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Bile Acid Derivatives of 5-Amino-1,3,4-thiadiazole-2-sulfonamide as New Carbonic Anhydrase Inhibitors: Synthesis and Investigation of Inhibition Effects

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Abstract—Bile acid amides (cholan-24-amides) of 5-substituted 1,3,4-thiadiazole-2-sulfonamide have been prepared from lithocholic, deoxycholic, cholic and dehydrocholic acids. Besides, the alcohol functional groups on the cholane ring systems were protected with acetyl group. Amides of the protected cholanes of lithocholic and cholic acids were also synthesized. Later, inhibition effects of these compounds on human carbonic anhydrase isozymes (HCA-I and II) have been investigated in vitro. For the most active compounds, inhibition constants ranged from 66 to 190 nM for HCA-II with I_{50} (molarity of inhibitor producing a 50% inhibition of CA activity). In addition, in vivo studies were performed for the synthesized compounds in Sprague–Dawley rats. The compounds (**11** and **18**) showed especially significant inhibition efficacy ($p < 0.001$).

Introduction

Carbonic anhydrases (EC. 4.2.1.1) are a family of zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide in a two-step reaction to yield bicarbonate and proton. A number of 14 different carbonic anhydrase (CA) isozymes were described up to now in higher vertebrates, including humans.[1] The isoenzymes relevant to the human eye are CA-I, CA-II and CA-IV. The isoenzymes CA-I and CA-II are cytosolic within the cell whereas CA-IV is membrane bound.[2]

Carbonic anhydrase inhibitors, which reduce aqueous production with a corresponding decrease in intraocular pressure (IOP), have been used as ocular hypotensive agents for the treatment of glaucoma.[3] Glaucoma is a term encompassing several diseases.[4] The risk factors for glaucoma disease include age, race, ocular hypertension, severe myopia and a family history of glaucoma. Of these, the strongest risk factors are age, race and ocular hypertension. An elevated IOP was formerly synonymous with glaucoma.[2a,5]

The 1,3,4-thiadiazole ring is also known to possess several biological activities and the antibacterial properties have been largely described.[6] Dorzolamide **1** and brinzolamide **2** are two topical CA inhibitors.[2a] Acetazolamide **3**, a CA inhibitor, possessing several biomedical applications, is also known to increase the cerebral blood flow when administered orally or intravenously.[7] The acetazolamide **3** as a therapeutically effective inhibitor of the enzyme has encouraged synthesis of further structural derivatives with the aim of studying the effects of these changes on its inhibitory activity.

In this paper, we report the synthesis of new CA inhibitors with 5-substituted-1,3,4-thiadiazole and both in vitro and in vivo inhibition study of them on CA-I and CA-II, because several important physiological and physio-pathological functions are played by CA isoenzymes.[8] Along with the use of CA inhibitors as glaucoma therapy reagents, a recent discovery is also connected with the involvement of inhibitors of CA isoenzymes in cancer.[9] Furthermore, some sulfonamides have also applications as diagnostic tools in positron emission tomography (PET) and magnetic resonance imaging (MRI).[10] Sulfonamides with the 1,3,4-thiadiazole ring are well known as inhibitors of the carbonic anhydrase enzyme.[11] We investigated the role of the substituted lipid moiety attached to a 1,3,4-thiadiazole ring on CA inhbition. Lithocholic (**5**), cholic (**6**), deoxycholic (**7**), and dehydrocholic (**8**) acids were chosen as lipid moieties. Bile acids have proved especially useful because of their availability and useful levels of functionalization.[12] On the other hand, the codirected hydroxyl groups present in most bile acids may be

