

AD-A157 126

R+D-2876-BC

2

THE USE OF SUGAR-OXIMES AND OTHER GLYCOSYLATED DRUGS IN TREATMENT AGAINST ORGANOPHOSPHATE POISONING

Final Report (June 1985)

Written by: E.S. Rachaman, Ph.D. Principal Investigator
E. Heldman, Ph.D. Principal Investigator

United States Army

Research & Standardization Group (Europe)

London, England

Contract No. DAJA 37-80-C-0187

Amendment No. P00001

Israel Institute for Biological Research

DTIC ELECTE
JUL 26 1985
S D E

DTIC FILE COPY

This document has been approved for public release and sale; its

85 7 15 071

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) The Use of Sugar-Oximes and Other Glycosylated Drugs in Treatment Against Organophosphate Poisoning		5. TYPE OF REPORT & PERIOD COVERED Final Technical Report 24 June 80 - 30 June 85
7. AUTHOR(s) E.S. Rachaman, Ph.D. Principal Investigator E. Heldman, Ph.D. Principal Investigator		6. PERFORMING ORG. REPORT NUMBER
9. PERFORMING ORGANIZATION NAME AND ADDRESS Israel Institute of Biological Research P.O. Box 19, Ness-Ziona, Israel.		8. CONTRACT OR GRANT NUMBER(s) DAJA37-80-C-0187
11. CONTROLLING OFFICE NAME AND ADDRESS USARDSG-UK Box 65, FPO NY 09510-1500		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 61102A 1T161102BH57-02
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)		12. REPORT DATE 30 June 1985
		13. NUMBER OF PAGES 29
		15. SECURITY CLASS. (of this report) unclassified
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE
16. DISTRIBUTION STATEMENT (of this Report) Approved for public release. Distribution unlimited.		
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)		
18. SUPPLEMENTARY NOTES		
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) Pyridinium aldoximes; Organophosphate poisoning; Sugar-Oximes; Acetylcholinesterase; Antidotes; Glycosylated Drugs; Structure- activity Relationship.		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) During the course of our work, mono- and bis-sugar oximes as well as glycosylated carbanates were synthesized, analyzed by various spectroscopic techniques and evaluated pharmacologically. We found that the clearance rate of the sugar conjugates was significantly slower and their toxicity was pronouncely lower than that of their parents' compounds. These two properties endow the sugar		

unclassified

SECURITY CLASSIFICATION OF THIS PAGE(When Data Entered)

20.) cont.
oximes with improved prophylactic potential as compared to their non-sugar analogues. Moreover, in some cases the sugar oximes exhibited better antidotal properties than their non-conjugated aldoximes.

Investigations into the mechanisms underlying the improved properties of the sugar oximes revealed the following:

- a) OH-free sugar form is important for the antidotal activity.
- b) Propyl bridge between the sugar and the pyridine ring appears to be superior to a direct linkage between the two moieties.
- c) Glucose seems to be more efficient than galactose.
- d) The glucose transport system does not seem to recognize the sugar oximes. Therefore, sites other than the glucose transporter should be considered as those responsible for their improved pharmacokinetics.

These results support our hypothesis that improvement in prophylactic and therapeutic features of drugs, can be achieved by conjugating a sugar moiety to the drug in question. However, the mechanism responsible for these phenomena is not yet clear. Therefore, in this final report we propose some future strategies which may contribute to our understanding of what is responsible for the improvement in the pharmacokinetic properties which characterize these drugs. This information may provide a rational way to design drugs which possess a greater prophylactic and antidotal potential.

unclassified

SECURITY CLASSIFICATION OF THIS PAGE(When Data Entered)

THE USE OF SUGAR-OXIMES AND OTHER GLYCOSYLATED DRUGS IN
TREATMENT AGAINST ORGANOPHOSPHATE POISONING

Final Report (June 1985)

Written by: E.S. Rachaman, Ph.D. Principal Investigator
E. Heldman, Ph.D. Principal Investigator

Accession For	
NTIS GRA&I	<input checked="" type="checkbox"/>
DTIC TAB	<input type="checkbox"/>
Unannounced	<input type="checkbox"/>
Justification	
By _____	
Distribution/ _____	
Availability Codes	
Dist	Avail and/or Special
A-1	

United States Army
Research & Standardization Group (Europe)
London, England
Contract No. DAJA 37-80-C-0187
Amendment No. P00001
Israel Institute for Biological Research.



In addition to the principal investigators, the following personnel contributed to one or more parts described in this report:

- Y. Ashani - Department of Organic Chemistry
- L. Raveh - Department of Pharmacology
- A. Vincze - Department of Analytical Chemistry
- D. Waysbort - Department of Physical Chemistry
- R. Sahar - Technical assistance

The authors wish to thank Mrs Z. Pittel for her valuable help during the preparation of this report, Drs. G. Amitai, A. Fisher and B. Green for useful discussion and suggestions and last but not least Mrs. D. Wallach for devoted secretarial assistance.

TABLE OF CONTENTS

	<u>Page</u>
1. Abstract.....	1
2. Introduction.....	2
3. Materials and methods.....	3
3.1. Chemical synthesis.....	3
3.1.1 Synthesis of <u>E-357</u>	3
3.1.2 Synthesis of <u>E-360</u>	3
3.1.3 Synthesis of <u>E-343a</u>	4
3.1.4 Synthesis of <u>E-345a</u>	4
3.2 Purification procedures.....	5
3.3 Spectrometric analysis.....	5
3.3.1 Positive Secondary Ion Mass Spectra (SIMS)....	5
3.3.2 NMR spectra.....	6
3.4. Biological evaluation.....	6
3.4.1 Animals.....	6
3.4.2 Toxicity determination.....	6
3.4.3 Pharmacokinetics.....	7
3.4.4 Glucose transport.....	7
3.4.5 Protection experiments.....	7
4. Results.....	8
4.1. Chemical synthesis and spectroscopic analysis.....	8
4.1.1. N-Pyridinium aldoximes and carbamates conjugated through C-6 of the sugar moiety... 8	8

4.1.2.	Synthesis of glucosyloxy propyl derivatives of 2'-PAM and pyridostigmine.....	9
4.1.3.	Synthesis of a glycosylated-TMB4 derivative..	10
4.1.4.	Mass spectral studies of E-290b.....	11
4.1.5.	NMR of pyridine aldoximes and their sugar derivatives.....	12
4.2.	Biological and Pharmacological evaluation.....	13
4.2.1.	Pharmacokinetics of sugar oximes.....	13
4.2.2.	Competition of sugar oximes with glucose for the glucose transporter.....	14
4.2.3.	Comparative therapeutic indexes and protec- tive ratios for 4-PAM and its sugar derivatives.....	15
4.2.4.	Experiments with a sugar derivative of TMB4..	16
5.	Discussion.....	18
6.	Research strategies for the future.....	20
6.1.	Synthesis of new compounds for structure- activity relationships.....	20
6.1.1.	Mono-oximes.....	20
6.1.2.	Bis-oximes.....	21
6.2.	Glycosylated derivatives of atropine.....	24
6.3.	Preparation of radioactively labelled derivatives....	25
7.	References.....	27
8.	Structural formulas.....	29

1. ABSTRACT

During the course of our work, mono-, and bis-sugar oximes as well as glycosylated carbamates were synthesized, analyzed by various spectroscopic techniques and evaluated pharmacologically.

We found that the clearance rate of the sugar conjugates was significantly slower and their toxicity was pronouncely lower than that of their parents' compounds. These two properties endow the sugar oximes with improved prophylactic potential as compared to their non-sugar analogues. Moreover, in some cases the sugar oximes exhibited better antidotal properties than their non-cojugated aldoximes.

