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Synthesis and Characterization of Methylbromoamiloride, a Potential Biochemical Probe of Epithelial Na^+ Channels

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Summary. We report the synthesis of a radioactive, methylated analog of bromoamiloride which inhibits the amiloride-sensitive, epithelial Na^+ channel reversibly and with high affinity. This synthesis was achieved by methylation of a nitrogen in the acylguanidinium moiety with tritiated methyliodide of high specific activity. This methylated bromoamiloride molecule (CH_3BrA) was purified by both thin layer and high performance liquid chromatography. Proton nuclear magnetic resonance and mass spectroscopy techniques were used to determine the structure of this analog. This compound inhibited both short-circuit current of *in vitro* frog skin and $^{22}Na^+$ influx into apical plasma membrane vesicles made from cultured toad kidney cells (line A6) with the same or lower apparent inhibitory dissociation constant as bromoamiloride. Irradiation with ultraviolet light rendered this inhibition irreversible in both A6 vesicles and frog skin. Preparation of radioactive CH_3BrA yielded specific activities in excess of 1 Ci/mmol. We suggest that this compound will be useful in the isolation and purification of this ubiquitous Na^+ channel.

Key Words amiloride · methylation · frog skin · A6 cells · Na^+ channel · epithelia

Introduction

The epithelial Na^+ channel is a protein of both clinical importance and intellectual interest. In tight epithelia it provides the rate-limiting step for Na^+ reabsorption and hence is a key control point in electrolyte and water balance. Its Na^+ selectivity and pharmacology define it as a unique ion channel, very different in behavior from the Na^+ channel of electrically excitable membranes (Sariban-Sohraby et al., 1984). While it is clearly a protein worthy of isolation and reconstitution, biochemical efforts have been difficult because of the lack of a specific ligand suitable for labeling the channel. The diuretic drug amiloride, although a potent and specific inhibitor of the epithelial Na^+ channel, is a poor label because of its rapid reversibility.

We have previously reported that one analog of amiloride, 6-bromoamiloride (BrA), irreversibly inhibits Na^+ transport across frog skin after irradiation with ultraviolet light (Benos & Mandel, 1978). This observation has been confirmed subsequently by Cobb and Scott (1981) in toad bladder and by Kleyman et al. (1983) for bromobenzamil in membrane vesicles prepared from toad bladder and mammalian renal cortex. However, ultraviolet irradiation at 254 nm on the apical surface of *Rana temporaria* skin by itself produces an irreversible inhibition of Na^+ transport (Cuthbert et al., 1982), unlike the effect in *R. catesbeiana* skin (Benos & Mandel, 1978).

A significant obstacle to be overcome in using bromoamiloride as a site-specific probe is to incorporate into the molecule a radiolabel of high specific activity, without altering the parent compound's potency. Tritium labeling via catalytic exchange or gas exposure is not feasible because all of the hydrogen atoms readily exchange with water. Carbon-14 labeling is possible (Cuthbert & Shum, 1976), but the final specific activity is too low to be useful in biochemical work. Our strategy, then, was to synthesize a radiolabeled analog of bromoamiloride which would irreversibly inhibit Na^+ entry under mild reaction conditions. This goal was accomplished by methylation of a nitrogen in the acylguanidine moiety with tritiated methyl iodide of high specific activity. This acylguanidine side chain seems to be the most permissive position of the amiloride molecule in that modifications can be made here that do not seriously influence drug potency (Cragoe et al., 1967; Shepard et al., 1969; Garcia-Romeu, 1970; Benos et al., 1976; Cuthbert & Fanelli, 1978). The methylated bromoamiloride molecule (CH_3BrA) was purified by both thin layer and high performance liquid chromatography. This compound inhibited both short-circuit current (I_{sc}) of isolated frog skin and ^{22}Na influx into apical plasma membrane vesicles made from A6 cells with the same or lower apparent inhibitory dissociation

constant (K_I') as bromoamiloride. Irradiation with UV light rendered this inhibition irreversible. Preparation of radioactive CH_3BrA yielded specific activities in excess of 1 Ci/mmol. We suggest that this compound will be useful in the isolation, purification, and reconstitution of this ubiquitous epithelial Na^+-conducting molecule.

Materials and Methods

Synthesis of Methylbromoamiloride

Methylations were performed using a modification of the procedures of Hakomori (1964). Methylation of bromoamiloride was accomplished by adding an excess of alkylhalide (CH_3I or CH_3Br) under basic conditions in aprotic solvents. A 10 mM solution of bromoamiloride in dimethylsulfoxide (DMSO) or dimethylformamide (DMF) was made fresh, and an excess (usually 5 to 10-fold) of CH_3I (10% vol/vol solution in methanol) was slowly added. The reaction was allowed to proceed at room temperature in the dark. The extent and time course of methylation was followed either by bromide ion release using a Br^- electrode or by thin layer chromatography (*see below*). In the radioactive synthetic procedure, DMF was the solvent of choice because the methyl groups of DMSO could exchange with the tritiated methyl group of CH_3I via the formation of the short-lived trimethyloxosulfonium iodide intermediate (Cotton et al., 1959), resulting in a greatly diminished radiolabel incorporation. For both time convenience and maximum specific activity of final methylated product, a 5:1 molar excess of CH_3I was employed. Prior to purification, the reaction mixture was dried under a stream of nitrogen at room temperature, in a fume hood, and redissolved in either DMSO or methanol.

