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UNIVERSITĀTE

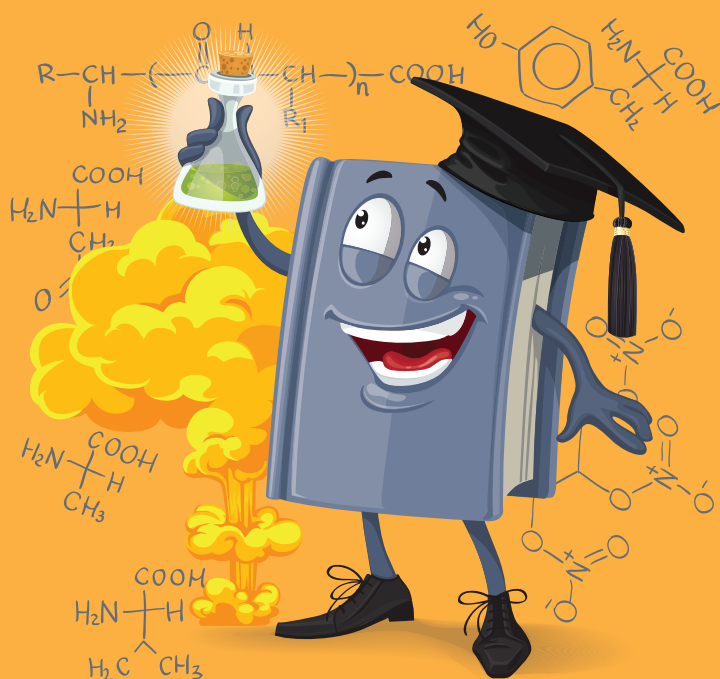
Konstantinos Grammatoglou

**JAUNU METOŽU IZSTRĀDE PLP ATKARĪGO ENZĪMU
INHIBITORU SINTĒZEI UN BAKTĒRIJU ENZĪMA
O-ACETILSERĪNA SULFHIDRILĀZES KOVALENTĀS
INHIBĪCIJAS POTENCIĀLA IZPĒTE**

Promocijas darba kopsavilkums

**THE DEVELOPMENT OF NEW METHODS FOR THE
SYNTHESIS OF PLP-DEPENDENT ENZYME INHIBITORS
AND EXPLORING COVALENT INHIBITION POTENTIAL OF
BACTERIAL ENZYME O-ACETYL SERINE SULFHYDRYLASE**

Summary of the Doctoral Thesis



RĪGAS TEHNISKĀ UNIVERSITĀTE

Materiālzinātnes un lietišķās ķīmijas fakultāte

Organiskās ķīmijas tehnoloģijas institūts

RIGA TECHNICAL UNIVERSITY

Faculty of Materials Science and Applied Chemistry

Institute of Technology of Organic Chemistry

Konstantinos Grammatoglou

Doktora studiju programmas “Ķīmija” doktorants

Doctoral Student of the Study Programme “Chemistry”

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Scientific supervisor

Profesors/Professor *Dr. chem.* Aigars Jirgensons

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PROMOCIJAS DARBS IZVIRZĪTS ZINĀTNES DOKTORA GRĀDA IEGŪŠANAI RĪGAS TEHNISKAJĀ UNIVERSITĀTĒ

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OFICIĀLIE RECENZENTI

Dr. chem. Mārtiņš Katkevičs,
Latvijas Organiskās sintēzes institūts, Latvija

Dr. chem. Artis Kinēns,
Latvijas Universitāte, Latvija

Dr. Marc Nazare,
Leibnīcas Molekulārās farmakoloģijas institūts, Vācija

APSTIPRINĀJUMS

Apstiprinu, ka esmu izstrādājis šo promocijas darbu, kas iesniegts izskatīšanai Rīgas Tehniskajā universitātē zinātnes doktora (*Ph. D.*) grāda iegūšanai. Promocijas darbs zinātniskā grāda iegūšanai nav iesniegts nevienā citā universitātē.

Konstantinos Grammatoglou(paraksts)

Datums

Promocijas darbs sagatavots kā tematiski vienota zinātnisko publikāciju kopa. Tajā ietvertas četras zinātniskās publikācijas, kas uzrakstītas angļu valodā, un kopsavilkums latviešu un angļu valodā. Kopējais apjoms, ieskaitot elektroniski pieejamo informāciju, ir 301 lappuse.

SAĪSINĀJUMI

AIBN	α,α' -azobisisobutironitrils
Boc	<i>tert</i> -butoksikarbonil-
CADD	datorizēta zāļu izstrāde
CAN	cerija amonija nitrāts
CDI	1,1'-karbonildiimidazols
DCE	1,2-dihloretāns
DCM	dihlormetāns
DDQ	5,6-diciāno-2,3-dihloro-1,4-benzohinons
DFSI	asparagīnskābes–fenilalanīna–serīna–izoleicīna peptīds
DIPEA	diizopropiletilamīns
DMAP	4-dimetilaminopiridīns
EDC	1-etil-3-(3-dimetilaminopropil)karbodiimīds
HATU	heksafluorfosfāts azabenzotriazols tetrametiluronijns
HPLC	augstas veiktspējas šķidrums hromatogrāfija
IC ₅₀	puse no maksimālās inhibējošās koncentrācijas
ist. t.	istabas temperatūra
LC/MS	šķidrums hromatogrāfija – masas spektrometrija
MeCN	acetoniitrils
NBS	<i>N</i> -bromsukcinimīds
<i>n</i> Bu	<i>n</i> -butil-
NMO	<i>N</i> -metilmorfolīna <i>N</i> -oksīds
OASS	<i>O</i> -acetilserīna sulfhidrilāze
PDB	proteīnu datu banka
Ph	fenil-
PLP	piridoksāla 5'-fosfāts (B6 vitamīns)
PMB	<i>p</i> -metoksibenzil-
RCSB	Strukturālās bioinformātikas pētījumu sadarbības centrs
PVO	Pasaules Veselības organizācija
SAR	struktūras–aktivitātes likumsakarības
SAT	serīna acetiltransferāze
TBA·BF ₄	tetrabutilamonija tetrafluorborāts
<i>t</i> Bu	<i>tert</i> -butil-
TBS	<i>tert</i> -butildimetilsilil-
TFA	trifluoretiķskābe
THF	tetrahidrofurāns
triF-Ala	3,3,3-trifluoralanīns

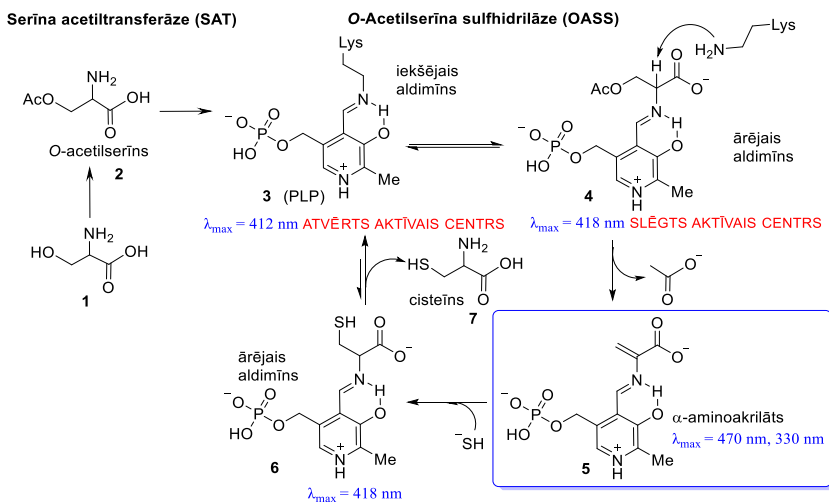
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izveidojās rezistence.^{1e} 2001. gadā Pasaules Veselības organizācija (PVO) pasludināja mikrobiālo rezistenci par “globālu sabiedrības veselības problēmu” un norādīja, ka zinātniskais izrāviens līdz mūsdienām šajā jomā nav noticis. Jaunatkāto antibiotiku skaits pēdējās desmitgadēs ir diezgan ierobežots, un lielākā daļa zāļu, kas nonāk klīnikā, strādā pēc tiem pašiem mehānismiem kā tradicionālās antibiotikas.^{1e}

Meklējot jaunas, efektīvas antibiotikas, ir izmantotas dažādas stratēģijas, piemēram, hibrīdu pretmikrobu līdzekļu, membrānas aktīvu zāļu un baktēriju virulences un patogēneses inhibitoru izstrāde, kā arī alternatīvu metožu, piemēram, bakteriofāgu izmantošana. Cita jaunu antibiotiku izstrādes metode ir vērsta uz galvenajām baktēriju vielmaiņas funkcijām. Mērķošana uz svarīgiem, bet nebūtiskiem gēnu produktiem var būt efektīvs līdzeklis baktēriju pielāgošanās un izdzīvošanās mazināšanai, kā rezultātā tiek uzlabota ārstēšana ar antibiotikām, saīsināti latentuma periodi, palielināta jutība pret tradicionālajām antibiotikām un tādējādi atvieglota saimnieka imūnsistēmas spēja izvadīt patogēnu.³

Sērs ir daudzu biomolekulu pamatkomponents, sākot no aminoskābēm, piemēram, cisteīnu, līdz kofaktoriem un savienojumiem, kas kontrolē redoks homeostāzi. Enzīmi, kas iesaistīti cisteīna biosintēzes ceļā un atrodami patogēnos un augos, bet nav sastopami zīdītājos, ir ļoti pievilcīgi antibakteriālie mērķi. Ir pierādīts, ka cisteīna biosintēzes inhibēšana ietekmē patogēnu spēju cīnīties ar oksidatīvo stresu, samazina to virulenci un rezistenci pret antibiotikām.^{3,4} Cisteīna biosintēzes pēdējie posmi ietver divu enzīmu darbību – serīna acetiltransferāzes (SAT), kas acetilē serīnu, un *O*-acetilserīna sulfhidrilāzes (OASS), no piridoksāla 5'-fosfāta (PLP) atkarīgu enzīmu, kas veic β-aizvietošanas reakciju acetilserīnā (1. shēma).

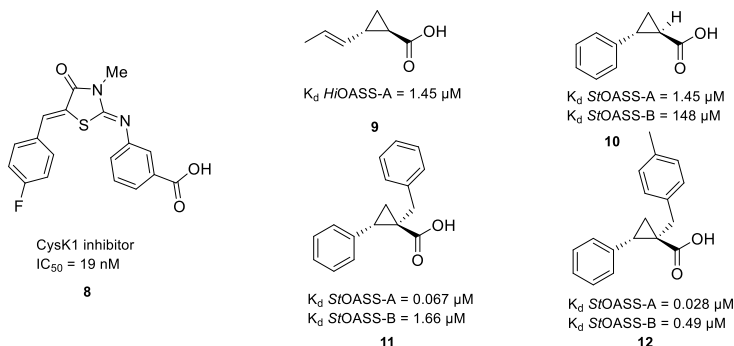


1. shēma. OASS katalītiskais cikls cisteīna (7) veidošanai.

OASS baktērijās atrodas divās izoformās, OASS-A un OASS-B, ko kodēšanas gēnos sauc arī par CysK un CysM. Serīna acetiltransferāze (SAT) spēj veidot augstas afinitātes kompleksu ar OASS-A, bet ne ar OASS-B.⁵

Lai izveidotu no PLP atkarīgu enzīmu inhibitorus, izmēģinātas vairākas pieejas, izmantojot struktūrā, ligandos vai mehānismā balstītu dizainu. Pirmie mēģinājumi bija vērsti

uz dabiska pentapeptīda struktūrelementu atdarināšanu, veidojot OASS ligandus ar afinitāti mikromolārā diapazonā. Tā kā peptīdiskiem inhibitoriem nereti nepiemīt zāļvielām raksturīgās īpašības, izmantojot *in silico* un sintētiskās ķīmijas metodes, tika izveidoti dažādi aktīvi mazmolekulāri savienojumi (2. att.).⁶



2. att. Zināmie OASS-A (CysK) inhibitori.

Mehānismā balstīti inhibitori ķīmiskas reakcijas rezultātā veido kovalentu neatgriezenisku proteīna–inhibitora kompleksu, kas kavē fermenta katalītisko darbību. Ir pētīts un ziņots par virkni kemotipu, kas darbojas kā mehānismā balstīti no PLP atkarīgu enzīmu inhibitori.⁷

Mikrobiālās rezistences pieaugums ir aktualizējis steidzamāku jaunu medikamentu mērķu noteikšanu. Lai gan PLP atkarīgie enzīmi veido apmēram 4 % no Enzīmu komisijas klasificētajiem enzīmiem, tikai neliels skaits no tiem ir identificēti kā potenciālie terapeitisko līdzekļu mērķi, un vēl mazāk ir tie, kuriem ir izstrādātas zāles. Šis ierobežotais skaits ir saistīts ar zināšanu trūkumu par PLP atkarīgo enzīmu saimi un to nozīmi dažādos bioloģiskos procesos. Tomēr tas, ka PLP piedalās dažādās reakcijās un ka dažādu PLP atvasinājumu spektrālās izmaiņas ievērojami palīdz kontrolēt reakcijas gaitu, ir tikai divas no iezīmēm, kas padara šos enzīmus kā interesantus pētījumu objektus.^{7d} Šajā aspektā enzīmi, kas iesaistīti cisteīna biosintēzes ceļā, ir ļoti aktuāli. OASS ir nozīmīgs cisteīna biosintēzes enzīms, kas ir potenciāls antibakteriālo zāļvielu mērķis, tāpēc ir svarīgi izstrādāt šī enzīma inhibitorus, lai izpētītu to lomu antibakteriālo zāļu atklāšanā. Ir nepieciešams identificēt jaunus kemotipus PLP atkarīgo enzīmu inhibēšanai un izstrādāt sintētiskās metodes šo jauno kemotipu iegūšanai.

Pētījuma mērķis un uzdevumi

Promocijas darba mērķis ir mehānismā balstītu no PLP atkarīgā enzīma *O*-acetilserīna sulfhidrilāzes (OASS) inhibitoru sintēze, balstoties esošajos kemotipos, jaunu kemotipu izpētē un jaunu sintētisko metožu izstrādē no PLP atkarīgu enzīmu potenciālo inhibitoru iegūšanai.

Darba mērķa īstenošanai definēti vairāki darba uzdevumi.

1. Izplānot un sintezēt fokusētu potenciālo OASS inhibitoru bibliotēku.
2. Izstrādāt efektīvu metodi kvaternāro alkīnīlglicīnolu sintēzei.

3. Izstrādāt sintētiskās metodes karbonskābes bioizostēra – tetrazola – ieviešanai molekulā.

Zinātniskā novitāte un galvenie rezultāti

Zinātnisko pētījumu gaitā pārbaudīti vairāki zināmi un jauni savienojumi kā potenciāli baktēriju *O*-acetilserīna sulfhidrilāzes (OASS) inhibitori. Trifluoralanīns atklāts kā pirmais mehānismā balstītais OASS inhibitors. Izpētītas trifluoralanīna atvasinājumu struktūras aktivitātes likumsakarības (SAL).

Izstrādātas vairākas jaunas metodes no PLP atkarīgo enzīmu inhibitoru sintēzei.

1. C-Kvaternāro alkīlilglicīnolu sintēzes metode.
2. Metode tiešai tetrazolu C–H funkcionalizēšanai, izmantojot turbo Grinjāra reaģentu.
3. Metode tetrazolu funkcionalizēšanai ar elektroķīmiski nošķeļamu *N*-aizsarggrupu.
4. Metode stereoselektīvai aminoskābju analogu sintēzei, kas satur tetrazolu kā karbonskābes aizvietotāju.

Darba struktūra un apjoms

Promocijas darbs ir tematiski vienota zinātnisku publikāciju kopa. Publikācijās aprakstīta trifluoralanīna analogu sintēze un no PLP atkarīgu enzīmu inhibitoru sintēzes metožu izstrāde.

Publikācijas un darba aprobācija

Darba galvenie rezultāti apkopoti četrās publikācijās. Pētījuma rezultāti prezentēti astoņās konferencēs.

Zinātniskās publikācijas

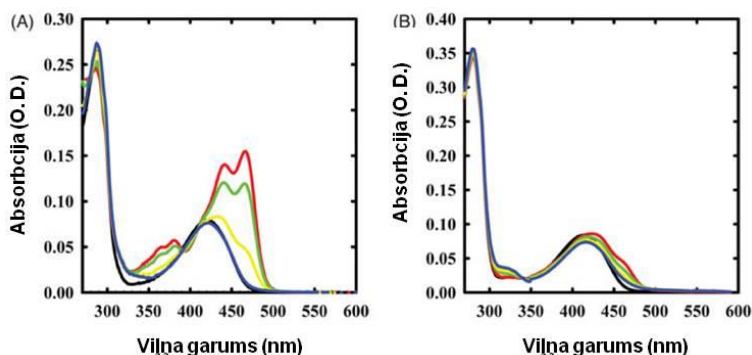
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3. **K. Grammatoglou**, A. Jirgensons. Functionalization of 1*N*-Protected Tetrazoles by Deprotonation with the Turbo Grignard Reagent. *J. Org. Chem.* **2022**, 87, 3810–3816.
4. **K. Grammatoglou**, M. Dārziņa, A. Jirgensons. Functionalization of Tetrazoles Bearing the Electrochemically Cleavable 1*N*-(6-Methylpyridyl-2-methyl) Protecting Group. *ACS Omega* **2022**, 7, 18103–18109.

Dalība konferencēs

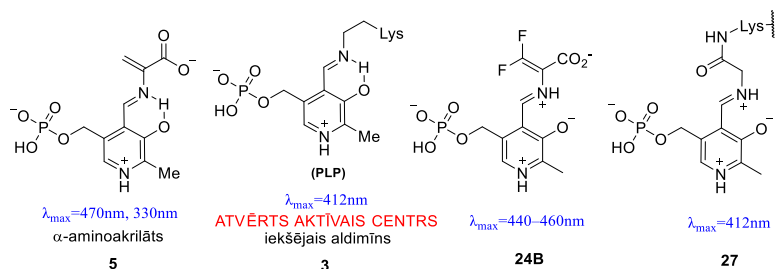
1. **K. Grammatoglou**, J. Sirotkina, A. Jirgensons. Synthesis of 4-Alkynyl 2-Oxazolines *via* the Ritter reaction. 9. *Balticum Organicum Syntheticum konference (BOS 2016)*, Rīga, Latvija, 3.–6. jūlijs, **2016**, postera prezentācija.
2. **K. Grammatoglou**, J. Sirotkina. C-Quaternary Alkynyl Glycinols *via* the Ritter Reaction of Cobalt Complexed Alkynyl Glycols. 10. *Paula Valdena simpozījs organiskajā ķīmijā*, Rīga, Latvija, 15.–16. jūnijs, **2017**, postera prezentācija.
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6. **K. Grammatoglou**. Functionalization of 1*N*-protected tetrazoles by deprotonation with turbo Grignard reagent. 11. *Paula Valdena simpozījs organiskajā ķīmijā*, Rīga, Latvija, 19.–20. septembris, **2019**, postera prezentācija.
7. **K. Grammatoglou**. Synthesis and Applications of Metalated 1*H*-tetrazoles. *Balticum Organicum Syntheticum konference (BOS 2022)*, Vilņa, Lietuva, 3.–6. jūlijs, **2022**, postera prezentācija.
8. **K. Grammatoglou**. Synthesis of PLP-dependent enzyme OASS inhibitors and the development of relevant synthetic methodologies. *Springboard vasaras skola “Major milestones in design and development of novel antimicrobials”*, Rīga, Latvija, 23.–25. augusts, **2022**, mutiska uzstāšanās.

saistītu un neaktīvu enzīma atvasinājumu **26A**. Ja nepiesātinātajam imīnam **26A** uzbrūk ūdens, tas pēc sekojošas HF izšķelšanas pārvēršas par amīdu **27**. Saskaņā ar B ceļu pēc sākotnējās transaldiminēšanas notiek HF eliminēšana, iegūstot starpproduktu **24B**, kas tiek pakļauts Maikla reakcijai ar aktīvā centra lizīnu un zaudē vēl vienu fluoridjonu, veidojot neaktīvu kompleksu **25B**. Pēc ūdens pievienošanās kompleksam **25B** un secīgas dekarboksilēšanas un fluora zaudēšanas tiek iegūts amīds **27** (2. shēma).^{5, 10}

TriF-Ala mijiedarbība ar abām OASS, OASS-A (vai CysK) un OASS-B (vai CysM), enzīma izomorfēm pētīta ar absorbcijas un fluorescences spektroskopiju. Fotoķīmiski pētījumi parādīja, ka β,β,β -trifluoralanīns (triF-Ala, **13**) veido kovalentu savienojumu ar enzīmu, kas izraisa absorbcijas spektra izmaiņas, salīdzinot ar iekšējā aldimīna spektriem miera stāvoklī esošam enzīmam. Tas norāda, ka reakcija notiek ar kofaktoru. Kā jau minēts, OASS-A un OASS-B absorbcijas spektros ir josla pie 412 nm, kas raksturīga iekšējam aldimīnam **3** (5. att.). Pēc triF-Ala pievienošanas OASS-A spektrā parādījās divi maksimumi pie 440 un 466 nm un divas nelielas joslas pie 360 un 380 nm (4. att.), kas liecina par starpsavienojumu ar garāku konjugācijas ķēdi (**24B**, 5. att.). Absorbcijas joslas pie 466 nm intensitāte lēnām samazinās, veidojoties joslai pie 412 nm. Mazāk intensīvas spektrālās izmaiņas novērotas triF-Ala reakcijā ar OASS-B diapazonā no 400 līdz 500 nm. Starpprodukta sadalīšanos, ko pavada joslas intensitātes samazinājums pie 457 nm un absorbcijas palielināšanos diapazonā no 300 līdz 350 nm, iespējams, var saistīt ar difluorpiruvāta veidošanos.

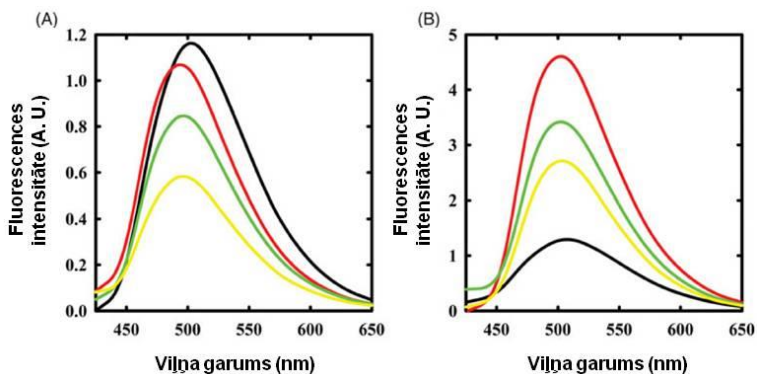


4. att. OASS absorbcijas spektri ar un bez 1 mM triF-Ala. A: OASS-A absorbcijas spektrs bez inhibitora (melna līnija), 1 min (sarkana līnija), 1 st (zaļa līnija), 3 st (dzeltēna līnija) un 7 st (zila līnija) pēc inhibitora pievienošanas. B: OASS-B absorbcijas spektrs bez inhibitora (melna līnija), 1 min (sarkana līnija), 1 st (zaļa līnija), 3 st (dzeltēna līnija) un 7 st (zila līnija) pēc inhibitora pievienošanas.



5. att. Savienojumi un to absorbcijas maksimumi, kas novēroti OASS inhibēšanas procesā.