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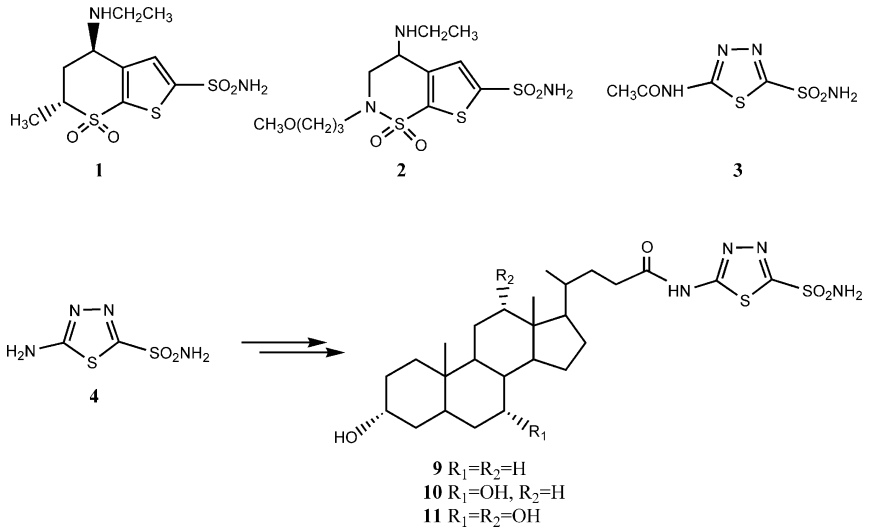
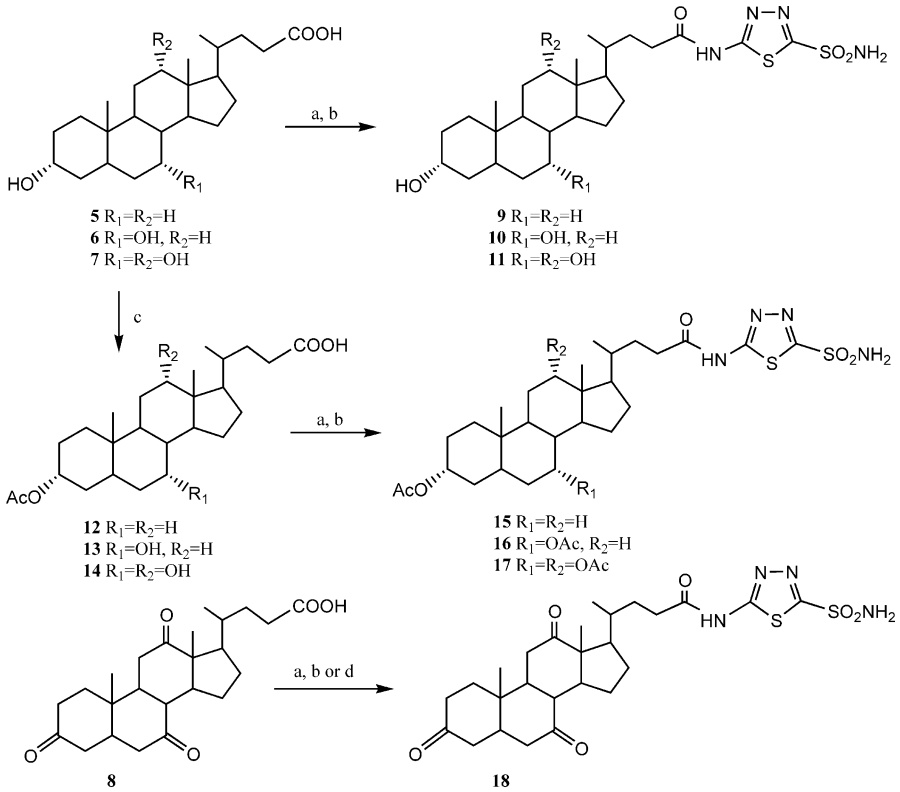


exploited in podand-type receptors,[13] linear dimeric hosts,[14] or 'facial amphiphiles'.[15] In addition, bile acids are natural ligands specifically recognized by hepatitic cells and are amphiphilic molecules that undergo a biological recycling during enterohepatic circulation.[16]

Synthesis

The key compound 2-amino-1,3,4-thiadiazole-5-sulfonamide (**4**) required for the synthesis of inhibitors was prepared by acidic hydrolysis of 2-acetilamino-1,3,4-thiadiazole-5-sulfonamide (**3**).[17] In order to approach the synthesis of the target inhibitors, the conversion of the bile acids to the concerning amides is required for activation of the side-chain carboxyls.[18] For activation, the bile acids were reacted by ethyl chloro formiate (ECF) in the presence of *N*-methyl morpholine (Scheme 1). Later, the activated carboxyl groups of the bile acids were treated with 2-amino-1,3,4-thiadiazole-5-sulfonamide to obtain the desired amides (**9**–**11**). In order to compare the inhibition effect on CA, the hydroxyl groups of the cholane ring were protected by using acetyl as a protecting group. For this reason, the bile acids **5**, **6** and **7** were acetylated using acetyl chloride at room temperature to afford the protected compounds **12**, **13** and



Scheme 1. (a) Ethyl chloro formate, *N*-methyl morpholine, THF; (b) **4**, reflux; (c) AcCl, rt; (d) $SOCl_2$, **4**, THF.

