The Synthesis of an Organophosphorus Analog of Acetylcholine

Jay-James R. Miller '96
Illinois Wesleyan University

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Jay-James R. Miller

Jeffrey A. Frick, Ph.D., Faculty Advisor


Illinois Wesleyan University
Approval Page
"The Synthesis of an Organophosphorus Analog of Acetylcholine"
by Jay-James R. Miller

A PAPER SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR CHEMISTRY 499 AND HONORS IN CHEMISTRY.

Approved, Honors Committee:

[Signatures]
Jeffrey A. Frick, Ph.D., Research Advisor
Forrest J. Frank, Ph.D.
L. Farah Sogo, Ph.D.
Wendy Woltzach, Ph.D.

Illinois Wesleyan University
1996
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Abstract

Acetylcholine (ACh) is the most widely studied of all neurotransmitter substances. For normal nerve function, the enzyme acetylcholinesterase (AChE) must hydrolyze ACh into its basic chemical constituents, choline and acetate. AChE is readily inhibited by organophosphorus (OP) compounds like sarin and soman—both nerve gases—as well as various pesticides. OP compounds also have been widely used to study the mechanism of ACh hydrolysis via AChE. In the 1980s, two studies using OP inhibitors examined the stereoselectivity of AChE hydrolysis. However, they yielded conflicting results. Hence, it is hoped that new studies with a novel OP compound will provide definitive information about the stereoselectivity of the mechanism of AChE action. Obviously, the first phase of this project must be the synthesis of that novel OP inhibitor. We present our efforts in that area and outline future directions.
Introduction

Acetylcholine

Acetylcholine (ACh) (see Figure 1, molecule 1) is synthesized by the enzyme choline acetyltransferase and is broken down after release into the synaptic cleft by the enzyme acetylcholinesterase (AChE, acetylcholine hydrolase, E.C. 3.1.1.7). These enzymes are synthesized in the neuronal cell body, and they are carried by axonal transport to the presynaptic terminal; synthesis of ACh, per se, occurs in the presynaptic terminal (see Figure 2).

ACh acts as a transmitter at a variety of sites in the peripheral nervous system (PNS) and the central nervous system (CNS). ACh, for example, is responsible for excitatory transmission at the neuromuscular junction (N-type, nicotinic ACh receptors). ACh is also the transmitter in autonomic ganglia and is released by preganglionic sympathetic and parasympathetic neurons. Postganglionic parasympathetic neurons, as well as one particular type of postganglionic sympathetic axon (i.e., the fibers innervating sweat glands), utilize ACh as their transmitter (M-type, muscarinic ACh receptors).

Within the CNS, several well-defined groups of neurons use ACh as a transmitter (1). These groups include neurons that project widely from the basal forebrain nucleus of Meynert to the cerebral cortex and from the septal nucleus to the hippocampus. It appears likely that, in fact, the basal forebrain nucleus is the primary source of cholinergic input to the cerebral cortex. Cholinergic neurons, located in the brain stem tegmentum, project to the hypothalamus and thalamus where they use ACh as a transmitter.

Considerable interest has focused recently on the role of cholinergic CNS neurons in neurodegenerative diseases. Cholinergic neurons in the basal forebrain
nucleus degenerate, and their cholinergic terminals in the cortex are lost as part of the pathology in Alzheimer's disease (1).

**Acetylcholinesterase**

There has been much investigation into AChE, and many of the major points of its hydrolytic mechanism are already known [for a review, see (2)]. Early kinetic studies indicated that the active site of AChE contained esteratic and anionic subsites (see Figure 3) (3, 4). The anionic subsite binds the quaternary nitrogen of ACh, and is believed to bind both quaternary ligands (5, 6) and quaternary oximes (7). The esteratic site is responsible for donating the electrons to the acetate portion of the ACh molecule and for cleaving it from the choline moiety. The actions at those two sites promote the hydrolysis of ACh.

