The Synthesis of O-alkylhydroxylamines

and the Potentiation of Histamine

in Canine Colonic Tissue

The Synthesis of O-alkylhydroxylamines and the Potentiation of Histamine in Canine Colonic Tissue

By

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TITLE: The Synthesis of O-alkylhydroxylamines and the Potentiation of Histamine in Canine Colonic Tissue

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ABSTRACT

Treatment of canine colonic tissue with some O-alkyl and O-benzyl hydroxylamines potentiated the response of the tissue to histamine in different experimental environments.

Sixteen O-substituted hydroxylamine compounds were synthesized using a modification of the Gabriel synthesis. These compounds were tested for their ability to potentiate histamine in canine colonic tissue through diamine oxidase inhibition.

Three procedures were used to determine their activity: (1) secondary rise - hydroxylamine derivatives were added to epithelial tissue preparations in Ussing chambers after an initial dose of histamine. Active compounds caused a secondary increase in the short circuit current across the tissue, (2) dose-response profiles were constructed for several hydroxylamine compounds to determine whether they caused a significant shift to the left of the normal histamine curve (potentiated response), and (3) diamine oxidase enzyme assays were performed to examine the ability of the hydroxylamine derivatives to inhibit partially purified diamine oxidase. This aided in determining if inactive compounds could not potentiate histamine due to an inability to access the enzyme in the epithelial preparation.

The structure-activity relation observed indicates that: (1) active

compounds are oxygen and not nitrogen substituted hydroxylamines, (2) branched compounds are less active than their straight chain analogues, (3) greater steric bulk of the alkyl substituent can decrease the activity of the compound, (4) the presence of a carbon-carbon double (allyl) or triple (4-pentynyl) bond does not affect the activity of the compound, (5) longer straight chain O-alkyl hydroxylamines are less active than shorter chain derivatives, (6) steric bulk of the benzyl compounds is not likely to be the cause of its inability to inhibit diamine oxidase since the cinnamyl derivative is active, and (7) meta- and para-oxygen substituents (-OH, $-OCH_3$) on O-benzyl hydroxylamines increase their diamine oxidase inhibiting properties.

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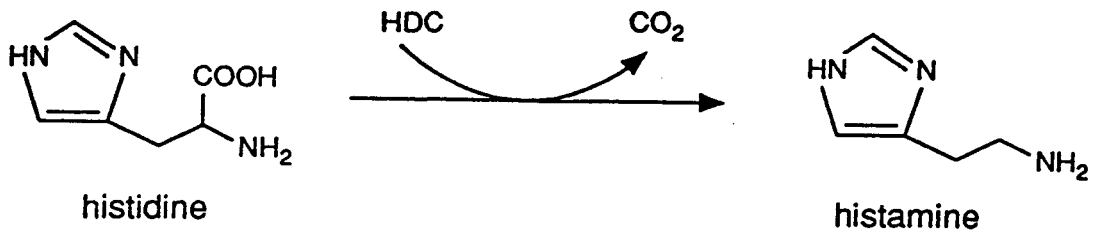
INTRODUCTION

1.1 Histamine

Histamine (β-iminazoylethylamine) is a primary amine that is stored in the granules of enterochromaffin-like cells and mast cells. It has a number of physiological effects that may be of major importance in clinical practice. Histamine affects major cardiac functions such as heart rate, force of contraction, and coronary blood flow. It has both contracting and dilating effects on the blood vessels and it stimulates the transport properties of the small intestines resulting in diarrhoeal disorders (Rangachari, 1992). There is growing evidence that histamine is important in cell growth, tissue repair (Mitsuhashi & Payan, 1992), platelet aggregation and tumour development since it can modify the surface of the cells making them less adherent to each other and therefore decreasing the close contact between them. This modifies many cellular interactions such as phagocytosis and tumour and metastasis development (Meretey et al., 1989). Histamine is also a neurotransmitter/neuromodulator in the brain (Mitsuhashi & Payan, 1992) and it is a well-known regulator of the lymphoid cells through H_1 and H_2 receptors (Meretey et al., 1989). Histamine has been called an "intracellular messenger".

Histamine is produced in the body by the enzymatic decarboxylation of histidine by either L-dopa decarboxylase or histidine decarboxylase (HDC).



histidine histamine

FIGURE 1

Histidine decarboxylase is present in a variety of cell types such as mast cells, skin, platelets, basophils and carcinoids. It is also found in some types of intestinal bacteria. Once histamine is produced, it is released from the cell after degranulation by either cytolytic release where the plasma membrane is damaged or non-cytolytic release where the plasma membrane remains intact. The former process is energy independent and does not require intracellular calcium while the latter process is energy dependent and requires calcium. There are many factors that affect the release of histamine such as pentagastrin, adenosine, prostaglandin-D_2 as well as histamine itself (Rangachari, 1992).

Once released, histamine can be metabolized by two enzymes: diamine oxidase (DAO) or histamine methyl transferase (HMT). Metabolism by diamine oxidase produces 4(5)-imidazolacetaldehyde which is converted to 4(5)-imidazolacetic acid which forms conjugates with riboside-phosphate while histamine methyl transferase catabolism produces N-methyl histamine which is

then oxidized to 1-methyl-4-imidazolacetic acid.

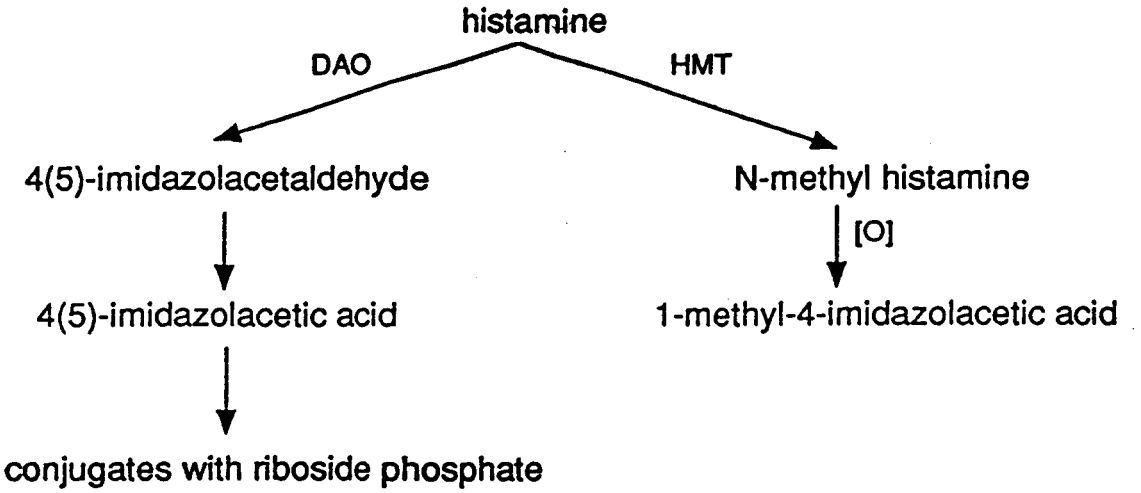


FIGURE 2

These metabolites are excreted in the urine, milk, sweat, sputum and feces. Histamine methyl transferase is widely distributed throughout the body and can be found in the stomach, thymus, lung, spleen, kidney and brain. Diamine oxidase is primarily found in the placenta, intestines, kidney and thymus (Rangachari, 1992).

1.2 Diamine Oxidase

Amine oxidases are divided into two types: flavins, which are associated with the mitochondria, and nonflavins, which contain copper and an organic cofactor (Kusche & Lorenz, 1983). Diamine oxidase is a nonflavin amine oxidase. Copper (nonflavin) amine oxidases catalyze physiologically important reactions such as the oxidative removal of biologically active amines from blood plasma, the cross-linking of collagen and elastin in connective tissue biogenesis and the regulation of intracellular spermine and spermidine (Janes et al., 1990).

Polyamines are essential for growth and replication of all living cells (Equi et al.,1991). Nonflavin amine oxidases are widely distributed in nature and are found in the eukaryotic cells of many species from fungus to mammals. Most nonflavin amine oxidases are composed of two identical subunits (M_r = 70,000 to 100,000) and they require one mole of copper per mole of subunit. They also require an organic cofactor. Amine oxidases catalyze the oxidative deamination of amines to produce aldehydes, ammonia and hydrogen peroxide (Gaugas, 1981).

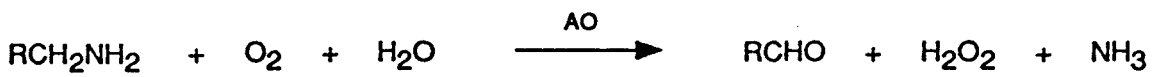


FIGURE 3

In 1929, Best discovered an enzyme that degraded histamine which he called histaminase. Zeller, in 1938, called this enzyme diamine oxidase. Diamine oxidase has been detected in micro-organisms, leguminous plants, fish, and various organs of mammals such as the kidney, intestinal mucosa, placenta, lung, skin, amniotic fluid and plasma of pregnant women, blood platelets, liver and cells (Kusche & Lorenz, 1983).

The identity of the organic cofactor has until recently been very uncertain. Early studies have shown that copper amine oxidases contain a reactive carbonyl group that can form a complex with phenylhydrazine. This suggested that pyridoxal phosphate was the cofactor but proof of this was never given. In 1984,

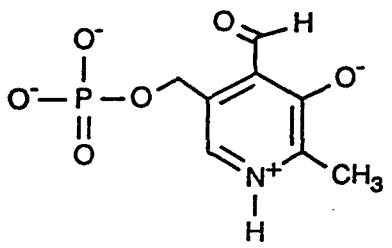
indirect evidence was presented indicating that pyrroloquinoline quinone (PQQ) was the cofactor. Resonance Raman studies concluded that pyridoxal phosphate was definitely not the cofactor therefore strongly implicating PQQ, but other experiments could still not provide enough information to prove that it was in fact the organic cofactor of copper amine oxidases (Janes et al., 1990).

In 1990, Janes et al. isolated an active site, cofactor containing peptide from bovine serum amine oxidase (BSAO) thus allowing a complete structural characterization. Their data showed that the cofactor is not PQQ and provided evidence that 6-hydroxydopa (topa) might be involved. The sequence of the isolated peptide fraction is shown below:

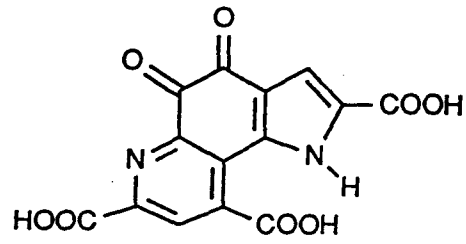
- leu - asn - X - asp - tyr -

where X is the organic cofactor. Characterization by mass spectrometry and UV/VIS spectroscopy showed that the cofactor X was indeed 6-hydroxydopa (topa) and final proof of this structure was given by proton nuclear magnetic resonance studies. Similarities between isomerized forms of topa and PQQ makes the initial assignment of PQQ as the cofactor understandable and studies of PQQ in bacteria implicate a topa-like intermediate as a precursor of PQQ. The authors hypothesize that topa arises in BSAO through either a posttranslational process involving the oxidation of an active site tyrosine or through direct incorporation of dopa into the growing protein chain via a special transfer RNA.

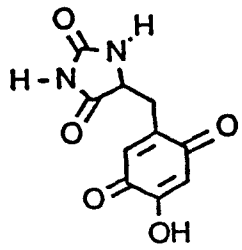
The structures of the compounds that have been considered as the organic cofactor are shown below (Janes et al., 1990).



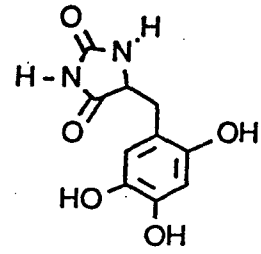
pyridoxal phosphate



pyrroloquinoline quinone (PQQ)



oxidized TOPA



reduced TOPA

FIGURE 4

The exact role of the copper present in diamine oxidase is still uncertain. Both coppers are present as Cu(II) and EPR shows that the majority of the copper remains as Cu(II) throughout the reaction. The two coppers do not interact and are thought to be located at the surface of the protein, possibly near the subunit interface. They are chemically distinct and may play two different roles in the catalytic mechanism: perhaps one copper ion is responsible for maintaining the tertiary structure of the protein while the other is involved in the catalytic mechanism (Knowles & Yadav, 1984). Kluetz & Schmidt (1977) have

shown that there is no binding between copper and nitrogen or oxygen indicating that copper is not directly involved in the oxidative deamination catalysis reaction but that it takes part in the reoxidation of the enzyme by oxygen.

The two electron deamination of primary amines is thought to occur by a ping-pong mechanism.

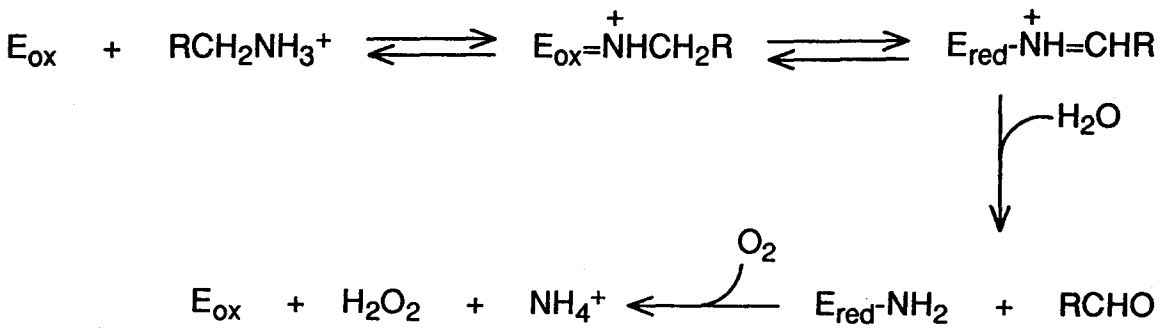


FIGURE 5

The oxidized form of the enzyme (E_{ox}) and the amine react to give a complex that equilibrates with a reduced form of the complex. This species reacts with water to liberate the aldehyde and the amine complexed with the reduced enzyme (E_{red}) in what is essentially a transamination process. Oxidation by O_2 gives back the oxidized form of the enzyme, hydrogen peroxide and protonated ammonia (Hartman & Klinman, 1991).

The nature of the two electron transfer is still unclear. Hartman and Klinman (1991) proposed a mechanism for the reaction of BSAO (figure 6).

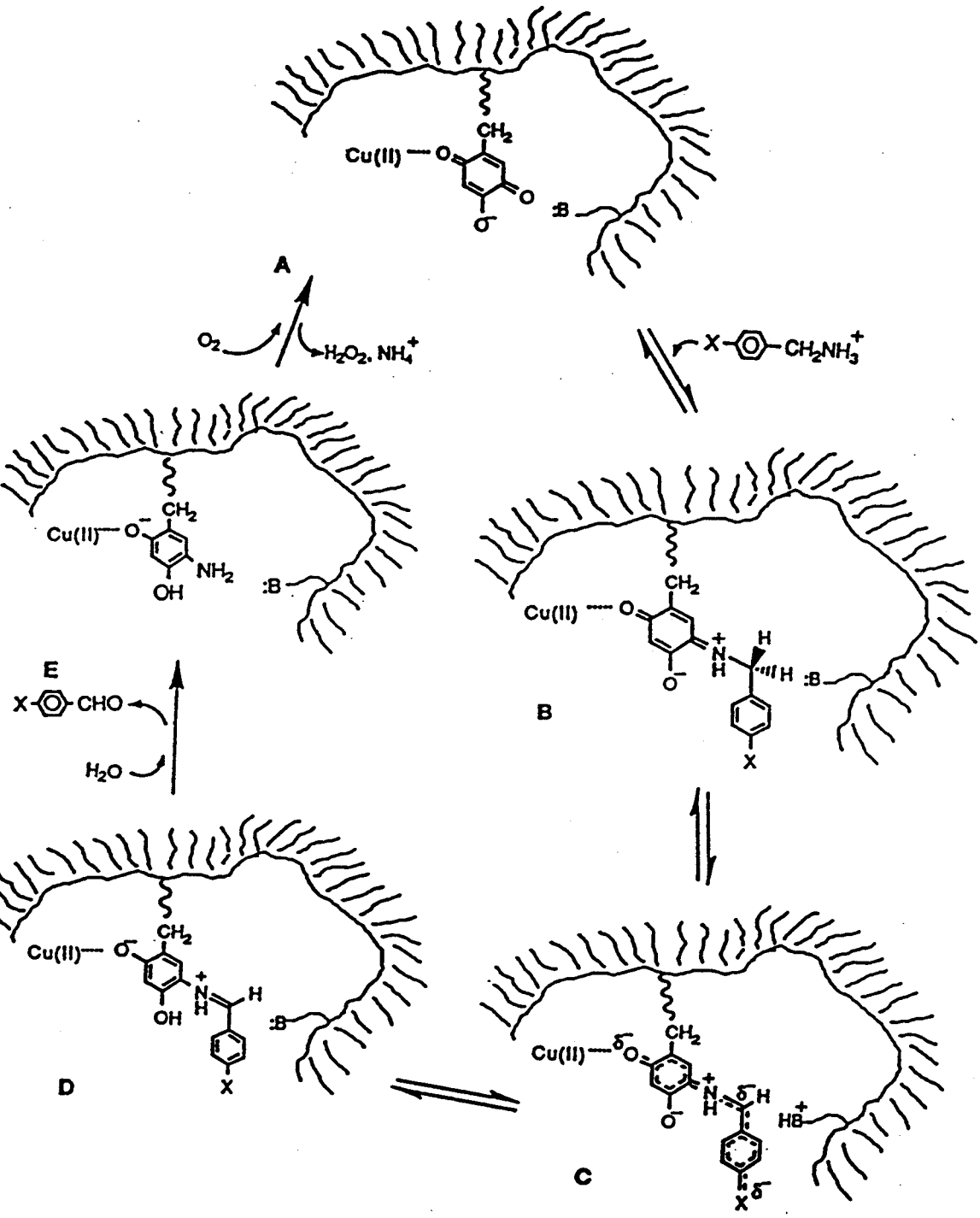
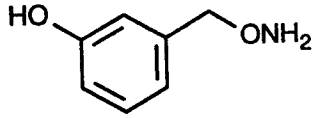


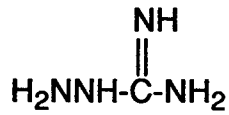
FIGURE 6

(A) shows the topa quinone cofactor near to the active site base and a copper atom. In (B), a covalent imine complex between the substrate and the cofactor is generated followed by formation of the carbanion intermediate (C) that forms on proton transfer from the substrate to the active site base. Transfer of the substrate-derived proton to the oxyanion at C-4 of the cofactor forms (D), the product-imine complex. Hydrolysis of the aldehyde from the product-imine complex gives the aminoquinol of the cofactor (E) which is oxidized back to species (A).

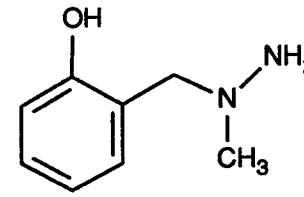
Diamine oxidase is inhibited by a number of compounds such as semicarbazide, hydroxylamine, methylene blue, pyocyanin, NSD 1039, NSD 1531, NSD 1024 (Gaugas, 1981), monoamidines, diamidines, guanidine derivatives, monoisothiourea derivatives, diisothiourea derivatives, stilbamidine, dimethyl stilbamidine, aneurin (Blaschko, Fastier & Wajda, 1951), cimetidine and imidazole (Metaye et al., 1988). Diamine oxidase inhibitors exhibit antimalarial, antitrypanosomal, antibacterial and antifungal activities (Equi et al., 1991). Structures of some of these compounds are given below.



NSD-1024



aminoguanidine



NSD-1039

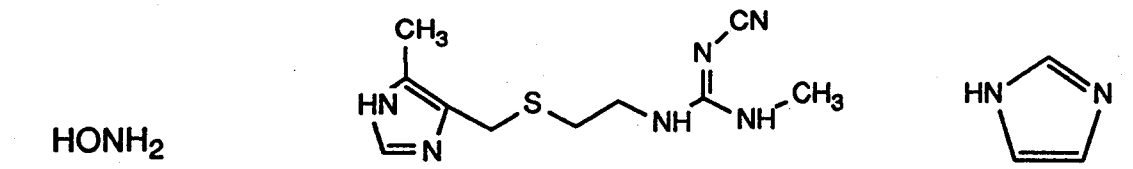


FIGURE 7

1.3 Hydroxylamines

1.3.1 Biological Aspects

Nitro compounds (-NO_2) in mammalian cells and colonic bacteria undergo metabolic transformations that produce nitroso (-N=O) and hydroxylamine (-NH_2OH) intermediates. These compounds, which have been associated with a

range of toxic effects such as carcinogenesis, aplastic anaemia and methaemoglobinaemia, are produced by intestinal bacteria as well as macrophages. Although they may not elicit any effects on their own, they can potentiate the responses of the intestine to other substances such as histamine (Rangachari, Prior, Bell & Huynh, 1992).

Hydroxylamine itself inhibits and stimulates a variety of enzymes. Hepatic phosphatases and reductases, sodium/potassium adenosine triphosphatase, diamine oxidase and cytochrome oxidase are all inhibited by hydroxylamine while guanylate cyclase, monoamine oxidase and adenylate cyclase are stimulated. Hydroxylamine also increases cyclic adenosine monophosphate (cyclic AMP) concentrations by inhibiting phosphodiesterases and has the potential to inactivate a number of coenzymes and intermediates of cell metabolism by reacting with carbonyl groups, activated alkene bonds (α,β-unsaturated) and esters (Rangachari, Bell, Prior & Huynh, 1992).

Hydroxylamine and its derivatives are important because they can shift the cumulative dose-response curve to histamine to the left with no significant increase in the maximal response suggesting that histamine is more "potent" in the presence of the hydroxylamine compound. This indicates that histamine is being potentiated by at least one mechanism. There are several possible actions of hydroxylamines that must be considered: (1) they facilitate the access of histamine to the receptor sites, (2) they increase histamine's affinity for its receptors, (3) they enhance post-receptor transduction mechanisms and (4) they

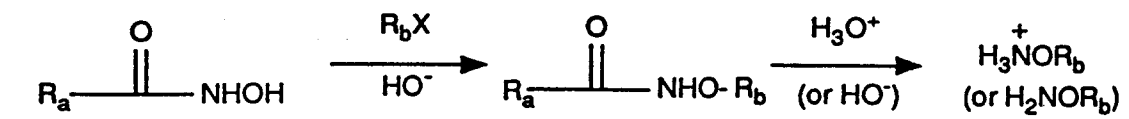
decrease the catabolism of histamine by diamine oxidase. Experiments have shown that the last consideration is the most likely; i.e. hydroxylamines can protect histamine from degradation by diamine oxidase. Additional experiments show that tissues that were clearly desensitized to histamine still produced sharp increases in the short circuit current after the addition of hydroxylamine at 10^{-4}M indicating that hydroxylamine must also permit access of preserved or excess histamine to "naive" sites that remain sensitive to the hydroxylamine agonist (Rangachari, Bell, Prior & Huynh, 1992).

1.3.2 Synthesis of O-substituted Hydroxylamines

A number of strategies for the synthesis of O-substituted alkylhydroxylamines have been published some of which are described below.

Nucleophilic displacement on an alkyl halide can be carried out using a variety of nucleophiles to obtain an intermediate that can be converted to the desired hydroxylamine:

(1) Displacement by the conjugate base of a hydroxamic acid derivative or N-hydroxyurethane or carbamate on an alkyl or benzyl halide followed by acid or base hydrolysis gives the protonated O-alkyl or O-benzyl hydroxylamine (Barton, 1979).



R_a = alkyl or aryl
(hydroxamic acid)
R_a = OCH_2CH_3
(N-hydroxyurethane or carbamate)

FIGURE 8

(2) Dipotassium hydroxylamine disulfonate in base will also displace the halide to give an intermediate which can be hydrolysed by acid to give the hydroxylamine derivative (McKay et al., 1960).

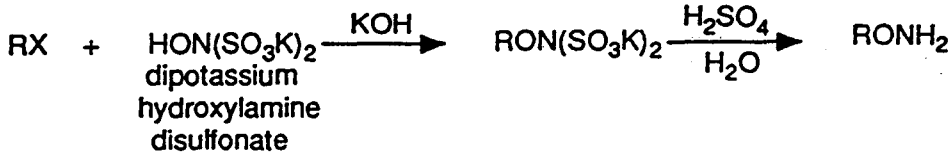


FIGURE 9

(3) N-hydroxyphthalimide in basic medium (triethylamine, potassium hydroxide or sodium hydride) will also undergo an S_n2 displacement as in the Gabriel synthesis (Gibson & Bradshaw, 1968). Hydrazinolysis (Ing & Manske, 1926) or acid hydrolysis cleaves the phthalimide portion to give the O-substituted hydroxylamine. This reaction can also be carried out using the potassium salt of N-hydroxyphthalimide in dimethylformamide (Sheehan & Bolhofer, 1950; McKay et al., 1960).

