STRADER, CHERILYN R., M.S. Fingolimod (Gilenya; FTY720): A Recently Approved Multiple Sclerosis Drug Based on a Fungal Secondary Metabolite and the Creation of a Natural Products Pure Compound Database and Organized Storage System. (2012)
Directed by Dr. Nicholas H. Oberlies, 78 pp.

Fingolimod (Gilenya; FTY720), a synthetic compound based on the fungal secondary metabolite, myriocin (ISP-I), is a potent immunosuppressant that was approved in September 2010 by the U.S. FDA as a new treatment for multiple sclerosis (MS). Fingolimod was synthesized by the research group of Tetsuro Fujita at Kyoto University in 1992 while investigating structure-activity relationships of derivatives of the fungal metabolite, ISP-I, isolated from *Isaria sinclairii*. Fingolimod becomes active *in vivo* following phosphorylation by sphingosine kinase 2 to form fingolimod-phosphate, which binds to specific G protein-coupled receptors (GPCRs) and prevents the release of lymphocytes from lymphoid tissue. Fingolimod is orally active, which is unique among current first-line MS therapies, and it has the potential to be used in the treatment of organ transplants and cancer. The first chapter reviews the discovery and development of fingolimod, from an isolated lead natural product, through synthetic analogues, to an approved drug.

Natural products play an important role in the pharmaceutical industry, accounting for approximately half of all drugs approved in the U.S. between 1981 and 2006. Given the importance of natural product research and the vast amount of data that is generated in the process, it is imperative that the pure compounds and data are stored in a manner that will allow researchers to derive as much value as possible. The field chemoinformatics, interchangeable termed cheminformatics, has set the standards for electronic storage of chemical data, as chemistry was one of the first scientific disciplines to pursue electronic means of data storage. In this

project an electronic database was created to capture all of the relevant data for each pure compound isolated via natural product research in Dr. Oberlies lab. A system was also implemented to catalogue and store the compounds in an organized manner.

FINGOLIMOD (GILENYA; FTY720): A RECENTLY APPROVED MULTIPLE SCLEROSIS DRUG BASED ON A FUNGAL SECONDARY METABOLITE AND THE CREATION OF A NATURAL PRODUCTS PURE COMPOUND DATABASE AND ORGANIZED STORAGE SYSTEM

by

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A Thesis Submitted to
the Faculty of the Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Greensboro
2012

Approved by

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Date of Final Oral Examination

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CHAPTER I

MULTIPLE SCLEROSIS: THE DISEASE AND ITS CURRENT TREATMENT

Over 2.5 million people worldwide suffer with the debilitating disease multiple sclerosis (MS). MS is a neurodegenerative disorder of the central nervous system that is estimated to affect twice as many women as men.[1] The disease causes irreversible nerve damage, resulting in a wide range of symptoms, including fatigue, depression, pain, motor weakness, visual disturbances, and vertigo.[1-3] The onset of symptoms is usually rapid and sudden, commonly appearing around age 30.[1] The clinical course of MS varies greatly, from quickly progressive, resulting in clinical disability and possibly death, usually within 25 years,[4,5] to recurring symptoms that reduce a patient's quality of life, but without resulting in decreased life span. If untreated, approximately 50% of patients with MS are incapable of walking unaided within 15 years of disease onset.[6,7]

The clinical course of MS is best predicted by the specific disease classification, either relapsing-remitting (RR), primary-progressive (PP), secondary-progressive (SP), or progressive-relapsing (PR).[8] Approximately 85-90% of patients are diagnosed with RR-MS,[9,10] defined by an ongoing and unpredictable cycle of acute episodes of symptoms followed by a distinguishable recovery period.[8] The disease eventually becomes progressive in 30-40% of those diagnosed with RR-MS, changing their classification to SP-MS.[6,10] For this, the relapse-recovery cycle may or may not continue, but the symptoms progressively worsen, which is atypical of RR-MS.[8] With PP-MS, the disease continually, albeit gradually, progresses from onset.[8] PR-MS is similar to PP-MS

in that the disease continually progresses in severity from onset, but with distinguishable relapses and possible recovery time in between episodes.[8]

Autoimmune disorders, such as MS, are marked by defective immune system responses. MS occurs when T- and B-lymphocytes cross the blood-brain barrier, progress into the central nervous system, and attack healthy cells.[11,12] Specifically, they destroy the myelin sheath and damage axons, resulting in inflammation.[11] This in turn causes irreversible nerve and tissue damage and accounts for the wide range of symptoms observed in MS.[11-13]

The cause of MS is unknown, but is believed to result from genetic predisposition in combination with environmental factors, which may include some type of infection.[12] However, to date, none of these factors have been proven to cause MS.[12] Diagnosis is often based solely on the presence of the attributed disease symptoms and patterns.[14] If a definitive diagnosis cannot be made from observable symptoms, magnetic resonance imaging (MRI) can aid in diagnosis.[14] MRI scans of patients with MS show distinct plaques in the brain and spinal cord,[12] usually measuring at least 3 mm in diameter.[14]

Current medicinal treatment options for patients with MS include immunomodulators, immunosuppressants, and anti-inflammatory agents.[15] Four different immunomodulators are approved by the U.S. Food and Drug Administration (FDA) for treatment of relapsing MS.[16] Three are interferon betas (IFNβ) and include IFNβ-1b (Betaseron, Bayer HealthCare Pharmaceuticals) approved in 1993, IFNβ-1a intramuscular once weekly (Avonex, Biogen Idec) approved in 1996, and IFNβ-1a subcutaneously thrice weekly (Rebif, EMD Serono, Inc. and Pfizer Inc.) approved in 2002. The fourth immunomodulator, glatiramer acetate (GA) (Copaxone, Teva Pharmaceutical Industries, Ltd.), was approved in 1997. These four treatments, administered via either

subcutaneous or intramuscular injection, only modestly affect disease progression, with a 30% reduction in disease relapse rates being reported.[15] The most commonly reported side effects include injection site reactions/necrosis and influenza-like symptoms.[17-19]

Immunosuppressants are also used for treatment, with cyclosporin A[20] (Neoral and Sandimmune, Novartis Pharmaceuticals), approved in 1983, and tacrolimus, also known as FK506,[21,22] (Prograf, Astellas Pharma US, Inc.), approved in 1994, being the two most common. However, these agents cause complete suppression of the immune system, leaving the patient susceptible to life-threatening secondary infections.[23] They also have toxic side effects at high doses, including renal impairment and kidney damage.[24,25] In 2002 the FDA approved mitoxantrone (Novantrone, EMD Serono, Inc.) for treatment of worsening MS. Mitoxantrone has the properties of both immunosuppressants and immunomodulators, although its use is limited by cardiotoxicity.[16]

In short, MS is a debilitating disease that often progresses, resulting in clinical disability. The current treatment options, while offering some benefits, are not ideal for myriad reasons. However, new treatment strategies may emerge due to the recent approval of fingolimod (**1**; Figure 1), given the trade name Gilenya by Novartis Pharma. The progenitor to fingolimod, myriocin (**2**, ISP-I; Figure 1), was discovered from a fungus and reported in 1972.[26] To date, fingolimod has shown unprecedented efficacy for reducing annual relapse rates and symptoms,[27-29] and combined with its oral bioavailability, it may become an important component in the arsenal to combat MS.

CHAPTER II

DISCOVERY

Cyclosporin A and FK506 were discovered initially as antifungal agents, and currently, both are clinically important immunosuppressants.[20-22] Cyclosporin A was reported in 1976 from the fungus *Trichoderma polysporum*, which was later re-identified as *Tolypocladium inflatum*.[20] FK506 was isolated from the bacterium *Streptomyces tsukubaensis* in 1987.[21,22] The discovery and development of these compounds supported and validated the screening of fungi and other microorganisms in pursuit of new immunosuppressants.[30]

During the late 1980s and early 1990s, Tetsuro Fujita and coworkers were also studying the fungus *Tolypocladium inflatum*.[31] In these studies they isolated a cyclic depsipeptide that was an active antibiotic, which was reported previously from the fungus *Isaria sinclairii*.[31] Fujita and colleagues thereafter focused their efforts on extracts of *I. sinclairii*.[31]

Isaria sinclairii is native to Asia, mainly China, Korea, and Japan and is classified as an entomopathogenic fungus.[32] It is the imperfect stage of *Cordyceps sinclairii* (Clavicipitaceae), and is closely related to *Cordyceps sinensis* Sacc., whose Chinese name, Dong Chong Xia Cao, means 'winter worm, summer grass;'[32] this species was reclassified recently to *Ophiocordyceps sinensis*.[33] Fungal spores infect the larvae of suitable insect hosts, including members of the order Hymenoptera and Lepidoptera; the fungus is parasitic, growing within the host and resulting in death of the insect.[32] The fungus completely colonizes the insect cadaver, and in the Spring and Summer white

fruiting bodies appear as stalks up to 6 cm in height.[34] Fungi at this stage of development are regarded as mysterious and mystical in some Asian cultures and have been used for thousands of years in traditional Chinese medicine, as they are believed to impart eternal youth.[32]

Fujita and colleagues[31] utilized two assays, one *in vitro* and one *in vivo*, to evaluate the fungus and its metabolites. To screen for immunosuppressive activity, they used a mouse allogeneic mixed lymphocyte reaction (MLR) assay. In this *in vitro* assay, spleen cells from two different strains of mice (BALB/c and C57BL/6) are co-cultured and alloantigen added to stimulate T-cell proliferation.[31] Samples were evaluated for inhibition of the proliferation of T-cells, with results reported as an IC_{50} value. The *in vivo* assay was performed by transplanting the dorsal skin of one rat (strain LEW) to the lateral thorax of a second rat (strain F344).[35] Test compounds were administered intraperitoneally daily until the skin grafts were rejected, as evidenced by 90% necrosis.[35] Compounds were scored based on their ability to prolong rat skin graft survival. Using these assays in concert was key to the eventual development of fingolimod.

This evaluation process guided the isolation of a compound with immunosuppressant activity, which Fujita *et al.*[31] termed ISP-I (**2**; Figure 1). Upon structure elucidation, they found ISP-I was identical to myriocin[26] and thermozymocidine,[36] which were isolated previously from *Myriococcum albomyces* and *mycelia sterilia*, respectively, via screening programs for antifungal agents.[26,36] ISP-I was shown by Fujita *et al.* to be 5- to 10-fold more potent than cyclosporin A in the mixed lymphocyte reaction (MLR) assay,[31] and at a dose of 0.1 mg/kg, ISP-I prolonged rat skin graft survival time by two days when compared to cyclosporin A at a dose of 1.0 mg/kg.[35] However, ISP-I had some

unfavorable properties, being toxic to rats at a dose of 1.0 mg/kg, compared to 100 mg/kg for cyclosporin A, and by poor solubility.[35]

Researchers began to study ISP-I, with the goal of both simplifying the structure and improving the biological properties.[37,38] Between 1995 and 1998, results from the evaluation of upwards of 50 analogues were reported.[35,37-42] In the published results from both the *in vitro* and *in vivo* assays, the activity of the analogues of ISP-I were compared to the activity of cyclosporin A. Structure-activity relationship studies guided the synthesis of compounds that had simplified structures, improved physical characteristics (i.e. solubility), and more potent activity (Table 1).[31,39]

The first analogue of interest was ISP-I-28 (**3**; Figure 1).[41] ISP-I-28 contained the following changes from ISP-I: reduction of the 6-7 double bond and reduction of the carboxylic acid and the 14-ketone[43] to alcohols. ISP-I-28 was less toxic than ISP-I (100 mg/kg compared to 1 mg/kg, respectively).[35] ISP-I-28 also prolonged rat skin graft survival time by two days, compared to cyclosporin A, both at a dose of 1.0 mg/kg, and ISP-I-28 was more soluble than ISP-I.[35] However, ISP-I-28 was less potent than ISP-I in the MLR assay, with an IC_{50} value of 1630 nM compared to 3 to 8 nM, respectively (Table 1).[35,42]

ISP-I-28 was simplified further by removing three hydroxy groups, leaving an 18-carbon alkyl chain and resulting in the compound ISP-I-36 (**4**; Figure 1), which had an improved IC_{50} value (12 nM) in the MLR assay (Table 1).[41] ISP-I-36 also had improved activity in the rat skin graft assay, increasing the survival time by five days, compared to ISP-I-28, at a dose of 1.0 mg/kg. By shortening the carbon alkyl chain of ISP-I-36 from 18 to 14 carbons, researchers generated ISP-I-55 (**5**; Figure 1), which was a more potent immunosuppressant in both the *in vivo* and *in vitro* assays. ISP-I-55 had an IC_{50}

value of 5.9 nM (MLR assay) and more than doubled the survival time observed with ISP-I-36 in the rat skin graft assay (37.3 days with a 1.0 mg/kg dose; Table 1).[35]

The final modification was the introduction of an aromatic moiety, which researchers believed would improve activity by restricting conformation,[44] thereby leading to fingolimod (**1**; Figure 1). Positioning of the aromatic unit was critical, as its placement was shown to either decrease or increase immunosuppressant activity.[39] By moving it one carbon position in either direction, there was greater than 10-fold loss of potency in the MLR assay.[39] Moreover, the absorbance of the aromatic moiety in fingolimod was easy to detect analytically, a point that became beneficial in preclinical development studies.[30] More importantly, when compared to ISP-I, fingolimod had improved activity, a more favorable toxicity profile, and more desirable physical properties, including increased solubility (Table 1).[39,44]

A critical point in the discovery of fingolimod (**1**) was the use of the MLR assay to evaluate immunosuppressant activity.[30] Alternatively, researchers could have used the serine palmitoyltransferase (SPT) inhibition assay, which evaluates immunosuppressant activity based on a compound's ability to inhibit the enzyme serine palmitoyltransferase. Cyclosporin A, FK506 and ISP-I (**2**) are active in both the SPT and MLR assays.[35,42] However, the analogues ISP-I-55 (**5**) and fingolimod (**1**) only show activity in the MLR assay, suggesting that they operate via a different mechanism of action.[35,42] If the SPT assay would have been used to evaluate the immunosuppressant capabilities of these compounds, both ISP-I-55 (**5**) and fingolimod (**1**) would have shown no activity[35,42] and may not have been pursued further.[30] The reported bioassay results for compounds **1-5** and cyclosporin A are compiled in Table 1.

Figure 1. Studied Compounds that Lead to the Development of Fingolimod (**1**).

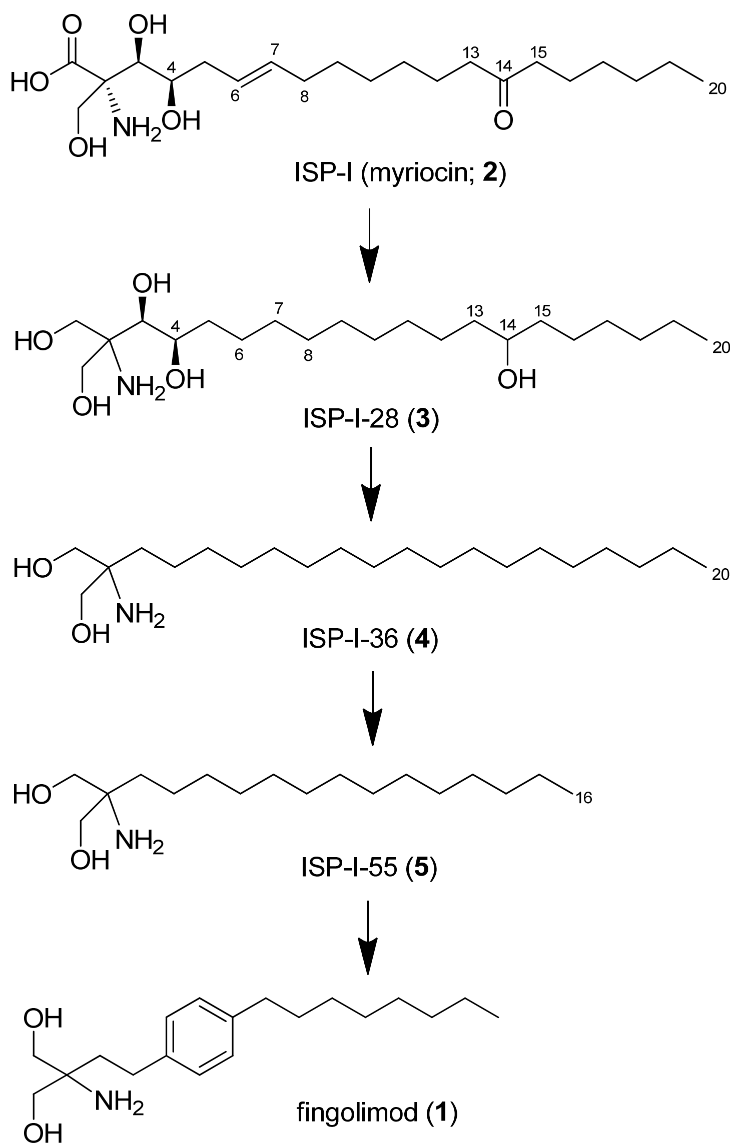


Table 1. Compiled Assay Results of Compounds of Interest in the Development of Fingolimod (**1**).

