergo different modifications that alter their functions. The take home message from this is clear: A human cell has the capacity to produce close to 100,000 different proteins, and many-fold more functionally distinct variants beyond that once the amino acid backbone becomes decorated with sugars, lipids, phosphates and other moieties. It is little surprising that, when offered this panoply of interesting targets, a drug will find more than one with which it can interact. Indeed, it would be astounding should a drug *only* find one target.

Thus, defining biological activity becomes a difficult task. It is necessary to identify how many proteins your molecule can interact with, and which of those interactions are truly responsible for any observed activity. This understanding also confounds the traditional medicinal chemistry approach to structure activity analysis since, in developing an analogue of your lead compound to improve its interaction with one target, its interactions with the potential remaining 99,999 proteins will also be changing - and the chance that your new compound will promiscuously acquire a new target becomes a significant probability.

## How to determine biologically relevant targets

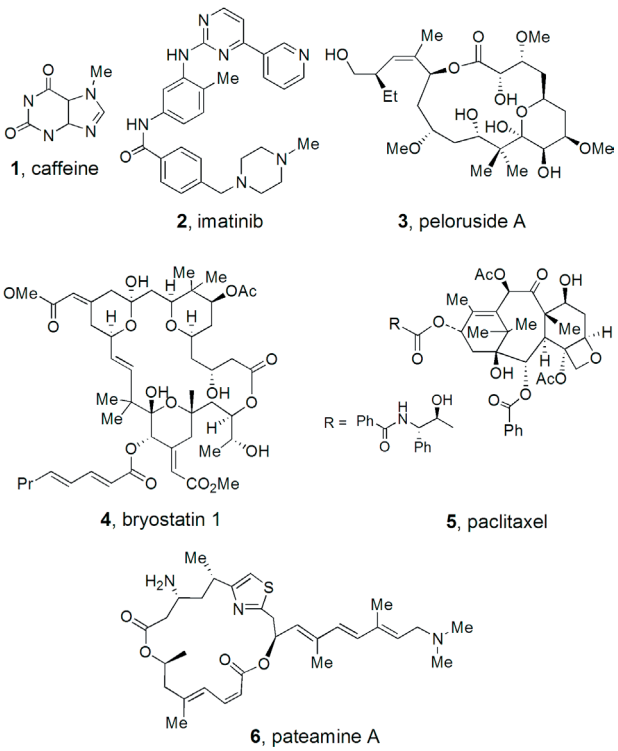
Unfortunately there is no single technique that can be used to reveal the target(s) of a biologically active compound. Probing biological activity is rather more like playing Cluedo. In this classic board game, players have to identify how Dr Black was killed, based on a set of six murder weapons, nine rooms, and six possible perpetrators. Cellular Cluedo is far more interesting! Whilst we know the *who* (our biologically active molecule), we do not know the *how* (which set of the ~100,000 proteins) or the *where* (which cellular compartment the drug reached). Consequently, there are far more possibilities in Cellular Cluedo than the 324 provided by the board game, and the questions asked have to be carefully planned. Even then, the evidence might be circumstantial only.

Two compounds, peloruside and pateamine, serve here to illustrate some of the methods available to identify cellular targets. These compounds come from the laboratory of the marine natural products group at Victoria University, led by Peter Northcote.

### *Peloruside A – serendipity in target discovery*

Peloruside A (**3**; Chart 1) was isolated by Linden West in Peter Northcote's laboratory, and first reported in 2000.[4] Aside from its interesting and novel structure, early work

***Chart 1***




revealed that it was a highly potent toxin to mammalian cells, leading to cell death at low nanomolar concentrations.

In itself, this toxicity was insufficient to make **3** an interesting molecule as there are many toxins in the world that are every bit as lethal. John Miller and then PhD student, Kylie Hood, set about defining the mode of action of **3** at Victoria's School of Biological Sciences. Whilst a daunting task, structural analysis suggested a similarity between **3** and bryostatin (**4**; Chart 1), a compound with a well-characterized mode of action. After more than a year of careful work, Kylie conclusively demonstrated that **3** and **4** did *not* share the same target.[5] Thus, one target down, 99,999 to go! Good luck then came to Kylie's aid as she stumbled upon a picture of cells treated with paclitaxel (Taxol®, **5**) and noticed similarities between those cells and the cells she had treated with **3**. She then showed that, despite no common structural features, peloruside (**3**), like **5**, stabilizes microtubules.[6] A microtubule is a dynamic assembly of tubulin proteins, which forms a rigid rod that a cell uses during cell division to help pull copies of DNA apart. Many well-established anti-cancer drugs, including **5**, interfere with microtubules.