OASS-A reakcija pētīta arī ar fluorescences emisiju (6. A att.), un josla, kas parādās pēc ierosināšanas pie 412 nm, nobīdījās uz spektra daļu līdz 495 nm. Tas apstiprina, ka izveidojusies struktūra nav α -aminoakrilāts **5** (5. att.). Emisijas josla lēnām samazinās, bet pēc sešu stundu inkubēšanas, atšķirībā no absorbcijas spektroskopijas, kur novērota sākotnējā emisijas spektra atgūšana, šajā gadījumā tas nenotika. Tas liecina, ka savienojuma struktūra, kas absorbē pie 412 nm, atšķiras no iekšēja aldimīna **27** (5. att.). Pēc triF-Ala reakcijas ar OASS-B un ierosināšanas pie 412 nm emisijas intensitāte sākumā palielinās un pēc tam lēnām samazinās (6. B att.). To pavada neliela nobīde zilajā spektra apgabalā līdz 501 nm, kas pēc septiņu stundu inkubēšanas lēnām pāriet atpakaļ uz 505 nm. Šīs izmaiņas var notikt ārējā aldimīna dēļ, un neliela nobīde uz zilo spektra daļu liecina par pārejoša starpprodukta veidošanos.

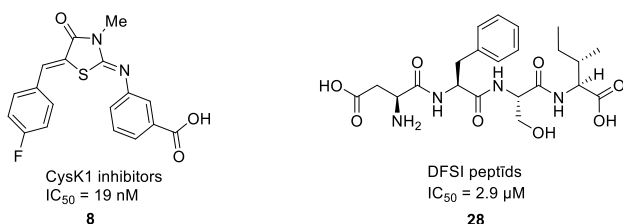


6. att. OASS fluorescences spektri ar vai bez 1 mM triF-Ala. Emisijas spektri reģistrēti pēc ierosināšanas pie 412 nm. A: OASS-A bez inhibitora (melna līnija), 1 min (sarkana līnija), 4 st (zaļa līnija) un 6 st (dzeltena līnija) pēc inhibitora pievienošanas. B: OASS-B bez inhibitora (melna līnija), 1 min (sarkana līnija), 3 st (zaļa līnija) un 7 st (dzeltena līnija) pēc inhibitora pievienošanas.

Turpinājumā tika veikts pētījums, lai novērtētu F-Ala un triF-Ala iespējamo inhibēšanas aktivitāti uz OASS-A un OASS-B izoformām. Šim nolūkam veikti divi dažādi eksperimenti. Vispirms abas enzīma izoformas pārbaudītas, apstrādājot ar pieaugošām F-Ala koncentrācijām. Noteiktās IC_{50} vērtības bija attiecīgi $480 \pm 50 \mu\text{M}$ uz OASS-A un $1290 \pm 230 \mu\text{M}$ uz OASS-B. TriF-Ala gadījumā rezultāti liecināja, ka $IC_{50}(\text{OASS-A}) = 130 \pm 10 \mu\text{M}$ un $IC_{50}(\text{OASS-B}) = 940 \pm 60 \mu\text{M}$. Lai noskaidrotu, vai triF-Ala ir neatgriezenisks

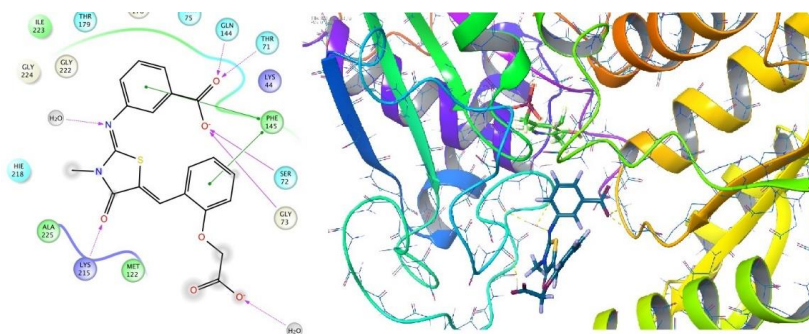
OASS inhibitors, kā liecina dati par citiem no PLP atkarīgiem enzīmiem, tika izpētīta OASS-A kinētika dažādās triF-Ala koncentrācijās, un rezultāti bija atbilstoši enzīma neatgriezeniskās inhibēšanas substrātiem. Otrajā eksperimentā pārbaudīts enzīms pēc 85 stundu reakcijas ar 10 mM triF-Ala, kam sekoja pilnīga liganda izvadīšana un inkubācija pie paaugstinātas PLP koncentrācijas. Eksperimentu rezultāti liecināja par enzīma inhibēšanu, ko izraisa kovalenta neatgriezeniska aktīvā centra atlikuma(-u) modificēšana triF-Ala dēļ.

Mūsu jaunie OASS inhibitoru meklējumi ietvēra datorizētās zāļvielu izveides (CADD) metožu izmantošanu. Tika izmantoti dati par jau identificētajiem OASS inhibitoriem un strukturālā informācija, kas iegūta no inhibitoru un enzīma kristalogrāfiskajiem datiem.^{6d} DFSI peptīds **28**, kas sastāv no pēdējiem četriem SAT (CysE) enzīma aminoskābju atlikumiem, ir iepriekš identificēts kā OASS-CysK inhibitors. *Sriram* grupa ziņoja par OASS-CysK inhibitora **8** pētījumiem, kuram piemīt uzlabota aktivitāte, salīdzinot ar dabisko peptīdu DFSI **28** (7. att.). Savienojumi **8** un **28** ir konkurējoši inhibitori, kas enzīma aktīvajā centrā saistās PLP kofaktora tuvumā.



7. att. OASS inhibitori, CysK1 inhibitors **8** un DFSI peptīds **28**.

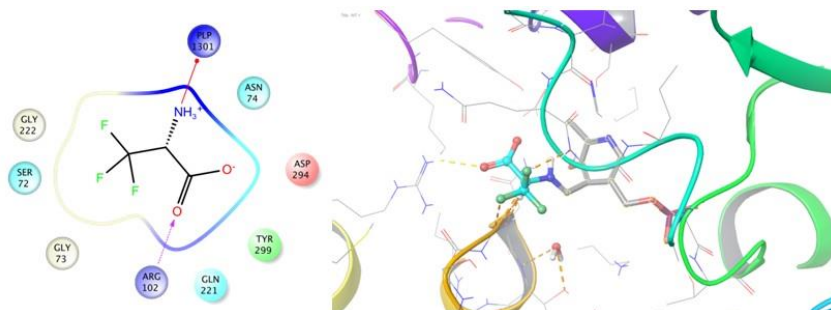
Pamatojoties uz šiem datiem, ar *Schrödinger Maestro* programmas palīdzību tika izveidots proteīna modelis, lai veiktu ligandu bibliotēku virtuālo skrīningu. Tika izvēlēti divi proteīni no RCSB proteīnu datu bankas (PDB) – OASS holoenzīma 2Q3C struktūra no *Mycobacterium tuberculosis* kompleksa ar inhibējošo peptīdu DFSI un *Mycobacterium tuberculosis* OASS-CysK 3ZEI struktūra kompleksā ar mazmolekulāro inhibitoru **8** (8. att.).



8. att. OASS-CysK komplekss ar inhibitoru **8**.

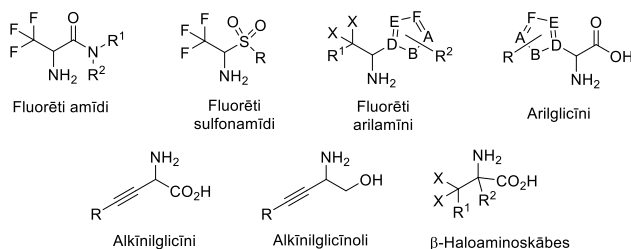
Abas proteīna struktūras apstrādātas, izmantojot *Schrödinger* proteīnu sagatavošanas veidni (*Schrödinger's Protein Preparation Wizard*). Par pētījuma pamatu izvēlējamies 3ZEI proteīnu, jo komplekss ar zināmo inhibitoru ļāva noteikt galvenās mijiedarbības enzīma aktīvajā centrā. Sekojot iepriekš noskaidrotajām mijiedarbībām dokinga eksperimentā ar

TriF-Ala, kas jau bija uzrādījis afinitāti pret PLP, noteicām tā svarīgākās mijiedarbības enzīma aktīvajā centrā un arī atšķirību no iepriekšējiem inhibitoriem, jo novērojām TriF-Ala mijiedarbību ar PLP (9. att.).



9. att. TriF-Ala mijiedarbība ar OASS proteīna atlikumiem un PLP.

Mūsu mērķis bija sintezēt OASS kovalentos inhibitorus, tāpēc ieprogrammētais reakcijas modelis bija jāizveido tā, lai tas ievēro reakciju starp substrātu un PLP, tāpēc tas bija jāuzstāda *Schrödinger* programmu komplektā modelēšanas eksperimentu veikšanai. Šim mērķim tika sagatavots jauns pielāgots reakcijas modeļa fails, kas veicina kovalentās saites veidošanos virtuālo eksperimentu laikā. Kad enzīms un reakcijas modelis bija sagatavoti, turpinājām ar komerciālo bibliotēku (kompānijas *Enamine* “Skrīninga savienojumu kolekcija”, *MolPort* “Skrīninga savienojumu datubāze” un *eMolecules* datubāze) un *Schrödinger* programmas nodrošināto fragmentu bibliotēku pielāgošanu eksperimentam. Tika apkopota 140 000 savienojumu bibliotēka, kur pēc savienojumu atlasēs izvēlējamies tikai tos, kas teorētiski var stāties mūs interesējošajā mijiedarbībā, un nonācām pie 200 savienojumu bibliotēkas. Šie savienojumi tika pakļauti kovalentā dokinga eksperimentiem, kas radīja aptuveni 2000 modelētās saistīšanās pozas. Apvienojot molekulārā dokinga *Glide* programmas rezultātus un struktūru fragmentu iedalījumu kategorijās, nonācām pie vispārīgām struktūrām kā potenciālajiem mērķiem mūsu sintētiskajam darbam (10. att.).

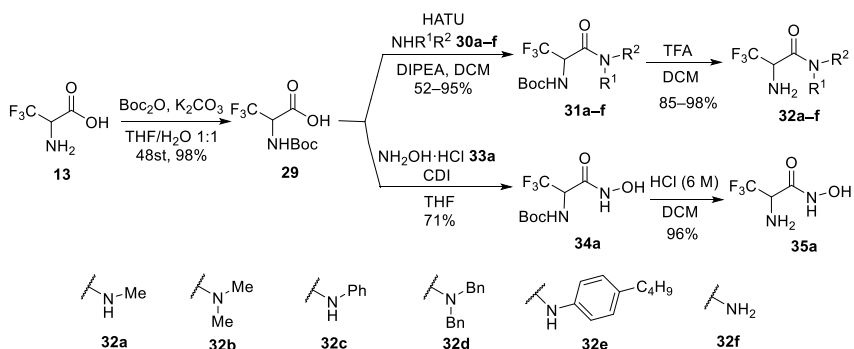


10. att. Potenciālo OASS inhibitoru struktūras.

2. Trifluoralanīna analoģu sintēze

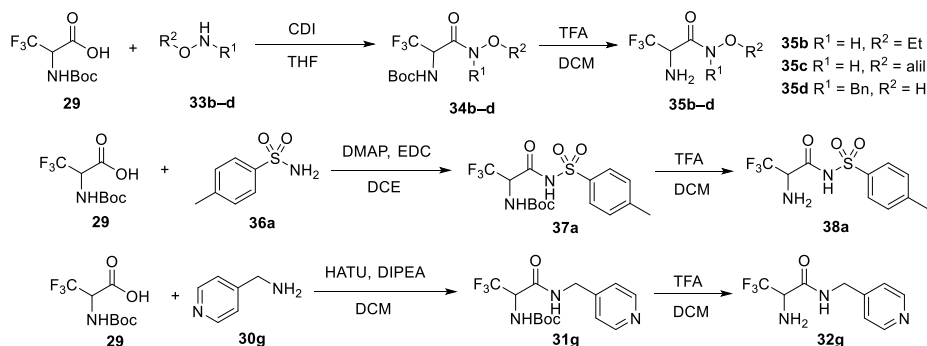
Vispirms veicām TriF-Ala (**13**) karbonskābes daļas aizvietošanu ar bioizostēriem, sintezējot vairākus amīdus **32a–f** un hidroksamskābi **35a** (3. shēma). Boc aizsargātais triF-Ala **29** tika kondensēts ar dažādiem amīniem, izmantojot HATU, un pēc Boc grupas

nošķelšanas skābā vidē ieguvām amīdus **32a–f** ar augstiem iznākumiem. Hidroksamskābes **35a** sintēzei vispirms ar izcilu iznākumu ieguvām starpproduktu **34a**, izmantojot CDI kā kondensēšanas reaģentu, kas pēc aizsarggrupas nošķelšanas deva savienojumu **35a**.



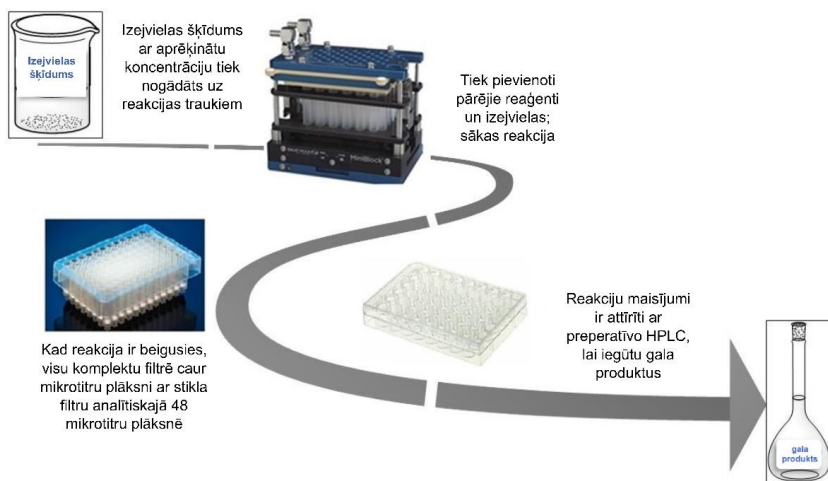
3. shēma. No TriF-Ala atvasinātu amīdu **32a–f** un hidroksamskābes **35a** sintēze.

Paplašinot triF-Ala analogu bibliotēku, pievērsāmies paralēlajai sintēzei. Sintētiskos protokolus, kas būtu piemēroti paralēlās sintēzes metodes izmatošanai, izveidojām, izmantojot modeļreakcijas. Hidroksamskābju **35b–d** sintēzei vislabākā izvēle izrādījās skābes **29** un hidroksilamīnu **33b–d** kondensācija CDI klātbūtnē. Acilsulfonamīda **38a** sintēzei izmantojām DMAP un EDC. Amīda **32g** sintēzei piemērojām agrāk izveidoto protokolu ar HATU un DIPEA (4. shēma).



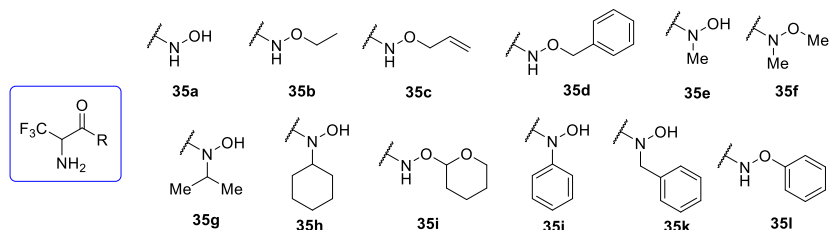
4. shēma. Modeļsavienojumu **35b–d**, **38a**, **32g** reakciju protokolu validācija paralēlajai sintēzei.

Kad protokoli bija izveidoti, turpinājām savienojumu sintēzi ar paralēlās sintēzes bloku. Šajā iekārtā varējām veikt vairākas reakcijas reizē 48 mēģeņu blokā, filtrēt visu komplektu caur mikrotitru plāksni ar stikla filtru analītiskajā 48 mikrotitru plāksnē. Iegūtos reakciju maisījumus pēc tam attīrījām ar preparatīvo HPLC (11. att.).



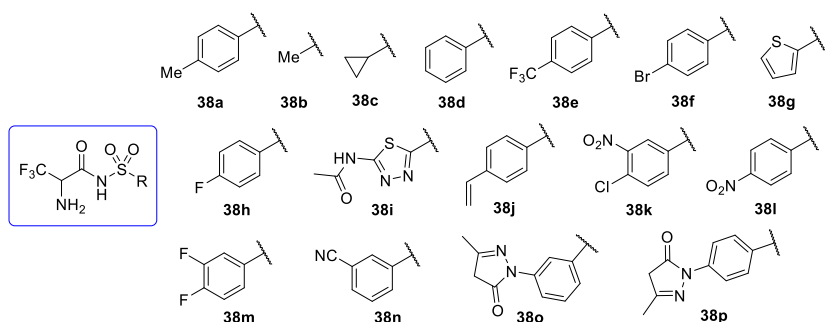
11. att. Paralēlās sintēzes procesa shēma.

Īstenojot šo procesu, īsā laikā izdevās sintetēt un izolēt kopumā 42 savienojumus. Starp tiem veiksmīgi tika sintetēti un izolēti 11 hidroksamskābju atvasinājumi **35b–l** (papildus iepriekš sintetētajai skābei **35a**), ietverot arī aromātiskos un alifātiskos *N*- un *O*-aizvietotos produktus (12. att.).



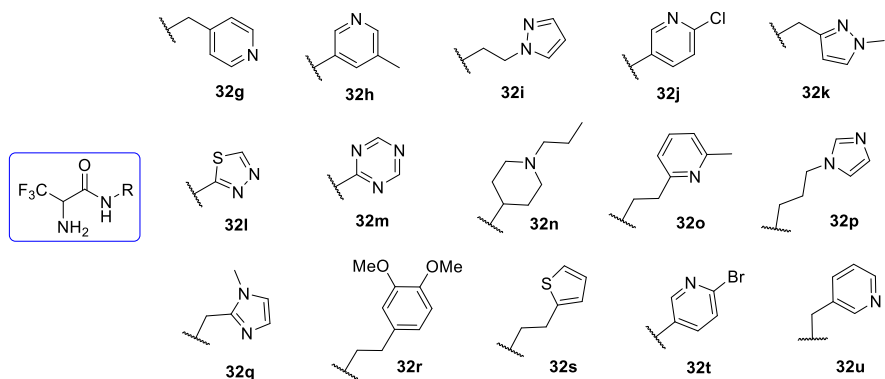
12. att. Hidroksāmskābes 35a–l, kas iegūtas ar paralēlās sintēzes pieeju.

Ieguvām arī dažādus *N*-acilsulfonamīdus **38b–p** (papildus iepriekš sintetētajam *N*-acilsulfonamīdam **38a**), vairums no tiem saturēja aromātisku vai heteroaromātisku fragmentu ar dažādiem aizvietotājiem, piemēram, halogēniem (**38e**, **38f**, **38h**, **38k**, **38m**), cianogrupu (**38n**) vai nitrogrupu (**38k**, **38l**) (13. att.).



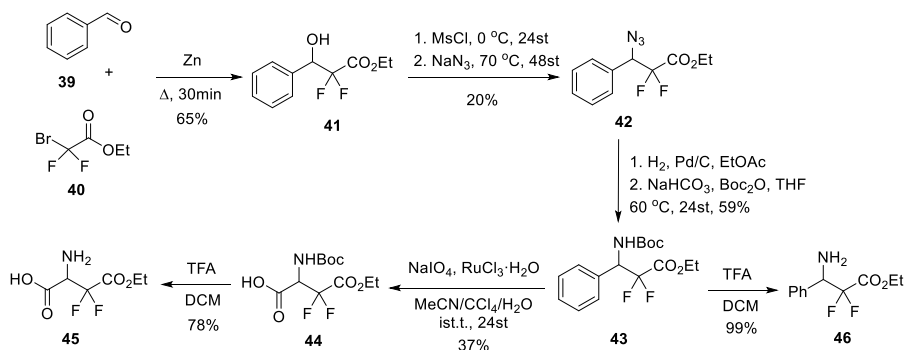
13. att. *N*-Acilsulfonamīdi 38a–p, kas iegūti ar paralēlās sintēzes pieeju.

Izmantojot paralēlās sintēzes metodi, tika sintezēti 15 amīdi **32h–u** (papildus iepriekš sintezētajam amīdam **32g**), veidojot daudzpusīgu savienojumu grupu, piemēram, iegūti amīdi ar halogēn- vai ar metilgrupu aizvietotu piridīnu (**32h**, **32j**, **32o**, **32t**), piridīnmetilamīdi (**32g**, **32u**), dažādi 5-locekļu heterociklus saturoši amīdi u. c. (14. att.).



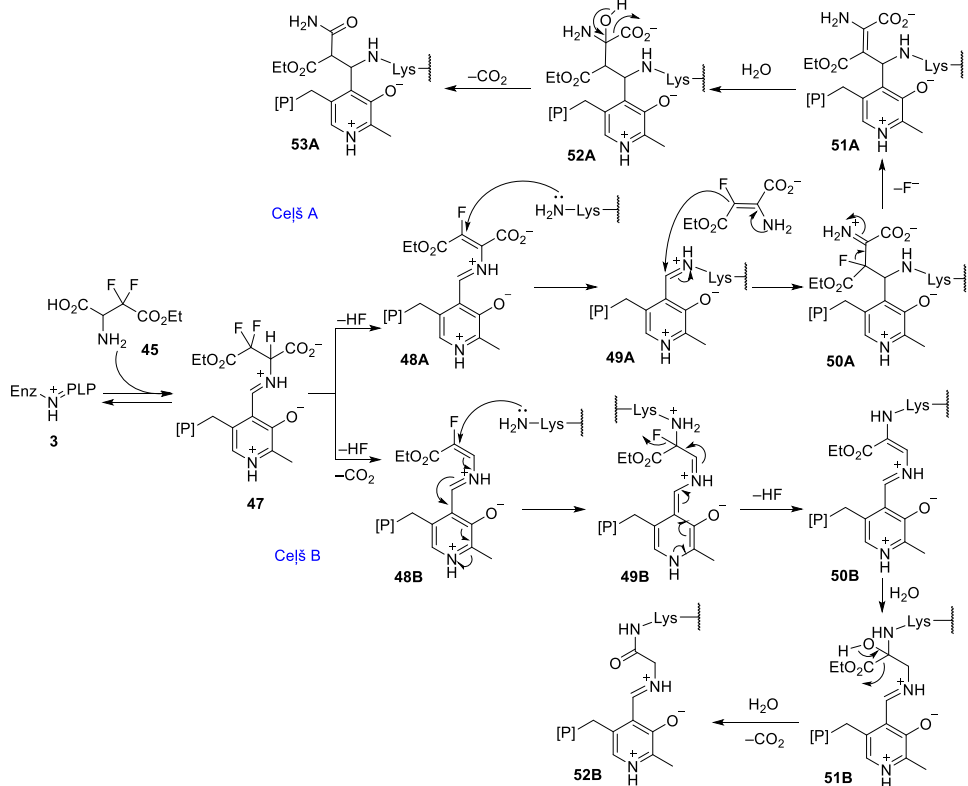
14. att. Amīdi 32g–u, kas iegūti ar paralēlās sintēzes pieeju.

TriF-Ala analoģu klāstā tika izvirzīts mērķis iegūt arī difluorasparģīnskābes monoesteri **45**. Šim nolūkam spirtu **41** sintezējām Barbjē tipa reakcijā starp benzaldehīdu (**39**) un bromdifluoretiķskābes etilesteri (**40**). Spirta **41** mezilēšana un aizvietošana ar azīdu nodrošināja starpprodukta **42** veidošanos. Azīda grupa tika reducēta par amīnu, kas tika aizsargāts ar Boc grupu *in situ*, lai iegūtu savienojumu **43**. Tas tika pakļauts aromātiskai oksidēšanai ar nātrija perjodātu un rutēnija hlorīdu, lai pārveidotu fenilgrupu par karboksilgrupu. No iegūtās skābes **44** nošķelta Boc aizsarggrupa, lai iegūtu vēlamo difluorasparģīnskābes monoesteri **45**. Lai paplašinātu triF-Ala analoģu bibliotēku, Boc aizsargātais amīns **43** tika pārvērsts par brīvu amīnu **46** (5. shēma).