	Assays Examined[a]				
Compound	**MLR Assay IC_{50} value (nM)**[35,39,41,42]	**Toxicity**[b] ***in vivo***[35,39,41,44]	**Rat skin graft survival time (days) at 1.0 mg/kg**[35,41,44]	**Rat skin graft survival time (days) at 3.0 mg/kg**[35,39,41,44]	**Active in SPT assay**[35,39,41,42,44]
ISP-I (**2**)	3 to 8	1.0	toxic	--	Yes
ISP-I-28 (**3**)	1630	100	9.2	11.0	NR[c]
ISP-I-36 (**4**)	12	10	14.8	17.6	NR
ISP-I-55 (**5**)	5.9	10	37.3	45.5	No
fingolimod (**1**)	6.1	C[d]	39.5	52.0	No
cyclosporin A	14	100	7.3	10.8	Yes

[a] Numerical values are as reported in the primary literature.

[b] Concentration (mg/kg) at which animals died in the *in vivo* rat skin graft assay.

[c] Not reported.

[d] Contradictory; the published toxicity data on fingolimod are inconsistent at 10 mg/kg in this assay, with one paper reporting it as non-toxic,[39] and a latter paper reporting it as toxic.[44]

CHAPTER III

SYNTHESIS

Fingolimod (**1**) was derived from ISP-I (**2**) (discussed above) but contains various synthetic alterations.[37] At least 13 methods for the synthesis of fingolimod (**1**) and fingolimod-phosphate (**6**) have been developed. A detailed analysis of all of these is beyond the scope of this review. However, Table 2 provides a synopsis of the various synthetic strategies that have been published to date, and brief descriptions of a few are provided below.

In 2004 Seidel *et al.*[45] published an eight step method utilizing iron-catalyzed cross-coupling reactions, starting from 2-(4-hydroxyphenyl)ethanol. They did not publish the overall yield of **1**, but syntheses with similar methods have resulted in overall yields of 6 to 24%.[39,44] In 2005 a shorter synthetic method was published by Sugiyama *et al.*[46] that required only five steps. The method was based on the Petasis reaction, which couples boronic acids, amines and carbonyls to give amino alcohols. The resulting overall yield of **1** was 28%.[46] A seven step approach was published subsequently by Kim *et al.*[47] that started with tris(hydroxymethyl)aminomethane (TRIS), which was converted to an aldehyde and then an alkyne. The alkyne was coupled to an aryl iodide via a Sonogashira reaction, hydrogenated, treated with acid and purified. This methodology was practical, inexpensive, and resulted in a 64% overall yield of fingolimod.[47]

Table 2. Highlights of Methods Used to Synthesize Fingolimod (**1**) and Fingolimod-P (**6**).

Year	Highlights of Method
1995	First published method for the synthesis of fingolimod[39]
2000	Synthesis of fingolimod and analogues to evaluate immunosuppressive and lymphocyte-decreasing activity; method begins with Friedel-Crafts acylation of phenylalkyl acetates[44]
2000	Efficient 5-step method beginning with Friedel-Crafts acylation of 1-phenyloctane; 13% yield[48]
2001	Method to synthesize fingolimod using $MgSO_4$/MeOH/$NaNO_2$ to regioselectively open the ring of an epoxide[49]
2004	Synthesis of both enantiomers of fingolimod-P; used L-serine derived oxazolizine to synthesize optically active intermediate, which was used to determine the absolute configuration of both enantiomers[50]
2004	Practical and scalable eight-step method based on iron-catalyzed cross-coupling, starting with 2-(4-hydroxyphenyl)ethanol;[45] similar methods have reported yields of 6–24%[39,44]
2005	Convenient five-step method based on Petasis reaction, using dihydroxyacetone, benzylamine and 2-(*p*-octylphenyl)vinylboronic acid; 28% yield[46]
2005	Efficient and practical method to synthesize fingolimod-P based on monophosphorylation, using silver (I) oxide, tetrabenzyl pyrophosphate (TBPP) and tetrahexylammonium iodide[51]
2005	Practical asymmetric synthesis of both enantiomers of fingolimod-P based on lipase-catalyzed acylation, starting with *N*-acetylated fingolimod[52]
2005	Determined that fingolimod is phosphorylated *in vivo* to form only the (*S*)-stereoisomer; performed efficient synthesis of both enantiomers of fingolimod-P in optically pure form, starting with fingolimod[53]
2006	Concise and practical seven-step method using palladium-catalyzed Sonogashira cross-coupling reaction; 64% yield[47]
2006	Convenient synthesis of both enantiomers of fingolimod-P from p-bromobenzaldehyde, using asymmetric Sharpless epoxidation[54]
2008	Convenient synthesis of immediate precursor of fingolimod, improving 1995 method;[39] precursor synthesized in 3 steps (vs. 6); yield of precursor 41% (vs. 18%)[55]

CHAPTER IV

MECHANISM OF ACTION

A recent review by Brinkmann *et al.*[56] summarizes nicely the details of the mechanism of action of fingolimod. Briefly, the activation cascade of T- and B-cells begins with the phosphorylation of the sphingolipid, sphingosine (**7**; Figure 2), by sphingosine kinase 2, to form sphingosine 1-phosphate (S1P) (**8**; Figure 2).[57] S1P is an activator of five different cell surface G-protein-coupled receptors (GPCRs), referred to as receptors $S1P_{1\text{-}5}$, which regulate a variety of cellular processes.[58] $S1P_{1\text{-}3}$ are expressed primarily on cells of the cardiovascular, immune, and central nervous systems, $S1P_4$ is expressed primarily in lymphoid tissue, and $S1P_5$ is expressed primarily in the spleen and central nervous system.[58] Activation of these GPCRs is necessary for the body's release of lymphocytes from the lymph nodes to the blood.[59]

The improper response of lymphocytes, as occurs with MS, causes internal inflammation, cell apoptosis, improper neuron firing, and severe pain.[15] Current medical treatments for MS, including cyclosporin A and FK506, act by inhibiting the enzyme serine palmitoyltransferase. Serine palmitoyltransferase is responsible for catalyzing the first step in sphingosine biosynthesis.[23] Inhibition of this enzyme results in the body's inability to produce sphingosine, preventing any activation of the GPCRs $S1P_{1\text{-}5}$. Ultimately this inhibits the release of T- and B-cells, rendering the body incapable of generating an immune response to any stimuli.[23]

Fingolimod (**1**) has a unique and novel mechanism of action. Once ingested, it is rapidly phosphorylated by sphingosine kinase 2 to form fingolimod-P (**6**; Figure 2).[57,60]

Fingolimod-P resembles the ligand S1P and competes with it to bind to four of the five S1P receptors.[59] Fingolimod-P has the highest binding affinity for $S1P_1$, binding to $S1P_{3-5}$ with slightly lower affinity, and has no affinity for $S1P_2$.[53,61,62] Blood samples show that after **1** has circulated throughout the body, the concentration of fingolimod-P is up to four times that of the parent.[62] This is essential for biological activity, as fingolimod itself has no binding affinity to any of the S1P receptors.[53,61,62]

A study was conducted to compare the pharmacokinetics of oral vs i.v. administration of fingolimod.[63] After i.v. administration, fingolimod (**1**) was present in the patient's blood, but fingolimod-P (**6**) was not.[63] Alternatively, during oral administration, presystemic phosphorylation of fingolimod to fingolimod-P may be a key, owning to the higher level of fingolimod-P measured after administration via this route.[63] These observations suggest that sphingosine kinase 2, which phosphorylates fingolimod, is active during either (or both) first pass metabolism through the liver and/or the adsorption processes;[63] it is known that sphingosine kinase 2 is highly expressed in the liver.[64,65]

Ultimately fingolimod is metabolized in the liver, specifically by the cytochrome P450 enzyme CYP4F, with a half-life of 5-6 days.[66] It is metabolized primarily by oxidation of the hydroxy moieties into carboxylic acid derivatives and excreted in urine.[67] Fingolimod undergoes a unique metabolic process, in which it is almost completely absorbed, slowly excreted and clearly favors oral administration of the drug. At present researchers are working to understand fingolimod's metabolic process more fully.[66,68]

Methods have been developed to synthesize fingolimod-P (**6**; Table 2); however, exploiting the body's natural phosphorylation processes for converting **1** to **6** may be the best option clinically. For instance, when **1** is phosphorylated *in vitro*, both the (*S*)- and

(*R*)- enantiomers of fingolimod-P are produced. However, only the (*S*)-configuration has binding affinity to the S1P receptors.[40,53,61,62] Fortunately, phosphorylation of fingolimod (**1**) *in vivo* results in only the biologically active (*S*)-configuration being formed.[53]

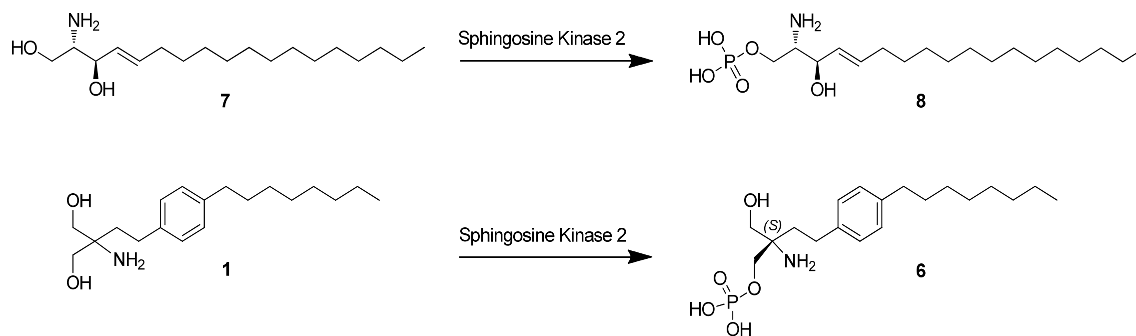
The novelty of the mechanism of action of fingolimod-P lies in its ability to redistribute the type of lymphocytes circulating in the blood, without reducing total lymphocytes. Central memory T-cells, which circulate regularly through lymph nodes,[69] are believed to be the subtype that are auto aggressive in MS patients.[58] Fingolimod-P (**6**) causes the lymphoid tissue to retain central memory T-cells, preventing them from entering into the blood.[70] Concomitantly, **6** also causes the concentration of effector memory T-cells in the blood to increase.[70] These latter T-cells do not circulate regularly through lymph nodes, and they are responsible for containing local pathogens and managing immune response memory.[69] This unique redistribution is crucial, as it prevents neurological damage by central memory T-cells, while preserving many necessary functions of the immune system carried out by other lymphocytes.

Fingolimod does not cause the destruction of any lymphocytes. Studies have shown that the overall count of lymphocytes circulating in the blood is reduced by approximately 70% during fingolimod treatment, compared with lymphocyte levels prior to treatment.[71] This decrease is due to the retention of lymphocytes in the lymphoid tissue. Once this redistribution occurs, lymphocyte counts in the blood remain stable throughout treatment and return to normal levels within 4 to 8 weeks after treatment discontinuation.[71]

Although fingolimod does not cause the destruction of lymphocytes, it does lead to degradation of the $S1P_{1,3\text{-}5}$ receptors.[72] Binding of either S1P or fingolimod-P to $S1P_{1,3\text{-}5}$ receptors causes internalization of the receptor, moving it from the plasma membrane into the cell.[73,74] Internalization of the receptor by S1P results in the receptor being

recycled to the cell surface within approximately 2 h.[72] However, internalization caused by the binding of fingolimod-P (**6**) blocks the receptor recycling pathway and leads to receptor degradation.[72,74,75] It takes 2 to 8 days after exposure to fingolimod-P for cells to recover normal expression of $S1P_{1,3\text{-}5}$ receptors.[74] $S1P_{1,3\text{-}5}$ receptor degradation likely contributes to the prolonged immunosuppressant activity observed with fingolimod.[74,76,77] This provides a benefit in that fingolimod's activity is not dependent on the long-term stability of the compound *in vivo*, as short exposure time to fingolimod results in prolonged immunosuppressant activity.[74]

Figure 2. Mechanism of Phosphorylation via Sphingosine Kinase 2 (Adapted from Chun *et al.*[58]). The top shows the natural conversion of sphingosine (**7**) to sphingosine 1-phosphate (**8**), while the bottom shows the formation of fingolimod-phosphate (**6**) from fingolimod (**1**).



CHAPTER V

PHARMACOLOGY

Fingolimod, given the trade name Gilenya by Novartis Pharma, is the first orally active treatment for MS.[27] Two study groups, FREEDOMS (FTY720 Research Evaluating Effects of Daily Oral therapy in Multiple Sclerosis),[29] and TRANSFORMS (Trial Assessing Injectable Interferon versus FTY720 Oral in Relapsing-Remitting Multiple Sclerosis),[28] published results of phase III clinical trials in February 2010. All of the completed clinical studies on fingolimod to date have been on those patients with RR-MS, although a phase III clinical trial (INFORMS study) was initiated in 2010 on those patients with PP-MS.[78]

The FREEDOMS trial ran for 24 months and included a total of 1272 patients, approximately 70% of whom were female.[29] All patients were 18 to 55 years of age and had relapsing-remitting (RR-MS). They were split into three treatment groups and administered daily either 0.50 mg of fingolimod (**1**), 1.25 mg of **1**, or placebo. The primary endpoint of the study was annualized relapse rates. The secondary endpoints were the time to disability progression and the growth or generation of lesions, as shown by MRI.[29] In this study the annualized relapse rate decreased 60% with a daily dose of 1.25 mg of **1**, and 54% with a daily dose of 0.5 mg of **1**, relative to placebo.[29] The probability of disability progression, confirmed after six months, was 12.5% for 0.5 mg of **1**, 11.5% for 1.25 mg of **1**, and 19.0% for placebo.[29] Similar proportions of patients reported adverse events in all three treatment groups.[29] A few adverse events were

reported more commonly with **1** treatment, including lower respiratory tract infections, macular edema, and elevated liver-enzyme levels.[29]

The TRANSFORMS study was conducted for 12 months and included 1292 patients between the ages of 18 and 55 who had RR-MS, approximately 67% of whom were female.[28] Patients were split into three treatment groups and received a daily dose of fingolimod (**1**) of either 0.50 mg or 1.25 mg or a weekly subcutaneous 30 μg dose of IFNβ-1a. This study[28] used the same endpoints as the FREEDOMS study.[29] Progression of the disease to disability was infrequent in all three treatment groups, as would be expected given the 12 month timeline.[28] Relapse rates were reduced by 38-52% in the fingolimod treatment groups *vs* the interferon IFNβ-1a treatment group.[28] There were also significantly fewer new lesions and less lesion growth in the fingolimod groups versus the interferon IFNβ-1a group.[28] Reported adverse events were similar to those reported in the FREEDOMS study, with the addition of localized skin cancer occurring in ten patients, eight of whom were undergoing treatment with fingolimod.[28] The 10 identified skin cancers were all excised successfully, did not appear to be related to the dose of fingolimod, and the data were not extensive enough to determine causality.[28]

Fingolimod has been shown in both Phase III clinical trials,[28,29] as well as in other clinical studies,[63] to lower the patient's heart rate by approximately 10% upon treatment initiation.[67] Heart rate usually returns to its pre-treatment rate within 7 to 14 days after the first dose of fingolimod, and does not change again during continued treatment.[67]

CHAPTER VI

OTHER PROSPECTIVE USES

Fingolimod has the potential to be used in organ transplant. Researchers have suggested that it can prevent strong lymphocyte-mediated immune reactions in response to the implantation of new organs.[59] Additionally, it has been suggested that fingolimod strengthens endothelial cells and preserves their function, although this has only been shown at concentrations that are, at a minimum, 5-fold higher than the dose administered normally.[59] Regardless, these are promising concepts, as it implies that fingolimod may have other *in vivo* effects apart from the regulation of lymphocyte circulation.[59]

Fingolimod (**1**) has been evaluated *in vitro* against three breast cancer cell lines (MCF-7, MDA-MC-231, and Sk-Br-3),[79,80] two colorectal cancer cell lines (HCT-116 and SW620),[79] and one prostate cancer cell line (LNCaP-AI).[80] Fingolimod inhibited the growth of these cell lines at IC_{50} values in the range of 5 – 20 μM.[79,80] However, treatment of the three breast and two colorectal cancer cell lines with fingolimod-P (**6**) did not inhibit their growth.[79] A new analogue, (*S*)-fingolimod vinylphosphonate, was evaluated in MCF-7 and LNCaP-AI cell lines and showed similar results to those of fingolimod.[80] This suggests that fingolimod's mechanism of action in cancer cell proliferation may not be the same as in MS, as only fingolimod-P is active in MS treatment. Fingolimod has also been evaluated *in vivo* in tumors derived from the MGC803 (gastric adenocarcinoma) cell line in nude mice.[81] Mice were treated with 10 mg/kg fingolimod daily and observed for 20 days.[81] Fingolimod inhibited tumor growth

and did not cause any notable side effects.[81] The potential of fingolimod to treat various types of cancer is an ongoing area of investigation.