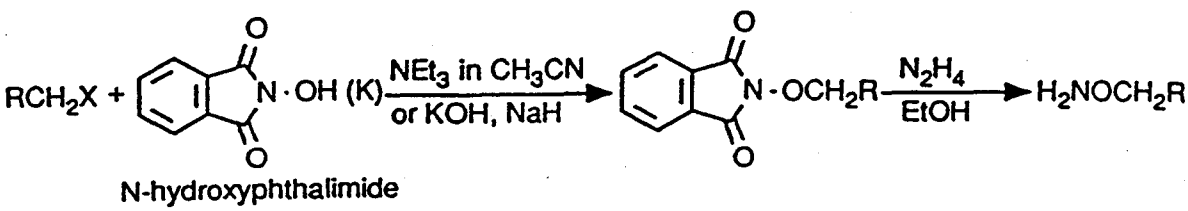


FIGURE 10

Another approach uses triphenylphosphine and diethyl azodicarboxylate to form a betaine. This will react with the appropriate alcohol and N-hydroxyphthalimide to produce the N-hydroxyphthalimide derivative which is again reacted with

hydrazine hydrate to give the O-substituted hydroxylamine (Grochowski & Jurczak, 1976).

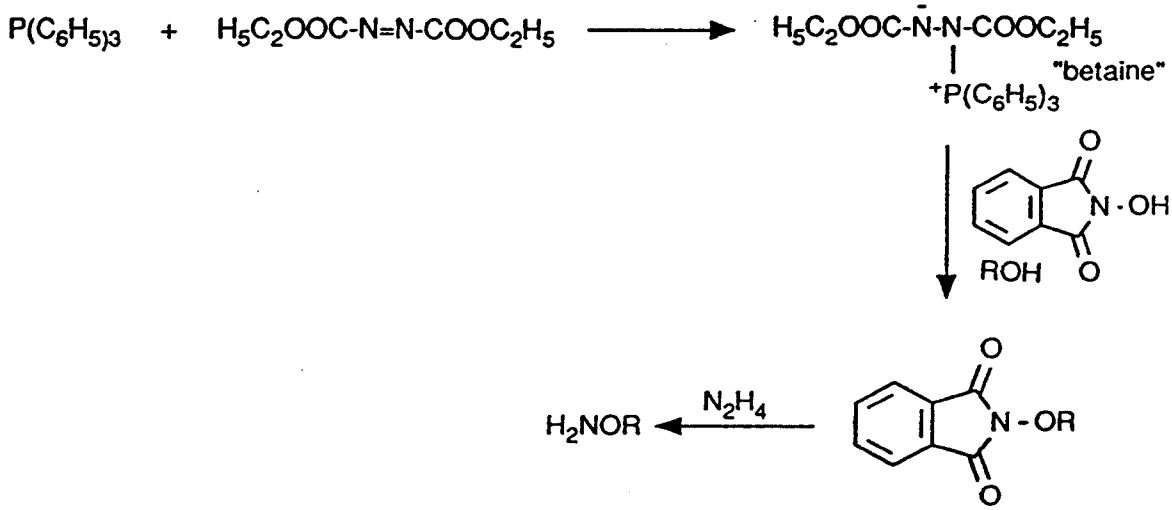


FIGURE 11

The compounds under investigation in this work were generally synthesized using the Gabriel method (Gibson & Bradshaw, 1968) followed by Ing-Manske hydrazinolysis (1926). Each compound was characterized by proton and carbon-13 nuclear magnetic resonance spectroscopy.

1.4 Measuring Diamine Oxidase Inhibition/Histamine Potentiation

1.4.1 Apparatus and Theory of the Method

The apparatus used to measure the potentiation of histamine in this work is called an Ussing chamber. Nerve-free epithelial tissue is bathed on both sides with oxygenated Kreb's solution at a fixed temperature. The trans-mucosal potential difference (PD) is measured with two open-ended agar-potassium chloride bridges positioned close to the tissue surface and connected to a high impedance millivoltmeter. First, sufficient voltage is applied to the tissue to

reduce PD to zero and the short-circuit current (I_{sc}) can then be used to measure the cells response to histamine (Huynh, 1990).

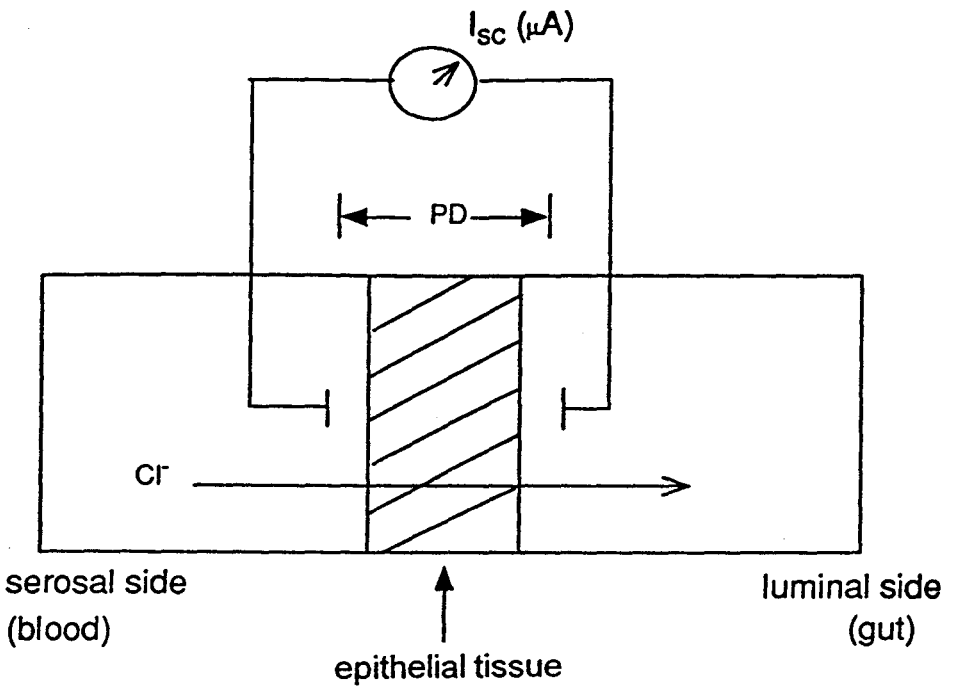


FIGURE 12

In the intestinal epithelium, histamine causes a net secretion of fluid in vivo and this secretion causes alterations in the electrical activity, observed as an *increase* in the short-circuit current, across the epithelium set up in vitro. These effects are mediated by occupation of H_1 receptors through chloride ion secretion. Serosally located sodium/potassium adenosine triphosphatase in conjunction with a sodium/potassium/chloride symport allows chloride ions to enter the cell. Accumulated chloride can then leave the cell across the apical membrane through specific channels regulated by cyclic AMP or calcium while basolateral potassium

efflux allows continued secretion of chloride across the apical surface. If any of these transport mechanisms are modified, chloride ion secretion is altered resulting in a change in I_{sc} (Rangachari, 1992).

1.4.2 Dose-Response Curves

Ideally, to quantitatively measure the potentiation of histamine by hydroxylamine compounds a dose-response relationship should be measured. These experiments involve adding histamine to the Ussing chamber apparatus to measure a control response followed by "washing" to remove the exogenously added histamine. The tissue is then treated with a known concentration of the hydroxylamine compound under investigation for a certain period of time. Histamine is then added in increasing concentrations so that a plot of the change in the short-circuit current (normalized to the initial histamine response) versus the log of the concentration of histamine can be constructed. By comparing the curve measured with the hydroxylamine pretreatment to a curve constructed with histamine alone, the potentiating effects of the hydroxylamine compound can be determined. Dose-response curves are frequently sigmoidal in shape. Two parameters that are easily measured from such curves are the maximal efficacy (E_{max}) and the ED_{50} value. The maximal efficacy is the maximum response elicited by the compound at any concentration. The ED_{50} value is a useful quantity for comparing two curves and is defined as the concentration at which the response is half of the maximal response (Conn & Gebhart, 1989). Thus, a compound that shifts the histamine curve to the left resulting in a lower ED_{50} value

is said to potentiate the tissue's response to histamine since a lower dose is required to cause half of the maximum response.

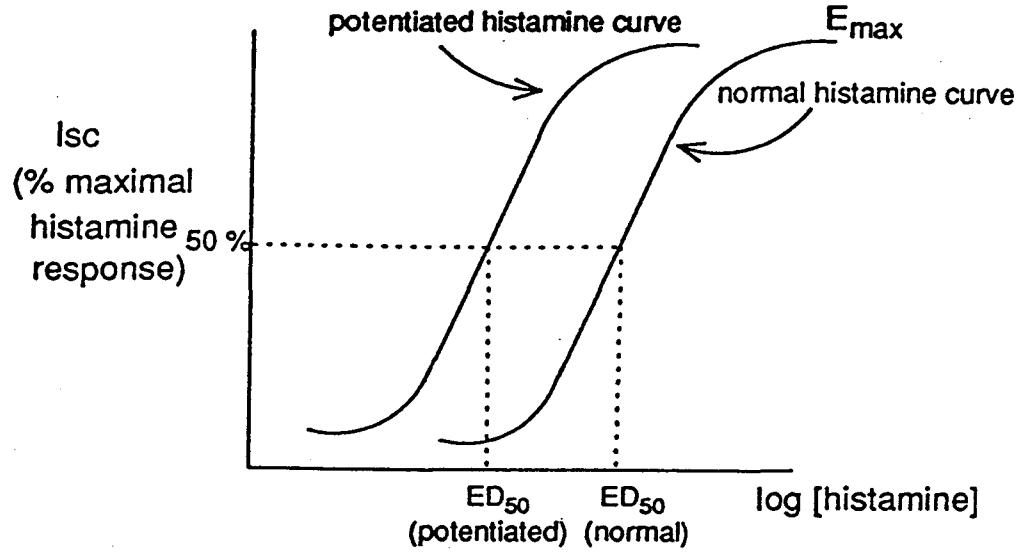


FIGURE 13

In this work, the ED_{50} values are expressed as their negative logarithms (pD_2 = -log ED_{50}) for convenience. The pD_2 values are more easily compared without statistical analysis.

1.4.3 Secondary Response Experiments

An experiment has been designed to perform a rapid, convenient screen for active compounds. In these so-called "secondary response" experiments, histamine is added to the serosal side of the tissue and the response is measured. The hydroxylamine compound is then added and any secondary response is measured. The compound is classified as active (+), inactive (-) or marginally active (+/-). The information obtained from these experiments is essentially qualitative. No significant quantitative values can be measured but it

is a useful method for determining how the compounds should behave in more rigorous tests.

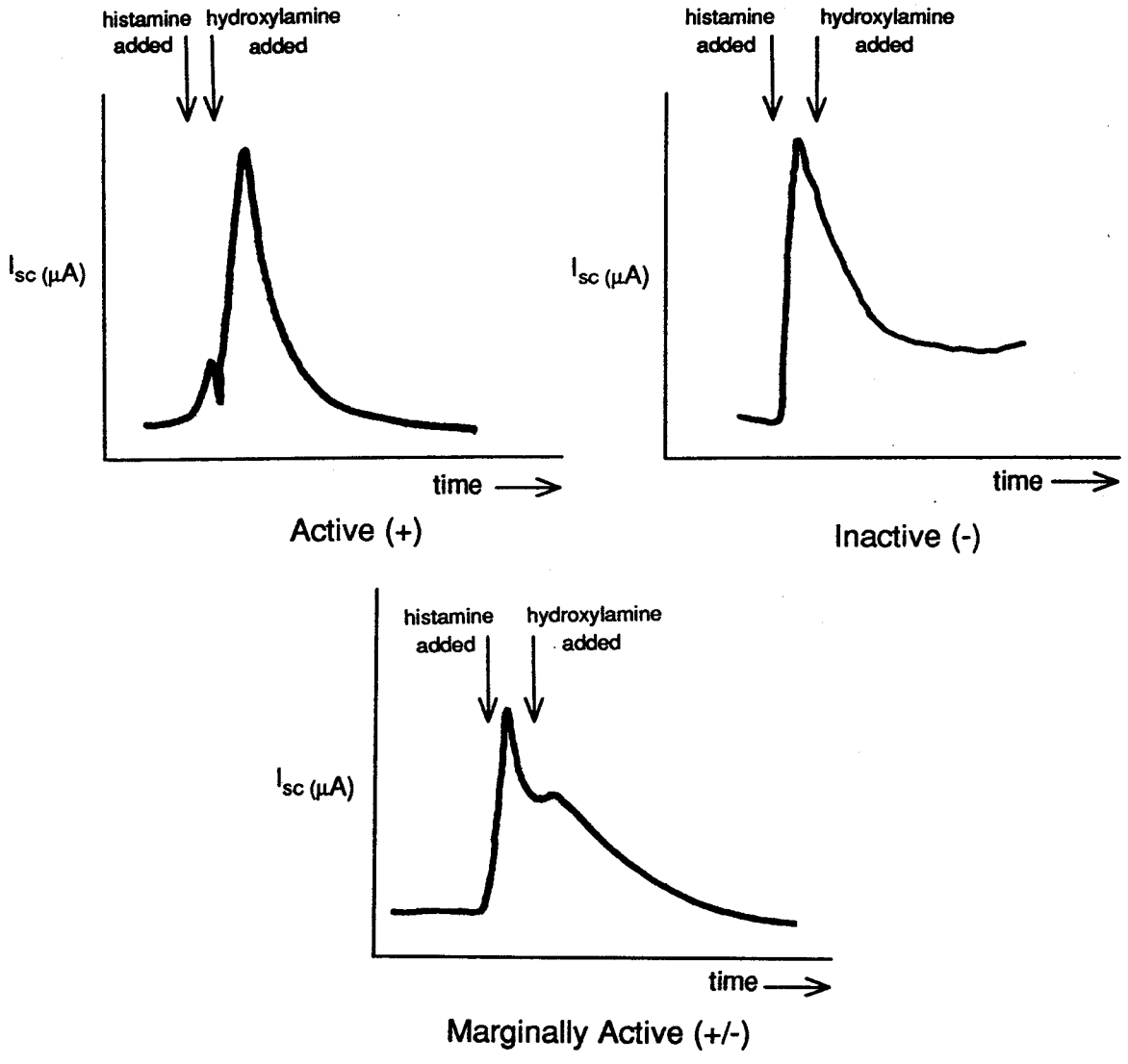


FIGURE 14

1.4.4 Enzyme Assay

The final step in assessing the effects of the hydroxylamine compounds in this system was to perform an enzyme assay on partially purified diamine oxidase. There are several methods available to carry out this task: (1) oxidative

deamination of p-dimethylaminomethylbenzylamine, (2) [β^3H] histamine test, (3) fluorometric vanillic acid method, (4) o-dianisidine test, (5) [^{14}C]-putrescine (radiometric) and (6) coupled NADH method (spectrophotometric) (Kusche & Lorenz, 1983).

The radiometric [^{14}C]-putrescine test developed by Okuyama and Kobayashi (1961) is highly sensitive and relatively simple to perform (Kusche et al., 1973). This method uses putrescine labelled with carbon-14 in positions 1 and 4 as the substrate for diamine oxidase. The oxidative deamination produces labelled γ-aminobutyraldehyde which undergoes spontaneous, non-enzymatic cyclization and polymerization to give labelled Δ_1-pyrroline and various polymers. By measuring the amount of labelled product extracted into toluene and using a liquid scintillation counter, the diamine oxidase activity can be measured.

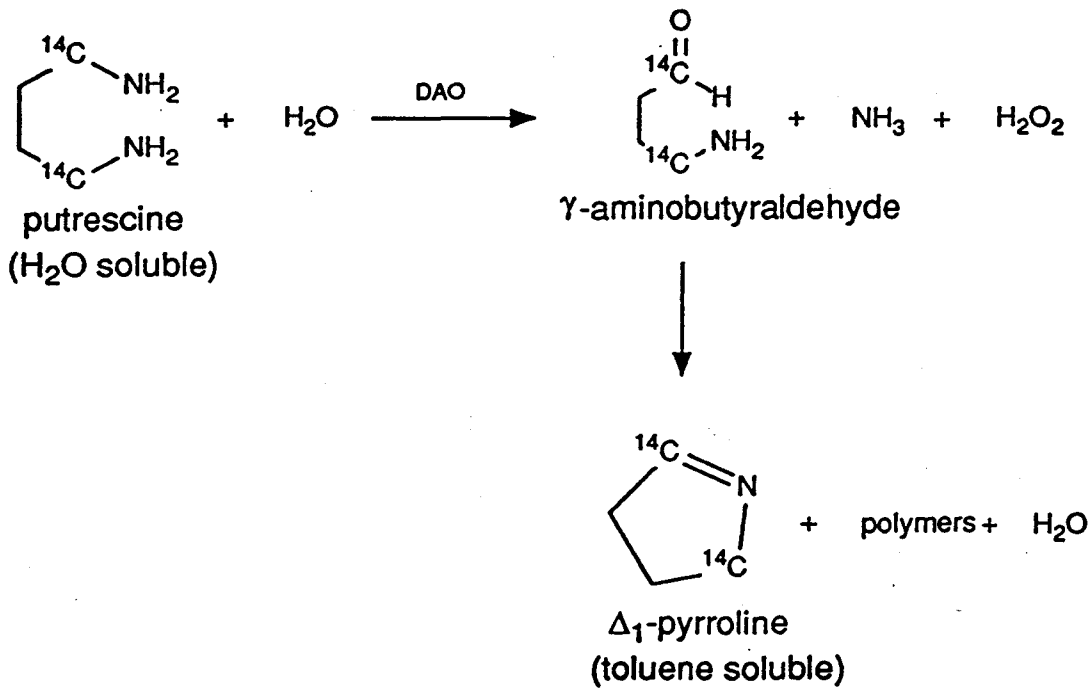


FIGURE 15

A hydroxylamine compound that inhibits diamine oxidase will result in fewer oxidative deamination products and therefore fewer labelled end products will be extracted for scintillation counting.

1.5 Previous Work

Several hydroxylamine compounds have already been tested to determine their ability to potentiate histamine in secondary response experiments. Hydroxylamine and O-methylhydroxylamine were shown to have little or no effect in the absence of exogenously added histamine. But when they were added to the Ussing chamber apparatus, either serosally or luminally, and then histamine added, the response to histamine was increased (potentiated). These effects are specific for histamine since they were not observed for serotonin or carbachol (Rangachari, Bell, Prior & Hunyh, 1992). The secondary response data measured previously is summarized below:

TABLE 1: Activity of Previously Tested Hydroxylamines

Compound	Structure	Activity	Concentration
hydroxylamine	$HONH_2$	+	10^{-4}M
O-methyl	H_3CONH_2	+	10^{-4}M
O-ethyl	$H_3CCH_2ONH_2$	+	10^{-4}M
O-propyl	$H_3C(CH_2)_2ONH_2$	+	10^{-4}M
O-butyl	$H_3C(CH_2)_3ONH_2$	+	10^{-4}M
O-isopropyl	$(CH_3)_2CHONH_2$	+/-	10^{-4}M
O-*tert*-butyl	$(CH_3)_3CONH_2$	-	10^{-4}M
O-benzyl	$(C_6H_5)CH_2ONH_2$	-	10^{-4}M
N-methyl	H_3CNHOH	-	10^{-4}M
N-tertbutyl	$(CH_3)_3CNHOH$	-	10^{-4}M
O-sulfonate	HO_3SONH_2	-	10^{-4}M
O-acetate	$HO_2CCH_2ONH_2$	-	10^{-4}M
O-(2-hydroxy)ethyl	$HO(CH_2)_2ONH_2$	+	10^{-4}M
methylamine	H_3CNH_2	-	10^{-4}M
dimethylamine[a]	$(CH_3)_2NH$	-	10^{-4}M
isopropylamine	$(CH_3)_2CHNH_2$	-	10^{-5}M

[a]All data from Rangachari, Bell, Prior & Hunyh, 1992 except dimethylamine from Hunyh, 1990.

From this work, some trends can be observed: (1) active compounds have the general structure H_2NOR; O-substituted compounds are active while N-substituted compounds are not (compare O-methyl and N-methyl hydroxylamines), (2) the branching of the side chain seems to be important; straight chain propyl and butyl compounds are active while their branched isobutyl and *tert*-butyl analogues are not, and (3) the size and charge of the alkyl substituent can affect

the activity of the compound; the O-benzylhydroxylamine is inactive possibly due to the steric bulk of the benzene ring and the sulfonate and acetate compounds are inactive possibly due to the charge on the substituent.

1.6 Objectives

The main objective of this thesis has been to further investigate the structure-activity relationship between O-alkyl and O-benzyl hydroxylamines and diamine oxidase in order to: (1) gather more information regarding diamine oxidase inhibition and the interactions between hydroxylamines and the active site of diamine oxidase and (2) gain additional insight into the mechanism of histamine action.

This work builds on that presented by Rangachari, Bell, Prior and Hunyh (1992) with the synthesis of sixteen O-alkyl and O-benzyl hydroxylamines and the physiological testing of these compounds to determine their abilities to potentiate histamine through diamine oxidase inhibition. We hypothesize from examination of the previous work that the O-alkyl substituted compounds should deactivate diamine oxidase and therefore potentiate histamine as O-methyl, O-ethyl, O-propyl and O-butyl hydroxylamines do. The O-benzyl hydroxylamines should not show any potentiating effects at least in the secondary response experiments.

To test this hypothesis, the compounds were synthesized and subjected to the following regimen of analysis:

(1) initial qualitative screen: secondary response (10^{-4}M) - measures potentiation of histamine

(2) quantitative measure of histamine potentiation: dose-response curves constructed for compounds chosen to test different functional groups (10^{-4}M)

(3) quantitative measure of diamine oxidase inhibition: enzyme assay (10^{-4} - 10^{-8}M).

The ultimate goal of this type of work is to crystallize diamine oxidase by itself and with a hydroxylamine inhibitor in place so that X-ray crystallographic structures can be measured. This would give us a much better picture of the active site on the diamine oxidase enzyme and hopefully make more clear the role of copper and the organic cofactor in the catalytic degradation of histamine.

CHAPTER 2

The Syntheses of O-alkyl and Simple O-benzyl hydroxylamines

2.1 Modified Gabriel Synthesis

The same general reaction was used to prepare all of the O-alkyl and O-benzyl hydroxylamines. The first step in each preparation is basically the Gabriel amine synthesis using deprotonated N-hydroxyphthalimide as the nucleophile (Drain et al., 1965).

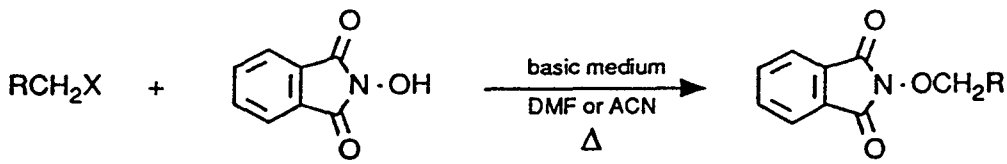


FIGURE 16

The reaction takes place by an S_n2 mechanism:

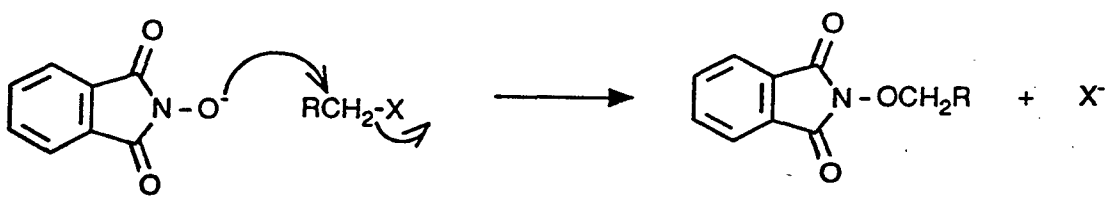


FIGURE 17

The trend in reaction time that is observed is that expected in an S_n2

reaction. The benzylic and allylic halides reacted in the least amount of time. The more steric bulk at the β-carbon of the electrophilic alkyl halides, the slower the reaction. For instance, the reaction of isobutyl bromide with N-hydroxyphthalimide is complete in less time than the reaction with *sec*-butyl bromide. Sodium iodide was added to facilitate the overall reaction rate since the iodide ion is a better nucleophile than the N-hydroxyphthalimide anion and it displaces the chloride or bromide giving an intermediate alkyl iodide. The N-hydroxyphthalimide anion then displaces the iodide at a faster rate since the iodide ion is a better leaving group than chloride or bromide. The N-hydroxyphthalimide compounds can be extracted into an organic solvent (chloroform) leaving any unreacted N-hydroxyphthalimide in the aqueous phase.

2.2 Ing-Manske Hydrazinolysis

As in the Gabriel synthesis, hydrazine hydrate is used to cleave the phthalimide portion (Ing & Manske, 1926).