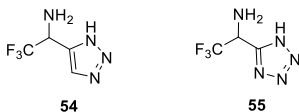
5. shēma. Difluoraspargīnskābes monoestera 45 sintēze.

Tika pieņemts, ka difluoraspargīnskābes monoesteris **45** varētu darboties kā mehānismā balstīts inhibitors (6. shēma) divos dažādos veidos: ar dekarboksilēšanu sākumposmā (**48B**–**52B**, ceļš B) vai beigās (**48A**–**53A**, ceļš A) saskaņā ar piedāvāto mehānismu.



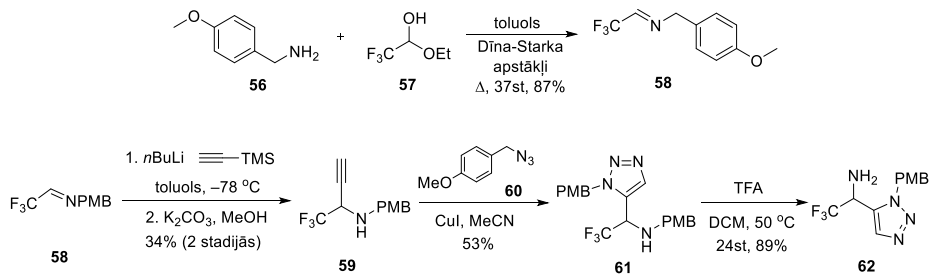
6. shēma. Iespējamie mehānismi OASS inhibēšanai ar difluoraspargīnskābes monoesteri **45**.

Citi sintētiskie mērķi triF-Ala analogu klāstā bija savienojumi, kuros karbonskābe aizstāta ar triazolu **54** un tetrazolu **55** (15. att.).



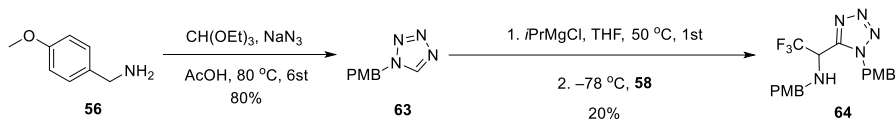
15. att. TriF-Ala triazola un tetrazola analogi.

Lai sintezētu mērķa savienojumus **54** un **55**, vispirms ieguvām imīnu **58**, kondensējot *p*-metoksibenzilamīnu (**56**) ar trifluoracetāldehīda hemiacetātu **57**. TMS-acetilēnīda jona nukleofilā pievienošanās imīnam **58** un desililēšana veidoja alkīnu **59**, kas stājās vara katalizētā [2+3] ciklopievienošanās reakcijā ar *p*-metoksibenzilazīdu (**60**), lai iegūtu aizsargāto triazolu **61**. PMB aizsarggrupas nošķelšana ar TFA diemžēl notika tikai no aminogrupas, kā rezultātā ieguvām triazolu **62** (7. shēma).



7. shēma. Triazola **62** sintēze.

Mērķa savienojuma **55** sintēzei sākotnēji vajadzēja iegūt PMB aizsargātu tetrazolu **63**, izmantojot klasiskos literatūrā aprakstītos reakcijas apstākļus. Starpprodukts **63** tika deprotonēts ar *i*PrMgCl un pievienots imīnam **58**, veidojot tetrazolu **64** ar zemu iznākumu (8. shēma). Mūsu centieni nošķelt aizsarggrupu tetrazolā **64** dažādos apstākļos (TFA/DCM, tīra TFA 60 °C, H₂/Pd, CAN, DDO) izraisīja tetrazola sadalīšanos (galvenais identificētais produkts bija *p*-metoksibenzilamīns **56**) vai sarežģīta produkta maisījuma veidošanos, kas sastāvēja no tetrazola **64** un produkta ar daļēji nošķeltu aizsarggrupu.

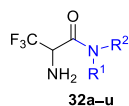


8. shēma. Tetrazola **64** sintēze.

3. Trifluoralanīna analogu inhibēšanas spēju noteikšana

Sintezētajiem savienojumiem **32a–u**, **35a–l**, **38a–p**, **45**, **46**, **62** (16. att.) tika noteikta reaģētspēja ar OASS-A un OASS-B izoformām, kā arī enzīmu inhibēšanas spēja un kinētika. Testi veikti ar *Salmonella Typhimurium* CysK un CysM, kas rekombinanti ekspresēti

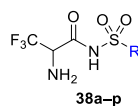
Escherichia coli. Enzīms inkubēts ar savienojumiem 1 mM koncentrācijā, enzimatiskā aktivitāte mērīta laika intervālos.



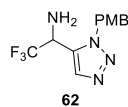
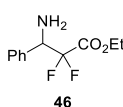
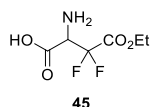
R¹ = H, Me, Bn
R² = H, Me, Ph, Bn
p-(C₄H₉)-C₆H₄, Hetaril-



R¹ = H, Me, *i*Pr, Cy, Ph, Bn
R² = H, Me, Et, alil-, Ph, Bn, THP



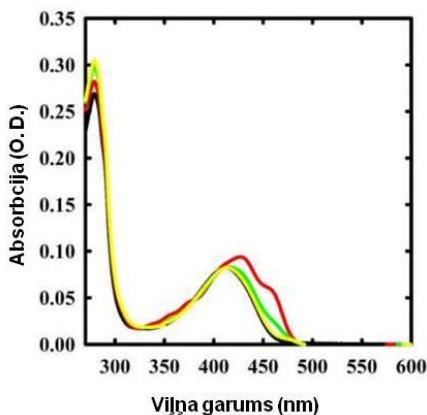
R = Me, ciklopropil, Ar, Hetaril-



16. att. Sintezēto savienojumu bibliotēka ar savienojumiem, kuriem pārbaudīta reaģētspēja ar OASS.

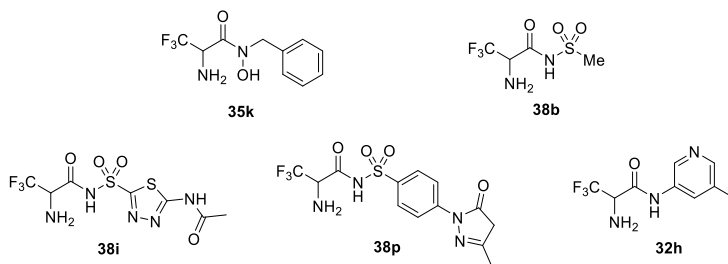
Lai izpētītu triF-Ala analoģu aktivitāti, tika analizēti savienojumu **3–5** (3. att.) un **45, 46** (5. shēma) reaģētspēja ar OASS-A un OASS-B izoformām. Neviens no šiem savienojumiem neuzrādīja reaģētspējas uzlabošanas, salīdzinot ar sākotnējo savienojumu TriF-Ala **13**.

Pēc tam pārbaudījām karbonskābes bioizostērās aizvietošanas ietekmi. Pārbaudot pirmo savienojumu grupu **32a–f** (16. att.) un **35a** (12. att.), tika novērota nenozīmīga ietekme uz reaģētspēju ar enzīmu. Jāatzīmē, ka hidroksāmskābe **35a** izraisījis nelielas izmaiņas OASS-A absorbcijas spektrā (17. att.) un aptuveni 14 % enzīma aktivitātes samazināšanos.⁵



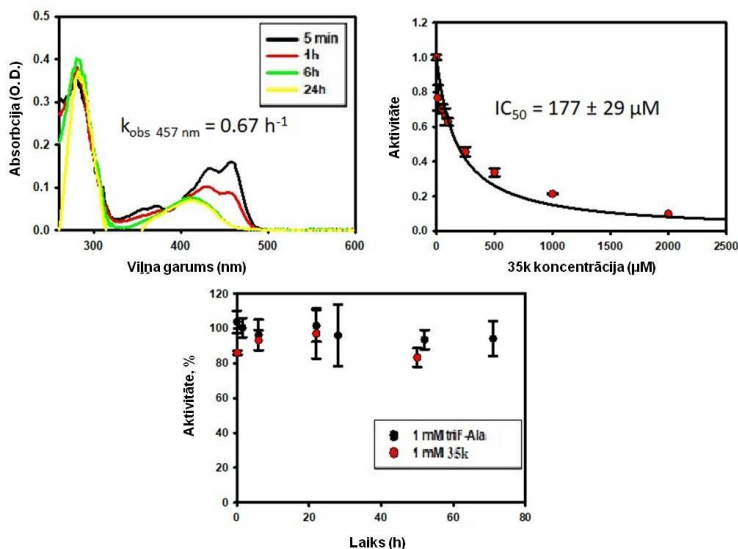
17. att. Spektrālās izmaiņas reakcijā starp OASS-A un 1 mM savienojuma **35a**. OASS-A bez inhibitora (melna līnija) un 1 min (sarkana līnija), 30 min (zaļa līnija) un 1 st (dzeltena līnija) pēc inhibitora **35a** pievienošanas.

No nākamās savienojumu grupas **35b–l, 38a–p** un **32g–u** (16. att.) pieci savienojumi uzrādīja vērā ņemamas OASS-A inhibēšanas īpašības (18. att.). Savienojumi **38i, 38p** un **32h** uzrāda vāju saistību ar enzīmu un neizraisa būtisku enzīma aktivitātes samazināšanos, tomēr hidroksāmskābes atvasinājums **35k** un sulfonamīds **38b** ir spēcīgākie savienojumi no šīs mazās bibliotēkas.



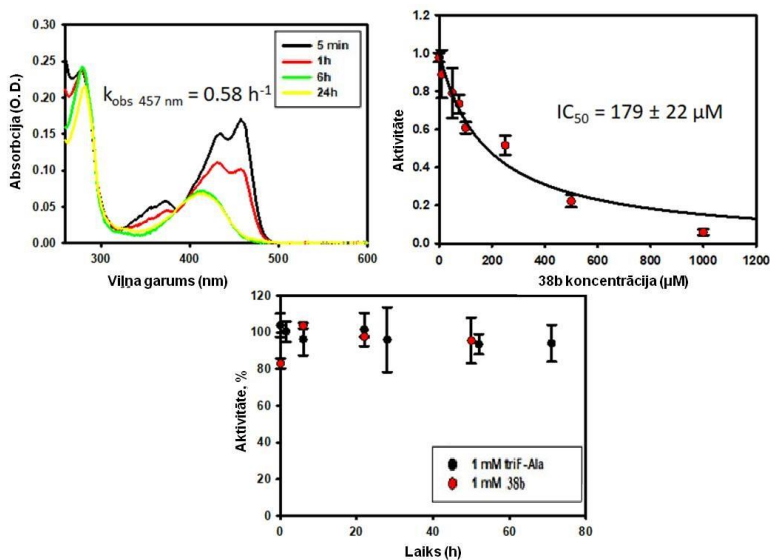
18. att. TriF-Ala analogi ar OASS inhibējošu aktivitāti.

Hidroksāmskābe **35k** uzrādīja OASS-A inhibēšanas spēju ar konstanti $IC_{50} = 177 \pm 29 \mu\text{M}$, kā arī inhibēšanas testā pirmajā laika punktā tika novērota aptuveni 15 % enzīma aktivitātes samazināšanās, lai gan vēlāk tā pazuda, iespējams, starpprodukta hidrolīzes rezultātā (19. att).



19. att. Inhibitors **35k** 1 μM koncentrācijā, testam 5000 reizes liels atšķaidījums (inhibitora koncentrācija testā 0,2 μM), divos atkārtojumos.

Sulfonamīds **38b** arī uzrādīja OASS-A inhibēšanu ar konstanti $IC_{50} = 179 \pm 22 \mu\text{M}$. Enzīma aktivitāte testā pirmajā laika punktā ir pazemināta par aptuveni 20 %, lai gan šis efekts vēlāk pazuda, iespējams, starpprodukta hidrolīzes rezultātā (20. att.).



20. att. Inhibitors **38b** 1 μM koncentrācijā, testam 5000 reizes liels atšķaidījums (inhibitora koncentrācija testā 0,2 μM), divos atkārtojumos.

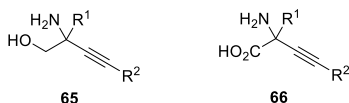
Pamatojoties uz spektrālajiem un kinētikas datiem, kas iegūti no TriF-Ala reaģētspējas eksperimentiem ar OASS, un ņemot vērā jau piedāvātos mehānismus triF-Ala reakcijai ar citiem no PLP atkarīgiem enzīmiem, izvirzījām hipotēzi, ka OASS-A inhibēšanas mehānisms ir līdzīgs iepriekš piedāvātajam (2. shēma). Tika pieņemts, ka mehānisms seko B ceļam, nevis A ceļam, jo OASS katalītiskajā ciklā dekarboksilēšana nenotiek.

Inhibitori **35k** un **38b** ir daudzsoļi bāzes savienojumi to turpmākai attīstībai. Atšķirībā no izejas inhibitora triF-Ala (**13**), šie savienojumi satur funkcionālās grupas, ko atvasinot var iegūt papildu mijiedarbības ar OASS. Darbs šajā virzienā tika apturēts, jo beidzās projekta termiņš un savienojumu bioloģiskā pārbaude vairs nebija pieejama.

4. Jaunu metožu izstrāde no PLP atkarīgu enzīmu inhibitoru sintēzei

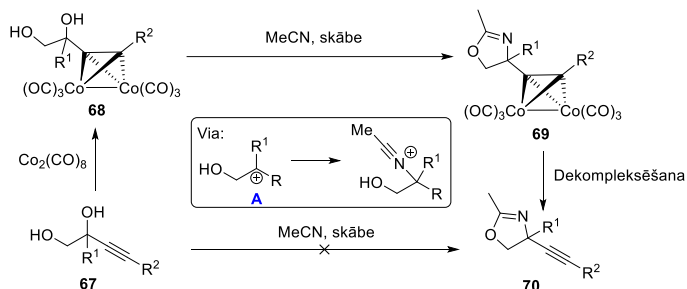
4.1. Jauna pieeja C-kvarternāro alkīnlglicīnolu sintēzei

Viens no kemitotiem, ko atklājām kā potenciālu no PLP atkarīgu enzīmu inhibitoru, bija alkīnlglicīnols **65**, kas kopā ar sintētiski līdzvērtīgiem alkīnlglicīna atvasinājumiem **66** (21. att.) ir ļoti noderīgi būvbloki sarežģītu bioloģiski aktīvu savienojumu sintēzei. Ir zināmas tikai dažas metodes alkīnlglicīnolu tiešai sintēzei, kurās netiek izmantota karboksilgrupas reducēšana glicīnos **66**.



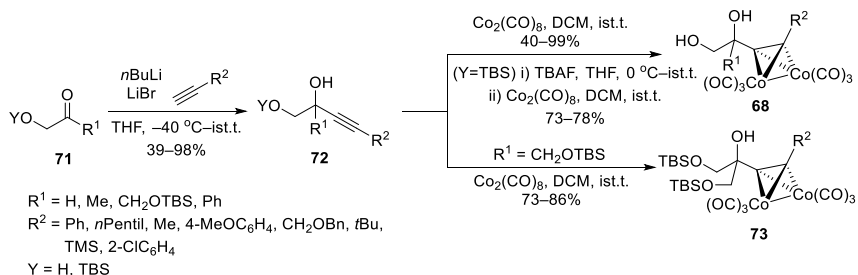
21. att. Alkīnlglicīnoli **65** un alkīnlglicīni **66**.

Kā viena no metodēm alkīnlglicīnolu **65** iegūšanai tika pārbaudīta 1,2-diolu Ritera reakcija, sākotnēji veidojot attiecīgos oksazolīnus. Pirmais mēģinājums iegūt oksazolīnu **70** deva sliktu produkta iznākumu, zem 10 % (9. shēma). Atklājām, ka kobalta kompleksētu alkīnlglicīnolu **68** izmantošana var efektīvi stabilizēt starpproduktu – karbēnija jonu A – un pēc kobalta kompleksa nošķelšanas nodrošināt veiksmīgu oksazolīnu **70** iegūšanu.



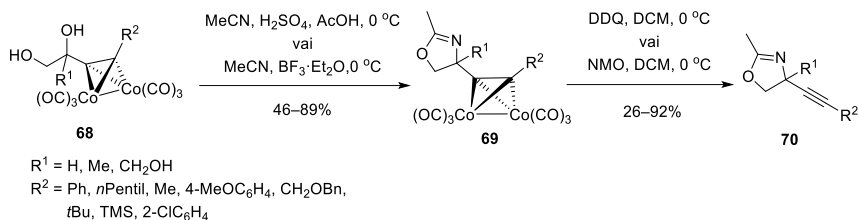
9. shēma. Oksazolīnu **70** sintēze Ritera reakcijā.

Kobalta kompleksētu alkīnlglicīnolu **68** un **73** sintēzi veicām divās stadijās, vispirms pievienojot litija acetilēnīdu α -hidroksiketona atvasinājumiem **71** un pēc tam apstrādājot iegūtos alkīnildiolus **72** ar $\text{Co}_2(\text{CO})_8$ (10. shēma).



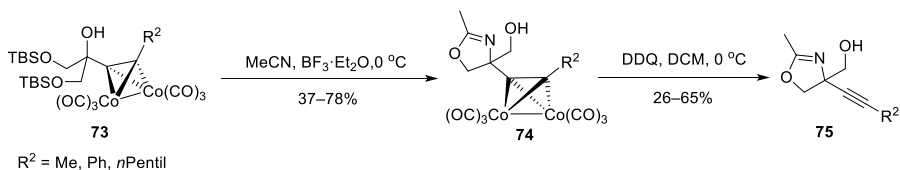
10. shēma. Alkīnlglicīnolu kobalta kompleksu **68** un **73** iegūšana.

Ritera reakcijā izmantojot H_2SO_4 vai $\text{BF}_3 \cdot \text{Et}_2\text{O}$, alkīnīlglikola kobalta kompleksi **68** reakcijā ar MeCN veidoja atbilstošos oksazolīnus **69**. Reakcijā iespējams izmantot plašu substrātu klāstu ar dažādiem aizvietotājiem R^2 pozīcijā, savukārt substrāti, kuriem R^1 pozīcijā bija fenilgrupa, nedeva gaidītos oksazolīnus (11. shēma).



11. shēma. Substrātu **68** Ritera reakcija un tai sekojoša kobalta kompleksa šķelšana oksazolīnu **70** iegūšanai.

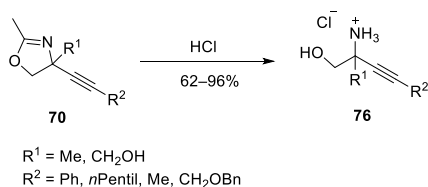
Lai gan galvenā sintēzes metode paredz spirta TBS aizsarggrupas nošķelšanu pirms kobalta kompleksa veidošanas, parādījām arī veiksmīgus piemērus, kuros aizsargāti spirti tiek pakļauti Ritera reakcijai ar vienlaicīgu TBS grupas šķelšanu (12. shēma).



12. shēma. Oksazolīnu **75** iegūšana no substrāta **73** Ritera reakcijas un kompleksa šķelšanas rezultātā.

Vairumā gadījumu alkīnīlglicīnolu kobalta kompleksa šķelšanai DDQ izmantošana izrādījās efektīvāka, salīdzinot ar NMO. Jāatzīmē, ka tas ir pirmais piemērs, kas demonstrē DDQ lietošanu šādas reakcijas veikšanai (11. un 12. shēma).

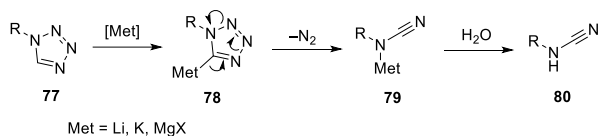
Izvēlētie oksazolīni tika pakļauti skābes ierosinātai hidrolīzei maigos apstākļos, lai iegūtu aminospirtus **76** ar labiem iznākumiem (13. shēma).



13. shēma. Oksazolīnu **70** hidrolīze par aminospirtiem **76**.

4.2. 1*N*-PMB aizsargātu tetrazolu funkcionalizēšana

Mēģinājums sintezēt tetrazolu saturošu TriF-Ala analogu **64** (8. shēma) bija sākumpunkts, lai izstrādātu jaunu metodi tiešai tetrazola C–H funkcionalizēšanai, kas dotu labāku iznākumu, salīdzinot ar mūsu sākotnējiem centieniem (20 %). No literatūras zināms, ka paralēli vēlamajai reakcijai notiek metalētā starpprodukta retro [2+3] ciklopievienošanās reakcija, kas veido ciānamīdu **80** (14. shēma). Metalēts tetrazols **78** var būt nestabils pat –98 °C temperatūrā, kas ir galvenais šķērslis tetrazola atvasinājumu iegūšanai C–H deprotonēšanas ceļā.

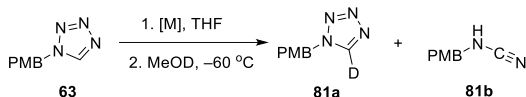


14. shēma. Metalētu tetrazolu retro [2+3] ciklopievienošanās reakcija.

Literatūrā ir zināmi tetrazolu saturoši Grinjāra reaģenti, kas iegūti halogēna–metāla apmaiņas rezultātā, un tie uzrāda augstāku stabilitāti, salīdzinot ar litijētajiem analogiem.¹¹ Tas rosināja mūs iegūt Grinjāra reaģentus C–H deprotonēšanas ceļā. Šim nolūkam sintezējām 1*N*-PMB aizsargātu tetrazolu **63**, kas tika pakļauts dažādiem deprotonēšanas apstākļiem (1. tab.). Lai noteiktu deprotonēšanās efektivitāti, pārbaudījām dažādus Grinjāra reaģentus, izmantojot reakciju ar deitērētu metanolu. Konstatējām, ka turbo Grinjārs (*i*PrMgCl·LiCl) ir visefektīvākais un nodrošina pat 99 % deitērija iekļaušanu molekulā (1. tab., 1., 2. aile). Jāatzīmē, ka šajos apstākļos retro [2+3] ciklopievienošanās produkts **81b** netika novērots pat pēc vienas stundas –60 °C temperatūrā. Izpētīti arī citi Grinjāra reaģenti, piemēram, *i*PrMgCl un *i*PrMgBr, tomēr tie izrādījās mazāk efektīvi, salīdzinot ar turbo Grinjāru (1. tab., 3.–6. aile).

1. tabula

Dažādu Grinjāra reaģentu deprotonēšanas spēju izvērtēšana



Nr. p. k.	[M]	Laiks, min	81a iznākums ^a , %	81b
1.	<i>i</i> PrMgCl·LiCl	15	98	n. n. ^b
2.	<i>i</i> PrMgCl·LiCl	60	99	n. n.
3.	<i>i</i> PrMgCl	15	53	n. n.
4.	<i>i</i> PrMgCl	60	76	n. n.
5.	<i>i</i> PrMgBr	15	57	n. n.
6.	<i>i</i> PrMgBr	60	69	n. n.

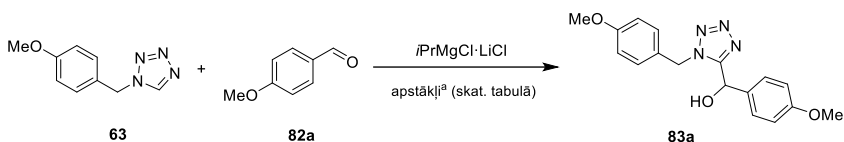
^a Iznākumi aprēķināti, pamatojoties uz sausā atlikuma svaru; KMR bez **81a** netika novēroti citi savienojumi.

^b n. n. = nav novērots.

Pēc tam pārbaudījām tetrazola **63** reakciju ar anīsaldehīdu (**82a**) dažādos šķīdinātājos. Izmantojot THF, spirts **83a** tika iegūts ar ļoti labu iznākumu (2. tab., 1. aile). Reakcijas iznākums būtiski nesamazinājās, ja reakcijas maisījumu tika uzsildīts līdz istabas temperatūrai pēc anīsaldehīda pievienošanas (2. tab., 2. aile). Citu šķīdinātāju (Et₂O, toluola) izmantošana pazemināja reakcijas iznākumu (2. tab., 3., 4. aile).

