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SUGAR CONJUGATES OF PYRIDINIUM ALDOXIMES AS ANTIDOTES AGAINST ORGANOPHOSPHATE POISONING*

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ABSTRACT

A series of pyridinium aldoximes having a sugar conjugated to the pyridine ring has been prepared as potential antidotes against organophosphate poisoning. The sugar residue was attached either directly through C-1 or C-6 of the pyranose ring or through a C_3 bridge between the glycosyl group and the nitrogen atom of the pyridine moiety. Attachment of a sugar group to the oxime derivative seems to increase the bioavailability of the antidote. The clearance rate of the sugar conjugates was significantly lower than that of their non-sugar analogs and thus they were retained longer in the blood circulation. The sugar derivatives were more potent in decreasing paraoxon-induced hypothermia (which is regulated within the central nervous system) than *N*-methyl-2-pyridiniumaldoxime methanesulfonate, one of the most commonly used mono-oximes. The sugar analogs were also less toxic than the non-sugar analogs; some also displayed higher efficacy. The mechanism underlying the improved features of the sugar oximes, and the structural requirements in relation to the sugar attachment to the oxime function, are discussed.

INTRODUCTION

Organophosphate poisoning is commonly treated by a combination of atropine and a quaternary oxime. Atropine is used for its antimuscarinic action, whereby it decreases the overstimulation of the muscarinic receptors that occurs during poisoning through accumulation of acetylcholine at synaptic terminals. Oximes are used in order to displace the phosphate group on the active site of acetylcholinesterase, and thus reactivate the inhibited enzyme.

As oximes may be given prophylactically, it is desirable for them to have a high reactivation rate constant and to persist for a long period in the blood circulation. Their toxicity should be low, and they should act without any side effects. In order to attain these important features in the antidotes currently available, we designed and synthesized conjugates of pyridinium aldoximes and sugars. It was thought

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that the sugar moiety might be recognized by sites responsible for sugar transport on the cell membrane, and thus make the nonpermeable quaternary oxime permeable. Improved permeability through biological membranes might make the sugar analogs more available at the critical target-sites.

The sugar oximes prepared, constitute a family of modified pyridinium nucleosides. Pyridinium nucleosides have attracted the attention of biologists because of their involvement in the function of NAD^+ as a redox coenzyme in various metabolic routes. It might thus be expected that oxidative detoxification of organophosphates would be assisted by the presence of such derivatives.

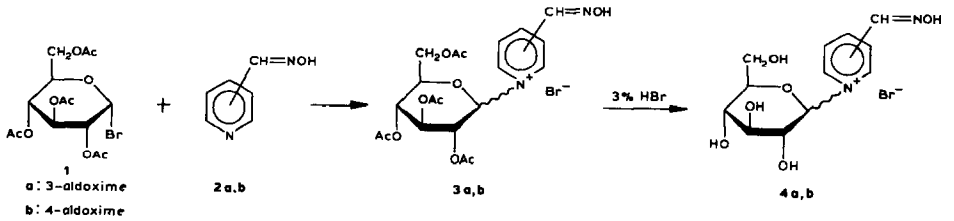
We have previously demonstrated that glycosylated oximes protect against poisoning by some organophosphates more efficiently than the parent compounds[1]. We have now extended this work and examined the bioavailability and the antidotal behavior of the sugar analogs, seeking the structural requirements for optimal reactivation potential and antidotal activity.

