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Carbonic anhydrase inhibitors: synthesis and inhibition of cytosolic/tumor-associated carbonic anhydrase isozymes I, II, and IX with bis-sulfamates

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Abstract—A series of bis-sulfamates incorporating aliphatic, aromatic, or betulinyl moieties in their molecules was obtained by reaction of the corresponding diols/diphenols with sulfamoyl chloride. The library of bis-sulfamates thus obtained was tested for the inhibition of three physiologically relevant human carbonic anhydrase (hCA, EC 4.2.1.1) isozymes, the cytosolic hCA I and II, and the transmembrane, tumor-associated hCA IX. The new compounds reported here inhibited hCA I with K_I s in the range of 79 nM–16.45 μM, hCA II with K_I s in the range of 6–643 nM, and hCA IX with K_I s in the range of 4–5400 nM. Several low nanomolar hCA IX inhibitors were detected, such as 1,8-octylene-bis-sulfamate or 1,10-decylene-bis-sulfamate (K_I s in the range of 4–7 nM), which showed good selectivity ratios (in the range of 3.50–3.85) for hCA IX over hCA II inhibition. The most selective hCA IX inhibitor was phenyl-1,4-dimethylene-bis-sulfamate (K_I of 61.6 nM), which was a 10.43 times better hCA IX than hCA II inhibitor. These derivatives are interesting candidates for the development of novel antitumor therapies targeting hypoxic tumors, since hCA IX is highly overexpressed in such tissues, and its presence is correlated with bad prognosis and unfavorable clinical outcome.

1. Introduction

In previous work from these laboratories,[1,2] we showed that the simple sulfamates possessing the general formula R–OSO_2NH_2 (R = aliphatic C_5–C_{16} saturated or unsaturated chains; simple aromatic-phenyl or substituted-phenyls—as well as polycyclic, mainly steroidal moieties) act as efficient inhibitors of several isozymes of carbonic anhydrase (CA, EC 4.2.1.1), among which is also the tumor-associated, transmembrane one CA IX, which is upregulated by hypoxia and overexpressed in many tumor types.[3–5] Indeed, it has only recently been discovered that invasive growth and metastatic spread of many tumors are closely associated with a reduced oxygenation of the tissue, and more precisely with hypoxia.[3] Tumor hypoxia is the result of the abnormal process of neoplastic growth and crucially depends on oxygen/nutrients supply from the host.[3] Thus, changes in tumor metabolism and microenvironment connected with adaptation of cells to hypoxia are important components of tumor progression.[3,4] Hypoxic conditions elicit cellular responses designed to improve cell oxygenation and survival by means of several mechanisms such as neoangiogenesis, improved glycolysis and enhanced energy production, as well as upregulation of molecules related to cell survival/apoptosis.[3] The most important molecule regulating the mammalian response to hypoxia is the heterodimeric protein hypoxia-inducible factor 1 (HIF-1), which in turn up-regulates genes involved in adaptation responses to hypoxic conditions.[3] Two such genes encode for the transmembrane hCA isozymes IX and XII, containing extracellular enzyme active sites. These hCAs appear to participate in tumorigenetic processes via their ability

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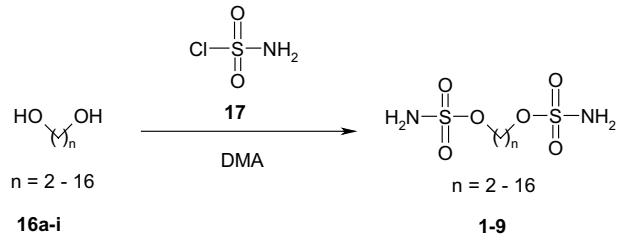
to catalyze hydration of CO_2 to bicarbonate and protons, regulating in this way the intratumoral pH.[4] In addition, hCA IX, possessing a unique N-terminal domain, has a capacity to perturb E-cadherin mediated cell–cell adhesion via interaction with β-catenin and may potentially contribute to tumor invasion.[4] hCA IX shows restricted expression in normal tissues but is tightly associated with different types of tumors, mostly due to its strong induction by tumor hypoxia that involves HIF-1 binding to a hypoxia response element in the *CA9* gene promoter.[3,4] hCA IX was proposed to serve as a marker of tumor hypoxia and its predictive and prognostic potential has been demonstrated in numerous clinical studies (reviewed in Ref. 4) hCA XII is present in many normal tissues and overexpressed in some tumors.[4] It is also induced by hypoxia, but the underlying molecular mechanism remains undetermined. Both hCA IX and hCA XII are negatively regulated by von Hippel Lindau tumor suppressor protein and their expression in renal cell carcinomas is related to inactivating mutation of *VHL* gene.[4] The high catalytic activity of these two CA isoforms supports their role in acidification of tumor microenvironment that facilitates acquisition of metastatic phenotypes.[3,4] Therefore, modulation of extracellular tumor pH via inhibition of CA activity represents a promising approach to anticancer therapy.[4–6] Sulfonamide CA inhibitors were shown to compromise tumor cell proliferation and invasion in vitro and improve the effect of conventional chemotherapy in vivo. [4–7] However, their precise targets are not known in detail at this moment, but it is presumed that these two tumor-associated CA isozymes, that is, hCA IX and XII, may represent important enzymes for targeting cancer cells, by an unconventional therapeutic approach.[4–7]