2. tabula

Reakcijas apstākļu optimizācija



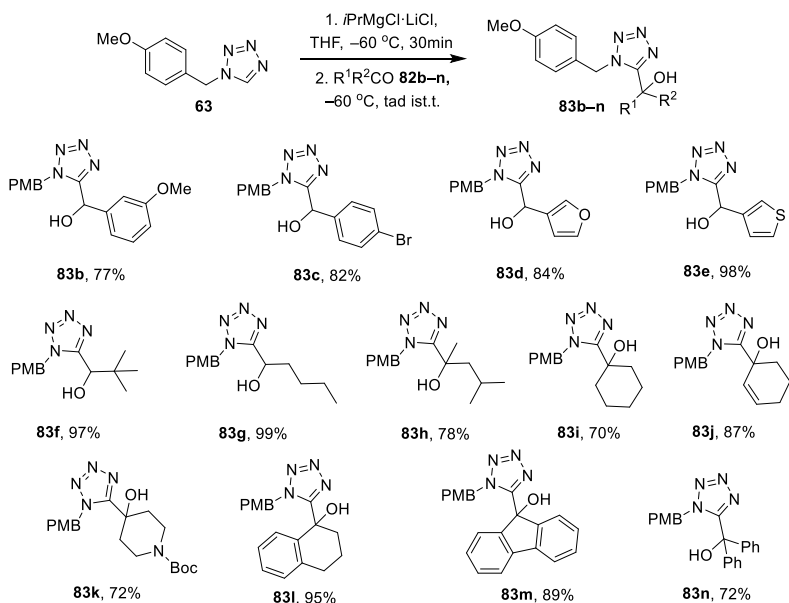
Nr. p. k.	Šķīdinātājs	Temperatūra, °C	Laiks, st	83a iznākums, %
1.	THF	-60 °C	5	78 ^b
2.	THF	ist. t.	24 ^c	76
3.	Et ₂ O	-60 °C	5	47
4.	Toluols	-60 °C	5	22

^a Tetrazols **63** (1,1 ekv.), *i*PrMgCl·LiCl (1,3 ekviv.) 2 mmol mērogā. Pēc *i*PrMgCl·LiCl pievienošanas reakciju maisa 30 min un tad pievieno aldehīdu **82a**.

^b Produkts **83a** iegūts ar 88 % iznākumu, veicot reakciju 5 mmol mērogā.

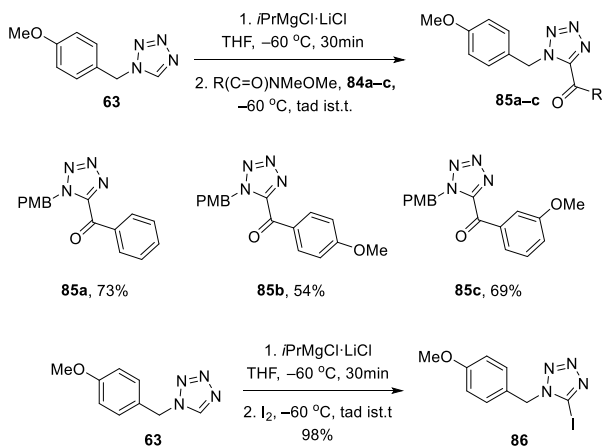
^c Aldehīda **82a** pievienošana veikta -60 °C, pēc tam reakcijas maisījums atšildīts līdz ist. t.

Substrāta klāsta izpētei izmantojām dažādus aromātiskus (**82b–e**) un alifātiskus (**82f–g**) aldehīdus, kā arī strukturāli daudzveidīgus ketonus (**82h–n**). Šo substrātu reakcijās ar Grinjāra reaģentu, kas atvasināts no tetrazola **63**, ieguvām atbilstošus spirtus **83b–n** ar labiem līdz izciliem iznākumiem (15. shēma).



15. shēma. Tetrazola **63** pievienošanas produkti aldehīdiem un ketoniem.

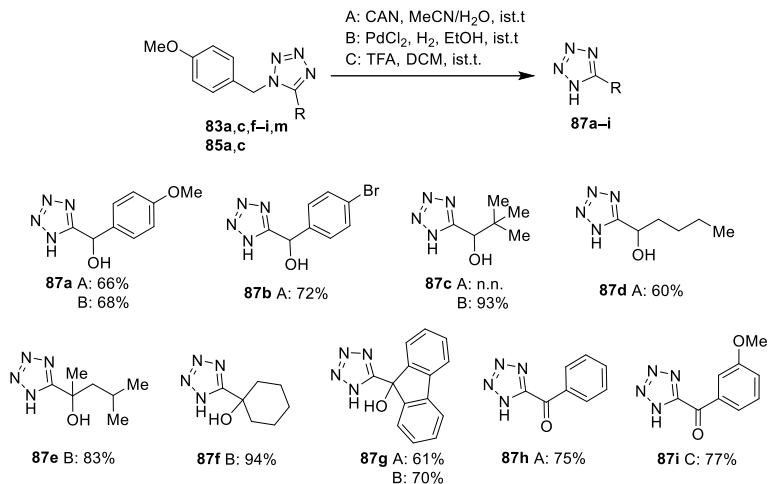
Izpētījām arī citus elektrofilus reakcijai ar Grinjāra reaģentiem, kas atvasināti no tetrazola **63**. Veinreba amīdus **84a–c** var veiksmīgi izmantot kā reakcijas komponentes, nodrošinot atbilstošo ketonus **85a–c** veidošanos ar labiem iznākumiem. Reakcija ar jodu deva atvasinājumu **86** ar gandrīz kvantitatīvu iznākumu (16. shēma).



16. shēma. Tetrazola **63** pievienošana Veinreba amīdiem un jodēšana.

1*N*-PMB aizsarggrupas nošķelšanai izmantojām trīs dažādas metodes – oksidējošo šķelšanu ar CAN MeCN/H₂O maisījumā (A metode), katalītisko hidrogenēšanu ar H₂/PdCl₂ etanolā (B metode) un šķelšanu ar trifluoretiķskābi dihlormetānā (C metode). Katra no šīm

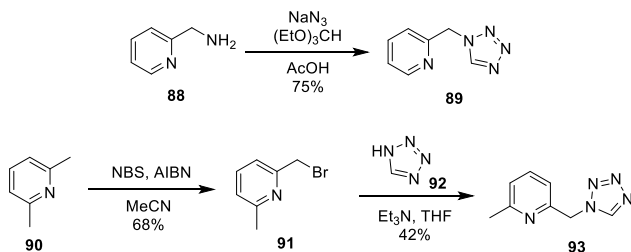
metodēm nodrošināja vēlamu neaizargāto tetrazolu **87a-i** iegūšanai ar labiem vai izciliem iznākumiem (17. shēma).



17. shēma. PMB aizsarggrupas šķelšanas apstākļi un iegūtie produkti **87a-i**.

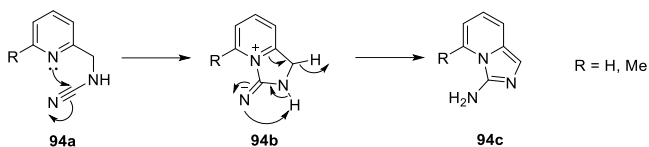
4.3. Elektroķīmiski nošķēlamas 1*N*-6-metilpiridil-2-metil- aizsarggrupas saturošu tetrazolu, funkcionalizēšana

Lai paplašinātu tetrazolu funkcionalizēšanas metodes lietojamību, centāmies ievadīt jaunu, elektroķīmiski nošķēlamu tetrazolu aizsarggrupu. Šim nolūkam sagatavojām divus substrātus – ar piridilmetilgrupu aizsargātu tetrazolu **89**, kas iegūts no atbilstošā 2-aminometilpiridīna (**88**), un ar 6-metilpiridilmetilgrupu aizsargātu tetrazolu **93**, kas iegūts no 2,6-lutidīna (**90**) ar bromēšanu un sekojošu tetrazola **92** alkilēšanu (18. shēma).



18. shēma. Ar piridilmetilgrupu aizsargātu tetrazolu sintēze.

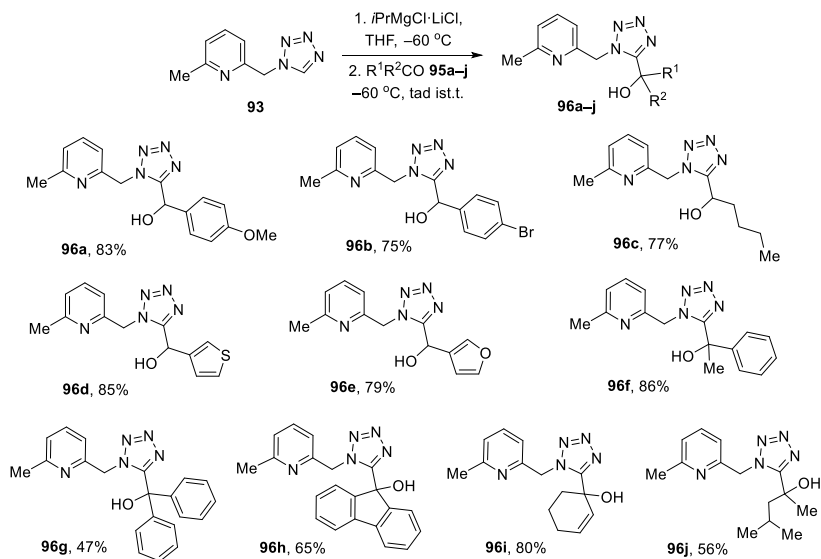
Turbo Grinjāra izmantošana tetrazolu **89** un **93** piektās pozīcijas deprotonēšanai rezultējās ar minimālu retrociklopievienošanās produktu veidošanās vai arī tie neveidojās vispār. Nelielos daudzumos izveidotais tetrazolu **89** un **93** sadalīšanās produkts, ciānamīds **94a**, ciklizējās par pirazolopiridīnu **94c** (19. shēma).



19. shēma. Piedāvātais blakusprodukta 94c veidošanās mehānisms.

Piridilmetil- aizsargāta tetrazola **89** metalēšanas/deiterēšanas reakcijā ieguvām nepilnīgu deitērija iekļaušanos tetrazola piektajā pozīcijā (40–60 %) kopā ar konkurējošu deitērešanas produktu CH₂ grupā un neizreaģējušu izejvielu. Deitērešanas selektivitāte ievērojami uzlabojās, kad deitērešanas eksperimentos izmantojām 6-metilpiridilmetil- aizsargātu tetrazolu **93** (deitērija iekļaušanās 97–98 %). Svarīgi, ka netika novērots ne konkurējošas deitērešanas, ne sadalīšanās produkts. Pieņemām, ka metilgrupa piridīna C-6 pozīcijā savienojumā **93** bloķē relatīvi skābo piridīna C–H funkciju, kas var izraisīt vairāku metalētu daļiņu līdzsvara maisījuma rašanos.

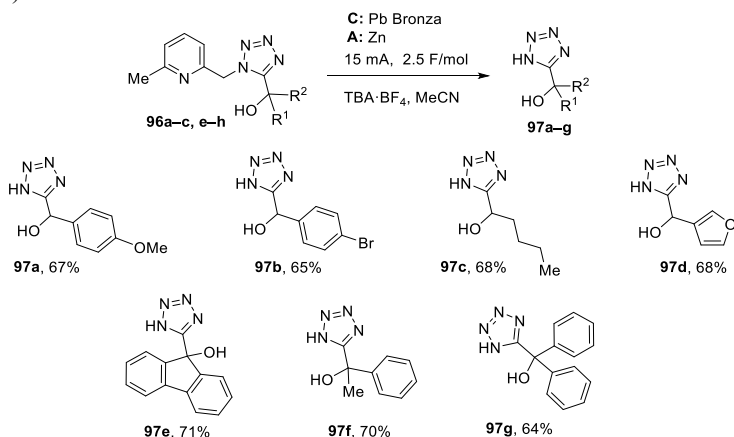
No 6-metilpiridilmetil-aizsargāta tetrazols **93** atvasināts Grinjāra reaģents stājās reakcijā ar aromātiskajiem, alifātiskajiem un heteroaromātiskajiem aldehīdiem **95a–e**, veidojot spirtus **96a–e** ar labiem līdz izciliem iznākumiem. Reakcijā ar ketoniem **95f–j** atbilstošie spirti **96f–j** tika iegūti ar mēreniem vai labiem iznākumiem (20. shēma).



20. shēma. Tetrazola **93** pievienošanas produkti aldehīdiem un ketoniem.

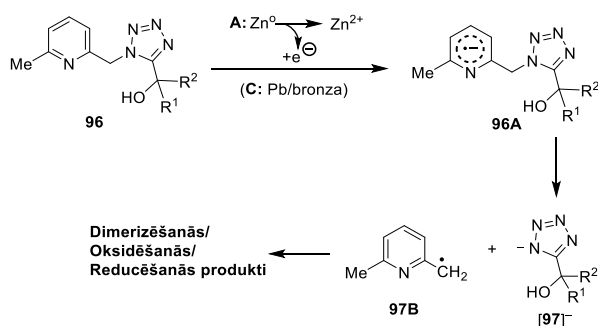
Literatūrā 6-metilpiridilmetilgrupa ir aprakstīta kā elektroķīmiski nošķelama aizsarggrupa no tioliem, karbonskābēm un spirtiem.¹⁴ Līdzīgus elektroķīmiskos apstākļus piemērojām arī 6-metilpiridilmetilgrupas reducējošai nošķelšanai no tetrazola, kā modeļsubstrātu izmantojot savienojumu **96a**. Izpētījām elektrodu un elektrolītu klāstu pie fiksētas strāvas un elektriskā lādiņa. Labākais rezultāts tika sasniegts, izmantojot svīnu saturošu bronzas elektrodu kā katodu un šķīstošo cinka elektrodu kā anodu, kā arī TBA·BF₄, nodrošinot neaizsargāta tetrazola **97a** veidošanos ar 67 % izolēto iznākumu. Šie optimizētie

aizsarggrupas nošķelšanas apstākļi tika piemēroti tetrazoliem **96a–c** un **96e–h**, lai iegūtu 1*H*-tetrazolus **97b–g** ar vidējiem iznākumiem. Augstākus tetrazolu iznākumus neizdevās sasniegt, jo to ļoti polārās dabas dēļ bija apgrūtināts produktu izolēšanas process (21. shēma).



21. shēma. 6-Metilpiridilmetil- aizsarggrupas elektroķīmiskā nošķelšana.

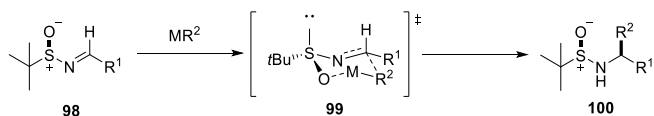
Piedāvātais mehānisms tetrazola **96** 6-metilpiridilmetil-aizsarggrupas elektroķīmiskai nošķelšanai redzams 22. shēmā. Tas sākas ar 6-metilpiridilmetilgrupas reducēšanu pie katoda, oksidējot Zn anodu, kas noved pie anjona radikāļa **96A**. Tas sadalās par radikāli **97B** un tetrazola anjonu **[97]⁻**. Radikālis **97B** tiek pakļauts turpmākām reakcijām, piemēram, ūdeņraža atoma abstrakcijai, dimerizēšanās, oksidēšanās un/vai reducēšanās reakcijām, kas veido blakusproduktu maisījumu. Piridilmetilradikāļa **97B** veidošanos apstiprina 2,6-lutidīna detektēšana LC/MS analizēs neapstrādātam reakcijas maisījumam. Šāds blakusprodukts var veidoties no piridilmetilradikāļa **97B** vai nu ūdeņraža atoma abstrakcijas rezultātā vai arī tad, ja to reducē par anjonu ar sekojošu protonēšanu.



22. shēma. 6-Metilpiridilmetil- aizsarggrupas elektroķīmiskas nošķelšanas iespējamais mehānisms.

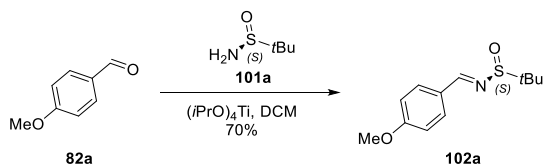
4.4. Tetrazolu pievienošana imīniem, aminoskābju analogu sintēze

Izpētījām metalētā tetrazola izmantošanu, lai iegūtu aminoskābju analogus, kas satur tetrazolu kā karbonskābes bioizostēru. Literatūrā ir zināms, ka metālorganiskos reaģentus var pievienot *t*-butilsulfinilimīniem diastereoselektīvā veidā (23. shēma).¹² Šāda tipa reakciju diastereoselektivitāte uzlabojas, ja kā šķīdinātāju izmanto DCM.



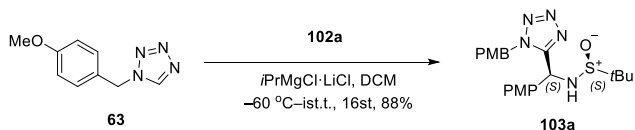
23. shēma. Metālorganisko savienojumu diastereoselektīva pievienošanās *t*-butilsulfinilimīniem **98**.

Kā modeļsubstrātu pirmajām testa reakcijām izvēlējamies imīnu **102a**, kas tika sintezēts saskaņā ar literatūrā aprakstīto metodi (24. shēma).¹³



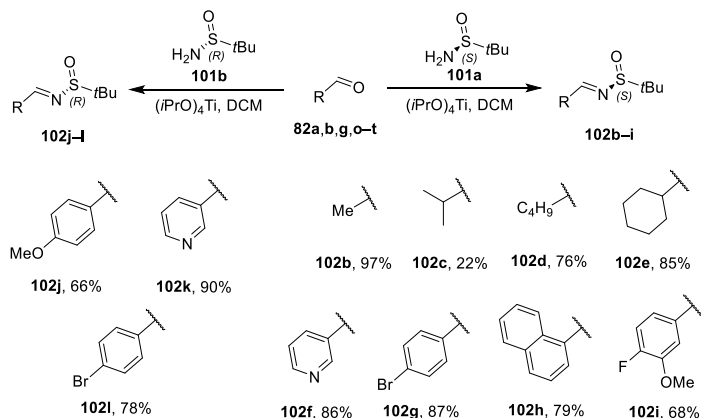
24. shēma. *t*-Butilsulfinilimīna **102a** sintēze.

1*N*-PMB aizsargātā tetrazola **63** deprotonēšanu veicām, izmantojot turbo Grinjāra reaģentu, kam sekoja metalētā starpprodukta pievienošana *t*-butilsulfinilimīnam **102a**, izmantojot DCM kā šķīdinātāju. Rezultātā ieguvām tikai pamatproduktu **103a** ar augstu iznākumu. ¹H un ¹³C KMR spektri metodes jutības robežās uzrādīja tikai viena diastereomēra klātbūtni (25. shēma). Saskaņā ar stereoindukcijas mehānismu, kas parādīts 23. shēmā, pieņemām, ka jaunizveidotā stereocentra konfigurācija produktā **103a** ir *S*, produkta konfigurācijas pierādīšanai ir nepieciešami papildu eksperimenti.



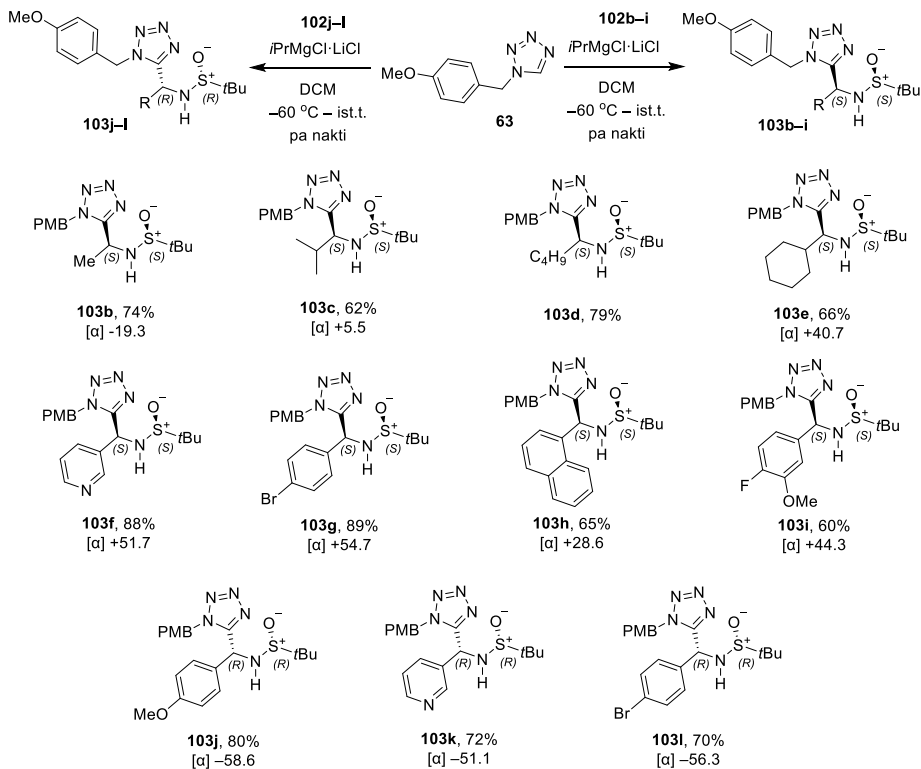
25. shēma. Tetrazola **63** pievienošana *t*-butilsulfinilimīnam **102a**.

Pēc pirmajiem daudzsološajiem rezultātiem pievērsāmies reakcijas produktu klāsta paplašināšanai, tāpēc sintezējām vairākus *t*-butilsulfinilimīnus **102b–l**, ko varētu izmantot tetrazola **63** pievienošanai (26. shēma).



26. shēma. *t*-Butilsulfinilimīnu **102b–l** sintēze.

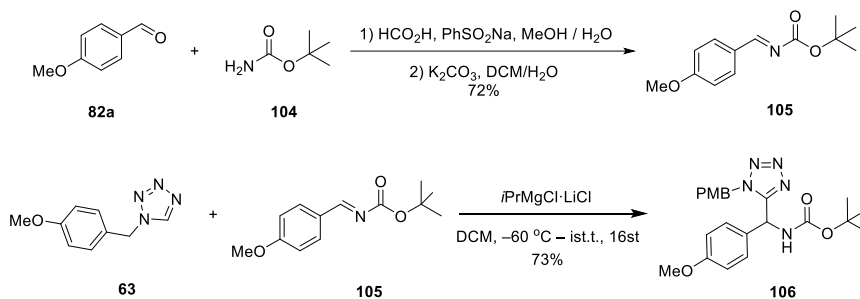
Piemērojot optimizētos reakcijas apstākļus *t*-butilsulfinilimīnu **102b–l** reakcijai ar tetrazolu **63**, ieguvām pievienošanās produktus **103b–l** ar labiem līdz izciliem iznākumiem (27. shēma).



27. shēma. Tetrazola **63** pievienošanās produkti *t*-butilsulfinilimīniem **102b–l**.

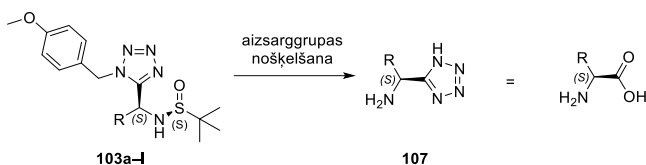
Noskaidrojām, ka tetrazolu **63** var pievienot arī *t*-butil- (*E*)-(4-metoksibenzilidēn)karbamātam (**105**), kas tika iegūts reakcijā starp *p*-anīsaldehīdu (**82a**) un

t-butylkarbamātu (**104**). Pievienošanas reakcijas rezultātā veidojās savienojums **106** (28. shēma).