Investigations into the mechanisms underlying the improved properties of the sugar oximes revealed the following:

- ~~a~~ OH-free sugar form is important for the antidotal activity;
- ~~b~~ Propyl bridge between the sugar and the pyridine ring appears to be superior to a direct linkage between the two moieties;
- ~~c~~ Glucose seems to be more efficient than galactose;
- ~~d~~ The glucose transport system does not seem to recognize the sugar oximes. Therefore, sites other than the glucose transporter should be considered as those responsible for their improved pharmacokinetics. *Originator Supplied keywords include:*

These results support our hypothesis that improvement in prophylactic and therapeutic features of drugs, can be achieved by conjugating a sugar moiety to the drug in question. However, the mechanism responsible for these phenomena is not yet clear. Therefore in this final report we propose some future strategies which may contribute to our undrestanding of what is responsible for the improvement in the pharmacokinetic properties which characterize these drugs. This information may provide a rational way to design drugs which possess a greater prophylactic and antidotal potential.

Key words

~~Pyridinium~~ aldoximes; Organophosphate poisoning; Sugar-oximes; Acetylcholinesterase; Antidotes; Glycosylated drugs; Structure-activity relationship

2. INTRODUCTION

A common therapy against organophosphorus poisoning is the use of a combination of atropine and a quarternary oxime. Atropine is used for its antimuscarinic action, by which it reduces the overstimulation of the muscarinic receptors, occurred during poisoning, by an excess of acetylcholine accumulating at the synaptic terminals. Oximes are used for their ability to displace the phosphoryl group on the active site of acetylcholinesterase (AChE) and thus reactivate the inhibited enzyme.

However, despite the similarity in the reactivation potential of some oximes, they may differ significantly in their antidotal properties. For example, 2-PAM and TMB4 possess similar reactivation values for Tabun-phosphorylated enzyme, but TMB4 is far more effective as an antidote against Tabun poisoning. This means that the reactivation constant in vitro, is not necessarily the important factor in determining the effectiveness of a therapeutic drug. Properties such as bioavailability, pharmacokinetics and relative toxicity might be important at least as much as the reactivation potency.

In order to improve these important features in the currently available antidotes, we designed and synthesized conjugates of pyridinium aldoximes and sugars. The sugar moiety may make the non-permeable quarternary oximes more permeable through biological membranes, as compared to non-sugar analogues. Improved permeability through biological membranes might make the sugar analogues more available at the critical target sites, where they are needed.

Various glucosyl derivatives which have already been synthesized in our laboratory met our expectations and exhibited improved pharmacokinetics as well as better antidotal characteristics (1, 2). In this report we summarize and discuss some of the important results which we obtained previously. We also include new results which were obtained with a glycosylated bis-oxime and we elaborate on possible mechanisms of action of the sugar oximes. Accordingly, we suggest future strategies for developing new glycosylated drugs which are related to treatment of OP poisoning.

3. MATERIALS AND METHODS

3.1. Chemical synthesis

3.1.1 Synthesis of N-[2-O(β -D-tetra-O-acetyl glucosyl)-propane]4'-bis-pyridinium aldoxime chloride (E-357):
Three grams (0.0054 moles) of compound 5 (1) and 4-pyridine aldoxime (5g, 0.04 mole) in a mixture of 2-methoxy ethanol (15 ml) and benzene (10 ml) were distilled under atmospheric pressure until most of the benzene was removed. In another flask a solution of sodium iodide (4g, 0.026 mole) in 2-methoxy ethanol (15 ml) and benzene (10 ml) was treated similarly in order to obtain dry reagents for quarternization. The two solutions were then combined and introduced into an ampule which was immediately sealed and heated to 120°C (external temperature) for 5 days.

Following the procedure mentioned above, the methoxy-ethanol was removed under reduced pressure. The residue was extracted with 10% methanol in chloroform and subjected to a purification process on a column of silica gel (100g). The crude quarternized products were eluted from the column when the polarity of chloroform-methanol mixture exceeded 25% of methanol. The iodide ion was exchanged with chloride by passing the crude product on a Dowex 1 anion exchange resin (30g). The aqueous fractions which were eluted from the resin, contained various mixtures of mono-, and bis-quarternized sugar oximes. However, one fraction that was crystallized from a mixture of ethanol and ether did indicate the presence of practically pure bis-oxime E-357, according to NMR and TLC data. (The product turned out to be a very hygroscopic material).

Cpd. E-357 ¹H nmr: (D₂O-DSS) 1.9-2.1 (12H, 4Ac); 2.6-4.0 (m, H₂ of the pyranose ring and CH of the propane chain); 4.3-5.2 (6H of the pyranose ring, CH₂-N⁺); 8.2-9.1 (m, 8H_{Ar}); 8.4 (s, CH=N-);

3.1.2. Synthesis of N-[2-O-(β -D-glucosyl)-propane]-4'-bis pyridinium aldoxime chloride (E-360):

The glycoside ester E-357 (600 mg) was dissolved in methanol and treated with several drops of 10% NaOH solution to adjust the pH to about 10.5. After 24 hours at room temperature the mixture was neutralized with acidic resin (Dowex 50 H⁺). The filtrate obtained after the removal of the resin was concentrated in vacuo. The residue was crystallized from a mixture of methanol-isopropanol leading to an amorphous hygroscopic material.

Cpd. E-360 ¹H nmr: (D₂O-DSS) 3.05-3.25 (m, H_{6A}, H_{6B}, H₅);
3.3 (m, CH); 3.5 (m, H₂, H₃ & H₄);
3.8 (d, H_p); 4.9-5.2 (m, CH₂-N⁺);
8.2-9.1 (m, 8H_{ar}); 8.45 (s, CH=N-);

UV: λ_{max} 0.1N-NaOH, 345 nm; [α]_D²⁰ = +2.7° (c 0.8, MeOH).

3.1.3. Synthesis of N-[6-(1,2:3,4-Di-O-isopropylidene)-α-D-galactopyranose]-3'-dimethyl carbamoyl pyridinium chloride (E-343a):

Compound 1 (8.2g, 0.02 mole) and 3-dimethyl carbamoyl pyridine (3.3g, 0.02 mole) in dichloroethane (40 ml) were stirred at room temperature for 48 hours. The reaction mixture was evaporated under reduced vacuum and the residue was purified on a silica gel column. Unreacted carbamate was eluted with chloroform.

The quarternized product E-343a was eluted by 10% methanol in chloroform. The crude yield was 6g (52%). Crystallization from a mixture of methanol-ether led to a crystalline material m.p. 187°.

Anal. Calcd. for C₂₆H₂₃N₂O₇.CF₃SO₃⁻: C, 45.17 H, 5.23

Found: C, 45.38 H, 5.34

¹H nmr: (CDCl₃-TMS) 1.23, 1.32, 1.4 (3s, Me groups);
3.08, 3.18 (2s, N(Me)₂ groups); 4.0-5.2 (6H of
the pyranose ring); 5.45 (d, 1H, H_p) 7.9-8.5 (m,
2 H_{ar}).

3.1.4. Synthesis of N-[3-O-(tetra-O-acetyl β-D glucosyl)-propane-1]-3-dimethyl carbamoyl pyridinium chloride (E-345a):

A solution of sodium iodide (10g) in 2-methoxy ethanol (40 ml) and benzene (20 ml) was distilled under atmospheric pressure. In another flask a mixture of 4 (4g) and 3-dimethyl carbamoyl pyridine (8.4g) was distilled similarly in order to obtain dry reagents for the quarternization. The two solutions were then combined and stirring continued for 48 hours at 110°C (external temperature).

The methoxy-ethanol was removed under reduced pressure and the residue was purified on a column of silica gel. The crude quarternized product was eluted with a mixture that contained at least 5% methanol in chloroform. The iodide anion was exchanged with chloride by passing the crude product on a Dowex 1 anion exchange resin. The aqueous eluate was distilled in a rotary evaporator. NMR analysis and TLC data provided supporting evidence that our product was structurally identical to E-345a. The compound was obtained as a syrup and did not crystallize.

¹H nmr: (CDCl₃-TMS), 1.95-2.1 (12H, 4Ac) 2.3 (m, CH₂); 3.05, 3.15 (2s, N(Me)₂); 3.23-5 (6H of the pyranose ring and 4H of 2 CH₂ groups); 5.1 (d, 1H_β); 8-8.4 (m, 2H_{αr}); 9-9.3 (m, 2H_{αr}).