14 which were converted into the amides **15** and **17** except for **16** as described in Scheme 1.

We next turned our attention to the preparation of the amide **18** of the amino thiadiazole **4** with the dehydrocholic acid (**8**) containing triketone. For the preparation of the amide, **8** was converted into the corresponding acyl chloride with $SOCl_2$, and was then reacted with **4** in dry THF. The structures of these compounds have been elucidated on the basis of IR, ^1H and ^{13}C NMR data, and satisfactory mass spectral analysis.

Biochemistry

The isoenzymes CA-I and CA-II were purified from human erythrocytes by Sepharose-4B-L-tyrosine-*p*-aminobenzensulfonamide affinity column.[19] In vitro inhibition study of newly synthesized compounds (**9**, **10**, **11**, **15**, **17** and **18**) and acetazolamide (**3**) are summarized in Table 1. Results are expressed as I_{50} value. In addition, a total of 42 adult rats (200–250 g) for in vivo studies were used. They were divided into six groups and each group had seven rats. The synthesized compounds were injected intraperitionally into each rat. Blood samples were taken from each rat at 2, 4 and 6 h after injection. As a result, hemolysate was prepared as in vivo studies. Carbonic anhydrase activity was assayed by the hydration of CO_2 according to the method of Wilbur–Anderson at in vitro and in vivo studies. Analyses of the data obtained from in vivo studies were made by test of *t*, and were given as $X \pm SD$.

Results and Discussion

Current therapeutic intervention for glaucoma is primarily directed toward physiological mechanism that leads to the reduction of IOP in an effort to prevent nerve fiber damage.[20] Carbonic anhydrase is intimately involved in the production of aqueous humor, and the inhibitors of this enzyme are effective in lowering intraocular pressure by reducing the production of aqueous humor.[21] Oral CA inhibitors have been in clinical use for over 40 years following the observation that orally administrated acetazolamide lowered the IOP of glaucoma patients.[2a,22] Systemically administered CA inhibitors are very effective ocular hypotensive agents. However, the extraocular inhibition of the enzyme results in a myriad of side effects, which include general malaise, fatigue, depression, loss of appetite, gastrointestinal disturbances, weight loss, parestheasias and renal calculi.[2a,23] Unfortunately, because systemic therapy with parenteral sulfonamides and their derivatives leads to significant side effects, these undesirable side effects obligate synthesis of new sulfonamide derivatives. There are two main approaches used for the drug design of effective antiglaucoma sulfonamides.[8] The first is 'ring' approach, used for the synthesis of dorzolamide and brinzolamide, consisted in exploring a great variety of ring systems on which the sulfonamide group have been attached.[8,24] The second approach is 'tail', consisted in attaching water solubilizing tails to different scaffolds of well-known aromatic/heterocylic sulfonamides possessing affinity for the CA active side.[8,25]

Table 1. I_{50} values which were obtained from in vitro studies for the synthesized compounds on carbonic anhydrase isozymes

Compound	I_{50} (nM)	
	HCA-I	HCA-II
9	No effect	3500
10	150	93
11	300	66
15	190	1500
17	1100	66
18	1900	1400
3	2600	2600

We have synthesized amides of 5-amino-1,3,4-thiadiazole-2-sulfonamide with some bile acids as candidate inhibitors for glaucoma therapy as shown in Scheme 1. Since the difference in interaction between the enzyme and inhibitors can be rationalized from the varied shape of inhibitors, three kind compounds having different functional group and different number were synthesized. The first framework contains mono, di and tri alcohol groups on a bile acid. As a second skeleton, in order to investigate the effect on inhibition activity, the alcohol functional group/groups were protected with acetyl. The last compound was also obtained from dehydrocholic acid containing tri ketone.