The serendipitous discovery of a functional target of **3** is but part of the story. Peloruside only has a future as a drug if it has a point of difference from existing drugs that bind to tubulin. In 2004 we were able to show that **3** has a binding site on tubulin that is different from paclitaxel,[7] and thus peloruside can be used synergistically with paclitaxel.[8]

The story of **3** serves to demonstrate that even when the target is known, there is more to discover about the biological activity of a compound; the location of the binding site and interaction with the cells' systems for dealing with foreign substances are important. There is one fur-

ther twist to this story. In work soon to be published, Anja Wilmes (with Miller and Jordan) has used proteomics (the study of the proteins produced by a cell under specific conditions) to investigate further the difference between peloruside and paclitaxel. While a human cell may have the potential to produce up to 100,000 different proteins, a particular cell only needs to produce a subset of these at any one time; this is referred to as its proteome. By looking at the proteome of cells with and without drug treatment, one can see which proteins the cell is calling upon to respond to the drug. Many of these proteins will be directly related to the drug's mode of action, *e.g*. many of the responding proteins in the experiment with **3** interact with tubulin. However, even though **3** and **5** appear to share a common mode of action (stabilising of microtubules) the proteome responses to the two compounds have relatively little in common. Our working hypothesis is that the differences point to as yet uncharacterized targets of either peloruside or paclitaxel.

### *Planning target discovery*

As the discovery of the target of **3** was serendipitous, it seemed reasonable to develop and exploit more systematic tools for such discovery. Four questions need addressing in order to be confident that the mode of action of a compound has been identified:

1. What characterizes the cellular response to the compound?
2. What cellular components *can* it interact with?
3. Which of these components does it interact with *functionally*?
4. Is it possible to validate the link between the proposed target and the cellular response?

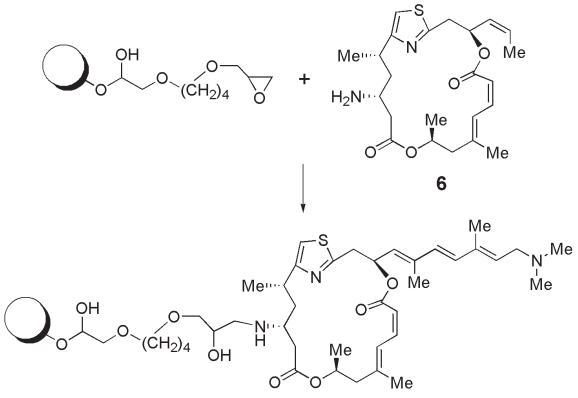
### *Pateamine – target discovery by design*

Pateamine A (**6**; Chart 1) is found within the same species of sponge that produces peloruside (**3**), and was first reported[9] from the Blunt and Munro laboratory in 1991. Like **3**, **6** is a potent toxin and a suggestion soon arose that pateamine had immunosuppressive activity.[10] Romo initiated a study probing the structure activity relationships of **6** and its analogues, but noted *Further analysis and understanding of these results in regard to their relevance for protein ligand interactions must await structural characterization of the interactions of the putative cellular protein receptor(s) with these PatA derivatives*.[11]

Although Romo's group remained interested in **6** for several years, it attracted little interest elsewhere, being one amongst a myriad of known toxins. The lack of a characterized target, the complexity of Romo's structure activity data, and its availability within our laboratories made **6** an ideal candidate to use in building a repertoire of target discovery skills. What follows stems from talented student, James Matthews who considered the questions proposed above. It shows how answers to these questions assisted in defining the biological activity of **6**.