28. shēma. Tetrazolu saturoša aminoskābes analoga **106** sintēze.

Sumējot iegūtos rezultātus, turbo Griņjāra izmantošana 1*N*-PMB aizsargāta tetrazola **63** deprotonēšanai un sekojoša pievienošana *t*-butilsulfīnilimīniem **102a–l** ļāva iegūt savienojumus **103a–l**, kas ir prekursori tetrazolu saturošiem aminoskābju analogiem **107** (29. shēma).



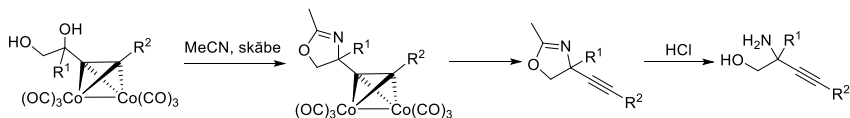
29. shēma. Piedāvātais aminoskābju analogu **107** sintēzes ceļš.

SECINĀJUMI

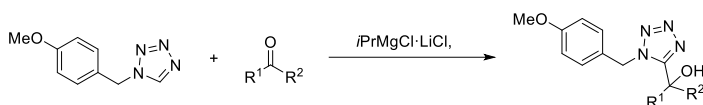
1. Identificēti jauni kemotipi kā potenciālie no PLP atkarīgu enzīmu kovalentie inhibitori. TriF-Ala analogi, kas satur hidroksāmskābi un sulfonamīdu, uzrādīja augstāko OASS inhibēšanas aktivitāti (mikromolārā līmenī).



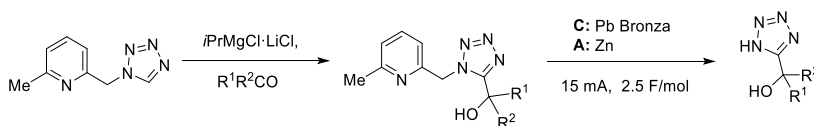
2. Izstrādāta jauna metode C-kvarternāro alkīnilglicīnolu sintēzei. Tā realizēta, izmantojot Ritera reakciju starp alkīnilglikola kobalta kompleksu un acetonitrilu, iegūstot oksazolīnu.



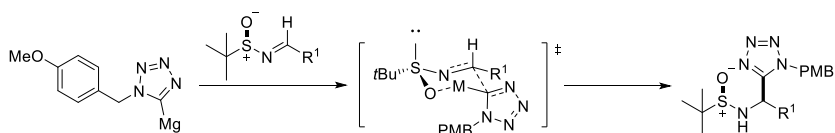
3. Tiešu tetrazolu C–H funkcionalizēšanu var panākt, izmantojot turbo Grīņjāra reaģentu (*i*PrMgCl·LiCl kompleksu). Turbo Grīņjāra reaģenta izmantošana nodrošina stabilāku metalētu starpproduktu, izvairoties no retro [2+3] ciklopievienošanās, kas ļauj realizēt reakcijas ar elektrofilēm, tai skaitā aldehīdiem un ketoniem.



4. 6-Metilpiridilmetil- aizsargātus tetrazolus var pakļaut C–H deprotonēšanai, izmantojot turbo Grīņjāra reaģentu, un sekojošai reakcijai ar elektrofilēm. 6-Metilpiridilmetilgrupu var nošķelt reducējošos elektroķīmiskos apstākļos.



5. Deprotonētu tetrazolu reakcijā ar enantiomēri bagātinātiem *t*-butilsulfīnilimīnīem var diastereoselektīvi iegūt atbilstošus pievienošanās produktus. Šī metode nodrošina piekļuvi aminoskābju analogiem, kas satur tetrazola fragmentu kā karbonskābes bioizostēru.



**DOCTORAL THESIS PROPOSED TO RIGA TECHNICAL
UNIVERSITY FOR THE PROMOTION TO THE SCIENTIFIC
DEGREE OF DOCTOR OF SCIENCE**

To be granted the scientific degree of Doctor of Sciences (Ph.D.), the present Doctoral Thesis has been submitted for the defence at the open meeting of RTU Promotion Council on June 14, 2023 at 14:00 at Riga Technical University Faculty of Materials Science and Applied Chemistry, Paula Valdena Street 3, Auditorium 272, Riga.

OFFICIAL REVIEWERS

Dr. chem. Mārtiņš Katkevičs
Latvian Institute of Organic Synthesis, Latvia

Dr. chem. Artis Kinēns
University of Latvia, Latvia

Dr. Marc Nazare
Leibniz-Institute for Molecular Pharmacology, Germany

DECLARATION OF ACADEMIC INTEGRITY

I hereby declare that the Doctoral Thesis submitted for the review to Riga Technical University for the promotion to the scientific degree of Doctor of Science (Ph. D.) is my own. I confirm that this Doctoral Thesis had not been submitted to any other university for the promotion to a scientific degree.

Konstantinos Grammatoglou
(signature)

Date

The Doctoral Thesis has been prepared as a collection of thematically related scientific publications. It unites four scientific publications that have been written in English, with a total volume of 301 pages, including supplementary data.

ABBREVIATIONS

AIBN	α,α' -azobisisobutyronitrile
Boc	<i>t</i> -butoxycarbonyl
CADD	computerassisted drug design
CAN	ceric ammonium nitrate
CDI	1,1'-carbonyldiimidazole
DCE	1,2-dichloroethane
DCM	dichloromethane
DDQ	2,3-dichloro-5,6-dicyano-1,4-benzoquinone
DFSI	aspartic acid–phenylalanine–serine–isoleucine peptide
DIPEA	diisopropylethylamine
DMAP	4-dimethylaminopyridine
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
HATU	hexafluorophosphate azabenzotriazole tetramethyl Uronium
HPLC	high performance liquid chromatography
IC ₅₀	half-maximal inhibitory concentration
LC/MS	liquid chromatography – mass spectrometry
MeCN	acetonitrile
NBS	<i>N</i> -bromosuccinimide
nBu	<i>n</i> -butyl
NMO	<i>N</i> -methylmorpholine- <i>N</i> -oxide
OASS	<i>O</i> -acetylserine sulfhydrylase
PDB	protein data bank
Ph	phenyl
PLP	pyridoxal 5'-phosphate (vitamin B6)
PMB	<i>p</i> -methoxybenzyl
RCSB	Research Collaboratory for Structural Bioinformatics
r.t.	room temperature
SAR	structure–activity relationship
SAT	serine acetyl transferase
TBA·BF ₄	tetrabutylammonium tetrafluoroborate
<i>t</i> Bu	tert-butyl
TBS	tert-butyldimethylsilyl
TFA	trifluoroacetic acid
THF	tetrahydrofuran
triF-Ala	3,3,3-trifluoro-alanine
WHO	World Health Organization

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GENERAL OVERVIEW OF THE THESIS

Introduction

The development of antibiotics in the first half of the 20th century, with penicillin discovery by Alexander Fleming in 1928 being the most prominent event, has signaled the beginning of a revolutionary era in medicine (Fig. 1).^{1a} Life expectancy rose significantly in all the developed countries, diseases that were until then untreatable and fatal were afterwards treatable by the use of antibiotics, the performance of complex surgical operations was made viable, people under immunosuppression or with chronic diseases managed to fight or prevent infections. Similar benefits from the use of antibiotics were observed in developing countries where food-borne and other poverty-related infections were treated, leading to decrease of morbidity and mortality.^{1a} Selman Waksman, in the late 30s, defined an antibiotic as “*a compound made by a microbe to destroy other microbes*”. Waksman identified soil-dwelling filamentous actinomycetes as producers of antimicrobial compounds, including neomycin and streptomycin, the first agent active against tuberculosis. Waksman’s work initiated the golden age of antibiotics, from the 40s until the late 60s, a period during which more than 20 new antibiotic classes were discovered. Amongst other macrolides, glycopeptides, cephalosporins, quinolones, azoles, etc., classes that include natural products or synthetic antibiotics inspired by natural products were developed (Fig. 1).^{1b,1c}

Alexander Fleming expressed his concerns about the improper use of penicillin as early as in 1945, he had already discovered staphylococci that were immune to penicillin and predicted the spread of resistant bacteria. The emergence of antibiotic resistance was caused by different factors like overuse of antibiotics, incorrect prescription of antibiotics, and extensive use in agriculture. Additionally, the decision of many companies to withdraw from the development of new antibiotics, as well as the increased difficulties in obtaining regulatory approval for new developments has led to diminished marketing of new antibiotics, adding to the established antibiotics resistance problem (Fig. 1).^{1a,1d,1e}

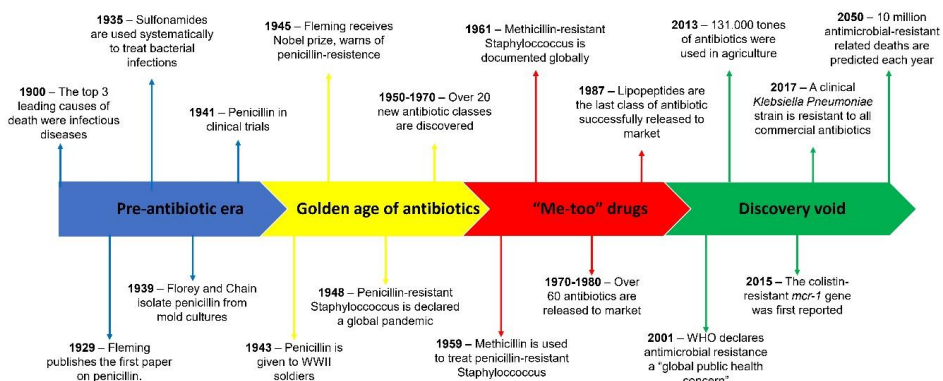
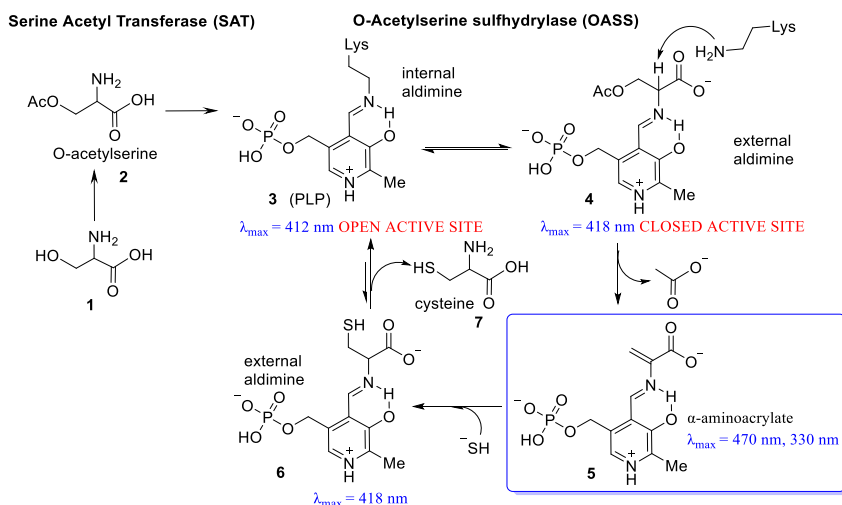


Fig. 1. The antibiotic-resistance timeline.

To overcome the ineffectiveness of certain antibiotics, “me-too” drugs were developed, which were the result of minor modifications made to existing antibiotics. The fact, however, that the mechanism of action of “me-too” drugs was, quite often, identical, resulted in rapid resistance to these compounds too.^{1e} The World Health Organization (WHO) declared antimicrobial resistance a “global public health concern” in 2001, and indicates a discovery void until today. The number of innovative new leads, during this period, is quite limited, and most of the drugs that enter the clinical pipeline aim to act on the same targets as traditional antibiotics.^{1e}

In the search of novel, effective antibiotics, various strategies have been exploited like the development of hybrid antimicrobial agents, membrane-active drugs, and inhibitors of bacterial virulence and pathogenesis, and exploitation of alternative methods like the use of bacteriophages.² Another strategy for the development of new antibiotics is focused on key metabolic functions of bacteria. Targeting important but non-essential gene products can be an effective means of reducing bacterial fitness, resulting in enhanced antibiotic treatments, shortened latency periods, increased susceptibility to traditional antibiotics and thereby facilitating clearance of the pathogen by the host immune system.³

Sulfur is a fundamental component of many biomolecules, from amino acids, such as cysteine, to cofactors and compounds that control the redox homoeostasis. Therefore, the enzymes involved in the cysteine biosynthetic pathway, which is present in pathogens and plants but absent in mammals, are very attractive antibacterial targets. Inhibition of cysteine biosynthesis has been proven to affect the ability of pathogens to fight oxidative stress, it reduces their virulence and leads to decreased antibiotic resistance.³⁻⁴ The final steps of the cysteine biosynthesis involve the action of two enzymes – serine acetyl transferase (SAT), which acetylates serine, and *O*-acetylserine sulfhydrylase (OASS), a pyridoxal 5'-phosphate (PLP) dependent enzyme that carries out a β -replacement reaction on acetyl serine (Scheme 1).



Scheme 1. Catalytic cycle of OASS to produce cysteine.

OASS is present in bacteria as two isoforms, OASS-A and OASS-B, also named CysK and CysM after the coding genes. Serine acetyltransferase (SAT) is able to form a high-affinity complex with OASS-A but not with OASS-B.⁵

Several efforts have been made to develop inhibitors of PLP-dependent enzymes, either following the structure-based, ligand-based, or mechanism-based design. The first attempts were focused in mimicking the nature developing pentapeptides with affinities in the micromolar range. In order to overcome the unfavorable drug-like properties of peptide inhibitors, and using *in silico* and synthetic chemistry techniques, various active small molecules were prepared (Fig. 2).⁶

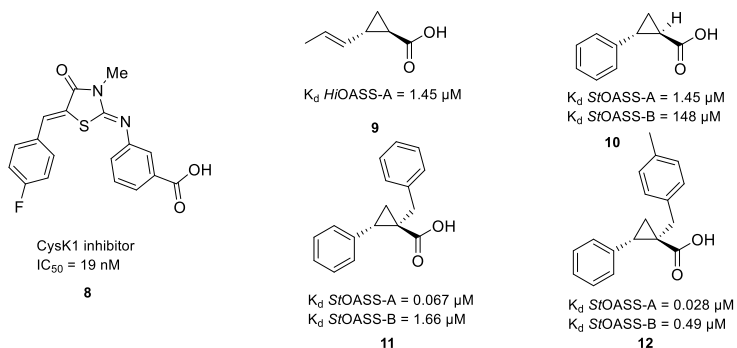


Fig. 2. Known inhibitors of OASS-A (CysK).

Mechanism-based inhibitors cause the inactivation of the enzyme through a chemical reaction that leads to an irreversible protein-inhibitor complex impeding the catalytic action of the enzyme. A range of chemotypes acting as mechanism-based inhibitors of PLP-dependent enzymes have been studied and reported.⁷

The rise of antimicrobial resistance made the identification of new druggable targets more urgent. Although PLP-dependent enzymes represent about 4 % of the enzymes classified by the Enzyme Commission, only a small number of them have been identified as potential targets for therapeutic agents, and even less are those for which drugs have been developed. These limited numbers are associated with the lack of knowledge regarding the family of PLP-dependent enzymes and the role played by them in a variety of biological processes. However, the fact that PLP carries out a variety of reactions and the spectral changes attributed to different PLP-substrate derivatives that help monitoring the reaction progress, are just two of the features that make PLP-dependent enzymes interesting targets.^{7d} In this aspect, the enzymes of the cysteine biosynthetic pathway attract enough interest. OASS is an important enzyme that could possibly be a target of antibacterials. Therefore, there is a need for the development of OASS inhibitors in order to investigate its importance for the antibacterial drug discovery. Furthermore, it is necessary to identify new chemotypes, from the unexplored chemical space, for PLP dependent enzymes' inhibitors. In addition, the requirement for the development of synthetic methods for these new chemotypes arises.

Aims and objectives

The aim of the Thesis is the synthesis of mechanism-based inhibitors-inactivators of PLP-dependent enzyme *O*-acetylserine sulfhydrylase (OASS) based on existing chemotypes, the investigation of new chemotypes, and the development of synthetic methods to facilitate the construction of potential PLP dependent enzyme inhibitors.

The following tasks were set:

1. Design and synthesis of small library of potential OASS inhibitors.
2. Development of efficient protocol for the synthesis of quaternary alkynyl glycinols.
3. Development of synthetic methods to introduce tetrazole as a carboxylic acid bioisoster.

Scientific novelty and main results

As part of scientific efforts, a number of known and new compounds were tested as potential inhibitors of bacterial *O*-acetylserine sulfhydrylase (OASS). Trifluoroalanine was found as the first mechanism-based inhibitor of OASS. SAR of triF-Ala derivatives was explored.

Additionally, several new methods were developed for the synthesis of PLP-dependent enzyme inhibitors:

1. Method for the synthesis of *C*-quaternary alkynyl glycinols.
2. Method for the direct C–H functionalization of tetrazoles using turbo Grignard reagent.
3. Introduction of electrochemically cleavable *N*-protecting group for tetrazole, facilitating its functionalization.
4. Stereoselective synthesis of amino acid analogues bearing tetrazole as carboxylic acid replacement.

Structure of the Thesis

The Thesis is a collection of scientific publications focused on the synthesis of triF–Ala analogues and the development of methods for the synthesis of PLP-dependent enzyme inhibitors.

Publications and approbation of the Thesis

Main results of the Thesis were summarized in four publications. In addition, results of the research were presented in seven conferences.

Scientific publications

1. **K. Grammatoglou**, J. Bolsakova, A. Jirgensons, C-Quaternary alkynyl glycinols *via* the Ritter reaction of cobalt complexed alkynyl glycols. *RSC Adv.* **2017**, *7*, 27530–27537.
2. N. Franko, **K. Grammatoglou**, B. Campanini, G. Costantino, A. Jirgensons, A. Mozzarelli, Inhibition of *O*-acetylserine sulfhydrylase by fluoroalanine derivatives. *J. Enzyme Inhib. Med. Chem.* **2018**, *33*, 1, 1343–1351.
3. **K. Grammatoglou**, A. Jirgensons, Functionalization of 1*N*-Protected Tetrazoles by Deprotonation with the Turbo Grignard Reagent. *J. Org. Chem.* **2022**, *87*, 3810–3816.
4. **K. Grammatoglou**, M. Dārziņa, A. Jirgensons, Functionalization of Tetrazoles Bearing the Electrochemically Cleavable 1*N*-(6-Methylpyridyl-2-methyl) Protecting Group. *ACS Omega* **2022**, *7*, 18103–18109.

Participation in conferences

1. **K. Grammatoglou**, J. Sirotkina, A. Jirgensons, Synthesis of 4-Alkynyl 2-Oxazolines *via* the Ritter reaction. *9th Balticum Organicum Syntheticum conference (BOS 2016)*, Riga, Latvia, July 3–6, **2016**, poster presentation.
2. **K. Grammatoglou**, J. Sirotkina, C-Quaternary Alkynyl Glycinols *via* the Ritter Reaction of Cobalt Complexed Alkynyl Glycols. *Paul Walden 10th Symposium on Organic Chemistry*, Riga, June 15–16, **2017**, poster presentation.
3. N. Franko, J. Magalhães, **K. Grammatoglou**, B. Campanini, M. Pieroni, E. Azzali, G. Annunziato, G. Costantino, A. Jirgensons, A. Mozzarelli, Targeting enzymes of the sulfur assimilation pathway for the development of new antibiotics. *Proceedings of the Proteine 2018 conference (PROTEINE2018)*, Verona, Italy, May 28–30, **2018**, poster presentation.
4. **K. Grammatoglou**, L. Levy, A. Jirgensons, Design and Synthesis of 3,3,3-trifluoroalanine Analogues as Potential Antibacterials. *10th Biannual Balticum Organicum Syntheticum conference (BOS 2018)*, Tallinn, Estonia, July 1–4, **2018**, poster presentation.
5. **K. Grammatoglou**, Design and synthesis of *O*-acetylserine sulfhydrylase inhibitors as potential antibacterials. *OUTREACH FINAL Conference ITN MSCA INTEGRATE “FIGHTING ESKAPE, THE BAD GANG”*, Parma, Italy, November 21–23, **2018**, oral presentation.
6. **K. Grammatoglou**, Functionalization of 1*N*-protected tetrazoles by deprotonation with turbo Grignard reagent. *Paul Walden 11th Symposium on Organic Chemistry*, Riga, September 19–20, **2019**, poster presentation.
7. **K. Grammatoglou**, Synthesis and Applications of Metalated 1*H*-tetrazoles. *Balticum Organicum Syntheticum conference (BOS 2022)*, Vilnius, Lithuania, July 3–6, **2022**, poster presentation.
8. **K. Grammatoglou**, Synthesis of PLP-dependent enzyme OASS inhibitors and the development of relevant synthetic methodologies. *Springboard Summer*

School “Major milestones in design and development of novel antimicrobials”,
Riga, August 23–25, **2022**, oral presentation.

MAIN RESULTS OF THE THESIS

1. Synthesis of mechanism-based OASS inhibitors

A focused library of amino acids – potential mechanism-based inhibitors of OASS as PLP-dependent enzyme was assembled (Fig. 3). Compounds **13–17** were purchased, as they were commercially available, while compounds **18–19** were synthesized for a different, recently published project,⁸ and compounds **20–22** were synthesized by our workgroup. The screening of the library against OASS revealed that triF-Ala (**13**) exhibited inhibitory potency with $IC_{50} = 132 \pm 13 \mu\text{M}$ (Fig. 3).

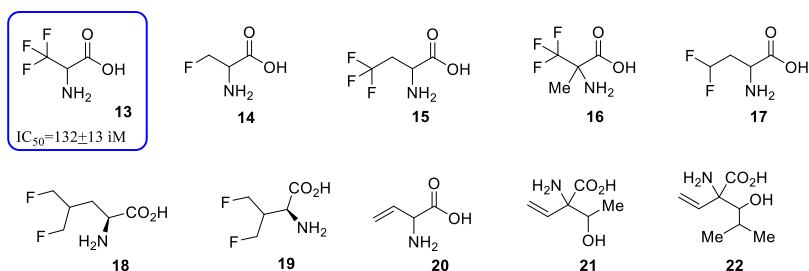
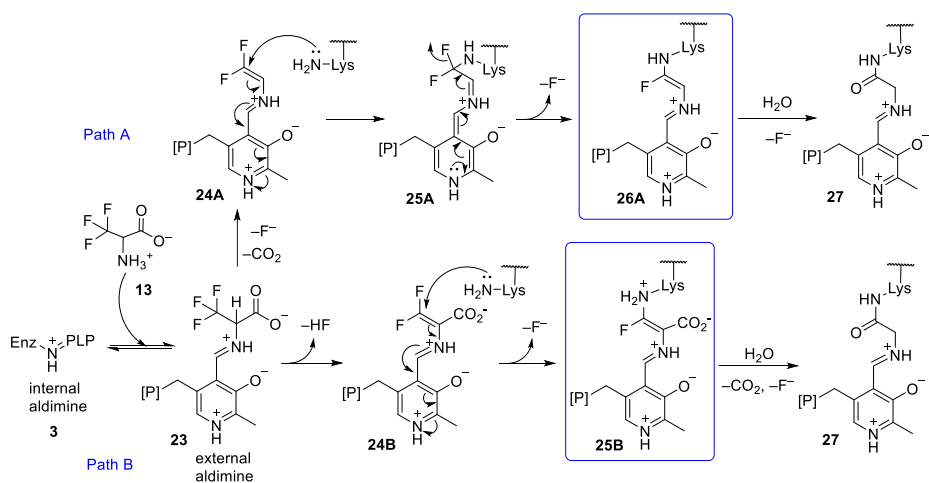


Fig. 3. Library of potential mechanism-based inhibitors of OASS.