**The Anionic Subsite**

The traditional anionic subsite was thought to contain multiple negative charges (8). The evidence for that site was based upon the interactions of the enzyme with various types of compounds (3, 9) including aromatic cations, tetraalkylammonium salts, aziridinium covalent modifying reagents (10, 11), bisquaternary ammonium compounds that span the anionic locus, and pyridinium reactivators.

These early studies recognized the possible transfer of the substrate from solution to a lipid-like environment, but there was still no conclusive evidence to suggest that a hydrophobic region was the binding site for the trimethylammonio moiety of the choline. Instead, it was believed that a negatively charged anionic subsite bound to the positively charged quaternary group of the choline moiety. The results of a later study involving both cationic and uncharged homologs, however, suggested that the anionic binding site actually might be uncharged and lipophilic
Acetylcholine

Based on those findings, Hasan and Cohen proposed that the enzyme binds to the choline moiety with a hydrophobic trimethyl binding site.

Recent crystallographic analysis by Sussman and co-workers (13) provides a reasonable explanation for the observations of both true anionic and hydrophobic anionic subsites. That analysis has revealed that the active site lies at the bottom of a deep narrow gorge that is about 20 Å long and that penetrates halfway into the enzyme. It appears that about forty percent of that gorge is lined by fourteen aromatic residues that may account for the previous observations of various hydrophobic and anionic sites separate from or overlapping the active site.

In addition to the main anionic subsite responsible for binding ACh, AChE possesses one or more additional binding sites for other quaternary ligands. Such peripheral anionic sites, clearly distinct from the choline-binding pocket of the active site gorge, were firmly established by Taylor and Lappi (14). These peripheral sites are the ones involved in much of the substrate inhibition characteristic of AChE.

Once trapped on top of the gorge, the aromatic lining could guide the ACh molecule rapidly toward the esteratic site where it is hydrolyzed (15). It is thought that the aromatic lining may permit the use of a mechanism involving initial absorption of ACh to low affinity sites, followed by two dimensional diffusion to the esteratic active site. Moreover, the depth of the gorge and the extent of the aromatic residues lead to many possible ways and places for substrate, agonists, and inhibitors to bind to AChE. The specific geometry of the gorge, therefore, may give clues how certain inhibitors behave by blocking or penetrating the gorge.
The Esteratic Subsite

The esteratic subsite contains the residues of three amino acids, glutamic acid (Glu), serine (Ser), and histidine (His), which likely function as an acid catalytic triad similar to that in the serine proteases (with the important differences of Glu replacing the aspartic acid residue found in the serine proteases and the position of the catalytic portion being a mirror image—as compared with the peptide backbone—of that in the serine proteases).

The major feature of the esteratic site is the amino acid serine. The serine hydroxyl attacks the carbonyl carbon of ACh and thus is ultimately responsible for the hydrolysis of ACh. The histidine residue plays an important role in the formation and cleavage of the tetrahedral intermediate during ACh hydrolysis. Various observations (3, 16, 17, 18, 19) support an acylenzyme mechanism for the catalysis of ACh in which the hydroxyl group of the serine acts as a nucleophile and the imidazole ring of the histidine likely functions as a general acid-base catalyst in the formation and decomposition of tetrahedral intermediates (see Scheme 1). Although this mechanism is not the only one that can be written which involves the active site histidine in general acid-base catalysis (20), it serves as a useful framework for the interpretation of most results. Since organophosphorus (OP) esters have a stable tetrahedral structure, it should not be surprising that these compounds function as competitive inhibitors of ACh (21). The tetrahedral OP inhibitors mimic the assumed geometries along the reaction coordinate involved in the hydrolysis of ACh and thus are transition-state analogs of ACh. Some examples of OP inhibitors of ACh are shown in Figure 4.
Phosphorylation

The serine hydroxyl group attacks the electrophilic phosphorous atom of OP compounds just as it does the carbonyl carbon of ACh. However, once an OP inhibitor is bound, the enzyme is phosphorylated and thus no longer capable of hydrolyzing ACh (see Scheme 2). After phosphorylation has occurred, there are three possible outcomes. One, the compound could be reactivated by the same mechanism as deacylation. Second, reactivation can either be spontaneous or induced by chemical means. Alternatively, the compound could simply remain phosphorylated indefinitely. In the third option, the compound may experience a process called aging in which one of the groups on the phosphorous atom is replaced by another group, usually a hydroxyl group. Aging results in a phosphorylated enzyme that is unable to undergo reactivation.

