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Synthesis, properties and biological activity of tritiated *N*-benzylamidino-3,5-diamino-6-chloro-pyrazine carboxamide — a new ligand for epithelial sodium channels

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A method is described for the synthesis and purification of tritiated *N*-benzylamidino-3,5-diamino-6-chloro-pyrazine carboxamide (benzamil). The tritium was inserted at the *meta* position of the benzyl ring, from which it apparently does not exchange with solvent hydrogen. When stored in ethanol at −4 °C the radioligand remains stable for at least 15 months. The pharmacology of benzamil is very similar to that of amiloride in terms of its effects on sodium transporting epithelia except that it has a higher affinity. The affinity of benzamil for sodium channels in amphibian epithelia in the absence of sodium is approximately 10^9 M^{-1}. The new ligand can be used to label sodium channels in epithelia, and may be useful in channel isolation procedures.

Understanding of the properties of membrane receptors in living tissues has been enormously advanced by the availability of specific, high affinity ligands of high specific activity. In recent years numerous ligands have been developed particularly for receptors for neurotransmitters, yet there have been few ligands available for ion channels in membranes.

In excitable membranes, radiolabelled tetrodotoxin and saxitoxin have been used extensively to probe the properties of the selectivity filter of the sodium channel (for example, Ritchie et al 1976) and scorpion toxin appears to label the gating mechanism of the same channels (Catterall & Morrow 1978). As yet there is no highly specific ligand for potassium channels in excitable membranes.

The apical membranes of some tight sodium transporting epithelia are selectively permeable to sodium ions which gain access to the cell by a translocation mechanism, probably an ion channel but which can also have characteristics typical of a carrier mechanism (Lindemann & van Dreissche 1977; Cuthbert & Shum 1978). The sodium ion translocation mechanism is blocked by some pyrazine carboxamides, such as amiloride. In recent years the density of the translocating sites in epithelial cells has been measured using [^{14}C]amiloride (Cuthbert 1973; Cuthbert & Shum 1974, 1975). The affinity and maximal specific activity of this ligand are insufficient to consider using this material for the isolation of the membrane macromolecule responsible for sodium ion movement.

Preliminary screening has already indicated that *N*-benzylamidino-3,5-diamino-6-chloro-pyrazine carboxamide (benzamil) has a higher affinity than amiloride (Cuthbert 1977; Cuthbert & Fanelli 1978) and that it might make a suitable radioligand. This paper reports on the synthesis, properties and biological activity of a new ligand which may be useful for biochemical isolation studies.

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METHODS

Biological assay method. Bioassay was carried out using the sodium transporting epithelium of frog skin. Abdominal skins of *Rana temporaria* were set up in Ussing type chambers and the short circuit currents recorded by conventional means as a measure of sodium transport. The Ringer solution used for bathing the tissues had the following composition, mM: NaCl, 111; KCl, 1; $CaCl_2$, 1; Tris buffer, pH 7·6, 5 and glucose 11·1. In some experiments low sodium Ringer was used to bathe the mucosal surface of the skins. This solution had the same composition as above except the NaCl concentration was reduced to 1·1 mM. In some instances this solution was adjusted to pH 6·5 by titration with HCl.

Thin layer chromatography

(a) Analytical. Camblab Polygram Sil G and Sil G/U_{254} plates were used (0·25 mm silica gel).

Compounds were visualized by their fluorescence at 350 nm.

(b) Preparative. A Kieselgel G (type 60) slurry was spread on glass plates to a thickness of 2 mm and dried before use. The chromatographic chambers were allowed to equilibrate for 1 h with solvents before the plates were introduced.

High voltage paper electrophoresis. A Shandon Southern (model L24) machine was used with Whatman No. 1 paper. The system was equilibrated for 1 h before use with a buffer of 0·1 M citric acid and 0·2 M Na_2HPO_4 (pH 3·05). Electrophoresis was carried out for 2 h at 40 V cm^{-1}.

Determination of radioactivity. Samples were counted in a Packard Tri-Carb liquid scintillation spectrometer. Where appropriate counts were converted to d min^{-1} using an external standard and calibration curve.

