CHAPTER 1

INTRODUCTION

1.1 OVERVIEW OF VANADIUM IN BIOLOGY

Vanadium was widely used as a therapeutic agent in the late 18th century, treating a variety of ailments including anemia, tuberculosis, rheumatism and diabetes.[1] Despite those early uses, studies on vanadium in biological systems was minimal until 1978, when Cantley and coworkers discovered that endogenous vanadium could act as an inhibitor for Na^+/ K^+ ATPase.[2,3] This led to an explosion of studies addressing the biochemical roles of vanadium and its complexes. Interesting discoveries along the way have included: two types of organisms which can be considered vanadium accumulators, two classes of enzymes with vanadium in the active site, and a variety of enzymes inhibited or activated by vanadium. Perhaps most significantly, vanadium complexes have shown promise as possible treatments for diabetes.

In 1911, Henze found that blood cells from ascidians (sessile marine creatures also called sea squirts) contained a high level of vanadium.[4] Further studies have shown that these levels may be as high as 100 mM[5,6] which is a million times that found in sea water (35 nM).[7,8] In 1993, Ishii and coworkers found that a type of fan worm contained vanadium in a similar concentration range.[9] While many studies have been carried out with both tunicates and fan worms, the biochemical function of vanadium in these organisms is as yet unknown. Proposals for this function include signal transport, energy storage, and defense against predation.[10,11]

Other organisms have been found to use vanadium in the active site of enzymes. In 1986, a vanadium nitrogenase was isolated from each of the two soil bacteria, *Azotobacter chroococcum*[12] and *A. vinelandii*.[13,14] The spectral studies done on this system suggest that vanadium substitutes for molybdenum in the iron-molybdenum cofactor of "normal" nitrogenase. Whether the role of the metal is structural or redox is as yet undetermined. However, differences in the efficiency of the vanadium and molybdenum enzymes under differing environmental conditions has led to the suggestion that the production of a vanadium nitrogenase may be an adaptation for low temperature or acidic environments.[15]

A second family of enzymes that involve vanadium in the active site is the haloperoxidases found in algae, fungi and lichens.[16] One member of this class, a chloroperoxidase from the fungus *Curvularia inaequalis,* has been characterized by X-ray crystallography.[17] While this answered many questions about the structure of the active site, the mechanism of its action is still poorly understood. One popular theory

about the mechanism is that vanadium is acting as a Lewis acid catalyst, activating peroxide to attack by a halide.[16]

Along with being essential in these two families of enzymes, vanadium can interact with other biomolecules in a number of ways. These interactions are frequently credited to the ability of vanadium to take either an anionic or a cationic form in aqueous solutions and the ease with which it reacts with biological redox reagents. The two most common oxidation states are IV and V, with an associated standard reduction potential of -0.34 V at pH=7.0.[18] This enables V^{IV} to be oxidized by dioxygen and V^{V} to be reduced by cellular reductants such as NADH. Aqueous V^{V} behaves similarly to phosphorus (P^{V}), forming a tetrahedral oxyanion called vanadate (VO_4^{3-}), which is analogous to phosphate. Differences between vanadate and phosphate include pK_a's, bond lengths and equilibrium constants for oligomerization reactions.[19] Vanadic acid H_3VO_4 (pK_a = 3.5, 7.8, 12.5) is a slightly weaker acid than phosphoric acid H_3PO_4 (pK_a = 1.7, 6.5, 12.1) and has bond lengths which are longer by approximately 0.2 Å (1.7 versus 1.5). In addition vanadate forms oligomers in lower concentration ranges than does phosphate. Vanadium(IV) often takes the form of the vanadyl cation, $(V^{IV}=O)^{2+}$, and as such can interact with proteins at cationic binding sites similar to divalent calcium, zinc or manganese.[20] Both V^{IV} and V^{V} also form a variety of coordination complexes that may interact with enzymes.