Aging thus provides a method to determine the potency of OP inhibitors: AChE first is inhibited with an OP compound; then the amount of activity restored to the enzyme after chemical reactivation is measured. Since an aged enzyme is no longer susceptible to reactivation, the activity of the enzyme after reactivation can be used to determine the extent of aging and thus the potency of the inhibitor. Berends and others were the first to use that method to examine the aging process in detail (22).

In the 1980s, similar studies of aging were conducted to examine the stereoselectivity of the phosphorylation of AChE (23, 24). Those studies showed that phosphorylation is highly stereoselective, but they failed to present a general rule that relates absolute configuration at the phosphorus atom to the toxicity of the inhibitor. In 1985, Eya and Fukuto (25) showed that the (R)p(+) isomer of 2,5-dichlorophenyl methyl phenylphosphonate (see Figure 4) was more toxic than the (S)p(+) isomer. However, in 1988, a study by Benschop and De Jong (24) showed that
nerve gases like isopropyl methylphosphonofluoridate (see Figure 4) also exhibit stereoselectivity in the phosphorylation of AChE. The results of that study indicated that compounds with the (-) configuration around the phosphorus atom are better inhibitors than the corresponding (+) isomers. Absolute configuration studies done on one of the compounds in the study indicate that the (+) isomer of isopropyl methylphosphonofluoridate has the R configuration. Obviously, much work still needs to be done to clarify the unanswered questions concerning aging.

Objective

The purpose of this project, consequently, is to first synthesize a novel OP analog of ACh (2) that closely resembles the tetrahedral transition state in the catalysis of ACh by AChE (see Figure 1), and then to use that compound to contribute to further knowledge of the stereoselectivity of AChE phosphorylation and to study the aging process in more detail. Since AChE itself is asymmetric, it should be most informative to examine OP compounds that are not only chiral at the phosphorus, but also have an asymmetric site elsewhere in the molecule, in this case at the β-carbon. The proposed internal asymmetric synthesis of compound 2 begins with the α-amino acid 1-proline (see Scheme 3). Since 1-proline contains a pyrrolidine ring, the amino portion of the molecule should be somewhat rigid and rotation about bonds adjacent to the chiral center should be restricted. Such conformational constraint should make it possible to study the effect of chirality at the β-carbon without the interference of steric effects from other portions of the molecule. That will provide better understanding of the conformation of the active site than would be possible from an acyclic compound, and will allow the stereoselectivity of the phosphorylation process to be examined in greater detail.
Results and Discussion

The synthesis of compound 2 is not complete, but the preliminary steps have been accomplished. The starting material for the synthesis was (S)-N-((benzyloxy)carbonyl) proline 4 which can be readily prepared from L-proline 3 and benzyloxy carbonyl chloride (25). The carboxylic acid group of 4 was selectively reduced to the alcohol by the addition of borane-THF (26). A study of the phosphorylation of 5 was begun.

A difficulty obtaining the high yields characteristic of the selective reduction reaction was a source of confusion throughout this project. In previous work (27), yields as high as ninety-two percent were reported when four moles of active hydride (each mole of borane contains three moles of active hydride) were added to each mole of 4. However, that ratio proved ineffective in nine trials. Expressly, only 0.12g (2.5%) of the alcohol was recovered from the combined product of those reactions. The purity of that alcohol product, moreover, was questionable even after our purification techniques, since it was a deep yellow color and the IR spectrum showed several unexpected carbonyl peaks (see Spectrum 2).

We first checked the purity of our 4. The melting point of the 4 being used was found to be 10-15°C below that of 99+% pure 4 (Aldrich; 76-78°C). The entire stock of 4, therefore, was repeatedly recrystallized until the melting point matched that for pure 4. The purification of 4, however, did not noticeably affect the yield of 5. So the purity of 4 was not the problem. The next course of action was to increase the amount of hydride added.

