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Synthesis and Properties of Two New Membrane-Impermeant High-Molecular-Weight Carbonic Anhydrase Inhibitors

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The synthesis and inhibitory properties and stability of two nontoxic high-molecular-weight carbonic anhydrase inhibitors (F 3500 and POBUMS) are reported. F 3500 was prepared by the covalent linkage of aminobenzolamide, a potent carbonic anhydrase (CA) inhibitor, to polyoxyethylene bisacetic acid (MW 3350). Linkage of the same inhibitor to polybutadiene maleic acid copolymer (MW 20,000) gave POBUMS. They contained on a mole percentage basis 0.11–0.28% unreacted aminobenzolamide as contaminant. F 3500 and POBUMS were approximately equipotent as carbonic anhydrase inhibitors on a weight basis with K_i at 37°C for CA II and CA IV of approximately 0.5 and 10 μg/ml, respectively. They showed a maximum release of 28% aminobenzolamide when heated at 70°C for 3 days at pH 10.5 and were completely stable toward enzymatic hydrolysis (protease and peptidase at 25°C). They were not membrane permeable as judged by their inability to bind to intracellular CA II in intact red cells, nor were they actively uptaken in rat kidney slices. Rats injected with F 3500 (200 mg/kg) showed no toxicity and excreted 93% of the polymer unchanged in the urine in 3 h. The two polymers should prove useful for *in vivo* and *in vitro* studies of selective CA IV inhibition in membranes.

INTRODUCTION

Secretory tissues, e.g., ciliary process (eye), choroid plexus (brain), parietal cell (stomach), and kidney tubule (*1, 2*), contain two forms of the zinc metalloenzyme carbonic anhydrase (CA II and IV).[2] Both forms effect the facile interconversion of CO_2 and HCO_3^- at physiological pH, and it is the latter ion, bicarbonate, that serves as counterion to Na^+ in fluid production. In addition to this role, CA isoenzymes are also responsible for proton secretion (kidney, stomach) and in bicarbonate reabsorption (kidney). The cytosolic form CA II is slightly more efficient in the catalysis of CO_2 hydration, $CO_2 + H_2O \leftrightharpoons HCO_3^- + H^+$, compared with the membrane-bound form CA IV (*3*) and, because of intracellular localization, is present in much higher concentration. Inhibition with potent and permeant sulfonamide carbonic anhydrase inhibitors (CAIs) invariably leads to an approximate 40% reduction in fluid production (*4*) due to reduced bicarbonate formation from

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[2] Abbreviations used: CA, carbonic anhydrase; CAI, carbonic anhydrase inhibitor; STZ, succinyl thiadiazolesulfonamide; EDC, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide; HOBT, hydroxybenzotriazole; DCC, dicyclohexylcarbodiimide; PAH, *p*-aminohippurate; PEG, polyethylene glycol.

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CO_2. Their effect on the kidney is to interfere with bicarbonate reabsorption in the proximal segment of the tubule, resulting in a bicarbonate-rich (100 mM) urine. This process is dependent both on brush border CA IV (bicarbonate reabsorption) and on cytosolic CA II as a source of H^+ (proximal acidification) (*5*).

All high-MW CAIs produced to date have consisted of heterocyclic sulfonamides [prontosil, succinyl thiadiazolesulfonamide (STZ), and aminobenzolamide (*p*-aminobenzenesulfonamido-1,3,4-thiadiazole-5-sulfonamide)] covalently bonded to derivatized dextrans of MW 5000–1,000,000 (*6–8*). They have been employed in perfusion studies in the isolated kidney tubule in the attempt to assess the relative roles of CA II and IV in bicarbonate reabsorption. The tendency to induce anaphylaxis in the living animal has restricted their use, however. Karlmark *et al.* (*6*) studied the effect of aminobenzolamide bonded to bromcyanide-activated dextran (MW 17,000), on acidification in the isolated tubule, but the failure to appreciate the effect of increasing K_i with temperature (*9*) leading to reduced potency likely led to use of too little compound.