We recently showed that hCA IX was a druggable target.[8–13] In previous works we have explored the design of potent and possibly selective sulfamate or sulfonamide hCA IX inhibitors belonging to various chemical classes.[1,2,8–13] Among the best hCA IX inhibitors detected so far were some aliphatic and aromatic simple sulfamates, such as the *n*-C_8–C_{16} alkylsulfamates, or the benzyl/phenethyl sulfamates, which showed inhibition constants against hCA IX in the range of 9–25 nM.[1,2] In these studies, only one aromatic bis-sulfamate and a steroidal-bis-sulfamate have been investigated for the inhibition of hCA IX, more precisely 5-*n*-pentyl-resorcinyl-bis-sulfamate, which showed effective hCA IX inhibitory properties (K_I of 43 nM),[1] and estradiol-3,17β-disulfamate (K_I of 58 nM),[1] whereas no such aliphatic compounds have been studied. Thus, we decided to extend our investigations to this type of compounds, and here we present the synthesis and hCA I, II, and IX inhibitory properties of a series of bis-sulfamates incorporating both aliphatic and aromatic as well as polycyclic moieties.

2. Chemistry

Bis-sulfamates **1**–**15** investigated here were prepared as reported earlier for the monosulfamates investigated as



Scheme 1.

CA inhibitors,[1,2] by reacting the appropriate diol/diphenol with sulfamoyl chloride in *N*,*N*-dimethylacetamide as solvent.[14–16] Sulfamoyl chloride has been generated from chlorosulfonyl isocyanate and formic acid.[16] Thus, for the aliphatic compounds **1**–**9**, reaction of the diols **16a**–**i** with sulfamoyl chloride **17** afforded the bis-sulfamates in high yields (Scheme 1).

By using the appropriate diphenol/diol, compounds **10**–**15** were prepared in a similar manner as **1**–**9** discussed above.[17]

3. Carbonic anhydrase inhibition

Inhibition data against three physiologically relevant isozymes, that is, the cytosolic isozymes hCA I and II and the membrane-bound, tumor-associated isozyme hCA IX (all of them of human origin) with sulfamates **1**–**15** as well as the standard, clinically used CA inhibitors acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, dichlorophenamide **DCP**, dorzolamide **DZA**, brinzolamide **BRZ**, and indisulam **IND** are shown in Table 1.[18]

The following SAR should be noted from data of Table 1: (i) sulfamates **1**–**15** inhibited the three investigated CA isozymes with different affinities, most of them behaving as medium potency hCA I inhibitors (K_I s in the range of 79 nM–16.45 μM), whereas against hCA II the K_I s varied in the range of 6–643 nM, and against hCA IX K_I s were in the range of 4–5400 nM; (ii) for hCA I, the best inhibitors in this series were resorcinyl-bis-sulfamate **13**, the catechol-bis-sulfamate derivative **11**, the betulinyl-bis-sulfamate **14** as well as the other resorcinyl derivative **15**, which showed inhibition constants in the range of 79–105 nM, being much more effective compared to most of the clinically used sulfonamides (except ethoxzolamide and indisulam). Among the *n*-aliphatic derivatives, good activity was shown by the C6 and C7 bis-sulfamates **2** and **3** (K_I s in the range of 136–145 nM), which was strongly diminished for the shorter (**1**), longer (**4**–**7**) or branched compounds (**8** and **9**). Phenyl-1,4-dimethylene-bis-sulfamate **10** was the most ineffective hCA I inhibitor in this series of derivatives (K_I > 20 μM); (iii) against hCA II, the best inhibitors were the aliphatic derivatives **1**–**4** (*n*-C4–C8 chain), the aromatic ones **11**–**13**, betulinyl-bis-sulfamate **14** as well as estradiol-3,17β-bis-sulfamate (previously investigated),[1] which showed inhibition constants in the range of 5–15 nM, of the same order of magnitude as the

