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Synthesis of New Sulfonamide Inhibitors of Carbonic Anhydrase

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Abstract—Four different derivatives of aromatic sulfonamides have been synthesized: 1,2-bis[(4-sulfonamidobenzamide)ethoxy]ethane (SBAM), 1,2-bis[(4-sulfonamidobenzoate)ethoxy]ethane, 1,2-bis[(2,4-dichloro-5-sulfonamidobenzamide)ethoxy]ethane, and 1,2-bis[(2,4-dichloro-5-sulfonamidobenzoate)ethoxy]ethane. SBAM is a most potent inhibitor on ciliary epithelium carbonic anhydrase and is approximately 13 times more active against carbonic anhydrase isoform II than against isoform I.

Key words: inhibition, carbonic anhydrase, glaucoma, sulfonamides

Carbonic anhydrase (CA, EC 4.2.1.1) is a ubiquitous zinc enzyme. Basically, there are several cytosolic forms (CA-I, CA-II, CA-III, and CA-VII), four membrane-bound forms (CA-IV, CA-IX, CA-XII, and CA-XIV), one mitochondrial form (CA-V), as well as a secreted CA form (CA-VI) [1, 2]. They all catalyze a very simple physiological reaction, the interconversion between carbon dioxide and the bicarbonate ion, and are thus involved in crucial physiological processes connected with respiration and transport of CO_2/bicarbonate between metabolizing tissues and the lungs, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (such as the gluconeogenesis, lipogenesis, and ureagenesis), bone resorption, calcification, tumorigenicity, and many other physiologic or pathologic processes [1-3].

Since the discovery, 61 years ago, that sulfonamides inhibit CA, powerful inhibitors of CA have been restricted to the structure RSO_2NH_2 where R is an aromatic or heteroaromatic residue [4]. Parenteral sulfonamides (i.e., acetazolamide, methazolamide, dichlorphenamide, and ethoxazolamide) have been used for 45 years to reduce intraocular pressure in glaucoma [5]. Their pharmacological effect is believed to be due to the inhibition of CA-II in the ciliary epithelium. Unfortunately, systemic therapy with parenteral sulfonamides and their derivatives leads to significant side effects [6, 7], many of which are probably due to inhibition of CA isoforms in other tissues. These undesirable side effects call for the synthesis of new derivatives of sulfonamides that are more selective against CA-II to be used in glaucoma treatment.

A new type of chemistry has been recently reported by Casini et al., who prepared 4-isothiocyanatobenzene-sulfonamide by treating sulfanilamide with thiophosgene [8]. This compound was then used for the preparation of a large number of thioureas, by reaction with amines, amino acids, as well as di-, tri-, and tetrapeptides [8]. Many of these derivatives are very potent CA inhibitors and effectively reduced intraocular pressure in both normotensive and glaucomatous rabbits. In addition, new sulfonamide compounds were also obtained by derivatizing 4-carboxybenzene-sulfonamide with aminoacyl, oligopeptidyl, or ethylene glycol moieties. These compounds were generally very effective CA-I and CA-II inhibitors [1].

In this paper we present the synthesis of new carbonic anhydrase inhibitors and an *in vitro* inhibition study of their effect on CA-I and CA-II.

MATERIALS AND METHODS

Chemicals were from Sigma (USA) and Aldrich (Germany) and were used without further purification. Benzene was dried over metallic sodium.

Purification of carbonic anhydrase I and II from human erythrocytes. Erythrocytes were isolated from human blood. The blood samples were centrifuged at 1500 rpm for 20 min, and the plasma and buffy coat were removed. After the packed red cells were washed with

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0.9% NaCl, the erythrocytes were hemolyzed with cold water. The ghost and intact cells were removed by centrifugation at 4°C, 20,000 rpm for 30 min. The pH of the hemolyzate was brought to 8.5 with solid Tris. The hemolyzate was applied to a Sepharose 4B-L-tyrosine-sulfanylamide affinity column equilibrated with 25 mM Tris-HCl/0.1 M Na_2SO_4 (pH 8.5). The affinity gel was washed with 25 mM Tris-HCl/22 mM Na_2SO_4 (pH 8.5), and CA-I and CA-II were eluted with 1 M NaCl/25 mM Na_2HPO_4 (pH 6.3) and 0.1 M CH_3COONa/0.5 M $NaClO_4$, respectively [9]. The purity of the enzyme preparation was monitored by SDS-polyacrylamide gel electrophoresis [10].

Measurement of CA activity. Carbonic anhydrase activity was measured by the Maren method [11], which is based on the determination of time required for pH to decrease from 10.0 to 7.4 due to CO_2 hydration. Phenol red was added to the assay medium as the pH indicator, and the buffer was 0.5 M Na_2CO_3/0.1 M $NaHCO_3$ (pH 10.0). All solutions were chilled to 0°C before use. One unit of CA activity is defined as the amount of the enzyme that reduces by 50% the time of CO_2 hydration measured without enzyme. In inhibition studies, CO_2 concentration was 70 mM and five different inhibitor concentrations were used. I_{50} values were calculated using computer regression analysis [12].