3.2. Purification procedures: Thin-layer chromatograms were performed on a silica gel G (using 9:1 or 4:1 CHCl₃:MeOH) and alumina (using mixtures of benzene, chloroform and ethyl acetate). The spots were visualized with iodine vapors and subsequently with 5% H₂SO₄ in ethanol solution. Column chromatograms were prepared from a silica gel 40, 70-230 mesh ASTM (E. Merck). All solvents were flash evaporated using an evaporator.

UV spectra were recorded on a Bausch and Lomb Spectronic 505 spectrophotometer. Optical rotations were determined with a Perkin Elmer model 141 polarimeter. Melting points were determined with Mettler FP5.

3.3 Spectrometric analysis

3.3.1. Positive Secondary Ion Mass Spectra (SIMS): Results were obtained with a Riber quadrupole mass spectrometer incorporating an energy prefilter prior to mass separation. A 5-KeV argon ion beam at a maximal current of 5x10⁻⁸ A cm⁻² or below was used to bombard the surface at a 45° angle. Secondary ions are extracted, normal to the surface, by a small voltage of around 100 V/cm. Ions pass through the energy and mass filters to an electron multiplier coupled to a pulse-counting detection system. The quadrupole was scanned at a rate of 3 s/amu, and the scan as well as the analog rate meter output voltages, were fed to an X-Y recorder. Operating pressure in the main chamber of the spectrometer was held at minimum vacuo of 1x10⁻⁸ torr.

Samples were prepared by burnishing the solid sample directly onto silver or copper foil supports (0.0125 cm thick) which had been abrasively cleaned. For high sensitivity studies, fresh solutions of known concentration were prepared, and a measured amount was placed onto the planchet with a syringe. Samples were introduced through a preparation chamber into the main analysis chamber. After analysis, the samples were retrieved and retained for future re-examination.

3.3.2. NMR Spectra: ^1H -nmr was recorded on Jeol C-60HL in deuterated solvent. ^{13}C nmr spectra were recorded at 25.2 MHz in 10 mm o.d. tubes on a Varian XL-100-FT spectrometer. The probe temperature was 26°C. The solvent D_2O was also used for locking the spectrometer. The proton-decoupled spectra were obtained by employing three methods:

(a) Irradiation in a noise band of 3KHz in order to cancel completely the coupling of protons with the carbon nuclei;

(b) Off resonance irradiation at a certain specified frequency;

(c) Gated-decoupling, where the decoupler was off during the acquisition period and on for the time delay between each pulse, chosen so as to retain the NOE;

The ^1H decoupled ^{13}C FT nmr spectra were measured under the following conditions: spectral width, 5000 Hz; acquisition time, 0.4 or 0.8s; pulse width, 18 μs ; pulse delay, 0.3s; sensitivity enhancement, 1.0A 10W H noise decoupling with 3 KHz band width, was used for the decoupling spectra. The gated decoupled spectra were recorded with the same pulse width but with a pulse delay of 2.0s. The chemical shifts are accurate to ± 0.2 ppm and the coupling constants are given with an accuracy of ± 5 Hz.

3.4. Biological evaluation

3.4.1. Animals: ICR (albino) male mice were maintained in a temperature-regulated (22°C-25°C) room and used when their body weight reached 22-25g.

3.4.2. Toxicity determination: Mice were injected subcutaneously or intramuscularly as specified in the results section with increasing doses of the tested drug. The injected mice were observed for signs of poisoning and death, up to 24 hours following the injection. LD_{50} was calculated according to Weil (3).

- 3.4.3. Pharmacokinetics: Mice were injected intramuscularly with the tested oxime. At various periods after the injection, the mice (at least four mice for each time point) were bled and oxime concentration in the serum was determined as previously described (4).
- 3.4.4. Glucose transport: Retinal tissue was quickly removed from mice, chopped to approximately 200 microns thick slices and kept in a phosphate-buffered saline (PBS) under stream of oxygen at 37°C. ¹⁴C-2-deoxy glucose (45mCi/mmole, 0.1 µCi/tube) was then added to an aliquot of the retina slices with or without the tested sugar oxime. The retina slices were incubated for additional 10 minutes, washed on millipore filter with PBS and the radioactivity which was retained on the filter was counted. For background counts, similar quantities of retina slices, were incubated with ¹⁴C-2-deoxy-glucose at 2°C.
- 3.4.5. Protection experiments: Mice were challenged subcutaneously in the neck region with either VX or soman. One minute following the poisoning, or upon appearance of poisoning signs (which ever came first), the mice were treated with a mixture of an oxime and atropine (15 mg/Kg of each unless otherwise specified), by intramuscular injections into the thigh region. LD₅₀ was calculated with and without protective treatment and the degree of protection was expressed in terms of protective ratio (PR).

$$PR = \frac{LD_{50} \text{ with protective treatment}}{LD_{50} \text{ without protective treatment}}$$

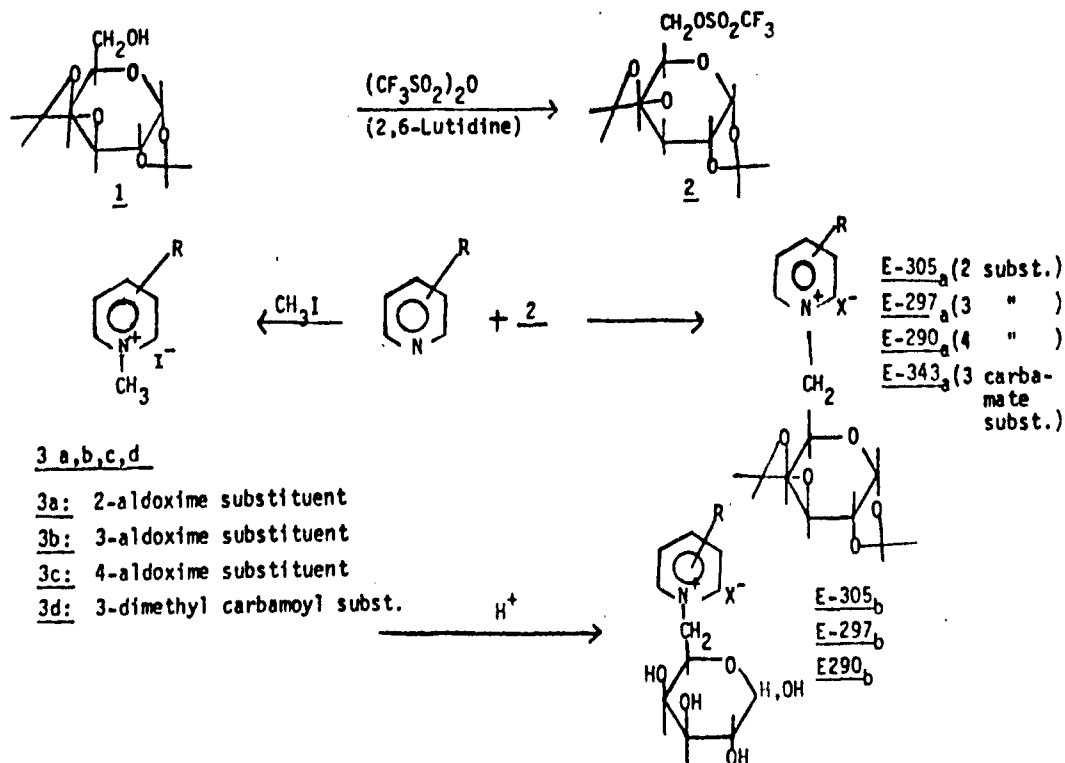
4. RESULTS

4.1. Chemical synthesis and spectroscopic analysis:

4.1.1. N-pyridinium aldoximes and carbamates conjugated through C-6 of the sugar moiety: In past annual reports we summarized the routes for obtaining sugar oximes in which the sugar residue is linked to the pyridinium ring via C-1 of the sugar moiety (2, 5). Another option to attach sugar residue to the aldoxime backbone is through the active C-6 position. This should yield sugar oximes with free anomeric position, in which the sugar residue might be more easily identified by the cellular sites that interact with sugars.