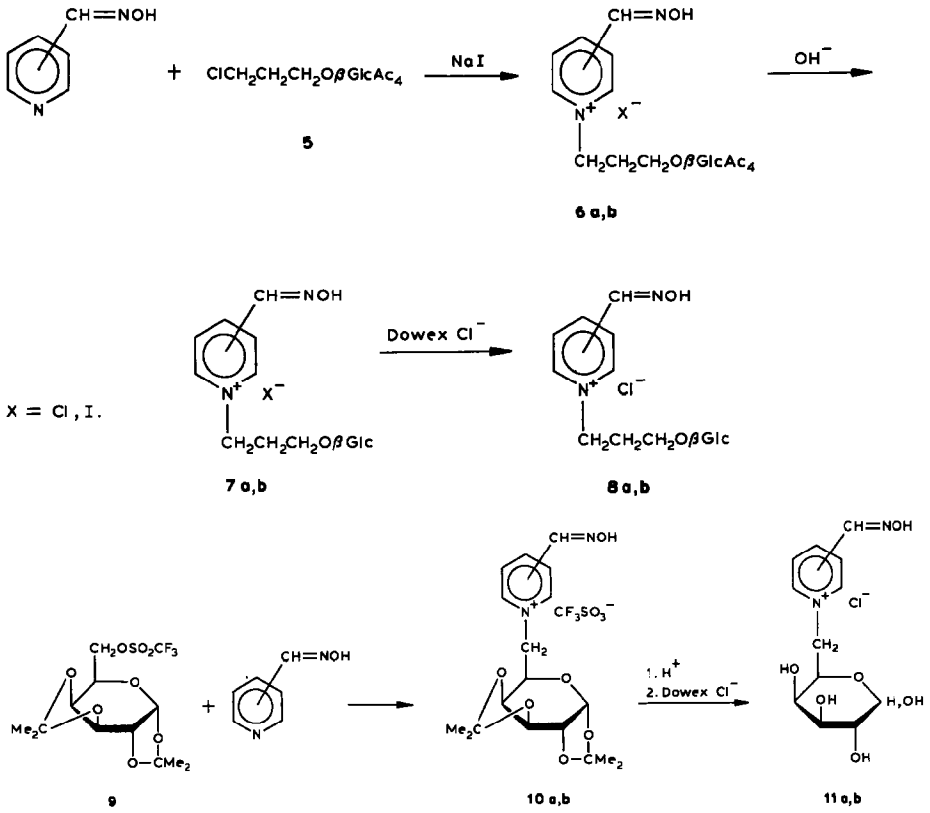
RESULTS AND DISCUSSION

Synthesis of sugar–oxime conjugates. — The compounds reported in this paper constitute the first nucleosides derived from pyridinium aldoximes. Direct *N*-glycosylation of the pyridine aldoxime derivatives[2] by the appropriate glycosyl halides gave 2:1 α,β anomeric mixtures as shown by ^{1}H-n.m.r. spectroscopy. This ratio was reversed following saponification because of the relative instability of the α anomer[2]. N.m.r. data in the literature[3] claim that the α anomer exists as a mixture of three conformers in rapid equilibrium. The anomeric mixture was evaluated pharmacologically without separation.

The synthesis of glucosyloxypropyl derivatives employed suitable halopropyl glucosides for the quaternization; the iodoalkyl glucosides gave the highest yield. The condensation could be effected with either protected or with free-hydroxyl glucosides. The nonprotected chloropropyl glucoside reacts only slowly with the aldoxime, and the involvement of NaI seems to be important. The purification procedure was simpler when acetylated glucosides were used. In addition, we found that the β-glycosylated pyridinium aldoximes were more stable during alkaline saponification than their non-sugar analogs[4] and thus provided an easy route for cleaving the ester group. This feature deserves further investigation.

The best approach to a pyridinium derivative having a sugar linked through C-6 was via a sugar 6-triflate derivative. A sterically hindered pyridine base





a: 3-aldoxime
b: 4-aldoxime

(collidine) was used to prevent interaction between the reactive triflyl group and the basic catalyst and to avoid unwanted quaternized products[5]. Barrette and Goodman[6] used lutidine as a catalytic base for the preparation of 1,2:3,4-di-*O*-isopropylidene 6-*O*-triflyl-α-D-galactopyranose[6] (**9**). This compound was then treated with the appropriate pyridine aldoxime in sulfolane at 40° to afford the quaternized products in reasonable yields. Removal of the protecting groups under acidic conditions led to an anomeric mixture of the expected sugar oxime derivatives, containing approximately equal amounts of the α and β anomers.