In 1991, Crans outlined five specific ways in which vanadium has been found to interact with proteins.[21]

1. Deactivation of the enzyme through oxidation of the essential sulfhydryl group.[22]
2. Vanadate monomer acting at a phosphate binding site.[19]
3. Inhibition through generation of organic-vanadate dead-end inhibitor complexes.[23-27]
4. Inhibition by oligomers.[28-31]
5. Conversion of spontaneously formed organic vanadate derivatives, by enzyme converting organic phosphates.[32,33]

Due to these (and probably other) vanadium-biomolecule interactions, vanadium can be used to elicit desired responses at the organism level. In particular, in 1985, the research teams of Dubyak and Kleinzeller[34] and of Shechter and Karlish[35] independently published reports that vanadium can function as

an insulin mimic. Administering vanadium salts to diabetic rats caused an alleviation of symptoms. Phase 1 clinical trials of the use of vanadium salts to treat diabetes began in 1995.[36,37]

The last fifteen years have seen an explosion in the number of studies on the biochemical roles of vanadium. New organisms that accumulate vanadium have been discovered, and new enzymes with vanadium in the active site have been found. Modes of interaction of vanadium with a wide variety of enzymes have been investigated, and clinical trials have begun on the use of vanadium to treat diabetes. Modern studies are beginning to bear witness to the wisdom of the 18th century doctors who believed in the use of vanadium as a therapeutic agent.

1.2 VANADIUM INTERACTIONS WITH BIOLOGICAL MATRIX ELEMENTS

Studies of interactions between vanadium and enzymes are complicated by the wide variety of complexes that vanadium can form and the diversity of chemistry that can occur. In aqueous solution, vanadate (VO_4^{3-}) will be present in any of three monomeric forms that differ in the extent of protonation.[38] If the concentration is in the millimolar or higher range, the vanadate will oligomerize to form dimers, tetramers and pentamers. There is also a decamer, which is stable between pH 2 and 6; it is unstable at higher pH's but, if formed, may persist for several days due to kinetic stability. The reduction potential for vanadate to vanadyl ($V^{IV}O^{2+}$) varies from 1.0 V in acidic solution to -0.74 V (vs. SHE) in basic solution.[18] Therefore, species in both oxidation states may be present in solution and can be readily interconverted by common small molecule redox agents. At pH < 4, vanadyl is mostly present in monomeric form; as the pH is increased, a dimer forms.[39] As the pH is raised still higher, vanadyl precipitates as an insoluble (K_{sp} = $1.1*10^{-22}$ M^3) hydroxide [$VO(OH)_2$] then finally (above pH 11) it takes on another monomeric form $[VO(OH)_3]^-$.

Even in relatively simple systems of vanadium and water, there are at least ten species that must be considered when performing an experiment. Experiments get even more complicated as other chemicals are added to the solution and additional possibilities for coordination chemistry are introduced. For example, if buffer is used to control the pH of a solution, it may complex with the vanadium. Buffers such as triethanolamine, tricine, bicine (N,N-bis[2-hydroxyethyl]glycine), citrate, and glycine interact so strongly with vanadium that they are not practical for use in studying vanadium interactions with enzymes.

Some buffers that interact weakly with vanadate and may be used in enzymatic studies include HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), acetate and MES (2-[N-morpholino]ethane sulfonic acid).[38]

Even with a weakly interacting buffer, enzymatic assays are complicated by vanadium interactions with additives to enzyme preparations. EDTA (ethylene-diamine-tetraacetic acid) is often added to protect enzymes from trace heavy metal ions; however, EDTA has a strong interaction with vanadate. Other frequent additives that interact with vanadium include thiols (added to keep the enzyme in a reducing environment) and glycerol (to prevent protein adsorption to glass and plastic). Buffer and assay components must be presumed to interact with vanadium until proven otherwise. Careful control reactions are necessary to ensure that the vanadium speciation in a specific matrix is fully understood so that the interactions with enzymes can be correctly analyzed.[38]