RESULTS

Synthesis of [^3H]benzamil. (a) [^{3}H]benzamil was prepared at the Radiochemical Centre, Amersham by exchange tritiation with a rhodium catalyst. Bioassay of the crude product showed that 75% of the material survived the synthesis assuming the reaction products were inactive. Pure [^{3}H]benzamil was separated from the reaction mixture by t.l.c. but the radioactivity profile did not confirm that the material was radiochemically pure when tested by high voltage electrophoresis.

It appeared that extensive tritium exchange with solvent hydrogen was occurring, particularly in the acidic buffer used for electrophoresis. This was confirmed as follows. A sample of [^{3}H]benzamil was prepared by t.l.c. in ethanol–triethylamine (9 : 1). The material was stored for 11 days either in aqueous or ethanolic solution at the same concentration. Aliquots of each were then run on t.l.c. using ethanol–triethylamine (9 : 1) as solvent. Fig. 1 shows the results. Both samples showed a single fluorescent spot, corresponding to pure benzamil, but a clear peak of radioactivity was shown only with the material stored in ethanol. Even so there was a good deal of radioactivity trailing the peak. From various other tests it appeared that the residual (non-exchangeable) activity was maximally 30 Ci mol^{-1}, far too low to be useful, and a synthetic approach was necessary.

(b) [^{3}H]benzamil was synthesized in a two stage process from [^{3}H]benzylamine HCl, with benzyl-

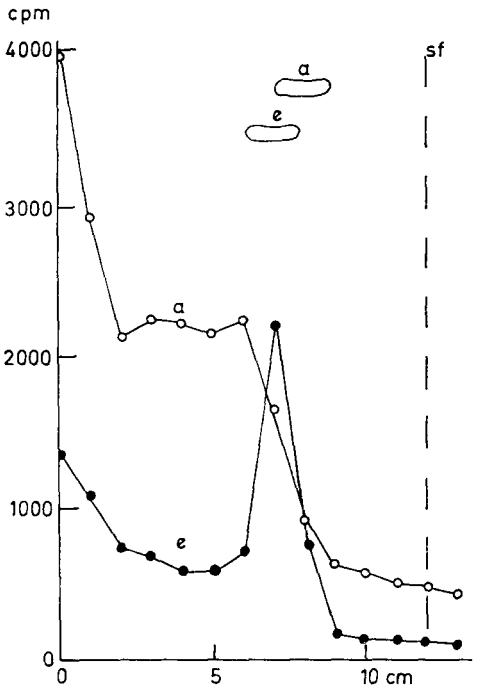
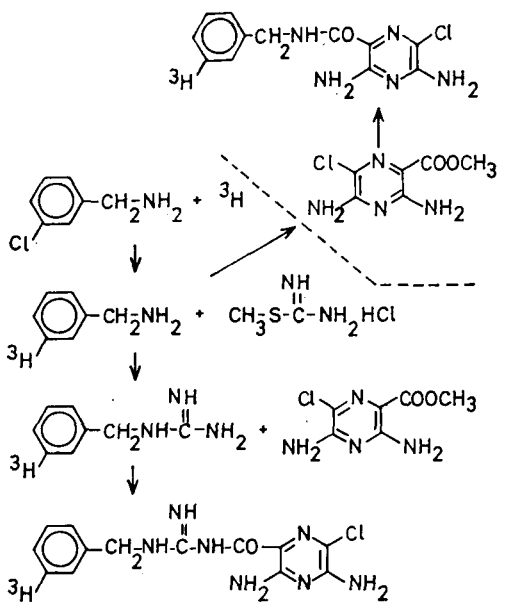


FIG. 1. Radioactivity profiles of two thin layer chromatograms of purified [^{3}H]benzamil prepared by catalytic exchange tritiation. The solvent was ethanol–triethylamine (9 : 1). In (a) the purified material was kept in aqueous solution for 11 days and in (b) in ethanolic solution for the same period before the chromatograms were run. The position of the fluorescent (350 nm) benzamil spots are shown. sf indicates the solvent front.