For the synthesized compounds, the inhibitor concentrations causing up to 50% inhibition (I_{50} values) were determined from regression analysis graphs. I_{50} values obtained for HCA-I and HCA-II purified by affinity chromatography are shown in Table 1. According to in vitro studies, while the compound **9** has higher I_{50} values than acetazolamide (**3**) on CA-I, the other compounds (**10**, **11**, **15**, **17** and **18**) have lower I_{50} values than **3** on CA-I. The compound **9** containing one hydroxyl group on cholane ring has no effect on CA-II. For this reason, we think that **10**, **11** and **17** in these compounds on CA-II have the best inhibitor effect. Although the compound **18** possessing tri ketone has lower I_{50} values than **3** on both CA-I and CA-II, **18** is a weaker inhibitor than the other inhibitors.

For the in vivo studies, six groups of 42 adult rats for each inhibitor were used. For compound **9**, it was observed that the activity of control, which did not contain any inhibitor, were determined as 70663.67 ± 3324 EU $(gHb)^{-1}$. Then the inhibitor injection was performed on the control groups intraperitoneally. The activities of the groups after the inhibitor injection were measured at 2, 4 and 6 h, and % inhibition of the corresponding activities were observed as 29% ($p < 0.001$), 12% ($p < 0.05$) and 14% ($p < 0.05$) (Table 2). These results indicated that compound **9** has inhibition effect. Thus, in vivo studies related to this inhibitor does not support the results of in vitro studies.

In an analogous manner for in vivo studies we have investigated the inhibition of the following compounds

Table 2. Statistical values obtained from in vivo studies for the synthesized compounds

Compound	Time (h)	X±SD EU $(gHb)^{-1}$	% Inhibition	*p*
9	Control	70663.67±3324	—	—
	2	50173.67±4335	29	<0.001
	4	62046.00±7386	12	<0.05
	6	61051.83±7903	14	<0.05
10	Control	75054.50±6573	—	—
	2	41259.67±6084	45	<0.001
	4	30 282.00±4773	60	<0.001
	6	4 4733.83±8712	41	<0.05
11	Control	6 8376.83±4256	—	—
	2	35 771.66±5996	48	<0.001
	4	39222.00±5356	43	<0.001
	6	39033.83±5484	43	<0.001
15	Control	73981.33±8567	—	—
	2	48970.83±5141	34	<0.001
	4	41650.17±5387	44	<0.001
	6	57222.67±4076	23	<0.05
17	Control	74158.83±2835	—	—
	2	43837.00±3020	41	<0.001
	4	44678.00±4643	40	<0.001
	6	73890.17±10382	1	>0.05
18	Control	78117.17±3137	—	—
	2	47479.17±6376	39	<0.001
	4	36341.67±7831	54	<0.001
	6	40998.50±5315	48	<0.001

10–**18**. In all cases, we have observed the different results for the corresponding compounds. These results indicated that the compounds **11** and **18** have similar inhibition effect and the found values for **11** and **18** at 2, 4 and 6 h ($p<0.001$) are significant. While **11** shows inhibition effect at both in vivo and in vitro, **18** only shows inhibition effect in vivo. In the current study, our data showed that the inhibitors (**10**, **15** and **17**) decrease the activation of CA enzyme in vivo studies during 2, 4 and 6 h. But the compound **17** has no inhibition effect at 6 h ($p>0.05$). We compared the results of in vivo and in vitro studies for **10**, **15** and **17**. In vivo studies related to **10**, **15** and **17** provided evidence similar to data observed in vitro studies.

In conclusion, in the present study, we report here the preparation of 5-(5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide derivatives substituted 3, 7 and 12α-. They were obtained by attaching bile acid moieties to the 5-amino-1,3,4-thiadiazole-2-sulfonamide incorporating free amino group. Also this work is aimed at the investigation of the structure–action relationship on in vitro and in vivo CA inhibition of the synthesized compounds. For the most active compounds, the in vitro data showed that inhibition constants ranged from 66 to 190 nM for HCA-II with I_{50}. Especially, the in vivo data showed that the presence of trihydroxy-, and triketone-groups on cholane ring resulted in better inhibition effect than the other substituents.

