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https://orcid.org/0000-0003-4682-4964

VILNIAUS UNIVERSITETAS

Audrius Zakšauskas

Karboanhidrazių izofermentų selektyvių slopiklių kūrimas ir sintezė

DAKTARO DISERTACIJA

Technologijos mokslai,
Chemijos inžinierija (T 005)

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Mokslinis konsultantas – prof. dr. Daumantas Matulis (Vilniaus universitetas, technologijos mokslai, chemijos inžinerija, T 005).

Gynimo taryba:
Pirmininkas – prof. dr. Rolandas Meškys (Vilniaus universitetas, technologijos mokslai, chemijos inžinerija – T 005).
Nariai:
prof. dr. Eglė Arbačiauskienė (Kauno technikos universitetas, gamtos mokslai, chemija – N 003),
doc. dr. Rima Budvytytė (Vilniaus Universitetas, gamtos mokslai, biochemija – N 004),
dr. Jonas Šarlauskas (Vilniaus Universitetas, technologijos mokslai, chemijos inžinerija – T 005),
dr. Saulius Šumanas (Pietų Floridos universitetas, JAV, gamtos mokslai, biochemija – N 004).

Disertacija ginama viešame Gynimo tarybos posėdyje 2026 m. liepos mėn. 2 d. 13 val. Vilniaus universiteto Gyvybės mokslų centro R401 auditorijoje. Adresas: Saulėtekio al. 7, Vilnius, Lietuva, tel. +370 5 2234419 ; el. paštas info@gmc.vu.lt

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VILNIUS UNIVERSITY

Audrius Zakšauskas

Design and Synthesis of Isozyme-Selective Carbonic Anhydrase Inhibitors

DOCTORAL DISSERTATION

Technological Sciences,
Chemical Engineering (T 005)

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The dissertation was prepared between 2013 and 2026 at the Institute of Biotechnology, Life Sciences Center of Vilnius University.

The dissertation is defended on an external basis

Academic consultant – Prof. Dr. Daumantas Matulis (Vilnius University, Technological Sciences, Chemical Engineering, T 005).

This doctoral dissertation will be defended in a public meeting of the Dissertation Defence Panel:

Chairman – Prof. Dr. Rolandas Meškys (Vilnius University, Technological Sciences, Chemical Engineering, T 005).
Members:
Prof. Dr. Eglė Arbačiauskienė (Kaunas University of Technology, Natural Sciences, Chemistry, N 003),
Assoc. Prof. Dr. Rima Budvytytė (Vilnius University, Natural Sciences, Biochemisty, N 004),
Dr. Jonas Šarlauskas (Vilnius University, Technological Sciences, Chemical engineering, T 005),
Dr. Saulius Šumanas (University of South Florida, USA, Natural Sciences, Biochemisty, N 004).

The dissertation shall be defended at a public meeting of the Dissertation Defence Panel at 13:00 on 2 July 2026 in Room R401 of the Life Sciences Centre (Vilnius University).
Address: Saulėtekio av. 7, Room No. R401, Vilnius, Lithuania
Tel. +370 5 2234419 ; e-mail: info@gmc.vu.lt

TURINYS

SANTRUMPOS

BMR	Branduolių magnetinis rezonansas
CA	Karboanhidrazė
CARP	Į karboanhidrazę panašus baltymas (CA related protein)
DFT	Tankio funkcionalo teorija (density functional theory)
DMF	Dimetilformamidas
DMSO	Dimetilsulfoksidas
HPLC	Didelio efektyvumo skysčių chromatografija
MeOH	Metanolis
NaOAc	Natrio acetatas
TEA	Trietilaminas
THF	Tetrahidrofuranas
ZBG	Su cinko jonu besijungianti grupė (angl. zinc binding group)

ĮVADAS

Mažamolekulinių cheminių vaistų kūrimas yra ilgas ir brangus procesas, kuris pastaraisiais dešimtmečiais tampa vis sudėtingesnis dėl padidėjusių reikalavimų veiksmingumui ir saugumui. Pirmasis etapas šiame procese yra potencialaus, galinčio būti naudingu gydant konkrečią ligą ar būklę, kandidato į vaistus nustatymas, organinė sintezė ir optimizavimas. Norint tai sėkmingai atlikti reikia suprasti kokiais būdais vaistai sąveikauja su biologinių sistemų tikslinėmis struktūromis (receptoriais, fermentais, medžiagų transporteriais ir pan.) ir kaip keičiant struktūrą pasiekti norimas vaistų savybes.

Siekiant suprasti bendrus vaistų kūrimo principus vieni iš patraukliausių tiriamųjų taikinių yra fermentai. Jie dažniausiai pasižymi selektyviu veikimu, t.y. dideliu specifiškumu substratams, todėl galima tikėtis sukurti tik konkretų procesą veikiančius vaistus. Be to, yra žinoma daug ligų, kurios siejamos su fermentų ekspresijos, ir tuo pačiu jų atliekamų funkcijų, pokyčiais. Taip pat, didelis fermentų katalizinis aktyvumas leidžia tikėtis, kad net nedidelis slopiklio kiekis stipriai paveiks aktyvumą ir sukels didelį terapinį efektą. Ir be viso to, tokie ryškūs aktyvumo pokyčiai dažniausiai palengvina laboratorinius tyrimus ir pagreitina patį tyrimų procesą. Todėl šioje disertacijoje pasirinkta viena tokių tiriamųjų taikinių šeima – karboanhidrazės.

Karboanhidrazės yra fermentai randami visuose gyvuose organizmuose, nuo bakterijų iki žinduolių, katalizuojantys plačiai sutinkamą anglies dioksido hidratacijos reakciją:

$$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^-$$

Kodėl gi tos karboanhidrazės tokios svarbios? Žiūrint į gazuotame gėrime kylančius burbuliukus sunku įsivaizduoti kam tos CA aplamai reikalingos. Pasirodo anglies dioksido hidratacijos reakcija yra ganėtinai lėta[1] (k-0,039 s^{-1}), o karboanhidrazės pagreitina ją iki ~10^6 s^{-1} ir tokiu būdu gali žymiai įtakoti įvairiausius organizme vykstančius procesus.

Vien žmogaus organizme aptinkama 12 katalitiškai aktyvių alfa šeimos karboanhidrazių, kurios pasižymi dideliu struktūriniu panašumu, bet skiriasi savo lokalizacija ląstelėje ir organuose, funkcija ir kataliziniu aktyvumu.

Karboanhidrazės žmogaus organizme atlieka skirtingas fiziologines funkcijas dalyvaudamos tokiuose procesuose kaip CO_2 pernaša, pH reguliacija, elektrolitų apykaita, kaulų rezobcija, vėžinių navikų susidarymas ir t.t. Tam tikrų karboanhidrazių veiklos pokyčiai (padidėjęs aktyvumas, ekspresija) yra siejami su įvairiomis ligomis, kurios gydomos CA slopikliais (glaukoma, epilepsija, idiopatinė intrakranijinė hipertenzija, aukštumos liga, migrena ir pan). Daugumos medicinoje naudojamų CA slopiklių trūkumas yra

šalutiniai poveikiai, kurie pirmiausia siejami su mažu atrankumu tikslinėms karboanhidrazėms. Todėl CA izofermentams atrankių slopiklių sukūrimas visą laiką išlieka aktualia užduotimi.

Ne mažiau svarbūs kiti CA slopiklių panaudojimo būdai, kai foto-, radioaktyviomis ir pan. grupėmis modifikuotas slopiklis atlieka vaizdinimo arba citotoksinę funkciją. Šios taikymo sritys tapo ypač aktualios pastaraisiais dešimtmečiais ir siejamos pagrinde su CA IX padidėjusia ekspresija vėžiniuose navikuose. Tokiu atveju CA aktyvumo pakeitimas nebūna svarbiausia užduotis, tačiau atrankus jungimasis prie tikslinių CA izofermentų yra dar svarbesnis.

Šiame **darbe iškeltas tikslas** sukurti ir susintetinti selektyvius CA izofermentams slopiklius, atskleisti selektyvumą nulemiančius faktorius ir panaudoti juos naujų junginių kūrimui.

Tikslui pasiekti iškelti šie **uždaviniai**:

- Susintetinti 3,4-dipakeistus-2-halobenzensulfonamidus ir atskleisti pakaitų įtaką slopiklių afiniškumui ir selektyvumui.
- Siekiant įvertinti kuo arčiau CA kofaktoriaus esančių inhibitorių pakaitų įtaką susintetinti 2-pakeistus-benzensulfonamidus.
- Nustatyti ir įvertinti pakaitų padėties, dydžio ir lankstumo įtaką CA slopiklių afiniškumui ir selektyvumui.
- Sukurti naujus su vėžiniais susirgimais siejamos CA IX didelio afiniškumo bei selektyvumo inhibitorius.

MOKSLINIS NAUJUMAS

Disertacijoje susintetintos ir palygintos dvi grupės benzensulfonamidinių slopiklių turinčių masyvius pakaitus 4,5- ir 2,5- padėtyse. Abiejose grupėse surasta slopiklių pasižyminčių išskirtinai geru sub-nanomoliniu afiniškumu atskiriems CA izofermentams.

Susintetinti junginiai įgalino nustatyti slopiklio–CA kompleksų kristalines struktūras. Tai padėjo suprasti slopiklių struktūros–biologinio aktyvumo sąryšius ir davė bendrą teigiamą tyrimų poslinkį, ypač su vėžiniais susirgimais siejamo karboanhidrazės IX izofermento slopiklių paieškoje.

Ištirtas 2,4-dihalo-5-sulfamoilbenzoatų turinčių identiškus halogenus nukleofilinio pakeitimo reakcijų regioelektyvumas ir surastas naujas būdas 2,5-dipakeistų-4-halo-benzensulfonamidų sintezei.

Nustatyta, kad 2,5-dipakeistų benzensulfonamidinių slopiklių grupėje ypač didelę įtaką slopiklio savybėms turi 2-padėtyje esantys pakaitai. 2- amino ir 2-sulfanilpakaitai pasižymi dideliu afiniškumu ir selektyvumu CA IX izofermentui. Tuo tarpu 2-sulfonilpakaitai sąlygoja labai didelį afiniškumo sumažėjimą visiems CA izofermentams. Taip pat parodyta, kad 2-pakeisti benzensulfonamidai gali būti perspektyvūs ir geresni CA IX slopikliai nei ligšioliniai panašios struktūros vaistai, tokie kaip bumetanidas ir piretanidas.

GINAMIEJI TEIGINIAI

1. Benzensulfonamidinių CA slopiklių pakaitų esančių *para*-padėtyje lankstumas įtakoja slopiklių selektyvumą atskiriems CA izofermentams.
2. Benzensulfonamidų *para*- sulfanil- ir sulfonilgrupės sąlygoja skirtingas pakaitų pozicijas CA aktyviajame centre, bei įtakoja didesnį slopiklio afiniškumą nei amino pakaitai.
3. Slopikliai sukurti į 5-[2-(benzimidazol-1-il)acetil]-2-chlor-benzensulfonamidą įvedant papildomus pakaitus, pasižymi didesniu afiniškumu visoms karboanhidrazėms, bet stipriai sumažina selektyvumą tikslinei CA VA.
4. Sulfonamidinių slopiklių *orto*-padėtyje esantys sulfanil- ir sulfonilpakaitai stipriai įtakoja afiniškumą ir selektyvumą tikslinei CA IX (sulfanil – didina, sulfonil – mažina).

1. LITERATŪROS APŽVALGA

1.1 CA paplitimas ir struktūra, konservatyvus centras

Karboanhidrazės (EC 4.2.1.1), tai metalo fermentai katalizuojantys vieną iš paprasčiausių reakcijų:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \overset{\text{labai greita}}{\rightleftharpoons} H^+ + HCO_3^- \quad (1)$$

$$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^- \quad (2)$$

$$CO_2 + OH^- \rightleftharpoons HCO_3^- \quad (3)$$

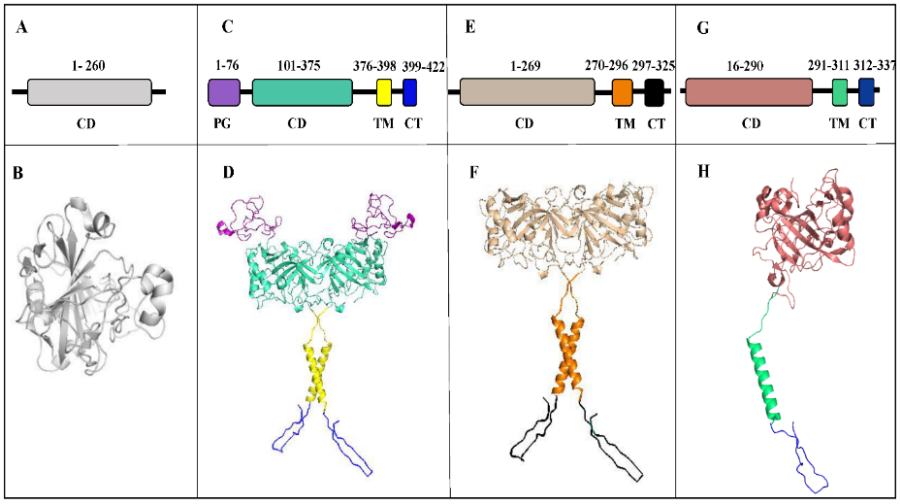
Karboanhidrazės šią ganėtinai lėtą reakciją gali pagreitinti nuo 0,039 s^{-1} iki ~10^6 s^{-1} ir tokiu būdu žymiai įtakoti įvairiausius organizme vykstančius procesus[1,2].

Dar neseniai buvo žinomos penkios CA šeimos: alfa, beta, gama, delta, zeta[3,4]. Šiuo metu jau žinomos septynios CA šeimos (prisidėjo eta ir theta[5,6]) Dauguma šių metalo fermentų aktyviajame centre turi Zn^{2+} joną, tačiau yra sutinkamos ir CA su Cd^{2+}, Fe^{2+}, Co^{2+} jonais[7–10]. Iš visų šeimų reikia išskirti gyvūnuose sutinkamą alfa-CA šeimą. Tuo pačiu tai vienintelė žmogaus organizme sutinkama CA šeima. Pastarąją šeimą sudaro 15 karboanhidrazių (1 lentelė), iš kurių 12 pasižymi kataliziniu aktyvumu, o 3 yra katalizinio aktyvumo neturintys į karboanhidrazes panašūs baltymai (angl. carbonic anhydrase related protein, CARP).

1 lentelė. Žmogaus CA izofermentų katalitinis aktyvumas, lokalizacija ir siejamumas su ligomis[7].

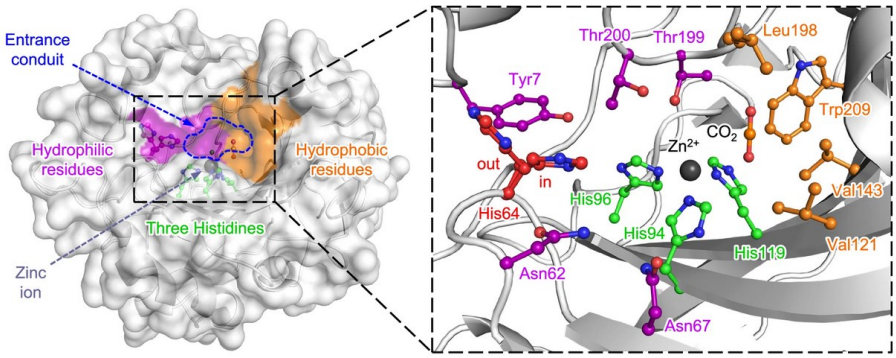
Izofermentas	CO_2 hidratacijos aktyvumas	Lokalizacija	Sąsajos
CA I	Mažas	Citozolyje	Hemolizinė anemija
CA II	Didelis	Citozolyje	Glaukoma, edema, aukštikalnių liga
CA III	Labai mažas	Citozolyje	-
CA IV	Didelis	Membraninis-neaišk.	Glaukoma
CA VA	Vidutinis-didelis	Mitochondrijose	Nutukimas
CA VB	Vidutinis	Mitochondrijose	-
CA VI	Vidutinis	Sekrecijoje	Kariesas
CA VII	Didelis	Citozolyje	Epilepsija, neuropatinis skausmas
CARP VIII	Neaktyvus	Citozolyje	-
CA IX	Didelis	Transmembraninis	Vėžys
CARP X	Neaktyvus	Citozolyje	-
CARP XI	Neaktyvus	Citozolyje	-
CA XII	Vidutinis-mažas	Transmembraninis	Glaukoma, vėžys
CA XIII	Mažas	Citozolyje	Nevaisingumas
CA XIV	Vidutinis	Transmembraninis	-

Visi 13 aktyvių α-CA izofermentų skiriasi savo lokalizacija ląstelėje ir organuose, katalitinių domenų skaičiumi (CAVI, CA IX ir CA XII turi 2 domenus), funkcija ir kataliziniu aktyvumu (1 pav.)[7,11].



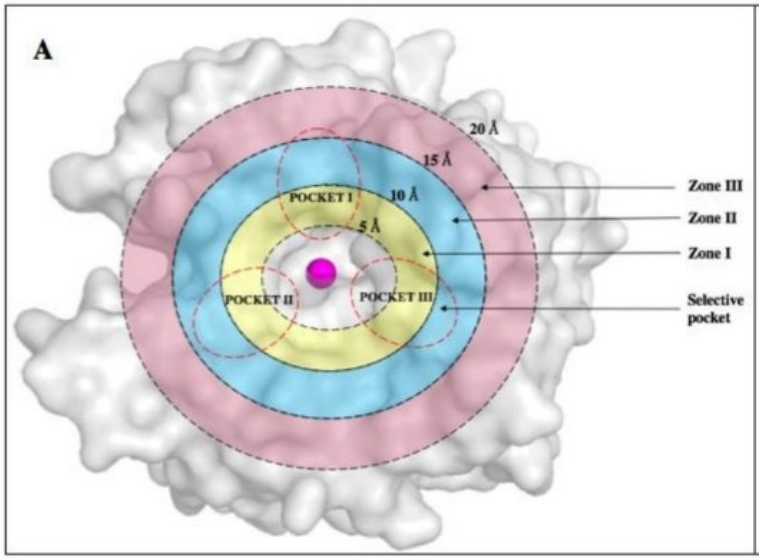
1 pav. CA izofermentų schemos ir struktūros. Viršutinė panelė, domenų schema: (A) CA II: katalitinis domenas (CD, pilka); (C) CA IX: proteoglikaninis domenas (PG, violetinė), katalitinis domenas (cianas), transmembraninis domenas (TM, geltona) ir C-galinis domenas (CT, mėlyna); (E) CA XII: katalitinis domenas (rusva), transmembraninis domenas (oranžinė) ir C-galinis domenas (juoda); (G) CA XIV: katalitinis domenas (varinė), transmembraninis domenas (žalia) ir C-galinis domenas (mėlyna). Apatinė panelė: (B) CA II, (D) CA IX, (F) CA XII ir (H) CA XIV piešinys[12].

Tačiau nepaisant skirtumų CA pasižymi tuo, kad jų katalitiniai domenai yra labai panašūs (konservatyvūs), ypač aktyvaus centro srityje. Visuose domenuose aktyvaus centro gilumoje yra cinko atomas koordinuotas tarp trijų histidino fragmentų (2 pav., His 19, 94, ir 96). Šonuose yra hidrofobinių fragmentų sritis, kuri palengvina substrato (CO_2) prisijungimą, ir hidrofilinė sritis, kuri dalyvaujant protonų šaudyklei His64 palaiko protonų perdavimą per tvarkingą vandens molekulių tinklą[13,14].



2 pav. Bendra katalitinio domeno forma (CAII). Hidrofobinė sritis (oranžinė), palengvinanti substrato (CO_2) prisijungimą, ir hidrofilinė sritis (violetinė), kurioje palaikomas protonų perdavimas per tvarkingą vandens molekulių tinklą[13].

Literatūroje išskiriamos 1-3 sritys (kišenės) esančios prie Zn-jono kurių aminorūgščių skirtumai yra svarbūs (3 pav., 2 lentelė)[15].



3 pav. CA II paviršiaus atvaizdavimas zonomis ir kišenėmis. Aktyviosios srities cinkas pavaizduotas raudona sfera. Įvairios zonos aktyviojoje srityje (atstumas nuo cinko): I zona (5–10 Å) – geltona, II zona (10–15 Å) – žydra, o III zona (15–20 Å) – rožinė. Trys kišenės aktyviojoje srityje ir aplink ją, apibrėžtos raudonais brūkšneliais[12].

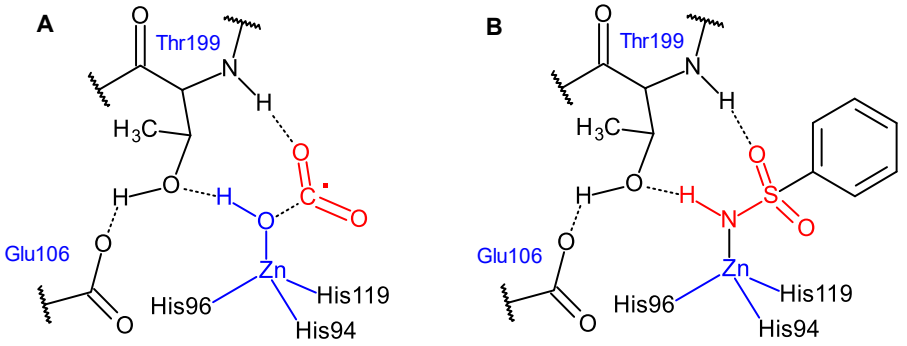
2 lentelė. CA izofermentų skirtumai(CA II numeracija)[12].

Eil. Nr *	Atstumas iki cinko (Å)	CA I	CA II	CA IX	CA XII
5-10 Å					
62	9.1	Val	Asn	Asn	Asn
65	6.9	Ser	Ala	Ser	Ser
67	7.3	His	Asn	Gln	Lys
10-15 Å					
60	13.7	Ile	Leu	Arg	Thr
69	13.8	Asn	Glu	Thr	Asn
91	11.1	Phe	Ile	Leu	Thr
131	10.4	Leu	Phe	Val	Ala
135	12.2	Ala	Val	Leu	Ser
204	13.7	Tyr	Leu	Ala	Asn
15-20 Å					
19	19.1	Leu	Asp	Val	Lys
20	15.2	Tyr	Phe	Ser	Tyr
57	19.6	Lys	Leu	Leu	Phe
58	16.3	Glu	Arg	Arg	Leu
71	21.1	Glu	Asp	Pro	Pro
72	15.9	Asp	Asp	Pro	Ser
123	15.4	Trp	Trp	Leu	Tyr
130	19.1	Ser	Asp	Arg	Asp
132	17.3	Ala	Gly	Asp	Ser
136	17.6	Ser	Gln	Gly	Asn
170	18.9	Lys	Lys	Ser	Lys
173	19.6	Arg	Ser	Glu	Glu

*CA II numeracija

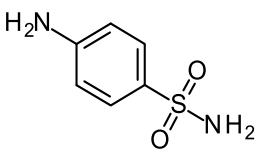
1.2 Klasikiniai CA slopikliai.

CA slopikliai pagal veikimo mechanizmą gali būti skirstomi į dvi grupes: a) su metalo jonu kompleksą sudarantys junginiai; b) nesudarantys tiesioginio ryšio su metalo jonu junginiai[7]. Pirmai grupei priklauso klasikiniai slopikliai – sulfonamidai ir jų dariniai (sulfamatai, sulfamidai ir pan.) (1 schema), bei kiti su metalais kompleksuojantys anijonai. Kitai grupei priklausantys slopikliai įvairiais būdais[16] jungiasi prie cinko jono koordinuotos vandens molekulės vandeniliniais ryšiais (fenoliai, poliaminai ir kt.) arba kitų CA aktyvaus centro vietų užkimšdami priėjimą prie aktyvaus centro.

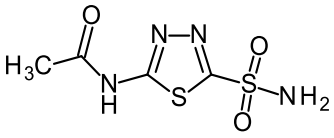


1 schema. Pirmos grupės CA inhibitorių veikimo mechanizmas. CA aktyvaus centro schemos: (A) CO_2 hidratacijos/dehidratacijos reakcijos pereinamoji būsena; (B) arilsulfonamido prisijungimas (Pagal[17])

Pirmi slopikliai buvo surasti netrukus po karboanhidrazių atradimo – buvo nustatyta, kad pirminiai benzensulfonamidai (sulfanilamidas ir kt.) inhibuoja CA aktyvumą, tuo tarpu antriniai benzensulfonamidai ir benzensulfonrūgštys „netrukdo“ CA veiklai [18]. Kiek vėliau buvo surasti CA slopikliai heterocikliniai sulfonamidai, tokie kaip acetozolamidas [19].

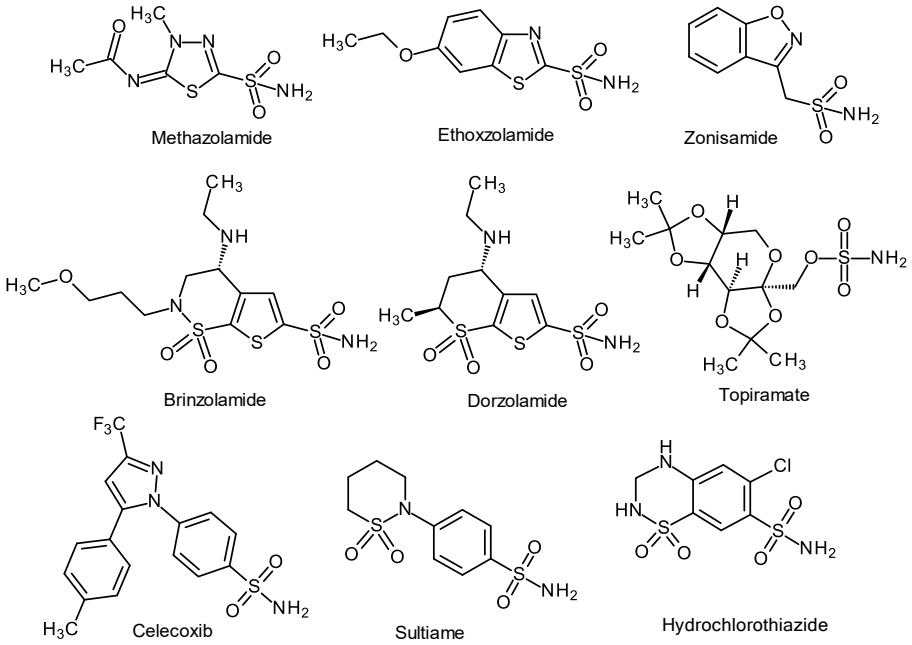


sulfanilamidas



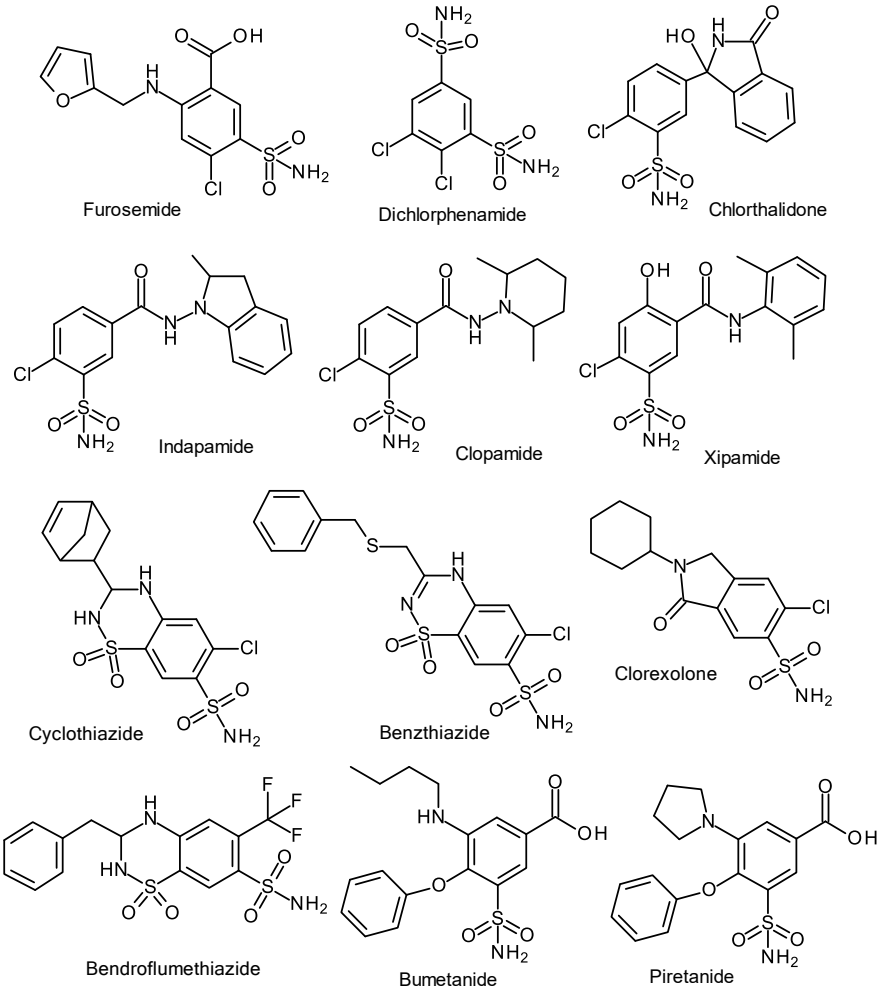
acetozolamidas

Vėliau buvo surasta dar ne viena nauja CA slopinančių junginių klasė[20–24], bet sulfonamidai taip ir liko plačiausiai naudojamais tiek tyrimuose, tiek farmacijoje. Vaistiniai sulfonamidiniai preparatai (4 ir 5 pav.) netgi priskiriami CA slopiklių klasei, nepriklausomai nuo to kokioms ligoms skiriami gydyti (https://go.drugbank.com/categories/DBCAT000727).



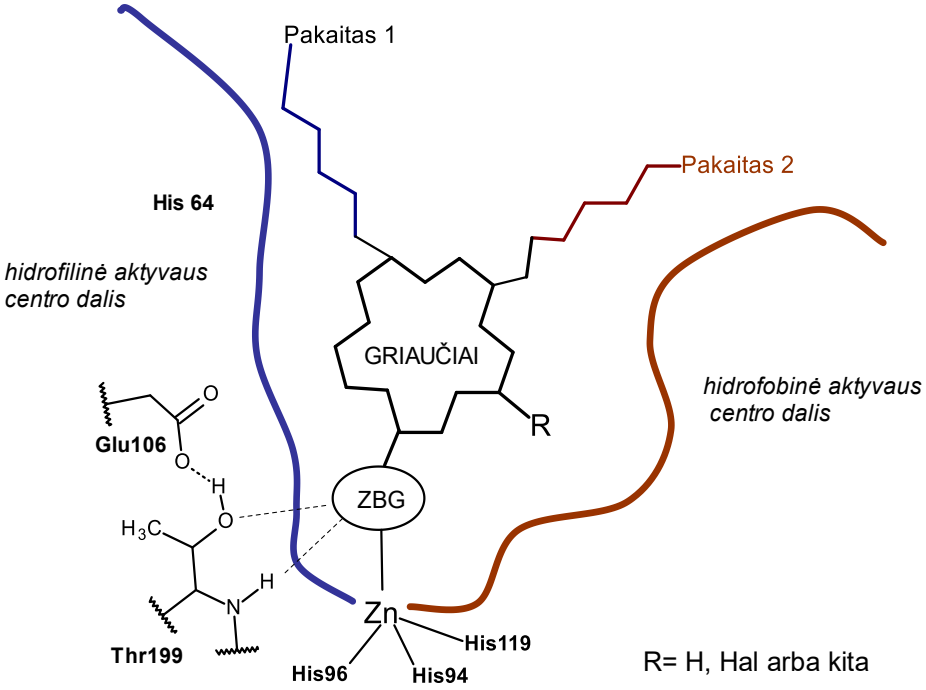
4 pav. Vaistų pasižyminčių CA slopinimu pavyzdžiai.

Taipogi reikia išskirti vieną grupę vaistinių junginių, pasižyminčių CA slopinimu, tai *orto*-pakeisti benzensulfonamidai (5 pav.). Šie junginiai *orto*-padėtyje dažniausiai turi nedidelius chloro- ar trifluorometilpakaitus, arba didesnius fenoksipakaitus bumetanido ir piretanido atveju. Jie buvo sugrupuoti ir aprašyti dar 2008 metais, bet autoriai, panašu nesuprato arba neįvertino pakaito svarbos[25]. Netgi ir 2025 metais parašytoje apžvalgoje prie pat sulfonamido esančioms grupėms neskirta dėmesio[26].



5 pav. *Orto*-pakeistų benzensulfonamidų pavyzdžiai.

Bendra selektyvių slopiklių kūrimo strategija pavaizduota 2 schemoje. Ji yra pagrįsta slopiklio jungimusi prie CA centre esančio metalo jono, bei prie griaučių (dažniausiai tai aromatinės ar heteroaromatinės sistemos) prijungtų įvairių grupių sąveikomis su CA aktyvaus centro paviršiuje esančiais aminorūgščių fragmentais. Schemoje pavaizduoti du pakaitai, kurie dėka individualių savybių gali sąveikauti su įvairiu atstumu esančiais hidrofobiniais arba hidrofiliniais paviršių fragmentais. Atskirai išskirtas pakaitas R, kuris yra arčiau aktyvaus centro nei kiti pakaitai ir dažniausiai įvardijamas kaip sąveikaujantis su konkrečiai hidrofobinėje dalyje esančia kišene. Pakaitas R gali būti riboto dydžio, dažniausia tai chloro atomas, nors yra išimčių (CF_3 ir fenoksigrupės 5 paveiksle ir kitur[27,28]).



2 schema. Schema apibūdinanti bendrą CA slopiklių kūrybos strategiją. ZBG (zinc binding group) – su cinko jonu besijungianti grupė, R – prie ZBG esanti grupė, dažniausiai chloro atomas.

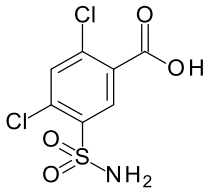
2. MEDŽIAGOS IR METODAI

Tyrimuose naudotos pradinės medžiagos ir reagentai įsigyti iš specializuotų tiekėjų ir naudoti be papildomo gryninimo. Lydymosi temperatūros nustatytos atviruose kapiliaruose IA9100 (*Electrothermal*) prietaisu. Reakcijų eiga sekta plonasluoksnės chromatografijos metodu naudojant TLC Silica gel 60 F_{254} (*Merck*) plokšteles, detektuojant 254/365 nm UV spinduliuote UV-6 S/L (*Herolab*) prietaisu. Chromatografiniam gryninimui naudotas Silica gel 60 (0,040-0,063 mm, *Merck*). Junginių ^{1}H, ^{13}C-BMR spektrai užrašyti spektrometru Ascend 400 (*Brucker*) (400 MHz ir 100 MHz), vidiniu standartu naudojant likutinius tirpiklių signalus. Aukštos skiriamosios gebos masių spektrai užrašyti Agilent TOF 6230 (*Agilent Technologies*) spektrometru ir Agilent Infinity 1260 HPLC sistema (*Agilent Technologies*).

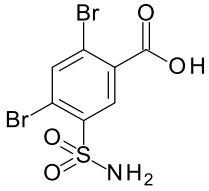
3. REZULTATAI

Šiame darbe susintetinta eilė benzensulfonamidinių CA slopiklių turinčių įvairius pakaitus 2-, 4- ir 5-padėtyse. Rezultatai pateikti 5 straipsniuose (**I-V**). Pirmi trys straipsniai skirti 2-halo-4,5-dipakeistiems benzensulfonamidiniams slopikliams. Ketvirtas straipsnis skirtas 2-halo-4-pakeistų ir 4-halo-2-pakeistų sulfamoilbenzoatų saveikų su karboanhidrazėmis palyginimui. Penktame straipsnyje ištirta prie sulfonamido grupės *orto*-padėtyje esančių pakaitų įtaka CA slopinimui.

Tyrimai pradėti nuo 4-aminopakeistų benzensulfonamidų 5-oje padėtyje turinčių karbonilamino grupes (**I str.**). Slopiklių sintezės pradėtos nuo 2,4-dichlor- ir 2,4-dibrom-5-sulfamoilbenzoinių rūgščių (**1**, **2**).



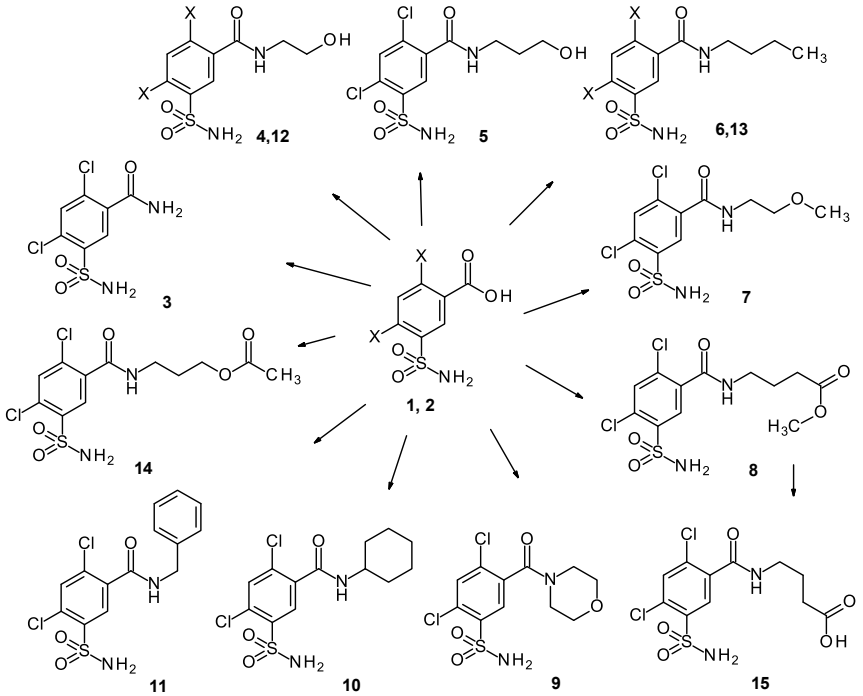
1



2

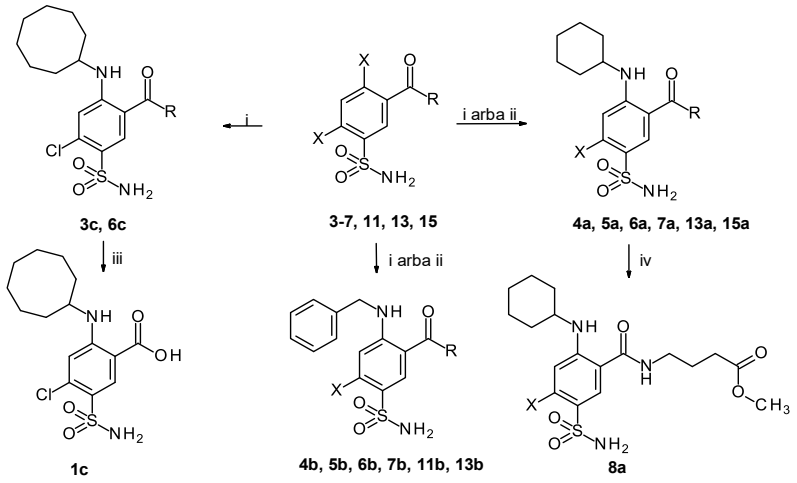
Iš šių dviejų benzoinių rūgščių per tarpinius chloranhidridus susintetinta eilė 2,4-dihalobenzamidų (**3-15**) su įvairaus ilgio amidopakaitais (3 schema). Gryninant 3-hidroksipropilbenzamidą **5** buvo pastebėtas pašalinio junginio susidarymas – etilacetatate vyko peresterifikavimo reakcija. Identifikuotas *O*-acetil benzamidas **14**, kuris vėliau resintetintas su 78 proc. išeiga, virinant etilacetate esant sieros rūgšties. Tuo tarpu su 2-hidroksietilbenzamidu **4** tokia reakcija nestebima.

Antras pakaitas įvestas pakeičiant halogeną įvairiais aminais. Reakcijos atliktos kaitinant 2,4-dichlorbenzamidus **3-7**, **11**, **15** alifatinių aminų pertekliuje 110-130 °C temperatūroje, be metalų katalizės [29]. Dibrombenzamido **13** reakcijos su aminais atliktos virinant dioksane ilgesnį laiką, taip išvengiant pašalinių produktų susidarymo (4 schema). Dalis junginių susintetinti hidrolizuojant (**1c**, **15**) arba modifikuojant amidus (**8a**)[30].



X: Cl (**1, 3-11, 14-15**), Br (**2, 12-13**)
R: NH_2 (**3**) $NH(CH_2)_2OH$ (**4,12**), $NH(CH_2)_3OH$ (**5**), $NH(CH_2)_3CH_3$ (**6,13**), $NH(CH_2)_2OCH_3$ (**7**), $NH(CH_2)_3CO_2CH_3$ (**8**), N-morfolinil- (**9**), NH-cikloheksil-(**10**), NH-benzil- (**11**), $NH(CH_2)_3OAc$ (**14**), $NH(CH_2)_3CO_2H$ (**15**).

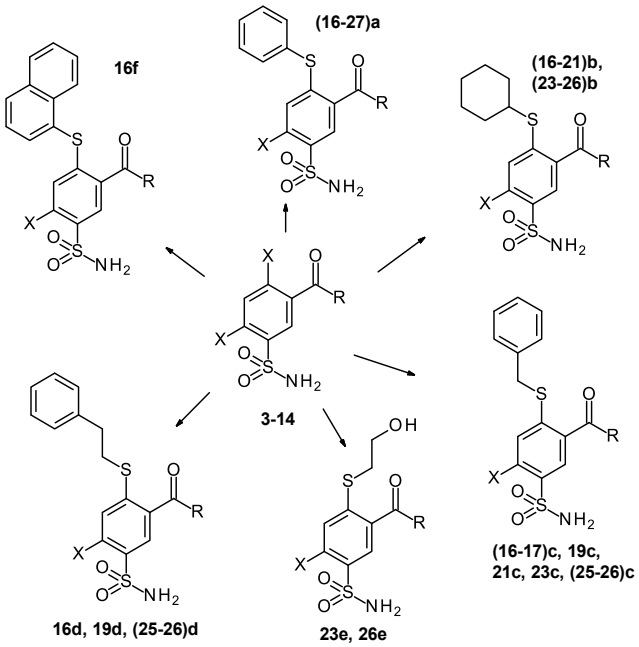
3 schema. 2,4-dihalobenzamidų **3-15** sintezė.



X: Cl (**3-8, 11, 15**), Br (**13**)
R: NH_2(**3**) $NH(CH_2)_2OH$ (**4**), $NH(CH_2)_3OH$ (**5**), $NH(CH_2)_3CH_3$ (**6, 13**), $NH(CH_2)_2OCH_3$ (**7**), $NH(CH_2)_3CO_2CH_3$ (**8**), NH-benzil- (**11**), $NH(CH_2)_3CO_2H$ (**15**),

4 schema. 4-aminopakeistų benzensulfonamidų sintezė.

Sekanti junginių serija (**II, IV str.**) turi per sieros atomą prijungtus įvairaus ilgio pakaitus. 2,4-Dihalosulfamoilbenzoinių rūgščių darinių reakcijos su tioliais literatūroje praktiškai neaprašytos, todėl tikėtasi, kad reakcijos vyks panašiai kaip su aminais, pakeičiant tolimesnį nuo sulfonamido halogeno atomą. Tokiu būdu iš atitinkamų benzamidų (**3-14**) susintetinta eilė junginių su įvairiais sulfanilpakaitais (5 schema).



X: Cl (**3-11, 14-24, 27-28**), Br (**12-13, 25-26**)
R: NH_2 (**3, 16**), $NH(CH_2)_2OH$ (**4, 12, 17, 25**), $NH(CH_2)_3OH$ (**5, 18**), $NH(CH_2)_3CH_3$ (**6, 13, 19, 26**), $NH(CH_2)_2OCH_3$ (**7, 20**), $NH(CH_2)_3CO_2CH_3$ (**8, 21**), N-morfolinil- (**9, 22**), NH-cikloheksil- (**10, 23**), NH-benzil- (**11, 24**), $NH(CH_2)_3OAc$ (**14, 27**), $NH(CH_2)_3CO_2H$ (**15, 28**).

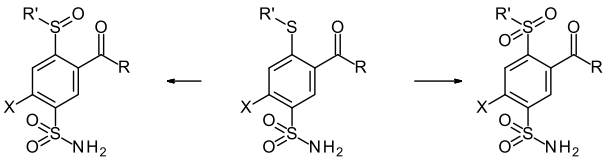
5 schema. 2-sulfanilbenzamidų sintezė.

Reakcijų sąlygų pasirinkimą įtakojo nukleofilo pobūdis. Junginių **3-8, 10-14** reakcijos su tiofenoliu arba 1-tionaftoliu vyko lengviausiai, virinant MeOH esant trietilamino. Su cikloheksantioliu šiomis reakcijos sąlygomis reakcijos vyko labai lėtai, todėl polinis protoninis tirpiklis buvo pakeistas į polinį aprotoninį DMSO, o trietilaminas – į bevandenį K_2CO_3. Tai leido atlikti junginių **3-8, 10-13** reakcijas per 2-6 valandas vietoje dienų ar savaičių, esant tai pačiai temperatūrai.

Naudojant kitus, vidutinio reaktingumo nukleofilus (pirminius alkiltiolius ir pan.) reakcijos atliktos poliniame aprotoniniame tirpiklyje (DMSO) esant TEA.

Vienintelė išimtis buvo morfolinildarinio **9** reakcija su tioliais, kai net su tiofenoliu reikėjo reakciją atlikti 120 °C temperatūroje, poliniame aprotoniniame tirpiklyje (DMSO) su Cs_2CO_3.