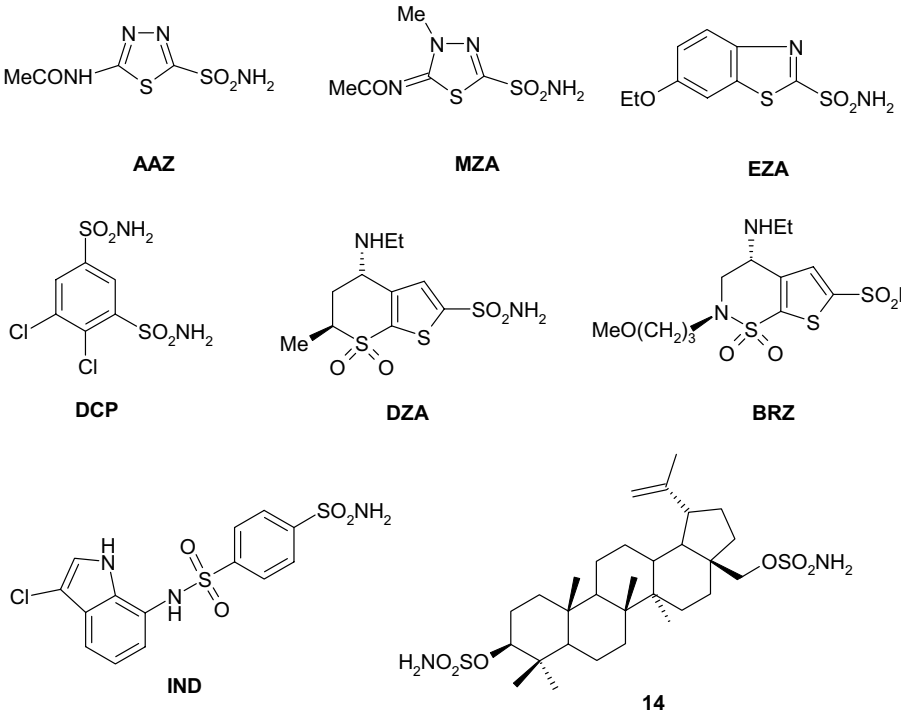


Table 1. Inhibition data for bis-sulfamates **1**–**15** investigated in the present paper and standard sulfonamide CA inhibitors, against isozymes I, II, and IX[24]

H_2NO_2S–O–R–O–SO_2NH_2

1–**15**

Inhibitor	R	K_I* (nM)			Selectivity ratio K_I(hCA II)/K_I(hCA IX)
		hCA I[a]	hCA II[a]	hCA IX[b]	
AAZ		900	12	25	0.48
MZA		780	14	27	0.52
EZA		25	8	34	0.23
DCP		1200	38	50	0.76
DZA		50,000	9	52	0.17
BRZ		NT	3	37	0.08
IND		31	15	24	0.62
1	$(CH_2)_4$	548	9.7	5400	0.0018
2	$(CH_2)_6$	136	8.3	13.2	0.63
3	$(CH_2)_7$	145	8.0	10.1	0.79
4	$(CH_2)_8$	378	14.6	4.0	3.85
5	$(CH_2)_{10}$	890	24.5	7.0	3.50
6	$(CH_2)_{12}$	1350	84.2	32.1	2.63
7	$(CH_2)_{16}$	16,450	563	525	1.07
8	MeCHCHMe	6490	417	1320	0.31
9	CH_2–CMe_2–CH_2	872	380	2020	0.18
10	4-CH_2–C_6H_4–CH_2	>20,000	643	61.6	10.43
11	4-*t*-Bu-1,2-phenylene	93	6.0	9.6	0.62
12	4-Me-1,2-phenylene	267	15.0	70.8	0.21
13	1,3-C_6H_4	79	9.4	100	0.09
14	Betulinyl[c]	108	14.7	28.0	0.52
15	5-Pentyl-1,3-C_6H_3[d]	105	76.3	43.1	1.76
Estradiol-3,17-disulfamate[d]		6000	5.0	58.0	0.08