Experimental

Solvents were concentrated at reduced pressure. Melting points were determined on a Thomas-Hoover capillary melting apparatus. Infrared spectra were obtained from KBr pellets for solids on a regular instrument. ^{1}H NMR and ^{13}C NMR spectra were recorded on 200 and 50 MHz Varian spectrometer and are reported in δ units with $SiMe_4$ as internal standard. Mass Spectrometry was performed on a Micromass'LCT using lockspray ionization.

5-(3α-Hydroxy-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (9). Lithocholic acid (**5**) (500 mg, 1.33 mmol) was dissolved in 50 mL of dry and freshly distilled THF. To this solution, *N*-methyl morpholin (148 mg, 1.46 mmol) and ethyl chloro formiate (ECF) (159 mg, 1.46 mmol) were added. After the solution was stirred for 24 h at room temperature, 263 mg (1.46 mmol) of 5-amino-1,3,4-thiadiazole-2-sulfonamide was added to the reaction mixture. The resulting mixture was refluxed for 48 h, cooled and the precipitated solid was filtrated. After removal of the solvent, the residue was dissolved in 50 mL of $CHCl_3$. The organic layer was washed with NaOH solution (1 M, 2×50 mL) and water (2×50 mL) and dried over $CaCl_2$. The chloroform was removed and the residue (750 mg) was crystallized from ethanol: colorless solid **9** (640 mg, 85%); mp 165 °C. ^{1}H NMR (200 MHz, DMSO-d_6): δ 8.35 (m, 2H), 4.50 (m, 1H), 1.93–0.82 (m, 30H), 0.89 (s, 3H), 0.63 (s, 3H); ^{13}C NMR (50 MHz, DMSO-d_6): δ 174.48, 166.08, 162.90, 71.68, 57.90, 57.30, 44.12, 43.35, 38.11, 37.21, 36.97, 36.70, 36.02 (2C), 33.76, 32.50, 32.20, 29.48, 28.70, 27.96, 25.64, 25.06 (2C), 22.23, 20.01, 13.67; IR (KBr, cm^{-1}): 3643, 3565, 3181, 2953, 2876, 1676, 1651, 1548, 1446, 1395, 1344, 1293, 1268, 1191, 1089, 1038, 961.

5-(3α,12α-Dihydroxy-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (10). The cholanamide **10** was prepared from deoxycholic acid (500 mg, 1.2 mmol) as described for the preparation of **9**. The residue (600 mg) was crystallized from ethanol: colorless solid **10** (500 mg, 90%); mp 155 °C. ^{1}H NMR (200 MHz, DMSO-d_6): δ 8.34 (m, 2H), 4.9 (m, 1H), 4.24 (m, 1H), 3.81 (m, 1H), 0.81–1.80 (m, 29H), 0.86 (s, 3H), 0.61 (s, 3H), ^{13}C NMR (50 MHz, DMSO-d_6): δ 174.54, 166.09, 162.90, 72.83, 71.78, 49.29, 47.96, 47.85, 43.46, 38.14, 37.49, 36.97, 36.78, 35.64, 34.78, 33.85, 32.61, 32.07, 30.43, 28.90, 28.81, 27.91, 25.27, 24.89, 18.83, 14.25, IR (KBr, cm^{-1}): 3565, 3514, 3412, 3259, 2953, 2876, 1753, 1676, 1651, 1574, 1446, 1395, 1370, 1319, 1268, 1191, 1114, 1063, 961, MS (CI) m/z 555.2617 (M + 1).

5-(3α,7α,12α-Trihydroxy-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (11). The cholanamide **11** was prepared from cholic acid (500 mg, 1.2 mmol) as described for the preparation of **9**. The residue (800 mg) was crystallized from ethanol: colorless solid **11** (600 mg, 87%); mp 182 °C. ^{1}H NMR (200 MHz, DMSO-d_6): δ 8.36 (m, 2H), 4.40 (m, 1H), 4.19 (m, 1H), 4.07 (m, 1H), 3.80 (m, 1H), 3.62 (m, 1H), 2.19–0.89 (m, 30H), 0.82 (s, 3H), 0.60 (s, 3H), ^{13}C NMR (50 MHz, DMSO-d_6): δ 174.59, 166.06, 162.90, 72.81, 72.25, 68.06, 47.84, 47.59, 43.32, 43.18, 37.11, 36.97, 36.87, 36.67, 36.18, 33.82, 32.63, 32.18, 30.36, 30.24, 29.01, 28.03, 24.59, 24.41,

18.84, 14.13, IR (KBr, cm^{-1}): 3412, 2953, 2876, 1753, 1676, 1651, 1548, 1472, 1446, 1370, 1319, 1268, 1191, 1089, 1038, 936, MS (CI) m/z 571.2617 (M + 1).