Pharmacological and toxicological evaluation. — The rationale for glycosylation of the oximes is to use the sugar moiety as a carrier of the drug. The sugar carrier should interact with various sugar-recognition sites and thus decrease the clearance rate of the attached drug. This should increase the time that the drug is available at the target organs and thus increase its efficacy.

We first studied the clearance rates of the glycosylated oximes and compared them to the non-sugar analogs. Table I summarizes the $t_{1/2}$ values of the glycosyl-

TABLE I

PHARMACOKINETIC PARAMETERS FOR 3- AND 4-PYRIDINIUM ALDOXIME DERIVATIVES

Oxime	$t_{1/2}$ *(min)*[a]	*Maximal time (min) for detectable plasma level*[b]
4-PAM[c]	15	60
6b	45	180
12	15	50
3-PAM[c]	20	60
4a	45	100

[a]Denotes the time in which half of the maximal oxime concentration was found in the serum. The first determination of the oxime concentration was performed 1 min after the injection and continued every 10 min for 60 min. Three additional determination were performed at 90, 180, and 360 min. [b]Lowest concentration detected was 20μM. [c]*N*-Methylpyridinium aldoxime chloride.

TABLE II

ATTENUATION OF PARAOXON-INDUCED HYPOTHERMIA BY SEVERAL SUGAR OXIMES AND *N*-METHYL-2-PYRIDINIUMALDOXIME METHANESULFONATE (P2S)

Oxime tested	*Minimal body temperature*[a]	*Hypothermia reduction*[b] *(%)*
None	34.6 (0.5)[c]	—
P2S	35.2 (0.1)[c]	16.3 (1.7)
6b	36.1 (0.2)[d]	35.5 (2.5)
3a	36.8[e]	42.0

[a]Lowest body temperature recorded during the experiment. [b]Percent hypothermia reduction = 100 [1 − (T with treatment)/(T without treatment)]. [c]Mean of three experiments (standard deviation in parenthesis). [d]Mean of two experiments (standard deviations in parenthesis). [e]Single experiment.

ated oximes tested and the maximal periods during which they could be detected in the blood stream. As may be seen, all of the sugar oximes tested showed increased $t_{1/2}$ values and could have been detected for a longer period than the non-glycosylated analogs. The sugar moiety seems to be the determinant factor in extending the clearance rate, as a parallel derivative (**12**) containing the hydroxypropyl moiety but not the sugar was identical in its pharmacokinetic behavior to the non-glycosylated analogs.

Another parameter that reflects bioavailability of oximes at target organs is their ability to attenuate paraoxon-induced hypothermia. As mentioned earlier, the oximes are used in the treatment of organophosphate poisoning because of their ability to dephosphorylate the inhibited acetylcholinesterase through nucleophilic displacement. The toxic signs of organophosphates include hypothermia through interference with the thermoregulation center, located in the central nervous system[7]. Therefore, the ability of an oxime to antagonize paraoxon-induced hypothermia may be used as an index of availability of the oxime at the central

nervous system. Table II shows that the glycosylated oximes were more efficient in decreasing hypothermia than *N*-methyl-2-pyridiniumaldoxime methanesulfonate (P2S), an oxime commonly used in treatment of organophosphate poisoning. As the reactivation coefficient of P2S is much higher than that of the glycosylated oximes (**3a, 6b**) tested, it may be concluded that the glycosylated derivatives were more available at the thermoregulation center than P2S.