**1. What characterizes the cellular response to 6?** - It has a number of biological activities associated with it as it is antiviral, antifungal and immunosuppressive. The key to these activities is its potent toxicity; it induces death in a number of cell lines at concentration in the very low nM range.[9,12] We have confirmed the relationship between the toxicity of **6** and its proposed mode of action, protein synthesis inhibition. If tritiated amino acids are introduced into the broth in which cells are grown, the radioactivity is incorporated into proteins. When cells are provided with both **6** and a tritiated amino acid, radioactivity is ***not*** incorporated; protein biosynthesis is stopped. This inhibition ***precedes*** the processes of cell death indicating that loss of protein biosynthesis is the cause, and not a consequence, of cell death.

**2. What cellular components can 6 interact with?** - Unpublished work within the Northcote group and Romo's structure activity analysis[11] both indicated that the $-NH_2$ moiety of **6** could be derivatized with little impact on global activity and provided a handle for chemical modification. Under mild basic conditions, **6** is alkylated with an epoxy-terminated agarose gel (Scheme 1). Cells, grown in culture were lysed (burst open) to provide a solution rich in cellular proteins, although lacking proteins remaining associated with cell membranes. This lysate was passed through agarose-immobilized **6** and led to the proteins that bind **6** being preferentially retained by the column. These proteins were subsequently eluted and their identity determined (MS methods) as actin, β-tubulin, and the charmingly named eukaryotic initiation factor 4A (eIF4A).




***Scheme 1***. Generating an affinity matrix with pateamine (**6**).

Using affinity chromatography to identify a target in this way has three weaknesses. Firstly, where the target is a membrane-bound protein it would not be isolated. Secondly, functionally-relevant binding proteins might be swamped by other proteins present in high concentrations within cells, and thirdly, the very act of derivatizing the compound may change its affinity for its cellular target. Two of these possibilities were evident from our results. No membrane associated proteins had been found and both actin and tubulin are present in very large amounts in cells. Realising the chance of important target information being lost, the functionally of **6** was tested for interaction with any of these three proteins.

**3. Which of these components does 6 interact with functionally?** - Actin and tubulin are important proteins in cells and contribute to the cytoskeleton - the protein filaments which give a cell its structure. To provide these filaments, monomeric actin or tubulin proteins assemble

into long fibres to give actin filaments or microtubules, respectively. Drugs that interact functionally with actin or tubulin generally change the position of the equilibrium between the protein monomers and filament/microtubule. Tubulin polymerization assays show that **6** has essentially no impact on microtubule formation or dissociation, even at concentrations many thousand fold in excess of those that lead to cell death. Also **6** is able to perturb actin polymerization, but again only at very high concentrations, albeit exhibiting a greater effect at lower concentrations than required for tubulin.

The third putative target, eIF4A, proved interesting. Collaboration Pelletier (McGill University) showed that **6** perturbs the function of eIF4A proteins at much lower concentration than was required for an effect with actin or tubulin.[13] Interestingly, **6** *stimulates* the activity of eIF4A in isolated systems. It is not until eIF4A activity is determined in its cellular context that inhibition becomes apparent – a salutary lesson on the risks of using isolated proteins to determine biological activity.

**4. Can the link between the proposed target and the cellular response be validated?** - Discovery of eIF4A as a potential target of **6** through the affinity chromatography analysis was very exciting. The DNA code is transcribed in the nucleus of a cell and creates a mobile RNA molecule that is used as a template for the production of a protein. The conversion of the nucleic acid sequence of RNA into the protein's amino acid sequence is *translation*. It is complex and requires a number of proteins to recognize an appropriate RNA molecule and then assemble into a functional machine, called a ribosome. The mammalian ribosome is an assemblage of over 80 proteins and large dedicated RNA molecules.[14] What is salient here is that eIF4A is one of the key proteins involved in ensuring that the ribosome is brought to the start of the coding section of an RNA molecule in eukaryotes (higher organisms), and thereby initiating translation. Any loss of eIF4A function will result in cessation of the majority of protein synthesis.