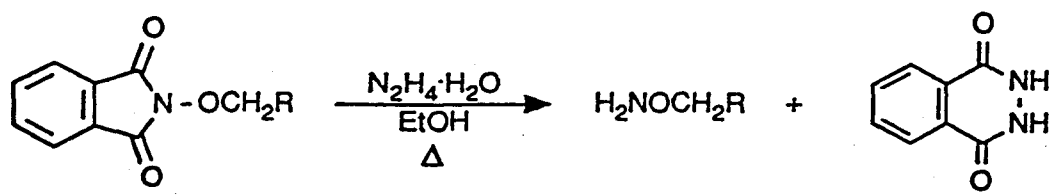


FIGURE 18

The proposed mechanism of this reaction is below:

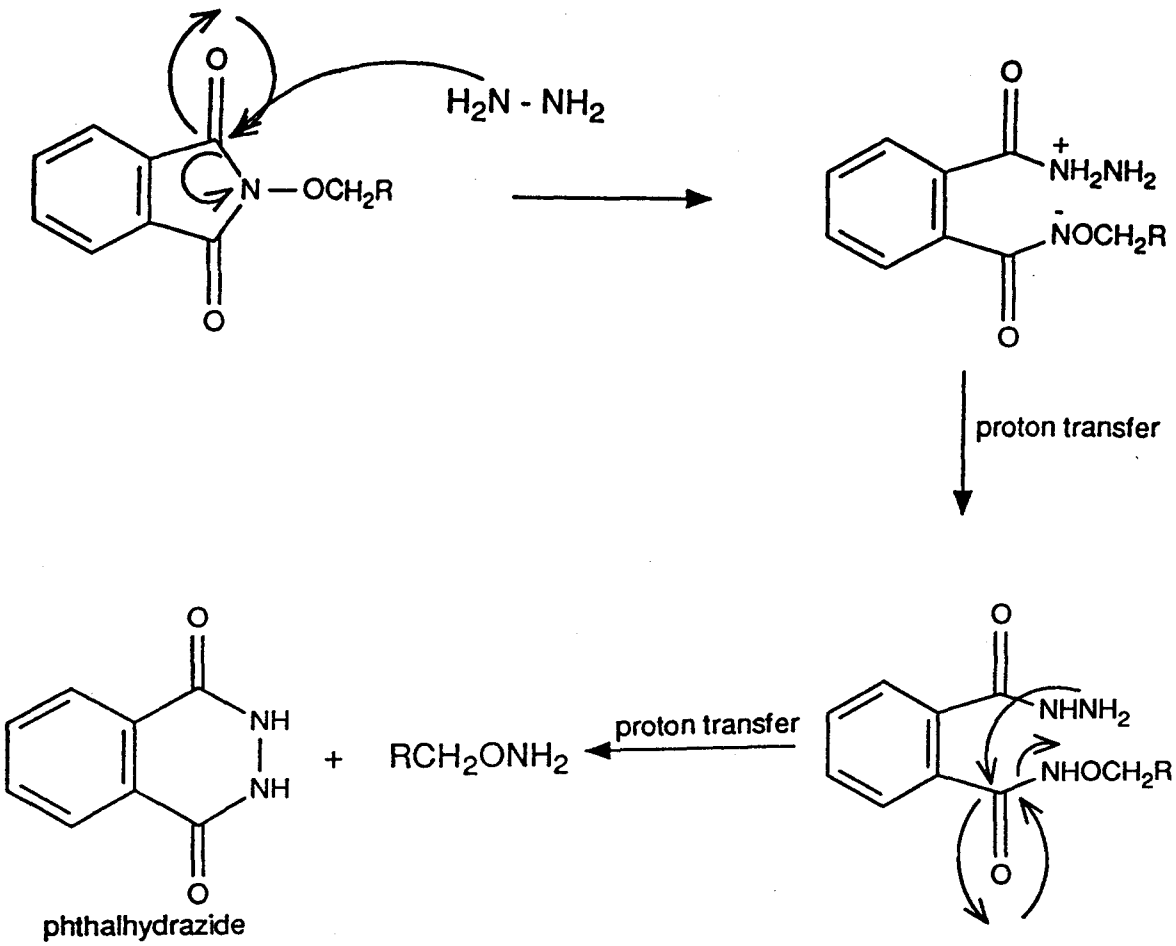


FIGURE 19

The majority of the phthalhydrazide is removed by virtue of the fact that it is less soluble in ethanol than the hydroxylamine compounds. Evaporating off the solvent slowly precipitates the phthalhydrazide which can then be filtered off leaving the impure hydroxylamine.

2.3 Removal of Residual Hydrazine and Hydrazides

Since hydrazine and hydrazide compounds can inhibit diamine oxidase, they must be removed before the pharmacological testing is performed. The pKa

of hydroxylamines is approximately 6.03 while hydrazine has a pKa of 8.23 (The CRC Handbook of Chemistry & Physics, 1968). In a buffer solution of pH 7.40, 96% of the hydroxylamine is present as the free-base and is therefore soluble in an organic solvent (methylene chloride). Hydrazine, on the other hand, exists mainly as the protonated species (87%) and is soluble in an aqueous phase. Two extractions with a KH_2PO_4/NaOH buffer with a pH of 7.40 will leave 1.7% of the hydrazine in the organic layer and 3.8% of the free hydroxylamine will be lost in the aqueous layer. The hydroxylamine can then be extracted into an aqueous phase as the hydrochloride salt with minimal hydrazine remaining.

2.4 Nuclear Magnetic Resonance Spectroscopy

The efficiency of the reaction can be determined by examining the chemical shift change of the methylene or methine protons adjacent to the oxygen atom. Typically, a downfield shift of 0.5 to 1 ppm is observed in going from the alkyl halide to the O-alkyl-N-hydroxyphthalimide. Moreover, measurement of the relative area of the methine or methylene protons adjacent to oxygen versus the area of the aromatic phthalimide signals enabled the purity of the isolated O-alkyl-N-hydroxyphthalimide to be assessed. The removal of the phthalimide portion can be monitored by observing the disappearance of the signal from the aromatic protons due to the phthalimide (typically a multiplet at approximately 7.76 ppm in $CDCl_3$).

Nuclear magnetic resonance spectroscopy is a useful tool for determining the presence of any organic contaminants such as side-products of the synthetic

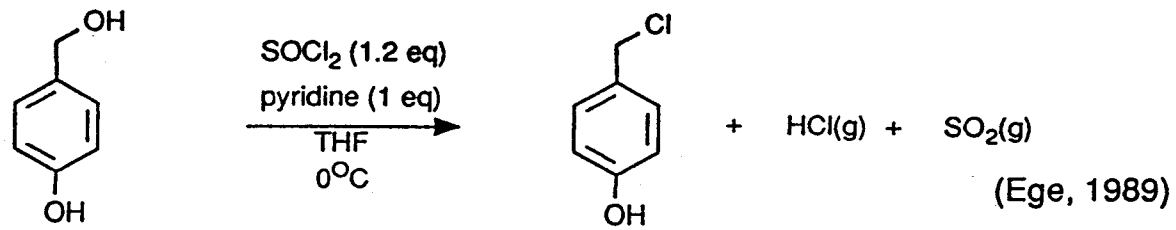
reactions except for residual hydrazine which does not usually produce signals due to rapid proton-deuterium exchange with the solvent.

CHAPTER 3

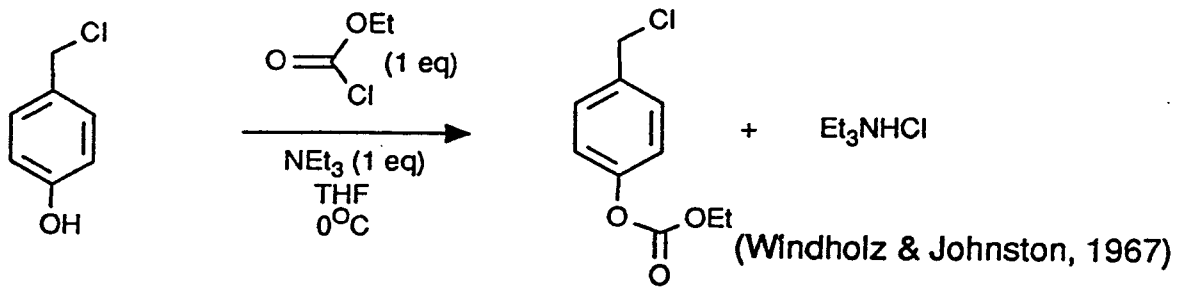
The Synthesis of p-hydroxybenzyl hydroxylamines

3.1 Problems With Direct Synthesis From p-hydroxybenzyl halides

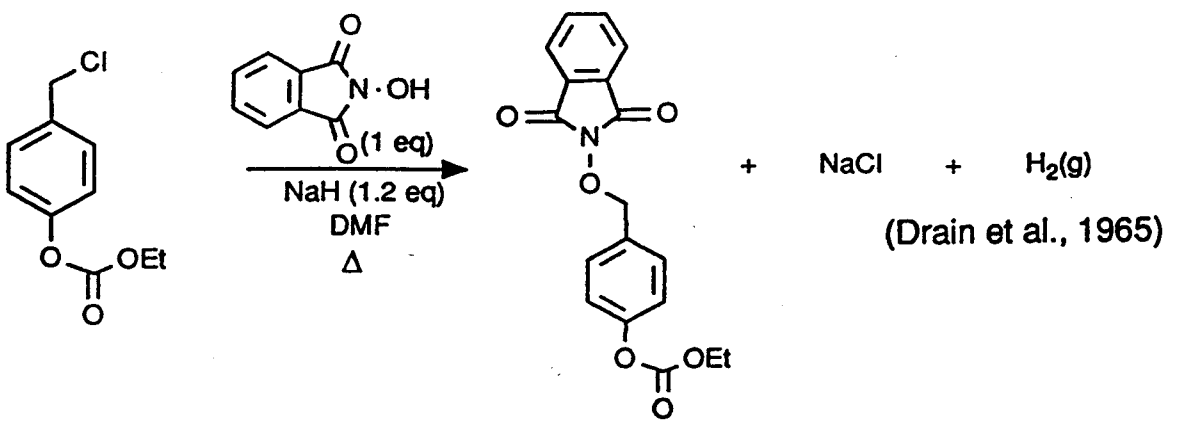
The proposed reaction scheme for the synthesis of p-hydroxybenzyl hydroxylamines was as follows:



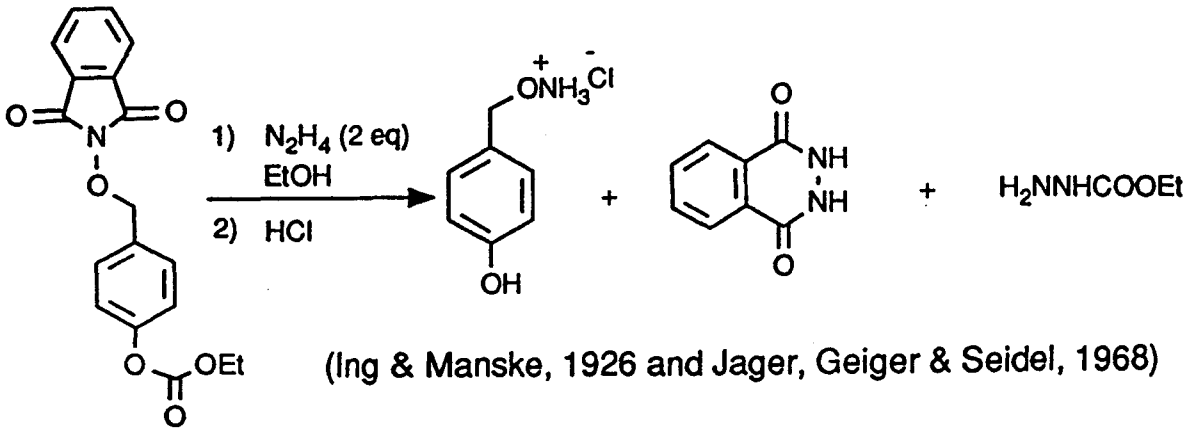
(Ege, 1989)



(Windholz & Johnston, 1967)



(Drain et al., 1965)

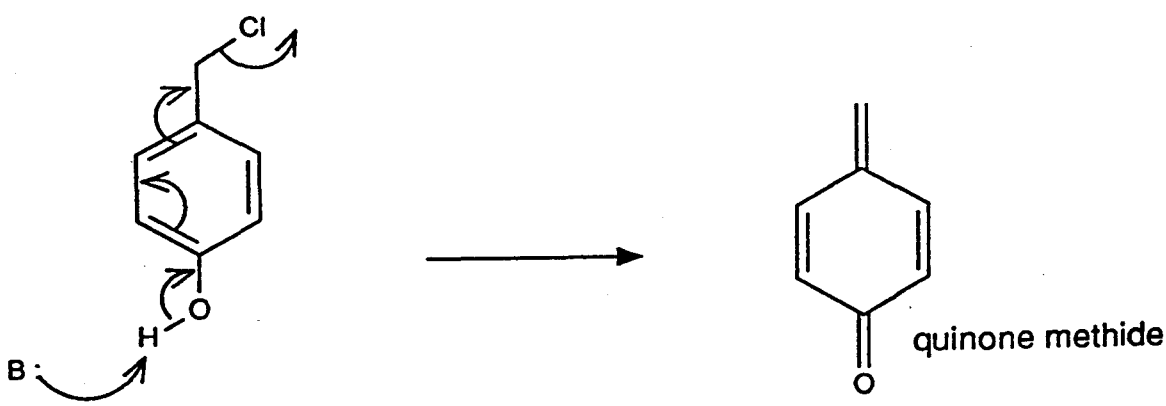


(Ing & Manske, 1926 and Jager, Geiger & Seidel, 1968)

FIGURE 20

The p-hydroxybenzyl chloride was isolated several times but problems were encountered in carrying the reaction further. Rapid polymerization was observed under various conditions and changing the solvent, temperature and amount of base added did not completely suppress the polymerization reaction.

The facile formation of the polymer can be rationalized by the formation of quinone-methide (Sanner et al., 1992) and is illustrated in figure 21:



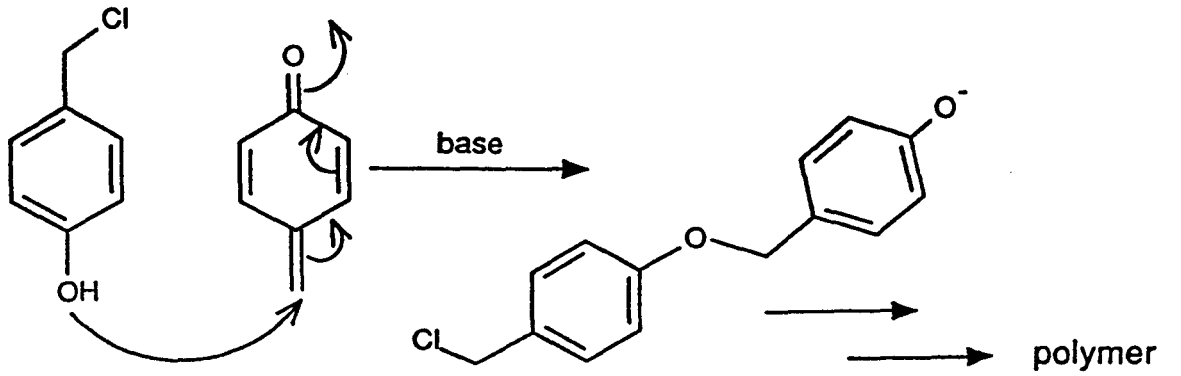
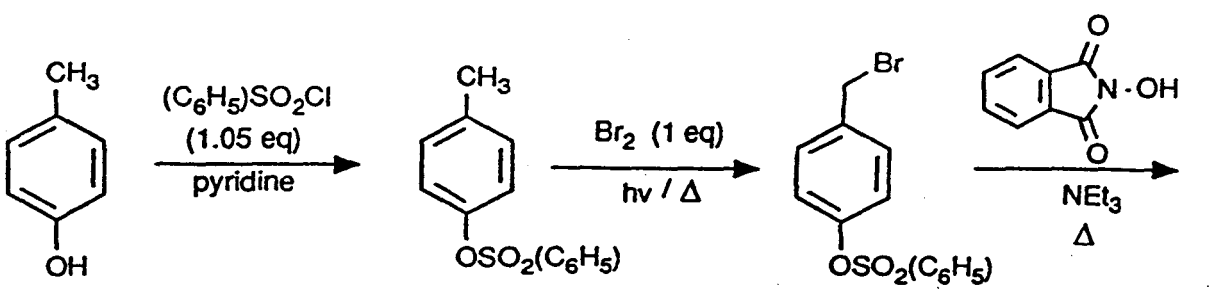


FIGURE 21

The synthesis of the benzyl chloride as well as the protection of the phenolic oxygen both occur in a basic medium thereby favouring the polymerization reaction.

3.2 Solution

This problem was circumvented by Drain et al. (1965) in a British Patent. They published the following scheme to synthesize various hydroxy-substituted benzyl hydroxylamines:



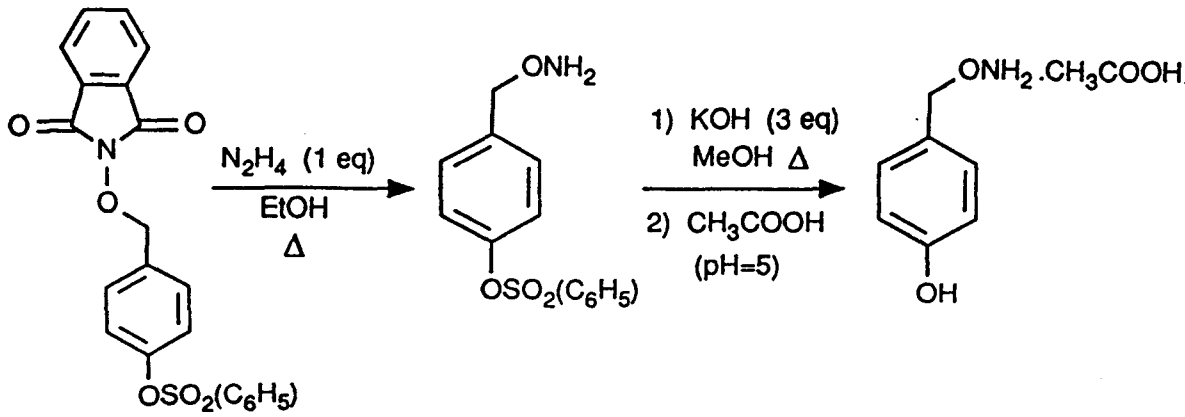


FIGURE 22

p-Hydroxybenzyl and p-hydroxy-m-bromobenzyl hydroxylamine were synthesized using this method with a few minor variations. All intermediates were characterized by proton nuclear magnetic resonance spectroscopy to observe the efficiency of each reaction step.

3.3 Purification

The residual hydrazine and hydrazides were removed as explained previously (see section 2.3) using a KH_2PO_4/NaOH buffer with a pH of 7.40.

The final product was purified by using an ion-exchange resin. The resin used was Amberlite IR-120 ion-exchange resin (Mallinckrodt). It is a strongly acidic, sulfonated polystyrene type, cation exchange resin of medium porosity. Initially, the resin is in its protonated form. The free hydroxylamine forms an ionic salt complex with the sulfonate groups. Rinsing the resin with water removes other contaminants that form weaker bonds to the sulfonate group ie. are less basic (specifically acidic or neutral species). The purified free hydroxylamine can

then be recovered by washing with ammonium hydroxide.

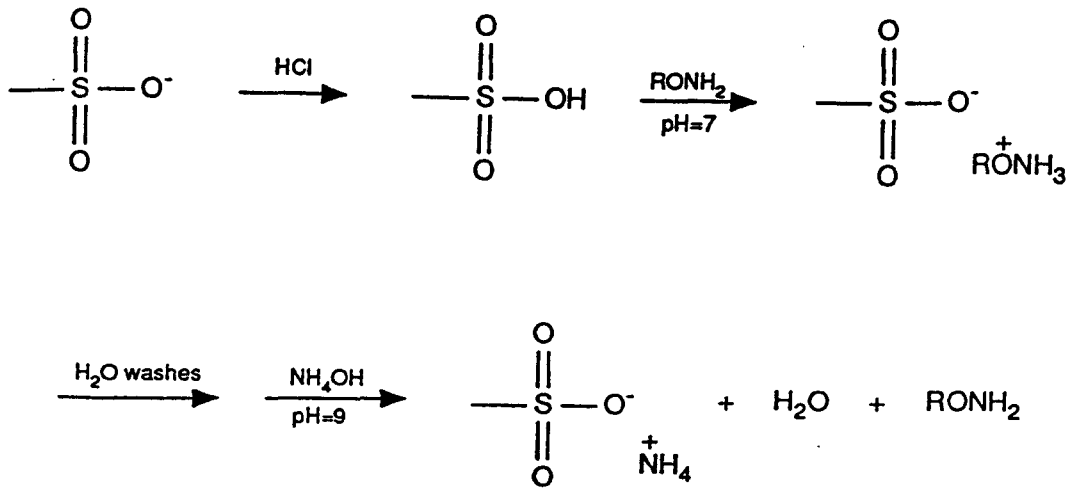


FIGURE 23

CHAPTER 4

PHARMACOLOGICAL TESTING

4.1 Definition of Activity

The results of each test were examined and each compound was classified as active, inactive or partially active. The criteria of each is dependant on the experiment.

The activity of each compound must be assessed from two perspectives. In secondary response and dose-response experiments, the diamine oxidase is located in the cells that compose the epithelium. Activity is manifested by the potentiation of histamine through an increase in the short-circuit current. If the compound cannot gain access to the diamine oxidase in the tissue preparation, no potentiation of histamine is observed. To alleviate this problem, the direct inhibition of diamine oxidase can be measured by preparing the partially purified enzyme. Access limitations are overcome in these experiments so that the ability of the hydroxylamine compound to inhibit diamine oxidase can be measured.

4.1.1 Secondary Response Experiments

This approach was used as a rapid qualitative screen for active compounds. The presence or absence of the secondary rise was an index of activity. If the secondary rise was larger than the initial response to histamine,

the compound was considered active (+). A smaller secondary rise or a flattening of the decaying histamine response was considered as partially active (+/-). Compounds that have no effect on the decaying curve are classified as inactive (-) and in one case (O-decyl), the hydroxylamine compound caused a sharp decrease in the short-circuit current. This compound was also called inactive.

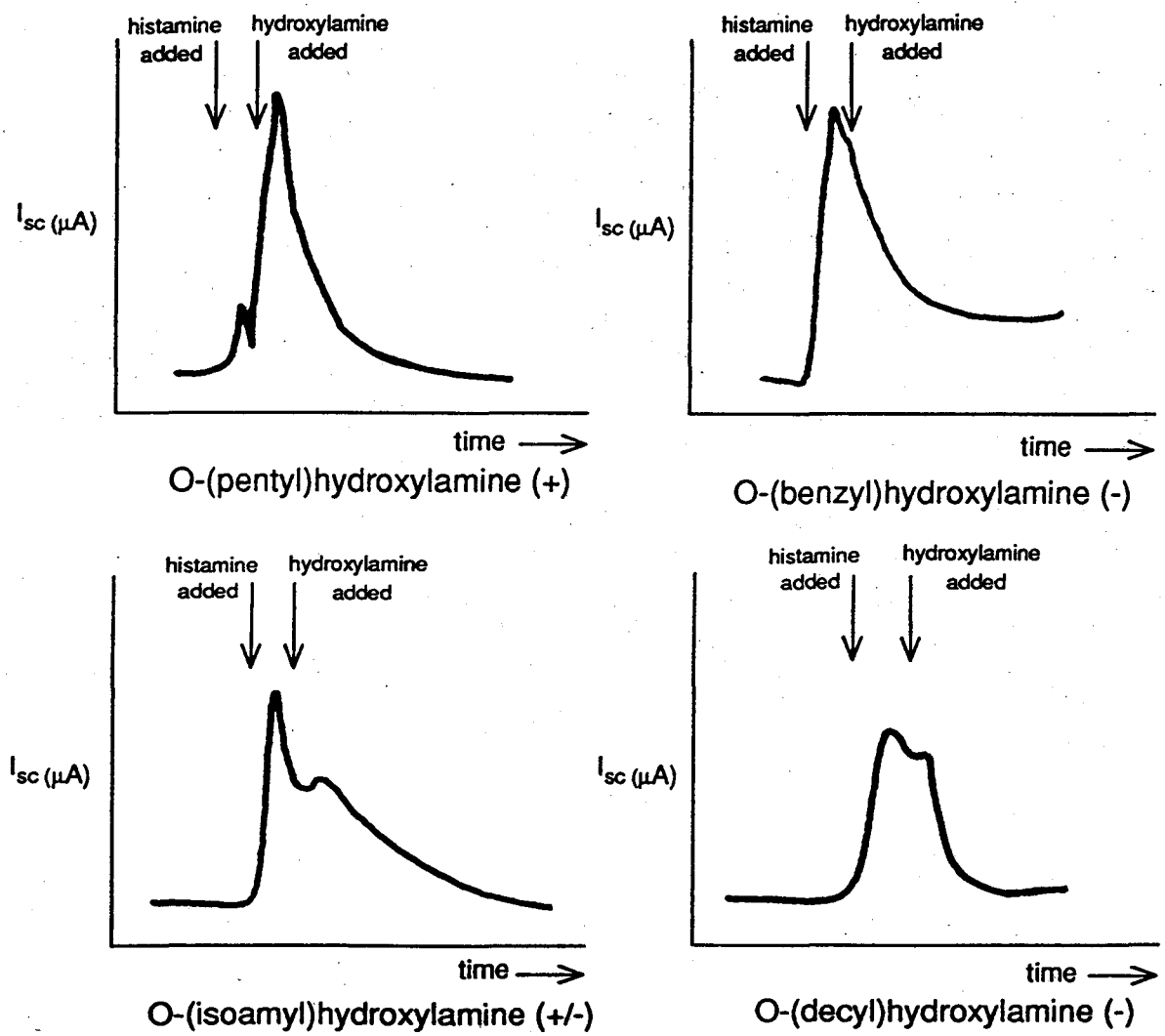


FIGURE 24

4.1.2 Dose-Response Experiments

Activity in these experiments was determined by examining the shift in the dose-response curve with hydroxylamine pretreatment relative to the normal histamine curve. The shift in the curve is detected by comparing the pD_2 value to histamine after pretreatment with the hydroxylamine to the control curve. Compounds that give a pD_2 value significantly different ($P < 0.05$) from the histamine control are considered active (+).