The amount of active hydride added was ultimately increased to a ratio of about seven moles of active hydride to one mole of 4. The result was yields about eighty percent (79.0% and 81.9%) purified 5 in two trials. The purity of 5 was checked with
IR (see Spectrum 3). That IR spectrum for 5 still showed evidence for two carbonyl peaks (see Spectrum 4). Hence the purity of 5 obtained from the last two reductions was significantly improved over that gathered from the earlier reductions, but still short of the high level of purity achieved in previous work (27). The NMR spectrum of purified 5, however, indicated that the alcohol was reasonably pure (see Spectrum 7). Since the selective reduction of 4 to 5 had previously been performed in high yields with an active hydride to 4 ratio of four-to-one (27), one can assume that the concentration of active hydride is not the same as would be calculated from the concentration of BH$_3$-THF claimed by the manufacturer (Acros). So, either the manufacturer mislabelled the product or, more likely, some of the borane had decomposed between the time of its packaging and usage.

Now that the alcohol 5 can be consistently obtained in high yields, it is possible to begin an investigation of possible methods of phosphorylating it. Currently, three methods of phosphorylation are being considered (28, 29, 30). Some of the differences between these methods are outlined in the next section.

The latest phase of this project was the preparation of 8 by the method of Oza and Corcoran (30) (see Scheme 6). That method was chosen because of the mildness of the procedure and because it has led to the formation of dimethyl phosphate esters from the corresponding alcohols in high yields (70-98%).

The IR spectrum of the crude product (see Spectrum 5) clearly showed a hydroxyl peak at 3450 cm$^{-1}$, and a carbonyl peak at 1700 cm$^{-1}$. Those peaks were also found on the IR spectrum of the alcohol product, but they should not appear in the IR spectrum for the phosphate ester. Hence at least some alcohol was present in the crude product. However, a second carbonyl peak at 1745 cm$^{-1}$ and the new, albeit weak, peak at 1280 cm$^{-1}$ indicated that there was also some phosphate ester present in
the sample.* Based on the intensities of those peaks the sample likely contained
mostly unreacted alcohol. Upon purification of the crude product, no change in the
IR spectrum was observed (see Spectrum 6).

There are two possible explanations for the disappearance/lack of formation
of the desired product. One is that the reaction afforded the desired product in low
yields and that the highly reactive phosphate group was removed from the
phosphoester during flash chromatography (31). All of the material collected from
the column, therefore, would be 5; even if the reaction produced some 8. This
scenario is least likely because Spectrum 6 still shows evidence of a phosphorus-
oxygen double bond.

The second possibility is that flash chromatography was unable to separate 8
from 5 as a result of the chosen solvent, ethyl acetate. In order for flash
chromatography to be effective, the difference in Rf values for the species to be
separated must be at least 0.15 (31). The difference in Rf values for the alcohol and
the presumed phosphate ester is between 0.10 and 0.15. Obviously, that difference
was not large enough to allow adequate separation of the compounds using ethyl
acetate, so another solvent system must be used.

Another interesting observation was made during the flash chromatography.
Most of the crude product remained on the top of the column after the purification
procedure was completed. That substance was a dark brown color and thus may have
contained the alkyl bromide product of 5. If that substance were the alkyl bromide
product of 5, then there was an alcohol exchange equilibrium established where one
of the methoxy groups on the phosphorus intermediate was replaced by a molecule of

* Peaks between 1250 and 1300 cm⁻¹ are associated with the phosphorus-oxygen
double bond.
5 (30). The result of that exchange was a combination of the desired product and the alkyl bromide product of 5.