In two subsequent studies (*7, 10*) in which STZ was bonded to aminoethyl-derived dextrans, MW 5000 and 100,000, a high degree of sulfonamide linkage was achieved, up to eight substitutions per molecule, despite the fact that the stoichiometry of drug binding to enzyme is 1:1. A later study using a series of weakly inhibitory prontosil dextrans (*8*), (MW 5000–1,000,000) yielded variable results in the washout of $H^{14}CO_3^-$ from the isolated rabbit hindlimb. The K_i values for the various inhibitors, however, were determined for inhibition of cytosolic CA II despite the fact that CA IV is the isoenzyme being inhibited by these compounds. Since inhibition by sulfonamides is weaker for CA IV by 7 to 33-fold compared with CA II (*3*), it is likely that too little compound was used. Additionally, it was determined (*8*) that the STZ dextrans of Tinker *et al.* (*7*) liberated appreciable quantities of free drug in solution.

We now report synthesis of two new high-MW CAIs based on nontoxic polymers, polybutadiene maleic acid (MW 20,000) and polyethylene glycol bisacetic acid (MW 3350), covalently bonded to aminobenzolamide **4** (Fig. 1), using the carbodiimide linking reaction. The first polymer has not previously been used for drug linkage. The second has been extensively employed for this purpose (*11*) and yields products that are water soluble, nontoxic, and generally well suited for *in vivo* studies and in this case of bicarbonate reabsorption in the kidney and now for the selective inhibition of CA IV *in vivo*.

MATERIALS AND METHODS

PEG dicarboxylate (polyoxyethylene glycol bisacetic acid, (Sigma, St. Louis, MO) and polybutadiene maleic acid copolymer (Polysciences, Warrington, PA) were used without further purification. CA II was obtained from hemolyzed dog red cells and CA IV from bovine kidney microsomes by ultracentrifugation (*12*). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), dicyclohexylcarbodiimide (DCC), hydroxybenzotriazole (HOBT), acetazolamide, trichloroacetic acid, ammonium sulfamate, sodium nitrite, bovine liver acetone powder, and bovine pancreas