[a] Human (cloned) isozymes, by the CO_2 hydration method.
[b] Catalytic domain of human, cloned isozyme, by the CO_2 hydration method.
[c] See structure in the text.
[d] From Ref. 1.
* Errors in the range of 5–10% of the reported value (from three different assays).

clinically used sulfonamides **AAZ–IND** (Table 1). It is interesting to note that the C-5 monosulfamate previously investigated behaved as a medium potency hCA II inhibitor (K_I of 58 nM),[2] whereas the C10–C12 monosulfamates were very effective inhibitors (K_I s in the range of 0.7–10 nM).[2] Thus, the presence of a second sulfamate moiety is beneficial for increasing hCA II inhibitory properties for compounds with a medium length chain (of four to eight carbon atoms) as compared to the corresponding monosulfamates, whereas for longer chain derivatives, the monosulfamates are better inhibitors than the corresponding bis-sulfamates (the entire series of C1–C18 monosulfamates has been investigated in the previous work).[2] Another group of bis-sulfamates, such as **5**, **6**, and **15** showed medium potency hCA II inhibitory properties, with K_I s in the range of 24.5–84.2 nM. The remaining compounds, possessing long alkyl chains (**7**), branched chains (**8** and **9**) as well as phenyl-1,4-dimethylene-bis-sulfamate **10** showed weak hCA II inhibitory effects (K_I s in the range of 380–643 nM); (iv) against hCA IX, the best inhibitors were the aliphatic derivatives **2**–**5** incorporating C6–C10 chains, and the aromatic bis-sulfamate **11** possessing a bulky *tert*-butyl tail, which showed K_I s in the range 4.0–13.2 nM, being much more effective than the clinically used sulfonamides **AAZ–IND** (K_I s in the range of 24–50 nM). Another group of bis-sulfamates including **6**, **10**, **12**–**15**, and estradiol-3,17β-bis-sulfamate behaved as medium potency hCA IX inhibitors, with K_I s in the range of 32–100 nM. Except **6**, all these compounds are either aromatic or polycyclic. The most ineffective hCA IX inhibitors were the C16 bis-sulfamate **7**, and the branched derivatives **8** and **9** (K_I s in the range of 525–2020 nM). Thus, SAR becomes rather clear: best hCA IX inhibition is achieved with aliphatic sulfamates incorporating C6–C10 *n*-alkyl chains (with the optimum of activity for the C8-bis-sulfamate) as well as with 1,2-phenylene-bis-sulfamates also possessing a bulky aliphatic moiety (the presence of the *tert-butyl* moiety in **11** leads to a 7-fold increase of hCA IX inhibitory properties as compared to the corresponding methyl-substituted compound **12**); (v) since both isozymes II and IX show good affinity for most of the sulfamates investigated here, the selectivity of these inhibitors for the tumor-associated isozyme IX is a critical issue for the drug design of such derivatives. As seen from data of Table 1, the clinically used sulfonamides **AAZ–IND** show better hCA II than hCA IX inhibitory properties, with selectivity ratios in the range of 0.08–0.62. Many of the bis-sulfamates investigated here, such as **1**–**3**, **8**, **9**, **11**–**14**, and estradiol-3,17β-bis-sulfamate also show selectivity ratios <1. However, six of the new derivatives have this parameter >1. Thus, the potent aliphatic hCA IX inhibitors **4**–**6** showed selectivity ratios in the range of 2.63–3.50, whereas compound **10** was a 10.43 times a better hCA IX than hCA II inhibitor (but it is a medium potency hCA IX inhibitor). Compounds **7** and **15** are only slightly better hCA IX than hCA II inhibitors (selectivity ratios of 1.07–1.76).