Susintetinti sulfanildariniai buvo toliau oksiduoti iki sulfonildarinių. Oksidacijos reakcijos atliktos geromis išeigomis (69-92 %), acto rūgštyje su vandenilio peroksidu *in situ* generuojant peracto rūgštį (6 schema). Oksiduojant hidroksilo grupę turinčius amidus buvo stebimas *O*-acetatų susidarymas ir siekiant to išvengti reakcijos produktų mišinys buvo papildomai veikiamas praskiesta druskos rūgštimi.

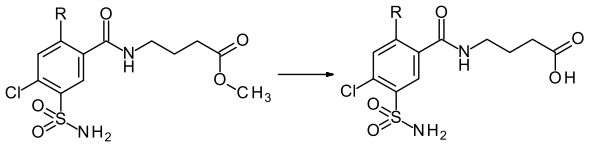


40a,c — **16d, 17a-c, 18a-b, 19a-b, 20a-b, 21b-c, 23a-c,e, 24a-b, 25a-c, 26a-b** — **30d, 31a-c, 32a-b, 33a-b, 34a-b, 35b-c, 36a-c,e, 37a-b, 38b-c, 39a-b.**

X: Cl (**16-24, 30-37, 40c**), Br (**25-26, 38-39, 40a**)
R: NH_2 (**16, 30**), $NH(CH_2)_2OH$ (**17, 25, 31, 38, 40a**), $NH(CH_2)_3OH$ (**18, 32**), $NH(CH_2)_3CH_3$ (**19, 26, 33, 39**), $NH(CH_2)_2OCH_3$ (**20, 34**), $NH(CH_2)_3CO_2CH_3$ (**21, 35**), NH-cikloheksil- (**23, 36, 40c**), NH-benzil- (**24, 37**)
R': a) fenil-, b) cikloheksil-, c) benzil-, d) 2-feniletil-, e) 2-hidroksietil-

6 schema. 4-sulfonil- ir 4-sulfinilbenzensulfonamidų sintezė.

Benzoinės rūgštys **29b, d** susintetintos iš atitinkamų benzamidų **16b, d** rūgštinės hidrolizės sąlygomis. Tačiau panašiai atlikus esterių **21b, 35c** hidrolizę (7 schema) buvo stebimas dviejų produktų susidarymas. Todėl tolimesnė esterių **21a-c** hidrolizė atlikta šarminėje terpėje, kambario temperatūroje, susidarant tik tiksliniams produktams.



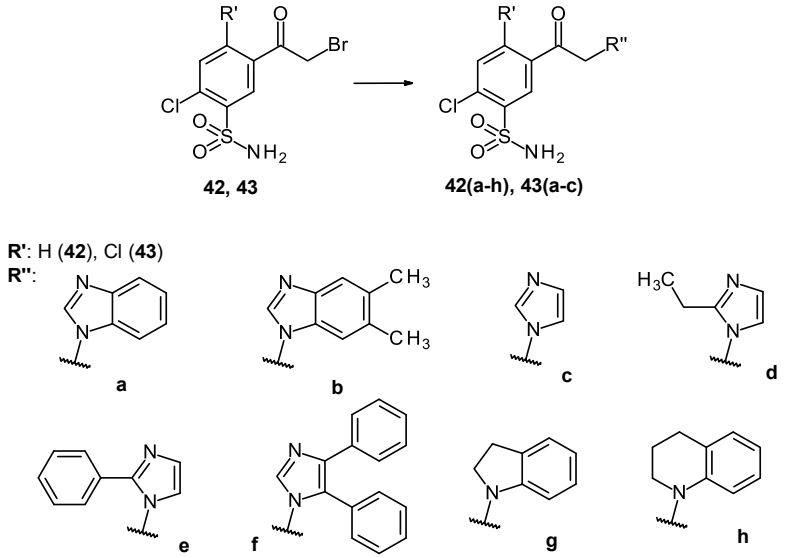
21a-c, 35c — **28a-c, 41c**

R: (**21a, 28a**), (**21b, 28b**), (**21c, 28c**), (**35c, 41c**).

7 schema. 4-aminobutaninių rūgščių darinių sintezė

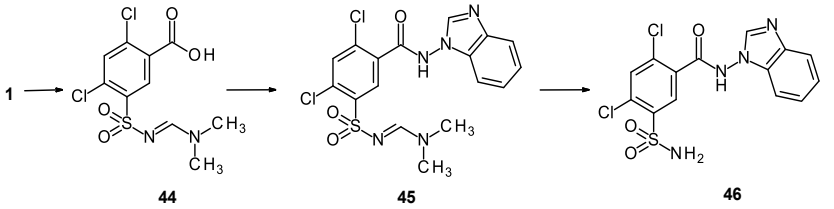
Sekanti junginių serija (**III str**.) buvo sukurta remiantis žiniomis[31] apie CA VA izofermentui selektyvius slopiklius. Susintetinti junginiai 2-chlor- ir 2,4-dichlorbenzensulfonamidų pagrindu, 5-oje padėtyje turintys įvairius heterociklus.

Junginiai **42a-h** ir **43a-c** susintetinti tikslinius heterociklus alkilinant (bromoacetil)-2-chlorobenzenesulfonamidu **42** arba 5-(bromoacetil)-2,4-dichlorobenzenesulfonamidu **43**. *N*-alkilinimai atlikti THF esant NaOAc kambario temperatūroje. Siekiant išvengti benzimidazolų dialkilinimo naudotas nedidelis heterociklo perteklius. Junginiai **42g** ir **42h** susintetinti naudojant 1,2,3,4-tetrahidrochinolino ir indolino perteklių, be bazės (8 schema).



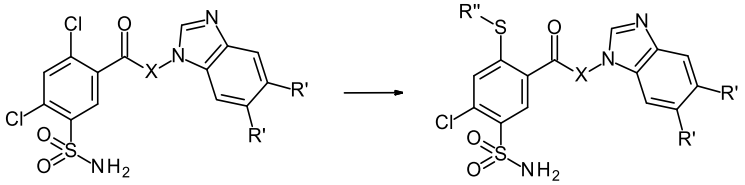
8 schema. Junginių **42a-h** and **43a-c** sintezė.

Junginio **46** nepavyko susintetinti tiesiogiai acilinant 1-aminobenzimidazolą 2,4-dichlor-5-sulfamoilbenzoilchloridu įvairiomis salygomis. Bet apsaugojus sulfonamido grupę *N,N'*-dimetilaminometiliden-grupe (9 schema, junginys **44**) pavyko išvengti pašalinių produktų susidarymo ir acilinimo reakcijos metu susintetintas junginys **45,** kurį deblokavus gautas tikslinis benzensulfonamidas **46**.



9 schema. Junginio **46** sintezė.

Dalis 2,4-dichlorjunginių (**43a,b, 46**) modifikuoti įvedant sieros tiltelį turinčius fragmentus pagal aprašytas (**II str**.) metodikas (10 schema). Taip susintetinti junginiai **47-50**.

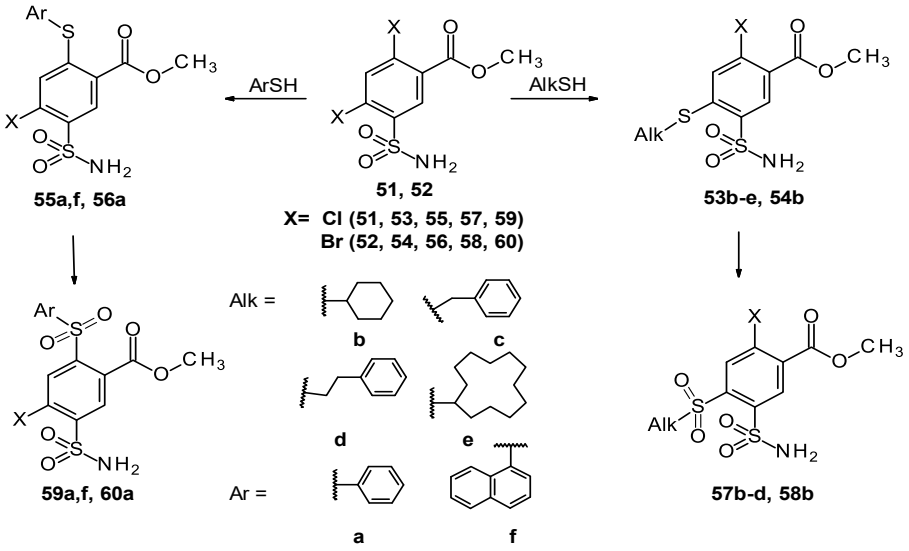


43a: X=CH_2, R'=H
43b: X=CH_2, R'=CH_3
46: X=NH, R'=H

47: X=CH_2, R'=H, R''=2-feniletil-
48: X=CH_2, R'=CH_3, R''=fenil-
49: X=NH, R'=H, R''=fenil-
50: X=NH, R'=H, R''=cikloheksil-

10 schema. 2-chlor-4,5-dipakeistų benzensulfonamidų **47-50** sintezė.

Straipsnyje **IV** aprašyta sekanti pakeistų halogenintų benzensulfonamidų grupė. Iš pradinių metil 2,4-dihalo-5-sulfamoilbenzoatų **51**, **52** susintetinti metil 2-halo-4-pakeisti ir 4-halo-2-pakeisti-5-sulfamoilbenzoatai (11 schema). Reakcijos sąlygos buvo parinktos remiantis ankstesniais darbais (**II** str.) kuriuose aprašytos 2,4-dihalo-5-sulfamoilbenzamidų reakcijos su sierą turinčiais nukleofilais. Atliekant reakcijas metanolyje aromatiniai tioliai (tiofenolis ir 1-tionaftolis) lengvai keitė halogeno atomą sudarydami *para*-pakeistus benzensulfonamidus **55a,f** ir **56a**. Tuo tarpu reakcijos su tioliais turinčiais CH_2 ar CH grupę tokiomis sąlygomis vyksta labai lėtai ar visai nevyksta. Siekiant paspartinti reakcijas polinis protoninis tirpiklis pakeistas į polinį aprotoninį DMSO ir susintetinti *orto*-pakeisti benzensulfonamidai **53b-e, 54b** (11 schema). Reakcijose taip pat stebėtas *para*- ir dipakeistų benzensulfonamidų susidarymas.



11 schema. Metil halo-2- ir 4-pakeistų-5-sulfamoilbenzoatų sintezė.

Buvo ištirtas 2,4-dihalo-5-sulfamoil-benzoatų **51, 52** nukleofilinio aromatinio pakeitimo reakcijų regioselektyvumas (3 lentelė). Visos reakcijos atliktos DMSO su tioliu ir trietilaminu, 60 °C temperatūroje 72h. Reakcijos konversija, produktų santykis ir struktūrų identifikavimas atliktas BMR ir HPLC/UV/MS pagalba.

2 lentelė. 2,4-dihalo-5-sulfamoilbenzoatų **51**, **52** reakcijos su tioliais išeigos ir produktų santykiais nustatyti pagal ^{1}H-BMR spektroskopijos ir HPLC/UV/MS tyrimų duomenis (*italic*). * 2- ir 4-pakeistų izomerų susidariusių junginių **51** ir **52** reakcijose su tiofenoliu atskirti HPLC būdu nepavyko.

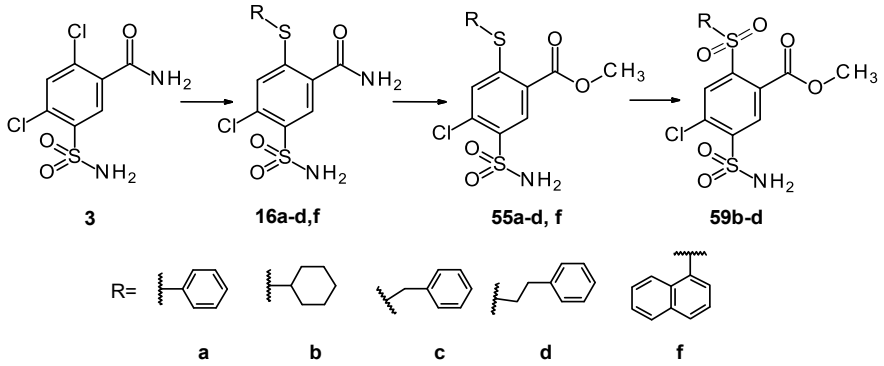
Pradinis junginys	Tiolis	Konversija (%)	2-pakeistas (%)	4-pakeistas (%)	2,4-dipakeisti (%)
51		30.07 *28.77*	18.70 *17.38*	81.30 *82.62*	-
52		15.71 *13.92*	9.09 *14.11*	90.91 *85.89*	-
51		89.47 *96.67*	58.82 *88.78**	35.29	5.88 *11.22*
52		95.07 *96.92*	51.81 *85.55**	40.42	7.77 *14.45*
51		72.73 *71.55*	23.53 *22.91*	73.53 *72.79*	2.94 *4.30*
51		72.15 *73.60*	12.28 *12.05*	87.72 *87.95*	-

Pradinis junginys	Tiolis	Konversija (%)	2-pakeistas (%)	4-pakeistas (%)	2,4-dipakeisti (%)
51		90.97 *95.25*	76.34 *78.84*	19.08 *15.72*	4.58 *5.44*
51		13.70 *18.19*	-	100.00 *100.00*	-

Tiolių reaktingumo mažėjimo seka yra tokia: fenil->naftil->benzil-≥2-etilfenil->cikloheksil-≥ciklododecil-. Dipakeistų produktų susidarymas stebimas reakcijose su fenil-, naftil- ir benziltioliais. 2-pakeistų produktų daugiau susidaro reakcijose su aromatiniais tioliais, kitais atvejais dominuoja 4-pakeisti produktai. Ciklododeciltiolio atveju stebimas tik vieno 2-pakeisto izomero susidarymas.

Trūkstami 2-pakeisti metilo esteriai **55a-d, f** susintetinti iš jau aprašytų 2-pakeistų benzamidų **16a-d, f** (12 schema). Amido grupė pakeista į esterio grupę vienos stadijos reakcijoje kaitinant metanolyje su tionilo chloridu.

Sulfanildarinių **53**(**b-d**), **54b**, **55**(**a-d**, **f**), **56a** oksidacija iki sulfonil darinių **57**(**b-d**), **58b, 59**(**a-d**,**f**), **60a** atlikta *in situ* generuojant peracto rūgštį (11 ir 12 schemos).



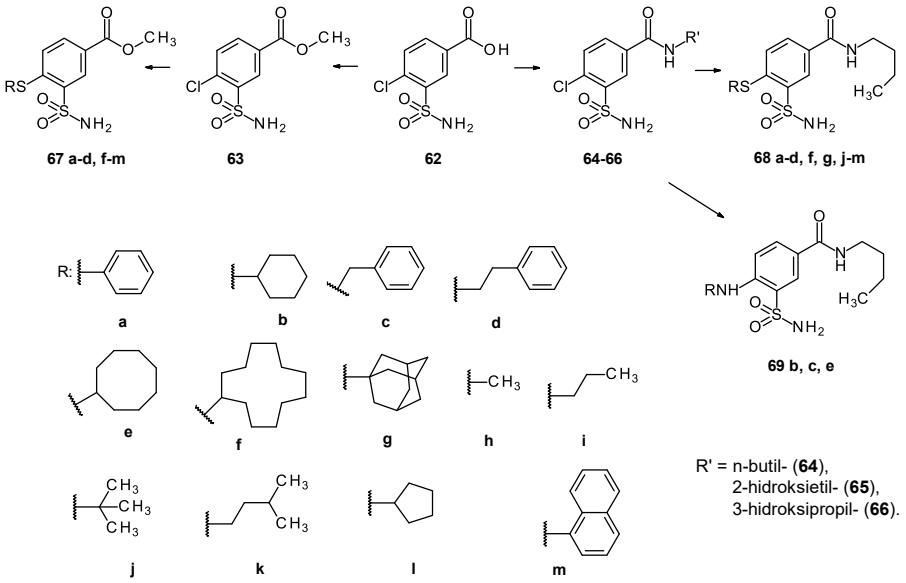
12 schema. Metil 4-chlor-2-pakeistų-5-sulfamoilbenzoatų sintezė.

Straipsnyje **V** siekiama ištirti arti sulfonamido grupės esančių pakaitų įtaką jungimuisi su karboanhidrazėmis. Tam susintetinti *orto*-pakeisti benzensulfonamidai su amino-, sulfanil-, sulfinil- ir sulfonilpakaitais.

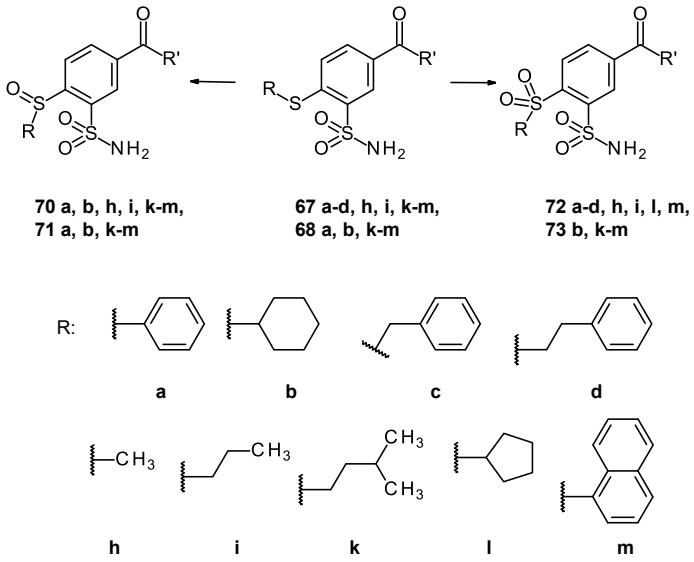
Kadangi 2,4-dihalodarinių reakcijose susidaro įvairūs produktai, buvo pasirinktas pradinis junginys su labiau nuspėjama elgsena – 4-chlor-3-sulfamoilbenzoinė rūgštis (**62**). Iš jos remiantis jau išbandytomis metodikomis susintetinti pradiniai junginiai – metilo esteris (**63**) ir eilė amidų (**64-66**) (13 schema).

Iš 4-chlor-3-sulfamoilbenzoinės rūgšties esterio **63** nukleofilinio aromatinio pakeitimo reakcijose susintetinti 4-sulfanilpakeisti junginiai **67a-d,f-m** (13 schema). Reakcijos atliktos šildant su įvairiais tioliais dimetilformamide, esant K_2CO_3. Tomis pačiomis reakcijos sąlygomis susintetinti 4-sulfanilpakeisti sulfamoilbenzamidai **68a-d, f, g, j-m**.

4-amino-pakeisti junginiai **69b, c** sintetinti benzamidą **64** kaitinant atitinkamo amino pertekliuje 130 °C. Junginys **69e** susintetintas virinant tolueno ir ciklooktilamino mišinyje.



13 schema. Metil 4-sulfanil-pakeistų-3-sulfamoil-benzoatų **67a-d, f-m**, 4-sulfanilpakeistų-3-sulfamoilbenzamidų **68 a-d, f, g, j-m** ir 4-amino-pakeistų-3-sulfamoilbenzamidų **69 b, c, e** sintezė.



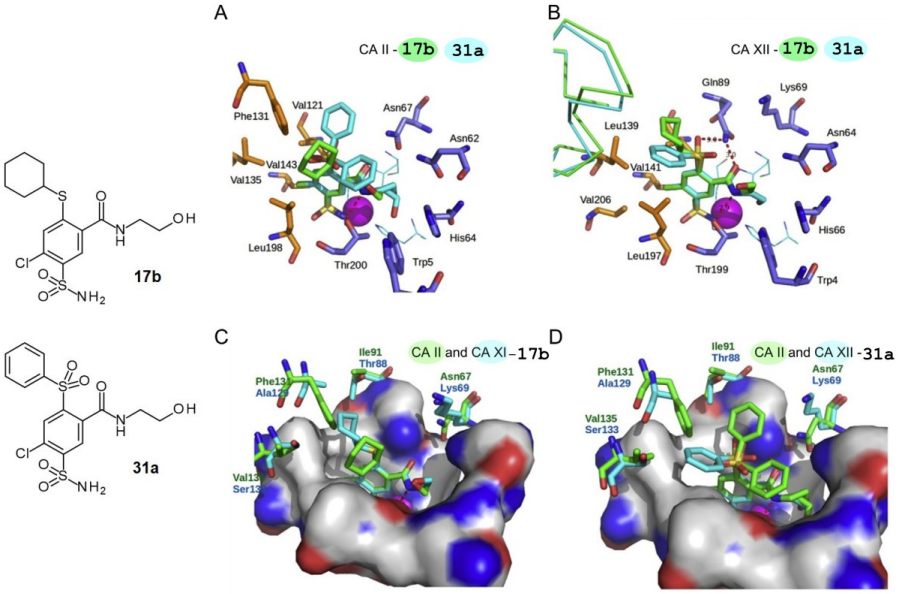
14 schema. Metil 4-sulfinilpakeistų-3-sulfamoilbenzoatų **70a, b, h, i, k-m**, *N*-butil-4- sulfinilpakeistų-3-sulfamoilbenzamidų **71a, b, k-m**, metil 4-sulfonilpakeistų-3-sulfamoilbenzoatų **72a-d, h, i, l, m ir** *N*-butil-4-sulfonilpakeistų-3-sulfamoilbenzamidų **73b, k-m** sintezė.

Esterių **67a-d, h, i, k-m** ir benzamidų **68a**, **b**, **k-m** oksidacijos iki sulfinil- ir sulfonil- junginių atliktos naudojant *in situ* generuotą peracto rūgštį (14 schema). Atliekant reakciją kambario temperatūroje susintetinti sulfinil-junginiai **70a, b, h, i, k-m**, **71a, b, k-m**, o kaitinant reakcijos mišinį 70 °C temperatūroje susintetinti sulfonil- junginiai **72a-d, h, i, l, m, 73b, k-m**.

4. REZULTATŲ APTARIMAS

Pirmame straipsnyje atlikta susintetintų junginių jungimosi su 12 α-CA izofermentų duomenų analizė. Pirma atlikus vieną pakaitą turinčių junginių (*N*-pakeistų 2,4-dihalo-5-sulfamoilbenzamidų **3-15**) afiniškumo ir selektyvumo analizę nustatyta, kad jie stipriausiai jungiasi su CA VII. Įvedus antrą pakaitą (2-aminopakeisti-4-halo-5-sulfamoilbenzamidai, 4 schema) daugumoje atvejų stebimas padidėjęs afiniškumas ir selektyvumas CA IX ir CA XIV izofermentams. Selektyvumas šioms CA priklauso nuo antrojo pakaito dydžio. Benzilamino grupė dėl savo lankstumo lengviau saveikauja su visų izofermentų aktyvaus centro fragmentais ir taip padidina afiniškumą, tačiau sumažina selektyvumą lyginant su mažiau lanksčia cikloheksilamino grupe. Kelios junginių ir karboanhidrazių kompleksų kristalografinės struktūros parodė kontaktus tarp slopiklio pakaitų ir baltymo aminorūgščių ir padidino supratimą apie tiriamų sistemų struktūros-aktyvumo sąryšius.

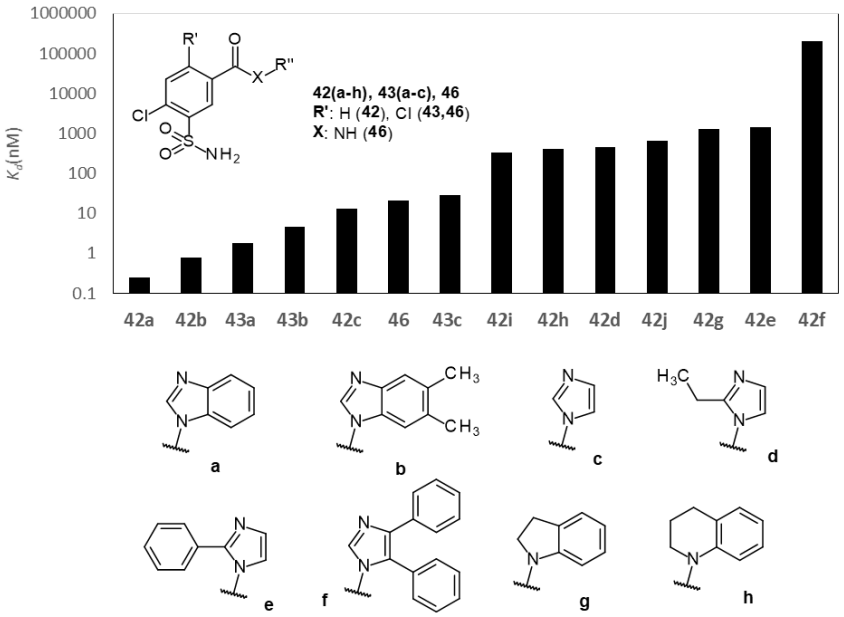
Antrame straipsnyje aprašyti susintetinti CA slopikliai turintys du pakaitus, vienas iš kurių prijungtas per sieros atomą turintį jungtuką (2-sulfanil- arba 2-sulfonilpakeisti-4-halo-5-sulfamoilbenzamidai). Šių junginių cheminių struktūrų ir jungimosi parametrų analizė parodė dviejų pakaitų svarbą ir įtaką afiniškumui bei selektyvumui konkretiems CA izofermentams. Kristalinės baltymo-slopiklio kompleksų struktūros (6 pav.) parodė struktūriškai panašių slopiklio pakaitų išsidėstymo karboanhidrazės izofermentų aktyviuosiuose centruose skirtumus bei galimas pakaitų ir aktyvių centrų sąveikos vietas.



6 pav. Junginių **17b** ir **31a** kristalografinės struktūros CA II ir CA XII aktyviuosiuose centruose. A ir B paveiksluose lyginamas dviejų panašios cheminės struktūros junginių (**17b** (žalia) ir **31a**(žydra)) prisijungimas prie CA II (A; junginys **17b** (PDB ID: 6R6F) ir **31a** (PDB ID: 6R6J)) ir CA XII (B; junginys **17b** (PDB ID: 6R6Y) ir **31a** (PDB ID: 6R71)). CA aktyviųjų centrų hidrofobinių aminorūgščių šoninės grandinės yra oranžinės, hidrofilinių – mėlynos spalvos. Cinko jonas pavaizduotas kaip violetinė sfera. C ir D paveikslėliuose lyginamas junginių **17b** ir **31a** prisijungimas prie CA II (žalia) ir CA XII (žydra). Panašios CA II ir CA XII aktyviųjų centrų aminorūgščių šoninės grandinės pavaizduotos kaip paviršiai.

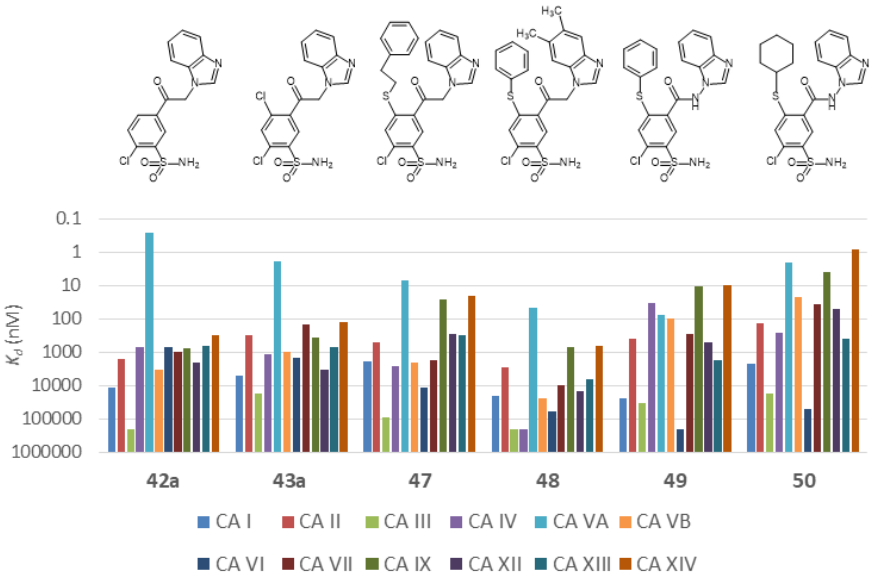
Taip pat reikia pažymėti, kad lyginant su pirmame straipsnyje aprašytais aminopakeistais junginiais pastarosios serijos junginiai pasižymi didesniu afiniškumu, o jungimosi su CA VII, CA IX, CA XII arba CA XIV atvejais stebimos subnanomolinės vertės.

Trečiame straipsnyje susintetinta selektyvių CA VA inhibitorių serija, pagrįsta anksčiau aprašytu junginiu 5-[2-(benzimidazol-1-il)acetil]-2-chlor-benzensulfonamidu (**42a**). Pirma ištirti 2-halo- ir 2,4-dihalobenzensulfonamidai su pakaitais 5-oje padėtyje ir jų afiniškumo priklausomybė nuo pakaite esančių heterociklų prigimties ir dydžio (7 pav.).



7 pav. Jungimosi su CA VA izofermentu stebimųjų disociacijos konstantų palyginimas. Junginiai išdėstyti mažėjančio afiniškumo tvarka.

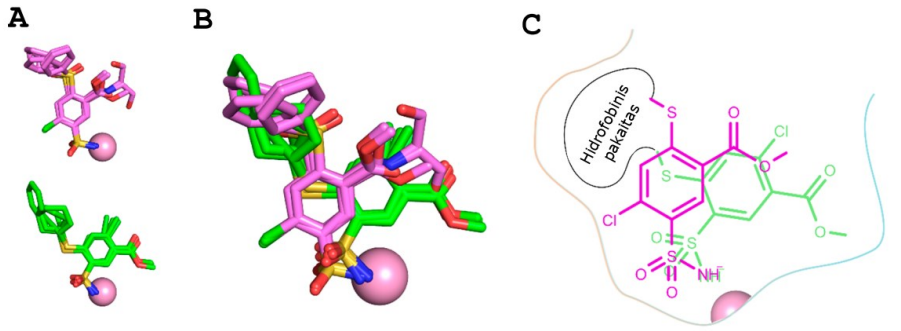
Nustatyta, kad benzimidazolo žiedas yra palankesnis nei imidazolo dėl aromatinės-aromatinės sąveikos su CA VA Tyr64. Benzimidazolo/imidazolo žiedo antras azoto atomas taip pat yra svarbus ir sudaro vandenilinius ryšius tarp azoto atomo ir Thr62 hidroksilo CA VA. Kai antro azoto atomo nėra, pavyzdžiui, indolino arba 3,4-dihidro-2H-chinolino pakaitų atveju (junginiai **42g, 42h**), selektyvumas CA VA atžvilgiu išnyksta. Gi antro pakaito įvedimas į benzensulfonamido *para*-padėtį padidina junginių afiniškumą visiems CA izofermentams ir stipriai sumažina selektyvumą tiksliniam CA VA (8 pav.).



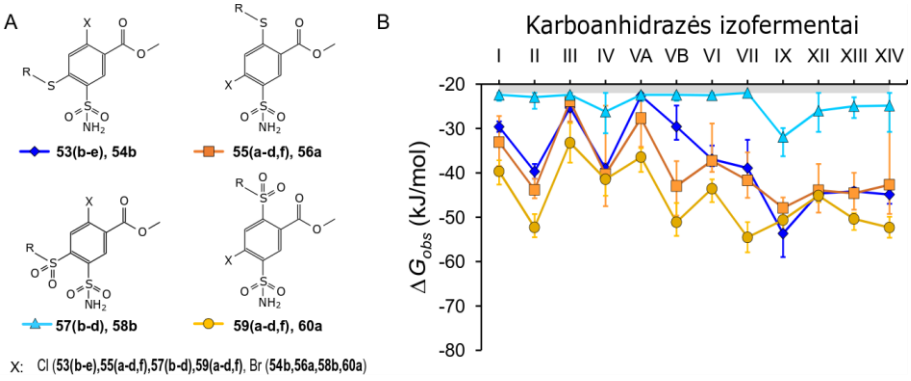
8 pav. Junginių **42a**, **43a** ir **47-50** sąveikos su 12 CA izofermentų K_d vertės selektyvumo CA VA izofermentui palyginimui.

Ketvirtame straipsnyje sukurtos kelios junginių serijos ir ištirta pakaitų bei jų padėčių poveikis metil- 2- arba 4-pakeistų-5-sulfamoilbenzoatų jungimuisi prie žmogaus CA izofermentų. Išsamiai išnagrinėti skirtingo dydžio sulfanilo (-S-) ir sulfonilo (-SO_2-) pakaitai, pateiktos šių dviejų junginių grupių prisijungusių prie CA aktyviųjų centrų kristalinės struktūros (9 pav.). *Para*-pakeisti 2-halobenzensulfonamidai, tiek sulfanil- (**55-56** grupės), tiek sulfonil- pakeisti (**59-60** grupės), jungėsi panašiai su įvairiomis CA izofermentų grupėmis ir nedemonstravo selektyvumo. Tuo tarpu *orto*-sulfanilpakeisti 4-halobenzensulfonamidai pasižymėjo itin geru afiniškumu ir selektyvumu su vėžiu siejamam CA IX izofermentui (junginių **53b, e** ir **54b** K_d artėjo prie 0,1 nM). Gi analogiški *orto*-sulfonilpakeisti 4-halobenzensulfonamidai (**57** ir **58** grupės) su visais CA izofermentais jungėsi silpnai (10 pav.).

Toks netikėtai didelis afiniškumo kritimas buvo priimtas kaip požymis, kad pasiekta ta riba, kuomet atlikus nedidelius pakitimus benzensulfonamido struktūroje *orto*-padėtyje, galima tikėtis esminių pokyčių slopiklio-baltymo saveikoje. Kas ir buvo atlikta sekančiame darbe.



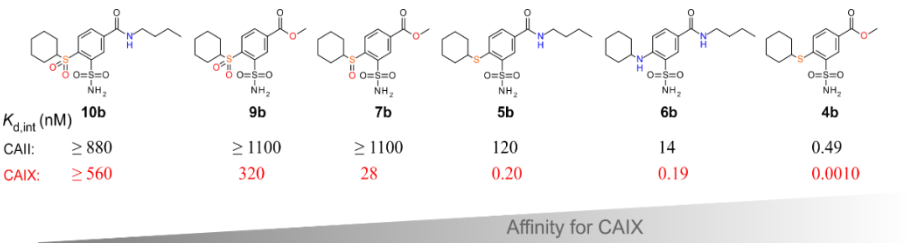
9 pav. Junginių cheminių struktūrų koreliacija ir CA izofermentų kristalinių struktūrų palyginimas. (A) Perkloti kristalinių struktūrų ligandai: *para*-pakeisti ligandai yra rausvos spalvos (PDB ID: 7PP9, 7PUW, 7POM, 7Q0C), *orto*-pakeisti ligandai yra žalios spalvos (PDB ID: 6R6F, 6R71, 7PUU, 7PUV, 7Q0E). (B) Palyginimui parodytos aukščiau paminėtos perklotos struktūros. (C) Schema, vaizduojanti B panelėje parodytų junginių padėtis, oranžinė linija žymi hidrofobiškesnę aktyviosios vietos pusę.



10 pav. (A) Keturių junginių grupių – metil halo- 2- arba 4-pakeistų 5-sulfamoilbenzoatų cheminės struktūros. (B) Vidutinis stebimos standartinės Gibso energijos pokytis junginiams, priklausantiems vienai iš keturių grupių. Stulpeliai rodo skirtumą tarp stipriausio ir silpniausio tos grupės junginių afiniškumo nurodytam CA izofermentui. Pirmos grupės (mėlyni rombai) junginiai pasižymi didžiausiu afiniškumu ir selektyvumu CA IX izofermentui.

Penktame straipsnyje tyrimams pasirinkta išsamiai išnagrinėti *orto*-padėtyje esančių benzensulfonamido pakaitų įtaką. Pasirinkti junginiai panašūs į geriausius ankstesnio straipsnio junginius, tik sąmoningai praleisti halogeno atomai, kad būtų lengviau susintetinti daugiau ir įvairesnių *orto*-pakeistų junginių. Buvo susintetintos serijos benzensulfonamidų su linijiniais

ir cikliniais pakaitais nuo metil- iki adamantanil-, prijungtais per sulfanil- (-S-), sulfinil- (-SO-) ir sulfonil-jungtukus (-SO_2-). Keičiant jungtuko oksidacijos laipsnį siekta dėl elektronų akceptorinių savybių sumažinti slopiklių sulfonamido grupės pK_a ir sustiprinti jungimąsi su CA. Deja, junginiai su -SO- arba -SO_2- jungtuku nepaisant sumažėjusio vienetu pK_a daug silpniau jungėsi su visomis CA nei junginiai su -S- jungtimi (11 pav.). Pakaitų mažinimo strategija nedavė norimų rezultatų – nors ir sulfinil- bei sulfonil-pakeistų slopiklių afiniškumas padidėjo, bet net junginiai su mažiausiu metilo pakaitu (**70h, 72h**) nepasiekė sulfanilo junginio **67h** afiniškumo. Pagrindinė to priežastis buvo leistinos junginių konformacijos, kurios buvo apskaičiuotos naudojant kvantinio tankio funkcionalo teoriją (DFT, atliko V. Kairys). Deguonies atomai junginiuose su –SO- ir –SO_2- riboja molekulės lankstumą randant optimalią padėtį aktyviajame baltymo centre ir veikia kaip sterinis trukdis.



11 pav. Junginiai išdėstyti didėjančio afiniškumo CA IX tvarka (**V** straipsnio numeracija).

Didžiausią afiniškumą CA IX šiame tyrime parodė metil 4-sulfanil-pakeisti-3-sulfamoilbenzoatai, ypač **67b**. Tuo tarpu *N*-butil-4-sulfanil-pakeisti-3-sulfamoilbenzamidai (**68-69** serijos) su visomis karboanhidrazėmis jungėsi silpniau, bet išlaikydami panašų selektyvumą CA IX. Bet didžiausiu afiniškumu CAIX pasižymėjo anksčiau (**IV** str.) sukurti panašūs junginiai, turintys 2-chlor- arba 2-brompakaitus (**53b, e** ir **54b)**.

IŠVADOS

1. 2-halo-benzensulfonamidų 4- ir 5- padėties pakaitai, dėl įvairioms sąveikoms palankios didelės aktyvaus centro erdvės, teigiamai įtakoja slopiklių afiniškumą daugumai CA-izofermentų. Kombinuotas pakaitų lankstumo sumažinimas sukeliant sterinius trukdžius įgalina pagerinti selektyvumą atskiriems izofermentams.
2. Norint pasiekti didžiausią slopiklio afiniškumą ir selektyvumą CA VA ir CA VII, uženka vieno masyvaus pakaito 5-oje 2,4- dihalobenzensulfonamido padėtyje.
3. Metil 2,4-dihalo-5-sulfamoil-benzoatų reakcijose su ariltioliais dominuoja 2-padėties halogeno atomo pakeitimas, o reakcijose su alkiltioliais – 4-os padėties halogeno atomo pakeitimas.
4. 2,5-dipakeisti benzensulfonamidai dėka masyvių 2-os padėties amino ir sulfanilpakaitų pasižymi dideliu afiniškumu ir selektyvumu CA IX izofermentui. Halogeno atomo įvedimas į 4-ą padėtį padidina afiniškumą CA IX neįtakodamas selektyvumo.
5. Sulfinil- ir ypač sulfonilpakaitams 2-oje padėtyje sąveikaujant su gretima sulfonamido grupe sudaromi steriniai trukdžiai stipriai sumažina slopiklių afiniškumą visiems CA izofermentams. Šiems junginiams 2-os padėties pakaito dydžio ir lankstumo įtaka CA izofermentų slopinimui yra nereikšminga.

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SUMMARY

INTRODUCTION

The development of small-molecule chemical drugs is a long and costly process that has become increasingly complex in recent decades due to higher requirements for efficacy and safety. The first stage of this process involves the identification, organic synthesis, and optimization of a potential drug candidate capable of being useful in the treatment of a specific disease or condition. Achievement of this stage requires an understanding of how drugs interact with target structures of biological systems (receptors, enzymes, transporters, etc.) and how desired drug properties can be obtained through structural modification.

In order to understand general principles of drug development, enzymes are among the most attractive research targets. They usually exhibit selective activity, i.e., high substrate specificity, which makes it possible to develop drugs that affect only a specific biological process. In addition, many diseases are known to be associated with changes in enzyme expression and, consequently, in the functions they perform. Furthermore, the high catalytic activity of enzymes allows even small amounts of an inhibitor to significantly affect enzyme activity and produce a strong therapeutic effect. Moreover, such pronounced changes in activity often facilitate laboratory studies and accelerate the research process itself. Therefore, in this dissertation, one such family of research targets—carbonic anhydrases—was selected.

Carbonic anhydrases are enzymes found in all living organisms, from bacteria to mammals, that catalyze the widely occurring carbon dioxide hydration reaction:

$$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^-$$

Why are carbonic anhydrases so important? When observing bubbles rising in a carbonated beverage, it is difficult to imagine why CAs are needed at all. However, the carbon dioxide hydration reaction is relatively slow ($k = 0.039\ s^{-1}$ (Khalifah, 1971)), and carbonic anhydrases accelerate it to approximately $10^6\ s^{-1}$, thereby significantly influencing a wide range of physiological processes.

In the human body alone, 12 catalytically active alpha-family carbonic anhydrases have been identified. These enzymes exhibit high structural similarity but differ in their cellular and organ localization, physiological function, and catalytic activity.

Carbonic anhydrases perform diverse physiological functions in the human body, participating in processes such as CO_2 transport, pH regulation,

electrolyte balance, bone resorption, and tumor development, among others. Changes in the activity of certain carbonic anhydrases (increased activity or expression) are associated with various diseases that are treated using CA inhibitors, including glaucoma, epilepsy, idiopathic intracranial hypertension, high-altitude sickness, migraine, and others. A major problem of most CA inhibitors used in medicine is their side effects, which are primarily associated with poor selectivity toward the target carbonic anhydrase isozymes. Therefore, the development of isozyme-selective CA inhibitors remains a continuously relevant challenge.

Equally important are other applications of CA inhibitors, in which inhibitors modified with photo-, radioactive, or similar groups are used for imaging or cytotoxic purposes. These applications have become particularly relevant in recent decades and are mainly associated with the overexpression of CA IX in cancerous tumors. In such cases, inhibition of CA activity itself is not the primary objective; however, selective binding to the target CA isozymes becomes even more critical.

The **aim of this work** was to design and synthesize selective inhibitors of CA isozymes, to identify the factors determining selectivity, and to apply this knowledge to the development of new compounds.

To achieve this aim, the following **objectives** were set:

- To synthesize 3,4-disubstituted-2-halobenzenesulfonamides and elucidate the influence of substituents on inhibitor affinity and selectivity.
- To synthesize 2-substituted benzenesulfonamides to evaluate the influence of inhibitor substituents located close to the CA cofactor.
- To determine and evaluate the influence of substituent position, size, and flexibility on the affinity and selectivity of CA inhibitors.
- To develop new high-affinity and selective inhibitors of the cancer-associated CA IX isozyme.

SCIENTIFIC NOVELTY

In this dissertation, two groups of benzenesulfonamide inhibitors bearing bulky substituents at the 4,5- and 2,5-positions were synthesized and compared. In both groups, inhibitors exhibiting exceptionally high subnanomolar affinity toward individual CA isozymes were identified.

The synthesized compounds enabled the determination of crystal structures of inhibitor–CA complexes. This facilitated understanding of structure–biological activity relationships and provided an overall positive advance in the research, particularly in the search for inhibitors of the cancer-associated carbonic anhydrase IX isozyme.

The regioselectivity of nucleophilic substitution reactions of 2,4-dihalo-5-sulfamoylbenzoates bearing identical halogens was investigated, and a new synthetic approach for 2,5-disubstituted-4-halobenzenesulfonamides was developed.

It was determined that, within the group of 2,5-disubstituted benzenesulfonamide inhibitors, substituents at the 2-position have a particularly strong influence on inhibitor properties. 2-Amino and 2-sulfanyl substituents exhibited high affinity and selectivity toward the CA IX isozyme. In contrast, 2-sulfonyl substituents resulted in a pronounced decrease in affinity toward all CA isozymes. It was also demonstrated that 2-substituted benzenesulfonamides may represent promising and superior CA IX inhibitors compared to previously used drugs of similar structure, such as bumetanide and piretanide.

DEFENDING STATEMENTS

1. The flexibility of *para*-position substituents in benzenesulfonamide CA inhibitors influences their selectivity toward individual CA isozymes.
2. *Para*-sulfanyl and sulfonyl groups in benzenesulfonamides lead to different substituent orientations in the CA active site and result in higher inhibitor affinity than amino substituents.
3. Inhibitors developed by introducing additional substituents into 5-[2-(benzimidazol-1-yl)acetyl]-2-chlorobenzenesulfonamide exhibit higher affinity for all carbonic anhydrases, but significantly reduce selectivity toward the target CA VA.
4. Sulfanyl and sulfonyl substituents at the *ortho* position of sulfonamide inhibitors strongly influence affinity and selectivity toward the target CA IX (sulfanyl positively, sulfonyl negatively).

SUMMARY OF RESULTS

In this work, a series of benzenesulfonamide carbonic anhydrase (CA) inhibitors bearing various substituents at the 2-, 4-, and 5-positions were synthesized. The results are presented in five publications (**I**–**V**). The first three papers focus on 2-halo-4,5-disubstituted benzenesulfonamide inhibitors. The fourth paper compares the interactions of 2-halo-4-substituted and 4-halo-2-substituted sulfamoyl benzoates with carbonic anhydrases. The fifth paper investigates the influence of *ortho*-substituents on the sulfonamide group on CA inhibition.

The studies were initiated with 4-amino-substituted benzenesulfonamides bearing carbonylamino groups at the 5-position (**I**). The synthesis of inhibitors was started from 2,4-dichloro- and 2,4-dibromo-5-sulfamoylbenzoic acids (**1**, **2**).

From these two benzoic acids, a series of 2,4-dihalobenzamides (**3**–**15**) with amide substituents of various lengths was synthesized via intermediate acid chlorides (Scheme 3). During the purification of 3-hydroxypropylbenzamide **5**, the formation of a side product was observed - transesterification occurred in ethyl acetate. *O*-acetyl benzamide **14** was identified and later resynthesized with a 78% yield by refluxing in ethyl acetate in the presence of acid. In contrast, such a reaction was not observed with 2-hydroxyethylbenzamide **4**.

