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(18) From Eastman Kodak. This material and other freshly distilled samples of (−)-nicotine gave $[\alpha]_D^{20-23}$ −170° (*c* 0.20–1.00, $CHCl_3$) and $[\alpha]_D^{25}$ −131° (*c* 0.98, MeOH); lit., K. D. Jackson, *Chem. Rev.* **24**, 162 (1941), $[\alpha]_D^{20}$ −157 and −118° in $CHCl_3$ and MeOH, respectively.
(19) From Aldrich.
(20) Room temperature.
(21) Generously supplied by Dr. T. S. Osdene, Philip Morris Research Center, Richmond, Va.
(22) Obtained initially by repeated recrystallization of a crude specimen of 1 which crystallized on cooling to 0 °C from a solution of (±)-nicotine and *d*-tartaric acid in MeOH.
(23) Occasional warming at first appeared to facilitate crystallization.
(24) With addition of 100 mg of *d*-tartaric acid.
(25) MeOH (4–6 mL) may be used instead of 95% EtOH, but crystallization is slower in MeOH and cooling to 0 °C is essential for good yields.
(26) A 3:2 or 2:1 mixture of MeOH–Me_2CO was always used to wash the crystals of 1.
(27) A sixfold scale-up in this resolution procedure increases the overall yield of 1 and 2 to 30–35%. Furthermore, evaporation to dryness of all filtrates from the nicotine salts and conversion of the latter to free base gives 20–25% more of the antipodes on resolution again.
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Synthesis of Pyridinium Analogues of Acetylcholine and Their Interactions with Intestinal Muscarinic Receptors

Dida Kuhnen-Clausen,*

Fraunhofer-Gesellschaft, Institut für Aerobiologie, D-5948 Schmallenberg-Grafschaft, West Germany

Ilse Hagedorn, and Rudolf Bill

Chemisches Laboratorium der Universität Freiburg, Albertstraße 21, D-7800 Freiburg i.Br., West Germany.
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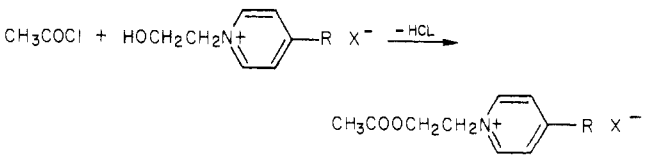
N-(β-Acetoxyethyl)pyridinium salts were synthesized and tested for muscarinic receptor interactions by the guinea pig ileum assay. Agonist activity indicates that receptor binding is substantially retained when the ammonium group of acetylcholine is formally replaced by a pyridinium ring. Introduction of alkyl groups into the ring yields antagonists. The 4-*tert*-butylpyridinium derivative is proved to have an activity superior to that of the 4-methylpyridinium salt. Competitive antagonism is favored by the more hydrophobic property of the *tert*-butyl group. A nonpolar area is suggested to be situated in the direct vicinity of the anionic binding sites of muscarinic receptors. The interaction of hydrophobic substituents with this area determines the antimuscarinic properties of pyridinium salts.

A large series of pyridinium salts was synthesized in a systematical search for drugs with antidotal properties against organophosphorus poisoning.[1-4] The most potent drugs against soman, *O*-(1,2,2-trimethylpropyl) methylfluorophosphonate, in vivo[5,6] and in vitro[6] are those which bear a hydrophobic group at the pyridinium ring. Their effect can be attributed to a protection of active sites of acetylcholinesterase (AChE, EC 3.1.1.7)[6] and to antimuscarinic properties.[7-10] Tentatively, it was assumed that hydrophobic surroundings of the anionic centers of AChE[11,12] and muscarinic receptors[13,14] are involved in the binding mechanisms of hydrophobic substituted pyridinium salts. The hypothesis was propounded that the pyridinium ring binds to the same site where the onium group of acetylcholine (ACH) can be attached.

To answer the question of whether the pyridinium ring can bind to the muscarinic receptor, ACh analogues were synthesized in which the quaternary ammonium group was formally replaced by a pyridinium ring. In order to test the significance of hydrophobic areas near the active site of the receptor, derivatives were substituted with alkyl groups in the ring. To our knowledge, these compounds were unknown until now. Their muscarinic or antimuscarinic activities were tested with the isolated longitudinal muscles of the guinea pig ileum. The interaction with other systems than cholinergic receptors cannot be excluded. This problem, however, was beyond the scope of this paper.