The phosphorylation of 5 may still be achieved via the reaction attempted in this study, however, if the reaction conditions are modified. For example, during the course of the reaction, the reaction mixture changed from yellow to red. Shortening the time allowed for the reaction, therefore, may influence the products obtained. The formation of the desired product may also be affected by using carbon tetraiodide instead of carbon tetrabromide. The original study (30) on this reaction did not study carbon tetriodide as a possible reagent because tetraiodide is more expensive and excellent results were obtained with tetrabromide. However, that study did compare carbon tetrachloride to carbon tetrabromide. The carbon tetrabromide was found to be significantly more effective than the carbon tetrachloride, and the authors of that study concluded from their results that carbon tetraiodide "likely . . . would be even more effective than carbon tetrabromide."

As has already been mentioned, there are many ways to phosphorylate 5. Should the suggested modifications to the "Oza-Corcoran" method fail to produce 8 in quantitative yields, then one of those other methods still might lead to the desired product. Therefore, the work on this project should be continued.
Future Work

The continuation of this project obviously must begin with a completion of the synthesis of 2, because only then will it be possible to use 2 to study the stereospecificity of the aging process. It is also important for the planned study of the phosphorylation of AChE that compound 2 be asymmetric at the phosphorus atom. The "Fletcher et al." (28) method of phosphorylation is the only one considered thus far that produces a product chiral at the phosphorus atom (see Scheme 4). However, the product using that method is a mixture of diastereomers, and an effective way to separate the two stereoisomers must be found. That method has been used to prepare O,O-diethyl O-p-nitrophenyl thiophosphate (parathion), an organophosphorus inhibitor of AChE (see Figure 4). Both the "Bannwarth-Trzeciak" (29) and the "Oza-Corcoran" (30) methods of phosphorylation produce a product that is achiral at the phosphorus atom (see Schemes 5 and 6, respectively). So, some method to induce chirality at the phosphorus atom must be developed if either of those methods are to be successful. Finally, since the "Fletcher et al." method of phosphorylation is unique in that it yields the thiophosphate equivalent of 2, it also might be possible to eventually compare the kinetic rates of phosphates to those of their thiophosphate equivalents.
Experimental

General Methods

Melting points were determined using a Mel-Temp melting point apparatus and are uncorrected. Proton spectra were taken in deuterated chloroform (CDCl₃) on a Jeol 270 MHz NMR. Pertinent ¹H NMR data are tabulated in the following order: chemical shift (ppm in delta), multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet), coupling constants (J in Hertz), and number of hydrogens. Infrared data (FTIR) were obtained on an ATI Mattson Genesis Series FTIR Model 9423-240-08061 spectrophotometer. Salient IR features are listed by decreasing wavenumber (cm⁻¹).

Analytical thin layer chromatography (TLC) was conducted with aluminum backed silica plate (Whatman). Visualization was affected with an ultraviolet lamp and/or with anisaldehyde stain (a 2.5% solution of p-anisaldehyde in 95:4:1 95% ethanol-concentrated sulfuric-glacial acetic acid). Flash chromatography refers to the method of Still and co-workers (31), and was conducted on Davisil Grade 643 Type 150A 230-425 mesh silica gel (Fisher).

When necessary, all solvents and reagents were purified prior to use (32). THF was distilled under nitrogen atmosphere from sodium/benzophenone. Pyridine was distilled over calcium hydride. Air and/or water sensitive techniques were conducted under a positive nitrogen atmosphere. The borane-THF [14044-65-6] was purchased from Acros Organics; 1-proline [147-85-3], carbon tetrabromide [558-13-4], and trimethyl phosphite [121-45-9] were purchased from Aldrich Chemical Company; and (S)-N-(benzyloxy carbonyl) proline [1148-11-4] was prepared using the method of Asami et al. (25). The (S)-N-(benzyloxy carbonyl) proline was recrystallized from warm ethyl acetate by the addition of hexane and dried thoroughly under high vacuum.
(S)-N-(benzyloxycarbonyl)-2-hydroxymethyl-pyrrolidine. 5.