The second substituent was introduced by replacing the halogen with various amines. Reactions were carried out by heating 2,4-dichlorobenzamides **3**–**7**, **11**, and **15** in excess aliphatic amines at 110–130 °C without metal catalysis [29]. Reactions of dibromobenzamide **13** with amines were performed by prolonged reflux in dioxane, thereby avoiding the formation of side products (Scheme 4).

The next series of compounds (**II**, **IV**) contains substituents of various lengths attached via a sulfur atom. Reactions of 2,4-dihalosulfamoylbenzoic acid derivatives with thiols are practically not described in the literature; therefore, it was expected that these reactions would proceed similarly to those with amines, substituting the halogen atom farther from the sulfonamide group. In this way, a series of compounds with various thioaryl substituents was synthesized from the corresponding benzamides (**3**–**14**) (Scheme 5).

Reaction conditions varied depending on the nucleophile. Reactions of compounds **3**–**8** and **10**–**14** with thiophenol or 1-thionaphthol proceeded most easily by refluxing in methanol in the presence of triethylamine. With cyclohexanethiol, reactions under these conditions were very slow; therefore, the polar protic solvent was replaced with polar aprotic DMSO, and triethylamine was replaced with anhydrous K_2CO_3. This allowed reactions of compounds **3**–**8** and **10**–**13** to be completed within 2–6 hours instead of days or weeks at the same temperature.

When other moderately reactive nucleophiles (primary alkyl thiols, etc.) were used, reactions were carried out in polar aprotic solvent (DMSO) in the presence of triethylamine.

The only exception was the morpholine derivative **9**, whose reactions with thiols required heating to 120 °C in DMSO with Cs_2CO_3, even with thiophenol.

The synthesized thioaryl derivatives were further oxidized to sulfonyl derivatives. Oxidation reactions were carried out with good yields (69–92%) in acetic acid using hydrogen peroxide, generating peracetic acid *in situ* (Scheme 6). During the oxidation of amides containing hydroxyl groups, the formation of *O*-acetates was observed; to avoid this, the reaction product mixture was additionally treated with dilute hydrochloric acid.

Benzoic acids **29b, d** were synthesized from the corresponding benzamides **16b, d** under acidic hydrolysis conditions. However, when ester hydrolysis of **21b** and **35c** was carried out under similar conditions (Scheme 7), the formation of two products was observed. Therefore, further hydrolysis of esters **21a**–**c** was performed under basic conditions at room temperature, yielding only the desired products.

The next series of compounds (**III**) was designed based on previous knowledge [31] about inhibitors selective for the CA VA isozyme. Compounds were synthesized based on 2-chloro- and 2,4-dichlorobenzenesulfonamides bearing various heterocycles at the 5-position.

In Scheme 8, compounds **42a–h** and **43a–c** were synthesized by alkylation of target heterocycles with (bromoacetyl)-2-chlorobenzenesulfonamide **42** or 5-(bromoacetyl)-2,4-dichlorobenzenesulfonamide **43**. *N*-alkylations were performed in THF with NaOAc at room temperature. Compounds **42g** and **42h** were synthesized using an excess of 1,2,3,4-tetrahydroquinoline and indoline without base.

Compound **46** could not be synthesized directly by acylation of 1-aminobenzimidazole with 2,4-dichloro-5-sulfamoylbenzoyl chloride under various conditions. However, protection of the sulfonamide group with an *N,N'*-dimethylaminomethylidene group (Scheme 9, compound **45**) prevented side reactions, allowing synthesis of compound **45** via acylation; deprotection yielded the target benzenesulfonamide **46**.

Some 2,4-dichloro compounds (**43a, b, 46**) were modified by introducing sulfur-bridged fragments according to methods described in paper **II** (Scheme 10), yielding compounds **47–50**.

Paper **IV** describes the next group of substituted halogenated benzenesulfonamides. From initial methyl 2,4-dihalo-5-sulfamoylbenzoates **51** and **52**, methyl 2-halo-4-substituted and 4-halo-2-substituted-5-sulfamoylbenzoates were synthesized (Scheme 11). Reaction conditions were selected based on previous work (paper **II**) describing reactions of 2,4-dihalo-5-sulfamoylbenzamides with sulfur-containing nucleophiles.

When reactions were carried out in methanol, aromatic thiols (thiophenol and 1-thionaphthol) readily substituted the halogen atom, forming *para*-substituted benzenesulfonamides **55a, f**, and **56a**. In contrast, reactions with

thiols containing CH_2 or CH groups proceeded very slowly or did not occur at all under these conditions. To accelerate reactions, the polar protic solvent was replaced with polar aprotic DMSO, yielding *ortho*-substituted benzenesulfonamides **53b–e** and **54b** (Scheme 11). *Para*- and disubstituted benzenesulfonamides were also observed.

The regioselectivity of nucleophilic aromatic substitution reactions of 2,4-dihalo-5-sulfamoylbenzoates **51** and **52** was investigated (Table 3). All reactions were performed in DMSO with thiol and triethylamine at 60 °C for 72 h. Reaction conversion, product ratios, and structure identification were determined by NMR and HPLC/UV/MS.

Table 3. Yields and product ratios of reactions of 2,4-dihalo-5-sulfamoylbenzoates 51 and 52 with thiols determined by ^{1}H NMR spectroscopy and HPLC/UV/MS data (italic). *The 2- and 4-substituted isomers formed in reactions of compounds **51** and **52** with thiophenol could not be separated by HPLC.

Starting compound	Thiol	Conversion (%)	2-substituted (%)	4-substituted (%)	2,4-disubstituted
51		30.07 *28.77*	18.70 *17.38*	81.30 *82.62*	-
52		15.71 *13.92*	9.09 *14.11*	90.91 *85.89*	-
51		89.47 *96.67*	58.82 *88.78**	35.29	5.88 *11.22*
52		95.07 *96.92*	51.81 *85.55**	40.42	7.77 *14.45*
51		72.73 *71.55*	23.53 *22.91*	73.53 *72.79*	2.94 *4.30*
51		72.15 *73.60*	12.28 *12.05*	87.72 *87.95*	-
51		90.97 *95.25*	76.34 *78.84*	19.08 *15.72*	4.58 *5.44*
51		13.70 *18.19*	-	100.00 *100.00*	-

The reactivity of thiols decreases in the following order: phenyl > naphthyl > benzyl ≥ 2-ethylphenyl > cyclohexyl ≥ cyclododecyl. Formation

of disubstituted products was observed in reactions with phenyl-, naphthyl-, and benzyl thiols. A higher proportion of 2-substituted products was obtained with aromatic thiols, whereas in other cases 4-substituted products predominated. In the case of cyclododecyl thiol, only one 2-substituted isomer was observed.

Missing 2-substituted methyl esters **55a–d, f** were synthesized from previously described 2-substituted benzamides **16a–d, f** (Scheme 12). The amide group was converted into an ester in a one-step reaction by heating in methanol with thionyl chloride.

Oxidation of sulfanyl derivatives **53**(**b–d**), **54b, 55**(**a–d, f**), and **56a** to sulfonyl derivatives **57**(**b–d**), **58b, 59**(**a–d, f**), and **60a** was performed by *in situ* generation of peracetic acid (Schemes 11 and 12).

Paper **V** aimed to investigate the influence of substituents located near the sulfonamide group on binding to carbonic anhydrases. For this purpose, *ortho*-substituted benzenesulfonamides with amino-, sulfanyl-, sulfinyl-, and sulfonyl substituents were synthesized.

Since reactions of 2,4-dihalo derivatives yield various products, a starting compound with more predictable behavior — 4-chloro-3-sulfamoylbenzoic acid (**62**) — was selected. Using previously established methods, initial compounds were synthesized from it: the methyl ester (**63**) and a series of amides (**64–66**) (Scheme 13).

From ester **63** of 4-chloro-3-sulfamoylbenzoic acid, nucleophilic aromatic substitution reactions yielded 4-sulfanyl-substituted compounds **67a–d, f–m** (Scheme 13). Reactions were performed by heating with various thiols in dimethylformamide in the presence of K_2CO_3. Under the same conditions, 4-sulfanyl-substituted sulfamoylbenzamides **68a–d, f, g, j–m** were synthesized.

4-Amino-substituted compounds **69b, c** were synthesized by heating benzamide **64** in an excess of the corresponding amine at 130 °C. Compound **69e** was synthesized by refluxing in a mixture of toluene and cyclooctylamine.

Oxidation of esters **67a–d, h, i, k–m** and benzamides **68a, b, k–m** to sulfinyl and sulfonyl compounds was performed using *in situ* generated peracetic acid (Scheme 14). Reactions at room temperature yielded sulfinyl compounds **70a, b, h, i, k–m** and **71a, b, k–m**, whereas heating the reaction mixture to 70 °C yielded sulfonyl compounds **72a–d, h, i, l, m** and **73b, k–m**.

DISCUSSION

In the first article, an analysis of binding data of synthesized compounds with 12 α-carbonic anhydrase (α-CA) isozymes was performed. Initially, affinity and selectivity studies of monosubstituted compounds (*N*-substituted 2,4-dihalo-5-sulfamoylbenzamides **3–15**) revealed that they bind most strongly to CA VII. Upon introduction of a second substituent (2-amino-substituted-4-halo-5-sulfamoylbenzamides, Scheme 4), increased affinity and selectivity toward CA IX and CA XIV isozymes were observed in most cases. Selectivity toward these CA isozymes depended on the size of the second substituent. Due to its flexibility, the benzylamino group interacts more readily with active-site fragments across all isozymes, thereby increasing affinity but reducing selectivity compared to the less flexible cyclohexylamino group. Several crystallographic structures of compound–carbonic anhydrase complexes revealed contacts between inhibitor substituents and protein amino acids, providing deeper insight into the structure–activity relationships of the studied systems.

In the second article, CA inhibitors bearing two substituents were synthesized, one of which was attached via a sulfur-containing linker (2-sulfanyl- or 2-sulfonyl-substituted-4-halo-5-sulfamoylbenzamides). Analysis of the chemical structures and binding parameters of these compounds demonstrated the importance and influence of both substituents on affinity and selectivity toward specific CA isozymes. Crystal structures of protein–inhibitor complexes revealed possible interaction sites between substituents and the active site, as well as pronounced differences in the positioning of sulfanyl and sulfonyl substituents within the CA active site (Figure 6).

It should also be noted that, compared to the amino-substituted compounds described in the first article, compounds from the latter series exhibited higher affinity, with subnanomolar binding values observed for CA VII, CA IX, CA XII, or CA XIV.

In the third article, a series of selective CA VA inhibitors was synthesized based on the previously described compound 5-[2-(benzimidazol-1-yl)acetyl]-2-chlorobenzenesulfonamide (**42a**). Initially, 2-halo- and 2,4-dihalobenzenesulfonamides bearing substituents at the 5-position were investigated, and the dependence of affinity on the nature and size of heterocycles present in the substituent was evaluated (Figure 7). It was determined that the benzimidazole ring is more favorable than imidazole due to aromatic–aromatic interactions with CA VA Tyr64. The nitrogen atom of the benzimidazole/imidazole ring was also shown to be important, forming hydrogen bonds with the Thr62 hydroxyl group of CA VA. In the absence of

this nitrogen atom, as in the case of indoline or 3,4-dihydro-2H-quinoline substituents (compounds **42g** and **42h**), selectivity toward CA VA was lost. Introduction of a second substituent into the *para* position of the benzenesulfonamide increased affinity toward all CA isozymes but markedly reduced selectivity for the target CA VA (Figure 8).

In the fourth article, several compound series were developed and the effects of substituents and their positions on the binding of methyl-2- or 4-substituted-5-sulfamoylbenzoates to human CA isozymes were investigated. Sulfanyl (-S-) and sulfonyl (-SO_2-) substituents of varying size were studied in detail, and crystal structures of these two compound classes bound to CA active sites were determined (Figure 9). *Para*-substituted 2-halobenzenesulfonamides, both sulfanyl-substituted (groups **55–56**) and sulfonyl-substituted (groups **59–60**), bound similarly to various CA isozymes and did not demonstrate selectivity. In contrast, *ortho*-sulfanyl-substituted 4-halobenzenesulfonamides exhibited exceptionally high affinity and selectivity toward the cancer-associated CA IX isozyme (K_d values of compounds **53b**, e, and **54b** approached 0.1 nM). Conversely, the analogous *ortho*-sulfonyl-substituted 4-halobenzenesulfonamides (groups **57** and **58**) bound weakly to all CA isozymes (Figure 10). This unexpectedly large decrease in affinity was interpreted as evidence that a threshold had been reached where small structural modifications at the *ortho* position of benzenesulfonamides can induce major changes in inhibitor–protein interactions. This hypothesis was further explored in subsequent work.

In the fifth article, the influence of *ortho*-substituents on benzenesulfonamides was investigated in greater detail. Compounds similar to the most potent inhibitors from article **IV** were selected, but halogen atoms were deliberately omitted to facilitate the synthesis of a larger and more diverse set of *ortho*-substituted compounds. A series of benzenesulfonamides bearing linear and cyclic substituents ranging from methyl to adamantyl, attached via sulfanyl (-S-), sulfinyl (-SO-), and sulfonyl (-SO_2-) linkers, were synthesized. By varying the oxidation state of the linker, the aim was to reduce the pK_a of the sulfonamide group through increased electron-accepting properties and thereby enhance CA binding. Unfortunately, despite a one-unit decrease in pK_a, compounds with -SO- or –SO_2- linkers bound much more weakly to all CA isozymes than those containing an -S- linker (Figure 11). The strategy of reducing substituent size did not yield the desired outcome: although affinity increased for sulfinyl- and sulfonyl-substituted inhibitors, even compounds with the smallest methyl substituent (**70h** and **72h**) did not reach the affinity of the corresponding sulfanyl compound **67h**. The primary reason for this was attributed to the accessible conformations of the

compounds, which were calculated using density functional theory (DFT; performed by V. Kairys). Oxygen atoms in compounds with -SO- and $–SO_2$- linkers restrict molecular flexibility in achieving an optimal orientation within the protein active site and act as a steric hindrance.

CONCLUSIONS

1. Substituents at the 4- and 5-positions of 2-halo-benzenesulfonamides positively influence inhibitor affinity toward most CA isozymes due to the large active-site space that is favorable for various interactions. Combined reduction of substituent flexibility by introducing steric hindrance enables improved selectivity toward individual isozymes.
2. For binding to CA VA and CA VII, a single bulky substituent at the 5-position of 2,4-dihalobenzenesulfonamides is sufficient to achieve the highest affinity and selectivity.
3. In reactions of methyl 2,4-dihalo-5-sulfamoylbenzoates with aryl thiols, substitution of the halogen atom at the 2-position predominates, whereas in reactions with alkyl thiols, substitution of the halogen atom at the 4-position predominates.
4. 2,5-Disubstituted benzenesulfonamides exhibit high affinity and selectivity toward the CA IX isozyme due to bulky amino and sulfanyl substituents at the 2-position. Introduction of a halogen atom at the 4-position increases affinity toward CA IX without affecting selectivity.
5. Steric hindrance formed by interactions between sulfinyl and especially sulfonyl substituents at the 2-position and the adjacent sulfonamide group strongly reduces inhibitor affinity toward all CA isozymes. For these compounds, the influence of the size and flexibility of the 2-position substituent on CA isozyme inhibition is negligible.

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Methyl 2-Halo-4-Substituted-5-Sulfamoyl-Benzoates as High Affinity and Selective Inhibitors of Carbonic Anhydrase IX

Audrius Zakšauskas [1], Edita Čapkauskaitė [1], Vaida Paketurytė-Latvė [1], Alexey Smirnov [1], Janis Leitans [2], Andris Kazaks [2], Elviss Dvinskis [2], Laimonas Stančaitis [1], Aurelija Mickevičiūtė [1], Jelena Jachno [1], Linas Jezepčikas [1], Vaida Linkuvienė [1], Andrius Sakalauskas [1], Elena Manakova [3], Saulius Gražulis [3], Jurgita Matulienė [1], Kaspars Tars [2] and Daumantas Matulis [1,*]

[1] Department of Biothermodynamics and Drug Design, Institute of Biotechnology, Life Sciences Center, Vilnius University, Saulėtekio al. 7, LT-10257 Vilnius, Lithuania; audrius.zaksauskas@bti.vu.lt (A.Z.); edita.capkauskaite@bti.vu.lt (E.Č.); vaida.paketuryte@gmc.vu.lt (V.P.-L.); alexeyus1@gmail.com (A.S.); laimonas.stancaitis@gmc.vu.lt (L.S.); aurelija.mickeviciute@bti.vu.lt (A.M.); jelena.jachno@bti.vu.lt (J.J.); linasje@gmail.com (L.J.); linkuviene.vaida@gmail.com (V.L.); andrius.sakalauskas@gmc.vu.lt (A.S.); jurgita.matuliene@bti.vu.lt (J.M.)

[2] Latvian Biomedical Research and Study Centre, Ratsupites 1 k-1, LV-1067 Riga, Latvia; janis.leitans@biomed.lu.lv (J.L.); andris@biomed.lu.lv (A.K.); elviss.dvinskis@protonmail.com (E.D.); kaspars@biomed.lu.lv (K.T.)

[3] Department of Protein—DNA Interactions, Institute of Biotechnology, Life Sciences Center, Vilnius University, Saulėtekio al. 7, LT-10257 Vilnius, Lithuania; elena.manakova@bti.vu.lt (E.M.); saulius.grazulis@bti.vu.lt (S.G.)

* Correspondence: daumantas.matulis@bti.vu.lt

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Abstract: Among the twelve catalytically active carbonic anhydrase isozymes present in the human body, the CAIX is highly overexpressed in various solid tumors. The enzyme acidifies the tumor microenvironment enabling invasion and metastatic processes. Therefore, many attempts have been made to design chemical compounds that would exhibit high affinity and selective binding to CAIX over the remaining eleven catalytically active CA isozymes to limit undesired side effects. It has been postulated that such drugs may have anticancer properties and could be used in tumor treatment. Here we have designed a series of compounds, methyl 5-sulfamoyl-benzoates, which bear a primary sulfonamide group, a well-known marker of CA inhibitors, and determined their affinities for all twelve CA isozymes. Variations of substituents on the benzenesulfonamide ring led to compound **4b**, which exhibited an extremely high *observed* binding affinity to CAIX; the K_d was 0.12 nM. The *intrinsic* dissociation constant, where the binding-linked protonation reactions have been subtracted, reached 0.08 pM. The compound also exhibited more than 100-fold selectivity over the remaining CA isozymes. The X-ray crystallographic structure of compound **3b** bound to CAIX showed the structural position, while several structures of compounds bound to other CA isozymes showed structural reasons for compound selectivity towards CAIX. Since this series of compounds possess physicochemical properties suitable for drugs, they may be developed for anticancer therapeutic purposes.

Keywords: methyl 5-sulfamoyl-benzoate; human carbonic anhydrase; *intrinsic* binding thermodynamics; enzyme inhibitor; X-ray crystallography; fluorescent thermal shift assay

1. Introduction

Low-molecular-weight compounds that strongly interact and inhibit the disease target enzymes may be developed as therapeutic drugs. The carbonic anhydrase (CA) family (CA, EC 4.2.1.1), consisting of twelve catalytically active human isozymes, performs the catalysis of reversible hydration of CO_2 into bicarbonate and acid protons. These enzymes participate in numerous physiological processes such as pH regulation and carbon metabolism and are also related to various diseases and conditions such as glaucoma, epilepsy, cancer, edema, osteoporosis, and obesity [1,2]. Since it has been observed that CAIX isozyme is highly

overexpressed in numerous cancers, it has been postulated that compounds inhibiting CAIX may possess the anticancer activity and may be used in tumor treatment [3–5].

Many CA inhibitors have been synthesized bearing flexible tails capable of finding an energetically favorable position in several CA isozymes, thus losing selectivity [6,7]. We have attempted to design inhibitors with a restrained position of the ring and two functional groups attached to the benzenesulfonamide [8–10]. This approach helped design inhibitors selective for CAVII, CAIX, CAXII, and CAXIV.

The introduction of a halogen in the *ortho*- position relative to sulfonamide constrained the benzenesulfonamide ring in a stable position in the CA active site. Additional substituents in the *meta*- and *para*-positions provided additional leverage for increased affinity and selectivity [11–13]. However, these compounds did not fully exploit differences in the CA active site and did not reach sufficient selectivity.

The isozymes CAIX and CAXII exhibit slightly more opened active site entrance than CAI and CAII because, at the entrance of the binding pocket, CAIX has Val131, and CAXII has Ala131, while CAI and CAII have bulky Tyr204 and Phe131 at isostructural positions [14]. Substituents in the *ortho*-position would be expected to interact with the narrower entrance to the active site closer to the Zn(II) and, therefore, would result in greater selectivity for CAIX and CAXII. We attempted to exploit these differences by designing inhibitors selective for CAIX and CAXII—*ortho*-substituted benzenesulfonamides. Two such compounds were designed to bear bulky groups in *ortho*- or *para*-positions to study the effect of substituent's position, size, and flexibility. The compounds were synthesized, and binding affinities and selectivities were determined for the entire set of 12 recombinant human CA isozymes. Key compounds were crystallized with several CA isozymes demonstrating their exact structural positions when bound to CAIX and other isozymes that lead to significant changes in affinity and selectivity for a particular isozyme. Several inhibitors possessed subnanomolar affinities for CAIX and greater than 100-fold selectivities over the remaining human CA isozymes. Thus, such compounds could be further developed for therapeutic purposes.

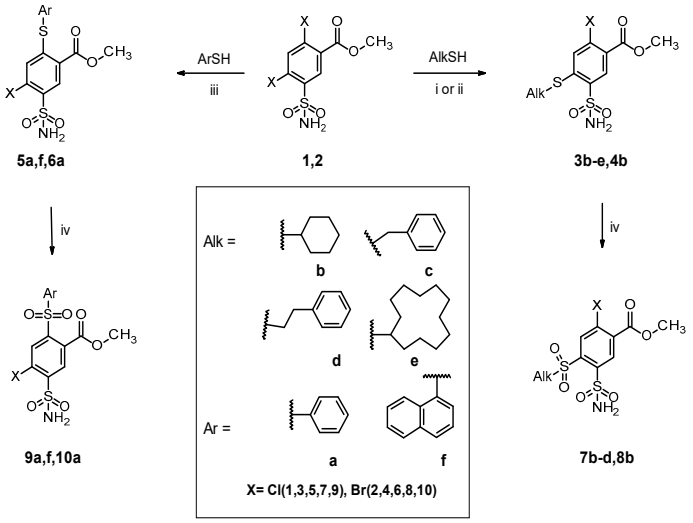
2. Results and Discussion

2.1. Organic Synthesis of Designed Compounds

Based on substituted halogenated benzenesulfonamide, a series of compounds were designed as potentially high-affinity and selective inhibitors of particular CA isozymes. Starting from methyl 2,4-dichloro-5-sulfamoyl-benzoate **1** and methyl 2,4-dibromo-5-sulfamoyl-benzoate **2**, the designed methyl halo 2- and 4-substituted-5-sulfamoyl-benzoates were synthesized (Scheme 1). Reaction conditions were first chosen according to our previous series where sulfur-containing nucleophiles reacted with dihalosulfamoylbenzamides [9]. The reactions of 2,4-dihalo-5-sulfamoyl-benzoates **1**, **2** with thiols were performed in boiling methanol with triethylamine. However, the reaction was successful only with aromatic thiols (benzenethiol and naphthalene-1-thiol), yielding the *para*-substituted benzenesulfonamides **5**(**a**,**f**) and **6a**. The reaction was very slow or absent, with thiols bearing aliphatic CH_2 or CH group next to the sulfur atom.

To improve the synthesis and based on the reaction of dihalobenzamide with cyclohexanethiol [9], the polar protic solvent MeOH was replaced with a polar aprotic solvent DMSO. This replacement enabled the synthesis of *ortho*-substituted benzenesulfonamides (**3b**–**e**), **4b** as the main product. However, by-products also formed, including *para*-substituted or even-disubstituted benzenesulfonamides.

The regioselectivity of the nucleophilic aromatic substitution of a halogen in methyl 2,4-dihalo-5-sulfamoyl-benzoates **1**, **2** was investigated. All reactions were repeatedly performed in DMSO with triethylamine at 60 °C temperature for 72 h. Determination of conversion yield, product ratio, and structure identification has been performed by NMR and HPLC/UV/MS (Table 1, and Supplementary material).



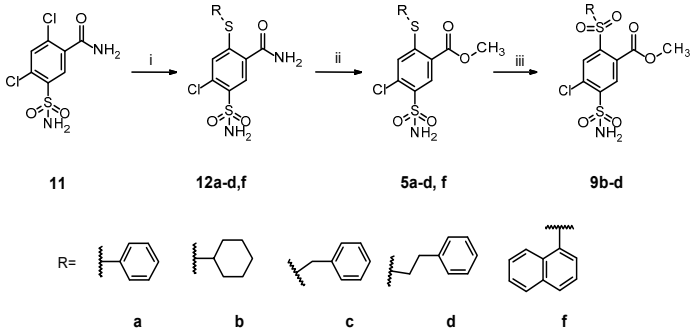
Scheme 1. The synthesis of methyl halo 2- and 4-substituted-5-sulfamoyl-benzoates: (i) TEA, DMSO, 60 °C; (ii) K_2CO_3, DMSO, 60 °C; (iii) TEA, MeOH, Δ; (iv) H_2O_2, AcOH, 75 °C. The legend in the middle explains the structures of alkyl (Ak; **b**, **c**, **d**, **e**) and aromatic (Ar; **a**, **f**) substituents and halogen atoms (Cl—compounds **1**, **3**, **5**, **7**, **9** or Br—**2**, **4**, **6**, **8**, **10**).

Table 1. The reaction of 2,4-dihalo-5-sulfamoyl-benzoates **1**, **2** with thiols and the ratios of the products as determined by NMR spectroscopy and HPLC/UV/MS system data (*italic*). * The separation of the 2- and 4-substituted isomers formed in the reaction of compounds **1** and **2** with phenylthiol using HPLC was unsuccessful.

Starting Compound	Thiol	Conversion (%)	2-Substituted (%)	4-Substituted (%)	2,4-Disubstituted (%)
1		30.07 *28.77*	18.70 *17.38*	81.30 *82.62*	-
2		15.71 *13.92*	9.09 *14.11*	90.91 *85.89*	-
1		89.47 *96.67*	58.82 *88.78 **	35.29	5.88 *11.22*
2		95.07 *96.92*	51.81 *85.55 **	40.42	*7.77* *14.45*
1		72.73 *71.55*	23.53 *22.91*	73.53 *72.79*	2.94 *4.30*
1		72.15 *73.60*	12.28 *12.05*	87.72 *87.95*	-
1		90.97 *95.25*	76.34 *78.84*	19.08 *15.72*	4.58 *5.44*
1		13.70 *18.19*	-	100.00 *100.00*	-

Thiols could be arranged in descending order by their reactivity: phenyl ≥ naphthyl > benzyl ≥ ethylphenyl > cyclohexyl ≥ cyclododecyl. The formation of a disubstituted product was observed in the reaction with phenyl-, naphthyl-, and benzyl- thiols. The 2-substituted product was mainly formed in the reactions with aromatic thiols (naphthyl- and phenylthiol), while in the remaining cases, the formation of the 4-substituted product was dominant. In the case of bulky dodecylthiol, only the formation of the 4-substituted isomer was observed.

A group of 2-substituted methyl benzoates **5**(**a**–**d**,**f**) was synthesized via 2,4-dichloro-5-sulfamoyl-benzamide (**11**) (Scheme 2) [9]. The nucleophilic substitution of chlorine occurred only in the *para*- position relative to the sulfonamide group. The amide group of the substituted products **12**(**a**–**d**,**f**) was converted to the ester group using thionyl chloride and MeOH to yield the 2-substituted benzenesulfonamide methyl esters **5**(**a**–**d**,**f**). Oxidation of sulfanyl derivatives **3**(**b**–**d**), **4b**, **5**(**a**–**d**,**f**), **6a** to sulfonyl derivatives **7**(**b**–**d**), **8b**, **9**(**a**–**d**,**f**), **10a** (Schemes 1 and 2) was carried out using in situ generated peracetic acid.



Scheme 2. Synthesis of 2-halo-4,5-disubstitutedbenzenesulfonamides: (i) TEA, DMSO, 60 °C; (ii) $SOCl_2$, MeOH, Δ; (iii) H_2O_2, AcOH, 75 °C.

2.2. Thermodynamics of Compound Binding to CA Isozymes

The chemical structures of 21 compounds used in this study are summarized in Figure 1A. The main goal was to determine the influence of the type and position of the sulfanyl or sulfonyl substituent on the affinity for human CA isozymes. The compounds were divided into four groups with different sulfanyl (-S-) or sulfonyl (-SO_2-) substituents in *para*- or *ortho*-positions with respect to the sulfonamide group. All compounds had the same ester group in the *meta*-position. The influence of the diversity of the *meta* group on the binding affinity to CAs has been investigated and described in greater detail in a previous publication [9]. We found no significant difference in binding for compounds with chlorine or bromine atoms in that study. Therefore, there is only one analogous brominated compound for comparison (**4b**, **6a**, **8b**, **10a**) in each of the four groups.

Dissociation constants (Table 2) of every compound for each of the 12 catalytically active human CA isozymes were measured using the fluorescent thermal shift assay (FTSA). Some experimental data of selected compounds are provided in Figures S1 and S2 in the Supplementary material. The observed constants (K_{d_obs}) were converted to the observed standard change in the Gibbs energy of binding ($\Delta G_{obs} = RT\ln K_{d\_obs}$, where R *is* the universal ideal gas constant and T *is* the temperature), shown in Figure 1B, upper panel, and Table S1. For visual simplicity, the affinity is averaged for all compounds belonging to one of the four groups and shown in the Figure by coloring the groups: **3**(**b**–**e**), **4b** are blue rhombs, **5**(**a**–**d**,**f**), **6a** are orange squares, **7**(**b**–**d**), **8b** are cyan triangles, and **9**(**a**–**d**,**f**), **10a** are yellow circles (Figure 1B). The bars show the range from the strongest to the weakest affinity for each CA isozyme in that group of compounds (not the error bars).

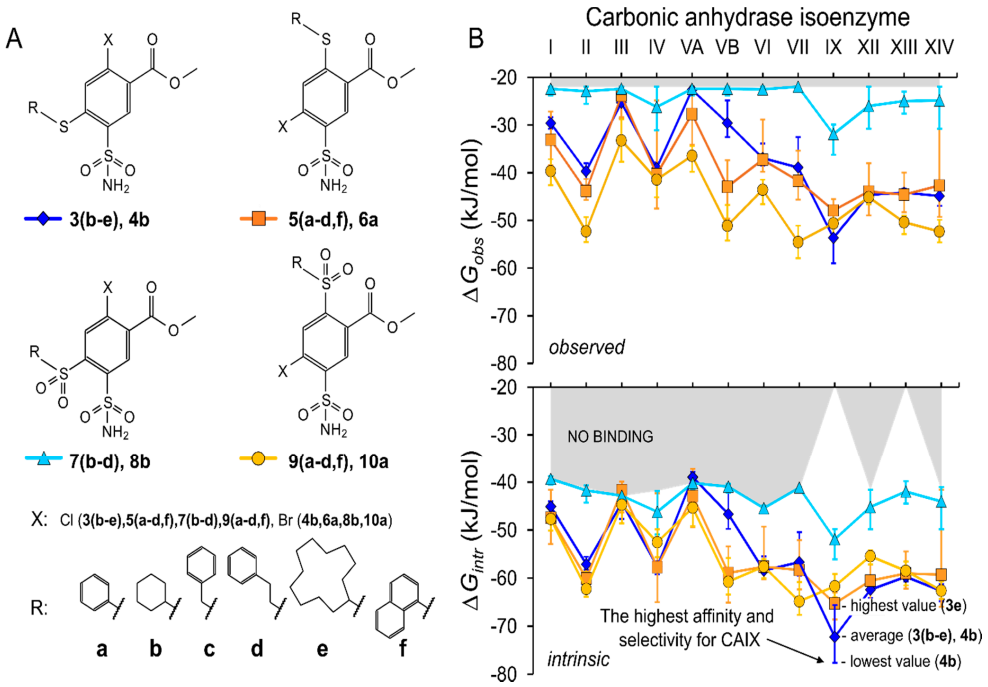


Figure 1. (**A**) Chemical structures of the four groups of compounds—methyl halo 2- or 4-substituted-5-sulfamoyl-benzoates containing chlorine or bromine and substituents listed at the bottom of the panel. (**B**) Averaged change in the standard *observed* (upper panel) or *intrinsic* (lower panel) Gibbs energy for compounds belonging to one of the four groups: **3**(**b**–**e**), **4b**—blue rhombs, **5**(**a**–**d**,**f**), **6a**—orange squares, **7**(**b**–**d**), **8b**—cyan triangles, and **9**(**a**–**d**,**f**), **10a**—yellow circles. The bars show the margin between the strongest and weakest affinity of compounds in that group for the indicated CA isozyme. The *intrinsic* binding affinity of compounds **3**(**b**–**e**), **4b** to CAIX is highlighted: the blue rhombus is the average of all five interactions, the bar above shows the weakest CAIX interaction with **3e**, and below is the strongest with **4b**. Compounds in this group have the highest selectivity and affinity for CAIX.

Interaction of CA with any primary sulfonamide is pH-dependent in identical shape. The affinity is greatest in the near-neutral range and decreases by exactly 10-fold with every pH unit in the acidic and alkaline pH regions. This is explained by the binding mechanism where the deprotonated sulfonamide compound replaces a water molecule (but not a hydroxide ion) coordinated by the Zn(II) in the active site of CA. The deprotonated state of the sulfonamide amino group bound to CA is directly observed by the neutron diffraction structures [15]. However, the sulfonamide state in the solution is predominantly in the protonated form because sulfonamide pK_a is usually in the region of 9–10. Both the compound and the protein have to undergo protonation–deprotonation reactions to be able to bind. These binding-linked reactions significantly reduce the affinity. Interaction energies of the binding-able states of the protein and ligand are termed *intrinsic*. However, since the protonation reactions occur indistinguishably from protein-ligand binding, we experimentally observe diminished energies, termed *observed*.

Table 2. *Intrinsic* (K_{d_intr}), calculated according to the model, and *observed* (K_{d_obs} at pH = 7.0), determined by the fluorescent thermal shift assay, dissociation constants, K_d (nM), of investigated compounds binding to all catalytically active human carbonic anhydrases (CAs) at 37 °C. pK_a values of the sulfonamide group were calculated from spectra at various pH as described in Methods and are listed to all compounds. Some compounds exhibited poorer solubility, which was observed visually and prompted in absorption spectra. Therefore, for such compounds, the pK_a value was assigned based on the average value of the other compounds in that group and is marked with *.

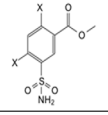
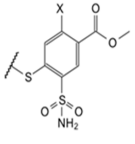
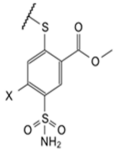
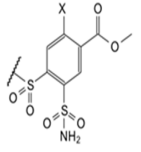
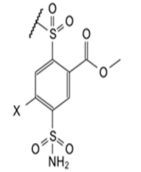
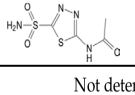
Compound (Lab. Name)	Chemical Structure	*ortho* Substituent	*para* Substituent	pK_a	K_d (nM)	CAI	CAII	CAIII	CAIV	CAVA	CAVB	CAVI	CAVII	CA IX	CAXII	CAXIII	CAXIV
1		Cl	Cl	8.9	*Observed*	14,000	71	50,000	180	6700	310	170	67	59	560	83	72
(*EA2-1*)					*Intrinsic*	160	0.39	150	0.65	55	1.9	0.19	0.32	0.21	2.7	0.94	0.35
2		Br	Br	8.8	*Observed*	20,000	91	$\geq 2 \times 10^5$	140	3700	33	290	56	120	330	63	40
(*LJ14-4*)					*Intrinsic*	290	0.63	≥750	0.63	38	0.26	0.41	0.34	0.52	2	0.89	0.24
3b		*cyclohexyl*	Cl	9.6	*Observed*	17,000	250	40,000	400	100,000	11,000	330	150	0.33	33	50	13
(*EA2-3*)					*Intrinsic*	39	0.28	24	0.29	170	14	0.080	0.15	0.00020	0.030	0.11	0.010
3c		*benzyl*	Cl	9.5	*Observed*	6900	110	80,000	120	$\geq 2 \times 10^5$	5000	360	150	2.9	22	38	40
(*EA2-4*)					*Intrinsic*	20	0.16	61	0.11	≥420	7.9	0.10	0.19	0.0030	0.030	0.11	0.050
3d		*2-phenylethyl*	Cl	9.6	*Observed*	13,000	170	15,000	430	110,000	10,000	630	100	13	53	38	43
(*EA2-5*)					*Intrinsic*	30	0.18	9.3	0.30	190	13	0.14	0.10	0.0090	0.050	0.090	0.040
3e		*cyclododecyl*	Cl		*Observed*	12,000	400	$\geq 2 \times 10^5$	200	$\geq 2 \times 10^5$	67,000	2000	3300	0.44	41	27	37
(*EA2-12*)					*Intrinsic*	27	0.44	≥120	0.14	≥330	84	0.46	3.2	0.00030	0.040	0.060	0.040
4b		*cyclohexyl*	Br	9.6 *	*Observed*	6700	200	48,000	330	$\geq 2 \times 10^5$	3300	560	220	0.12	16	33	20
(*LJ15-12*)					*Intrinsic*	15	0.22	29	0.24	≥330	4.2	0.13	0.22	0.000080	0.020	0.080	0.020
5a		Cl	*phenyl*	9.4 * LS	*Observed*	3300	36	17,000	14	$\geq 2 \times 10^5$	250	200	50	2.5	5.7	20	19
(*EA2-2*)					*Intrinsic*	12	0.060	16	0.020	≥530	0.50	0.070	0.080	0.0030	0.0090	0.070	0.030
5b		Cl	*cyclohexyl*	9.4	*Observed*	330	20	$\geq 2 \times 10^5$	67	4000	5.7	210	21	7.5	13	10	5.1
(*EA1-3N*)					*Intrinsic*	1.2	0.040	≥190	0.080	11	0.010	0.070	0.030	0.0080	0.020	0.040	0.0080
5c		Cl	*benzyl*	9.4 * LS	*Observed*	2000	33	$\geq 2 \times 10^5$	120	1800	470	290	130	14	400	100	47
(*EA1-4N*)					*Intrinsic*	7.3	0.060	≥190	0.13	4.8	0.92	0.10	0.19	0.020	0.61	0.36	0.070
5d		Cl	*2-phenylethyl*	9.3	*Observed*	3400	27	89,000	1000	3800	4.4	850	24	12	180	7.4	17
(EA1-5N)					*Intrinsic*	16	0.060	110	1.5	13	0.010	0.38	0.050	0.020	0.34	0.030	0.030
5f		Cl	1-naphthyl	9.4 * LS	*Observed*	27,000	110	$\geq 2 \times 10^5$	66,000	96,000	510	14,000	1100	22	66	57	63,000
(EA2-NF)					*Intrinsic*	98	0.20	≥190	75	250	1.0	5.0	1.7	0.020	0.10	0.21	97
6a		Br	phenyl	9.4 * LS	*Observed*	2500	80	$\geq 2 \times 10^5$	40	86,000	31	420	210	9.8	20	16	13
(LJ15-11)					*Intrinsic*	9.2	0.14	≥190	0.050	230	0.060	0.15	0.32	0.010	0.030	0.060	0.020

Table 2. *Cont.*

Compound (Lab. Name)	Chemical Structure	*ortho* Substituent	*para* Substituent	pK_a	K_d (nM)	CA I	CA II	CA III	CA IV	CA VA	CA VB	CA VI	CA VII	CA IX	CA XII	CA XIII	CA XIV
7b		cyclohexyl	Cl	9.8	*Observed*	100,000	50,000	100,000	41,000	100,000	100,000	190,000	$\geq 2 \times 10^5$	9200	67,000	69,000	69,000
(EA2-3o)					*Intrinsic*	150	35	38	19	110	79	28	≥120	4.2	41	99	42
7c		benzyl	Cl	9.8* LS	*Observed*	$\geq 2 \times 10^5$	190,000	$\geq 2 \times 10^5$	5900	$\geq 2 \times 10^5$	$\geq 2 \times 10^5$	130,000	$\geq 2 \times 10^5$	790	6700	140,000	6700
(EA2-4o)					*Intrinsic*	≥290	130	≥76	2.7	≥210	≥160	19	≥120	0.36	4.1	200	4.1
7d		2-phenylethyl	Cl	9.8 * LS	*Observed*	$\geq 2 \times 10^5$	$\geq 2 \times 10^5$	$\geq 2 \times 10^5$	$\geq 2 \times 10^5$	$\geq 2 \times 10^5$	$\geq 2 \times 10^5$	130,000	$\geq 2 \times 10^5$	6700	34,000	22,000	$\geq 2 \times 10^5$
(EA2-5o)					*Intrinsic*	≥300	≥140	≥76	≥90	≥210	≥160	19	≥120	3.0	21	32	≥120
8b		cyclohexyl	Br	9.9	*Observed*	$\geq 2 \times 10^5$	$\geq 2 \times 10^5$	$\geq 2 \times 10^5$	$\geq 2 \times 10^5$	$\geq 2 \times 10^5$	$\geq 2 \times 10^5$	$\geq 2 \times 10^5$	$\geq 2 \times 10^5$	6700	$\geq 2 \times 10^5$	71,000	$\geq 2 \times 10^5$
(LJ15-12o)					*Intrinsic*	≥230	≥110	≥60	≥16	≥170	≥130	≥23	≥97	2.4	≥97	82	≥97
9a		Cl	phenyl	8.2	*Observed*	140	0.91	3300	25	770	13	56	0.45	3.3	22	5.6	0.83
(EA2-2o)					*Intrinsic*	7.9	0.020	48	0.42	30	0.40	0.30	0.010	0.060	0.51	0.30	0.020
9b		Cl	cyclohexyl	8.2	*Observed*	67	0.67	2800	200	500	0.75	42	0.18	1	14	2.7	0.64
(EA1-3No)					*Intrinsic*	3.7	0.020	40	3.4	20	0.020	0.22	0.0040	0.020	0.33	0.15	0.010
9c		Cl	benzyl	8.2	*Observed*	560	5	450	100	830	1.1	14	2.5	2.5	20	5	2.6
(EA1-4No)					*Intrinsic*	31	0.13	6.5	1.7	33	0.030	0.080	0.060	0.040	0.46	0.27	0.060
9d		Cl	2-phenylethyl	8.4	*Observed*	330	1.7	920	140	200	1.1	26	0.85	2.5	48	3	1.3
(EA1-5No)					*Intrinsic*	12	0.030	8.5	1.6	5.1	0.020	0.090	0.010	0.030	0.72	0.10	0.020
9f		Cl	1-naphthyl	8.4	*Observed*	140	1.3	14,000	50	1400	3.4	100	0.67	10	50	1.3	4
(EA2-Nfo)					*Intrinsic*	5.1	0.020	130	0.55	36	0.060	0.35	0.010	0.11	0.74	0.040	0.060
10a		Br	phenyl	8.4	*Observed*	330	2.3	4800	370	1600	5.3	110	0.67	3.3	16	4.3	2
(LJ15-11o)					*Intrinsic*	12	0.040	44	4.0	41	0.10	0.37	0.010	0.040	0.24	0.15	0.030
Acetazolamide				7.0	*Observed*	2400	46	40,000	87	840	140	220	13	21	130	120	63
					Intrinsic	1100	9.8	4600	12	270	34	9.8	2.4	2.9	24	53	12

Not determined, ND; low solubility, LS. * pK_a value was assigned based on the average value of the other compounds in that group. The measurement limit is $\geq 2 \times 10^5$. The highest used ligand concentration was 200 µM. *Intrinsic* values are calculated according to the model described in the Methods section.

The *intrinsic* dissociation constants were calculated according to the model described in the Methods section and Supplementary material using measured pK_a values of the sulfonamide group of the compounds (Table 2). It is more appropriate to use the *intrinsic* parameters in interpreting the interactions, as they help separate the linked protonation reactions that can lead to erroneous conclusions [16]. The *intrinsic* standard change in the Gibbs energy of binding is shown in Figure 1B, lower panel, and Table S2. These parameters were also used in the analysis of crystal structures.

2.3. X-ray Crystal Structures and Correlations with Compound Binding Thermodynamics to CA Isozymes

Eight crystal structures of CA complexes with compounds are presented in this study: **9a** bound to CAI and CAII, **3b**, **3d**, **5b**, and **9a** bound to CAXII, **3b** bound to CAIX and mimic-CAIX (mutant of CAII containing amino acids in the active site as in CAIX: A65S, N67Q, I91L, F130V, V134L, and L203A). The electron density maps of the ligands bound in the active site of CA are shown in Figure S3. The data collection and refinement statistics are presented in Table S3. The crystal structures of CAI, CAII (and mimic-CAIX), CAIX, and CAXII complexes in the asymmetric unit contained 2, 1, 4, and 4 protein subunits, respectively.

The structure–thermodynamics correlations that determine the recognition between the CA active site and the ligand will be analyzed in three sections:

(i) methyl 2-halo-4-substituted-5-sulfamoyl-benzoates, abbreviated *ortho*- by substituent at the *ortho*- position relative to sulfonamide group;
(ii) methyl 4-halo-2-substituted-5-sulfamoyl-benzoates, abbreviated as *para*-,
(iii) the position of the substituent in benzenesulfonamide is compared in these two groups of compounds.