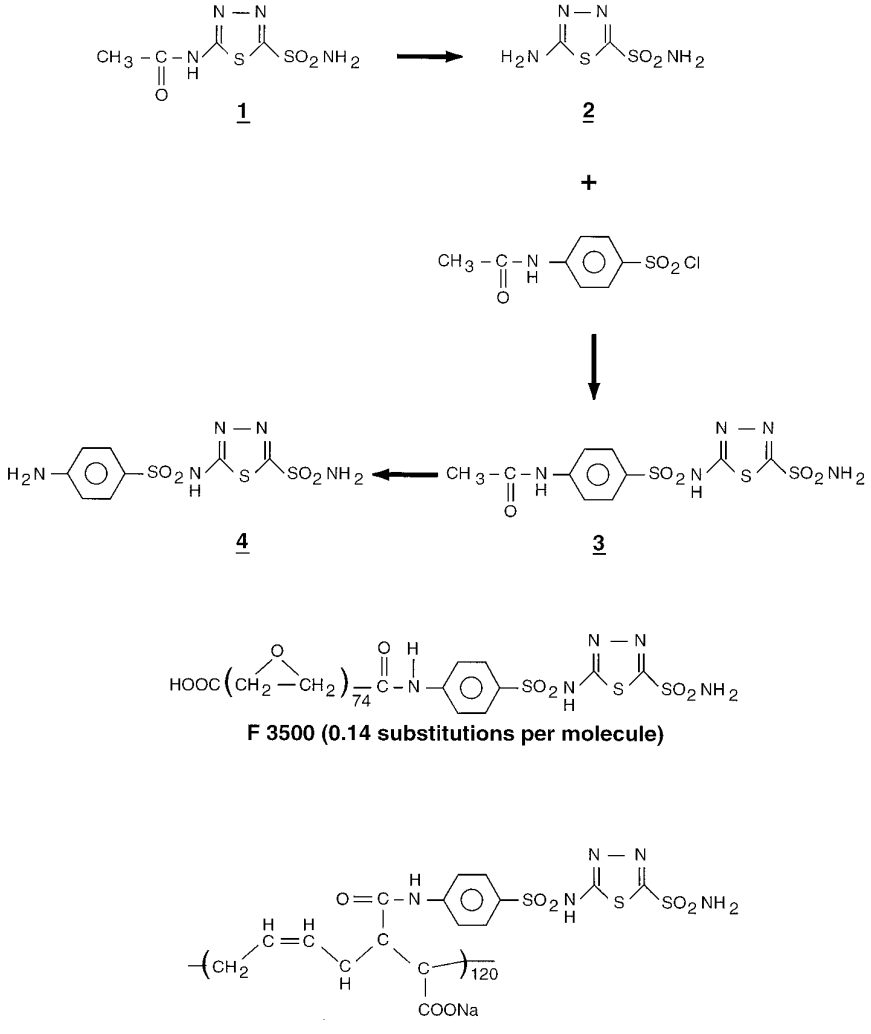

FIG. 1. Synthesis of aminobenzolamide and structures of POBUMS and F 3500.

protease were obtained from Sigma and used without further purification. *N*-Acetylsulfanilyl chloride (Aldrich) and *N*-(1-naphthyl)ethylenediamine dihydrochloride (Kodak) were used without further purification.

Analytical Methods

The inhibitory potency (K_i) of POBUMS and F 3500 against CA II and CA IV was determined at 0 and 23°C by a changing pH-indicator method (*13*) using barbital buffer and bromthymol blue as indicator. K_i was related to the equilibrium constant

(K_{eq}) by the relationship $K_{eq} = 1/K_i$. Arrhenius plots of ln K_{eq} vs $1/T$(°K) were then used to obtain estimates of K_i at physiological temperature (37°C). Degrees of fractional inhibition of CA isoenzymes (i) were calculated from the relationship

$$i = \frac{I_f}{I_f + K_i},$$

where I_f is the unbound drug concentration in tissue or fluids.

The presence of unreacted aminobenzolamide in preparations of F 3500 and POBUMS was determined by a modification of the Bratton–Marshall procedure (*14*) in which the pH of the trichloroacetic acid/sodium citrate solution is raised to pH 4 by addition of 6 N NaOH. Results were expressed as mole percentages (mole free arylamine/mole polymer) × 100.

The degree of drug substitution on polymers was determined by hydrolysis of F 3500 and POBUMS in 1 N HCl at 100°C for 30 min prior to assay for free arylamine by the Bratton–Marshall method. This number was also confirmed based on the uv absorption at 270–290 nm.

The stability of drug polymers toward hydrolysis was determined both enzymatically (*in vivo* and *in vitro*) and nonenzymatically. In the latter procedure 10 mM solutions were heated at 70°C in pH 3.5, 7, and 10.5 phosphate buffer (50 mM) for 72 h. These solutions were monitored for free aminobenzolamide using the Bratton–Marshall method. The stability of POBUMS and F 3500 toward enzymatic hydrolysis was tested *in vitro* by incubation of 5 mg polymer in 1 ml pH 7.5 phosphate buffer at 25°C for 1 h with 50 enzyme units of bovine pancrease protease or with 50 mg bovine acetone powder followed by analysis for free arylamine. In the *in vivo* procedure, rats were administered F 3500 (200 mg/kg), and the urine was collected for 3 h, pooled, and tested for arylamine. Washed red cells collected at 3 h were tested in an analogous manner.

The membrane permeability of POBUMS and F 3500 was determined by incubation of drug polymers (150 μM) with intact rabbit red cells for periods up to 3 h according to Maren *et al.* (*15*). Red cells were washed three times against saline, lysed, and analyzed for drug. The active uptake of both polymers in kidney slices was studied by the method of Cross and Taggert (*16*) at 25°C in Ringer's solution containing 10 mM acetate in an O_2 atmosphere and compared with that of benzolamide, aminobenzolamide, and PAH.