The most critical test for the sugar oximes is their ability to confer protection against organophosphate poisoning. Tables III and IV summarize the protective ratios obtained with the various oximes synthesized here. All of the glycosylated oximes, when used in combination with atropine, improved the antidotal properties against poisoning by both VX* and paraoxon when compared to the parent compounds. Changes in the reactivation constants alone could not explain the improved antidotal properties, at least in the case of the 4-aldoximes (Table IV). However, some correlation between the changes in the reactivation constants and the antidotal properties can be demonstrated in the series of 3-aldoximes. It still remains to be established whether the improved antidotal properties are associated with the improved bioavailability that characterizes at least some of the glycosylated oximes. In addition to the improved properties of the sugar oximes already discussed, their relative toxicity seems to be lower than that of the non-glycosylated derivatives in all examples tested (Table IV). The decreased toxicity of the sugar conjugates endows them with a greater prophylactic potential.

TABLE III

PHARMACOLOGICAL DATA FOR SUGAR DERIVATIVES OF 3-PYRIDINIUMALDOXIMES

Oxime	*Protective ratio (PR)*[a]				*Reactivation of diethyl phosphoryl acetylcholinesterase (M^{-1} min^{-1})*
	VX[b]		*Paraoxon*		
	PR	*Optimal dose of oxime (mg/kg)*	*PR*	*Optimal dose of oxime (mg/kg)*	
3-PAM	4.2 (3.6–5.0)	120	4.5 (3.8–5.4)	120	0.5
6a	5.6 (3.8–8.5)	15	5.2 (3.5–7.9)	11	1.0
8a	18.6 (15.4–22.4)	180	6.2 (6.1–6.3)	180[c]	
4a	13.3 (11.4–15.7)	87	14.5	174	2.0
11a	12.1	231	5.5	154[c]	

[a]PR = LD_{50} with treatment/LD_{50} without treatment (numbers in parenthesis are 95% confidence limits). [b]*O*-Ethyl *N,N*-diisopropylaminoethyl methylphosphonothiolate. [c]Optimal dose has not yet been determined.

*O-Ethyl *N,N*-diisopropylaminoethyl methylphosphonothiolate.

TABLE IV

PHARMACOLOGICAL DATA FOR SUGAR DERIVATIVES OF 4-PYRIDINIUMALDOXIMES

Oxime	*Protective ratio (PR)*[a]				*Toxicity of the oxime (LD_{50} in mg/kg)*	*Reactivation of diethyl phosphoryl acetyl-cholinesterase (M^{-1} min^{-1})*
	VX[b]		*Paraoxon*			
	PR	*Optimal dose of oxime (mg/kg)*	*PR*	*Optimal dose of oxime (mg/kg)*		
4-PAM	5.1 (4.4–5.6)	30	4.1 (3.9–4.2)	30	145.5 (127.8–165,7)	24.4
12	9.8 (9.1–10.5)	50	5.7 (5.4–6.4)	67		
6b	4.6 (4.0–5.3)	16	3.5 (3.2–3.9)	16	478.5 (417.1–549.0)	12.7
8b	23.0 (20.4–26.3)	90	15.1 (10.5–21.6)	90	434.9 (383.0–493.8)	6.1
4b	14.3 (11.4–17.9)	22	7.5 (6.8–8.2)	22[c]		
11b	10.8 (10.1–11.5)	231	2.7	77[c]		

[a–c]For remarks, see Table III.

Structure–activity relationships may be drawn for the various oximes, synthesized and tested. Comparison of two derivatives of the same family, one with a direct linkage between the sugar residue and the pyridine backbone (e.g. **4b**) and the other with a propyl bridge between the two moieties (e.g. **8b**) shows the propyl-bridged compounds to be superior in terms of protecive ratios (see Tables III and IV), for both the 3- and the 4-aldoximes. The protective ratios were lower when the sugar was acetylated (compare **6b** with **8b**). Finally, glucose seems to be superior to galactose (compare **11b** with **4b**), although this last feature was pronounced only for the 4-substituted pyridine aldoximes and was merely a tendency for the 3-substituted pyridine aldoximes. Comparison between the monosaccharides needs further investigation, as the foregoing comparisons were made with sugar oximes in which galactose was attached through the C-6, whereas glucose was linked via C-1.