2.3.1. Methyl 2-Halo-4-Substituted-5-Sulfamoyl-Benzoates Binding to CA Isozymes

In the group of *ortho*- sulfanyl– **3**(**b**–**e**), **4b**, and sulfonyl– **7**(**b**–**d**), **8b** substituted benzenesulfonamides, the chlorinated **3b**, and brominated **4b** bound similarly. Their K_d differed by less than 3-fold; not a significant difference within the error limits (Table 2). Compounds **7** bound most isozymes with barely detectable affinity, except CAIX, where chlorinated and brominated bound similarly (K_{d_obs} (**7b**) = 9200 nM and K_{d_obs} (**8b**) = 6700 nM). Sulfonyl (-SO_2-, **7**(**b**–**d**), **8b**) compounds bound CA significantly weaker than the parent compound **1** or analogous sulfanyl compounds **3b**–**e**. The strongest interaction with CAIX was likely due to its wider active site than in other CAs [10]. The sulfonyl compounds likely were in an unfavorable conformation for binding and prevented an optimal formation of the sulfonamide group-nitrogen and protein-Zn(II) coordination bond due to steric hindrance.

The pK_a values of the sulfonamide amino group of a series of these compounds were determined. Although the compounds showed limited solubility, the absorption spectra of **7b** and **8b** at different pH were used to determine their pK_a values, 9.8 and 9.9, respectively (Supplementary material, Figure S5). The same, 9.8, value was assigned to other compounds (**7c** and **7d**) in the same group. *Para*-substituted sulfonyl (-SO_2-) compounds **9**(**a**–**d**,**f**), and **10a** (pK_a = 8.2–8.4) had lower pK_a values than sulfanyl (-S-) compounds **5**(**a**–**d**,**f**), and **6a** (pK_a = 9.3–9.4), while *ortho*-substituted compounds showed the opposite (Figure 1B, upper panel and Table 2). The pK_a values were 9.9 for sulfonyl (-SO_2-) compounds **7**(**b**–**d**,**f**), **8b**, and 9.5-9.6 for sulfanyl (-S-) compounds **3**(**b**–**e**), **4b**. Therefore, oxidation did not reduce the pK_a of the sulfonamide group in all cases. The pK_a values were well correlated with chemical shifts determined by NMR (Supplementary material, Figure S6). Chemical shifts show the same trend: values of *ortho*-sulfonyl compounds were lower than sulfanyl, and, in contrast, *para*-sulfonyl compounds had higher values than sulfanyl.

Sulfanyl- compounds **3b**–**e** and **4b** bound weakly to CAI, CAIII (narrow entrance to the active site due to Phe198), CAVA, and CAVB. However, compounds had μM affinity to CAII, CAIV, CAVI, CAVII and even approximately 10-fold stronger (K_{d_obs} is 13–53 μM) to CAXII, CAXIII, and CAXIV (the measured affinity to CAIX is up to 0.12 μM (Table 2 and Figure 1B).

Comparison between parent compound **1** and the *ortho*-substituted sulfanyl compounds showed that the hydrophobic substituent had a significant effect (Figure 2). The intrinsic affinity for CAXII (−11.4 kJ/mol), CAXIII (−5.4 kJ/mol), CAXIV (−8.6 kJ/mol), and especially for CAIX (−17.5 kJ/mol or about 1000-fold) significantly increased, and CAVA (2.9 kJ/mol) and CAVB (5.1 kJ/mol) decreased (**1**→**3b**). The affinity strongly depended on the flexibility of the substituent and shorter substituents in compounds **3b** and **3e** had higher affinity to CAIX than longer and more flexible substituents in compounds **3c** and **3d**: the difference was at least about 6 kJ/mol or about 10-fold (ΔG_{intr} (**3b**) = −75.0 kJ/mol, ΔG_{intr} (**3c**) = −68.8 kJ/mol, ΔG_{intr} (**3d**) = −65.6 kJ/mol, ΔG_{intr} (**3e**) = −74.2 kJ/mol). The affinity for CAVII decreased significantly (9.0 kJ/mol) from compound **3d** to compound **3e**, possibly due to the size of the substituent. In other cases, the differences were within the error margin.

We compared the crystal structures of several compounds in two different CA isozymes (Figure 2B–D). Figure 2B shows the binding mode of **3b** and **3d** in the active site of CAXII. These compounds are structurally similar and differ only in the size of large hydrophobic *ortho* substituents; compound **3b** has the octyl, while **3d** has the 2-phenylethyl moiety. The binding modes of both ligands in the active site of CAXII were the same: *ortho* groups were directed to the hydrophobic part of the active site, while *meta* substituents were orientated to the hydrophilic part. The binding affinities and enthalpy changes to CAXII were almost identical (ΔG_{intr} = −62.3 kJ/mol, ΔH_{intr} = −48.0 kJ/mol (**3b**) vs. ΔG_{intr} = −61.1 kJ/mol, ΔH_{intr} = −50.1 kJ/mol (**3d**), Figure 2B). Experimental data of the ΔH_{obs} are shown in the Supplementary material in Figure S8.

Figure 2C shows the superposition of **3b** in the active sites of CAIX and CAXII. The positions of the compounds were similar. However, the binding affinities of **3b** to CAIX and CAXII differed significantly and were 150 times stronger to CAIX than CAXII (K_{d_intr} = 0.030 nM or ΔG_{intr} = −62.3 kJ/mol to CAXII and K_{d_intr} 0.20 pM or ΔG_{intr} = −75.0 kJ/mol to CAIX, Table 2 and Table S2). Again, the side chains interacting with **3b** in CAIX and CAXII were different. They were more hydrophobic in CAIX (A203, L135, V131, L91, and Q67) and more hydrophilic in CAXII (N204, S135, V131, T91, and K68). Thus, these differences could be related to the solvation entropy because binding to CAIX was much more entropy-driven than to CAXII (ΔH_{intr} = −29.9 kJ/mol, $-T\Delta S_{intr}$ = −45.1 kJ/mol to CAIX and ΔH_{intr} = −48.0 kJ/mol, $-T\Delta S_{intr}$ = −14.3 kJ/mol to CAXII).

The binding of **3b** to CAIX and mimic-CAIX is compared in Figure 2D. Mimic-CAIX is CAII with multiple point mutations that replace the side chains of the active site of CAII with the corresponding residues of CAIX. It has been shown that six-point mutations (A65S, N67Q, I91L, F130V, V134L, and L203A) are sufficient to switch the binding mode of the selective ligand [17–19] (mutations slightly different A65S, N67Q, E69T, I91L, F131V, K170E, and L204A). The ligand positions were similar except for the shift of the *ortho* cyclooctyl group. Nevertheless, the native CAIX bound **3b** 10-fold more strongly than the mimic-CAIX (Supplementary material Figure S1). The crystal structure could not answer this question with certainty, but the differences in binding affinities between CAIX and CAII were even greater: ΔG_{intr} = −56.7 kJ/mol to CAII, ΔG_{intr} = −67.4 kJ/mol to mimic-CAIX and ΔG_{intr} = −75.0 kJ/mol to CAIX. The mutations introduced in CAII mimicked the active site of CAIX. The binding affinities were more similar to CAIX than CAII, and very similar enthalpy changes between CAIX and mimic-CAIX were observed: ΔH_{intr} = −29.9 kJ/mol to CAIX and ΔH_{intr} = −31.2 kJ/mol to mimic-CAIX).

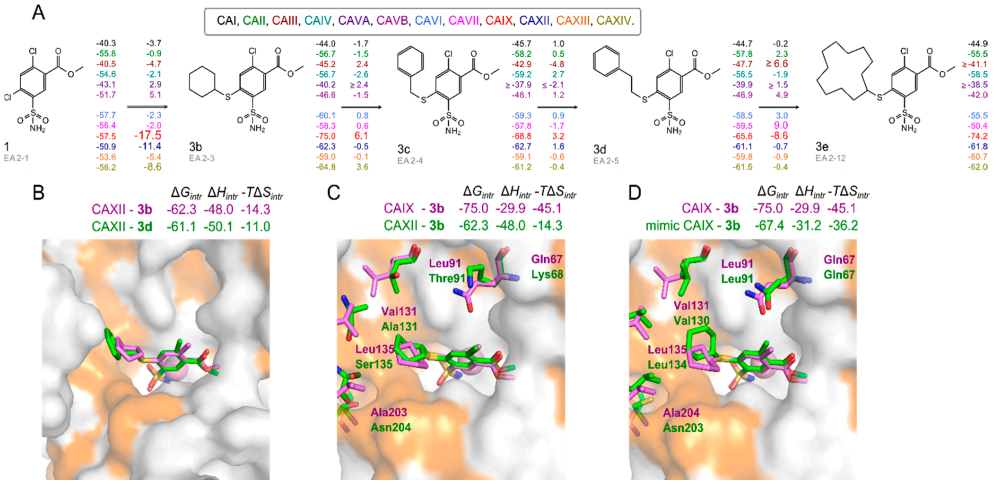


Figure 2. Correlation of compound chemical structures with the changes in standard intrinsic Gibbs energy upon binding and the comparison of the crystal structures between CA isozymes. (**A**) Differences of ΔG_{intr} between neighboring compounds are listed on the connecting arrows. All values have units of kJ/mol. Colors represent CA isozymes. (**B**) Compounds **3b** (magenta, PDB ID: 7PP9) and **3d** (green, PDB ID: 7PUW) bound to CAXII. (**C**) Compound **3b** bound to CAXII (green, PDB ID: 7PP9) and CAIX (magenta, PDB ID: 7POM). (**D**) Compound **3b** bound to CAIX (magenta, PDB ID: 7POM) and mimic-CAIX (green, PDB ID: 7Q0C). Zinc ion is shown as a pink sphere. The notable nonconservative residues between active sites are shown in the "stick" mode. The protein surface of the CA active site is colored orange for hydrophobic residues (V, I, L, F, M, A, G, and P) and gray for the residues with charged and polar side chains (R, D, N, E, Q, H, K, S, T, Y, W, and C).

2.3.2. Methyl 4-Halo-2-Substituted-5-Sulfamoyl-Benzoates Binding CA Isozymes

The compounds that contain *para*- and *ortho*- substituents are similar in the weak binding affinity to CAI, CAIII, and CAVA. The binding of *ortho* **3b–f**, **4b**, and *para* **5a–f**, **6a**, **9a–f**, and **10a** was similar to CAII, CAVI, and CAXIII. The affinity of **5a–f** (*para*-sulfanyl) to CAVB was even 10-fold higher than of *ortho*-substituted **3b–e**, but no significant differences between *para*-substituted sulfanyl (-S-), **5a–f**, **6a**, and sulfonyl (-SO_2-), **9a–f**, **10a**, compounds was observed while comparing the intrinsic constants. Thus, only in some cases the oxidation of the sulfanyl group in the *para*- position had an effect on the affinity for several isozymes, namely, CAVII, CAIX, CAXII, and CAXIV (Figure 1B, lower graph). The binding affinity was quite similar for CAII, CAXII, and other isozymes, containing similar amino acids in the active site.

Comparing the influence of chlorine and bromine, the binding affinities to all CA isozymes were practically identical between compounds **5a** and **6a**, **9a** and **10a** (Table 2). The influence of the R substituent could not be clearly compared, but compounds with more hydrophobic substituents were weaker binders, e.g., **5d** and **5f**. This was possibly due to solubility issues that were visually apparent at the highest concentrations used in the experiment. There was a slight trend that the compounds bearing less flexible substituents, **5a** and **5b** or **9a** and **9b**, often had higher affinity than **5c**, **5d**, **5f**, and **9c**, **9d**, **9f**. The greater effect was seen for CAVB going from phenyl to cyclohexyl (**5a** → **5b** −9.7 kJ/mol and **9a** →**9b** −7.4 kJ/mol) in Figure 3A. The affinity similarly decreased in vertical transition for CAIV (**5a** → **9a** 8.4 kJ/mol, **5b** → **9b** 9.8 kJ/mol, **5c** → **9c** 6.5 kJ/mol), CAIX (**5b** → **9b** 1.8 kJ/mol, **5c** → **9c** 2.5 kJ/mol, **5d** → **9d** 1.2 kJ/mol).

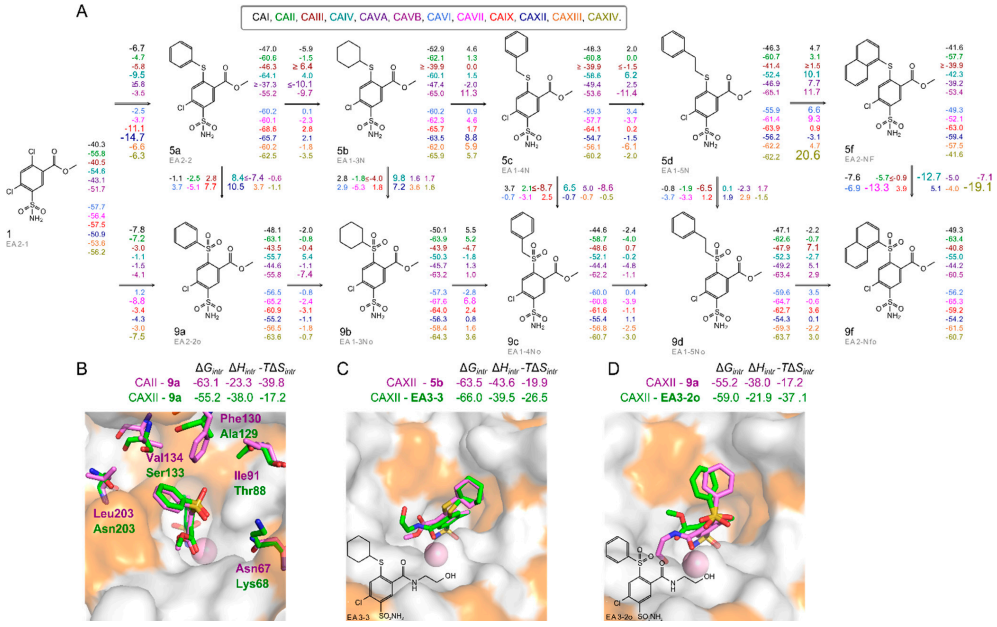


Figure 3. Correlations of compound chemical structures with the changes in the standard intrinsic Gibbs energies of binding and the crystal structures of CAII and CAXII isozymes. (**A**) Differences of ΔG_{intr} (kJ/mol) between neighboring compounds are listed on the connecting arrows. Colors represent CA isozymes. (**B**) Compound **9a** bound to CAII (PDB ID: 7Q0E) and CAXII (PDB ID: 7PUV). Protein side chains and ligands bound to CAII are colored magenta, CAXII green. The notable nonconservative residues between active sites are shown in the "stick" mode. (**C**) Compounds **5b** (magenta, PDB ID: 7PUU) and **EA3-3** (green, previously studied, PDB ID: 6R6Y) in the active site of CAXII. (**D**) Compounds **9a** (green, PDB ID: 7PUV) and **EA3-2o** (magenta, previously described, PDB ID: 6R71) in the active site of CAXII. Zinc ion is shown as a pink sphere. The protein surface of the CA active site is colored orange for hydrophobic residues (V, I, L, F, M, A, G, and P) and gray for the residues with charged and polar side chains (R, D, N, E, Q, H, K, S, T, Y, W, and C).

The X-ray crystal structures showed the energetically best pose of the compound when bound to a CA isozyme. Compound **9a** was found at an identical position in the active sites of CAII and CAXII (Figure 3B). On the other hand, the binding affinity for CAXII differed from CAII by a factor of 26 (K_{d_intr} = 0.020 nM or ΔG_{intr} = −63.1 kJ/mol for CAII vs. K_{d_intr} = 0.51 nM or ΔG_{intr} = −55.2 kJ/mol for CAXII, Table 2 and Figure 3B). The same binding mode of **9a** in CAII and CAXII could be explained by the same interactions between the compound and conservative residues of these active sites. The active site of CAII is more hydrophobic than CAXII (see residues in "stick" mode shown in Figure 3B). For this reason, the binding of this compound to CAII was 26-fold stronger than for CAXII. The interaction of the phenyl group with the hydrophobic side chains was more favorable. The solvation of the ligand during the dissociation process in the presence of a more hydrophobic environment is more difficult. In this case, the stronger interaction was characterized by a lower enthalpy contribution (ΔH_{intr} = −23.3 kJ/mol to CAII, ΔH_{intr} = −38.0 kJ/mol to CAXII, Figure 3B). The structure of **9a** was also determined in the active site of CAI and compared with the positions determined in CAII and CAXII (Figure S4). In this case, the results were somewhat inconclusive because two alternative positions of the compound were found in CAI. One position is partially similar to the positions determined in CAII

and CAXII, and the alternative is rotated (Figure S4). The **9a** is unlikely to occupy a well-defined position and had a weak binding to CAI (ΔG_{intr} = −48.1 kJ/mol). Still, it showed a significant change in the enthalpy of binding (ΔH_{intr} = −39.7 kJ/mol).

We divided the compounds bound to CAXII into pairs according to their similar binding affinity (Figure 3C,D). Compounds **5b** and **EA3-3** in the active site of CAXII were compared in Figure 3C. Compound **EA3-3** was described in our previous study [9] and was named "4b". The **5b** is well defined in the crystal structure (Figure S3D). The binding affinities of the two compounds for CAXII differed by a factor of 2.5, which is insignificant: K_{d_intr} = 0.020 nM, ΔG_{intr} = −63.5 kJ/mol (**5b**) and K_{d_intr} = 0.0080 nM, ΔG_{intr} = −66.0 kJ/mol (**EA3-3**). These compounds differ structurally by *meta*-substituents; **EA3-3** has a longer and more flexible substituent (-$NH(CH_2)_2OH$), but both *meta*- groups were mostly exposed to the solvent, so they did not contribute much to the positioning in the active site. Both compounds are chlorinated at the *ortho*- position of the benzenesulfonamide, and chlorine is known to restrict the position of the ligand in the active site [11,20]. In addition, the *para*-cyclooctyl groups of both compounds occupied the same position. Since the chlorinated compounds had less freedom in the active site, the differences in entropy and enthalpy of the slightly different binding of the *meta*- groups compensated each other: ΔH_{intr} = −43.6 kJ/mol, $-T\Delta S_{intr}$ = −19.9 kJ/mol (**5b**) ΔH_{intr} = −39.5 kJ/mol, $-T\Delta S_{intr}$ = −26.5 kJ/mol (**EA3-3**).

The comparison of **9a** and **EA3-2o** bound to CAXII (described in [9] and named "16a") is shown in Figure 3D. Like the previous pair, these compounds differ only by their *meta*- groups: compound **EA3-2o** contains -$NH(CH_2)_2OH$, while **9a** contains -OCH_3. The positions of both compounds were not similar in CAXII; the *para*-benzene rings had slightly different orientations, and they were directed into the center of the active site cavity. Their orientation was defined by the bulky SO_2 group of the linker, which was in contact with the protein surface. The **EA3-2o** bound to CAXII with 3.4-fold greater affinity than **9a** (K_{d_intr} = 0.12 nM, ΔG_{intr} = −59.0 kJ/mol vs. K_{d_intr} = 0.51 nM ΔG_{intr} = −55.2 kJ/mol). The differences of binding modes between compounds were found in the position of both *meta*- and *para*- groups, but the different terms of the binding reaction mutually compensated each other: ΔH_{intr} = −38.0 kJ/mol, $-T\Delta S_{intr}$ = −17.2 kJ/mol (**9a**) ΔH_{intr} = −21.9 kJ/mol, $-T\Delta S_{intr}$ = −37.1 kJ/mol (**EA3-2o**).

The pairs of compounds bound to the same CA isozyme analyzed in Figures 2D and 3C,D had the following common features: the positions and the binding affinities of the compounds in the pairs were the same or similar. Analogous observations were made previously [21], and such pairs were called "similar" binders. In pairs of "similar" binders, the additional hydrophobic surface did not produce additional interactions with the active site of CA. The possible origin of the entropy–enthalpy compensation was the changes in the solvation–desolvation processes of ligands and the active sites.

Comparison of compound binding to different isozymes when the differences of binding affinities were experimentally significant (Figure 2C,D and Figure 3A) showed some similarities. The compound occupied a similar position in the active site of different isozymes, and the binding of the ligand to a more hydrophobic site was stronger.

2.3.3. Comparison of Methyl Halo 2- and 4-Substituted-5-Sulfamoyl-Benzoates Binding to CA Isozymes

The crystal structures discussed below suggested that the binding interactions between *ortho*- and *para*-sulfanyl/sulfonyl compounds with several CA were quite different. Various *ortho*- and *para*-substituted compounds had different affinities for CA isozymes. Figure 4A compares the affinity of analogous sulfanyl compounds for CA isozymes, and panels B and C compare the positions of these compounds in different crystal structures. The transition from **1** to **3b** and **5b** shows different changes in affinity and selectivity; compound **3b** bound distinctly more strongly to CAIX (−17.5 kJ/mol), CAXII (-11.4 kJ/mol), whereas compound **5b** bound more strongly to CAI (−12.6 kJ/mol), CAVB (−13.3 kJ/mol) and CAXII (−12.6 kJ/mol). The influence of the hydrophobic R substituent in the same group

(transition from left to right (→) has already been discussed above, but in some cases, the transition from *para*- to *ortho*-positions (↑) showed similar trends and was in all cases favorable only for binding to CAIX. However, a change in the position of more flexible substituents (**5c**→**3c**: 2.6 kJ/mol and **5d**→**3d**: 1.6 kJ/mol) had a lower negative effect on the binding affinity to CAI than a rigid cyclohexyl (**5b**→**3b**: 8.9 kJ/mol).

Figure 4B upper part shows 5 superimposed structures: CAXII—**EA3-3** (PDB ID: 6R6Y), CAXII—**EA3-2o** (PDB ID: 6R71), CAXII—**5b** (PDB ID: 7PUU), CAXII—**9a** (PDB ID: 7PUV), CAII—**9a** (PDB ID: 7Q0E); and 4 structures in the lower part: CAXII—**3b** (PDB ID: 7PP9), CAXII—**3d** (PDB ID: 7PUW), CAIX—**3b** (PDB ID: 7POM), mimic CAIX—**3b** (PDB ID: 7Q0C). Therefore, regardless of the compound, all *ortho*-compounds occupied a similar position in CAIX and CAXII, and *para*- occupied another but similar position to each other in CAII and CAXII (Figure 4C,D, respectively). Despite all the differences in the substituents and amino acids in the active sites, the main frame of the compound depicted in the scheme in Figure 4D maintained a similar position. The position of hydrophobic substituents varied, but in the optimal conformation, it was close to the hydrophobic part of the active site. The *meta* substituents were not analyzed in this study, but a comparison with previously published compounds shows that the hydrophilic substituents in these cases did not result in a different binding mode and were flexible. Thus, the hydrophobic effect plays a key role in enabling the compounds to be in the optimal position.

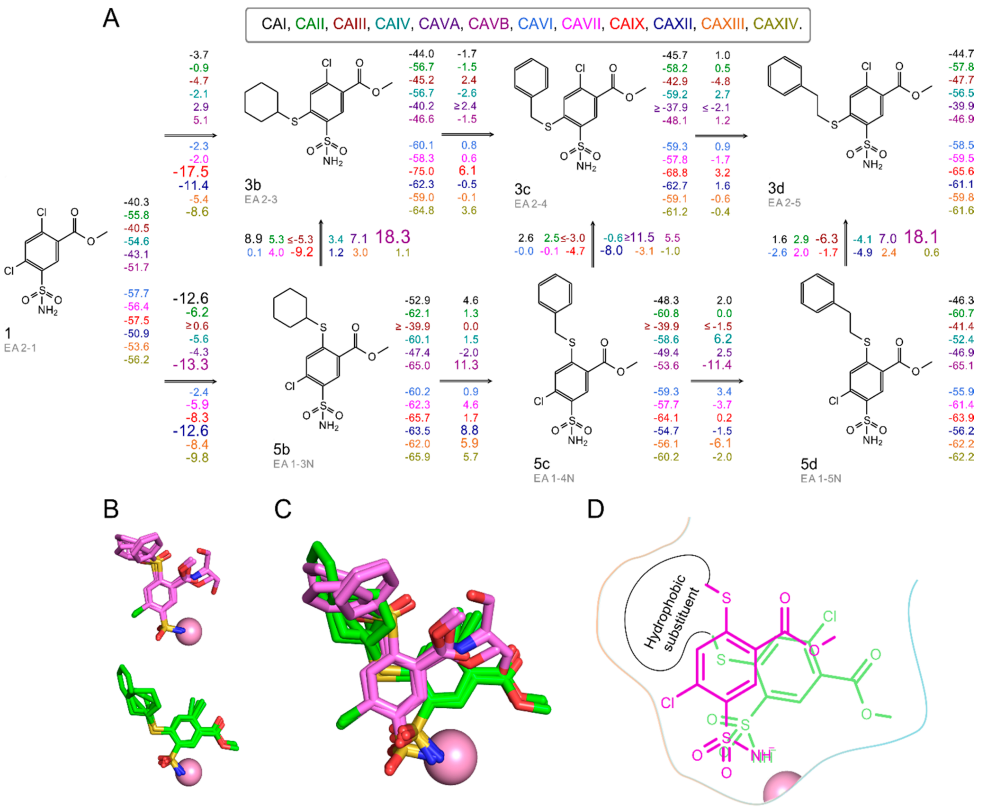


Figure 4. Correlation of compound chemical structures with the changes in standard intrinsic Gibbs energy upon binding and comparison of the crystal structures between CA isozymes. (**A**) Differences of ΔG_{intr} (kJ/mol) between neighboring compounds are listed on the connecting arrows. Colors represent CA isozymes. (**B**) Superimposed ligands of these crystal structures: (upper panel) *para*-substituted ligands are colored magenta: CAXII—**3b** (PDB ID: 7PP9), CAXII—**3d** (PDB ID: 7PUW), CAIX—**3b** (PDB ID: 7POM), mimic-CAIX—**3b** (PDB ID: 7Q0C), (lower panel) *ortho*-substituted ligands are colored green: CAXII—**EA3-3** (PDB ID: 6R6F), CAXII—**EA3-2o** (PDB ID: 6R71), CAXII—**5b** (PDB ID: 7PUU), CAXII—**9a** (PDB ID: 7PUV), CAII—**9a** (PDB ID: 7Q0E). (**C**) The abovementioned superimposed structures are shown for comparison. (**D**) Scheme representing compound position shown in panel C. Orange line represents more hydrophobic side of the active site. Zinc ion is shown as a pink sphere.

3. Conclusions

We have designed a series of compounds and investigated the effect of substituents and their positions in methyl halo 2- and 4-substituted-5-sulfamoyl-benzoate binding to human CA isozymes. The sulfanyl (-S) and sulfonyl (-SO_2-) substituents of different sizes and hydrophobicity were examined in greater detail. We provided a crystallographic position of these two groups of compounds bound in the active sites of CA. Although there were differences in the orientation of the substituents and the thermodynamic parameters, the positions in the same group of compounds remained similar among CA isozymes.

The strongest binding *ortho*-sulfanyl-substituted benzenesulfonamides, especially compounds **3b** and **4b**, showed extremely high femtomolar *intrinsic* affinity (80 fM, cor-

responding to very high, 120 pM *observed* affinity) and selectivity for tumor-associated CAIX. Meanwhile, analogous sulfonyl compounds bound weakly. Other *para*-substituted compounds had completely different binding profiles and bound similarly to several CA isozymes not demonstrating such selectivity.

The crystal structures of the complexes containing compounds bound to CAI, CAII, mimic-CAIX (mutant of CAII$^{A65S, N67Q, I91L, F130V, V134L, L203A}$), CAIX, and CAXII provided a broader understanding of the differences and similarities in the thermodynamic parameters of binding. We observed a tendency that interaction, where there was a higher contribution of the hydrophobic effect and higher entropy contribution, usually also had a higher affinity. This may be explained by the fact that (i) the hydrophobic side chains and bulky hydrophobic substituents of the ligand prevented water molecules from entering the cavity, leading to stronger interaction, and (ii) the water molecules could penetrate between the ligand and the active site cavity through the hydrophilic surface part of the active site. Water molecules in the deeper part of the active site could compete with the compound and cause weaker interaction than in the first case.

4. Materials and Methods

4.1. Organic Synthesis

All starting materials and reagents were commercial products and were used without further purification. Melting points of the compounds were determined in open capillaries on a Thermo Scientific 9100 Series and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a (400 and 100 MHz, respectively) spectrometer in DMSO-d_6 using residual DMSO signals (2.52 ppm and 40.21 ppm for ^{1}H and ^{13}C NMR spectra, respectively) as the internal standard. TLC was performed with silica gel 60 F254 aluminum plates (Merck) and visualized with UV light. Column chromatography was performed using silica gel 60 (0.040–0.063 mm, Merck). High-resolution mass spectra (HRMS) were recorded on a Dual-ESI Q-TOF 6520 mass spectrometer (Agilent Technologies). The purity of final compounds was verified by HPLC to be >95% using the Agilent 1290 Infinity instrument with a Poroshell 120 SB-C18 (2.1 mm × 100 mm, 2.7 µm) reversed-phase column. Analytes were eluted using a linear gradient of water/methanol (20 mM ammonium formate in both phases) from 60:40 to 30:70 over 12 min, then from 30:70 to 20:80 over 1 min, and then 20:80 over 5 min at a flow rate of 0.2 mL/min. UV detection was at 254 nm.

4.2. General Procedure for the Syntheses of **1**, **2**

The appropriate 2,4-dichloro-5-sulfamoylbenzoic acid or 2,4-dibromo-5-sulfamoylbenzoic acid [8] (10.0 mmol) was refluxed in MeOH (100 mL) with concentrated H_2SO_4 (1 mL) for 20 h. The reaction mixture was concentrated under reduced pressure.

*Methyl 2,4-dichloro-5-sulfamoyl-benzoate (***1**, *EA2-1).* Recrystallization was accomplished from MeOH. Yield: 2.70 g, 95%, mp 202 °C. ^{1}H NMR δ ppm: 3.91 (3H, s, CH_3), 7.87 (2H, s, SO_2NH_2), 8.04 (1H, s, C_3-H), 8.40 (1H, s, C_6-H). ^{13}C NMR δ ppm: 53.5, 128.8, 131.7, 134.0, 135.0, 136.8, 140.5, 164.1. HRMS calculated for $C_8H_7Cl_2NO_4S$ [$(M+H)^+$]: 283.9546, found: 283.9546.

*Methyl 2,4-dibromo-5-sulfamoyl-benzoate (***2**, *LJ14-4).* Recrystallization was accomplished from MeOH. Yield: 2.35 g, 63%, mp 201–203 °C. ^{1}H NMR δ ppm: 3.90 (3H, s, CH_3), 7.84 (2H, s, SO_2NH_2), 8.33 (1H, s, C_6-H), 8.35 (1H, s, C_3-H). ^{13}C NMR δ ppm: 53.6, 123.6, 125.2, 131.3, 131.6, 140.2, 142.8, 164.9. HRMS calculated for $C_8H_7Br_2NO_4S$[$(M+H)^+$]: 373.8515 (100%), found: 373.8514 (100%).

4.3. General Procedure for the Syntheses of **3b**, **3e**, *and* **4b**

The mixture of appropriate methyl 2,4-dihalogeno-5-sulfamoylbenzoate (compounds **1**,**2**) (1.00 mmol), DMSO (2 mL), appropriate cyclohexanethiol or cyclododecanethiol (1.10 mmol), and K_2CO_3 (553 mg, 4.00 mmol) was heated at 60 °C temperature for 4–6 h. The mixture was cooled to room temperature, and brine was added. The product was

extracted with EtOAc (3 × 7 mL). The organic layer was washed with H_2O, dried over anhydrous $MgSO_4$, filtered, and concentrated.

*Methyl 2-chloro-4-(cyclohexylsulfanyl)-5-sulfamoylbenzoate (***3b**, *EA2-3).* The product was purified by chromatography on a column of silica gel with $CHCl_3$:EtOAc (10:1), R_f = 0.40. Yield: 131 mg, 36%, mp 112–114 °C. ^{1}H NMR δ ppm: 1.21–1.31 (1H, m, Cy-H), 1.38–1.50 (4H, m, Cy-H), 1.60–1.63 (1H, m, Cy-H), 1.70–1.77 (2H, m, Cy-H), 1.93–2.01 (2H, m, Cy-H), 3.71–3.77 (1H, m, Cy-H), 3.88 (3H, s, CH_3), 7.56 (2H, s, SO_2NH_2), 7,71 (1H, s, C_3-H), 8.33 (1H, s, C_6-H).^{13}C NMR δ ppm: 25.6, 25.7, 32.4, 44.3, 53.2, 125.1, 130.3, 131.5, 136.4, 140.2, 142.9, 164.4. HRMS calculated. for $C_{14}H_{18}ClNO_4S_2$[(M+H)$^+$]: 364.0439, found: 364.0440.

*Methyl 2-chloro-4-(cyclododecylsulfanyl)-5-sulfamoylbenzoate (***3e**, *EA2-12).* The product was purified by chromatography on a column of silica gel with $CHCl_3$:EtOAc (5:1), R_f = 0.65, then recrystallization was accomplished from toluene:EtOAc (8:1). Yield: 210 mg, 47%, mp 185–187 °C. ^{1}H NMR δ ppm: 1.25–1.64 (20H, m, cyclododecyl-H), 1.71–1.83 (2H, m, cyclododecyl-H), 3.71–3.79 (1H, m, cyclododecyl-H), 3.88 (3H, s, CH_3), 7.56 (2H, s, SO_2NH_2), 7.67 (1H, s, C_3-H), 8.33 (1H, s, C_6-H). ^{13}C NMR δ ppm: 22.1, 23.3, 23.4, 23.8, 24.1, 29.2, 43.2, 53.2, 124.9, 130.3, 131.4, 136.3, 140.3, 143.5, 164.4. HRMS calculated for $C_{20}H_{30}ClNO_4S_2$[(M+H)$^+$]: 448.1378, found: 448.1370.

*Methyl 2-bromo-4-(cyclohexylsulfanyl)-5-sulfamoylbenzoate (***4b**, *LJ15-12).* The product was purified by chromatography on a column of silica gel with $CHCl_3$:EtOAc (15:1), R_f = 0.29. Yield: 180 mg, 44%, mp123–125 °C. ^{1}H NMR δ ppm: 1.24–1.31 (1H, m, Cy-H), 1.38–1.50 (4H, m, Cy-H), 1.60–1.63 (1H, m, Cy-H), 1.70–1.75 (2H, m, Cy-H), 1.95–1.97 (2H, m, Cy-H), 3.72–3.77 (1H, m, Cy-H), 3.88 (3H, s, CH_3), 7.54 (2H, s, SO_2NH_2), 7.85 (1H, s, C_6-H), 8.27 (1H, s, C_3-H). ^{13}C NMR δ ppm: 25.6, 25.7, 32.4, 44.5, 53.2, 125.1, 127.5, 131.0, 133.6, 140.1, 142.5, 165.0. HRMS calculated for $C_{14}H_{18}BrNO_4S_2$[(M+H)$^+$]: 409.9913 (100%), found: 409.9915 (100%).

4.4. General Procedure for the Syntheses of **3c** *and* **3d**

The mixture of methyl 2,4-dichloro-5-sulfamoylbenzoate (**1**) (284 mg, 1.00 mmol), DMSO (2 mL), appropriate phenylmethanethiol or 2-phenylethanethiol (1.10 mmol), and TEA (121 mg, 1.20 mmol) was heated at 50 °C temperature for 6–12 h. The progress of the reaction was monitored by TLC. The mixture was cooled to room temperature, and brine was added. The product was extracted with EtOAc (3 × 7 mL). The organic layer was washed with H_2O, dried over anhydrous $MgSO_4$, filtered, and concentrated.

*Methyl 4-(benzylsulfanyl)-2-chloro-5-sulfamoylbenzoate (***3c**, *EA2-4).* The product was purified by chromatography on a column of silica gel with $CHCl_3$:EtOAc (4:1), R_f = 0.48. Yield: 119 mg, 32%, mp 125–126 °C. ^{1}H NMR δ ppm: 3.87 (3H, s, CH_3), 4.50 (2H, s, CH_2), 7.29 (1H, t, *J* = 7.2 Hz, Ph-H), 7.36 (2H, t, *J* = 7.2 Hz, Ph-H), 7.51 (2H, d, *J* = 7.2 Hz, Ph-H), 7.64 (2H, s, SO_2NH_2), 7.69 (1H, s, C_3-H), 8.31 (1H, s, C_6-H). ^{13}C NMR δ ppm: 36.3, 53.2, 124.9, 128.0, 129.1, 129.2, 129.8, 131.3, 135.8, 136.4, 139.1, 143.7, 164.3. HRMS calculated for $C_{15}H_{14}ClNO_4S_2$ [(M+H)$^+$]: 372.0126, found: 372.0125.

*Methyl 2-chloro-4-[(2-phenylethyl)sulfanyl]-5-sulfamoylbenzoate (***3d**, *EA2-5).* The product was purified by chromatography on a column of silica gel with $CHCl_3$:EtOAc (5:1), R_f = 0.67. Yield: 97 mg, 25%, mp 111–112 °C. ^{1}H NMR δ ppm: 2.97 (2H, t, *J* = 7.6 Hz, $\underline{CH_2}$Ph), 3.46 (2H, t, *J* = 7.6 Hz, CH_2S), 3.88 (3H, s, CH_3), 7.23 (1H, m, Ph-H), 7.29–7.33 (4H, m, Ph-H), 7.59 (2H, s, SO_2NH_2), 7.65 (1H, s, C_3-H), 8.31 (1H, s, C_6-H). ^{13}C NMR δ ppm: 33.3, 34.1, 53.2, 124.8, 126.9, 128.9, 129.1, 129.2, 131.2, 136.5, 139.4, 140.0, 143.9, 164.4. HRMS calculated for $C_{16}H_{16}ClNO_4S_2$ [(M+H)$^+$]: 386.0282, found: 386.0282.

4.5. General Procedure for the Syntheses of **5a**, **5f**, *and* **6a**

The mixture of appropriate 2,4-dihalogeno-5-sulfamoylbenzoate (compounds **1**,**2**) (1.00 mmol), MeOH (5 mL), thiophenol or 1-thionaphthol (1.10 mmol,) and TEA (121 mg, 1.20 mmol) was refluxed for 2–6 h. MeOH was evaporated under reduced pressure, and the resultant precipitate was washed with H_2O.

*Methyl 4-chloro-2-(phenylsulfanyl)-5-sulfamoylbenzoate (***5a**, *EA2-2).* The product was purified by chromatography on a column of silica gel with $CHCl_3$:EtOAc (10:1), R_f = 0.32. Yield: 161 mg, 45%, mp 223–226 °C. ^{1}H NMR δ ppm: 3.94 (3H, s, CH_3), 6.68 (1H, s, C_3-H), 7.62–7.67 (5H, m, Ph-H), 7.74 (2H, s, SO_2NH_2), 8.50 (1H, s, C_6-H). ^{13}C NMR δ ppm: 53.3, 124.3, 128.7, 130.1, 131.2 (2C), 131.8, 135.2, 136.2, 137.5, 149.6, 164.8. HRMS calculated for $C_{14}H_{12}ClNO_4S_2$ [(M+H)$^+$]: 357.9969, found: 357.9970.

*Methyl 4-chloro-2-(1-naphthylsulfanyl)-5-sulfamoyl-benzoate (***5f**, *EA2-NF).* The product was purified by chromatography on a column of silica gel with $CHCl_3$:EtOAc (5:1), R_f = 0.77. Yield: 265 mg, 65%, mp 235–237 °C. ^{1}H NMR δ ppm: 3.99 (3H, s, CH_3), 6.34 (1H, s, C_3-H), 7.61–7.68 (2H, m, Naph-H), 7.70 (2H, s, SO_2NH_2), 7.71–7.74 (1H, m, Naph-H), 8.04 (1H, dd, *J* =7.2 Hz, *J* =1.2 Hz, Naph-H), 8.08–8.15 (2H, m, Naph-H), 8.26 (1H, d, *J* = 8.4 Hz, Naph-H), 8.54 (1H, s, C_6-H). ^{13}C NMR δ ppm: 53.4, 124.3, 125.0, 126.7, 127.2, 127.6, 128.5, 128.8, 129.8, 132.0, 132.6, 133.8, 134.6, 135.1, 136.9, 137.5, 148.9, 164.9. HRMS calculated for $C_{18}H_{14}ClNO_4S_2$ [(M+H)$^+$]: 408.0126, found: 408.0131.

*Methyl 4-bromo-2-(phenylsulfanyl)-5-sulfamoylbenzoate (***6a**, *LJ15-11).* The product was purified by chromatography on a column of silica gel with $CHCl_3$:EtOAc (15:1), R_f = 0.20, then recrystallization was accomplished from toluene. Yield: 145 mg, 36%, mp 202–204 °C. ^{1}H NMR δ ppm: 3.93 (3H, s, CH_3), 6.89 (1H, s, C_3-H), 7.58–7,63 (5H, m, Ph-H), 7.71 (2H, s, SO_2NH_2), 8.52 (1H, s, C_6-H). ^{13}C NMR δ ppm: 53.3, 124.3, 124.8, 130.2, 131.1(2C), 131.7, 132.3, 136.1, 139.3, 149.2, 165.0. HRMS calculated for $C_{14}H_{12}BrNO_4S_2$[(M+H)$^+$]: 403.9443 (100%), found: 403.9437 (100%).

4.6. General Procedure for the Syntheses of **7b**–**d**, **8b**, **9a**–**d**, **9f**, **10a**

The 30% H_2O_2(aq) (1.50 mmol, 0.148 mL) was added to a solution of appropriate benzenesulfonamide (compounds **3b**–**d**,**4b**,**5a**–**d**,**f**,**6a**) (0.500 mmol) in AcOH (1.7 mL) at 70 °C and allowed stirring for 2–3h. The solvent was removed under reduced pressure, and the resultant precipitate was washed with H_2O.

*Methyl 2-chloro-4-cyclohexylsulfonyl-5-sulfamoylbenzoate (***7b**, *EA2-3o).* Yield: 164 mg, 83%, mp 184–187 °C. ^{1}H NMR δ ppm: 1.14–1.21 (3H, m, Cy-H), 1.42–1.50 (2H, m, Cy-H), 1.63 (1H, br s, Cy-H), 1.81–1.87 (4H, m, Cy-H), 3.89–3.92 (1H, m, Cy-H), 3.96 (3H, s, CH_3), 7.48 (2H, s, SO_2NH_2), 8.11 (1H, s, C_3-H), 8.57 (1H, s, C_6-H). ^{13}C NMR δ ppm: 24.9 (2C), 25.1, 53.9, 62.2, 133.3, 135.1, 135.5, 136.7, 138.6, 142.1, 163.9. HRMS calculated for $C_{14}H_{18}ClNO_6S_2$ [(M+H)$^+$]: 396.0337, found: 396.0336.

*Methyl 4-benzylsulfonyl-2-chloro-5-sulfamoylbenzoate (***7c**, *EA2-4o).* Recrystallization was accomplished from MeOH. Yield: 123 mg, 61%, mp 154–156 °C. ^{1}H NMR δ ppm: 3.95 (3H, s, CH_3), 5.04 (2H, s, CH_2), 7.24–7.26 (2H, m, Ph-H), 7.33–7.40 (3H, m, Ph-H), 7.58 (2H, s, SO_2NH_2), 7.77 (1H, s, C_3-H), 8.57 (1H, s, C_6-H).^{13}C NMR δ ppm: 53.9, 60.9, 127.5, 129.1, 129.5, 131.7, 132.9, 135.0, 135.4, 136.3, 139.2, 142.0, 163.8. HRMS calculated for $C_{15}H_{14}ClNO_6S_2$ [(M+H)$^+$]: 404.0024, found: 404.0023.

*Methyl 2-chloro-4-(2-phenylethylsulfonyl)-5-sulfamoylbenzoate (***7d**, *EA2-5o).* Recrystallization was accomplished from MeOH. Yield: 161 mg, 77%, mp 132–134 °C. ^{1}H NMR δ ppm: 3.03 (2H, t, *J* = 7.8 Hz, CH_2), 3.95 (3H, s, CH_3), 4.08 (2H, t, *J* = 7.8 Hz, CH_2), 7.13–7.27 (5H, m, Ph-H), 7.50 (2H, s, SO_2NH_2), 8.07 (1H, s, C_3-H), 8.49 (1H, s, C_6-H). ^{13}C NMR δ ppm: 28.0, 53.9, 55.8, 127.1, 128.9(2C), 132.8, 135.0, 135.3, 136.7, 137.7, 140.0, 141.6, 163.9. HRMS calculated for $C_{16}H_{16}ClNO_6S_2$ [(M+H)$^+$]: 418.0180, found: 418.0175.

*Methyl 2-bromo-4-cyclohexylsulfonyl-5-sulfamoylbenzoate (***8b**, *LJ15-12o).* Yield: 172 mg, 78%, mp 217–219 °C. ^{1}H NMR δ ppm: 1.16–1.24 (3H, m, Cy-H), 1.42–1.50 (2H, m, Cy-H), 1.57–1.63 (1H, m, Cy-H), 1.79–1.86 (4H, m, Cy-H), 3.89–3.92 (1H, m, Cy-H), 3.95 (3H, s, CH_3), 7.47 (2H, s, SO_2NH_2), 8.25 (1H, s, C_3-H), 8.50 (1H, s, C_6-H). ^{13}C NMR δ ppm: 24.9(2C), 25.1, 53.9, 62.2, 125.3, 132.8, 137.7, 138.1, 138.4, 142.6, 164.6. HRMS calculated for $C_{14}H_{18}BrNO_6S_2$[(M+H)$^+$]: 441.9811 (100%), found: 441.9806 (100%).

*Methyl 2-(benzenesulfonyl)-4-chloro-5-sulfamoylbenzoate (***9a**, *EA2-2No).* The product was purified by chromatography on a column of silica gel with $CHCl_3$:EtOAc (4:1), R_f = 0.33. Yield: 158 mg, 81%, mp 166–168 °C. ^{1}H NMR δ ppm: 3.86 (3H, s, CH_3), 7.68 (2H, t, *J* = 7.6 Hz,

Ph-H), 7.78 (1H, t, *J* = 7.6 Hz, Ph-H), 8.03 (2H, s, SO_2NH_2), 8.07 (2H, d, *J* = 7.6 Hz, Ph-H), 8.23 (1H, s, C_3-H), 8.51 (1H, s, C_6-H). ^{13}C NMR δ ppm: 53.8, 128.5, 130.1, 130.3, 131.7, 133.5, 134.2, 134.9, 140.0, 142.3, 146.0, 165.7. HRMS calculated for $C_{14}H_{12}ClNO_6S_2$ [$(M+H)^+$]: 389.9867, found: 389.9869.

