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Homologues of benzilylcholine mustard

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Seven homologues of the alkylating muscarinic antagonist benzilylcholine mustard (BCM) have been prepared. The rate of reaction of the aziridinium ion derivatives with water is largely independent of the structure, whereas the rate of reaction with the muscarinic receptor drops markedly when the *N*-alkyl group is larger than n-propyl. PrBCM is practically equiactive with BCM. Recovery from block caused by PrBCM was first-order with a half-time of 32 h at 37°.

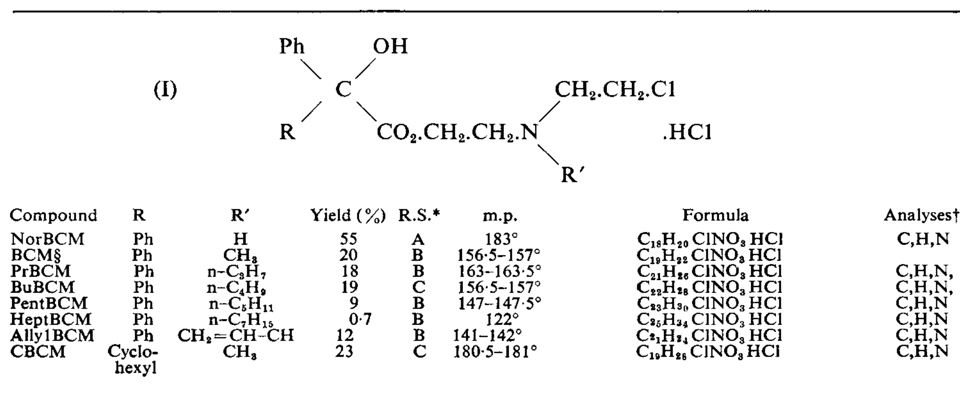
Benzilylcholine mustard (BCM)* was introduced by Gill & Rang (1966) as an alkylating antagonist for the muscarinic receptor. It has proved to be of high specificity and affinity and is a valuable tool in receptor pharmacology. Although commonly called an irreversible antagonist, the (presumed) covalent bond to the receptor is apparently unstable, the half-time of recovery of pharmacological activity being about 12 h at 37°. To investigate this phenomenon and for studies in receptor isolation a highly radioactive derivative is required, preferably with the label on the nitrogen side of the ester bond. The synthesis of such a compound would be easier if the *N*-methyl group could be substituted by a larger alkyl residue without loss of pharmacological activity. We have therefore prepared a series of *N*-alkyl homologues of BCM and we report here their activity relative to BCM as irreversible muscarinic antagonists.

Preparation of compounds

The *N*-alkyl-*N*-2′-chloroethyl-2-aminoethyl esters (I) were synthesized by thermal rearrangement of the benzilic or cyclohexylphenylglycollic acid salt of the appropriate *NN*-bis(2-chloroethyl)alkylamine (Horenstein & Pählicke, 1938; Gill & Rang, 1966). The acid and amine were refluxed in isopropanol for 12–24 h after which the ester hydrochloride was isolated by fractional crystallization from isopropanol: ether mixtures. Yields were low (9–23%), in part due to further reaction to give an *NN*-bis (benzilyloxyethyl)alkylamine, although none of this by-product was isolated from the reaction mixture containing BCM. The required mustard hydrochlorides became increasingly more difficult to isolate as they became more lipid soluble, e.g. HeptBCM was isolated in a yield of only 0·7% and only when acetone was used as a solvent, whilst the bisester by-product separated readily. To minimize formation of the by-product and to allow cleaner fractionations, the *NN*-bis(2-chloroethyl)amine was routinely added in excess, even though in reaction mixtures containing the bischloroethylmethylamine some of the piperazine dimer (Hanby & Rydon, 1947) was formed. The general procedure is illustrated by the preparation of CBCM and BuBCM. The preparation of NorBCM via hydrogenolysis of AllylBCM is also described. The compounds prepared and the analytical data are listed in Table 1.