Analytical Procedures

Thin Layer Chromatography

The methylated reaction products and BrA were separated using silica gel thin layer plates containing calcium sulfate as binder (Analtech, Inc., Newark, DE). The solvent system consisted of chloroform/methanol/10 N NaOH (4:1:0.1). The plates were prewashed and activated by heating at 80°C for 30 min prior to use. Samples were spotted on the plates and then run. Plates with radioactive products were sprayed with ENHANCE (New England Nuclear, Boston, MA), and exposed to unflashed Kodax XAR-2 X-ray film for 1–2 days at −70°C. The resulting fluorographs were developed by autoprocessing (Kodax X-OMAT M-60).

High Pressure Liquid Chromatography

In some cases, the methylated reaction products were separated and purified by HPLC using a silica preparative column and a solvent system of chloroform/methanol/10 N NaOH (4:1:0.1). The instrument was a Micromeritics model 9000 chromatograph equipped with a Waters Associates model 440 detector that monitored UV absorbance at 254 and 313 nm. The solvent flow rate was 1–5 ml/min, depending upon the amount of sample injected. UV spectra of the purified products were obtained in water on a Beckman Acta CIII UV-visible recording spectrophotometer.

Nuclear Magnetic Resonance Spectroscopy

All analyses were performed at 90 MHz on a Nicolet NT-360 wide base spectrometer. ^1H NMR spectra were determined in deuterated dimethylsulfoxide (Me_2SO_4-d_6). The spectrometer was operated using a 4000-Hz spectral width, a data line of 8000, and a 5–7 μsec pulse width. ^1H chemical shifts were expressed as hertz values relative to tetramethylsilane as internal standard. The chemical shifts of the methylated products generated via the reaction indicated above were compared with those determined for known methylated model pyrazine compounds dissolved in Me_2SO_4-d_6.

Mass Spectroscopy

The computer-assisted mass spectroscopy analyses were performed using a Hewlett-Packard 5985 GC-MS system, running in the electronic ionization mode.

Frog Skin Experiments

The abdominal skin of the bullfrog, *Rana catesbeiana*, was mounted as a flat sheet (3.14 cm^2 in area) between Lucite chambers equipped with glass solution reservoirs. The solutions (12 ml in each chamber) were stirred and oxygenated by bubbling with room air. All experiments were performed at 19°C.

The open circuit potential across the skin was measured with calomel electrodes, and current was passed through the skin via platinum electrodes. The potential sensing electrodes were connected to the solution reservoirs through 2% agar bridges having a composition identical to that of the bathing solution in the chambers. An automatic voltage clamp was used to pass and monitor the appropriate amount of current through the skin to clamp the transepithelial membrane potential to 0 mV. The voltage clamp circuit also compensated for the resistance of the solution between the agar bridges. In all experiments both sides of the skin were bathed with identical solutions. Under these conditions, the magnitude of the I_{sc} and the net active transport of sodium are equivalent (Cereijido et al., 1974; Candia & Reinach, 1977; Benos, Mandel & Balaban, 1979).

A6 Cell Culture and Apical Membrane Vesicle Preparation

A6 cells were purchased from American Type Culture Collection (Rockville, MD) in the 68th plating. Stock cultures were carried as described previously (Sariban-Sohraby, Burg & Turner, 1983), except that 5% rather than 10% fetal bovine serum was used in the growth medium. Lowering the serum concentration did not appear to alter the growth pattern of the cells or the development of transepithelial voltage. Vesicles were prepared from A6 cells grown in filter-bottomed cups. The cups were made from rings of acrylic tubing 1 cm high and 10.7 cm in diameter. A Millipore filter (0.45 μm, HAWP) was attached to

one end of the cup in the following way. The filters were soaked for 10 min in a solution of 30% ethanol in water (vol/vol) then removed and placed on a flat surface. The acrylic rings were dipped for 5 sec in methylene chloride and firmly applied to the wet filters for 10 sec. Three small polycarbonate plastic strips 0.25 mm thick were glued to the underside of each cup along its outer edge with chloroform to elevate it from the underlying surface. Filter bottomed cups were sterilized in 70% ethanol (vol/vol) for 20 min, then rinsed in sterile distilled water and left overnight in phosphate buffered saline. A few hours before seeding with cells each filter-bottomed cup was placed in a 14-cm tissue culture dish (Falcon No. 1013) and soaked in growth medium. Cells were fed twice weekly. The volumes of growth medium (25 ml on the serosal side and 20 ml on the mucosal side) were chosen to equalize the fluid levels inside and outside the cup. Transepithelial voltage was measured using sterile salt bridges placed in the fluid inside and outside the cups (Sariban-Sohraby et al., 1983). Cells were grown at 28°C in a humidified incubator gassed with 1% CO_2 in air. The growth medium consisted of Dulbecco's modified Eagle medium modified for amphibian cells to contain 75 mM NaCl, 8 mM $NaHCO_3$, 1 mM Na pyruvate, 0.4 mM L-glutamine, 5.56 mM glucose, and 0.0173 mg/liter selenium, final osmolality = 220 mOsm/kg (Gibco Laboratories, Grand Island, NY, Formulation #84-5022).