Halogenated alanines have been exploited as inhibitors of various PLP-dependent enzymes.^{5, 9} Based on previously reported mechanisms for the inactivation of PLP-dependent enzymes, addition of triF-Ala (**13**) to the resting enzyme leads to initial transaldimination forming the external aldimine (**23**) (Scheme 2).



Scheme 2. Possible inactivation pathways of PLP-dependent enzymes by triF-Ala.

Two possible paths have been proposed after that, path A describes the loss of fluoride and decarboxylation to intermediate **24A**. Elimination of the second fluoride ion and concomitant attack of an enzyme active site lysine on the olefinic terminus of the electrophilic unsaturated imine leads to the enzyme inactivation. The resultant inactive complex **26A** would be covalently bound to the enzyme through the lysine residue. Unsaturated imine **26A** is attacked by water and after further loss of HF it gives the amide **27**. According to path B after the initial transaldimination, elimination of HF occurs to give intermediate **24B**, which is more active towards Michael attack by active site lysine. Further elimination of the second fluoride ion gives rise to the inactive complex **25B**, which would be covalently bound to the enzyme through the lysine residue. **25B** is attacked by water and after further loss of HF and CO₂, it gives the amide **27** (Scheme 2).^{5, 10}

Photochemical investigations showed that β,β -trifluoroalanine (triF-Ala, **13**), a known suicide substrate of PLP-dependent enzymes, forms an adduct with the enzyme, which causes changes to the absorbance spectra compared to the spectra of the internal aldimine of the resting enzyme, an indication of the reaction taking place on the co-factor. The interactions of triF-Ala with both isozymes of OASS, OASS-A (or Cys-K) and OASS-B (or Cys-M) were investigated by absorbance and fluorescence spectroscopy.

As it has already been noted, the absorption spectra of OASS-A and OASS-B exhibit a band at 412 nm, attributed to the internal aldimine (Fig. 5, **3**). After addition of triF-Ala to OASS-A two peaks appeared at 440 and 466 nm, and two minor bands at 360 and 380 nm (Fig. 4), indicating a species with extended conjugation (Fig. 5, **24B**). The intensity of 466 nm absorbance slowly decreased with the formation of a band at 412 nm. Less intense spectral changes were observed at the reaction of triF-Ala with OASS-B in the range of 400–500 nm. The decomposition of the intermediate along with the decrease at 457 nm was accompanied by the increase in absorbance in the range of 300–350 nm, possibly attributed to the production of difluoropyruvate.

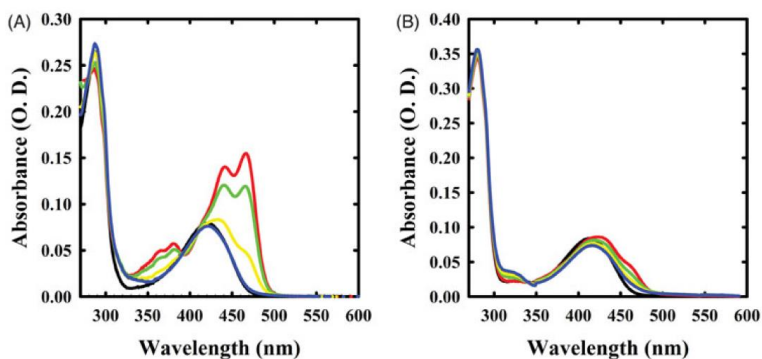


Fig. 4. Absorbance spectra of OASS in the absence and presence of 1 mM triF-la. Panel A: absorbance spectrum of OASS-A in the absence of reagent (black line), 1 min (red line), 1 h (green line), 3 h (yellow line), and 7 h (blue line) after addition of the reagent. Panel B: absorbance spectrum of OASS-B in the absence of reagent (black line), 1 min (red line), 1 h (green line), 3 h (yellow line), and 7 h (blue line) after addition of the reagent.

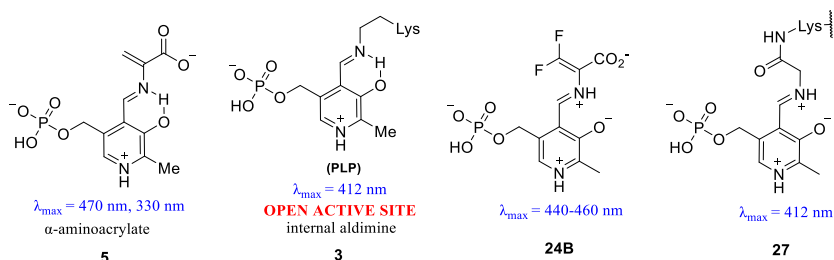


Fig. 5. Structures attributed to absorbance bands observed during OASS inactivation process.

Next, the reaction of OASS-A was monitored by fluorescence emission (Fig. 6), and the band measured upon excitation at 412 nm was blue shifted to 495 nm. This confirms that the species formed is not the α -aminoacrylate (Fig. 5, **5**). The emission band slowly decreased but after 6 hours incubation, in contrast to what was observed by absorbance spectroscopy, there was no recovery of the initial emission spectrum. The latter suggests that this species that absorbs at 412 nm is different than the internal aldimine **27** (Fig. 5). Upon excitation at 412 nm of the reaction of triF-Ala with OASS-B, the intensity of emission is primarily increased and then slowly decreased. This is accompanied by a small blue shift to 501 nm, which slowly shifts back to 505 nm after incubation of 7 hours. These changes may refer to an external aldimine, and the small blue shift suggests the formation of a transient species.

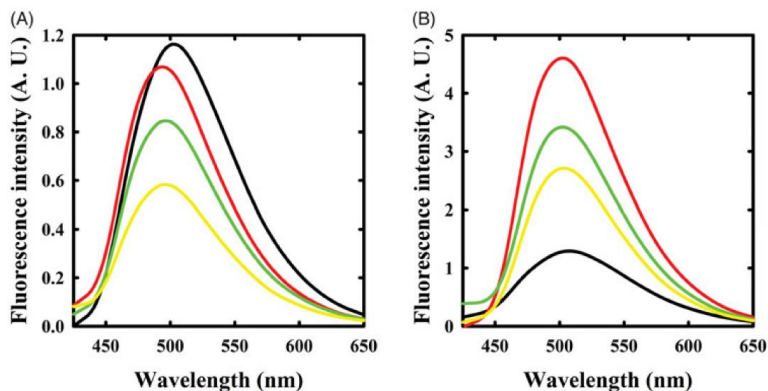


Fig. 6. Fluorescence emission spectrum of OASS in the absence and presence of 1 mM triF-Ala. Emission spectra were recorded upon excitation at 412 nm. Panel A: OASS-A in the absence of reagent (black line), 1 min (red line), 4 h (green line), and 6 h (yellow line) after addition of the reagent. Panel B: OASS-B in the absence of reagent (black line), 1 min (red line), 3 h (green line), and 7 h (yellow line) after addition of the reagent.

Further investigation was performed to evaluate the potential inhibitory action of F-Ala and triF-Ala on OASS-A and OASS-B. Two different experiments were conducted for this purpose; first the two isozymes were assayed after exposure to increasing concentrations of F-Ala determining IC_{50} values of $480 \pm 50 \mu\text{M}$ and $1290 \pm 230 \mu\text{M}$ for OASS-A and OASS-B respectively, and for triF-Ala IC_{50} values of $130 \pm 10 \mu\text{M}$ and $940 \pm 60 \mu\text{M}$ were determined. To detect whether triF-Ala is irreversible inhibitor of OASS, as it is reported for

other PLP-dependent enzymes, the kinetics of OASS-A in the presence of different concentrations of triF-Ala were monitored, and the results were in agreement to what is expected by the suicide substrates. The second experiment involved assaying the enzyme after 85-hour reaction with 10 mM triF-Ala, the complete removal of ligand, and incubation in the presence of saturating PLP concentration. The results of the latter experiment were indicative of the inactivation caused by irreversible covalent modification of active site residue(s) that was brought about by triF-Ala.

Our research for new OASS inhibitors involved the use of computer assisted drug design (CADD) methods. We relied on the data already reported about identified OASS inhibitors and the structural information retrieved from the crystallographic data of the inhibitors and the enzyme.^{6d} The natural peptide DFSI **28**, which consists of the last four residues of SAT (CysE) enzyme, has been previously identified as OASS-CysK inhibitor. Sriram et al. reported the development of OASS-CysK inhibitor **8** with improved activity compared to the natural peptide, DFSI (Fig. 7, **28**). Inhibitor **8**, as well as **28**, are competitive inhibitors, which bind in the proximity of PLP cofactor in the active site cleft of the enzyme.

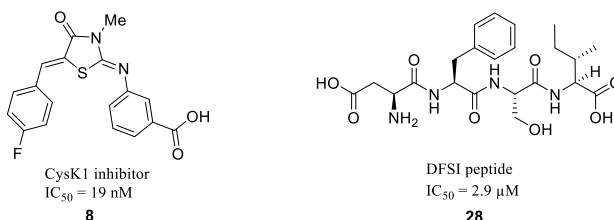


Fig. 7. OASS inhibitors, CysK1 inhibitor **8** and DFSI peptide **28**.

Based on these data we proceeded to construction of a protein model on Schrödinger Maestro suite in order to perform virtual screening of ligand libraries. Two proteins were chosen from RCSB Protein Data Bank (PDB), 2Q3C crystal structure of OASS holoenzyme from *mycobacterium tuberculosis* in complex with the inhibitory peptide DFSI and 3ZEI structure of the *mycobacterium tuberculosis* OASS-CysK in complex with the small molecule inhibitor **8** (Fig. 8).

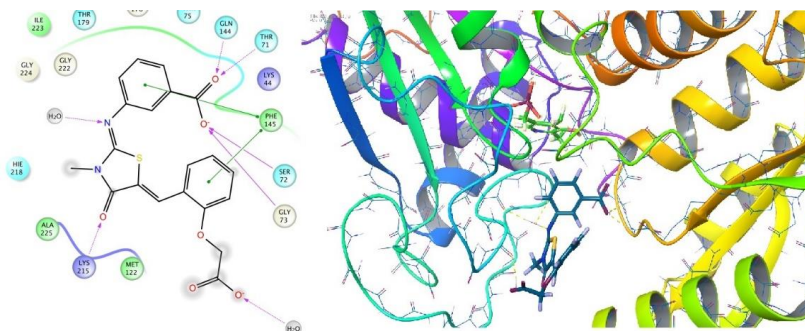


Fig. 8. OASS-CysK complex with inhibitor **8**.

Both protein structures were prepared using Schrödinger's protein preparation wizard. The 3ZEI protein was chosen as the base for our study, as the complex with the known

inhibitor allowed us to identify the main interactions in the enzyme pocket. Docking of triF-Ala, that had already shown affinity to PLP, followed the interactions revealed above, pointed out the most important ones and also the difference from previous inhibitors, as the interaction with PLP is observed (Fig. 9).

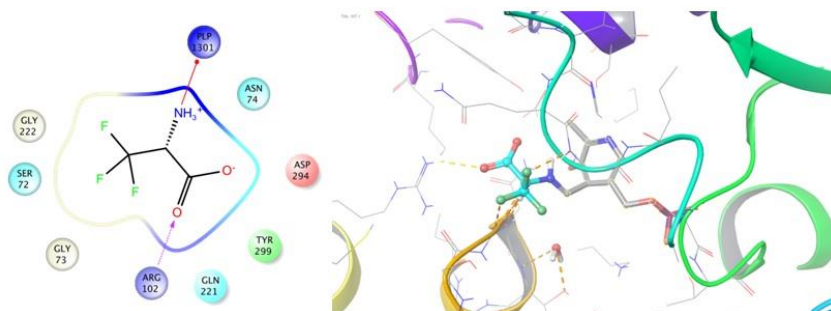


Fig. 9. Interactions of triF-Ala with OASS protein residues and PLP.

As our aim was to synthesize covalent inhibitors of OASS, we aimed for the substrates to perform a reaction with PLP. This had to be programmed in Schrödinger suite in order to perform the docking experiments with this function. Therefore, the docking motif had to be reprogrammed to address the reaction between the substrate and PLP, and a new custom reaction file was prepared to facilitate this covalent bond during the virtual experiments. Having the protein and the reaction motif prepared we proceeded to the covalent docking of commercial libraries (Enamine's "Stock Screening Compounds Collection", MolPort's "Screening Compound Database", and eMolecules database) and the fragment library provided by Schrödinger software. A library containing 140000 compounds was compiled and after selection of the compounds that fulfilled the verified interactions, we ended up with a library of 200 compounds. These compounds were submitted to covalent docking experiments producing approximately 2000 docking poses. Combining the scoring given by the molecular docking program Glide, and the categorization based on structural motifs, we ended up with generic structures as indicative targets for our synthetic work (Fig. 10).

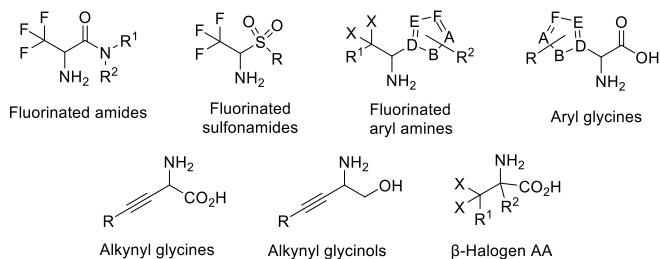
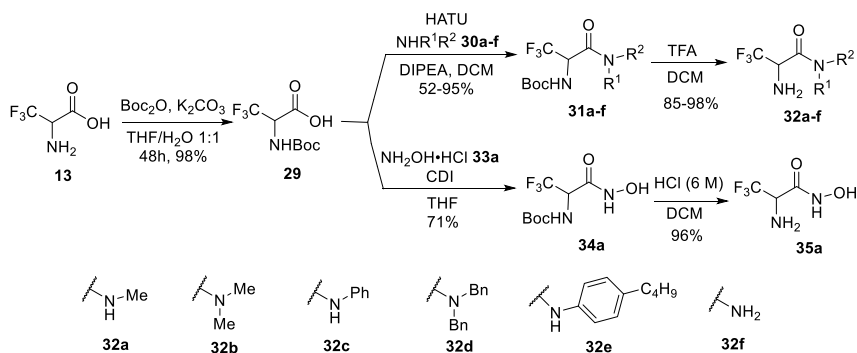


Fig. 10. Chemotypes promoted for synthetic realization.

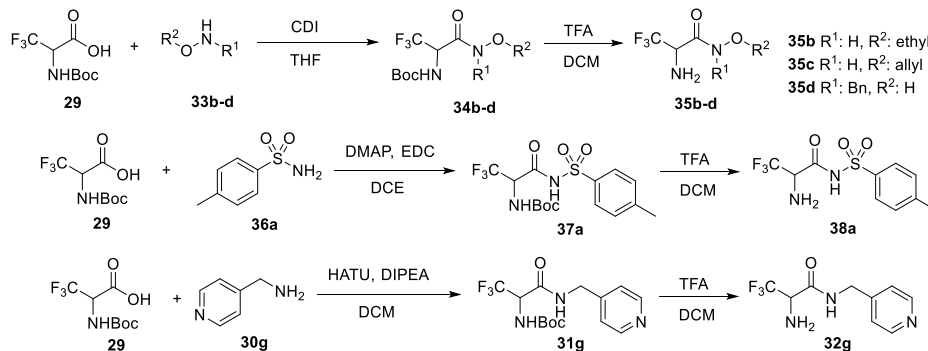
2. Synthesis of trifluoroalanine analogues

The substitution of the carboxylic acid moiety of triF-Ala (**13**) with bioisosters was attempted first by synthesizing several amides and hydroxamic acid (Scheme 3). Boc-protected triF-Ala **29** underwent coupling with different amines using HATU, and after acidic removal of Boc group amides **32a-f** were obtained in high yields. For the synthesis of hydroxamic acid **35a** the use of CDI as a coupling agent afforded the required product **34a** in excellent yield, which was deprotected to give **35a**.



Scheme 3. Synthesis of triF-Ala derived amides **32a-f** and hydroxamic acid **35a**.

In a further effort to expand the library of triF-Ala analogues, we proceeded to parallel synthesis. Initially, the synthetic protocols for the application in parallel synthesis setup were established using model reactions. For the synthesis of hydroxamic acids **35b-d** the CDI coupling method proved to be a good choice. For the synthesis of acylsulfonamide **38a** the use of DMAP and EDC coupling was appropriate. For the synthesis of amide **32g** the already established protocol with HATU and DIPEA was applicable (Scheme 4).



Scheme 4. Validation of reaction protocols for parallel synthesis.

Having the protocols established we proceeded to the synthesis of several compounds using the parallel synthesis blocks. In this setup we could run multiple reactions in 48 tubes' block, then filter the whole set through fritted filter plates to an analytical 48 well plate, which was submitted to purification with preparative HPLC (Fig. 11).

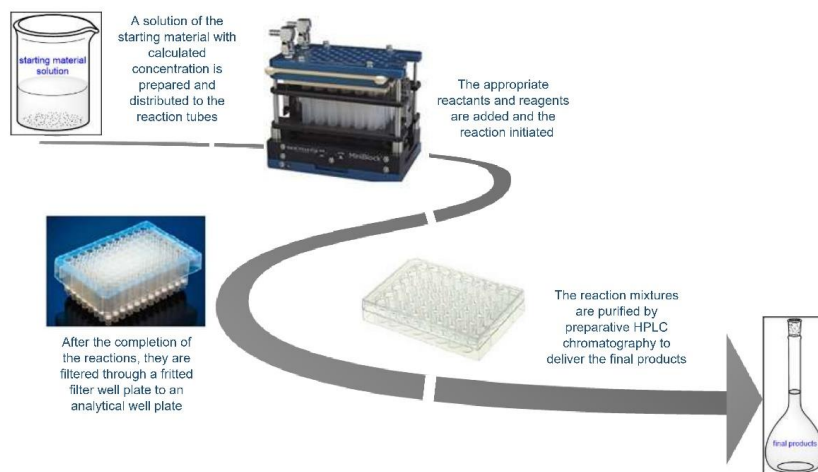


Fig. 11. Parallel synthesis workflow.

Following this workplan we managed to synthesize and isolate 42 compounds in short period. 11 hydroxamic acids were successfully synthesized and isolated, **35b–l** (additional to **35a** previously synthesized too), including aromatic and aliphatic N- and O- substituted compounds (Fig. 12).

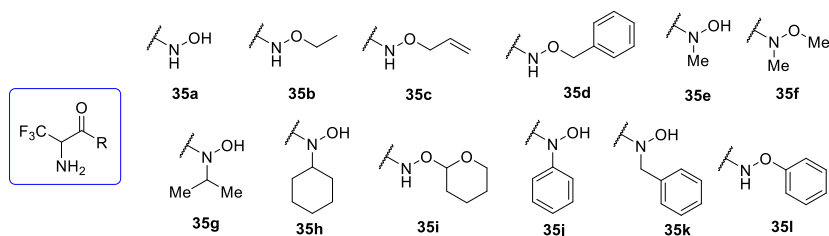


Fig. 12. Hydroxamic acids synthesized by parallel chemistry approach.

Different acylsulfonamides **38b–p** (plus **38a**) were prepared, most of them containing an aromatic or heteroaromatic moiety with various substituents like halogen (**38e**, **38f**, **38h**, **38k**, **38m**), cyano (**38n**), nitro (**38k**, **38l**), etc. (Fig. 13).

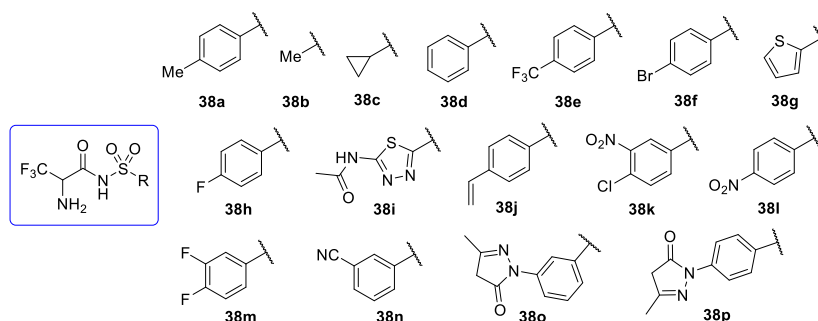


Fig. 13. *N*-acylsulfonamides **38a–p** synthesized by parallel chemistry approach.

Finally, 15 amides **32h–u** (plus **32g**) were synthesized using parallel synthesis setup compiling a versatile group of compounds, like pyridine with halogen or methyl substitution (**32h**, **32j**, **32o**, **32t**) substrates, pyridine-methyl amides (**32g**, **32u**), and various 5-membered heterocyclic amides (Fig. 14).

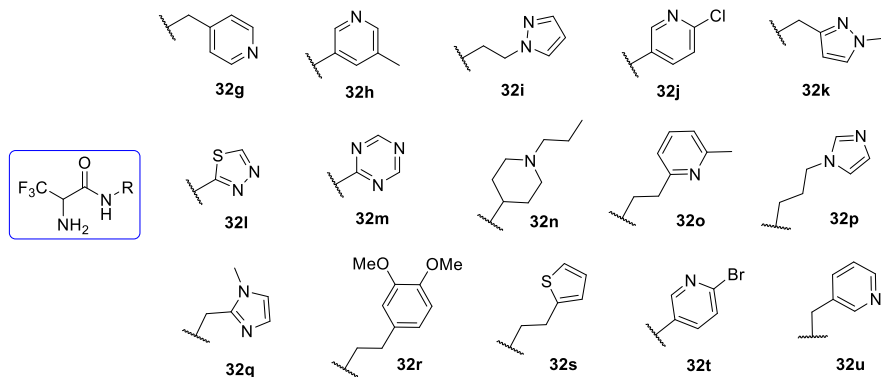
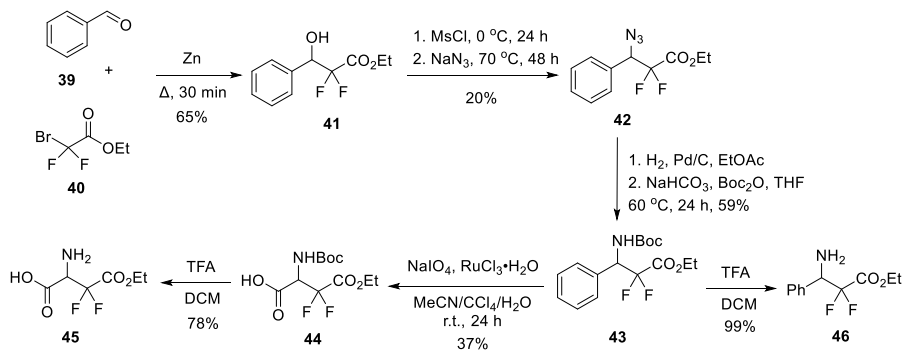


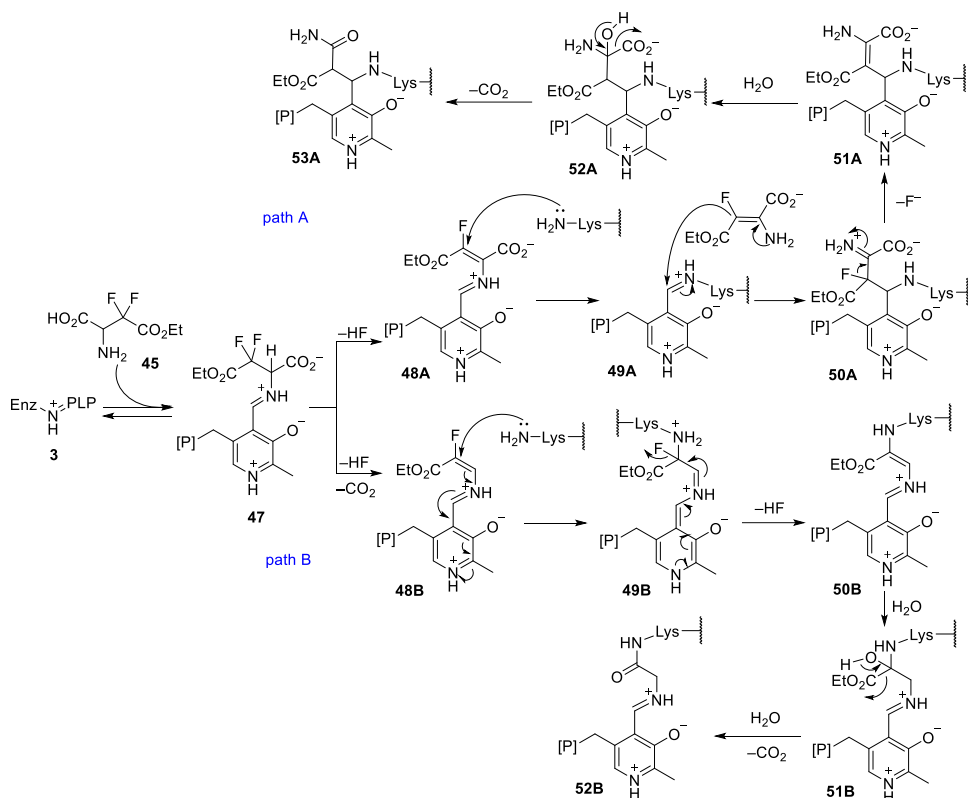
Fig. 14. Amides **32g–u** synthesized by parallel chemistry approach.