CHAPTER VII

CONCLUSION

The discovery of fingolimod (**1**) was due largely to the persistence of the research group of Tetsuro Fujita. The lead natural product, ISP-I (**2**), which was isolated from *Isaria sinclairii*,[31] gave them a framework to explore, particularly coupled with their clever and productive bioassay methodologies.[31,35] Many analogues of ISP-I were tested before fingolimod was discovered with improved physical properties,[37] a short synthetic method,[47] and a novel mechanism of action.[56,58] Fingolimod (**1**), under the trade name Gilenya, was first approved for use in Russia on September 10, 2010, and was subsequently approved by the U.S. FDA in September 2010 and by Europe's European Medicines Agency (EMA) in March 2011.Europe's EMA did initiate a review of Gilenya in January 2012 due to 11 reported patient deaths, but a causal relationship has not been established.[82] European regulators have advised doctors to increase their level of monitoring of patients after administering the first dose of Gilenya, mainly due to the reported decrease in heart rate.[82]

As Gilenya is the only current FDA approved first-line treatment for MS that is orally available, it has the potential to revolutionize the therapy of this debilitating disease.[27-29] Novartis reported in October 2011 that more than 25,000 patients have been treated with Gilenya and it has been lauded by some as one of the top ten medical inventions of 2011.[83] Given this impact, it was estimated that Novartis may profit upwards of $1 billion annually from sales of Gilenya,[84] adding it to the long list of natural product inspired blockbuster drugs.[85,86] Future research on **1** will likely focus on more detailed analysis of

its mechanism of action, while continuing to test it for the treatment of other diseases. Moreover, there are certainly other research groups looking to develop second- and third-generation analogues that further enhance potency and/or minimize side effects.

CHAPTER VIII

INTRODUCTION AND BACKGROUND

Natural products play an important role in the pharmaceutical industry.[85] Natural products have many chemical advantages, including being good scaffolds for drug design, as they are known to provide molecular diversity.[87] Additionally, when compared to synthetic drugs, natural products tend to be more readily absorbed *in vivo*.[87]

It has been estimated that approximately 25-50% of all drugs marketed in the U.S. in 2010 originated as natural products.[88] More specifically, approximately half of all drugs approved in the U.S. between the years 1981 and 2006 were either natural products, or based on natural products.[85] Natural products have made a significant contribution to the treatment options of many widespread diseases, particularly bacterial infections and cancer.[85] Of all antibacterial and anticancer drugs approved between the years 1981 and 2006, approximately 78% of them were either derived from, based on, or itself a natural product.[85]

Natural products are derived from a wide range of source organisms, including fungi, bacteria, plants, amphibians and marine organisms. Fungi produce secondary metabolites that have a long history of providing the scientific community with biologically active and structurally diverse chemical compounds. It has been estimated conservatively that 1.5 million species of fungi exist in the world today,[89] with more aggressive estimates predicting up to 5.1 million species.[90] Of these species, only approximately 100,000 have been described taxonomically.[91] This leaves the majority of

fungal species as yet to be studied, pointing to the importance of natural products research programs aimed at studying fungi and their secondary metabolites.

According to a search conducted on February 12, 2012 in the Dictionary of Natural Products, there are 243,250 natural product compounds that have been characterized worldwide. Of these, 13,298 were isolated from fungi. To date, approximately 150 pure compounds have been isolated from fungi in Dr. Oberlies lab, equating to roughly one percent of the world's supply of fungal secondary metabolites.

Conducting natural product research generates a large amount of data. First, extractions are done to remove secondary metabolites from the organism serving as the source. The extractions contain a mixture of compounds, which go through bioassays to screen for biological activity. Approximately five percent of the extractions show biological activity and make it past this stage of research. The extractions that present biological activity must then undergo fractionation to begin untangling the web of mixed compounds. Another round of bioassays are conducted to evaluate the individual compound's activity. The compounds that show activity in the bioassays are then purified and their structure elucidation process begins. This phase of research includes many techniques, such as mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy, high performance liquid chromatography (HPLC) and liquid chromatography/mass spectrometry (LC/MS). The use of these tools often results in the elucidation of a lead structure, which is then studied and refined for many years before it likely can be considered as a potential drug.

Given the importance of natural product research and the vast amount of data that is generated in the process, it is imperative that the compounds and data are stored in an organized manner that will allow researchers to derive as much value as possible.

When determining the method for data storage, it is important to consider many factors. First, the data should be stored in a manner that will allow it to be readily accessible to those who need it, in this case being all of those working in Dr. Oberlies research group. Second, once stored, it should be easy to retrieve and make use of any particular piece of data. Moreover, as people frequently come and go from University research groups, it is important to have a standard data storage process that is easy to learn and use.

Keeping detailed laboratory notebooks is a universal data storage method for chemists. However, they are organized chronologically, and after only a few years of research the amount of time required to find one specific piece of data is extensive and impractical. Likewise, from a time and feasibility standpoint, it is not possible to build relationships between similar types of data generated over the lifetime of the lab. Therefore, it is now widely accepted that electronic storage of chemical research data is necessary to allow the data to be easily shared, retrieved and ultimately useful.[92]

One of the first scientific disciplines to pursue electronic means of data storage was chemistry.[92] Development in this field was undoubtedly motivated by constant growth in the amount of data generated by chemists. The earliest example comes from a publication in 1946 by King et al.[93] that describes a method of using punched cards to solve long calculations that ultimately aided them in generating and evaluating band spectra of asymmetrical rotors.[94] The Chemical Abstract Service (CAS) of the American Chemical Society was instrumental in furthering research in using computers to store chemical data throughout the 1950s.[94]

One key challenge that presented itself was the need for a method to store chemical structures electronically, as early computers operated solely with textual “alpha numeric” information.[95] CAS is credited with being the first to store chemical structures as

computer files that were searchable, in the 1960s. Progress throughout the 1970's included the development of methods to search databases for compounds based on their crystallographic data, an advancement that allowed researchers to select compounds based on their three-dimensional motif.[96]

The American Chemical Society first dedicated a journal to the importance of computerized storage of chemistry-related information in 1961 titled the *Journal of Chemical Documentation*.[95] As the field has evolved, the name of the journal has changed to the *Journal of Chemical Information and Computer Sciences* in 1975, and in 2005 to the *Journal of Chemical Information and Modeling*.[95]

The change in name of the journal representing this field is characteristic of the evolving name of the discipline itself. Until the late 1990's, the discipline was referred to interchangeably as chemometrics, computer chemistry and chemical informatics.[94] Societies of chemometrics and universities with "computer chemistry" in their name coexisted[94] and were focused mainly on making predictions about chemical data by applying statistical analysis to chemistry databases.[96] The term chemical informatics was used to describe the merging fields of chemistry and information technology.[96]

Concurrently, in 1976 the discipline of biology began using the term bioinformatics to describe the application of information technology to biology.[94] In 1998 Dr. Frank Brown first introduced the term chemoinformatics and defined it as "the mixing of those information resources to transform data into information and information into knowledge for the intended purpose of making better decisions faster in the arena of drug lead identification and optimization".[97] Today, chemoinformatics is used interchangeably with the term cheminformatics. However, the original definition has been expanded so as not to be limited to the process of drug discovery.[96]

A common example of utilizing chemoinformatics to aide in drug design is screening a virtual library of compounds for their ability to perform a targeted activity. In this example, researchers would build a virtual library of compounds that have been or could be synthesized. If they have information such as the crystal structure of a targeted enzyme, they could let a computer virtually screen the library for a compound that would bind to the active site. With their lead compound identified, they could synthesize it and begin the next step in their drug discovery process.

Using natural products as a source of drug discovery adds another dimension to the use of chemoinformatics. In this process, researchers conduct field collections to obtain a variety of organisms. Secondary metabolites from the organism are extracted in complex mixtures and usually undergo multiple fractionations until the compounds are purified. They are then assayed, and researchers may not determine the full chemical structure until after they receive assay results.

In this instance, chemoinformatics can be applied as a method to store all of the data generated in the discovery process, while easily revealing relationships between the data that might not otherwise be obvious, hence transforming “data into information and subsequently into knowledge”. This application of chemoinformatics can likewise guide the research being conducted. A major goal of the project described herein was to apply the principles of chemoinformatics to the research conducted by Dr. Oberlies lab group through the design and implementation of a computer database.

The secondary goal was to create an organized system for storing all of the pure compounds in Dr. Oberlies lab. Currently, each chemist in this lab follows their own method of compound storage, as shown in figures 3 and 4. This has led to disorganization, as only the original chemist can find any isolated pure compound. Each

of the isolated pure compounds is the product of weeks, if not months, of research time and tens of thousands of dollars of research grants. If a pure compound cannot be located within the lab, all of the research time and money is lost. Given the resources expended while isolating and characterizing these compounds, it is also critical to derive as much value as possible.

Dr. Oberlies research group pursues the discovery of new anticancer drug leads, and therefore screens compounds using a battery of anticancer bioassays. Other researchers around the world are studying a wide variety of human and animal diseases and utilize hundreds of other bioassays. It is very probable that in the future Dr. Oberlies lab may be interested in looking for drug leads for different diseases or may be using different bioassays to screen for anticancer activity.

Having the already isolated pure compounds stored in an organized manner will allow them to be leveraged into different bioassays in the future, increasing the productivity of the time and money spent on natural products research. An example of this was published by Dr. Matthias Hamburger and collaborators as they looked for natural product compounds that displayed potent HIV inhibition activity.[98] As a result of previous research, the authors had a library of over 800 plant and fungal extracts readily accessible. Instead of trying to duplicate years of expensive and time-intensive lab work extracting secondary metabolites from various organisms, they screened small aliquots of each extract in their cell-based antiretroviral bioassay.[98] This produced four daphnane diterpene compounds that inhibited the replication of HIV-1 while displaying no cytotoxicity at the concentrations tested.[98] More importantly, having a physical library of extracts on-hand allowed them to capitalize on research already conducted and derive the greatest value from their research.

Figure 3**.** Various Storage Methods Currently Utilized in Dr. Oberlies Lab.

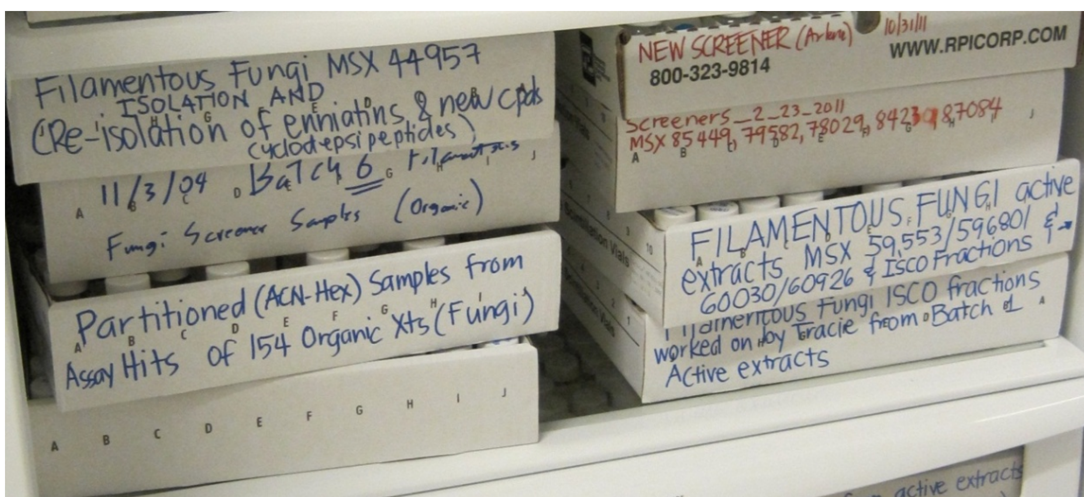


Figure 4. Example of Hand-written Labels on Boxes of Pure Compounds in Dr. Oberlies Lab.

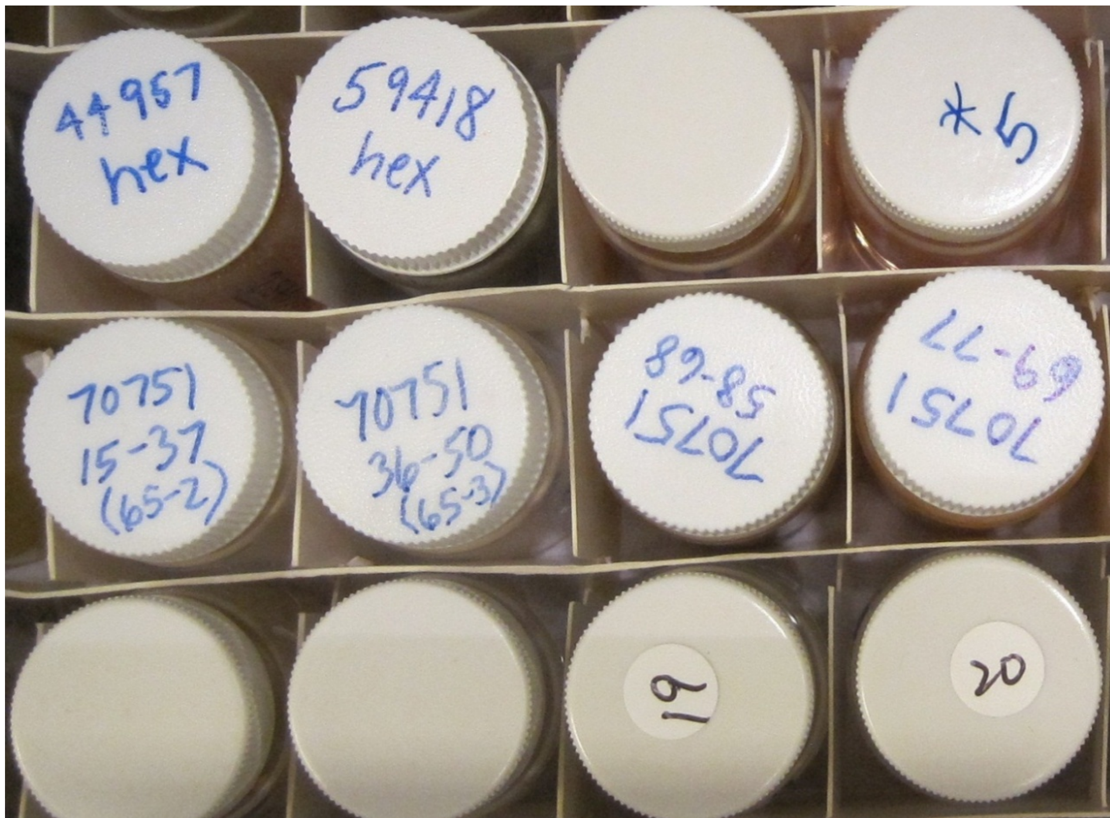


Figure 5. Example of Various Vial Labeling Methods Currently Utilized in Dr. Oberlies Lab.