4.1.3 Enzyme Assay

The O-alkyl and benzyl hydroxylamines synthesized were tested at five concentrations in this set of experiments. The optimal concentration range for determining activity appeared to be between 10^{-5}M and 10^{-6}M.

The data were expressed as a percentage of the control activity:

raw dpm with inhibitor/raw dpm without inhibitor X 100

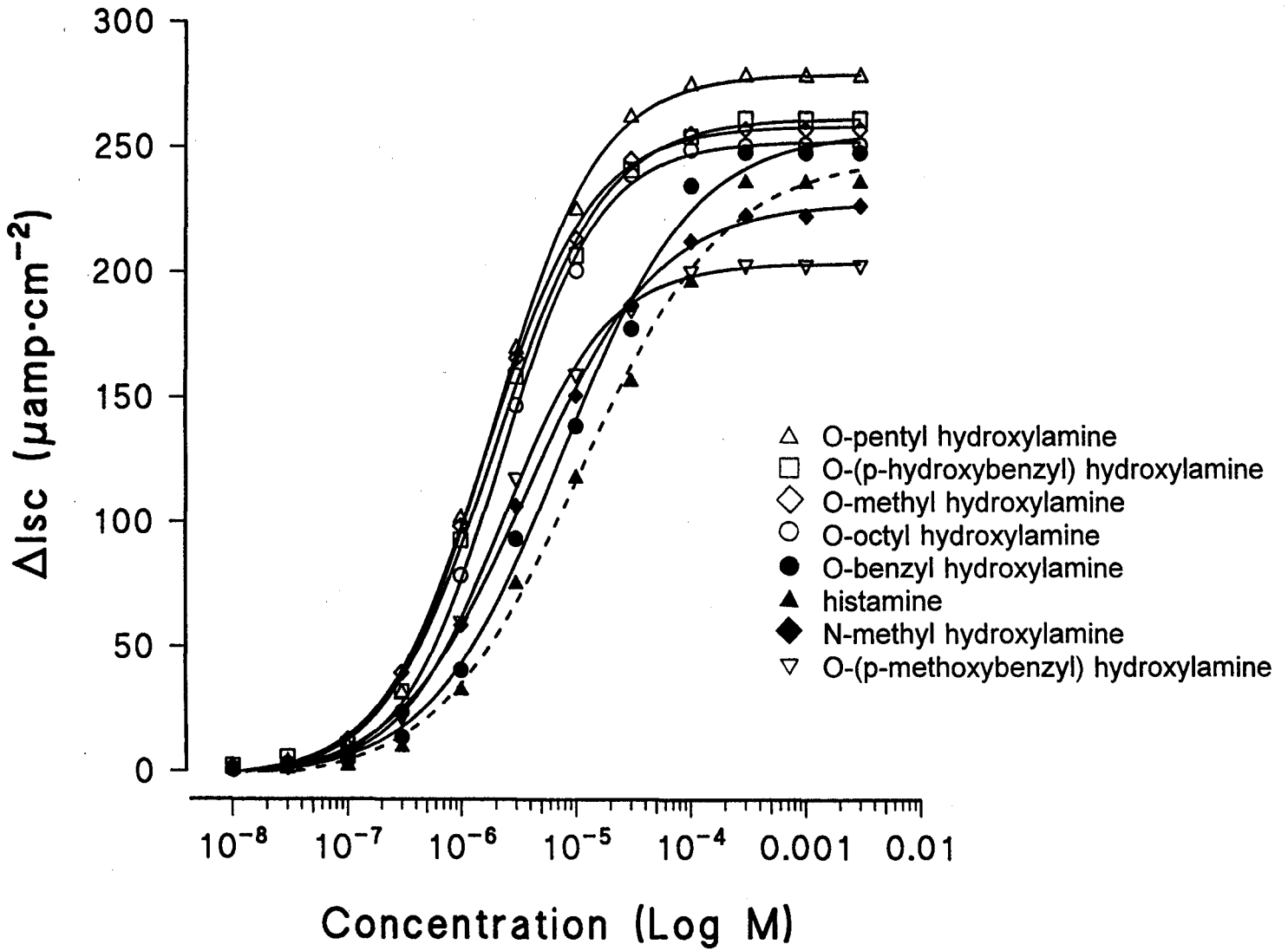
The larger the number of decays per minute, and consequently the larger the percentage, the less active the hydroxylamine compound in inhibiting diamine oxidase. At 10^{-5}M and 10^{-6}M, compounds giving percentages less than approximately 30% were considered active (+), greater than approximately 70% were inactive (-) and between 30 and 70% were called partially active (+/-).

4.2 Comparing the Dose-Response Curves

Dose-response relationships were constructed for seven compounds at 10^{-5}M. Only five of the sixteen compounds synthesized were tested because of the time and number of tissue preparations required to complete the dose-

response experiments. The compounds that were tested were chosen to examine the different types of alkyl substituents. O-pentyl and O-octyl hydroxylamines represent short and long straight chain hydrocarbons. O-benzyl, O-(p-hydroxybenzyl) and O-(p-methoxybenzyl) hydroxylamines were studied to examine the effect on activity of an oxygen substituent of the benzene ring. O-methyl hydroxylamine is a known diamine oxidase inhibitor and was used as a positive check while N-methyl hydroxylamine is known to be inactive and was used as a negative check. The results presented below are the average of five repetitions of the experiment.

FIGURE 25


300
250
200
150
100
50
0
ΔIsc (μamp·cm⁻²)
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
0.001
0.01
Concentration (Log M)
O-pentyl hydroxylamine
O-(p-hydroxybenzyl) hydroxylamine
O-methyl hydroxylamine
O-octyl hydroxylamine
O-benzyl hydroxylamine
histamine
N-methyl hydroxylamine
O-(p-methoxybenzyl) hydroxylamine

By examining the relative positions of the curves, it appears that a shift has occurred in the cases of O-pentyl, O-methyl, O-(p-hydroxybenzyl), O-octyl and possibly O-(p-methoxybenzyl)hydroxylamines (open symbols on graph in figure 25). This indicates that these compounds may be active.

The curves have been fit using a Fig.P software package (Fig. P, version 6.0, Biosoft, Milltown, NJ) that calculates the maximum of each curve as well as a pD_2 value for each compound tested. These two parameters are typically used in pharmacology to compare dose-response curves. A one-way analysis of variance was performed on the curve maxima and on the pD_2 values. The maxima did not show any significant variations ($P = 0.762$), as expected for simple potentiation of histamine through diamine oxidase inhibition, and therefore no further analysis of these data was necessary. The pD_2 values found for the eight curves did however show significant differences ($P = 0.001$) in the analysis of variance test and so Newman-Keuls tests (Rangachari, Bell, Prior & Hunyh, 1992) were performed for each compound. From these tests, a P value was determined and the difference between the test compound curve and the histamine control curve was considered significant if P was less than 0.05.

TABLE 2: pD_2 Values for Histamine in the Presence of Hydroxylamines

Pretreatment	pD_2 (histamine)	P
none	4.87 ± 0.25	-
O-methylhydroxylamine	5.71 ± 0.13	0.004*
N-methylhydroxylamine	5.34 ± 0.14	0.074
O-pentylhydroxylamine (12)	5.72 ± 0.16	0.005*
O-octylhydroxylamine (16)	5.59 ± 0.09	0.008*
O-benzylhydroxylamine (20)	5.14 ± 0.18	0.237
O-(p-methoxybenzyl) hydroxylamine (26)	5.73 ± 0.07	0.010*
O-(p-hydroxybenzyl) hydroxylamine (33)	5.68 ± 0.06	0.004*

*These compounds are considered active in these experiments since $P < 0.05$.

As seen in the above table, the compounds O-methyl, O-pentyl, O-octyl, O-(p-methoxybenzyl) and O-(p-hydroxybenzyl)hydroxylamine showed significant shifts in the dose response curve and can therefore be considered as active potentiators of histamine.

4.3 Summary of Pharmacological Test Results

The activities, as defined previously, of each compound in the secondary response and dose response experiments are summarized in the following table:

TABLE 3: Results of Pharmacological Testing

Compound	[a]2° Response (10^{-4}M)	Characteristics of 2° Rise	[a]Shift in pD_2
sec-butyl (2)	+	- sharp, larger response	NT
isobutyl (4)	+	- sharp, larger response	NT
isoamyl (6)	+/-	- slow, smaller response	NT
allyl (8)	+	- sharp, larger response	NT
4-pentynyl (10)	+	- sharp, larger response	NT
pentyl (12)	+	- sharp, larger response	+
hexyl (14)	+	- sharp, slightly larger	NT
octyl (16)	+/-	- slow, minimally larger	+
decyl (18)	-[b]	- dips below baseline	NT
benzyl (20)	-[c]	- no effect on decay	-
cinnamyl (22)	+/-	- slow, smaller response	NT
p-bromobenzyl (24)	+/-	- flattens curve	NT
p-methoxybenzyl (26)	+	- sharp, larger response	+
m-methoxybenzyl (28)	+	- slow, larger response	NT
p-hydroxybenzyl (33)	+	- sharp, larger response	+
p-hydroxy-m-bromobenzyl (38)	+/-	- slow, smaller response	NT

[a]Activities of the hydroxylamine compounds are "+" active, "-" inactive or "+/-" partially active (see section 4.1)

[b]O-decyl hydroxylamine causes a quick decrease in the measured short-circuit current in 2° response experiments resulting in a dip below the baseline of the curve.

[c]Tissues used to test compounds that appeared to be inactive (-) in the 2° response experiments were tested with O-methylhydroxylamine, a compound that is known to be active in such experiments, to ensure that the measured activity was not due to a problem with the tissue.

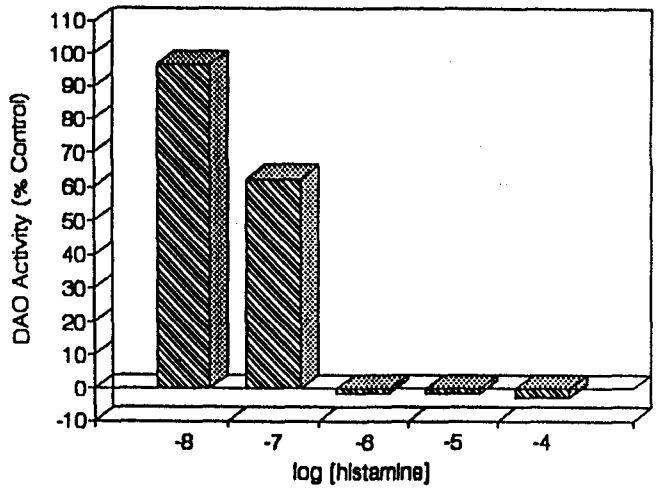
NT = not tested due to amount of time and number of tissue preparations required for dose-response experiments (see section 4.2)

The observed activities are consistent between the two sets of experiments. So, we can conclude that some of the hydroxylamine compounds synthesized in this work do in fact potentiate histamine responses in the colonic tissue preparation. This is in all likelihood through the inhibition of diamine oxidase. Other hydroxylamines do not appear to potentiate histamine in these types of experiments. To exclude the possibility that this failure is due to limitation of access to the active enzyme, the effects were studied on partially purified diamine oxidase.

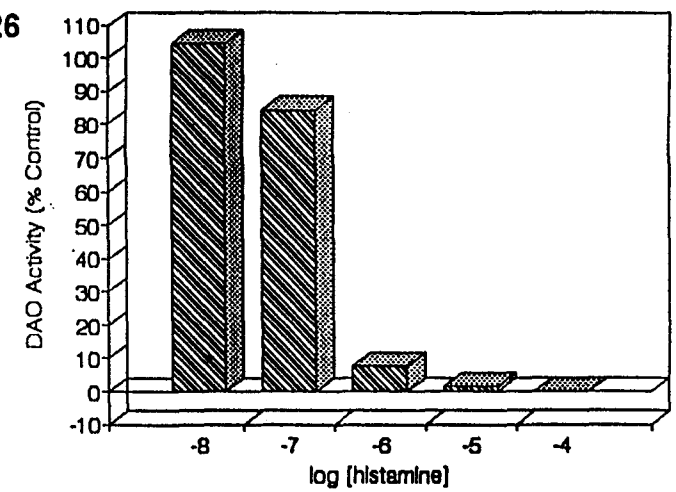
4.4 Enzyme Assay Activity-Concentration Profiles

Based on the results of the pharmacological tests, it appears that some of the O-alkyl hydroxylamines are able to inhibit diamine oxidase (observed as potentiation of histamine in the epithelial preparation). These results were confirmed by performing the enzyme assays. The activities of the hydroxylamine compounds as a function of histamine concentration is shown in the following bar graphs (figure 26):

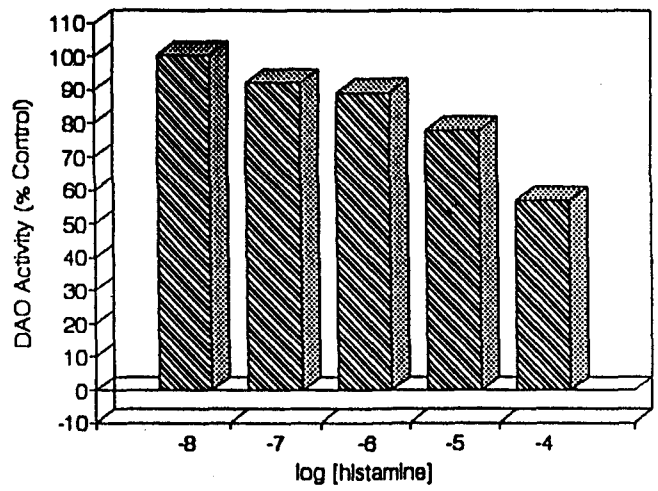
FIGURE 26



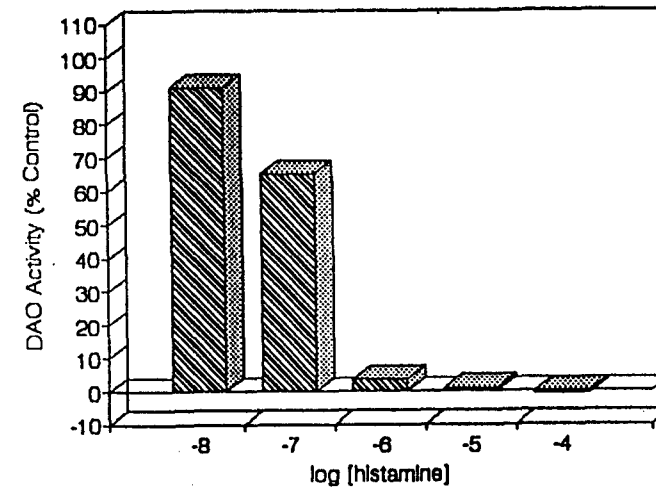
Aminoguanidine



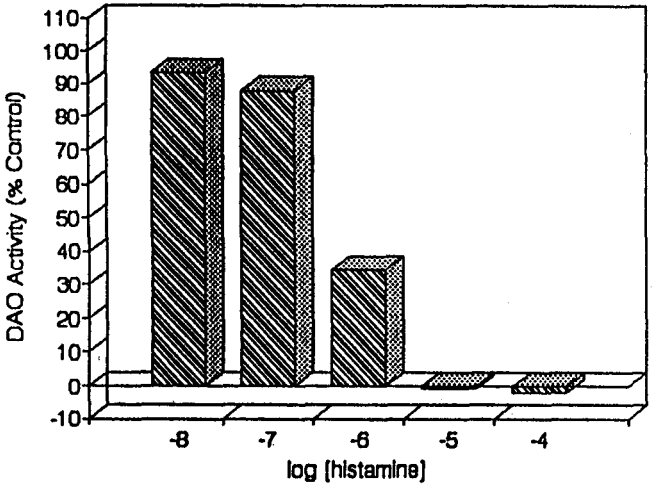
Hydroxylamine



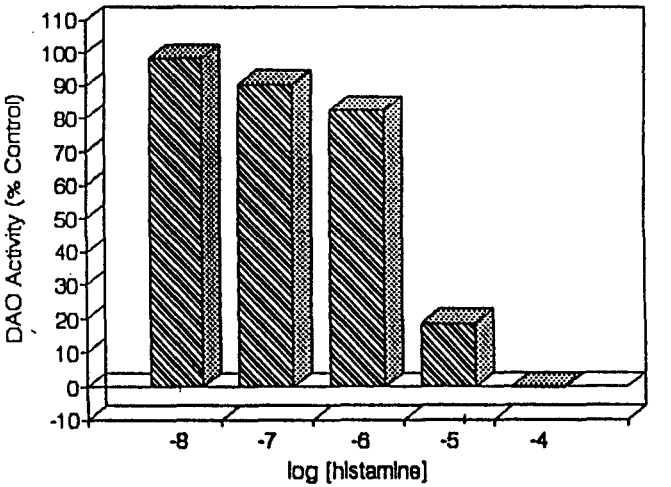
N-(methyl)hydroxylamine



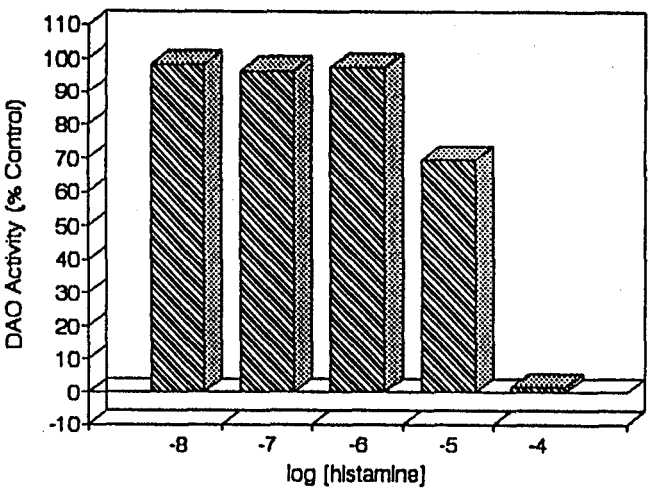
O-(methyl)hydroxylamine



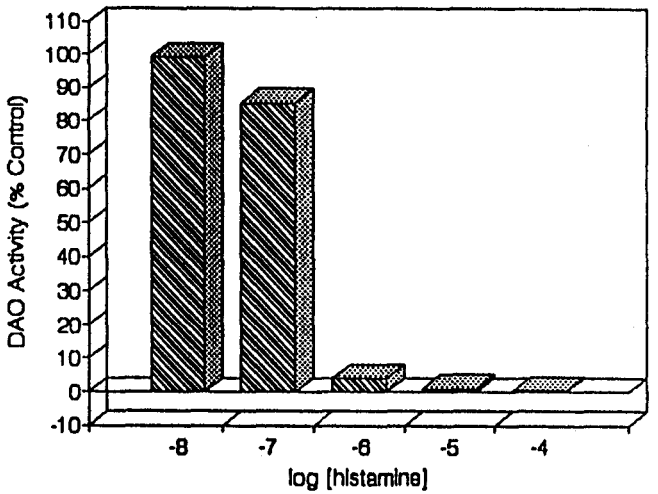
O-(*sec*-butyl)hydroxylamine hydrochloride (2)



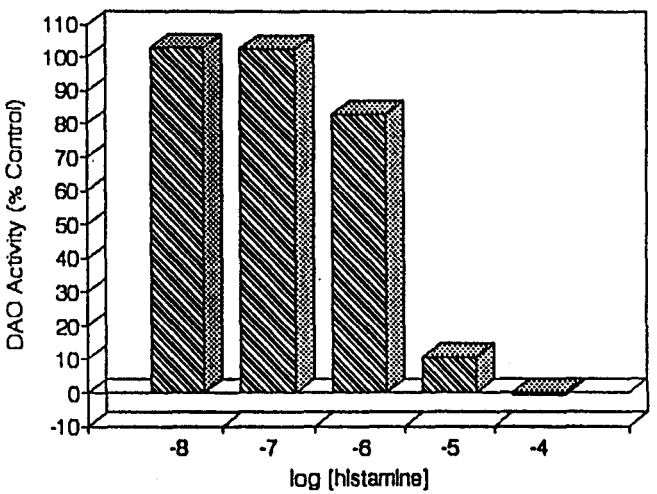
O-(isobutyl)hydroxylamine hydrochloride (4)



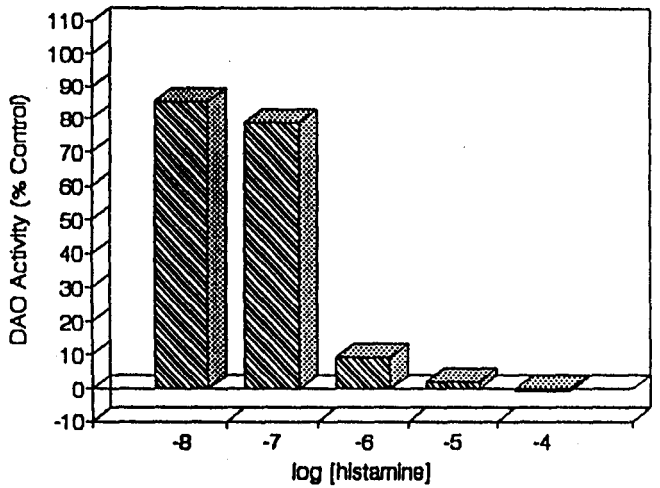
O-(isoamyl)hydroxylamine hydrochloride (6)



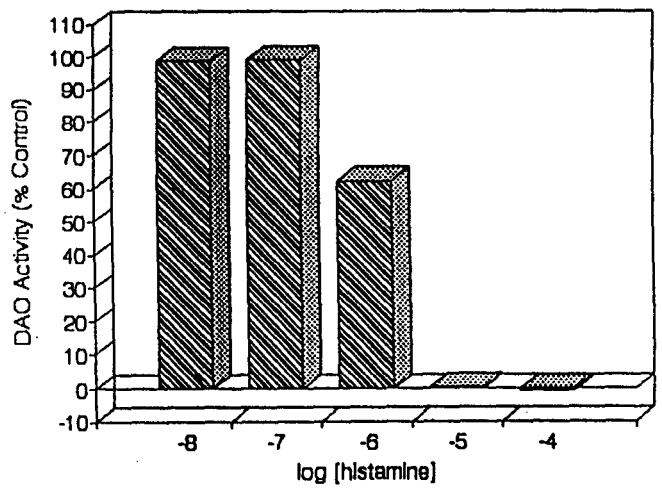
O-(allyl)hydroxylamine hydrochloride (8)



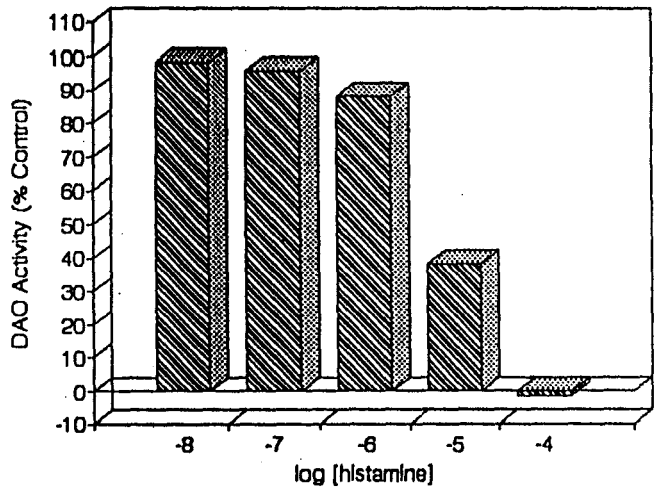
O-(4-pentynyl)hydroxylamine hydrochloride (10)