As part of triF-Ala analogues synthesis we aimed to obtain difluoroaspartic acid monoester **45**. For that, the synthesis of alcohol **41** was realized through a Barbier type reaction between benzaldehyde (**39**) and ethyl bromodifluoroacetate (**40**). The mesylation of alcohol **41** and substitution with azide provided intermediate **42**. The latter was reduced to amine, which was Boc-protected *in situ* to give amine **43**. This was subjected to aromatic oxidation with sodium periodate and ruthenium chloride to transform the phenyl group to carboxyl group. The resulting acid **44** was deprotected to give the desired difluoro aspartic acid monoester **45**. To expand the library of triF-Ala analogues, Boc-protected amine **43** was transformed to a free amine **46** for testing against OASS (Scheme 5).



Scheme 5. Synthesis of difluoro aspartic acid monoester **45**.

We also assume that difluoro aspartic acid monoester **45** might possibly act as mechanism-based inhibitor (Scheme 6) following two different pathways: with decarboxylation taking place early (**48B–52B**, path B) or late (**48A–53A**, path A) in the route, according to the proposed mechanism.



Scheme 6. Plausible mechanisms of inactivation of OASS by difluoro aspartic acid monoester 45.

Other targets within the triF-Ala analogues program were the compounds in which carboxylic acid was substituted with triazole **54** and tetrazole **55** (Fig. 15).

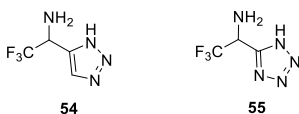
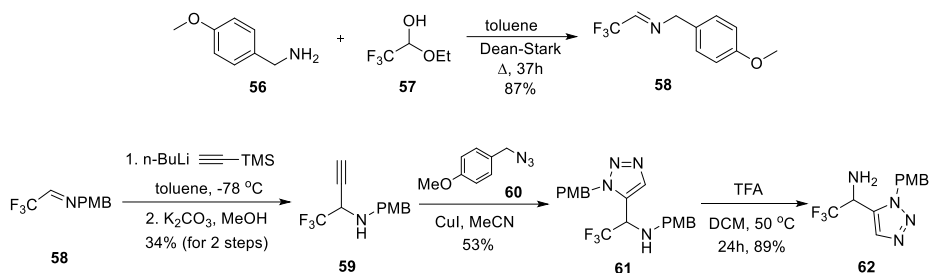


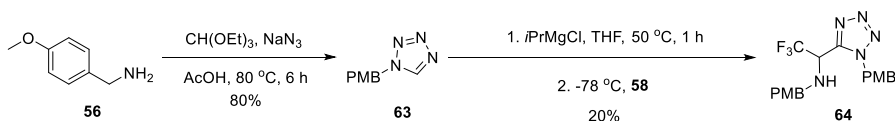
Fig. 15. Triazole and tetrazole analogues of triF-Ala.

To prepare the target compounds **54** and **55** first, we synthesized imine **58** by the condensation of *p*-methoxy benzylamine **56** with trifluoro acetaldehyde ethyl hemiacetal **57**. The nucleophilic addition of TMS-acetylene to imine **58** followed by desilylation provided alkyne **59**. The latter underwent copper mediated [2 + 3] cycloaddition with *p*-methoxy benzylazide **60** to give the protected triazole **61**. The PMB deprotection with TFA, to our surprise, occurred only from amino group providing triazole **62** (Scheme 7).



Scheme 7. Triazole 62 synthetic route.

The synthesis of the target compound **55** began by the preparation of PMB protected tetrazole **63** following the classic conditions reported in literature. Intermediate **63** was C–H metalated using *i*PrMgCl for the deprotonation and added to the imine **58** providing tetrazole **64** in a low yield (Scheme 8). Efforts to deprotect tetrazole **64** under various conditions (TFA/DCM, neat TFA 60 °C, H₂/Pd, CAN, DDQ) led either to decomposition of the tetrazole (main product identified was *p*-methoxy benzylamine **56**) or to an inseparable mixture of tetrazole **64** with partially deprotected product.



Scheme 8. Synthesis of tetrazole 64.

3. Evaluation of inhibitory potency of trifluoroalanine analogues

The compounds **32a–u**, **35a–l**, **38a–p**, **45**, **46**, **62** (Fig. 16) synthesized as described herein, were evaluated for their reactivity with OASS-A and OASS-B isozymes as well as for enzyme inhibition potency and inactivation kinetics. Assays were carried out on CysK and CysM of *Salmonella Typhimurium* recombinantly expressed in *Escherichia coli*. The enzyme was incubated with compounds at 1 mM concentration and the enzymatic activity was measured at time intervals.

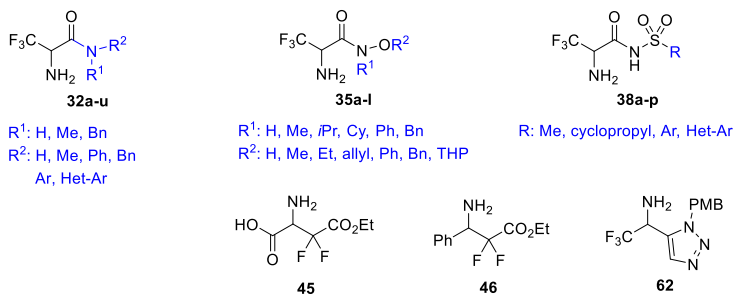


Fig. 16. Library of synthesized compounds that were tested for their reactivity with OASS.

To study the effect of the modification in the chain of triF-Ala, compounds **3–5** (Fig. 3) and **45–46** (Scheme 5) were investigated for reactivity with OASS-A and OASS-B. None of these compounds showed measurable improvement in reactivity compared to parent compound **13**, triF-Ala ($IC_{50} = 132 \mu\text{M}$).

Next, the effect of bioisosteric replacement of the carboxylic acid moiety was examined. The first set of compounds, **32a–f** (Fig. 16) and **35a** (Fig. 12) were tested observing again only negligible effects on reactivity with an enzyme. Noteworthy, that hydroxamic acid **35a** caused small absorbance changes in OASS-A (Fig. 17), and about 14 % decrease in enzyme activity.⁵

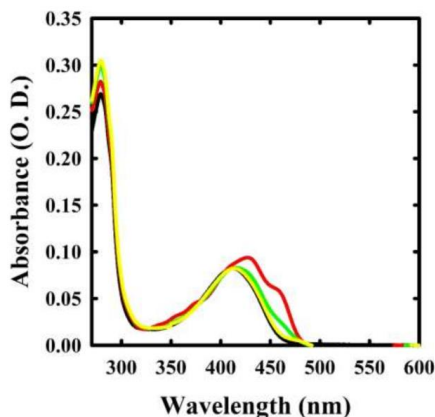


Fig. 17. Spectral changes for the reaction between OASS-A and 1 mM compound **35a**. OASS-A in the absence of reagent (black line) and 1 min (red line), 30 min (green line) and 1 h (yellow line) after compound **22a** addition.

From the next set of compounds, **35b–l**, **38a–p**, and **32g–u** (Fig. 16) 5 compounds showed measurable OASS-A inhibitory properties (Fig. 18). Compounds **38i**, **38p** and **32h** proved to be weak binders of the enzyme and did not cause significant inactivation, however, hydroxamic acid derivative **35k** and sulfonamide **38b** were the most potent compounds from this small library.

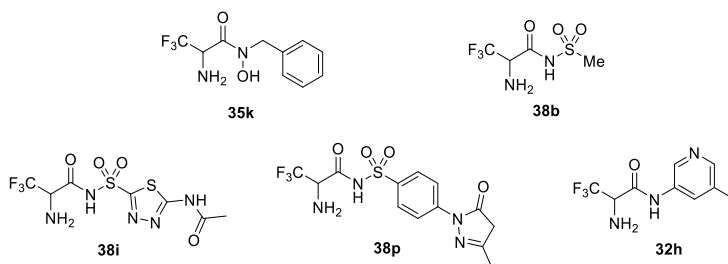


Fig. 18. Highest performing compounds.

Hydroxamic acid **35k** exhibited IC_{50} ($177 \pm 29 \mu\text{M}$) and in inactivation assay, approximately 15 % inhibition was observed at the first time point, although it is lost later, probably due the hydrolysis of the intermediate (Fig. 19).

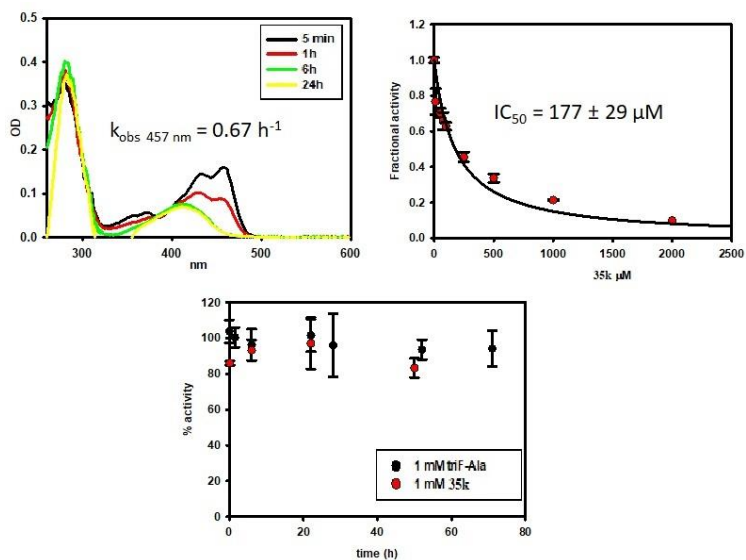


Fig. 19. 1 mM inhibitor 35k, following 5000 times dilution for the assay (0.2 μM inhibitor in the assay) in two replicates.

Sulfonamide **38b** also showed IC_{50} ($179 \pm 22 \mu\text{M}$) for the inhibition of OASS-A and approximately 20 % inhibition was observed during the inactivation assay at the first time point, although it is lost later, probably due the hydrolysis of the intermediate (Fig. 20).

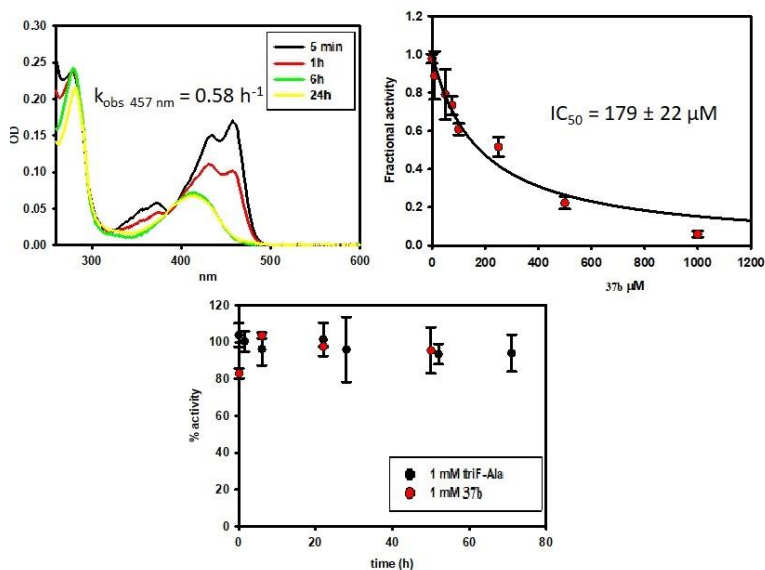


Fig. 20. 1 mM inhibitor 38b, following 5000 times dilution for the assay (0.2 μM inhibitor in the assay) in two replicates.

Based on the spectral and kinetics data collected from the reactivity experiments of triF-Ala with OASS and taking into consideration the mechanistic schemes already proposed for the reaction of triF-Ala with other PLP-dependent enzymes, we hypothesized that the inactivation mechanism of OASS-A is similar to the one proposed previously (Scheme 2). We propose that the mechanism follows path B rather than path A, as decarboxylation does not take place in the catalytic cycle of OASS.

Inhibitors **35k** and **38b** comprise promising scaffolds for further development, as these, in contrast to the parent triF-Ala (**13**), contain substructures amenable for derivatization to pick up additional interactions with OASS. The work in this direction was suspended due to expiration of the program making the biological testing temporarily unavailable.

4. Development of new methods for the synthesis of PLP-dependent enzyme inhibitors

4.1. Novel approach to C-quaternary alkynyl glycinols

One of the chemotypes that emerged as potential inhibitors of PLP-dependent enzymes was the alkynyl glycinols **65**. These, along with synthetically equivalent alkynyl glycine derivatives **66** (Fig. 21), are very useful building blocks for the synthesis of complex bioactive molecules. However, there are only a few methods for the direct synthesis of alkynyl glycinols avoiding the reduction of carboxyl groups in glycines **66**.

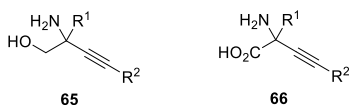
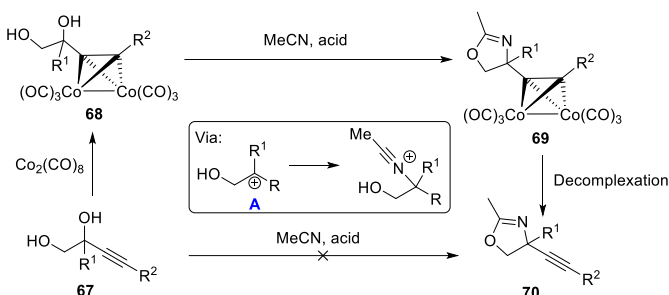


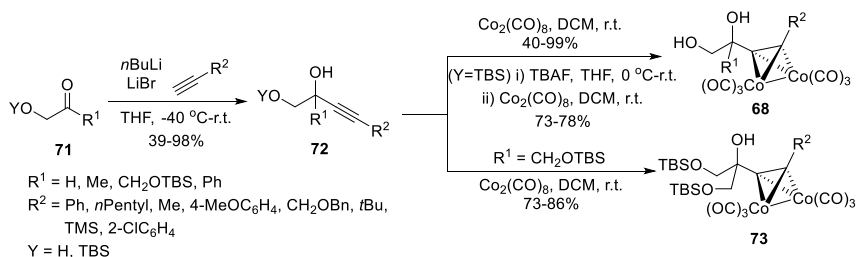
Fig. 21. Alkynyl glycinols **65** and alkynyl glycines **66**.

The Ritter reaction of 1,2-diols was examined as method for the synthesis of alkynyl glycinols through the corresponding oxazolines. The first attempt for the direct access to oxazoline **70** only delivered a poor <10 % yield (Scheme 9). The use of cobalt complexed alkynyl glycinols **68**, which can effectively stabilize the intermediate carbenium ion **A**, provided oxazolines **70**, that serve as a precursor of alkynyl glycinol derivatives **65**.



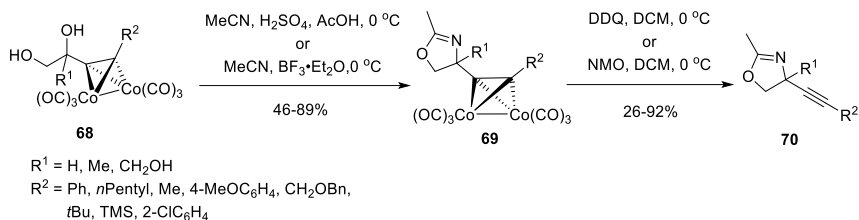
Scheme 9. Synthesis of oxazolines by Ritter reaction.

Preparation of cobalt complexed alkynyl glycols **68** and **73** was carried out in two steps, first, by the addition of lithium acetylenides to hydroxy ketone derivatives **71** and then by the treatment of the provided alkynyl diols **72** with $\text{Co}_2(\text{CO})_8$ (Scheme 10).



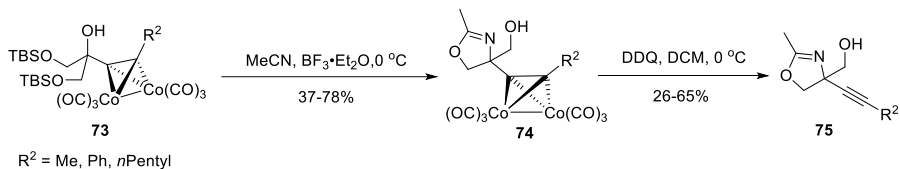
Scheme 10. Synthesis of cobalt complexed glycols **68** and **73**.

Using either H_2SO_4 or $\text{BF}_3 \cdot \text{Et}_2\text{O}$ as acid promoters, cobalt complexed alkynyl glycols **68** gave the corresponding oxazolines **69** *via* the Ritter reaction with acetonitrile. The reaction tolerated a wide range of substituents at R^2 position, while substrates bearing a phenyl moiety at R^1 position did not give the expected oxazoline (Scheme 11).



Scheme 11. The Ritter reaction and cobalt decomplexation towards oxazolines **70**.

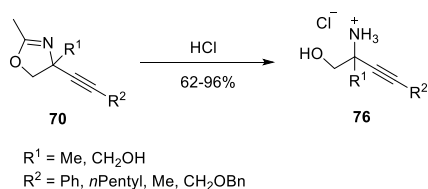
While the main route involved the deprotection of TBS protected alcohols before the formation of the cobalt complex, successful examples of protected alcohols undergoing the Ritter reaction with concomitant removal of the TBS group are reported (Scheme 12).



Scheme 12. The Ritter reaction and cobalt decomplexation towards oxazolines **75**.

For the Co-decomplexation the use of DDQ proved to be more effective compared to NMO in most cases. Notably it is the first demonstration of DDQ as a reagent for the decomplexation of alkyne cobalt complexes (Schemes 11 and 12).

Selected oxazolines were submitted to acidic hydrolysis in mild conditions to obtain the final amino alcohols **76** with good yields (Scheme 13).

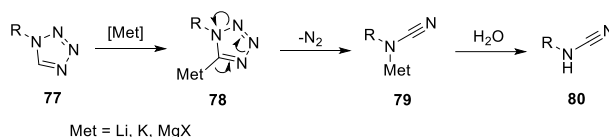


Scheme 13. Hydrolysis of oxazolines **70** to amino alcohols **76**.

4.2. Functionalization of 1*N*-PMB-protected tetrazoles

Our efforts to synthesize tetrazole containing analogue of triF-Ala **64** were the starting point to develop a new method for the direct C–H functionalization of tetrazole with improved yield compared to our initial effort presented in Scheme 8.

Our target was to raise the reaction yield (20 %) of the addition of tetrazole to imine which suffered from the retro [2+3] cyclization reaction of metalated intermediate that leads to the cyanamide **80** (Scheme 14). The instability of metalated tetrazole **78** can take place even at temperature as low as $-98\text{ }^{\circ}\text{C}$, which constitutes the major problem for the derivatization of tetrazole by C–H deprotonation.

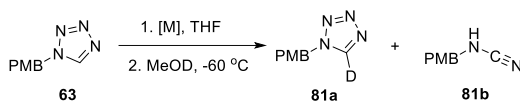


Scheme 14. Retro [2+3] cyclization of metalated tetrazoles.

It was known from the literature that tetrazole-derived Grignard reagents made by halogen-metal exchange showed improved stability.¹¹ This urged us to turn our research towards preparation of these reagents by C–H deprotonation. For these purposes we prepared 1*N*-PMB-protected tetrazole **63**, which served as our test substrate (Table 1). We examined Grignard and turbo Grignard reagents using deuterium quench to determine the efficiency of the deprotonation. Range of bases were investigated, and it was the turbo Grignard (*i*PrMgCl·LiCl) that proved to be the most efficient, giving 99 % of deuteration (Table 1, entries 1–2). Notably, under these conditions, the product of the retro [2+3] cycloaddition compound **81b** was not detected even after 1 hour at $-60\text{ }^{\circ}\text{C}$. Other Grignard reagents such as *i*PrMgCl and *i*PrMgBr were investigated; however, these turned out to be less efficacious compared to the turbo Grignard reagent (Table 1, entries 3–6).

Table 1

Evaluation of Deprotonating Reagents



Entry	[M]	Time (min)	81a ^a yield %	81b
1	<i>i</i> PrMgCl·LiCl	15	98	n.d. ^b
2	<i>i</i> PrMgCl·LiCl	60	99	n.d.
3	<i>i</i> PrMgCl	15	53	n.d.
4	<i>i</i> PrMgCl	60	76	n.d.
5	<i>i</i> PrMgBr	15	57	n.d.
6	<i>i</i> PrMgBr	60	69	n.d.

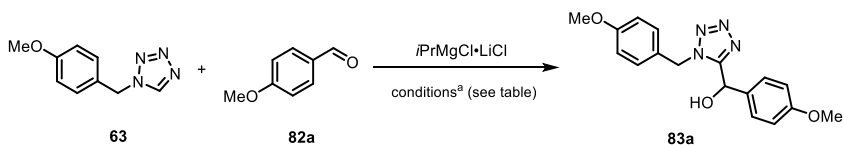
^a Yields were calculated based on the weight of crude material; NMR did not reveal any other compounds apart from **81a** and **81b**.

^b n.d. = not detected.

The reaction of tetrazole **63** with anisaldehyde **82a** was then examined. Using THF as a solvent, the expected product, alcohol **83a**, was obtained in very good yield (Table 2, entry 1). The reaction yield was not decreased when the reaction was warmed to room temperature after the addition of anisaldehyde (Table 2, entry 2). The use of different solvents (Et₂O, Toluene) lowered the reaction outcome, and they were excluded.

Table 2

Optimization of Reaction Conditions



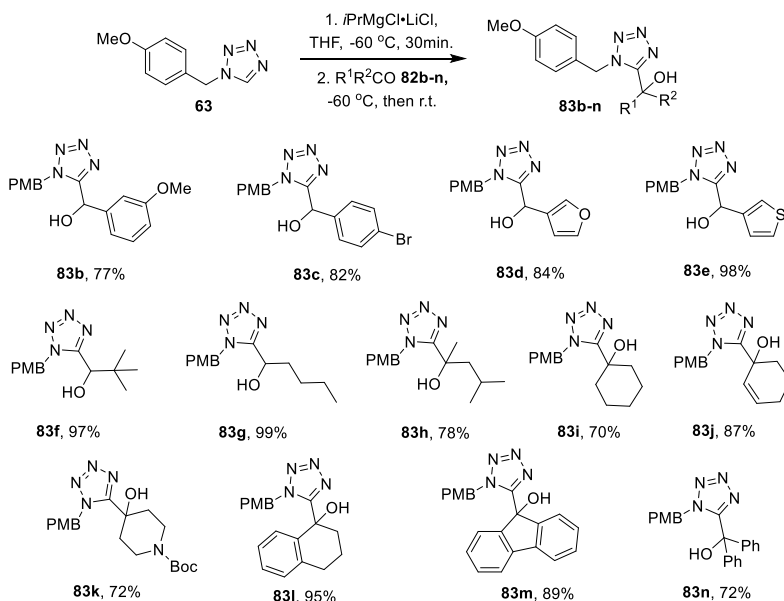
Entry	Solvent	Temperature (°C)	Time (h)	83a , yield %
1	THF	-60 °C	5	78 ^b
2	THF	r.t.	24 ^c	76
3	Et ₂ O	-60 °C	5	47
4	Toluene	-60 °C	5	22

^a Tetrazole **63** (1.1 equiv), *i*PrMgCl·LiCl (1.3 equiv), 2 mmol scale. After the addition of *i*PrMgCl·LiCl, the reaction mixture was stirred for 30 min, and then aldehyde **82a** was added.