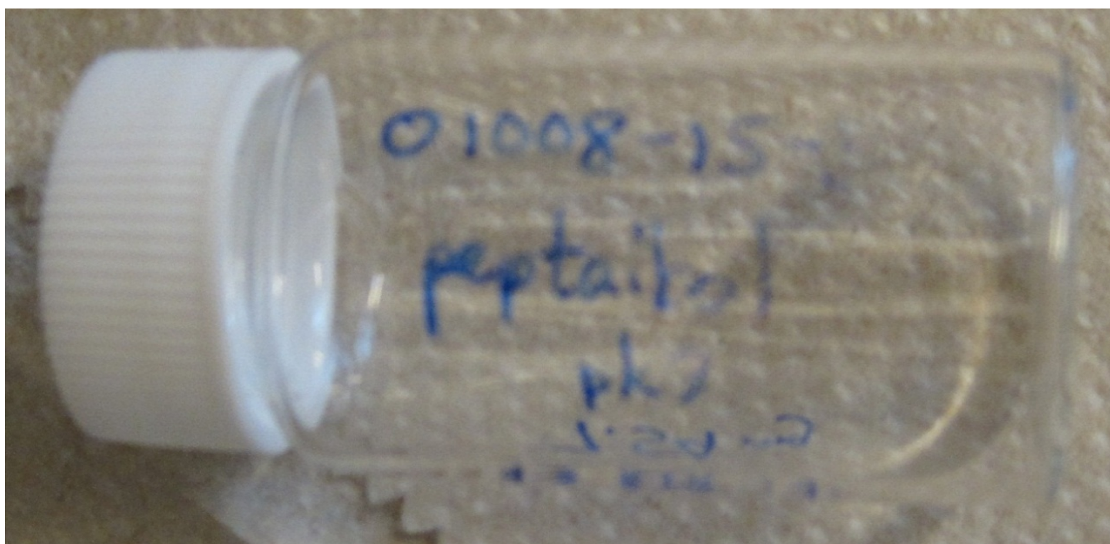


Figure 6. Example of Hand-written Labeling by Chemist in Dr. Oberlies Lab.

CHAPTER IX

METHODS

Database: All software used to populate the database was purchased from Advanced Chemistry Development, Inc. (Toronto, Ontario, Canada). The program in which the database was created was ACD/Spectrum Database, Product Version 12.5, ACD/Labs Release 12.00. Structures in the database were uploaded from ACD/ChemSketch. ACD/ SpecManager was used to process and upload spectroscopic data into the database.

Fields in the Record User Data and Document User Data windows of ADC/Spectrum Database were customized to capture the information that is specifically relevant to natural product research. The customized fields are as follows:

Record User Data

Compound Name	1H NH	Compound Storage
Notebook ID	13C	Crystal Structure
Specimen Number	COSY	Publication
Compound Amount	HSQC	Chemist
Project	HMBC	Entered by
Chromatogram	NOESY	Date
PDA Spectrum	ROESY	Date Modified
1H	Crystal Picture	

Document User Data

Sample No.	Order	IC50 SF268
Collection Site	GenBank Accession No.	IC50 HT-29
Collection Country/State	IC50 MCF-7	IC50 MDA-MB-435
Genus_species	IC50 H460	IC50 NF-kB

Compound Storage: The pure compounds are stored in 8 mL clear glass E-Z Ex-Traction vials, product W224618, purchased from Wheaton Industries, Inc. via Fisher Scientific. These vials were selected because they are the appropriate size, fit properly into vial files and are uniquely different from any other vial currently used in Dr. Oberlies lab. The vials are stored in an M-T Vial File, product W228790, purchased from Wheaton Industries, Inc. via Fisher Scientific. The vials were labeled with labels created via LabelView software produced by Teklynx International.

CHAPTER X

DATABASE RESULTS

As of February 17, 2012 the database contained 116 records, each of which represents one pure compound isolated in Dr. Oberlies lab. Multiple telephone calls were made to Advanced Chemistry Development, Inc. to determine how to fix minor software issues and adjust ACD/Spectrum Database settings. Approximately thirty-five compounds were initially entered to allow for testing and configuration of the database. Next, a protocol was written detailing how the database should be populated. Researchers in the lab who had compounds to be entered into the database were provided with the written protocol and one-on-one support to facilitate the entry of all data. Researchers gave feedback on the protocol, which was revised and clarified where needed. The final version of the written protocol is as follows.

Written Protocol for Entry of Data into Database

1 BEFORE YOU BEGIN

****IMPORTANT: BACK-UP DATABASE**** **NEVER SKIP THIS STEP**

The database is currently stored on the N drive, accessible only to the Oberlies research group.

To get to the database:

1. From your computer tabs, click on Computer.

2. Click on N directory (ChemBioChem)

3. Click on Custom folder. Here you will see the Oberlies lab group folder. Click on it and you will see the ACD database folder.

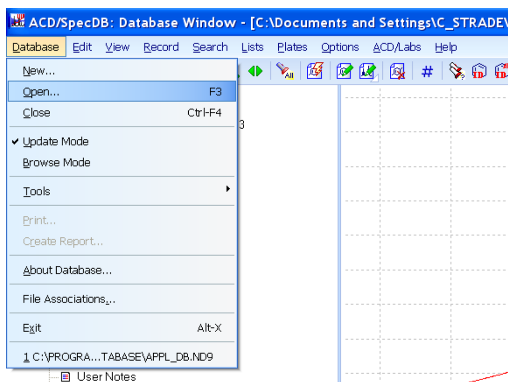
To back-up the database:

1. Right click on the database file and select “Copy”
2. While in the same folder on the N directory, again right click and select “Paste”
3. The file name should become “Copy of Oberlies Lab”. Right click this file and select “Rename”. Rename the file “Backup mm-dd-yr Oberlies Lab” with dd-mm-yr indicating the current date.

Note: Once you create a back-up file of the database, leave it alone. Work from the original “Oberlies Lab” file. The back-up file will be kept indefinitely and is to be used only to correct unfixable mistakes (such as the deletion of a record).

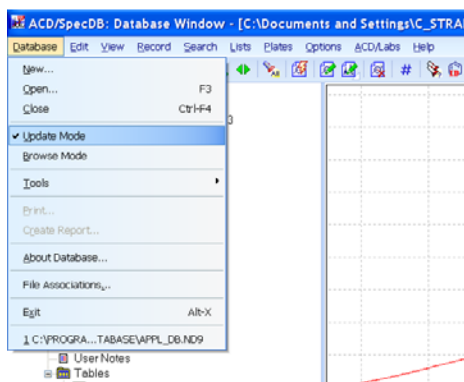
2 OPENING DATABASE

Open ACD’s Spectrum Database. Click “Database” on the toolbar, and then “Open”. To locate the database file, select the N drive, open the Custom Folder and then Oberlies Lab Group folder.

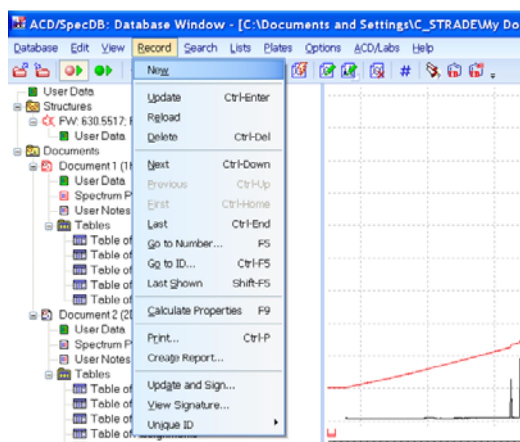


3 ADDING A NEW RECORD

Database always opens in Browse Mode (read only). To edit database, click “Database” on the toolbar, then “Update Mode”.



Click “Record” on the toolbar, and then “New”.



You now have a blank record. Each record should correspond to 1 pure compound. You will need to import all available data for this compound (NMR, MS, etc.), import a structure (if known), populate the “Document User Data” fields and the “Record User Data” fields for your compound in this 1 record. If any of this information is

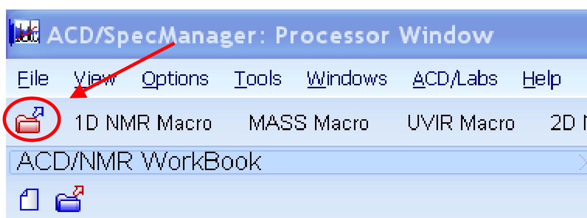
unknown, leave the field blank. **<u>NEVER</u> add information to this database that you do not believe is 100% accurate!**

4 ADDING SPECTRA TO A RECORD

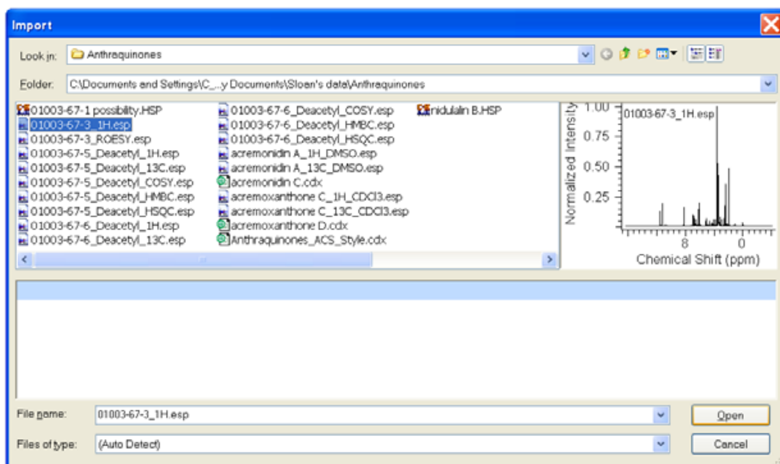
On the bottom left of the database window, select "Processor".

When the window shown below appears, make sure that "Open an Existing File" is selected, and then double click "More files".

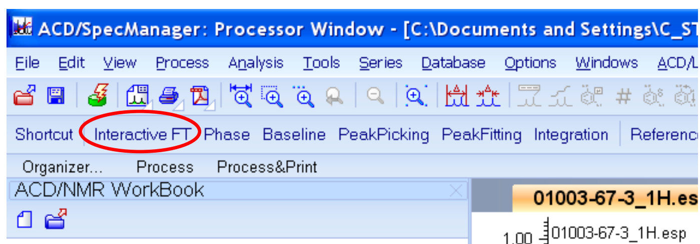
Alternatively, to open a data file when this window does not appear, click the red file icon in the upper left corner of the processor screen.



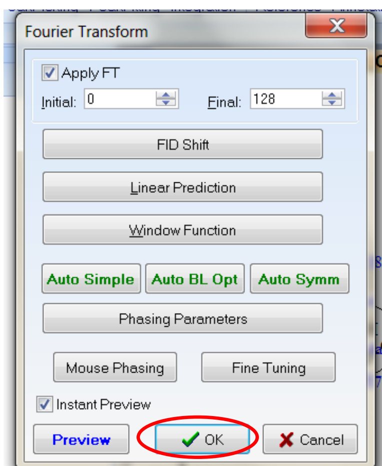
Locate the desired file and then click “Open”.



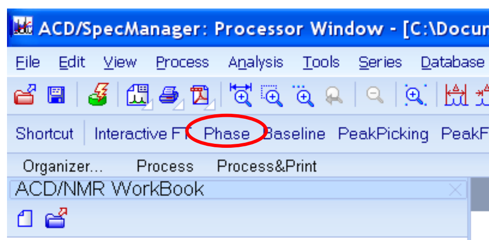
**If 1H spectra appears as FID (unprocessed), click “Interactive FT”.

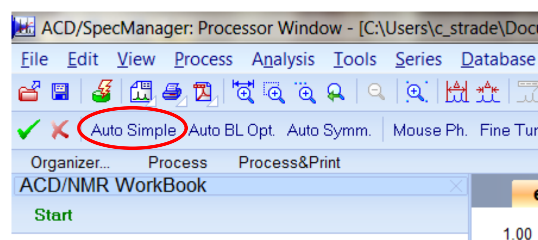


When the box below appears, click “OK”.

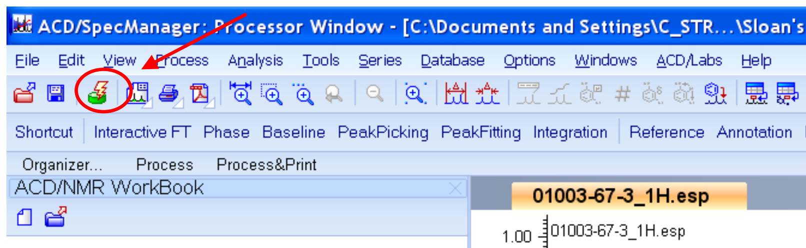


Next, click “Phase” and then “Autosimple” as shown below.



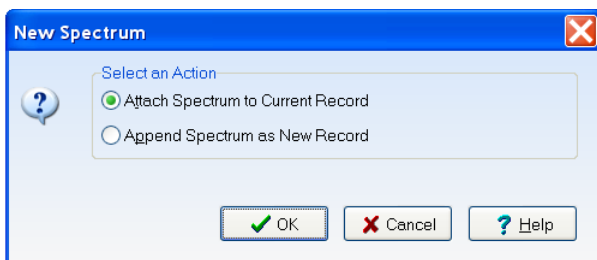


Once the file is opened and processed, click the green icon shown below to upload the file to the database.



If this warning appears, click “OK”:

When the box shown below appears, be very intentional about your selection.



(a) **Attach Spectrum to Currect Record** adds the file to the record you are viewing in the database

(b) **Append Spectrum as New Record** creates a new, blank record and adds your selected file. If you have already created a new record, you should choose option (a).

Note: If you are uncertain about which record you are currently viewing in the database, do not proceed. Click cancel, go back to the database window (by clicking the Database tab on the bottom left) and make sure you are uploading the correct file to the correct record.

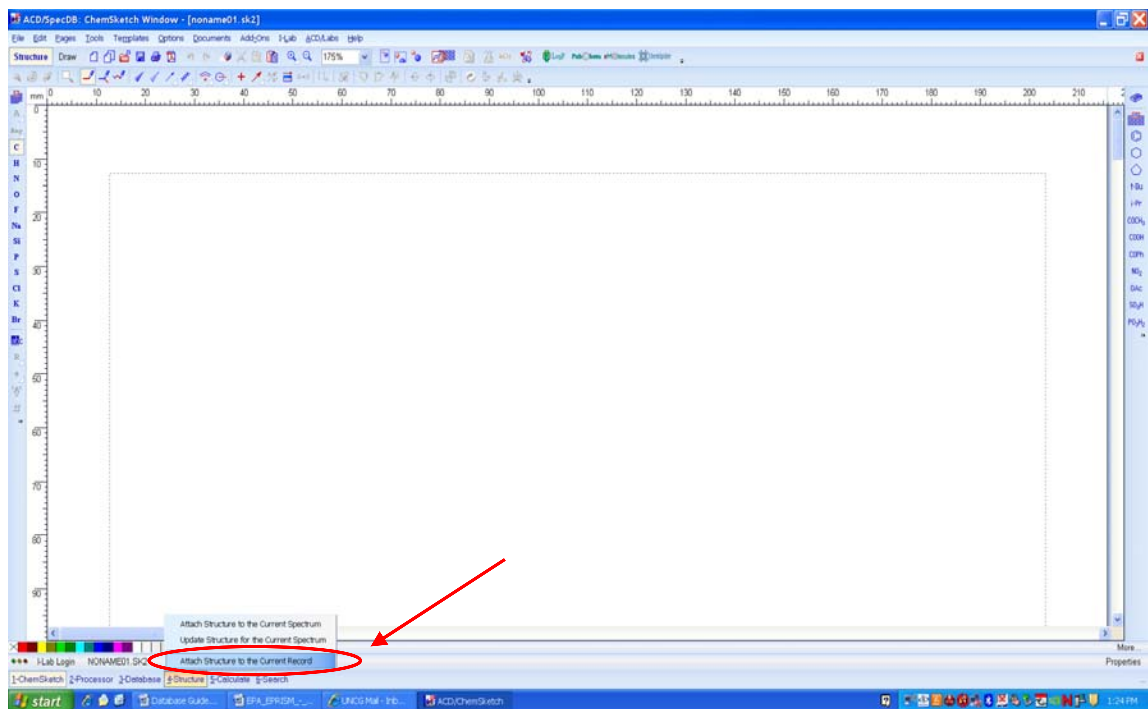
Once you click “OK”, you will automatically go from the Processor to the Database and should see your uploaded file. Repeat these steps to add all files relevant to your compound.