As a model study we started with the easily available diisopropylidene galactopyranose. By inserting triflyl ester at position 6 of galactose, we obtained an excellent starting agent for quarternizing any pyridine aldoxime derivative. Thus we synthesized 2', 3' and 4' galactose-PAM analogues as well as sugar pyridostigmine derivative as follows:

Scheme A

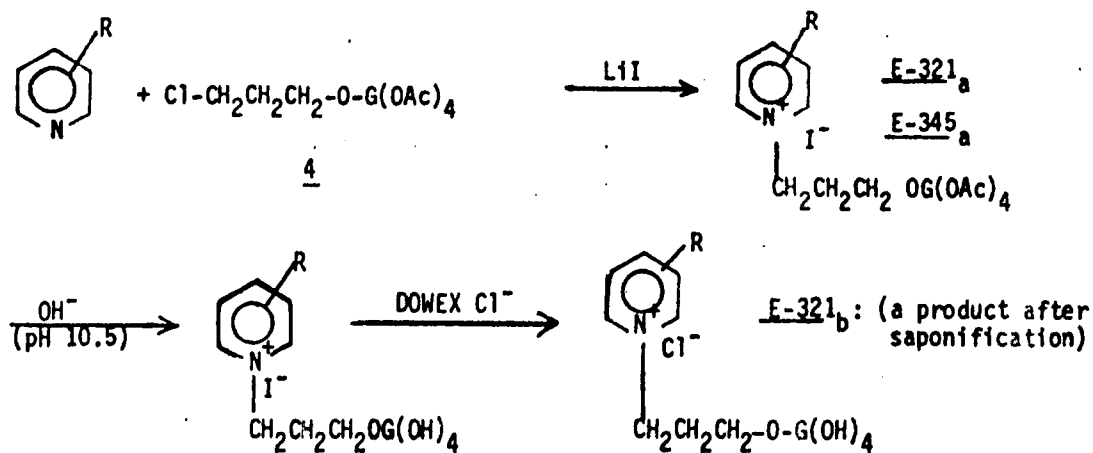


Using this technique we were also able to synthesize a first sugar-carbamate by just substituting the aldoxime function with dimethyl-carbamoyl residue.

4.1.2. Synthesis of glucosyloxy propyl derivatives of 2'-PAM and pyridostigmine:

For preparing the 2'-PAM glucosyloxypropyl analogue we employed iodopropyl β -D-glucosyl tetraacetate. On the other hand for preparing the 3' and 4' PAM analogues in this series as well as for the preparation of the pyridostigmine sugar analogue, we could accomplish the synthesis even with the less reactive agent- chloropropyl β -D-glucosyl tetraacetate.

Scheme B



E-321 : R=2-aldoxime substituent.

E-345 : R=3-dimethyl carbamoyl subst.

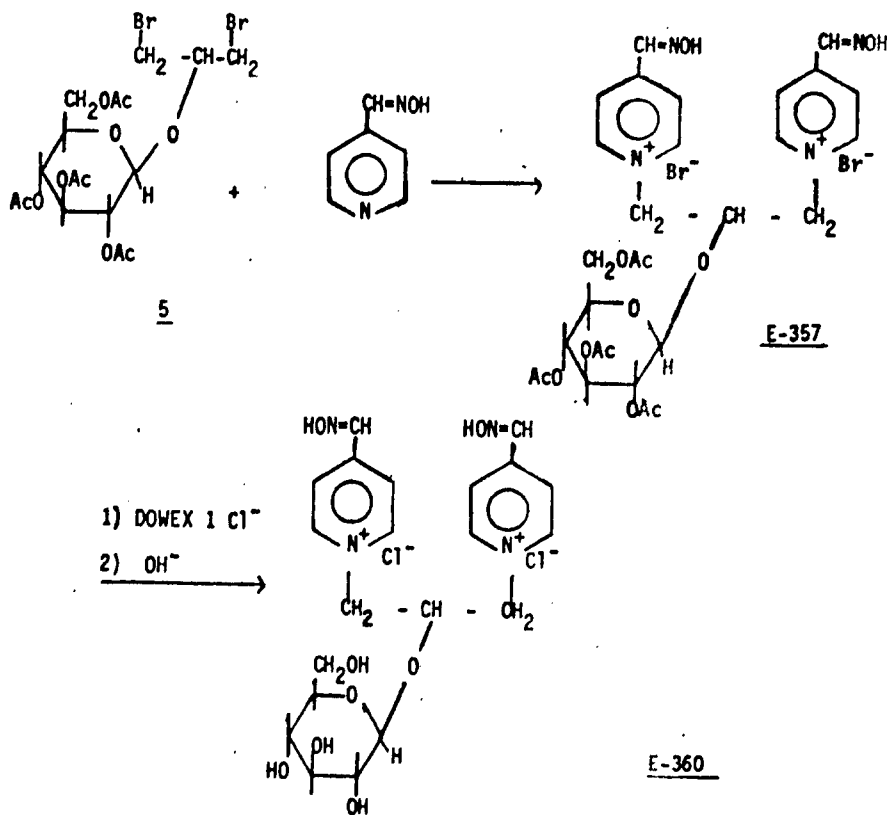
G(OAc)₄ : Glucosyl tetraacetate .

It is of interest to note that during the synthesis of the various sugar oximes, we observed that the new series of oximes displayed a relative higher stability in an alkaline medium than the corresponding methiodides (6, 7, 8). Thus, we were able to purify compounds E-212, E-308 and E-321 in their ester form, by using column chromatography and less polar mixtures of eluents.

4.1.3. Synthesis of a glycosylated TMB4 derivative:

In analogy to the synthetic procedures which led to the formation of TMB4 or Toxogonin we have looked into the possibility of making a sugar bis-oxime of type E-360 by the route described in scheme C .

Scheme C



It turned out that in this case, unlike the non-sugar bis-oxime (9), the yield of the bis-oxime sugar conjugate E-357 was relatively low (20%). Subsequent saponification (aqueous methanol, pH 10.5) of E-357 was performed to yield E-360.

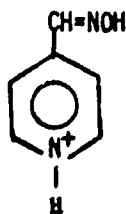
4.1.4. Mass spectral studies of E-290b:

Compound E-290b was studied by a relatively new technique known as "Organic Secondary Ion Mass Spectroscopy" (SIMS), which is useful for organic salts and onium compounds (10, 11).

In order to obtain additional structural conformation for the newly synthesized conjugates we employed mass-spectral analysis of the oximes. To date we performed complete analysis of 1'-(2-D-galactopyranose-6-yl) - 4'-hydroxyimino methyl pyridinium chloride (compound E-290b). We also currently analyze the bisoxime derivative E-360.

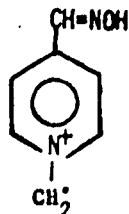
The intact cation is observed at m/z 285 along with fragment ions at m/z 105, 123, 136, 147, 149 and 165. The ion at 105 has resulted from dehydration of the ion at m/z 123 (see a bellow) which itself results from loss of the sugar unit with a concomittant H transfer to the pyridine ring.

a



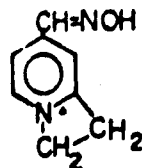
The ion at m/z 136 probably has the structure represented by (b)

b



Which resulted from a simple cleavage of E-290b

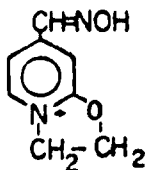
c



The structure of (c) may account for the presence of an ion at m/z 149. Following loss of 2H from ion (c), a new fragment at m/z 147 is formed.

The ion at m/z 165 is particularly interesting and is assumed to possess the structure represented by (d), in which both carbon and oxygen from the sugar are incorporated into the cyclic pyridinium structure.

d



Analogous fragmentations resulting in a cleavage of the sugar in the nucleosides have been described (12, 13).

This study indicates that even a quaternary compound such as E-290b and other sugar analogues can be analyzed accurately either by SIMS technique or possibly by the FAB technique. These techniques will be valuable for future studies.