guanidinium HCl as an intermediate. Large scale versions of both stages have already been described (Bicking et al 1965; Cragoe et al 1967). A small scale procedure was devised so that the whole synthesis could be carried out with only one transfer between reaction vessels. Benzylamine HCl (50 mg) was dissolved in 240 μl of NaOH solution (5 g in 85·7 ml water) to which 2-methyl-2-pseudothiouronium chloride (44 mg) was added. The mixture was stood at room temperature (20 °C) for 36 h, and then evaporated by vacuum to give benzylguanidinium HCl. To this was added 190 μl sodium methoxide in methanol (5·75 g Na dissolved in 150 ml dry methanol) and the mixture agitated until there was a NaCl precipitate. The supernatant was removed by hypodermic syringe and transferred to another dry vessel. The NaCl was washed with a further 200 μl of dry methanol which was added to the first portion. To the liquor was added methyl-3,5-diamino-6-chloropyrazine carboxylate, and the mixture refluxed for 1 h at 70 °C (Scheme 1).



Scheme 1. The synthesis of benzamil. The dotted line separates the main reaction from the side reaction.

The yields were 70% for the production of benzylguanidium HCl (gravimetrically) and 65% for the second stage (determined by bioassay). The overall reaction yield was 45% (by bioassay) in a separate experiment. 2-Methyl-2-pseudothiouronium chloride was prepared from the sulphate by titration with $BaCl_2$ and removal of the $BaSO_4$ precipitate.

For the radioactive synthesis [^{3}H]benzylamine HCl was prepared from metachlorobenzylamine HCl by reduction with tritium gas and chromatographic purification to separate any remaining chloro compound. This was carried out at the Radiochemical Centre, Amersham to give a product with a specific activity of 21·5 Ci mmol^{-1}.

None of the reactants or reaction products except benzamil have any effect on the SCC in frog skin bathed on both sides with Ringer solution. Benzylguanidinium HCl has a minor effect when the sodium concentration of the mucosal bathing solution is reduced. Thus bioassay on isolated frog skin was a powerful aid when following the purification of the labelled compound. The radioactive synthesis was carried out at Amersham but unfortunately the yield was much less than for the non-radioactive synthesis. This was almost certainly because of a poor yield at the first stage, so that the pyrazine reagent reacted with unreacted [^{3}H]benzylamine HCl to give *N*-benzyl-3,5-diamino-6-chloro-pyrazine carboxamide. In a subsequent non-radioactive synthesis it was

Table 1. R_F values of compounds by thin layer chromatography. System I ethanol–triethylamine (9 : 1); II chloroform–triethylamine (9 : 1); III ethanol–water–acetic acid (4 : 1 : 0·5).

Compound	R_F value in system: I	II	III
Benzamil	0·50	0·05	0·65
Amiloride	0·20	0·01	0·58
Methyl-3,5-diamino-6-chloro-pyrazine carboxylate	0·70	0·25	0·71
Benzylamine	0·62	0·38	0·81
Benzylamine hydrochloride	0·71	0·32	0·69
Benzylguanidine hydrochloride	0·42	0·04	0·81
Synthesis side product	0·05	—	—

shown that benzylamine would react with the pyrazine reagent in dry sodium methoxide to produce a compound having the same R_F characteristics on thin layer and high voltage electrophoresis as the side reaction product (Scheme 1).

The R_F values for the reagents and reaction products of this synthesis using 3 solvent systems are given in Table 1. Purification of the crude [^{3}H]-benzamil was by t.l.c. in eight stages using ethanol–triethylamine (9 : 1) (solvent I); chloroform–triethylamine (9 : 1) (solvent II) and ethanol–water–acetic acid (4 : 1 : 0·5) (solvent III). The eight stages were using the solvent systems in the following sequence I, I, II, I, II, III, III, I. At each stage the spot corresponding to benzamil was scraped off and eluted with ethanol. At stage 3 an aliquot of the eluate was run on high voltage electrophoresis. There was a single fluorescent spot, corresponding to benzamil and a major peak of radioactivity. A minor peak (ratio 1 : 11) of lower mobility was also discernible. Failure of subsequent purification stages to alter the pattern seen on high voltage electrophoresis suggests that [^{3}H]benzamil may be slightly degraded under the conditions of electrophoresis.