3α-Acetoxy-5-β-cholan-24-oic acid (12). Lithocholic acid (500 mg, 1.33 mmol) was dissolved in 5 mL of acetyl chloride and the resulting solution was stirred for 12 h at room temperature. The excessive acetyl chloride was removed under reduced pressure and the residue (680 mg) was recrystallized from ethanol as a white solid **12** (510 mg, 92%); mp 80 °C. ^{1}H NMR (200 MHz, DMSO-d_6): δ 4.71 (m, 1H), 2.92–2.65 (m, 1H), 2.02 (s, 3H), 2.22–0.61 (m, 30H), 0.92 (s, 3H), 0.64 (s, 3H), ^{13}C NMR (50 MHz, $CDCl_3$): δ 176.10, 172.54, 76.36, 58.50, 57.89, 46.40, 44.82, 43.91, 42.45, 42.15, 37.82, 37.05, 36.97, 36.60, 34.29, 33.11, 30.15, 29.01, 28.66, 28.32, 26.14, 25.30, 23.41, 22.84, 20.24, 14.04, IR (KBr, cm^{-1}): 3565, 3463, 3438, 2953, 2876, 1753, 1651, 1625, 1446, 1395, 1370, 1319, 1268,1217, 1191, 1089, 1038, 987.

5-(3α-Acetoxy-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (15). The acetoxy cholanamide **15** was prepared from 3α-acetoxy-5-β-cholan-24-oic acid (**12**) (500 mg, 1.20 mmol) as described for the preparation of **9**. The residue (800 mg) was crystallized from ethanol/H_2O: white solid **15** (510 mg, 92%); mp 165 °C. ^{1}H NMR (200 MHz, DMSO-d_6): δ 8.32 (m, 2H), 4.61 (m, 1H), 1.98 (s, 3H), 1.92–1.01 (m, 31H), 0.91 (s, 3H), 0.63 (s, 3H), ^{13}C NMR (50 MHz, DMSO-d_6): δ 174.45, 171.49, 166.10, 162.90, 75.31, 57.71, 57.33, 44.12, 43.03, 42.10, 41.68, 37.15, 36.68, 36.35, 35.98, 33.80, 33.73, 32.52, 29.47, 28.41, 28.09, 27.75, 25.61, 24.81, 22.85, 22.24, 20.02, 13.67, IR (KBr, cm^{-1}): 3285, 3208, 3080, 2953, 2876, 1753, 1702, 1548, 1472, 1370, 1268, 1191, 1114, 1038, 987, 936.

3α,12α-Diacetoxy-5-β-cholan-24-oic acid (13). The diacetoxy cholanoic acid **13** was prepared from deoxycholic acid (500 mg, 1.20 mmol) as described for the preparation of **12**. The residue (720 mg) was crystallized from ethanol/H_2O: white solid **13** (450 mg, 75%); mp 235 °C. ^{1}H NMR (200 MHz, DMSO-d_6): δ 4.97 (s, 1H), 4.59 (m, 1H), 2.05 (s, 3H), 1.98 (s, 3H), 2.56–0.69 (m, 29H), 0.89 (s, 3H), 0.70 (s, 3H), ^{13}C NMR (50 MHz, DMSO-d_6): δ 174.94, 171.55, 171.40, 76.72, 75.11, 50.85, 48.84, 46.35, 42.89, 36.89, 36.02, 35.87, 35.68, 35.39, 33.69, 32.35, 32.24, 28.60, 28.23, 27.95, 27.36, 26.92, 24.81, 24.51, 22.88, 22.72, 18.97, 13.86, IR (KBr, cm^{-1}): 3464, 2953, 2876, 1753, 1472, 1395,1268, 1140, 1114, 961, 783.