*Methyl 4-chloro-2-cyclohexylsulfonyl-5-sulfamoyl-benzoate (***9b**, *EA1-3No).* Yield: 150 mg, 76%, mp 181–183 °C. ^{1}H NMR δ ppm: 1.18–1.30 (3H, m, Cy-H), 1.41–1.50 (2H, m, Cy-H), 1.62–1.66 (1H, m, Cy-H), 1.80–1.91 (4H, m, Cy-H), 3.64–3.70 (1H, m, Cy-H), 3.90 (3H, s, CH_3), 8.06 (2H, s, SO_2NH_2), 8.12 (1H, s, C_3-H), 8.30 (1H, s, C_6-H). ^{13}C NMR δ ppm: 24.8, 25.0, 25.1, 54.0, 62.9, 130.5, 132.6, 133.8, 134.1, 140.1, 145.9, 165.9. HRMS calculated for $C_{14}H_{18}ClNO_6S_2$ [$(M+H)^+$]: 396.0337, found: 396.0341.

*Methyl 2-benzylsulfonyl-4-chloro-5-sulfamoylbenzoate (***9c**, *EA1-4No).* Yield: 162 mg, 80%, mp 224–225 °C. ^{1}H NMR δ ppm: 3.97 (3H, s, CH_3), 4.97 (2H, s, CH_2), 7.27–7.30 (2H, m, Ph-H), 7.37–7.42 (3H, m, Ph-H), 7.79 (1H, s, C_3-H), 8.07 (2H, s, SO_2NH_2), 8.31 (1H, s, C_6-H). ^{13}C NMR δ ppm: 54.2, 61.7, 127.7, 129.1, 129.4, 130.4, 131.7, 132.1, 133.6, 133.9, 140.9, 145.9, 166.0. HRMS calculated for $C_{15}H_{14}ClNO_6S_2$ [$(M+H)^+$]: 404.0024, found: 404.0018.

*Methyl 4-chloro-2-(2-phenylethylsulfonyl)-5-sulfamoylbenzoate (***9d**, *EA1-5o).* Recrystallization was accomplished from MeOH. Yield: 142 mg, 68%, mp 156–158 °C. ^{1}H NMR δ ppm: 3.06 (2H, t, *J* = 7.6 Hz, CH_2), 3.91 (3H, s, CH_3), 4.00 (2H, t, *J* = 8.0 Hz, CH_2), 7.16–7.20 (1H, m, Ph-H) 7.21–7.27 (4H, m, Ph-H), 8.03 (1H, s, C_3-H), 8.04 (2H, s, SO_2NH_2), 8.26 (1H, s, C_6-H). ^{13}C NMR δ ppm: 28.2, 54.1, 56.5, 127.2, 128.89, 128.92, 130.3, 131.8, 133.8, 133.9, 137.8, 141.5, 145.8, 165.9. HRMS calculated for $C_{16}H_{16}ClNO_6S_2$ [$(M+H)^+$]: 418.0180, found: 418.0178.

*Methyl 4-chloro-2-(1-naphthylsulfonyl)-5-sulfamoylbenzoate (***9f**, *EA2-NFo).* Yield: 180 mg, 82%, mp 244–245 °C. ^{1}H NMR δ ppm: 3.72 (3H, s, CH_3), 7.66–7.75 (2H, m, Naph-H), 7.81 (1H, t, *J* = 7.6 Hz, Naph-H), 8.00 (2H, s, SO_2NH_2), 8.17 (1H, d, *J* = 8.0 Hz, Naph-H), 8.23 (1H, s, C_3-H), 8.35 (1H, s, C_6-H), 8.36 (2H, t, *J* = 8.0 Hz, Naph-H), 8.41 (1H, d, *J* = 8.4 Hz, Naph-H).^{13}C NMR δ ppm: 53.8, 123.5, 125.2, 127.7, 127.8, 129.5, 130.1, 130.6, 131.5, 131.6, 132.4, 133.9, 134.0, 134.2, 136.4, 142.4, 145.8, 165.4. HRMS calculated for $C_{18}H_{14}ClNO_6S_2$ [$(M+H)^+$]: 440.0024,found: 440.0020.

*Methyl 2-(benzenesulfonyl)-4-bromo-5-sulfamoylbenzoate (***10a**, *LJ15-11o).* Yield: 189 mg, 87%, mp 191–193 °C. ^{1}H NMR δ ppm: 3.86 (3H, s, CH_3), 7.67-7.79 (3H, m, Ph-H), 7.98 (2H, s, SO_2NH_2), 8.04–8.07 (2H, m, Ph-H), 8.22 (1H, s, C_6-H), 8.62 (1H, s, C_3-H). ^{13}C NMR δ ppm: 53.8, 122.7, 128.5, 130.1, 130.2, 132.2, 134.9, 136.7, 140.0, 141.9, 147.8, 165.8. HRMS calculated for $C_{14}H_{12}BrNO_6S_2$[$(M+H)^+$]: 435.9342 (100%), found: 435.9345 (100%).

4.7. General Procedure for the Syntheses of **12a**–**d**,**f**

The mixture of methyl 2,4-dichloro-5-sulfamoylbenzamide (**11**) [8] (269 mg, 1.00 mmol), DMSO (1.5 mL), appropriate thiol(1.10 mmol), and TEA (121 mg, 1.20 mmol) was heated at 60 °C temperature for 12 h. The mixture was cooled to room temperature, and brine (15 mL) was added. The product was extracted with EtOAc (3 × 15 mL). The organic layer was washed with H_2O, dried over anhydrous $MgSO_4$, filtered, and concentrated.

*4-chloro-2-(1-naphthylsulfanyl)-5-sulfamoylbenzamide (***12f**, *EA1A-NF).* The product was crystallized from toluene:MeOH (7:1).Yield: 277 mg, 70%, mp 277–280 °C.^{1}H NMR δ ppm: 6.38 (1H, s, C_3-H), 7.59 (2H, s, SO_2NH_2), 7.61–7.66 (2H, m, Naph-H), 7.67–7.71(1H, m, Naph-H), 7.85 (1H, s, $CONH_2$), 8.00 (1H, dd, *J* = 7.2 Hz, *J* =1.2 Hz, Naph-H), 8.10 (1H, s, C_6-H), 8.10–8.14 (2H, m, Naph-H), 8.21 (1H, d, *J* = 8.4 Hz, Naph-H), 8.34 (1H, s, $CONH_2$). ^{13}C NMR δ ppm: 125.2, 127.0, 127.5, 127.7, 128.5 128.6, 128.9, 129.7, 131.1, 132.1, 132.3, 133.7, 134.6, 135.0, 136.6, 137.5, 168.0. HRMS calculated for $C_{17}H_{13}ClN_2O_3S_2$ [$(M+H)^+$]: 393.0129, found: 393.0133.

*4-chloro-2-phenylsulfanyl-5-sulfamoylbenzamide (***12a**, *EA1A-2)* [9].

*4-chloro-2-cyclohexylsulfanyl-5-sulfamoylbenzamide (***12b**, *EA1A-3)* [9].

*2-benzylsulfanyl-4-chloro-5-sulfamoylbenzamide (***12c**, *EA1A-4)* [9].

*4-chloro-2-(2-phenylethylsulfanyl)-5-sulfamoylbenzamide (***12d**, *EA1A-5)* [9].

4.8. General Procedure for the Syntheses of **5a**–**d**,**f**

The mixture of appropriate 2-substituted 4-chloro-5-sulfamoylbenzamide (compounds **12a–d,f**) (0.500 mmol), methanol (5 mL), and thionyl chloride (0.200 mL, 2.76 mmol) was heated at 50 °C temperature for 48–96 h. The progress of the reaction was monitored by TLC. The mixture was concentrated under reduced pressure.

Methyl 4-chloro-2-phenylsulfanyl-5-sulfamoylbenzoate (**5a**, *EA2-2).* The product was recrystallized from H_2O. Yield: 122 mg, 68%, mp 223-226 °C.

Methyl 4-chloro-2-cyclohexylsulfanyl-5-sulfamoylbenzoate (**5b**, *EA1-3N).* The product was recrystallized from H_2O. Yield: 120 mg, 66%, mp 150–151 °C. ^{1}H NMR δ ppm: 1.20–1.51 (5H, m, Cy-H), 1.58–1.66 (1H, m, Cy-H), 1.69–1.77 (2H, m, Cy-H), 1.93–2.02 (2H, m, Cy-H), 3.60–3.69 (1H, m, Cy-H), 3.86 (3H, s, CH_3), 7,65 (1H, s, C_3-H), 7.71 (2H, s, SO_2NH_2), 8.39 (1H, s, C_6-H). ^{13}C NMR δ ppm: 25.6, 25.7, 32.5, 42.9, 53.1, 126.3, 129.0, 131.6, 135.0, 136.9, 146.9, 165.0. HRMS calculated for $C_{14}H_{18}ClNO_4S_2$[(M+H)$^+$]: 364.0439, found: 364.0444.

Methyl 2-benzylsulfanyl-4-chloro-5-sulfamoylbenzoate (**5c**, *EA1-4N).* The product was recrystallized from H_2O. Yield: 145 mg, 78%, mp 211–212 °C. ^{1}H NMR δ ppm: 3.86 (3H, s, CH_3), 4.42 (2H, s, CH_2), 7.30 (1H, t, *J* = 7.2 Hz, Ph-H), 7.37 (2H, t, *J* = 7.2 Hz, Ph-H), 7.47 (2H, d, *J* = 6.8 Hz, Ph-H) 7.70 (1H, s, C_3-H), 7.73 (2H, s, SO_2NH_2), 8.44 (1H, s, C_6-H). ^{13}C NMR δ ppm: 36.0, 53.1, 124.7, 128.0, 128.5, 129.1, 129.7, 131.6, 135.2, 136.0, 136.8, 148.4, 164.9. HRMS calculated for $C_{15}H_{14}ClNO_4S_2$ [(M+H)$^+$]: 372.0126, found: 372.0129.

Methyl 4-chloro-2-(2-phenylethylsulfanyl)-5-sulfamoylbenzoate (**5d**, *EA1-5N).* The product was recrystallized from H_2O:acetone (3:1). Yield: 166 mg, 85%, mp 135–137 °C. ^{1}H NMR δ ppm: 2.96 (2H, t, *J* = 7.6 Hz, $\underline{CH_2}$Ph), 3.39 (2H, t, *J* = 7.6 Hz, CH_2S), 3.87 (3H, s, CH_3), 7.24 (1H, m, Ph-H), 7.29–7.33 (4H, m, Ph-H), 7.63 (1H, s, C_3-H), 7.73 (2H, s, SO_2NH_2), 8.44 (1H, s, C_6-H). ^{13}C NMR δ ppm: 33.7, 33.8, 53.1, 125.1, 126.9, 128.3, 128.9, 129.0, 131.6, 135.3, 136.7, 140.1, 148.5, 164.9. HRMS calculated for $C_{16}H_{16}ClNO_4S_2$ [(M+H)$^+$]: 386.0282, found: 386.0286.

Methyl 4-chloro-2-(1-naphthylsulfanyl)-5-sulfamoylbenzoate (**5f**, *EA2-NF).* The product was recrystallized from H_2O:MeOH (4:1). Yield: 143 mg, 70%, mp 235–237 °C.

4.9. Protein Preparation

Recombinant human carbonic anhydrases (CAs) were expressed in *E. coli* (CAI, CAII, mutant CAII$^{A65S, N67Q, I91L, F130V, V134L, L203A}$, CAIII, CAIV, CAVA, CAVB, CAVII, CAXII, CAXIII, CAXIV), yeast (CAIX used to obtain only PDB ID: 7POM crystal structure) or mammalian cells (CAVI and CAIX used for all remaining experiments). Proteins were chromatographically purified following published protocols: CAI [22], CAII [23], CAII$^{A65S, N67Q, I91L, F130V, V134L, L203A}$ (mimic-CAIX), CAIII, CAIV, CAVA, CAVB, CAVI, CAIX (except CAIX (PDB ID: 7POM) crystal was obtained using yeast-expressed protein [24], and [17], CAVII, CAXIII- [25], CAXII [26]. The protein stock solutions were stored at −80 °C. The protein concentration was determined by UV absorption at 280 nm using Beer-Lambert law and extinction coefficient determined by amino acid analysis. Proteins used for crystallization were additionally purified by affinity chromatography and concentrated.

4.10. Determination of Binding Parameters

4.10.1. Fluorescent Thermal Shift Assay (FTSA)

Observed affinities (dissociation constant, $K_{d,obs}$, proportional to the change in standard Gibbs energy upon binding, $\Delta G = RT\ln K_d$) of the compounds binding to CAs were determined by the fluorescent thermal shift assay [27,28], using a QIAGEN's real-time PCR cycler the "Rotor-Gene Q" and Rotor-Gene® Style 4-strip tubes from STARLAB. Ligands were dissolved in DMSO stock solutions to concentrations of 10 mM or 20 mM and used for the serial dilution in DMSO of the dilution factor 2. These samples were diluted with buffer solution and mixed with a prepared protein solution, consisting of protein stock, buffer solution, and 8-anilino-1-naphthalene sulfonate (ANS; solvatochromic dye). All final samples typically contained up to 10 μM CA, compound solutions of serial dilution from 0 μM to 200 μM at 8 different concentrations differing by two times, 50 μM ANS,

50 mM sodium phosphate buffer at pH 7.0, 100 mM sodium chloride, and 2.0% (*v/v*) DMSO. Samples preparation is explained in detail in [28]. Protein denaturation was monitored by determining the fluorescence of ANS as a function of temperature[29]. The excitation and emission wavelengths of ANS are 365 ± 20 and 460 ± 15 nm. Samples were heated from 25 °C to 99 °C at the rate of 1 °C/min. The curve-fitting procedure has been explained previously [30] and was performed at 37 °C.

The dissociation constants of each compound with all 12 catalytically active human CAs are listed in Table 2, and some experimental data are provided in Supplementary material in Figures S1 and S2.

4.10.2. Isothermal Titration Calorimetry (ITC)

Additionally, a selected set of compounds binding to CAs were tested by ITC [31,32] to determine changes in binding enthalpies. ITC experiments were carried out using a MicroCal PEAQ-ITC calorimeter (Northampton, MA, USA). The protein solution in the cell contained a constant concentration of CA (10–20 μM), while the concentration of compound loaded in the ITC syringe was ten times higher than the protein concentration (100–200 μM). Both cell and syringe solutions were diluted in the buffer: 50 mM sodium phosphate at pH 7.0 and containing 100 mM sodium chloride and 2.0 % (*v/v*) of DMSO. Samples were centrifuged prior to the experiment to improve quality. A typical experiment consisted of 19 injections with 150 s spacing between injections; the volume of the first injection was 0.4 μL (duration 0.8s) and 2.0 μL (duration 4.0 s) for the remaining injections. All experiments were performed at 37 °C with reference power 4.0 μcal/s.

The enthalpy changes of binding are listed in Table S8, and experimental data are provided in Supplementary material in Figure S6. A comparison of the affinity determined by FTSA and ITC is shown in Supplementary material in Figure S9. ITC shows weaker affinity than FTSA for strong interactions. Thus, the affinity determined by the FTSA was used due to the ITC detection limit as the more reliable.

4.10.3. Calculation of Intrinsic Binding Parameters

All experiments allow the determination of the *observed* binding parameters of the sulfonamide binding to CA. However, the change in standard Gibbs energy (or dissociation constant), enthalpy, and entropy upon binding depend on buffer and pH because sulfonamide binding to CAs is linked to several reactions: (i) Zn^{2+}-bound hydroxide ion protonate into water molecule in the active site of CA; (ii) Sulfonamide amino group ($R\text{-}SO_2NH_2$) deprotonate ($R\text{-}SO_2NH^-$); (iii) Buffer molecule protonate or deprotonate depending on the protons' balance; (iv) After these reactions, the negatively charged sulfonamide ($R\text{-}SO_2NH^-$) replaces the Zn^{2+}-bound water molecule in the active site of CA and binds to the protein in its position [15]. The sum of these reactions is the *observed* binding. However, only the fourth reaction represents the actual binding reaction, called *intrinsic* [16]. Observed binding parameters are important to obtain the compound's affinity under certain conditions, for example, in the human body. Nevertheless, these parameters are less important in the drug design to estimate the energy for the binding affinity of each substituent. However, the contribution of protonation reactions can be subtracted. Equations of intrinsic dissociation constant (K_{d_intr}) or standard Gibbs energy (ΔG_{intr}) and enthalpy change (ΔH_{intr}) are listed in Table 2 and Table S7 in Supplementary materials.

4.11. Determination of Protonation Parameters

4.11.1. Determination of pK_a Values

The pK_a values of water molecules bound to Zn^{2+} in the active site of CAs, p$K_{a_CAZn(II)H2O}$, were taken from [33] and of compounds, p$K_{a_RSO2NH2}$, were determined as described in [34]. Figure 2D shows the intrinsic parameters of the binding of mimic-CAIX (mutant $CAII^{A65S, N67Q, I91L, F130V, V134L, L203A}$) and compound **3b**, which were calculated using the p$K_{a_CAZn(II)H2O}$ of CAII.

We used a constant concentration of sulfonamide (25–400 μM) and 2.0% (*v*/*v*) of DMSO in universal buffer (50 mM sodium acetate, 25 mM sodium borate, and 50 mM sodium phosphate) at different pH values (in the range from pH 6 to 12 at every half pH unit). UV–VIS spectra of compound solution were recorded at 37 °C using the spectrophotometer "Agilent 89090A". To determine $pK_{a\_RSO2NH2}$, a plot of the normalized ratio of two absorbancies (approximately 10 nm above and 10 nm below the isosbestic point) vs. buffer pH and fitted Henderson–Hasselbach curve using the least-square method. The midpoint of this fitted curve is equal to $pK_{a\_RSO2NH2}$. Experimental data are provided in Supplementary material in Figure S5 and Table 2.

4.11.2. Determination of Protonation Enthalpy

The enthalpy changes of sulfonamide amino group (RSO_2NH_2) protonation, $\Delta_{p\_RSO2NH2}H$, were determined by titration of 0.25 mM sulfonamide and 0.375 mM sodium hydroxide with 2.75 mM nitric acid using a MicroCal PEAQ-ITC calorimeter (Northampton, MA, USA). DMSO concentrations in the syringe and the sample cell were 2% (*v*/*v*). Experimental parameters: total number of injections was 40, the volume of each injection was 0.9 μL, spacing between injections was 120 s, the temperature was 37 °C. $\Delta_{p\_CAZn(II)H2O}H$ values of the water molecule in the active site CAs were taken from [33]. Figure 2D shows the change in binding enthalpy of mimic-CAIX (mutant $CAII^{A65S, N67Q, I91L, F130V, V134L, L203A}$) and compound **3b**, which was calculated using the $\Delta_{p\_CAZn(II)H2O}H$ of CAII. Experimental data are provided in Supplementary material in Figure S7 and Table S5.

4.12. X-ray Crystallography: Crystallization, Data Collection, and Structure Determination

The proteins were ultrafiltered to the indicated concentration in Table S4. The same table lists the crystallization conditions and solutions. Crystals of CAIX (PDB ID: 7POM) and CAXII (PDB ID: 7PP9) were achieved by co-crystallization and others by soaking. The crystal soaking ligand solutions were produced by combining 50 μL of matching reservoir solution with 1 μL of 50 mM ligand solution in DMSO. The data were processed and scaled using XDS [35], MOSFLM [36], and SCALA [37]. Except CAXII dataset was processed and scaled using SAINT [38] and SADABS [39]. MOLREP [40] was used for molecular replacement with an initial model of 1CAB for CAI, 4HT0 for CAII, 3HLJ for mimic-CAIX, 6FE2 for CAIX, and 6QNL for CAXII. The model was refined with REFMAC [41] and fitted in the electron density map using COOT [42]. The 3D models of compounds were constructed by AVOGADRO [43] program, and ligand parameter files were created using LIBCHECK [44,45]. Coordinates and structure factors have been deposited to PDB. The PDB IDs, data collection, and refinement statistics are shown in Supplementary material in Table S3. The PyMOL program was used to create graphics.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms23010130/s1.

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Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare that they may have authored patent applications on carbonic anhydrase inhibitors.

Abbreviations

CA	carbonic anhydrase
FTSA	fluorescent thermal shift assay (or differential scanning fluorimetry, DSF)
intr	intrinsic
ITC	isothermal titration calorimetry
obs	observed
VARIABLES	
ΔG_{intr}	change of the intrinsic standard Gibbs energy upon binding
ΔG_{obs}	change of the observed standard Gibbs energy upon binding
ΔH_{intr}	change of the intrinsic standard enthalpy upon binding
ΔH_{obs}	change of the observed standard enthalpy upon binding
K_{d_intr}	the intrinsic equilibrium dissociation constant
K_{d_obs}	the observed equilibrium dissociation constant
T_m	melting temperature (midpoint of the unfolding transition)
$T\Delta S_{intr}$	change of the intrinsic standard entropy upon binding multiplied by absolute temperature

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Article

Affinity and Selectivity of Protein−Ligand Recognition: A Minor Chemical Modification Changes Carbonic Anhydrase Binding Profile

Audrius Zakšauskas, Vaida Paketurytė-Latvė, Alberta Jankūnaitė, Edita Čapkauskaitė, Yann Becart, Alexey Smirnov, Klára Pospíšilová, Janis Leitans, Jiří Brynda, Andris Kazaks, Lina Baranauskienė, Elena Manakova, Saulius Gražulis, Visvaldas Kairys, Kaspars Tars, Pavlína Řezáčová, and Daumantas Matulis*

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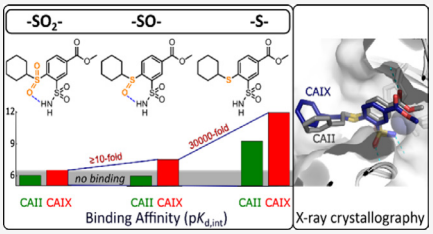
ABSTRACT: Discovery of small-molecule drugs relies on their strong binding affinity compared to nontarget proteins, thus possessing selectivity. Minor chemical structure changes usually exhibit little change in the compound efficacy, with rare exceptions. We developed a series of nearly 50 *ortho*-substituted benzenesulfonamides and experimentally measured their interactions with the 12 catalytically active human carbonic anhydrase (CA) isozymes. Inhibitors were designed using seven different substituent groups, including 4-*sulfanyl*-substituted 3-sulfamoyl benzoates and benzamides, 4-*sulfinyl*-substituted 3-sulfamoyl benzoates and benzamides, 4-*sulfonyl*-substituted 3-sulfamoyl benzoates and benzamides, and 4-amino-substituted benzamides. The oxidation state of sulfur at the *ortho* position significantly influenced the compound's affinity for CAIX, a target for anticancer drugs, demonstrating affinities hundreds of thousands of times stronger than related compounds. Coupled with X-ray crystal structures and molecular docking, the relationship between structure and thermodynamics offers insights into how small changes in the structure lead to significant changes in affinity for drug design.



INTRODUCTION

A detailed understanding of protein−ligand recognition is an essential goal in small-molecule drug discovery.[1] Any drug-like chemical compound should bind the disease-related target protein with sufficiently high affinity. Furthermore, the compound must bind with high selectivity and thus not interact strongly with other nontarget proteins whose inhibition or binding could cause undesired side effects.[2] Rational design of such compounds is complex because of the limited understanding of the underlying energies of binding and how a compound recognizes and binds to the target protein.

As a model system, we study sulfonamide compound binding to human carbonic anhydrases (CA), zinc-containing enzymes.[3] Humans have 12 catalytically active CA isozymes (EC 4.2.1.1).[4−6] The enzyme catalyzes the reversible hydration of carbon dioxide and has many essential physiological functions. Since these enzymes function in pH and electrolyte homeostasis and regulation, many drugs target CA isozymes to treat diseases like glaucoma, edema, obesity, epilepsy, infertility, and cancer.[7−9] Primary sulfonamides are the most investigated CA inhibitors.[10−12] Their amino group binds directly to the catalytic zinc in the active site by forming a coordination bond and inhibits the activity of all CA isozymes. However, the binding affinity may be low or high and depends on small details of each compound arrangement on the protein surface, possible steric hindrances, or attraction due to hydrogen bonds or hydrophobic interactions.[13]

The 12 catalytically active human CA isozymes have nearly identical beta-sheet folds. Their active sites are highly similar in shape, but several amino acids in the active site vary among the isozymes.[14−16] Because the differences in the active site amino acid composition are small, it is difficult to design compounds that would bind one isozyme with high affinity while all other isozymes with low affinity, thus leading toward high selectivity for only one isozyme. The active site of CAs is funnel-shaped and has hydrophobic and hydrophilic sides for some isozymes. Differences in a few amino acids determine the selectivity of inhibitors for particular isozymes. Introducing various scaffolds on the aromatic sulfonamide ring targets unique residues in the active site.[17] In most studies, the tails are relatively distant from

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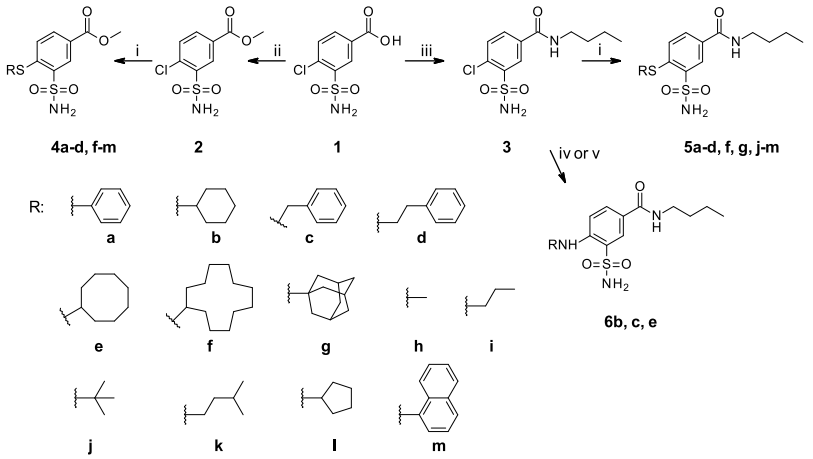
Scheme 1. Synthesis of Methyl 4-Substituted-3-Sulfamoylbenzoates 4a−d, f−m, 4-Substituted 3-Sulfamoylbenzamides 5a−d, f, g, j−m, and 4-Amino-Substituted Benzamides 6b, c, e[a]



[a]Reagents and conditions: (i) RSH, K_2CO_3, DMF, 80 °C; (ii) H_2SO_4, MeOH, Δ; (iii) (a) $SOCl_2$, toluene, Δ, (b) RNH_2, THF, 0 °C; (iv) RNH_2, 130 °C; (v) RNH_2, TEA, toluene, Δ.

the sulfonamide group, resulting in weak interactions with peripheral amino acids. The affinity profile depended mostly on the substituents located close to the sulfonamide headgroup.[18−20] This is also dependent on the flexibility of the substituents, which help adjust to the protein shape.

Depending on the variation of the substituents while investigating doubly substituted compounds,[20−22] several high-affinity compounds exhibiting picomolar K_d were obtained for CAVII, CAIX, CAXII, and CAXIV. A significant achievement was a *LJ15-12* compound that exhibited 0.08 pM intrinsic K_d for CAIX.[20] In this study, we investigate the functional groups in the *ortho* position by varying 13 substituents from small methyl to bulky adamantyl. Sulfinyl and amino substituents were also synthesized to examine the influence of the linking atoms. Interestingly, the substitution of only one atom, an addition of an oxygen atom in the *ortho* position, decreased compound affinity for nontarget isozymes by a million-fold, significantly improving the selectivity, which is one of the main goals in small-molecule drug discovery.

RESULTS

Organic Synthesis of Designed Compounds. The synthesis of 2,5-disubstituted benzenesulfonamides was performed starting from 4-chloro-3-sulfamoylbenzoic acid **1** (Scheme 1). Methyl ester **2** was obtained from 4-chloro-3-sulfamoylbenzoic acid **1** by reflux in methanol in the presence of a catalytic amount of sulfuric acid. Amide **3** was synthesized by refluxing acid **1** with thionyl chloride in toluene and subsequent treatment, the resulting anhydride with an appropriate amine, using as the base excess of amine according to the procedure reported in ref 21.

The synthesis of 4-substituted sulfamoylbenzoic acid derivatives **4a−d, f−m** was achieved by aromatic nucleophilic substitution of the chlorine substituent with various thiols under an inert atmosphere in dimethylformamide using potassium carbonate as the base (Scheme 1). All thiols were commercially available except cyclododecylthiol, which was synthesized by subjecting cyclododecanone to reaction with 1,2-ethanedithiol and subsequent reduction of intermediate dithiolane with *n*-butyllithium.[23]

The 4-substituted 3-sulfamoylbenzamides **5a−d, f, g, j−m** were synthesized using the same reaction conditions as 4-sulfanyl-substituted esters **4a−d, f−m**. Substitution of the chlorine group with amines required harsher reaction conditions than thiols. Therefore, 4-amino-substituted benzamides **6b** and **c** were synthesized by heating amide **3** over the appropriate amine at 130 °C. Compound **6e** was synthesized by boiling amide **3** in toluene with 2 equiv of cyclooctylamine and 2 equiv of triethylamine.

The oxidation of esters **4a−d, h, i, k−m** and benzamides **5a, b, k−m** to the sulfinyl and sulfonyl compounds was performed using in situ generated peracetic acid (Scheme 2). The reaction was carried out at room temperature and produced 4-sulfinyl compounds **7a, b, h, i, k−m**, **8a, b, k−m**, and heating the reaction mixture at 70 °C yielded the corresponding 4-sulfonyl compounds **9a−d, h, i, l, m**, **10b,** and **k−m**.

Compound Binding to CA Isozymes. All synthesized compounds were divided into 7 groups: **4(a−d, f−m)**, **5(a−d, f, g, j−m)**, **6(b, c, e)**, **7(a, b, h, i, k−m)**, **8(a, k−m)**, **9(a−d, h, i, l, m)**, and **10(b, k−m)** (Figure 1). Compounds that started with numbers **4**, **7**, and **9** were 4-sulfanyl-, 4-sulfinyl, and 4-sulfonyl-substituted esters, respectively. Compounds starting with the numbers **5**, **8**, and **10** were 4-sulfanyl-, 4-sulfinyl, and 4-sulfonyl-substituted benzamides analogous to the previous series. Compounds starting with the number **6** were 4-amino-substituted benzamides. Various linear, branched, and cyclic aliphatic and aromatic substituents at the *ortho* position were tested to assess whether size, flexibility, and hydrophobicity affect affinity. Compound affinities for the 12 catalytically active human CA isozymes are listed in Table 1.

As one of the important findings in this manuscript, Figure 2 arranges the compounds in the order of their affinity for CAIX

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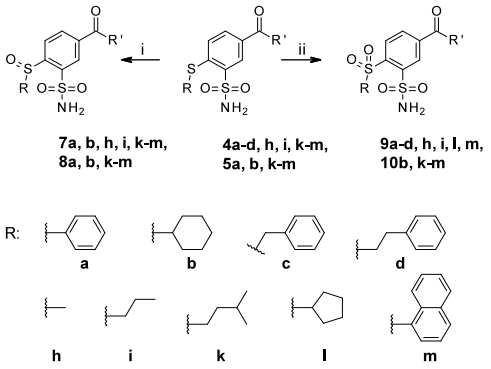
Scheme 2. Synthesis of Methyl 4-Sulfinyl-Substituted-3-Sulfamoyl Benzoates 7a, b, h, i, k−m, *N*-Butyl-4-sulfinyl-Substituted-3-Sulfamoylbenzamides 8a, b, k−m, Methyl 4-Sulfonyl-Substituted-3-Sulfamoylbenzoates 9a-d, h, i, l, m, and *N*-Butyl-4-sulfonyl-Substituted-3-Sulfamoylbenzamides 10b, k−m[a]



[a]Reagents and conditions: (i) 30% H_2O_2, AcOH, r. t.; (ii) 30% H_2O_2, AcOH, 70 °C.

and compared to undesired target CAII. A high affinity for CAIX is desired, because CAIX is implicated in various types of cancer.[24] However, CAII is abundant in erythrocytes, thus an off-target for anticancer inhibitors.

Compound affinities for human CA isozymes were analyzed using fluorescence-based thermal shift assay (FTSA) and the enzymatic activity stopped-flow-based inhibition assay (SFA) (Figure 3). The FTSA determined the observed dissociation constants for all compounds with all 12 CA isozymes (Table 1 and Supplementary Figure S1). From these experimentally determined $K_{d,obs}$, the *intrinsic* dissociation constants $K_{d,int}$ were calculated, and the results are primarily focused on them. Figure 3B,C shows two compounds with strong and weak affinity for CAII and CAIX by FTSA. A single oxygen atom strongly diminished affinity for both isozymes. The stopped-flow assay of CO_2 hydration enzymatic activity inhibition confirmed that the compounds inhibited CAIX and CAII (Figure 3D,E). Inhibition K_i values are presented in Table 2 and Figure S2. The FTSA is a convenient technique covering a significantly wider K_d range than the SFA.[25]

The experimental conditions slightly differed between FTSA and SFA, and it is therefore not appropriate to directly compare the $K_{d,obs}$ and K_i. However, the measured affinities were similar in both techniques. Further analysis is based on FTSA data due to its wider limits in identifying strong binders. Note that the enzyme concentration limits the SFA's ability to determine high-affinity binders. For example, if we use a 10 nM concentration of a CA isozyme, the lowest IC_{50} is 5 nM, half of the protein concentration. Any compound with an IC_{50} stronger than 5 nM would exhibit a dosing curve that appears as an IC_{50} of 5 nM. Thus, compounds that possess single-digit nM or picomolar K_d, cannot be distinguished by SFA.

Sulfonamide binding of CA is a pH-dependent reaction.[6,26,27] The water molecule bound to the CA zinc ion is replaced by the deprotonated form of sulfonamide upon binding.[28,29] The protonation forms required for the interaction exist at different pHs: the CA-Zn(II)-H_2O has the largest fraction at acidic pH and the deprotonated sulfonamide has the largest fraction at alkaline pH. Therefore, the measured affinity is always lower than the intrinsic affinity. The intrinsic parameters are calculated (see equations in the Experimental section) by knowing the pK_a of the CA zinc-bound water and the pK_a of the sulfonamide group (Table 1 and Figure S3). Experimental data of the sulfonamide group pK_a determination for compound **4b** are shown in Figure 4. Figure S3 shows the graphs of pK_a determination for the remaining compounds.

Intrinsic affinities are especially important in rational drug design. It is not rare when a stronger affinity is observed not because of the formed bonds but because of the substitutions that lower the pK_a of the sulfonamide group and thus increase the fraction of the ready-to-bind form.[30] Intrinsic parameters are used to avoid misleading conclusions when comparing the affinity of compounds for CAs.

Esters vs Benzamides. 4-sulfanyl-substituted esters **4a−d, f−m** were the largest (12 compounds) studied group of compounds with the same framework. Compounds of this group, even almost independently of the substituent, interacted strongly and selectively with several CAs. $K_{d,int}$ of **4b** for CAIX was 0.0010 nM and it was the strongest interaction measured in this study, CAXIII—0.090 nM and CAXIV—0.020 nM, with others interacting much weaker. The size of the substituent was critical in this interaction. CAIX has a larger active site than the rest of the CAs.[31] It was interesting that compound **4g** (*adamantyl*), which has a similar affinity for CAIX ($K_{d,int}$ =

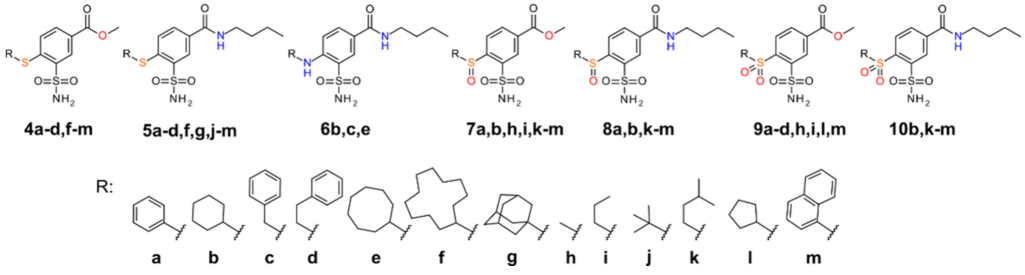


Figure 1. Chemical structures of the compounds synthesized and investigated in this study. Compounds **1**, **2**, and **3** are the starting compounds of the synthesis shown in Scheme 1.

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Table 1. ***Observed*** **and** ***Intrinsic*** **($K_{d,obs}$ and $K_{d,int}$) Dissociation Constants (in nM Units) of Investigated Compounds to All Catalytically Active Human CAs at 37 °C Obtained by Fluorescent Thermal Shift Assay (FTSA)**[a]

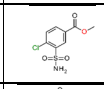
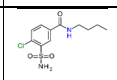
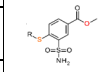
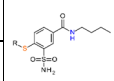
					CAI	CAII	CAIII	CAIV	CAVA	CAVB	CAVI	CAVII	CAIX	CAXII	CAXIII	CAXIV
					$pK_{a,ca}$	8.1	6.9	6.5	6.6	7.3	7.0	6.0	6.8	6.6	6.8	8.0	6.8
Compound	A framework of the structure	$pK_{a,sa}$	fraction at pH		7.0	92.6%	44.3%	24.0%	28.5%	66.6%	50.0%	9.1%	38.7%	28.5%	38.7%	90.9%	38.7%
2 *JA17-1*		9.2	0.63%	$K_{d,obs}$	21000	460	≥200000	500	770	42	300	240	240	92	220	170
				$K_{d,int}$	120	1.3	≥300	0.90	3.2	0.13	0.17	0.58	0.43	0.22	1.3	0.41
3 *JA17-3*		9.1	0.79%	$K_{d,obs}$	36000	140	44000	99	1800	70	340	94	190	10	880	290
				$K_{d,int}$	260	0.50	83	0.22	10	0.28	0.24	0.29	0.43	0.030	6.3	0.89
4a *JA17-1-1*		9.8	0.16%	$K_{d,obs}$	≥200000	87	≥200000	460	≥200000	120	430	140	4.0	260	12	10
				$K_{d,int}$	≥290	0.060	≥76	0.21	≥210	0.10	0.060	0.090	0.0020	0.16	0.020	0.0060
4b *JA17-1-2*		9.7	0.20%	$K_{d,obs}$	≥200000	560	≥200000	810	≥200000	440	710	680	1.8	280	52	22
				$K_{d,int}$	≥370	0.49	≥96	0.46	≥270	0.44	0.13	0.52	0.0010	0.22	0.090	0.020
4c *JA17-1-3*		9.8	0.16%	$K_{d,obs}$	≥200000	590	≥200000	110	140000	930	660	960	10	350	52	90
				$K_{d,int}$	≥290	0.42	≥76	0.050	150	0.70	0.10	0.60	0.0050	0.21	0.080	0.050
4d *JA17-1-4*		9.6	0.25%	$K_{d,obs}$	160000	580	≥200000	1100	97000	190	1300	360	19	450	27	20
				$K_{d,int}$	380	0.64	≥120	0.79	160	0.23	0.29	0.34	0.010	0.43	0.060	0.020
4f *JA18-29*		9.7[a]	0.20%	$K_{d,obs}$	≥200000	1000	≥200000	2100	≥200000	1800	6500	18000	11	68	100	100
				$K_{d,int}$	≥370	0.90	≥96	1.2	≥270	1.8	1.2	14	0.0060	0.050	0.18	0.080
4g *E19-3*		9.7[b]	0.20%	$K_{d,obs}$	≥200000	3300	≥200000	100000	≥200000	≥200000	13000	96000	4.8	1500	1200	1200
				$K_{d,int}$	≥370	2.9	≥96	57	≥270	≥200	2.3	74	0.0030	1.1	2.2	1.0
4h *JA19-17*		9.8	0.16%	$K_{d,obs}$	≥200000	2200	≥200000	960	120000	680	500	1000	650	6500	570	440
				$K_{d,int}$	≥290	1.6	≥76	0.43	130	0.54	0.070	0.61	0.29	4.0	0.80	0.27
4i *JA18-30*		9.6	0.25%	$K_{d,obs}$	≥200000	1000	≥200000	400	91000	990	700	490	23	1800	98	70
				$K_{d,int}$	≥460	1.1	≥120	0.28	150	1.2	0.16	0.48	0.020	1.7	0.22	0.070
4j *JA18-33*		9.6	0.25%	$K_{d,obs}$	≥200000	2400	≥200000	10000	≥200000	4900	4600	15000	290	120	220	370
				$K_{d,int}$	≥460	2.7	≥120	7.4	≥330	6.1	1.0	14	0.21	0.12	0.51	0.36
4k *JA18-32*		9.8	0.16%	$K_{d,obs}$	40000	370	≥200000	160	85000	310	300	210	2.7	190	40	33
				$K_{d,int}$	58	0.26	≥76	0.070	89	0.25	0.040	0.13	0.0010	0.11	0.060	0.020
4l *E19-1*		9.6	0.25%	$K_{d,obs}$	≥200000	670	≥200000	1000	180000	6700	560	180	3.3	670	110	20
				$K_{d,int}$	≥460	0.74	≥120	0.71	300	8.4	0.13	0.18	0.0020	0.65	0.25	0.020
4m *E19-2*		9.7	0.20%	$K_{d,obs}$	≥200000	50	11000	2000	≥200000	1300	500	8.7	2.5	500	1.4	25
				$K_{d,int}$	≥370	0.040	5.3	1.1	≥270	1.2	0.090	0.0070	0.0010	0.39	0.0030	0.020
5a *JA17-3-1*		9.7	0.20%	$K_{d,obs}$	≥200000	16000	≥200000	≥200000	120000	9000	19000	53000	360	17000	17000	60000
				$K_{d,int}$	≥370	14	≥96	≥110	160	9	3.5	41	0.20	13	30	46
5b *JA17-3-2*		9.8	0.16%	$K_{d,obs}$	≥200000	180000	100000	≥200000	≥200000	33000	≥200000	≥200000	450	140000	150000	≥200000
				$K_{d,int}$	≥290	120	38	≥90	≥210	26	≥29	≥120	0.20	83	220	≥120
5c *JA17-3-3*		9.8	0.16%	$K_{d,obs}$	≥200000	12000	≥200000	40000	68000	33000	43000	77000	2100	8600	43000	50000
				$K_{d,int}$	≥290	8.1	≥76	18	72	26	6.2	47	0.93	5.3	62	31
5d *JA17-3-4*		9.9	0.13%	$K_{d,obs}$	≥200000	58000	≥200000	≥200000	110000	17000	98000	≥200000	3200	46000	110000	≥200000
				$K_{d,int}$	≥230	32	≥60	≥72	95	10	11	≥97	1.2	22	130	≥97
5f *JA19-16*		9.8[b]	0.16%	$K_{d,obs}$	≥200000	100000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	840	≥200000	≥200000	62000
				$K_{d,int}$	≥290	70	≥76	≥90	≥210	≥160	≥29	≥120	0.38	≥120	≥290	38
5g *YB19-4*		9.8[b]	0.16%	$K_{d,obs}$	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	2000	≥200000	≥200000	≥200000
				$K_{d,int}$	≥290	≥140	≥76	≥90	≥210	≥160	≥29	≥120	0.90	≥120	≥290	≥120
5j *YB19-3*		9.8	0.16%	$K_{d,obs}$	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	25000	≥200000	≥200000	≥200000
				$K_{d,int}$	≥290	≥140	≥76	≥90	≥210	≥160	≥29	≥120	11	≥120	≥290	≥120