The final purified sample of [^{3}H]benzamil was stored in alcohol, and was assayed by uv absorption as well as by bioassay and radioassay. Benzamil absorbs at 280 and 363 nm, but the latter is more useful since at 280 nm there is interference from unknown materials extracted from the silica gel by ethanol. From the concentration dependence of absorption at 363 nm it was estimated that the ethanolic solution contained $2{\cdot}8 \times 10^{-5}$ M [^{3}H]-benzamil. Bioassay of an aliquot of this solution gave a value of $2{\cdot}4 \times 10^{-5}$ M. Radioassay of the sample of [^{3}H]benzamil gave a specific activity of 21·0 Ci mmol^{-1} (using the concentration given by

uv absorption) which agrees closely with the specific activity of the starting material (21·5 Ci mmol^{-1}).

The final product was divided into 50 μl aliquots and stored in sealed ampoules at −4 °C.

Biological activity and stability of [^{3}H]benzamil

It is already known for the parent compound, amiloride, that the concentration which causes 50% inhibition of SCC in *R. temporaria* skin is dependent upon the sodium concentration. This is true too for benzamil. To illustrate: values of EC50 for a single tissue at sodium concentrations of 10, 20 and 110 mM (pH 6·5) were respectively 3·5, 5·0 and 13·2 nM. The serosal solution was Ringer at pH 7·6. The points fell on a straight line corresponding to:

$$B = K_B + [Na]\frac{K_B}{K_{Na}}$$

where B is the concentration of benzamil and K_B and K_{Na} are the dissociation constants for benzamil and sodium with the membrane receptors.

This relationship is indicative of a competitive type of interaction between sodium ions and benzamil. At each sodium concentration the activity of benzamil is approximately 10 times greater than that expected for amiloride. At a sodium concentration of 1·1 mM the mean value for the EC50 was 1·2 ± 0·3 nM (7) (range 0·5–2·6 nM) when measured at pH 6·5. The tritiated compound is apparently stable for long periods in ethanolic solution kept at −4 °C. After 9 months storage under these conditions benzamil retained both its radiochemical purity and its biological activity. Fig. 2 shows a concentration response curve for pure benzamil measured at pH 7·6 and at a sodium concentration of 1·1 mM. In this instance the EC50 was 1·8 nM. Dilutions of [^{3}H]benzamil produced responses which indicated that the concentration of the stock solution was 2·7 × 10^{-6} M (original value was 2·8 × 10^{-5} M by uv absorption). More importantly t.l.c. showed (Fig. 2) that the radioactive peak corresponded exactly with the location of the fluorescent spot with an R_F equal to that of benzamil, indicating that the tritiated compound was radiochemically pure.

A further test for stability was made after 15 months, and by a different approach. A sample of the original tritiated material was counted and the concentration calculated assuming an initial specific activity of 21·0 Ci mmol^{-1}. The material was then bioassayed on frog skin together with a fresh solution of benzamil. If no degradation had occurred then the concentration response curves for benzamil and

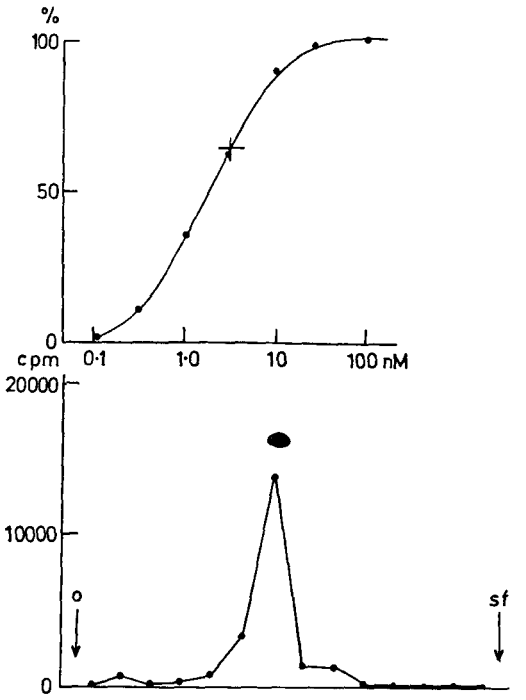


FIG. 2. (a) Inhibition of SCC in frog skin by benzamil applied to the mucsoal surface. The mucosal solution contained 1·1 mM Na, pH 7·6. The serosal solution was normal Ringer at pH 7·6. A 9000 times dilution of the stock [^{3}H]benzamil solution gave a response indicated by the cross, indicating a concentration of 2·7 × 10^{-5} M. The initial SCC of the skin was 27 μA for 9·6 cm^2.
(b) Thin layer chromatogram of [^{3}H]benzamil run in ethanol–triethylamine (9 : 1). O indicates origin and sf solvent front. The position of the fluorescent spot (R_F = 0·5) is indicated. Both experiments were performed 9 months after preparation of [^{3}H]benzamil.