3α,7α,12α-Triacetoxy-5-β-cholan-24-oic acid (14). The triacetoxy cholanoic acid **14** was prepared from cholic acid (500 mg, 1.22 mmol) as described for the preparation of **12**. The residue (680 mg) was crystallized from ethanol/H_2O: white solid **14** (520 mg, 79%); mp 115 °C. ^{1}H NMR (200 MHz, DMSO-d_6), δ 4.98 (m, 1H), 4.80 (m, 1H), 4.45 (m, 1H), 2.09 (s, 3H), 2.02 (s, 3H), 1.99 (s, 3H), 2.31–0.74 (m, 27H), 0.91 (s, 3H), 0.70 (s, 3H), ^{13}C NMR (50 MHz, DMSO-d_6), δ 174.93, 171.57, 171.50, 171.41, 76.36, 75.12, 71.97, 48.72, 46.41, 44.82, 42.01, 41.60, 38.66, 36.15, 35.84, 35.71 32.56, 32.32, 32.16, 30.12, 28.45, 28.25, 26.88, 24.03, 23.94, 23.02, 22.93, 22.77, 18.96, 13.66, IR (KBr, cm^{-1}): 3464, 3412, 2953, 2876, 1753, 1651, 1446,1395,1268, 1038, 961.

5-(3α,7α,12α-triacetoxy-5-β-cholanamido)-1,3,4-Thiadiazole-2-sulfonamide (17). The cholanamide **17** was prepared from 3α,7α,12α-triacetoxy-5-β-cholan-24-oic acid (**14**) (500 mg, 0.94 mmol) as described for the preparation of **9**. The residue (700 mg) was crystallized from ethanol: yellow solid **17** (470 mg, 72%); mp 185 °C. ^{1}H NMR (200 MHz, $CDCl_3$), δ 5.04 (m, 1H), 4.85 (m, 1H), 4.50 (m, 1H), 2.11 (s, 3H), 2.07 (s, 3H), 2.01 (s, 3H), 2.29–0.80 (m, 27H), 0.90 (s, 3H), 0.71 (s, 3H), ^{13}C NMR (50 MHz, DMSO-d_6), δ 181.01, 174.91, 171.80 (2C), 171.69, 171.62, 77.15, 75.83, 72.48, 49.36, 47.07, 45.40, 42.99, 39.82, 36.67, 36.58, 36.33, 33.54, 33.27, 32.74, 32.57, 30.84, 29.17, 28.88, 27.50, 24.83, 24.57, 23.41, 23.25, 23.18, 19.53, 14.25, IR (KBr, cm^{-1}): 3489, 3412, 2978, 2876, 1753, 1651, 1625, 1548, 1472, 1446, 1395, 1268, 1191, 1089, 1038, MS (CI) m/z 697.2941 (M + 1).

5-(3,7,12-Trioxo-5-β-cholanamido)-1,3,4-thiadiazole-2-sulfonamide (18). Method A: The cholanamide **18** was prepared from dehydrocholic acid (500 mg, 1.24 mmol) as described for the preparation of **9**. The residue (450 mg) was crystallized from ethanol: yellow solid **18** (340 mg, 60%); mp 180 °C. **Method B:** A solution of $SOCl_2$ (31 mg, 0.26 mmol) in THF (5 mL) was added dropwise into a stirred solution of dehydrocholic acid (100 mg, 0.25 mmol) and 5-amino-1,3,4-thiadiazole-2-sulfonamide (**4**) (45 mg, 0.25 mmol) in dry THF (15 mL) during 10 min. After the addition was complete, stirring was continued for 12 h. The THF was removed under reduced pressure and the residue was crystallized from ethanol: **18** (115 mg, 82%). ^{1}H NMR (200 MHz, DMSO-d_6): δ 2.68–0.73 (m, 27H), 1.32 (s, 3H), 0.99 (s, 3H), ^{13}C NMR (50 MHz, DMSO-d_6): δ 213.64, 211.28, 211.21, 176.50, 174.93, 173.43, 58.05, 53.00, 49.78, 47.78, 47.19, 46.34, 45.87, 44.33, 40.17, 37.94, 37.43, 36.77, 36.38, 32.82, 32.14, 29.02, 26.39, 22.95, 20.42, 13.22, IR (KBr, cm^{-1}): 3565, 3489, 3438, 3259, 2978, 2876, 1727, 1651,1625, 1548, 1472, 1395, 1293, 1191, 1114, 1063, 961.