1.3 SIGNAL TRANSDUCTION, DIABETES AND VANADIUM

Cellular signal transduction relies on the transfer of inorganic phosphate among various proteins. These transfers are collectively termed a phosphate signal cascade.[40,41] By interfering with phosphate transfers, extracellular reagents can affect cellular processes. Disruption of the phosphate signal cascade is associated with diseases such as cancer and diabetes mellitus. Diabetes affects 15 million people in the United States and is the 7th leading cause of death is this country.[42] Diabetes results when organisms fail to make or fail to utilize the signaling hormone insulin. Insulin normally regulates the uptake and storage of nutrients such as amino acids, fatty acids and glucose; and the conversion of glucose to glycogen.[43] In performing its regulatory task, insulin first binds to the insulin receptor kinase (IRK) as shown in Figure 1-1. The IRK is a membrane-spanning protein; the insulin binds extracellularly and causes the IRK to autophosphorylate on several regulatory tyrosine residues of the intracellular domain.[44,45] Once the regulatory tyrosines of the IRK are phosphorylated, the IRK's kinase activity toward other substrates is increased manyfold. Antagonizing the action of the IRK are protein tyrosine phosphatases (PTP's), which catalyze the dephosphorylation of tyrosine. PTP's can act both on the substrates that have been phosphorylated by the IRK and on the IRK itself. Together, the IRK and the PTP's regulate the transfers of phosphate that ultimately lead to the insulin response. In the case of diabetes, when the insulin is either

absent or fails to initiate the phosphate signal cascade, it is conceivable that another compound could elicit a similar response by either activating the IRK or inhibiting the PTP's.