(S)-N-(benzyloxycarbonyl) proline (1.00 g, 4.0 mmol) was placed in a dry round-bottom flask equipped with a magnetic stirring bar and a rubber septum. After addition of THF (20 mL) the resulting solution was placed under a positive nitrogen atmosphere and cooled to 0°C and borane-THF (1 M, 11.0 mmol, 11.0 mL) was added to it in a dropwise manner. Upon completion of the addition of borane-THF, the reaction mixture was allowed to warm to room temperature. During that time, the progress of the reaction was monitored via TLC. When TLC indicated that all the starting material was consumed, the reaction mixture was partitioned between 50 mL diethyl ether and 50 mL deionized water. The aqueous layer was extracted once more with 50 mL diethyl ether. The combined organic layers then were extracted with a saturated sodium bicarbonate solution to remove any excess acid that might be present. The organic layer then was extracted with brine, dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure to a clear, pale yellow oil which was purified by flash chromatography to give 0.77g (81.9%) of the desired product as an oil (Rf 0.4 with ethyl acetate). H NMR: δ 1.53-2.04 (m, 4H), 3.32-3.67 (m, 4H), 3.92-4.04 (m, 1H), 5.11 (s, 2H), 7.27-7.40 (m, 5H). FTIR: 3420, 1680 cm⁻¹. Phosphoric acid. (S)-N-(benzyloxycarbonyl)-2-hydroxymethyl-pyrrolidinyl dimethyl ester, 8.

(S)-N-(benzyloxycarbonyl)-2-hydroxymethyl-pyrrolidine (0.41 g, 1.7 mmol) and carbon tetrabromide (0.64 g, 1.9 mmol) were added to a dry round-bottom flask equipped with a magnetic stirring bar and a rubber septum. After addition of pyridine (1.0 mL) the resulting solution was placed under a positive nitrogen atmosphere and cooled to 0°C and trimethyl phosphite (0.30 mL, 2.5 mmol) was added to it in a dropwise manner. Upon completion of the addition of trimethyl phosphite,
the solution was allowed to warm to room temperature. During that time, the progress of the reaction was monitored via TLC. After 20 hours the reaction mixture was transferred into a separatory funnel, diluted with ether (10 mL), and then washed with 5% HCl (2 x 3 mL), saturated sodium bicarbonate (1 x 3 mL), and brine (1 x 3 mL). Each of the aqueous layers was collected in a separate flask, and any remaining traces of product were individually extracted back into the organic phase using ether (3 x 10 mL). The combined organic extracts were dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure to a brown oil which was purified by flash chromatography to give 0.03g (4.9%) of the desired product and unreacted alcohol as a yellow oil (Rf 0.5 with ethyl acetate). $^1$H NMR: $\delta$ 1.44-2.19 (m, 4H), 3.33-3.72 (m, 4H), 3.86-4.41 (m, 1H), 5.13 (s, 1H), 7.22-7.50 (m, 5H). FTIR: 1740, 1280 cm$^{-1}$. 
Acetylcholine

References

Acetylcholine

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27. J. A. Frick, unpublished work.
Appendix I:

Figures and Schemes
FIGURE 1
Acetylcholine and the Proposed Novel Analog
FIGURE 2

The ACh Cycle at the Neuromuscular Junction

A schematic representation of some of the events involved in ACh synthesis, release, and action at a prototypic synapse, the neuromuscular junction. Part of the nerve terminal is shown lying in close apposition to a muscle end-plate. Synthesis of ACh occurs locally, in the presynaptic terminal, from acetyl-CoA and choline (a). ACh is then incorporated into membrane-bound synaptic vesicles (b). Release of ACh occurs by exocytosis, which involves fusion of the vesicles with the presynaptic membrane (c). This process is triggered by an influx of Ca^{2+} that occurs in response to propagation of the action potential into the presynaptic axons. The contents of approximately 200 synaptic vesicles are released into the synaptic cleft in response to a single action potential. The released ACh diffuses rapidly across the synaptic cleft (d) and binds to postsynaptic ACh receptors where it triggers a conformational change that leads to an influx of Na^+ ions that depolarizes the membrane. When the channel closes, the ACh dissociates and is hydrolyzed by AChE (e). The choline moiety then is reabsorbed by the presynaptic neuron (f), and the cycle starts again.
FIGURE 3
A Schematic View of the AChE Active Site
Acetylcholine