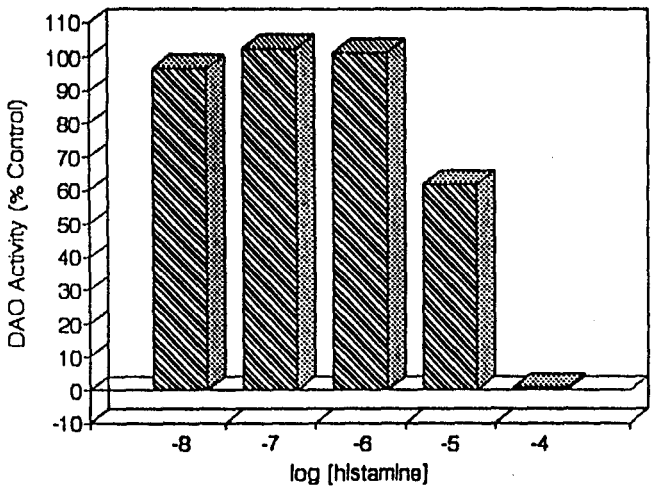
O-(pentyl)hydroxylamine hydrochloride (12)



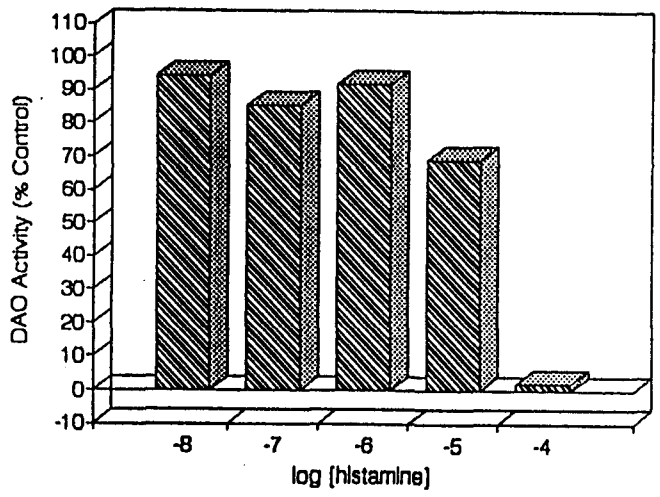
O-(hexyl)hydroxylamine hydrochloride (14)



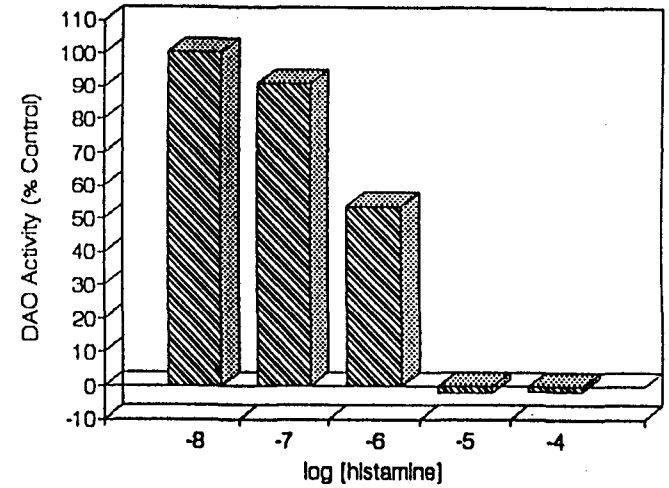
O-(octyl)hydroxylamine hydrochloride (16)



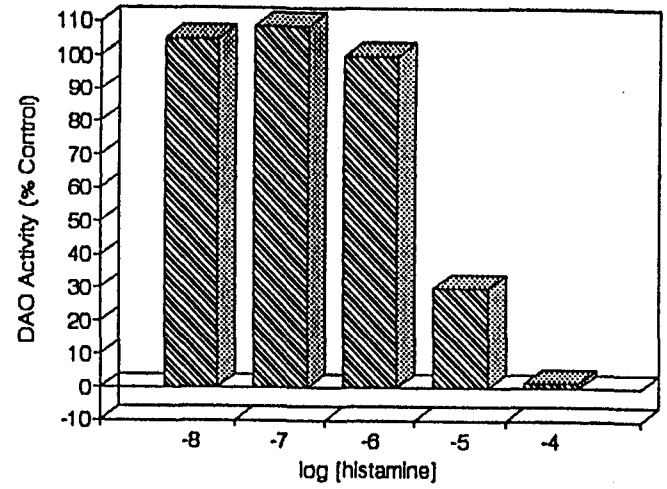
O-(decyl)hydroxylamine hydrochloride (18)



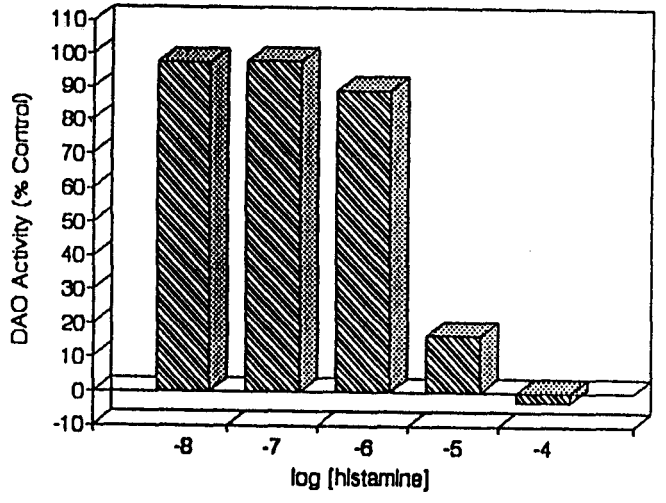
O-(benzyl)hydroxylamine hydrochloride (20)



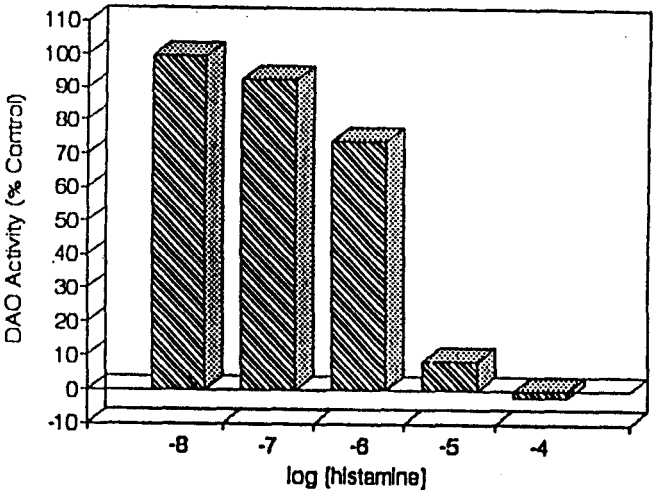
O-(cinnamyl)hydroxylamine hydrochloride (22)



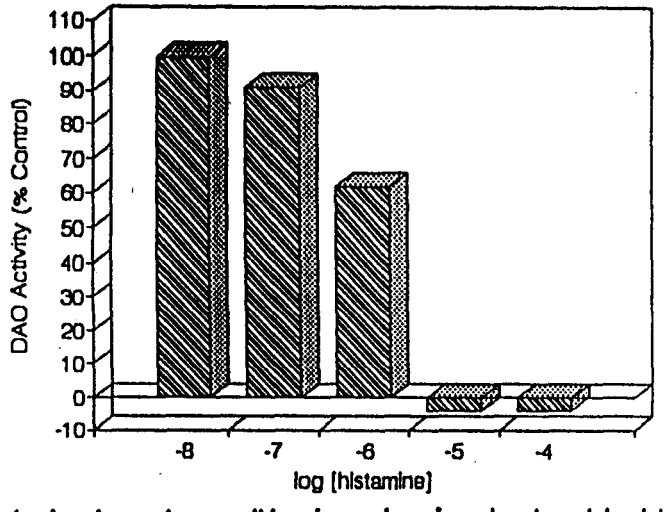
O-(p-bromobenzyl)hydroxylamine hydrochloride (24)



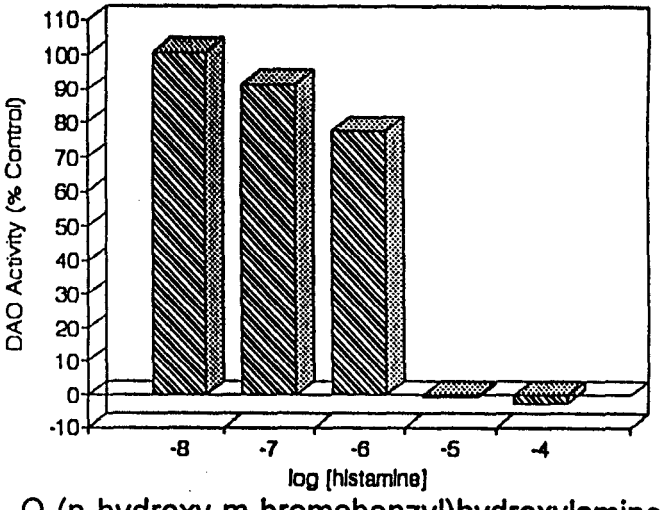
O-(p-methoxybenzyl)hydroxylamine hydrochloride (26)



O-(m-methoxybenzyl)hydroxylamine hydrochloride (28)



O-(p-hydroxybenzyl)hydroxylamine hydrochloride (33)



O-(p-hydroxy-m-bromobenzyl)hydroxylamine hydrochloride (38)

4.5 Comparing the Enzyme Assay Data to the Pharmacological Test Results

To facilitate comparison of all the data measured in the three types of experiments, the pharmacological data will be presented again in addition to the observed activities of the hydroxylamine compounds in the diamine oxidase enzyme assay experiments.

TABLE 4: Results of Pharmacological Tests and Enzyme Assays

Compound	[a]Secondary Response (10^{-4}M)	[a]Dose-Response (10^{-4}M)	[a]Enzyme Assay	
			low conc. 10^{-6}M	high conc. 10^{-5}M
sec-butyl (2)	+	NT	+	+
isobutyl (4)	+	NT	-	+
isoamyl (6)	+/-	NT	-	-
allyl (8)	+	NT	+	+
4-pentynyl (10)	+	NT	-	+
pentyl (12)	+	+	+	+
hexyl (14)	+	NT	+/-	+
octyl (16)	+/-	+	-	+
decyl (18)	-[b]	NT	-	-
benzyl (20)	-[c]	-	-	-
cinnamyl (22)	+/-	NT	+/-	+
p-bromobenzyl (24)	+/-	NT	-	+
p-methoxybenzyl (26)	+	+	-	+
m-methoxybenzyl (28)	+	NT	-	+
p-hydroxybenzyl (33)	+	+	+/-	+
p-hydroxy-m-bromobenzyl (38)	+/-	NT	+/-	+

[a]Activities of the hydroxylamine compounds are "+" active, "-" inactive or "+/-" partially active (see section 4.1)

[b]O-decyl hydroxylamine causes a quick decrease in the measured short-circuit current in 2° response experiments resulting in a dip below the baseline of the curve.

[c]Tissues used to test compounds that appeared to be inactive (-) in the 2° response experiments were tested with O-methylhydroxylamine, a compound that is known to be active in such experiments, to ensure that the measured activity was not due to a problem with the tissue.

NT = not tested due to amount of time and number of tissues required to perform dose-response experiment (see section 4.2).

Table 4 shows that the activities of all sixteen hydroxylamine compounds tested were, in general, consistent in all three experiments. It is apparent that the enzyme assay is a much more sensitive method for testing the diamine oxidase inhibiting ability of a compound. Compounds such as O-(p-bromobenzyl) or O-cinnamylhydroxylamine which are only marginally active in secondary response experiments at 10^{-4}M show significant activity in the enzyme assay at 10^{-5} and even 10^{-6}M. Both O-decyl and O-benzylhydroxylamines are inactive in all three test conditions at the concentrations examined but they do begin to exhibit some inhibitory activity in the diamine oxidase assay at 10^{-4}M. Thus, they may not be truly "inactive", but simply much less active than compounds such as O-pentyl and O-allylhydroxylamines which are quite inhibitory at 10^{-6}M.

Ideally, the secondary response and dose-response experiments should be performed over a range of concentrations. With these data, a correlation between concentration and activity could be constructed in different diamine oxidase environments. Further enzyme assay testing is also desirable to determine the lowest concentration that exhibits activity, a so called "activity threshold". The hydroxylamines should be tested at additional concentrations that cover the transition from active to inactive to pinpoint the lowest concentration of the compound that can inhibit diamine oxidase.

CHAPTER 5

DISCUSSION

5.1 Objectives Reviewed

The goals of this work were to synthesize several oxygen substituted hydroxylamines and to test their abilities to potentiate histamine in canine colonic tissue through diamine oxidase inhibition to further examine the structure-activity relationship. The activities of the hydroxylamines were determined using a rapid secondary response screen, followed by a more involved dose-response regimen on selected compounds and finally, assays on partially purified diamine oxidase enzyme.

5.2 Syntheses

The general method used to prepare the O-alkyl and O-benzyl hydroxylamines was a modification of the Gabriel amine synthesis using N-hydroxyphthalimide instead of phthalimide as the source of the nucleophile. This method is a fairly simple S_n2 displacement reaction and the problems incurred are those typical of the S_n2 reaction type.

Steric hinderance at the electrophilic carbon can slow down the reaction considerably and at higher temperatures, elimination products are possible.

Compounds that contain other groups that are susceptible to S_n2 reactions

can produce side-products that must be removed before testing. The use of sodium hydride also necessitates that there are no base sensitive components in the starting material. This was the problem faced in the synthesis of p-hydroxybenzyl hydroxylamines. The presence of the base caused polymerization, rather than S_n2 displacement, to occur. Therefore, the base sensitive substituent must be protected before the Gabriel-type reaction can be carried out.

Purification of the hydroxylamines by removing hydrazides was also not simple in all cases. Since the procedure depends on the extraction of the free hydroxylamine into an organic solvent, polar substituents (i.e. hydroxyl groups) that will decrease the solubility of the compound in solvents such as methylene chloride will decrease the effectiveness of the purification process. Compounds of this type must therefore be purified using another method such as an ion-exchange resin.

One additional problem was encountered in attempting to synthesize the longer chain alkyl hydroxylamines. Once the number of carbons in the hydrocarbon chain reaches seven or eight, the product hydroxylamine hydrochloride can form micelles which are difficult, if not impossible, to extract into organic solvents. Impurities can be trapped inside the micelle and are therefore not removed under normal conditions. The surfactant properties of these compounds also makes removing the solvent difficult. Using a reduced pressure rotoevaporator results in the solution "foaming up". So, the solvent must be removed from these solutions using a freeze-drying apparatus. For these

reasons, a less pure final product may be isolated, and this was especially so in the case of O-dodecyl hydroxylamine which could not be adequately purified in this work.

5.3 Examining the Structure-Activity Relationship

The observed activities indicate the following:

(1) As shown previously (Rangachari, Bell, Prior & Hunyh, 1992), the active hydroxylamine compounds are oxygen and not nitrogen substituted. N-methyl hydroxylamine is still not active in any of the experiments performed while O-methyl, along with many of the other O-substituted compounds, is active.

(2) Branched compounds are less active than their straight chain analogues. Rangachari, Bell, Prior and Hunyh (1992) have shown that the O-(isobutyl) and O-(*sec*-butyl) derivatives are less active than O-butyl and that O-(*tert*-butyl) is not active at all. In this work, we see that similarly, O-(isoamyl)hydroxylamine is considerably less active than O-pentyl hydroxylamine. Thus, it appears that more steric bulk at the ß-carbon decreases diamine oxidase inhibiting ability.

(3) The presence of a double or triple carbon-carbon bond does not appear to significantly decrease the activity of straight chain hydroxylamines. O-allyl and O-(4-pentynyl) hydroxylamines both inhibit diamine oxidase to a degree similar to their respective saturated analogues, O-propyl and O-pentyl hydroxylamines.

(4) The longer the hydrocarbon chain of a straight chain alkyl substituent,

the less able it is to inhibit diamine oxidase. The shorter chain O-methyl, ethyl, propyl, butyl and pentyl hydroxylamines are quite active. By the time the number of carbons has increased to six, we begin to observe a slight decrease in activity and as the chain length increases to eight, and subsequently ten, the activity becomes insignificant.

In the tissue preparations, longer chain hydrocarbons may be getting trapped in the lipid layer of the cells and are therefore unable to access the diamine oxidase. However, O-decyl hydroxylamine is inactive even on the partially purified enzyme indicating that access is not the major problem. It is possible that the O-decyl hydroxylamine is forming micelles causing steric bulk around the hydroxylamine moiety. Since the hydroxylamine must interact with the active site on the enzyme, this added steric bulk could hinder approach to the active site enough that diamine oxidase inhibition is not observed.

(5) In examining the benzyl compounds, we see that steric bulk at the ß-carbon may again be important. O-benzyl hydroxylamine is not an active compound but O-cinnamyl hydroxylamine is. The cinnamyl compound is larger in overall size than the benzyl and so, if size is the determining factor, O-benzyl hydroxylamine should be the more active species. If, however, the steric bulk at the ß-carbon is what affects the ability of the hydroxylamine to inhibit diamine oxidase, it is understandable that the cinnamyl compound is more active. O-benzyl hydroxylamine has a benzene ring bonded to the ß-carbon while the benzene ring in O-cinnamyl hydroxylamine is two carbons (approximately 5Å)

further away. The decrease in steric bulk at the ß-position might allow the hydroxylamine group to get closer to the active site of diamine oxidase enabling it to "deactivate" the enzyme more effectively.

(6) O-benzyl hydroxylamines that have an oxygen substituent (hydroxy, methoxy) on the benzene ring are more active than O-benzyl and O-(p-bromobenzyl) hydroxylamines. This may be due to the ability of the oxygen substituents to hydrogen bond to atoms at or near the active site of diamine oxidase. The hydrogen bonding increases the interaction between the hydroxylamine and the active site to a large enough extent that the effect of the steric bulk at the ß-carbon is diminished making these compounds able to inhibit diamine oxidase.

EXPERIMENTAL METHODS

Melting points were measured on a Gallenkamp capillary tube melting point apparatus and are uncorrected.

Proton and carbon-13 nuclear magnetic resonance spectra were recorded on a Bruker AC-200 (200 MHz) or Bruker AC-300 (300 MHz) spectrometer. Solvents used were chloroform-d ($CDCl_3$), dimethylsulfoxide-d_6 (DMSO-d_6), or deuterium oxide (D_2O) with tetramethylsilane (TMS) as an internal standard. The abbreviations (s)=singlet, (d)=doublet, (t)=triplet, (qr)=quartet, (pn)=pentet, (sx)=sextet, (sp)=septet and (m)=multiplet are used to describe spin-spin coupling patterns. Proton spectra were collected in sixteen scans in 16K data points. The free-induction decay (FID) patterns were processed using exponential multiplication (line broadening = 0.3) and were zero-filled to 32K before Fourier transformation. The carbon-13 spectra were collected in adequate scans to determine all carbon signals in 16K data points. The FID patterns were processed as with the proton spectra (line broadening = 3.0) and zero-filled to 32K before Fourier transformation.

N,N-dimethylformamide (DMF) was dried by distilling a known volume of benzene to remove the water as an azeotrope. The DMF was then distilled under reduced pressure and stored over molecular sieves under a nitrogen atmosphere. Acetonitrile (ACN) was distilled from CaH_2 under dry nitrogen immediately prior

to use. All other solvents used were of reagent grade. NMR solvents ($CDCl_3$, DMSO-d_6) were stored over molecular seives (4Å) prior to use.

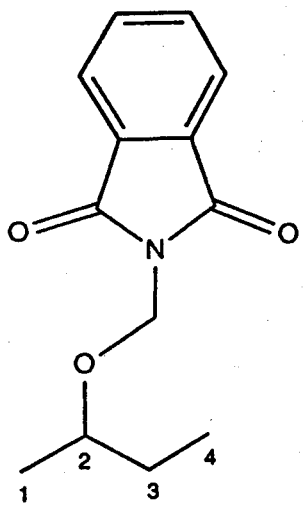
PREPARATION OF O-ALKYL AND SIMPLE O-BENZYL HYDROXYLAMINES BY A MODIFIED GABRIEL SYNTHESIS

Most of the O-substituted hydroxylamines were prepared in a similar manner following a Gabriel type of synthesis (Drain et al., 1965). O-(*sec*-butyl)hydroxylamine is described in detail as a typical example.

Synthesis of O-(*sec*-butyl)-N-hydroxyphthalimide (1)

A sample of N-hydroxyphthalimide (3.0094 g, 18.4 mmol) was dissolved in dry dimethylformamide (DMF) under a nitrogen atmosphere. Sodium hydride in a 60% mineral oil suspension (0.778 g suspension, 19.5 mmol of NaH) was washed with approximately 20 mL of pentane that had been dried over molecular sieves and 10 mL of dry dimethylformamide. The N-hydroxyphthalimide in DMF was then added to the NaH suspended in DMF under an N_2 atmosphere, yielding a red opaque solution. Two equivalents (5.0690 g, 36.8 mmol) of *sec*-butyl bromide and 1-2% NaI in DMF were added dropwise via a dry syringe over approximately fifteen minutes. The total volume of DMF used was 50 mL. The reaction mixture was heated to approximately 70°C and stirred magnetically for 22 hours. During this time, the red colour diminished. The DMF solvent was removed at reduced pressure by use of a Büchi rotoevaporator, the residue dissolved in 50 mL $CHCl_3$ and the whole added to 50 mL H_2O in a separatory funnel. The $CHCl_3$ layer was

then washed with two 50 mL portions of H_2O while the aqueous layer was washed with two 50 mL portions of $CHCl_3$. The combined $CHCl_3$ layers were evaporated down to 50 mL and washed with 50 mL aliquots of H_2O, $NaHSO_3/Na_2CO_3$(aq) (approximately 5% each) and H_2O. The $CHCl_3$ layer was dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure yielding the phthalimide derivative.



Physical Data: yellow brown liquid
yield = 2.075 g (51.4%)

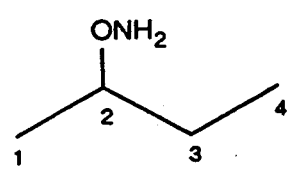
Spectral Data: ^1H NMR (200 MHz); ($CDCl_3$) δ 0.98 (t, 3H, $J_{3,4}$ 7.2 Hz, H_4), 1.28 (d, 3H, $J_{1,2}$ 6.2 Hz, H_1), 1.55 (pn, 1H, $J_{2,3}$ 6.6 Hz, $J_{3,4}$ 7.2 Hz, H_3), 1.78 (pn, 1H, $J_{2,3}$ 6.6 Hz, $J_{3,4}$ 7.2 Hz, $H_{3'}$), 4.26 (sx, 1H, $J_{1,2}$ 6.2 Hz, $J_{2,3}$ 6.6 Hz, H_2), 7.73 (m, 4H, phthalimide).

Synthesis of O-(*sec*-butyl)hydroxylamine hydrochloride (2)

The phthalimide group was removed by reaction with hydrazine (Ing & Manske, 1926). The phthalimide derivative (2.075 g, 9.464 mmol) was dissolved in 29 mL absolute ethanol (3 mL/mmol). Hydrazine solution (0.3987 g of a 95% solution in H_2O, 1.25 equivalents of N_2H_4) was added dropwise over approximately

one minute. The reaction mixture was heated under reflux with magnetic stirring for approximately 2 hours at which point a voluminous white precipitate had appeared. The mixture was cooled and concentrated HCl was added to bring the pH to approximately 1. The solid precipitate (phthalhydrazide) was removed by vacuum filtration and the resulting mother liquor concentrated to about one-half of its volume by evaporation at reduced pressure (Büchi rotoevaporator) to precipitate more solid. The solution was filtered again and then the remaining solvents removed at reduced pressure. The semi-solid residue was dissolved in 10 mL H_2O and filtered through Celite. The mother liquor was evaporated to dryness yielding the hydrochloride salt of the hydroxylamine.

The hydroxylamine hydrochloride was treated to remove residual hydrazine by dissolving it in 10 mL H_2O. To this solution, 10 mL of CH_2Cl_2 were added in a separatory funnel and the pH brought to around 10 with NaOH and the aqueous solution extracted. The organic layer was separated and then washed with two 10 mL portions of a KH_2PO_4/NaOH buffer (pH = 7.4), 3-4 mL H_2O, and 10 mL 10% HCl. The acidic aqueous layer was evaporated to dryness at reduced pressure (Büchi rotoevaporator) yielding the purified hydroxylamine hydrochloride.



Physical Data: before purification - transparent yellow liquid
yield = 0.653 g (54.9%)
after purification - yellow-white crystalline solid
yield = 0.249 g (20.9%)
melting point = 60 - 70°C

Spectral Data: ^{1}H NMR (200 MHz); (DMSO-d_6) δ 0.85 (t, 3H, $J_{3,4}$ 7.3 Hz, H_4), 1.20 (d, 3H, $J_{1,2}$ 6.1 Hz, H_1), 1.45 (pn, 1H, $J_{2,3}$ 6.6 Hz, $J_{3,4}$ 7.3 Hz, H_3), 1.64 (pn, 1H, $J_{2,3}$ 6.6 Hz, $J_{3,4}$ 7.3 Hz, H_3), 4.15 (sx, 1H, $J_{1,2}$ 6.1 Hz, $J_{2,3}$ 6.6 Hz, H_2).