4.1.5. NMR of Pyridine Aldoximes and their sugar Derivatives

Another powerful technique for elucidating a structural configuration is nmr. During the course of our studies we have employed ¹³C-nmr and ¹H-nmr. With ¹³C-nmr, in addition to the chemical shifts, we were also able to obtain important information concerning the preferable conformers which existed in the tested samples. This additional data will provide us with a useful information for the study of structure-activity relationships.

The assignment of the absorption peaks in the ¹³C nmr spectra is based primarily on the comparison between the ¹³C spectra of the methiodides (Scheme A, 3a - 3c) with the spectra of some sugar-oximes (E-305a, E-297a & E-290a). The chemical shifts (δ) and the coupling constants (¹J(CH)) for the starting material, 1, and for the sugar oximes mentioned above, were previously reported (2).

In conclusion, the carbons in the sugar residue were not significantly altered in their proton coupling constants, indicating that the sugar residue maintained its basic configuration within the conjugate. Therefore it should be expected that the recognition of the sugar moiety, by the sugar recognition sites (within biological tissues), would not be altered either. However, we found

changes in the chemical shifts, indicating that the electronic environment within the pyridine ring was influenced by the coupling between the sugar moiety and the oxime. Nevertheless, these changes should not affect the sugar conformation and therefore should not change the affinity of the sugar moiety to the sugar recognition sites.

The results of the ^{13}C -nmr spectrum of the pyridine ring of N-glucosyloxypropyl-2-pyridinium aldoxime chloride were as follows (in ppm relative to TMS):

C-2' = 147.9; C-3' = 127.4; C-4' = 142.7
C-5' = 128.3; C-6' = 146.6; C-7' = 146.1

These values are closer to those of 3a than the values found for E-305a (2). Such results are expected from the inductive electron donating property of the methylene groups where their larger number overtakes their decreased efficiency of electron donation, compared with the hyperconjugative donation of the N-methyl group. Thus, it may be concluded that the paramagnetic shifts of the carbons of the pyridine residue in E-305a, E-297a, & E-290a, compared to those in 3a - 3c, are a result of the hyperconjugation of the N-methyl group existing in 3a - 3c. Therefore it might be expected that some changes in the functional properties (such as reactivation values) of the pyridine ring would take place as indeed we previously demonstrated (2).

4.2. BIOLOGICAL AND PHARMACOLOGICAL EVALUATIONS

4.2.1. Pharmacokinetics of sugar oximes: We examined two sugar oximes for their pharmacokinetic characteristics and compared them to their non-sugar analogues. Table 1 summarizes the $t_{1/2}$ and the maximal time that the tested oximes were detected in the blood circulation after intramuscular injection of the oximes into mice.

Table 1: pharmacokinetic parameters for 3' and 4'-aldoximes derivatives

<u>SUGAR OXIME (code)</u>	<u>t_{1/2} (min)</u>	<u>TIME (min.) OXIME WAS DETECTED IN CIRCULATION</u>
<u>4-PAM</u>	15	60
<u>E-212</u>	45	180
<u>3-PAM</u>	20	60
<u>E-168</u>	45	100

The data presented in table 2 clearly indicate that the sugar conjugates offer much better pharmacokinetic features than their non-sugar analogues.

4.2.2 Competition of sugar oximes with glucose for glucose-transporter: When slices of rat retina were incubated with ¹⁴C-2-deoxy-glucose, rapid accumulation of the labelled sugar within the tissue was observed. The uptake was temperature-dependent. Thus at 37°C significant amount of ¹⁴C-2-deoxy-glucose was taken up, while at 2°C very little incorporation of glucose was observed. When D-glucose was added to the reaction mixture, significant reduction in the temperature-dependent ¹⁴C-2-deoxy-glucose incorporation was observed (table 2). However, D-galactose affected only slightly the incorporation of the ¹⁴C-2-deoxy-glucose to the retina slices (table 2).

Table 2: Effect of glucose, galactose and glycosylated oximes on ¹⁴C-2-deoxy-glucose uptake into retina slices.

<u>Compound* added to the incubation medium</u>	<u>Incorporated ¹⁴C-2-deoxy-glucose CPM/ng/min ± SD</u>
none	581.0 ± 84.3
D-glucose	51.5 ± 42.6
D-galactose	504.0 ± 60.5
4-PAM	605.8 ± 23.5
<u>E-193b</u>	582.9 ± 84.7
<u>E-212</u>	630.3 ± 51.9

*The concentration of each tested compound was 10mM.

It seems therefore that the 2-deoxy-glucose transport system specifically recognizes D-glucose and transport it into the cell. We then examined if the sugar oximes can also be recognized by this glucose transporter.

As can be seen from table 2, all the tested sugar oximes, even when used in high concentrations, did not alter the incorporation of the ¹⁴C-2-deoxy-glucose into the retina slices. We concluded that the sugar oximes are not recognized by this glucose transporter and therefore did not compete with ¹⁴C-2-deoxy-glucose for the uptake system as does free glucose.

4.2.3. Comparative therapeutic indexes and protective ratios for 4-PAM and its sugar derivatives: In the last annual report (2) we described some toxicological values for various 4-PAM-sugar analogues. Evaluation of the optimal doses for each of the oxime, when used against VX poisoning, revealed that as a rule, there was no steep dose-dependent curve for any of the tested oximes. Therefore, it can be expected that each oxime with reduced toxicity would yield a higher therapeutic index. Indeed, it can be seen in table 3 that E-193b yielded significantly higher therapeutic index than its non sugar analogue. In addition, some of the sugar conjugates protected more efficiently against OP poisoning, as demonstrated for E-212, E-196, and E-290b. Table 3 also summarizes the toxicological values (in terms of LD₅₀'s) for the 4-aldoximes and some of its sugar derivatives against VX poisoning.

TABLE 3: Therapeutic indexes and protective ratios of 4-PAM and its sugar analogues

<u>OXIME</u> <u>(code)</u>	<u>TOXICITY</u> <u>(LD₅₀)</u>	<u>OPTIMAL DOSE</u> <u>(mg)</u>	<u>THERAPEUTIC</u> <u>INDEX</u>	<u>PROTECTIVE</u> <u>RATIO</u>
<u>4-PAM</u>	145.5	30	4.85	5.1
<u>E-193b</u>	478.5	16	29.90	4.6
<u>E-212</u>	434.9	90	4.83	23.0
<u>E-196</u>	-	22	-	14.3

When two derivatives of the same family, one with a direct linkage between the sugar residue and the pyridine backbone (E-196) and the other with a propyl bridge between the two moieties (E-212) were compared, we found that the propyl bridge provided an antidotal advantage in terms of protective ratio. This phenomenon was observed also among the family members of the 2'- and the 3'-substituted pyridine aldoximes (2). When the OH groups of the sugar moiety were acetylated, a significant reduction in the protective ratio was noticed (compare E-123b with E-212 in table 3).

Finally, glucose seemed to be superior to galactose (compare E-290L with E-196 in table 3). However, this last feature was expressed pronouncely, only in the case of 2'-and 4'- substituted pyridine aldoximes, and was less remarkable in the case of 3' pyridine aldoxime-sugar analogue. The comparizon between the monosacharide residues needs further investigations since the above observations were made with sugar oximes in which the galactose was attached to C-6, whereas glucose was linked through the anomeric position.

4.2.4 Experiments with a sugar derivative of TMB4: We compared the recently synthesized sugar bis-oxime conjugate, glucose-TMB4 (E-360), with its non-conjugated analogue for their ability to protect mice against VX and soman poisoning. Results of this study are presented in table 4.

Table 4: Protective ratios and toxicity of bis-oximes and a sugar derivative used against organophosphate poisoning in mice.