* The abbreviations employed are given in Table 1.

Table 1. *Homologues of BCM prepared*



Compound	R	R′	Yield (%)	R.S.*	m.p.	Formula	Analyses†
NorBCM	Ph	H	55	A	183°	$C_{18}H_{20}ClNO_3\,HCl$	C,H,N
BCM§	Ph	CH_3	20	B	156·5–157°	$C_{19}H_{22}ClNO_3\,HCl$	
PrBCM	Ph	n-C_3H_7	18	B	163–163·5°	$C_{21}H_{26}ClNO_3\,HCl$	C,H,N,
BuBCM	Ph	n-C_4H_9	19	C	156·5–157°	$C_{22}H_{28}ClNO_3\,HCl$	C,H,N,
PentBCM	Ph	n-C_5H_{11}	9	B	147–147·5°	$C_{23}H_{30}ClNO_3\,HCl$	C,H,N
HeptBCM	Ph	n-C_7H_{15}	0·7	B	122°	$C_{25}H_{34}ClNO_3\,HCl$	C,H,N
Ally1BCM	Ph	CH_2=CH–CH	12	B	141–142°	$C_{21}H_{24}ClNO_3\,HCl$	C,H,N
CBCM	Cyclo-hexyl	CH_3	23	C	180·5–181°	$C_{19}H_{28}ClNO_3\,HCl$	C,H,N

* Recrystallization solvent: A, ethanol; B, acetone-ether; C, ethanol-ether.
† Microanalyses by Dr. F. B. Strauss, 10 Carlton Road, Oxford—all fell within the usual limits for the theoretical values.
§ Identical (infrared spectrum and mixed m.p.) with a sample kindly provided by Dr. E. W. Gill.

CBCM (I, R = cyclohexyl, R′ = methyl) — A mixture of *NN*-bis(2-chloroethyl) methylamine (6·16 g) and cyclohexylphenylglycollic acid (7·4 g) in isopropanol (150 ml) was refluxed for 16 h. The piperazine dimer which had separated was filtered off and the isopropanol removed *in vacuo*. The residue was taken up as far as possible in acetone, then hyflo supercel added and the solution filtered. The filtrate was concentrated to a syrup *in vacuo*, redissolved in isopropanol (20 ml) and ether (30 ml) added slowly. The crystals of impure CBCM which separated after 30 h at 4° were recrystallized once from acetone–ether and then three times from ethanol–ether.

BuBCM (I, R = phenyl, R′ = n-butyl) — A mixture of *NN*-bis(2-chloroethyl)-n-butylamine (6·0 g) and benzilic acid (5·0 g) in isopropanol (120 ml) was refluxed for 13 h. The solvent was then removed in a vacuum, the residue taken up in isopropanol (12·5 ml) and ether (60 ml) added slowly. After 6 h at 4° the crystals of BuBCM which had formed were filtered and recrystallized five times from ethanol–ether. Further addition of ether to the filtrate yielded first a second crop of BuBCM and then *NN*-bis(2-benzilyloxyethyl)-n-butylamine hydrochloride (1·15 g), m.p. 168–169·5° (from ethanol–ether) (Found: C,69·9; H, 6·6; N,2·3. $C_{36}H_{40}ClNO_6$ requires C, 69·9; H,6·5; N,2·3%.

NorBCM (I, R = phenyl, R′ = H) — A mixture of AllylBCM (0·4 g) and 5% Pt on charcoal catalyst (30 mg) in dioxan (16 ml) was hydrogenated at atmospheric pressure and room temperature (20°) for 20 min, when uptake had ceased. The catalyst was filtered and the filtrate evaporated in a vacuum. Trituration of the residue with acetone, followed by recrystallization from ethanol yielded pure NorBCM.