Apical plasma membrane vesicles were prepared as follows. Six filter-bottomed cups containing confluent epithelia were rinsed twice with ice-cold homogenization medium (10 mM Tris-HEPES, pH 7.4, containing 30 mM mannitol and 10 mM $CaCl_2$). The cells were scraped into homogenization medium from the filters with a rubber policeman and diluted to a total volume of 30 ml. The scraped cells were left on ice for 10 min then homogenized at 4° in a VirTis model 23 homogenizer for 10 min at setting 4 and for 4 min at top speed. The homogenate was left on ice for 10 min and then centrifuged for 15 min at 5500 × g. The supernatant was saved and centrifuged for 20 min at 43,000 × g. The resulting pellet was suspended in 3 ml of a buffer appropriate for the experiment and recentrifuged at 43,000 × g. This pellet was resuspended in the appropriate buffer, passed successively through 22, 25, and 30 gauge needles and stored on ice until use. This procedure typically yielded 2.0–2.5 mg membrane protein. Aliquots of the homogenate and the vesicle preparation were frozen in a dry ice/ethanol slush and stored above liquid nitrogen. We generally used fresh material for transport studies, but found no systematic difference in the amiloride-sensitivity of vesicles frozen for less than one month.

Unless otherwise stated, $^{22}Na^+$ influx measurements were carried out under equilibrium exchange conditions in 10 mM Tris-HEPES, 100 mM mannitol, and 1 mM NaCl, pH 7.4. The incubation medium contained 10 μCi $^{22}Na^+$/ml, and 5 μCi [^{3}H] mannitol/ml. A 50 μl aliquot of vesicles (approx. 1 mg protein/ml) was placed in a 12 × 75 mm glass test tube, and at time zero a 100 μl aliquot of incubation medium was added. After an appropriate time the reaction was stopped by adding a 10-fold volume of ice-cold stop solution (A6 Ringer containing 10 mM mannitol and 10^{-4} M amiloride). The vesicles were then immediately applied to a Millipore filter (0.45 μm HAWP) under light suction. The filter, which retained the vesicles, was then washed with a further 4.5 ml of cold stop solution, dissolved in 0.5 ml ethylacetate and 10 ml of liquid scintillation fluid, and counted for radioactivity along with samples of the incubation medium and appropriate standards. The entire stopping and washing procedure took less than 30 sec, during which the vesicles were in contact with the stop solution for less than 20 sec.

All experimental points are averages of at least three determinations at room temperature (25–28°C). The simultaneously measured "uptake" of [^{3}H] mannitol has been used to correct $^{22}Na^+$ fluxes for extravesicular trapping by the membranes and filters. Sodium equilibrium exchange fluxes were calculated from 15-sec points. Control experiments (Sariban-Sohraby et al., 1984) have shown that $^{22}Na^+$ uptake into the vesicles is linear with time over this interval.

Photolysis Experiments

Vesicles were photolyzed with 254 nm UV light emitted from a low pressure mercury lamp (Oriel Optics Corp., Stamford, CT; energy output = 14 μW/cm^2, emission maximum = 254 nm). The A6 vesicles (0.125 mg/ml protein) were passed through a narrow bore (0.1 mm inside diameter) quartz coil surrounding the arc lamp by a peristaltic pump at a flow rate of 3 ml/min. The experiments were conducted on ice, and the vesicles were exposed to the UV light for no more than 5 min. Preliminary experiments have shown that longer exposure times (>10 min) result in significant destruction of Na^+ channels and CH_3BrA. The vesicles were suspended in a 1 mM NaCl, 10 mM Tris-HEPES (pH 7.4) buffer solution containing 1–2 μM BrA or CH_3BrA in the presence or absence of a 100-fold excess of amiloride. After photolysis, the vesicles were collected, the coil was washed with 4 ml of fresh buffer solution, and the mixture ultracentrifuged for 1 hr at 30,000 RPM's (Beckman L8-80, SW 41 Rotor). After centrifugation, the supernatant was removed by aspiration, and the vesicles resuspended in an appropriate volume of buffer for measurement of $^{22}Na^+$ influx. Irradiation experiments in the frog skin were performed essentially as described by Benos and Mandel (1978).

Chemicals

All chemicals were of reagent grade, and all solutions were made with doubly glass distilled water which was first passed through an ultra high purity demineralizer cartridge (Corning 3508A). Bromoamiloride and other analogs were obtained as a gift from Merck Sharp, & Dohme Research Laboratories (West Point, PA). All other organic compounds were obtained from Aldrich Chemical Company (Milwaukee, WI).