5 ADDING A STRUCTURE TO THE RECORD

On the bottom left of the database window, select “ChemSketch”

Once in ChemSketch, either open a file of your structure, draw the structure, or copy the structure and paste into ChemSketch.

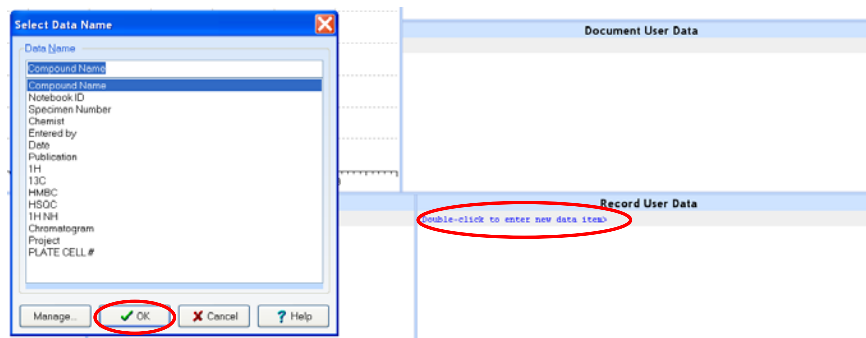
To upload the structure to the database, select the tab labeled “Structure” on the bottom left of the screen. Then select “Attach Structure to the Current Record”.



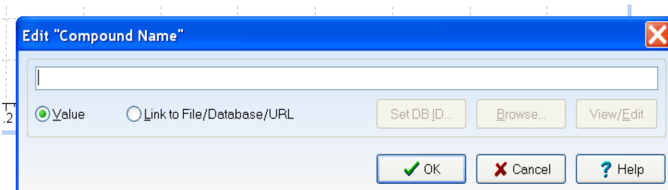
Note: If you are uncertain about which record you are currently viewing in the database, do not proceed. Go back to the database window (by clicking the Database tab on the bottom left) and make sure you are uploading the correct structure to the correct record. Once you click “Attach Structure to the Current Record”, you will automatically go from ChemSketch to the Database and should see your uploaded structure.

6 ADDING RECORD USER DATA

Under the “Record User Data” heading, double click the line that says “Double-click to enter new data item”. In the pop-up box that says “Select Data Name”, click the field that you wish to enter and then click “OK”.



The following box will appear.



(a) Value: to enter any alpha-numeric information, leave the selection as "Value", enter the information and click "OK".

(b) Link to File/Database/URL: only link files (not URL) in this database. Files to be linked include a pdf of the publication corresponding to the compound or any NMR/MS/Chromatogram files that are a pdf.

(c) To link a file to the database:

1. Find the appropriate file on your computer. Right-click the file and select "copy". Then, navigate into the N directory, click on "Custom Folder", "Oberlies Lab Group" and then "Linked Database Files".
2. If there is not a folder for the Fungus Number of the organism for the compound you are entering, right click and select "New", "Folder" and enter the Fungus Number as the folder name

3. In that folder, again right click and select “New”, “Folder” and enter the Notebook ID as the folder name.
4. Right-click and paste your file in the folder you created for the Notebook ID. Repeat this process for all files you would like to link.
5. In the database, select “Link to File/Database/URL” instead of “Value”
6. Click “Browse”. Browse to the file you pasted on the N directory. Select “OK”

Repeat this process to enter all of the known pieces of information in the Record User Data window. If a particular field is unknown, do not add it to the record. If you are entering multiple new records containing a lot of the same record user data, it is possible to right-click in the record user data window, select copy and then paste into your next record.

Example and format of Record User Data Fields:

Compound Name: Zeaenol

Notebook ID: 01003-47-2 (if 2, separate by a comma and space)

Specimen Number: MSX63935 (if 2, separate by a comma and space)

Compound Amount: 5.26 mg 12/06/2011 (total amount of compound in lab & date)

Chemist: Sloan Ayers (person in lab that isolated compound)

Entered by: Cherilyn Strader (your name, or person that is entering compound in database)

Date: 7/26/2011 (date compound is entered into database)

Compound Storage: 2 vials 1B3 2E8 **(**number of and location of vials in lab)

Publication: (Link)

1H: (Link)

13C: (Link)

HMBC: (Link)

HSQC: (Link)

1H NH: (Link)

Chromatogram: (Link)

Project: KinghornP01 CA125066

Plate Cell #: Do not enter

7 ADDING DOCUMENT USER DATA

Under the “Document User Data” header, follow the same process as entering Record User Data. If a particular field is unknown, do not add it to the record. If you are entering multiple new records containing a lot of the same document user data, it is possible to right-click in the document user data window, select copy and then paste into your next record.

Example and format of Document User Data Fields:

Sample No: G12345

Collection Site: Agricultural Farms, Univ of Nigeria

Collection Country/State: Nigeria

Genus_species: Phoma clematidina

Order: Pleosporales

GenBank Accession No: JF767207

8 HELPFUL INFORMATION ABOUT EDITING DATABASE

Saving: The database automatically saves every change you make. When you are finished working, simply click the red “X” in the top right-hand corner to close the database.

<u>Appearance of Structure</u>: Sometimes the database displays the structure a little differently than you may have entered it into ChemSketch. For example, a terminal CH_3 may be displayed as a straight line or two labeled –OH groups that are close together in the ChemSketch structure may overlap each other in the database view. You can right-click on the structure in the database and click "delete", tweak your drawn structure in ChemSketch and then upload it again to make small changes.

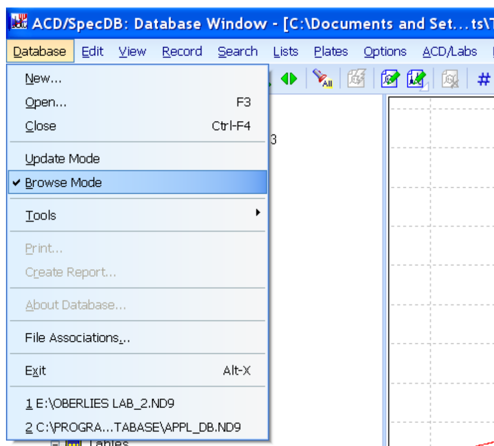
<u>Structure User Data</u>: When you upload a structure, the database automatically displays the formula and calculates the formula weight. If you know the compounds exact mass, enter it in the Structure User Data window.

A user-guide was also written that details how to perform searches within the database. This user guide is as follows.

Written User-Guide to Facilitate Searching the Database

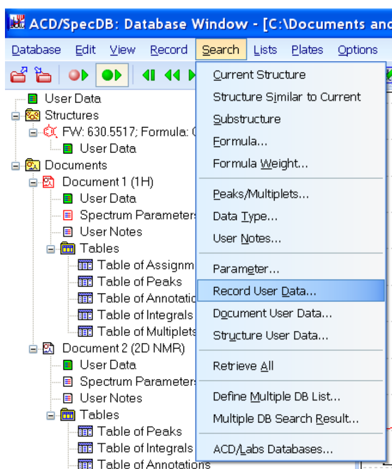
1 SEARCHING BY ALPHA-NUMERIC INFORMATION

First, ensure that the database is in "Browse Mode". To do this, click "Database" on the toolbar. You should see a check beside "Browse Mode". If you do not, click "Browse Mode".



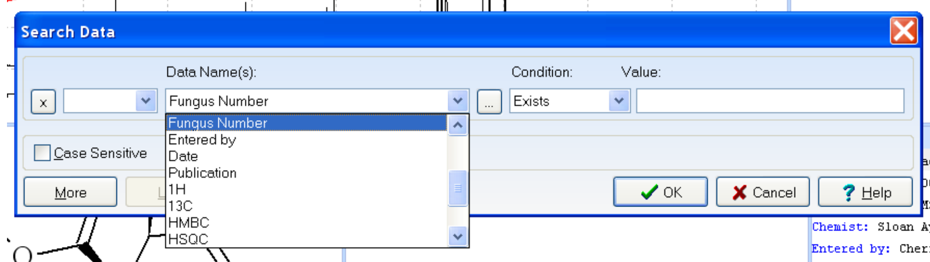
To search by a Record User Data or Document User Data field, on the top menu select

"Search" and then either "Record User Data" or "Document User Data".

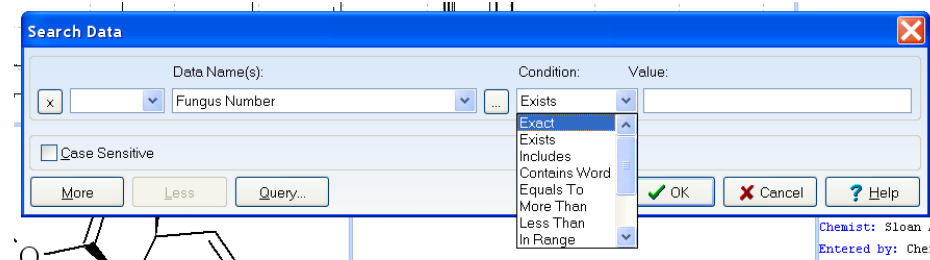


Multiple search options are then presented. Under "Data Name(s)" select the field you

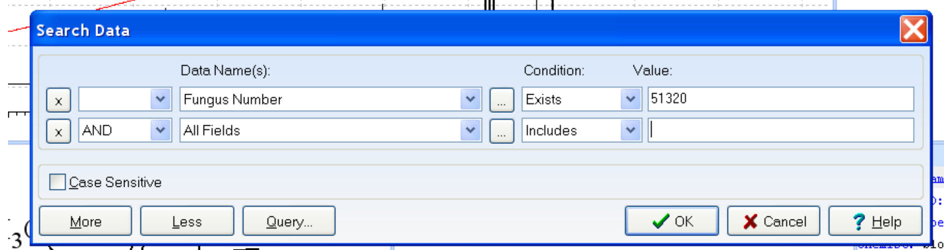
wish to search, or select "All Fields".



Then select the search condition. If you are looking for a specific piece of information, select “Exact”. The database will then find exact matches of the alpha-numeric text you enter in the specified Data Name (field).

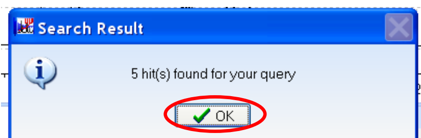


To search by multiple conditions, select “More”. To undo, select “Less”.

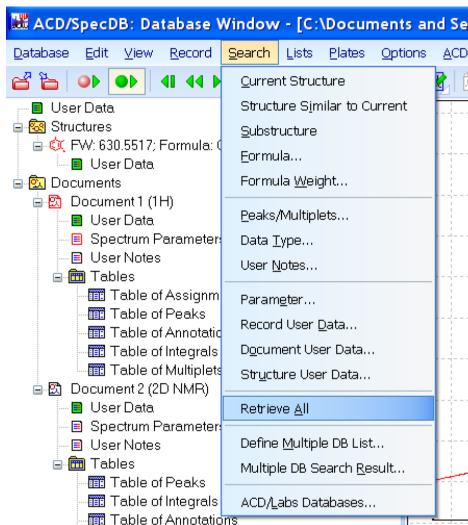


When you are ready to execute the search, select “OK”.

A pop-up box will display, showing how many results your search produced. Select “OK” to scroll through the results.

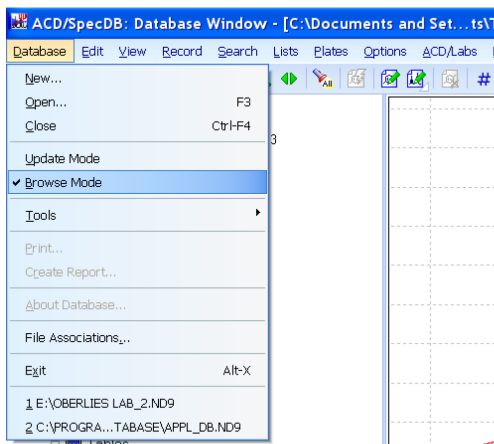


When you are finished viewing your search results, on the top menu select “Search” and then “Retrieve All” to view all records in the database.



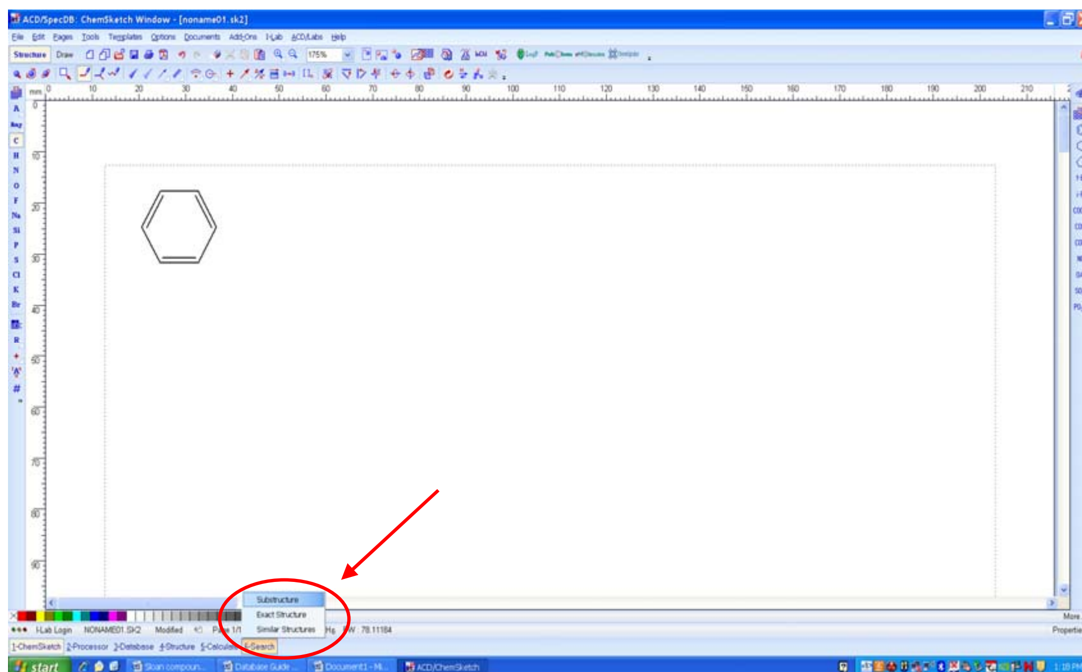
2 SEARCHING BY STRUCTURE

First, ensure that the database is in “Browse Mode”. To do this, click “Database” on the toolbar. You should see a check beside “Browse Mode”. If you do not, click “Browse Mode”.

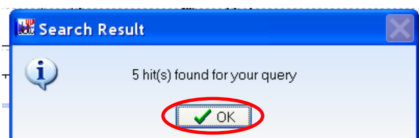


On the bottom left of the database window, select “ChemSketch”

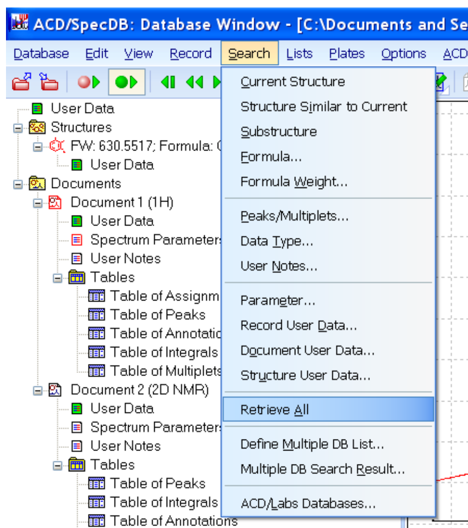
Once in ChemSketch, draw the full structure or sub-structure that you want to search for. When completed, click the “Search” tab on the bottom of the screen, and then select either “Substructure”, “Exact structure” or “Similar Structure”.