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Table 1. continued

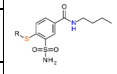
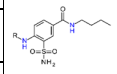
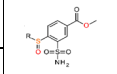
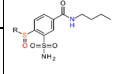
Compound lab. name	A framework of the structure	$pK_{a,SA}$	fraction at pH 7.0		CAI	CAII	CAIII	CAIV	CAVA	CAVB	CAVI	CAVII	CAIX	CAXII	CAXIII	CAXIV
				$pK_{a,CA}$	8.1	6.9	6.5	6.6	7.3	7.0	6.0	6.8	6.6	6.8	8.0	6.8
					92.6%	44.3%	24.0%	28.5%	66.6%	50.0%	9.1%	38.7%	28.5%	38.7%	90.9%	38.7%
5k *YB19-2*		9.7	0.20%	$K_{d,obs}$	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	670	≥200000	≥200000	≥200000
				$K_{d,int}$	≥370	≥180	≥96	≥110	≥270	≥200	≥36	≥150	0.38	≥150	≥360	≥150
5l *YB19-5*		9.8	0.16%	$K_{d,obs}$	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	800	≥200000	≥200000	≥200000
				$K_{d,int}$	≥290	≥140	≥76	≥90	≥210	≥160	≥29	≥120	0.36	≥120	≥290	≥120
5m *YB19-1*		9.9	0.13%	$K_{d,obs}$	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	500	≥200000	10000	≥200000
				$K_{d,int}$	≥230	≥110	≥60	≥72	≥170	≥130	≥23	≥97	0.18	≥97	11	≥97
6b *IA17-3-11*		9.8	0.16%	$K_{d,obs}$	180000	20000	≥200000	68000	80000	6500	26000	62000	420	27000	44000	46000
				$K_{d,int}$	260	14	≥76	31	84	5.2	3.7	38	0.19	16	64	28
6c *IA17-3-9*		9.8	0.16%	$K_{d,obs}$	≥200000	22000	≥200000	63000	83000	10000	25000	62000	1500	6500	32000	33000
				$K_{d,int}$	≥290	16	≥76	29	88	8.0	3.6	38	0.66	4.0	47	20
6e *IA17-3-10*		9.8	0.16%	$K_{d,obs}$	≥200000	110000	≥200000	≥200000	190000	≥200000	≥200000	≥200000	1200	64000	150000	20000
				$K_{d,int}$	≥290	79	≥76	≥90	200	≥160	≥29	≥120	0.53	39	220	12
7a *IA19-11*		8.9[a]	1.24%	$K_{d,obs}$	≥200000	340	93000	900	≥200000	910	970	79	130	2900	120	99
				$K_{d,int}$	≥2300	1.8	280	3.2	≥1700	5.6	1.1	0.38	0.45	14	1.3	0.48
7b *IA19-10*		8.9	1.24%	$K_{d,obs}$	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	7800	≥200000	≥200000	≥200000
				$K_{d,int}$	≥2300	≥1100	≥600	≥710	≥1700	≥1200	≥230	≥960	28	≥960	≥2300	≥960
7h *IA19-19*		8.9	1.24%	$K_{d,obs}$	≥200000	4300	≥200000	5500	≥200000	33000	1200	18000	5200	15000	720	3300
				$K_{d,int}$	≥2300	24	≥600	19	≥1700	200	1.3	87	18	74	8.1	16
7i *IA19-3*		8.9	1.24%	$K_{d,obs}$	≥200000	≥200000	≥200000	≥200000	100000	≥200000	111000	≥200000	43000	≥200000	26100	45000
				$K_{d,int}$	≥2300	≥1100	≥600	≥710	830	≥1200	130	≥960	150	≥960	290	220
7k *IA19-8*		8.8	1.56%	$K_{d,obs}$	≥200000	2400	≥200000	9000	≥200000	14000	13000	9300	140	7600	400	2000
				$K_{d,int}$	≥2900	16	≥750	40	≥2100	110	18	56	0.63	46	5.6	12
7l *E19-6*		8.9	1.24%	$K_{d,obs}$	27000	3600	≥200000	12000	≥200000	130000	9800	68000	200	18000	1600	510
				$K_{d,int}$	310	20	≥600	42	≥1700	780	11	330	0.72	86	18	2.5
7m *E19-7*		9.1	0.79%	$K_{d,obs}$	52000	2900	≥200000	1200	≥200000	58000	65000	5400	110	2600	700	180
				$K_{d,int}$	380	10	≥380	2.8	≥1000	230	47	16	0.24	8.1	5.0	0.54
8a *IA19-15*		9.0	0.99%	$K_{d,obs}$	180000	13000	≥200000	50000	≥200000	13000	≥200000	100000	14000	≥200000	50000	≥200000
				$K_{d,int}$	1600	55	≥480	140	≥1300	62	≥180	380	40	≥770	450	≥770
8k *YB19-10*		9.0	0.99%	$K_{d,obs}$	≥200000	160000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	3300	≥200000	180000	≥200000
				$K_{d,int}$	≥1800	710	≥480	≥560	≥1300	≥990	≥180	≥770	9	≥770	1600	≥770
8l *YB19-11*		9.0[b]	0.99%	$K_{d,obs}$	33000	14000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	13000	≥200000	≥200000	10000
				$K_{d,int}$	310	63	≥480	≥560	≥1300	≥990	≥180	≥770	35	≥770	≥1800	38
8m *YB19-7*		8.9	1.24%	$K_{d,obs}$	≥200000	141800	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	4600	≥200000	146300	≥200000
				$K_{d,int}$	≥2300	780	≥600	≥710	≥1700	≥1200	≥230	≥960	16.0	≥960	1700	≥960
9a *IA17-1-5*		9.0	0.99%	$K_{d,obs}$	≥200000	56000	≥200000	≥200000	≥200000	≥200000	110000	57000	76000	≥200000	16000	42000
				$K_{d,int}$	≥1800	250	≥480	≥560	≥1300	≥990	94	220	220	≥770	140	160
9b *IA17-1-7*		8.9	1.24%	$K_{d,obs}$	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	28000	90000	≥200000	100000	140000
				$K_{d,int}$	≥2300	≥1100	≥600	≥710	≥1700	≥1200	≥230	130	320	≥960	1100	680
9c *IA17-1-8*		8.9	1.24%	$K_{d,obs}$	≥200000	≥200000	≥200000	68000	≥200000	≥200000	≥200000	70000	11000	≥200000	56000	29000
				$K_{d,int}$	≥2300	≥1100	≥600	240	≥1700	≥1200	≥230	340	37	≥960	640	140
9d *IA17-1-6*		9.0	0.99%	$K_{d,obs}$	≥200000	≥200000	≥200000	140000	≥200000	≥200000	160000	≥200000	≥200000	≥200000	25000	54000
				$K_{d,int}$	≥1800	≥880	≥480	390	≥1300	≥990	150	≥770	≥560	≥770	220	210
9h *IA19-20*		8.8	1.56%	$K_{d,obs}$	≥200000	82000	≥200000	89000	140000	≥200000	64000	160000	73000	170000	22000	59000
				$K_{d,int}$	≥2900	570	≥750	400	1400	≥1600	91	960	320	1100	310	350

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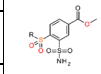
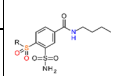
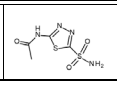
Table 1. continued

Compound lab. name	A framework of the structure	$pK_{a,SA}$	fraction at pH 7.0		CAI	CAII	CAIII	CAIV	CAVA	CAVB	CAVI	CAVII	CAIX	CAXII	CAXIII	CAXIV
		$pK_{a,CA}$			8.1	6.9	6.5	6.6	7.3	7.0	6.0	6.8	6.6	6.8	8.0	6.8
					92.6%	44.3%	24.0%	28.5%	66.6%	50.0%	9.1%	38.7%	28.5%	38.7%	90.9%	38.7%
9i *IA19-2*		8.9	1.24%	$K_{d,obs}$	≥200000	1700	≥200000	6200	≥200000	9000	1500	≥200000	900	16000	250	920
				$K_{d,int}$	≥2300	10	≥600	22	≥1700	56	1.7	≥960	3.2	76	2.8	4.4
9l *E19-4*		9.0	0.99%	$K_{d,obs}$	≥200000	71000	≥200000	190000	≥200000	≥200000	≥200000	≥200000	9100	≥200000	22000	20000
				$K_{d,int}$	≥1800	310	≥480	540	≥1300	≥990	≥180	≥770	26	≥770	200	78
9m *E19-5*		9.0	0.99%	$K_{d,obs}$	≥200000	5900	≥200000	≥200000	≥200000	≥200000	35000	6800	4000	≥200000	630	6300
				$K_{d,int}$	≥1800	26	≥480	≥560	≥1300	≥990	31	26	11	≥770	5.6	24
10b *IA19-14*		9.0	0.99%	$K_{d,obs}$	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000
				$K_{d,int}$	≥1800	≥880	≥480	≥560	≥1300	≥990	≥180	≥770	≥560	≥770	≥1800	≥770
10k *YB19-8*		9.0	0.99%	$K_{d,obs}$	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	160000
				$K_{d,int}$	≥1800	≥880	≥480	≥560	≥1300	≥990	≥180	≥770	≥560	≥770	≥1800	620
10l *YB19-9*		9.1	0.79%	$K_{d,obs}$	≥200000	≥200000	≥200000	≥200000	≥200000	150000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000
				$K_{d,int}$	≥1500	≥700	≥380	≥450	≥1000	610	≥140	≥610	≥450	≥610	≥1400	≥610
10m *YB19-6*		8.9	1.24%	$K_{d,obs}$	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000	≥200000
				$K_{d,int}$	≥2300	≥1100	≥600	≥710	≥1700	≥1200	≥230	≥960	≥710	≥960	≥2300	≥960
AZM		7.0	50%	$K_{d,obs}$	2400	46	40000	87	840	140	220	13	21	130	79	63
				$K_{d,int}$	1100	9.8	4600	12	270	34	9.8	2.4	2.9	24	35	12

[a]Observed values were determined using 50 mM sodium phosphate buffer at pH 7.0 containing 100 mM sodium chloride, 50 μM ANS dye, and 2% (v/v) DMSO. $K_{d,int}$s were calculated according to eq 1 (see the Experimental Section). Values with a "≥" sign show that they are at the detection limit of ≥200 000 nM of $K_{d,obs}$ according to the highest used ligand concentration. The intrinsic value limits vary for different CAs and compounds due to differences in pK_a. $pK_{a,SA} - pK_a$ of the sulfonamide group; $pK_{a,CA} - pK_a$ value of water molecule bound to Zn(II) in the active site of CA; AZM, acetazolamide (a standard inhibitor). [b]Not determined due to solubility issues or low intensity of the spectrum curves. The $pK_{a,SA}$ value was assigned based on similarities in chemical structure.

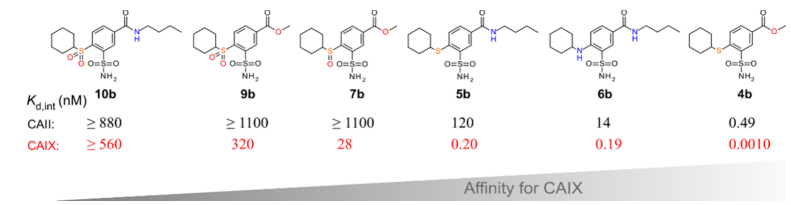


Figure 2. Compounds are arranged in the order of increasing affinity for CAIX. Compound **4b** had the highest affinity and selectivity for CAIX. The intrinsic dissociation constants (in nM units) are compared for CAII and CAIX, while values for the remaining CAs are listed in Table 1.

0.0030 nM) to most of the compounds in this group, is a few hundred times more selective for CAIX, whereas, for example, **4b** (*cyclohexyl*) is only a few tens of times more selective for CAIX and can be considered a strong binder to several undesired CAs. On the other hand, the 4-methylsulfanyl- substituted compound **4h** is no longer selective. Thus, the hydrophobic interaction made a significant contribution to affinity for CAIX, and selectivity was mainly obtained by the size of the substituent when the binding to CAIX was optimal and binding to other CAs was limited by steric interference. Analogous benzamides were much weaker binders of CAs. Most benzamides exhibited no interaction with CAs. Nevertheless, all compounds in this series bound to CAIX and were, in most cases, at least several dozen times more selective for it than for other CAs.

Sulfanyl vs Sulfinyl vs Sulfonyl Compounds. Different forms of sulfur oxidation led to drastically different affinities for CAs. A higher degree of oxidation in these compounds weakened the affinity. However, in our opinion, it was not the oxidation itself that had the main influence, but rather the conformation of the compound. The affinity of all sulfinyl- compounds with all CAs was significantly weaker than analogous sulfanyl- compounds. The decrease in affinity varied depending on the CA isozyme and the substituents. Therefore, no generalized observations could be made. For example, compound **7a** did not bind to CAI and CAVA, but the weak affinity was determined for CAIII, with all other CAs the interactions were similar and did not exceed more than 10-fold in most cases and there was no selectivity for CAIX. Compounds **7b**, **7h**, and **7i** did not bind or bind weakly and nonselectively to all CAs. Except for **7b**, it is bound only to CAIX with 28 nM. The $K_{d,int}$ of compounds **7k**, **7l**, and **7m** were 0.63, 0.72, and 0.24 nM, respectively. Also, there was a similar affinity for CAXIII and CAXIV and weaker for the other CAs. From compounds **5(a–d, f, g, j–m)** to **8(a, k–m)** decreased affinity for all CAs.

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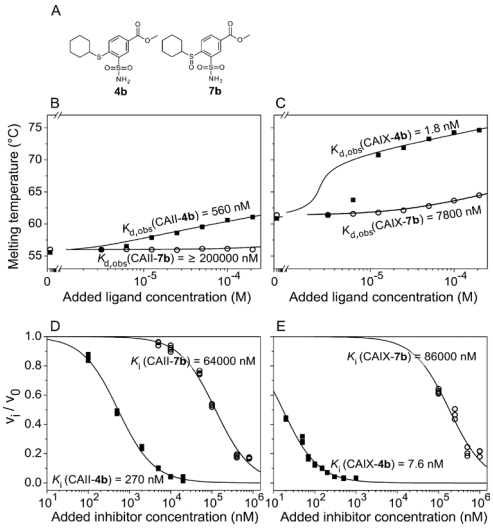

Figure 3. Compound affinity was determined by two assays in this study. (A) Chemical structures of two compounds whose binding data are shown below. (B) and (C) Fluorescent thermal shift assay data (FTSA) of compounds **4b** (closed squares) and **7b** (open circles) binding to CAII and CAIX, respectively. (D) and (E) Stopped-flow carbon dioxide hydration assay (SFA) data of compounds **4b** (closed squares) and **7b** (open circles) inhibition of CAII and CAIX, respectively. The dissociation constants (K_d) or inhibition constants (K_i) are given next to the corresponding curves. It is important to note that the experimental conditions of the methods were slightly different: FTSA, pH 7.0, 37 °C, while for SFA, pH 7.5, 25 °C.

Switching to sulfonyl compounds reduced the affinity and abolished the selectivity for CAIX. **9b** only bound to CAVII, CAIX, CAXIII, and CAXIV with $K_{d,int}$ 130, 320, 1100, and 680 nM, respectively. The most strongly interacting compound in this series was **9i**, e.g., $K_{d,int}$ of CAVI—1.7 nM, CAIX—3.2 nM but also bound to other CAs quite similarly. Meanwhile, compounds **10(b, k−m)** did not bind to any isozymes, only a couple of measurements showed a weak interaction.

4-Sulfanyl- vs 4-Amino-Substituted. Comparing **5b** vs. **6b** and **5c** vs. **6c**, in most cases, the dissociation constants differed

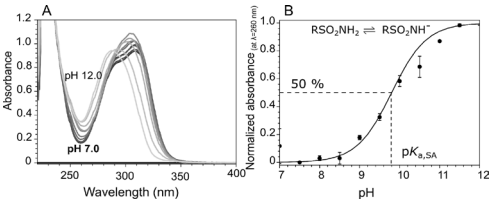

Figure 4. Spectrophotometric determination of the sulfonamide group deprotonation pK_a for compound **4b**. (A) Absorption spectra of compound **4b** in solutions at various pH at intervals of 0.5 pH units at 37 °C. (B) Normalized absorbance at 260 nm was plotted as a function of pH, and the pK_a value was determined as a midpoint of the curve. The data points are the mean points of two repeats ((A) shows one repeat for simplicity), with standard deviations. The pK_a value of compound **4b** was 9.74 ± 0.13 (±1.3%) with a confidence interval [9.62−9.87] of 95%.

only a few times, and the constants for CAIX did not exceed the margin of error. The S or N atom in the same position of the compound almost did not change the affinity for all isozymes. Compound **6e** (cyclooctyl-substituted) selectively interacted with CAIX, $K_{d,int}$ of CAIX—0.53 nM, CAXII—39 nM, and CAXIV—12 nM. It did not bind to other CAs. Most likely the selectivity was due to the size of the active site pocket.

X-ray Crystal Structures of Compound Binding to CAII and CAXIII. Nine crystal structures were determined by X-ray crystallography, the complexes of CAII with compounds **2**, **3**, **4c**, **4d**, **4h**, CAIX with **4d** and **5b**, and CAXIII with **4c** and **4d**. Table 3 lists the data collection and refinement statistics. Figure 5 shows the electron density maps of these compounds in the active site of CAs. Two molecules of compound **4h** were identified in the active site of CAII, one conventionally formed a coordination bond between the sulfonamide group and zinc, and the other was independently located near the periphery of the active site. The localization of both separated molecules relative to the same zinc is shown in Figure 5E,F. The main highlights of the identified protein−ligand interactions are described below and illustrated in Figure 6.

*Structures of **2** vs **3** Bound to CAII.* Starting compounds **2** and **3** used in the synthesis differed by one substitution at the *meta* position relative to the sulfonamide group, ester vs. amide. Figure 6A,B shows the position of these compounds in the active site of CAII. Both compounds retained the same position of the sulfonamide group and the chlorine atom in the active site of

Table 2. Inhibition Constants and IC_{50} (in nM Units) of CAII and CAIX with Compounds Obtained by Stopped-Flow Carbon Dioxide Hydration Assay (SFA) at 25 °C[a]

	K_i (nM)		IC_{50} (nM)	
compound	CAII	CAIX	CAII	CAIX
4a	25 ± 1.3	5.4 ± 0.5	47 ± 2.6	12 ± 0.5
4b	270 ± 20	7.6 ± 0.5	530 ± 40	16 ± 1.3
4c	210 ± 30	37 ± 3.5	400 ± 50	79 ± 7
4d	120 ± 10	51 ± 6	230 ± 20	110 ± 12
4h	9200 ± 500	1300 ± 200	18,000 ± 1000	2700 ± 400
7a	63 ± 6	360 ± 30	120 ± 12	760 ± 60
7b	64,000 ± 7500	86,000 ± 16,000	100,000 ± 10,000	200,000 ± 30,000
7h	3400 ± 200	7600 ± 700	6500 ± 300	16,000 ± 1600
9a	34,000 ± 4000	3700 ± 600	65,000 ± 7000	7800 ± 1300

[a]Experiments were performed using 20 mM HEPES Na at pH 7.5, 20 mM Na_2SO_4, and 0.2 mM Phenol Red.

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Table 3. X-ray Diffraction Data Collection and Refinement Statistics

isozyme—ligand	CAII—**2**	CAII—**3**	CAII—**4c**	CAII—**4d**	CAII—**4h**	CAIX—**4d**	CAIX—**5b**	CAXIII—**4c**	CAXIII—**4d**
PDB ID	9FPT	9FPU	9FPQ	9FPR	9FPS	9R8X	9R8Y	9FPV	9FPW
				Data Collection Statistics					
space group	$P12_11$	$P12_11$	$P12_11$	$P12_11$	$P12_11$	H3	H3	$P2_12_12_1$	$P2_12_12_1$
unit-cell parameters (a, b, c (Å); α, β, γ (°))	a = 42.1, b = 40.9, c = 71.7, $\alpha = \gamma$ = 90, β = 104.0	a = 42.0, b = 41.1, c = 71.9, $\alpha = \gamma$ = 90, β = 104.2	a = 42.3, b = 41.3, c = 72.2, $\alpha = \gamma$ = 90, β = 104.2	a = 42.3, b = 41.3, c = 72.1, $\alpha = \gamma$ = 90, β = 104.5	a = 42.3, b = 41.2, c = 72.0, $\alpha = \gamma$ = 90, β = 104.4	$a = b$ = 152.5, c = 172.4, $\alpha = \beta$ = 90, γ = 120	$a = b$ = 151.9, c = 173.7, $\alpha = \beta$ = 90, γ = 120	a = 55.1, b = 58.0, c = 160.2, $\alpha = \beta = \gamma$ = 90	a = 55.5, b = 58.4, c = 160.7, $\alpha = \beta = \gamma$ = 90
resolution range (Å)	69.6−1.2	40.7−1.1	70.0−1.5	40.0−1.5	39.9−1.4	47.95−2.0	47.8−1.95	80.1−1.7	40.2−1.9
unique reflections number	75321	87594	38824	41447	47284	101012	108927	57402	42102
R_{merge}, overall (outer shell)	0.04 (0.38)	0.05 (0.31)	0.05 (0.25)	0.05 (0.39)	0.04 (0.38)	0.08 (1.18)	0.21 (1.30)	0.09 (0.32)	0.12 (0.31)
I/σ overall (outer shell)	16.9 (3.5)	13.7 (3.8)	15.9 (4.9)	19.6 (4.6)	24.2 (4.7)	21.1 (2.3)	7.1 (1.5)	16.8 (7.4)	13.0 (7.6)
multiplicity overall (outer shell)	6.8 (6.7)	6.6 (6.0)	6.6 (6.4)	7.0 (6.9)	7.0 (6.8)	10.4 (10.5)	10.4 (9.9)	13.2 (13.1)	13.1 (13.4)
completeness (%) overall (outer shell)	96.7 (94.0)	96.0 (91.3)	99.6 (99.2)	98.7 (97.7)	97.6 (94.9)	100.0 (100.0)	100.0 (100.0)	100 (100)	100 (100)
Wilson B-factor	12.8	11.4	17.8	15.5	17.0	28.0	22.4	16.9	22.5
				Refinement Statistics					
R_{work}	0.14	0.15	0.17	0.14	0.14	0.17	0.21	0.16	0.19
R_{free}	0.18	0.17	0.20	0.21	0.19	0.21	0.25	0.19	0.23
RMSD bond lengths (Å)/ angles (°)	0.02/2.13	0.02/2.26	0.01/1.80	0.01/2.04	0.01/2.02	0.01/1.81	0.01/1.73	0.01/1.90	0.01/1.57
average B factors (Å^2): all atoms/inhibitors	22.7/17.2	19.6/16.9	21.6/21.9	20.1/15.7	21.8/21.7	31.9/50.4	30.7/39.4	21.6/32.5	25.9/35.6
Ramachandran statistics (%): favored/allowed/outliers	97/3/ 0	97/3/ 0	97/3/ 0	96/4/ 0	96/4/ 0	96/4/ 0.11	94/5/ 0.32	chain A: 98/2/ 0 chain B: 97/3/ 0	chain A: 97/3/ 0 chain B: 97/3/ 0

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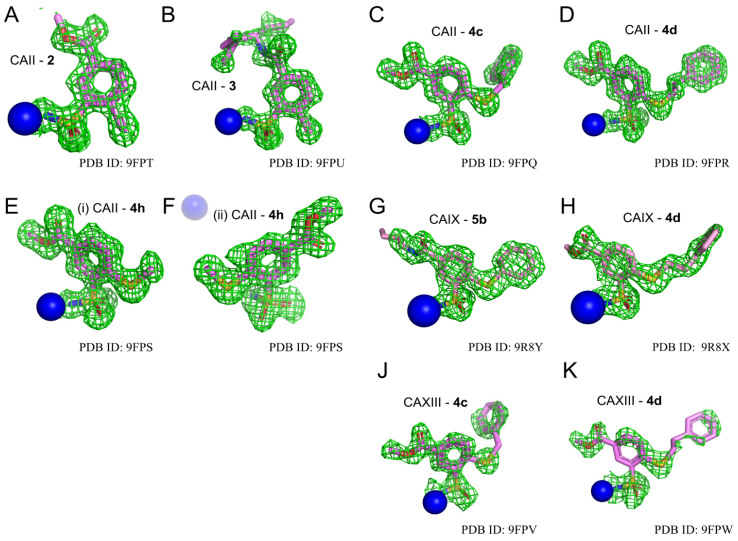

Figure 5. $|F(o) - F(c)|$ omit maps at 3σ in green for the investigated ligands in the active site of CAs. (A) CAII—**2** (PDB ID: 9FPT), (B) CAII—**3** (PDB ID: 9FPU), (C) CAII—**4c** (PDB ID: 9FPQ), (D) CAII—**4d** (PDB ID: 9FPR), (E) and (F) CAII—**4h** (PDB ID: 9FPS; two ligand molecules were identified, one bound directly to Zn in a conventional position, while the second was seen located nearby toward the edge of the active site), (G) CAIX—**5b** (PDB ID: 9R8Y), (H) CAIX—**4d** (PDB ID: 9R8X), (**J**) CAXIII—**4c** (PDB ID: 9FPV), (**K**) CAXIII—**4d** (PDB ID: 9FPW). Omit maps were taken from a refinement run of the final model without the ligand. Zinc is shown in blue.

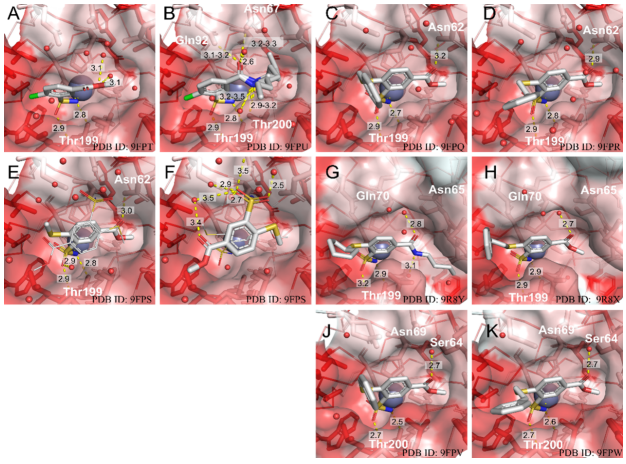

Figure 6. X-ray crystal structures of (A) CAII—**2** (PDB ID: 9FPT), (B) CAII—**3** (PDB ID: 9FPU), (C) CAII—**4c** (PDB ID: 9FPQ), (D) CAII—**4d** (PDB ID: 9FPR), (E) and (F) CAII—**4h** (PDB ID: 9FPS; two ligand molecules were identified, one bound directly (E) to Zn in a conventional position, while the second is seen located nearby toward the edge (F) of the active site), (G) CAIX—**5b** (PDB ID: 9R8Y), (H) CAIX—**4d** (PDB ID: 9R8X), (**J**) CAXIII—**4c** (PDB ID: 9FPV), (**K**) CAXIII—**4d** (PDB ID: 9FPW). The yellow dashed line represents the hydrogen bond; the distances are given in angstroms. The amino acids directly involved in hydrogen bond formation are labeled. Amino acids are colored according to hydrophobicity:[52] the most intense red color represents the most hydrophobic amino acids.

CAII. The amide substituent formed multiple hydrogen bonds with the amino acid side groups, while the ester was stabilized by a network of hydrogen bonds through water molecules. Presumably, a different network of hydrogen bonds pulled the entire molecule slightly, so a partially rotated benzene ring was observed in the crystal structure. No significant conformational changes were observed in the amino acid chains of the active site of CAII. However, the amide substituent of the compound was

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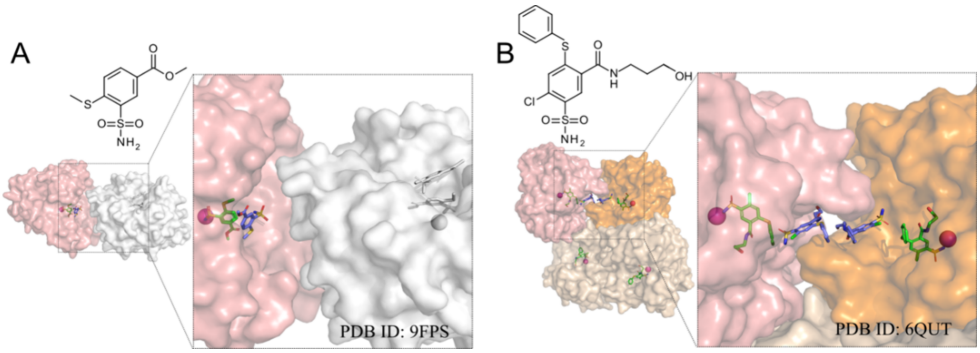

Figure 7. Unusual ligand positions in crystal structures of CAII (PDB ID: 9FPS) and CAIX (PDB ID: 6QUT, published previously[2]). The chemical structures of the ligands are shown above the crystal structures. The crystal structures represent a view of the monomers and a zoomed-in view of the active site. Zn(II) is shown as a pink sphere. Bound to Zn(II) inhibitor molecules are green and others are blue. (A) Monomer of CAII is shown in surface mode and colored salmon. The symmetric chain is colored white. (B) In the case of CAIX, only the asymmetric unit is represented. Six inhibitor molecules are bound to 4 CA IX chains. Chain C (orange) and chain D (salmon) have two inhibitor molecules, and chains A and B (beige) have one molecule. In this case, the interaction of ligands between separate chains is visible.

not in one fixed position. Instead, 3 alternative conformations of the substituent were identified in the structure. However, it should be noted that this substituent has a poor electron density, likely due to its high flexibility. Consequently, its exact arrangement cannot be determined.

*Structures of CAII with Chlorine-Substituted (**2**, **3**) vs Sulfanyl-Substituted (**4c**, **4d**, and **4h**).* The position of *ortho-sulfanyl* and chlorine-substituted compounds in the CAII active site differed significantly. The entire sulfanyl-substituted molecule was shifted to avoid steric interference but maintained a similar distance between the sulfonamide group's nitrogen and the enzyme's zinc compared to the chlorine-substituted compound. Meanwhile, the studied *ortho*-substituted compounds occupied similar positions, and the ester groups of all three compounds formed a hydrogen bond with Asn62. Notably, the electron density of all inhibitor molecules is well-defined.

*Structures of CAII vs CAIX and CAXIII with Bound Sulfanyl-Substituted (**4c** and **4d** or **5b**).* Figure 6 shows the interactions between CAII with compounds **4c** and **4d** (Figure 6C,D), CAIX with **4d** (Figure 6H), and CAXIII with compounds **4c** and **4d** (Figure 6J,K). One of the main differences was in the formed hydrogen bonds. The oxygen of the ester group of the compound formed a direct hydrogen bond with Asn62 in the active site of CAII. Meanwhile, a hydrogen bond is formed in the active site of CAIX and CAXIII through a water molecule with Gln70 and Asn65 or Ser64 and Asn69, respectively. Asn62 in CAII, Asn65 in CAIX, and Asn69 in CAXIII differ only by the numbering but correspond to the same position.

Incidentally, compound **5b** CAIX also forms the same hydrogen bond through a water molecule, and the entire molecule adopts a similar conformation. Notably, the electron density of the 17 amino acids at the N-terminus of both X-ray crystal structures of CAIX is not defined as expected. These amino acids fold in their 3D structure to form the active site of the protein. This side of the active site is called the hydrophilic part. This suggests that the bound ligand pushes these amino acids to fit fully into the active site. When comparing these structures (PDB ID: 9R8X and 9R8Y) with those existing in the PDB (e.g., PDB ID: 3iai), the clash between ligand and Tyr7 is seen without changing their arrangement.

It is important to emphasize that both **4c** and **4d** have very poor electron density in the CAXIII active center, so the arrangement of the compound should be evaluated more cautiously. On the other hand, a comparison of the structures shows that compound **4d** is more similarly located in the active sites of CAII and CAXIII, while in CAIX, it is shifted toward the hydrophilic side, which, as mentioned, is not fully visible in the X-ray structure.

Atypical Binding Position. The structure 9FPS was unique because two ligand molecules were identified as bound in the active site. One molecule was bound classically and formed a coordination bond with the zinc ion. The second formed a hydrophobic interaction with the first molecule, and hydrogen bonds with water molecules located toward the edge of the active site. This could be a crystallographic artifact or a secondary interaction with the protein.[33] A previous publication[2] identified a similar case with CAIX (PDB ID: 6QUT) (Figure 7). In that case, the molecules interacted in the active site and among themselves between the chains in an asymmetric unit. This case with CAII was different because the two chains had no interaction between ligand molecules. The active sites of symmetric chains were not oriented face-to-face. The atoms of the second nonclassical ligand were further than 5Å from the amino acids of the symmetric chain.

Molecular Docking. To understand the differences in the binding affinities of series **4**, **7**, and **9** ligands, they were docked into CAII, except for **4e** and **4f**, containing large, flexible rings that are challenging to dock. Series **4** ligands were also docked into CAIX and CAXIII, to compare with crystal structures and thus assess the docking accuracy. To reduce bias, different receptors (PDB IDs – CAII: 3HS4; CAIX: 6G9U, chain A; CAXIII: 4KNN, chain A) were chosen instead of the new X-ray structures presented in this paper.

To mimic the donor–acceptor bond between the zinc ion and the sulfonamide nitrogen of the ligand, we employed constrained docking using the Smina program.[34] The constraint forced the sulfonamide nitrogen to maintain its original position in the X-ray structure. The generated poses were afterward

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rescored using the Vinardo scoring function.[35] Vina[36] and GNINA[37] Machine-learning-based scoring functions were found to be inferior to Vinardo for this system, and therefore only the latter was employed for further analysis. The pose with the best rescored affinity that matched the X-ray conformation of the benzenesulfonamide moiety was then chosen as the representative best docked structure (in all cases, for ligand **4** series, it was ranked 1 by Vinardo, except for **4j**, where the correct benzenesulfonamide conformation was ranked 3). The best docked poses for CAII are shown in Figure 8A. The Pearson

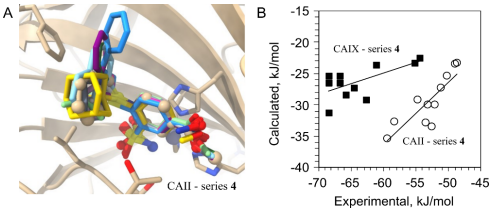

Figure 8. (A) Docked series **4** ligands (see main text for details) superposed onto the X-ray structure of **4c** (PDB ID: 9FPQ; rendered using ball-and-stick representation) in a complex with CAII. Note that hydrophobic substituents stack against the Phe131 side chain. (B) Computed ligand binding affinities to CAII (circles) and CAIX (squares) using Vinardo scoring function plotted against the experimental binding affinities—intrinsic Gibbs energy changes. The Pearson correlations R of the plotted points are 0.87 for CAII and 0.66 for CAIX. The large difference between the computed and experimental binding affinities is because the Vinardo scoring function does not take into account the interaction with zinc. However, it can be assumed to be approximately equal for all ligands.

correlation coefficient R with the experimental *intrinsic* binding affinities to CAII for the best docked poses was 0.87. Figure 8B plots the corresponding computed and experimental binding affinities for CAII and CAIX (values listed in Table S1 in the Supporting Information). Notably, for CAII, the scoring function correctly predicts the best binder, **4m**, and the four worst binders (**4g**–**j**). The panel also shows the correspondence between the experimental and predicted binding affinities for CAIX, with $R = 0.66$.

Validation of the Docking Protocol via Comparison with the X-ray Structures. The proposed docking and scoring protocol was further validated by comparing the predicted docked poses of some of the ligands with their conformations in the newly reported X-ray structures. Figure S4A–C shows the ranked poses of ligands **4c**, **4d**, and **4h** compared against their X-ray conformations in the complex with CAII. The heavy atom Root Mean Square Deviation (RMSD) between the docked and X-ray conformation is 2.03, 3.13, and 0.46 Å, respectively. For the first two ligands, however, the rank 2 conformations are close to the X-ray conformation (**4c**: 0.61 Å; **4d**: 0.67 Å, see Figure S4A,B).

To further validate the chosen docking protocol, **4d** and **5b** were docked into CAIX (PDB ID: 6G9U, chain A). The best-scored poses after reranking using Vinardo are shown in Figure S5. The corresponding heavy atom RMSDs for **4d** and **5b** are 2.18 and 0.92 Å, respectively, and despite the RMSD for **4d** being above 2 Å, the binding mode is captured by the docking reasonably well, especially since the receptor used for docking was originally bound to a ligand from a chemically different series.

Compounds **4c** and **4d** were also docked into the CAXIII receptor (PDB ID: 4KNN, chain A). Similarly to CAIX, the docking protocol picked the docked pose with the approximately correct binding mode (with RMSDs equal to 1.90 and 0.95 Å, respectively) (Figure S6).

Docking of Series 7 and 9 Compounds. Since series **4** compounds using the docking protocol described above can reproduce the scaffold rather well and generally seem to stack the hydrophobic substituents reasonably, we applied a similar procedure by docking series **9** ligands into CAII as a model protein, in hopes that it will help to explain a difference between the binding affinities of series **4** and **9**. Docking using the same protocol led to comparable binding affinities between the two series (not shown). However, experiments indicate that **9** binds worse by several orders of magnitude, and rescored constrained pose affinities for this series exhibit practically no correlation with the experiment ($R \cong 0.12$). A careful examination of the docking results for series **9** revealed the source of the poor binding affinities and the poor performance of the docking score. Figure 9A displays the best ranking pose of **9a**. While the phenyl substituent of **9a** stacks well with Phe131 side chain, the two sulfonyl moieties exhibit an apparent clash against each other.

To further explore this clash, we ran simulations of a simple compound 2-(methylsulfonyl)benzenesulfonamide containing sulfonamide and methylsulfonyl groups at the *ortho*-positions on a benzene ring. The lowest energy conformer for this compound was generated using CREST software[38] using semiempirical GFN2-xTB wave function,[39] and afterward reoptimized in the implicit solvent using the Density Functional Theory (DFT) approach with the GAMESS-US program.[40] In the lowest energy conformer, sulfonamide nitrogen forms a hydrogen bond with the sulfonyl oxygen at the *ortho* position (Figure 9B, bottom). The approximate conformational energy of the docked conformation was also computed using DFT, keeping frozen the sulfonamide S–N and methylsulfonyl S–C bond torsional angles for the benzene ring, and optimizing the rest of the 2-methylsulfonylbenzenesulfonamide molecule (Figure 9B, top). The freezing of the select torsions was necessary because the DFT calculation lacks the protein environment that keeps them at certain values seen in docking. The energy difference between the lowest energy and the docked conformers in Figure 9B was 53.8 kJ/mol. It became clear that the used scoring function did not report a correct binding energy of the conformation in Figure 9A because the Vinardo scoring function neither takes into account the change of the ligand conformation when going from the solution into the receptor, nor the ligand intramolecular energy (in fact, none of the several built-in scoring functions in Smina do). This means that in the conformation shown in Figure 9A the 2-methylsulfonyl group must rotate to avoid the rather severe clash with the sulfonamide oxygens (Figure 9B, top), and by doing so the hydrophobic substituent rotates away from Phe131, potentially yielding other clashes with the protein and leading to the poor binding energies.

Series **7** compounds are more complex to investigate because two enantiomers of the S atom of sulfinyl exist (Figure 9C,D). We will explore the behavior of sulfinyl-containing series **7** using compound **7a** as an example. One of its docked enantiomers, (*S*)-**7a** forms sulfonyl-sulfinyl clash (Figure 9C and the top left part of Figure 9D) similar to what we found for **9a**. The docked (*R*)-**7a** enantiomer is about 1.7 times more stable compared to the (*S*)- enantiomer because of the lack of the clash (Figure 9D,F). Calculations also show that in the absence of the protein,

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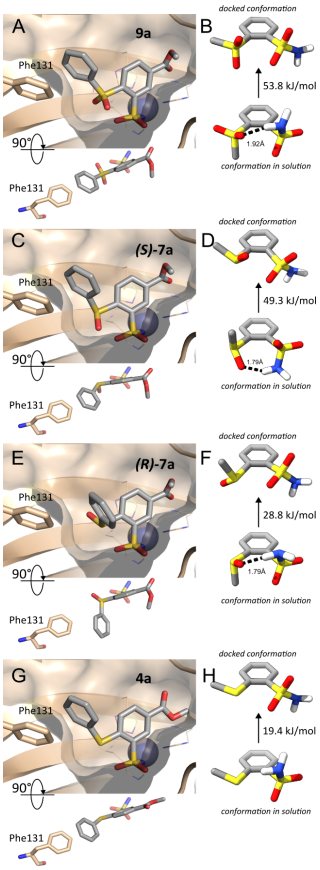

Figure 9. Best ranking docked pose of **9a**, (S)-**7a**, (R)-**7a**, and **4a** in the active site of CAII as a model protein. (A) Best-ranking docked pose of **9a**. While the phenyl substituent stacks well with the Phe131 side chain, the two SO_2 moieties presumably clash. (B) Two conformations of 2-(methylsulfonyl)benzenesulfonamide: Top: conformation with two benzene-to-S bond dihedrals constrained so that it is similar to the docked conformation in (A); bottom: lowest energy conformation with an intramolecular H-bond that is likely to be found in solution. The energy difference between the two rotamers computed using density functional theory is 53.8 kJ/mol. The fact that the used scoring function does not take into account this difference explains why the calculated affinities (not shown) do not match the experimental values. (C), (E) Best-ranked docked conformations of *(S)*- and *(R)*-**7a**, correspondingly. (D), (F) Top: the conformations 2-(methylsulfinyl)-benzenesulfonamide, matching the docked geometries in (C) and (E). Bottom: The lowest energy conformer with an intramolecular H-bond, presumably existing in solution. The docked *(R)*- enantiomer is much more stable due to the lack of clashes present in the docked *(S)*-enantiomer. (G) Best-ranking docked pose of **4a**. (H) Two conformations of 2-methylsulfanylbenzenesulfonamide: Top: the optimized with constraints conformation matching the docked conformation; bottom: The lowest energy conformer. Compared with the sulfinyl- and sulfonyl-forms, the docked conformation for the sulfanyl analog is the most stable, which is also reflected in the binding affinities.

the preferred conformation **7a** (of either chirality) has an intramolecular H-bond (Figure 9D,F, bottom).

For comparison, we also used DFT calculations of the 2-methylsulfanylbenzenesulfonamide molecule to estimate the stability of the docked **4a** (Figure 9G), which does not seem to exhibit clashes. Comparison of Figure 9B,D,F,H shows that the docked conformer **4a** is ∼1.5 times more stable than the most stable docked conformer of **7a** and nearly 3 times more stable than docked **9a**, corresponding to the experimentally determined binding affinities that are best in series **4**, followed by **7** and **9**. Interestingly, calculations suggest that one reason for the relative stability of docked **4** is the lack of the intramolecular hydrogen bond in solution (Figure 9H, bottom), and this observation could be potentially used in designing better binders for carbonic anhydrases, or even other receptors.

DISCUSSION

Recognition of the pockets on the protein surface by small-molecule ligands is still rather poorly understood, and it is not possible to accurately calculate the thermodynamic binding parameters.[41] Primary sulfonamide compounds are known to inhibit CA isozymes by binding to the catalytic Zn(II). Chemical variation of the remaining molecule often has a limited influence on binding affinity and selectivity. However, some chemical changes made near the sulfonamide group have a significant impact and may change the behavior from strong binder to completely undetectable binding. To investigate this phenomenon, we synthesized *ortho*-substituted benzenesulfonamides, determined their binding affinity to all catalytically active isoforms of human carbonic anhydrases, obtained crystal structures with CAII, CAIX, and CAXIII, and performed molecular docking. Different oxidation forms of the linker at the *ortho* position were used: −S−, −SO−, and $-SO_2-$. Compounds with −SO− or $-SO_2-$ linker were much weaker binders to any CA isozyme than compounds with the −S− linker. Several reasons could cause this. First, a substituent with a higher oxidation state exhibits a stronger electron-withdrawing effect, thus lowering the pK_a of the amine of the sulfonamide group and causing stronger binding. Second, the linker oxygen atoms may prevent the easier rotation of the ligand molecule bonds and prevent conformational changes needed for the ligand to adapt to the protein surface. Third, the oxygen atoms take additional space, and the steric hindrance could be an essential factor in diminishing the binding affinity.

To test the electron-withdrawing effect, the pK_a values of compounds were determined experimentally. The pK_a of compounds containing −S− linker was almost an entire pH unit higher than compounds with linkers −SO− or $-SO_2-$. Sulfonamides bind to CA in their negatively charged deprotonated form.[42] Therefore, the lowering of sulfonamide pK_a increases the fraction of the binding-ready deprotonated sulfonamide and thus increases the observed affinity. This effect is simply the effect of compound availability in the proper form. To eliminate this misleading increase in affinity, we subtract the fraction effects and calculate the *intrinsic* affinity. The intrinsic dissociation constants of all compounds are provided next to the observed values in the table. In all cases, the intrinsic affinities are higher than the observed ones. The intrinsic values show the 'real' affinities between the binding components in the binding-ready protonation state. These values should be used in drug design to explain the structure−function relationship of the compound effects and not the experimentally observed ones. However, the experimentally observed affinity values show the

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affinities observed by any experimental technique, and the values are biologically relevant. They should be used to calculate the bound fractions and drug effect at particular conditions.

Furthermore, the compounds with a higher oxidation state (**7a, b, h, i, k−m** and **9a−d, h, i, l, m**) did not bind or bind with lower affinity than compounds **4a−d, f−m**. Even the compounds **7h** and **9h** with the smallest methyl substituent did not match the sulfanyl compound **4h** in affinity. The main reason for this was the allowed conformations of the compounds, which were calculated using quantum Density Functional Theory (DFT). The oxygen atoms limited both the flexibility of the molecule in finding the optimal position and acted as a steric hindrance. The present study shows that the *ortho* modifications had a more significant effect than the *para* variations.[21]

The highest affinity for CAIX in this study was exhibited by compound **4b** (methyl 4-cyclohexylsulfanyl-3-sulfamoylbenzoate). Previously we have designed similar compounds bearing 2-chloro or 2-bromo substituents that exhibited slightly higher affinity for CAIX.[20] Here, we have intentionally omitted the halogen atoms to synthesize the *ortho*-substituted compounds more easily and explore the binding without halogens. It was also easier to synthesize compounds with an amide substituent in the *meta* position. The length of these amide-substituted compounds was of limited importance in the previous study but had a significant influence on this study, likely due to reasons of steric hindrance.

Sulfonamide compounds usually bind to CA isozymes by forming a coordination bond between the Zn(II) and sulfonamide amino groups. This bond significantly increases the affinity, but is not necessary for binding to occur.[43] Removal of the metal or change of the metal with another demonstrated that the coordination bond contribution is additive and metal-dependent. Strongly binding compounds like brinzolamide to CAII also bind to the Zn-free apoCAII. The energy contribution of the coordination bond could be determined by using a metal-exchange approach.

In the crystal structure of compound **4h** bound to CAII, two well-resolved compound molecules were bound in the active site. The first was bound in a conventional way forming a coordination bond with the Zn(II), but the second was bound to the protein residues without forming a coordination bond with the Zn(II). This second compound molecule did not bind solely due to crystal-forming effects because it did not bind to the second protein molecule. Therefore, the binding of the second molecule is likely not an artifact. However, the presence of the second molecule in the crystal structure does not mean that it is bound as strongly, nor that we can measure its binding affinity experimentally. Binding assays showed that the stoichiometry here was 1:1, and the affinity of the second molecule was likely weak compared to the first one. Compound concentration in the crystallization experiments was relatively high, millimolar, thus the second molecule could be seen in the crystal structure even if the K_d was in the millimolar range and therefore not interfering in any binding assays. This also means that the second molecule is biologically irrelevant and would not play an essential role in drug design.

CONCLUSIONS

The strategy to acidify the pK_a of *ortho* sulfanyl-substituted benzenesulfonamides by oxidation, with the goal of increased affinity to CA, yielded an unexpected drop of affinity in the order of hundreds of thousands of times. Small changes in chemical structures influenced the flexibility of the molecule's substituents and steric restrictions on interactions with proteins. Furthermore, some minor changes led to the discovery of novel CAIX inhibitors with high affinity and selectivity.