Results

The rate of methylation of bromoamiloride is strongly dependent upon a number of factors, including the concentration of methyliodide, the nature of the solvent, and the pH. Using model pyrazine ring compounds and methylbromide as the methylating agent (so as to monitor reaction extent from the appearance of free Br^- in solution with a Br^--ion sensitive electrode), we found that aprotic solvents like DMSO and DMF support the methylation of BrA, while methanol, 1, 3-dioxane, or acetonitrite do not. Further, the reaction only occurred at basic pH (pH optimum = 9.8); no measurable methylation occurred when the pH was below 7. These initial observations were confirmed using the

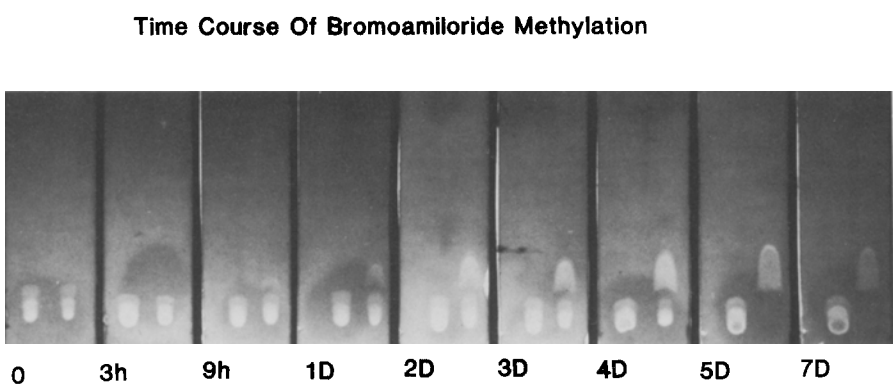

Fig. 1. Thin layer chromatography showing time course of methylation reaction between bromoamiloride and methyliodide. Exactly 2 μl of either a 10 mM solution in dimethylformamide of bromoamiloride or the methylation reaction mixture were spotted on an activated analytical TLC silica G plate and run 7 cm with a solvent system of chloroform/methanol/10 N NaOH (4 : 1 : 0.1). The dried plates were photographed under ultraviolet light. Bromoamiloride was spotted on the left in all plates

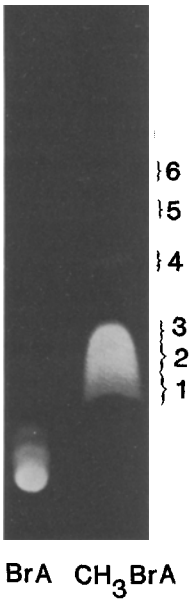

Fig. 2. Thin layer chromatograph of bromoamiloride and the completely reacted methylation reaction mixture (10 days of reaction time). Numbers on right indicate position of isolated reaction products. Conditions were the same as indicated in the legend to Fig. 1

techniques described below for CH_3I and for the substrates BrA and amiloride.

Because CH_3I reacts primarily with amino groups and because there are potentially eight methylation sites, a reliable method for monitoring the extent of reaction as well as separating the reaction products had to be devised. We chose high performance thin layer chromatography for this purpose. The solvent system which gave the most efficient separation was chloroform/methanol/10 N NaOH (4 : 1 : 0.1). Figure 1 shows the time course of the methylation of bromoamiloride in the presence of a fivefold molar excess of CH_3I (10 mM BrA; 50 mM CH_3I). At 9 hr, reaction product(s) is (are) beginning to appear. After 5 days, the reaction is complete as assessed by no further changes in the thin layer chromatography pattern. A 10-fold molar excess of CH_3I resulted in the methylation reaction reaching completion in 2 days. Any reaction mixture below a fivefold molar excess of CH_3I did not reach completion for at least two weeks.

Figure 2 shows a more detailed thin layer separation of the methylated bromoamiloride products. At least six reaction products can be distinguished. These bands have been labeled products *1* through *6*. Their average R_f (defined as ratio of distances run by sample over solvent) values were (beginning at #1): 0.21, 0.28, 0.33, 0.47, 0.56, 0.65. These compounds were scraped from preparative Silica G plates, extracted in methanol and, after evaporation, redissolved in DMSO. The concentration of each sample was determined spectrophotometrically at 365 nm using a molar absorption coefficient of 28,200 units cm^{-1} $mole^{-1}$. This calculation assumed that the methylated products had the same absorbance characteristics at this wavelength as the parent BrA (*see below*).

Figure 3 presents log dose-response curves of bromoamiloride and methylated bromoamiloride reaction product inhibition of short-circuit current (I_{sc}) in *in vitro* frog skin epithelia (concentration range: 5×10^{-8} to 10^{-6} M). The inhibition of I_{sc} by products 1, 2, and 3 was similar to that produced by bromoamiloride (apparent inhibitory dissociation constant, K_i', 0.1 μM). Compounds 4, 5, and 6, on the other hand, had only a very slight effect on I_{sc} in the concentration range studied. Thus, the methylated reaction products 1, 2 and 3 retained biological activity, while compounds 4–6 did not.