EXPERIMENTAL

General methods. — T.l.c. was performed on Silica Gel G and alumina (E. Merck A.G.). The spots were made visible with 5% ethanolic H_2SO_4 or iodine vapor. Column chromatography was performed on silica gel 40, 70–230 mesh ASTM (E. Merck). All solvents were flash evaporated using a water aspirator. The ^{1}H-n.m.r. spectra were measured with a JEOL C-60HL spectrometer. U.v. spectra were recorded with a Bausch and Lomb Spectronic 505 instrument. Melting points are uncorrected (melting points not specified indicate amorphous products or broad melting-ranges).

N-*(Tetra*-O-*acetyl*-α,β-D-*glucopyranosyl)*-*3*-*pyridiniumaldoxime bromide* (**3a**). — A solution of 3-pyridinealdoxime (2.0 g) and tetra-*O*-acetyl-α-D-glucopyranosyl bromide (**1**, 6.2 g) in 25 mL of sulfolane was stirred for 24 h at 50°. The product was purified on a column of silica gel; the sulfolane was eluted first with chloroform and then the non-quaternized sugar derivatives were eluted by raising gradually the content of methanol in the chloroform eluent. The quaternized acetylated nucleosides were eluted with 20% methanol in chloroform. Compound **3a** crystallized from ethanol (2.5 g, 30%); m.p. 164°, $[\alpha]_D$ +16.8° (*c* 1.3, MeOH); ^{1}H-n.m.r. (D_2O–DSS): δ 1.9–2.1 (12 H, 4 OAc), 4.3–5.6 (6 H of pyranose ring), 6.2 (d, Hβ), 6.8 (d, Hα), 8.14 (m, 1 Ar-H), 8.36 (s, 1 methine H), 8.8 (d, 1-Ar-H), 9.05 (d, 1 Ar-H), and 9.3 (s, 1 Ar-H).

Anal. Calc. for $C_{20}H_{25}BrN_2O_{10} \cdot H_2O$ (551.3): C, 43.55; H, 4.90; N, 5.08. Found: C, 43.58; H, 5.30; N, 5.06.

N-*(α,β*-D-*Glucopyranosyl)*-*3*-*pyridiniumaldoxime bromide* (**4a**). — The acetylated nucleoside (**3a**) was dissolved in 3% aqueous HBr and stirred[8] for 16 h at 45°. The solution was then evaporated several times *in vacuo* (<40°) after adding 2-propanol each time. The residue crystallized from methanol–2-propanol–ether (yield 35–40%); $[\alpha]_D$ +24.5° (*c* 1.4, MeOH). The ^{1}H-n.m.r. spectrum was similar to that of **3a** except that the anomeric signals were shifted ~0.5 p.p.m. upfield in comparison with the acetylated precursors.

Anal. Calc. for $C_{12}H_{17}BrN_2O_6$ (365.16): C, 39.46; H, 4.69. Found: C, 39.60; H, 4.98.

N-*(Tetra*-O-*acetyl*-α,β-D-*glucopyranosyl)*-*4*-*pyridiniumaldoxime bromide* (**3b**). — The 4-aldoxime **3b** was obtained as an amorphous material in the same manner and with a similar yield as **3a**; its ^{1}H-n.m.r. spectrum differed from that of **3a** only in the aromatic resonances as follows: δ 8.20 (d, Ar-H), 8.31 (s, 1 methine H), and 8.92 (d, 2 Ar-H).

Anal. Calc. for $C_{20}H_{25}BrN_2O_{10}$ (533.3): C, 45.07; H, 4.73; Br, 15.0. Found: C, 45.58; H, 4.71; Br, 14.68.