EXPERIMENTAL SECTION

Organic Synthesis. All starting materials and reagents were commercial products used without further purification. Melting points of the compounds were determined in open capillaries on a Thermo Scientific 9100 Series and are uncorrected. ^{1}H and ^{13}C NMR spectra (Figure S7) were recorded on a (400 and 100 MHz, respectively) spectrometer in DMSO-d_6 using residual DMSO signals (2.52 and 40.21 ppm for ^{1}H and ^{13}C NMR spectra, respectively) as the internal standard. TLC was performed with silica gel 60 F254 aluminum plates (Merck) and visualized with UV light. Column chromatography used silica gel 60 (0.040−0.063 mm, Merck). High-resolution mass spectra (HRMS) were recorded by an Agilent TOF 6230 equipped with an Agilent Infinity 1260 HPLC system (Agilent Technologies). HPLC verified the purity of final compounds to be >95% (Figure S8) using the Agilent Infinity 1260 instrument with a ZORBAX Eclipse Plus C18 Rapid Resolution 4.6 × 100 mm 3.5 μm column, ZORBAX Eclipse Plus-C18 4.6 × 12.5 mm 5.0 μm analytical guard column, eluents A − 20 mM ammonium acetate (pH = 6.9 of unadjusted solution) and B − 100% MeOH was used. HPLC gradient elution with a flow rate of 0.80 mL/min was used, B gradient: 0−10 min 55−95%, 10−14 min 95%. UV detection was recorded at 254 nm. Figure S9 contains ESI-MS spectra of representative compounds.

*Methyl 4-Chloro-3-sulfamoyl-benzoate (**2**).* 4-chloro-3-sulfamoyl-benzoic acid **1** (1.78 g, 7.55 mmol, Sigma-Aldrich) was refluxed in MeOH (30 mL) with concentrated H_2SO_4 (0.3 mL) for 8 h. The reaction mixture was concentrated under reduced pressure. Residue was filtered, washed with H_2O and crystallized from H_2O:MeOH(4:1). Yield: 1.70 g, 91%, mp 129−130 °C (Literature[44] mp 124−125 °C).

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 3.91 (s, 3H, C**H**$_3$O), 7.80 (d, J = 8.4 Hz, 1H, Ar**H**), 7.84 (s, 2H, $SO_2N\mathbf{H}_2$), 8.14 (dd, J = 8.4 Hz, J = 2.1 Hz, 1H, Ar**H**), 8.52 (d, J = 2.1 Hz, 1H, Ar**H**).

*N-Butyl-4-chloro-3-sulfamoyl-benzamide (**3**).* The mixture of 4-chloro-3-sulfamoylbenzoic acid **1** (500 mg, 2.12 mmol), $SOCl_2$ (0.616 mL, 8.48 mmol), and one drop DMF in toluene (6.0 mL) was refluxed for four h. Excess $SOCl_2$ and toluene were removed by distillation under reduced pressure. The crude acid chloride was dissolved in THF (20 mL) and added dropwise to a solution of *N*-butylamine (0.591 mL, 6.0 mmol) in THF (20 mL) at 0 °C and allowed stirring for one h. The mixture was warmed to room temperature and stirred for another 4 h. THF was removed under reduced pressure. The crude product was crystallized from H_2O. Yield: 492 mg, 80%, mp 172−173 °C (lit.[45] mp 171−172 °C).

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 0.92 (t, J = 7.3 Hz, 3H, C**H**$_3$CH$_2$), 1.37 (sextet, J = 7.3 Hz, 2H, C**H**$_2$CH$_3$), 1.54 (quint, J = 7.3 Hz, 2H, CH$_2$C**H**$_2$CH$_2$), 3.29 (q, J = 6.8 Hz, 2H, C**H**$_2$NH), 7.72 (s, 2H, $SO_2N\mathbf{H}_2$), 7.77 (d, J = 8.2 Hz, 1H, Ar**H**), 8.04 (dd, J = 8.2 Hz, J = 2.0 Hz, 1H, Ar**H**), 8.45 (d, J = 2.0 Hz, 1H, Ar**H**), 8.77 (t, J = 5.5 Hz, 1H, N**H**).

*General Procedure for the syntheses of **4a−d, f, g, i−m**.* The mixture of methyl 4-chloro-3-sulfamoylbenzoate **2** (285 mg, 1,14 mmol), DMF (4.0 mL), appropriate thiol (1.25 mmol), and K_2CO_3 (630 mg, 4,56 mmol) was heated at 80 °C for 4−6 h in an inert atmosphere (argon). The mixture was cooled to room temperature, and 10 mL of H_2O was added. The product was extracted with EtOAc (3 × 8 mL). The organic layer was washed with H_2O, dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure.

*Methyl 4-Phenylsulfanyl-3-sulfamoyl-benzoate (**4a**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 6:1). Yield: 303 mg, 82%, mp 155−156 °C. (lit.[46] mp 154−157).

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 3.86 (s, 3H, C**H**$_3$O), 7.00 (d, J = 8.4 Hz, 1H, Ar**H**), 7.55−7.62 (m, 5H, Ar**H**), 7.74 (s, 2H, $SO_2N\mathbf{H}_2$), 7.93 (dd, J = 8.4 Hz, J = 1.9 Hz, 1H, Ar**H**), 8.46 (t, J = 1.9 Hz, 1H, Ar**H**).

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^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 52.9, 126.8, 129.0, 129.2, 130.5, 130.8, 131.0, 132.6, 135.5, 140.8, 144.2, 165.4.

HRMS calcd for $C_{14}H_{13}NO_4S_2$ [(M+H)$^+$]: 324.0359, found: 324.0354.

*Methyl 4-Cyclohexylsulfanyl-3-sulfamoyl-benzoate (**4b**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 7:1). Yield: 315 mg, 84%, mp 119−120 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 1.26−1.99 (m, 10H, C**H** cyclohexyl), 3.61 (m, 1H, C**H**S), 3.88 (s, 3H, CH_3O), 7.47 (s, 2H, SO_2NH_2), 7.74 (d, J = 8.4 Hz, 1H, Ar**H**), 8.04 (dd, J = 8.3 Hz, J = 1.9 Hz, 1H, Ar**H**), 8.43 (d, J = 1.9 Hz, 1H, Ar**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 25.7, 25.8, 32.5, 44.4, 52.9, 100.0, 125.9, 129.1, 132.3, 141.7, 142.8, 165.6.

HRMS calcd for $C_{14}H_{19}NO_4S_2$ [(M+H)$^+$]: 330.0828, found: 330.0833.

*Methyl 4-Benzylsulfanyl-3-sulfamoyl-benzoate (**4c**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 6:1). Yield: 211 mg, 55%, mp 135−136 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 3.88 (s, 3H, CH_3O), 4.43 (s, 2H, CH_2S), 7.29−7.37 (m, 3H, Ar**H**), 7.51−7.53 (m, 2H, Ar**H**), 7.58 (s, 2H, SO_2NH_2), 7.74 (d, J = 8.3 Hz, 1H, Ar**H**), 8.02 (dt, J = 8.3 Hz, J = 1.7 Hz, 1H, Ar**H**), 8.42 (t, J = 2.2 Hz, 1H, Ar**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 36.4, 52.9, 125.9, 127.7, 128.0, 128.9, 129.0, 129.8, 132.2, 136.0, 140.7, 143.7, 165.6.

HRMS calcd for $C_{15}H_{15}NO_4S_2$ [(M+H)$^+$]: 338.0515, found: 338.0517.

*Methyl 4-(2-Phenylethylsulfanyl)-3-sulfamoyl-benzoate (**4d**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 5:1). Yield: 332 mg, 83%, mp 146−147 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 2.99 (t, J = 7.5 Hz, 2H, CH_2Ph), 3.41 (t, J = 7.5 Hz, 2H, CH_2S), 3.88 (s, 3H, CH_3O), 7.23−7.34 (m, 5H, Ar**H**), 7.51 (s, 2H, SO_2NH_2), 7.73 (d, J = 8.4 Hz, 1H, Ar**H**), 8.05 (dd, J = 8.4 Hz, J = 1.8 Hz, 1H, Ar**H**), 8.43 (d, J = 1.8 Hz, 1H, Ar**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 33.4, 34.3, 52.9, 125.8, 126.9, 127.8, 128.8, 128.9, 129.1, 132.4, 140.2, 141.,0, 143.6, 165.6.

HRMS calcd for $C_{16}H_{17}NO_4S_2$ [(M+H)$^+$]: 352.0672, found: 352.0675.

*Methyl 4-Cyclododecylsulfanyl-3-sulfamoyl-benzoate (**4f**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 10:1). Yield: 203 mg, 43%, mp 167−168 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 1.32−1.59 (m, 20H, C**H** cyclododecyl), 1.76 (m, 2H, C**H** cyclododecyl), 3.66 (m, 1H, C**H**S), 3.88 (s, 3H, CH_3O), 7.47 (s, 2H, SO_2NH_2), 7.70 (d, J = 8.4 Hz, 1H, Ar**H**), 8.07 (dd, J = 8.4 Hz, J = 2.0 Hz, 1H, Ar**H**), 8.44 (d, J = 2.0 Hz, 1H, Ar**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 22.0, 23.2, 23.3, 24.0, 24.2, 29.2, 43.5, 52.9, 125.9, 129.0, 129.2, 132.3, 142.0, 143.2, 165.6.

HRMS calcd for $C_{20}H_{31}NO_4S_2$ [(M+H)$^+$]: 414.1767, found: 414.1773.

*Methyl 4-(1-Adamantylsulfanyl)-3-sulfamoyl-benzoate (**4g**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 10:1). Yield: 173 mg, 40%, mp 189−190 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 1.63 (br s, 6H, C**H** adamantanyl), 1.98 (br s, 6H, C**H** adamantanyl), 2.00 (br s, 3H, C**H** adamantanyl), 3.90 (s, 3H, OCH_3), 7.40 (s, 2H, SO_2NH_2), 7.87 (d, J = 8.0 Hz, 1H, Ar**H**), 8.08 (dd, J = 8.0 Hz, J = 2.0 Hz, 1H, Ar**H**), 8.53 (d, J = 2.0 Hz, 1H, Ar**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 30.1, 35.9, 43.8, 52.2, 53.0, 128.8, 128.9, 131.8, 137.7, 137.9, 147.0, 165.5.

HRMS calcd for $C_{18}H_{23}NO_4S_2$ [(M+H)$^+$]: 382.1141, found: 382.1144.

*Methyl 4-Propylsulfanyl-3-sulfamoyl-benzoate (**4i**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 5:1). Yield: 202 mg, 61%, mp 111−112 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 1.05 (t, J = 7.3 Hz, 3H, CH_3CH_2), 1.71 (quint, J = 7.3 Hz, 2H, CH_3CH_2), 3.11 (t, J = 7.3 Hz, 2H, CH_2S), 3.88 (s, 3H, CH_3O), 7.54 (s, 2H, SO_2NH_2), 7.65 (d, J = 8.3 Hz, 1H, Ar**H**), 8.04 (dd, J = 8.3 Hz, J = 1.9 Hz, 1H, Ar**H**), 8.43 (d, J = 1.9 Hz, 1H, Ar**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 13.8, 21.7, 33.9, 52.8, 125.6, 127.5, 128.9, 132.3, 140.9, 144.1, 165.6.

HRMS calcd for $C_{11}H_{15}NO_4S_2$ [(M+H)$^+$]: 290.0515, found: 290.0519.

*Methyl 4-tert-Butylsulfanyl-3-sulfamoyl-benzoate (**4j**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 5:1). Yield: 214 mg, 62%, mp 124−125 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 1.43 (s, 9H, CH_3), 3.90 (s, 3H, CH_3O), 7.43 (s, 2H, SO_2NH_2), 7.91 (d, J = 8.2 Hz, 1H, Ar**H**), 8.11 (dd, J = 8.2 Hz, J = 2.0 Hz, 1H, Ar**H**), 8.52 (d, J = 2.0 Hz, 1H, Ar**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 31.6, 49.6, 53.0, 128.4, 128.9, 132.0, 136.2, 140.0, 146.1, 165.5.

HRMS calcd for $C_{12}H_{17}NO_4S_2$ [(M+H)$^+$]: 304.0672, found: 304.0671.

*Methyl 4-Isopentylsulfanyl-3-sulfamoyl-benzoate (**4k**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 5:1). Yield: 265 mg, 74%, mp 92−93 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 0.94 (d, J = 6.6 Hz, 6H, CH_3), 1.57 (td, J = 7.8 Hz, J = 6.8 Hz, 2H, CH_2CH), 1.76 (m, 1H, C**H**), 3.11 (t, J = 7.8 Hz, 2H, CH_2S), 3.88 (s, 3H, CH_3O), 7.52 (s, 2H, SO_2NH_2), 7.67 (d, J = 8.4 Hz, Ar**H**), 8.05 (dd, J = 8.4 Hz, J = 2.0 Hz, 1H, Ar**H**), 8.42 (d, J = 2.0 Hz, 1H, Ar**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 22.6, 27.5, 30.2, 36.9, 52.8, 125.6, 127.5, 128.9, 132.3, 140.9, 144.1, 165.6.

HRMS calcd for $C_{13}H_{19}NO_4S_2$ [(M+H)$^+$]: 318.0828, found: 318.0833.

*Methyl 4-Cyclopentylsulfanyl-3-sulfamoyl-benzoate (**4l**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 8:1). Yield: 259 mg, 72%, mp 106−107 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 1.55−1.65 (m, 4H, C**H** cyclopentyl), 1.73−1.78 (m, 2H, C**H** cyclopentyl), 2.18−2.23 (m, 2H, C**H** cyclopentyl), 3.84−3.92 (m, 4H, CH_3O and C**H**S), 7.50 (s, 2H, SO_2NH_2), 7.72 (d, J = 8.4 Hz, 1H, Ar**H**), 8,.04 (dd, J = 8.4 Hz, J = 2.0 Hz, 1H, Ar**H**), 8.42 (d, J = 2.0 Hz, 1H, Ar**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 25.1, 33.3, 43.9, 52.8, 125.5, 128.4, 128.9, 132.3, 140.8, 144.7, 165.6.

HRMS calcd for $C_{13}H_{17}NO_4S_2$ [(M+H)$^+$]: 316.0672, found: 316.0677.

*Methyl 4-(1-Naphthylsulfanyl)-3-sulfamoyl-benzoate (**4m**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 8:1). Yield: 281 mg, 66%, mp 187−188 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 3.83 (s, 3H, CH_3O), 6.64 (d, J = 8.4 Hz, 1H, Ar**H**), 7.54−7.64 (m, 2H, Ar**H**), 7.69 (m, 1H, Ar**H**), 7.74 (dd, J = 8.4 Hz, J = 2.0 Hz, 1H, Ar**H**), 7.93 (s, 2H, SO_2NH_2), 8.04−8.15 (m, 3H, Ar**H**), 8.22 (d, J = 8.3 Hz, 1H, Ar**H**), 8.49 (d, J = 2.,0 Hz, 1H, Ar**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 52.9, 125.6, 126.7, 126.9, 127.3, 127.5, 128.4, 128.5, 129.1, 129.6, 132.2, 132.5, 133.8, 134.8, 136.8, 140.4, 143.9, 165.4.

HRMS calcd for $C_{18}H_{15}NO_4S_2$ [(M+H)$^+$]: 374.0515, found: 374.0511.

*Methyl 4-Methylsulfanyl-3-sulfamoyl-benzoate (**4h**).* The mixture of methyl 4-chloro-3-sulfamoylbenzoate **2** (308 mg, 1,23 mmol), DMSO (4.0 mL), sodium methanethiolate (259 mg, 3.69 mmol), and K_2CO_3 (509 mg, 3.69 mmol) was heated at 80 °C temperature for 12 h in inert atmosphere(argon). The mixture was cooled to room temperature and 20 mL brine was added. The product was extracted with EtOAc (3 × 10 mL). The organic layer was washed with H_2O, dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The product was crystallized from H_2O:MeOH(4:1). Yield: 203 mg, 64%, mp 157−158 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 2.59 (s, 3H, CH_3S), 3.89 (s, 3H, CH_3O), 7.54 (s, 2H, SO_2NH_2), 7.61 (d, J = 8.3 Hz, 1H, Ar**H**), 8.07 (dd, J = 8.3 Hz, J = 1.4 Hz, 1H, Ar**H**), 8.42 (d, J = 1.4 Hz, 1H, Ar**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 15.5, 52.9, 125.4, 126.6, 128.8, 132.4, 140.4, 145.1, 165.7.

HRMS calcd for $C_9H_{11}NO_4S_2$ [(M+H)$^+$]: 262.0202, found: 262.0199.

*General Procedure for the Syntheses of **5a−d, f−g, j−m**.* The mixture of *N*-butyl-4-chloro-3-sulfamoyl-benzamide **3** (111 mg, 0.381

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mmol), DMF (4.0 mL), appropriate thiol (0.572 mmol), and K_2CO_3 (210 mg, 1.52 mmol) was heated at 80 °C temperature for 12 h in an inert atmosphere(argon). The mixture was cooled to room temperature and 20 mL H_2O was added. The product was extracted with EtOAc (3 × 8 mL). The organic layer was washed with H_2O, dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure.

*N-Butyl-4-phenylsulfanyl-3-sulfamoyl-benzamide (**5a**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 1:1). Yield: 71 mg, 51%, mp 109−110 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 0.90 (t, J = 7.3 Hz, 3H, CH_3), 1.33 (sext, J = 7.3 Hz, 2H, $CH_2CH_2CH_3$), 1.52 (quint, J = 7.3 Hz, 2H, $CH_2CH_2CH_2$), 3.27 (dt, J = 6.9 Hz, J = 5.7 Hz, 2H, CH_2NH), 6.99 (d, J = 8.3 Hz, 1H, ArH), 7.51−7.58 (m, 5H, ArH), 7.60 (s, 2H, SO_2NH_2), 7.82 (dd, J = 8.3 Hz, J = 2.0 Hz, 1H, ArH), 8.38 (d, J = 2.0 Hz, 1H, ArH), 8.59 (t, J = 5.7 Hz, 1H, NH).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 14.2, 20.1, 31.6, 39.4, 127.5, 129.6, 129.9, 130.5, 130.6, 132.3, 132.4, 134.9, 140.7, 141.2, 164.9.

HRMS calcd for $C_{17}H_{20}N_2O_3S_2$ [(M+H)$^+$]: 365.0988, found: 365.0985.

*N-Butyl-4-cyclohexylsulfanyl-3-sulfamoyl-benzamide (**5b**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 1:1). Yield: 70 mg, 50%, mp 114−115 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 0.92 (t, J = 7.3 Hz, 3H, CH_3), 1.23−1.63 (m, 10H, CH cyclohexyl and butyl), 1.72 (m, 2H, CH cyclohexyl), 1.90−1.96 (m, 2H, CH cyclohexyl), 3.29 (q, J = 6.6 Hz, 2H, CH_2NH), 3.55−3.60 (m, 1H, CHS), 7.34 (s, 2H, SO_2NH_2), 7.70 (d, J = 8.2 Hz, 1H, ArH), 7.96 (dd, J = 8.2 Hz, J = 1.2 Hz, 1H, ArH), 8.36 (d, J = 1.2 Hz, 1H, ArH), 8.64 (t, J = 5.3 Hz, 1H, NH).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 14.2, 20.1, 25.7, 25.8, 31.6, 32.5, 39.4, 44.8, 127.6, 129.7, 130.3, 131.6, 139.0, 142.1, 165.1

HRMS calcd for $C_{17}H_{26}N_2O_3S_2$ [(M+H)$^+$]: 371.1458, found: 371.1463.

*4-Benzylsulfanyl-N-butyl-3-sulfamoyl-benzamide (**5c**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 1:1). Yield: 65 mg, 45%, mp 185−186 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 0.91 (t, J = 7.2 Hz, 3H, CH_3), 1.36 (sext, J = 7.2 Hz, 2H, CH_2CH_3), 1.53 (quint, J = 7.2 Hz, 2H, $CH_2CH_2CH_2$), 3.28 (q, J = 6.9 Hz, 2H, CH_2NH), 4.40 (s, 2H, CH_2S), 7.29 (t, J = 7.1 Hz, 1H, ArH), 7.36 (t, J = 7.1 Hz, 2H, ArH), 7.44 (s, 2H, SO_2NH_2), 7.52 (d, J = 7.1 Hz, 2H, ArH), 7.66 (d, J = 8.3 Hz, 1H, ArH), 7.94 (dd, J = 8.3 Hz, J = 1.9 Hz, 1H, ArH), 8.36 (d, J = 1.9 Hz, 1H, ArH), 8.62 (t, J = 5.6 Hz, 1H, NH).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 14.2, 20.1, 31.6, 36.5, 39.4, 127.6, 127.7, 127.8, 128.9, 129.7, 130.2, 131.2, 136.5, 140.2, 140.7, 165.0.

HRMS calcd for $C_{18}H_{22}N_2O_3S_2$ [(M+H)$^+$]: 379.1145, found: 379.1140.

*N-Butyl-4-(2-phenylethylsulfanyl)-3-sulfamoyl-benzamide (**5d**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 1:1). Yield: 95 mg, 63%, mp 116−117 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 0.92 (t, J = 7.3 Hz, 3H, CH_3), 1.37 (sext, J = 7.3 Hz, 2H, CH_2CH_3), 1.55 (quint, J = 7.3 Hz, 2H, $CH_2CH_2CH_2$), 2.98 (t, J = 7.3 Hz, 2H, CH_2Ar), 3.28 (q, J = 6.6 Hz, 2H, CH_2NH), 3.34−3.38 (m, 2H, CH_2S), 7.25−7.27 (m, 1H, ArH), 7.31−7.33 (m, 4H, ArH), 7.35 (s, 2H, SO_2NH_2), 7.69 (d, J = 8.3 Hz, 1H, ArH), 7.99 (d, J = 8.3 Hz, 1H, ArH), 8.38 (s, 1H, ArH), 8.65 (t, J = 5.4 Hz, 1H, NH).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 14.2, 20.1, 31.6, 33.7, 34.4, 39.4, 126.9, 127.5, 127.9, 128.9, 129.0, 130.4, 131.3, 140.1, 140.3, 141.2, 165.1.

HRMS calcd for $C_{19}H_{24}N_2O_3S_2$ [(M+H)$^+$]: 393.1301, found: 393.1297.

*N-Butyl-4-cyclododecylsulfanyl-3-sulfamoyl-benzamide (**5f**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 3:1). Yield: 76 mg, 44%, mp 166−168 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 0.93 (t, J = 7.3 Hz, 3H, CH_3), 1.24−1.59 (m, 24H, $CH_2CH_2CH_3$ and CH cyclododecyl), 1.72−1.75 (m, 2H, CH cyclododecyl), 3.29 (dt, J = 6.8 Hz, J = 5.6 Hz, 2H, CH_2NH), 3.68 (s, 1H, CHS), 7.33 (s, 2H, SO_2NH_2), 7.66 (d, J = 8.3 Hz, 1H, ArH), 7.99 (dd, J = 8.3 Hz, J = 2.0 Hz, 1H, ArH), 8.37 (d, J = 2.0 Hz, 1H, ArH), 8.64 (t, J = 5.6 Hz, 1H, NH).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 14.2, 20.1, 22.1, 23.4, 23.8, 23.9, 24.2, 29.4, 31.6, 39.4, 43.7, 127.6, 129.5, 130.3, 131.6, 139.7, 142.3, 165.1.

HRMS calcd for $C_{23}H_{38}N_2O_3S_2$ [(M+H)$^+$]: 455.2397, found: 455.2399.

*4-(1-Adamantylsulfanyl)-N-butyl-3-sulfamoyl-benzamide (**5g**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 1:1). Yield: 64 mg, 40%, mp 187−189 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 0.91 (t, J = 6.8 Hz, 3H, CH_3), 1.34 (sext, J = 7.2 Hz, 2H, CH_2CH_3), 1.52 (quint, J = 7.2 Hz, 2H, $NHCH_2CH_2$), 1.63 (br s, 6H, CH adamantanyl), 1.95 (br s, 6H, CH adamantanyl), 2.01 (br s, 3H, CH adamantanyl), 3.28 (q, J = 6.8 Hz, 2H, $NHCH_2$), 7.28 (s, 2H, SO_2NH_2), 7.78 (d, J = 8.0 Hz, 1H, ArH), 7.98 (dd, J = 8.0 Hz, J = 2.0 Hz, 1H, ArH), 8.44 (d, J = 2.0 Hz, 1H, ArH), 8.71 (t, J = 5.6 Hz, 1H, NH).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 14.2, 20.1, 30.1, 31.6, 36.0, 39.5, 43.9, 51.8, 127.4, 129.8, 134.0, 134.5, 138.2, 147.3, 165.2.

HRMS calcd for $C_{21}H_{30}N_2O_3S_2$ [(M+H)$^+$]: 423.1771, found: 423.1777.

*N-Butyl-4-tert-butylsulfanyl-3-sulfamoyl-benzamide (**5j**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 1:1). Yield: 55 mg, 42%, mp 150−152 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 0.91 (t, J = 7.6 Hz, 3H, CH_3), 1.30−1.37 (m, 2H, $CH_2CH_2CH_3$), 1.41 (s, 9H, $C(CH_3)_3$), 1.52 (quint, J = 7.2 Hz, 2H, $NHCH_2CH_2$), 3.28 (q, J = 6.0 Hz, 2H, $NHCH_2$), 7.31 (s, 2H, SO_2NH_2), 7.82 (d, J = 7.6 Hz, 1H, ArH), 7.99 (dd, J = 8.0 Hz, J = 1.6 Hz, 1H, ArH), 8.44 (d, J = 2.0 Hz, 1H, ArH), 8.71 (t, J = 5.8 Hz, 1H, NH).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 14.2, 20.1, 31.6, 31.7, 39.5, 49.4, 127.5, 130.1, 134.3, 136.2, 137.0, 146.7, 165.2.

HRMS calcd for $C_{15}H_{24}N_2O_3S_2$ [(M+H)$^+$]: 345.1301, found: 345.1295.

*N-Butyl-4-isopentylsulfanyl-3-sulfamoyl-benzamide (**5k**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 1:1). Yield: 98 mg, 72%, mp 130−132 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 0.89−0.94 (m, 9H, CH_3), 1.33 (sext, J = 7.2 Hz, 2H, $CH_2CH_2CH_3$), 1.48−1.57 (m, 4H, $NHCH_2CH_2$ and SCH_2CH_2), 1.75 (hept, J = 7.6 Hz, 1H, $CH(CH_3)_2$), 3.09 (t, J = 7.8 Hz, 2H, SCH_2), 3.30 (q, J = 6.4 Hz, 2H, $NHCH_2$), 7.38 (s, 2H, SO_2NH_2), 7.61 (d, J = 8.4 Hz, 1H, ArH), 7.97 (dd, J = 8.3 Hz, J = 1.6 Hz, 1H, ArH), 8.36 (d, J = 1.7 Hz, 1H, ArH), 8.62 (t, J = 5.3 Hz, 1H, NH).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 14.2, 20.1, 22.6, 27.5, 30.4, 31.7, 37.2, 39.4, 127.6(2C), 130.4, 131.1, 140.6, 141.0, 165.1.

HRMS calcd for $C_{16}H_{26}N_2O_3S_2$ [(M+H)$^+$]: 359.1458, found: 359.1464.

*N-Butyl-4-cyclopentylsulfanyl-3-sulfamoyl-benzamide (**5l**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 1:1). Yield: 84 mg, 62%, mp 122−124 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 0.91 (t, J = 7.2 Hz, 3H, CH_3), 1.33 (sext, J = 7.2 Hz, 2H, $CH_2CH_2CH_3$), 1.48−1.67 (m, 6H, $NHCH_2CH_2$ and CH cyclopentyl), 1.71−1.81 (m, 2H, CH cyclopentyl), 2.12−2.21 (m, 2H, CH cyclopentyl), 3.28 (q, J = 6.4 Hz, 2H, $NHCH_2$), 3.92 (quint, J = 6.0 Hz, 1H, SCH), 7.36 (s, 2H, SO_2NH_2), 7.66 (d, J = 8.4 Hz, 1H, ArH), 7.96 (d, J = 7.6 Hz, 1H, ArH), 8.36 (s, 1H, ArH), 8.61 (t, J = 5.2 Hz, 1H, NH).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 14.2, 20.1, 25.1, 31.7, 33.3, 39.4, 44.2, 127.6, 128.5, 130.3, 131.0, 141.0, 141.2, 165.1.

HRMS calcd for $C_{16}H_{24}N_2O_3S_2$ [(M+H)$^+$]: 357.1301, found: 357.1307.

*N-Butyl-4-(1-naphthylsulfanyl)-3-sulfamoyl-benzamide (**5m**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 1:1). Yield: 96 mg, 61%, mp 216−218 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: (DMSO−D_6): 0.86 (t, J = 7.6 Hz, 3H, CH_3), 1.28 (sext, J = 7.6 Hz, 2H, $CH_2CH_2CH_3$), 1.45 (quint, J = 7.2 Hz, 2H, $NHCH_2CH_2$), 3.21 (q, J = 6.8 Hz, 2H, $NHCH_2$), 6.57 (d, J = 8.4 Hz, 1H, ArH), 7.53−7.63 (m, 3H, ArH and, naphthyl-H), 7.68 (t, J = 8.0 Hz, 1H, naphthyl-H), 7.83 (s, 2H,

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SO_2NH_2), 8.03 (d, J = 6.8 Hz, 1H, naphthyl-**H**), 8.08 (d, J = 8.0 Hz, 1H, naphthyl-**H**), 8.15 (d, J = 8.4 Hz, 1H, naphthyl-**H**), 8.19 (d, J = 8.4 Hz, 1H, naphthyl-**H**), 8.41 (d, J = 2.0 Hz, 1H, Ar**H**), 8.50 (t, J = 5.6 Hz, 1H, N**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 14.1, 20.0, 31.6, 39.4, 125.8, 126.9, 127.4, 127.7, 128.1, 128.2(2C), 129.5, 130.4, 131.8, 132.1, 133.7, 134.8, 136.5, 140.4, 140.7, 165.1.

HRMS calcd for $C_{21}H_{22}N_2O_3S_2$ [(M+H)$^+$]: 415.1145, found: 415.1151.

*General Procedure for the Syntheses of **6b, c, e**.* The mixture of *N*-butyl-4-chloro-3-sulfamoyl-benzamide **3** (100 mg, 0.344 mmol) and appropriate amine (2.0 mL) was heated at 130 °C for 24 h in an inert atmosphere. The mixture was cooled to room temperature and 2 N HCl(aq) (5 mL) was added. The product was extracted with EtOAc (3 × 5 mL). The organic layer was washed with H_2O, dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure.

*N-Butyl-4-(cyclohexylamino)-3-sulfamoyl-benzamide (**6b**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 1:1). Yield: 64 mg, 53%, mp 89−91 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 0.91 (t, J = 7.3 Hz, 3H, CH_3), 1.23−1.60 (m, 10H, $CH_2CH_2CH_3$ and C**H** cyclohexyl), 1.66−1.69 (m, 2H, C**H** cyclohexyl), 1.90−1.93 (m, 2H, C**H** cyclohexyl), 3.25 (dt, J = 6.8 Hz, J = 5.7 Hz, 2H, CH_2NH), 3.49−3.52 (m, 1H, C**H**NH), 6,17 (d, J = 7.6 Hz, 1H, N**H**-cyclohexyl), 6.84 (d, J = 8.8 Hz, 1H, Ar**H**), 7.43 (s, 2H, SO_2NH_2), 7.85 (dd, J = 8.8 Hz, J = 2.1 Hz, 1H, Ar**H**), 8.21 (d, J = 2.1 Hz, 1H, Ar**H**), 8.25 (t, J = 5.7 Hz, 1H, N**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 14.2, 20.1, 24.4, 25.8, 31.9, 32.3, 39.2, 50.5, 111.8, 120.9, 124.5, 129.2, 132.3, 146.3, 165.4.

HRMS calcd for $C_{17}H_{27}N_3O_3S$ [(M+H)$^+$]: 354.1846, found: 354.1840.

*4-(Benzylamino)-N-butyl-3-sulfamoyl-benzamide (**6c**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 1:1). Yield: 40 mg, 32%, mp 132−133 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 0.88 (t, J = 7.2 Hz, 3H, CH_3), 1.35 (sext, J = 7.2 Hz, 2H, CH_2CH_3), 1.49 (quint, J = 7.2 Hz, 2H, $CH_2CH_2CH_2$), 3.23 (dt, J = 6.8 Hz, J = 5.9 Hz, 2H, CH_2NH), 4.55 (d, J = 5.8 Hz, 2H, $PhCH_2NH$), 6.69 (d, J = 8.8 Hz, 1H, Ar**H**), 6.82 (t, J = 5.8 Hz, 1H, N**H**-benzyl), 7.23−7.39 (m, 5H, Ar**H**), 7.49 (s, 2H, SO_2NH_2), 7.77 (dd, J = 8.8 Hz, J = 2.0 Hz, 1H, Ar**H**), 8.23 (m, 2H, Ar**H** and N**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 14.2, 20.1, 31.8, 39.2, 46.5, 111.9, 121.5, 125.1, 127.4, 128.9, 128.9, 132.1, 139.2, 146.8, 165.4.

HRMS calcd for $C_{18}H_{23}N_3O_3S$ [(M+H)$^+$]: 362.1533, found: 362.1539.

*N-Butyl-4-(cyclooctylamino)-3-sulfamoyl-benzamide (**6e**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 1:1). Yield: 55 mg, 42%, brownish oily residue.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 0.91 (t, J = 7.3 Hz, 3H, CH_3), 1.35 (sext, J = 7.3 Hz, 2H, CH_2CH_3), 1.44−1.73 (m, 14H, C**H** cyclooctyl and $CH_2CH_2CH_2$), 1.81−1.86 (m, 2H, C**H** cyclooctyl), 3.25 (dt, J = 6.9 Hz, J = 5.9 Hz, 2H, CH_2NH), 3.70−3.73 (m, 1H, C**H**NH cyclooctyl), 6.19 (d, J = 7.6 Hz, 1H, N**H**-cyclooctyl), 6.75 (d, J = 8.8 Hz, 1H, Ar**H**), 7.43 (s, 2H, SO_2NH_2), 7,.86 (dd, J = 8.8 Hz, J = 2.1 Hz, 1H, Ar**H**), 8.19 (d, J = 2.1 Hz, 1H, Ar**H**), 8.24 (t, J = 5.9 Hz, 1H, N**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 14.2, 20.1, 23.6, 25.5, 27.2, 31.7, 31.9, 39.2, 51.9, 111.9, 120.8, 124.6, 129.3, 132.4, 146.1, 165.5.

HRMS calcd for $C_{19}H_{31}N_3O_3S$ [(M+H)$^+$]: 382.2159, found: 382.2155.

*General Procedure for the Syntheses of **7(a, b, h, i, k−m), 8(a, b, k−m)**.* The mixture of appropriate methyl 4-sulfanilsubstituted-3-sulfamoyl-benzoate (**4a, b, h, i, k−m**) or appropriate *N*-butyl-4-sulfanilsubstituted-3-sulfamoyl-benzamide (**8a, b, k−m**) (0.200 mmol), acetic acid (2.0 mL) and 30% H_2O_2 (0.090 mL, 1.15 mmol) was stirred for 12 h. Then 1% Na_2SO_3(aq) (6.0 mL) was added to the mixture and the product was extracted with EtOAc (3 × 5 mL). The organic layer was washed with H_2O, dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure.

*Methyl 4-(Benzenesulfinyl)-3-sulfamoyl-benzoate (**7a**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 3:1). Yield: 56 mg, 82%, mp 107−108 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 3,91 (s, 3H, CH_3O), 7.48−7.53 (m, 3H, Ar**H**), 7.75−7.78 (m, 2H, Ar**H**), 8.09 (s, 2H, SO_2NH_2), 8.32 (d, J = 8.2 Hz, 1H, Ar**H**), 8.37 (dd, J = 8.2 Hz, J = 1.7 Hz, 1H, Ar**H**), 8.47 (d, J = 1.7 Hz, 1H, Ar**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 53.3, 125.6, 126.9, 128.5, 129.9, 131.6, 132.8, 133.9, 142.3, 145.9, 149.7, 164.9.

HRMS calcd for $C_{14}H_{13}NO_5S_2$ [(M+H)$^+$]: 340.0308, found: 340.0311.

*Methyl 4-Cyclohexylsulfinyl-3-sulfamoyl-benzoate (**7b**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 3:1). Yield: 52 mg, 75%, mp 188−189 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 1.14−2.07 (m, 10H, CH_2 cyclohexyl), 2.96−3.04 (m, 1H, CHSO), 3.94 (s, 3H, CH_3O), 7.97 (s, 2H, SO_2NH_2), 8.11 (d, J = 8.2 Hz, 1H, Ar**H**), 8.38 (dd, J = 8.2 Hz, J = 1.7 Hz, 1H, Ar**H**), 8.51 (d, J = 1.7 Hz, 1H, Ar**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 20.9, 25.1, 25.4, 26.1, 28.6, 53.3, 60.9, 127.3, 129.1, 132.5, 132.6, 142.1, 146.5, 165.1.

HRMS calcd for $C_{14}H_{19}NO_5S_2$ [(M+H)$^+$]: 346.0777, found: 346.0783.

*Methyl 4-Methylsulfinyl-3-sulfamoyl-benzoate (**7h**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 5:1). Yield: 50 mg, 90%, mp 258−259 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 2.81 (s, 3H, CH_3SO), 3.95 (s, 3H, CH_3O), 7.97 (s, 2H, SO_2NH_2), 8.34 (d, J = 8.2 Hz, 1H, Ar**H**), 8.43 (dd, J = 8.2 Hz, J = 1.6 Hz, 1H, Ar**H**), 8.47 (d, J = 1.6 Hz, 1H, Ar**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 44.6, 53.3, 125.7, 128.5, 132.6, 133.8, 141.5, 151.2, 165.1.

HRMS calcd for $C_9H_{11}NO_5S_2$ [(M+H)$^+$]: 278.0151, found: 278.0155.

*Methyl 4-Propylsulfinyl-3-sulfamoyl-benzoate (**7i**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 3:1). Yield: 40 mg, 66%, mp 144−145 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 0.99 (t, J = 7.4 Hz, 3H, CH_3CH_2), 1.59−1.62 (m, 1H, $CH_AH_BCH_3$); 1,77−1.81 (m, 1H, $CH_AH_BCH_3$), 2.66−2.69 (m, 1H, CH_AH_BSO), 3.10−3.13 (m, 1H, CH_AH_BSO), 3.93 (s, 3H, CH_3O), 7.97 (s, 2H, SO_2NH_2), 8.26 (d, J = 8.2 Hz, 1H, Ar**H**), 8.41 (dd, J = 8.2 Hz, J = 1.7 Hz, 1H, Ar**H**), 8.48 (d, J = 1.7 Hz, 1H Ar**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 13.2, 16.5, 53.3, 58.9, 126.3, 128.7, 132.5, 133.4, 141.7, 149.2, 165.1.

HRMS calcd for $C_{11}H_{15}NO_5S_2$ [(M+H)$^+$]: 306.0464, found: 306.0470.

*Methyl 4-Isopentylsulfinyl-3-sulfamoyl-benzoate (**7k**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 5:1). Yield: 37 mg, 55%, mp 98−99 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 0.86 (d, J = 6.5 Hz, 3H, CH_3), 0.89 (d, J = 6.5 Hz, 3H, CH_3), 1.40−1.45 (m, 1H, CH_AH_BCH), 1.63−1.68 (m, 2H, $CHCH_3$ and CH_AH_BCH), 2.66−2.69 (m, 1H, CH_AH_BSO), 3.15−3.19 (m, 1H, CH_AH_BSO), 3.94 (s, 3H, CH_3O), 7.97 (s, 2H, SO_2NH_2), 8.26 (d, J = 8.2 Hz, 1H, Ar**H**), 8.41 (dd, J = 8.2 Hz, J = 1.7 Hz, 1H, Ar**H**), 8.49 (d, J = 1.7 Hz, 1H, Ar**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 22.4, 22.8, 27.2, 31.4, 53.3, 55.1, 126.4, 128.7, 132.5, 133.4, 141.7, 149.1, 165.1.

HRMS calcd for $C_{13}H_{19}NO_5S_2$ [(M+H)$^+$]: 334.0777, found: 334.0771.

*Methyl 4-Cyclopentylsulfinyl-3-sulfamoyl-benzoate (**7l**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 4:1). Yield: 51 mg, 77%, mp 109−111 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 1.08−1.18 (m, 1H, C**H** cyclopentyl), 1.43−1.53 (m, 1H, C**H** cyclopentyl), 1.54−1.66 (m, 3H, C**H** cyclopentyl), 1.80−1.94 (m, 2H, C**H** cyclopentyl), 2.00−2.11 (m, 1H, C**H** cyclopentyl), 3.53 (quint, J = 7.6 Hz, 1H, C**H** cyclopentyl), 3.94 (s, 3H, OCH_3), 7.97 (s, 2H, SO_2NH_2), 8.16 (d, J = 8.4 Hz, 1H, Ar**H**), 8.36 (dd, J = 8.0 Hz, J = 1.6 Hz, 1H, Ar**H**), 8.49 (d, J = 1.3 Hz, 1H, Ar**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 21.3, 25.7, 26.3, 29.6, 53.3, 61.7, 126.6, 128.9, 132.4, 132.9, 141.7, 148.0, 165.1.

HRMS calcd for $C_{13}H_{17}NO_5S_2$ [(M+H)$^+$]: 332.0621, found: 332.0615.

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*Methyl 4-(1-Naphthylsulfinyl)-3-sulfamoyl-benzoate (**7m**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 1:1). Yield: 47 mg, 60%, mp 125−127 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 3.93 (s, 3H, CH_3), 7.61−7.68 (m, 4H, naphthyl-**H**), 8.04−8.08 (m, 1H, naphthyl-**H**), 8.11 (s, 2H, SO_2NH_2), 8.14 (d, J = 7.6 Hz, 1H, naphthyl-**H**), 8.22 (d, J = 8.0 Hz, 1H, Ar**H**), 8.39 (dd, J = 1.6 Hz, J = 8.0 Hz, 1H, Ar**H**), 8.49−8.52 (m, 1H, naphthyl-**H**), 8.54 (d, 1H, J = 1.6 Hz, Ar**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 53.4, 123.5, 125.5, 126.3, 127.5, 128.0, 128.7, 128.8, 129.2, 129.8, 132.5, 133.2, 133.8 (2C), 141.9, 143.1, 146.7, 165.0.

HRMS calcd for $C_{18}H_{15}NO_5S_2$ [(M+H)$^+$]: 390.0464, found: 390.0460.

*4-(Benzenesulfinyl)-N-butyl-3-sulfamoyl-benzamide (**8a**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 1:1). Yield: 61 mg, 80%, mp 168−171 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 0.89 (t, J = 7.4 Hz, 3H, CH_3),), 1.32 (sext, J = 7.8 Hz, 2H, NH(CH_2)$_2$$CH_2CH_3$), 1.50 (quint, J = 7.2 Hz, 2H, $NHCH_2CH_2$), 3.27 (q, J = 7.2 Hz, 2H, $NHCH_2$), 7.47−7.53 (m, 3H, Ar**H**), 7.75 (d, J = 6.4 Hz, 2H, Ar**H**), 7.97 (s, 2H, SO_2NH_2), 8.20 (s, 2H, Ar**H**), 8.35 (s, 1H, Ar**H**), 8.77 (t, J = 5.4 Hz, 1H, N**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 14.1, 20.1, 31.5, 39.6, 125.5, 126.4, 127.2, 129.8, 131.5, 131.7, 138.0, 142.0, 146.3, 147.3, 164.8.

HRMS calcd for $C_{17}H_{20}N_2O_4S_2$ [(M+H)$^+$]: 381.0937, found: 381.0933.

*N-Butyl-4-cyclohexylsulfinyl-3-sulfamoyl-benzamide (**8b**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 1:1). Yield: 63 mg, 82%, mp 195−196 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 0.93 (t, J = 7.3 Hz, 3H, CH_3), 1.07−1.18 (m, 3H, C**H** cyclohexyl), 1.29−1.43 (m, 4H, C**H** cyclohexyl and CH_2CH_3), 1.49−1.60 (m, 4H, C**H** cyclohexyl and $CH_2CH_2CH_2$), 1.71−1.74 (m, 1H, C**H** cyclohexyl), 1.81−1.87 (m, 1H, C**H** cyclohexyl), 2.01−2.05 (m, 1H, C**H** cyclohexyl), 3.01 (tt, J = 12.3 Hz, J = 3.6 Hz, 1H, C**H**SO), 3.24−3.29 (m, 2H, CH_2NH), 7.84 (s, 2H, SO_2NH_2), 8.02 (d, J = 8.2 Hz, 1H, Ar**H**), 8.24 (dd, J = 8.2 Hz, J = 1.7 Hz, 1H, Ar**H**), 8.41 (d, J = 1.7 Hz, 1H, Ar**H**), 8.82 (t, J = 5.7 Hz, 1H, N**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 14.2, 20.1, 21.0, 25.1, 25.4, 26.1, 28.5, 31.6, 39.5, 60.9, 126.6, 127.9, 130.3, 137.7, 141.7, 143.8, 164.9.

HRMS calcd for $C_{17}H_{26}N_2O_4S_2$ [(M+H)$^+$]: 387.1407, found: 387.1401.