^b 88 % yield of the product **83a** was obtained from the reaction performed on a 5 mmol scale.

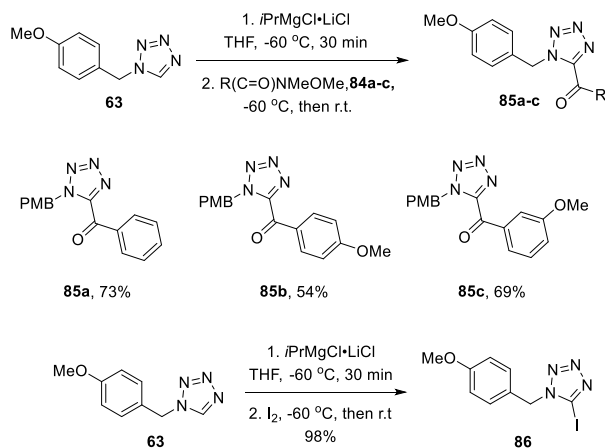
^c Addition of aldehyde **82a** performed at -60 °C, then warmed to r.t.

The scope of substrates was then explored. Using various aromatic (**82b–e**) and aliphatic (**82f–g**) aldehydes, as well as structurally diverse ketones (**82h–n**) the corresponding alcohols **83b–n** were obtained in good to excellent yields (Scheme 15).



Scheme 15. Addition of tetrazole **63** to carbonyl electrophiles.

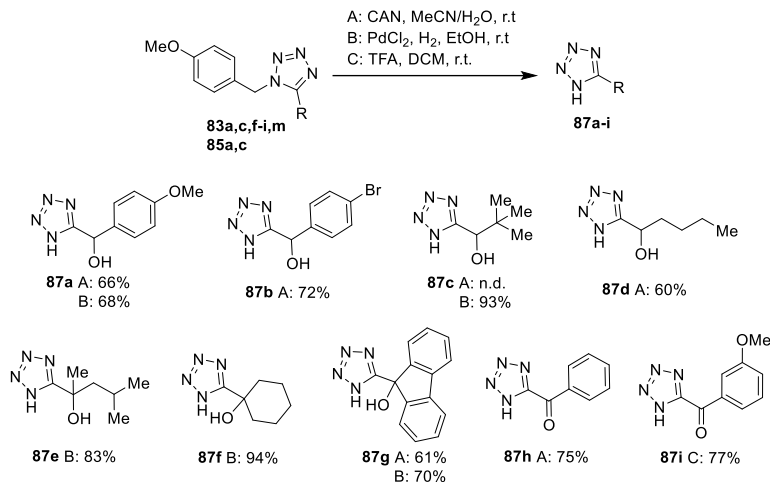
Weinreb amides **84a–c** and iodine were also successfully used as electrophiles, providing the corresponding ketones **85a–c** in good yields and the 5-iodo derivative **86** in almost quantitative yield (Scheme 16).



Scheme 16. Addition of tetrazole **63** to Weinreb amides and iodination of compound **63**.

For the deprotection of 1*N*-PMB group three different methods were used, oxidative cleavage using CAN in MeCN/H₂O (method **A**), catalytic hydrogenation with PdCl₂ and H₂ in EtOH (method **B**), and acidic cleavage using TFA in DCM (method **C**). Each of these

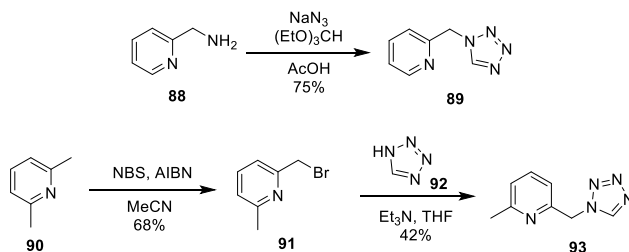
methods successfully delivered the desired free tetrazoles **87a-i** in good to excellent yield (Scheme 17).



Scheme 17. Removal of the PMB protecting group.

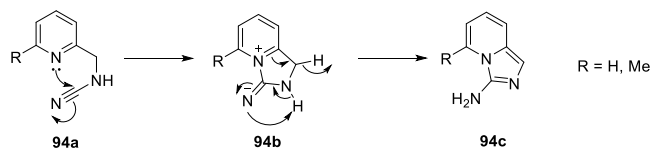
4.3. Functionalization of tetrazoles bearing an electrochemically cleavable 1*N*-6-methylpyridyl-2-methyl protecting group

In order to broaden the utility of our tetrazole functionalization method, we aimed to incorporate a new electrochemically cleavable protecting group for tetrazole. Two substrates were prepared to be tested for that purpose, pyridylmethyl protected tetrazole **89** from the corresponding 2-aminomethyl pyridine (**88**), and 6-methyl-pyridylmethyl protected tetrazole **93** from 2,6-lutidine (**90**) through bromination followed by tetrazole **92** alkylation (Scheme 18).



Scheme 18. Synthesis of pyridyl-protected tetrazoles.

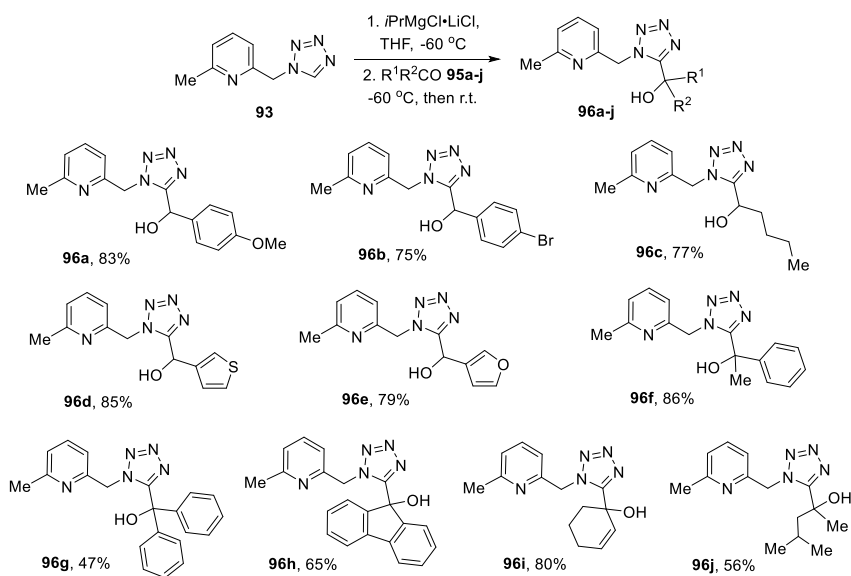
The use of the turbo Grignard reagent for the deprotonation of the fifth position of the tetrazole resulted again in minor or complete absence of retro cyclisation-decomposition product, which in the case of tetrazoles **89** and **93** proved to be **94c** instead of the cyanamide **94a** (Scheme 19).



Scheme 19. Proposed mechanism for the formation of side product 94c.

Pyridylmethyl protected tetrazole **89** gave moderate deuterium incorporation at the fifth position (40–60 %), along with the competitive deuteration product on CH₂ group and high recovery of tetrazole derivative **89**. Significant improvement in deuteration experiments was observed using 6-methyl-pyridylmethyl protected tetrazole **93** (97–98 %). Importantly, no competitive deuteration or decomposition product was detected. Our assumption is that the methyl group at the C-6 of pyridine in compound **93** resulted in blockage of the relatively acidic C-H, which can cause an equilibrium mixture of several metalated species.

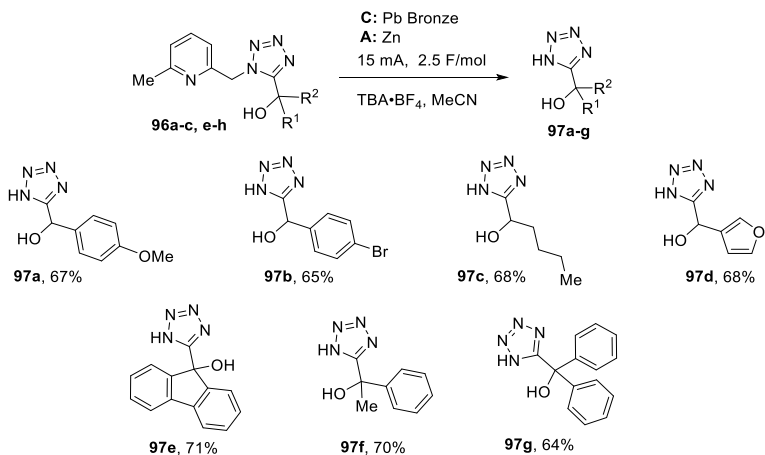
6-methyl-pyridylmethyl-protected tetrazole **93** underwent metalation and reaction with aromatic, aliphatic and heteroaromatic aldehydes **95a–e**. The resulting alcohols **96a–e** were obtained in good to excellent yields. The reaction with ketones **95f–j** was also successful providing the corresponding alcohols **96f–j** in moderate to good yields (Scheme 20).



Scheme 20. Addition of tetrazole 93 to carbonyl electrophiles.

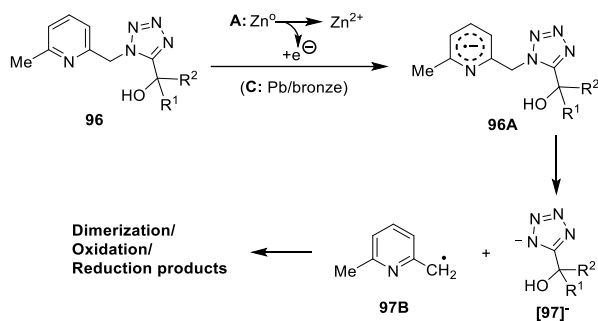
6-methyl-pyridylmethyl group has been demonstrated as an electrochemically cleavable protection for thiols, carboxylic acids, and alcohols¹⁴. Similar electrochemical conditions were applied for the reductive cleavage of 6-methyl-pyridylmethyl group from tetrazole using compound **96a** as the model substrate. Range of electrodes and electrolytes was investigated at the fixed current and electric charge. The best result was achieved using leaded bronze electrode as cathode, sacrificial zinc as anode, and TBA·BF₄ as electrolyte providing deprotected tetrazole **97a** in 67 % isolated yield. These optimized deprotection

conditions were further applied to tetrazoles **96a–c** and **96e–h** to obtain the 1*H*-tetrazoles **97b–g** in fair isolated yields as a result of the very polar nature of tetrazoles, which made the isolation rather complicated (Scheme 21).



Scheme 21. Electrochemical removal of 6-methyl-pyridylmethyl protecting group.

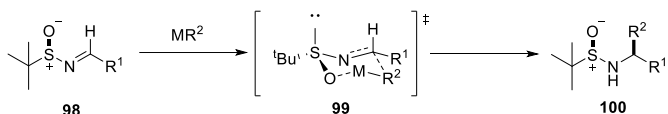
The proposed mechanism for the electrochemical cleavage of 6-methyl-pyridylmethyl group from tetrazole **96** is depicted in Scheme 22. It starts with the reduction of 6-methyl-pyridylmethyl group at the cathode by sacrificing the Zn anode, which leads to an anion radical **96A**. The latter fragments to radical **97b** and tetrazole anion [**97**]⁻. The radical **97b** undergoes further reactions, like hydrogen abstraction, dimerization, oxidation and/or reduction to give a mixture of byproducts. The formation of pyridylmethyl radical **97b** is supported by the observation of 2,6-lutidine by LC/MS analysis of crude reaction mixture, which can form either by hydrogen abstraction or a reduction followed by protonation.



Scheme 22. Proposed mechanism for the electrochemical cleavage of 6-methyl-pyridylmethyl.

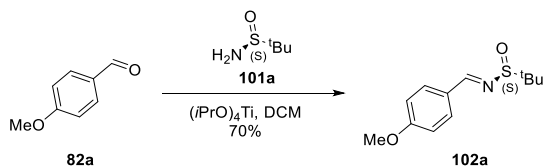
4.4. Addition of tetrazoles to imines, synthesis of amino acids' bioisosters

We turned our attention to the application of metalated tetrazole to produce amino acids' analogues bearing tetrazole as a bioisoster of the carboxylic acid. It has been reported in the literature that organometallic reagents can be added to *t*-butanesulfinyl imines in diastereoselective manner (Scheme 23).¹² The diastereoselectivity of this type of reactions is improved when DCM is used as a solvent.



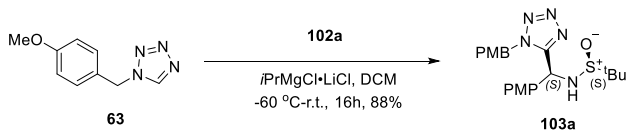
Scheme 23. Diastereoselective addition of organometallics to *t*-butanesulfinyl imines.

For the first test reactions enantiomeric imine **102a** was chosen, which was synthesized based on the literature procedure (Scheme 24).¹³



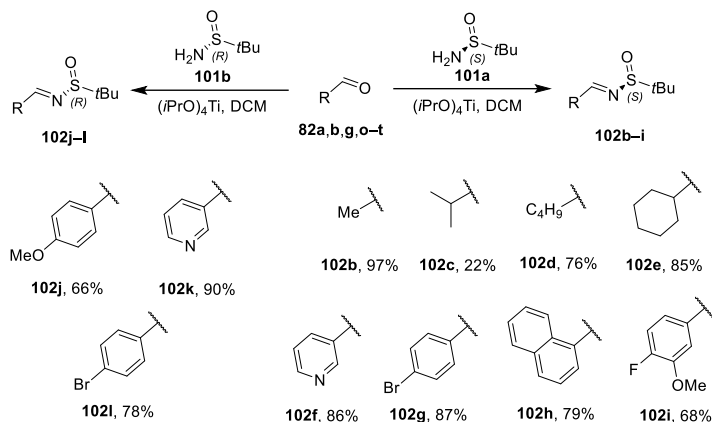
Scheme 24. Synthesis of *t*-butanesulfinyl imine **102a**.

Then, we proceeded to the deprotonation of 1*N*-PMB protected tetrazole **63** using the turbo Grignard reagent followed by the addition of the metalated intermediate to *t*-butanesulfinyl imine **102a** using DCM instead of THF as a solvent. The outcome was a single product, **103a**, with high yield, and only one diastereomer could be detected by H- and C-NMR (Scheme 25). The stereochemistry of newly formed center in product **103a** was assumed to be *S*-configuration according to the stereo-induction mechanism shown in the Scheme 23; however, an unambiguous proof is still needed.



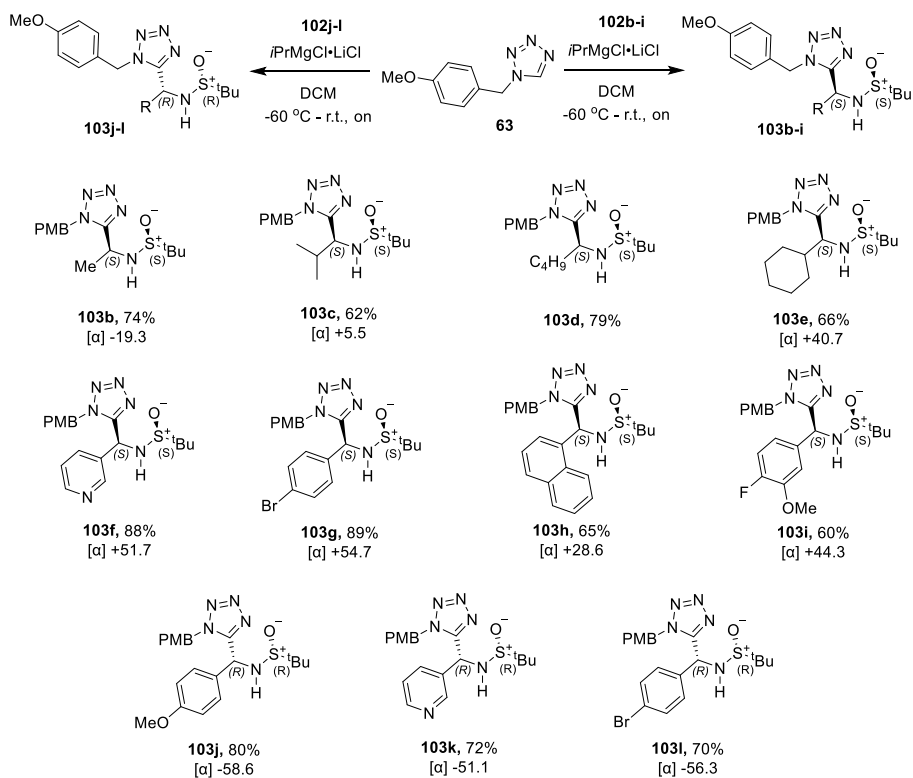
Scheme 25. Addition of tetrazole **63** to *t*-butanesulfinyl imine **102a**.

After the first encouraging results we moved to expanding the scope of the reaction. A number of *t*-butanesulfinyl imines **102b–k** were synthesized as substrates for tetrazole **63** addition (Scheme 26).



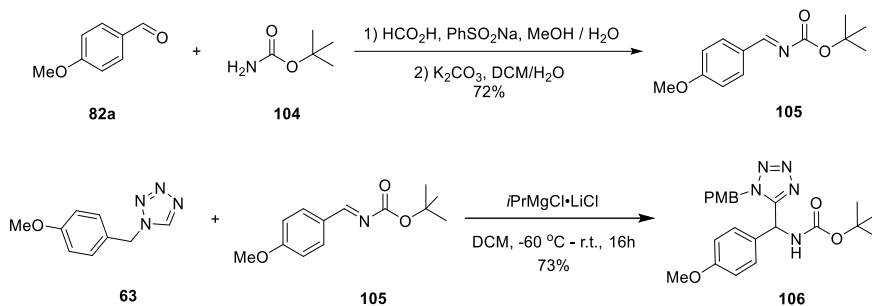
Scheme 26. Synthesis of *t*-butanesulfinyl imines **102b-l**.

Applying the already optimized reaction conditions to the *t*-butanesulfinyl imines **102b-l** provided the desired tetrazole addition products **103b-l** in good to excellent yields (Scheme 27).



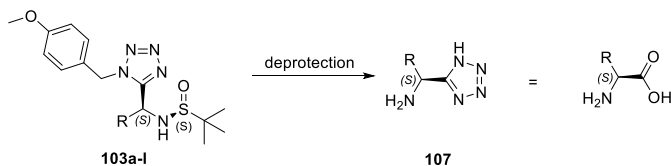
Scheme 27. Addition of tetrazole **63** to *t*-butanesulfinyl imines **102b-l**.

The tetrazole **63** could be added to *t*-butyl (*E*)-(4-methoxybenzylidene) carbamate **105**, prepared by condensation of *p*-anisaldehyde (**82a**) and *t*-butyl carbamate (**104**), resulting in a single product, compound **106** (Scheme 28), extending the applicability of the reaction to different imines.



Scheme 28 Synthesis of tetrazole **106**.

Concluding, the use of the turbo Grignard for the deprotonation of *1N*-PMB protected tetrazole **63** and subsequent addition to *t*-butanesulfinyl imines **102a–l** gave access to compounds **103a–l** that upon deprotection would provide amino acid analogues **107**, bearing tetrazole as carboxylic acid bioisoster (Scheme 29).



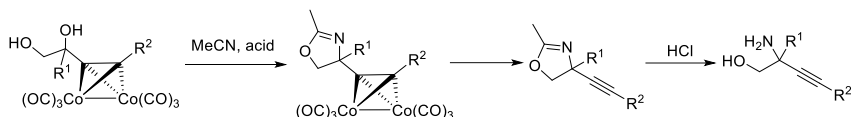
Scheme 29. Proposed route to amino acid analogues **107**.

Conclusions

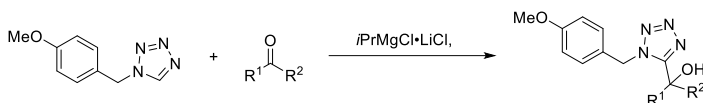
1. New chemotypes were identified as potential covalent inhibitors of PLP dependent enzymes. Hydroxamic acid and sulfonamide analogues of triF-Ala were the most potent compounds, though further study is required.



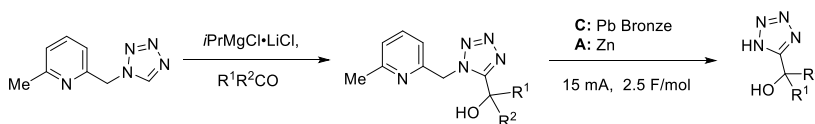
2. A new method was developed for the synthesis of *C*-quaternary alkynyl glycinols. The synthesis was realized *via* the Ritter reaction of cobalt complexed alkynyl glycols with acetonitrile to give oxazolines.



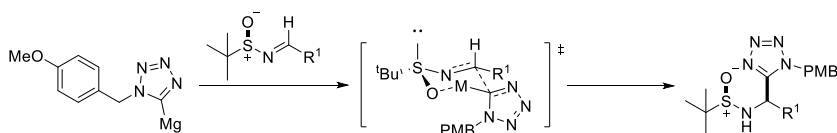
3. Direct C–H functionalization of tetrazoles can be achieved using the turbo Grignard reagent (*i*PrMgCl·LiCl complex). The use of turbo Grignard results in more stable metalated intermediate, avoiding the retro [2+3] cyclization and enabling the efficient addition of tetrazoles to electrophiles.



4. 6-methyl-pyridylmethyl protected tetrazoles can undergo C–H deprotonation using the turbo Grignard reagent and participate in reactions with electrophiles. 6-methyl-pyridylmethyl group can be removed under reductive electrochemical conditions.



5. The reaction of deprotonated tetrazoles with enantiomeric *t*-butanesulfinyl imines can deliver the corresponding addition products in diastereoselective manner. This method can give access to amino acids' analogues bearing tetrazole moiety as carboxylic acid replacement.



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Konstantinos Grammatoglou dzimis 1980. gadā Grieķijā, Salonikos. Aristoteļa Universitātē (Saloniki) ieguvis bakalaura (2013) un maģistra (2015) grādu ķīmijā. No 2015. līdz 2022. gadam strādājis Latvijas Organiskās sintēzes institūta Organiskās sintēzes metodoloģijas grupā. Patlaban ir pētnieks *Symeres* Groningenā, Nīderlandē. Zinātniskās intereses saistītas ar organisko ķīmiju, mazo molekulu un bioloģiski aktīvu savienojumu sintēzi. Ir četru oriģinālpublikāciju, kas publicētas starptautiski citējamos žurnālos (impaktfaktors 5,756), līdzautors.

Konstantinos Grammatoglou was born in 1980 in Thessaloniki, Greece. He obtained his Bachelor's degree in 2013 and his Master's degree in 2015 from Aristotle University of Thessaloniki. From 2015 until 2022, he has been a Research Assistant at Latvian Institute of Organic Synthesis in Organic Synthesis Methodology group, Riga. He is currently a Research Scientist at *Symeres* in Groningen, Netherlands. His research interests are organic chemistry research, synthesis of small molecules and synthesis of bioactive compounds. He is a co-author of four original publications in international journals (IF up to 5.756).