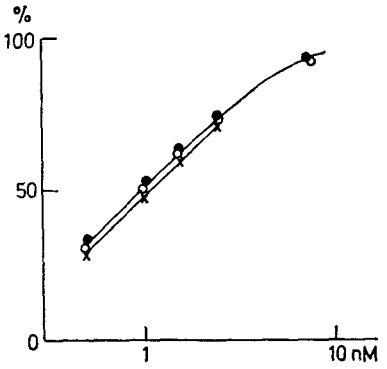


FIG. 3. Bioassay of [^{3}H]benzamil and benzamil on frog skin. The mucosal solution contained 1·1 mM at pH 6·5. The serosal solution was normal Ringer at pH 7·6. Percentage inhibition versus concentration (or calculated concentration) is shown. The circles represent responses to benzamil and the crosses represent responses to [^{3}H]benzamil. The responses to benzamil were obtained before (closed circles) and after (open circles) the responses to [^{3}H]benzamil.

[^{3}H]benzamil should be superposed. The result is given in Fig. 3 from which it can be calculated that the radioactive material had 91% of the activity of the unlabelled compound, assuming the initial specific activity was correct. Thus very little, if any, degradation had occurred in ethanolic solution over a period of 15 months at −4 °C.

As far as we can tell, the actions of benzamil are identical with those of amiloride except for potency. It is known, for example, that when the SCC in frog skin is stimulated by antidiuretic hormone the apparent affinity for amiloride is decreased (Cuthbert & Shum 1974) and the possible reasons for this have been discussed (Cuthbert 1974). We felt this would be a sensitive test for the identity of the action of benzamil. Fig. 4 shows the result where it can be seen that the hormone causes a threefold reduction in the affinity of benzamil.

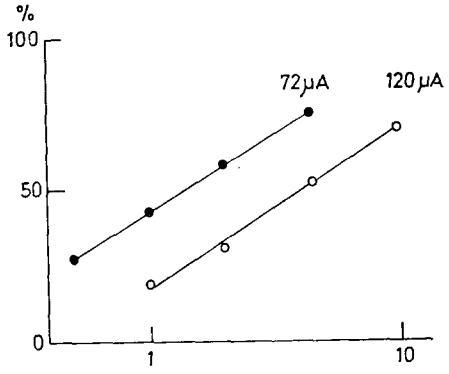


FIG. 4. Percentage inhibition of SCC vs benzamil concentration (nM) in frog skin. The serosal solution was normal Ringer at pH 7·6, while the mucosal solution contained 10 mM Na at pH 6·5. The area was 9·6 cm^2. Initial current was 72 μA. After transport was stimulated by ADH (50 mu ml^{-1}) the current increased to 120 μA. Closed circles show the responses to benzamil before, and open circles after, hormone treatment.

The partition coefficient of benzamil between chloroform and low sodium Ringer was measured. At pH 6·5 benzamil had a value of 0·43 compared with 0·003 for amiloride.

DISCUSSION

We have shown that under a variety of conditions benzamil behaves similarly to amiloride, but has an affinity at least ten times greater. A method is described for the synthesis of tritiated benzamil, with the tritium in a position which does not exchange with solvent hydrogen. In addition the material appears to be very stable in dilute solution stored in ethanol at −4 °C in the dark. The specific activity (originally 21·0 Ci mmol^{-1}) is 400 times greater than was possible with [^{14}C] amiloride (54 Ci mol^{-1}). This factor alone will increase the usefulness of benzamil as a radioligand for binding to sodium entry sites in epithelia, by reducing the amount of tissue required. On the other hand the high lipid solubility is an undesirable character of a ligand for binding studies. A preliminary report (Aceves et al 1978) has shown that [^{3}H]benzamil can be used to measure the density of sodium entry sites in frog skin epithelium.

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