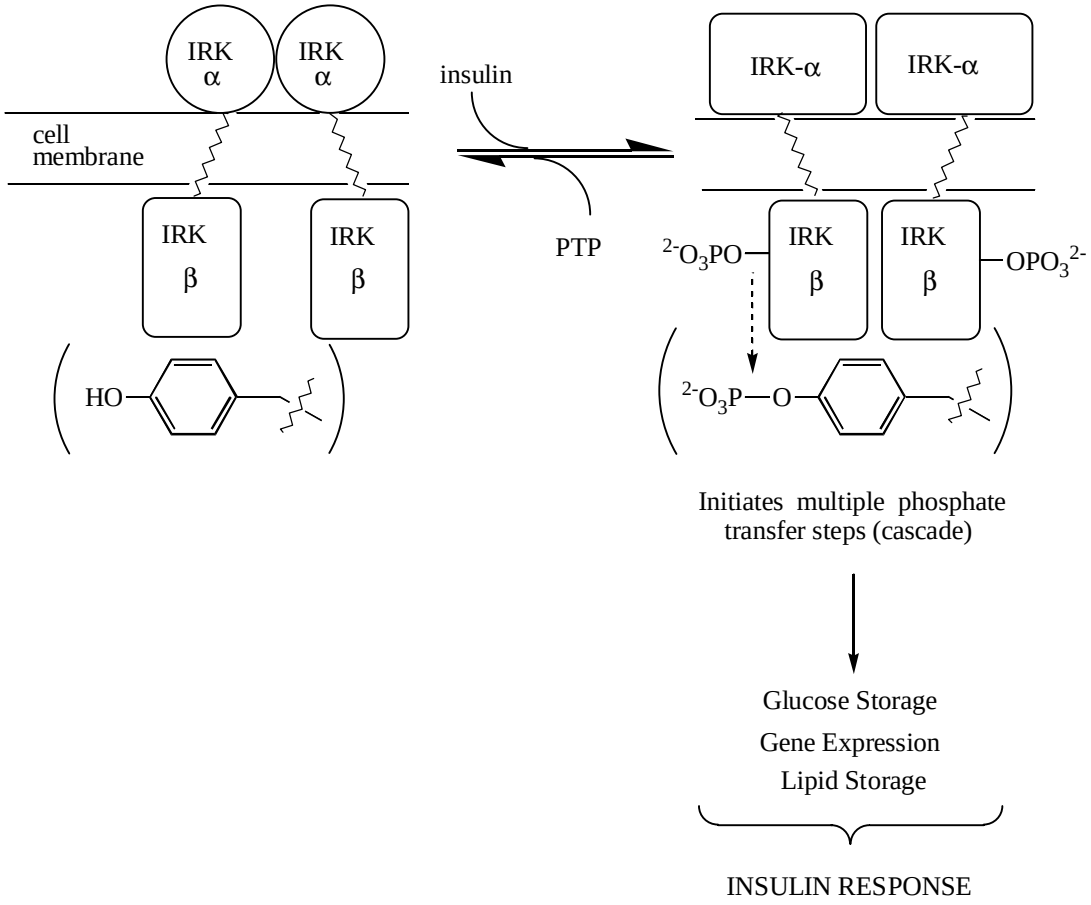

Figure 1-1. Insulin and the phosphate signal cascade.

Studies over the last several years have shown that a variety of vanadium molecules, both salts and coordination complexes, have insulin-like effects on cells, tissues and organisms. In rat adipocytes, vanadium stimulates hexose uptake, lipogenesis and glucose oxidation; and inhibits lipolysis.[46] It also stimulates hexose transport in skeletal muscle, K^+ uptake in cardiac muscle,[46] and glycogen synthesis in rat diaphragm and liver cells.[43] In studies of perfused rat liver, vanadate inhibits glucose release.[43] *In vivo* studies have shown that when administered orally to streptozotocin-treated diabetic rats, vanadate lowers the average blood-glucose level and alleviates the diabetic symptoms.[47] In 1995, clinical trials of vanadium use in diabetic humans were reported with favorable results.[36,37] The exact method by which vanadate

elicits the euglycemic response is unknown; possibilities include activation of the IRK and inhibition of PTP's. While some studies have shown that vanadate is capable of stimulating the kinase activity of the insulin receptor,[48,49] others have shown that such stimulation is not requisite for insulin mimicry.[50] This suggests a postreceptor role for vanadate, perhaps inhibiting PTP's.[43]

The majority of studies have focused on the use of vanadium salts such as sodium orthovanadate and vanadyl sulfate to mimic the actions of insulin. By using coordination complexes, the efficacy of vanadium to mimic insulin can be modified. To effect delivery of the vanadium to the cellular environment, ligands can be used to enhance transmembrane transport. Within the cell, ligands may modify the ability of vanadium to interact with enzymes (such as PTP), altering the equilibrium constant for reversible binding of vanadium to enzyme or allowing the vanadium to covalently modify the enzyme and change its catalytic activity.

So far, only peroxide, hydroxylamine, and maltol have been studied with respect to their effect on the ability of vanadium to modulate PTP activity. Molecules of oxovanadium(V) coordinated to one or two peroxide molecules are collectively called pervanadates. These molecules have been shown to have insulin-mimetic activity whether administered as pervanadate[51,52] or generated *in situ* by administering a mixture of vanadates and hydrogen peroxide.[53-55] Posner's group has demonstrated a correlation between insulin-mimetic activity and the ability of pervanadates to inhibit protein tyrosine phosphatases (PTP's).[56-58] Gresser and coworkers have studied the kinetics of pervanadate interactions with PTP1B and found that the pervanadate irreversibly inactivated the PTP by oxidizing the active-site thiolate to a sulfonate.[59]

Hydroxylamine complexes are electronically similar to peroxide complexes and are currently under investigation by Nxumalo and Tracey.[60] They have shown that these complexes can increase the phosphotyrosine levels in cell cultures that overexpress the human insulin receptor. Their kinetic studies have shown that these molecules reversibly inhibit PTP's in a competitive manner.