SCHEME 1
The Mechanism of AChE Hydrolysis

Acylation

Deacylation

Tetrahedral Intermediate

Acylenzyme

Tetrahedral Intermediate

RCO₂⁻ + H⁺
FIGURE 4
Examples of Organophosphorus Inhibitors of AChE

2,5-dichlorophenyl methyl phenylphosphonate

Isopropyl methylphosphonofluoridate (sarin)

O,O-diethyl O-p-nitrophenyl thiophosphate (parathion)
SCHEME 2

Phosphorylation of AChE

Acetylcholinesterase

"REACTIVATION"

"AGING"

Acetylcholine
SCHEME 3
A Synthetic Overview

\[ 3 \xrightarrow{\text{CBZ-Cl, NaOH, H}_2\text{O} \ 5^\circ \text{C}} 4 \xrightarrow{\text{BH}_3\cdot\text{THF} \ \text{THF} \ 0^\circ \text{C}} 5 \]

\[ 5 \xrightarrow{\text{H}_2, \text{Pd}} 6 \]

\[ 6 \xrightarrow{2 \text{CH}_3\text{I}} 2 \]
SCHEME 4
The "Flether et al." Method of Phosphorylation

\[
\begin{align*}
\text{5} \quad \text{Na} & \quad \rightarrow \\
\text{5} \quad \text{Na}^+ & \quad + \quad \frac{1}{2} \text{H}_2
\end{align*}
\]

\[
\begin{align*}
\text{6} \quad \text{CH}_3\text{OPCl}_2 & \quad \rightarrow \\
\text{6} \quad \text{Cl} & \quad + \quad \text{OCH}_3
\end{align*}
\]

\[
\begin{align*}
\text{7} \quad \text{CH}_3\text{MgBr} & \quad \rightarrow \\
\text{7} \quad \text{CH}_3 & \quad + \quad \text{OCH}_3
\end{align*}
\]
SCHEME 5

The "Bannworth-Trzeciak" Method of Phosphorylation

\[ \text{CH}_3\text{OH} + \text{PCl}_3 \rightarrow \text{CH}_3\text{OPCl}_2 \rightarrow \text{CH}_3\text{OPCl}_2 + 2 \text{HN} \rightarrow \text{CH}_3\text{OPCl}_2 + \text{N} \rightarrow \text{CBZ} 5 \]

\[ \text{CH}_3\text{OH} \rightarrow \text{N}-\text{P} \rightarrow \text{OCH}_3 \rightarrow \text{N}-\text{P} \rightarrow \text{OCH}_3 \]

\[ \text{CBZ} \]

\[ \text{O}_2 \rightarrow \text{CBZ} \]

\[ \text{8} \]
SCHEME 6
The "Oza-Corcoran" Method of Phosphorylation

\[
\begin{align*}
\text{5} & \quad \xrightarrow{(\text{CH}_3\text{O})_3\text{P}/ \text{CBr}_4 \text{ pyridine, } 0^\circ\text{C}} \quad \text{8} \\
& \quad + \quad \text{HCB}_3 + \text{CH}_3\text{Br}
\end{align*}
\]
Appendix II:
IR and NMR Spectra
Spectrum 1
An IR Spectrum of 99+% Pure Acetylcholine
Spectrum 2
An IR Spectrum of Impure 5
Acetylcholine

Spectrum 3
An IR Spectrum of Purified 5

![Graph of IR Spectrum](image)
Spectrum 4
A Closer Inspection of the Carbonyl Peak in Spectrum 3
Acetylcholine

Spectrum 5

An IR Spectrum of Impure 8

![IR Spectrum of Impure 8](image_url)
Spectrum 6
An IR Spectrum of Purified 8
Spectrum 7
An NMR Spectrum of Purified 5

Acetylcholine
Spectrum 8

An NMR Spectrum of Purified 8

Acetylcholine

\[ 1H \]