<u>poisoning</u> <u>by</u>	<u>pre-treatment</u> <u>*</u>	<u>treatment</u> <u>**</u>	<u>protective</u> <u>ratio ***</u>	<u>toxicity of</u> <u>corresponding</u> <u>oxime</u> <u>(mg/kg ± SD)</u>
GD	none	E-360+ atropine	1.8(1.4-2.3)	>300
"	pyrido- stigmine	E-360+ atropine	2.6(1.8-3.9)	-
"	none	TMB4+ atropine	1.6(1.3-1.9)	100 ± 6
"	none	toxogonin +atropine	1.6(1.2-2.0)	128 ± 12
"	pyrido- stigmine	toxogonin +atropine	2.1	-
VX	none	E-360 +atropine	19.3(13.3-28.0)	-
VX	none	TMB4+ atropine	31.4(25.5-38.6)	-
VX	none	toxogonine +atropine	22.4(15.1-33.1)	-

*100ug/Kg, 15 min before poisoning.

**Treatment was given 1 min after poisoning, with a dose of 15mg/Kg of each compound.

***Numbers in parentheses represent 95% confidence limits.

As can be seen from table 4, the most pronounced advantage of E-360 is its low toxicity as compared to the non-glycosylated bis-oximes TMB4 and toxogonin. Its protective ratio against VX or GD poisoning is not significantly different than that of the non-glycosylated bis-oximes. However it is not certain at this stage whether the linkage position of the sugar residue to the oxime backbone is optimal. We plan to synthesize additional members of these group and test their antidotal activity. (see chapter on future strategies).

5. DISCUSSION

In this report we summarized some of the significant findings concerning the possibility of improving the antidotal efficacy of oximes, by conjugating them with sugar moieties. In addition, we have also presented some experiments, not previously reported, in which we attempted to gain an insight into the mechanism of action of the glycosylated drugs.

We selected the sugar conjugates of 3- and 4-PAM, for initial synthesis, even though they are known to be weak antidotes. This choice was done not only because the synthesis of 3- and 4-PAM sugar analogues is simple, but also because we believed that an improvement in antidotal efficacy could have been better detected when the basal activity is low. Indeed as we described here, compound E-212, a 4-PAM derivative, exhibited over four fold increase in the antidotal activity, as compared to its parent compound. By contrast, the sugar analogue of a more potent antidote, 2-PAM, (compound E-321), exhibited only two fold increase in the antidotal activity against VX, as compared to its non-sugar analogue. Moreover, while E-212 yielded an increased antidotal activity against paraoxon, as compared to 4-PAM, E-321 was less effective against paraoxon than its parent compound (2). In the case of bis-oximes the difference between the sugar derivative E-360 and toxogonin or TMB4 was even less striking, but in this case we tested only one sugar derivative and the position of the sugar attachment might not have been ideal.

Inconsistencies were also found when reactivation factors were compared to antidotal activities pointing out that in some cases sugar oximes demonstrated better antidotal properties than could have been expected from their reactivation factors (2).

These results strongly suggest that the sugar moiety provides the oxime with some advantages which are unrelated to their ability to merely reactivate the inhibited ChE. Our pharmacological experiments suggested that these advantages might be associated with their improved bioavailability and also related to their low toxicity. If so, then one could predict that other drugs such as atropine, or pyridostigmine, which are also used in treatments against OP poisoning, might be more effective when conjugated to glycosyl residue. As we presented in this report we succeeded to synthesize two glycosylated derivatives of pyridostigmine and we are now ready to evaluate them biologically. Improved pharmacokinetics in this case is of particular importance since these kind of drugs are used in pre-treatment and therefore need to be maintained in the blood circulation for an extended period.

Our success to synthesize the glycosylated pyridostigmine marks a milestone in the efforts to synthesize other glycosylated drugs. The routes for these syntheses and their importance will be discussed in the next chapter concerning future strategies.

For the purpose of drug design, as far as the glycosylated drugs are concerned, it is of extreme importance to understand why the sugar moiety improves the antidotal efficacy. We demonstrated here, and in previous reports (1), that sugar oximes prevail in the blood circulation for a longer period than their non-sugar analogues and that some glycosylated oximes are more efficient in reducing hypothermia induced by paraoxon than P2S (1). These results strongly suggest that some sugar-oximes are characterized by an increased bioavailability. We tested whether this increase in bioavailability may be related to the sugar recognition site on the cell membrane. For this purpose we selected retina cells since their neuronal origin makes them one of the relevant target site for AChE inhibitors. The fact that sugar oximes did not compete with 2-deoxyglucose for the glucose transport system, may suggest that the sugar residue which is attached to the sugar oximes is not recognized by the glucose transporter. The lack of recognition at one site does not necessarily exclude the possibility that the sugar residue could interact with an alternative mechanism which involves high affinity sites for sugars, and thus crosses the cell membrane more easily than its non-sugar analogue. Heterogeneity of the glucose transport system (14) may explain why such possible recognition would vary in different tissues. Routes other than the glucose transporter should also be considered in relation to the oxime bioavailability. However, the lack of labelled sugar oximes prevented us from examining this question more thoroughly. The availability of a labelled sugar oxime will provide us with a tool to investigate the distribution of the sugar-oxime in the body before and after poisoning and thus judge about their availability at critical sites. Therefore we also included in the next chapter (concerning future strategies) a proposal of how to synthesize ³H-sugar-oximes.

6. RESEARCH STRATEGIES FOR THE FUTURE

As we mentioned in the discussion, one important point which has not been solved to date, is the mechanism by which the sugar moiety provides the oxime with an increased antidotal activity and with a better antidotal potential. We believe that further understanding of how the sugar residue improves the antidote, will enable us to design better drugs than those reported so far. For this purpose, we consider two experimental set-ups: 1) Synthesis of new compounds that will shed light on structural requirements. 2) Tracing the distribution, metabolism and site of action of the glycosylated drugs by labelling them with a radioactive tag.

6.1. Synthesis of new compounds for studying structure-activity relationships:

6.1.1. Mono-oximes: The following series of studies are suggested for the mono-oximes:

A) Comparison between sugar oximes of various monosaccharides

So far, it was found that a glucose moiety which is linked through its anomeric center, either directly or through a propyl bridge, bears an improved antidotal potential (1, 2, 5). On the other hand, galactosyl derivatives bound through the C-6 position, were found to be weaker antidotes compared to the glucosyl sugar oximes. We think that it is important to prepare a series of sugar oximes, composed of either, glucose, galactose or mannose, linked to the oxime backbone through identical positions, and then compare their pharmacological behavior. Thus, we will ascertain whether glucose is indeed the preferred sugar for glycosyl containing antidotes, or it is the position through which the sugar moiety is linked to the pyridine backbone, which determines how efficient the glycosylated antidote would be.

For that purpose, we intend to synthesize an analogue of E-212 in which the glucose residue would be replaced by galactose. In parallel, an analogue of E-290b, in which the galactose residue would be replaced by glucose, will also be prepared. Both pairs will then be evaluated pharmacologically, for pharmacokinetics, toxicity and protective ratios. Such data may provide us with an important information concerning structure activity relationships.

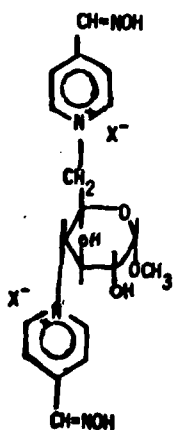
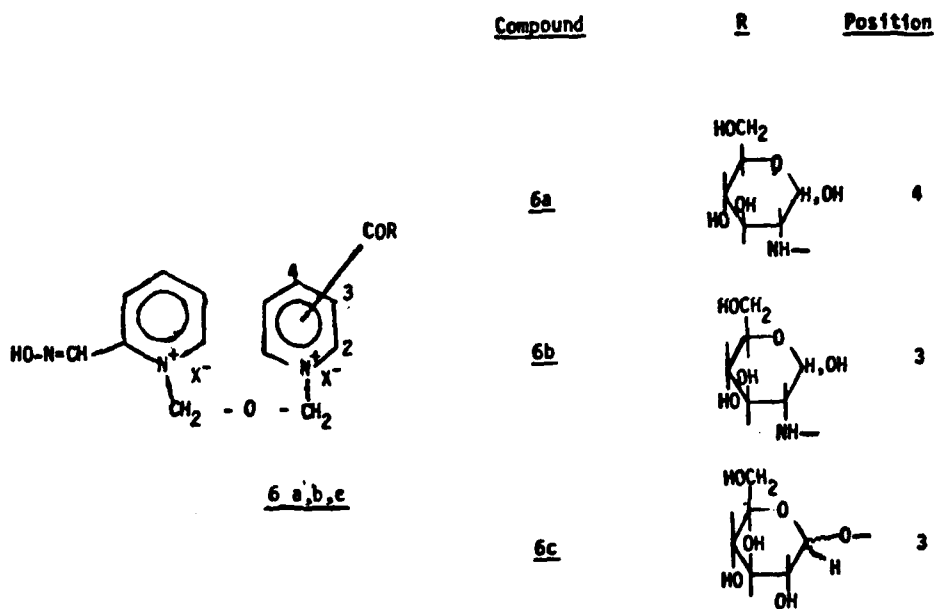
B) The relations between the linkage position of the sugar moiety and the identification of the glycosylated oxime by the sugar recognition sites.