In vitro studies

Purification of carbonic anhydrase I and II from human erythrocytes. Erythrocytes were purified from human blood. The blood samples were centrifuged at 1500 rpm for 20 min and plasma and buffy coat were removed. After the packed red cells were washed with NaCl (0.9%), the erythrocytes were hemolyzed with 1.5 volumes of ice-cold water. Cell membranes were removed by centrifugation at 4 °C, 20,000 rpm for 30 min. The pH of hemolysate was brought to 8.7 with solid Tris. The hemolysate was applied to affinity column having a structure of Sepharose-4B-L-tyrosine-*p*-aminobenzensulfonamide and equilibrated with 25 mM Tris–HCl/0.1 M Na_2SO_4 (pH 8.7). The affinity gel was washed with solution of 25 mM Tris–HCl/22 mM Na_2SO_4 (pH 8.7). CA-I and CA-II isozymes were eluted with the solution of 1 M NaCl/25 mM Na_2HPO_4 (pH 6.3) and 0.1 M $NaCH_3COO$/0.5 M $NaClO_4$ (pH 5.6),

respectively. The absorbance at 280 nm was used to monitor protein in the column effluents. For protein content estimation, the method of Coomassie Brillant Blue was used with bovine serum albumin as a standard.

Measurement of CA activity. CO_2-hydratase activity of the enzyme was determined at 0 °C in a veronal buffer (pH = 8.15) with Brom Timol Blue as indicator and saturated carbon dioxide solution as substrate in a final volume of 4.2 mL. The time (in seconds) taken for the solution to change from blue to green was measured. The enzyme unit (EU) is the enzyme amount resulting in 50% decreased of the nonenzymatic reaction time. Activity as an enzyme unit was calculated by using the equation (t_0-t_c/t_c) where t_o and t_c are times for pH change of the nonenzymatic and enzymatic reactions, respectively.

Determination of I_{50}. Carbonic anhydrase activity % values were assayed by following the hydration of CO_2. I_{50} values for the synthesized compounds and acetazolamide (**3**) were determined on CA-I and CA-II. In order to determine I_{50} values, a saturated solution of CO_2 was used as substrate. To a total volume of 1.7 mL of various aliquots (10–250 μL) of a suitable concentration of inhibitors and water were added to 1 mL of Tris–HCl, 0.1 mL of Brom Timol Blue and 0.1 mL enzyme solution. The reaction was started by adding 2.5 mL of CO_2 solution. The activity was determined by using the equation (t_0-t_c/t_c) where t_0 and t_c are times for pH change of enzymatic reactions for each inhibitor concentration, respectively. Regression analysis graphs were drawn by using inhibitor concentration values by a statistical packing program on a computer. I_{50}, the inhibitor concentration resulting in 50% inhibition of the enzyme activity, was obtained from the plot of % activity against inhibitor concentration.

In vivo inhibitor studies

Forty-two adult male Spargue–Dawley rats with a weight from 200 to 250 g were used for the experiment. All of the animals were fed with standard laboratory chow and water before the experiment. The animal laboratory was windowless with automatic temperature (22 ± 1 °C) and lighting controls (14 h light/10 h dark). Twenty-four hours before the experiments, the rats were fasted and allowed access to water ad lib. All of the animals, seven in each group, were housed in different cages.

For the control, 100 μL of blood samples were taken from tail vein before inhibitor administration, which were placed into test tubes containing EDTA. Then, 250 mg/kg inhibitors (**9**–**11**, **15**–**18**) were injected intraperitoneally into each rat group. Blood samples were taken from each rat at 2, 4 and 6 h after injection. Hemolysate was prepared as described for in vitro studies. CA activity was measured with the Wilbur and Anderson method.

Statistical analysis. Results were given as mean ± SD. Data were analyzed by using the paired *t*-test, *p* values less than 0.001 were considered as indicative of significance.

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