Synthesis of new compounds as inhibitors. The structures of synthesized compounds were identified by IR spectra (Perkin Elmer Spectrum BX II), 400 MHz ^{1}H-NMR (Bruker GmbH DPX-400), 60 MHz ^{13}C-NMR (Bruker-AC) and mass spectra at 70 eV (Hitachi RMU-6E). Melting points were measured on an Electrothermal 9200 instrument.

General methods for SBAM and CSBAM. Sulfonamide (0.002 mol) and 1,2-bis(aminoethoxy) ethane (0.001 mol) were heated with stirring at 155°C for 2 h, cooled, and the resulting solid product was crystallized from ethanol.

1,2-Bis[(4-sulfonamidobenzamide)ethoxy]ethane (SBAM). 4-Carboxybenzene-sulfonamide was used as the starting compound. Yield, 75%; mp, 186°C; white crystal. IR (KBr), ν (cm^{-1}): 3299, 2992, 1605, 1557, 1385, 1321, 1299, 1163, 1092. ^{1}H-NMR ($CDCl_3$), δ (ppm): 7.75 (d, Ar-H), 7.95 (d, Ar-H), 6.9 (br, CO-NH), 2.95 (t, NH-CH_2), 2.95 (t, CH_2-O), 3.6 (t, O-CH_2). ^{13}C-NMR (DMSO), δ_C (ppm): 170, 146, 143, 130, 126, 70, 68, 44. Analysis ($C_{20}H_{26}N_4O_8S_2$). Mass: M^+ ($C_7H_6NSO_3$): 184 (100%).

1,2-Bis[(2,4-dichloro-5-sulfonamidobenzamide) ethoxy]ethane (CSBAM). 2,4-Dichloro-5-carboxybenzene-sulfonamide was used as the starting compound. Yield, 60%; mp, 49°C; white crystal. IR (KBr), ν (cm^{-1}): 3364, 3347, 3254, 2990, 1691, 1345, 1190, 1112. ^{1}H-NMR ($CDCl_3$), δ (ppm): 7.8 (s, Ar-H), 8.2 (s, Ar-H), 6.6 (s, CO-NH), 2.5 (t, NH-CH_2), 2.9 (t, CH_2-O), 3.6 (t, O-CH_2). ^{13}C-NMR (DMSO), δ_C (ppm): 163, 141, 136, 135, 133, 131, 127, 70, 68, 43. Analysis ($C_{20}H_{22}N_4O_8S_2Cl_4$). Mass, M^+ ($C_7H_5Cl_2N_2SO_3$): 268 (100%).

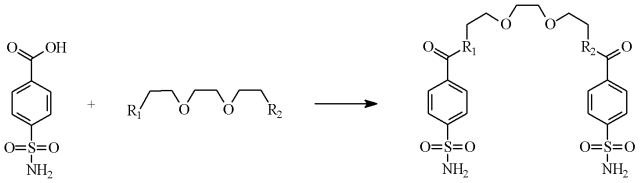
General methods for SBO and CSBO. Sulfonamide (0.002 mol), triethylene glycol (0.001 mol), and benzene (80 ml) were stirred and refluxed for 4 h in a Dean–Stark apparatus. The mixture was cooled and the obtained solid was crystallized from ethanol.

1,2-Bis[(4-sulfonamidobenzoate)ethoxy]ethane (SBO). 4-Carboxybenzene-sulfonamide was used as the starting compound. Yield, 70%; mp, 182°C; brown crystal. IR (KBr), ν (cm^{-1}): 3281, 3232, 1621, 1596, 1339, 1247, 1168, 1078. ^{1}H-NMR ($CDCl_3$), δ (ppm): 7.95 (d, Ar-H), 8.05 (d, Ar-H), 3.5 (m, O-CH_2-CH_2-O), 3.4 (t, O-CH_2). ^{13}C-NMR (DMSO), δ_C (ppm): 168, 149, 135, 131, 127, 73, 71, 61. Analysis ($C_{20}H_{24}N_2O_{10}S_2$). Mass: M^+ ($C_7H_6NSO_4$): 200 (100%).