In our previous studies we could not demonstrate competition between free glucose and glycosylated oximes for the glucose transporter. However, it is possible that when the linkage of the sugar residue will result in a derivative with a free anomeric position, the recognition of the glycosylated oxime, will be manifested. We propose therefore, to prepare several glycosylated pyridinium aldoximes, in which the attachment of the sugar residue to the pyridine backbone, will be through positions other than the anomeric site. This will be done by synthesizing a family of sugar oximes, in which the sugar moiety will be linked through position 2, 3, 4, or 6 of the sugar. For that purpose, we will start with a suitable protected sugar, in which the OH group will be condensed with the pyridine component, by the methods which were employed successfully for obtaining the previously described sugar oxime. Alternatively, we may use an activated ester like triflate for achieving the required linkage. The members of this family will be tested for their affinity to the sugar transporter and for the pharmacokinetic profile as well as for their antidotal properties.

6.1.2 Bis-oximes

Bis-quarternized pyridinium oximes include among their various members much more powerful antidotal agents compared to mono quarternized analogues. We expect that glycosylated bis-oximes will exhibit improved prophylactic and pharmacokinetic advantages, as well as an increase in their antidotal efficacy, compared to non glycosylated bis-oximes. Confirmation of this expectation will mark a new class of potent antidotes.

The following bis-quarternized sugar oximes are planned:

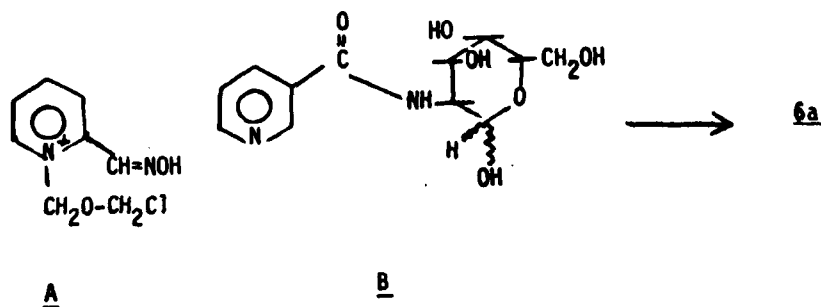


Synthetic approach for 6a

a) The first step involves the synthesis of an amide or an ester of nicotinic acid (or isonicotinic acid) derivative. For this purpose, we prefer to work with nicotinic anhydride, rather than with nicotinyl chloride which is less stable in solution (15).

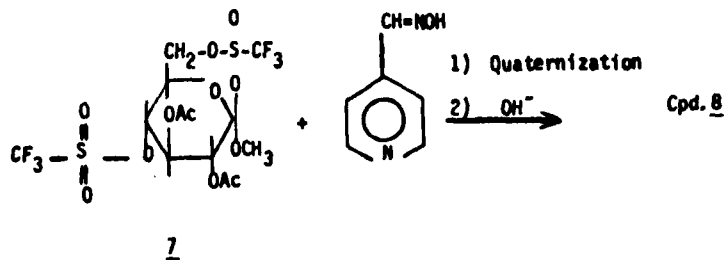
b) N-Nicotinyl glucosamine may be obtained by reacting a methanolic solution of glucosamine with nicotinic anhydride dissolved in the same solvent. At this stage, it is not clear if there is an advantage to esterify the hydroxyls of the amide B in order for all the reactants to be soluble in aprotic solvents. This seems to be important for having all the reacting components in solution, when considering quaternization with A, as described in scheme D.

Scheme D



For obtaining compound 8 we intend to employ the glucosyl bis-triflate derivative represented by 7 and to condense it with 4-pyridine aldoxime. After alkaline treatment we expect to obtain 8 (see scheme E).

Scheme E

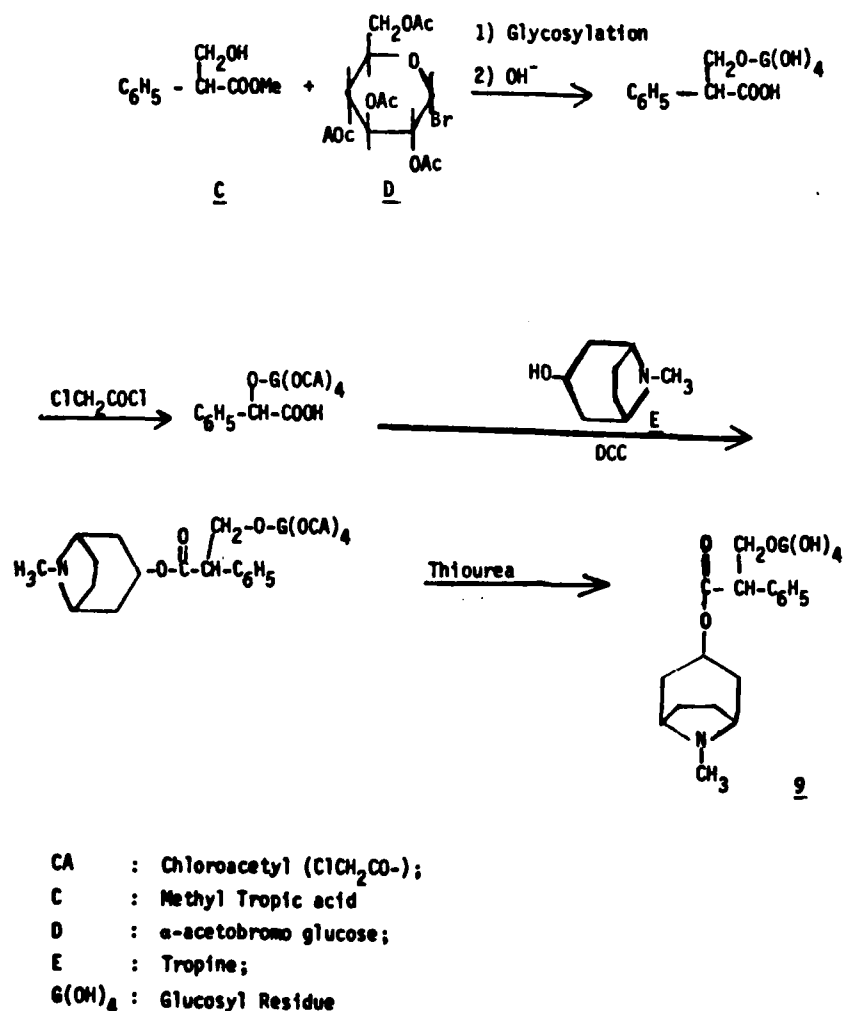


6.2 Glycosylated Derivatives of Atropine

As mentioned earlier, the introduction of a glycosylated drug may enhance its permeability through cell membranes and increase its availability at the sites of actions, relative to a non-sugar derivative. In order to test this hypothesis we suggest to synthesize drugs other than oximes, but which are used in combination with oximes for OP poisoning therapy. We selected as a test compound glycosylated atropine (compound 9).