There have been several reports of insulin mimicry with vanadium complexed with organic ligands.[61] Bis(maltolato)oxovanadium(IV) (BMOV) is a particularly effective insulin-mimetic compound,[62-66] requiring one third the amount of BMOV to achieve euglycemia as vanadate or vanadyl.[62,63] BMOV has been shown to be a highly selective inhibitor of PTP's,[67] but no details have been published with regard to the inhibition mechanism.

1.4 PROTEIN TYROSINE PHOSPHATASES

Phosphate-ester bond formation and cleavage are critical processes in the cellular activities of living organisms. Phosphate ester chemistry is involved in genetic polymerization (DNA and RNA), biological membranes and the interactions between biological macromolecules. Formation of phosphate monoesters (phosphorylation) on the protein components serine, threonine, and tyrosine; and the hydrolysis of those ester bonds (dephosphorylation) is central to signal transduction pathways. While the properties of the enzymes that regulate the level of phosphorylation on serine and threonine have been investigated in great detail, the study of the enzymes that catalyze the hydrolysis of tyrosine-phosphate-ester bonds (PTP's) is still in its infancy. The first PTP was isolated in 1988. Since then more than forty other PTP's have been characterized.[68] They are known to be responsible for regulation of cell growth and proliferation; they act as tumor suppressors for brain, breast and prostate cancers,[69] and they act as T-cell activators.[70] PTP's have also been implicated in biological pathogenesis. Bacteria of the genus *Yersinia*[71,72] (responsible for the bubonic plague) and *Salmonella*[73] encode a PTP that is essential for the virulence of these bacteria.[74]

One feature that is common to all members of the PTP family is a conserved active-site motif (I/V)HCXAGXGR(S/T)G (X = any amino acid).[68] The importance of different amino acids in this sequence to the activity of the enzyme has been addressed in a number of studies. The Cys is absolutely essential for activity (mutation of the Cys to a Ser leads to total loss of activity), and it has been shown to form a covalent bond with the phosphate of the substrate.[74] This Cys has an unusually low pK_a of 4.7 while a normal Cys has a pK_a of 8.3.[75] This pK_a is affected by the adjacent His. Mutation of this His to Asn or Ala increases the Cys pK_a to 5.99 or 7.35, respectively,[75] and causes the enzyme activity to decrease by 90%.[76] The fact that the His is required for the low pK_a of Cys could mean there is a direct interaction between them, as in the cysteine proteases, or an indirect interaction through water molecules or a hydrogen bonding network. However, the X-ray structures of several PTP's rule out the possibility of direct interaction between the two residues since the two side chains are not in close proximity to each other.[77] According to these structures, the negative charge of the Cys thiolate anion is stabilized by eight hydrogen bonds to the amide hydrogens of the protein backbone and one to the side chain hydroxyl hydrogen of Ser or Thr. Since the His is involved in its own hydrogen-bonding network, its effect on the

pK_a must be secondary; it is not interacting directly, but rather through a complicated network of hydrogen bonds that stabilizes the tertiary structure of the active site.

Kinetic studies support the catalytic cycle that is shown in Figure 1.2. Studies of the pH dependence of the reaction mechanism have revealed that both acid and base catalysis are required for optimal enzyme activity. Mutation studies have implicated an Asp (Asp-356 in the *Yersinia* PTP) as the acid catalyst and a Glu (Glu-290 in the *Yersinia* PTP) as the base catalyst.[76] Mechanistic studies have further shown that the reaction proceeds through two distinct steps. The first is the cleavage of the tyrosine-phosphate ester bond and formation of the cysteine thiolate-phosphate ester bond. The second and rate limiting step (under acidic conditions) is the hydrolysis of the thiolate-phosphate bond.[78] Both steps proceed through a three-coordinate "busted tetrahedron" transition state.[79] The Asp serves as a proton donor to the Tyr in the first step of the reaction while Glu serves as a proton acceptor from the water in the second step. Also helping to stabilize charge are the conserved Arg and Ser/Thr. The Arg is believed to have hydrogen bonding interactions with two of the phosphate oxygen atoms helping to stabilize the charge on the phosphate in the transition state of both reactions.[72, 80] The hydroxyl proton of the conserved Ser/Thr stabilizes the negative charge on the thiolate in the transition state during the second part of the reaction.[81]