In conclusion, we report the synthesis and CA I, II, and IX inhibitory properties for a series of aliphatic, aromatic, and polycyclic bis-sulfamates. SAR showed that each isozyme is best inhibited by a certain structural type of such compounds, with hCA I being best inhibited by resorcinyl-1,3-bis-sulfamate (K_I of 79 nM), hCA II by estradiol-3,17β-bis-sulfamate, 4-*t*-butyl-1,2-phenylene-bis-sulfamate and the aliphatic C4–C7 bis-sulfamates (K_I s in the range of 5–9.7 nM) and CA IX by the C7–C10 aliphatic bis-sulfamates (K_I s in the range of 4-10 nM). Some of these derivatives are also more selective CA IX compared to CA II inhibitors (selectivity ratios in the range of 2.63–10.43). Since CA IX is highly overexpressed in many hypoxic tumors, such compounds represent valuable candidates for the potential development of novel non-classical anti-tumor therapies.

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17. General procedure for the preparation of sulfamates **1**–**15**. Sulfamates **1**–**15** were prepared by reacting the commercially available appropriate diol or biphenol (1 equiv) with sulfamoyl chloride (5 equiv) in *N*,*N*-dimethylacetamide.[1,14] Sulfamoyl chloride was prepared from chlorosulfonyl isocyanate and formic acid as described in Ref. 15. After completion of the reaction (TLC monitoring), the mixture was diluted with ethyl acetate, and washed several times with water. The organic extract was dried ($MgSO_4$) and concentrated under vacuum. The residue was purified either by crystallization from ether/pentane or by chromatography on silica gel.
1,4-Butylene-bis-sulfamate **1**: mp 134–135 °C (lit. 126–129 °C);[16] ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.45 (s, 4H), 4 (s, 4H), 1.7 (s, 4H); ^{13}C NMR (DMSO-d_6, 400 MHz): δ 68.9, 25.3; MS ESI$^+$ *m*/*z* 271 (M+Na)$^+$, 519 (2M+Na)$^+$. ESI$^-$ *m*/*z* 247 (M−H)$^-$, 495 (2M−H)$^-$.
1,6-Hexylene-bis-sulfamate **2**: mp 128–129 °C; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.4 (s, 4H), 4 (t, 4H, J = 5.8 Hz), 1.65 (m, 4H), 1.35 (m, 4H); ^{13}C NMR (DMSO-d_6, 400 MHz): δ 69.4, 28.6, 25; MS ESI$^+$ *m*/*z* 299 (M+Na)$^+$, 575 (2M+Na)$^+$. ESI$^-$ *m*/*z* 275 (M−H)$^-$, 551 (2M−H)$^-$.
1,7-Heptylene-bis-sulfamate **3**: mp 94–95 °C; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.4 (s, 4H), 4 (t, 4H, J = 6.5 Hz), 1.65 (m, 4H), 1.3 (m, 6H); ^{13}C NMR (DMSO-d_6, 400 MHz): δ 69.4, 28.7, 28.4, 25.4; MS ESI$^+$ *m*/*z* 313 (M+Na)$^+$, 603 (2M+Na)$^+$. ESI$^-$ *m*/*z* 289 (M−H)$^-$, 579 (2M−H)$^-$.
1,8-Octylene-bis-sulfamate **4**: mp 97–98 °C; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.4 (s, 4H), 4 (t, 4H, J = 6.4 Hz), 1.6 (m, 4H), 1.3 (m, 8H); ^{13}C NMR (DMSO-d_6, 400 MHz): δ 69.4, 28.8, 28.7, 25.4; MS ESI$^+$ *m*/*z* 327 (M+Na)$^+$, 631 (2M+Na)$^+$. ESI$^-$ *m*/*z* 303 (M−H)$^-$, 607 (2M−H)$^-$.
1,10-Decylene-bis-sulfamate **5**: mp 111–112 °C (lit. 102–105 °C)[16]; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.4 (s, 4H), 4 (t, 4H, J = 6.4 Hz), 1.6 (m, 4H), 1.3 (m, 12H); ^{13}C NMR (DMSO-d_6, 400 MHz): δ 69.4, 29.3, 28.9, 28.7, 25.5; MS ESI$^+$ *m*/*z* 355 (M+Na)$^+$, 687 (2M+Na)$^+$. ESI$^-$ *m*/*z* 331 (M−H)$^-$, 663 (2M−H)$^-$.
1,12-Dodecylene-bis-sulfamate **6**: mp 121–122 °C; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.4 (s, 4H), 4 (t, 4H, J = 6.4 Hz), 1.6 (m, 4H), 1.3 (m, 16H); ^{13}C NMR (DMSO-d_6, 400 MHz): δ 69.4, 29.45, 29.4, 29, 28.7, 25.5; MS ESI$^+$ *m*/*z* 383 (M+Na)$^+$. ESI$^-$ *m*/*z* 359 (M−H)$^-$, 719 (2M−H)$^-$.