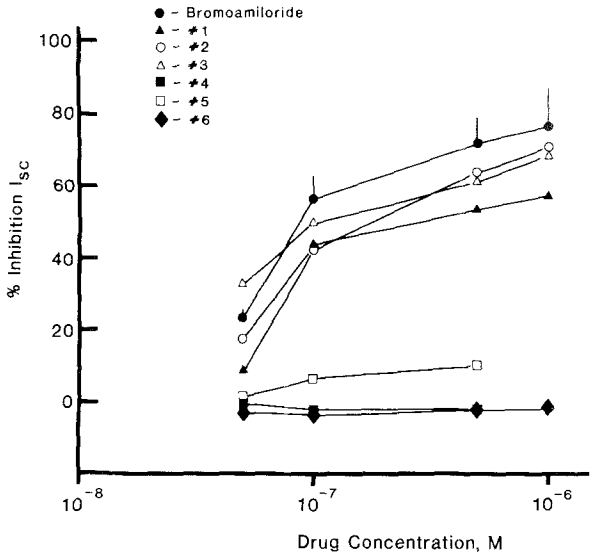

Fig. 3. Log dose response curves of the inhibition of short-circuit current (I_{sc}) of frog skin produced by bromoamiloride and different methylated bromoamiloride derivatives. Each product was isolated from a preparative thin layer plate by extracting the compound from the silica with methanol. The methanol was evaporated under nitrogen and the compound redissolved in DMSO. The final concentration was estimated by absorbance measurements at 365 nm. Each point represents the mean value of six experiments. The standard error bars (vertical lines) were omitted for clarity, but were of comparable magnitude to those shown for bromoamiloride

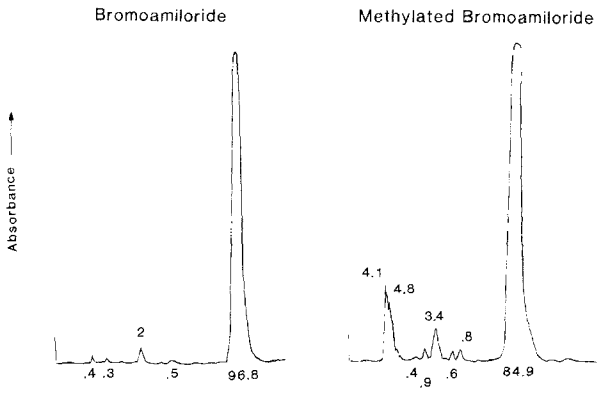

Fig. 4. HPLC separation of bromoamiloride and the methylation reaction mixture. A silica column with chloroform/methanol/10 N NaOH (4 : 1 : 0.1) as the solvent system was used. A 10-μl sample of a 10-mM solution was injected; flow rate was 1 ml/min. The numbers above or below each peak represent the area % of that peak

We next wanted to obtain some structural information concerning the site or sites of methylation of the active reaction products. For these studies we decided to separate our reaction mixture by high pressure liquid chromatography (HPLC). Typical chromatographs of this separation are shown as Fig. 4. Bromoamiloride separated as one major peak, while the methylated reaction mixture displayed one major and several minor components. The major peaks for bromoamiloride and the methylated compound separated in the same place in the chromatograph. The major methylated bromoamiloride peak inhibited I_{sc} of bullfrog skin epithelium in a comparable manner as the parent BrA (K_i of 0.72 ± 0.11 μM versus 0.50 ± 0.13 μM, respectively, $n = 5$).

Proton nuclear magnetic resonance of this peak demonstrated that this molecule contained a single methyl group at 190 Hz (Fig. 5). ^1H HMR spectra of BrA (*not shown*) displayed no proton peaks at all. The large signal at 420 Hz also appeared in BrA spectra and was due to H_2O contamination in each sample. The position of the methyl signal in the spectra indicated that the CH_3 group was probably contained within a secondary amine nitrogen. Comparisons of ^1H NMR spectra of different amiloride structural analogs containing methyl groups at known positions suggested that this major reaction product was not methylated on the pyrazine ring amino groups, nor on the terminal acylguanidine amino groups. Hence, we thought that methylation of the nitrogen adjacent to the carbonyl group was most likely. Definitive proof of this possibility was achieved by mass spectroscopy.

Figure 6 presents mass spectra of BrA and the major methylated BrA derivative as separated by HPLC. Mass spectra were obtained by electronic ionization of the parent compounds. The intensity of each signal reflects the relative abundance of the fragmented ion producing the signal. Analysis of these spectra is consistent with placement of the methyl group on the acylguanidinium nitrogen adjacent to the carbonyl moiety as shown, and inconsistent with any other placement. This placement was determined based upon the following observations. First, the preservation of peaks at 187,189 and 215,217 in cuts 2 and 3 in both molecules without the appearance of peaks at values 14 greater than these in the methylated derivative shows that methylation did not occur on any of the nitrogens associated with the pyrazine ring. [Note: Any fragment containing a single bromide atom will show a double peak because bromide has two equally abundant, naturally occurring isotopes, one with an atomic mass number of 79 and the other 81.] Second, the doublet at 231,233 disappeared and a new doublet appeared at 246,248 in the methylated product. These changes most likely result from a cut at position 1 as shown, and are suggestive of the addition of a methyl group to the nitrogen adjacent to the carbonyl group. Strict addition, however, predicts a doublet at 230,232 in bromoamiloride and at 244,246