For **6**, this combination of a cellular activity dominated by protein synthesis inhibition and its ability to bind to and inhibit eIF4A make a compelling case that eIF4A is the primary target. However, while compelling, the evidence is somewhat circumstantial. Additional confidence that eIF4A is the primary target of **6** came by using yeast as a model organism. It was grown in increasing concentrations of **6** over many generations, eventually, to give colonies that could grow in concentrations of **6** lethal to normal yeast. Each of these yeast colonies could have acquired resistance to **6** through a number of mechanisms but, by using some elegant molecular biology, James copied only the gene that codes for eIF4A from the resistant yeast colonies. These eIF4A genes were sequenced and each was found to contain the same mutation. Furthermore, when this gene was placed into normal yeast, it became resistant to **6**. These yeast cells were not trained to cope with high concentrations of **6** so that the only route for their resistance is through the new copy of the eIF4A gene. This proves that eIF4A is the primary functional target of pateamine.

There is one further complication relating the interaction of **6** with eIF4A. Humans carry three variants of eIF4A, two of which (eIF4A1 and eIF4A2) fulfil essentially identical roles to each other in translation initiation. The third variant, imaginatively denoted eIF4A3, has an entirely different role. This protein is involved in checking RNA for errors in a process called nonsense-mediated decay. This removes faulty RNA molecules before they are used as a template for the production of proteins. Nonsense-mediated decay is an essential process in cells, and interfering with it leads to cell death. We have shown that **6** can bind to eIF4A3 and inhibit its activity. However, it is not clear just how significant this inhibition is to the biological activity.

In the time that it has been studied, **6** has moved from being a mildly interesting toxin with a range of valuable activities to yet another toxic protein synthesis inhibitor, to one that interacts with a number of cellular targets, including actin (albeit weakly), the eIF4As associated with initiation of protein synthesis and, in humans, the eIF4A3 protein central to mediating the error checking of RNA. Not surprisingly, structural modification of **6** will alter its affinity for these targets, and even allow it to interact with completely new partners in a cell.

Finally, it needs to be noted that **6** was the first drug-like molecule shown to interact with the eIF4A proteins opening up this protein as a new drug target. As a result of these studies and that presented subsequently by Liu,[15] marine natural product **6** has attracted substantial international interest and become the target of review articles in two quite disparate publications, ACS's *Chemical Biology* and *Chemical and Engineering News*.[16]

## Upcoming developments in identifying biological activity

This discussion has highlighted a number of valuable tools, old and new, in identifying the target of a biologically active compound. These include simple microscopy, affinity chromatography, classical biochemical assays with an isolated target, proteomics, and developing and sequencing resistant strains. However, this is but a glimpse of the approaches now available to identify drug targets. Many groups use transcriptomics (the study of RNA levels in a cell using microarray technology), which should be representative of the proteins that a cell is intending to make in response to a compound. The emerging technique of chemical genetics allows for understanding the interaction of a biologically active compound with a cell on a gene-by-gene basis.[17] Using this technique with **6** has opened up new avenues for exploration in both the mode of action of the compound and in understanding some fundamental cell biology. In addition to using chemical genetics to understand the detail of the biological interactions of the substrate, it can be used to show what other compounds it shares a mode of action with. The growing database of compounds with well characterized gene-by-gene interaction profiles allows the results from a new compound to be clustered with those whose mode of action is known and provide insight into the function of the new compound.[18]

The yeast biology community have provided many tools that are useful in tracing biological activity. Yeast is a simple organism, having about a quarter of the number of genes that are found in humans. However, the majority of the cellular processes found in yeast are also found in humans, and *vice versa*, and much of the detail of what we know about how our cells function comes from study in yeast. This combination of relative simplicity and shared fundamental pathways makes yeast a surprisingly good model organism for biological activity research. Amongst the available tools are the yeast gene deletion libraries that provide the basis of chemical genetics [17] and the yeast green fluorescent protein (GFP) library.[19] GFP is a fluorescent protein produced by the bioluminescent jellyfish *Aequorea victoria*. The GFP library contains over 4,000 yeast strains. Each strain has the genetic code for the green fluorescent protein inserted into a different gene, so that when transcribed and translated the protein produced has a GFP marker at its carboxyl terminal. Using this powerful tool, the location of each of these proteins within the cell can be identified.[19]