^{13}C NMR (50 MHz); (DMSO-d_6) δ 8.91 (C_4), 17.70 (C_1), 26.72 (C_3), 81.30 (C_2).

Other O-Alkyl Hydroxylamines

Several other O-substituted hydroxylamines were prepared using the above general method. The amounts used in each preparation are summarized in the following tables where:

A = the amount of N-hydroxyphthalimide used in grams and in millimoles

B = the mass of 60% NaH suspension used and the molar equivalents of NaH added relative to the amount of N-hydroxyphthalimide

C = the amount of the alkyl halide added in grams or millilitres and the equivalents of the alkyl halide relative to the N-hydroxyphthalimide

D = the length of time the reaction mixture was left to heat at approximately 70°C

E = the amount of the phthalimide derivative used in the hydrazinolysis reaction

F = the mass of 95% N_2H_4 solution used and the molar equivalents of N_2H_4 added.

Table 5 gives the data for N-hydroxyphthalimide formation and Table 6 data for the hydrazinolysis reaction.

Physical and spectral data for each compound is given on pages 65 - 77.

TABLE 5: Data for N-hydroxyphthalimide Formation

Compound	A(g) mass N-hydroxy phthalimide	A(mmol)	B(g) mass NaH suspension	B equiv. of NaH	C(g or mL) amount of RX used	C equiv. of RX	D(hrs) reflux time
sec-butyl (1)	3.0094	18.4	0.778	1.06	5.0690 g	2.01	22
isobutyl (3)	1.5030	9.21	0.645	1.75	2.7803 g	2.20	42[a]
isoamyl (5)	1.8578	11.4	0.647	1.53	1.65 mL	1.21	18
allyl (7)	1.5002	9.196	0.757	2.06	1.0 mL	1.26	4.5[b]
4-pentynyl (9)	1.5540	9.526	0.419	1.10	1.2 mL	1.19	23.5
pentyl (11)	1.5022	9.209	0.798	2.20	3.0742 g	2.21	12.5[a]
hexyl (13)	1.5030	9.214	0.769	2.10	2.6 mL	2.01	23[a]
octyl (15)	1.5098	9.255	0.518	1.40	2.0 mL	1.25	23
decyl (17)	1.7102	10.50	0.780	1.90	2.4 mL	1.10	48
benzyl (19)	1.5002	9.196	0.605	1.64	1.3 mL	1.23	2.5[b]
cinnamyl (21)	1.5570	9.545	0.598	1.57	2.2573 g	1.20	15
p-Br-benzyl (23)	1.7484	10.70	0.649	1.52	2.9567 g	1.11	1
p-CH_3O-benzyl (25)	1.5081	9.245	0.867	2.34	1.2 mL	0.96	2.5[b]
m-CH_3O-benzyl (27)	1.5010	9.202	0.524	1.43	1.61 mL	1.20	3.5

[a]In these cases, the alkyl halide was added in two portions; isobutyl: 1.2 equivalents refluxed for 22 hours then 1 additional equivalent for 20 hours; n-pentyl: 1.21 equivalents for 10.5 hours then 1 equivalent for 2 hours; n-hexyl: 1.24 equivalents for 21.5 hours then 0.77 equivalents for 1.5 hours.

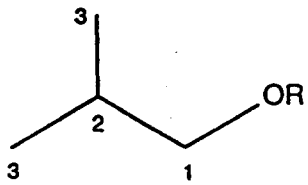
[b]The phthalimide preparation was followed by thin-layer chromatography. (TLC) for these compounds using 1% methanol, 25% ethyl acetate, 74% hexanes as the eluent (see physical data for R_f values).

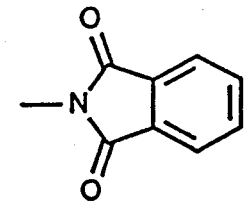
TABLE 6: Data for Hydrazinolysis Reaction

Compound	E(g) mass phthalimide derivative used	F(g) mass N_2H_4 solution used	F equivalents of N_2H_4
sec-butyl (2)	2.075	0.3987	1.25
isobutyl (4)	1.110	0.2259	1.32
isoamyl (6)	1.116	0.1953	1.21
allyl (8)	1.005	0.2251	1.35
4-pentynyl (10)	1.050	0.1853	1.20
pentyl (12)	1.205	0.2352	1.30
hexyl (14)	1.322	0.2264	1.30
octyl (16)	1.159	0.1869	1.30[c]
decyl (18)	3.200	0.3948	1.10[c]
benzyl (20)	0.999	0.1591	1.20
cinnamyl (22)	1.492	0.2262	1.26
p-Br-benzyl (24)	3.037	0.3614	1.17
p-CH_3O-benzyl (26)	1.100	0.1765	1.34
m-CH_3O-benzyl (28)	1.557	0.2492	1.34

[c]The hydroxylamine hydrochloride is a surfactant and evaporation on a normal Büchi rotoevaporator was not possible. In the case of the octyl compound, both the impure and pure hydroxylamines were freeze-dried on a Labconco Cascade Freeze Dryer 8 (model #4451F). The impure decyl hydroxylamine was not isolated. Instead, the aqueous layer was carried right into the procedure for removing residual hydrazine. The final product was isolated by freeze-drying as noted above.

Isobutyl



where R = 

or R = $-NH_2 \cdot HCl$

O-(isobutyl)-N-hydroxyphthalimide (3):

Physical Properties: - transparent yellow liquid
yield = 2.946 g

Spectral Data: ^1H NMR (200 MHz); (DMSO-d_6) δ 0.96 (m, 6H, $J_{2,3}$ 6.7 Hz, H_3), 1.97 (sp, 1H, $J_{1,2}$ 6.7 Hz, $J_{2,3}$ 6.7 Hz, H_2), 3.90 (d, 2H, $J_{1,2}$ 6.7 Hz, H_1), 7.89 (m, 4H, phthalimide).

O-(isobutyl)hydroxylamine hydrochloride (4):

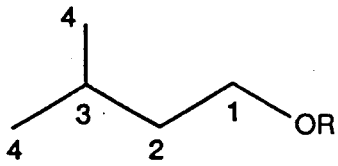
Physical Properties: before purification - yellow crystalline solid
yield = 0.390 g (61.3 %)
after purification - yellow-white solid
yield = 0.203 g (31.9 %)
melting point = 113-125°C

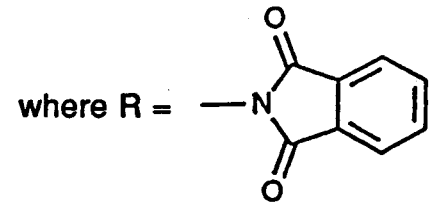
Spectral Data:

^1H NMR (200 MHz); (DMSO-d_6) δ 0.87 (d, 6H, $J_{2,3}$ 6.7 Hz, H_3), 1.90 (sp, 1H, $J_{1,2}$ 6.6 Hz, $J_{2,3}$ 6.7 Hz, H_2), 3.78 (d, 2H, $J_{1,2}$ 6.6 Hz, H_1).

^{13}C NMR (50 MHz); (DMSO-d_6) δ 18.73 (C_3), 26.59 (C_2), 79.97 (C_1).

Isoamyl





O-(isoamyl)-N-hydroxyphthalimide (5):

Physical Properties: - yellow crystalline solid
yield = 2.462 g (92.7 %)
melting point = 41-44°C

Spectral Data: ^{1}H NMR (300 MHz); ($CDCl_3$) δ 0.88 (d, 6H, $J_{3,4}$ 6.7 Hz, H_4), 1.60 (qr, 2H, $J_{1,2}$ 6.8 Hz, $J_{2,3}$ 6.8 Hz, H_2), 1.79 (sp, 1H, $J_{2,3}$ 6.8 Hz, $J_{3,4}$ 6.7 Hz, H_3), 4.15 (t, 2H, $J_{1,2}$ 6.8 Hz, H_1), 7.70 (m, 4H, phthalimide).

O-(isoamyl)hydroxylamine hydrochloride (6):

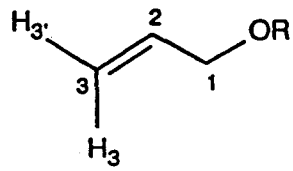
Physical Properties: before purification - yellow-white solid
yield = 0.764 g
after purification - white solid
yield = 0.577 g (86.4 %)
melting point = 112-127°C

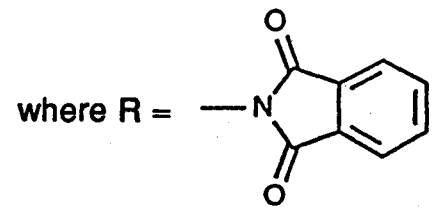
Spectral Data:

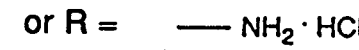
^{1}H NMR (300 MHz); (DMSO-d_6) δ 0.87 (d, 6H, $J_{3,4}$ 6.4 Hz, H_4), 1.45 (qr, 2H, $J_{1,2}$ 6.6 Hz, $J_{2,3}$ 6.7 Hz, H_2), 1.61 (sp, 1H, $J_{2,3}$ 6.7 Hz, $J_{3,4}$ 6.4 Hz, H_3), 4.01 (t, 2H, $J_{1,2}$ 6.6 Hz, H_1).

^{13}C NMR (75 MHz); (DMSO-d_6) δ 22.21 (C_4), 24.33 (C_3), 35.74 (C_2), 72.45 (C_1).

Allyl







O-(allyl)-N-hydroxyphthalimide (7):

Physical Properties: - brown crystalline solid
yield = 1.660 g (88.8 %)
melting point = 45-50°C
R_f = 0.94 (R_f of N-hydroxyphthalimide = 0.13)

Spectral Data: ^{1}H NMR (200 MHz); (DMSO-d_6) δ 4.55 (d, 2H, $J_{1,2}$ 6.6 Hz, H_1), 5.35 (d/d/t, 1H, $J_{2,3'}$ 10.3 Hz, $J_{3,3'}$ 1.0 Hz, $H_{3'}$), 5.38 (d/d, 1H, $J_{2,3}$ 17.1 Hz, $J_{3,3'}$ 1.0 Hz, H_3), 6.03 (d/d/t, 1H, $J_{1,2}$ 6.6 Hz, $J_{2,3}$ 17.1 Hz, $J_{2,3'}$ 10.3 Hz, H_2), 7.84 (s, 4H, phthalimide).

O-(allyl)hydroxylamine hydrochloride (8):

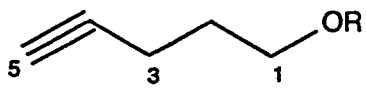
Physical Properties: before purification - yellow solid
yield = 0.304 g (56.1 %)
after purification - transparent yellow film
yield = 0.046 g (8.49 %)

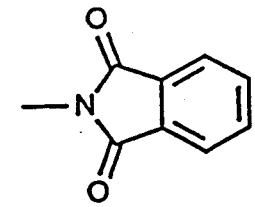
Spectral Data:

^{1}H NMR (200 MHz); (DMSO-d_6) δ 4.55 (d, 2H, $J_{1,2}$ 6.1 Hz, H_1), 5.35 (d/d/t, 1H, $J_{2,3'}$ 10.4 Hz, $J_{3,3'}$ 1.4 Hz, $H_{3'}$), 5.45 (d/d, 1H, $J_{2,3}$ 17.2 Hz, $J_{3,3'}$ 1.4 Hz, H_3), 5.93 (d/d/t, 1H, $J_{1,2}$ 6.1 Hz, $J_{2,3}$ 17.2 Hz, $J_{2,3'}$ 10.4 Hz, H_2).

^{13}C NMR (50 MHz); (DMSO-d_6) δ 74.58 (C_1), 121.26 (C_3), 130.76 (C_2).

4-pentynyl



where R = 

or R = —— $NH_2 \cdot HCl$

O-(4-pentynyl)-N-hydroxyphthalimide (9):

Physical Properties: - yellow solid
yield = 1.368 g (62.6 %)
melting point = 77-85°C

Spectral Data: ^1H NMR (200 MHz); ($CDCl_3$) δ 1.82 (m, 2H, $J_{2,3}$ 6.8 Hz, $J_{1,2}$ 6.4 Hz, H_2), 1.89 (t, 1H, $J_{3,5}$ 2.6 Hz, H_5), 2.33 (t/d, 2H, $J_{2,3}$ 6.8 Hz, $J_{3,5}$ 2.6 Hz, H_3), 4.15 (t, 2H, $J_{1,2}$ 6.4 Hz, H_1), 7.65 (m, 4H, phthalimide).

O-(4-pentynyl)hydroxylamine hydrochloride (10):

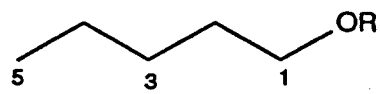
Physical Properties: before purification - yellow solid
yield = 1.222 g
after purification - yellow-white solid
yield = 0.514 g (82.8 %)
melting point = 126-131°C

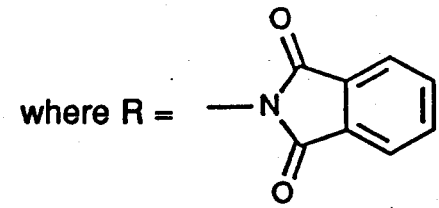
Spectral Data:

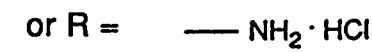
^1H NMR (200 MHz); ($DMSO-d_6$) δ 1.76 (pn, 2H, $J_{2,3}$ 6.9 Hz, $J_{1,2}$ 6.9 Hz, H_2), 2.22 (t/d, 2H, $J_{2,3}$ 6.9 Hz, $J_{3,5}$ 2.6 Hz, H_3), 2.82 (t, 1H, $J_{3,5}$ 2.6 Hz, H_5), 4.06 (t, 2H, $J_{1,2}$ 6.9 Hz, H_1).

^{13}C NMR (50 MHz); ($DMSO-d_6$) 14.24 (C_3), 26.28 (C_2), 71.84 (C_1), 72.78 (C_5), 83.29 (C_4).

Pentyl







O-(pentyl)-N-hydroxyphthalimide (11):

Physical Properties: - orange-yellow liquid
yield = 2.608 g

Spectral Data: ^{1}H NMR (200 MHz); ($CDCl_3$) δ 0.89 (t, 3H, $J_{4,5}$ 6.9 Hz, H_5), 1.37 (m, 4H, $J_{4,5}$ 6.9 Hz, $H_{3,4}$), 1.72 (pn, 2H, $J_{1,2}$ 6.9 Hz, H_2), 4.16 (t, 2H, $J_{1,2}$ 6.9 Hz, H_1), 7.75 (m, 4H, phthalimide).

O-(pentyl)hydroxylamine hydrochloride (12):

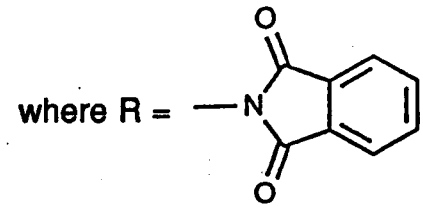
Physical Properties: before purification - yellow solid
yield = 0.403 g (55.9 %)
after purification - white solid
yield = 0.205 g (28.4 %)
melting point = 138-143°C

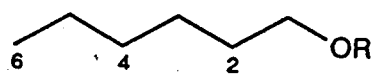
Spectral Data:

^{1}H NMR (200 MHz); (DMSO-d_6) δ 0.86 (t, 3H, $J_{4,5}$ 6.6 Hz, H_5), 1.27 (m, 4H, $J_{4,5}$ 6.6 Hz, $H_{3,4}$), 1.56 (pn, 2H, $J_{1,2}$ 6.5 Hz, H_2), 3.97 (t, 2H, $J_{1,2}$ 6.5 Hz, H_1).

^{13}C NMR (50 MHz); (DMSO-d_6) δ 13.69 (C_5), 21.61 (C_4), 26.70 (C_3), 27.18 (C_2), 73.95 (C_1).

Hexyl





or R = —— $NH_2 \cdot HCl$

O-(hexyl)-N-hydroxyphthalimide (13):

Physical Properties: - brown-yellow liquid
yield = 2.141 g (94.0 %)

Spectral Data: ^1H NMR (200 MHz); ($CDCl_3$) δ 0.85 (t, 3H, $J_{5,6}$ 6.7 Hz, H_6), 1.27 (m, 6H, $J_{2,3}$ 7.1 Hz, $J_{5,6}$ 6.7 Hz, $H_{3\text{-}5}$), 1.73 (pn, 2H, $J_{1,2}$ 6.9 Hz, $J_{2,3}$ 7.1 Hz, H_2), 4.14 (t, 2H, $J_{1,2}$ 6.9 Hz, H_1), 7.75 (m, 4H, phthalimide).

O-(hexyl)hydroxylamine hydrochloride (14):

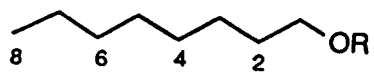
Physical Properties: before purification - yellow crystalline solid
yield = 1.776 g
after purification - white crystalline solid
yield = 1.404 g
melting point = 141-147°C

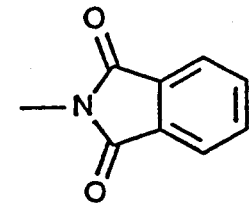
Spectral Data:

^1H NMR (200 MHz); (DMSO-d_6) δ 0.83 (t, 3H, $J_{5,6}$ 6.5 Hz, H_6), 1.23 (m, 6H, $J_{2,3}$ 6.3 Hz, $J_{5,6}$ 6.5 Hz, $H_{3\text{-}5}$), 1.53 (pn, 2H, $J_{1,2}$ 6.4 Hz, $J_{2,3}$ 6.3 Hz, H_2), 3.98 (t, 2H, $J_{1,2}$ 6.4 Hz, H_1).

^{13}C NMR (50 MHz); (DMSO-d_6) δ 14.03 (C_6), 22.09 (C_5), 24.91 (C_4), 27.20 (C_3), 30.92 (C_2), 74.16 (C_1).

Octyl



where R = 

or R = —— $NH_2 \cdot HCl$

O-(octyl)-N-hydroxyphthalimide (15):

Physical Properties: - brown-yellow crystalline solid
yield = 2.431 g (95.4 %)

Spectral Data: ^1H NMR (300 MHz); ($CDCl_3$) δ 0.86 (t, 3H, $J_{7,8}$ 6.7 Hz, H_8), 1.42 (m, 10H, $J_{2,3}$ 7.2 Hz, $J_{7,8}$ 6.7 Hz, $H_{3\text{-}7}$), 1.77 (pn, 2H, $J_{1,2}$ 7.0 Hz, $J_{2,3}$ 7.2 Hz, H_2), 4.18 (t, 2H, $J_{1,2}$ 7.0 Hz, H_1), 7.77 (m, 4H, phthalimide).

O-(octyl)hydroxylamine hydrochloride (16):

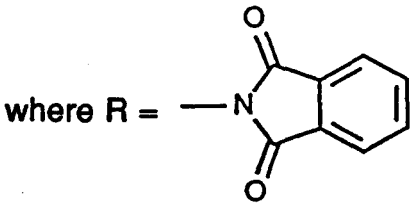
Physical Properties: before purification - yellow-white solid
yield = 0.643 g (84.1 %)
after purification - yellow crystalline solid
yield = 0.323 g (42.2 %)
melting point = 139-143°C

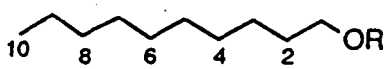
Spectral Data:

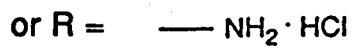
^1H NMR (200 MHz); ($DMSO\text{-}d_6$) δ 0.84 (t, 3H, $J_{7,8}$ 5.9 Hz, H_8), 1.24 (m, 10H, $J_{2,3}$ 5.9 Hz, $J_{7,8}$ 5.9 Hz, $H_{3\text{-}7}$), 1.55 (pn, 2H, $J_{1,2}$ 6.2 Hz, $J_{2,3}$ 5.9 Hz, H_2), 3.95 (t, 2H, $J_{1,2}$ 6.2 Hz, H_1).

^{13}C NMR (50 MHz); ($DMSO\text{-}d_6$) δ 13.95 (C_8), 22.06 (C_7), 25.12 (C_6), 27.10 (C_5), 28.54 ($C_{3,4}$), 31.19 (C_2), 74.03 (C_1).

Decyl







O-(decyl)-N-hydroxyphthalimide (17):

Physical Properties: - brown-yellow solid
yield = 3.200 g
melting point = 42-47°C

Spectral Data: ^{1}H NMR (200 MHz); ($CDCl_3$) δ 0.84 (t, 3H, $J_{9,10}$ 6.3 Hz, H_{10}), 1.43 (m, 14H, $J_{2,3}$ 7.5 Hz, $J_{9,10}$ 6.3 Hz, $H_{3\text{-}9}$), 1.72 (pn, 2H, $J_{1,2}$ 7.1 Hz, $J_{2,3}$ 7.5 Hz, H_2), 4.16 (t, 2H, $J_{1,2}$ 7.1 Hz, H_1), 7.76 (m, 4H, phthalimide).

O-(decyl)hydroxylamine hydrochloride (18):

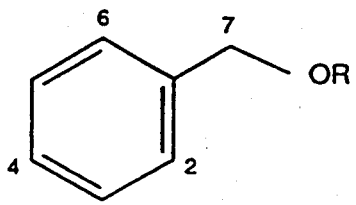
Physical Properties: after purification - yellow-white film
yield = 0.040 g (1.82 %)

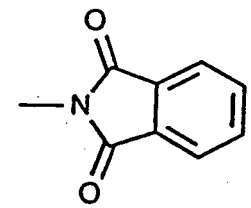
Spectral Data:

^{1}H NMR (200 MHz); (DMSO-d_6) δ 0.84 (t, 3H, $J_{9,10}$ 6.3 Hz, H_{10}), 1.24 (m, 14H, $J_{2,3}$ 6.6 Hz, $J_{9,10}$ 6.3 Hz, $H_{3\text{-}9}$), 1.55 (pn, 2H, $J_{1,2}$ 6.5 Hz, $J_{2,3}$ 6.6 Hz, H_2), 3.96 (t, 2H, $J_{1,2}$ 6.5 Hz, H_1).

^{13}C NMR (50 MHz); (DMSO-d_6) δ 22.10 (C_{10}), 25.09 (C_9), 27.07 (C_8), 28.90/28.67 ($C_{3\text{-}7}$), 31.28 (C_2), 73.99 (C_1).

Benzyl



where R = 

or R = $NH_2 \cdot HCl$

O-(benzyl)-N-hydroxyphthalimide (19):

Physical Properties: - yellow crystalline solid
yield = 2.467 g
melting point = 92-110°C
R_f = 0.67 (R_f of N-hydroxyphthalimide = 0.10)

Spectral Data: ^{1}H NMR (200 MHz); (DMSO-d_6) δ 5.16 (s, 2H, H_7), 7.39 (m, 5H, $H_{2\text{-}6}$), 7.85 (s, 4H, phthalimide).

O-(benzyl)hydroxylamine hydrochloride (20):

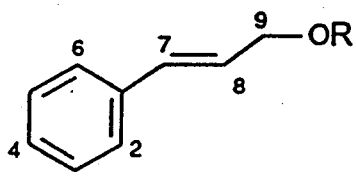
Physical Properties: before purification - white crystalline solid (recrystallized: isopropanol)
yield = 0.315 g (50.1 %)
after purification - white solid
yield = 0.046 g (7.31 %)
melting point = 200°C (decomposes)

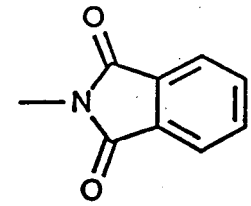
Spectral Data:

^{1}H NMR (200 MHz); (DMSO-d_6) δ 5.03 (s, 2H, H_7), 7.41 (s, 5H, $H_{2\text{-}6}$).

^{13}C NMR (50 MHz); (DMSO-d_6) δ 75.71 (C_7), 128.58 ($C_{2,6}$), 129.07 (C_4), 129.18 ($C_{3,5}$).

Cinnamyl



where R = 

or R = —$NH_2 \cdot HCl$

O-(cinnamyl)-N-hydroxyphthalimide (21):

Physical Properties: - yellow solid
yield = 2.490 g (93.4 %)
melting point = 132-138°C

Spectral Data: ^{1}H NMR (300 MHz); ($CDCl_3$) δ 4.85 (d, 2H, $J_{8,9}$ 7.0 Hz, H_9), 6.44 (d/t, 1H, $J_{7,8}$ 15.9 Hz, $J_{8,9}$ 7.0 Hz, H_8), 6.65 (d, 1H, $J_{7,8}$ 15.9 Hz, H_7), 7.29 (m, 5H, $H_{2\text{-}6}$), 7.75 (m, 4H, phthalimide).