A pop-up box will display, showing how many results your search produced. Select “OK” to scroll through the results.

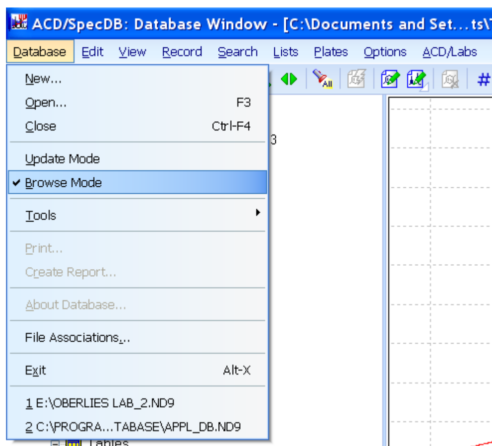


When you are finished viewing your search results, on the top menu select “Search” and then “Retrieve All” to view all records in the database.

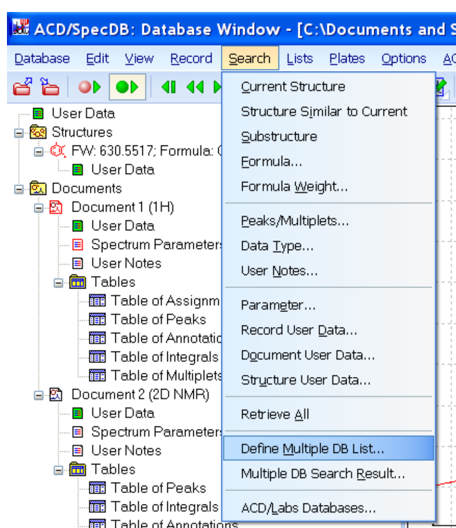


3 SEARCHING MULTIPLE DATABASES

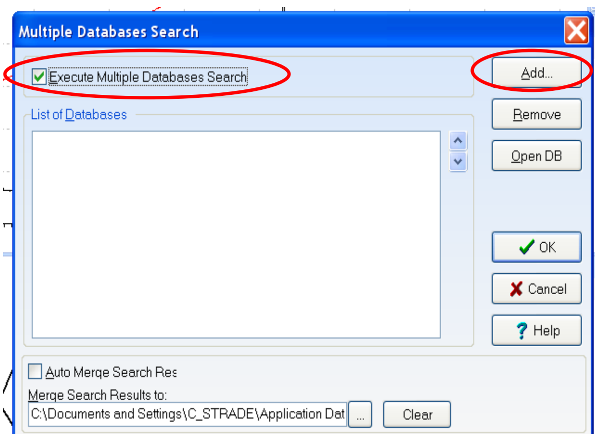
First, ensure that the database is in “Browse Mode”. To do this, click “Database” on the toolbar. You should see a check beside “Browse Mode”. If you do not, click “Browse Mode”.



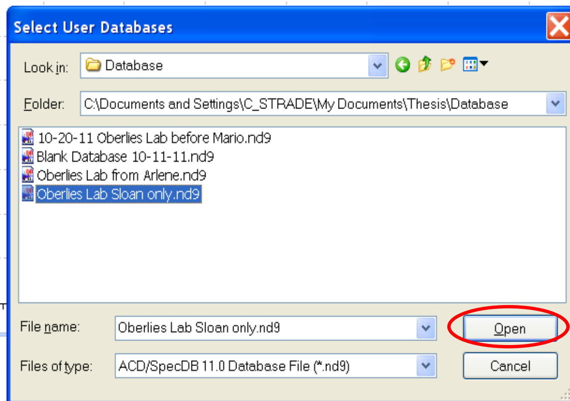
On the top menu, select “Search” and then “Define Multiple DB List”.



A pop-up box will display. Make sure that there is a check in the box to the left of "Execute Multiple Databases Search". If there is not, click that box. Next, click "Add".



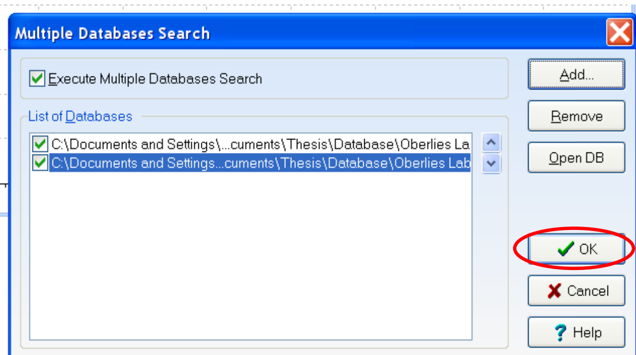
Locate the first database that you wish to search, select it and click "Open".



Repeat this process by clicking “Add”, selecting the second database you wish to search, and clicking “Open”.

There is no limit to the number of databases you can search at one time. However, the more databases you search simultaneously, the slower the search will be processed.

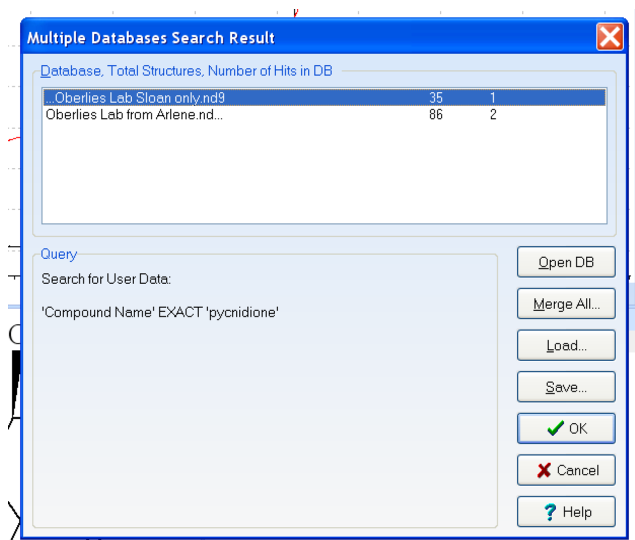
You should see all of the databases that you selected to search listed as shown in the box to the below.



When you are satisfied with the list of databases, click “OK”.

Next, select “Search” on the top toolbar and proceed by searching for any alpha-numeric information or by structure as described in the appropriate sections of this guide (Sections 9 and 10, respectively).

Once your search is processed, a box as shown below will appear displaying the number of results found in each database.

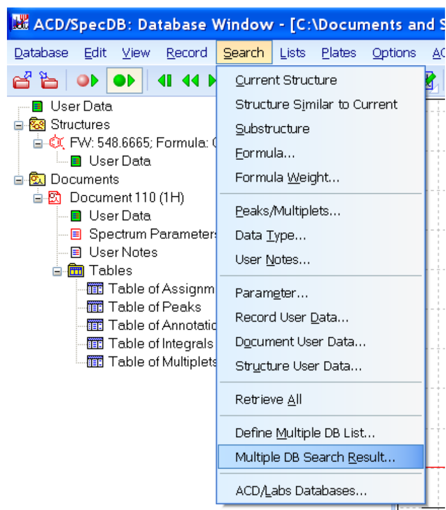


There are four options for viewing the search results, as described below.

(a) Open DB: recommended if you want to view the search results from one database, and then view the search results from the next database individually.

To view the results found in the first database, select it and click "Open DB". The first database will open and only the records found in that database matching your search criteria will be displayed.

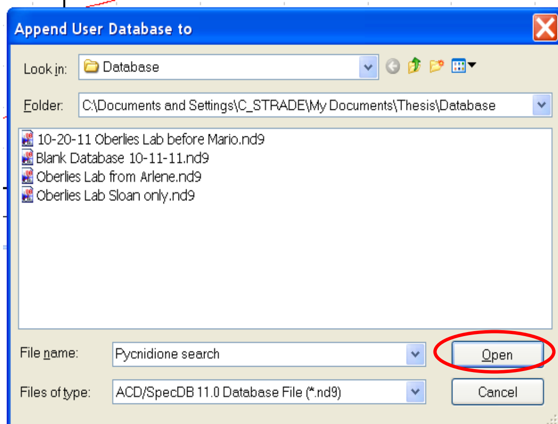
To subsequently view the search results found in the other database(s) searched, on the top toolbar select "Search" and then "Multiple DB Search Result". When the box reappears displaying the number of results found in each database, select the second database instead of the first.



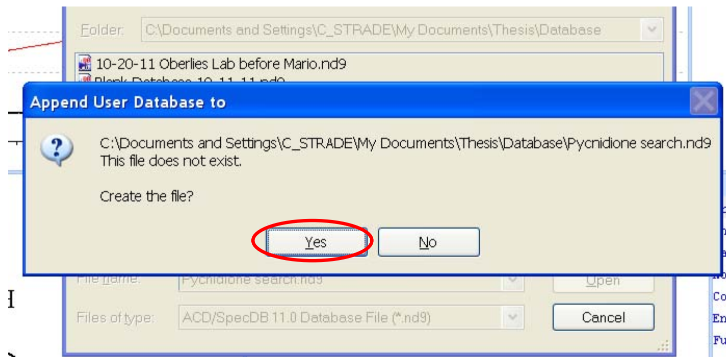
Then click “Open DB”. The second database will open and only the records found in that database matching your search criteria will be displayed.

(b) Merge All: recommended if you would like to merge all of the results into one file as a new database. It will require you to save the file of the merged results before you can view them.

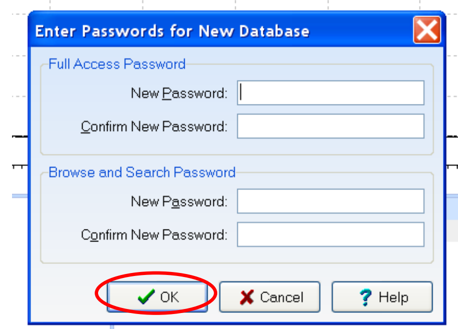
A box as shown below will appear. To merge the results into a new database, use the “Look in” drop-down menu to open the folder in which you wish to save the new database.



Change the “File name:” to reflect the name you wish to give the database, and then click “Open”. When the box shown below appears, click “Yes”.



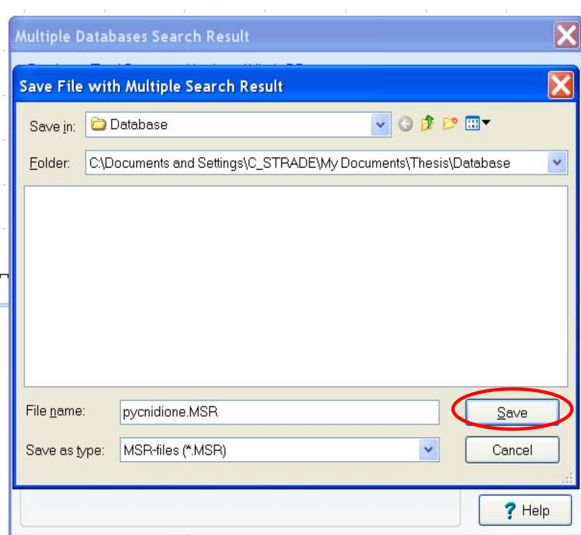
Next, a box will appear prompting you to set a password to either edit (“Full Access Password”) or view (“Browse and Search Password”) the new database you created containing your search results.



If you wish to set a password to edit or view the database, enter it and click “OK”. If you do not wish to set a password, leave all of the password fields blank and click “OK”.

To view the new database you created that contains only your search results, on the top toolbar select “Database”, “Open”, browse for the file and click “Open”.

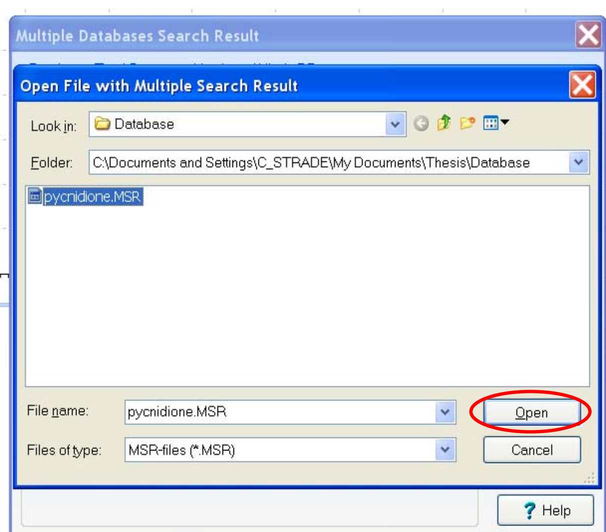
(c) Save: recommended if you will want to quickly load the same search results at another time.



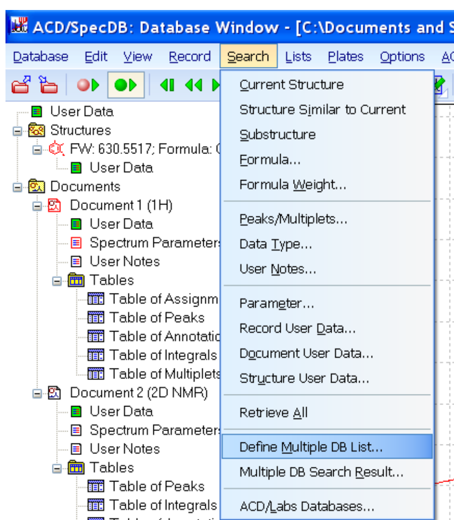
A box as shown above will appear. Use the “Save in” drop-down menu to open the folder in which you wish to save the search. Change the “File name:” to reflect the Name you wish to give the saved search, and then click “Save”.

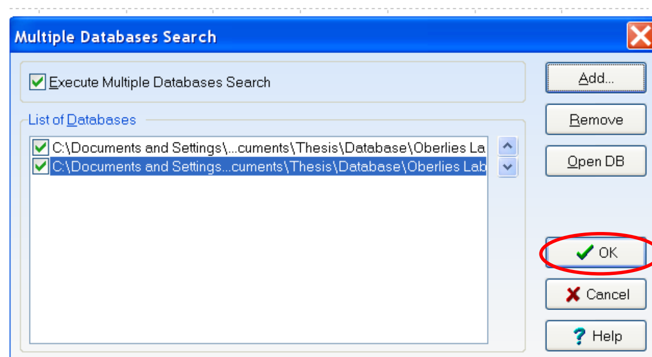
Note: the default file name that will be shown before you change it is *.MSR. In order to save the file, you must remove the *. If you do not remove the *, when you click “Save” nothing will happen.

(d) **Load:** used to load a previously saved search. Locate the saved search file that you wish to open, select it and click “Open”.

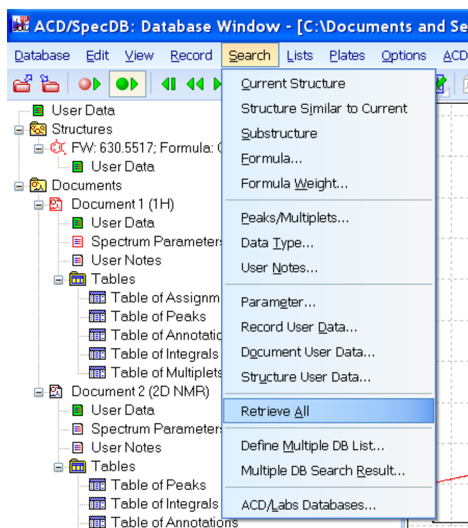


Important: When you are finished searching multiple databases, go back to "Search" on the top toolbar and select "Define Multiple DB List". Highlight each listed database and select "Remove". When finished, select "OK". If you do not do this, any search you perform will continue to be searched through the multiple databases you previously selected.