1,16-Hexadecylene-bis-sulfamate **7**: mp 126–127 °C; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.4 (s, 4H), 4 (t, 4H, J = 6.4 Hz), 1.6 (m, 4H), 1.3 (m, 24H); ^{13}C NMR (DMSO-d_6, 400 MHz): δ 69.4, 29.56, 29.54, 29.48, 29.42, 29, 28.8, 25.5; MS ESI$^+$ *m*/*z* 439 (M+Na)$^+$, 855 (2M+Na)$^+$. ESI$^-$ *m*/*z* 415 (M−H)$^-$, 831 (2M−H)$^-$.
2,3-Butylene-bis-sulfamate **8**: mp 153–155 °C (lit. 115–117 °C)[16]; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.5 (s, 4H), 4.5 (m, 2H), 1.35 (d, 6H, J = 6 Hz); ^{13}C NMR (DMSO-d_6, 400 MHz): δ 77.7, 16.8; MS ESI$^+$ *m*/*z* 271 (M+Na)$^+$, 519 (2M+Na)$^+$. ESI$^-$ *m*/*z* 247 (M−H)$^-$, 495 (2M−H)$^-$.
2,2-Dimethyl-1,3-propylene-bis-sulfamate **9**: mp <30 °C; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.5 (s, 4H), 3.8 (s, 4H), 1 (s, 6H); ^{13}C NMR (DMSO-d_6, 400 MHz): δ 73.6, 35.2, 21.2; MS ESI$^+$ *m*/*z* 285 (M+Na)$^+$, 547 (2M+Na)$^+$. ESI$^-$ *m*/*z* 261 (M−H)$^-$, 523 (2M−H)$^-$.
Phenyl-1,4-dimethylene-bis-sulfamate **10**: mp >220 °C decomp.; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.6 (s, 4H), 7.45 (s, 4H), 5.1 (s, 4H); ^{13}C NMR (DMSO-d_6, 400 MHz): δ 135.5, 128.9, 70.3; MS ESI$^+$ *m*/*z* 319 (M+Na)$^+$. *4-tert-Butyl-1,2-phenylene-bis-sulfamate* **11**: mp 127–128 °C; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.1 (s, 4H), 7.5 (m, 1H), 7.4 (m, 2H), 1.4 (s, 9H); ^{13}C NMR (DMSO-d_6, 400 MHz): δ 150.4, 142, 140, 124.5, 123.3, 121.1, 34.9, 31.5; MS ESI$^+$ *m*/*z* 347 (M+Na)$^+$, 670 (2M+Na)$^+$. ESI$^-$ *m*/*z* 323 (M−H)$^-$, 647 (2M−H)$^-$.
4-Methyl-1,2-phenylene-bis-sulfamate **12**: mp 149–151 °C; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.1 (s, 2H), 8.05 (s, 2H), 7.35 (d, 1H, J = 3.8 Hz), 7.3 (d, 1H, J = 1.6 Hz), 7.15 (dd, 1H, J = 3.8 Hz, J = 1.6 Hz); ^{13}C NMR (DMSO-d_6, 400 MHz): δ 142.3, 140.3, 137.3, 127.8, 124, 123.6, 20.9; MS ESI$^+$ *m*/*z* 305 (M+Na)$^+$, 587 (2M+Na)$^+$. ESI$^-$ *m*/*z* 280 (M−H)$^-$, 562 (2M−H)$^-$.
Resorcinyl-bis-sulfamate **13**: mp 117–118 °C; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.1 (s, 4H), 7.25 (m, 3H), 6.7 (m, 1H); ^{13}C NMR (DMSO-d_6, 400 MHz): δ 158.9, 130.8, 121, 116.8; MS ESI$^+$ *m*/*z* 291 (M+Na)$^+$, 559 (2M+Na)$^+$. ESI$^-$ *m*/*z* 267 (M−H)$^-$, 534 (2M−H)$^-$.
Betulinyl-bis-sulfamate **14**: mp 116–117 °C; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.4 (s, 2H), 7.35 (s, 2H), 4.7 (s, 1H), 4.6 (s, 1H), 4.1 (d, 1H, J = 9.3 Hz), 4 (dd, 1H, J = 14.1 Hz, J = 9.6 Hz), 3.75 (d, 1H, J = 9.2 Hz), 2.95 (s, 1H), 2.8 (s, 1H), 2.45 (m, 1H), 2–0.8 (m, 42H); ^{13}C NMR (DMSO-d_6, 400 MHz): δ 170, 150.1, 110.5, 88, 67.5, 55.4, 50, 48.6, 47.6, 46.4, 42.7, 38.6, 37.9, 37.6, 36.9, 34.9, 34, 29.3, 28, 26.8, 25.1, 24.3, 21.8, 21.2, 20.8, 19.7, 19.2, 18.2, 16.6, 16.2, 16.1, 15.1, 14.8; MS ESI$^+$ *m*/*z* 624 (M+Na)$^+$. ESI$^-$ *m*/*z* 599 (M−H)$^-$.
5-Pentyl-resorcinyl-bis-sulfamate **15** was prepared as described in Ref. 1.
18. Human CA I and CA II cDNAs were expressed in *Escherichia coli* strain BL21 (DE3) from the plasmids pACA/hCA I and pACA/hCA II described earlier by Behravan, G.; Jonsson, B. H.; Lindskog, S. *Eur. J. Biochem.* **1990**, *190*, 351–357, and enzymes were purified by affinity chromatography according to the method of Khalifah et al.[19] Enzyme concentrations were determined spectrophotometrically at 280 nm, utilizing a molar