Synthesis of Aminobenzolamide

Aminobenzolamide was synthesized by a variation of the method of Vaughan *et al.* (*17*), as shown in Fig. 1. Acetazolamide **1** (2-acetylamino-1,3,4-thiadiazole-5-sulfonamide (111 g 0.5 mol) was refluxed for approximately 1 h in 1 liter 0.1 *N* HCl. Complete solution was effected in 45 min followed by an additional 15 min at reflux. The solution was cooled and adjusted to pH 5 by the careful addition of sodium carbonate. The precipitate that formed after standing overnight at 5°C was washed with 100 ml water. Recrystallization from boiling water gave 90 g (80% yield) of 2-amino-1,3,4-thiadiazole-5-sulfonamide **2**.

Fifty-four grams (0.3 mol) of **2** was dissolved in 155 ml 2.5 N NaOH and cooled

to 10°C in an ice bath. *N*-Acetylsulfanilyl chloride (14 g, 0.06 mol) and 30 ml 5 N NaOH were added simultaneously as the temperature was maintained at 10°C. These additions were repeated four more times and the solution was refrigerated overnight. The solution was then adjusted to pH 4 with 6 N HCl and chilled in ice. The precipitate, *p*-acetylaminobenzenesulfonamido-1,3,4-thiadiazole-5-sulfonamide **3**, was not purified but deacetylated by dissolving in 300 ml of 6 N HCl and refluxing for 45 min. The cooled solution was taken to pH 4 with 1 N NaOH and the precipitate collected. The crude aminobenzolamide was recrystallized from 20% EtOH/H_2O, giving 110 g of pure **4** mp 247–248°C (dec.) in 65% yield.

Synthesis of POBUMS

Polybutadiene maleic acid (2.5 g, 40% solids in water, representing 1 g polymer or 0.05 mmol) was dissolved in 30 ml 50% acetone/water. The pH was raised to 4.9 by addition of 1 N NaOH. A 25-fold molar excess of aminobenzolamide (1.25 mmol, 418 mg) was dissolved in boiling ethanol/water and added to the stirred solution above. The pH was readjusted to 4.9. A 10-fold molar excess of EDC (0.5 mmol, 96 mg) was dissolved in water and added slowly overnight while the pH was maintained at 4.9. Additional EDC (96 mg) was added on Days 2 and 3. After 5 days of reaction the crude reaction product was placed in benzolated dialysis tubing (MW cutoff, 3500) and dialyzed for 1 week, first against dilute dibasic phosphate (to facilitate removal of unreacted drug), then against distilled water. The product was finally dried in air to an amorphous yellow waxy solid.

Synthesis of F 3500

The synthesis of F 3500 was the same as used by Johansson for the synthesis of $(Et_3NEtO)_2$ Ph-NH-azelate-poly(EtO), a polymer used in the affinity partitioning of synaptic membrane domains (*18*). Polyoxyethylene bisacetic acid (1 g 0.3 mmol), HOBT (0.08 g, 0.6 mmol), DCC (0.14 g, 0.7 mmol), and aminobenzolamide (0.20 g, 0.6 mmol) were dissolved in 10 ml pyridine. The flask was sealed and heated in a water bath at 45°C for 2 days during which time a white precipitate presumed to be the corresponding dicyclohexyl urea formed. The solution was evaporated in vacuo to remove all traces of pyridine and the resultant waxy solid was extracted with three 10-ml volumes of CH_2Cl_2. The CH_2Cl_2 was evaporated and the solid taken up in 5 ml H_2O, adjusted to pH 8.5 with 0.1 N NaOH, and carefully filtered to remove all insoluble matter. This was then applied to a 50 × 600-mm Sephadex G-50 column and eluted with 25 mM ammonium carbonate. Fractions corresponding to OD > 0.2 at 280 nM were pooled and dried in vacuo to yield F 3500 in 70% yield as a white waxy solid.

RESULTS

Table 1 shows the inhibitory potency of aminobenzolamide, POBUMS, and F 3500 against CA II and CA IV isoenzymes as a function of temperature. Inhibition data obtained at 0°C for these compounds were used to construct standard curves