Scheme I



R = H, $-CH_3$, $-C(CH_3)_3$
X = Cl^-, Br^-, I^-

Chemistry. The synthesis of the ACh analogues succeeded according to the reaction in Scheme I. The *N*-(hydroxyethyl)pyridinium salts **1–3** (Table I) could be prepared by the reaction of the respective pyridine derivative with chloro- or bromoethanol.[15] In order to synthesize **4** (HB 14, Table I), it is suitable to prepare

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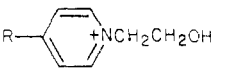
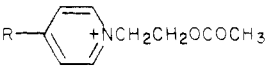
Table I. List of Compounds Synthesized

compd	code	R	X⁻	formula	yield, %	mp, °C	anal.
							
1		H	Cl	$C_7H_{10}ClNO$	64	127[a]	C, H, N, Cl
1a		H	I	$C_7H_{10}INO$	58	72.5	C, H, N, I
2		CH_3	Br	$C_8H_{12}BrNO$	98	79.5	C, H, N, Br
3		$C(CH_3)_3$	Br	$C_{11}H_{18}BrNO$	99	115.5	C, H, N, Br
							
4	HB 14	H	I	$C_9H_{12}INO_2$	80	70-71	C, H, N, I
5	HB 5	CH_3	Br	$C_{10}H_{14}BrNO_2$	97	137-138	C, H, N, Br
6	HB 6	$C(CH_3)_3$	Br	$C_{13}H_{20}BrNO_2$	69	148-148.5	C, H, N, Br

[a] Astle and Donat,[16] 127 °C

Table II. Effect of 4-6 on the Activity of Electric Eel AChE and on the Isolated Longitudinal Muscles of the Guinea Pig Ileum [a]

compd	concn, M	AChE,[b] % inhib	ileum musc, % max contract.	n[c]
4	10^{-6}	23.9 ± 1.2	0	29
	10^{-4}	30.0 ± 0.8	89 ± 5	29
5	3×10^{-5}	42.5 ± 0.9	0	8
	10^{-3}	77.4 ± 1.1	0	8
6	10^{-5}	32.3 ± 1.3	0	16
	3×10^{-4}	66.2 ± 0.6	0	16

[a] Values are means ± SD. [b] Each value was derived from six experiments with 1 mM ACh as substrate.[18] [c] Number of experiments with the ileal muscle preparation.

firstly the chloride 1 and then to transform this into the iodide 1a by ionic exchange with NaI.

Biological Assays. In preliminary experiments, the compounds 4-6 were tested for possible spasmogenic activities. Within a concentration range from 1 μM to 1 mM, only compound 4 induced contractions of the ileal muscles, while 5 and 6 were inactive. A few enzymological experiments ruled out that the ACh analogues hydrolyzed neither spontaneously nor in the presence of AChE (electric eel, Boehringer, Mannheim), when measured with the pH-stat method as described by Kuhnen.[17] On the other hand, the three compounds are weak inhibitors of AChE, as could be determined by the decreased decomposition rate of 1 mM ACh[18] (Table II). The inhibitory effects of the compounds tested do not correlate with their spasmogenic activities and are, therefore, not supposed to interfere with the results presented below.

5 and 6 were tested in vivo in female NMRI mice with respect to their toxicity and their possible protective effect against soman intoxication, with methods described elsewhere.[5,6] Their toxicity is low when compared with other pyridinium salts,[5] the LD_{50} being about 2 mM/kg in mice. The derivatives are completely ineffective in the prophylaxis against soman.

The pharmacological assays in vitro have been carried out in the presence of 10 μM hexamethonium chloride in order to avoid ganglionic stimulation of the ileal muscle preparation. Acetyl-β-methylcholine chloride (MeCh) and ethyl(2-hydroxyethyl)dimethylammonium chloride benzylate (Lachesine[20]) served as reference compounds for muscarinic agonists and antagonists, respectively. MeCh was chosen because of its weak nicotinic activity and its resistance to hydrolysis. Lachesine was prefered rather than atropine for its structural close relationship to ACh and the pyridinium analogues with respect to the $COOCCN^+$ chain. On the other hand, its hydrophobic moiety is opposite to the nitrogen but approaches the ester group, whereas the alkyl groups of 5 and 6 are near the pyridinium nitrogen but opposite to the ester group. From a comparison of the mode of action of Lachesine with that of 5 and 6, data were expected regarding the influence of hydrophobic substituents on the interaction with muscarinic receptors.