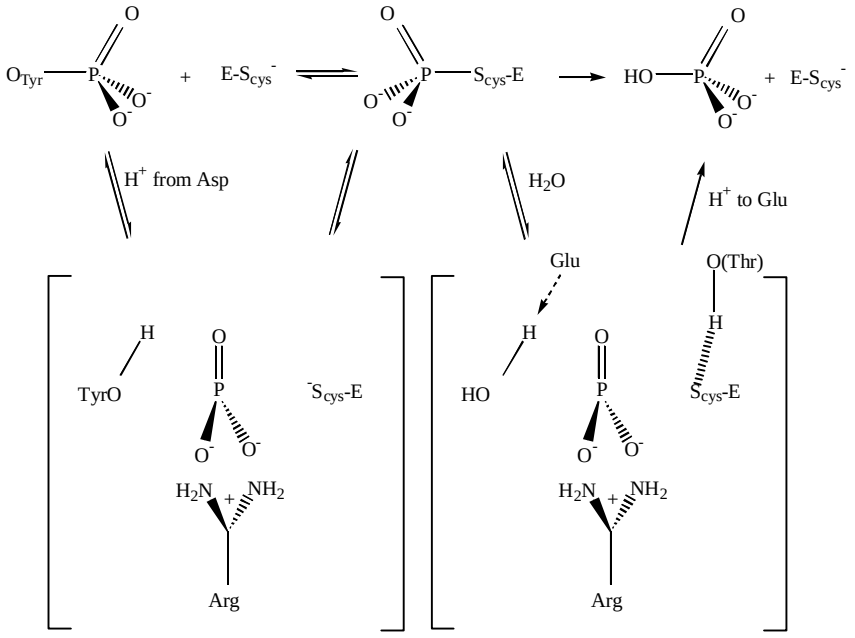

Figure 1-2. Mechanism for protein tyrosine phosphatases.

There are numerous ways in which this catalytic cycle may be disrupted by an inhibitor. The inhibitor may bind to either the Asp or Glu that is needed for acid/base catalysis; it may interact with the Arg or Thr/Ser that function as transition state stabilizers, or it may bind to the active site nucleophile (Cys). Any of these possibilities would lead to a reduction in enzyme activity. Vanadate (VO_4^{3-}) is a polyoxoanion that has been shown to inhibit PTP activity with K_i of <100 μM for all members of the PTP family.[68] Studies into the mode of vanadate-based inhibitions of PTP's have only recently (1997) appeared.

In early 1997, Van Etten and coworkers reported that vanadate is a competitive inhibitor of the low molecular weight PTP obtained from bovine heart,[82] with $K_{i,c} = 5.4 \pm 0.8$ μM at pH=5.0 and $K_{i,c} = 1.0 \pm 0.6$ μM at pH=7.5. In the same work, a crystal structure of vanadate bound to the PTP was also obtained. It showed that vanadate forms a covalent bond with the active site cysteine to generate a five-coordinate adduct. This X-ray structure is similar to that for the vanadium adduct of the *Yersinia* PTP recently reported by Dixon *et al.*[83]

In another recent study, Ramachandran and coworkers have shown that vanadate can have two separate effects on the activity of PTP1B. [84] As seen with the low molecular weight PTP from bovine-heart, vanadate can act as a competitive inhibitor of PTP1B ($K_{i,c} = 0.38 \pm 0.02$ μM at pH=7.3). Additionally, vanadate can interact with adventitious peroxides to oxidize the active-site cysteine to a cysteine-sulfonate.