Formation and decay of aziridinium ion derivatives

The pharmacologically active form of BCM is the aziridinium ion formed in aqueous solution at neutral pH. The formation and decay of this ion can be followed by using the quantitative reaction with thiosulphate (Golumbic, Fruton & Bergmann, 1946; Gill & Rang, 1966) and the results from kinetic studies with some of the mustards are summarized in Table 2. No aziridinium ion was detected from NorBCM.

Table 2. *Formation and decay of aziridinium ion derivatives in 10* mM *phosphate buffer, pH 7·5.* The mustard, dissolved in a small volume of acetone or ethanol, was added to 10 mM phosphate buffer, pH 7·5, to give a final concentration of approximately 0·8 mM, the initially cloudy solution becoming clear as cyclization proceeded. The concentration of aziridinium ion was measured as described by Gill & Rang (1966). Values are means ± standard error with the number of determinations in parentheses. The half-time of decay has been calculated from the first-order rate constant obtained from the slope of a plot of log [(aziridinium ion concentration)/(aziridinium ion concentration at zero time)] *vs* time.

Compound	Temp.	Max. yield of aziridinium ion (%)	At time (min)	Half-time of decay (min)
BCM	30°	84 ± 1	20	141 ± 3 (3)
PrBCM	20°	91 ± 0·5	50	457 ± 17 (3)
PrBCM	30°	84·5 ± 1·5	20	133 ± 1 (5)
PrBCM	37°	84 ± 1	7	56 ± 2 (3)
BuBCM	30°	79·5 ± 2	35	149 ± 2 (3)
PentBCM	30°	65 ± 1	100	157 ± 6 (5)
CBCM	30°	74	55	148 (1)

The rate of cyclization falls with increasing size of the *N*-alkyl group and with the substitution of a phenyl by a cyclohexyl residue, but both of these effects are largely a consequence of the decreasing solubility of the parent chloroethylamine in aqueous solution at pH 7·5, only BCM being completely soluble at the concentration employed. In contrast the aziridinium ion derivatives are all soluble and the rate of decay, presumably hydrolysis, was little affected by the size of the *N*-alkyl substituent. The smaller maximum yields with the more slowly cyclizing mustards can be ascribed to increased loss through hydrolysis. The rate of hydrolysis is markedly temperature-dependent (for the ion from PrBCM $E_a = 22$ kcal mol, 92 kJ mol^{-1}); and at 0° is practically zero.

The rate was also slower in Krebs-Henseleit medium ($t_{\frac{1}{2}} = 223 \pm 9$ min at 30°) than in 10 mM phosphate buffer ($t_{\frac{1}{2}} = 133 \pm 1$ min). Approximately half of this decrease is the result of a stabilizing effect by the 128 mM chloride ion present, nucleophilic attack on the aziridinium ion reforming the parent chloroethylamine, but we have not investigated what factor(s) are responsible for the remainder.

The activity of BCM homologues as alkylating muscarinic antagonists

The potency of the antagonists was measured on strips of guinea-pig ileum longitudinal muscle suspended in Krebs solution bubbled with 5% carbon dioxide in oxygen, using carbachol (Koch-Light) as agonist. Contractions were recorded isotonically. Nominal receptor availability, γ, was obtained from the relation (Paton, 1961)

$$\gamma = 1/\text{dose ratio}$$

and the rate constant for formation of the reversible antagonist-receptor complex, k_1, was determined from the slope, $k_1 \times$[aziridinium ion], of a plot of log γ against time (Gill & Rang, 1966). This plot was linear for all the mustards tested (three examples are shown in Fig. 1), implying that in all cases once the reversible complex is formed the rate at which it dissociates is slower than the rate of chemical reaction to form the