The pharmacological activities of agonists and antagonists were determined as pD_2 and pA_2 values, respectively. Average values are noted as means ± standard deviations.

Results and Discussion

Cumulative dose-response curves to MeCh and to 4 were constructed alternatively with one ileal muscle preparation every 20 min. The curves of both agonists have similar shapes and maxima; i.e., the drugs display the same intrinsic activity. However, 250-fold higher concentrations of 4 than of MeCh are needed to induce equivalent responses. The respective pD_2 values of MeCh and 4 are 7.02 ± 0.14 and 4.62 ± 0.13 (n = 15 for each drug.) The omission of the competitive nicotinic antagonist, hexamethonium, from the organ bath or the elevation of its concentration from 10 to 100 μM affected neither the intrinsic activity nor the pD_2 value of compound 4 or of MeCh. Both agonists are potentiated by 0.05 μM physostigmine to the same extent: the log dose-response curves are shifted parallelly to the left for about half a logarithmic unit.

Apparently, the action of 4 is very similar to that of MeCh. Therefore, it is assumed that the muscarinic effect is the predominant feature of 4. The difference in the pD_2 values of the ACh analogue and MeCh is thought to originate in the low degree of complementarity between the pyridinium ring and the onium binding site. The high intrinsic activity of 4 probably derives from the correct orientation of the acidic moiety to the ester binding site of the muscarinic receptor.

In order to examine possible spasmolytic effects of 5 and 6, the muscle preparations were conditioned with different single doses of the respective derivative for 10 min before the cumulative dose-response curves to MeCh were determined. Lachesine was tested by the same procedure. The three derivatives shifted the log dose-response curves to MeCh to the right without changing their shape or maxima. This points to competitive antagonism against the muscarinic agonist. The respective pA_2 values are listed in Table III.

The introduction of alkyl groups into the pyridinium ring of 4 abolishes the intrinsic activity. The antagonistic

Table III. pA_2 Values of Antagonists Against Acetyl-β-methylcholine (MeCh) and 4

antagonist	MeCh pA_2	n^a	4 pA_2	n^a
Lachesine	8.62 ± 0.05	8	8.66 ± 0.10	6
5	3.43 ± 0.08	8		
6	4.43 ± 0.09	8	4.52 ± 0.09	8

[a] Number of experiments.

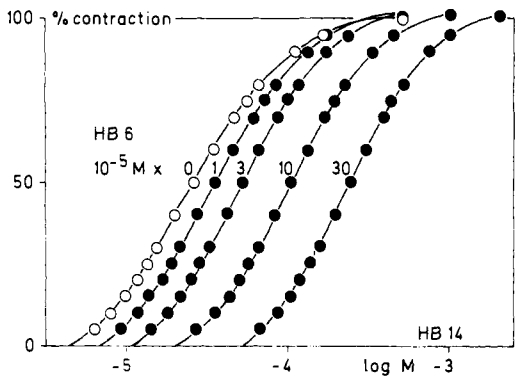


Figure 1. Log dose–response curves to 4 (HB 14) constructed in the absence (open circles) or presence (filled circles) of different single doses of 6 (HB 6). The symbols are means of equipotent agonist concentrations producing standard responses[9] from eight experiments. For sake of clarity, standard deviations are not indicated.

activity depends upon the size and hydrophobicity of the alkyl group. The substitution of the methyl group of **5** with a *tert*-butyl group results in **6** with a pA_2 value increased by 1 unit (Table III), possibly due to a certain complementarity to a nonpolar area near the onium group binding site.

The low pA_2 value of **5** does not allow the examination of its antagonism in sufficiently high concentrations. Therefore, clear-cut evidence is not obtainable for its action at muscarinic receptors. Further experiments were carried out exclusively with **6**.