N-*(α,β*-D-*Glucopyranosyl)*-*4*-*pyridiniumaldoxime bromide* (**4b**). — This compound was prepared by the procedure used for **4a**; $[\alpha]_D$ +39.5° (*c* 1.5, MeOH); its ^{1}H-n.m.r. spectrum was similar to that of **4a** except that the aromatic resonances gave values similar to those of **3b**.

Anal. Calc. for $C_{12}H_{17}BrN_2O_6 \cdot H_2O$ (383.16): C, 37.60; H, 4.96. Found: C, 37.84; H, 5.03.

N-[*3*-*(Tetra*-O-*acetyl*-β-D-*glucopyranosyloxy)propyl*]-*4*-*pyridiniumaldoxime chloride* (**6b**). — A mixture of 3-chloropropyl tetra-*O*-acetyl-β-D-glucopyranoside[9] (**5**, 3 g), 4-pyridinealdoxime (1.2 g), and NaI (2.1 g) dissolved in 2-methoxyethanol (20 mL) was stirred and boiled under reflux (120°) for 20 h. The solvent was removed under vacuum and the residue was purified on a column of silica gel. The product (**6b**) was eluted with 20% methanol in chloroform. The iodide ion was exchanged with chloride by passing **6b** on Dowex 1 (Cl^-). Compound **6b** was then eluted with water and evaporated to an amorphous, hygroscopic material (1.9 g,

50%); $[\alpha]_D$ −10° (*c* 1.5, MeOH); ^{1}H-n.m.r. ($CDCl_3$–Me_4Si): δ 2.0–2.2 (12 H, 4 OAc), 2.4 (M, 2 H, -CH_2-), 3.3–5.2 (7 H of the pyranose ring and 4 H of two CH_2 groups), 8.05 (d, 2 Ar-H), 8.17 (1 methine H), and 8.8 (d, 2 Ar-H).

Anal. Calc. for $C_{23}H_{31}ClN_2O_{11} \cdot H_2O$ (564.9): C, 48.93; H, 5.85. Found: C, 48.55; H, 6.07.

N-[*3-(Tetra-*O*-acetyl-β-*D*-glucopyranosyloxy)propyl*]*3-pyridiniumaldoxime* (**6a**). — This compound, an amorphous hygroscopic material, was obtained in a similar manner and with a similar yield to that described for **6b**; $[\alpha]_D$ —6.3° (*c* 1.0, $CHCl_3$): its ^{1}H-n.m.r. spectrum was similar to that of **6b**, except for the aromatic resonances as follows: δ 7.85 (m, 1 Ar-H), 8.08 (s, 1 methine H), 8.5 (d, 1 Ar-H), 8.8 (d, 1 Ar-H), and 9.1 (s, 1 Ar-H).

N-[*(3-β-*D*-Glucopyranosyloxy)propyl*]*-3-pyridiniumaldoxime chloride* (**8a**). — An aqueous methanolic solution of **6a** was brought to pH 10.5. After 24 h at room temperature the mixture was made neutral in the presence of Dowex 50W (H^+). Following conventional processing, **8a** was isolated as a hygroscopic, amorphous material; $[\alpha]_D$ +10.4° (*c* 1.5, MeOH); its ^{1}H-n.m.r. spectrum was closely related to that of **6a**.

N-[*(3-β-*D*-Glucopyranosyloxy)propyl*]*-4-pyridiniumaldoxime chloride* (**8b**). — This compound was obtained as an amorphous hygroscopic material in a similar manner to that described for **8a**; $[\alpha]_D$ +4.4° (*c* 1.0, MeOH); its ^{1}H-n.m.r. spectrum was closely related to that of **6b**.

Alternative method for preparation of **8a** *and* **8b**. — Compounds **8a** and **8b** were also prepared by conjugating the saponified product of **5** (obtained conventionally) with the corresponding pyridine aldoxime in a manner similar to that described earlier for **6a** and **6b**. The products were purified on a column of silica gel and were eluted with a 20% chloroform in methanol; conventional isolation gave compounds identical to those already described (yield 16%, based on the saponified product of **5**).