*N-Butyl-4-isopentylsulfinyl-3-sulfamoyl-benzamide (**8k**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 1:1). Yield: 55 mg, 74%, mp 121−124 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 0.83 (d, J = 6.4 Hz, 6H, S(CH_2)$_2$CH(CH_3)$_2$), 0.92 (t, J = 7.2 Hz, 3H, NH(CH_2)$_3$$CH_3$), 1.35 (sext, J = 7.2 Hz, 2H, $NHCH_2CH_2CH_2$), 1.45−1.66 (m, 5H, $NHCH_2CH_2$, SCH_2CH_2CH), 3.31 (q, J = 6.8 Hz, 2H, $NHCH_2$), 3.66−3.70 (m, 2H, SCH_2), 7.33 (s, 2H, SO_2NH_2), 8.25 (d, J = 8.0 Hz, 1H, Ar**H**), 8.30 (dd, J = 8.0 Hz, J = 1.6 Hz, 1H, Ar**H**), 8.60 (d, J = 1.6 Hz, 1H, Ar**H**), 8.96 (t, J = 5.6 Hz, 1H, N**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 14.2, 20.1, 22.3, 27.0, 30.7, 31.5, 39.6, 53.4, 129.6, 131.5, 133.8, 138.1, 140.4, 143.1, 164.2.

HRMS calcd for $C_{16}H_{26}N_2O_4S_2$ [(M+H)$^+$]: 375.1407, found: 375.1411.

*N-Butyl-4-cyclopentylsulfinyl-3-sulfamoyl-benzamide (**8l**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 1:1). Yield: 47 mg, 63%, mp 103−107 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 0.92 (t, J = 7.2 Hz, 3H, CH_3), 1.09−1.17 (m, 1H, C**H** cyclopentyl), 1.35 (sext, J = 7.2 Hz, 2H, NH(CH_2)$_2$$CH_2CH_3$), 1.45−1.63 (m, 6H, $NHCH_2CH_2$, C**H** cyclopentyl), 1.82−1.94 (m, 2H, C**H** cyclopentyl), 2.01−2.10 (m, 1H, C**H** cyclopentyl), 3.26−3.33 (m, 2H, $NHCH_2$), 3.52 (quint, J = 7.6 Hz, 1H, C**H** cyclopentyl), 7.85 (s, 2H, SO_2NH_2), 8.07 (d, J = 8.4 Hz, 1H, Ar**H**), 8.23 (dd, J = 8.0 Hz, J = 1.6 Hz, 1H, Ar**H**), 8.39 (d, J = 1.6 Hz, 1H, Ar**H**), 8.80 (t, J = 5.6 Hz, 1H, N**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 14.2, 20.1, 21.4, 25.8, 26.3, 29.6, 31.5, 39.6, 61.7, 125.9, 127.7, 130.6, 137.7, 141.3, 145.4, 164.9.

HRMS calcd for $C_{16}H_{24}N_2O_4S_2$ [(M+H)$^+$]: 373.1250, found: 373.1255.

*N-Butyl-4-(1-naphthylsulfinyl)-3-sulfamoyl-benzamide (**8m**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 1:1). Yield: 28 mg, 33%, mp 232−235 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 0.95 (t, J = 7.2 Hz, 3H, CH_3), 1.38 (sext, J = 7.2 Hz, 2H, NH(CH_2)$_2$$CH_2CH_3$), 1.56 (quint, J = 7.2 Hz, 2H, $NHCH_2CH_2$), 3.28−3.37 (m, 2H, $NHCH_2$), 7.63−7.73 (m, 4H, naphthyl-**H**), 8.08 (s, 2H, SO_2NH_2), 8.11−8.13 (m, 2H, naphthyl-**H**, Ar**H**), 8.18−8.22 (m, 1H, naphthyl-**H**), 8.26 (dd, J = 1.6 Hz, J = 8.0 Hz, 1H, Ar**H**), 8.47 (d, 1H, J = 1.6 Hz, Ar**H**), 8.52−8.54 (m, 1H, naphthyl-**H**), 8.86 (t, J = 5.6 Hz, 1H, N**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 14.2, 20.1, 31.5, 39.6, 123.5, 125.3, 126.2, 127.4, 127.5, 128.0, 128.3, 129.2, 129.7, 131.5, 132.3, 133.8, 138.4, 142.0, 142.8, 144.3, 164.8.

HRMS calcd for $C_{21}H_{22}N_2O_4S_2$ [(M+H)$^+$]: 431.1094, found: 431.1088.

*General Procedure for the Syntheses of **9(a−d, h, i, l, m)** and **10(b, k−m)**.* The 30% H_2O_2(aq) (1.08 mmol, 0.110 mL) was added in small portions over 3 h to a solution of appropriate methyl 4-sulfanilsubstituted-3-sulfamoyl-benzoate (**4a−d, h, i, l, m**) or appropriate *N*-butyl-4-sulfanilsubstituted-3-sulfamoyl-benzamide (**10b, k−m**) (0.200 mmol) and acetic acid (2.0 mL) at 70 °C and allowed stirring for 6−8h. The solvent was removed under reduced pressure and the resultant precipitate was filtered, and washed with H_2O.

*Methyl 4-(Benzenesulfonyl)-3-sulfamoyl-benzoate (**9a**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 5:1). Yield: 57 mg, 80%, mp 212−213 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 3.94 (s, 3H, CH_3O), 7.55 (s, 2H, SO_2NH_2), 7.61 (t, J = 7.4 Hz, 2H, Ar**H**), 7.70 (t, J = 7.4 Hz, 1H, Ar**H**), 7.95 (d, J = 7.4 Hz, 2H, Ar**H**), 8.43 (dd, J = 8.2 Hz, J = 1.7 Hz, 1H, Ar**H**), 8.61 (d, J = 8.2 Hz, 1H, Ar**H**), 8,64 (d, J = 1.7 Hz, 1H, Ar**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 53.6, 128.4, 129.6, 130.7, 133.9, 134.3, 134.4, 135.1, 140.6, 141.6, 143.4, 164.5.

HRMS calcd for $C_{14}H_{13}NO_6S_2$ [(M+H)$^+$]: 356.0257, found: 356.0266.

*Methyl 4-Cyclohexylsulfonyl-3-sulfamoyl-benzoate (**9b**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 5:1). Yield: 61 mg, 85%, mp 155−156 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 1.17−1.81 (m, 10H, CH_2 cyclohexyl), 3.86−3.91 (m, 1H, C**H**SO_2), 3.95 (s, 3H, CH_3O), 7.42 (s, 2H, SO_2NH_2), 8.25 (d, J = 8.1 Hz, 1H, Ar**H**), 8.42 (dd, J = 8.1 Hz, J = 1.7 Hz, 1H, Ar**H**), 8.70 (d, J = 1.7 Hz, 1H, Ar**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 24.8, 24.9, 25.1, 53.6, 61.8, 130.9, 133.6, 134.9, 135.2, 138.6, 143.8, 164.6.

HRMS calcd for $C_{14}H_{19}NO_6S_2$ [(M+H)$^+$]: 362.0727, found: 362.0721.

*Methyl 4-Benzylsulfonyl-3-sulfamoyl-benzoate (**9c**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 5:1). Yield: 66 mg, 89%, mp 189−190 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 3.94 (s, 3H, CH_3O), 5.03 (s, 2H, CH_2SO_2), 7.19−7.21 (m, 2H, Ar**H**), 7.30−7.34 (m, 3H, Ar**H**), 7.53 (s, 2H, SO_2NH_2), 7.88 (d, J = 8.0 Hz, 1H, Ar**H**), 8.25 (dd, J = 8.0 Hz, J = 1.8 Hz, 1H, Ar**H**), 8.70 (d, J = 1.8 Hz, Ar**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 53.6, 60.9, 127.9, 129.0, 129.3, 130.6, 131.2, 133.4, 134.5, 135.1, 139.4, 143.7, 164.5.

HRMS calcd for $C_{15}H_{15}NO_6S_2$ [(M+H)$^+$]: 370.0414, found: 370.0419.

*Methyl 4-(2-Phenylethylsulfonyl)-3-sulfamoyl-benzoate (**9d**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 5:1). Yield: 64 mg, 83%, mp 186−187 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 2.98 (t, J = 8.0 Hz, 2H, CH_2Ph), 3.94 (s, 3H, CH_3O), 4.02 (t, J = 8.0 Hz, 2H,CH_2SO_2), 7.18−7.27 (m, 5H, Ar**H**), 7.44 (s, 2H, SO_2NH_2), 8.30 (d, J = 8.0 Hz, 1H, Ar**H**), 8.40 (d, J = 8.0 Hz, 1H, Ar**H**), 8.67 (s, 1H, Ar**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 28.1, 53.6, 55.9, 127.1, 128.9, 129.0, 130.7, 133.9, 134.3, 135.1, 137.9, 140.0, 143.5, 164.5.

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HRMS calcd for $C_{16}H_{17}NO_6S_2$ [(M+H)$^+$]: 384.0570, found: 384.0578.

*Methyl 4-Methylsulfonyl-3-sulfamoyl-benzoate (**9h**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 5:1). Yield: 55 mg, 95%, mp 187−188 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 3.51 (s, 3H, CH_3SO_2), 3.96 (s, 3H, CH_3O), 7.42 (s, 2H, SO_2NH_2), 8.36 (d, J = 8.2 Hz, 1H, Ar**H**), 8.45 (dd, J = 8.2 Hz, J = 1.6 Hz, 1H, Ar**H**), 8.67 (d, J = 1.6 Hz, 1H, Ar**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 44.1, 53.6, 130.5, 133.4, 134.1, 135.1, 141.6, 143.2, 164.6.

HRMS calcd for $C_9H_{11}NO_6S_2$ [(M+H)$^+$]: 294.0101, found: 294.0106.

*Methyl 4-Propylsulfonyl-3-sulfamoyl-benzoate (**9i**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 5:1). Yield: 57 mg, 88%, mp 169−170 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 0.94 (t, J = 7.4 Hz, 3H, CH_3CH_2), 1.63−1.66 (m, 2H, CH_2CH_3), 3.66 (t, J = 7.7 Hz, 2H, CH_2SO_2), 3.96 (s, 3H, CH_3O), 7.43 (s, 2H, SO_2NH_2), 8.33 (d, J = 8.1 Hz, 1H, Ar**H**), 8.44 (dd, J = 8.1 Hz, J = 1.6 Hz, 1H, Ar**H**), 8.69 (d, J = 1.6 Hz, 1H, Ar**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 13.1, 16.2, 53.6, 56.7, 130.7, 133.9, 134.2, 135.2, 140.2, 143.5, 164.6.

HRMS calcd for $C_{11}H_{15}NO_6S_2$ [(M+H)$^+$]: 322.0414, found: 322.0422.

*Methyl 4-Cyclopentylsulfonyl-3-sulfamoyl-benzoate (**9l**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 5:1). Yield: 63 mg, 90%, mp 199−200 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 1.55−1.65 (m, 2H, C**H** cyclopentyl), 1.68−1.76 (m, 2H, C**H** cyclopentyl), 1.77−1.86 (m, 2H, C**H** cyclopentyl), 1.88−1.96 (m, 2H, C**H** cyclopentyl), 3.96 (s, 3H, OCH_3), 4.44 (quint, J = 7.2 Hz, 1H, C**H** cyclopentyl), 7.41 (s, 2H, SO_2NH_2), 8.34 (d, J = 8.0 Hz, 1H, Ar**H**), 8.42 (dd, J = 8.0 Hz, J = 1.6 Hz, 1H, Ar**H**), 8.70 (d, J = 1.6 Hz, 1H, Ar**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 26.0, 27.0, 53.6, 62.7, 130.9, 133.8, 134.6, 135.1, 139.7, 143.6, 164.6.

HRMS calcd for $C_{13}H_{17}NO_6S_2$ [(M+H)$^+$]: 348.0570, found: 348.0560.

*Methyl 4-(1-Naphthylsulfonyl)-3-sulfamoyl-benzoate (**9m**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 8:1). Yield: 63 mg, 78%, mp 223−224 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 3.92 (s, 3H, OCH_3), 7.63−7.67 (m, 4H, naphthyl**H** and SO_2NH_2), 7.74 (t, J = 8.0 Hz, 1H, Ar**H**), 8.12−8.14 (m, 1H, naphthyl**H**), 8.25 (d, J = 8.0 Hz, 1H, Ar**H**), 8.31−8.37 (m, 4H, naphthyl**H**), 8.70 (s, 1H, Ar**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 53.6, 123.9, 125.0, 127.5, 127.8, 129.1, 129.9, 130.8, 131.0, 133.0, 133.8, 134.2, 134.9, 135.2, 135.9, 141.8, 143.5, 164.5.

HRMS calcd for $C_{18}H_{15}NO_6S_2$ [(M+H)$^+$]: 406.0414, found: 406.0423.

*N-Butyl-4-cyclohexylsulfonyl-3-sulfamoyl-benzamide (**10b**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 3:1). Yield: 68 mg, 84%, mp 155−156 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 0.93 (t, J = 7.3 Hz, 3H, CH_3), 1.17−1.81 (m, 14H, $CH_2CH_2CH_3$ and C**H** cyclohexyl), 3.30−3.32 (m, 2H, CH_2NH), 3.90−3.93 (m, 1H, $CHSO_2$), 7.33 (s, 2H, SO_2NH_2), 8.19 (d, J = 8.1 Hz, 1H, Ar**H**), 8.29 (d, J = 8.1 Hz, 1H, Ar**H**), 8.61 (s, 1H, Ar**H**), 8.96 (t, J = 5.3 Hz, 1H, N**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 14.1, 20.1, 24.9, 24.9, 25.1, 31.5, 39.6, 61.8, 129.8, 131.2, 134.4, 136.5, 140.4, 143.4, 164.2.

HRMS calcd for $C_{17}H_{26}N_2O_5S_2$ [(M+H)$^+$]: 403.1356, found: 403.1351.

*N-Butyl-4-isopentylsulfonyl-3-sulfamoyl-benzamide (**10k**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 1:1). Yield: 53 mg, 68%, mp 160−162 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 0.83 (d, J = 6.4 Hz, 6H, $S(CH_2)_2CH(CH_3)_2$), 0.92 (t, J = 7.6 Hz, 3H, $NH(CH_2)_3CH_3$), 1.35 (sext, J = 7.2 Hz, 2H, $NHCH_2CH_2CH_2$), 1.46−1.66 (m, 5H, $NHCH_2CH_2$, SCH_2CH_2CH), 3.31 (q, J = 6.8 Hz, 2H, $NHCH_2$), 3.66−3.70 (m, 2H, SCH_2), 7.33 (s, 2H, SO_2NH_2), 8.25 (d, J = 8.0 Hz, 1H, Ar**H**), 8.30 (dd, J = 8.0 Hz, J = 1.6 Hz, 1H, Ar**H**), 8.60 (s, 1H, Ar**H**), 8.97 (t, J = 5.2 Hz, 1H, N**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 14.2, 20.1, 22.3, 27.0, 30.7, 31.5, 39.7, 53.4, 129.6, 131.5, 133.8, 138.1, 140.4, 143.1, 164.2.

HRMS calcd for $C_{16}H_{26}N_2O_5S_2$ [(M+H)$^+$]: 391.1356, found: 391.1359.

*N-Butyl-4-cyclopentylsulfonyl-3-sulfamoyl-benzamide (**10l**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 1:1). Yield: 57 mg, 73%, mp 143−145 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 0.92 (t, J = 7.6 Hz, 3H, CH_2CH_3), 1.35 (sext, J = 7.6 Hz, 2H, $CH_2CH_2CH_3$), 1.54 (quint, J = 7.2 Hz, 2H, $NHCH_2CH_2$), 1.57−1.65 (m, 2H, C**H** cyclopentyl), 1.68−1.85 (m, 4H, C**H** cyclopentyl), 1.88−1.96 (m, 2H, C**H** cyclopentyl), 3.31 (q, J = 6.8 Hz, 2H, $NHCH_2$), 4.44 (quint, J = 7.2 Hz, 1H, SO_2CH cyclopentyl), 7.32 (br. s, 2H, SO_2NH_2), 8.26−8.31 (m, 2H, Ar**H**), 8.62 (s, 1H, Ar**H**), 8.96 (t, J = 5.6 Hz, 1H, N**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 14.2, 20.1, 26.0, 27.0, 31.5, 39.6, 62.6, 129.7, 131.4, 134.1, 137.7, 140.3, 143.2, 164.2.

HRMS calcd for $C_{16}H_{24}N_2O_5S_2$ [(M+H)$^+$]: 389.1199, found: 389.1205.

*N-Butyl-4-(1-naphthylsulfonyl)-3-sulfamoyl-benzamide (**10m**).* The product was purified by flash chromatography on silica gel ($CHCl_3$:EtOAc, 1:1). Yield: 22 mg, 24%, mp 266−268 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 0.89 (t, J = 7.6 Hz, 3H, CH_2CH_3), 1.32 (sext, J = 7.2 Hz, 2H, $CH_2CH_2CH_3$), 1.51 (quint, J = 7.2 Hz, 2H, $NHCH_2CH_2$), 3.29 (q, J = 6.4 Hz, 2H, $NHCH_2$), 7.52 (s, 2H, SO_2NH_2), 7.62−7.68 (m, 2H, naphthyl**H**), 7.74 (t, J = 8.0 Hz, 1H, naphthyl**H**), 8.13−8.15 (m, 1H, naphthyl**H**), 8.19−8.23 (m, 2H, Ar**H**), 8.30 (d, J = 8.4 Hz, 1H, naphthyl**H**), 8.34−8.39 (m, 2H, naphthyl**H**), 8.60 (d, J = 1.6 Hz, 1H, Ar**H**), 8.92 (t, J = 5.2 Hz, 1H, N**H**).

^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 14.1, 20.1, 31.4, 39.6, 124.0, 125.0, 127.5, 127.8, 129.0, 129.8, 129.9, 130.7, 131.4, 132.7, 134.2, 135.5, 135.8, 139.8, 140.2, 143.1, 164.3.

HRMS calcd for $C_{21}H_{22}N_2O_5S_2$ [(M+H)$^+$]: 447.1043, found: 447.1048.

Protein Preparation. Recombinant human carbonic anhydrases (CAI, CAII, CAIII, CAIV, CAVA, CAVB, CAVI, CAVII, CAIX, CAXII, CAXIII, CAXIV) were expressed and chromatographically purified according to previously published protocols[47] and were used for FTSA experiments. Proteins (CAII and CAXIII) used for crystallization were additionally purified by affinity chromatography and concentrated. CAIX used for crystallization were expressed and purified according to this protocol.[48] Production of recombinant CAII and the extracellular part of CAIX comprising PG and CA domains (residues 38−391) used in SFA experiments is described in ref 49.

Determination of Observed Binding Parameters. *Fluorescent Thermal Shift Assay (FTSA).* Dissociation constants, $K_{d,obs}$, (listed in Table 1) of the compounds binding to CAs were determined by the fluorescent thermal shift assay using a QIAGEN's real-time PCR cycler the "Rotor-Gene Q" and Rotor-Gene Style 4-strip tubes from STARLAB. Ligands were dissolved in DMSO stock solutions to concentrations of 10 mM or 20 mM and used for the serial dilution of the dilution factor 2 in DMSO. These samples were diluted with buffer solution and mixed with a prepared protein solution, consisting of protein stock, buffer solution, and solvatochromic dye 8-anilino-1-naphthalenesulfonate (ANS). All final samples typically contained up to 10 μM CA, compound solutions of serial dilution from 0 μM to 200 μM at 8 different concentrations differing by 2 times, 50 μM ANS, 50 mM sodium phosphate buffer at pH 7.0, 100 mM sodium chloride, and 2.0% (v/v) DMSO. Samples preparation is explained in detail in.[50] The excitation and emission wavelengths of ANS were 365 ± 20 and 460 ± 15 nm. Samples were heated from 25 to 99 °C at the rate of 1 °C/min. The curve-fitting procedure was performed by Thermott[51] at 37 °C. Data are deposited in the public database: Protein−Ligand Binding Database[52] (Database URL: https://plbd.org/).

Stopped-Flow Carbon Dioxide Hydration Assay. Recombinant CAII and the extracellular part of CAIX comprising PG and CA domains (residues 38−391) were used in inhibition assays. A stopped-flow instrument (BioLogic) was used for measuring the CA-catalyzed CO_2 hydration activity in the presence of inhibitors.[53] The assay buffer

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Table 4. Crystallization Conditions Used to Grow Protein Crystals in This Study

PDB ID	isozyme—compound	cystallization buffer	sitting drop
9FPT	CAII—**2**	0.1 M sodium bicine (pH 9.0) and 2 M sodium malonate (pH 7.0)	2 μL of 41 mg/mL CAII solution and 2 μL of crystallization buffer
9FPU	CAII—**3**	0.1 M sodium bicine (pH 9.0) and 2 M sodium malonate (pH 7.0)	2 μL of 41 mg/mL CAII solution and 2 μL of crystallization buffer
9FPQ	CAII—**4c**	0.1 M sodium bicine (pH 9.0), 0.2 M ammonium sulfate, and 2 M sodium malonate (pH 7.0)	2 μL of 41 mg/mL CAII solution and 2.5 μL of crystallization buffer
9FPR	CAII—**4d**	0.1 M sodium bicine (pH 9.0), 0.2 M ammonium sulfate, and 2 M sodium malonate (pH 7.0)	3 μL of 12 mg/mL CAII solution and 3 μL of crystallization buffer
9FPS	CAII—**4h**	0.1 M sodium bicine (pH 9.0) and 2 M sodium malonate (pH 7.0)	3 μL of 12 mg/mL CAII solution and 3 μL of crystallization buffer
9R8X	CAIX—**4d**	1.0 M diammonium hydrogen phosphate and 0.1 M sodium acetate (pH 4.5)	10 mg/mL CAIX solution
9R8Y	CAIX—**5b**	1.0 M diammonium hydrogen phosphate and 0.1 M sodium acetate (pH 4.5)	10 mg/mL CAIX solution
9FPV	CAXIII—**4c**	0.1 M sodium citrate (pH 5.5), 0.1 M sodium acetate (pH 4.5), and 26% of PEG4000	1 μL of 23 mg/mL CAXIII solution and 0.4 μL of crystallization buffer
9FPW	CAXIII—**4d**	0.1 M sodium citrate (pH 5.5), 0.1 M sodium acetate (pH 4.5), and 26% of PEG4000	1 μL of 23 mg/mL CAXIII solution and 0.4 μL of crystallization buffer

consisted of 0.2 mM Phenol Red (pH indicator used in absorbance maximum of 557 nm), 20 mM HEPES Na (pH 7.5), and 20 mM Na_2SO_4. The concentration of CAII and CAIX in the enzyme assay was 4 nM and 1 nM, respectively. To stabilize CAIX during the measurements, 0.0025% dodecyl-β-D-maltopyranoside (DDM, Anatrace, and Anagrade purity) was included in the reaction mixture.

The substrate (CO_2) concentration in the reaction was 8.5 mM. Rates of the CA-catalyzed CO_2 hydration reaction were followed for 30 s at 25 °C. Four traces of substrate conversion in the reaction were fitted by the exponential function to determine the rate for each inhibitor concentration. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitors (100 mM) were prepared in dimethyl sulfoxide, and dilutions of up to 100 nM were made thereafter in DMSO. Apparent K_i' values were obtained from dose−response curves recorded for at least six different concentrations of the test compound by the nonlinear least-squares method using an Excel spreadsheet fitting the Williams-Morrison equation.[54] K_i values were then derived using the Cheng-Prusoff equation.[55] The K_m values used in the Cheng−Prusoff equation were 9.3 mM for CAII and 7.5 mM for CAIX. Inhibition data are provided in Figure S2.

Calculation of the Intrinsic Binding Parameters. The detailed description of the importance and calculation of the intrinsic values have been previously described.[27] For the calculation of the *intrinsic* dissociation constants, the experimentally measured *observed* dissociation constants determined by the FTSA, the pK_a of the sulfonamide group of the compound, and the pK_a of the water molecule bound to the zinc cation by the CA were used.

The intrinsic dissociation constant, $K_{d,int}$, is equal to

$$K_{d,int} = K_{d,obs} \times f_{RSO_2NH^-} \times f_{CAZnH_2O} \quad (1)$$

The fraction of deprotonated sulfonamide:

$$f_{RSO_2NH^-} = \frac{10^{pH-pK_{a,SA}}}{1 + 10^{pH-pK_{a,SA}}} \quad (2)$$

The fraction of Zn(II)-bound water form of CA:

$$f_{CAZnH_2O} = \frac{10^{pK_{a,CA}-pH}}{1 + 10^{pK_{a,CA}-pH}} \quad (3)$$

- $K_{d,obs}$ − observed dissociation constant;
- $f_{RSO_2NH^-}$ and f_{CAZnH_2O} − fractions of deprotonated sulfonamide and Zn(II)-bound water molecule;
- $pK_{a,SA}$ − pK_a of the sulfonamide group;
- $pK_{a,CA}$ − pK_a value of water molecule bound to Zn(II) in the active site of CA.

In this study, the pH value was equal to 7.0.

Determination of pK_a Values of the Compound Sulfonamide Group. The pK_a values of the water molecule bound to Zn^{2+} in the active site of CAs, $pK_{a,CA}$, were taken from ref 42 and of compounds, $pK_{a,SA}$, (Figure S3) were experimentally determined as described in ref S6.

We used a constant concentration of sulfonamide (25−400 μM) and 2.0% (v/v) or 20% (v/v; but only for very poorly soluble ones) of DMSO in universal buffer (50 mM sodium acetate, 25 mM sodium borate, and 50 mM sodium phosphate) at different pH values (in the range from pH 6.0 to 12.0 with 0.5 pH increment). UV/vis spectra of the compound solution were recorded at 37 °C using BMG Labtech CLARIOstarPlus plate reading spectrophotometer. The pK_a values were calculated by normalizing the absorbance and plotting it as a function of pH, then fitting it to the Henderson−Hasselbalch equation using the least-square method. The midpoint of this fitted curve is equal to the sulfonamide group $pK_{a,SA}$.

X-ray Crystallography. *Crystallization.* Crystals of CAII, CAIX, and CAXIII were obtained using the sitting-drop vapor diffusion method at room temperature. Table 4 lists the concentrations of proteins and buffers used for crystallization.

Ligand Soaking. The crystal structures of CAII and CAXIII with ligands were obtained by soaking. A 50 mM solution of each ligand was prepared in dimethyl sulfoxide. One μL of this solution was then diluted using 50 μL of matching reservoir solution corresponding to the conditions under which the crystal was formed. Crystals were incubated for up to 1 week in the soaking solution.

Cocrystallization. The crystal structures of CAIX with ligands were obtained by cocrystallization. Table 4 lists crystallization conditions. The ligand used for cocrystallization was in 5−10 mM concentration, while the stock solution contained 100 mM ligand dissolved in dimethyl sulfoxide.

Data Collection and Structure Determination. Data for CAII and CAXII were collected at PETRA III BEAMLINE P13 (MX1) and for CAIX at BESSY II beamline 14.1.

The data were processed and scaled using XDS,[58] MOSFLM,[59,60] and SCALA.[61] The crystal structures were solved by molecular replacement using MOLREP.[62] The initial model for molecular replacement−CAII: 3HLJ; CAIX: 8Q18,[63] CAXIII: 2NNO. The structure was refined by REFMAC[64] and remodeled using COOT.[57] The 3D models of compounds were constructed by the AVOGADRO[65] program and ligand parameter files were created using LIBCHECK.[66,67]

The data diffraction and final model refinement statistics and PDB IDs are summarized in Table 3. All graphics were created using PyMOL Molecular Graphics System. Authors will release the atomic coordinates upon article publication.

Molecular Docking. The following receptors PDB IDs were chosen for docking: CAII: 3HS4; CAIX: 6G9U, chain A; CAXIII: 4KNN, chain A. The receptors selected from the Protein Data Bank

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differed from the new X-ray structures presented in this Paper to decrease bias. The proteins were prepared for docking using ChimeraX (version 1.9).[68−70] The ligands were optimized using the MMFF94s force field[71−76] within Avogadro molecular viewer (version 1.2.0).[65] For series 7 ligands, two enantiomers of the chiral sulfur were created and docked separately. The format conversions were performed using OpenBabel (The Open Babel Package, version 3.1.1, http://openbabel.org).[77] The docking was performed using the Smina program (version master:dc3dfab+).[34] Smina is based on Autodock Vina.[36] The constrained optimization was done using Smina, using a custom scoring function with a quadratic bias function with weight w=−10 added to the default Vina scoring function.[36] The quadratic constraint forced sulfonamide nitrogen to adhere to its position in the X-ray structure. A cubic docking box of size (24 Å),[3] exhaustiveness 100, and energy range 10 kcal/mol was set as docking parameters. The resulting poses were rescored with Smina using the Vinardo scoring function[35] without the constraint. Only one of the symmetry equivalent poses (e.g., phenyl ring flip) was included when evaluating pose ranking after docking. Heavy atom Root Mean Square Deviations (RMSD) were calculated using DockRMSD software (version 1.1).[78]

The quantum Density Functional Theory (DFT) optimizations were performed using GAMESS-US (version 2019.R2)[40] using DFT functional ωB97X-D[79] with the cc-pVDZ basis set[80,81] and the C-PCM implicit solvation model for water.[82] The conformational search was performed using CREST software (version 3.0.2)[38] using xTB (version 6.7.1)[83] computational engine, employing the GFN2-xTB semiempirical tight binding method[39] and the ALPB implicit solvation model for water.[84]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.5c01421.

FTSA data of compound binding to CA isozymes; SFA data of CA isozyme inhibition by compounds; determination of pK_a values of compound − RSO_2NH_2; NMR spectra of compounds; HPLC chromatograms of representative compounds; ESI-MS spectra of representative compounds; and comparison of docking and crystallographic binding poses (PDF)

SMILE strings of the synthesized compound and corresponding biological activity: *observed* and *intrinsic* ($K_{d,obs}$ and $K_{d,int}$) dissociation constants (in nM units) of investigated compounds to all catalytically active human CAs at 37 °C obtained by FTSA (CSV)

■ AUTHOR INFORMATION

Corresponding Author

Daumantas Matulis − *Department of Biothermodynamics and Drug Design, Institute of Biotechnology, Life Sciences Center, Vilnius University, Vilnius LT-10257, Lithuania;* orcid.org/0000-0002-6178-6276; Email: daumantas.matulis@bti.vu.lt

Authors

Audrius Zakšauskas − *Department of Biothermodynamics and Drug Design, Institute of Biotechnology, Life Sciences Center, Vilnius University, Vilnius LT-10257, Lithuania*

Vaida Paketurytė-Latvė − *Department of Biothermodynamics and Drug Design, Institute of Biotechnology, Life Sciences Center, Vilnius University, Vilnius LT-10257, Lithuania;* orcid.org/0000-0003-0919-7826

Alberta Jankūnaitė − *Department of Biothermodynamics and Drug Design, Institute of Biotechnology, Life Sciences Center, Vilnius University, Vilnius LT-10257, Lithuania*

Edita Čapkauskaitė − *Department of Biothermodynamics and Drug Design, Institute of Biotechnology, Life Sciences Center, Vilnius University, Vilnius LT-10257, Lithuania*

Yann Becart − *Department of Biothermodynamics and Drug Design, Institute of Biotechnology, Life Sciences Center, Vilnius University, Vilnius LT-10257, Lithuania*

Alexey Smirnov − *Department of Biothermodynamics and Drug Design, Institute of Biotechnology, Life Sciences Center, Vilnius University, Vilnius LT-10257, Lithuania*

Klára Pospíšilová − *Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences, Prague 6 16610, Czech Republic*

Janis Leitans − *Latvian Biomedical Research and Study Centre, Riga LV-1067, Latvia*

Jiří Brynda − *Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences, Prague 6 16610, Czech Republic*

Andris Kazaks − *Latvian Biomedical Research and Study Centre, Riga LV-1067, Latvia*

Lina Baranauskienė − *Department of Biothermodynamics and Drug Design, Institute of Biotechnology, Life Sciences Center, Vilnius University, Vilnius LT-10257, Lithuania;* orcid.org/0000-0002-9924-9177

Elena Manakova − *Department of Protein−DNA Interactions, Institute of Biotechnology, Life Sciences Center, Vilnius University, Vilnius LT-10257, Lithuania*

Saulius Gražulis − *Sector of Crystallography and Cheminformatics, Institute of Biotechnology, Life Sciences Center, Vilnius University, Vilnius LT-10257, Lithuania*

Visvaldas Kairys − *Department of Bioinformatics, Institute of Biotechnology, Life Sciences Center, Vilnius University, Vilnius LT-10257, Lithuania;* orcid.org/0000-0002-5427-0175

Kaspars Tars − *Latvian Biomedical Research and Study Centre, Riga LV-1067, Latvia;* orcid.org/0000-0001-8421-9023

Pavlína Řezáčová − *Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences, Prague 6 16610, Czech Republic*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jmedchem.5c01421

Author Contributions

A.Z. and V.P.-L. contributed equally to this paper.

Notes

The authors declare the following competing financial interest(s): Authors declare that they have patents and patent applications pending on CA inhibitors.

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■ ABBREVIATIONS

CA, human carbonic anhydrase; FTSA, fluorescent thermal shift assay (or differential scanning fluorimetry DSF); int, intrinsic; $K_{d,int}$, intrinsic equilibrium dissociation constant; $K_{d,obs}$, observed equilibrium dissociation constant; obs, observed; $pK_{a,CA}$, pK_a value of water molecule bound to Zn(II) in the active site of CA; $pK_{a,SA}$, pK_a of the sulfonamide group; SFA, stopped-flow carbon dioxide hydration assay

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GYVENIMO APRAŠYMAS

Vardas Pavardė Audrius Zakšauskas

Išsilavinimas

1995-1997 Vilniaus Universitetas, Chemijos fakultetas, magistro studijos. Suteiktas chemijos magistro kvalifikacinis laipsnis. Magistro darbo tema: „Pakeistų titanilo ftalocianinų sintezė“

1991-1995 Vilniaus Universitetas, Chemijos fakultetas, bakalauro studijos. Suteiktas chemijos bakalauro kvalifikacinis laipsnis. Bakalauro baigiamojo darbo tema: “Biciklo[3.3.1]nonan-2,6-diono oksidacija talio(III) druskomis

Pagrindinės mokslinės veiklos kryptys

2013-iki dabar Vilniaus universitetas, Biotechnologijos Institutas, Biotermodinamikos ir vaistų tyrimo skyrius. Fermentų slopiklių sintezė.

2002-2013 Vilniaus universitetas, Taikomųjų mokslų institutas, Skystųjų kristalų laboratorija. Organinių junginių sintezė.

1994-2001 Vilniaus universitetas, Chemijos fakultetas, Organinės chemijos katedra. Organinių junginių sintezė. Darbo vadovai doc. P.Kadziauskas, prof. A.Undzėnas.

Mokymo patirtis

1997-1999 Vilniaus universitetas, Chemijos fakultetas, Organinės chemijos katedra. Magistro baigiamojo darbo vadovas

2015-2016 Vilniaus universitetas, Biotechnologijos Institutas, Biotermodinamikos ir vaistų tyrimo skyrius. Bakalauro baigiamojo darbo vadovas

Darbo patirtis

2013- iki dabar Vilniaus universitetas, Biotechnologijos Institutas, Biotermodinamikos ir vaistų tyrimo skyrius, j.m. darbuotojas

2007-2013 UAB „Tikslioji sintezė“, chemikas.

2002-2013 Vilniaus universitetas, Taikomųjų mokslų institutas, Skystųjų kristalų laboratorija, vyresnysis inžinierius

1994-1998 Vilniaus universitetas, Chemijos fakultetas, Organinės chemijos katedra, laborantas

Dalyvavimas projektuose

1. Junginių efektyvumo slopinant SARS CoV-2 rekombinantinius virusinius fermentus įvertinimo technologijos prototipo sukūrimas (296 127 Eur.,

2021-2023, 3.1.1-LMT-K-718-05-0001. Vadovas J. Matulienė, Vykdytojas VU).

2. Vėžinių auglių išplitimo diagnostikos ir metastazių vaizdinimo operacijos metu sistemų vystymas naudojant CA IX biožymenį.(140 000 Eur., 2020-2021. Sveikas senėjimas Nr. S-SEN-20-10. Vadovas J. Matulienė. Vykdytojas VU)
3. Karboanhidrazių-slopiklių atpažinimo mechanizmas – priešvėžinės terapijos link.(100 000 Eur., 2017-2020. MIP-17-87. Vadovas D. Matulis. Vykdytojas VU)
4. Žmogaus karboanhidrazės IX, kaip vėžinių ląstelių žymens, taikymo onkologinių ligų diagnostikai, vaizdinimui bei prognozei, tyrimas.(Eur., 2015-2017. Sveikas senėjimas Nr. SEN-04/2015. Vadovas J. Matulienė. Vykdytojas VU)
5. Nuo 2014-04 iki 2014-12-31. Karboanhidrazės hCA XII, kaip vėžinių ląstelių žymens, diagnostinio potencialo įvertinimas. (590 500 Lt, 2012 05 02 - 2014 12 31, ES struktūrinių fondų lėšos, Lėtinės neinfekcinės ligos LIG-09/2012 , vadovas D. Matulis. Vykdytojas VU)
6. Nuo 2013-05-24 iki 2013-10-09. Nauji lanksčios struktūros bifluoreno junginiai optoelektronikos pramonei (BiFluorenas). (1 460 000 Lt, 2013-2015, VP1-3.1-ŠMM-10-V-02-023/LSS-23000-933, vykdytojas VU)
7. Inovatyvūs tiofeno junginiai OLED technologijai bei fotovoltainei konversijai (saulės baterijoms). **(**706 090 Lt, 2009-2012, „Intelektas LT“ VP2-1.3-ŪM-02-K-01-011, vykdytojas UAB “Tikslioji sintezė“).
8. Organinės elektronikos medžiagos ir prietaisai energiją taupančioms technologijoms**.** (2007-2009, VMSF remiamas Aukštųjų technologijų plėtros programos projektas, vykdytojas VU).

Mokslinės publikacijos

1. **Zakšauskas, A.,** Paketurytė-Latvė, V., Jankūnaitė, A., Čapkauskaitė, et al. Affinity and Selectivity of Protein–Ligand Recognition: A Minor Chemical Modification Changes Carbonic Anhydrase Binding Profile. *Journal of Medicinal Chemistry*. 2025, 68(16), 17752-17773. doi: 10.1021/acs.jmedchem.5c01421
2. Bagdonas, M., Stančaitis, L., Urniežius, E., **Zakšauskas**, **A**., Mickevičiūtė, A., Kananavičiūtė, R., et al. Design, synthesis, and binding analysis of target-specific covalent inhibitors of SARS-CoV-2 papain-like protease. *European Journal of Medicinal Chemistry Reports*. 2025, 15, 100306. doi.org/10.1016/j.ejmcr.2025.100306
3. Petrosiute, A., **Zakšauskas, A**., Lučiūnaitė, A., Petrauskas, V., Baranauskienė, L., Kvietkauskaitė, A., et al. Carbonic anhydrase IX

inhibition as a path to treat neuroblastoma. *British Journal of Pharmacology*, 2025, 182(7), 1610-1629. doi.org/10.1111/bph.17429

4. Paketurytė-Latvė, V., Smirnov, A., Manakova,E., Baranauskiene, L., Petrauskas, V., Zubrienė, A., Matulienė, J., Dudutienė, V., Čapkauskaitė, E., **Zakšauskas, A**., et al. From X-ray crystallographic structure to intrinsic thermodynamics of protein–ligand binding using carbonic anhydrase isozymes as a model system. *IUCrJ*, 2024, 11|, 4, 556-569. doi.org/10.1107/S2052252524004627
5. Žvinys, G., Petrosiute, A., **Zakšauskas, A**., Zubrienė, A., et al. High-Affinity NIR-Fluorescent Inhibitors for Tumor Imaging via Carbonic Anhydrase IX. *Bioconjugate Chem*, 2024, 35, 6, 790–803. doi.org/10.1021/acs.bioconjchem.4c00144
6. Lingė, D., Gedgaudas, M., Merkys, A., Petrauskas, V., Vaitkus, A., Grybauskas, A., Paketurytė, V., Zubrienė, A., **Zakšauskas, A**., et al. PLBD: protein–ligand binding database of thermodynamic and kinetic intrinsic parameters, *Database*, Volume 2023, 2023, baad040.doi.org/10.1093/database/baad040
7. Matulienė, J., Žvinys, G., Petrauskas, V., Kvietkauskaitė, A., **Zakšauskas, A**., et al. Picomolar fluorescent probes for compound affinity determination to carbonic anhydrase IX expressed in live cancer cells. *Scientific Reports,* 2022**,** 12, 17644. doi.org/10.1038/s41598-022-22436-1
8. **Zakšauskas, A.,** Čapkauskaitė, E., Paketurytė-Latvė, V., Smirnov, A., Leitans, J., Kazaks, A., Dvinskis, E., Stančaitis, L., Mickevičiūtė, A., Jachno, J., Jezepčikas, L., Linkuvienė, V., Sakalauskas, A., Manakova, E., Gražulis, S., Matulienė, J., Tars, K., Matulis, D. Methyl 2-Halo-4-Substituted-5-Sulfamoyl-Benzoates as High Affinity and Selective Inhibitors of Carbonic Anhydrase IX. *Int. J. Mol. Sci*. 2022, 23, 130. doi.org/10.3390/ijms23010130
9. Smirnovienė, J., Smirnov, A., **Zakšauskas, A**., Zubrienė, A., Petrauskas, V., Mickevičiūtė, A., Michailovienė, V., Čapkauskaitė, E., Manakova, E., Gražulis, S., Baranauskienė, L., Wen-Yih Chen, Ladbury, J.E., Matulis, D. Switching the Inhibitor-Enzyme Recognition Profile via Chimeric Carbonic Anhydrase XII. *ChemistryOpen*, 2021, 10(5), 567-580. doi:10.1002/open.202100042
10. **Zakšauskas, A**., Čapkauskaitė, E., Jezepčikas, L., Linkuvienė, V., Paketurytė, V., Smirnov, A., Leitans, J., Kazaks, A., Dvinskis, E., Manakova, E., Gražulis, S., Tars, K., Matulis, D. Halogenated and Di-Substituted Benzenesulfonamides as Selective Inhibitors of Carbonic Anhydrase Isoforms. *European Journal of Medicinal Chemistry*, 2020, 185, 111825. Doi:org/10.1016/j.ejmech.2019.111825.

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13. Čapkauskaitė, E., **Zakšauskas, A**., Ruibys, V., Linkuvienė, V., Paketurytė, V., Gedgaudas, M., Kairys, V., Matulis. D. Benzimidazole design, synthesis, and docking to build selective carbonic anhydrase VA inhibitors. *Bioorganic & Medicinal Chemistry*. 2018, 26(3), 675-687. DOI: 10.1016/j.bmc.2017.12.035.
14. Zubrienė, A., Smirnov, A., Dudutienė, V., Timm, D. D., Matulienė, J., Michailovienė, V., **Zakšauskas, A**., Manakova, E., Gražulis, S., Matulis, D. Intrinsic Thermodynamics and Structures of 2,4- and 3,4-Substituted Fluorinated Benzenesulfonamides Binding to Carbonic Anhydrases. *ChemMedChem*, 2017, 12 (2), 161-176. DOI: 10.1002/cmdc.201600509.
15. Juozapaitienė, V., Bartkutė, B., Michailovienė, V., **Zakšauskas, A**., Baranauskienė, L., Satkūnė, S., Matulis, D. 2016. Purification, enzymatic activity and inhibitor discovery for recombinant human carbonic anhydrase XIV. *Journal of Biotechnology*. 2016, 240, 31-42. DOI: 10.1016/j.jbiotec.2016.10.018
16. V. Dudutienė, A. Zubrienė, A. Smirnov, D. D. Timm, J. Smirnovienė, J. Kazokaitė, V. Michailovienė, **A. Zakšauskas**, E. Manakova, S. Gražulis and D. Matulis. Functionalization of Fluorinated Benzenesulfonamides and Their Inhibitory Properties toward Carbonic Anhydrases. *ChemMedChem*, 2015, 10 (4), 662–687. DOI: 10.1002/cmdc.201402490
17. Dudutienė, V., Matulienė, J., Smirnov, A., Timm, D., Zubrienė, A., Baranauskienė, L., Morkūnaitė, V., Smirnovienė, J., Michailovienė, V., Juozapaitienė, V., Mickevičiūtė, A., Kazokaitė, J., Bakšytė, S., Kasiliauskaitė, A., Jachno, J., Revuckienė, J., Kišonaitė, M., Pilipuitytė, V., Ivanauskaitė, E., Milinavičiūtė, G., Smirnovas, V., Petrikaitė, V., Kairys, V., Petrauskas, V., Norvaišas, P., Lingė, D., Gibieža, P., Čapkauskaitė, E., **Zakšauskas, A**., Kazlauskas, E., Manakova, E., Gražulis, S., Ladbury, J., Matulis, D. Discovery and characterization of

novel selective inhibitors of carbonic anhydrase IX. *Journal of Medicinal Chemistry,* 2014, 57 (22), 9435–9446. DOI**:** 10.1021/jm501003k
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19. **Zakšauskas, A**.; Kadziauskas, P.; Undzėnas, A. Hydroxygallium phthalocyanine and its forms. *Environmental and chemical physics*., 2000, Vol. 22, no 3-4. ISSN 1392-740X p. 145-150

Patentinės paraiškos

1. EP4255886(A1) D. Matulis, **A. Zakšauskas**, A. Zubrienė, L. Baranauskienė, J. Matulienė, V. Dudutienė, E. Čapkauskaitė, V. Paketurytė, J. Smirnovienė. Carbonic anhydrase inhibitors synthesized on interconnecting linker chains
2. US11312682 (B2) E. Čapkauskaitė, **A. Zakšauskas**, V. Morkūnaitė, D. Matulis. Selective inhibitors of carbonic anhydrase.
3. Lietuvos patentas Nr. 5510B Ona Adomėnienė, Povilas Adomėnas, **Audrius Zakšauskas**, Virginija Žvinytė. 2-(4'-fenilbenzoil)benzoinės rūgšties gamybos būdas. Paraiškos numeris 2006 084. Patento paskelbimo data 2008-07-25 Process for preparing of 2-(4'-phenylbenzoyl)benzoic acid.

Vilniaus universiteto leidykla
Saulėtekio al. 9, III rūmai, LT-10222 Vilnius
El. p. info@leidykla.vu.lt, www.leidykla.vu.lt
bookshop.vu.lt, journals.vu.lt
Tiražas 15 egz.