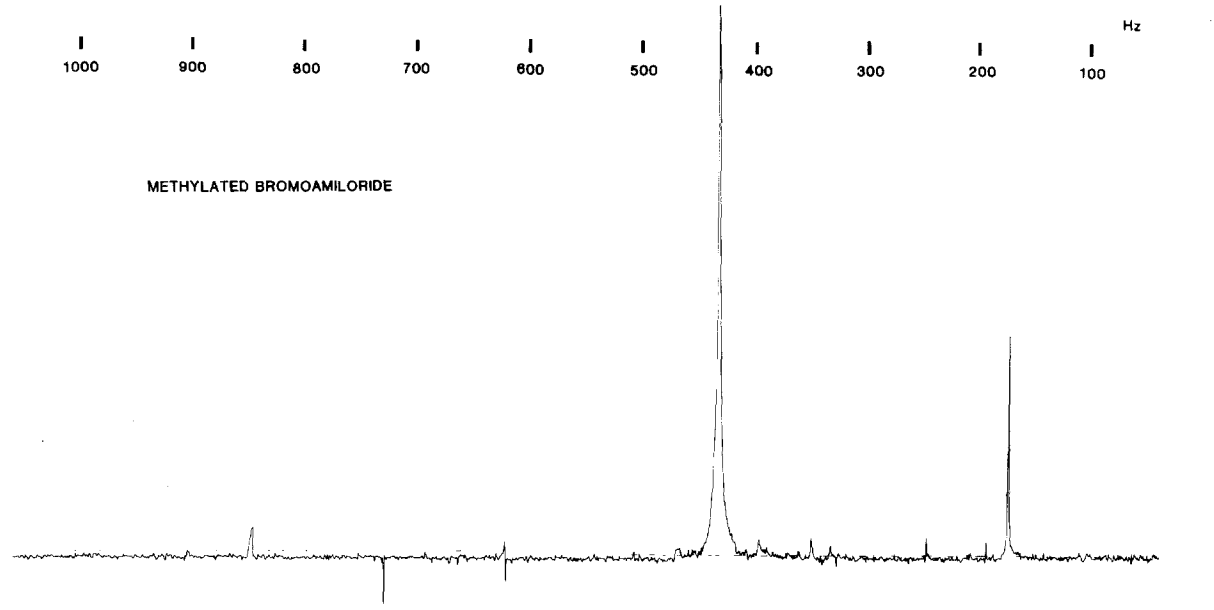

Fig. 5. Proton NMR spectrum of the major methylated bromoamiloride reaction product. The large peak at 430 Hz is from water contamination and hence also appeared in the bromoamiloride spectrum. The single peak at 185 Hz is consistent with a single methyl group located on the product. The magnetic field strength was 90 MHz, and 70 scans were taken