N-*(6-Deoxy-1,2:3,4-di-*O*-isopropylidene-α-*D*-galactopyranose-6-yl)-3-hydroxyiminomethylpyridinium triflate* (**10a**). — Compound **9** (ref. 6) (3.9 g) and 3-pyridincaldoxime (2.4 g) in acetonitrile (30 mL) were stirred at room temperature for 48 h. The mixture was concentrated *in vacuo* and the residue was purified on a column of silica gel that was eluted with 4:1 chloroform–methanol. The product was crystallized from methanol–ether (3.6 g, 70%); m.p. 195°, $[\alpha]_D$ −10.2° (*c* 1.35, $CHCl_3$); λ_{max} (0.1M NaOH) 290 nm; ^{1}H-n.m.r. (Me_2SO-d_6): δ 1.21, 1.32, 1.39 (3 s, four CH_3), 4.0–5.0 (6 H of pyranose ring), 5.45 (d, Hα), 8.2 (m, Ar-H), 8.37 (s, 1 methine H), 8.79 (d, Ar-H), 9.05 (d, Ar-H), 9.3 (s, Ar-H), and 12.2 (s, 1 H, N-OH).

Anal. Calc. for $C_{18}H_{25}N_2O_6 \cdot CF_3SO_3^-$ (514.6): C, 44.35; H, 4.89. Found: C, 44.41; H, 4.95.

N-*(6-Deoxy-1,2:3,4-di-*O*-isopropylidene-α-*D*-galactopyranose-6-yl)-4-hydroxyiminomethylpyridinium triflate* (**10b**). — This compound was prepared and crystallized as for **10a**, with a similar yield; m.p. 218°, $[\alpha]_D$ −35° (*c* 1.0, MeOH);

λ_{max} (0.1M NaOH) 345 nm; its ^{1}H-n.m.r. spectrum differed from that of **10a** only in the aromatic resonances whose shifts resemble those described for **3b**.

Anal. Calc. for $C_{18}H_{25}N_2O_6 \cdot CF_3SO_3^- \cdot 0.5\ H_2O$ (523.6): C, 43.59; H, 5.01. Found: C, 43.33; H, 5.39.

N-*(6-Deoxy-α,β-*D*-galactopyranose-6-yl)-3-pyridiniumaldoxime chloride* (**11a**). — Compound **10a** (3.5 g) was dissolved in a mixture of 1,4-dioxane (20 mL) and water (14 mL). Concentrated HCl (several drops) was added until the solution reached pH 1.1. The mixture was stirred for 20 h at 80–90°, cooled to room temperature, made neutral with $NaHCO_3$ solution to pH 6.0, and then concentrated under vacuum. The residue was dissolved in water and passed over an anion-exchange resin (Dowex 1, Cl^-) to afford the pyridinium chloride derivative. The product (**11a**) crystallized from methanol–ethanol–2-propanol (1.5 g, 65%); $[\alpha]_D$ +53.7° (*c* 1.14, MeOH); its ^{1}H-N.m.r. spectrum differed from that of **10a** by the absence of the isopropylidene residues and by exhibiting two anomeric resonances as follows: δ 4.5 (d, Hβ), 5.2 (d, Hα).

N-*(6-Deoxy-α,β-*D*-galactopyranose-6-yl)-4-pyridiniumaldoxime chloride* (**11b**). — Prepared as for **11a**, this hygroscopic, amorphous product crystallized from abs. ethanol–methanol and was precipitated by 2-propanol and ether; $[\alpha]_D$ +62.6° (*c* 1.3, MeOH); its ^{1}H-n.m.r. spectrum differed from that of **10b** by the absence of the isopropylidene residues and by exhibiting two anomeric protons as described for **11a**.