Antagonists that are supposed to act at common receptors are expected to produce a simple additive action when they are applied simultaneously. Single doses of **6**, ranging from 10 to 300 μM, were administered together with 0.01 μM Lachesine. The combined antagonistic effect against MeCh corresponds to the addition of each drug's concentration. This is suggestive for an action of **6** at muscarinic receptors.

For agonists that are supposed to produce their effects on common receptors, more definite arguments are obtained from their combined action with competitive antagonists. The pA_2 values of these antagonists should be independent on the respective agonist. Figure 1 shows the antagonism of **6** against **4**. The log dose–response curves to **4** are shifted in nearly parallel planes to the right. The height of the maximum responses to the agonist remains unchanged. The pA_2 value of **6** against **4** agrees with that against MeCh (Table III). The similarity of the antagonism of **6** against **4** and MeCh favors the assumption made for an action of both angonists and the antagonist at muscarinic receptors.

The action of Lachesine against **4** appears to be a combination of competitive and noncompetitive antagonism (Figure 2). The competitive antagonism is indicated by the parallel fashion by which the log dose–response curves to **4** are shifted to the right. The pA_2 value of Lachesine is the same as that found against MeCh (Table III). This lends further support to the assumption that **4** acts at the muscarinic receptor. The noncompetitive

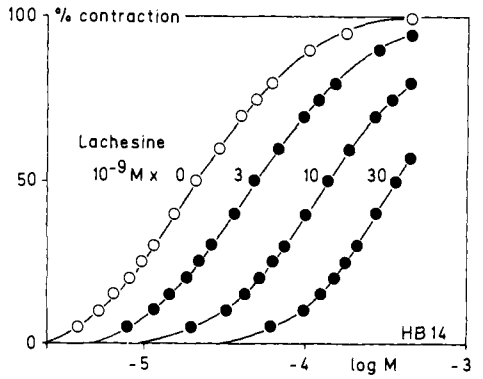


Figure 2. Log dose–response curves to 4 (HB 14) constructed in the absence (open circles) or presence (filled circles) of different single doses of Lachesine. The symbols are means of equipotent agonist concentrations producing standard responses[9] from six experiments. For sake of clarity, standard deviations are not indicated. Note the combination of the parallel shift of the curves and the depression of maximum responses.

antagonism of Lachesine manifests itself only when the concentration of **4** comes up to 0.4 mM. The maximum responses to **4** decreases then gradually with the increase of the dose of Lachesine (Figure 2). A dose of 0.1 μM Lachesine abolishes practically all responses to the agonist.

The differences between the actions of Lachesine and **6** against MeCh or **4** are thought to stem from the antagonists' hydrophobic moieties. Lachesine apparently binds through its benzylate moiety to an area near the ester binding site[13,21,22] and inhibits the propagation of receptor stimulation by high doses of **4**—but not of MeCh—to the contraction-inducing processes. However, **6** seemingly binds through its *tert*-butyl group to a nonpolar area close to the onium group binding site.[14,21,23] This may favor the correct orientation of the pyridinium ring at this site so that **6** blocks the receptor activation by MeCh or **4** by competition.

Conclusions

The results reported here support the hypothesis that the pyridinium ring of **4** and **6** binds to the same site of the muscarinic receptor, where the cationic moiety of cholinergic agonists can be attached. The spasmogenic or spasmolytic activities of the pyridinium analogues of ACh depend on the spatial arrangement around the nitrogen. The nonsubstituted pyridinium ring of **4** apparently conserves a certain complementarity to the onium group binding site. This allows the correct orientation of the acyl moiety to the ester binding site. Compound **4**, therefore, is an agonist. The antimuscarinic activity of the *tert*-butyl-substituted **6** lends support to the assumption that a nonpolar area is located in the direct vicinity of the anionic center of the receptor.[14,23] The space-filling *tert*-butyl group enables a better fit to this nonpolar region than the introduction of one methyl group into the pyridinium ring. Consequently, **6** is a more potent cholinergic antagonist than **5**. Its mechanism of action can be regarded as a competitive antagonism against MeCh and **4**.

Experimental Section

Melting points were determined on a Mettler FP 1 apparatus and were corrected. IR spectra were recorded on a Perkin-Elmer Infracord 137 spectrophotometer. NMR spectra were measured on a Varian A-60. Elemental analyses were performed by the organic-analytical laboratory of the Chemisches Laboratorium der Universität Freiburg. The analyses are indicated by symbols of the elements (Table I).