Although apparently not exciting enough for the world of chemistry it has profound implications. To date, biological activity at the cellular level has been largely limited to answering questions like *Does it kill cells?* and *Does it inhibit this enzyme*. Use of the yeast GFP library allows far more subtle biological effects to be revealed than simple toxicity. Image recognition software and powerful automated microscopes allow almost any change that we choose to follow within a cell to be monitored. In combination with chemical genetics profiling, almost all biological activities can be revealed. For example, Fig. 1 shows four microscopy images with a) and b) the *before and after* treatment with dithiothreitol. These show both upregulation and relocalization of a protein that is drawn upon to assist with the protein unfolding the dithiothreitol causes. Panels c) and d) show the upregulation of metallothionein, a copper binding protein, in response to treatment with $Cu^{2+}$ ions. For both treatments, the yeast cells seen in the micrographs are healthy, but the treatments cause marked biological activity revealed by the responses in key proteins. This ability to pick up subtle changes in abundance and location for almost any protein in a cell is a tremendous step forward in assay methods.

Those who make or isolate new molecules will frequently have experienced the disappointment of poor biological activity from their compounds. Usually, all that has been learned is that the compound interacts weakly with just one of the proteins in a cell or is not particularly toxic. This is no bad thing as many good drugs are not outstandingly toxic either! Phillip von Hohenheim, born in 1493 and later known as Paracelsus, had a mixed impact on scientific development with his ideas often clouded by the beliefs and practices of his day. He is reputed to be the originator of the name zinc and is recognized for his contributions to pharmacology and toxicology. He stated *All things are poison and nothing is without poison, only the dose permits something not to be poisonous*.[20] Were biological activity analysis limited to studying toxicity, we would be little further forward than the practitioners

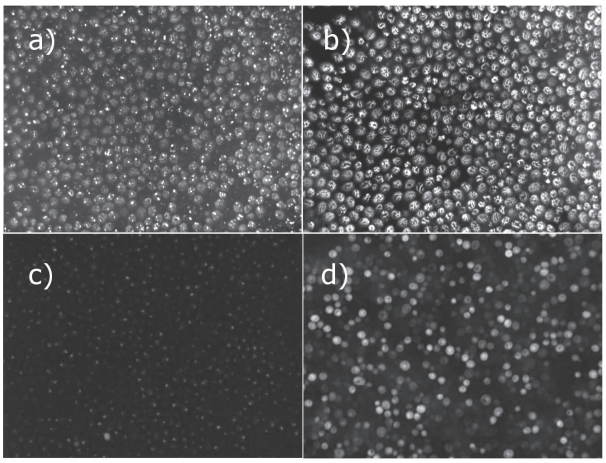



***Fig. 1.*** Changes in the localization of selected proteins in yeast on treatment with dithiothreitol (a and b) and copper (c and d); images reproduced with permission, courtesy of Peter Bircham, School of Biological Sciences, VUW and Dr David Maass, ESR Ltd.

of the early 16th century. Below the dose that achieves toxicity lies a panoply of other, potentially beneficial, biological effects, *e.g.* as is seen with sub-lethal doses of caffeine. The yeast GFP library allows the visualization of any number of these effects.

## Conclusion

Biological activity extends well beyond the inhibition of a single, isolated protein target or toxicity in cell culture. Identifying how a compound acts within a cell is possible using a range of classical and modern methodologies. Biological sciences are in an era of investigating interacting networks, and large scale high-throughput analyses – the -omics era. Genomics, transcriptomics, and proteomics can all be used in one way or another to look holistically at what a biologically active compound does, and to determine or infer a target or targets responsible for the activity. There are more emerging technologies, such as the use of following protein localization in a cell, that will reveal subtle cellular responses to compounds at sub-lethal levels. As Paracelsus reminds us, everything is a toxin, it is the subtle responses that are likely to make a compound functionally useful.