Lastly, Pregel and Storer have shown that vanadate can interact with truncated forms of SHP-1 and PTP1B in such a way as to prevent reaction of the active-site cysteine with an aromatic disulfide.[85] This is in contrast to phosphate, which merely slowed the reaction between the enzyme and the disulfide. This difference requires the vanadate to do something other than competitively inhibit the enzyme. If it were merely functioning as a competitive inhibitor, it would slow the reaction (as observed with phosphate), not totally prevent it.

1.5 SCOPE OF THIS WORK

In this work, the interactions of vanadate with the modified *Yersinia* PTP, Yop51*Δ162,[86] will be investigated, and the mode of inhibition and relevant inhibition constants will be determined. The enzyme's activity will be measured using p-nitrophenylphosphate as a substrate. This small molecule has

the requisite ester bond between an aryl group and the phosphate, and it also is easily probed by uv-vis spectroscopy due to a strong absorbance at 410 nm ($\varepsilon \cong 18{,}000\ M^{-1}cm^{-1}$).[87] The interactions of vanadate with the buffer components EDTA and catalase will also be addressed.

To date, no detailed study of the use of an organic ligand to modify vanadium interactions with PTP has been published. This work intends to assay a variety of ligands to determine if they can increase the inhibition of Yop51*Δ162 by vanadate. For the ligands that do enhance that inhibition, it will be determined if the chemistry is reversible or irreversible, and appropriate rate and equilibrium constants will be determined. Ligands studied will include salicylic acid derivatives, substituted *o*-aminophenols, substituted *o*-catechols, maltol, ethyldiethanolamine, and adenosine triphosphate.

Most of the ligands selected for study are derivatized phenols (phenol being the side chain of tyrosine) as shown in Figure 1-3. Since a tyrosine phosphate is the enzyme substrate, the active site of PTP must be designed to readily accommodate phenols. If such a phenol were coordinated to vanadium and that complex in turn formed a covalent bond with the cysteine, the result might be an enhancement of vanadium's inhibitory ability with regard to PTP. To promote coordination of phenol to vanadium, chelating groups were placed in the ortho position. Variations were also made para to the phenol oxygen in order to modulate the pK_a of the phenol and thus affect its ability to interact with vanadate.

Other ligands used were maltol due to the insulin-mimetic properties of BMOV and adenosine triphosphate because of the high portion of vanadium bound to nucleotides in the cellular environment.[88]

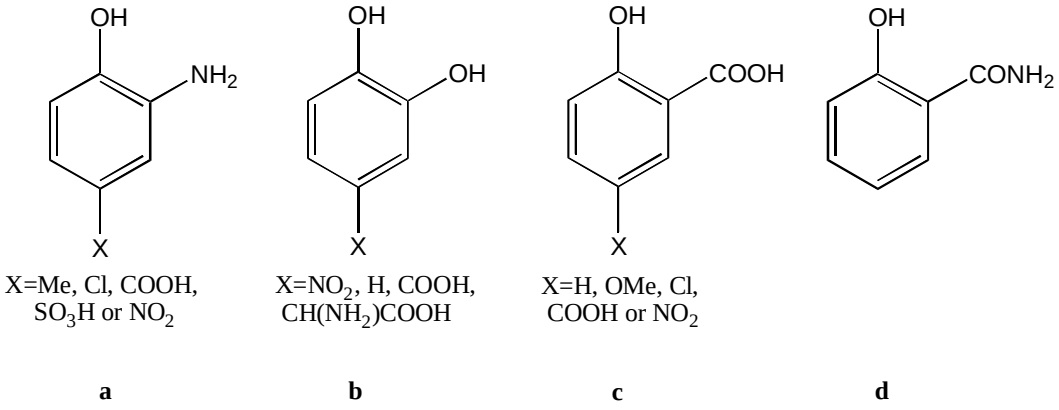

Figure 1-3. Phenol based ligands proposed for study. a)Aminophenol derivatives, b) Catechol derivatives, c) Salicylic acid derivatives d) Salicylamide.

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