General Procedure for the Preparation of the *N*-(Hydroxyethyl)pyridinium Salts 1–3. Chloro- or bromoethanol (0.1 mol) was refluxed for 4 h, with stirring and all moisture

excluded, with an excess of the respective pyridine component. The precipitate which formed when the mixture cooled was quickly sucked off and dried under vacuum (≤1 mmHg) at 60 °C. After several hours of drying, recrystallization from a small volume of ethanol yielded a colorless hygroscopic product. The reaction of 1 (Table I) with NaI in absolute ethanol yielded compound 1a.

General Procedure for the Preparation of the *N*-(β-Acetoxyethyl)pyridinium Salts 4–6. 1a, 2, or 3 (0.1 mol) was dissolved or suspended in acetonitrile. Acetyl chloride (0.15 mol), also dissolved in a small amount of acetonitrile, was dropped into this mixture, which was being refluxed. This mixture was stirred, and all moisture was excluded. After a maximum of 4 h of heating, the solvent and the excess of acetyl chloride were evaporated under reduced pressure. The residue was solid or oleaginous. It was ground with dry ether or acetone. After washing, it was crystallized from ethanol, 2-propanol, or acetone, if necessary, with the addition of ethyl ester.

Pharmacology. Longitudinal muscle strips of the guinea pig ileum were isolated according to Paton and Rang.[24] They were derived from animals of 350–600 g and suspended in an organ bath at 37 °C, containing 10 mL of Tyrode's solution of the following composition (mM): NaCl, 137; KCl, 3.7; $CaCl_2$, 1.8; $MgCl_2$, 1.05; NaH_2PO_4, 0.2; $NaHCO_3$, 11.9; glucose, 5.5; hexamethonium chloride, 0.01. The bath was gassed with 5% CO_2 in O_2, the pH was 7.4 ± 0.5. Isotonic contractions were recorded on a kymograph with a magnification of 1:8. The load of the lever was 150 mg.

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Studies on 3-Substituted 1,2-Benzisoxazole Derivatives. 6. Syntheses of 3-(Sulfamoylmethyl)-1,2-benzisoxazole Derivatives and Their Anticonvulsant Activities

Hitoshi Uno,* Mikio Kurokawa, Yoshinobu Masuda, and Haruki Nishimura

Research Laboratories, Dainippon Pharmaceutical Company, Ltd., 33-94, Enoki-cho, Suita, Osaka, Japan.
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Several 3-(sulfamoylmethyl)-1,2-benzisoxazole derivatives were synthesized from 3-(bromomethyl)-1,2-benzisoxazole by the reaction with sodium bisulfite followed by chlorination and amination. Some of them displayed marked anticonvulsant activity in mice. The introduction of a halogen atom to the 5 position of the benzisoxazole ring caused increased activity and neurotoxicity; the substitution of a sulfamoyl group caused decreased activity. The activity of monoalkylated compounds might be the result of biotransformation. Among these compounds, 3-(sulfamoylmethyl)-1,2-benzisoxazole (1a) was thought to be the most promising as an anticonvulsant from the ratio of NTD_{50} and ED_{50}.

During the course of routine testing, it was noted that 3-(sulfamoylmethyl)-1,2-benzisoxazole[1] (1a, Scheme I) exerted a potent anticonvulsant effect as measured by protection against maximal electroshock (MES) seizure.

The present paper deals with the syntheses of several 3-(sulfamoylmethyl)-1,2-benzisoxazole derivatives and the results of their biological evaluation.

It is well known that some arylsulfonamides show anticonvulsant activity which is supposed to be due to the inhibition of carbonic anhydrase. However, 1a showed only a weak effect on carbonic anhydrase in vitro.[2] Therefore, derivatives of 1a might be of interest as novel anticonvulsants.

Chemistry. In the early work,[1] 1a was prepared by the chlorosulfonation and the successive amination of 1,2-benzisoxazol-3-acetic acid (11). In these reactions, 5-sulfamoyl-3-(sulfamoylmethyl)-1,2-benzisoxazole was also obtained as a side product, and the yield of 1a was very poor (7%). Therefore, another method was chosen to prepare derivatives of 1a.

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