O-(cinnamyl)hydroxylamine hydrochloride (22):

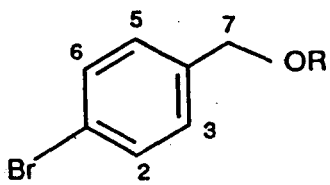
Physical Properties: before purification - yellow solid
yield = 3.501 g
after purification - white solid
yield = 3.012 g
melting point = 164-166°C

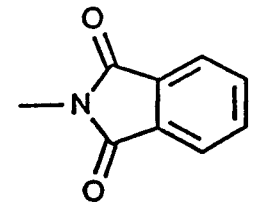
Spectral Data:

^{1}H NMR (300 MHz); (DMSO-d_6) δ 4.68 (d/d, 2H, $J_{7,9}$ 0.6 Hz, $J_{8,9}$ 6.7 Hz, H_9), 6.35 (d/t, 1H, $J_{7,8}$ 15.9 Hz, $J_{8,9}$ 6.7 Hz, H_8), 6.75 (d, 1H, $J_{7,8}$ 15.9 Hz, $J_{7,9}$ 0.6 Hz, H_7), 7.37 (m, 5H, $H_{2\text{-}6}$).

^{13}C NMR (75 MHz); (DMSO-d_6) δ 74.69 (C_9), 121.73 (C_8), 126.98 ($C_{3,5}$), 128.64 (C_4), 128.88 ($C_{2,6}$), 135.79 (C_1), 136.49 (C_7).

p-bromobenzyl



where R = 

or R = —— $NH_2 \cdot HCl$

O-(p-bromobenzyl)-N-hydroxyphthalimide (23):

Physical Properties: - yellow-white solid
yield = 5.055 g
melting point = 122-130°C

Spectral Data: ^1H NMR (200 MHz); ($CDCl_3$) δ 5.13 (s, 2H, H_7), 7.38 (s, 2H, $J_{2,3}/J_{5,6}$ 7.6 Hz, $H_{3,5}$), 7.48 (d, 2H, $J_{2,3}/J_{5,6}$ 7.6 Hz, $H_{2,6}$), 7.75 (m, 4H, phthalimide).

O-(p-bromobenzyl)hydroxylamine hydrochloride (24):

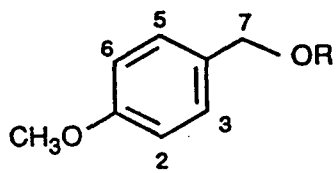
Physical Properties: before purification - yellow-white solid
yield = 0.287 g (13.2 %)
after purification - white crystalline solid
yield = 0.082 g (3.76 %)
melting point = 183-205°C (decomposes)

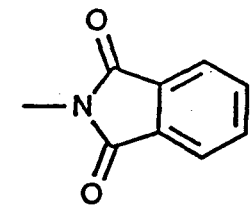
Spectral Data:

^1H NMR (200 MHz); (DMSO-d_6) δ 5.00 (s, 2H, H_7), 7.37 (d, 2H, $J_{2,3}/J_{5,6}$ 8.3 Hz, $H_{3,5}$), 7.62 (d, 2H, $J_{2,3}/J_{5,6}$ 8.3 Hz, $H_{2,6}$).

^{13}C NMR (50 MHz); (DMSO-d_6) δ 74.82 (C_7), 122.35 (C_4), 131.36 ($C_{3,5}$), 131.59 ($C_{2,6}$), 133.44 (C_1).

p-methoxybenzyl



where R = 

or R = —— $NH_2 \cdot HCl$

O-(p-methoxybenzyl)-N-hydroxyphthalimide (25):

Physical Properties: - yellow solid
yield = 3.518 g
melting point = 109-122°C
R_f = 0.68 (R_f of N-hydroxyphthalimide = 0.11)

Spectral Data: ^{1}H NMR (200 MHz); ($CDCl_3$) δ 3.78 (s, 3H, -OC$\underline{H}_3$), 5.13 (s, 2H, H_7), 6.87 (d, 2H, $J_{2,3}/J_{5,6}$ 8.7 Hz, $H_{3,5}$), 7.43 (d, 2H, $J_{2,3}/J_{5,6}$ 8.7 Hz, $H_{2,6}$), 7.74 (m, 4H, phthalimide).

O-(p-methoxybenzyl)hydroxylamine hydrochloride (26):

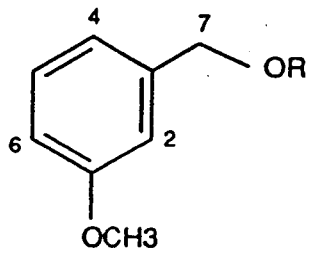
Physical Properties: before purification - yellow-grey solid
yield = 0.186 g (25.3 %)
after purification - yellow-white solid
yield = 0.040 g (5.43 %)
melting point = 162-165°C

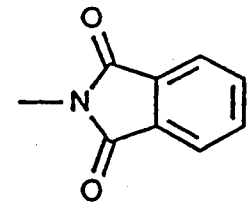
Spectral Data:

^{1}H NMR (200 MHz); (DMSO-d_6) δ 3.76 (s, 3H, -OC$\underline{H}_3$), 4.94 (s, 2H, H_7), 6.96 (d, 2H, $J_{2,3}/J_{5,6}$ 8.6 Hz, $H_{3,5}$), 7.34 (d, 2H, $J_{2,3}/J_{5,6}$ 8.6 Hz, $H_{2,6}$).

^{13}C NMR (50 MHz); (DMSO-d_6) δ 55.14 (-O$\underline{C}H_3$), 75.39 (C_7), 113.95 ($C_{2,6}$), 125.56 (C_4), 131.08 ($C_{3,5}$).

m-methoxybenzyl



where R = 

or R = —— $NH_2 \cdot HCl$

O-(m-methoxybenzyl)-N-hydroxyphthalimide (27):

Physical Properties: - yellow crystalline solid
yield = 3.167 g
melting point = 94-102°C

Spectral Data: ^{1}H NMR (300 MHz); ($CDCl_3$) δ 3.81 (s, 3H, -OC$\underline{H}_3$), 5.18 (s, 2H, H_7), 6.89 (m, 1H, $J_{4,5}/J_{5,6}$ 8.1 Hz, H_6), 7.08 (m, 2H, $J_{2,5}$ 2.8 Hz, $J_{4,5}/J_{5,6}$ 8.1 Hz, $H_{2,4}$), 7.26 (d/t, 1H, $J_{2,5}$ 2.8 Hz, $J_{4,5}/J_{5,6}$ 8.1 Hz, H_5), 7.75 (m, 4H, phthalimide).

O-(m-methoxybenzyl)hydroxylamine hydrochloride (28):

Physical Properties: before purification - yellow solid
yield = 1.065 g
after purification - white solid
yield = 0.984 g (94.4 %)
melting point = 113-119°C

Spectral Data:

^{1}H NMR (300 MHz); (DMSO-d_6) δ 3.74 (s, 3H, -OC$\underline{H}_3$), 5.03 (s, 2H, H_7), 6.95 (m, 3H, $J_{4,5}/J_{5,6}$ 8.0 Hz, $H_{2,4,6}$), 7.31 (t, 1H, $J_{4,5}/J_{5,6}$ 8.0 Hz, H_5).

^{13}C NMR (75 MHz); (DMSO-d_6) δ 55.24 (-O$\underline{C}H_3$), 75.64 (C_7), 114.57 (C_6), 114.79 (C_4), 121.35 (C_2), 129.86 (C_5), 135.21 (C_3), 159.39 (C_1).

ATTEMPTED SYNTHESIS OF P-HYDROXYBENZYL HYDROXYLAMINE

The initial approach to the synthesis of p-hydroxybenzyl hydroxylamine used p-hydroxybenzyl alcohol as the starting material and followed the reaction scheme illustrated in figure 20 (see section 3.1). In a typical trial experiment, the p-hydroxybenzyl alcohol (0.5565 g, 4.48 mmol) was dissolved in 50 mL of tetrahydrofuran and the thionyl chloride (1.5353 g, 5.38 mmol) was added dropwise at 0°C. The pyridine (0.36 mL, 4.48 mmol) was added and the reaction stirred at approximately 0°C for thirty minutes. The reaction was followed by thin layer chromatography (TLC) using 10 % methanol in chloroform as the eluent.

R_f of p-hydroxybenzyl alcohol = 0.43
R_f of product = 0.71

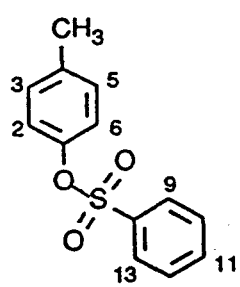
The reaction was discontinued when the starting material was no longer visible in the reaction mixture on the developed silica TLC plate. The resulting opaque yellow solution was filtered through Cellite (sinter funnel) and the solvent removed under reduced pressure (Büchi rotoevaporator) giving a transparent brown-yellow liquid. Within twelve hours at 2 - 5°C, the p-hydroxybenzyl chloride product turned into a sticky brown mass. It was determined using solubility properties and proton nuclear magnetic resonance spectroscopy that the compound had undergone polymerization.

In order to avoid this polymerization, the amount of pyridine was reduced and in one trial completely omitted. The synthesis was repeated several times but each attempt resulted in the formation of the brown polymeric compound. Keeping the product in dilute solution reduced the rate of polymerization but did not suppress it completely.

Synthesis of O-(p-Hydroxybenzyl)hydroxylamine Hydrochloride

This compound was synthesized according to the contents of British Patent #984,305 as shown in figure 22 (Drain et al., 1965).

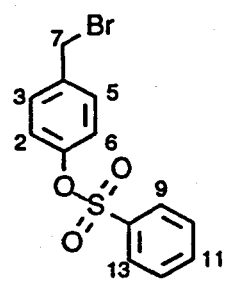
p-Cresol (10.0 mL, 0.0956 mol) was dissolved in 20 mL of pyridine. Phenyl sulfonyl chloride (13 mL, 0.102 mol) was added dropwise. The mixture was heated for thirty minutes and then poured into 215 mL water. The water was decanted and the resulting yellow oil was washed with two 50 mL portions of water to remove the pyridine. Upon standing at 2-5°C for one hour, a yellow-white crystalline solid formed. This solid was washed with approximately 10 mL portions of 2M HCl (aq) and water yielding the benzenesulfonate of p-cresol (29) as a damp yellow-white solid (34.11 g).



melting point = 39-42°C

^{1}H NMR (200 MHz); ($CDCl_3$) δ 2.28 (s, 3H, -C$\underline{H}_3$), 6.82 (d/t, 2H, $J_{2,3}/J_{5,6}$ 8.4 Hz, $J_{2,5}/J_{3,6}$ 2.4 Hz, $H_{2,6}$), 7.04 (d, 2H, $J_{2,3}/J_{5,6}$ 8.4 Hz, $J_{2,5}/J_{3,6}$ 2.4 Hz, $H_{3,5}$), 7.49 (d/t, 2H, $J_{10,11}/J_{11,12}$ 7.4 Hz, $J_{9,10}/J_{12,13}$ 7.3 Hz, $H_{10,12}$), 7.64 (t/t, 1H, $J_{10,11}/J_{11,12}$ 7.4 Hz, $J_{9,11}/J_{11,13}$ 1.7 Hz, H_{11}), 7.81 (d/t, 2H, $J_{9,10}/J_{12,13}$ 7.3 Hz, $J_{9,11}/J_{11,13}$ 1.7 Hz, $H_{9,13}$).

The benzylic position of compound #29 was brominated by a radical bromination reaction. The protected p-cresol (11.87 g, 0.0478 mol) was dissolved in 58 mL carbon tetrachloride with gentle refluxing. Bromine (0.0692 mol) was added dropwise under illumination with a tungsten lamp (100 W) over a period of two days. The reaction mixture was stirred magnetically for an additional day. The carbon tetrachloride solution was extracted with two 25 mL portions of water to remove a brown, water-soluble oil that had formed. The organic layer was then dried over anhydrous sodium sulfate and the solvent removed by evaporation under vacuum using a Büchi rotoevaporator yielding a yellow oil (19.18 g, >100 %). This oil was shown by proton nuclear magnetic resonance to contain unbrominated, monobrominated and dibrominated species. The desired monobrominated compound (30) was present as 75 % of the mixture and was used as such in subsequent reactions.

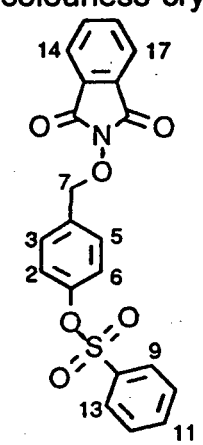


literature mp = 86-87°C (recrystallized three times from methanol) (Drain et al., 1965)

^{1}H NMR (200 MHz); ($CDCl_3$) δ 4.64 (s, 2H, H_7), 7.17 (d/t, 2H, $J_{2,3}/J_{5,6}$ 8.6 Hz, $J_{2,5}/J_{3,6}$ 2.2 Hz, $H_{2,6}$), 7.53 (d/t, 2H, $J_{2,3}/J_{5,6}$ 8.6 Hz, $J_{2,5}/J_{3,6}$ 2.2 Hz, $H_{3,5}$), 7.75 (m, 2H, $H_{10,12}$), 7.89 (m, 1H, H_{11}), 8.05 (m, 2H, $H_{9,13}$).

The phthalimide displacement reaction was carried out on compound #30 as in the previous cases using the modified Gabriel method (Sheehan & Bolhofer, 1950). The crude bromide (0.0586 mol) was dissolved in 90 mL of dry

acetonitrile. N-hydroxyphthalimide (7.18 g, 0.75 eq) and triethylamine, dried over 4 Å molecular sieves, (4.51 g, 0.75 eq) were added in dry acetonitrile under a nitrogen atmosphere. The mixture was refluxed for approximately twenty hours with magnetic stirring and then left to stir at room temperature for an additional twenty-four hours. A white precipitate formed after the refluxing period. The reaction mixture was filtered under vacuum and the solvent was removed from the filtrate by evaporation under vacuum (Büchi rotoevaporator). The solid residue was dissolved in 20 mL of methylene chloride and the methylene chloride was washed with 20 mL water and dried over anhydrous sodium sulfate. Evaporation of the solvent under reduced pressure gave a yellow-white solid half of which was recrystallized from acetone/water giving the phthalimide derivative (31) as a colourless crystalline solid (4.63 g, 38.6 %).



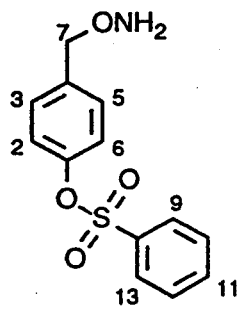
melting point = 102-114°C

literature mp = 138.5-139.5°C
(recrystallized from ethanol)
(Drain et al., 1965)

^{1}H NMR (200 MHz); ($CDCl_3$) δ 5.14 (s, 2H, H_7), 6.96 (d/t, 2H, $J_{2,3}/J_{5,6}$ 8.6 Hz, $J_{2,5}/J_{3,6}$ 2.3 Hz, $H_{2,6}$), 7.45 (d, 2H, $J_{2,3}/J_{5,6}$ 8.6 Hz, $H_{3,5}$), 7.64 (d/t, 2H, $J_{10,11}/J_{11,12}$ 7.5 Hz, $H_{10,12}$), 7.75 (overlapping multiplets, 7H, H_{11}, $H_{9,13}$, $H_{14\text{-}17}$).

The phthalimide group of compound #31 was removed by reacting the phthalimide derivative (4.63 g, 0.0113 mol) with hydrazine (0.40 g of a 95 %

solution, 1.05 eq N_2H_4) in 28 mL absolute ethanol. The reaction mixture was refluxed for approximately two hours and the resulting opaque white solution was cooled and vacuum filtered. The ethanol was removed by evaporation under reduced pressure (Büchi rotoevaporator) yielding a white solid. This solid was dissolved in 50 mL of chloroform and filtered again. The filtrate was washed with two 20 mL portions of buffer ($NaOH/KH_2PO_4$, pH = 7.4) and once with 10-15 mL water to remove residual hydrazine. The chloroform layer was dried over anhydrous sodium sulfate and the solvent removed on the Büchi rotoevaporator giving compound #32 as a slightly translucent yellow liquid (2.40 g, 76 %).

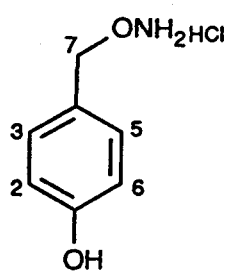


^{1}H NMR (200 MHz); ($CDCl_3$) δ 4.80 (s, 2H, H_7), 7.13 (d/t, 2H, $J_{2,3}/J_{5,6}$ 7.9 Hz, $J_{2,5}/J_{3,6}$ 2.3 Hz, $H_{2,6}$), 7.44 (d/t, 2H, $J_{2,3}/J_{5,6}$ 7.95 Hz, $J_{2,5}/J_{3,6}$ 2.3 Hz, $H_{3,5}$), 7.68 (d/t, 2H, $J_{10,11}/J_{11,12}$ 7.4 Hz, $J_{9,10}/J_{12,13}$ 7.3 Hz, $H_{10,12}$), 7.83 (t/t, 1H, $J_{10,11}/J_{11,12}$ 7.4 Hz, $J_{9,11}/J_{11,13}$ 1.6 Hz, H_{11}), 8.00 (m, 2H, $J_{9,10}/J_{12,13}$ 7.3 Hz, $J_{9,11}/J_{11,13}$ 1.6 Hz, $H_{9,13}$).

The sulfonate group was removed by reacting the protected hydroxylamine compound #32 (2.40 g, 8.60 mmol) with potassium hydroxide (1.45 g, 0.0258 mol) in 10 mL of methanol. The mixture was refluxed for five minutes and allowed to cool to room temperature yielding a white solid and a transparent yellow solution. Glacial acetic acid was added to bring the pH to 5 and the mixture was filtered under vacuum. The filtrate was evaporated under reduced pressure and 40 mL

of water were added to the residue. This solution was then purified using an ion-exchange resin.

Approximately 10 g of Amberlite IR-120 ion-exchange resin were washed with three 50 mL portions of methanol and five 50 mL portions of water by stirring the resin for 30 minutes with each wash. To ensure all sites on the resin were protonated, 30 mL of 0.25 M HCl (aq) were added to the washed resin and allowed to stir for 15 minutes. The pH of the solution containing the impure hydroxylamine was brought to approximately 7 with 1M NaOH (aq) and one-half of this solution was added to the resin and stirred for approximately 20 hours. The solution was decanted and the resin washed with 50 mL portions of water until the washings were of pH 5-6. The resin was then washed four times with 30-40 mL water and sufficient ammonium hydroxide to make the pH = 9 to remove the product from the resin. The ammonium hydroxide solution was evaporated under reduced pressure and aqueous HCl (50 %) added immediately to minimize oxidation of the free hydroxylamine. The acidified solution was filtered through Cellite to remove any insoluble impurities and the filtrate evaporated to dryness to yield 0.24 g (15.9 %) of a yellow-white solid (33).



melting point = 127-140°C

literature mp = 128.5-129.5°C (free hydroxylamine recrystallized twice from isopropanol) (Drain et al., 1965)

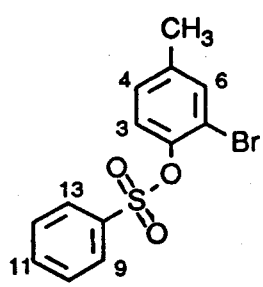
^{1}H NMR (200 MHz); (D_2O/TMS) δ 4.89 (s, 2H, H_7), 6.87 (d, 2H, $J_{2,3}/J_{5,6}$ 8.4 Hz, $H_{3,5}$), 7.28 (d, 2H, $J_{2,3}/J_{5,6}$ 8.4 Hz, $H_{2,6}$).

^{13}C NMR (50 MHz); (D_2O/TMS) δ 77.49 (C_7), 116.58, ($C_{3,5}$), 125.15 (C_4), 125.68 (C_1), 132.56 ($C_{2,6}$).

Synthesis of O-(m-Bromo-p-hydroxybenzyl)hydroxylamine Hydrochloride

This compound was synthesized according to the contents of British Patent #984,305 as shown in figure 22 (Drain et al., 1965).

m-Bromo-p-cresol (4.0 mL, 0.0331 mol) was dissolved in 7.5 mL of pyridine. Phenyl sulfonyl chloride (4.5 mL, 0.0353 mol) was added dropwise. The mixture was heated for thirty minutes and then poured into 74 mL water. The water was decanted and the resulting yellow oil was washed with two 50 mL portions of water to remove the pyridine. Upon standing at 2-5°C for one hour, a yellow-white crystalline solid formed. This solid was washed with approximately 10 mL portions of 2M HCl (aq) and water yielding the benzenesulfonate of m-bromo-p-cresol (34) as a yellow-white solid (10.18 g, 94.0 %)

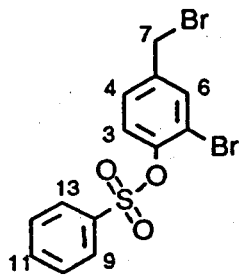


melting point = 39-44°C
literature mp = 63-65°C (recrystallized twice from methanol)
(Drain et al., 1965)

^{1}H NMR (200 MHz); ($CDCl_3$) δ 2.37 (s, 3H, -C$\underline{H}_3$), 7.14 (d, 1H, $J_{3,4}$ 7.7 Hz, H_4), 7.26 (d, 1H, $J_{3,4}$ 7.7 Hz, H_3), 7.35 (d, 1H, H_6), 7.57 (d/d, 2H, $J_{10,11}/J_{11,12}$ 7.3 Hz, $J_{9,10}/J_{12,13}$ 7.2 Hz, $H_{10,12}$), 7.77 (d/d, 1H, $J_{10,11}/J_{11,12}$ 7.3 Hz, H_{11}), 7.97 (d, 2H, $J_{9,10}/J_{12,13}$ 7.2 Hz, $H_{9,13}$).

The benzylic position of compound #34 was brominated by a radical bromination reaction. The protected bromocresol (8.05 g, 0.0246 mol) was

dissolved in 25 mL carbon tetrachloride with gentle refluxing. Bromine (0.0386 mol) was added dropwise under illumination with a tungsten lamp (100 W) over a period of two days. The reaction mixture was stirred magnetically for an additional day. The solvent was removed by evaporation under vacuum using a Büchi rotoevaporator yielding a mushy yellow solid (12.92 g, >100 %). This solid was shown by proton nuclear magnetic resonance to contain unbrominated, monobrominated and dibrominated species. The desired monobrominated compound (35) was present as 75 % of the mixture and was used as such in subsequent reactions.

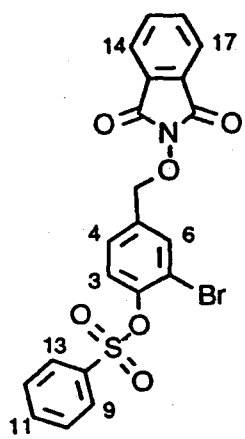


literature mp = 96-98°C (recrystallized twice from methanol) (Drain et al., 1965)

^{1}H NMR (200 MHz); ($CDCl_3$) δ 4.31 (s, 2H, H_7), 7.24 (s, 1H, H_4) 7.25 (s, 1H, H_3), 7.29 (s, 1H, H_6), 7.47 (m, 2H, $H_{10,12}$), 7.64 (m, 1H, H_{11}), 7.85 (m, 2H, $H_{9,13}$).

The phthalimide displacement reaction was carried out on compound #35 as in the previous cases using the modified Gabriel method (Sheehan & Bolhofer, 1950). The crude bromide (0.0246 mol) was dissolved in 50 mL of dry acetonitrile. N-hydroxyphthalimide (3.0163 g, 0.75 eq) and triethylamine, dried over 4 Å molecular sieves, (1.8750 g, 0.75 eq) were added in dry acetonitrile under a nitrogen atmosphere. The mixture was refluxed for two hours with magnetic stirring and then left to stir at room temperature for an additional

nineteen hours. A white precipitate formed after ten hours. The cooled reaction mixture was filtered under vacuum and the precipitate washed with approximately 10 mL of water. The solvent was removed from the filtrate by evaporation under vacuum (Büchi rotoevaporator) and the solid residue dissolved in 20 mL of methylene chloride. The methylene chloride was washed with 20 mL water and dried over anhydrous sodium sulfate. Evaporation of the solvent under reduced pressure gave a thick brown oil which was recrystallized twice from absolute ethanol giving the phthalimide derivative (36) as a colourless solid (7.90 g, 87.4 %).

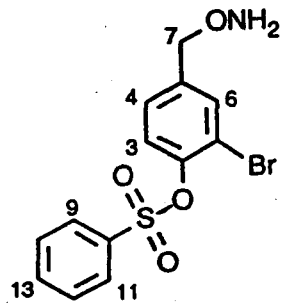


literature mp = 149-150°C (recrystallized from 50:50 acetone/methanol) (Drain et al., 1965)

^{1}H NMR (200 MHz); ($CDCl_3$) δ 5.12 (s, 2H, H_7), 7.34 (d, 1H, H_4), 7.45-7.88 (overlapping multiplets, 11H, H_3, H_6, $H_{9,13}$, $H_{10,12}$, H_{11}, H_{14-17}).