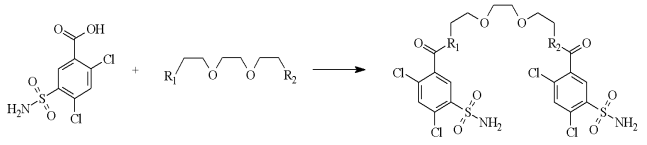
1,2-Bis[(2,4-dichloro-5-sulfonamidobenzoate)-ethoxy]ethane (CSBO). 2,4-Dichloro-5-carboxybenzene-sulfonamide was used as the starting compound. Yield, 62%; mp, 120.5°C; white crystal. IR (KBr), ν (cm^{-1}): 3430, 3283, 3102, 2982, 2919, 1694, 1580, 1359, 1296, 1257, 1171, 1127, 1078. ^{1}H-NMR ($CDCl_3$), δ (ppm): 7.9 (s, Ar-H), 8.35 (s, Ar-H), 3.5 (m, O-CH_2-CH_2-O-CH_2). ^{13}C-NMR (DMSO), δ_C (ppm): 166, 141, 137, 135.6, 135.2, 132, 131, 73, 71, 69. Analysis ($C_{20}H_{20}N_2O_{10}S_2Cl_4$). Mass: M^+ ($C_7H_4Cl_2NSO_4$): 251 (100%).

RESULTS AND DISCUSSION

The synthesis of the inhibitors is given in Scheme. Bis(triethyleneglycol)-bridged benzene-sulfonamides were prepared from 4-carboxybenzene-sulfonamide and 2,4-dichloro-5-carboxybenzene-sulfonamide. Compound (**a**) was reacted with triethyleneglycol and 1,2-bis(aminoethoxy)ethane to produce 1,2-bis[(4-sulfonamidobenzamide)ethoxy]ethane (SBAM) and 1,2-bis[(4-sulfonamidobenzoate)ethoxy]ethane (SBO), respectively. Compound (**b**) was reacted with triethyleneglycol and 1,2-bis(aminoethoxy)ethane to produce 1,2-bis[(2,4-dichloro-5-sulfonamidobenzamide) ethoxy]ethane (CSBAM) and 1,2-bis[(2,4-dichloro-5-sulfonamidobenzoate)ethoxy]ethane (CSBO), respectively [13]:



a) (SBAM) R_1 : -NHR_2 : -NH
(SBO) R_1 : -OR_2 : -O



b) (CSBAM) R_1 : -NHR_2 : -NH
(CSBO) R_1 : -OR_2 : -O

Scheme

Carbonic anhydrase inhibitors lower intraocular pressure by reducing bicarbonate formation in the ciliary process, thus lowering Na^+ transport and flow of aqueous humor: this is the basis for their use in glaucoma treatment. Unfortunately, systemic therapy with parenteral sulfonamides and their derivatives leads to significant side effects, many of them being probably due to inhibition of CA isoforms in other tissues. Acetazolamide is the most widely used inhibitor and has advantages over the others because it is 20 times less active against CA-I than against CA-II in erythrocytes. But the inhibition of various CA isoforms present in tissues other than eye leads to an entire range of side effects, the most prominent being numbness and tingling of extremities, metallic taste, depression, fatigue, malaise, weight loss, decreased libido, gastrointestinal irritation, metabolic acidosis, renal calculi and transient myopia [2, 5, 14].

CA-I and CA-II were purified from human erythrocytes by Sepharose 4B-L-tyrosine-sulfanylamide affinity column. Specific activities of CA-I and CA-II were found to be 0.67 and 1.7 U/mg, respectively. The purified carbonic anhydrase isozymes migrated as a single band during SDS polyacrylamide gel electrophoresis (data not shown). We determined the inhibition constants the CO_2-hydration activity of carbonic anhydrase, which is the primary physiological function of this enzyme. Results expressed as I_{50}, i.e., inhibitor concentration that reduces enzyme activity by 50%.

In vitro inhibitory effects of SBAM, SBO, CSBAM, and CSBO on CA-I and CA-II purified by affinity chromatography were measured to obtain the I_{50} values given in the table. SBAM was slightly a more potent inhibitor than SBO with respect to CA-II and a weaker inhibitor than SBO with respect to CA-I. The inhibitory effect of CSBAM on CA-II is similar to that of CSBO. As seen in the table, SBAM exhibited an approximately ninefold higher inhibition than CSBAM on CA-II in the ciliary epithelium.

The synthesized compounds have a lower affinity to CA-II compared to acetazolamide, which is used in the treatment of glaucoma, and moreover it has been found that acetazolamide is a more potent inhibitor of CA-I than our four synthesized compounds [14]. One of the most important findings of this study is that SBAM is approximately 13 times less active against CA-I by comparison with CA-II. Therefore, SBAM may have fewer side effects on the body as discussed above. This compound should be tested *in vivo* as a candidate for the treatment of glaucoma.

In vitro inhibitory effects of SBAM, SBO, CSBAM, and CSBO on CA-I and CA-II purified by affinity chromatography

Inhibitor	I_{50}, μM		
	CA-I	CA-II	CA-I/CA-II
SBAM	8.99	0.69	13.1
SBO	7.14	1.05	6.8
CSBAM	14.2	6.44	2.2
CSBO	33.7	8.75	3.8

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