N-*(3-Hydroxypropyl)-4-pyridiniumaldoxime chloride* (**12**). — This compound was prepared from 3-chloro-1-propanol (5 g) and 4-pyridinealdoxime (3.6 g), by a conventional technique similar to that already described. The final product was dissolved in ethanol and precipitated from ethyl acetate; yield 5.0 g (77%); m.p. 165°; ^{1}H-n.m.r. (D_2O–DSS): δ 2.3 (m, $-CH_2-$), 3.7 (t, CH_2-OH), 4.72 (t, CH_2-N^+), 8.1 (d, 2 Ar-H), 8.22 (s, 1 methine H), and 8.8 (d, 2 Ar-H).

Anal. Calc. for $C_9H_{13}ClNO_2$ (216.3): C, 49.93; H, 6.05. Found: C, 50.40; H, 6.21.

Pharmacological and toxicological evaluation. — ICR (albino) male mice used for most of the biological evaluations were maintained in a temperature-regulated (22–25°) room and were used when their body weight reached 22–25 g.

Sprague–Dawley rats were used for measuring the effect of the oximes on paraoxon-induced hypothermia. The following pharmacological and toxicological tests were carried out.

1. Pharmacokinetics. Mice were injected intramuscularly with the tested oxime. At various periods after the injection, the mice (at least four for each time point) were bled and oxime concentration in the serum was determined according to Creasey and Green[10].

2. Protection experiments. Mice were challenged subcutaneously in the neck region with either *O*-ethyl *N,N*-diisopropylaminoethyl methylphosphonothiolate (VX), or *O,O*-diethyl *O*-(*p*-nitrophenyl) phosphate (paraoxon). One min following the poisoning, or upon appearance of poisoning signs (whichever came first), the

mice were treated with a mixture of an oxime and atropine (15 mg/kg of each, unless otherwise specified) by intramuscular injection into the thigh region. The LD_{50} values were calculated according to Weil[11] and protective ratios (PR) were calculated as follows:

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

3. Toxicity of oximes. Mice were injected intramuscularly with increasing doses of the tested drug. The injected mice were observed, up to 24 h following the injection, for signs of poisoning and death. The LD_{50} values were calculated as described in the foregoing section.

4. Protection against paraoxon-induced hypothermia. The following modification of the method described by Meeter *et al.*[12] was used in this study. (a) Rats were not anaesthetized (as hexobarbital used in the original procedure was found to induce slight hypothermia). (b) Sprague–Dawley male rats weighing 200–340 g were injected subcutaneously with 0.04–0.07 mL paraoxon solution (1 mg/mL in 0.9% NaCl). Control animals were injected with 0.9% NaCl. Tested oximes (0.1–0.2 mL) were injected intraperitoneally 30 min prior to injection of paraoxon. Body core temperature was continuously recorded with a thermistor and checked frequently with a clinical thermometer, both inserted 5 cm into the rectum. After injection of paraoxon (80 min) the rats were placed in controlled cold environment (10°) for 60 min and then returned to room temperature.

5. Determination of reactivation constants of diethyl phosphoryl–acetylcholinesterase conjugate. Reactivation was initiated by adding 10–50 μL of an aqueous solution of the tested oxime (10–100mM) into 0.5 mL of enzyme solution previously incubated for 5 min at 30° with or without 1.6μM *O,O*-diethyl phosphorochloridate. Under these conditions 85–90% of the original activity of the enzyme was inhibited. The rate of reactivation was monitored by the Ellman procedure[13], by diluting 25 μL of the assayed solution directly into the mixture of reagents. The following controls were performed in parallel to the reactivation experiments: non-inhibited enzyme (including the oxime), inhibited enzyme, and reaction mixture that did not contain the enzyme (blank). The values for enzyme reactivation as a function of incubation time were corrected for the oxime-catalyzed hydrolysis of acetylcholine whenever the reactivator concentration showed such activity.

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