The phthalimide group of compound #36 was removed by reacting the phthalimide derivative (7.90 g, 0.0162 mol) with hydrazine (1.0880 g of a 95 % solution, 1.99 eq N_2H_4) in 41 mL absolute ethanol. The reaction mixture was refluxed for approximately two hours and the resulting opaque yellow-white solution was cooled and vacuum filtered. The ethanol was removed by evaporation under reduced pressure (Büchi rotoevaporator) yielding a yellow oil.

The oil was added to 23 mL of chloroform and filtered again. The filtrate was washed with two 20 mL portions of buffer ($NaOH/KH_2PO_4$, pH = 7.4) and once with 10-15 mL water to remove residual hydrazine. The chloroform layer was dried over anhydrous sodium sulfate and the solvent removed on the Büchi rotoevaporator giving compound #37 as a translucent yellow-brown oil (3.17 g, 52.0 %). This material was used immediately in the next reaction step without purification.

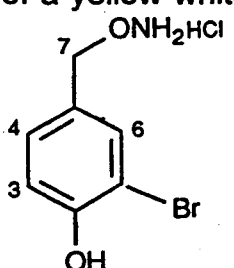


^{1}H NMR (200 MHz); ($CDCl_3$) δ 4.58 (s, 1H, H_7), 5.45 (br s, 2H, -N$\underline{H}_2$), 7.25 (br s, 2H, $H_{3,4}$), 7.48 (d/t, 1H, H_6), 7.54 (m, 2H, $J_{10,11}/J_{11,12}$ 7.4 Hz, $H_{10,12}$), 7.66 (t/t, 1H, $J_{9,11}/J_{11,13}$ 1.6 Hz, $J_{10,11}/J_{11,12}$ 7.4 Hz, H_{11}), 7.88 (m, 2H, $J_{9,11}/J_{11,13}$ 1.6 Hz, $H_{9,13}$).

The sulfonate group was removed by reacting the protected hydroxylamine compound #37 (3.17 g, 8.852 mmol) with potassium hydroxide (1.49 g, 26.6 mmol) in 9.1 mL of methanol. The mixture was refluxed for five minutes and allowed to cool to room temperature yielding a yellow solid and a transparent yellow solution. Glacial acetic acid was added to bring the pH to 5 and the mixture was filtered under vacuum. The filtrate was evaporated under reduced pressure and 5 mL of water were added to the residue. This solution was vacuum filtered through a sinter funnel and the filtrate left below 0°C for twelve hours to induce crystallization. No crystals formed during this time so the solution

was basified to pH 7 with 10 % aqueous sodium hydroxide and then purified using an ion-exchange resin.

Approximately 9 g of Amberlite IR-120 ion-exchange resin were washed with three 50 mL portions of methanol and five 50 mL portions of water by stirring the resin for 30 minutes with each wash. To ensure all sites on the resin were protonated, 20 mL of 0.25 M HCl (aq) were added to the washed resin and allowed to stir for 15 minutes. One-half of the compound in basic solution was then added and stirred for approximately 20 hours. The solution was decanted and the resin washed with 50 mL portions of water until the washings were of pH 5-6. The resin was then washed four times with 30 mL water and sufficient ammonium hydroxide to make the pH = 9 to remove the product from the resin. The ammonium hydroxide solution was evaporated under reduced pressure and aqueous HCl (50 %) added immediately to minimize oxidation of the free hydroxylamine. The acidified solution was filtered through Cellite to remove any insoluble impurities and the filtrate evaporated to dryness to yield 0.04 g (4.14 %) of a yellow-white solid (38).



literature mp = 135-137°C
(dihydrogen phosphate salt)
(Drain et al., 1965)

^{1}H NMR (200 MHz); (D_2O/TMS) δ 5.06 (s, 2H, H_7), 7.12 (d, 1H, $J_{3,4}$ 8.5 Hz, H_4), 7.42 (d, 1H, $J_{3,4}$ 8.5 Hz, H_3), 7.75 (s, 1H, H_6).

There was insufficient sample available to collect a carbon-13 nuclear magnetic resonance spectrum.

Pharmacological Testing of O-Alkylhydroxylamines

Secondary Response Experiments

The colonic epithelial preparations were obtained using procedures described by Rangachari and McWade (1986). Briefly, adult dogs of either sex were killed with pentobarbital sodium (100 mg/kg). The proximal colon was quickly excised and immersed in oxygenated Krebs solution warmed to 37°C. A quick initial dissection of the tissue was carried out to remove the circular and longitudinal muscles followed by a careful dissection to remove the muscularis mucosa. The resulting tissue preparation, which was functionally "nerve-free", was set up in conventional Ussing chambers for recording short-circuit currents (Rangachari & McWade, 1987).

The tissues were bathed on both sides with warm (37°C) oxygenated Krebs solution having the following composition (in mM): 116 NaCl, 4.6 KCl, 1.2 $MgCl_2$, 1.5 $CaCl_2$, 22 $NaHCO_3$, 1.2 NaH_2PO_4 and 10 glucose (pH of approximately 7.2). Short-circuit currents (μA) were measured using a World Precision Instruments (Sarasota, Florida) dual voltage clamp. The data were collected using a computerized data acquisition system (IBM PC 486, Intel; MP100 DAS/Acknowledge version 2.02 software, Biopac).

The secondary response curves were obtained by treating each tissue with 10^{-5}M histamine to measure a control response. When the initial response to the

serosally added histamine had begun to fade, the hydroxylamine compound was added at a concentration of 10^{-4}M and the secondary response measured. N-methyl hydroxylamine was used as a negative check and hydroxylamine or O-methyl hydroxylamine were used as positive checks.

Dose-Response Curves

The colonic epithelial preparations were obtained and the short-circuit current measured as described for the secondary response experiments.

With each tissue, two concentration-response curves were measured. The measured response was the increase in short-circuit current (I_{sc}). A control histamine dose-response curve was obtained early in the day by adding successive increments of histamine at the peak of the previous response. The tissues were then extensively washed and, after a sixty minute recovery period, the second dose-response curves to histamine were constructed. Each tissue was pretreated for 15 minutes with a hydroxylamine compound at 10^{-4}M and successive increments of histamine were again added at the peak of the previous response For all curves, the increases in current were normalized to the maximal changes obtained under control conditions for each particular tissue and are expressed as a percentage of the histamine control curve.

All statistical procedures were performed on a microcomputer, using Kwikstat (version 2.0) software (Texasoft, Cedar Hill, TX). All data were analyzed using distribution-free methods [analysis of varience (ANOVA) - Kruskal Wallis] to avoid the necessity for assumptions based on the distribution of the data.

Mean effective dose values (ED_{50} and PD_2 values) were determined by nonlinear regression analysis. The analysis was completed on a microcomputer using a curve-fitting procedure in Fig.P (version 6.0; Biosoft, MA). A four-parameter logistic equation of the following form was used:

$$Y = \frac{(A - D)}{1 + (X/C)^{-B}} + D$$

where Y represents the magnitude of the response to the agonist, and X is the concentration of the agonist at which that response occurs. A - D are computer generated parameters reflecting maximal response (A), slope factor (B), PD_2 (C) and minimal response (D), respectively. Based on this expression, PD_2 values were estimated from the value of C obtained for each dose-response curve. pD_2 values thus obtained were tested against the pD_2 values obtained from the control curve value to determine whether the values were significantly different.

Diamine Oxidase Enzyme Assay

The diamine oxidase enzyme assay was performed using a modification of a procedure described by Kusche et al. (1973). The enzyme was prepared by homogenizing epithelial tissue in nine volumes of phosphate buffer (vol:wet weight) and then centrifuging for thirty minutes (48,000 g, 4°C). The supernatant was collected and used as the source of the enzyme. The supernatants from five dogs were combined and frozen in 1 mL aliquots for use as the test solution in these experiments and the enzyme was found to remain stable for at least six weeks. Each test was performed in duplicate.

The assay mixture for the test consisted of (1) 0.6 mL of 0.1 M sodium phosphate buffer (pH 7.2), (2) 0.1 mL of test-sample solution (diamine oxidase ± inhibitors), (3) 0.05 mL of substrate solution (containing 4.5 mM putrescine dihydrochloride and 1 μCi/mL of [1,4-^{14}C] putrescine dihydrochloride). The enzymatic activity was determined using an incubation period of ten minutes at 37°C at which time the reaction was stopped by adding 0.2 mL of perchloric acid (1.7 M). The pH was adjusted by adding 1 mL of alkaline buffer (0.8 M NaOH, 0.6 M $NaHCO_3$; pH 12.2) and the labelled reaction product ([^{14}C]-Δ_1-pyrroline) directly extracted into 6 mL of scintillation fluid (toluene containing 3.5 g/L of 2,5-diphenyloxazole). The radio activity present in 5 mL of this scintillation fluid was measured using a Beckman LS-5801 liquid scintillation counter. The three blanks used were: (1) sample blank - perchloric acid added before substrate solution (0 minute incubation), (2) enzyme blank - 0.1 mM aminoguanidine added to inactivate the enzyme and (3) reagent blank - buffer added instead of enzyme solution.

The data in these experiments were expressed as percentages of the control diamine oxidase activity (disintegrations or decays per minute of $^{14}C \cdot min^{-1} \cdot mg\ protein^{-1}$) (Rangachari, Prior, Bell & Huynh, 1992).

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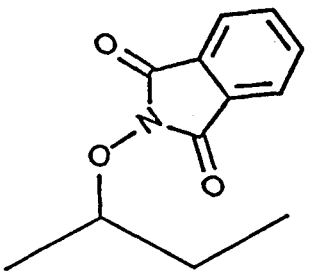
APPENDIX 1: ABBREVIATIONS USED

ACN	acetonitrile
(cyclic) AMP	(cyclic) adenosine monophosphate
AO	amine oxidase
asn	asparagine
asp	aspartic acid
BSAO	bovine serum amine oxidase
DAO	diamine oxidase
DMF	N,N-dimethylformamide
DMSO	dimethylsulfoxide
dpm	decays or disintegrations per minute
E_{max}	maximum efficacy
E_{ox}	oxidized form of enzyme
E_{red}	reduced form of enzyme
ED_{50}	concentration of agonist required to give one-half of E_{max}
EPR	electron paramagnetic resonance
FID	free-induction decay
HDC	histidine decarboxylase
HMT	histamine methyl transferase
I_{sc}	short-circuit current
leu	leucine
NADH	β-nicotinamide-adenine dinucleotide
NMR	nuclear magnetic resonance
PD	(trans-mucosal) potential difference
pD_2	the negative logarithm of the ED_{50} value
ppm	parts per million
(toluene)-PPO	(toluene-2,5)-diphenyloxazole
PQQ	pyrroloquinoline quinone
RNA	ribonucleic acid
THF	tetrahydrofuran
TLC	thin-layer chromatography
TMS	tetramethylsilane
topa	6-hydroxydopa(trihydroxyphenylalanine)
tyr	tyrosine
UV/VIS (spectroscopy)	ultra-violet/visible (spectroscopy)

APPENDIX 2: STRUCTURES OF SYNTHESIZED COMPOUNDS



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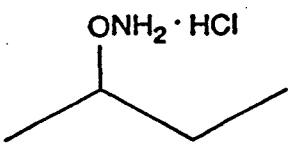
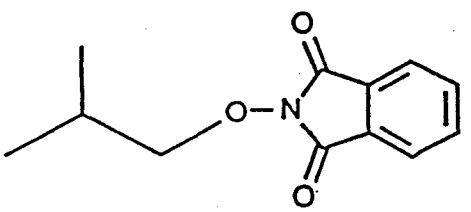


O-(*sec*-butyl)hydroxylamine hydrochloride (2)



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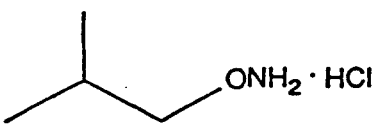

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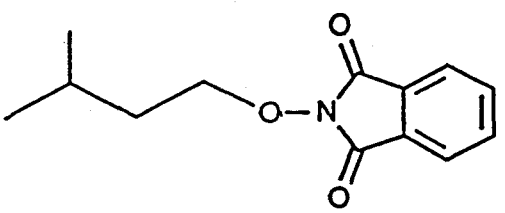

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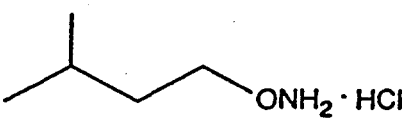

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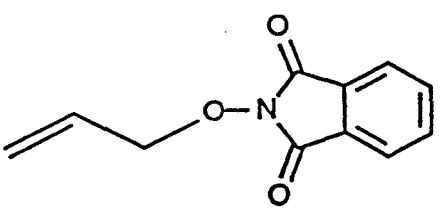
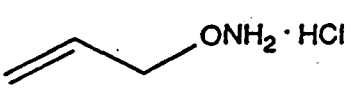
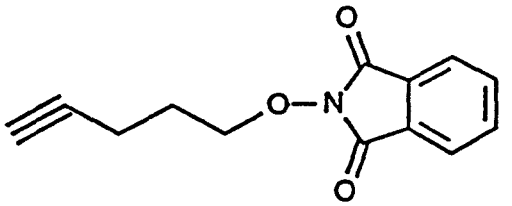


O-(allyl)-N-hydroxyphthalimide (7)



O-(allyl)hydroxylamine hydrochloride (8)



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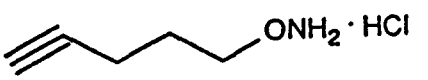

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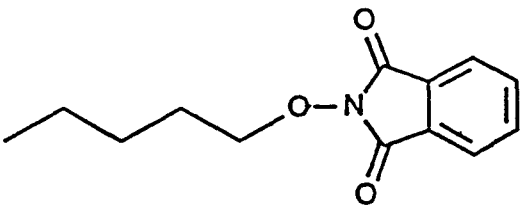

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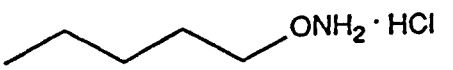

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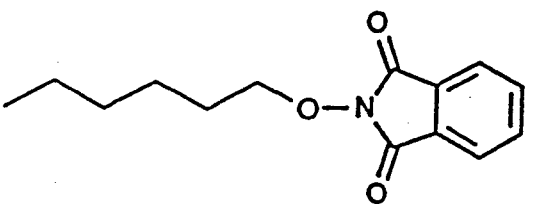

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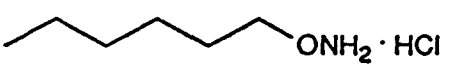

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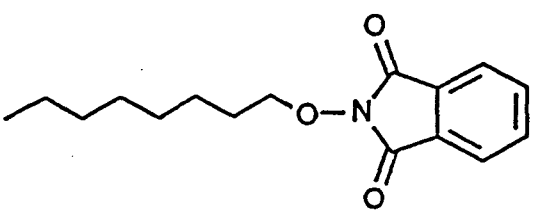

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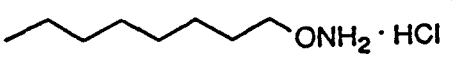

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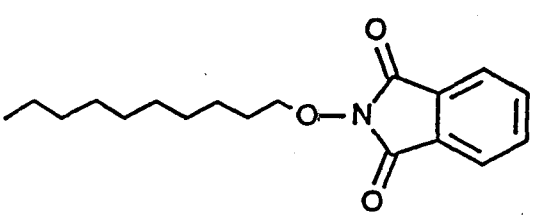

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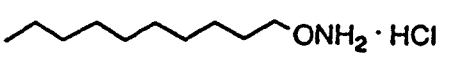

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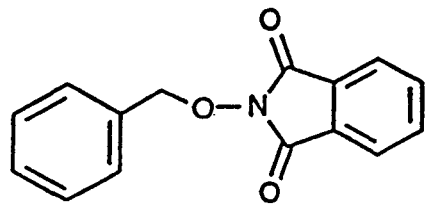

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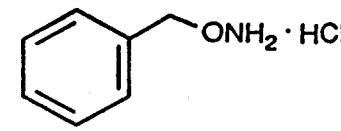

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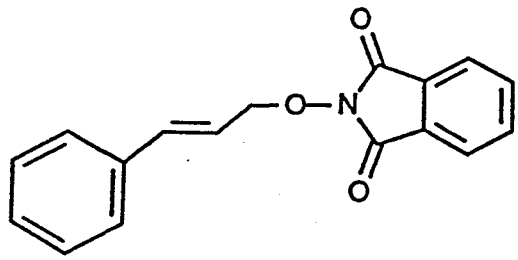

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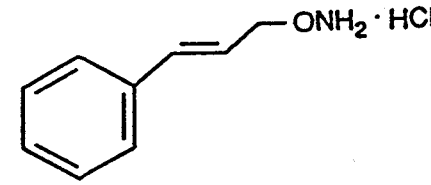
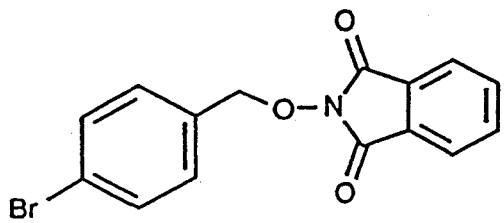


O-(cinnamyl)hydroxylamine hydrochloride (22)



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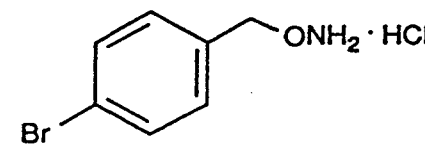

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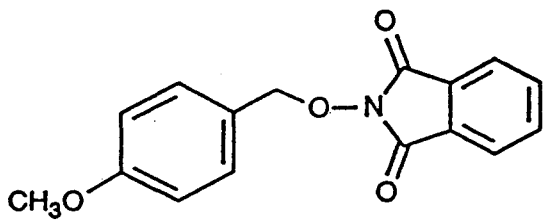

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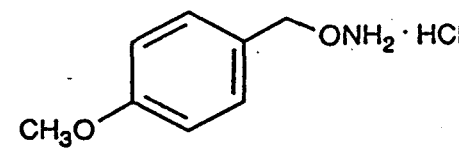

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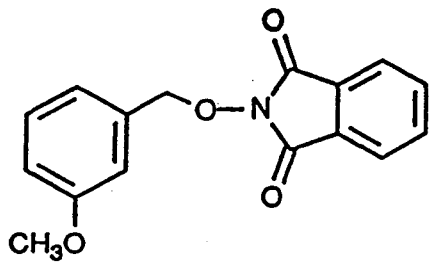

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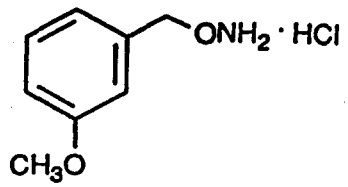

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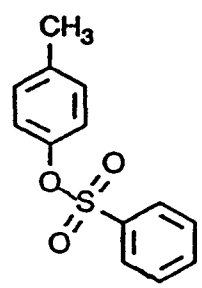
hydroxylamine hydrochloride (28)



Benzene sulfonate
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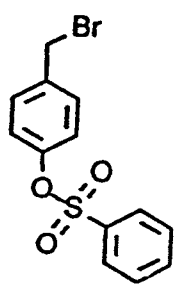

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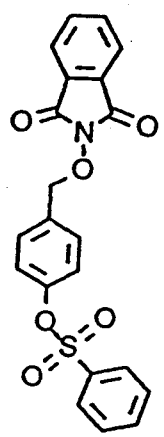

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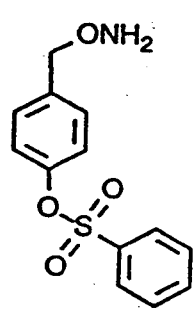

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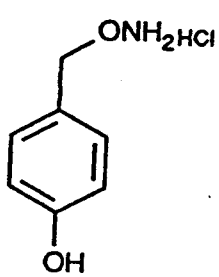
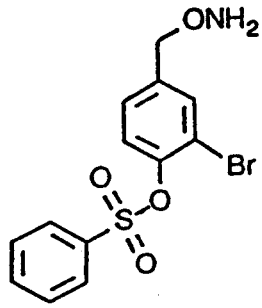
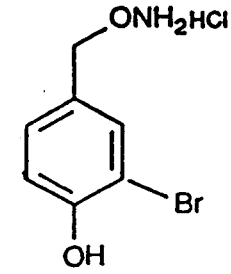


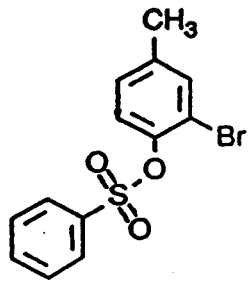
O-(p-hydroxybenzyl)
hydroxylamine hydrochloride (33)



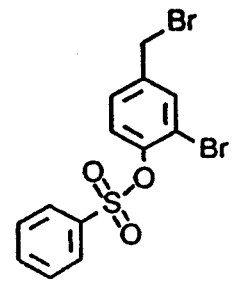
(37)



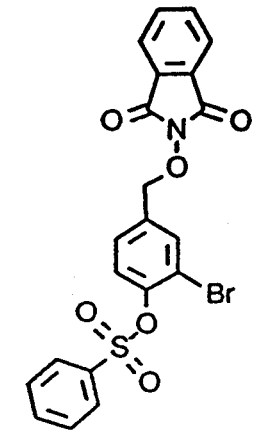
O-(p-hydroxy-m-bromobenzyl) hydroxylamine hydrochloride (38)



Benzene sulfonate of m-bromo-p-cresol (34)



(35)



(36)

APPENDIX 3: DOSE-RESPONSE DATA

Summary Table of Means

	MAX mean	MAX n	MAX std. dev.	PD_2 mean	PD_2 n	PD_2 std. dev.
histamine	485.32	5	72.02	4.87	5	0.56
O-methyl	508.66	5	149.83	5.71	5	0.29
N-methyl	449.54	5	154.20	5.34	5	0.32
O-pentyl (12)	547.66	5	192.13	5.72	5	0.36
O-octyl (16)	497.76	5	216.65	5.59	5	0.20
O-benzyl (20)	502.37	4	91.92	5.14	4	0.35
O-(p-methoxybenzyl) (26)	367.23	4	59.07	5.73	4	0.13
O-(p-hydroxybenzyl) (33)	518.32	5	152.78	5.68	5	0.14
All Groups	487.23	38	143.49	5.48	38	0.42

These data are the average maximum and pD_2 values from either four or five (n) repetitions of the dose-response experiments. The maximum and pD_2 values are defined in section 1.4.2 (pages 16-17). The results of the dose-response experiments are graphed in figure 25 (page 38).

Newman-Keuls Data for PD_2 Values

Hydroxylamine Compound	P Value (compared to histamine)
O-methylhydroxylamine	0.003936
N-methylhydroxylamine	0.073546
O-pentylhydroxylamine (12)	0.004525
O-octylhydroxylamine (16)	0.007915
O-benzylhydroxylamine (20)	0.236653
O-(p-methoxybenzyl)hydroxylamine (26)	0.01037
O-(p-hydroxybenzyl)hydroxylamine (33)	0.004053

Note: A "P" value smaller than 0.05 indicates that the two numbers are significantly different.

APPENDIX 4: ENZYME ASSAY DATA

Values given are the raw dpm (decays per minute) of ^{14}C expressed as a percentage of the control enzyme activity.

Compound	10^{-4}M	10^{-5}M	10^{-6}M	10^{-7}M	10^{-8}M
sec-butyl (2)	-2.43	-0.94	34.52	87.93	93.51
isobutyl (4)	-0.50	18.51	82.44	90.41	98.27
isoamyl (6)	1.65	69.29	97.58	96.54	98.49
allyl (8)	0.08	0.83	3.86	85.48	99.06
4-pentynyl (10)	-0.91	10.40	82.90	102.47	102.74
pentyl (12)	-1.11	1.73	9.19	78.60	85.15
hexyl (14)	-1.06	0.06	62.40	99.26	98.90
octyl (16)	-1.52	38.29	88.08	95.97	98.48
decyl (18)	0.64	61.63	101.17	102.49	96.75
benzyl (20)	1.85	69.20	91.72	85.28	94.19
cinnamyl (22)	-1.70	-2.00	53.77	91.18	100.29
p-Br-benzyl (24)	1.28	29.88	100.02	108.37	105.09
p-OCH_3-benzyl (26)	-3.13	16.37	89.20	97.98	97.66
m-OCH_3-benzyl (28)	-2.12	8.47	73.97	92.61	99.52
p-OH-benzyl (33)	-4.04	-4.26	61.54	91.00	99.57
p-OH-m-Br-benzyl (38)	-2.85	-0.80	77.62	91.53	100.90
aminoguanidine	-3.09	-1.92	-1.48	62.32	96.72
hydroxylamine	-0.14	1.45	7.64	84.21	104.43
N-methylhydroxylamine	56.87	77.91	89.25	92.35	100.45
O-methylhydroxylamine	-0.92	0.89	3.61	65.03	90.78