Important: When you are finished viewing your search results, on the top menu select "Search" and then "Retrieve All" to view all records in the database.



CHAPTER XI

DATABASE CHALLENGES

There were and will be challenges in populating and maintaining this database. Because multiple people use and populate the database, the highest risk is error in data entry. At present there are eight different people adding data to the database. These eight people represent five different countries and native languages. This diversity is not expected to change and only further complicates the task of getting specific scientific information entered into the database in a precise and systematic way.

If data is entered into the database incorrectly, the integrity and therefore the utility of the database will be jeopardized. If the information in the database is inaccurate, searches will not return accurate results, resulting in limited functionality. Because it is necessary to allow for flexibility in the format of data entered into the database, it is not possible to check for errors in an automated manner. Therefore, this risk is best mitigated by peer review.

An unanticipated challenge of this project was multiple entries, or records, being entered for one compound. Each chemist in the lab is responsible for entering the compounds that they isolate into the database. As an example, Dr. Sloan Ayers isolated the compound pycnidione from the fungus coded MSX50044 and a record was created in the database for pycnidione capturing all of the data he generated. Dr. Arlene Sy-Cordero also isolated the compound pycnidione, but from a different fungus coded MSX60926. She created a record in the database for pycnidione which includes all of the data that she generated for the compound.

Today, a search performed in the database for the compound pycnidione will produce two hits. As the lab continues to isolate and characterize pure compounds, it is impossible to predict how many different chemists will isolate the same compound from different organisms. Careful consideration was given to whether all of the data for one compound should be put into one record in the database, regardless of the chemist and source organism. It was concluded, however, that the best method of organization is to continue having each chemist enter their data about each compound they isolate separately, even if this results in the database containing multiple records for the same compound. Some records in the database may appear to be redundant, but each captures a unique set of data. Users of the database will therefore have to thoughtfully select the appropriate data for their specific needs if a compound search produces more than one hit.

Another database was created simultaneously by a mycologist in the same lab (Dr. Huzefa Raja) to capture all of the information and data relating to the collection of organisms. A sample of the fields in the database include the collector, collection site, country, state, latitude, longitude, temperature, habitat, pH and elevation. Once an organism is isolated, this database also includes details such as the fungus number, GenBank accession number for the organism, and the organism's genus and species. Some of the information is redundant, because details such as the fungus number and organism's taxonomy are entered into the ACD/Spectrum Database.

Careful consideration was given to whether or not the databases should be combined, but it was concluded that the two different databases will be maintained, independent of one another. The ACD/Spectrum Database will be used to locate data and information about any pure compound in the lab. If more information about source

of a pure compound is needed, a search will have to be executed in the other database. Neither of these searches require a significant amount of time, and keeping the databases separate should help reduce the burden of having to enter an extensive quantity of information in either database. Moreover, we concluded that the integrity of two specific data bases would be easier to maintain. This could be considered akin to the saying that “you should not put all your eggs in one basket”.

CHAPTER XII

COMPOUND STORAGE RESULTS

To start this process, each vial was quickly cleaned via blowing air into the vial to remove any dust. A label was prepared and placed on the outside of the vial, as shown in figure 5. Labels specify the compound name, notebook ID, specimen number, and a barcode which, when scanned, provides the notebook ID. Empty vials with labels affixed were weighed and the tare weight was neatly written on the vial. The tare weight was also entered into an Excel spreadsheet in the event that it is ever removed or becomes illegible on the vial.

Pure compounds were dissolved in either MeOH or $CHCl_3$ and transferred into the appropriately labeled vial. They were then vacuum-dried to evaporate the MeOH or $CHCl_3$. Vials containing the pure compounds were stored in vial file boxes, as shown in figure 6.

The vial file boxes have been numbered sequentially. Within each box, horizontal rows have been assigned a letter A through F and vertical rows have been assigned a letter 1 through 9. This provides each pure compound with a unique letter and number combination, such as 1A5, indicating box 1, horizontal row A, vertical row 5. This code allows all of the pure compounds to be catalogued and easily located. These codes have been entered into the database and the vial file boxes were placed in the freezer for long-term storage.

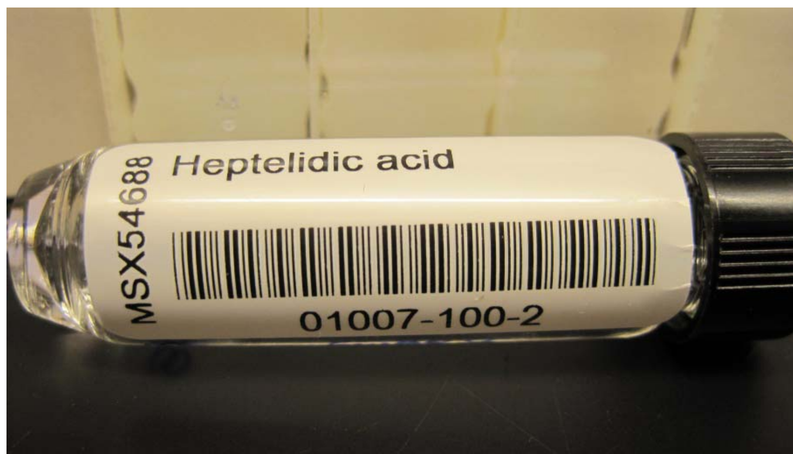


Figure 7. Labeled Vial for Pure Compound Storage.

Figure 8. Vial File Box of Pure Compounds.

As of February 20, 2012 there are approximately 161 appropriately labeled vials containing pure compounds that have been stored in a vial file box. The number of vials stored is higher than the number of records in the database, mainly because some compounds have been stored in more than one vial. This has occurred when, for example, a chemist takes a sample of the pure compound to perform an NMR experiment, and does not return the sample to the original vial. Likewise, when the amount of a pure compound in the lab exceeds the 8 mL storage capacity of one vial, the compound is stored in two identically labeled vials. The difference in the number of vials and the number of records in the database also occurs, to a lesser extent, because some compound data has not yet been uploaded to the database by the researcher.

The biggest challenge of developing and implementing this storage system was locating all of the pure compounds in the lab. Previously, all of the chemists used their own method for storing the pure compounds that they isolated. Some of the chemists who have isolated pure compounds in the lab have since left the research group, making it a challenge to determine where and how their compounds were stored. Additionally, as can be seen in figure 6, most of the chemists identified their compounds by writing on the vial with a Sharpie marker. However, over time some of the writing has been smudged or erased, making it very difficult to determine what was in each vial.

A protocol has been written for this process and is as follows.

OBERLIES LAB PURE COMPOUND PROTOCOL

1. Print a label for the vial.
    a. In the Label View software, open the “Cherilyn-mini-label” template

b. The field lengths will likely need adjusted. To do this, click on the barcode. Right click and select “Edit”. Next, select “Define Field Lengths” on the lower right side of the pop-up box.

c. Adjust the field lengths and enter the information in the fields as below:

Field	**Field Length**	**Information to Enter in Field**
Notebook ID	13	Notebook ID
Lengthy Text	23	Compound Name
Weight	8	Fungus Number

2. Make sure the inside of the pure compound vial is clean, and affix your label to it.
3. Weigh the clean, empty, labeled vial and neatly write the weight in grams on the lower part of the vial with a Sharpie.
4. Open the excel work book titled “Vial Weights” in the Oberlies Lab Group folder on the N drive. Enter your compound name, notebook ID and the weight in grams of your empty, labeled vial there.
5. Transfer your pure compound into the vial.
6. Weigh the vial with your dry, pure compound in it. Write the amount of the compound, in milligrams, neatly on the middle of the vial with a Sharpie.
7. Place the vial into a vial file box.
8. Note the Box Number (vial file box should say “Box 1”, for example, on top). If it is a new vial file box, use a permanent marker to write a box number onto the paper insert so that it is viewable from the outside. Boxes are to be numbered sequentially.
9. Note the lettered horizontal row and numbered vertical row in which your compound vial is placed.

10. Enter the box number, row letter and row number into the pure compound database in that order. Example for formatting: 1A5 would be the compound in box 1, horizontal row A, vertical row 5.
11. Place the box in the appropriate freezer, where all other pure compound vial file boxes are stored.

CHAPTER XIII

CONCLUSION

Using ACD/Spectrum Database software, a library of natural product pure compounds was created. While challenges with the database, such as redundant or erroneous entries, will continue to be present, they should be less frequent if the protocol is properly followed and are able to be mitigated through peer-review. The compounds were also physically stored in an organized and systematic manner that will allow the compounds to be leveraged into other bioassays in the future. Protocols were written that will aide in the long-term maintenance of the database and compound storage system. Moreover, the frame work of the database can be leveraged into other needs that develop over time.

REFERENCES

(1) Anonymous, *Atlas Multiple Sclerosis Resources in the World 2008*. WHO Press: Geneva, 2008; pp 56.

(2) Motl, R. W.; Suh, Y.; Weikert, M. *J. Pain Symptom Manage.* **2010**, *39*, 1025-1032.

(3) McAlpine, D. *Br. Med. J.* **1957**, *1*, 475-480.

(4) Riise, T.; Grønning, M.; Aarli, J. A.; Nyland, H.; Larsen, J. P.; Edland, A. *J. Clin. Epidemiol.* **1988**, *41*, 1031-1036.

(5) Phadke, J. G. *J. Neurol. Neurosurg. Psychiatry* **1987**, *50*, 523-531.

(6) Weinshenker, B. G.; Bass, B.; Rice, G. P. A.; Noseworthy, J.; Carriere, W.; Baskerville, J.; Ebers, G. C. *Brain* **1989**, *112 (Pt 1)*, 133-146.

(7) Weinshenker, B. G. *Ann. Neurol.* **1994**, *36*, S6-S11.

(8) Lublin, F. D.; Reingold, S. C. *Neurology* **1996**, *46*, 907-911.

(9) Hafler, D. A. *J. Clin. Invest.* **2004**, *113*, 788-794.

(10) Confavreux, C.; Vukusic, S.; Moreau, T.; Adeleine, P. *N. Engl. J. Med.* **2000**, *343*, 1430-1438.

(11) Compston, A.; Coles, A. *Lancet* **2002**, *359*, 1221-1231.

(12) Frohman, E. M.; Racke, M. K.; Raine, C. S. *N. Engl. J. Med.* **2006**, *354*, 942-955.

(13) Peterson, L. K.; Fujinami, R. S. *J. Neuroimmunol.* **2007**, *184*, 37-44.

(14) McDonald, W. I.; Compston, A.; Edan, G.; Goodkin, D.; Hartung, H. P.; Lublin, F. D.; McFarland, H. F.; Paty, D. W.; Polman, C. H.; Reingold, S. C.; Sandberg-Wollheim,

M.; Sibley, W.; Thompson, A.; van den Noort, S.; Weinshenker, B. Y.; Wolinsky, J. S. *Ann. Neurol.* **2001**, *50*, 121-127.

(15) Goodin, D. S.; Frohman, E. M.; Garmany, G. P.; Halper, J.; Likosky, W. H.; Lublin, F. D.; Silberberg, D. H.; Stuart, W. H.; van den Noort, S. *Neurology* **2002**, *58*, 169-178.

(16) Rizvi, S. A.; Agius, M. A. *Neurology* **2004**, *63 (Suppl. 6)*, S8-S14.

(17) Duquette, P.; Girard, M.; Despault, L.; Dubois, R.; Knobler, R. L.; Lublin, F. D.; Kelley, L.; Francis, G. S.; Lapierre, Y.; Antel, J.; Freedman, M.; Hum, S.; Greenstein, J. I.; Mishra, B.; Muldoon, J.; Whitaker, J. N.; Evans, B. K.; Layton, B.; Sibley, W. A.; Laguna, J.; Krikawa, J.; Paty, D. W.; Oger, J. J.; Kastrukoff, L. F.; Moore, G. R. W.; Hashimoto, S. A.; Morrison, W.; Nelson, J.; Goodin, D. S.; Massa, S. M.; Gutteridge, E.; Arnason, B. G. W.; Noronha, A.; Reder, A. T.; Martia, R.; Ebers, G. C.; Rice, G. P. A.; Lesaux, J.; Johnson, K. P.; Panitch, H. S.; Bever, C. T.; Conway, K.; Wallenberg, J. C.; Bedell, L.; van den Noort, S.; Weinshenker, B.; Weiss, W.; Reingold, S.; Pachner, A.; Taylor, W. *Neurology* **1993**, *43*, 655-661.

(18) Jacobs, L. D.; Cookfair, D. L.; Rudick, R. A.; Herndon, R. M.; Richert, J. R.; Salazar, A. M.; Fischer, J. S.; Goodkin, D. E.; Granger, C. V.; Simon, J. H.; Alam, J. J.; Bartoszak, D. M.; Bourdette, D. N.; Braiman, J.; Brownscheidle, C. M.; Coats, M. E.; Cohan, S. L.; Dougherty, D. S.; Kinkel, R. P.; Mass, M. K.; Munschauer, F. E.; Priore, R. L.; Pullicino, P. M.; Scherokman, B. J.; Weinstock-Guttman, B.; Whitham, R. H. *Ann. Neurol.* **1996**, *39*, 285-294.

(19) Ebers, G. C.; Rice, G.; Lesaux, J.; Paty, D.; Oger, J.; Li, D. K. B.; Beall, S.; Devonshire, V.; Hashimoto, S.; Hooge, J.; Kastrukoff, L.; Krieger, C.; Mezei, M.; Seland, P.; Vorobeychi, G.; Morrison, W.; Nelson, J.; Freedman, M. S.; Chrisie, S.; Nelson, R.;

Rabinovitch, H.; Freedman, C.; Hartung, H. P.; Rieckmann, P.; Archelos, J.; Jung, S.; Weilbach, F.; Flachenecke, P.; Sauer, J.; Hommes, O.; Jongen, P.; Brouwer, S.; McLeod, J.; Pollard, J.; Ng, R.; Sandberg-Wollheim, M.; Kallen, K.; Nilsson, P.; Ekberg, R.; Lundgren, A.; Jadback, G.; Wikstrom, J.; Multanen, J.; Valjakka, M.; Carton, H.; Lissoir, F.; Declerq, I.; Vieren, M.; Peeters, E.; Dubois, B.; Dekeersmaeker, E.; Van Herle, A.; Hughes, R. A. C.; Sharrack, B.; Soudain, S.; Panelius, M.; Eralinna, J.; Soilu-Hanninen, M.; Murto, S.; Medaer, R.; Broeckx, J.; Vanroose, E.; Bogaers, A.; Blumhardt, L. D.; Edwards, S.; Liu, C.; Orpe, V.; Barnes, D.; Schwartz, M.; Stoy, N.; Harraghy, C.; Bertelsmann, F.; Uitdehaag, B.; Nasseri, K.; Chofflon, M.; Roth, S.; Kappos, L.; Huber, S.; Bellaiche, Y.; Senn, C.; King, J.; Jubert, J.; Whitten, S.; Newsom-Davis, J. M.; Palace, J.; Lee, M.; Evangelou, N.; Pinto, A.; Cavey, A.; Sindic, C. J. M.; Monteyne, P.; Verougstraete, D.; Van Doorn, P. A.; Moll, W.; Visser, L.; Willems, M.; Martina, I.; Buljevac, D.; Loman, L.; Bates, D.; Pandit, D.; Irving, J.; Rhodes, B.; Riddehough, A.; Zhao, G. J.; Wang, X.; Cheng, Y.; Ammoury, N.; Dupont, F.; Galazka, A.; Hyde, R.; Olson, M.; Pernin, M. O.; Abdul-Ahad, A. K.; Hommes, O.; Noseworthy, J.; Borden, E.; O'Brien, P.; Wolinsky, J. *Lancet* **1998**, *352*, 1498-1504.

(20) Dreyfuss, M.; Härri, E.; Hofmann, H.; Kobel, H.; Pache, W.; Tscherter, H. *Eur. J. Appl. Microbiol.* **1976**, *3*, 125-133.

(21) Kino, T.; Hatanaka, H.; Hashimoto, M.; Nishiyama, M.; Goto, T.; Okuhara, M.; Kohsaka, M.; Aoki, H.; Imanaka, H. *J. Antibiot.* **1987**, *40*, 1249-1255.