The glycosylated atropine derivative 9 will be prepared via glycosylation of the hydroxyl function according to the following scheme:

Scheme F



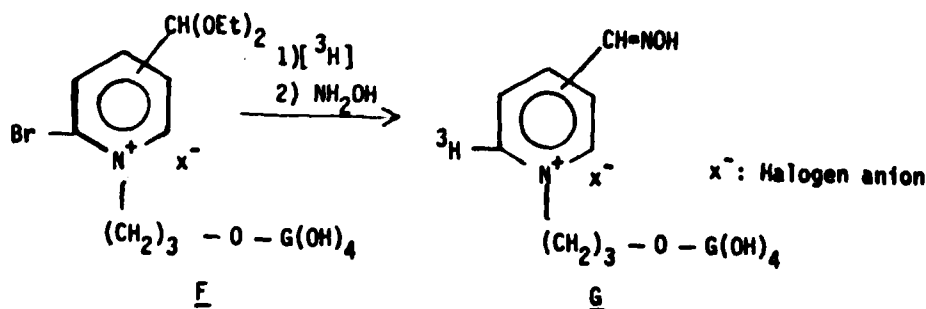
The chloroacetyl group (CA) is chosen as a selective protective group (16) due to the possibility of its preferential removal without affecting the ester linkage between tropine and tropic acid. The hydroxyl function of the atropine is regarded as an important feature of its pharmacological potency (17). However, we postulate that the hydroxyls of glucose in compound 9, will compensate for the loss of the original one that was substituted with the sugar.

In order to monitor the syntheses detailed above, we will also employ mass spectroscopy (17), ¹H-nmr and ¹³C-nmr (4) to illuminate any specific structural features associated with the newly suggested compounds. The efficacy of compound 9, in treatment against OP poisoning, in combination with free or glycosylated oximes, will be tested in mice.

6.3 Preparation of Radioactively Labelled Derivatives

In order to achieve a better understanding of the mechanism of action of sugar oximes, we plan to prepare radioactive analogues. Such analogues will enable us to trace the distribution of the oxime in the body and to study its metabolism and transport properties as well. We propose to synthesize radio-labelled compounds derived from E-212 and E-321.

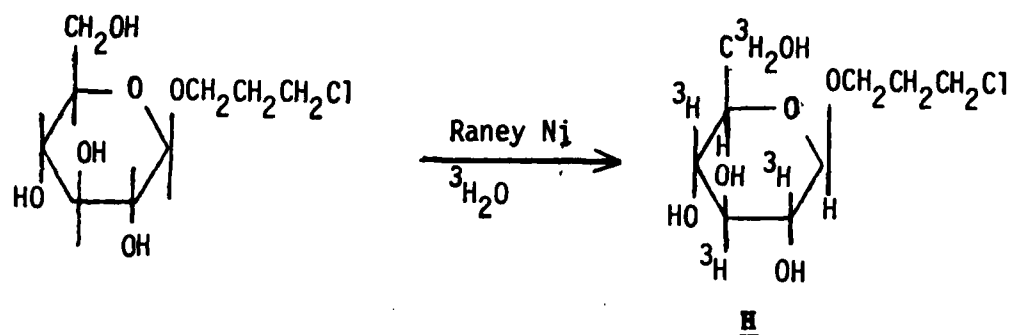
Scheme G



As described above (scheme G), we shall start with the 2-halo analogue of the appropriate pyridine ketal and after completing the original procedure (1, 5), the bromo derivative F will be obtained. A suitable catalytic reduction with tritium and a subsequent oximation should then lead to the formation of the required radiolabelled compound G.

Alternatively, ^3H can be introduced directly into the sugar moiety by the method of Koch & Stewart (18) as suggested in scheme H.

Scheme H



The radio-labelled compound H will serve as a precursor for quaternization with various pyridine aldoximes to form ^3H -glycosylated antidotes.

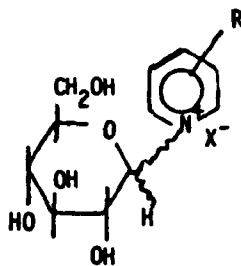
We intend to use the labelled compounds described above for whole body autoradiography, before and after poisoning, and for pharmacokinetic studies. In addition we shall conduct in vitro studies to examine transport as well as binding properties of the glycosylated oximes, to the sugar recognition sites. By gathering such information we hope to shed light on the mechanism of action underlying the antidotal activity of glycosylated drugs.

7. R E F E R E N C E S

1. Rachaman, E.S., Ashani, Y., Heldman, E., Barness, I. and Raveh, L.: Grant No. DAERO-79-G-001. Final Technical Report (1980).
2. Heldman, E., Rachaman, E.S., Ashani, Y., Waysbort, D. and Raveh, L. Contract No. DAJA 37-80-C-0187, Annual Report (Sept. 1981).
3. Weil, C. S., Biometrics **8**, 249-263, (1952).
4. Creasey, N. H., Green, A. L., J. Pharmacol **11**, 485, (1959).
5. Rachaman, E. S., Contract No. DAJA 37-75-C-2213, Final Technical Report (1976).
6. Ellin, R. I., Carless, J. S. and Kondritzer, A. A., J. Pharmac. Sci. **51**, 141-146, (1962).
7. Ellin, R. I., J. Am. Chem. Soc. **80**, 6588-6590, (1958).
8. Kosower, E. W., Patton, J. W., Tetrahedron **22** 2081-2093, (1966).
9. Poziomek, E. J., Hackley, B. E. and Steinberg, G. M., J. Org. Chem. **23**, 714, (1958).
10. Day, R. J., Unger, S. E. and Cooks, R. G., J. Am. Chem. Soc. **101**, 501 (1979).
11. Unger, S. E., Schoen, A. E., Chang, R. G., J. Org. Chem. **46**, 4765 (1981).
12. Unger, S. E., Vincze, A., Cooks, R.G., Chrisman, R., and Rothman, L. D., Anal Chem. **53**, 976 (1981).
13. Rachaman, E. S., Heldman, E., Vincze, A.: Second Half-Yearly Interim Report (Oct 81 - April 82).
14. Ritchfield, R. W. and Wells, W. W., Infect. Immun. **16**, 189-197, (1977).
15. Schrecker, A. W. and Maury, P. B., J. Am. Chem. Soc. **76**, 580, (1954).
16. Roy, H. and Glaudemans, C. P. J., Carbohydr. Res. **45** 299 (1975).

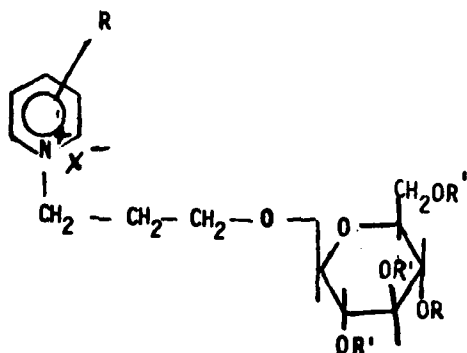
17. Weiner, N., in: The Pharmacological Basis of Therapeutics. Edited by Goodman & Gilman. Sixth Ed. p. 121 (1980). Mamillan Publishing Co. Inc. N.Y.
18. Koch, H. J. and Stuart, R. S. S., Carbohyd. Res. 59, C7-C11, (1977).

8. STRUCTURAL FORMULAS



E-168: R=3-aldoxime subst.

E-196: R=4- " "

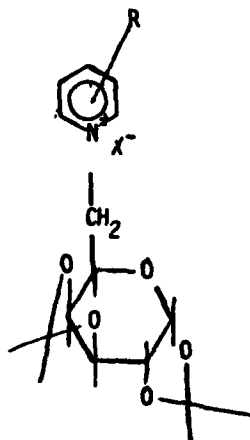


E-193_b: R'=Ac;4-ald. subst.

E-212: R'=H;4- " "

E-321_b: R'=H;3- " "

E-345_a: R'=OAc;3-dimethyl carbamoyl subst.



E-305: R-2 -ald. subst.

E-297_a: R-3 - " "

E-290_a: R-4 - " "

E-343_a: R-3 -dimethyl carbamoyl subst.