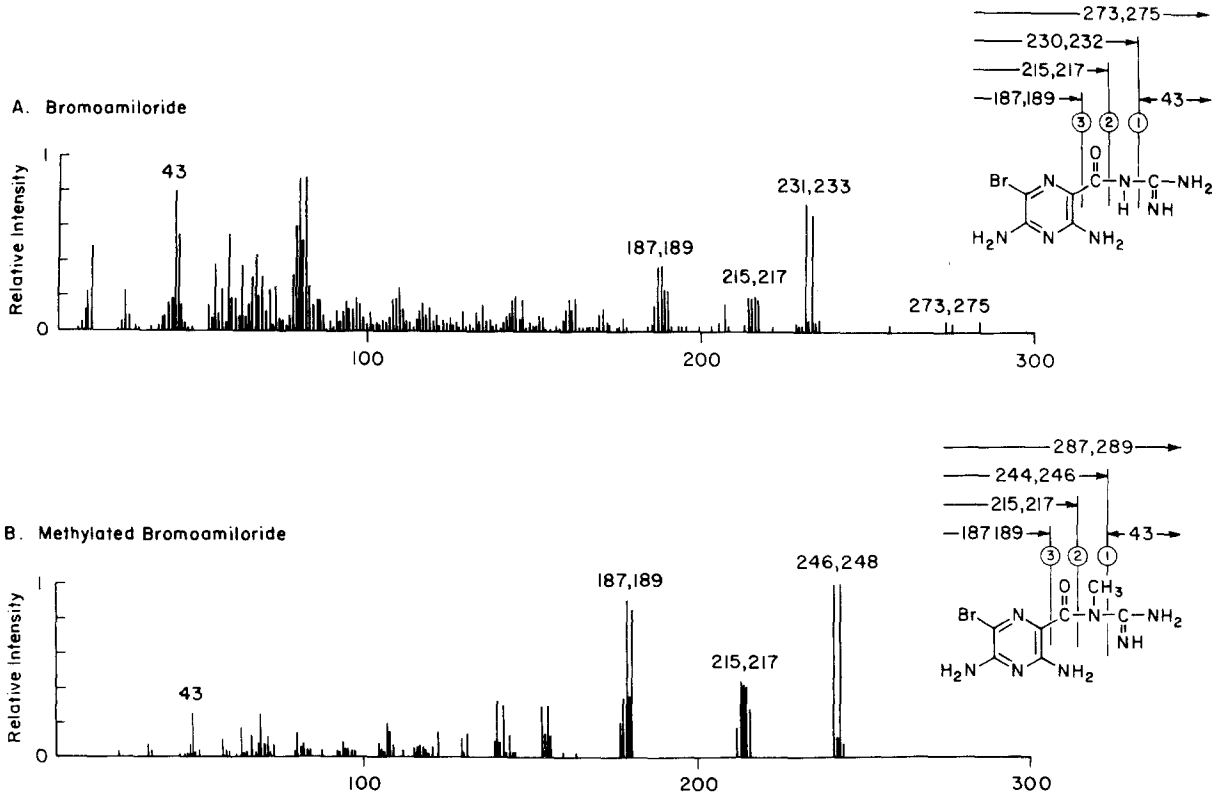

Fig. 6. Mass spectra of bromoamiloride (*A*) and the major methylated bromoamiloride reaction product. *See* text for details

in the methyl product rather than the 231, 233, and 246, 248 observed. This discrepancy most likely results from post-fragmentation modification of these ions by the addition of protons to increase molecular stability. Third, the single peak at mol wt 43, which represents the fragmented guanidinium group appearing from cut 1, is preserved in both spectra. The preservation of this peak, with the dis-

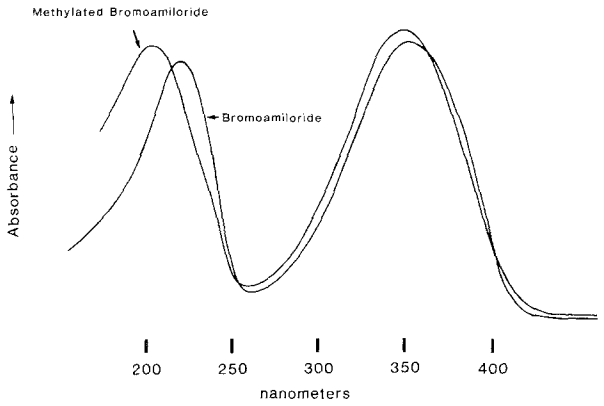

Fig. 7. Ultraviolet absorption spectra of amiloride and methylated bromoamiloride. The concentration of each compound was adjusted to be 50 μM from absorbance measurements at 365 nm

appearance of the 231,233 peak as described previously, makes the placement of the methyl on a terminal guanidinum nitrogen unlikely.

We have also compared the mass spectra of compounds 1–6 isolated from TLC plates (*cf.* Fig. 2) with the spectrum of CH_3BrA from HPLC. It appears that compound 3 is identical to the major CH_3BrA component as resolved by HPLC. This finding is reasonable in that compound 3 is the major product recovered from the thin layer plate.

Figure 7 compares the UV absorbance spectra of BrA and the purified CH_3 BrA product. Compound 3 from TLC had identical UV absorbance characteristics as the major HPLC separated compound.

Table 1 summarizes the results of experiments where $^{22}Na^+$ uptake into A6 apical membrane vesicles was measured in the absence or presence of 100 μM bromoamiloride and 35 μM methylated bromoamiloride. At these concentrations, BrA inhibited $^{22}Na^+$ uptake by 53.4% while CH_3BrA inhibited uptake by 82.9%. In other experiments, the suitability of this vesicle preparation as a model system for photolysis experiments was examined (Table 2). Both amiloride and bromoamiloride inhibited $^{22}Na^+$ influx into these vesicles (31.5% and 45.7% at 100 μM, respectively). Irradiation of the vesicles themselves in the absence of bromoamiloride produced no inhibition of the $^{22}Na^+$ influx. However, a 10-min irradiation period in the presence of 1.7 μM bromoamiloride (an additional 1.7 μM bromoamiloride was added to the medium halfway through the irradiation period) resulted in a 55% irreversible inhibition of $^{22}Na^+$ influx. We interpret this inactivated portion of the $^{22}Na^+$ influx to be the result of the irreversible binding of BrA to the Na^+ channels present in the membrane. Preliminary experiments using methylbromoamiloride yielded essentially comparable results. No irreversible inhibition of

Table 1. The effect of bromoamiloride and methylbromoamiloride on $^{22}Na^+$ influx into A6 apical membrane vesicles

Condition	J^i_{Na} (nmol Na^+/mg protein · min)
Control	2.34 ± 0.58
100 μM bromoamiloride	1.09 ± 0.17
35 μM methylbromoamiloride	0.40 ± 0.03

Table 2. Sodium influx into A6 apical membrane vesicles under different experimental conditions

Experimental condition	J^i_{Na} (nmol Na^+/mg protein · min)
Control	1.40 ± 0.05
100 μM amiloride	0.94 ± 0.05
100 μM bromoamiloride	0.76 ± 0.19
UV irradiation (10 min, 254 nm)	1.48 ± 0.08
UV irradiation + 1.7 μM bromoamiloride	0.62 ± 0.05