(22) Tanaka, H.; Kuroda, A.; Marusawa, H.; Hatanaka, H.; Kino, T.; Goto, T.; Hashimoto, M.; Taga, T. *J. Am. Chem. Soc.* **1987**, *109*, 5031-5033.

(23) Brinkmann, V.; Pinschewer, D.; Chiba, K.; Feng, L. *Trends Pharmacol. Sci.* **2000**, *21*, 49-52.

(24) Busuttil, R. W.; McDiarmid, S.; Klintmalm, G. B.; Goldstein, R.; Miller, C. M.; Schwartz, M.; Shaw, B. W.; Roberts, J. P.; Hebert, M. F.; Esquivel, C. O.; Nakazato, P.; Wiesner, R. H.; Krom, R. A. F.; Kalayoglu, M.; D'Alessandro, A. M.; Marsh, J. W.; Peters, M. G.; Burdick, J.; Klein, A.; Lewis, W. D.; Jenkins, R.; Thistlethwaite, J. R.; Emond, J. C.; Jusko, W. J.; D'Ambrosio, R.; Buell, D.; Fitzsimmons, W. E. *N. Engl. J. Med.* **1994**, *331*, 1110-1115.

(25) Neuhaus, P.; Pichlmayr, R.; Williams, R.; Bechstein, W. O.; Blumhardt, G.; McMaster, P.; Mayer, D.; Buckels, J.; Calne, R.; Friend, P.; Joughin, C.; Winkler, M.; Ringe, B.; Otto, G.; Bleyl, J.; Devlin, J.; O'Grady, J.; Groth, C.; Ericzon, B.; Duraj, F.; Bismuth, H.; Samuel, D.; Rucay, P. *Lancet* **1994**, *344*, 423-428.

(26) Kluepfel, D.; Bagli, J.; Baker, H.; Charest, M. P.; Kudelski, A.; Sehgal, S. N.; Vezina, C. *J. Antibiot.* **1972**, *25*, 109-115.

(27) O'Connor, P.; Comi, G.; Montalban, X.; Antel, J.; Radue, E. W.; de Vera, A.; Pohlmann, H.; Kappos, L. *Neurology* **2009**, *72*, 73-79.

(28) Cohen, J. A.; Barkhof, F.; Comi, G.; Hartung, H. P.; Khatri, B. O.; Montalban, X.; Pelletier, J.; Capra, R.; Gallo, P.; Izquierdo, G.; Tiel-Wilck, K.; de Vera, A.; Jin, J.; Stites, T.; Wu, S.; Aradhye, S.; Kappos, L. *N. Engl. J. Med.* **2010**, *362*, 402-415.

(29) Kappos, L.; Radue, E. W.; O'Connor, P.; Polman, C.; Hohlfeld, R.; Calabresi, P.; Selmaj, K.; Agoropoulou, C.; Leyk, M.; Zhang-Auberson, L.; Burtin, P. *N. Engl. J. Med.* **2010**, *362*, 387-401.

(30) Adachi, K.; Chiba, K. *Perspect. Medicin. Chem.* **2007**, *1*, 11-23.

(31) Fujita, T.; Inoue, K.; Yamamoto, S.; Ikumoto, T.; Sasaki, S.; Toyama, R.; Chiba, K.; Hoshino, Y.; Okumoto, T. *J. Antibiot.* **1994**, *47*, 208-215.

(32) Im, D. S. *Trends Pharmacol. Sci.* **2003**, *24*, 2-4.

(33) Sung, G. H.; Hywel-Jones, N. L.; Sung, J. M.; Luangsa-Ard, J. J.; Shrestha, B.; Spatafora, J. W. *Stud. Mycol.* **2007**, *57*, 5-59.

(34) Petch, T. *Transactions of the British Mycological Society* **1924**, *10*, 28-45.

(35) Fujita, T.; Hirose, R.; Yoneta, M.; Sasaki, S.; Inoue, K.; Kiuchi, M.; Hirase, S.; Chiba, K.; Sakamoto, H.; Arita, M. *J. Med. Chem.* **1996**, *39*, 4451-4459.

(36) Aragozzini, F.; Manachini, P. L.; Craveri, R.; Rindone, B.; Scolastico, C. *Tetrahedron* **1972**, *28*, 5493-5498.

(37) Fujita, T.; Inoue, K.; Yamamoto, S.; Ikumoto, T.; Sasaki, S.; Toyama, R.; Yoneta, M.; Chiba, K.; Hoshino, Y.; Okumoto, T. *J. Antibiot.* **1994**, *47*, 216-224.

(38) Fujita, T.; Hamamichi, N.; Kiuchi, M.; Matsuzaki, T.; Kitao, Y.; Inoue, K.; Hirose, R.; Yoneta, M.; Sasaki, S.; Chiba, K. *J. Antibiot.* **1996**, *49*, 846-853.

(39) Adachi, K.; Kohara, T.; Nakao, N.; Arita, M.; Chiba, K.; Mishina, T.; Sasaki, S.; Fujita, T. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 853-856.

(40) Kiuchi, M.; Adachi, K.; Kohara, T.; Teshima, K.; Masubuchi, Y.; Mishina, T.; Fujita, T. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 101-106.

(41) Fujita, T.; Yoneta, M.; Hirose, R.; Sasaki, S.; Inoue, K.; Kiuchi, M.; Hirase, S.; Adachi, K.; Arita, M.; Chiba, K. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 847-852.

(42) Fujita, T.; Hirose, R.; Hamamichi, N.; Kitao, Y.; Sasaki, S.; Yoneta, M.; Chiba, K. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 1857-1860.

(43) To the best of our knowledge, the configuration at position 14 in ISP-I-28 (**3**) was not determined.

(44) Kiuchi, M.; Adachi, K.; Kohara, T.; Minoguchi, M.; Hanano, T.; Aoki, Y.; Mishina, T.; Arita, M.; Nakao, N.; Ohtsuki, M.; Hoshino, Y.; Teshima, K.; Chiba, K.; Sasaki, S.; Fujita, T. *J. Med. Chem.* **2000**, *43*, 2946-2961.

(45) Seidel, G.; Laurich, D.; Fürstner, A. *J. Org. Chem.* **2004**, *69*, 3950-3952.

(46) Sugiyama, S.; Arai, S.; Kiriyama, M.; Ishii, K. *Chem. Pharm. Bull.* **2005**, *53*, 100-102.

(47) Kim, S.; Lee, H.; Lee, M.; Lee, T. *Synthesis* **2006**, 753-755.

(48) Durand, P.; Peralba, P.; Sierra, F.; Renaut, P. *Synthesis* **2000**, 505-506.

(49) Kalita, B.; Barua, N. C.; Bezbarua, M. S.; Bez, G. *Synlett* **2001**, 1411-1414.

(50) Hale, J. J.; Yan, L.; Neway, W. E.; Hajdu, R.; Bergstrom, J. D.; Milligan, J. A.; Shei, G. J.; Chrebet, G. L.; Thornton, R. A.; Card, D.; Rosenbach, M.; Rosen, H.; Mandala, S. *Bioorg. Med. Chem.* **2004**, *12*, 4803-4807.

(51) Takeda, S.; Chino, M.; Kiuchi, M.; Adachi, K. *Tetrahedron Lett.* **2005**, *46*, 5169-5172.

(52) Kiuchi, M.; Adachi, K.; Tomatsu, A.; Chino, M.; Takeda, S.; Tanaka, Y.; Maeda, Y.; Sato, N.; Mitsutomi, N.; Sugahara, K.; Chiba, K. *Bioorg. Med. Chem.* **2005**, *13*, 425-432.

(53) Albert, R.; Hinterding, K.; Brinkmann, V.; Guerini, D.; Müller-Hartwieg, C.; Knecht, H.; Simeon, C.; Streiff, M.; Wagner, T.; Welzenbach, K.; Zécri, F.; Zollinger, M.; Cooke, N.; Francotte, E. *J. Med. Chem.* **2005**, *48*, 5373-5377.

(54) Lu, X.; Bittman, R. *Tetrahedron Lett.* **2006**, *47*, 825-827.

(55) Matsumoto, N.; Hirose, R.; Sasaki, S.; Fujita, T. *Chem. Pharm. Bull.* **2008**, *56*, 595-597.

(56) Brinkmann, V.; Billich, A.; Baumruker, T.; Heining, P.; Schmouder, R.; Francis, G.; Aradhye, S.; Burtin, P. *Nat. Rev. Drug Discov.* **2010**, *9*, 883-897.

(57) Kharel, Y.; Lee, S.; Snyder, A. H.; Sheasley-O'Neill, S. L.; Morris, M. A.; Setiady, Y.; Zhu, R.; Zigler, M. A.; Burcin, T. L.; Ley, K.; Tung, K. S. K.; Engelhard, V. H.;

Macdonald, T. L.; Pearson-White, S.; Lynch, K. R. *J. Biol. Chem.* **2005**, *280*, 36865-36872.

(58) Chun, J.; Hartung, H. P. *Clin. Neuropharmacol.* **2010**, *33*, 91-101.

(59) Brinkmann, V. *Yonsei Med. J.* **2004**, *45*, 991-997.

(60) Sanchez, T.; Estrada-Hernandez, T.; Paik, J. H.; Wu, M. T.; Venkataraman, K.; Brinkmann, V.; Claffey, K.; Hla, T. *J. Biol. Chem.* **2003**, *278*, 47281-47290.

(61) Mandala, S.; Hajdu, R.; Bergstrom, J.; Quackenbush, E.; Xie, J.; Milligan, J.; Thornton, R.; Shei, G. J.; Card, D.; Keohane, C.; Rosenbach, M.; Hale, J.; Lynch, C. L.; Rupprecht, K.; Parsons, W.; Rosen, H. *Science* **2002**, *296*, 346-349.

(62) Brinkmann, V.; Davis, M. D.; Heise, C. E.; Albert, R.; Cottens, S.; Hof, R.; Bruns, C.; Prieschl, E.; Baumruker, T.; Hiestand, P.; Foster, C. A.; Zollinger, M.; Lynch, K. R. *J. Biol. Chem.* **2002**, *277*, 21453-21457.

(63) Kovarik, J. M.; Hartmann, S.; Bartlett, M.; Riviere, G. J.; Neddermann, D.; Wang, Y. B.; Port, A.; Schmouder, R. L. *Biopharm. Drug Dispos.* **2007**, *28*, 97-104.

(64) Liu, H.; Sugiura, M.; Nava, V. E.; Edsall, L. C.; Kono, K.; Poulton, S.; Milstien, S.; Kohama, T.; Spiegel, S. *J. Biol. Chem.* **2000**, *275*, 19513-19520.

(65) Hannun, Y. A.; Luberto, C.; Argraves, K. M. *Biochemistry* **2001**, *40*, 4893-4903.

(66) Zollinger, M.; Gschwind, H. P.; Jin, Y.; Sayer, C.; Zecri, F.; Hartmann, S. *Drug Metab. Dispos.* **2011**, *39*, 199-207.

(67) Kovarik, J. M.; Schmouder, R. L.; Slade, A. J. *Ther. Drug Monit.* **2004**, *26*, 585-587.

(68) Jin, Y.; Zollinger, M.; Borell, H.; Zimmerlin, A.; Patten, C. J. *Drug Metab. Dispos.* **2011**, *39*, 191-198.

(69) Sallusto, F.; Geginat, J.; Lanzavecchia, A. *Ann. Rev. Immunol.* **2004**, *22*, 745-763.

(70) Mehling, M.; Brinkmann, V.; Antel, J.; Bar-Or, A.; Goebels, N.; Vedrine, C.; Kristofic, C.; Kuhle, J.; Lindberg, R. L. P.; Kappos, L. *Neurology* **2008**, *71*, 1261-1267.

(71) Schmouder, R.; Aradhye, S.; O'Connor, P.; Kappos, L. *Mult. Scler.* **2006**, *12*, S101.

(72) Oo, M. L.; Thangada, S.; Wu, M. T.; Liu, C. H.; Macdonald, T. L.; Lynch, K. R.; Lin, C. Y.; Hla, T. *J. Biol. Chem.* **2007**, *282*, 9082-9089.

(73) Liu, C. H.; Thangada, S.; Lee, M. J.; Van Brocklyn, J. R.; Spiegel, S.; Hla, T. *Mol. Biol. Cell* **1999**, *10*, 1179-1190.

(74) Gräler, M. H.; Goetzl, E. J. *FASEB J.* **2004**, *18*, 551-553.

(75) Jo, E.; Sanna, M. G.; Gonzalez-Cabrera, P. J.; Thangada, S.; Tigyi, G.; Osborne, D. A.; Hla, T.; Parrill, A. L.; Rosen, H. *Chem. Biol.* **2005**, *12*, 703-715.

(76) Matloubian, M.; Lo, C. G.; Cinamon, G.; Lesneski, M. J.; Xu, Y.; Brinkmann, V.; Allende, M. L.; Proia, R. L.; Cyster, J. G. *Nature* **2004**, *427*, 355-360.

(77) Brinkmann, V.; Cyster, J. G.; Hla, T. *Am. J. Transplant.* **2004**, *4*, 1019-1025.

(78) Buck, D.; Hemmer, B. *Journal of Neurology* **2011**, *258*, 1747-1762.

(79) Nagaoka, Y.; Otsuki, K.; Fujita, T.; Uesato, S. *Biol. Pharm. Bull.* **2008**, *31*, 1177-1181.

(80) Tonelli, F.; Lim, K. G.; Loveridge, C.; Long, J.; Pitson, S. M.; Tigyi, G.; Bittman, R.; Pyne, S.; Pyne, N. J. *Cell. Signal.* **2010**, *22*, 1536-1542.

(81) Zheng, T.; Meng, X.; Wang, J.; Chen, X.; Yin, D.; Liang, Y.; Song, X.; Pan, S.; Jiang, H.; Liu, L. *J. Cell. Biochem.* **2010**, *111*, 218-228.

(82) Stovall, S., EU Regulator Reviews Novartis MS Pill. *Wall Street Journal* January 23, 2012.

(83) Zeltner, B., Detector for Alzheimer's Leads This Year's List of Top Medical Innovations: Health Care Leaders Gather to Discuss Advances. *Plain Dealer* November 3, 2010, p A1.

(84) Whalen, J., Novartis Gets Nod For Oral MS Drug. *Wall Street Journal* September 23, 2010, p B.1.

(85) Newman, D. J.; Cragg, G. M. *J. Nat. Prod.* **2007**, *70*, 461-477.

(86) Butler, M. S. *J. Nat. Prod.* **2004**, *67*, 2141-2153.

(87) Harvey, A. L. *Drug Discov. Today* **2008**, *13*, 894-901.

(88) Kingston, D. G. I. *J. Nat. Prod.* **2010**, *74*, 496-511.

(89) Hawksworth, D. L. *Mycol. Res.* **1991**, *95*, 641-655.

(90) O'Brien, H. E.; Parrent, J. L.; Jackson, J. A.; Moncalvo, J.-M.; Vilgalys, R. *Appl. Environ. Microbiol.* **2005**, *71*, 5544-5550.

(91) Blackwell, M. *Amer. J. Bot.* **2011**, *98*, 426-438.

(92) Engel, T. *Journal of Chemical Information and Modeling* **2006**, *46*, 2267-2277.

(93) King, G. W.; Cross, P. C.; Thomas, G. B. *J. Chem. Phys.* **1946**, *14*, 35-42.

(94) Chen, W. L. *Journal of Chemical Information and Modeling* **2006**, *46*, 2230-2255.

(95) Willett, P. *Journal of Information Science* **2008**, *34*, 477-499.

(96) Bunin, B. A.; Siesel, B.; Morales, G. A.; Bajorath, J., *Chemoinformatics Theory, Practice and Products.* Springer: Dordrecht, The Netherlands, 2007.

(97) Brown, F. K. In *Annual Reports in Medicinal Chemistry*, James, A. B., Ed., Academic Press, 1998; Vol. Volume 33, pp 375-384.

(98) Vidal, V.; Potterat, O.; Louvel, S.; Hamy, F.; Mojarrab, M.; Sanglier, J.-J.; Klimkait, T.; Hamburger, M. *J. Nat. Prod.* **2011**.