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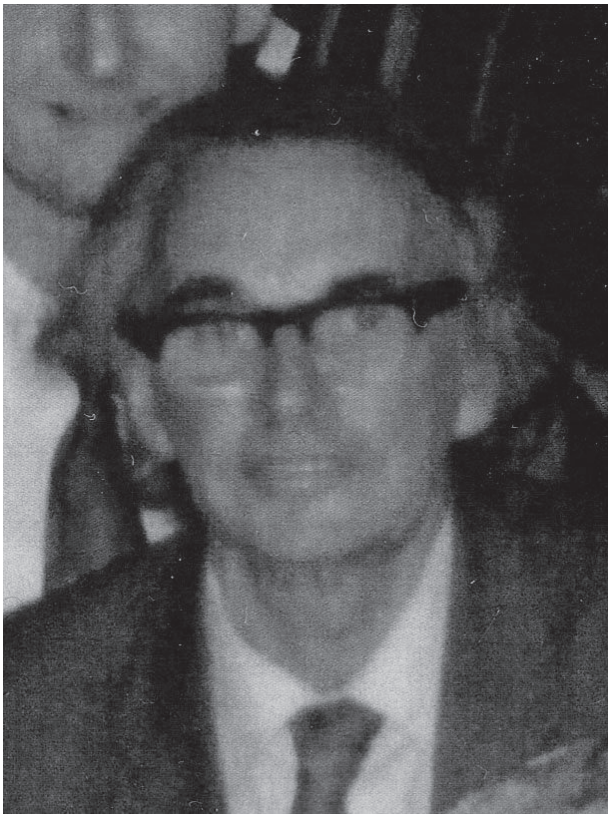
# Walter Sidney Metcalf (1928-2008)



Walter Metcalf, a long-time member of the Chemistry Department at Canterbury University, passed away on Thursday July 24. He arrived at Canterbury University College (now the Arts Centre), from what is now Victoria University of Wellington, in 1954 as a senior lecturer in charge of physical chemistry, replacing Hugh Parton who had just moved to Otago. He retired as a Reader 21 years later.

Though not driven to publish prolifically in his own right, Walter initiated into original research a number of people whose careers provide ample testimony to the blindingly obvious truth that universities are about more than just putting your name into print. Amongst these are *Robin* ***Clark*** (FRS), *Leon* ***Phillips***, *Sally* ***Page***, *Murray* ***McEwan***, *Ward* ***Robinson***, *David* ***Natusch*** (Rhodes Scholar) and the late *Terry* ***Quickenden*** (a unique and eccentric academic stimulator in the Metcalf tradition). His early career at Victoria involved *Hugh* ***Melhuish***, who subsequently joined DSIR and became NZ's foremost physical photochemist, and *Laird* ***Ward***. Laird made his career in US industry but remained loyally fond of Walter, for whom he had synthesized many large round-bottomed flasks of brilliantly fluorescing organic liquids.

Walter was an unorthodox and often inspiring lecturer who, on one occasion, cleared everybody out of the chemistry lecture room with an *aluminium-powder-plus-finely-ground-iodine-plus-a-few-drops-of-water* demonstration that produced rather more iodine vapour than anticipated, and on another occasion (a beautiful spring morning) gave a lucid and highly memorable lecture on Förster non-radiative energy transfer in solution while leading the class of six or seven honours students on a hike around Lake Victoria and the botanic gardens. His main research field was photochemistry, for which work he won the RSNZ T.K. Sidey Medal. He did his DPhil at Oxford University with E.J. Bowen, author of *Chemical Aspects of Light* which was one of the first books on photochemistry, and subject of an oft-repeated rhyme: Says E.J. Bowen, '*I always empty my pipette by blowin*'. In later years Walter branched out into calcium metabolism.

Walter also had a bachelor's degree in music (his instrument was the viola), which he obtained simultaneously with his first science degree. He had wanted only to sit in on a few courses but his scholarship would not cover the fees for a course that was not aimed at gaining a degree, so he went ahead and completed the degree. Subsequently, he played in a quartet with friends and became chief rescuer of old violins and cellos for use by pupils of the Christchurch School of Instrumental Music.

Sally Page has commented that she only began to appreciate the quality of the advice given to her by Walter when she had to advise her own graduate students at UC London. Ward Robinson comments that: *I was privileged to participate in a modest MSc project with Walter and this had a lot to do with my reaching for the research career which is still thrilling me 48 years later. I am sure we all would want to record our deep appreciation of all the different ways in which his long life impacted upon us*, and Leon Phillips adds: *Walter was a Quaker, an exceptionally kind person and an enthusiastic individualist on almost any topic; I miss him*.

*Leon Phillips & Ward Robinson*