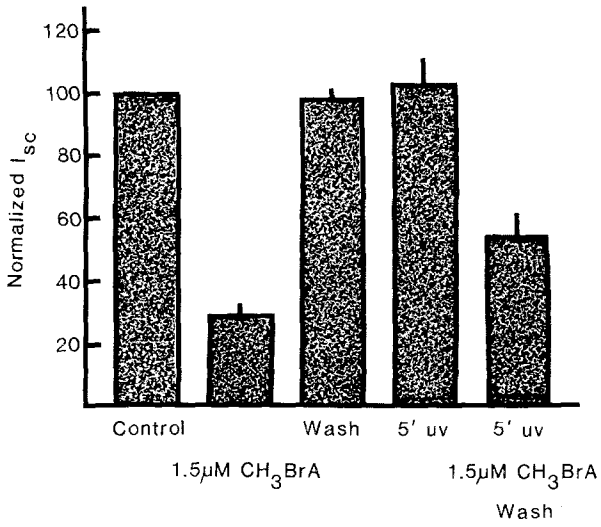

Fig. 8. The effect of methylbromoamiloride (CH_3BrA) and ultraviolet irradiation on the short-circuit current (I_{sc}) of *in vitro* bullfrog skin epithelium. The results are expressed as the mean normalized I_{sc} (vertical bars indicate 1 SEM, $n = 4$). The mean transepithelial conductances before any experimental manipulation, in the presence of 1.5 μM CH_3BrA, after washing, after a 5-min UV irradiation in the presence of 1.5 μM CH_3BrA, and subsequent to exhaustive washing with drug-free Ringer solution were (in mS/cm^2): 0.611 ± 0.026, 0.252 ± 0.016, 0.655 ± 0.028, 0.597 ± 0.064, and 0.429 ± 0.081, respectively

$^{22}Na^+$ influx was noted if amiloride was used in the irradiation experiment (*data not shown*).

We also performed experiments designed to test whether this methylated bromoamiloride analog could irreversibly inhibit sodium transport in *in vitro* frog skin after photoirradiation. The results of these experiments are summarized in Fig. 8. Addi-

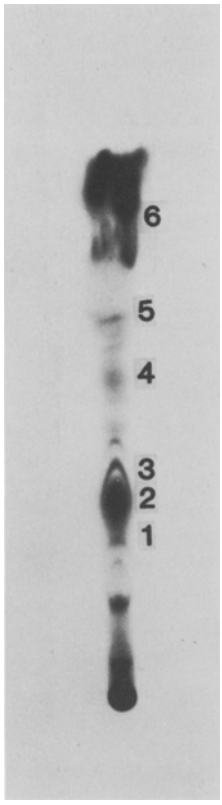

Fig. 9. Fluorograph of ^{3}H-methyl bromoamiloride products after 12 days of reaction. The numbers correspond to the R_f values determined from TLC as shown in Fig. 2. Two microliters of the radioactive reaction mixture were spotted onto an analytic TLC plate and run as described in Fig. 1. After the plate was dried, it was sprayed with ENHANCE, covered with X-ray film, and exposed for 1 day at −70°C

tion of 1.5 μM CH_3BrA reversibly inhibited I_{sc} by 70.5 ± 2.4%. A 5-min period of ultraviolet irradiation of the apical surface of the frog skin did not by itself affect I_{sc}. Photoirradiation in the presence of 1.5 μM amiloride likewise caused no irreversible inhibition of I_{sc} (*data not shown*). These results contrast with the irreversible inhibition (46.2 ± 7.1%) produced after a 5-min ultraviolet exposure in the presence of 1.5 μM CH_3BrA. In two experiments, a second 5-min exposure to UV in the presence of 1.5 μM CH_3BrA produced an additional 50 and 39.8% irreversible inhibition of I_{sc}.

We have performed six successful radioactive syntheses. An autoradiogram of a thin layer chromatography run of the radioactive mixture is shown in Fig. 9. Radioactive methylated products are evident. By day 12, all six reaction products that were identified by fluorescence are radioactively labeled. Not surprisingly, there appears to be other products as well. Isolation of product 3 from the thin layer plate and determination of its specific activity yielded a value of 1.1 Ci/mmol.

Discussion

The purpose of our present work was to synthesize an analog of bromoamiloride which would (i) retain the ability to inhibit Na^+ transport through epithelial channels, (ii) bind irreversibly under appropriate conditions, and (iii) possess a high and stable radioactive specific activity. Also we wanted to have the analog synthesized and purified in a relatively simple and straightforward fashion. Our approach was to methylate one of amiloride's nitrogens with tritiated methyliodide.

Our results show that we can obtain a methylated product with the above mentioned characteristics. Proton NMR studies reveal that our active analog contains a single methyl group and, through mass spectroscopic analysis, we conclude that the methylation occurs on the nitrogen lying immediately adjacent to the carbonyl carbon in the acylguanidine side chain. This methylated molecule specifically and reversibly inhibits both short-circuit current in frog skin and $^{22}Na^+$ uptake into A6 apical membrane vesicles. Radioactive (tritium) methylation of BrA up to approximately 1 Ci/mmol has been achieved. Because of the ease of the reaction conditions and the simplicity of purification of the methylated derivative, this product can be made and utilized by many laboratories. Because of its irreversible binding to apical membrane sodium channels after irradiation, this molecule should serve as a useful biochemical probe of epithelial Na^+ channels.

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