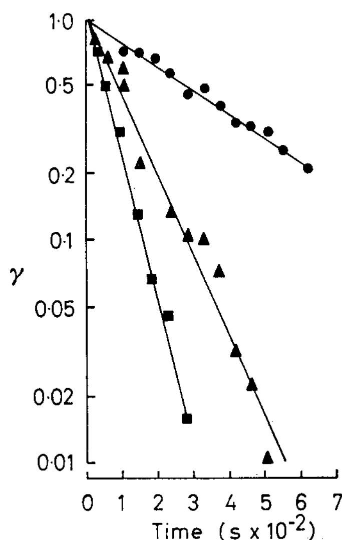


FIG. 1. Rate of decrease of the fraction of free receptors (γ) in longitudinal muscle strips from guinea-pig ileum in the presence of homologues of BCM. The compounds were cyclized in 10 mM phosphate buffer, pH 7·5, at 30° before addition to the bathing solution. Curves are for the aziridinium ions from PrBCM (-■-), 33·6 nM; BuBCM (-▲-), 102 nM; and PentBCM (-●-), 202 nM.

Table 3. *Rate of inactivation of muscarinic receptors by homologues of BCM.*

Compound	k_1 (M^{-1} s^{-1})	
	30°	37°
BCM	$1{\cdot}8 \pm 0{\cdot}2 \times 10^5$ (5)	$2{\cdot}4 \pm 0{\cdot}2 \times 10^5$ (6)
PrBCM	$1{\cdot}7 \pm 0{\cdot}2 \times 10^5$ (6)	$2{\cdot}0 \pm 0{\cdot}3 \times 10^5$ (4)
BuBCM	$2{\cdot}7 \pm 0{\cdot}5 \times 10^4$ (5)	
PentBCM	$1{\cdot}2 \pm 0{\cdot}1 \times 10^4$ (5)	
CBCM	$7{\cdot}2 \pm 1{\cdot}0 \times 10^4$ (5)	

Values are means ± standard error with the number of determinations in parentheses.

covalently bonded complex. The value of k_1 is thus an index of the rate of inactivation of the receptor. The values obtained (Table 3) were independent of the concentration of antagonist employed, which in the case of the ion from PrBCM ranged between 7 and 200 nM. PrBCM reacts as fast as BCM, but the rate falls off sharply with BuBCM and PentBCM; CBCM also reacts more slowly. The temperature dependence of k_1 is relatively small ($E_a \sim 6$ kcal mol^{-1}; 25 kJ mol^{-1}). The rate constant for BCM at 37° is considerably lower than that, $1{\cdot}1 \times 10^6$ $M^{-1}s^{-1}$, found by Gill & Rang (1966), but the reason for this discrepancy is not clear.

Recovery from the block produced by these antagonists is very slow and we have made accurate measurements only for strips inactivated by PrBCM, where the process followed first order kinetics with a rate constant of $6 \pm 1 \times 10^{-6}$ s^{-1} at 37°, equivalent to a half time of 32 h. This is somewhat longer than the 12 h reported for the recovery from block by BCM (Gill & Rang, 1966).

DISCUSSION

The results show that, of the *N*-alkyl homologues of BCM, only PrBCM has a similar rate of reaction with the muscarinic receptor; further increase in chain length leads to a marked fall off in the rate of reaction. Interestingly, k_1 for CBCM was significantly lower than for BCM. We prepared CBCM because the choline ester of cyclohexylphenylglycollic acid has a higher affinity than benzilylcholine for the muscarinic receptor (Abramson, Barlow & others, 1969) and it seemed probable that the higher affinity compound would show a rate of complex formation that was at least as fast, if not faster, than benzilylcholine. For this reason and assuming that the binding properties of aziridinium and trimethylammonium are similar, we had expected that k_1 for CBCM might be larger than k_1 for BCM, whereas the reverse is observed.

As a potential label for use in the isolation of muscarinic receptors PrBCM has one particular advantage over BCM in that it is easier to introduce tritium to high specific activity in a known position on the nitrogen side of the ester bond and in a subsequent publication we shall describe the preparation and properties of such a derivative.

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