absorptivity of 49 mM^{-1} cm^{-1} for CA I and 54 mM^{-1} cm^{-1} for CA II, respectively, based on M_r = 28.85 kDa for CA I, and 29.3 kDa for CA II, respectively.[20,21] A variant of the previously published[1,2,11] CA IX purification protocol has been used for obtaining high amounts of hCA IX needed in these experiments. The catalytic domain of hCA IX cloned into pGEX-4T-1 vector (details described previously in Zatovicova et al.[22]) was expressed in *Escherichia coli* BL21 Codon Plus bacterial strain (Stratagene). The bacterial cells were resuspended in the lysis buffer (10 mM Tris pH 7.5, 1 mM EDTA pH 8, 150 mM NaCl and 0.2% Triton X-100) and incubated for 20 min on ice with lysozyme (Sigma) in final concentration 1 mg/mL. To suspension was added COMPLETE cocktail of protease inhibitors (Roche) and bacterial cells were sonicated (5 × 30 s). The obtained lysate was centrifuged for 30 min at 10,000 rpm, at +4 °C and the supernatant was then applied to a prepacked Glutathione Sepharose 4B column (Amersham), extensively washed with the lysis buffer followed by phosphate buffer-saline, pH 7.4, and the fusion protein was eluted with a buffer consisting of 5 mM reduced glutathione in 50 mM Tris–HCl, pH 8.0. Finally the GST part of the fusion protein was cleaved with thrombin (Sigma). The advantage of this method over the previous one,[1,2,11] is that CA IX is not precipitated in inclusion bodies from which it has to be isolated by denaturing–renaturing in the presence of high concentrations of urea, when the yields in active protein were rather low, and the procedure much longer. The obtained CA IX was further purified by sulfonamide affinity chromatography,[19] the amount of enzyme being determined by spectrophotometric measurements and its activity by stopped-flow experiments, with CO_2 as substrate.[23] The specific activity of the obtained enzyme was the same as the one previously reported,[1,2] but the yields in active protein were 5–6 times higher per liter of culture medium.

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24. An SX.18MV-R Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity.[23] Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. Saturated CO_2 solutions in water at 20 °C were used as substrate.[23] Stock solutions of inhibitor (1 mM) were prepared in distilled–deionized water with 10–20% (v/v) DMSO (which is not inhibitory at these concentrations) and dilutions up to 0.01 nM were done thereafter with distilled–deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. Triplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results.